

The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats

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Rats were fed a grape seed extract (GSE) containing (+)-catechin, (–)-epicatechin and dimers, trimers, tetramers and polymeric procyanidins. Liver, kidney, brain and gastrointestinal (GI) tract together with plasma, urine and faeces were collected over a 24 h period and their flavan-3-ol content was analysed by HPLC with tandem mass spectrometry and diode array detection. Small amounts of the GSE flavan-3-ols moved out of the stomach and into the duodenum/jejunum, and to a greater extent the ileum 1 h after ingestion, and into the caecum after 2 h with relatively small amounts being detected in the colon after 3 h. The GI tract contained the parent GSE flavan-3-ols and procyanidins with only trace amounts of metabolites and there were no indications that proanthocyanidins were depolymerised in the GI tract releasing monomeric flavan-3-ols. Plasma contained exclusively catechin glucuronides and methylated glucuronide metabolites which were also detected in the liver and kidneys. These metabolites were also present in urine together with sulphated metabolites and low amounts of the procyanidin dimers B₁, B₂, B₃ and B₄ as well as the trimer C₂ and an unknown GSE trimer. The amounts of (+)-catechin and (–)-epicatechin metabolites excreted in urine relative to the quantity of the monomers ingested were 27 and 36 %, respectively, after 24 h. This is similar to the levels of urinary excretion reported to occur by other investigators after feeding (–)-epicatechin to rats and provides further, albeit indirect, evidence that the procyanidin oligomers in the GSE were not depolymerised to monomers to any extent after ingestion. No convincing analytical data were obtained for the presence of flavan-3-ol metabolites in the brain.

Flavan-3-ols: Procyanidins: Absorption: Metabolism: Excretion: Rats: HPLC–MS–MS

Flavan-3-ols are a complex subclass of flavonoids encompassing the simple monomers (+)-catechin and its isomer (–)-epicatechin, and the oligomeric and polymeric procyanidins, commonly known as condensed tannins (Fig. 1). The procyanidins are formed from the condensation of monomeric units, between two and five units for oligomers and over five units for polymers. Procyanidins differ in their position and configuration of their monomeric linkages, with the dimers B₁, B₂, B₃ and B₄ being detected most frequently. Besides forming complexes with other flavan-3-ols, the monomeric flavan-3-ols are hydroxylated to form the gallo catechins, and also undergo esterification with gallic acid (Crozier, 2003).

Flavan-3-ols are widely dispersed in the human diet and are one of the most abundant dietary flavonoids with red wine, tea, berries, apples and chocolate providing the richest food sources (Sanoner *et al.* 1999; Foo *et al.* 2000; Auger *et al.* 2004;

Del Rio *et al.* 2004). Although there are no accurate estimates of flavan-3-ol intake levels, it has been speculated that consumption ranges between 0.1 and 0.5 g/d (de Pascual-Teresa *et al.* 2000; Scalbert & Williamson, 2000). The data of Arts *et al.* (2001) imply that flavan-3-ols may be one of the more effective groups of dietary phenolics in reducing the risk of coronary heart disease. In keeping with this possibility (+)-catechin and procyanidins have been shown to reduce platelet aggregation (Ruf *et al.* 1995) and to act as powerful inhibitors of LDL oxidation *in vitro* (Teissedre *et al.* 1996; Steinberg *et al.* 2003) and *ex vivo* (Kondo *et al.* 1996), events believed to play a crucial role in preventing the onset of atherogenesis (Steinberg *et al.* 1989). It has also been demonstrated that consumption of a procyanidin-rich grape seed extract (GSE) reduces the incidence of cataracts in the eyes of hereditary cataractous (ICR/f) rats (Yamakoshi *et al.* 2002).

Abbreviations: amu, atomic mass unit; GI, gastrointestinal; GSE, grape seed extract; [M-H][–], negatively charged molecular ion; MS–MS, tandem MS; RDA-F, retro-Diels-Alder fission; SIM, selected ion monitoring; SRM, selected reaction monitoring; t_R, retention time.

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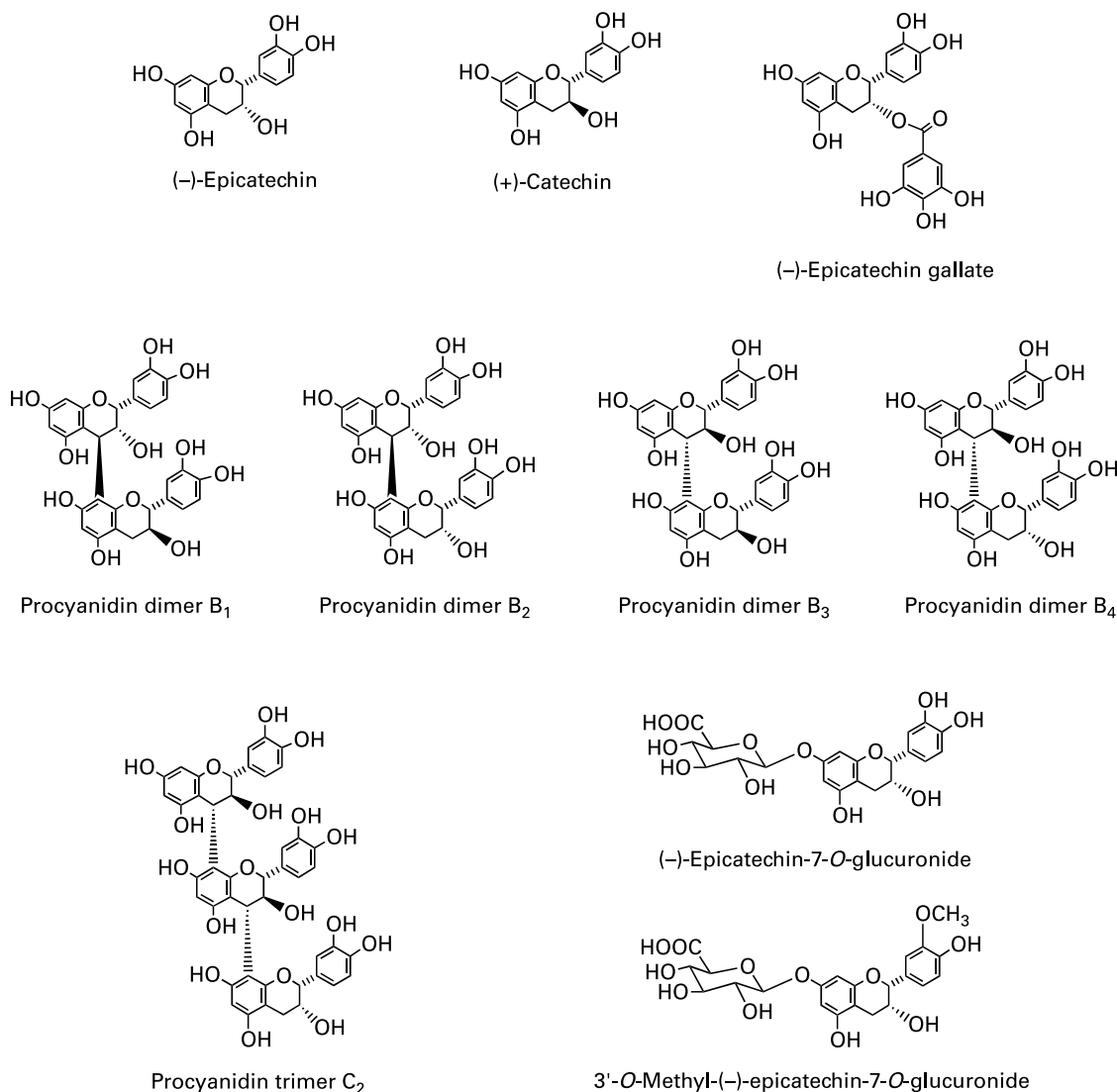


Fig. 1. Structures of flavan-3-ols and procyanidin dimers and trimers.

(+)-Catechin and (-)-epicatechin are absorbed in humans and animals, appearing in plasma and urine primarily as glucuronidated, methylated and sulphated metabolites following the ingestion of chocolate (Rein *et al.* 2000; Wang *et al.* 2000; Baba *et al.* 2001), black and green tea (Piskula & Terao, 1998; Yang *et al.* 1998; Warden *et al.* 2001) and red wine (Donovan *et al.* 1999; Bell *et al.* 2000). There is, nevertheless, conflicting evidence on the absorption and metabolism of the oligomeric and polymeric flavan-3-ols in humans and animals. Koga *et al.* (1999) observed the presence of (+)-catechin and (-)-epicatechin and an absence of dimers in the plasma of rats following ingestion of a GSE. Extending this study, Donovan *et al.* (2002) fed rats GSE, (+)-catechin and procyanidin B₃ meals. While conjugated metabolites of (+)-catechin were detected in plasma and urine after both the (+)-catechin and GSE meals, there was no evidence for absorption of the procyanidins. However, in another study (-)-epicatechin, (+)-catechin and trace amounts of procyanidin dimer B₂ were detected in sulphatase- and β -glucuronidase-treated human plasma collected 30 min after ingestion of a cocoa beverage rich in flavan-3-ol monomers and procyanidins (Holt *et al.* 2002). In keeping with this report, it has

been shown that after oral administration of B₂ to rats, the dimer is absorbed and excreted in urine with a portion of the procyanidin being converted to (-)-epicatechin, which undergoes post-ingestion conjugation and methylation (Baba *et al.* 2002).

In this paper we report on a comprehensive study of the absorption, excretion and sequestration in body tissues of (+)-catechin, (-)-epicatechin, their metabolites and procyanidin dimers and a trimer following the oral intake of a GSE by rats.

Methods

Materials

(+)-Catechin, (-)-epicatechin, (-)-epicatechin-3-O-gallate and procyanidin B₃ were obtained from AASC Chemicals (Southampton, UK). HPLC-grade acetonitrile was supplied by Rathburn Chemicals (Walkerburn, Peebleshire, UK). Standards of the procyanidin dimers B₁, B₂ and B₄ were obtained from a GSE using procedures described by Teissedre *et al.* (1996). Other laboratory reagents were purchased from Sigma Aldrich (Poole, UK).

The GSE was produced by Partoeno (Bordeaux, France). It was oenological international grade (tannin indices 94.7%, mineral ashes 1.01% (m/m), insoluble residue 0.62% (m/m)) produced from Chardonnay and Pinot Noir grape seeds by water and alcohol extraction *in vacuo* at low temperature.

Animal and sample preparation

Sprague–Dawley male rats ($n = 24$), weighing 250 ± 5 g, were housed in metabolic cages allowing the collection of 24 h urine and faecal samples. Rats were deprived of food for 16 h before being fed by gavage a GSE (1 g/kg body weight) containing monomers, oligomers and polymers of flavan-3-ols dissolved in water (1 g GSE per 6 ml). Three animals were terminally anaesthetised with pentobarbital 0, 1, 2, 3, 4, 6, 12 and 24 h after administration of the GSE. Blood was removed by cardiac puncture with heparin-moistened syringes and plasma was obtained by centrifugation at 2000 g for 10 min at 4°C. Liver, kidney, brain, stomach, duodenum/jejunum, ileum, caecum, colon, urine and faeces were collected at each time point. All samples were immediately frozen in liquid N₂ and stored at –80°C prior to analysis.

Extraction of phenolics from rat tissues

Samples from three individual rats were combined and extracted according to the method of Mullen *et al.* (2002). In brief, 4 g liver, kidney and brain, and 2.5 g stomach and sections of the gastrointestinal (GI) tract, including contents, were macerated using an Ultraturax homogeniser and extracted by continuous shaking with 15 ml methanol in 0.1 M-phosphate buffer (pH 7; 1:1, v/v) containing 20 mM-sodium diethyldithiocarbamate for 10 min. The mixture was centrifuged at 2000 g for 20 min. The methanolic supernatant was decanted and the pellet re-extracted twice. The three methanolic supernatants were combined and the methanol was removed *in vacuo*. The remaining aqueous phase was adjusted to pH 3.0 with 5% aqueous HCl and partitioned three times with an equal volume of ethyl acetate. The ethyl acetate extracts were combined and stored overnight at –20°C prior to filtration to remove ice. The filtered ethyl acetate extract was reduced to dryness *in vacuo*, and the extract was dissolved in 50 µl methanol in 450 µl aqueous 1% formic acid before analysis by HPLC–tandem MS (HPLC–MS–MS).

After partitioning, the residual aqueous phase was placed *in vacuo* to remove ethyl acetate before being loaded on to a 2 g Sep-Pak C₁₈ cartridge (Waters, Milford, MA, USA). The cartridge was washed with acidified water (pH 3.0) before elution of polar compounds with 10 ml methanol followed by 10 ml methanol containing 0.1% HCl. The methanolic eluates were combined and reduced to a volume of 1 ml *in vacuo* before analysis using HPLC–MS–MS. As there was a wide range of phenolic compounds present it was impossible to add a specific internal standard for each compound analysed. Therefore, the overall extraction efficiency was calculated as described by Mullen *et al.* (2004) using [2-¹⁴C]quercetin-4'-glucoside.

Extraction of phenolics from plasma

Proteins in plasma were precipitated and intact phenolics extracted as described by Mullen *et al.* (2002). Briefly, 1 ml plasma was spiked with an internal standard of [2-¹⁴C]quercetin-4'-glucoside

then 2.5 ml acetonitrile were added and the sample was vortexed for 30 s every 2 min for 10 min. The sample was then centrifuged at 3000 g at 4°C for 10 min, the supernatant removed and the pellet extracted as outlined above using methanol instead of acetonitrile. The two supernatants were then combined, reduced to dryness and redissolved in a known volume of 5% methanol in water containing 1% formic acid. An aliquot of this was taken for scintillation counting to allow the extraction efficiency to be calculated, which was typically about 86%.

HPLC with diode array detection and tandem mass spectrometry

All samples were analysed in triplicate on a Surveyor HPLC system comprising of a HPLC pump, diode array detector scanning from 250 to 700 nm, and an autosampler set at 4°C (Thermo Finnigan, San Jose, USA) with separation carried out using a 250 × 4.6 mm internal diameter 4 µm Synergi RP-Max column (Phenomenex, Macclesfield, UK) eluted at a flow rate of 1 ml/min. A mobile phase consisting of a 5–40% gradient over 60 min of 1% formic acid and acetonitrile was used for the analysis of all samples except urine, for which a 5–20%, 60 min gradient was utilised. After passing through the flow cell of the diode array detector the column eluate was split and 0.3 ml was directed to a LCQ Deca XP ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan). Analysis was carried out in negative ion mode operating in full-scan mode from 100 to 2000 atomic mass units (amu).

The tuning of the mass spectrometer was optimised by infusing a standard of (+)-catechin dissolved in 5% acetonitrile in water containing 1% formic acid into the source at a flow rate of 0.25 ml/min. Capillary temperature was 350°C, sheath gas and auxiliary gas were 80 and 20 arbitrary units, respectively, source voltage was 5 kV and capillary voltage was –39 V. Maximum scan time for MS–MS, single ion monitoring (SIM) and selected reaction monitoring (SRM) was 50, 200 and 400 ms, respectively. The automatic gain control was on, which ensures the optimum number of ions are collected in the ion trap.

Identification of flavan-3-ols and/or their metabolites in all samples was carried out using full-scan data-dependent MS–MS. Quantitative estimates of the flavan-3-ols and procyanidins in the GSE were based on the absorbance response at 280 nm. (+)-Catechin and (–)-epicatechin were quantified by reference to authentic standards while the levels of all dimers, trimers and tetramers were expressed as dimer B₃ equivalents. The extensive purification of extracts of body tissues also enabled flavan-3-ols and their metabolites to be quantified in (+)-catechin equivalents based on the absorbance response at 280 nm with peak identification confirmed by full-scan MS–MS. Quantitative estimates of metabolite levels in plasma and urine made use of the selectivity of the mass spectrometer operating in SIM and SRM modes.

The quantities of procyanidins in urine were determined by SRM. The conditions used were as outlined above except that the mass spectrometer was set up to monitor specific ions. When measuring the levels of dimers the parent ion was m/z 577, the collision energy 35%, and product ions used for quantification were m/z 425, 407 and 289. In the case of trimers, the parent ion was m/z 865 and product ions used for quantification were m/z 695 and 577. Dimer B₃ was used as a calibration standard. Estimated levels of trimers are therefore quoted in B₃ equivalents. When an ion trap mass spectrometer is used in the

SRM mode, there is no loss in sensitivity by increasing the range of the product ions scanned. In practice, the mass range that can be scanned is from the parent ion down to an m/z value around one-third of the mass of the parent ion. Hence, with the flavan-3-ol dimers the product ions arising from the parent ion at m/z 577 can be scanned from m/z 577 to m/z 190. Quantification was carried out on the selected authentic ions, in this case m/z 425, 407 and 289. This allows for accurate quantification while the occurrence of additional spurious ions can be monitored so precluding false positive identifications.

Quantification of (–)-epicatechin glucuronide, (+)-catechin glucuronide, methyl(–)-epicatechin glucuronide and methyl(+)-catechin glucuronide in plasma and urine used the conditions outlined above with the mass spectrometer in the SIM mode. The parent ions were m/z 465 and m/z 479. Quantitative analyses of (–)-epicatechin sulphate and (+)-catechin sulphate, and methyl(–)-epicatechin sulphate and methyl(+)-catechin sulphate were also based on SIM using m/z 369 and m/z 389, respectively, as the parent ions. No standards of these compounds were available so (+)-catechin was used as the calibration standard. Our experience in previous studies has demonstrated that if the mass spectrometer is set up using quercetin, the same parameters work equally well for a quercetin glucoside conjugate and vice versa. Likewise calibration files created using quercetin or quercetin-4'-glucoside in SIM mode produce identical results.

Results

Analysis of the grape seed extract

A 1 mg/ml solution of GSE in 25% methanol in water containing 1% formic acid was prepared and 20 μ l aliquots were analysed by gradient reversed-phased HPLC with absorbance and MS–MS detection. In total 13 phenolic compounds were identified in the GSE by HPLC–MS–MS and quantified by absorbance detection at 280 nm. The data obtained are illustrated in Fig. 2 and summarised in Table 1. Where no reference compounds were available to confirm identifications by co-chromatography, identifications were facilitated by previously published data on the analyses of flavan-3-ols in grape seeds and GSE as cited in the text.

Peak 1. Peak 1 ($t_R = 6.2$ min, $\lambda_{max} = 280$ nm) had a $[M-H]^-$ at m/z 865 with MS^2 yielding major fragment ions at m/z 713, 695, 577, 425 and 407. The first fragment ion at m/z 713, a loss of 152 amu, comes from retro-Diels-Alder fission (RDA-F) of

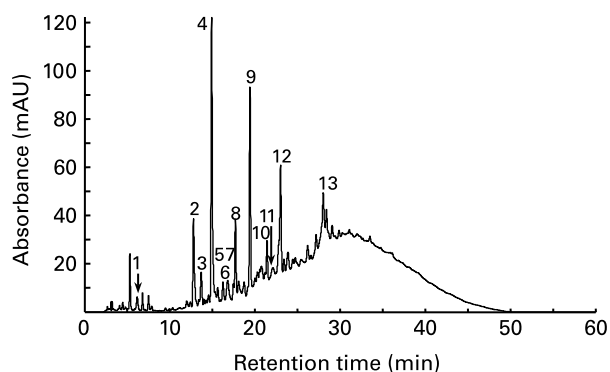


Fig. 2. Reversed-phase HPLC of a grape seed extract using a 60 min 5–40% gradient of acetonitrile in 1% aqueous formic acid with detection at 280 nm. For MS^2 data and identification and quantification of peaks 1–13, see Table 1.

the heterocyclic ring system. The base ion at m/z 695, a loss of 170 amu, is from RDA-F with an additional loss of water. The ion at m/z 577, a loss of 288 amu, is from interflavanic bond cleavage. The ions at m/z 425 and m/z 407 are from further RDA-F after interflavanic bond cleavage (Friedrich *et al.* 2000; Shui & Leong, 2004). Based on the mass spectral data this compound is a procyanidin trimer. Its elution prior to procyanidin dimer B_1 (peak 2) (Santos-Buelga *et al.* 1995) indicates that it is probably C_2 , a known component of grape seeds (Romeyer *et al.* 1986).

Peak 2. Peak 2 ($t_R = 12.1$ min, $\lambda_{max} = 280$ nm) had a $[M-H]^-$ at m/z 577 with MS^2 yielding three fragment ions at m/z 425, 407 and 289. This mass spectrum and co-chromatography with an authentic standard established that peak 2 is procyanidin dimer B_1 , which has previously been detected in grape seeds (Ricardo da Silva *et al.* 1991).

Peak 3. Peak 3 ($t_R = 13.1$ min, $\lambda_{max} = 280$ nm) also had a $[M-H]^-$ at m/z 577 which fragmented to produce MS^2 ions at m/z 425, 407 and 289. Its elution prior to (+)-catechin (Sun *et al.* 1999) and co-chromatography with an authentic standard demonstrates that peak 3 is procyanidin dimer B_3 .

Peak 4. Peak 4 ($t_R = 14.3$ min, $\lambda_{max} = 280$ nm) had a $[M-H]^-$ at m/z 289 and MS^2 produced fragment ions at m/z 245 and 205. This mass spectrum and co-chromatography with an authentic standard identifies peak 4 as (+)-catechin.

Peak 5. Peak 5 ($t_R = 15.0$ min, $\lambda_{max} = 280$ nm) produced a $[M-H]^-$ at m/z 1153 which on MS^2 yielded ions at m/z 865 and 577. This sequential loss of 288 amu is typical of a procyanidin oligomer. Although a reference compound was not available, the early elution of this peak close to (+)-catechin and the mass spectral data indicate that it is a procyanidin tetramer, the presence of which has been previously reported in a GSE (Escrībana-Bailón *et al.* 1992).

Peak 6. Peak 6 ($t_R = 15.5$ min, $\lambda_{max} = 280$ nm) had a $[M-H]^-$ at m/z 865 with MS^2 producing major fragment ions at m/z 695, 577, 425 and 407. Based on the mass spectral data, this compound is a procyanidin trimer. Its elution between (+)-catechin (peak 4) and the dimer B_4 (peak 7) indicates that it may be epicatechin-(4 β -8)-epicatechin-(4 β -8)-catechin previously identified in a GSE by Santos-Buelga *et al.* (1995).

Peak 7. Peak 7 ($t_R = 16.1$ min, $\lambda_{max} = 280$ nm) had a $[M-H]^-$ at m/z 577 which on MS^2 yielded three ions at m/z 425, 407 and 289. This mass spectrum and co-chromatography with an authentic standard identifies peak 7 as procyanidin B_4 , a known component of grape seeds (de Pascual-Teresa *et al.* 2000).

Peak 8. Peak 8 ($t_R = 16.9$ min, $\lambda_{max} = 280$ nm) produced a $[M-H]^-$ at m/z 577 which produced MS^2 fragments at m/z 425, 407 and 289. The mass spectral data indicated the presence of a procyanidin dimer and co-chromatography with an authentic standard identified it as the B_2 dimer.

Peak 9. Peak 9 ($t_R = 18.7$ min, $\lambda_{max} = 280$ nm) had a $[M-H]^-$ at m/z 289 which on MS^2 yielded two ions at m/z 245 and 205. This mass spectrum and co-chromatography with an authentic standard established that peak 9 is (–)-epicatechin.

Peak 10. Peak 10 ($t_R = 20.6$ min, $\lambda_{max} = 280$ nm) produced a $[M-H]^-$ at m/z 865, which yielded MS^2 fragments at m/z 695, 577, 425 and 407. Based on the mass spectral data this compound is a procyanidin trimer.

Peak 11. Peak 11 ($t_R = 21.3$ min, $\lambda_{max} = 275$ nm) had a $[M-H]^-$ at m/z 1153, and MS^2 yielded major ions at m/z 865 and 577. This is in keeping with peak 11 being a procyanidin tetramer.

Table 1. HPLC and mass spectral characteristics of flavan-3-ols in a grape seed extract and amounts ingested per rat*(Values are means with their standard errors (*n* 3))

| Peak | <i>t_R</i> (min) | Compound | [M-H] ⁻ (<i>m/z</i>) | MS ² ions (<i>m/z</i>) | Amount ingested (mg per rat) | |
|------|----------------------------|---|-----------------------------------|-------------------------------------|------------------------------|-----|
| | | | | | Mean | SE |
| 1 | 6.2 | Procyanidin trimer C ₂ | 865 | 577, 289 | 0.3 | 0.0 |
| 2 | 12.1 | Procyanidin dimer B ₁ | 577 | 425, 407, 289 | 1.5 | 0.2 |
| 3 | 13.1 | Procyanidin dimer B ₃ | 577 | 425, 407, 289 | 0.6 | 0.2 |
| 4 | 14.3 | (+)-Catechin | 289 | 245 | 5.8 | 0.2 |
| 5 | 15.0 | Procyanidin tetramer | 1153 | 577, 289 | 0.2 | 0.0 |
| 6 | 15.5 | Procyanidin trimer | 865 | 577, 289 | 0.3 | 0.1 |
| 7 | 16.1 | Procyanidin dimer B ₄ | 577 | 425, 407, 289 | 0.7 | 0.1 |
| 8 | 16.9 | Procyanidin dimer B ₂ | 577 | 425, 407, 289 | 1.5 | 0.2 |
| 9 | 18.7 | (-)-Epicatechin | 289 | 245 | 3.6 | 0.1 |
| 10 | 20.6 | Procyanidin trimer | 865 | 577, 289 | 0.7 | 0.1 |
| 11 | 21.3 | Procyanidin tetramer | 1153 | 577, 289 | 0.5 | 0.2 |
| 12 | 22.2 | Procyanidin B ₁ -3- or B ₂ -3'- <i>O</i> -gallate | 729 | 577, 407 | 2.8 | 0.1 |
| 13 | 27.2 | (-)-Epicatechin-3- <i>O</i> -gallate | 441 | 289 | 0.7 | 0.2 |

[M-H]⁻, negatively charged molecular ion.*Identifications based on retention times (*t_R*), and full-scan negative ionisation MS-MS data. Peak numbers and *t_R* values refer to peaks in Fig. 2.

Peak 12. Peak 12 (*t_R* = 22.2 min, λ_{\max} = 275 nm) had a [M-H]⁻ at *m/z* 729, with MS² ions at *m/z* 577, 425, 407 and 289. These ions are similar to those observed with the procyanidin dimers in peaks 8 and 9 with the *m/z* 577 fragment resulting from a 154 amu loss from the [M-H]⁻ which is indicative of the cleavage of a gallate unit. On the basis of the MS² spectrum, this compound is, therefore, a gallated procyanidin dimer. Although a reference compound was not available, the elution order of this peak after (-)-epicatechin (peak 9) and a procyanidin trimer (peak 10) along with the mass spectral data indicate that it is either B₁-3-*O*-gallate or B₂-3'-*O*-gallate, both of which have been detected in grape seeds. Under similar chromatographic conditions both gallated dimers elute after (-)-epicatechin and a procyanidin trimer (Santos-Buelga *et al.* 1995).

Peak 13. Peak 13 (*t_R* = 27.2 min, λ_{\max} = 275 nm) had a [M-H]⁻ at *m/z* 441 with MS² producing a fragment at *m/z* 289. This mass spectrum and co-chromatography with an authentic standard established that peak 3 is (-)-epicatechin-3-*O*-gallate.

The levels of the 13 flavan-3-ols that were ingested when each rat was fed the GSE are presented in Table 1 together with the summarised analytical data. As well as the flavan-3-ol monomers, dimers, trimers and tetramers listed in Table 1, each rat also consumed a substantial but undetermined quantity of high-molecular weight polymeric procyanidins whose presence in the GSE is

evident from the increasing baseline and the broad unresolved band of components that elute across the HPLC-A₂₈₀ chromatogram illustrated in Fig. 2.

Identification of flavan-3-ol metabolites in body tissues and fluids

Using a 5–40% acetonitrile gradient HPLC-MS-MS in the full-scan mode detected four flavan-3-ol metabolites in the GI tract, plasma, liver and kidneys. The results of these analyses are summarised below and in Table 2.

Peak M-1. Peak M-1 (*t_R* = 11.6 min, λ_{\max} = 280 nm) and M-2 (*t_R* = 14.5 min, λ_{\max} = 280 nm) both had a [M-H]⁻ at *m/z* 465 which yielded MS² fragments at *m/z* 289. MS² of the ion at *m/z* 289 provided a mass spectrum that matched that of (+)-catechin/(-)-epicatechin. The [M-H]⁻-176 loss is in keeping with the cleavage of a glucuronyl unit. The MS fragmentation pattern, together with the HPLC elution order, indicates that the earlier eluting M-1 peak is a (+)-catechin glucuronide while M-2 is a (-)-epicatechin glucuronide.

Peak M-3. Peak M-3 (*t_R* = 17.7 min, λ_{\max} = 280 nm) and M-4 (*t_R* = 20.7 min, λ_{\max} = 280 nm) also had identical mass spectra with a [M-H]⁻ at *m/z* 479. The loss of 176 amu (cleavage of a glucuronyl unit) yielded an MS² ion at *m/z* 303. MS² of the ion at *m/z* 303 provided a mass spectrum that matched that of (+)-catechin/(-)-epicatechin though 14 amu higher. The parent and

Table 2. HPLC and mass spectral properties of flavan-3-ol metabolites detected in rats after oral ingestion of a grape seed extract

| Peak | <i>t_R</i> (min) | Metabolite | [M-H] ⁻ (<i>m/z</i>) | MS ² ions (<i>m/z</i>) | Location |
|------|----------------------------|------------------------------------|-----------------------------------|---|--------------------|
| M-1 | 11.6* | (+)-Catechin glucuronide | 465 | 289 [C] ([M-H] ⁻ -GlcUA) | P, U, D/J, I, L, K |
| M-2 | 14.5* | (-)-Epicatechin glucuronide | 465 | 289 [EC] ([M-H] ⁻ -GlcUA) | P, U, D/J, I, L, K |
| M-3 | 17.7* | Methyl-(+)-catechin glucuronide | 479 | 303 [C] ([M-H] ⁻ -GlcUA) | P, U, D/J, I, L, K |
| M-4 | 20.7* | Methyl-(-)-epicatechin glucuronide | 479 | 303 [EC] ([M-H] ⁻ -GlcUA) | P, U, D/J, I, L, K |
| U-1 | 24.6† | (+)-Catechin sulphate | 369 | 289 [C] ([M-H] ⁻ -SO ₃) | U |
| U-2 | 27.9† | (-)-Epicatechin sulphate | 369 | 289 [EC] ([M-H] ⁻ -SO ₃) | U |
| U-3 | 29.2† | Methyl-(+)-catechin sulphate | 383 | 303 [MC] ([M-H] ⁻ -SO ₃) | U |
| U-4 | 38.1† | Methyl-(-)-epicatechin sulphate | 383 | 303 [ME] ([M-H] ⁻ -SO ₃) | U |

t_R, retention time; C, (+)-catechin; D/J, duodenum/jejunum; EC, (-)-epicatechin; GlcUA, glucuronyl unit; I, ileum; L, liver; K, kidneys; [M-H]⁻, negatively charged molecular ion; MC, methyl-(+)-catechin; ME, methyl-(-)-epicatechin; P, plasma; U, urine.

*HPLC gradient 5–40%, 60 min.

†HPLC gradient 5–20%, 60 min.

fragment ions 14 amu higher than the equivalent ions seen in the spectra of *M-1* and *M-2* indicate the presence of methylated flavan-3-ol glucuronides. *M-3* is therefore probably a methyl-(+)-catechin glucuronide and the later eluting *M-4* is probably a methyl-(−)-epicatechin glucuronide.

Without reference compounds it was not possible to determine the positions on the flavan-3-ol skeleton of the methyl and glucuronic acid substituents. The most likely candidates for *M-2* and *M-4* are (−)-epicatechin-7-*O*-glucuronide and 3′-*O*-methyl-(−)-epicatechin-7-*O*-glucuronide, which have been identified in rat plasma and urine after ingestion of (−)-epicatechin (Natsume *et al.* 2003). (+)-Catechin is converted to 3′-*O*-methyl-(+)-catechin in rats (Nakamura & Tonogai, 2003), so *M-3* may be a 3′-*O*-methyl-(+)-catechin glucuronide. However, no (+)-catechin glucuronides have been structurally elucidated so it is not possible to speculate on the position of the glucuronide moiety in *M-1* and *M-3*.

Urine contained impurities that interfered with metabolite identification when HPLC was carried out with a 5–40% gradient of acetonitrile. This was overcome with the use of a 5–20% gradient which enhanced resolution and revealed the presence of the four previously detected metabolites (*M-1*, *M-2*, *M-3* and *M-4* at retention times of 14.1, 19.0, 26.0 and 30.2 min, respectively) together with four additional urinary metabolites, *U-1*, *U-2*, *U-3* and *U-4*, that were not present in other samples (Table 2).

Peaks *U-1* and *U-2*. Peaks *U-1* ($t_R = 24.6$ min, $\lambda_{max} = 280$ nm) and *U-2* ($t_R = 27.9$ min, $\lambda_{max} = 280$ nm) both had a $[M-H]^-$ at m/z 369 yielding an MS^2 ion at m/z 289. The $[M-H]^-$ -80 loss is in keeping with the cleavage of a SO_3 unit. MS^2 of the ion at m/z 289 also provided a mass spectrum that corresponded to that of (+)-catechin/(−)-epicatechin. The MS fragmentation pattern, together with the HPLC elution order, indicates that the earlier eluting *U-1* peak is a (+)-catechin sulphate while *U-2* is an (−)-epicatechin sulphate.

Peaks *U-3* and *U-4*. Peaks *U-3* ($t_R = 29.2$ min, $\lambda_{max} = 280$ nm) and *U-4* ($t_R = 38.1$ min, $\lambda_{max} = 280$ nm) had a $[M-H]^-$ at m/z 383 which on loss of 80 amu (cleavage of a SO_3 unit) yielded an MS^2 ion at m/z 303. MS^2 of the ion at m/z 303 provided a mass spectrum that matched that of (+)-catechin/(−)-epicatechin though 14 amu higher. The fragmentation patterns seen in the spectra of *U-1* and *U-2* indicate the presence of methylated flavan-3-ol glucuronides. Unlike human subjects, which produce 4′-*O*-methylated derivatives, rats accumulate predominantly 3′-*O*-methylated metabolites of flavan-3-ols (Nakamura & Tonogai, 2003; Natsume *et al.* 2003), *U-3* is therefore probably a 3′-*O*-methyl-(+)-catechin sulphate and the later eluting *U-4*, a 3′-*O*-methyl-(−)-epicatechin sulphate. This is the first direct evidence for the occurrence of sulphated flavan-3-ol metabolites in rats and as yet the position of the sulphate moiety remains undetermined.

Quantification of plasma flavan-3-ol metabolites

(+)-Catechin glucuronide (*M-1*), (−)-epicatechin glucuronide (*M-2*), methyl-(+)-catechin glucuronide (*M-3*) and methyl-(−)-epicatechin glucuronide (*M-4*) were detected in plasma (Table 2). This is shown in the HPLC traces in Fig. 3, which illustrate data obtained with plasma collected 4 h after GSE ingestion. The $A_{280\text{ nm}}$ trace contains a number of impurities in addition to the four flavan-3-ol metabolites and it is evident that *M-3* is a shoulder on a contaminant peak. None the less, full-scan MS^2 spectra, as summarised in

Table 2, were obtained for all four metabolites. However, it was evident from the 280 nm trace that the presence of co-eluting compounds would adversely influence quantification using the diode array detector. These compounds were, therefore, quantified using the enhanced selectivity of SIM by monitoring the response at m/z 465 for *M-1* and *M-2* and m/z 479 for *M-3* and *M-4* (Fig. 3). Unmetabolised (+)-catechin and (−)-epicatechin were not present in the plasma samples in detectable quantities and neither were procyanidin dimers, trimers or tetramers.

The plasma pharmacokinetic profiles based on HPLC-SIM analyses are illustrated in Fig. 4. Three hours after GSE ingestion, (+)-catechin glucuronide and (−)-epicatechin glucuronide attained peak plasma concentrations of 10.3 (SE 0.7) and 9.9 (SE 0.1) $\mu\text{g/ml}$, respectively, which corresponds to 36.0 (SE 2.5) and 34.4 (SE 0.2) $\mu\text{mol/l}$. The glucuronide derivatives of methyl-(+)-catechin and methyl-(−)-epicatechin also peaked after 3 h at 5.5 (SE 0.1) and 3.3 (SE 0.2) $\mu\text{g/ml}$, respectively. There was an approximately 20% reduction in the concentrations of all four plasma flavan-3-ol metabolites at 4 h after which the levels declined more rapidly (Fig. 4). Assuming 12 ml of plasma per rat, the peak plasma concentrations of the two (+)-catechin metabolites correspond to around 3% of the (+)-catechin ingested and the (−)-epicatechin metabolites equate with about 4% of intake.

Quantification urinary flavan-3-ol metabolites

A total of eight flavan-3-ol metabolites, methyl-glucuronides, sulphates and methyl-sulphates of the monomers (+)-catechin

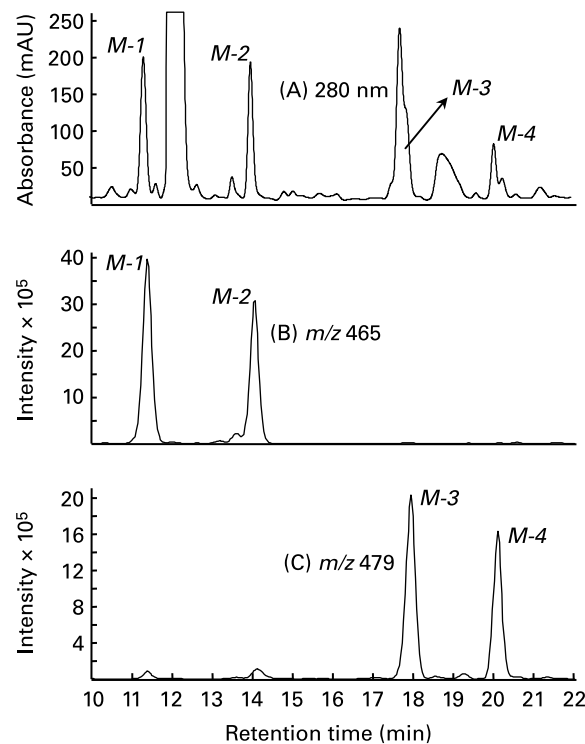


Fig. 3. Reversed-phase HPLC analysis, with diode array and MS-selected ion monitoring detection, of the flavan-3-ol metabolites (+)-catechin glucuronide (*M-1*), (−)-epicatechin glucuronide (*M-2*), methyl-(+)-catechin glucuronide (*M-3*), methyl-(−)-epicatechin glucuronide (*M-4*) in rat plasma collected 4 h after the ingestion of a grape seed extract. Extract analysed using a 60 min 5–40% gradient of acetonitrile in 1% aqueous formic acid with detection at 280 nm (A), m/z 465 (B) and m/z 479 (C). For MS^2 data, see Table 2.

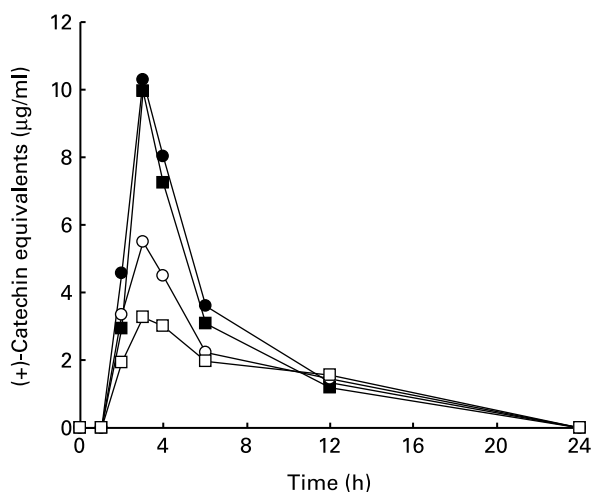


Fig. 4. Pharmacokinetic profile of flavan-3-ol metabolites (●, catechin glucuronide; ■, epicatechin glucuronide; ○, methyl-catechin glucuronide; □, methyl-epicatechin glucuronide) detected in rat plasma collected over a 0–24 h period after the ingestion of a grape seed extract. Results are expressed as μg (+)-catechin equivalents/ml. Values are means and in all instances standard error bars are smaller than symbols.

and (–)-epicatechin, whose LC–MS–MS identifications were outlined earlier and in Table 3, were detected in urine. HPLC data obtained with a 0–24 h urine sample are illustrated in Fig. 5. The $A_{280\text{ nm}}$ trace contained numerous impurities (Fig. 5(A)) so the flavan-3-ol metabolites were quantified using SIM. A SIM trace at m/z 383 illustrating the detection of the methylated flavan-3-ol sulphates, *U-3* and *U-4*, is illustrated in Fig. 5(B). Information on the cumulative excretion of the eight metabolites over 0–2, 0–4 and 0–24 h time periods is presented in Table 3. The levels of (+)-catechin and (–)-epicatechin metabolites excreted, relative to the quantity of the monomers ingested, were 1 and 2%, respectively, after 2 h, 13% after 4 h, and 27 and 36% after 24 h. No (+)-catechin and (–)-epicatechin were detected in the urine samples.

When HPLC–MS–MS in the SRM mode was used to analyse urine collected up to 4 h after ingestion of the GSE, the procyanidin dimers B_1 , B_2 , B_3 and B_4 were detected (Fig. 6). Also analysis at m/z 865 revealed the presence of the trimer C_2 , peak 1 in the GSE, and an additional trimer that eluted at the same retention time as the trimer peak 10 in the GSE. All these procyanidins were detected in low μg amounts (Table 4), which is much less than the levels of the urinary

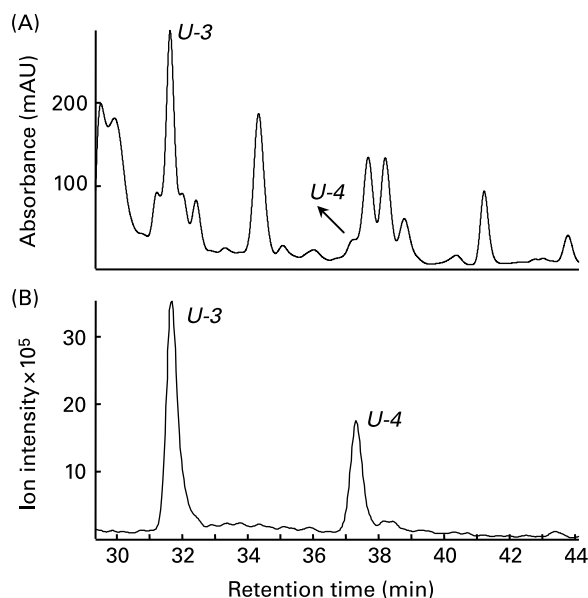


Fig. 5. Reversed-phase HPLC analysis, with diode array and MS-selected ion monitoring detection, of methyl-(+)-catechin sulphate (*U-3*) and methyl-(–)-epicatechin sulphate (*U-4*) in rat urine collected 4 h after the ingestion of a grape seed extract. Extract analysed using a 60 min 5–20% gradient of acetonitrile in 1% aqueous formic acid with detection at 280 nm (A) and m/z 383 (B). For MS^2 data, see Table 2.

metabolites of (+)-catechin and (–)-epicatechin that were excreted in the first 4 h after ingestion of the GSE (Table 3).

Quantification of flavan-3-ols and metabolites in the gastrointestinal tract

The levels of flavan-3-ols and their metabolites that were detected in the various sections of the GI tract are presented in Table 5. The amounts fluctuated somewhat from time point to time point, presumably because of rat-to-rat variations. While this precludes a detailed analysis of the data, certain trends are none the less apparent. One hour after GSE supplementation (+)-catechin, (–)-epicatechin and other flavan-3-ols were found in the GI tract. The highest quantities were present in the stomach with low levels in the duodenum/jejunum and ileum. Trace quantities of metabolites, detected in the duodenum/jejunum and ileum 1 h after the ingestion of the GSE, were identified as glucuronides

Table 3. Cumulative excretion of flavan-3-ol metabolites in rat urine 0–2, 0–4 and 0–24 h after oral ingestion of a grape seed extract

(Values are means with their standard errors (n 3))

| Peak | Metabolite | Metabolite excretion (μg per rat) | | | | | |
|------------|------------------------------------|---|-----|-------|-----|--------|-----|
| | | 0–2 h | | 0–4 h | | 0–24 h | |
| | | Mean | SE | Mean | SE | Mean | SE |
| <i>M-1</i> | (+)-Catechin glucuronide | 40 | 0.2 | 324 | 8.2 | 814 | 49 |
| <i>M-2</i> | (–)-Epicatechin glucuronide | 11 | 2.2 | 201 | 1.0 | 635 | 23 |
| <i>M-3</i> | Methyl-(+)-catechin glucuronide | 66 | 7.7 | 332 | 9.0 | 534 | 25 |
| <i>M-4</i> | Methyl-(–)-epicatechin glucuronide | 6.3 | 0.2 | 193 | 0.7 | 391 | 48 |
| <i>U-1</i> | (+)-Catechin sulphate | 2.5 | 0.1 | 27 | 0.4 | 78 | 6.1 |
| <i>U-2</i> | (–)-Epicatechin sulphate | ND | | 39 | 0.2 | 139 | 13 |
| <i>U-3</i> | Methyl-(+)-catechin sulphate | 2.3 | 0.2 | 45 | 11 | 120 | 1.1 |
| <i>U-4</i> | Methyl-(–)-epicatechin sulphate | 1.4 | 0.1 | 29 | 1.4 | 152 | 1.3 |

ND, not detected.

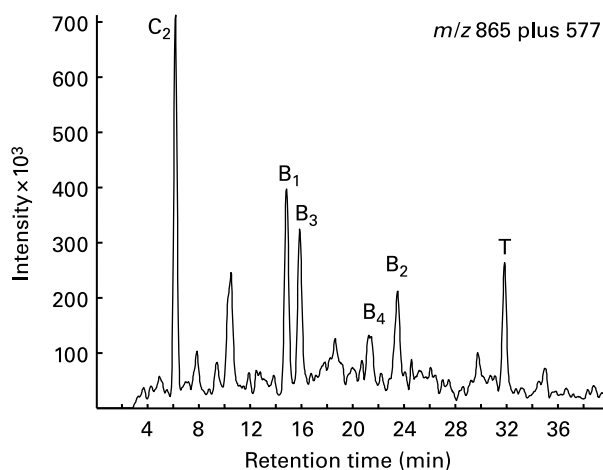


Fig. 6. Reversed-phase HPLC analysis, with diode array and MS-selected reaction monitoring detection of procyanidin dimers (B_1 , B_2 , B_3 and B_4) and trimers (C_2 and T (unknown trimer)) in rat urine collected 4 h after the ingestion of a grape seed extract. Extract analysed using a 60 min 5–40% gradient of acetonitrile in 1% aqueous formic acid with detection at m/z 577 and 865.

of the monomers (+)-catechin and (–)-epicatechin and their methylated analogues. The amounts present in these organs at 2 h corresponded to only 1.2% and 2%, respectively, of the (–)-epicatechin and (+)-catechin in the ingested GSE. Small amounts of flavan-3-ol metabolites were present in the duodenum/jejunum and ileum up to 6 h after ingestion after which they were not detected. No metabolites were detected in the stomach, caecum and colon at any time point throughout the 24 h collection period although relatively small quantities of unmetabolised flavan-3-ols, including the B_1 , B_2 , B_3 and B_4 dimers, were present up to 12 h after ingestion of the GSE. All the GSE flavan-3-ols were eliminated from the GI tract 24 h after ingestion.

Quantitative analysis of flavan-3-ol metabolites in the liver, kidneys and brain

HPLC–MS–MS analyses, as described above, identified glucuronidated and methylated metabolites of (–)-epicatechin and (+)-catechin in liver extracts collected 1 and 4 h after ingestion of the GSE. The methylated conjugates were the major metabolites (Table 6). None of the metabolites were present in subsequent liver samples collected 6, 12 and 24 h after ingestion. The levels of metabolites detected in the kidney were low, reaching a maximum by 4 h post-ingestion with 134 and 28 μg of total (+)-catechin and (–)-epicatechin, respectively.

Table 4. Cumulative excretion of procyanidin dimers in rat urine 0–4 h after oral ingestion of a grape seed extract

(Values are means with their standard errors (n 3))

| Procyanidin | Procyanidin excretion (μg per rat)* | | |
|----------------|---|-----|-----|
| | Mean | % | SE |
| Dimer B_1 | 7.5 | 0.5 | 0.3 |
| Dimer B_2 | 4.2 | 0.3 | 0.2 |
| Dimer B_3 | 6.4 | 1.0 | 0.1 |
| Dimer B_4 | 3.0 | 0.4 | 0.4 |
| Trimer C_2 | 13.0 | 4.3 | 0.1 |
| Unknown trimer | 5.1 | 0.7 | 0.4 |

* Percentage values represent amount excreted as a percentage of intake.

No metabolites were detected in brain extracts using full-scan MS–MS, although El Mohsen *et al.* (2002) have reported the presence of glucuronyl and methyl-glucuronyl metabolites of (–)-epicatechin in the brain of rats that had been fed 100 mg (–)-epicatechin per kg body weight for a period of 10 d. We, therefore, pooled the remaining brain extracts and attempted to detect the presence of both of these metabolites with the HPLC conditions used for urine analysis and the mass spectrometer operating in the SRM mode. Interestingly, a strong peak was observed using SRM of parent ion m/z 479 and product ion m/z 303, which was not seen in the control brain sample. Inspection of the product ion spectrum revealed an additional major ion at m/z 411, which was not present in the equivalent spectra obtained with $M-3$ and $M-4$ in other tissues, plasma and urine (Table 3). The retention time of this peak was also different from those of the methylated flavan-3-ol glucuronides detected in other samples. The spectra shown by El Mohsen *et al.* (2002) contained many ions that could not have originated from the fragmentation of the putative flavan-3-ol metabolites. Ryan *et al.* (1999) and de Rijke *et al.* (2003) have reported that MS–MS provides better limits of detection though less structural information than positive ionisation, which is in agreement with our own observations (Mullen *et al.* 2004). This makes the presence of additional ions in our spectra all the more significant. When a flavonoid O -conjugate undergoes MS^2 fragmentation the resultant base ion should be that of the flavonoid aglycone or radical aglycone formed from heterolytic or homolytic cleavage (Hvattum & Ekeberg 2002), which is also in agreement with our findings in the current study. Apart from the complete loss of the conjugate moiety, O -conjugated compounds have the same spectra as their corresponding aglycone (Waridel *et al.* (2001). This was not the case with the putative methyl-flavan-3-ol glucuronide SRM spectrum obtain with our rat brain extract or in the spectra reported by El Mohsen *et al.* (2002). We therefore believe that more convincing analytical data need to be obtained before it can be claimed that flavan-3-ol metabolites cross the blood–brain barrier.

Metabolites identified in faeces

HPLC–MS–MS with SRM detected trace non-quantifiable amounts of (+)-catechin glucuronide, (–)-epicatechin glucuronide and their methyl derivatives in a few of the faecal samples collected over a 24 h period after GSE ingestion. They were, however, not detected in the majority of samples that were analysed.

Discussion

In the present study rats were fed a single acute dose of a flavan-3-ol-rich GSE (Table 1). The mean amounts fed to each rat by gavage were 5.8 mg (+)-catechin, 3.6 mg (–)-epicatechin, 28.4 mg procyanidin dimers, 5.2 mg trimers and 2.8 mg tetramers (Table 1). In addition, the GSE contained a substantial, but undetermined, quantity of high-molecular weight polymeric procyanidins. Relatively high levels of the various components in the GSE remained in the stomach for 6 h, declined by 12 h and had disappeared after 24 h. Small amounts of GSE phenolics appeared in the duodenum/jejunum and to a greater extent the ileum after 1 h and in both instances had disappeared by 12 h. Phenolics were detected in the caecum 2 h after ingestion and only trace amounts remained after 12 h. Smaller amounts were found in the colon 3–12 h after gavage. The vast majority of compounds

Table 5. Flavan-3-ols and their metabolites detected in the gastrointestinal tract of rats over a 24 h period after ingestion of a grape seed extract* (Values are means (µg per organ) with their standard errors (n 3))

| Time (h) | C | | EC | | B ₁ | | B ₂ | | B ₃ | | B ₄ | | ECG | | PCBG | | CGlcUA | | ECGlcUA | | MeCGlcUA | | MeECGlcUA | | | | | | | |
|-------------------------|------|----|------|-----|----------------|----|----------------|-----|----------------|----|----------------|----|------|----|------|-----|--------|----|---------|----|----------|----|-----------|----|------|----|----|----|----|----|
| | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | Mean | SE | | | | |
| Stomach | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 1173 | 43 | 926 | 7.2 | 367 | 7 | 549 | 18 | 212 | 4 | 152 | 13 | 265 | 1 | 866 | 3 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | |
| 2 | 1856 | 99 | 1265 | 94 | 1061 | 3 | 782 | 107 | 719 | 6 | 578 | 71 | 724 | 3 | 1351 | 11 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | |
| 3 | 715 | 89 | 635 | 61 | 357 | 2 | 329 | 52 | 186 | 1 | 140 | 3 | 290 | 13 | 594 | 1 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | |
| 4 | 1711 | 98 | 1755 | 96 | 1819 | 9 | 1706 | 7 | 749 | 2 | 355 | 8 | 1503 | 89 | 1204 | 187 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | |
| 6 | 1468 | 10 | 1120 | 75 | 612 | 3 | 517 | 1 | 338 | 3 | 279 | 10 | 694 | 3 | 121 | 64 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | |
| 12 | 515 | 53 | 313 | 48 | 109 | 11 | 103 | 21 | 68 | 4 | 56 | 9 | 256 | 21 | 266 | 34 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | |
| 24 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | | |
| Duodenum/jejunum | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 119 | 1 | 73 | 3 | 14 | 1 | 22 | 1 | 18 | 1 | 11 | 1 | 21 | 8 | 21 | 4 | 3 | 0 | 0 | ND | ND | 9 | 2 | 9 | 1 | 1 | 1 | 1 | | |
| 2 | 35 | 2 | 26 | 1 | 42 | 1 | 16 | 6 | 7 | 1 | 7 | 1 | 26 | 5 | 13 | 3 | 7 | 0 | 0 | 6 | 1 | 26 | 1 | 11 | 1 | 1 | 1 | 1 | | |
| 3 | 46 | 1 | 29 | 0 | 16 | 1 | 6 | 1 | 5 | 1 | 5 | 1 | 7 | 1 | ND | ND | 9 | 1 | 1 | ND | 30 | 1 | 12 | 1 | 1 | 1 | 1 | 1 | | |
| 4 | 71 | 2 | 45 | 1 | 6 | 3 | 7 | 0 | 9 | 1 | 5 | 0 | 12 | 4 | 11 | 1 | 1 | 1 | 1 | ND | 4 | 0 | 2 | 1 | 1 | 1 | 1 | 1 | | |
| 6 | 45 | 18 | 30 | 11 | 6 | 3 | 4 | 0 | 5 | 0 | 3 | 0 | 7 | 2 | ND | ND | ND | ND | ND | ND | 3 | 0 | 2 | 1 | 1 | 1 | 1 | 1 | | |
| 12 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | |
| 24 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | |
| Ileum | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | 208 | 2 | 156 | 7 | 91 | 3 | 73 | 2 | 49 | 2 | 31 | 2 | 26 | 4 | 97 | 1 | 8 | 1 | 0 | 8 | 0 | 30 | 1 | 33 | 1 | 1 | 1 | 1 | 1 | |
| 2 | 196 | 1 | 142 | 8 | 82 | 1 | 73 | 6 | 44 | 1 | 30 | 1 | 21 | 2 | 83 | 4 | 8 | 0 | 0 | 6 | 1 | 21 | 2 | 26 | 0 | 0 | 0 | 0 | 0 | |
| 3 | 204 | 4 | 141 | 3 | 72 | 1 | 30 | 0 | 37 | 3 | 69 | 1 | 20 | 3 | 79 | 3 | 7 | 4 | 4 | ND | 33 | 2 | 36 | 2 | 2 | 2 | 2 | 2 | 2 | |
| 4 | 271 | 10 | 188 | 3 | 96 | 3 | 79 | 1 | 41 | 1 | 40 | 4 | 41 | 25 | 117 | 3 | ND | 1 | 1 | ND | 15 | 2 | 36 | 2 | 2 | 2 | 2 | 2 | 2 | |
| 6 | 178 | 8 | 121 | 6 | 79 | 1 | 60 | 2 | 41 | 1 | 29 | 2 | 34 | 10 | 96 | 2 | 11 | 1 | 1 | ND | 46 | 4 | 23 | 2 | 2 | 2 | 2 | 2 | 2 | |
| 12 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 24 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Caecum | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 2 | 58 | 3 | 66 | 7 | 29 | 1 | 18 | 2 | 17 | 1 | 44 | 1 | ND | ND | 32 | 11 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 3 | 122 | 1 | 245 | 3 | 156 | 6 | 40 | 2 | 23 | 1 | 239 | 12 | ND | ND | 47 | 1 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 4 | 437 | 81 | 271 | 20 | 339 | 3 | 307 | 25 | 56 | 5 | 88 | 3 | ND | ND | 396 | 4 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 6 | 761 | 6 | 428 | 45 | 443 | 2 | 394 | 39 | 137 | 6 | 188 | 7 | ND | ND | 489 | 37 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 12 | ND | ND | ND | ND | 67 | 4 | ND | ND | 11 | 1 | 64 | 9 | ND | ND | 105 | 36 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 24 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Colon | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 2 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 3 | 78 | 1 | 106 | 5 | 56 | 1 | 80 | 2 | 35 | 1 | 150 | 40 | ND | ND | 95 | 1 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 4 | 129 | 4 | 69 | 1 | 66 | 1 | 63 | 1 | 16 | 0 | 21 | 1 | ND | ND | 76 | 10 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 6 | 120 | 3 | 79 | 19 | 66 | 9 | 69 | 9 | 22 | 1 | 32 | 1 | ND | ND | 84 | 3 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 12 | ND | ND | 36 | 2 | 93 | 0 | 92 | 3 | 20 | 1 | 32 | 3 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 24 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |

B₁, B₂, B₃, B₄, procyanidin dimers; C, (+)-catechin; CgIcUA, (+)-catechin glucuronide; EC, (-)-epicatechin; ECG, (-)-epicatechin gallate; ECGlcUA, (-)-epicatechin glucuronide; MeCGlcUA, methyl(-)-epicatechin glucuronide; PCBG, procyanidin B₁-3- or B₂-3'-O-gallate.

Table 6. Levels of flavan-3-ol metabolites in liver and kidney of rats over a 24 h period after the ingestion of a grape seed extract*(Values are means (μg per organ) with their standard errors (n 3))

| Organ | Time (h) | CGlcUA | | ECGlcUA | | MeCGlcUA | | MeECGlcUA | |
|--------|----------|--------|-----|---------|-----|----------|-----|-----------|-----|
| | | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Liver | 1 | 4.3 | 0.1 | 2.5 | 2.0 | 57 | 1.3 | 21 | 0.1 |
| | 2 | NA | | NA | | NA | | NA | |
| | 3 | NA | | NA | | NA | | NA | |
| | 4 | 2.3 | 0.1 | 1.3 | 1.0 | 45 | 10 | 20 | 0.9 |
| | 6 | ND | | ND | | ND | | ND | |
| | 12 | ND | | ND | | ND | | ND | |
| | 24 | ND | | ND | | ND | | ND | |
| Kidney | 1 | ND | | ND | | ND | | ND | |
| | 2 | 7.6 | 0.1 | 4.1 | 0.2 | 87 | 0.1 | 9.1 | 0.3 |
| | 3 | 2.7 | 0.2 | 1.2 | 0.3 | 8.4 | 0.4 | 2.7 | 0.4 |
| | 4 | 3.1 | 0.2 | 2.1 | 0 | 131 | 1.0 | 26.3 | 2.5 |
| | 6 | 2.7 | 0.6 | 2.3 | 0.1 | 58 | 0.3 | 8.2 | 0.3 |
| | 12 | ND | | ND | | ND | | ND | |
| | 24 | ND | | ND | | ND | | ND | |

CGlcUA, (+)-catechin glucuronide; ECGlcUA, (-)-epicatechin glucuronide; MeCGlcUA, methyl-(+)-catechin glucuronide; MeECGlcUA, methyl(-)-epicatechin glucuronide; NA, not analysed; ND, not detected.

detected in the GI tract were the original GSE flavan-3-ols with only trace levels of four metabolites occurring in the duodenum/jejunum and ileum. The data presented in Table 5 do not show either a sizable increase in either (+)-catechin or (-)-epicatechin or a concomitant decrease in the relative amounts of dimers which supports the view that oligomeric proanthocyanidins are not depolymerised into monomeric flavan-3-ols to any extent, if at all, during passage through the stomach and GI tract (Donovan *et al.* 2002; Rios *et al.* 2002; Nakamura & Tonogai, 2003).

HPLC retention data and MS² fragmentation patterns indicated that the trace levels of flavan-3-ol metabolites appearing in the GI tract were a (+)-catechin glucuronide and a methyl-(+)-catechin glucuronide together with the corresponding (-)-epicatechin derivatives which, in view of earlier studies with rats (Natsume *et al.* 2003), were tentatively identified as (-)-epicatechin-7-*O*-glucuronide and 3'-*O*-methyl(-)-epicatechin-7-*O*-glucuronide. The fate of the GSE flavan-3-ols in the GI tract of rats is, therefore, very different from that of quercetin-4'-glucoside which undergoes very rapid conversion to a mixture of glucuronide, methyl and sulphated metabolites with only 25% of the parent glycoside remaining after 1 h (Mullen *et al.* 2002).

In marked contrast to the GI tract where the parent GSE flavan-3-ols were the major components, the circulatory system contained only the four previously mentioned flavan-3-ol metabolites in detectable quantities. Three hours after ingestion a peak plasma concentration of approximately 35 $\mu\text{mol/l}$ was attained with the (+)-catechin and (-)-epicatechin glucuronides with their methylated derivatives present at 2–3-fold lower levels (Fig. 3). The peak plasma concentrations of the two (+)-catechin metabolites correspond to about 3% of the (+)-catechin ingested and the (-)-epicatechin metabolites equate with about 4% of intake. The levels of the metabolites in the bloodstream thus greatly exceeded the amounts present in the GI tract. This is in keeping with the methylation and glucuronidation of (+)-catechin and (-)-epicatechin occurring on the luminal side of the endoplasmic reticulum of the small intestine during transport into the blood stream (Kuhnle *et al.* 2000; Donovan *et al.* 2001). The small quantities of metabolites in the GI tract could be due

to either low-level efflux back into the lumen of the intestine or enterohepatic recirculation via the bile.

Trace levels of the methylated and glucuronidated flavan-3-ol metabolites were also detected in liver and kidney extracts (Table 2) but they were excreted in much higher amounts in urine along with two sulphated metabolites and two methyl sulphated metabolites that were not present in either the GI tract, the liver and kidneys or the circulatory system. In contrast to other studies, no free (+)-catechin or (-)-epicatechin was detected outside the GI tract, although trace quantities of the procyanidin B₁, B₂, B₃ and B₄ dimers and the C₂ trimer were detected in urine (Fig. 6; Table 4). The levels of (+)-catechin and (-)-epicatechin metabolites excreted relative to the quantity of the monomers ingested were 27 and 36%, respectively, after 24 h. This is similar to the 37% urinary excretion reported to occur after feeding (-)-epicatechin to rats (Donovan *et al.* 2002). This provides further, albeit indirect, evidence that the procyanidin oligomers in the GSE were not depolymerised to monomers to any extent after ingestion. If this had occurred, the level of recovery of the monomeric metabolites in urine is likely to have been substantially higher than the 37% obtained by Donovan *et al.* (2002) after feeding rats with (-)-epicatechin.

The approximate 30% recovery of the ingested (+)-catechin and (-)-epicatechin as metabolites in urine is well in excess of comparable figures of about 2–3% and 0.1% that are typically obtained with flavonols and anthocyanins, respectively (Prior, 2003). However, this still leaves 70% of the ingested monomer unaccounted for and, although seemingly not absorbed to any extent, only trace amounts of the procyanidins in the GSE reach the colon (Table 5) and are excreted in faeces. The most likely fate of these compounds is that they are converted to low-molecular weight phenolic acids (Déprez *et al.* 2000; Gonthier *et al.* 2003), most notably 3-hydroxyphenylpropionic acid (Ward *et al.* 2004). These compounds were not analysed in the current study. They have a low extinction coefficient and a λ_{max} below 250 nm and as a result are not readily detected with a diode array detector and, in addition, they do not ionise readily when subjected to MS with an electrospray interface.

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