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ORIGINAL CONTRIBUTION

Intestinal transit and systemic metabolism of apple polyphenols

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Abstract
Background Apples are the most widely consumed fruits in Germany and various other countries. Positive health effects of apple-derived polyphenols in vivo depend on their absorption, metabolism, distribution, and elimination from the body after consumption. Data on the metabolism of these polyphenols in humans are scarce. In order to study the intestinal transit and metabolism of apple polyphenols in humans, a variety of experiments were carried out.
Methods Polyphenols were incubated with saliva (for 5 min), simulated gastric or duodenal juice (4 or 10 h, respectively), or rat hepatocytes (4 h) under aerobic conditions, and with ileostomy fluid under aerobic conditions for 10 h. The polyphenol profile in human serum (8 h later) and renal elimination in urine (24 h later) were also investigated after consumption of 1 L apple juice. Polyphenols and their metabolites were identified and quantified by high-performance liquid chromatography with diode array detection (HPLC–DAD), HPLC–electrospray ionization–tandem mass spectrometry (ESI-MS/MS), and gas chromatography (GC)-MS.
Results In the presence of native saliva or ileostomy fluid, β-glycosides of phloretin and quercetin were hydrolyzed, to varying degrees depending on the sugar moiety, and to much lesser degrees in the presence of antibiotics. In the gastric milieu, almost complete degradation of procyanidin B_2 to (−)-epicatechin was observed. In the presence of artificial duodenal juice flavan-3-ol epimerization occurred. Quercetin was completely converted to phloroglucinol, 3,4-dihydroxybenzoic acid, and 2,4,6-trihydroxybenzoic acid. Formation of isomeric products of hydroxycinnamic acid esters and their corresponding methyl esters was also observed, and similar results were obtained after incubation with rat hepatocytes. Products of phase II metabolism, two phloretin *O*-glucuronides and eight (methyl) quercetin *O*-glucuronides, were identified in the hepatocyte samples. Following enzymatic hydrolysis, 5-caffeoylquinic acid, 4-*p*-coumaroylquinic acid, caffeic acid, (−)-epicatechin, phloretin, and quercetin were recovered in both serum and urine (5.3% and 3.5% of the amounts consumed, respectively). In addition, 19.5% of the polyphenols consumed were identified in the urine in the form of hydroxylated phenolic and hippuric acids.
Conclusion The findings relating to the absorption, metabolism, and systemic availability of polyphenols in vivo should contribute to our understanding of their biological effects, and the characterization of newly formed metabolites should facilitate further studies.

Keywords Metabolism · Bioavailability · Apple · Polyphenols · Intestinal transit · Urine · Plasma

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Introduction

Numerous epidemiological studies published toward the end of the twentieth century provided evidence that increased consumption of fruit and vegetables provides protection against cardiovascular diseases (CVD), various kinds of cancer, and other chronic diseases [1]. Apples are the most widely consumed fruits in Germany and various other countries with moderate climates and thus make a substantial contribution to overall fruit consumption. In addition to consumption of the raw fruit, apple juice makes a highly significant contribution, with a per-capita consumption of 11.4 L per year. Indeed, apple juice is the most popular fruit juice in Germany [2].

The antioxidative effectiveness of apple extracts and apple juices has been demonstrated in numerous in vitro studies and animal models. They have been shown to increase antioxidative capacity, reduce oxidative cell damage, and inhibit lipid peroxidation [3–9].

Secondary plant metabolites, particularly polyphenols, are thought to be partially responsible for these beneficial effects [4]. In apples, the phenolic substances present in substantial concentrations are the hydroxycinnamic acids and their esters, flavonols, flavan-3-ols, dihydrochalcones and anthocyanidins, precise quantities of each class depending on the apple variety [10–12].

The antioxidative potential of the polyphenols, their bioactivity in vivo, and thus their positive health effects in vivo depend on their absorption, metabolism, distribution, and elimination from the body after consumption. However, data on the metabolism of apple-derived polyphenols after subjects have drunk apple juice or apple cider are scarce [13].

In this paper, results obtained from both in vitro and ex vivo studies of the intestinal transit and metabolism of apple polyphenols in humans (using human saliva, simulated gastric and duodenal juice, rat hepatocytes, and human ileostomy fluids) are reported. In addition, we report the polyphenol contents observed in blood samples collected over 8 and 24 h after healthy subjects consumed 1 L of cloudy apple juice.

Materials and methods

Chemicals

All chemicals and solvents were of analytical grade. Solvents were redistilled before use, and water for HPLC analysis was filtered through a Millipore Milli-Q ion exchange system from Millipore S.A. (Molsheim, France). ACN (Lichrosolv®) was from Merck (Darmstadt, Germany); dihydrocaffeic acid (DHCA), *p*-coumaric acid (*p*-CouA), 3,4-dihydroxybenzoic acid (3,4-DHBA), 3-hydroxyphenylacetic acid (3-HPAA), 3-hydroxyphenylpropionic acid (3-HPPA), formic acid, 2,4,6-trihydroxybenzoic acid (2,4,6-THBA), D-(−)-quinic acid (QA), phloroglucinol, trichloroacetic acid (TCA), and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Fluka (Deisenhofen, Germany). Methyl *p*-coumarate was purchased from Apin Chemicals Ltd. (Abingdon, U.K.). Phloretin (Phl, 4,2′,4′,6′,-tetrahydroxydihydrochalcone), quercetin 3-*O*-glucoside (Q-3-glu, isoquercitrin), quercetin 3-*O*-galactoside (Q-3-gal, hyperoside), (+)-catechin ((+)-C), (−)-epicatechin ((−)-EC), 5-caffeoylquinic acid (5-CQA, chlorogenic acid), and caffeic acid (CA) were from Roth (Karlsruhe, Germany). 1- (1-CQA) and 4-caffeoylquinic acid (4-CQA) were kindly provided by Michael Sefkow (Leipzig, Germany). DMSO was obtained from Gruessing (Filsum, Germany). The compounds 3,4,5-*trans*-trimethoxycinnamic acid (IS), ferulic (FA), and isoferulic acid (IFA), 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), phloretin 2′-*O*-glucoside (Phl-glu), quercetin (Q, 3,5,7,3′,4′-pentahydroxyflavone), quercetin 3-*O*-rutinoside (Q-3-rut, rutin), quercetin 3-*O*-rhamnoside (Q-3-rham, quercitrin), *β*-glucuronidase (EC 3.1.6.1) and sulfatase (EC 3.2.1.31) from *Helix pomatia* (type H-1), pepsin (EC 3.4.1), pancreatin (4 × USP specifications), and bile extract were purchased from Aldrich (Steinheim, Germany). Penicillin/streptomycin antibiotic was obtained from Biochrom AG (Berlin, Germany), while 4-hydroxybenzoic acid (4-HBA), hippuric acid (HA), 3-hydroxyhippuric acid (3-HHA), and 4-hydroxyhippuric acid (4-HHA) were acquired from Sigma (Steinheim, Germany). Quercetin 3-*O*-xyloside (Q-3-xyl, reynoutrine), quercetin 3-*O*-arabinoside (Q-3-ara, avicularin), phloretin 2′-*O*-xyloglucoside (Phl-xylglu), and procyanidins B_1 and B_2 were kindly provided by Prof. Dr. H. Becker (Saarbruecken, Germany) and Prof. Dr. P. Winterhalter (Braunschweig, Germany), and trypan blue was obtained from Invitrogen Corp. (Eggenstein, Germany). 3-Caffeoylquinic acid (3-CQA), methyl caffeate, and phloretin 2′-*O*-glucuronide (Phl-glcA) were synthesized and isolated as previously described by us [14].

Subjects

Saliva samples were collected in the morning from three healthy subjects (two men and one woman; 24–29 years old) without brushing their teeth since the previous evening. Ileostomy effluents were provided by three healthy female subjects (34–39 years old) who had undergone colectomy with terminal ileostomy 5–6 years prior to the study (all suffered from Crohn's disease without involvement of the ileum). Due to each individual's medical history, no surgical ileal resection had been performed. Blood samples were collected from five healthy subjects

(two men and three women; 24–33 years old), and in a separate study, urine samples were collected from six healthy subjects (two men and four women; 24–33 years old). All volunteers were medically normal. None of them had taken antibiotics during the preceding 4 weeks. Human intervention studies were approved by the local ethics committee no. 124/04 (Medical Faculty, University of Wuerzburg, Germany), and all participants gave written consent.

Incubation conditions

Saliva

For incubations, collected saliva was diluted 1:1 with distilled water and shaken to reduce viscosity. The diluted saliva was divided into three aliquots (A–C). A was centrifuged at 5,000×*g* for 5 min, and the supernatant was used as the incubation medium. To C, 1% penicillin/streptomycin antibiotic was added. A–C was preincubated at 37 °C for 5 min.

Diluted saliva A (1.0 mL) was added to pregassed (N_2) incubation vessels containing an aliquot of 20 µL from a stock solution of each substrate (25 mM in DMSO). The incubation vessels were sealed tightly and stored at 37 °C for 0, 0.5, 1, 2, and 5 min. To stop the enzymatic reaction, vessels were placed in liquid nitrogen and the samples were lyophilized immediately. Saliva aliquots B and C as well as controls (20 µL DMSO and diluted saliva; flavonoid and buffer) were subjected to the procedure described above. Experiments were performed in triplicate.

Simulated gastric juice

To prepare a volume of 500 mL simulated gastric juice (according to Gillatt et al. [15]), 2.10 g sodium chloride (70 mM), 1.87 g potassium chloride (50 mM), and 0.69 g pepsin (0.14 mM) were dissolved in 500 mL water. The pH was adjusted to 1.81 using HCl. Portions (1.25 mL) of simulated gastric juice (pH 1.8) were added to pregassed (N_2) incubation vessels containing a 20 µL portion of a stock solution of each substrate (25 mM in DMSO), sealed tightly, and stored at 37 °C for 0, 20, and 40 min, and 1, 1.5, 2, and 4 h. Reactions were stopped by liquid nitrogen, and then the samples were lyophilized. Experiments were performed in duplicate. Controls (20 µL DMSO and gastric juice; flavonoid and buffer) were treated identically to experimental samples.

Simulated duodenal juice

To prepare a volume of 500 mL simulated duodenal juice (according to Glahn et al. [16]), 1.0 g pancreatin and 6.0 g bile extract were diluted in 300 mL of 0.1 mM sodium hydrogen carbonate in an ultrasonic bath. Finally, 0.1 mM sodium hydrogen carbonate was added to 500 mL and filtered to remove insoluble particles. Portions (1.25 mL) of simulated duodenal juice were added to pregassed (N_2) incubation vessels containing a 20 µL portion of a stock solution of each substrate (25 mM in DMSO) and 4.98 mL distilled water. The pH was adjusted to pH 7.2 ± 0.1 with HCl, and the volume was made up to 7.5 mL with a solution of NaCl (60 mM) and KCl (2.3 mM), sealed tightly, and stored at 37 °C for 0, 0.5, 1, 2, 4, 6, 8, 10, and 24 h. Reactions were stopped by liquid nitrogen, and then the samples were lyophilized. Experiments were performed in duplicate. Controls (20 µL DMSO and duodenal juice; flavonoid and buffer) were treated identically to other samples.

Ileostomy fluids

After removal of the ileostomy bag from each subject, it was immediately placed in an anaerobic jar containing AnaeroGen™ from Oxoid Limited (Hampshire, UK) and then transported directly to the laboratory into an anaerobic chamber and flushed with a N_2/CO_2 gas mixture (Aligal, 80:20; v/v). The ileostomy fluid was diluted with an equal volume of anaerobic carbonate–phosphate buffer (pH 6.3) according to Lebet et al. [17] and mixed, and coarse particles were removed by filtration using glass wool (inoculum). Portions (2.5 mL) were added to pregassed (N_2) incubation vessels, containing a 20 µL portion of a stock solution of each substrate (50 mM in DMSO). The incubation vessels were sealed tightly and stored at 37 °C for 0, 0.5, 1, 2, 4, 6, 8, 10, and 24 h in the dark. Reactions were stopped by placing the vessels in liquid nitrogen, and then the samples were lyophilized. Experiments were performed in triplicate. Control vessels (20 µL DMSO and inoculum; flavonoid and buffer) were treated identically.

Hepatocytes

Hepatocyte samples were prepared by in situ perfusion of the whole livers of male Wistar rats. According to trypan blue exclusion assays, 91% of the hepatocytes were viable. Fresh cells were seeded onto collagen-coated plates at a density of 4×10^6 viable cells and covered in 2 mL of William's E culture medium (Sigma–Aldrich). The cultivation was performed in an incubator at 37 °C (5% CO_2). After cell attachment, 2 mL culture medium containing 100 µL of a stock solution of each substrate (4 mM in DMSO) was added, and plates were stored at 37 °C for 0, 0.5, 1, 2, and 4 h in an incubator. Supernatants were collected into plastic tubes and immediately frozen in liquid nitrogen. Intracellular samples were obtained by scraping the cells into 1 mL of DMSO–water (1:1; v/v), which was then also frozen with liquid nitrogen and stored at −80 °C until analyses. Experiments were performed in duplicate.

Control samples without substrate or without cells were treated identically.

Distribution of polyphenols in the body

Healthy subjects avoided polyphenol-containing food for 2 days prior to the study. After an overnight fast, they consumed 1 L of cloudy apple juice (Hofmann, Nuedlingen, Germany) within 15 min, and venous blood was withdrawn from blood vessels at the back of the hands immediately before and 1, 2, 4, and 8 h after they drank. Standard Sarstedt® Monovettes (Sarstedt, Nuembrecht, Germany) were used for sample collection. Serum was isolated by centrifugation (5,000×*g* for 5 min) of the blood samples. To avoid polyphenol degradation, ascorbic acid was added and serum was acidified (to pH 5) using 0.58 M acetic acid. Samples were stored in glass tubes at −80 °C until analyses. Controls without substrate (human serum; Blood Donation Service of the Bavarian Red Cross, Munich, Germany) or without serum were treated identically.

Urine samples were collected on the previous day (−12 to 0 h) and during two periods after apple juice consumption (0–12 h, 12–24 h). After acidification (with HCl_{conc} to pH 5), the samples were stored in glass tubes at −24 °C until analyzed. Controls without substrate or without urine were treated identically.

Sample preparation

Saliva, simulated gastric juice, simulated duodenal juice, and ileostomy fluid

Freeze-dried and control samples were extracted twice, using 1.25 mL (2.5 mL for ileostomy fluid samples) of 70% (v/v) methanol in water containing 1% (v/v) acetic acid. After centrifugation (5,000×*g* for 10 min), supernatants were pooled and filtered (through polyvinylidene difluoride, pore size 0.45 μm). Standards were added, and samples (50 μL) were analyzed by HPLC–DAD and HPLC–ESI–MS/MS.

Hepatocytes

The freeze-dried samples and controls were dissolved in 1 mL of 70% (v/v) methanol in water containing 1% (v/v) acetic acid. Samples of 5-CQA and 4-*p*-CouQA were dissolved in 1 mL acetone. Standards were added and samples (50 μL) were analyzed by HPLC–DAD and HPLC–ESI–MS/MS. For identification of Q glucuronides, powdered sodium acetate was added to dissolved Q samples until a precipitate formed at the bottom of the tubes. The precipitate is a degradation product of Q-7-O-glucuronide but is not formed from 3′-*O*- or 4′-*O*-glucuronides. After 5 min, the supernatant was filtered (polyvinylidene difluoride, 0.45 μm) and analyzed by HPLC–DAD. Glucuronides were identified by comparing UV spectra before and after sodium acetate addition according to Day et al. [18].

Serum samples

Using 1.5 mL DMSO–methanol (1:4; v/v), 2-mL portions of human serum were extracted twice. After centrifugation (5,000×*g* for 10 min), the supernatants were pooled and extracted twice with 1 mL DMSO–methanol (1:4; v/v) to ensure complete protein precipitation. After centrifugation (5,000×*g*) for 10 min, supernatants were pooled and solvent was evaporated at 35 °C under nitrogen. The residue was redissolved in 150 μL of 70% (v/v) methanol in water containing 1% (v/v) acetic acid and was then filtered (polyvinylidene difluoride, pore size 0.45 μm). Standards were added, and samples (50 μL) were analyzed by HPLC–DAD and HPLC–ESI–MS/MS.

Urine samples

A volume of 5.4 mL portions of filtered (polyvinylidene difluoride, pore size 0.45 μm) human urine were acidified with 0.6 mL of 85% (v/v) phosphoric acid to pH 1.5 and centrifuged (5.000×*g* for 5 min). The supernatants were then subjected to solid-phase extraction (Oasis® HLB, 3 cc, 60 mg adsorbent; Waters, Milford, USA), preconditioned, and activated sequentially with 2 mL methanol and 2 mL 0.01% (v/v) HCl. The cartridges were loaded with 5 mL of the urine supernatants, and each washed with 2 mL 0.01% (v/v) HCl, dried, and then eluted with 2 mL HCl (0.01% v/v) in methanol into glass tubes. The solvent was then evaporated at 35 °C under nitrogen, and the residues (prepared in duplicate and designated A and B) were stored at −80 °C until analyzed. Each sample was prepared in duplicate (residue A and B). For identification of phenolic compounds in urine, residue A was silylated by 50 μL of MSTFA, and the glass tubes were then sealed and heated at 75 °C for 30 min. After cooling, the phenolics in 1 μL of each mixture were analyzed by GC–MS. Residue B was quantified by redissolving it in 500 μL of 70% (v/v) methanol in water containing 1% (v/v) acetic acid, adding standards, and then analyzing samples (99 μL) by HPLC–ESI–MS/MS in selected reaction monitoring (SRM) mode.

Apple juice

Apple polyphenols in the apple juice used in the experiments were quantified by HPLC–DAD and HPLC–ESI–MS/MS as previously described [11, 14].

β-Glucuronidase/sulfatase treatment of human serum and urine samples

Portions of 1 mL serum or 5.4 mL urine samples (each pH 5.0) were treated with β-glucuronidase (50 μL, 250 units) and sulfatase (50 μL, 50 units) or no enzymes (controls) at 37 °C for 2 h [19]. The reaction was stopped by the addition of 100 μL (20% w/v) TCA. Serum and urine sample preparations were then treated as described above.

HPLC–DAD analysis

For details of the HPLC system used, see [14]. A symmetry^{TM} C_{18} column (150 × 3.9 mm, 5 μm particle size; Waters, Milford, USA) was used. The mobile phase consisted of aqueous 0.1% (v/v) formic acid (A) and ACN (B), applied in a gradient from 1 to 40% B over 40 min at a flow rate of 1 mL/min. The peaks were identified by comparing their retention times and UV spectra (200–600 nm) with those of authentic standards as shown previously [14, 20]. For serum samples, limits of quantitation (LOQs) ranged from 0.04 to 0.06 μmol/L, and limits of detection (LODs) from 0.02 to 0.03 μmol/L defined as S/N ratios of 3:1, respectively [14]. For stereoselective analysis of monomeric flavan-3-ols, a Nucleodex β-PM (200 × 4.0 mm, with a 5 μm particle size) column was used, with an isocratic mobile phase of 40% (v/v) methanol at a flow rate of 0.5 mL/min. To evaluate the hydrolysis rates of each substrate (in μmol per unit time), their concentrations in the incubating mixtures were plotted against time, lines were draw through the linear regions of the resulting hydrolysis curves, and their hydrolysis rates were calculated from the slopes of the lines.

HPLC–ESI–MS/MS analysis

For the HPLC–ESI–MS/MS used, see [14]. The deprotonated ions obtained and mass spectra produced by ions were compared to those of pre-analyzed reference compounds [11, 14, 20]. For QA quantitation, see Kahle et al. [14].

Phenolic compounds in urine samples were quantitatively analyzed by MS in negative ionization, SRM mode with 3,4,5-*trans*-trimethoxycinnamic acid as an internal standard, by means of calibration curves, again as described by Kahle et al. [14]. LOQs ranged from 18 to 43 nmol/L, and LODs from 14 to 37 nmol/L, defined as S/N ratios of 10:1 and 3:1.

GC–MS analysis

Derivatized samples were analyzed using a gas chromatograph (Fisons Instruments GC 8060) equipped with a DB-1MS-fused silica capillary column (30 m × 0.32 mm i.d.; d_f 0.25 μm; J&W, Agilent, Waldbronn, Germany) coupled to a mass spectrometer (Fisons Instruments MD 800 supplied by Thermo Electron, Dreieich, Germany). Data were acquired using Xcalibur Qual Browser Software 1.2 (Thermo Electron Corp., Dreieich, Germany). Samples were injected directly (splitless) into the GC injection port, set at 250 °C, with helium (2.0 mL/min) as the carrier gas, and analytes were separated using a temperature program consisting of 2-min isothermal at 80 °C, followed by a 15 °C/min gradient to 155 °C, 2 min isothermal, a 6 °C/min gradient to 185 °C, then 15 °C/min to 320 °C, which was held for 5 min. The transfer line temperature was 300 °C, while the temperature of the interface was 280 °C. Eluting analytes were detected mass selectively (70 eV, electron impact) in scanning mode (70–600 amu). Phenolic compounds in urine and phloroglucinol were identified by comparing their linear retention indices and mass spectra with those of appropriate reference compounds.

Results

Saliva

The influence of human saliva on apple polyphenols (5-CQA, CA, 4-*p*-CouQA, Phl, Phl-glu, Phl-xylglu, (+)-C, (−)-EC, procyanidin B_2, Q, Q-3-glu, Q-3-gal, Q-3-ara, Q-3-xyl, Q-3-rham, and Q-3-rut) was investigated in three series of tests under aerobic conditions at 37 °C for 5 min (A–C). In test series A, the supernatant obtained after centrifuging saliva was used as inoculum. Under these conditions, all the polyphenols investigated were stable and recoveries ranged between 94.4 and 100.3%, indicating that the activity of soluble salivary enzymes was very low. In series B, the samples were incubated in uncentrifuged saliva. Hydrolysis of the flavonoid β-glycosides (Phl-glu, Phl-xylglu, Q-3-glu, Q-3-gal, Q-3-xyl, and Q-3-rut) into the corresponding aglycones and sugar moieties was observed within 5 min. Phl-glu exhibited the highest hydrolysis rate (0.11 μmol/min) followed by Q-3-glu (0.05 μmol/min), Q-3-gal (0.04 μmol/min), Phl-xylglu, and Q-3-xyl (each 0.01 μmol/min). Q-3-rut was found to be almost stable (<0.01 μmol/min). In series C, incubations were performed with uncentrifuged saliva in the presence of antibiotics (penicillin/streptomycin) to determine the importance of bacterial flora. Compared to hydrolysis rates in test series B, hydrolysis rates of the flavonoid glycosides were clearly lower (Fig. 1). These results demonstrate that oral hydrolysis of flavonoid glycosides in humans depends on oral bacterial flora.

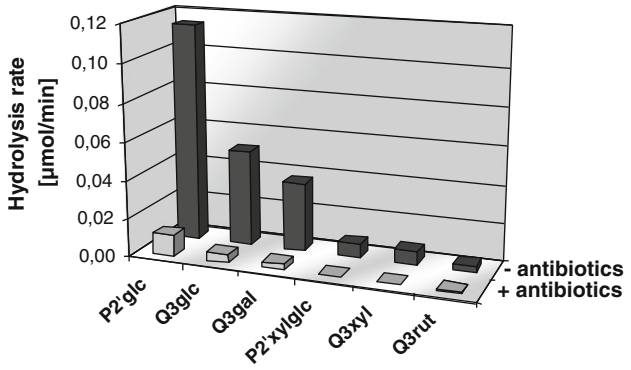

Fig. 1 Hydrolysis rates [µmol/min] of phloretin 2′-*O*-glucoside (P2′glc), quercetin 3-*O*-glucoside (Q3glc), quercetin 3-*O*-galactoside (Q3gal), phloretin 2′-*O*-xyloglucoside (P2′xylglc), quercetin 3-*O*-xyloside (Q3xyl), and quercetin 3-*O*-rutinoside (Q3rut) after incubation with saliva (with no centrifugation prior to incubation) for 5 min in the presence (*open square*) and absence (*filled square*) of penicillin/streptomycin antibiotics. Values are means of triplicate subsamples

Simulated gastric juice

Apple polyphenols were incubated with simulated gastric juice (pH 1.8) at 37 °C for up to 4 h. Results of subsequent HPLC analysis demonstrated that hydroxycinnamic acid derivatives, and flavonols, dihydrochalcones and monomeric flavan-3-ols are stable under these conditions (recoveries: 98.0–100.4%). In contrast, procyanidin B_2 was almost completely degraded into (−)-EC within 1.5 h, exhibiting a hydrolysis rate of 0.23 µmol/h (Fig. 2) appearing rapidly and reaching a maximum level of 0.41 µmol after 4 h. Per decomposed dimer, only one monomeric molecule was detected. This may be due to the formation of larger oligomers from the monomer under acidic conditions. Experiments using buffer solution (pH 1.8) showed similar results, suggesting that cleavage of procyanidin B_2 is caused by the acid in gastric juice.

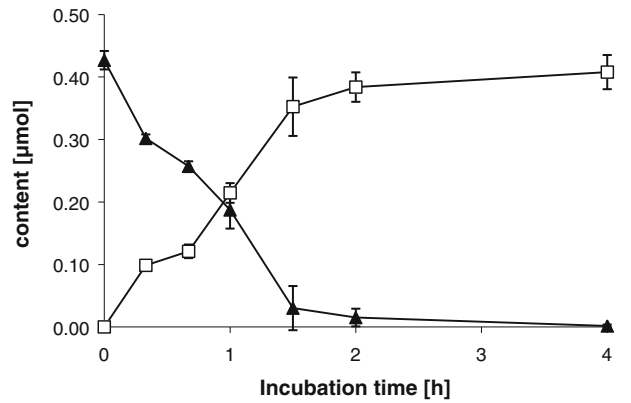

Fig. 2 Time course of procyanidin B_2 (20 µL, 25 mM stock solution) incubation with simulated gastric juice (pH 1.8) measured by HPLC–DAD (280 nm). Procyanidin B_2 (*filled triangle*), (−)-epicatechin (*open square*). Values are means ± SE of duplicate subsamples

Simulated duodenal juice

Among the 16 apple polyphenols investigated, the dihydrochalcones (Phl, Phl-glu and phl-xylglu) as well as the flavonol glycosides (Q-3-glu, Q-3-gal, Q-3-ara, Q-3-xyl, Q-3-rut) were stable in simulated duodenal juice (pepsin, pancreatin and bile extract) over the entire 24-h incubation period. In contrast, 5-CQA, CA, 4-*p*-CouQA, (+)-C, (−)-EC, procyanidin B_2, and Q underwent varying modifications in simulated duodenal juice (pH 7.2). Interestingly, in control experiments (buffer solution, pH 7.2, without pancreatin, bile extract, and pepsin), similar results were obtained for these phenolic substances, indicating that the observed effects were mostly due to the chemical conditions of the assay. Spectroscopic data (retention time, UV max, and HPLC–ESI–MS/MS fragmentation patterns) of products detected in artificial duodenum samples are summarized in Table 1.

Slow decomposition of 4-*p*-CouQA from an initial amount of 4.42 µmol with 0.02 µmol/h was observed (Fig. 3), and after 24 h, 56.3% of this initial amount was still detectable in duodenal samples. The products (*p*-CouA, QA, 3- and 5-*p*-CouQA, and methyl *p*-coumarate) were identified by HPLC–DAD and HPLC–MS/MS analysis. Liberated *p*-coumaric acid was first detectable after 6 h of incubation and reached a maximum (0.01 µmol) after 24 h. The other products reached their respective maximum amounts of 0.045 µmol (3-*p*-CouQA), 0.024 µmol (5-*p*-CouQA), 0.033 µmol (methyl *p*-coumarate), and 0.009 µmol (QA) after 10-h incubation. Comparable results were obtained from the incubation of 5-CQA (initial amount, 0.44 µmol), which underwent 37% degradation at a rate of 0.014 µmol/h. The ring fission products, CA and QA, in addition to its isomers 3- and 4-CQA, and methyl caffeate, were found in duodenal samples, reaching peak amounts of 0.002, 0.023, 0.044, 0.015, and 0.022 µmol, respectively, after 6 h (CA: 10 h). Formation of methyl caffeate following the incubation of caffeic acid has also been demonstrated (data not shown). In addition, ferulic and dihydrocaffeic acid were identified as metabolites. To examine the influence of methyl ester formation via sample preparation, hydroxycinnamic acid derivatives were extracted with acetone in a second incubation series. No methyl esters were identified after this sample preparation, indicating that its formation is dependent on the pH value. Thus, neither in the acidic milieu of the stomach (pH 1.8) nor under conditions present in the small intestine (pH 6.3) methyl esters were generated under the study, although these samples were also extracted with methanol.

Table 1 Spectroscopic data (retention time, UV maximum, and HPLC-ESI$_{neg}$-MS/MS fragmentation patterns) of polyphenol products in simulated duodenal juice

	t_R^a [min]	λ_{max}^a [nm]	[M-H]$^-$ (*m/z*)	MS/MS (*m/z* (relative abundance, %))	eV
3-Caffeoylquinic acid	8.4	326	353.0	190.8 (100), 178.8 (58), 134.8 (7)	20
4-Caffeoylquinic acid	10.4	326	353.1	172.8 (100), 178.8 (72), 190.8 (23)	20
D-(−)-quinic acid	2.0	ND	190.6	84.7 (100), 92.8 (35), 126.6 (13)	30
Methyl caffeate	16.7	324	192.7	134.3 (100), 160.7 (35)	35
Ferulic acid	14.0	324	192.6	133.8 (100), 177.8 (68), 148.9 (45)	20
Dihydrocaffeic acid	9.9	280	180.7	136.9 (100)	30
3-*p*-Coumaroylquinic acid	11.2	326	337.0	162.8 (100), 190.6 (10)	25
5-*p*-Coumaroylquinic acid	11.8	325	337.0	190.8 (100), 172.9 (5)	25
p-Coumaric acid	13.1	310	162.8	118.8 (100)	20
Methyl *p*-coumarate	19.8	311	176.8	116.9 (100), 144.8 (35)	30
(−)-Catechin	9.9	278	289.0	245.1 (100), 205.1 (35)	20
Phloroglucinol	5.2	228	124.9	56.5 (100), 82.8 (9)	20
3,4-Dihydroxybenzoic acid	9.5	224	152.8	108.7 (100)	30
2,4,6-Trihydroxybenzoic acid	14.9	256	168.9	124.9 (100)	25

For details see "Materials and methods"

ND not determinable

[a] Retention time (t_R) and wavelength (λ_{max}) determined by HPLC–DAD

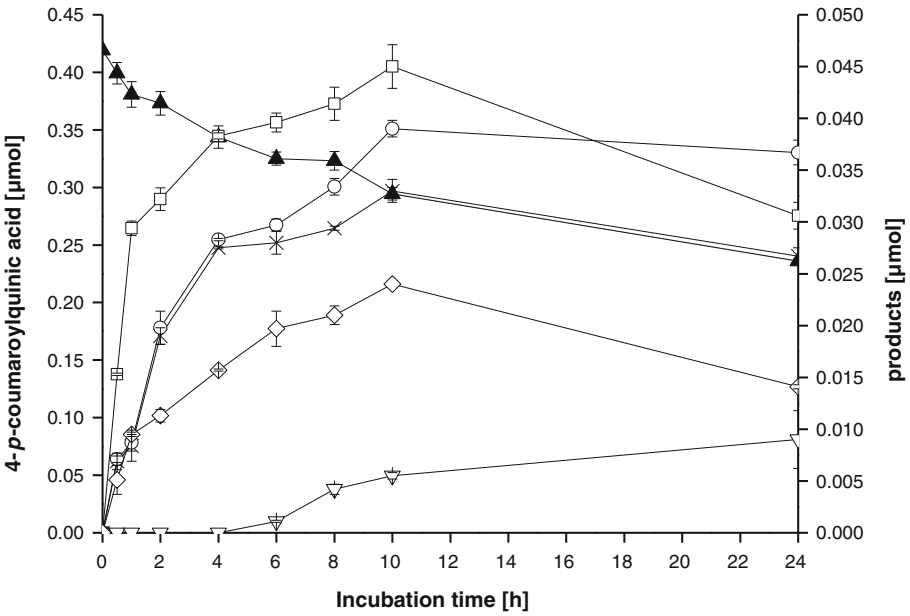

Fig. 3 Time course of 4-*p*-coumaroylquinic acid (20 µL, 25 mM stock solution) incubation with simulated duodenal juice (pH 7.2) measured by HPLC–DAD (320 nm). 4-*p*-Coumaroylquinic acid (*filled triangle*), 3-*p*-coumaroylquinic acid (*open square*), 5-*p*-coumaroylquinic acid (*open diamond*), D-(−)-quinic acid (*open circle*), *p*-coumaric acid (*open down triangle*), methyl *p*-coumarate (*multisymbol*). Values are means ± SE of duplicate subsamples

Within 8 h of incubation, the monomeric flavan-3-ols (+)-C and (−)-EC were almost completely converted, at rates of 0.11 and 0.14 µmol/h, respectively. These findings indicate that the 2,3-*trans* configuration confers greater stereochemical stability than the 2,3-*cis* configuration. In addition, HPLC analysis using a chiral stationary phase revealed epimerization of (+)-C to (−)-EC and (−)-EC to (−)-C. The amounts of formation products increased up to 0.15 and 0.13 µmol after 2 h, followed by an almost complete decrease after 10 h. Complete degradation of the dimeric procyanidin B_2 occurred within 8 h of incubation (0.11 µmol/h). No (−)-EC was detectable.

In the presence of simulated duodenal juice, Q was almost completely degraded within 2 h (0.31 µmol/h) with the formation of the intermediate 2,4,6-THBA and the two end products 3,4-DHBA and phloroglucinol. The identity of phloroglucinol was confirmed by GC–MS after silylation. The radical cation and the production [M-15]$^+$ had *m/z* values of 342 and 327, respectively.

Ileostomy incubations

All substances studied were chemically stable for incubation times of up to 10 h; therefore, any changes observed in ileostomic samples were attributable to the action of ileal enzymes (data not shown).

No significant degradation was detected of the hydroxycinnamic acids (5-CQA, CA and 4-*p*-CouQA), the flavan-3-ols ((+)-C, (−)-EC, and procyanidin B_2), or the aglycones (Phl and Q) (all recoveries: 96.3–100.2%). In contrast, the flavonoid glycosides (Phl-glu, Phl-xylglu, Q-3-glu, Q-3-gal, Q-3-ara, Q-3-xyl, Q-3-rham, and Q-3-rut) were hydrolyzed at rates that depended on their sugar moiety (glucoside > galactoside > xyloside > arabinoside > rhamnoside > rutinoside > xyloglucoside). Phl-glu was hydrolyzed fastest at 0.44 µmol/h, while Phl-xylglu was almost stable (0.002 µmol/min).

Hepatocytes

In order to study the metabolic processes in the liver affecting the studied polyphenols, experiments modeling hepatic conditions using freshly isolated rat hepatocytes were carried out. The investigated flavan-3-ols ((+)-C, (−)-EC), procyanidin B_2, and *p*-CouA were all found to be stable over the whole 4-hour incubation period. Conversion of CA to trace amounts of FA and IFA was observed, while 30.3% of the 4-*p*-CouQA was degraded at a hydrolysis rate of 0.041 µmol/h (Fig. 4). The metabolites identified were its 3- and 5-isomers, QA and *p*-coumaric acid methyl ester. Maximum amounts of 0.052 µmol for 3-*p*-CouQA, 0.027 µmol for 5-*p*-CouQA, and 0.022 µmol for the methyl ester were obtained after 4 h. The time course (4 h) and amount of QA, measured by HPLC-ESI_{neg}-MS/MS in SRM mode, were comparable to those of the methyl esters. Incubations of 5-CQA showed similar results. After 4-h incubation, 72.9% of the initial amount was detectable; 1-, 3-, and 4-CQA as well as QA and CA methyl ester were generated, with peak contents of 0.035, 0.019, and 0.021 µmol for the isomers and 0.015 µmol for both QA and the methyl ester.

The aglycones, Phl and Q, underwent intensive conjugation reactions. Phl was conjugated via phase II metabolism to two glucuronides (conversion rate: 0.13 µmol/h). Their identities were confirmed using HPLC-ESI_{neg}-MS/MS; in both cases, their fragmentation patterns showed the presence of a deprotonated ion with an *m/z* value of 449 and a characteristic product ion with an *m/z* of 273. Treatment of Phl samples with *β*-glucuronidase resulted in complete degradation of both metabolites with a simultaneous increase in the expected products (data not shown). One of the glucuronides was identified as phloretin 2′-*O*-glucuronide (for details, see [14]). Structural analysis of the second glucuronide by ^{1}H-NMR was not possible, since too low amounts were present in the samples, but it is conceivable that glucuronidation at position 4′-OH or 4-OH occurred.

After rapid degradation of Q (0.22 µmol/h), quercetin 4′-*O*-glucuronide and lesser amounts of quercetin 7-O-glucuronide, 3′-*O*-methylquercetin 3-*O*-glucuronide, quercetin 3-*O*-glucuronide, 3′-*O*-methylquercetin 7-*O*-glucuronide, quercetin 3′-*O*-glucuronide, and 3′-*O*-methylquercetin were detected in the studied samples. Compounds present were identified according to methods described in the literature [18, 21]. After 4 h of incubation, only 7.4% of the initial Q was detected, and more than 30% had been converted to quercetin 4′-*O*-glucuronide.

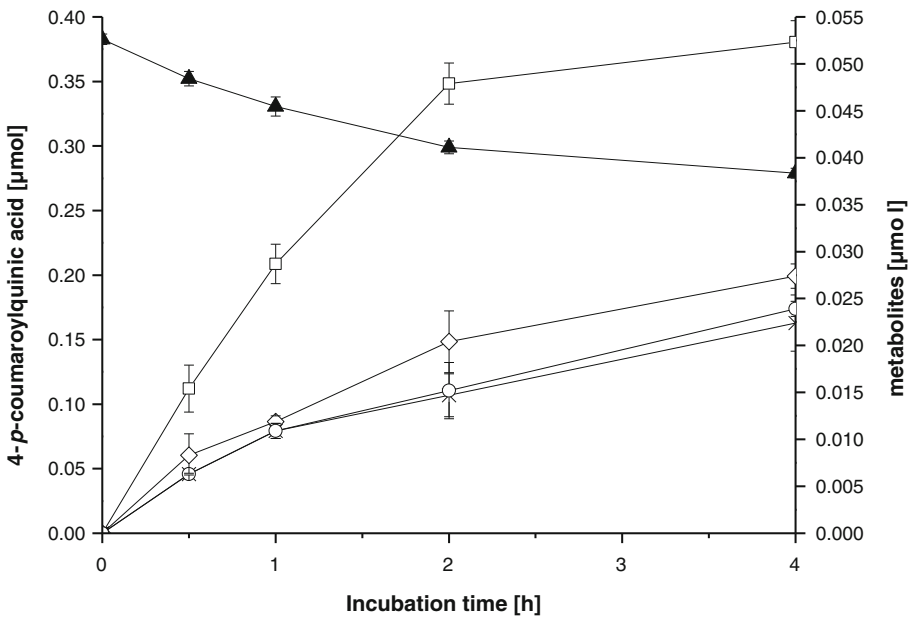

Fig. 4 Time course of 4-*p*-coumaroylquinic acid [0.42 µmol] incubation (4 h) with rat hepatocytes measured by HPLC–DAD (320 nm). 4-*p*-Coumaroylquinic acid (*filled triangle*), 3-*p*-coumaroylquinic acid (*open square*), 5-*p*-coumaroylquinic acid (*open diamond*), D-(−)-quinic acid (*open circle*), methyl *p*-coumarate (*multisymbol*). Values are means ± SE of duplicate subsamples

Identification and quantification of apple juice polyphenols in human serum

Five healthy volunteers each consumed one liter of cloudy apple juice, and blood samples were taken 1, 2, 4, and 8 h later. The serum samples were analyzed by HPLC–DAD after centrifugation, acidification, and glucuronidase/sulfatase treatment. A chromatogram (obtained by monitoring at 280 nm) of a serum sample taken 2 h after juice intake is shown in Fig. 5. 5-CQA, 4-CouQA, CA, Phl, (−)-EC, and Q were detected following enzymatic treatment (with variations between individuals). Results are summarized in Table 2. 5-CQA and 4-*p*-CouQA were only detectable after enzymatic treatment of the serum samples. CA, Phl, (−)-EC, and Q were detectable before and after enzymatic treatment but more strongly after glucuronidase/sulfatase treatment. The highest plasma concentrations of 5-CQA (0.39–0.59 μmol/L) were reached in three of the six probands after 1 h (see Fig. 6). In the serum samples of four probands, 4-*p*-CouQA was detected at plasma concentrations of 0.21–0.37 μmol/L. However, in one proband, neither 5-CQA nor 4-*p*-CouQA were detectable, but in the serum of this, as well as the other probands, caffeic acid was detected in both conjugated and free forms. (−)-EC (in three of the five probands) was the only measurable flavan-3-ol. Phl and Q were present, mostly in the bound form, in all samples. For phloretin, 8.3–11.6% of the amount measured was determined to be in the free form (maximum concentration, 0.72 μmol/L). In addition to 5-CQA (with serum concentrations of up to 1.91 μmol/L), high amounts of Q were present, with concentrations of 1.13 μmol/L measured in one proband (0.13–1.13 μmol/L). In total, up to 5.3% of the ingested polyphenols were recovered in the serum samples within 8 h.

Identification and quantification of apple juice polyphenols in human urine

Urine samples were collected 12 h prior to the consumption of one liter of cloudy apple juice (see 3.6) and over a period of 24 h (0–12 h and 12–24 h) after its consumption. The metabolites were identified and quantified after glucuronidase/sulfatase treatment. Between 94.8 and 98.6% of apple-derived phenolic acids and 90.3–95.1% of polyphenols were recovered. The results are summarized in Table 3. Prior to enzymatic treatment, only free CA was detected. The aglycones (Q, CA, 5-CQA, 4-*p*-CouQA, Phl, and (−)-EC) were quantified after juice intake (at 3.5% of the dose). In three out of six subjects, most of the 5-CQA and 4-*p*-CouQA was observed (averages of 0.87 and 0.17 μmol; 0.5% of the ingested dose) within the first 12 h after juice consumption. All probands excreted conjugated and unconjugated CA, whereas Phl and Q (0.6 and 0.4% of

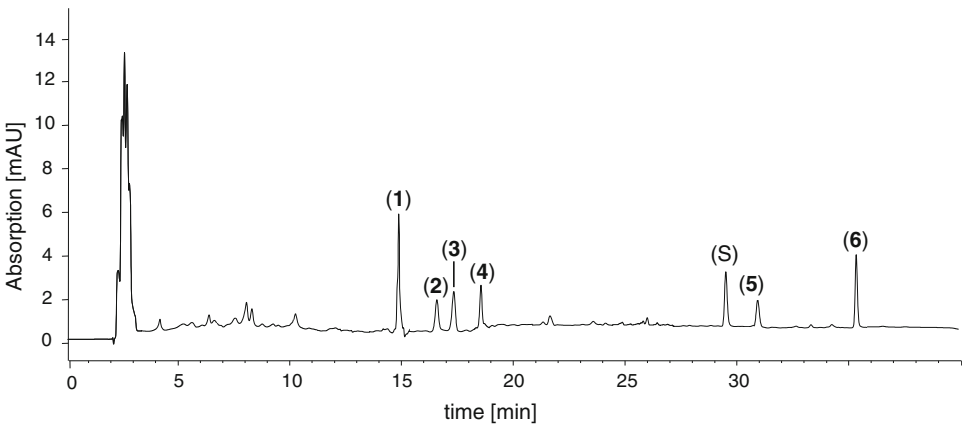

Fig. 5 HPLC–DAD elution profile (280 nm) of a serum sample, taken 2 h after cloudy apple juice consumption, following enzymatic treatment with *β*-glucuronidase and sulfatase. (*1*) 5-Caffeoylquinic acid, (*2*) caffeic acid, (*3*) (−)-epicatechin, (*4*) 4-*p*-coumaroylquinic acid, (*5*) quercetin, (*6*) phloretin, (*S*) standard (3,4,5-trans-trimethoxycinnamic acid). For details, see "Materials and methods"

Table 2 Polyphenol contents, c_{max} [μmol/L], t_{max} [h], $t_{1/2}$ [h], and $AUC_{0-8\ h}$ [(μmol*h)/L] of human serum samples ($n = 6$) after enzymatic treatment with *β*-glucuronidase and sulfatase following consumption of cloudy apple juice

	c_{max} [μmol/L]	t_{max} [h]	$t_{1/2}$ [h]	$AUC_{0-8\ h}$ [(μmol*h)/L]
5-Caffeoylquinic acid	0.73 ± 0.28	0.7 ± 0.8	3.8 ± 2.9	1.52 ± 1.43
Caffeic acid	0.09 ± 0.03	2.0 ± 1.2	6.0 ± 2.4	0.47 ± 0.20
4-*p*-Coumaroylquinic acid	0.09 ± 0.07	1.3 ± 1.0	5.2 ± 3.4	0.37 ± 0.25
Phloretin	0.17 ± 0.09	2.1 ± 0.5	5.6 ± 1.2	0.86 ± 0.37
(−)-Epicatechin	0.05 ± 0.06	0.9 ± 1.1	2.3 ± 2.5	0.22 ± 0.23
Quercetin	0.25 ± 0.12	1.1 ± 0.4	3.7 ± 1.2	1.02 ± 0.65

Values are means ± SD of five probands ($n = 5$)

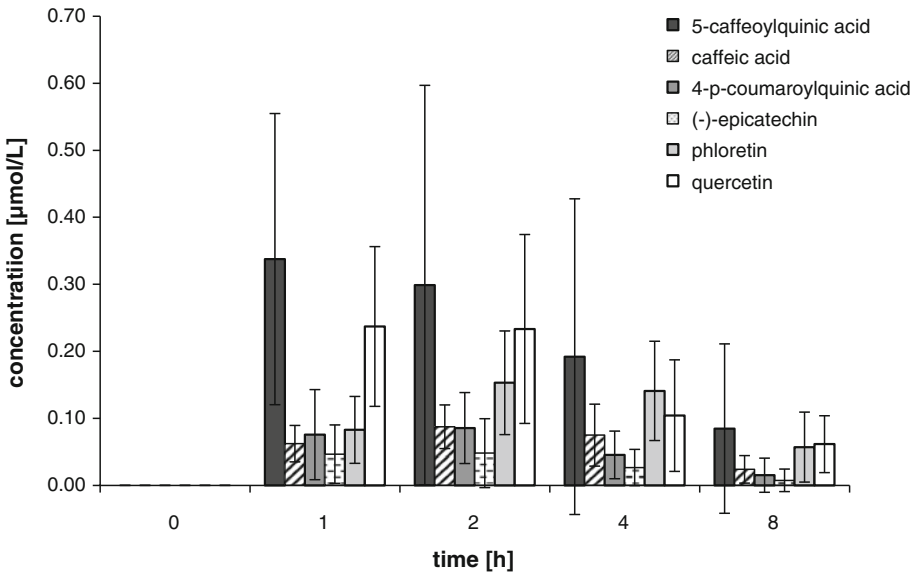

Fig. 6 Time course (0–8 h) of excretion of the apple polyphenols [µmol/L]; 5-caffeoylquinic acid, caffeic acid, 4-*p*-coumaroylquinic acid, (−)-epicatechin, phloretin, and quercetin measured from the serum of studied subjects ($n = 5$). Data are expressed as means ± SD

the ingested dose, respectively) were measureable only after enzymatic treatment.

Degradation products of the apple-derived polyphenols were identified using GC–MS analysis following derivatization to obtain additional structural information. Using GC–MS, HPLC–MS, and HPLC–MS/MS FA, DHFA, *p*-Cou, DHBA, DHCA, 4-HBA, 3-HPAA, 3,4-DHPAA, 3-HPPA, HA, 3-HA, and 4-HA were detected in the urine samples. Up to 19.5% of the ingested polyphenols were excreted as phenolic acids. The lowest amounts recovered were of FA, *p*-Cou, and IFA (0.2 µmol) and the highest of hippuric acids (HA, 3-HHA, and 4-HHA), DHCA and 3-HPPA. No flavan-3-ols were detected in the urine samples collected.

Discussion

Polyphenols are secondary metabolites of plants and thus are ingested as part of our daily diet, and various health-promoting properties have been attributed to them on the basis of effects observed in diverse in vitro assays, experiments with animals, and epidemiological studies (as summarized by Boyer and Liu [4]). Prerequisites for these compounds to have any in vivo effects are that they must be absorbed from the gastrointestinal tract after food consumption and subsequently reach sufficiently high plasma concentrations in the systemic circulation to induce biological activity, i.e., they must have sufficient oral bioavailability. In the work reported here, a number of experiments were carried out to model conditions in vivo and thus gain greater understanding of the steps involved in apple polyphenol digestion in humans. Their distribution and renal elimination were also investigated following apple juice consumption by healthy human subjects.

Table 3 Amounts of polyphenols and their metabolites [µmol/12 h] in human urine samples ($n = 6$) after enzymatic treatment with β-glucuronidase and sulfatase following consumption of cloudy apple juice

	−12 to 0 h	0–12 h[a]	12–24 h[a]	0–24 h[a]
5-Caffeoylquinic acid	ND	0.87	0.15	1.02
Caffeic acid	ND	0.09	0.02	0.11
4-*p*-Coumaroylquinic acid	ND	0.17	0.01	0.18
(−)-Epicatechin	ND	0.26	0.03	0.29
Phloretin	ND	0.47	0.07	0.54
Quercetin	ND	0.08	0.02	0.10
Ferulic acid	ND	0.19	0.02	0.21
Dihydrocaffeic acid	1.67	11.75	5.72	17.47
p-Coumaric acid	ND	0.18	0.03	0.22
3,4-Dihydroxybenzoic acid	0.88	7.31	2.24	9.56
Isoferulic acid	ND	0.16	0.02	0.18
4-Hydroxybenzoic acid	0.76	6.84	2.60	9.44
3-Hydroxyphenylacetic acid	0.93	4.32	1.85	6.17
3,4-Dihydroxyphenylacetic acid	0.54	4.78	2.29	7.07
3-Hydroxyphenylpropionic acid	2.04	15.86	7.23	23.09
Hippuric acid	6.87	32.85	12.45	45.30
3-Hydroxyhippuric acid	2.21	10.67	6.32	16.99
4-Hydroxyhippuric acid	2.34	8.34	5.22	13.56

Values are means of duplicates

ND not detectable

[a] Value after substraction of the blind value (−12 to 0 h)

Upon oral consumption, apple polyphenols first come into contact with saliva. In our study, the hydrolysis of Phl and Q β-glycosides was demonstrated to depend on the

sugar moiety, while quercetin α-glycosides (Q-3-ara and Q-3-rham) were found to be stable in the presence of saliva. These results agree with the current literature [22–25]. Our findings on the stability of monomeric and dimeric flavan-3-ols are also in accordance with reported results of other authors [25]; however, no information on the stability of the other studied phenolics in the presence of saliva was previously available. Furthermore, we confirmed that enzymatic activity depends on oral bacterial flora, as illustrated in Fig. 1. The glycosidic activity of mouth flora is well documented [22, 24, 25].

Our incubations with simulated gastric juice confirmed the previously documented stability of monomeric flavan-3-ols [26, 27], Q, and its glycosides [28], CA and CQAs [28–30] in the presence of pepsin at pH 1.8. As shown in our studies (Fig. 2), the decomposition of procyanidins under mildly acidic conditions is a well-characterized chemical process [31, 32] or in the presence of simulated gastric juice [26] and has been observed in ileostomy subjects [14]. Information is not yet available in the literature on modification reactions that 4-*p*-CouQA undergoes in the mildly alkaline conditions found in the duodenum, but Farah et al. [33] observed isomerization and hydrolysis reactions of 5-CQA after incubation with both artificial and human duodenal juice. In addition, Bermudéz-Soto et al. [28] discovered that a rearrangement of 5-CQA into the 3-isomer occurred in the presence of simulated duodenal juice (pH 7.5). However, free CA was not detected, probably due to the lack of esterase activity in the pancreatin used.

Zhu et al. [26] reported complete conversion of monomeric flavan-3-ols into their isomers within 10 h of incubation with artificial duodenal juice. However, configuration analysis was not carried out. Using HPLC with a chiral stationary phase, we demonstrated the epimerization of (−)-EC into (−)-C and (+)-C into (−)-EC. The first conversion agrees with previous findings [34, 35], but no signs of a reported epimerization from (+)-C to (+)-EC in an alkaline environment [61] were detected here.

The rapid degradation of procyanidin dimer B_2 by artificial duodenal juice demonstrated here agrees with earlier observations, as did our failure to detect any degradation products [26].

Our findings on the stability of Phl and its glycosides in duodenal juice are novel, but in vitro degradation of Q by duodenal fluid has already been described [28]. The formation of its degradation products (phloroglucinol, 3,4-DHBA and 2,4,6-THBA) may occur via oxidative decarbonylation or oxidative decarboxylation [36], whereas C3 substitution completely suppresses oxidative decarboxylation. So Q 3-*O*-glycosides remained stable while they were incubated with duodenal juice.

In the small intestine, most flavonoid glycosides are completely degraded to their corresponding aglycone and sugar moiety. The aglycone then enters enterocytes by passive diffusion where it undergoes various conjugation reactions [37]. Lactase phloridzin hydrolase (LPH), a β-glycosidase located on the brush border of the mammalian small intestine, catalyzes deglycosylation [38, 39]. Since LPH is membrane-bound, its influence on the hydrolytic activity of the ileostomy fluids used would be low; the influence on the hydrolytic activity arises from the intestinal microflora and depends not only on the sugar structures but also on the aglycone structures [40]. However, no human ileal microflora with esterase activity capable of hydrolyzing 5-CQA or 4-*p*-CouQA has been described yet. Therefore, findings on the liberation of CA or *p*-CouA and QA in the studies presented here using ileal effluents and studies with ileostomists consuming cloudy apple juice [14] might be due to the pH and esterase activity in the duodenal region. The stability of monomeric flavan-3-ols we observed also agrees well with previous demonstrations of their absorption without prior cleavage into smaller molecules [41]. Interestingly, procyanidin B_2 was not degraded in the ileostomy fluid either, which highlights its stability in a neutral milieu (pH 6.3).

For the first time, the metabolism of apple-derived polyphenols in the liver was studied in detail using rat hepatocytes. During the first 4 h of incubation, (+)-C, (−)-EC, procyanidin B_2, and *p*-coumaric acid were not affected by phase I or II metabolism. This is in partial agreement with previous findings; Vaidyanathan and Walle [42] did not detect any metabolites of (+)-C and (−)-EC in the liver, whereas other authors have identified glucuronides [42]. No data are available in the literature on the metabolism of procyanidin B_2, but procyanidin B_3 has been found to be methylated by homogenates of human liver [41]. The hydroxycinnamic acid esters of *p*-CouA and CA were isomerized and partly liberated to QA and the corresponding acid in both free and methylated form. Here, for the first time, we report the formation of 1-CQA from 5-CQA and the rapid isomerization of 4-*p*-CouQA into 5- and 4-*p*-CouQA by hepatocytes. In addition, FA and IFA were identified in trace amounts after CA incubation, in agreement with previous expressions of this methylation [43].

The glucuronidations and methylations of Q observed here have also been previously reported [21, 44], but no information on the metabolization of Phl in the liver was previously available. Interestingly, our findings correlate well with recent reports of Phl conjugates identified in human plasma and urine after apple cider consumption [45]. Phl-glu was the main metabolite identified in plasma, together with other glucuronides and sulfates. Therefore, the positions of glucuronidation or sulfation in the serum

samples obtained after apple juice consumption remain under investigation.

There have been a few relevant studies on plasma concentrations of polyphenols and their metabolites. Some detected increases in phenolic plasma concentrations following the consumption of apple cider [45, 46]. In addition, after the ingestion of pure 5-CQA, several authors have detected minor concentrations of the unaltered compound in the plasma and urine of probands. These authors concluded that the ester was cleaved and metabolized [29, 47]. In support of this conclusion, *p*-CouA and CA, in addition to metabolites such as DHCA and FA, have been found in the plasma and urine of probands following coffee consumption [46, 48–50].

The profile of metabolites generated from CQAs varies and seems to depend on the quantities ingested. Protein binding plays an important role in the recovery of hydroxycinnamic acids from plasma, with up to 90% of caffeic acid binding to human albumin, while less than 10% of ferulic and *p*-CouAs appear to bind in vivo [51]. No data on plasma concentrations of 4-*p*-CouQA are currently available, but there is some information on plasma concentrations of 5-CQA following its consumption. Following the consumption of artichoke extract, apple cider and coffee neither free nor bound 5-CQA has been detected in plasma by several authors [46, 52, 53]. However, Stalmach et al. [49] identified small amounts of 5-CQA in plasma samples after coffee consumption, and 5-CQA has been detected in plasma, along with its 3- and 4-isomers, following consumption of high quantities (≥850 μmol) of each isomer. Maximal plasma concentrations (after enzymatic hydrolysis) up to 3.14 μmol/L were detected, between 1.75 and 2.33 h after ingestion. Following coffee consumption, a minor amount of CA in its free form has also been detected in plasma by Nardini et al. [53], and the percentage of conjugated caffeic acid in plasma (relative to total free and conjugated levels) fell from 77.1 to 67.7% following treatment with *β*-glucuronidase and sulfatase. Interestingly, Nardini et al. [53] found plasma concentrations to be maximal an hour after caffeic acid consumption. The difference in kinetics may be due to the fact that coffee contains a readily soluble form of 5-CQA, since caffeic acid in plasma may originate from the consumption of either free caffeic acid or its esters, such as 5-CQA. Following the consumption of 5-CQA, Azuma [54] did not recover 5-CQA in plasma but detected glucuronidated and sulfated caffeic and ferulic acid in samples. Following the consumption of artichoke extract, no 5-CQA was found, but caffeic acid, ferulic acid, and dihydroxylation products were all detected [29].

The bioavailability of chlorogenic acids after the consumption of 200 ml of instant coffee in eleven volunteers has been investigated by Stalmach et al. [49]. Without enzymatic treatment, the sulfates of DHCA, DHFA, CA, and FA and small amounts of CQA lactone sulfates were detected in the plasma samples. The highest amounts measured were of DHCA 3-*O*-sulfate and DHFA, with only small amounts of 3-, 4-, and 5-feruloylquinic acids. Compounds such as CA 3-O-sulfate occurred in the plasma within the first 3 h, whereas the concentration of the colonic metabolite DHFA was maximal after 6 h. In agreement with our study, conjugated DHCA was recovered as the main metabolite in the urine samples at the same concentration range (35 μmol/24 h) in addition to other conjugated and non-conjugated metabolites.

In a recently published study, the bioavailability of dihydrochalcones following the consumption of apple cider was investigated [45] in ileostomists and healthy subjects. The results showed that the Phl glycosides, Phl-xylglu and Phl-glu, are metabolized to Phl-2′-*O*-glcA in probands with and without a colon. Glucuronide was detected in ileal fluid, urine, and plasma samples in addition to minor amounts of Phl conjugates (glucuronides and sulfates) and unconjugated Phl in the ileal samples.

Several studies have suggested that low molecular weight polyphenols from apple juice and colonic degradation products are likely to be absorbed and metabolized [37]. Furthermore, intervention studies indicate that low molecular weight flavan-3-ols (mono and dimers), but not larger forms of these compounds, can be absorbed and are subsequently detectable in the plasma [55]. Following oral administration of 5.1 mg flavan-3-ols (1.1 mg C, 2.4 mg EC, 0.66 mg 3′-*O*-methylcatechin and 0.93 mg 3′-*O*-methyl epicatechin) in apple cider, by DuPont et al. [46], no epicatechin was detected in any plasma samples from probands. However, after the consumption of tea, chocolate, and cocoa, both catechin and epicatechin have been identified following the enzymatic hydrolysis of plasma samples from probands with concentrations peaking (at 0.4 μmol/L) after 1.8 h [56].

It has been reported that Q glycosides do not occur in plasma but appear in other forms as metabolites [37]. For instance, following oral ingestion of Q glycosides, the metabolites Q 3-O-glucuronide, 3′-methylquercetin 3-O-glucuronide, and 3′-O-quercetin sulfate have been detected in human plasma [56, 57].

In the urine samples we examined, most of the polyphenols derived from apple juice were present in their conjugated forms. Only CA in its free form was detected, whereas after liberation by glucuronidase and sulfatase treatment, the aglycones Q, CA, 5-CQA, 4-*p*-CouQA, Phl, and (−)-EC were quantified—at 3.5% of the ingested dose. These findings agree with data provided by other researchers, who detected no free 5-caffeoylquinic acid or caffeic acid after coffee consumption, although FA, IFA, DHFA, and vanillic acid have been identified in the urine

Table 4 Summary of the results on the metabolization of apple-derived polyphenols obtained from digestion models and intervention studies

Method	Saliva	Stomach	Duodenum	Ileum	Liver	Serum	Urine**
pH		1.8	7.2	6.3		5.0	5.0
Caffeic acid	NA	NA	Metabolization	NA	NA	Free and conjugated*	Free and conjugated*
p-Coumaric acid	–	–	Metabolization	–	NA	Free and conjugated*	ND
5-Caffeoylquinic acid	NA	NA	Isomerization and hydrolysis	NA	Isomerization and hydrolysis	Conjugates	Free and conjugated*
4-*p*-Coumaroylquinic acid	NA	NA	Isomerization	NA	Isomerization and hydrolysis	Conjugates	Free and conjugated*
Phloretin	NA	NA	NA	NA	Glucuronides	Free and conjugated*	Free and conjugated*
Phloretin 2′-*O*-glucoside	Hydrolysis	NA	NA	Hydrolysis	–	ND	ND
Phloretin 2′-*O*-xyloglucoside	Hydrolysis	NA	NA	Hydrolysis	–	ND	ND
(+)-Catechin	NA	NA	Isomerization	NA	NA	Free and conjugated*	ND
(−)-Epicatechin	NA	NA	Isomerization	NA	NA	Free and conjugated*	Free and conjugated*
Procyanidin B_1	–	–	–	–	–	ND	ND
Procyanidin B_2	NA	Hydrolysis	Degradation	NA	NA	ND	ND
Quercetin	NA	NA	Degradation	NA	(methyl) Glucuronides	Free and conjugated*	Free and conjugated*
Quercetin 3-*O*-glucoside	Hydrolysis	NA	Hydrolysis	Hydrolysis	–	ND	ND
Quercetin 3-*O*-galactoside	Hydrolysis	NA	NA	Hydrolysis	–	ND	ND
Quercetin 3-*O*-arabinoside	NA	NA	NA	Hydrolysis	–	ND	ND
Quercetin 3-*O*-xyloside	Hydrolysis	NA	NA	Hydrolysis	–	ND	ND
Quercetin 3-*O*-rhamnoside	NA	NA	NA	NA	–	ND	ND
Quercetin 3-*O*-rutinoside	NA	NA	NA	Hydrolysis	–	ND	ND
Polymeric procyanidins	–	–	–	–	–	–	–

NA not affected, – not analyzed, *ND* not detectable

* Analyzed after glucuronidase/sulfatase treatment

** Hydroxycinnamic acid metabolites measured (see Table 3)

of probands [53]. Similar results have been observed following artichoke consumption [52]. Interestingly, these groups found higher amounts (up to 5%) of these metabolites in urine samples than we detected (0.6%). No data are available on the urinary excretion of 4-*p*-CouQA as yet, but after coffee consumption, 2.3% of the 5-CQA consumed has been recovered in urine [58–60]. In these studies, additional metabolites were also observed. Interestingly, high amounts of phenolic acids (19.5% of the ingested polyphenols), which were mostly hippuric acids (HA, 3-HHA, and 4-HHA), DHCA, and 3-HPPA were recovered in the urine samples. In agreement with our findings, up to 50% of the hydroxycinnamic acids were excreted as HA [59, 60]. Some authors have suggested that HA formation (by colonic microflora and the liver) represents a significant step in the metabolism of polyphenols [61], QA or benzoic acids [62]. In our study, an average of 0.6% of the ingested phloretin glycosides (Phl-xylglu, but not the Phl-glu) were recovered in all urine samples ($n = 6$), whereas DuPont et al. [46] detected 21.5% of the administered dose of phloretin in human urine following enzymatic hydrolysis. As early as 1984, Monge et al. [62] identified 50% of the Phl administered in the urine of rats as Phl, 3-(4-hydroxyphenyl)-propionic acid, and phloroglucinol, and this finding was supported by others [37].

Following the consumption of quercetin and its conjugates in apples, onions, buckwheat, tomato juice, or as pure Q [56], 0.3–6.4% of the consumed dose of Q has been detected in urine samples in the conjugated form. In the present study, we found the average amount to be 0.4% of the ingested dose.

In the case of the flavan-3-ols, no monomeric or dimeric compound, except (−)-EC, was detectable in the urine samples of the probands. This is consistent with the findings of other authors, who have identified (−)-EC in urine after the consumption of cocoa (289 mg flavan-3-ols in total) [58]. Other research groups have not identified any flavan-3-ols in samples of human urine after the consumption of cider [46] black tea [60] or chocolate [63]. From these results, it could be postulated that monomeric, dimeric, and oligomeric flavan-3-ols are degraded to hydroxylated phenylacetic acids or phenylvalerolactones by colonic microflora [64, 65].

The overall results obtained in this study are summarized in Table 4. The findings relating to the absorption, metabolism, and systemic availability of polyphenols in vivo should contribute to our understanding of their biological effects, and the characterization of newly formed metabolites should facilitate further studies.

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