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Polyphenols Are Intensively Metabolized in the Human Gastrointestinal Tract after Apple Juice Consumption

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Polyphenols are secondary plant compounds showing anticarcinogenic effects both in vitro and in animal experiments and may thus reduce the risk of colorectal cancer in man. The identification of polyphenol metabolites formed via their passage through the small intestine of healthy ileostomy subjects after apple juice consumption is presented. Identification and quantification of polyphenols and their metabolites were performed using HPLC-DAD as well as HPLC-ESI-MS/MS. Total procyanidin content (TPA) was measured, and additionally the mean degree of polymerization (DP_m) of the procyanidins was determined in the apple juce and ileostomy effluents. As products of polyphenol metabolism, D-(−)-quinic acid and methyl esters of caffeic acid and *p*-coumaric acid are liberated from the corresponding hydroxycinnamic acid esters. 1-Caffeoylquinic acid and 3-caffeoylquinic acid were determined as products of isomerization. Phloretin 2′-*O*-glucoside (phloridzin) and phloretin 2′-*O*-xyloglucoside were metabolized into the corresponding aglycons phloretin and phloretin 2′-*O*-glucuronide and all were found in the ileostomy effluent. Ninety percent of the consumed procyanidins were recovered in the ileostomy effluent and therefore would reach the colon under physiologic circumstances. The DP_m was reduced (DP_m of apple juice = 5.7) and varied depending on the time point of excretion. The gastrointestinal passage seems to play an important role in the colonic availability of apple polyphenols.

KEYWORDS: Apple juice; phloretin glucuronide; caffeoylquinic acids; quinic acid; procyanidins; hydroxycinnamic acids; ileostomy; metabolism; small intestine; colon

INTRODUCTION

Flavonoids and hydroxycinnamates are widespread phenolic secondary plant metabolites. The daily flavonoid intake has been estimated to range between 0.15 and 1 g of total phenols (*1, 2*). Dietary polyphenols have received considerable interest because of their possible role in the prevention of various degenerative diseases (*3, 4*). They have shown antioxidant activities in vitro (*5, 6*) and protective effects in diseases associated with oxidative stress, such as cardiovascular diseases, cancer, inflammation, and others (*7–9*). The extent of their protective effect in vivo depends on their bioavailability and metabolism (*10, 11*).

Apples, *Malus domestica* L., are an important source of polyphenols (*1*). Several classes of phenolics are present such as hydroxycinnamates, flavan-3-ols, flavonols, dihydrochalcones, and anthocyanins depending on the apple variety (*11–13*). Other important apple phenolics are the hydroxycinnamate ester chlorogenic acid (5-caffeoylquinic acid), flavan-3-ol oligomers, that is, procyanidins, and dihydrochalcones such as phloridzin (phloretin 2′-*O*-glucoside) and phloretin 2′-*O*-xyloglucoside. Found in minor amounts are quercetin glycosides such as quercetin 3-*O*-glucoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-arabinoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-rhamnoside, and quercetin 3-*O*-rutinoside as well as anthocyanins such as cyanidin 3-*O*-galactoside, which are found in the skin of certain red apple cultivars (*14, 15*). The polyphenol profiles of apples are influenced by different factors such as variety, storage, and climatic factors as well (*16*).

Apple juice, in particular, has shown protective effects on low-density lipoprotein oxidation in humans in vitro (*17*). Breinholt et al. (*18*) have demonstrated an increase of antioxidant capacity in rat plasma by apple polyphenols. The increase in plasma phenolic concentrations after the consumption of apple cider was reported by DuPont et al. (*19*). Dose-dependent

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antiproliferative activities in colon as well as liver cancer cells have been observed after administration of extracts made from fresh apples (*20*). Recently, Gossé and co-workers (*21*) as well as Barth et al. (*22*) have demonstrated the reduction of aberrant crypt foci in the rat colon carcinogenesis model by the intervention with apple-derived procyanidins or cloudy apple juice. Therefore, it is important how many and especially in what form apple polyphenols reach the colon. Only a few studies have investigated the availability of food-derived polyphenols in the colon of humans so far (*23–26*). Data on the identification and quantification of major polyphenols from apples in the ileostomy fluids were published previously (*25, 26*). In this study (*25, 26*) subjects with a terminal ileostomy consumed 1 L of cloudy apple juice. Ileostomy bags were removed 0, 1, 2, 4, 6, and 8 h after juice consumption. Polyphenols present in apple juice, namely, chlorogenic acid, 4-caffeoylquinic acid, 3-*p*-coumaroylquinic acid, 4-*p*-coumaroylquinic acid, 5-*p*-coumaroylquinic acid, (–)-epicatechin, quercetin 3-*O*-arabinoside, quercetin 3-*O*-rhamnoside, and phloretin 2′-*O*-xyloglucoside, were determined.

In the current study new metabolites of hydroxycinnamic acids and dehydrochalcones were characterized and quantified in the ileostomy effluent. Additionally, procyanidin amounts and degrees of polymerization were measured in ileostomy contents.

MATERIALS AND METHODS

Chemicals. All chemicals and solvents were of analytical grade. Solvents were redistilled before use. Acetonitrile (Lichrosolv) and dimethyl sulfoxide-d_6 (DMSO-d_6, 99.8% D) were from Merck (Darmstadt, Germany); formic acid, 1,4-dithio-DL-threitol (DTT), trichloroacetic acid (TCA), and D-(−)-quinic acid were purchased from Fluka (Deisenhofen, Germany); and methyl *p*-coumarate was purchased from Apin Chemicals Ltd. (Abingdon, U.K.). Phloretin (4,2′,4′,6′,-tetrahydroxydihydrochalcone), quercetin 3-*O*-glucoside (isoquercitrin), and quercetin 3-*O*-galactoside (hyperoside) as well as (+)-catechin, (−)-epicatechin, and Tris-HCl buffer solution were from Roth (Karlsruhe, Germany). 1-Caffeoylquinic acid was kindly provided by Michael Sefkow (Leipzig, Germany). The standard 3,4,5-trimethoxycinnamic acid, phloridzin (2′-*O*-glucosyl-4,6′,4′-trihydroxydihydrochalcone), quercetin (3,5,7,3′,4′-pentahydroxyflavone), quercetin 3-*O*-rutinoside (rutin), quercetin 3-*O*-rhamnoside (quercitrin), butanol, and methanol-d_3 (99.8% D) were acquired from Sigma (Steinheim, Germany). α-Toluene thiol, β-glucuronidase (EC 3.1.6.1), and sulfatase (EC 3.2.1.31) from *Helix pomatia* (type H-1) as well as uridine 5′-diphosphoglucuronic acid (UDPGA) were purchased from Aldrich (Steinheim, Germany). Human recombinant UDP-glucuronosyl transferase (UGT 1A9) was from BD Bioscience (Heidelberg, Germany). D_2O (99.97% D) was from Eurisotop (Gif sur Yvette, France). Quercetin 3-*O*-xyloside (reynoutrine), quercetin 3-*O*-arabinoside (avicularin), and phloretin 2′-*O*-xyloglucoside as well as procyanidins B_1 and B_2 were kindly provided by Prof. Dr. H. Becker (Saarbrücken, Germany) and Prof. Dr. P. Winterhalter (Braunschweig, Germany).

Subjects and Study Design. Information on the performance of the ileostomy study and data on the preparation of ileostomy bags has been published previously (*25, 26*). The study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. In brief, 11 healthy ileostomy subjects consumed 1 L of cloudy apple juice (Hofmann, Nüdlingen, Germany; produced mainly from cider apples). Ileostomy bags were removed 0, 1, 2, 4, 6, and 8 h after juice consumption and submitted to freeze-drying. No artifact formation of the polyphenols under study was observed by this procedure (data not shown).

Preparation of Ileostomy Fluids. Freeze-dried aliquots of the ileostomy bag contents (*25, 26*) (range of 0.5–2 mg) were extracted three times with 20 mL of methanol as published before (*23, 24*). For the quantification of D-(−)-quinic acid extraction was performed with water/methanol (70:30, v/v). The pooled solvent was evaporated at 37 °C, and the extract was dissolved in 2 mL of methanol. For the determination of recovery of the sample preparation, reference compounds were added to a polyphenol-free extract (sample removed directly before apple juice intake; 0 h, blind value). Sample preparations were performed as described above. Recoveries of new metabolites ranged between 93 and 104%.

Synthesis and Isolation of 3-Caffeoylquinic Acid. 3-Caffeoylquinic acid, the isomer of chlorogenic acid (5-caffeoylquinic acid) was produced by incubation of an aqueous solution of 5-caffeoylquinic acid (pH 8) at 100 °C for 30 min (*27*). The product was separated from 4- and 5-caffeoylquinic acid by preparative RP-HPLC. Preparative separation of 3-caffeoylquinic acid was carried out on a Knauer HPLC gradient pump and a Knauer UV−vis spectral photometer (Berlin, Germany) at 326 nm. A Eurospher-100 C_{18} column, 250 × 8 mm, with 5 μm particle size (Knauer, Berlin, Germany) was used. The mobile phase consisted of aqueous 0.1% formic acid (A) (v/v) and acetonitrile (B). The gradient applied was 5–50% B in 40 min at a flow rate of 12 mL/min. The injection volume was 0.5 mL. 3-Caffeoylquinic acid was obtained as a pale yellow powder with a purity of 95% by HPLC-DAD; mp 204–206 °C. ^{1}H NMR data were consistent with previously published data (*28*).

Synthesis and Isolation of Methyl Caffeate. In the presence of catalytic amounts of sulfuric acid, caffeic acid and methanol were heated under reflux for 10 h. Methanol was evaporated at 37 °C. The residue was dissolved in ethyl acetate (EtOAc), washed with water, and dried over anhydrous $MgSO_4$. Purification of methyl caffeate was performed by column chromatography on silica gel (EtOAc/*n*-hexane, 1:2, v/v). The ester was obtained as a white solid of 99% purity by HPLC−diode array detection (HPLC-DAD); mp 151 – 152 °C. ^{1}H NMR data were consistent with those previously reported (*29*).

Synthesis and Characterization of Phloretin 2′-*O*-β-Glucuronide. One millimolar UDPGA (3.23 mg), 50 μM phloretin (0.25 mL) (from a 1 mM stock solution of 2.743 mg/10 mL DMSO), 20 mM $MgCl_2$ (9.53 mg), and 1 mM DTT (0.772 mg) were dissolved in 50 mM Tris-HCl buffer (end volume = 5 mL at pH 7.4). An aliquot of 900 μL was preincubated for 5 min, and then 100 μL of UGT 1A9 (0.5 mg/mL) was added (end volume = 1 mL), and the solution was incubated at 37 °C for 2 h. The reaction was stopped by the addition of 20% TCA (25 μL) (*30*). HPLC-ESI-MS/MS analysis showed two peaks; each with a deprotonated ion of $[M - H]^-$ *m/z* 449 (both with a product ion of *m/z* 273) at retention times of 14.1 and 14.7 min. The retention time of the first peak at 14.1 min was identical to that of the phloretin glucuronide in the ileostomy samples under study. This peak at 14.1 min was preparatively collected by analytical HPLC (see below), and ^{1}H NMR measurement of the compound confirmed C-2′ as the position of glucuronidation. Phloretin 2′-*O*-β-glucuronide was identified on a Bruker Avance 600 MHz NMR instrument at ambient temperature in 0.4 mL of DMSO-d_6 and additionally in 0.4 mL of DMSO with 2 μL of deuterated water (D_2O). Chemical shift was referenced to a DMSO solvent signal. ^{1}H NMR (600 MHz, DMSO-d_6): δ 2.77 (t, J = 7.3 Hz, β-CH_2), 3.21–3.63 (m, glucuronide-H, α-CH_2), 4.56, 5.02, 5.16 (3 × glucuronide-OH), 4.83 ($H^{1''}$), 5.88 (br, H-5′), 6.09 (br, H-3′), 6.62 (d, J = 8.4 Hz, H-3, H-5), 7.02 (d, J = 8.4 Hz, H-2, H-6), 9.08 (OH-4), 10.67 (br, OH-4′), 13.59 (OH-6′). ^{1}H NMR (600 MHz, DMSO-d_6 + D_2O): δ 2.77 (t, J = 7.3 Hz, β-CH_2), 3.21–3.63 (m, glucuronide-H, α-CH_2), 4.83 (d, J = 7.0 Hz, $H^{1''}$), 5.88 (br, H-5′), 6.14 (br, H-3′), 6.62 (d, J = 8.4 Hz, H-3, H-5), 7.02 (d, J = 8.1 Hz, H-2, H-6). NMR data were analyzed by Bruker 1D WINNMR software.

HPLC−Diode Array Detection (DAD) Analysis. The HPLC system used was a Hewlett-Packard 1100 HPLC gradient pump and a Hewlett-Packard 1100 photodiode array detector (Waldbronn, Germany), equipped with a Wisp 712b autosampler (Waters, Eschborn, Germany). Data acquisition and analysis were performed with Hewlett-Packard ChemStation software. A Hypersil Gold C_{18} column, 100 × 4.6 mm, with 3 μm particle size (Thermo, Runcorn, U.K.) was used. The mobile phase consisted of aqueous 0.1% (v/v) formic acid (A) and acetonitrile (B). The gradient applied was 1–99% B in 40 min at a flow rate of 1 mL/min, and a 25 μL injection volume was used. The peaks were identified by comparison of the retention time and UV spectra (200−600 nm) with authentic references [for limit of detection (LOD) and limit of quantification (LOQ) see ref *12*]. Phloretin 2′-*O*-β-glucuronide was determined at 280 nm, and hydroxycinnamic acid

derivatives were determined at 320 nm. Calibration curves (at the appropriate wavelengths according to the absorption maximum of the compounds) were used for quantification. Polyphenols were quantified by means of calibration curves [peak area divided by internal standard area versus quotient of polyphenol and the internal standard concentration (*31*)].

HPLC-ESI-MS(MS) Analysis. HPLC-ESI-MS/MS was performed with a TSQ 7000 tandem mass spectrometer system equipped with an ESI interface (Finnigan MAT, Bremen, Germany) and an Applied Biosystems 140b pump (BAI, Bensheim, Germany). Data acquisition and analysis were conducted using Xcalibur Qual Browser Software 1.2/1.3 (Thermo Electron Corp., Dreieich, Germany). HPLC chromatographic separations were carried out on a Hypersil Gold C_{18} column, 100 × 2.1 mm, with 3 μm particle size (Thermo). The mobile phase consisted of aqueous 0.1% formic acid (A) (v/v) and acetonitrile (B). The gradient applied was 5–99% B in 40 min at a flow rate of 0.2 mL/min, and 10 μL injection volume was used. The analysis was performed in the negative ionization mode. The spray capillary voltage was set to 3.2 kV, and the temperature of the heated capillary was 200 °C. Nitrogen served both as sheath (70 psi) and as auxiliary gas (10 L/min). For verification of compounds, the mass spectrometer was operated in the full-scan mode, *m/z* 150–700, with a total scan duration of 1.0 s and a dwell time of 2 ms. MS/MS experiments were performed at a collision energy of 20–40 eV, with argon (2.0 mTorr) serving as collision gas. The obtained deprotonated ions and product ion mass spectra were compared to those of references measured before (*12*).

D-(−)-Quinic acid quantification was performed in the negative ionization mode using selected reaction monitoring (SRM) mode with 3,4,5-trimethoxycinnamic acid as a standard. For SRM measurement of D-(−)-quinic acid the deprotonated ion ($[M - H]^-$) in Q1 was *m/z* 191 and the characteristic product ion determined in Q3 was *m/z* 85, at a collision energy of 30 eV and 266 mPa argon as collision cell pressure, with a total scan duration of 1.0 s and a dwell time of 2 ms. For 3,4,5-trimethoxycinnamic acid the precursor ion of *m/z* 237 and the product ion of *m/z* 103 (20 eV, 266 mPa) were used. LOQ was 0.2 μg/L, and LOD was 0.09 μg/L.

Headspace Solid Phase Microextraction GC-MS (HS-SPME-GC-MS) Analysis of Methanol. Samples of 13 mL of apple juice spiked with 1.25 μg of methanol-d_3 and 6 g of $(NH_4)_2SO_4$ were equilibrated at 70 °C in an aluminum block heater for 15 min. HS-SPME sampling was performed using a 65 μm fiber coated with carbowax/divinylbenzene (Supelco, Steinheim, Germany). A desorption time of 1 min was used. An HP Agilent 6890 series gas chromatograph with split injection (1:20) was directly coupled to an HP Agilent 5973 network mass spectrometer (Agilent Technologies Inc.) using a DB-Wax fused silica capillary column (J&W, Agilent, Waldbronn, Germany) (30 m × 0.25 mm i.d.; d_f = 0.25 μm) and helium (1.0 mL/min) as carrier gas. The temperature program was 10 min isothermal at 30 °C and then raised at 25 °C/min to 240 °C. The injector and interface temperatures were 220 °C. Mass-selective detection (70 eV, electron impact) was performed in the scan mode (10–50 amu). Identification of methanol in apple juice was performed by comparison of linear retention indices and mass spectral data of sample constituent with that of authentic reference compounds. Methanol concentrations were calculated by plotting the peak area ratios (normal to deuterated forms) for base ion pairs (*m/z* 32/35$_d$) over the indicated range of concentration ratios.

Quantification of Apple Juice and Ileostomy Fluids. Data on the quantification of apple polyphenols by HPLC-DAD in apple juice as well as in ileostomy bags were published previously (*12, 25*).

***β*-Glucuronidase/Sulfatase Treatment of Ileostomy Fluids.** Redissolved ileostomy samples (3 mL) were treated with β-glucuronidase (50 μL, 250 units) or sulfatase (50 μL, 50 units) in sodium acetate buffer (NaAc buffer, pH 5) or without (control) enzymes at 37 °C for 2 h. The reaction was stopped by the addition of methanol (3 mL), and the mixtures were stored at −24 °C (*32*) until lyophilization, extraction, and analysis as described for ileostomy sample preparation using a Waters Symmetry C_{18} column (150 × 3.9 mm, 5 μm; Eschborn, Germany).

Acid Butanol Assay (Total Proanthocyanidin Content, TPA). TPA was analyzed similarly to that previously described (*33*). Seventy-five microliters of apple juice or ileostomy extract solution (2 mg/mL methanol) (preparation of apple juice extract; see ref *34*) was added to 1.4 mL of a solution of 1-butanol/hydrochloric acid (95:5, v/v). The reaction mixture was heated at 95 °C for 2 h and then cooled to room temperature. Absorption was measured at 555 nm on a Spectronic GenesysTM 2PC UV−vis spectrophotometer (Spectronic Instruments, Rochester, NY). TPA content was quantified using a purified standard of procyanidins (a mixture of different tetramers of B-procyanidins) from tamarind seeds, isolated preparatively according to the method in ref *35*.

Thiolysis of Procyanidins (Mean Degree of Polymerization, DP_m). The performed thiolysis method was modified after that in ref *36*. Briefly, in a 1.5 mL vial (CZT, Kriftel, Germany) 200 μL of a solution of apple juice extract or ileostomy extract (2 mg/mL methanol) was mixed with 400 μL of α-toluene thiol solution (5% in methanol, v/v), acidified with concentrated HCl (1.1%, v/v), and sealed with an inert Teflon cap (CZT). The reaction was carried out at 40 °C for 30 min to ensure complete degradation of the polymers. Reaction mixtures were cooled and stored in a freezer to minimize epimerization and any side reactions prior HPLC analysis. Thiolysis samples were analyzed on a Waters SymmetryC_{18} column (150 × 3.9 mm, 5 μm; Eschborn, Germany), the gradient applied was 1–40% B in 40 min at a flow rate of 1 mL/min, and an injection volume of 50 μL was used. The DP_m was calculated from the ratio of all flavan-3-ol units (thioether adducts and terminal units) to (+)-catechin and (−)-epicatechin.

RESULTS

Identification of Caffeic and *p*-Coumaric Acid Ester. Neither unbound caffeic nor *p*-coumaric acids released from caffeoyl- and *p*-coumaroylquinic acid were detected in the ileostomy fluids, but in 9 of 11 subjects their esterified forms were determined. To exclude methyl ester formation via sample preparation, aliquots of ileostomy extracts were extracted with water and with ethanol. Only the methyl esters (no free acids or ethyl esters) were identified after this sample preparation. For this reason, the methanol content of the apple juice consumed by the volunteers was measured by stable isotope dilution analysis (SIDA) with headspace SPME GC-MS. In the 11 samples 23.5 ± 2.16 mg/L methanol was determined.

Identification of Phloretin 2′-*O*-*β*-Glucuronide. The aglycon phloretin was identified in ileostomy bag contents, and additionally HPLC-ESI-MS/MS measurement indicated a glucuronide of phloretin. To confirm the presence of a glucuronide, β-glucuronidase treatment of ileostomy extract was carried out and resulted in the disappearance of the phloretin glucuronide peak and an increase of the corresponding phloretin peak. In contrast, sulfatase treatment showed no change in the HPLC-DAD profile of ileostomy fluid (**Figure 1**). The incubation of the phloretin with UDP-glucuronosyl transferase (UGT) 1A9 revealed the formation of two phloretin glucuronides. The retention time of the first peak at 14.1 min was identical to that of the phloretin glucuronide in the ileostomy samples under study. The phloretin glucuronide showed a characteristic UV spectrum, a deprotonated ion of *m/z* 449, and a characteristic product ion of *m/z* 273 identical to that of a UGT conjugate. ^{1}H NMR analysis of the preparatively isolated compound had shown that phloretin 2′-*O*-β-glucuronide was formed. To achieve a distinct structural identification, the replaceable H-atoms of the substance were exchanged by adding 2 μL of D_2O. A complete assignment of all protons was given.

Identification and Quantification of D-(−)-Quinic Acid. D-(−)-Quinic acid was identified by HPLC-ESI-MS and HPLC-ESI-MS/MS analysis of D-(−)-quinic acid reference in the negative mode, revealing the characteristic precursor ion of *m/z* 190.6 at 2.0 min and product ion spectra (30 eV) with *m/z* 84.7, *m/z* 92.8 ($[M - H - HCOOH - 3OH]^-$) and *m/z* 126.6 ($[M - 4O - H]^-$) as shown by Ng et al. (*37*). For quantification the

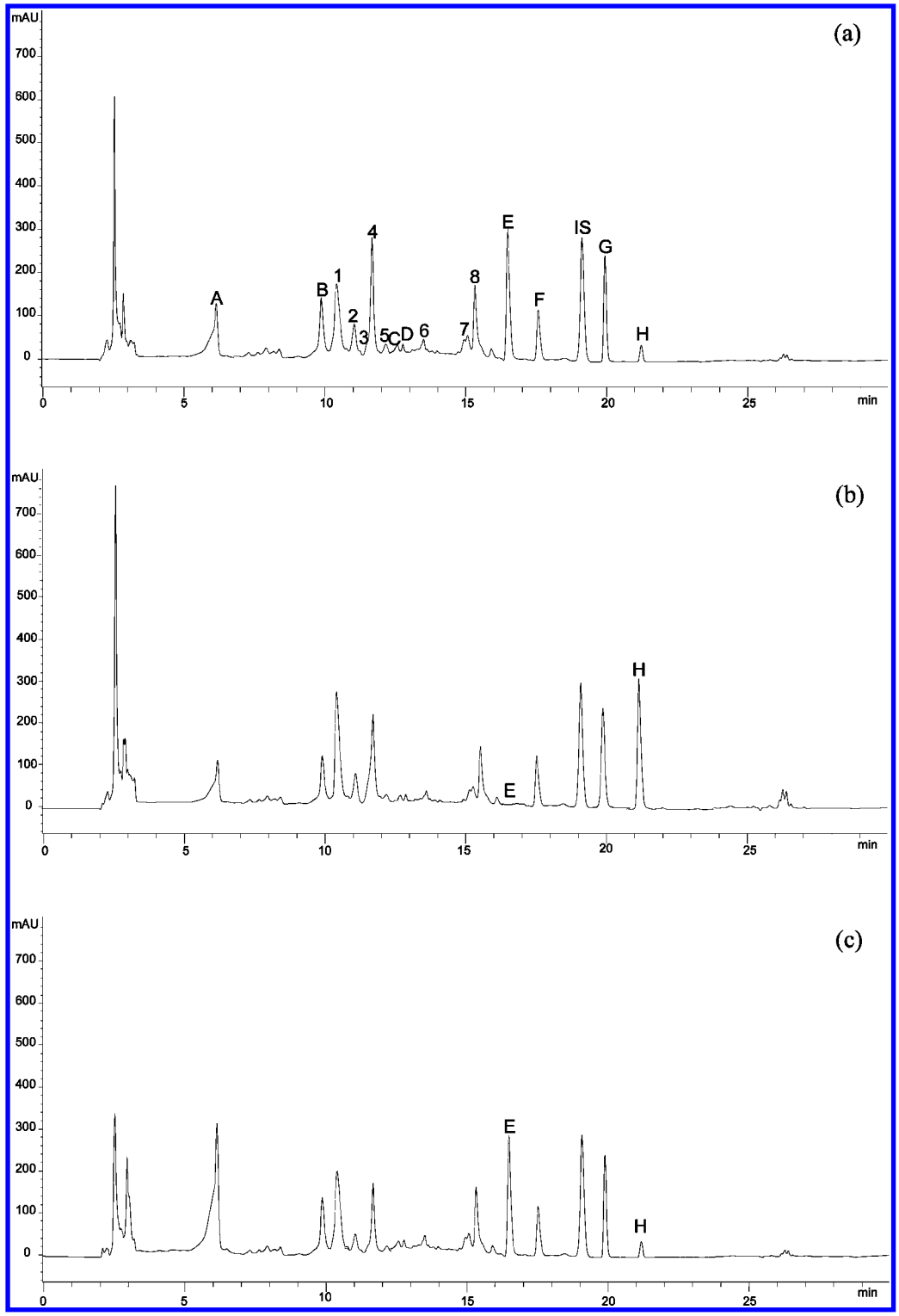

Figure 1. High-performance liquid chromatography−diode array (HPLC-DAD) chromatograms (280 nm) of an extract of ileostomy fluid 2 h after apple juice consumption before (**a**) and after enzymatic hydrolysis by β-glucuronidase (**b**) and sulfatase (**c**). Peaks: 1, 5-caffeoylquinic acid; 2, 4-caffeoylquinic acid; 3, 3-*p*-coumaroylquinic acid; 4, 4-*p*-coumaroylquinic acid; 5, 5-*p*-coumaroylquinic acid; 6, quercetin 3-*O*-arabinoside; 7, quercetin 3-*O*-rhamnoside; 8, phloretin 2′-*O*-xyloglucoside; A, 3-caffeoylquinic acid; B, 1-caffeoylquinic acid; C, D, unidentified ileal substances; E, phloretin 2′-*O*-β-glucuronide; F, methyl caffeate; G, methyl *p*-coumarate; H, phloretin; IS, internal standard (3,4,5-trimethoxycinnamic acid). For details see Materials and Methods.

characteristic precursor ion *m*/*z* 190.6 and the product ion of *m*/*z* 84.7 were used in the selected reaction monitoring (SRM) mode. Here, amounts of 67.6 ± 8.08 μmol D-(−)-quinic acid were determined in the ileostomy bags of the 11 volunteers under study.

Recovery of Apple Juice Polyphenols in Ileostomy Fluid. Spectroscopic data (retention time, UV maximum, and HPLC-ESI-MS/MS fragmentation patterns) of new polyphenol metabolites in ileostomy fluid under study are summarized in **Table 1**, and the structures of the new metabolites are presented in **Figure 2**. All substances under study were quantified by HPLC-DAD measurements at their appropriate wavelength using calibration curves generated with authentic reference compounds. D-(−)-Quinic acid was determined by HPLC-MS/MS

Table 1. Spectroscopic Data (Retention Time, UV Maximum, and HPLC-ESI-MS/MS Fragmentation Patterns) of New Polyphenol Metabolites in Ileostomy Fluid under Study (For Details See Materials and Methods)

	t_R[a] (min)	λ_{max}[a] (nm)	$[M - H]^-$ (*m/z*)	MS^2 (*m/z* (relative abundance, %))	eV
D-(−)-quinic acid	2.0	ND[b]	190.6	84.7 (100); 92.8 (35); 126.6 (13)	30
3-caffeolyquinic acid	7.4	326	353.0	190.8 (100); 178.8 (58); 134.8 (7)	20
1-caffeoylquinic acid	8.3	326	353.0	190.8 (100); 178.8 (10)	20
phloretin 2′-*O*-*β*-glucuronide	13.2	284	449.0	272.9 (100)	20
methyl caffeate	14.2	324	192.7	133.3 (100); 160.7 (35)	35
methyl *p*-coumarate	16.6	311	176.8	116.9 (100); 117.2 (35)	30
phloretin	18.2	284	273.1	167.0 (100)	35

[a] Retention time (t_R) and wavelength (λ_{max}) determined by HPLC-DAD. [b] ND, not determinable.

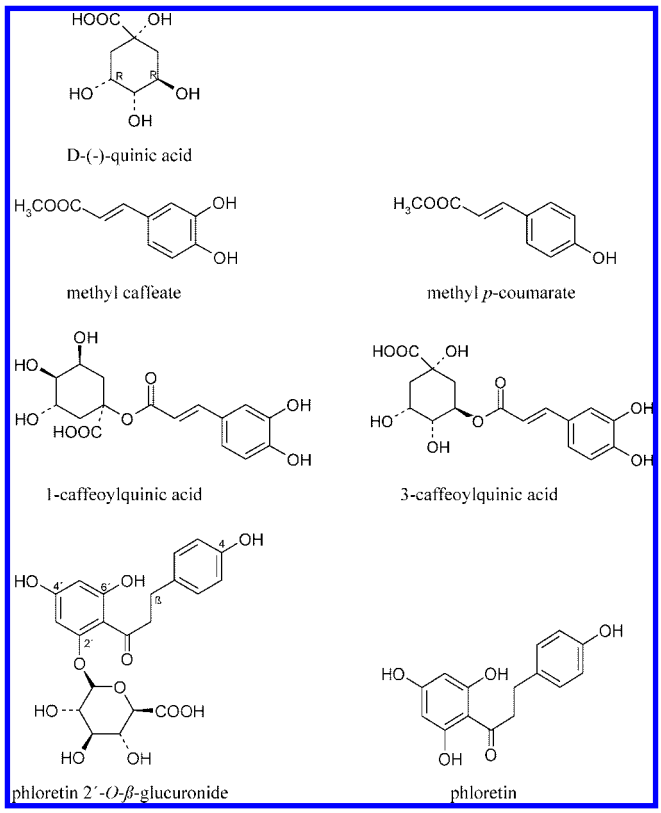

Figure 2. Chemical structures of the metabolites found in the ileostomy fluids.

measurements. The total amounts are given in **Table 2**. In **Figure 3** the time courses of methyl caffeate, methyl *p*-coumarate, 3-caffeoylquinic acid, 1-caffeoylquinic acid, D-(−)-quinic acid, phloretin 2′-*O*-*β*-glucuronide, and phloretin occurring in the ileostomy fluids of all 11 subjects in a period of 8 h are shown. None of the substances were present at 0 h. Maximum amounts of all compounds emerge in the ileostomy bags at 2 h. Both methyl esters, methyl caffeate and methyl *p*-coumarate, determined in the ileostomy bags had passed the small intestine completely 8 h after apple juice consumption, whereas small amounts of phloretin (0.06 ± 0.13 *μ*mol), phloretin 2′-*O*-*β*-glucuronide (0.03 ± 0.07 *μ*mol), 3-caffeoylquinic acid (0.04 ± 0.11 *μ*mol), and 1-caffeoylquinic acid (0.01 ± 0.03 *μ*mol) and high amounts of D-(−)-quinic acid (0.62 ± 1.41 *μ*mol) were still being excreted. In 2 of 11 ileostomy samples no methyl esters could be detected. One volunteer showed maximum polyphenol excretion at 4 h, whereas all other subjects under study showed a maximum at 2 h.

Total amounts of polyphenols determined in 1 L of the cloudy apple juice in relation to the polyphenol amounts detected in ileostomy fluids (micromoles) are shown in **Table 2**. In

Table 2. Polyphenol Content of Cloudy Apple Juice and Polyphenol Amounts Detected in Ileostomy Fluids after Cloudy Apple Juice Consumption[a]

	apple juice (mg/L)	apple juice (*μ*mol)	total excretion (*μ*mol)
1-caffeoylquinic acid	ND[b]	ND	16.5 ± 2.4
3-caffeoylquinic acid	ND	ND	33.7 ± 8.2
4-caffeoylquinic acid	14.1 ± 2.0	39.8 ± 5.6	9.2 ± 3.0
5-caffeoylquinic acid	112.8 ± 3.5	318.4 ± 9.9	32.5 ± 7.7
caffeic acid	5.3 ± 1.7	29.4 ± 9.4	ND
methyl caffeate	ND	ND	11.9 ± 8.5
3-*p*-coumaroylquinic acid	7.1 ± 0.4	21.0 ± 1.2	6.4 ± 0.45
4-*p*-coumaroylquinic acid	18.4 ± 0.3	54.4 ± 0.89	9.9 ± 0.95
5-*p*-coumaroylquinic acid	4.2 ± 0.1	12.4 ± 0.30	1.4 ± 0.33
methyl *p*-coumarate	ND	ND	12.3 ± 8.8
D-(−)-quinic acid	ND	ND	67.6 ± 8.1
phloretin 2′-*O*-xyloglucoside	36.9 ± 1.8	64.9 ± 3.2	12.7 ± 0.90
phloretin 2′-*O*-glucoside	7.1 ± 0.7	16.3 ± 1.6	ND
phloretin 2′-*O*-*β*-glucuronide	ND	ND	5.6 ± 1.2
phloretin	ND	ND	1.8 ± 0.86
(+)-catechin	3.0 ± 0.5	10.3 ± 1.7	ND
(−)-epicatechin	15.0 ± 2.1	51.7 ± 7.2	8.8 ± 1.7
procyanidin B_1	5.3 ± 1.6	9.2 ± 2.8	ND
procyanidin B_2	9.9 ± 0.3	17.1 ± 0.52	ND
procyanidin B_3	ND	ND	ND
procyanidin B_4	ND	ND	ND
oligomeric procyanidins	157.0 ± 2.6	97.9 ± 1.6	88.4 ± 4.5
quercetin 3-*O*-glucoside	1.8 ± 0.2	3.9 ± 0.43	ND
quercetin 3-*O*-galactoside	1.5 ± 0.3	3.2 ± 0.65	ND
quercetin 3-*O*-xyloside	3.9 ± 0.1	9.0 ± 0.24	ND
quercetin 3-*O*-arabinoside	0.9 ± 0.1	2.1 ± 0.24	0.06 ± 0.1
quercetin 3-*O*-rhamnoside	2.7 ± 0.3	6.0 ± 0.67	0.63 ± 0.3
quercetin	ND	ND	0.03 ± 0.1
anthocyanins	ND	ND	ND
total polyphenol amount	406.9	767.0	319.5

[a] Values are means of all time points with their standard deviation (SD); $n = 11$. [b] ND, not determined.

ileostomy fluid under study 41.7% of polyphenols were recovered, whereas 58.3% seemed to be absorbed or degraded. The recovery of caffeoylquinic acids from apple juice was 26.8%, calculated as the sum of 1-, 3-, 4-, and 5-caffeoylquinic acid as well as free caffeic acid and its methyl ester. Recovery of *p*-coumaroylquinic acids was 34.2%, calculated as the sum of 3-, 4-, and 5-*p*-coumaroylquinic acid as well as methyl *p*-coumarate. Considering that D-(−)-quinic acid arose as a metabolite of hydroxycinnamates, its recovery was 15.2%. For the two dihydrochalcone derivatives in apple juice, namely, phloretin 2′-*O*-glucoside and phloretin 2′-*O*-xyloglucoside, in the ileostomy effluents phloretin 2′-*O*-*β*-glucuronide and its aglycon phloretin were measured within a total phloretin recovery of 24.8%. No phloretin 2′-*O*-glucoside was determined in the ileostomy samples. In general, a total of 10.0% of the monomeric and dimeric flavan-3-ols was recovered as (−)-

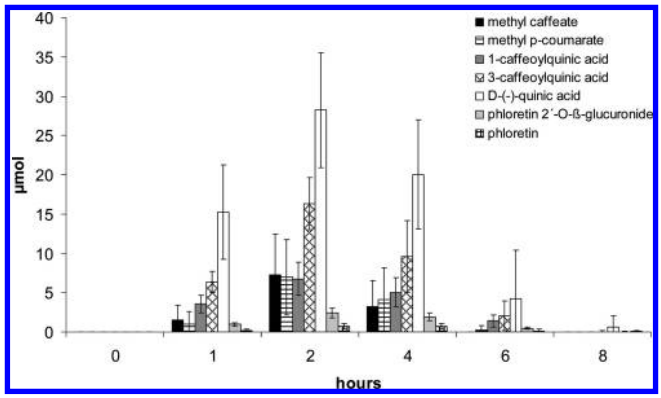

Figure 3. Time course of excretion (0−8 h) of the metabolites (micromoles) methyl caffeate, methyl *p*-coumarate, 1-caffeoylquinic acid, 3-caffeoylquinic acid, D-(−)-quinic acid, phloretin 2′-*O*-glucuronide, and phloretin in the ileostomy fluids of subjects ($n = 11$) under study. Data are expressed as means with their standard deviation (SD) of triplicate subsamples.

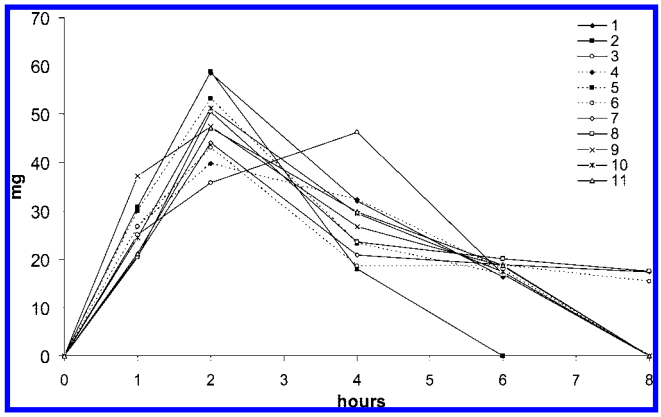

Figure 4. Total procyanidin content (TPA) of ileostomy samples ($n = 11$) excreted within 8 h (milligrams per bag). The amount in 1 L of apple juice was 157.0 mg. Values are means with their standard deviation (SD) of triplicates.

epicatechin in ileostomy fluid. Quercetin and quercetin glycosides showed the highest absorption and appeared with a recovery of 3.0%.

Total Procyanidin Content (TPA). The acid butanol assay is based on the hydrochloric acid catalyzed depolymerization of procyanidins in butanol to yield colored anthocyanidins, which are measured spectrophotometrically (*33*). As a procyanidin standard, a mixture of different tetramers of B-procyanidins isolated from tamarind seeds (*Tamarindus indica*) were used (for details see ref *34*). The total procyanidin content of the cloudy apple juice used for the study was determined to be 157.0 mg/L. Time courses of the total apple juice procyanidins occurring in the ileostomy fluids for all 11 volunteers in a period of 8 h are shown in **Figure 4**. Maximum amounts emerge in the ileostomy bag at 2 h. Only one volunteer showed maximum procyanidin excretion at 4 h. For 8 of 11 probands the procyanidins had passed the small intestine completely 8 h after apple juice consumption.

Determination of the Mean Degree of Polymerization (DP_m) of Procyanidins. Solutions of ileostomy extracts (0, 1, 2, 4, 6, and 8 h samples of 11 subjects) as well as the apple juice extract (preparation of apple juice extract see ref *34*) were converted with α-toluene thiol to the corresponding benzylthioethers. HPLC-DAD analysis was performed to characterize the formed procyanidins. The cloudy apple juice extract showed a DP_m of 5.7 ± 0.8. As expected, in all ileostomy samples at

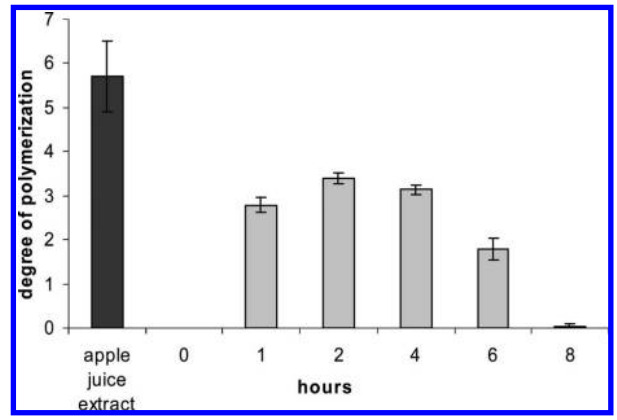

Figure 5. Mean degree of polymerization (DP_m) of procyanidins in apple juice and ileostomy samples under study ($n = 11$) within 8 h after apple juice consumption. Values are means with their standard deviation (SD) of triplicates.

0 h, neither (+)-catechin, (−)-epicatechin, nor their benzyl thioethers could be detected and, therefore, no DP_m could be determined. In contrast, DP_m of the ileostomy samples 2 h after the consumption of apple juice when maximum procyanidin excretion was observed revealed an average DP_m of 3.4 ± 0.13 (from 2.9 ± 0.15 to 4.0 ± 0.35) (**Figure 5**). Thus, the DP_m of the apple juice procyanidins was reduced during the passage through the gastrointestinal tract and into the ileostomy fluid. The DP_m was highest (3.4 ± 0.13) 2 h after juice intake, whereas it was reduced (1.78 ± 0.25) 6 h after the intake.

DISCUSSION

The polyphenols that are the most common in the human diet are not necessarily "the most active structures" within the body. They may either have a lower intrinsic activity or be poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. The question of colonic availability of orally consumed polyphenols and their metabolites can be studied in subjects who have undergone colectomy with terminal ileostomy. In this work, 11 ileostomy subjects consumed 1 L of a cloudy apple juice containing 407.0 mg/L (767.0 µmol) of total polyphenols. In our study, 41.7% (319.5 µmol) of the ingested phenolic substances and their corresponding metabolites were recovered in the ileostomy effluent.

Most of the polyphenols in the apple juice and the ileostomy effluent were of polymeric nature (*38, 39*). We have determined a total procyanidin amount of 157.0 mg/L in the apple juice used for our study. These findings are in good agreement with the literature data reporting procyanidin polymers as the predominant fraction in apple juices (*38, 39*). These procyanidins differ from most other plant polyphenols because of their polymeric nature and high molecular weight. Recent studies suggest that procyanidins have chemopreventive properties (*21, 40*), but only the low molecular weight oligomers (DP ≤ 3) are absorbed intact in the gastrointestinal tract (*41*). For the first time in our in vivo study data on the colonic availability of food-derived procyanidins in human subjects are given. In our study, 90.3% of the ingested oligomeric procyanidins were detected in the ileostomy fluids with a maximum DP_m of 3.4 after 2 h in comparison to a DP_m of 5.7 in the apple juice. Our findings suggest that oligomeric procyanidins were cleaved into smaller units and that minor parts were absorbed. In our recent observations no (+)-catechin and dimeric procyanidins (B_1 and B_2) were recovered in the ileostomy bags (*25*). This is in accordance with the findings of Déprez et al. (*41*) demonstrating

that (+)-catechin and procyanidin dimers and trimers were permeable through monolayers of Caco-2 human intestinal cell line. Data based on absorption and degradation of procyanidins are, however, somewhat inconsistent. In vitro experiments have shown that procyanidins from cocoa were cleaved into monomeric units during their passage through the gastrointestinal tract (small intestine) (*42, 43*). In contrast, Rios et al. (*44*) and Donovan et al. (*45*) have reported that most of the ingested procyanidins reach the small intestine intact. Manach et al. (*11*) have stated that procyanidins are poorly absorbed and that their action is therefore limited to the small and large intestine. Our data supported the fact that polymeric procyanidins ($DP_m > 2$) could reach the colon under physiological circumstances and may provide protective effects (*21*). Our results indicate a minor degradation of the oligomeric procyanidins. We suppose the release of dimeric procyanidins to be cleaved into monomers or eventually the absorbed in their intact forms.

Our data provide evidence for the isomerization of caffeoylquinic acids in the human gastrointestinal tract after the consumption of 5- or 4-caffeoylquinic acid. In apple juice, only 4- and 5-caffeoylquinic acids were determined, whereas in the ileostomy fluid, 1-caffeoylquinic acid and 3-caffeoylquinic acid were also identified. The isomerization of caffeoylquinic acids to a mixture of 3-, 4-, and 5-caffeoylquinic acid is well established in the literature (*24, 46–49*). These results are in good agreement with our data concerning the formation of 3-caffeoylquinic acid. Additionally, in our study, 1-caffeoylquinic acid was detected in the human small intestine. We suppose that this isomerization product is formed by esterase activity of the enterocytes (*32*).

One of the metabolites found in ileostomy fluid in high amounts was D-(−)-quinic acid, which occurs in the apple juice as caffeoyl- and *p*-coumaroylquinic acid esters. D-(−)-Quinic acid arose as a result of ester bond cleavage of caffeoylquinic or *p*-coumaroylquinic acid during passage through the upper intestinal tract. Various groups have reported that chlorogenic acid (5-caffeoylquinic acid) is stable during the passage through the small intestine and will be subsequently hydrolyzed by the gut microflora (*1, 49, 50*). Couteau et al. (*51*) reported that only a small number of bacterial species of the colonic microflora such as *Escherichia coli*, *Bifidobacterium lactis*, and *Lactobacillus gasseri* would be capable of hydrolyzing chlorogenic acid. An esterase activity identified in human colonic microflora that can hydrolyze chlorogenic acid has been demonstrated (*52*). The esterase activity can deesterify methyl esters of ferulic acids and diferulic acids, suggesting catabolism of hydroxycinnamates by feruloyl esterase (*53, 54*). Using an in situ perfusion model to perfuse chlorogenic acid into a segment of ileum and jejunum, the in vivo hydrolysis of chlorogenic acid in the gut mucosa was demonstrated (*55*). Lafay et al. (*47*) found traces of caffeic acid in the stomach and the small intestine after feeding rats a diet supplemented with chlorogenic acid. Recently, Azzini et al. (*57*) have reported the bioavailability of hydroxycinnamic acids in humans after the ingestion of cooked artichokes. On the other hand, data are available suggesting that cleavage of cinnamoyl esters via carboxylesterases can occur in the enterocytes of the small intestine (*32, 57*). Our findings support the assumption that in the small intestine, esterase activity catalyzes the liberation of D-(−)-quinic acid by hydrolysis of 5- and 4-caffeoylquinic acids or *p*-coumaroylquinic acids.

With the use of ileostomy subjects, it can be shown that hydroxycinnamic acid esters of D-(−)-quinic acid were cleaved during their passage through the small intestine. In fact, 15.2% of the originally esterified D-(−)-quinic acid was measured as free D-(−)-quinic acid and 24.4% occurred in the ileal fluid as hydroxycinnamic esters. This means that high amounts of D-(−)-quinic acid could have been absorbed or lost in the small intestine and might be excreted as hippuric acid as shown before (*49*). Caffeic and *p*-coumaric acid were released through deesterification. In this work, we identified methyl caffeate and methyl *p*-coumarate as hydroxycinnamic acid metabolites. Both were detected in 9 of 11 subjects showing interindividual differences in human metabolism, whereas their unmethylated forms did not occur in any ileostomy effluent.

No free *p*-coumaric acid was detected in apple juice under study; therefore, its methyl ester must result from the *p*-coumaroylquinic acid esters, namely, 3-, 4-, and 5-*p*-coumaroylquinic acid. Both, caffeic acid and *p*-coumaric acid were stable when incubated with rat hepatocytes for 4 h or with ileostomy fluid for 24 h (data not shown). Incubations with ileostomy effluents, chlorogenic acid and 4-*p*-coumaroylquinic acid, did not give rise to methyl esters, but after incubation with rat hepatocytes, both methyl esters were observed (data not shown). This indicates that methyl ester formation takes place in the liver and would reach the small intestine via enterohepatic circulation. Our results suggest the formation of methyl caffeate and methyl coumarate via the liver metabolism or transesterification by the carboxyl esterase of the enterocytes. Still the question arises: where does the methanol come from? Methanol is formed in the human body for different reasons. Lindinger et al. (*58*) have demonstrated its continuous endogenous accumulation in the C_1-metabolism and calculated amounts daily ranging between 300 and 600 mg per person. Additionally, apple pectin could be a possible source of methanol, because it is released from pectin during the processing of fruits to juice. Up to 200 mg/L methanol was detected in apple juices, whereas 89% of the analyzed juices ranged between 0 and 50 mg/L (*59*); pectinase treatment of mash can yield 300–400 mg/L (*60*). In our study, the cloudy apple juice consumed by the volunteers contained 23.5 ± 2.16 mg/L methanol detected by SIDA with headspace SPME GC-MS.

In previous studies, Crespy et al. (*61*) showed that phloretin and phloridzin (phloretin 2′-*O*-glucoside) were absorbed in the small intestine, using an in situ intestinal perfusion model. After phloridzin perfusion, the corresponding aglycon as well as conjugated derivatives (glucuronide and/or sulfate) appeared in the lumen; in this, 80% of phloridzin was hydrolyzed to the aglycon. In another study, Crespy et al. (*62*) tested the bioavailability of phloretin and phloridzin in rats. Phloretin was recovered in plasma in small amounts, and no phloridzin was detected, indicating that this glucoside must be hydrolyzed before absorption and metabolism. These results are in agreement with our data showing that no phloridzin but the aglycon phloretin and phloretin 2′-*O*-*β*-glucuronide were detected. We provide evidence on the position of phloretin glucuronidation by NMR analysis. Whereas phloretin 2′-*O*-*β*-xyloglucoside is partially stable in the small intestine, phloridzin is cleaved into phloretin and *β*-D-glucose (data not shown). Human hepatic UGT1A9 treatment of phloretin showed that the glucuronidation takes place in the liver and the conjugate reaches the small intestine through the enterohepatic cycle. Otherwise, glucuronidation could take place in the enterocytes of the small intestine as reported by Lewinsky et al. (*63*) or Mizuma and Awazu (*64*). As Kern et al. (*32*) have reported, small intestinal epithelium Caco-2 cells are able to glucuronidate hydroxycinnamic acid esters. The findings that demonstrate that phloretin and its conjugated forms (phloretin 2′-*O*-xyloglucoside and 2′-*O*-*β*-glucuronide) are present in the ileostomy effluents and

therefore would reach the colon show the importance of such studies to determine the metabolic pathway of flavonoids in animals and humans. Information on its in vivo as well as in vitro health-related bioactivity is not available for the 2′-*O*-β-glucuronide of phloretin.

The sulfatase and glucuronidase treatment of the ileostomy effluent supported the fact that no glucuronide other than phloretin 2′-*O*-β-glucuronide was formed and excreted. In contrast to the literature (*1, 31, 50, 65*), where the formation of quercetin glucuronides or sulfates as well as conjugates of (+)-catechin, (−)-epicatechin, and hydroxycinnamic acids has been demonstrated, we have not identified such metabolites. Additionally, HPLC-MS/MS experiments showed no evidence of the formation of methylated metabolites of phloretin in the ileostomy samples.

Our results show that after apple juice intake in healthy ileostomy subjects some of the polyphenols reach the end of the small intestine unmetabolized (84.2 μmol; 12.7%) and a greater part can be recovered as metabolites (148.8 μmol; 22.3%). These metabolites, specifically phloretin 2′-*O*-β-glucuronide, 1-caffeoylquinic acid, methyl caffeate, and methyl coumarate, were identified and quantified for the first time in humans. It is important to keep these figures in mind when in vitro studies with polyphenols are devised. Furthermore, the influence of conjugation and metabolism has to be considered as well as the concentrations actually present in vivo.

Information about the physiological polyphenol conjugates and metabolites of hydroxycinnamic acids, chalcones, and procyanidins is given. Up to now nothing is known about the biological impact of new metabolites identified. Better knowledge about the exact amounts and forms of polyphenols reaching the colon is of importance for further studies. In the future, experiments using in vitro models of the colonic mucosa to study the biological responses will be able to use physiologically relevant flavonoids and their conjugates at appropriate in vitro concentrations.

ABBREVIATIONS USED

CQA, caffeoylquinic acid; DAD, diode array detection; ESI, electrospray ionization; DP_m, mean degree of polymerization; GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; RP, reversed phase; SRM, selected reaction monitoring; TPA, total proanthocyanidins (total procyandin content); UDPGA, uridine 5′-diphosphoglucuronic acid; UGT, UDP-glucuronosyl transferase.

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