RESEARCH ARTICLE

Colonic availability of polyphenols and D-(−)-quinic acid after apple smoothie consumption

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Scope: The aim of this study was to determine the amounts of polyphenols and D-(−)-quinic acid reaching the ileostomy bags of probands (and thus the colon in healthy humans) after ingestion of apple smoothie, a beverage containing 60% cloudy apple juice and 40% apple puree.

Methods and results: Ten healthy ileostomy subjects each ingested 0.7 L of apple smoothie (a bottle). Their ileostomy bags were collected directly before and 1, 2, 4, 6 and 8 h after smoothie consumption, and the polyphenol and D-(−)-quinic acid contents of the ileostomy fluids were examined using HPLC-DAD and HPLC-MS/MS. The total polyphenol and D-(−)-quinic acid content of the apple smoothie was determined to be 1955.6 ± 124.6 mg/0.7 L, which is very high compared to cloudy apple juices. The most abundant substances found in the ileostomy bags were oligomeric procyanidins (705.6 ± 197.9 mg), D-(−)-quinic acid (363.4 ± 235.5 mg) and 5-caffeoylquinic acid (76.7 ± 26.8 mg). Overall recovery of ingested polyphenols and D-(−)-quinic acid in the ileostomy bags was 63.3 ± 16.1%.

Conclusions: The amounts of polyphenol and D-(−)-quinic acids reaching the ileostomy bags are considerably higher after apple smoothie consumption than after the consumption of cloudy apple juice or cider. These results suggest that the food matrix might affect the colonic availability of polyphenols, and apple smoothies could be more effective in the prevention of chronic colon diseases than both cloudy apple juice and apple cider.

Keywords:
Apple smoothie / Chlorogenic acid / D-(−)-Quinic acid / Ileostomy / Phloretin

1 Introduction

Apples (*Malus domestica* Borkh.) have been shown to have significant cancer preventive effects in several studies, and apple constituents have shown corresponding activities both *in vitro* (summarized by [1]) and in humans [2]. Possible apple constituents responsible for the positive effects may include vitamins, minerals and secondary plant compounds like polyphenols. The polyphenol content of apples ranging between 662 and 2119 mg/kg fresh weight [3] is considerably higher than their calcium, magnesium and vitamin C concentrations (70, 60 and 120 mg/kg, respectively) [4]. Polyphenols are known to exhibit antioxidative and anti-inflammatory effects, as well as the ability to chelate transition metals *in vitro* [5–7]. Therefore, polyphenols are likely to play an important role in the cancer preventive potential of apples [8].

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Abbreviations: **DP_m,** mean degree of polymerization; **FD,** fluorescence detection; **IS,** internal standard

Apples and apple juices contain four main classes of polyphenols: hydroxycinnamic acids (*e.g.* chlorogenic acid), flavan-3-ols ((epi)catechin, procyanidins), flavonols (quercetin glycosides) and dihydrochalcones (phloretin glycosides) [9]. Additionally, anthocyanidins can be found in small amounts in the skin of red apples [10].

Many studies have demonstrated protective effects of apple constituents or apple juice extracts *in vitro* [1], but it is difficult to transfer the results of these studies to *in vivo* situations, since factors like bioavailability and metabolism have to be taken into account. Polyphenols can be absorbed, degraded or metabolized in the small intestine as well as in the colon [11]. Thus, to evaluate the *in vivo* effectiveness of a specific polyphenol-rich food or beverage information on the fate of polyphenols in the gastrointestinal tract are required. To determine the amount of polyphenols reaching the colon, studies with ileostomy probands are valuable because it can be assumed that all substances detected in the ileostomy bags would reach the colon in healthy human subjects [12].

In a previous study with ileostomy subjects consuming 1 L of cloudy apple juice, we recovered up to 33% of the ingested monomeric polyphenols in the ileostomy bags, indicating that at least 67% were absorbed, degraded or metabolized in the small intestine [9]. Marks *et al.* [13] have examined polyphenol contents of ileostomy bags from five subjects, after they had consumed 500 mL of apple cider, and found that bags collected over 24 h contained 38.6% of the ingested dihydrochalcones.

In the study presented here, ileostomy subjects drank 0.7 L of apple smoothie containing 60% cloudy apple juice and 40% apple puree produced from cider apples (Winesap), which generally have higher polyphenol contents than dessert apple varieties [14]. Further, since apple puree is produced from whole fruits with lesser processing steps, such smoothies are likely to have much higher polyphenol contents than respective cloudy apple juices. However, the higher content of cell wall constituents in the apple smoothies compared to cloudy apple juice or cider, might affect the bioavailability of polyphenolic constituents.

The objectives were to determine whether the ingestion of higher amounts of polyphenols leads to higher polyphenol concentrations in the ileostomy bags, and to elucidate potential influences of the beverage matrix on the recovered amounts. If so, apple smoothies could be more potent preventatives of chronic diseases of the colon than cloudy apple juices. To determine the polyphenol amounts in the ileostomy fluids, the samples were freeze-dried, extracted and submitted to HPLC-DAD analysis. Oligomeric procyanidins were measured after phloroglucinolysis by HPLC with fluorescence detection (HPLC-FD). Additionally, HPLC-MS/MS was used to detect unknown metabolites and to quantify D-(−)-quinic acid in the smoothie and ileostomy fluids.

2 Materials and methods

2.1 Chemicals

Methanol, ACN (both of analytical grade) and hydrochloric acid were purchased from J.T. Baker (Deventer, The Netherlands). Phloroglucinol, caffeic acid, (−)-epicatechin, 5-caffeoylquinic acid, D-(−)-quinic acid and 3,4,5-trimethoxycinnamic acid were from Sigma (Steinheim, Germany). Ascorbic acid and formic acid were from Geyer (Renningen, Germany), sodium acetate and quercetin (3,3′,4′,5,7-pentahydroxyflavone) were purchased from Merck (Darmstadt, Germany). (+)-Catechin, quercetin 3-*O*-rhamnoside, phloretin and phloretin 2′-*O*-glucoside were obtained from Roth (Karlsruhe, Germany). Procyanidin B_1 and B_2 were purchased from Extrasynthèse (Genay, France), *p*-coumaric acid was from Fluka (Neu-Ulm, Germany), and methyl *p*-coumarate from Apin Chemicals (Abingdon, UK), ^{13}C-U-D-(−)-quinic acid from IsoLife (Wageningen, The Netherlands). Methyl caffeate and phloretin 2′-*O*-xyloglucoside were synthesized as described by Kahle *et al.* [12] or isolated from a laccase extract of apple juice [15] using a preparative HPLC system. 1–caffeoylquinic acid was synthesized from caffeic acid and D-(−)-quinic acid according to published protocols [16, 17]. 3-CQA (purity 99%) and 4-CQA (purity 95%) were obtained by inter-esterification of 5-CQA in alkaline medium using the method described by Trugo and Macrae [18] and isolated by analytical HPLC. Characterizations were carried out with standards provided by the department of Food Chemistry, University of Wuerzburg.

2.2 Study design

The study protocol was approved by the Ethics Commission of Rhineland-Palatinate (no. 837.292.07 (5827)). Ten healthy ileostomy patients (five male, five female) took part in the study. They were aged between 39 and 72 years (mean 52.9 ± 10.6 years), and their body mass index was $24.8 \pm 2.7\,kg/m^2$. Their underlying conditions were Crohn's disease ($n = 5$), ulcerative colitis ($n = 1$) or colon cancer ($n = 4$), leading to a temporary or permanent stoma of the terminal ileum. Three of the patients were not under medication, whereas the others took Omeprazol ($n = 2$), 5-aminosalicylic acid ($n = 1$) or Ramipril ($n = 2$) and one patient Tavegil/Allopurinol. The subjects were requested to follow a diet free of flavonoids and phenolic compounds (no fruits, vegetables, tea, fruit juices or coffee) for 24 h prior to the study. After an overnight fast, the volunteers each drank 0.7 L of apple smoothie, produced at the Geisenheim Research Centre (Section of Wine Analysis and Beverage Technology) from Winesap apples (an old American cider apple variety (1875)), harvested in the region of Glantal in October 2007. The ileostomy bags were collected directly before and 1, 2, 4, 6 and 8 h after smoothie consumption. During the study, the volunteers remained on a polyphenol-

free diet. After collecting and weighting the ileostomy bags, the contents were freeze-dried, weighed again, homogenized and stored at −20°C prior to extraction and analysis.

2.3 Analysis of procyanidins (phloroglucinolysis)

Portions of freeze-dried ileostomy fluid (10–15 mg) or 200 μL of the freeze-dried and homogenized apple smoothie were mixed with 400 μL of a methanolic solution containing 250 g/L phloroglucinol and 45 g/L ascorbic acid. Then, 200 μL of a hydrochloric acid solution (0.3 M in methanol) were added and the mixtures were incubated at 50°C for 30 min. After that, reaction mixtures were cooled (−20°C) and 600 μL of a sodium acetate solution (0.2 M in methanol) were added. After centrifugation (2 min, 12 000 g, 4°C) and filtration (through a 0.45-μm polyvinylidene fluoride membrane, Buddeberg, Mannheim, Germany), the mixtures were stored at room temperature for 10 h before analysis by HPLC-FD (see Section 2.6 for details). To determine the concentration of monomeric flavan-3-ols before phloroglucinolysis, the procedure described above was repeated with the addition of 400 μL of a methanolic solution containing 45 g/L ascorbic acid instead of a solution containing ascorbic acid and phloroglucinol (modified after a method by Jan Oszmianski, personal communication).

Calibration curves were set for flavan-3-ol monomers and phloroglucinol adducts using procyanidin B_2 for phloroglucinolysis, which showed linearity from 1 to 1000 mg/L, with limits of detection and quantification (LOD and LOQ, 3:1 and 10:1 signal to noise ratios) of 1 and 2 mg/L, respectively. In addition, calibration curves for (+)-catechin and (−)-epicatechin were produced (linear range, 1–1000 mg/L; LOD, 1 mg/L; LOQ, 2 mg/L) to quantify each monomer separately. Average recoveries were 86.0 ± 0.4% for the phloroglucinol-adducts and 104.0 ± 0.1% for the flavan-3-ol monomers.

The total procyanidin content was calculated from the sum of flavan-3-ols and phloroglucinol adducts after phloroglucinolysis (flavan-3-ols$_a$) minus the amount of flavan-3-ols before phloroglucinolysis (flavan-3-ols$_b$). The mean degree of polymerization (DP_m) was calculated from the following equation: adducts/(flavan-3-ols$_b$–flavan-3-ols$_a$)+1. The experiments were performed in triplicate unless insufficient amounts of ileostomy fluid were available.

2.4 Extraction and quantification of polyphenols in the ileostomy fluids and apple smoothie

Twenty-five-milligram portions of the freeze-dried ileostomy fluid and 300 μL portions of the freeze-dried apple smoothie were each dissolved in 1 mL methanol containing 1% v/v formic acid. After homogenization in an ultrasonic bath (Bandelin, Berlin, Germany) and centrifugation (10 min, 10 000 rpm, 20°C), each supernatant was transferred into a pear-shaped flask. This procedure was repeated twice, and the combined supernatants were dried using a rotary evaporator (Büchi, Essen, Germany). The residue was dissolved in 500 μL of a methanol–water mixture (70:30, v/v) and filtered through a 0.45-μm polyvinylidene fluoride membrane (Pall Corporation, Port Washington, USA). Three hundred microlitre of the filtrate were mixed with 30 μL of 3,4,5-trimethoxycinnamic acid (internal standard, IS; 100 mg/L in methanol) and used for HPLC measurement (see Section 2.7 for details). The experiments were performed in triplicate unless insufficient amounts of ileostomy fluid were available.

Calibration curves for quantifying the polyphenols (assuming that analyte to IS peak area ratios correspond to their concentration ratios) were generated by preparing and analyzing solutions containing caffeic acid, *p*-coumaric acid, methyl caffeate, methyl *p*-coumarate, 5-caffeoylquinic acid, phloretin, phloretin 2′-*O*-glucoside, phloretin 2′-*O*-xyloglucoside, quercetin and quercetin 3′-*O*-rhamnoside in methanol–water (30:70, v/v) and adding IS (300 μL sample plus 30 μL IS). The resulting calibration curves were linear for concentrations from 0.04 to 1000 mg/L, LODs ranged from 0.04 to 0.46 mg/L and LOQs from 0.1 to 0.82 mg/L. 1-caffeoylquinic acid, 3-caffeoylquinic acid, 4-caffeoyquinic acid and all *p*-coumaroylquinic acids were quantified as 5-caffeoylquinic acid equivalents, phloretin 2′-*O*-glucuronides as phloretin 2′-*O*-xyloglucoside equivalents, (epi)catechin *O*-sulfates as (−)-epicatechin equivalents, and quercetin 3-*O*-galactoside, quercetin 3-*O*-xyloside and quercetin 3-*O*-arabinoside as quercetin 3-*O*-rhamnoside equivalents. Recoveries ranged between 60 and 100% for all analytes except for phloretin and phloretin 2′-*O*-glucoside (45 and 25%, respectively).

2.5 Quantification of D-(−)-quinic acid

To determine the amounts of D-(−)-quinic acid in the apple smoothie and ileal fluids, the extraction steps were identical to those described in 2.4. After filtration, the samples were diluted 1:1000 with a methanol–water mixture (35:65, v/v) and 30 μL of ^{13}C-U-D-(−)-quinic acid (IS, 200 ng/mL) was added to 30 μL of the sample. The measurement was described before [19]. The recovery was determined to be 82.0 ± 7.1%. The D-(−)-quinic acid contents were quantified using a calibration curve, and the linearity, LOD and LOQ were identical to those described by [19].

2.6 HPLC-FD analysis

The system used for the HPLC-FD analyses consisted of a DG-2080-53 Degasser, a PU-2080 Pump, an AS-2055/2057 Autosampler and a FP-2020plus Fluorescence Detector (all

from Jasco, Groß-Umstadt, Germany), equipped with a Symmetry C_{18} column, 4.6 × 250 mm, with 5-μm particle size (Waters, Eschborn, Germany) and ChromPass software for data acquisition and evaluation. The mobile phase consisted of aqueous 0.1% v/v formic acid (A) and methanol (B), applied in a linear gradient from 2% B to 22% B over 22 min, followed by a rise over 3 min to 26% B, which was held isocratically for 17 min. The flow rate was 0.8 mL/min and in each case the injection volume was 20 μL. The excitation wavelength of the fluorescence detector was 276 nm, and emissions were monitored at 316 nm.

2.7 HPLC-DAD analysis

The HPLC-DAD System consisted of an Agilent Technologies 1200 Series chromatograph equipped with a G 1379 B Degasser, a G 1312 A Binary Pump, a G 1329 A ALS Autosampler, a G 1315 D DAD (Agilent Technologies, Santa Clara, CA, USA), the same column as described in Section 2.6, and ChemStation software for data acquisition and evaluation. The mobile phase consisted of a linear gradient of aqueous 0.1% v/v formic acid (A) and methanol (B), rising from 10 to 67.5% B over 57 min, with an injection volume of 20 μL and a flow rate of 0.8 mL/min. Dihydrochalcones were determined at 280 nm, hydroxycinnamic acid derivatives at 320 nm and quercetin derivatives at 360 nm. Peaks were identified using retention times and UV spectra determined by HPLC-DAD, as well as masses and fragmentation patterns determined by HPLC-MS/MS of reference compounds. The masses, fragmentation patterns and chronological orders of the retention times of the analytes were in agreement with literature data [12, 13, 20].

2.8 HPLC-MS/MS analysis

The D-(−)-quinic acid contents in ileostomy fluids and apple smoothie were determined as described by [19], using the same column, gradient and HPLC-MS/MS-system.

The other metabolites were identified using an HPLC-MS/MS system consisting of a Series 200 Micro Pump, a Series 200 Autosampler, a 785A UV-VIS-Detector (all from Perkin Elmer, Waltham, MA, USA) and an API 2000 Triple Quadrupole Mass Spectrometer (PE Sciex, Darmstadt, Germany). The data acquired were compared to those obtained for reference compounds and literature information [12, 13, 20]. Both the HPLC parameters and the column were identical to those described in Section 2.7. The analysis was performed in negative ionization mode, with the spray capillary voltage set to −4.5 kV, and the temperature of the capillary set to 450°C. Nitrogen served as both curtain gas (45 psi) and collision gas. The collision energy was set to −20 V, and the scan range was 20–580 amu.

3 Results

3.1 Polyphenol contents of the apple smoothie

HPLC-DAD and HPLC-MS/MS were used to identify and quantify polyphenols and D-(−)-quinic acid in the apple smoothie. Peaks in the chromatograms were identified using both reference substances and literature data [12, 13, 20].

Table 1 shows the amounts of polyphenols and D-(−)-quinic acid in the apple smoothie used in the human intervention study (mean ± SD, $n = 9$). Four classes of polyphenols were detected: the flavan-3-ols (+)-catechin, (−)-epicatechin and oligomeric procyanidins; the hydroxycinnamic acids 5-caffeoylquinic acid, 4-caffeoylquinic acid, caffeic acid, 3-*p*-coumaroylquinic acid, 4-*p*-coumaroylquinic acid, 5-*p*-coumaroylquinic acid); the quercetin derivatives

Table 1. Polyphenol and D-(−)-quinic acid contents (mg/L and μmol/L) of the apple smoothie used in the human intervention study determined by HPLC-DAD, and HPLC-MS/MS for D-(−)-quinic acid (mean ± SD, $n = 9$)

	(mg/L)	(μmol/L)
5-Caffeoylquinic acid	156.6 ± 15.3	442.0 ± 43.2
4-Caffeoylquinic acid	13.0 ± 0.6	36.7 ± 1.7
3-Caffeoylquinic acid	n.d.	n.d.
1-Caffeoylquinic acid	n.d.	n.d.
Caffeic acid	0.5 ± 0.3	7.7 ± 1.7
p-Coumaric acid	n.d.	n.d.
3-*p*-Coumaroylquinic acid	16.6 ± 0.4	46.1 ± 1.2
4-*p*-Coumaroylquinic acid	21.2 ± 0.6	62.7 ± 1.8
5-*p*-Coumaroylquinic acid	61.0 ± 2.6	180.5 ± 7.7
Σ Hydroxycinnamic acids	268.9 ± 15.6	775.7 ± 44.0
Phloretin	n.d.	n.d.
Phloretin 2′-*O*-xyloglucoside	45.5 ± 4.3	80.1 ± 7.6
Phloretin 2′-*O*-glucoside	71.6 ± 3.4	164.1 ± 7.8
Phloretin 2′-*O*-glucuronide	n.d.	n.d.
Phloretin glucuronide (2)	n.d.	n.d.
Phloretin glucuronide (3)	n.d.	n.d.
Σ Dihydrochalcones	117.0 ± 5.5	244.2 ± 10.9
Quercetin	2.0 ± 0.2	6.6 ± 0.7
Quercetin 3-*O*-rhamnoside	8.9 ± 0.6	19.9 ± 1.3
Quercetin 3-*O*-galactoside	13.8 ± 1.0	29.7 ± 2.2
Quercetin 3-*O*-xyloside	7.3 ± 0.3	16.8 ± 0.7
Quercetin 3-*O*-arabinoside	13.0 ± 0.9	29.9 ± 2.1
Σ Flavonols	45.0 ± 1.5	102.9 ± 3.5
Catechin	1.7 ± 0.2	5.9 ± 0.7
Epicatechin	1.9 ± 0.1	6.6 ± 0.3
(Epi)catechin *O*-sulfate (1)	n.d.	n.d.
(Epi)catechin *O*-sulfate (2)	n.d.	n.d.
(Epi)catechin *O*-sulfate (3)	n.d.	n.d.
(Epi)catechin *O*-sulfate (4)	n.d.	n.d.
Oligomeric procyanidines	1623.4 ± 131.6	2812.5 ± 228.0
Σ Flavan-3-ols	1627.0 ± 131.6	2825.0 ± 228.0
D-(−)-Quinic acid	735.9 ± 118.7	3828.8 ± 617.6
Σ Polyphenols and D-(−)-quinic acid	2793.8 ± 178.0	7776.6 ± 659.9

n.d., not detectable.

quercetin 3-*O*-rhamnoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-xyloside and quercetin 3-*O*-arabinoside; and the dihydrochalcones phloretin 2′-*O*-xyloglucoside and phloretin 2′-*O*-glucoside. Furthermore, high amounts of D-(−)-quinic acid were detected (735.9 ± 118.7 mg/L).

Oligomeric procyanidins were the most abundant polyphenols (1623.4 ± 131.6 mg/L), followed by D-(−)-quinic acid, 5-caffeoylquinic acid (156.6 ± 15.3 mg/L) and phloretin 2′-*O*-glucoside (71.6 ± 3.4 mg/L). The amounts of quercetin and quercetin glycosides were determined to be between 2.0 ± 0.2 and 13.8 ± 1.0 mg/L; therefore, this class was the least abundant class of polyphenols found in the smoothie. The total amount of polyphenols and D-(−)-quinic acid was determined to be 2793.8 ± 178.0 mg/L.

3.2 Polyphenol and D-(−)-quinic acid contents in ileostomy bags

Ten healthy ileostomy subjects consumed 0.7 L of the apple smoothie, and ileostomy bags were collected immediately before (0 h) and 1, 2, 4, 6 and 8 h afterwards. Polyphenols and D-(−)-quinic acid were identified and quantified in the ileostomy bag contents using HPLC-DAD and HPLC-MS/MS. The analytes were identified by comparing their retention times, UV absorption maxima and *m/z* fragmentation patterns to those of reference substances and literature data. Figure 1 shows three HPLC-DAD chromatograms obtained from one ileostomy fluid sample after extraction (recorded by monitoring the eluate at 280, 320 and 360 nm). In these chromatograms, all polyphenols detected in the ileostomy bags are labelled with lowercase letters at the wavelength at which they were quantified. Unlabelled peaks correspond to unknown ileal constituents of the ileostomy bags.

Four classes of polyphenols were detected in the ileostomy bags: flavan-3-ols, represented by (+)-catechin, (−)-epicatechin, four (epi)catechin *O*-sulfates (with sulphate groups at unknown positions) and oligomeric procyanidins; the hydroxycinnamic acids 5-caffeoylquinic acid, 4-caffeoylquinic acid, 3-caffeoylquinic acid, 1-caffeoylquinic acid, *p*-coumaric acid, 3-*p*-coumaroylquinic acid, 4-*p*-coumaroylquinic acid and 5-*p*-coumaroylquinic acid); the quercetin derivatives quercetin 3-*O*-rhamnoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-xyloside and quercetin 3-*O*-arabinoside; and the dihydrochalcones phloretin, phloretin 2′-*O*-xyloglucoside and three phloretin *O*-glucuronides (the glucuronidation positions are unknown in phloretin *O*-glucuronides 2 and 3).

Figures 2 and 3 show time courses of polyphenol and D-(−)-quinic acid excreted into the ileostomy bags in the 8 h sampling period (0–2 h, 2–4 h, 4–6 h, 6–8 h). For six of the ten subjects, excretion was maximal between 0 and 2 h (see Fig. 2), while the excretion of the remaining four probands was maximal between 2 and 4 h (see Fig. 3). Prior to

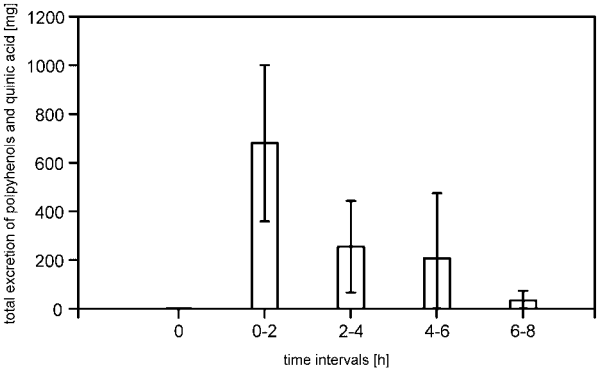

Figure 2. Time course of polyphenol excretion in the ileostomy bags of probands ($n = 6$) for whom excretion was maximal between 0 and 2 h (mean ± SD); 0 h is the time the ileostomy bag was removed before apple juice consumption; the ileostomy bag of one of the probands was collected after 1.5 h instead of after 2 h.

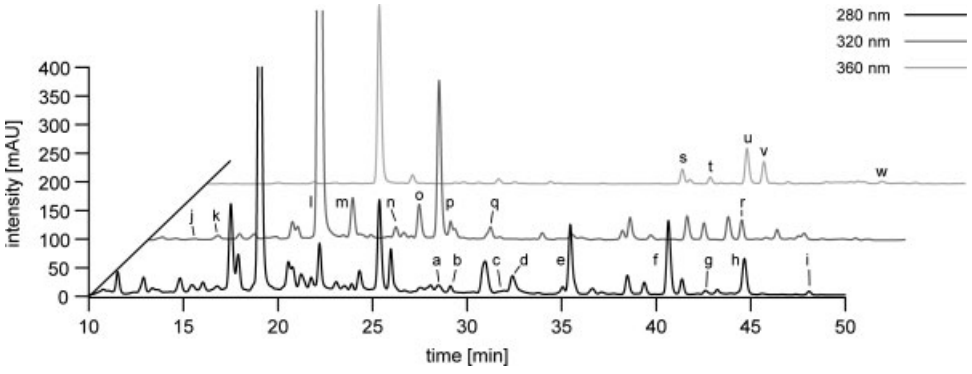

Figure 1. HPLC-DAD chromatograms (λ = 280, 320 and 360 nm) of an extracted ileostomy fluid sample 2–4 h after apple smoothie consumption; peaks are labelled at the wavelength at which the substance was quantified; unlabelled peaks are intestinal constituents. 280 nm: a–d, (epi)catechin *O*-sulfates; e, phloretin 2′-O-xyloglucoside; f–h, phloretin *O*-glucuronides (f, phloretin 2′-*O*-glucuronide); i, phloretin. 320 nm: j, 1-caffeoylquinic acid; k, 3-caffeoylquinic acid; l, 5-caffeoylquinic acid; m, 4-caffeoylquinic acid; n, 3-coumaroylquinic acid; o, 4-coumaroylquinic acid; p, 5-coumaroyquinic acid; q, *p*-coumaric acid; r, 3,4,5-trimethoxycinnamic acid (IS). 360 nm: s, quercetin 3-*O*-galactoside; t, quercetin 3-*O*-xyloside; u, quercetin 3-*O*-arabinoside; v, quercetin 3-*O*-rhamnoside; w, quercetin.

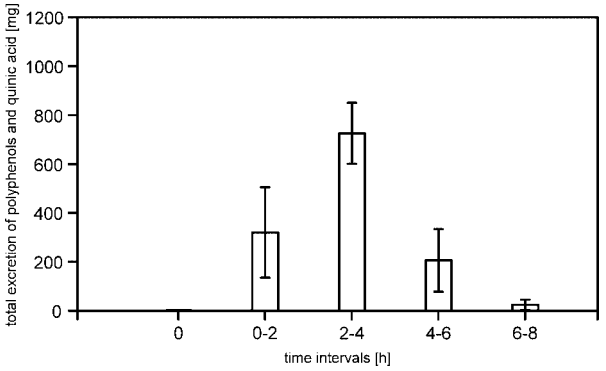

Figure 3. Time course of polyphenol excretion into the ileostomy bags of probands ($n = 4$) for whom excretion was maximal between 2 and 4 h (means ± SD); 0 h is the time the ileostomy bag was removed before apple smoothie consumption.

smoothie consumption (0 h), no polyphenols were detected in the bags, except for two subjects, excreting small amounts. In the 6- to 8-h time interval, most of the ingested polyphenols and D-(−)-quinic acid had already passed the small intestine; in the bags collected after 8 h, only small amounts (< 34 mg) were detected.

Table 2 lists the means ($n = 10$) of total amounts of polyphenols and D-(−)-quinic acid detected in the ileostomy bags between 0 and 8 h after apple smoothie consumption. Oligomeric procyanidins were the most abundant polyphenols (705.6 ± 197.9 mg), followed by D-(−)-quinic acid (363.4 ± 235.5 mg) and 5-caffeoylquinic acid (76.7 ± 26.8 mg). The amounts of all other polyphenols were below 20 mg. Taken together, the most abundant class of polyphenols in the ileostomy bags was the flavan-3-ol derivatives, followed by D-(−)-quinic acid, hydroxycinnamic acids, dihydrochalcones and flavonols.

3.3 Comparison of polyphenol contents in the apple smoothie and ileostomy bags

Comparing the polyphenols detected in the apple smoothie to those recovered in the ileostomy bags revealed certain differences: caffeic acid and phloretin 2′-*O*-glucoside were present in the smoothie, but not in the ileostomy bags. In contrast, 3-caffeoylquinic acid, 1-caffeoylquinic acid, coumaric acid, phloretin, the three phloretin *O*-glucuronides and the four (epi)catechin *O*-sulfates were found in the ileostomy bags, but not in the smoothie. All other polyphenols and D-(−)-quinic acid were detected in the smoothie and in the ileostomy bags as well. The ratios between polyphenol and D-(−)-quinic acid amounts in the ileostomy bags and those ingested in the apple smoothie are shown in Table 3. Ingested polyphenols were recovered in the ileostomy bags to a strongly varying degree (0–106%); indicating compounds specific degradation and/or absorption in the small intestine.

Table 2. Total polyphenol and D-(−)-quinic acid contents of total ileostomy bags (0–8 h) determined by HPLC-DAD and HPLC-MS/MS (mean ± SD, $n = 10$)

	Total excretion (mg)	Total excretion (μmol)
5-Caffeoylquinic acid	76.7 ± 26.8	216.5 ± 75.6
4-Caffeoylquinic acid	9.6 ± 5.5	27.1 ± 15.5
3-Caffeoylquinic acid	5.0 ± 4.7	14.1 ± 13.3
1-Caffeoylquinic acid	0.7 ± 0.6	2.0 ± 1.7
Caffeic acid	n.d.	n.d.
p-Coumaric acid	0.4 ± 0.2	2.4 ± 1.2
5-Coumaroylquinic acid	14.8 ± 5.1	43.8 ± 15.0
4-Coumaroylquinic acid	5.5 ± 1.3	16.3 ± 3.8
3-Coumaroylquinic acid	1.9 ± 0.7	5.6 ± 2.1
Σ Hydroxycinnamic acids	114.6 ± 28.3	327.8 ± 79.9
Phloretin	1.6 ± 3.1	5.8 ± 11.3
Phloretin 2′-*O*-xyloglucoside	17.9 ± 5.8	31.5 ± 10.2
Phloretin 2′-O-glucoside	n.d.	n.d.
Phloretin 2′-*O*-glucuronide	11.9 ± 2.6	26.4 ± 5.8
phloretin *O*-glucuronide (2)	0.8 ± 0.4	1.8 ± 0.9
Phloretin *O*-glucuronide (3)	6.9 ± 3.2	15.3 ± 7.1
Σ Dihydrochalcones	39.1 ± 7.8	80.8 ± 17.8
Quercetin	1.5 ± 1.2	5.0 ± 4.0
Quercetin 3-*O*-rhamnoside	3.9 ± 1.2	8.7 ± 2.7
Quercetin 3-*O*-galactoside	2.0 ± 1.1	4.3 ± 2.4
Quercetin 3-*O*-xyloside	1.7 ± 1.4	3.9 ± 3.2
Quercetin 3-*O*-arabinoside	5.6 ± 1.9	12.7 ± 4.4
Σ Flavonols	14.7 ± 3.1	34.6 ± 7.7
Catechin	0.4 ± 0.3	1.4 ± 1.0
Epicatechin	0.7 ± 0.5	2.4 ± 1.7
(Epi)catechin *O*-sulfate (1)	0.4 ± 0.2	1.1 ± 0.5
(Epi)catechin *O*-sulfate (2)	0.3 ± 0.2	0.8 ± 0.5
(Epi)catechin *O*-sulfate (3)	0.3 ± 0.4	0.8 ± 1.1
(Epi)catechin *O*-sulfate (4)	0.7 ± 0.5	1.9 ± 1.4
Oligomeric procyanidines	705.6 ± 197.9	1222.5 ± 342.9
Σ Flavan-3-ol derivatives	708.4 ± 197.9	1230.9 ± 342.9
D-(−)-Quinic acid	363.4 ± 235.5	1890.7 ± 1225.2
Σ Polyphenols and D-(−)-quinic acid	1240.2 ± 309.0	3564.8 ± 1228.0

n.d. = not detectable.

To obtain a better overview, the amounts of four polyphenol classes (hydroxycinnamic acids, dihydrochalcones, flavonols, flavan-3-ols) and D-(−)-quinic acid detected in the apple smoothie and the ileostomy bags were summed and compared (Fig. 4). Altogether, between 45 and 71% of the ingested polyphenols and D-(−)-quinic acid were excreted in the ileostomy bags. The recovery rates of dihydrochalcones and flavonols ranged between 46 and 48%, hydroxycinnamic acids (61%), and flavan-3-ols (62%) were recovered in clearly large extent in the ileostomy bags but highest rates were observed for D-(−)-quinic acid (71%). Overall 63.3 ± 16.1% of the ingested polyphenols was excreted.

Table 3. Ratio (%) of total excretion of polyphenols and quinic acid in the ileostomy bags to the polyphenol and quinic acid contents of the ingested apple smoothie (mean ± SD; $n = 10$ for ileostomy bags, $n = 9$ for apple smoothie)

	[%]
5-Caffeoylquinic acid	70.0 ± 26.3
4-Caffeoylquinic acid	106.1 ± 61.3
3-Caffeoylquinic acid	–
1-Caffeoylquinic acid	–
Caffeic acid	0.0
p-Coumaric acid	–
5-Coumaroylquinic acid	34.6 ± 12.1
4-Coumaroylquinic acid	36.8 ± 9.0
3-Coumaroylquinic acid	16.2 ± 6.2
Σ Hydroxycinnamic acids	60.9 ± 15.9
Phloretin	–
Phloretin 2′-*O*-glucoside	0.0
Phloretin 2′-*O*-xyloglucoside	56.3 ± 18.9
Phloretin 2′-*O*-glucuronide	–
Phloretin *O*-glucuronide (2)	–
Phloretin *O*-glucuronide (3)	–
Σ Dihydrochalcones	47.7 ± 9.7
Quercetin	106.7 ± 87.3
Quercetin 3-*O*-rhamnoside	62.2 ± 19.8
Quercetin 3-*O*-galactoside	20.7 ± 11.9
Quercetin 3-*O*-xyloside	32.7 ± 26.5
Quercetin 3-*O*-arabinoside	60.9 ± 21.1
Σ Quercetin derivatives	46.3 ± 10.0
Epicatechin	50.7 ± 35.1
Catechin	29.8 ± 22.6
(Epi)catechin *O*-sulfate (1)	–
(Epi)catechin *O*-sulfate (2)	–
(Epi)catechin *O*-sulfate (3)	–
(Epi)catechin *O*-sulfate (4)	–
Oligomeric flavan-3-ols	62.0 ± 18.1
Σ Flavan-3-ol derivatives	62.2 ± 18.1
D-(−)-Quinic acid	70.6 ± 47.1
Σ Polyphenols + D-(−)-quinic acid	63.3 ± 16.1

–, not determinable.

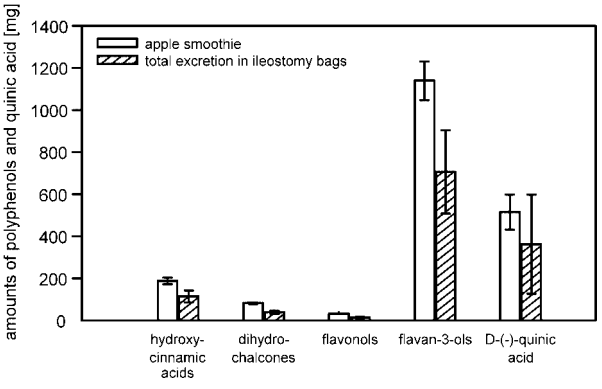

Figure 4. Comparison of polyphenol and D-(−)-quinic acid amounts determined in the apple smoothie and excreted into ileostomy bags between 0 and 8 h after apple smoothie consumption (mean ± SD, $n = 9$ for apple smoothie, $n = 10$ for ileostomy bags).

4 Discussion

To assess the potential health effects of food and food constituents, knowledge on intestinal absorption and/or metabolism in the gastrointestinal tract is required. If a substance, for example, a polyphenol, exhibits constitutional effects *in vitro* but is not absorbed from the gastrointestinal tract *in vivo*, it will not reach the bloodstream and thus other body compartments. Food matrices or the amounts ingested can influence absorption and metabolism of polyphenols (and other substances) either positively or negatively, respectively. Studies with ileostomy subjects are appropriate to obtain such knowledge because analysis of ileostomy bag contents provides information on substances that are absorbed in the small intestine, those that are metabolized before reaching the colon and those that reach the colon unaltered. However, few studies in which the ileostomy bags of subjects who have consumed specific foods or beverages have been performed to date [12, 13, 20].

In the present study, the Winesap apple smoothie used had a total polyphenol content of 2793.8 ± 178.0 mg/L; much higher than those reported for cloudy apple juice (406.9 mg/L, [12]) or an apple smoothie produced from Boskoop apples (600 mg/L, [21]). The Winesap variety was chosen because of its high polyphenol content. Winesap contains high amounts of polyphenols, but the amounts of total titratable acids are only in the upper range of table apples, thus leading to a sensory harmony and a better consumer acceptance.

In this study, ten healthy ileostomy probands each consumed 0.7 L of the apple smoothie containing 1955.5 ± 124.6 mg of polyphenols and D-(−)-quinic acid. Shortly before and 1, 2, 4, 6 and 8 h after ingesting the smoothie, ileostomy bags were collected and their polyphenol and D-(−)-quinic acid contents were analysed. During the collection period of 8 h, a total of 63.3 ± 16.1% of the ingested substances was excreted into the ileostomy bags. Excretion was maximal between 0 and 2 h for six subjects and between 2 and 4 h for the other four. These results are largely consistent with findings of a study by Kahle *et al.* [9], in which 11 ileostomy subjects each drank 1 L of cloudy apple juice (total polyphenol contents, 406.9 mg/L), and polyphenol excretion was maximal 2 h later. Similarly, Marks *et al.* [13] found that dihydrochalcone excretion of ileostomy subjects peaked 2–5 h after consumption of 500 mL of cider containing 46.0 ± 0.3 μmol dihydrochalcones *per* litre. As mentioned, six of our subjects showed maximal excretion between 0 and 2 h, in accordance with results presented by Kahle *et al.* [9]. The slightly retarded excretion maximum of the other four (between 2 and 4 h) could have been due to the effects of the greater viscosity and more abundant matrix constituents of the apple smoothie (compared to apple juice) on the passage time through the gastrointestinal tract.

As shown in Table 3, 70.0 ± 26.3% of the ingested 5-caffeoylquinic acid and 106.1 ± 61.3% of the ingested 4-caffeoylquinic acid were recovered in the ileostomy bags.

Furthermore, 3-caffeoylquinic acid and 1-caffeoylquinic acid were found, indicating that extensive metabolism of caffeoylquinic acids *via* isomerization [22, 23] and esterase activity [24] in the small intestine occurred, as previously reported by our group [12]. Coumaroylquinic acids were also metabolized (recoveries ranged between $16.2 \pm 6.2\%$ and $36.8 \pm 9.0\%$) or cleaved, leading to the appearance of *p*-coumaric acid, which was not present in the apple smoothie but was detected in the ileostomy fluids. $70.6 \pm 47.1\%$ of D-(−)-quinic acid was recovered in the ileostomy bags. Hydrolysis of caffeoylquinic acids and *p*-coumaroylquinic acids liberated additional D-(−)-quinic acid, indicating that a certain amount of D-(−)-quinic acid was absorbed in the small intestine. Kahle *et al.* [12] also detected 4- and 5-caffeoylquinic acid as well as 3-, 4- and 5-coumaroylquinic acid in both apple juice and ileostomy bags. 1- and 3-caffeoylquinic acid, D-(−)-quinic acid and coumaric acid were only found in the ileostomy bags, whereas caffeic acid was present only in the apple juice. Apart from D-(−)-quinic acid, which was found in both the smoothie and ileostomy bags, Kahle's findings concerning hydroxycinnamic acids are similar to the results presented in this paper. However, while Kahle *et al.* found methyl caffeate and methyl *p*-coumarate in the ileostomy bags they examined, and proposed that these two substances may be synthesized in the gastrointestinal tract after the consumption of apple smoothie, we detected neither methyl caffeate nor methyl *p*-coumarate in the ileostomy bags. A possible reason for this difference may be the high concentration of methanol (23.5 mg/L) in the cloudy apple juice used by Kahle *et al.*, which might have led to the formation of methyl caffeate and methyl *p*-coumarate [12]. The methanol content of the apple smoothie in the present study was only 5.3 mg/L and thus probably too low to allow formation of the methyl esters.

Olthof *et al.* [23] performed a study in which ileostomy subjects consumed 500 mg of pure caffeic acid and 1000 mg of pure 5-caffeoylquinic acid. Recoveries in the ileostomy bags (5% for the caffeic acid and 67% for the 5-caffeoylquinic acid) were very similar to the recoveries we found (0% for caffeic acid and 70.0% for 5–caffeoylquinic acid). Kahle *et al.* [9] also recovered no caffeic acid, but only 10.2% of 5-caffeoylquinic acid. Table 4 shows the total amounts (μmol) of polyphenols and D-(−)-quinic acid in the apple smoothie and the cloudy apple juice used by Kahle *et al.* [12] as well as in the ileostomy bags, and the amounts absorbed or metabolized in the gastrointestinal tract. These data indicate that the amounts of hydroxycinnamic acids absorbed or metabolized are smaller after consumption of apple smoothie than after consumption of apple juice.

In Olthof's study, the subjects consumed 10–100 times more of the substances than in Kahle's study, which might have led to an overload of absorption mechanisms and thus to decreased absorption. The recoveries obtained from our apple smoothie study are closer to the results obtained by Olthof *et al.* Since the apple smoothie contained very high amounts of polyphenols, the situation was probably similar to that in Olthof's investigation; other polyphenols like flavan-3-ols may have competed with the hydroxycinnamic acids for cleavage and transport into the enterocytes, leading to less absorption and metabolism than after the consumption of cloudy apple juice. Another factor influencing the polyphenol absorption may have been the high amount of cell wall constituents present in the smoothie. Polyphenols might bind to those constituents and thus reduce their bioavailability, resulting in higher polyphenol excretion in the ileostomy bags.

Among the flavonols, recovery rates in the ileostomy bags for quercetin, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-arabinoside, quercetin 3-*O*-xyloside and quercetin 3-*O*-galactoside amounted to 106.7, 62.2, 60.9, 32.7 and 20.7%, respectively (Table 3). These findings suggest degradation of all quercetin glycosides in the small intestine, at rates depending on the nature of the sugar moiety to yield free quercetin, which was either absorbed or excreted into the ileostomy bags. Kahle and co-workers [8] detected quercetin 3-*O*-rhamnoside, 3-*O*-galactoside, 3-*O*-glucoside, 3-*O*-xyloside and 3-*O*-arabinofuranoside in their cloudy apple juice, but of these substances only quercetin 3-*O*-rhamnoside and quercetin 3-*O*-arabinofuranoside were found in the ileostomy bags (recovery rates 10.3 and 6.3%, respectively). Since the amounts of quercetin glycosides in the cloudy apple juice were much smaller than in the apple smoothie, it seems likely that only the glycosides with the highest recovery rates (3-*O*-rhamnoside, 3-*O*-arabinofuranoside) were detected in the ileostomy bags [12]. The recovery rates determined by Kahle *et al.* were much lower than those presented in this paper (see Table 4).

Amongst the flavan-3-ols, (+)-catechin (29.8%), (−)-epicatechin (50.7%) and oligomeric flavan-3-ols (62.0%) were detected in both the smoothie and ileostomy bags. In addition, four different (epi)catechin *O*-sulfates were found (at relatively low concentrations; 0.8–1.9 μmol) in the ileostomy bags, which were presumably formed after uptake of (epi)catechin into the enterocytes of the small intestine [25]. Methylation and sulphatation may have occurred before some of the metabolites were transported back into the lumen of the small intestine. In contrast, Kahle *et al.* [12] detected no (epi)catechin-sulfates in the ileostomy bags of their subjects after consumption of cloudy apple juice. However, these substances, and methyl-(epi)catechin *O*-sulfates, were detected in the ileostomy bags of probands who had drunk green tea in a study by Stalmach *et al.* [20]. Green tea contains high amounts of monomeric flavan-3-ols; therefore, high amounts of metabolites are created in the gastrointestinal tract, which can be detected in the ileostomy fluids. The apple smoothie used in our study did not contain much monomeric flavan-3-ols, but high amounts of oligomeric flavan-3-ols, particularly dimeric flavan-3-ols which were partially cleaved in the small intestine [26]. The resulting monomeric flavan-3-ols can be absorbed in the enterocytes and metabolized, resulting in

Table 4. Comparison of polyphenol and D-(−)-quinic acid contents in apple smoothie/cloudy apple juice and the ileostomy bags in the study performed by Kahle *et al.* [12] and the apple smoothie study presented in this paper (smoothie).

	(μmol/0.7 L apple smoothie)	Recovery in the ileostomy bags (%) (smoothie)	(μmol/1 L cloudy apple juice) [12]	Recovery in the ileostomy bags (%) [12]
Hydroxycinnamic acid derivatives	541.9	60.9	475.4	28.1
D-(−)-Quinic acid	2680	70.6	n.d.	–
Dihydrochalcones	170.9	47.7	81.2	21.1
Flavonols	72.1	46.3	24.2	2.9
Monomeric, dimetric and oligomeric flavan-3-ols	1977.4	62.2	186.2	52.2
Total	5392.4	63.3	767.0	41.7

n.d., not detectable; –, not determinable.

methylated and sulphated compounds. Kahle [12] reported a recovery rate of 90.3% for oligomeric procyanidins. This rate exceeded the recovery rate determined in our study (62.0%, see Table 4), but as the flavan-3-ol concentration in the apple smoothie is much higher than in the cloudy apple juice, the total amount appearing in the ileostomy bags is still higher than after apple smoothie consumption.

The DP_m ranged between 1.5 and 3 for the procyanidins recovered in the ileostomy bags (data not shown). For the procyanidins in the apple smoothie under study, Oszmianski determined a DP_m of 4.8 (personal communication). These results indicate that degradation of procyanidins occurs during passage through the gastrointestinal tract.

The dihydrochalcone phloretin 2′-*O*-xyloglucoside was detected in both the apple smoothie and ileostomy bags (recovery, 56.3 ± 18.9%). Phloretin 2′-*O*-glucoside was found only in the apple smoothie, whereas phloretin and three phloretin *O*-glucuronides were detected only in the ileostomy bags. These results are consistent with findings by Kahle *et al.* [9, 12], who detected phloretin 2′-*O*-xyloglucoside in both cloudy apple juice and ileostomy bags (recovery rate 19.6 ± 0.2% in the bags), phloretin 2′-*O*-glucoside only in the juice, and phloretin together with two phloretin *O*-glucuronides only in the ileostomy effluents. Our results are also in line with those of Marks *et al.* [13] who found three different phloretin *O*-glucuronides (two with unidentified glucuronidation positions) in the ileostomy bags of subjects who had each drunk 500 mL of cider, and an overall recovery of dihydrochalcones of 38.6% (compared to 47.7% in our investigation, see Table 3).

To evaluate possible health effects of polyphenols in a food or beverage, it is important to know how much of each class of polyphenols that is ingested is absorbed from the gastrointestinal tract and how much reaches the colon. Original polyphenols or metabolites reaching the colon may exhibit preventive effectiveness against colon cancer [27, 28]. To obtain a better overview, the percentage recoveries of four classes of polyphenols (hydroxycinnamic acids, dihydrochalcones, flavonols and flavan-3-ols) in the ileostomy bags that we observed in our smoothie study are summarized in Table 4 in comparison to the data obtained by Kahle *et al.* in their juice study [12]. The apple smoothie contained about 86% more polyphenols than the cloudy apple juice used by [12], mainly due to much higher concentrations of flavan-3-ols and D-(−)-quinic acid. Moreover, the percentages of hydroxycinnamic acids, D-(−)-quinic acid, dihydrochalcones and flavonols recovered in the ileostomy bags were considerably higher after apple smoothie consumption. For the flavan-3-ols, Kahle *et al.* observed a higher percentage recovery [12], but the total amounts recovered were still significantly higher after apple smoothie consumption (not shown). Altogether, the polyphenol and D-(−)-quinic acid amounts reaching the colon were significantly higher after apple smoothie consumption. Additionally, the apple smoothie contained about 60% more dihydrochalcones than the cider used by Marks *et al.*, and the amount of these substances reaching the colon was 66% higher after apple smoothie consumption than after consumption of cider [13].

Taken together, comparison of the results of the three studies demonstrate that substantially more polyphenols and D-(−)-quinic acid reached the ileostomy bags (and therefore the colon in healthy humans) after consumption of apple smoothie compared to cloudy apple juice or cider. This might indicate a higher preventive potential of apple smoothies against chronic colon diseases compared to cloudy apple juice and cider. Reasons for the differences in bioavailability of polyphenols and D-(−)-quinic acid in the small intestine and colon after consumption of the three beverages might be related to the differences in the amounts of cell wall constituents they contain or ingested amounts of polyphenols. The apple smoothie contains much more of these matrix components than both apple juice and cider, hence they probably bind more polyphenols and thus reduce the bioavailability of the substances in the small intestine. In addition, the considerably higher polyphenol amounts present in the apple smoothie could further reduce their percentage bioavailability. Therefore, it can be concluded that the consumption of apple smoothie or puree might increase the colonic availability of apple-derived polyphenols.

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