Total phenolics, flavonoids, anthocyanins and antioxidant activity following simulated gastro-intestinal digestion and dialysis of apple varieties: Bioaccessibility and potential uptake

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ABSTRACT

In the present study, an *in vitro* model simulating gastrointestinal (GI) digestion, including dialysability, was adapted to assess free soluble polyphenols from apples (four varieties). Results indicated that polyphenol release was mainly achieved during the gastric phase (*ca.* 65% of phenolics and flavonoids), with a slight further release (<10%) during intestinal digestion. Anthocyanins present after the gastric phase (1.04–1.14 mg/100 g) were not detectable following intestinal digestion. Dialysis experiments employing a semipermeable cellulose membrane, presenting a simplified model of the epithelial barrier, showed that free soluble dialysable polyphenols and flavonoids were 55% and 44% of native concentrations, respectively, being approximately 20% and 30% lower than that of the GI digesta. Similar results were found for the antioxidant capacity of dialysable antioxidants, being 57% and 46% lower compared to total antioxidants in fresh apples (FRAP and ABTS test, respectively). It is suggested that some polyphenols are bound to macromolecular compounds that are non-dialysable, that the presented method allowed the study of free soluble polyphenols available for further uptake, and that both chemical extraction and concentrations in final digesta would overestimate polyphenol availability.

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1. Introduction

Owing to their availability on the market during the whole year, apples constitute the most frequently consumed fruits in many westernised diets, and are therefore among the main dietary sources of exogenous antioxidants in the human body (Brat et al., 2006; Lamperi et al., 2008; Lee, Kim, Kim, Lee, & Lee, 2003). The major part of the apple production – over 62 million metric tons worldwide in 2005 (Lamperi et al., 2008) – is consumed in fresh form; the remainder as juices, concentrates, and purees (Wojdylo, Oszmianski, & Laskowski, 2008). In the US, for example, apples rank second after banana among consumed fresh fruits, providing 22% of total polyphenol intake (Brat et al., 2006; Lee et al., 2003). However, considering both fresh and processed products combined, apples rank first in consumption (Lee et al., 2003). In France, apples represent 30% of total consumed fruits (*ca.* 57 g per capita and day), providing almost 37% of total polyphenol intake from fruits and vegetables (Brat et al., 2006).

Polyphenols constitute the majority of secondary plant metabolites and also of dietary antioxidants (Bouayed, 2010; Bouayed & Bohn, 2010). The preventive effect of apples against many chronic diseases, such as lung cancer, asthma and cardiovascular diseases, found in several epidemiological investigations (reviewed by Bouayed & Bohn, 2010; Woods et al., 2003) is at least in part attributed to its polyphenol constituents. As a consequence, much data has been generated on polyphenol constituents in a variety of food items, including apple, measuring total phenolics, flavonoids (the major phenolics in apples) and related total antioxidant activities based on chemical extraction, typically using methanol or water/methanol mixtures (Lamperi et al., 2008; Lee et al., 2003; Neveu et al., 2010; Wojdylo et al., 2008). However, under *in vivo* conditions, polyphenols from the diet have to be extracted following gastrointestinal (GI) digestion. The nature of extractable phytochemicals, their stability and their antioxidant activity depend on many factors, such as the food matrix, the pH, the temperature, presence of inhibitors or enhancers of absorption, presence of enzymes, host, and other related factors (McDougall, Dobson, Smith, Blake, & Stewart, 2005; Saura-Calixto, Serrano, & Goni, 2007; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). The GI tract may be considered as an efficient extractor, where part of the phytochemicals contained in food matrices is extracted and becomes

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available for uptake in the intestine (Saura-Calixto et al., 2007; Tagliazucchi et al., 2010).

Despite their limitations, such as typically constituting only a static model of digestion, *in vitro* GI models have been developed for bioaccessibility assessment, allowing the study of changes in dietary components during the gastric and the intestinal stage, due to factors impacting their availability discussed above (Biehler & Bohn, 2010; Cilla, González-Sarrías, Tomás-Barberán, Espín, & Barberá, 2009; McDougall et al., 2005). Furthermore, it has been demonstrated that the evaluation of bioaccessibility by *in vitro* models can be well correlated with results from human studies and animal models (reviewed by Biehler & Bohn, 2010). Based on an *in vitro* study, it has been demonstrated that the digestion process decreased the phenolic content by at least 47% in digested fruit beverages compared to undigested ones (Cilla et al., 2009). In another study, following gastric and intestinal digestion, it was found that only 62% of originally present polyphenols in grapes were bioaccessible (Tagliazucchi et al., 2010). The transition from the acidic gastric to the mild alkaline intestinal environment caused a decrease in the amount of bioaccessible total polyphenols, flavonoids and especially anthocyanins. Anthocyanins, one of the five major groups of polyphenol compounds in apple (Wojdylo et al., 2008), are highly unstable at intestinal pH (McDougall et al., 2005; Tagliazucchi et al., 2010). Furthermore, Tagliazucchi et al. (2010) have also shown that radical-scavenging activities of polyphenols may be pH-dependent, suggesting greater scavenging capacity in the intestine than in the stomach.

In the present study, we intended to study total phenolics, total flavonoids, total anthocyanins and total antioxidant activity of apples at different phases of simulated digestion (gastric and intestinal phase), using an extended *in vitro* model. This model also allowed the study of the free soluble fraction of polyphenols potentially available for further uptake, following dialysis through a semipermeable cellulose membrane. The concentrations of phenolic constituents in these fractions were then compared to those natively present in apples, determined by chemical extraction using an ultrasound-assisted method employing methanol.

2. Materials and methods

2.1. Chemicals

All products were of analytical grade or superior, and 18 MΩ water (Millipore, Brussels, Belgium) was used throughout. Methanol was obtained from Biosolve (Valkenswaard, The Netherlands). Iron(III)chloride 6-hydrate and iron(II)sulphate 7-hydrate were procured from Merck (Darmstadt, Germany) and VWR (Leuven, Belgium), respectively. Aluminium chloride ($AlCl_3$), 2-2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt (ABTS), 2-2'-azobis(2-methylpropionamidine)dichloride (AAPH), (+)-catechin, gallic acid, Folin–Ciocalteu's phenol reagent, phosphate buffer saline (PBS), porcine pepsin (800–2500 U/mg protein), porcine bile mixture, porcine pancreatin (4× US Pharmacopeia specifications), sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) and vitamin C were purchased from Sigma–Aldrich (Bornem, Belgium). Dialysis bags (pre-washed and wetted, type P/N 128418, with a molecular weight cut-off of 10,000 Da, thickness of 0.002 in. and a flat width of 18 mm) and clips (P/N 132735 with a width of 23 mm) were obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA).

2.2. Sample preparation

Four Luxembourgish apple varieties were procured at commercial maturity from a local farmer (Kehlen, Luxembourg) during February 2010: Jonaprinz, Jonagold, Golden and Mutzu, the first two varieties being of red, the others of green skin. For each variety, six apples were rapidly cut into thin slices with peel, put into transparent polyethylene bags, dipped into liquid nitrogen, and frozen at −80 °C. Samples were then further homogenised at 4 °C with a Gastroback robot mixer (Gastroback GmbH, Hollenstedt, Germany) and aliquots stored at −80 °C in 50-mL plastic centrifuge tubes until analysis.

2.3. Extraction of phenolics

2.3.1. Chemical extraction

Three independent extractions for each cultivar were carried out as described previously by Kim, Heo, Kim, Yang, and Lee (2005), using ultrasound-assisted liquid extraction. For this objective, a mixture of *ca.* 10 g (fresh weight) of composite fruit tissue samples prepared from six fruits and 20 mL of methanol was sonicated for 20 min under argon gas to prevent possible oxidative degradation of polyphenolics. The mixture was then filtered through a filter paper (MN615. Ø70 mm, Macherey–Nagel, Düren, Germany) using a chilled Buchner funnel and rinsed with 10 mL of 100% methanol. Extraction of the residue was repeated using 80% aqueous methanol, using the same conditions for sonication and filtration. The two filtrates were combined and transferred into a 200-mL flask. The solvent was then removed using a rotary evaporator (Laborota 4000, Heidolph, Schwabach, Germany) at 40 °C to a final volume of *ca.* 4 mL. The remaining phenolic concentrate was first dissolved in 10 mL of 100% methanol and diluted to a final volume of 20 mL with water. The mixture was centrifuged at 4 °C and 5500*g* for 20 min (Harrier 18/80 refrigerated centrifuge, MSE, London, UK), and the supernatant was then stored at −80 °C until analysis within the next days.

2.3.2. Simulated in vitro gastrointestinal (GI) digestion

To mimic GI digestion conditions *in vivo*, namely gastric and intestinal phases, and to determine the amount of free soluble polyphenols potentially available for further uptake, the original (Miller, Schricker, Rasmussen, & Van Campen, 1981) and later modified protocol (Luten et al., 1996) of iron dialysability from solid food matrices was followed with slight modifications.

To monitor the release of phytochemicals (phenolics, flavonoids and anthocyanins) from apple matrices, at different stages of digestion, aliquots from gastric digesta, GI digesta and dialysable content were analysed, respectively. Antioxidant activities were also determined. Individual experiments were conducted to measure bioaccessible polyphenols at each of the different stages of digestion.

2.3.2.1. Gastric phase.
Into a 125-mL screw-top polypropylene tube (VWR, Leuven, Belgium), a mixture of 50 mL NaCl (0.9%), 8 mL HCl (0.1 M), 4 mL of pepsin solution (40 mg/mL in HCl 0.1 M) and 10 g of homogenised complete apple material was incubated for 1 h in a shaking water bath at 37 °C and 100 rpm. A blank was prepared with identical chemicals but without food matrix, and underwent the same conditions as the samples. Before and after gastric digestion, the pH was verified (target pH 2–2.5). Aliquots of the gastric phase were stored at −20 °C until further analysis.

2.3.2.2. Intestinal phase with dialysis.
Segments of dialysis bags were cut to a specified length (15.5 cm), rinsed (outer and inner surface) with 0.9% NaCl solution and then one end of each segment was sealed with clips. Bags were filled bubble-free with 5.5 mL NaCl (0.9%) and 5.5 mL $NaHCO_3$ (0.5 M), sealed with clips, and completely immersed into the gastric digesta immediately after digestion. The amount of $NaHCO_3$ in the dialysis tubes corresponded to the amount of NaOH consumed (in mol) to neutralise

the gastric samples. The samples were then incubated for 45 min in a shaking water bath at 37 °C and 100 rpm. After this step, reflecting the transition from the gastric phase to the intestinal phase, the pH was increased to *ca.* 6.5. Afterwards, 18 mL of a mixture of pancreatin and porcine bile extract (2 mg/mL pancreatin and 12 mg/mL bile extract dissolved in 0.1 M $NaHCO_3$) were added to the digesta, which was further incubated in a shaking water bath for an additional 2 h at 37 °C. At the end of the incubation period, the pH in the digesta was measured at 7–7.5. Aliquots of the intestinal phase were stored at −20 °C until further analysis. The dialysis bags were recovered, rinsed with water, carefully dried using a paper cloth and weighed. The content of each dialysis bag was then transferred quantitatively into a Falcon tube and diluted to a final volume of 14 mL with 0.9% NaCl and stored at −20 °C until analysis.

2.3.2.3. Further sample preparation for analysis. Dialysed samples were not processed further. For aliquots of gastric and intestinal phase, prior to storage at −20 °C, samples from individual experiments were filtered through a filter paper (MN615. Ø70 mm, Macherey–Nagel) and aliquots kept for further analysis.

2.4. Analyses of total phenolics, flavonoids, and total anthocyanins

2.4.1. Determination of total phenolics

Total phenolic content was determined with Folin–Ciocalteu's phenol reagent (Singleton & Rossi, 1965) using spectrophotometric analysis (DU 800 UV/Visible spectrophotometer, Beckman Coulter, Palo Alto, CA). Briefly, an aliquot (1 mL) of standard solution of gallic acid at concentrations between 0 and 100 mg/L (external calibration with $n = 6$ concentrations) in 50% aqueous methanol or sample (appropriately diluted extracts or aliquots from the various fractions following GI digestion) was added to a 25-mL volumetric flask containing 9 mL of water. One millilitre of Folin–Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 mL of 7% aqueous Na_2CO_3 solution was added. The solution was then immediately diluted to a final volume of 25 mL with water and mixed thoroughly. After incubation for 90 min at 23 °C, the absorbance *versus* the prepared blanks was read at 750 nm. Total phenolic content in apples was expressed as mg gallic acid equivalents (GAE) per 100 g fresh weight.

2.4.2. Determination of total flavonoids

Total flavonoid content was measured according to a colorimetric assay (Zhishen, Mengcheng, & Jianming, 1999). A 1-mL aliquot of standard solution of catechin at different concentrations (0–100 mg/L, external calibration with $n = 6$ concentrations) or sample (appropriately diluted extracts or aliquots from GI digestion) was added to 10-mL volumetric flasks containing 4 mL water. At the onset of the experiment, 0.3 mL of 5% $NaNO_2$ was added to the flask. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. At 6 min, 2 mL of 1 M NaOH was added to the mixture. Immediately, the solution was diluted to a final volume of 10 mL with water and mixed thoroughly. Absorbance of the mixture was determined at 510 nm *versus* the prepared blanks. Total flavonoid content in apples was expressed as mg catechin equivalents (CE) per 100 g fresh weight.

2.4.3. Determination of total anthocyanins

The quantification of total anthocyanins was evaluated by the pH differential method (Giusti & Wrolstad, 2001). Phenolic extracts of apples or aliquots from GI digestion in both 0.025 M potassium chloride solution (pH 1.0; 1.86 g KCl in 1 L water adjusted to pH with concentrated HCl) and 0.4 M sodium acetate buffer (54.43 g $CH_3CO_2Na\cdot3H_2O$ in 1 L water adjusted to pH 4.5 with concentrated HCl) were measured both at 510 and 700 nm after 15 min of incubation at 23 °C. The content of total anthocyanins was expressed as

mg cyanidin 3-glucoside equivalents (CGE)/100 g of fresh apple. A molar absorption coefficient of 26,900 L mol^{-1} cm^{-1} was used to calculate the concentration of cyanidin 3-glucoside in solution.

2.5. Methods to estimate antioxidant capacity

2.5.1. ABTS radical-scavenging capacity assay

The vitamin C equivalent antioxidant capacity (VCEAC) test developed by Lee et al. (2003) was used in this study. Total antioxidant activity of apples was determined by scavenging blue-green ABTS radicals and was expressed as mg vitamin C equivalents (VCE) per 100 g fresh weight. Vitamin C was used as a standard to quantify total radical-scavenging activity, as this method allows for optimal comprehension of results for both the general public and researchers.

In brief, 1 mM AAPH, a radical initiator, was mixed with 2.5 mM ABTS in PBS (pH 7.4). The mixture was heated in a water bath at 68 °C for *ca.* 15 min. The resulting blue-green ABTS radical solution was adjusted to an absorbance of 0.650 ± 0.020 at 734 nm with additional PBS. Twenty microlitres of sample was then added to 980 µL of the ABTS radical solution. The mixture was incubated in a 37 °C water bath under restricted light for 10 min. The control consisted of 20 µL 50% methanol and 980 µL of ABTS radical solution. The decrease of absorbance at 734 nm was measured after 10 min. An external standard (vitamin C) calibration curve (range: 0–0.2 mg/mL, $n = 5$ calibration points) was prepared to determine concentrations in solution.

2.5.2. Ferric-reducing antioxidant power assay

The FRAP assay was carried out according to the procedure of Benzie and Strain (1996). Briefly, the FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM iron(III)chloride solution in proportions of 10:1:1 (*V/V/V*), respectively. The FRAP reagent was freshly prepared on a daily basis and was warmed to 37 °C in a water bath prior to use. Fifty microlitres of sample (appropriately diluted extracts or aliquots from GI digestion) was added to 1.5 mL of the FRAP reagent. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min. The standard curve was constructed using iron(II)sulphate solution (range 100–2000 µM with $n = 7$ concentrations), and the results were expressed as µmol Fe(II) per 100 g fresh weight.

2.6. Statistical analyses

Data were collected from three independent extractions for each cultivar and reported as mean ± SD. For multiple comparisons, ANOVA was carried out and followed by Bonferroni *post hoc* tests, after verification of normality of distribution and equality of variance *via* Q–Q plots and box plots, respectively. To compare anthocyanins present following gastric digestion with chemical extraction, Student's unpaired *t*-tests were used. Mean differences with a $p < 0.05$ (2-tailed) were considered statistically significant. The statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL). For all polyphenolic-related comparisons following various digestion steps, concentrations corrected for the respective dilutions comprised within the digestion procedure were compared.

3. Results

3.1. Total phenolics

The impact of GI digestion on total phenolics is shown in Fig. 1. In general, the amount of released phenolics (corrected for the varying
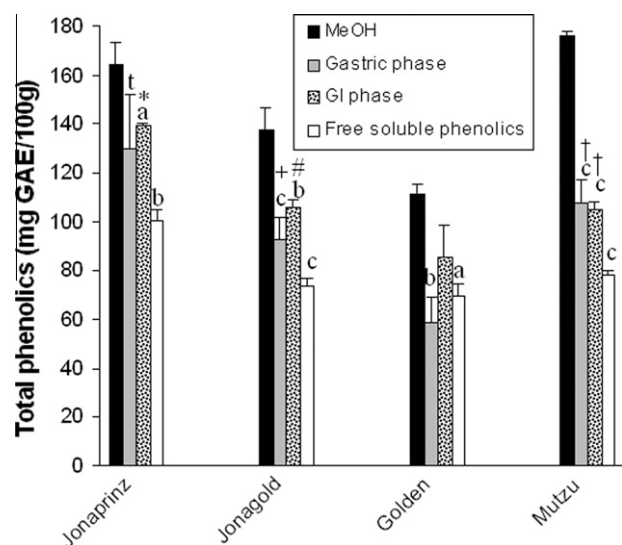
Fig. 1. Amount of total phenolics, expressed as gallic acid equivalents (GAE), as determined from fresh apple matrix (100 g) using methanolic extraction (MeOH) and after simulated gastro-intestinal digestion, i.e., phenolics remaining after the gastric phase, intestinal phase, and free soluble (dialysable through cellulose membrane) phenolics. Data shown represent means ± SD ($n = 3$ independent experiments). $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ significantly different from apple methanolic extracts, "t" indicating tendency, i.e., $0.05 < p < 0.1$. $^{*}p < 0.05$, $^{#}p < 0.01$, $^{†}p < 0.001$ significantly different from dialysable phenolics, "+" indicating tendency, i.e., $0.05 < p < 0.1$. All p-values are 2-tailed following ANOVA and Bonferroni *post hoc* test.

Fig. 2. Amount of flavonoids, expressed as catechin equivalents (CE), as determined from fresh apple matrix (100 g) using methanolic extraction (MeOH) and after simulated gastro-intestinal digestion, i.e., flavonoids remaining after the gastric phase, intestinal phase, and free soluble (dialysable through cellulose membrane) flavonoids. Data shown represent means ± SD ($n = 3$ independent experiments). $^{b}p < 0.01$, $^{c}p < 0.001$ significantly different from apple methanolic extracts. $^{†}p < 0.001$ significantly different (2-tailed, following ANOVA and Bonferroni *post hoc* test) from dialysable flavonoids.

volumes of digesta) increased stepwise from gastric to intestinal digesta for all apple varieties, albeit the effect was not significant for all apple varieties. The amount of dialysable phenolics was finally found to be lower compared to intestinal digesta, albeit again not significantly for all apple varieties, i.e., Golden. All amounts were lower when compared to chemical extraction, indicating incomplete release or degradation of phenolics. This decreased recovery was most pronounced when comparing dialysable phenolics compared to phenolics extracted from apples directly, with recoveries varying between 44.6% and 62.7%, and more pronounced losses for Mutzu > Jonagold > Golden > Jonaprinz.

3.2. Total flavonoids

The pattern of release was similar to total phenolics, with stepwise release from the gastric phase to the intestinal phase, while not all flavonoids were dialysable. Amounts of flavonoids detected after the gastric and the intestinal phase as well as free soluble (dialysable phenolics) were significantly lower than those determined by chemical extraction for all apple varieties (Fig. 2). For all varieties, the content of free soluble dialysable flavonoids was also significantly lower than the content of flavonoids found in the gastric as well as the intestinal digesta ($p < 0.001$), except for Golden.

3.3. Total anthocyanins

Two of the four apple varieties (Golden and Mutzu) contained non-detectable amounts of anthocyanins. Unpaired Student's *t*-test revealed that there were no significant differences between the amount of anthocyanins detected by chemical extraction and following gastric digestion for both Jonaprinz and Jonagold varieties (Table 1). However, after GI digestion, anthocyanins were no longer detectable in the intestinal digesta.
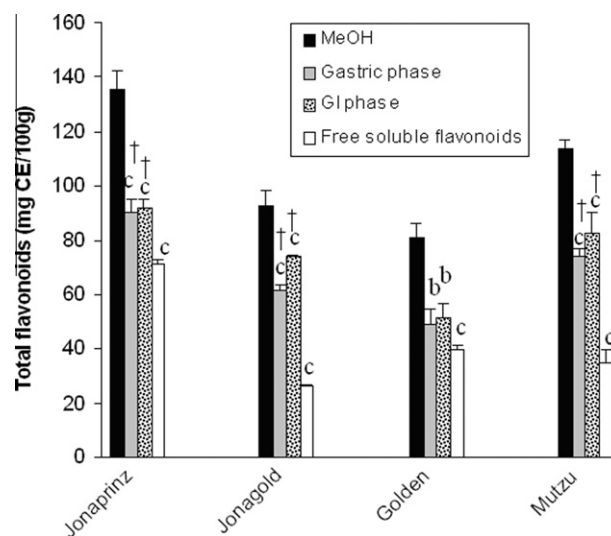
3.4. Total reducing antioxidant capacity comparisons

The overall trend of reducing antioxidant capacity followed the concentrations of total phenolics and flavonoids. Reducing antioxidant capacity natively present in apple varieties, as determined in methanolic extracts by the FRAP test, was significantly higher compared to those found in gastric digesta for all apple varieties with exception for Jonagold (Fig. 3), to those recorded in intestinal digesta ($p < 0.001$ for all varieties), and compared to free soluble antioxidants crossing the cellulose membrane ($p < 0.001$ for all varieties), with the latter being significantly lower compared to the gastric phase for all varieties, and partly when compared to the intestinal phase (Jonagold: $p < 0.05$, Mutzu: $p < 0.001$; Fig. 3).

3.5. Total radical-scavenging activity comparisons

Total radical-scavenging activity by ABTS followed, in general, concentrations of phenolics and flavonoids. Radical-scavenging activities natively present in apple varieties, as determined from methanolic extracts by the ABTS test, were found to be significantly higher, compared to those displayed by gastric digesta for some apple varieties (Jonaprinz: $p < 0.001$, Golden: $p < 0.05$; Fig. 4), but not compared to the intestinal digesta, with the exception of Jonaprinz. However, free soluble antioxidants displayed an antioxidant activity significantly reduced compared to their corresponding native antioxidant activity in apples, except for Jonagold, and compared to both gastric digesta (Jonaprinz and Mutzu) and intestinal digesta (all apple varieties).

3.6. Overall comparison with combined results

As seen from pooled data obtained from the four studied varieties (Fig. 5), amounts of released phytochemicals (both phenolics and flavonoids) after both the gastric phase and the intestinal phase were significantly lower than initial concentrations determined by chemical extraction using methanol ($p < 0.001$), indicating incomplete release or degradation. Pooled results from dialysis

Table 1
Recovery and concentration of total anthocyanins expressed as mg cyanidin 3-glucoside equivalents (CGE)/100 g fresh apples in simulated gastric digesta, compared to initial concentrations found by methanolic extraction.

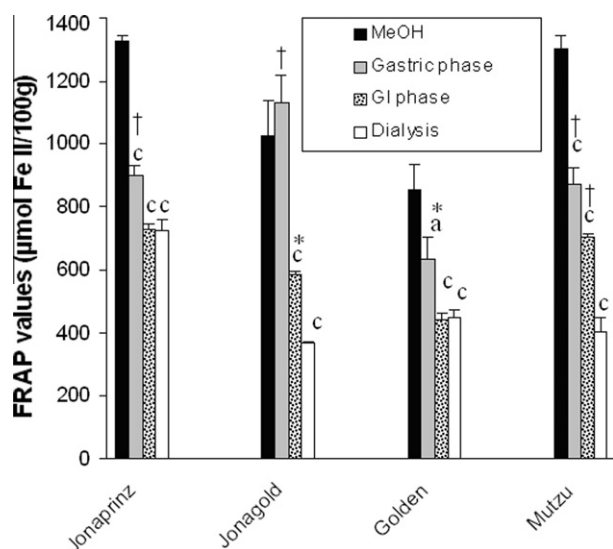| Apple variety	Chemical extraction (mg CGE/100 g)	SD	Gastric digestion (mg CGE/100 g)	SD	Gastric recovery (%)	SD	Intestinal digestion (mg CGE/100 g)
Jonaprinz	1.14	0.08	1.04	0.01	91.2	0.36	ND
Jonagold	1.04	0.12	1.11	0.06	107	5.54	ND
Golden	ND	–	ND	–	–	–	ND
Mutzu	ND	–	ND	–	–	–	ND

ND, not detected.

Fig. 3. Reducing antioxidant capacity assessed by the FRAP test, expressed as µmol Fe II/100 g, of gastric and intestinal digesta, and free soluble (dialysable) antioxidants, compared to those obtained by chemical extraction using methanol (MeOH). Data shown represent means ± SD ($n = 3$ independent experiments). [a]$p < 0.05$, [c]$p < 0.001$ significantly different from antioxidant activity of apple methanolic extracts. *$p < 0.05$, †$p < 0.001$ significantly different (2-tailed, following ANOVA and Bonferroni *post hoc* test) from reducing activity of dialysable antioxidants.

Fig. 4. Radical-scavenging activity assessed by the ABTS test, expressed as vitamin C equivalents (VCE/100 g), of gastric and intestinal digesta, and free soluble (dialysable) antioxidants, compared to those obtained by chemical extraction using methanol (MeOH). Data shown represent means ± SD ($n = 3$ independent experiments). [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$ significantly different from antioxidant activity of apple methanolic extracts. *$p < 0.05$, #$p < 0.01$, †$p < 0.001$ significantly different (2-tailed, following ANOVA and Bonferroni *post hoc* test) from scavenging activity of dialysable antioxidants, " + " indicating tendency, i.e., $0.05 < p < 0.1$).

experiments showed that the contents of free soluble dialysable phenolics and flavonoids, through an epithelial barrier modelled by a semipermeable cellulose membrane, were significantly lower than contents of phenolics and flavonoids found in the gastric as well as the intestinal digesta ($p < 0.001$).

Compared to the native antioxidant activity of apples (pooled results), the FRAP test showed that reducing activity of digesta in both phases of GI digestion (i.e., gastric and intestinal phases), was significantly lower ($p < 0.001$); however, the ABTS test showed that only reducing activity of gastric digesta was significantly lower ($p < 0.01$, Fig. 5). With regard to antioxidant activity of free soluble dialysable phenolic constituents, their antioxidant activities, as assessed by both the FRAP test and the ABTS test, were significantly lower than that natively existing in apples ($p < 0.001$), and that remaining after apple digestion in both phases (gastric and intestinal phases) ($p < 0.001$).

4. Discussion

In the present study, we investigated the availability of polyphenols from apple fruits, following stages during simulated GI digestion, i.e., gastric phase, intestinal phase, and free soluble dialysable polyphenols. Our results highlight that the majority of polyphenols and flavonoids were already available during the gastric phase, but suggest that not all detected polyphenols present in the intestinal digesta were available for uptake under our conditions as simulated by dialysis experiments, emphasising that
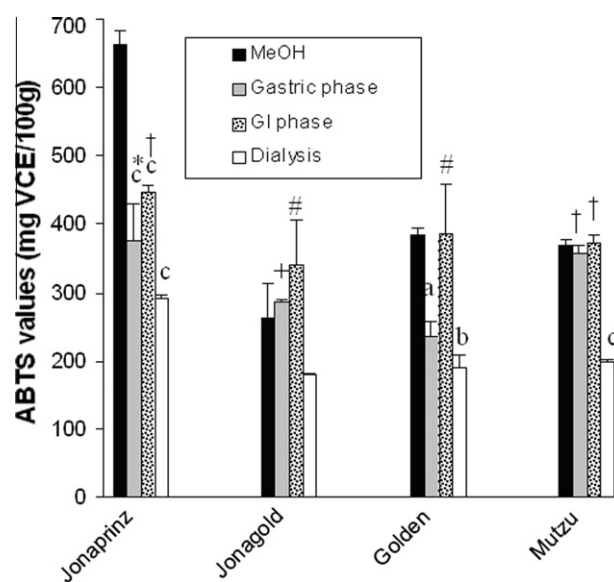
merely chemical extraction and determination of polyphenols in the intestinal digesta can overestimate antioxidants and antioxidant activity. To our knowledge, this is the first study investigating the impact of individual digestion stages on polyphenols, integrating the dialysable fraction.

To exert their bioactivity, antioxidants have first to be bioaccessible, i.e., released from the food matrix and solubilised. In this study, results have shown that bioaccessible total phenolics and total flavonoids in gastric phases were *ca.* 35% lower on average compared to values obtained by chemical extraction (Fig. 5). Interestingly, this reduction was not observed for anthocyanins (Table 1), suggesting that during the gastric phase either extraction of skin phenolics from apples is more efficient than flesh phenolic constituents or that anthocyanins were more stable under these conditions. However, the present results also demonstrate that the release of polyphenols from apples following simulated GI digestion is mainly achieved during the gastric phase (Figs. 1 and 2). The following intestinal phase resulted in a further increase (<10%) in total phenolics and total flavonoids compared to amounts released after the gastric phase; however, they were still significantly lower compared to those determined by chemical extraction using methanol, by *ca.* 25% and 30%, respectively (Fig. 5). The increase of total phenolics and flavonoids after the intestinal phase could be explained by the additional time of extraction (plus 2 h 45 min) and/or the effect of intestinal digestive
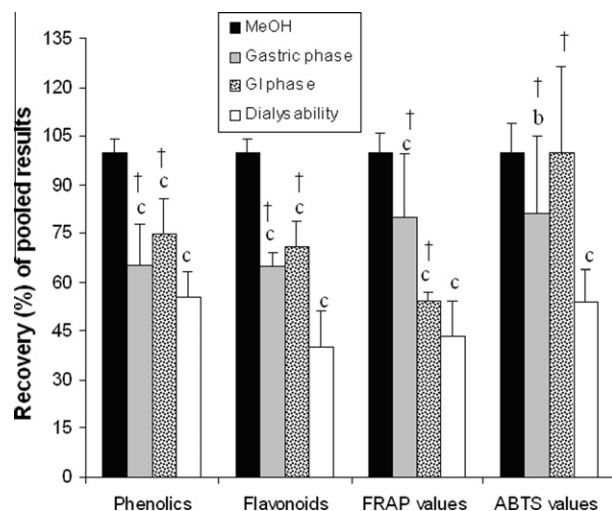
Fig. 5. Percentage (%) of recovery from fresh apple matrix (100 g) of phenolics, flavonoids and related antioxidant activities, assessed by both the FRAP test and the ABTS test, after simulated gastro-intestinal digestion, i.e., gastric phase, intestinal phase, and fraction following dialysis through a cellulose membrane. Data obtained by using methanolic extraction (MeOH) were considered as 100% of phenolics, flavonoids and antioxidant activity contained in apple food matrices. Results of four apple varieties were pooled. Data shown represent means ± SD ($n = 12$ independent experiments). [b]$p < 0.01$, [c]$p < 0.001$ significantly different (2-tailed, following ANOVA and Bonferroni *post hoc* test) from apple methanolic extracts. [†]$p < 0.001$ significantly different from dialysable antioxidants.

enzymes (lipase and pancreatin, with the latter also having amylase and protease activity) on the complex food matrix, facilitating the release of phenolics bound to the matrix.

No anthocyanins could be measured following the gastric and intestinal digestion, suggesting their degradation after the transition from the acidic gastric to the mild alkaline intestinal environment, and the impact of bile acids and pancreatin. Sensitivity of anthocyanins to neutral or slightly alkaline pH has already been pointed out in several studies (McDougall et al., 2005; Tagliazucchi et al., 2010). For instance, *ca.* 80% of bioaccessible grape anthocyanins during gastric digestion were lost after intestinal digestion (Tagliazucchi et al., 2010). The complete loss (100%) of anthocyanins in the present study (Table 1) is possibly due to lower amounts of anthocyanins present in apples (1.04–1.14 mg/ 100 g) compared to grapes (*ca.* 6 mg/100 g). In a recent study, it has been reported that most apple-derived polyphenols appear to be stable in the gastric medium, with the exception of procyanidin B_2 (a flavanol). However, anthocyanins were not studied within this investigation (Kahle et al., 2011).

Despite that health beneficial effects of anthocyanins have been questioned due to their instability, it has also been suggested that under *in vivo* conditions, anthocyanins could be absorbed directly from the stomach (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004), a condition which could not be simulated by the *in vitro* model used. However, even when unabsorbed, the bioaccessibility of antioxidants including anthocyanins in the stomach producing a wide variety of reactive species such as reactive oxygen species (ROS) is of great interest (Halliwell, Rafter, & Jenner, 2005). In general, both stomach and GI tract are constantly exposed to ROS produced, amongst others, by host-related factors, such as by respiratory bursts of immune cells following their activation by diet-derived bacteria and toxins (Halliwell et al., 2005). Therefore, antioxidants may play a role in the GI tract by maintaining redox equilibrium against harmful oxidants, preventing GI tract diseases linked to ROS generation during digestion processes. Our results have demonstrated that phenolics released during simulated GI digestion were

able to reduce free radicals either by hydrogen donation, as assessed by ABTS test, or by electron donation, as assessed by the FRAP test (Figs 3 and 4), possibly favouring hydrogen atom transfer mechanisms, requiring lower energy (Leopoldini, Russo, & Toscano, 2011). In the present study, total antioxidant activities in both gastric and intestinal digesta were lower than those determined by methanolic extracts, presumably due to the lower concentrations of polyphenols present compared to chemical extraction (Fig. 5). This was emphasised also in earlier studies indicating positive correlations between total phenolics and flavonoids, and total antioxidant activity (Bouayed, Rammal, Dicko, Younos, & Soulimani, 2009; Bouayed et al., 2007).

In addition to their concentration, pH could also play a role in the antioxidant activity of phenolics (Bouayed & Bohn, 2010). It has been suggested in a previous *in vitro* study that grape phenolics exert increased protective effects in the intestine compared to the stomach, as individual phenolics including quercetin and resveratrol in grape extracts displayed higher antioxidant activities as measured by ABTS test in the neutral to slightly alkaline condition of the intestine compared to the gastric phase (Tagliazucchi et al., 2010). It is thought that the transition from acidic to alkaline environment enhances the antioxidant power of phenolics by causing deprotonation of the hydroxyl moieties present on their aromatic rings. Regarding the pH condition of antioxidant activity tests performed in our study, we hypothesise that the FRAP test (conducted at a buffered pH of 3.6) could be more appropriate to evaluate antioxidant activity in the gastric digesta, rather than intestinal digesta. However, the ABTS assay (conducted at a buffered pH of 7.4) could be more appropriate to evaluate the intestinal digesta with a pH of *ca.* 7–7.5.

In addition to the dependency of phenolic activity on surrounding conditions, such as pH, interaction of phenolics with other dietary constituents released during digestion, such as iron, other minerals, dietary fibre or proteins may occur, all of which are known to impact polyphenol solubility and availability (Argyri, Komaitis, & Kapsokefalou, 2006; Manach et al., 2004; Saura-Calixto et al., 2007). Likewise, these factors would impact their antioxidant potential; for instance, it was shown that antioxidant activity of free phenols is higher than iron–phenol chelates (Argyri et al., 2006; El Hajji, Nkhili, Tomao, & Dangles, 2006). Furthermore, the chemical structure of phenolics also plays a role in the free radical-scavenging activity, which is mainly depending on the number and position of hydrogen-donating hydroxyl groups on the aromatic rings of the phenolic molecules (Rice-Evans, Miller, & Paganga, 1996). In this sense, it is well known that aglycones display an antioxidant power higher than their glycosides (Lee et al., 2003).

Based on our dialysis experiments, we noted a further significant reduction of phenolics and flavonoids following dialysability through cellulose membranes, chosen as a model of mechanical aspects of the epithelial barrier, by about 25% and 43%, respectively, compared to their concentration in the final GI digesta (Fig. 5). It is thus suggested that bioaccessible phenolics and flavonoids in the final GI digesta were present both in soluble free form as well as partly bound to other constituents, such as proteins or polysaccharides (Manach et al., 2004), or perhaps forming mineral complexes with reduced solubility and therefore dialysability. Compared to amounts natively present in apples, the percentage of dialysable phenolic constituents was 55% for free soluble phenolics and 40% for flavonoids, respectively (Fig. 5). It cannot be entirely excluded that the limited time of interaction between digesta and the membrane, as would be the case *in vivo*, limited further the fraction of dialysable phenolics.

Data from chemical extractions have often been used to estimate the amount of available nutrients and phytochemicals (e.g., polyphenols) from human daily diets or portions (Bouayed et al.,

2009; Brat et al., 2006). Many recommendations on nutritional intake are based on mere content data, not taking into account changes happening during GI digestion. Our results emphasise that chemical extraction could overestimate the availability of active food constituents, i.e., total phenolics and flavonoids, when compared to the free soluble fraction following GI digestion, here by *ca.* 45% and 60%, respectively (Fig. 5). When incorporating the limited availability of polyphenols based on the present results, it may be estimated, for example, that the consumption of 100 g of apples (a portion) can provide free soluble antioxidants equivalent to approx. 180–290 mg vitamin C.

Despite constituting an extension of previous models, our *in vitro* model possesses limitations when compared to true *in vivo* conditions. In spite of constituting a static model (see Section 1), we used a single cellulose membrane as a model for the epithelial barrier, with an arbitrarily chosen molecular cut-off and non-physiological material, which could compromise diffusion also by molecular interactions, such as electrostatic interactions, or polar–polar interactions with the membrane. The feasibility of using a cellulose membrane as a model for the epithelial barrier, similar to that used earlier for minerals, would be further based on the assumption that the majority of polyphenols is taken up by the epithelial gut cells unchanged.

Indeed, cellular uptake of aglycones has been suggested to occur in their native form by passive diffusion (Manach et al., 2004), but may occur also by a sodium-dependent saturable transport mechanism, at least for some phenolics, such as for cinnamic and ferulic acid (Ader, Grenacher, Langguth, Scharrer, & Wolffram, 1996). Before further transportation, native forms of polyphenols (esters, glycosides or polymers) are hydrolysed by intestinal enzymes, such as β-glucosidase (Hollman, de Vries, van Leeuwen, Mengelers, & Katan, 1995; Manach et al., 2004). Absorption of glucosides has been suggested to occur in their native form by using the sodium-dependent glucose transporter SGLT1, prior to their further cleavage by the cytosolic β-glucosidase (Hollman et al., 1995; Manach et al., 2004). Alternatively, extracellular hydrolysis of glucosides by the brush-border-active phloridzin hydrolase has also been proposed by Day et al. (2000).

Thus, as in all *in vitro* tests, our model only simulated several *in vivo* aspects; however, we suggest that the model presented constitutes a useful approach for determining the free soluble fraction of polyphenols potentially available for further uptake and could more realistically reflect the bioaccessible fraction than merely determining recovery of remaining polyphenols following GI digestion. In addition, our extended model for simulating GI digestion including dialysability could be a useful tool for coupling the dialysable fraction with cell lines without further purification steps, such as filtration or centrifugation.

In conclusion, results following *in vitro* GI digestion including dialysability, using apple fruit as food matrix, were statistically significantly lower than those obtained by chemical extraction; about 30% and 45% for phytochemicals and antioxidant capacity, respectively, in the GI phase, being of similar magnitude as reported in an earlier study with grapes (Tagliazucchi et al., 2010). Furthermore, free soluble antioxidants crossing a cellulose membrane and thus potentially available for further uptake, were significantly lower than the amount of antioxidants bioaccessible in the intestinal digesta, by approximately an additional 25%. Although the data obtained with this extended model of simulated *in vitro* GI digestion cannot be directly extrapolated to human *in vivo* conditions, we suggest that this model could be helpful for investigating mechanistic effects, such as food matrix, or host-related factors, such as enzyme concentrations impacting polyphenol bioaccessibility and to some extent uptake. Further investigations with phenolic constituents, employing dialysability, perhaps coupled together with cellular models such as Caco-2, are warranted.

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