Bioaccessibility, bioactivity and cell metabolism of dark chocolate phenolic compounds after in vitro gastro-intestinal digestion

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Abstract

The bioaccessibility of phenolic compounds after *in vitro* gastro-intestinal digestion of dark chocolate, dark chocolate enriched with Sakura green tea and dark chocolate enriched with turmeric powder was studied. The phenolic profile, assessed by accurate mass spectrometry analysis, was modified during *in vitro* gastro-intestinal digestion, with a considerable decrease of total and individual phenolic compounds. Phenolic acids showed the highest bioaccessibility with hydroxycinnamic acids displaying higher bioaccessibility (from 41.2% to 45.1%) respect to hydroxybenzoic acids (from 28.1% to 43.5%). Isomerisation of caffeoyl-quinic acids and galloyl-quinic acids as well as dimerization of (epi)gallocatechin were also observed after *in vitro* gastro-intestinal digestion. Antioxidant activity increased after the gastric step and rose further at the end of the digestion. Furthermore, *in vitro* digested phenolic-rich fractions showed anti-proliferative activity against two models of human colon adenocarcinoma cell lines. Cell metabolism of digested phenolic compounds resulted in the accumulation of coumaric and ferulic acids in the cell media.

Keywords: mass spectrometry, *in vitro* digestion, cell metabolism, functional foods, Caco-2, SW480

1. Introduction

Cocoa and cocoa-based products, such as dark chocolate, are widely consumed in several countries and significantly contribute to the daily intake of antioxidants and phenolic compounds in adults and children (Rusconi, & Conti). Recently, our research group comprehensively analysed the phenolic profile of dark chocolate (Martini, Conte, & Tagliazucchi, 2018). More than 140 individual phenolic compounds were identified by accurate mass spectrometry analysis. Flavan-3-ols are the most abundant phenolic compounds in dark chocolate, accounting for around the 64% of total phenolics (Martini et al., 2018).

There are several *in vivo* studies suggesting that cocoa-derived polyphenols may have beneficial effects on markers of cardiovascular disease risk (Del Rio et al., 2013). Short-term randomized clinical trials have demonstrated that dark chocolate intake reduced blood pressure, improved flow-mediated dilation and ameliorated the lipid profile in healthy and hypertensive subjects (Grassi, Lippi, Necozione, Desideri, & Ferri, 2005a; Grassi et al., 2005b; Lin et al., 2016). These effects have been partially attributed to the high flavan-3-ols content of dark chocolate (Engler et al., 2004). Furthermore, dark chocolate intake has been shown to reduce the number of pre-neoplastic lesions in azoxymethane-induced colonic cancer in rats (Hong, Nulton, Shelechi, Hernández, & Nemposeck, 2013; Rodríguez-Ramiro et al., 2011a). The protective effect of dark chocolate against colon cancer may be due to the biological activities of its phenolic compounds through the regulation of several signal transduction pathways and the modulation of gene expression (Carnésecchi et al., 2002; Granado-Serrano et al., 2010; Martín et al., 2010; Rodríguez-Ramiro, Ramos, Bravo, Goya, & Martín, 2011b).

The bioavailability of phenolic compounds differs widely among the different classes. Some phenolic compounds are poorly absorbed (Del Rio et al., 2013) and/or are unstable under the gastro-intestinal tract conditions (Bouayed, Deußer, Hoffmann, & Bohn, 2012; Juániz et al., 2017). Indeed, dark chocolate phenolic compounds are entrapped in a solid food matrix and only the released

compounds are potentially bioavailable and able to exert their beneficial effects in the gastro-intestinal tract or at systemic level (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010; Tagliazucchi, Verzelloni, & Conte, 2012). Therefore, studies carried out with cell culture models using pure phytochemicals (Carnésecchi et al., 2002) or cocoa/chocolate extracts (Rodríguez-Ramiro, et al., 2011b) are unrealistic unless the bioaccessibility and gastro-intestinal tract stability of the phenolic compounds have been well defined. Furthermore, *in vitro* studies did not take into account the stability of tested molecules in cell cultures and their metabolic fate within the cells (Aragonès, Danesi, Del Rio, & Mena, 2017).

This work aimed to investigate the effect of *in vitro* gastro-intestinal digestion on the bioaccessibility of phenolic compounds in dark chocolate and dark chocolate functionalized with Sakura green tea leaves or turmeric powder. In addition, the antioxidant and anti-proliferative activities of *in vitro* digested dark chocolates phenolic compounds against two models of human colonic cell lines were assessed. Finally, the last task was to identify and quantify the main metabolites derived from incubation of *in vitro* digested dark chocolate phenolic compounds with cells.

2. Materials and methods

2.1. Materials

Phenolic compound standards, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS*),* 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ), Folin-Ciocalteau phenol reagent were purchased from Sigma (Milan, Italy). Methanol and formic acid were obtained from Carlo Erba (Milan, Italy). All MS/MS reagents were from Bio-Rad (Hercules, CA, U.S.A.). Chemicals and enzymes for the digestion procedure were purchased from Sigma-Aldrich (Milan, Italy). All the materials and chemicals for cell culture were from Euroclone (Milan, Italy). MTS cell proliferation assay kit was purchased from Promega (Milan, Italy). Solid phase extraction (SPE) columns (C18, 50 μm, 60 Å, 500 mg) were supplied by Waters (Milan, Italy). Three different types of chocolate (dark 70% cocoa (DC), dark 70% cocoa and 8% turmeric (TDC), dark 70% cocoa and 2% Sakura green tea (GTDC)) were bought from a local shop in Modena (Italy). The chocolates were all from the same manufacturer and had the same composition. The ingredients were cocoa mass, sugar, cocoa butter, soya lecithin and natural flavour vanilla. GTDC and TDC were enriched with 2% Sakura green tea leaves and 8% turmeric powder, respectively. Three chocolate bars for each sample were used in this study.

2.2. In vitro gastro-intestinal digestion of dark chocolates and preparation of the chemical extract

For the *in vitro* digestion, the protocol previously developed within the COST Action INFOGEST was followed (Minekus et al., 2014). The procedure consisted of three consecutive steps: oral, gastric and intestinal phases. The three steps were carried out in absence of light. Simulated salivary, gastric, and intestinal fluids (SSF, SGF and SIF) were employed for each step and prepared according to Minekus et al. (2014). Five grams of each type of dark chocolate were melted at 37°C for 10 minutes and then 5 mL of the stock SSF solution and 150 U/mL of porcine α-amylase were added (oral phase of digestion). The samples were shaken for 5 min at 37°C. The

second step of the digestion (gastric phase) was carried out by adding to the bolus 10 mL of SGF. The pH was adjusted to 2.0 with 6 mol/L HCl and supplemented with porcine pepsin (2000 U/mL of simulated gastric fluid). After 2 h of incubation at 37°C, the final intestinal step was carried out by adding 15 mL of SIF (prepared by mixing 10 mL of pancreatic fluid and 5 mL of bile salts). Then, the pH was adjusted to 7.0, supplemented with pancreatin and the samples were incubated at 37°C for 2 h. All samples were immediately cooled on ice, centrifuged at 10000*g* for 20 min at 4°C to eliminate insoluble materials and the supernatant frozen at –80°C for further analysis. The digestions were performed in triplicate.

In addition, phenolic compounds were extracted from each dark chocolate (chemical extract) as reported in Martini et al. (2018). The extractions were performed in triplicate.

Dark chocolate chemical extracts and samples collected at the end of each stage of the *in vitro* digestion procedure were then used for total phenolic compounds and antioxidant activity determinations.

2.3. Identification and quantification of phenolic compounds by liquid chromatography mass spectrometry (LC-ESI-QTOF-MS/MS)

Dark chocolate chemical extracts and *in vitro* digested samples were analysed on Agilent HPLC 1200 Infinity (Agilent Technologies, Santa Clara, CA) equipped with a C18 column (HxSil C18 Reversed phase, 250×4.6 mm, 5 μm particle size, Hamilton Company, Reno, Nevada, USA) as reported in Martini et al. (2018). The mobile phases consisted of (A) H_2O/formic acid (99:1, v/v) and (B) acetonitrile/formic acid (99:1, v/v). After 0.5 min at 4% B, the gradient linearly rose up to 30% B in 60 min. The mobile phase composition was ramped up to 100% B in 1 min and maintained for 5 min in order to wash the column before returning to the initial condition. The flow rate was established at 1 mL/min. After passing to the column, the eluate was split and 0.3 mL/min were directed to a 6520 accurate Q-TOF mass spectrometer (Agilent Technologies, Santa Clara,

CA). Identification of phenolic compounds in all samples was carried out using full scan, data-dependent MS^2 scanning from m/z 100 to 1700. MS operating conditions, calibration curve equations, linearity ranges and limit of quantifications (LOQ) for the different standards are reported in Martini et al. (2018).

Quantitative results were expressed as μmol of compounds per 100 g of chocolate.

2.4. Total phenolic compounds and antioxidant activity assays

Folin-Ciocalteau assay was performed as reported by Singleton, Orthofer, & Lamuela-Raventós (1999). The results were expressed as μmol of gallic acid per 100 g of chocolate.

The antioxidant properties of dark chocolate chemical extracts and *in vitro* digested samples were evaluated performing two different assays. The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and ferric reducing power (FRAP) assays were performed according to the protocols described by Re et al. (1999) and Benzie, & Strain (1996), respectively. The ABTS scavenging capacity and FRAP values were expressed as mmol of trolox equivalent per 100 g of chocolate.

2.5. Preparation of dark chocolate phenolic-rich fractions

Samples collected at the end of the *in vitro* digestion were then passed through a SPE column preconditioned with 4 mL of acidified methanol (containing 0.1% of formic acid), followed by 5 mL of acidified water (containing 0.1% of formic acid). Elution was carried out with acidified water (6 mL) to eliminate the unbound material. Phenolic compounds were then desorbed by elution with 3 mL of acidified methanol. The obtained phenolic-rich extracts were diluted in the cell media and used for the anti-proliferative activity determination. Each sample was extracted in triplicate.

2.6. Cell cultures and anti-proliferative activity of in vitro digested dark chocolate phenolic-rich fractions

132 Human adenocarcinoma Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium
133 (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% antibiotic mix (streptomycin and
134 penicillin) and 2 mmol/L L-glutamine. Caco-2 cells were used for experiments between passage 57
135 and 58. Human adenocarcinoma SW480 cells were cultured in Leibowitz medium supplemented
136 with 10% FBS, 1% antibiotic mix (streptomycin and penicillin) and 2 mmol/L L-glutamine. SW480
137 cells were used for experiments between passage 33 and 34. Cells were maintained at 37°C in a
138 humidified atmosphere of 5% CO_2.
139 Cells were seeded at $5x10^3$/100 µL and $10x10^3$/100 µL for Caco-2 and SW480, respectively, in 96-
140 well plates 24 h before the assay to allow cell adhesion to the bottom of the wells.
141 For the anti-proliferative assays a colorimetric method for the sensitive quantification of viable cells
142 was performed, using MTS assay kit. Different amounts of the *in vitro* digested phenolic-rich
143 fractions were diluted in cell culture media and added to the cell plates for 24 h. At the end of the
144 treatments, the medium was refreshed with 180 µL of culture medium and 20 µL of MTS reagent
145 were added to each well. After 4 h of incubation at 37°C, the absorbance was measured at the
146 wavelength of 490 nm using a microplate reader and results were expressed as IC_{50}. IC_{50} was
147 defined as the concentration of phenolic compounds required to inhibit 50% cell proliferation and
148 expressed as µmol of total phenolic compounds/100 g of chocolate. The IC_{50} values were
149 determined using nonlinear regression analysis and fitting the data with the log (inhibitor) vs.
150 response model generated by GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The
151 amount of phenolic compounds was determined by LC-ESI-QTOF MS/MS analysis as described in
152 section 2.3.
153
154 ***2.7. Liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis of cell media***
155 Caco-2 and SW480 cell lines were incubated with *in vitro* digested dark chocolate (DC) at a
156 concentration corresponding to IC_{50}. After 24 h of incubation at 37°C, cell culture supernatants

were collected and analysed by LC-MS/MS to determine the stability and the metabolism of the dark chocolate phenolic compounds in the cell media. Cell media were extracted according to Sala et al. (2015) and investigated according to Martini, Conte, & Tagliazucchi (2017). Briefly, samples were analysed using a HPLC Agilent 1200 Series system equipped with an Agilent 6300 ion trap mass spectrometer. Separations were performed using a C18 column (HxSil C18 Reversed phase, 250×4.6 mm, 5 μm particle size, Hamilton Company, Reno, Nevada, USA), with an injection volume of 40 μL and elution flow rate of 1 mL/min. The mobile phase composition, the gradient and MS operating conditions are the same as reported in Martini et al. (2017). MS experiments were performed in ESI negative ion mode. Identification of phenolic compounds and metabolites in all samples was carried out using full scan, data-dependent MS^2 scanning from m/z 100 to 1700.

2.8. Statistic

All data are presented as mean ± SD for three replicates for each prepared sample. One-way analysis of variance (one-way ANOVA) with Tukey's post-hoc test was applied using Graph Pad prism 6.0 (GraphPad software, San Diego, CA, U.S.A.). The differences were considered significant with $P<0.05$.

3. Result and discussion

3.1. In vitro bioaccessibility of phenolic compounds in different types of dark chocolate

In our previous work we identified and quantified by high-resolution mass spectrometry 141, 155 and 142 phenolic compounds in dark chocolate (DC), dark chocolate enriched with Sakura green tea (GTDC) and dark chocolate enriched with turmeric powder (TDC), respectively (Martini et al., 2018). In this work, we present data regarding the release and bioaccessibility of dark chocolate total and individual phenolic compounds following *in vitro* gastro-intestinal digestion. **Figure 1** shows the impact of *in vitro* gastro-intestinal digestion on total phenolic compounds. The chemical extract of GTDC showed a significant higher amount ($P<0.05$) of total polyphenols (20090.58 ± 760.92 μmol gallic acid equivalent/100 g of dark chocolate) respect to TDC (17887.63 ± 556.33 μmol gallic acid equivalent/100 g of dark chocolate) and DC (15425.27 ± 660.47 μmol gallic acid equivalent/100 g of dark chocolate). After salivary phase, only 8.9%, 7.8% and 10.2% of total phenolic compounds were released from the food matrices in DC, GTDC and TDC, respectively. The amount of bioaccessible total phenolic compounds increased by 31%, 26.5% and 20.1% in DC, GTDC and TDC, respectively, after two hours of gastric digestion (**Figure 1**). The incubation with pancreatic solution further increased the bioaccessibility of total compounds in the different samples but to a different extent (**Figure 1**). At the end of the entire phase of digestion, the 68.7%, 68.2% and 40.1% of total phenolic compounds from DC, GTDC and TDC, respectively, were bioaccessible, while the remaining were degraded or not extracted from the solid matrices. From a quantitative point of view, GTDC showed a significant higher amount ($P<0.05$) of total bioaccessible polyphenols (13709.31 ± 377.47 μmol gallic acid equivalent/100 g of dark chocolate) respect to DC (10599.88 ± 213.43 μmol gallic acid equivalent/100 g of dark chocolate). TDC showed the lowest amount ($P<0.05$) of total bioaccessible phenolic compounds (7172.14 ± 512.02 μmol gallic acid equivalent/100 g of dark chocolate). These results are in agreement with previously reported data showing that the gastro-intestinal tract behaved as an extractor promoting the release

of phenolic compounds from solid food matrices (Blancas-Benitez, Pérez-Jiménez, Montalvo-González, González-Aguilar, & Sáyago-Ayerdi, 2018; Tagliazucchi et al., 2010; Tagliazucchi et al., 2012). However, other studies found a decrease in bioaccessible total phenolic compounds during the intestinal digestion (Bouayed et al., 2012; Lingua, Wunderlin, & Baroni, 2018). The different results can be related to the higher stability of dark chocolate phenolic compounds to the intestinal conditions respect to the other foods tested or to a different food matrix effect. However, it should be taken into account that the Folin-Ciocalteau assay is strongly subject to interferences, especially from sugars and vitamin C (Singleton et al., 1999). On the other hand, dark chocolate is rich in Maillard reaction products that react in a concentration-dependent manner with the Folin-Ciocalteau reagent, possibly resulting in an overestimation of bioaccessible total phenolic compounds (Verzelloni, Tagliazucchi, & Conte, 2007).

Figure 2 and **Tables 1-6** show how the *in vitro* gastro-intestinal digestion modified the phenolic compounds profile in the samples from a qualitative and quantitative point of view. The MS data of the individual phenolic compounds are reported in Martini et al. (2018). A total of 78, 122 and 86 phenolic compounds were identified by accurate mass spectrometry analysis after *in vitro* gastro-intestinal digestion of DC, GTDC and TDC, respectively. This means that 45%, 21% and 39% of individual phenolic compounds were not bioaccessible in DC, GTDC and TDC, respectively. A significant lower amount of phenolic compounds was observed in all the samples after simulated gastro-intestinal digestion respect to the chemical extracts. In TDC, only 17.6% of the total amount of phenolic compounds was released from the food matrix or not degraded during digestion. In DC and GTDC, the amount of bioaccessible total phenolic compounds at the end of the digestion was 23.0% and 23.2%, respectively (**Figure 2H** and **Table 6**).

The apparent lowest bioaccessible value of phenolic compounds in TDC was ascribed to the poor bioaccessibility (0.24%) of curcuminoids (**Table 4**).

222 Among the different phenolic classes, phenolic acids showed the highest bioaccessibility (**Figure**
223 **2B** and **2G)** with hydroxycinnamic acids displaying higher bioaccessibility (from 41.2% to 45.1%)
224 than hydroxybenzoic acids (from 28.1% to 43.5%). These compounds were efficiently released
225 from the food matrices and stable under gastro-intestinal conditions. When the effect of gastro-
226 intestinal digestion in coffee and cardoon was studied, chlorogenic acids were proved to be quite
227 stable (Juániz et al., 2017; Monente et al., 2015). Similarly, Tagliazucchi et al. (2010) and Bouayed
228 et al. (2012) found that caffeic and coumaric acids were quite stable during *in vitro* gastro-intestinal
229 digestion. On the other hand, Bouayed et al. (2012) observed a bioaccessibility of 31.6%-56.5% of
230 hydroxycinnamic acids in selected apple varieties following *in vitro* gastro-intestinal digestion.
231 Hydroxycinnamic acid-aspartate derivatives were the most bioaccessible hydroxycinnamic acids in
232 the tested dark chocolates (**Table 2**). Ferulic acid (the most abundant hydroxycinnamic acid in dark
233 chocolates) was detected in lower concentration in the intestinal environment respect to
234 hydroxycinnamic acid-aspartate derivatives (**Table 2**). Coumaric acid was the only
235 hydroxycinnamic acid recovered in the intestinal media at higher concentrations than its initial
236 content in the samples. Coumaric acid and in general simple hydroxycinnamic acids are known to
237 be strongly bound to fibers, such as cellulose, hemicellulose, lignin and pectin (Juaniz et al., 2016
238 and 2017). However, Blancas-Benitez et al. (2015) found that hydroxycinnamic and
239 hydroxybenzoic acids were efficiently released from mango dietary fiber during *in vitro* gastro-
240 intestinal digestion. Indeed, a loss of an OH-group in the phenolic ring of di-hydroxycinnamic acid
241 isomers resulting in the formation of coumaric acid could be hypothesized, as already suggested by
242 Juaniz et al. (2017).
243 In addition, some isomerization reactions took place during *in vitro* gastro-intestinal digestion.
244 Isomerization from 5-caffeoylquinic acid to 3-caffeoylquinic acid and 4-caffeoylquinic acid is
245 highly pH-dependent and may occur during the intestinal step of the digestion process (alkaline pH)
246 (Bermúdez-Soto, Tomás-Barberán, & García-Conesa, 2007; Juaniz et al., 2017). This may explain

247 the appearance of 4-caffeoylquinic acid in all the dark chocolates after *in vitro* gastro-intestinal
248 digestion (**Table 7**). Similarly, other isomerization reactions might take place among galloylquinic
249 acid isomers as observed in GTDC subjected to *in vitro* digestion (**Table 5**).
250 Flavan-3-ols were the dominant class of phenolic compounds in the tested dark chocolates.
251 However, due to their low bioaccessibility, hydroxycinnamic acids dominated the phenolic profile
252 in *in vitro* digested chocolates, with the only exception of GTDC (**Figure 2A** and **Table 1**). While
253 the monomeric flavan-3-ols appeared to be in some way bioaccessible, the recovered amount of
254 procyanidins was extremely low and most of them were not found in the intestinal environment.
255 (Epi)gallocatechin isomers were only detected after *in vitro* digestion of GTDC probably because
256 they were present in higher concentration in GTDC respect to the other dark chocolate samples. The
257 high instability of catechins and procyanidins had been reported earlier (Bouayed et al. 2012). In a
258 previous study, procyanidin B2 was almost completely degraded into the monomeric epicatechin
259 during gastric digestion (Kahle et al., 2011). The degradation of procyanidin B2, epicatechin and
260 catechin into unknown degradation products in artificial intestinal conditions was also observed
261 (Kahle et al., 2011; Zhu et al., 2002; Bouayed et al. 2012). In another study, epigallocatechin and
262 epigallocatechin gallate were found to be sensitive to gastro-intestinal digestion with less than 10%
263 recovery after *in vitro* digestion of green tea (Green, Murphy, Schulz, Watkins, & Ferruzzi, 2007).
264 Some new compounds appearing in the intestinal environment may be indicative of catechin
265 monomers degradation (**Table 7**). For example, trihydroxybenzene may be originated from the B-
266 ring of (epi)gallocatechin and epigallocatechin gallate. Indeed, after *in vitro* digestion of GTDC two
267 new compounds were detected and identified as (epi)gallocatechin homodimers (theasinensin
268 isomer and P2 analogue) (Neilson et al., 2007). Finally, the highest bioaccessibility of
269 (epi)gallocatechin isomers and the higher content of gallic acid observed after digestion of GTDC,
270 respect to the contents found in the chemical extract, could be explained as a consequence of
271 hydrolysis of epigallocatechin gallate.

3.2 Effect of in vitro digestion on antioxidant activities

In order to study how the antioxidant activity of the dark chocolate samples was modified throughout the digestive process, antioxidant activity was determined by FRAP and ABTS assays at each stage of the *in vitro* gastro-intestinal digestion and in the chemical extracts (**Figure 3A and B**). The GTDC chemical extract was the sample with the highest activity for both the assays (15.4 ± 1.4 mmol trolox/100 g chocolate in ABTS and 15.4 ± 0.8 mmol trolox/100 g chocolate in FRAP). In general, it was observed that the two different assays gave similar trends for the distinct tested samples during the gastro-intestinal digestion. For all the tested samples, the antioxidant activity after the salivary phase was significantly lower than the antioxidant activity of the chemical extracts, in accordance with the low total phenolic content extracted after this step. Antioxidant activity increased after the gastric step of the digestion and further rose after the intestinal step. The 91.5%, 74.0% and 80.1% of DC, GTDC and TDC antioxidant activities, respectively, was observed after intestinal digestion respect to the chemical extracts with the ABTS assay. The FRAP assay recovered lower antioxidant activity than those observed with the ABTS assay and equal to 37.6%, 35.1% and 38.0% of DC, GTDC and TDC, respectively. Beside phenolic compounds, dark chocolate also contains other well-known antioxidants such as Maillard reaction products that can be formed during chocolate high temperature processes such as drying, roasting and conching (Quiroz-Reyes, & Fogliano, 2018). Differences between FRAP and ABTS values could be explained by considering that Maillard reaction products show a high chain-breaking activity despite their low reducing potential (Di Mattia, Sacchetti, Mastrocola, & Serafini, 2017). At the end of the gastro-intestinal digestion, GTDC displayed the highest amount of antioxidant activity in both the assays (11.4 ± 0.1 mmol trolox/100 g chocolate in ABTS and 5.4 ± 0.5 mmol trolox/100 g chocolate in FRAP).

These results are consistent with previous studies, where an increase in antioxidant activity was observed during digestion of grapes (Tagliazucchi et al., 2010), fruits (Tagliazucchi et al., 2012) and fruit extracts (Pavan, Sancho, & Pastore, 2014). However, other authors reported a large decrease in antioxidant activity after digestion of different foods (Garbetta et al., 2018; Lingua et al., 2018; Wang, Amigo-Benavent, Mateos, Bravo, & Sarriá, 2017). Multiple factors such as assay conditions, solubility and matrix effect may affect the antioxidant activity of foods and phenolic compounds during *in vitro* digestion. In any case, the antioxidant potential of dietary phenolic compounds in the intestinal tract, independently from their bioavailability, could offer protection by scavenging reactive oxygen species and reducing the oxidative stress at the intestinal cells level (Lingua et al., 2018 Tagliazucchi et al., 2010).

3.3 Anti-proliferative activity of in vitro digested dark chocolate and dark chocolate enriched with Sakura green tea or turmeric powder phenolic-rich fractions on human colon adenocarcinoma cell lines

The anti-proliferative activity of DC, GTDC and TDC phenolic-rich fractions extracted at the end of the *in vitro* gastro-intestinal digestion on the growth of human colon adenocarcinoma Caco-2 and SW-480 cells was investigated. Caco-2 and SW480 cells were incubated with different concentrations of phenolic-rich extracts ranging from 7 to 170 μmol/100 g of chocolate for 24 h. As shown in **Figure 4**, the inhibition was similar between DC and GTDC phenolic-rich fractions against Caco-2 cells. This result was not surprising, considering the similar phenolic profile of the two dark chocolates after in *vitro* gastro-intestinal digestion. However, when TDC phenolic-rich fraction was tested against Caco-2 cell line, a significantly lower IC_{50} value (which means a higher anti-proliferative activity) was found in comparison with the other two tested dark chocolates. All of the samples showed a higher anti-proliferative activity against SW480 respect to Caco-2.

320 According to the literature, there are no reports regarding the anti-proliferative activity of dark
321 chocolate after *in vitro* digestion.
322 Previous *in vitro* studies have shown anti-proliferative properties of procyanidin and procyanidin-
323 enriched extract isolated from cocoa powder in colon cancer Caco-2 cells (Carnesecchi et al., 2002;
324 Martin and Ramos, 2017). However, 24 h treatment of Caco-2 and SW480 with catechin,
325 epicatechin or procyanidin B2 did not affect cell growth, suggesting that other compounds rather
326 than flavan-3-ols can be responsible for the observed anti-proliferative effect of phenolic-rich
327 fractions extracted at the end of the *in vitro* gastro-intestinal digestion (Ramos, Rodríguez-Ramiro,
328 Martín, Goya, & Bravo, 2011). The highest effect of TDC phenolic-rich fraction against Caco-2 cell
329 line can be ascribed to the anti-proliferative activity of curcuminoids or a synergistic effect between
330 curcuminoids and other phenolic compounds (Iwuchukwu, Tallarida, & Nagar, 2011).
331
332 ***3.4. In vitro metabolism of digested dark chocolate phenolic-rich fraction in cell cultures***
333 In order to verify the cell metabolism of dark chocolate phenolic compounds, Caco-2 and SW480
334 media were analysed by LC-MS ion trap after incubation (24 h) with *in vitro* digested dark
335 chocolate (at concentration corresponding to IC_{50}). Different metabolic reactions, including
336 (de)hydroxylation, (de)hydrogenation, and conjugation with methyl, glucuronide, sulphate, and
337 glutathione moieties were monitored. Some parent compounds and newly formed metabolites were
338 detected in both cell types and reported in **Table 8**.
339 In addition to the parent compounds catechin and epicatechin, two newly formed metabolites were
340 tentatively identified. Methyl-(epi)catechin was found in the cell media of both the cell lines
341 whereas dimethyl-(epi)catechin was found only in Caco-2 medium. Previous studies identified
342 methyl-epicatechin and sulphate-epicatechin as the main metabolites in Caco-2 experiments with a
343 prevalence of methylation (Aragonès et al., 2017; Sanchez-Bridge et al., 2015). The lack of
344 identification of sulphate metabolites of (epi)catechin could be due to their low concentration in the

media (i.e. they could be formed but were below the limit of detection) or to the inhibition of the specific enzymes as a consequence of the presence of other phenolic compounds. Sanchez-Bridge et al. (2015) showed that the co-administration of epicatechin with flavonols, flavones and isoflavones reduced the metabolism of epicatechin (especially sulphation) in Caco-2. Despite the appearance *in vivo* of glucuronidated epicatechin metabolites, we did not find these substituted metabolites under our experimental conditions. Previous studies suggested the absence of specific uridine 5'-diphospho-glucuronosyl-transferase isoforms able to form glucuronic acid conjugate of epicatechin in Caco-2 cells (Actis-Goretta et al., 2013; Sanchez-Bridge et al., 2015).

The main hydroxycinnamic acid derivatives found after *in vitro* gastro-intestinal digestion of dark chocolate were the conjugated forms with amino acids (such as aspartate and tyrosine, **Table 2**). With the exception of trace amounts of feruloyl-aspartate (found in the media of both cell lines), we were not able to identify these compounds after incubation with the two cell lines. Diversely, we found ferulic and coumaric acids in the media of both cell lines and caffeic acid only after incubation with SW480 cells. A sulphated form of coumaric acid and dihydro-ferulic acid were tentatively identified as newly formed metabolites in the cell culture media after incubation with SW480 and Caco-2, respectively. The concentration of coumaric acid increased from 5.10 ± 0.12 after *in vitro* digestion to 86.14 ± 3.19 and 96.80 ± 4.96 μmol/100 g of chocolate after 24 h of incubation with Caco-2 and SW480, respectively (**Table 2** and **Table 8**). The increased amount of coumaric acid may derive from hydroxylation of cinnamic acid (which was present in the dark chocolate after *in vitro* gastro-intestinal digestion but not in the cell culture media, data not shown), dehydroxylation of caffeic acid or dehydrogenation of dihydro-coumaric acid, as already suggested by Poquet, Clifford, & Williamson (2008) for dihydro-ferulic acid. Alternatively, a hydrolysis of coumaroyl-aspartate and/or coumaroyl-tyrosine, catalysed by membrane-bound carboxypeptidases, may be hypothesized. Indeed, coumaric acid has been found particularly stable when incubated with Caco-2 or rat hepatic cells (Kahle et al., 2011; Kern et al., 2003). After 24 h of incubation with

370 Caco-2 an increased amount of ferulic acid respect to the concentration found at the end of the
371 digestion was detected (**Table 2** and **Table 8**). Methylation of caffeic acid by catechol-*O*-
372 methyltransferase may account for the increase in ferulic acid concentration (Kern et al., 2003).
373 This conclusion is supported also by the evidence of the disappearance of caffeic acid from the
374 medium. Indeed, methylation of di-hydro-caffeic acid may account for the appearance of di-hydro-
375 ferulic acid in the medium, as already suggested in Caco-2 cells by Poquet et al. (2008).
376 The same conclusions can not be drawn for SW480. In the medium of this cell line we found some
377 residual caffeic acid and the amount of ferulic acid did not increase during incubation (**Table 2** and
378 **Table 8**). Indeed, we did not identify di-hydro-ferulic acid in the medium of SW480. This evidence
379 suggested that caffeic and di-hydro-caffeic acid were not substrates for the catechol-*O*-
380 methyltransferase in SW480, despite its presence as indicated by the appearance of methylated
381 (epi)catechin as reported above. Therefore, hydroxycinnamic acids metabolism under our
382 experimental conditions resulted in the accumulation of coumaric and ferulic acids in cell media
383 with only minor phase II metabolism. **Figure 5** reported the hypothetical pathways of
384 hydroxycinnamic acids metabolism leading to the accumulation of coumaric and ferulic acids.
385 Quercetin-hexoside and quercetin-pentoside were tentatively identified after 24 h of incubation with
386 SW480 cell line, despite their low concentration in the sample after *in vitro* gastro-intestinal
387 digestion. This is indicative of their relative stability in cell culture medium as already suggested by
388 Xiao, & Högger (2015). Instead, quercetin-hexoside was not identified after 24 h of incubation with
389 Caco-2. The aglycone quercetin, which was not present in dark chocolate after *in vitro* gastro-
390 intestinal digestion, appeared after incubation with Caco-2, suggesting that this cell line was able to
391 de-glycosylate quercetin-hexoside releasing the corresponding aglycone. De-glycosylation of
392 flavonoid glycosides can be catalysed by the action of membrane-bound lactase phloridzin
393 hydrolase and/or cytosolic β-glucosidase (Németh et al., 2003). Some previous studies failed to
394 detect de-glycosylation of quercetin-glucoside by using Caco-2 cells (del Mar Contreras, Borrás-

395 Linares, Herranz-López, Micol, & Segura-Carretero, 2015; Walgren, Walle, & Walle, 1998).
396 However, Caco-2 cells express both lactase phloridzin hydrolase and cytosolic β-glucosidase
397 (Németh et al., 2003). This discrepancy can be due to the shorter incubation time in the previous
398 studies (1-2 h vs 24 h in our study). Quercetin was not identified in SW480 cell culture medium,
399 suggesting that this cell line was not able to hydrolyse quercetin-hexoside.
400 Finally, one methylated derivative of ellagic acid was tentatively identified only in the SW480 cell
401 medium.
402

403 **4. Conclusions**

404 Bioactivity of phenolic compounds is primarily conditioned by their bioaccessibility in the gastro-
405 intestinal tract, and secondly by their cellular uptake and internal transformation. The present study
406 determined the amounts of bioaccessible dark chocolate phenolic compounds after gastro-intestinal
407 digestion. We have demonstrated that gastro-intestinal digestion modified the phenolic profile in the
408 samples from a qualitative and quantitative point of view.
409 We have also demonstrated that Caco-2 and SW480 cell lines showed metabolic activity resulting
410 in a partial modification of dark chocolate phenolic compounds leading to the accumulation of
411 coumaric and ferulic acids in the cell media. The observed anti-proliferative activity could be
412 related to the accumulation of these simple hydroxycinnamic acids. The presence of ferulic acid and
413 quercetin in Caco-2 cell medium at higher concentration than in SW480 cell medium or the
414 presence of caffeic acid only in SW480 cell medium may suggest the intrinsic differences between
415 the two cell lines and the metabolic mechanisms involved. Further studies are necessary in order to
416 confirm the proposed pathways of metabolism of hydroxycinnamic acids during incubation with
417 cell lines and their potential anti-proliferative activity.
418 The addition of green tea leaves or turmeric powder in dark chocolate recipe lead to a modification
419 of dark chocolate healthy properties. Functionalization with green tea leaves resulted in a higher

amount of flavan-3-ols and flavonols after *in vitro* digestion than dark chocolate, achieving a more efficient antioxidant activity. Similarly, the addition of turmeric powder may lead to an increased anti-proliferative activity against adenocarcinoma cell lines respect to DC and GTDC.

In this way, the potential healthy effect of dark chocolate consumption could be maximized, reducing the amount of energy and calories introduced with chocolate itself and resulting in a lower intake to achieve the same biological effects.

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References

Actis-Goretta, L., Lévèques, A., Rein, M., Teml, A., Schäfer, C., Hofmann, U., Li, H., Schwab, M., Eichelbaum, M., & Williamson, G. (2013). Intestinal absorption, metabolism, and excretion of (–)-epicatechin in healthy humans assessed by using an intestinal perfusion technique. *American Journal of Clinical Nutrition, 98*, 924-933.

Aragonès, G., Danesi, F., Del Rio, D., & Mena, P. (2017). The importance of studying cell metabolism when testing the bioactivity of phenolic compounds. *Trends in Food Science & Technology, 69*, 230-242.

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry, 239*, 70-76.

Bermúdez-Soto, M. J., Tomás-Barberán, F. A., & García-Conesa, M. T. (2007). Stability of polyphenols in chokeberry (*Aronia melanocarpa*) subjected to in vitro gastric and pancreatic digestion. *Food Chemistry, 102*, 865–874.

Blancas-Benitez, F. J., Mercado-Mercado, G., Quíros-Sauceda, A. E., Montalvo-González, E., González-Aguilar, G. A., & Sáyago-Ayerdi, S. G. (2015). Bioaccessibility of polyphenols associated with dietary fiber and in vitro kinetics release of polyphenols compounds in Mexico "Ataulfo" mango (*Mangifera indica* L.) by-products. *Food and Function, 6*, 859-868.

Blancas-Benitez, F. J., Pérez-Jiménez, J., Montalvo-González, E., González-Aguilar, G. A., & Sáyago-Ayerdi, S. G. (2018). *In vitro* evaluation of the kinetics of the release of phenolic compounds from guava (*Psidium guajava* L.) fruit. *Journal of Functional Foods, 43*, 139-145.

Bouayed, J., Deußer, H., Hoffmann, L., & Bohn, T. (2012). Bioaccessible and dialyzable polyphenols in selected apple varieties following *in vitro* digestion vs their native pattern. *Food Chemistry, 131*, 1466-1472.

Carnésecchi, S., Schneider, Y., Lazarus, S. A., Cohelo, D., Gossé, F., & Raul, F. (2002). Flavanols and procyanidins of cocoa and chocolate inhibit growth and polyamine biosynthesis of human colonic cancer cells. *Cancer Letters, 175*, 147-155.

del Mar Contreras, M., Borrás-Linares, B., Herranz-López, M., Micol, V., & Segura-Carretero, A. (2015). Further exploring the absorption and enterocyte metabolism of quercetin forms in the Caco-2 model using nano-LC-TOF-MS. *Electrophoresis, 37*, 998-1006.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants & Redox Signaling*, *18*, 1818-1892.

Di Mattia, C. D., Sacchetti, G., Mastrocola, D., & Serafini, M. (2017). From cocoa to chocolate: the impact of processing on in vitro antioxidant activity and the effects of chocolate on antioxidant markers in vivo. *Frontiers in Immunology, 8*, 1207.

Engler, M. B., Engler, M. M., Chen, C. Y., Malloy, M. J., Browne, A., Chiu, E. Y., Kwak, H. K., Milbury, P., Paul, S. M., Blumberg, J., & Mietus-Snyder, M. L. (2004). Flavonoid-rich dark chocolate improves endothelial function and increases plasma epicatechin concentrations in healthy adults. *Journal of American College of Nutrition, 3*, 197–204.

Garbetta, A., Nicassio, L., D'Antuono, I., Cardinali, A., Linsalata, V., Attolico, G., & Minervini, F. (2018). Influence of in vitro digestion process on polyphenolic profile of skin grape (cv. Italia) and on antioxidant activity in basal or stressed condition of human intestinal cell line (HT-29). *Food Research International, 106*, 878-884.

Granado-Serrano, A. B., Martín, M. A., Haegeman, G., Goya, L., Bravo, L., & Ramos, L. (2010). Epicatechin induces NF-kappaB, activator of protein-1 (AP-1) and nuclear transcritpion factor erythroid 2p45-related factor-2 (Nrf2) via phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and extracellular regulated kinase (ERK) signalling in HepG2 cells. *British Journal of Nutrition, 103*, 168-179.

Grassi, D., Lippi, C., Necozione, S., Desideri, G., & Ferri, C. (2005a). Short-term administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons. *American Journal of Clinical Nutrition, 8*, 611–614.

Grassi, D., Necozione, S., Lippi, C., Croce, G., Valeri, L., Pasqualetti, P., Desideri, G., Blumberg, J. B., & Ferri, C. (2005b). Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives. *Hypertension, 46*, 398-405.

Green, R. J., Murphy, A. S., Schulz, B., Watkins, B. A. & Ferruzzi, M. G. (2007). Common tea formulations modulate in vitro digestive recovery of green tea catechins. *Molecular Nutrition and Food Research, 51*, 1152-1162.

Hong, M. Y., Nulton, E., Shelechi, M., Hernández, L. M., & Nemposeck, T. (2013). Effects of dark chocolate on azoxymethane-induced colonic aberrant crypt foci. *Nutrition and Cancer, 65*, 677-685.

Iwuchukwu, O. F., Tallarida, R. J., & Nagar, S. (2011). Resveratrol in combination with other dietary polyphenols concomitantly enhances antiproliferation and UGT1A1 induction in Caco-2 cells. *Life Sciences, 88*, 1047-1053.

Juániz, I., Ludwig, I. A., Bresciani, L., Dall'Asta, M., Mena, P., Del Rio, D., Cid, C., & de Peña, M. P. (2017). Bioaccessibility of (poly)phenolic compounds of raw and cooked cardoon (*Cynara cardunculus* L.) after simulated gastrointestinal digestion and fermentation by colonic microbiota. *Journal of Functional Foods, 32*, 195-207.

Kahle, K., Kempf, M., Schreier, P., Scheppach, W., Schrenk, D., Kautenburger, T., Hecker, D., Huemmer, W., Ackermann, M., & Richling, E. (2011). Intestinal transit and systemic metabolism of apple polyphenols. *European Journal of Nutrition, 50*, 507-522.

Kern, S. M., Bennett, R. N., Needs, P. W., Mellon, F. A., Kroon, P. A., & García-Conesa, M. T. (2003). Characterization of metabolites of hydroxycinnamates in the in vitro model of human

small intestinal epithelium Caco-2 cells. *Journal of Agricultural and Food Chemistry, 51*, 7884-7891.

Lin, X., Zhang, I., Li, A., Manson, J. E., Sesso, H. D., Wang, L., & Liu, S. (2016). Cocoa flavanol intake and biomarkers for cardiometabolic health: A systematic review and meta-analysis of randomized controlled trials. *Journal of Nutrition, 146*, 2325-2333.

Lingua, M. S., Wunderlin, D. A., & Baroni, M. V. (2018). Effect of simulated digestion on the phenolic components of red grapes and their corresponding wines. *Journal of Functional Foods, 44*, 86-94.

Martín, M. A., Serrano, A. B., Ramos, S., Pulido, M. I., Bravo, L., & Goya, L. (2010). Cocoa flavonoids up-regulate antioxidant enzyme activity via the ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. *Journal of Nutritional Biochemistry, 21*, 196-205.

Martini, S., Conte, A., & Tagliazucchi, D. (2017). Phenolic compounds profile and antioxidant properties of six sweet cherry (*Prunus avium*) cultivars. *Food Research International*, *97*, 15-26.

Martini, S., Conte, A., & Tagliazucchi, D. (2018). Comprehensive evaluation of phenolic profile in dark chocolate and dark chocolate enriched with Sakura green tea leaves or turmeric powder. *Food Research International*, *112*, 1-16.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., et al. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food & Function*, 5, 1113 –1124.

Monente, C., Ludwig, I. A., Stalmach, A., de Peña, M. P., Cid, C, & Crozier, A. (2015). In vitro studies on the stability in the proximal gastrointestinal tract and bioaccessibility in Caco-2 cells of chlorogenic acids from spent coffee grounds. *International Journal of Food Sciences and Nutrition, 66*, 657-664.

Neilson, A. P., Hopf, A. S., Cooper, B. R., Pereira, M. A., Bomser, J. A., & Ferruzzi, M. G. (2007). Catechin degradation with concurrent formation of homo- and heterocatechin dimers during *in vitro* digestion. *Journal of Agricultural and Food Chemistry, 55*, 8941-8949.

Németh, K, Plumb, G. W., Berrin, J. G., Juge, N., Jacob, R., Naim, H. Y., Williamson, G., Swallow, D. M., & Kroon, P. A. (2003). Deglycosylation by small intestinal epithelial cell beta-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. *European Journal of Nutrition, 42*, 29–42.

Pavan, V., Sancho, R. A. S., & Pastore, G. M. (2014). The effect of in vitro digestion on the antioxidant activity of fruit extracts (*Carica papaya*, *Artocarpus heterophillus* and *Annona marcgravii*). *LWT-Food Science and Technology, 59*, 1247-1251.

Poquet, L., Clifford, M. N., & Williamson, G. (2008). Investigation of the metabolic fate of dihydrocaffeic acid. *Biochemical Pharmacology, 75*, 1218-1229.

Quiroz-Reyes, C., N., & Fogliano, V. (2018). Design cocoa processing towards healthy cocoa products: The role of phenolics and melanoidins. *Journal of Functional Foods, 45*, 480-490.

Ramos, S., Rodríguez-Ramiro, I., Martín, M. A., Goya, L., & Bravo, L. (2011). Dietary flavanols exert different effects on antioxidant defenses and apoptosis/proliferation in Caco-2 and SW480 colon cancer cells. *Toxicology in Vitro, 25*, 1771-1781.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26,* 1231-1237.

Rodríguez-Ramiro, I., Ramos, S., López-Oliva, E., Agis-Torres, A., Gómez-Juaristi, M., Mateos, R., Bravo, L., Goya, L., & Martín, M. A. (2011a). Cocoa-rich diet prevents azoxymethane-induced colonic preneoplastic lesions in rats by restraining oxidative stress and cell proliferation and inducing apoptosis. *Molecular Nutrition and Food Research, 55*, 1895-1899.

Rodríguez-Ramiro, I., Ramos, S., Bravo, L., Goya, L., & Martín, M. A. (2011b). Procyanidin B2 and a cocoa polyphenolic extract inhibit acrylamide-induced apoptosis in human Caco-2 cells by preventing oxidative stress and activation of JNK pathway. *Journal of Nutritional Biochemistry, 22*, 1186-1194.

Rusconi, M., & Conti, A. (2010). *Theobroma cacao* L., the Food of the Gods: A scientific approach beyond myths and claims. *Pharmacological Research, 61*, 5-13.

Sala, R., Mena, P., Savi, M., Brighenti, F., Crozier, A., Miragoli, M., Stilli, D., & Del Rio, D. (2015). Urolithins at physiological concentrations affect the levels of pro-inflammatory cytokines and growth factor in cultured cardiac cells in hyperglucidic conditions. *Journal of Functional Foods, 15*, 97-105.

Sanchez-Bridge, B., Lévèques, A., Li, H., Bertschy, E., Patin, A., & Actis-Goretta, L. (2015). Modulation of (-)-epicatechin metabolism by coadministration with other polyphenols in Caco-2 cell model. *Drug Metabolism and Disposition, 43*, 9-16.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology, 299,* 152-178.

Tagliazucchi, D., Verzelloni, E., Bertolini, D., & Conte, A. (2010). In vitro bio-accessibility and antioxidant activity of grape polyphenols. *Food Chemistry, 120*, 599-606.

Tagliazucchi, D., Verzelloni, E., & Conte, A. (2012). The first tract of alimentary canal as an extractor. Release of phytochemicals from solid food matrices during simulated digestion. *Journal of Food Biochemistry, 36*, 555-568.

Verzelloni, E., Tagliazucchi, D., & Conte, A. (2007). Relationship between the antioxidant properties and the phenolic and flavonoid content in traditional balsamic vinegar. *Food Chemistry, 105*, 564-571.

Walgren, R. A., Walle, U. K., & Walle, T. (1998). Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells. *Biochemical Pharmacology, 55*, 1721-1727.

Wang, S., Amigo-Benavent, M., Mateos, R., Bravo, L., & Sarriá, B. (2017). Effects of in vitro digestion and storage on the phenolic content and antioxidant capacity of a red grape pomace. *International Journal of Food Sciences and Nutrition, 68*, 188-200.

Xiao, J., & Högger, P. (2015). Stability of dietary polyphenols under the cell culture conditions: avoiding erroneous conclusions. *Journal of Agricultural and Food Chemistry, 63*, 1457–1557.

Zhu, Q. Y., Holt, R. R., Lazarus, S. A., Ensunsa, J. L., Hammerstone, J. F., Schmitz, H. H., & Keen, C. L. (2002). Stability of the flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from cocoa. *Journal of Agricultural and Food Chemistry, 50*, 1700–1705.

Figure captions

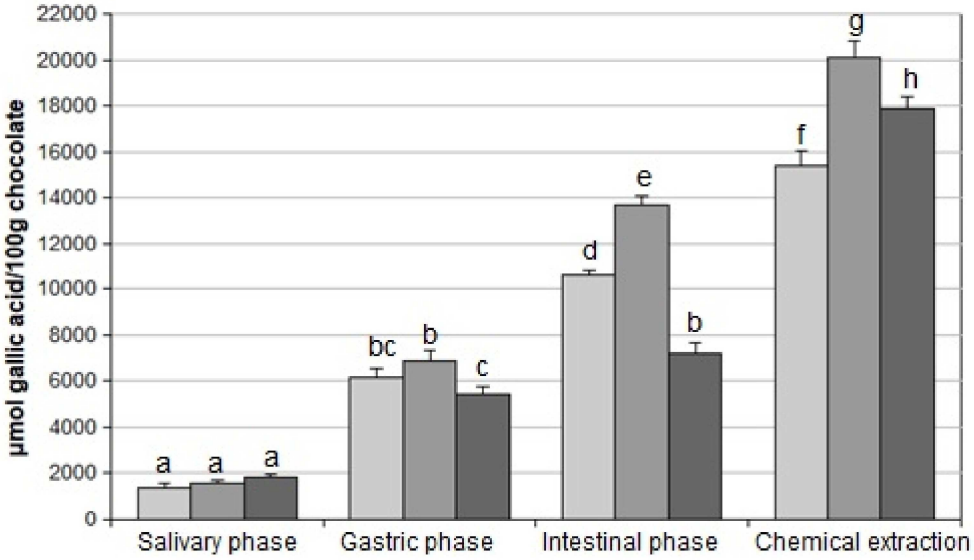
Figure 1. Changes in total phenolic content during *in vitro* gastro-intestinal digestion. Total phenolic content was determined with the Folin-Ciocalteau assay and expressed as μmol of gallic acid equivalents/100 g of chocolate. Light grey columns represent the changes detected during *in vitro* digestion of dark chocolate. Grey columns represent the changes detected during *in vitro* digestion of dark chocolate enriched with Sakura green tea leaves. Dark grey columns represent the changes detected during *in vitro* digestion of dark chocolate enriched with turmeric powder. Results are expressed as mean ± standard deviation. Different letters refer to statistically significant differences ($P<0.05$) in total phenolic compounds content among samples.

Figure 2. Bioaccessibility of individual phenolic compounds identified and quantified by LC-ESI-QTOF MS/MS grouped by classes. (A) Flavan-3-ols; (B) hydroxycinnamic acids; (C) flavonols; (D) other phenolics; (E) flavones; (F) ellagitannins; (G) hydroxybenzoic acids; (H) sum of the different classes. DC identify dark chocolate; GTDC identify dark chocolate enriched with Sakura green tea leaves; TDC identify dark chocolate enriched with turmeric powder. Black columns represent the amount of the individual classes found in the chemical extract whereas grey columns the amount at the end of the digestion.

Figure 3. Changes in antioxidant activity during *in vitro* gastro-intestinal digestion. Antioxidant capacity (expressed as mmol of trolox equivalent/100 g of chocolate), measured by ABTS (A) and FRAP (B) assays. Light grey columns represent the changes detected during *in vitro* digestion of dark chocolate. Grey columns represent the changes detected during *in vitro* digestion of dark chocolate enriched with Sakura green tea leaves. Dark grey columns represent the changes detected during *in vitro* digestion of dark chocolate enriched with turmeric powder digestion. Results are expressed as mean ± standard deviation. Values in the same graph with different lowercase letters are significantly different ($P < 0.05$).

Figure 4. Anti-proliferative activity of phenolic-rich fractions extracted at the end of the *in vitro* digestion. DC identify dark chocolate; GTDC identify dark chocolate enriched with Sakura green tea leaves; TDC identify dark chocolate enriched with turmeric powder. IC_{50} is defined as the concentration of phenolic compounds required to inhibit 50% of cell proliferation. The amount of phenolic compounds was determined by LC-ESI-QTOF MS/MS analysis as described in material and methods. Dark grey columns represent the IC_{50} versus Caco-2 cells. Grey columns represent the IC_{50} versus SW480 cells. Values in with different lowercase letters are significantly different ($P < 0.05$).

Figure 5. Proposed pathways for hydroxycinnamic acids metabolism after incubation with Caco-2 and SW480 cell lines of dark chocolate phenolic-rich fractions extracted at the end of the *in vitro* digestion. COMT: catechol-*O*-methyl transferase; CPase: carboxypeptidase; RA: reduction; DHY: de-hydroxylation; HYD: hydroxylation; SULT: sulfotransferase. Compounds in dark grey boxes were detected both in *in vitro* digested samples and after incubation with cells; compounds in light grey boxes were detected only in *in vitro* digested samples; compounds in white boxes were detected only after incubation with cells. The unbroken arrows indicate previously demonstrated pathways whereas dotted arrows indicate pathways hypothesized in this study. Steps not found in SW480 are indicated. Please note that caffeic acid was found only after incubation with SW480 whereas di-hydro-ferulic acid was found only after incubation with Caco-2.


µmol gallic acid/100g chocolate
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
a
a
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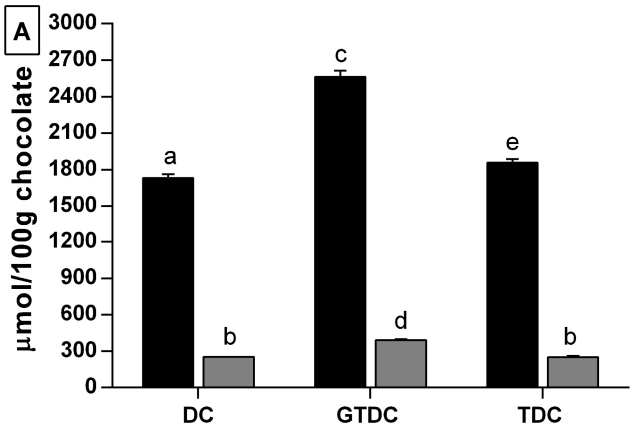
bc
b
c
d
e
b
f
g
h
Salivary phase
Gastric phase
Intestinal phase
Chemical extraction


A
µmol/100g chocolate
3000
2700
2400
2100
1800
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1200
900
600
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a
b
c
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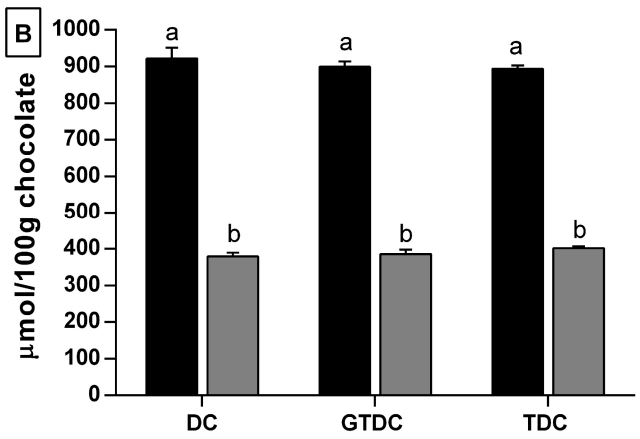
e
b
DC
GTDC
TDC


B
µmol/100g chocolate
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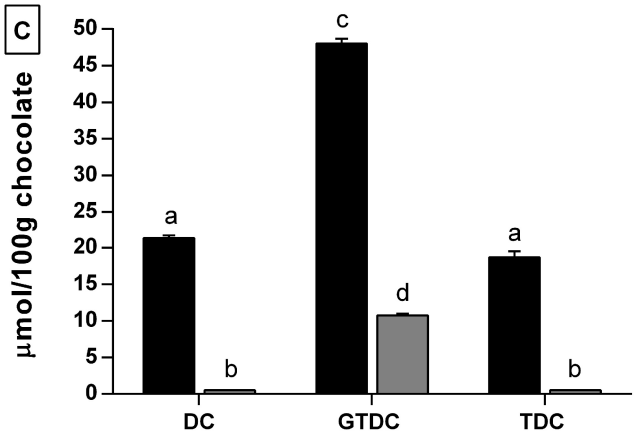
DC
GTDC
TDC


C
µmol/100g chocolate
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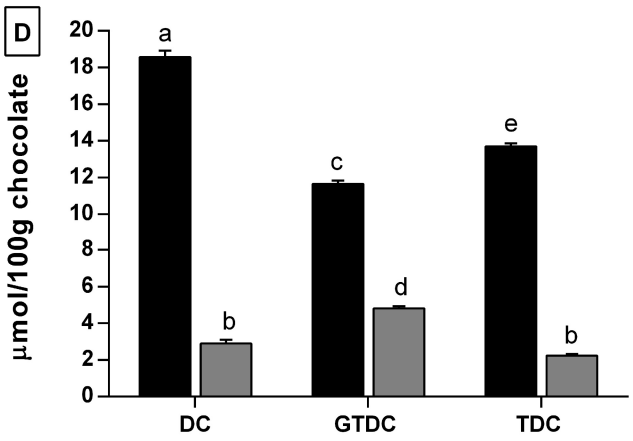
DC
GTDC
TDC


D
µmol/100g chocolate
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18
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12
10
8
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DC
GTDC
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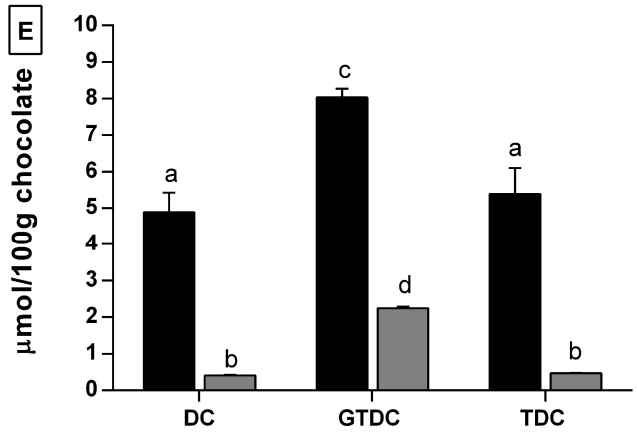
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µmol/100g chocolate
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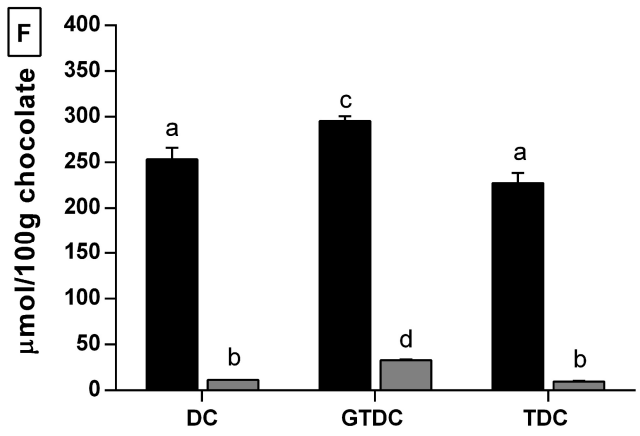
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µmol/100g chocolate
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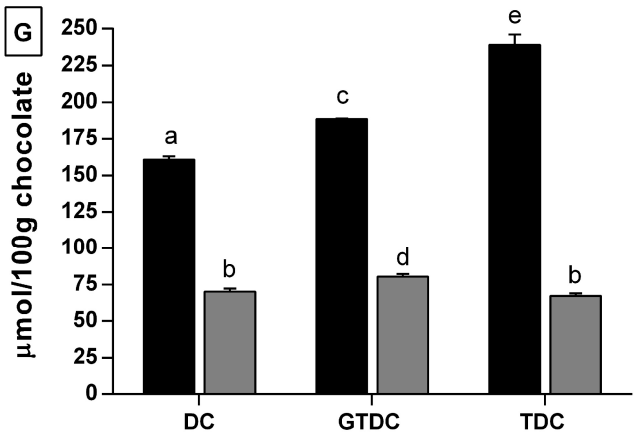
TDC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225
200
175
150
125
100
75
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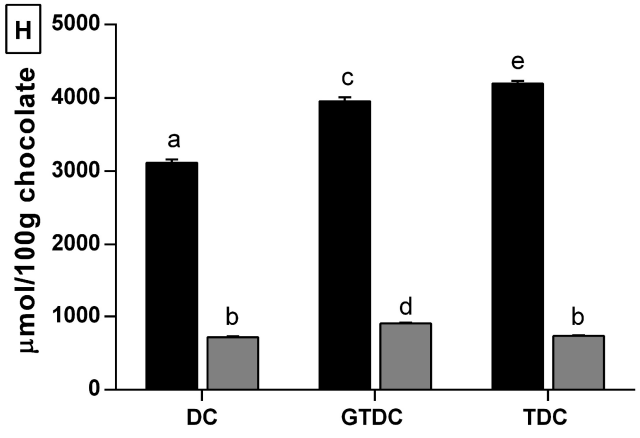
DC
GTDC
TDC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µmol/100g chocolate
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4000
3000
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a
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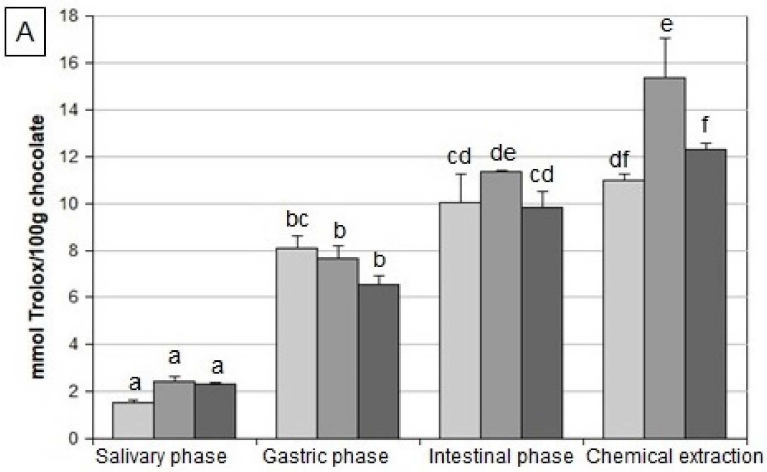
DC
GTDC
TDC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0
2
4
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8
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12
14
16
18
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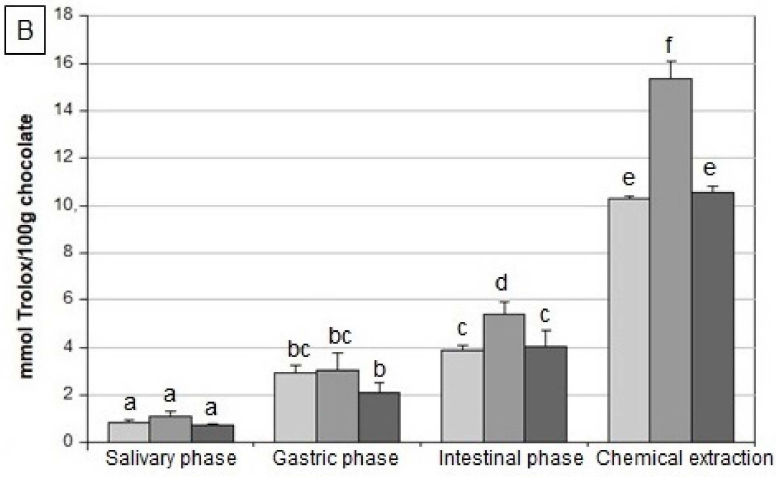
cd
df
e
f
Salivary phase
Gastric phase
Intestinal phase
Chemical extraction


B
mmol Trolox/100g chocolate
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8
10,
12
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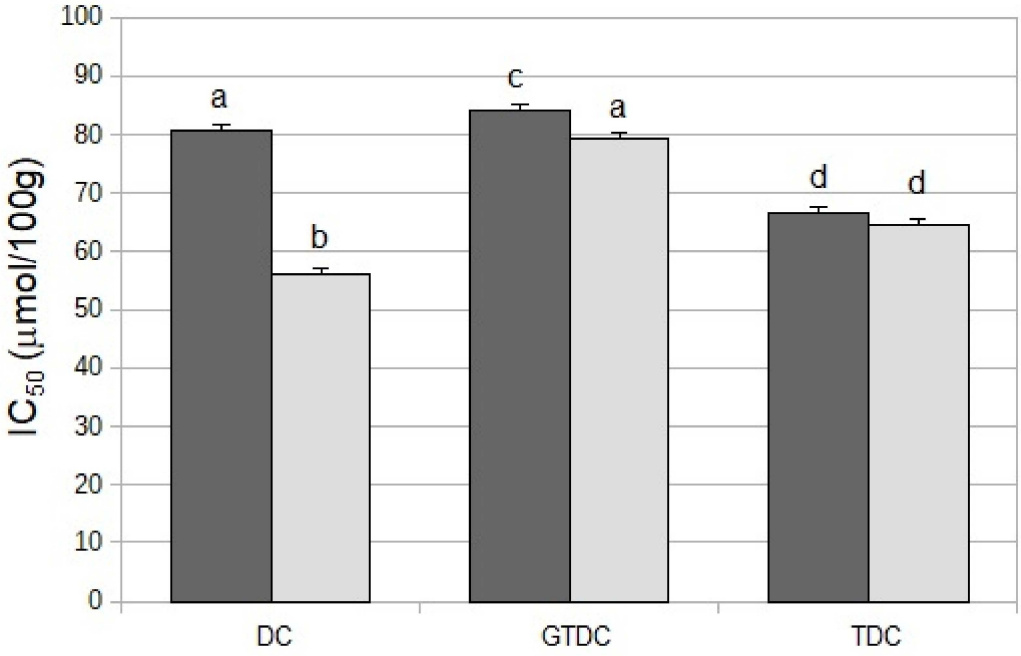
16
18
a
a
a
bc
bc
b
c
d
c
e
f
e
Salivary phase
Gastric phase
Intestinal phase
Chemical extraction


100
90
80
70
60
50
40
30
20
10
0
IC50 (µmol/100g)
a
b
c
a
d
d
DC
GTDC
TDC

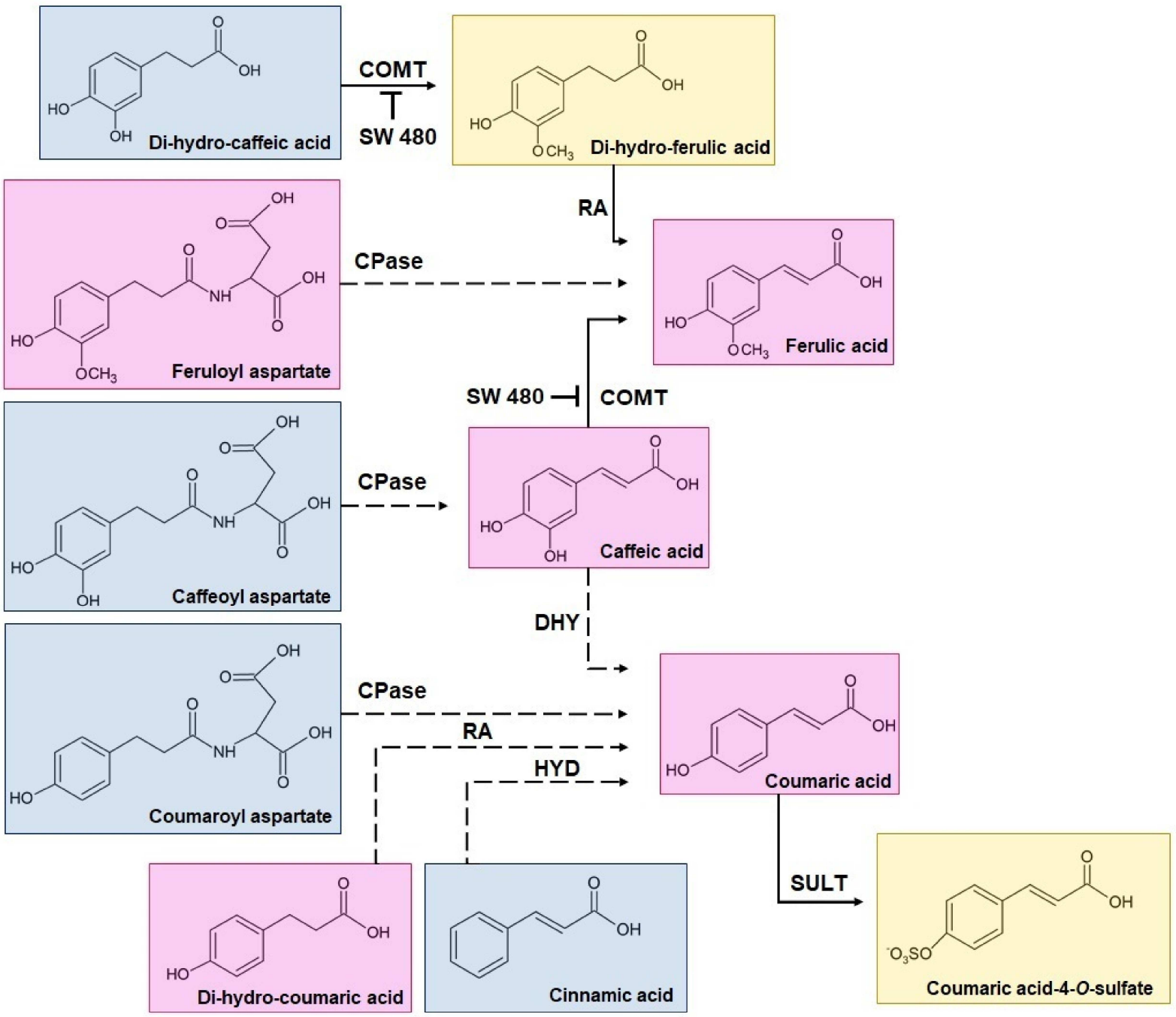

Di-hydro-caffeic acid
COMT
SW 480
Di-hydro-ferulic acid
RA
Feruloyl aspartate
CPase
Ferulic acid
SW 480
COMT
Caffeoyl aspartate
CPase
Caffeic acid
DHY
Coumaroyl aspartate
CPase
RA
HYD
Coumaric acid
SULT
Di-hydro-coumaric acid
Cinnamic acid
Coumaric acid-4-O-sulfate

Table 1. Quantitative results (μmol/100 g of chocolate) for flavan-3-ols identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

Compound	Dark chocolate 70%		Sakura green tea dark chocolate 70%		Turmeric dark chocolate 70%	
	Chemical extraction	After digestion	Chemical extraction	After digestion	Chemical extraction	After digestion
Flavan-3-ols						
Catechin	228.28 ± 6.86^{a}	42.00 ± 6.65^{b}	240.07 ± 18.28^{a}	47.18 ± 2.12^{b}	245.28 ± 3.97^{a}	40.21 ± 5.40^{b}
Epicatechin	701.00 ± 26.48^{a}	124.75 ± 7.79^{b}	1047.21 ± 40.17^{c}	175.59 ± 6.55^{d}	753.21 ± 27.86^{a}	126.66 ± 8.23^{b}
Gallocatechin	1.48 ± 0.03^{a}	n.d.	7.21 ± 0.48^{b}	6.58 ± 0.20^{b}	< LOQ	n.d.
Epigallocatechin	54.93 ± 0.34^{a}	n.d.	102.62 ± 6.00^{b}	53.64 ± 0.78^{a}	52.10 ± 2.76^{a}	n.d.
(Epi)catechin-*O*-sulphate isomer	7.10 ± 0.45^{a}	4.21 ± 0.23^{b}	5.97 ± 0.69^{a}	3.75 ± 0.13^{b}	10.59 ± 1.07^{c}	4.81 ± 0.11^{d}
(Epi)catechin-*O*-sulphate isomer	24.66 ± 1.21^{a}	14.13 ± 1.01^{b}	24.69 ± 0.41^{a}	14.65 ± 0.93^{b,d}	35.28 ± 2.86^{c}	16.10 ± 0.61^{d}
(Epi)catechin-3-*O*-trihydroxybenzene	16.97 ± 1.72^{a,d}	1.11 ± 0.01^{b}	16.07 ± 0.34^{a}	1.68 ± 0.06^{c}	20.48 ± 1.93^{d}	1.08 ± 0.01^{b}
(Epi)catechin-3-*O*-trihydroxybenzene	12.45 ± 0.21^{a}	1.01 ± 0.05^{b}	15.79 ± 0.90^{c}	1.33 ± 0.02^{d}	16.69 ± 1.45^{c}	0.65 ± 0.02^{e}
(Epi)catechin-7-*O*-trihydroxybenzene	8.97 ± 0.52^{a}	0.82 ± 0.02^{b}	13.21 ± 1.93^{c}	1.10 ± 0.08^{b}	11.69 ± 0.48^{c}	0.40 ± 0.01^{d}
(Epi)catechin-*C*-pentoside isomer	1.45 ± 0.17^{a}	0.74 ± 0.01^{b}	1.03 ± 0.24^{a}	0.86 ± 0.01^{b}	2.86 ± 0.38^{c}	0.90 ± 0.03^{b}
Catechin-3-*O*-gallate*	n.d.	n.d.	< LOQ	< LOQ	n.d.	n.d.
Epicatechin-3-*O*-gallate *	n.d.	n.d.	31.45 ± 0.45^{a}	1.16 ± 0.11^{b}	n.d.	n.d.
(Epi)catechin-3-*O*-hexoside isomer	43.59 ± 0.69^{a}	10.95 ± 0.52^{b}	33.72 ± 4.62^{c}	13.16 ± 0.62^{d}	12.10 ± 1.79^{d}	7.13 ± 0.42^{e}
(Epi)catechin-*C*-hexoside isomer	6.59 ± 0.14^{a}	4.90 ± 0.19^{b}	5.59 ± 0.34^{c}	6.17 ± 0.27^{a,c}	4.55 ± 0.66^{b}	6.74 ± 0.34^{a}
(Epi)catechin-7-*O*-hexoside	14.17 ± 1.00^{a}	3.64 ± 0.16^{b}	14.69 ± 1.03^{a}	4.30 ± 0.17^{c}	18.07 ± 0.10^{d}	1.41 ± 0.02^{e}
(Epi)catechin-*C*-hexoside isomer	14.10 ± 0.72^{a}	8.11 ± 0.54^{b}	12.00 ± 2.76^{a}	9.67 ± 0.37^{c}	12.83 ± 0.01^{a}	9.02 ± 0.40^{b,c}
(Epi)catechin-3-*O*-hexoside isomer	4.69 ± 0.72^{a}	2.18 ± 0.10^{b}	6.24 ± 0.76^{a}	3.10 ± 0.16^{c}	5.10 ± 0.28^{a}	1.81 ± 0.01^{d}
Epigallocatechin-3-*O*-gallate *	n.d.	n.d	115.66 ± 7.45^{a}	3.20 ± 0.34^{b}	n.d	n.d.
Gallocatechin-3-*O*-hexoside	0.31 ± 0.03^{a}	1.41 ± 0.02^{b}	0.21 ± 0.01^{a}	1.52 ± 0.02^{b}	0.52 ± 0.03^{c}	1.49 ± 0.02^{b}
Epigallocatechin-3-*O*-hexoside	0.31 ± 0.03^{a}	1.27 ± 0.01^{b}	0.24 ± 0.01^{a}	1.80 ± 0.05^{c}	0.59 ± 0.03^{d}	1.41 ± 0.01^{e}
Procyanidin dimer A type	3.79 ± 0.21^{a}	n.d.	4.52 ± 0.28^{a}	n.d.	4.17 ± 0.24^{a}	n.d.
Procyanidin dimer B type isomer	29.83 ± 2.90^{a}	2.09 ± 0.23^{b}	28.93 ± 1.17^{a}	2.08 ± 0.05^{b}	36.52 ± 5.10^{a}	1.48 ± 0.11^{c}
Procyanidin dimer B type isomer	10.52 ± 0.76^{a,c}	1.12 ± 0.24^{b}	9.28 ± 0.14^{a}	< LOQ	12.48 ± 0.66^{c}	1.04 ± 0.13^{b}
Procyanidin dimer B type isomer	128.38 ± 11.86^{a}	n.d.	180.86 ± 18.76^{b}	2.29 ± 0.15^{c}	121.10 ± 0.14^{a}	n.d.
Procyanidin dimer B type isomer	117.52 ± 6.83^{a}	19.56 ± 0.54^{b}	155.14 ± 3.97^{c}	24.26 ± 1.03^{d}	131.90 ± 9.52^{a}	23.60 ± 1.66^{d}
Procyanidin dimer B type isomer	18.34 ± 1.03^{a}	n.d.	22.97 ± 2.21^{b}	n.d.	23.90 ± 2.55^{b}	< LOQ
Procyanidin dimer B type isomer	35.07 ± 0.45^{a}	n.d.	42.59 ± 1.21^{b}	1.92 ± 0.11^{c}	45.24 ± 3.07^{b}	< LOQ

(Epi)catechin-(Epi)gallocatechin *	n.d.	n.d.	0.41 ± 0.03[a]	n.d.	n.d.	n.d.
(Epi)catechin-3-*O*-dihexoside isomer	2.28 ± 0.03[a]	n.d.	2.52 ± 0.03[a]	< LOQ	1.52 ± 0.03[b]	< LOQ
(Epi)catechin-3-*O*-dihexoside isomer	5.69 ± 0.07[a]	n.d.	3.59 ± 1.76[b]	n.d.	4.62 ± 0.10[b]	n.d.
(Epi)catechin-3-*O*-gallate-7-*O*-glucuronide isomer	1.14 ± 0.03[a]	n.d.	0.62 ± 0.03[b]	< LOQ	0.83 ± 0.10[b]	n.d.
(Epi)catechin-3-*O*-gallate-7-*O*-glucuronide isomer	1.07 ± 0.07[a]	n.d.	0.48 ± 0.03[b]	< LOQ	0.79 ± 0.07[b]	n.d.
(Epi)catechin derivative isomer	4.00 ± 0.14[a,b]	< LOQ	3.21 ± 0.34[a]	0.37 ± 0.01	4.48 ± 0.48[b]	< LOQ
(Epi)catechin derivative isomer	3.52 ± 0.14[a]	< LOQ	3.66 ± 0.14[a]	0.28 ± 0.02	2.66 ± 0.17[b]	n.d.
Procyanidin dimer A type pentoside isomer	4.90 ± 0.03[a]	n.d.	4.45 ± 0.83[a]	n.d.	5.79 ± 0.14[b]	< LOQ
Procyanidin dimer A type pentoside isomer	3.76 ± 0.03[a]	n.d.	4.03 ± 0.28[a]	n.d.	3.59 ± 0.14[a]	< LOQ
Procyanidin dimer A type pentoside isomer	6.03 ± 0.10[a]	n.d.	7.72 ± 0.72[b]	n.d.	6.28 ± 0.38[a]	< LOQ
Procyanidin dimer A type pentoside isomer	5.07 ± 0.38[a]	n.d.	9.45 ± 0.38[b]	n.d.	4.14 ± 0.21[a]	< LOQ
Procyanidin dimer A type hexoside isomer	5.10 ± 0.21[a]	n.d.	5.24 ± 0.79[a,b]	n.d.	6.59 ± 0.24[b]	< LOQ
Procyanidin dimer A type hexoside isomer	3.48 ± 0.10[a]	0.42 ± 0.01[b]	4.28 ± 0.38[c]	0.45 ± 0.01[b]	5.45 ± 0.17[d]	n.d.
Procyanidin dimer A type hexoside isomer	7.10 ± 0.31[a]	n.d.	10.66 ± 1.28[b]	< LOQ	8.03 ± 0.17[a]	< LOQ
Procyanidin dimer A type hexoside isomer	5.07 ± 0.10[a]	n.d.	8.90 ± 0.52[b]	< LOQ	7.10 ± 0.10[c]	< LOQ
Procyanidin dimer B type hexoside isomer	2.76 ± 0.14[a]	1.78 ± 0.08[b]	3.07 ± 0.24[a]	1.93 ± 0.03[b]	3.52 ± 0.34[a]	1.68 ± 0.08[b]
Procyanidin dimer B type hexoside isomer	3.59 ± 0.07[a]	0.92 ± 0.01[b]	3.66 ± 0.14[a]	1.22 ± 0.07[c]	4.21 ± 0.14[d]	1.03 ± 0.07[a]
Procyanidin dimer B type hexoside isomer	1.76 ± 0.28[a]	1.08 ± 0.02[b]	1.90 ± 0.10[a]	1.29 ± 0.02[b]	1.86 ± 0.14[a]	1.21 ± 0.02[b]
Procyanidin dimer B type derivative	6.41 ± 0.10[a]	2.88 ± 0.09[b]	6.48 ± 1.62[a]	2.75 ± 0.15[b]	7.83 ± 0.45[a]	1.75 ± 0.11[c]
Procyanidin trimer A type	2.41 ± 0.17[a]	n.d.	2.31 ± 0.14[a]	n.d.	3.34 ± 0.14[b]	n.d.
Procyanidin trimer B type isomer	3.21 ± 0.17[a]	n.d.	3.17 ± 0.10[a]	< LOQ	3.83 ± 0.38[a]	n.d.
Procyanidin trimer B type isomer	14.83 ± 1.07[a]	0.34 ± 0.02[b]	24.59 ± 1.76[c]	0.73 ± 0.01[d]	17.10 ± 1.41[a]	n.d.
Procyanidin trimer B type isomer	11.07 ± 0.24[a]	0.20 ± 0.01[b]	14.48 ± 0.86[b]	< LOQ	12.59 ± 0.38[b]	n.d.
Procyanidin trimer B type isomer	38.00 ± 1.55[a]	n.d.	52.28 ± 3.10[b]	n.d.	40.00 ± 2.21[a]	< LOQ
Procyanidin trimer B type isomer	30.69 ± 0.59[a]	0.46 ± 0.01[b]	60.31 ± 4.62[c]	n.d.	33.24 ± 0.17[a]	n.d.
Procyanidin trimer B type isomer	12.41 ± 0.66[a]	n.d.	12.66 ± 0.62[a]	n.d.	12.07 ± 0.76[a]	n.d.

Procyanidin trimer B type isomer	7.07 ± 0.28[a]	n.d.	7.41 ± 0.24[a]	n.d.	10.03 ± 0.52[b]	n.d.
Procyanidin trimer B type isomer	3.83 ± 0.10[a]	n.d.	5.79 ± 0.28[b]	n.d.	4.76 ± 0.07[c]	n.d.
Procyanidin trimer A type hexoside isomer	2.41 ± 0.21[a]	n.d.	3.41 ± 0.10[b]	n.d.	2.90 ± 0.24[a]	n.d.
Procyanidin trimer A type hexoside isomer	1.86 ± 0.07[a]	n.d.	3.34 ± 0.10[b]	n.d.	2.41 ± 0.14[c]	n.d.
Procyanidin tetramer A type isomer	4.97 ± 0.03[a]	n.d.	4.76 ± 0.24[a]	< LOQ	4.90 ± 0.28[a]	< LOQ
Procyanidin tetramer A type isomer	6.97 ± 0.45[a]	n.d.	12.69 ± 1.97[b]	n.d.	7.03 ± 0.55[a]	< LOQ
Procyanidin tetramer A type isomer	6.97 ± 0.10[a]	n.d.	13.93 ± 2.83[b]	< LOQ	9.76 ± 0.28[c]	< LOQ
Procyanidin tetramer A type isomer	2.72 ± 0.24[a,b]	n.d.	3.83 ± 1.00[a]	< LOQ	2.28 ± 0.21[b]	n.d.
Procyanidin tetramer B type isomer	1.28 ± 0.03[a]	n.d.	6.28 ± 0.17[b]	n.d.	1.28 ± 0.17[a]	n.d.
Procyanidin tetramer B type isomer	4.62 ± 0.14[a]	n.d.	6.24 ± 0.76[b]	n.d.	6.38 ± 0.17[b]	n.d.
Procyanidin tetramer B type isomer	3.21 ± 0.10[a]	n.d.	6.28 ± 0.24[b]	n.d.	5.90 ± 0.55[b]	< LOQ
Procyanidin tetramer B type isomer	4.10 ± 0.28[a]	n.d.	8.90 ± 0.10[b]	n.d.	5.69 ± 0.03[c]	n.d.
Procyanidin pentamer B type isomer	2.07 ± 0.07[a]	n.d.	1.90 ± 0.14[a]	n.d.	2.31 ± 0.02[a]	n.d.
Procyanidin pentamer B type isomer	2.83 ± 0.14[a]	n.d.	5.34 ± 0.48[b]	n.d.	3.41 ± 0.01[c]	n.d.
Procyanidin pentamer B type isomer	4.03 ± 0.28[a]	n.d.	11.97 ± 0.97[b]	n.d.	6.03 ± 0.14[c]	n.d.
Procyanidin hexamer A type	< LOQ	n.d.	< LOQ	n.d.	< LOQ	n.d.
Procyanidin hexamer B type isomer	1.07 ± 0.03[a]	n.d.	3.38 ± 0.07[b]	n.d.	1.28 ± 0.07[a]	n.d.
Procyanidin hexamer B type isomer	1.50 ± 0.14[a]	n.d.	4.66 ± 0.14[b]	n.d.	1.00 ± 0.03[c]	n.d.
Procyanidin hexamer B type isomer	0.76 ± 0.07[a]	n.d.	6.38 ± 0.69[b]	n.d.	1.59 ± 0.14[c]	n.d.

< LOQ means the compound was detected but it was below the limit of quantification; **n.d.** means not detected

***** mean the compounds were detected only in green tea dark chocolate.

Different superscript letters within the same row indicate that the values are significantly different (*P<0.05*).

Flavan-3-ols were quantified as epicatechin equivalent.

Data from chemical extraction were from Martini et al. (2018).

Table 2. Quantitative results (μmol/100 g of chocolate) for hydroxycinnamic acids identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

Compound	Dark chocolate 70%		Sakura green tea dark chocolate 70%		Turmeric dark chocolate 70%	
	Chemical extraction	After digestion	Chemical extraction	After digestion	Chemical extraction	After digestion
Hydroxycinnamic acids						
Coumaric acid	2.07 ± 0.06[a]	5.10 ± 0.12[b]	2.26 ± 0.30[a]	5.02 ± 0.20[b]	3.35 ± 0.06[c]	5.34 ± 0.42[b]
Di-hydro-coumaric acid	49.57 ± 0.85[a]	31.29 ± 2.09[b]	27.87 ± 0.49[c]	17.06 ± 0.94[d]	68.17 ± 2.50[e]	38.31 ± 2.10[b]
Di-hydroxycinnamic acid isomer	5.61 ± 0.06[a]	4.96 ± 0.27[b]	6.95 ± 0.12[c,e]	7.31 ± 0.32[c]	14.21 ± 0.18[d]	6.41 ± 0.31[e]
Di-hydroxycinnamic acid isomer	2.93 ± 0.12[a]	1.05 ± 0.21[b]	2.20 ± 0.06[c]	1.15 ± 0.02[b]	3.48 ± 0.06[d]	1.05 ± 0.16[b]
Caffeic acid	6.59 ± 0.06[a]	2.16 ± 0.06[b]	10.24 ± 0.12[c]	2.82 ± 0.14[d]	6.65 ± 0.12[a]	2.78 ± 0.18[d]
Di-hydroxycinnamic acid isomer	2.93 ± 0.06[a]	1.35 ± 0.10[b]	2.07 ± 0.01[c]	1.13 ± 0.08[b]	3.66 ± 0.24[d]	1.82 ± 0.04[c]
Di-hydroxycinnamic acid isomer	3.54 ± 0.06[a]	0.46 ± 0.01[b]	3.84 ± 0.18[a]	1.02 ± 0.02[c]	3.54 ± 0.18[a]	0.57 ± 0.01[b]
Di-hydro-caffeic acid	11.46 ± 0.98[a]	1.79 ± 0.06[b]	1.46 ± 0.01[c]	< LOQ	5.37 ± 0.24[d]	1.47 ± 0.05[e]
Ferulic acid	315.62 ± 19.28[a]	27.89 ± 3.21[b]	299.43 ± 12.01[a]	16.92 ± 1.25[c]	285.05 ± 7.01[a]	17.37 ± 0.93[c]
Coumaroyl aspartate	95.85 ± 1.40[a]	69.18 ± 2.65[b]	85.79 ± 0.30[c]	65.26 ± 3.42[b]	102.62 ± 1.04[d]	79.55 ± 1.22[e]
Di-hydroxycinnamic aspartate isomer	52.38 ± 3.17[a]	22.70 ± 1.37[b]	41.83 ± 0.98[c]	21.47 ± 1.04[b]	42.20 ± 0.24[c]	25.11 ± 0.48[b]
Di-hydroxycinnamic aspartate isomer	218.78 ± 17.62[a]	119.70 ± 8.28[b]	204.15 ± 1.52[a]	121.05 ± 11.06[b]	201.95 ± 3.54[a]	130.79 ± 3.69[b]
Feruloyl aspartate	42.16 ± 0.31[a]	36.91 ± 2.09[b]	48.96 ± 0.36[c]	26.49 ± 1.25[d]	46.19 ± 2.11[a,c]	38.66 ± 1.36[b]
Ferulic acid-4-*O*-pentoside	2.06 ± 0.01[a]	1.24 ± 0.04[b]	2.47 ± 0.01[c]	1.92 ± 0.01[a]	2.42 ± 0.10[c]	1.27 ± 0.02[b]
Di-deoxyclovamide (Coumaroyl-DOPA)	28.41 ± 1.28[a]	14.85 ± 1.02[b]	28.96 ± 0.12[a]	15.32 ± 1.06[b]	27.87 ± 1.46[a]	16.82 ± 1.07[b]
3-Coumaroylquinic acid *cis*[*]	n.d.	n.d.	9.21 ± 0.10[a]	6.59 ± 0.66[b]	n.d.	n.d.
3-Coumaroylquinic acid *trans*[*]	n.d.	n.d.	4.15 ± 0.10[a]	3.84 ± 0.40[b]	n.d.	n.d.
4-Coumaroylquinic acid *cis*[*]	n.d.	n.d.	12.50 ± 0.61[a]	8.47 ± 0.66[b]	n.d.	n.d.
4-Coumaroylquinic acid *trans*[*]	n.d.	n.d.	21.46 ± 0.55[a]	17.62 ± 1.41[b]	n.d.	n.d.
Mono-deoxyclovamide (Caffeoyl-DOPA / Coumaroyl-tyrosine) isomer	3.54 ± 0.01[a]	1.78 ± 0.11[b]	2.93 ± 0.24[c]	1.38 ± 0.20[b]	3.05 ± 0.01[c]	1.47 ± 0.03[b]
Mono-deoxyclovamide (Caffeoyl-DOPA / Coumaroyl-	17.87 ± 0.12[a]	9.06 ± 0.78[b]	13.72 ± 0.61[c]	9.16 ± 0.57[b]	12.87 ± 0.79[c]	9.12 ± 0.21[b]

tyrosine) isomer						
5-Caffeoylquinic acid	1.40 ± 0.12[a]	0.76 ± 0.01[b]	3.96 ± 0.12[c]	3.44 ± 0.14[d]	1.52 ± 0.18[a]	0.81 ± 0.01[b]
3-Caffeoylquinic acid	0.73 ± 0.12[a]	0.50 ± 0.01[b]	1.89 ± 0.12[c]	1.78 ± 0.07[c]	1.28 ± 0.06[d]	0.50 ± 0.01[b]
Clovamide (caffeoyl-tyrosine) isomer	11.04 ± 0.30[a]	3.16 ± 0.12[b]	8.66 ± 0.12[c]	3.34 ± 0.17[b]	7.20 ± 0.43[d]	2.63 ± 0.11[e]
Clovamide (caffeoyl-tyrosine) isomer	47.13 ± 3.29[a]	23.71 ± 0.93[b]	54.51 ± 2.20[a]	25.64 ± 1.28[b]	50.67 ± 0.06[a]	20.02 ± 1.23[b]

< LOQ means the compound was detected but it was below the limit of quantification; **n.d.** means not detected

***** mean the compounds were detected only in green tea dark chocolate.

Different superscript letters within the same row indicate that the values are significantly different ($P<0.05$).

Hydroxycinnamic acids were quantified as coumaric acid or ferulic acid equivalent.

Data from chemical extraction were from Martini et al. (2018).

Table 3. Quantitative results (µmol/100 g of chocolate) for flavonols identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

Compound	Dark chocolate 70%		Sakura green tea dark chocolate 70%		Turmeric dark chocolate 70%	
	Chemical extraction	After digestion	Chemical extraction	After digestion	Chemical extraction	After digestion
			Flavonols			
Quercetin	1.77 ± 0.02[a]	n.d.	2.66 ± 0.05[b]	< LOQ	2.25 ± 0.02[c]	< LOQ
Quercetin-3-*O*-pentoside	5.43 ± 0.34[a]	0.15 ± 0.01[b]	4.59 ± 0.23[c]	0.29 ± 0.01[b]	6.80 ± 0.66[d]	0.20 ± 0.01[b]
Kaempferol-7-*O*-hexoside	0.23 ± 0.01[a]	n.d.	0.39 ± 0.02[a]	n.d.	0.25 ± 0.02[a]	n.d.
Kaempferol-3-*O*-galactoside	0.07 ± 0.01[a]	n.d.	0.46 ± 0.01[b]	< LOQ	< LOQ	n.d.
Kaempferol-3-*O*-glucoside	0.08 ± 0.01[a]	< LOQ	0.61 ± 0.05[b]	< LOQ	< LOQ	n.d.
Quercetin-3-*O*-rhamnoside	0.08 ± 0.01[a]	n.d.	0.82 ± 0.03[b]	< LOQ	< LOQ	n.d.
Dihydro-kaempferol-7-*O*-hexoside	0.41 ± 0.02[a]	n.d.	0.34 ± 0.02[a]	n.d.	0.38 ± 0.02[a]	n.d.
Quercetin-3-*O*-galactoside	0.80 ± 0.02[a]	0.10 ± 0.01[b]	2.74 ± 0.14[c]	n.d.	4.00 ± 0.07[d]	0.09 ± 0.01[b]
Quercetin-3-*O*-glucoside	3.21 ± 0.03[a]	0.28 ± 0.01[b]	3.75 ± 0.11[a]	0.49 ± 0.03[b]	5.00 ± 0.44[c]	0.24 ± 0.01[b]
Myricetin-3-*O*-galattoside	0.49 ± 0.01[a]	n.d.	3.85 ± 0.07[b]	0.39 ± 0.01[a]	< LOQ	n.d.
Myricetin-3-*O*-glucoside	0.75 ± 0.02[a]	n.d.	3.02 ± 0.23[b]	0.52 ± 0.03[a]	< LOQ	n.d.
Kaempferol-3-*O*-rutinoside	0.43 ± 0.01[a]	< LOQ	1.07 ± 0.03[b]	0.41 ± 0.01[a]	< LOQ	n.d.
Quercetin-3-*O*-rutinoside *	n.d.	n.d.	6.89 ± 0.31[a]	1.19 ± 0.08[b]	n.d.	n.d.
Myricetin-3-*O*-rutinoside *	n.d.	n.d.	0.95 ± 0.02[a]	0.33 ± 0.03[b]	n.d.	n.d.
Myricetin-3-*O*-(*O*-galloyl) hexoside	0.18 ± 0.01[a]	n.d.	1.02 ± 0.03[b]	0.11 ± 0.01[a]	< LOQ	n.d.
Kaempferol-7-*O*-rhamnoside-3-*O*-rutinoside	0.05 ± 0.01[a]	n.d.	0.15 ± 0.02[b]	0.04 ± 0.01[a]	< LOQ	n.d.
Quercetin-7-*O*-rhamnoside-3-*O*-rutinoside	0.23 ± 0.01[a]	n.d.	0.33 ± 0.03[a]	0.16 ± 0.01[a]	< LOQ	n.d.
Kaempferol-7-*O*-hexoside-3-*O*-rutinoside isomer	0.93 ± 0.01[a]	n.d.	2.08 ± 0.03[b]	0.95 ± 0.04[a]	< LOQ	n.d.
Kaempferol-7-*O*-hexoside-3-*O*-rutinoside isomer	0.72 ± 0.01[a]	n.d.	2.26 ± 0.02[b]	1.16 ± 0.06[c]	< LOQ	n.d.
Quercetin-7-*O*-hexoside-3-*O*-rutinoside isomer	1.84 ± 0.01[a]	n.d.	3.67 ± 0.02[b]	1.62 ± 0.13[a]	< LOQ	n.d.
Quercetin-7-*O*-hexoside-3-*O*-rutinoside isomer	3.43 ± 0.21[a]	n.d.	5.98 ± 0.43[b]	2.93 ± 0.16[a]	< LOQ	n.d.
Myricetin-7-*O*-hexoside-3-*O*-rutinoside	0.20 ± 0.01[a]	n.d.	0.38 ± 0.03[a]	0.16 ± 0.01[a]	< LOQ	n.d.

< LOQ means the compound was detected but it was below the limit of quantification; **n.d.** means not detected

* mean the compounds were detected only in green tea dark chocolate.
Different superscript letters within the same row indicate that the values are significantly different ($P<0.05$).
Flavonols were quantified as quercetin-3-*O*-rutinoside equivalent.
Data from chemical extraction were from Martini et al. (2018).

Table 4. Quantitative results (μmol/100 g of chocolate) for flavones, ellagitannins, curcuminoids and other phenolics identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

Compound	Dark chocolate 70%		Sakura green tea dark chocolate 70%		Turmeric dark chocolate 70%	
	Chemical extraction	After digestion	Chemical extraction	After digestion	Chemical extraction	After digestion
Other phenolics						
Vanillin	8.64 ± 0.32^{a}	2.24 ± 0.19^{b}	2.97 ± 0.18^{c}	2.10 ± 0.12^{b}	3.03 ± 0.13^{c}	1.51 ± 0.08^{d}
Phloretin-*C*-hexoside isomer	0.25 ± 0.01^{a}	< LOQ	0.26 ± 0.01^{a}	0.07 ± 0.01^{b}	1.03 ± 0.05^{c}	n.d.
Eriodictyol-7-*O*-hexoside	0.16 ± 0.02^{a}	< LOQ	0.31 ± 0.02^{b}	0.58 ± 0.01^{c}	0.87 ± 0.05^{d}	n.d.
Cinchonain isomer	6.24 ± 0.69^{a}	0.66 ± 0.02^{b}	3.90 ± 0.03^{c}	0.87 ± 0.06^{b}	7.21 ± 0.10^{a}	0.73 ± 0.01^{b}
Cinchonain isomer	2.66 ± 0.21^{a}	n.d.	2.72 ± 0.07^{a}	n.d.	2.59 ± 0.07^{a}	n.d.
Naringenin-*C*-hexoside-7-*O*-hexoside isomer	0.38 ± 0.01^{a}	< LOQ	1.03 ± 0.03^{b}	0.73 ± 0.01^{c}	< LOQ	n.d.
Eriodictyol-*C*-hexoside-7-*O*-hexoside isomer	0.25 ± 0.01^{a}	n.d.	0.46 ± 0.02^{b}	0.45 ± 0.01^{b}	< LOQ	n.d.
Flavones						
Apigenin-*C*-hexoside isomer	0.16 ± 0.02^{a}	< LOQ	0.69 ± 0.02^{b}	0.19 ± 0.01^{a}	0.31 ± 0.0^{a}	n.d.
Apigenin-*C*-hexoside-*C*-pentoside isomer	1.56 ± 0.02^{a}	0.12 ± 0.01^{b}	3.46 ± 0.11^{c}	0.35 ± 0.02^{d}	1.36 ± 0.05^{a}	0.12 ± 0.01^{b}
Apigenin-*C*-hexoside-2''-*O*-rhamnoside isomer	2.15 ± 0.52^{a}	n.d.	1.46 ± 0.21^{b}	0.30 ± 0.01^{c}	2.80 ± 0.70^{a}	n.d.
Apigenin-6,8-*di*-*C*-hexoside isomer	0.28 ± 0.02^{a}	n.d.	0.77 ± 0.02^{b}	< LOQ	0.38 ± 0.02^{a}	0.35 ± 0.03^{a}
Apigenin-6,8-*di*-*C*-hexoside isomer	0.33 ± 0.02^{a}	0.29 ± 0.01^{a}	0.89 ± 0.01^{b}	1.00 ± 0.05^{b}	0.54 ± 0.02^{c}	n.d.
Apigenin-*C*-hexoside-2''-*O*-hexoside isomer	0.41 ± 0.01^{a}	n.d.	0.77 ± 0.04^{b}	0.40 ± 0.01^{a}	< LOQ	n.d.
Ellagitannins						
Ellagic acid	185.96 ± 11.85^{a}	11.55 ± 0.05^{b}	176.95 ± 3.38^{a}	10.27 ± 0.74^{b}	167.22 ± 10.93^{a}	9.62 ± 0.88^{b}
Ellagic acid-galloyl-hexoside	15.40 ± 0.89^{a}	n.d.	36.85 ± 1.42^{b}	4.34 ± 0.14^{c}	13.54 ± 0.50^{a}	n.d.
HHDP-galloyl-hexose	52.28 ± 3.97^{a}	n.d.	81.62 ± 3.34^{b}	18.32 ± 0.56^{c}	46.72 ± 2.28^{a}	n.d.
Curcuminoids						
Bisdemethoxy-curcumin **	n.d.	n.d.	n.d.	n.d.	398.45 ± 7.45^{a}	0.81 ± 0.08^{b}
Demethoxy-curcumin **	n.d.	n.d.	n.d.	n.d.	284.97 ± 4.59^{a}	1.48 ± 0.03^{b}
Curcumin **	n.d.	n.d.	n.d.	n.d.	257.07 ± 1.62^{a}	n.d.

< LOQ means the compound was detected but it was below the limit of quantification; **n.d.** means not detected

****** mean the compounds were detected only turmeric dark chocolate.

Different superscript letters within the same row indicate that the values are significantly different ($P<0.05$).

Flavones were quantified as quercetin-3-*O*-rutinoside equivalent.

Ellagitannins were quantified as ellagic acid equivalent.

Curcuminoids were quantified as curcumin equivalent.

Data from chemical extraction were from Martini et al. (2018).

Table 5. Quantitative results (μmol/100 g of chocolate) for hydroxybenzoic acids identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

Compound	Dark chocolate 70%		Sakura green tea dark chocolate 70%		Turmeric dark chocolate 70%	
	Chemical extraction	After digestion	Chemical extraction	After digestion	Chemical extraction	After digestion
Hydroxybenzoic acids						
Hydroxybenzoic acid isomer	1.36 ± 0.06^{a}	0.58 ± 0.09^{b}	1.30 ± 0.26^{a}	0.45 ± 0.06^{b}	1.75 ± 0.13^{c}	0.60 ± 0.02^{b}
Hydroxybenzoic acid isomer	0.84 ± 0.06^{a}	0.60 ± 0.05^{a}	0.52 ± 0.06^{a}	0.63 ± 0.04^{a}	2.66 ± 0.13^{b}	0.83 ± 0.01^{a}
Hydroxybenzoic acid isomer	35.52 ± 0.19^{a}	10.72 ± 0.71^{b}	41.95 ± 0.39^{c}	10.11 ± 0.85^{b}	48.70 ± 3.05^{c}	10.55 ± 0.47^{c}
Hydroxybenzoic acid isomer	2.01 ± 0.06^{a}	0.72 ± 0.09^{b}	8.57 ± 0.13^{c}	0.99 ± 0.04^{b}	2.14 ± 0.13^{a}	0.89 ± 0.12^{b}
Hydroxybenzoic acid isomer	4.03 ± 0.26^{a}	1.00 ± 0.09^{b}	6.10 ± 0.71^{c}	1.93 ± 0.03^{d}	5.00 ± 0.19^{c}	0.98 ± 0.12^{b}
Protocatechuic acid	69.87 ± 1.95^{a}	34.75 ± 2.09^{b}	59.16 ± 0.58^{c}	21.58 ± 0.86^{d}	118.90 ± 6.30^{e}	29.02 ± 1.76^{b}
Vanillic acid isomer	2.53 ± 0.2 ^{a}	2.58 ± 0.11^{a}	2.40 ± 0.06^{a}	2.88 ± 0.04^{a}	2.92 ± 0.13^{a}	3.11 ± 0.11^{a}
Vanillic acid isomer	4.87 ± 0.84^{a}	1.01 ± 0.09^{b}	3.57 ± 0.32^{a}	1.25 ± 0.05^{b}	7.27 ± 0.06^{c}	1.14 ± 0.08^{b}
Gallic acid*	n.d.	n.d.	0.91 ± 0.01^{a}	1.00 ± 0.02^{a}	n.d.	n.d.
Syringic acid	0.84 ± 0.13^{a}	0.63 ± 0.01^{a}	1.69 ± 0.19^{b}	0.96 ± 0.07^{a}	0.91 ± 0.13^{a}	0.95 ± 0.03^{a}
Protocatechuic acid-4-*O*-hexoside	0.65 ± 0.06^{a}	n.d.	2.01 ± 0.19^{b}	1.22 ± 0.09^{c}	1.23 ± 0.01^{c}	0.68 ± 0.01^{a}
Vanillic acid-4-*O*-hexoside isomer	1.62 ± 0.06^{a}	1.70 ± 0.07^{a}	1.30 ± 0.13^{a}	1.65 ± 0.20^{a}	1.95 ± 0.06^{a}	2.49 ± 0.18^{b}
Vanillic acid-4-*O*-hexoside isomer	11.49 ± 0.06^{a}	5.95 ± 0.24^{b}	10.84 ± 0.14^{a}	6.02 ± 0.19^{b}	17.08 ± 0.06^{c}	6.68 ± 0.41^{b}
Vanillic acid-4-*O*-hexoside isomer	12.47 ± 0.13^{a}	2.09 ± 0.13^{b}	7.86 ± 0.06^{c}	2.43 ± 0.26^{b}	11.23 ± 0.01^{d}	2.31 ± 0.14^{b}
Galloyl glucose isomer *	n.d.	n.d.	1.88 ± 0.10^{a}	1.75 ± 0.09^{a}	n.d.	n.d.
Galloylquinic acid isomer *	n.d.	n.d.	11.95 ± 0.52^{a}	< LOQ	n.d.	n.d.
Galloylquinic acid isomer *	n.d.	n.d.	13.57 ± 0.32^{a}	18.74 ± 0.98^{b}	n.d.	n.d.
Syringic acid-4-*O*-hexoside isomer	6.10 ± 0.26^{a}	< LOQ	5.26 ± 0.24^{a}	< LOQ	6.95 ± 0.06^{b}	n.d.
Syringic acid-4-*O*-hexoside isomer	4.81 ± 0.13^{a}	4.20 ± 0.27^{a}	4.61 ± 0.26^{a}	4.55 ± 0.28^{a}	6.75 ± 0.71^{b}	4.98 ± 0.24^{a}
Vanillic acid derivative	2.01 ± 0.01^{a}	2.71 ± 0.12^{b}	3.18 ± 0.06^{c}	2.38 ± 0.01^{b}	3.64 ± 0.26^{c}	1.97 ± 0.11^{a}

< LOQ means the compound was detected but it was below the limit of quantification; **n.d.** means not detected

***** mean the compounds were detected only in green tea dark chocolate.

Different superscript letters within the same row indicate that the values are significantly different ($P<0.05$).

Hydroxybenzoic acids were quantified as protocatechuic acid equivalent.

Data from chemical extraction were from Martini et al. (2018).

Table 6. Quantitative results (μmol/100 g of chocolate) for phenolic compounds grouped by classes identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

Compound	Dark chocolate 70%		Sakura green tea dark chocolate 70%		Turmeric dark chocolate 70%	
	Chemical extraction	**After digestion**	**Chemical extraction**	**After digestion**	**Chemical extraction**	**After digestion**
Total flavan-3-ols	1732.09 ± 30.97^{a}	252.07 ± 10.34^{b}	2563.56 ± 50.12^{c}	389.99 ± 7.07^{d}	1857.63 ± 31.02^{a}	251.62 ± 10.03^{b}
Total hydroxycinnamic acids	921.67 ± 29.28^{a}	379.05 ± 9.94^{b}	899.48 ± 14.55^{a}	385.20 ± 12.00^{b}	893.29 ± 9.91^{a}	401.85 ± 4.79^{b}
Total flavonols	21.33 ± 0.35^{a}	0.53 ± 0.02^{b}	48.00 ± 0.64^{c}	10.75 ± 0.24^{d}	18.70 ± 0.79^{e}	0.53 ± 0.01^{b}
Total other phenolics	18.57 ± 0.35^{a}	2.90 ± 0.19^{b}	11.65 ± 0.19^{c}	4.80 ± 0.14^{d}	13.70 ± 0.16^{e}	2.24 ± 0.08^{f}
Total flavones	4.89 ± 0.53^{a}	0.41 ± 0.01^{b}	8.03 ± 0.24^{c}	2.24 ± 0.05^{d}	5.39 ± 0.71^{a}	0.47 ± 0.01^{b}
Total ellagitannins	253.64 ± 12.53^{a,d}	11.55 ± 0.05^{b}	295.43 ± 14.96^{a}	32.93 ± 0.94^{c}	227.48 ± 21.17^{d}	9.62 ± 0.88^{e}
Total hydroxybenzoic acids	161.04 ± 2.20^{a}	70.04 ± 2.25^{b}	188.64 ± 0.19^{c}	80.51 ± 1.64^{d}	239.09 ± 7.05^{e}	67.19 ± 1.91^{b}
Total curcuminoids	n.d.	n.d.	n.d.	n.d.	940.48 ± 8.90^{a}	2.29 ± 0.09^{b}
Total phenolic compounds	**3113.22 ± 44.48^{a}**	**716.55 ± 14.52^{b}**	**3954.78 ± 52.43^{c}**	**906.45 ± 14.06^{d}**	**4195.76 ± 36.26^{e}**	**735.81 ± 11.31^{b}**

Different superscript letters within the same row indicate that the values are significantly different ($P<0.05$).
Data from chemical extraction were from Martini et al. (2018).

Table 7. Mass spectral and quantitative data of newly formed phenolic compounds identified in different dark chocolates after *in vitro* gastro-intestinal digestion. DC identify dark chocolate; GTDC identify dark chocolate enriched with Sakura green tea leaves; TDC identify dark chocolate enriched with turmeric powder. Data are expressed as μmol/100 g of chocolate.

Compounds	*Molecular formula*	*Exp [M-H]⁻*	*Calc [M-H]-*	*ppm*	*Fragment ions*	*DC*	*GTDC*	*TD*
4-Caffeoylquinic acid[a]	$C_{16}H_{18}O_9$	353.0951	353.0950	-0.05	179.0433, 173.0049	1.10 ± 0.09	1.04 ± 0.02	1.20 ± 0.04
Trihydroxybenzene[b]	$C_6H_6O_3$	125.0311	125.0317	4.71	81.0290	0.19 ± 0.01	0.45 ± 0.03	0.21 ± 0.02
Theasinensin isomer[c]	$C_{30}H_{26}O_{14}$	609.1335	609.1322	-2.04	471.0657, 453.0536, 427.0777, 333.0453, 167.0299	n.d.	0.73 ± 0.04	n.d.
Epigallocatechin dimer isomers (P2-analogue)[c]	$C_{29}H_{24}O_{13}$	579.1198	579.1216	3.26	543.1247, 423.1119, 405.0840, 167.0453, 125.0292	n.d.	<LOQ	n.d.

<LOQ means the compound was detected but it was below the limit of quantification.
n.d. means not detected
[a]quantified as coumaric acid equivalent
[b]quantified as protocatechuic acid equivalent
[c]quantified as epicatechin equivalent

Table 8. Phenolic compounds identified in the cell media after 24 h of incubation with Caco-2 and SW480 of dark chocolate phenolic-rich fraction extracted at the end of the *in vitro* digestion. Data are expressed as μmol/100 g of chocolate.

Compounds	*[M-H]⁻*	*Fragment ions*	*Caco-2*	*SW480*
Catechin	289	245	< LOQ	< LOQ
Epicatechin	289	245	< LOQ	< LOQ
Methyl-(epi)catechin	303	288	< LOQ	< LOQ
Di-methyl-(epi)catechin	317	287	< LOQ	n.d.
Ferulic acid[a]	193	178	34.91 ± 1.67	9.87 ± 0.27
Feruloyl-aspartate[a]	308	290, 264, 246, 220	< LOQ	1.69 ± 0.02
Dihydro-ferulic acid[a]	195	177, 136	4.25 ± 0.25	n.d.
Coumaric acid[b]	163	119	86.14 ± 3.19	96.80 ± 4.96
Coumaroyl-sulphate	243	163, 158, 119	n.d.	< LOQ
Dihydro-coumaric acid[b]	165	147, 119	n.d.	9.41 ± 0.58
Caffeic acid[c]	179	135	n.d.	1.00 ± 0.06
Quercetin-glucoside[d]	463	301, 179, 151	n.d.	0.31 ± 0.01
Quercetin-pentoside[d]	433	301, 179, 151	< LOQ	0.16 ± 0.01
Quercetin[d]	301	271, 255	0.06 ± 0.01	n.d.
Methyl-ellagic acid[e]	315	257, 229	n.d.	0.40 ± 0.02

<LOQ means the compound was detected but it was below the limit of quantification.
n.d. means not detected
[a]quantified as ferulic acid equivalent
[b]quantified as coumaric acid equivalent
[c]quantified as caffeic acid equivalent
[d]quantified as quercetin-3-*O*-glucoside equivalent
[e]quantified as ellagic acid equivalent