(Article begins on next page)

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

Serena Martini*, Angela Conte, Davide Tagliazucchi

Department of Life Sciences, University of Modena and Reggio Emilia, Via Amendola 2, 42100 Reggio Emilia, Italy

* Corresponding author. Tel.: +39-0522-522060; fax: +39-0522-522053

E-mail address: serena.martini@unimore.it (S. Martini)

1   **Abstract**

2   The bioaccessibility of phenolic compounds after *in vitro* gastro-intestinal digestion of dark

3   chocolate, dark chocolate enriched with Sakura green tea and dark chocolate enriched with turmeric

4   powder was studied. The phenolic profile, assessed by accurate mass spectrometry analysis, was

5   modified during *in vitro* gastro-intestinal digestion, with a considerable decrease of total and

6   individual phenolic compounds. Phenolic acids showed the highest bioaccessibility with

7   hydroxycinnamic acids displaying higher bioaccessibility (from 41.2% to 45.1%) respect to

8   hydroxybenzoic acids (from 28.1% to 43.5%). Isomerisation of caffeoyl-quinic acids and galloyl-

9   quinic acids as well as dimerization of (epi)gallocatechin were also observed after *in vitro* gastro-

10 intestinal digestion. Antioxidant activity increased after the gastric step and rose further at the end

11 of the digestion. Furthermore, *in vitro* digested phenolic-rich fractions showed anti-proliferative

12 activity against two models of human colon adenocarcinoma cell lines. Cell metabolism of digested

13 phenolic compounds resulted in the accumulation of coumaric and ferulic acids in the cell media.

14

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

16 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

42   compounds are potentially bioavailable and able to exert their beneficial effects in the gastro-

43   intestinal tract or at systemic level (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010;

44   Tagliazucchi, Verzelloni, & Conte, 2012). Therefore, studies carried out with cell culture models

45   using pure phytochemicals (Carnésecchi et al., 2002) or cocoa/chocolate extracts (Rodríguez-

46   Ramiro, et al., 2011b) are unrealistic unless the bioaccessibility and gastro-intestinal tract stability

47   of the phenolic compounds have been well defined. Furthermore, *in vitro* studies did not take into

48   account the stability of tested molecules in cell cultures and their metabolic fate within the cells

49   (Aragonès, Danesi, Del Rio, & Mena, 2017).

50   This work aimed to investigate the effect of *in vitro* gastro-intestinal digestion on the

51   bioaccessibility of phenolic compounds in dark chocolate and dark chocolate functionalized with

52   Sakura green tea leaves or turmeric powder. In addition, the antioxidant and anti-proliferative

53   activities of *in vitro* digested dark chocolates phenolic compounds against two models of human

54   colonic cell lines were assessed. Finally, the last task was to identify and quantify the main

55   metabolites derived from incubation of *in vitro* digested dark chocolate phenolic compounds with

56   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

82   second step of the digestion (gastric phase) was carried out by adding to the bolus 10 mL of SGF.

83   The pH was adjusted to 2.0 with 6 mol/L HCl and supplemented with porcine pepsin (2000 U/mL

84   of simulated gastric fluid). After 2 h of incubation at 37°C, the final intestinal step was carried out

85   by adding 15 mL of SIF (prepared by mixing 10 mL of pancreatic fluid and 5 mL of bile salts).

86   Then, the pH was adjusted to 7.0, supplemented with pancreatin and the samples were incubated at

87   37°C for 2 h. All samples were immediately cooled on ice, centrifuged at $10000g$ for 20 min at 4°C

88   to eliminate insoluble materials and the supernatant frozen at –80°C for further analysis. The

89   digestions were performed in triplicate.

90   In addition, phenolic compounds were extracted from each dark chocolate (chemical extract) as

91   reported in Martini et al. (2018). The extractions were performed in triplicate.

92   Dark chocolate chemical extracts and samples collected at the end of each stage of the *in vitro*

93   digestion procedure were then used for total phenolic compounds and antioxidant activity

94   determinations.

95

96   *2.3. Identification and quantification of phenolic compounds by liquid chromatography mass*

97   *spectrometry (LC-ESI-QTOF-MS/MS)*

98   Dark chocolate chemical extracts and *in vitro* digested samples were analysed on Agilent HPLC

99   1200 Infinity (Agilent Technologies, Santa Clara, CA) equipped with a C18 column (HxSil C18

100   Reversed phase, 250×4.6 mm, 5 μm particle size, Hamilton Company, Reno, Nevada, USA) as

101   reported in Martini et al. (2018). The mobile phases consisted of (A) H_2O/formic acid (99:1, v/v)

102   and (B) acetonitrile/formic acid (99:1, v/v). After 0.5 min at 4% B, the gradient linearly rose up to

103   30% B in 60 min. The mobile phase composition was ramped up to 100% B in 1 min and

104   maintained for 5 min in order to wash the column before returning to the initial condition. The flow

105   rate was established at 1 mL/min. After passing to the column, the eluate was split and 0.3 mL/min

106   were directed to a 6520 accurate Q-TOF mass spectrometer (Agilent Technologies, Santa Clara,

107 CA). Identification of phenolic compounds in all samples was carried out using full scan, data-

108 dependent MS[2] scanning from m/z 100 to 1700. MS operating conditions, calibration curve

109 equations, linearity ranges and limit of quantifications (LOQ) for the different standards are

110 reported in Martini et al. (2018).

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

112

113 **2.4. Total phenolic compounds and antioxidant activity assays**

114 Folin-Ciocalteau assay was performed as reported by Singleton, Orthofer, & Lamuela-Raventós

115 (1999). The results were expressed as µmol of gallic acid per 100 g of chocolate.

116 The antioxidant properties of dark chocolate chemical extracts and *in vitro* digested samples were

117 evaluated performing two different assays. The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-

118 sulfonic acid) and ferric reducing power (FRAP) assays were performed according to the protocols

119 described by Re et al. (1999) and Benzie, & Strain (1996), respectively. The ABTS scavenging

120 capacity and FRAP values were expressed as mmol of trolox equivalent per 100 g of chocolate.

121

122 **2.5. Preparation of dark chocolate phenolic-rich fractions**

123 Samples collected at the end of the *in vitro* digestion were then passed through a SPE column

124 preconditioned with 4 mL of acidified methanol (containing 0.1% of formic acid), followed by 5

125 mL of acidified water (containing 0.1% of formic acid). Elution was carried out with acidified water

126 (6 mL) to eliminate the unbound material. Phenolic compounds were then desorbed by elution with

127 3 mL of acidified methanol. The obtained phenolic-rich extracts were diluted in the cell media and

128 used for the anti-proliferative activity determination. Each sample was extracted in triplicate.

129

130 **2.6. Cell cultures and anti-proliferative activity of in vitro digested dark chocolate phenolic-rich**

131 *fractions*

7

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 5×10^3/100 μL and 10×10^3/100 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

157   were collected and analysed by LC-MS/MS to determine the stability and the metabolism of the

158   dark chocolate phenolic compounds in the cell media. Cell media were extracted according to Sala

159   et al. (2015) and investigated according to Martini, Conte, & Tagliazucchi (2017). Briefly, samples

160   were analysed using a HPLC Agilent 1200 Series system equipped with an Agilent 6300 ion trap

161   mass spectrometer. Separations were performed using a C18 column (HxSil C18 Reversed phase,

162   250×4.6 mm, 5 μm particle size, Hamilton Company, Reno, Nevada, USA), with an injection

163   volume of 40 μL and elution flow rate of 1 mL/min. The mobile phase composition, the gradient

164   and MS operating conditions are the same as reported in Martini et al. (2017). MS experiments were

165   performed in ESI negative ion mode. Identification of phenolic compounds and metabolites in all

166   samples was carried out using full scan, data-dependent MS^2 scanning from m/z 100 to 1700.

167

168   *2.8. Statistic*

169   All data are presented as mean ± SD for three replicates for each prepared sample. One-way

170   analysis of variance (one-way ANOVA) with Tukey's post-hoc test was applied using Graph Pad

171   prism 6.0 (GraphPad software, San Diego, CA, U.S.A.). The differences were considered

172   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

10

198 of phenolic compounds from solid food matrices (Blancas-Benitez, Pérez-Jiménez, Montalvo-

199 González, González-Aguilar, & Sáyago-Ayerdi, 2018; Tagliazucchi et al., 2010; Tagliazucchi et al.,

200 2012). However, other studies found a decrease in bioaccessible total phenolic compounds during

201 the intestinal digestion (Bouayed et al., 2012; Lingua, Wunderlin, & Baroni, 2018). The different

202 results can be related to the higher stability of dark chocolate phenolic compounds to the intestinal

203 conditions respect to the other foods tested or to a different food matrix effect. However, it should

204 be taken into account that the Folin-Ciocalteau assay is strongly subject to interferences, especially

205 from sugars and vitamin C (Singleton et al., 1999). On the other hand, dark chocolate is rich in

206 Maillard reaction products that react in a concentration-dependent manner with the Folin-Ciocalteau

207 reagent, possibly resulting in an overestimation of bioaccessible total phenolic compounds

208 (Verzelloni, Tagliazucchi, & Conte, 2007).

209 **Figure 2** and **Tables 1-6** show how the *in vitro* gastro-intestinal digestion modified the phenolic

210 compounds profile in the samples from a qualitative and quantitative point of view. The MS data of

211 the individual phenolic compounds are reported in Martini et al. (2018). A total of 78, 122 and 86

212 phenolic compounds were identified by accurate mass spectrometry analysis after *in vitro* gastro-

213 intestinal digestion of DC, GTDC and TDC, respectively. This means that 45%, 21% and 39% of

214 individual phenolic compounds were not bioaccessible in DC, GTDC and TDC, respectively. A

215 significant lower amount of phenolic compounds was observed in all the samples after simulated

216 gastro-intestinal digestion respect to the chemical extracts. In TDC, only 17.6% of the total amount

217 of phenolic compounds was released from the food matrix or not degraded during digestion. In DC

218 and GTDC, the amount of bioaccessible total phenolic compounds at the end of the digestion was

219 23.0% and 23.2%, respectively (**Figure 2H** and **Table 6**).

220 The apparent lowest bioaccessible value of phenolic compounds in TDC was ascribed to the poor

221 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.

272

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.

According to the literature, there are no reports regarding the anti-proliferative activity of dark chocolate after *in vitro* digestion.

Previous *in vitro* studies have shown anti-proliferative properties of procyanidin and procyanidin-enriched extract isolated from cocoa powder in colon cancer Caco-2 cells (Carnesecchi et al., 2002; Martin and Ramos, 2017). However, 24 h treatment of Caco-2 and SW480 with catechin, epicatechin or procyanidin B2 did not affect cell growth, suggesting that other compounds rather than flavan-3-ols can be responsible for the observed anti-proliferative effect of phenolic-rich fractions extracted at the end of the *in vitro* gastro-intestinal digestion (Ramos, Rodríguez-Ramiro, Martín, Goya, & Bravo, 2011). The highest effect of TDC phenolic-rich fraction against Caco-2 cell line can be ascribed to the anti-proliferative activity of curcuminoids or a synergistic effect between curcuminoids and other phenolic compounds (Iwuchukwu, Tallarida, & Nagar, 2011).

3.4. *In vitro metabolism of digested dark chocolate phenolic-rich fraction in cell cultures*

In order to verify the cell metabolism of dark chocolate phenolic compounds, Caco-2 and SW480 media were analysed by LC-MS ion trap after incubation (24 h) with *in vitro* digested dark chocolate (at concentration corresponding to IC_{50}). Different metabolic reactions, including (de)hydroxylation, (de)hydrogenation, and conjugation with methyl, glucuronide, sulphate, and glutathione moieties were monitored. Some parent compounds and newly formed metabolites were detected in both cell types and reported in **Table 8**.

In addition to the parent compounds catechin and epicatechin, two newly formed metabolites were tentatively identified. Methyl-(epi)catechin was found in the cell media of both the cell lines whereas dimethyl-(epi)catechin was found only in Caco-2 medium. Previous studies identified methyl-epicatechin and sulphate-epicatechin as the main metabolites in Caco-2 experiments with a prevalence of methylation (Aragonès et al., 2017; Sanchez-Bridge et al., 2015). The lack of 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

420 amount of flavan-3-ols and flavonols after *in vitro* digestion than dark chocolate, achieving a more

421 efficient antioxidant activity. Similarly, the addition of turmeric powder may lead to an increased

422 anti-proliferative activity against adenocarcinoma cell lines respect to DC and GTDC.

423 In this way, the potential healthy effect of dark chocolate consumption could be maximized,

424 reducing the amount of energy and calories introduced with chocolate itself and resulting in a lower

425 intake to achieve the same biological effects.

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.

Di-hydro-caffeic acid

COMT

SW 480

Di-hydro-ferulic acid

RA

Feruloyl aspartate

CPase

Ferulic acid

Caffeoyl aspartate

CPase

SW 480 **COMT**

Caffeic acid

DHY

Coumaroyl aspartate

CPase

RA

HYD

Coumaric acid

Di-hydro-coumaric acid

Cinnamic acid

SULT

Coumaric acid-4-*O*-sulfate
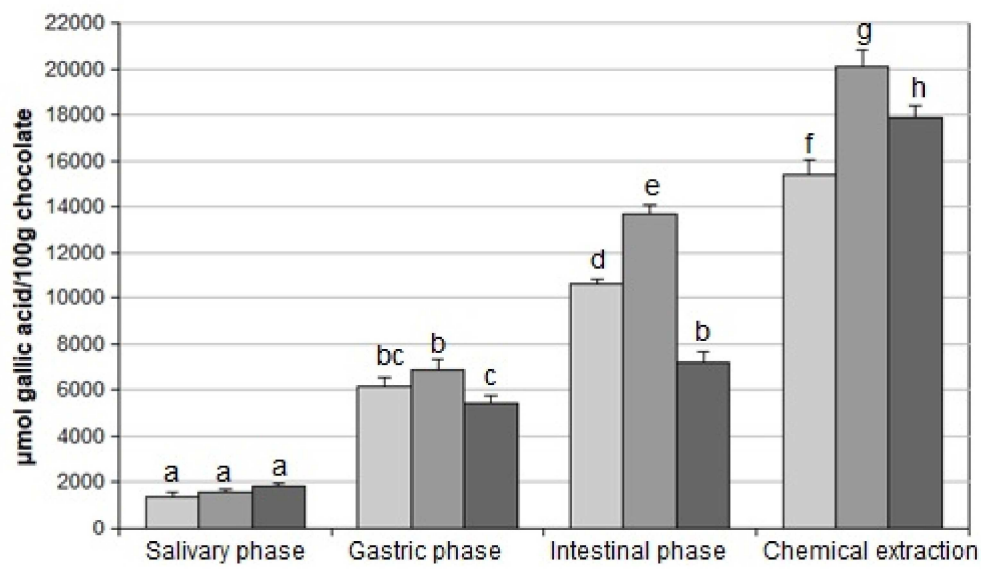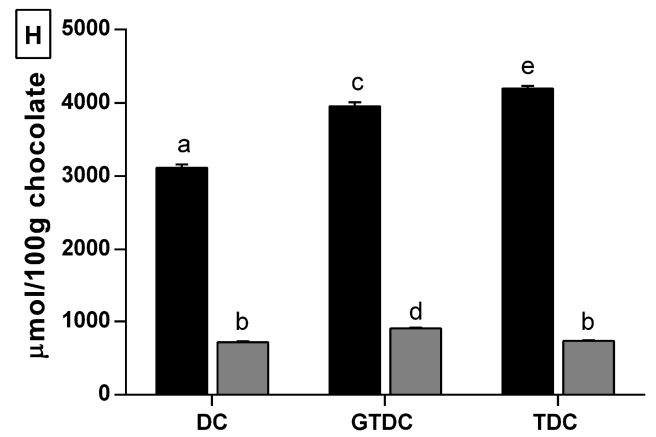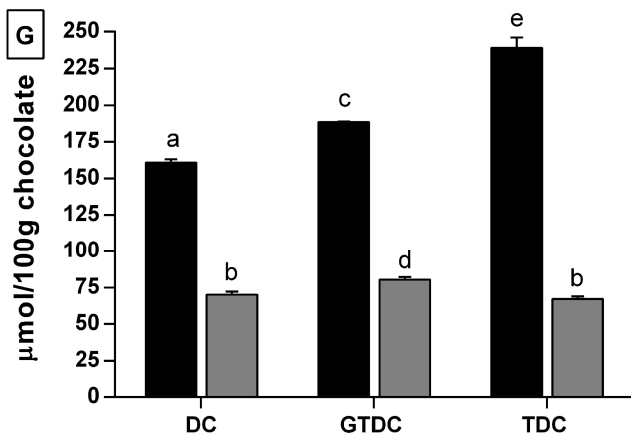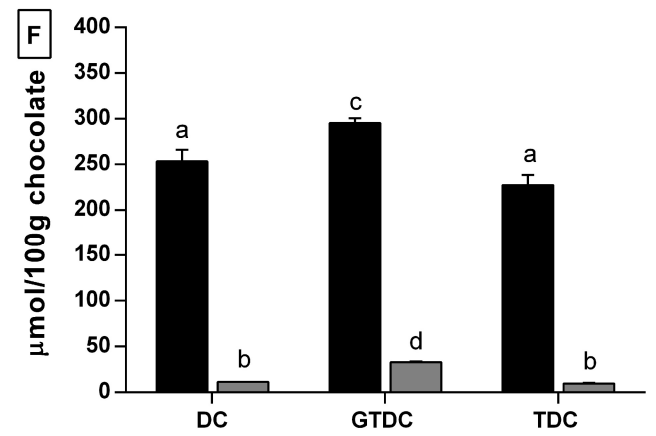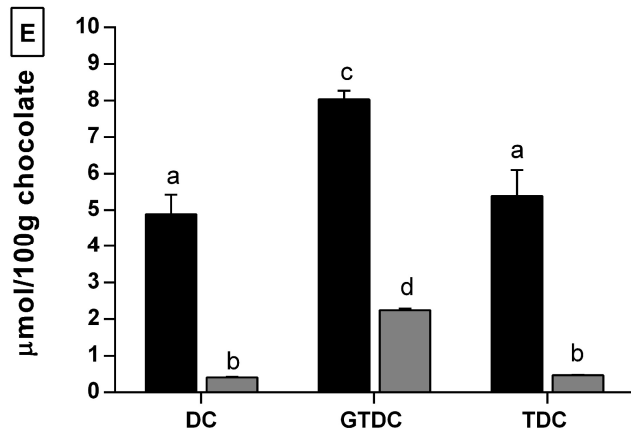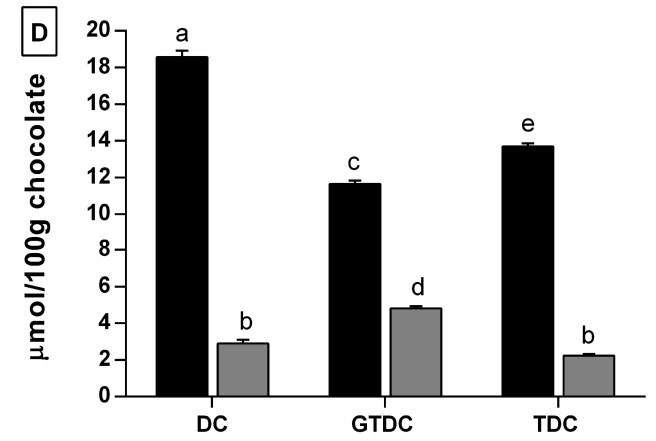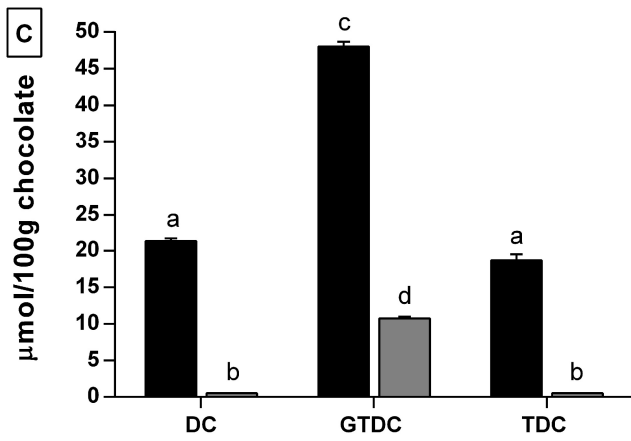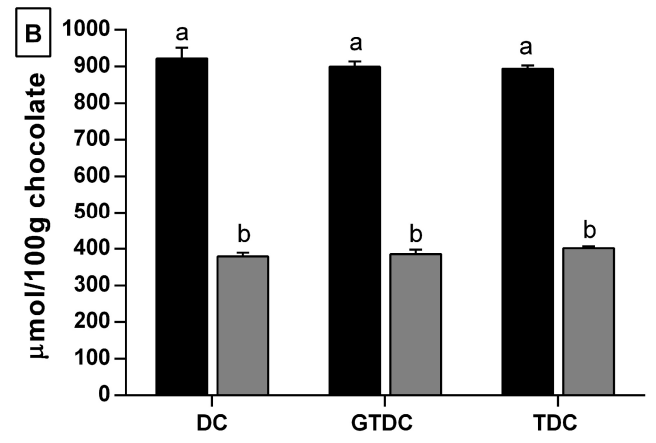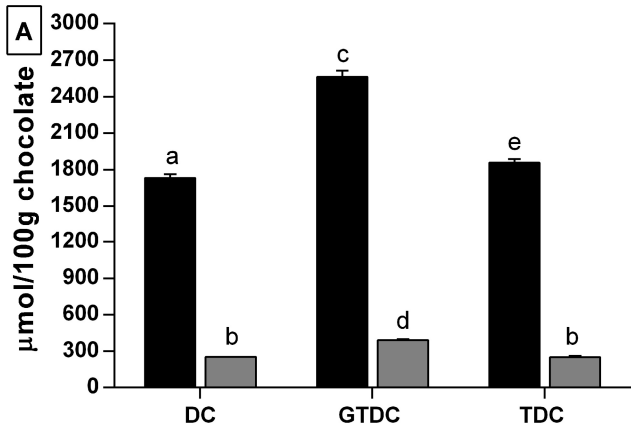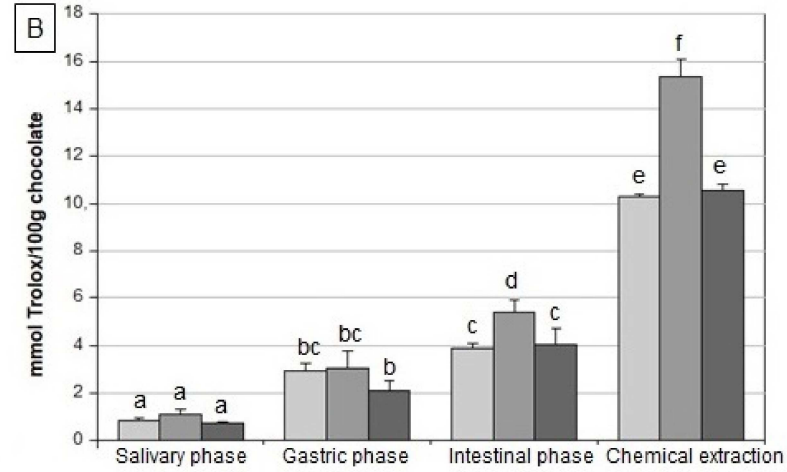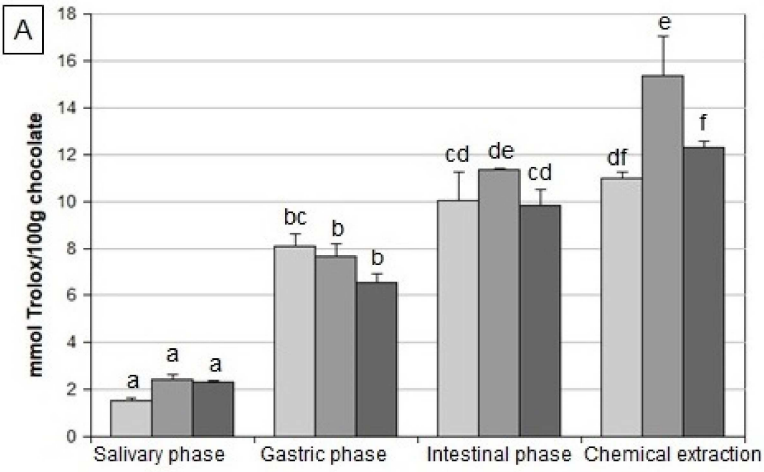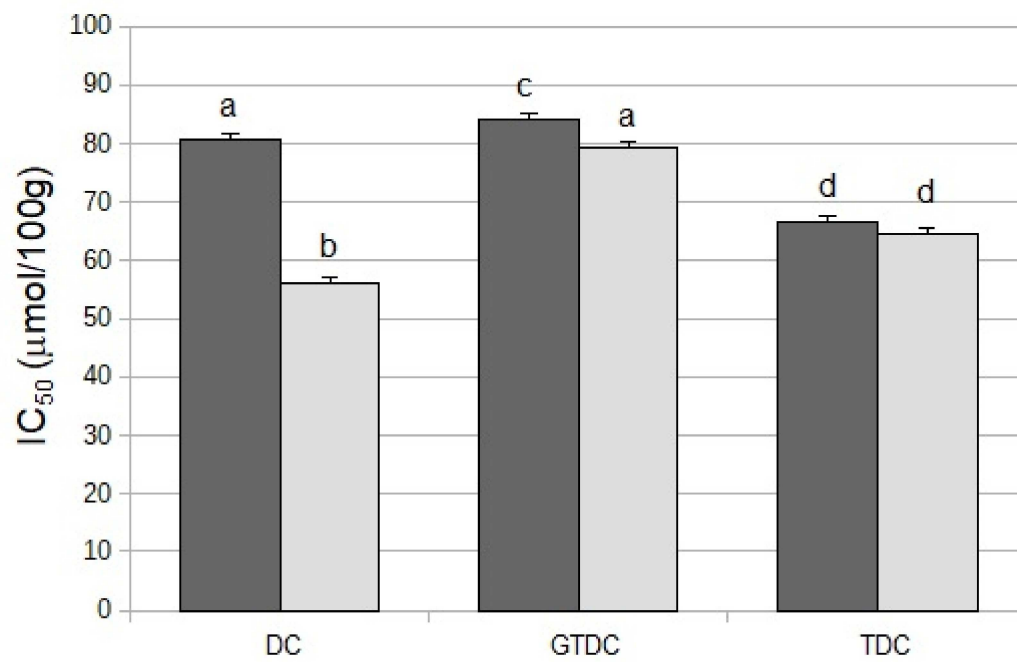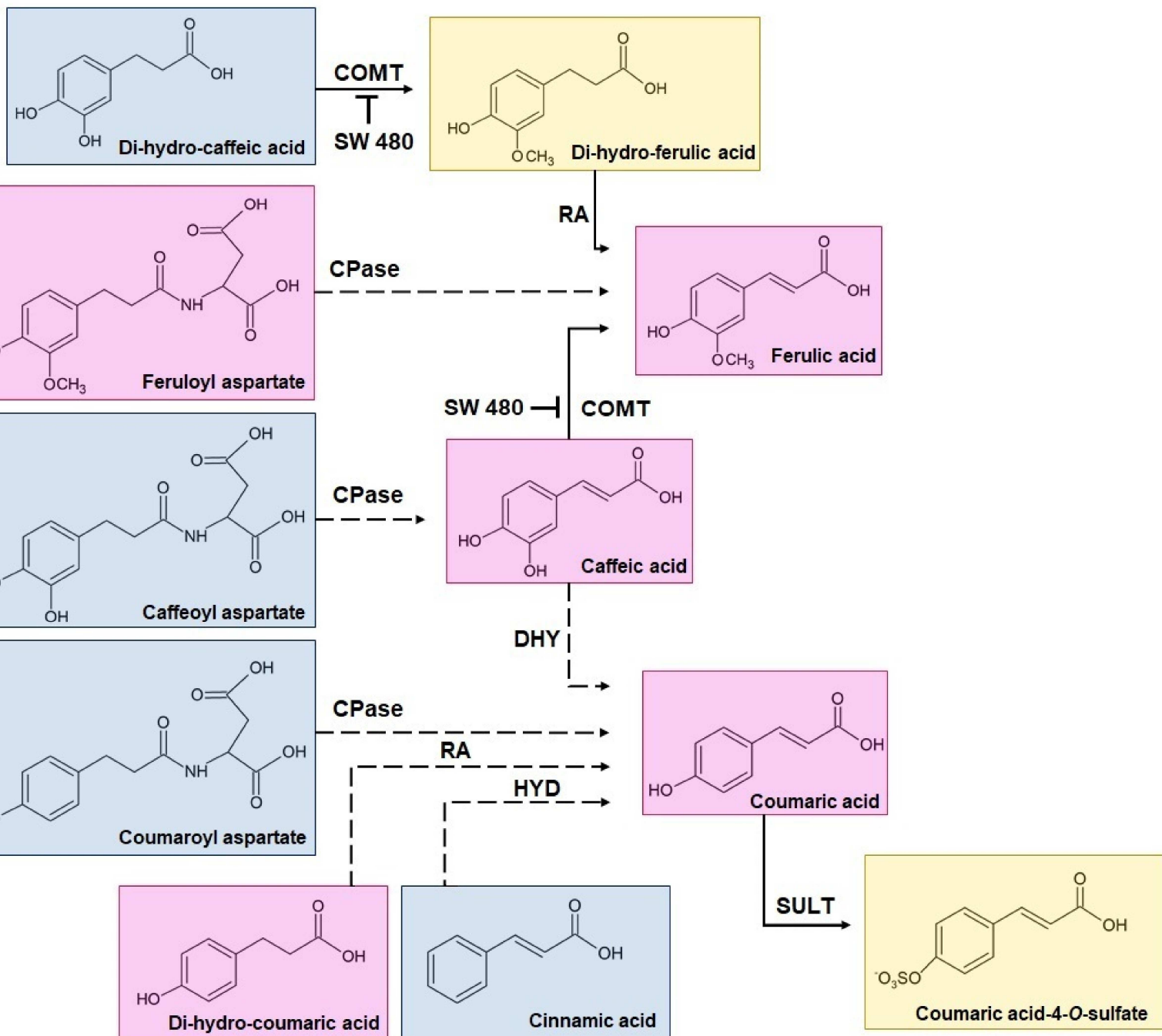
Table 1. Quantitative results (μmol/100 g of chocolate) for flavan-3-ols identified in dark chocolate after chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.

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.

Compound					
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