Bioactivity and cell metabolism of *in vitro* digested sweet cherry (*Prunus avium*) phenolic compounds

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Abstract

In this study, the bioaccessibility of phenolic compounds after *in vitro* gastro-intestinal digestion of two cherry cultivars was assessed. The phenolic profile was modified during *in vitro* digestion, with a considerable decrease of total and individual phenolic compounds. Hydroxycinnamic acids and especially coumaroylquinic acids showed the highest bioaccessibility. Isomerisation of caffeoylquinic and coumaroylquinic acids was observed after *in vitro* digestion. Modification of the phenolic profile after digestion resulted in an increased or decreased scavenging activity depending on the assay. *In vitro* digested phenolic-rich fractions also showed anti-proliferative activity against SW480 but no effect against Caco-2 cell lines. Both Caco-2 and SW480 cell lines were able to metabolize cherry phenolic compounds with remarkable differences. An accumulation of glycosylated flavonols was observed in SW480 medium. In conclusion, phenolic compounds from cherries and especially hydroxycinnamic acids were efficiently released and remained bioaccessible after *in vitro* digestion, resulting in antioxidant and anti-proliferative activities.

Keywords: mass spectrometry, bioaccessibility, anti-proliferative activity, hydroxycinnamic acids, Caco-2, SW480

1. Introduction

Sweet cherry (*Prunus avium* L., Rosacea) is a commercially important table fruit highly appreciated by consumers due to its taste, colour and sweetness, but also for its nutritional properties and beneficial effects (Kelley et al. 2018). Sweet cherry fruits contain different phenolic compounds, including anthocyanins, flavan-3-ols and hydroxycinnamic acids that have been related to the healthy properties of these fruits (Ferretti et al. 2010). Indeed, cherries were considered as the main dietary source of anthocyanins within the context of the Mediterranean Diet (Godos et al. 2017). Recently, our research group thoroughly investigated the phenolic profile of different sweet cherry varieties, from pale yellow flesh and slightly reddish skin to dark red cultivars, using mass spectrometry (Martini et al. 2017). Among the 86 identified compounds, 3-coumaroylquinic acid, epicatechin and cyanidin-3-rutinoside were present at the highest concentrations (Martini et al. 2017). The amount of individual phenolic compounds in sweet cherries is variable and strongly dependent on the cultivars (Martini et al. 2017; Picariello et al. 2016). The dark red cultivars are rich in anthocyanins, whereas the slightly reddish cultivars are rich in hydroxycinnamic acids and/or flavan-3-ols (Martini et al. 2017; Picariello et al. 2016).

There are several human intervention studies suggesting that cherry consumption may have beneficial effects on markers of cardiovascular disease risk (Kelley et al. 2018). Short-term supplementation of cherries reduced both systolic and diastolic blood pressure in diabetic women or hypertensive subjects (Keane et al. 2016; Kent et al. 2016). The effect on blood pressure correlated in time with changes in plasma cyanidin-3-glucoside metabolites such as vanillic and protocatechuic acids (Keane et al. 2016). Furthermore, cherry intake ameliorated the lipid profile of overweight and obese subjects by reducing the level of very-low density lipoproteins and increasing the amount of high-density lipoproteins (Martin et al., 2011). Some mechanisms have been proposed to explain the protective effect of cherries such as antioxidant activity, increased

41 expression of nitric oxide synthase and decreased expression of endothelin-1 (Kelley et al. 2013;
42 Edwards et al. 2015).
43 Cherry intake also resulted in an anti-cancer activity at gastro-intestinal tract level (Ferretti et al.
44 2010). Apc^{Min} mice consuming cherries, anthocyanins or cyanidin had significantly fewer and
45 smaller intestinal adenomas than Apc^{Min} mice consuming the control diet (Kang et al. 2003). The
46 protective effect of cherries against intestinal cancer has been attributed to the biological activities
47 of anthocyanins. Extracts of sweet cherries as well as anthocyanins and cyanidin aglycone showed
48 *in vitro* anti-proliferative activity against human cancer cells from the colon (HT-29 and HCT-15)
49 and stomach (MKN45) (Bastos et al. 2015; Serra et al. 2011; Kang et al. 2003). These *in vitro*
50 studies with cell culture models, carried out with pure phenolic compounds or cherry extracts, did
51 not take into account the bioaccessibility and gastro-intestinal tract stability of the phenolic
52 compounds. Some phenolic compounds, and especially anthocyanins, are unstable under the gastro-
53 intestinal tract conditions (Yang et al. 2018; Bouayed et al. 2012; Juániz et al. 2017). Indeed, cherry
54 phenolic compounds are entrapped in a solid food matrix and only the compounds released from the
55 food matrix may exert their beneficial effects in the gastro-intestinal tract or at systemic level
56 (Tagliazucchi et al. 2010). Furthermore, only few recent *in vitro* studies investigated the metabolism
57 and/or the stability of phenolic compounds in cell cultures (Aragonès et al. 2017; Mele et al., 2016;
58 Sala et al., 2015).
59 Therefore, this work aimed to investigate the effect of *in vitro* gastro-intestinal digestion on the
60 bioaccessibility and antioxidant properties of cherry phenolic compounds from two different
61 cultivars (Celeste and Durone Nero I). In addition, the anti-proliferative activity and the cell
62 metabolism of *in vitro* digested cherry phenolic compounds against two models of human colon
63 adenocarcinoma cell lines were assessed.

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) column (C18, 50 μm, 60 Å, 500 mg) were supplied by Waters (Milan, Italy). The absorbance was read using a Jasco V-550 UV/Vis spectrophotometer.

2.2. Cherry cultivars

Two sweet cherry (*Prunus avium*) cultivars (Celeste and Durone Nero I) were harvested at full maturity in Vignola (Modena province, Italy). For each variety, about 2 kg of cherries were randomly sampled from several trees and processed immediately or frozen within 1 h after harvesting and stored at -80°C until used. The two cherry cultivars were selected according to the different skin and flesh colours and phenolic composition (Martini et al. 2017). Celeste cultivar presented a pale red flesh and a bright red skin and predominantly contained hydroxycinnamic acids and flavan-3-ols. Durone Nero I instead was a dark red cultivar rich in anthocyanins.

2.3. In vitro gastro-intestinal digestion of cherry cultivars and preparation of the chemical extracts

For the *in vitro* digestion, the protocol previously developed within the COST Action INFOGEST was followed (Minekus et al. 2014). The fluids (simulated salivary, gastric and intestinal fluids)

89 were prepared according to Minekus et al. (2014). Salivary phase was simulated by homogenizing
90 with a laboratory blender five grams of each cherry cultivar in presence of 5 mL of the stock
91 simulated salivary fluid solution. After homogenization, 150 U/mL of porcine α-amylase were
92 added and the samples were incubated for 5 min at 37°C in a rotating wheel. The gastric phase of
93 the digestion was carried out by adding to the bolus 10 mL of simulated gastric fluid. After pH
94 adjustment to 2.0 with 6 mol/L HCl, 2000 U/mL of pepsin were added. After 2 h of incubation at
95 37°C, the final intestinal step was carried out by adding 15 mL of simulated intestinal fluid. Then,
96 the pH was adjusted to 7.0, supplemented with pancreatin and the samples were incubated at 37°C
97 for 2 h. All samples were immediately cooled on ice and frozen at –80°C for further analysis. The
98 digestions were performed in triplicate.
99 In addition, phenolic compounds were extracted from each cherry cultivar (chemical extract) as
100 reported in Martini et al. (2017). The extractions were performed in triplicate.
101
102 ***2.4. Preparation of the phenolic-rich fractions***
103 Chemical extracts and samples collected at the end of the *in vitro* digestion were then passed
104 through a SPE column preconditioned with 4 mL of acidified methanol (containing 0.1% of formic
105 acid), followed by 5 mL of acidified water (containing 0.1% of formic acid). Elution was carried
106 out with acidified water (6 mL) to eliminate the unbound material and phenolic compounds were
107 then desorbed with 3 mL of acidified methanol. The obtained phenolic-rich extracts were then used
108 for the subsequent analysis. Each sample was extracted in triplicate.
109
110 ***2.5. Identification and quantification of phenolic compounds by liquid chromatography***
111 ***electrospray ionization ion trap mass spectrometry (LC-ESI-IT-MS)***
112 Phenolic-rich fractions from chemical extracts and *in vitro* digested samples were analysed on
113 HPLC Agilent 1200 Series system 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. (2017). The mobile phase composition was (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 ramped up to 30% B in 60 min. The mobile phase composition was raised 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 set at 1 mL/min. The samples were injected in the amount of 20 µL. After passing through the column, the eluate was split and 0.3 mL/min were directed to an Agilent 6300 ion trap mass spectrometer. Two MS experiments were performed, one in negative ion mode and one using positive ionization (for anthocyanins), under the same chromatographic conditions. Identification of phenolic compounds in all samples was carried out using full scan, data-dependent MS^2 scanning from *m/z* 100 to 800 and selected reaction monitoring. MS operating conditions, limits of detection (LOD) and limits of quantification (LOQ) for the different standards are reported in Martini et al. (2017). Quantitative results were expressed as µmol of compounds per 100 g of cherry.

2.6. Bioaccessibility calculation

The percentage bioaccessibility of phenolic compounds after *in vitro* gastro-intestinal digestion was calculated as follow:

$$Bioaccessibility = \frac{\mathrm{CC}d}{\mathrm{CC}e} * 100$$

Where CC*d* is the concentration of total or individual phenolic compounds in the phenolic-rich fraction from *in vitro* digested sample and CC*e* is the concentration in the phenolic-rich fraction from the chemical extract.

2.7. Antioxidant activity assays

The antioxidant properties of the phenolic-rich fractions obtained from chemical extracts and *in vitro* digested samples were detailed by using four different assays.

139 The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and ferric reducing power
140 (FRAP) assays were performed according to the protocols described by Re et al. (1999) and Benzie
141 and Strain (1996), respectively. The capacity to scavenge hydroxyl and superoxide anion radicals
142 were evaluated according to the methods reported by Martini et al. (2017).
143 The results were expressed as μmol of trolox equivalent per mmol of total phenolic compounds.
144
145 ***2.8. Cell cultures and anti-proliferative activity of cherry phenolic-rich fractions***
146 Human adenocarcinoma Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium
147 (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% antibiotic mix (streptomycin and
148 penicillin) and 2 mmol/L L-glutamine. Human adenocarcinoma SW480 cells were cultured in
149 Leibowitz medium supplemented with 10% FBS, 1% antibiotic mix (streptomycin and penicillin)
150 and 2 mmol/L L-glutamine. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.
151 Cells were seeded at $5x10^3$/100 μL and $10x10^3$/100 μL for Caco-2 and SW480, respectively, in 96-
152 well plates for 24 h to allow cell adhesion to the bottom of the wells.
153 For the anti-proliferative assays, a colorimetric method for the sensitive quantification of viable
154 cells was performed, using MTS assay kit. 100 μL of digested and un-digested cherry phenolic-rich
155 fractions diluted in cell culture media at different concentrations were added to the cell plates and
156 incubated for 24 h. At the end of the treatments, media were refreshed with 180 μL of culture media
157 and 20 μL of MTS reagent were added to each well. After 4 h of incubation at 37°C, the absorbance
158 was measured at the wavelength of 490 nm using a microplate reader and results were expressed as
159 IC_{50}. IC_{50} was defined as the concentration of phenolic compounds required to inhibit 50% cell
160 proliferation and expressed as μmol of total phenolic compounds/L. The IC_{50} values were
161 determined using nonlinear regression analysis and fitting the data with the log (inhibitor) vs.
162 response model generated by GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).
163

2.9. Liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis of cell media

Cell culture supernatants were collected at the end of the experiments and analysed by LC-MS/MS to determine the cell metabolism of the cherry phenolic compounds. Cell media were extracted according to Sala et al. (2015) and analysed using a HPLC Agilent 1200 Series system equipped with an Agilent 6300 ion trap mass spectrometer as reported above. 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 above.

2.10. 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. Results and discussion

3.1. In vitro bioaccessibility of phenolic compounds in cherry cultivars

Table 1 shows data about the bioaccessibility of individual phenolic compounds in the two analysed cherry cultivars. The MS data of the individual phenolic compounds are reported in Martini et al. (2017).

A total of 57 and 56 phenolic compounds were identified and quantified in the chemical extracts of cherry cultivars Celeste and Durone Nero I, respectively (**Table 1**). In both the cultivars, 3-coumaroylquinic acid (found as *cis* and *trans* isomers) was the main phenolic compound accounting for 40.6% and 31.3% of the total compounds in Celeste and Durone Nero I, respectively. In general, hydroxycinnamic acids were the most representative class of phenolic compounds in both the cultivars (54.2% and 42.2% of the total phenolic compounds in Celeste and Durone Nero I, respectively). From a quantitative point of view, flavan-3-ols were the second class of phenolic compounds in both the cultivars (40.7% and 33.4% of the total phenolic compounds in Celeste and Durone Nero I, respectively). Anthocyanins accounted for the 15.4% of total compounds in the cultivar Durone Nero I whereas their incidence was about 3% in the cultivar Celeste. Finally, small amounts of others flavonoids were also detected in both the cultivars.

The phenolic compounds profile was significantly modified from a qualitative and quantitative point of view after *in vitro* gastro-intestinal digestion (**Figure 1** and **Table 1**). Only 25 and 39 of the original phenolic compounds were identified after simulated digestion of the cultivars Celeste and Durone Nero I, respectively. This meant that 56% and 30% of individual phenolic compounds were not bioaccessible.

Total phenolic compounds determined by LC-ESI-IT-MS/MS in the cherry cultivars chemical extracts were significantly higher ($P<0.05$) than those found after *in vitro* gastro-intestinal digestion (**Figure 1A** and **Table 1**). The bioaccessibility of total phenolic compounds was 39.7% and 29.9% in the cultivars Celeste and Durone Nero I, respectively. Hydroxycinnamic acids were the most

abundant phenolic compounds released from cherry matrices with a bioaccessibility of 71.7% and 46.5% in the cultivars Celeste and Durone Nero I, respectively (**Figure 1B** and **Table 1**). At the end of the digestion, hydroxycinnamic acids represented the 98.0% and 65.5% of total phenolic compounds in the cultivars Celeste and Durone Nero I, respectively (**Figure 1H**). These compounds were particularly stable under gastro-intestinal conditions and easily released from the cherry matrices. The stability of hydroxycinnamic acids under gastro-intestinal conditions has been already reported. For example, Tagliazucchi et al. (2012) found that only the 30% of hydroxycinnamic acids were degraded during *in vitro* digestion of a coffee beverage whereas Monente et al. (2015) assessed a 14% decrease during *in vitro* digestion of a spent coffee water extract. Previous studies with solid food matrices confirmed the easy release of hydroxycinnamic acids during *in vitro* digestion. The bioaccessibility of hydroxycinnamic acids was 60-67% in cooked cardoon, 32-57% in apple and about 77% in frozen sweet cherries subjected to gastro-intestinal digestion (Juániz et al. 2017; Fazzari et al. 2008; Bouayed et al. 2012). This is also in accordance with the recorded recovery of caffeoylquinic acids (59-77%) in ileal fluid after coffee or apple ingestion by ileostomists (Erk et al. 2014; Stalmach et al. 2010). The highest release after incubation with digestive fluids was found for coumaroylquinic acids, followed by feruloylquinic and caffeoylquinic acids (**Table 1** and **Figure 2**). These results are in accordance with Monente et al. (2015) and Tagliazucchi et al. (2012) who found that feruloylquinic and caffeoylquinic acids were quite stable under gastro-intestinal conditions. No data about the stability or bioaccessibility of coumaroylquinic acids have been published until now. Otherwise, the minor hydroxycinnamic acids identified in cherries (such as di-hydroxycinnamic acids and lactone derivatives) were not bioaccessible probably because of their instability in the gastro-intestinal environment (Monente et al. 2015; Tagliazucchi et al. 2012). The differences between the recoveries of hydroxycinnamic acids in the two different cherry cultivars could be indicative of a food matrix impact (**Figure 2** and **Table 1).**

As reported above, overall coumaroylquinic, caffeoylquinic and feruloylquinic acids were easily released from food matrices, although changes mainly due to variations in isomers level were recorded after *in vitro* digestion. While the 5- and 3-caffeoylquinic acid isomers were found at lower concentrations after *in vitro* digestion compared to the chemical extracts, the 4-caffeoylquinic acid isomers concentration increased significantly (**Figure 2A** and **2B** and **Table 1**). Acyl migration of caffeoylquinic acids has been already demonstrated both *in vitro* with pure standard compounds or after digestion of coffee and *in vivo* in ileostomy volunteers (Erk et al. 2014; Monente et al. 2015; Tagliazucchi et al. 2012). Similar results were observed for feruloylquinic acids (**Figure 2C** and **2D** and **Table 1**). Otherwise, a different behaviour was observed for coumaroylquinic acids. In this case, we found remarkable higher concentrations of 4- and 5-coumaroylquinic acid isomers after simulated digestion (**Figure 2E** and **2F** and **Table 1**). On the contrary, the concentration of 3-coumaroylquinic acid isomers was lower after digestion than that recorded after chemical extraction. Previously, Kahle et al. (2011) reported isomerization of 4-coumaroylquinic acid to 3- and 5-coumaroylquinic acids after incubation of standard compounds with intestinal fluids. These results, instead, suggested that coumaroylquinic acids were subjected to isomerization from the 3-acyl to 4- and 5-acyl structures.

Simple hydroxycinnamic acids (coumaric, caffeic and ferulic acids) were not found after intestinal digestion, suggesting that relevant hydrolysis of ester bond did not occur during *in vitro* digestion. On the other hand, incubation of caffeoylquinic and coumaroylquinic acids standards resulted in the appearance of simple acids after 6 h of incubation in intestinal fluids (Kahle et al. 2011).

Flavan-3-ols, which were the second most abundant class of phenolic compounds in cherry chemical extracts, were characterized by a very low bioaccessibility (**Figure 1C** and **Table 1**). In the cultivar Celeste, only (epi)catechin-3-gallate was detected after gastro-intestinal digestion. In the cultivar Durone Nero I we also found monomeric and dimeric (epi)catechins, although (epi)catechin-3-gallate was still the flavan-3-ol with the highest bioaccessibility. The

bioaccessibility of monomeric catechins seems to be highly dependent on the food matrix. During *in vitro* digestion of green tea, Green et al. (2007) found that only the 32% of epicatechin was degraded. High stability of monomeric catechins was also described when the standard compounds were incubated with gastro-intestinal fluids (Tagliazucchi et al. 2010). However, *in vitro* digestion of apple varieties, grape or wine resulted in an important degradation of monomeric catechins (Bouayed et al. 2012; Lingua et al. 2018). Whether catechin and epicatechin were degraded or not released from cherry matrices remained to be established.

Cultivar Durone Nero I was also characterized by a high content of anthocyanins. In this cherry cultivar, anthocyanins represented the phenolic class with highest bioaccessibility (55.0%), constituting the 28.2% of total phenolic compounds after *in vitro* digestion (**Figure 1G** and **1H** and **Table 1**). Anthocyanins are generally considered very unstable compounds under slightly alkaline conditions as found in the intestinal milieu. For example, *in vitro* digestion of red wine, chokeberry, and pomegranate juice resulted in a substantial degradation of monomeric anthocyanins (Bermúdez-Soto et al. 2007; Lingua et al. 2018; Perez-Vicente et al. 2002). Anthocyanins stability in the digestive system appeared to be related to their molecular structure. The presence of a methoxy group in the B-ring stabilized the anthocyanin structure (Yang et al. 2018). The acetylated derivatives of anthocyanins showed relatively higher stability during the gastro-intestinal transit than simple anthocyanins (Yang et al. 2018). As shown in **Table 1**, the rutinoside derivatives of cherry anthocyanins were more stable than the corresponding glycosidic forms. These results are in agreement with Fazzari et al. (2008). It can be speculated that fruits containing high levels of rutinoside derivatives are likely to provide larger amounts of anthocyanins in the colon. Indeed, peonidin-3-*O*-rutinoside, which is characterized by the presence of a rutinose moiety and a methoxy group in the B-ring showed 100% of bioaccessibility.

Some newly identified compounds were indicative of anthocyanins degradation (**Table 1**). Protocatechuic acid appeared after *in vitro* digestion of cherry cultivars and plausibly came from the

279 degradation of cyanidin-3-*O*-glucoside. Moreover, cyanidin-3-*O*-glucoside chalcone was also found
280 after *in vitro* digestion of Durone Nero I due to the presence of its $[M+H]^+$ precursor ion at 467 and
281 product ions at *m/z* 305 (-162 amu; loss of a glucosyl moiety), *m/z* 449 (-18 amu; loss of water) and
282 *m/z* 287 (-18-162 amu; loss of water and glucosyl moiety) (Lopes et al. 2007). After *in vitro*
283 digestion of Durone Nero I, a new compound with a $[M+H]^+$ mass signal at 737 was detected. In
284 ESI positive experiments, it gave a base peak product ion at *m/z* 575 (-162 amu) corresponding to
285 the loss of a glucosyl moiety. Additional product ions were detected at *m/z* 557 (loss of water from
286 *m/z* 575) and *m/z* 287 (loss of a (epi)catechin unit from *m/z* 575). Finally, a product ion at *m/z* 423
287 was detected corresponding to the loss of 152 amu from *m/z* 575 resulting from the retro Diels-
288 Alder decomposition of the flavan-3-ol unit. The described fragmentation pattern corresponded to
289 that previously observed for a (epi)catechin-cyanidin-3-*O*-glucoside dimer (Sentandreu et al. 2010).
290 The bioaccessibility of the other phenolic classes was very low with the exception of flavonols
291 (52.2% and 39.3% of bioaccessibility in the cultivars Celeste and Durone Nero I, respectively)
292 (**Figure 1D** and **Table 1**). However, due to their low concentration in the cherries, they represented
293 only the 1.5% and 4.4% of total phenolic compounds determined after *in vitro* digestion in the
294 cultivars Celeste and Durone Nero I, respectively (**Figure 1H** and **Table 1**).
295
296 ***3.2 Effect of in vitro digestion on the antioxidant properties***
297 To fully characterize the antioxidant properties of the phenolic-rich fractions of cherries, the
298 ability to scavenge some physiologically relevant radicals (superoxide anion and hydroxyl radical)
299 and the organic nitro-radical ABTS as well as the reducing power were evaluated before and after
300 digestion (**Figure 3**).
301 The hydroxyl radical scavenging capacity of phenolic-rich extracts decreased after *in vitro* digestion
302 of both the cherry cultivars (**Figure 3A**). This means that cherry phenolic compounds in the
303 chemical extracts were more efficient scavengers of hydroxyl radical than the phenolic compounds

304 present in the digested cherries. This decrease may reflect the different composition of the chemical
305 and *in vitro* digested phenolic-rich extracts. In both the cherry cultivars, the *in vitro* digested
306 extracts were enriched in hydroxycinnamic acids (**Table 1** and **Figure 1H**) that showed the lowest
307 hydroxyl scavenging activity among phenolic compounds (Özyürek et al. 2008). On the contrary,
308 ferric reducing power of the phenolic-rich extracts increased after *in vitro* digestion (**Figure 3B**).
309 This clearly reflected the different composition of the chemical and digested extracts, since the
310 ferric reducing ability of hydroxycinnamic acids is considered higher than that of flavan-3-ols
311 (Pulido et al. 2000). The superoxide anion scavenging activity instead was similar between the
312 phenolic-rich extracts before and after *in vitro* digestion (**Figure 3C**). Finally, the ABTS radical
313 scavenging activity assay displayed a different behaviour in the two cherry cultivars (**Figure 3D**).
314 In Celeste cultivar, the phenolic-rich extract obtained after *in vitro* digestion showed a lower
315 scavenging ability than the extract before digestion. This is in keeping with the observed percent
316 increase in hydroxycinnamic acids that showed lower ABTS radical scavenging activity respect to
317 flavan-3-ols (Rice-Evans et al. 1996). Instead, in the cultivar Durone Nero I we observed an
318 increase in the ABTS radical scavenging activity in the phenolic-rich extract after *in vitro* digestion.
319 This can be related to the percentage increase of anthocyanins and flavonols in the digested extracts
320 (**Table 1** and **Figure 1H**), which displayed greater ABTS radical scavenging activity than flavan-3-
321 ols (Rice-Evans et al. 1996).
322 Indeed, it is important to note that the different incidence of the phenolic classes in the digested
323 phenolic-rich extracts gave rise to significant different behaviours in the scavenging capacity or
324 reducing power in relation to the type of assay and mechanism involved, leading to an increase or
325 decrease of the considered activities.
326
327 ***3.3 Anti-proliferative activity of cherry phenolic-rich fractions on human colon adenocarcinoma***
328 ***cell lines***

329 The effect of cherry phenolic-rich fractions extracted before and after *in vitro* gastro-intestinal
330 digestion on the proliferation of human colon adenocarcinoma Caco-2 and SW480 cells was
331 investigated. Caco-2 and SW480 cells were incubated with different concentrations of phenolic-rich
332 extracts for 24 h. Cherry phenolic-rich extracts did not affect the proliferation of Caco-2 (data not
333 shown). Instead, the phenolic compounds from the chemical extracts of both the cultivars were able
334 to inhibit in a dose-dependent manner the proliferation of SW480 cell line (**Figure 4**). The chemical
335 extract of cultivar Durone Nero I was about 2.5 times more efficient than Celeste with IC_{50} values
336 of 48.01 ± 3.02 and 121.90 ± 5.03 μmol/L, respectively. *In vitro* digestion positively affected the
337 anti-proliferative activity of Celeste phenolic-rich fraction. As reported in **Figure 4**, the IC_{50} value
338 of phenolic compounds extracted after digestion of cultivar Celeste halved to 61.22 ± 4.02 μmol/L.
339 No significant differences were found between the IC_{50} values calculated for cultivar Durone Nero I
340 before and after digestion.
341 According to the literature, this is the first report investigating the anti-proliferative activity of
342 sweet cherry phenolic-rich extracts against human colon adenocarcinoma Caco-2 and SW480 cell
343 lines and the influence of *in vitro* digestion processes.
344 Previous *in vitro* studies have shown anti-proliferative properties of sweet cherry extracts against
345 colon cancer cell lines such as HCT-15 and HT29 (Bastos et al. 2015; Serra et al. 2011). This effect
346 has been attributed to the anthocyanin content of the cherry extracts (Wang and Stoner 2008).
347 However, Olsson et al. (2008) found no correlation between proliferation of HT29 cells and
348 the concentration of anthocyanins in ten fruits and berries (including cherries). In accordance, also
349 in this study we did not find any correlation between the anthocyanin content and the anti-
350 proliferative activity. In fact, Durone Nero I chemical extract contained five times more
351 anthocyanins than Celeste chemical extract but exhibited only 2.5 times more anti-proliferative
352 activity. Indeed, *in vitro* digested phenolic fraction from Celeste was as effective as Durone Nero I
353 chemical extract although it did not contain anthocyanins.

354 Our results suggested that other compounds rather than anthocyanins can be responsible for the
355 observed anti-proliferative effect of phenolic-rich fractions extracted before and at the end of the *in*
356 *vitro* gastro-intestinal digestion.
357
358 ***3.4. Metabolism of cherry phenolic compounds in cell cultures***
359 In an attempt to unravel the metabolism of cherry phenolic compounds by Caco-2 and SW480, cell
360 media were collected after 24 h of incubation with *in vitro* digested Durone Nero I phenolic-rich
361 fraction and subjected to LC-MS ion trap analysis. Some parent compounds and newly formed
362 metabolites were detected in both cell types and reported in **Table 2**.
363 Total coumaroylquinic acids final concentration accounted for the 13.9 and 14.7% of the initial
364 concentration in the cultured Caco-2 and SW480, respectively. In addition to the coumaroylquinic
365 acid isomers, one newly formed metabolite was tentatively identified as coumaroylquinic acid
366 sulphate in the cell media of both the cell lines. The rate of sulphation of coumaroylquinic acid
367 isomers in SW480 was 0.5%, while in Caco-2 coumaroylquinic acid sulphate was found only at
368 trace levels. To the best of our knowledge, this is the first report showing sulphation of
369 coumaroylquinic acids by Caco-2 and SW480. Coumaric and (iso)ferulic acids were found in the
370 media after incubation with both cell lines, whereas caffeic acid only after incubation with SW480
371 (**Table 2**). The appearance of these simple hydroxycinnamic acids, which were not present in the
372 digested sample, was accompanied by the disappearance of caffeoylquinic and feruloylquinic acids
373 (with the exception of trace amounts of 3-feruloylquinic acid) and the decrease in concentration of
374 coumaroylquinic acids. These results can be indicative of hydroxycinnamoyl-quinic acids
375 degradation in the media or to the hydrolysis catalysed by esterases. Considering the initial
376 concentration of feruloylquinic acids, their complete hydrolysis during incubation with cells should
377 result in a (iso)ferulic acid concentration of 10.47 μmol/L. However, the amount of (iso)ferulic acid
378 found in cell medium after 24 h of incubation with Caco-2 was about 5.5 times higher (**Table 2**).

379 Previous studies have shown that Caco-2 possess catechol-*O*-methyltransferase able to metabolize
380 caffeic acid to (iso)ferulic acid (Monente et al. 2015; Farrell et al. 2011; Kern et al. 2003).
381 Therefore, the exceeded amount of (iso)ferulic acid can be a consequence of the methylation of
382 caffeic acid released from hydrolysis of caffeoylquinic acids. The mass balance recovery of
383 (iso)ferulic acid also considering the initial amount of caffeoylquinic acids present in the digested
384 sample was 35.6%, suggesting the partial methylation of caffeic acid released after hydrolysis of
385 caffeoylquinic acids. This conclusion is also supported by the lack of identification of caffeic acid
386 in the medium after 24 h of incubation with Caco-2.
387 The same conclusions can not be drawn for SW480. In the medium of this cell line, we found some
388 residual caffeic acid and the amount of (iso)ferulic acid was 1.2 times higher respect than the
389 amount found at the end of the digestion (**Table 2**). It can be speculated that SW480 expressed
390 lower amount of catechol-*O*-methyltransferase or that the isoform in these cells had a lower affinity
391 for caffeic acid respect to the isoform in Caco-2 cells.
392 Another difference between the two cell lines was related to the ability to metabolize glycosylated
393 flavonols. Caco-2 cells were found to be able to hydrolyse quercetin-3-*O*-glucoside to the
394 corresponding aglycone. Furthermore, a newly formed metabolite, quercetin-3-*O*-glucoside
395 sulphate appeared in the cell medium after 24 h of incubation with Caco-2. The ability of Caco-2
396 cells to hydrolyse glycosylated flavonols was previously reported (Boyer et al. 2004). Finally,
397 glycosylated flavonols tended to be accumulated after 24 h of incubation with SW480 cell line.
398
399 **4. Conclusions**
400 Bioactivity of phenolic compounds is primarily conditioned by their bioaccessibility in the gastro-
401 intestinal tract, and secondly on their cellular uptake and internal transformation. The present study
402 determined the amounts of bioaccessible phenolic compounds from two cherry cultivars after *in*
403 *vitro* gastro-intestinal digestion. Results showed that the digestion process modified from a

qualitative and quantitative point of view the profile of phenolic compounds in the samples. Hydroxycinnamic acids and flavonols showed the highest bioaccessibility. Isomerisation of caffeoylquinic and coumaroylquinic acids was observed after *in vitro* gastro-intestinal digestion. We have also demonstrated that Caco-2 and SW480 cell lines showed metabolic activity resulting in a partial modification of cherry phenolic compounds. Methylation, sulphation, de-glycosylation and hydrolysis of esters were the main pathways of phenolic compounds metabolism in the tested cell lines. Moreover, we demonstrated that bioaccessible cherry phenolic compounds showed anti-proliferative activity against two models of human colon adenocarcinoma cell lines. The presence of (iso)ferulic acid in Caco-2 cell medium at higher concentration than in SW480 cell medium or the presence of caffeic acid only in SW480 cell medium and quercetin and quercetin-3-*O*-glucoside sulphate only in Caco-2 cell medium may suggest the intrinsic differences among the two cell lines and the metabolic mechanisms involved. The different effects on the two tested cell lines can be related to the accumulation of glycosylated flavonols in the cell medium of SW480 or to a different sensitivity of the cell lines to the phenolic compounds. Nevertheless, it could be interesting to underline how the sensitivity to the presence of phenolic-rich extracts increased after digestion, where the hydroxycinnamic acids were the major phenolic class present.

Further studies are necessary in order to confirm the proposed pathways of metabolism of cherry phenolic compounds during incubation with cell lines and the potential role of hydroxycinnamic acids.

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Figure captions

Figure 1. Bioaccessibility of individual phenolic compounds identified and quantified by LC-ESI-IT MS/MS grouped by classes. (A) Sum of the different classes; (B) hydroxycinnamic acids; (C) flavan-3-ols; (D) flavonols; (E) other flavonoids; (F) hydroxybenzoic acids; (G) anthocyanins. Dark grey columns represent the amount of the individual classes found in the chemical extract whereas light grey columns the amount at the end of the digestion. (H) Percent incidence of the different classes. UC: un-digested Celeste; DC: digested Celeste; UDN: un-digested Durone Nero I; DDN: digested Durone Nero I. ( ) Anthocyanins; ( ) other flavonoids; ( ) flavan-3-ols; ( ) flavonols; ( ) hydroxycinnamic acids; ( ) hydroxybenzoic acids. Results are expressed as mean ± standard deviation. Values in the same graph with different lowercase letters are significantly different ($P < 0.05$).

Figure 2. Bioaccessibility of individual hydroxycinnamic acids isomers. (A, C and E) Celeste cultivar; (B, D and F) Durone Nero I cultivar. Black columns represent the amount of the individual isomers found in the chemical extracts whereas grey columns the amount at the end of the digestion. CQA: caffeoylquinic acids; FQA: feruloylquinic acids; CoQA: coumaroylquinic acids. Results are expressed as mean ± standard deviation. Values in the same graph with different lowercase letters are significantly different ($P < 0.05$).

Figure 3. Antioxidant properties of un-digested and *in vitro* digested cherry phenolic-rich fractions. Antioxidant capacity measured as hydroxyl radical scavenging capacity (A), ferric reducing power (FRAP) (B), superoxide anion scavenging capacity (C) and ABTS radical scavenging activity (D). Black columns show data from the chemical extracts whereas grey columns from the extracts at the end of the 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 un-digested and *in vitro* digested cherry phenolic-rich fractions. 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-IT MS/MS analysis. Dark grey columns represent the activity of the chemical extracts whereas light grey columns the activity of the extracts at the end of the digestion. Results are expressed as mean ± standard deviation. Values in the same graph with different lowercase letters are significantly different ($P < 0.05$).

Table 1. Quantitative results (μmol/100 g fresh weight fruit) for phenolic compounds identified in the cherry phenolic-rich fractions obtained from both chemical extraction and after gastro-intestinal digestion. Values represent means ± standard deviation of triplicate determination.*

Compound	*Celeste*		*Durone Nero I*	
	Chemical extraction	**After digestion**	**Chemical extraction**	**After digestion**
Hydroxycinnamic acids				
3-Caffeoylquinic acid *cis*	31.90 ± 1.76^{b}	8.44 ± 0.45^{c}	37.87 ± 0.28^{a}	3.54 ± 0.51^{d}
3-Caffeoylquinic acid *trans*	123.91 ± 5.43^{b}	33.06 ± 0.72^{c}	197.17 ± 0.75^{a}	33.36 ± 0.40^{c}
4-Caffeoylquinic acid *cis*	1.44 ± 0.03^{c}	6.23 ± 0.45^{a}	2.51 ± 0.22^{b}	2.19 ± 0.01^{b}
5-Caffeoylquinic acid *trans*	85.03 ± 3.50^{b}	17.23 ± 0.31^{c}	170.27 ± 6.56^{a}	14.64 ± 0.17^{c}
4-Caffeoylquinic acid *trans*	2.75 ± 0.07^{b}	37.85 ± 0.42^{a}	n.d.	36.57 ± 0.17^{a}
5-Caffeoylquinic acid *cis*	11.91 ± 0.08^{b}	3.70 ± 0.06^{c}	26.37 ± 0.11^{a}	2.05 ± 0.01^{d}
3-Coumaroylquinic acid *cis*	464.38 ± 11.74^{a}	89.38 ± 4.12^{c}	383.90 ± 20.47^{b}	54.94 ± 2.66^{d}
3-Coumaroylquinic acid *trans*	551.01 ± 5.45^{b}	212.71 ± 3.28^{d}	1278.30 ± 14.14^{a}	351.02 ± 5.85^{c}
4-Coumaroylquinic acid *cis*	2.09 ± 0.04^{d}	136.06 ± 3.46^{a}	38.18 ± 0.96^{c}	102.24 ± 1.10^{b}
4-Coumaroylquinic acid *trans*	29.45 ± 1.43^{d}	287.86 ± 2.17^{b}	50.91 ± 0.76^{c}	319.44 ± 2.67^{a}
5-Coumaroylquinic acid *trans*	1.54 ± 0.06^{c}	78.95 ± 1.25^{a}	4.32 ± 0.16^{b}	81.85 ± 2.69^{a}
5-Coumaroylquinic acid *cis*	2.07 ± 0.11^{c}	49.25 ± 0.72^{a}	2.72 ± 0.11^{c}	32.77 ± 0.36^{b}
3-Feruloylquinic acid *cis*	5.30 ± 0.07^{a}	3.05 ± 0.03^{b}	4.94 ± 0.09^{a}	1.84 ± 0.05^{c}
3-Feruloylquinic acid *trans*	6.98 ± 0.19^{a}	2.59 ± 0.07^{b}	2.03 ± 0.01^{c}	2.13 ± 0.02^{c}
4-Feruloylquinic acid *cis*	0.97 ± 0.07^{c}	2.79 ± 0.05^{a}	1.10 ± 0.03^{c}	1.96 ± 0.03^{b}
5-Feruloylquinic acid *trans*	0.39 ± 0.02^{c}	1.02 ± 0.06^{a}	0.69 ± 0.03^{b}	n.d.
5-Feruloylquinic acid *cis*	5.59 ± 0.05^{b}	0.69 ± 0.01^{c}	8.24 ± 0.24^{a}	0.35 ± 0.01^{c}
Caffeoylquinic acid-hexoside isomer	1.21 ± 0.03^{a}	n.d.	1.20 ± 0.03^{a}	n.d.
Caffeoylquinic acid-hexoside isomer	1.05 ± 0.02^{b}	1.04 ± 0.08^{b}	2.07 ± 0.07^{a}	0.78 ± 0.02^{c}
3,5-diCaffeoylquinic acid	3.38 ± 0.03^{b}	n.d.	5.60 ± 0.12^{a}	n.d.
4,5-diCaffeoylquinic acid	1.64 ± 0.02^{b}	n.d.	2.06 ± 0.17^{a}	n.d.
Caffeoylshikimic acid isomer	1.58 ± 0.04^{a}	n.d.	0.75 ± 0.09^{b}	n.d.
3- and 4-Caffeoylquinic lactone	1.99 ± 0.01^{a}	n.d.	2.26 ± 0.15^{a}	n.d.
Caffeoylshikimic acid isomer	0.67 ± 0.03	n.d.	n.d.	n.d.
3-Coumaroylquinic lactone	1.09 ± 0.03	n.d.	n.d.	n.d.
4-Coumaroylquinic lactone	1.90 ± 0.04^{b}	n.d.	5.46 ± 0.10^{a}	n.d.
3-Coumaroyl-5-caffeoylquinic acid	0.94 ± 0.05^{b}	n.d.	1.78 ± 0.08^{a}	n.d.
3-Caffeoyl-4-coumaroylquinic acid	0.90 ± 0.07	n.d.	n.d.	n.d.

Caffeoyl-hexose isomer	0.57 ± 0.02[b]	n.d.	2.21 ± 0.21[a]	n.d.
Caffeic acid-hexoside isomer	2.94 ± 0.13[b]	n.d.	8.70 ± 0.26[a]	n.d.
Caffeoyl alcohol 3/4-O-hexoside	2.21 ± 0.11	n.d.	n.d.	n.d.
Feruloyl-hexose isomer	6.17 ± 0.05	n.d.	n.d.	n.d.
Sinapoyl-hexose isomer	n.d.	n.d.	0.94 ± 0.02[a]	0.82 ± 0.02[b]
Sinapic acid-hexoside	0.93 ± 0.02	n.d.	n.d.	n.d.
Total hydroxycinnamic acids	***1354.99 ± 4.49[b]***	***971.91 ± 3.22[d]***	***2242.57 ± 5.69[a]***	***1042.51 ± 2.87[c]***
		Flavan-3-ols		
Catechin	127.92 ± 3.20[b]	n.d.	229.51 ± 6.86[a]	1.86 ± 0.08[c]
Epicatechin	772.30 ± 4.82[b]	n.d.	1369.61 ± 12.58[a]	11.16 ± 0.17[c]
(Epi)catechin-3-gallate	10.75 ± 0.23[a]	4.30 ± 0.22[c]	8.41 ± 0.09[b]	4.86 ± 0.03[c]
(Epi)catechin-hexoside isomer	16.03 ± 0.59[b]	n.d.	26.78 ± 0.42[a]	1.35 ± 0.05[c]
Procyanidin tetramer B type isomer	3.40 ± 0.19[b]	n.d.	10.17 ± 0.14[a]	n.d.
Procyanidin dimer B type isomer	13.17 ± 1.80[b]	n.d.	21.66 ± 2.58[a]	0.77 ± 0.01[c]
Procyanidin dimer B type isomer	65.66 ± 1.08[b]	n.d.	91.27 ± 1.98[a]	1.05 ± 0.04[c]
Procyanidin dimer B type isomer	7.11 ± 0.46[b]	n.d.	12.22 ± 0.15[a]	0.94 ± 0.02[c]
Propelargonidin dimer isomer	n.d.	n.d.	2.65 ± 0.06	n.d.
Procyanidin pentamer B type isomer	2.59 ± 0.21[b]	n.d.	5.79 ± 0.23[a]	n.d.
Total flavan-3-ols	***1018.94 ± 6.21[b]***	***4.30 ± 0.22[d]***	***1778.07 ± 14.71[a]***	***21.99 ± 0.20[c]***
		Flavonols		
Quercetin-3-*O*-rutinoside	15.69 ± 0.42[c]	6.09 ± 0.07[d]	102.25 ± 2.09[a]	29.72 ± 0.14[b]
Quercetin-3-*O*-glucoside	1.40 ± 0.03[c]	0.64 ± 0.02[d]	40.90 ± 0.22[a]	26.41 ± 0.15[b]
Quercetin-7-*O*-hexoside-3-*O*-rutinoside	7.53 ± 0.06[b]	6.07 ± 0.14[b]	11.90 ± 0.13[a]	3.53 ± 0.07[c]
Kaempferol-3-*O*-hexoside	n.d.	n.d.	2.93 ± 0.07[a]	2.39 ± 0.05[a]
Kaempferol-3-*O*-rutinoside	2.97 ± 0.06[c]	1.31 ± 0.02[d]	17.53 ± 0.14[a]	7.04 ± 0.06[b]
Kaempferol-7-*O*-hexoside-3-*O*-rutinoside	1.44 ± 0.07[b]	1.05 ± 0.01[c]	2.33 ± 0.02[a]	0.76 ± 0.01[c]
Total flavonols	***29.02 ± 0.44[c]***	***15.16 ± 0.16[d]***	***177.83 ± 2.11[a]***	***69.85 ± 0.23[b]***
		Other flavonoids		
Naringenin-hexoside	0.81 ± 0.01[b]	n.d.	4.18 ± 0.24[a]	n.d.
Taxifolin-rutinoside isomer	10.01 ± 0.26[b]	n.d.	151.92 ± 11.33[a]	2.09 ± 0.08[c]
Taxifolin-rutinoside isomer	6.70 ± 0.12[b]	n.d.	38.12 ± 0.19[a]	2.02 ± 0.05[c]
Taxifolin-hexoside isomer	n.d.	n.d.	78.53 ± 1.02[a]	2.27 ± 0.03[b]
Taxifolin-hexoside isomer	n.d.	n.d.	24.13 ± 0.09[a]	2.10 ± 0.04[b]

Total other flavonoids	***17.53 ± 0.29*** [b]	***n.d.***	***296.88 ± 11.38*** [a]	***8.49 ± 0.10*** [c]
		Hydroxybenzoic acids		
Protocatechuic acid	n.d.	0.13 ± 0.01 [b]	n.d.	0.34 ± 0.01 [a]
Protocatechuoyl-hexose	0.52 ± 0.01 [b]	n.d.	1.05 ± 0.08 [a]	n.d.
Hydroxybenzoic acid-hexoside	0.41 ± 0.01	n.d.	n.d.	n.d.
Vanillic acid-hexoside	2.96 ± 0.10 [b]	n.d.	5.24 ± 0.24 [a]	n.d.
Total hydroxybenzoic acids	***3.89 ± 0.10*** [b]	***0.13 ± 0.01*** [d]	***6.29 ± 0.25*** [a]	***0.34 ± 0.01*** [c]
		Anthocyanins and derivatives		
Cyanidin-3-*O*-glucoside	2.49 ± 0.03 [c]	n.d.	317.02 ± 12.32 [a]	86.91 ± 0.56 [b]
Cyanidin-3-*O*-rutinoside	68.77 ± 0.74 [c]	0.59 ± 0.02 [d]	480.05 ± 4.82 [a]	346.74 ± 2.14 [b]
Cyanidin-5-*O*-hexoside-3-*O*-coumaroyl-hexoside	n.d.	n.d.	0.97 ± 0.01	< LOQ
Cyanidin-3-O-glucoside chalcone	n.d.	n.d.	n.d.	0.07 ± 0.01
(Epi)catechin-cyanidin-3-O-glucoside dimer	n.d.	n.d.	n.d.	0.09 ± 0.01
Peonidin-3-*O*-rutinoside	5.07 ± 0.09 [b]	n.d.	13.43 ± 0.14 [a]	13.94 ± 0.17 [a]
Pelargonidin-3-*O*-rutinoside	0.27 ± 0.01 [c]	n.d.	3.85 ± 0.50 [a]	1.47 ± 0.04 [b]
Pelargonidin-3-*O*-hexoside	n.d.	n.d.	1.59 ± 0.20 [a]	0.32 ± 0.01 [b]
Total anthocyanins	***76.60 ± 0.74*** [c]	***0.59 ± 0.02*** [d]	***816.92 ± 13.24*** [a]	***449.53 ± 2.22*** [b]
Total phenolic compounds	***2500.96 ± 3.99*** [b]	***992.10 ± 3.23*** [d]	***5318.56 ± 23.61*** [a]	***1592.71 ± 3.67*** [c]

[*]Hydroxycinnamic acids were quantified as 5-caffeoylquinic acid equivalent; flavan-3-ols were quantified as epicatechin equivalent; flavonols and other flavonoids were quantified as quercetin-3-glucoside equivalent; hydroxybenzoic acids were quantified as gallic acid equivalent; anthocyanins and derivatives were quantified as cyanidin-3-glucoside equivalent.

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

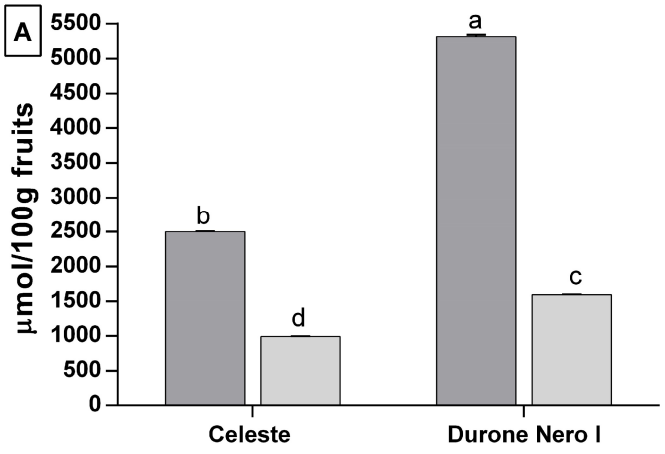
<LOQ means the compound was detected but it was below the limit of quantification.

n.d. means not detected

Table 2. Phenolic compounds extracted from *in vitro* digested Durone Nero I identified in the cell media after 24 h of incubation with Caco-2 and SW480. Results are expressed as µmol/L*. In bracket the percentage of recovery respect to the time zero (before incubation with cells).

Compounds	$[M-H]^-$	Fragment ions	Caco-2 µmol/L	SW480 µmol/L
3-Coumaroylquinic acid *cis*	337	163, 119, 191	19.56 ± 0.20 (21.4)^b	55.49 ± 0.16 (60.6)^a
3-Coumaroylquinic acid *trans*	337	163, 119, 191	47.18 ± 2.79 (8.1)^a	44.35 ± 0.06 (7.6)^a
4-Coumaroylquinic acid *cis*	337	173, 163	22.69 ± 0.47 (4.3)^b	48.80 ± 2.29 (9.2)^a
4-Coumaroylquinic acid *trans*	337	173, 163	59.96 ± 4.77 (32.8)^a	37.31 ± 0.32 (21.9)^b
5-Coumaroylquinic acid *trans*	337	191, 173, 163	45.44 ± 1.26 (33.3)^a	21.49 ± 0.55 (15.8)^b
5-Coumaroylquinic acid *cis*	337	191, 163, 173	27.50 ± 0.51 (50.4)^a	23.71 ± 1.22 (43.4)^a
Total coumaroylquinic acids	/	/	218.33 ± 5.71 (13.9)^b	231.14 ± 7.30 (14.7)^a
Coumaroylquinic acid sulphate	417	337, 163	< LOQ	7.83 ± 0.54
Caffeoylquinic acid-hexoside isomer	515	341, 353, 179	< LOQ	n.d.
3-Feruloylquinic acid	367	193	< LOQ	n.d.
Caffeic acid	179	135	n.d.	< LOQ
(Iso)ferulic acid	193	178	58.47 ± 4.40^a	12.62 ± 0.52^b
Coumaric acid	163	119	4.11 ± 5.71	< LOQ
Dihydro-caffeic acid	181	137	n.d.	< LOQ
Quercetin-3-*O*-rutinoside	609	301	n.d.	26.84 ± 0.15 (54.2)
Quercetin-3-*O*-glucoside	463	301	n.d.	9.80 ± 0.19 (22.3)
Quercetin-3-*O*-glucoside sulphate	543	381, 301	< LOQ	n.d.
Quercetin	301	179, 151	< LOQ	n.d..
Kaempferol-3-*O*-rutinoside	593	285, 255	5.99 ± 0.08 (51.0)^b	9.17 ± 0.49 (78.1)^a

*Hydroxycinnamic acids were quantified as 5-caffeoylquinic acid equivalent, whereas flavonols were quantified as quercetin-3-glucoside equivalent.
Different superscript letters within the same row indicate that the values are significantly different ($P<0.05$).
<LOQ means the compound was detected but it was below the limit of quantification.
n.d. means not detected


A
µmol/100g fruits
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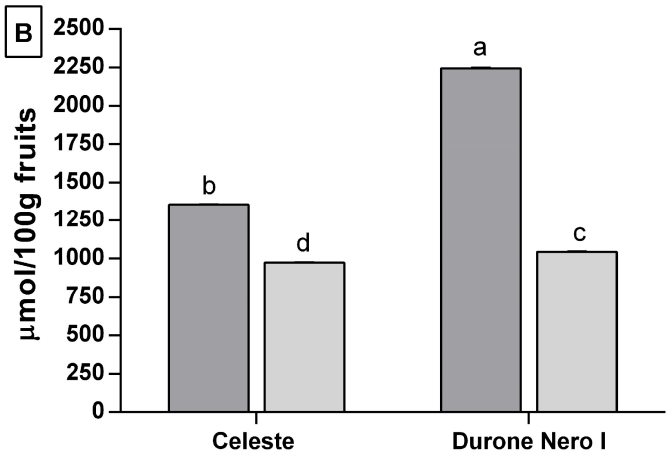
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
a
b
c
d
Celeste
Durone Nero I


B
µmol/100g fruits
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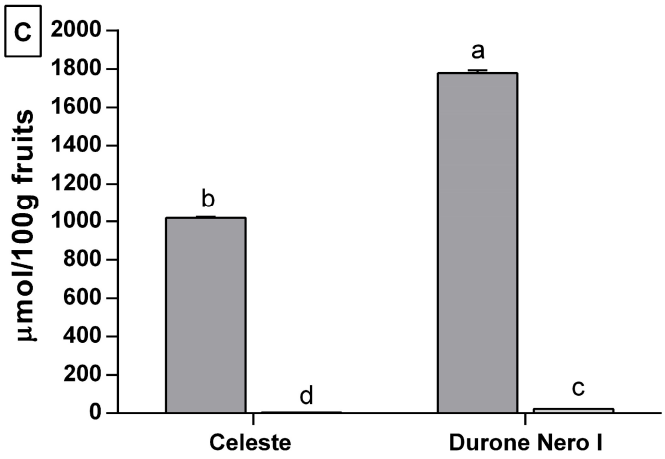
2000
1750
1500
1250
1000
750
500
250
0
a
b
c
d
Celeste
Durone Nero I


C
µmol/100g fruits
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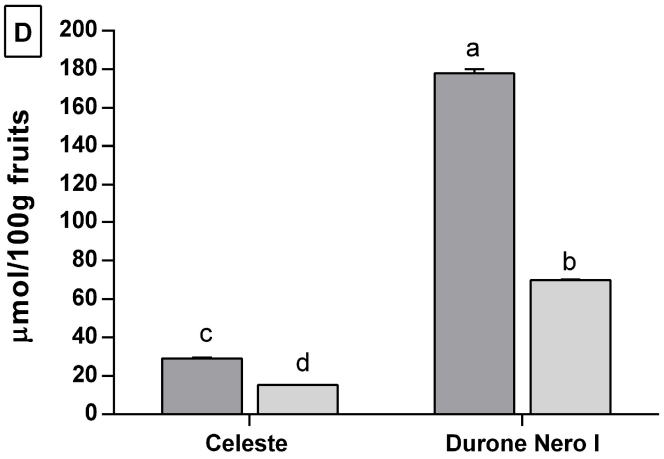
1800
1600
1400
1200
1000
800
600
400
200
0
a
b
c
d
Celeste
Durone Nero I


D
µmol/100g fruits
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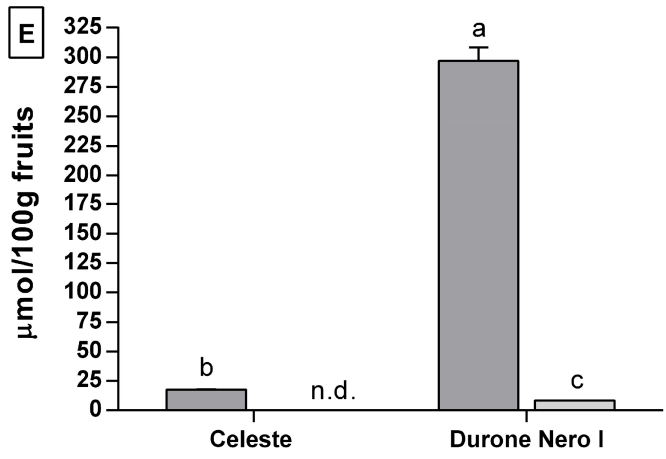
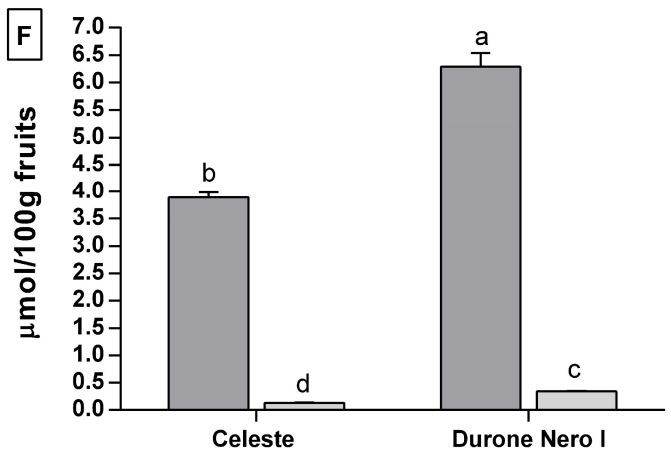
180
160
140
120
100
80
60
40
20
0
a
b
c
d
Celeste
Durone Nero I


E
µmol/100g fruits
325
300
275
250
225
200
175
150
125
100
75
50
25
0
a
b
c
n.d.
Celeste
Durone Nero I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µmol/100g fruits
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6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
d
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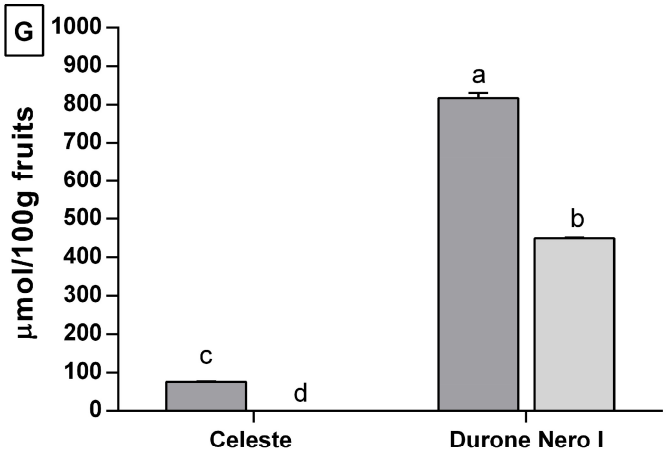
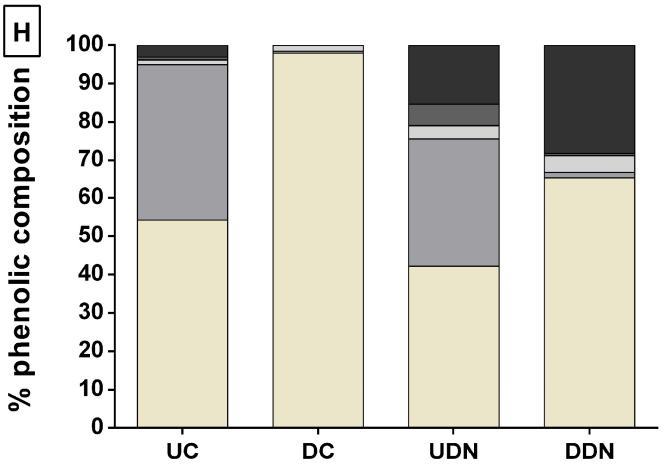
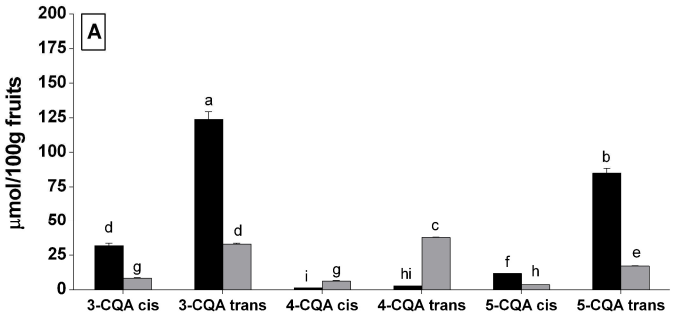
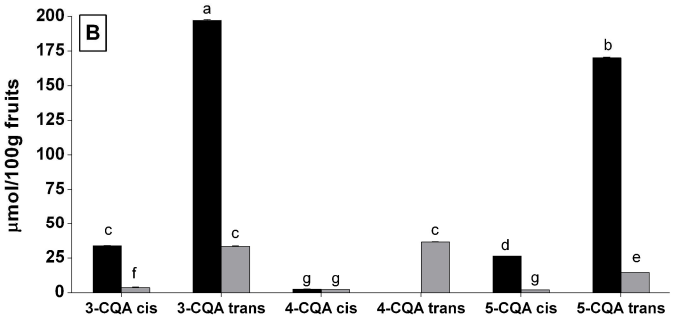
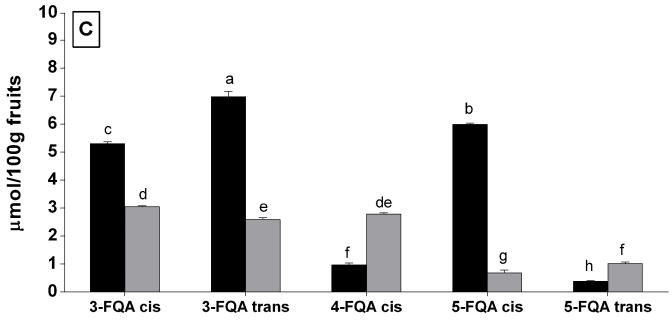
Durone Nero I

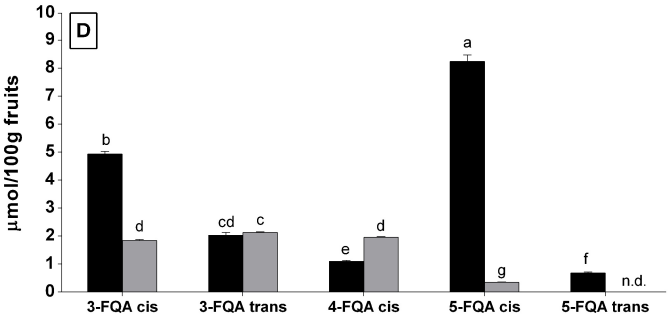

G
µmol/100g fruits
1000
900
800
700
600
500
400
300
200
100
0
a
b
c
d
Celeste
Durone Nero I

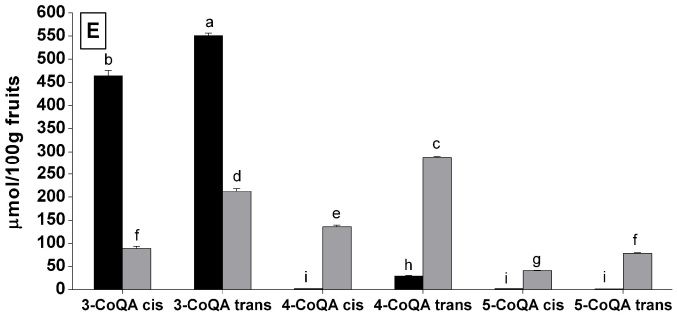

H
% phenolic composition
100
90
80
70
60
50
40
30
20
10
0
UC
DC
UDN
DDN

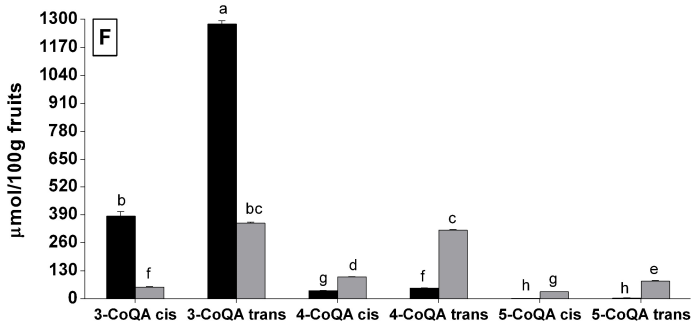

A
µmol/100g fruits
200
175
150
125
100
75
50
25
0
d
g
a
d
i
g
hi
c
f
h
b
e
3-CQA cis
3-CQA trans
4-CQA cis
4-CQA trans
5-CQA cis
5-CQA trans

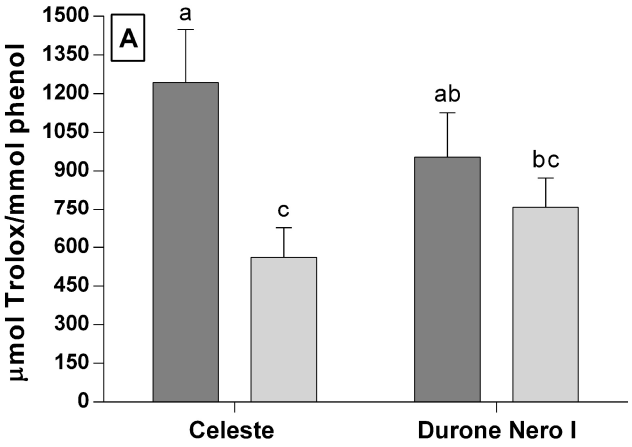

B
µmol/100g fruits
200
175
150
125
100
75
50
25
0
c
f
a
c
g
g
c
d
g
b
e
3-CQA cis
3-CQA trans
4-CQA cis
4-CQA trans
5-CQA cis
5-CQA trans

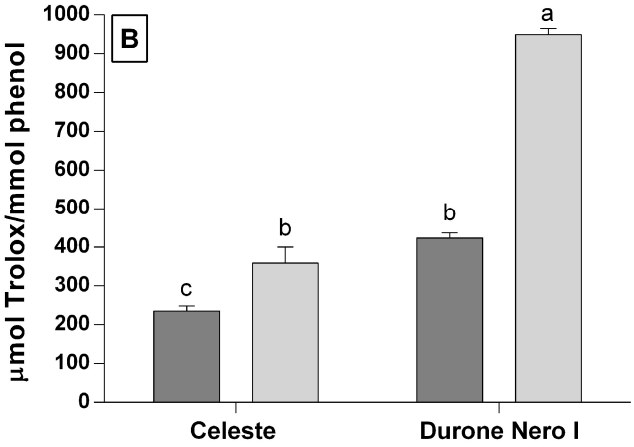

C
µmol/100g fruits
10
9
8
7
6
5
4
3
2
1
0
c
d
a
e
f
de
b
g
h
f
3-FQA cis
3-FQA trans
4-FQA cis
5-FQA cis
5-FQA trans

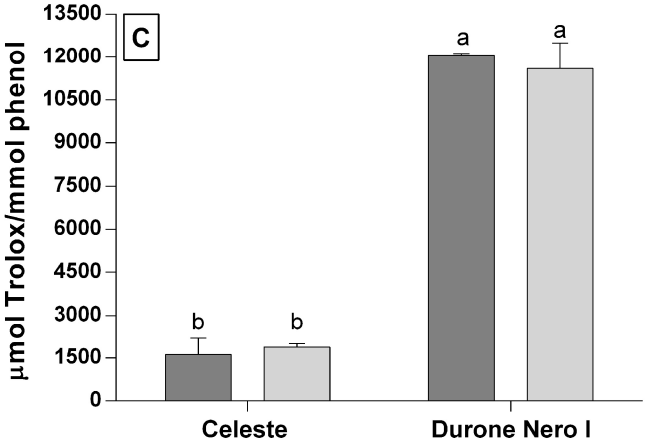

D
µmol/100g fruits
10
9
8
7
6
5
4
3
2
1
0
b
d
cd
c
e
d
a
g
f
n.d.
3-FQA cis
3-FQA trans
4-FQA cis
5-FQA cis
5-FQA trans

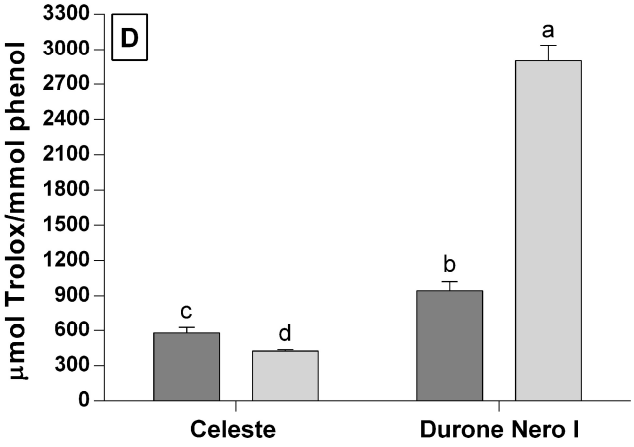

E
µmol/100g fruits
600
550
500
450
400
350
300
250
200
150
100
50
0
b
f
a
d
i
e
h
c
i
g
i
f
3-CoQA cis
3-CoQA trans
4-CoQA cis
4-CoQA trans
5-CoQA cis
5-CoQA trans

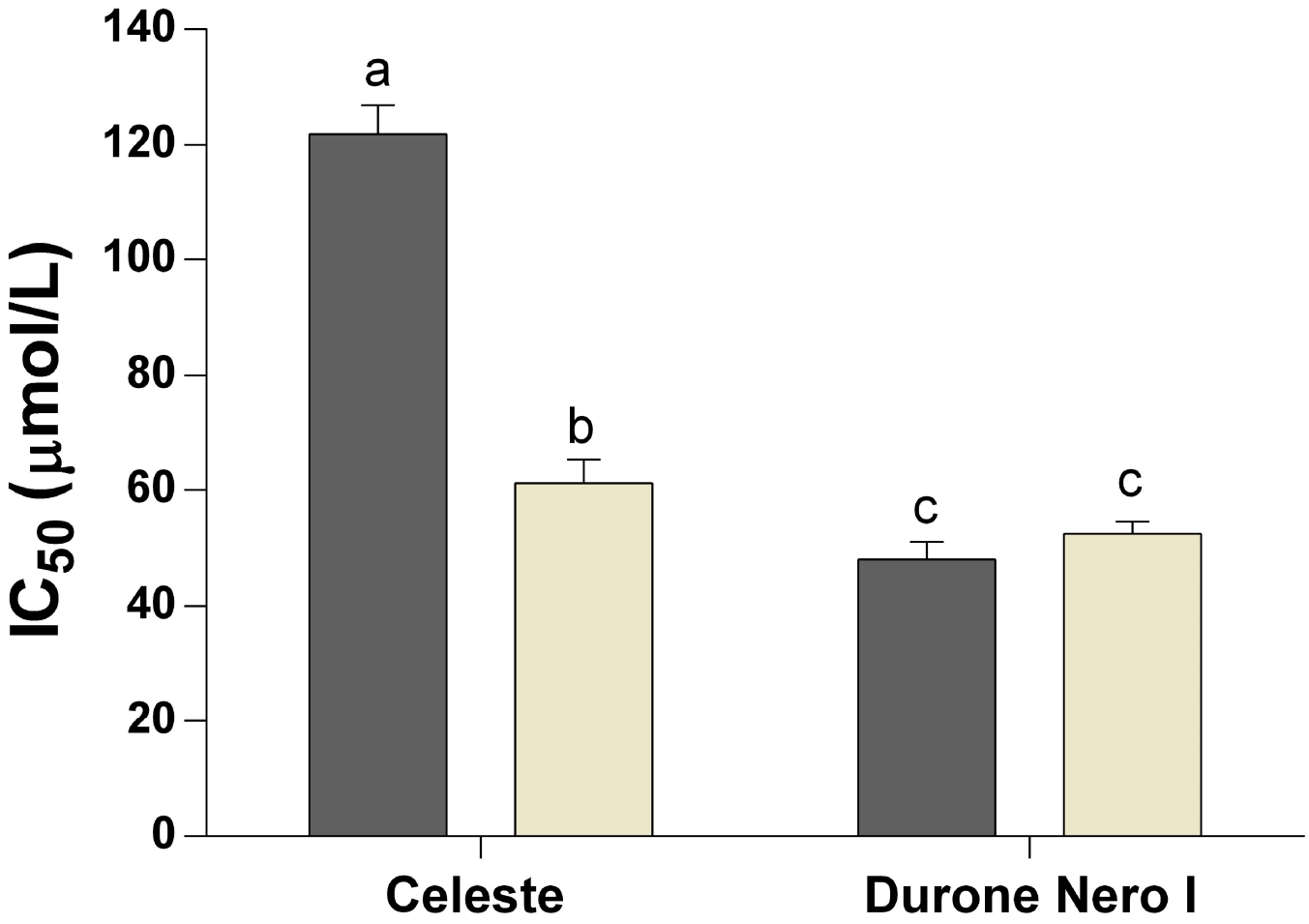

F
µmol/100g fruits
1300
1170
1040
910
780
650
520
390
260
130
0
b
f
a
bc
g
d
f
c
h
g
h
e
3-CoQA cis
3-CoQA trans
4-CoQA cis
4-CoQA trans
5-CoQA cis
5-CoQA trans


A
µmol Trolox/mmol phenol
1500
1350
1200
1050
900
750
600
450
300
150
0
a
c
ab
bc
Celeste
Durone Nero I


B
µmol Trolox/mmol phenol
1000
900
800
700
600
500
400
300
200
100
0
c
b
b
a
Celeste
Durone Nero I


C
µmol Trolox/mmol phenol
13500
12000
10500
9000
7500
6000
4500
3000
1500
0
b
b
a
a
Celeste
Durone Nero I


D
µmol Trolox/mmol phenol
3300
3000
2700
2400
2100
1800
1500
1200
900
600
300
0
c
d
b
a
Celeste
Durone Nero I


140
120
100
80
60
40
20
0
IC50 (µmol/L)
a
b
c
c
Celeste
Durone Nero I