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Anti-proliferative activity and cell metabolism of hydroxycinnamic acids in human colon adenocarcinoma cell lines

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1 **Abstract**

2 In this study, we investigated the anti-proliferative activity and the stability and metabolic fate of
3 the main dietary hydroxycinnamates, using two colonic adenocarcinoma cell models (Caco-2 and
4 SW480). Di-hydro-caffeic and di-hydro-ferulic acids were the most effective against cell
5 proliferation in both cell lines with IC₅₀ values of 71.7 ± 1.1 and 83.1 ± 1.1 µmol/L, respectively
6 (*P*<0.05) in Caco-2. At 200 µmol/L, caffeic and ferulic acids inhibited SW480 proliferation by 40.8
7 ± 1.6 and 59.9 ± 1.3%, respectively. Hydroxycinnamic acids with a catechol-type structure were
8 degraded in Caco-2 cell medium resulting in the production of H₂O₂. Intracellular Caco-2 UDP-
9 glucuronosyltransferases and catechol-*O*-methyltransferases were able to form glucuronide and
10 methyl conjugates. However, only the sulphate conjugates were detected after incubation with
11 SW480. In addition, simple hydroxycinnamates were released from quinic and aspartic-conjugates.
12 The remarkable effect of di-hydro-caffeic and di-hydro-ferulic acids against cell proliferation is of
13 paramount importance since these compounds are the main metabolites detectable at colonic level.

14
15 **Keywords:** mass spectrometry, metabolism, anti-proliferative activity, phenolic compounds, colon
16 cancer

17 **1. Introduction**

18 Dietary polyphenols have gained increasing interest because of their numerous biological effects
19 such as free-radical scavenging and metal chelation activities, modulation of enzymatic activity,
20 inhibition of cellular proliferation, and altering signal transduction pathways.¹⁻² In this context,
21 emerging evidence is confirming the ever-increasing importance of hydroxycinnamic acids in the
22 prevention of cardiovascular and other chronic diseases.³ Hydroxycinnamic acids are widespread
23 dietary components found, for instance, in coffee, cherries, apples, chocolate, yerba mate and
24 several vegetables and herbs.³ The interest in these compounds is related to their beneficial health
25 effects *in vivo*.⁴⁻⁵ However, the extent of their protective effect depends on their bioavailability after
26 intestinal absorption, metabolism, and subsequent interaction with target tissues.¹

27 More interestingly, highly and regularly consumed foods rich in hydroxycinnamic acids are
28 particularly intriguing for their potential beneficial effects in the gastro-intestinal tract where
29 hydroxycinnamic acids can reach concentrations of hundreds of $\mu\text{mol/L}$.^{3,6,7} To this purpose, recent
30 epidemiological studies on colorectal cancer risk have demonstrated some evidence of protection
31 after ingestion of hydroxycinnamates-rich foods or beverages such as coffee and apples.⁸⁻¹⁰
32 Furthermore, dark chocolate and cherry intake have been shown to inhibit tumour development in
33 azoxymethane-induced colonic cancer in rats.¹¹⁻¹²

34 Recently, our research group thoroughly investigated the effect of *in vitro* gastro-intestinal digestion
35 on the bioaccessibility of phenolic compounds from two different cherry cultivars (Celeste and
36 Durone Nero I) and three different types of dark chocolates.¹³⁻¹⁴ Phenolic acids and especially
37 hydroxycinnamic acids showed the highest bioaccessibility in all the food matrices. The highest
38 release after incubation of cherries with digestive fluids was found for coumaroylquinic acids,
39 followed by feruloylquinic and caffeoylquinic acids; a similar trend was seen for the conjugate
40 forms of hydroxycinnamic acids with aspartic acid after *in vitro* digestion of dark chocolate. We
41 also demonstrated that hydroxycinnamic acids tended to accumulate in cell media suggesting a

42 possible role in the anti-proliferative activity of *in vitro* digested cherry and dark chocolate against
43 colon adenocarcinoma cell lines.¹³⁻¹⁴

44 The stability of phenolic compounds in cell culture media as well as their cellular metabolism are
45 two aspects to be critically addressed when testing their anti-proliferative activity. Their instability
46 in cell media may hinder their biological activity, severely changing the concentrations and/or the
47 chemical structures of the tested compounds.¹⁵ Indeed, the cellular metabolism of phenolic
48 compounds may evidently alter the bioactivity of the tested molecules.¹⁶ Therefore, to get a
49 complete picture of the scenario, the stability of the parent compounds and the formation of their
50 cellular metabolites should be evaluated.

51 Thus, the aim of this work was to study the stability and the cell metabolism of cinnamic acid, the
52 main dietary hydroxycinnamic acids (*o*-coumaric acid, *p*-coumaric acid, caffeic acid, ferulic acid
53 and 5-*O*-caffeoylquinic acid) as well as the two most important colonic metabolites (di-hydro-
54 caffeic acid and di-hydro ferulic acid) using Caco-2 and SW480 cell lines. Moreover,
55 coumaroylquinic acids and hydroxycinnamoyl aspartates extracted from sweet cherry (Celeste
56 cultivar) and dark chocolate, respectively, were also included in the analysis (**Figure 1**). In addition,
57 the anti-proliferative effect against two models of human colonic adenocarcinoma cell lines (Caco-2
58 and SW480) was assessed. The main metabolic pathways were drawn through the detection and
59 identification of newly-formed metabolites by LC-ESI-IT-MS/MS analysis after 24 h of incubation.

60

61 **2. Materials and methods**

62 **2.1. Chemicals**

63 Phenolic compound standards (*o*-coumaric acid, *p*-coumaric acid, caffeic acid, ferulic acid, di-
64 hydro-caffeic acid, di-hydro ferulic acid, cinnamic acid and 5-*O*-caffeoylquinic acid) and reagents
65 for analytical determinations were purchased from Sigma (Milan, Italy). Methanol, formic acid and
66 acetonitrile were obtained from Carlo Erba (Milan, Italy). All MS/MS reagents were from Bio-Rad

67 (Hercules, CA, U.S.A.). All the materials and chemicals for cell culture were from VWR
68 International (Milan, Italy). MTS cell proliferation assay kit was purchased from Promega (Milan,
69 Italy). Solid phase extraction (SPE) column (C18, 50 μm , 60 \AA , 500 mg) were supplied by Waters
70 (Milan, Italy).

71

72 **2.2. Hydroxycinnamoyl aspartates and coumaroylquinic acids enriched extracts preparation**

73 Coumaroylquinic acids and hydroxycinnamoyl aspartates enriched fractions were extracted from *in*
74 *vitro* digested sweet cherry (Celeste cultivar) and dark chocolate, respectively, following the
75 protocol reported in Tagliacruzchi et al. with some modifications.¹⁷ The two food matrices were
76 digested as reported in Martini et al.¹⁴ Firstly, SPE column were preconditioned with 4 mL of
77 methanol, followed by 5 mL of water adjusted to pH 7.4 with NaOH 1 mol/L. Then, 1 mL of the *in*
78 *vitro* digested fractions were passed through the pre-conditioned C-18 column to adsorb neutral
79 phenolic compounds. Non-adsorbed phenolic acids were washed with 3 mL of water at pH 7. After
80 acidification of the column with 5 mL of acidified water (containing 0.1% of formic acid) the
81 adsorbed phenolic compounds were eluted with 4 mL of an acetonitrile solution (10% acetonitrile
82 and 90% acidified water). During elution with acetonitrile, four fractions of 1 mL each were
83 collected separately. These fractions were called ACN1, ACN2, ACN3 and ACN4. The remaining
84 bound compounds were then eluted with 3 mL of acidified methanol.

85 Three digested fractions for each food matrix were extracted in triplicate.

86 Fractions collected from SPE were analyzed by using mass spectrometry to identify phenolic

87 compounds. Firstly, all of the fractions (2 μL) were directly injected on an ion trap mass

88 spectrometer coupled with an electrospray ionizator (ESI). ESI operating conditions are reported in

89 Martini et al.¹⁸ The direct infusion experiments were carried out to detect the *m/z* signals present in

90 each fraction. Specific fractions containing the *m/z* signals corresponding to coumaroylquinic acids

91 (*m/z* 337) and hydroxycinnamoyl aspartates (*m/z* 278, 294 and 308 for coumaroyl-aspartate,

92 caffeoyl-aspartate and feruloyl-aspartate, respectively) were analyzed by liquid chromatography
93 electrospray ionization ion trap mass spectrometry (LC-ESI-IT-MS/MS) on HPLC Agilent 1200
94 Series system equipped with a C18 column (HxSil C18 Reversed phase, 250×4.6 mm, 5 µm particle
95 size, Hamilton Company, Reno, Nevada, USA). The mobile phase composition was (A)
96 H₂O/formic acid (99:1, v/v) and (B) acetonitrile/formic acid (99:1, v/v). After 0.5 min at 4% B, the
97 gradient linearly ramped up to 30% B in 60 min. The mobile phase composition was raised up to
98 100% B in 1 min and maintained for 5 min in order to wash the column before returning to the
99 initial condition. The flow rate was set at 1 mL/min. The samples were injected in the amount of 40
100 µL. After passing through the column, the eluate was split and 0.4 mL/min were directed to an
101 Agilent 6300 ion trap mass spectrometer. Identification of phenolic compounds in all samples was
102 carried out using full scan and data-dependent MS² scanning from *m/z* 100 to 1700. MS operating
103 conditions, limits of detection (LOD) and limits of quantification (LOQ) for the different standards
104 are reported in Martini et al.¹⁸ Coumaroylquinic acids and coumaroyl-aspartate were quantified as
105 coumaric acid equivalent whereas caffeoyl-aspartate and feruloyl-aspartate were quantified as
106 caffeic and ferulic acids equivalents, respectively.
107 Results were expressed as µmol/L of standard equivalents.

108

109 ***2.3. Cell cultures and anti-proliferative activity***

110 Human adenocarcinoma Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium
111 (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic mix (streptomycin and
112 penicillin) and 2 mmol/L L-glutamine. Human adenocarcinoma SW480 cells were cultured in
113 Leibowitz medium (L-15) supplemented with 10% FBS, 1% antibiotic mix (streptomycin and
114 penicillin) and 2 mmol/L L-glutamine. Cells were maintained at 37°C in a humidified atmosphere
115 of 5% CO₂.

116 Cells were seeded at $4 \times 10^3/\text{cm}^2$ and $8 \times 10^3/\text{cm}^2$ for Caco-2 and SW480, respectively, in 96-well
117 plates for 24 h to allow cell adhesion to the bottom of the wells.

118 The anti-proliferative activity was assayed by a colorimetric method for the sensitive quantification
119 of viable cells, using MTS assay kit on Caco-2 and SW480 cell lines as reported in Martini et al.¹⁶

120 Coumaric acids (*o*- and *p*-isomers), caffeic, ferulic, di-hydro-caffeic, di-hydro ferulic, 5-*O*-
121 caffeoylquinic and cinnamic acids standards were dissolved in methanol at concentration of 20
122 mmol/l and then diluted in both the two cell lines culture media (Caco-2 and SW480) at a final
123 concentration of 200 $\mu\text{mol/L}$. A control solution of 1% methanol in cell medium was used as 100%
124 proliferation. Coumaroylquinic acids and hydroxycinnamoyl aspartates enriched fractions from SPE
125 were diluted twice in both the two cell lines culture media and the pH was brought to 7.4 with
126 concentrated NaOH. An appropriate control solution consisting of the elution phase of SPE (10%
127 acetonitrile and 90% acidified water containing 0.1% of formic acid) diluted twice in the cell
128 medium and bring to pH 7.4 was used as 100% proliferation. 100 μL of each sample were added to
129 the cell plates and incubated for 24 h. At the end of the treatment, the media were collected for
130 detection of newly-formed metabolites and 180 μL of fresh culture medium and 20 μL of MTS
131 reagent were added to each well. After 4 h of incubation at 37°C, the absorbance was measured at
132 the wavelength of 490 nm using a microplate reader and results were expressed as percentage of
133 inhibition of cell proliferation. When possible, the IC_{50} values, representing concentration of
134 compound required to inhibit 50% cell proliferation, were determined by incubating cells with
135 different concentrations of the phenolic compound. IC_{50} values were expressed as μmol of phenolic
136 compound/L and were determined using nonlinear regression analysis and fitting the data with the
137 log (inhibitor) vs. response model generated by GraphPad Prism 6.0 (GraphPad Software, San
138 Diego, CA, USA).

139

140 ***2.4. Stability of hydroxycinnamic acids in cell culture media***

141 Hydroxycinnamic acids were diluted to 200 $\mu\text{mol/L}$ with DMEM or L-15 before testing.
142 Coumaroylquinic acids and hydroxycinnamoyl aspartates enriched fractions were diluted twice in
143 both the two cell lines culture media. 100 μL of the diluted hydroxycinnamic acids solution were
144 incubated at 37 $^{\circ}\text{C}$ in 5% CO_2 for 24 h in 96-well plates. At the end of incubation, the samples were
145 collected from the plates and analyzed by LC-MS/MS analysis as reported in section 2.5.

146

147 ***2.5. Liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis of cell media***

148 Cell culture supernatants as well as cell-free media containing hydroxycinnamic acids were
149 collected after 24 h to test the stability of hydroxycinnamic acids in cell culture media and the
150 presence of newly-formed metabolites. Cell media were extracted according to Sala et al. and
151 analyzed using the same LC-ESI-IT-MS/MS system as reported above, with an injection volume of
152 40 μL and elution flow rate of 1 mL/min .¹⁹ The mobile phase composition, the gradient and MS
153 operating conditions are the same as reported in Martini et al.²⁰

154

155 ***2.6. Measurement of the concentration of H_2O_2 in cell media***

156 The generation of H_2O_2 after 24 h of incubation of hydroxycinnamic acids with cell-free media was
157 assessed with the FOX assay as reported in Martini et al.²⁰ The concentration of H_2O_2 was
158 calculated from a standard curve obtained with different concentrations of H_2O_2 .

159

160 ***2.7. Statistic***

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

165

166 **3. Results**

167 **3.1. Anti-proliferative activity of hydroxycinnamic acid standards and colonic metabolites**

168 Treatment for 24 h with *p*-coumaric, *o*-coumaric, cinnamic and 5-caffeoylquinic (5-CQA) acids at
169 200 µmol/L did not affect cell proliferation in both colon adenocarcinoma cell lines (**Table 1**).

170 Caffeic and ferulic acids showed some effects, decreasing cell proliferation of SW480 cancer cells
171 after 24 h treatment at 200 µmol/L. The anti-proliferate activity of ferulic acid against SW480 cell
172 line was significantly higher than that of caffeic acid ($P<0.05$) however, neither of the compounds
173 reduced cell proliferation in Caco-2 cells. The microbial metabolites di-hydro-caffeic (DHC) and
174 di-hydro-ferulic (DHF) acids were able to notably reduce cell proliferation in both the cell lines.

175 The effect was significantly higher in Caco-2 respect to SW480 cell line ($P<0.05$). Contrary to
176 ferulic acid, the reduced methylated molecule, DHF, was less effective than DHC in SW480 cancer
177 cells. Indeed, DHC showed higher anti-proliferative activity than caffeic acid. This, however, was
178 not observed for DHF, which was less efficient than ferulic acid in inhibiting SW480 cell
179 proliferation. IC₅₀ values for DHC and DHF against Caco-2 cell proliferation were calculated
180 revealing that DHF was significantly more effective than DHC (71.7 ± 1.1 and 83.1 ± 1.1 µmol/L,
181 respectively; $P<0.05$).

182

183 **3.2. Stability of hydroxycinnamic acid standards and colonic metabolites in cell-free media**

184 Identification of the standard compounds and metabolites in cell media was based on LC-ESI-
185 MS/MS analyses, and a summary of mass spectrometry data in negative ionization mode is
186 presented in **Table 2**.

187 Results about the stability of individual hydroxycinnamic acids after 24 h incubation with DMEM
188 (Caco-2 medium) and L-15 (SW480 cell medium) cell-free media are given in **Table 3**. Some
189 hydroxycinnamic standards, such as *p*-coumaric, *o*-coumaric, ferulic and cinnamic acids, were very
190 stable even after 24 h incubation in both the cell media. Whereas, caffeic acid was stable during

191 incubation with L-15 but significantly degraded in DMEM after 24 h of incubation ($P < 0.05$).
192 However, the other tested compounds such as DHC, DHF and 5-CQA were significantly degraded
193 during incubation with both cell-free media ($P < 0.05$). A new peak with m/z value of 300 appeared
194 after incubation of DHC in L-15 but not in DMEM (**Figure S1**). The fragmentation pattern
195 observed in the LC-MS/MS experiments (fragment ions at m/z 213, 181, 135 and 107) was coherent
196 with the formation of a DHC-cysteine adduct.²¹ The concentration of the DHC-cysteine adduct
197 (quantified as DHC equivalent) accounted for the 40.9% of the initial DHC dose, explaining the
198 loss of DHC in L-15.

199 Regarding 5-CQA, after 24 h of incubation in both cell media, two new peaks at m/z 353 were
200 observed (**Figure S2**). These new formed compounds showed the typical fragmentation pattern of
201 3-caffeoylquinic acid (MS^2 fragment ions at m/z 191 and 179) and 4-caffeoylquinic acid (MS^2
202 fragment ions at m/z 179, 173, 135 and 191) suggesting that this compound underwent
203 isomerization during 24 h incubation.¹⁸ The sum of the percentage recovery of the three different
204 isomers of caffeoyl-quinic acid accounted for the 100% of the original standards in L-15 whereas in
205 DMEM the total recovery was 85.9% (**Figure 2A and B**).

206 The production of H_2O_2 was measured after 24 h of incubation of hydroxycinnamic acids with cell-
207 free media at concentration of 200 $\mu\text{mol/L}$. In DMEM caffeic acid, DHC and 5-CQA degradation
208 was accompanied to the generation of H_2O_2 . The highest amounts of H_2O_2 were found in caffeic
209 acid and DHC (199.1 ± 17.4 and 241.3 ± 12.2 $\mu\text{mol/L}$, respectively) followed by 5-CQA ($155.3 \pm$
210 6.3 $\mu\text{mol/L}$). No evidence of H_2O_2 production was observed in the other hydroxycinnamic acids
211 incubated with DMEM. Indeed, no compounds led to the production of H_2O_2 during incubation in
212 L-15. The H_2O_2 production was also tested after 24 h incubation of DHC in DMEM at 100 $\mu\text{mol/L}$
213 (corresponding to about the IC_{50} value against Caco-2 as reported above), resulting in a H_2O_2
214 concentration of 173.7 ± 16.0 $\mu\text{mol/L}$.

215

216 **3.3. Metabolism of hydroxycinnamic acid standards and colonic metabolites in human**
217 **adenocarcinoma colon cell lines**

218 All the tested compounds underwent considerable biotransformations in Caco-2 and SW480 cancer
219 cells and several newly-formed metabolites were identified (**Table 4**). In Caco-2, *o*-coumaric acid
220 was extensively glucuronidated with the appearance of two isomers of coumaric acid-glucuronide
221 showing a $[M - H]^-$ deprotonated ion at m/z 339, fragmenting to m/z 163 (loss of glucuronide
222 moiety) and with the characteristic fragmentation pattern of coumaric (m/z 119) and glucuronic
223 acids (m/z 175 and 113) (**Table 2** and **Figure S3**). The glucuronide-derivative of *p*-coumaric acid
224 was instead found only at trace levels (**Table 4**). Two additional metabolites were found in Caco-2
225 after incubation with *p*-coumaric acid: cinnamic acid (identified by comparison with its
226 corresponding standard) and di-hydro-coumaric acid (m/z 165, fragmenting to m/z 121
227 corresponding to the loss of CO_2). Metabolism by Caco-2 of ferulic acid resulted mainly in the
228 formation of ferulic acid-glucuronide (m/z 369, fragmenting to m/z 193, corresponding to the loss of
229 the glucuronide moiety, 175 and 113). Lower amounts of ferulic acid-sulphate (m/z 273,
230 fragmenting to m/z 193 corresponding to the loss of sulphate group) and DHF (identified by
231 comparison with its corresponding standard) were also found (**Table 4**). The most important
232 metabolites found after incubation of caffeic acid and 5-CQA were (iso)ferulic acid (identified by
233 comparison with its corresponding standard) and 5-feruloylquinic acid (m/z 367, fragmenting to m/z
234 179 and 135) while DHF was found only in trace amounts after incubation of DHC with Caco-2
235 cells (**Table 4**). Evidence of oxidation of the microbial metabolites DHC and DHF in caffeic and
236 ferulic acids (identified by comparison with standard compounds) was observed after incubation
237 with Caco-2 cells. Small amounts of DHF-sulphate (m/z 275, fragmenting to m/z 195 corresponding
238 to the loss of sulphate group) and DHF-glucuronide (m/z 371, fragmenting to m/z 195,
239 corresponding to the loss of the glucuronide moiety, and 113) were found after incubation of DHF
240 with Caco-2. 5-CQA underwent isomerization in 4- and 3-caffeoylquinic acids as observed also

241 after incubation with cell-free medium (**Table 4** and **Figure 2A**). The appearance of caffeic acid in
242 the cell medium after incubation of 5-CQA with Caco-2 was indicative of the presence of esterase
243 activity. Hydroxylation of cinnamic acid to coumaric acid (identified by comparison with standard
244 compounds) was the only metabolic pathway observed after incubation of cinnamic acid with Caco-
245 2.

246 A slightly different metabolic profile was observed with SW480 since the major metabolites found
247 after incubation with *o*-coumaric, caffeic and ferulic acids as well as DHC and DHF were the
248 sulphate conjugates (**Table 4**). Caffeic acid-sulphate and DHC-sulphate were identified from the
249 respective parent ions at m/z 259 and 261 producing daughter ions at m/z 179 and 181 (loss of
250 sulphate group), the un-conjugated forms, by MS² analysis. Coumaric acid-sulphate (m/z 243)
251 yielded MS² fragment ions at m/z 163 and 119, corresponding to the loss of the sulphate moiety
252 generating coumaric acid and CO₂ from the unconjugated molecule, respectively. Trace amounts of
253 coumaric acid were observed after incubation of caffeic acid with SW480. Instead, no sulphate
254 conjugates were found for *p*-coumaric acid, which was extensively reduced to di-hydro-coumaric
255 acid. The latter was also detected after incubation of *o*-coumaric acid with SW480 but only in trace
256 levels. As observed after incubation with cell-free medium, 5-CQA underwent isomerization with
257 the formation of the 3- and 4-acyl isomers (**Table 4** and **Figure 2B**). Evidence of SW480 esterase
258 activity should be confirmed by the appearance of free hydroxycinnamic acids after incubation with
259 5-CQA. Moreover, cinnamic acid was particularly stable with only trace amounts of coumaric acid
260 detected in the supernatant after incubation with SW480, highlighting the hydroxylating ability of
261 these cells.

262

263 **3.4. Preparation of coumaroylquinic acids and hydroxycinnamoyl aspartates enriched fractions**

264 The application of the solid phase extraction method on *in vitro* digested Celeste cherry cultivar and
265 dark chocolate allowed us to obtain fractions enriched in coumaroylquinic acids and
266 hydroxycinnamoyl aspartates, respectively.

267 **Figure 3A** shows the extracted ion chromatograms at m/z 337 (corresponding to the deprotonated
268 coumaroylquinic acid molecules) after direct infusion in the mass spectrometer of the different
269 fractions obtained from the extraction procedure with *in vitro* digested Celeste cherry cultivar. As
270 shown, cherry ACN2 was the most enriched fraction in coumaroylquinic acids. The total ion
271 chromatogram of this fraction determined by direct infusion mass spectrometry revealed the
272 presence of a prominent signal at m/z 337 and others minor signals at m/z 353 and 413 (**Figure 3B**).

273 The LC-MS/MS experiments carried out on fraction ACN2 revealed the presence of seven resolved
274 peaks (**Figure 3C**). **Table 5** lists the compounds identified in the different observed peaks. The
275 identification was based on previously reported paper.¹⁸ The amount of recovered 4-
276 coumaroylquinic and 5-coumaroylquinic acids (sum of the two isomers) were 72.3 ± 0.9 and $27.7 \pm$
277 $0.2 \mu\text{mol/L}$, respectively.

278 The direct infusion mass spectrometry of the different fractions obtained with solid phase extraction
279 from *in vitro* digested dark chocolate revealed the presence of the typical m/z values of
280 hydroxycinnamoyl aspartates (m/z 278, 294 and 308 for coumaroyl-aspartate, caffeoyl-aspartate and
281 feruloyl-aspartate, respectively) in both the chocolate ACN1 and ACN2 fractions (data not shown).
282 These fractions were further analyzed by liquid chromatography mass spectrometry (LC-MS/MS).
283 The extracted ion chromatograms of the m/z values corresponding to the hydroxycinnamoyl
284 aspartates (**Figure 4A-C**) showed that fraction ACN2 contained higher amount of these compounds
285 respect to fraction ACN1. The LC-MS/MS experiments carried out on fraction ACN2 revealed the
286 presence of several peaks as detailed in the base peak chromatogram showed in **Figure 4D**.
287 Compounds identified in the observed peaks are reported in **Table 5**. The identification was based
288 on previously reported paper.²² The amount of recovered coumaroyl-aspartate, caffeoyl-aspartate

289 and feruloyl-aspartate (sum of the two isomers) were 448.2 ± 73.5 , 110.3 ± 16.6 and 66.1 ± 9.9
290 $\mu\text{mol/L}$, respectively.

291

292 ***3.5. Anti-proliferative activity, stability in cell-free media and cell metabolism of coumaroylquinic*** 293 ***acids and hydroxycinnamoyl aspartates enriched fractions***

294 Coumaroylquinic acids and hydroxycinnamoyl aspartates enriched fractions were tested for their
295 anti-proliferative activity after two-fold dilution in cell media. Both the fractions showed notably
296 anti-proliferative activity against SW480 cells whereas only the hydroxycinnamoyl aspartates
297 enriched fraction was able to slightly inhibit Caco-2 cell proliferation (**Table 1**). **Table 3** shows
298 data about the stability of coumaroylquinic acids and hydroxycinnamoyl aspartates after 24 h
299 incubation with DMEM (Caco-2 medium) and L-15 (SW480 cell medium) cell-free media. Results
300 showed that coumaroylquinic acids were subject to isomerization in both the cell media (**Figure 2C**
301 and **D**). After 24 h incubation in DMEM, the amount of 4-coumaroylquinic acid decreased by about
302 43%, whereas the amount of 5-coumaroylquinic acid increased slightly but significantly ($P < 0.05$).
303 A new signal at m/z 337 was recorded after incubation in DMEM with MS² product ions at 163, 119
304 and 191 characteristic of the 3-acyl isomer of coumaroylquinic acid.¹⁸ The sum of the percentage
305 recovery of the three different isomers accounted for about 83% of the original amount. In L-15, the
306 amount of both the 4- and 5-acyl isomers decreased with the concurrent formation of the 3-acyl
307 isomer. Incubation of coumaroylquinic acids enriched fraction with Caco-2 resulted in the
308 formation of some metabolites (**Table 4**). Hydrolysis of the quinic acid-derivatives by cell esterases
309 led to the formation of the free coumaric acid. Both coumaroylquinic and coumaric acids were also
310 detected in the glucuronidated forms. Coumaroylquinic acid-glucuronide showed a precursor ion at
311 m/z 513 and fragmented at m/z 337 (loss of the glucuronide moiety). The appearance of di-hydro-
312 coumaric acid may be a consequence of the reduction of coumaric acid released from the quinic

313 acid-derivative. Isomerization to 3-coumaroyl-quinic acid was also observed as reported after
314 incubation with cell-free medium (**Table 3** and **Figure 2C**).

315 The total recovery considering the three isomers was 92.4%. Coumaroyl-aspartate was quite stable
316 during incubation with both the cell-free media whereas the feruloyl- and caffeoyl-derivatives were
317 partially degraded (**Figure 2E** and **F**). The only metabolites found after incubation of
318 hydroxycinnamoyl aspartates enriched fraction with Caco-2 cells were the corresponding free
319 hydroxycinnamic acids. However, feruloyl-aspartate was detected in higher amounts after
320 incubation with Caco-2 respect to the incubation in cell-free medium suggesting the possible
321 methylation of the caffeoyl derivative (**Table 4** and **Figure 2E**). Finally, the only metabolite
322 detected after incubation of the coumaroyl-quinic acid and hydroxycinnamoyl aspartates enriched
323 fractions with SW480 cells were coumaric and caffeic acids, respectively.

324

325 **4. Discussion**

326 Degradation of phenolic compounds during incubation in cell media and their putative cellular
327 metabolism may represent a limitation in their biological activity such as the anti-proliferative
328 activity.

329 In this study, we found that the stability of hydroxycinnamic acids in Caco-2 cell medium (DMEM;
330 pH 7.4) was strongly correlated with structural motifs of the molecules. The presence of a catechol-
331 type structure in the aromatic ring weakened the stability of hydroxycinnamic acids in DMEM. For
332 example, caffeic acid bearing a catechol-type structure was clearly less stable than the mono-
333 hydroxylate coumaric acid or ferulic acid where one of the two hydroxyl groups is methylated.
334 Similarly, DHC was less stable than DHF in Caco-2 cell medium. Hydrogenation of the $C_7=C_8$
335 double bond of hydroxycinnamic acids to corresponding di-hydro-hydroxycinnamic acids also
336 decreased the stability of the compounds. Phenolic compounds with a catechol-type structure are
337 easily oxidized at alkaline or slightly alkaline pH values in presence of O_2 via the semiquinone

338 intermediate formation.^{15,23} The oxidative degradation of these compounds generate $O_2\bullet^-$ which
339 enhance the rate of autoxidation resulting in the formation of H_2O_2 .²³ In agreement, we found that
340 the decrease of caffeic acid and DHC in DMEM resulted in the production of H_2O_2 suggesting that
341 the oxidative degradation pathway was the main one of these compounds in DMEM. The stability
342 of 5-CQA in DMEM was similar to that of caffeic acid; however, the degradation pathways were
343 slightly different. In addition to the oxidative degradation, the positional isomers 3-CQA and 4-
344 CQA were also produced during incubation. This finding agrees with the results obtained in
345 previous studies.^{13,24}

346 The stability profile of the hydroxycinnamic acids in the SW480 cell medium (L-15; pH 7.4) was
347 quite different respect to those observed after incubation in DMEM. In fact, caffeic acid was stable
348 during incubation in L-15 suggesting that the oxidative degradation did not occur. Indeed, no H_2O_2
349 production was detected in L-15. Surprisingly, DHC was partially degraded after 24 h of incubation
350 in L-15 medium. However, this was not a consequence of the oxidative degradation pathway (no
351 formation of H_2O_2 was observed) but was related to the formation of a DHC-cysteine adduct. This
352 adduct was not formed in DMEM since cysteine was absent from the medium.

353 DHF was partially degraded in both the media thorough a not yet identified pathway that did not
354 involve oxidative degradation, as suggested by the absence of H_2O_2 in the cell media.

355 Our data provide evidence that Caco-2 and SW480 cells are able to metabolize hydroxycinnamic
356 acids by means of phase I and phase II enzymes. **Figures 5** and **6** summarize the metabolic
357 reactions detected in Caco-2 and SW480 cell lines, showing pathways for phase I de-esterification
358 and hydroxylation and phase II glucuronidation, sulphation, and *O*-methylation of the various
359 hydroxycinnamic acids substrates used in this study.

360 In both the cell lines, small amounts of caffeoylquinic and coumaroylquinic acids were hydrolyzed
361 resulting in the appearance of free hydroxycinnamic acids. Kern and colleagues reported the
362 presence of both intracellular and extracellular esterases in Caco-2.²⁵ Farrel et al.²⁶ suggested that

363 serine hydrolases might be responsible for the cinnamoyl esterase activity of cultured gastric cells
364 since these carboxylesterases share similar structural homology and mechanism of hydrolysis with
365 cinnamoyl esterases. This class of enzymes was detected in Caco-2 cells, as well as in the intestinal
366 mucosa.²⁶⁻²⁸ Esterases activity was more heightened in Caco-2 cells respect to SW480. Williamson
367 et al.²⁸ evidenced that Caco-2 esterases were active against 3-*O*-caffeoylquinic acid but not versus
368 5-*O*-caffeoylquinic and feruloylquinic acids. Here we gave evidence, for the first time, of the
369 activity of cell esterases against coumaroylquinic acids. We also reported for the first time the
370 ability of SW480 and, especially, Caco-2 cells to release simple acids (such as caffeic, coumaric
371 and ferulic acids) from hydroxycinnamoyl-aspartates. In this sense, a hydrolysis of
372 hydroxycinnamoyl-aspartates catalyzed by membrane bound carboxypeptidases may be
373 hypothesized.

374 Our data showed that other phase I reactions might be involved in the metabolism of
375 hydroxycinnamic acids. Hydroxylation by cytochrome P450 of cinnamic acid resulted in the
376 formation of coumaric acid. Moreover, de-hydroxylation by phase I reductases brought about the
377 formation of cinnamic and coumaric acids from coumaric and caffeic acids, respectively.
378 Whereas, phase II metabolism was found to be cell line- and compound-dependent and varied
379 notably between the two cell lines and the compounds considered. Major pathways in Caco-2 cells
380 were glucuronidation and methylation whereas in SW480 sulphation was the predominant one.
381 Caco-2 UDP-glucuronosyltransferase activity was strongly depended on the type of substrate.
382 Glucuronidation was a major pathway in ferulic acid metabolism whereas caffeic acid was only
383 slightly glucuronidated. This result suggested that the presence of the catechol group hinder the
384 action of the UDP-glucuronosyltransferase. This observation was also supported by the fact that *o*-
385 coumaric acid was a good substrate for UDP-glucuronosyltransferase. The activity of UDP-
386 glucuronosyltransferase was also dependent on the position of the OH group in the aromatic ring as
387 evidenced from the missing identification of glucuronidated metabolites of *p*-coumaric acid.

388 Methylation by catechol-*O*-methyl transferase was the preferential pathway for caffeic acid
389 metabolism (both when present as free acid and when conjugate with quinic acid or aspartate),
390 while ferulic and coumaric acids, lacking the catechol structure, could not be methylated by
391 catechol-*O*-methyltransferase. Reduction of ferulic acid to DHF as well as oxidation of DHC to
392 caffeic acid and DHF to ferulic acid were observed, as already reported by Poquet et al.²⁹
393 Previous studies with Caco-2 found di-hydro-ferulic acid and ferulic acid-sulphate as the main
394 metabolite originated from ferulic acid whereas the glucuronidated forms were detected only at
395 trace levels.^{25,30,31} Farrel et al.³¹ and Monente et al.³⁰ also detected caffeic acid-sulphate after
396 incubation of caffeic acid with Caco-2. Differences between our study and previous works could be
397 related to the hydroxycinnamic acids incubation with differentiated Caco-2. On the other hand,
398 incubation for 23 h of ferulic and caffeic acids with non-differentiated Caco-2 (as in our study)
399 resulted in the appearance of the glucuronidated derivatives without any evidence of sulphation.³²
400 Altogether, these results pointed out a different metabolism between differentiated and non-
401 differentiated Caco-2 cells. No previous studies were available in literature concerning the
402 metabolism of hydroxycinnamic acids with SW480.
403 The instability of some hydroxycinnamic acids in Caco-2 cell media may partially explain the
404 observed results regarding the anti-proliferative activity of hydroxycinnamic acids. During
405 incubation with Caco-2, caffeic acid was drastically degraded by the oxidative pathway hindering
406 the detection of any biological activities. Furthermore, caffeic acid was definitely more stable in L-
407 15 than DMEM and was able to reduce SW480 cells proliferation. In addition, 5-CQA was partially
408 degraded in both the cell media and was devoid of any activity.
409 The correlation between the observed effects and the stability in the cell media is not always
410 evident. This is the case of DHC, which was particularly unstable in DMEM resulting in the
411 formation of H₂O₂. Despite degradation, DHC was able to reduce Caco-2 cell proliferation in a
412 concentration-dependent manner. This effect could be related to the production of H₂O₂. However,

413 caffeic acid degradation resulted in a H₂O₂ production similar to that observed when DHC was
414 incubated at 100 µmol/L (corresponding at about the IC₅₀ value against Caco-2) without anti-
415 proliferative activity. These observations suggest that H₂O₂ did not have a key role in the anti-
416 proliferative action of DHC. Similarly, DHF was quite unstable in both the media but was able to
417 exert anti-proliferative effect.

418 Phase II enzymes in Caco-2 cells extensively metabolized some hydroxycinnamic acids, such as
419 coumaric, ferulic and coumaroyl-quinic acids. Previous studies demonstrated that phase II
420 metabolites of phenolic compounds presented reduced biological activities respect to the parent
421 compounds.^{33,34} González-Sarrías and co-worker demonstrated that glucuronidation of urolithins
422 during incubation with HT29 cells limits their anti-proliferative activity.³³ Similarly,
423 glucuronidation of hesperetin by Raw264.7 cells reduced its anti-inflammatory activity.³⁵ In this
424 study, we found that SW480 cells displayed lower metabolic activity and the majority of the
425 hydroxycinnamic acids showed appreciable anti-proliferative activity. Ferulic acid was clearly
426 stable in both the cell media but active only against SW480 proliferation. During incubation with
427 Caco-2 ferulic acid was extensively glucuronidated and, to a lesser extent, sulphated. The Caco-2
428 metabolism of ferulic acid may have hampered their anti-proliferative activity against this cell line.
429 Some of the tested standard compounds and metabolites were recovered in significantly lower
430 amount after incubation with cells (**Table 4**) respect to the amount observed after incubation with
431 cell-free media (**Table 3**) such as *p*-coumaric acid, caffeic acid and DHF in Caco-2 and caffeic acid,
432 *o*-coumaric acid, ferulic acid and 5-CQA in SW480. These compounds could be metabolized in un-
433 identified compounds or transported inside the cells. Some studies suggest that hydroxycinnamic
434 acids can be transported in Caco-2 cells. For example, Konishi and co-worker reported that *p*-
435 coumaric and caffeic acids but not 5-CQA were efficiently transported across Caco-2 membrane
436 *via* the monocarboxylic acid transporter (MCT).³⁶ Moreover, Monente et al. found that the
437 transepithelial transport of DHF in Caco-2 cells was more pronounced than that of DHC.³⁰ No

438 studies about the possible transport of phenolic compounds across SW480 are present in literature.
439 However, these colon cancer cells express high level of MCT.³⁷
440 It is useful mentioning that the observed different susceptibility of the two studied cancer cell lines
441 could reflect their different gene expressions, differentiation stages, proliferative rates, nevertheless
442 their distinct capacity to metabolize and accumulate hydroxycinnamic acids.
443 Despite their stability in the upper gastro-intestinal tract, hydroxycinnamic acids are thoroughly
444 modified in the colon by the action of colonic microbiota. Ludwig and colleagues identified a total
445 of 11 metabolites after *in vitro* colonic fermentation by human microbiota of coffee caffeoylquinic
446 and feruloylquinic acids.³⁸ However, basing on quantitative data, they observed that major
447 degradation pathways led to the accumulation of DHC and DHF.³⁸ Further *in vivo* studies have
448 confirmed free and sulphated DHC and DHF as main metabolites in plasma and urine after
449 ingestion of coffee.^{39,40} DHC and DHF have also been identified after *in vitro* colonic fermentation
450 of blueberries, oat and wheat bran, orange and apple juices as well as black and green tea.⁴¹
451 Therefore, it is important to investigate the potential anti-proliferative effect of hydroxycinnamic
452 acid colonic metabolites in colon adenocarcinoma cell systems.⁴² The results with the reduced
453 microbial metabolites, DHC and DHF, showed that they were more effective than the parent
454 compounds in inhibiting Caco-2 proliferation. DHC was also more active than caffeic acid against
455 SW480 whereas ferulic acid was more effective than DHF in inhibiting SW480 proliferation.
456 In addition to standard hydroxycinnamic acids and colonic metabolites, we also tested two different
457 extracts obtained from *in vitro* digested cherry and dark chocolate with the aim to increase our
458 previous findings and identify the possible compounds involved in the already reported anti-
459 proliferative activity.^{13,14} The two extracts were enriched in coumaroylquinic acids (from digested
460 cherry) and hydroxycinnamoyl aspartates (from digested dark chocolate). Similarly to what was
461 observed for *in vitro* digested cherry, the coumaroylquinic acids enriched extract was effective
462 against SW480 but not on Caco-2 cell proliferation. These results pointed out that this fraction

463 contained the causative compounds of the anti-proliferative activity of *in vitro* digested cherry.¹³
464 This extract was enriched in coumaroylquinic acids but also contained other compounds such as 3-
465 and 4-caffeoylquinic acids and roseoside. In this study, we demonstrated that caffeoylquinic acids
466 were not effective against SW480 cells proliferation. Concerning roseoside, no studies about its
467 effect on colon cancer Caco-2 and SW480 cells were found in literature. Differently, the
468 hydroxycinnamoyl aspartates extract was able to inhibit the proliferation of both the colon cancer
469 cell lines but was three-times more effective against SW480. These results fitted with the data
470 obtained with *in vitro* digested dark chocolate and suggested that this fraction contained the active
471 compounds.¹⁴ Besides hydroxycinnamoyl aspartates, the extract also contained (epi)catechin
472 sulphate and 12-hydroxy jasmonic acid sulphate. Ramos et al. reported that 24 h treatment of Caco-
473 2 and SW480 with catechin, epicatechin or procyanidin B2 did not affect cell growth.⁴³ No studies
474 about the effect of 12-hydroxy jasmonic acid sulphate on colon cancer Caco-2 and SW480 cells or
475 against others cancer cell lines were found in literature. However, methyl jasmonic acid was found
476 to be able to inhibit *in vitro* cancer cell proliferation and to induce cell death in several cancer cell
477 lines, including colon cancer HT29.⁴⁴
478 These results indicated that some hydroxycinnamic acids at a concentration close to those which
479 they can achieve in the gastro-intestinal tract after the consumption of hydroxycinnamic acid-rich
480 foods and beverages, may exert anti-proliferative activity. In particular, the remarkable effect of di-
481 hydro-caffeic and di-hydro-ferulic acids against cell proliferation is of paramount importance since
482 these compounds are the main metabolites detectable at colonic level after the consumption of
483 hydroxycinnamates-rich foods. This study suggests that these metabolites could be the real active
484 molecules involved in the reported biological effects of hydroxycinnamic acid-rich foods, especially
485 those effects related to gastrointestinal pathologies such as colon cancer.
486 Our results highlighted that the drastic biotransformations of hydroxycinnamic acids occurring in
487 cell cultures should be carefully taken into account in order to fully understand the real compounds

488 exerting the observed bioactivities. Some compounds were drastically degraded in culture medium
489 whereas some other compounds were extensively metabolized in phase II metabolites, which could
490 have resulted in a reduction of their anti-proliferative activity. This integrated approach may
491 therefore be relevant to understand the molecules and mechanisms responsible for the biological
492 effects of phenolic metabolites.

493 Finally, hydroxycinnamic acids and especially their colonic metabolites di-hydro-caffeic and di-
494 hydro-ferulic acids might have interesting anti-proliferative potential in colon cancer prevention,
495 warranting further studies to confirm this relationship as well as the possible mechanisms of action.

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Figure captions

Figure 1. Structure of hydroxycinnamic acids used in this study. R1=OH, R2=H: *o*-coumaric acid. R1=H, R2=OH: *p*-coumaric acid.

Figure 2. Recovery and isomerization of hydroxycinnamoyl-quinic acids and hydroxycinnamoyl aspartates. (A, C, E) Caco-2; (B, D, F) SW480. Black columns represent the percentage of the different isomers at the beginning of incubation (time 0). Light grey columns represent the percentage of the different isomers after 24 h of incubation in cell-free media. Dark grey columns represent the percentage of the different isomers after 24 h of incubation with cells. CQA: caffeoylquinic acid; CoQA: coumaroylquinic acid. Values in the same graph with different lowercase letter are significantly different ($P<0.05$). Coumaroylquinic acids enriched fraction was obtained from *in vitro* digested cherry as reported in materials and methods section.

Hydroxycinnamoyl aspartates enriched fraction was obtained from *in vitro* digested dark chocolate as reported in materials and methods section.

Figure 3. Preparation of coumaroylquinic acids enriched fraction from *in vitro* digested cherry. (A) Extracted ion chromatogram at m/z 337 from direct infusion of ACN fractions obtained from solid phase extraction of *in vitro* digested Celeste cherry cultivar. Dashed line represents ACN1 fraction. Solid line represents ACN2 fraction. Dotted line represents ACN3 fraction. Light grey solid line represents ACN4 fraction. Grey solid line represents methanolic fraction. (B) Total ion chromatogram obtained from direct infusion of fraction ACN2. (C) Base peak chromatogram of fraction ACN2.

Figure 4. Preparation of hydroxycinnamoyl aspartates enriched fraction from *in vitro* digested dark chocolate. Extracted ion chromatograms at m/z 278 (A), 294 (B) and 308 (C) from fraction ACN1 (solid black line) and ACN2 (solid grey line). (D) Base peak chromatogram of fraction ACN2.

Figure 5. Proposed pathways for hydroxycinnamic acids metabolism after incubation with Caco-2 cell line. COMT: catechol-*O*-methyl transferase; CPase: carboxypeptidase; EST: esterase; RA: reduction; OX: oxidation; DHY: de-hydroxylation; HYD: hydroxylation; SULT: sulfotransferase; UDP-GT: UDP-glucuronosyltransferase. Bold arrows indicate major routes. Percentages indicate the extent of biotransformation.

Figure 6. Proposed pathways for hydroxycinnamic acids metabolism after incubation with SW480 cell line. CPase: carboxypeptidase; EST: esterase; OX: oxidation; DHY: de-hydroxylation; HYD: hydroxylation; SULT: sulfotransferase. Bold arrows indicate major routes. Percentages indicate the extent of biotransformation.

Table 1. Effect of hydroxycinnamic acids and coumaroyl-quinic acids and hydroxycinnamoyl-aspartates-enriched fractions on the proliferation of colon adenocarcinoma human cell lines.

| Compound | Caco-2 % inhibition | SW 480 % inhibition |
|---|--------------------------------|--------------------------------|
| <i>Standards</i> | | |
| Cinnamic acid | n.a. | n.a. |
| <i>p</i> -Coumaric acid | n.a. | n.a. |
| <i>o</i> -Coumaric acid | n.a. | 7.23 ± 3.86 ^e |
| Caffeic acid | n.a. | 40.8 ± 1.63 ^c |
| Ferulic acid | n.a. | 59.9 ± 1.29 ^e |
| 5-Caffeoylquinic acid | n.a. | n.a. |
| Di-hydro-caffeic acid | 101 ± 1.70 ^a | 46.8 ± 1.05 ^b |
| Di-hydro-ferulic acid | 100 ± 2.11 ^a | 37.3 ± 1.21 ^{cd} |
| <i>Extracts</i> | | |
| Coumaroylquinic acids-enriched fraction | n.a. | 41.4 ± 3.31 ^{bc} |
| Hydroxycinnamoyl aspartates enriched fraction | 10.7 ± 1.92 ^e | 34.2 ± 1.55 ^d |

Hydroxycinnamic acids were tested at 200 µmol/L. Coumaroylquinic acids and hydroxycinnamoyl aspartates enriched fractions were tested after two-fold dilution in cell media. Coumaroylquinic acids final concentration in the cell media were 36.2 and 13.9 µmol/L for 4-coumaroyl-quinic and 5-coumaroyl-quinic acids, respectively. Hydroxycinnamoyl-aspartates concentration in the cell media were 224, 55.2 and 33.1 µmol/L for coumaroyl-aspartate, caffeoyl-aspartate and feruloyl-aspartate, respectively.

n.a. means no activity

Values with different uppercase letters are significantly different ($P < 0.05$).

Table 2. Mass spectral characteristics of hydroxycinnamic acid standards and their metabolites identified in this study

| Compound | [M-H] ⁻ (<i>m/z</i>) | MS ² ion fragments (<i>m/z</i>) |
|-----------------------------------|--------------------------------------|--|
| Caffeic acid* | 179 | 135 (100%) |
| Caffeic acid-glucuronide | 355 | 179 (100%) |
| Caffeic acid-sulphate | 259 | 179 (100%), 135 (19%) |
| 3- <i>O</i> -Caffeoylquinic acid | 353 | 191 (100%), 179 (43%), 135 (9%) |
| 4- <i>O</i> -Caffeoylquinic acid | 353 | 179 (100%), 173 (44%), 135 (40%), 191 (27%) |
| 5- <i>O</i> -Caffeoylquinic acid* | 353 | 191 (100%), 173 (3%) |
| Cinnamic acid * | 147 | 103 (100%) |
| <i>o</i> -Coumaric acid* | 163 | 119 (100%) |
| <i>p</i> -Coumaric acid* | 163 | 119 (100%) |
| Coumaric acid-glucuronide | 339 | 113 (100%), 163 (35%), 119 (21%), 175 (7%) |
| Coumaric acid-sulphate | 243 | 163 (100%), 119 (14%) |
| Di-hydro-caffeic acid* | 181 | 137 (100%) |
| Di-hydro-caffeic acid-cysteine | 300 | 213 (100%), 135 (76%), 181 (65%), 107 (40%) |
| Di-hydro-caffeic acid-sulphate | 261 | 181 (100%) |
| Di-hydro-coumaric acid | 165 | 121 (100%) |
| Di-hydro-ferulic acid* | 195 | 177 (100%), 136 (99%), 151 (11%), 175 (7%) |
| Di-hydro-ferulic acid-glucuronide | 371 | 195 (100%), 113 (65%) |
| Di-hydro-ferulic acid-sulphate | 275 | 195 (100%) |
| Ferulic acid* | 193 | 178 (100%), 134 (94%), 149 (79%) |
| Ferulic acid-glucuronide | 369 | 175 (100%), 193 (42%), 113 (40%) |
| Ferulic acid-sulphate | 273 | 193 (100%) |
| 5- <i>O</i> -Feruloylquinic acid | 367 | 179 (100%), 135 (30%) |

*Identified by comparison with the corresponding standard

Table 3. Stability of hydroxycinnamic acids and coumaroylquinic acids and hydroxycinnamoyl aspartates enriched fractions after 24 h of incubation in Caco-2 and SW480 cell media without cells. Results are expressed as % recovery respect to the initial dose.

| Compounds | Caco-2 medium % recovery | SW 480 medium % recovery |
|---|-----------------------------|-----------------------------|
| <i>Standards</i> | | |
| Cinnamic acid | 100 ± 1.57 | 100 ± 1.01 |
| <i>p</i> -Coumaric | 100 ± 0.07 | 84.7 ± 1.44* |
| <i>o</i> -Coumaric | 96.1 ± 0.51 | 96.4 ± 0.94 |
| Caffeic acid | 56.3 ± 1.34* | 97.2 ± 1.44 |
| Ferulic acid | 93.1 ± 2.27 | 100 ± 0.58 |
| 5-Caffeoylquinic acid | 35.3 ± 0.33* | 63.2 ± 2.36* |
| Di-hydro-caffeic acid | 35.3 ± 0.52* | 70.8 ± 0.42* |
| Di-hydro-ferulic acid | 71.1 ± 0.33* | 69.7 ± 1.34* |
| <i>Coumaroylquinic acids enriched fractions</i> | | |
| 4-Coumaroylquinic acid | 56.8 ± 1.32* | 64.6 ± 0.34* |
| 5-Coumaroylquinic acid | 124.2 ± 2.06* | 47.3 ± 0.92* |
| <i>Hydroxycinnamoyl aspartates enriched fractions</i> | | |
| Coumaroyl-aspartate | 90.4 ± 4.31 | 94.6 ± 0.88 |
| Caffeoyl-aspartate | 57.2 ± 0.74* | 47.6 ± 0.41* |
| Feruloyl-aspartate | 62.3 ± 0.39* | 88.4 ± 1.27* |

Hydroxycinnamic acids were tested at 200 µmol/L. Coumaroylquinic acids and hydroxycinnamoyl aspartates enriched fractions were tested after two-fold dilution in cell media. Coumaroylquinic acids final concentration in the cell media were 36.2 and 13.9 µmol/L for 4-coumaroyl-quinic and 5-coumaroyl-quinic acids, respectively. Hydroxycinnamoyl-aspartates concentration in the cell media were 224, 55.2 and 33.1 µmol/L for coumaroyl-aspartate, caffeoyl-aspartate and feruloyl-aspartate, respectively.

*means significantly different from the initial amount

Table 4. Recovery (% of initial dose) of free hydroxycinnamic acids and their metabolites in the Caco-2 and SW480 extracellular culture medium after 24 h of incubation with cells.

| Compounds | Caco-2 % of initial dose | SW 480 % of initial dose |
|--|-----------------------------|-----------------------------|
| <i>Substrate</i> | | |
| Cinnamic acid | 101 ± 1.46 | 100 ± 1.01 |
| <i>Metabolites</i> | | |
| Coumaric acid | Traces | Traces |
| <i>Total</i> | 101 ± 1.46 | 100 ± 1.01 |
| <i>Substrate</i> | | |
| <i>p</i> -Coumaric acid | 76.3 ± 0.73 | 72.9 ± 2.70 |
| <i>Metabolites</i> | | |
| <i>p</i> -Coumaric acid glucuronide | Traces | n.d. |
| Cinnamic acid | Traces | n.d. |
| Di-hydro-coumaric acid | Traces | 13.8 ± 0.40 |
| <i>Total</i> | 76.3 ± 0.73* | 86.7 ± 2.73 |
| <i>Substrate</i> | | |
| <i>o</i> -Coumaric acid | 63.3 ± 0.31 | 55.2 ± 1.05 |
| <i>Metabolites</i> | | |
| <i>o</i> -Coumaric acid glucuronide isomer 1 | 0.55 ± 0.02 | n.d. |
| <i>o</i> -Coumaric acid glucuronide isomer 2 | 43.6 ± 1.11 | n.d. |
| Di-hydro-coumaric acid | n.d. | Traces |
| Coumaric acid sulphate | n.d. | 32.0 ± 0.95 |
| <i>Total</i> | 107 ± 1.15 | 87.2 ± 1.42* |
| <i>Substrate</i> | | |
| Caffeic acid | 46.4 ± 0.40 | 57.7 ± 2.62 |
| <i>Metabolites</i> | | |
| Ferulic acid | 3.08 ± 0.07 | n.d. |
| Coumaric acid | Traces | Traces |
| Caffeic acid glucuronide | 0.11 ± 0.01 | n.d. |
| Caffeic acid sulphate | n.d. | 0.24 ± 0.02 |
| <i>Total</i> | 49.6 ± 0.41* | 57.9 ± 2.62* |
| <i>Substrate</i> | | |
| Ferulic acid | 82.0 ± 0.21 | 48.2 ± 1.00 |
| <i>Metabolites</i> | | |
| Ferulic acid glucuronide | 21.9 ± 0.82 | n.d. |
| Di-hydro-ferulic acid | Traces | n.d. |
| Ferulic acid sulphate | 4.01 ± 0.08 | 2.90 ± 0.24 |
| <i>Total</i> | 107 ± 0.85 | 51.1 ± 1.03* |
| <i>Substrate</i> | | |
| 5-Caffeoylquinic acid | 41.2 ± 1.50 | 45.5 ± 0.41 |
| <i>Metabolites</i> | | |
| 3-Caffeoylquinic acid | 14.5 ± 0.21 | 2.52 ± 0.13 |
| 4-Caffeoylquinic acid | 19.7 ± 1.90 | 43.1 ± 1.02 |
| Caffeic acid | 0.27 ± 0.01 | Traces |
| 5-Feruloylquinic acid | 17.0 ± 0.57 | n.d. |
| <i>Total</i> | 92.7 ± 2.50 | 91.1 ± 1.11* |
| <i>Substrate</i> | | |
| Di-hydro-caffeic acid | 30.7 ± 0.27 | 76.9 ± 0.95 |

| | | |
|-----------------------------------|--------------|--------------|
| <i>Metabolites</i> | | |
| Di-hydro-ferulic acid | Traces | n.d. |
| Caffeic acid | 0.87 ± 0.01 | n.d. |
| Di-hydro-caffeic acid cysteine | n.d. | 23.8 ± 1.12 |
| Di-hydro-caffeic acid sulphate | n.d. | 4.77 ± 0.12 |
| <i>Total</i> | 31.6 ± 0.27 | 105 ± 1.47 |
| <i>Substrate</i> | | |
| Di-hydro-ferulic acid | 46.9 ± 1.45 | 63.2 ± 2.17 |
| <i>Metabolites</i> | | |
| Di-hydro-ferulic acid glucuronide | 0.53 ± 0.01 | n.d. |
| Ferulic acid | 0.22 ± 0.01 | n.d. |
| Di-hydro-ferulic acid sulphate | 0.71 ± 0.01 | 2.98 ± 0.03 |
| <i>Total</i> | 48.4 ± 1.45* | 66.2 ± 2.17 |
| <i>Substrate</i> | | |
| 4-Coumaroylquinic acid | 25.9 ± 0.19 | 31.0 ± 0.64 |
| 5-Coumaroylquinic acid | 26.6 ± 0.11 | 20.5 ± 2.08 |
| <i>Metabolites</i> | | |
| 3-Coumaroylquinic acid | 30.4 ± 0.15 | 23.9 ± 0.24 |
| Coumaroylquinic acid glucuronide | 2.29 ± 0.04 | n.d. |
| Di-hydro-coumaric acid | 3.26 ± 0.09 | n.d. |
| Coumaric acid glucuronide | 6.67 ± 0.12 | n.d. |
| Coumaric acid | 3.47 ± 0.02 | Traces |
| <i>Total</i> | 98.6 ± 0.31 | 75.4 ± 2.19* |
| <i>Substrate</i> | | |
| Caffeoyl-aspartate | 7.62 ± 0.95 | 42.6 ± 8.78 |
| Coumaroyl-aspartate | 27.2 ± 2.87 | 8.31 ± 0.58 |
| Feruloyl-aspartate | 9.56 ± 1.08 | 9.54 ± 0.25 |
| <i>Metabolites</i> | | |
| Caffeic acid | 1.55 ± 0.21 | 0.52 ± 0.02 |
| Ferulic acid | 0.42 ± 0.02 | n.d. |
| Coumaric acid | 0.41 ± 0.02 | n.d. |
| <i>Total</i> | 46.8 ± 3.22* | 61.0 ± 8.80* |

Hydroxycinnamic acids were tested at 200 µmol/L. Coumaroylquinic acids and hydroxycinnamoyl aspartates enriched fractions were tested after two-fold dilution in cell media. Coumaroylquinic acids final concentration in the cell media were 36.2 and 13.9 µmol/L for 4-coumaroyl-quinic and 5-coumaroyl-quinic acids, respectively. Hydroxycinnamoyl-aspartates concentration in the cell media were 224, 55.2 and 33.1 µmol/L for coumaroyl-aspartate, caffeoyl-aspartate and feruloyl-aspartate, respectively.

n.d. means not detected

*means significantly different from the amount recovered after incubation with cell-free media (**Table 3**).

Table 5. Mass spectral characteristics of compounds identified in the coumaroyl quinic acids and hydroxycinnamoyl aspartates enriched extracts

| Peak | Compound | [M-H] ⁻ (<i>m/z</i>) | MS ² ion fragments (<i>m/z</i>) |
|--|--|--------------------------------------|--|
| <i>Coumaroylquinic acids enriched fraction</i> | | | |
| 1 | 3-Caffeoylquinic acid | 353 | 191 (100%), 179 (43%), 135 (9%) |
| 2 | 4-Caffeoylquinic acid | 353 | 179 (100%), 173 (44%), 135 (40%), 191 (27%) |
| 3 | 4-Coumaroylquinic acid <i>cis</i> | 337 | 173 (100%), 163 (6%) |
| 4 | Roseoside | 431 | 385 (100%), 153 (11%), 223 (6%) |
| 5 | 4-Coumaroylquinic acid <i>trans</i> | 337 | 173 (100%), 163 (4%) |
| 6 | 5-Coumaroylquinic acid <i>trans</i> | 337 | 191 (100%), 173 (3%), 163 (2%) |
| 7 | 5-Coumaroylquinic acid <i>cis</i> | 337 | 191 (100%), 163 (7%), 173 (2%) |
| <i>Hydroxycinnamoyl aspartates enriched fraction</i> | | | |
| 1 | Caffeoyl aspartate isomer 1 | 249 | 132 (100%), 179 (79%), 227 (36%), 276 (34%), 250 (14%) |
| 2 | Caffeoyl aspartate isomer 2 | 249 | 132 (100%), 276 (54%), 179 (31%), 232 (2%) |
| 3 | Coumaroyl aspartate isomer 1 | 278 | 132 (100%), 163 (48%), 234 (19%), 119 (3%) |
| 4 | Feruloyl aspartate isomer 1 | 308 | 193 (100%) |
| 5 | Coumaroyl aspartate isomer 2 | 278 | 132 (100%), 163 (74%), 119 (24%), 234 (6%) |
| 6 | Feruloyl aspartate isomer 2 | 308 | 193 (100%), 149 (65%), 132 (35%) |
| 7 | (Epi)catechin-O-sulphate | 369 | 289 (100%), 245 (9%), 125 (9%) |
| 8 | 12-hydroxy jasmonic acid sulphate isomer 1 | 305 | 97 (100%), 225 (35%) |
| 9 | 12-hydroxy jasmonic acid sulphate isomer 2 | 305 | 97 (100%), 225 (33%) |

Coumaroylquinic acids enriched fraction was obtained from *in vitro* digested cherry as reported in material and methods section. Peaks are numbered as reported in **Figure 3C**. Hydroxycinnamoyl aspartates enriched fraction was obtained from *in vitro* digested dark chocolate as reported in material and methods section. Peaks are numbered as reported in **Figure 4D**.

Figure 1

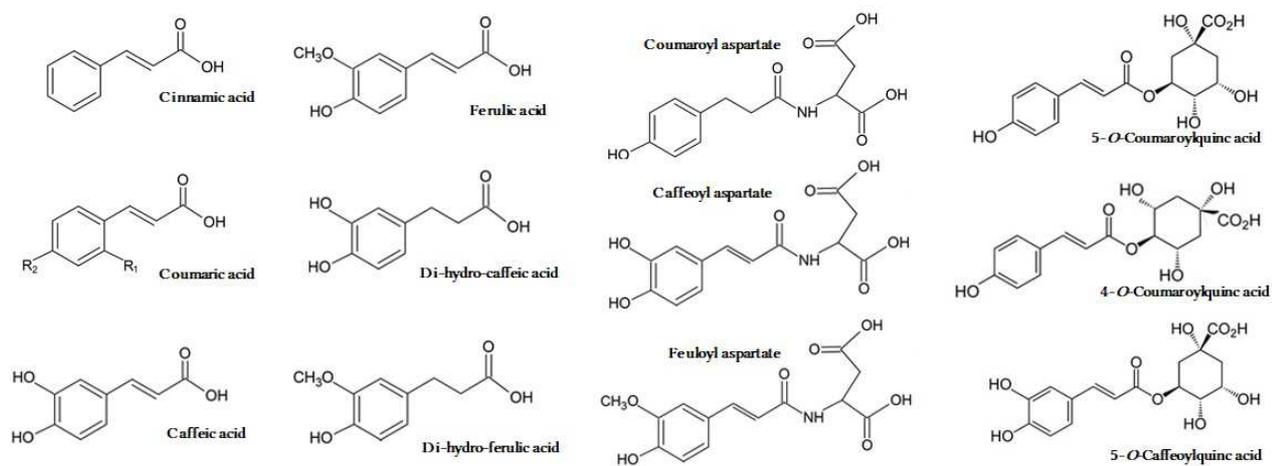


Figure 2

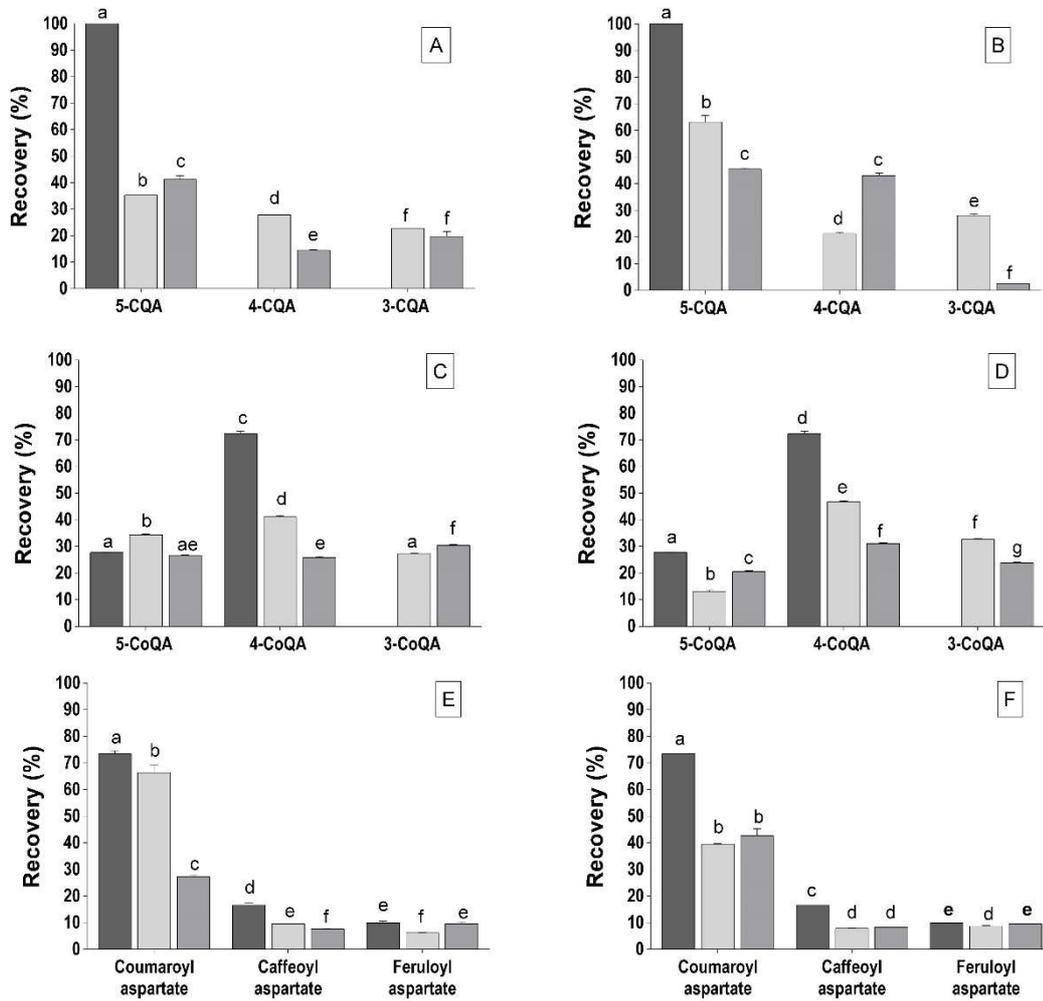


Figure 3

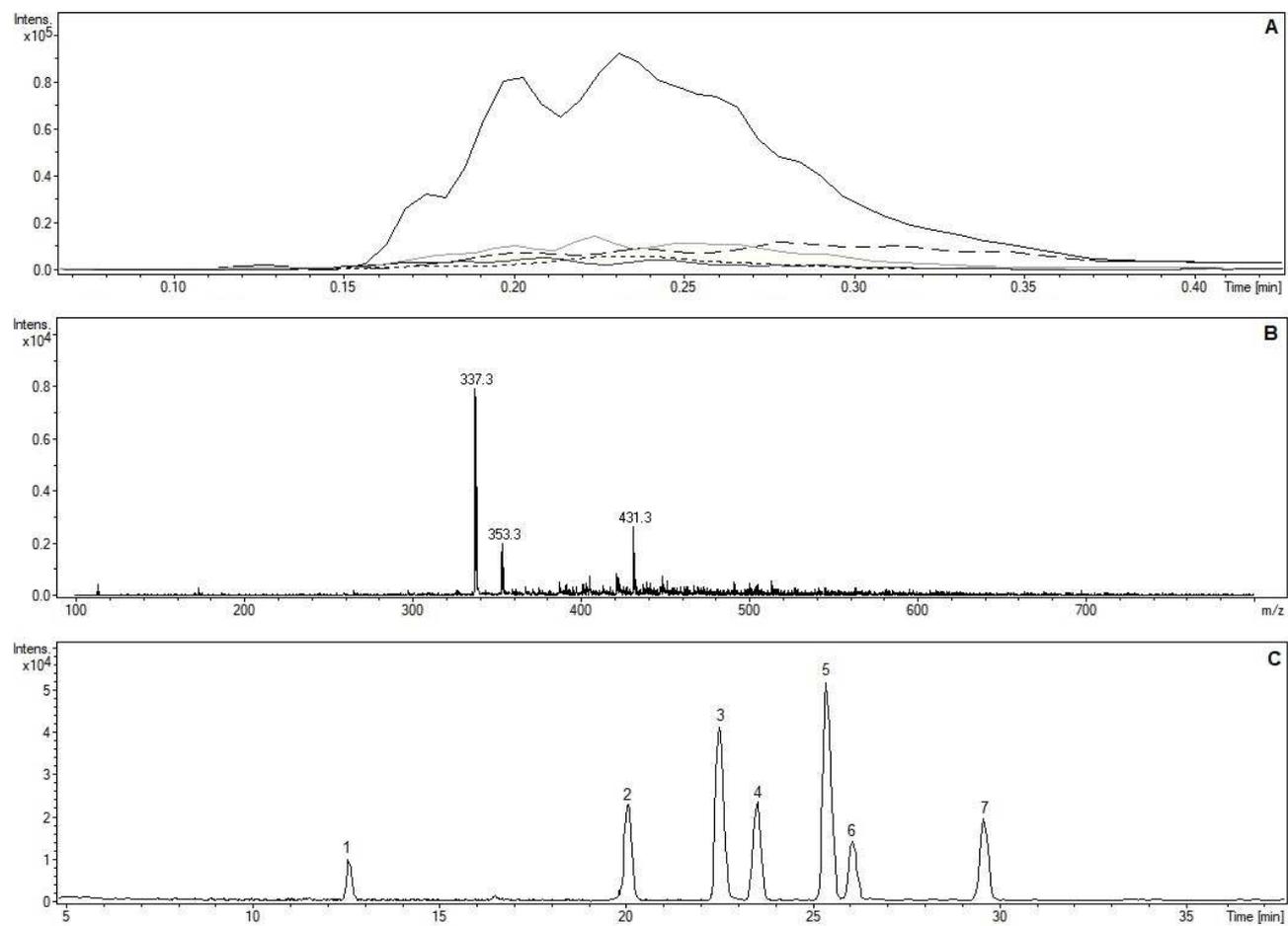


Figure 4

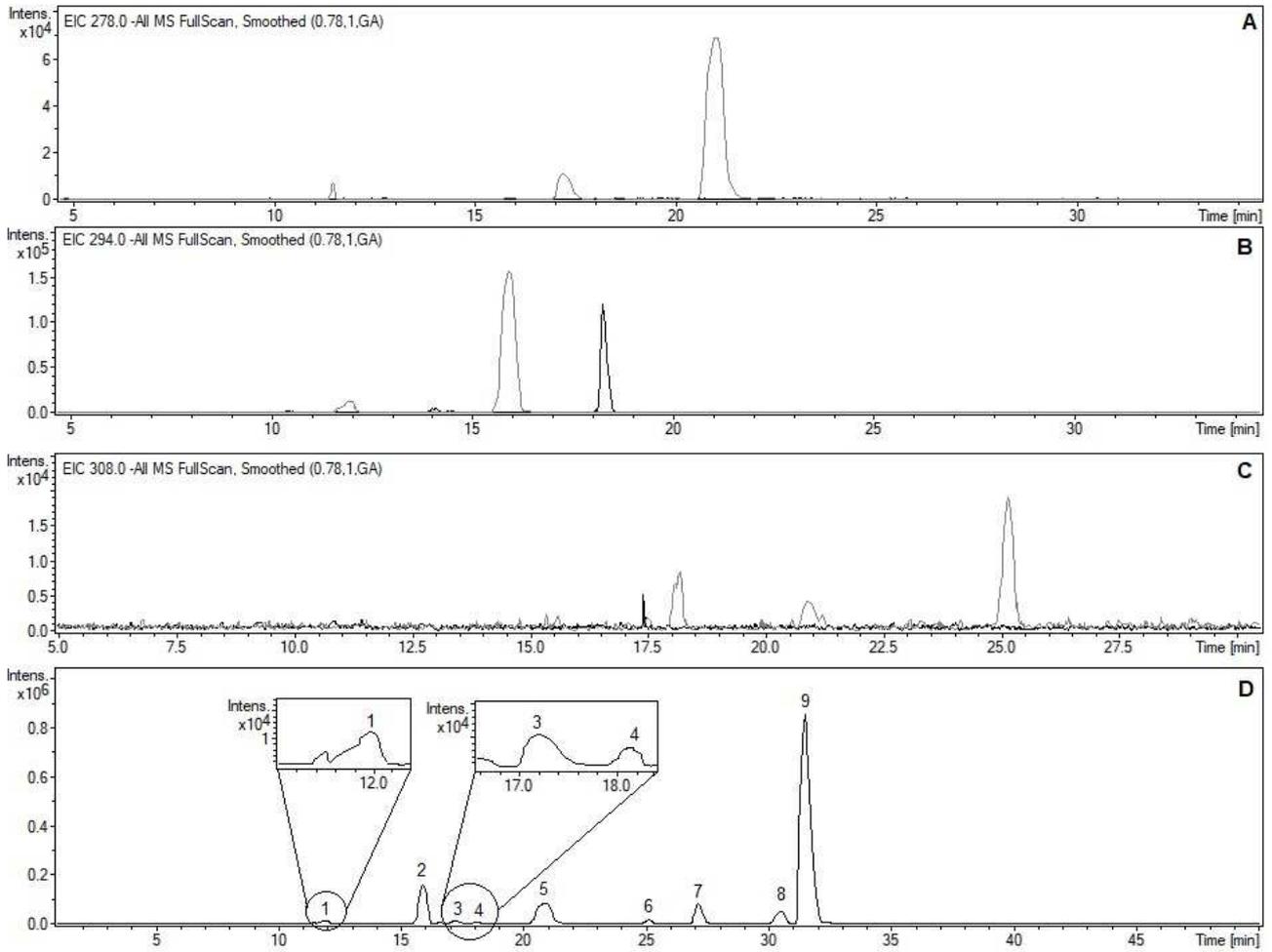


Figure 5

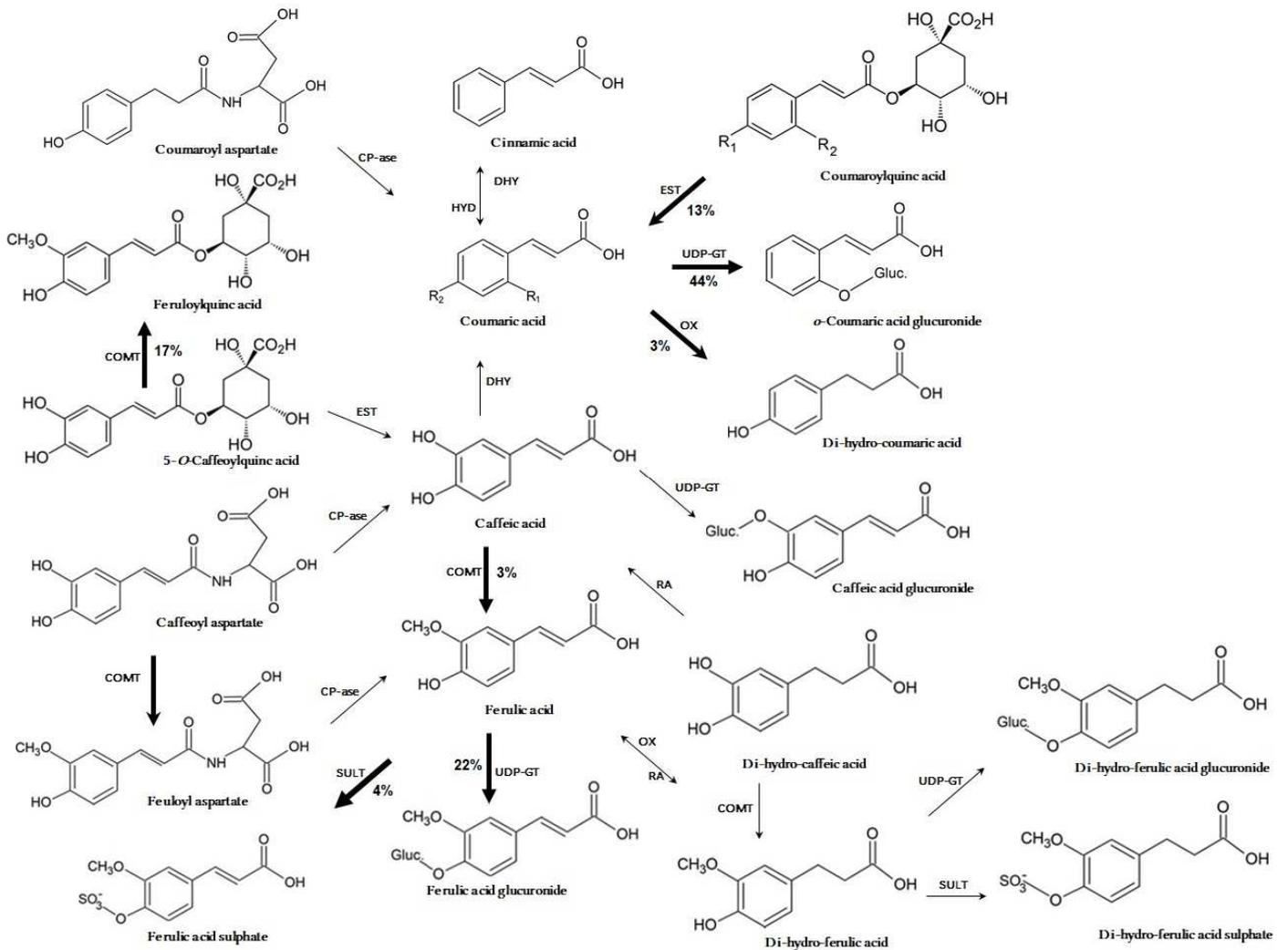
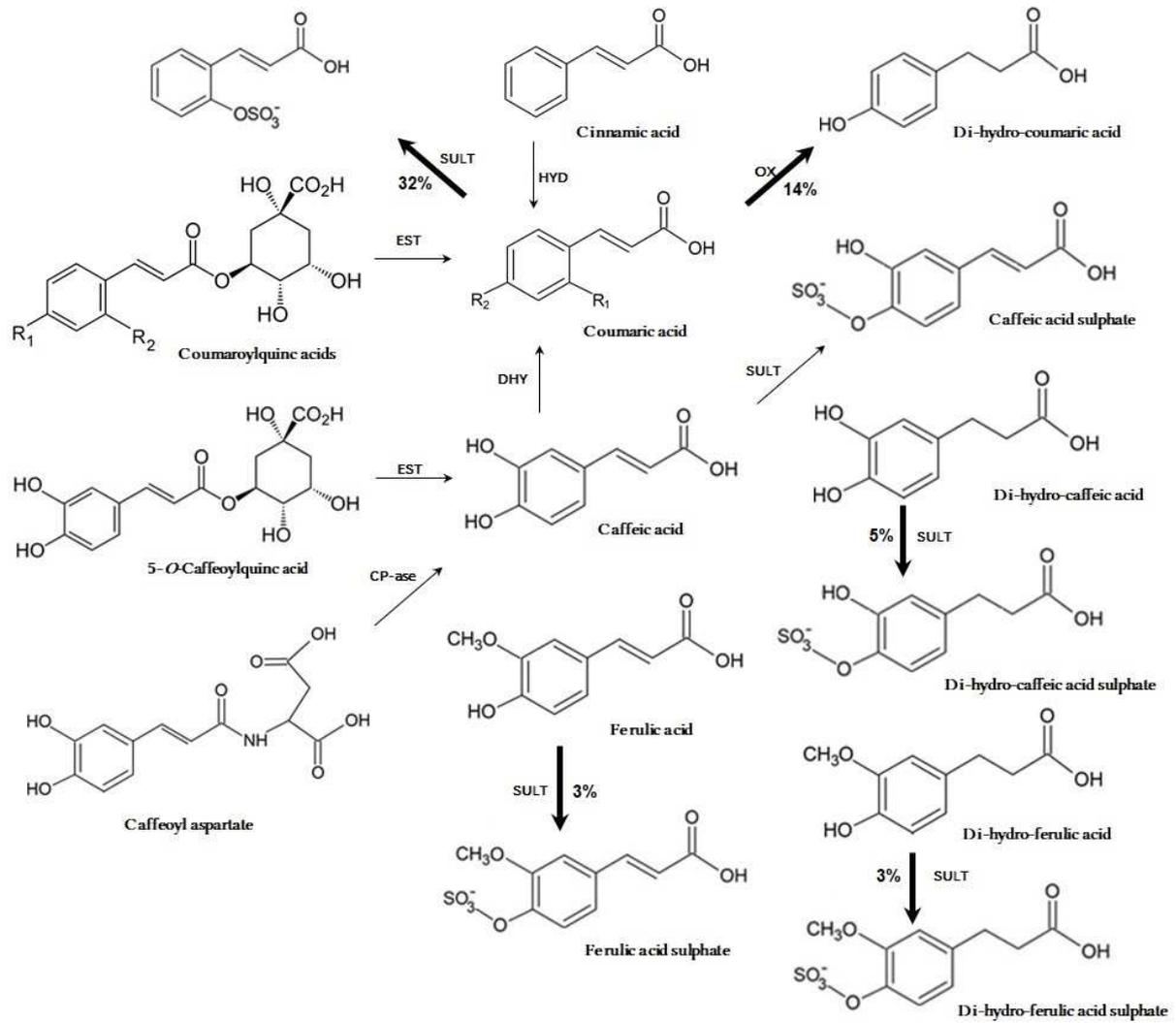


Figure 6



Graphic for table of contents

