Effect of Foods and β-Cyclodextrin on the Bioaccessibility and the Uptake by Caco-2 Cells of Hydroxytyrosol from Either a Pure Standard or Alperujo

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Research Article

Effect of foods and β-cyclodextrin on the bioaccessibility and the uptake by Caco-2 cells of hydroxytyrosol from either a pure standard or alperujo

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Abstract

Hydroxytyrosol bioaccessibility and absorption by the intestinal cells have been studied using an *in vitro* digestion model and Caco-2 TC7 monolayers cells in culture, in the presence or absence of β-cyclodextrin and foods. Hydroxytyrosol was either provided as a pure standard or in an alperujo powder. The presence of foods significantly decreased hydroxytyrosol bioaccessibility and absorption (-20% and -10%, respectively), while β-cyclodextrin had no effect. Moreover, the presence of other compounds from alperujo in the intestine compartment reduced hydroxytyrosol absorption by Caco-2 cells compared to pure standard (-60%). The final bioavailability of hydroxytyrosol, defined as its quantity at the basolateral side of cultured cell monolayers compared to the initial amount in the test meal, was 6.9±0.4%, 31.1±1.1% and 40.9±1.5% when hydroxytyrosol was from alperujo, or a standard administered with or without food, respectively. Our results show that conversely to foods, β-cyclodextrin does not alter hydroxytyrosol bioavailability.

Chemical compounds

Hydroxytyrosol (PubChem CID: 82755)

β-cyclodextrin (PubChem CID: 444041)

1 Introduction

Two-phase olive pomace, also called alperujo, is one of the most abundant industrial Mediterranean pollutants. It is produced in large quantity in two-phase centrifuge mills and is composed of olive vegetation water and solid olive pieces [1]. Two-phase centrifuge mills allow reducing the water consumption and then the quantity of olive mill wastes compared to both traditional and three-phase centrifuge systems. Alperujo pollutant character is especially due to its high phenolic content. Interestingly, previous studies have demonstrated the great interest of these phenolic compounds because of their high health benefits [2].

In order to valorize these co-products, the phenolic composition of alperujo has been extensively characterized, thus confirming that it can be an interesting source of valuable compounds for the nutraceutical, cosmetic and food industries. The major phenolic compounds identified into olive mill wastes were hydroxytyrosol (HT) and tyrosol, both belonging to the phenyl alcohol family, as well as *p*-hydroxycinnamic acids such as caffeic acid and derivatives [3, 4]. These molecules display a catechol unit that confers them a reducing (electron-donating) character tightly related to their bioactivity (e.g., their antioxidant potential) [5]. HT has received a health claim by the European Food Safety Agency (EFSA) due to its high ability to scavenge reactive oxygen species and to reduce the risks of cardiovascular disease [6-8]. However, the electron-donating properties of olive phenols make them sensitive to oxidation. Hence, investigating the influence of the food matrix, including food ingredients used for formulation purposes, on the stability and bioavailability of olive phenols is a relevant issue.

Cyclodextrins (CDs) are natural cyclic oligosaccharides made of D-glucose units bound by α-1,4 linkages and mainly used in the agro-food and pharmaceutical industries to form inclusion complexes (IC) with bioactive compounds, to enhance their stability and solubility [9, 10]. β-CD (7 D-glucose units) is the most used CD because of its low price, its availability and its ability to

66 form inclusion complexes with a large range of medium-sized compounds (MM < 800 g/mol) [11].
67 β-CD can also be used to facilitate polyphenol extraction from plants, such as resveratrol from
68 grape pomace [12-14], and were suggested to be suitable to extract bioactives such as triterpenes from
69 alperujo [15]. However, its ability to interact with polyphenol bioavailability is not known.
70 *In vitro* digestion studies can be carried out to assess the bioaccessibility of a given compound, *i.e*.
71 the fraction of the ingested dose that is transferred from the food matrix to the aqueous phase or to
72 mixed micelles (combining bile acids and lipid digestion products). This fraction is considered as
73 available for subsequent absorption by the enterocytes, which can be investigated using the Caco-
74 2 cell model. Bioaccessibility and intestinal absorption are two critical steps governing a
75 compound bioavailability, *i.e*. the fraction of the ingested dose (native forms + metabolites) that
76 reaches the general blood circulation and/or target tissues [16].
77 In this work, we investigated the effects of β-CD, alperujo matrix and foods (represented by a test
78 meal containing pureed potatoes, minced beef and refined olive oil) on the bioaccessibility and the
79 intestinal absorption of HT (from a standard powder and from a local alperujo).
80

81 **2 Materials and Methods**

82 2.1. Supplies

83 β-CD was given from Roquette Freres (Lestern, France). HT (purity > 98%) was kindly provided
84 by Pr. Francesco Visioli (IMDEA, Madrid, Spain). Tyrosol and gallic acid were supplied from
85 Sigma-Aldrich Co (St Louis, USA). Pepsin, porcine pancreatin, porcine bile extract, water, formic
86 acid, ethanol and acetonitrile were purchased from Sigma-Aldrich (Fontenay sous Bois, France).
87 Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and trypsin-EDTA (500
88 mg/L and 200 mg/L, respectively), non-essential amino acids, penicillin/streptomycin and PBS
89 were purchased from Life Technologies (Illkirch, France). Fetal bovine serum (FBS) came from

PAA (Vélizy Villacoublay, France). Olive pomace was collected from the Castelas mill equipped with a two-phase centrifuge system (Baux-de-Provence, France). Foods were purchased from a local supermarket.

2.2. Preparation of the alperujo sample

Alperujo (Aglandau variety, 72% of moisture, stored in cheesecloth canvas) was manually pressed, then filtered on celite and passed through 0.45µm and 0.2µm paper filters (VWR). Ethanol was added to a final proportion of 42% to precipitate proteins (removed by centrifugation). After ethanol evaporation under vacuum, the protein-free aqueous phase from alperujo was frozen at -20°C.

2.3. Preparation of the inclusion complexes

In aqueous solution, HT is known to bind β-CD with a 1:1 stoichiometry and a binding constant ≈ 40 M^{-1} [17]. Hence, the inclusion complex was prepared from an equimolar solution of HT and β-CD (5 mM) in water. The concentration was chosen high enough to allow a substantial formation of the inclusion complex (≈ 20%), despite the relatively low affinity of HT for β-CD in aqueous solution. The equimolar HT:β-CD solution was stirred at 200 rpm for 1h at room temperature, then freeze-dried. The inclusion complex in a solid form (powder) was kept in amber flask at -20°C until use.

The total phenol concentration of the protein-free aqueous phase of alperujo was assessed using Folin-Ciocalteau method and diluted to reach a total phenol concentration of 5 mM in gallic acid equivalent [18]. Then, β-CD was added to the sample in the same concentration and the solution was stirred at 200 rpm for 1h at room temperature. After freeze-drying, the aqueous phase of alperujo

113 + β-CD sample was stored as a powder at -20°C in amber glass. For comparison, an alperujo
114 extract without β-CD was also freeze-dried and store as a powder in similar conditions.
115
116 2.4. Simulated digestion
117 The test meal, when present, was composed of pureed potatoes (6.7 g), cooked minced beef (1.2
118 g) and refined olive oil (0.2 g). HT and alperujo samples with or without β-CD were added so as
119 to reach 7 mg of HT in the meal. The simulated digestion was carried out as described previously
120 (Figure 1) [19]. All analyses were run in quadruplicate. Aliquots from oral, gastric and duodenal
121 steps were taken up and frozen at -80°C until use.
122
123 2.5. Cell culture and uptake experiments
124 The human colon adenocarcinoma cell line Caco-2 TC7 was cultured on transwell membrane (six-
125 well plate, 1 mm pore size polycarbonate membrane; Becton Dickinson) to obtain confluent and
126 differentiated cell monolayers as previously described [20].
127 Cytotoxicity of digestion samples on Caco-2 TC7 was primarily evaluated to determine the
128 suitable dilution of the phenolic aqueous fractions from in vitro digestion in HBSS before adding
129 them to the apical side of cell monolayers. These results showed that 1/20 and 1/10 dilutions were
130 required for HT and alperujo samples, respectively. To avoid any interference with DMEM or
131 serum components, the phenolic aqueous fractions were diluted in HBSS and Caco-2 cells received
132 HBSS in both chambers 12h before the experiments. At the beginning of each experiment, cell
133 monolayers were washed twice with 1 mL of PBS and received 1 mL of diluted aqueous fraction.
134 Finally, cell monolayers were incubated at 37°C for 2h, 4h and 6h. After the incubation period,
135 apical and basolateral solutions were harvested. Cell monolayers were washed twice with 1 mL of
136 PBS and scraped in 0.5 mL of PBS. All samples were stored at -80°C until use.

137

138 2.6. Analyses of HT and alperujo samples

139 2.6.1. Extraction of HT and alperujo samples

140 Phenolic compounds were extracted from salivary, gastric and duodenal steps as follows: 0.3 mL
141 ethanol containing the internal standard was added to 0.2 mL of sample. The internal standards
142 were tyrosol and gallic acid for HT and alperujo samples, respectively, as gallic acid was not found
143 in the alperujo extracts [21]. n-Hexane (0.2 mL) was then added and the mixture was homogenized
144 for 10 min using a vortex blender at maximal speed. After centrifugation (2500 rpm for 10 min at
145 4°C), the lower phase was collected and the sediment further extracted with 0.3 mL ethanol and
146 additional vortexing for 10 min. The two ethanol phases were pooled, evaporated to dryness using
147 a Speed-Vac®, and the dried extracts dissolved in 200 µL H_2O and frozen at -80°C before analysis.
148 Apical media from cell culture experiments were directly injected into UHPLC-DAD-MS system
149 for analysis. Basolateral media (1900 µL) were primarily dried using a Speed-Vac® and the
150 residues dissolved in 80 µL H_2O.
151 The PBS fractions containing harvested cells (500 µL) were sonicated with 50 µL of internal
152 standard for 10 min at room temperature and centrifuged at 7000 rpm for 10 min at 4°C [22].
153 Supernatants were recovered and evaporated to dryness, then dissolved in 50 µL H_2O and frozen
154 at -80°C before analysis.

155

156 2.6.2. Chromatographic analysis

157 All extracts were analyzed by UHPLC-DAD-MS using an Acquity UPLC® system linked to both
158 a diode array detector and a Bruker Daltonics HCT Ultra Ion Trap mass spectrometer equipped
159 with an Electron Spray Ionization (ESI) source operating in negative mode. The separation was
160 performed on an Acquity C18 BEH column (50x2.1 mm i.d., 1.7 µm). The solvents were (A)

161 water/formic acid (99.5/0.5) and (B) acetonitrile. For alperujo analyses, the proportions of solvent
162 B used were: 0-10 min: 1-20%, 10-12 min: 20-30%, 12-14 min: 30-100%. The injection volume
163 was 1 µL for all samples and 10 µL for cells and basolateral extracts. The column temperature was
164 kept at 35°C. Along the 3 steps of the gradient, the flow rate was 0.30, 0.35 and 0.40 mL/min.
165 Chromatograms were acquired at 280 nm. The spectroscopic detection was performed in the range
166 200-800 nm with a resolution of 1.2 nm. HT concentrations were estimated from a calibration
167 curve (peak area vs. concentration) constructed with HT standard with R^2 values greater than 0.99.
168 Homovanillyl alcohol and homovanillyl alcohol glucuronide were quantified as HT equivalent [21].
169 For HT analyses, the same conditions were used and the flow rate was constant at 0.30 mL/min.
170 The proportions of B were: 0 – 2.4 min: 1-30%, 2.4 – 3 min: 30-100%.
171
172 2.6.3. Mass spectrometry
173 ESI mass spectra were obtained in the following conditions: ionization energy = 50 or 100 eV,
174 capillary voltage = 2 kV, source temperature = 365°C. The drying gas was introduced at a flow
175 rate of 10 L/min and the skimmer voltage was 40V. Scans were performed in the *m/z* range 100 –
176 2000.
177
178 2.6.4. Calculation and statistics
179 All the *in vitro* experiments were run in quadruplicate. Results were expressed as means and
180 standard deviations. Differences between means were assessed using ANOVA followed by the
181 post-hoc Tukey test for parametric data. P values under 0.05 were considered significant. The
182 bioavailability was assessed by the ratio between the amount of phenolic compounds in the
183 basolateral side and the initial amount added to the apical side or to the meal.
184

3 Results

3.1. HT bioaccessibility in the oral, gastric and duodenal compartments

The digestion of the HT in the 3 compartments was assessed with free HT and β-CD-bound HT (HTCD). The influence of the meal on HT bioaccessibility was also evaluated with the HTCD complex. Figure 2 shows the percentage of remained HT in each compartment. In the mouth compartment, β-CD seems to act as a protective agent for HT within the meal, HT recovery being 87.6 % (± 1.2) and 96.4 % (± 1.1) for HT and HTCD respectively ($p < 0.0001$). In the absence of food (HTCD-FF), β-CD provided a weaker but still significant protective effect. In the gastric compartment, HT recoveries in this step were 91.3 % (± 2.7), 94.9 % (± 1.9) and 97.7 % (± 2.7) for HT, HTCD and HTCD-FF, respectively. No significant benefit of β-CD was observed. Conversely, the presence of food had a negative effect on HT recovery (HTCD *vs* HTCD-FF, $p < 0.05$). Finally, except for the HTCD condition, the stability of HT was not significantly different in the duodenal compartment at pH 6 compared to the gastric one. The total apparent losses in the aqueous fractions were 37.2 % (± 1.0), 33.6 % (± 0.9) and 13.5 % (± 3.2) for HT, HTCD and HTCD-FF respectively. There was thus no difference regarding HT bioaccessibility between free HT and its β-CD complex. However, the absence of food significantly improved HT bioaccessibility compared to the other conditions ($p < 0.0001$).

Figure 3 presents the bioaccessibility of HT from an alperujo powder during the same digestion steps. There was no significant influence of β-CD on HT recovery from alperujo in the different compartments. As for pure HT, no degradation was observed in the mouth while an important loss was observed in the duodenal compartment. HT recovery from alperujo samples in the gastric compartment and in the aqueous phase decreased from 77.2 % (± 2.8) to 52.6 % (± 2.3) and from 76.4 % (± 2.7) to 50.3 % (± 1.2) for alperujo and alperujo-CD, respectively. Overall, HT recovery

208 from alperujo samples decreased along digestion ($p < 0.0001$). The stability of HT into the alperujo
209 samples appeared less important than for pure HT ($p < 0.001$).
210
211 3.2. HT absorption by Caco-2 TC7 cells
212 The absorption and metabolism of HT were studied using differentiated Caco-2 TC7 cell
213 monolayers. Bioavailability was determined as the quantity of targeted compounds in the
214 basolateral side *vs*. the initial amount added to the meal. The aqueous fractions obtained from the
215 precedent digestion studies were used to study HT absorption into the enterocytes after a 1/10 or
216 a 1/20 dilution for alperujo and HT samples, respectively. Thus, the cells received about 5.1-5.3,
217 6.0 and 6.7 µg per well of HT from meals containing HT, HTCD or alperujo and from HTCD-FF,
218 respectively. Chromatograms of the different fractions are presented on Figure 4A. UPLC-DAD-
219 MS analyses allowed to identify homovanillyl alcohol (HVA) as a O-methylether metabolite of
220 HT, giving a parent ion $[M-H]^-$ at *m/z* 167 and a fragment ion at *m/z* 153 (HT). Figure 4 (B, C, D,
221 E panels) shows the quantity of HT and HVA recovered at the apical and basolateral sides of the
222 cells. Each quantity was expressed as a percentage of the initial HT concentration at the apical
223 side. For all conditions, a significant decrease of the HT content was observed at the apical side
224 (more than 85%, $p > 0.05$) after 6h incubation. Concomitantly, a significant increasing quantity of
225 HT was recovered at the basolateral side (>40%). The curve profiles highlight the time-dependent
226 transport of HT from the apical to basolateral side. No significant difference was observed at the
227 basolateral side between the three HT samples. HVA was the only HT metabolite observed in our
228 conditions. It was mainly recovered at the apical side reaching about 32.8± 1.2%, 37.5± 4.1% and
229 40.3± 2.5% of the initial HT content for the HTCD-FF, HTCD and HT conditions, respectively.
230 The lower percentage of HVA that appeared in the aqueous fraction without meal was due to the
231 higher initial quantity of HT in this condition.

232 In the case of HTCD-FF, the initial HT amount was higher (+26%) compared to other conditions.
233 The final HT amounts at the basolateral side were about 2.2 µg for HT and HTCD and 2.9 µg for
234 HTCD-FF, respectively. Thus, the absorption of native HT from HTCD-FF increased over 30%
235 ($p < 0.0001$) compared to conditions containing foods. The transport of HT through the intestinal
236 barrier was also concentration-dependent. The same amount of HVA was approximately produced
237 whatever the initial HT concentration. The amount of native HT at the basolateral side reached
238 65.7± 2.1%, 63.9± 1.4% of the initial apical content for HT and HTCD, and 71.0± 0.8% for HTCD-
239 FF. The analysis of cell contents revealed neither HT nor HT metabolite: more than 99% of HT
240 and its metabolite were recovered in the apical and basolateral compartments.
241 The absorption of HT from alperujo samples by Caco-2 cells was also evaluated, as shown in
242 Figure 5. Chromatograms of the different fractions are presented on Figure 5A. The initial HT
243 amount was around 6 µg per well at the apical side of the cells. The general curve profile indicates
244 a time-dependent transport of HT. No significant difference regarding the amounts of both HT and
245 its metabolite was found between alperujo and alperujo-CD samples in all culture media over time.
246 About 0.88 ± 0.05 µg and 0.91 ± 0.04 µg of HT were recovered at the basolateral side for alperujo
247 and alperujo-CD conditions, respectively. So, 14.4 ± 0.8% and 15.0 ± 0.5% of the initial apical
248 HT amount from alperujo samples crossed the cell monolayers ($p > 0.05$). Despite the higher HT
249 load in the alperujo conditions (about 6 µg *vs* 5.1-5.3 µg for alperujo conditions, HT and HTCD),
250 the amount of HT absorbed was about 2.5-fold less important than for HT and HTCD conditions,
251 *i.e.* the absorption rate decreases by more than 60% ($p < 0.0001$). Homovanillyl alcohol
252 glucuronide (HVA-GlcU) was identified according to its molecular ion $[M-H]^-$ at *m/z* 343 and its
253 fragment ions $[M-H-GlcU]^-$ at *m/z* 167 (HVA) and 153 (HT), characteristic of the homovanillyl
254 moiety. This HT metabolite was only found in the basolateral compartment and was estimated at
255 6.7 ± 0.8% and 7.3± 0.9% of the initial apical HT amount for alperujo and alperujo-CD,

256 respectively. The total amount of unmetabolized HT at the basolateral side was 68.8 ± 1.5% and
257 69.8 ± 2.6 % for alperujo and alperujo-CD, respectively. So, β-CD had no significant effect on the
258 HT metabolism rate. In these conditions and conversely to the standard samples, a low amount of
259 HT was recovered into Caco-2 cells. Moreover, the total recovery of HT in these three
260 compartments exceeds 110 % after 6h of incubation ($p < 0.0001$). This may be explained by the
261 fact that a partial metabolization of other compounds from alperujo could generate HT.
262
263 **4 Discussion**
264
265 This work evaluated for the first time in a comprehensive manner the effect of the presence of β-
266 CD and/or foods on both HT bioaccessibility and HT uptake by intestinal cells.
267 The first step of HT digestion occurs in the mouth. Mastication favors interactions between the
268 phenolic compounds, food, saliva and dioxygen. In this compartment, the protective effect of β-
269 CD observed in our study may be linked to its ability to build a protective shell around HT, thereby
270 limiting its contact with potential food prooxidants such as iron species. In the stomach, food
271 disintegration intensifies due to the periodic and synchronized contractions of its wall, the acidic
272 environment and the enzymatic activity [23]. The *in vitro* digestion of HT standard confirmed that
273 HT is stable in the acidic conditions of the gastric compartment, which is in accordance with
274 previous data [24]. The recovery of HT after the gastric step was almost total, in agreement with
275 Pereira-Caro et al. who obtained a recovery rate higher than 99 % in their *in vitro* digestion study
276 without food [25]. In our work, the small loss (lower than 5%) in the gastric compartment may be
277 due to interactions between HT and the food matrix.
278 In our duodenal conditions, pure HT recovery did not significantly decrease. Several studies have
279 shown that HT was not stable in neutral or mildly alkaline conditions. In their work on the

digestion of phenolic compounds from olive oil, Soler et al. observed a loss of HT in alkaline conditions [26]. Corona et al. also observed that the amounts of HT and its derivatives progressively decreased during digestion by pancreatin (pH 7.5), reaching a total apparent loss of 20.3% after 2h for HT. This value took into account the formation of 3,4-dihydroxyphenylacetic acid (DOPAC) as a side-product of HT [27]. In our cases, no DOPAC formation was observed. We suggest that the total apparent losses in the aqueous fractions were the result of the partition of HT after the centrifugation step.

The comparative study of the HT standard and the HTCD sample showed a slight effect of β-CD on the final bioaccessibility of HT. β-CD is mainly used in the pharmaceutical industry to protect bioactive compounds and increase their water solubility and consequently their bioavailability [28]. As a cyclic starch derivative, β-CD may be partially hydrolyzed during digestion. However *in vivo*, β-CD only partly digested in the upper gastrointestinal tract and can reach the large intestine where it is metabolized by the microflora fermentation [29, 30]. Besides possible β-CD digestion, dilution is the major factor triggering the release of the guest compound from a CD complex [31]. This factor should be very important in our study because HT has only a weak affinity for β-CD (binding constant $< 10^2$ M^{-1} [17], data not shown). Overall, the bioaccessibility of HT from HTCD was not significantly increased compared to the free HT standard without β-CD.

In the absence of food, the bioaccessibility of HT was increased by almost 20% compared to the same sample in the presence of food. Many macromolecular food components can bind phenolic compounds and retain them within the food matrix. In this work, potato is a source of starch, which is known to retain phenolic compounds [32]. Similarly, beef is rich in proteins, which have a general affinity for phenols [33, 34]. The clear influence of the food matrix on the bioaccessibility of dietary plant phenols such as HT outlines the importance of including real meal components in *in vitro* digestion studies.

304 The study with alperujo samples was carried out in the presence of food. In each compartment,
305 HT from alperujo samples was generally less bioaccessible than from free standard. Indeed,
306 although alperujo samples are protein-free, they contain fibers and sugars, which could interact
307 with the meal components and the digestive enzymes (possibly slowing down protein and starch
308 digestion), thereby reducing the bioaccessibility of phenolic compounds [2]. The apparent loss of
309 HT bioaccessibility from alperujo powders (compared to the standard) was also higher (+10%).
310 Again, no significant effect of β-CD was observed.
311 Caco-2 TC7 cells were then chosen as a suitable model to follow the absorption of target
312 compounds through the intestinal barrier. HT was brought to the cells as an aqueous fraction
313 obtained from our previous digestion study. The study of HT and its β-CD complex within a meal
314 revealed that HT was largely absorbed through Caco-2 cells and partly metabolized into
315 homovanillyl alcohol due to the catechol-O-methyltransferase (COMT) activity of the enterocytes.
316 Most of the HT recovered at the basolateral side was unmetabolized (over 60%) and HVA was
317 recovered in the two culture media, especially in the apical compartment. These data are in
318 agreement with previous results. Indeed, Manna et al. (2000) observed that 25% of HT reached
319 the basolateral side of Caco-2 cells after 1h, 90% of which being unmetabolized. They also
320 identified HVA as a metabolite. They determined that HT reaches the basolateral side through
321 passive diffusion and that this transport was time- and concentration-dependent [35]. Corona et al.
322 also observed 90% of unmetabolized HT at the basolateral side of the cells after the phenolic
323 compounds were added to the apical side from a standard buffered solution [27]. Finally, Mateos et
324 al. observed that 59% of HT from apical side (initial concentration = 50 μM) reached the
325 basolateral side after 4h of incubation, with almost 20% recovered as O-methylether and 80% as
326 unmetabolized HT [36]. In our study, for HT, HTCD and HTCD-FF conditions, the transport of
327 unmetabolized HT from the apical to the basolateral side of Caco-2 cells ranged from 25.4 ± 1.5%

328 to 42.3 ± 0.8% over 2 to 6h. The difference regarding HT absorption rates in our study compared
329 to previous data is likely explained by the fact that HT was just dissolved in an aqueous buffer
330 (HBSS or PBS) in previous studies, while we used a more complex mixture obtained from *in vitro*
331 digestion.
332 In the case of HT from alperujo powders, we observed the appearance of HVA-GlcU as a HT
333 metabolite. Moreover, the transport of HT from the apical to basolateral side ranged from 8.4 ±
334 0.6% to 14.7± 0.5% over 2 to 6h. This lower transport rates compared to HT standard conditions
335 can be the result of a competition between HT and other alperujo components to cross the intestinal
336 cells. The same phenomenon was observed when comparing the absorption of pure diosmetin and
337 diosmetin from a rosemary extract [37]. Interestingly, a small amount of HT from alperujo was
338 recovered into the harvested Caco-2 cells and the total recovery of HT exceeded 100%. If
339 metabolized, HT-glucoside that is also present in alperujo extract could be a source of HT, which
340 would explain this result.
341 Finally, it is interesting to compare two methods to calculate the *in vitro* bioavailability of HT, by
342 making a ratio with either the initial HT apical content during the absorption experiments (Table
343 1) or the initial HT amount brought via the meal (Table 2). The second method allows to correct
344 the common overestimation of the *in vitro* bioavailability when considering uniquely the
345 absorption step and not the whole digestion process. The second values were decreased by more
346 than 25% and 50% for HT standard and alperujo samples, respectively. This analysis confirmed
347 that HT was more bioavailable when it was brought as a pure standard form than as a plant extract.
348 The absence of food also participated in increasing the final HT bioavailability. HT bioavailability
349 from the alperujo samples was lower, likely because of possible interactions between HT and other
350 alperujo components and/or competition between them for absorption by Caco-2 cells [26].
351

In summary β-CD, which can be used to enhance phenol stability during storage, did not modified the bioaccessibility and the bioavailability of HT from alperujo, in the presence or in the absence of foods. It would be interesting to extend this result to modified β-CDs such as 2-hydroxypropyl-β-CD, which can also be used to complex bioactive compounds [15]. Conversely to β-CD, interactions with food components (probably potato starch and beef proteins) were shown to decrease HT bioaccessibility. These interactions had a strong impact on HT final bioavailability, the HT amount absorbed by the intestinal cells being strongly dependent on the bioaccessible HT content. Besides, HT was more bioaccessible and better absorbed by enterocytes from a pure form than from an alperujo powder, in which it likely competes with other phenolic compounds at different steps of the digestion-absorption process. HVA was the only metabolite observed when HT was from a pure standard and HVA-GlcU was detected when HT was provided via alperujo powders. The low bioavailability of HT reflected its high metabolization in the intestine.

Our data have dietary significance as plant phenol supplements are usually consumed within a meal and under the form of complex mixtures, rather than individual supplements taken at fast.

The authors have declared no conflicts of interest.

Abbreviations: β-CD, β-cyclodextrin; **HT,** hydroxytyrosol; **HTCD**: HT:β-CD inclusion complex; **HTCD-FF**: HT:β-CD inclusion complex-food free condition; **HVA**: homovanillyl alcohol; **HVA-GlcU**: homovanillyl alcohol glucuronide

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380

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Tables

Table 1. Bioavailability of phenolic compounds as percentage of HT initial apical amount.

Samples	HT	HVA	HVA-GlcU
HT	42.6 ± 2.5	22.2 ± 2.5	
HTCD	41.4 ± 3.0	23.4 ± 2.0	
HTCD-FF	43.0 ± 0.7	17.5 ± 0.6	
Alperujo	14.4 ± 0.8		6.7 ± 0.8
Alperujo-CD	15.0 ± 0.5		7.3 ± 0.9

Values are expressed as mean ± SD of quadruplicate measurements.

Table 2. Bioavailability of the phenolic compounds as percentage of HT initial amount in the test meal.

Samples	HT	HVA	HVA-GlcU
HT	31.1 ± 1.1	16.2 ± 1.3	
HTCD	30.9 ± 1.8	17.5 ± 1.4	
HTCD-FF	40.9 ± 1.5	16.7 ± 0.6	
Alperujo	6.9 ± 0.4		3.2 ± 0.3
Alperujo-CD	7.3 ± 0.3		3.6 ± 0.4

Values are expressed as mean ± SD of quadruplicate measurements.

Figure legends

Figure 1. In vitro digestion procedure

HT = hydroxytyrosol, β-CD = β-cyclodextrin.

Figure 2. Bioaccessibility of hydroxytyrosol from standard powder in each digestive compartment

Samples were taken at the beginning of the experiment (T0) and at the end of the oral, gastric and duodenal digestion steps.

HT = hydroxytyrosol standard added to a meal, HTCD = HT-β-cyclodextrine complex added to a meal, HTCD-FF = HTCD complex without food. Values are expressed as mean ± SD of quadruplicate measurements. Different letters indicate a significant difference according to Tukey test ($p \leq 0.05$) between all conditions for each compartment. Different symbols indicate a significant difference according to Tukey test ($p \leq 0.05$) between all compartments for each condition.

Figure 3. Bioaccessibility of hydroxytyrosol from alperujo powder in each digestive compartment

Samples were taken at the beginning of the experiment (T0) and at the end of the oral, gastric and duodenal digestion steps.

Alperujo = hydroxytyrosol from alperujo. Alperujo-CD = hydroxytyrosol from alperujo phenolic compound - β-cyclodextrine complex. Values are expressed as mean ± SD of quadruplicate measurements. Different letters indicate a significant difference according to Tukey test ($p \leq 0.05$) between all conditions for each compartment. Different symbols indicate a significant difference according to Tukey test ($p \leq 0.05$) between all compartments for each condition.

Figure 4. Absorption and metabolism by Caco-2TC7 cells of hydroxytyrosol from standard samples

The aqueous fractions obtained from *in vitro* digestion of pure hydroxytyrosol (HT) samples were added to the apical side of cell monolayers. HT and its metabolites were monitored over time.

(A) UHPLC chromatograms of cells culture media: (a) Apical media at T=0h; (b) Apical media at t= 6h; (c) Basolateral media at 6h. HVA = homovanillyl alcohol, IS = internal standard.

(B) Quantity of HT at the apical side; (C) Quantity of HT at the basolateral side; (D) Quantity of HVA at the apical side; (E) Quantity of HVA at the basolateral side. All results are expressed in percent of the initial HT amount at the apical side.

(◆) HT meal; (●) HTCD meal; (▲) HTCD-FF. Values are expressed as mean ± SD of quadruplicate measurements.

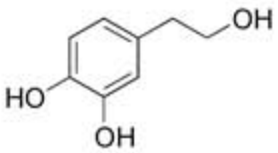
Figure 5. Absorption and metabolism by Caco-2 TC7 cells of hydroxytyrosol from alperujo samples

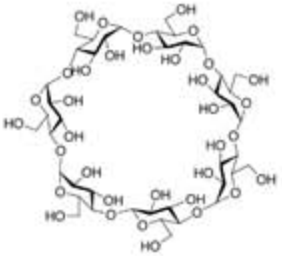
The aqueous fractions obtained from *in vitro* digestion of alperujo samples were added to the apical side of the cell monolayers. Hydroxytyrosol (HT) and its metabolites were monitored over time.

(A) UHPLC chromatograms of cells culture media: (a) Apical media at T=0h; (b) Cell monolayer content at t=6h; (c) Basolateral media at 6h. HVA-GlcU: homovanillyl alcohol glucuronide, IS = internal standard.

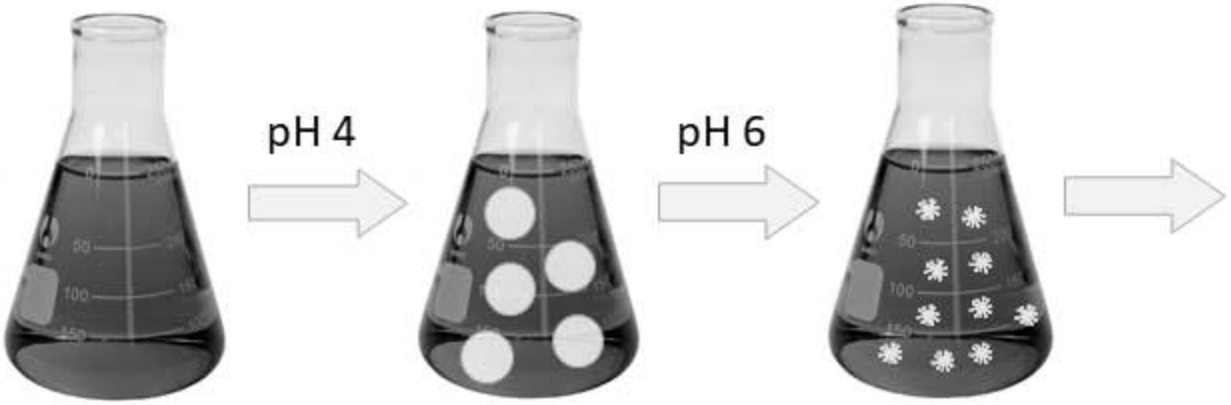
(B) Quantity of HT at the apical side; (C) Quantity of HT and homovanillyl alcohol glucuronide (HVA-GlcU) at the basolateral side; (D) Quantity of HT in the cytosolic compartment of Caco- 2 TC7 cells. All results are expressed in percent of the initial HT amount at the apical side. (●) HT from alperujo, (◆) HT from alperujo-CD; (▲) HVA-GlcU from alperujo (■) HVA-GlcU from alperujo-CD. Values are expressed as mean ± SD of quadruplicate measurements.

Figure 1





Test meal and/or **pure HT** or **HT from alperujo** complexed or not with **β-CD**



Isolation of the aqueous phase from the digesta

Oral phase
NaCl solution + artificial saliva
10 min under agitation at 37°C

Gastric phase
+ pepsine
30 min under agitation at 37°C

Duodenal phase
+ bile
+ pancreatine
30 min under agitation at 37°C

Figure 2

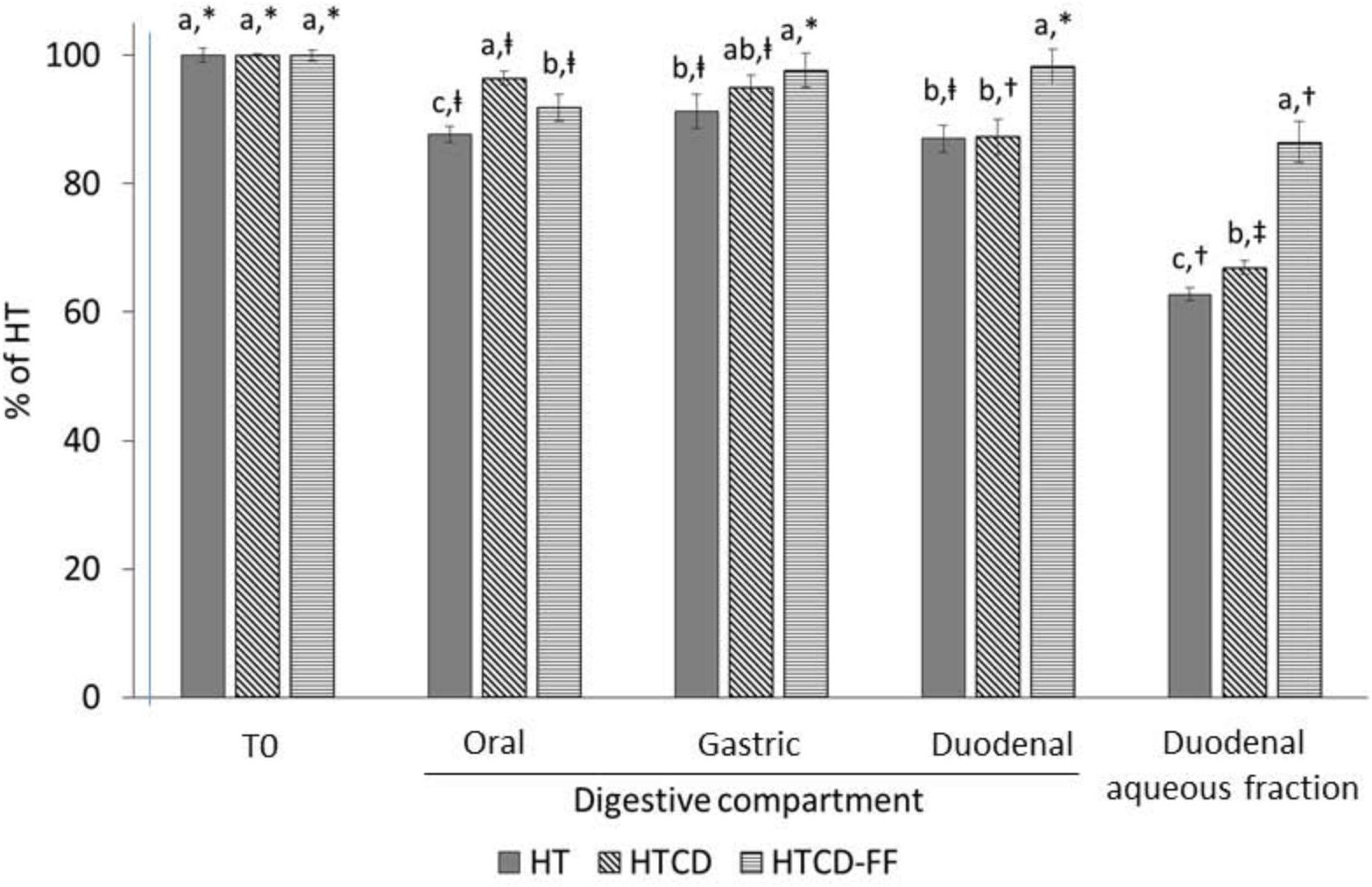
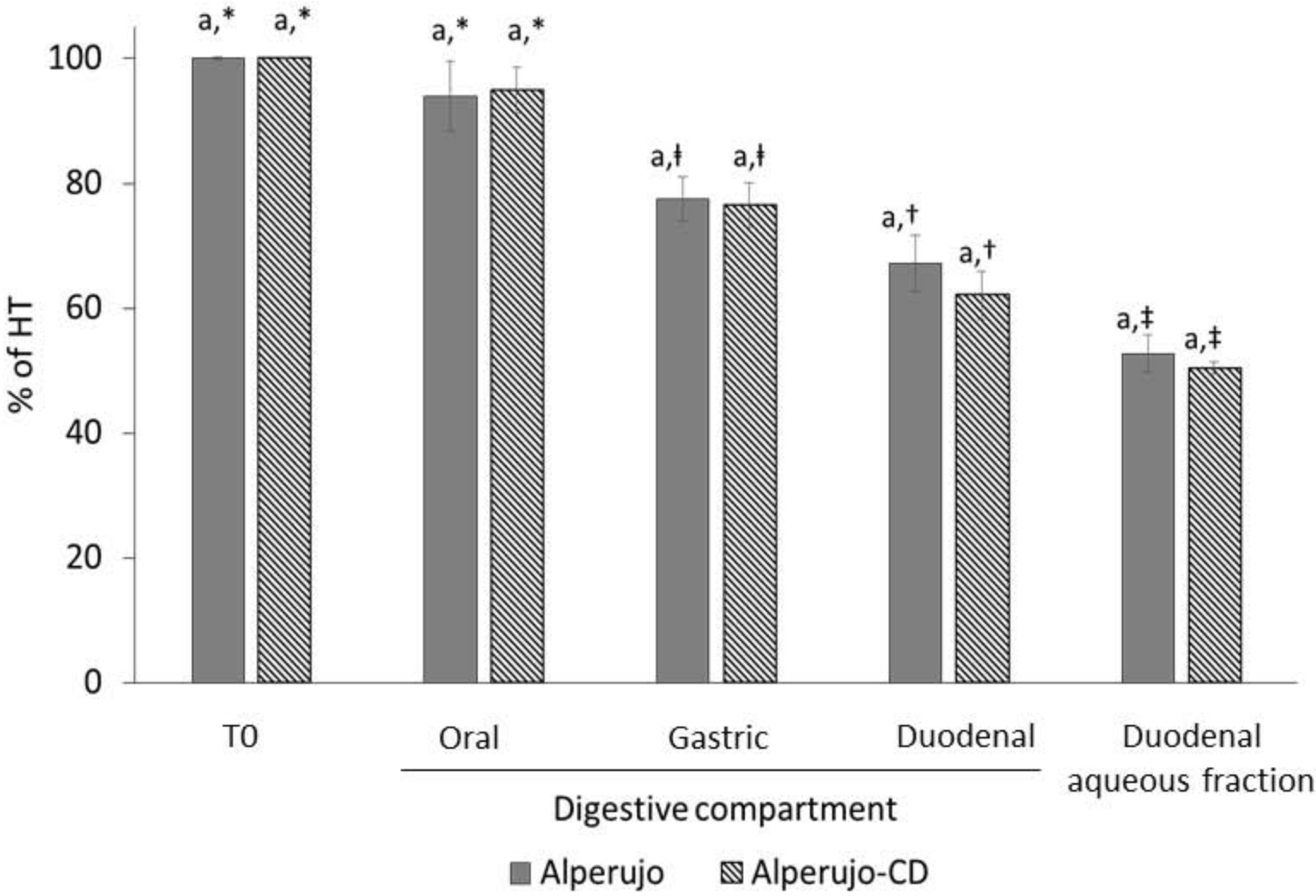

a,* a,* a,*
c,‡ a,‡ b,‡
b,‡ ab,‡ a,*
b,‡ b,† a,*
c,† b,‡ a,†
100
80
60
40
20
0
% of HT
T0
Oral
Gastric
Duodenal
Duodenal aqueous fraction
Digestive compartment
HT
HTCD
HTCD-FF

Figure 3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% of HT
T0
Oral
Gastric
Duodenal
Duodenal aqueous fraction
Digestive compartment
Alperujo
Alperujo-CD

Figure 4

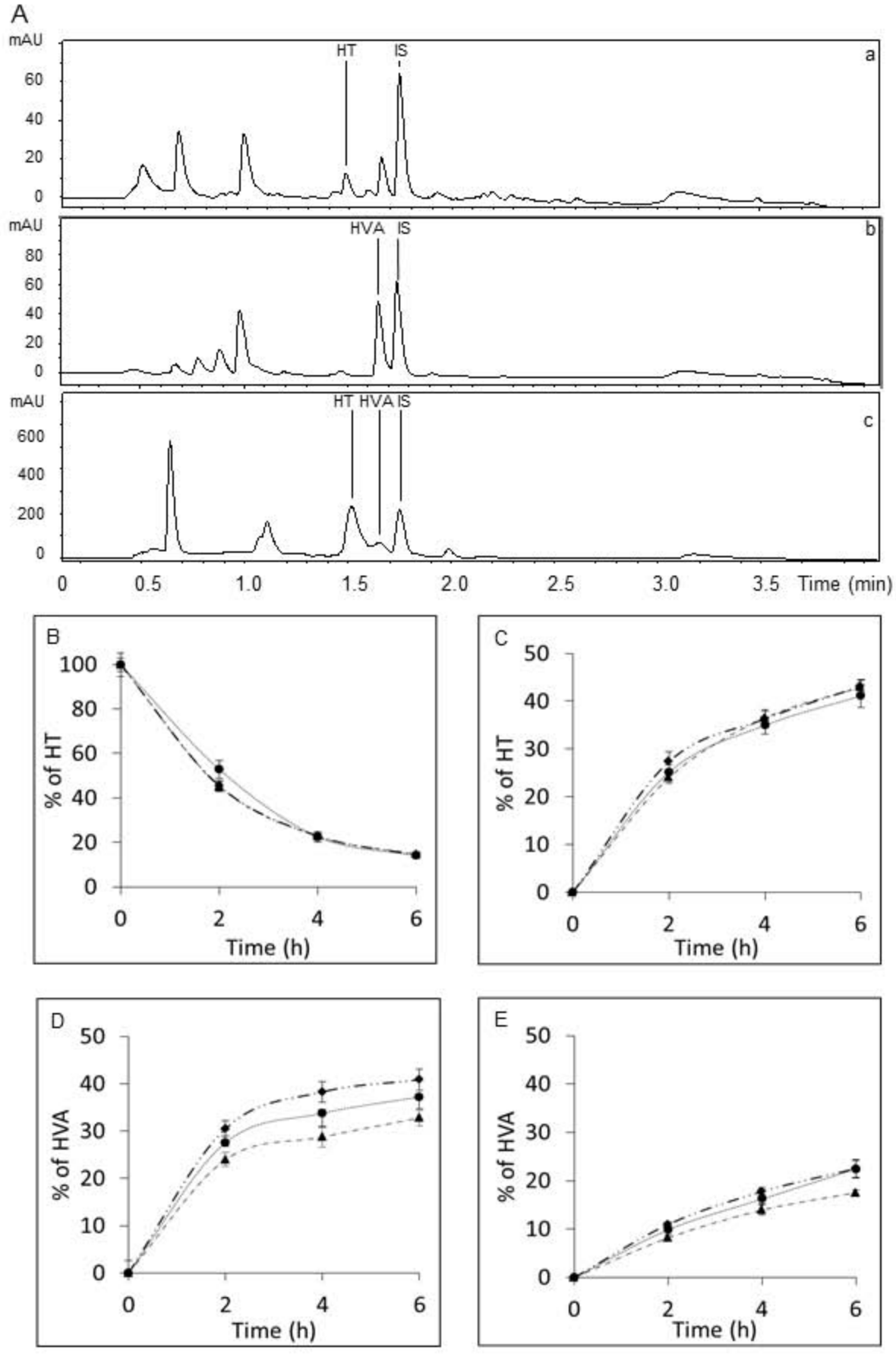
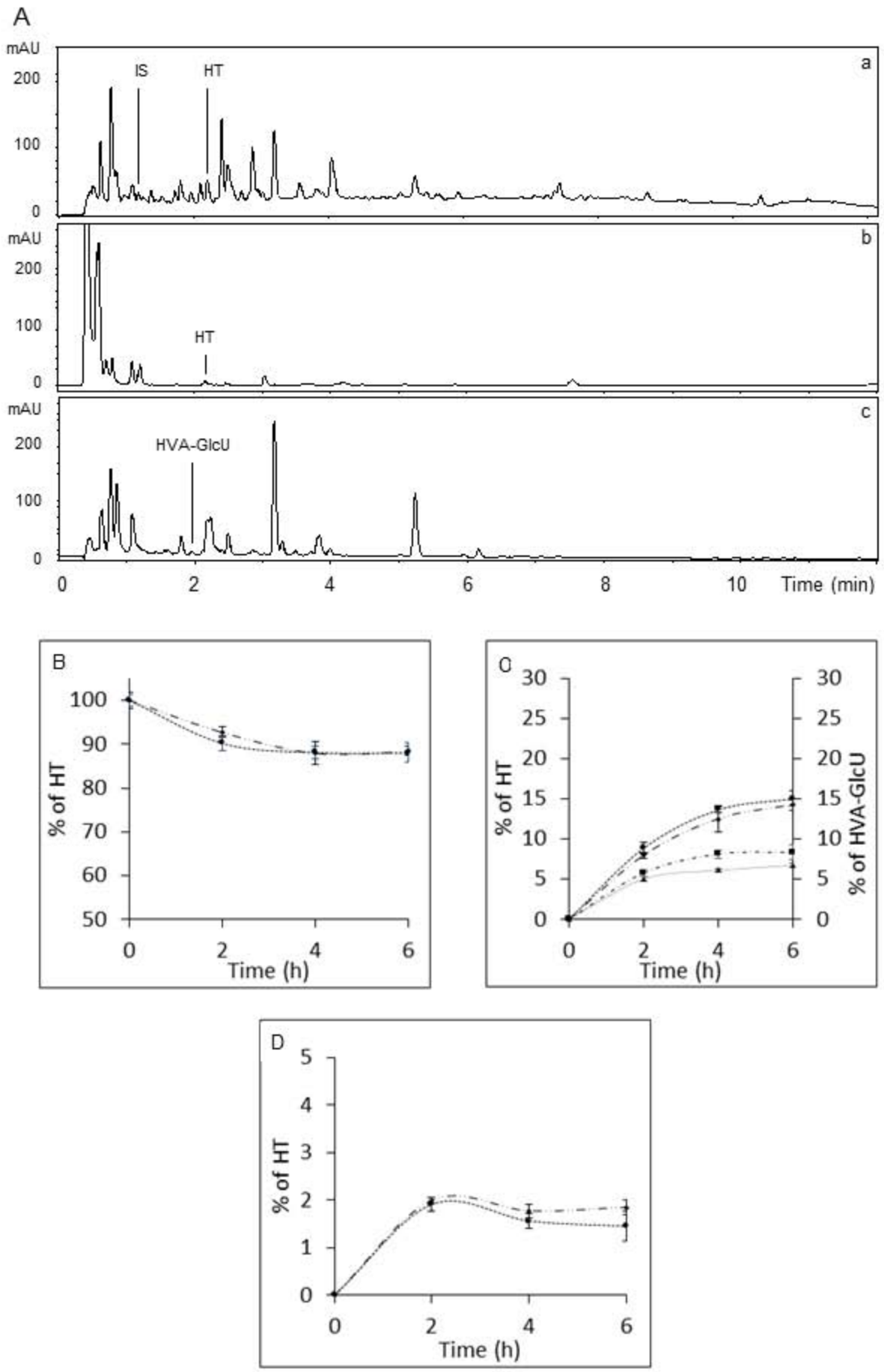


Figure 5


A
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100
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IS
HT
a
b
c
HVA-GlcU
0
2
4
6
8
10
Time (min)
B
% of HT
100
90
80
70
60
50
Time (h)
C
% of HT
% of HVA-GlcU
30
25
20
15
10
5
0
Time (h)
D
% of HT
5
4
3
2
1
0
Time (h)