

1 **In vitro digestion and lactase treatment influence uptake of quercetin and quercetin**
2 **glucoside by the Caco-2 cell monolayer**

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21

21 **ABSTRACT**

22 **Background**

23 Quercetin and quercetin glycosides are widely consumed flavonoids found in many fruits
24 and vegetables. These compounds have a wide range of potential health benefits, and
25 understanding the bioavailability of flavonoids from foods is becoming increasingly
26 important.

27 **Methods**

28 This study combined an in vitro digestion, a lactase treatment and the Caco-2 cell model
29 to examine quercetin and quercetin glucoside uptake from shallot homogenates.

30 **Results**

31 The in vitro digestion alone significantly decreased quercetin aglycone recovery from the
32 shallot digestate ($p < 0.05$), but had no significant effect on quercetin-3-glucoside
33 recovery ($p > 0.05$). Digestion increased the Caco-2 cell uptake of shallot quercetin-4'-
34 glucoside by 2-fold when compared to the non-digested shallot. Despite the loss of
35 quercetin from the digested shallot, the bioavailability of quercetin aglycone to the Caco-
36 2 cells was the same in both the digested and non-digested shallot. Treatment with lactase
37 increased quercetin recovery from the shallot digestate nearly 10-fold and decreased
38 quercetin-4'-glucoside recovery by more than 100-fold ($p < 0.05$), but had no effect on
39 quercetin recovery from apple digestates. Lactase treatment also increased shallot
40 quercetin bioavailability to the Caco-2 cells approximately 14-fold, and decreased shallot
41 quercetin-4'-glucoside bioavailability 23-fold ($p < 0.05$). These Caco-2 cells had lactase
42 activity similar to that expressed by a lactose intolerant human.

43 **Conclusions**

44 The increase in quercetin uptake following treatment with lactase suggests that dietary
45 supplementation with lactase may increase quercetin bioavailability in lactose intolerant
46 humans. Combining the digestion, the lactase treatment and the Caco-2 cell culture

47 model may provide a reliable in vitro model for examining flavonoid glucoside
48 bioavailability from foods.
49

49 **Background**

50 Cardiovascular disease and cancer are the two most common causes of death in the
51 United States and most industrialized nations. A diet high in fruits and vegetables has
52 been correlated with a reduced risk for both cancer and heart disease [1, 2]. It is thought
53 that the phytochemicals found in fruits and vegetables may be responsible in part for
54 these health benefits [3]. Phytochemicals from fruits and vegetables may inhibit cell
55 proliferation, protect against oxidative stress, influence cell-signaling pathways, and
56 reduce inflammation. Because these compounds appear to have such beneficial effects,
57 interest has been raised in examining the bioavailability of these compounds.

58

59 A phytochemical of particular interest is quercetin, a strong antioxidant that is widely
60 consumed in many fruits and vegetables. Quercetin has potential protective effects
61 against both cancer and heart disease. Briefly, quercetin has been found to down regulate
62 expression of mutant p53 in breast cancer cells, arrest human leukemic T-cells in G1,
63 inhibit tyrosine kinase, and inhibit heat shock proteins [4]. Quercetin has been shown to
64 decrease lipid peroxidation, inhibit cell proliferation, induce apoptosis, and inhibit
65 platelet aggregation [5-8]. Because quercetin exhibits such a wide array of positive
66 health effects, it is especially important to understand quercetin bioavailability from
67 whole foods. Two widely consumed, good food sources of quercetin are apples and
68 onions [9-13]. In most foods, quercetin does not exist in the aglycon form, but is,
69 instead, conjugated. The type of sugar moiety to which quercetin is bound affects
70 quercetin bioavailability. For example, quercetin in the apple is bound mainly to
71 galactosides, rhamnosides, and arabinosides, and these quercetin conjugates are not well
72 absorbed by the small intestine. The onion contains mainly quercetin glucosides, which
73 are well absorbed by the small intestine [14].

74

75 More work is needed to understand the bioavailability of quercetin and other flavonoids
76 from foods. The Caco-2 cell culture model is a well-established *in vitro* technique,
77 extensively used to study intestinal cell absorption of compounds such as
78 pharmaceuticals and nutrients; it is an excellent *in vitro* tool to study bioavailability of
79 specific compounds. We have previously used the Caco-2 cell culture model to examine
80 the uptake of quercetin from apple and onion extracts (Boyer, 2004). Using this model,
81 we found that absorbed quercetin from onion extracts was significantly greater than from
82 apple extracts, as expected (Boyer, 2004). Others have used the Caco-2 cell culture
83 model to evaluate cell transport and/or accumulation of pure phytochemicals such as
84 quercetin, quercetin glucosides, chrysin, flavone, epicatechin, proanthocyanidin, and
85 carotenoids [15-20, 21, Liu, et al., 2004].

86

87 To further understand quercetin bioavailability, it is important to examine the effects of
88 digestion on foods prior to intestinal uptake. An *in vitro* digestion has been paired
89 successfully with the Caco-2 cell culture model to study iron and carotenoid
90 bioavailability [22, 23]. Use of the *in vitro* digestion with the Caco-2 cell culture model
91 could be quite useful in more specifically analyzing quercetin bioavailability from foods.
92 At this time there is little information available describing the effects of digestion on
93 flavonoids from foods. Vallejo et al. [24] found that over 80% of total flavonoids were
94 lost during an *in vitro* digestion of broccoli. In a study of healthy subjects, Walle et al.
95 (2000) estimated that the intestine might absorb 65-81% of major forms of dietary
96 flavonoids after enzymatic hydrolysis [25].

97

98 A good *in vitro* model would aid in evaluating bioavailability of phytochemicals from
99 foods by offering a simple method to screen for factors that may affect intestinal
100 absorption of quercetin and quercetin glucosides, such as the food matrix, food
101 processing, digestion, and interactions with other foods. Human and animal models can

102 be expensive and time consuming, while a cell culture model allows for rapid,
103 inexpensive screenings. The Caco-2 model has the potential to be a good model to
104 measure quercetin absorption, however there are some drawbacks. Caco-2 cells have
105 been shown to express significantly less lactase phlorizin hydrolase (LPH) than the
106 average human small intestine. Since this enzyme is most likely responsible for the first
107 step in the metabolism of quercetin glucosides [26], this deficiency would clearly limit
108 the ability of the Caco-2 cells to ability to metabolize and absorb quercetin from
109 quercetin glucosides.

110

111 Caco-2 cells used in our lab have expressed greater LPH activity (3 mU/mg protein) than
112 other Caco-2 cells (0.3 mU/mg protein) (Boyer et al., 2004), resulting in lactase activity
113 similar to that expressed by enterocytes from a lactose intolerant human (2-10 mU/mg
114 protein)[27]. The compound forskolin induced lactase phlorizin hydrolase activity
115 fourfold in Caco-2 cells [28]. In weanling rats, lactose consumption increased lactase
116 activity in the jejunum by threefold [29]. Thus we hypothesized that treating Caco-2
117 cells with either forskolin or lactase may raise lactase expression to rates comparable to
118 humans, making the Caco-2 cell model a more valid model for screening quercetin
119 glucoside bioavailability from food. If lactase activity cannot be induced in Caco-2 cells,
120 treating food samples with lactase following the digestion procedure and prior to cell
121 bioavailability assays may give more comparable results to humans.

122

123 The objectives of this study were (1) to develop an optimized in vitro digestion method
124 for examining quercetin and quercetin glucoside recovery from digestates using onions
125 and apples (2) to examine the effect of lactase on shallot digestates and (3) to examine
126 Caco-2 cellular uptake of quercetin and quercetin glucosides from digested and lactase
127 treated shallot.

128

129 **Methods**

130 **Chemicals and materials:**

131 Shallots (Lake Ontario variety) and onions (New York Bold variety) were obtained from
132 a local grocery store. Apples (Red Delicious and Cortland varieties) were obtained from
133 the Cornell Orchards (Cornell University, Ithaca, NY). Porcine pepsin, bile extract,
134 pancreatin, lactase (beta-galactosidase, from *Kluyveromyces lactis*, activity of 3000
135 units/mL), quercetin, and quercetin-4'-glucoside were purchased from Sigma Chemical
136 Company (St. Louis, MO). Quercetin-3-glucoside was purchased from Indofine
137 Chemical Company, Inc (Hillsborough, NJ). Caco-2 cells were obtained from the
138 American Type Culture Collection (Rockville, MD) and were cultured in Dulbecco's
139 Modified Eagle Medium (DMEM; Gibco Life Technologies, Grand Island, NY)
140 supplemented with 5% fetal bovine serum (Gibco Life Technologies, NY), 10 mM
141 HEPES, 50 units/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL gentamicin and
142 were maintained at 37° C in 5% CO₂.

143

144 **In vitro digestion**

145 Two hundred grams of each food sample were chopped, blended for 5 min with 200 mL
146 saline (140 mM NaCl, 5 mM KCl) using a Waring blender, and then homogenized using
147 a Virtis 45 homogenizer. The total homogenates were aliquotted in 15 mL centrifuge
148 tubes and stored at -20° C until use.

149

150 For the digestion treatment, 2 g aliquots of the food sample were placed in a centrifuge
151 tube with an equal amount of saline. The pH was decreased to 2.0 by drop-wise addition
152 of 1M HCl, and porcine pepsin was added to a final concentration of 1.3 mg/mL. The
153 digestate was incubated in a shaking water bath at 37° C for 30 minutes. The pH of the
154 digestate was then increased to 5.8 with the drop-wise addition of 1M NaHCO₃. Porcine
155 bile extract and pancreatin were added to a final concentration of 1.1 and 0.175 mg/mL,

156 respectively. The pH was increased to 6.5 by drop-wise addition of 1M NaHCO₃, and the
157 samples were incubated for 1 hour in a water bath at 37° C. Following digestion the pH
158 was decreased to 2 by addition of HCl and the digestates were stored at -80° C for further
159 analysis.

160

161 To examine and optimize the effects of digestion time and pH on the recovery of
162 compounds from the digestate, the above parameters were varied. To examine the effects
163 of pepsin digestion time, the pepsin digestates were incubated for 0, 30, 60 or 90 minutes
164 and then incubated with the intestinal digestion enzymes for 60 minutes. To examine the
165 effects of intestinal digestion, the samples were incubated with pepsin for 30 minutes,
166 then incubated with pancreatin and bile for 0, 30, 60, or 90 minutes.

167

168 The effects of 100 µM ascorbic acid and a nitrogen environment on quercetin and
169 quercetin glucoside recovery from onion and apple digestates were examined.

170 Following homogenization and prior to digestion, the food samples were mixed 1:1 with
171 saline containing 200 µM ascorbic acid, leaving a final sample concentration of 100 µM
172 ascorbic acid. During the digestion procedure described above, the samples were flushed
173 constantly with nitrogen.

174

175 The effect of pH of either 6.5 or 7.0 during intestinal digestion on quercetin and
176 quercetin-3-glucoside recovery were compared. The effect of the storage pH was
177 examined by comparing digested samples having either a final pH of 2.0 or 6.5. The
178 effect of storage pH on 20 µM pure quercetin and 20 µM quercetin-3-glucoside was
179 examined by comparing recoveries from samples stored at pH = 2.0, 3.5, 5.0 or 7.0. All
180 samples were stored overnight at -80° C. Prior to HPLC analysis samples were thawed
181 and extracted 4 times with acidified ethyl acetate (pH 2.0), evaporated to dryness and
182 reconstituted in 2 mL acidified methanol (pH 2.0).

183

184 **Lactase digestion**

185 Doses of lactase (0.5 units-3000 units per gram sample) were applied to 1 gram shallot
186 extract and incubated for 15 minutes at 37° C. The final pH was brought to 2.0 and the
187 samples were stored at -80° C. The time kinetics were examined by incubating 1 gram
188 shallot extract with 100 units of lactase for 0, 15, 30, 60, 90, 120, 240, and 720 minutes.

189

190 The effect of both lactase and digestion were examined by digesting the samples with
191 pepsin and pancreatin as described above, then incubating the samples with 100 units of
192 lactase for 30 minutes at 37° C. Samples were stored overnight at -80° C.

193

194 Prior to HPLC analysis, all digestate samples were thawed and extracted 4 times with
195 acidified ethyl acetate (pH 2.0), evaporated to dryness and reconstituted in 2 mL
196 methanol.

197

198 **Uptake of quercetin-4'-glucoside and quercetin from shallot digestates by Caco-2**
199 **cells**

200 Caco-2 cells were seeded at a density of 5×10^5 cells per well in a collagen coated 6-well,
201 flat bottom plate and incubated at 37° C in a 5% CO₂ environment. Caco-2 cells were
202 used between passages #10-25, and the cells reached confluency approximately 5 days
203 post seeding. Culture media was changed three times a week. On day 14 post seeding,
204 the DMEM was removed and the cells were rinsed three times with phosphate buffered
205 saline (PBS).

206

207 Shallot homogenates were digested as previously described and were placed directly on
208 the 14 day old Caco-2 cells, or the samples were diluted 1:2 or 1:4 in HBSS (Hank's
209 Balanced Salt Solution). Cells were also incubated with non-digested shallot

210 homogenates for comparison. For each treatment, two wells of cells were used for each
211 sample, and each treatment was repeated in triplicate. To examine the effect of lactase
212 on quercetin uptake from shallots, shallots were digested then incubated with lactase (50,
213 100, 300, and 1000 units/g shallot) for 20 minutes at 37° C. The digested shallot
214 homogenates and the digested plus lactase treated shallot homogenates were diluted 1:2
215 in HBSS and placed on the cells. In all experiments, Caco-2 cells were incubated with
216 treatment for 30 minutes at 37° C in 5% CO₂. The shallot treatment and HBSS was
217 removed and the cells were rinsed three times with 20% methanol in PBS. Cells were
218 scraped in acidified methanol (pH= 2.0) and the wells were rinsed three times with
219 methanol. The scraped cells were sonicated for 15 minutes, centrifuged at 1600 g for 5
220 minutes, and the methanol supernatant was collected. The cells were rinsed three more
221 times with methanol, the supernatants were collected and the methanol extracts were
222 evaporated to dryness under nitrogen and reconstituted in 400 µl acidified methanol for
223 HPLC analysis.

224

225 **Induction of lactase activity in Caco-2 cells**

226 Caco-2 cells were seeded at a density of 5×10^5 cells per well in a 6-well flat bottom
227 plate. The cells were cultured in DMEM spiked with different doses of either lactose (10,
228 50, 100, 500, and 1000 µM), or forskolin (1, 10, 50, 100, and 200 µM). Media was
229 changed every two days, and cells were harvested and lactase activity measured at 14
230 days post-seeding. Lactase activity of Caco-2 cells was measured using a method
231 adapted from Dahlqvist [30]. Cells were trypsinized, collected, centrifuged and
232 resuspended in homogenization buffer (50 mM sodium phosphate; 1 mM EDTA; 10 mM
233 DTT; protease inhibitor cocktail, Sigma Chemical Co., St. Louis, MO). Cells were
234 homogenized 5 times for 30 seconds with 1 minute of cooling between bursts using a
235 benchtop homogenizer. Homogenates were treated with 56 mM lactose and incubated at
236 37° C for 60 minutes. Glucose oxidase, peroxidase, and o-dianisidine were applied to

237 the cell homogenates and the final colored products were measured at 420 nm using a
238 spectrophotometer [30]. The results were compared to a glucose standard curve to
239 determine the amount of glucose released by lactase in the Caco-2 cell monolayer.
240 Protein was determined from crude cell homogenates colorimetrically using the Biuret
241 reagent and Folin/Ciocalteu's phenol reagent with comparisons to a bovine serum
242 albumin standard curve. Results are expressed as milliunits/mg of protein, and one unit is
243 defined as the lactase activity that hydrolyzes 1 μ mole of lactose per minute at 37 °C.

244

245 **HPLC analysis**

246 Quercetin and quercetin-3-glucoside content of untreated homogenates, digestates, and
247 Caco-2 cell extracts were determined using an RP-HPLC procedure with a Supelcosil
248 LC-18-DB column (150 mm x 4.6 mm, and 3 μ m pore size). A Waters 515 HPLC pump
249 (Waters Corp., Milford, MA) and a Waters 2487 dual wavelength absorbance detector
250 (Waters Corp., Milford, MA) set at 370 nm were used for all HPLC analysis. Quercetin,
251 quercetin-3-glucoside, and quercetin-4'-glucoside were used as standards. For quercetin,
252 quercetin-3-glucoside, quercetin-4'-glucoside apple peel extract shallot extract, and
253 digestate extract analyses, the solvent system used was (A) acidified water (pH 2.0;
254 trifluoroacetic acid) and (B) acetonitrile. The gradient method was the following: 0.0
255 min, flow rate = 1.4, (A) 90% and (B) 10%; 53 min, flow rate = 1.5, (A) 80% and (B)
256 20%; 58 min, flow rate = 1.7, (A) 65% and (B) 35%; 64 min, flow rate = 1.4, (A) 90%
257 and (B) 10%. Twenty μ l injections were made for each sample. Quercetin, quercetin-3-
258 glucoside, and quercetin-4'-glucoside concentrations in the apple peel extract, shallot
259 extract, and in the digestates were extrapolated from the pure quercetin and quercetin-3-
260 glucoside standard curves.

261

262 **Statistical analysis**

263 All data were reported as means \pm SD for three replicates of each treatment. An analysis
264 of variance (ANOVA) was used to compare results between treatment groups, and
265 pairwise multiple comparisons were performed using Fisher's LSD with an individual
266 error rate of 0.05. The statistical analysis was completed using Minitab Release 12
267 software (State College, PA).

268

269 **Results**

270 **In vitro gastrointestinal digestion**

271 The total pepsin digestion time and pancreatin/bile digestion time had little to no effect
272 on recovery of both quercetin and quercetin-3-glucoside from apple and onion
273 homogenates when treated for up to 60 minutes (data not shown). After 90 minutes of
274 pepsin digestion and 90 minutes of pancreatic digestion, quercetin and quercetin 3-
275 glucoside in both the apple and the onion decreased slightly. Based on these results we
276 chose to use a 30-minute pepsin digestion and a 60-minute pancreatin/bile digestion. In
277 past studies, quercetin from onion and quercetin from quercetin-4-glucoside supplements
278 reached the plasma in less than an hour following consumption by human volunteers
279 [31]. Therefore, long in vitro digestion times were not necessary to mimic human
280 digestion of quercetin compounds from apples and onions.

281

282 The presence of ascorbic acid and nitrogen had no effect on quercetin or quercetin-3-
283 glucoside recoveries from the digestates (data not shown). Quercetin and quercetin-3-
284 glucoside recoveries from digested samples treated with ascorbic acid, nitrogen or both
285 ascorbic acid and nitrogen were not different from the recoveries from the untreated
286 digested samples.

287

288 The factor that had the greatest effect on recovery was pH (Figure 1A). Quercetin is less
289 stable at higher pH, therefore the effect of pH during intestinal digestion was examined.

290 Recoveries of quercetin and quercetin-3-glucoside after intestinal digestion at pH 7.0,
291 when compared to pH 6.5, were not significantly different ($p > 0.05$). However,
292 following overnight storage at -80°C , the samples stored at pH 2.0 had much higher
293 quercetin and quercetin-3-glucoside recoveries than samples stored at pH 6.5 and 7.0 ($p <$
294 0.05). Pure quercetin and quercetin-3-glucoside were also more stable at lower storage
295 pH following digestion (Figure 1B). At pH 2.0, the recoveries for pure quercetin and
296 quercetin-3-glucoside were 74.8% and 86.2% compared to the control. At the highest pH
297 (7.0), recoveries for quercetin and quercetin-3-glucoside were 46.5% and 13.9%,
298 respectively.

299

300 Based on these results, optimal digestion conditions were decided to be: pepsin digestion
301 at pH 2.0 for 30 minutes, pancreatin/bile digestion at pH 6.5 for 60 minutes, and a final
302 storage at pH 2.0. Ascorbic acid and nitrogen treatments were not continued. Using
303 these conditions, the effect of digestion on quercetin and quercetin-3-glucoside recovery
304 from apples, onions, and pure quercetin and quercetin-3-glucoside was examined (Figure
305 2). Following digestion, recoveries of pure quercetin-3-glucoside, apple quercetin-3-
306 glucoside, and onion quercetin-3-glucoside were similar to recoveries from non-digested
307 samples ($p > 0.05$). Quercetin-3-glucoside recoveries from the digested apple, onion, and
308 pure compound were 87.7, 89.5, and 86.4%, respectively. Quercetin recovery was
309 significantly reduced in digested pure quercetin and digested onion samples, when
310 compared to non-digested samples ($p < 0.05$). Quercetin recovery was lower than
311 quercetin-3-glucoside recovery and tended to vary more, depending on the food matrix:
312 52.5% and 74.3%, from the onion, and pure compound, respectively. There was no
313 significant difference in quercetin recovery between to the non-digested and digested
314 apple homogenates. There was only trace or no quercetin in non-digested apple
315 homogenates, so the appearance of any quercetin following digestion resulted in a net
316 increase.

317

318 **Uptake of quercetin-4'-glucoside and quercetin from shallot digestates by Caco-2**
319 **cells**

320 Quercetin-4'-glucoside and quercetin were absorbed by Caco-2 cells following treatment
321 with both digested shallot and non-digested shallot homogenates (Figure 3). Quercetin-3-
322 glucoside was not detected in any sample. Quercetin-4'-glucoside uptake by the Caco-2
323 cells increased by approximately 2-fold following digestion. Caco-2 cells treated with
324 shallot homogenate absorbed approximately 2.9 ± 0.65 nmol of quercetin-4-glucoside,
325 and Caco-2 cells treated with digested shallot absorbed 5.4 ± 0.04 nmol. Quercetin
326 aglycon recovery from the digested shallot extract was only 47% that of the non-digested
327 homogenate (Figure 3B insert), however quercetin uptake from the digested samples was
328 similar to the non-digested samples ($p > 0.05$). Caco-2 cells absorbed 2.8 ± 0.4 nmol and
329 2.7 ± 0.2 nmol quercetin from the non-digested and digested shallot homogenate,
330 respectively. Absorption of both quercetin-4-glucoside and quercetin from digested
331 shallot followed a dose response. The Caco-2 cells absorbed quercetin 4'-glucoside and
332 quercetin incrementally less from the digested samples that were diluted 1:2 or 1:4 in
333 HBSS.

334

335 **Induction of lactase activity in Caco-2 cells**

336 Addition of lactose and forskolin, a specific inducer of lactase (Hauri et al., 1994), to
337 Caco-2 cells did not significantly increase lactase activity of Caco-2 cells. The lactase
338 activity of all cells ranged from 2-4 mU/mg protein in all treatments.

339

340 **Lactase Digestion**

341 Treatment with 100 units lactase/g sample had a significant effect on both quercetin and
342 quercetin-3-glucoside recovery from the shallot ($p < 0.05$; Figure 4). Quercetin recovery
343 from shallot digestates increased 5.5 fold, from 47.5 ± 7.6 $\mu\text{g/g}$ sample in the untreated

344 digestate to $262.2 \pm 17.6 \mu\text{g/g}$ sample in the lactase treated sample. The lactase plus
345 digestion treatment resulted in a nonsignificant decrease in quercetin recovery compared
346 to the lactase only treated samples. Quercetin-3-glucoside from shallot digestates also
347 increased approximately 5 fold, from $17.3 \pm 1.7 \mu\text{g/g}$ sample to $80.0 \pm 10.3 \mu\text{g/g}$ sample
348 following the lactase treatment. The effect of lactase on the apple samples was not as
349 great. Quercetin-3-glucoside recovery decreased slightly, while changes in quercetin
350 levels were not significant ($p > 0.05$).

351

352 Because treating shallots with lactase increased quercetin recovery so greatly without
353 significantly decreasing quercetin 3-glucoside recovery, the effect of lactase on
354 quercetin-4'-glucoside recovery was also examined. More quercetin-4'-glucoside is
355 found in shallots when compared to quercetin-3-glucoside. As the dose of lactase
356 increased, quercetin-3-glucoside recovery increased from 18.2 ± 3.7 up to 175.5 ± 48.1
357 $\mu\text{g/g}$ shallot at the 1000 unit dose and then decreased to $60.2 \pm 2.0 \mu\text{g/g}$ at the 3000 unit
358 dose. As the dose of lactase increased, quercetin recovery increased and quercetin-4'-
359 glucoside decreased (Figure 5A). The increase in quercetin was quite dramatic.

360 Recovery of quercetin from untreated shallot samples was $93.7 \pm 2.2 \mu\text{g}$ quercetin per
361 gram sample, and at the highest lactase dose, recovery of quercetin from shallot samples
362 was $958.8 \pm 76.1 \mu\text{g}$ quercetin per gram shallot. Quercetin-4'-glucoside recovery
363 decreased from $518.2 \pm 10.7 \mu\text{g/g}$ shallot from the untreated sample to $3.2 \pm 0.8 \mu\text{g/g}$
364 shallot at the highest treatment dose. A similar trend was seen for all compounds in the
365 kinetic experiment. As the incubation time increased, recoveries of quercetin increased
366 and quercetin-4'-glucoside decreased (Figure 5B). Quercetin-3-glucoside increased
367 through two hours, and then decreased following four and eight hours of incubation with
368 lactase. In both the dose response and kinetic experiments, increases in quercetin
369 recoveries were greater than decreases in quercetin-3-glucoside or quercetin-4'-glucoside
370 recoveries.

371

372 **Caco-2 cell uptake of quercetin and quercetin glucosides following lactase treatment**

373 The addition of lactase following the pepsin and pancreatin/bile digestion significantly
374 increased the amount of quercetin and significantly reduced the amount of quercetin-4'-
375 glucoside absorbed by the Caco-2 cells ($p < 0.05$, Figure 6). Quercetin uptake increased
376 from 0.98 ± 0.67 nmol from the digested sample up to 14.1 ± 1.6 nmol from the digested
377 plus 1000 units lactase treated sample. Quercetin-4'-glucoside uptake by Caco-2 cells
378 decreased as the dose of lactase increased, however the increase in quercetin was more
379 dramatic than the decrease in quercetin-4'-glucoside. Quercetin-3-glucoside uptake was
380 not detected.

381

382 **Discussion**

383 **In vitro digestion**

384 Previously, our laboratory determined that our Caco-2 cells had the potential to be used
385 as a model to study quercetin bioavailability from onions and apples [32]. In the present
386 study, we modified an *in vitro* digestion procedure and combined it with the Caco-2 cell
387 model to give a more comprehensive examination of quercetin and quercetin glucoside
388 bioavailability in Caco-2 cells.

389

390 The digestion procedure modified in these experiments resulted in quercetin-3-glucoside
391 recoveries that ranged from 78.9 to 89.5% and quercetin recoveries that ranged from 47.3
392 to 74.3%. In both cases, the lowest digestate recoveries were from the shallot.

393 Interestingly, quercetin recovery from both the onion and shallot was considerably lower
394 than from the pure compound. This difference is not yet explained, but could potentially
395 be due to interactions with other compounds found in the shallot.

396

397 In all cases, quercetin recovery from digestates was lower than quercetin-3-glucoside.
398 The glucoside moiety may lend stability to quercetin-3-glucoside during digestion, and
399 could contribute to its greater bioavailability *in vivo* as well. Quercetin aglycone may be
400 more susceptible to oxidation or other degradation during exposure to both the digestive
401 enzymes and the variations in pH in the stomach and intestine. Ascorbic acid and
402 nitrogen, both added to help decrease oxidation, had no effect on quercetin or quercetin-
403 3-glucoside recoveries from digested onions.

404

405 The factor that had the greatest effect on both quercetin and quercetin-3-glucoside
406 stability in the digestate was pH. Recoveries of digested pure compounds and digested
407 compounds from the onion homogenates were significantly less if stored at a pH of 6.5 or
408 7.0 than if stored at pH 2.0 at -80° C (Figure 1). Vallejo et. al [24] used an *in vitro*
409 digestion method to measure the effect of digestion on a variety of compounds from
410 broccoli. Following the pepsin and pancreatic digestion, they recovered only 16% of
411 total flavonoids, and the main flavonoids found in the broccoli were quercetin and
412 kaempferol glycosides. The recovery of quercetin and quercetin-3-glucosides, flavonoids
413 common to the foods in our study, was much higher than 16% from the digested shallot,
414 onion and apple. It has been estimated that quercetin glucoside bioavailability may be as
415 high as 80% in humans and quercetin bioavailability may range from 35-53% [25, 33].
416 The Vallejo estimate that only 16% of broccoli flavonoids are available following
417 digestion appears to be very low. Our results would appear to be more reasonable
418 estimates, if indeed, quercetin and quercetin glucoside bioavailability lies within the
419 approximated ranges found by Walle et. al [25, 33].

420

421 **Effect of digestion on quercetin and quercetin glucoside uptake by Caco-2 cells**

422 Digestion of the shallot resulted in decreased amounts of both quercetin and quercetin-3-
423 glucoside, therefore it was expected that digestion might decrease the bioavailability of

424 these compounds as well. Following digestion, quercetin aglycone in the shallot was
425 decreased by approximately 50%; however, quercetin bioavailability was unchanged
426 following digestion compared to the non-digested samples. This means that the digestion
427 procedure must degrade quercetin, and simultaneously enhances the bioavailability of
428 quercetin, bringing the uptake back to comparable levels with the non-digested samples.
429 Quercetin-4'-glucoside uptake by the Caco-2 cells from the shallot increased following
430 the *in vitro* digestion. We hypothesize that the digestion procedure may have released
431 more compounds from the food matrix leaving them more available for uptake by the
432 Caco-2 cells. The digestion procedure may also have improved solubility of the
433 compounds, increasing their absorption by the Caco-2 cells.

434

435 Quercetin-3-glucoside was not detected in the Caco-2 cells following treatment with
436 shallot. Quercetin-3-glucoside is a minor compound in the shallot, and it is believed that
437 the levels were below our detection limit. In the past, we found that Caco-2 cells did
438 absorb trace amounts of quercetin-3-glucoside from shallot extracts [32]. In the previous
439 experiments, shallots were first extracted with ethanol and ethyl acetate, and finally
440 reconstituted and concentrated in methanol. This procedure produced more concentrated
441 shallot extracts for cell treatment than with our current procedure. In the current study,
442 shallot homogenates and digestates were too dilute to detect small changes in initial
443 concentrations or in cellular uptake of quercetin-3-glucoside, which was below the
444 detection limit.

445

446 Strong evidence suggests that quercetin glucosides are more bioavailable in humans than
447 the quercetin aglycone, however it has not yet been determined why this is the case.
448 Based on the digestion data, quercetin glucoside is more stable following the *in vitro*
449 digestion conditions than quercetin and is therefore more likely to reach the intestine
450 intact. Quercetin-4'-glucoside bioavailability in Caco-2 cells was increased nearly 2-fold

451 following digestion and quercetin absorption was not changed (Figure 3). It has been
452 hypothesized that absorbed intact quercetin glucosides are then quickly hydrolyzed by
453 cytosolic β -glucosidase to quercetin aglycone [34]. It has also been hypothesized that the
454 major pathway for quercetin glucoside absorption begins with hydrolysis by LPH.
455 Deglycosylation of quercetin glucosides at the brush border membrane positions the
456 resulting aglycone in a prime position for diffusion across the brush border. The
457 deglycosylation of the quercetin glucoside would result in a higher concentration of
458 aglycone at the apical enterocyte membrane and potentially increase the rate of
459 absorption [35].

460

461 **Effect of lactase on quercetin and quercetin-4'-glucoside uptake by Caco-2 cells**

462 The potential pathways for quercetin glucoside and quercetin metabolism and absorption
463 can be seen in Figure 7. Quercetin aglycone passively diffuses across the apical
464 membrane and is then glucuronidated. Evidence strongly suggests that quercetin
465 glucosides are first hydrolyzed by the lactase site of lactase phlorizin hydrolase prior to
466 diffusion across the apical membrane [36]. Quercetin glucosides may also be transported
467 into the cell by the sodium-dependent glucose transporter1 (SGLT1) and then hydrolyzed
468 by the cytosolic beta-glucosidase. Quercetin-3-glucoside is not a good substrate for
469 cytosolic beta-glucosidase [37]. Since research has shown that both quercetin-3-
470 glucoside and quercetin-4'-glucoside are similarly bioavailable in humans [14, 38], this
471 could be an indication that hydrolysis by LPH and the subsequent passive diffusion of
472 quercetin into the cell is the main pathway for quercetin glucoside absorption across the
473 brush border. Following hydrolysis and incorporation into the cells, quercetin aglycone
474 is then glucuronidated. Quercetin glucosides and possibly quercetin glucuronides are
475 then transported back into the lumen by multidrug resistance protein 2 (MRP2).
476 Conjugated quercetin metabolites also eventually reach circulation, but the transporter
477 involved in transporting them across the basolateral side is still unknown.

478

479 Since lactase is an important enzyme in the metabolism and subsequent absorption of
480 quercetin glucosides, lactose intolerant individuals may have a reduced capability to
481 hydrolyze quercetin glucoside for further absorption across the small intestinal wall.
482 Many lactose intolerant people use commercial lactase to break down lactose. Not only
483 might this enzyme help improve digestibility of lactose, but it may also increase
484 bioavailability of quercetin from foods. Initial lactase treatments were applied both to
485 shallot and apple homogenates and digestates. Lactase had little to no effect on apple
486 samples. Apples contain quercetin bound mainly to galactosides, rhamnosides, and
487 xylosides, conjugates that would not be readily hydrolyzed by lactase. However, lactase
488 treatment had great effects on shallot digestates. The shallot is high in quercetin
489 glucosides, mainly quercetin-4'-glucoside, quercetin-3-glucoside, and quercetin-3,4'-
490 diglucoside, compounds readily hydrolyzed by lactase. Treatment with lactase in the
491 range of 15 units/mg sample up to 1000 units/mg sample, significantly increased both
492 quercetin and quercetin-3-glucoside recovery in shallot homogenates and digestates. The
493 increase in quercetin-3-glucoside is most likely a result of deglycosylation of quercetin-
494 3,4'-diglucoside. Rhodes et al. [39] found that over time quercetin-3,4'-diglucoside in
495 chopped onion will autolyze to monoglucosides, and within 24 hours the diglucoside will
496 completely disappear. This may also explain the increase in quercetin-3-glucoside over
497 time. Quercetin-4'-glucoside decreased following treatment with lactase as expected.
498 Results from this work suggest that quercetin-4'-glucoside is utilized by lactase prior to
499 quercetin-3-glucoside. Interestingly, the increase in total quercetin was greater than the
500 decrease in quercetin-4'-glucoside. Digestion with lactase may release quercetin from the
501 food matrix as well, making it more available for absorption.

502

503 Following hydrolysis of quercetin glycosides, it has been hypothesized that quercetin is
504 quickly glucuronidated, and quercetin glucuronides are then found circulating in the

505 plasma [40]. In the current studies, these Caco-2 cells showed no signs of
506 glucuronidating quercetin following quercetin absorption, but do express some lactase
507 phlorizin hydrolase activity as was evident by the increased quercetin uptake from
508 shallots and from the lactase activity assays [32]. Thus, these Caco-2 cells have the
509 potential to be a good model of quercetin absorption, but not of further metabolism.

510

511 A good model of quercetin glucoside bioavailability should incorporate lactase activity.
512 The Caco-2 cells used for these experiments expressed lactase activity similar to that of a
513 lactose intolerant human. These cells had approximate lactase activity of between 2-4
514 mU/mg protein, consistent with what we previously reported [32]. Lactose tolerant
515 humans tend to have intestinal lactase activity that ranges from 20-80 mU/mg protein
516 [41]. Forskolin and lactose did not induce lactase activity in our Caco-2 cells. The
517 lactase activity of our cells was nearly 10 times higher than previously reported values for
518 Caco-2 cells of 0.3 mU/mg protein of lactase activity [27], and it is quite possible that the
519 lactase enzyme in our Caco-2 cells is already expressed to the fullest extent.

520

521 Since lactase activity could not be increased in the Caco-2 cells, we combined a lactase
522 treatment with digestion to provide an intestinal uptake model that is more comparable to
523 a lactose tolerant human. This would be similar to a lactose intolerant human ingesting a
524 lactase digestive aid to help improve lactose digestion. Not only did lactase increase the
525 amount of quercetin in digested shallot homogenates, but it also increased the amount of
526 quercetin taken up by the Caco-2 cells from the digested shallot extracts. This suggests
527 that a lactase containing digestive aid may aid in absorption of quercetin from onions in
528 lactose intolerant humans. Combining the Caco-2 cell model with an in vitro digestion,
529 simulating stomach and small intestinal digestion, and a lactase digestion may make a
530 more useful model to examine and screen for bioavailability of flavonoids glucosides
531 from common foods (Figure 8).

532

533 **Conclusions**

534 Following an in vitro stomach and small intestinal digestion, the recovery of quercetin
535 and quercetin-3-glucoside is much greater if stored at a low pH. Storage at pH above 3.5
536 results in loss of most of the compounds. The in vitro digestion increased the uptake of
537 shallot quercetin-4'-glucoside by the Caco-2 cells. Quercetin uptake by the Caco-2 cells
538 was similar between digested and non-digested samples, despite the fact that
539 approximately 50% of shallot quercetin is lost during digestion. Treating shallot
540 digestates with lactase increased the recovery of quercetin aglycone 10-fold and
541 decreased the recovery of quercetin-4'-glucoside. Lactase treatment increased the
542 bioavailability of quercetin aglycone 14-fold and decreased the bioavailability of
543 quercetin-4'-glucoside to the Caco-2 cells. Combining pepsin, pancreatin/bile, and
544 lactase digestions with the Caco-2 cell culture monolayer may results in a useful model
545 for studying flavonoid bioavailability from foods.

546

547 Many advances have been made in understanding flavonoid bioavailability, however
548 many questions still remain unanswered. Food processing, interactions with other
549 compounds, and interactions with other foods are all factors that may affect
550 bioavailability of flavonoids. A simple and inexpensive screening model would be
551 beneficial as a first step in examining many of these factors. The Caco-2 cell model has
552 the potential to be a good model for analyzing quercetin, quercetin glucoside and other
553 flavonoid intestinal uptake. Caco-2 cells will never be an exact replica of a human small
554 intestine, but it could be a valuable model for screening large quantities of samples
555 relatively quickly and inexpensively. In this study, it was found that a simple digestive
556 aid such as lactase, may increase quercetin bioavailability. This may be of importance to
557 lactose intolerant people. Interesting and significant trends such as this one may then be
558 examined in more detail using human or animal models.

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709

709 **Figure Legends**

710 Figure 1. The effects of intestinal digestion pH and acidic storage on quercetin and
711 quercetin-3-glucoside recovered from digested onions (A) and the effects of storage pH
712 on digested pure quercetin and quercetin-3-glucoside (B). Samples were digested for 30
713 minutes with pepsin, 60 minutes with pancreatin and bile, and then stored at -80°C. Each
714 point represents the mean \pm standard deviation of triplicate observations within the same
715 experiment. Different letters indicate significantly different observations within each
716 compound ($p < 0.05$).

717

718 Figure 2. The effects of digestion on pure quercetin and quercetin-3-glucoside and
719 quercetin and quercetin-3-glucoside from apple and onion. Each point represents the
720 mean \pm standard deviation of triplicate observations within the same experiment. An
721 asterisk indicates a significant difference between the control and the digestate ($p < 0.05$).

722

723 Figure 3. Caco-2 uptake of quercetin-4-glucoside (A) and quercetin (B) from digested
724 and non-digested shallot homogenates. Shallot homogenates were digested for 30
725 minutes with pepsin at pH 2.0 and for 60 minutes with pancreatin/bile at pH 6.5.
726 Digestates were directly placed on cells or diluted 1:2 or 1:4 in HBSS and then placed on
727 cells. Cells were incubated with digestates for 30 minutes at 37° C. The imbedded graph
728 in (B) shows quercetin recovery from shallots following the digestion procedure only.
729 Each bar represents the mean \pm standard deviation of triplicate observations within the
730 same experiment. Different letters indicate significantly different observations within
731 each compound ($p < 0.05$).

732

733 Figure 4. The effects of lactase and a combined lactase and digestion treatment on
734 quercetin and quercetin-3-glucoside recovery from shallot (A) and apple (B)
735 homogenates. Each point represents the mean \pm standard deviation of triplicate

736 observations within the same experiment. Different letters indicate significantly different
737 observations within each compound ($p < 0.05$).

738

739 Figure 5. Dose response (A) and kinetics (B) of lactase on quercetin and quercetin
740 glucoside recovery from shallots. Homogenized shallots were incubated 60 for minutes
741 with 10, 50, 100, 300, 500, 1000, or 3000 units of lactase/mL sample. Homogenized
742 shallots were incubated with 100 units lactase/mL sample for 15, 30, 60, 90, 120, 240,
743 and 720 minutes. Each point represents the mean \pm standard deviation of triplicate
744 observations within the same experiment.

745

746 Figure 6. Caco-2 uptake of quercetin-4-glucoside and quercetin from digested shallot
747 and digested plus lactase treated shallot. Shallot homogenates were digested for 30
748 minutes with pepsin at pH=2.0 and for 60 minutes with pancreatin/bile at pH=6.5.
749 Digested shallots were then treated with either 50, 100, 300, or 1000 units lactase/mL
750 sample for 20 minutes. Samples were diluted 1:2 and then placed on the cells for 30
751 minutes. Each point represents the mean \pm standard deviation of triplicate observations
752 within the same experiment.

753

754 Figure 7. Potential mechanism of quercetin and quercetin glucosides uptake by
755 enterocytes. LPH, lactase phlorizin hydrolase; SGLT1, sodium-dependent glucose
756 transporter 1; CBG, cytosolic B-glucosidase; MRP2, multi-drug resistance protein 2;
757 UDP-GT, UDP glucuronosyl transferase; QUE, quercetin.

758

759 Figure 8. Caco-2 cell culture model for examining quercetin bioavailability from foods.

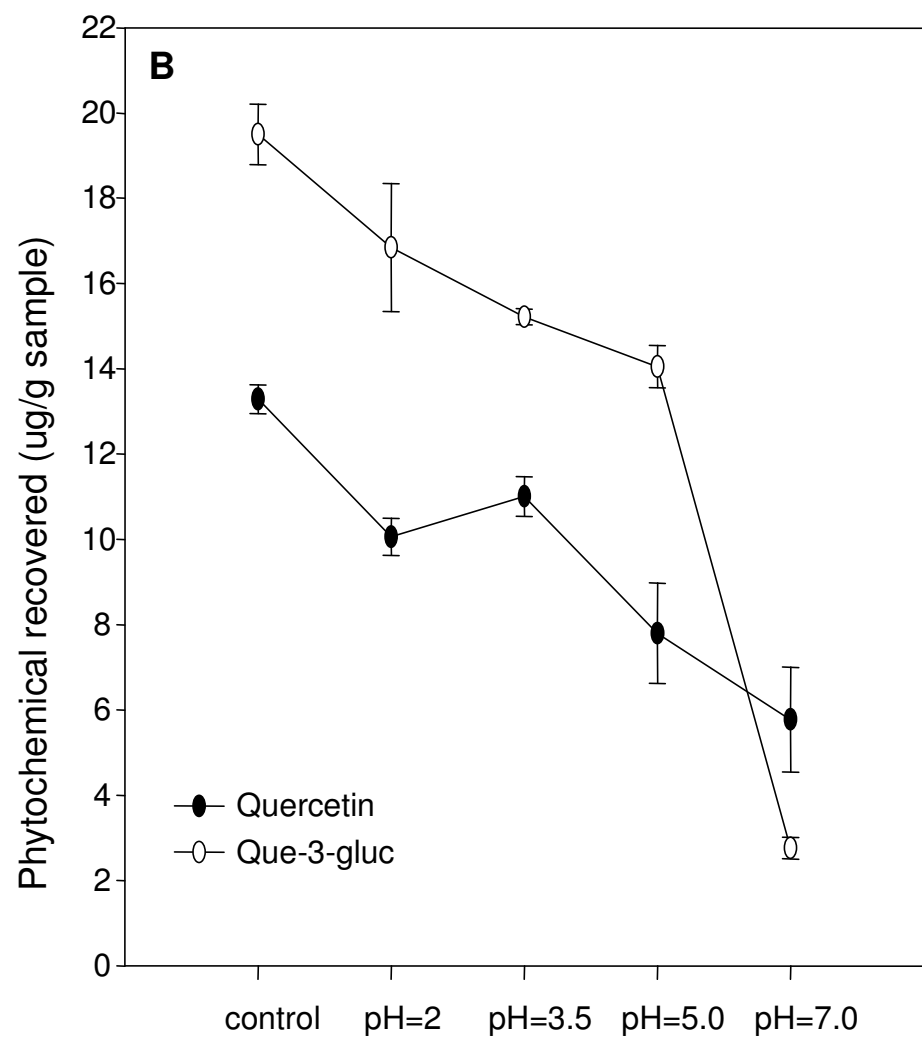
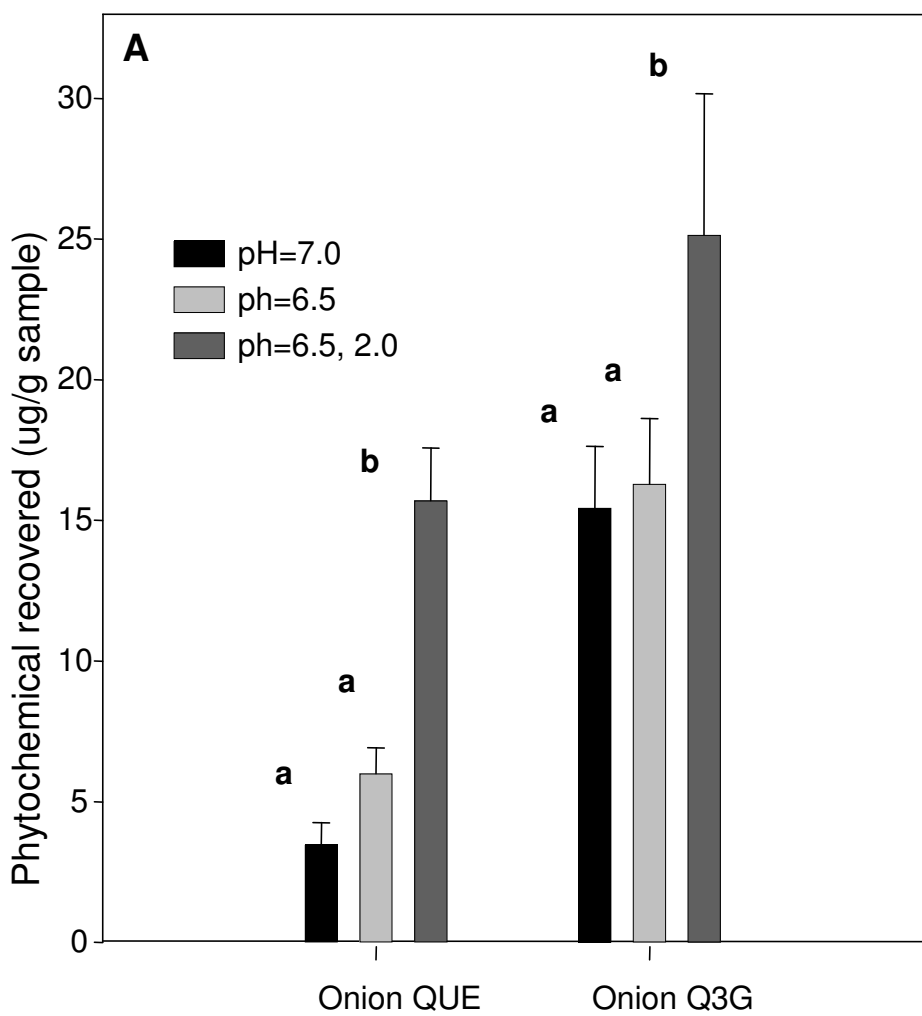


Figure 1

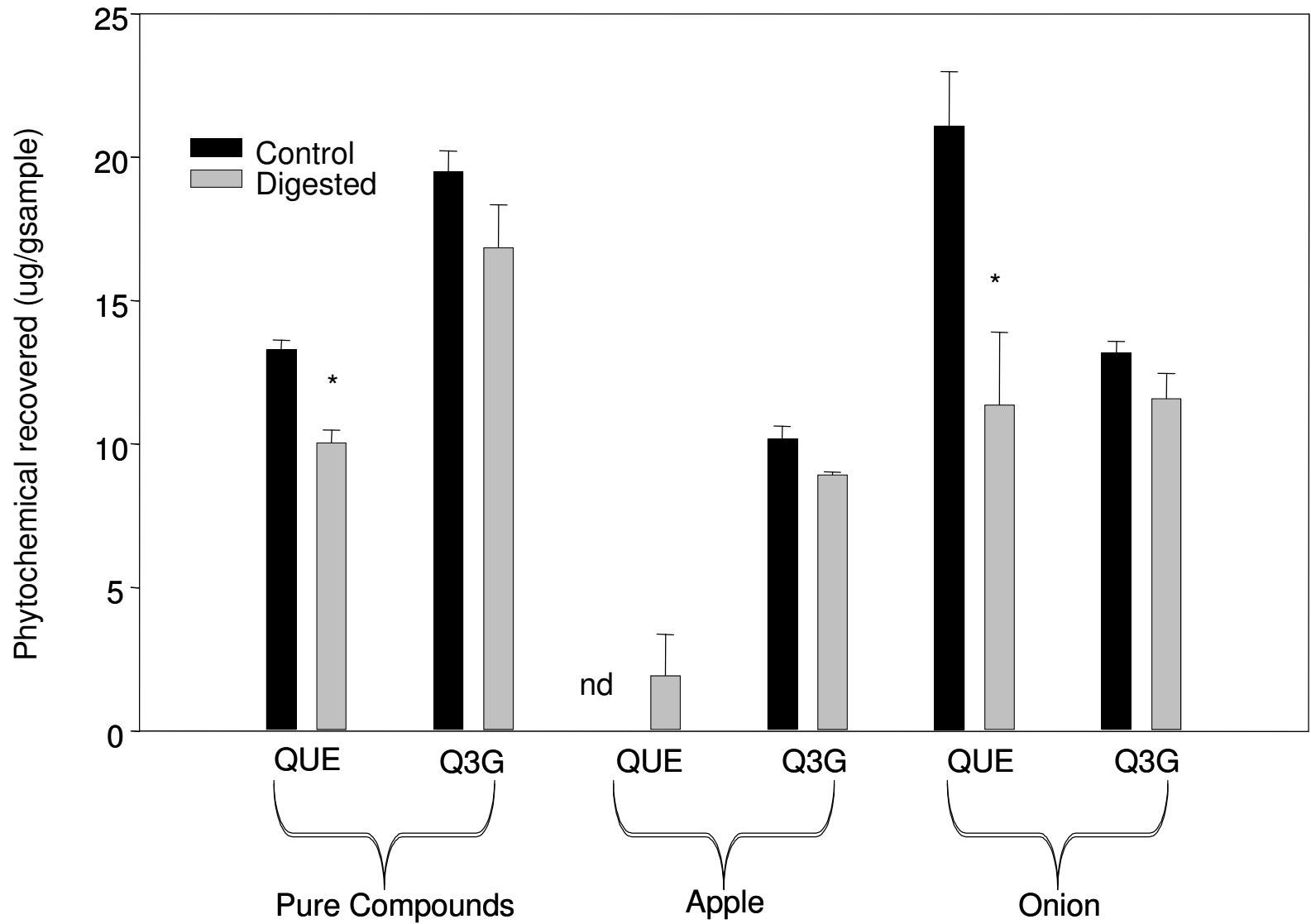


Figure 2

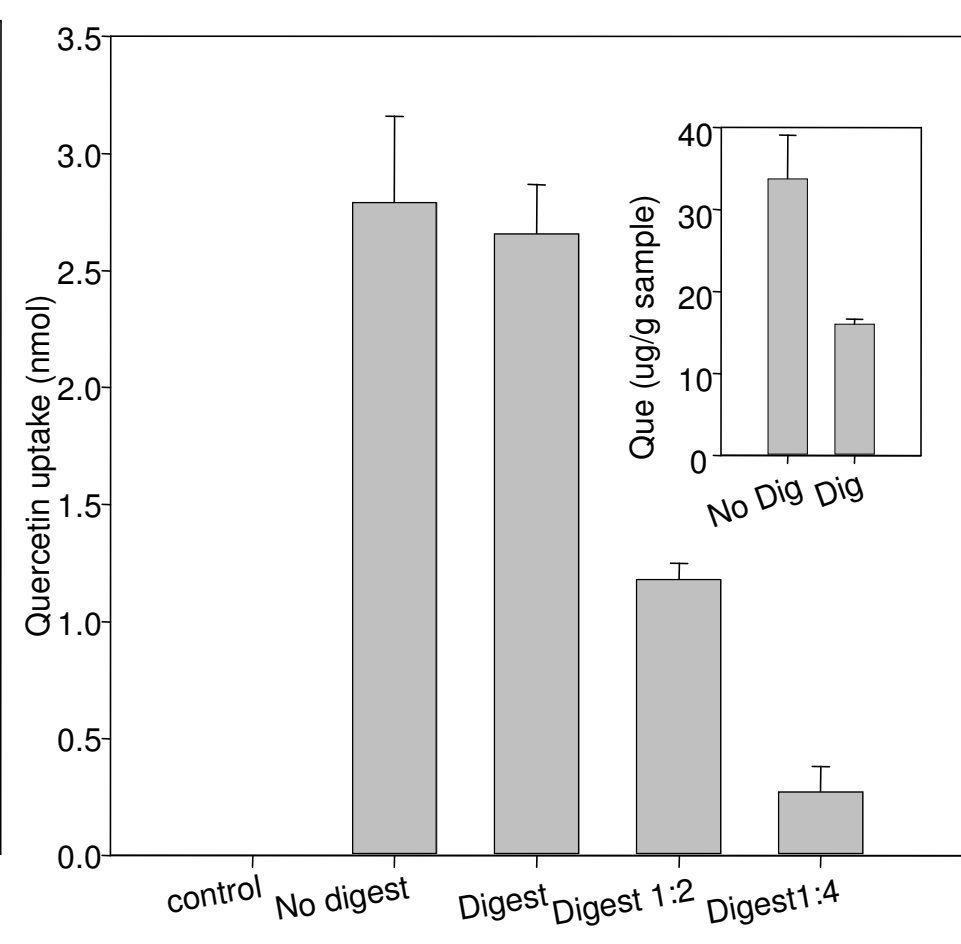
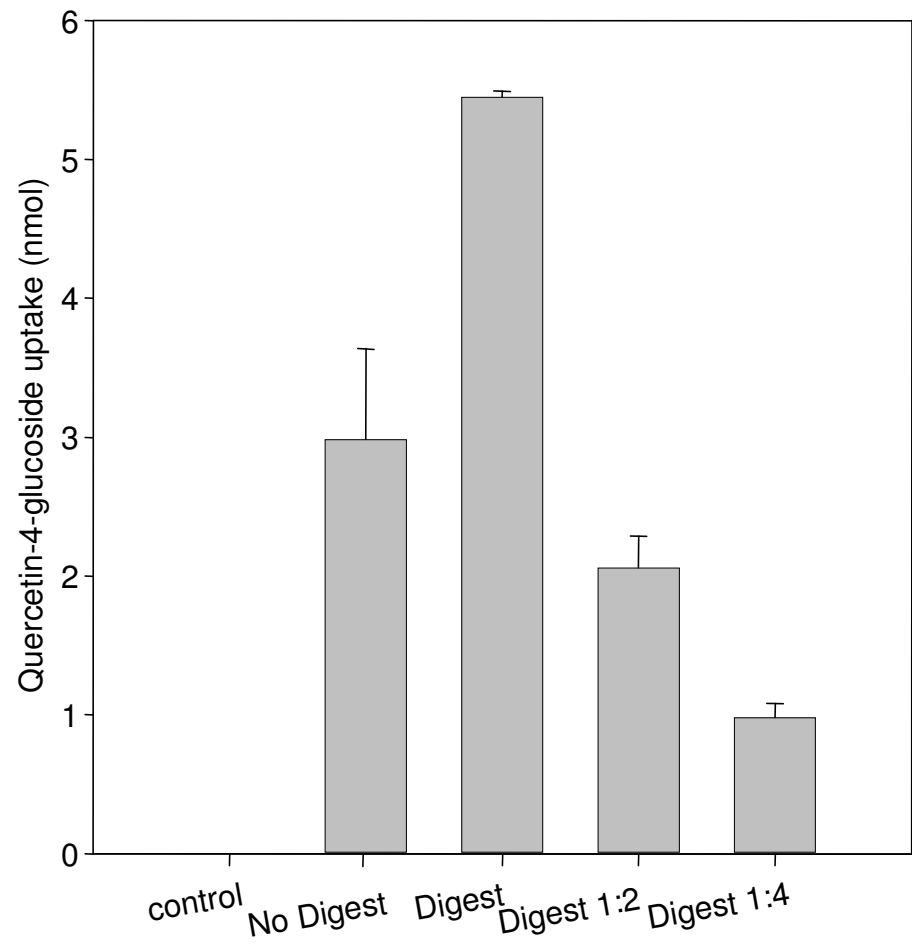


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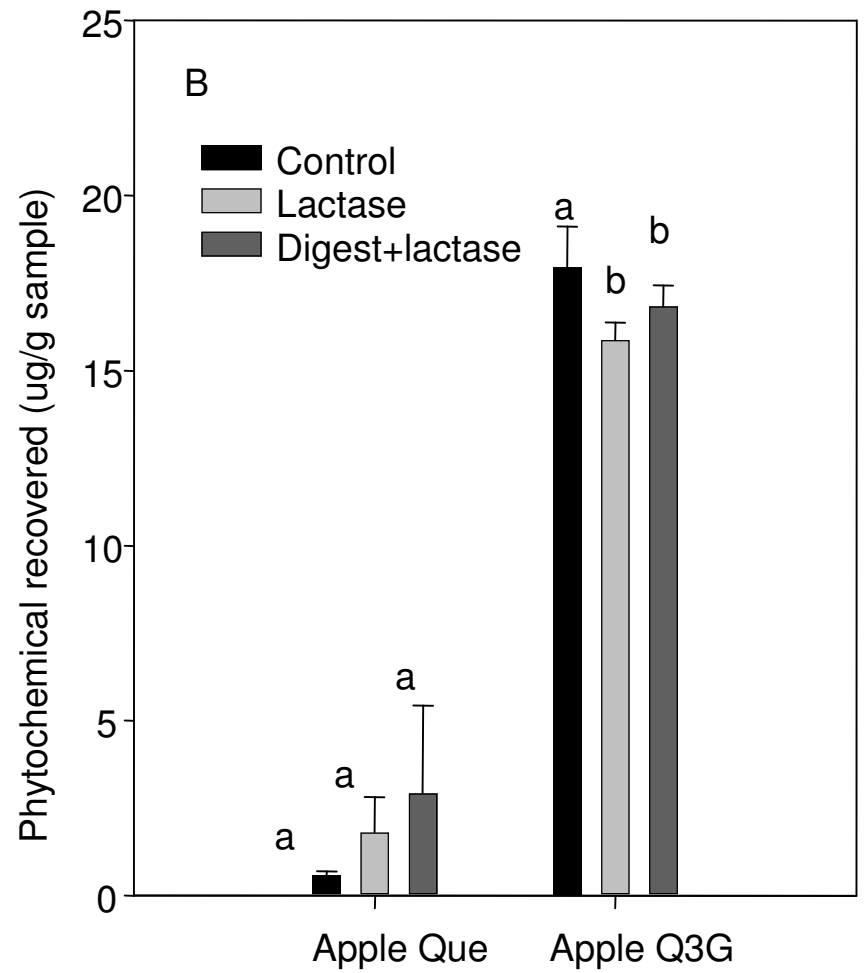
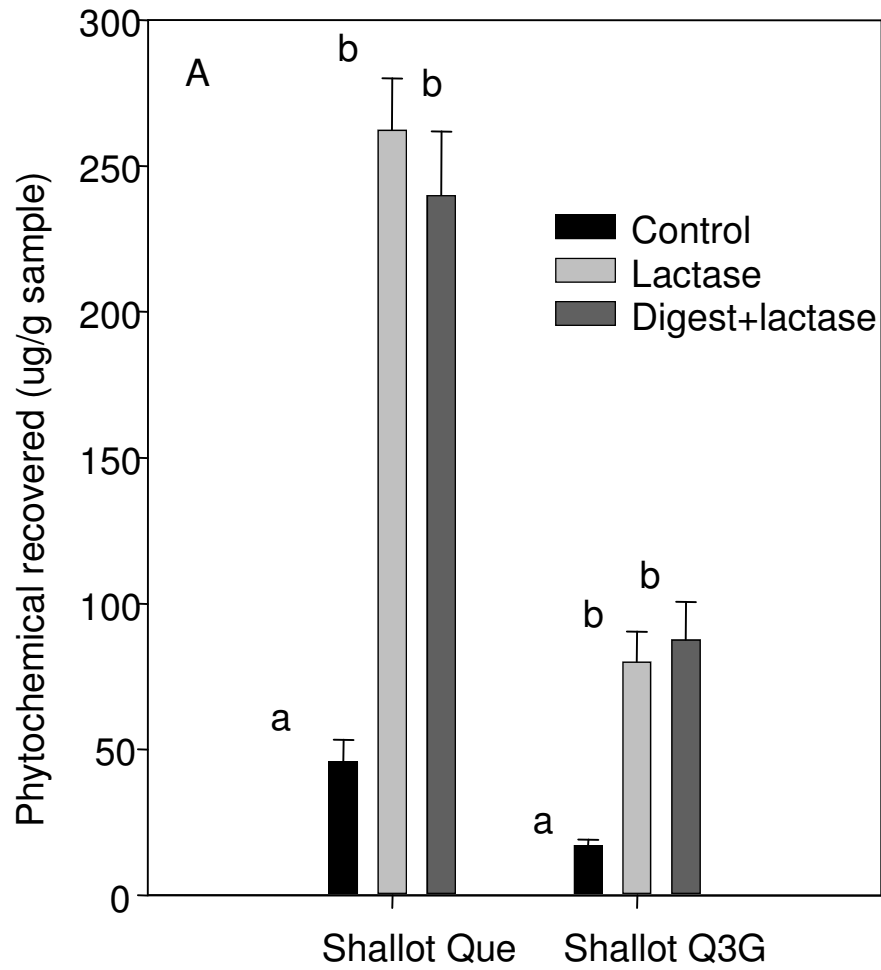


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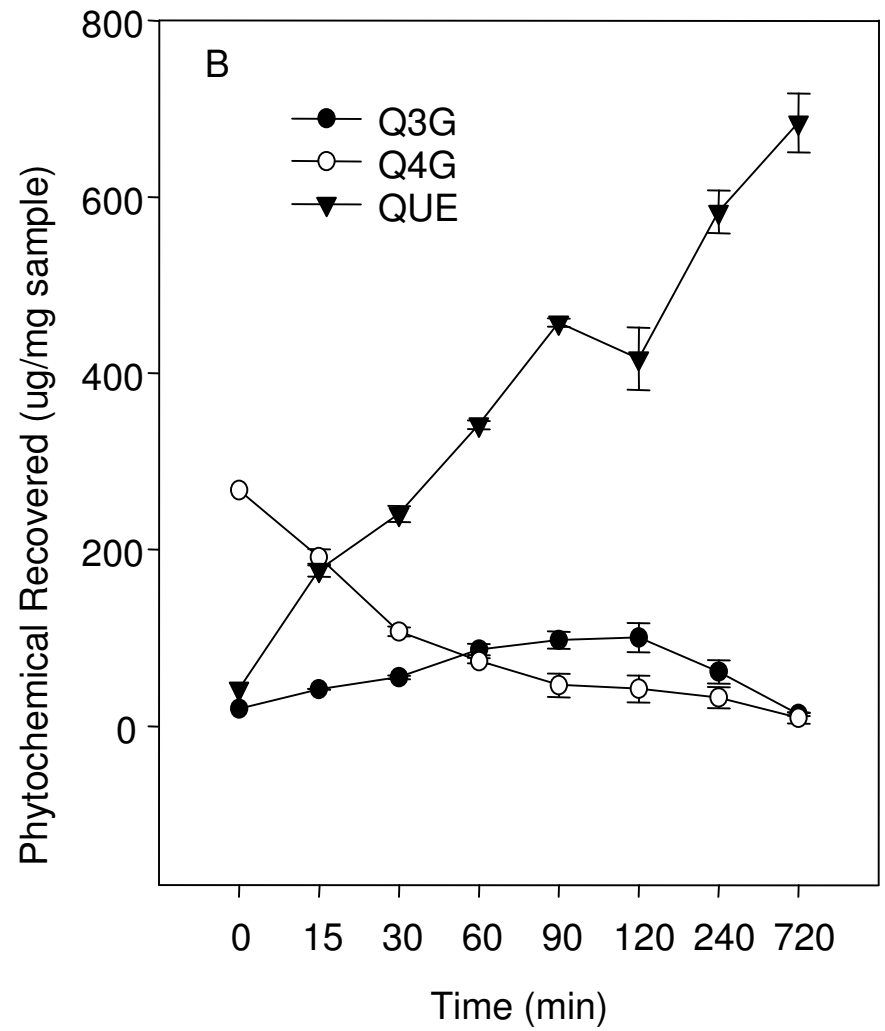
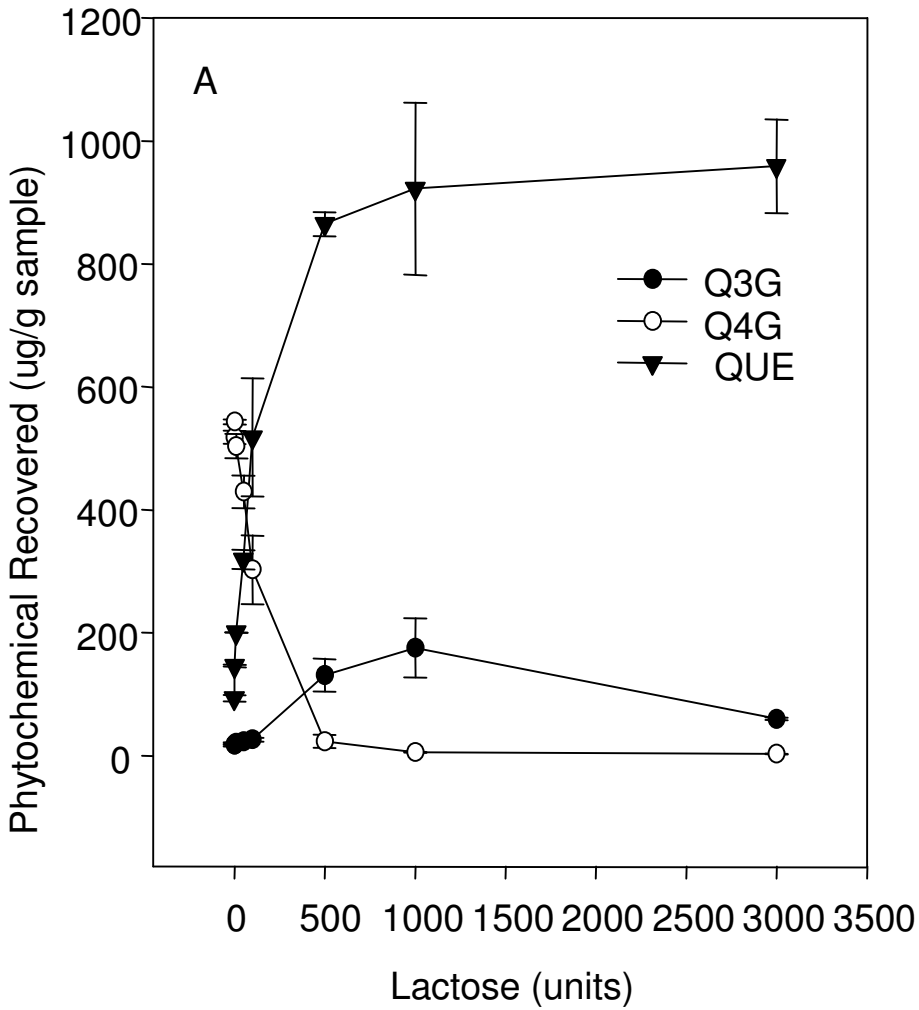


Figure 5

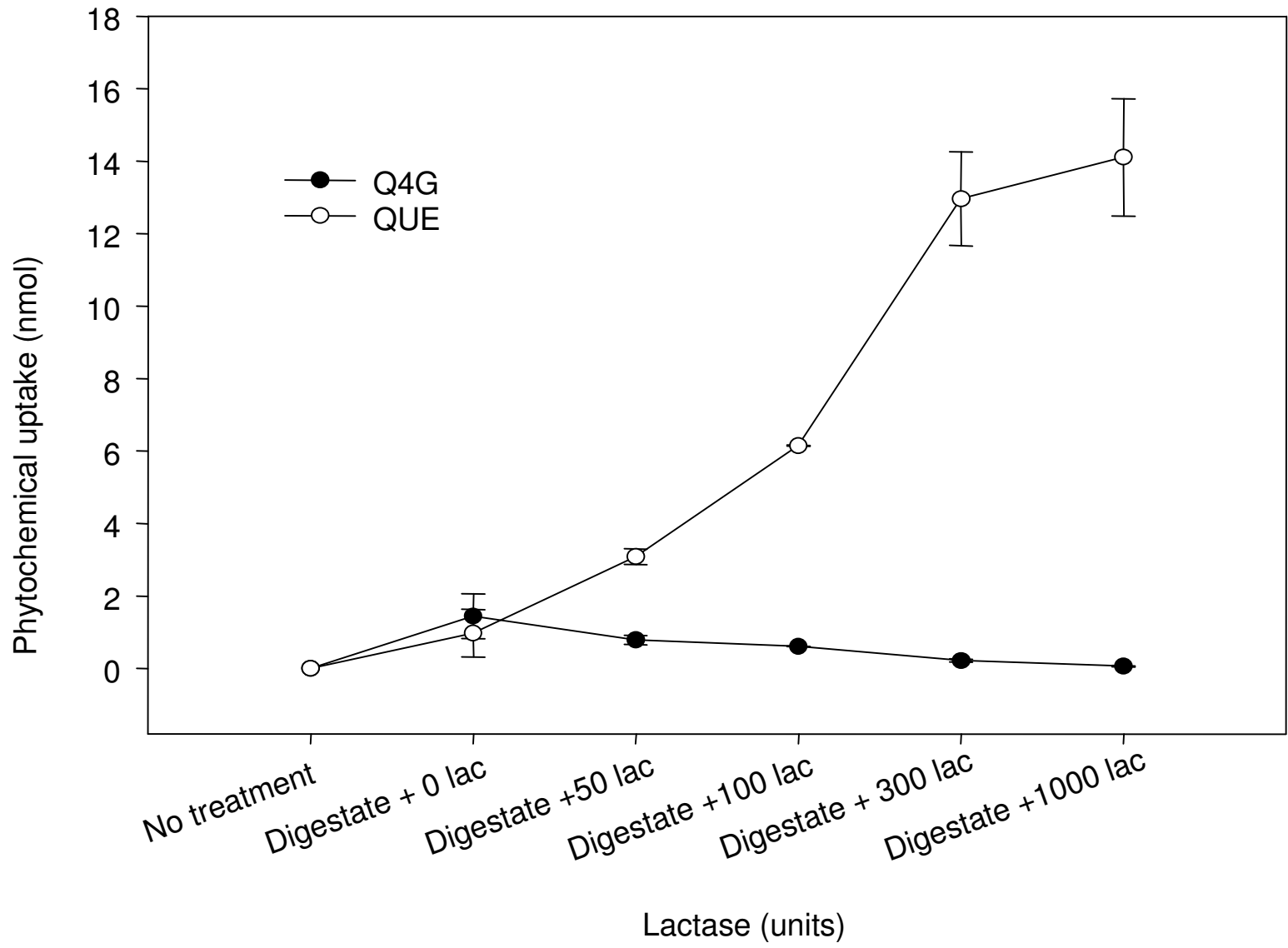


Figure 6

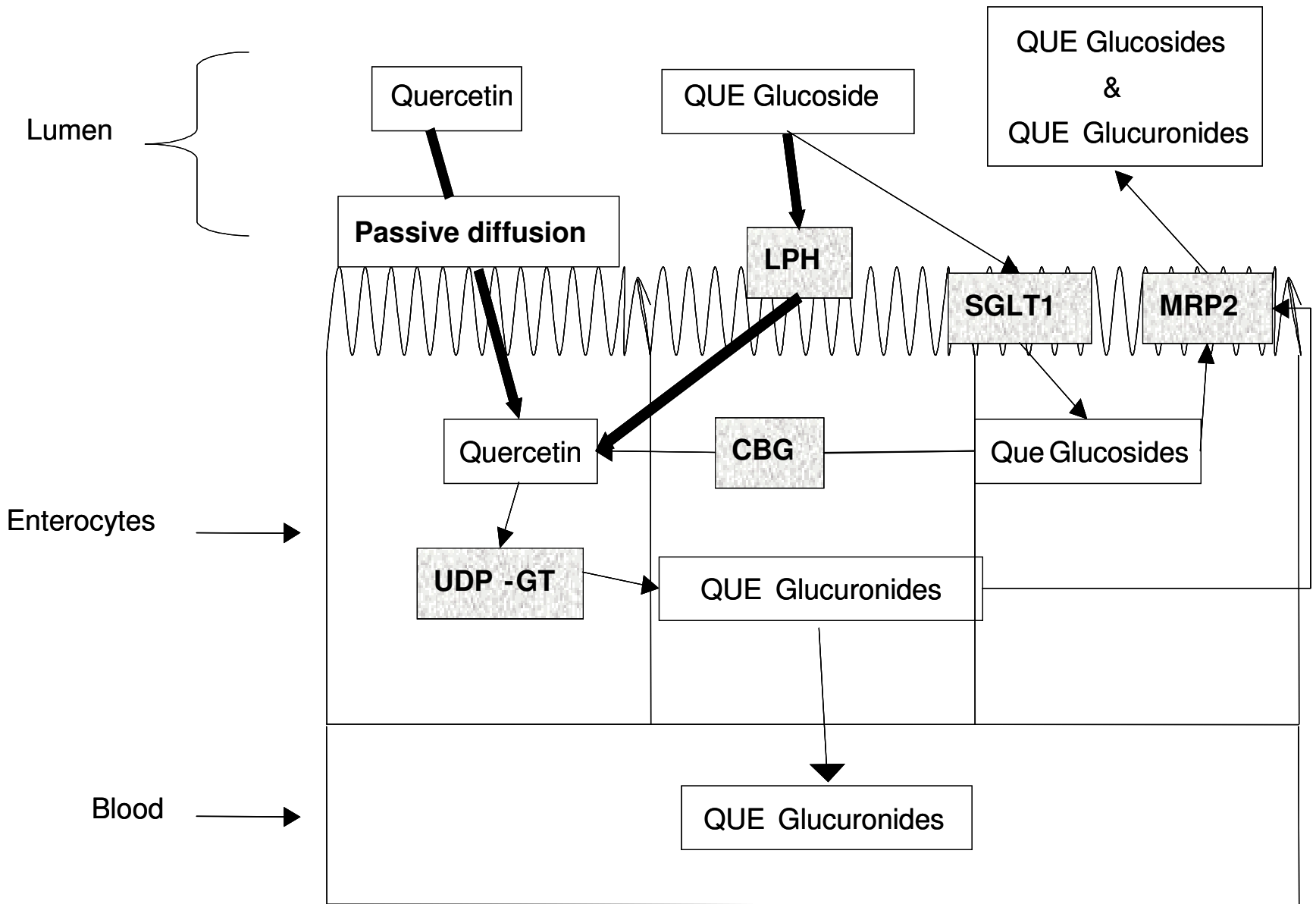


Figure 7

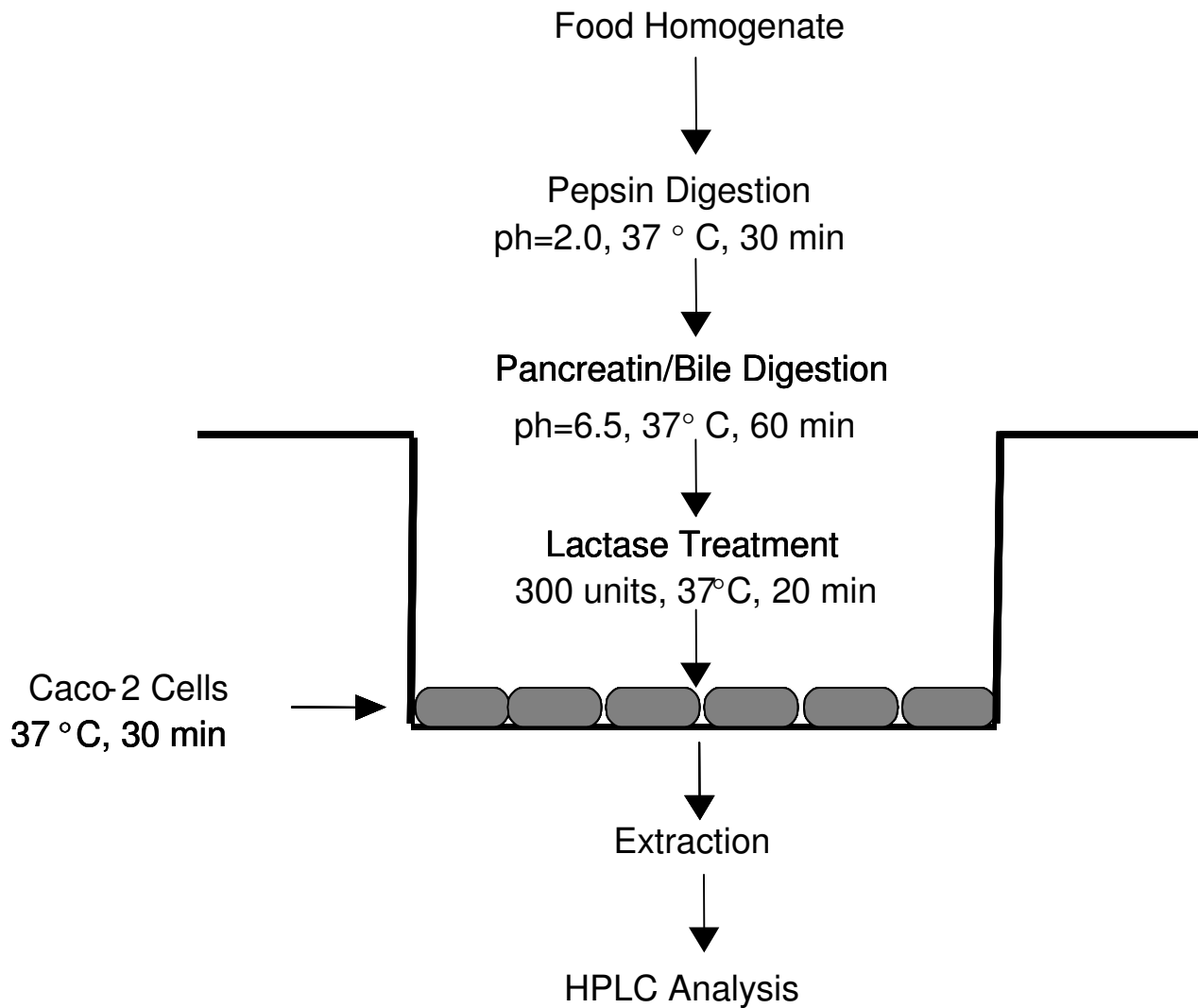


Figure 8