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1 **Lipid protection by polyphenol-rich apple matrices is modulated by pH and pepsin in *in***
2 ***vitro* gastric digestion**

3

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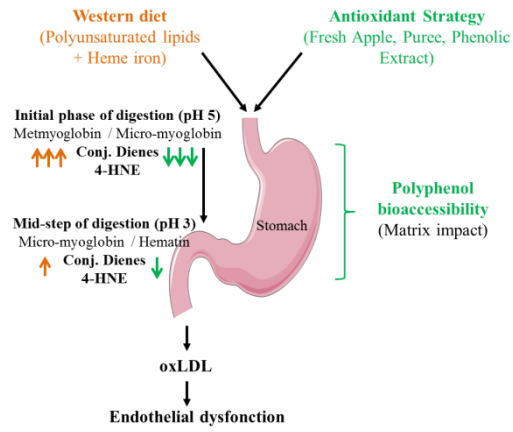
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18

19 **Abstract**

20 Lipid oxidation takes place in the gastric tract after the ingestion of a Western diet rich in ω -6
21 polyunsaturated fatty acids (PUFA) and red meat (heme iron). The incorporation of oxidation
22 products such as 4-hydroxy-2-nonenal (4-HNE) into low-density lipoproteins is further
23 correlated to endothelial dysfunction. Gastric postprandial stress could thus be reduced by
24 antioxidant phytonutrients. The aim of this study was to investigate dietary lipid
25 oxidation and its inhibition by apple polyphenols under different matrix forms (fresh fruit,
26 puree, extract) in *in vitro* gastric digestion conditions. A deep insight was given into the two
27 factors pH and pepsin governing the metmyoglobin-initiated lipid oxidation of sunflower oil-
28 in-water emulsions simulating the physical state of dietary lipids. Our results first showed that
29 pepsin accelerated lipid oxidation at pH 5 through the formation of a micro-metmyoglobin
30 form likely displaying a higher accessibility to lipids. Spectroscopic studies further
31 highlighted the formation of a reversible unfolded metmyoglobin form at pH 3 which was
32 shown to be more pro-oxidant in the absence of pepsin. At nutritional levels, the three apple
33 matrices inhibited less efficiently the accumulation of lipid-derived conjugated dienes and 4-
34 HNE at pH 5 when pepsin was present whereas at pH 3 the opposite was true. High initial
35 bioaccessibilities of monomeric phenolic compounds were evidenced for both puree (57-74%)
36 and the phenolic extract (79-96%) compared to fresh apple (1-14%) supporting their greater
37 antioxidant capacity. By contrast, the bioaccessibility of dimer B2 was low for all matrices
38 suggesting non-covalent binding to apple pectins.



39

40

41 **Keywords:** heme iron, lipid oxidation, 4-hydroxy-2-nonenal, antioxidant, apple,

42 proanthocyanidins.

43

44

45 **Introduction**

46 The gastric tract has been proposed as a major site for diet-related oxidative stress and
47 antioxidant activity of plant micronutrients.^{1, 2} The Western diet brings elevated levels of ω -6
48 polyunsaturated fatty acids (ω -6 PUFA) which are markedly prone to oxidation. Indeed, the
49 heme iron-initiated oxidation of oil-in-water emulsions used to simulate the physical state of
50 dietary lipids in gastric digestion has resulted in the production of lipid-derived conjugated
51 dienes and short-chain aldehydes and alcohols.^{3, 4} The ingestion of a Western type meal by
52 minipigs further led to accumulating levels of TBARS in the gastric tract.² Lipid oxidation
53 products are readily absorbed in the gastrointestinal tract (GIT) and incorporated into
54 chylomicrons and then LDL as shown for humans, pigs and rats.⁵⁻⁷ Among lipid oxidation
55 products, 4-hydroxy-2-nonenal (4-HNE) is a genotoxic and cytotoxic α,β -unsaturated
56 hydroxyalkenal specifically generated from ω -6 PUFA. 4-HNE administered to rats proved to
57 be absorbable and largely metabolized as shown by the identification of 22 urinary
58 metabolites.⁸ 4-HNE contributes to the atherogenicity of oxidized LDL (oxLDL) by forming
59 4-HNE-apoB adducts that deviate the LDL metabolism to the scavenger receptor pathway of
60 macrophagic cells then leading to the formation of foam cells. oxLDL has been clearly
61 identified as a risk factor for the endothelial function and a key step in the atheromatous
62 plaque formation.^{9, 10} Additionally, 4-HNE is more effective at modifying the affinity for the
63 apoB receptor than smaller aldehydes like MDA.¹¹

64 On the other hand, meta-analyses have repeatedly indicated an inverse relationship between
65 coronary artery disease or stroke and the consumption of fruit and vegetables (F&V).¹²⁻¹⁴ In
66 recent controlled trials with apple, cocoa or other flavonoid-rich F&V, the vascular function
67 was improved along with the plasma NO status and inflammation-related markers.^{15, 16 17}
68 Monomeric and oligomeric proanthocyanidins appear as potential mediators in reducing
69 blood pressure and improving endothelium mediated-dilation¹⁸⁻²⁰ when their circulating

70 metabolites were shown to reduce monocyte adhesion to endothelial cells through modulation
71 of gene expression and TNF- α .^{21, 22} Proanthocyanidins represent the second polyphenol group
72 in the French diet although this class is largely underestimated due to uncomplete
73 extractability.^{23, 24} Apple is the major contributor to this class and its phenolic content can
74 vary by a 5-fold factor depending upon apple variety and growth conditions.²⁵⁻²⁷ Monomeric
75 and oligomeric proanthocyanidins represent more than 80% of apple phenolic compounds
76 followed by hydroxycinnamic acids, flavonols, dihydrochalcones and anthocyanins in
77 decreasing order. After ingestion, native form of polyphenols may be released in the chyme
78 and exert their antioxidant capacity directly in the gastric tract. The vegetable matrix, food
79 processing, and constituents from both the diet and bolus may all be factors affecting the
80 bioaccessibility of polyphenols. As a matter of fact, the strong affinity between polyphenols
81 and either dietary proteins or fibers largely decreases polyphenol *in vivo* bioaccessibility in
82 the gastric tract.^{28, 29} During gastric digestion, partial proteolysis and lipolysis take place
83 under the action of pepsin and gastric lipase, respectively.³⁰ In addition, gastric pH sharply
84 increases to values between 5 and 7 after meal intake before slowly returning to a basal pH
85 close to 2 after gastric emptying.^{31, 32} One aim of this study is to evaluate the effect of pH and
86 pepsin on lipid oxidation and its inhibition by apple polyphenols under different matrix forms
87 in the *in vitro* digestion of an oil-in-water emulsion simulating a Western type diet. Because
88 lipid oxidation is triggered by heme iron, this study also gives an insight into the myoglobin
89 forms present at the different pH and after the action of pepsin to shed light on the initiation
90 and inhibition mechanisms for lipid oxidation in emulsion systems.

91

92 **Materials and methods**

93 **Chemicals and Solvents**

94 *Chemicals:* Horse heart myoglobin (M1882, type II), porcine hematin
95 (hydroxyprotoporphyrin IX), egg yolk L- α -phosphatidylcholine (P3556) (PL), porcine pepsin
96 (P6887), 2,4-dinitrophenylhydrazine (DNPH), toluene- α -thiol and (\pm)- α -tocopherol were
97 purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Polyphenol standards, (-)-
98 epicatechin, chlorogenic acid, *p*-coumaric acid, and phloridzin dihydrate were from Sigma-
99 Aldrich. Analysis of the PL showed the presence of phosphatidylcholine (33%),
100 phosphatidylethanolamine (13%), sphingomyelin (3%), phosphatidylinositol (2%), and
101 lysophosphatidylcholine (2%) along with a neutral fraction containing triacylglycerols (47%).
102 Isoquercitrin was purchased from Extrasynthèse (Genay, France) and 4-hydroxy-2-nonenal
103 from Bertin Pharma (Montigny le Bretonneux, France).

104 *Solvents:* HPLC-MS grade methanol, acetonitrile, acetone, hexane, toluene, 2-propanol were
105 from Fisher Scientific (Illkirch, France), formic acid from Merck (Darmstadt, Germany), and
106 hydrochloric acid from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ultrapure water
107 (resistivity 18.2 M Ω .cm⁻¹ at 25 °C) was obtained with a Millipore OPak 2 (Bedford, MA,
108 USA).

109

110 **Characterization of sunflower oil**

111 To determine the fatty acid composition of sunflower oil (Rustica from Leclerc, lot A21815),
112 15 mg of oil were solubilized in 200 μ L of toluene before transesterification at 100 °C during
113 1 h by addition of 2 mL of sulfuric acid/methanol (0.025/0.975, v/v). Then, 2 mL of water and
114 2 mL of hexane were added and homogenized before injection of 1 μ L of the organic phase
115 onto a gas-chromatograph equipped with a flame ionization detector (Clarus 500 GC, Perkin
116 Elmer) using a DB-225ms column (thickness 0.25 μ m, height 0.25 μ m, length 30 m,
117 Agilent). Chromatographic conditions were as follows: carrier gas was H₂ at 37 cm/s,
118 injection temperature was 250 °C in the split mode (ratio 20), oven temperature was held at

119 60 °C for 1 minute and then ramped up at 20 °C/min to 180 °C and then at 3 °C/min to 230
120 °C. Fatty acid identification was made by comparing the retention time of FAME peaks from
121 samples with standard solutions (FAMES 37, Supelco). The results are expressed in relative
122 percentage of each fatty acid, calculated by internal normalization of the chromatographic
123 peak area. This sunflower oil contained 9.8% of saturated fatty acids (6.3% C16; 3.3% C18;
124 0.2% C20), 27.8% of monounsaturated fatty acids (0.1% C16:1; 27.0% C18:1n-9; 0.6%
125 C18:1n-7; 0.1% C20:1) and 62.4% of polyunsaturated fatty acids (62.4% C18:2; 0.01%
126 C18:3). α -Tocopherol amounted to 665 ppm.

127

128 **Preparation of the different apple matrices**

129 Fresh apples (*Reinette de Flandre*) were collected from an organic orchard in Northern France
130 in autumn 2015. Apples were stored in a cold room at 4 °C during 3 months without any
131 change in the phenolic content.

132 The apple puree was obtained by first cutting the fresh apples into 12 pieces with concomitant
133 removal of the core (skin preserved). The apple pieces were further halved for puree
134 preparation or quick-freezing followed by freeze drying for extraction. The apple pieces (2396
135 g) dispersed in 550 mL of water were rapidly cooked in a saucepan to avoid oxidation on an
136 induction cooker set at 2000 W and 120 °C during 20 min keeping the temperature at the
137 heart of the puree at 80 °C. After cooking, the puree was homogenized in a blender for 5
138 seconds. The puree was poured into 250 g-jam jars before thermal treatment in an autoclave at
139 100 °C for 20 min. The jars were stored at 4 °C until use. The apple polyphenol extract was
140 obtained by extraction of the homogenized freeze-dried apple powder (200 g by blender) by
141 hexane (600 ml) once followed by acetone/water (60/40, v/v, 1.3 L) three times under
142 magnetic stirring during 15 min at room temperature. Between each extraction, the apple
143 powder was filtered on Whatman filter paper (grade 40) using a vacuum pump. The pooled

144 acetone/water phases containing polyphenols were concentrated on a rotary evaporator prior
145 to freeze-drying. The phenolic extract containing soluble sugars was stored at -20 °C before
146 use.

147

148 **Qualitative analyses of apple phenolic compounds by UPLC/DAD/ESI-MS**

149 Separation and identification of apple phenolic compounds were performed by using a Waters
150 ACQUITY UPLC chromatograph (Milford, MA) coupled to an UV-vis diode-array detector
151 and a HCT ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped
152 with an electrospray ionization source. Separation was carried out using a reverse-phase
153 Acquity BEH C18 column (100 mm x 2.4 mm i.d., 1.7 µm; Waters) at 35 °C. The mobile
154 phase was constituted by a binary solvent system with water/formic acid (99.95/0.05, v/v,
155 solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 mL/min. The elution gradient was
156 as follows: 0-3 min, isocratic 3% B; 3-7 min, linear 3-6% B; 7-13 min, linear 6-10% B; 13-20
157 min, linear 10-30% B; 20-22 min, linear 30-100% B; 22-23 min, isocratic 100% B; 23-23.5,
158 linear 100-3% B; 23.5-25 min, isocratic 3% B. The volume injected was 2 µL of aqueous
159 solutions of freeze-dried apple (10 mg/mL). Mass detection was conducted in the negative
160 ionization mode from m/z 100 to 1400. MS conditions were as follows: capillary voltage of 2
161 kV, dry gas flow rate at 12 L/min, desolvation temperature at 365 °C and nebulization
162 pressure at 60 psi.

163

164 **Analysis of procyanidins by thioacidolysis and polyphenol quantification.**

165 Quantification of (+)-catechin, (-)-epicatechin, phloretin glycosides, quercetin glycosides was
166 performed before thioacidolysis as follows. Freeze-dried apple matrices (50 mg) were
167 dissolved in 800 µL of anhydrous methanol and 400 µL of 1% acetic acid in anhydrous
168 methanol. Extraction was conducted at 40 °C during 18 min in an US bath with vortexing

169 every 5 min. The supernatant was filtered at 0.45 μm (PTFE) before injection onto an Ultra
170 Fast LC Prominence system (Shimadzu, Kyoto, Japan). Separation conditions were as in Le
171 Bourvellec et al. (2011).³³ (+)-catechin, (-)-epicatechin, quercetin and phloridzin were used as
172 standards and response factors were obtained from 6 independent 40 mg/L solutions.
173 Procyanidin analysis was achieved after thioacidolysis. For this, freeze-dried apple matrices
174 (50 mg) were dissolved in 800 μL of a 5% solution of toluene- α -thiol in anhydrous methanol
175 then added with 400 μL of 0.4 N HCl in anhydrous methanol. The reaction was conducted as
176 above (no US) during 30 min before cooling on ice, filtration and injection. 5-Caffeoylquinic
177 acid and 4-*p*-coumaroylquinic acid were more accurately quantified as their methylated
178 derivatives in the thiolysis experiment at 320 nm using 5-caffeoylquinic acid and *p*-coumaric
179 acid as standards. Epicatechin benzylthioether was expressed as epicatechin at 280 nm.
180 Procyanidins were characterized by their subunit composition and average degree of
181 polymerization (mDP). The mDP was calculated as the molar ratio of all the flavanol units
182 (thioether adducts plus terminal units) to (-)-epicatechin and (+)-catechin corresponding to
183 terminal units. Analyses were run in triplicate for each matrix.

184

185 **Preparation of gastric model emulsions**

186 The physical state of the gastric content was modeled by 10% oil-in-water emulsions
187 stabilized by egg yolk phospholipids (PL). In a 60 mL short-necked glass bottle, 100 mg of
188 PL were dispersed in 36 mL of either citrate-phosphate buffer at pH 3 (10 mM citric acid and
189 20 mM sodium hydrogenphosphate) or an acetate buffer at pH 5 (10 mM) using a rotor stator
190 homogenizer (SilentCrusher M-01, Heidolph) at 24000 rpm for 2 min at room temperature.
191 Then, 4 g of sunflower oil were added and the biphasic mixture was homogenized again at
192 24000 rpm for 2 min. The resulting emulsion was sonicated for 8 periods of 30 s with a rest
193 interval of 30 s and an amplitude of 40% on ice (Q700, QSonica, 20 kHz). For the study of

194 lipid oxidation, 13.5 mL (13.35 mL in the presence of pepsin) of the fine emulsion were
195 transferred in a 50 mL round-bottom flask. Pepsin was diluted in the appropriate buffer at a
196 concentration of 6.25, 25 and 100 mg/mL prior to use. When needed, 150 μ L of pepsin were
197 added to the emulsion to obtain final concentrations of 0.0625, 0.25, and 1 mg/mL (2828 \pm 159
198 U/mg, pepsin activity measured as in Minekus et al., 2014).³⁰ For antioxidant evaluation, 150
199 μ L of a 10 mM (-)-epicatechin solution in methanol were added to the emulsion (100 μ M
200 final) or replaced by 150 μ L of methanol in control experiments. For the phenolic extract, 111
201 mg of the dry extract were first solubilized in 1 mL of the appropriate buffer (10 mM in total
202 phenolic compounds) before the addition of 150 μ L to the emulsion. Puree (125 mg) was
203 directly added in the emulsion to reach 100 μ M final in phenolic compounds. Mastication of
204 fresh apple as in the oral phase was obtained by grinding during 3 seconds 60 g of 2 apples
205 freshly cut in 24 pieces as described above in a domestic grinder (Valentin, Seb) in the
206 presence of the appropriate buffer (30 g). A final concentration of 133 μ M in phenolic
207 compounds was obtained by adding 215 mg of this mixture to the emulsion. Lipid oxidation
208 was next initiated by adding 1.5 mL of a 200 μ M solution of metmyoglobin (MbFe^{III}) (ϵ =
209 7700 M⁻¹ cm⁻¹ at 525 nm)³⁴ prepared in ultrapure water leading to a 20 μ M final
210 concentration. Round-bottom flasks were protected by punched parafilm and incubated in an
211 oven at 37 °C under constant magnetic agitation at 280 rpm. All the experiments were run at
212 least in triplicate.

213

214 **Measurement of lipid-derived conjugated dienes**

215 Every hour, emulsion samples (200 μ L) were diluted in 2-propanol (1000 μ L) before
216 centrifugation (5 min at 16 200g, 4 °C). After further dilution of the supernatant (200, 100 or
217 50 μ L) in iPrOH (2 mL), the concentration in conjugated dienes (CD) was determined by
218 measuring the absorbance at 234 nm (HP 8453 diode-array spectrometer; optical path length 1

219 cm). The molar absorption coefficient used for conjugated linoleyl hydroperoxides was 27
220 $000 \text{ M}^{-1} \text{ cm}^{-1}$.³⁵

221

222 **Measurement of 4-hydroxy-2-nonenal (4-HNE)**

223 Emulsion samples (200 μL) were diluted in 2-propanol (400 μL) before centrifugation (5 min
224 at 16 200g, 4 $^{\circ}\text{C}$) and stored at -20 $^{\circ}\text{C}$ until analysis of 4-HNE. Derivatization with DNPH
225 was conducted as follows: equal volumes (400 μL) of the reagent (50 mg DNPH in 20 mL of
226 acetonitrile and 0.4 mL of formic acid) and the supernatant were mixed in a 1.5 mL HPLC
227 vial and incubated for 1 h at room temperature under stirring. Separation and quantification of
228 derivatized 4-HNE was performed by using the UPLC/DAD/ESI-MSn system described
229 above for apple phenolics with the same column at 40 $^{\circ}\text{C}$. For chromatographic separation,
230 the mobile phase was constituted by a binary solvent system with water/formic acid
231 (99.95/0.05, v/v, solvent A) and acetonitrile (solvent B) at the flow rate of 0.4 mL/min. The
232 volume injected was 2 μL . The elution gradient was as follows: 0-1 min, isocratic 50% B; 1-9
233 min, linear 50-98% B; 9-10 min, isocratic 98% B; 10-10.1 min, linear 98-50% B and 10.1-11
234 min, isocratic 50% B. MS conditions were: : ionization in negative mode, capillary voltage of
235 4 kV, end plate offset -500 V, dry gas flow rate at 12 L/min; desolvation temperature at 360
236 $^{\circ}\text{C}$ and nebulization pressure at 55 psi. Quantification was performed in the multiple reaction
237 monitoring (MRM) mode. Quantification was based on the transition between parent ion at
238 m/z 335 and fragment ion at m/z 167 (retention time 3.96 min) for derivatized 4-HNE. Five
239 point-calibrations (1.02 μM to 0.064 μM) were run with 4-HNE diluted in acetonitrile and
240 reacted with DNPH as described above. The stability of the adduct between DNPH and 4-
241 HNE was evaluated by injection of the same sample every hour during 6 hours. The variation
242 was less than 5% (data not shown).

243

244 **Analyses of α -tocopherol and (-)-epicatechin**

245 The emulsion sample diluted twice in isopropanol as described for 4-HNE analysis was also
246 used for the simultaneous analysis of (-)-epicatechin and α -tocopherol. Separation and
247 quantification were performed by using an ACQUITY UPLC system coupled to a DAD and a
248 fluorescence detector (Waters, Milford, MA). Separation was carried out using an Acquity
249 BEH C18 column (50 mm x 2,1 mm i.d., 1.7 μ m; Waters) at 35°C. Mobile phase was
250 constituted by a binary solvent system with water/formic acid (99.95/0.05, v/v, solvent A) and
251 methanol (solvent B) at a flow rate of 0.2 mL/min. The volume injected was 2 μ L. The elution
252 gradient was as follows: 0-5 min, linear 5-60% B; 5-6.5 min, linear 60-100% B; 6.5-11.5 min,
253 isocratic 100% B; 11.5-12 min, linear 100-5% B, and 12-16 min, isocratic 5% B. (-)-
254 Epicatechin was detected by absorption at 280 nm, α -tocopherol by fluorometry (excitation
255 290 nm; emission 330 nm) and both were quantified after calibration with the appropriate
256 standards.

257

258 **Determination of polyphenol bioaccessibility**

259 Polyphenol bioaccessibility was determined as the content in free phenolic compounds in the
260 aqueous phase of the emulsions at the beginning of the simulated gastric digestion. A 500 μ L
261 aliquot of the emulsion was centrifuged at 16 000g for 5 min at 4 °C. The aqueous phase was
262 removed via syringe, filtered (Phenex RC 0.45 μ m) and stored at -20 °C until analysis of the
263 phenolic compounds as described above. For quantification, 5 point-calibrations were run in
264 UPLC/DAD/MS with phenolic standards in methanol. Monomeric and dimeric procyanidins
265 were quantified as epicatechin equiv. (280 nm), chlorogenic acid at 320 nm; *p*-coumaroyl
266 derivatives as *p*-coumaric acid equiv. (320 nm); dihydrochalcones as phloridzin equiv. (280
267 nm) and flavonols as isoquercitrin equiv. (350 nm).

268

269 **Impact of pH, pepsin and (-)-epicatechin on the stability of metmyoglobin**

270 The impact of pH on the stability of metmyoglobin was investigated using UV-vis
271 spectroscopy in the kinetic mode with spectral recording every 30 s (HP 8453 diode-array
272 spectrophotometer with optical path length 1 cm). In 1950 μL of a 10 mM citrate/ 20 mM
273 phosphate buffer at pH 7 placed in a quartz cell were added 50 μL of 200 μM metmyoglobin
274 prepared in the same buffer (5 μM final). A Mettler Toledo microelectrode was introduced
275 just below the surface of the solution. After warming at 37 °C under stirring at 1000 rpm,
276 acidification was conducted from pH 7 to 3 by stepwise addition of small volumes of 1 N HCl
277 (0.8-10 μL). When pH was at 3 for 6 or 30 min, incremental volumes of 1 N NaOH were
278 added to increase pH to 6.25. Stability was reached at 410 nm (Soret band) before the next
279 HCl or NaOH addition. Measured pH values at 37 °C were lower by 0.05 pH unit from values
280 at 25 °C.

281 The effect of pepsin and (-)-epicatechin on the metmyoglobin stability was evaluated as
282 follows. To 1910 μL of 10 mM citrate/20 mM phosphate buffer acidified at pH 5, 4.75, 4.5,
283 and 4.25 were added 20 μL of (-)-epicatechin (2.5 mM solution in MeOH, 25 μM final) or 20
284 μL MeOH (absence of epicatechin), followed 30 s later by 50 μL of MbFe^{III} (200 μM in
285 water, 5 μM final), then 3 min later by 20 μL of pepsin (6.25 mg/mL in 10 mM acetate buffer
286 at pH 5, 0.0625 mg/mL final, activity 2041 ± 177 U/mg pepsin determined as in Minekus et al.,
287 2014).³⁰ Spectra were recorded every 1 to 5 s and first-order kinetics data extracted using the
288 internal software. All experiments were run under constant magnetic stirring at 1000 rpm and
289 at 37 °C in triplicate.

290

291 **Statistical Analyses**

292 All the results are expressed as the mean \pm standard deviation (SD). One-way analysis of
293 variance (ANOVA) was performed to test the effect of variation factors. If significant effects

294 were found at a 95% confidence level, ANOVA was followed by a Tukey-Kramer post hoc
295 test to identify differences among groups (XLStat software, version 2013, Addinsoft SARL,
296 Paris, France).

297

298 **Results**

299 **Characterization of the different apple matrices**

300 A total of 46 compounds was identified by UPLC/DAD/ESI-MS (Suppl. Table 1) and found
301 to belong to the main four phenolic classes usually present in dessert apples: flavan-3-ols,
302 hydroxycinnamic acids, dihydrochalcones and flavonols. The quantification of the major
303 compounds, represented by oligomeric procyanidins and chlorogenic acid, points to a
304 statistically similar distribution for all three matrices (Table 1). Monomeric and oligomeric
305 procyanidins account for 5-7% and 70% of all the phenolic compounds, respectively. Only
306 epicatechin was identified as extension units in oligomeric procyanidins whereas both
307 catechin and epicatechin appeared as terminal units. This constitution and a relatively low
308 mean degree of polymerization (mDP = 5) are classically observed in dessert apple.³⁶ The
309 UPLC separation conducted on the apple matrix allowed the identification of various B-type
310 oligomers up to heptamers. Hydroxycinnamic acids, the second most abundant class, are
311 represented by 5-caffeoylquinic acid (19-20%) and 4-*p*-coumaroylquinic acid as a minor
312 compound (0.5-0.8%). 5-*p*-Coumaroylquinic acid was also found in half the amount
313 compared to 4-*p*-coumaroylquinic acid (not shown). Dihydrochalcones were present as
314 phloretin-2'-O-glucoside (1.8-2.3%) and phloretin-2'-O-xyloglucoside (0.6-0.9%). Flavonols,
315 constituted mainly by quercetin monohexosides, were weak contributors (1.4-1.9%). This
316 composition is in agreement with the average composition for dessert apples and location of
317 flavonols in apple skin only.³³ Additionally, the occurrence of taxifolin hexoside (**10**) and
318 cinchonains I, II, and III (**5**, **32**, **40**) was demonstrated for the first time in an apple genotype.

319 Moreover, the total polyphenol content was markedly high for the cultivar *Reinette de*
320 *Flandre* used in this study (4.3 g per kg FM) compared to Golden, Braeburn, and Granny
321 Smith (1 g max per kg)³⁶ and is closer to contents evidenced in cider apple.³⁷

322

323 **Impact of pH, pepsin and apple matrices on lipid peroxidation**

324 Metmyoglobin-initiated lipid oxidation of 10% oil-in-water emulsions stabilized by egg yolk
325 phospholipids was investigated at pH 5 and pH 3 in the absence and presence of pepsin. An
326 insight was then given into the antioxidant capacity of different apple matrices with
327 polyphenol concentration higher for fresh apple (133 μ M total polyphenols) than for puree or
328 phenolic extract (100 μ M) to account for an expected lower bioaccessibility. Apple matrices
329 were also compared with epicatechin (100 μ M), the main constitutive unit of monomeric and
330 oligomeric procyanidins.

331 At pH 5 in the absence of pepsin and antioxidant, lipid-derived conjugated dienes (CD)
332 accumulated in an almost linear pattern whereas the secondary lipid oxidation marker 4-HNE
333 tended to plateau after 3 h (Fig. 1A-B). α -Tocopherol was consumed within 15 min (Suppl.
334 Fig. 1D) in agreement with the observed slower rates for CD and 4-HNE formations during
335 the first hour. Epicatechin appeared by far as the best inhibitor, totally inhibiting the
336 accumulation of both CD and 4-HNE for 3 h (Fig. 2A-B). In addition, epicatechin increased
337 the lifetime of α -tocopherol from 15 min to 3 h (Suppl. Fig. 1E) and was finally consumed
338 within 4 h at which stage lipid oxidation initiated. With apple products, the lag-phase
339 durations were the same considering both lipid oxidation markers, ranging from 1 h for fresh
340 apple to 2 h for puree and the phenolic extract. The phenolic extract appeared more efficient
341 than puree and fresh apple during the first 3 h. In the second half of the kinetics, the protective
342 capacities of fresh apple and puree may appear opposite when addressing both lipid oxidation
343 markers. Marker 4-HNE should be more reliable as it is specifically quantified by mass

344 spectrometry. By contrast, CD were quantified at 234 nm in the presence of potential residual
345 substances absorbing at this wavelength such as amino acids and sugars which may have
346 diffused from puree during the 6-h long digestion. Although fresh apple brought 33% more
347 phenolic compounds it proved to be the least efficient matrix for preventing the formation of
348 4-HNE.

349 The addition of pepsin at the levels of 1, 0.25 and 0.0625 mg/mL (2800, 700 and 175 U/mL)
350 significantly enhanced the early accumulation of CD for non-inhibited lipid oxidation ($p <$
351 0.05) (Suppl. Fig. 1A). No lag-phase could be observed suggesting the presence of a different
352 initiating myoglobin form. While a sustained increase of CD accumulation was observed with
353 the lowest pepsin concentrations 0.25 and 0.0625 mg/mL, lipid oxidation tended to level off
354 after 3 h in the presence of 1 mg pepsin per mL. A pepsin concentration of 0.25 mg/mL,
355 simulating mid-digestion conditions was further used to study the antioxidant capacity of the
356 various apple products and the stability of epicatechin and α -tocopherol. In the presence of
357 pepsin, α -tocopherol disappeared in less than 15 min as rapidly as in the absence of pepsin
358 (Suppl. Fig. 1D). By contrast, the 4-HNE formation rate was halved in the presence of pepsin
359 (Fig. 1B). Apple matrices showed a rather similar pattern with total and significantly high
360 inhibitions of CD and 4-HNE accumulations after 1 and 2 h, respectively (Fig. 3). Puree and
361 the phenolic extract proved to be better inhibitors than fresh apple when addressing 4-HNE.
362 After 2 h, lipid oxidation proceeded at a faster rate leading rapidly to an inhibition loss for 4-
363 HNE and apparently higher CD amounts compared to the pepsin control. Epicatechin
364 displayed contrasting effects. While as weakly efficient as apple products for CD inhibition, it
365 markedly retarded the appearance of 4-HNE (up to 3 h) and increased the lifetime of α -
366 tocopherol from 15 to 45 min (Suppl Fig. 1E). By contrast, no protection was afforded to α -
367 tocopherol by apple products. Additionally, epicatechin was more rapidly degraded in the
368 presence (3 h) than in the absence of pepsin (4 h) (Fig.2A and 3A).

369 During human gastric digestion, pH almost linearly decreases from 5 to 3 at a rate depending
370 on the consistency of food.²⁹ Interestingly, the pattern for CD accumulation at pH 3
371 superimposed rather closely with that observed at pH 5 in the presence of pepsin (Fig. 1A).
372 As stated earlier, there was no lag-phase and significantly higher amounts of CD were
373 observed during the first 3 hours compared to lipid oxidation at pH 5. Furthermore, the
374 patterns for 4-HNE production were also markedly similar between pH 3 without pepsin and
375 pH 5 with pepsin (Fig. 1B). Although epicatechin was found to totally inhibit lipid oxidation
376 for 3 hours at pH 5, it appeared poorly efficient at pH 3 with a 50% decrease in CD at 2 h
377 followed by a major loss of the activity at 3 hours (Fig. 2C). Epicatechin retained only a
378 partial activity when focusing on 4-HNE (Fig. 2D). Whereas no 4-HNE was produced until 3
379 h of lipid oxidation at pH 5, epicatechin reduced by ca. 37% the extent of 4-HNE formation at
380 pH 3. Fresh apple, puree and the phenolic extract behaved more or less as epicatechin
381 displaying a weak antioxidant capacity towards CD formation while almost no inhibition of 4-
382 HNE formation. Last, α -tocopherol lifetime was not extended by any of the antioxidant
383 studied (not shown).

384 At pH 3, the addition of pepsin (0.25 mg/mL) had a marked impact on the formation of both
385 markers (Fig. 1). Lipid oxidation only started after 2 h of incubation and its extent was largely
386 reduced with concentrations of only 1.2 mM for CD and 2.7 μ M for 4-HNE after 6 h. At pH
387 3, in the absence of pepsin, the CD and 4-HNE levels were 9.6 mM and 6.6 μ M, respectively.
388 Epicatechin, apple puree and the phenolic extract inhibited almost totally the formation of
389 both CD and 4-HNE during the 6 h-long kinetics. By contrast, fresh apple proved to be rather
390 inefficient. The lifetime of α -tocopherol was found to be less than 15 min for all conditions at
391 pH 3 with pepsin. Finally, epicatechin disappeared in 15 min as already observed in the
392 absence of pepsin.

393

394 **Effect of the matrix on the bioaccessibility of apple phenolic compounds**

395 The bioaccessibility of apple phenolic compounds was evaluated for fresh apple, puree, and
396 the phenolic extract at the initial time of the simulated gastric digestion before addition of
397 metmyoglobin to prevent oxidation (Table 2). Bioaccessible phenolic compounds are the
398 molecules freely present in the aqueous phase after separation of the aqueous and lipid phases
399 of the emulsion. When monomeric flavanols, flavonols, hydroxycinnamic acids and
400 dihydrochalcones were brought as a hydroacetic extract, they were recovered in high
401 yields. Recoveries of 96 and 95% were obtained at pH 5 in the absence and presence of
402 pepsin while these values were 89 and 79% at pH 3, respectively. Chlorogenic acid, phloretin-
403 2'-xyloglucoside and epicatechin proved to be more highly bioaccessible than 4-*p*-
404 coumaroylquinic acid, phloretin-2'-glucoside and flavonols. Additionally, dimer B2, the
405 major procyanidin oligomer in apple, was found to be present in the range 14-19% whatever
406 the conditions. When puree was probed for antioxidant capacity, the recovery of phenolic
407 compounds appeared lower, 74 and 60% at pH 5 while 57 and 60% at pH 3 in the absence and
408 presence of pepsin, respectively. The three more highly bioaccessible compounds were
409 chlorogenic acid, phloretin-2'-xyloglucoside and 4-*p*-coumaroylquinic acid. Dimer B2 was
410 recovered in yields ranging between 7 and 23%. With fresh fruit, the bioaccessibility of
411 phenolic compounds markedly decreased, being 14 and 3% without and with pepsin at pH 5
412 while 1 and 12% at pH 3. Surprisingly, phloretin-2'-xyloglucoside, whose content is
413 commonly higher in apple skin than flesh, was similarly bioaccessible than chlorogenic acid
414 for which the favored location site is reversed.^{33, 36}

415

416 **Impact of pH and pepsin on the structure of metmyoglobin**

417 The structure of metmyoglobin was examined between pH 7 and 3 using visible spectroscopy
418 (Fig. 4A). No spectral change was recorded for metmyoglobin between pH 7 and 4.79.

419 However, a drastic decay of the Soret band absorption peaking at 409 nm was evidenced for
420 pH lower than 4.5. At pH 4.2, the intensity of the Soret band is reduced by half while a further
421 pH decrease to 4.0 led to a loss of the Soret band with the flattening of the absorption band
422 between 370 and 410 nm. Between 3.8 and 3.0, the absorption spectrum slightly evolved
423 towards a band peaking at 370 nm. Isobestic points at 380 and 433 nm suggest the appearance
424 of a single new form only. Although this form had a spectrum more closely related to that of
425 hematin at pH 5 than 3, it proved to be reversible. Indeed, when small increments of a NaOH
426 solution were added, the Soret band was slowly restored (Fig. 4B). Intermediate spectra at pH
427 4.0, 4.3 and 4.6 were observed as in the first part devoted to pH decrease. Further NaOH
428 addition and long equilibration time did not allow to fully recover the initial metmyoglobin
429 spectrum. As a matter of fact, 15 to 18% of the initial structure were lost when the time spent
430 at pH 3 was between 6 min (Fig. 4B) and 30 min (not shown).

431 Pepsin addition to metmyoglobin at pH between 5 and 4 led to a pseudo-first order decrease
432 of the Soret band yielding a hematin-like band peaking at 370 nm (Fig. 4 CD). The presence
433 of epicatechin at pH 5 slightly changed the pattern of this new band towards a more flat one
434 while being without any visible effect at pH 4 (Suppl. Fig. 2). Epicatechin appears to slow
435 down pepsinolysis by 10-20% at pH 5, 4.75 and 4.5 when this process is still slow (Suppl.
436 Table 2 and Suppl. Fig. 3).

437

438 **Discussion**

439 In this study, we first gave an insight into the two main factors influencing lipid oxidation in
440 gastric digestion, *i.e.* pH and pepsin. In Western countries, fat daily consumption comprises
441 100-150 g of triacylglycerols and 2-10 g of phospholipids. In the presence of dietary
442 emulsifiers and following gastric antral contractions, triacylglycerols are recovered as lipid
443 droplets with 50% (initial) and 30% (after 4 h) of the particles in the 1-10 μm diameter

444 range.³⁸ Thus, 10% sunflower oil-in-water emulsions stabilized by 0.25% (w/w)
445 phospholipids were designed to model the physical state of lipids during gastric digestion.
446 Particle size characterization by laser light scattering gave a mode of $11.6 \pm 1.1 \mu\text{m}$ (not
447 shown). The oxidative stress was brought by addition of metmyoglobin to the emulsion at the
448 $20 \mu\text{M}$ level that corresponds to the consumption of 57 g of steak (ca. $20 \mu\text{g}$ heme iron/g
449 cooked meat)^{2, 39} and a chyme volume of 1 L. α -Tocopherol, the main antioxidant in
450 sunflower oil, slowed down lipid oxidation during the first hour when compared to the same
451 reaction with sunflower oil stripped of α -tocopherol.⁴ α -tocopherol, which is located in the
452 lipid droplets, rather acts as an inhibitor of the propagation step. Low concentrations (0.5
453 mM) of conjugated dienes (CD), involving lipid-derived hydroperoxides, were initially found
454 in the emulsion. The latter are required to activate metmyoglobin (MbFe^{III}) into
455 perferrylmyoglobin which auto-reduces to ferrylmyoglobin ($\text{MbFe}^{\text{IV}}=\text{O}$), the prooxidant
456 species involved in the propagation step through the production of lipid-derived peroxy
457 radicals.⁴⁰ This reaction likely takes place at the droplet interface between the lipid and
458 aqueous phases. Besides, the above-described mechanism has been mainly described at pH
459 5.8 for meat in post-mortem conditions. In gastric digestion, metmyoglobin will face a
460 decrease in pH from 6-5 to 2 and pepsin secretion and these two factors can affect the
461 metmyoglobin-initiated oxidation of dietary lipids.³¹

462

463 **Metmyoglobin structures involved in lipid oxidation**

464 Using kinetic monitoring by spectroscopy, it was possible to investigate the effect of pH on
465 metmyoglobin stability and to give an insight into its three dimensional structure. No major
466 structural change could be observed between pH 7 and 4.5. However, the Soret band with a
467 λ_{max} at 409 nm typical of the myoglobin structure largely decayed between pH 4.25 and 4,
468 indicating an exposure of the porphyrin ring to a different surrounding environment. Between

469 pH 3.75 and 3 a larger and more flat absorption band has formed displaying a high analogy
470 with that for hematin. At this stage, the bond between the proximal histidine nitrogen and the
471 iron center could be disrupted. It is worth noting that reversibility to the initial metmyoglobin
472 structure can be obtained upon pH increase. This process requires more time for full structure
473 recovery when compared to the pH decrease process. Unfolding of the protein may result
474 from the change in protonation status (pI ca. 7). A high reversibility (> 82%) was observed
475 when metmyoglobin was left at pH 3 for periods shorter than 30 min. This is of particular
476 interest as these unfolded forms may move to the duodenum upon gastric emptying. The pH
477 in duodenum is ca. 6.5 and metmyoglobin could still play a role as a prooxidant. In our study,
478 lipid oxidation, which was rather initiated by this open form of metmyoglobin than by
479 hematin, proved to be faster at pH 3 compared to pH 5 as shown by the 3-fold excess in CD
480 after 1 h (Fig. 1). By contrast, 4-HNE accumulated more slowly at low pH.

481 Upon addition of pepsin, the initial structure of metmyoglobin was lost as shown by the pH-
482 dependent first-order decay of the Soret band (Suppl. Fig. 3). It is worth noting that pepsin is
483 active at pH 5 although proteolysis appears quite slow at the beginning of the digestion.
484 Decreasing the pH to 4.75, 4.5 then 4.25 speeds proteolysis by factors in the range 5-7, 3-5
485 and finally 2-4, respectively (Suppl. Table 2). As a matter of fact, the activity of pepsin was
486 calculated to increase *ca.* 72 times between pH 5 and pH 4.25. Recently, Sayd et al. identified
487 15 different peptides from myoglobin in the *in vivo* digestion of beef meat by minipigs.⁴¹
488 These peptides mainly mapped the C- and N-terminal regions and one central region not
489 implicated in the binding site of the porphyrin ring. In particular, the bonding between the
490 porphyrin iron center and both His 64 and 93 could be preserved in the remaining non-
491 digested peptide. These results are consistent with data from Schwarzinger and coll. who
492 named micro-myoglobin A the peptide Ile 20-Phe 106 principally obtained from pepsinolysis
493 of sperm whale myoglobin.⁴² Additionally, this peptide exhibited heme-binding activity as

494 demonstrated by the shift for the Soret band from 385 nm to 404 nm attributed to the
495 coordination of the fifth and sixth coordination sites of iron. Whether hematin is ultimately
496 released after prolonged pepsinolysis is still unclear in our study. However, the formation of a
497 micro-metmyoglobin form may no doubt increase the accessibility of surrounding lipids to the
498 iron center. This would result in a higher catalytic activity of MbFe^{III} as observed through the
499 faster rate for CD accumulation. As a matter of fact, the CD level was 5-fold higher after 1 h
500 in the presence of pepsin at pH 5. Carlsen et al. similarly observed faster oxidation rates in
501 methyl linoleate emulsions stabilized by Tween 20 after the addition of MbFe^{III} proteolyzed
502 by pepsin at pH 3.9.⁴³ The prooxidant species also retained partly the initial Soret band in
503 agreement with a heme-peptide structure. By contrast, no accelerated 4-HNE formation could
504 be observed. This α,β -unsaturated aldehyde is highly susceptible to the nucleophilic addition
505 of amino acids such as cysteine, lysine and histidine. Indeed, LC-MS evidenced the coupling
506 of 4-HNE to up to seven histidine residues in myoglobin.⁴⁴

507

508 **A role for apple phenolic compounds as antioxidants in gastric digestion**

509 The 100 and 133 μ M polyphenol concentrations used in this study correspond to the
510 respective intake of only 8 and 9 g of puree and fresh apple per liter of chyme, which is
511 nutritionally relevant. The metmyoglobin-initiated lipid oxidation of phospholipid-stabilized
512 emulsions with pH set at 5 and no pepsin closely simulates the initial stage of gastric
513 digestion when pepsin has not already diffused from the stomach wall into the chyme.
514 Polyunsaturated lipids are totally protected from oxidation by epicatechin and the three
515 studied apple matrices as shown by lag-phases between 1 and 3 h for both CD and 4-HNE
516 markers (Fig. 2AB). Epicatechin, or any other antioxidant phenolic compound located at the
517 interface or in the aqueous phase of the emulsion can interact with heme iron reducing the
518 MbFe^{IV}=O to the MbFe^{III} form, thus inhibiting the initiation step of the radical chain

519 mechanism of lipid oxidation. Furthermore, the presence of epicatechin was found to reduce
520 by ca. 10-20% the proteolysis rate of metmyoglobin at pH 5 and 4.75. Phenolic compounds
521 displaying an affinity for pepsin and/or metmyoglobin could thus slow down lipid oxidation
522 in the initial stage of gastric digestion through a non-antioxidant mechanism.

523 During gastric digestion, pepsinogen and HCl are respectively secreted by chief and parietal
524 cells located in the stomach body and fundus. Upon acidic hydrolysis, pepsinogen releases
525 pepsin which diffuses into the chyme following antral contractions. A physiological pepsin
526 level has been suggested to be 2000 U/mL digesta at mid-digestion.³⁰ In our work, the
527 addition of pepsin at 175, 700, and 2800 U/mL was found to boost initial lipid oxidation by
528 factors 3 to 5. This was ascribed to the formation of a more active micro-metmyoglobin form
529 after partial globin pepsinolysis. In the presence of pepsin (700 U/mL) at pH 5, the
530 antioxidant capacity of the various apple matrices was partly retained (Fig. 3 AB). Polyphenol
531 may bind to pepsin or have a decreased affinity for micro-metmyoglobin, which may prevent
532 them to reduce efficiently ferrylmyoglobin. Despite lipid protection is limited to 3 h in our
533 model system, it remains relevant in human gastric digestion.

534 Decreasing the pH to 3 to simulate mid-digestion was found to influence lipid oxidation and
535 its inhibition by apple matrices rather similarly to pepsin addition at pH 5. Indeed, lipid
536 oxidation initiated faster as shown by increased CD contents after 1 and 2 h (Fig. 1A). In
537 addition, apple matrices only weakly retarded the formation of CD while having no effect on
538 4-HNE formation (Fig. 2AB). Actually, digestion conditions with a low pH and no pepsin are
539 not physiologically relevant due to the concomitant secretion of pH and pepsinogen by the
540 same stomach areas. More interesting for human digestion, lipid oxidation was drastically
541 reduced at pH 3 in the presence of pepsin (Fig. 1AB). A reversible unfolding of globin is
542 expected at this pH. However, pepsinolysis of this open form may be extremely fast likely
543 leading to the release of heme. Although heme- and metmyoglobin-initiated lipid

544 oxidations were found earlier to proceed at comparable rates at both pH 4 and pH 5.8 in
545 phospholipid-stabilized emulsions,³ this is clearly not the case in this work in the presence of
546 pepsin. This difference could be accounted by hematin binding to pepsin. The resulting
547 hematin-pepsin complex may be unable to interact with polyunsaturated lipids owing to its
548 conformation or location in the triphasic emulsion system. Inactivation of iron forms may
549 only be partial as lipid oxidation could start after 3 h. It is worth noting that epicatechin, puree
550 and the phenolic extract strongly inhibited the accumulation of CD and 4-HNE while fresh
551 apple was inactive (Fig. 3CD).

552 Actually, our results obtained *in vitro* by decomposing gastric digestion stages are fully
553 consistent with results obtained *in vivo* with minipigs.² Indeed, CD were found to accumulate
554 following a bell-shaped kinetics and this is supported by the higher oxidizability of PUFA at
555 pH 5 compared to pH 3 in the presence of pepsin. Furthermore, TBARS, as secondary lipid
556 oxidation markers, only slowed down at the end of the digestion as does 4-HNE. Artichoke,
557 plum and apple, added to the meal containing sunflower oil and beef meat, played a protective
558 role by totally and largely inhibiting CD and TBARS formation, respectively. These defrosted
559 or cooked fruit and vegetables are structurally related to puree displaying a loss of plant cell
560 integrity with the subsequent release of phenolic compounds.

561 Our study finally outlined a considerable effect of the matrix on the bioaccessibility of
562 phenolic compounds. Indeed, phenolic compounds were almost fully bioaccessible when
563 provided as an extract (79-96%) and only slightly less bioaccessible when brought as puree
564 (57-74%). Polyphenols may noncovalently bind apple proteins and cell wall
565 polysaccharides.³³ As a matter of fact, higher affinities are exhibited by procyanidins as
566 shown here with dimer B2 whose bioaccessibility remains below 20%. By contrast, almost no
567 phenolic compounds were recovered in the aqueous phase for fresh apple (1-14%) suggesting
568 that mastication is by far less efficient than thermal processing for cell membrane breakdown.

569 Our bioaccessibility data are in line with the general trend observed for the antioxidant
570 capacity of the three matrices, i.e., the phenolic extract and puree similarly inhibited lipid
571 oxidation and, as a matter of fact, more efficiently than does fresh apple.

572

573 **Conclusion**

574 The present study shed some light on the role of pH and pepsin on heme iron-initiated lipid
575 oxidation taking place in the gastric tract after the ingestion of a Western type diet. In
576 particular, it evidenced the formation of two reversibly unfolded or partly proteolyzed forms
577 of metmyoglobin retaining pro-oxidant activities. Because these forms can move forward to
578 the intestinal tract along with PUFA, more work should be done to assess their activity
579 towards mixed micelles of bile salts, free fatty acids and monoacylglycerols after the action of
580 intestinal lipase. The role of trypsin or chymotrypsin on newly formed heme-iron forms
581 should also be evaluated.

582 Although most apple dessert brings 3 to 4 times less polyphenols, the consumption of an
583 apple associated to a Western type diet could provide health benefits directly in the
584 gastrointestinal tract by limiting the formation of deleterious and absorbable lipid oxidation
585 products. Procyanidins are also known to be metabolized by the colon microbiota providing
586 circulating metabolites endowed with the capacity to protect the vascular endothelium. Hence,
587 the reintroduction of such healthy fruits into the Western diet should be encouraged for a
588 better protection towards cardiovascular diseases.

589

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594

595 **Conflicts of interest**

596 There are no conflicts of interest to declare.

597

598

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Table 1. Relative composition (% weight) for main phenolic compounds and classes, characterization of flavan-3-ols and overall polyphenol content for the different apple matrices.

Matrix	Monomeric procyanidins		Oligomeric procyanidins	mDP	Procyanidin characterization				Hydroxycinnamic acids		Dihydrochalcones		Flavonols	Overall content in g/kg (mmol/kg)
	CAT	EPI			Term. units (%)		Ext. units (%)		5-CQ	4-pCou	Phln	Phlx		
Fresh Apple	1.2 ± 0.2	5.6 ± 0.2	70.0 ± 2.2	5.4 ± 0.1	2.6 ± 0.4	8.0 ± 0.2	-	89.4 ± 0.6	18.9 ± 1.0	0.52 ± 0.01	1.8 ± 0.4	0.75 ± 0.03	1.4 ± 0.1	4.31 ± 0.11 (14.2 ± 0.4)
Puree	1.2 ± 0.2	3.8 ± 0.6	70.6 ± 4.1	5.3 ± 0.2	1.9 ± 0.3	11.1 ± 0.9	-	86.9 ± 1.2	18.8 ± 0.4	0.66 ± 0.01	2.1 ± 0.2	0.9 ± 0.1	1.9 ± 0.1	3.75 ± 0.16 (12.2 ± 0.5)
Phenolic extract	0.43 ± 0.04	4.3 ± 0.4	70.2 ± 3.3	4.7 ± 0.1	3.7 ± 0.1	12.5 ± 0.3	-	83.7 ± 0.3	19.8 ± 0.7	0.76 ± 0.03	2.3 ± 0.2	0.55 ± 0.05	1.7 ± 0.2	27.2 ± 1.2 (88.9 ± 4.0)

CAT: (+)-catechin, EPI: (-)-epicatechin, flavonols in equivalent quercetin, mDP: mean degree of polymerization of monomeric and oligomeric procyanidins, 5-CQ: 5-caffeoylquinic acid, 4-pCou: 4-*p*-coumaroylquinic acid, Phln: Phloretin-2'-O-glucoside, Phlx: Phloretin-2'-O-xyloglucoside. Term. units: Terminal units, Ext. units: Extension units. Overall content in g or mmol/kg FM for apple fruit and puree and in g or mmol/kg DM for the extract. Values represent Mean ± SD (*n* = 3).

Table 2. Initial bioaccessibility of apple phenolic compounds for the different matrices in phospholipid-stabilized emulsions at pH 5 and 3 in the absence or presence of pepsin (0.25 mg/mL). Recovery yields given at T0 in %. Polyphenol concentration = 100 μ M for puree, phenolic extract, epicatechin and 133 μ M for fresh apple. Values represented mean \pm SD ($n = 3$).

Matrix	Conditions	Flavanols		Hydroxycinnamic acids		Dihydrochalcones		Flavonols	Total polyphenols
		Epi	Dimer B2	5-CQ	4-pCou	Phln	Phlx		
Fresh	pH 5	0.5 \pm 0.4	34 \pm 7	21 \pm 0	5 \pm 3	6 \pm 3	19 \pm 9	-	14 \pm 0
Apple	pH 5 + pepsin	6 \pm 2	13 \pm 3	-	5 \pm 2	27 \pm 1	-	-	2.9 \pm 0.4
	pH 3	2 \pm 1	1 \pm 0	-	4 \pm 1	1 \pm 0	10 \pm 1	-	0.8 \pm 0.3
	pH 3 + pepsin	1 \pm 0	47 \pm 10	17 \pm 2	8 \pm 6	5 \pm 2	29 \pm 3	-	12 \pm 1
Puree	pH 5	44 \pm 4	23 \pm 13	95 \pm 6	50 \pm 2	32 \pm 14	48 \pm 3	47 \pm 11	74 \pm 3
	pH 5 + pepsin	13 \pm 3	7 \pm 1	85 \pm 10	43 \pm 8	15 \pm 3	27 \pm 3	22 \pm 3	60 \pm 6
	pH 3	25 \pm 9	15 \pm 7	76 \pm 6	39 \pm 5	18 \pm 1	43 \pm 5	23 \pm 6	57 \pm 3
	pH 3 + pepsin	29 \pm 19	10 \pm 2	81 \pm 6	40 \pm 8	15 \pm 2	41 \pm 2	21 \pm 18	60 \pm 6
Phenolic extract	pH 5	40 \pm 26	19 \pm 4	125 \pm 2	57 \pm 20	30 \pm 3	87 \pm 5	41 \pm 15	96 \pm 4
	pH 5 + pepsin	82 \pm 5	16 \pm 1	115 \pm 2	51 \pm 2	31 \pm 0	70 \pm 6	43 \pm 9	95 \pm 1
	pH 3	76 \pm 7	17 \pm 0	104 \pm 4	49 \pm 4	28 \pm 1	116 \pm 1	65 \pm 28	89 \pm 2
	pH 3 + pepsin	47 \pm 27	14 \pm 2	96 \pm 0	40 \pm 6	28 \pm 0	108 \pm 5	51 \pm 16	79 \pm 4

Abbreviations Epi, 5-CQ, 4-pCou, Phln and Phlx are defined in Table 1.

Figure 1. Metmyoglobin-initiated lipid oxidation of phospholipid-stabilized emulsions in the absence or presence of pepsin (0.25 mg/mL) at pH 5 and 3. Accumulation of (A) conjugated dienes ($n = 4 - 5$) and (B) 4-HNE ($n = 3$). Values represent mean \pm SD.

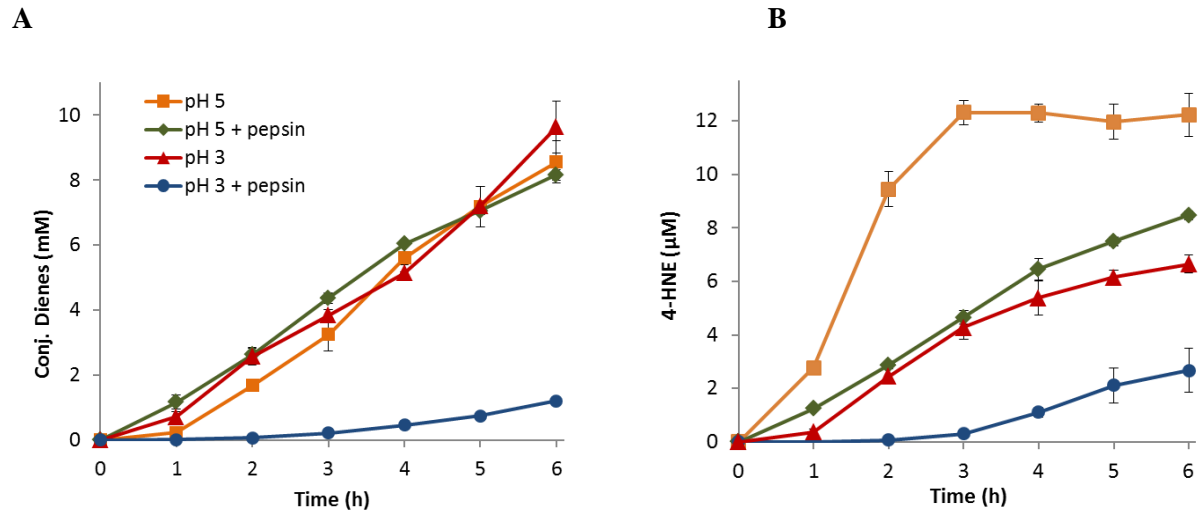


Figure 2. Metmyoglobin-initiated lipid oxidation and its inhibition by different apple matrices in phospholipid-stabilized emulsions. Accumulation of conjugated dienes (A) and 4-HNE (B) at pH 5. Accumulation of conjugated dienes (C) and 4-HNE (D) at pH 3. Dotted red lines are for the consumption of epicatechin. Polyphenol concentration = 100 μM for puree, phenolic extract, epicatechin and 133 μM for fresh apple. Values represent mean \pm SD ($n = 3$ to 5 for CD and $n = 3$ for 4-HNE and epicatechin).

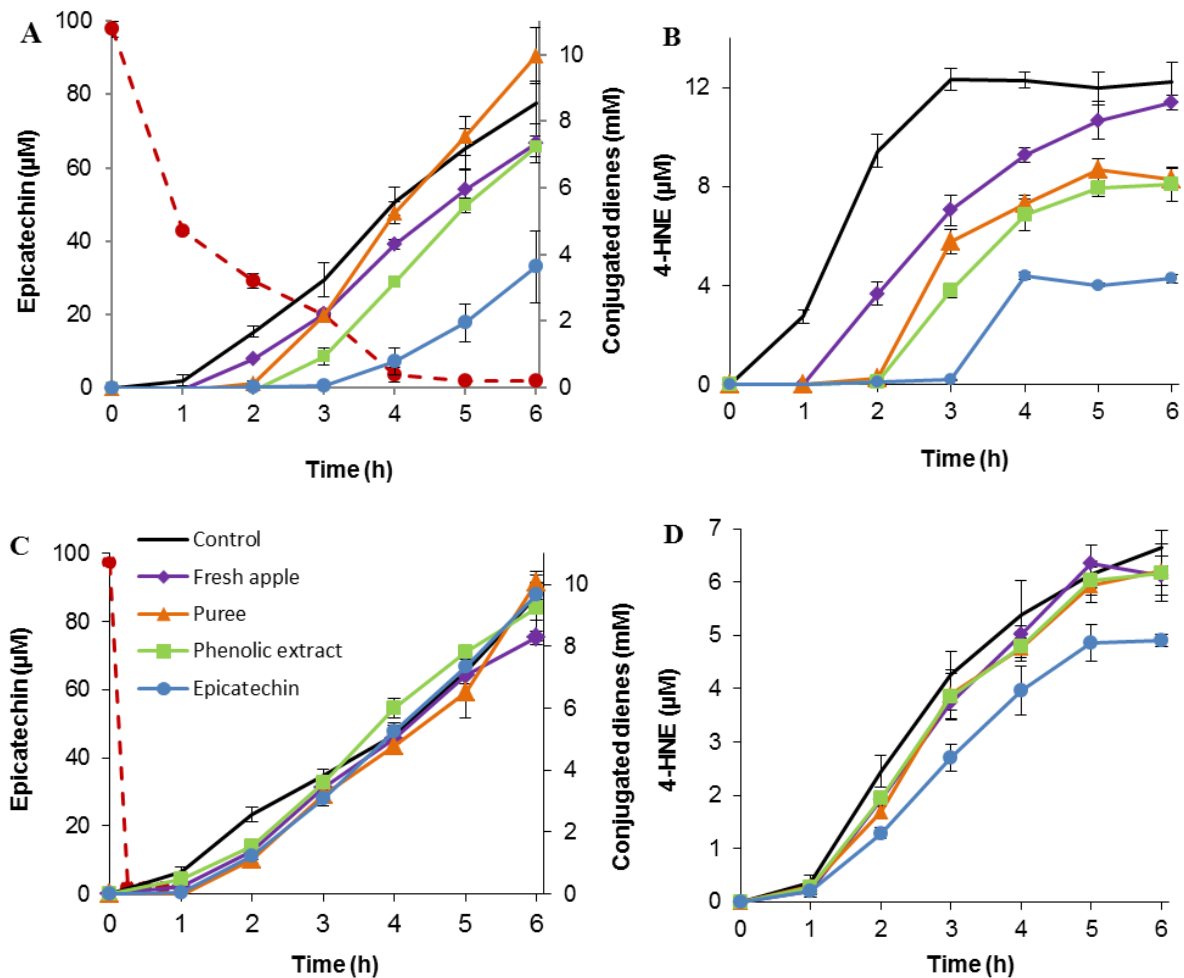


Figure 3. Metmyoglobin-initiated lipid oxidation and its inhibition by different apple matrices in phospholipid-stabilized emulsions in the presence of pepsin (0.25 mg/mL). Accumulation of conjugated dienes (A) and 4-HNE (B) at pH 5. Accumulation of conjugated dienes (C) and 4-HNE (D) at pH 3. Dotted red lines are for the consumption of epicatechin. Polyphenol concentration = 100 μ M for puree, phenolic extract, epicatechin and 133 μ M for fresh apple. Values represent mean \pm SD ($n = 3$ to 5 for CD and $n = 3$ for 4-HNE and epicatechin).

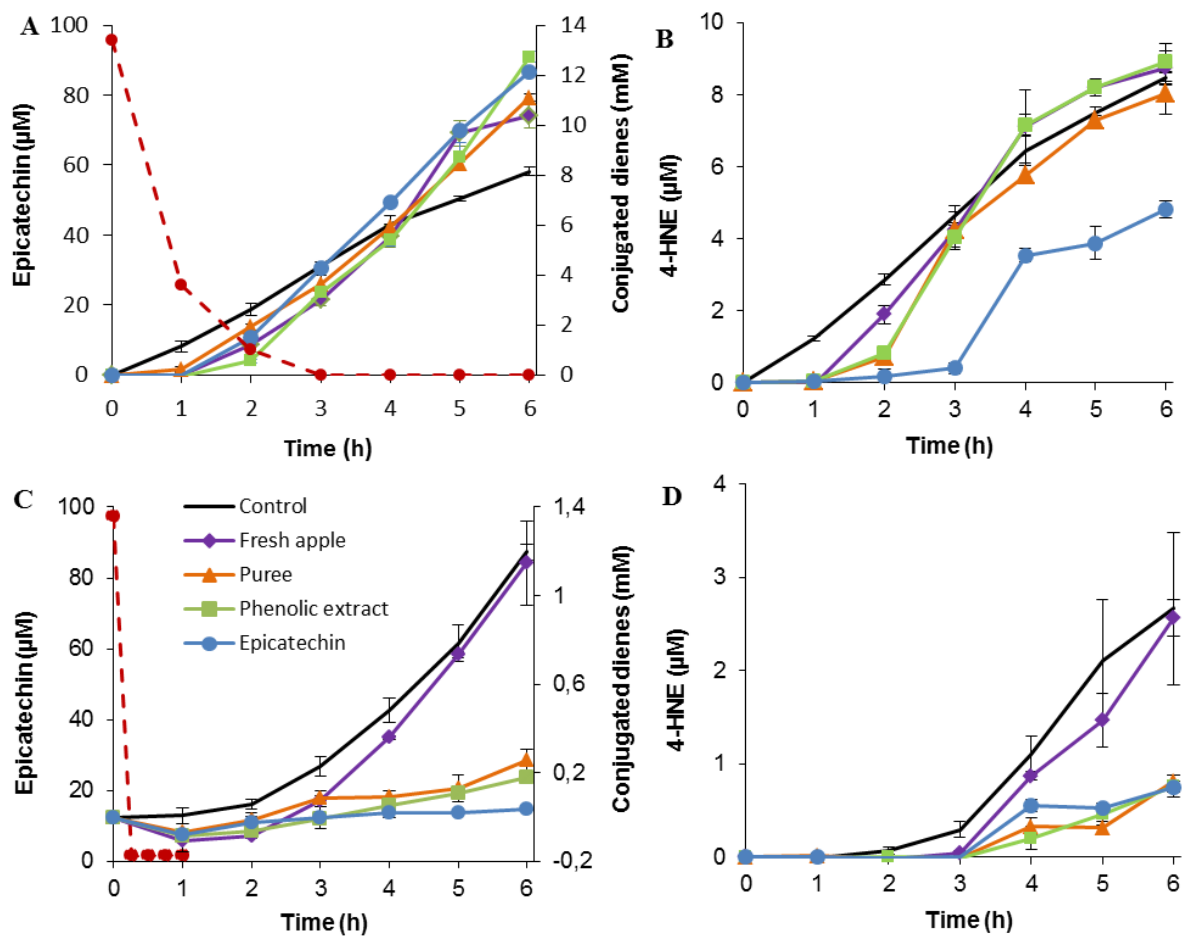


Figure 4. Effect of pH and pepsin on the structure of metmyoglobin (5 μ M). (A) Stepwise pH decrease from pH 7.0 to 3.0; dotted lines are hematin at pH 3.0. (B) Stepwise pH increase from pH 3.0 to 6.27; dotted lines are hematin at pH 5.0 (C) Addition of pepsin (0.0625 mg/mL) on metmyoglobin at pH 5. (D) Addition of pepsin (0.0625 mg/mL) on metmyoglobin at pH 4.

