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2	vitro gastric digestion
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19 Abstract

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



41 Keywords: heme iron, lipid oxidation, 4-hydroxy-2-nonenal, antioxidant, apple,
42 proanthocyanidins.

45 Introduction

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

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

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

91

92 Materials and methods

93 Chemicals and Solvents

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

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

109

110 Characterization of sunflower oil

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

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

127

128 Preparation of the different apple matrices

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

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

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

147

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

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

163

164 Analysis of procyanidins by thioacidolysis and polyphenol quantification.

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

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

184

185 **Preparation of gastric model emulsions**

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

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

213

214 Measurement of lipid-derived conjugated dienes

Every hour, emulsion samples (200 μ L) were diluted in 2-propanol (1000 μ L) before centrifugation (5 min at 16 200*g*, 4 °C). After further dilution of the supernatant (200, 100 or 50 μ L) in iPrOH (2 mL), the concentration in conjugated dienes (CD) was determined by measuring the absorbance at 234 nm (HP 8453 diode-array spectrometer; optical path length 1 cm). The molar absorption coefficient used for conjugated linoleyl hydroperoxides was 27
000 M⁻¹ cm⁻¹.³⁵

221

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

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

243

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

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

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258 Determination of polyphenol bioaccessibility

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

268

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

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

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

290

291 Statistical Analyses

All the results are expressed as the mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) was performed to test the effect of variation factors. If significant effects were found at a 95% confidence level, ANOVA was followed by a Tukey-Kramer post hoc
test to identify differences among groups (XLStat software, version 2013, Addinsoft SARL,
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 to belong to the main four phenolic classes usually present in dessert apples: flavan-3-ols, 301 hydroxycinnamic acids, dihydrochalcones and flavonols. The quantification of the major 302 compounds, represented by oligomeric procyanidins and chlorogenic acid, points to a 303 statistically similar distribution for all three matrices (Table 1). Monomeric and oligomeric 304 procyanidins account for 5-7% and 70% of all the phenolic compounds, respectively. Only 305 epicatechin was identified as extension units in oligomeric procyanidins whereas both 306 catechin and epicatechin appeared as terminal units. This constitution and a relatively low 307 mean degree of polymerization (mDP = 5) are classically observed in dessert apple.³⁶ The 308 UPLC separation conducted on the apple matrix allowed the identification of various B-type 309 oligomers up to heptamers. Hydroxycinnamic acids, the second most abundant class, are 310 311 represented by 5-caffeoylquinic acid (19-20%) and 4-p-coumaroylquinic acid as a minor compound (0.5-0.8%). 5-p-Coumaroylquinic acid was also found in half the amount 312 313 compared to 4-p-coumaroylquinic acid (not shown). Dihydrochalcones were present as phloretin-2'-O-glucoside (1.8-2.3%) and phloretin-2'-O-xyloglucoside (0.6-0.9%). Flavonols, 314 constituted mainly by quercetin monohexosides, were weak contributors (1.4-1.9%). This 315 composition is in agreement with the average composition for dessert apples and location of 316 flavonols in apple skin only.³³ Additionally, the occurrence of taxifolin hexoside (10) and 317 cinchonains I, II, and III (5, 32, 40) was demonstrated for the first time in an apple genotype. 318

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

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

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

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

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

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

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

At pH 3, the addition of pepsin (0.25 mg/mL) had a marked impact on the formation of both 384 markers (Fig. 1). Lipid oxidation only started after 2 h of incubation and its extent was largely 385 reduced with concentrations of only 1.2 mM for CD and 2.7 µM for 4-HNE after 6 h. At pH 386 3, in the absence of pepsin, the CD and 4-HNE levels were 9.6 mM and 6.6 µM, respectively. 387 Epicatechin, apple puree and the phenolic extract inhibited almost totally the formation of 388 both CD and 4-HNE during the 6 h-long kinetics. By contrast, fresh apple proved to be rather 389 inefficient. The lifetime of α -tocopherol was found to be less than 15 min for all conditions at 390 pH 3 with pepsin. Finally, epicatechin disappeared in 15 min as already observed in the 391 392 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 the phenolic extract at the initial time of the simulated gastric digestion before addition of 396 397 metmyoglobin to prevent oxidation (Table 2). Bioaccessible phenolic compounds are the molecules freely present in the aqueous phase after separation of the aqueous and lipid phases 398 of the emulsion. When monomeric flavanols, flavonols, hydroxycinnamic acids and 399 dihydrochalcones were brought as a hydroacetonic extract, they were recovered in high 400 yields. Recoveries of 96 and 95% were obtained at pH 5 in the absence and presence of 401 pepsin while these values were 89 and 79% at pH 3, respectively. Chlorogenic acid, phloretin-402 2'-xyloglucoside and epicatechin proved to be more highly bioaccessible than 4-p-403 coumaroylquinic acid, phloretin-2'-glucoside and flavonols. Additionally, dimer B2, the 404 major procyanidin oligomer in apple, was found to be present in the range 14-19% whatever 405 406 the conditions. When puree was probed for antioxidant capacity, the recovery of phenolic compounds appeared lower, 74 and 60% at pH 5 while 57 and 60% at pH 3 in the absence and 407 408 presence of pepsin, respectively. The three more highly bioaccessible compounds were chlorogenic acid, phloretin-2'-xyloglucoside and 4-p-coumaroylquinic acid. Dimer B2 was 409 recovered in yields ranging between 7 and 23%. With fresh fruit, the bioaccessibility of 410 phenolic compounds markedly decreased, being 14 and 3% without and with pepsin at pH 5 411 while 1 and 12% at pH 3. Surprisingly, phloretin-2'-xyloglucoside, whose content is 412 commonly higher in apple skin than flesh, was similarly bioaccessible than chlorogenic acid 413 for which the favored location site is reversed.^{33, 36} 414

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 spectroscopy418 (Fig. 4A). No spectral change was recorded for metmyoglobin between pH 7 and 4.79.

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

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

437

438 Discussion

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

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

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

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

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

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

507

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

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

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

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

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

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

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

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

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

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

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

589

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Conflicts of interest

596 There are no conflicts of interest to declare.

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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		zation	Hydroxycinnamic acids		Dihydrochalcones		Flavonols	Overall content in g/kg (mmol/kg)	
					Term. units (%)		Ext. units (%)							
	CAT	EPI			CAT	EPI	CAT	EPI	5-CQ	4-pCou	Phln	Phlx		
Fresh	1.2 ± 0.2	5.6 ± 0.2	70.0 ± 2.2	5.4	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
Apple				± 0.1										(14.2 ± 0.4)
Puree	1.2 ± 0.2	3.8 ± 0.6	70.6 ± 4.1	5.3	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
				± 0.2										(12.2 ± 0.5)
Phenolic	0.43 ± 0.04	4.3 ± 0.4	70.2 ± 3.3	4.7	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
extract				± 0.1										(88.9 ± 4.0)

CAT: (+)-catechin, EPI: (-)-epicatechin, flavonols in equivalent quercetin, mDP: mean degree of polymerization of monomeric and oligomeric procyanidins, 5-CQ: 5caffeoylquinic 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 \pm 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 ± SD (*n* = 3).

Matrix	Conditions Flavanols			Hydroxyc	innamic acids	Dihydro	ochalcones	Flavonols	Total
									polyphenols
		Epi	Dimer B2	5-CQ	4-pCou	Phln	Phlx		
Fresh	рН 5	0.5 ± 0.4	34 ± 7	21 ± 0	5 ± 3	6 ± 3	19 ± 9	-	14 ± 0
Apple	pH 5 + pepsin	6 ± 2	13 ± 3	-	5 ± 2	27 ± 1	-	-	2.9 ± 0.4
	рН 3	2 ± 1	1 ± 0	-	4 ± 1	1 ± 0	10 ± 1	-	0.8 ± 0.3
	pH 3 + pepsin	1 ± 0	47 ± 10	17 ± 2	8 ± 6	5 ± 2	29 ± 3	-	12 ± 1
Puree	рН 5	44 ± 4	23 ± 13	95 ± 6	50 ± 2	32 ± 14	48 ± 3	47 ± 11	74 ± 3
	pH 5 + pepsin	13 ± 3	7 ± 1	85 ± 10	43 ± 8	15 ± 3	27 ± 3	22 ± 3	60 ± 6
	рН 3	25 ± 9	15 ± 7	76 ± 6	39 ± 5	18 ± 1	43 ± 5	23 ± 6	57 ± 3
	pH 3 + pepsin	29 ± 19	10 ± 2	81 ± 6	40 ± 8	15 ± 2	41 ± 2	21 ± 18	60 ± 6
Phenolic	рН 5	40 ± 26	19 ± 4	125 ± 2	57 ± 20	30 ± 3	87 ± 5	41 ± 15	96 ± 4
extract	pH 5 + pepsin	82 ± 5	16 ± 1	115 ± 2	51 ± 2	31 ± 0	70 ± 6	43 ± 9	95 ± 1
	рН 3	76 ± 7	17 ± 0	104 ± 4	49 ± 4	28 ± 1	116 ± 1	65 ± 28	89 ± 2
	pH 3 + pepsin	47 ± 27	14 ± 2	96 ± 0	40 ± 6	28 ± 0	108 ± 5	51 ± 16	79 ± 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 ± 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 μ M for puree, phenolic extract, epicatechin and 133 μ M for fresh apple. Values represent mean ± 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 ± 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.

