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Gaëtan Boléa[1,2], Christian Ginies[1], Marie-José Vallier[1], Claire Dufour[1*]

[1]UMR408 SQPOV "Safety and Quality of Plant Products", INRA, University of Avignon, F-84000 Avignon, France.

[2] EA4278 LaPEC "Laboratory of Cardiovascular Pharm-ecology ", University of Avignon, F-84000 Avignon, France.

Corresponding author

C. DUFOUR

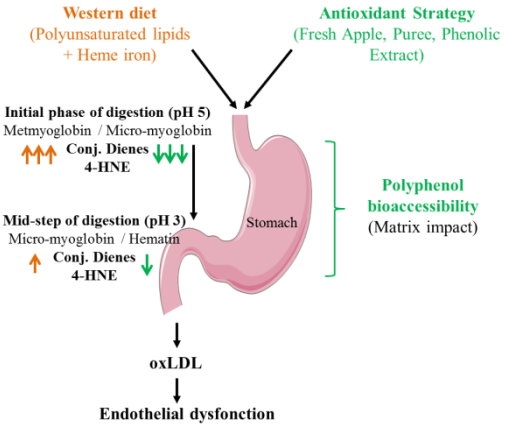
INRA, UMR408 SQPOV, 228 route de l'aérodrome, CS 40509 - 84914 Avignon Cedex 9

Phone: (+ 33) 4 32 72 25 15 /Fax: (+ 33) 4 32 72 24 92

E-mail: claire.dufour@inra.fr

Abstract

Lipid oxidation takes place in the gastric tract after the ingestion of a Western diet rich in ω-6 polyunsaturated fatty acids (PUFA) and red meat (heme iron). The incorporation of oxidation products such as 4-hydroxy-2-nonenal (4-HNE) into low-density lipoproteins is further correlated to endothelial dysfunction. Gastric postprandial stress could thus be reduced by antioxidant phytomicronutrients. The aim of this study was to investigate dietary lipid 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 factors pH and pepsin governing the metmyoglobin-initiated lipid oxidation of sunflower oil-in-water emulsions simulating the physical state of dietary lipids. Our results first showed that pepsin accelerated lipid oxidation at pH 5 through the formation of a micro-metmyoglobin form likely displaying a higher accessibility to lipids. Spectroscopic studies further highlighted the formation of a reversible unfolded metmyoglobin form at pH 3 which was shown to be more pro-oxidant in the absence of pepsin. At nutritional levels, the three apple 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 bioaccessibilities of monomeric phenolic compounds were evidenced for both puree (57-74%) 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 suggesting non-covalent binding to apple pectins.



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

42 proanthocyanidins.

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Introduction

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

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-dilation[18-20] when their circulating

metabolites were shown to reduce monocyte adhesion to endothelial cells through modulation of gene expression and TNF-α.[21, 22] Proanthocyanidins represent the second polyphenol group in the French diet although this class is largely underestimated due to uncomplete extractability.[23, 24] Apple is the major contributor to this class and its phenolic content can vary by a 5-fold factor depending upon apple variety and growth conditions.[25-27] Monomeric and oligomeric proanthocyanidins represent more than 80% of apple phenolic compounds followed by hydroxycinnamic acids, flavonols, dihydrochalcones and anthocyanins in decreasing order. After ingestion, native form of polyphenols may be released in the chyme and exert their antioxidant capacity directly in the gastric tract. The vegetable matrix, food 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 and either dietary proteins or fibers largely decreases polyphenol *in vivo* bioaccessibility in the gastric tract.[28, 29] During gastric digestion, partial proteolysis and lipolysis take place under the action of pepsin and gastric lipase, respectively.[30] In addition, gastric pH sharply increases to values between 5 and 7 after meal intake before slowly returning to a basal pH close to 2 after gastric emptying.[31, 32] One aim of this study is to evaluate the effect of pH and pepsin on lipid oxidation and its inhibition by apple polyphenols under different matrix forms 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 forms present at the different pH and after the action of pepsin to shed light on the initiation and inhibition mechanisms for lipid oxidation in emulsion systems.

Materials and methods

Chemicals and Solvents

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

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 $M\Omega.cm^{-1}$ at 25 °C) was obtained with a Millipore OPak 2 (Bedford, MA, USA).

Characterization of sunflower oil

To determine the fatty acid composition of sunflower oil (Rustica from Leclerc, lot A21815), 15 mg of oil were solubilized in 200 µL of toluene before transesterification at 100 °C during 1 h by addition of 2 mL of sulfuric acid/methanol (0.025/0.975, v/v). Then, 2 mL of water and 2 mL of hexane were added and homogenized before injection of 1 µL of the organic phase onto a gas-chromatograph equipped with a flame ionization detector (Clarus 500 GC, Perkin Elmer) using a DB-225ms column (thickness 0.25 µm, height 0.25 µm, length 30 m, Agilent). Chromatographic conditions were as follows: carrier gas was H_2 at 37 cm/s, injection temperature was 250 °C in the split mode (ratio 20), oven temperature was held at

60 °C for 1 minute and then ramped up at 20 °C/min to 180 °C and then at 3 °C/min to 230 °C. Fatty acid identification was made by comparing the retention time of FAME peaks from samples with standard solutions (FAMEs 37, Supelco). The results are expressed in relative 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; 0.2% C20), 27.8% of monounsaturated fatty acids (0.1% C16:1; 27.0% C18:1n-9; 0.6% 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.

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.

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

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.

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

Separation and identification of apple phenolic compounds were performed by using a Waters ACQUITY UPLC chromatograph (Milford, MA) coupled to an UV-vis diode-array detector and a HCT ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source. Separation was carried out using a reverse-phase Acquity BEH C18 column (100 mm x 2.4 mm i.d., 1.7 µm; Waters) at 35 °C. The mobile phase was constituted by a binary solvent system with water/formic acid (99.95/0.05, v/v, solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 mL/min. The elution gradient was 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, 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 ionization mode from *m/z* 100 to 1400. MS conditions were as follows: capillary voltage of 2 kV, dry gas flow rate at 12 L/min, desolvation temperature at 365 °C and nebulization pressure at 60 psi.

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 µL of anhydrous methanol and 400 µL 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 Fast LC Prominence system (Shimadzu, Kyoto, Japan). Separation conditions were as in Le Bourvellec et al. (2011).[33] (+)-catechin, (-)-epicatechin, quercetin and phloridzin were used as standards and response factors were obtained from 6 independent 40 mg/L solutions. Procyanidin analysis was achieved after thioacidolysis. For this, freeze-dried apple matrices (50 mg) were dissolved in 800 µL of a 5% solution of toluene-α-thiol in anhydrous methanol 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 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 acid as standards. Epicatechin benzylthioether was expressed as epicatechin at 280 nm. Procyanidins were characterized by their subunit composition and average degree of polymerization (mDP). The mDP was calculated as the molar ratio of all the flavanol units (thioether adducts plus terminal units) to (-)-epicatechin and (+)-catechin corresponding to terminal units. Analyses were run in triplicate for each matrix.

Preparation of gastric model emulsions

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

lipid oxidation, 13.5 mL (13.35 mL in the presence of pepsin) of the fine emulsion were transferred in a 50 mL round-bottom flask. Pepsin was diluted in the appropriate buffer at a concentration of 6.25, 25 and 100 mg/mL prior to use. When needed, 150 µL of pepsin were added to the emulsion to obtain final concentrations of 0.0625, 0.25, and 1 mg/mL (2828±159 U/mg, pepsin activity measured as in Minekus et al., 2014).[30] For antioxidant evaluation, 150 µL of a 10 mM (-)-epicatechin solution in methanol were added to the emulsion (100 µM final) or replaced by 150 µL of methanol in control experiments. For the phenolic extract, 111 mg of the dry extract were first solubilized in 1 mL of the appropriate buffer (10 mM in total phenolic compounds) before the addition of 150 µL to the emulsion. Puree (125 mg) was 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 freshly cut in 24 pieces as described above in a domestic grinder (Valentin, Seb) in the 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 was next initiated by adding 1.5 mL of a 200 µM solution of metmyoglobin ($MbFe^{III}$) (ε= 7700 M^{-1} cm^{-1} at 525 nm)[34] prepared in ultrapure water leading to a 20 µM final concentration. Round-bottom flasks were protected by punched parafilm and incubated in an oven at 37 °C under constant magnetic agitation at 280 rpm. All the experiments were run at least in triplicate.

Measurement of lipid-derived conjugated dienes

Every hour, emulsion samples (200 µL) were diluted in 2-propanol (1000 µL) before centrifugation (5 min at 16 200*g*, 4 °C). After further dilution of the supernatant (200, 100 or 50 µL) 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]

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

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

Analyses of α-tocopherol and (-)-epicatechin

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 quantification were performed by using an ACQUITY UPLC system coupled to a DAD and a fluorescence detector (Waters, Milford, MA). Separation was carried out using an Acquity BEH C18 column (50 mm x 2,1 mm i.d., 1.7 µm; Waters) at 35°C. Mobile phase was 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 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. (-)-Epicatechin was detected by absorption at 280 nm, α-tocopherol by fluorometry (excitation 290 nm; emission 330 nm) and both were quantified after calibration with the appropriate standards.

Determination of polyphenol bioaccessibility

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 aliquot of the emulsion was centrifuged at 16 000*g* for 5 min at 4 °C. The aqueous phase was 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 UPLC/DAD/MS with phenolic standards in methanol. Monomeric and dimeric procyanidins were quantified as epicatechin equiv. (280 nm), chlorogenic acid at 320 nm; *p*-coumaroyl derivatives as *p*-coumaric acid equiv. (320 nm); dihydrochalcones as phloridzin equiv. (280 nm) and flavonols as isoquercitrin equiv. (350 nm).

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

The effect of pepsin and (-)-epicatechin on the metmyoglobin stability was evaluated as follows. To 1910 µL of 10 mM citrate/20 mM phosphate buffer acidified at pH 5, 4.75, 4.5, 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 water, 5 µM final), then 3 min later by 20 µL of pepsin (6.25 mg/mL in 10 mM acetate buffer at pH 5, 0.0625 mg/mL final, activity 2041±177 U/mg pepsin determined as in Minekus et al., 2014).[30] Spectra were recorded every 1 to 5 s and first-order kinetics data extracted using the internal software. All experiments were run under constant magnetic stirring at 1000 rpm and at 37 °C in triplicate.

Statistical Analyses

All the results are expressed as the mean ± 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).

Results

Characterization of the different apple matrices

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, hydroxycinnamic acids, dihydrochalcones and flavonols. The quantification of the major compounds, represented by oligomeric procyanidins and chlorogenic acid, points to a statistically similar distribution for all three matrices (Table 1). Monomeric and oligomeric procyanidins account for 5-7% and 70% of all the phenolic compounds, respectively. Only epicatechin was identified as extension units in oligomeric procyanidins whereas both catechin and epicatechin appeared as terminal units. This constitution and a relatively low mean degree of polymerization (mDP = 5) are classically observed in dessert apple.[36] The UPLC separation conducted on the apple matrix allowed the identification of various B-type oligomers up to heptamers. Hydroxycinnamic acids, the second most abundant class, are 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 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, constituted mainly by quercetin monohexosides, were weak contributors (1.4-1.9%). This composition is in agreement with the average composition for dessert apples and location of flavonols in apple skin only.[33] Additionally, the occurence of taxifolin hexoside (**10**) and cinchonains I, II, and III (**5**, **32**, **40**) was demonstrated for the first time in an apple genotype.

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]

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 µM total polyphenols) than for puree or phenolic extract (100 µM) to account for an expected lower bioaccessibility. Apple matrices were also compared with epicatechin (100 µM), the main constitutive unit of monomeric and oligomeric procyanidins.

At pH 5 in the absence of pepsin and antioxidant, lipid-derived conjugated dienes (CD) accumulated in an almost linear pattern whereas the secondary lipid oxidation marker 4-HNE tended to plateau after 3 h (Fig. 1A-B). α-Tocopherol was consumed within 15 min (Suppl. Fig. 1D) in agreement with the observed slower rates for CD and 4-HNE formations during the first hour. Epicatechin appeared by far as the best inhibitor, totally inhibiting the accumulation of both CD and 4-HNE for 3 h (Fig. 2A-B). In addition, epicatechin increased the lifetime of α-tocopherol from 15 min to 3 h (Suppl. Fig. 1E) and was finally consumed 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 apple to 2 h for puree and the phenolic extract. The phenolic extract appeared more efficient 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 markers. Marker 4-HNE should be more reliable as it is specifically quantified by mass

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) significantly enhanced the early accumulation of CD for non-inhibited lipid oxidation ($p < 0.05$) (Suppl. Fig. 1A). No lag-phase could be observed suggesting the presence of a different initiating myoglobin form. While a sustained increase of CD accumulation was observed with 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, simulating mid-digestion conditions was further used to study the antioxidant capacity of the various apple products and the stability of epicatechin and α-tocopherol. In the presence of pepsin, α-tocopherol disappeared in less than 15 min as rapidly as in the absence of pepsin (Suppl. Fig. 1D). By contrast, the 4-HNE formation rate was halved in the presence of pepsin (Fig. 1B). Apple matrices showed a rather similar pattern with total and significantly high inhibitions of CD and 4-HNE accumulations after 1 and 2 h, respectively (Fig. 3). Puree and 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-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 markedly retarded the appearance of 4-HNE (up to 3 h) and increased the lifetime of α-tocopherol from 15 to 45 min (Suppl Fig. 1E). By contrast, no protection was afforded to α-tocopherol by apple products. Additionally, epicatechin was more rapidly degraded in the presence (3 h) than in the absence of pepsin (4 h) (Fig.2A and 3A).

During human gastric digestion, pH almost linearly decreases from 5 to 3 at a rate depending on the consistency of food.[29] Interestingly, the pattern for CD accumulation at pH 3 superimposed rather closely with that observed at pH 5 in the presence of pepsin (Fig. 1A). 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 patterns for 4-HNE production were also markedly similar between pH 3 without pepsin and pH 5 with pepsin (Fig. 1B). Although epicatechin was found to totally inhibit lipid oxidation for 3 hours at pH 5, it appeared poorly efficient at pH 3 with a 50% decrease in CD at 2 h followed by a major loss of the activity at 3 hours (Fig. 2C). Epicatechin retained only a 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 pH 3. Fresh apple, puree and the phenolic extract behaved more or less as epicatechin 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 studied (not shown).

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

Effect of the matrix on the bioaccessibility of apple phenolic compounds

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 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 of the emulsion. When monomeric flavanols, flavonols, hydroxycinnamic acids and dihydrochalcones were brought as a hydroacetonic extract, they were recovered in high yields. Recoveries of 96 and 95% were obtained at pH 5 in the absence and presence of pepsin while these values were 89 and 79% at pH 3, respectively. Chlorogenic acid, phloretin-2'-xyloglucoside and epicatechin proved to be more highly bioaccessible than 4-*p*-coumaroylquinic acid, phloretin-2'-glucoside and flavonols. Additionally, dimer B2, the major procyanidin oligomer in apple, was found to be present in the range 14-19% whatever 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 presence of pepsin, respectively. The three more highly bioaccessible compounds were chlorogenic acid, phloretin-2'-xyloglucoside and 4-*p*-coumaroylquinic acid. Dimer B2 was recovered in yields ranging between 7 and 23%. With fresh fruit, the bioaccessibility of phenolic compounds markedly decreased, being 14 and 3% without and with pepsin at pH 5 while 1 and 12% at pH 3. Surprisingly, phloretin-2'-xyloglucoside, whose content is commonly higher in apple skin than flesh, was similarly bioaccessible than chlorogenic acid for which the favored location site is reversed.[33, 36]

Impact of pH and pepsin on the structure of metmyoglobin

The structure of metmyoglobin was examined between pH 7 and 3 using visible spectroscopy (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 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 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 of a single new form only. Although this form had a spectrum more closely related to that of 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 4.0, 4.3 and 4.6 were observed as in the first part devoted to pH decrease. Further NaOH 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 at pH 3 was between 6 min (Fig. 4B) and 30 min (not shown).

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).

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

Metmyoglobin structures involved in lipid oxidation

Using kinetic monitoring by spectroscopy, it was possible to investigate the effect of pH on metmyoglobin stability and to give an insight into its three dimensional structure. No major structural change could be observed between pH 7 and 4.5. However, the Soret band with a λmax at 409 nm typical of the myoglobin structure largely decayed between pH 4.25 and 4, 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 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 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 from the change in protonation status (pI ca. 7). A high reversibility (> 82%) was observed when metmyoglobin was left at pH 3 for periods shorter than 30 min. This is of particular interest as these unfolded forms may move to the duodenum upon gastric emptying. The pH in duodenum is ca. 6.5 and metmyoglobin could still play a role as a prooxidant. In our study, 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 after 1 h (Fig. 1). By contrast, 4-HNE accumulated more slowly at low pH.

Upon addition of pepsin, the initial structure of metmyoglobin was lost as shown by the pH-dependent first-order decay of the Soret band (Suppl. Fig. 3). It is worth noting that pepsin is 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 and finally 2-4, respectively (Suppl. Table 2). As a matter of fact, the activity of pepsin was 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] These peptides mainly mapped the C- and N-terminal regions and one central region not implicated in the binding site of the porphyrin ring. In particular, the bonding between the porphyrin iron center and both His 64 and 93 could be preserved in the remaining non-digested peptide. These results are consistent with data from Schwarzinger and coll. who 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

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 µM 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 hematin. Although hematin- 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.

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

Conclusion

The present study shed some light on the role of pH and pepsin on heme iron-initiated lipid oxidation taking place in the gastric tract after the ingestion of a Western type diet. In particular, it evidenced the formation of two reversibly unfolded or partly proteolyzed forms of metmyoglobin retaining pro-oxidant activities. Because these forms can move forward to 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 intestinal lipase. The role of trypsin or chymotrypsin on newly formed heme-iron forms 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.

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Conflicts of interest

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				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 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 µM for puree, phenolic extract, epicatechin and 133 µM for fresh apple. Values represented mean ± 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 ± 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
	pH 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	**pH 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
	pH 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	**pH 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
	pH 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.

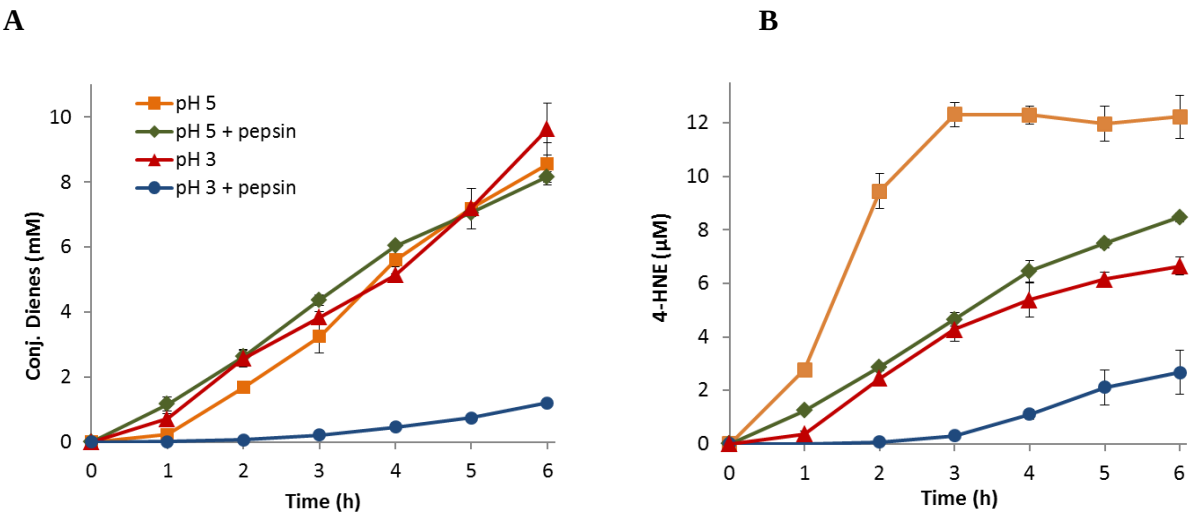

Figure 2. Metmyoglobin-initiated lipid oxidation and its inhibition by different apple matrices in phospholipid-stabilized emulsions. Accumulation of conjugated dienes (A) and 4-HNE (B) at pH 5. Accumulation of conjugated dienes (C) and 4-HNE (D) at pH 3. Dotted red lines are for the consumption of epicatechin.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

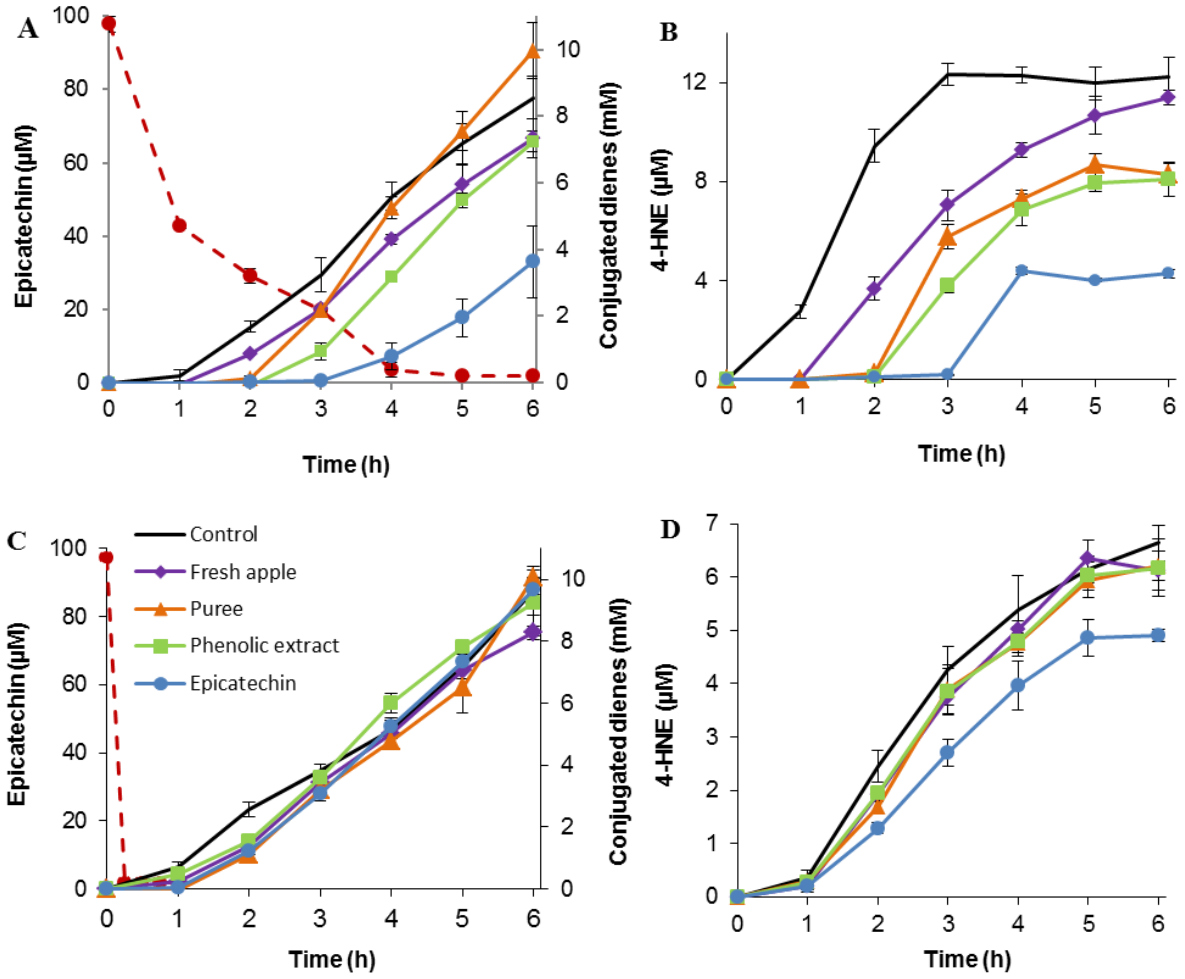

Figure 3. Metmyoglobin-initiated lipid oxidation and its inhibition by different apple matrices in phospholipid-stabilized emulsions in the presence of pepsin (0.25 mg/mL). Accumulation of conjugated dienes (A) and 4-HNE (B) at pH 5. Accumulation of conjugated dienes (C) and 4-HNE (D) at pH 3. Dotted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

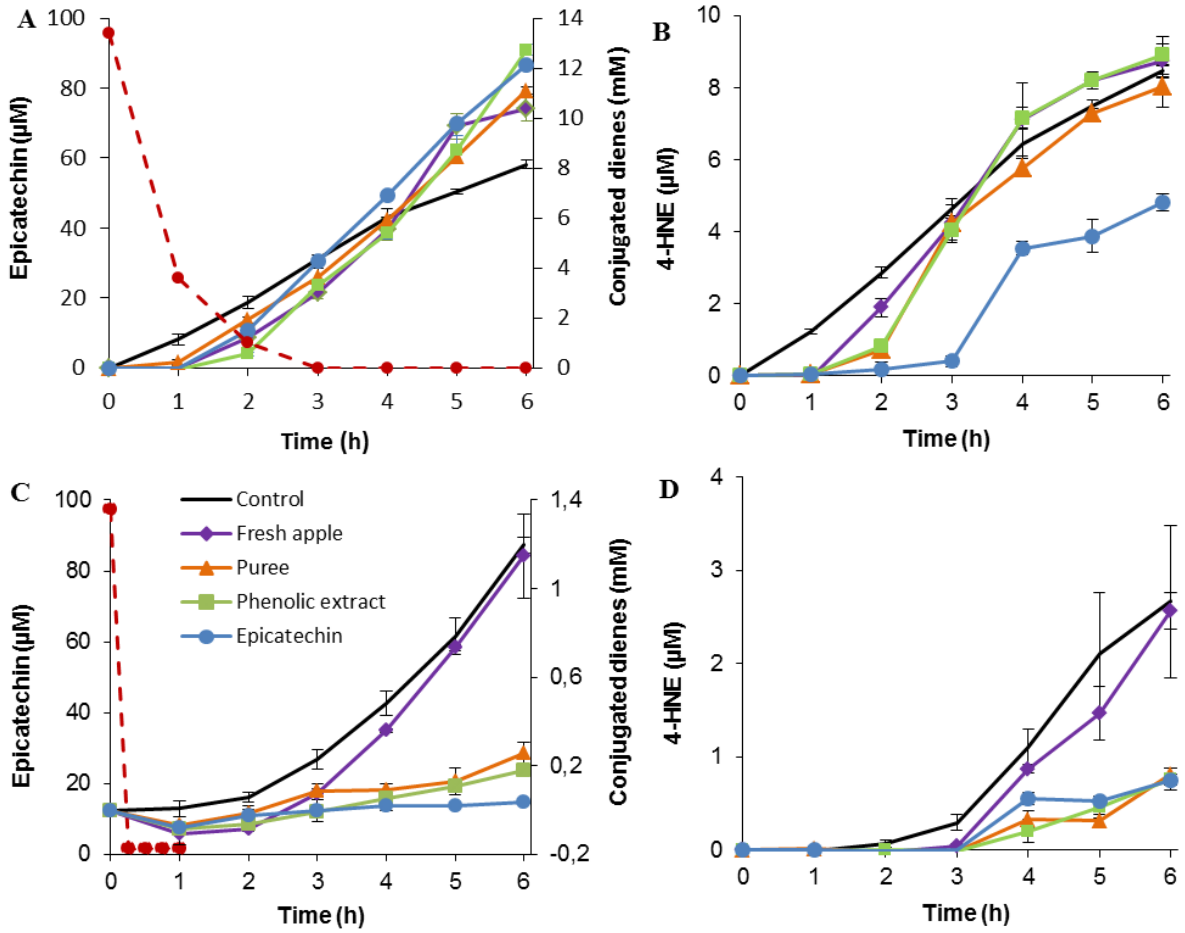

Figure 4. Effect of pH and pepsin on the structure of metmyoglobin (5 µM). (A) Stepwise pH decrease from pH 7.0 to 3.0; dotted lines are hematin at pH 3.0. (B) Stepwise pH increase from pH 3.0 to 6.27; dotted lines are hematin at pH 5.0 (C) Addition of pepsin (0.0625 mg/mL) on metmyoglobin at pH 5. (D)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