Dietary Iron-Initiated Lipid Oxidation and Its Inhibition by Polyphenols in Gastric Conditions

Bénédicte Lorrain,[†,‡] Olivier Dangles,[†,‡] Michèle Loonis,[†,‡] Martine Armand,[§,‖,⊥] and Claire Dufour*[,†,‡]

[†]UMR408 Safety and Quality of Plant Products, INRA, F-84000 Avignon, France
[‡]UMR408 Safety and Quality of Plant Products, University of Avignon, F-84000 Avignon, France
[§]U476 Human Nutrition and Lipids, INSERM, F-13385 Marseille, France
[‖]UMR1260 Lipidic Nutrients and Prevention of Metabolic Diseases, INRA, F-13385 Marseille, France
[⊥]Faculty of Medicine, University of Aix-Marseille, F-13385 Marseille, France

ABSTRACT: The gastric tract may be the first site where food is exposed to postprandial oxidative stress and antioxidant activity by plant micronutrients. After food intake, dietary iron, dioxygen, and emulsified lipids come into close contact and lipid oxidation may take place. This study investigated lipid oxidation and its inhibition by dietary polyphenols in gastric-like conditions. Lipid oxidation induced by heme and nonheme iron was studied in acidic sunflower oil-in-water emulsions. The emulsifier type (bovine serum albumin, phospholipids), pH, and iron form were found to be factors governing the oxidation rates. Quercetin, rutin, and chlorogenic acid highly inhibited the metmyoglobin-initiated lipid oxidation in both emulsified systems at pH 5.8. Additionally, quercetin inhibited nonheme iron-initiated processes, while it was inefficient with hematin as an initiator. The presence of human gastric juice did not influence lipid oxidation, although it diminished the antioxidant activity of phenolics. Model emulsions may thus be valuable tools to study the gastric stability of polyunsaturated lipids.

KEYWORDS: antioxidant, heme iron, lipid oxidation, oil-in-water emulsion, polyphenols

■ INTRODUCTION

There is now compelling evidence that dietary lipid oxidation products may play a key role in the development of cardiovascular diseases.[1] Indeed, ingestion of oxidized oils may result in the impairment of the endothelial function.[2] At a molecular level, lipid oxidation products were recovered first in chylomicrons, and then in low-density lipoprotein (LDL), when humans were fed oxidized corn oil.[3] These minimally modified LDL particles were found to be more susceptible to oxidation and are likely precursors of highly oxidized LDL, which are taken up by macrophages in the subintimal space of arteries during the development of fatty streaks. On the other hand, covalent binding of aldehydes to apolipoprotein is strongly implicated in the atherogenicity of LDL.[4] Potentially toxic electrophiles such as aldehydes but also epoxides may arise from the decomposition in the stomach of dietary lipid hydroperoxides.[5]

Degradation during food processing and that during food storage may not be the only routes for the formation of dietary lipid oxidation products. The latter can be generated in vivo, and the gastric tract has been proposed as a major site for diet-related oxidative stress.[6] Indeed, after food intake, dietary iron, elevated levels of dioxygen, and emulsified lipids closely interact in the gastric tract where substantial lipid oxidation can take place. Lipid oxidation initiated by metmyoglobin, the principal heme iron form in the diet, was first investigated in micelles containing free fatty acids.[6−8] However, protein- or phospho-lipid-stabilized emulsions of triacylglycerols, the main forms of dietary lipids, appear to be more physiologically relevant models of the gastric content.[9,10]

On the other hand, cardiovascular diseases are inversely associated with the intake of flavonoids, a class of polyphenols largely distributed in fruit, vegetables, and cereals.[11] An increase in plasma antioxidant capacity, inhibition of LDL oxidation, a decrease in platelet aggregation, and improvement of endothelial vasorelaxation are the main mechanisms proposed for the health benefit of flavonoids.[12] On the other hand, wine polyphenols were suggested to prevent the formation of cytotoxic and genotoxic lipid oxidation products before their absorption in the gastrointestinal tract.[13,14] After ingestion of a meal rich in plant products, native forms of polyphenols (1.1−1.3 g/d) are indeed recovered in large concentrations in the gastric tract.[15] Hydroxycinnamic acids are the most largely consumed polyphenols in the French diet (599 mg/d), with chlorogenic acid being the most important contributor (216 mg/d). Proanthocyanidins (227 mg/d) is the next class for polyphenol intake, followed in decreasing order by catechins (99 mg/d), anthocyanins (57 mg/d), and flavonols (51 mg/d).

The aim of the present study is the in vitro investigation of lipid oxidation possibly taking place in the gastric compartment and its inhibition by dietary polyphenols. Polyphenols were evaluated at a concentration of 100 μM corresponding to the ingestion of 35 mg of chlorogenic acid, 29 mg of (+)-catechin, or 61 mg of rutin with a mean stomach volume of 1 L. Two major steps of digestion will be considered: after meal intake (pH 5.8) and at the midcourse of digestion (pH 4).[16] Dietary

lipids in the gastric tract are modeled by slightly acidic oil-in-water emulsions stabilized either by bovine serum albumin or by egg yolk phospholipids. Oxidative stress is triggered by dietary iron. Meat provides heme iron with hemoglobin and myoglobin, the dioxygen transporters in blood and muscle cells, respectively, as well as nonheme iron with ferritin, the storage protein of ferric iron. Beef is one of the richest sources of iron with total levels between 20 and 29 μg/g of raw meat.[17−19] Myoglobin (MbFe[II]), the main contributor to total iron, is readily autoxidized in postmortem processes to yield metmyoglobin (MbFe[III]). Besides, free iron was shown to be released from heme iron upon cooking and storage. On the other hand, fruit and vegetables, with a special contribution of cereals, are significant dietary sources of free iron. Digestion of a standard meal may thus lead to the release in a 1 L stomach volume of heme and nonheme iron at levels higher than 20 μM, which is the concentration used in this study.

Both lipid oxidation and its inhibition by polyphenols are investigated as a function of the pH and emulsifier type. Last, an insight into real gastric digestion is brought through the addition of human gastric juice into the bovine serum albumin (BSA)-stabilized digestion model.

■ MATERIALS AND METHODS

Materials. Quercetin, rutin, chlorogenic acid, L-ascorbic acid, iron(III) nitrate nonahydrate, iron(II) sulfate heptahydrate, BSA (fraction V A-9647), myoglobin (from horse heart), hematin (porcine), and L-α-phosphatidylcholine from egg yolk (type X-E) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Commercial L-α-phosphatidylcholine (PL) contained phosphatidylcholine (47 wt %), phosphatidylethanolamine (18%), sphingomyelin (4%), and lysophosphatidylcholine (4%) along with a neutral fraction containing triacylglycerols (24%).[10] All solvents (2-propanol, pentane, acetonitrile, dimethyl sulfoxide, methanol) were purchased from Fisher Scientific Bioblock (Illkirch, France) and were of analytical or HPLC/MS grade. Sodium phosphate (5 mM, pH 5.8) and sodium acetate (5 mM, pH 4) buffers, prepared with Millipore Q-100 Plus water, were stirred with CHELEX-100 chelating resin (Bio-Rad) to remove contaminating metal ion traces.

Commercial sunflower oil (Carrefour store) was stripped of tocopherols using adsorption chromatography on alumina as described before.[10] Stripped sunflower oil contained around 63% 18:2n−6, 25% 18:1n−9, 7% 16:0, and 5% 18:0 as major fatty acids. It was stored under N_2 at −20 °C.

Gastric juices were collected from adults for diagnostic purposes at fasting or after pentagastric stimulation (6 μg/kg) and selected from subjects presenting no gastric pathology and no duodenal reflux (a gift from Dr. J. Peyrot, Gastroenterology Department, Hôpital Nord, Marseille, France). The gastric juice after stimulation was characterized for lipase activity (70 U/mL on tributyrin at pH 5.4), proteolytic activity (533 U Anson on hemoglobin at pH 1.8), free acidity (168 mequiv/mL), and total protein concentration (1.0 g/L).

Gastric Model Emulsions. The gastric content was modeled by 10% oil-in-water emulsions stabilized either by BSA (1.4%, w/w) or by egg PL (2%, w/w). In a 50 mL round-bottom flask, 1 g of stripped sunflower oil was added to 7.9 mL of either BSA (140 mg) or PL (200 mg) previously dispersed in a pH 4 or 5.8 buffer. The PL dispersion was best achieved using a rotor stator homogenizer (UltraTurrax T25, IKA) at 24000 rpm for 1.5 min twice (room temperature). The oil and aqueous phases were then homogenized at 24 000 rpm for 2 min. The coarse emulsion was swept with N_2 for 5 min before probe sonication: two periods of 30 s with a resting interval of 30 s for the BSA-stabilized emulsions and six periods of 30 s in ice with intervals of 30 s for the PL-stabilized emulsions (130 W, 20 kHz, Bioblock Vibracell). A volume of 0.1 mL of the antioxidant in acetonitrile/DMSO (4:1, v/v) was added via syringe to ensure final concentrations of 25 and 100 μM.

Particle size distributions were determined by laser light scattering (Mastersizer 2000, Malvern Instruments, Orsay, France) as described before.[10]

Lipid Oxidation in Model Emulsions. Lipid oxidation was initiated by adding heme and nonheme iron forms at a 20 μM final concentration. For this, 1 mL of a 200 μM solution of MbFe[III] (ε = 7700 M^{-1} cm^{-1} at 525 nm) prepared in the appropriate buffer was added to the emulsion.[10] Hematin was dissolved in methanol/DMSO (1:1, v/v) while solutions of Fe[III], Fe[II], and ascorbic acid were prepared in Milli-Q water to final concentrations of 2 mM prior to use. Volumes of 0.1 mL of the latter solutions were added to start lipid oxidation and completed by 0.8−0.9 mL of buffer. Ascorbic acid (0.1 mL) was added 30 s prior to the Fe[III] solution. The system was protected by punched Parafilm before being placed in an oven at 37 °C under constant magnetic stirring.

Every hour, aliquots of emulsions (200 μL) were diluted in 2-propanol (1.8 mL) before centrifugation (4 min at 5600 rpm, 5 °C). Concentrations of conjugated dienes (CDs) were determined after additional dilution of the supernatant by measuring the absorbance at 233 nm (HP 8453 diode-array spectrometer; optical path length 1 cm). The molar absorption coefficient used for CDs was that for conjugated linoleyl hydroperoxides (27000 M^{-1} cm^{-1}).[10] The antioxidant capacity was expressed as the relative area under the curve (AUC for 100 μM antioxidant/control AUC) × 100. The area under the CD concentration vs time curve (AUC) was integrated over 7 h by a polyhedral method. For heme and nonheme iron-initiated lipid oxidation, control and 100 μM antioxidant experiments were run at least in triplicate whereas 25 μM antioxidant experiments were acquired in duplicate.

Quantitative and Qualitative Analyses of Polyphenols and Their Oxidation Products. A 100 μL aliquot of the emulsion was diluted in 2-propanol (200 μL) and centrifuged at 10 000 rpm for 2 min. The supernatant was frozen at −25 °C until ultraperformance liquid chromatography (UPLC)/MS analysis. UPLC/ESI-MS^n analyses were carried out using a Waters ACQUITY UPLC system (Waters, Milford, MA) coupled to a UV−vis diode-array detector and an HCT ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Mass analyses were performed in the negative electrospray ionization mode with a capillary voltage of 2 kV, from m/z = 100 to m/z = 1000. Nitrogen was used as the drying and nebulizing gas with a flow rate of 10 L min^{-1}. The desolvation temperature was set at 365 °C and the nebulization pressure at 60 psi. Separation was obtained at 35 °C using an ACQUITY UPLC HSS T$_3$ column (1.8 μm, 2.1 × 50 mm, Waters). The solvent system was a gradient of A (0.05% aqueous HCO$_2$H) and B (MeOH) at a flow rate of 0.6 mL min^{-1}. Initial B was set at 5%. For quercetin and catechin, the gradient was 60% B at 1.7 min and 100% B at 2.3 min. For rutin, the gradient was 35% B at 1.7 min, 80% B at 2.3 min, and 100% B at 2.83 min. Quercetin and rutin were detected at 365 nm and catechin and all new products at 280 nm.

Lipid Oxidation in the Presence of Human Gastric Juice. Human gastric juice (3.5 mL, pH 5.4) was added to 1.5 mL of a BSA-stabilized emulsion prepared as above and placed in an open vial. The pH was adjusted to 5.8, and lipolysis was conducted for 1 h in an oven at 37 °C under constant magnetic stirring. To 1.4 mL of this new emulsion was added 80 μL of quercetin or (+)-catechin in acetonitrile/DMSO (4:1, v/v) via syringe to ensure a final concentration of 760 μM. Lipid oxidation was started by adding 150 μL of the metmyoglobin solution in the pH 5.8 buffer to a final concentration of 20 μM. The system was protected by punched Parafilm before being placed in an oven at 37 °C under stirring. For comparison, experiments were run by replacing the gastric juice by the same volume of buffer. Sampling was performed to evaluate the particle size, lipolysis rate before and after 1 h of gastric juice addition, CD accumulation, and polyphenol consumption every 2 h for 8 h. The stability of the phenolic antioxidants in the gastric juice-containing emulsion was checked by adding buffer instead of the metmyoglobin solution.

Lipids from digestion tests were extracted following a modified Folch method using chloroform/methanol (2:1, v/v). Dried lipid extract pellets were resuspended in 2-propanol, and free fatty acids
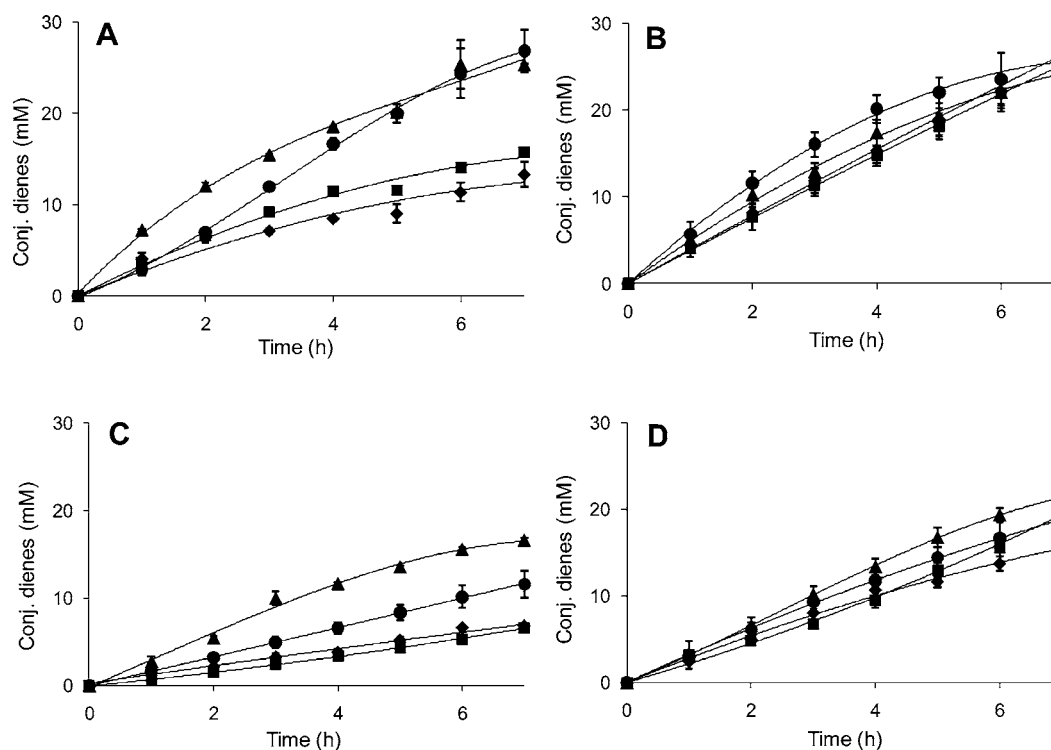
Figure 1. Lipid oxidation initiated by heme iron (metmyoglobin (●), hematin (▲)) and nonheme iron (Fe^{II} (■), Fe^{III}/Asc (◆)) in BSA-stabilized emulsions at pH 5.8 (A) and 4 (B) and in PL-stabilized emulsions at pH 5.8 (C) and 4 (D). Bars are the SDs.

were quantified using enzymatic assay (kit NEFA Randox, Crumlin, U.K.). Lipolysis rates were calculated as the percentage of triglyceride (TG) hydrolyzed, from micromoles of released free fatty acids (FFA) at a given time (FFAt) relative to the total micromoles of physiologically releasable FFA (Sn-1 and Sn-3 positions), based on the initial amount of TG, using the following equation: $[FFAt/(TG \times 2)] \times 100$.

Statistical Analyses. 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 interval, ANOVA was followed by a Tukey−Kramer honestly significant difference (HSD) post hoc test to identify differences among groups (XLStat software, version 2008.3.02, Addinsoft SARL, Paris, France).

■ RESULTS AND DISCUSSION

Heme vs Nonheme Iron as an Initiator of Lipid Oxidation. Heme iron (metmyoglobin, hematin) and non-heme iron (Fe^{II}, Fe^{III}/ascorbate) were evaluated as initiators of lipid oxidation through monitoring of CDs. CDs are primary markers consisting mainly of highly reactive lipid-derived hydroperoxides formed in the propagation step of the lipid oxidation process. Minor compounds are the corresponding alcohol derivatives produced by homolytic cleavage of the hydroperoxides followed by reduction.[20] At pH 5.8, the CD accumulation for $MbFe^{III}$-initiated lipid oxidation was nearly linear with a rate of 3.5 mM CD/h in the BSA model and 1.6 mM CD/h in the PL model (Figure 1A,C). When $MbFe^{III}$ was replaced by hematin, the quasi-linear CD accumulation changed for a two-phase kinetic profile. The initial fast CD accumulation was followed by a much slower step as already observed in an acidic micelle solution.[7] Besides, the initial rate of the hematin-initiated oxidation was found to exceed that of the $MbFe^{III}$-initiated process by nearly a factor of 2, suggesting a higher catalytic activity of hematin in the production of lipid

peroxyl radicals from lipid hydroperoxide traces.[21] Similarly, in hematin-initiated lipid oxidation of methyl linoleate dispersed in Tween 20 micelles, dioxygen depletion rates were found to be twice as fast as for the corresponding $MbFe^{III}$-induced process.[22] In our models, this rate difference could also be ascribed to a better anchoring to the interfacial layer of the smaller and more hydrophobic hematin (Fe^{III}−porphyrin complex) compared to $MbFe^{III}$. After this fast initial step, the CD accumulation tended to level off, indicating an inefficient regeneration of the catalytically active hematin. At this stage, both heme iron-induced processes displayed similar rates, in agreement with lipid oxidation being controlled by the propagation step.

At pH 4, the CD concentration vs time curves were no longer discriminated for both hematin- and $MbFe^{III}$-induced peroxidation processes (Figure 1B,D). Their shape was also close to that observed for the hematin-initiated lipid oxidation at pH 5.8 in both PL- and BSA-emulsified systems. This is consistent with the denaturation of $MbFe^{III}$, leading to the release of the heme cofactor and a process purely initiated by hematin.

Postprandial-like oxidative stress by nonheme iron was induced either by Fe^{II} alone or by Fe^{III} in the presence of L-ascorbate (1:1 Fe^{III}:ascorbate molar ratio). In both BSA and PL models and for each pH, lipid oxidation proceeded at nearly similar rates, thus outlining the involvement of ascorbate in the reduction of Fe^{III} into Fe^{II}. Furthermore, at pH 5.8 and in both emulsion models, free iron forms appeared to have half the prooxidant activity of heme iron forms (Figure 1A,C). The lower oxidation rates in the presence of free iron may be attributed to the fast accumulation of relatively inert Fe^{III} ions resulting from insufficient concentrations of reducing species. However, it is noteworthy that the pH drop benefits free iron, which appeared as aggressive as heme iron at pH 4 (Figure

1B,D). This change is consistent with an increased concentration of prooxidant Fe^{II} species. Indeed, at low pH, Fe^{II} is much less susceptible to autoxidation into Fe^{III} and also more easily regenerated from Fe^{III} due to the increased oxidizing character of the latter.

The pH drop from 5.8 to 4 does not modify the charges of the zwitterionic heads of phosphatidylcholine and phosphatidylethanolamine, the main phospholipids in the PL model, whereas BSA switches from a negatively charged to a positively charged particle (pH_i 4.7−4.9). On the other hand, the overall charge of hematin changes from negative to positive owing to the protonation of the carboxylic side chains. Nonheme iron is also expected to increase its positive charge when the pH decreases due to the protonation of hydroxyl ligands. Thus, decreasing electrostatic interactions are anticipated between the iron initiators and the protein interface upon pH lowering. Actually, initiators Fe^{II} and Fe^{III}/ascorbate led to higher oxidation rates, whereas the hematin-initiated process was unaffected when the pH decreased from 5.8 to 4. This clearly rules out a role for electrostatic interactions in the lipid oxidation of BSA-stabilized-emulsions.

By contrast, the interfacial composition was found to drastically influence lipid oxidation. Indeed, the CD accumulation rate was nearly twice as fast in the BSA-emulsified model as in the PL-emulsified one. This can hardly be explained by the weak differences in surface-weighted diameters d_{32} (2.4 ± 0.2 and 2.8 ± 0.3 μm for the BSA and PL emulsions, respectively, at both pH values) and modes (7 and 2 μm for the BSA and PL models, respectively). The study of the Fe^{II}/EDTA-promoted oxidation of protein- and surfactant-stabilized emulsions of comparable sizes suggests that surfactants give more compact and more homogeneous interfaces than proteins, probably hindering the access of initiators to the oxidizable lipids.[23]

Mechanisms implied in the initiation of lipid oxidation are rather different depending mostly on the prooxidant form. Initiation by Fe^{II} ions is based on a Fenton-like mechanism with a homolytic cleavage of lipid hydroperoxides (LOOH) present as traces in most lipid systems (Scheme 1). Fe^{III} is produced along with an oxyl radical ($LO^{\bullet}$). This radical and/or radicals derived from it are involved in the abstraction of a bisallylic hydrogen atom from a polyunsaturated fatty acid (PUFA) chain with subsequent formation of the propagating peroxyl radicals ($LOO^{\bullet}$). By contrast, experiments with purified linoleic acid hydroperoxides suggest that MbFe^{III} reacts with LOOH to yield an unstable and ill-defined one-electron-oxidized iron species, which then abstracts a H-atom from a second LOOH molecule, or possibly from a PUFA residue itself, to form $LOO^{\bullet}$ (Scheme 2).[24] The accumulation of ketones (L=O), a major product of $LO^{\bullet}$ rearrangement, confirms that the initial step is a homolytic cleavage of LOOH but also suggests that, in this system, the $LO^{\bullet}$ radicals rapidly evolve toward nonradical products rather than initiate lipid peroxidation themselves.

For hematin, homolytic cleavage of simple hydroperoxides has been reported to be favored with formation of ferrylhematin (iron−oxo form) and $LO^{\bullet}$ radicals.[25]

Dietary Polyphenols as Antioxidants. *Inhibition by Quercetin of Heme and Nonheme Iron-Initiated Lipid Oxidation.* Lipid oxidation initiated by heme or nonheme iron was inhibited by the flavonol quercetin (Figure 2), already demonstrated as one of the best polyphenolic antioxidant due to its labile H-atoms at positions 3 and 4′.[26] Although quercetin is usually found as glycosides in the diet, it has been shown to
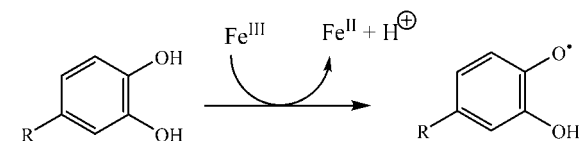
Scheme 1. Lipid Peroxidation Induced by Nonheme Iron and the Action of Polyphenols[a]
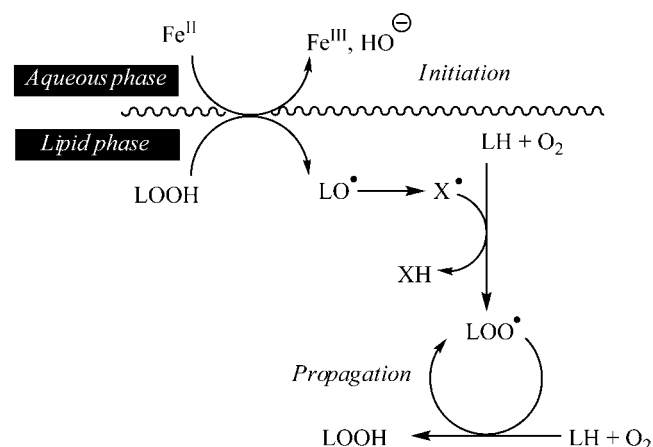


Antioxidant activity (pH 5.8)
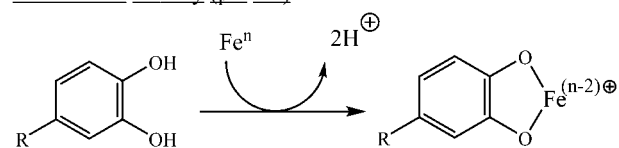
Underlying prooxidant activity (pH 4)

[a]$X^{\bullet}$ = undefined radical derived from an initial lipid oxyl radical.

be released in the oral cavity through hydrolysis of quercetin O-β-D-glucosides by saliva and shedded epithelial cells.[27]

At pH 5.8, quercetin very strongly inhibited lipid oxidation induced by MbFe^{III} and free iron (Figure 3). A remarkably high activity is exhibited toward metmyoglobin, with inhibition levels reaching 75% and 92% for 25 and 100 μM quercetin concentrations, respectively, in the BSA model (Figure 3A). A similar trend was observed in PL-stabilized emulsions, although the inhibition of the MbFe^{III}-initiated process appeared significantly less efficient (Figure 3B). Additionally, the inhibition by quercetin was shown to proceed by decreasing the CD accumulation rate in a concentration-dependent manner. In an earlier work, pseudo lag phases (periods of very slow peroxidation) were observed in the BSA model while not in the PL model with initiator MbFe^{III}.[10] Interestingly, the pseudo lag phases lasted up to 3 and 4 h for respective quercetin concentrations of 50 and 100 μM, whereas quercetin was consumed much earlier. This observation and the monitoring of transient quercetin oxidation products during inhibited lipid peroxidation both strongly support an important role of some quercetin oxidation products in the antioxidant protection.[10] In contrast, no antioxidant effect was observed in the BSA or in the PL model when hematin was used as the initiator, which is not consistent with previous reports in mildly acidic micelle solutions.[7] This strong dependence of the inhibitory properties of quercetin on the initiator clearly

Scheme 2. Lipid Peroxidation Induced by Heme Iron and the Action of Polyphenols[a]
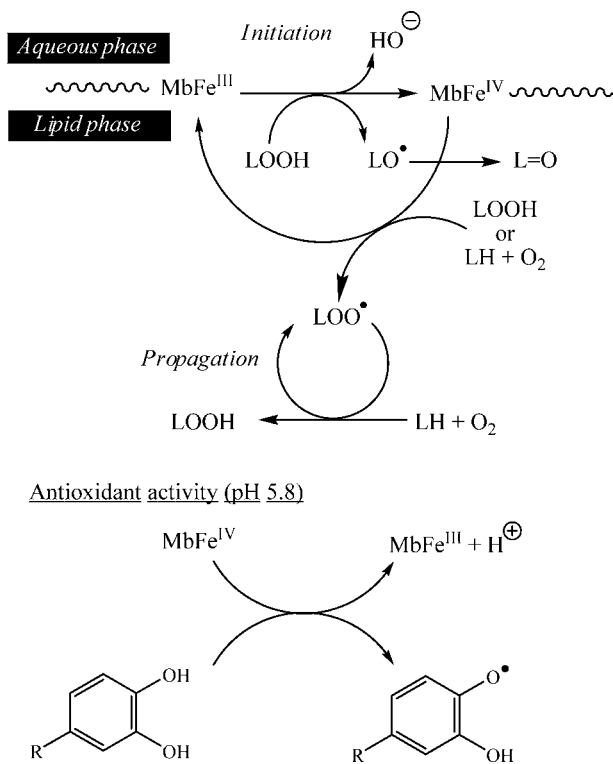
[a]MbFe[IV] = undefined one-electron-oxidized metmyoglobin distinct from ferrylmyoglobin.
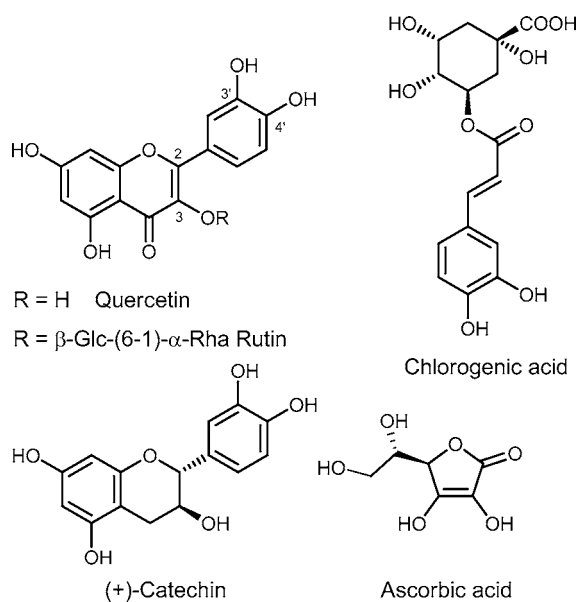

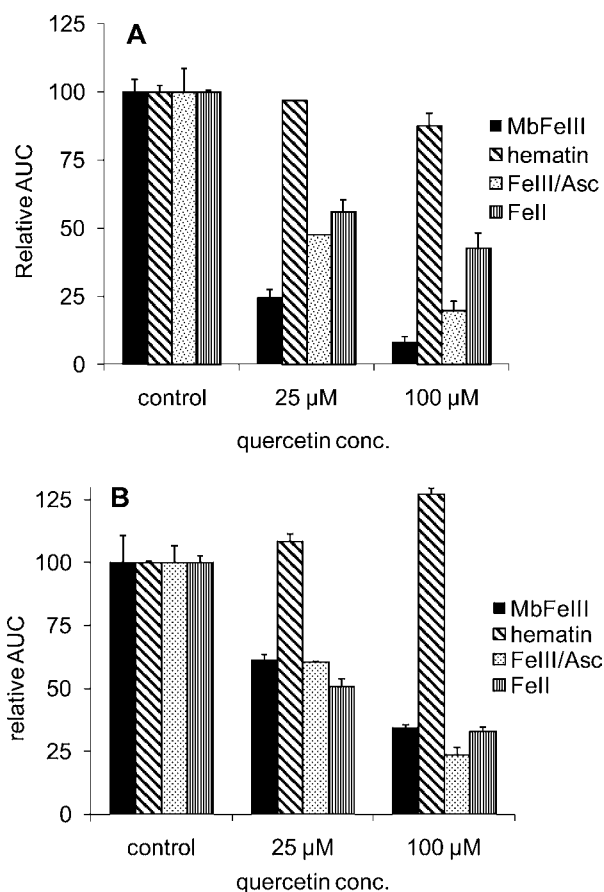Figure 2. Chemical structures of the studied antioxidants.

Figure 3. Inhibition by quercetin (25 and 100 μM) of conjugated diene formation for lipid oxidation initiated by various iron forms at pH 5.8: (A) in the BSA model, (B) in the PL model. Error bars represent the SDs for $n = 3$ (control, 100 μM) and $n = 2$ (25 μM).

shows that quercetin mainly reacts with the initiator rather than by direct scavenging of the propagating lipid peroxyl radicals, which are common to all systems. Actually, quercetin has been demonstrated to rapidly reduce ferrylmyoglobin (MbFe[IV]═O, previously prepared by reacting MbFe[III] with H_2O_2) even in the presence of BSA.[24] The inability of quercetin to inhibit hematin-induced lipid peroxidation may reflect a different

partition for quercetin and hematin that prevents the reduction of hypervalent iron by quercetin.

A decrease in pH mainly reduced the inhibitory effect of quercetin. At pH 4, quercetin only moderately inhibited CD formation when the peroxidation was induced by MbFe[III] or free iron in the BSA model (Figure 4). The loss of antioxidant activity in the MbFe[III]-initiated peroxidation is consistent with the release of hematin from MbFe[III] at lower pH. However, the inhibitory differences persisting between the MbFe[III] and hematin initiators in the BSA model suggest that this release is only partial. Remarkably, quercetin proved totally inefficient in the PL model whatever the initiator, which again outlines the major influence of the emulsifier.

Unlike the heme-induced process, lipid peroxidation induced by nonheme iron cannot be inhibited by reduction of high-valence iron (in this case Fe[III]) as this would mean regeneration of the prooxidant Fe[II] form. Instead, inhibition must proceed by the formation of inert iron−quercetin complexes (Scheme 1). However, iron binding is associated with the removal of protons from the chelating sites and is thus thermodynamically less favorable at low pH.[28] We can thus propose that the low efficiency of quercetin at inhibiting nonheme-induced lipid peroxidation at low pH reflects weaker iron binding and/or underlying prooxidant effects due to quercetin reducing Fe[III] (a reaction that is more favorable at low pH as Fe[III] becomes a stronger oxidant and Fe[II] less sensitive to autoxidation). In this view, it is worth noting that both caffeic acid and rutin proved
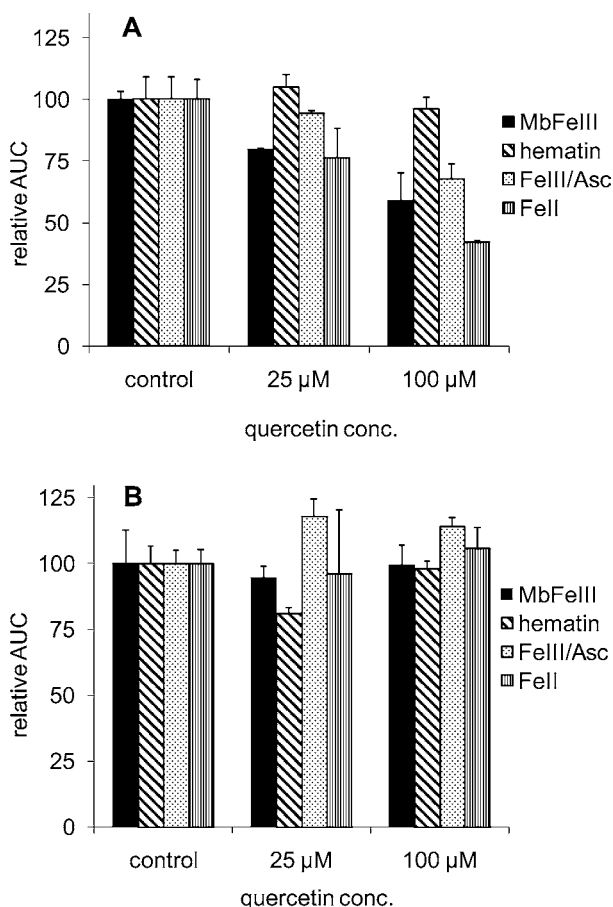
Figure 4. Inhibition by quercetin (25 and 100 μM) of conjugated diene formation for lipid oxidation initiated by various iron forms at pH 4: (A) in the BSA model, (B) in the PL model. Error bars represent the SDs for $n = 3$ (control, 100 μM) and $n = 2$ (25 μM).

Figure 5. Inhibition of metmyoglobin-initiated lipid oxidation by selected polyphenols and ascorbic acid in the BSA and PL models.: (A) at pH 5.8, (B) at pH 4. The antioxidant concentration was 100 μM. The same letters (capital letters for the BSA model and small letters for the PL model) indicate no significant differences between the antioxidants ($p < 0.05$).

prooxidant in Citrem-stabilized oil-in-water (o/w) emulsions at pH 3.[29] Low gallic acid concentrations were also found to enhance lipid oxidation in o/w emulsions at pH 3 while being protective at pH 7.[30]

Dietary Polyphenols as Antioxidants in MbFe[III]-Initiated Lipid Oxidation. The antioxidant capacity of rutin and chlorogenic acid was also assessed in BSA and PL models with MbFe[III] as the initiator. At pH 5.8, the three polyphenols proved largely more efficient inhibitors than ascorbate (Figure 5). Quercetin and rutin (quercetin 3-β-O-rutinoside) were not discriminated, suggesting that the presence of a glycosyl moiety may not prevent the reduction of activated MbFe[III] (Figure 5A). Similarly, caffeic acid and chlorogenic acid (5-caffeoyl-quinic acid) were found equally potent in both models at pH 5.8.[10] The hydrophobic/hydrophilic balance known to influence the antioxidant capacity of polyphenols in bulk oil or micelle solutions may not be the main factor governing their lipid-protecting capacity in o/w emulsions. Again, a significant reduction of the inhibitory effect was observed when replacing BSA by PL, with a decrease from 91% to 65% for quercetin, from 75% to 65% for rutin, and from 60% to 39% for chlorogenic acid. Additionally, the weak protection demonstrated by ascorbate in the BSA model was clearly annihilated in the PL model.

The pH drop from 5.8 to 4 dramatically affected the antioxidant capacity of quercetin, rutin, and chlorogenic acid (Figure 5B). The weak protection that persisted in the BSA
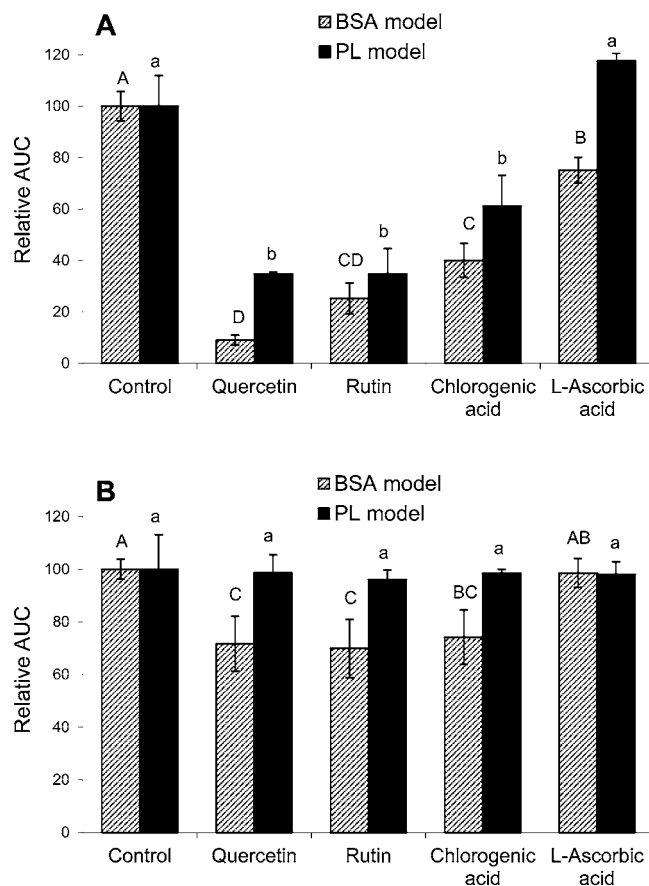
model was abolished in the PL model at pH 4. It is worth noting that the decay of the three phenolic compounds was accelerated by pH lowering. In the BSA model, quercetin, rutin, and chlorogenic acid disappeared in 2, 5, and 4 h, respectively, while in 3, 7, and 8 h at pH 5.8. Surprisingly, none of the antioxidants could be detected after 1 h in the PL model, while the decay times were 3 h for quercetin, 6 h for rutin, and above 8 h for chlorogenic acid at pH 5.8.

As suggested for quercetin, hydrophilic antioxidants do not interact with the iron center of hematin released by denaturation of MbFe[III] but rather decay through other oxidative pathways. It can be noted that BSA–metmyoglobin binding has been demonstrated and that this interaction could partially prevent the release of hematin at pH 4 and consequently the complete collapse of the antioxidant activity.[24] Inhibition by ascorbate and reducing polyphenols (bearing a catechol group) must proceed by reduction of hypervalent heme iron (formed by reaction between MbFe[III] and the lipid hydroperoxides), in agreement with their fast reaction with MbFe[IV]=O (prepared by reacting MbFe[III] with H$_2$O$_2$).[31,32]

Binding Affinity of Quercetin and Rutin to BSA. The affinity of quercetin and rutin for BSA was investigated to refine the antioxidant location in the corresponding oil-in-water system. Assuming a single site of high affinity, the corresponding binding constants K were obtained as described

earlier: $K = (57 \pm 5) \times 10^3$ M^{-1} for quercetin and $K = (1.7 \pm 0.1) \times 10^3$ M^{-1} for rutin at pH 5.8.[10,33] Rutin, the bulky 3-glycoside of quercetin, is more loosely bound to BSA than the aglycon. At pH 4, the K values, $(33 \pm 2) \times 10^3$ M^{-1} for quercetin and $(0.3 \pm 0.1) \times 10^3$ M^{-1} for rutin, point to a slight decrease in the affinity of BSA for flavonols. For 100 μM concentrations, the percentage values of bound quercetin can be calculated to be 87% and 80% vs only 24% and 6% for rutin, at pH 5.8 and 4, respectively. With an estimated BSA adsorption of 3 mg/m^2 at the interface, it can be calculated that only 5% of BSA is located at the interface.[34] The colocation of quercetin at this site is thus rather limited, whereas that of rutin is nearly null. Free and BSA-bound polyphenols may either act in the aqueous phase (e.g., by binding nonheme iron) or be in a fast equilibrium with the small fraction at the interface. Additionally, previous results obtained in aqueous solutions at pH 5.8 demonstrated that the quercetin–BSA complex could interact with MbFe^{III} ($K = 24 \times 10^3$ M^{-1}) and reduce MbFe^{IV}=O (activation by H$_2$O$_2$).[24] A similar reaction could occur at the interface of the BSA-stabilized emulsion at pH 5.8, with BSA-bound quercetin reducing the one-electron-oxidized form of MbFe^{III} involved in the initiation of lipid peroxidation.

Lipid Oxidation and Inhibition in the Presence of Human Gastric Juice. The incubation of human gastric juice with the standard BSA-stabilized emulsion (pH 5.8) promoted the lipolysis of triacylglycerols with rates in the 30−40% range. This is in the upper range of the gastric lipolysis rates (5−40%) reported after the ingestion of emulsified test meals, in agreement with an optimal activity for gastric lipase between pH 3 and pH 6.[35] In addition, the lipid droplet size did not change upon addition of gastric juice, even during the course of the MbFe^{III}-initiated lipid oxidation of this new 2.8 g/L oil-in-water emulsion. It is worth noting that the presence of gastric juice did not significantly affect the rate of CD formation (Figure 6), thus suggesting that the catalytic activity of MbFe^{III} was unaltered and that the fatty acids released were not more prone to oxidation than the initial triacylglycerols. This result supports a prooxidant activity of metmyoglobin toward lipids in real human gastric digestion.

By contrast, inhibition of lipid peroxidation at a 100 μM quercetin concentration proved to be inefficient in the presence of gastric juice. The addition of 760 μM quercetin (i.e., 230 mg of this phenolic compound in a mean stomach volume of 1 L) led to a 36% inhibition level, while this value was 58% in the absence of gastric juice (Figure 6). It can be noted that inhibition by quercetin in the absence of gastric juice is much weaker than anticipated from its efficiency at 100 μM (91% inhibition, Figure 5A). This unexpected difference could point to a prooxidant activity of phenolic antioxidants at high concentration. Indeed, saturation was observed in our previous work when quercetin, catechin, and chlorogenic acid were found barely more active at 100 μM than at 50 μM.[10]

As with quercetin, a loss of antioxidant activity in the presence of gastric juice was also observed for (+)-catechin. This altered efficiency could be attributed to interactions with juice components such as mucins or other proteins, thus hindering the access of polyphenols to the heme cavity.

In the absence of metmyoglobin, quercetin and catechin proved to be stable in the BSA-stabilized emulsion supplemented with gastric juice. Upon addition of MbFe^{III}, quercetin disappeared in less than 4 h while catechin slowly decayed over the 8 h long monitoring. Identified oxidation products for quercetin were 3,4-dihydroxybenzoic acid, a typical benzofuranone derivative (formed by two-electron oxidation of quercetin followed by water addition and rearrangement), along with quercetin dimers as observed in the absence of gastric juice. In the case of catechin, colored dimers (m/z 575, λ_{max} = 410 and 390 nm) resulting from further oxidation of unobserved colorless dimers could be evidenced as described earlier.[10]

In conclusion, o/w emulsions could be used as valuable models of the gastric content to study the stability of lipids in the course of digestion. However, lipid oxidation and its inhibition by polyphenols appear far more complex when studied in emulsions since the interface constitutes a third physical phase where reactivity is mainly driven by factors such as the emulsifier type, pH, and droplet size.

More physiological significance can be brought through the addition of human gastric juice. In the early phase of digestion at pH 5.8, lipase activity, which is optimal, did not affect MbFe^{III}-induced lipid oxidation of emulsified triacylglycerols. However, insight should be given into the late and more acidic phase of digestion to take into account protein hydrolysis. Indeed, the optimal pH for pepsin activity is in the range of 1−3, and fractions of myoglobin mildly hydrolyzed under pepsin catalysis were shown to display a strongly enhanced prooxidant effect on methyl linoleate oxidation compared to native metmyoglobin.[22] Realistic in vitro models of gastric digestion should continue to be developed to take into account pH variations and physiological processes including pepsin and lipase activities.
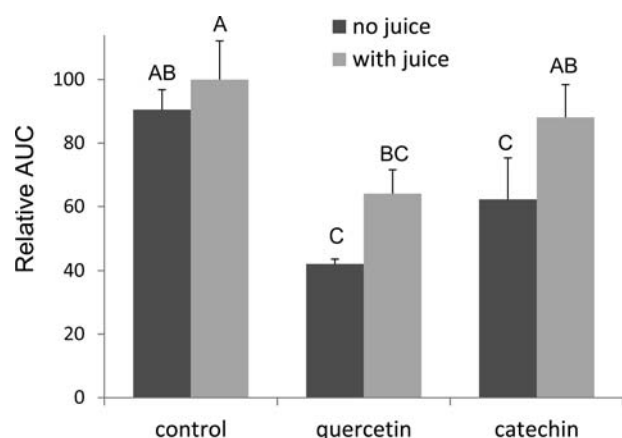

Figure 6. Inhibition of lipid oxidation by quercetin and (+)-catechin in the presence and absence of human gastric juice in the BSA model at pH 5.8. The polyphenol concentration was 760 μM (n = 3; bars are the SDs).

■ AUTHOR INFORMATION

Corresponding Author

*Phone: + 33 432 72 25 15. Fax: + 33 432 72 24 92. E-mail: claire.dufour@avignon.inra.fr.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Staprans, I.; Pan, X. M.; Rapp, J. H.; Feingold, K. R. The role of dietary oxidized cholesterol and oxidized fatty acids in the development of atherosclerosis. *Mol. Nutr. Food Res.* **2005**, *49*, 1075−1082.

(2) Williams, M. J. A.; Sutherland, W. H. F.; McCormick, M. P.; de Jong, S. A.; Walker, R. J.; Wilkins, G. T. Impaired endothelial function following a meal rich in used cooking fat. *J. Am. Coll. Cardiol.* **1999**, *33*, 1050−1055.

(3) Staprans, I.; Rapp, J. H.; Pan, X. M.; Kim, K. Y.; Feingold, K. R. Oxidized lipids in the diet are a source of oxidized lipid in chylomicrons of human serum. *Arterioscler. Thromb.* **1994**, *14*, 1900−1905.

(4) Uchida, K. Role of reactive aldehyde in cardiovascular diseases. *Free Radical Biol. Med.* **2000**, *28*, 1685−1696.

(5) Kanazawa, K.; Ashida, H. Catabolic fate of dietary trilinoleoyl-glycerol hydroperoxides in rat gastrointestines. *Biochim. Biophys. Acta, Lipids Lipid Metab.* **1998**, *1393*, 336−348.

(6) Kanner, J.; Lapidot, T. The stomach as a bioreactor: dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants. *Free Radical Biol. Med.* **2001**, *31*, 1388−1395.

(7) Goupy, P.; Vulcain, E.; Caris-Veyrat, C.; Dangles, O. Dietary antioxidants as inhibitors of the heme-induced peroxidation of linoleic acid: mechanism of action and synergism. *Free Radical Biol. Med.* **2007**, *43*, 933−946.

(8) Vulcain, E.; Goupy, P.; Caris-Veyrat, C.; Dangles, O. Inhibition of the metmyoglobin-induced peroxidation of linoleic acid by dietary antioxidants: action in the aqueous vs. lipid phase. *Free Radical Res.* **2005**, *39*, 547−563.

(9) Armand, M.; Borel, P.; Dubois, C.; Senft, M.; Peyrot, J.; Salducci, J.; Lafont, H.; Lairon, D. Characterization of emulsions and lipolysis of dietary lipids in the human stomach. *Am. J. Physiol.* **1994**, *266*, G372−G381.

(10) Lorrain, B.; Dangles, O.; Genot, C.; Dufour, C. Chemical modeling of heme-induced lipid oxidation in gastric conditions and inhibition by dietary polyphenols. *J. Agric. Food Chem.* **2010**, *58*, 676−683.

(11) Arts, I. C. W.; Hollman, P. C. H. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr.* **2005**, *81*, 317S−325.

(12) Williamson, G.; Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.* **2005**, *81*, 243S−255S.

(13) Gorelik, S.; Ligumsky, M.; Kohen, R.; Kanner, J. A novel function of red wine polyphenols in humans: prevention of absorption of cytotoxic lipid peroxidation products. *FASEB J.* **2008**, *22*, 41−46.

(14) Natella, F.; Ghiselli, A.; Guidi, A.; Ursini, F.; Scaccini, C. Red wine mitigates the postprandial increase of LDL susceptibility to oxidation. *Free Radical Biol. Med.* **2001**, *30*, 1036−1044.

(15) Pérez-Jiménez, J.; Fezeu, L.; Touvier, M.; Arnault, N.; Manach, C.; Hercberg, S.; Galan, P.; Scalbert, A. Dietary intake of 337 polyphenols in French adults. *Am. J. Clin. Nutr.* **2011**, *93*, 1220−1228.

(16) Tyssandier, V.; Reboul, E.; Dumas, J. F.; Bouteloup-Demange, C.; Armand, M.; Marcand, J.; Sallas, M.; Borel, P. Processing of vegetable-borne carotenoids in the human stomach and duodenum. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **2003**, *284*, G913−G923.

(17) Min, B.; Cordray, J. C.; Ahn, D. U. Effect of NaCl, myoglobin, Fe(II), and Fe(III) on lipid oxidation of raw and cooked chicken breast and beef loin. *J. Agric. Food Chem.* **2010**, *58*, 600−605.

(18) Lombardi-Boccia, G.; Martinez-Dominguez, B.; Aguzzi, A. Total heme and non-heme iron in raw and cooked meats. *J. Food Sci.* **2002**, *67*, 1738−1741.

(19) Schricker, B. R.; Miller, D. D.; Stouffer, J. R. Measurement and content of nonheme and total iron in muscle. *J. Food Sci.* **1982**, *47*, 740−743.

(20) Dufour, C.; Loonis, M. Regio- and stereoselective oxidation of linoleic acid bound to serum albumin: identification by ESI-mass spectrometry and NMR of the oxidation products. *Chem. Phys. Lipids* **2005**, *138*, 60−68.

(21) Roginsky, V.; Zheltukhina, G. A.; Nebolsin, V. E. Efficacy of metmyoglobin and hemin as a catalyst of lipid peroxidation determined by using a new testing system. *J. Agric. Food Chem.* **2007**, *55*, 6798−6806.

(22) Carlsen, C. U.; Skibsted, L. H. Myoglobin species with enhanced prooxidative activity is formed during mild proteolysis by pepsin. *J. Agric. Food Chem.* **2004**, *52*, 1675−1681.

(23) Berton, C.; Ropers, M.-H.; Viau, M.; Genot, C. Contribution of the interfacial layer to the protection of emulsified lipids against oxidation. *J. Agric. Food Chem.* **2011**, *59*, 5052−5061.

(24) Lorrain, B.; Dufour, C.; Dangles, O. Influence of serum albumin and the flavonol quercetin on the peroxidase activity of metmyoglobin. *Free Radical Biol. Med.* **2010**, *48*, 1162−1172.

(25) Dix, T. A.; Marnett, L. J. Conversion of linoleic acid hydroperoxide to hydroxy, keto, epoxyhydroxy, and trihydroxy fatty acids by hematin. *J. Biol. Chem.* **1985**, *260*, 5351−5357.

(26) Goupy, P.; Dufour, C.; Loonis, M.; Dangles, O. Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J. Agric. Food Chem.* **2003**, *51*, 615−622.

(27) Walle, T.; Browning, A. M.; Steed, L. L.; Reed, S. G.; Walle, U. K. Flavonoid glucosides are hydrolyzed and thus activated in the oral cavity in humans. *J. Nutr.* **2005**, *135*, 48−52.

(28) El Hajji, H.; Nkhili, E.; Tomao, V.; Dangles, O. Interactions of quercetin with iron and copper ions: complexation and autoxidation. *Free Radical Res.* **2006**, *40*, 303−320.

(29) Sorensen, A. D. M.; Haahr, A. M.; Becker, E. M.; Skibsted, L. H.; Bergenstahl, B.; Nilsson, L.; Jacobsen, C. Interactions between iron, phenolic compounds, emulsifiers, and pH in omega-3-enriched oil-in-water emulsions. *J. Agric. Food Chem.* **2008**, *56*, 1740−1750.

(30) Mei, L.; McClements, D. J.; Decker, E. A. Lipid oxidation in emulsions as affected by charge status of antioxidants and emulsion droplets. *J. Agric. Food Chem.* **1999**, *47*, 2267−2273.

(31) Carlsen, C. U.; Kroger-Ohlsen, M. V.; Bellio, R.; Skibsted, L. H. Protein binding in deactivation of ferrylmyoglobin by chlorogenate and ascorbate. *J. Agric. Food Chem.* **2000**, *48*, 204−212.

(32) Laranjinha, J.; Almeida, L.; Madeira, V. Reduction of ferrylmyoglobin by dietary phenolic-acid derivatives of cinnamic acid. *Free Radical Biol. Med.* **1995**, *19*, 329−337.

(33) Dufour, C.; Dangles, O. Flavonoid−serum albumin complexation: determination of binding constants and binding sites by fluorescence spectroscopy. *Biochim. Biophys. Acta, Gen. Subj.* **2005**, *1721*, 164−173.

(34) Kong, L.; Beattie, J. K.; Hunter, R. J. Electroacoustic study of BSA or lecithin stabilised soybean oil-in-water emulsions and SDS effect. *Colloids Surf., B* **2003**, *27*, 11−21.

(35) Armand, M. Lipases and lipolysis in the human digestive tract: where do we stand? *Curr. Opin. Clin. Nutr. Metab. Care* **2007**, *10*, 156−164.