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# Fruits, vegetables and their polyphenols protect dietary lipids from oxidation during gastric digestion†

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Previous studies indicate that the ingestion of oxidized vegetable oils leads to the incorporation of chemically reactive molecules issued from the decomposition of the initial lipid hydroperoxides into lipoproteins. The aim of the present study is to investigate the oxidation of dietary lipids in the gastric compartment and their inhibition by plant polyphenols provided either as fruit and vegetables (F&V) or an extract. Six minipigs received a standard Western diet containing primarily sunflower oil, ground beef meat, and starch. Polyphenols in different matrix forms were ingested either as cubed F&V or as the corresponding hydroacetonic extract. Sampling of the gastric digesta allowed the kinetic investigation of pH, heme and non-heme iron forms, total lipids, lipid-derived conjugated dienes (CD) and TBARS. F&V and the corresponding polyphenol extract delayed the gastric digestion process as shown for total lipid and heme iron contents. This study also demonstrated the occurrence of *in vivo* oxidation of dietary lipids in the presence of meat iron. Interestingly, F&V played a protective role by totally inhibiting the accumulation of CD while largely decreasing the formation of TBARS. The polyphenol extract similarly slowed down the TBARS formation although it had no effect on the CD accumulation.

## 1 Introduction

Accumulating evidence suggests that lipid oxidation products present in the diet may contribute to the pathogenesis of atherosclerosis.<sup>1</sup> Among others, the intake of oxidized oils was shown to induce endothelial dysfunction.<sup>2</sup> At the molecular level, lipid oxidation products appear to be absorbed by the small intestine before their incorporation into chylomicrons and then LDL as shown for humans and pigs.<sup>3,4</sup> Besides, LDL postprandial modifications such as aldehyde binding to apolipoproteins are reported to be strongly implicated in the atherogenicity of LDL.<sup>5,6</sup>

Food processing or 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 compartment has been proposed as a major site for diet-related oxidative stress.<sup>7</sup>

Indeed, after food intake, dietary iron could trigger lipid oxidation during gastric digestion. This assumption was substantiated *in vitro* using oil-in-water emulsions to model the physical state of dietary lipids.<sup>8,9</sup> In this work, lipid-derived conjugated dienes and short-chain aldehydes and alcohols were produced concomitantly. Their accumulation rates were found to be drastically influenced by pH, the emulsifier type (proteins *vs.* phospholipids), and iron forms (heme *vs.* non-heme iron). To the best of our knowledge, very little is known on the *in vivo* gastric fate of lipid hydroperoxides. Only two studies reported the decomposition of trilinolein and linoleic acid hydroperoxides to aldehyde, epoxyketone and alcohol derivatives in the stomach of rats fed intragastrically.<sup>10,11</sup> Nonetheless, the gastric stability of dietary lipids after the ingestion of a complex meal remains unknown and should be further elucidated.

On the other hand, various meta-analyses have revealed that the consumption of fruit and vegetables (F&V) was associated with a reduced rate of coronary artery disease<sup>12</sup> and stroke.<sup>13</sup> Besides, the development of coronary artery disease was inversely associated with the consumption of flavonoids, a class of polyphenols largely distributed in fruit and vegetables.<sup>14</sup> Increase in plasma antioxidant capacity, inhibition of LDL oxidation, decrease in platelet aggregation and improvement of the endothelial function are the main mechanisms proposed for the health benefit of flavonoids.<sup>15,16</sup> In a recent controlled trial, flavonoid-rich apples independently augmented the nitric oxide status, enhanced endothelial function, and lowered blood

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pressure acutely, outcomes that may benefit the cardiovascular health.<sup>17</sup> Similarly, the ingestion of a Western-type meal enriched in wine polyphenols led to a reduced elevation of malondialdehyde in plasma<sup>18</sup> and decreased the susceptibility of postprandial LDL to oxidation.<sup>19</sup>

Dietary intakes in polyphenols have been reliably evaluated for the British (0.9 g per day)<sup>20</sup> and for the French people (1.2 g per day).<sup>21</sup> A deeper insight revealed that tea, coffee and fruit juices are the major contributors for both groups. After ingestion of a meal rich in plant products, native forms of polyphenols could thus be recovered in elevated concentrations in the gastric tract. However, the bioaccessibility of polyphenols, which is defined as the amount of polyphenols released and solubilized in the chyme, can be modulated by several parameters such as plant matrix, processing, bolus constituents and physiological conditions.<sup>22</sup>

The present study aims at assessing lipid oxidation in the gastric tract after the consumption of a typical Western diet. Because lipid oxidation is triggered by dietary iron forms which themselves show pH dependency, the contents in heme and non-heme iron forms will be kinetically monitored along with pH. Additionally, the lipid protective capacity of polyphenols either embedded or extracted from their natural F&V matrix will be compared. The reported digestion study is conducted with minipigs as the relevance of this model animal has already been established for the digestion of proteins.<sup>23</sup>

## 2 Experimental section

### 2.1 Test meals

**2.1.1 Fruit and vegetables (F&V) and the phenolic extract (PE).** Frozen artichoke hearts (Camus de Bretagne var., Picard) were cooked in a microwave oven for 5 min (8 hearts at a time) then cut into 6 pieces and quick-frozen before freezing at  $-20^{\circ}\text{C}$ . Fresh rennet apples were purchased from a local market. The central part was removed before cutting apples into 12 or 24 pieces for meal or extraction, respectively. Quick freezing of apple pieces was followed by freezing at  $-20^{\circ}\text{C}$  until use. Frozen quetsche plums (halves) were purchased from Picard and kept at  $-20^{\circ}\text{C}$  until needed. Each F&V portion was made of 120 g of apple, 40 g of artichoke heart and 40 g of plum as prepared above.

For the extraction of phenolic compounds from frozen F&V, apple (2.4 kg), cooked artichoke (800 g) and plum (800 g) were ground separately in liquid nitrogen for 3 min at 3000 rpm  $\text{min}^{-1}$  using a PM-400 ball grinder (Retsch GmbH, Germany). The resulting powders were freeze-dried and kept at  $-20^{\circ}\text{C}$ . The combined powders were divided into four portions and each one was extracted as follows. One powder batch (ca. 170 g) was homogenized with 800 mL of acetone-water (70 : 30) for 2 min at 24000 rpm (Ultra-Turrax T25, IKA) before addition of 2.2 L of the same solvent system and stirring for 30 min at RT. After Buchner filtration on Whatman paper no. 3, the powder was extracted once more with 3 L of this solvent system for 30 min. The combined liquid phases were concentrated *in vacuo* using a rotative evaporator at  $30^{\circ}\text{C}$ . The obtained aqueous extract was distributed into plastic trays, freeze-dried and kept

at  $-20^{\circ}\text{C}$  until needed. One F&V portion contained the same amount of polyphenols as 22.8 g of the phenolic extract (PE).

**2.1.2 Meal preparation.** Each meal contained primarily 40 g of sunflower oil (Lesieur “Coeur de Tournesol” from local market) as a source of lipids and 120 g of ground beef meat as a source of protein (Table 1). The meat (*Triceps brachii* muscle) was obtained from a 15 month old Charolais bull and aged 15 days. It was minced with an 8 mm diameter grind before cooking in a vacuum packing at  $70^{\circ}\text{C}$  (water bath) for 30 min and finally freezing at  $-20^{\circ}\text{C}$ . The meals were prepared by quickly mixing in a food processor (KM336 Kenwood) the defrosted meat, the sunflower oil, egg yolk phospholipids (Sigma-Aldrich, St Quentin-Fallavier, France) and either the frozen F&V cut into cubes (2, 5 and 8 mm-edge lengths for apple, plum and artichoke, respectively) or the phenolic extract. F&V defrosted during the mixing step were thus protected as long as possible from browning and polyphenol degradation. When F&V were absent from the meal, starch, cellulose, and apple pectin (all from Sigma-Aldrich, St Quentin-Fallavier, France) were added to simulate complex sugars and cell wall materials as found in the F&V matrix along with water.

### 2.2 Study design

All procedures were conducted in accordance with the guidelines formulated by the European Community for the use of experimental animals (L358-86/609/EEC), and the study was approved by the Local Committee for Ethics in Animal Experimentation (no. CE24-10; Comité d’Ethique en Matière d’Expérimentation Animale d’Auvergne, Aubière, France).

**2.2.1 Animals.** The study involved 6 female Göttingen minipigs (Ellegaard, Denmark) (12–16 months old; 20–25 kg body weight). At least 3 weeks before initiating the study, minipigs were surgically fitted with a permanent cannula (silicone rubber; 12 mm i.d., 17 mm o.d.) in the body of the stomach, in the middle of the long axis of the greater curvature. The cannula

Table 1 Qualitative and quantitative compositions for the three test meals fed to minipigs

	Beef meal	Beef meal + F&V <sup>a</sup>	Beef meal + PE <sup>b</sup>
Ground cooked beef (g)	120	120	120
Sunflower oil (g)	40	40	40
Egg yolk phospholipids (g)	3	3	3
Quetsche plum (g)	—	40	—
Apple (g)	—	120	—
Artichoke heart (g)	—	40	—
Hydroacetic extract (g)	—	—	22.8
Cellulose (g)	7	—	7
Starch (g)	70	—	70
Pectin (g)	1	—	1
Water	30	—	30
Expected meal weight (g)	271	363	293.8
Measured meal weight (g) $\pm$ SD	$255.8 \pm 1.6$	$344.5 \pm 5.6$	$273.5 \pm 2.3$

<sup>a</sup> F&V: fruit and vegetables. <sup>b</sup> PE: polyphenol extract.

was ventrally exteriorized on the left flank, just after the last rib. Surgical procedures, as well as post-surgical care, have been previously described in detail by Rémond *et al.*<sup>24</sup> Minipigs were housed in individual pens (1 × 1.5 m), separated by Plexiglass walls, in a ventilated room with controlled temperature (20–23 °C). Apart from sampling days, they were fed once daily, at 0815, with 400 g of a commercial feed [18% protein, 2% fat, 5% cellulose, 6% ash] (Porcyprima, Sanders Nutrition Animale, France), and had free access to water. In order to ensure a rapid and complete ingestion of the test meals during the sampling days, they were accustomed to receive this type of meal before starting the experiment.

**2.2.2 Experimental protocol.** The three test meals were randomly tested on each minipig. For a given minipig, the days of sampling were separated by at least 3 days. On days in between, minipigs received the commercial feed. The evening before the day of sampling, stomach was flushed by intragastric injection of 200 mL of water followed by free evacuation of the chyme through the cannula. On the day of sampling, minipigs did not receive the commercial feed and were exclusively offered test meals (at 0815). They always consumed the whole meal in less than 15 min. Minipigs had continuous access to water during the sampling period. Digesta (average volume 60 mL) were gravimetrically collected in a graduated beaker 30 min before and 15, 45, 90, 150, 240, and 330 min after test meal delivery. The exact digesta volume was recorded before mixing with 10 mL of water for better consistency. Then the diluted digesta were halved. One part was homogenized for 30 s with an Ultra-Turrax (IKA25, 20 000 rpm) and pH was immediately recorded. The homogenized digesta were subsampled for the remaining analyses (TBARS, lipid-derived conjugated dienes, iron forms). All aliquots were immediately frozen in liquid nitrogen and kept at –80 °C until analysis.

## 2.3 Analyses of the meals and digesta

**2.3.1 Fatty acid chemical analysis.** Total lipids of oil, bovine meat and whole meals were extracted from 6 g of ground samples with chloroform–methanol (2 : 1, v/v) according to the method reported by Folch *et al.*, and then assayed gravimetrically.<sup>25</sup> Lipids were converted into fatty acid methyl esters (FAME) at room temperature using 1 M Na methanolate followed by 14% (vol/vol) BF<sub>3</sub>–methanol for 2 × 20 min. FAME analysis was performed by gas chromatography as described previously.<sup>26</sup> FAME were quantified using C19:0 as internal standard (Supelco, Bellefonte, PA, USA). The identification and calculation of the response coefficient for each individual FAME were achieved using the Supelco quantitative mix C4–C24 FAME.

### 2.3.2 Determination of lipid oxidation

**Determination of total lipids.** Total lipids from freeze-dried homogenized digesta and meals (1–1.5 g) were extracted twice with chloroform–methanol (2 : 1, v/v) according to the method reported by Folch *et al.* using 4 mL per g of fresh matter.<sup>25</sup> The combined organic phases were washed with 0.9% aq. NaCl, dried on sodium sulfate and concentrated first *in vacuo* and then under nitrogen. Total lipids were assayed gravimetrically

and the results are expressed in grams of lipids per 100 g of fresh sample.

**Measurement of conjugated dienes.** Total lipids were dissolved in 2-propanol (2 mL). The concentration of conjugated dienes (CD) was determined by measuring the absorbance at 234 nm (HP 8453 diode-array spectrometer equipped with a magnetically stirred cell; optical path length = 1 cm) and by using 27 000 M<sup>–1</sup> cm<sup>–1</sup> as the molar absorption coefficient for conjugated linoleyl hydroperoxides. Results are expressed in micromoles of CD per gram of lipids.

**Determination of TBARS.** Thiobarbituric acid-reactive substances (TBARS) were evaluated according to Lynch & Frei<sup>27</sup> with slight modifications. Freeze-dried samples of meat, meal and gastric digesta (1 g) were homogenized for 30 s with 10 mL of 0.15 M KCl containing 0.1 mM butylated hydroxytoluene (BHT) using an Ultra-Turrax homogenizer (IKA25, 15 000 rpm). Homogenates (0.5 mL) were incubated with 1% (w/v) 2-thiobarbituric acid in 50 mM NaOH (0.25 mL) and 2.8% (w/v) trichloroacetic acid (0.25 mL) for 10 min in a boiling water bath. After cooling at room temperature for 30 min, the aqueous phase was added with *n*-butanol (2 mL) under stirring, and then centrifuged (4000g, 10 min). The absorbance of the extracted pink chromogen in *n*-butanol was measured at 535 nm with deduction of potential turbidity at 760 nm. TBARS concentrations were calculated using 1,1,3,3-tetraethoxypropane as a standard, and expressed as μmole of equivalent malondialdehyde (MDA) per g of lipids.

**2.3.3 Determination of total iron, heme iron, free iron and Fe<sup>2+</sup>.** Total iron in bovine meat, meals and gastric digesta was evaluated by wet mineralization to extract all iron forms including chelated forms. Measurements were performed by inductively-coupled plasma mass spectrometry (ICP-MS) and expressed as μg of Fe per g of fresh sample.

Free iron and Fe<sup>2+</sup> were measured by the ferrozine assay according to Stolze *et al.*<sup>28</sup> with slight modifications. Samples (1 g) of frozen meat powder, frozen meal powder and defrosted gastric content were homogenized with 10 mL of 140 mM NaCl and 10 mM sodium citrate buffer for 30 s using an Ultra-Turrax homogenizer (20 000 rpm). The dialysis membrane (12 kDa MWCO) was used to form two rolls filled each with 1 mL of the previous buffer and dipped into the first mixture. After 3 to 4 h of dialysis at room temperature under agitation, the contents of the dialysis tubing were centrifuged (4000 rpm, 4 °C, 10 min). The first dialysate was added with 1 mM ferrozine and the second one with 1 mM ascorbate and 1 mM ferrozine, allowing the determination of the Fe<sup>2+</sup> form and free iron, respectively. Iron was determined spectrophotometrically at 562 nm using iron sulfate for calibration. The level in Fe<sup>3+</sup> is the difference between [free iron] and [Fe<sup>2+</sup>]. The heme iron level was deduced by subtracting the free iron content from that of the total iron. All iron form levels are expressed in μg of Fe per g of sample.

## 2.4 Statistical analysis

All data are presented as mean ± SEM (*n* = 6 per group). The postprandial evolutions of pH, CD and TBARS were compared by one-way ANOVA for repeated measures (Tukey *post-hoc* test



for statistical effects of (1) time alone over the postprandial period (15–330 min), (2) meal alone independently of the time in the postprandial period, and (3) interaction of both factors, time and meal) (XLStat software, version 2008.3.02, Addinsoft SARL, Paris, France).

## 3 Results

### 3.1 Evolution of pH during gastric digestion

After the ingestion of a standard Western diet containing principally beef meat and sunflower oil (beef meal, Table 1), the gastric pH increased sharply from 2.1 in the fasting state to 5.6 after 15 min (Fig. 1). When F&V or the phenolic extract (PE) were added to the meal, this pH was found to be 4.5 in both cases outlining a significant effect of meal ( $p < 0.05$ ). The postprandial pH decayed faster during the first 150 min for the beef meal compared to the F&V- and PE-added meals and similarly for the last part of the digestion. At 45 min, the gastric pH after ingestion of the PE meal was still significantly different from pH for the beef meal. After 330 min, pH has not yet returned to the fasting pH suggesting that a period of 5 h 30 min was not sufficient for completion of gastric digestion by minipigs. Finally, a significant effect of time on gastric pH was found during the digestion of the three meals ( $p < 0.0001$ ).

### 3.2 Iron forms

The content in total iron for cooked beef (23  $\mu\text{g}$  per g FW, Table 2) was in the range of data reported for total iron for raw beef (19.5–26.1  $\mu\text{g}$  per g) and cooked beef (24.1  $\mu\text{g}$  per g).<sup>31</sup> In the beef, F&V and PE meals, total iron levels were respectively 10, 8 and 9  $\mu\text{g}$  per g FW as a result of the dilution by the different meal constituents. There is no apparent contribution of F&V although artichoke, apple and plum could theoretically contribute for 2.1 and 2.6  $\mu\text{g}$  per g FW to the F&V and PE meals (USDA National Nutrient Database for Standard Reference, Release 25. Nutrient Data Laboratory Home Page, <http://www.ars.usda.gov/ba/bhnrc/ndl>, accessed 15/7/2013). A small

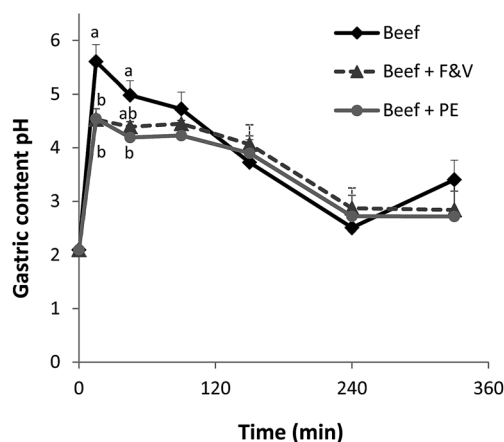


Fig. 1 Evolution of the *ex vivo* gastric pH after the ingestion by minipigs of beef- and sunflower oil-based test meals (mean  $\pm$  SEM,  $n = 6$ ). Different letters indicate statistical differences ( $p < 0.05$ ).

dilution by gastric juice (12–15%) may be observed after 15 min with total iron contents of 8.5, 6.8, and 7.9  $\mu\text{g}$  per g of FW for the beef, F&V and PE meals, respectively. Additionally, levels in free iron and the  $\text{Fe}^{2+}$  form were measured using the ferrozine assay while levels in the  $\text{Fe}^{3+}$  form and heme iron were deduced through calculations. The larger contribution of heme iron (19.8  $\mu\text{g}$  per g FW) compared to free iron (3.2  $\mu\text{g}$  per g FW) is found in raw beef meat or after light cooking<sup>31</sup> indicating that the steam cooking procedure used in this study did not induce iron release. Free iron is dominated by the  $\text{Fe}^{3+}$  form (2.5  $\mu\text{g}$  per g FW) compared to the reduced and unstable  $\text{Fe}^{2+}$  form (0.7  $\mu\text{g}$  per g FW). The gastric digestion of the beef meal led to a rapid

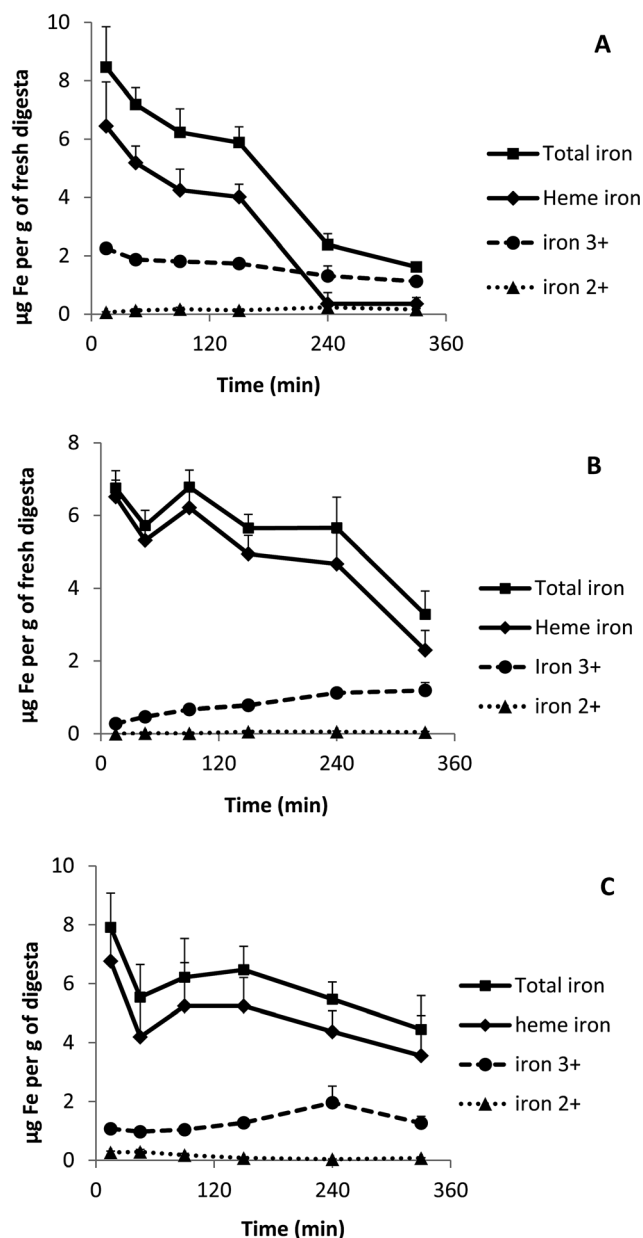


Fig. 2 Evolution of the gastric concentration in total iron, heme iron,  $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$  after ingestion of the beef meal (A), the F&V meal (B) and the PE meal (C). (Mean  $\pm$  SEM,  $n = 6$ ).

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Table 2 Contents in lipids, lipid oxidation products and iron forms in the beef meat and the three test meals

	Bovine <i>Triceps brachii</i>	Beef meal	Beef meal + F&V	Beef meal + PE
Measured lipid content <sup>a</sup> (g per 100 g FW)	2.1 ± 0.1	12.1 ± 0.1	9.4 ± 0.2	12.8 ± 0.5
Theoretical lipid content (g per 100 g FW)		15.7	11.7	14.5
Conj. dienes <sup>a</sup> (μmol per g lipid)	39.4 ± 0.2	18.7 ± 3.5	10.4 ± 2.0	10.1 ± 1.7
TBARS (μmol per g lipid)		0.156	0.106	0.105
Total iron (μg Fe per g FW)	23.0	10.0	8.0	9.0
Free iron (μg Fe per g FW)	3.2	3.1	0.4	1.8
Heme iron <sup>b</sup> (μg Fe per g FW)	19.8	6.9	7.6	7.2

<sup>a</sup>  $n = 2$  or  $3$ , mean ± SD. <sup>b</sup> Heme iron is the difference between total iron and free iron.

decrease in heme iron which became nearly undetectable after 240 min (Fig. 2A). Free iron was mostly recovered in the form of  $\text{Fe}^{3+}$  in both the meal and the digesta suggesting a more oxidizing environment compared to meat. The  $\text{Fe}^{3+}$  form decayed more slowly than heme iron suggesting that part of the heme iron atoms could be released from the protoporphyrin ring of metmyoglobin. For the F&V and the PE meals, slower decreases for total and heme irons were observed while  $\text{Fe}^{3+}$  was even shown to accumulate in agreement with the suggested conversion of heme iron into free iron (Fig. 2B and C). It is worth noting that the content in the  $\text{Fe}^{3+}$  form is unexpectedly low in the F&V meal (0.4 μg per g) and the corresponding T15 min digesta (0.28 μg per g) compared to the contents in the PE meal (1.8 μg per g) and corresponding T15 min digesta (1.07 μg per g). This could be attributed to a strong complexation of free iron by unidentified F&V components and its subsequent lack of dialysability, thus making free iron unavailable for titration by ferrozine.

### 3.3 Lipid stability in the gastric tract

**3.3.1 Total lipids.** The decrease in total lipids in the gastric digesta was almost linear over the 330 min long period of monitored digestion for the three test meals (Fig. 3) in agreement with dilution by gastric juices and simultaneous gastric emptying. Although the effect of meals cannot be statistically assessed owing to the difference in meal size, a faster rate for the decay in total lipids was observed during the digestion of the beef meal. This trend is similar to the one observed for the gastric pH and suggests a slowdown role in digestion parameters for some biomolecules present in both the extract and F&V.

**3.3.2 Lipid oxidation in the gastric digesta.** The oxidative state of lipids was first probed by analyzing the gastric digesta for lipid-derived conjugated dienes (CD) as primary markers. Forty five minutes after the ingestion of the beef meal, CD started to accumulate following a bell-shaped kinetics (Fig. 4A). The maximal content, observed between 150 and 240 min, corresponds to a 35% increase in CD. The addition of the phenolic extract to the beef meal (PE meal) had no effect on the CD accumulation. However, with the F&V meal, CD levels were found significantly higher at the initial stage of the digestion (T15 and T45 min). Nevertheless, there was no noticeable CD accumulation within this meal during the 330 min-long digestion process.

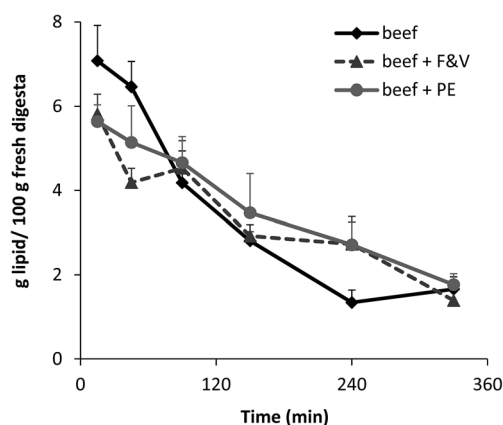


Fig. 3 Evolution of total lipids in the course of gastric digestion (mean ± SEM,  $n = 6$ ).

TBARS were next followed as secondary lipid oxidation products. Their evolution was clearly different from that of CD (Fig. 4B). Indeed, TBARS accumulated continuously for the three meals for at least 240 min. The F&V and PE meals markedly slowed down the formation of TBARS. ANOVA with repeated measures revealed significant effects for meal ( $p = 0.03$ ), time ( $p < 0.0001$ ) and meal × time ( $p = 0.0003$ ). At 240 min, the TBARS level per gram of lipids was significantly lower ( $p < 0.05$ ) for both the F&V and the PE meals compared to the beef meal. At this stage, TBARS have increased by a 5-fold factor for the beef meal, while only by a two-fold factor for both the F&V and PE meals.

## 4 Discussion

In the few intervention studies investigating gastric pH for complex meals, liquid test meals were classically fed to nasogastrically intubated humans. By contrast, solid ingredients of human consumption such as beef meat, sunflower oil and fruit and vegetables (F&V) were used in our study after classical home processing including grinding, cooking and mixing. The ratio between triglycerides and phospholipids is representative of an average Western adult consumption (100–150 g triglycerides and 2–10 g phospholipids each day) (Table 1). The Western diet

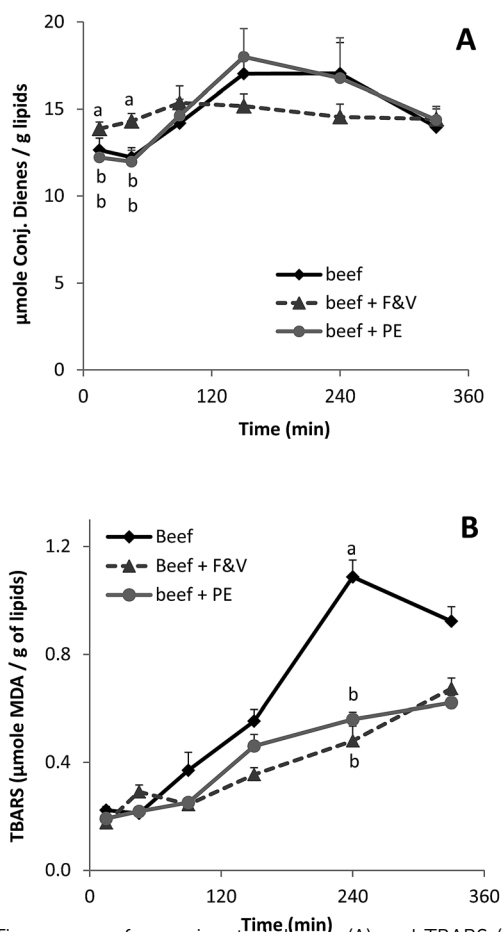


Fig. 4 Time course for conjugated dienes (A) and TBARS (B) in the gastric digesta after the ingestion of the beef, F&V and PE meals (mean  $\pm$  SEM,  $n = 6$ ). Different letters indicate statistical differences at a given time ( $p < 0.05$ ).

compared to linolenic acid as evidenced here with the commonly consumed sunflower oil, egg yolk phospholipids and beef meat.

It was reported that gastric pH reached 6.4 thirty minutes after the consumption of Ensure Plus<sup>R</sup> (a nutrient-rich emulsion with an intrinsic pH of 6.6), pH between 5.4 and 6.2 twenty minutes after the ingestion of a liquid Western-type diet enriched in vegetable purees and pH 5.4 only three minutes after the consumption of a cocoa beverage (intrinsic pH 6.4).<sup>32-34</sup> The pH variations recorded in the minipig stomach during digestion appear thus to be similar to those observed in humans with a very rapid rise after food ingestion followed by a nearly linear decay to return to the fasting pH. The high pH values reached after a few minutes are mainly related to food intrinsic pH and its buffering capacity. In meat, proteins and carnosine play this role. In this study, initial gastric pHs were found to be 5.6, 4.5, and 4.5 after the ingestion of the beef, F&V and PE meals, respectively. A significant effect of the F&V and PE matrices is highlighted, resulting possibly from the additional presence of soluble sugars, amino-acids, small peptides or polyphenols. Hence, pH kinetic data obtained for gastric

digestion in this study, like data previously reported for meat and milk protein digestibility,<sup>35,36</sup> support well the use of the minipig as an animal model for digestion studies.

#### 4.1 The fate of lipids during digestion

**4.1.1 Total lipids.** The contents in total lipids were evaluated for beef meat, meals and the corresponding gastric digesta over 330 min (Table 2 and Fig. 3). The measured total lipid contents were 12.1, 9.4 and 12.8 g per 100 g FW in the initial meals in agreement with a higher dilution of the lipids by F&V than PE during meal preparation. In the T15 min sampling arising from the beef, F&V and the PE meals, lipids represent 7.1, 5.8 and 5.6 g per 100 g of FW, respectively. These concentrations in total lipids correspond to only 59, 62 and 44% of the total lipids initially present in the beef, F&V and PE meals, respectively. Part of this difference in concentrations could be explained by the dilution of the chyme by both saliva and gastric juices as shown by the 12–15% decrease in total iron. Indeed, the viscous aspect observed for the chyme reveals the presence of mucins known to be present in both fluids. However, the loss at 15 min of nearly half of the lipids could also be accounted for by the formation of a lipid layer on top of the chyme in the upper part of the gastric compartment. This hypothesis could not be confirmed as sampling was performed on the greater curvature of the stomach, *i.e.* at the mid-height of the full stomach. Nevertheless, gastric digesta that were sampled did not exhibit phase separation, only a continuous decrease in viscosity upon time (ESI, S1†). Additionally, light microscopy and granulometry revealed perfectly circular objects which could be assessed to emulsified oil droplets (unpublished data). Egg yolk phospholipids and meat proteins or their hydrolysates are known to be efficient dietary emulsifiers helping thus to the early emulsification of sunflower oil triacylglycerols.

**4.1.2 Lipid oxidation.** CD correspond to early lipid oxidation products which share a conjugated dienyl system and diversely oxygenated functions. Unstable lipid hydroperoxides are known to give rise to related alcohols, epoxides and ketones through intramolecular radical and non-radical rearrangements.<sup>37</sup> In the presence of metmyoglobin, a heme iron form, linoleic acid hydroperoxides were found to be mainly converted into the corresponding ketones.<sup>38</sup> Other pathways including carbon–carbon cleavage lead to short-chain aldehydes, unsaturated aldehydes or alcohols among others.<sup>39</sup> Most of these short-chain derivatives were shown to be produced during the storage of sunflower oil or in the thermal treatment of vegetable oils rich in linoleic and linolenic acids.<sup>40,41</sup> Among them, malondialdehyde (MDA) is a typical marker for secondary lipid oxidation and is classically assessed as thiobarbituric acid-reactive substances (TBARS). Although CD are mostly produced through lipid oxidation of linoleic acid, a fatty acid largely found in sunflower oil, MDA may be a more suitable marker for more highly polyunsaturated fatty acids such as linolenic and arachidonic acids<sup>42,43</sup> mostly provided by meat and egg yolk phospholipids (Table 3). For the complete oxidation of polyunsaturated fatty acids, the yield in TBARS is 0.55% (mol/mol) for linoleic acid, 4.9% for linolenic acid and 8.6% for

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Table 3 Composition in main fatty acids of sunflower oil, beef meat and test meals

	Sunflower oil	Beef meat	Beef meal	Beef meal + F&V	Beef meal + PE
C16:0	5.8 <sup>a</sup>	19.7	8.5	8.3	7.3
C18:0	4.0	15.2	6.5	5.2	4.9
C18:1 <i>cis</i> 9	53.9	32.4	48.9	42.7	52.7
C18:2 <i>n</i> -6	34.3	11.4	30.5	31.8	32.4
C18:3 <i>n</i> -3	0.12	2.1	0.26	0.32	0.28
C20:4 <i>n</i> -6	—	1.7	0.33	0.36	0.25
Σ saturated FA	11.4	37.8	16.8	15.0	13.6
Σ monounsaturated FA	54.2	39.4	51.7	53.4	53.2
Σ polyunsaturated FA	34.5	17.7	31.3	32.9	34.4

<sup>a</sup> Results are expressed as percentage of total FA (*n* = 1).

arachidonic acid.<sup>43</sup> For the meals under study, the relative composition in these fatty acids is 100 : 1 : 1, respectively (Table 3). Although unexpected, linoleic acid could thus produce 4-fold more TBARS than combined linolenic and arachidonic acids.

The initial oxidation state of the test meals was evaluated right before serving to minipigs. CD were present at the levels of 18.7, 10.4 and 10.1 μmol per g lipids in the beef, F&V and PE test meals, respectively (Table 2). TBARS were also identified with again higher levels for the initial beef meal (0.156 μmol per g lipids) compared to both F&V and PE meals (0.106 and 0.105 μmol per g lipids respectively). F&V and the phenolic extract may thus exert a protective effect during meal preparation when polyunsaturated fatty acids and prooxidant iron species from meat are brought into contact. In the same way, a polyphenol-rich grape seed extract was reported to inhibit the onset of lipid oxidation during the storage of minced fish.<sup>44</sup> This difference in favor of a higher oxidation level in the beef meal was also outlined in the T15 min sample of gastric digesta with TBARS values of 0.222, 0.175 and 0.191 μmol per g lipids (Fig. 4B). Similarly, CD evolved rapidly between meal preparation and T15 min with values of 12.6, 13.9 and 12.2 μmol per g lipids for the beef, F&V and the PE meals, respectively (Fig. 4A). It is noteworthy that a significantly higher CD content was observed for the F&V meal (+10%, *p* = 0.0004) although this content did not increase further during the course of the digestion process.

A steady-state pattern for CD usually accounts for identical rates for the formation and degradation of lipid oxidation products sharing a conjugated dienyl moiety. Thus, the bell-shaped kinetics observed for CD (Fig. 4A) indicates faster rates of formation than decomposition during the period between 15 and 150 min. After 150 min, the CD content tends to level off before apparently decreasing. The assumption of a continuous accumulation of CD is supported by recent reports of *in vitro* digestion. TBARS and lipid hydroperoxides were shown to dramatically increase when cod hemoglobin was added to cod liver oil<sup>45</sup> or when cooked turkey meat was digested with simulated gastric juices.<sup>46</sup> The low stability of the lipid hydroperoxyl group under gastric conditions was investigated by Kanazawa and Ashida. These authors found that, when partly peroxidized trilinolein was intragastrically administered to rats, the stomach content in trilinolein hydroperoxides decayed over 4 h.<sup>10</sup> Linoleic acid hydroperoxides and the corresponding

alcohols were recovered in the stomach probably through the action of gastric lipase. Besides, neither trilinolein hydroperoxides nor linoleic acid hydroperoxides reached the intestine but only cleavage products. These data support the decomposition of lipid conjugated dienes which mainly consist of linoleyl hydroperoxides owing to the abundance of linoleic acid residues in the meals. Whatever the meal ingested, TBARS levels increased during the whole process of gastric digestion in agreement with the continuous degradation of primary lipid oxidation products (Fig. 4B). These results substantiate the occurrence of lipid oxidation in gastro and validate previous results obtained in static *in vitro* models of gastric digestion.<sup>8,9,47</sup> Lorrain *et al.* reported a quasi-linear accumulation of both CD and short-chain volatile compounds upon addition of metmyoglobin, the heme iron form of beef, to sunflower oil-in-water emulsions. The emulsifier type (BSA, phospholipids), pH and the iron form were demonstrated to be key factors governing the lipid oxidation rates. Overall, the extent of lipid oxidation was markedly depressed when egg yolk phospholipids were present.<sup>8,9,47</sup> This small-sized surfactant gives more homogeneous interfaces, limiting the access to the prooxidant species. Additionally, in the early step of the *in vitro* digestion at pH 5.8, heme iron forms (metmyoglobin, hematin) had twice a prooxidant activity than free iron forms (Fe<sup>2+</sup> and Fe<sup>3+</sup>/ascorbate). When the pH was set at 4, the free and the heme iron forms were found as aggressive. Moreover, metmyoglobin undergoes denaturation at pH 4 with the concomitant release of its protoporphyrin nucleus. In this study, pH is above 4 between T15 and T150 min and the prooxidant iron form is thus mainly metmyoglobin or digested metmyoglobin. After 150 min, pH decreases below 4 and the main iron forms may be hematin and Fe<sup>3+</sup> (Fig. 2). A redox Fe<sup>3+</sup>/Fe<sup>2+</sup> cycle in the presence of ascorbic acid may lead to transient Fe<sup>2+</sup> concentrations, an iron form which cleaves lipid hydroperoxides through the Fenton reaction.

The amount of TBARS compared to CD can be calculated. The CD/TBARS ratio evolved from 60 to 70 in the initial stage of the digestion (T15 min) to 15 for the beef meal and 30 for the F&V and PE meals, respectively, at T240 min. This difference is thus in favor of the primary marker of oxidation and is similar to that observed for lipoproteins where TBARS were found to accumu-



individuals.<sup>48</sup> Free MDA may be underevaluated in the presence of proteins as it reacts with the  $\epsilon$ -amino group of lysine leading to the formation of covalent adducts such as Schiff bases.<sup>49</sup> Additional routes for the formation of secondary oxidation products may also be responsible for the measured low levels in TBARS. Indeed, the formation of covalent adducts between proteins and either 4-hydroxy-2-nonenal or 4-hydroxy-2-hexenal, arising from the respective oxidation of *n*-6 and *n*-3 polyunsaturated fatty acids, was evidenced in the three meals with a noticeable increase starting after 150 min (unpublished results).

#### 4.2 Lipid protection by F&V and the corresponding phenolic extract

The sunflower oil used in this study contained 900 ppm of vitamin E. The main constituent of vitamin E in sunflower oil,  $\alpha$ -tocopherol, is thus unable to totally protect emulsified lipids from oxidation during gastric digestion as also observed for the *in vitro* digestion of cod liver oil.<sup>45</sup> F&V and their flavonoid constituents exert coronary and vascular protection as demonstrated by epidemiologic studies.<sup>12-14</sup> Although the causal mechanism of these associations needs to be demonstrated, these studies provide a strong support for the recommendations to consume more than five servings of F&V per day. In this study, minipigs were fed with a Western-type diet associated with half of the recommended portion, *i.e.* 2.5 servings or 200 g of F&V. Both cubed F&V and the corresponding hydroacetic extract contained 154 mg of identified monomeric phenolic compounds (ESI, S2†), 79 mg of oligomeric flavanols (average degree of polymerization = 3) along with F&V soluble sugars and amino acids (22.6 g). As expected, apple (120 g) was a source of monomeric and oligomeric flavanols, flavonols as well as dihydrochalcones. Quetsche plum (40 g) contributed to the different classes of phenolic compounds. As to the artichoke heart (40 g), it provided 61% (p/p) of the monomeric phenolic pool mainly as hydroxycinnamic acids. In the French diet, hydroxycinnamic acids are the most largely consumed polyphenols (599 mg per day) followed by proanthocyanidins (227 mg per day).<sup>21</sup> Actually, caffeoylquinic acids are the main contributors (74%, p/p) to the extract with chlorogenic acid being the most abundant compound. Caffeoylquinic acids and flavanols, the second major group, display the typical 1,2-dihydroxyphenyl moiety that is critical to the reducing capacity of phenolic compounds. It has been reported that ferrylmyoglobin (MbFe(IV)=O), produced upon activation of metmyoglobin MbFe(III) by lipid hydroperoxides or hydrogen peroxide, is efficiently reduced by hydroxycinnamic acids<sup>50,51</sup> and flavonoids.<sup>38,52</sup> Thus, the phenolic compounds brought by F&V and the extract may protect lipids by reduction of hypervalent iron forms as well as by chelation of free iron forms, all involved in the initiation step of lipid oxidation.

In the evaluation of the lipid protection, different effects were unexpectedly observed on the accumulation kinetics of CD. The polyphenol extract had no influence on the CD pattern whereas F&V, although increasing the initial level in the primary marker, prevented totally and significantly ( $p < 0.05$ ) their apparent formation (Fig. 4A). By contrast, when TBARS were assessed, both F&V and the corresponding extract proved to be

highly protective of lipids, limiting TBARS accumulation by a 2.5 to 3-fold factor (Fig. 4B). Significance ( $p < 0.05$ ) was only reached at T240 min owing to a large inter-individual variability. Similarly, Gorelik *et al.* found a marked inhibition of lipid hydroperoxide and MDA formation when heated turkey meat was digested *in vitro* in the presence of red wine polyphenols.<sup>53</sup> Additionally, the inclusion of a polyphenol-rich grape seed extract during the digestion of minced fish in a dynamic *in vitro* digestion model decreased the formation of CD in both the gastric and intestinal compartments.<sup>44</sup> In a static *in vitro* digestion model, Lorrain *et al.* established that catechol-bearing quercetin, (+)-catechin and chlorogenic acid highly inhibited the accumulation of CD and short chain volatiles in the initial step of gastric digestion (pH 5.8), although only slightly when human gastric juice was added or pH set at 4.<sup>8</sup> By contrast, in this *in vivo* study, the inhibitory capacity of F&V and the corresponding phenolic extract appeared conserved throughout the digestion process.

## 5 Conclusion

In conclusion, the present study clearly demonstrates the occurrence of *in vivo* oxidation of dietary lipids in the presence of meat iron and suggests that F&V and their polyphenols can play a protective role. The chemical structure of the antioxidant microconstituents and their respective bioaccessibility are key determinants to the antioxidant capacity of F&V. Because data on the metabolism of polyphenols in the human gastrointestinal (GI) tract are scarce and mainly from ileostomy patients, efforts should now be devoted to the evaluation of the polyphenol bioaccessibility in the GI tract.

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