CHARACTERISATION OF THE FREE RADICAL CHEMISTRY IN PHYSICOCHEMICAL CONDITIONS OF THE DIGESTIVE TRACT

P. Gatellier[1], A. Meynier[2], C. Dufour[3, 4], C. Caris[3, 4], P. Goupy[3, 4], J. Do. Daudin[1], M. Delosière[1], C. Ferreira[1] and V. Santé-Lhoutellier[1]

[1] Quality of Animal Products UR 370, INRA, F-63122 Saint Genès Champanelle, France

[2] Biopolymers Interactions Assemblies UR 1268, INRA, F-44316 Nantes, France

[3] Safety and Quality of Plant Products UMR 408, INRA, F-84000 Avignon, France

[4] Safety and Quality of Plant Products UMR 408, University of Avignon, F-84000 Avignon, France

Abstract – The free radical reactions were evaluated in an aqueous model system which mimics the physicochemical conditions of the digestive tract (37°C and pH 3.5, 5, 6.5). Oxidants (Fe^{2+}/H_2O_2) and antioxidants were added in concentrations which can be found in a bolus after ingestion of a balanced meal composed of meat and vegetables. Detection of the free radicals was achieved using two specific probes (Nitroblue Tetrazolium for superoxide radicals, $O_2^{\circ-}$, and Terephthalate for hydroxyl radicals, OH°). The level of the detected radicals increased with increasing oxidant concentrations. The pH effect varied according to the free radical. Detected $O_2^{\circ-}$ decreased with increasing pH while a biphasic effect of pH was observed with OH°, with a maximum of detected OH° at pH 5. Antioxidants had various impacts on the free radicals. Plant-derived antioxidants; β-carotene and polyphenols (caffeic acid, rutin, chlorogenic acid and quercetin) had antagonist effects on the two radicals. They increased the level of detected $O_2^{\circ-}$ and decreased OH° detection. Trolox C (a water soluble analogue of vitamin E) did not significantly impact the free radical detection. From these data a predictive mathematic model of oxidations in the digestive tract will be built.

Key Words – antioxidant, digestion, oxidation

I. INTRODUCTION

Meat is an important source of iron in the human diet. The total iron content and the ratio between heminic and non heminic iron (also called free iron) depend on animal species [1]. Increasing temperature and decreasing pH favour the transformation of heminic iron into free iron. Free iron is the main catalyst of oxidation in meat. It reacts with oxygen and its peroxide derivatives to give oxygenated free radicals. These reactions belong to the Fenton chemistry. Superoxide radical ($O_2^{\circ-}$) and hydroxyl radical (OH°) are the precursors of the cascade of reactions leading to lipid and protein oxidation. Meat is almost always cooked before consumption. By increasing the free radical production heating accelerates greatly lipid and protein oxidation [2] thus leading to a negative impact on the nutritional value of meat. Meat oxidation generated by processes has long been studied but little is known about oxidation during digestion. Nevertheless, the digestive tract, and especially the stomach, is a favourable medium for the propagation of oxidation [3]. The digestive tract is characterized by important changes in pH and by the enzymatic degradation of nutrients. Under enzymatic processes, lipids and proteins are released from the meat matrix and transformed into fatty acids and amino acids, rendering them more sensitive to the free radical attack. Moreover, the initial stage of digestion takes place in aerobic conditions which favours the formation of oxygenated free radicals. Therefore, the oxidative process, which has been initiated during cooking, can develop during digestion. In balanced meals, plant foods also provide iron, but its irreversible chelation with phytate almost completely blocks the generation of free radicals via the Fenton reactions [4]. Fruits and vegetables are important source of various antioxidants, like vitamins, polyphenols and carotenoids, while only a few are provided by meat, like thiols and antioxidant peptides (carnosine, anserine). The efficiency of these antioxidants in the digestive tract still remains poorly documented. The aim of this study was to characterize the free radical chemistry under

some selected physicochemical conditions which can be observed in the digestive tract, and with various levels of oxidants and antioxidants, representative of a balanced meal.

II. MATERIALS AND METHODS

The free radical production was evaluated in a saline solution KCl/NaCl/$CaCl_2$ (2/120/6 mM) with 20 mM sodium phosphate added. pH was fixed to 3.5, 5 and 6.5. Four concentrations (0.02, 0.05, 0.10 and 0.20 mM) of oxidants, consisting in an equimolecular mixture of ferrous iron ($FeSO_4$) and H_2O_2, were tested. Two concentrations (0.01, and 0.1 mM) of antioxidants (trolox C, β-carotene and various polyphenols) were tested. Temperature was fixed at 37°C and all kinetics were performed during 3 hours. The initial concentration of dissolved oxygen at this temperature was from 0.21 +/- 0.01 mM (measured with a luminescent dissolved oxygen probe). The free radical production was evaluated with two specific probes. $O_2^{\circ -}$ production was evaluated by the reduction of nitroblue tetrazolium NBT (0.5 mM) into formazan, measured by absorbance of formazan at 530 nm [5]. OH° formation was evaluated by the hydroxylation of terephthalate (1mM) into hydroxy-terephtalate, measured by fluorescence spectroscopy (λex = 320 nm and λem = 420 nm) [6]. When antioxidants were added, their absorbance or fluorescence was subtracted.

III. RESULTS AND DISCUSSION

III.1. Choice of experimental conditions:

The different conditions (temperature, pH, oxidant and antioxidant concentrations) were chosen in order to represent some of the physicochemical conditions observed, at different stages of the digestion, in a bolus stemming from a balanced meal. pH 5 and 3.5 were representative of the pH at the beginning and at the mid-step of the gastric digestion and pH 6.5 was chosen to represent the pH in the small intestine. Concentrations of iron (from 0.02 to 0.2 mM) were representative of meals composed of different meats after application of a dilution factor taking into account dilution in the bolus by the digestive juices. Hydrogen peroxide content was more difficult to estimate. H_2O_2 results essentially from superoxide radical dismutation. In turkey ground muscle, Harel and Kanner [7] estimated the production of H_2O_2 to 0.045 mM/h at 37°C and at pH 5.6. This production is probably higher in red meat due to higher level of iron responsible of the $O_2^{\circ -}$ production. So, after taking into account the species effect, dilution factor, and digestion time, we decided to add hydrogen peroxide at the same concentration than iron. Antioxidants were also added at realistic levels. These conditions are of course not intended to be exhaustive of all conditions to be observed with different meals. This is why we develop, in parallel, a mathematic model of the Fenton chemistry. When achieved, this mathematic tool will allow extrapolating the kinetic results to different conditions that exist in practice.

III.2. Superoxide radical ($O_2^{\circ -}$) detection:

First measurements were conducted at the 3 pHs with oxidants (Fe^{2+}/H_2O_2) only. In these conditions superoxide radical was formed by reaction of ferrous iron with dissolved oxygen according to the reaction:

$$(1)\ Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\circ -}$$

And $O_2^{\circ -}$ reacted with the probe according to:

$$(2)\ NBT + 2\ O_2^{\circ -} \rightarrow Formazan + 2\ O_2$$

Figure 1 shows the cumulative amount of $O_2^{\circ -}$ which had reacted with the probe during 3 hours of incubation at pH 3.5. The level of $O_2^{\circ -}$ detected increased significantly with time ($p<0.001$) and with the oxidant concentration ($p<0.001$). The same developments were observed at the two higher pHs (results not shown). We also noted that increasing pH from 3.5 to 6.5 decreased significantly ($p<0.001$) the level of detected $O_2^{\circ -}$. This pH effect can be explained by the decrease of the reaction constant k NBT/$O_2^{\circ -}$ with increasing pH [5]. The combined effect of oxidants and pH on the final level of detected $O_2^{\circ -}$ is given by the following equation:

$$[O_2^{\circ -}]\ mM = (0.195 - 0.025\ pH) \times [Oxidants]$$

Many reactions of the Fenton chemistry can compete with reaction (2) thereby decreasing the formazan accumulation. For example, in

acidic medium, an important fraction of $O_2^{\circ-}$ can disproportionate into hydrogen peroxide and oxygen according to the reaction:

(3) $2\ O_2^{\circ-} + 2\ H^+ \rightarrow\ H_2O_2 + O_2$

$O_2^{\circ-}$ can also be deactivated by reaction with Fe^{3+} in a recycling reaction:

(4) $O_2^{\circ-} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$

The mathematical model of the Fenton chemistry, which is in progress in our team, will be used to determine the kinetic constants of each reaction implicated in the $O_2^{\circ-}$ chemistry.

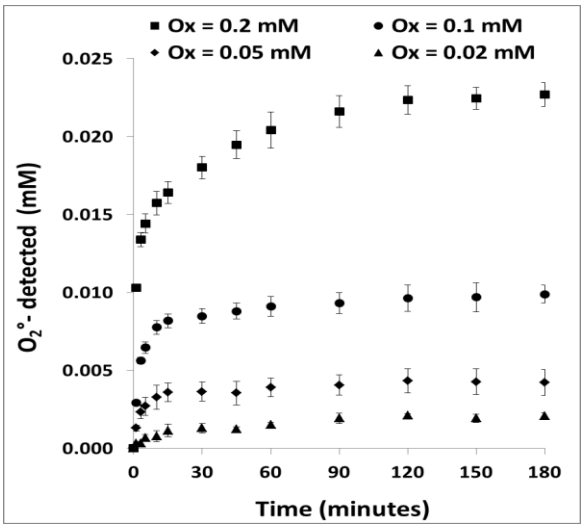

Figure 1: Effect of different concentrations of oxidants (Ox) on the detection of superoxide radicals at pH 3.5. Values are means +/- sd of 4 determinations.

III.3. Hydroxyl radical (OH°) detection:

First measurements were also conducted at the 3 pHs with oxidants only. In these conditions hydroxyl radical was formed by reaction of ferrous iron with hydrogen peroxide according to the Fenton reaction:

(5) $Fe^{2+} + H_2O_2\ \rightarrow\ Fe^{3+} + OH^\circ + OH^-$

And OH° reacted with the probe according to:

(6) Terephtalate + OH° -> Terephtalate-OH

Figure 2 shows the cumulative amount of OH° which had reacted with the probe during 3 hours of incubation at pH 3.5. As observed with superoxide radical, the level of OH° detected increased significantly with time ($p<0.001$) and with the oxidant concentration ($p<0.001$). The same developments were observed at the two higher pHs (results not shown). Contrary to $O_2^{\circ-}$, the evolution with pH was biphasic with the highest detection of OH° at pH 5. This pH effect can be explained by the yield of conversion of OH° from $O_2^{\circ-}$, via the coupling of reactions (3) and (5), which is optimum at pH 4.8 [8].

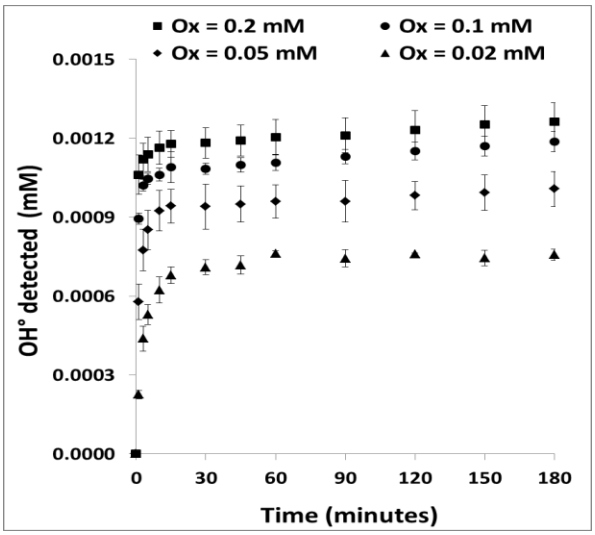

Figure 2: Effect of different concentrations of oxidants (Ox) on the detection of hydroxyl radicals at pH 3.5. Values are means +/- sd of 4 determinations.

III.4. Assessment of antioxidants on the free radical production:

In a second time, different antioxidants were tested on the superoxide and hydroxyl radical formation. Results presented in table 1 were obtained in conditions which reflect the beginning of the gastric digestion (at pH 5 for 30 minutes). We chose these conditions because we can presume that antioxidants are still effective at the beginning of the digestion. Studies are lacking to assert that it is the same after many hours of digestion. The effect of vegetable antioxidants, β−carotene and polyphenols, was surprising. Indeed, these antioxidants had antagonistic effects on the two radicals tested. Table 1 shows an increase of the $O_2^{\circ-}$ detection while an important scavenging effect was observed on OH°. Studies performed at longer periods of time (till 3 h) confirm these results (data not shown). Polyakov *et al.* [9] have demonstrated that carotenoids could decrease or increase (by reducing Fe^{3+} to Fe^{2+}) the free

radical production, depending on the nature of the radical. Iron recycling could promote reaction (1) and disadvantage reaction (4) with, as a result, the increase of $O_2^{\circ -}$ detection. Iron recycling could also promote reaction (5) but plant antioxidants scavenged effectively OH° resulting in a decreased detection. Similar effects were described in literature for other reducing agents. Finally, as OH° is considerably more reactive than $O_2^{\circ -}$ against lipids and proteins, all the plant-derived antioxidants tested here would act globally as antioxidants in the digestive tract, at least at the beginning of the digestion. Table 1 shows that trolox C had no significant effect on the detected radicals. The result on $O_2^{\circ -}$ was expected as vitamin E is poorly reactive with this radical. The low effect on OH° is more surprising as trolox C has a phenol structure similar to the four polyphenols tested here. This lower effect of trolox C could be due to the steric hindrance presented by the methyl groups of the phenol moiety responsible of the antioxidant activity. These results were in good accordance with Apak *et al.* [10] who showed, by using various total antioxidant capacity assays, that the four polyphenols tested had always higher antioxidant activity than trolox C. Studies are in progress to describe the antioxidant effects at other pHs.

Table 1: Effect of antioxidants on the radical detection after 30 minutes at 37°C, pH 5, with 0.2 mM oxidants.

Antioxidants	$O_2^{\circ -}$		OH°	
Carotene 0.01 mM	+18.8	NS	-88.7	***
Carotene 0.1 mM	+113	***	-99.0	***
Caffeic acid 0.01 mM	+53.5	***	-98.2	***
Caffeic acid 0.1 mM	+68.7	***	-98.9	***
Rutin 0.01 mM	+18.7	NS	-85.2	***
Rutin 0.1 mM	+74.0	***	-99.4	***
Chlorogenic acid 0.01 mM	+89.3	***	-98.2	***
Chlorogenic acid 0.1 mM	+95.5	***	-99.7	***
Quercetin 0.01 mM	+89.4	***	-84.2	***
Quercetin 0.1 mM	+491.1	***	-99.4	***
Trolox C 0.01 mM	-3.5	NS	-7.4	NS
Trolox C 0.1 mM	-6.3	NS	-14.2	NS

Values are expressed as percentage of increase or decrease when compared to control with oxidants only. NS : non-significant, *** $p<0.001$.

IV. CONCLUSIONS

In this work we have demonstrated the possibility to characterize the free radical chemistry in a model system which mimics the physicochemical environment of the digestive tract. This work shows the relative contribution of pH, oxidants and antioxidants on the free radical production. The results reported here will be integrated into our data-base to develop a mathematical tool for predicting oxidations during digestion.

REFERENCES

1. Lombardi-Boccia, G., Martinez-Dominguez, B. & Aguzzi, A. (2002). Total heme and non-heme iron in raw and cooked meats. Journal of Food Science 67: 1738–1741.
2. Gatellier, P., Santé-Lhoutellier, V., Portanguen, S. & Kondjoyan, A. (2009). Use of meat fluorescence as a marker of oxidation promoted by cooking. Meat Science 83: 651-656.
3. Kanner, J. & Lapidot, T. (2001). The Stomach as a Bioreactor: Dietary Lipid Peroxidation in the Gastric Fluid and the Effects of Plant-Derived Antioxidants. Free Radical in Biology and Medicine 31: 1388-1395.
4. Graf, E., Empson, K.L. & Eaton, J.W. (1987). Phytic acid. A natural antioxidant. Journal of Biological Chemistry 262: 11647-11650.
5. Auclair, C. & Voisin, E. (1985). Nitroblue tetrazolium reduction. In: Greenwald RA, editor. Handbook of Methods for Oxygen Radical Research (pp 123–132). Boca Raton, FL, USA: CRC Press, Inc.
6. Gomes, A., Fernandes, E. & Lima, J. (2005). Fluorescence probes used for detection of reactive oxygen species. Journal of Biochemical and Biophysical Method 65: 45-80.
7. Harel, S. & Kanner J. (1985). Hydrogen peroxide generation in ground muscle tissues. Journal of Agricultural and Food Chemistry 33: 1186-1188.
8. Backer**,** M. S. & Gebicki, J. M. (1984). The effect of pH on the conversion of superoxide to hydroxyl free radicals. Archives of Biochemistry and Biophysics 25: 234-258.
9. Polyakov, N.E, Leshina, T.V., Konovalova, T.A. & Kispert, L.D. (2001). Carotenoids as scavengers of free radicals in a Fenton reaction: antioxidants or pro-oxidants. Free Radical Biology & Medecine 31: 398-404.
10. Apak, R., Güclü, K., Demirata, B., Ozyürec, M., Celik, S.E., Bektasoglu, B., Berker, K.I. & Ozyurt, D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. Molecules, 12: 1496-1547.