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Diane de La Pomélie, Véronique Santé-Lhoutellier, Thierry Sayd, Laetitia Théron and Philippe Gatellier*

INRA, UR370 Qualité des Produits Animaux, F-63122 Saint Genès-Champanelle, France

*Corresponding author: Philippe Gatellier, tel. +33 473 62 41 98; email address: philippe.gatellier@inra.fr

## Abstract

The International Agency for Research on Cancer recently classified cured meats as carcinogenic for humans and red meats as probably carcinogenic. Mutagens can be formed during meat process or digestion. In a previous study, we used a dynamic artificial digestive system (DIDGI®) to investigate protein oxidation and N-nitrosation during bovine meat digestion. This new paper completes the previous one by focusing on the endogenous heme iron nitrosylation. Low nitrosylation due to nitrate initially present in meat and to ammonia oxidation in the stomach was observed in the digestive tract even in conditions in which no nitrite was added to the model. The endogenous addition of nitrite (1 mM) considerably increased heme iron nitrosylation while a significant decrease was observed with prior meat cooking (30 minutes at 60 and 90°C).

## Keywords

Nitrite; nitrosylation; nitrosylheme; meat; myoglobin

## 1 Introduction

Numerous studies (Larsson and Wolk, 2006; Norat, Lukanova, Ferrari, & Riboli, 2002) indicated that high consumption of processed meat and red meat increases the risk of colorectal cancer, thus leading the International Agency for Research on Cancer to classify processed meat as carcinogenic for humans and red meat as probably carcinogenic (Bouvard et al., 2015). Mutagens can be formed during meat process but also in the digestive tract (Bechaux, de La Pomélie, Théron, Santé-Lhoutellier & Gatellier, 2018; Demeyer, Mertens, De smet & Ulens, 2016; Papuc, Goran, Predescu, & Nicorescu, 2017). The chemical changes leading to mutagen formation during digestion are not as well documented as those occurring in stored and processed food.

In a recent paper (de La Pomélie, Santé-Lhoutellier, Sayd, & Gatellier, 2018) we used an artificial digestive system (DIDGI®) to investigate the level of protein oxidation and N-nitrosation during bovine meat digestion. In the present paper, we provide results obtained during the same experiments, on the endogenous formation of nitrosylheme. Heme iron nitrosylation has been reported as an important factor in the promotion of gastro-intestinal cancer (Santarelli, Pierre, & Corpet, 2008; Santarelli et al., 2010). Heme iron nitrosylation occurs during the curing process by reaction of nitrite with myoglobin but a gastro-intestinal nitrosylation is also suspected. Indeed, it has been demonstrated that a red meat diet increased the level of nitrosylheme at ileal level and in the stools of volunteers (Kuhnle and Bingham, 2007; Kuhnle et al., 2007; Lunn et al., 2007).

The aim of this paper is to provide a better knowledge of the mechanisms and kinetics of heme iron nitrosylation in gastrointestinal conditions for evaluating the risk associated with red meat consumption.

## 2 Materials and methods

*2.1 Reagents*

All the reagents used in this study were purchased from Sigma Aldrich France.

*2.2 Meat cooking*

The experiment was carried out on bovine M. *semimembranosus*. To overcome animal variability experiments were carried out on samples taken from only one bovine muscle. Muscle was aged 13 days under vacuum at 4°C after which it was cooked in vacuum bags (50 g of meat per bag with a thickness of 1cm) by immersion in water for 30 minutes at 60 and 90°C. After cooking, the meat was minced with a meat grinder through 8 mm diameter holes to mimic bolus formation. The cooking juices were reincorporated in the minced meat to form *in vitro* food boluses.

*2.3 Determination of the level of soluble myoglobin and its oxidation state in meat samples*

Soluble myoglobin was determined in a 40 mM phosphate buffer at pH 6 to simulate the pH and ionic strength of meat. To do this, 1g of raw or cooked meat was homogenized in 9 ml of cold phosphate buffer. The extract was then centrifuged at 4000 rpm for 10 minutes. The visible absorbance spectra of supernatants were recorded on a Jasco V-770 spectrometer (figure 1). The level of soluble myoglobin was estimated by measuring specific absorbance at 418 nm with an absorption coefficient of 136 $mM^{-1}$ $cm^{-1}$ (Millar, Moss, & Stevenson, 1996). The three forms of the pigment (deoxymyoglobin, oxymyoglobin, and metmyoglobin) were evaluated by the method of Krzywicki (1979).

*2.4 Determination of nitrite and nitrate content*

Nitrite and nitrate were determined in meat before digestion by performing a Griess reaction with a Sigma-Aldrich colorimetric assay kit (ref: 23479-1KT-F).

77 *2.5 In vitro dynamic digestion of meat*

78 Digestion was performed in an *in vitro* dynamic system (DIDGI®, INRA, Paris, France)
79 controlled by computer. This system can reproduce the main digestion parameters
80 (temperature, pH changes, and enzymatic secretions) with good reliability. This system and
81 the operating conditions were described in detail in our previous paper (de La Pomélie, Santé-
82 Lhoutellier, Sayd et al., 2018). Digestion took place in the presence of a mixture of sodium
83 nitrite (1 mM) and sodium ascorbate (1 mM), or in presence of ascorbate (1 mM) only, added
84 directly in powder form in the initial simulated gastric fluid. Samples were collected at
85 different times after the beginning of digestion, namely: 0, 20, 60, and 120 minutes in the
86 gastric compartment (G), 60, 120, and 150 minutes in the duodenal/jejunal compartment
87 (D/J), and 120, 150, 210 minutes in the ileal compartment (I). Digestion experiments were
88 performed in triplicate.

89

90 *2.6 Evaluation of heme iron nitrosylation*

91 Samples were filtered on gauze. pH was raised to 7 by the addition of a small amount of
92 NaOH 2.5 N. The selective extraction of nitrosylheme was achieved by adding 4 volumes of
93 acetone to 1 volume of the reaction medium. The visible absorbance spectrum of
94 nitrosylheme is provided in Figure 2. This spectrum was similar to that previously observed
95 by Hornsey (1956) in an 80% acetone extract of cooked cured pork gammon and to
96 synthesized nitrosylheme pigments (Soltanizadeh and Kadivar, 2012). The level of
97 nitrosylheme formed was evaluated by measuring specific absorbance at 540 nm with an
98 absorption coefficient of 11.3 $mM^{-1}$ $cm^{-1}$ (Hornsey, 1956). In parallel, the total heme iron was
99 estimated in the form of acid haematin by extraction in acidic acetone (Hornsey, 1956). The
100 nitrosylation of heme iron was expressed as the percentage of nitrosylheme to total heme iron.

101

102 *2.7 Statistical analysis*

To assess the effect of cooking and nitrite addition on the endogenous nitrosylation, data were analysed by the non-parametric statistical test of Friedman, based on the chi-square test $X^2$, with the statistical analysis software from Statistica (V12, Statsoft Inc. Tulsa, USA). The Friedman analysis was preferred to the parametric ANOVA because in the dynamic digestive model, the measurement scale of the different variables was ordinal and not continuous due to the presence of different compartments and to emptying from one compartment to another. As experiments were carried out on a single animal, the muscle was not considered as a factor in our statistical analysis. Only variability inherent to the measurement methods and to the digestive process was taken into account in this study.

## 3 Results

### *3.1 Characterization of meat samples before digestion*

The reactives implicated in the heme iron nitrosylation were assessed before meat digestion. Table 1 and Figure 1 show that cooking significantly decreased the level of soluble myoglobin. This decrease was mainly pronounced at 90°C. Such a dramatic decrease at the highest temperature could be attributed to heme release from the globin moiety or to iron release from heme. This result was in good agreement with those of the literature. Indeed, Purchas, Simcock, Knight, and Wilkinson (2003) and Kristensen and Purslow (2001) reported that heating converts a large part of the myoglobin heme iron into insoluble heme iron (haematin) and accelerates in parallel the release of iron from heme. Protein aggregation reported during meat cooking (Kajak-Siemaszko et al., 2011) could also explain this loss of soluble myoglobin. Oxymyoglobin (the oxygenated form of myoglobin) was the main pigment observed in raw and in 60°C cooked meats (table 1). Only a small increase of metmyoglobin (oxidized myoglobin) was observed at 60°C. After the 90°C cooking, myoglobin oxidoreduction state was unmeasurable. The total heme iron, extracted in acidic

acetone, also decreased with meat cooking, showing iron release from heme under thermal treatment.

A very low level of nitrite was assessed only in the 90°C cooked meat. Nitrate levels significantly decreased with cooking. These results confirmed those of Iammarino and di Taranto (2012) who did not detect nitrite residues and reported nitrate levels around 120 µM, in fresh bovine meats. Table 1 shows that no nitrosylheme was detected in meat samples after acetone extraction.

*3.2 Justification of the experimental model*

The design of the experimental model was chosen to reflect what can be observed in a diet containing meat and vegetables. The ascorbate level (1 mM) used in this study corresponded to level observed in many plant food. Common plant foods contain high concentrations of nitrate (from 100 to 2000 mg/kg) (Thomson 2004). Saliva is able to convert a large part, from 5% to 25%, of nitrate into nitrite before swallowing (Maanen, van Geel, & Kleinjans, 1996). Consequently, plant foods are the main source of nitrite in human. The value of 1 mM in sodium nitrite used in this study corresponded to vegetables containing around 400 mg/kg of sodium nitrate (thus corresponding to approximately 60 mg/kg of equivalent sodium nitrite, after buccal endogenous reduction of 20%). In order to evaluate the impact of thermal processing on protein changes during digestion, raw meat was compared to cooked meats (60 and 90°C for 30 minutes). These cooking conditions reflected core temperatures for medium rare and overcooked meat respectively (Green, 2005). Meat was minced in particle sizes that corresponded to the grinding process of mastication in which muscle fibres are subjected to mechanical constraints, cutting and pressure.

*3.3 Heme iron nitrosylation during meat digestion*

153 Nitrite is poorly reactive towards heme iron and it is generally admitted that the nitric oxide
154 radical (°NO) is the main nitrosylating agent (Skibsted, 2011). Under the acidic and reducing
155 conditions of the stomach, nitric oxide is produced by reactions 1 and 2 with nitrous acid
156 ($HNO_2$) as the reaction intermediate (Skibsted, 2011). Ascorbate added in the medium favors
157 reaction 2.

158 (1) $NO_2^- + H^+ \leftrightarrows HNO_2$ ($k_{forward} = 1.10\ 10^9\ M^{-1}s^{-1}$; $k_{reverse} = 6.18\ 10^5\ s^{-1}$) (pKa = 3.25)

159 (2) $HNO_2 + H^+ + e^- \rightarrow {}^\circ NO + H_2O$ ($k = 2\ 10^3\ s^{-1}$)

160 In the upper gastro intestinal tract, the pigment is mainly in the form of oxymyoglobin or
161 metmyoglobin. Oxymyoglobin mainly reacts with °NO to produce metmyoglobin and nitrate,
162 although in some conditions a small amount of nitrosylheme has been measured in this
163 reaction (Doyle and Hoekstra, 1981). In the reducing conditions of the digestive tract,
164 metmyoglobin can be partially reduced into deoxymyoglobin that can then react with °NO to
165 form nitrosylheme in reaction 3.

166 (3) $Mb(Fe^{2+}) + {}^\circ NO \leftrightarrows Mb(Fe^{2+})\text{-}NO$ ($k_{forward} = 10^7\ M^{-1}\ s^{-1}$, $k_{reverse} = 1.2\ 10^{-4}\ s^{-1}$)

167 Nevertheless, due to the high oxygen tension encountered in the upper digestive tract, reaction
168 3 is seriously limited by deoxymyoglobin oxygenation. Thus, reaction 3 is more probable in
169 acidic conditions observed at the end of gastric digestion, due to intensive °NO production via
170 reactions 1 and 2, and at the end of intestinal digestion, as in the ileum and colon, when media
171 becomes depleted of oxygen. Metmyoglobin can also react directly with °NO or $NO_2^-$ to form
172 nitroso-metmyoglobin, which can then be reduced into nitrosomyoglobin, but the rate
173 constants of these reactions are very low (Copper, 1999; Skibsted, 2011).
174 Figure 3 shows the levels of nitrosylation in the different compartments of the digestive
175 system.
176 Surprisingly, nitrosylheme was formed even in conditions where no nitrite was added to the
177 model. Various hypothesis could explained this basal nitrosylation. Nitrate measured in meat

178 samples before digestion (table 1) can be partially reduced into nitrite by the muscle xanthine
179 oxidoreductase that display nitrate reductase activity (Piknova et al., 2015). Nitric oxide
180 synthase can also produce nitric oxide by oxidation of arginine (Stuehr, 2004). Nevertheless,
181 due to the rapid degradation of the muscle enzymes during cooking and in the digestive tract
182 these two processes cannot probably entirely explain the basal nitrosylation. Another source
183 of nitric oxide in our model could be endogenous ammonia oxidation. Indeed, to mimic
184 gastric fluid, 0.5 mM of ammonium carbonate $(NH_4)_2CO_3$ was added in the gastric
185 compartment (Minekus et al., 2014) that can produce ammonia in acidic condition. In
186 humans, the production of ammonia in the stomach is mainly due to *Helicobacter pylori*
187 urease activity. Levels around 0.9 mM have been reported in the gastric juice of healthy
188 people and around 2.3 mM in the case of H. pylori infection (Chakrabarti et al., 2002).
189 Moreover, the considerable oxidation that occurs in the gastro-intestinal tract can also produce
190 ammonia *via* the oxidative deamination of lysine (de La Pomélie, Santé-Lhoutellier, Sayd et
191 al., 2018). It has been reported that ammonia can be oxidized by the hydroxyl radical (HO°)
192 into unstable nitrogen intermediates ($NH_2OH$ and NOH) that are then converted into nitrite.
193 Nevertheless, this mechanism reported in the rat and *in vitro* by Saul and Archer (1984), and
194 more recently in wastewater by Huang, Li, Dong, & Hou (2008) has to be confirmed in the
195 digestive tract.
196 In order to confirm and to estimate the production of nitrite by ammonia oxidation in the
197 acidic conditions of the stomach we realized a simple test consisting in incubating oxidants
198 ($FeSO_4$, 25 µM; $H_2O_2$, 25 µM; ascorbate, 1 mM) with ammonium carbonate (0.5 mM) in the
199 simulated gastric fluid at pH 3.5. These concentrations in oxidants reflected what can be
200 observed in the digestive tract (Bechaux et al., 2018). In these conditions, we were not able to
201 measure nitrite by the Griess method described in section 2.4, probably because of the
202 oxidative degradation of the sulfanilamide used in this method (Khankhasaeva,

Dashinamzhilova, & Dambueva, 2017), and so we used an indirect method based on the tryptophan nitrosation described by de La Pomélie, Santé-Lhoutellier, & Gatellier (2017). In these oxidative and acidic conditions, we found that 0.5 mM of ammonium carbonate had the same nitrosating power on tryptophan than 0.06 mM nitrite. Consequently, even if the rate of conversion is low, nitrite produced by ammonia oxidation could participate to the nitrosylation observed in this study in absence of added nitrite.

Although the endogenous nitrosylation observed in the absence of added nitrite was relatively low, with a maximum value of 30% and a value of around 10% observed at the end of the ileum digestion, it should be taken into account when trying to evaluate the mutagenic risk of red meat consumption. These results were in good agreement with those of Khunle et al. (2007) who reported a maximum nitrosylation of 10% in feces, and in the fecal waters of humans fed with red meat without added nitrite.

In the second set of experiments, nitrite was added to the model at a level of 1 mM. The addition of nitrite in the model significantly increased ($p < 0.001$, ***) the level of endogenous nitrosylation (figure 3). A global increase from 3.2 was observed in samples with added nitrite when compared to samples without nitrite. A maximum nitrosylation level of 76% was observed after 150 minutes of the ileal digestion of raw meat in the presence of nitrite, approaching the nitrosylation levels generally observed during meat curing processes. This percentage of nitrosylation corresponded to a "true" concentration of 9.2 µM nitrosylheme. This value was comparable to those reported by Chenni et al. (2013). These authors reported concentrations of nitrosylheme of around 10 and 90 µM respectively in the fecal waters of rats given diets with 1% haemoglobin, and drinking water with nitrate (2.7 mM) or a mixture of nitrate (2.7 mM) and nitrite (2.5 mM).

Prior meat cooking significantly decreased ($p < 0.001$, ***) the level of endogenous nitrosylation. This effect was most pronounced between the raw meat and that cooked at

60°C, with a global decrease in the nitrosylation level of 26.2% ($p = 0.002$, **). Increasing cooking temperatures beyond 60°C had less effect on the level of nitrosylation and we observed only a decrease from 16.4% ($p = 0.036$, *) between the samples cooked at 60 and 90°C. When the samples cooked at 90°C were compared to raw meat a global decrease of 38.2% ($p<0.001$, ***) was observed. This decrease was almost the same whether nitrite was added or not to the food boluses. The effect of prior meat cooking could be due to the release of heme from myoglobin, leading to insoluble haematin that polymerizes in the conditions of the digestive tract (Hooda, Shah, & Zhang, 2014), or to protein denaturation and further aggregation. These two phenomena would hinder the access of nitric oxide to heme iron. Iron release from heme at high temperature could also decrease the formation of nitrosylheme. Nevertheless, modifications of myoglobin under heating, as reported in Table 1, reflect only partially this decrease of nitrosylation. Indeed, the slight modifications of protein state observed between raw meat and the 60°C cooked meat were linked to higher impact on the level of nitrosylation than the large modifications observed between the 60°C and the 90°C cooked meats. Structural modifications at the cellular level, not taken into account in this study, could explain this result. For example, disintegration of myofibrillar and membrane structures at high temperature could facilitate nitric oxide diffusion in the meat bolus. We must note that in our previous paper (de La Pomélie, Santé-Lhoutellier, Sayd et al., 2018) we also observed a decrease of the digestive protein N-nitrosation after meat cooking. This effect of prior cooking was attributed to protein aggregation and to a lower level of proteolysis in the cooked samples, resulting in less amino acid N-nitrosation.

Figure 3 shows that heme iron nitrosylation began rapidly in the gastric compartment with about half of the maximum nitrosylation already observed after 20 minutes of digestion. Nevertheless, nitrosylation continued to slowly increase in the intestine and the highest levels were observed in the duodenum/jejunum and ileum compartments. A considerable decrease of

nitrosylation was always observed at the end of digestion (I 210), which could be due to the degradation of nitrosylheme into various compounds (heme-peroxynitrite, ferric or perferryl derivatives) or to denitrosylation, as described in the reverse reaction 3.

The causes of gastro-intestinal cancer are multifactorial. The genetic predisposition was demonstrated in certain forms of cancers but the importance of nutritional factors is now recognized unanimously. This study in model system demonstrated the feasibility of the heme iron nitrosylation under gastro-intestinal conditions. Therefore, future studies will have to take into account this phenomenon when trying to evaluate the mutagenic risk of nitrite and heme iron. However, it is important to note that this experiment was performed in a simplified model. In the case of a balanced meal, the level of nitrosylation would probably have been lower because of the protective effect of antioxidants, mainly provided by vegetables. Indeed, in a previous study (de La Pomélie, Santé-Lhoutellier, & Gatellier, 2018) we clearly demonstrated that antioxidants can inhibit the nitrosylation of heme iron.

## Conclusion

This study using a dynamic artificial digestive system clearly demonstrated that the digestive tract favors heme iron nitrosylation. Therefore, such data must be taken into account to estimate the cancer risk associated with the consumption of meat products. We also demonstrated that ammonia oxidation might contribute to human exposure to nitrite, and therefore increase endogenous nitrosylation. In the future, experiments will take into account diet complexity and interactions between different nutrients, by adding to the model certain plant foods that provide various antioxidants. The role of the gut microbiota on nitrite chemistry and NOC production will be also considered.

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375

376

377 **Legends to figures and tables:**

378

379 **Table 1:** Characterisation of myoglobin chemical state and level of nitrite and nitrate in meat
380 samples before digestion. Values are mean +/- sem of three independent determinations.
381 Values not bearing common superscripts differ significantly ($p < 0.05$). nm = not measurable.

382

383 **Figure 1:** Visible absorbance spectra of myoglobin extracted in 40 mM phosphate buffer at
384 pH 6 from raw or cooked (60°C and 90°C) meats before digestion.

385

386 **Figure 2:** Example of visible absorbance spectrum of nitrosylheme formed during digestion
387 of raw meat in presence of nitrite (1 mM). The level of nitrosylheme was evaluated by
388 absorbance measurement at 540 nm.

389

**Figure 3:** Heme iron nitrosylation levels measured during *in vitro* digestion of raw and cooked meats in the presence or absence of nitrite. Samples were collected at different times after the beginning of digestion, namely: 20, 60, and 120 minutes in the gastric compartment (G), 60, 120, and 150 minutes in the duodenal/jejunal compartment (D/J), and 120, 150, 210 minutes in the ileal compartment (I). Values are means ± sem of 3 independent determinations. Significance of the nitrite effect is noticed as: $p>0.05$, NS ; $p<0.05$, * ; $p<0.01$, ** and $p<0.001$, ***.

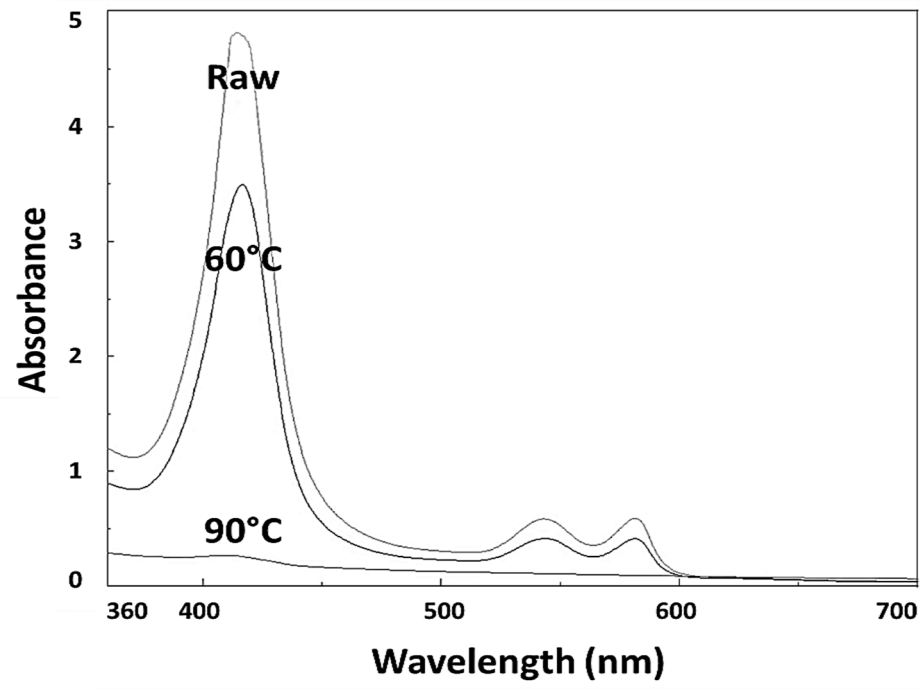


**Figure 1**

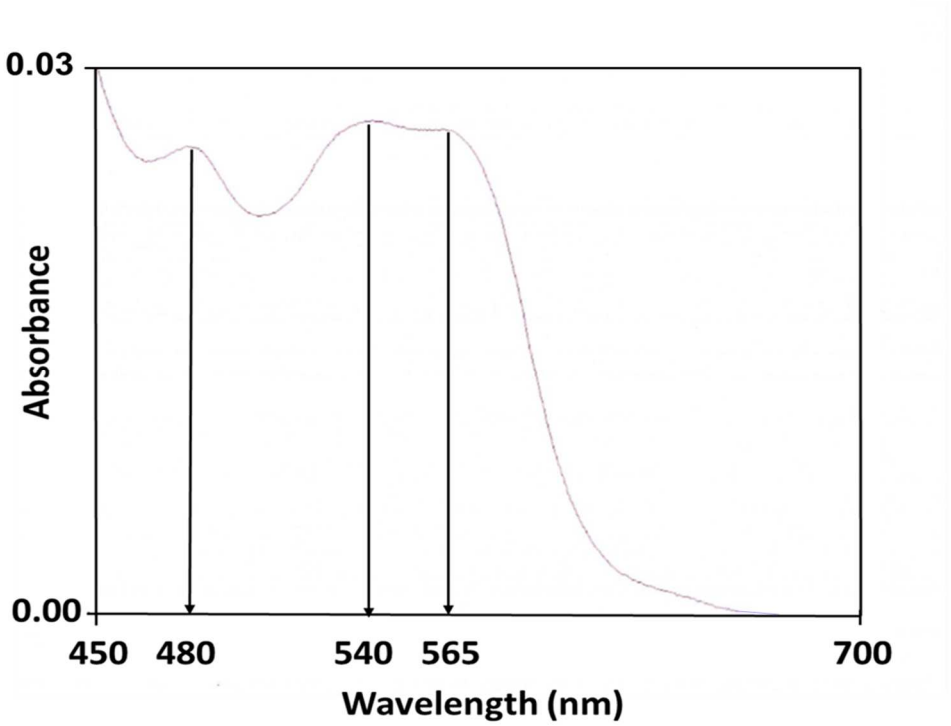


**Figure 2**

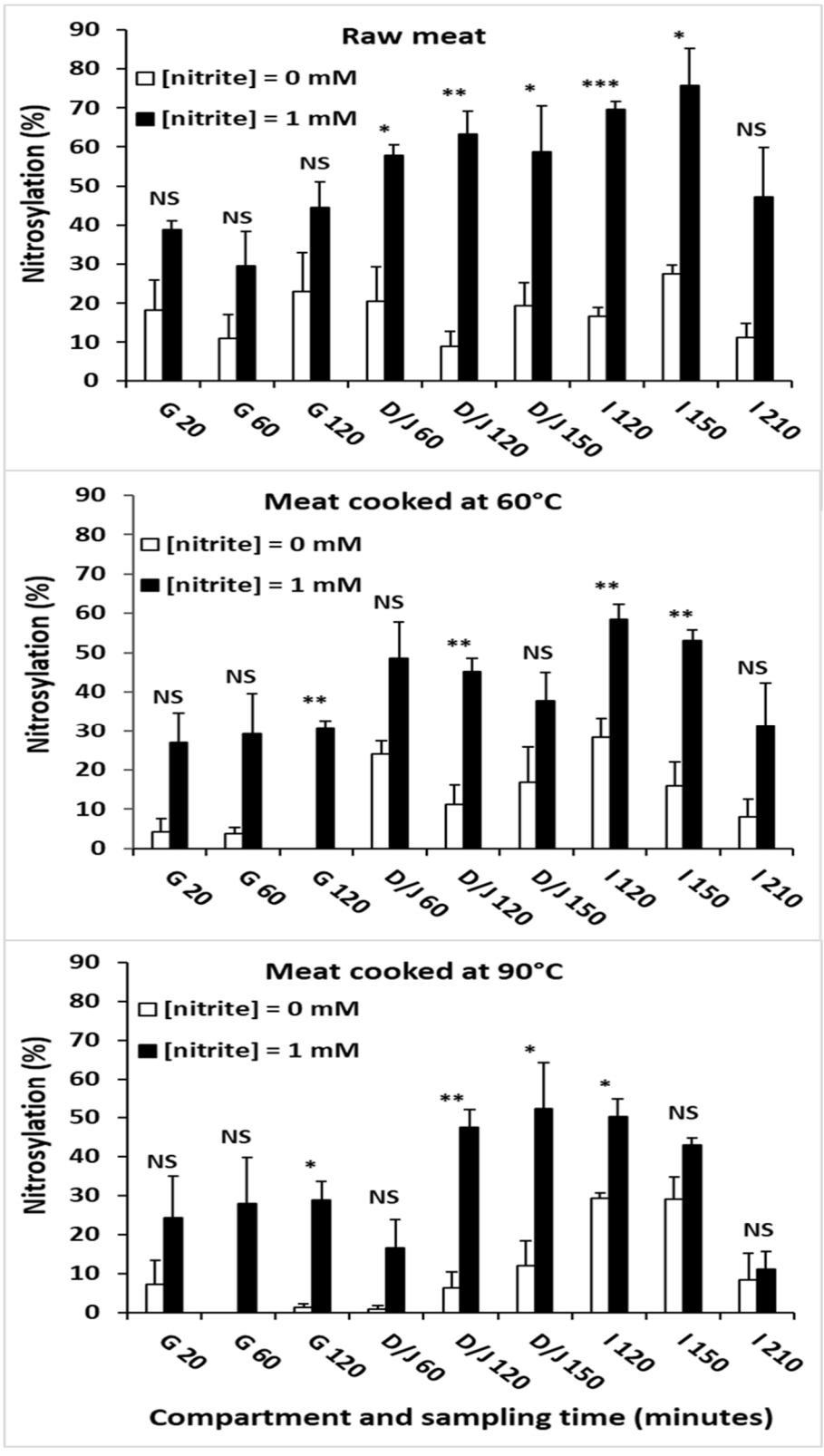
Raw meat
[nitrite] = 0 mM
[nitrite] = 1 mM
Nitrosylation (%)
NS
NS
NS
*
**
*
***
*
NS
G 20
G 60
G 120
D/J 60
D/J 120
D/J 150
I 120
I 150
I 210
Meat cooked at 60°C
[nitrite] = 0 mM
[nitrite] = 1 mM
Nitrosylation (%)
NS
NS
**
NS
**
NS
**
**
NS
G 20
G 60
G 120
D/J 60
D/J 120
D/J 150
I 120
I 150
I 210
Meat cooked at 90°C
[nitrite] = 0 mM
[nitrite] = 1 mM
Nitrosylation (%)
NS
NS
*
NS
**
*
*
NS
NS
G 20
G 60
G 120
D/J 60
D/J 120
D/J 150
I 120
I 150
I 210
Compartment and sampling time (minutes)

**Figure 3**

| | Raw meat | Meat cooked at 60°C | Meat cooked at 90°C |
|---|---|---|---|
| Soluble Myoglobin (μM) | 352.9 ± 12.1 (a) | 257.3 ± 7.8 (b) | 19.1 ± 2.1 (c) |
| Deoxymyoglobin (%) | nm | nm | nm |
| Oxymyoglobin (%) | 83.4 ± 0.9 (a) | 78.1 ± 1.1 (b) | nm |
| Metmyoglobin (%) | 16.6 ± 0.9 (a) | 21.9 ± 1.1 (b) | nm |
| Total heme iron (μM) | 342.1 ± 13.3 (a) | 325.4 ± 11.3 (a) | 268.1 ± 8.2 (b) |
| Nitrosylheme (μM) | nm | nm | nm |
| $NO_2^-$ (μM) | nm | nm | 2.7 ± 0.1 |
| $NO_3^-$ (μM) | 53.0 ± 0.2 (a) | 33.1 ± 0.1 (b) | 17.8 ± 0.2 (c) |

**Table 1**