Formation of naturally occurring pigments during the production of nitrite-free dry fermented sausages

Hannelore De Maere[1, 2*], Ilse Fraeye[1], Eveline De Mey[1], Lore Dewulf[1], Chris Michiels[3], Hubert Paelinck[1], Sylvie Chollet[2]

[1]*Research Group for Technology and Quality of Animal Products, Department M²S, member of Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven Technology Campus Ghent, Gebroeders De Smetstraat 1, B-9000 Ghent, Belgium*

[2]*Groupe ISA, Food Quality Laboratory, Boulevard Vauban 48, F-59046 Lille Cedex, France*

[3]*Centre for Food and Microbial Technology, Department M²S, member of Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Kasteelpark Arenberg 23 box 2457, B-3001 Leuven, Belgium*

*corresponding author: Tel.: +32 9 265 87 28; fax: +32 9 265 87 24

E-mail address: hannelore.demaere@kuleuven.be

Formation of naturally occurring pigments during the production of nitrite-free dry fermented sausages

ABSTRACT

This study investigates the potential of producing red coloured dry fermented sausages without addition of nitrite and/ or nitrate. Therefore, the formation of zinc protoporphyrin IX (Zn(II)PPIX) as naturally occurring pigment, and the interrelated protoporphyrin IX (PPIX) and heme content were evaluated during nitrite-free dry fermented sausage production at different pH conditions. Zn(II)PPIX was only able to form in dry fermented sausages at pH conditions higher than approximately 4.9. Additionally, the presence of Zn(II)PPIX increased drastically at the later phase of the production process (up to day 177), confirming that in addition to pH, time is also a crucial factor for its formation. Similarly, PPIX also accumulated in the meat products at increased pH conditions and production times. In contrast, a breakdown of heme was observed. This breakdown was more gradual and independent of pH and showed no clear relationship with the formed amounts of Zn(II)PPIX and PPIX. A statistically significant relationship between Zn(II)PPIX formation and product redness was established.

Keywords: zinc protoporphyrin IX; protoporphyrin IX; heme; natural colouring; pH condition

1. Introduction

Myoglobin is a globular sarcoplasmic protein with a non-protein iron protoporphyrin IX (known as heme) macromolecule enclosed, and is considered as the major responsible for the colour in meat and meat products. Heme is formed in the mitochondria from protoporphyrin IX (PPIX) by ferrochelatase (FECH) as terminal enzymatic reaction step in the heme biosynthetic pathway. Hereby, FECH inserts ferrous iron into the protoporphyrin ring structure (Ajioka, Phillips, & Kushner, 2006). Depending on the redox state of the heme iron and the ligand bound to its sixth coordination place, colour changes may occur in meat, ranging from purplish red deoxymyoglobin (DMb) in anaerobic conditions and no ligand present, cherry red oxymyoglobin (OMb) whereby ferrous iron is bound to oxygen, to brown metmyoglobin (MMb) if ferrous iron is oxidized to ferric iron, having water as a ligand (Lindahl, 2005).

Sodium nitrite is traditionally used in meat products for multiple purposes, such as antimicrobial, antioxidant and colour formation properties. The latter implies the appearance of the typical cured red (if uncooked) or pink (if cooked) colour of meat products in the form of nitrosylmyoglobin (NOMb) or nitrosylhemochromogen, respectively, with a, from nitrite reduced NO molecule coordinated to iron (Honikel, 2008). However, the use of nitrite is controversial due to its direct toxicity and its involvement in the formation of carcinogenic *N*-nitrosamines (De Mey, 2014).

When nitrite is omitted, the majority of the heme pigments oxidizes to MMb, resulting in the formation of an unacceptable dull brown colour of the meat products (Adamsen, Møller, Laursen, Olsen, & Skibsted, 2006). In Parma ham, a North Italian traditional dry cured ham made without the addition of nitrates or nitrites, zinc protoporphyrin IX (Zn(II)PPIX) is regarded as the major colour forming pigment instead of nitrosylheme, as was thought

previously (Wakamatsu, Nishimura, & Hattori, 2004). Moreover, as nitrite distinctly inhibits its formation, Zn(II)PPIX can only be detected in dry cured meat products without addition of nitrite or nitrate (Adamsen et al., 2006; Wakamatsu, Hayashi, Nishimura, & Hattori, 2010).

The formation mechanisms of this fluorescent pigment in meat products are not completely unraveled yet, however, it is generally ascribed to the interchange of heme iron and zinc. Three possible mechanisms for this metal substitution have been suggested: (1) a non-enzymatic reaction; (2) an enzymatic reaction linked to the activity of endogenous FECH; and (3) an enzymatic reaction with FECH formed by bacteria (Adamsen et al., 2006; Becker, Westermann, Hansson, & Skibsted, 2012; Wakamatsu et al., 2004). It is known that FECH is not only responsible for the insertion of ferrous iron into the PPIX moiety, but also the removal of iron and the insertion of zinc belong to its capabilities (Chau, Ishigaki, Kataoka, & Taketani, 2010; Taketani, Ishigaki, Mizutani, Uebayashi, Numata, Ohgari, & Kitajima, 2007). On the contrary, Wakamatsu, Okui, Hayashi, Nishimura, and Hattori (2007) claimed that Zn(II)PPIX could be formed immediately from PPIX without any involvement of heme, with FECH inserting zinc directly into the PPIX ring. Becker et al. (2012) suggested that enzymatic formation of Zn(II)PPIX dominated initially during meat production, while the non-enzymatic substitution reactions mostly occurs in later stages of the production processes. In earlier studies, Zn(II)PPIX formation was already followed during production of meat products, in particular in dry cured hams. Parolari, Benedini, and Toscani (2009) noticed little or no formation of Zn(II)PPIX in Parma ham during the first 3 months of cold resting stage, whereas a gradual increase of fluorescence intensity, ascribed to the presence of Zn(II)PPIX, occurred during the further processing. Wakamatsu, Uemura, Odagiri, Okui, Hayashi, Hioki, Nishimura, and Hattori (2009b) also observed the formation of Zn(II)PPIX in Parma ham, but only after ca. 40 weeks of maturation. The latter ascribed this delay in Zn(II)PPIX formation to the processing conditions of dry cured ham, including temperature, salt concentrations, or

free zinc content. Grossi, do Nascimento, Cardoso, and Skibsted (2014) concluded eventually that the delayed formation of Zn(II)PPIX in dry cured hams is concomitant with the globin denaturation due to proteolytic activities. They suggested that after globin denaturation ferrous iron is removed from heme, oxidized to the ferric ion forming non heme colloidal complexes, whereby the insertion reaction of zinc into the PPIX ring can take place.

Until now, no studies could be found about the formation of Zn(II)PPIX during the production process of dry fermented sausages. In contrast to the relatively high pH values in dry cured hams, the pH decline during fermentation of dry fermented meat products is expected to be a potential problem for Zn(II)PPIX formation, mostly related to the optimal pH values of FECH. These optima vary from pH 5.5 to 8.0 depending on the origin of FECH, including bacteria, yeast, or mammalian FECH (Camadro & Labbe, 1982; Hansson & Hederstedt, 1994; Nunn, Norris, Hawk, & Cox, 1988; Ishikawa, Yoshihara, Baba, Kawabuchi, Sato, Numata, & Matsumoto, 2006). The enzyme activity of porcine heart extract exhibits an optimum pH of 5.5 (Ishikawa et al., 2006). Moreover, Wakamatsu et al. (2007) found in a porcine meat based *in vitro* model also an optimum pH of 5.5. The optimum pH for Zn(II)PPIX formation differs depending on various internal organs, with pH optima of porcine heart, liver and kidney of 5.0–5.5, 4.5 and 5.5–6.0, respectively (Wakamatsu, Murakami, & Nishimura, 2015). The pH optima also vary according to the function of FECH, ranging from pH 5.5 – 6.0 for iron removal activities to pH 7.5 – 8.0 for zinc insertion activities investigated using an *in vitro* model with porcine mitochondria (Chau et al., 2010).

Semi-dry fermented Northern type sausages normally have an end-pH of 4.8 – 5.0 and are ready to eat after a few weeks drying, reaching approx. 20% weight loss (Toldra, 2008). As these conditions are not preferable for optimal Zn(II)PPIX formation, our hypothesis that Zn(II)PPIX formation might improve in sausages with a higher pH and a longer ripening time, was investigated in this study. The general objective was to evaluate the potential of

115 producing red coloured dry fermented sausages without addition of the undesirable nitrite
116 and/ or nitrate. However, it is generally known that, next to the lack of colour formation,
117 omission of nitrite in dry fermented sausages leads to reduced microbial safety, especially
118 with regard to *Clostridium botulinum* which can cause food poisoning, and antioxidant
119 conditions. Moreover, an increasing pH will require the reorienting of the custom hurdles
120 (Leistner & Gorris, 1995) to ensure food safety. Therefore, a sufficiently low water activity
121 (a_w), obtained by a prolonged drying period, was aspired in this study, however, further
122 investigation on food safety was outside the scope of this work.
123

2. Material and methods

2.1. Dry fermented sausage preparation

Different preparations of nitrite-free dry fermented sausages were made using pork shoulder meat (69.6 %), Italian pork back fat (26.8 %), sodium chloride (2.8 %), sodium ascorbate (0.05 %), spices (white pepper and nutmeg) and a commercial starter culture Texel SA306, containing *Lactobacillus sakei, Staphylococcus xylosus* and *S. carnosus* (Danisco, Dangé-Saint-Romain, France). The meat and fat fractions were purchased at local meat wholesale suppliers, all other ingredients and additives were bought at Solina Group Belgium (Eke-Nazareth, Belgium). The meat batter was prepared by cutting a portion of frozen pork meat, equally inoculated with the starter culture, and frozen backfat into a bowl cutter (Kilia, Neumünster, Germany) until particles of ca. 3 mm were achieved. The meat-fat fraction was seasoned with sodium ascorbate and spices. Finally the binding was achieved by mixing a portion of refrigerated meat and sodium chloride into the meat batter. After the cutting and mixing process, the meat batter was stuffed in collagen casings with 90 mm diameter (Naturin, Weinheim, Germany) using a sausage stuffing machine (Industrial Fuerpla, Valencia, Spain). The sausages were fermented for 3 days (24 °C/ 95RH %) and subsequently dried (14 °C/ 87RH %) until day 21, 45, 64 and 177 in a climate chamber (Kerres Anlagensysteme GmbH, Backnang, Germany).

In total, 4 pH variations were made in triplicate, by adding different concentrations of dextrose simultaneously with the seasonings to the meat batter, i.e., 0.00 % (1), 0.25 % (2), 0.50 % (3) and 0.75 % (4). The latter concentration is normally used for the production of Northern type dry fermented sausages.

Core samples of sausages of each pH variation, derived from the 3 individually prepared batches, were taken at different points of time during the production process, more

specifically at day 0 (production day of the meat batter), day 3 (after the fermentation process), day 21 (after the initial drying period, characterised by a weight loss of approx. 20 % as normally applied for semi-dry Northern type dry fermented sausages), day 45, 64 and at day 177 (extended drying process). General analyses for process monitoring, by means of weight losses, pH, dry matter (DM) and a_w, were performed immediately at each sampling day. Also immediately after sampling, colour was measured and Zn(II)PPIX and/ or PPIX formation was screened. Other samples were frozen at -24°C until quantitative analysis was performed of PPIX, Zn(II)PPIX and total heme.

2.2. General analyses

Weight losses (%) were calculated as percentages of differences in weight of the whole sausages between day 0 and at each sampling day. The pH in the sausage samples was measured by inserting the glass pH electrode in the meat portion (Knick Portamess®, Houston, USA), the a_w was determined using a dewpoint hygrometer (AquaLab, Decagon Devices, Pullman, USA). DM (%) was determined by drying a homogenized test portion to constant mass at 103°C (ISO 1442, 1997).

2.3. Colour measurements

The instrumental colour analysis was based on the 3-dimensional CIELAB colour scale recommended by CIE (1976). A Miniscan EZ 4500L 45°/0° (Hunterlab, Murnau, Germany) with 8 mm viewing area size, illuminant D65 and 10° standard observer was used to register the L^* (lightness), a^* (redness), and b^* (yellowness) values, whith one channel for lightness (L^*) and two colour channels (a^*), going from red (a^*+) to green (a^*-), and (b^*), going from yellow (b^*+) to blue (b^*-). The brightness varies from black ($L^* = 0$) to white ($L^* = 100$).

2.4. Screening method for a fast detection of zinc protoporphyrin IX and/or protoporphyrin IX formation

A screening method, for the fast detection of the fluorescent Zn(II)PPIX and/or PPIX on transverse slices of meat products was assessed according to Wakamatsu, Odagiri, Nishimura, and Hattori (2006) with some modifications. This screening method offers the opportunity to easily assess the formation of the two natural pigments qualitatively prior to the more time consuming and expensive quantitative HPLC method.

Summarized, 12 light-emitting diodes (LEDs) of 420 nm (Roithner Lasertechnik, Vienna, Austria) were connected in a well-sealed darkened room (44×44×30 cm). On top a macro convertor (Olympus MCON-P01, Tokyo, Japan) and a Kodak Wratten Colour Gelatin Filter No.12 (Edmund Optics Inc, Barrington, USA), allowing to transmit only wavelengths higher than 500 nm, were fixed. As such, the fluorescence emission of Zn(II)PPIX (590 nm) and/or PPIX (630 nm) is transmitted while avoiding the interference of the irradiated purple LED light (420 nm).

Meat slices (12mm) were put on the bottom of the darkened room immediately after slicing. RAW images were taken with a digital camera (Olympus PEN E-PL3, Tokyo, Japan), connected externally on the macro converter. Image analysis was performed on all RAW pictures, using OlympusViewerII for the RAW development and ImageJ (image processing program, http://imagej.net) for splitting the RGB colour channels. The fluorescence emission in the R(red) channel is regarded as autofluorescence of Zn(II)PPIX and/ or PPIX. Inversed pictures are obtained with Gimp2.8 (GNU image manipulation program, http://www. gimp. org) for better visibility.

2.5. *Determination of total heme pigments by spectrophotometry*

Total heme content was determined based on the method described by Lombardi-Boccia, Martinez-Dominguez, and Aguzzi (2002) with minor modifications, using an acidified acetone solution that extracts heme from all heme proteins in the form of hemin. An aliquot (4 g) of thawed and minced meat samples was weighted in 50 ml centrifuge tubes with the addition of 25 ml 75% v/v acidified acetone solution (3.15% v/v HCl). Subsequently, the mixture was vigorously mixed for 5 min using an ultra-turrax T25 homogenizer (IKA®, Staufen, Germany), continuously shaken for 1 hour (nutating mixer, VWR International, West Chester, PA, USA), centrifuged for 10 min at 557 g (Hettich© Universal 320R, Sigma Aldrich, Diegem, Belgium) and filtered through filter paper (Machery-Nagel MN 616, Filter Service, Eupen, Belgium). Absorbances were measured at 640 nm via spectrophotometry (Cary 100 Bio, Agilent Technologies, CA, US). Hemin (Sigma Aldrich, Diegem, Belgium) was used as a standard. Data are expressed as nmol/g DM. All reagents were appropriate for analytical use.

2.6. *Determination of protoporphyrin IX and zinc protoporphyrin IX by high performance liquid chromatography with fluorescence detection*

After thawing and mincing, 1 g of meat sample was accurately weighted into a 14 ml centrifuge tube. Extraction was carried out by adding 5 volumes of the extraction solvent (75% v/v acetone solution) and homogenizing it for 1 min using an ultra turrax T18 homogenizer (IKA®, Staufen, Germany). The meat sample was centrifuged (Heraeus Labofuge 200, Fisher Scientific, Tournai, Belgium) for 5 min at 2697 g. This extraction was repeated 5 times. After each extraction the supernatant was filtered and collected into dark volumetric flasks. The resulting volume was diluted up to 25 ml with 75% v/v acetone solution. The obtained solution was filtered using a syringe filter with 0.20 µm pore size

(Machery-Nagel Cromafil® RC-20/15 MS, Filter Service, Eupen, Belgium) and was transferred into dark vials. During all extraction operations, direct contact with light was avoided as much as possible.

The chromatographic method was based on Wakamatsu, Odagiri, Nishimura, and Hattori (2009a) with some modifications. The fluorescence properties of PPIX (ex./em. 410/630 nm) and Zn(II)PPIX (ex./em. 420/590 nm) were used for their determination, using a Hitachi LaChrom Elite® high performance liquid chromatograph (HPLC) equipped with a model L2200 autosampler, a model L 2485 fluorescence detector (VWR International, Leuven, Belgium). Using an Altima™ C18 5 µm, 150 mm × 4,6 mm chromatographic column (Grace Davision Discovery Sciences, Lokeren, Belgium), the porphyrins were separated by isocratic elution using methanol/ammonium acetate (80:20, v/v, pH = 5.16) at a flow rate of 1 ml/min at 35°C. Forty microliters of each sample was injected. PPIX and Zn(II)PPIX (Sigma Aldrich, Diegem, Belgium) were used as standards. Data are expressed as nmol/g DM. All reagents were appropriate for analytical use.

2.7. Statistical analysis

Differences in pH, weight losses and a_w between the different pH variations at each sampling day were assessed using a one-way ANOVA at a significance level of $P < 0.05$. Post-hoc pairwise testing between all pH variations was performed using a Tukey correction to account for multiple testing (IBM SPSS Statistics 21.0, Chicago, USA).

Zn(II)PPIX, PPIX and total heme were analyzed using a linear mixed model that included factors for pH variation, time and their interaction. Correlations between the different measurements are taken into account using random intercepts for the batch effect and batch by pH effect. At each sampling day, an F-test ($P < 0.05$) was performed to assess whether there was an overall effect of pH variation. Post-hoc pairwise testing between all pH

variations was performed using a Tukey correction to account for multiple testing. Similar analyses were performed for each pH variation to assess differences between the sampling days.

For the analyses of *L**, *a** and *b**, a random intercept for the batch effect was added to the model. In addition, an unstructured variance-covariance matrix was used to model correlations between the sampling days and a compound-symmetry matrix was used to account for correlations between the repeated measurements on each day. Analyses for *L**, *a** and *b** were adjusted for Zn(II)PPIX, PPIX, total heme and for all three simultaneously. Only models in which the pigments (Zn(II)PPIX, PPIX, total heme) showed significant effects, were included in the discussion of the results. If deviations from the linearity assumption were observed for these variables, they were included using restricted cubic splines.

Model assumptions of normality and constant variance of the residuals were assessed using visual inspection of residual plots. All these analyses were performed with SAS version 9.4 with SAS/STAT 13.2.

3. Results

3.1. Evolution of pH, weight losses and water activity in nitrite-free dry fermented sausages at different pH conditions

Lactic acid bacteria present in the starter culture are able to acidify the sausages during the fermentation process, by the production of lactic acid, whereby dextrose is acting as the power supply for a better growth of these bacteria (Toldra, 2008). In order to achieve different pH conditions during processing, different concentrations of dextrose were added to the meat batter: 0.00% (1), 0.25% (2), 0.50% (3) and 0.75% (4). This approach was successful as, after fermentation, significantly different pH values were obtained between the different dextrose variations. At day 3, pH values of 5.32 ± 0.02, 4.932 ± 0.004, 4.75 ± 0.01 and 4.55 ± 0.01 were achieved for variations 1, 2, 3 and 4, respectively (Table 1). The re-increase of pH during further manufacturing is probably related to the generation of biogenic amines and ammonia as a result of proteolysis (Toldra, 2008). However, the significant differences in pH between the 4 dextrose variations were maintained, even during the further extensive drying period. In the following, dextrose variations will be interpreted in terms of pH conditions.

In addition to pH, weight losses and a_w are also shown in Table 1. As a function of time, the weight losses of the sausages increased and a_w decreased due to the continuous drying conditions, whereby the weight losses were more pronounced during the initial drying period while the a_w decrease was more obvious at the later stage of the processing. The negative values of weight losses at day 3 indicate a slight increase of weight in the sausages due to the high relative humidity conditions (95 % RH) during fermentation. Significant differences in weight losses were observed between the 4 pH variations, with lower values in dry fermented sausages with higher pH levels. This can be explained by the coagulation of meat proteins at lower pH which encourages drying and weight loss of the product (Ockerman & Basu, 2008).

279 However, with exception of day 64, no significant differences in a_w were observed between
280 the 4 pH variations, or, the different pH conditions had no influence on the a_w-decline during
281 drying.

282 ### *3.2. Changes in zinc protoporphyrin IX, protoporphyrin IX and total heme content in nitrite-*
283 ### *free dry fermented sausages at different pH conditions*

284 #### *3.2.1. Sreening of zinc protoporphyrin IX and/or protoporphyrin IX formation*

285 Fig. 1 shows the evolution of the red fluorescence emission, ascribed to the formation of
286 Zn(II)PPIX and/ or PPIX, of the 4 pH variations at different stages of the production process.
287 At day 0, almost no red fluorescence was observed for all variations. At day 21, clear
288 fluorescence appeared in variations 1 and 2, and its intensity remained similar during the
289 longer drying period up to 64 days. Only after 177 days the sampling reveiled a remarkable
290 increase of red fluorescence. Grossi et al. (2014) claimed that the formation of Zn(II)PPIX in
291 Parma ham occurs when myoglobin denatures due to proteolytic activities, which is most
292 pronounced at the end of the maturation process. It is assumed that also in this study the factor
293 time, related to myoglobin denaturation, plays an important role in the formation of
294 Zn(II)PPIX and/ or PPIX.
295 In pH variations 1 and 2, the red fluorescence intensity is much higher than in pH variations 3
296 and 4. Based on these results, it can be concluded that the formation of Zn(II)PPIX and/ or
297 PPIX is more pronounced in circumstances with higher pH levels, and more specifically equal
298 to or higher than approximately 4.9. It seems that this pH level acts as a treshold for the
299 formation of the natural pigments, dividing the variations into 2 groups. When the pH
300 decreased to lower pH values during the production process, fluorescence formation was
301 reduced to a minimum. In variation 1 and 2, the pH remained above 4.9 uninteruptedly. As for
302 variations 3 and 4, the pH was only exceeding 4.9 at day 45 and 64, respectively (see Table 1).

Despite the pH increase during processing, resulting in more optimal pH conditions for Zn(II)PPIX and/ or PPIX formation in variations 3 and 4, their delay in pigment formation could not be catched up with respect to variations 1 and 2. The influence of pH can be explained by the pH dependence of porcine FECH activity, with pH optima around 5.5 (Ishikawa et al., 2006; Wakamatsu et al., 2007).

This screening method offers the opportunity to easily assess the formation of Zn(II)PPIX and/ or PPIX qualitatively. But in order to gain more insight into the formation of the individual components in dry fermented meat products at different pH conditions, Zn(II)PPIX, PPIX and additionally also total heme were quantified.

3.2.2. Evolution of zinc protoporphyrin IX, protoporphyrin IX and total heme content

The concentrations of Zn(II)PPIX, PPIX and total heme as a function of production time and varying pH conditions in nitrite-free dry fermented sausages, are presented in Table 2.

Only small amounts of Zn(II)PPIX and PPIX were measured in the fresh meat batters. Zn(II)PPIX formation occured during the first 21 days of processing for pH variation 1 and 2, but then stabilized. For pH variation 3 and 4, however, no significant Zn(II)PPIX formation was seen during this initial phase. But for all pH variations, a significant increase of Zn(II)PPIX formation was observed after the longer drying period of 177 days. As for PPIX, formation started in the first 2 pH variations somewhat later during processing, between day 21 and 45, again followed by a stabilization and a significant increase at day 177. For pH variation 3, significant PPIX formation was only seen at day 177. For pH variation 4, no PPIX formation was seen at all.

Significant differences in Zn(II)PPIX and PPIX formation between the 4 pH variations were observed starting from day 21 and day 45, respectively, with increased pigment formation at higher pH values. The pH variations can be divided into 2 groups with significantly more

Zn(II)PPIX formation in variations 1 and 2, exhibiting pH levels equal to or higher than approx. 4.9. However, this treshold was not seen for PPIX formation.

These results are in accordance to the observed fluorescence intensities assessed with the fast screening method, with time and pH as crucial factors concerning the formation of PPIX and Zn(II)PPIX. The delayed formation of PPIX and Zn(II)PPIX can be assigned to globin denaturation as also suggested to occur during the production of Parma ham (Grossi et al., 2014). Influence of pH can be explained by the pH dependence of porcine FECH activity (Ishikawa et al., 2006; Wakamatsu et al., 2007).

The total heme content decreased significantly during processing, regardless of the pH variation. Reductions between 35 % and 45 % are recorded between day 0 and 177. Decreasing total heme concentrations were already observed by Wakamatsu et al. (2009b) in dry cured ham and by Chasco, Lizaso, and Biriain (1996) in nitrite-cured dry fermented sausages. This could partly be explained by increasing salt concentrations due to the dehydratation of the product, as Sakata and Nagata (1992) found in minced porcine skeletal muscle during refrigerated storage a decreased heme protein content of 50% and 80% with increasing salt concentrations of 2% and 10%, respectively. Besides the known thermal degradation of heme (Garcia, Martinez-Torres, Leets, Tropper, Raminez, & Layrisse, 1996; Gomez-Basauri, & Regenstein, 1992; Lombardi-Boccia et al., 2002; Turhan, Ustun, & Altunkaynak, 2004), Estevez and Cava (2004) also observed a significant increase in non heme iron in liver paste during refrigeration storage. These results suggest that some disruption of the porphyrin ring could have occurred during storage which led to the release of iron (Gomez-Basauri et al., 1992; Miller, Gomez-Basauri, Smith, Kanner, & Miller, 1994). Additionally, it must be noted that the heme breakdown mechanisms are also pH dependent, with optima at more alkaline pH values around 8 (Ukpabi, 2012). In this study however, the

different (acid) pH conditions within the 4 variations did not have a determining influence on the total heme content in the nitrite-free dry fermented sausages.

By determining the total heme concentration as a function of production time and pH, an attempt was made to gain better insight into the formation mechanisms of Zn(II)PPIX in nitrite-free dry fermented sausages. However, no clear relationship could be seen between the breakdown of the heme pigments and the formation of Zn(II)PPIX and PPIX. The total heme breakdown is independent of pH and is more gradually decreasing during production, whereas the formation of Zn(II)PPIX has proved to be influenced by pH and is formed drastically at the later stage of the production process. Based on these results, no conclusion could be drawn concerning the possible substitution reactions within the different metalloporphyrins.

In comparison with Parma ham (Wakamatsu et al., 2009b), similar or even higher concentrations of Zn(II)PPIX could be measured in the dry fermented sausages with limited pH declines (variation 1 and 2) and whereby an extensive drying period of more than 64 days or at least 177 days was carried out. In Parma ham, Zn(II)PPIX amounts of 27.7 – 47.0 µg/ g or 104.11 – 176.65 nmol/g DM (calculated with 42.5% DM, as reported by Adamsen et al., 2006) were found in the different muscles.

In contradiction to the delayed formation of Zn(II)PPIX in Parma ham, the formation of Zn(II)PPIX in the dry fermented sausages with limited pH declines started immediately and continued up to day 21. Probably, the ambient temperature of 24°C during fermentation is determinative (Wakamatsu et al., 2007), but also the addition of salt and additives, such as sodium ascorbate, in combination with the greatly enhanced direct contact of these constituents with the meat due to severe mincing, could play an important role. Sodium chloride concentrations up to 3% increase Zn(II)PPIX formation due to the increased solubility of the proteins. With higher concentrations of sodium chloride inhibition of Zn(II)PPIX formation was observed, although FECH has been shown to be active in fresh

meat extracts at sodium chloride concentrations up to 8% (Becker et al., 2012). Due to its reducing capacities, sodium ascorbate also promotes Zn(II)PPIX formation (Ishikawa et al., 2006). After day 21 however, the formation rate decreased and stabilized during the further processing. In the later phase of the drying process, the rate increased again as was also seen during the production process of Parma ham (Parolari et al, 2009; Wakamatsu et al., 2009b).

PPIX on the other hand was present in much higher amounts in the dry fermented sausages with higher pH (up to 83.73 ± 15.02 nmol/ g DM in variation 1) in comparison to Parma ham (0.4 – 1.1 µg/ g, or 1.67 - 4.60 nmol/g DM). The accumulation of PPIX during the production of meat products has never been described before. Some possible causes could be the absence of free zinc ions (Ishikawa, Kawabuchi, Kawakami, Sato, Numata, & Matsumoto, 2007) or the presence of chelating constituents (Benedini, Raja, Parolari, 2008), which are able to inhibit Zn(II)PPIX formation. Although this can be an important factor for better understanding the formation mechanisms of the natural pigments, no clarification can be given based on the results obtained in this study.

3.3. Colour formation in nitrite-free dry fermented sausages at different pH conditions

*L**, *a** and *b** values as a function of production time and pH in nitrite-free dry fermented sausages are shown in Table 3.

A significant decrease of *L** was observed during the production process, and was most pronounced during the extensive drying period. This can be attributed to the strong decrease in moisture content, resulting in a darker product. No clear differences, however, could be observed for *L** between the different pH conditions within each sampling day.

In contrast to *L**, the results on the colour scales *a** and *b** were drastically influenced by the fermentation process, whereby the sausages evolved to a less red (decrease of *a**) and yellow (decrease of *b**) colour, probably related to the formation of higher concentrations of MMb

(Adamsen et al., 2006). During the further production process, *a** gradually increased again, which corresponds with an increase of the redness, while *b** remained more or less stable as a function of time (only in exception of day 64 for pH variation 3 and 4). As for variation 1, the decrease of *a** immediately after fermentation was more limited in comparison with the other variations. Also during the further processing, significantly higher *a** values were observed for pH variation 1. Also *b** was higher for the batches with higher pH immediately after fermentation, but during the further processing the differences between the *b** values became smaller and even negligible after extensive drying.

As presented in the material and methods section, analyses for *L**, *a** and *b** were adjusted for Zn(II)PPIX, PPIX, total heme and for all three simultaneously. Interestingly, Zn(II)PPIX had a highly significant effect ($P < 0.0001$) on *a** values. The fact that the redness of the sausages (*a**) is significantly related to the content of the natural red pigment Zn(II)PPIX, may indicate a causal connection. However, other underlying simultaneous reactions during the production process may play a role, complicating the relationship between Zn(II)PPIX and colour formation.

Additionally, Zn(II)PPIX was found to have a significant effect ($P = 0{,}0439$) on *L** and both PPIX and Zn(II)PPIX had a significant effect ($P = 0.0054$ and $P = 0.0456$, respectively) on *b**. Nevertheless, the decrease of *L** will probably mainly be attributed to the dehydration of the meat products as a function of time. Since *b** is more or less stable as a function of time and no clear differences could be observed between the different pH variations, the significant effect of PPIX and Zn(II)PPIX on *b** is also difficult to explain. In all cases, total heme showed no significant effect on the instrumental colour parameters.

Also in earlier studies, it was not evident for researchers to correlate Zn(II)PPIX formation with instrumental colour measurements during production of meat products. Parolari et al. (2009) for instance reported that the colour heterogeneity between muscles of green hams

425 disappeared with paler muscles becoming more red and vice versa during the production of
426 dry cured meat products, in spite of increasing Zn(II)PPIX formation. However, it was
427 generally stated that the redness of nitrite-free dry cured hams is attributed to Zn(II)PPIX
428 formation during processing, with Zn(II)PPIX accounting for 60% - 70% of all porphyrins
429 measured (Wakamatsu et al., 2004; Wakamatsu et al., 2009a).
430

4. Conclusion

In this study, the formation of the naturally occurring pigments Zn(II)PPIX and PPIX was demonstrated in nitrite-free dry fermented sausages with higher pH values (pH > 4.9) and a longer production time (up to 177 days). Zn(II)PPIX formation was already described in Parma ham like products, but the accumulation of PPIX was never observed before and could be a determining factor concerning the further elucidation of the formation mechanisms of Zn(II)PPIX in meat products. The total heme content decreased more gradually, irrespective of the different pH conditions. As such, no clear conclusion about the relation between total heme breakdown and Zn(II)PPIX formation could be drawn.

Interestingly, a statistically significant relationship between Zn(II)PPIX formation and product redness was established. Additionally, an effect of Zn(II)PPIX on L^* and an effect of both PPIX and Zn(II)PPIX on b^* were seen. Total heme, however, did not play any significant role in the instrumental colour parameters.

These results are promising for producing red coloured dry fermented sausages without addition of undesirable nitrite and/ or nitrate. However, it is important to stress that nitrite omission in meat products raises concern with regard to food safety. This issue still needs further study.

Acknowledgements

This work was performed in the framework of the MeCagrO_2 project “Safe products, sustainable processes and employment increased attractiveness for companies from the 2 Seas agro-food Area”. Note: “The document reflects the author’s views. The interreg IVA 2 Seas Programme Authorities are not liable for any use that may be made of the information contained therein.” E. De Mey is a postdoctoral fellow supported by the Postdoctoral Mandate (PDM) from KU Leuven.

References

Adamsen, C. E., Møller, J. K. S., Laursen, K., Olsen, K., & Skibsted, L. H. (2006). Zn-porphyrin formation in cured meat products: Effect of added salt and nitrite. *Meat science, 72*(4), 672-679.

Ajioka, R. S., Phillips, J. D., & Kushner, J. P. (2006). Biosynthesis of heme in mammals. *Biochimica et Biophysica Acta, 1763*, 723-736.

Becker, E. M., Westermann, S., Hansson, M., & Skibsted, L. H. (2012). Parallel enzymatic and non-enzymatic formation of zinc protoporphyrin IX in pork. *Food Chemistry, 130*(4), 832-840.

Benedini, R., Raja, V., Parolari, G. (2008). Zinc-promoting activity in pork muscle. *LWT-Food Science and Technology 41*, 1160-1166.

Camadro, J-M. & Labbe, P. (1982). Kinetic studies of ferrochelatase in yeast: Zinc or iron as competing substrates. *Biochimica et Biophysica Acta, 707*, 280-288.

Chasco, J., Lizaso, G., & Beriain, M. J. (1996). Cured colour development during sausage processing. *Meat Science, 44*(3), 203-211.

Chau, T., Ishigaki, M., Kataoka, T., & Taketani, S. (2010). Porcine ferrochelatase: The relationship between iron-removal reaction and the conversion of heme to zn-protoporphyrin. *Bioscience, Biotechnology, and Biochemistry*, *74*(7), 1415-1420.

De Mey, E. (2014). N-nitrosamines in dry fermented sausages: Occurence and formation of N-nitrosopiperidine. PhD dissertation, KU Leuven, Groep Wetenschap & Technologie, Ghent, Belgium.

Estevez, M., & Cava, R. (2004). Lipid and protein oxidation, release of iron from heme molecule and colour deterioration during refrigerated storage of liver pâté. *Meat Science, 68*(4), 551-558.

Garcia, M. N., Martinez-Torres, C., Leets, I., Tropper, E., Raminez, J., & Layrisse M. (1996). Heat treatment on heme iron and iron-containing proteins in meat: iron absorption in humans from diets containing cooked meat fractions. *Journal of Nutritional Biochemistry, 7*(1), 49-54.

Gomez-Basauri, J. V., & Regenstein, J. M. (1992). Processing and frozen storage effects on the iron content of cod and mackerel. *Journal of food science,* 57(6), 1332-1336.

Grossi, A. B., do Nascimento, E. S. P., Cardoso, D. R., & Skibsted, L. H. (2014). Proteolysis involvement in zinc–protoporphyrin IX formation during Parma ham maturation. *Food Research International,* 56, 252-259.

Hansson, M. & Hederstedt, L. (1994). Purification and characterisation of a water-soluble ferrochelatase from Bacillus subtilis. *European Journal of Biochemistry, 220*, 201-208.

Honikel, K.-O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science, 78*(1-2), 68-76.

Ishikawa, H., Kawabuchi, T., Kawakami, Y., Sato, M., Numata, M., & Matsumoto, K. (2007). Formation of zinc protoporphyrin IX and protoporphyrin IX from oxymyoglobin in porcine heart mitochondria. *Food Science and Technology Reseach, 13*(1), 85-88.

Ishikawa, H., Yoshihara, M., Baba, A., Kawabuchi, T., Sato, M., Numata, M., & Matsumoto, K. (2006). Formation of Zinc Protoporphyrin IX from Myoglobin with Pork Loin Extract. *Journal of the Faculty of Agriculture, Kyushu University, 53*(1), 93-97.

500 International Organization for Standardization (1997). Determination of moisture content,
501 ISO 1442:1997 standard.

502 Leistner, L., & Gorris, L. G. M. (1995). Food preservation by hurdle technology. *Trends in*
503 *Food Science and Technology, 6*, 41-46.

504 Lindahl, G. (2005). Colour Characteristics of Fresh Pork. Doctor's dissertation, Swedish
505 University of Agricultural Sciences, Uppsala, Sweden.

506 Lombardi-Boccia, A., Martinez-Dominguez, G., & Aguzzi, B. (2002). Total Heme and Non-
507 heme Iron in Raw and Cooked Meats. *Food chemistry and Toxicology, 67*(5), 1738-
508 1741.

509 Miller, D. K., Gomez-Basauri, J. V., Smith, V. L., Kanner, J., & Miller D. D. (1994). Dietary
510 iron in swine rations affects nonheme iron and TBARS in pork skeletal muscles.
511 *Journal of food science, 59*(4), 747-750.

512 Nunn, A. V. W., Norris, P., Hawk, J. L. M., & Cox, T. M. (1988). Zinc chelatase in human
513 lymphocytes: Detection of the enzymatic defect in erythropoietic protoporphyria.
514 *Analytical Biochemistry, 174*, 146-150.

515 Ockerman, H.W. & Basu, L. (2008). Fermented Meat Products: production and consumption.
516 In Toldra, F., Handbook of fermented meat and poultry. Spain: Blackwell Publishing.

517 Parolari, G., Benedini, R., & Toscani, T. (2009). Color formation in nitrite-free dried hams as
518 related to zn-protoporphyrin IX and zinc chelatase activity. *Journal of Food Science,*
519 *74*(6), 413-418.

520 Sakata, R., & Nagata, Y. (1992). Heme pigment content in meat as affected by the addition of
521 curing agents. *Meat Science, 32*(3), 343-350.

522 Taketani, S., Ishigaki, M., Mizutani, A., Uebayashi, M., Numata, M., Ohgari, Y., & Kitajima,
523 S. (2007). Heme synthase (ferrochelatase) catalyzes the removal of iron from heme
524 and demetalation of metalloporphyrins. *Biochemistry, 46*, 15054–15061.

525 Toldra, F. Handbook of Fermented Meat and Poultry (2008). Spain: Blackwell Publishing.

526 Turhan, S., Ustun, N. S., & Altunkaynak, T. B. (2004). Effect of cooking methods on total
527 and heme iron contents on anchovy (Engraulis encrasicholus). *Food Chemistry, 88*(2),
528 169-172.

529 Ukpabi, G. N. (2012). Structural basis for heme degradation in Staphylococcus aureus. Master
530 thesis, University of British Columbia, Vancouver, Canada.

531 Wakamatsu, J, Odagiri, H, Nishimura, T, & Hattori, A. (2006). Observation of the distribution
532 of Zn protoporphyrin IX (ZPP) in Parma ham by using purple LED and image
533 analysis. *Meat Science, 74*(3), 594-599.

534 Wakamatsu, J., Hayashi, N., Nishimura, T., & Hattori, A. (2010). Nitric oxide inhibits the
535 formation of zinc protoporphyrin IX and protoporphyrin IX. *Meat Science, 84*(1), 125-
536 128.

537 Wakamatsu, J., Murakami, N, & Nishimura, T. (2015). A comparative study of zinc
538 protoporphyrin IX-forming properties of animal by-products as sources for improving
539 the color of meat products. *Animal Science Journal, 86*, 547-552.

540 Wakamatsu, J., Nishimura, T., & Hattori, A. (2004). A Zn-porphyrin complex contributes to
541 bright red color in Parma ham. *Meat Science, 67*(1), 95-100.

542 Wakamatsu, J., Odagiri, H., Nishimura, T., & Hattori, A. (2009a). Quantitative determination
543 of Zn protoporphyrin IX, heme and protoporphyrin IX in Parma ham by HPLC. *Meat*
544 *Science, 82*(1), 139-142.

545 Wakamatsu, J., Okui, J., Hayashi, N., Nishimura, T., & Hattori, A. (2007). Zn protoporphyrin
546 IX is formed not from heme but from protoporphyrin IX. *Meat Science, 77*(4), 580-
547 586.

548 Wakamatsu, J., Uemura, J., Odagiri, H., Okui, J., Hayashi, N., Hioki, S., Nishimura, T., &
549 Hattori, A. (2009b). Formation of zinc protoporphyrin IX in Parma-like ham without
550 nitrate or nitrite. *Animal science journal, 80*(2), 198-205.

551

552 **Table 1 pH (n = 9), weight losses (n=3) and a_w (n = 6) during the production of nitrite-free dry fermented sausages. Different pH conditions are obtained by adding**

553 **different concentrations of dextrose to the meat batter, 0.00% (1), 0.25% (2), 0.50% (3) and 0.75% (4).**

		Day 0	Day 3	Day 21	Day 45	Day 64	Day 177
	dextrose	meat batter	after fermentation	after initinal drying period	extensive drying period		
pH (-)	1) 0.00%	5.69±0.01^{ab}	5.32±0.02^{d}	5.33±0.01^{d}	5.49±0.01^{d}	5.647±0.003^{d}	5.63±0.00^{d}
	2) 0.25%	5.66±0.01^{a}	4.932±0.004^{c}	4.956±0.002^{c}	5.20±0.01^{c}	5.30±0.01^{c}	5.44±0.01^{c}
	3) 0.50%	5.70±0.01^{b}	4.75±0.01^{b}	4.83±0.02^{b}	4.94±0.03^{b}	5.01±0.01^{b}	5.197±0.003^{b}
	4) 0.75%	5.72±0.01^{b}	4.55±0.01^{a}	4.66±0.01^{a}	4.73±0.01^{a}	4.913±0.003^{a}	5.13±0.01^{a}
Weight losses (%)	1) 0.00%	0.00±0.00^{a}	-0.63±0.55^{ab}	17.74±0.40^{a}	23.71±0.55^{a}	26.18±0.34^{a}	55.43**
	2) 0.25%	0.00±0.00^{a}	-1.54±0.11^{a}	18.70±0.30^{b}	24.83±0.25^{ab}	26.85±0.35^{ab}	39.49*
	3) 0.50%	0.00±0.00^{a}	-0.00±0.26^{b}	18.56±0.44^{ab}	25.62±0.30^{b}	27.79±0.26^{b}	48.17*
	4) 0.75%	000±0.00^{a}	-1.04±0.08^{ab}	20.86±0.43^{c}	27.85±0.81^{c}	30.10±0.39^{c}	40.52**
a_w (-)	1) 0.00%	0.963±0.002^{a}	0.964±0.002^{a}	0.956±0.002^{a}	0.930±0.006^{a}	0.894±0.005^{a}	0.843±0.005^{a}
	2) 0.25%	0.961±0.001^{a}	0.970±0.002^{a}	0.958±0.004^{a}	0.933±0.009^{a}	0.926±0.007^{b}	0.857±0.005^{a}
	3) 0.50%	0.960±0.002^{a}	0.966±0.001^{a}	0.957±0.002^{a}	0.915±0.003^{a}	0.898±0.005^{a}	0.852±0.002^{a}
	4) 0.75%	0.964±0.001^{a}	0.966±0.001^{a}	0.955±0.003^{a}	0.919±0.004^{a}	0.905±0.007^{ab}	0.862±0.006^{a}

554 Data are expressed as means ± SE. Different letters indicate significant differences ($P < 0.05$) between pH variations within sampling day.
555 NOTE: * based on only 1 measurement, ** based on only 2 measurements

556

557

558

559 **Table 2 Zn(II)PPIX, PPIX and total heme evolution (n = 3) during the production of nitrite-free dry fermented sausages. Different pH conditions are obtained by**
560 **adding different concentrations of dextrose to the meat batter, 0.00% (1), 0.25% (2), 0.50% (3) and 0.75% (4).**

		Day 0	Day 3	Day 21	Day 45	Day 64	Day 177
		meat batter	after fermentation	after initinal drying period	extensive drying period		
Zn(II)PPIX	**1) 0.00%**	1.31±0.03^{a,1}	7.28±0.98^{a,12}	13.15±4.13^{b,23}	15.28±1.02^{b,3}	16.49±1.35^{b,3}	125.69±5.66^{b,4}
(nmol/g DM)	**2) 0.25%**	1.307±0.003^{a,1}	5.87±0.72^{a,1}	12.79±1.25^{b,2}	13.80±3.36^{b,2}	16.60±1.08^{b,2}	113.83±24.85^{b,3}
	3) 0.50%	1.31±0.01^{a,1}	4.82±0.21^{a,1}	5.66±0.77^{a,1}	5.18±0.31^{a,1}	4.45±0.21^{a,1}	35.11±4.42^{a,2}
	4) 0.75%	1.30±0.04^{a,1}	4.32±1.47^{a,1}	5.47±0.23^{a,1}	4.79±0.26^{a,1}	4.84±0.35^{a,1}	26.58±0.00^{a,2}
PPIX	**1) 0.00%**	0.76±0.03^{a,1}	1.65±0.18^{a,1}	5.42±2.66^{a,1}	16.77±2.18^{c,2}	17.07±2.52^{b,2}	83.73±8.67^{d,3}
(nmol/g DM)	**2) 0.25%**	0.76±0.04^{a,1}	1.90±0.05^{a,1}	4.88±1.10^{a,12}	9.04±4.00^{b,23}	14.31±3.73^{b,3}	45.04±8.12^{c,4}
	3) 0.50%	0.77±0.03^{a,1}	1.70±0.11^{a,1}	2.45±0.46^{a,1}	2.42±0.23^{a,1}	2.50±0.16^{a,1}	15.35±1.06^{b,2}
	4) 0.75%	0.81±0.02^{a,1}	3.69±1.97^{a,1}	1.82±0.06^{a,1}	1.58±0.04^{a,1}	1.81±0.06^{a,1}	4.84±0.00^{a,1}
Total heme	**1) 0.00%**	181.33±10.52^{a,3}	180.23±3.36^{a,3}	154.38±11.76^{a,2}	128.48±6.94^{a,1}	127.06±9.90^{a,1}	114.51±24.30^{a,12}
(nmol/g DM)	**2) 0.25%**	186.50±1.56^{a,2}	173.63±3.30^{a,2}	178.07±10.71^{b,2}	139.30±6.94^{ab,1}	132.56±1.32^{ab,1}	121.32±5.11^{a,1}
	3) 0.50%	189.58±4.71^{a,3}	188.54±10.88^{a,3}	177.46±4.00^{b,3}	165.92±4.26^{c,23}	149.28±1.90^{b,2}	112.50±12.38^{a,1}
	4) 0.75%	217.82±10.53^{b,4}	194.22±4.16^{a,34}	188.69±6.34^{b,3}	159.41±4.27^{bc,2}	145.09±3.50^{ab,12}	120.90±15.82^{a,1}

561 Data are expressed as means ± SE. Different letters indicate significant differences ($P < 0.05$) between pH variations within sampling day. Different numbers indicate significant
562 differences ($P < 0.05$) between sampling days within pH variation.
563
564
565

Table 3 Changes in L*, a* and b* (n = 18) during the production of nitrite-free dry fermented sausages. Different pH conditions are obtained by adding different concentrations of dextrose to the meat batter, 0.00% (1), 0.25% (2), 0.50% (3) and 0.75% (4).

		Day 0	Day 3	Day 21	Day 45	Day 64	Day 177
		meat batter	after fermentation	after initinal drying period	extensive drying period		
L*	**1) 0.00%**	61.73±0.60^{a,34}	61.62±0.18^{a,4}	60.48±0.27^{a,4}	59.19±0.28^{a,3}	58.08±0.25^{a,2}	55.84±0.34^{a,1}
(-)	**2) 0.25%**	63.04±0.29^{a,23}	60.89±0.12^{a,3}	61.22±0.25^{a,3}	61.08±030^{b,3}	59.75±0.17^{b,12}	55.41±0.19^{a,1}
	3) 0.50%	62.77±0.20^{a,234}	62.21±0.23^{a,4}	61.15±0.13^{a,34}	60.31±0.17^{b,3}	58.77±0.22^{ab,2}	55.01±0.25^{a,1}
	4) 0.75%	61.56±0.32^{a,234}	61.34±0.22^{a,4}	61.31±0.20^{a,4}	59.69±0.20^{ab,3}	58.00±0.24^{ab,2}	52.63±0.39^{a,1}
a*	**1) 0.00%**	16.24±0.21^{a,4}	8.61±0.23^{b,12}	7.75±0.15^{b,1}	9.15±0.08^{b,23}	9.61±0.09^{b,3}	13.03±0.19^{c,4}
(-)	**2) 0.25%**	16.19±0.17^{a,5}	5.45±0.09^{a,1}	6.72±0.19^{a,2}	7.93±0.08^{a,3}	9.04±0.08^{ab,4}	9.93±0.27^{ab,234}
	3) 0.50%	16.37±0.14^{a,5}	5.10±0.09^{a,1}	6.21±0.09^{a,2}	7.62±0.09^{a,3}	9.60±0.10^{b,4}	10.30±0.13^{b,4}
	4) 0.75%	17.31±0.19^{a,4}	4.93±0.23^{a,1}	6.07±0.07^{a,1}	6.96±0.08^{a,2}	8.20±0.15^{a,3}	8.96±0.21^{a,3}
b*	**1) 0.00%**	24.82±0.23^{a,4}	16.21±0.19^{d,3}	13.47±0.11^{c,2}	12.45±0.07^{a,1}	12.15±0.08^{b,1}	11.55±0.10^{a,12}
(-)	**2) 0.25%**	25.10±0.16^{ab,4}	12.47±0.08^{c,3}	12.05±0.12^{b,23}	11.65±0.12^{a,12}	11.60±0.09^{b,1}	12.06±0.19^{a,123}
	3) 0.50%	25.27±0.14^{ab,3}	11.50±0.13^{b,2}	11.74±0.11^{ab,2}	11.42±0.16^{a,2}	7.60±0.06^{a,1}	12.10±0.12^{a,2}
	4) 0.75%	25.82±0.18^{b,3}	10.85±0.10^{a,2}	10.97±0.09^{a,2}	11.20±0.10^{a,2}	6.96±0.12^{a,1}	12.13±0.15^{a,2}

Data are expressed as means ± SE. Different letters indicate significant differences ($P < 0.05$) between pH variations within sampling day. Different numbers indicate significant differences ($P < 0.05$) between sampling days within pH variation.

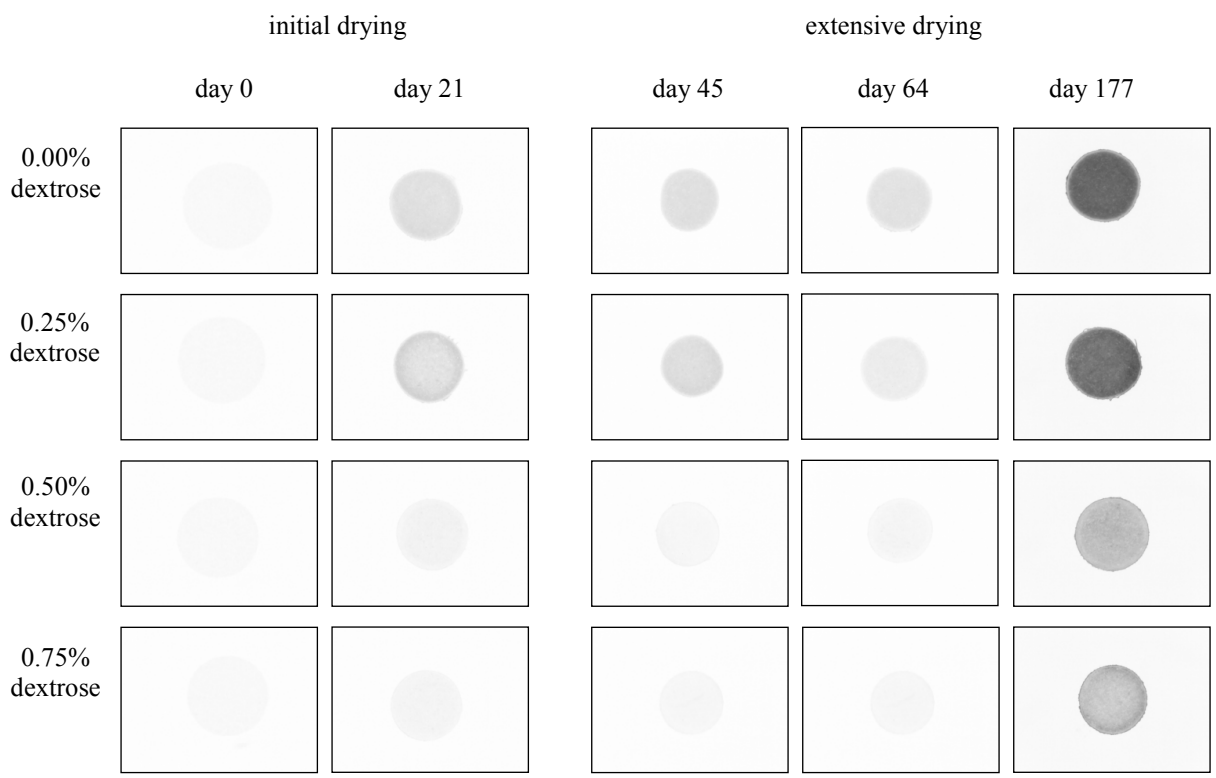

Figure 1 Evolution of (zinc) protoporphyrin IX during the production of nitrite-free dry fermented sausages using a fast screening method (red fluorescence is visualized as inversed red channels via image analysis after irradiation with purple LED light). Different pH conditions are obtained by adding different concentrations of dextrose to the meat batter, 0.00% (1), 0.25% (2), 0.50% (3) and 0.75% (4).