Influence of pH on (zinc) protoporphyrin IX formation in dry fermented sausages assessed using a fast screening method

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Abstract- The aim of this study is to investigate the influence of pH on the formation of zinc protoporphyrin IX and/ or protoporphyrin IX in nitrite-free dry fermented sausages using a fast, qualitative screening method, based on the fluorescence properties of the respective molecules. Four batches of dry fermented sausages with different pH are obtained by adding different concentrations of dextrose at production day. A higher formation of zinc protoporphyrin IX and protoporphyrin IX is observed in dry fermented sausages with higher pH levels. The fluorescence intensities during production initially remain relatively stable. Only after an extensive drying process, a strong increase in pigment formation can be observed. Further quantitative determination of the amount of zinc protoporphyrin IX and protoporphyrin IX in the samples using HPLC is relevant.

Key Words – Zn(II)PPIX, PPIX, pH, nitrite-free dry fermented sausages

I. INTRODUCTION

Porphyrins are naturally occurring macrocyclic compounds, which play an important role in a lot of biological activities. Due to their aromaticity, they typically have intense absorption bands in the visible region and are strongly coloured. Moreover, some porphyrins also show strong fluorescence properties. More specifically, zinc protoporphyrin IX (Zn(II)PPIX) is characterized by a red fluorescence emission at 590 nm in response to the excitation of blue light at 420 nm, for protoporphyrin IX (PPIX) fluorescence emission is obtained at 630 nm with 410 nm excitation wavelength [1].

Zn(II)PPIX is able to form in meat tissue, but until now the mechanisms are still unclear. However, it is known that Ferrochelatase (FECH), the final enzyme in the heme-biosynthetic pathway, is also involved in the formation of Zn(II)PPIX [2] [3]. In dry cured meat products prepared without the use of additional nitrite or nitrate, such as Parma ham, Zn(II)PPIX is even able to form during the long maturation process in such an amount that a stable red colour is obtained [4]. From the moment this pigment was identified, researchers have been interested to investigate the formation of Zn(II)PPIX for realizing the production of other nitrite-free meat products with a shorter production process, such as dry fermented sausages. This interest is fueled by the direct and indirect toxicity of nitrite in meat products.

In vitro studies already revealed the importance of pH for the formation of Zn(II)PPIX. Wakamatsu et al. [1] found an optimum pH of 5.50 for the formation of Zn(II)PPIX in a liquid model, using pork meat homogenized in distilled water with a mix of antibiotics and which was anaerobically stored at 25° during 5 days. Chau et al. [5] [6] however showed that the zinc insertion reaction had a high activity at pH 7.50-8.00. FECH activity was determined by measuring the zinc insertion of mesoporphyrin IX at anaerobic conditions.

In this study the influence of pH on the formation of Zn(II)PPIX and PPIX is investigated in nitrite-free dry fermented sausages. Different pH evolutions are obtained by adding different amounts of dextrose to the meat batter.

Similar to Wakamatsu *et al.* [7] a non-invasive screening method, based on the fluorescence properties of Zn(II)PPIX and PPIX, is optimized in order to observe the presence of these pigments during the production of dry fermented sausages. 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.

II. MATERIALS AND METHODS

Sausage production and sampling

Four variations of dry fermented sausages are made using pork meat (69.6 %), pork back fat (26.8 %), salt (2.8 %), sodium ascorbate (0.05 %), spices and a commercial starter culture containing *Lactobacillus sakei, Staphylococcus xylosus* and *Staphylococcus carnosus* (Texel SA306, Danisco, Belgium). The variation in pH after fermentation and at the end of the drying process is achieved by adding different concentrations of dextrose, namely 0.00% for batch A, 0.25% for batch B, 0.50% for batch C and 0.75% for batch D. All variations are made in triplicate. The meat batter is filled in casings (Ø 90 cm), and fermented during three days (24°C/ 95RH %), smoked and subsequently dried (14°C/ 87RH %).
Samples (transverse sections) are taken at day 0 (production day of the meat batter) and day 21 (end of production with 20% weight loss). An extensive drying process is carried out, during which samples are taken at day 45, 64 and 177. pH is measured in triplicate in all samples at each sampling day.

Screening method

A screening method, for the fast detection of Zn(II)PPIX and PPIX on transverse slices of meat products, is optimized based on Wakamatsu *et al.* [7] with minor modifications.

Optimization of the black box set-up.
A well-sealed black box is made in such a way that no external light can enter. 12 LED's H2A1-H420, 420 nm 130 mW 350 mA HEX (Roithner Lasertechnik GmbH, Vienna, Austria) are connected to 3 driver modules for 1W LEDs (VM 143,gentronics, gent-BE). On top a macro convertor (Fotografie Deknudt, Drongen-Belgium) is fixed. A digital camera Olympus PEN E-PL3 (Fotografie Deknudt, Drongen-Belgium) can be connected externally, which facilitates taking the photographs. Meat slices are put on the bottom at a distance of 33 cm to the macro convertor.
With Kodak Wratten Color Gelatin Filters No.12 only wavelengths higher than 500 nm are transmitted and can be detected by the digital camera. As such, the fluorescence emission of Zn(II)PPIX (590 nm), is transmitted while avoiding the 'noise' of the blue LED light (420 nm, which is the excitation wavelength of Zn(II)PPIX) and other unknown signals below 500 nm. Also PPIX, which has an emission wavelength of 630 nm, is detected.

Image analysis.
Red fluorescence of Zn(II)PPIX is observed by using image analysis of RAW images. The width of images is downsized to 250 pixels, and the colours of the images are divided in RGB channels by using OlympicViewerII. The images are saved as Microsoft Windows Bitmap Images. The red emission in the R (red) channel is regarded as autofluorescence of ZnPPIX and PPIX by using ImageJ and inversed pictures are obtained using Gimp2.

III. RESULTS AND DISCUSSION

Dry fermented sausages are made using different concentrations of dextrose, respectively 0.00%, 0.25%, 0.50% and 0.75%.

Evolution of pH in dry fermented sausages

By adding different concentrations of dextrose, different pH evolutions during the production of dry fermented sausages are pursued based on the availability of nutrients for the lactic acid bacteria (LAB). Lack of dextrose, thus lack of nutrients for LAB, results in a lower production of lactic acid, which is responsible for the pH decline during the fermentation process (table 1).

Significant differences in pH are observed between the different variations within each sampling day, meaning that the addition of varying concentrations of dextrose results in a significantly different pH decline during fermentation (first three days of the production process). After fermentation, the pH remains stable until the end of production (usually after 21 days or 20% weight loss). During the prolonged drying process, an increase of pH is observed, which is probably attributed to proteolytical processes [8]. Nevertheless the differences in pH between the four variations also remain after this extensive drying period.

Table 1 Evolution of pH at day of batter production (day 0), end of production (day 21) and after 45, 64 and 177 days of drying of four variations dry fermented sausages, using 0.00% (A), 0.25% (B), 0.50% (C) and 0.75% (D) dextrose. Data are expressed as means ±stdev (n = 9), different superscripts indicate significant differences at p<0.05 within sampling days.

	Variation			
	A	B	C	D
Day0	5.69±0.03 ^{a,b}	5.66±0.02^{a}	5.70±0.03^{b}	5.72±0.02 ^{b}
Day21	5.33±0.03 ^{d}	4.96±0.01 ^{c}	4.83±0.07 ^{b}	4.66±0.02 ^{a}
Day45	5.49±0.07 ^{d}	5.22±0.03 ^{c}	4.95±0.07 ^{b}	4.72±0.04 ^{a}
Day64	5.29±0.05 ^{d}	5.06±0.03 ^{c}	4.79±0.05 ^{b}	4.61±0.06 ^{a}
Day177	5.60±0.02 ^{d}	5.37±0.05 ^{c}	5.25±0.07 ^{b}	5.09±0.04 ^{a}

Zn(II)PPIX and PPIX formation in nitrite-free dry fermented sausages assessed using a fast screening method

A fast screening method, based on the fluorescence properties of Zn(II)PIX and PPIX is used to investigate the influence of the four different pH evolutions obtained in dry fermented sausages. The Red channels of the four variations (2 photographs per variation) at sampling day 0, 21, 45, 64 and 177 are shown in figure 1.

At day 0 almost no fluorescence is observed in the Red channels. At the end of production (day 21), clear fluorescence appears, and is remaining stable during the longer drying period until sampling day 64. At sampling day 177 however, a large increase is seen in the Red channels.

In a recent study wherein the formation of Zn(II)PPIX is investigated in Parma ham, it is claimed that the formation of Zn(II)PPIX occurred when myoglobin denatures, which happens during the long ripening process of the product. Iron is removed from the heme, forming non heme colloidal ferric hydroxide and a substitution reaction from heme to Zn(II)PPIX can take place [9]. It is assumed that also in this study the factor time plays an important role in the formation of Zn(II)PPIX.

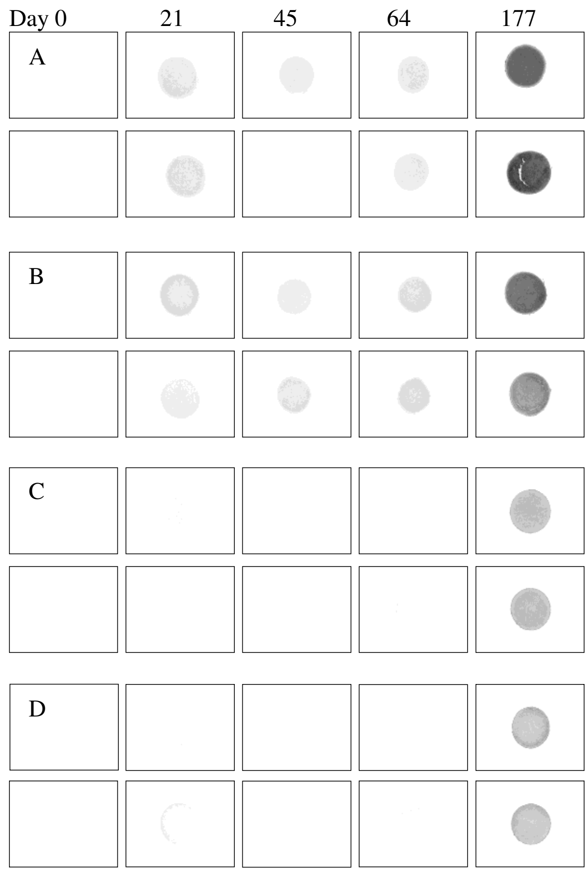

Figure 1. Evolution of Zn(II)PPIX and PPIX formation at day of production (day 0), end of production (day 21) and after 45, 64 and 177 days of drying of four variations dry fermented sausages, with 0.00% (A), 0.25% (B), 0.50% (C) and 0.75% (D) dextrose, using a fast screening method.

In variations A (0.00% dextrose) and B (0.25% dextrose) the intensity of the fluorescence is much higher than in variations C (0.50% dextrose) and D (0.75% dextrose). Based on these results it can be concluded that the formation of Zn(II)PPIX and / or PPIX is more pronounced in circumstances with pH levels similar to or higher than 5.00. When the pH decreases to lower pH values during the production process, formation of the natural pigments is much more difficult. After 177 days of extensive drying however, the pH in these low-pH sausages increases due to proteolysis and formation of the pigments seems to be possible again [8] [9]. The importance of pH for the formation of Zn(II)PPIX can be explained by the optimum pH for the activity of FECH, the enzyme responsible for its formation [1] [5] [6].

IV. CONCLUSION

Formation of (zinc) protoporphyrin IX is observed in dry fermented sausages with higher pH levels. If the pH remains higher than 5.00 during the production process of dry fermented sausages, formation occurs and remains stable even when an extensive drying process is taking place (until sampling day 64). Only if an extremely long drying period is carried out (day 177), large increases of fluorescence intensities can be observed.

Based on this screening, it is concluded that further investigation will be performed to quantify the formation of Zn(II)PPIX and PPIX in the samples using HPLC. Moreover, it is interesting to investigate which concentrations of natural pigments are necessary to achieve a red colour in dry fermented sausages and which product and process conditions are able to fasten this process, since it is recommended to avoid an extreme long production time for nitrite-free dry fermented sausages.

ACKNOWLEDGEMENTS

This investigation was done in the framework of an European Interreg IV a 2 seas program. The research project MeCagrO_2 involves safe products, sustainable processes and employment increased attractiveness for companies from the 2 Seas agro-food area.

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