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Determination of chicken meat contamination by porphyrin fluorescence

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ABSTRACT

Meat quality is normally defined by a combination of factors such as visual appearance, smell, firmness, succulence, tenderness, and flavor. Contamination of poultry meat with pathogens remains an important public health issue since it can cause illnesses due to negligence in handling, cooking or post-cooking storage. Conventionally, meat quality tests are based on visual evaluation or chemical analysis, which have the disadvantages of being subjective and time-consuming. To improve the biological contamination detection accuracy and productivity, the evaluation of porphyrin contents in meat by fluorescence spectroscopy is proposed, considering that most microorganisms and animal cells excrete porphyrins. For this purpose, chicken meat was cut into small pieces and separated in three groups: a control group where the meat was conserved under refrigeration, and two experimental groups in which the pieces were kept for 24 and 30 h at room temperature. Porphyrin was extracted from the meat samples and the fluorescence was measured in the range 550–750 nm, under excitation around 400 nm. The fluorescence lifetime was also studied. To ensure porphyrin synthesis, a concentration of 9.3 mM of δ-Aminolevulinic acid (ALA) was added to each sample, 2 h before porphyrin extraction. The results show that, in meat kept at room temperature and incubated with ALA, the porphyrin fluorescence increased, and a short-lived component was enhanced due to the action of microorganisms, indicating a potential new method to test meat quality.

1. Introduction

Chicken is the most common type of poultry in the world. The two most important quality attributes for poultry meat are its appearance and texture, and the quality grade is a composite evaluation of factors that affect the meat palatability (tenderness, juiciness, and flavor)[1]. Contamination of poultry meat with microorganisms is an important public health concern, since it can lead to human illnesses due to negligence in handling, cooking or post-cooking storage of the product.

Myoglobin present in chicken meat is responsible for transporting oxygen and also gives meat its color [2]. The prosthetic group in myoglobin is the protoporphyrin IX (PPIX), in which center resides an iron ion [2,3]. Porphyrins are a group of organic molecules consisting of four pyrrole rings linked by methane bridges, and some of those have been related to oxidative stability in meat and meat products [4]. Protoporphyrin IX, which is a sub-group of the porphyrins, has its formation attributed to the action of endogenous enzymes in meat [4], and is mainly produced under anaerobic conditions, while oxygen inhibits its formation [5]. PPIX also accumulates in meat products at increased pH conditions and production times [6]. Porphyrins exhibit fluorescence between 500 and 750 nm after excitation at 420 nm [7], and since PPIX is a significant photosensitizer that can produce singlet oxygen upon light exposure [8], and may be involved in the process of photo-oxidation in foods, it is of high interest to investigate its presence in meats.

Additionally, porphyrins can be produced by different microorganisms [9]. In fresh pork, PPIX and zinc PPIX fluorescence intensities increased with storage time and temperature [10] also when stored under different atmospheric conditions or after a freezing process [11].

The porphyrin present in meat can be detected by conventional fluorescence spectroscopy [12], which is extremely sensitive, rapid, non-destructive and has relatively low cost. For these reasons, fluorescence spectroscopy is a promising method for probing contamination in poultry meat [13]. Recently Durek et al. investigated a new non-invasive mobile system for fresh pork and lamb meat quality monitoring based on the fluorescence of NADPH (nicotinamide adenine dinucleotide phosphate) and two porphyrins that could be correlated to the total viable count [14].

Aminolevulinic acid (ALA) is a naturally occurring intermediate in the heme synthesis pathway [15], and is a precursor of porphyrins that can be biosynthesized in nearly all aerobic cells. It is synthesized in

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mitochondria and converted into PPIX by several reactions in the cytosol, and then is modified to heme in mitochondria [16]. In situ detection of ALA-stimulated porphyrin metabolic products in bacteria by fluorescence was already demonstrated in the literature [15,17].

In this work, the PPIX molecules present in chicken meat were evaluated by steady state and lifetime fluorescence spectroscopy, and the ALA effects on the meat PPIX fluorescent emission were studied. The results can help in characterizing meat quality methods in terms of their potential impacts on human health.

2. Materials and methods

To perform the study presented here, chicken meat stored in common condition was acquired at a local supermarket. The meat extracted PPIX and synthetic PPIX solutions fluorescence behaviors were investigated in the absence and in the presence of δ-Aminolevulinic acid. The experimental details are given below.

2.1. Synthetic PPIX solutions

A synthetic PPIX solution with 100 μM concentration was prepared dissolving Protoporphyrin IX (Sigma, CAS Number 553-12-8) in acetone P.A. (Synth).

2.2. Chicken meat extracted PPIX

Chicken meat (boneless thigh) was cut into small pieces (~0.3 g). The samples were placed in closed falcon tubes. Six samples were refrigerated (0 °C − 4 °C) and 12 samples were kept at the room temperature (20 °C − 25 °C) for 24 (6 samples) and 30 h (6 samples). PPIX was extracted from the meat pieces by the addition of 3 mL of acetone P.A. to the falcon tubes containing them, followed by centrifugation at 4000 rpm for 15 min. The clear supernatant of the mixtures was separated and stored in glass tubes.

2.3. δ-Aminolevulinic acid (ALA) solution

0.0122 g of ALA was dissolved in 10 mL of miliQ water and the solution pH was adjusted to 7.0.

100 μl of prepared ALA solution was added to the ~0.3 g meat pieces and incubated for 2 h before PPIX extraction with the above procedure.

2.4. Optical measurements

The fluorescence measurements were performed using a RF-5301 fluorimeter (Shimadzu Scientific Instruments). The samples fluorescence spectra were obtained for wavelengths between 420 and 780 nm, under excitation at 400 nm. All measurements were carried out at room temperature using a quartz cuvette with four polished faces and 1 mm of optical path.

The PPIX fluorescence lifetimes were obtained with a pulsed diode laser (PDL 800-B, PicoQuant) that provides 45 ps pulses, centered at 403 nm, in an 8 Hz repetition rate pulse train, with the average power fixed at 0.1 mW. Detection was performed using a photomultiplier (Hamamatsu PMA 182-PM) and a RG610 longpass colored glass filter, and a reflective ND filter ND30A (ThorLabs) was used to reduce the background noise. This setup yields a (248 ± 1) ps FWHM temporal instrument response. The obtained data was processed using PicoQuant PicoHarp 300 (TCSPC system connected to a PC through a USB 2.0 interface).

The fluorescence decay normalized intensity was averaged for the three samples in each group, and the resulting curve was fitted with a three-component exponential decay function:

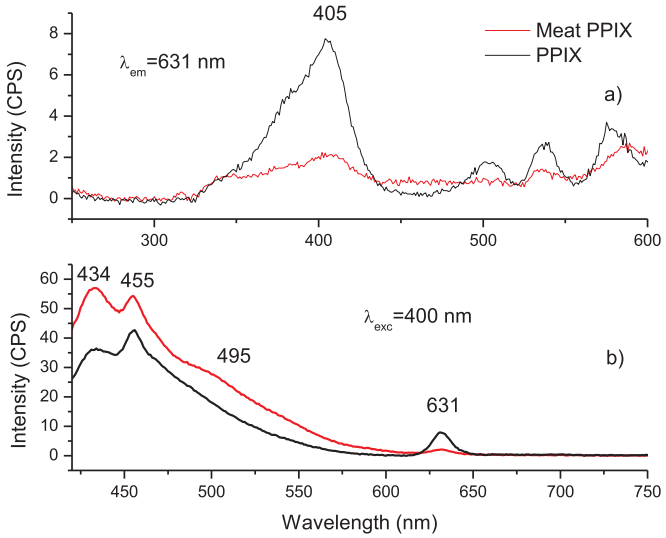

Fig. 1. Excitation (a) and fluorescence (b) spectra of synthetic PPIX and PPIX extracted from meat porphyrin (MP). The fluorescence spectra were obtained by excitation at 400 nm. Excitation spectra were obtained fixing the emission at 631 nm.

$$I(t) = bg + A_1 exp\left(-\frac{t}{\tau_1}\right) + A_2 exp\left(-\frac{t}{\tau_2}\right) + A_3 exp\left(-\frac{t}{\tau_3}\right) \quad (1)$$

where bg is the background noise, and τ_i and A_i are the components lifetimes and their relative weights (amplitudes), respectively. The quality of the fit was judged by the reduced-χ^2 value and inspection of the residuals distribution. The amplitude-weighted lifetime $< \tau >$ was calculated according to:

$$\langle \tau \rangle = \frac{\sum A_i \tau_i^2}{\sum A_i \tau_i} \quad (2)$$

3. Results and discussion

3.1. PPIX characterization

Fig. 1a shows the excitation (emission at λ_{em} = 631 nm) and Fig. 1b shows the fluorescence (excitation at λ_{exc} = 400 nm) spectra for both the synthetic and the chicken meat extracted PPIX solutions. In the excitation measurements, the Soret and Q bands can be observed in the 350–450 nm and 500–600 nm ranges, respectively. The emission band around 631 nm, along with a weak shoulder located around 700 nm, are due to the porphyrin fluorophore.

3.2. Chicken meat PPIX fluorescence

The chicken meat fluorescence spectra were obtained from meat samples conserved under refrigeration (Control) and samples kept at room temperature for 24 and 30 h. Fig. 2 shows the average of three spectra of each group. It is possible to observe an increase in the PPIX fluorescence intensity for the 24 h and 30 h groups and the presence of more defined bands around 630 nm and 700 nm for the samples kept at room temperature. The spectra of these samples have also a peak around ~ 590 nm attributed to Zn–porphyrin [5].

In Fig. 3, the bar chart shows the integrated emission (between 550 and 750 nm) for each studied group (Control, 24 h, 30 h, Control + ALA, 24 h + ALA e 30 + ALA), obtained after subtracting the background fluorescence over the baseline region of all the emission curves in the groups with and without ALA, followed by the integration of the porphyrin emission signal. This graph shows an increase in the porphyrin fluorescence for the groups in which meat was kept at room temperature and incubated with ALA. The kinetics of the PPIX fluorescence was

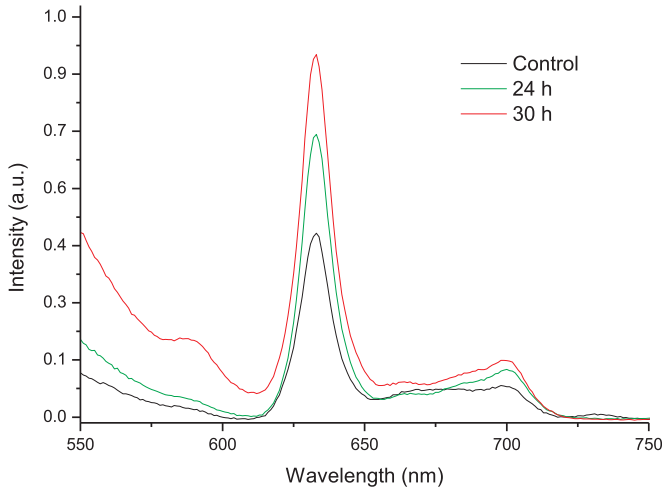

Fig. 2. Fluorescence spectra of PPIX extracted from chicken meat (each curve is the average of three spectra), from refrigerated samples (Control), 24 h at RT (24 h) and 30 h at RT (30 h).

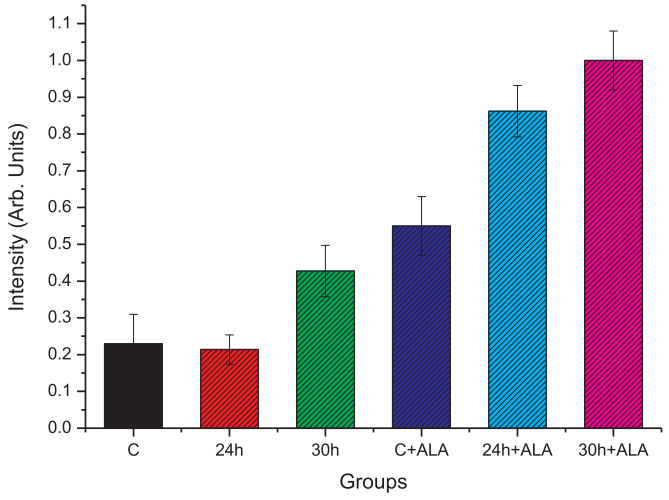

Fig. 3. Changes in the PPIX emission intensity for groups control 24 h and 30 h, with and without ALA incubation.

assessed using ANOVAl test to identify values deviating from the linear relationship, and a p-value < 0.05 was considered statistically significant for all groups, except between C and 24 h ones.

The fluorescence lifetimes for refrigerated and not refrigerated (30h) meat samples were obtained exciting samples at 403 nm. The results are shown in Fig. 4.

The comparisons between the groups for τ_1, τ_2, τ_3 and $< \tau >$ are shown in Fig. 5. All samples exhibit a short lifetime component, around 0.2 ns (τ_3), attributed to the acetone, and a longer decay time, τ_2, between 8 and 10 ns attributed to the PPIX [18]. Samples kept at room temperature present an intermediate lifetime, $\tau_1 \sim 2.0$ ns, due to the S1-T1 intersystem crossing of the zinc porphyrin [19,20]. ALA treated samples do not present the same behavior, as it is shown in the Fig. 5b.

4. Discussions

Hemoglobin and myoglobin proteins are important factors to determine meat quality. They contain heme groups, i.e., prosthetic groups of iron containing porphyrins that are able to bind oxygen, giving these heme proteins their typical color [21].

Ferrous iron is a strong inhibitor of zinc insertion; thus, it is often assumed that Zn-porphyrin is just formed in meat products when iron availability decreases [22,23]. It is also assumed that a higher

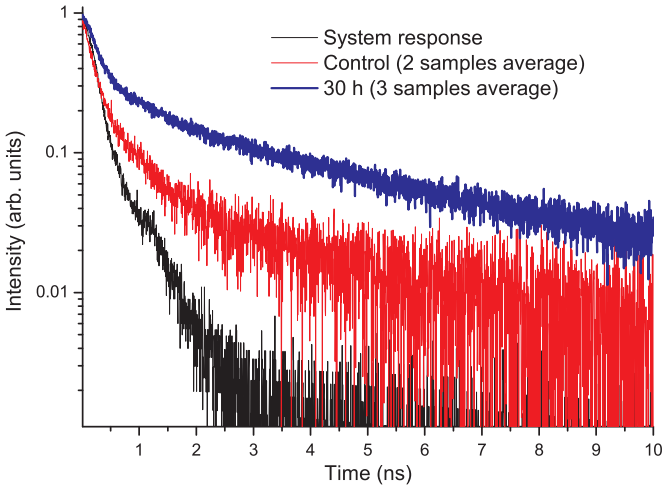

Fig. 4. Samples fluorescence lifetime under excitation at 403 nm and integrating emission with a filter RG630 for control groups (refrigerated until measurement), and 30 h. The dashed curve is the measurement system response function.

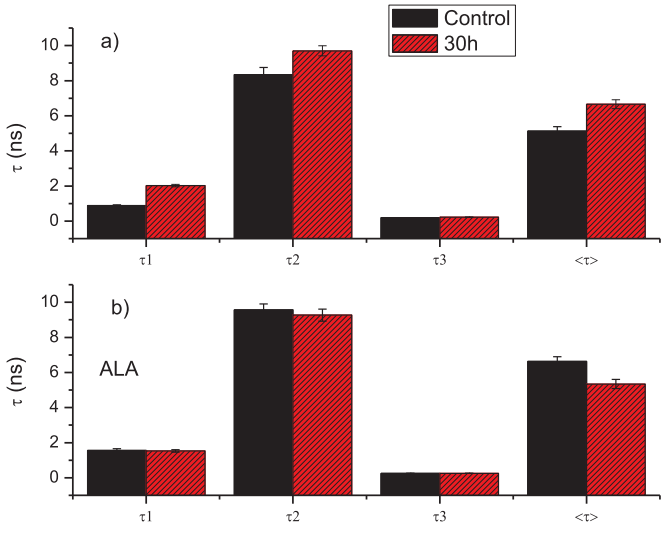

Fig. 5. Lifetime components and amplitude-weighted lifetimes, $< \tau >$, obtained for the decay curves for refrigerated and 30 h at room temperature samples a) without ALA and b) with ALA.

concentration of zinc may promote Zn-porphyrin formation, however, it has been shown that the profit of extra zinc addition is limited as it acts as a substrate inhibitor of mammalian ferrochelatase [24]. A meat based in vitro model was established by Da Maere et. al [6] with the addition of antibiotics to exclude the influence of microorganisms and to focus on the endogenous enzymatic pathway of Zn-porphyrin X formation. Only trace amounts of Zn-porphyrin and PPIX could initially be found in chicken meat.

Microbiological changes are the most important modifications that occur in food because certain microorganisms are pathogenic and directly affect consumer health. The ambient temperature range (20–25 °C) and storage time are the factors that may be the causes for increased number of microorganisms. Refrigeration between 0 °C and 7 °C halts the growth of most pathogens.

Fig. 2 shows an increase in the PPIX emission band from chicken meat stored at room temperature for 24 and 30 h. To verify if this fluorescence increase is due to the presence of microorganisms, the meat was incubated with ALA. Alive cells can synthesize their own heme, but microorganisms require this compound from an exogenous source. This acquired heme can be directly used for heme protein biosynthesis or serve as a source of iron. Bacteria in meat encounter

extremely low free iron concentrations and find an abundance of heme in the form of hemoglobin and myoglobin [25]. When an excessive amount of exogenous ALA is administrated, PPIX accumulates in bacteria.

A reddening of Parma ham due to the formation of Zn-porphyrin, caused by the action of bacteria such as *staphylococci* [9], has been reported. In chicken meat kept at room temperature, an emission band at 589 nm, attributed to Zn–porphyrin, was observed. This increase in Zn- porphyrin band is related to a bacterial enzymatic reaction where bacterial growth naturally degrades meat proteins [5,26]. The contribution of Zn porphyrin in the fluorescence lifetime was quantified by the τ_1 component, being around 2.0 ns.

The administration of ALA increases the number of available PPIX molecules in the medium, allowing a process of aggregation, and consequently modifying the PPIX decay time. Samples with ALA administration present shortened PPIX fluorescence lifetime, particularly for samples kept at room temperature.

We demonstrated here that both steady state and lifetime fluorescence techniques can distinguish fresh and non-appropriately refrigerated chicken meat. The advantage of optical spectroscopic methods when compared to microbiological ones for meat quality control is their simplicity and facility. Spectroscopic methods do not require specialized instrumentations and technicians and take at most a few minutes to deliver results after PPIX extraction. Fluorescence lifetime is more appropriate than the measurement of fluorescence intensity, since it does not depend on the intensity of excitation nor on the concentration of the fluorophores. The presence of Zn porphyrin and the presence of microorganisms in meat can be confirmed by the presence of $\tau_1 \sim 2$ ns.

Although the ALA administration results in more intense PPIX emission, the high price of this compound do not justify its use.

5. Conclusions

The critical factors for food quality and safety are temperature and pH. High-risk foods occur when bacteria multiply, and this can be caused by storing food at the wrong temperature. We demonstrated that protoporphyrin IX and zinc protoporphyrin fluorescence increase in chicken meat kept at room temperature for long periods. The result was confirmed by incubation of meat with Aminolevulinic acid, indicating the proliferation of microorganisms in meat. The presence of an elevated number of microorganism in meat can be confirmed by the presence of an intermediate lifetime component around 2 ns, and by an emission peak in $\sim$ 589 nm.

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