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Effect of pH on the stability and molecular structure of nitrosyl hemochromogen

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ABSTRACT

This study explored the effect of pH on the stability and molecular structure of nitrosyl hemochromogen (NO-Heme) using ultraviolet (UV), electron paramagnetic resonance (EPR) and Fourier transform infrared (FT-IR) spectroscopy. The NO-Heme was extracted from pre-cooked cured beef and was dissolved in an 80% acetone solution pre-adjusted to different pH values with 2 mol/L HCl and 2 mol/L NaOH. The results show that pH significantly influences the optical properties and stability of NO-Heme. Strong acidic and weak basic conditions favor the red stability of NO-Heme. The conjugate structure of NO-Heme changed under strong basic conditions, as the NO^- group was shown to be still associated with the iron porphyrin, but the color of NO-Heme changed from pink to yellow. NO-Heme is extremely unstable at weakly acidic pH, and this may be the why pH affects the apparent color of cured meat products during storage.

1. Introduction

Nitrite-cured meat products are widely consumed in modern society. Although the color of cured meats is notably more stable than that of fresh meat, discoloration during storage is a common problem that limits the shelf-life of this type of product (Møller et al., 2003; Sun & Xiong, 2015). The apparent color of cured meat is known to be influenced by many extrinsic conditions (e.g., lighting, oxygen, temperature, pH, microorganisms, and non-meat ingredients) (Brooke et al., 2011; Kathrine, Gunilla, Anders, Karlsson, & Pere, 2013; King & Whyte, 2007; Suman & Joseph, 2014). Among the different variables, pH is recognized to be one of the factors that promotes color fading, resulting in a reduced consumer acceptability of cured meat.

The predominant pigment in cooked, cured meat is nitrosyl hemochromogen (NO-Heme). Myoglobin in meat products treated with nitrate or nitrite is converted into the reddish-pink NO-Heme, in which the ferrous ion is coordinated to nitric oxide, after the meat has been cured and cooked (Cassens, Greaser, Ito, & Lee, 1979). Based on a plethora of spectroscopic investigations of nitrosyl-heme complexes, it is believed that the globin portion of nitrosylmyoglobin denatures and subsequently detaches itself from the heme moiety to form NO-Heme during the thermal processing of nitrite-cured meat (Pegg & Shahidi, 1996; Sun, Zhou, Xu, & Peng, 2009). At present, a lot of modern techniques, such as electron paramagnetic resonance (EPR) spectroscopy, Fourier transform infrared (FT-IR) spectroscopy (Liu et al., 2011) and fluorescence polarizing angle spectroscopy (Mu, Chen, Zhang, Chen, & Guo, 2014; Mu, Chen, Zhang, Guo, & Chen, 2015) are widely used to analyze the molecular structure of pigments.

The effect of pH on the apparent color of meat products has been reported. For example, Lien et al. (2002) and Mancini, Kropf, Hunt, and Johnson (2005) reported that high pH not only maintains the red color of cooked pork products, but also prolongs the time of fading during cold storage. Honikel (2008) reported that decreasing a product's pH dramatically increases the rate of nitrite reduction to nitric oxide during meat curing. However, Zhong (2004) synthesized NO-Heme from ferroheme and nitric oxide, and reported on the storage stability of the synthetic NO-Heme under different pH conditions (pH 7 > pH 6 > pH 8 > pH 5 > pH 4).

Our interest in the effect of pH on extracted NO-Heme started during our investigations into the mechanism of color fading of cooked cured meat products during storage. Previous studies, which are mostly focussed on the apparent color of the meat, analyze the pH values of the meat matrix system, which contains many buffer materials. The effect of partial pH change may be quite significant. In addition, for Chinese traditional sour meat, the pH

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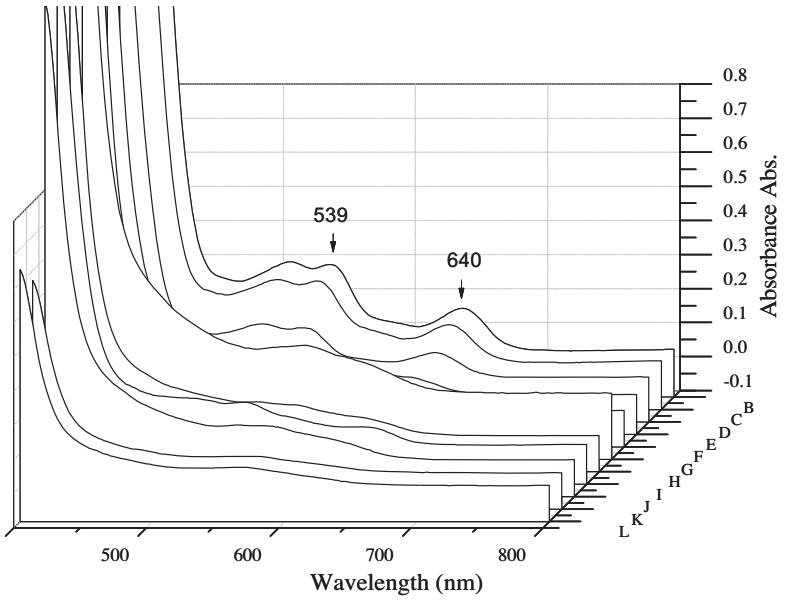

Fig. 1. The UV spectrum of NO-Heme dissolved in acetone at different pH values (B, pH 1.5; C, pH 2.0; D, pH 3.4; E, pH 4.6; F, pH 5.7; G, pH 6.3; H, pH 7.5; I, pH 8.6; J, pH 9.4; K, pH 10.3; L, pH 11.3).

Table 1
Effect pH on the properties of NO-Heme.

pH	Color (0 d)	λ_{max} (nm)	$A_{\lambda_{max}}$	A_{540}	Color (1 d)
1.5	Red	509, 539, 640	0.290	0.289	Red
2.0	Red	510, 539, 639	0.278	0.277	Red
3.4	Light red	539, 638	0.180	0.176	Light red
4.6	Pink	539	0.130	0.131	Light Pink
5.7	Light pink with little yellow	–	–	0.115	Yellow
6.3	Pink	398, 540	0.230	0.227	Light pink with little yellow
7.5	Pink	398, 542	0.110	0.105	Light pink with little yellow
8.6	Pink	396.5, 539, 628.5	0.141	0.14	Light Pink
9.4	Light pink with dark yellow	398, 547.5	0.110	0.109	Yellow
10.3	Light claybank	398.5, 554.5	0.060	0.054	Beige
11.3	Yellow	398.5, 565	0.060	0.057	Light Yellow

value may be reduced to 3.6 from 7.2 during curing and fermentation (Zhang, Guo, Zhang, Fan, & Ren, 2013). The government now strongly supports the development of traditional local characteristic products due to their unique flavor. In the processing of salted and emulsified meat products, the international standard of the pH value of the brine is at least 8.8, and in general 8.8–9.5 to achieve high yields. Sometimes the use of sodium tripolyphosphate makes the local pH more alkaline.

In the present study, a three-step gradational extraction procedure was developed to isolate NO-Heme, and its stability and molecular structure at different pH values were investigated by ultraviolet (UV), electron paramagnetic resonance (EPR) and Fourier transform infrared (FT-IR) spectroscopy.

2. Materials and methods

2.1. Materials

Acetone, ethyl acetate, petroleum ether and sodium nitrite were of analytical grade and were obtained from Kermel Chemical Reagent Ltd. (Tianjin, China). All solvents used were flushed with nitrogen before use. Sodium ascorbate and sodium polyphosphate were of food grade and obtained from Tianjin Dongda Chemical Group Co., Ltd. (Tianjin, China).

2.2. Preparation of cooked cured beef

Pre-cooked cured beef used for extracting NO-Heme was produced at the China Yurun Food Group Ltd. (Nanjing, China). Beef hindquarter muscles, after having all of the external fat and connective tissue removed, were aseptically divided into halves and cured with 900 mg nitrite/kg meat, which was above standard, to obtain more NO-Heme molecules. The muscles were injected with curing brine (the curing solution contained appropriate amounts of sodium chloride, sodium ascorbate and sodium tripolyphosphate to obtain final concentrations of 2%, 0.04%, and 0.3%, respectively, in the beef sample) to 115% of their initial weight using a multi-needle injector pump (Dick, F., Postfach 209, 73703 Esslingen, Germany), and tumbled for 10 min every 30 min at a temperature of 4 ± 1 °C (TM-902C food center thermometer, Nanjing Wanda Instrument Factory) for a total of 16 h at a speed of 10 rpm. The meat was vacuum packed (DZQ500/2SB vacuum inflating packing machine, Zhejiang Baochun packaging machinery co., Ltd.) and held at 4 ± 1 °C for 36 h. The cured muscle was then stuffed into boiling bags, closed under vacuum, placed in molds and heated in a water bath (SHW.W-600/420 type intelligent electric constant temperature water tank, 85 °C, ca. 55 min) until a center temperature of 72 ± 1 °C was reached. After heat-treatment, the cooked cured beef was cooled to 4 ± 1 °C for 6 h, then sliced and vacuum

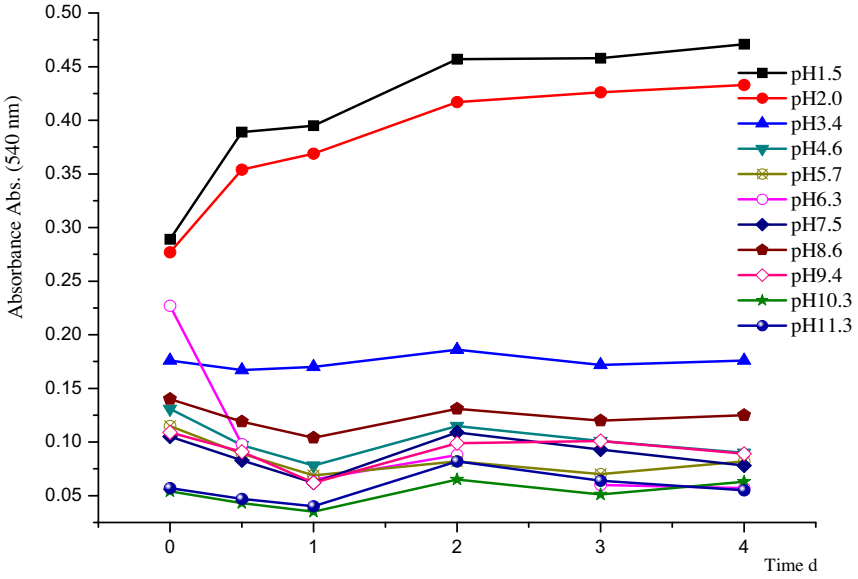

Fig. 2. The stability curve of NO-Heme at different pH values.

packed (50 g/package) in high oxygen barrier platinum film (oxygen transmission rate <1 $cm^3/m^2/24$ h/atm). The packages were stored at 4 ± 1 °C for pigment extraction.

2.3. Extraction and isolation of NO-Heme from pre-cooked cured beef

NO-Heme was extracted and isolated using a three-step gradational extraction procedure according to Sun, Zhou, Xu, and Peng (2010). Pre-cooked cured beef (ca. 200 g) was defatted by extracting with petroleum ether flushed with a stream of nitrogen gas in the dark for 28 h. The defatted samples were then extracted using acetone (ca. 400 ml) for 10 min and filtered through Whatman No. 4 filter paper, resulting in a bright red solution. The acetone was then removed on a rotary evaporator, leaving residual aqueous extract and pigment. This was extracted with ethyl acetate (ca. 30 ml), forming two layers of bright red solution and ivory turbid liquid. The bright red solution was collected, the ethyl acetate was removed on a rotary evaporator, and the red particles (NO-Heme) were obtained. The isolated NO-Heme was dissolved in 2 ml acetone, deoxygenated with nitrogen and transferred to a brown centrifuge tube with a cap. The headspace gases were purged from the tube with nitrogen for ca. 30 s, and then centrifuged at 1200×*g* for 5 min. The supernatant was used for analysis. All the procedures were carried out under strict anaerobic conditions in a dark room illuminated by a weak red light (ca. 1.04 lx). All solvents were deoxygenated by flushing with a stream of nitrogen gas (DC-12H nitrogen blowing instrument, ANPEL Laboratory Technologies (Shanghai) Inc.).

2.4. Preparation of NO-Heme at different pH values

The extracted NO-Heme (50 μl) was dissolved in a 5 ml 80% aqueous acetone solution at different pH values (pH = 1.5, 2.0, 3.4, 4.6, 5.7, 6.3, 7.5, 8.6, 9.4, 10.3 and 11.3), pre-adjusted with 2 mol/L HCl and 2 mol/L NaOH. Samples were then kept static for 20 min at room temperature prior to analysis. All samples were scanned spectrophotometrically from 350 to 800 nm (UV-2450, Shimadzu Inc., Columbia, MD, USA), then further analyzed by EPR and FT-IR spectroscopy. The stability of NO-Heme at different pH values was inspected by a single wavelength at 540 nm.

2.5. EPR analysis

The EPR measurements were taken on a Bruker EMX 10/12 X-band spectrometer (Bruker Instruments, Germany) at 110 K. Experimental conditions were as follows: modulation frequency, 100 kHz; receiver gain, 5.02 e + 003; time constant, 40.96 ms and microwave power, 20 mW. The g factor was calculated using the formula:

$$g = h\upsilon/\beta B$$

where h is Planck's constant ($h = 6.62620 \times 10^{-27}$), υ is the spectrometer frequency, β is the Bohr magneton ($\beta = 9.27410 \times 10^{-21}$), and B is the resonance magnetic field (magnetic field corrections in this experiment, $\Delta B = 6$ Gs).

2.6. FT–IR analysis

FT–IR spectra were recorded from 4000 to 400 cm^{-1} on a Bruker TENSOR27 IR spectrometer (Bruker Instruments, Germany).

3. Results and discussion

3.1. UV spectrum characteristics and stability of NO-Heme at different pH

Fig. 1 and Table 1 show that pH has a great impact on the optical properties of the NO-Heme. Both the absorption peak and the color of the NO-Heme aqueous acetone solutions change significantly as the pH changes gradually. NO-Heme stays pink to red under strongly acidic or weakly basic conditions, but the red color of NO-Heme fades to yellow or colorless under weakly acidic or strongly basic conditions.

Fig. 1 shows that under strongly acidic (pH 1.5, 2.0 and 3.4) conditions, the UV spectrum of NO-Heme exhibits absorption peaks at about 640 nm and 539 nm which correspond to a red color. Ginevra (2002) postulates that extreme acidity causes NO-Heme to break down into heme, which produces a red characteristic absorption peak at 640 nm and 540 nm. The strongly acidic NO-Heme pigment acetone solutions (pH 1.5, 2.0 and 3.4) that we studied were all bright red, which supports this theory. In the weak acid (pH 5.7) environment, the characteristic red absorption of

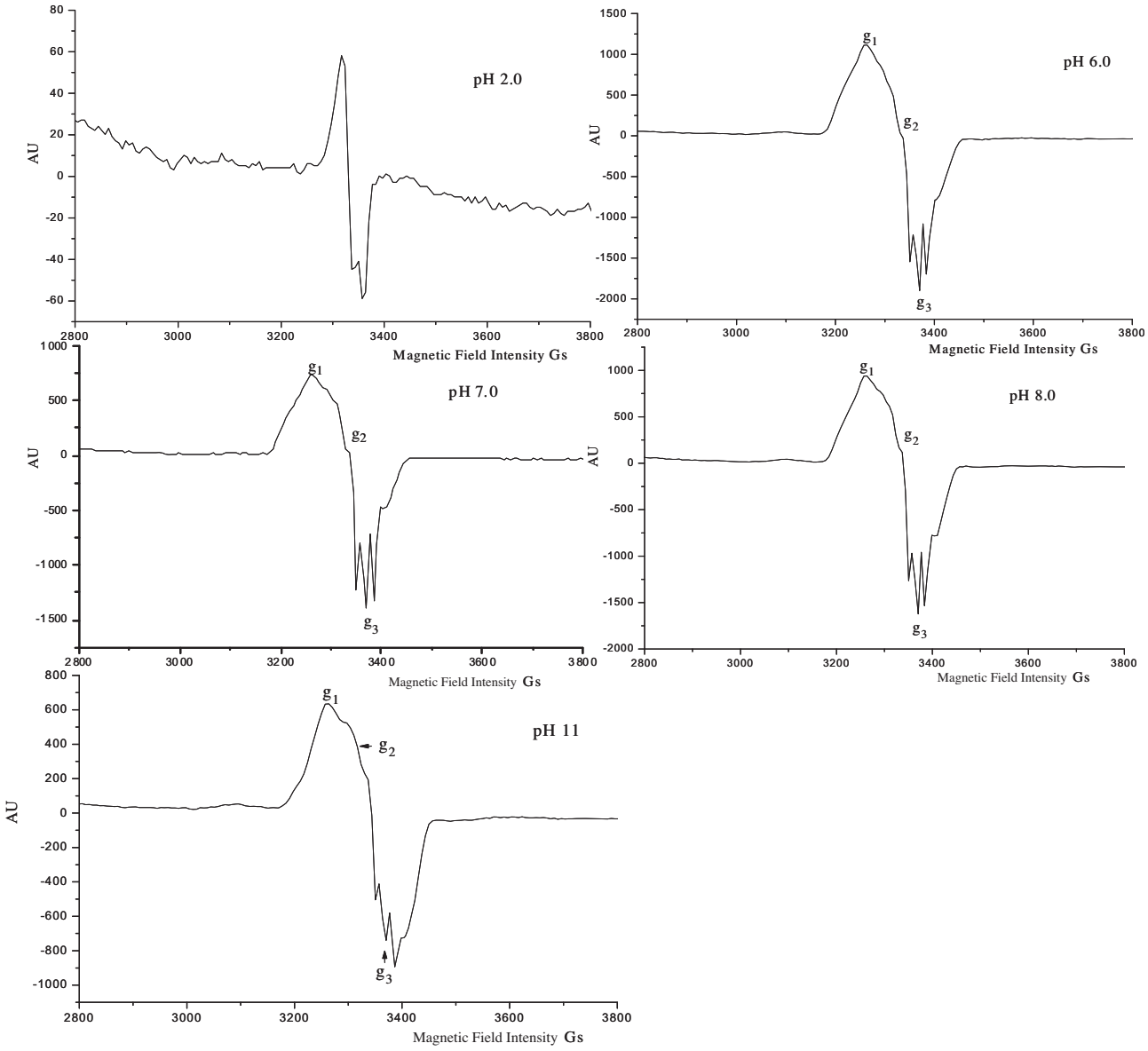

Fig. 3. The EPR spectra of NO-Heme dissolved in acetone and water at different pH values (pH 2.0, pH 6.0, pH 7.0, pH 8.0 and pH 11.0).

NO-Heme at 640 nm and 540 nm disappeared, and the color of the pigment solutions were yellow.

Fig. 2 shows that the absorbance of the NO-Heme solution at 540 nm gradually increases over time in the pH 1.5 and pH 2.0 environments, but remains stable at pH 3.4. The results illustrate that acidic conditions are beneficial for maintaining the red color, which is probably because NO-Heme is relatively stable, NO-Heme may also be transformed into another red pigment in strongly acidic conditions (described later). Therefore it is suggested that red pigments other than NO-Heme probably contribute to the absorbance value at 540 nm. However, at pH 6.3, the absorbance value of NO-Heme at 540 nm was not very stable and decreased significantly on storage after 0.5 days. The above results show that the red color of NO-Heme easily fades in weakly acidic and neutral conditions. As the pH value of cooked cured-meat products is between 5.5 and 6.5 during storage, this may be an important factor affecting the discoloration of this type of product. The absorbance value of NO-Heme solution at 540 nm decreased significantly after the first day of storage in an alkaline environment, but the curve was smooth during the later period of storage, when the color of the NO-Heme in acetone solution had become yellow or yellow–brown (Table 1). This suggests that NO-Heme may change into another relatively stable claybank pigment under alkaline conditions.

Hornsey (1956) report that a NO-Heme acetone solution will gradually change from red to yellow–brown in air with natural light, and propose that the yellow–brown degradation products may be alkaline hemoglobin. However, our previous studies (Sun et al., 2009) on the NO-Heme oxidation characteristics show that

Table 2
Parameters of EPR spectra of extracted NO-Heme in 80% acetone solution at different pH values (pH 2.0, pH 6.0, pH 7.0, pH 8.0, and pH 11.0).

pH	Color	g_1	g_2	g_3	a_3 (mT)
2	Red	–	–	–	–
6	Pink	2.0771	2.0377	2.0092	1.66
7	Pink	2.0769	2.0386	2.0100	1.67
8	Pink	2.0779	2.0390	2.0090	1.69
11	Yellow	2.0762	2.0245	2.0090	1.86

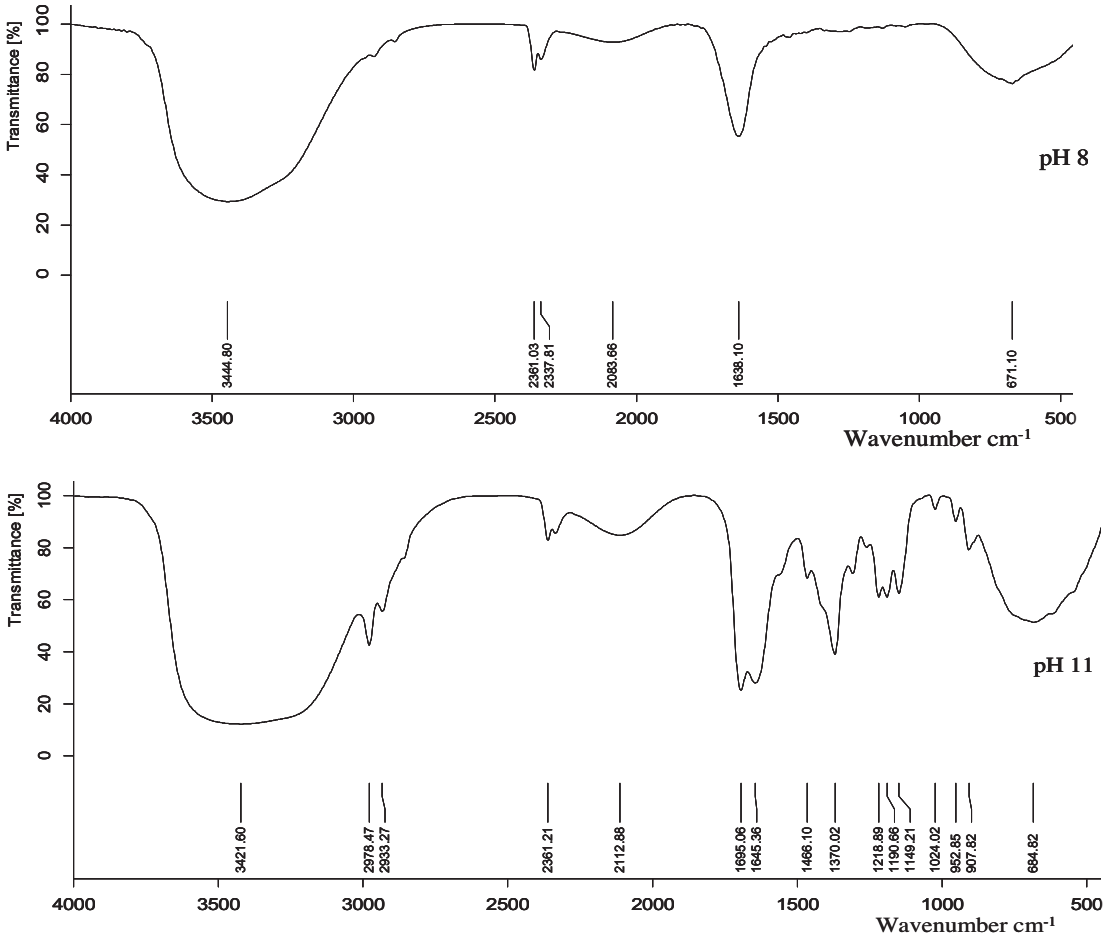

Fig. 4. The FT-IR spectra of NO-Heme dissolved in 80% acetone solution at pH 8 and pH 11.

the yellow–brown degradation products, formed from a NO-Heme acetone solution in air with natural light, resulted from another conjugate structure of NO-Heme, where the NO group may not have detached from the iron porphyrin. The present study involves further research about the change of molecular structure of NO-Heme through EPR and FT-IR spectral analysis at different pH values.

3.2. EPR spectra of NO-Heme at different pH values

The EPR spectra of NO-Heme at pH 6, pH 7 and pH 8 exhibit triplet hyperfine splitting in the g_3 region (Fig. 3), which results from the coupling of the electron spin of unpaired electrons on the iron (II) ion 3d orbital, and the nuclear spin of the ^{14}N atom from NO ligation (Pegg, Shahidi, Gogan, & DeSilva, 1996). As shown in Table 2, the g factors and coupling constant (a_3) of the EPR spectra are different at pH 6, pH 7 and pH 8. This indicates that the chemical bonds between the iron porphyrin and NO have not been broken, but the molecular structure of the NO-Heme has simply changed slightly as the pH value changed. At pH 2, significant changes were visible in the EPR spectrum of the NO-Heme. The triplet hyperfine splitting signal in the g_3 region has disappeared and the spectral line is not very smooth. Based on the results described above, it is postulated that the NO group may have dissociated from the iron porphyrin of NO-Heme, which causes the coupling between the nuclear spin of the ^{14}N atom with the electron spin of unpaired electrons on the 3d orbital of iron(II) ion to disappear. The iron porphyrin may have combined with H^+ at a low pH to form acidified hemoglobin (Pegg et al., 1996; Santhosh Kumar, Schwidder, Grünert, & Brückner, 2004; Tsai, Tsou, & Liaw, 2015).

However, the triplet hyperfine splitting does not disappear at pH 11, where the location of g_1, g_2 and g_3 became closer than that at pH 8, and the a_3 in the g_3 region was the biggest. This suggests that in a strongly alkaline environment, the NO ligand in NO-Heme had not detached from the iron–porphyrin ring, but simply shifted in the conjugated structure. Furthermore, Fig. 3 and Table 2 show that the location of g_1, and g_3 at pH 11 became closer than that at pH 8, and the a_3 was even higher at pH 11, suggesting that the molecule exhibited axial symmetry, the bond length or bond angle of NO-Fe^{2+} has changed, the steric hindrance around the porphyrin iron is reduced, and the coupling ability between the electron spin from Fe^{2+} and the nuclear spin from the ^{14}N atom have increased. Therefore the structure of NO-Heme at pH 11 is relatively more stable, but appears as a yellow color.

These results are consistent with our previous studies (Sun et al., 2009) on the structure changes of NO-Heme during autoxidation in air. It can therefore be suggested that the yellow–brown substance formed from NO-Heme natural oxidation in air is likely to be alkaline NO-Heme rather than alkaline hemoglobin, as reported by Hornsey (1956). In order to prove the presence of the NO group in the NO-Heme molecules under alkaline conditions, the FT-IR spectra of NO-Heme were further analyzed at pH 8 and pH 11.

3.3. FT-IR spectra of NO-Heme at different pH values

The FT-IR spectra of NO-Heme at pH 8 and pH 11 are presented in Fig. 4. The FT-IR spectrum of NO-Heme shows a band at

1638.10 cm^{-1} at pH 8, and bands at 1645.36 cm^{-1} and 1695.06 cm^{-1} at pH 11. According to the literature, the stretching frequency of the bent Fe–NO moiety in NO-Heme is in the range of 1600–1700 cm^{-1}. (Maxwell & Caughey, 1976). Within this range, Pegg and Shahidi (1996) reported the stretching frequency (1659 cm^{-1}) of pre-formed NO-Heme was consistent with the first nitrosyl group bound to the ferrous atom of the heme molecule, and Killday, Tempests, Bailey, and Climaco (1988) report that the infrared spectrum of extracted NO-Heme had a nitrosyl stretch at 1656 cm^{-1}, which is consistent with a bent NO^- ligand state.

In our previous studies on the structure analysis of NO-Heme, the FT-IR spectrum of a NO-Heme ethyl acetate solution had a nitrosyl stretch at 1653.31 cm^{-1}, which is consistent with the bent Fe–NO moiety of NO-Heme (Sun et al., 2009). Considering the influence of different solvents and an alkaline environment, the stretching frequency of the bent Fe–NO moiety of NO-Heme is likely to shift. The stretching vibration of the free nitrosyl ion generally occurs around 1550 cm^{-1}, so the observed stretching frequency in 80% acetone solution must be due to the bent Fe–NO moiety of NO-Heme; 1638.10 cm^{-1} and 1645.36 cm^{-1} at pH 8 and pH 11, respectively.

The results suggest that the NO^- ligand in NO-Heme was not detached from the iron–porphyrin ring under the conditions, but that the conjugated structure had changed.

4. Conclusions

The optical properties and stability of NO-Heme were significantly influenced by pH. Strong acidic and weak basic conditions are favorable for maintaining the red color of NO-Heme. Under strong alkaline conditions, the NO-Heme structure changed into another conjugated form, and its color changed from pink to yellow. The NO-Heme is extremely unstable under weak acid conditions, which may be the main reason why pH significantly affects the apparent color of these cured meat products. This research has demonstrated that a range of pH values can be used to design final products in order to obtain the optimal sensory effect in the meat industry.

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