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Studies on the structure and oxidation properties of extracted cooked cured meat pigment by four spectra

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ABSTRACT

The structure and oxidation properties of cooked cured meat pigment (CCMP) were investigated by comparing the change in spectra of CCMP before and after oxidisation. CCMP was extracted using petroleum ether/acetone/ethyl acetate step by step from precooked cured beef. The extracted sample was oxidised by being exposed to air with normal lighting or adding 1.5 ppm H_2O_2, respectively. The structure of CCMP was identified as a pentacoordinate mononitrosylheme complex by electron paramagnetic resonance (EPR), HPLC/ESI-HR-MS, Raman and FT–IR spectra. The changed EPR spectra of CCMP in acetone oxidised under different conditions suggested a new proposal that the NO^- group might not detach itself from iron porphyrin during oxidation in air with normal lighting, but changed in conjugated structure, and the structure tended to axial symmetry by analysis of the changes in *g* factor. This hypothesis was further supported by the results of the HPLC/ESI-HR-MS and Raman spectrum.

1. Introduction

Colour stability is one of the important characteristics of meat and meat products. It is the primary quality attribute seen by the consumer, who may use it as an indication of freshness and wholesomeness. The red colour of meat is due to myoglobin, a heme protein with the iron complex of porphyrin, and the colour of myoglobin depends on the kind of molecule coordinating to iron (Fox, 1966; Ledward, 1992; Sakata, 2000). Myoglobin in meat products with added nitrate and/or nitrite is converted into red nitrosylmyoglobin coordinated to nitric oxide, and nitrosylmyoglobin changes into pink-reddish nitrosohemochromogen (cooked cured meat pigment, CCMP) after the meat has been cooked. Exposure of CCMP to oxygen, even at low oxygen levels, promotes its oxidation that imposes a dull greyness on the meat surface (Efthimia, John, & Eleftherios, 2002). However, the pathway of oxidation and even the exact identity of the CCMP still remain unknown.

Isolation of the CCMP from precooked cured meats is difficult due to its instability in the presence of air and light. Few data (Hornsey, 1956; Killday, Tempests, Bailey, & Climaco, 1988) were reported about the nature of actual extracted CCMP. Many researchers (Killday et al., 1988; Pegg & Shahidi, 1996; Shahidi, Rubin, Diosady, Chew, & Wood, 1984; Shahidi, Rubin, Diosady, & Wood, 1985) attempted to characterise nitrosohemochromogen of cooked cured meat in comparison with synthetic pigments. However, the structure of CCMP is very controversial up to the present. Based on previous correlative studies, it was believed that, during thermal processing of nitrite-cured meat, the globin part of nitrosylmyoglobin was denatured and subsequently detached itself from the haem moiety. The resultant pigment was either a five-coordinate mononitrosylhaem complex (Jankiewicz, Kwasny, Wasylki, & Graczyk, 1994; Killday et al., 1988; Pegg & Shahidi, 1996; Pegg, Shahidi, Gogan, & DeSilva, 1996) or a six-coordinate dinitrosylhaem compound (Lee & Cassens, 1976; Renerre & Rougie, 1979; Shahidi et al., 1984, 1985) (see Fig. 1).

>Our interest in the structure and oxidation properties of the actual extracted CCMP stems from our investigations of the mechanism of colour fading of cooked cured meat products during storage. In the present study, a three-step gradational extraction procedure was developed to isolate the CCMP, and the structure and oxidation properties of actual CCMP were investigated by EPR, high performance liquid chromatography/electrospray ionisation high resolution mass spectra (HPLC/ESI-HR-MS), FT–IR and Raman spectra.

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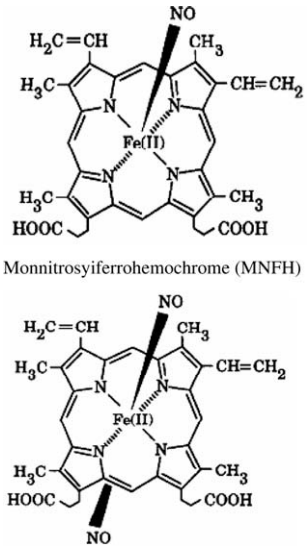

Fig. 1. Proposed chemical structures of the CCMP, MNFH or DNFH (Pegg and Shahidi, 1996).

2. Materials and methods

2.1. Materials

Acetone, ethyl acetate, petroleum ether and hydrogen peroxide were of analytical grade and were obtained from Kermel Chemical Reagent Ltd. (Tianjin, China). The acetonitrile was HPLC grade and was obtained from Tedia company, Inc. (USA). All solvents used were flushed with nitrogen before use.

2.2. Preparation of cooked cured beef

Precooked cured beef used for extracting CCMP experiment were 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 were cured with 900 mg nitrite/kg meat, which was above standard to obtain more CCMP. The muscles were injected with curing brine to 115% of initial weight using a multi-needle injector pump (Dick, F., Postfach 209, 73703 Esslingen, Germany) and tumbled for 10 min in every 30 min, at a temperature of 4 ± 1 °C for a total of 16 h at a speed of 10 rpm. The meat was vacuum packed and held at 4 ± 1 °C for 36 h to allow colour development. After that, the cured muscle were stuffed into boiling bags and closed under vacuum, were placed in moulds and heated in a water bath to a centre temperature of 72 ± 1 °C. After heat-treatment, cooked cured beef were cooled at 4 ± 1 °C for 6 h, sliced and vacuum 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 the CCMP from precooked cured beef

CCMP was extracted and isolated using a three-step gradational extraction procedure according to Killday et al. (1988) with modifications. Precooked cured beef was defatted by extracting for 28 h with petroleum ether flushed with a stream of nitrogen gas in darkness. After which, samples were extracted for CCMP using acetone and then ethyl acetate. 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. The finally isolated CCMP was dissolved in the acetone deoxygenated with nitrogen and transferred to a set of brown centrifuge tube with cap, the headspace gases were purged from each tube with nitrogen for ca. 30 s. Every tube was centrifuged at 3000 rpm for 5 min and the supernatant was used for analysis.

2.4. Preparation of oxidised CCMP

Discolouration in retail cooked cured meats is substantially a combining effect of light, oxygen, hydrogen peroxide from bacteria and display time (Efthimia et al., 2002; Hornsey, 1956). Therefore, different oxidation conditions were designed as follows: (a) Control (0 h), analysis was conducted just after CCMP extraction; (b) Air (48 h), briefly, 3 mL isolated CCMP was transferred to a 7 mL transparent centrifuge tube which was covered with an oxygen permeable polyethylene film (oxygen transmission rate > 1000 cm^3/m^2/atm/24 h), and then placed in air under natural light condition until the redness was entirely faded (ca. 48 h); (c) Air (10 d), the samples of group b were kept at the same condition for a further eight days till pigment was transformed into another stable state; (d) H_2O_2-treated: 1.5 ppm H_2O_2 (30%, 15 μL) was added to 3 mL isolated CCMP, and the mixture was shaken for ca. 30 s and then stood until the redness was entirely faded. The colour of CCMP was fully changed to bright yellow after about 5 min. All of the spectra measure was carried out at least two times on each sample to ensure the information of spectrum were correct and reliable.

2.5. Instruments and techniques

2.5.1. EPR spectra measurements

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; microwave power, 20 mW. The g factor was calculated using the formula:

$$g = h\upsilon/\beta B \quad (1)$$

where h is Planck's constant ($h = 6.62620 \times 10^{-27}$), υ is the spectrometer frequency, β is 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).

2.5.2. FT–IR analysis

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

2.5.3. Raman spectra analysis

The Raman spectra were recorded on a Labram HR800 Laser Raman spectrometer (Jobin Yvon Co., France). Experimental conditions were as follows: laser wavelength, 785 nm; Raman displacement, 700–2000 cm^{-1}; focusing lens, X50LMP; hole, 200 μm; accumulation time, 30 sec.

2.5.4. HPLC/ESI-HR-MS analysis

Extracted CCMP were separated by the HPLC system from Waters Co. (USA) consisted of a WATERS 996 detector and a Sunfire C-18 column (2.1 × 150 mm), and were detected at 400 nm. The mobile phase A consisted of 100% HPLC grade acetonitrile, whereas mobile phase B was 100% distilled water. Separation of CCMP was carried out for 30 min. The elution profile was a linear gradient elution with 20% solvent A from 0 to 15 min, 100% from 15 to 30 min. The HPLC temperature was at 30 °C, the flow rate was 0.3 mL/min, and the injection volume was 5 μL.

ESI-HR-MS analysis of the CCMP separated by HPLC was carried out using a LCQ DECA XP Plus (Thermo Finnigan Co., USA) equipped with an ESI ion source. The source voltage and capillary voltage were 5 KV and 32 V, respectively. The capillary temperature was set to 275 °C.

3. Results and discussion

3.1. EPR spectra analysis of extracted CCMP

The EPR spectra of extracted CCMP and its oxidised form are presented in Fig. 2 and the parameters of EPR spectra are shown in Table 1.

EPR spectra of extracted CCMP exhibited hyperfine splitting in the g_3 region from NO ligation with a_3 of 1.69 mT. Hyperfine coupling by the single nitrogenous ligand ($I = 1$) produced a signal split into a triplet of equal line intensities (Fig. 2(1) a). The EPR parameters of extracted CCMP possessed characteristics recognised as a pentacoordinate nitrosylheme system. According to Pegg et al. (1996) this ^{14}N hyperfine coupling provided evidence for placing the odd electron in a molecular orbital with substantial iron dz^2 character. The odd electron of NO became highly delocalised onto the iron atom. If a second NO coordinated to nitrosylprotoheme and the free electrons in the π^* orbitals of each NO molecule occupied separate orbitals (*i.e.* no pairing in a dz^2 orbital), the hyperfine structure at g_3 should reveal a quintet (for $I = 1$ and $n = 2$) with line intensities in the ratio 1:2:3:2:1. However, this was not observed in this study. In addition, the EPR spectra of extracted CCMP in acetone showed g_1, g_2, and g_3 values characteristic of a rhombic g tensor due to the anisotropic nature of the system.

The acetone solution of extracted CCMP turned yellow–brown in light and air after 48 h, turned green after being exposed for 10 days, and the solution turned bright yellow by the addition of 1.5 ppm H_2O_2 within 5 min (Table 1). It is the first time that the EPR spectra of extracted CCMP oxidised under different conditions was reported. The EPR spectra of extracted CCMP oxidised in air with normal lighting conditions in the laboratory also exhibited a triplet hyperfine splitting in the g_3 region from NO ligation (Fig. 2(1) b and c), but the profile of EPR spectra showed the location of g_1, g_2 and g_3 increasingly became closer. Fig. 2(1) b showed a small shoulder with hyperfine splitting of low intensity in the g_2 = ca. 2.04 region, whereas Fig. 2(1) c showed $g_{//} = 2.0675$, $g_{\perp} = 2.0136$, and g_2 nearly disappeared. Based on the results described above, it was concluded that the NO^- group might not dissociate from iron porphyrin of extracted CCMP, but that the conjugated system structure changed during oxidation in air with normal lighting conditions. Furthermore, the structure tended to axial symmetry of the molecule. This hypothesis would be further supported by the following HPLC/ESI-HR-MS spectrum analysis. The EPR spectra (Fig. 2(1) d) of extracted CCMP changed dramatically by addition of 1.5 ppm H_2O_2, and the hyperfine structure in the g_3 region from NO ligation were no longer observed. Loss of resolution of the hyperfine splitting in the EPR spectrum (Fig. 2(1) d) suggested that the NO group might interact with free radicals leaving the iron porphyrin in the system to quench free radicals in the medium, and subsequently the iron(II) was converted to iron(III) since it lost the protection of NO. And even the iron porphyrin of nitrosylheme complex was also broken down when the extracted CCMP was in the process of oxidation with H_2O_2. Further evidence was provided by the information from Fig. 2(2), in which a notable signal of Fe^{3+} in half magnetic field

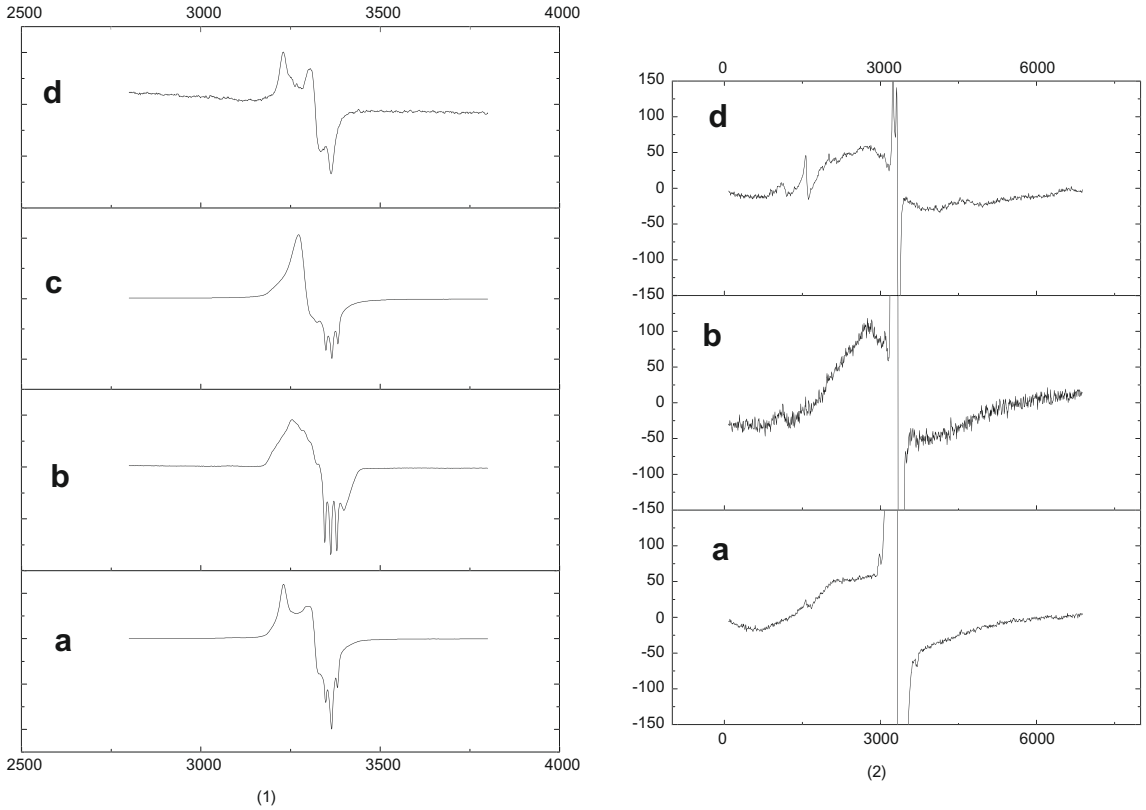

Fig. 2. EPR spectra with sweep width of (1) 1000 Gs and (2) 6800 Gs of extracted CCMP in acetone at 110 K. (a) Extracted CCMP in acetone; (b) extracted CCMP in acetone after standing for 48 h in air under natural lighting conditions in the laboratory; (c) extracted CCMP in acetone after standing for 10 days in air under natural lighting conditions in the laboratory; (d) extracted CCMP in acetone oxidised by the addition of 1.5 ppm H_2O_2.

Table 1
Parameters of EPR spectra of extracted CCMP in acetone at 110 K.

Samples	Colour of sample solution	g_1	g_2	g_3	a_2 (mT)	a_3 (mT)
1[a]	Bright red	2.0972	2.0398	2.0123	–	1.69
2[b]	Yellow-brown	2.0790	2.0435	2.0139	≈1.035	1.70
3[c]	Green	$g_{//}$ = 2.0675	–	$g_{\perp}$ = 2.0136	–	1.70
4[d]	Bright yellow	2.0975	2.0405	2.0151	–	–

[a] Extracted CCMP in acetone.
[b] Extracted CCMP in acetone after standing for 48 h under natural lighting conditions in the laboratory.
[c] Extracted CCMP in acetone after standing for 10 days under natural lighting conditions in the laboratory.
[d] Extracted CCMP in acetone oxidised by addition of 1.5 ppm H_2O_2.

(ca. 1600 Gs) was observed due to the electronictransition in EPR spectra of CCMP oxidised by the addition of H_2O_2 (Fig. 2(2) d), but not observed in EPR spectra of CCMP oxidised in air and light (Fig. 2(2) b).

3.2. HPLC/ESI-HR-MS results of CCMP and analysis

ESI-HR-MS spectrum of extracted CCMP is shown in Fig. 3a. It is the first time that HPLC/ESI-HR-MS was used to analyse the extracted CCMP. The extracted CCMP was eluted after 10.50 min in this experiment. The major fragments in the ESI-HR-MS spectrum (Fig. 3a) were identified according to related references (Killday et al., 1988; Wakamatsu, Nishimura, & Hattori, 2004) and assigned as follows: *m/z* 657.3 (100%), the molecular ion peak of iron porphyrin coordinated with an acetonitrile acting as a mobile phase; *m/z* 616.7 (85%), the molecular ion peak of iron protoporphyrin IX; and *m/z* 647.5 (28%), the molecular ion peak of iron porphyrin coordinated with a NO^- group. The peak pattern (cluster peak of *m/z* = 617.7, 618.5, 619.9, 621.9; *m/z* = 652.3, 654.7, 656.3, 657.3 and *m/z* = 657.3, 658.1, 659.9, 660.8) agreed well with that of Fe protoporphyrin because Fe has four isotopes (*m/z* = 54, 56, 57 and 58) and the isotopic ratio is characteristic. The molecular ion peak (*m/z* 677.5) of the dinitrosyl complex was not observed in this ESI-HR-MS spectrum. These results also indicated that the CCMP was a pentacoordinate nitrosylheme complex.

Fig. 3b and c showed the ESI-HR-MS spectrum of extracted CCMP oxidised in air and light for 48 h and 10 days, respectively. This indicated that the major molecular ion peak of oxidised CCMP with normal lighting possessed the same *m/z* as that of the fresh extracted CCMP, although the retention time of oxidised CCMP in air and light was different from that of the fresh extracted CCMP. These results provided further proof for the above-mentioned consequence that the structure of oxidised CCMP in air and light might

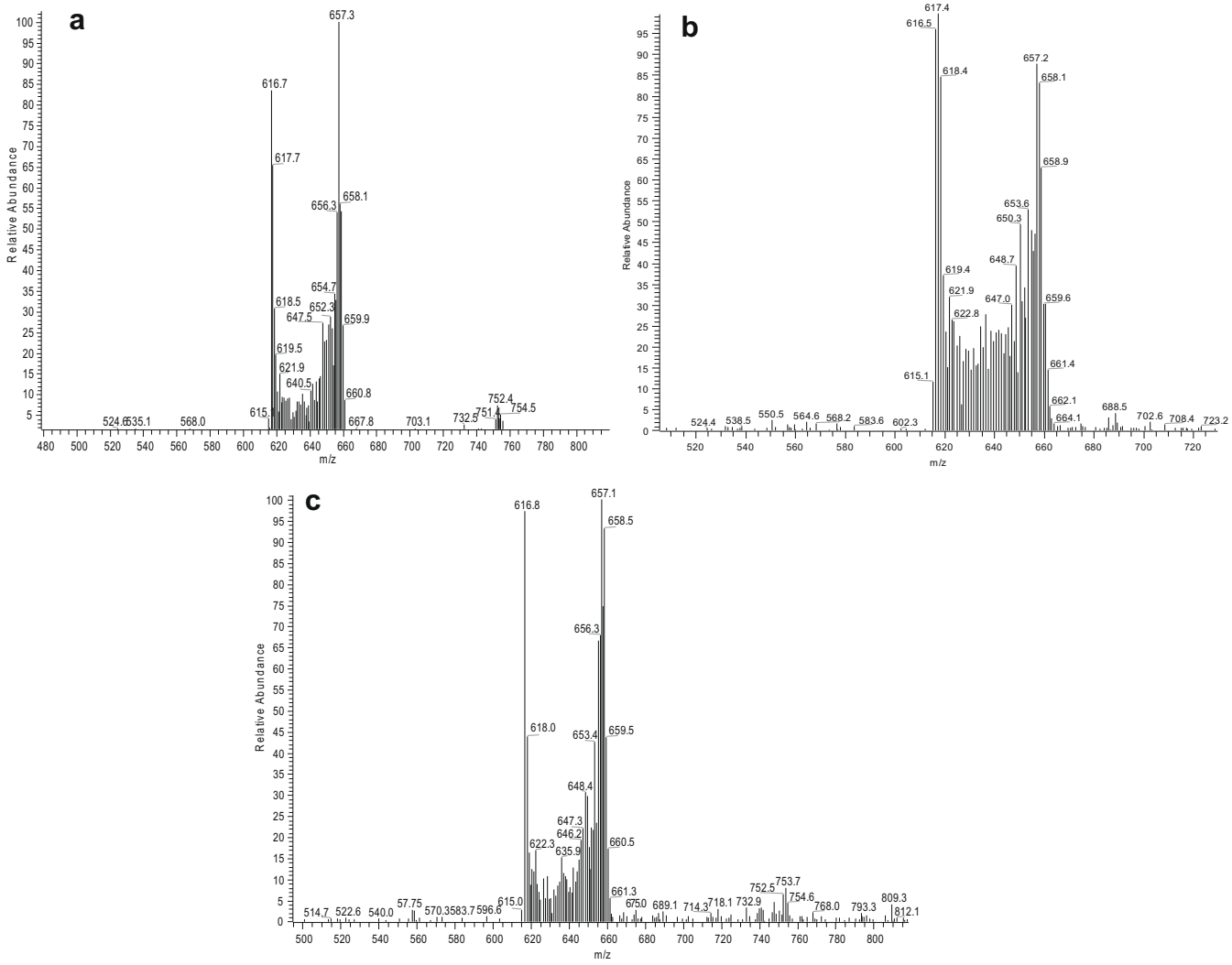

Fig. 3. ESI-HR-MS spectra of extracted CCMP. (a) Extracted CCMP in acetone; (b) extracted CCMP in acetone after standing for 48 h in air under natural lighting conditions in the laboratory; (c) extracted CCMP in acetone after standing for 10 days in air under natural lighting conditions in the laboratory.

be a changed conjugated system and the molecular weight was not changed compared with that of fresh extracted CCMP. The results disagree with those of Efthimia et al. (2002), who reported that light can catalyse the dissociation of nitric oxide from cured meat pigments and cause discolouration especially when oxygen is present. Trout (1991) also reported that the discolouration of cooked cured meat product was exhibited in the presence of light and oxygen, which was the result of metmyoglobin accumulation due to the CCMP oxidation. However, these reports did not analyse the change in structure of the pigment. The HPLC–MS spectra analysis was also carried out on the oxidised CCMP with H_2O_2. However, it is difficult to exactly identify fragments in absence of references. Therefore, its HPLC–MS spectrum was not shown and further studies are still needed.

3.3. FT–IR and Raman spectra analysis of extracted CCMP

The FT–IR and Raman spectra of extracted CCMP were shown in Figs. 4 and 5.

Fig. 4a showed that the FT–IR spectrum of extracted CCMP exhibited a band at v = 1653.31 cm^{-1} which corresponded to the presence of a bent Fe–NO moiety and a pentacoordinate complex. The result was similar to that previous reported, in which Pegg and Shahidi (1996) and Jankiewicz et al. (1994) reported the stretching frequency (v = 1659 cm^{-1}) of preformed CCMP was consistent with the first nitrosyl group bound with the ferrous atom of the haem molecule; Killday et al. (1988) reported that the infrared spectrum of extracted CCMP had a nitrosyl stretch at 1656 cm^{-1}, which was consistent with a bent NO^- ligand state; Maxwell and Caughey (1976) had reported that the stretching frequency of bent Fe–NO moiety of CCMP was in the range of 1600 cm^{-1}–1700 cm^{-1}. In addition, the stretching band at v = ca. 1900 cm^{-1}, which would indicate the presence of a second nitrosyl group in CCMP according to Killday et al. (1988), was not observed in this study (Fig. 4a).

Further evidence for a mononitrosylhaem complex as the pigment of cooked nitrite-cured meat can be seen from the Raman spectra, which was first reported in this study. The Raman spectra of extracted CCMP (Fig. 5a) also exhibited a band at v = 1656.69 cm^{-1} which corresponded to the first nitrosyl group bound with the ferrous atom of the haem molecule, and no stretching band corresponding to a second coordinated nitrosyl ligand were present in the 1900 cm^{-1} region.

Fig. 4b showed that the band at v = 1653.31 cm^{-1} in the FT–IR spectra of extracted CCMP disappeared after standing for 48 h in air under normal lighting conditions in the laboratory. The similar results had been reported by Killday et al. (1988). However, the

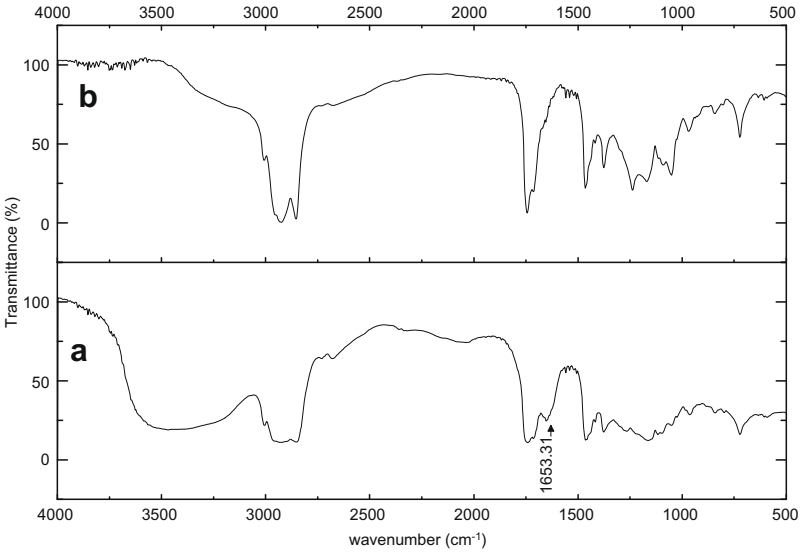

Fig. 4. Infrared spectra (IR) of extracted CCMP. (a) Extracted CCMP in acetone; (b) extracted CCMP in acetone after standing for 48 h in air under natural lighting conditions in the laboratory.

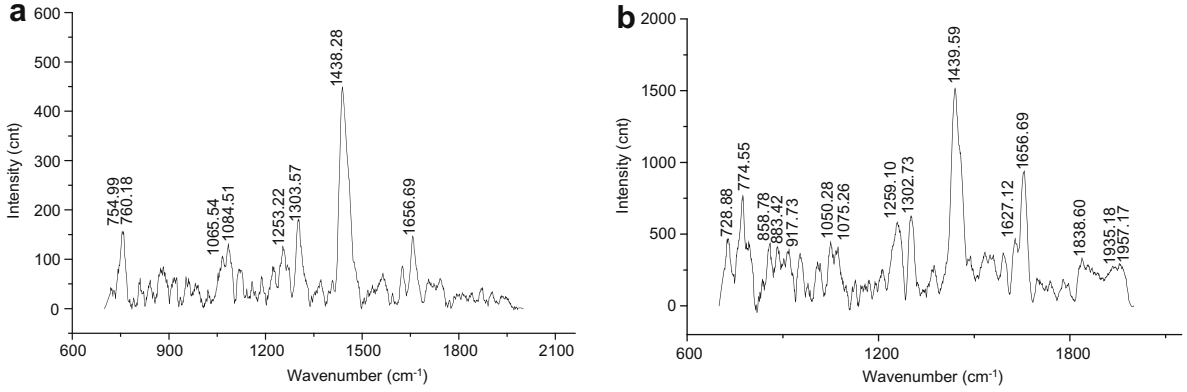

Fig. 5. Raman spectra of extracted CCMP. (a) Extracted CCMP acetone glass; (b) extracted CCMP acetone glass after standing for 48 h in air under natural lighting conditions in the laboratory.

infrared laser Raman spectra (Fig. 5b) of CCMP oxidised in air and light did not change at $\nu = 1656.69\ cm^{-1}$, which supplied evidence that the NO group might not detach itself from the haem moiety. The photochemical information come from IR and Raman spectra were complementary to each other. According to the above results, it would be enough to elucidate the mechanism of discolouration of CCMP in air and light. Therefore, IR and Raman spectra of the 10 days oxidised CCMP and H_2O_2-added CCMP were not analysed.

4. Conclusion

In conclusion, the structure of extracted CCMP was identified as monnitrosyiferrohemochrome (MNFH), which is a pentacoordinate complex. The changed spectra of oxidised CCMP under different conditions suggested that the NO^- group might not detach itself from iron porphyrin, but that the conjugated structure changed during oxidation in air and light. The dissociation of NO^- group as well as the decomposition of iron porphyrin of CCMP might occur only in the presence of a strong oxidant such as H_2O_2. In the absence of the knowledge of the precise structure of oxidised CCMP, preliminary studies should be carried out to establish photochemical characteristics of CCMP before and after oxidation. It is also of interest to analyse the specific structure of oxidised CCMP as well as the effect of pH and meat matrix on its change and obtain absolute proof to elucidate the mechanism of colour fading of cooked cured meat.

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