Investigation of nitrite alternatives for the color stabilization of heme–iron hydrolysates

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Abstract This study investigates the potential of novel heme–ligand complexes, derived from heme–iron isolated from porcine hemoglobin by enzymatic hydrolysis, to use as pigments for meat products. Five alternatives to sodium nitrite were identified as possible heme ligands and stabilizing agents of the red conformation of heme. The effects of 4-methylimidazole, methyl nicotinate, pyrrolidine, piperidine, pyrazine and sodium nitrite (as comparative benchmark) on the color of heme–iron extract and pure hemin standard were studied in solution. The ligand affinity and heme–ligand stability was assessed over time in solution by UV–Vis absorbance spectroscopy and CIELAB color space parameters. The CIE redness score a^* was used as a single measurement to propose a predictive model based on the following parameters: heme source (heme–iron extract or hemin standard), heme-to-ligand molar ratio (1:20 to 1:300), and storage time (up to 32 days). The optimal concentration at which each ligand can be added to either heme source, as well as the stability of the red color of the formed heme–ligand complexes in-solution was determined. Heme–iron extract-derived samples showed increased redness and color stability as compared to their hemin counterparts. No ligand showed as much affinity for heme as sodium nitrite. As the most promising ligand candidates, methyl nicotinate and 4-methylimidazole started to show color changes at a 1:50 molar ratio, but higher amounts (1:100 and 1:300, respectively) were required to

attain the maximum redness possible with the highest stability.

Keywords Heme · Sodium nitrite · Ligands · Color stability · Animal side-streams

Introduction

Porcine blood is a by-product generated in great volume in abattoirs, and is minimally used in side-stream products (Wismer-Pedersen 1988). Recent advances in blood collection and processing techniques, coupled with the environmental, nutritional and economic benefits obtained from a maximal utilization of animal blood have led to a myriad of blood ingredients (Ofori and Hsieh 2012). For example, blood plasma obtained after centrifugation of whole blood is used as a protein source in food and dietary supplements. The remaining cellular fraction, consisting mainly of hemoglobin, is underutilized due to instability of the color, as it tends to decay into an unwanted brown hue upon oxidation of the heme–iron. Recent approaches using enzymatic hydrolysis have permitted further processing of the blood cellular fraction, and allow for a separation of the globin moiety from the heme group (Robinson 2013; Toldrà et al. 2011). There is a good opportunity to add value to isolated heme–iron hydrolysate extract due to the role of the heme group in coloration of meat products.

Nitrates or nitrites are commonly added to meat products as curing agents, usually in the form of sodium nitrite; in addition to have a strong antimicrobial effect and to help controlling lipid oxidation, they impart the characteristic cured meat flavor and color to products (Martin 2012; Sindelar and Milkowski 2012). Color of meat products is dictated by the redox state of the heme–iron. Nitrite added

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to cured meat preparations binds and temporarily stabilizes the ferrous (Fe^{2+}) heme–iron and as such, causes the desirable red color of cured meat products (Faustman and Suman 2017; Honikel 2008). However, there have been concerns for several decades about the usage of nitrate and nitrite in meat products related to their role in the formation of carcinogenic nitrosamine compounds (Ruiz-Carrascal 2016; Santarelli et al. 2008). Despite the inherent health risks associated with microbial growth, especially *Clostridium botulinum*, the consumer demand for reduced nitrite or nitrite-free products is high. Use of other hurdles such as plant-based extracts, novel starter cultures or emerging technologies are typically used in such products (Alahakoon et al. 2015; Holck et al. 2017). One drawback of the elimination of nitrite in meat products is the color. As such, several attempts have been made to stabilize the color of hemoglobin in meat products using sodium nitrite alternatives (Fontes et al. 2004; Saguer et al. 2003; Salvador et al. 2009). Few studies used the isolated heme group, or any heme–iron hydrolysate as starting material, and most of those efforts were conducted with using nitrites as ligands (Shahidi et al. 1984, 1985; Zhou et al. 2014).

Ultraviolet–visible (UV–Vis) absorbance spectroscopy is the most practical instrumental analysis to assess the effectiveness of the tested ligands to form a stable, red heme–ligand complex. A characteristic UV–Vis spectrum of a heme–iron will exhibit two distinct regions of interest. One is a broad, intense peak around the 360–430 nm range, called the Soret band, while the other region displays weaker peaks, called Q-bands, spanning across 500–750 nm. Heme ligation causes spectral shifts in both of those regions due to conformational changes in the molecule (Giovannetti 2012; Nienhaus and Nienhaus 2005). Particularly, heme–iron samples exhibiting a desirable red hue will show more intense Q-bands in the 500–600 nm range. In order to assign a numerical score to the redness of the samples, the obtained UV–Vis spectra can be converted to the three-dimensional Lab color-space, also called CIELAB 1976 color space, as defined by the International Commission on Illumination (CIE 2004). From the spectral data in the visible range (380–780 nm), the redness index a^* can be calculated, along with the other color parameters L^* (lightness), b^* (yellowness), C^* (chroma) and h_{ab} (hue angle). The mathematical model to allow for such conversion is described in the aforementioned report (CIE 2004). Instruments to measure CIELAB values for solid or liquid samples are available and usually used in color studies (Cofrades et al. 2000; Fontes et al. 2010). Here, however, the inherent vulnerability to oxidation of most heme–ligand complexes, and associated color instability, did not allow for the usage of such instruments, even more so when the samples of interest are present in

aqueous solutions with disadvantageous surface-to-volume ratio.

In this study, we present novel heme–ligand complexes and evaluate their potential as red pigments for meat products through use of spectroscopic analyses and storage studies, in solution. We suggest that ligand groups such as imidazoles and N-heterocycles known to bind heme in hemoglobin (Akoyunoglou et al. 1963; Gallagher and Elliott 1967) bind as strongly with the isolated heme group. The potential of using heme–iron extract obtained by enzymatic hydrolysis as a pigment source in meat products is also discussed by comparing with a commercially available heme source. Using predictive models, this study demonstrates, for each heme ligand candidate, the optimal concentration at which they can be added to different heme sources, as well as the color stability of the heme–ligand complex stored in aqueous solution. Additionally, we evaluate the feasibility for a single, good measurement of redness obtained from UV–Vis absorbance spectra.

Materials and methods

Materials

Frozen porcine red blood cell fraction was obtained from Danish Crown Ingredients, and collected from their slaughterhouse situated in Ringsted, Denmark. The enzyme Savinase[®] was provided by Novozymes, Denmark. Hemin, sodium hydroxide (NaOH), sodium ascorbate, sodium nitrite (SN), 4-methylimidazole (4-MeI), methyl nicotinate (MeN), pyrrolidine (PyrI), piperidine (PipD) and pyrazine (PyrZ) were purchased from Sigma-Aldrich. All chemicals were analytical grade commercial products.

Preparation of starting material

Heme–iron extract was isolated from the blood source after enzymatic hydrolysis, subsequently freeze-dried, and stored until experiments were performed. The frozen red blood cell fraction was first thawed, and then hemolyzed by adding water at a 2:1 ratio and thorough mixing. The enzymatic hydrolysis was carried out by adding the endo-protease Savinase[®] (Novozymes, Denmark) to the hemolyzed red blood cells at a 0.5% w/v content. The temperature and pH were adjusted to the optimal conditions for the enzyme (65 °C, pH at 8.5, adjusted with NaOH). The reaction was allowed to proceed for 3 h, and subsequent separation of the heme extract was achieved by lowering the mixture pH to 4 with 2 M sulfuric acid. Under low pH, isolated heme precipitated along with insoluble peptides. The mixture was centrifuged for 10 min at 5000 g and the near-colorless supernatant was discarded.

The obtained extract was recovered, freeze-dried and subsequently stored in the dark, at 5 °C. Heme content was determined by re-solubilizing the freeze-dried extract in 20 mM NaOH, and reading the absorbance at 384 nm and comparing with a standard curve for commercially-obtained, pure heme dissolved in 20 mM NaOH (Shaklai et al. 1985).

Formation of the heme–ligand complexes

Formation of heme–ligand complexes was performed following a modified protocol from Shahidi et al. (1984). Solubilized heme extract and heme in 20 mM NaOH were diluted to a heme concentration of 0.1 mg/mL before being bubbled under nitrogen gas in order to remove dissolved oxygen. Sodium ascorbate was added to a 1:100 heme-to-ascorbate molar ratio. 1800 µL of the two heme solutions were aliquoted in 2 mL-tubes. Different solutions of the six ligands tested, SN, MeN, 4-MeI, PyrI, PipD and PyrZ, were prepared in water. The concentrations were adjusted such that 200 µL of the solutions could be added to each heme aliquot to obtain mixtures with a heme-to-ligand molar ratio of 1:0 (control), 1:20, 1:50, 1:100, 1:200, and 1:300, in triplicates. The test tubes were vortexed, and the ligation process was allowed to proceed in the absence of oxygen and light.

Absorption spectra and conversion to CIELAB values

Absorption spectra in the range 350 to 780 nm were recorded on a SpectraMax i3x multiplate reader (5 nm step intervals) (Molecular Device, UK) at the following time-points: 3 h, 1, 2, 8, 15 days and when possible, 32 days after preparation. For each heme–ligand complex, data collection was halted when no perceived redness could be further observed. Samples were taken from the closed test tubes and measured within 5 min of being pipetted into the multiplate. The color coordinates L^* (lightness), a^* (redness), and b^* (yellowness) and color parameters C^* (chroma) and h_{ab} (hue angle) were derived from the spectra generated according to standard guidelines. The investigated spectral range was chosen to include the two distinct regions characteristic for the heme–iron UV–Vis absorption spectrum. Calculations were carried out with the illuminant D65 as a reference.

The conversion of the UV–Vis spectral data to the CIE 1976 ($L^*a^*b^*$) values was done according to the guidelines published by the International Commission on Illumination (CIE 2004). The coordinates and parameters are defined within the three-dimensional color space as such:

$$L^* = 116 \cdot (Y/Y_n)^{1/3} - 16$$

$$a^* = 500 \cdot \left[(X/X_n)^{1/3} - (Y/Y_n)^{1/3} \right]$$

$$b^* = 200 \cdot \left[(Y/Y_n)^{1/3} - (Z/Z_n)^{1/3} \right]$$

$$C^* = (a^{*2} + b^{*2})^{1/2}$$

$$h_{ab} = \arctan(b^* / a^*)$$

where X , Y and Z are the tristimulus values for the measured sample, and X_n , Y_n and Z_n are the tristimulus values for a standard white object. These are given by the following equations:

$$X = \sum_{\lambda=380}^{780} (\% \tau \cdot D65 \cdot x_{10})_{\lambda} \quad \text{and} \quad X_n = \sum_{\lambda=380}^{780} (D65 \cdot x_{10})_{\lambda}$$

$$Y = \sum_{\lambda=380}^{780} (\% \tau \cdot D65 \cdot y_{10})_{\lambda} \quad \text{and} \quad Y_n = \sum_{\lambda=380}^{780} (D65 \cdot y_{10})_{\lambda}$$

$$Z = \sum_{\lambda=380}^{780} (\% \tau \cdot D65 \cdot z_{10})_{\lambda} \quad \text{and} \quad Z_n = \sum_{\lambda=380}^{780} (D65 \cdot z_{10})_{\lambda}$$

where $\% \tau$ is the transmittance at each obtained wavelength after conversion from absorbance values, D65 is the power distributions for the standard illuminant of the same name, and xyz_{10} corresponds to the color-matching function. The tristimulus values X , Y , Z and X_n , Y_n , Z_n are given as single value, which is the sum of the expressions calculated for the wavelength $\lambda = 380$ to 780 nm, taken at a 5 nm intervals. The values for D65 and xyz_{10} are standardized for such wavelengths and given in the CIE guideline (CIE 2004).

Data analyses

To illustrate the effectiveness of each tested ligand candidate, the redness index a^* from the CIELAB color space was used for fitting individual statistical models to each ligand, for all heme sources, heme-to-ligand molar ratio and storage periods. Statistical analyses were done with JMP® software (v13.0.0), from SAS Institute Inc. (USA).

Results and discussion

Absorbance spectra

Like all porphyrin-type compounds, the highly conjugated π -electron system of the heme–iron molecular structure dictates its color and intensity. When changes in the symmetry and conjugation pathway of the porphyrin arise, the perceived color can be affected as well; these changes

are easily detectable by a UV–Vis absorption spectrum (Gouterman 1961). Sodium nitrite alternative candidates as color stabilizing compounds were thus assessed via the recording of their absorbance spectra in the UV–Vis region. To measure effectiveness in terms of intensity and stability, spectra were recorded for different heme-to-ligand molar ratio added to either standard hemin or heme–iron extract, over different periods of storage.

The full UV–Vis spectra (350–780 nm) for each heme–ligand complex are given in Fig. 1, along with the spectra of the heme–iron extract. Those spectra were recorded for the same heme source (extract), storage timepoint (1 day), and heme-to-ligand molar ratio (1:100), excluding the blank for the heme source. The peak shifts for both the Soret and the Q-bands are visible for each ligand. It can be seen that for the same reaction conditions, different ligands show different absorbance intensities and maxima shape. The flattening of the maxima in the Q-bands region is associated with a loss of the desirable red color and a lower redness index a^* value, while a peak increase would signify a stronger perceived red hue (Ahn and Maurer 1990; Hornsey 1956).

Redness index a^* and individual ligands

A global visual representation of the calculated redness index a^* for all samples at all timepoints is shown in Fig. 2. The heme–4-MeI complex was the only pigment candidate to exhibit an intense red color after more than 15 days of storage in sealed Eppendorf tubes. PyrZ was not accounted for on the 15th day of storage, since no heme–pyrazine complex showed any red color after 7 days. Data collection for the other heme–ligand complexes was halted

after 15 days. Samples exhibiting the largest a^* values almost exclusively originate from the heme–iron extract; such samples would correlate with a more intense and more stable red color. This result is coherent with previous studies stating that porcine blood hydrolysates typically have strong reducing power (Chang et al. 2007; Liu et al. 2010). The presence of reductants in the extract would allow for an increased stabilization of the Fe^{2+} conformation upon ligation of the heme, as well as an improvement of the oxidation equilibrium points towards the reduced, red state of the ferrous heme–iron, explaining the more intense color and enhanced chromatic stability in heme–iron extract samples.

The reducing effect of the heme–extract on the redness intensity and stability of the samples is particularly visible in the case of 4-MeI, MeN and PipD, for which the a^* values of the higher heme-to-ligand ratios are markedly higher than the hemin standard equivalent. As well, three of the ligands tested (MeN, PyrI and PipD) did not give any red coloration when mixed with the pure hemin standard, but yielded some satisfactory coloration using the heme source derived from porcine blood. Another general trend that can be observed is the time needed for the reaction to yield the maximum redness index which was generally high, albeit variable: two of the ligands, MeN and PyrI, reached their maximum redness index within the first reading (3 h after mixing heme source and ligands), while it took 24 h or more for the other ligands. The reaction speed could be modulated by varying the amount of sodium ascorbate added to the heme source prior to adding the ligand candidates (not shown). Additionally, the addition of reductant is the reason behind the increase in the a^* value for all blank samples (1:0 molar ratio). As the central heme iron gets reduced to its ferrous form, the heme solution gets redder, if only temporarily. With no ligands to coordinate with the central iron and stabilize its ferrous form, the solution oxidizes to a duller, brown color (Faustman and Suman 2017; Mancini and Hunt 2005). Thus for some ligands tested, and within the first two days of the ligation reaction (see MeN and PipD in heme standard solution), the blank sample looks redder than its heme–ligand complex counterpart. The poor repeatability between the blank samples is due to the unfavorable surface-to-volume ratio of the 96-wells multiplate system, combined to the measurement time required and the high sensitivity to oxidation of the ligand-less reduced heme.

It is important to note that at lower a^* value, looking solely at the redness index, becomes increasingly less accurate as other dominant colors take over. In those cases, a general overview taking into account the other color coordinates would be needed to properly assess the perceived hue of the samples. However, and in this particular case, at relatively stable L^* and b^* values (12 and 16%

Fig. 1 Ultraviolet–visual absorption spectra of all heme–ligand complexes tested (heme source: heme–iron extract/molar ratio: 1:100/storage period: 1 day), and heme–iron extract

Fig. 2 Redness index a^* evolution over time, for all ligands at all molar ratio, using both heme sources (standard = heme standard; extract = heme extract from hemoglobin)

coefficient variations across all samples, respectively, opposed to 94% variation in the a^* value), a subjective cutoff value for perceived visual redness could be made at $a^* = 10$. Over this threshold, the red hue becomes dominant enough to serve as a single value to evaluate relative redness across samples of the same ligand.

Predictive models

The heme–ligation reaction for each ligand candidate is described in more detail by modelling the redness index a^*

response against the different experimental factors (heme source, heme-to-ligand molar ratio, storage time, replicates; all treated as categorical variables) via a linear additive statistical model. For all ligands the starting point was a full model that included all main effects and all first order interactions (e.g. the interaction of molar ratio and storage time). The models for all ligands showed that all main effects and interactions to be highly significant ($p < 0.0001$), except for the effect of replicates. The linear model for SN is shown in Table 1 as an example. It can be seen that as a consequence of treating the experimental

Table 1 *F*-test report summarizing the effect and significance of the different parameters tested for SN

Effects tests (<i>F</i> -test)					
Source	<i>DF</i>	Sum of squares	Mean square	<i>F</i> ratio	Prob > <i>F</i>
Heme source	1	174.20	174.20	202.67	< 0.0001
Molar ratio	5	522.59	104.52	121.60	< 0.0001
Time (d)	3	2311.87	770.62	896.57	< 0.0001
[Source] × [Molar ratio]	5	56.46	11.29	13.14	< 0.0001
[Source] × [Time (d)]	3	229.72	76.57	89.09	< 0.0001
[Molar ratio] × [Time]	15	93.38	6.22	7.25	< 0.0001
Error	108	92.83	0.860		
C. Total	140	3501.47		< 0.0001	

factors as categorical variables, the number of individual parameters to test becomes appreciably high. However, treating the variables as categorical rather than numerical was necessary as each ligand shows its own effect on concentration and time.

Rather than writing out all the parameter estimates of the models for each ligand, an interaction profiler is used to illustrate the effects. In essence, the interaction profilers are realizations of the prediction models with all combinations of inputs. For the purpose of having the redness index a^* as a single measurement for redness, individual models for each ligand were preferred over one global statistical model. This approach was favored since the conversion method for the calculated a^* value heavily depends on the baseline value of the full UV–Vis spectra, which differs from ligand to ligand as well as between different molar ratios. In other words, the spectral shifts induced by the ligands changes the a^* values for each ligand so a^* cannot be directly compared across ligands. Hence, while obtained a^* values give a good indication for the presence or absence of perceived redness, evaluating this lone value is impractical to compare one ligand to another. Even so, the calculated redness indexes for all ligand would be correlated together, but this single parameter will not give a complete color overview of the different samples, since any color change affects all CIELAB coordinates (Hernández et al. 2016). In such case, when the redness a^* is not enough to explain a color change, the other color coordinates and parameters are needed to complete the information. The overview for the interaction profilers can be seen in Fig. 3, where each line of charts refers to a single heme–ligand complex, and each column corresponds to a specific parameter interaction. The aforementioned reducing effect of the heme–iron extract is clearly visible in the columns A and B.

Sodium nitrite data corresponds to Fig. 3, line 1. An important aspect of its effectiveness as a heme ligand is the lower heme-to-ligand molar ratio required to induce a red hue. Here, a ratio of 1:20 is sufficient to reach the maximum a^* value attainable by the heme–SN complex. This is

consistent with the previous work from Shahidi et al. (1984), which reported the production of a heme–SN pigment at a 1:13 ratio. It is worth noting that while adding high concentrations of ligand does not influence the redness, color stability in solution is slightly increased in higher heme-to-ligand molar ratio (1:200 and 1:300). Also, using SN as a ligand seems to mitigate the reducing power of the heme–iron extract, as the response difference between the two heme source is less pronounced compared to the other ligands, particularly 4-MeI and MeN.

In addition to having a markedly stronger reaction when using heme–iron extract rather than pure hemin standard, 4-MeI (Fig. 3, line 2) exhibits a steep increase between from the 1:20 until the effects plateau, at 1:100 molar ratio. Hence, the most important variable for 4-MeI seems to be the heme-to-ligand ratio. Interestingly, when combined with heme extract, this ligand candidate was the only ligand which redness index did not decay under the speculated boundary of $a^* = 10$ for acceptable color, even after an appreciably longer storage time period. As such, and although some degradation could be recorded, 4-MeI was the only ligand candidate for which the storage experiment could be spanned over 32 days, as opposed to the other tested ligands which red color all completely faded away by the 15th day of storage.

The MeN-containing samples (Fig. 3, line 3) behaved similarly to 4-MeI: a marked redness increase with a increasing heme-to-ligand molar ratio, and a more efficient ligation reaction when added to heme–iron extract rather than a pure standard. However, where 4-MeI reached a plateau at 1:100 molar ratio, the highest a^* value for MeN was attained at a 1:300 molar ratio. Additionally, the pure hemin standard did not yield any red coloration when conjugated with MeN. It is surprising, as MeN exhibit a pyridine ring as the most probably ligation target for heme and was expected to be effective. Since pyridine is a well-known heme ligand, and the reaction of pyridine hemochromes is typically used in heme concentration assays (Barr and Guo 2015; Berry and Trumpower 1987), it would be expected to react with hemin standard. Nonetheless,

Fig. 3 Interaction profiler for all heme–ligand complexes. Lines 1–6 are for, in order, SN, 4-MeI, MeN, PyrI, PipD and PyrZ. Different columns correspond to different interaction parameters (A = $a^* \times$ molar ratio $\times$ heme source; B = $a^* \times$ time (d) $\times$ heme source; C = $a^* \times$ time (d) $\times$ molar ratio). All statistically significant except for 4A and 6C

Fig. 4 UV-Vis spectra for PyrZ (heme source: heme standard; storage period: 3 h). Maxima increases with the higher heme-to-ligand molar ratio

redness scores for this particular heme–ligand complex were satisfactory, even more so when factoring in the relative high stability of the compound. At 14 days of storage, high molar ratio (1:200 and 1:300) samples could retain some redness and stay above the a^* cutoff value.

The ligand PyrI (Fig. 3, line 4) yielded no notable redness in presence of ferrous heme–iron. The maximum a^* index value attained was slightly higher from its basic value, with little to no amount of ligand added. Its counterpart PipD (Fig. 3, line 5), performed noticeably better, again with a stronger reaction when coupled with heme–iron extract, but no noticeable redness effect while using hemin standard. However, the piperidine–heme complex needed a higher amount of ligand to exhibit redness. A heme-to-ligand molar ratio of 1:300 was required to achieve satisfactory color; even so, the compound showed

relatively low stability in solution, and the desirable color faded after 24 h of storage period.

The ligand PyrZ (Fig. 3, line 6) demonstrated some of the limitations aforementioned about the spectra mathematical conversion to CIELAB values. In that case, solely looking at the a^* index value is insufficient to properly judge the redness of the heme–ligand compound, as demonstrated by the relatively stable redness index at all molar ratio. Paradoxically, when looking at the UV–Vis spectra for the hemin standard-based complex, a maxima increase in the region of interest 500–600 nm can be witnessed, even with the redness a^* values being stable (Fig. 4). Only at 1:20 molar ratio was the pure hemin exhibiting noticeably lower maxima in the 500–600 nm spectral region. This discrepancy between the observed spectra and the calculated redness index a^* illustrates the sensitivity of the mathematical model towards baseline shifts, which are not necessarily color-relevant. This sensitivity is due to the method of conversion, which calculates the color coordinates $L^*a^*b^*$ from the tristimulus values XYZ summed across the visible region of the spectra. Hence, a detectable absorption band can be offset by a baseline shift of roughly the same magnitude, as seen in the example of the heme-pyrazine compound (Fig. 4).

As such, for PyrZ, the other CIELAB color attributes are needed to better describe the color. In addition to the two other color coordinates, L^* (lightness) and b^* (yellowness), the chroma C^* , corresponding to the vividness of a color (higher the chroma, more vivid the color) and hue angle h_{ab} (for redness, when $0 \leq h_{ab} \leq 90^\circ$, the smaller the hue angle the more red the color) can be calculated following the CIE guidelines, as previously described (CIE 2004; Hernández et al. 2016). As seen in Table 2, the computed values show what could be expected from a ligated ferrous heme, corresponding to a darker, duller, redder color with increased heme-to-ligand molar ratio, at the same storage

Table 2 CIELAB color coordinates for PyrZ, at storage time = 1 day

Molar ratio	Source	L^*	a^*	b^*	C^*	h_{ab} ($^\circ$)
1:0	Hemin	59.4 ± 0.5	10.0 ± 0.9	40.6 ± 0.2	41.9 ± 0.4	76.2 ± 1.2
	HiE	n/a	n/a	n/a	n/a	n/a
1:20	Hemin	55.8 ± 0.9	10.7 ± 1.0	37.3 ± 0.8	38.8 ± 1.0	74.0 ± 1.1
	HiE	50.1 ± 0.2	12.3 ± 0.9	41.6 ± 0.6	43.4 ± 0.7	73.5 ± 1.0
1:50	Hemin	51.7 ± 0.6	10.5 ± 1.1	32.9 ± 1.6	34.6 ± 1.8	72.4 ± 1.0
	HiE	51.5 ± 0.3	12.3 ± 0.1	39.0 ± 0.3	40.8 ± 0.3	72.9 ± 1.0
1:100	Hemin	49.8 ± 1.4	11.4 ± 0.3	31.7 ± 1.8	33.6 ± 1.7	70.3 ± 0.7
	HiE	50.3 ± 1.4	12.0 ± 0.9	36.2 ± 1.1	38.3 ± 1.3	71.0 ± 0.1
1:200	Hemin	48.3 ± 0.5	11.0 ± 0.1	29.9 ± 0.6	31.9 ± 0.6	69.7 ± 0.6
	HiE	51.9 ± 0.6	12.5 ± 0.2	31.2 ± 0.5	33.4 ± 0.5	69.0 ± 0.2
1:300	Hemin	46.3 ± 0.8	10.9 ± 0.1	27.4 ± 0.6	29.5 ± 0.6	68.4 ± 1.1
	HiE	52.9 ± 1.2	11.5 ± 0.7	28.4 ± 0.3	30.6 ± 0.2	67.9 ± 1.3

All values are in units $\pm$ SD. HiE: Heme–iron extract

time. The difference between the values corresponding to the two ligand sources is also minimal for all color parameters, which makes pyrazine stands out as compared to the other nitrite alternative candidates, since most ligands reacted less strongly with the pure heme source. This could be explained by a spectral change between the different heme sources, which heme-PyrZ is the only complex to display amongst the other pigment candidates. In the Q-bands region, the two peaks present are exhibiting a shift of about 10 nm; an additional peak starting at 670 nm and continuing into the infrared region (> 800 nm) is also present when the heme–iron extract is used as a heme source. This peak was previously reported as being the result of the formation of polymeric heme structures linked by PyrZ, as its symmetric and aromatic structure allows for (Bartocci et al. 1987). The authors reported that the formed structure had a high sensitivity to oxidation, which would explain the relatively poor stability exhibited by the heme-PyrZ complex in the present study. Despite this shortcoming, with a perceived redness at a relatively low heme-to-ligand molar ratio of 1:50, PyrZ may prove a promising nitrite alternative candidate.

Conclusion

The ligand effectiveness was demonstrated and analyzed for the six compounds: sodium nitrite, 4-methylimidazole, methyl nicotinate, pyrrolidine, piperidine and pyrazine. While tested in-solution, 4-MeI and MeN exhibited a more intense color and a greater stability than the comparative benchmark SN, and showed promise to be used as nitrite alternatives as coloring agent in meat products. In addition, it was shown that the use of heme–iron extract isolated from porcine blood by enzymatic hydrolysis is generally more efficient at stabilizing a desirable red hue than pure heme, probably due to the presence of reducing capacity of the hydrolysates in the extract. The two notable exceptions to this discrepancy are SN and PyrZ, which leaves doubts as to whether the tested ligands would react similarly in a meat matrix. Additionally, the minimum heme-to-ligand molar ratio required to obtain a suitable red color is higher for all tested compounds than for SN, with only PyrZ as an exception.

Due to the CIELAB conversion method's sensitivity towards spectral changes, and the fact that the ligands do indeed shift the ultraviolet–visible absorption spectra, makes direct comparison of redness between different ligands impossible. This highlights that these a^* values should be interpreted with care and may indicate the need for another method for objectively assessing redness in aqueous solutions, especially for weaker red hue. Complementing the single value a^* with the other color

coordinates obtained through the same conversion method provides a more reliable overview in the case of baseline shifts and weaker redness.

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