Stability of nitrosylmyoglobin in presence of low oxygen concentrations estimated by constrained bi-linear curve resolution

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The colour of cured meat products is known to fade quickly when exposed to light even in presence of low oxygen concentrations (Rikert et al., 1957; Andersen et al., 1988). This problem has been recognised for different types of cured meat products packed in vacuum or modified atmospheres (MAP) during retail display. Common to both packaging principles is that low residual levels of oxygen cannot be avoided. At present the meat industry and retailers in Denmark are aware of the problem, and precautions are taken by dark storage of packed cured meat products for a few days prior to light exposure in the supermarkets. This storage period is not well controlled and undesirable from a number of viewpoints: Storage capacity, logistic of transportation and keeping quality of the product.

Consequently, there is need for more detailed knowledge about the stability of the primary pigment in cured meats, nitrosylmyoglobin (MbNO), under conditions resembling MAP. Preliminary experiments have proved that MbNO becomes more susceptible to photodegradation with increasing partial pressure of oxygen (Andersen & Skibsted, 1992).

Moreover, studies of the light stability of MbNO have until now only employed a limited number of spectral regions to evaluate the rate of degradation. Methods that incorporate all spectral data ($450 < \lambda < 800$ nm) will be applied when assessing the kinetics of MbNO degradation under various experimental conditions. Bi-linear curve resolution of the data will be applied as a mean to assess the stability of MbNO flushed with different gas mixtures. Deriving *spectral profiles* and corresponding *time profiles* can capture the systematic variations of data in a few factors. Interpreting such factors that represent the whole array of observations rather than local and discrete observations, the qualitative and quantitative information outcome gain in stability. Bi-linear curve resolution schemes based on least squares optimisation have lately been extended with a multitude of constraints (e.g., de Juan 1997). Although early curve resolution techniques not based on least squares optimisation have been proposed, the least square approach is preferred due to the precision with which the goal function under optimisation can be defined (Lawton, 1971; Karjalainen, 1991).

Objectives:
The objective is to develop a method making it possible to follow kinetics of MbNO degradation at very low oxygen concentrations. In addition, the applicability of bi-linear curve resolution is assessed when evaluating the stability of MbNO submitted to different conditions relevant for MAP of cured meat products.

Methods:
Synthesis of MbNO: MbNO was synthesised from purified equine metmyoglobin (MMb), $NaNO_2$ and ascorbic acid in an array of three Zwickel flasks according to the method described by Andersen & Skibsted (1992). Subsequently, excess $NaNO_2$ and ascorbic acid were removed by eluation with 5 mM phosphate buffer (pH 7.0, I = 0.16 M) on a PD-10 column held at 10°C. The purified MbNO was identified by its visible absorption spectra and used for kinetic experiments within the day of synthesis.

Flushing of MbNO with gas mixtures: The isolated MbNO in buffer was transferred to a single Zwickel flask and flushed with various gas mixtures. The flushing was performed with gas cooled in a spiral submerged in ice water. MbNO was flushed for 5 minutes with a flow of 100 ml per minute. The flushed MbNO was hereafter purged into a quartz cuvette (3 ml) through a glass transition connecting the outlet of the Zwickel flask to the cuvette. This glass transition had two vents that when closed secured a completely sealed closure of the cuvette. These vents were closed following transfer of MbNO, and the glass transition was left in the cuvette during recording of spectral data.

Spectrophotometric measurements of thermal oxidation of MbNO: Visible spectra ($450 < \lambda < 800$) are recorded with a HP 8452A (Hewlett-Packard, Palo Alto, CA, USA) diode array spectrophotometer at 25.0°C. The time intervals between data recording varied from 2 to 15 minutes, and measurements proceeded 3-15 hours depending on the gas mixtures (with/without oxygen) investigated.

Bi-linear curve resolution: Since we expect the presence of two absorbing species, two sets of spectral profiles and time profiles have been estimated using a non-negativity constrained alternating least squares algorithm implemented in MATLAB 5.2 running on an DELL Optiplex GxPro under MS Windows NT 4.0. For each scenario (flushed with oxygen or atmospheric air) a number of 77 absorbance spectra representing 192 wavelengths were measured at an interval of two minutes.

Results and Discussions:
Measurements without oxygen present: Kinetic measurements of MbNO flushed with pure gasses (N_2 or CO_2) or mixtures hereof (20%/80%, 40%/60% and vice versa) show no changes in spectral data over time indicating a complete exclusion of atmospheric air and thereby oxygen. These findings are further supported by principal component analysis (PCA) of the spectral data using time intervals as samples and recorded wavelengths as variables. In PCA of data from these measurements the first principal component (PC) describes 100% of the variance within the data set. This indicates the presence of only one component in the system, hence no degradation of MbNO occurs. Inspections of the first PC's profile show a high resemblance to the absorption spectrum of native MbNO.

Measurements with oxygen present: Data from kinetic experiments in which MbNO were flushed with atmospheric air or pure oxygen are analysed via a non-negativity constrained bi-linear model based on alternating least squares regression. Figure 1a depicts the resolved temporal profiles and Figure 1b depicts the resolved normalised underlying spectral profiles. It is important to notice that the estimated spectral profile for MbNO is identical to its absorption spectrum, whereas the estimated spectral profile for MMb on the other hand shows

limited resemblance to the absorption spectrum of pure MMb. The profiles in Figure 1a expresses the relative contents of the two components, MbNO and MMb. The relative concentration of MbNO decreases from approx. 8.8 to 4.4, while the concentration of MMb increases from 0 to 3.0 during the recording of spectral data. It is important to note that both sets of profiles are derived from all observations, rather than only a few discrete ones. Correspondingly, in the scenario with pure oxygen the relative concentration of MbNO decreases from approx. 8.5 to 3.8 in the same time interval, whereas the concentration of the formed MMb increases to 3.5 (Figure 2a). To summarise, the thermal oxidation of MbNO over the same period of time is slightly higher for the scenario where pure oxygen is used. This is verified by the slightly higher relative concentration of the formed MMb. In previous findings a slightly higher rate constant (k_{obs}) is established for thermal oxidation of MbNO in presence of pure oxygen compared to atmospheric air (Andersen & Skibsted, 1992). The temporal profiles for the two experiments indicate a more extensive degradation of MbNO when pure oxygen is used during flushing, whereas the residual level of MbNO is higher in the system flushed with atmospheric air. This can be explained by differences in the ratio of [O2]:[MbNO] in the two systems.

Conclusion:

The application of non-negativity constrained bi-linear curve resolution can prove very useful when judging the rate of degradation and mechanistic aspects of MbNO/MMb chemistry in model systems resembling MAP systems. Major advantage are i) the ease at which the significant variations in the data can be presented and discussed and ii) the increased stability of the data on which kinetic analysis is performed, due to the better estimates of the time profiles which exploit the full spectral information. In future investigations handling of data collected from an experimental design will apply multi-linear models for curve resolution. In light of the success of applying non-negativity constraints, a future approach will be based on linear constraints applied to the time profiles and non-negativity spectral profiles for the curve resolution model. The method will also be applied to search for peroxynitrite as a reaction intermediate.

Pertinent literature:

Andersen; H.J.; G. Bertelsen; L. Boegh-Soerensen; C.K. Shek; L.H. Skibsted (1988). Effect of light and packaging conditions on the colour stability of sliced ham. Meat Science **22**, 283-292

Andersen, H.J. & L.H. Skibsted (1992). Kinetics and mechanism of thermal oxidation and photooxidation of nitrosylmyoglobin in aqueous solution. J. Agric. Food Chem. **40**, 1741-1750

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Karjalainen, E. J. (1991). Component reconstruction in the primary space of spectra and concentrations. Alternating regression and related direct methods. Analytica Chimica Acta **250**, 169-179

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Rikert, J.A.; L. Bressler; C.O. Ball; E.F. Stier (1957). Factors affecting quality of prepacked meat. Food Technology 11, 625-632

Data:

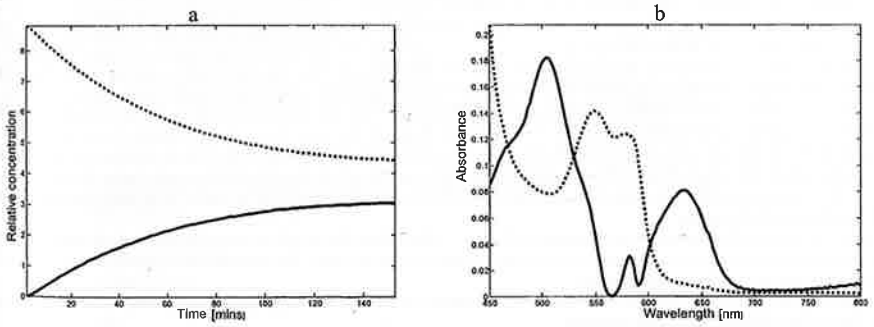

Figure 1. Profiles from bi-linear curve resolution describing the degradation of MbNO in atmospheric air. a) Time profiles describing the concentration of MbNO (dotted line) and MMb (solid line), and b) Absorbance spectra of pure MbNO (dotted line) and MMb (solid line) estimated from the model.

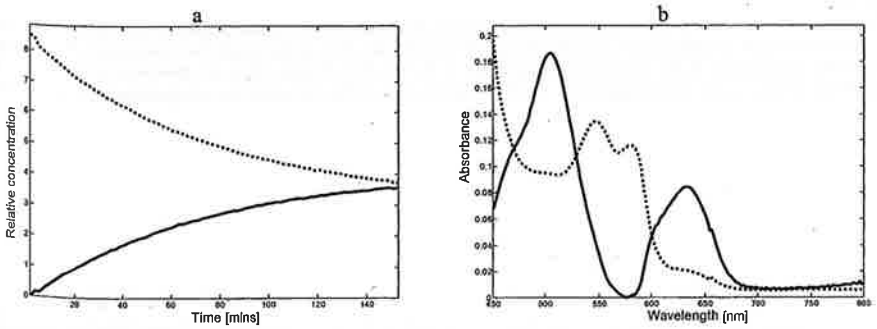

Figure 2. Profiles from bi-linear curve resolution describing the degradation of MbNO in pure oxygen. a) Time profiles describing the concentration of MbNO (dotted line) and MMb (solid line), and b) Absorbance spectra of pure MbNO (dotted line) and MMb (solid line) estimated from the model.