Comparison between two different methods to obtain the proportions of myoglobin redox forms on fresh meat from reflectance measurements

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Abstract Two procedures based on reflectance (R) measurements to calculate the proportions of deoxymyoglobin (DMb), oxymyoglobin (OMb) and metmyoglobin (MMb) in meat are recommended by the American Meat Science Association (AMSA). One uses the K/S ratios (K and S are the absorption and scattering coefficients) and the other method (Krzywicki 1979) uses the reflex attenuance $A = \log(1/R)$. Both methods were compared in: a) synthetic sets of two pigment mixtures and b) 15 samples of beef Longissimus Lumborum measured after 24 h, 4 and 7 days of exposure to air. It was found that K/S and Krzywicki methods gave different values of pigment proportions. However both methods exhibited a high linear correlation ($R^2 = 0.8733$ in DMb, $R^2 = 0.9771$ in OMb and $R^2 = 0.9390$ in MMb, $p < 0.0001$). This makes them equivalent for statistical analysis based on differences in pigment proportions.

Keywords Beef · Myoglobin states · Pigment proportions · Reflectance · Kubelka-Munk theory · Krzywicki method

Highlights

- We compare the AMSA methods to calculate myoglobin proportions from reflectance.
- K/S and Krzywicki methods give different absolute values of pigment proportions.
- Both methods give highly correlated values of pigment proportions.
- Both methods are equivalent for statistical analysis based on pigment differences.

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Introduction

Myoglobin is the pigment that imparts color to meat, a color that depends on the myoglobin oxidation state on the surface. It is a water-soluble protein that contains iron in its structure. In fresh meat it is principally found in three basic states: deoxymyoglobin (DMb), oxymyoglobin (OMb) and metmyoglobin (MMb) (Mancini and Hunt 2005). The characteristic colors of these three myoglobin forms are a consequence of their distinctive reflectance spectra. In meat surface these three states are in a dynamic equilibrium driven by the oxygen availability and a number of other biochemical factors. During oxygenation the changing proportions of the three myoglobin forms follow a certain depth profile, several mm in depth, as oxygen proceeds through the sample affecting the surface reflectance (Sáenz et al. 2008). In the absence of oxygen, deoxymyoglobin is the dominant state imparting a purplish-red color typically associated with vacuum packaged meat or with fresh pieces immediately after cutting. When meat is exposed to oxygen, oxymyoglobin is formed and accompanied by the development of a bright cherry red color. Color deterioration in meat surface is due to the appearance of metmyoglobin, the myoglobin oxidized state. As the proportion of metmyoglobin increases meat color turns to brown, this induces a negative response in consumers and when the proportion of MMb reaches 20 % it has been found that the product is rejected by half of the potential consumers (Hood and Riordan 1973; Renerre and Mazuel 1985).

Since knowing the proportions of these pigments on the meat surface is very important, having a reliable method to obtain such proportions becomes a crucial point. The procedure to evaluate the proportion of each myoglobin form has been the subject of a long standing debate. Based on available research results, the AMSA (American Meat Science Association) published in 1991 a set of guidelines in

this respect (AMSA 1991). Recently a revised, second edition of the “Meat Color Measurement Guidelines” has been published (AMSA 2012). These guidelines describe two different methods to calculate the proportions of myoglobin redox forms. Both methods are based on reflectance measurements on a meat surface using a set of wavelengths that are found to be particularly important to describe each pigment and the relationship among them. In the first method, the surface reflectance at four isobestic wavelengths (474, 525, 572 and 610 nm) is used to calculate the K/S ratios (K is the absorption coefficient and S the scattering coefficient) that are further related with pigment proportions. The second method, transforms the reflectance values at four wavelengths (473, 525, 572 and 730 nm) to reflex attenuation, the logarithm of the reciprocal of reflectance (Shibata 1966), which is then used to compute the proportion of each pigment (Krzywicki 1979).

The method based on the calculation of K/S ratios from direct reflectance measurements on meat surface can be traced back to the Kubelka-Munk theory developed to explain the behavior of light on tinted or pigmented artificial samples (Kubelka and Munk 1931). This theory is widely used in paint and textile industries (Francis and Clydesdale 1975; Wysecky and Stiles 2000). In the case of meat samples, the method considers the three redox states of myoglobin as three different pigments. The K/S ratios obtained from a given sample are compared with the K/S ratios obtained from samples where the pigment was found only in one of the redox forms. These K/S ratios obtained for “purely” DMb, OMb and MMb samples serve as reference values. Determining these reference values is a delicate issue and the AMSA guidelines establish the recommended procedures to obtain them.

The second method (Krzywicki 1979) also makes use of reflectance values at a particular set of wavelengths. From these reflectance values the so called reflex attenuation (A) is calculated as the logarithm of the inverse of the reflectance, $A = \log(1/R)$, with $0 \leq R \leq 1$. The main hypothesis of the method is that myoglobin forms do not contribute to the reflectance, and thus to the reflex attenuation, at 730 nm. Therefore this value can be regarded as a reference for each particular sample. In this method the measurement of reference samples having 100 % of each of the three pigments is not required.

The main objective of this work is to compare both methods and analyze the relationship between them. Two different but complementary perspectives have been used. In the first one the three myoglobin states: DMb, OMb and MMb are used to construct synthetic reflectance data with carefully controlled pigment content in the framework of standard Kubelka-Munk theory. In the second approach, a set of reflectance spectra measured real meat samples corresponding to a variety of oxygenation times, and therefore a variety in pigment proportions, is used.

Materials and methods

Meat samples

Meat samples were obtained from the veal Longissimus Lumborum (LL) muscle of 15 different animals in a local slaughterhouse (Pamplona, Spain). All animals were male then slaughtered when they were about 12 months old. They had an average left side carcass weight of 132 ± 19 kg and pH values 24 h post mortem between 5.6 and 5.7. The Longissimus Lumborum muscles were removed from the left carcass side 24 h post mortem. Meat samples were sliced into steaks about 2 cm thick. Steaks were individually placed in plastic foam trays overwrapped with oxygen permeable film and stored at 4 °C in the dark. Measurements were made directly on the meat surface without the film.

Reflectance measurements

A Minolta CM-2002 Spectrophotometer, d/8 (diffuse illumination / 8° viewing angle) geometry, and 8 mm diameter circular aperture, was used to record the meat spectra from 400 to 700 nm, at 10 nm intervals. The methods used in this work require the values of the reflectance at wavelengths not directly provided by the instrument. According to the AMSA guidelines these values were obtained by linear interpolation of the measured reflectance values at the two nearest wavelengths (AMSA 1991). In order to avoid pillowing effects and to prevent dirtying the instrument's internal integrating sphere, all measurements were made through a protecting glass cover supplied with the spectrophotometer (cover set CM-A40). Calibration was performed with the glass, using a white calibration plate (CM-A21) and a light trap (Zero Calibration Box CM-A32). Each measurement was the average of five different readings in five non-overlapping zones of each steak, changing the instrument orientation each time to reduce the effect of possible inhomogeneities such as fat and blood. Measurements were performed on each sample after 1, 4 and 7 days of exposure to air.

Calculation of the percentage of myoglobin forms

Quantifying the proportion of myoglobin forms from K/S values (method M1)

In order to apply the K/S method to calculate the proportions of each myoglobin form in a meat sample we need the reference values obtained from samples having all its myoglobin content in one of the redox forms. They were obtained according to the procedures described in AMSA 2012, section IX.

Samples with 100 % of MMb in its surface were created by chemical induction with potassium ferricyanide (section IX, B1a). Samples with 100 % of DMb were obtained making a

fresh cut surface on the sample's interior and taking a reflectance measurement immediately after cutting (section IX, B2b). In this case, and according to the guidelines, a single reading was taken on each sample to prevent the influence of the OMb that forms once the fresh cut is exposed to air. Finally samples with 100 % of OMb were obtained by rapid oxygenation in enriched oxygen atmosphere (section IX, B3a).

The proportion of each pigment form in a particular sample is calculated from the K/S values at the isobestic points, i.e. selected wavelengths where two or more pigments have the same reflectance value. The K/S value is obtained from the reflectance values as $K/S = (1-R)^2/2R$ (with R given in the range 0–1). Once we have these values we compute the proportion (in %) of each pigment using the following relations:

$$\%MMb = \frac{\frac{K/S \ 572}{K/S \ 525} \text{ for } 100\%DMb - \frac{K/S \ 572}{K/S \ 525} \text{ for sample}}{\frac{K/S \ 572}{K/S \ 525} \text{ for } 100\%DMb - \frac{K/S \ 572}{K/S \ 525} \text{ for } 100\%MMb} \times 100 \quad (1)$$

$$\%DMb = \frac{\frac{K/S \ 474}{K/S \ 525} \text{ for } 100\%OMb - \frac{K/S \ 474}{K/S \ 525} \text{ for sample}}{\frac{K/S \ 474}{K/S \ 525} \text{ for } 100\%OMb - \frac{K/S \ 474}{K/S \ 525} \text{ for } 100\%DMb} \times 100 \quad (2)$$

Similarly %OMb could be calculated using an analogous expression using the K/S ratio at 610 nm. However it can be also obtained as 100 minus the proportion of the two other pigments (Mancini et al. 2003) i.e.:

$$\%OMb = 100 - (\%MMb + \%DMb) \quad (3)$$

This is also the equation used in the Krzywicki method. Since our main objective is to compare both methods, Eq. (3) has been used to compute %OMb. In the rest of the work this method of calculating the proportions of myoglobin forms from the K/S values will be referred as method 1 or M1.

Quantifying myoglobin forms from reflex attenuation (method M2)

This method was proposed by Krzywicki (1979) and does not require the determination of the reflectance curves of pure pigments. For a given sample the proportions of each pigment are obtained from the reflectance values at specific wavelengths by means of the so called reflex attenuation. This term was proposed by Shibata (Shibata 1966) to distinguish between attenuation of light due to both absorption and diffusion and the attenuation caused by light absorption by transparent materials (absorbance) only. The reflex attenuation (A) is defined as $A = \log(1/R)$ and according

to Krzywicki can be used to compute the proportions of each pigment using the relations:

$$\%MMb = \left[1.395 - \left(\frac{A_{572} - A_{730}}{A_{525} - A_{730}} \right) \right] \times 100 \quad (4)$$

$$\%DMb = \left[2.375 \times \left(1 - \frac{A_{473} - A_{730}}{A_{525} - A_{730}} \right) \right] \times 100 \quad (5)$$

$$\%OMb = 100 - (\%MMb + \%DMb) \quad (6)$$

A practical common difficulty with this method is that 730 nm is slightly beyond the range of common spectrophotometers that usually measure up to 700 nm. In this case the AMSA (2012) suggest using the reflectance at 700 nm instead. In this work we will follow this recommendation and use the reflectance values at 700 nm.

In the rest of the work we will refer to this method of calculating the proportions of myoglobin forms from the values of the reflex attenuation as method 2 or M2.

Construction of synthetic reflectance spectra corresponding to samples of known pigment proportions

The Kubelka-Munk theory provides a framework to obtain the reflectance of a complex sample composed by a set of pigments on a substrate from the reflectance of the individual pigments and the values of their concentrations (proportions) on the substrate (Kubelka and Munk 1931). For opaque samples the ratio of the light absorption coefficient (K) to the light scattering coefficient (S), is proportional to the concentration of colorant in the sample. Since the K/S factors for each colorant at a given wavelength are additive, the Kubelka-Munk principle provides a basis for computing the amounts of each colorant necessary to match a given standard (Francis and Clydesdale 1975). This can be used to construct synthetic or artificial reflectance curves corresponding to samples with known proportions of each pigment. It is only required the reflectance data of pure pigments from which the K/S values are calculated. The synthetic reflectance is constructed in K/S space by a weighted addition of K/S values of pure pigments according to:

$$(K/S)_{\text{mixture}} = a(K/S)_{DMb} + b(K/S)_{OMb} + c(K/S)_{MMb} \quad (7)$$

Where a, b and c are respectively the proportions of DMb, OMb and MMb expressed in the 0–1 range. Since proportions are relative values, the coefficients a, b and c can be taken, without loss of generality, to verify:

$$a + b + c = 1 \quad (8)$$

This simply says that, taken in %, the proportions of the three pigments add to 100 %. Once K/S values have been

calculated, the reflectance is obtained as (Wysecky and Stiles 2000):

$$R_{mixture} = 1 + \left(\frac{K}{S} \right)_{mixture} + \left(\frac{K^2}{S^2} + 2 \frac{K}{S} \right)_{mixture}^{1/2} \quad (9)$$

This expression gives the reflectance R expressed in the 0–1 range. The synthetic reflectance spectrum of any desired sample, with known pigment proportions, can be then constructed by fixing appropriate values for the coefficients a , b and c .

Following this procedure two sets of synthetic spectra have been constructed. The first set (synthetic set 1) corresponds to mixtures of only DMb and OMb. This set simulates the oxygenation process in freshly cut meat. In normal conditions oxygenation is a relatively fast process and therefore this set represents the evolution of meat reflectance during the first hours of exposure to air, when the proportion of MMb is still negligible.

The second set (synthetic set 2) corresponds to mixtures of only OMb and MMb. This set simulates the progressive deterioration of meat due to oxidation and the development of MMb. In this process DMb accounts for a very small fraction of the total pigment content and its presence remains undetected to the naked eye (Seideman et al. 1984). This process is slower, taking several days or even weeks, and never reaches a point of complete oxidation because meat is rejected well before MMb reaches 100 % due to the great deterioration of the product.

These two sets approximately represent the principal stages of the evolution of meat during exposure to air that can be described with good approximation with the contribution of only two myoglobin forms, thus simplifying the analysis and interpretation of the results.

In each set there are 21 reflectance spectra. The proportion of one pigment varies from 0 to 100 % at 5 % increments through the set. The proportions of the second pigments are 100 % minus the proportion of the first one. Notice that in each synthetic set the reflectance spectrum corresponding to 100 % of one of the pigments is not a “synthetic” spectrum but an actually measured spectrum corresponding to one of the reference curves of pure myoglobin forms.

Statistical analysis

Statistical analysis including curve fitting and regression, correlation, ANOVA and t-Student tests have been performed with Minitab (Minitab Inc.) statistical software.

Results and discussion

Reference curves of pure myoglobin redox forms

The reflectance spectra of the three myoglobin forms, obtained as described before, are shown in Fig. 1. As recommended by

Fig. 1 Reflectance spectra of the three myoglobin forms: Deoxymyoglobin (DMb, black solid line); Oxymyoglobin (OMb, red short-dashed line) and Metmyoglobin (MMb, blue long-dashed line) obtained according to AMSA 2012 recommendations

AMSA, curves have been normalized to have the same value at 525 nm, an isobestic point for all the pigments. This required only a minute scaling of the original data.

Obtained reference curves can be qualitatively compared with those published by AMSA. The AMSA 1991 reference curves are extracted from Snyder (1965). This author measured the reference curves on beef, but he used a different muscle (round steak) instead of the Longissimus Lumborum used in this work. The AMSA 2012 curves on the other hand are not referenced and no indication about the muscle or type of meat is given. In fact it can be seen by simple inspection that the example curves published in the 1991 and 2012 guidelines are different in absolute value, with the 2012 curves having an obviously greater reflectance. Nonetheless both share the same spectral shapes, in particular the characteristic peaks and depressions as well as the location of the isobestic points, also published by Strange et al. (1974).

Qualitatively our curves in Fig. 1 are close to AMSA curves, being the absolute numerical values similar to the 1991 curve set, surely related with sample similarities. In particular, the isobestic points are located at the same wavelengths and the curves show the same distinctive spectral features characteristic of each pigment. These spectral features are very useful to evaluate the pureness of each reference curve.

Regarding the OMb spectrum, it has a characteristic peak between 530 and 590 nm that is a good indicator of the presence of this pigment. It can be seen in Fig. 1 that this peak is totally absent in the DMb curve, indicating that there is little contamination of OMb, if any. The MMb spectrum has a characteristic depression, a small valley, around 630 nm. This depression is not visible neither in the OMb or DMb data. It can be then affirmed that the DMb reference curve has no significant traces of OMb or MMb and faithfully represents a 100 % DMb sample. It can be also said that the OMb reference curve has no significant contribution of MMb and that OMb is the major contribution to its reflectance spectra although it

could have some contribution of DMb. Similarly the MMb reference curve mainly corresponds to MMb but small contribution of OMb and DMb cannot be completely discarded.

Comparison between method 1 and method 2 using synthetic set 1: DMb-OMb mixtures

The results of applying methods M1 and M2 to calculate the proportions of DMb and OMb in synthetic set 1 are graphically summarized in Fig. 2. While method M1 gives values almost identical to the theoretical values, as expected by construction, values given by method M2 greatly differ. These differences are as great as 51 units (in the 0–100 % scale) in case of OMb (36 on the average) and as great as 40 in case of DMb (26 on the average).

Consider for instance the sample that represents a fully oxygenated meat surface, having 100 % of OMb and 0 % of DMb. This sample is the reference curve of OMb and it is an actually measured sample, shown in Fig. 1. In Fig. 2 it corresponds to the rightmost point in the OMb data and to the leftmost point in the DMb data. In this sample method M1 gives 100 % of OMb and 0 % of DMb as expected. Method M2 on the other hand, estimates a mixture of 49 % of OMb, 39.7 % of DMb and 11.3 % of MMb. However, the well developed characteristic features of oxmyoglobin in the spectrum (Fig. 1) make difficult to accept that there is a remaining 40 % of DMb and a none negligible amount of MMb (11.3 %), taking into account that all samples had normal pH. Although no MMb was introduced in these synthetic samples, method M2 gives a slightly varying, positive proportion of MMb in all samples, going from 8.7 to 11.3 % while M1 always gives 0 % of MMb as expected by construction.

Let us now consider the sample having 100 % of DMb, i.e. the DMb reference curve. In Fig. 2 this sample corresponds to the leftmost point in the OMb data and to the rightmost point in the DMb data. Again method M1 gives almost exactly 100 % of DMb and 0 % of OMb and MMb. When we apply method M2 to this sample we obtain more than 100 % of DMb

(111.6 %), a negative proportion of OMb (−20.3 %) and a non negative value of MMb (8.7 %). The problem of proportions outside the 0–100 % range has been noticed by several authors (Aporta et al. 1996; Beriain et al. 2009). Mancini et al. (2003) recommend transforming the results to fit them in the 0–100 % range, an approach followed by some authors (Li et al. 2012). Although not necessary for statistical tests, it provides a more easy evaluation of the state of conservation of a meat sample. We agree with this suggestion in the sense that, for instance, one readily interprets a 0 % MMb as a meat showing a healthy aspect but on the other hand one feels somewhat uncomfortable interpreting a negative value. Even if we correct for out of range values in M2, results are incoherent with those given by M1. Method M2 gives negative values for OMb for all samples in synthetic set 1 having less or equal than 30 % of OMb by construction.

It is noticeable that even though M1 and M2 give different absolute values, both methods exhibit a perfect mutual linear correlation with $R^2=1.000$ ($p<0.0001$) for both OMb and DMb proportions. Notice that here corrections of out of range values affect M2 only and, if applied, the linear relationship would have been partially masked.

Comparison between method 1 and method 2 in the synthetic set 2: OMb-MMb mixtures

The proportions of OMb and MMb obtained by method M2 versus the corresponding values obtained by method M1 have been plotted in Fig. 3. Like in the previous case, method M1 applied to any of these samples gives proportions that are close to the theoretical ones. Method M2 on the contrary gives values that are not in agreement with M1 with differences that are as great as 51 units (27 on average) in case of OMb and as great as 32 (13 on average) in case of MMb.

The case of a sample having 100 % OMb (0 % MMb) has been already discussed in the previous section. For the theoretically 100 % oxidized sample (MMb reference curve) method M2 gives only 68.3 % of MMb, a small 2.5 % of OMb but a

Fig. 2 Pigment proportions given by method M1 and M2 in synthetic set 1 for DMb (squares) and OMb (triangles). Linear regression fits are shown as solid lines

Fig. 3 Pigment proportions given by method M1 and M2 in synthetic set 2 for MMb (circles) and OMb (triangles). Linear (dashed lines) and quadratic (solid lines) fits are also shown

Fig. 4 Average reflectance spectra of all measured samples after 24 h (solid gray line), 4 days (dashed line) and 7 days (solid black line) of exposure to air

quite large 29.2 % of remnant DMb. As the proportion of OMb decreases and the proportion of MMb increases method M2 gives a slowly decaying, non negligible amount of DMb that goes from 39.7 % in the 100 % OMb sample to 29.2 % in case of the 100 % MMb sample.

Although absolute values given by M1 and M2 greatly differ, Fig. 3 shows an evident relationship between them. While the relationship is close to a linear one, the influence of a small quadratic term can be seen for both pigments. In case of OMb proportions, a linear fit of method M2 values versus method M1 has $R^2=0.9860$ while a quadratic fit has $R^2=0.9999$. In the case of MMb proportions the linear fit has $R^2=0.9910$ while a quadratic fit has $R^2=0.9999$. All fitting models are significant at $p<0.0001$.

Comparison between method M1 and method M2 in real meat samples 1, 4 and 7 days of exposure to air

Figure 4 shows the average reflectance spectra of all samples at each measurement time. It can be seen that after 24 h of exposure to air the characteristic peaks of OMb in the central region of the spectra are already visible and no depression is visible in the 610–650 nm region, meaning that MMb is absent. After 4 days of exposure to air the MMb depression can be identified and after 7 days it is clearly marked. Similar behavior can be seen in other works although in a different kind of samples (Mancini et al. 2005). This increase in the

proportion of MMb is related with the deterioration process (Insausti et al. 1999).

Sample average over measurement days can be used to analyze M1 and M2 methods respect to the importance of the correction of out of range values carried out in their application (Table 1). The results obtained with method 1 after applying the correction of out of range values are shown as M1*. Since measurements at different dates have been performed on the same samples a pair wise t-Student test has been performed to analyze statistical differences between pigment content averages for each method and measurement day.

As shown in Table 1, in case of M1 and for the three measurement times %DMb is negative when one should expect vanishing concentrations of DMb. MMb is (slightly) negative at 24 h, again in a situation in which the contribution of MMb is expected to be small. When out of range values are corrected (M1*) the values of the proportions of each pigment form are in accordance with the values we would expect according to the times of exposure to air. OMb steadily decreases as MMb increases from 24 h to 7d while DMb content is negligible.

Respect to method M2, within the range of pigment proportions in our sample set Krzywicki method does not give out of range values. However, the proportions given by method M2, as seen in the table, are in conflict with what it is expected during the oxygenation/oxidation processes through the studied period of exposure to air. DMb remains almost constant with a small variation from 38.5 % at 24 h to 32.7 % at 7 days. Similarly OMb proportions change very little, from 50.9 to 47.5 %, and finally MMb has a doubtfully high value at 24 h. None of these values appear to be correct. Krzywicki himself (Krzywicki 1979) shows as an example that normal pH beef, 30 min after being cut, has no DMb and a proportion of MMb ranging from 14 to 38 %. The situation resembles what it was found in the synthetic sets, although M2 makes no use of reference curves or reference values of any kind and these results are independent of the results obtained in the previous sections.

It is noticeable that after 7 days of exposure to air the MMb proportion is 18.0 % according to M1 and 19.8 % according to M2. These values are close to the 20 % of MMb that has been

Table 1 Average proportions of myoglobin forms (in %) given by methods M1, M1* and M2 after 24 h, 4 and 7 days of exposure to air

Method	24 h			4d			7d		
	DMb	OMb	MMb	DMb	OMb	MMb	DMb	OMb	MMb
M1	−1.4 ^a	105.4 ^a	−4.0 ^a	−5.7 ^a	100.9 ^{a,b}	4.8 ^b	−13.4 ^b	95.4 ^b	18.0 ^c
M1*	2.2 ^a	95.4 ^a	2.4 ^a	3.1 ^a	90.5 ^a	6.4 ^a	0.6 ^a	81.0 ^b	18.4 ^c
M2	38.5 ^a	50.9 ^a	10.6 ^a	37.1 ^a	49.3 ^{a,b}	13.6 ^b	32.7 ^b	47.5 ^b	19.8 ^c

For each pigment and method, means at different measurement days having the same letters are not significantly different ($p>0.05$)

used as a cut off for consumer rejection (Hood and Riordan 1973; Renerre and Mazuel 1985). However we cannot be sure that this concordance is casual or it can be considered an expected behavior in any situation. On the other hand, both methods M1 and M2 exhibit exactly the same ability to detect significant changes in pigment content during the measurement days, suggesting that they are equivalent to trace pigment changes despite the differences in absolute values.

The results obtained here are similar to those obtained with method M2 in a variety of applications, with different types of meat and regarding different treatments. These works usually focus in changes or differences in the proportions of a particular pigment but sometimes the absolute values of those proportions are also given (Gatellier et al. 2001; Li et al. 2011; Realini et al. 2013).

Pigment proportions given by M1 and M2 in individual samples at each measurement time are shown in Figs. 5, 6 and 7. As we expect from the previous analysis, there is a clear disagreement in absolute values between both methods. However, as with synthetic sets, the linear or quasi linear relationship between them is still visible, not hidden by the natural statistical dispersion in values associated to the samples. In each figure the least squares linear fit has been also plotted. It can be seen that the linear relationship is a good approximation to the relationship between both methods. The coefficients of determination are $R^2=0.8733$ for DMb, $R^2=0.9771$ for OMb and $R^2=0.9390$ for MMb. In all cases they are highly significant ($p<0.0001$). The effect of correcting out of range values can be seen here, affecting only M1. If we plot the results of corrected method M1 (M1*), then samples with negative proportions would clutter at 0 % in the horizontal axis only, keeping their values in method M2, the vertical axis. This would reduce the values of the coefficients of determination to $R^2=0.65$ for DMb, $R^2=0.61$ for OMb and $R^2=0.87$ for MMb, clearly showing how correcting out of range values hides the linear relationship between the equation sets used in both methods.

Fig. 5 Deoxymyoglobin (DMb) proportions given by methods M1 and M2 after 24 h (triangles), 4 days (circles) and 7 days (squares) of exposure to air. Linear regression fit is shown as solid line

Fig. 6 Oxymyoglobin (OMb) proportions given by methods M1 and M2 after 24 h (triangles), 4 days (circles) and 7 days (squares) of exposure to air. Linear regression fit is shown as solid line

Conclusions

Both studied methods have been compared from different points of view and it has been shown that they gave pigment proportions that greatly differ in absolute value. Such large differences cannot be regarded as arising from experimental uncertainties or statistical artifacts. At least one method is giving incorrect pigment proportions.

In the K/S method the necessity of fixing the reference reflectance curves of pure pigments provides an opportunity to check the qualitative and quantitative characteristics of the reflectance curves of each myoglobin form. The distinctive spectral signatures of each myoglobin form help us to be confident on the successful determination of these reference curves. The need of reference curves is not a weakness of the method, on the contrary is one of its principal strengths. It provides adaptation to a particular type of meat or muscle because the reference curves are obtained for that type of sample.

Actually, it is difficult to understand a method that does not need any kind of calibration. Variations in total pigment content, fiber structure, pH and other factors are expected to influence the details of the whole process of determination of

Fig. 7 Metmyoglobin (MMb) proportions given by methods M1 and M2 after 24 h (triangles), 4 days (circles) and 7 days (squares) of exposure to air. Linear regression fit is shown as solid line

each pigment proportion. In this sense the Krzywicki method has no possibility to adapt itself to the characteristics of a particular meat product. This fact may be an important factor in the observed mismatch between both methods. From different perspectives, our comparison indicates that Krzywicki method provides pigment proportions that are not consistent with the expected values and we are forced to admit that it fails in giving the correct absolute values of these proportions.

Nonetheless, despite the mismatch between the absolute values of pigment proportions, both methods appear to be highly correlated. This is an important fact, since frequently one is not concerned with the determination of absolute values of pigment proportions but with the changes or differences in these proportions caused by some treatment or procedure applied on the samples. From the perspective of having a parameter that is able to track such changes or differences, the Krzywicki method retains a similar ability than the K/S method. Although the relationship between both methods is not exactly linear, differences do not appear to be large enough to pose a fundamental objection and any method could be used in practice. In particular any of these methods can be used as an indicator of the progressive deterioration of meat. This is interesting in the sense that Krzywicki method does not require the delicate procedure for the precise determination of the reflectance curves of pure pigments, making it easier to implement. In this respect, those works using the K/S method could easily incorporate the results of the Krzywicki method and provide further evidence on this practical equivalence, extending it to other muscles or types of meat. Furthermore, in such applications there is little need to correct out of range values, since we can regard the proportions not as true proportions but as parameters correlated with such proportions. It has been shown that this correction could even mask the desired correlation with true proportions.

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