A rapid method for measuring pigment concentration in porcine and other low pigmented muscles.

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SUMMARY: In this paper a rapid method for measuring total pigment concentration which is applicable to porcine and other low pigmented muscles is described. The method involves: 1) Extraction of total pigments by homogenising the muscle in 0.04M pH 6.5 phosphate buffer [sample to buffer ratio 1:10]; 2) Filtration through No 1 Whatman filter paper; 3) Clarification of the filtered extract by addition of 10% Triton X100 [final concentration 2.5%]; 4) Oxidation of the pigment with 65mM sodium nitrite [final concentration 1.2mM); and 5) Measuring absorbance of the oxidised pigments at 409 nm [corrected for turbidity, measured at 730nm]. This method when used with porcine muscle had the same accuracy and precision as the Hornsey, cyanomet and $absorbance_{525}$ methods but was much more rapid and did not involve the use of toxic and/or flammable reagents. Additionally, the method was more accurate than the alkaline haematin method.

The greater accuracy and precision obtained with this method in muscles with low pigment levels is due to the fact that the absorbance is measured in the Soret band where the absorbance is 5-10 times greater than at the wavelengths used with other methods.

INTRODUCTION: Meat colour is one of the most important characteristics consumers use when selecting and purchasing meat. Colour is determined, to varying extents, by the pigment concentration in the meat. Consequently, methods for measuring pigment concentration are important in the evaluation of meat quality.

Although several methods are available for measuring total pigment levels in muscle the methods currently available have their drawbacks. For example, the cyanomet method, the recognised standard method for determination of haemoprotein concentration (Drabkin et al.,1950; Warriss, 1979), has the main drawback that it uses cyanide which is extremely toxic. The Hornsey method (Hornsey, 1956), another widely used procedure for haemoprotein determination, uses acetone/hydrochloric acid to quantitatively extract the haem from the haem pigments. The drawbacks with this method are 1) because of the volatile reagents used the results are not always reliable and 2) the solvent also extracts protein and fat from the sample which causes turbidity in the extracts and overestimation of pigment level (Karlsson and Lundstrom, 1988). A method described by Krzywicki (1982) which involves extraction of the pigments in phosphate buffer, clarification by centrifugation and filtration, and measurement of absorbance at 525 nm suffers from the disadvantages that 1) turbidity in extracts causes overestimation of pigment concentrations, 2) the absorbance at 525 nm is very low for light pigmented muscle and 3) the method is too slow for large numbers of samples. An alkaline haematin method developed by Karlsson and Lundstrom (1988) overcomes many of the disadvantages in the methods described previously; however, the method does have disadvantages in that with porcine extracts 1) the alkaline haematin is not stable and 2) the absorbance of the alkaline haematin is very low (Trout, 1991).

The method described in this paper is a rapid procedure for measuring total pigment concentration in porcine and other low pigmented muscles which has none of the disadvantages described above. The accuracy and precision of the method described in this paper is the same or better than the methods described in the previous paragraph.

MATERIAL AND METHODS:

Reagents: All reagents used were Analytical Reagent grade or equivalent except the Triton X-100 which was Scintillation grade (Merck, Darmsadt, FRG). Horse muscle myoglobin and haematin hydrochloride were obtained from Sigma Chemical Company (St Louis, MO, USA).

Sample preparation: Muscles used in this research were taken from the shoulder, loin and leg of market weight pigs and were chosen so as to give a range of pigment concentrations. The meat was obtained 24-48 hr post-mortem trimmed of all visible fat and connective tissue and ground

through a 3mm plate. The meat was used within 24 hours of collection.

New method for total pigment concentration: For each sample assayed, triplicate 3-g aliquots of ground pork muscle were homogenised in 30 ml cold (0°C) 0.04M phosphate buffer pH 6.5 for 20 sec using a probe type homogeniser set at 15,000 rpm (Chu et al., 1987). The homogenate was filtered through a 12.5 cm Whatman No. 1 filter paper until 10 ml of filtrate was collected. Immediately after filtration, to 4.0 ml of filtrate was added 1.4 ml of 10% Triton X-100 (final concentration 2.5%) and 100 ul of 65 mM sodium nitrite (final concentration 1.2 mM) and the solution was gently mixed. After 60 minutes at 22°C the absorbance was read at 730, 525 and 409 nm using a double-beam Hitachi 150-20 spectrophotometer (Tokyo, Japan). The total pigment concentration was calculated as follows using the MAC_{409} (molar absorbance coefficient at 409nm), dilution factor and the turbidity correction procedure suggested by Goldbloom and Brown (1966):

$$\text{Pigment (mg/g)} = ((A_{409}-A_{730}\times 2.68)\times \text{dilution factor}\times MW_{myoglobin})/(MAC_{409}\times 1000)$$
$$= ((A_{409}-A_{730}\times 2.68)\times 14.78\times 17{,}500)/(79.6\times 1000)$$
$$= (A_{409}-A_{730}\times 2.68)\times 3.249 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

The $MW_{myoglobin}$ was taken as 17,500 since it was assumed that most of the pigment present in meat is myoglobin. MAC_{409} was determined to be 79.6 mM and was obtained by the procedure described by Bowen (1949), i.e., by measuring the absorbance of the extract at 409 nm using the above procedure and determining the haemoprotein concentration on another portion of the original homogenate using the standard cyanomet procedure after the homogenate had been centrifuged at 50,000 x g for 30 min (Warriss, 1979). A similar approach was used for determining total pigment concentration using the absorbance value at 525 nm.

Comparison of methods: The Nit409 method (i.e., the method described above) was compared to the following methods to determine the accuracy and repeatability of each method: Hornsey (1956) method (absorbance measured at both 640 and 512 nm), cyanomet method (Warriss, 1979), $absorbance_{525}$ method described by Krzywicki (1982), and the alkaline haematin method (Karlsson and Lundstrom, 1988). With the alkaline haematin method and Nit409 method, portions of the homogenates were centrifuged at 50,000 x g for 30 min to determine if centrifugation reduced turbidity in the extracts. Additionally, with the alkaline haematin method absorbance was measured both at the suggested wavelength of 575 nm and at the wavelength maxima in the Soret region (400 nm) to determine if the higher absorbance at the lower wavelength increased the sensitivity of the method. With the alkaline haematin method, both haematin hydrochloride and purified horse myoglobin were used as standards to determine the MAC which was then in turn used to calculate the pigment concentration in the extracts. This approach was used because it was found that alkaline haematin derivatives of the pigments extracted from pork muscle were unstable and could not be used to determine the MAC using the approach described for the Nit409 procedure.

For all methods and procedure combinations evaluated, Abs_{730} was measured as an indicator of turbidity in the extracts, this absorbance was multiplied by the correction factors described by Goldbloom and Brown (1966) and the resulting value was subtracted from the absorbance obtained at the wavelength used to quantify the pigment.

Experimental design and statistical analysis: On each of three days three separate lots of pork muscle (i.e, a total of nine lots) were analysed in triplicate using all procedures and procedure variations described in the previous section and as outlined in Table 1. Data were analysed by analysis of variance using Genstat V (Payne et al., 1987). When F values were significant, Fisher's Least Significance Difference test was used to determine differences between treatment means (Steel and Torrie, 1960).

RESULTS AND DISCUSSION:

Nit409 method: The main drawbacks with procedures for determining total pigment concentration in porcine muscle are that they either: 1) Use toxic reagents; 2) Are too slow for routine analysis; 3) Use specialised equipment such as high speed centrifuges; 4) Lack sensitivity because of low pigment concentration and hence low absorbance; or 5) Overestimate pigment

concentration because of turbidity in the extracts. The alkaline haematin method described by Karlsson and Lundstrom (1988) overcomes the first three disadvantages of these methods: It overcomes the first by using nontoxic chemicals and increases the speed of the analysis and obviates the need for a high speed centrifugation by using a combination of filtration and addition of a solubilising agent (Triton X100). However, on initial evaluation of this procedure in this laboratory, it was found that the alkaline haematin, the compound produced in this analysis, is unstable when used with porcine extracts (but not with purified myoglobin, haemoglobin and haematin) and did not give consistent results.

Because of the shortcomings of the alkaline haematin method, the method outlined in this paper was developed. This method uses similar extraction, filtration and clarification procedures as the alkaline haematin method. To increase the sensitivity of the method the absorbance of the extracts was measured at the Soret band where the absorbance is 10-15 times higher than at the higher wavelengths (the absorbance at 525 nm, the only other suitable analytical wavelength, is very low [0.015-0.030] relative to the absorbance due to turbidity in the extracts and spectrophotometric variation). Since the wavelength maxima in the Soret region for myoglobin varies with the oxygenation and oxidation state of the pigment, the myoglobin must be fully oxidised or fully reduced before the Soret band can be used for quantification. In this procedure the pigments were oxidised to metmyoglobin using a modification of the procedure outlined by Smith and Nunn (1984); i.e., by oxidising the pigments with 1.2 mM sodium nitrite for 60 minutes. Preliminary research showed that under these conditions the pigments were fully oxidised and were stable for at least 120-180 minutes and that the wavelength maxima was 409 nm.

Turbidity is a major problem in quantifying pigment concentration in porcine muscle extracts. The suggested approach for overcoming this problem is to measure turbidity in the wavelength range where haemoproteins do not absorb (i.e., 690-730 nm) and to subtract this value from that obtain at the wavelength used for quantifying the haemoproteins (Karlsson and Lundstrom (1988). This approach is only partly successful since absorbance due to turbidity increases exponentially as the wavelength decreases (Goldbloom and Brown, 1988). For example, at 409 nm the absorbance due to turbidity is 2.7 times the value obtained at 730 nm. To compensate for this the effect, the absorbance at 730 nm must be multiplied by the appropriate correction factor before it is subtracted from absorbance due to the pigment. This procedure greatly improves both the accuracy and repeatability of the method.

Comparison of methods: The mean total pigment concentration obtained with the Nit409 method (at either 409 or 525 nm) was not significantly different ($p<0.05$) from the values obtained with the cyanomet and absorbance$_{525}$ methods (Table 1). However, all three methods gave slightly but significantly lower values ($p<0.05$) than obtained with the Hornsey method, indicating that the latter procedure is more efficient at extracting haem pigments from porcine muscle. The values obtained with the noncentrifuged alkaline haematin method were consistently lower ($p<0.05$) than those obtained with the other methods. Centrifugation increased the accuracy of alkaline haematin method and caused an overestimation by the Nit409 method. The standard used for the alkaline haematin method had little effect on its accuracy. The within sample standard deviation, a measure of the repeatability of the method, was low for all methods and varied little between methods. The turbidity of the extracts as measured by the absorbance at 730 nm was appreciable for all methods indicating the need to correct for turbidity in calculating total pigment concentration.

CONCLUSION: The Nit409 method is a safe, rapid, accurate and repeatable method for determining total pigment concentration in porcine muscle.

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Table 1. Mean total pigment concentration, within sample variation and turbidity of the 16 procedures and procedure combinations investigated.

METHOD	CENTRIFUGED	WAVELENGTH nm	CONVERSION FACTOR - STANDARD#	TOTAL PIGMENT CONCENTRATION (mg/g)	WITHIN SAMPLE STANDARD DEVIATION	TURBIDITY Abs_{730}
HORNSEY[1]	NO	640	Literature	1.36*	0.049	0.008
HORNSEY	NO	512	Literature	1.40*	0.056	0.008
WARRISS[2]	YES	540	Literature	1.28	0.094	0.009
KRZYWICKI[3]	YES	525	Literature	1.32	0.050	0.010
This work	YES	409	Calculated	1.37*	0.059	0.004
This work	YES	525	Calculated	1.29	0.065	0.004
This work	NO	409	Calculated	1.25	0.056	0.004
This work	NO	525	Calculated	1.27	0.045	0.004
ALK. HAEM.[4]	YES	400	Haematin	1.17*	0.032	0.006
ALK. HAEM.	YES	575	Haematin	1.25	0.070	0.006
ALK. HAEM.	YES	400	Myoglobin	1.00*	0.027	0.006
ALK. HAEM.	YES	575	Myoglobin	1.25	0.070	0.006
ALK. HAEM.	NO	400	Haematin	1.12*	0.044	0.006
ALK. HAEM.	NO	575	Haematin	1.13*	0.066	0.006
ALK. HAEM.	NO	400	Myoglobin	1.09*	0.045	0.006
ALK. HAEM.	NO	575	Myoglobin	1.12*	0.066	0.006

* Mean values which are significantly different ($p<0.05$) from the average of the Warriss and Krzywicki procedures.
[1] Hornsey (1956); [2] Warriss (1979); [3] Krzywicki (1982); [4] Karlsson and Lundstrom (1988)
Source of conversion factor or standard used to calculate total pigment concentration.