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# Rapid and quantitative determination of hexanal in cooked muscle foods

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**A simple and rapid method was developed to quantify hexanal in cooked muscle foods. The method involves extraction of the 2,4-dinitrophenylhydrazones of carbonyls with hexane and their separation by reversed-phased high performance liquid chromatography. The method compared well with the classical thiobarbituric acid test. The method was successfully used to quantify hexanal in cooked beef and pork burgers during storage at 4 °C for up to 1 week as well as in cooked turkey burgers after diet supplementation with vitamin E. After 7 days' storage at 4 °C, the contents of hexanal increased from 0.71 to 22.50 μmol/kg in beef burgers, from 0.89 to 32.75 μmol/kg in pork burgers and from 1.31 to 52.16 μmol/kg in turkey burgers (20 mg vitamin E per kg feed). Supplementation of turkey feeds with 600 mg vitamin E per kg resulted in a 24% reduction in hexanal content in cooked turkey burgers after storage for 7 days at 4 °C, compared to the control (unsupplemented) group.**

*Keywords*: Hexanal; lipid oxidation; TBARS; vitamin E

## Introduction

Lipid oxidation is a free-radical mediated, autocatalytic process involving the removal of labile hydrogen from a lipid molecule followed by the addition of oxygen to the resultant lipid radical. The lipid peroxy free radicals undergo a complex series of reactions leading to the formation of unstable hydroperoxides which are degraded to yield a variety of products, including aldehydes, ketones and organic acids which, in turn, contribute to the sensory properties of meats, particularly after cooking (Mottram, 1987; Morrissey and Apte, 1988).

Processed muscle foods are highly sus-

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ceptible to lipid oxidation during refrigerated and frozen storage. The processes involved in the production and storage of muscle foods enhance the degradation of polyunsaturated fatty acid hydroperoxides into secondary aldehydes, such as pentanal, hexanal, 4-hydroxynonenal and deca-2,4-dienal (St. Angelo *et al.*, 1987; Spanier, Edwards and Dupuy, 1988). These aldehydes, individually and in combination, are highly correlated with flavour scores of muscle foods. No simple method is available for the determination of trace amounts of aldehydes and other carbonyls.

Several methods have been used to follow lipid oxidation in muscle foods (Melton, 1983). The thiobarbituric acid (TBA) method is one of the oldest and most frequently used because of its simplicity and sensitivity. However, it has considerable limitations, such as lack of specificity and speed. In recent years, two other methods have been developed to detect carbonyls in meats. The first one involves the isolation of carbonyls by solvent extraction or distillation, followed by analysis without derivitisation using gas chromatography and mass spectrometry (Bailey and Swain, 1973; Bailey, Dupuy and Legendre, 1980). The second method involves reacting the carbonyls with 2,4-dinitrophenylhydrazine (DNPH) followed by chromatographic separation and determination of the resulting dinitrophenylhydrazones. Satisfactory separation of the hydrazones has been achieved using reversed-phase high performance liquid chromatography (HPLC) (Reindl and Stan, 1982a,b). In contrast to the free aldehydes, the hydrazones are stable and non-volatile, which greatly facilitates subsequent work-up procedures. Moreover, the DNPH derivatives have a strong yellow colour which facilitates their detection by thin layer chromatography or by HPLC. The latter has many of the advantages of gas chromatography such as speed and accurate quantitation and has become a powerful technique for the analysis of trace components. However, most of the methods involve a number of tedious and time consuming steps. For example, the prolonged time periods required to separate the volatiles from the non-volatile matrix, to form the hydrazones and to remove the large excess of unreacted reagents, have all limited the application of HPLC to the routine determination of carbonyls. Therefore, a simple, direct and sensitive method is required for fast determination of rancidity in muscle foods.

Of the many compounds involved, hexanal, a secondary reaction product of linoleic acid oxidation, is the most consistent by-product and has been suggested by many researchers as a marker to follow lipid oxidation in meat products (Tichivangana and Morrissey, 1984; St. Angelo *et al.*, 1987; Hwang, Bowers and Kropf, 1990). To date, most studies have focused on methodology and there are few studies on hexanal production in cooked muscle foods during storage. There are limited reports on the effect of antioxidants/prooxidants on hexanal production. Morrissey and Apte (1988) reported that the highest levels of hexanal were found in $Fe^{2+}$-catalysed muscle systems, with haemoglobin and ferritin being less effective catalysts than $Fe^{2+}$. Beef, pork, fish and chicken cured with nitrite showed significant reductions in hexanal production (Morrissey and Apte, 1988; Ramarathnan, Rubin and Diosady, 1991a,b). Vitamin E is an ideal natural antioxidant and has been demonstrated to be extremely efficient in protecting against lipid peroxidation in muscle subcellular membranes, based on thiobarbituric acid reacting substances (TBARS), colour stability, drip loss and other lipid oxidation-related criteria (Morrissey *et al.*, 1994; Buckley, Morrissey and Gray, 1995). However, no studies have been carried out on the effect of vitamin E on hexanal production. The objectives of this study were to develop a simple, rapid and sensitive method for routine analysis of hexanal in muscle foods and to use it to investigate the effect of species, storage time and dietary vitamin E supplementation on hexanal production.



## Materials and Methods

### *Chemicals*

All the chemicals used were 'AnalaR' grade obtained from British Drug Houses, Poole, Dorset, UK; Sigma Chemical Co. Ltd., Poole, Dorset, UK and Rathburn Chemical Co. Ltd., Walkerburn, Peebleshire, UK. Hexanal was purchased from Aldrich Chemical Co. Ltd., Dorset, UK.

### *Preparation of muscle samples*

Beef and pork muscles were obtained from local commerical slaughtering facilities. Turkey breast muscles were from turkeys fed either 20 (control) or 600 (supplemented) mg $\alpha$-tocopberyl acetate/kg feed for 21 weeks prior to slaughter.

Visible fat was removed from each muscle which was then minced with added water (5 ml/100 g) and NaCl (1g/100 g), using a conventional meat grinder. The minced muscle samples were immediately made into burgers using a conventional burger-maker (Ministeak, O.L. Smith & Co. Ltd., Italy). Burgers (~ 50 g) were immediately placed in polythene bags and cooked at 70 °C × 30 min in a hot water bath, before storage at 4 °C. Oxidative changes were monitored over a 7-day period.

### *Extraction of hexanal and formation of hexanal-dinitrophenylhydrazone*

One g of meat was homogenised (Ultraturrax, Model IKA T50 fitted with a S25N-18G shaft, Janke and Kunkel GmbH, Germany) with 2.5 ml of 2,4-DNPH [0.17 g/100 ml $H_2SO_4$ (30% w/v)]. The shaft of the homogeniser was then rinsed with 2.5 ml of 2,4-DNPH, the washings transferred to the homogenate, which was left to stand in the dark for 4 h at room temperature to complete the reaction. To the reaction mixture, 10 ml of hexane was added. After thorough mixing by hand for about 30 sec, the reaction mixture was centrifuged at 3000 g for 10 min, and the hexane layer removed. The extraction was repeated a further two times to achieve optimum recovery. The pooled hexane was evaporated to dryness on a rotary evaporator at 35 °C and the residue re-dissolved in 10 ml hexane.

### *Preparation of hexanal-dinitrophenylhydrazone standard*

The 2,4-dinitrophenylhydrazone derivative of hexanal was prepared as a reference by reacting 10 $\mu$moles of hexanal with 10 ml of the 2,4-DNPH [0.17 g/100 ml $H_2SO_4$ (30% w/v)] solution. The contents were thoroughly mixed and held in the dark for up to 24 h at room temperature to complete formation of the hexanal-dinitrophenylhydrazone complex. After the specified incubation periods, 10 ml of hexane were added and the solution was thoroughly mixed for about 30 sec. As much of the hexane layer as possible was transferred to a glass tube and the extraction was repeated a further two times. The pooled hexane layers were evaporated to dryness on a rotary evaporator at 35 °C, the residue was re-dissolved in 10 ml of hexane and the concentration of the derivitised hexanal was

determined colorimetrically using a molar extinction coefficient of $2.25 \times 10^4$/M/cm (Schwartz, Haller and Keeney, 1963). The stock solution was diluted 1:500 in hexane to prepare a working standard. Serial dilutions of the working standard were made and a calibration curve was constructed, ranging from 0 to 0.156 nmol/20 $\mu$l injection.

*High performance liquid chromatography*

HPLC analyses were performed on a system consisting of a Waters Model S10 pump, a Waters Model 717 autosampler, a Macheray-Nagel Nucleosil 100-5 $C_{18}$ ($250 \times 0.4$ mm) reverse phase column and a Waters Model 486 UV-visible wavelength detector set at 360 nm. The eluent was acetonitrile:water:tetrahydrofuran (75:24:1) at a flow rate of 1 ml/min. The injection volume was 20 $\mu$l. Data were recorded and evaluated on a Millipore Millenium Chromatography Management system.

*Measurement of lipid oxidation*

The extent of lipid oxidation in the muscle samples was assessed by the TBA method of Ke et al. (1977). The TBARS values were expressed as mg malonaldehyde/kg meat.

*Statistical analysis*

All samples were assayed in duplicate. Statistical analysis was performed using the SAS statistical package (SAS, 1989). The significance of the difference between two means was determined by the Student's t-test; Duncan's multiple range test (1955) was used to identify differences between more than two means when significant ($P < 0.05$) treatment effects were determined. Linear regression was employed to assess the relationship between TBARS and hexanal during storage.

## Results and Discussion

*Effect of reaction time on formation of hydrazones*

Using a two-phase (organic and aqueous) system, almost complete formation of hydrazones of aldehydes with more than five carbons was achieved after a 3 h reaction time (Reindl and Stan, 1982b). In a one-phase reaction medium, Selim (1977) observed that an equilibrium for the 2,4-DNPH derivative of propionaldehyde in the aqueous phase was reached after 15 h, when 70.6% of the derivative had been formed. Morrissey and Apte (1988) held the mixture of hexanal and 2,4-DNPH overnight at room temperature to complete the formation of the hyrazone of hexanal. It was considered that the reaction time in the one-phase system could be reduced. To test this hypothesis, 100 $\mu$moles of hexanal were added to 100 ml of 2,4-DNPH [0.17 g in 100 ml of 30% (w/v) $H_2SO_4$] and 5 ml aliquots of the solution were stored at room temperature. Duplicate samples were extracted using 5 ml of hexane (as detailed above) after 4, 8, 16 and 24 h incubations and five replicates from each sample were injected into the HPLC. Data in Table 1 show that the reaction was about 90% complete after 4 h and

**Table 1. Conversion of hexanal (100 $\mu$mol) to its 2,4-dinitrophenylhydrazone at room temperature**

| Reaction time (h) | Chromatogram[1,2] ($\times 10^6$) |
|---|---|
| 4 | $3.56 \pm 0.211^a$ |
| 8 | $3.91 \pm 0.046^b$ |
| 16 | $3.70 \pm 0.086^{ab}$ |
| 24 | $3.04 \pm 0.398^c$ |

[1]Mean ($\pm$ s.d.) for five replicate samples.
[2]Arbitrary units.
[abc]Means without a common superscript are significantly ($P < 0.01$) different.



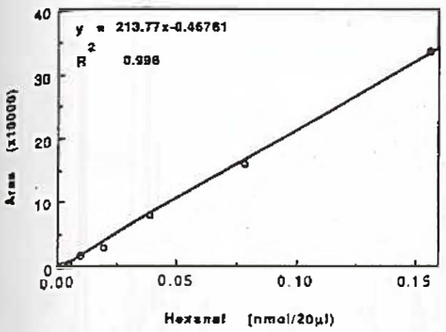


*Figure 1: Calibration curve for 2,4-dinitrophenylhydrazone of hexanal. Absorbance was measured at 360 nm.*

probably complete after 8 h. Allowing the reaction to proceed for 16 and 24 h resulted in a reduction in the peak area for the hydrazone. Although the peak area was higher after the 8 h incubation, it was decided to use the 4 h reaction time for analysis, since the main consideration was that the method would be suitable for routine analysis of lipid oxidation and, consequently, should be completed in 1 day.

*Linearity of detector response*

The standard curve obtained on injection of various concentrations of hexanal-2,4-DNPH ranging from 0.001 to 0.156 nmol/20 $\mu$l is shown in Figure 1. The detector response was linear over the entire concentration range. Linear regression analysis yielded an equation of $y = 213.77x - 0.46761$, with a correlation coefficient of 0.998, where y is the peak area and x is the amount of hexanal 2,4-DNPH (nmol/20 $\mu$l) injected.

*Recovery of hexanal and reproducibility of method*

The recovery of hexanal from meat samples was established as follows: (a) 5 $\mu$moles of hexanal were reacted directly with 5 ml of 2,4-DNPH solution to form the hydrazone of hexanal; (b) the same amount of hexanal was homogenised with 1 g of fresh pork and derivitised with 5 ml of 2,4-DNPH solution; (c) 1 g of fresh pork was homogenised with 5 ml of 2,4-DNPH. The samples were stored for 4 h at room temperature and then extracted with hexane as outlined above. Concentrations of the hexanal derivative in each case were determined by HPLC. The percentage recovery of the hexanal was calculated from $(B\text{-}C)/A \times 100$. The average recovery was 71.6 (s.d. 8.8, n = 5).

The reproducibility of the procedure was determined by adding hexanal (5 $\mu$mol/g) to fresh meat and extracting and derivitising as outlined above. Ten individual samples were analysed and the variation (standard deviation) calculated. The average reproducibility of the procedure was found to be $\pm 8.2\%$. Esterbauer, Zollner and Schaur (1990) developed a somewhat similar procedure for determining hexanal in liver microsomes and reported a reproducibility of $\pm 10$ to 15%.

*HPLC profiles in cooked meat systems*

The profiles of the 2,4-dinitrophenylhydrazones of carbonyl compounds in heated beef, pork and turkey burgers after refrigerated storage for up to 7 days are shown in Figure 2. It is evident from the chromatogram that the hexanal ($C_6$) content increased in the order: turkey > pork > beef. The present and earlier studies (Morrissey and Apte, 1988) indicate that hexanal formation increased with storage time while the other components (peaks) did not show a consistent pattern of increase during storage. The inconsistencies observed for some of the compounds are probably due to further oxidation or degradation of these compounds to other carbonyls or derivatives (Schieberle and Grosch, 1981).

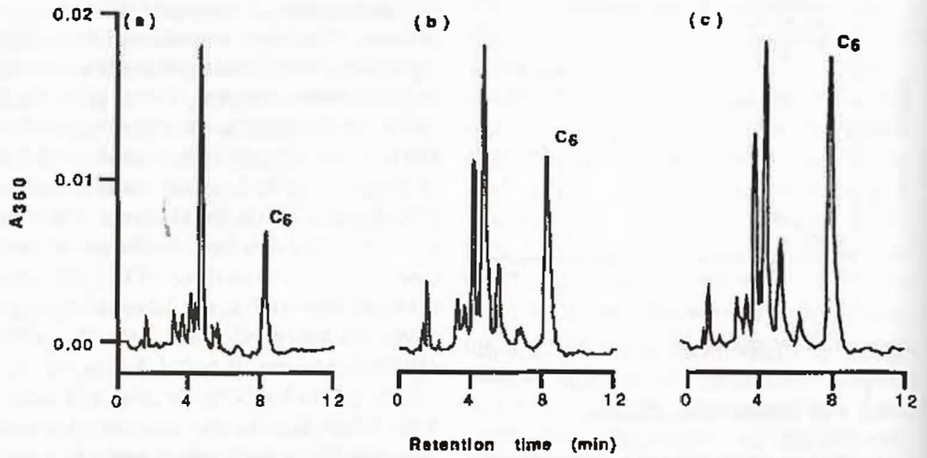


*Figure 2: HPLC profiles of carbonyl hydrazones from beef (A), pork (B) and turkey (C) after heating and storage at 4 °C for 7 days. $C_6$ indicates the 2,4-dinitrophenylhydrazone of n-hexanal.*

*Hexanal and TBARS production in cooked meat systems*

The hexanal-hydrazone method was used to measure the production of hexanal due to oxidation in cooked pork, beef and turkey burgers and the trend of oxidation was compared with TBARS (Figures 3 to 5).

After 7 days of storage at refrigeration temperature, hexanal increased in concentration from 0.71 to 22.5 μmol/kg in beef burgers and from 0.99 to 32.8 μmol/kg in pork burgers. In the case of turkey burgers, the hexanal content increased from 0.9 to 39.5 μmol/kg in the supplemented (600 mg α-tocopheryl acetate/kg feed) group and from 1.3 to 52.2 μmol/kg in the control (20 mg) group. Vitamin E supplementation resulted in a reduction ($P < 0.01$) in hexanal of approximately 24% relative to the control after 7 days storage at refrigeration temperature. The hexanal content of all samples rapidly increased with storage time, especially in the early stages of storage. After 1 day's storage the hexanal contents were 8.4, 10.2, 14.9 and 15.3-fold higher than the levels at the commencement of storage in beef, pork and high- and low-vitamin E fed turkeys, respectively. The corresponding TBARS values of the above samples only increased 1.7, 1.9, 1.6 and 1.6-fold, respectively, over the same period. Measurement of hexanal, therefore, appears to be more sensitive and better than TBARS as an indicator of the oxidative state of cooked meats, especially in the early stages of storage. Overall, the rate of the hexanal and TBARS production was in the order: turkey > pork > beef, which supports our earlier findings (Morrissey and Apte, 1988) that the rate and extent of lipid oxidation is primarily dependent on the level of polyunsaturated fatty acids present in the meat system, and hexanal is one of the major secondary products of linoleic acid oxidation.

For the two turkey groups, both hexanal and TBARS values decreased slightly after storage from 1 to 3 days. Similar reductions in TBARS values were also



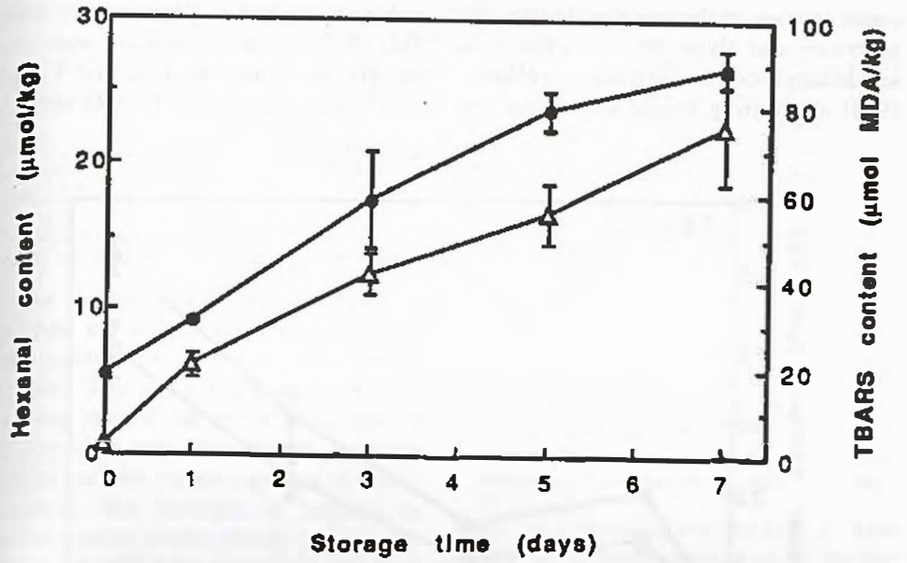


*Figure 3: Changes in hexanal concentrations (Δ) and TBARS values (•) in cooked beef burgers during refrigerated storage. Mean (± s.d.) from three analyses performed in duplicate.*

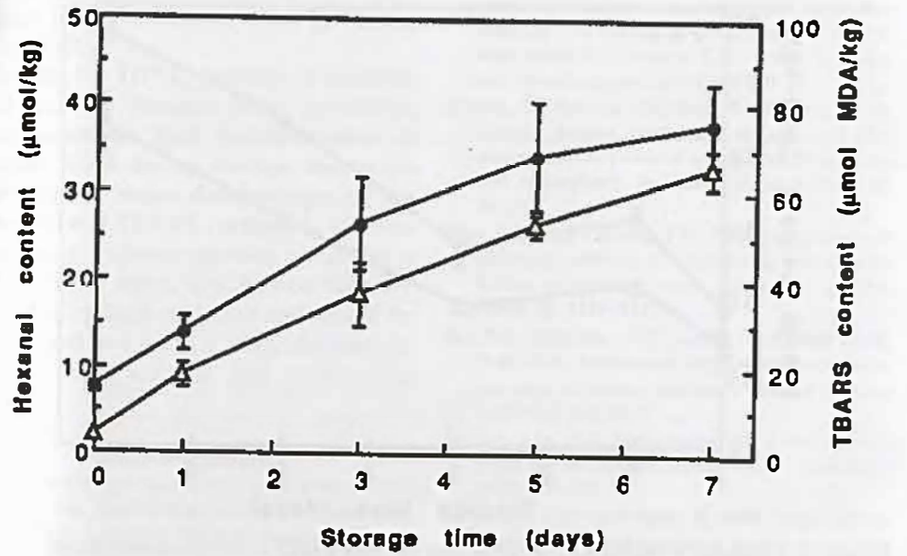


*Figure 4: Changes in hexanal concentrations (Δ) and TBARS values (•) in cooked pork burgers during refrigerated storage. Mean (± s.d.) from three analyses performed in duplicate.*

found in cooked chicken muscle (Sheehy, Morrissey and Flynn, 1993), cooked meat and fishery products (Tarladgis and Watts, 1960) and during frozen storage of raw and cooked turkey (Wen *et al.*, 1996). This observation is consistent with other reports. According to Kim and LaBella (1987), the reduction in TBARS observed

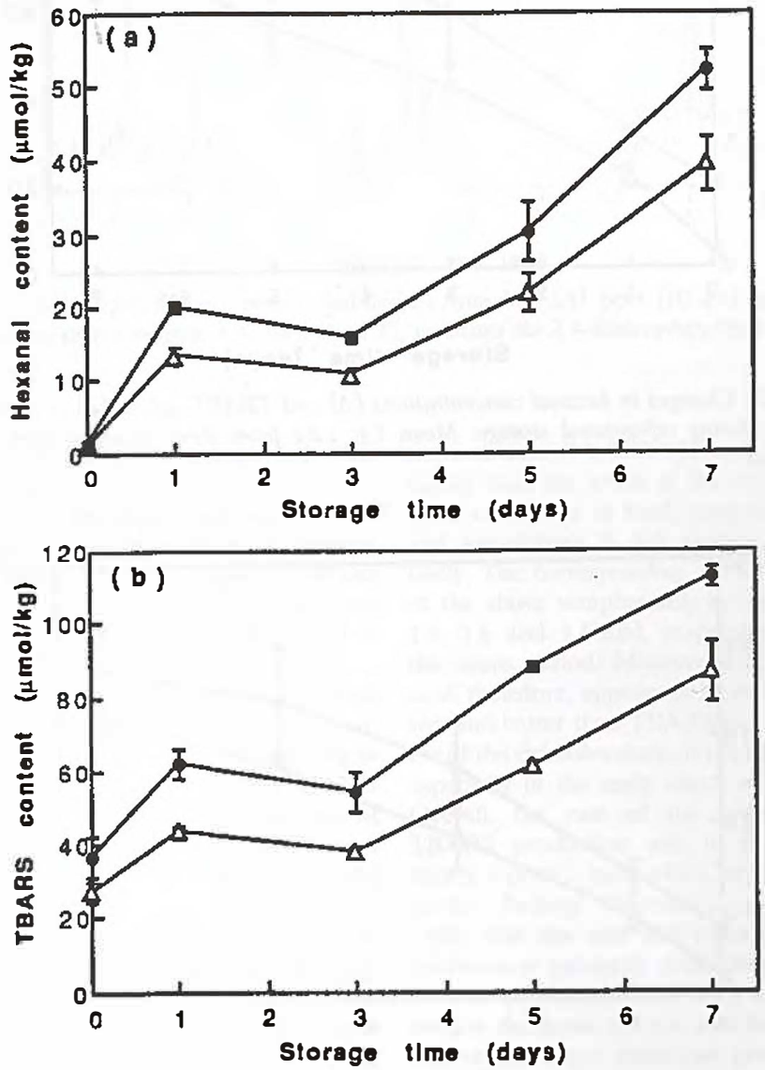


*Figure 5: Changes in hexanal concentrations (a) and TBARS (b) of cooked burgers made from turkeys, which were fed diets supplemented with 20 (●) and 600 (Δ) mg α-tocopheryl acetate / kg diet, during refrigerated storage. Mean (± s.d.) from three analyses performed in duplicate.*



**Table 2. Regression analysis of hexanal (µmol / kg) on TBARS (mg malonaldehyde / kg) in cooked burgers held at 4 °C for up to 7 days**

| Species | n | Intercept | Slope | $R^2$ | Significance |
|---|---|---|---|---|---|
| Beef | 8 | 0.2527 | 1.0266 | 0.98 | < 0.001 |
| Pork | 8 | 0.1454 | 0.8652 | 0.99 | < 0.001 |
| Turkey (600)[a] | 8 | 0.1175 | 1.6744 | 0.99 | < 0.001 |
| Turkey (20)[b] | 8 | 0.1102 | 2.4625 | 0.97 | < 0.003 |

[a] Fed 600 mg α-tocopheryl acetate/kg feed for 21 weeks.
[b] Fed 20 mg α-tocopheryl acetate/kg feed for 21 weeks.

in the latter stages of oxidation is probably associated with increased polymerisation of secondary oxidation products. The slight decrease in hexanal observed may be due to the formation of an Amadori-type compound between hexanal and the ε-amino groups of lysine. Generally, the increase in hexanal in cooked muscle foods closely follows the pattern of TBARS values, and this was also evident for the low- and high-vitamin E samples. The relationship between the two methods is shown in Table 2. Correlation coefficients between the two methods ranged from 0.97 to 0.99 and were higher than the 0.81 reported by Hwang *et al.* (1990).

Using the HPLC method to separate and quantify hexanal from breakdown compounds of lipid hydroperoxides of muscle foods during storage overcomes some of the major disadvantages of the conventional TBARS method. In the present study, a simple one-step procedure is shown to be rapid, specific and sensitive in following lipid oxidation and should be considered as a routine analytical method for the meat scientist and quality controller.

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# A note on a non-destructive method of chlorophyll determination in wheat (*Triticum aestivum* L.)

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**The relationship between the chlorophyll content of wheat flag leaves estimated by a chemical method in the laboratory and that obtained using a Minolta chlorophyll meter was evaluated. A statistically significant ($r^2$ 0.85, $P < 0.001$) linear relationship was found to exist between extractable chlorophyll and meter readings when chlorophyll content was expressed on a leaf area basis. A further improvement in the relationship was obtained when a quadratric function was fitted to the data ($r^2$ 0.88). The use of the quadratic equation for predicting chlorophyll concentrations from meter readings was validated using an independent data set. Predicted values of chlorophyll content on this data set were highly correlated ($r^2$ 0.91) with the measured chlorophyll concentrations. The results indicate that this rapid technique for chlorophyll determination can play a useful role in many areas of crop science especially where estimates of leaf senescence are required.**

*Keywords*: Chlorophyll; flag leaves; spring wheat

## Introduction

Chlorophyll is the pigment responsible for the green colour of plants. As the light capturing molecule in pbotosystems I and II (Bidwell, 1974), chlorophyll occupies a unique role in the physiology of green plants. Modern crop species and varieties are greener, establisb green tissues faster, retain them longer and are visibly more responsive to agricultural inputs than old crop species (Thomas and Smart, 1993). The amount of cblorophyll per unit area is influenced by nutrient availability (Hewitt, 1963) and environmental stress (Palta, 1990). Chlorophyll is thus a sensitive indicator of plant physiological status and its quantification has always been of special interest to plant scientists.

Analysis of chlorophyll in plant tissues is typically achieved in the laboratory by time-consuming grinding and centrifuga-