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Hydroperoxide formation in different lean meats

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ABSTRACT

Peroxide is one of the compounds that are indicated to be toxic in the human digestion system. Lean fresh meat samples were collected from beef, lamb, pork and chicken to investigate their hydroperoxide formation potential. Total peroxides of fresh comminuted raw meat were determined by analysing protein-bound peroxides and hydroperoxide compounds in water–methanol and chloroform extracted phases. The amount of total peroxides was ranked as: beef > pork > lamb > chicken. Hydroperoxide formation was examined at different pH values and at different incubation times, using beef and chicken samples. All peroxides were transient, with a maximum value after 2–4 h of incubation at 37 °C. When pH fell from 7 to 1.5, the different peroxides fell by 10–20%. Non-polar peroxide formation could largely (70%) be described by variation in fatty acid composition and hemin content of the meat, while protein-bound peroxide variation was less explained by these variables. Liposome addition increased (40%) the amount of protein-bound peroxides.

1. Introduction

Meat consumption from some land-based animals has come under attack due to unclear status regarding many diseases. Colon cancer is among these diseases, and it is one of the major causes of death in western countries (Sesink, Termont, Kleibeuker, & Van der Meer, 1999). It has been recognised that many genetic factors are involved as determinants of colorectal cancer (Fearon & Jones, 1992), but environmental factors have appeared to contribute to the incidences of colon cancer (MacLennan, 1997). The World Cancer Research Fund panel has judged that the evidence of red meat and processed meat being a cause of colon cancer is convincing (WCRF, 2007), and a western style diet with a high red meat consumption is suggested as a risk factor for colon cancer (Sesink et al., 1999). Increased consumption of meat can be due to improved efficiency in agriculture, which has then created sufficient amounts of relatively cheap meat products. Animal breeding has so far given most priority to rapid animal growth and cost-effective feeds. But meat should also have a good oxidative and microbial shelf life. Sufficient oxidative stabilization is paramount for meat flavour. A present understatement is that oxidised food can be consumed as long as the microbiology and sensory quality are acceptable to consumers. Compounds that could increase the genetic instability of colon cells and the appearance of cancer have received much attention (Ferguson, 2010). Lipid or lipid-derived peroxides are a major source of dietary pro-oxidants speculated to be of toxicological importance (Halliwell & Chirico, 1993).

An *in vitro* study on intake of fat and derived peroxides has identified this as one of many important factors in colon cancer (Angeli et al. 2011). Lipid peroxides are set with an acceptable upper level of 5–10 mmol/kg in oil or fat (Sattar & Deman, 1976). Peroxide limits are normally not defined for products other than oil/fats. However, it is more common to eat larger amounts of lean meat than of pure oil/fats in a meal. Heated turkey meat has been reported to have 1 mmol of lipid hydroperoxide/kg wet weight (Kuffa, Priesbe, Krueger, Reed, & Richards, 2009). This suggests a high peroxide value in the endogenous lipids (~100 mmol/kg lipid). In addition, proteins may also carry peroxides equal to 3–22 mmol/kg of protein (Salminen and Heinonen, 2008). Proteins damaged by free radicals in the presence of oxygen can yield relatively long-lived protein peroxides (Davies, Fu, & Dean, 1995; Gebicki & Gebicki, 1993), which have been shown to readily degrade to free radicals upon reaction with iron (II) complex. It is therefore necessary to include them in an assay for hydroperoxide measurements, in particularly in lean meat where the lipid content is low relative to the protein content.

With sufficient amounts of efficient antioxidants, meat should be a homoeostatic system which remains reduced or without oxidised compounds and reactive components. The aim of this study was: (1) to set up a new model system for measuring total hydroperoxide values of lean meat and the reactivity of lean meat towards liposomes, (2) to discover if the lipid peroxides were always dominant over the protein-bound peroxides, (3) to investigate whether the peroxides were stable when incubated

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over time and at different pH values, (4) to establish the hydroperoxide formation ability in some Norwegian regular diet meats.

2. Material and methods

2.1. Meat samples

Chicken muscles (*Musculus pectoralis major*) were collected on the day of slaughter from a hot boning line, vacuum-packed and frozen at −80 °C. The chicken-SO group was chicken fed with a wheat-based diet containing 4% soybean oil and 0.003% selenium-enriched yeast (Ultra Bio-logics., Inc., O.S.Y. 2000× containing 2.15 g Se/kg), whereas the chicken-LO group was fed with a wheat-based diet with 2.4% linseed oil, 1.6% rapeseed oil, and 0.04% selenium yeast. Beef muscles (*Musculus semimembranosus*) were obtained on the day of slaughter from a hot boning line, vacuum-packed and frozen at −40 °C until they could be brought to −80 °C (after 5 days). Pork muscles (*Musculus gluteus medius*) were collected 1 day after slaughter from the cold boning line, vacuum-packed, and frozen at −80 °C. The pig group was homogeneous, as all pigs were of the crossbreed Noroc that was produced to give higher intramuscular fat content than the regular Norwegian Landrace/Yorkshire crossbreed. All the pig samples were from the same farm. Lamb muscles (*Musculus psoas major*) were obtained 1 day after slaughter from a cold boning line, vacuum-packed, frozen at −40 °C until they could be brought to −80 °C (after 5 days). Each group contained 10 animals. These beef (*M. semimembranosus)*, pork (*M. gluteus medius*) and lamb (*M. psoas major*) muscles were randomly chosen from different Norwegian feeding farms from a local meat supplier (Nortura SA, Lillehammer, Norway).

2.2. Chemicals

L-α-Phosphatidylcholine 95% (egg, chicken) powder was purchased from Avanti Polar Lipids, Inc., (Alabaster, USA). Water was purified by a purification system (Millipore, Sydney, Australia). Chloroform (AR grad), sulphuric acid, methanol, acetone, iron (II) sulphate, hexane and Ringer's solution tablets were from Merck (Darmstadt, Germany). Guanidine hydrochloride, hydrochloric acid (37%), streptomycin and C13:0 internal standard were supplied by Sigma–Aldrich Chemical (Sydney, Australia). Butylated hydroxytoluene, xylenol orange sodium salt and triphenylphosphine (99% in purify) were purchased from Alfa Aesar (Lancashire, UK). Sorbitol and hemin were bought from Sigma–Aldrich (St. Louis, USA). Sodium dithionite and KOH were purchased from VWR Inc., (Oslo, Norway). All the other chemicals were of analytical grade as supplied.

2.3. Generation of liposomes

L-α-Phosphatidylcholine 95% (egg, chicken) powder (1 g) was first dissolved and mixed in 50 ml of chloroform to assure a homogeneous mixture of lipids. The organic solvent was evaporated to 1 ml by using a rotary evaporator (R215, Buchi Rotavapor, Switzerland). The solution was dried thoroughly by nitrogen gas to a lipid residue at room temperature. Hydration of the dry lipid cake was accomplished by adding 50 ml of Ringer's solution in a 60 °C water bath for 60 min. Liposomes were produced by using an extrusion technique, which yielded a polydisperse suspension of multilamellar liposomes. The mini-extruder was assembled by inserting two internal membrane filters and one polycarbonate membrane filter (0.1 μm pore size, Avanti polar lipids, Inc. Alabama, USA), and then the system was heated to 60 °C before use. One gas-tight syringe (Hamilton, Bonaduz, Switzerland) was loaded with 1 ml of solution and applied to one end of the mini-extruder while the other end of the mini-extruder was supported with an empty gas-tight syringe so that the fluid could be circulated through filters from both sides. This resulted in large, unilamellar liposome vesicles defined by the pore size of the membrane.

The lipid solution was completely transferred between the original and alternative syringes by gently pushing the plunger (1 min each time) 10 times (20 passes through the membranes). A successfully prepared liposome solution had no sediment after storage at 4 °C overnight. Liposome solutions were stored at −80 °C after preparation for later use.

2.4. Hydroperoxide value (PV) measurements by using the ferric–xylenol orange (FOX) method

Meat cuts were trimmed of all visible fat, frozen in liquid nitrogen and homogenised by blender (800 W Home blender, Invite) to meat powder. Hydroperoxide measurements were made on meat, with or without added liposomes. Triplicates of meat samples (0.1 g) were incubated in 1 ml of Ringer's solution and quadruplicate meat samples were incubated in 200 μl of liposomes (4 mg/ml) and 800 μl of Ringer's solution. To all systems, 10 μl of 20 g/l streptomycin was added and the systems were incubated for 2 h in a 37 °C water bath.

The measurements without added liposomes served to identify endogenous ability to produce peroxides, while the other measurement served to verify the potential of the meat samples to induce peroxides in liposomes (as an *in vitro* model for cell membranes). The samples were mixed with 1 ml of chloroform and methanol (2:1, volume-ratio), vortexed and centrifuged at 24,462*g* for 10 min at 4 °C. After centrifugation the system separated into three phases which were 1.33 ml of polar upper phase (25% methanol + 75% Ringer's solution, pH 7), an interphase (the meat protein aggregate) and 0.67 ml of non-polar lower phase (chloroform) containing soluble lipids. Each of the three phases was removed for separate hydroperoxide measurements. Upper phase (700 μl) was removed and the following chemicals were added immediately in this order: 5 μl of 4 mM BHT, 4 μl of 2 M H_2SO_4, 40 μl of H_2SO_4 at pH 1.8, 30 μl of 5 mM XO + 5 M sorbitol mixture at pH 1.8 and 40 μl of 1.67 mM $FeSO_4$ at pH 1.8. A blank containing the upper phase reduced with 10 μl of 1 M sodium dithionite and subjected to an identical protocol was used as a negative control. The protein aggregate at the interphase was washed three times with 2:1 chloroform:methanol before 1.7 ml of 6 M GuHCl were added to resolubilise the protein for optimal hydroperoxide exposure. The protein aggregate did not always solubilise to a transparent solution, but it swelled to an open system that allowed for low molecular weight diffusion (*i.e.* diffusion of the chemicals added). After 30 min of solubilisation, all chemicals were added immediately in this order: 12 μl of 4 mM BHT, 97 μl of H_2SO_4 at pH 1.8, 73 μl of 5 mM XO + 5 M sorbitol mixture at pH 1.8 and 73 μl of 1.67 mM $FeSO_4$ at pH 1.8. A blank containing suspended protein phase reduced with 10 μl of 1 M sodium dithionite and subjected to identical protocol was used as a negative control. Lower phase (50 μl chloroform) was removed and chemicals were added immediately in this order: 200 μl of chloroform, 460 μl of methanol, 5 μl of 4 mM BHT, 12 μl of 2 M H_2SO_4, 26 μl of 10 mM XO at pH 1.8 and 54 μl of 1.67 mM $FeSO_4$ at pH 1.8. A blank containing the lower phase reduced with 10 μl of 1 M triphenylphosphine and subjected to identical protocol was used as a negative control. All the samples were incubated for 60 min in enclosed Eppendorf tubes at room temperature to ensure colour development. The upper phases and the suspended protein interphases were centrifuged at 24,462*g* for a further 10 min at 4 °C to secure transparency before the measurements by the spectrophotometer, while the lower phases were measured spectrophotometrically at 590 nm immediately after the incubation. The initially obtained hydroperoxide

values were calculated by first subtracting the negative control, then the absorbance was divided by the pigments' molar absorptivities of 14,840 (1 cm pathway) and 87,583 (1 cm pathway) for the upper phase/inter phase and the lower phase, respectively, before correcting for dilution. Our procedure is a modification of Gay and Gebicki (2002a), but adapted to meat instead of serum and with reduced volumes to adapt the technique to Eppendorf tubes. The peroxides of the upper, inter and lower phase are hereafter called polar, protein-bound and non-polar peroxides, respectively.

In order to check the effect of pH on hydroperoxide formation in meat, pH values from 1.5 to 7.0 were examined. Ringer's solution was adjusted to the required pH with 2 M H_2SO_4 before incubation. The FOX method is based on oxidation by hydroperoxide under certain acidic conditions (pH 1.8) for a maximum response at room temperature (Bou, Codony, Tres, Decker, & Guardiola, 2008; Gay, Collin, & Gebicki, 1999). Normally when the samples were incubated at pH 7, a final pH 1.8 (pH of maximum absorbance) was obtained when absorbances were read. But when the samples were incubated at pH 5.5, 3.5 and 1.5, the final pH was <pH 1.8, so the absorbances were lower. So we used the absorbance ratios at pH 7 to pH 5.5 (1.0134), pH 7 to pH 3.5 (1.0321) and pH 7 to pH 1.5 (1.124) to correct absorbances below pH 1.8 back to absorbance at pH 1.8.

The ratio of endogeneous meat fatty acids to the liposome fatty acids varied with the amount of fat in the lean meat, but was always less than 1:2 (weight ratio). The initial peroxide value of the liposomes added was less than 0.037 mmol/kg of phospholipids.

2.5. Conjugated compounds (CC)

The amounts of CC in water–methanol and chlorofrom produced during PV measurements were measured. Both the polar and non-polar phases were removed for CC measurements. Polar phase (100 μl) was removed and diluted 10 times by adding 900 μl of 75% methanol and 25% water solution and the non-polar phase was removed (50 μl) and diluted 20 times by adding 950 μl of chloroform. Both phases were measured spectrophotometrically in the UV range (240–340 nm). The obtained absorbances were multiplied by the dilution factor (×10 in polar phases and ×20 in non-polar phases) then divided by the molar absorptivity of conjugated trienes of 36,300 (1 cm pathway) at 268 nm.

2.6. Hemin distributions among extracted phases

In order to check which phase hemin remained in during hydroperoxide analysis, 1 ml of hemin solution (0.31 mg/ml) was blended with 1 ml of 2:1 chloroform:methanol solution. The same procedure was also carried out for extraction of the three phases for hydroperoxide determination. After centrifugation, undissolved hemin particles were found to appear between polar phase and non-polar phase. The polar phase showed an average absorbance of 0.01 at 407 nm. The non-polar phase had its absorbance tested against chloroform as a blank. By using the molar absorbitivity of 36,000 (1 cm pathway) (Uc, Stokes, & Britigan, 2004), an upper limit of 1.8% of the added hemin was identified as presented in the non-polar phase if the initial solution contained 8 g/l of myoglobin. Therefore hemin, in meat homogenates during the PV assay, was distributed mainly to the interphase with the proteins.

2.7. Hemin analysis

The analyses were carried out on meat samples, following the analytical method described by Ginevra et al. (2002) with some optimizations. Meat cuts were trimmed of all visible fat, frozen in lipid nitrogen and homogenised to meat powder. Meat homogenates (0.155 g) were dissolved in 233 μl of distiled Millipore water, 1.55 ml of acetone and 63 μl of concentrated HCl (37%) in capped Eppendorf tubes. The mixture was vortexed vigorously and then centrifuged at 24,462*g* for 10 min at 4 °C. The supernatant was extracted and the absorbance was measured at 407 nm against a reagent blank. Two replicates were measured, myoglobin solutions were used to make a linear standard curve and hemin concentrations were read from the standard curve.

2.8. Fatty acid analysis by gas chromatography (GC)

Meat samples were placed into 16 × 125 mm screw-cap Pyrex culture tubes and 0.8 ml of the C13:0 internal standard, 0.56 ml of 10 N KOH in water, and 4.24 ml of MeOH were added. All tubes were incubated in a 55 °C water bath for 1.5 h with hand-shaking for 5 s every 20 min to properly permeate, dissolve and hydrolyse the samples. The samples were cooled to below room temperature and 0.464 ml of 24 N H_2SO_4 was added. All the tubes were incubated again in a 55 °C water bath for 1.5 h with hand-shaking for 5 s every 20 min; then the tubes were cooled again in a cold water bath and 2.4 ml of *n*-hexane were added to each tube. All the tubes were vortex-mixed for 5 min and centrifuged for 5 min in a table top centrifuge. The hexane layer, containing the fatty acid methyl esters, was transferred into a GC vial, capped and kept at −20 °C prior to GC analysis (O'Fallon, Busboom, Nelson, & Gaskins, 2007).

The fatty acid composition of the meat samples was determined by gas chromatography on a fused capillary column. The oven temperature was 70 °C at the start, held there for 4 min and then increased to 160 °C at a rate of 20 °C/min. Thereafter the temperature was held for a further 15 min, then the temperature was further increased at 3 °C per minute to 230 °C. Helium was used as the carrier gas at a flow rate of 68.4 ml/min at a temperature of 280 °C and the column head pressure was 309.4 kPa. Both the injector and the detector were set at 260 °C. The split ratio was 30:1. The flame ionisation detector temperature was 290 °C with H_2, air and N_2 make-up gas flow rates of 40, 450 and 45 ml/min, respectively. The run time for a single sample was 92 min. C13:0 was added as an internal standard and used to calculate the amounts of fatty acids in muscle (mg/100 g). The fatty acids were identified by comparing their retention times with the fatty acid methyl standards.

2.9. Statistics

Minitab (version 16; Minitab Inc., State College PA, USA) was used for univariate regression analysis (incl. stepwise regression) and one way ANOVA. The unscrambler (version X 10.2 CAMO Software AS, Oslo, Norway) was used for principal component analysis (PCA), as well as partial least square (PLS) regression. Evaluation of the PLS regression model was with full cross-validation.

3. Results

3.1. Time of maximum content of peroxides at pH 7

Beef and chicken meat samples were incubated for different times, with or without liposomes, to examine when the largest amount of peroxides was formed. The peroxides in raw beef and chicken homogenates increased rapidly during the first 2 h of incubation at 37 °C. Thereafter, the amount of peroxides declined by more than 90% in all three extracted phases. Due to the presence of the largest amount of peroxides after 2 h of incubation, this time point was chosen as a standard incubation time for all meat samples. Beef homogenates showed 1- to 1.5-fold higher amounts of peroxides than did chicken samples for all three extracted phases incubated for 2 h, with or without liposomes (Fig. 1).

3.2. Effect of liposomes addition on hydroperoxides of the three extracted phases at pH 7

Meat homogenates incubated with liposomes showed higher PV in all three extracted phases than did those without liposomes. The increase in PV with liposome addition was significantly ($P < 0.05$) independent of extracted phase. The average increase in polar PV over time, with liposome addition, was 6% ($P < 0.001$, linear regression). For the protein-bound peroxides, the average increase over time was 40% ($P < 0.001$, linear regression) whereas, for lipid hydroperoxides, the average increase in PV over time was only 3% ($P < 0.001$) with liposome addition. Although the PVs of the two systems (with and without liposomes) were correlated, the increased PV with liposome addition of non-polar peroxides

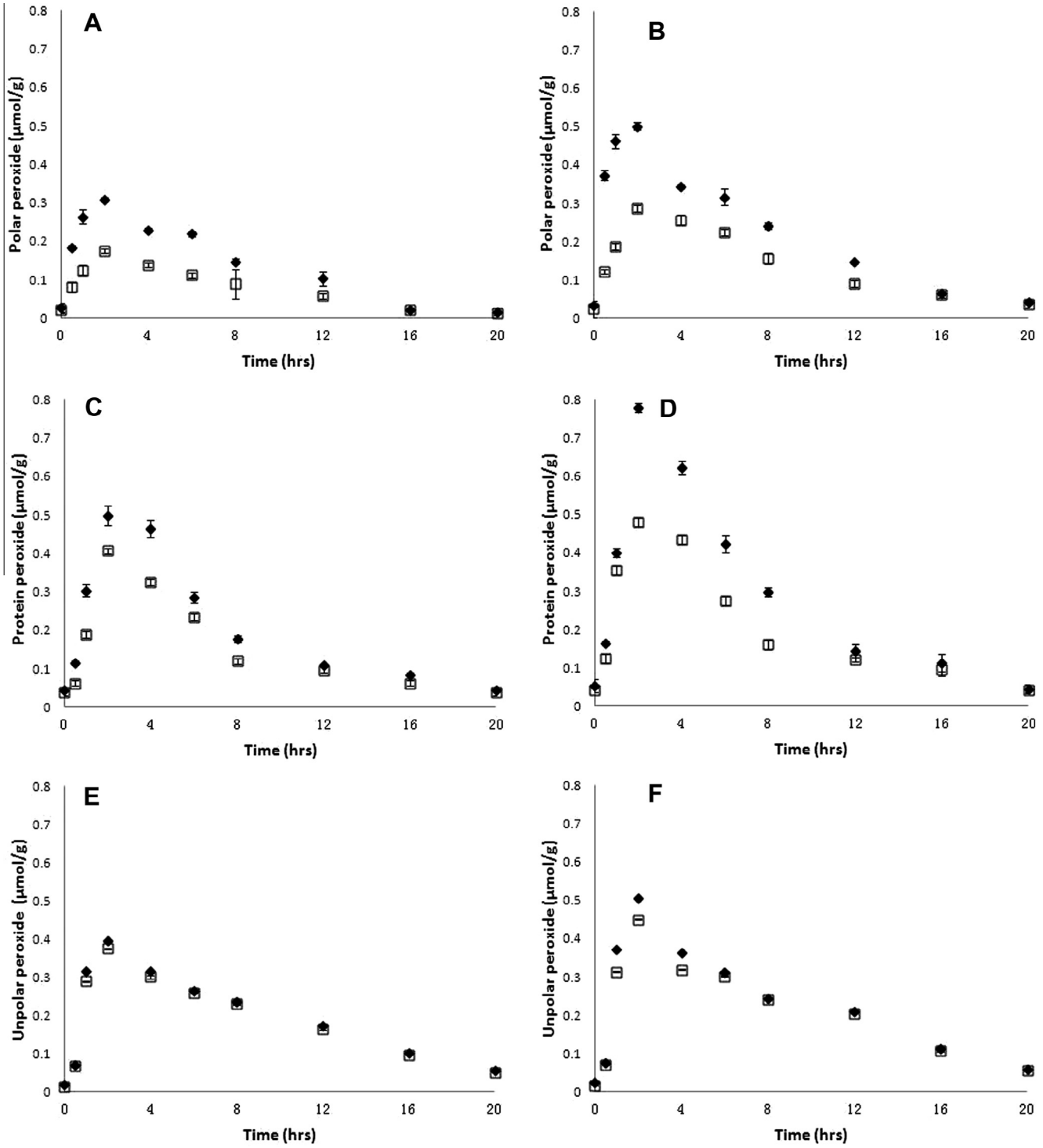

Fig. 1. Peroxides changes of phases over time in raw beef and chicken samples. Closed rhombuses are beef samples and opened squares are chicken samples. (A) Polar peroxide changes over time in polar phase without lipsomes. (B) Polar peroxide changes over time in polar phase with liposomes. (C) Protein-bound peroxide changes over time in interphase without liposomes. (D) Protein-bound peroxide changes over time in interphase with lipsomes. (E) Non-polar peroxide changes over time in non-polar phase without liposomes. (F) Non-polar peroxide changes over time in non-polar phase with lipsomes.

was on average higher (>25%) than at the other incubation time points (Fig. 1). However, the polar peroxides increased the most (~30%, at average) with liposomes addition after 2–4 h. Addition of liposomes gave higher hydroperoxide values when added up to 12 h of incubation.

3.3. Effect of pH on hydroperoxide formation

Both beef and chicken homogenates were incubated for 2 h at pH 1.5, 3.5, 5.5 and 7, with or without liposomes, at 37 °C. Samples that were incubated at lowest pH had the lowest amount of peroxides for

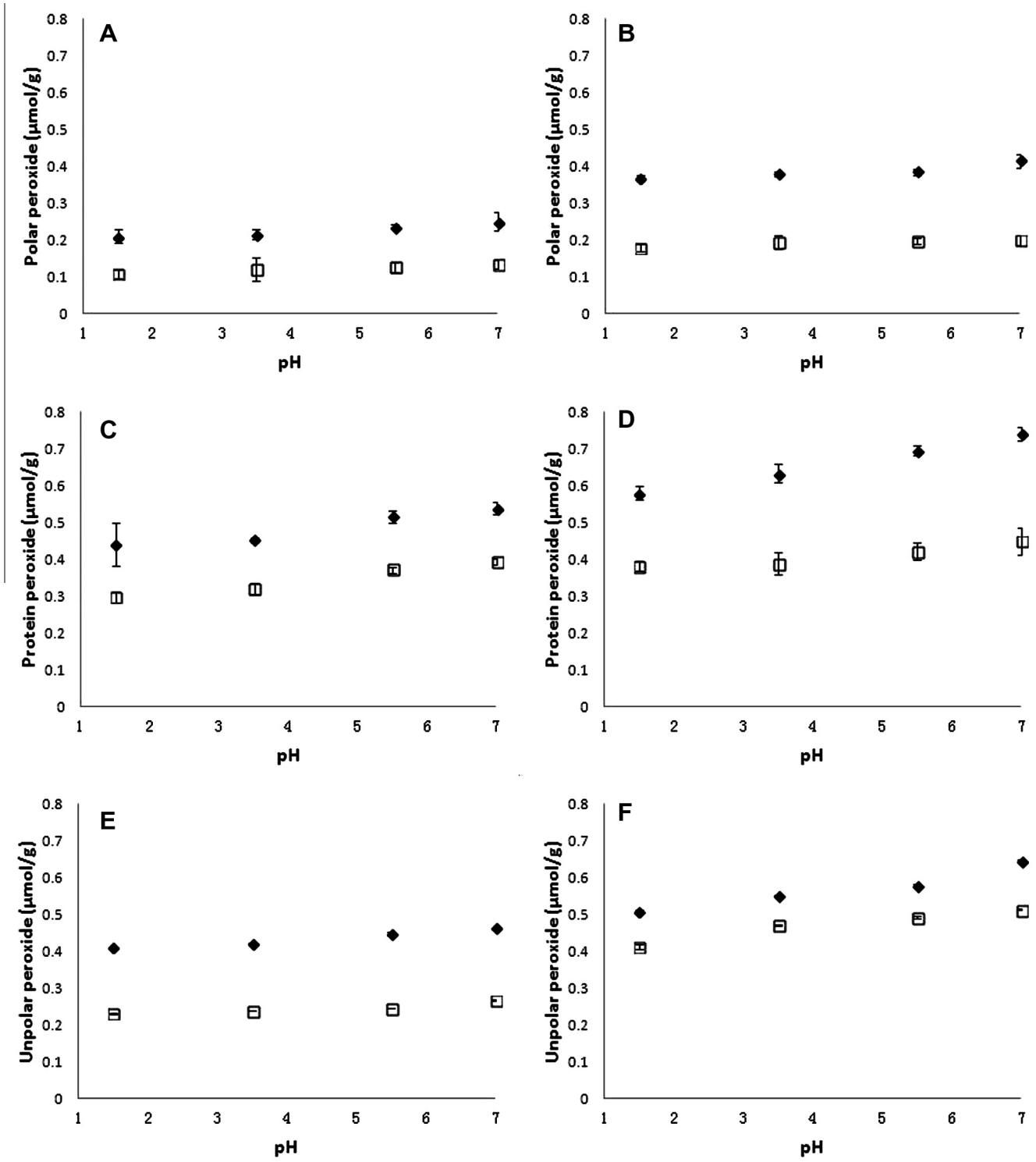

Fig. 2. Peroxide changes among phases at different pH in raw beef and chicken samples. Closed rhombuses are beef samples and open squares are chicken samples. (A) Polar peroxide changes at different pH in polar phase without liposomes. (B) Polar peroxide changes at different pH in polar phase with liposomes. (C) Protein-bound peroxide changes at different pH of interphase without liposomes. (D) Protein-bound peroxide changes at different pH of interphase with liposomes. (E) Non-polar peroxide changes at different pH in non-polar phase without liposomes. (F) Non-polar peroxide changes at different pH in non-polar phase with liposomes.

all phases (Fig. 2). The decrease in peroxides with pH was almost linear for both raw beef and chicken homogenates. In all extracted phases, incubated with or without liposomes, beef homogenates showed 1- to 2-fold higher hydroperoxide value than did chicken homogenates. All the meat homogenates samples incubated with liposomes showed 1.25- to 2-fold higher hydroperoxide values than did the extracted phases without liposomes. As reported previously, the addition of liposomes increased the amount of polar peroxides and protein-bound peroxides more than non-polar peroxides. The protein-bound peroxides depended most on pH, while the polar peroxides were the least pH-dependent.

3.4. Effect of washing times on the amount of protein-bound hydroperoxide

Washing of the protein interphase reduced the peroxide values. The reduction of peroxides by increasing washings in the system without liposomes was larger than the system with addition of liposomes. It should be noted that the reduction in protein-bound peroxides with 6 washings was 8% for systems with liposomes and 3.5% for systems without liposomes (Fig. 3).

3.5. Total peroxides in different species

The total amount of peroxides in meat was ranked as follows: beef > pork > lamb > chicken-LO group = chicken-SO group (Fig. 4). The peroxide values of the three extracted phases were correlated. This relationship (data from all species included) was stronger for the polar and protein-bound peroxides than for the non-polar peroxides. The hydroperoxide distribution varied from 13.9% to 22.3% in the polar phase, from 38.5% to 41.5% in the protein interphase and from 39.2% to 45.6% in the non-polar phase, using data from all five animal groups without liposomes. The hydroperoxide distribution varied between 17.3% and 22.6% in the polar phase, between 36.4% and 44.4% in the protein interphase and between 35.4% and 45.5% in the non-polar phase in all five animal groups with liposomes. Polar peroxides were the lowest while the non-polar peroxides were the highest ($P < 0.001$).

The total hydroperoxide contents in the pork, lamb and beef muscles were 1.4- to 1.8-fold and 1.2- to 1.9-fold higher (with liposomes) than the average total amount of hydroperoxide in chicken muscles. Since the weight-ratio of protein to lipid was approximately 1.5:20, this suggested that the amount of peroxides

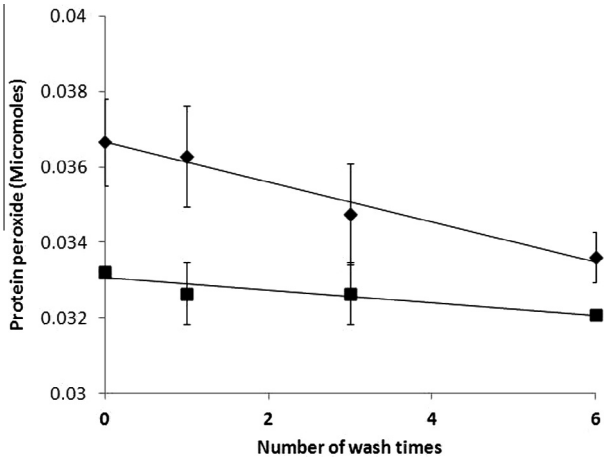

Fig. 3. Effect of multiple washings (x) on the protein-bound hydroperoxide values (y) without liposomes; rhombuses are beef samples ($y = -0.005x + 0.036$, $R^2 = 0.9768$) and squares are chicken samples ($y = -0.0002x + 0.0331$, $R^2 = 0.8571$). Note that the y-axis is not starting at 0.

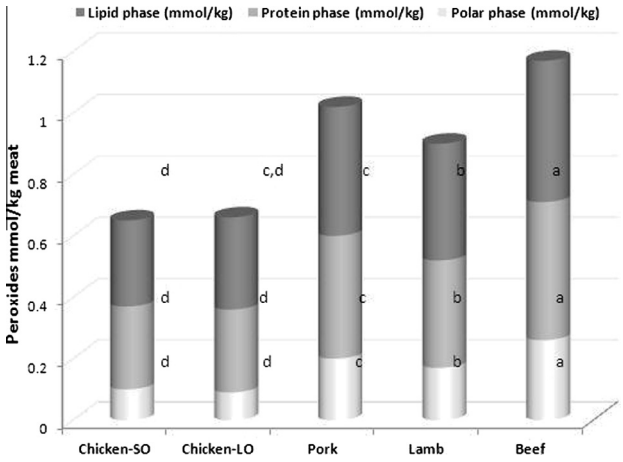

Fig. 4. Total amount of peroxides (mmol/kg meat) without liposomes in different animal species and their distributions among extracted phases. Peroxides from different extracted phases with different letters, across species, were significantly ($P < 0.05$) different. The statistics were only between species.

would be 10- to 15-fold higher per kg of lipid than per kg of protein. As the fat content, on average, was 1 mmol/kg (10 g/kg), Fig. 4 suggests that the lipid peroxides could be induced to contain 20–40 mmol peroxides/kg of meat lipid.

3.6. Conjugated dienes

Conjugated compound measurements of the polar phase at 268 nm were the only measurements that differed between the two chicken groups (Table 1). There were more conjugated compounds in the chicken-LO group that was fed on the diet that included 2.6% linseed oil, which is a rich source to generate more LC-PUFAs (Cleveland, Francis, & Turchini, 2012; Haug, Nyquist, Mosti, Andersen, & Hoestmark, 2012). There was also a tendency for the same chicken-LO group to give more lipid peroxides ($P = 0.067$).

3.7. Hemin and fat composition of the selected meat

The hemin contents of the muscles were in the following order: beef > lamb > pork > chicken-SO group = chicken-LO group (Table 1). The PUFA contents (g/100 g meat) of the muscles were as follows: chicken-LO > pork > chicken-SO = lamb > beef (Table 1). For long chain PUFAs the order was: chicken-LO group > chicken-SO group > lamb > beef = pork. There were some differences in fat content: pork had the highest amount and chicken-SO group had the lowest amount of fat (Table 1). When liposomes were added before incubation for PV measurements, the endogenous fat varied from 38% (pork samples) to 18% (chicken-SO group samples).

3.8. Relationships between peroxides, hemin and fatty acid compositions

The PCA plot (Fig. 5) was calculated with the amounts of unsaturated fatty acids, the more frequent monounsaturated fatty acids, total amount of fat, conjugated compounds, hemin concentrations and the determined peroxide values. The outlier was a pork sample which had a high content of intramuscular fat and belonged to the heaviest pig of the group.

Total amount of fat was, however, not a robust predictor of peroxides; *i.e.* Fig. 5 would not be different, whether the pork sample with the highest fat content was included in the regression or not. Hemin, conjugated compounds, peroxides and C20:5 *n*-3 plus

Table 1
Fat content, selected fatty acid variables, hemin concentration of lean meat and phospholipids used, plus conjugated trienes of polar (water/methanol) phase of meat.

Species	Fat (g/100 g)	PUFAs (g/100 g fat)	PUFAs (g/100 g meat)*	C18:2*n*-6 (g/100 g meat)	C18:3*n*-3 (g/100 g meat)	LC-PUFAs** (g/100 g meat)	CC 268 nm*** (μmol/g)	Hemin (g/kg meat)
Chicken-SO	0.90 ± 0.15[b]	34.6	0.312 ± 0.047[a,b]	0.189 ± 0.039[a,b]	0.012 ± 0.004[b]	0.110 ± 0.007[a,b]	0.188 ± 0.002[a]	0.04 ± 0.004[d]
Chicken-LO	1.19 ± 0.54[b]	32.4	0.387 ± 0.156[a]	0.176 ± 0.084[b]	0.089 ± 0.070[a]	0.121 ± 0.010[a]	0.191 ± 0.002[b]	0.04 ± 0.004[d]
Pork	2.43 ± 1.10[a]	15.3	0.372 ± 0.116[a]	0.266 ± 0.095[a]	0.014 ± 0.008[a]	0.090 ± 0.014[c]	0.209 ± 0.002[c]	0.07 ± 0.004[c]
Lamb	1.50 ± 0.43[b]	20.8	0.312 ± 0.060[a,b]	0.151 ± 0.038[b,c]	0.043 ± 0.013[a,b]	0.116 ± 0.016[a]	0.211 ± 0.002[d]	0.17 ± 0.004[b]
Beef	1.33 ± 0.66[b]	17.0	0.227 ± 0.032[b]	0.099 ± 0.022[c]	0.029 ± 0.013[b]	0.097 ± 0.015[b,c]	0.237 ± 0.002[e]	0.24 ± 0.004[a]
Egg	95	20.7	19.7	16.3	0	3.4	N.m.*****	N.m.*****
PC****								

* Data in each column with different superscripts are significantly different ($P < 0.05$).
** LC-PUFA is fatty acid with chain length >20 *cis* with two or more double bonds).
*** Conjugated trienes of polar (water/methanol) after 2 h of incubation.
**** Used in liposomes (Avanti polar lipids, Inc., Alabama, USA).
***** N.m. = not measured.

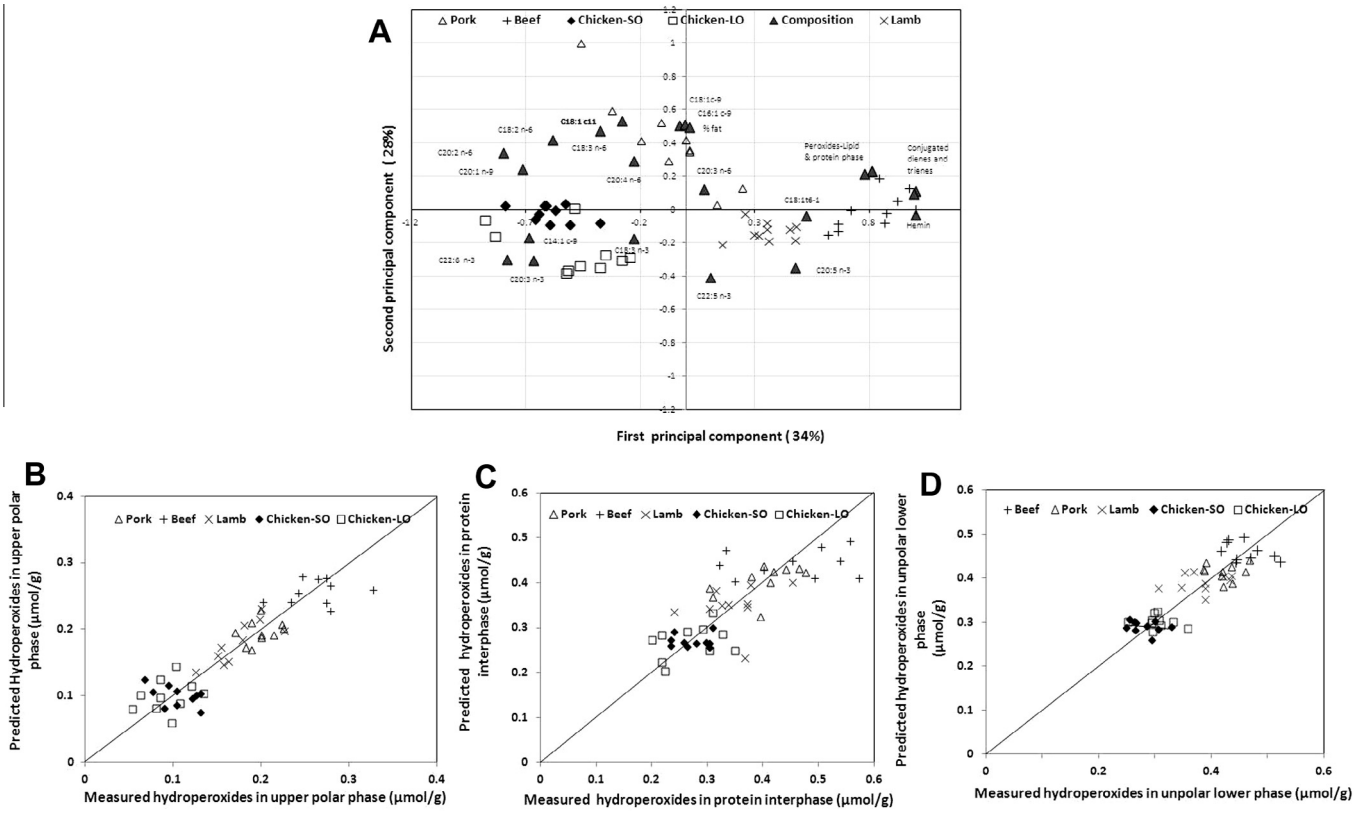

Fig. 5. (A) Principal component (PC) analysis biplot of fatty acids, total fat, hemin, peroxides and conjugated compounds, as well as samples (rhombuses are chicken-SO, opened triangles are pork, plus signs are beef, opened squares are chicken-LO and crosses are lamb species). (B–D) Measured versus predicted values (from the partial least square regression model with six factors) for the peroxides of the different extracted phases: polar peroxides, protein-bound peroxides and non-polar peroxides, respectively. The variables were weighted by 1/standard deviation before carrying out the regression.

C18:1 t6–t11 were the most characteristic components clustering closest to beef meat when the first principal component was plotted against the second principal component (Fig. 5A).

The amount of peroxides was significantly related to hemin level for all extracted phases. For the polar peroxides, 60.7% of the variation in peroxides could be attributed to variation in hemin content. The variation in the protein-bound and lipid peroxides (as opposed to the polar peroxides) depended relatively more on the presence of specific (amounts of) fatty acids.

There were only significant ($P < 0.05$) univariate relationships between induced peroxides (all extracted phases) for a few fatty acids. For example, between the level of C22:6 *n*-3 and the amount of polar peroxides a significant and negative relationship was found. But the level of C22:6 *n*-3 correlated negatively ($P < 0.001$) with hemin level (Fig. 5A, hemin concentration is located opposite to C22:6 *n*-3 concentration) as the species (beef) highest in hemin was also lowest in C22:6 *n*-3. It is possible that C22:6 *n*-3 oxidation is hemin-catalysed, but in order to identify these meat samples with more C22:6, *n*-3 in combination with high hemin levels might be necessary, *i.e.* designed samples, to reduce/eliminate confounding patterns. This was somewhat different for C20:5 *n*-3 due to its higher (up to 0.029 g/100 g of meat) concentration in beef meat (Fig. 5A), as opposed to chicken meat (1/10 of beef value). Thus, the level of C20:5 *n*-3 related significantly and positively ($P > 0.001$) to the hemin level. C20:5 *n*-3 also related significantly to polar peroxides and protein-bound peroxides ($P = 0.013$ and

$P = 0.002$, respectively) while its relation to lipid peroxides in the non-polar phase was on the border of being significant ($P = 0.052$).

Many fatty acids were interrelated, as shown in Fig. 5A, and these made it difficult to identify specific fatty acids as important for peroxide formation in meat using univariate regression methods.

Multivariate regression (partial least square regression) was thus attempted between peroxides and fatty acid composition and hemin (Fig. 5B–D). Polar peroxides correlated with fatty acids and hemin, as indicated by the plotting predicted and measured values of polar peroxides (Fig. 5B; correlation $r = 0.91$). Hemin, C22:6 *n*-3 and C20:3 *n*-6 levels were important predictors of polar hydroperoxide formation. The non-polar peroxides gave similar results but included the fatty acid C20:5 *n*-3 (and C20:1n9) as a predictor of higher hydroperoxide levels (Fig. 5C, $r = 0.87$). The protein-bound peroxides were less well explained ($r = 0.76$) by measured variables but still with hemin as a dominant explanatory variable of peroxide formation. The pork sample had an indicated outlier sample (high in intramuscular fat) that was not removed. Despite the pork meat's limited variation in hemin, this variable (as content) still gave the largest influence on hydroperoxide formation, when studied in a separate pork model. The lamb samples were different from the others and their hemin level was no longer the largest predictor of hydroperoxide levels, and this system alone (10 samples) would not give any significant model to hemin level.

4. Discussion

4.1. FOX hydroperoxide assay adaptations

The FOX method, as set up in this study, can provide a convenient way to measure both lipid and protein-bound peroxides. The method used for hydroperoxide determination was adapted from that of Gay and Gebicki (2002a), with some modifications. The drying (concentration) step for non-polar phase was omitted, as there was no need for it. Also, perchloric acid was replaced with H_2SO_4, due to safety requirements in the laboratory. The assay was adapted to use a 2 ml Eppendorf tube due to the efficiency and convenience during the assay. Effendorf tubes were stable without chemical reactions and did not affect the optical readings in this assay (Ewald, 2010). The assay was designed to make it possible to calculate the total amount of peroxides in meat, as opposed to only the peroxides extracted in one specific solvent (Miyazawa, Yasuda, Fujimoto, & Kaneda, 1988; Schmedes & Hølmer, 1989). Thus, polar peroxides and protein-bound peroxides were included. The assay used in this study relates to the approach described by Volden et al. (2011), where the protein is left as an interphase between extracting solvents.

4.2. Hydroperoxide content of meat

Peroxides can be formed on several amino acid side chains but also on the protein backbone following exposure to reactive oxygen species. Detection of peroxides in a pure protein model system, using the FOX method, has been demonstrated (Gay & Gebicki, 2002a). These authors reported the presence of 0.44 mmol of peroxides/kg of ovalbumin when Rose Bengal was used to generate reactive oxygen species. They also reported that the amount of peroxides/kg of protein depended on the type of protein. There is, to our knowledge, no comparison between the method used by Morgan, Li, Jang, el Sayed, and Chan (1989) and ours regarding the amount of peroxides to be formed on proteins, but the amount of protein-bound peroxides measured here is in a range comparable to their values.

With regard to lipid peroxides, our values were on the high side if compared to the values normally given as 20–40 meqv peroxide/kg of oil (we only had, on average, about 1.5% w/w fat in the samples). But the determination of hydroperoxide is challenging because different types of hydroperoxide can be produced during the oxidation procedure (Bou et al., 2008). Many methods have been carried out to investigate lipid hydroperoxide in biological materials and foods (Dobarganes & Velasco, 2002; Gray & Monahan, 1992; Moore & Roberts, 1998) but the analysis is sensitive to different laboratory details (Bou et al., 2008). Thus our higher non-polar peroxide values could relate to the choice of analytical method. It has been claimed that the more traditional peroxide measurement loses peroxides during the assay (Meisner & Gebicki, 2009). This may explain why our values are relatively high. Regarding polar peroxides, it makes sense that these are the lowest, since the dry matter content of the water–methanol phase will be low.

4.3. The composition of the three extracted phases used for hydroperoxide determination

The polar phase contains degradation products from lipids (Volden et al., 2011) but it should also contain most of the water-soluble low molecular weight compounds (amino acids and peptides) in meat. For example, phenolic antioxidants would be present here. Most proteins will be present in the so-called protein-interphase, but also components that fail to dissolve in any of the other two (polar and non-polar) phases, or have a density that would be intermediate between the densities of the polar and non-polar phases (*i.e.* hemin). Highly non-polar components (lipids), plus components derived through oxidation that are still not soluble in the polar phase, will remain in the non-polar phase.

4.4. Hydroperoxide stability

Transient stability of lipid peroxides has been reported numerous times (Reeder & Wilson, 2001; Takahashia, Shibata, & Niki, 2001). Here we also report that protein-bound peroxides are transient, having a maximum value at 2–4 h from being subjected to oxygen. Since these samples were fresh meat kept at −80 °C under vacuum, we have to consider the sample as being kept anaerobic until incubated with access to oxygen at 37 °C. Addition of extra lipids as liposomes did not affect the transient nature of the peroxides. It should be pointed out that, with extended incubation time, the protein became more difficult to resolubilise, in agreement with the fact that protein crosslinking becomes likely when the peroxides decline (Gay & Gebicki, 2002b). When proteins crosslink, meat becomes tougher and the activities of proteases are reduced. Both processes will be negative for meat quality.

Incubations at lower pH gave consistently lower hydroperoxide values. The effect was largest on the protein-bound peroxides. Both the kinetics of formation and the stability of peroxides may change with lower pH (Gay & Gebicki, 2002b; Reeder & Wilson, 2001). Hemin-catalysed peroxidation is expected to provide more peroxides with lower pH (Gao, Song, Li, & Gao, 2009). The fact that this was not observed here may be due to the fact that the peroxide value had started to decline before 2 h had passed at the lower pH values. The pH effect was also smaller when compared with the effect of incubation time from 1 to 24 h.

4.5. Liposome addition

Addition of liposomes to meat systems is interesting because the liposomes can mimic cell membranes. The fact that protein-bound peroxides increased the most upon liposome addition, may suggest that the added phospholipids interacted with the liposomes. Similar interactions have been reported and ascribed to

electrostatic and hydrophobic interactions (Alipour, Suntres, Halwani, Azghani, & Omri, 2009). It is possible that the effect of multiple washings was partly due to peroxides in the liposomes that were removed, along with other components. However, the effect of washing was nevertheless small and even 10 washes with their removal of peroxides would only explain 1/3 of the increase in protein-bound peroxides upon incubation with liposomes.

4.6. Hydroperoxide formation in different meat species

Five groups of meat homogenates were incubated for 2 h, with or without liposomes. It became apparent that, for the lean meat used here, measuring only the lipid peroxides will give about 40% of the absolute value of peroxides. If there is a need to know the total amount of peroxides, at least protein-bound peroxides should be included.

This experiment was set up to examine fatty acids and hemin levels and to use these variables as predictors of oxidation. This is done in several model systems for accelerating oxidation (Bishov, Henick, & Koch, 1961; Oszmianski & Lee, 1991; Van Dyck, Verleyen, Dooghe, Teunckens, & Adams, 2005). The hemin content emerged as a significant predictor of peroxide formation. However, due to the fact that hemin level was correlated with the amount of many unsaturated fatty acids, it was difficult to identify the importance of specific fatty acids for hydroperoxide formation. This can be exemplified by the fact that C22:6 *n*-3 was a reducer of peroxides in some models due to its correlation with low hemin levels of the biological samples. Nevertheless, our data suggested that the hemin level alone would account for about 60% of the variation in peroxides in commercial meat. By including information about the variation in fatty acid composition, close to 70% of the variation was accounted for. This can explain why beef meat produced more peroxides than did chicken meat, despite the fact that the latter meat had a higher amount of polyunsaturated lipids.

The difference between lamb and pork seemed due to either more efficient fat-soluble antioxidants in lamb meat or a lamb myoglobin that is less pro-oxidative than is pork myoglobin. In addition, the pork samples contained more fat than did lamb samples and that tended to be important for peroxidation of the pork samples.

4.7. Relevance for meat quality and health issues

The peroxide formation ability is relevant to meat quality as it precedes off-flavour formation and protein crosslinking to give tougher meat. In addition, peroxide formation could exhaust the presence of antioxidants in the reduced state.

Angeli et al. (2011) indicated that peroxides originating from lipids and the heme group could be factors that could contribute to cell cytotoxicity. These authors suggested that concentrations higher than 0.1 mM of lipoperoxides would exert toxic effects on cells. According to our data, this concentration is exceeded in all our meat systems, even if only lipid peroxides are accounted for. However, when meat is consumed, it is normally diluted and, in addition, it is heat-treated, except for dried meat products. Other factors, such as processing, storage, added ingredients, pro-oxidants, and antioxidants, are also very important for lipid oxidation (Ladikos & Lougovois, 1990). On the other hand, the results suggest that, in particular for Norwegian pork meat quality, it should be possible to improve it with respect to peroxide levels compared to lamb meat that had a higher hemin level.

5. Conclusion

The fraction of non-lipid hydroperoxide was 40–50% in lean meat. The FOX method ranked the total peroxide as follows: beef > pork > lamb > chicken groups. The lipid peroxide variations could largely (70%) be explained by the hemin level and the variation in fatty acid composition, while the protein-bound peroxides were less well explained by the hemin concentration.

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References

Alipour, M., Suntres, Z. E., Halwani, M., Azghani, A. O., & Omri, A. (2009). Activity and interactions of liposomal antibiotics in presence of polyanions and sputum of patients with cystic fibrosis. *PLoS ONE, 4*(5), 9.

Angeli, J. P., Garcia, C. C., Sena, F., Freitas, F. P., Miyamoto, S., Medeiros, M. H., et al. (2011). Lipid hydroperoxide-induced and hemoglobin-enhanced oxidative damage to colon cancer cells. *Free Radical Biology & Medicine, 51*(2), 13.

Bishov, S. J., Henick, A. S., & Koch, R. B. (1961). Oxidation of fat in model systems related to dehydrated foods. II. Composition and position of dispersed lipid components and their effect on oxidation rates. *Journal of Food Science, 26*(2), 6.

Bou, R., Codony, R., Tres, A., Decker, E. A., & Guardiola, F. (2008). Determination of hydroperoxides in foods and biological samples by the ferrous oxidation–xylenol orange method: A review of the factors that influence the method's performance. *Analytical Biochemistry, 377*(1), 15.

Cleveland, B. J., Francis, D. S., & Turchini, G. M. (2012). Echium oil provides no benefit over linseed oil for (*n*-3) long-chain PUFA biosynthesis in rainbow trout. *Journal of Nutrition, 142*(8), 7.

Davies, M. J., Fu, S. L., & Dean, R. T. (1995). Protein hydroperoxides can give rise to reactive free radicals. *Journal of Biochemistry, 305*(Pt 2), 7.

Dobarganes, M. C., & Velasco, J. (2002). Analysis of lipid hydroperoxides. *European Journal of Lipid Science and Technology, 104*(7).

Ewald, K. (2010). Chemical stability of consumables. *Liquid Handling, 5*(023), 4.

Fearon, E. R., & Jones, P. A. (1992). Progressing toward a molecular description of colorectal cancer development. *The FASEB Journal, 6*(10), 8.

Ferguson, L. R. (2010). Dietary influences on mutagenesis—Where is this field going? *Environmental and Molecular Mutagenesis, 51*(8–9), 10.

Gao, P., Song, Y., Li, H., & Gao, Z. (2009). Efficiency of methemoglobin, hemin and ferric citrate in catalyzing protein tyrosine nitration, protein oxidation and lipid peroxidation in a bovine serum albumin–liposome system: Influence of pH. *Journal of Inorganic Biochemistry, 105*(5), 8.

Gay, C. A., Collin, J., & Gebicki, J. M. (1999). Determination of iron in solutions with the ferric–xylenol orange complex. *Analytical Biochemistry, 273*(2), 6.

Gay, C. A., & Gebicki, J. M. (2002a). Measurement of protein and lipid hydroperoxides in biological systems by the ferric–xylenol orange method. *Analytical Biochemistry, 315*(1), 7.

Gay, C. A., & Gebicki, J. M. (2002b). Perchloric acid enhances sensitivity and reproducibility of the ferric–xylenol orange peroxide assay. *Analytical Biochemistry, 304*(1), 5.

Gebicki, S., & Gebicki, J. M. (1993). Formation of peroxides in amino acids and proteins exposed to oxygen free radicals. *Journal of Biochemistry, 289*(Pt 3), 7.

Ginevra, L. B., Beatriz, M. D., Altero, A., & Francisco, R. L. (2002). Optimization of heme iron analysis in raw and cooked red meat. *Food chemistry, 78*(4), 6.

Gray, J. I., & Monahan, F. J. (1992). Measurement of lipid oxidation in meat and meat products. *Trends in Food Science & Technology, 3*, 5.

Halliwell, B., & Chirico, C. (1993). Lipid peroxidation: Its mechanism, measurement, and significance. *American Journal of Clinical Nutrition, 57*(5), 10.

Haug, A., Nyquist, N. F., Mosti, T. J., Andersen, M., & Hoestmark, A. T. (2012). Increased EPA levels in serum phospholipids of humans after four weeks daily ingestion of one portion chicken fed linseed and rapeseed oil. *Lipids in Health and Disease, 11*(104), 11.

Kuffa, M., Priesbe, T. J., Krueger, C. G., Reed, J. D., & Richards, M. P. (2009). Ability of dietary antioxidants to affect lipid oxidation of cooked turkey meat in a simulated stomach and blood lipids after a meal. *The Journal of Functional Foods, 1*(2), 9.

Ladikos, D., & Lougovois, V. (1990). Lipid oxidation in muscle foods: A Review. *Food Chemistry, 35*(4), 20.

MacLennan, R. (1997). Diet and colorectal cancer. *International Journal of Cancer Supplement, 10*, 3.

Meisner, P., & Gebicki, J. L. (2009). Determination of hydroperoxides in aqueous solutions containing surfactants by the ferrous oxidation–xylenol orange method. *Acta Biochimica Polonica, 56*(3), 5.

Miyazawa, T., Yasuda, K., Fujimoto, K., & Kaneda, T. (1988). Chemiluminescence-high performance liquid chromatography of phosphatidylcholine hydroperoxide in human blood plasma. *Basic Life Sciences, 49*(4), 191.

Moore, K., & Roberts, L. J. (1998). Measurement of lipid peroxidation. *Free Radical Research, 28*, 13.

Morgan, J. E., Li, P. M., Jang, D. J., el Sayed, M. A., & Chan, S. I. (1989). Electron transfer between cytochrome A and copper A in cytochrome C oxidase: A perturbed equilibrium study. *Biochemistry, 28*(17), 9.

O'Fallon, J. V., Busboom, J. R., Nelson, M. L., & Gaskins, C. T. (2007). A direct method for fatty acid methyl ester synthesis: Application to wet meat tissues, oils and feedstuffs. *Journal of Animal Science, 85*(6), 11.

Oszmianski, J., & Lee, C. Y. (1991). Enzymatic oxidation of phloretin glucoside in model system. *Journal of Agriculture and Food Chemistry, 39*(6), 3.

Reeder, B. J., & Wilson, M. T. (2001). The effects of pH on the mechanism of hydrogen peroxide and lipid hydroperoxide consumption by myoglobin: A role for the protonated ferryl species. *Free Radical Biology & Medicine, 30*(11), 8.

Salminen, H., & Heinonen, M. (2008). Plant phenolics affect oxidation of tryptophan. *J Agric Food Chem, 56*(16), 10.

Sattar, A. J. M., & Deman, J. C. A. (1976). Light-induced oxidation of edible oils and fats. *Lebensmittel-Wissenschaft and Technologie, 9*(3), 4.

Schmedes, A., & Hølmer, G. (1989). A new thiobarbituric acid (TBA) method for determining free malondialdehyde (MDA) and hydroperoxides selectively as a measure of lipid peroxidation. *Journal of the American Oil Chemists Society, 66*(6), 5.

Sesink, A. L., Termont, D. S., Kleibeuker, J. H., & Van der Meer, R. (1999). Red meat and colon cancer: The cytotoxic and hyperproliferative effects of dietary heme. *Cancer Research, 59*(22), 6.

Takahashia, M., Shibata, M., & Niki, E. (2001). Estimation of lipid peroxidation of live cells using a fluorescent probe, diphenyl-1-pyrenylphosphine. *Free Radical Biology & Medicine, 31*(2), 11.

Uc, A., Stokes, J. B., & Britigan, B. E. (2004). Heme transport exhibits polarity in Caco-2 cells: Evidence for an active and membrane protein-mediated process. *American Journal of Physiology. Gastrointestinal and Liver Physiology, 287*(6), 8.

Van Dyck, S. M. O., Verleyen, T., Dooghe, W., Teunckens, A., & Adams, C. A. (2005). Free radical generation assays: New methodology for accelerated oxidation studies at low temperature in complex food matrices. *Journal of Agriculture and Food Chemistry, 53*(4), 6.

Volden, J., Bjelanovic, M., Vogt, G., Slinde, E., Skaugen, M., Nordvid, B., et al. (2011). Oxidation progress in an emulsion made from metmyoglobin and different triacylglycerols. *Food Chem, 128*(4), 10.

WCRF (2007). *Food, nutrition, physical activity, and the prevention of cancer: A global perspective*. Washington, DC: WCRF and American Institute for Cancer Research (p. 537).