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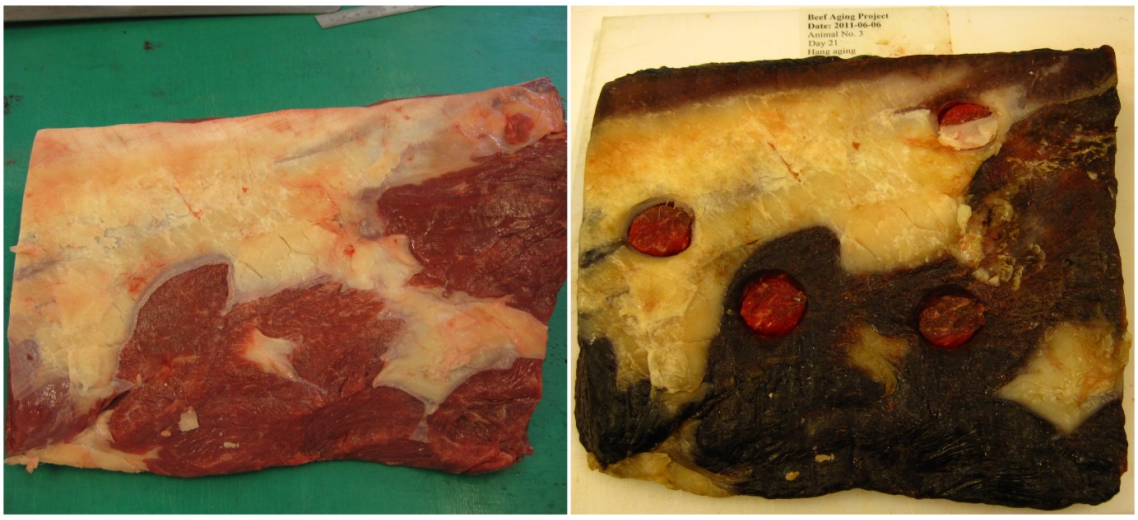

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Lipidoxidation i nötkött under olika lagringsbetingelser: Mätningar med statisk head-space gaskromatografi och 2-tiobarbitursyrareaktiv substans (TBARS)

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Lipid oxidation in differently aged beef: use of static headspace gas chromatographic and 2-thiobarbituric acid reactive substance (TBARS) methods

Abstract
This thesis assesses and compares lipid oxidation in beef samples from different ageing approaches by using static headspace gas chromatographic method and 2-thiobarbituric acid reactive substances. Beef samples were compared between three different ageing methods: hang ageing (traditional dry ageing); vacuum bag ageing (wet ageing); and tublin bag ageing and two ageing durations, 10 days or 21 days. Gas chromatographic and spectrophotometric methods were utilized for quantifying volatile lipid oxidation products and TBARS, respectively. Results showed that beef from hang ageing had lower TBARS values and volatile lipid oxidation products than the other two ageing methods irrespective of ageing time. The longer ageing time yielded higher TBARS values and volatile compounds in all ageing methods. Among the volatile lipid oxidation aldehydes, hexanal was the most predominant one.

Key words: lipid oxidation, beef ageing, TBARS, static headspace gas chromatography
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Introduction

The flavor of beef is dependent upon many factors. The post-mortem ageing process is an important process in which many flavor components are formed and proteins are broken down which results in increased tenderness during ageing of meat (Spanier *et al.*, 1997). In the traditional dry ageing method, the beef carcass is hung or the more valuable muscles are placed on a rack to age after the animal is slaughtered and cleaned, which requires control over temperature, humidity and air-flow (Ahnström *et al.*, 2006). Since the 1960s, vacuum ageing (wet ageing) has become the prevailing ageing method because of its convenience during storage and transport and low ageing loss (Warren & Kastner, 1992). Sensory analyses between dry ageing and vacuum ageing have turned out mixed results. Some studies have shown that dry ageing induces more desirable flavor (Warren & Kastner, 1992; Campbell *et al.*, 2001), whereas the same conclusion was not found by others (Oreskovich *et al.*, 1988; Parrish *et al.*, 1991). Nevertheless, vacuum aged beef inclines to have a bloody flavor, whereas dry aged beef has a brown roasted beefy flavor (Hodges *et al.*, 1974). The reason for these differences might be that consumers are more familiar with the vacuum aged beef since it is the most commonly available type of aged meat. However, in some studies, no significant tenderness differences were found between vacuum and hang aged meat (Warren & Kastner, 1992; Minks & Stringer, 1972). Generally, the traditional dry ageing products have higher price due to the strict environment control and the higher weight loss during the process (DeGeer *et al.*, 2009). Recently a water vapor-permeable dry ageing bag was introduced to the market, which intends to improve the traditional unpackaged dry ageing process (Ahnström *et al.*, 2006). Beef aged with the ageing bag are expected to have the same sensory quality as beef from traditional unpackaged dry ageing method, yet with less weight loss, lower risk of microbial contamination, and brings the advantages of convenient storage (Ahnström *et al.*, 2006; DeGeer *et al.*, 2009).

Lipids are important constituents in meat and meat products: they enhance the flavor and aroma characteristics of meat products and also enhance the tenderness and juiciness of meat.

On the other hand, lipid oxidation and the changes associated with lipids are the major cause of meat food quality deterioration. These quality losses are related to nutrient loss, food safety, biological damage, flavor deterioration, aging and functional property, hence they have gained much attention in food science studies (Frankel, 1984). In addition, end products of lipid oxidation may be mutagenic and carcinogenic, such as malondialdehyde (MDA), which can react with DNA and form MDA adducts (Marnett, 1999). The development of lipid oxidation in meat starts at the time of slaughter and continues during storage. Low temperature and oxygen free containers cannot stop the oxidation completely, but may however retard the process (Jensen *et al.*, 1998).

The mechanism of lipid oxidation can be interpreted as a chain reaction where lipid radicals play a key role. During initiation, a labile hydrogen from a fatty acyl chain is abstracted by a hydroxyl radical, which produces a free lipid radical that rapidly reacts with oxygen and forms a peroxyl radical. The peroxyl radical then abstracts a labile hydrogen from another hydrocarbon chain yielding a hydroperoxide and a new free radical which can proceed and sustain the chain reaction (Pearson et al., 1977; Ladikos & Lougovois, 1990). With further free radical propagation and non-radical products formed, the decomposition of lipid hydroperoxides yields rancidity-causing mixtures including aldehydes, hydrocarbons, ketones, esters and lactones (Frankel, 1987). Linoleic, linolenic and oleic are the main unsaturated fatty acids in animal tissues and their autoxidation together with other different decomposition pathways result in a variety of hydroperoxides, which lead to formation of volatile compounds (Love & Pearson, 1971).

There are various analytical methods that have been developed to determine lipid oxidation products in foods. Among them, gas chromatographic (GC) techniques are substantially more accurate and specific for certain compounds of interest. However, these techniques are more time consuming, require precise control of the experimental practice and the data processing is complex (Barriuso *et al.*, 2013). Several approaches may be adopted to recover volatile oxidation compounds before chromatographic analysis, such as solvent extraction, static headspace and dynamic headspace (Robards *et al.*, 1988). Same authors also suggested that gas chromatographic headspace analysis determines volatile lipid oxidation products, which are highly correlated to flavor deterioration. In static GC headspace analysis, volatile lipid oxidation products from the heated sample reach equilibrium between the sample and gas phase in a sealed vial, then an aliquot of headspace is injected into a gas chromatography for

analysis (Kolb & Ettre, 2006). While in dynamic headspace, instead of reaching the equilibrium between sample and gas phase, the sample is constantly purged by inert gas and passes through a porous trap that collects volatile compounds before the final chromatography analysis (Nielsen & Jacobsen, 2009). Given its advantages of being easy to perform, not requiring solvent extraction and being cheaper than dynamic headspace, static headspace is a very advisable method when there are many samples to be analyzed (Joaquin et al., 2008). However, applying static headspace, the sensitivity is limited as when equilibrium is established between the volatile compounds and remaining in the sample, hence comparatively low quantities of compounds are actually recovered (Barriuso *et al.*, 2013).

The 2-thiobarbituric acid reactive substance (TBARS) test is one of the most frequently employed methods for assessing lipid oxidation in muscle foods. Reacting with minor lipid oxidation products (predominantly malonaldehyde), 2-thiobarbituric acid produces a colored complex with an absorption maximum at 530-532nm (Melton, 1983). The intensity of the color complex detected from the reaction of TBA reagent and meat sample is adopted as the indicator of lipid oxidation. The TBARS test has limitations because many substances other than lipid oxidation products react with the reagent (Gutteridge & Halliwell, 1990). However, if all TBARS analyses are performed by the same method for all samples in a batch, the change in TBARS result can show the relative amount of lipid oxidation among the samples from different treatments (Gray & Monahan, 1992). Notably, correlative data from other indices of lipid oxidation would be profoundly helpful to interpret TBARS test in food matrixes (Janero, 1990). The objective of the current study was to analyze the lipid oxidation products in differently aged beef using static headspace gas chromatographic and 2-thiobarbituric acid reactive substance methods.

Materials and methods

Sampling and preparation of beef meat

Eleven Swedish Red breed steers were slaughtered at a commercial slaughter plant with the average age of 24 months. In all animals the average conformation score was “O” and fatness score was “3” according to the EUROP schemes modified to the Swedish system. After storage at 4 °C for 48 h, the muscle *longissimus thoracis et lumborum* (LTL), which was approximately 55 cm in length, was removed from both sides of each carcass. LTL was further cut into 6 pieces (about 16 cm long for each) and assigned to three different ageing methods (tublin ageing bag, traditional ageing or vacuum package) and two different aging

times (10 or 21 days). In total, there were thus 66 samples from 6 treatments (three different ageing methods and two ageing duration) and eleven biological replicates from 11 steers. 11 replicates were analyzed for TBARS while only 5 and 1 replicates were used in headspace gas chromatography and fatty acid acids analysis, respectively.

Tublin bag ageing employed bags made out of polyamide mix and were 50 µm thick with water vapor transmission rate of 5000 g/m^2 (50µ pore size, /24h, 38 °C, 50% relative humidity). In traditional dry ageing treatment, samples were aged in the cooling room (4 °C) without packaging. Vacuum bags were 68 µm thick with the permeability (24 h, 23 °C, 0% relative humidity) of 20 cm^3/m^2 for O_2 and 100 cm^3/m^2 for CO_2. During the ageing process, all samples were placed on stainless steel gratings in darkness in a cooling room with an average temperature of 2.9 °C and an average humidity of 91%. All samples were flipped and rotated among shelf positions every day to minimize location effects. After completing the respective ageing process samples were stored at -80 °C until analyses.

Beef samples were removed one day before usage from -80 °C and thawed at 4 °C overnight prior to analyses. Samples were cut into small pieces and ground using homogenizer at the speed of level 4 for one minute (Robot Coupe, France,) in darkness and in a container covered with ice and stored in falcon tubes at -80 °C until analyses.

The 2-thiobarbituric acid reactive substances (TBARS) procedure

The TBARS method was performed according to a modified version of the method described by Salih *et al.*(1987) and Pikul *et al.* (1989). Before weighing, samples in falcon tubes were thawed at 4 °C overnight. Around one gram of ground beef was homogenized with 0.5 mL 0.2% butylated hydroxytoluene (Sigma, USA) and 5.0 mL of 10% TCA (Sigma, USA) in 0.2 M H_3PO_4 (Merck, Germany) for 30 seconds with the speed set of red using a homogenizer (Janke & Kunkel, France,). Homogenates were filtered (Munktell, 00K) into clean glass tubes and kept in darkness for 30 minutes. Duplicates samples (A and B) of 1.0 mL of filtrate were transferred into separate screw cap glass tubes, after which 1.0 mL of 0.02 M 2-thiobarbituric acid (Sigma, USA) was added to sample A as test sample and 1.0 mL of deionized water (Millipore Synergy 185, Germany) was added to sample B as blank sample. All tubes were thoroughly vortexed for about 10 seconds before placing in a water-bath (Grant instruments SS40-2, England) at 85 °C for 45 minutes. The absorbance of samples at 532 nm against air was recorded after they were cooled to room temperature. The absorbance values of TBA-MDA adduct in the sample were subtracted from the blank (Sample B) value. TBARS values

were calculated using 1,1,3,3-tetramethoxypropane (TMP) as a standard precursor of malonaldehyde. The standard curve was prepared using solution of 25 µM 1,1,3,3-tetramethoxypropane (Sigma, USA) was used to prepare dilutions ranging from 8.2 * 10^{-9} g/mL to 2.1 * 10^{-7} g/mL of concentration. Each TMP dilution was added with 10% (w/v) trichloroacetic acid (TCA) up to 5 mL, and mixed with 5 mL of 0.02 M TBA or Millipore water (blank). After incubation under the same condition of the samples, the absorbance values were measured at 532 nm and the blank values were subtracted from the sample absorbance values and used to plot the standard curve (Figure 1). MDA concentration was expressed as milligrams per kilogram meat as follows:

$$MDA\ concentration\ = \frac{MDA\ absorbance\ *\ total\ volume\ of\ sample}{sample\ weight}$$

The static headspace gas chromatography procedure

The volatile lipid oxidation products analysis was performed with a headspace autosampler (CTC analytics, MH 01-00B) attached to a gas chromatographic system (Varian CP-3800, Sweden) equipped with a 30 m*0.320 mm inner diameter, 1.00µm film DB-5 fused capillary column (Agilent Technologies, USA) and a flame ionization detector (FID).

Five grams of meat samples were sealed into a 20 mL headspace vial (Chromacol, USA) with 5 mL deionized water (Millipore Synergy 185, Germany) and agitated in the static headspace auto sampler at a constant temperature of 120 °C for 15 minutes, which is the temperature for which equilibrium in the gas phase was reached. An aliquot of the vial's gas phase was introduced by a heated 120 °C syringe (10 µl, SGE, Australia) into the carrier gas stream (helium) which carried it onto the column. Column temperature was increased linearly with 40 °C during 3 minutes, followed by an increase of 5 °C per minute until 180 °C; injector and FID temperatures were set at 250 °C and 270 °C, respectively. Flow rate of the helium carrier gas was 1.5 mL/min with an inlet pressure of 10 psi and a split injection ratio of 1:10. Blank vials were run between each sample to clean the column and alleviate carry-over effects.

Individual volatile compounds were tentatively identified by comparing their relative GC retention times with those of commercially available standards. To identify standard volatile compounds retention times, five microliter aliquots of each standard solutions (Sigma, USA) were diluted to 5 mL methanol (Sigma, USA) and 0.05 mL of which was further diluted with

0.5 mL deionized water (Millipore Synergy 185, Germany) to prepare a working solution of standard aliquot (100 ng/µL). Sample solution for gas chromatography was made by adding 60 µL of a working solution into 5 mL of deionized water in a headspace vial which gave a final concentration of 600 ppb for each standard.

To quantify the volatile lipid oxidation products in the meat samples, 20 µL of internal standard (4-methyl-2-pentanone) from working solution was added into 5 mL of deionized water in a headspace vial with 5 g of meat sample, yielded the internal standard concentration of 0.1602 µg/mL. Peak areas of internal standard and volatile compounds were integrated employing Galaxie chromatography data system software version 1.9 (Varian AB, Sweden). All samples were analyzed in duplicates with 11 biological replicates for each treatment.

Quantification of ***volatile lipid oxidation products***

Volatile lipid oxidation products were quantified using 4-methyl-2-pentanone as an internal standard with the assistance of 4-heptanone as a secondary reference to check analysis system stability. Different compounds have varied purge ability thus appear different heights of peaks in the gas chromatography machine even when they are in the same concentration. To compensate this purgibility variation, a calibration factor was calculated between each target compounds and the internal standard with known concentration. Calibration factors were calculated using the following formula:

$$Fi = \frac{Ci}{Cs} * \frac{As}{Ai}$$

Fi = Calibration factor of target compound; Ci = concentration of target compound; Cs = concentration of internal standard; As = area of internal standard; Ai = area of target compound

With the detected area of target compounds and added known amount internal standard, the amount of target compounds were calculated with the assistance of calibration factor employing the following formula:

$$Concentration = \frac{Fi * Ai * Cs}{As}$$

Lipid analysis

Extraction of lipids from meat

Total lipids of meat samples were extracted in hexane:isopropanol (3:2 v/v) with a modified method adapted from a previous study (Hara & Radin, 1978). One gram of ground meat sample was weighed and placed in glass tube with 10 mL hexane:isopropanol (HIP) (Sigma, USA) and homogenized for 3*30 seconds (5411 g) (ULTRA-TURRAX T25, IKA). The homogenizer was rinsed with HIP between samples. The homogenate was then quantitatively transferred to teflon centrifuge tubes using 5 mL HIP and 6.5 mL Na_2SO_4 (6.67% w/v). Samples were centrifuged at 716 g, 18 °C, for 5 min, after which the upper phase was removed to pre-weighed evaporation tubes using glass Pasteur pipettes. One milliliter of hexane was added to the centrifuge bottle and centrifugation was repeated. The upper phase from both centrifugations were then combined and evaporated at 40 °C with N_2 flushing for approximately 20 minutes until dried. Evaporation tubes were reweighed and the amounts of fat extracted were calculated. 0.5 mL hexane was added into the evaporation tubes before storage at -18 °C.

Methylation of fatty acids

The concentration of lipids dissolved in hexane was calculated before methylation, by microbalance weighing (Metller type UMT2, Switzerland). The methylation of fatty acids was done according to a modified method of Appelqvi(1968). Based on the microbalance lipid concentrations, required volumes of lipid solutions with 2 mg content were transferred to glass tubes with 2 mL methanol and 15 µL standard fatty acid solution (STD) (17:1), where STD (1.44 µg/µL) was used as an internal standard for gas chromatography. The glass tubes were vortexed and incubated in a heating block at 60 °C for 10 minutes. Three milliliters of BF3 were added to tubes and followed by incubation under the same conditions. Afterwards, the samples were cooled in ice box for 15 minutes, after which 2 mL 20% NaCl and 2 mL hexane were added. After 10 second vortexing, the tubes were stored at 4°C for 20 minutes. The upper phase was transferred to small glass vial with Pasteur pipettes and again stored at 4°C for 20 minutes. Transfer of the upper phase was repeated once more with 1 mL hexane added to the tube. The tubes were evaporated at 40 °C with N_2 until dried (approx 20 minutes). Finally, 300 µL aliquots of lipids were transferred into test tubes and kept at -80 °C until GC analysis.

Gas chromatographic analysis of fatty acids

Fatty acids were analyzed with a gas chromatographic system (Varian CP-3800, Sweden) with a flame ionization detector (FID) equipped with a 50 m*0.22 mm inner diameter, 0.25 μm film DB-5 fused capillary column (Agilent Technologies, USA). The column temperature was programmed to initiate at 158 °C for 5 minutes and increased by 2 °C/ minute up to 220 °C and remained for 8 minutes. The makeup gas was nitrogen and carrier gas was helium (0.8 mL/min). The injector and detector temperatures were 230 and 250 °C, respectively. Fatty acids were analyzed by comparing with the standard fatty acid solution (STD) and retention time. Chromatograms were analyzed using Galaxie chromatography data system software version 1.9 (Varian AB, Sweden).

Validation of methodology

The static headspace gas chromatography method and TBARS were validated by calculating linearity and repeatability.

Linearity suggests the ability of the method to acquire test results proportional to the concentration of the analyte. Linearity was calculated measuring the response of 6 concentration levels covering different range in gas chromatography or TBARS. Different analyte concentrations were plotted against the instrument response and the fitted line was characterized by a correlation coefficient (r). Two examples were shown in Figure 1 and Figure 4, which showed clear linearity in the concentration range tested.

The precision of the method was also evaluated by calculating the repeatability. To calculate repeatability, the internal standard or TMP solution were measured 10 or 6 times by the same experimental facilities, respectively.

Statistical analyses

The Statistical Analysis System (Version 9.2, SAS Institute, USA) was employed for statistical analyses. Experimental design was completely randomized. Unless otherwise stated, each animal (n=11) for each treatment (n=6) was considered a biological replicate, thus resulting in a total of 66 replicates. No statistical analysis employed for fatty acid analysis since the primary purpose of the procedure is to study the fatty acid composition. The MIXED procedure was used to calculate overall effect and pair-wise comparisons. Pearson and Spearman correlations were calculated with the CORR procedure using all individuals. All concentrations were log-transformed before statistical analysis in order to normalize residual

distributions. Data are reported as back-transformed least squares (LS) means with 95% confidence interval.

Results and discussion

Levels of TBARS in aged beef

The standard curve obtained for tublin bag day 10 samples is shown in Figure 1. One standard curve was plotted before the measurement of each treatment. TBARS values were calculated by substituting the absorbance from samples into the equation derived from the stand curve.

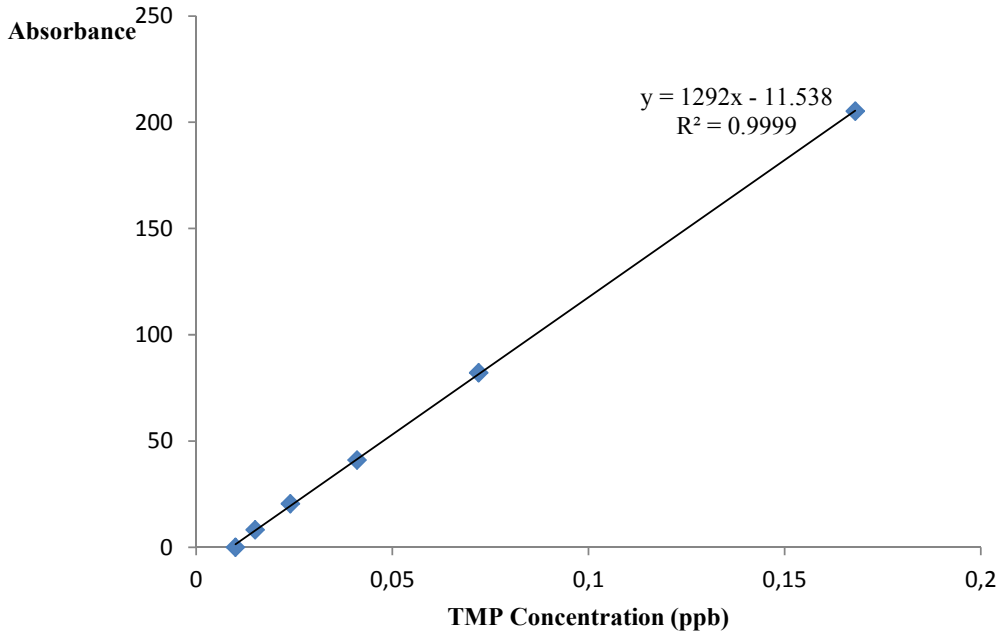


Figure 1. Standard curve obtained by using 1,1,3,3-tetramethoxypropane for beef aged according to the Tublin bag method

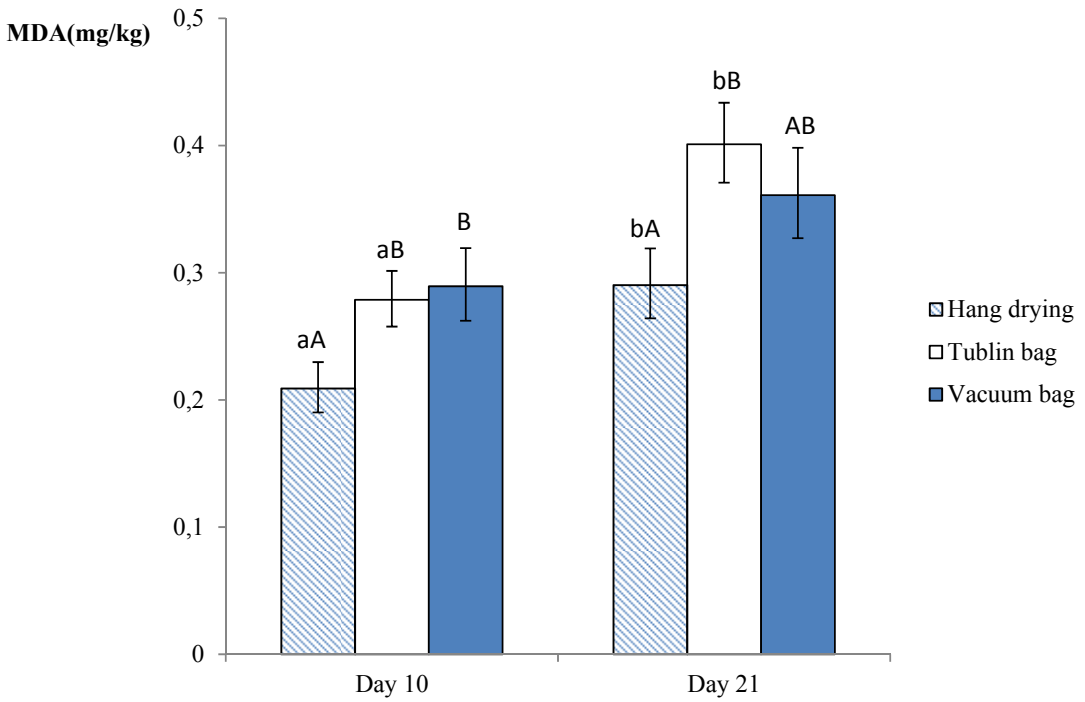
TBARS numbers were expressed as milligrams of malonaldehyde per kilogram of sample. The TBARS values varied from 0.212 to 0.421 mg/kg in the six treatments, with the lowest from traditional hang ageing on day-10 and highest from tublin bag ageing on day-21, respectively. The coefficient variation (CV) between duplicates was generally within acceptable range. 17 out of 66 (25.76%) samples had a CV higher than 15 (Table 1).

Table 1. *TBARS values (mg/kg) of beef aged according to three methods and two time periods*

	Day 10			Day 21		
ID	Vacuum	Tublin	Hang	Vacuum	Tublin	Hang
1	0.221	0.223	0.181	0.275	0.292	0.293*
2	0.244*	0.201	0.178	0.288*	0.314*	0.404
3	0.328	0.356*	0.254*	0.465	0.442	0.471
4	0.210	0.281	0.216	0.904	0.389	0.392
5	0.454	0.343*	0.271	0.330	0.513	0.591
6	0.285	0.323	0.238	0.353	0.591	0.239
7	0.190*	0.265*	0.161*	0.304*	0.738	0.173
8	0.297	0.278	0.264	0.317	0.380	0.201*
9	0.405	0.239	0.187*	0.314	0.274*	0.186*
10	0.244*	0.272	0.185*	0.278	0.286	0.230
11	0.444	0.330	0.199	0.415	0.410	0.268
Mean	0.302	0.283	0.212	0.386	0.421	0.314

Reported values represent an average of duplicates.
*CV ranged from 15 to 40, between duplicates.

As shown in Figure 2, TBARS values of beef aged according to the hang ageing method was significantly lower than that of other two treatments at day 10 ($P<0.05$). In day 21, hang ageing was significantly lower than tublin bag ageing ($P<0.05$) but not to vacuum ageing. Differences between two time durations for given method were significant in hang ageing and tublin bag ageing ($P<0.05$) but not in vacuum bag ageing. Similar conclusion was observed from King's study (1995), which showed lower hexanal, octanal and nonanal in hanging aged beef than vacuum packaged beef after heat treatment. The least square means of the six treatments values were shown in Figure 2.



[AB] Different superscripts in capital letters indicate significant difference between treatment at one single time at $p<0.05$. [ab] Different superscripts in simple letters indicate significant difference between ageing time for a given treatment at $p<0.05$.

Figure 2. Least square means of TBARS values from beef samples aged for 10 days and 21 days.

Static headspace volatile compounds in differentially aged beef

The retention time of each compound differs due to their own chemical properties and can thus be used to identify them by gas chromatography. Change in GC parameters can affect the retention times. Hence the method was optimized for separation, reproducibility and analytical response (see Materials and Methods). Investigated parameters included isothermal vs gradient heating, split ratio and injection volume. Retention times and peak areas of 14 commercially available standards were used as outcome parameters for the optimization (Figure 3). Though some peaks were not be able to identify in the chromatogram.

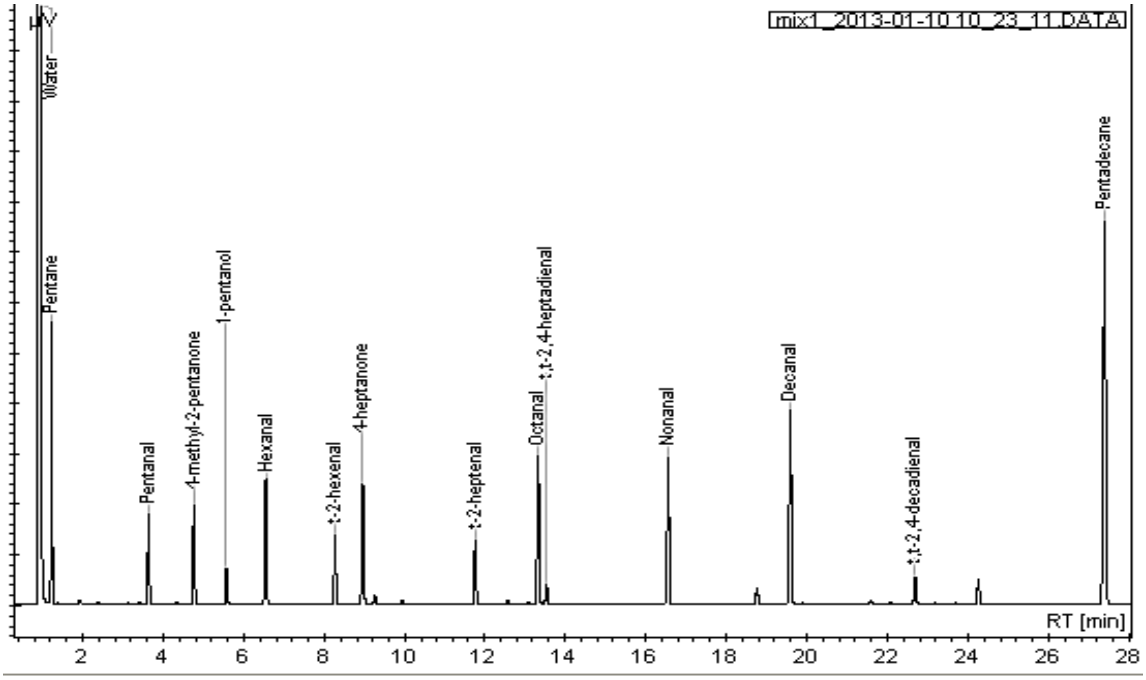


Figure 3. GC static headspace chromatogram of standards used for lipid oxidation volatiles analysis in beef.

4-methyl-2-pentanone was employed as internal standard in the static headspace gas chromatographic method. Since the internal standard is not naturally present in meat, the target analysis compounds could be normalized against the amount of internal standard measured in each sample to improve analytical accuracy and precision. In this case, a good linear response of internal standard is vital for accurate qualification. As shown in Figure 4, linear response was observed with an acceptable linearity.

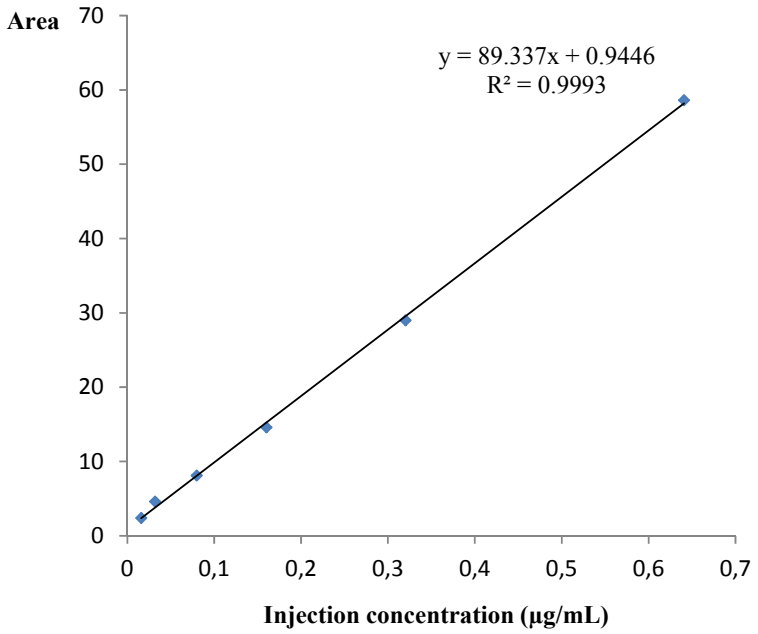


Figure 4. Linearity of 4-methyl-2-pentanone as internal standard for GC-headspace analysis of lipid oxidation volatiles analysis in beef.

Calibration factors

Calibration factor was induced to calculate the proportion between target analytes and the internal standard, which were adopted in the formula for compound concentration calculation. The calibration factors relative to each compound were shown in Table 2.

Table 2. *Calibration factors of standard compounds in relative to internal standard.*

Compound	Calibration factor[a]
4-Methyl-2-pentanone	1.00*
pentane	3.949
pentanal	1.668
1-pentanol	2.964
hexanal	0.754
t-2-hexenal	1.317
t-2-heptenal	1.279
octanal	0.513
nonanal	0.416
decanal	0.378
t,t-2,4-decadienal	1.792
pentadecane	0.382

*Internal standard

[a] Results from triplicates

Optimize internal standard concentration

The analytical protocol was optimized with regard to concentration of internal standard (Table 3). Addition of 20 uL of working solution provided satisfactory number of target peaks and accuracy.

Table 3. *Amount of internal standard added in samples and corresponding numbers of target volatile compounds detected.*

Amount of working solution (µL)	Numbers of target compounds detected
100	1
60	2
40	4
20	6
10	6
5	6

Quantification of volatile lipid oxidation products in beef samples

Due to the limited time and access to facility, five out of eleven biological replicates from each treatment were analyzed with the static headspace gas chromatographic method. Among all tested standard analytes, only pentane, hexanal, octanal and nonanal were detected and quantified in meat samples corresponding to respective retention times.

As shown in Table 4, except for pentane, all other volatile lipid oxidation products such as hexanal, octanal, nonanal were higher ($P<0.0001$) in vacuum bag aged beef samples compared to other ageing methods on day 10. The same trend was observed on day 21 samples with the probability of $P<0.05$. Hexanal, octanal, nonanal contents were higher ($P<0.05$) in vacuum bag aged beef samples on day 10, compared to day 21. The corresponding values were not significantly different in beef aged according to tublin bag and hang ageing methods.

Table 4. *Effect of ageing method and time on beef lipid oxidation (ppm).*

Compound		Vacuum	Hanging	Tublin	p-value (treatment)
Pentane	10 days	13.7[a] (12.3-15.4)	29.8[b] (27.5-32.3)	12.7[a] (12.1-13.3)	<0.0001
	21 days	10.9[a] (9.2-12.9)	25.7[b] (24.9-26.5)	15.7[a] (14.6-16.8)	0.0002
	p-value (time)	0.2023	0.1667	0.0060	
Hexanal	10 days	2.62[aA] (2.34-2.94)	0.134[b] (0.102-0.176)	0.150[b] (0.106-0.212)	<0.0001
	21 days	0.506[aB] (0.390-0.656)	0.137[b] (0.097-0.193)	0.154[b] (0.106-0.224)	0.0257
	p-value (time)	0.0002	0.9693	0.9636	
Octanal	10 days	1.79[aA] (1.60-2.00)	0.072[b] (0.054-0.096)	0.103[b] (0.059-0.182)	<0.0001
	21 days	0.450[aB] (0.356-0.473)	0.091[b] (0.064-0.131)	0.098[b] (0.068-0.141)	0.0065
	p-value (time)	0.0004	0.6859	0.9360	
Nonanal	10 days	1.45[aA] (1.29-1.62)	0.113[b] (0.094-0.137)	0.115[b] (0.095-0.138)	<0.0001
	21 days	0.476[aB] (0.414-0.546)	0.130[b] (0.117-0.145)	0.174[b] (0.163-0.186)	0.0002
	p-value (time)	0.0003	0.6134	0.0840	

Data are presented as back-transformed least square means with 95% confidence interval within parentheses. [ab] Different superscripts in simple letters within row indicate significant difference between treatment at one single time at $p<0.05$. [AB] Different superscripts in capitals within column for a specific lipid oxidation product indicate significant difference between ageing time for a given treatment at $p<0.05$.

Correlation with TBA values

Correlation analysis was performed between MDA numbers from the TBA method and observed target compounds from static GC-headspace. No significant correlations were found between MDA numbers and volatile analytes from GC analysis, while correlations between volatile lipid oxidation compounds were significant (Table 5). This could be due to TBARS is not as sensitive as gas chromatographic method, not only malonaldehyde but also other none specific compounds which are present in meat samples could react with 2-thiobarbituric acid hence could generate pink color yielding high absorbance value from the TBARS method.

Table 5. *Correlations between MDA numbers and volatile lipid oxidation products on log-transformed data. Pearson and Spearman correlations above and below the diagonal, respectively*

	MDA[a]	Pentane	Hexanal	Octanal	Nonanal
MDA[a]	-	-0.197	0.014	0.022	0.028
Pentane	-0.247	-	-0.285	-0.316†	-0.359†
Hexanal	0.105	-0.262	-	0.967***	0.929***
Octanal	0.135	-0.313†	0.956***	-	0.924***
Nonanal	0.247	-0.348†	0.871***	0.910***	-

[a] MDA = malonaldehyde

Correlations were significant at †: $p<0.10$; ***: $p<0.0001$.

Fatty acid composition

To better understand the mechanism of lipid oxidation, fatty acid composition in beef samples were analyzed using the methods described in material and method section. One representative sample from the same animal from each treatment was analyzed to generate an outline of fatty acid composition between ageing methods and ageing time. The most predominant fatty acids in the beef samples were oleic acid (C18:1(n-9)), palmitic acid (C16:0) and stearic acid (C18:0) (Table 6). Notably, linoleic acid (C18:2), which plays an important role in generating volatile oxidation products, was the predominant polyunsaturated fatty acid in all samples.

Table 6. *Fatty acid composition (%) in beef samples subjected to different ageing methods and times.*

Fatty acids	H10	T10	V10	H21	T21	V21
C14:0	2.26	2.09	1.91	1.95	2.15	1.81
C14:1	0.53	0.54	0.43	0.51	0.54	0.35
C15:0	0.34	0.35	0.34	0.31	0.39	0.31
C16:0	24.30	24.54	26.45	24.03	24.45	24.17
C16:1(n-7)	2.59	2.88	2.47	2.87	2.80	2.37
anteiso C17:0	0.58	0.50	0.54	0.57	0.65	0.49
C17:0	0.88	0.79	0.84	0.83	0.94	0.76
C18:0	14.94	13.89	14.49	14.36	15.00	14.34
C18:1(n-9 *cis*)	0.54	1.14	0.83	0.77	0.74	1.31
C18:1(n-11)	1.38	1.28	1.43	1.24	1.62	1.29
C18:1(n-9 *trans*)	38.37	37.30	38.00	40.31	38.56	37.66
CLA	1.23	1.18	1.16	1.23	1.13	1.17
C18:2(n-6)	3.26	3.94	2.98	2.78	2.71	4.27
C18:3(n-3)	1.18	1.38	1.16	1.08	1.11	1.50
C20:0	0.34	0.31	0.33	0.31	0.41	0.27
C20:3(n-6)	0.21	0.32	0.23	0.22	0.21	0.31
C20:4(n-6)	1.14	1.47	0.85	0.91	0.86	1.24
C20:5(n-3)	0.39	0.52	0.29	0.33	0.30	0.46
C22:5(n-3)	0.60	0.71	0.46	0.53	0.48	0.68
Total identified	95.06	95.13	95.19	95.13	95.04	94.76
SFA	43.65	42.47	44.89	42.37	43.99	42.15
MUFA	43.40	43.15	43.17	45.69	44.26	42.98
PUFA	8.01	9.52	7.13	7.07	6.79	9.63
PUFA n-3	2.17	2.62	1.91	1.93	1.88	2.64
PUFA n-6	4.61	5.73	4.07	3.91	3.78	5.82
n-6/n-3	2.13	2.19	2.13	2.03	2.01	2.20

[a] H, T, V represents hanging, tublin bag and vacuum bag, respectively. 10 and 21 represents ageing days in corresponding ageing method.

Comparison of volatile lipid oxidation products in differentially aged beef.

The MDA numbers of hang ageing samples were much lower than samples aged with tublin and vacuum bag, it was consistent with the results from GC-headspace procedure where aldehydes (hexanal, octanal and nonanal) quantity from hang ageing were much lower than those from tublin and vacuum bag ageing. Aldehydes such as hexanal and malonaldehyde are major secondary products of lipid oxidation from hydroperoxide outcome of polyunsaturated fatty acid such as linoleic acid (Figure 5), which are responsible for off-flavor and odors (Tims & Watts, 1958). It explains the consistency of lower MDA and aldehydes numbers from hang ageing method compared to other two methods. It may contrast to speculation that hang ageing should induce more oxidation products since samples were exposed to air in this condition, while the reason might be that the oxidized beef surface were removed before further preparation for technical analyze. Similar results can be concluded from a study (King

et al., 1995) which found lower hexanal, octanal and nonanal in hanging aged beef than vacuum packaged beef after microwave cooked. Furthermore, in the study of Xin (2013) which employed the same meat samples as in this project, higher sensory test scores were graded for hang ageing samples compared to the other two methods. It shows consistency because aldehydes are predominant volatile lipid oxidation compounds in meat products which are related to off-flavor. Other studies found few sensory panel differences among traditional and vacuum ageing (Dikeman *et al.*, 2013; Laster *et al.*, 2008; Warren & Kastner, 1992; Parrish *et al.*, 1991; Oreskovich *et al.*, 1988) and some found consumer preference for vacuum packaged beef over traditional aged one (Sitz *et al.*, 2006)

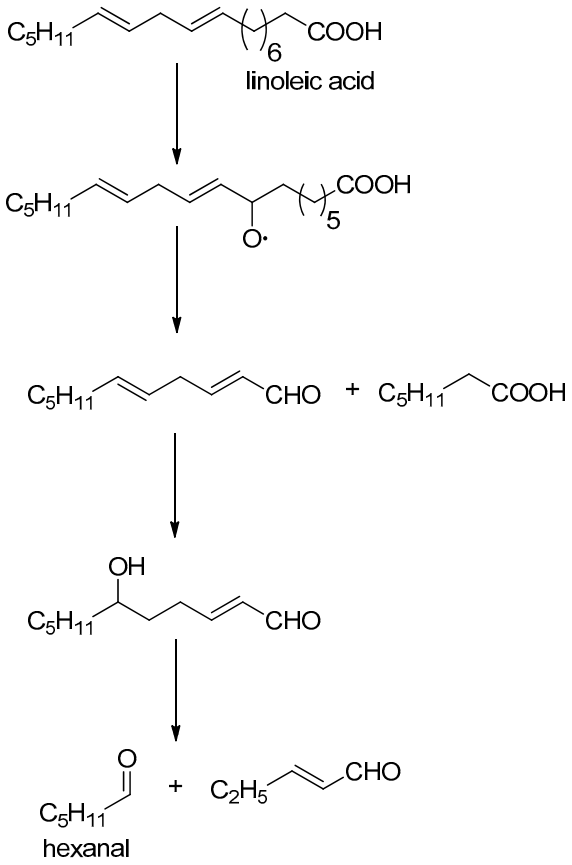


Figure 5. Possible pathway for generation of hexanal during oxidation of linoleic acid in beef (adapted from Spiteller, 2001)

Meanwhile, pentane value from hang ageing was significantly higher than from tublin and vacuum bags ageing. This can be explained by the mechanism of thermal release of pentane from unsaturated fatty acid in the meat. Unsaturated fatty acid such as linoleic acid develops

hydroperoxide through autoxidation, which further yields pentane on thermal decomposition (Evans *et al.*, 1969). The thermal decomposition happened assumingly in incubation process before purging volatile compounds into GC column, which was 120 °C for 15 minutes (Figure 6). The unsaturated fatty acids were close between samples of varied treatments judging from the fatty acid analysis result (Table 7), in hang ageing treatment, less unsaturated fatty acid were degraded into aldehydes, and more unsaturated fatty acid were left for thermal degradation to yield pentane. In gas chromatographic procedure, significantly lower aldehydes and higher pentane values from hang ageing compared to the other two ageing method could be explained with that theory. Thus it is conclusive that even though the yield of pentane is higher than other volatile compounds, it is not an ideal biomarker of lipid oxidation product.

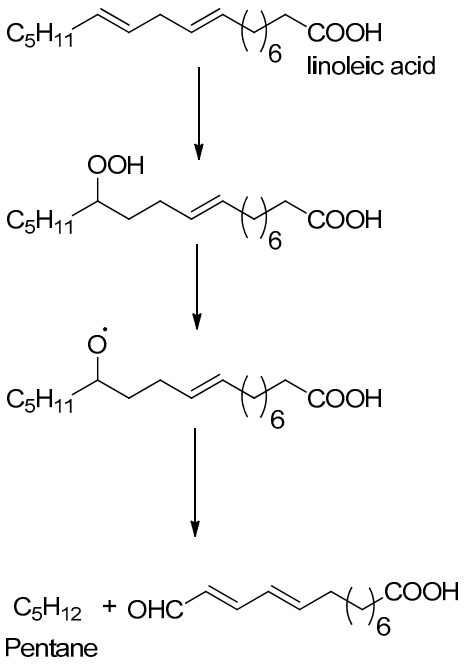


Figure 6. Possible pathway for thermal degradation of linoleic acid and generation of pentane (adapted from Spiteller, 2001)

Conclusions

In order to assess the effect of ageing methods on beef lipid oxidation, TBARS and static headspace gas chromatographic procedures were employed to quantify MDA and volatile lipid oxidation products, respectively. Fatty acid composition analyses were performed to elucidate oxidation mechanisms. Lower MDA and volatile aldehydes detected from hang ageing compared to vacuum and tubling bag ageing, clearly indicated less lipid oxidation, and this could be due to the presence of hard out layer in hang ageing beef. Higher yield of pentane from hang ageing samples observed by GC-headspace analysis could be explained by thermal decomposition of polyunsaturated fatty acid and should not be treated as a candidate for lipid oxidation indication.

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