# THE FORMATION OF 4-HYDROXY-2-TRANS-NONENAL (HNE), A TOXIC ALDEHYDE, IN BEEF, PORK AND CHICKEN FATS HEATED AT FRYING TEMPERATURE (185°C)

A THESIS

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# Abstract

4-Hydroxy-2-trans-nonenal (HNE), a secondary lipid oxidation product, is of special interest because of its abundance and toxicity. In this study, the HNE formation and secondary lipid oxidation of varies types of beef, pork and chicken fats undergoing heat treatment at 185°C frying temperature was investigated.

Beef, pork and chicken fats were selected based on their different degrees of unsaturation and fatty acid distribution. Beef, pork and chicken fats from different sources were chosen to give comprehensive result. These fats were heated at 185°C for up to 6 hours. Lipid oxidation and secondary oxidation product formation of these fats were measured every hour by the thiobarbituric acid reactive substances (TBARS) assay. The formation of HNE in fats heated at 185°C for 0, 1, 3 and 5 hours were measured as 2,4-dinitrophnyhydrazone derivatives using HPLC system.

The results show that 1) the formation of HNE and total secondary lipid oxidation products from all fat sources were increased with the heating time; 2) HNE formation and total secondary lipid oxidation products levels in beef, pork and chicken fats are correlated with their linoleic acid and PUFA percentage, respectively; 3) the HNE formation and total secondary lipid oxidation products of fat extracted from ground meats were lower than that from fat tissues, this may be related to antioxidants content in the meat.

Overall, beef fats produce less HNE and total secondary lipid oxidation products under thermal treatment, compared to pork and chicken fats as well as most vegetable oils.

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# Introduction

Oils and fats act as a transfer medium during the frying process, a widely-used cooking method. Under thermal treatment, oils and fats provide sensory properties that are popularly accepted by consumers. However, lipid oxidation happens in the meantime. Lipid oxidation causes chemical changes in oils and fats and produces primary and secondary lipid oxidation products, such as alkanals, alkenals, alkadienals, α, β-unsaturated-4-hydroxyaldehydes and related carbonyl compounds. 4-Hydroxy-2-trans-nonenal (HNE) is one of secondary lipid oxidation products [1]. HNE is of particular interest because of its high toxicity. Recently, numerous studies have shown that HNE is related to liver diseases, atherosclerosis, stroke, Parkinson's, Alzheimer's, and Huntington's diseases [2-4]. It has been shown that HNE is cytotoxic and mutagenic because it can react with the thiol (SH) and amino (NH2) groups and this reaction results in DNA and protein modification [5-9]. ω-6 Polyunsaturated fatty acids, like linoleic acid, have been proved to be the precursor of HNE [10]. Previously in our lab, experiments have been conducted to prove that HNE formed in frying fats or oils, and it can be incorporated into the fried foods [11]. Some recent studies also indicate that dietary HNE can be distributed to major organs of consumers, like liver, kidney and brain [12]. Therefore, it is important to know how much HNE can be formed in frying oils and fats. The HNE formation in some commonly used vegetable oils has been extensively studied, such as sunflower, olive, corn and soybean oils. However, the study of HNE formation

in animal fats, which contain lower percentage of linoleic acid than vegetable oils, is very limited.

The objective of this study is to determine the influence of heating time at 185°C frying temperature and the unsaturation of fatty acids on the formation of HNE in beef, pork and chicken fats.

# Part I: Literature Review

Lipid oxidation, the oxidative degradation of lipid, is a problem commonly seen in lipid containing food during storage and processing, especially in foods rich in polyunsaturated fatty acids (PUFA). Over the past decades, there has been an increasing attention on HNE, the secondary lipid oxidation products, because of its abundance and high toxicity.

In this part, basis of lipid oxidation processes is introduced at first, including primary lipid oxidation and secondary lipid oxidation. And then follows the formation of HNE. Furthermore, some factors that affect the HNE formation are listed. In the end, reaction mechanisms and biological effects of HNE are introduced.

## 1. Lipid oxidation in deep-frying oil

Deep frying is a widely-used cooking method which is common and popular in home and commercial cooking. The frying temperature is usually between 150°C and 190°C [13]. During frying process, the culinary oils and fats act as a transfer medium and the physical and chemical changes in oils and fats could provide sensory properties that are popularly accepted by consumers [14]. However, undesirable changes occur during frying, like thermal oxidation. Oils and fats fried at elevated temperatures with atmospheric oxygen for long time or repeated use could produce oxidative compounds, including volatile and non-volatile compounds [15, 16]. For example, dimeric and polymeric triglycerides and low molecular weight decomposition products are formed. Some studies have reported that highly oxidized and heated lipids have carcinogenic properties because of these

potentially toxic compounds [17-21]. In the meantime, lipid oxidation causes the losses of some nutrients such as essential fatty acids during frying [22].

### 1.1 Primary oxidation and its chemical reactions

When oils and fats are subjected to thermal treatment, lipid is oxidative degraded. This process is called lipid oxidation. This is the process in which fatty acids react with free radicals and produce hydroperoxides, aldehydes, related carbonyl compounds and polymers. Once started by reacting with free radical, this process is an automatic chain reaction. The cascade of this free-radical chain involves three steps: initiation, propagation and termination [23, 24].

Figure 1 shows the lipid oxidation mechanism. In the initiation step, the hydrogen on the carbon of fatty acid will be attacked to form an alkyl free radical (R·). When the carbon-hydrogen bond is weak, the hydrogen will be removed easily. And the location of attacked hydrogen depends on the structure of fatty acids. Then, in the propagation step, a lipid peroxy radical (ROO·) is formed after a molecule of oxygen is added on an alkyl free radical. This peroxy radical is very strong and it will take a hydrogen from another free fatty acid (RH) to form hydroperoxide (ROOH) and a new alkyl radical (R·). The new alkyl radical (R·) reacts with a molecule of oxygen and the reactions are repeated, forming a chain reaction. Termination involves the reaction of free radicals to form noninitiating and nonpropagating products.

Initiation: $RH \rightarrow R\cdot + H\cdot$

Propagation: $R\cdot + O_2 \rightarrow ROO\cdot$

$ROO\cdot + RH \rightarrow ROOH + R\cdot$

Termination: $ROO\cdot + R\cdot \rightarrow ROOR$

$ROO\cdot + ROO\cdot \rightarrow ROOR + O_2$

$R\cdot + R\cdot \rightarrow RR$

R: lipid alkyl

**Figure 1. The free radical chain cascade of lipid oxidation [25].**

The mechanism of lipid oxidation described above is the summary of common steps when oils and fats are oxidized. However, when free radical attacks different positions on different type of fatty acids (FA), different hydroperoxides are formed as explained below.

### *1.1.1 Primary oxidation mechanism of saturated fatty acid*

Free radical attacks the methylene group, which is next to the end. And hydroperoxides is formed by adsorbing oxygen. This reaction needs very high energy. Therefore, it is not autocatalytic.

### *1.1.2 Primary oxidation mechanism of monounsaturated fatty acid*

When free radical attacks monounsaturated fatty acid, double bond is activated. The hydroperoxide is formed on α-methylene group (next to double bond), and double bond shifts generally. Take Oleic acid for example, double bond is between C-9 and C-10.

Because of resonance, the double bond can be on any positions between carbon 8 to 11, and hydroperoxide group can be attached to the 8, 9, 10, 11 positions.

### *1.1.3 Primary oxidation mechanism of non-conjugated polyunsaturated fatty acid*

When free radical attacks non-conjugated polyunsaturated fatty acid, methylene bridge is activated. Because of resonance, methylene shifts with double bond, the structure becomes conjugated. Take Linoleic acid for example (shown in Figure 2), the two double bonds are in carbon 9-10 and carbon 12-13. Free radical attacks methylene of C-11. Fatty acid free radical forms, then the radical may shift to carbon 14 with the double bond reforming between carbons 11 and 12. The radical may also shift to carbon 9 with the double bond forming between carbons 10 and 11. Both cases result in conjugated structures that are at lower energies than the non-conjugated structures they derived from. For this reason, the oxidation of linoleic acid yields approximately equal amounts of the C-13 and C-9 radical with only traces level of the original C-11 radical present.

### *1.1.4 Primary oxidation mechanism of conjugated polyunsaturated fatty acid*

Oxidation occurs by addition of oxygen to the diene system, conjugation disappears. Polymeric noncyclic peroxides are formed. This reaction is not autocatalytic.

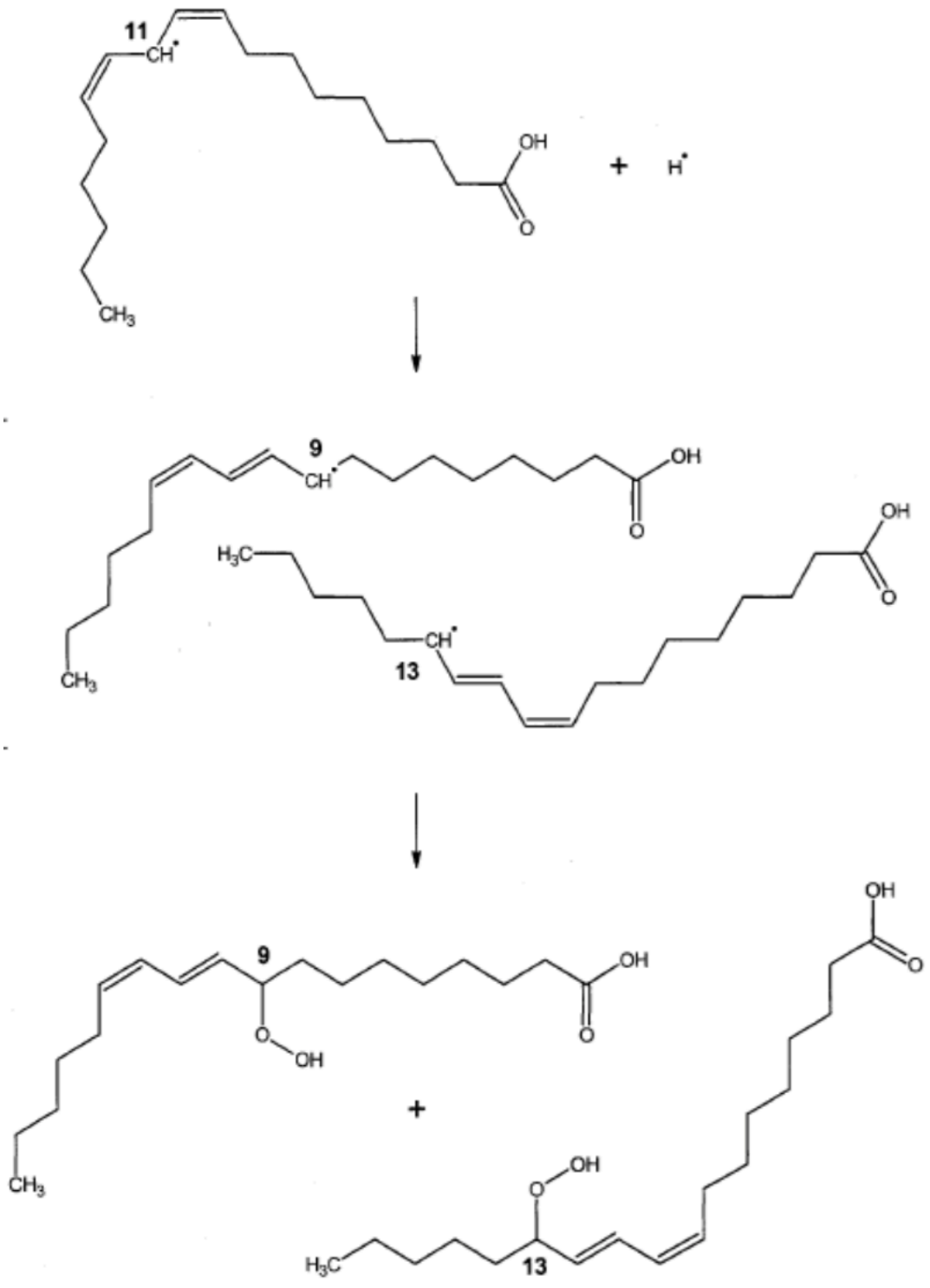

11
CH
OH
O
+
H
CH3
9
CH
OH
O
H3C
CH
13
OH
O
CH3
9
OH
O
O
OH
+
CH3
OH
O
H3C
13
OH
O

**Figure 2. Primary oxidation mechanism of linoleic acid [26].**

## 1.2 Secondary oxidation and the formation of aldehydes in thermal oxidation.

Hydroperoxide, the primary oxidation products, are easily decomposed to a variety of compounds, named secondary lipid oxidation products. The secondary lipid oxidation products include aldehydes, ketones and some short-chain hydrocarbons [27, 28] (shown in Figure 3).

There are two major steps in secondary oxidation. The first step is homolytic cleavage at oxygen-oxygen bond in the hydroperoxide to form a hydroxyl radical and an alkoxy radical. The second step, called β-cleavage or β-scission reaction, is the cleavage of carbon-carbon bond on either side of alkoxy group to produce more radicals. The general mechanism of hydroperoxide decomposition is shown in Figure 4. Secondary lipid oxidation products are formed after numbers of electron rearrangements [29].

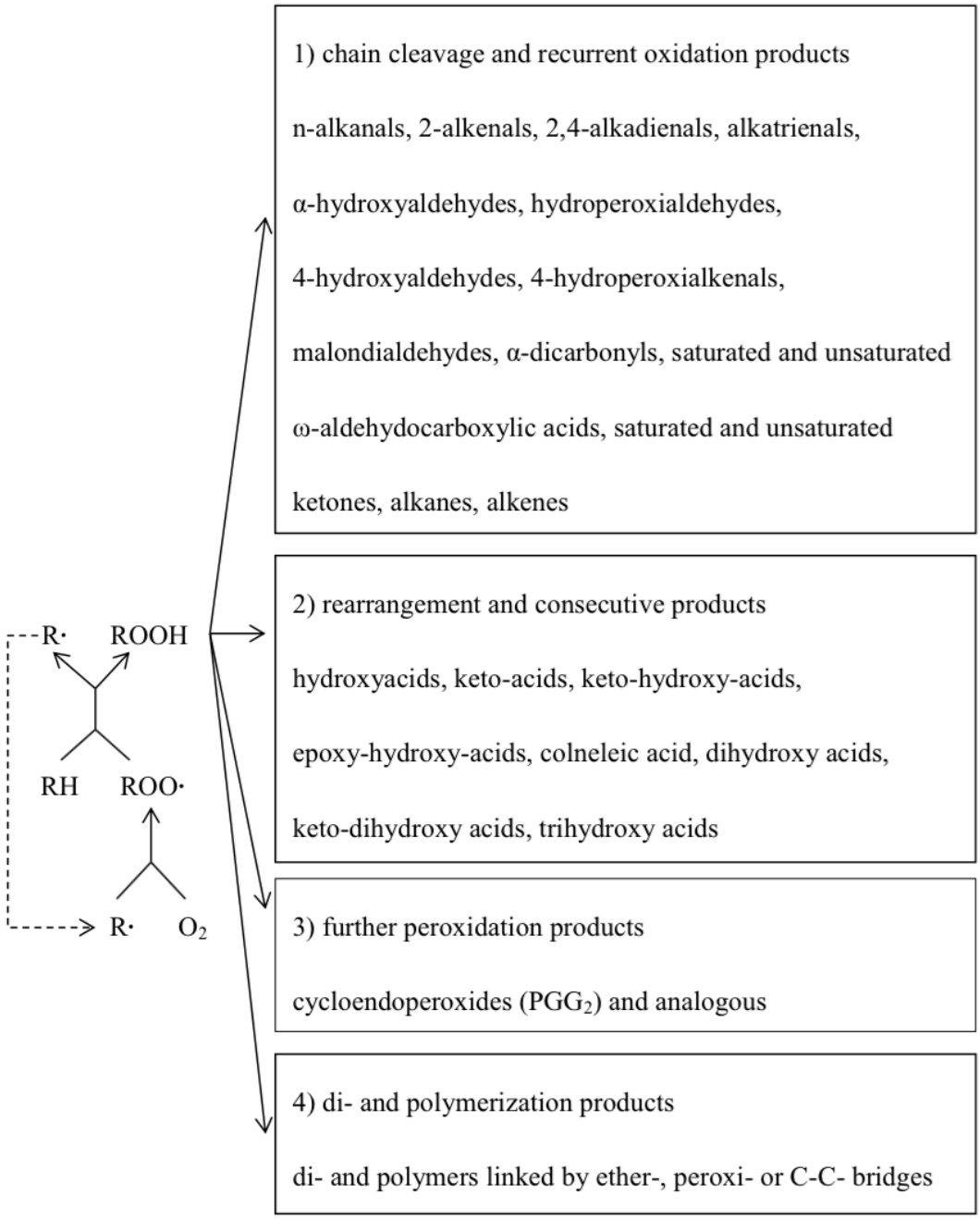



**Figure 3. The classes of substances formed by enzymatic or non-enzymatic oxidation of PUFA [30].**

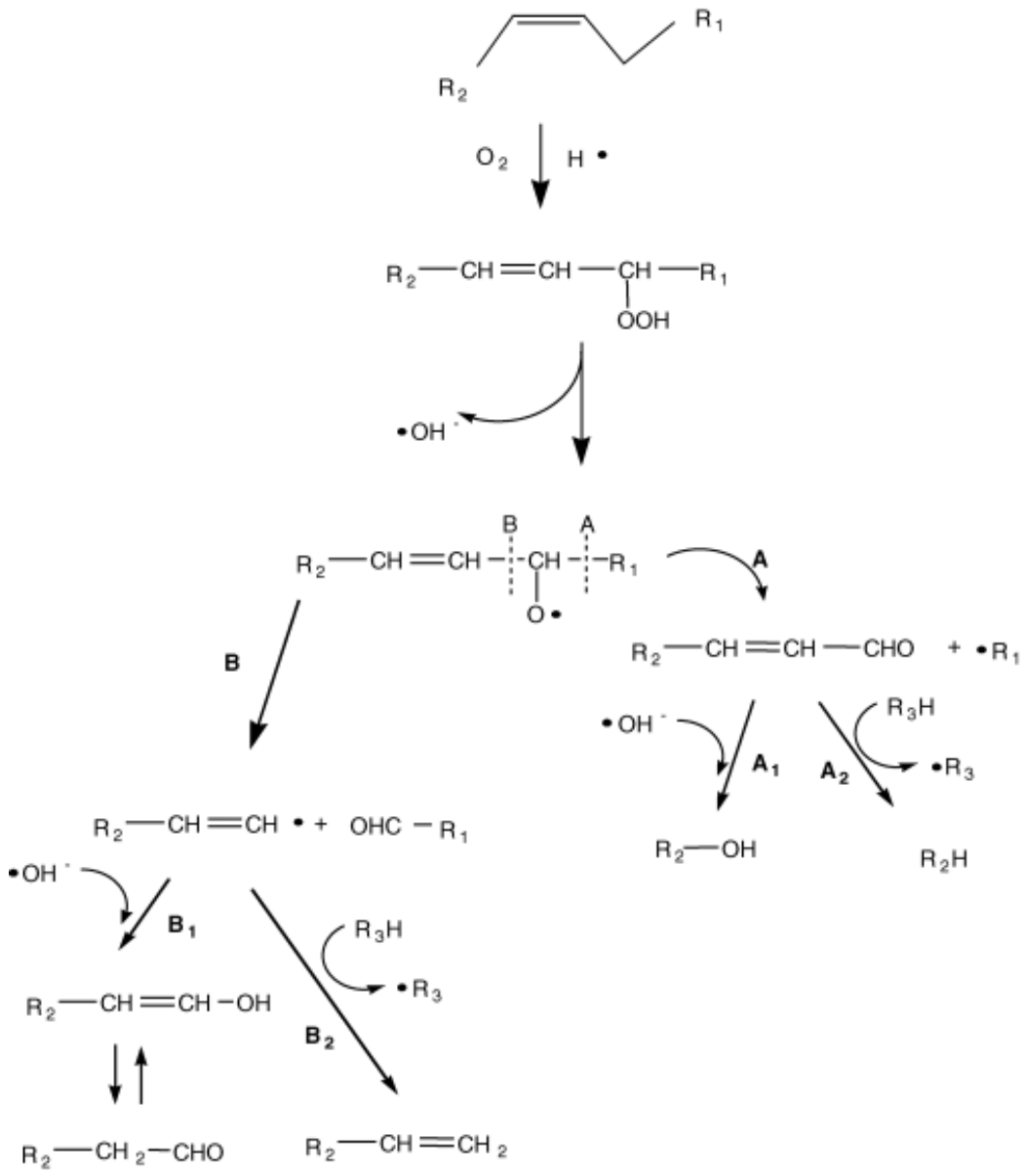



**Figure 4. Mechanisms of hydroperoxide decomposition to form secondary oxidation products [29].**

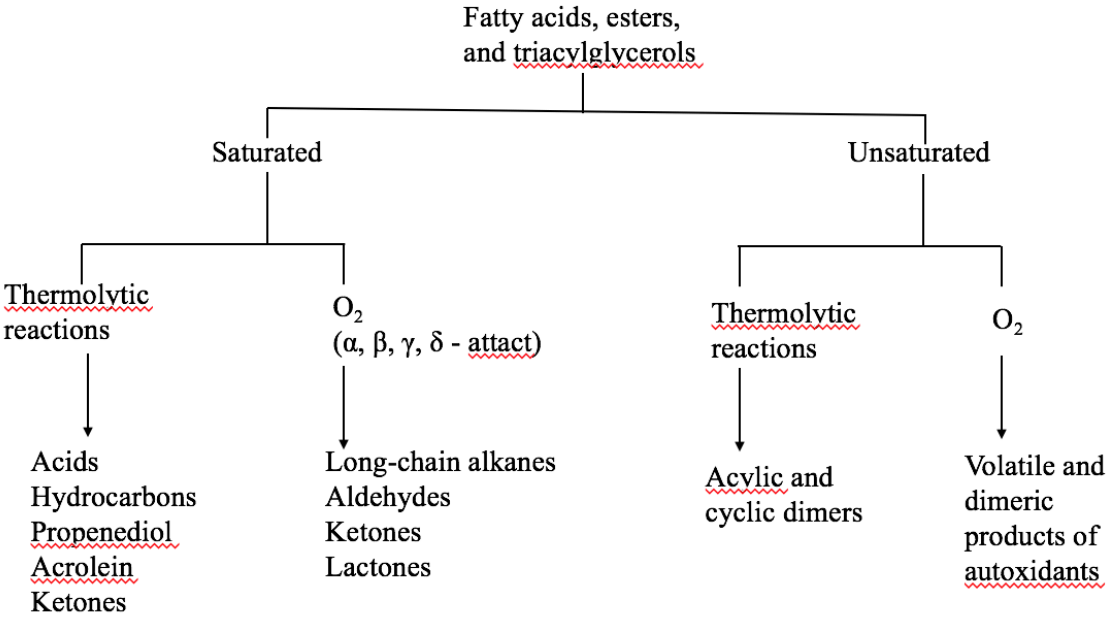


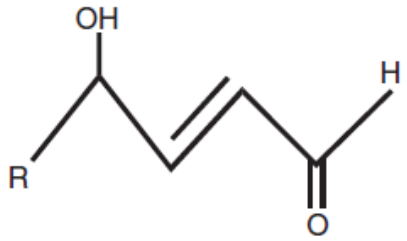

**Figure 5. Generalized scheme for thermal decomposition of lipids [31].**

Overall, lipid oxidation is very complex, especially at elevated temperature. Both saturated and unsaturated fatty acids can react with oxygen and be decomposed to secondary lipid oxidation products when subjected to heating treatment. A summary of these pathways is shown in Figure 5.

## 2. Formation of HNE

Lipid oxidation of polyunsaturated fatty acids yields a broad array of secondary oxidation products. Aldehyde is one major class among these products. Based on the structure, these short-chain aldehydes formed from lipid oxidation can be classified into three families: 2-alkenals, 4-hydroxy-2-alkenals and ketoaldehydes. And the group of 4-hydroxy-2-alkenals is the most prominent lipid oxidation specific aldehydes [1]. 4-Hydroxy-2-alkenals includes 4-hydroxy-2-trans-hexenal (HHE), 4-hydroxy-2-trans-octenal (HOE),

4-hydroxy-2-trans-nonenal (HNE) and 4-hydroxy-2-trans-decenal (HDE). Figure 6 shows the basic structure of 4-Hydroxy-2-alkenals, which has a double bond between position two and three carbons, a hydroxyl group in position four and a carbonyl group in the end. HHE, HOE, HNE and HDE have same basic structure but different chain length.




R = $C_2H_5$: 4-hydroxy-2-hexenal, HHE; R = $C_4H_9$: 4-hydroxy-2-octenal, HOE

R = $C_5H_{11}$: 4-hydroxy-2-nonenal, HNE; R = $C_6H_{13}$: 4-hydroxy-2-decenal, HDE

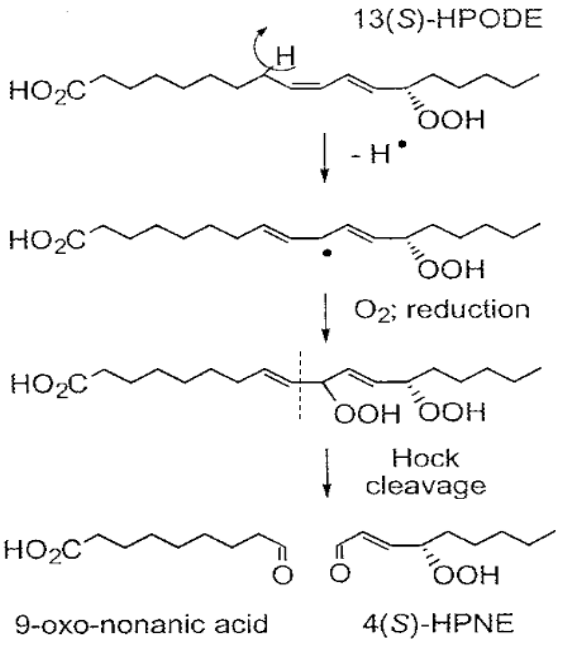
**Figure 6. The basic structure of 4-Hydroxy-2-alkenals.**

Of these compounds, HNE is of particular interest because it's discovered as a major cytotoxic product of lipid oxidation [32] and it's abundant in high PUFA oils and fats after heat treatment. It has been found that the precursor of HNE is ω-6 polyunsaturated fatty acids, like linoleic acid and arachidonic acid [33]. In 1993, Grein and his colleagues suggested that 2,4-decadienal is also the precursor of HNE [10]. But later, Han and Csallany in our laboratory have proved that HNE cannot be formed from 2,4-decadienal [33]. They also discovered that the precursor of HHE is mainly from linolenic acid, because the break of three double bonds leads to shorter chain secondary lipid oxidation

product. HOE comes from both linoleic and linolenic acids. However, it's still unclear about the precursor of HDE.

The mechanism of HNE formation has been a matter of debate since it was described as a major cytotoxic product of lipid oxidation [34]. Based on previous studies, the nine carbon HNE are assumed to represent the last nine carbons of the omega-6 polyunsaturated acids or acyl group [35]. The first mechanism proposed for the HNE formation was published in 1990 by Pryor and Porter [36]. They used araquidonic or linoleic acids in 15- or 13-hydroperoxides, respectively, as the starting materials. HNE are yielded by transformation of these two materials by reduction in alkoxyl radicals, which in subsequent through oxidation, reduction and scission steps. Later, many other studies show different pathways for the formation of HNE. More recently, Brash and his colleagues showed two different pathways leading to the formation of 4-hydroperoxy-2E-nonenal (4-HPNE) by using 9- and 13-hydroperoxides of linoleic acid as starting materials. The formation of 9- and 13-hydroperoxides of linoleic acid is shown in Figure 2. One pathway is that allylic hydrogen abstraction at C-8 of 13S-hydroperoxyoctadecenoic acid (HPODE) leads to the formation of 10, 13-dihydroperoxide that undergoes cleavage between C-9 and C-10 to yield 4S-HPNE (Figure 7A). The other one is 9S-HPODE cleaves directly to form 3Z-nonenal. 3Z-nonenal can be converted to 4-HPNE by 3Z-alkenal oxygenase (Figure 7B). Once 4-HPNE is formed, it can be subsequently converted to HNE [37]. Two pathways are shown in Figure 7.

**A: 13(S)-HPODE**




**B: 9(R)-HPODE**

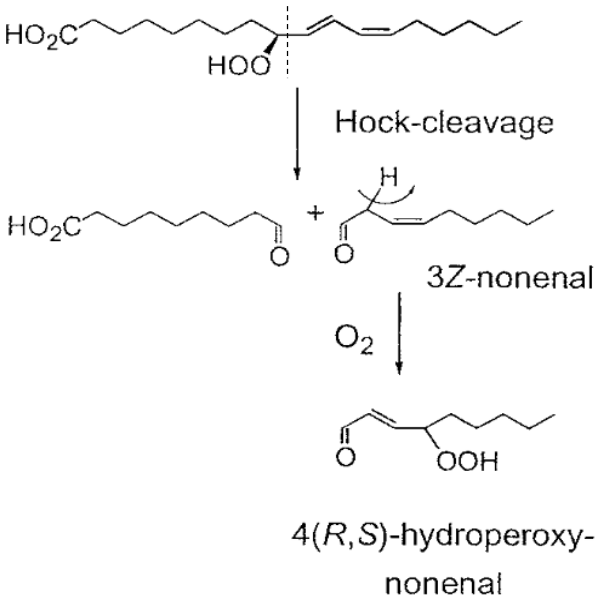



**Figure 7. Mechanism of formation of HNE from 13(S)-HPODE (A) and 9(R)-HPODE (B) [37].**

## 3. Factors that influence the formation of HNE

### 3.1 Heating temperature and time

Like most chemical reactions, the formation of HNE increases with the increasing temperature and the heating time. Previous experiments in our laboratory have found that HNE formation was temperature dependent [38]. Han and Csallany tested corn, soybean and butter oils heated at 190 °C for 0, 0.5, 1, 2 and 3 hours, and 218 °C for 0, 5, 15 and 30 min, respectively. They found that the concentration of HNE at 218 °C was higher at the same heating time compared with that at 190 °C for all three oils. At 30 min of heating, for corn, soybean and butter oils, the HNE concentration of corn, soybean and butter oils at higher temperature was 4.9, 3.7, and 8.7 times higher than at the lower temperature, respectively. Also in the study conducted by Seppanen and Csallany, they heated the soybean oil at frying temperature (185°C) for 0, 2, 4, 6, 8, and 10 hours [39]. An increased formation of HNE was observed with increased heating time and reached the highest amount at 6 hours of heating and then decreased, probably due to the degradation of HNE. Besides, many other researchers have found that the higher temperature and the longer heating time lead to the greater lipid oxidation, providing another layer of evidence that HNE formation depends on the heating time and temperature. Coscione and Artz studied the thermoxidative stability of partially hydrogenated soybean oil. They heated the samples at 120, 160, 180, and 200 °C for 72 hours and sampled every 12 hours. They found that the acid value, p-anisidine value, dielectric constant and the triacylglycerol polymer content increased with an increase in temperature and heating time, indicating the increase of lipid oxidation [40].

### 3.2 FA composition

In addition, the fatty acid composition of lipids affects the formation of HNE due to the precursor of HNE is ω-6 polyunsaturated fatty acids. Previous study in this laboratory proved that the linoleic acid (LA) level is correlated with the HNE production. In the study mentioned before [38], Han and Csallany tested high-LA-containing oils such as corn and soybean, and low-LA-containing oil such as butter heated at 190 °C and 218 °C for different time. They found that butter oil has significantly lower HNE formation compared to corn and soybean oils at both heating temperature in any heating time. They also studied the formation of toxic α, β- unsaturated 4-hydroxy-aldehydes, such as HHE, HOE, HNE and HDE, in thermally oxidized methyl stearate (MS), methyl oleate (MO), methyl linoleate (ML) and methyl linolenate (MLN) [33]. HHE was detected in ML and MLN, with higher concentration in MLN. HOE was also found in both ML and MLN. However, HNE was only found in ML and HDE was not detected in any of the four fatty acid methyl esters. Table 1 shows that the composition of animal fats. Due to the high percent of linoleic acid in chicken fats and low in beef fats, we expect that the HNE formation order from low to high are beef tallow, lard and chicken fat.

**Table 1. Percentage of fatty acids of some commonly used animal fats**

| Fats | C16:0 | C18:0 | C18:1 | C18:2 | C18:3 |
|---|---|---|---|---|---|
| Chicken fat | 25.3 | 6.5 | 37.7 | 20.6 | 0.8 |

| | | | | | |
|---|---|---|---|---|---|
| Lard | 26 | 13.5 | 43.9 | 9.5 | 0.4 |
| Beef tallow | 24.3 | 18.6 | 42.6 | 2.6 | 0.7 |

### 3.3 Myoglobin

Furthermore, in meat, hemoglobin and myoglobin are in relative high concentration. They both have catalytic activity because of iron protoporphyrin structure, as shown in Figure 8.

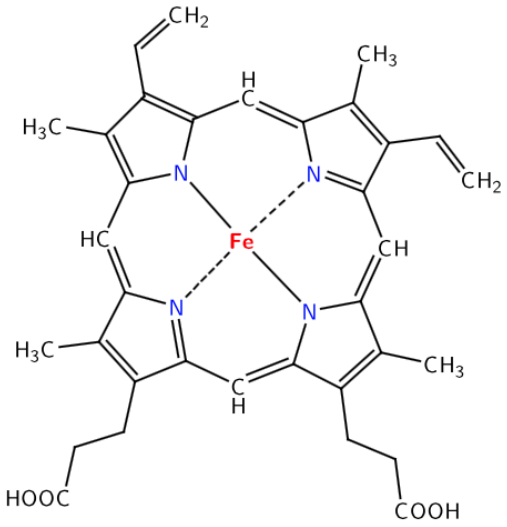



**Figure 8. The structure of iron protoporphyrin.**

In meat, myoglobin oxidation and lipid oxidation are coupled and influence each other [23]. Myoglobin is a major contributor to the color of muscle and its structure contains heme group. The ferrous iron in heme group can be oxidized at the present of oxygen by Fenton reaction, which is also the oxidation of ferrous-oxymyoglobin to ferric-metmyoglobin. The equation of Fenton reaction is shown in Figure 9. Ferrous iron ($Fe^{2+}$)

can react with oxygen molecular to produce ferric iron ($Fe^{3+}$) and singlet oxygen ($O_2^{\bullet -}$). The singlet oxygen is a very strong free radical and highly reactive, it converts to hydrogen peroxide ($H_2O_2$) by dismutase. Ferrous iron reacts with hydrogen peroxide to produce hydroxyl radical. The hydroxyl radical initiates the autoxidation of lipid. On the other hand, aldehyde lipid oxidation products, such as HNE, affect the redox stability of myoglobin [41]. It has been shown that α, β-unsaturated aldehydes, such as HNE, accelerates metmyoglobin formation and subsequent lipid oxidation [42]. In the meantime, the combination of HNE and low hemin affinity facilitates rapid decomposition of pre-formed lipid hydroperoxides to secondary lipid oxidation products (HNE-Myoglobin).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -}$$

$$2O_2^{\bullet -} + 2H^{+\bullet -} \rightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

**Figure 9. Reactive oxygen species generated by the Fenton reaction [43].**

### 3.4 Sodium Chloride

Sodium Chloride (NaCl) is added to muscle foods for a variety of purpose, including flavor and inhibition of microorganisms. NaCl, nevertheless, has been shown to have an accelerating effect on lipid oxidation in muscle tissue [44]. In 1991, Kenner and his colleagues [45] conducted a study in which they added iron ions or iron ions with NaCl

to minced muscle. In the control group, the lipid oxidation rate of muscle with only iron ions increased slightly. However, in the present of NaCl, the same treatment enhanced lipid oxidation approximately 3-5 folds more than control. The free iron ions may participate in the initiation of lipid oxidation. So, their results suggest that a large part of the added iron ions bind with the protein and NaCl has ability to disturb this interaction, freeing the iron ions for lipid oxidation initiation [43]. Later, the Sakai group studied the effect of NaCl on HNE formation in minced pork and beef [46]. Pork and beef containing NaCl were stored at 0 °C and HNE content were measured after 0, 3, 7 and 10 days of storage. The results showed that samples with NaCl have higher HNE content than those without NaCl, indicating that NaCl may act as a pro-oxidant in pork and beef.

# 4. HNE reaction mechanisms

HNE has toxicity due to its high reactivity. Figure 6 shows the structure of HNE. As shown in this figure, three functional groups of HNE make its high reactivity: carbonyl group, C=C double bond and hydroxyl group [47]. Below introduce the detailed reaction mechanisms of these three functional groups.

## 4.1 Reactions of the carbonyl group

### *4.1.1 Schiff-base formation*

The carbonyl group can react with primary amines, e.g. lysine (Figure 10). This reaction is a competitive reaction to the Michael addition of amines, which causes the crosslink of proteins. Also, because the carbonyl group can react with $NH_2$ groups, 2,4-dinitrophenyl

hydrazine (DNPH) can be used to react with HNE to forms 2,4-dinitrophenyl hydrazone, a stable derivative for analytical purposes [1].

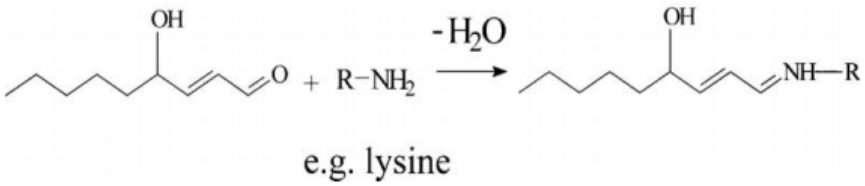



**Figure 10. Schiff-base formation with primary amines [47].**

### *4.1.2 Acetal formation*

The carbonyl group in HNE can react with alcohols, such as methanol, by two steps to form an acetal (Figure 11). This reaction is commonly used in synthetic chemistry to storage HNE since the stability of acetals is higher than the HNE. And the free aldehyde can be released again in acidic medium.

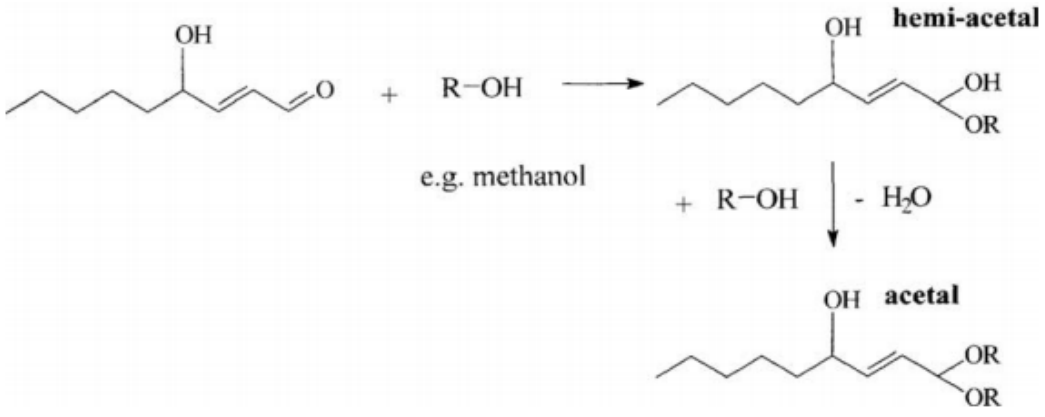



**Figure 11. Acetal formation with alcohols [47].**

### *4.1.3 Oxidation*

The carbonyl group can be oxidized in present of aldehyde dehydrogenase (ALDH) with NAD+ as cofactor, to yield 4-hydroxy-nonenoic acid (Figure 12). Studies indicate that the ALDH in human is not the right class of ALDH that can react with HNE, which play an important role of protecting cell from the HNE toxicity [48].

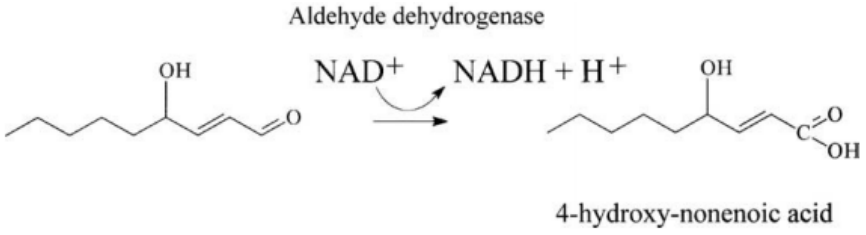



**Figure 12. Oxidation of the carbonyl group [47].**

### *4.1.4 Reduction*

In the opposite, the carbonyl group can be reduced with alcohol dehydrogenase and NADH (Figure 13). It is also one of the ways of HNE metabolism.

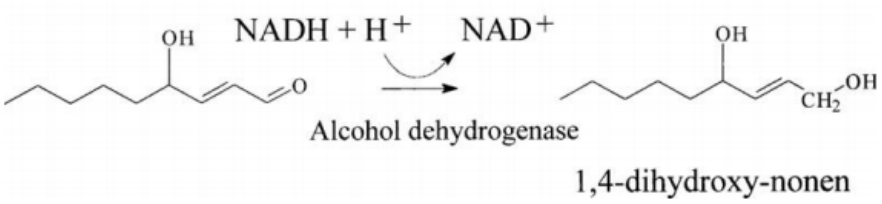



**Figure 13. Reduction of the carbonyl group [47].**

### 4.2 Reactions of the C=C double bond

#### *4.2.1 Michael additions*

In Michael additions, amino compounds which contains thiols are added to the C=C double bond. For example, cysteine or glutathione (GSH) can be added to the C=C double bond through Michael additions. This reaction is enhanced in present of enzyme glutathione-S-transferase (GST) (Figure 14). GST mediated conjugation of HNE to GSH accounts for approximately 60% of the total HNE degradation [49, 50]. In addition to GSH, other amino compounds, such as lysine, ethanol amine, guanine and the imidazole group of histidine can also react with HNE [51].

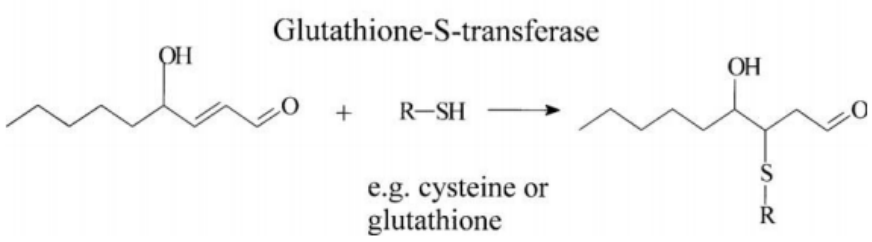



**Figure 14. Michael addition of thiols [47].**

#### *4.2.2 Reduction*

The C=C double bond can be reduced by an alkenal/one oxidoreductase with NAD(P)H as cofactor to produce 4-hydroxynonanal (Figure 15).

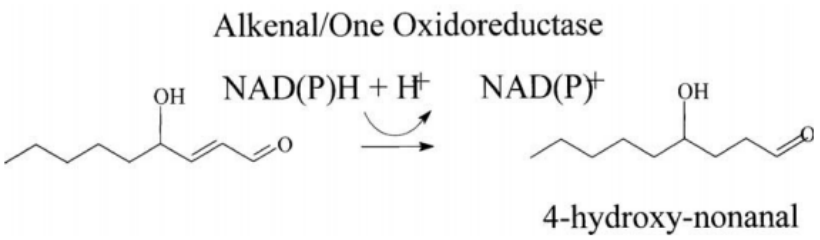



**Figure 15. Reduction of the C=C double bond [47].**

*4.2.3 Epoxidation*

An oxirane ring can be formed into HNE through epoxidation with a hydroperoxide (Figure 16). The precise mechanism for this reaction is not known yet.

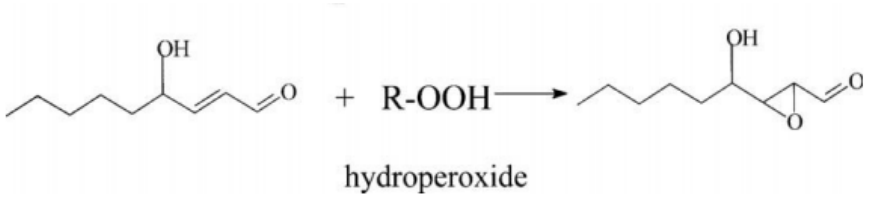



**Figure 16. Epoxidation in presence of a hydroperoxide [47].**

**4.3 Reactions of the hydroxy group**

*4.3.1 Hemi-acetal formation*

After the Michael addition in C=C double bond, the hydroxy group can form a hemi-acetal as a secondary reaction (Figure 17).

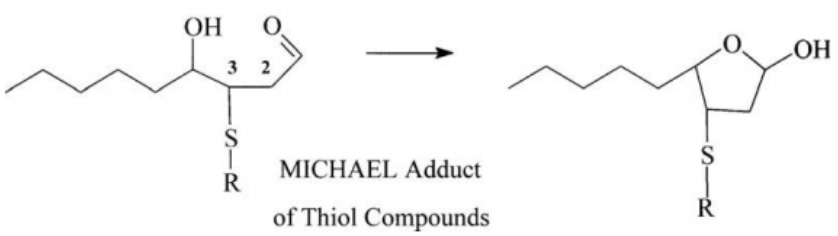



**Figure 17. Hemi-acetal formation of a Michael adduct of HNE [47].**

### *4.3.2 Oxidation*

The hydroxy group can be oxidized to yield ketone (Figure 18).

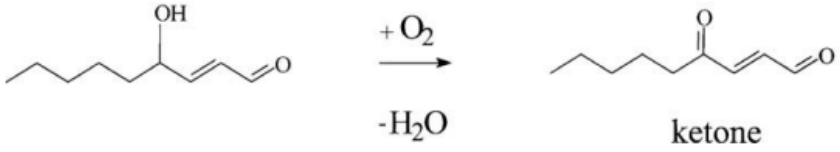



**Figure 18. Oxidation of the hydroxy group [47].**

## 5. Biological effect of HNE

Many researchers have investigated the biological activity of HNE. HNE has been shown to be cytotoxic and mutagenic [52]. Its toxicity comes from the high reactivity of three main functional groups: the carbonyl group, the C=C double bond, and the hydroxyl group [47]. As mentioned above, the conjugated double bond group reacts with thiol (SH) and amino (NH2) groups by Michael addition results in protein and DNA modifications. HNE forms conjugates with proteins containing cysteine, histidine, and lysine residues and can damage DNA by inducing gene mutations and can alter the structure and function of cancer-related proteins [5-7]. Besides, the carbonyl group can react with primary amine groups in amino acids, phospholipids and proteins to produce Schiff bases, which modify

the functionality of these molecules in biological systems [9, 53]. In addition, the third functional group, hydroxyl group, is able to be transformed to oxo group [54]. In a nut shell, HNE is considered as a marker of LDL oxidation, liver damage, stroke, Parkinson's, Alzheimer's, and Huntington's diseases [2, 55]; causative agent of atherosclerosis [56] and of liver cirrhosis [57]; as well as a cytotoxic, mutagenic, and carcinogenic factor [58, 59].

The level of HNE determines its toxicity. It has been shown that a low level of oxidation exists in normal tissues. It is estimated that free HNE concentrations in the plasma of healthy individuals is between 0.3 and 0.7 μM [60]. At this level, HNE displays several activities referring to cell multiplication and differentiation, such as stimulation of neutrophil chemotaxis, or activation of membranes [35]. However, when the concentration is increased to between 1 to 20 μM, HNE partially inhibits DNA and protein synthesis [34]. At cellular concentrations >100 μM, partly or fully inhibition of catabolic effects (mitochondrial respiration), and anabolic effects (DNA, RNA, protein synthesis) were observed, which lead to cell death [1]. Concentration of HNE would be raised in vivo by oxidative stress or consumption of fried foods.

## 6. Metabolism of HNE

In many tissues, HNE can be rapidly metabolized to less cytotoxic, water soluble forms, and then excreted [61]. In addition, HNE modified proteins can also be cleared by autophagic and proteasomal degradation pathways [62-64]. It has been shown that hepatocytes, thymocytes and enterocytes from small intestine are known to be

particularly efficient at metabolizing HNE [65]. Three major routes of HNE metabolism are forming glutathionyl-HNE, corresponding alcohol 1,4-dihydroxy-2-nonene (DHA) and corresponding acid 4-hydroxy-2-nonenoic (HNA). Under physiological condition, the major pathway of HNE detoxification is to conjugate with the cellular antioxidant glutathione (GSH) by glutathione-S-transferases, especially GSTA4-4, forming glutathionyl-HNE [66]. It can be further reduced by aldose reductases or oxidized by aldehyde dehydrogenases to form DHA – GSH or HNA – GSH, respectively. Free HNE can also be reduced by aldose reductases or oxidized by aldehyde dehydrogenases to form DHA and HNA, respectively [67]. HNA can be future metabolized by cytochrome P450 (CYP) to form 9-hydroxy-HNA (9-OH-HNA). These water-soluble HNE metabolism are eventually excreted in urine, in the form of mercapturic acid conjugates [68]. Although most HNE can be removed from cells, approximately 1 to 8% of HNE still remains as HNE adducts to functional amino acid groups, including cysteine, histidine and lysine [50].

## 7. Studies of HNE production in food

Because of high toxicity of HNE, many studies have been done to figure out how much HNE can be formed in food system and how the HNE in food affect human's health.

The first study of determining HNE applied to foods was published by Land et al. in 1985 [69]. In this study, some edible oils, fried mushrooms, and roasted meats were analyzed. Oils and foodstuffs are extracted with distilled water containing 2,6-diterbutyl-*para*-cresol (or BHT) and Desferal (quelant agent of metallic ions) to avoid oxidation of the

sample. After additional sample cleanup by solid-phase extraction on a disposable octadecyl silica gel (ODS) extraction column, the sample was analyzed by HPLC. The authors found variable concentrations of HNE in all samples. In the roasted pork cutlet and roasted chicken, HNE concentration were 162 and 117μg/kg food, respectively.

The second study applied to determine HNE in pork and beef meat was conducted by Sakai *et al* [70]. In this study, HNE and ω-6 fatty acids contents were analyzed in pork and beef. First, the samples were reacted with DNPH, and then clean up on a silica gel extraction column. After separated by HPLC, the derived compounds were identified by electrochemical detection. The results show that the HNE contents in beef (n=4) and pork (n=7) were 14.0-150 nmol/g and 1.0-152 nmol/g, respectively. Furthermore, a linear correlation between the content of HNE and the content of total ω-6 fatty acids, linoleic acid, or arachidonic acid was observed in pork.

Later, the same research group investigated the relationships between 4-hydroxy-2-nonenal (HNE), 2-thiobarbituric acid reactive substances (TBARS) and n-6 polyunsaturated fatty acids (n-6 PUFAs) for pork stored at 0, -20 and -80°C by using the same method mentioned above [71]. In samples stored at 0 °C the increase in the concentration of HNE was observed from day 7 to day 12, reaching 27.96±5.59 nmol/g meat; in samples stored at -20 °C, a clear increase was observed only after 8 months; and no significant difference was detected in samples stored at -80 °C. However, the author found that the content of ω-6 PUFAs remained unchanged during storage.

In our laboratory, Seppanen and Csallany conducted a study to investigate the formation of HNE in soybean oil, which was heated at frying temperature 185°C for 2, 4, 6, 8 and 10 hours [39]. Results showed that unheated soybean oil contained no HNE and a very low concentration of polar lipophilic secondary oxidation products. A great increase in the concentration of both HNE and the total lipophilic polar oxidation products was observed with increased heating time at frying temperature. A considerable concentration of HNE had already formed at 2 hours and the concentration continued to increase upon 4 and 6 hours of heating time in soybean oil which contains a high level of linoleic acid (45-52%). After 6 hours, the concentration of HNE started to decrease probably due to thermal decomposition. In addition, it was found that the tocopherol concentration decreased as the lipid oxidation and the secondary oxidation products increased.

The above-mentioned authors also found that the HNE formation is temperature dependence [38]. In this study, corn oil, soybean oil, and butter were heated at 190°C or 218°C. The concentration of HNE at 218°C increased more significantly for all the three oils compared to the heating at 190°C for the same period. In comparison, HNE concentration of corn, soybean and butter oils after only 30 min of heating at higher temperature (218°C) was 4.9, 3.7, and 8.7 times higher than at the lower temperature (190°C), respectively. This proved that HNE formation was temperature dependent in the tested oils.

The same authors conducted another study to investigate the effect of intermittent and continuous heating of soybean oil at frying temperature on the formation of HNE [72]. In this study, Soybean oil samples were heated either for 1 h each day for five sequential

days or for 5 h continuously at 185±5°C. HNE, HHE, HOE and HDE were quantified in thermally oxidized samples. The results show that the concentration of these four compounds increased similarly under intermittent and continuous heating. This indicate that no matter the oils are heated continuously or intermittently, just like cooking in many restaurants, the formation of HNE is accumulated.

The formation of α, β-unsaturated-4-hydroxyaldyhydes was investigated in fatty acid methyl ester format by the same authors [33]. Fatty acid methyl esters (FAMEs) of stearic, oleic, linoleic and linolenic acids were heated separately at 185°C for 0 to 6 hours. As a result, methyl stearate (MS) and methyl oleate (MO) did not produce any of the α, β-unsaturated- 4-hydroxyaldehydes after thermally induced lipid oxidation. The formation of HHE was detected in both methyl linoleate (ML) and methyl linolenate (MLN), with concentration higher in MLN than in ML. HOE was detected in both ML and MLN, too. HNE was found only in ML and HDE was not detected in any of the four heat treated FAMEs. This results proved that the precursor of HNE is ω-6 fatty acids.

The above-mentioned authors also found that HNE was incorporated into fried food from frying oil which had been heated at 185 °C for 5 hours [11]. Similar concentration of HNE was found in frying soybean oil and oil extracted from fried potatoes. This results indicate that HNE was readily incorporated into food fried in thermally oxidized oil and large consumption of these fried foods could be a health concern due to the toxicity of HNE.

The determination of HNE in patties, from ground beef stored in the presence of air at 4°C for 9 days was conducted by Lynch group [5]. The method used was similar like Sakai group. The results showed that the concentration of HNE increased with the increasing storage time.

Keller and his colleagues investigated the metabolism of HNE after oral administration [12]. They use radioactive and stable isotopes of HNE to identify and quantify urinary HNE metabolites after oral administration in rats. Radioactivity distribution revealed that after 24 hours, 48% of the administered radioactivity was excreted into urine and 15% into feces, while 3% were measured in intestinal contents and 2% in major organs, mostly in the liver.

In conclusion, HNE can be produced in foods during processing or storage. Many factors can influence the formation of HNE when heating the oils and fats, such as the heating temperature, the heating time and FA composition. And once HNE is formed in oils and fats, it can be absorbed through the diet and then distributed to humans' major organs. If the concentration of HNE is high in human body, health problem would be concerned. Therefore, it is important to know which types of oils and fats are good to use when frying and to control the frying temperature and time.

## 8. Objectives

The HNE formation in some commonly used vegetable oils has been extensively studied, such as sunflower, olive, corn and soybean oils. However, the study of HNE formation in animal fats, which contain lower percentage of linoleic acid than vegetable oils, is very limited. The objective of the present experiments was to determine the influence of heating time and unsaturation on formation of HNE in frying beef, pork and chicken fats.

# Part II: Experiments

## Methods and Materials

### Chemicals and Materials

2.4-dinitrophenylhydrazine (DNPH), 2-thiobarbituric acid, thichloroacetic acid, HPLC-grade methanol, HPLC-grade water, HPLC-grade dichloromethane, ACS-grade methanol, ACS-grade ethanol, boron trifluoride-methanol solution, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), potassium persulfate (di-potassium peroxdisulfate) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich Company (St. Louis, MO); sodium thiosulfate and ACS-grade hexane was purchased from Fisher Scientific (Fair Lawn, NJ), hydrochloric acid was from Mallinckrodt Baker Inc. (Paris, KY). HNE was from Cayman Chemicals Co. (Ann Arbor, MI). Thin layer chromatography (TLC), No.1 filter paper and 0.45 um syringe filters were purchased from Whatman Ltd. (Kent, England). Syringe was purchased from Terumo Medicare Cop (Somerset, NJ).

Beef back fat, beef suet, ground beef, pork back fat, leaf lard and ground pork were obtained from meat laboratory of the University of Minnesota; commercial beef suet, commercial lard, chicken thigh were purchased from local stores; commercial chicken fats were purchased from Glatt Organic LLC (Brooklyn, NY).

### Fat Extraction

Fifty grams of animal fats or ground meats were each washed with 100mL distilled water three time. For those unwashed samples, this step was skipped. Then, fifty grams of fat

tissues or ground meats were each homogenized with 100mL of hexane. The hexane supernatant was collected, and the slurry was extracted two subsequent times with 100mL and 60mL of hexane, respectively. The hexane portions were combined. About 25g of sodium thiosulfate was added twice into hexane portions and shaken for 10 min, respectively. Then, the hexane/$Na_2S0_4$ portions was filtered to remove $Na_2S0_4$ and other animal tissues. Ultimately, the filtered hexane supernatants were evaporated under vacuum using rotary evaporator to obtain animal fats. All samples were stored at -70 °C freezer until analyzed. Types of samples were listed in Table 2.

**Table 2. List of all animal fats used in this project.**

| **Beef Fats** | **Pork Fats** | **Chicken Fats** |
|---|---|---|
| Fat Extracted from Washed Ground Beef (GB) | Fat Extracted from Washed Ground Pork (GP) | Chicken fat scraped from surface of thigh (Chicken Thigh Fat) |
| Fat Extracted from Unwashed Ground Beef (GB) | Fat Extracted from Unwashed Ground Pork (GP) | Chicken fat scraped from surface of Skin (Chicken Skin Fat) |
| Beef Back Fat | Pork Back Fat | Commercial Chicken Fat |
| Beef Suet | Leaf Lard | |
| Commercial Beef Suet | Commercial Lard | |

**Fatty Acids Distribution by Gas Chromatography**

For the analysis of the fatty acid methyl esters (FAME) of various beef fats, pork fats and chicken fats, Gas Chromatography (GC) was used by the method of Metcalfe and Schmitz [29]. Three mL of boron trifluoride (BF3)-methanol (14% of BF3 in methanol) was added into two drops of unheated animal fats in 20mL test tubes, and vigorously shanked the mixture. Then, the mixture was put in the boiling water for 1 hour. Three mL of distilled water and 10mL of hexane were added into the mixture after cooling. The test tubes were shake well for 10min. When the mixture was separated well, the top hexane layer was saved to a new test tube and dried with 2-3 grams of sodium sulfate. The FAME in hexane were stored at -20 until use. Two to five μL of hexane samples were injected at split-mode of injection into GC.

A HP 5830A GC with flame ionization detection (FID) and a Carbowax capillary column (All Tech Econo Cap# 119563, Deerfield, IL, USA), 15m length, 0.53 mm i.d, 1.2IJ film thickness, was used for GC system. Helium was used as the carrier gas at a velocity of 70mL/min as well as the make-up gas at a flow rate of 30mL/min. The injection temperature was 230°C and the FID temperature was 250°C. The oven condition was a temperature gradient from 150°C to 220°C with 5°C /min.

**Thiobarbituric Acid Reactive Substances (TBARS) Assay**

TBARS assay is a common, simple and sensitive colorimetric method used to measure the secondary lipid oxidation. This method is based on the measurement of chromogen

that is formed by reaction of 2-thiobarbituric acid (TBA) and malondialdehyde (MDA). Therefore, in this study, MDA is used to standardize the test and the results are expressed as MDA equivalents. [73, 74].

Each type of animal fats was measured 2g in duplicate in open test tubes (16×150mm) and was heated continuously at sand bath at 185 °C for 1, 2, 3, 4, 5, and 6 hours. After each heating, the TBARS value was measured. The TBARS assay was done by the method of Buege and Aust [75]. The TCA/TBA/HCl solution was made by combing the equal volumes of 15% w/v thichloroacetic acid (TCA), 0.375% w/v TBA, and 0.25N HCl. Before samples were tested, the standard curve was determined by combining 200 μL of various concentrations of MDA with 4 mL of TCA/TBA/HCl solution. The various concentrations of MDA of made by diluting with distilled water as listed in Table 3 below. The mixture of MDA and TCA/TBA/HCl solution was heated for 15 minute in a boiling water bath. After cooling, the absorbance of the sample was measured at 535 nm with a UV/Vis spectrophotometer. The standard curve is shown in Figure 19.

Measurement of samples: 200 μL of unheated or heated animal fats was combined with 4 mL of TCA/TBA/HCl solution. The mixture was heated for 15 minute in a boiling water bath. After cooling down, the mixtures were centrifuged at 2000 rpm for 5min. The aqueous layer was transferred to the test tube and the absorbance was measured at 535 nm with a UV/Vis spectrophotometer (Milton Roy Spectronic 20D).

**Table 3. The concentration of MDA for TBARS standard curve.**

| Tube Number | MDA(5×10-5M) (µL) | Water (µL) | Concentration of MDA (µg/mL) |
|---|---|---|---|
| 1 | 0 | 200 | 0 |
| 2 | 10 | 190 | 0.0086 |
| 3 | 25 | 175 | 0.0214 |
| 4 | 50 | 150 | 0.0429 |
| 5 | 100 | 100 | 0.086 |
| 6 | 150 | 50 | 0.129 |
| 7 | 200 | 0 | 0.171 |

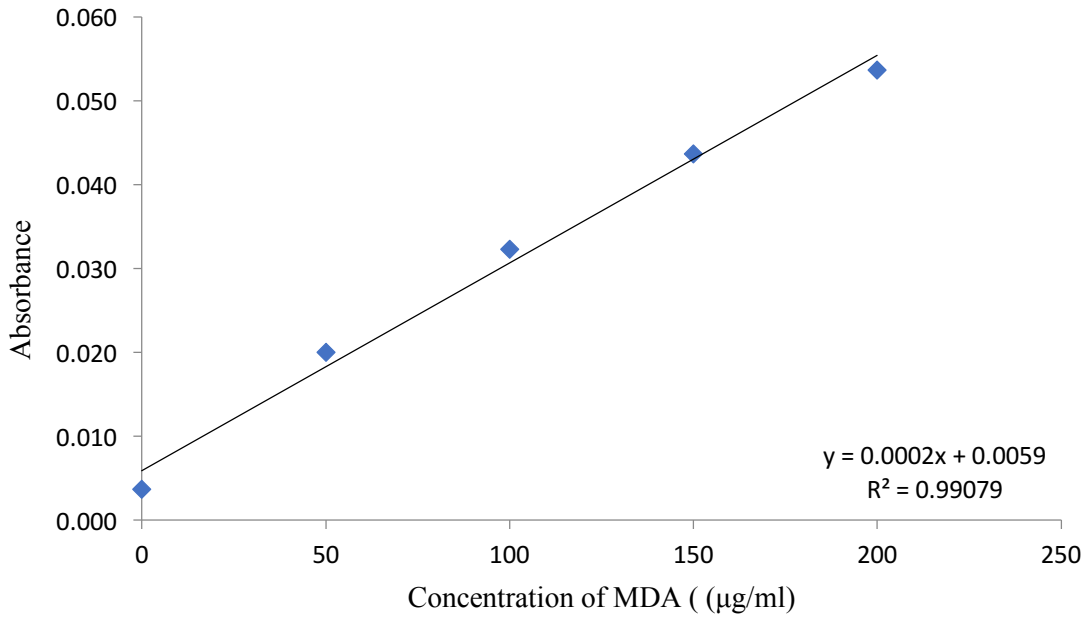



**Figure 19. The standard curve of MDA concentration**.

**Method to Determine HNE in Samples**

The method was developed by Seppanen and Csallany [76]. The principle of this method is that 2, 4-dinitrophenylhydrazones (hydroxyalkenal-DNPH derivatives) is produced after hydroxyalkenals react with 2, 4-dinitrophenylhydrazine (DNPH) by formation of Schiff base. The 2, 4-dinitrophenylhydrazones can be measured by ultraviolet (UV) light at 378nm.

- *Preparation of thermally oxidized animal fat samples*.

  Two grams of animal fats was weighed in 13 × 100 mm open test tubes. Each fat sample was heated in sand bath separately at 185 °C for 1, 3 and 5 hours. Each heating condition was prepared in duplicate. Fifteen minutes was needed for sample to reach up to 185 °C in the sand bath.

- *Preparation and isolation of DNPH-HNE*.

1. *Recrystallization of DNPH*

   Five grams of DNPH was dissolved in 100 mL methanol and heated at 60°C for 30 minutes. The dissolved DNPH methanol was put in an ice bath for at least 18h for crystallization. Then crystallized DNPH was filtered by No.1 filter paper and dissolved in 100 mL methanol. Repeated this step at least two more times until DNPH crystals were got. The DNPH crystals was dried in desiccator for 3 days.

2. *Preparation of DNPH Reagent*

   DNPH reagent was prepared freshly for every assay. Then 10 mg of DNPH crystal was dissolved in 20mL 1 N hydrochloric acid and heated at 50 °C for 1 h. After cooling, the

DNPH reagent was washed with 10mL of HPLC-grade hexane to remove impurities. The bottom layer (DNPH layer) was saved and washed three more times.

3. *Preparation of the DNPH reagent blank and the acetone-DNPH standard*

Three mL of HPLC-grade water or 3mL of 1% acetone were added into 3mL of freshly prepared DNPH reagent, to make DNPH reagent blank and the acetone–DNPH standard, respectively. The mixture was shaken at the speed of 120 oscillations/min overnight in the dark at room temperature. Then, the mixture was washed three times with 5mL dichloromethane. The dichloromethane extracts were combined and evaporated under $N_2$ gas to a volume of 0.5mL. The condensed standards were covered with Parafilm tightly and stored at 0 °C until use.

4. *Preparation of HNE-DNPH standard*

One hundred μL (5mg/500μL of ethanol) of HNE was added to 10mL freshly made DNPH reagent. The mixture was shaken at the speed of 120 oscillations/min to react overnight in the dark at room temperature. The HNE-DNPH derivatives was washed three times with 10mL dichloromethane. The dichloromethane extracts were combined and evaporated under $N_2$ gas to a volume of 1.5mL. The condensed HNE-DNPH derivatives was applied to two Thin Layer Chromatography (TLC) plates with DNPH reagent blank and the acetone-DNPH standard to remove impurities. The polar regain was cut into small pieces and was extracted three times with 10mL methanol. The combined methanol extract was evaporated under $N_2$ gas to an exact volume of 10mL. The condensed HNE-DNPH standard was covered with Parafilm tightly and stored at -20°C until use.

5. *Preparation of DNPH-hydrazones of lipophilic aldehydes and related carbonyl compounds from animal fats*

   One gram of unheated or heat-treated animal fats was reacted with 5mL freshly prepared DNPH reagent in 50mL Erlenmeyer flask in duplicate. The mixture was incubated at about 40°C in the shaker with 120 oscillations/min to react overnight in the dark. After incubation, the DNPH derivatives were extracted with 10mL methanol/water (75:25, vol/vol) and separated by centrifugation at 2000 rpm for 10 min. The aqueous layer was removed and the oil layer was extracted with 10mL methanol/water (75:25, vol/vol) two more times as before. The combined aqueous layer was further extracted three times with 10mL dichloromethane in separating funnel. The dichloromethane layer was combined and evaporated under $N_2$ gas until the sample volume was about 1mL.

6. *Preliminary separation of DNPH-hydrazones of lipophilic aldehydes and related carbonyl compound by Thin Layer Chromatography (TLC)*

   The concentrated dichloromethane extract (about 1mL) was applied equally onto two silica gel thin-layer chromatographic plates for preliminary separation. The sample was applied in a very thin straight line across the bottom of plates by using a 250μL micropipette attached to a Hamilton syringe with a piece of flexible rubber tubing. To identify the position of the polar and nonpolar aldehyde-DNPH in samples, the DNPH reagent blank and acetone-DNPH standard were also spotted on the plates in the same line next to sample. The plates were developed in HPLC-grade dichloromethane for 45min.

Figure 20 shows a diagram of a typical TLC plate. With the location of DNPH reagent and acetone-DNPH standards, the nonpolar and polar aldehydes and other related carbonyl compounds can be identified. Polar carbonyl (PC) compounds, including the HNE-DNPH, were in the bottom region between the origin and the DNPH reagent band ($R_f = 0.25$). Nonpolar carbonyl (NPC) compounds, such as alkanals, alkenals, alkadienals and ketones, were in the top region between the acetone-DNPH band ($R_f = 0.75$) and the solvent front. Between the acetone-DNPH band and the DNPH reagent band were some osazones. The polar and nonpolar regions were cut from the plates and then cut into small pieces, and placed in 125mL Erlenmeyer flasks. The compounds were eluted from TLC plate three times with 10mL methanol. The combined PC and NPC methanol extracts were evaporated under $N_2$ gas to about 0.5mL and centrifuged at 2000 rpm for 5 min to separate the silica gel residue. The clear supernatants were transferred to 1mL volumetric flasks and diluted with methanol to exact 1mL. The final samples were transferred by disposable syringe with a 0.2 μm PVDF filter into amber vials tightly covered with Parafilm at -20°C until HPLC analysis.

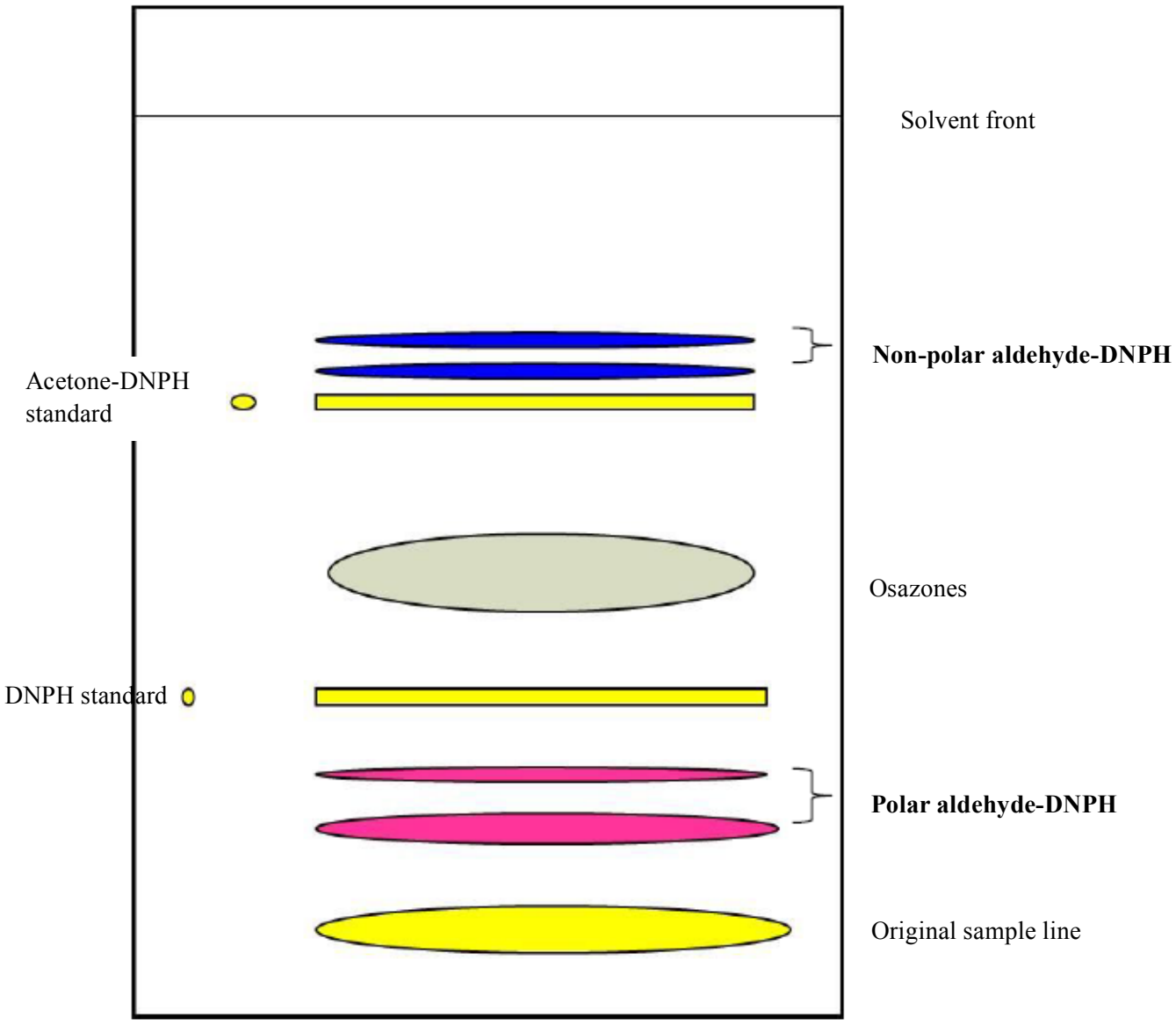



**Figure 20. Display of polar and non-polar aldehydes-DNPH derivatives after development**

7. *Separation and identification of DNPH derivatives of PC and NPC lipophilic aldehydes and related carbonyl compounds from animal fats by HPLC*

Fifty μL aliquots of PC-DNPH derivatives and NPC-DNPH derivatives were injected into an HPLC reverse-phase C18 column, equipped with a guard column. For DNPH derivatives of the PC, started with 10 min of isocratic elution with methanol/water (50:50, vol/vol), then a linear gradient to 100% methanol for next 20 min followed by 100% methanol for an additional 10 min. For DNPH derivatives of the NPC, started with 10 min of isocratic elution with methanol/water (75:25, vol/vol), then a linear gradient to 100% methanol for next 20 min followed by 100% methanol for an additional 10 min. For both, the total elution time was 40 min at a flow rate of 0.8mL/min. Absorbance was measured at 378 nm. A HNE-DNPH standard was injected daily to give a reference retention time and to check the reproducibility of the HPLC system.

Identification of HNE-DNPH from animal fats was accomplished a) by comparing the retention times of HNE-DNPH standard and the retention times of peaks derived from samples, and b) by co-chromatography of sample mixed with a certain amount of pure HNE-DNPH standards. Sample, the standard and the mixture of sample and standard were injected, respectively. The ratio of the peak area of the mixture sample compared with expected peak area was calculated.

The HPLC peak area of DNPH-HNE was converted to hexanal equivalent (1 ng of hexanal standard equals 22182 peak area), with the unite of μg hexanal equivalent/g fat. Then, the quantity of HNE (ng HNE/ g fat) was calculated based on the molecular weight.

**Trolox Equivalent Antioxidant Capacity (TEAC) Assay**

TEAC measures the antioxidant capacity of a given substance by comparing to the standard, trolox. TEAC assay is based on the converting ABTS radical cation ($ABTS^{\cdot+}$) into a colorless non-radical product [77]. The TEAC value of a sample is determined by the ratio of decolorization induced by samples over that induced by trolox.

This method is developed according to Pellergrini *et al* [78, 79]. ABTS was dissolved in water to make 7mM ABTS stock solution. Then, The ABTS radical cation solution was prepared by reacting ABTS stock solution with 2.45 mM potassium persulfate in the dark at room temperature for 12-16 hours before use. The ABTS radical cation solution was diluted by ethanol with final absorbance of 0.7 (±0.02) at 734nm. Fats were melted in water bath at 35~45 °C, then 1g of each fat sample was weighted and dissolved in 5mL hexane in triplicate and mixed well. Then 40μL of sample was added into 4.0mL of diluted ABTS radical cation solution. After 6 min, the discoloration was measured by UV-Vis Spectrophotometer at 734 nm. Before samples were measured, the standard curve was determined by adding 4.0mL of diluted ABTS radical cation solution to 40μL of various concentrations of trolox standards in triplicate and mixed well. The various concentrations of trolox standards was made by diluting 2.5mM trolox with ethanol as listed below in Table 4. The absorbance of the mixture was measured by UV-Vis Spectrophotometer at 734 nm after 6 min. The standard curve is shown in Figure 21.

**Table 4. The concentration of trolox for TEAC assay.**

| Tube Number | 2.5mM Trolox (μL) | Ethanol (μL) | Total volume (μL) | Concentration of Trolox standard solution (mM) | Final concentration of Trolox (after adding 4.0mL $ABTS^{\cdot+}$ solution) (μM) |
|---|---|---|---|---|---|
| 1 | 0 | 1000 | 1000 | 0.00 | 0.00 |
| 2 | 100 | 900 | 1000 | 0.25 | 2.48 |
| 3 | 200 | 800 | 1000 | 0.50 | 4.95 |
| 4 | 300 | 700 | 1000 | 0.75 | 7.43 |
| 5 | 400 | 600 | 1000 | 1.00 | 9.90 |
| 6 | 500 | 500 | 1000 | 1.25 | 12.38 |

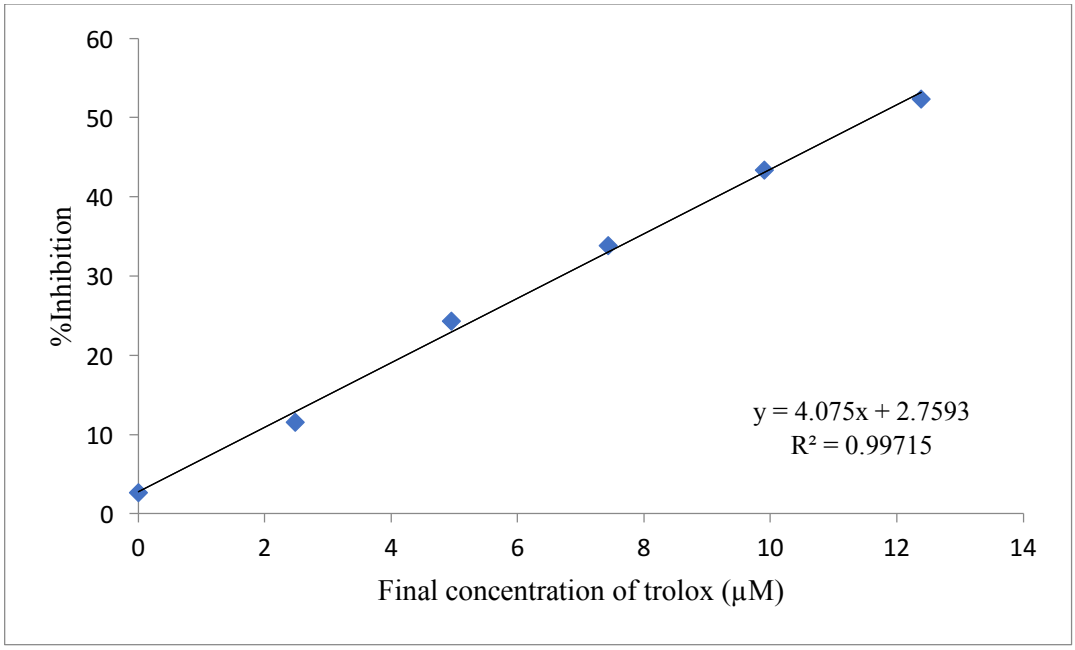



**Figure 21. Trolox Standards Curve.**

**(X axis: final concentration of Trolox (μM); Y axis: % inhibition= (Abs control – Abs sample)/Abs control × 100; Solvent blanks to calibrate zero: ethanol)**

### Statistical Analysis

The average and standard error of the mean were calculated for HNE-DNPH. ANOVA was used to determine the significant differences between groups. Statistic differences were determined at $p \leq 0.05$.

## Results

### Fatty Acid Distribution

Animal fats from different sources have different fatty acids composition, with different lipid oxidation capacity. Therefore, the fatty acid distribution of fats from beef, pork and chicken were analyzed by GC. Table 5 shows the retention time of pure fatty acids standards measured by GC method described above.

**Table 5. Retention time of fatty acids standards.**

| Fatty Acid | Retention time (min) |
|---|---|
| Palmitic acid | 24.3 |
| Stearic acid | 18.6 |
| Oleic acid | 42.6 |
| Linoleic acid | 2.6 |
| Linolenic acid | 0.7 |

The fatty acid percentage of beef, pork and chicken fats are shown in Table 6 – 8. The identification of fatty acids was determined by comparing the retention times of fatty acids in samples and standards.

**Table 6. The fatty acids percentage of beef fats.**

| Fatty Acids | Average Percentage | | | | |
|---|---|---|---|---|---|
| | Fat from Washed GB | Fat from Unwashed GB | Beef Back Fat | Beef Suet | Commercial Beef Suet |
| Palmitic acid | 25.69 | 26.06 | 21.88 | 28.81 | 24.02 |
| Stearic acid | 14.38 | 18.37 | 7.25 | 20.99 | 22.41 |
| Oleic acid | 51.46 | 47.49 | 56.13 | 40.80 | 45.19 |
| Linoleic acid | 1.91 | 1.36 | ND | 1.72 | 2.58 |

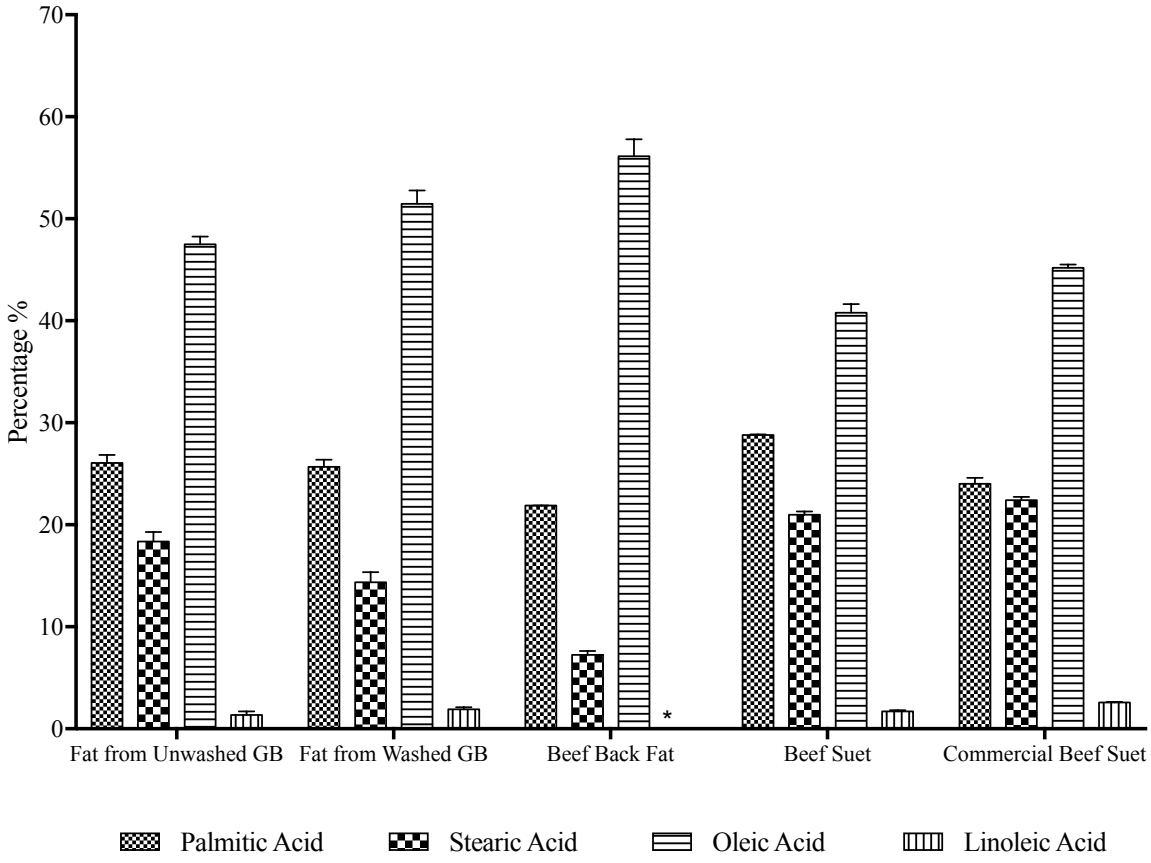



**Figure 22. Comparison of fatty acids distribution of beef fats from different regions.**

*** Means no detection.**

Figure 22 shows that different beef regions have different fatty acids compositions. However, all beef fats contain high percentage of oleic acid, then followed by palmitic acid and stearic acid. The percentage of linoleic acid in beef fats is very small. Linoleic acid wasn't detected in beef back fat.

**Table 7. The fatty acids percentage of pork fats.**

| Fatty Acids | Average Percentage |
|---|---|

| | Fat from Washed GP | Fat from Unwashed GP | Pork Back fat | Leaf Lard | Commercial Lard |
|---|---|---|---|---|---|
| Palmitic acid | 28.63 | 22.84 | 22.72 | 32.60 | 28.54 |
| Stearic acid | 19.44 | 10.39 | 13.16 | 18.16 | 24.52 |
| Oleic acid | 43.90 | 49.94 | 47.26 | 22.91 | 37.31 |
| Linoleic acid | 8.04 | 12.20 | 13.95 | 9.41 | 9.62 |

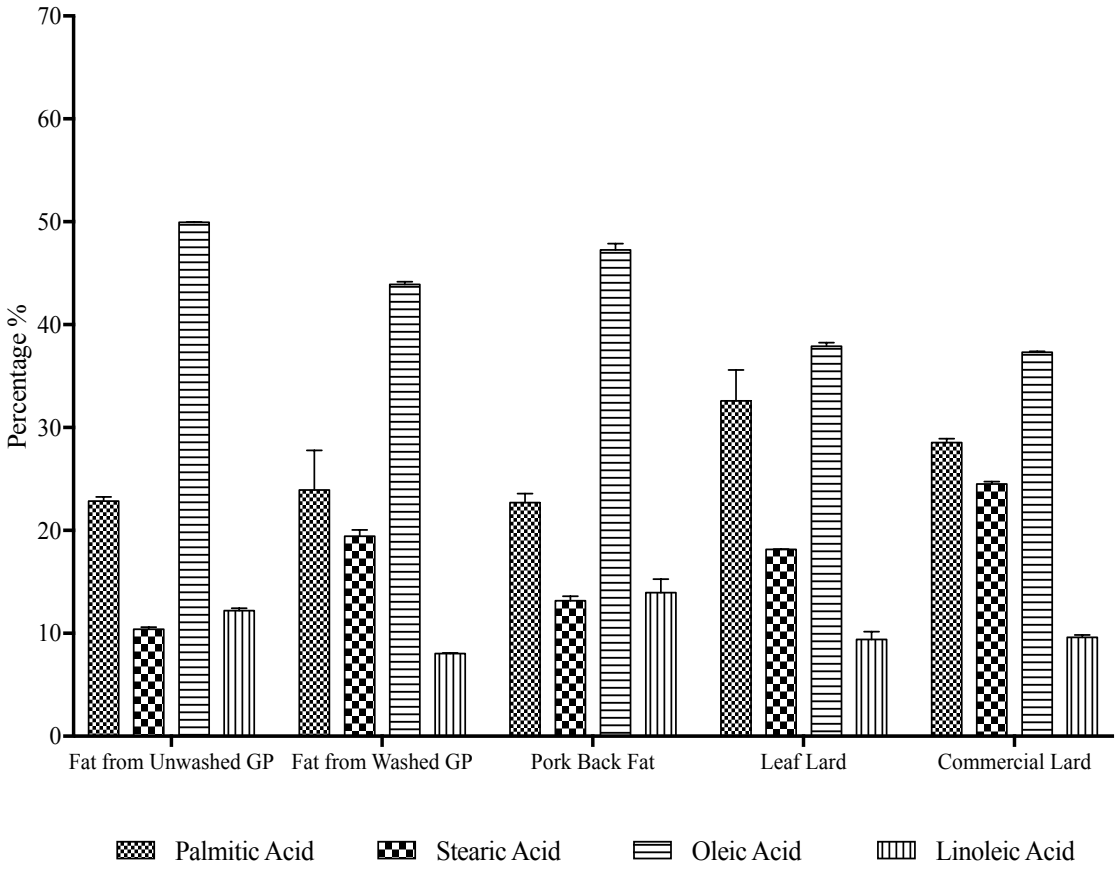



**Figure 23. Comparison of fatty acid distribution of pork fats from different regions.**

Figure 23 shows that different pork regions have different fatty acid compositions. Similar with beef fats, pork fats also contain high level of oleic acid and then followed by palmitic acid. However, in leaf lard, the percentage of palmitic acid is higher than oleic acid. In addition, the linoleic acid content in pork fats is higher than that in beef fat.

**Table 8. The fatty acids percentage of chicken fats.**

| Fatty Acids | Average Percentage | | |
|---|---|---|---|
| | Chicken Thigh Fat | Chicken Skin Fat | Commercial Chicken Fat |
| Palmitic acid | 24.91 | 23.36 | 23.46 |
| Stearic acid | 6.08 | 5.18 | 4.71 |
| Oleic acid | 39.02 | 36.45 | 30.02 |
| Linoleic acid | 25.33 | 28.69 | 33.71 |
| Linolenic acid | 1.25 | 1.40 | 3.54 |

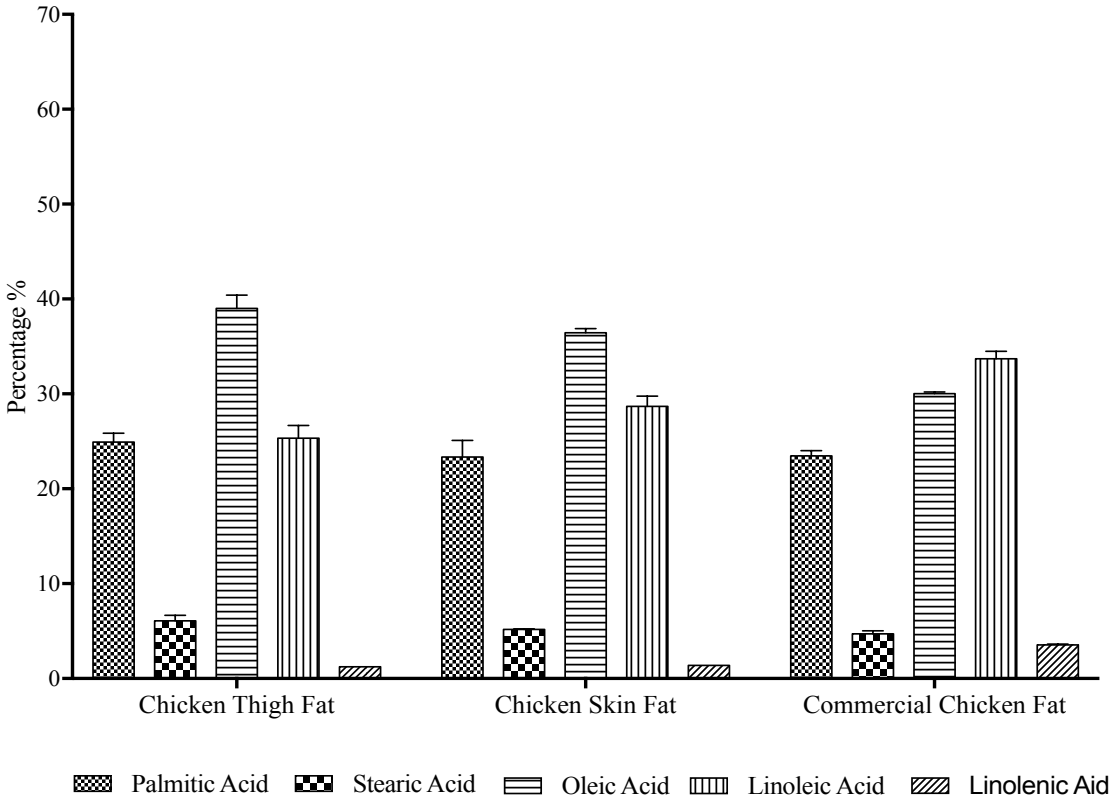



**Figure 24. Comparison of fatty acids distribution of chicken fats from different regions.**

Figure 24 shows that different chicken regions have different fatty acids compositions. All chicken fats contain high level of linoleic acid. A significant amount of linolenic acid was also detected in chicken fats from all sources.

The concentration of linoleic acid is of particular interest since it is the precursor of HNE. Figure 25 shows the comparison of linoleic acid percentage in all beef, pork and chicken fats. Chicken fats have the highest linoleic acid percentage, while beef fats contain lowest amount of linoleic acid.

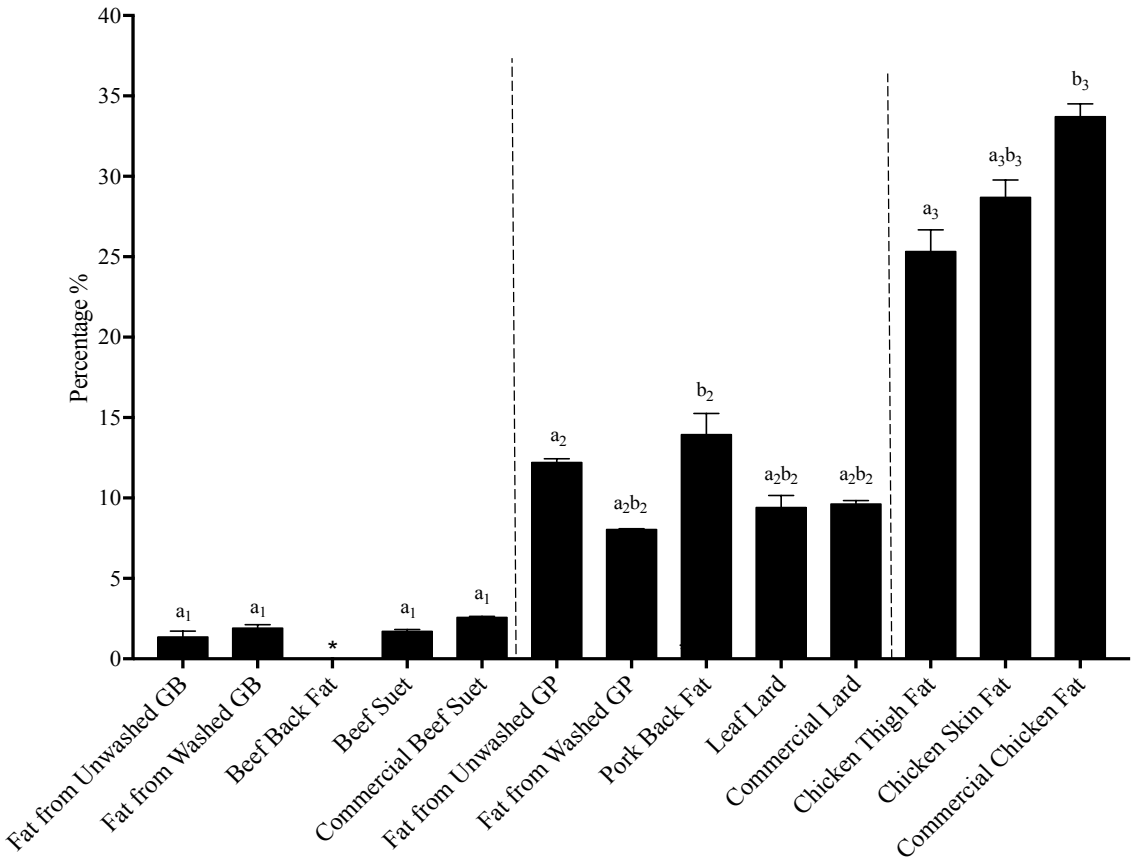



**Figure 25. Comparison of linoleic acid percentage in beef, pork and chicken fats.**

*** Means no detection**.

**Thiobarbituric Acid Reactive Substances (TBARS) Assay**

To measure the oxidation trend of beef, pork and chicken fats, TBARS was conducted as a primary experiment. Results of TBARS shows the total secondary lipid oxidation products in beef, pork and chicken fats heated at 185℃ for 0, 1, 2, 3, 4, 5 and 6 hours. It gives a basic idea on how it's oxidized under heat treatment for time periods.

Figure 26 shows the TBARS analysis results in washed ground beef fat, beef suet, beef back fat and commercial beef suet heated at 185 °C from 0 to 6 hours. This figure clearly

shows that the secondary lipid oxidation products of beef suet, beef back fat and commercial fat were increased with the increasing heating time at first and reached the plateau after 1 hour of heating. However, the amount of secondary lipid oxidation products of washed ground beef fat was decreased during the period when temperature remained at 185 °C. This's probably due to the evaporation of some short-chain compounds. Then, the amount of secondary lipid oxidation products of washed ground beef fat was increased with the increasing heating time and lower than the other three beef fat sources. The lower level of product may attribute to the antioxidants extracted from ground beef meat.

Figure 27 shows the TBARS analysis results in washed ground pork fat, pork back fat, leaf lard and commercial lard heated at 185 °C from 0 to 6 hours. The TBARS results in all pork fats increased with the increasing heating time during first 2 hours of heating, and then reached the plateau during the following hours of heating. Additionally, the amount of secondary lipid oxidation products of washed ground pork fat was lower than other pork fat sources, probably due to the antioxidants co-extracted from ground pork.

Figure 28 shows the TBARS analysis results in chicken thigh fat, chicken skin fat and commercial chicken fat heated at 185 °C from 0 to 6 hours. The total secondary lipid oxidation products in all chicken fats were increased with the increasing heating time and reached the highest level at 1 or 2 hours of heating. The secondary lipid oxidation products were then remained at the high level during the following hours of heating.

Figure 29 shows the comparison of all secondary oxidation products among beef, pork and chicken fats heated at 185 °C from 0 to 6 hours. As shown in this figure, beef fats in general produced lowest level of secondary lipid oxidation products compared with pork and chicken fats, which may attribute to the low percentage of polyunsaturated fatty acids. The TBARS result of fat extracted from meat is lower than the other fat sources, which may due to the lipid soluble antioxidant co-extracted from ground meat, like α-tocopherol. There is no difference between chicken fats and pork fats even though chicken fats contain higher amount of polyunsaturated fatty acids. This may be due to the antioxidants effects. It's interesting to find out the antioxidants capacity in all samples.

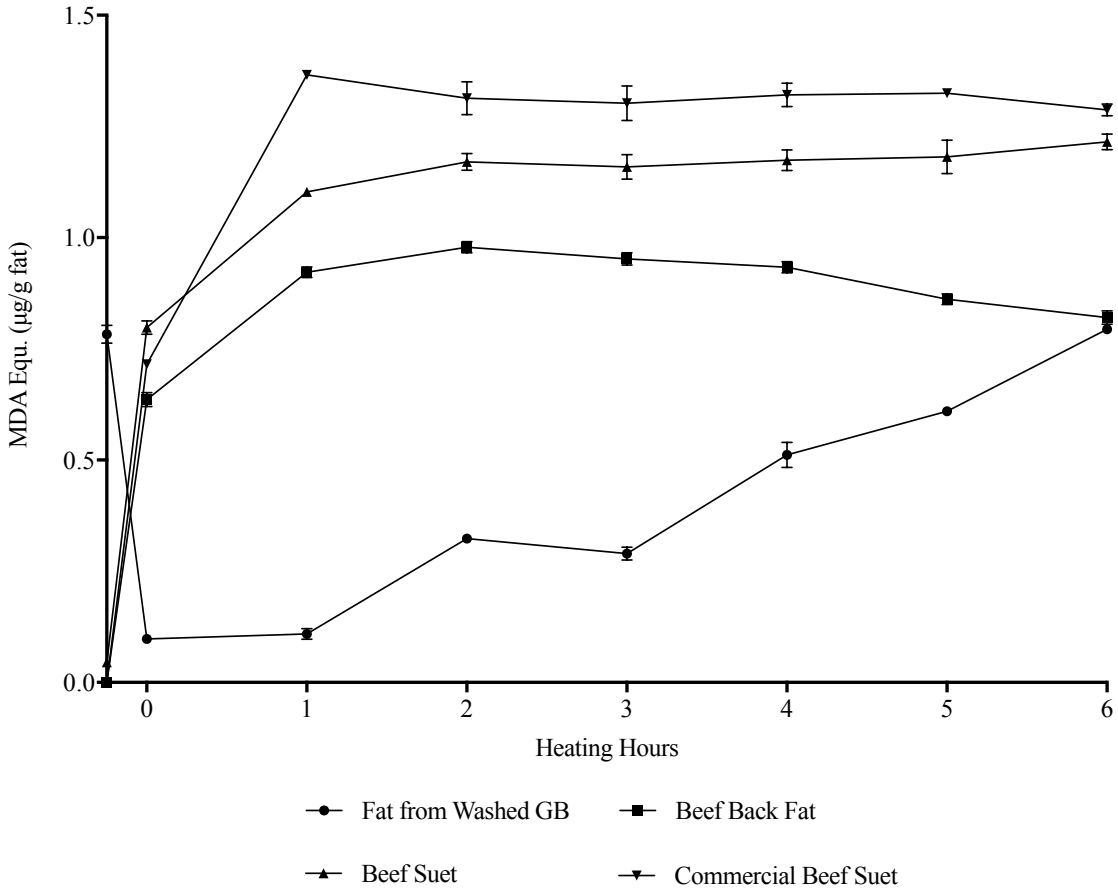



**Figure 26. Comparison of TBARS analysis among different beef fats heated at 185 °C for 0, 1, 2, 3, 4, 5 and 6 hours.**

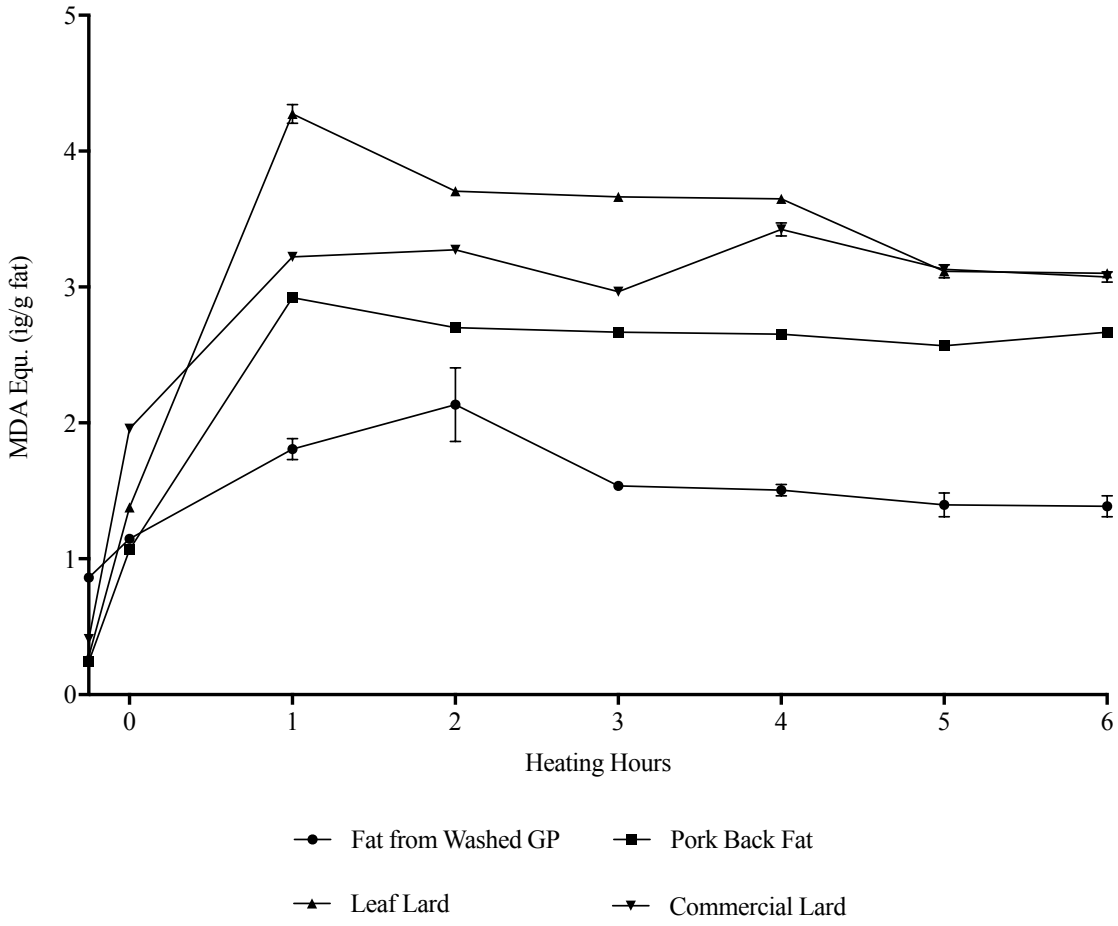



**Figure 27. Comparison of TBARS analysis among different pork fats heated at 185 °C for 0, 1, 2, 3, 4, 5 and 6 hours.**

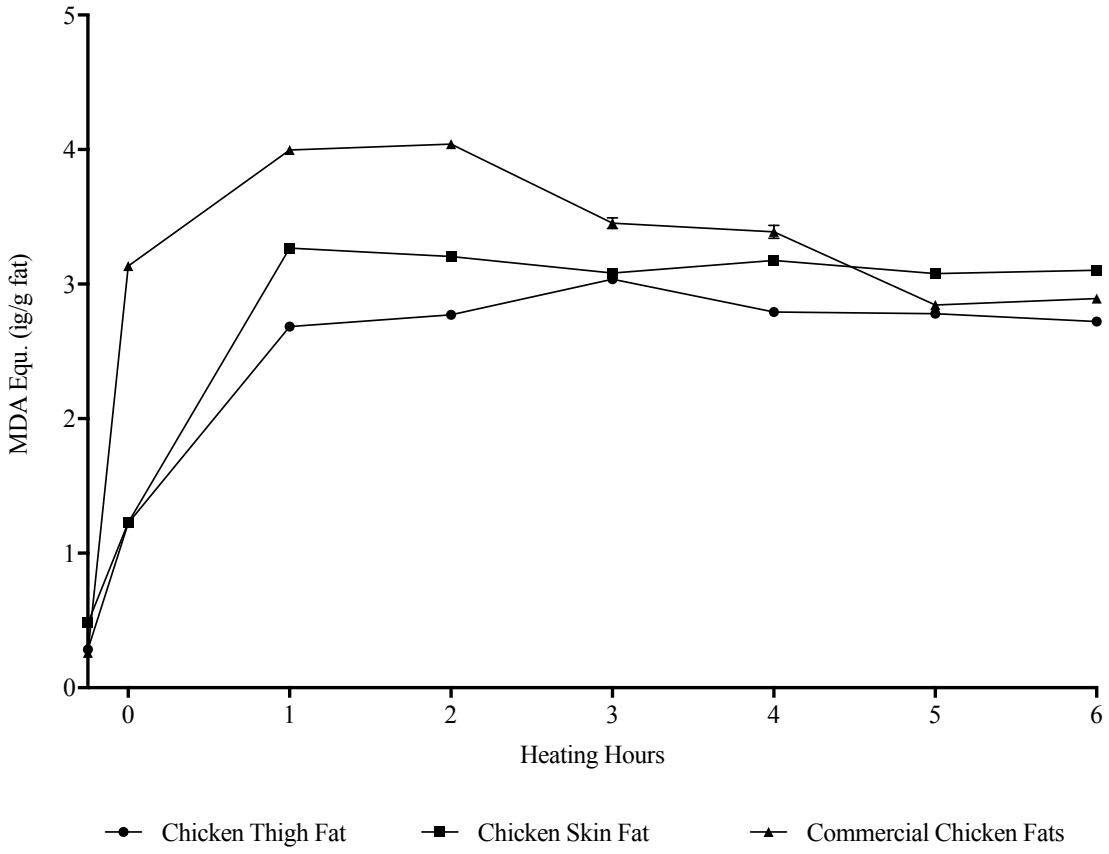



**Figure 28. Comparison of TBARS analysis among different extracted chicken fats heated at 185 °C for 0, 1, 2, 3, 4, 5 and 6 hours.**

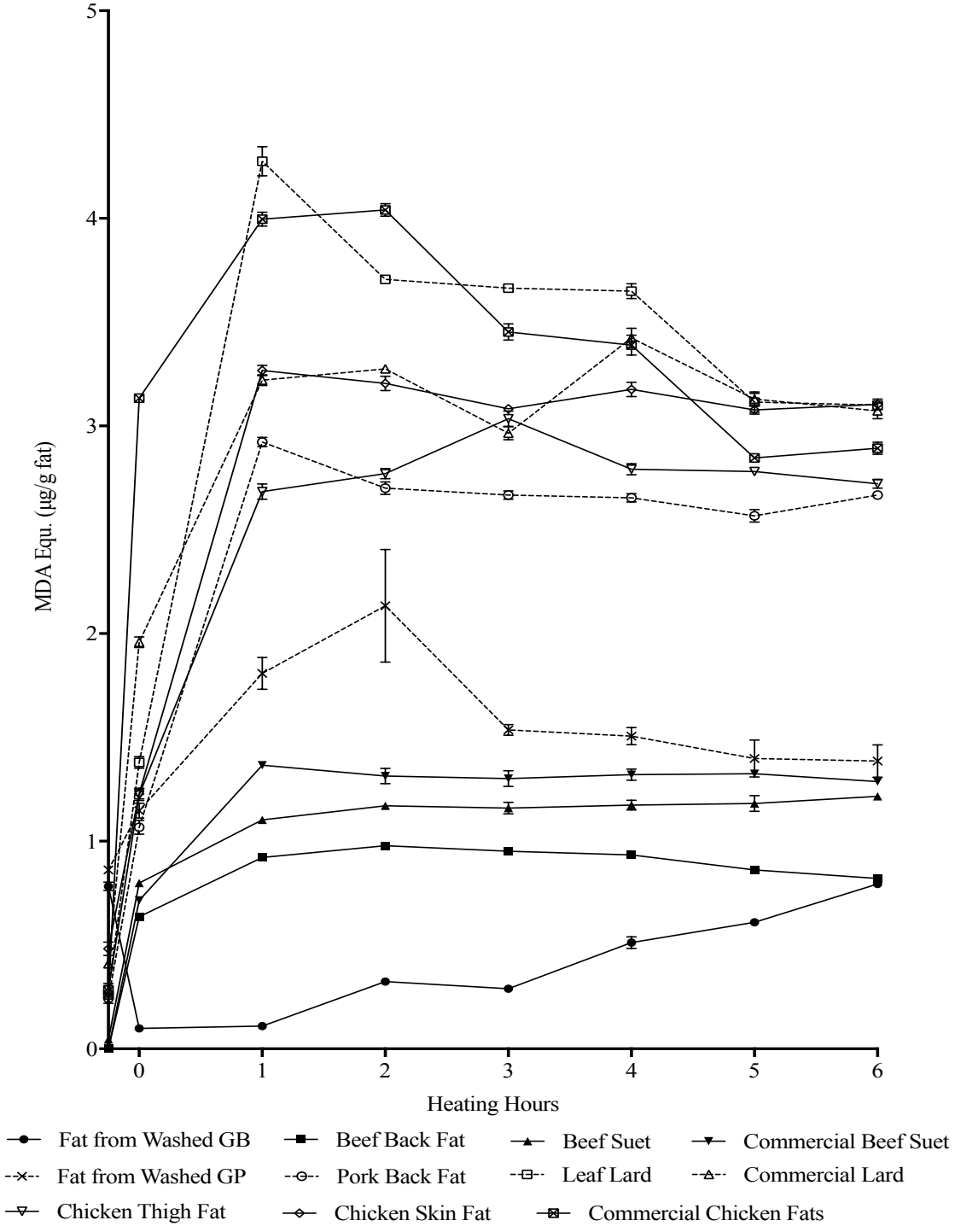



**Figure 29. Comparison of all secondary oxidation products among beef, pork and chicken fats heated at 185 °C from 0 to 6 hours.**

**HNE formation in beef, pork and chicken fats heated at 185°C for 0, 1, 3 and 5 hours**

The formation of HNE was measured in all samples, including washed ground beef fat, unwashed ground beef fat, beef back fat, beef suet, commercial beef suet, washed ground pork fat, unwashed ground pork fat, pork back fat, leaf lard, commercial lard, chicken thigh fat, chicken skin fat and commercial chicken fat. Each of the above-mentioned fats was heated at 185°C for 0, 1, 3 and 5 hours. Figure 30 to 36 show the HNE formation in samples. Representatives of HPLC chromatography were shown in Appendix.

*Beef fats*

Figure 30 shows the heating time related HNE formation in fat extracted from unwashed ground beef heated at 185°C for 0, 1, 3 and 5 hours. The concentration of HNE was increased with the heating time. The HNE concentration after 0, 1, 3 and 5 hours of heating were 0.49, 0.80, 3.32 and 5.17 μg /g fat, respectively. The HNE formation increased slightly during the first hour of heating, and then increased remarkably during the next 4 hours of heating. There was significant difference among HNE concentration at 1, 3 and 5 hours of heating.

Figure 31 shows the heating time related HNE formation of fat extracted from washed ground beef heated at 185°C. Similar with unwashed ground beef fat, the HNE concentration increased with the increasing heating time. The HNE concentration after 0, 1, 3 and 5 hours of heating were 0.70, 0.87, 3.95 and 4.48 μg /g fat, respectively. The HNE formation in washed ground beef fat increased slightly during the first hour of heating, and then increased remarkably during the next 4 hours of heating. There was significant difference among HNE concentration of 1, 3 and 5 hours of heating.

Figure 32 shows the heating time related HNE formation of beef suet heated at 185°C. It can be seen from this figure that the HNE formation increased with the increasing heating time. The HNE concentration of 0, 1, 3 and 5 hours of heating were 0.86, 3.81, 6.94 and 8.62 μg /g fat, respectively. There was significant difference among 0, 1, 3 and 5 hours of heating.

Figure 33 shows the heating time related HNE formation of beef back fat heated at 185°C. It shows that the HNE concentration significantly increased during 1 and 3 hours of heating. However, there is no significant difference between 3 and 5 hours of heating. The HNE concentration of 0, 1, 3 and 5 hours heating were 0.35, 4.80, 7.21 and 6.79 μg /g fat, respectively.

Figure 34 shows the heating time related HNE formation of commercial beef suet heated at 185°C. The HNE concentration of 0, 1, 3 and 5 hours of heating were 0.43, 4.34, 4.81 and 5.04 μg /g fat, respectively. From this figure, during 1 hour of heating, the HNE concentration increased significantly. After the next 3 and 5 hours of heating, the HNE formation increased slightly and there was no significant difference among 1, 3 and 5 hours of heating.

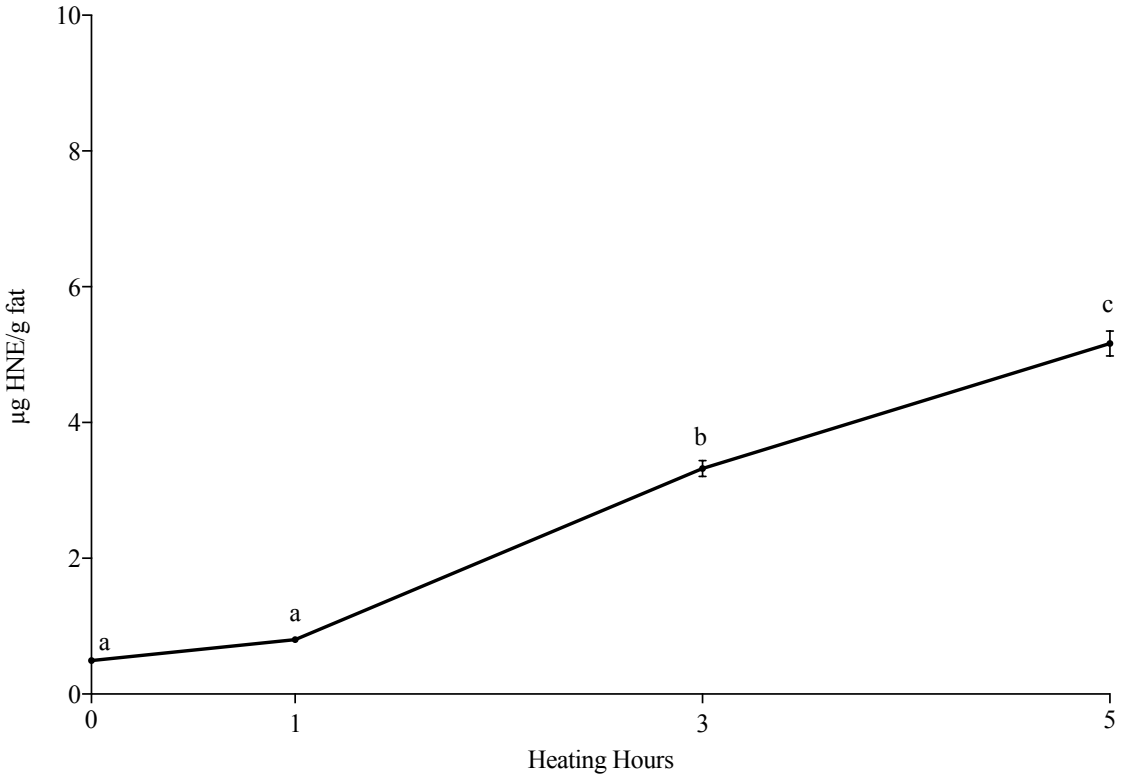



**Figure 30. Heating time related HNE formation of fats extracted from unwashed ground beef meat heated at 185°C.**

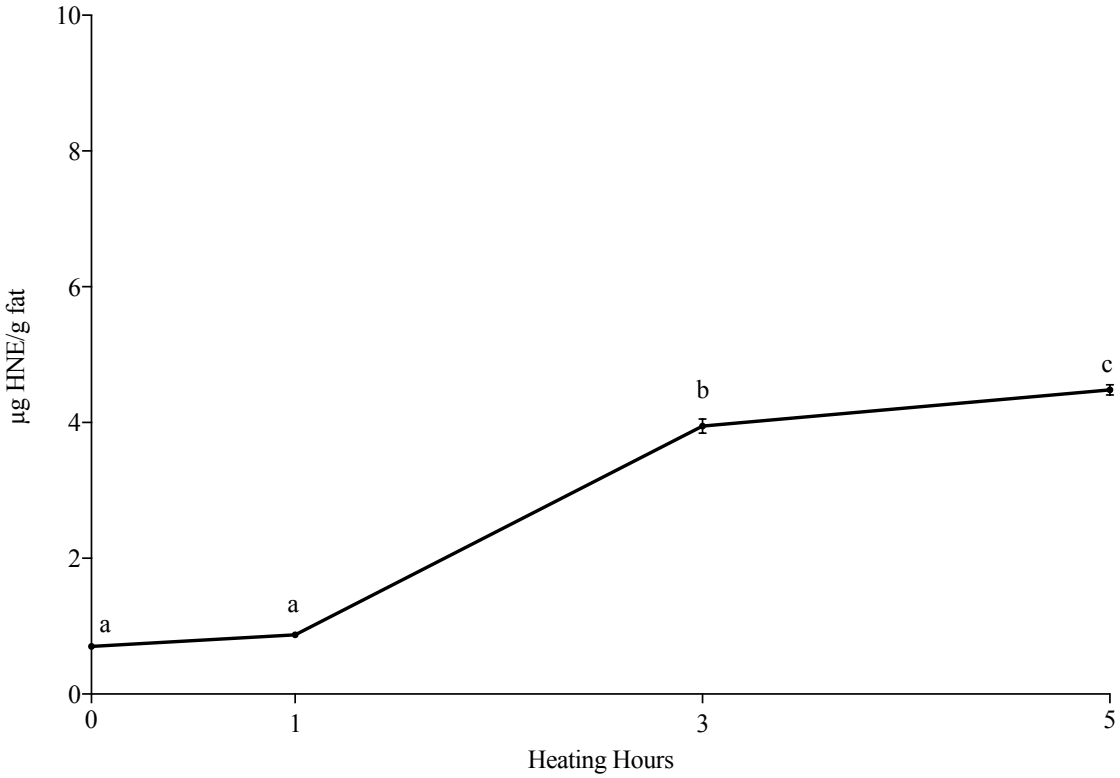



**Figure 31. Heating time related HNE formation of fats extracted from washed ground beef meat heated at 185°C.**

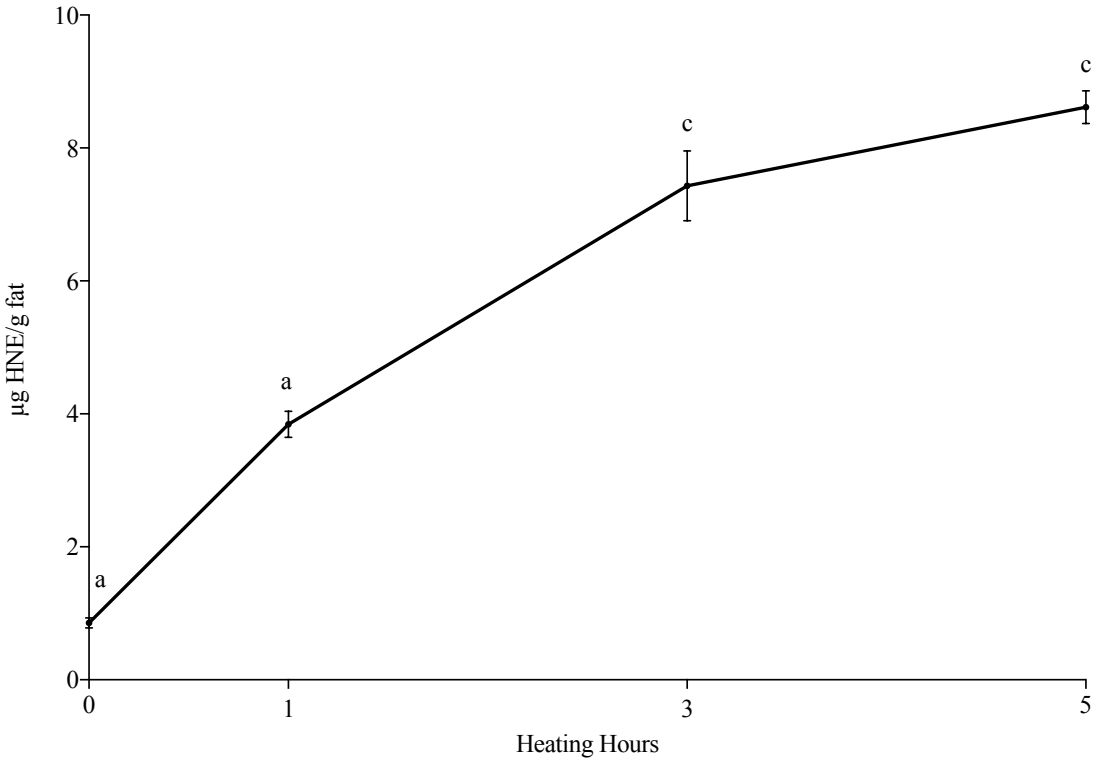



**Figure 32. Heating time related HNE formation of beef suet heated at 185°C.**

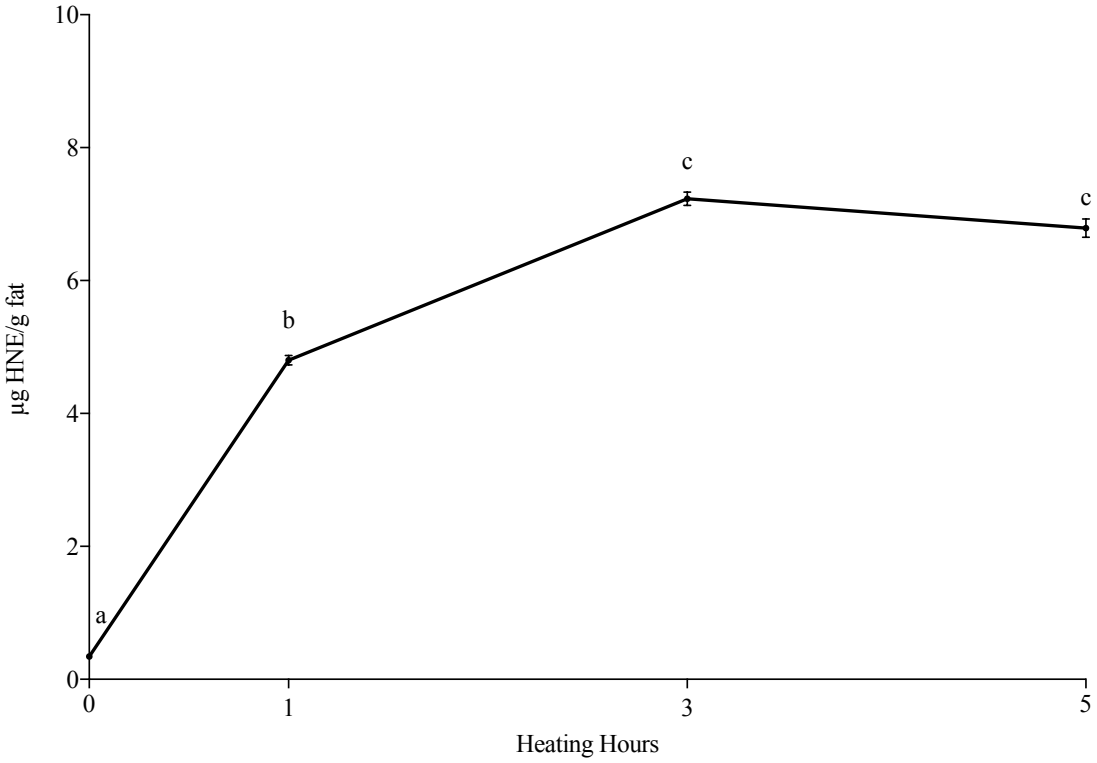



**Figure 33. Heating time related HNE formation of beef back fat heated at 185°C.**

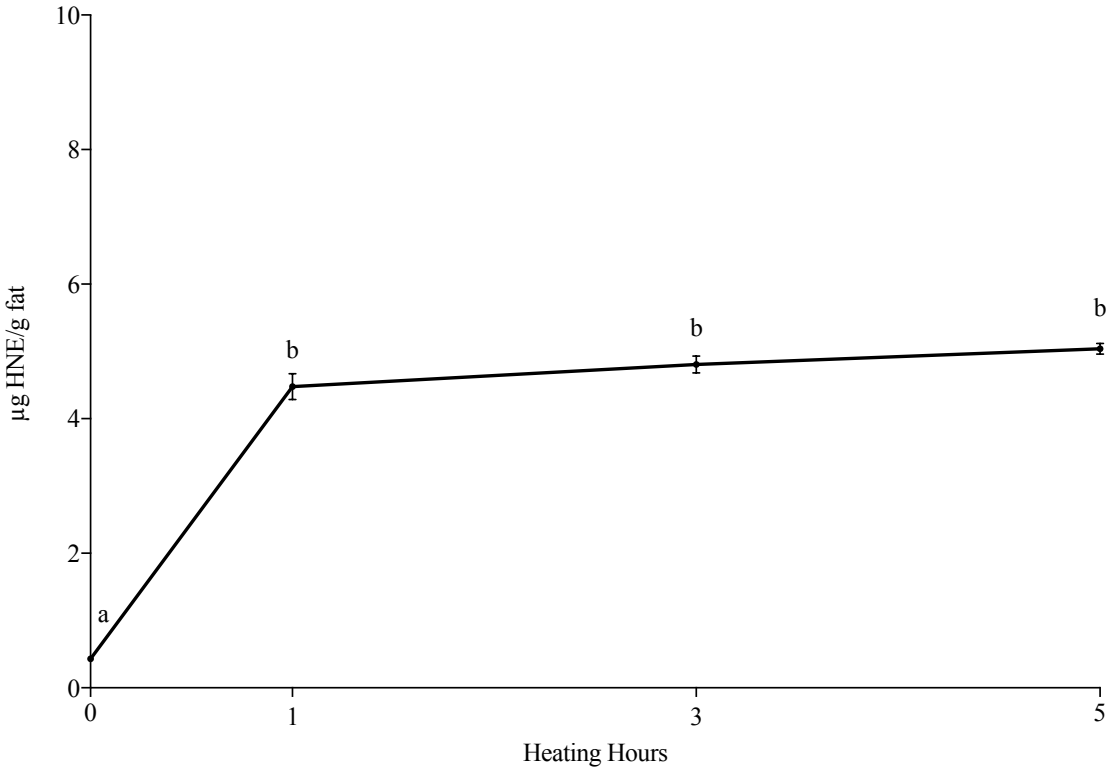



**Figure 34. Heating time related HNE formation of commercial beef suet heated at 185°C.**

Figure 35 shows the HNE formation in all sources of beef fat heated at 185°C. In general, the HNE concentration increased with the increasing heating time. The HNE formation in fat extracted from washed and unwashed ground beef started to increase remarkably at the third hours of heating, while that in other beef fat sources started to increase significantly during the first hour of heating. The HNE formation in fat extracted from ground beef increased later and slower than other beef fat sources. It may be explained by the reaction between HNE and the extracted fat-soluble compounds from ground beef.

Figure 36 also shows the HNE formation in heated beef fats at 185°C, clustered by heating hours. There was no significant difference between washed and unwashed ground beef fat during the heating periods. Also, there was no significant difference among all unheated beef fat sources. However, the HNE concentration in washed and unwashed ground beef fats were lower than other beef fat scours at 1, 3 and 5 hours heating. Beef suet has the highest HNE formation at 5 hours of heating among all different beef fat sources, which is 8.62 µg HNE/g fat.

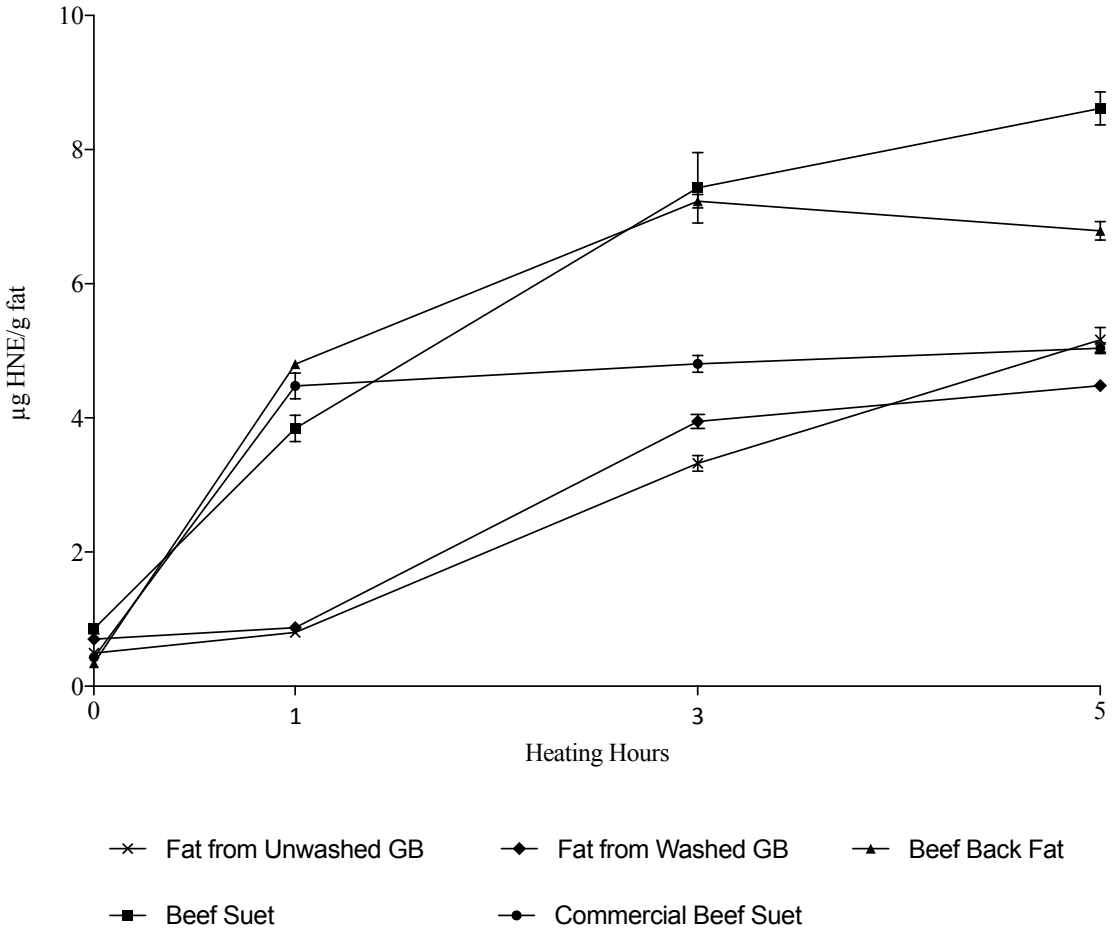



**Figure 35. Change of HNE formation of different beef fats heated at 185°C.**

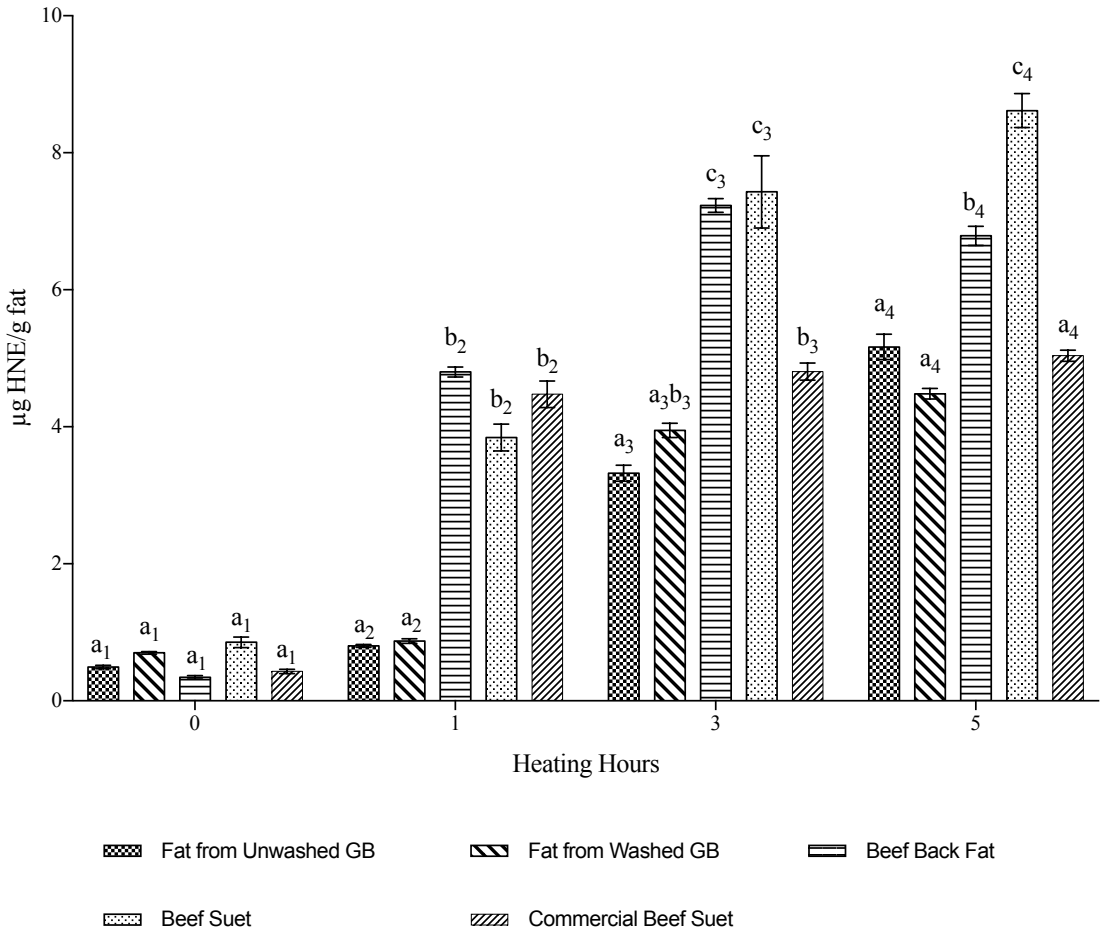



**Figure 36. Change of HNE formation of beef fats heated at 185°C for 0, 1, 3 and 5 hours.**

*Pork fats*

Figure 37 - 43 show the HNE formation in unheated and heated pork fats heated at 185°C for 1, 3 and 5 hours. Figure 36 shows the heating time related HNE formation of fats extracted from unwashed ground pork meat heated at 185°C. The formation of HNE increased with the heating time. And there was a significant increase among the heating

period. The HNE concentration of 0, 1, 3and 5 hours of heating are 0.51, 1.98, 8.34 and 9.71 μg /g fat, respectively.

Figure 37 shows the heating time related HNE formation of fats extracted from washed ground pork heated at 185°C. It has the same pattern as the unwashed ground pork. The formation of HNE increased with the heating time. The HNE concentration increased slightly after 1 hour of heating and increased significantly at the next 3 and 5 hours of heating. The HNE concentration of 0, 1, 3 and 5 hours heating were 0.44, 1.48, 7.57 and 11.48 μg /g fat, respectively.

Figure 38 shows the heating time related HNE formation of leaf lard heated at 185°C. It shows that the formation of HNE increased with the heating time. The HNE concentration increased significantly after 1 and 3 hours of heating. At 5 hours of heating, it slightly increased but there was no significant difference between 3 and 5 hours of heating. The HNE concentration of 0, 1, 3 and 5 hours of heating were 0.30, 11.44, 18.08 and 21.47 μg /g fat, respectively.

Figure 39 shows the heating time related HNE formation of pork back fat heated at 185°C. It shows that the formation of HNE increased significantly with the heating time until 3 hours of heating. However, it decreased at 5 hours of heating. We do not think the HNE concentration at 3 hours of heating is a reproducible data, since the HNE formation in all the other fat sources were increased with the heating time, The HNE concentration of 0, 1, 3 and 5 hours of heating are 1.11, 11.99, 19.50 and 15.04 μg /g fat, respectively.

Figure 40 shows the heating time related HNE formation of commercial lard heated at 185°C. It shows that the formation of HNE increased significantly with the increasing heating time. The HNE concentration of 0, 1, 3 and 5 hours of heating were 0.53, 19.15, 30.42 and 38.82 µg /g fat, respectively.

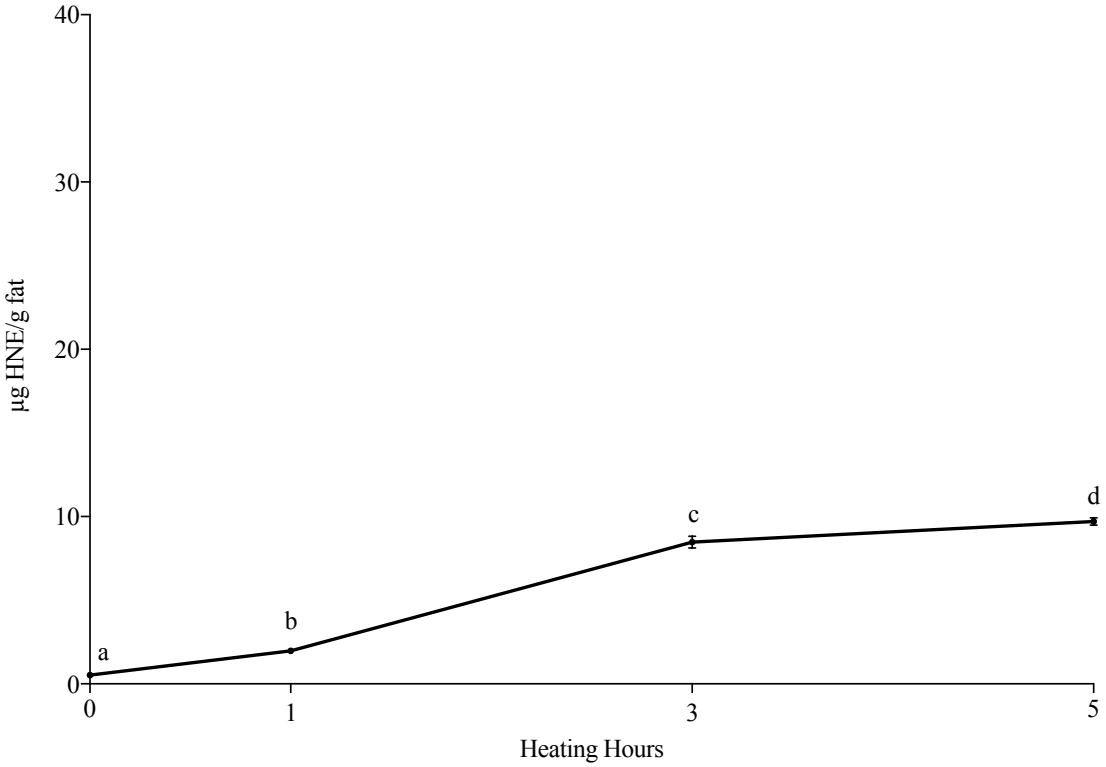



**Figure 37. Change of HNE formation of beef fats heated at 185°C for 0, 1, 3 and 5 hours.**

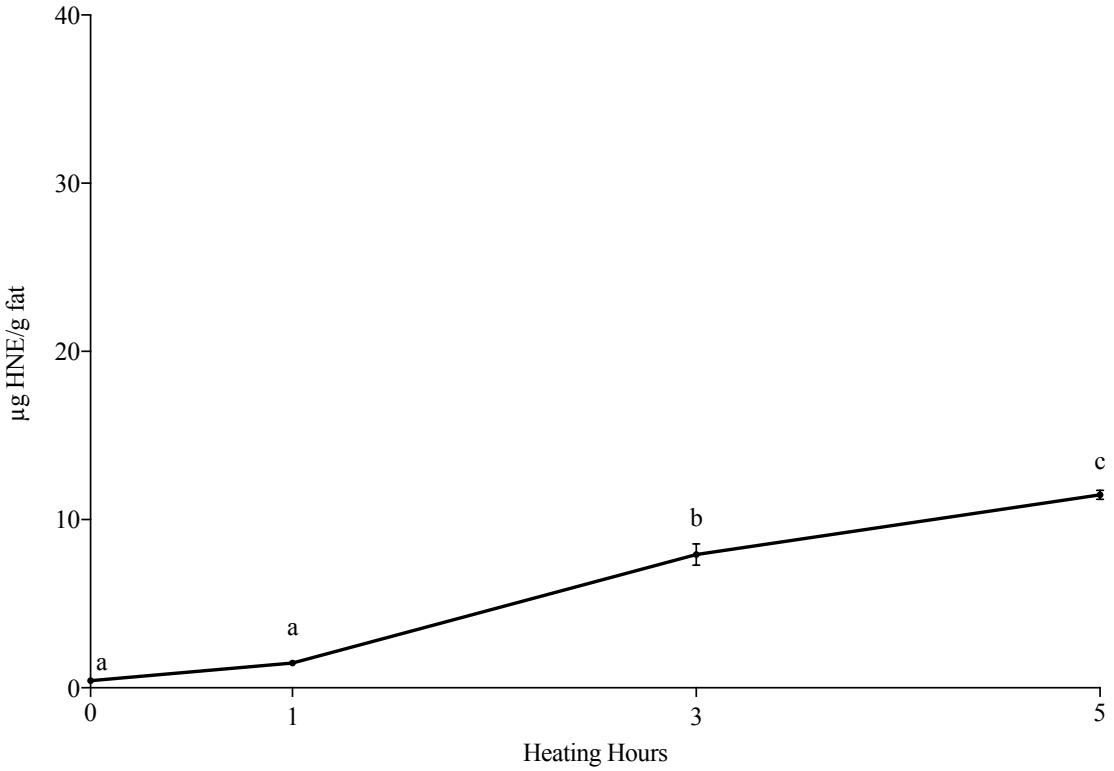



**Figure 38. Heating time related HNE formation of fats extracted from washed ground pork meat at 185°C.**

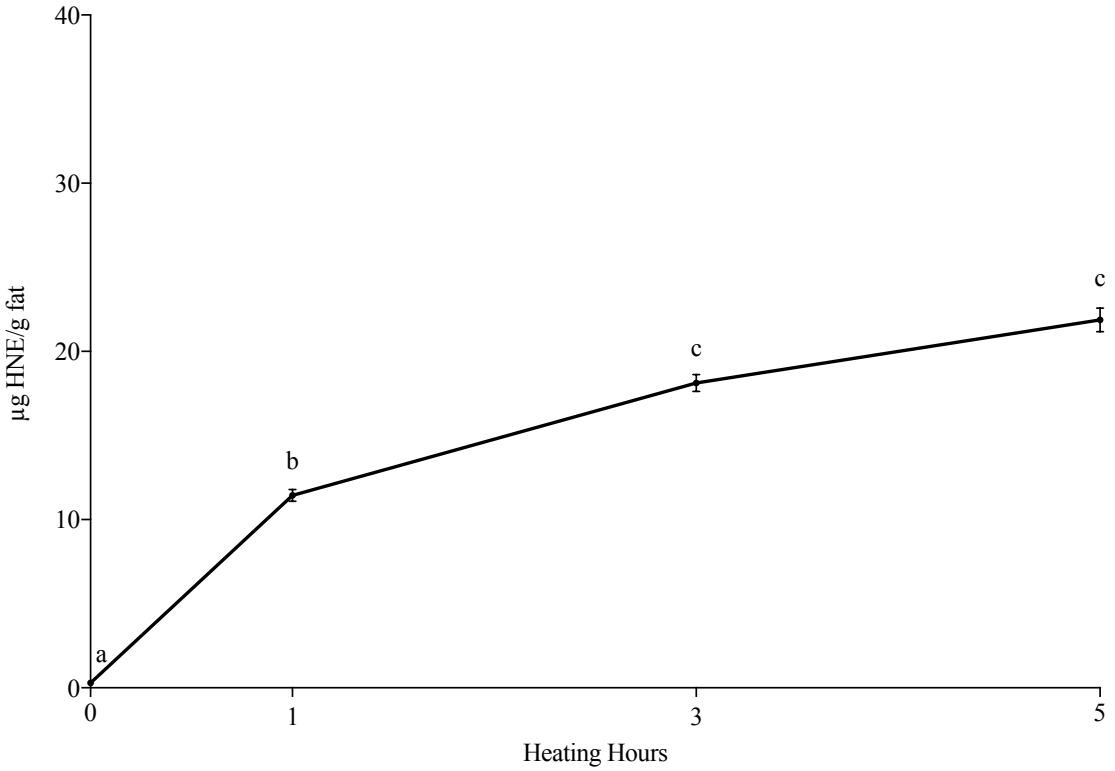



**Figure 39. Heating time related HNE formation of leaf lard heated at 185°C.**

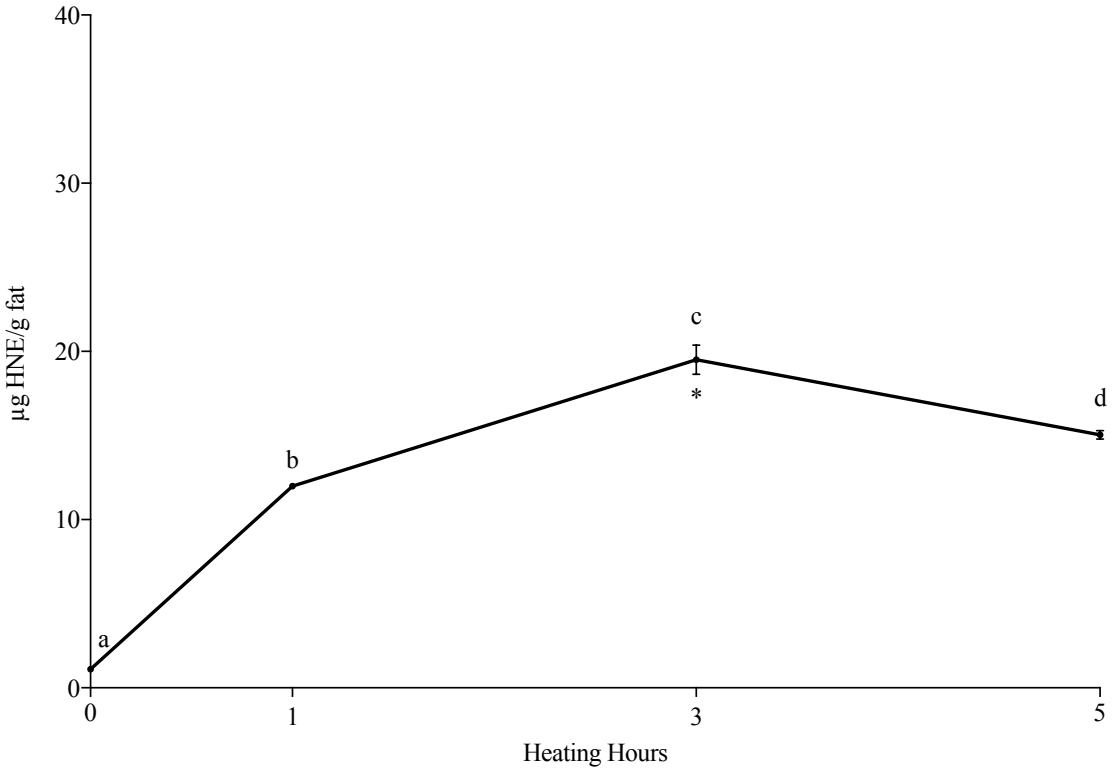



**Figure 40. Heating time related HNE formation of pork back fats heated at 185°C.**

*** Not reproducible data.**

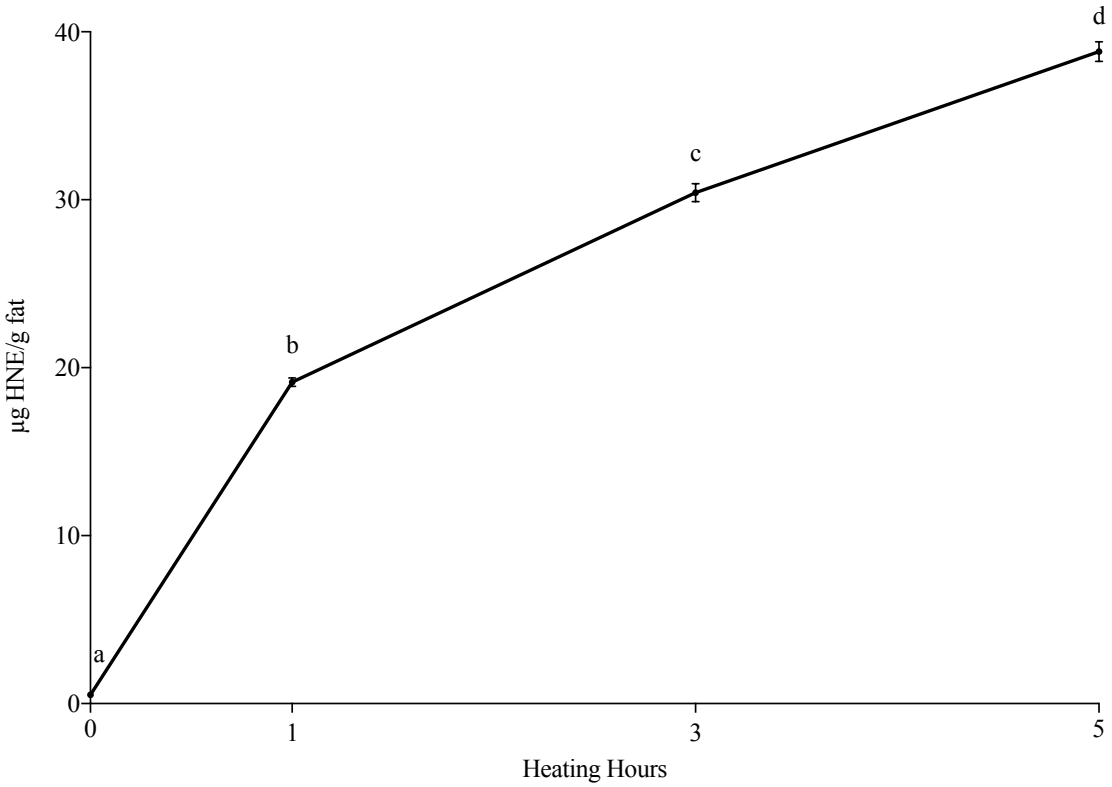



**Figure 41. Heating time related HNE formation of commercial lard heated at 185°C.**

Figure 42 shows the HNE formation in various pork fat sources heated at 185°C. In general, the HNE concentration increased with the heating time. The HNE concentration of fats extracted from washed and unwashed ground pork increased remarkably at the third hour of heating. However, it started to increased remarkably after 1 hour of heating for other pork fat sources. This probably due to the reaction between HNE and the extracted fat-soluble compounds from ground pork.

Figure 43 also shows the HNE formation in different pork fats clustered by heating hours. There was no significant difference between washed and unwashed ground pork fat during the heating periods. Also, the HNE formations in washed and unwashed ground pork fats were lower and slower than that of other pork fat scours at 1, 3 and 5 hours of heating. Commercial lard had the highest HNE formation at 1, 3 and 5 hours of heating compared with other pork fat sources. It reached the highest HNE concentration after 5 hours of heating, which is 38.82 µg HNE/g fat.

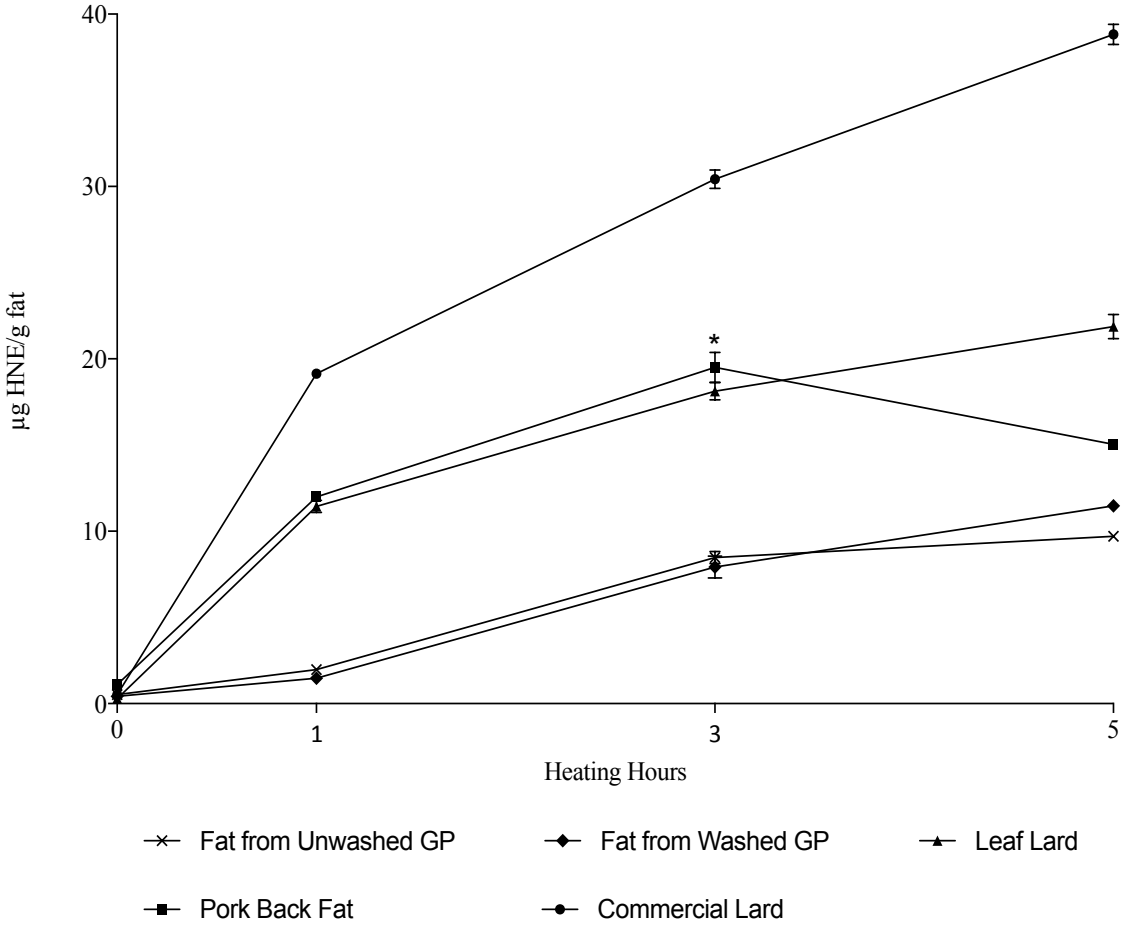



**Figure 42. Change of HNE formation of different pork fats heated at 185°C.**

***Not reproducible data.**

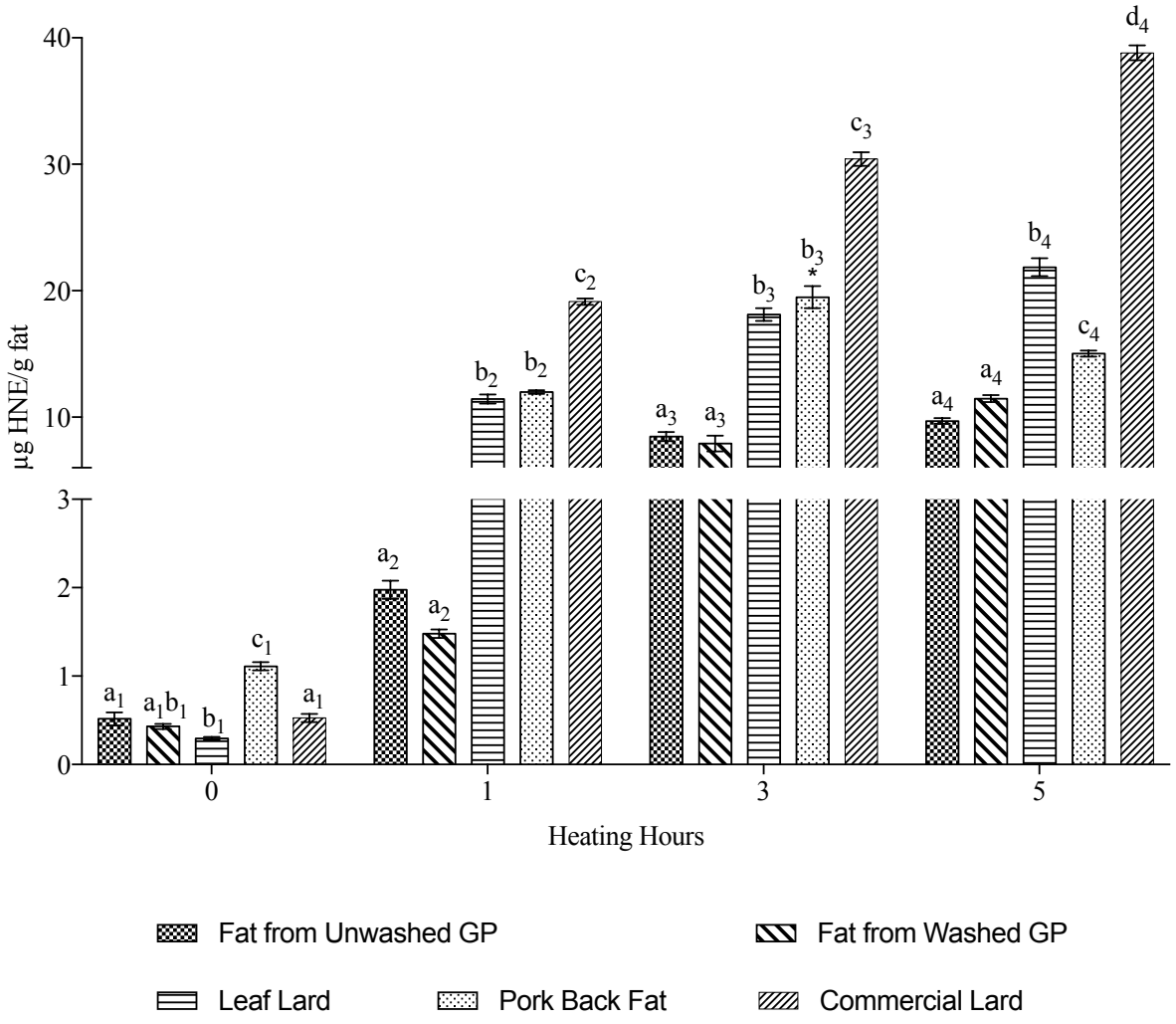



**Figure 43. Change of HNE formation of pork fats heated at 185°C for 0, 1, 3 and 5 hours.**

***Not reproducible data.**

*Chicken Fats*

Figure 44 - 48 show the HNE formation in chicken fats heated at 185°C for 0, 1, 3 and 5 hours. Figure 44 shows the heating time related HNE formation of chicken fats from surface of chicken thigh heated at 185°C. The formation of HNE increased significantly with the heating time. The HNE concentration of 0, 1, 3 and 5 hours of heating were 1.53, 12.23, 25.65 and 29.19 μg /g fat, respectively.

Figure 45 shows the heating time related HNE formation of chicken fats from surface of chicken skin heated at 185°C. The formation of HNE increased significantly with the heating time. The HNE concentration of 0, 1, 3 and 5 hours of heating were 0.97, 14.05, 28.07 and 37.84 μg /g fat, respectively.

Figure 46 shows the heating time related HNE formation of commercial chicken fats heated at 185°C. It shows the HNE formation increased significantly at 1 and 3 hours of heating. However, there was no significant difference between 3 and 5 hours of heating. The HNE concentration at 0, 1, 3 and 5 hours heating were 0.78, 13.87, 42.77 and 43.87 μg /g fat, respectively.

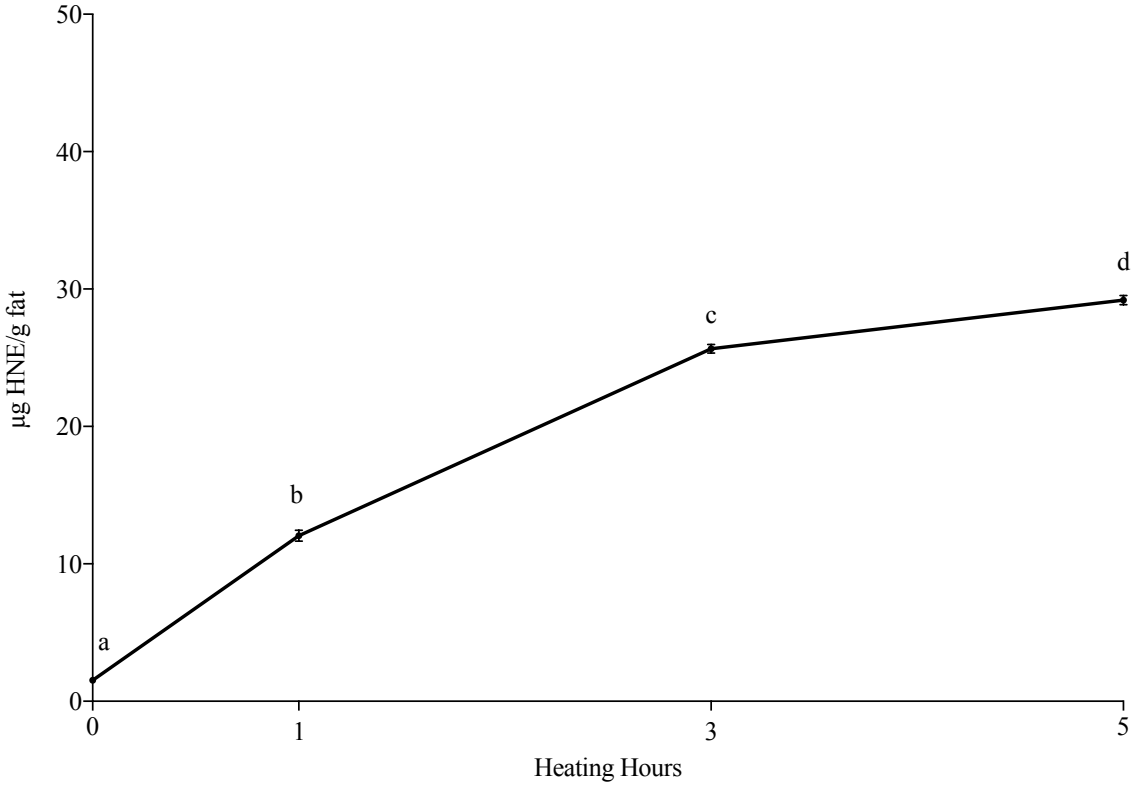



**Figure 44. Heating time related HNE formation of chicken fats from surface of chicken thigh heated at 185°C.**

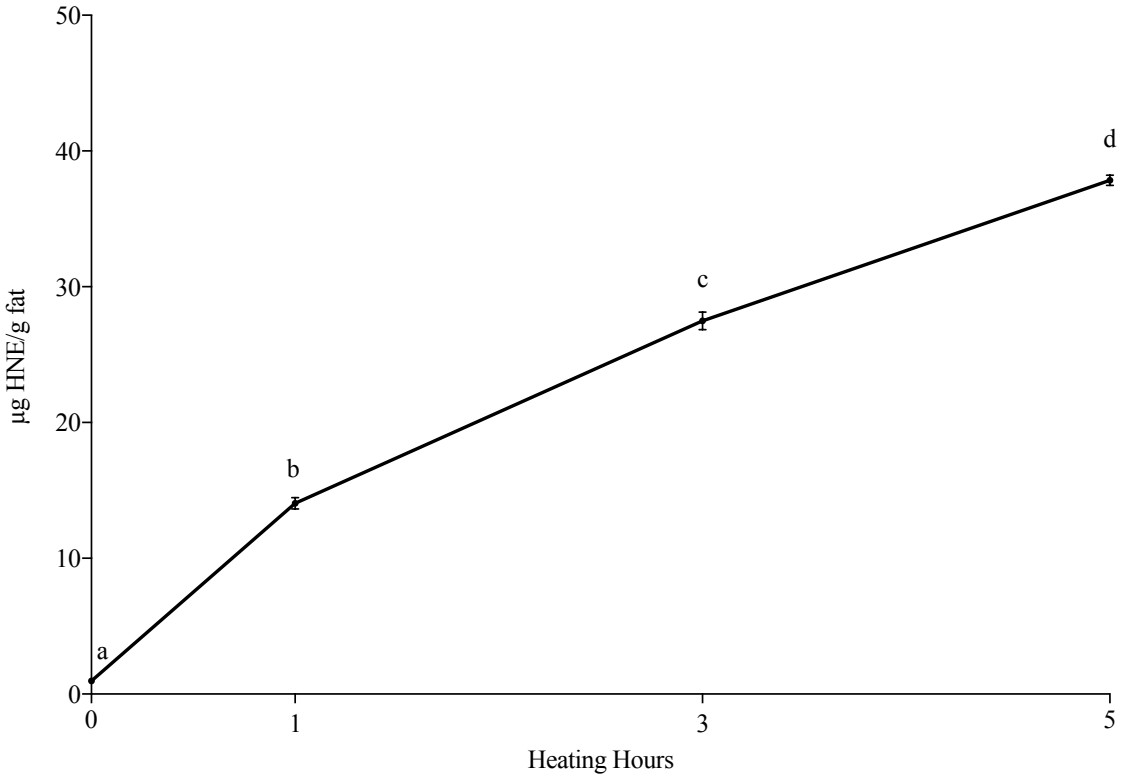



**Figure 45. Heating time related HNE formation of chicken fats from surface of chicken skin heated at 185°C.**

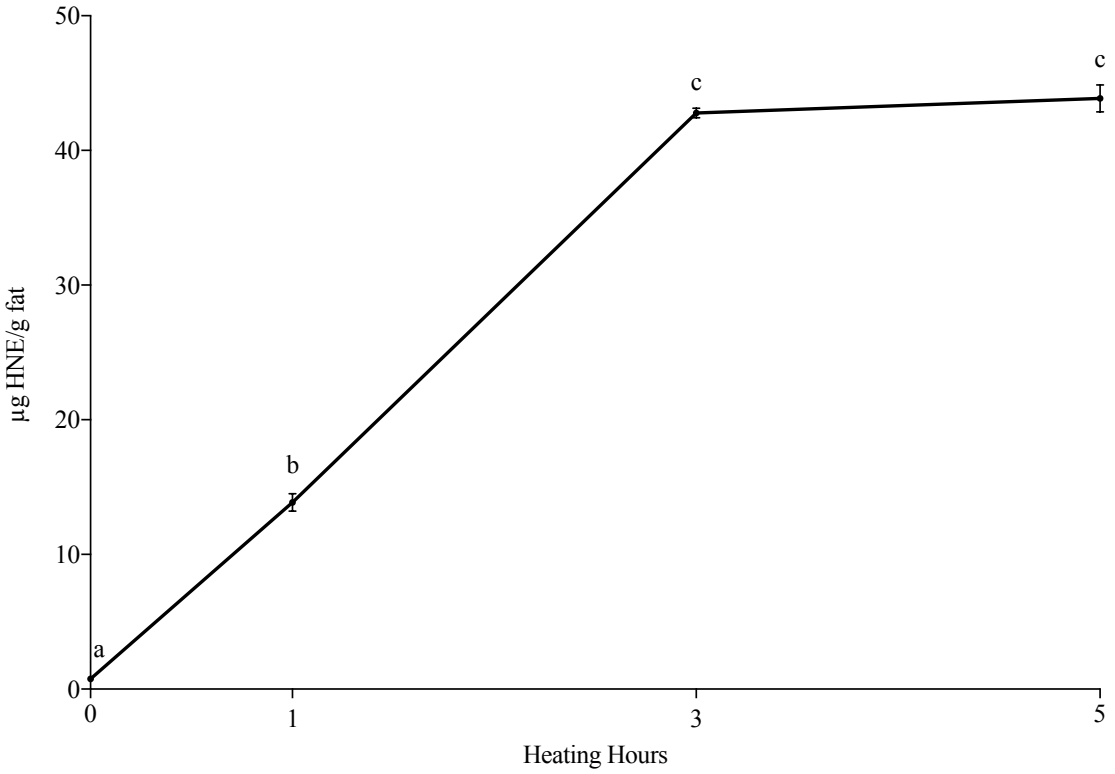



**Figure 46. Heating time related HNE formation of commercial chicken fat heated at 185°C.**

Figure 47 shows the HNE formation in all chicken fats heated at 185°C.The HNE concentration increased with the heating time for all fats. The increasing rates of chicken thigh fat and chicken skin fat were stable. However, the increasing rates of commercial chicken fat was higher at 3 hours of heating.

Figure 48 also shows the HNE formation in different chicken fats clustered by heating hours. Although the HNE formation of unheated chicken thigh fat was significantly higher than commercial chicken fat, there was no significant difference among samples

after 1 hour of heating. In addition, the formation of HNE in commercial chicken fat after 3 and 5 hours were significantly higher than the other two chicken fats. After 5 hours of heating, the HNE concentration of commercial chicken fat reached the highest number, which was 43.87μg HNE/g fat.

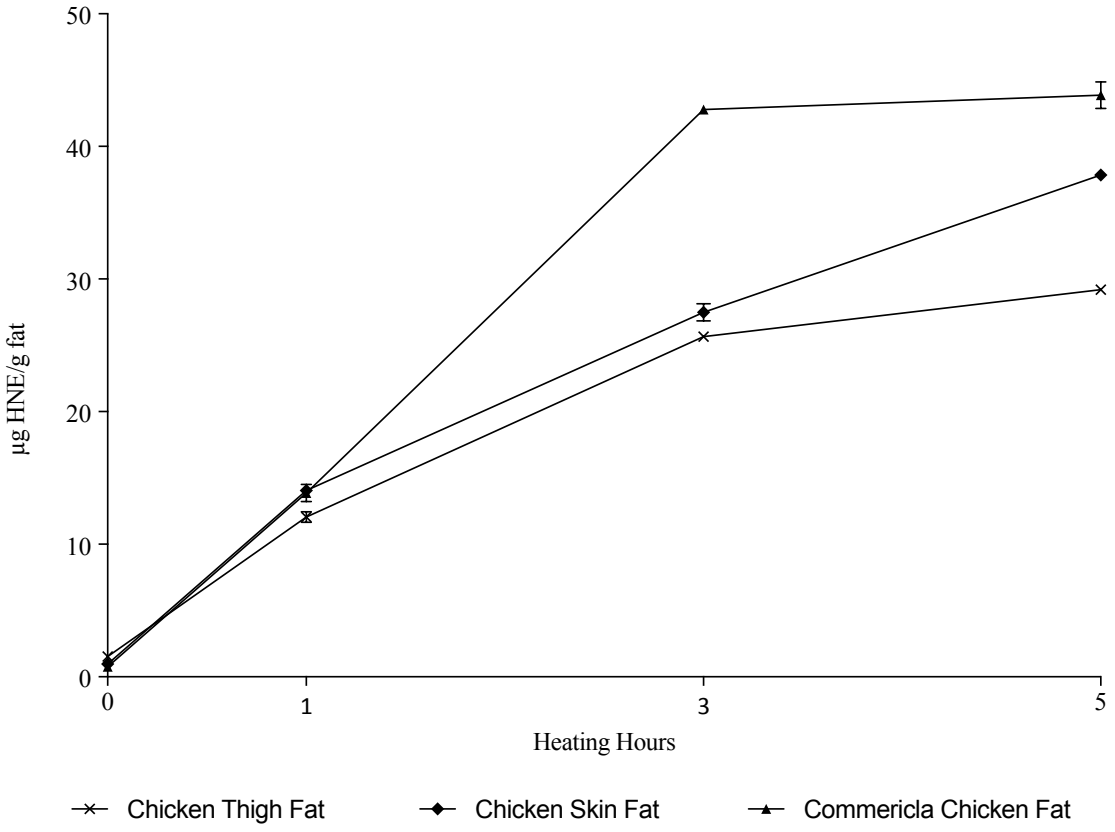



**Figure 47. Change of HNE formation of different chicken fats heated at 185°C.**

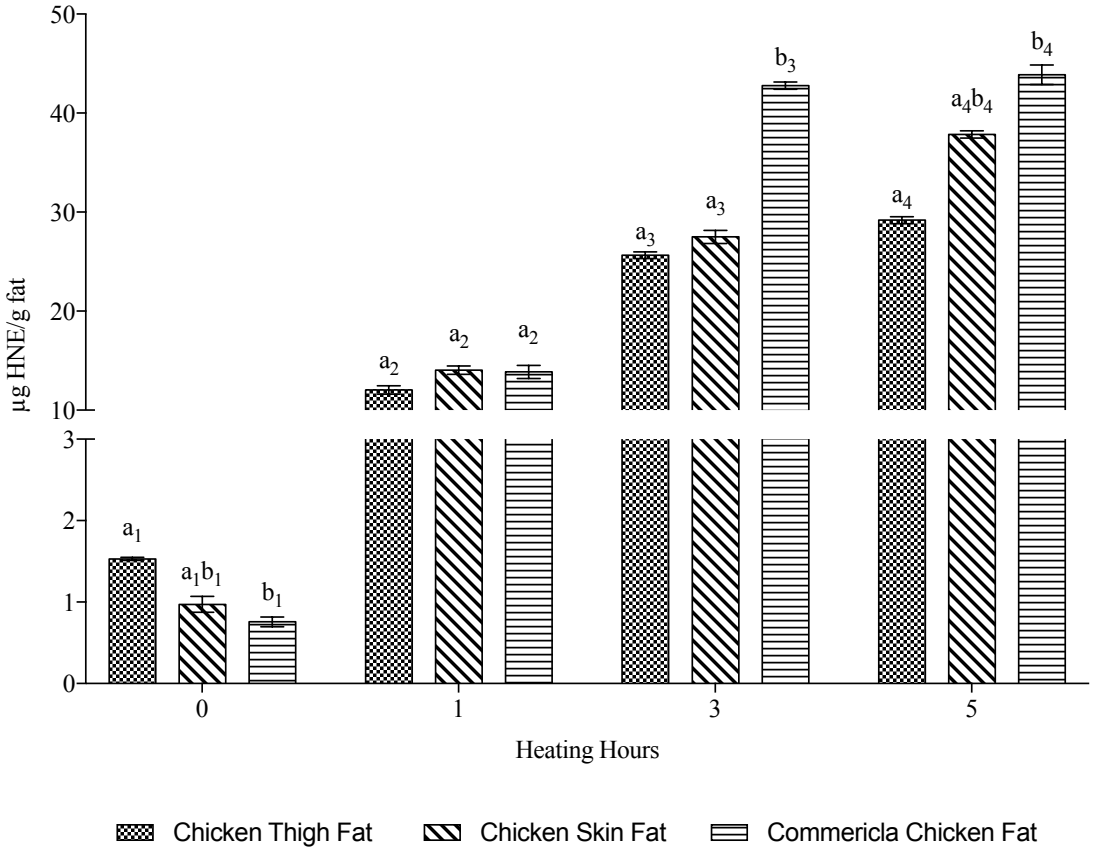



**Figure 48. Change of HNE formation of chicken fats heated at 185°C for 0, 1, 3 and 5 hours.**

Figure 49 and Table 9 show the HNE formation in all animal fat samples heated at 185°C for 0, 1, 3 and 5 hours. In general, beef fats had the lowest HNE concentration compared with pork and chicken fats due to low percentage of linoleic acid, which is the precursor of HNE. Fats that extracted from ground meats had less HNE formation than other fat sources. That may be due to some extracted antioxidants from ground beef or pork. It is of interest to measure the antioxidant capacity in samples. Although chicken fats had

higher linoleic acid level than pork fats, the HNE formation in commercial lard was higher than that in chicken thigh fat. That may be attributed to the production process of commercial lard.

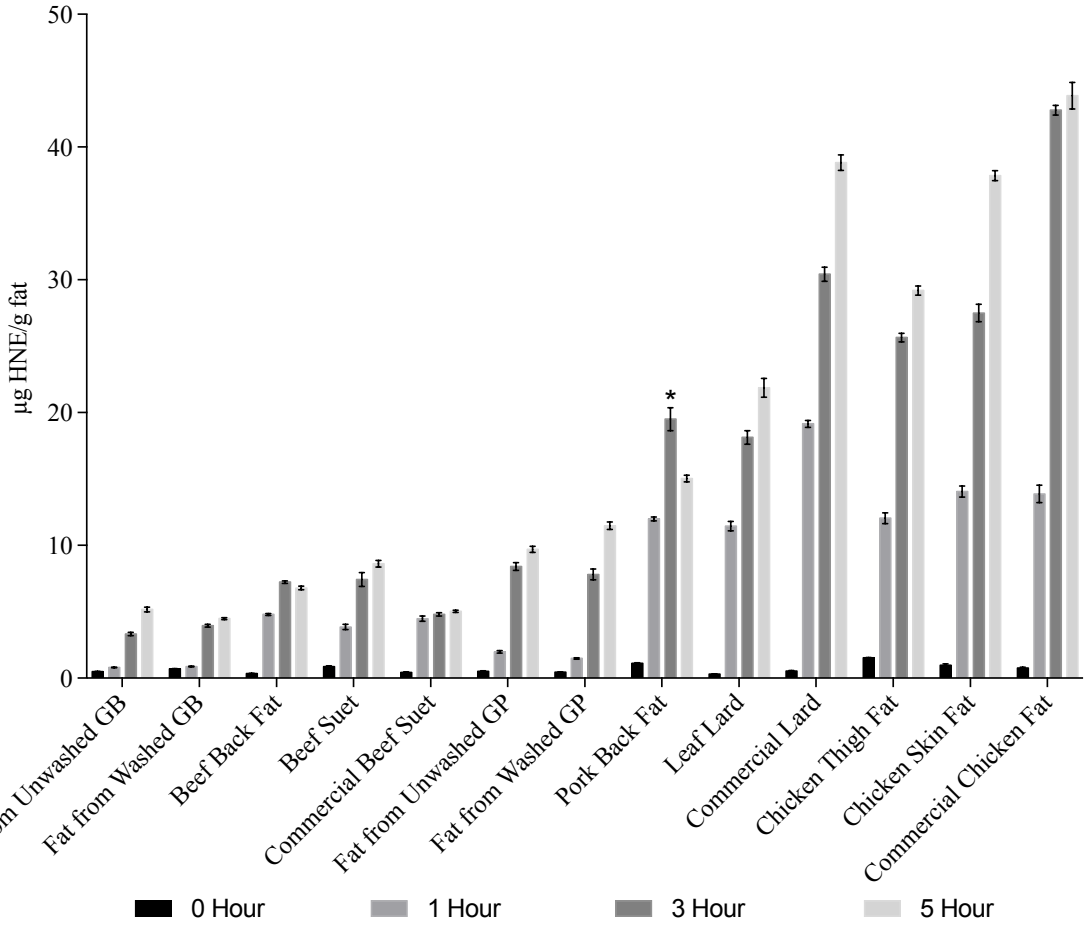



**Figure 49. Comparison of HNE formation of beef, pork and chicken fats heated at 185°C.**

*** Not reproducible data.**

**Table 9. HNE formation (µg HNE/g fat) in all beef, pork and chicken fats heated at 185°C for 0, 1, 3 and 5 hours.**

| Heating Hour / Samples | 0 | 1 | 3 | 5 |
|---|---|---|---|---|
| Fat from Washed Ground Beef | 0.70 ± 0.02 | 0.87 ± 0.05 | 3.95 ± 0.01 | 4.48 ± 0.12 |
| Fat from Unwashed Ground Beef | 0.49 ± 0.01 | 0.80 ± 0.04 | 3.32 ± 0.02 | 5.17 ± 0.30 |
| Beef Suet | 0.35 ± 0.13 | 4.80 ± 0.42 | 7.21 ± 0.31 | 6.79 ± 0.32 |
| Beef Back Fat | 0.86 ± 0.19 | 3.81 ± 0.08 | 6.94 ± 0.06 | 8.62 ± 0.16 |
| Commercial Beef Suet | 0.43 ± 0.04 | 4.34 ± 0.27 | 4.81 ± 0.02 | 5.04 ± 0.05 |
| Fat from Washed Ground Pork | 0.44 ± 0.04 | 1.4 8± 0.08 | 7.57 ± 0.71 | 11.48 ± 0.42 |
| Fat from Unwashed Ground Pork | 0.51 ± 0.01 | 1.98 ± 0.16 | 8.34 ± 0.36 | 9.71 ± 0.25 |
| Leaf Lard | 0.30 ± 0.02 | 11.44 ± 0.49 | 18.08 ± 0.72 | 21.47 ± 0.57 |
| Pork Back Fat | 1.11 ± 0.02 | 11.99 ± 0.13 | 19.50 ± 0.64 | 31.89 ± 3.99 |
| Commercial Lard | 0.53 ± 0.02 | 19.15 ± 0.16 | 30.42 ± 0.72 | 38.82 ± 0.31 |
| Chicken Thigh Fat | 1.53 ± 0.01 | 12.23 ± 0.73 | 25.65 ± 0.25 | 29.19 ± 0.51 |
| Chicken Skin Fat | 0.97 ± 0.16 | 14.05 ± 0.19 | 28.07 ± 0.60 | 37.84 ± 0.08 |
| Commercial Chicken Fat | 0.78 ± 0.01 | 13.87 ± 0.73 | 42.77 ± 0.25 | 43.87 ± 0.51 |

To give a general order of beef, pork and chicken fats in terms of their HNE formation capacity, the HNE concentration in different regions of beef, pork and chicken fats are averaged respectively, as shown in figure 50. The average HNE formations of beef and pork fats were excluded fat extracted from washed and unwashed ground beef and pork, and commercial lard. Results showed that fats extracted from ground meats produced much less HNE. We assumed that they may contain antioxidants, which prevent the HNE formation. Besides, the production process of commercial lard promotes its lipid oxidation and HNE formation. Therefore, they are different from other fats and were exclude. It easy to say that the HNE formation capacity from high to low are chicken, pork and beef fats, which are the order of linoleic acid percentage.

Figure 51 shows the comparison of HNE formation in fat extracted from washed and unwashed ground beef and pork. During heating period, fats extracted from ground pork produced higher level of HNE than that in ground beef. And the difference became larger with the heating time.

Figure 52 shows the comparison of HNE formation in fats extracted from ground meats and average HNE formation of three beef, two pork and three chicken fats. During heating period, fats extracted from ground beef and ground pork produced lower HNE than the average beef and pork fats, respectively.

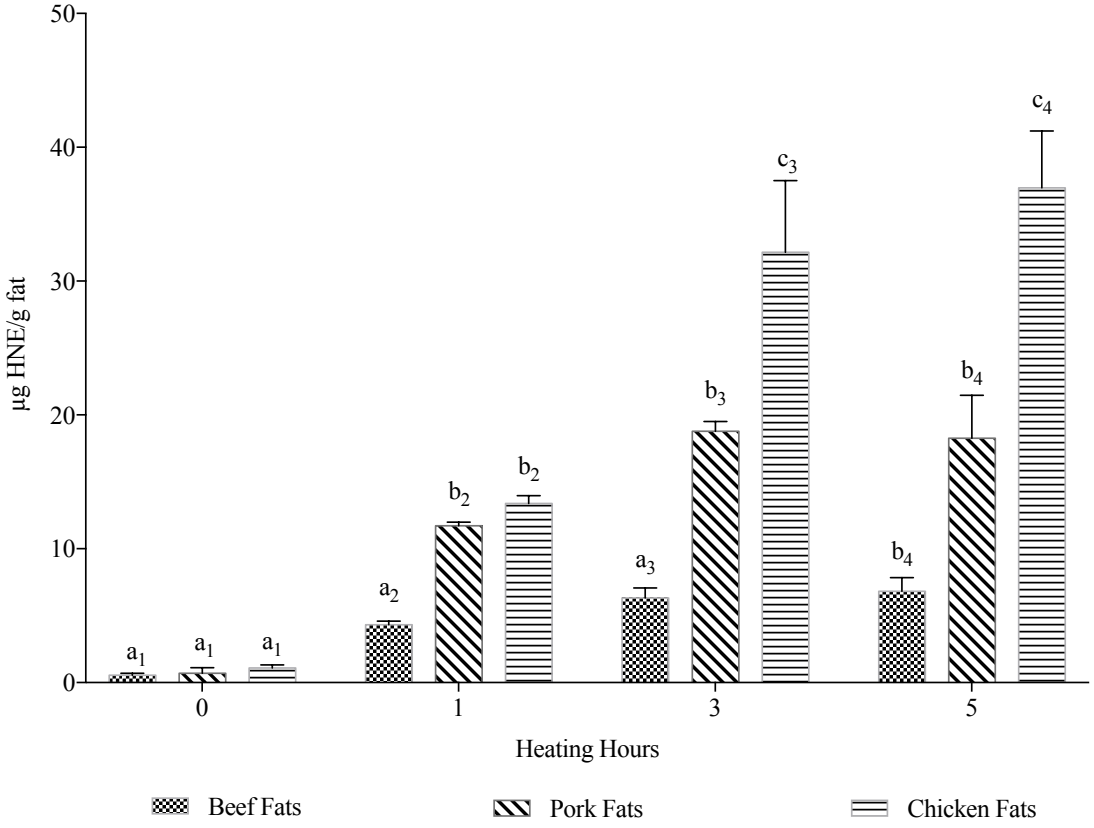



**Figure 50. Comparison of average HNE formation of three beef, two pork and three chicken fats samples heated at 185°C.**

*** Exclude fat extracted from ground beef and ground pork, and commercial lard.**

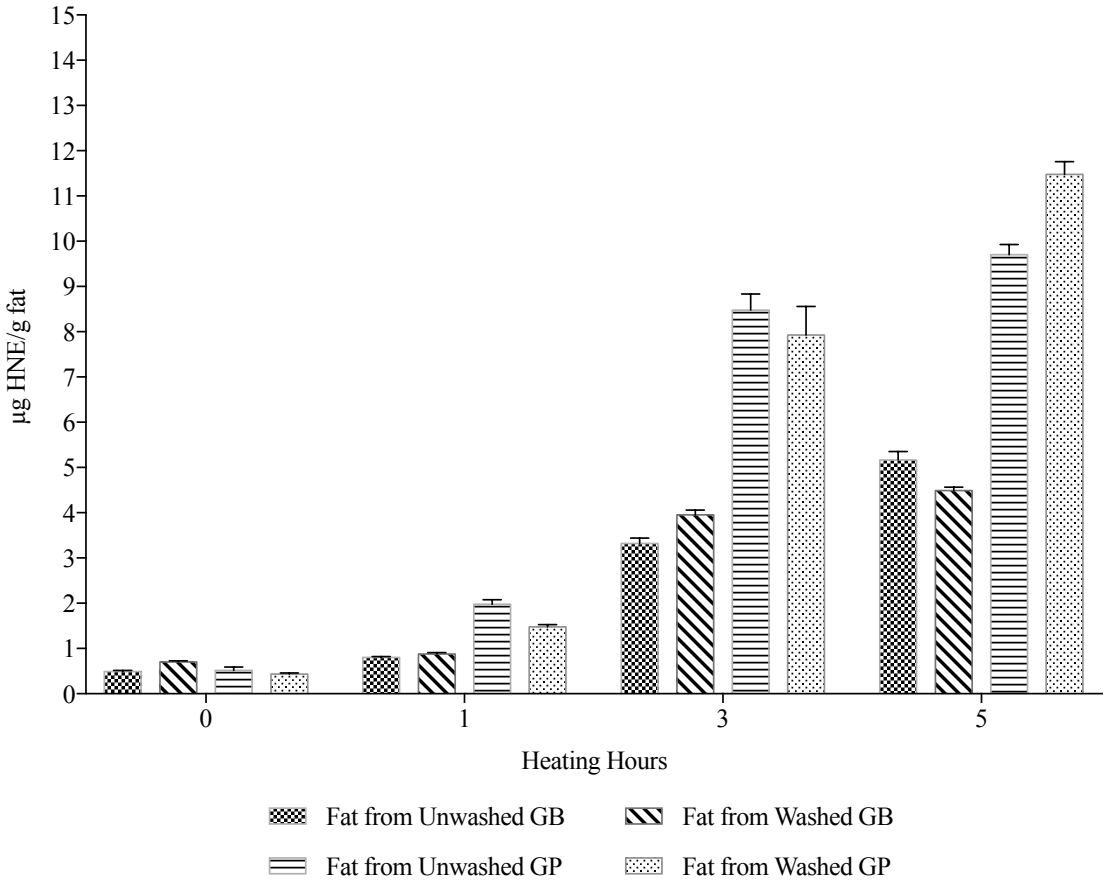



**Figure 51. Comparison of HNE formation of fat extracted from washed and unwashed ground beef and ground pork heated at 185°C.**

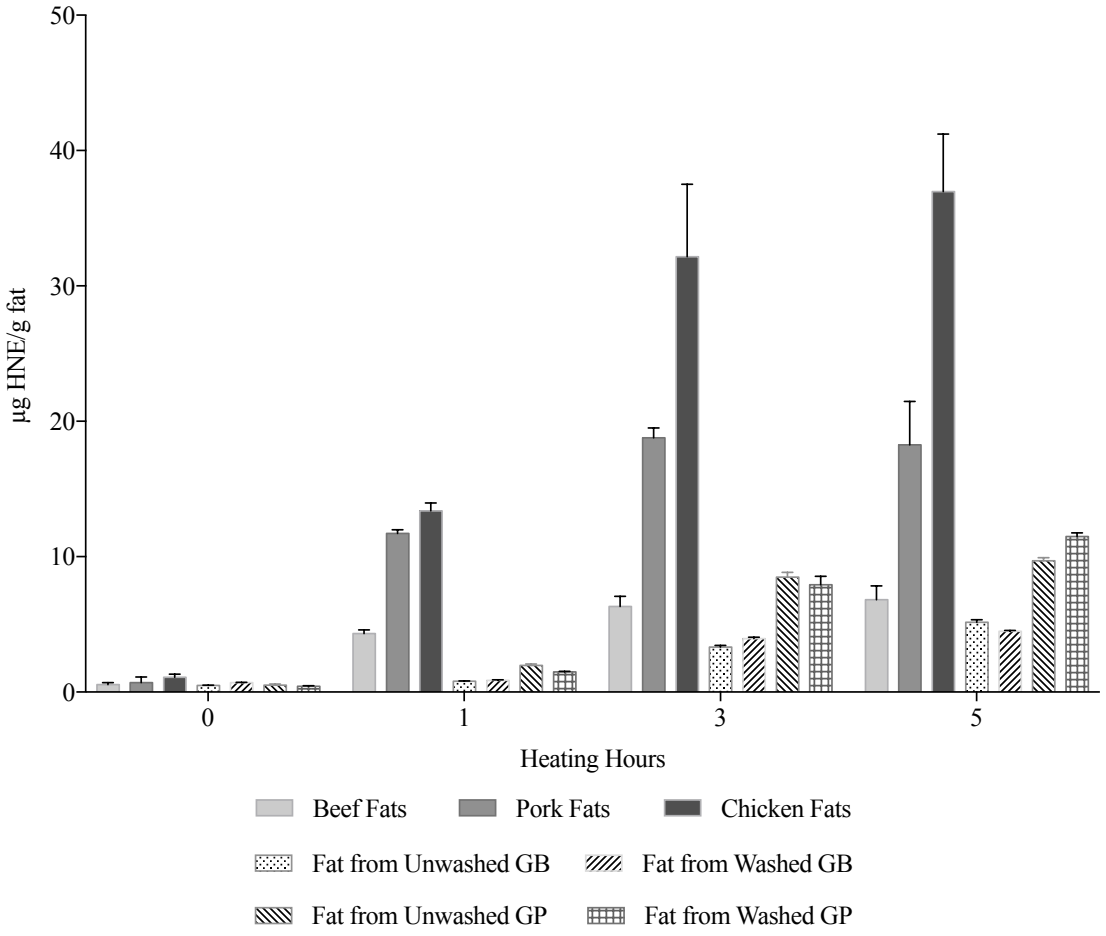



**Figure 52. Comparison of HNE formation of fats extracted from ground meats and average HNE formation of three beef, two pork and three chicken fats samples heated at 185°C.**

*** The average HNE formation exclude fat extracted from ground beef and ground pork, and commercial lard.**

**Trolox Equivalent Antioxidant Capacity**

The antioxidant capacity was measured in fat from washed ground beef, fat from unwashed ground beef, beef back fat, beef suet, commercial beef suet, fat from washed ground pork, fat from unwashed ground pork, pork back fat, leaf lard, commercial lard, chicken thigh fat, chicken skin fat and commercial chicken fat. The results were expressed as concentration of trolox (μM) and listed in Table 10.

**Table 10. Trolox equivalent antioxidant capacity (TEAC) of fats from all sources of beef, pork and chicken**

| Samples | Trolox Equivalents (μM) |
|---|---|
| Washed Ground Beef | 5.28 ± 0.396 |
| Unwashed Ground Beef | 1.59 ± 0.372 |
| Beef Suet | 1.76 ± 0.149 |
| Beef Back Fat | 1.61 ± 0.149 |
| Commercial Beef Suet | 1.21 ± 0.024 |
| Washed Ground Pork | 1.28 ±0.095 |
| Unwashed Ground Pork | 1.12 ±0.041 |
| Leaf Lard | 1.66 ±0.063 |
| Pork Back Fat | 1.71 ±0.156 |
| Commercial Lard | 0.76 ±0.539 |
| Chicken Thigh Fat | 1.80 ±0.063 |
| Chicken Skin Fat | 1.92 ±0.024 |
| Commercial Chicken Fat | 1.76 ±0.071 |

## Discussion

Lipid oxidation generates reactive aldehydes, such as MDA and HNE. Currently, lipid oxidation products receive significant public attention because they are considered as critical risks for numerous diseases, such as chronic inflammation, atherosclerosis, diabetes and different types of cancer. The lipid oxidation process happens both in cells and tissues of living organisms and in foods during processing or storage [80]. HNE from food can be absorbed and distributed into human's major organs, like liver, kidney and brain [12]. However, with a previous study focused more on the endogenous HNE, the research on the exogenous HNE from frying oil is limited.

Since epidemiological and clinical studies have suggested potential benefits of PUFA on brain development and prevention of cardiovascular disease, there is a trend of using PUFA-rich vegetable oils to replace animal fats for cooking. However, PUFA are very easy to be oxidized and generate HNE [81]. Therefore, nowadays, there are questions on the overall benefits of highly unsaturated vegetable oils, and it calls for a more thorough evaluation on them. In this study, the oxidation capacity of varies types of animal fats undergoing heating treatment to mimic frying conditions were investigated. We found that beef fats produced less HNE and total lipid oxidation products under thermal treatment, compared to pork and chicken fats, which suggest it a good option for cooking.

In this study, beef, pork and chicken fats were selected based on their different degrees of unsaturation and fatty acid distributions. However, even if fats come from the same type of animal, different fat sources have different fatty acid compositions. For example,

beef suet (fat around loins and kidneys) is softer than beef back fat, which contain more PUFA. Therefore, various fat sources were chosen to have more comprehensive results. In the present study, TBARS assay and quantification of HNE by HPLC in thermal treated fats were conducted. TBARS assay measures carbonyl-containing secondary lipid oxidation products that can react with TBA, including HNE and other aldehydes, such as alkanals, alkenals, hydroxyalkenals, various ketones and related carbonyl compounds. Therefore, it gives a general idea of secondary lipid oxidation products from beef, pork and chicken fats. Next, HPLC was used to identify and quantify HNE, which is a toxic secondary lipid oxidation product. Combining of these two results can comprehensively reflect lipid oxidation and toxicity in fats. The trends of HNE formation in beef, pork and chicken fats in this study were consistent with the results of TBARS assay. The results show that 1) the formation of HNE and the total secondary lipid oxidation products measured by TBARS from all three fat sources were increased with the time of heating; 2) the HNE formation and total secondary lipid oxidation products levels in beef, pork and chicken fats were correlated with their linoleic acid and PUFA percentage, respectively; 3) the HNE formation and total secondary lipid oxidation products of fat extracted from ground beef and ground pork were lower than other beef and pork fats, respectively; 4) the HNE formation of commercial lard was higher than other pork fats, which may be attributed to its production process. The details of each point are discussed below.

1) The formation of HNE and total secondary lipid oxidation products measured by TBARS from all fat sources were increased with the time of heating.

The results show that, in general, the HNE formation and total lipid oxidation products in all beef, pork and chicken fats were increased with the increasing heating time. This is consistent with previous reports targeted on different types of oils. In the study conducted by Seppanen and Csallany, they heated the soybean oil at frying temperature (185°C) for 0, 2, 4, 6, 8, and 10 hours [39]. An increased formation of HNE was observed with increased heating time and reached the highest amount at 6 hours of heating and then decreased, probably due to the degradation of HNE. In another study conducted by Marzena and his colleges, lipid oxidation of hardened frying fat was evaluated by the TBARS assay [82]. The samples were heated at 180°C for up to 24 hours and evaluated after every 2 h of heating time. Results showed that TBARS value of hardened frying fat increased first with the heating time and reached the highest level at 16 hours of heating. In summary, the formation of HNE and total secondary lipid oxidation products in each type of oil and fat are time dependent. However, oils and fats from different sources have differently increase their rates, which is carefully discussed below.

2) The HNE formation and the level of total secondary lipid oxidation products levels in beef, pork and chicken fats are correlated with their linoleic acid and PUFA percentage level, respectively.

It was found that HNE formation and total secondary lipid oxidation products of beef fats were lower than that in pork and chicken fats, while chicken fats produced the highest amounts of HNE and total secondary lipid oxidation products measured by TBARS.

In the present experiment, the fatty acids composition results show that beef fats contain the lowest percentage of linoleic acid (1.36~2.58%), compared with pork fats (8.04~13.95%) and chicken fats (25.33~33.71%). This is correlated with the HNE formations and total secondary lipid oxidation in three types of animal fats. Similar correlation has been reported previously in the literature. In 1995, Sakai *et al* has found a linear correlation between the content of HNE and the content of total ω-6 fatty acids in pork [70]. The same group also investigated the relationships between HNE, TBARS and ω-6 PUFA content for pork stored at 0, -20 and -80°C. A significant linear correlation was found between HNE and TBARS for pork stored at each temperature [71]. Later, Xiaoyu Liu in this laboratory compared linoleic acid percentage and the HNE formation in different oils and fats, including corn oil, soybean oil, peanut oil and canola oil. She also confirmed that in most cases, the HNE formations is correlated with the linoleic acid concentration (submitted to). The only exception is that commercial lard had low linoleic acid percentage but high level of HNE production.

In another study [82], rapeseed oil (33.99% PUFA), soybean oil (59.13% PUFA) and hardened frying fat (16.40% PUFA) were heated at 180 ± 2 °C for up to 24 hours. They found that hardened frying fat has lower intensity of hydrolytic and oxidative changes during heating, as measured by acid value, peroxide value and carbonyl value. This indicates that hardened frying fat is better for frying than rapeseed and soybean oils. However, the author did not mention the type of hardened frying fat. At 6 hours of heating, the TBARS value of hardened frying fat was 1.62 ± 0.03 μg/g fat, which is about the same value with beef fats in the present study.

3) The HNE formation and secondary lipid oxidation products of fat extracted from meat sources were significantly lower than other fats.

In beef and pork fats, the fats extracted from ground meats has slower HNE formation rate and TBARS value when compared with other pure fats from the same animal source. This may attribute to the effect of antioxidants that co-extracted from ground meat. During hexane extraction, the lipophilic antioxidants may be extracted from muscle together with the fat. It has been shown that muscle tissue possesses several endogenous antioxidant compounds and these antioxidants continue to function even after animal being slaughtered [83]. The major lipid-soluble free radical scavengers are tocopherol, ubiquinone and carotenoids. α-Tocopherol has been found in significant level in beef and pork muscle tissues, with concentration of 3.4 and 5.8 mg/kg muscle, respectively [84]. And a study has been done to show that the concentration of carnosine in beef (shoulder) and pork (shoulder) were 1500 and 2800 mg/kg muscle, respectively [85]. It's very likely that lipophilic antioxidants, such as α-tocopherol, which can be co-extracted with fats, cause the lower levels of lipid oxidation.

To test whether antioxidants were in the fats, we performed TEAC assay to measure the antioxidant capacity in samples. The method is developed first for the measurement of water-soluble antioxidants [79]. Later, some other researchers used this method to measure the antioxidants capacity in lipids as well. In this study, no significant difference was found between the different fats. However, we only performed TEAC assay in unheated fat samples. Different fats may contain different antioxidants, and they may have different heat stability. So, the antioxidant capacity in different fats may change

differently after a period of heating treatment. Therefore, it would be better to measure the antioxidant capacity of these fats at different time of heating in the future.

4) The HNE formation of commercial lard is higher than the other pork fats, which may attribute to its production process.

In this study, the HNE formation of commercial lard is higher than other pork fat sources, which can be explained probably by its production processes. The production of commercial lard involves either the wet or the dry process. The wet process involves boiling the fat at high temperature and skimming the surface to obtain the lard. During dry process, no water is used however high temperature is applied on the fat. No matter in which process, commercial lard is treated at high temperature. This treatment causes changes in commercial lard, and may make it more sensitive to thermal treatment.

Myoglobin in meats can enhance HNE formation. Previously, Sakai *et al* [70] tested the HNE contents in unheated beef and pork meats. They found that beef has higher HNE concentration (2.187-23.430 μg HNE/g food) than pork (0.156-23.742 μg HNE/g food). This was attributes to the higher myoglobin level in beef. To eliminate myoglobin in our fat extracted from ground meats, the HNE formation between washed and unwashed fat extracted from ground beef and pork was compared. It turned out that they have similar HNE formation, indicating that the myoglobin level in fats extracted from ground meats maybe negligible.

In summary, in this study, lipid oxidation measured by TBARS and HNE formation in different sources of beef, pork and chicken fats were compared. Next, their correlation

with linoleic acid percentages was examined. The result confirms some general rules, including 1) HNE formation and lipid oxidation are dependent on the heating time 2) in this study, the HNE formation in fats from low to high are beef, pork and chicken fats, respectively, which is consistent with the order of linoleic acid percentage in these samples. Interestingly, fats extracted from ground beef and ground pork were oxidized less and formed less HNE. These results indicate 1) beef fats were the best for frying compared with pork and chicken fats 2) ground meats may contain some antioxidants that prevent lipid oxidation in the extracted fats.

It is of interest in the future to identify the fat-soluble antioxidants in fats extracted from ground meats. It is assumed some antioxidants may co-extract with fats from meats, such as tocopherols.

## Conclusion:

The oxidation capacity of varies types of animal fats, such as beef, pork and chicken fats undergoing heating treatment to frying temperature 185 °C were investigated. TBARS assay and HNE by HPLC in thermal treated fats were also measured. Then I correlated

these results with their linoleic acid percentage, which is determined by GC. The results show that 1) the formation of HNE and total secondary lipid oxidation products measured by TBARS from all fat sources were increased with the heating time; 2) the HNE formation and total secondary lipid oxidation products levels in beef, pork and chicken fats were correlated with their linoleic acid and PUFA percentage, respectively; 3) the HNE formation and total secondary lipid oxidation products of fat extracted from ground beef and ground pork were lower than other fat sources. Overall, beef fats produce less HNE and total secondary lipid oxidation products under thermal treatment, compared to pork and chicken fats as well as most vegetable oils reported in the literature, which suggest that fat sources are good for cooking.

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# Appendix

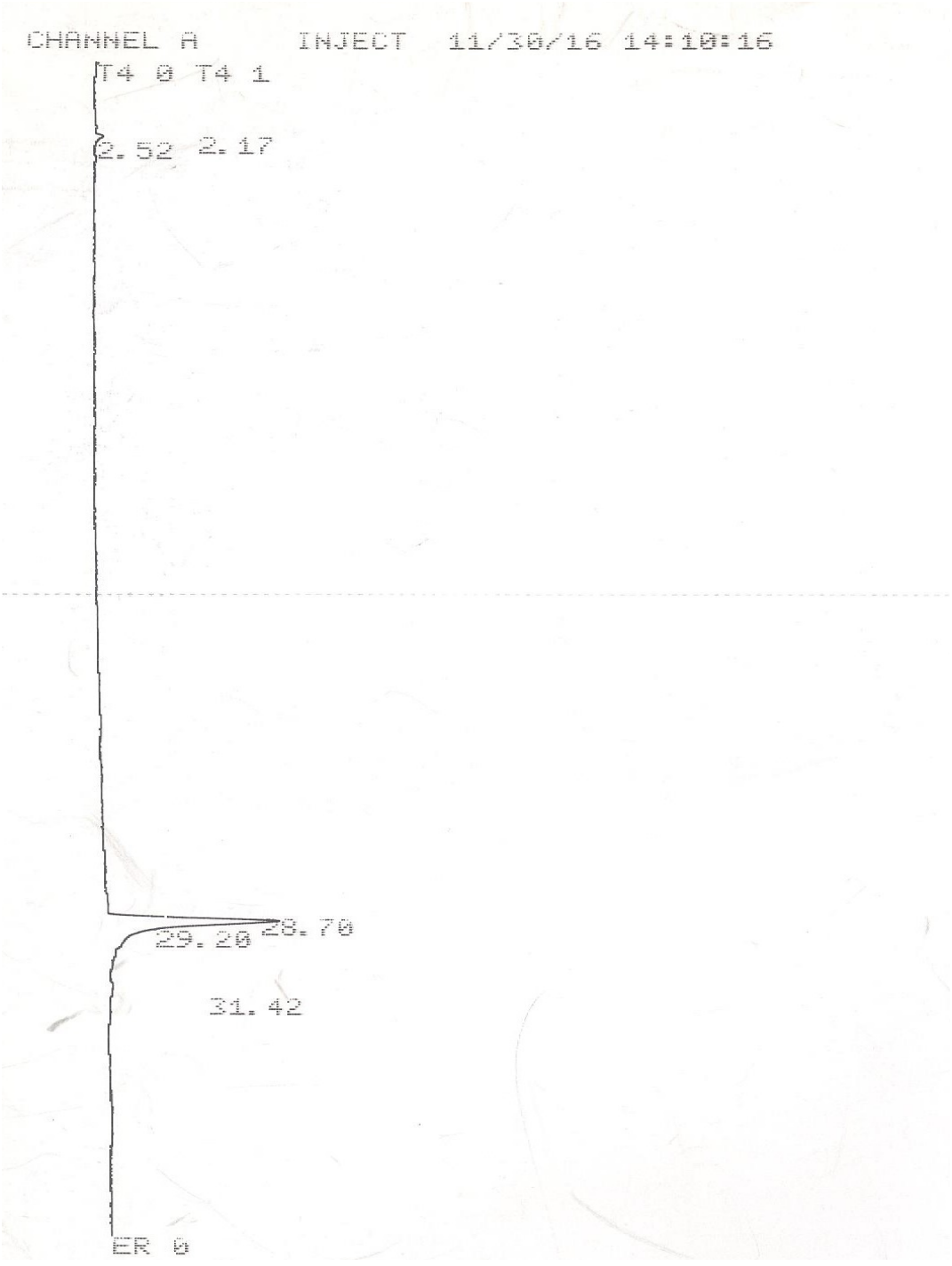


**Figure 53. HPLC chromatograph of HNE standard. (Attenuation = 512)**

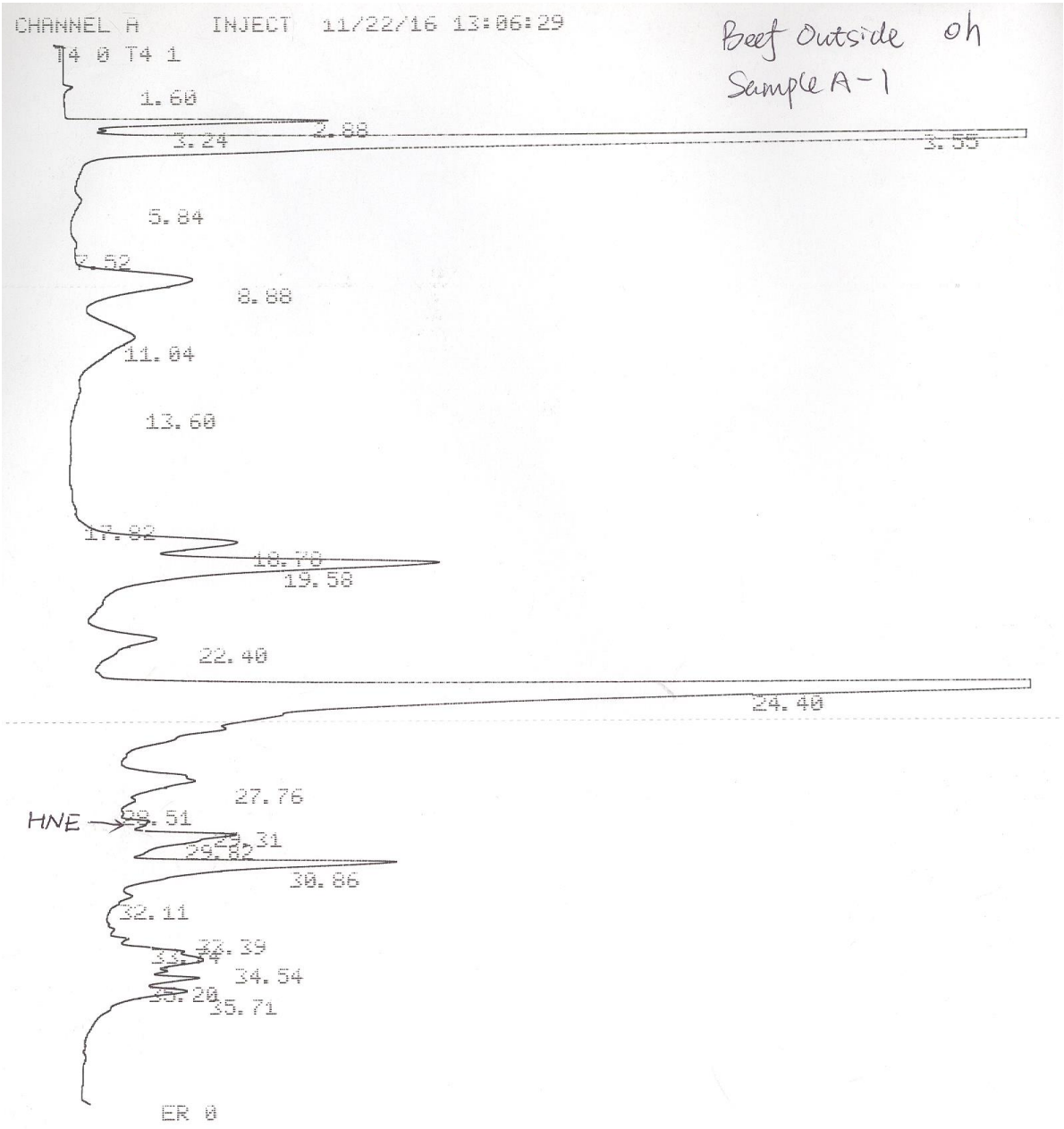


**Figure 54. HPLC chromatograph of unheated beef back fat. (Attenuation = 128)**

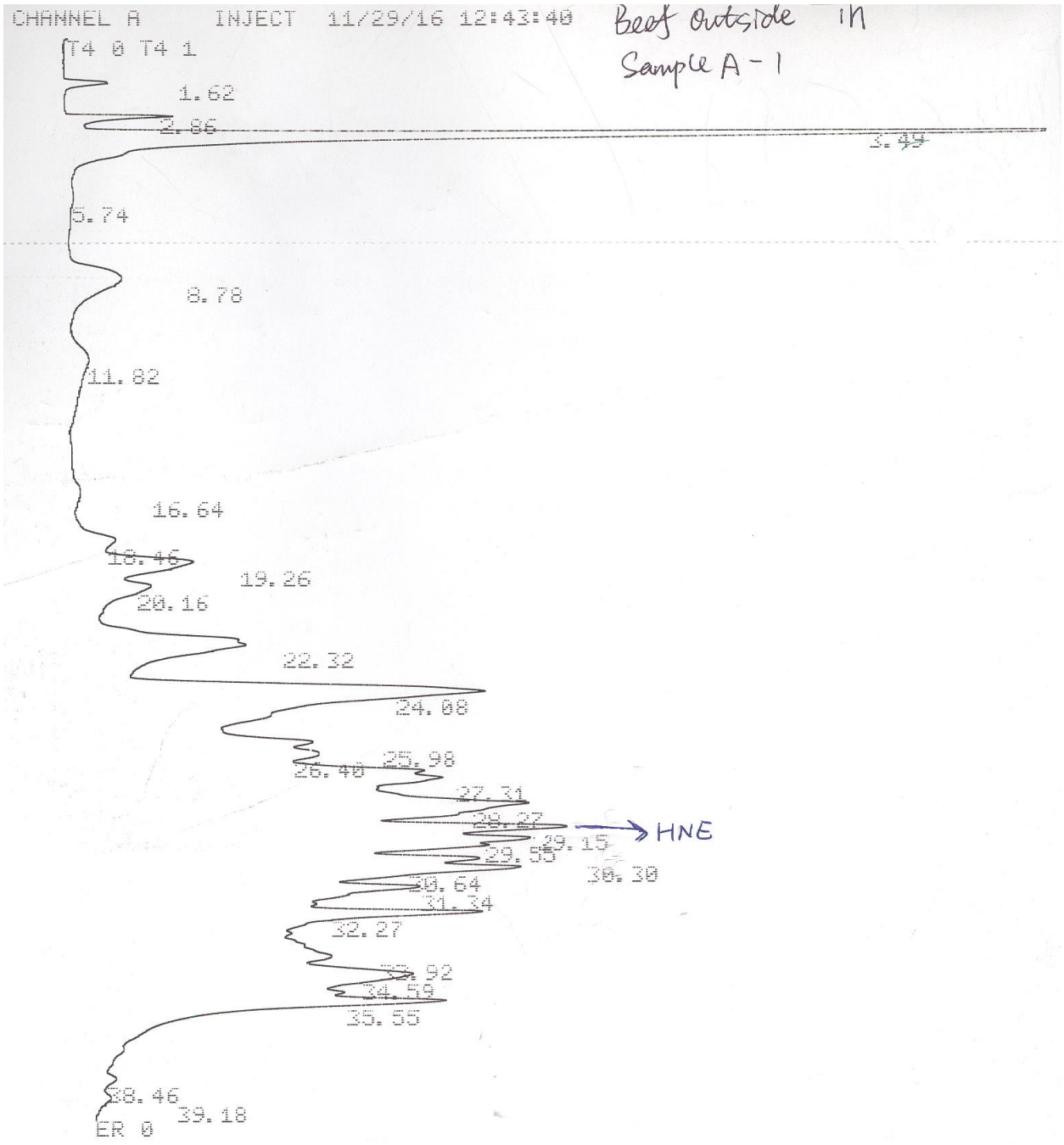


**Figure 55. HPLC chromatograph of beef back fat heated at 185°C for 1 hour. (Attenuation = 128)**

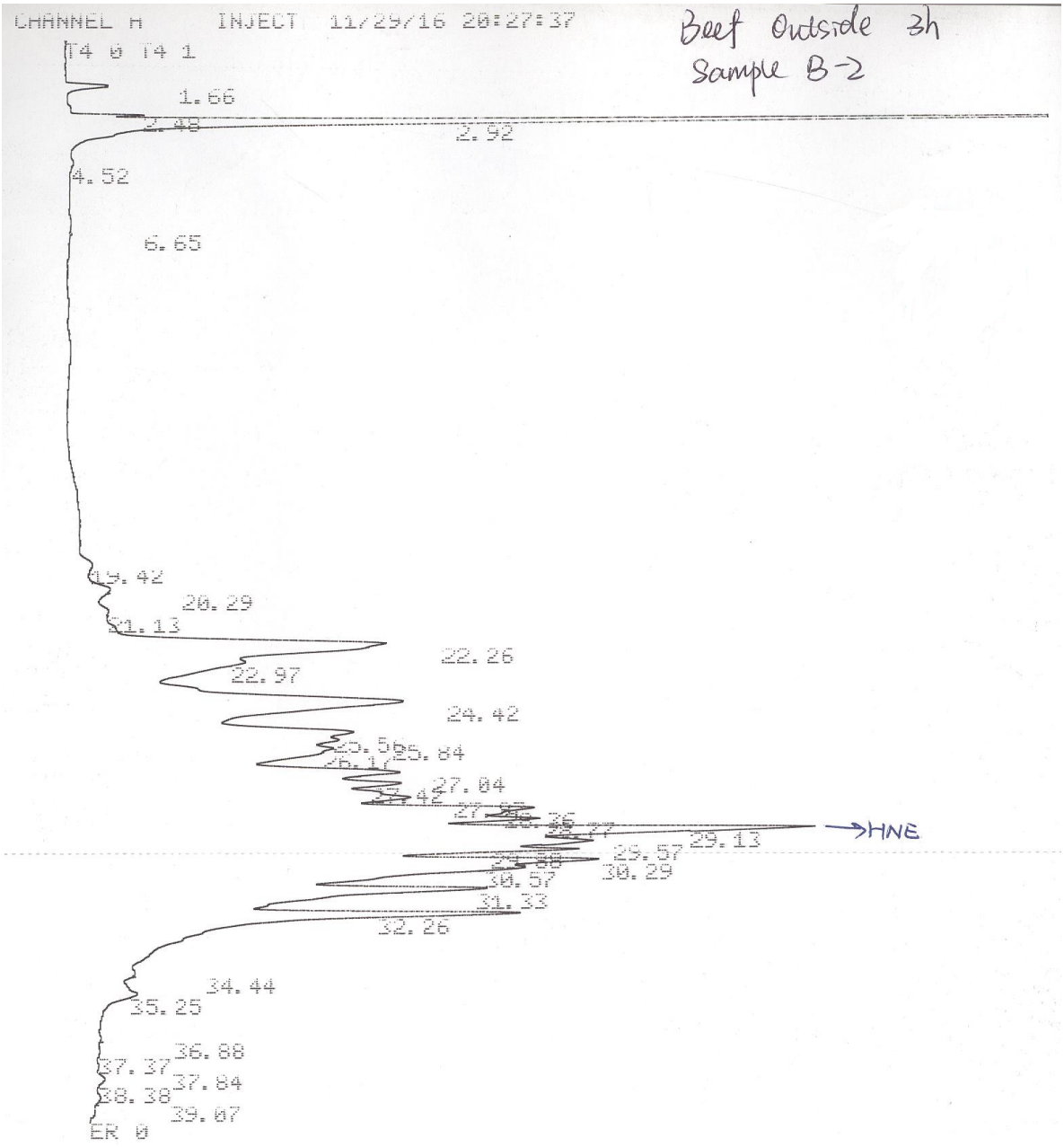


**Figure 56. HPLC chromatograph of beef back fat heated at 185°C for 3 hour. (Attenuation = 128)**

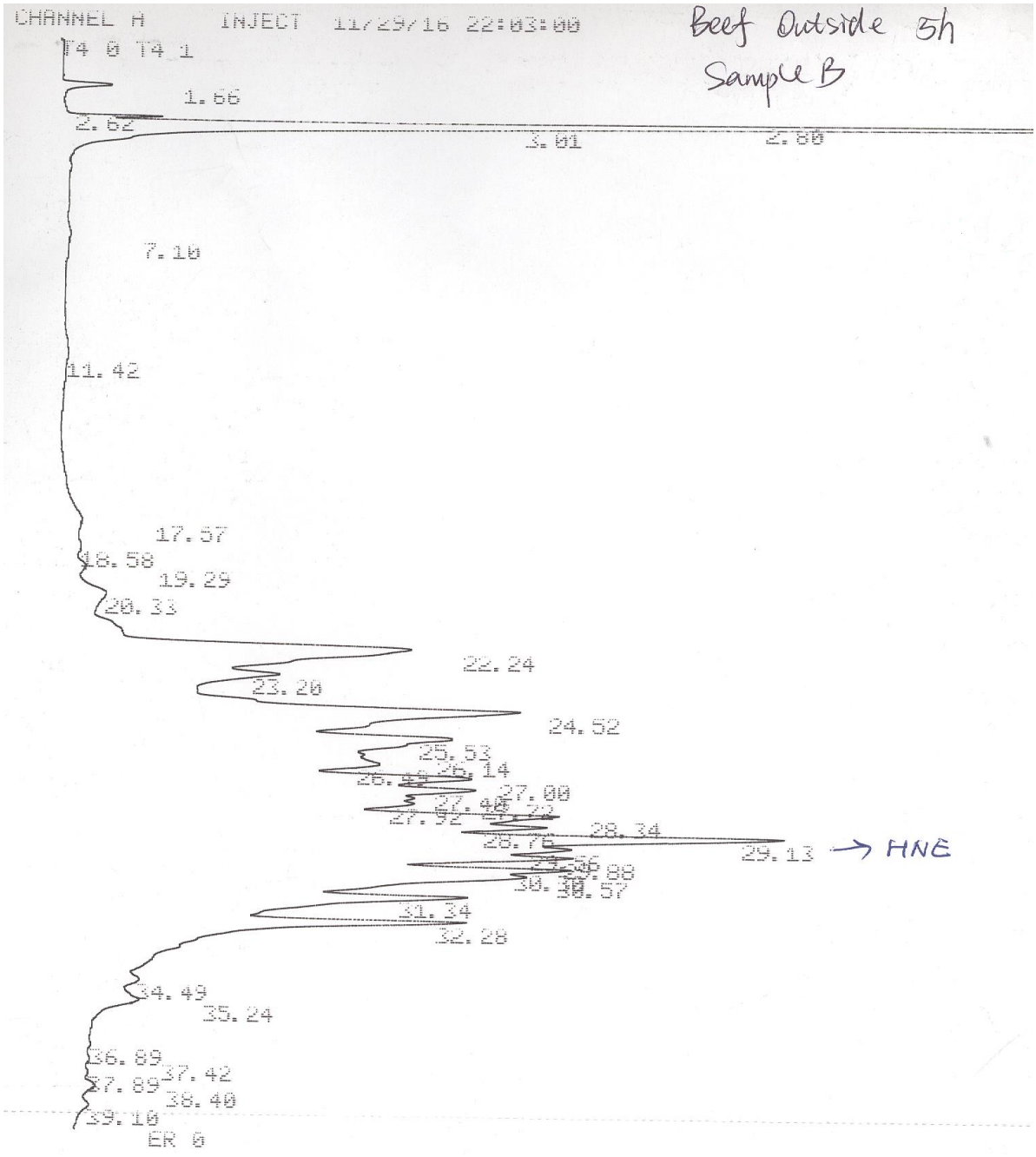


**Figure 57. HPLC chromatograph of beef back fat heated at 185°C for 5 hour. (Attenuation = 128)**

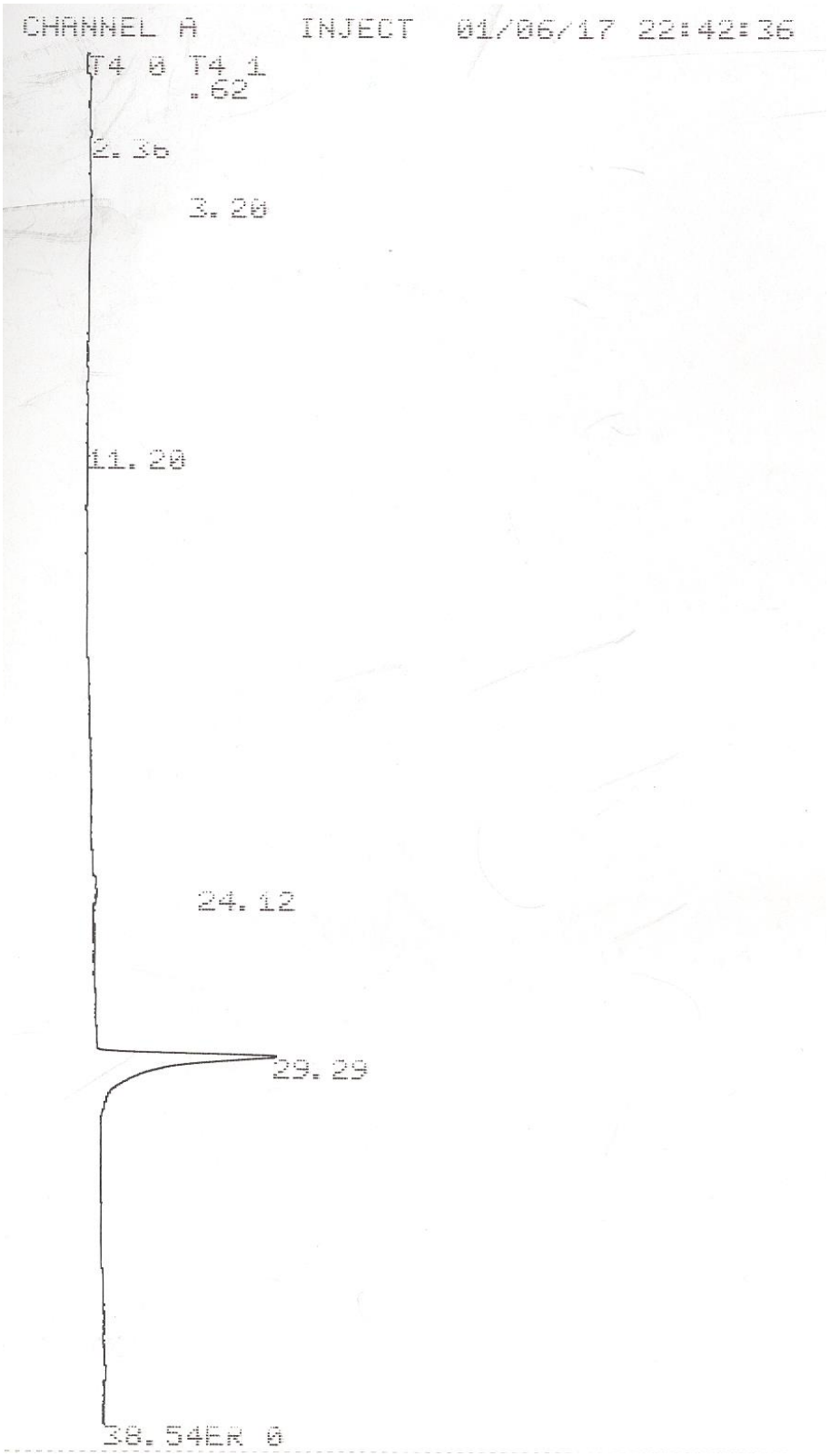


**Figure 58. HPLC chromatograph of HNE standard. (Attenuation = 512)**

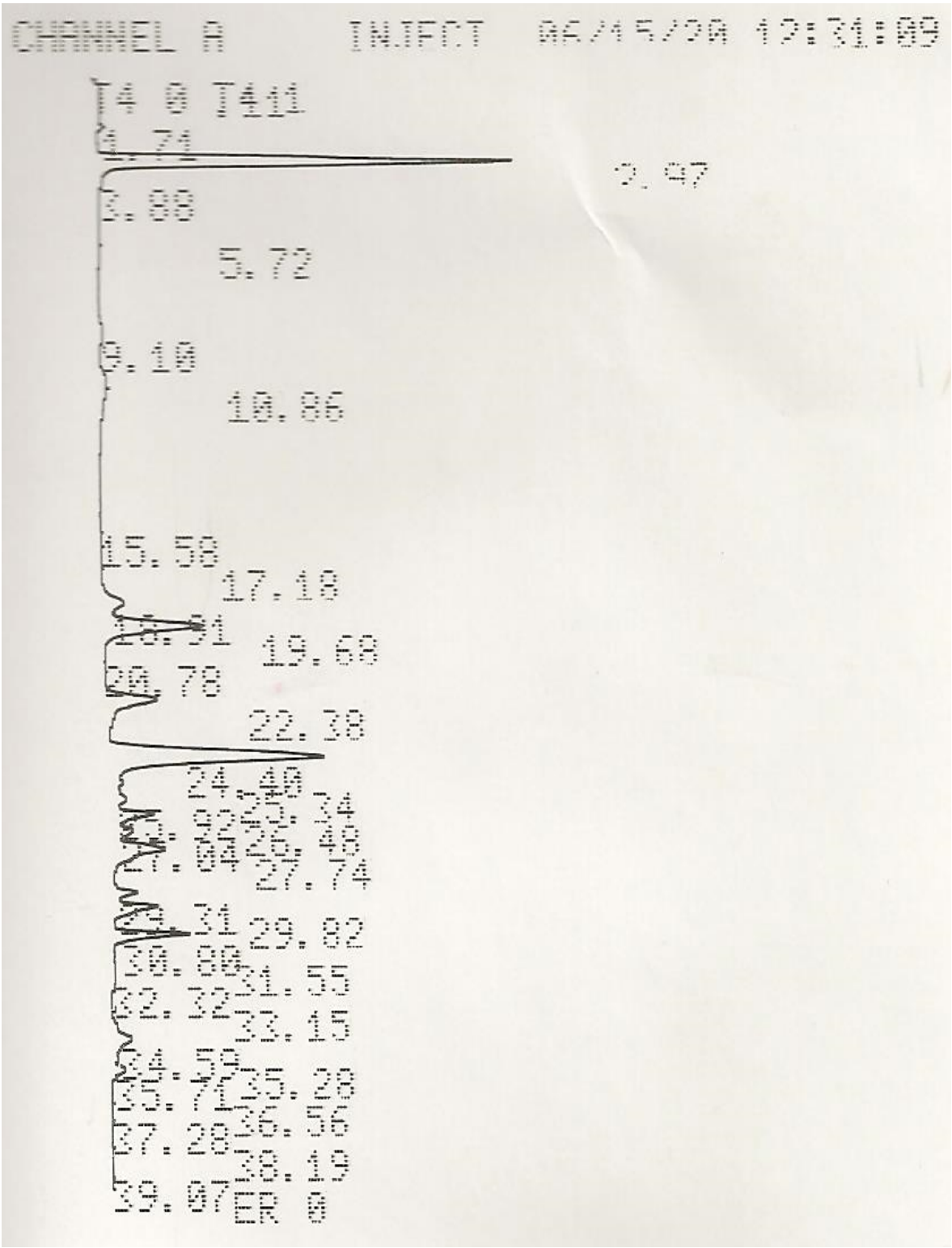


**Figure 59. HPLC chromatograph of unheated pork back fat. (Attenuation = 512)**

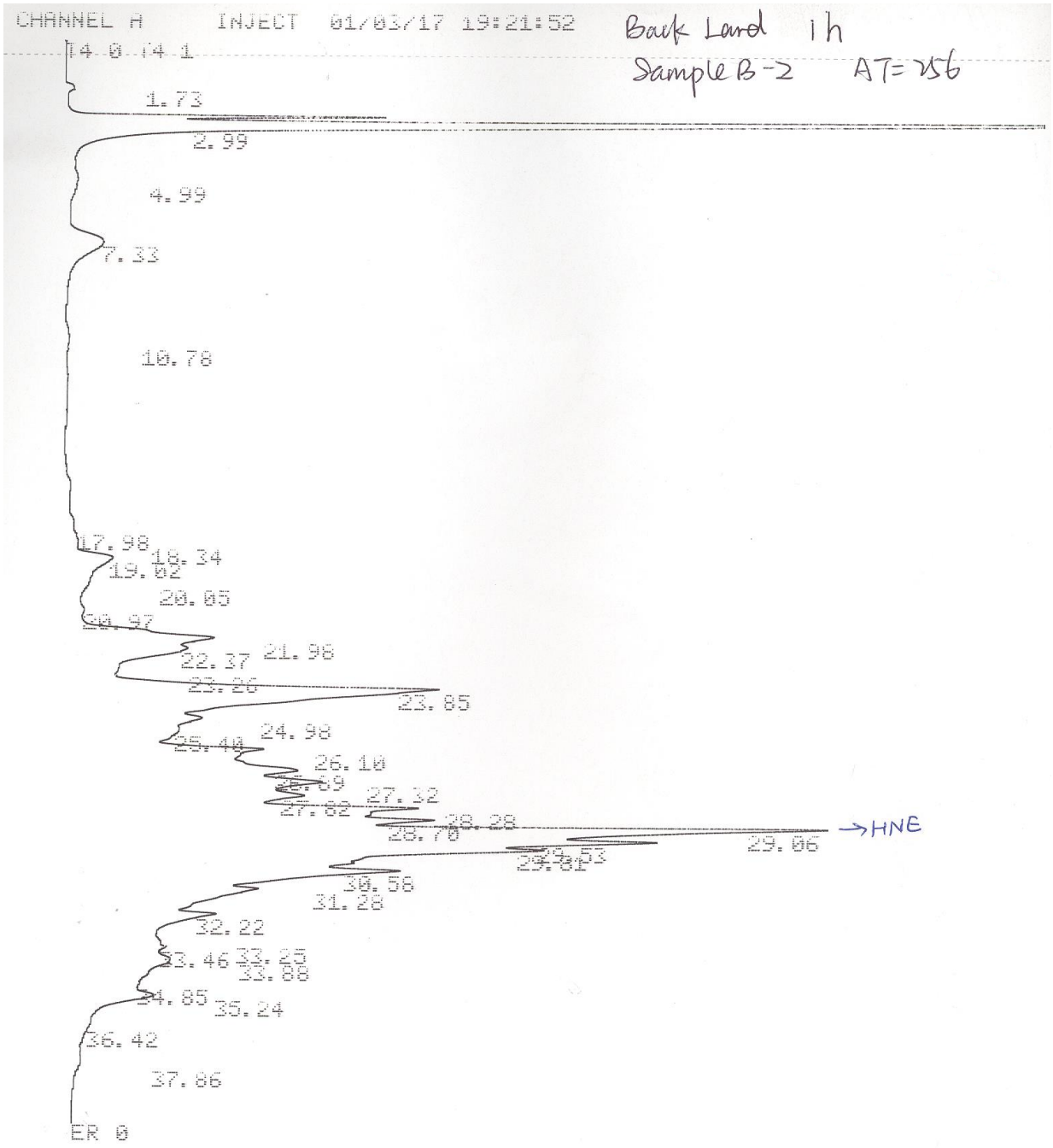


**Figure 60. HPLC chromatograph of pork back fat heated at 185°C for 1 hour. (Attenuation = 256)**

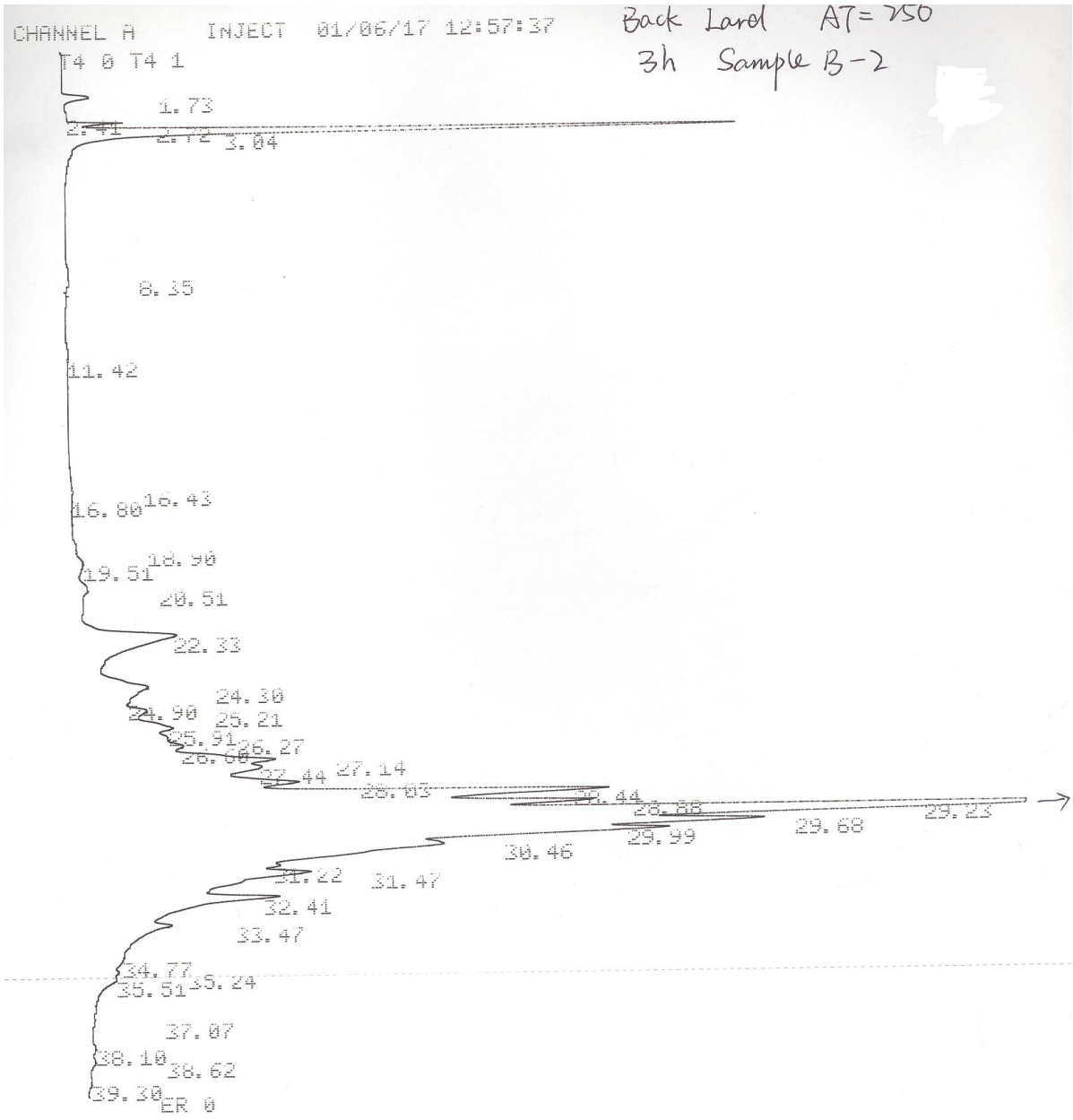


**Figure 61. HPLC chromatograph of pork back fat heated at 185°C for 3 hour. (Attenuation = 256)**

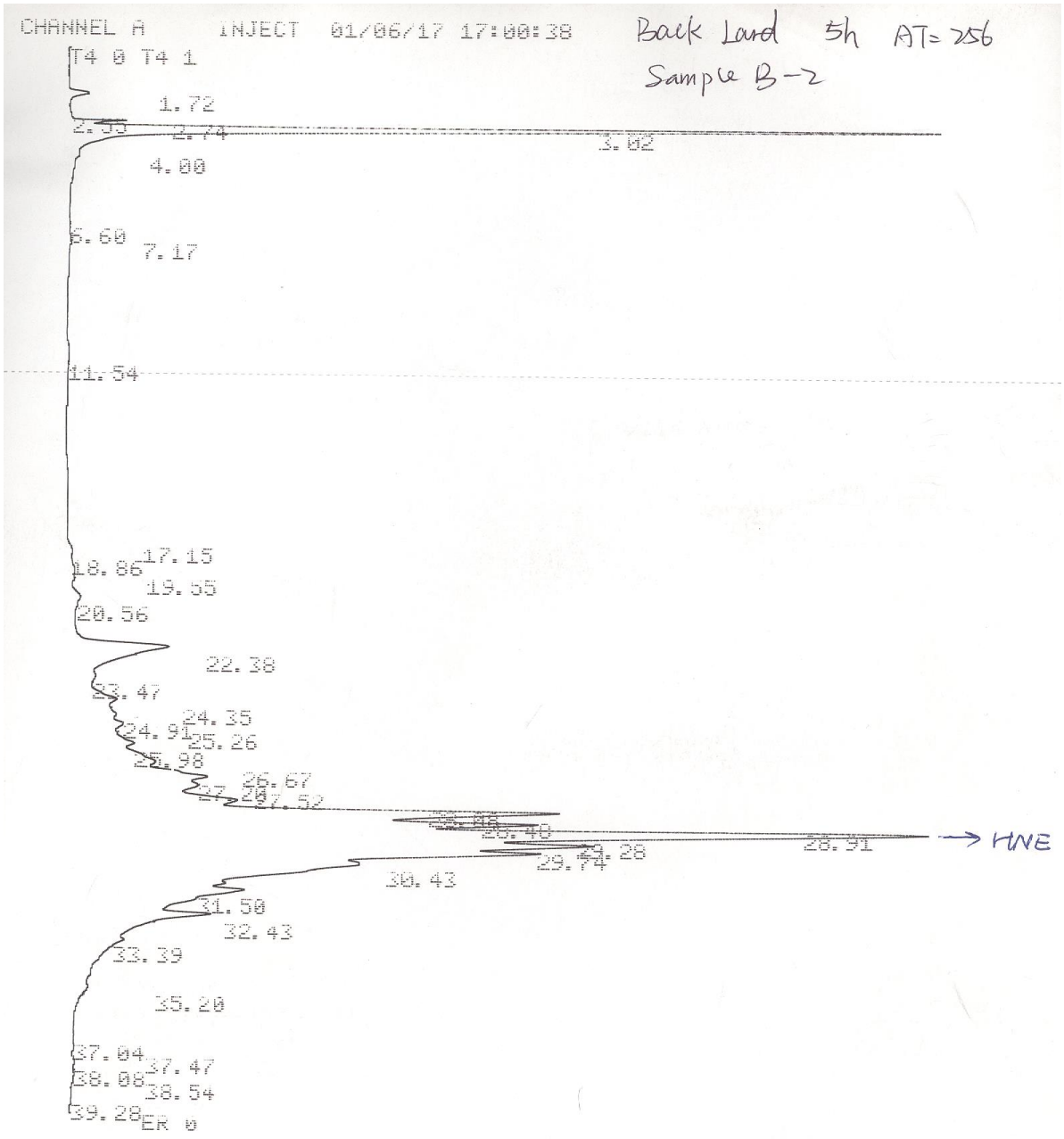


**Figure 62. HPLC chromatograph of pork back fat heated at 185°C for 5 hour. (Attenuation = 256)**

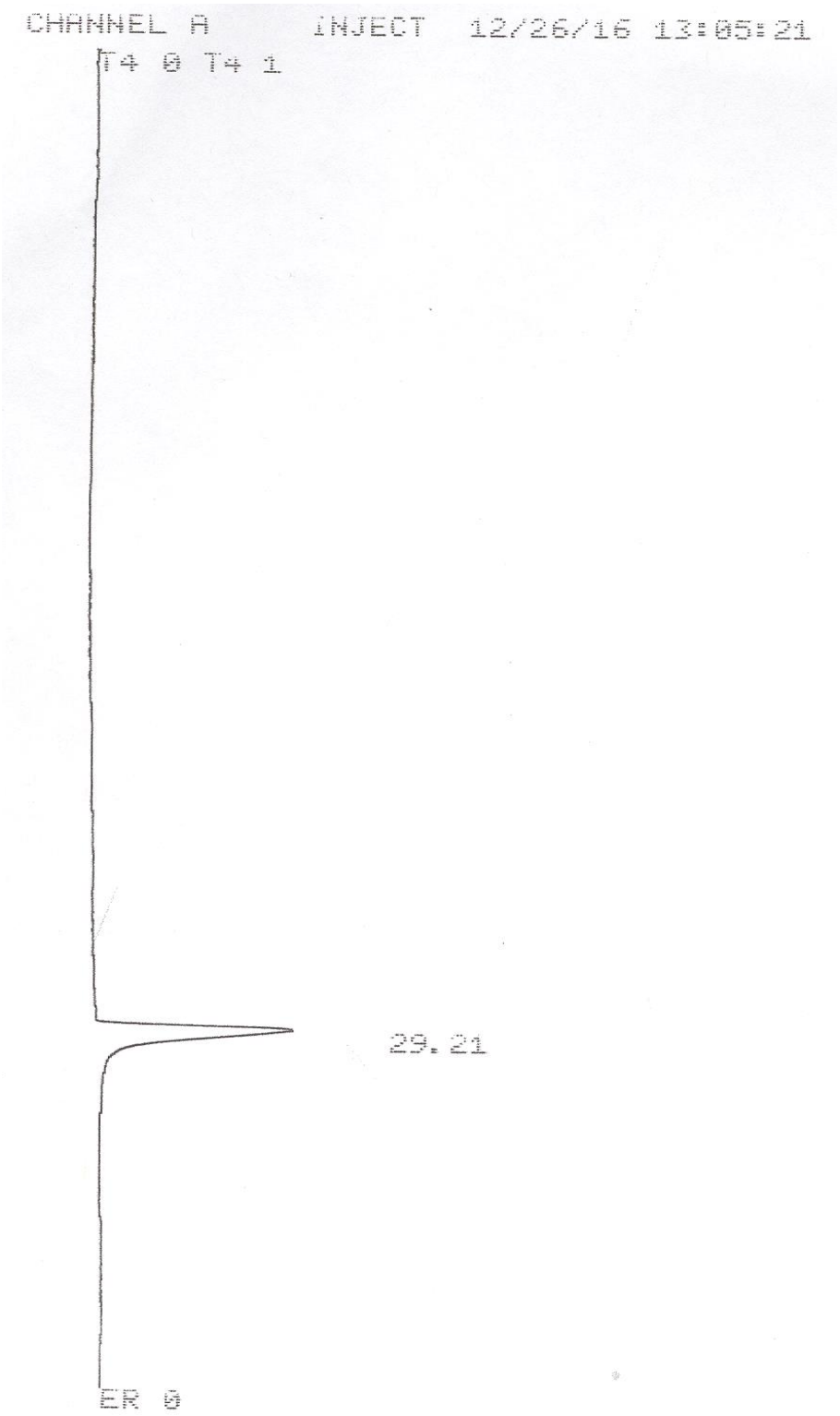


**Figure 63. HPLC chromatograph of HNE standard. (Attenuation = 512)**

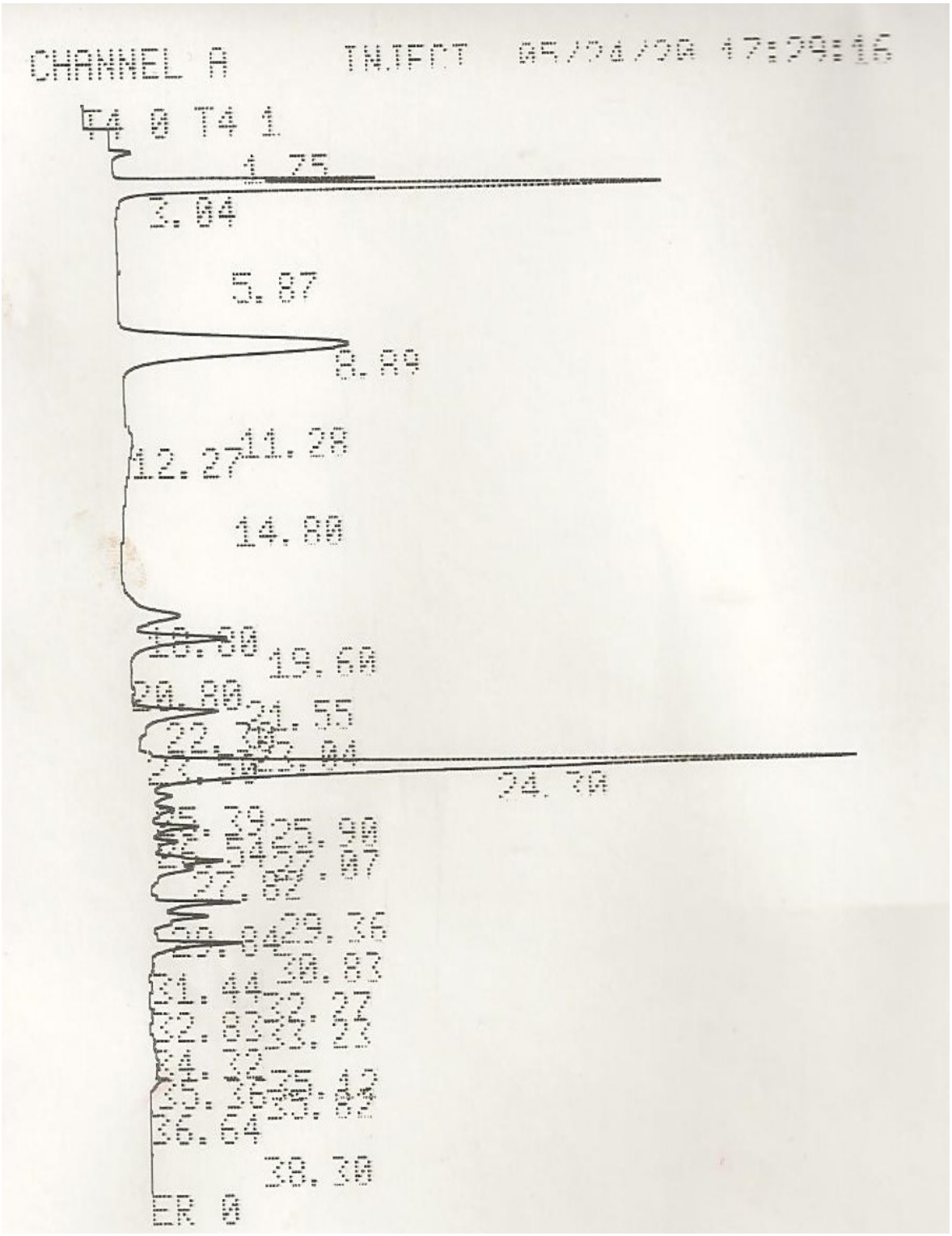


**Figure 64. HPLC chromatograph of unheated chicken thigh fat. (Attenuation = 512)**

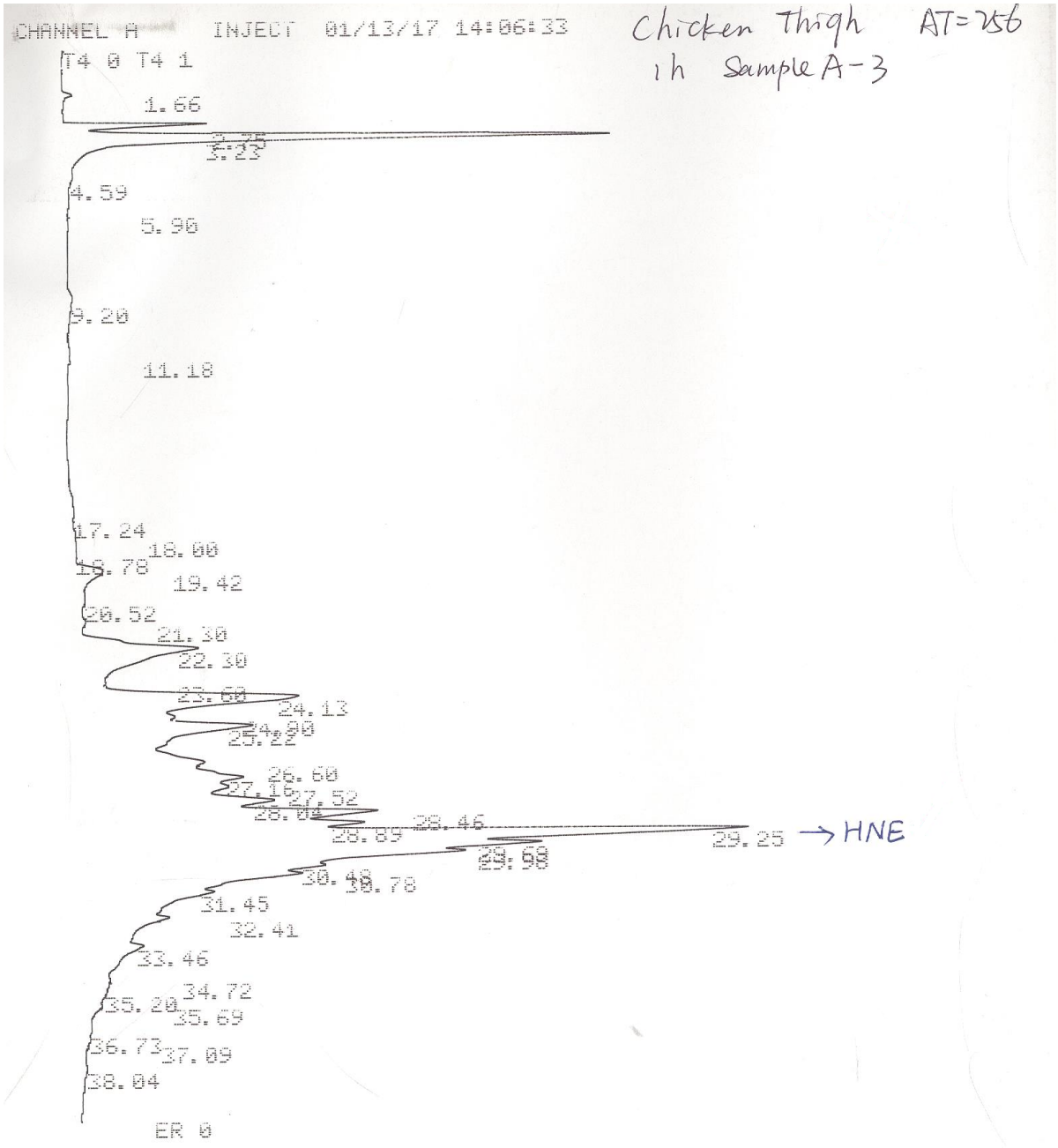


**Figure 65. HPLC chromatograph of chicken thigh fat heated at 185°C for 1 hour. (Attenuation = 256)**

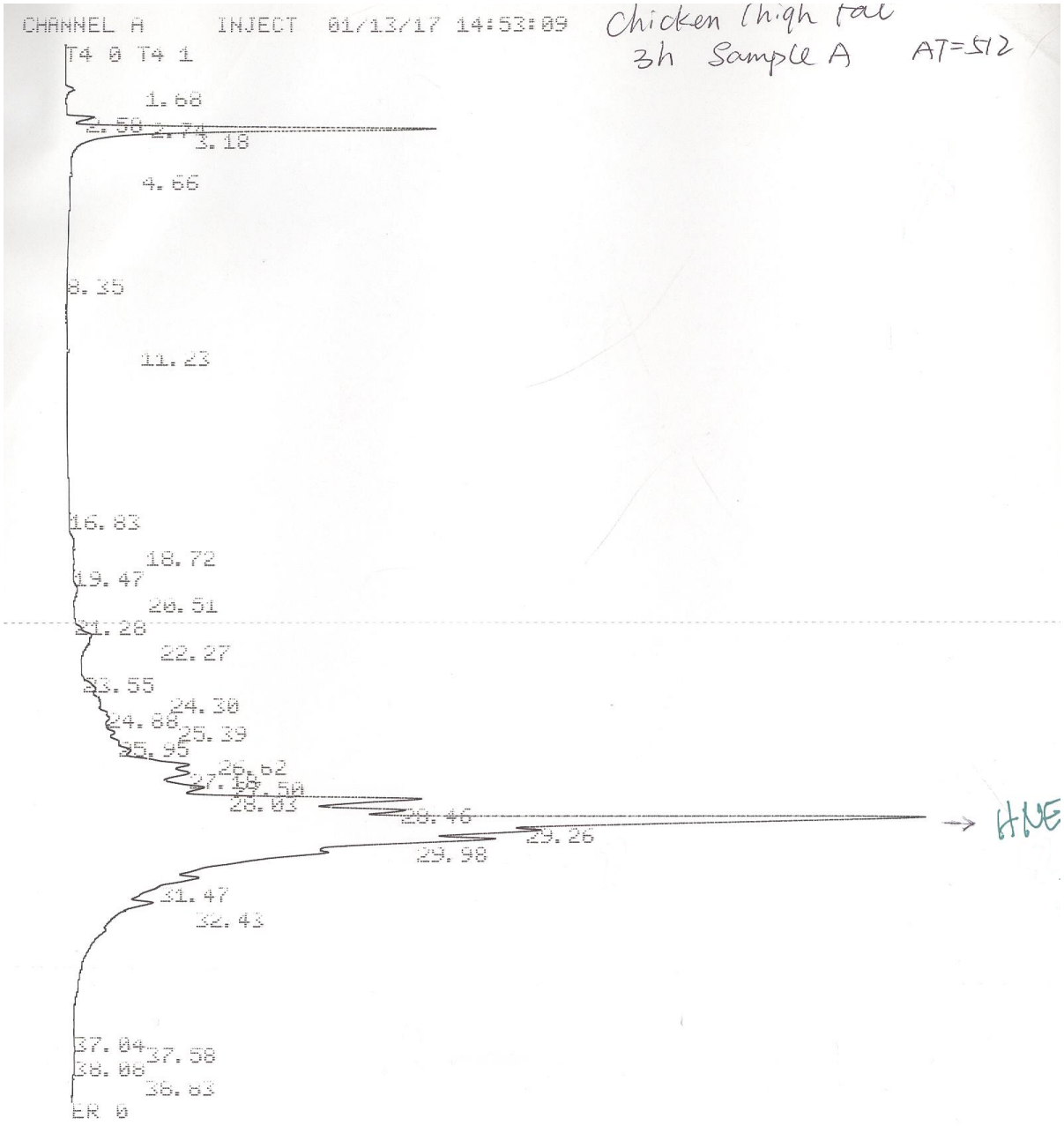


**Figure 66. HPLC chromatograph of chicken thigh fat heated at 185°C for 3 hour. (Attenuation = 512)**

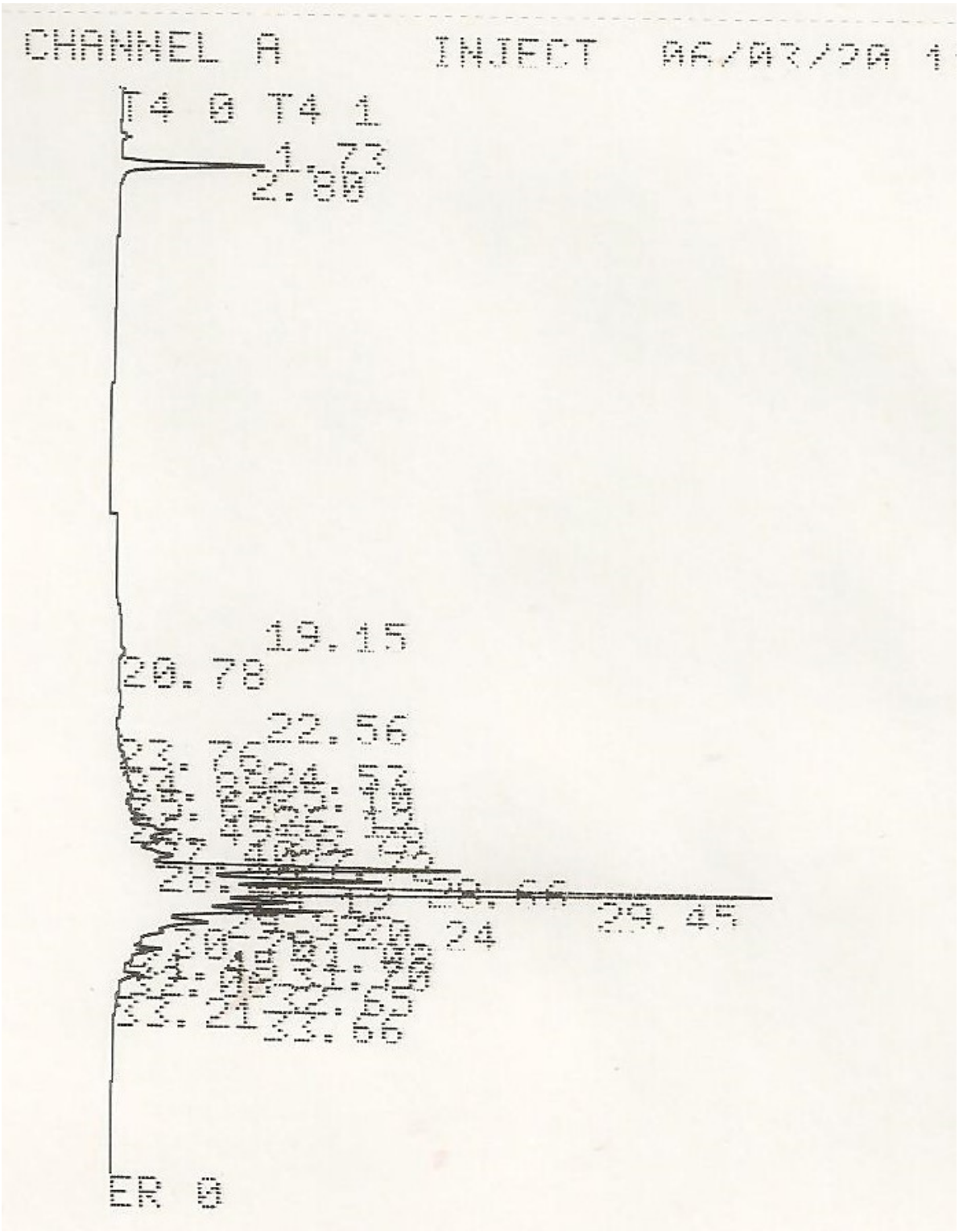


**Figure 67. HPLC chromatograph of chicken thigh fat heated at 185°C for 5 hour.**