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**RAPID QUANTIFICATION OF TRYPSIN INHIBITORS IN FOOD AND FEED  
FORMULATIONS BY ELECTROSPRAY IONIZATION MASS  
SPECTROMETRY**

by

**RADHESHYAM PANTA**

**A DISSERTATION**

**Submitted to the Faculty of the Graduate School of the  
MISSOURI UNIVERSITY OF SCIENCE AND TECHNOLOGY**

**In Partial Fulfillment of the Requirements for the Degree**

**DOCTOR OF PHILOSOPHY**

in

**CHEMISTRY**

**2017**

**Approved by:**

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## **PUBLICATION DISSERTATION OPTION**

This dissertation contains two articles that have been published or submitted for publication as follows:

Paper I, pages 61-79 has been published in CURRENT TREND IN MASS SPECTROMETRY, Vol 14(2), 36-41, 2016.

Paper II, pages 80-100 has been accepted for publication in IEEE TRANSACTION ON DIELECTRICS AND ELECTRICAL INSULATION.

## ABSTRACT

Presence of anti-nutrients deteriorate the nutritional value of animal foods and feed formulations. The important one being trypsin inhibitors. Trypsin inhibitors in food and feed formulations lower the protein supplement and hence the ultimate consequence is the decrease in growth and development of animals. It is therefore, crucial to monitor trypsin inhibitors in animal food and feed formulations. Research work mentioned in this dissertation is based on the development of rapid, more accurate and robust method for determination of trypsin inhibitor in food and feed formulations. The developed method includes extraction of trypsin inhibitor from food and feed formulation and monitoring the decrease in hydrolysis of Hydroxy Methyl Thio Butanoic Acid (HMTBA) derived oligo lysine with the help of electrospray ionization mass spectrometry (ESI-MS). HMTBA capped oligo lysine was synthesized through a chymotrypsin catalyzed reaction in phosphate buffer. The developed method was favorably compared with the existing method recommended by American Association of Cereal and Chemists (AACC). The second part discusses on the test methods and specifications of unused natural ester oils developed for transformers and similar electrical equipment. Because of lack of internationally recognized test methods to study oxidation stability of natural ester oils for use in transformers and similar electrical equipment, this research work has been aimed at developing appropriate oxidation stability test methods for natural esters and synthetic esters. The test methods include various round robin tests participated by different laboratories from seven countries around the world. The natural ester oils in this work refers to the vegetable oils obtained from seed or other suitable biological methods. These specifications have been adopted in new IEC standard test method (IEC 62770).

## ACKNOWLEDGEMENTS

At first, I would like to thank my advisor Dr. Shubhender Kapila for his constant support and guidance during my PhD work. It's my privilege to be his graduate student. I am thankful to him for his time, patience, encouragement, trainings and teachings that made my PhD work fruitful. I was really impressed with his way of teaching and trainings. His inspirational and motivational speech with wonderful guidance made it possible to come out this dissertation.

I would also like to express my sincere thanks to my committee members Dr. Michael Van De Mark, Dr. Prakash Reddy, Dr. Paul Nam and Dr. K. Chandrashekhara for their willingness to be my graduate research committee member. I appreciate their valuable time and useful suggestions. Also, my special thanks go to Dr. Rachadaporn seemamahanoop for her special guidance and suggestions. It was wonderful experience to work with her. I would like to acknowledge the department of Chemistry at Missouri University of Science and Technology for providing this opportunity. I am indebted to all staffs at this department who helped me directly or indirectly.

I would like to thank my parents, my elder and younger brothers and all family members in my country Nepal who supported me on this journey. Finally, my deep sense of gratitude goes to my wife and son for their unconditional love and support. Their love and support encouraged me constantly throughout my PhD degree. I dedicate this dissertation to my parents and family.

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## SECTION

### 1. INTRODUCTION

#### 1.1 QUANTIFICATION OF TRYPSIN INHIBITORS IN FOOD AND FEED FORMULATION

**1.1.1. Background.** In mammals, nutrients uptake involves three steps: catabolism, absorption, and anabolism (1). Among them, enzymatic hydrolysis of food occurs during catabolism inside gastrointestinal tract. Many enzymes involve in this process of food digestion. Trypsin is the one of the important enzymes for protein digestion. In fact, trypsin is a serine protease which helps to digest protein inside the digestive tract through the breakdown of larger proteins and polypeptides into smaller and simple peptides. Then human and animal body can uptake these smaller peptides. In general, Trypsin helps to cleave peptide bond of protein at the carboxylic acid side of basic amino acids such as Lysine and arginine except when either is followed by proline (2-4). Legume plant seeds like soybean, pea, black eyed pea, Lima bean are chief sources of proteins for humans and animals. These plant seeds contain significant amount of proteins and peptide (5-9). But the presence of anti-nutritional factors deteriorates the nutritional value of food. The important one is trypsin inhibitor (TI) which affects the full utilization of legumes in human diet and animal feeds (5,10-12). The ultimate impact of such inhibitor is on growth and development of animals. It is therefore very important to remove or deactivate trypsin inhibitor from protein rich legume. The most common way of deactivating Trypsin inhibitor is by soaking, de hulling and heat treatment (13). Recently extrusion cooking is also popular choice for removing the TI from food and feed formulation (14). However, these methods are good only for small scale production and not suitable for large scale production.

Extrusion cooking method can be used as potential large scale method to remove TI. The seed and meal that have undergone heat treatment have shown the significant reduction of inhibition activity (5,15). Despite their anti- nutritional behavior, anti-nutrients are essential chemicals present in plants that help to protect plants from environment. In fact, they protect plant from sun's radiation, animals and microorganisms like bacteria, viruses and fungi (16). According to Deshpande, anti-nutrients present in plants are result of secondary metabolism and protect plants from viral and fungal attack (17). In other words, anti-nutrients act like immune system of animals. Anti-nutrients are also produced because of some adverse environment condition. All most of plants have some types of antinutrients and such anti-nutrients present in legumes are studied widely due to their importance as food and feed. The most common anti-nutrients found in legumes are trypsin inhibitor, phenolic substances, non-protein amino-acids, and lecithin (18).

**1.1.2. Animal Nutrition.** Nutrition is the study of nutrients in foods and their utilization in the body of living beings for proper health and wellbeing. A nutrient is a chemical substance that involves in metabolism and is required for proper growth and maintenance of any organisms (19). Good nutrition is essential for all animals to work and function their system properly. Animals require different kinds of nutrients to fulfill the daily needs. These different kinds of nutrients include carbohydrate, protein, lipids (fats and oils), vitamins and minerals. Deficiency of any one of these nutrients hinder proper functioning of the body. Animals obtain these nutrients through foods. However, some foods are rich in a specific nutrient while they lack other nutrients. In such a case, deficient nutrients are supplied to animals through the dietary supplement. The dietary supplement

is a food item intended to supply specific nutrient to animals. Therefore, food supplement is very essential part of balanced diet of animals.

One of the important nutrient is protein which is necessary for proper growth, development and maintenance of body (20). In fact, proteins are building block of animals. The digested proteins inside the body are absorbed through the enantio-specific transport system (21,22). The anabolism of protein consists of deamination followed by transamination reaction as shown in the Figure 1.1.

The deamination of amino acids consists of removal of amine group leading to the formation of alpha-keto acid. The second stage transamination reaction consists of transfer of amine group from other amino acids to alpha-keto acid formed in first step. These alpha-keto acids are then utilized by liver cells as energy sources and raw materials for production of fatty acids (23).

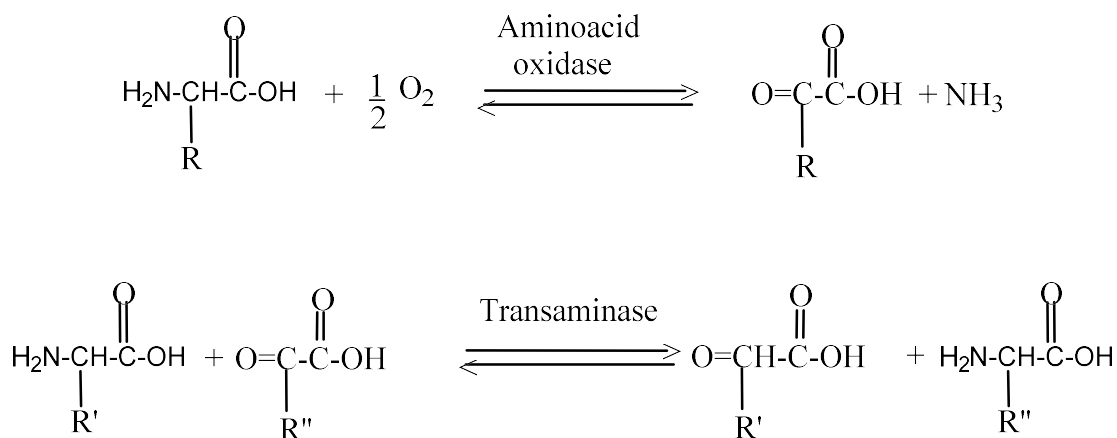


Figure 1.1: Schematic of amino-acid digestion

In monogastric animals, amino acid requirement is supplied through either direct absorption or degraded amino acids or from transamination reaction. But in ruminating animals, protein requirements are supplied through a bioconversion process which converts nitrogenous compounds to amino acids (20). Based upon the production of amino acids inside the body, there are two types of amino acids namely essential and non-essential amino acids. The former type is the amino acids that the animals cannot produce and later type is that the animals can produce inside their body. The former type includes Lysine, Isoleucine, Histidine, Methionine, Threonine, Valine, Leucine, Tryptophan and Phenylalanine. The essential amino acids whose supplementation to animals is low from diet are termed as limiting amino acids. The two-major limiting amino acids for livestock especially for cows and poultry are methionine and lysine (24-26).

**1.1.3. Animal Feed.** An understanding of chemical and nutritional composition of feed is very important to develop animal feed. Many factors need to be considered while formulating animal feed. These factors are nutrient content, calorie to protein (C/P) ratio, digestibility, palatability, bioavailability, presence of toxins and handling of feed (25). The protein content of food is measured in terms of the (C/P) ratio. The feed with high C/P ratio has less protein and high calorie. Diet having low C/P ratio results in high utilization of proteins in the body of animals. To meet the required supplement of protein, feed formulations are fortified with protein supplement. Some of the important sources of protein supplements are seed meals, legumes grain, whey, fishmeal etc. Among them, soybean meal is the chief source of protein supplement for livestock in USA. Although, soybean meal contains lysine and tryptophan, it does not contain important amino acid called methionine. The best alternative source of methionine supplement is HMTBA.



**1.1.4. HMTBA.** HMTBA is the short form for the chemical 2-Hydroxy 4- (Methyl Thio) Butyric Acid. It is a hydroxyl acid soluble in water. It is naturally occurring methionine precursor. It has been found in yeast, plants, chicks, and other organisms. The HMTBA molecule is analogous to methionine except  $-\text{NH}_2$  group in methionine is replaced by  $-\text{OH}$  group (Figure 1.2). In animal feed industry, HMTBA is extensively used as feed supplement. When HMTBA enters the animal body, it is converted into L-methionine which is then taken up by animals (27).

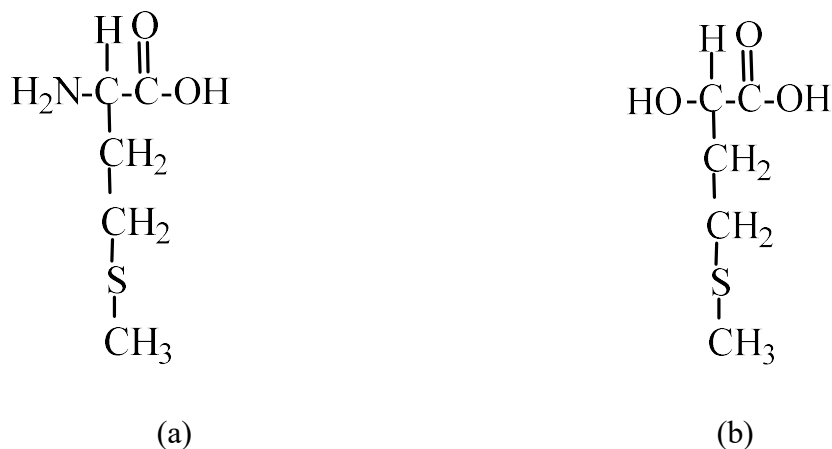


Figure 1.2: Molecular structure of (a) Methionine (b) HMTBA

**1.1.5. HMTBA as Methionine Supplement.** HMTBA is an alternate methionine supplement to animals. It is not an amino acid but its D and L form of isomers can be converted into L-methionine through stereo specific reaction pathway. Then L- methionine is utilized for synthesis of proteins (28,29). It is believed that HMTBA is absorbed in animal bodies differently than methionine. The relative absorption rate of these two types of supplement by animals has been reported in many literatures (30-32). Several in vivo

and in vitro techniques have been used to assess the uptake of these food supplements in animals.

**1.1.6. Trypsin Inhibitor.** It is a serine protease inhibitor which inhibits the activity of an enzyme called trypsin in the body of human or animals. These protease inhibitors are ubiquitous in nature. They are widely distributed in plant seeds especially in legume seeds (5,33,34). Presence of Trypsin inhibitor can either bind the active site of the trypsin or block the approach of enzymes to the protein and thereby making trypsin ineffective on digestion of the protein. Study has also shown that trypsin inhibitor can cause enlargement of pancreas (35). This leads to the hypersecretion of digestive enzymes leading to the loss of protein containing sulfur. In general, legume seeds containing Trypsin inhibitors belong to two families: Kunitz type and Bowman-Birk type trypsin inhibitor. The former TI has an approximate molecular weight of 20 to 24 K Dalton while later TI has molecular weight 7 to 8 K Daltons (36). Some legumes contain only one whereas other legumes contain both types. For example, soybean contains both types of inhibitors (Figure 1.3) while beans, lentils contain only Bowman-Birk type trypsin inhibitor. Table 1.1 shows trypsin inhibitor content of some legume seeds (10).



Figure 1.3: Structure of (a) Kunitz type (left) and Bowman Birk type (right) trypsin inhibitor (Source: [https://en.wikipedia.org/wiki/Bowman%E2%80%93Birk\\_protease\\_inhibitor](https://en.wikipedia.org/wiki/Bowman%E2%80%93Birk_protease_inhibitor))

Table 1.1: Trypsin inhibitor content of some legume seeds

Legume	Trypsin inhibitor (mg/g)
<i>Glycine max</i> (soya bean)	26.2
<i>Phaseolus vulgaris</i> (Canadian wonder)	10.5
<i>Phaseolus vulgaris</i> (Red kidney)	11.6
<i>Phaseolus vulgaris</i> (pinto)	3.4
<i>Phaseolus vulgaris</i> (Navy)	10.8
<i>Phaseolus lunatus</i> (lima bean)	20.2
<i>Pisum sativum</i> (white prolific)	2.0
<i>Pisum sativum</i> (Rovar)	1.9
<i>Pisum sativum</i> (Huka)	3.5
<i>Pisum sativum</i> (garden pea, wrinkled)	0.72
<i>Vicia faba</i> (broad beans)	2.7
Soybean meal, defatted commercial	0.84
Popped soya 1	0.76
Popped soya 2	1.17
Jet sploded soya	1.25
<i>Lupinus mutabilis</i> 1011	0.47
<i>Lupinus mutabilis</i> A2792	0.38
Beans and peas (boiled for 1 hr.)	0

The amount of trypsin inhibitor in legume seed varies with species and cultivar. Some legumes contain high amount of trypsin inhibitor whereas other legumes contain less amount of trypsin inhibitor. Lima bean contains higher amount of trypsin inhibitor than other legumes as shown in table-1. The amount of trypsin inhibitor is lowest in *Lupines mutabilis*. Also, the amount of trypsin inhibitor is dropped to zero after boiling beans and peas for 1 hour.

**1.1.7. Important Sources of Trypsin Inhibitor.** There are mainly four natural sources of trypsin inhibitor. These can bind with serine protease to form inactive complex and hence inhibit the activity of trypsin.

**1.1.7.1. Bovine pancreatic juice.** Bovine pancreatic trypsin inhibitor is a kind of protein found in many tissues in the body. It can inhibit many serine proteases like trypsin, chymotrypsin. Trypsin inhibitors separated from bovine pancreatic juice are two types. The first one is Kazal type and the other is Kunitz type. These trypsin inhibitors are produced by acinar cells in a pancreas. The function of these inhibitors is to provide safety against accidental trypsinogen activity. The first type TI was isolated by Kazal and et al in 1948 for the first time while the second type was isolated by Kunitz and Northrop in 1936 for the first time. The former differs from later by forming a less stable complex and eventually get digested by trypsin. These trypsin inhibitors are stable in acid or neutral media. The approximate molecular weight of these TI is 6500 Da.

**1.1.7.2. Soybean.** TI from soybean was first isolated by Kunitz in 1945 from soybean. It contains single polypeptide chain crosslinked by two disulfide bridge. It inhibits trypsin in mole to mole ratio. It has been found that soybean trypsin inhibitor can inhibit leukocytic

proteases but not esterolytic, proteolytic or elastolytic activities of porcine elastase. This type of TI has molecular weight of  $21500 \pm 800$  Da.

**1.1.7.3. Ovomuroid.** These inhibitors were isolated from avian egg white. According to Feeney and et al, different ovomucoid have different specificities having identical physical properties. The major one is chicken ovomucoid which is glycoprotein. One type of this trypsin inhibitor acts upon ficin and papain whereas other type ovoidinhibitor acts upon bovine trypsin and chymotrypsin. Also, chicken ovomucoid inhibits bovine trypsin but not human trypsin. This type of TI has approximate molecular weight of 28000 Da.

**1.1.7.4. Lima bean.** Trypsin inhibitor derived from lima bean can inhibit bovine and human trypsin. They have specific binding sites. Like other trypsin inhibitors, it can do reversible hydrolysis of peptide bond. Different variety of lima bean trypsin inhibitor have been separated using chromatography technique and characterized four types having similar amino acid composition. Trypsin inhibitors from lima beans have molecular weight in the range 8000-10000 Da (2).

**1.1.8. Methods of Removal of Anti-nutrients.** Removal of these anti-nutrients is essential to enhance the dietary quality of food and feed formulation. Many traditional food processing methods can be applied to remove these anti-nutrients. However, all traditional methods may not be effective on removing all anti-nutrients from food. There are few studies about the industrial process of dehydration after soaking and cooking treatments have been carried out on legume flour to improve nutritional value (37). Oluseyi and et.al have demonstrated the effect of fermentation, roasting and germination in enhancement of

nutritional properties of tamarind seed flour as a good food item (38). The important methods for removing trypsin inhibitor are as follows;

**1.1.8.1. Soaking.** the most primitive and easiest way of removing anti-nutrients from food and feed is by soaking. It is the simple method which is based on leaching of anti-nutrients to the soaking medium. This process involves the simple steps that consist of soaking the seed in medium (generally in water) for certain period. Many factors play role on this process. These factors are temperature, time of soaking, medium, type and permeability of seed. The soaking efficiency of seeds can be enhanced by adding little alkali or salt to the water. Alkali or salt solution increases the permeability of seed membrane and hence increases the leaching of anti-nutrients into the medium (39).

**1.1.8.2. Dehulling.** It is the simple process of removing outer coat of seeds. This process is carried out in a machine called huller. Dehulling of seed can remove some of the antinutrients like polyphenols significantly. However, soaking and dehulling have less effect on removing trypsin inhibitors (40).

**1.1.8.3. Heating.** Heating is the one of the efficient way of removing anti- nutrients. In general, boiling of seeds for different time can remove part of trypsin inhibitor Heating can be carried out in different ways. For example, boiling, roasting, microwave heating and autoclaving. These different heating methods have different effect on anti-nutritional factors. It has been found that microwave heating cause slight loss of minerals whereas boiling and autoclaving causes significant loss of minerals (41). Similarly, boiling of different beans i.e. soybean, mung bean and kidney bean for different time periods showed

the reduction in trypsin inhibition activity and no trypsin inhibition activity was found at all after boiling the sample for 90 minutes (42).

**1.1.8.4. Germination.** Germination is the process in which reserved nutrients present in seed are consumed for growth and development of embryo. Germination process causes mobilization of reserved nutrients in seed and thereby helps in elimination of some of the trypsin inhibitor. It has been found that dry beans showed significant reduction in trypsin inhibitory activity on germination (43,44).

**1.1.8.5. Fermentation.** Fermentation helps to reduce certain anti-nutrients. This process reduces raffinose oligosaccharides and phytic acids found in beans. Microorganisms required for fermentation play an important role (39). These microorganisms produce enzymes that help to reduce anti nutritional factor. It has been shown that fermentation reduces trypsin inhibitor from foods (45,46).

**1.1.8.6. Radiation.** Irradiation of seeds with gamma radiation can destroy trypsin and chymotrypsin. The time of irradiation determine the extent of destruction of these anti nutrients.

The combination of above methods has been found very effective on removing anti-nutrients from seeds, foods, and feeds.

**1.1.9. Monitoring Trypsin Inhibition Activity.** Until now, there are two methods available for monitoring the trypsin inhibition. The most common way of monitoring TI is through a method called American Association of Cereal and Chemist (AACC) method 22-40.01 or International organization for standardization (ISO) 14902. Both method involves the same fundamental working principle. The major difference between AACC and

ISO is the substrate. AACC uses enantiomeric mixture of substrate called N- $\alpha$ -benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPA) while ISO uses only L form of substrate i.e. N- $\alpha$ -benzoyl-L-arginine-p-nitroanilide hydrochloride. AACC method is based on monitoring the decrease in trypsin induced hydrolysis of a synthetic substrate called N- $\alpha$ -benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPA) due to the presence of TI. Hydrolysis of BAPA produce P-nitroaniline which has tendency of strong absorption in blue region ( $\lambda=410$  nm) due to the presence of chromophore. This is shown in the Figure 1.4.

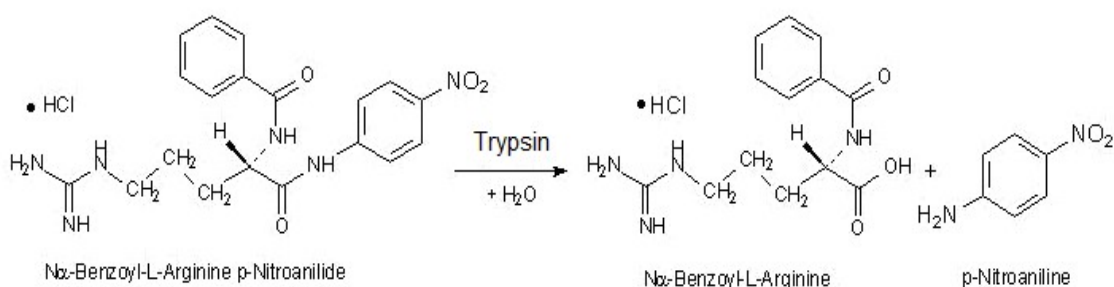


Figure 1.4: Trypsin catalyzed hydrolysis of BAPA

Presence of trypsin inhibitor in the aqueous reaction medium leads to poisoning of the enzyme leading to the retardation or complete cessation of enzyme activity. The ultimate result of this enzyme poisoning leads to reduced release of p-nitro aniline which in turn lower the absorption at wavelength 410 nm. The amount of p-nitro aniline formed during a 10-minutes reaction is measured spectrophotometrically at the wavelength 410 nm in the presence and absence of legume meal extracts. The experimental procedure for this assay is discussed below.



**1.1.9.1. Extraction of trypsin inhibitor from feed formulation.** About 1.0 g of finely ground feed formulation was extracted with 50 mL of 0.01N NaOH. The extraction is performed by stirring the contents for about 3 hours.

**1.1.9.2. Procedure for trypsin inhibitor assay.** The procedure for the assay of trypsin inhibitor is given below:

- (a) Take five clean glass test tubes and mark them as tube 1, 2, 3, 4 and 5.
- (b) Add 2 mL of diluted feed formulation extracts to tubes 1, 2, 3 and 4.
- (c) Add 2 mL of nano pure water to tube 5.
- (d) Add 2 mL of trypsin solution to tube 2, 3, 4 and 5.
- (e) Place tubes 2, 3, 4 and 5 in an oven and maintain the temperature at 37 °C for 10 minutes.
- (f) Add 5 mL of BAPA solution (pre-warmed at 37°C) to tubes 2, 3, 4 and 5.
- (g) Stir the contents (vortex) and place at 37 °C for 10 minutes.
- (h) Terminate the reaction by adding 1 mL of 30% acetic acid. Vortex the contents.
- (i) Use tube 1 as sample blank. The preparation procedure is same except that trypsin solution is added after terminating the reaction by adding 30% acetic acid.

**1.1.9.3. Calculation of trypsin inhibitor content.** The amount of trypsin inhibitor can be determined by measuring the absorbance of the trypsin standard and sample solution at wavelength 410 nm. For this, a spectrophotometer is used to measure the absorbance of

standards as well as samples. It is very important to filter each solution prior to each measurement. The absorbance values of the legumes sample extracts are subtracted from the trypsin standard (differential absorbance). The values are averaged and calculated using the formula;

$$\text{TI (mg/g) of sample} = \frac{(\text{Abs}_{\text{trypsin standard}} - \text{Abs}_{\text{formulation sample}}) \times \text{Dilution factor}}{0.019 \times 1000}$$

Where, 0.019 = the activity of 1 µg of pure trypsin is 0.019 absorbance units)

**1.1.9.4. Shortcomings of the current trypsin inhibitor assay.** Recently a lot of improvements and modifications have been made to the current TI assay method (16). The changes have been made to improve the reproducibility and linearity of the method. The modified method still does not yield accurate and precise results. The major problem of the method is that it works only in the certain specified concentration regime. At high concentration of the reacting mixture, deviation from linearity is prevalent. Therefore, it is tedious to choose accurate concentration in restricted regime to get precise and accurate results. On the other hand, even at low concentration, there is still a possibility of error. The variations in the results are caused due to the presence of suspended particles in the sample solutions. These particles are extracted along with the TI from the feed formulation and cannot be eliminated through membrane filtration. The presence of these suspended particles is not consistent and it varies from batch to batch. These suspended particles cause scattering of light during the spectrophotometer detection and cause the variation in the results.

**1.1.10. New Approach of Trypsin Inhibitor Determination.** To overcome shortcomings of the current TI assay listed above, a new assay has been developed. The main objective of this research work is to develop an assay method which is more accurate and precise using electrospray ionization mass spectrometry (ESI-MS). The method relies on relatively inexpensive target reagents and is immune from the presence of micro-particulates but it yields accurate and precise quantification of TI in food and feed formulations. This objective can be met through a series of experiments designed to fulfil the specific sub-objectives listed below:

1. Synthesis of lysine (K) and arginine homopeptides.
2. End capping of oligolysines and oligo-arginines with Hydroxy methyl thiobutyric acid (HMTBA).
3. Efficient extraction of TI from food and feed formulations
4. Optimization of Trypsin catalyzed hydrolysis of (Lys)<sub>n</sub>, (Arg)<sub>n</sub>, HMTBA-(Lys)<sub>n</sub> and Monitoring of trypsin catalyzed hydrolysis with ESI-MS.
5. Quantification of trypsin catalyzed hydrolysis of (Lys)<sub>n</sub>, (Arg)<sub>n</sub>, HMTBA-(Lys)<sub>n</sub> and HMTBA-(Arg)<sub>n</sub> in the presence of TI inhibitors.
6. Monitoring of TI activity with the BAPA based assay.
7. Validate results.

A new method for monitoring Trypsin Inhibition Activity has been evaluated. The method relies on Trypsin catalyzed hydrolysis of a synthetic substrates HMTBA capped Lys-Met [HMTBA-Lys-Met] and oligolysines HMTBA-(Lys)<sub>n</sub>. Lys-Met and oligolysines can be synthesized through a *Papain* catalyzed reaction with Lysine – ethyl ester (Lys – EE) and Methionine ethyl ester (Met-EE) as the substrates. The HMTBA capped Lys-Met

and oligolysines can be synthesized through a *Chymotrypsin* catalyzed reaction with Lys-Met, oligolysines and HMTBA-ethyl ester as the substrates. It was hypothesised that HMTBA-[Lys-Met], and HMTBA-[Lys]<sub>n</sub> should be suitable substrates for monitoring trypsin activity, because trypsin is known to cleave peptide bond at the carboxylic end of basic amino acids Arginine and Lysine unless the amino acid down side of the Arginine or Lysine is Proline. Therefore substrate used in this new method is more natural to study proteins hydrolysis than that used in classical AACC method. Furthermore, ESI-MS is very famous and suitable technique for analysis of proteins nowadays. Hydrolysis of HMTBA-[LysMet] or HMTBA – [Lys]<sub>n</sub> results in the release of a free methionine and lysine leading to a decrease in the concentration of the selected HMTBA-[Lys-Met] or HMTBA-[Lys]<sub>n</sub> substrate. HMTBA-Lys-Met and HMTBA-[Lys]<sub>n</sub> yield characteristic ions in ESI-MS so that their concentrations can be readily monitored. Trypsin activity can thus be measured as the extent of hydrolysis of the HMTBA-[Lys-Met] or HMTBA-[Lys]<sub>n</sub> (Figure 1.5).

**1.1.10.1. HMTBA capped Lys-Met.** Decrease in the rate of hydrolysis in the presence of TIs can be used to assess their concentration in the sample. Greater specificity for [Lys]<sub>n</sub> and HMTBA-(Lys)<sub>n</sub> can be obtained with ESI-MS/MS; however, results obtained with ESI-MS showed no interference. Therefore ESI-MS/MS was used in this study.

**1.1.10.2. General methods of synthesis of peptides.** Peptides are formed by linkage between amino group of one amino acid and carboxyl group of other amino acid. There are several methods available for synthesis of polypeptides. In general, peptides are usually synthesized by chemical reactions like solid phase synthesis, ring opening polymerization of N-carboxyanhydrides and recombinant bacterial synthesis (47-49). All

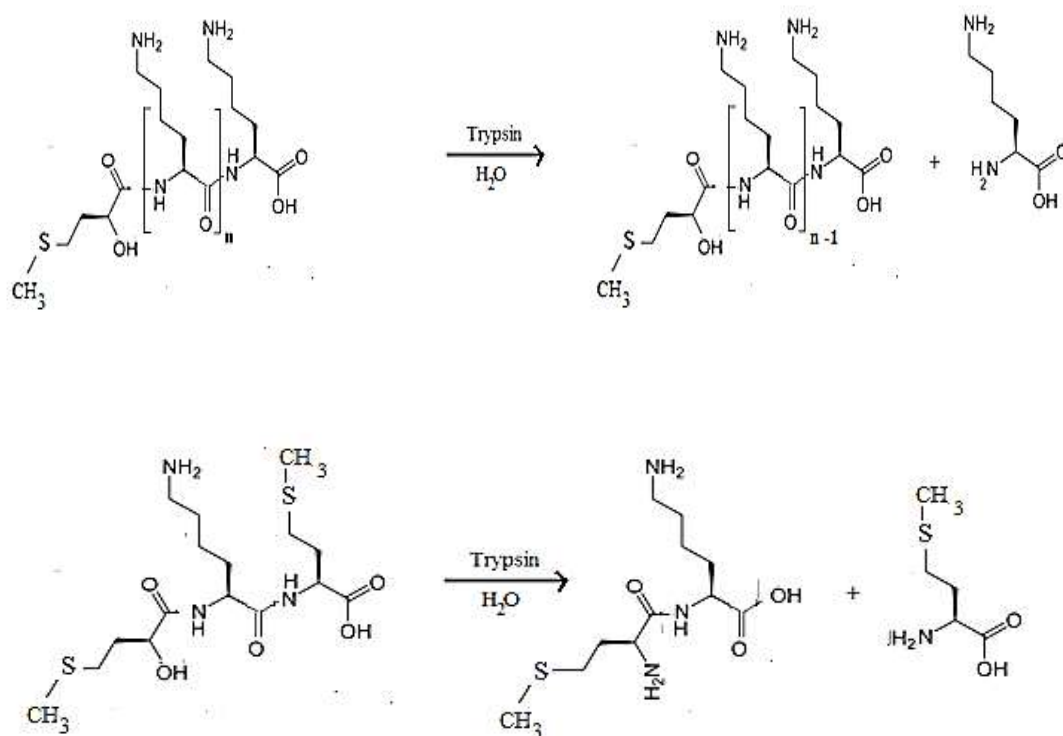


Figure 1.5: Trypsin catalyzed hydrolysis of HMTBA-[Lys]<sub>n</sub>

these methods offer both advantages and disadvantages. One of the best alternative to these methods is protease catalyzed chemo enzymatic synthesis. The advantage of this method is that it is flexible and specific for peptide synthesis, requires limited or no side chain protection and leads to no racemization of products.

The function of such enzyme is specific. However, many factors affect the enzyme catalysis like temperature, concentration of substrate, P<sup>H</sup>, solvent. The protease can do hydrolysis as well as aminolysis of polypeptides. These two processes compete during the reaction. But under specific condition like high concentration of substrate, reaction occurs through aminolysis as a major choice. According to Schechter and Berger, acyl donor needs

to be recognized in the subsite of enzyme, an active site of an enzyme (Figure 1.6). On the other hand, nucleophiles are recognized in S region. The specificity of enzyme towards acyl donor determines the rate of reaction. The specific binding of nucleophile to the S subsite of an enzyme is determining factor for overall yield of the product (50).

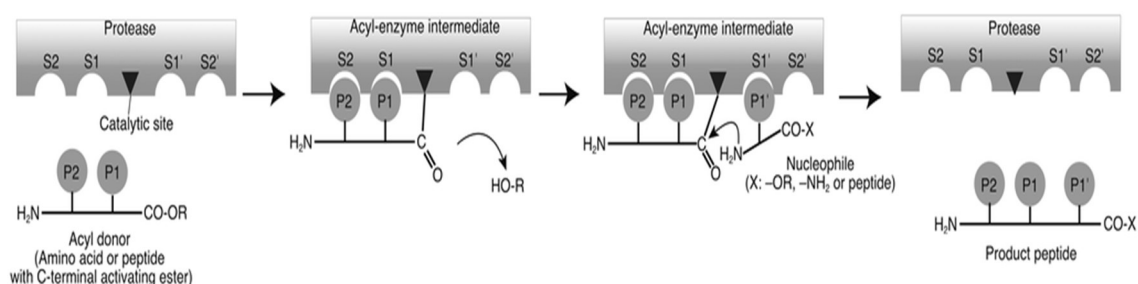


Figure 1.6: Mechanism of protease catalyzed synthesis of peptide

There are two types of proteases; endopeptidase and exopeptidase. The use of endopeptidase as catalyst leads to the extensive hydrolysis of polypeptide and thus result the low molecular weight polypeptide. The exopeptidase catalyst causes the breakdown of terminal peptide bond leading to the formation of high molecular weight polypeptide.

**1.1.10.3. Papain and papain catalyzed reaction.** papain is the cysteine protease, an enzyme present in a fruit papaya. Papain consists of a single polypeptide chain with three disulfide bridges and a sulfhydryl group necessary for activity of the enzyme. It exhibits endopeptidase, amidase and esterase activity. Papain contains enzyme that help to breakdown proteins into smaller peptides. Therefore, papain can also be used as digestive aid. It is used to make medicines that can be used for treatment of pain and swelling,

inflammation of throat and pharynx, diarrhea, running nose etc. It contains antioxidant which is essential for human body. According to Memorial Sloan-Kettering Cancer Center, papain may help to support immune system of body to fight against cancer. In addition to this, papain has been utilized to make cosmetics, toothpaste, lens cleaner and meat tenderizer in industrial area (51). Papain can also hydrolyze esters and amides. Therefore, Papain has also been used in the enzymatic synthesis of amino acids, peptides, and other molecules (Figure 1.7).

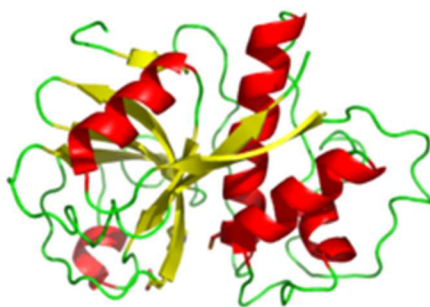


Figure 1.7: Papain from papaya (*Carica papaya*)  
(Source: <https://en.wikipedia.org/wiki/Papain>)

Papain is one of the important catalysts in chemo enzymatic peptide synthesis. Papain is stable and active under wide range of conditions from  $P^H$  4 to  $P^H$  12 and temperature up to  $80^{\circ}C$ . The  $P^H$  control of reaction is very important because at high  $P^H$ , degree of polymerization is higher than at neutral medium (50). In the past, papain has been used as catalyst for oligomerization of Glycine, methionine, leucine, tyrosine and ethyl ester derivative of amino acids (52-56). papain can cause the hydrolysis of peptide bond that is one amino acid away from hydrophobic aromatic residues of peptides. It means papain can

oligomerize hydrophobic amino acids. According to kinetic model, reaction proceeds through kinetically controlled method. The enzyme forms acyl-enzyme reaction intermediate with amino acid. Then intermediate undergoes deacylation by nucleophile or water. If water acts as nucleophile, reaction stops further and if N-terminal end of amino acid acts as a nucleophile, a new peptide bond is formed. Therefore, water is favorable nucleophile for low molecular weight peptide synthesis whereas other reagent is favorable nucleophile for high molecular weight peptide synthesis (50, 52, 57-59).

The protease catalyzed polymerization of amino acids produces oligomers. Both water soluble oligomers of acidic amino acids and water insoluble oligomers of some non-polar amino acids have been synthesized using papain or alpha Chymotrypsin in aqueous media (54,60-63). Also, the polymerizations of amino acids have been successfully carried out in organic reaction medium containing little water (64). Uyama et al. reported protease catalyzed regioselective polymerization of diethyl L-glutamate hydrochloride using papain, bromelain and alpha chymotrypsin (65). Similarly, polymerization of ester hydrochloride of methionine, threonine, phenyl alanine and tyrosine were carried by using papain catalyst in buffer medium. (53,61,64). Schwab et al. have carried out papain catalyzed co oligomerization of some alpha amino acids (66).

The mild selective method of polymerization is the method of choice when polypeptide product having specific stereochemistry and composition are desired. The common one is the protease catalyzed oligomerization of amino acids. The advantage of such protease catalyzed polymerization is that it does not form racemic mixture, reduce the protection and de protection steps, easy to handle as being the mild reaction and leads to



the synthesis of renewable and cost effective amino acids (52,67,68). In this research work, papain catalyzed reaction was performed by using Ethyl ester of Lysine as monomer.

**1.1.10.4. Synthesis of Lys-Met and Oligo-lysines [Lys]<sub>n</sub>.** Lys-Met and oligolysines was synthesized through a papain catalyzed reaction with Lys-EE and Met-EE as the substrates in a three phase reaction media, comprised of water, n-octane (C<sub>8</sub>H<sub>18</sub>) and decafluoropentane (C<sub>5</sub>H<sub>2</sub>F<sub>10</sub>).

High yields of [Lys]<sub>n</sub>, higher than 80% of the substrate can be obtained with reaction mixture comprised of CH<sub>3</sub>CN - 90% and H<sub>2</sub>O – 10% with incubation temperature maintained at 37<sup>0</sup>C. Number of Lys residues in the homo-oligomer can be determined by the incubation period. Predominant products after a two-hour incubation period will be di and tri Lysine.

Optimal yields of Lys – Met with substrate (Lys-EE and Met-EE) ratio of 1:1 can be obtained in reaction mixture comprised of CH<sub>3</sub>CN 80% and H<sub>2</sub>O 20%; with incubation temperature maintained at 37<sup>0</sup>C. The predominant product after a two-hour incubation period was Lys-Met (K-M). Product was monitored with ion pair liquid chromatography (LC) and electrospray ionization mass spectrometry (ESI-MS).

**1.1.10.5. Synthesis of HMTBA capped Lys-Met and oligo-lysines [Lys]<sub>n</sub>.** HMTBA capped peptides was synthesized through a chymotrypsin catalyzed reaction in phosphate buffer. Lys-Met and Lys oligomer and HMTBA-EE were added to a reaction vial containing 5mL of 50mM sodium phosphate (dibasic) buffer (pH 7.8). Then 250μL (50 units) of enzyme suspension (3.33mg/mL of enzyme in sodium phosphate (dibasic) buffer) was added to the vial. The reaction mixture was incubated in a shaker for 180 min. After the reaction, 250μL aliquot was taken for analysis. This aliquot was diluted

proportionately, centrifuged, filtered and 5 $\mu$ L aliquot of the filtered product was injected directly into ESI -MS for determining the product profile.

**1.1.10.6. Isolation of HMTBA [Lys]<sub>n</sub> with Semi-preparative RPLC.** The pure fractions of HMTBA [Lys]<sub>n</sub> can be isolated using a semi preparatory RPLC system. The semi preparatory RPLC separation was achieved with a C18 (25 cm x 20 mm I.D) column using water and acetonitrile as the mobile phase. The composition of the mobile phase was set to 20% water and 80% acetonitrile with the flow rate of 10 ml/min. The sample injection volume was set to 100  $\mu$ l with the total analysis time of 50 minutes. The fractions were collected from 10 to 50 minutes using the fraction collector. The isolated fractions were rota evaporated to dryness and analyzed with RPLC to evaluate the purity of isolated HMTBA-[Lys]<sub>n</sub>. Isolated HMTBA-[Lys]<sub>4</sub> was used in the assay.

**1.1.10.7. Characterization of HMTBA capped Lys-Met and oligo-lysines (lys)<sub>n</sub>.** The synthesized oligomers can be characterized with the help of high pressure liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS). For the ease of analysis, the oligo lysines can be separated by semi preparative reverse phase liquid chromatography (RPLC) technique.

**1.1.10.8. Preparation of reagents for new method of trypsin inhibitor assay.** Reagents for HMTBA capped peptides based trypsin inhibitor assay were prepared in a manner similar to the one used in the BAEE based trypsin inhibitor assays.

Sodium phosphate buffer, pH 7.6 at 25 °C (1.67 mM sodium phosphate – 8g anhydrous sodium phosphate monobasic in 1L deionized water), pH was adjusted with 1N NaOH.

1. Substrate (HMTBA-Lys-Met, HMTBA-(Lys)<sub>n</sub> 0.25 mM, prepared by dissolving 10.2 mg of HMTBA-Lys-Met; 13.1 mg HMTBA- (Lys)<sub>4</sub> were dissolved in 100 mL water. Solution is stable for up to one week.
2. 1 mM HCl was prepared by dissolving 1mL of 1N HCl in 1L of deionized water.
3. Trypsin solution was prepared by dissolving 1.0 mg mL<sup>-1</sup> of Trypsin in 100 mL 1mM HCl. The solution was stored in refrigerator at 4<sup>0</sup>C for up to 3 weeks without loss of significant activity.
4. Matrix extract (Soybean meal / Jatropha meal): Meal was ground and passed through a 100-mesh sieve. One gram of sieved meal was extracted with 50mL of 0.01N NaOH for 2 hours, pH of the extract was maintained at 8.4.

## **1.2. STUDIES ON OXIDATION STABILITY OF NATURAL ESTERS FOR TRANSFORMERS AND SIMILAR ELECTRICAL EQUIPMENT**

**1.2.1. Background.** Transformer is an electrical device for transferring electrical energy from one circuit to another by electromagnetic induction. It is a very important electrical device in an electric power system which helps in transmission and distribution of electricity (69). It is obvious that power transformers generate heat continuously while operating. The generated heat if not dissipated, can cause failure of transformer which results great economic loss. The insulating fluid takes the heat away from the core of transformer and dissipates it into the surrounding. Besides heat dissipation, insulating fluids helps to damp the noise in transformers. Not only this, the insulating fluid provides a convenient way of routine evaluation of condition of transformers during its service period. The condition of the insulating fluid provides important information about the

electrical and mechanical failure of transformer. This way insulating fluid helps to enhance electrical power transmission and distribution and minimize the risk of power failure. Therefore, the role of insulating fluid is critical for optimal performance of the transformer (70, 71). In general, the insulating materials used in liquid filled transformers are mineral oils, cellulose papers and pressboard. Insulating liquids are used either alone or in combination with impregnate cellulose paper to insulate electrical conductors from each other. The oil penetrates the small gap in the insulating paper and effectively removes air thereby filling the pore with oil having high dielectric strength. Several billion liters of such insulating fluids are used in transformers worldwide. From the historical point of view, several dielectric insulating fluids have been introduced into the market by different manufacturers. Some of those fluids have been discontinued with time. Mineral oils have been the primary insulating liquids in power transformers since 1900 due to its excellent insulation, heat dissipation, resistance to electrical and thermal ageing, low viscosity, availability and relatively low cost (72-74). However, the major drawbacks of mineral oil are that it has low flash and fire point and is non- biodegradable. The large transformers contain several thousand liters of mineral oil and therefore represents significant fire hazard especially in sensitive locations like public place, shopping mall, school, rail station. According to research, burning of 1000 kg of mineral oil releases approximately 10 kg of harmful gases (70). In such sensitive locations where fire safety is utmost important, Polychlorinated biphenyls (PCBs) were developed under the trade name Askarel, Aroclor, pyranol etc. in early 1930 and available in the market until mid of 1970s. These synthetic halogenated hydrocarbons based dielectric liquids were more stable to oxidation and does not form sludge and acid while the transformer is in operation. During 1970s, it was found

that PCBs are not safe to the environment. In case of fire and incomplete combustion, PCBs can form harmful gas called dioxin under chemical modification. PCBs itself is harmful and can remain persistent in the environment for long time. So, the manufacturing of PCBs was banned after 1978 (71, 75,76). Then other dielectric insulating fluids such as Silicone oils, high temperature hydrocarbons, tetrachloroethylenes and synthetic esters were introduced for application in transformers. Although these alternative fluids possessed superior fire resistance than mineral oils, most of these alternative oils except silicone oils possess low biodegradability (77).

In a quest of safer, fire resistant and environmentally benign insulating liquid for use in transformer, researchers have investigated many alternative insulating oils with significant modification during last four decades (79,80). Now a day, different types of insulating fluids accepted in the market are mineral oils, silicone oil, synthetic natural ester oils and natural ester oil.

**1.2.2. Types of Commercial Dielectric Insulating Fluids.** In general, there are mainly 4 types of commercial insulating fluids used in transformers as dielectric fluid.

**1.2.2.1 Mineral oil.** Mineral oil is the most common insulating oil used in transformer. It is obtained by fractional distillation and subsequent refinement of petroleum crude oil. Chemically, mineral oil is the mixture of different kinds of hydrocarbon having 15-30 carbons (naphthenic  $C_nH_{2n}$  e.g. cyclohexane, paraffin  $C_nH_{2n+2}$  e.g. hexane and aromatic  $C_nH_n$  e.g. benzene). Actually, refined mineral oils are very complex blend of more than 3000 hydrocarbons; paraffinic (40-60%), naphthenic (30-50%) and aromatic (5-20%). Generally mineral oils for transformers are divided into two types.

i. Paraffin rich oil

ii. naphthene rich oil

The latter is used dominantly in USA and other countries due to their better heat transfer capacity and low temperature characteristics. In the past three decades, the improvement in the refining technology led to the development of uninhibited and inhibited oils. The choice of the oil depends primarily on the power rating of transformer. In general, uninhibited oils are used in low power transformer and inhibited oils are used in high power transformer. Inhibited oils are more resistant to oxidation due to the addition of antioxidant. The two most common antioxidants used in transformer oils are 2, 6-ditertiary-butyl-para-cresol (DBPC or BHT) and 2,6-ditertiary-butyl-phenol (DBP) (70,81, 82).

Mineral oils have been used in transformers since the invention of transformer due to its good dielectric property and thermal conductivity. Moreover, its availability and low cost made its use widespread. However, its low flash point has raised a fire issue in sensitive locations where fire safety is essential. Also, the sources of mineral oils are getting exhausted since mineral oil is non- renewable material. In recent decades, use of mineral oils has raised the environmental concern since they are poorly biodegradable. In case of leakage or spillage, the oil can contaminate surrounding soil and water. The large amount of oil spillage from the transformer puts the surrounding ecology on danger. Also, the aromatic content of the oil on leakage causes immediate threat to human health as some of the aromatic compounds (Benzene, Toluene, Xylene) are carcinogenic (70, 83). So, mineral oil filled transformers needs installation of dripping pan to prevent any leakage or spillage of oil into the soil. In case of leakage or spillage, removing the oil from soil makes the overall cost of transformer expensive. Therefore, in quest of an insulating fluid for transformers, natural ester oil has been found as good alternative.

**1.2.2.2. Silicone oil.** Silicone oil was introduced in transformer in mid of 1970 for the first time. This oil is environmentally friendly with less fire hazards. This type of oil has high fire point and have self- extinguishing property when fire is removed. Silicone oils have better thermal stability than mineral oils. There are both IEC and ASTM specifications guidelines for silicone based oil for transformers. Silicone oils have good thermal stability. Chemically silicone oil is siloxane polymer having organic side chains. The most common silicone oil is polydimethylsiloxane (Figure 1.8). Silicone oil has been used for many years in both outdoor and indoor applications. However, at high temperature (around 300 F) silicone oil emit formaldehyde, a chemical which is skin and respiratory sensitizer, eye and throat irritant and potential carcinogen (84).

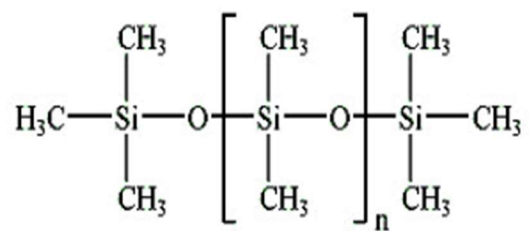


Figure 1.8: Molecular structure of Polydimethylsiloxane

**1.2.2.3. Synthetic ester oil.** synthetic ester oils are famous for lubricant which can be synthesized by reaction between alcohol and carboxylic acid. This type of oils has good oxidative stability and is biodegradable. Several kinds of oils can be synthesized from different combination of acids and alcohols. In transformers, saturated polyol esters are

preferred because of operation at high temperature. The most common one is Trimethylolpropane ester whose stability can be explained by absence of beta hydrogen in a molecule. (Figure 1.9). These polyol esters have fire point above 300 °C and possess better oxidation stability than natural ester oil (85).

**1.2.2.4 Vegetable oil.** In the frame of green and sustainable insulating fluid, natural ester oils derived from vegetable are gaining popularity recent decades. These vegetable oils are biodegradable, nontoxic liquid. They offer the advantage of having high fire point

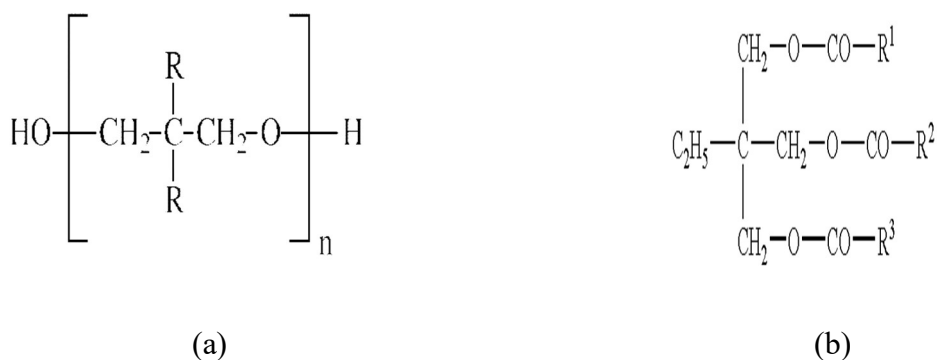


Figure 1.9: Molecular structure of (a) polyol ester and (b) Tri-methylol propane ester

and good dielectric behavior. Initially, vegetable oils were considered as dielectric fluids for capacitors only. But, considering the environmental impact of mineral oils, vegetable oils had been considered as dielectric fluid alternative to mineral oil (86-88). The major source of such oil is from plant crops. Plant crops especially seed contains significant amount of oil and fat. The oil is obtained by pressing crops or seeds and collected after



refinement. However, oils obtained from all the plant crops do not qualify for electro-technical application. The chemical composition of vegetable oils determines their selection as a dielectric fluid for transformers. Some of the common vegetable seeds for such applications are soybean, canola, rapeseed, and sunflower (Figure 1.10) due to their availability, performance, and cost (87).



Soybean seeds



sunflower seeds



Canola seed



Rapeseed

Figure 1.10: Picture of seeds of (a) soybean (b) sunflower (c) canola and (d) rapeseed

Comparative test done by Cigre group (Table 1.2) have shown that vegetable oil has properties comparable with mineral oil and silicone oil (88). Due to the comparable properties, natural ester oils derived from vegetables and suitable biogenic materials are considered as potential substitute for mineral oils in transformers.

**1.2.3. Natural Ester (Vegetable) Oil.** vegetable oils are triglyceride (triacylglycerol) molecules which are naturally formed in plants by esterification reaction between glycerol and fatty acids (Figure 1.11). Triglycerides have less density than water and remain as solid or liquid at room temperature. Those triglycerides which remain solid at room temperature are fats and those which remain liquid at room temperature are oils (89). In fact, both oils and fats belong to the group of biological substance called lipid. In oil, fatty acids may be saturated or unsaturated. The unsaturated fatty acid is generally mono, di or tri unsaturated. The fatty acid chains can have different number of carbon atoms (ranging from 8-22). The most common five fatty acid chains found in vegetable oils are palmitic acid, steric acid, oleic acid, linoleic acid and linolenic acid. (Figure 1.12).

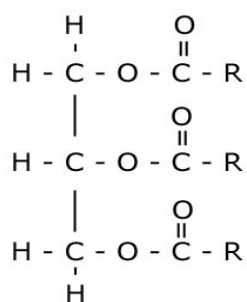


Figure1.11: Molecular structure of Triglyceride molecule

Table 1.2: Comparison of properties of different oils

properties	Unit	Mineral oil	Silicone oil	Synthetic ester	Vegetable oil	Test method
Dielectric breakdown	KV	30/85	35/60	45/70	82/99	IEC60156
Rel.permittivity at 25 <sup>0</sup> C		2.1/2.5	2.6/2.9	3.0/3.5	2.7/3.0	IEC60247
Viscosity at 40 <sup>0</sup> C	mm <sup>2</sup> S <sup>-1</sup>	3/16	35/40	14/29	15/37	ISO3104
Pour point	<sup>0</sup> C	-30/-60	-50/-60	-40/-50	-19/-33	ISO3016
Fire point	<sup>0</sup> C	110/185	340/350	300/310	350/360	ISO2592
Flash point	<sup>0</sup> C	110/170	300/310	250/270	315/328	ISO2592
Density at 20 <sup>0</sup> C	Kgdm <sup>3</sup>	0.83/0.89	0.96/1.1	0.9/1.00	0.87/0.92	ISO3675
Specific heat	Jg <sup>-1</sup> K <sup>-1</sup>	1.6/2.0	1.5	1.8/2.3	1.5/2.1	ASTM E1296
Thermal conductivity	Wm <sup>-1</sup> K <sup>-1</sup>	0.11/0.16	0.15	0.15	0.16/0.17	DCS
Exp. Coeff.	10 <sup>-4</sup> K <sup>-1</sup>	7/9	10	605/10	5.5/5.9	ASTM D1903

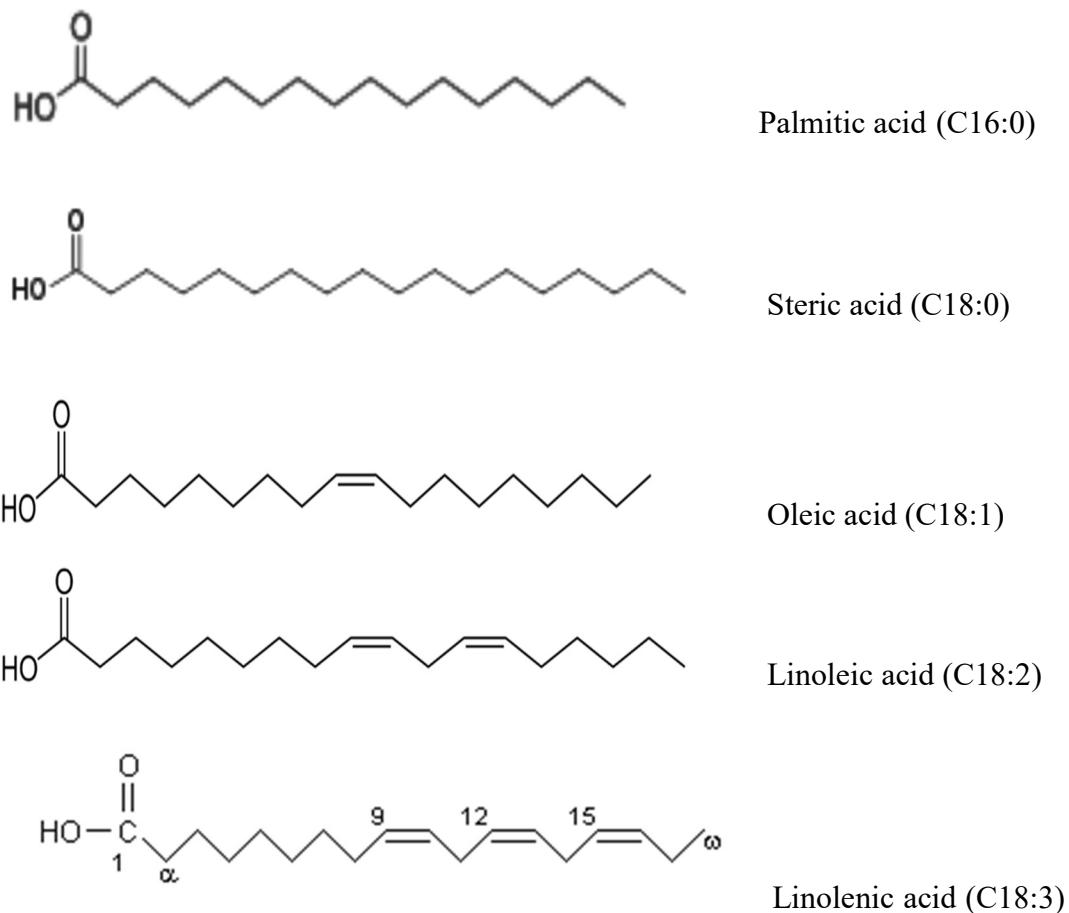


Figure 1.12: Molecular structure of five types of fatty acids

These fatty acids for example oleic acid can be conveniently expressed as C18:0 where two number represents number of carbon atoms and number of carbon-carbon double bonds in a fatty acid molecule respectively. The relative amount of fatty acids in oil depends on type of plant seed. The physicochemical properties of oil basically depend on fatty acid composition (87,88). Table 1.3 shows fatty acid composition of some vegetable oils (90,91).

Table 1.3: Fatty acid composition of some oils

FAs (%)	Sunflower	Palm oil	Rapeseed	Olive	Canola	Soybean
C16:0	6.2	44.4	4.6	8.7	3.75	9.9
C18:0	2.8	4.5	1.7	3.47	1.87	3.94
C18:1	28.0	39.2	63.44	76.34	62.41	21.35
C18:2	62.2	10.1	19.6	8.64	20.12	56.02
C18:3	0.16	0.4	1.2	0.75	8.37	7.15
C20:1	0.18		9.1	0.34	1.54	0.22
Others	0.46	1.4	0.36	1.76	1.94	1.42

**1.2.4. Properties of Vegetable Oil as Dielectric Insulating Fluid.** In order to qualify the vegetable oil as dielectric insulating oil, some of the critical properties of vegetable oils should be comparable to those commercial insulating fluids used in transformers.

**1.2.4.1. Breakdown voltage.** Breakdown voltage of the oil is the minimum voltage required to breakdown its insulation. At breakdown voltage, disruptive discharge begins and oil start to conduct electricity. There are standard test methods available to measure breakdown voltage e.g. IEC 60165. Breakdown voltage is measured by applying increasing voltage between small portions of oil. In transformers, insulating oils are designed to withstand the insulation under high electric field. Vegetable oils have breakdown voltage values that are in the acceptable range as insulating fluid for transformers. Many factors

can affect the value of breakdown voltage. The most important factors are purity and moisture content. Presence of moisture decreases the breakdown voltage of oil. Although, Vegetable oils tend to have more moisture than mineral oils, the breakdown voltage of oil depends on relative humidity of oil.

**1.2.4.2. Viscosity and pour point.** Vegetable oils are more viscous than mineral oils. This is because of difference in molecular structure between two oils. Oils having higher viscosity are not preferred for the transformer in which cooling is based on natural convection. The reason for this is because more viscous liquids are not efficient in dissipating the heat from transformer. Vegetable oils having higher percentage of saturated fatty acid have high viscosity. So, it is desirable that for low viscosity, the oil should have higher percentage of unsaturated fatty acids. However, high degree of unsaturation makes the oil more susceptible to the oxidation. The other property pour point is the minimum temperature at which oil just flows. Vegetable oils have higher pour point than mineral oils. Pour point of oil is important especially when transformer is installed in cold regions. For transformer oil, it is generally recommended that oil should have pour point 10 k less than IEC lowest cold start energizing temperature (70,76, 88).

**1.2.4.3. Flash point and fire point.** one of the major problem of the transformers containing mineral oils is high flammability because mineral oils have low flash and fire points. This problem can be resolved by replacing mineral oils by vegetable oils owing to their high flash and fire points. Generally, vegetable oils have fire points greater than 300<sup>0</sup>C and hence classified as K class as per IEC 61100 classification (78,92).

**1.2.4.4. Chemical and thermal stability.** The chemical and thermal stability of vegetable oil is the most important key parameter for its utilization as dielectric insulating

fluid. One of the requirements of good insulating fluid is that it should not react with another component of the transformer. Vegetable oil does not react with copper and insulating paper and other parts of transformer under normal conditions. It has been reported in many literatures that thermal degradation of insulating paper is 5-8 times less in vegetable oil than in mineral oil (93,94). Although oxidative stability of vegetable oils is poor compared to mineral oils, former does not form sludge whereas later forms sludge as oxidative byproducts. The oxidation of vegetable oil depends on degree of unsaturation. Higher degree of unsaturation poses greater possibility of oxidation. The oxidation phenomenon leads to the thickening of vegetable oil due to polymerization reaction that leads to the increase in viscosity.

Due to the thermal and electrical stress, insulating oil undergoes decomposition leading to the production of different gases. Natural ester oils produce carbon dioxide, carbon monoxide, hydrogen, acetylene and methane. As compared to mineral oil, natural ester oils produce more carbon monoxide and carbon dioxide due to the presence of carbonyl group. However, the total gas production from natural ester fluid is less than that of mineral oil (92,95-97).

There are numbers of industry standards and guides for the use of natural ester oil in transformers. The most common standards available are ASTM (American Society For Testing Materials), IEC (International Electro-technical Commission) and IEEE guide. The general comparison between transformers having different insulating oil is shown in Table 1.4 (77).

**1.2.5. Oxidation Stability.** Oxidation stability refers to the oil's ability to resist oxidation under specified conditions. Oxidation stability of oil can be assessed by

Table 1.4. Relative comparison of different types of liquid filled transformer types

Attributes	Mineral oil	Hydrocarbon oil	Silicon oil	Natural ester oil
Fire resistance	Poor	Excellent	Excellent	Excellent
Environmental impact	Moderate	Moderate	Moderate	Excellent
Life expectancy at maximum temperature rating	Good	Good	Good	High
Efficiency	High	high	High	High
Sound level	Low	Low	Low	Low
Operating temperature	Low	low	Low	Low
Contamination resistance	Excellent	Excellent	Excellent	Excellent
Overload capacity	good	excellent		Excellent
First cost	Low	Low/moderate	High	Moderate
Energy cost	Low	Low	Low	Low
Recycle/disposal cost	low	low	high	Low



measuring many parameters like acid formation, dissolved gas generation, sludge formation and even change in color of oils. Several factors affect the rate of oxidation. The important factors that determine the oxidation of oil are temperature, catalyst, time, composition of oil, presence of water and acids. The oxidation of oil leads to the deterioration of quality of oil. The important byproducts of oxidative degradation of natural ester oils are acids, gases, alcohol, aldehyde and ketones. These oxidative byproducts help to accelerate the oxidation. Not only this, oxidation of oils leads to the increase in viscosity of oil. This increase in viscosity of oil is undesirable as it lowers the thermal conductivity of oil. Therefore, the stability of dielectric insulating fluids towards oxidation is very important for the transformer's life.

Natural ester fluids are less stable toward oxidation as compared to mineral oils and silicone oils. Therefore, natural esters are not suitable dielectric insulating fluid for free breathing transformers. However, oxidation stability of commercial natural ester oil has been improved by adding very small quantity of chemicals called antioxidants. Natural ester oils are suitable for sealed transformer where there is no or minimal chance of oxidation. There are various standard test methods available to study oxidation stability of natural ester oil. In this work, oxidation stability of oils was studied per IEC 61125 C.

**1.2.6. Oxidation Stability of Some Commercial Natural Ester Oil.**  $25 \pm 0.5$  gm of unused commercial natural ester based oil has been taken in a borosilicate glass tube of about 20 cm length and 8.5 cm outer diameter. A copper coil of surface area  $28.6 \text{ cm}^2$ . (having about 5 cm length and 1.5 cm diameter) was dipped in each oil. Each tube was purged with Argon gas and then closed tightly with cork having two bores fitted with tubes. Clean and dry air was passed through one tube at the flow rate of 0.15 Liter per hour. The

other glass tube was used for collecting any volatile gases produced during the experiment. Each glass tube was labelled properly with the time that the oil is being heated i.e. 12 hrs., 24 hrs., 48 hrs., 96 hrs. and 164 hrs. After that, each labelled tube containing oil was put in a thermostatic aluminum block that was already heated at 120°C with the help of heating block (Figure 1.18). The temperature of oil was monitored with thermometer. The time when oil attained 120 °C was recorded. Then each tube containing oil was heated for respective time as labelled. The temperature and air flow rate was monitored each day. After heating for corresponding time, each tube was taken out of the heating block and then oil was allowed cool down to the room temperature. Then the cooled oil was poured in a clean glass bottle (100 ml amber glass bottle) and purged with Argon before close it. Some portion of oil was taken out for transesterification to assess the change in oil composition.

The reason for transesterification reaction is that gas chromatography cannot analyze oil due to its low vapor pressure and high boiling point. Therefore, oil was converted into methyl ester derivative so that it can be analyzed easily in gas chromatography. The most common way of derivatization is transesterification. By doing transesterification, more volatile fatty acid methyl esters will be formed which can be easily run in GC/FID for studying change in composition of oil due to oxidation.

**1.2.6.1. Transesterification of oil.** 10 ml of natural ester oil was taken in a 3-necked clean round bottom flask fitted with condenser and thermometer. The temperature of oil was raised to 50<sup>0</sup> C. After that 10 ml of 0.5% sodium methoxide (in methanol) was poured slowly and then the flask was closed. The reaction mixture was allowed heating at 65<sup>0</sup> C for about 75 minutes under constant stirring. The schematic of reaction is shown in the Figure 1.13. The progress of reaction was checked by TLC from time to time. After

completion of reaction, the content of the flask was transferred to the separating funnel where it forms two layers. The upper layer was discarded and lower layer containing fatty acid methyl ester was collected, washed with deionized water many times. The washed fatty acid methyl ester was collected in a beaker. Any moisture present in ester was removed by adding little (1 gm) sodium sulfate.

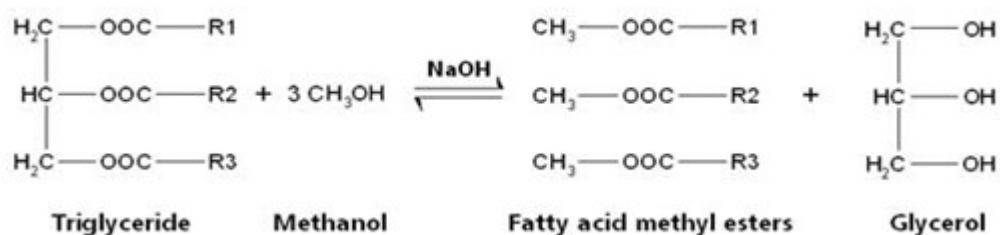


Figure 1.13: General Transesterification reaction

**1.2.6.2. TLC monitoring of the transesterification reaction.** The overall completion of reaction can be monitored with thin layer chromatography (TLC). It is the simple analytical technique in which sample (mixture) is resolved into different spots with the help of thin plate coated with silica. The reaction mixture taken out at each time interval was dissolved in hexane (1 drop sample in about 1 ml hexane). Then two small spot of sample was made on the TLC plate with the help of capillary. These spots were reaction mixture (sample), oil. Then the TLC plate was developed in a development chamber containing mixture of ether, hexane and acetic acid (90:10:1). Finally, the plate was put

into the iodine chamber where separated species on the plate were identified as yellow spots (Figure 1.14).

**1.2.6.3. Analysis of change in composition of oil.** During the oxidation of oil, some oil molecules undergo oxidative degradation that cause change in composition of



Figure 1.14: TLC separation of reaction mixture showing (a) incomplete reaction (b) completion of reaction

Oil. The change in composition of oil can be seen visually by change in color of oil (Figure 1.15). In the figure, we can see that oxidized oils have color different than un-oxidized oil and this change in color is more prominent when period of oxidation is increased. This change in color is due to the presence of different degradation products of oil. The quantitative change in composition of oil can be analyzed by gas chromatography having flame ionization detector. Series of standard solution of FAME mixture containing (C16:0, C17:0, C18:0, C18:1, C18:2 and C18:3) was made in Isooctane. The concentration of

standard solution was in the range from 10 ppm to 500 ppm. C17:0 FAME was used as internal standard. In the next step, standard FAME solution (say 100 ppm) was prepared in isooctane having C17:0 as an internal standard (note: make sure that same amount of internal standard is added to each sample). After that 1  $\mu$ L of each standard and sample solution was injected into GC/FID. The Varian gas chromatograph was equipped with DB wax column (30 m length X 25 mm i.d. X 0.25  $\mu$  thicknesses) and flame ionization detector having ultrapure He as carrier gas. The air and pure hydrogen was used as fuel for detector. The temperature of injector and detector was set at 2300C. The column oven temperature was set as: initial 50  $^{\circ}$ C, ramp to 180  $^{\circ}$ C at the rate 25 $^{\circ}$ C with no hold time and finally ramp to 230  $^{\circ}$ C at the rate 10  $^{\circ}$ C with hold time of 2 min. The obtained chromatogram will be analyzed for any change in fatty acid composition of oil heated for different period under different conditions. The oxidized oil was characterized by evaluating loss of unsaturated fatty acids.

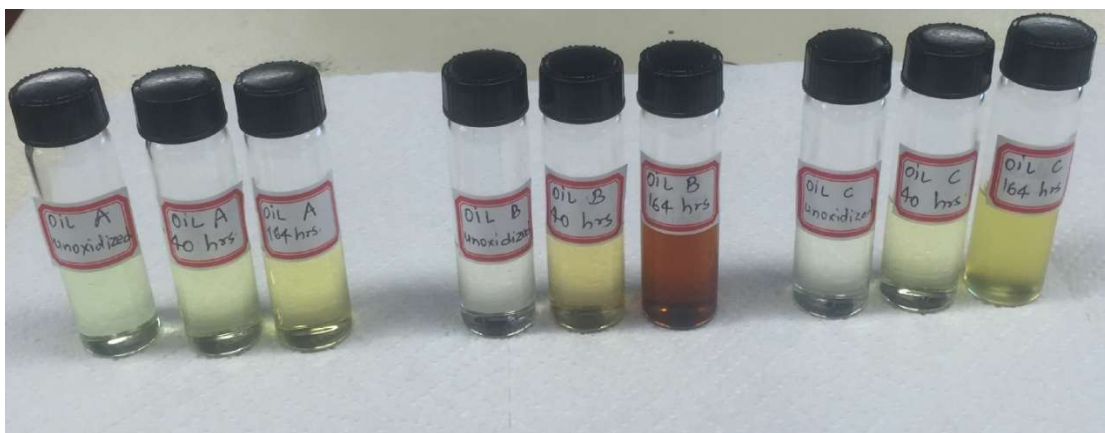


Figure 1.15: Change in color of commercial oils due to oxidation

**1.2.7. Autooxidation of Oil.** Because of presence of unsaturated fatty acid moieties like oleic acid, linoleic acid, linolenic acid chains, vegetable derived oils are prone to oxidation even at normal room temperature. The rate of oxidation depends on degree of unsaturation in fatty acid chains. The oxidation of oils leads to the formation of many oxidation byproducts leading to the degradation of oil (Figure 1.16). This phenomenon leads to the decrease in dielectric strength and insulating properties of oil.

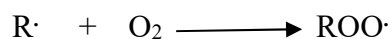
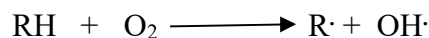
The overall mechanism of oxidation of oil contains three steps.

i) Chain initiation- In this step, formation of free radicals take place from oil due to the effect of heat, light, catalyst or stress.

ii) Chain propagation- Free radicals formed in the first step carry on reaction with oil leading to the formation of other free radicals as well as hydroperoxide. The hydroperoxide is unstable molecule undergo decomposition to form hydroxyl radical and alkoxide radical. These secondary radicals react with other oil molecules and continue the chain reaction.

iii) Chain termination- In this step, different free radicals combine together to form non radical molecules.

Initiation:



Propagation:



Termination:

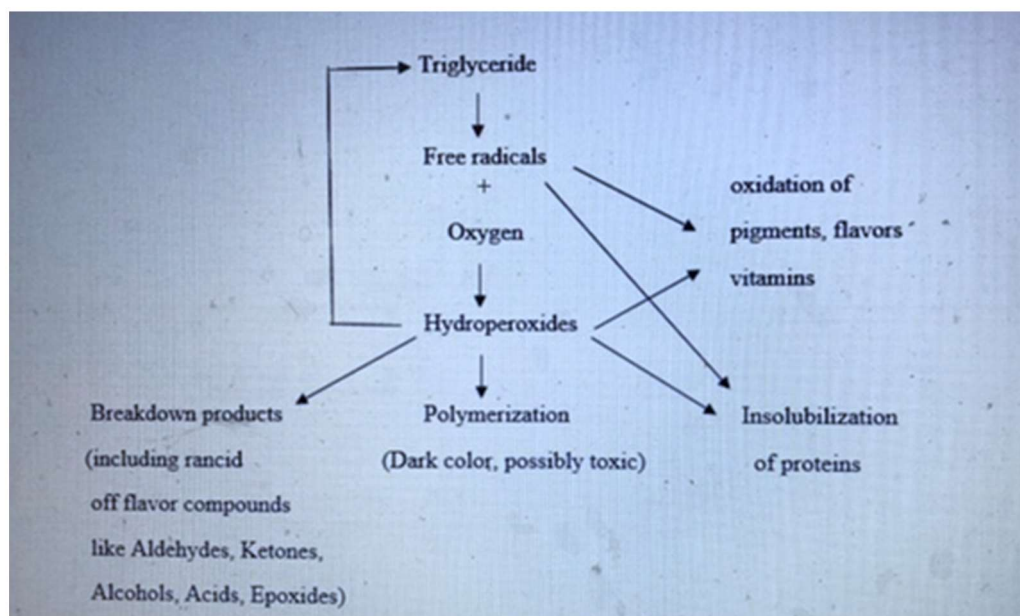
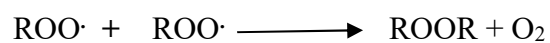
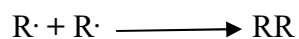


Figure 1.16: Basic pathways for oxidation of oils

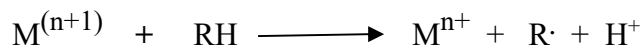
**1.2.8. Factors Affecting Oxidation of Oil.** There are several factors that affect oxidation of oil. The major factors that affect oxidation of oils are as follows;

**1.2.8.1. Oxygen availability.** Oxidation of oil occurs in presence of oxygen. Therefore oxidation of oil is directly related to the oxygen availability. High oxygen availability increases the chance of oxidation oil.

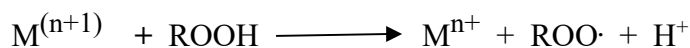
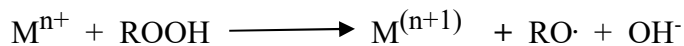
**1.2.8.2. Temperature.** Generally increase in temperature increases the rate of reaction. So the rate of oxidation of oil increases with increasing the temperature. The mechanism of oxidation is also affected by change in temperature. In general, above 60<sup>0</sup>C oxidation of oil leads to the decompositon of hydroperoxide into the volatile oxidation products.

**1.2.8.3. Copper to oil ratio.** Presence of metals like copper greatly enhance the oxidation of oil. Copper is an active metal that can act as catalyst for oxidation of oil. Generally Copper can act as catalyst either by electron transfer or by catalyzing decompositon of hydroperoxide (104).

Electron transfer:



Hydroperoxide decomposition:



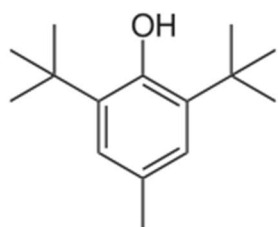
**1.2.8.4. Chemical composition of oils.** Chemical composition is the major factor that determine the oxidation stability of oil. Oils having higher degree of unssaturation are more prone to oxidation than those having low degree of unsaturation. Therefore, oils having higher degree of unssaturation are not suitable for transformers as dielectric fluid.

**1.2.9. Anti-oxidants.** Antioxidants are chemical compounds that prevent or inhibit the oxidation of other chemical compounds by scavenging free radicals or chelating metals. Antioxidants are commonly used as food additives to extend the life of oils and oils derived foods (98). The role of antioxidants in vegetable oil is to slow down the oxidation by

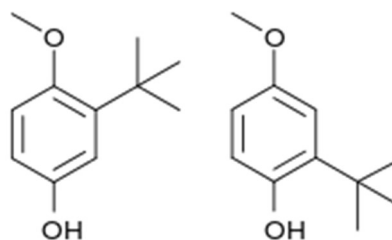


scavenging free radicals (99,100). As long as antioxidants are present, oils remain protected from oxidation. But the rate of oxidation goes high upon the complete consumption of antioxidant. Therefore, it is essential to estimate the antioxidant in oils for the stability of oil. There are two major class of antioxidants; natural and synthetic antioxidants. Some of the important natural antioxidants are vitamin C (Ascorbic acid) and vitamin E (tocopherol) and citric acid. The important class of commercial antioxidant is phenolic type including Tertiary Butyl Hydro Quinine (TBHQ), Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA), propyl gallate (PG) and aniline type including aniline, phenyldiamine, aminophenol (101-103). Some of Them are shown in figure 1.17. In transformer, the dielectric fluids (vegetable oils in this case) get heated that increase the chance of oxidation along with heat degradation. Therefore, dielectric fluids are fortified with antioxidants to prolong oil's life. The commonly used antioxidants in commercial oils are phenolic compounds like BHT, BHA, TBHQ. (Figure 1.22). According to Cooper power, level of antioxidants in the in- service transformer is between 3550 ppm to 4595 ppm. According to the ASTM, type I oil contain 0.08% antioxidant while type II oil contain 0.3% antioxidant. The level of antioxidant should not be less than 0.02% to obtain proper function of transformer oil. The antioxidant in different oil can be separated by gel permeation chromatography and analyzed by gas chromatography with FID and electrospray ionization mass spectrometry.

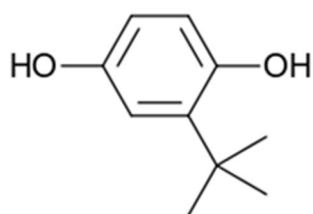
**1.2.10. Mechanism of Antioxidant Activity.** Antioxidants play their role through many basic mechanisms; scavenging free radicals, quenching the singlet oxygen, chelating metals, destroying free radical chain reaction and reducing oxygen concentration (Figure 1.18). Effectiveness of antioxidants depends on nature of antioxidant. phenolic type of



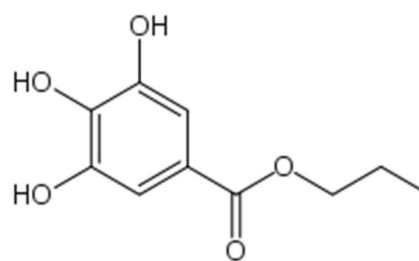
(a)



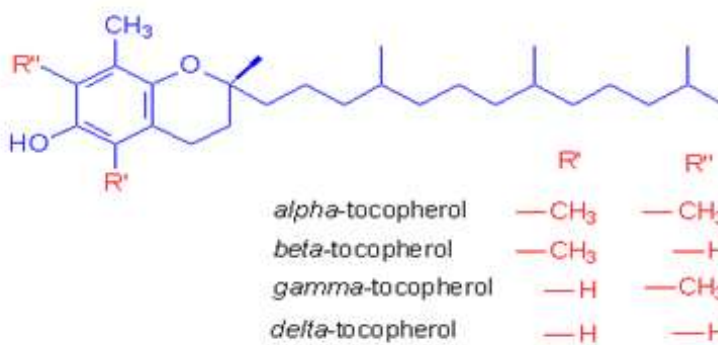
(b)



(c)



(d)



(e)

Figure 1.17: Molecular structure of (a) BHT (b) BHA (c) TBHQ (d) Propyl gallate and (e) Tocopherols

antioxidants are powerful radical scavengers but not effective on chelating metals. But flavonoids can do both efficiently (105,106). Antioxidants which can block free radical chain reactions are effective antioxidants. Such antioxidants have phenolic or aromatic rings in them. But, flavonoids can do both efficiently. Antioxidants which can block free radical chain reactions are effective antioxidants. Such antioxidants have phenolic or aromatic rings in their molecules. For such antioxidants having phenolic and aromatic rings, termination of free radicals in chain propagation step is the major dominant mechanism. Their free radicals formed during intermediate step are stable due to resonance stabilization.

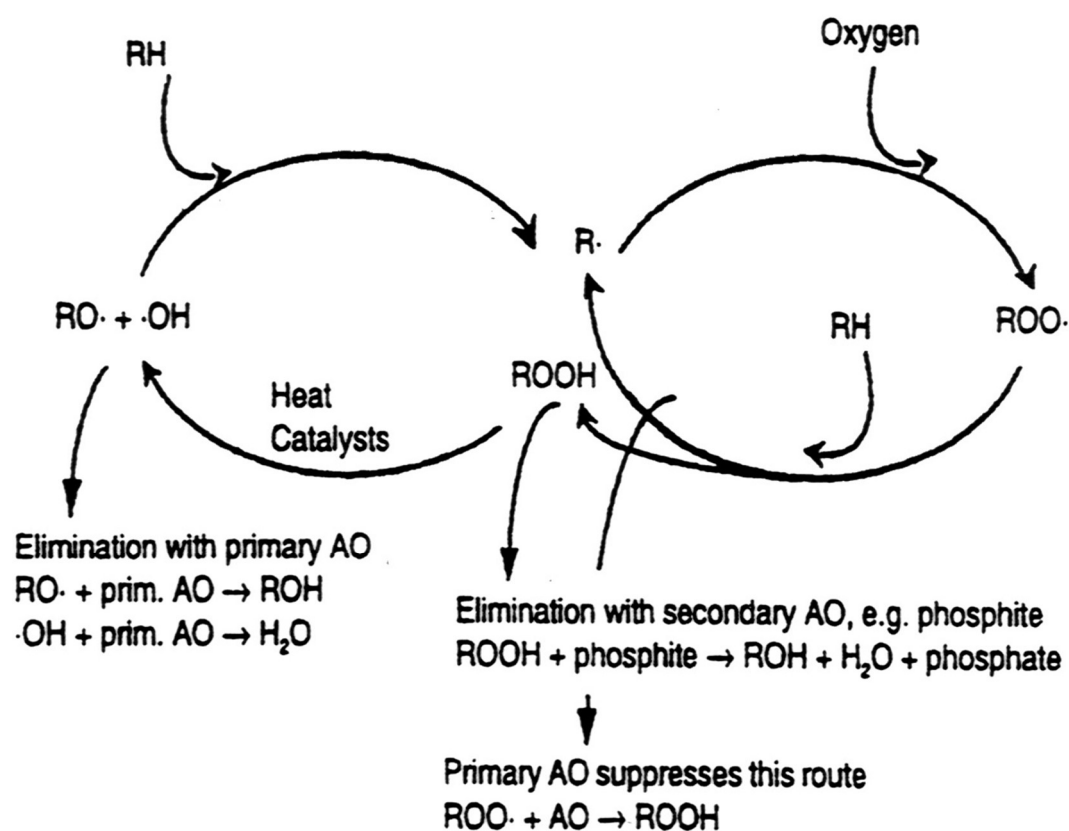


Figure 1.18: Different pathways showing mechanism of antioxidants

### **1.2.11. Chromatographic Techniques for Analysis of Antioxidants.**

Chromatography encompasses separation technique in which solutes are resolved due to the differential migration that arises from differences in distribution of solute between two immiscible phases namely stationary phase and mobile phase. Solute that prefer to distribute in mobile phase move rapidly through the column than the solute that prefers to distribute in stationary phase. Chromatography is the very common method for analysis of antioxidants presents in foods, drinks, fruits, juices, nectars, commercial oils. The quantitative analysis of antioxidants involves running series of standards followed by running the sample solution. The accuracy and precision of the analysis is very good using chromatographic methods.

**1.2.11.1. Gas Chromatography (GC).** Gas Chromatography is the most commonly used column chromatography for analysis of volatile and semi volatile compounds that are thermally stable during volatilization. The instrument has five major components; carrier gas, injector, column, oven and detector. Samples are introduced into the carrier gas (inert gas) through injector where samples are volatilized prior to mixing with carrier gas. The sample along with carrier gas get into the capillary column where samples are distributed between stationary phase and mobile phase. The stationary phase is usually very thin layer of liquid or polymer on inert solid support. Analytes emerging from the exit end of column are monitored with detector which gives a signal for the presence of analytes in the carrier gas. The most common detectors are flame ionization detector (FID), thermal conductivity detector (TCD) and electron capture detector (ECD). In this research, flame ionization detector was used to monitor analytes.

**1.2.11.2. High Performance Liquid Chromatography (HPLC).** HPLC shares some common theoretical concepts with gas chromatography and in many ways, it is complementary to gas chromatography. In HPLC mobile phase is liquid solvent at ambient temperature and high pressure. The important parts of HPLC are solvent reservoir, injector, pump, precolumn, column and detector. The analyte that has finite solubility with mobile phase can be separated by using this technique. Therefore, HPLC has much wider application than GC and is more versatile technique.

**1.2.11.3. Gas Chromatography Mass Spectrometry (GC/MS).** GC/MS is very useful technique to get orthogonal nature of information from the instrument. GC separates analytes according to vapor pressure and separated eluents from GC are sent to mass spectrometer which ionizes analyte molecules, separates ions according to mass to charge ratio. Then MS monitors separated ions which can be presented in tabular form or in bar graph called mass spectrum. This mass spectrum provides important information about mass and structure of the molecule.

**1.2.11.4. Liquid Chromatography Mass Spectrometry (LC/MS).** LC hyphenated with mass spectrometry is versatile analytical tool for analysis of polar and thermally labile compounds. The two components of LC/MS system provide orthogonal information about analytes. Separation of analytes are achieved through LC system due to the differential migration of analytes between stationary phase and mobile phase. The separated analytes are monitored with mass spectrometry through generation and separation of ions under electric and magnetic field. In LC/MS, ionization and transfer of ions occur in a component called ionization interface. There are variety of interface available for LC/MS system. Some important ionization interfaces are electrospray

ionization interface (ESI), atmospheric pressure chemical ionization interface (APCI), sonic spray interface (SSI). Ions produced in ion interface are introduced into mass analyzer through series of sampling cone and skimmers. There are three important mass analyzers namely ion trap mass analyzer, triple quadrupole mass analyzer and time of flight mass analyzer.

**1.2.12. Gel Permeation Chromatography for Separation of Antioxidants.** 10 grams of Biobead SX3 (200-400 mesh, 37-74  $\mu$ ) is weighed and placed in a clean borosilicate beaker. 25 ml of Cyclohexane and Methylene chloride [C: D] mixture (50:50) is added to the beaker and beads are allowed to swell in the solvent mixture for approximately 4 hours. Beads along with solvent are then poured into a clean chromatography column (50 cm, 8 mm id) with PTFE frit at the exit end clamped to a column stand. Beads in solvent are allowed to settle in the column for 2 hours. 50 ml of [C: D] mixture is allowed to pass through beads by opening stop cock at the exit end of column. Approximately 1 cm layer of solvent on the top of beads is left.

Natural ester insulating samples from suppliers are weighed ( $0.2 \pm 0.02$  g) and dissolved in 2 ml of [C: D] mixture and added to column (make sure that there is some solvent above the top of bead before one loads the sample). After that, sample is eluted with approximately 45 ml of [C: D]. The first 30 ml of [C: D] is collected in pre-weighed 50 ml beaker. The remaining 15 ml is collected in a second 50 ml beaker. The [C: D] fraction in first beaker is allowed to evaporate to constant weight in a fume hood. The beaker is weighed to constant weight and weigh of natural ester is determined gravimetrically from difference in the weight of beaker. The [C: D] in the second beaker is allowed to evaporate to near dryness in the fume hood. The separated antioxidant is

reconstituted in 1 ml of isooctane. Finally, 1  $\mu\text{L}$  of aliquot of the solution is introduced into an electrospray ionization mass spectrometry.

To make calibration standards in iso-octane, at first 1 mg/ml of stock solution of BHT in isooctane is prepared. Series of standard BHT solutions are prepared in isooctane to get 500  $\mu\text{g mL}^{-1}$ , 250  $\mu\text{g mL}^{-1}$ , 100  $\mu\text{g mL}^{-1}$  and 50  $\mu\text{g mL}^{-1}$  respectively. Solutions are stored in vials having PTFE lined screw caps.

BHT fortified vegetable oil aliquots: 25 $\pm$ 0.1 mg of BHT is weighed in a 30 mL of vial with PTFE lined screw cap. 20  $\pm$  0.01g of vegetable oil is added to the vial. The vial is shaken well to ensure that BHT is completely dissolved in the oil. Series of dilutions is carried out to make BHT fortified oil having concentration 500  $\mu\text{g g}^{-1}$ , 100  $\mu\text{g g}^{-1}$ , 25  $\mu\text{g g}^{-1}$  and 5  $\mu\text{g g}^{-1}$ . BHT content of oil after ESI-MS analysis has been tabulated in Table 1.5.

Table 1.5: BHT content of commercial oil

Std/Oil ppm	Response			Average	Concentration ppm
	Rep 1	Rep 2	Rep 3		
200	1.006 x 10 <sup>6</sup>	1.086x10 <sup>6</sup>	1.032x10 <sup>6</sup>	1.059E+6	
500	2.754x10 <sup>6</sup>	2.746x10 <sup>6</sup>	3.079x10 <sup>6</sup>	2.86E+6	
1250	1.263x10 <sup>7</sup>	1.346x10 <sup>7</sup>	1.286x10 <sup>7</sup>	1.298E+7	
Unoxidized oil	4.485x10 <sup>7</sup>	4.303x10 <sup>7</sup>	4.375x10 <sup>7</sup>	4.387E+7	4225
12hr	1.404x10 <sup>7</sup>	1.269x10 <sup>7</sup>	1.292x10 <sup>7</sup>	1.32E+07	1271
24 hr	4.485x10 <sup>6</sup>	3.966x10 <sup>6</sup>	4.056x10 <sup>6</sup>	4.17E+06	729
36 hr	743447	77998	813244	5.45E+05	103
48 hr	500616	456387	459246	4.72E+05	89
96 hr	x	x	x	x	x

The ESI-MS response for BHT at low concentration (<50 ppm) was not so good. Therefore, data plot for 50  $\mu\text{g mL}^{-1}$  and less than that concentration was not included. In

order to validate the method, we fortified the vegetable oil with BHT to make standard. Then BHT was separated from the oil by GPC column described earlier. While doing this type of analysis, the volume of the fraction of the eluent collected for separation of antioxidant was optimized.

The result of an antioxidant (BHT) analysis of one of the commercial insulating oil under simulated oxidation was studied which showed that the amount of BHT goes decreasing with increasing the time for oxidation of oil. This indicates that oxidation of oil cause the consumption of antioxidant. As the oxidation period increases, oxidation of oil keep going on leading to the production of secondary oxidation by products. These oxidation byproducts help to catalyze the oxidation of oil. Therefore, rate of consumption of BHT increases. It has been found that rate of oxidation depends on availability of oxygen, type of oil, concentration of antioxidant, temperature, and catalyst.



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## PAPER

### **I. Rapid Quantification of Trypsin Inhibitors in Food and Feed Formulation with Electrospray Ionization Mass Spectrometry**

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A rapid, accurate and precise method for the quantification of trypsin inhibitor activity was evaluated. The method uses alpha-hydroxyl acid-capped oligolysine (hydroxy acid [lys]<sub>n</sub>) or alpha-hydroxyl acid-capped oligolysine-methionine (hydroxy acid [lys-met]) as substrates. Hydrolysis of oligopeptides yields unique chemical residues that were readily quantified with electrospray ionization mass spectrometry (ESI-MS). Accuracy and precision of the approach compared favorably with that of the standard test method.

## **Introduction**

The nutritional importance of legumes as an economic source of proteins is well recognized [1]. Legumes provide a good balance of proteins, amino acids and essential lipids, resulting in high overall nutritional value. Legumes are the leading source of protein in human diet in many countries and they are also important sources of protein in animal feed [2]. However, legumes also contain many anti-nutritional factors (ANFs), the principle one being the trypsin inhibitors (TIs), which are a serious obstacle to full utilization of legumes in human diet and animal feed, because these proteins deactivate (inhibit) trypsin catalyzed hydrolysis of proteins and peptides [3]. TIs are widespread which are present in many plants and some animal species. TIs have been reported in various legumes such as soybeans [4], lima beans [5], navy bean [6], black eyed peas [7] and *Jatropha curcas* [8]. Trypsin is a serine protease that breaks down proteins in the digestive tract of animals. Trypsin cleaves peptide chains at the carboxyl side of the basic amino acids lysine and arginine, except when either is followed by proline [9]. The enzyme thus plays an important role in the digestion of proteins and uptake of amino acid. TIs manifest their action in two ways, either by blocking the substrate from the enzyme active site or by modifying the amino acid residues of the enzyme, thus reducing its activity. The end result is decreased utilization of proteins and thereby reducing the nutritional value of grain and legumes. To counteract these effects, processing of the seeds before ingestion is often recommended and widely practiced. Heat treatment is perhaps the most commonly used and effective method for eliminating or reducing TIs content. Traditional heat treatment processing includes de-hulling, soaking and heating [10]. The use of moderate heat treatment causes the partial denaturation of proteins and generally has a beneficial effect on the nutritional

value; by facilitating enzyme access it makes proteins more digestible [11, 12]. Measuring the TIs in legumes is of importance to the food and feed processors, who are concerned with providing a high-quality product for animal feed. Therefore, it is important to do accurate measurements for the TI content in the food and feed formulations.

### Classical Methods for the Quantification of Trypsin Inhibitors

At present TI content of soy products is determined through the standard American Association of Cereal Chemists (AACC) method 22-40.01 or a similar method ISO 14902 [13, 14]. The AACC method is based on monitoring the decrease in trypsin induced hydrolysis of a synthetic substrate *N*- $\alpha$ -benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA) in the presence of TIs. BAPA hydrolysis leads to the release of *p*-nitroaniline, a chromophore that absorbs radiation in the blue region at  $\lambda_{410}$  nm. BAPA hydrolysis is depicted in Figure 1.

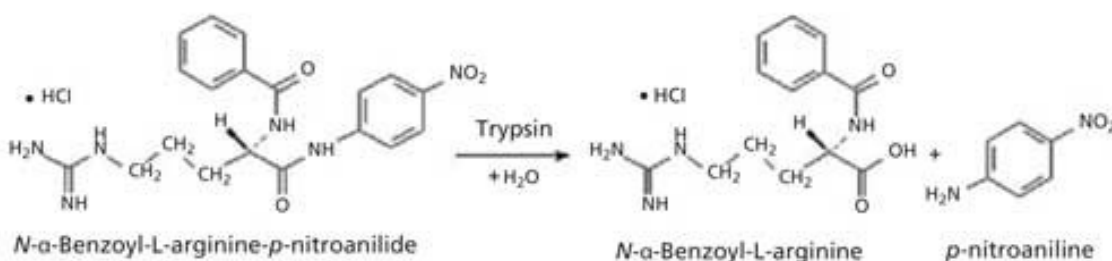


Figure 1: Trypsin-catalyzed hydrolysis of BAPA

Presence of TIs in the aqueous reaction medium leads to “poisoning” of trypsin leading to retardation or complete cessation of enzyme activity leading to reduced release of *p*-nitroaniline and lower absorption at  $\lambda_{410}$  nm. The amount of *p*-nitroaniline formed during a specified time period is measured spectrophotometrically at  $\lambda_{410}$  nm. The original procedure was reported by Kakade *et al.* and adopted as the AACC standard method 71-

10 [15, 16]. Further modifications in the method were suggested by Hamerstrand *et al.* and incorporated into the current AACC standard method 22-40.01 [17].

However, the modified method still suffers from problems stemming from inherent non-linearity of absorption which can affect accuracy and precision unless the procedure is adhered within very restrictive regime [18]. Discrepancies in results obtained with two BAPA based standard methods ISO 14902 and AACC 22-40.01 were reported recently by Sueiro *et al.* [19]. The ISO 14902 differs from AACC 22-40.0, primarily in the choice of the substrate. The ISO method requires the use of enantiomerically pure N- $\alpha$ -benzoyl-L-arginine-p-nitroanilide, whereas AACC method recommends the use of racemic N- $\alpha$ -benzoyl-DL-arginine-p-nitroanilide. Thus both methods are subject to non-linearity issue related to absorption based determinations. To overcome this shortcoming of the spectrophotometry based methods, an ESI-MS based approach was evaluated. The approach relies on lysine containing synthetic substrate, whose concentration in feed suspensions can be readily monitored with ESI-MS. The approach is immune to inherent non-linearity of absorption based methods and should yield accurate and precise quantification of TIs in food and feed formulations.

The method relies on trypsin catalyzed hydrolysis of a synthetic substrates HMTBA-(Lys)<sub>2</sub>, which were synthesized through a *Papain* catalyzed reaction with Lysine – ethyl ester (Lys – EE) as the substrates. The HMTBA capped dilysine was synthesized through a *Chymotrypsin* catalyzed reaction of di-lysine and HMTBA-ethyl ester [20, 21]. It was hypothesised that HMTBA-[Lys]<sub>n</sub> should be suitable substrates for monitoring trypsin activity, because trypsin is known to cleave peptide bond at the carboxylic end of basic amino acids arginine and lysine unless the amino acid down side of the arginine or

lysine is Proline. Hydrolysis of HMTBA – [Lys]<sub>n</sub> results in the release of a free lysine leading to a decrease in the concentration of the selected HMTBA-[Lys]<sub>n</sub> substrate. HMTBA-[Lys]<sub>n</sub> yield characteristic ions in ESI-MS and their concentrations can be readily monitored. Trypsin activity can be determined by monitoring the extent of hydrolysis of suitable substrates such as HMTBA-dilysine hydrolysis, Figure 2.

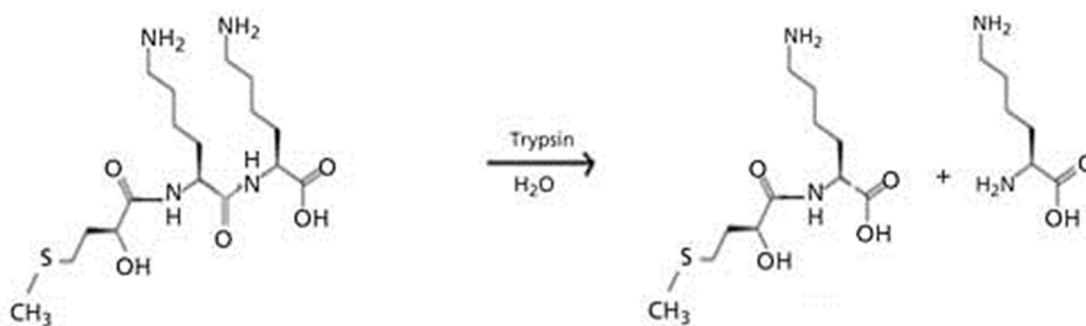


Figure 2: Trypsin-catalyzed hydrolysis of HMTBA-(Lys)<sub>2</sub>.

Decrease in the rate of HMTBA-dilysine hydrolysis in the presence of trypsin inhibitors (TIs) extracted from soybean meal and other plant materials with ESI-MS can be used to assess concentration of TIs in plant materials, thus offering an approach which is less susceptible to the nonlinearity issue.

## Experimental

The overall experimental approach involved following steps:

- Synthesis of Hydroxy methyl thiobutyric acid (HMTBA) capped di-lysine [HMTBA-(Lys)<sub>2</sub>].

- Optimization of Trypsin catalyzed hydrolysis of HMTBA-(Lys)<sub>2</sub> and monitoring of trypsin catalyzed hydrolysis with ESI-MS.
- Extraction of TIs from soybean and Jathropha meals
- Monitoring of TI activity in soybean and Jathropha meals samples with AACC 22-40.01 and with ESI-MS with HMTBA – (Lys)<sub>2</sub> as the substrate.

### **Synthesis of Di-lysine [Lys]<sub>2</sub>**

Di-lysine was synthesized through a papain catalyzed reaction with lysine ethyl ester as the substrates in a three phase reaction media, comprised of water, n-octane (C<sub>8</sub>H<sub>18</sub>) and decafluoropentane (C<sub>5</sub>H<sub>2</sub>F<sub>10</sub>), incubation temperature was set at 37°C. Number of lysine residues in the oligomer depends on the incubation period. Predominant product after a two hour incubation period was found to be di-lysine. Product yield was monitored with ion pair liquid chromatography (LC) and ESI-MS.

### **Characterization of Di-lysine**

Di-lysine prepared through papain catalyzed reaction was characterized with ESI-MS and ion pair chromatography on a C-18 column.

### **Synthesis of HMTBA Capped Di-lysine**

HMTBA capped peptides were synthesized through a chymotrypsin catalyzed reaction in phosphate buffer. Di-lysine and HMTBA ethyl ester were added to a reaction flask containing 50mM sodium phosphate (dibasic) buffer (pH 7.8). An enzyme suspension (3.33 mg/mL of enzyme in sodium phosphate-dibasic buffer) was added to the flask. The reaction mixture was incubated at 37 °C in an incubator-shaker for 180 min. After the

reaction an aliquot was taken for analysis. The aliquot was diluted proportionately, centrifuged, filtered, 5 $\mu$ L of the filtered product was introduced into ESI-MS for determining the product profile.

### **Preparation of Reagents for Determining Trypsin Inhibitors**

Reagents for trypsin inhibitor determinations with HMTBA-(Lys)<sub>2</sub> as the substrate were prepared in a manner similar to that described in the AACC 22-40.01 standard method for determination of trypsin inhibitors in soybean meal.

1. Sodium phosphate buffer, pH 7.6 at 25°C, (1.67 mM sodium phosphate, pH was adjusted with 1N NaOH).
2. Substrate (HMTBA-(Lys)<sub>2</sub>) 1.0 mM, prepared by dissolving 406 mg HMTBA-(Lys)<sub>2</sub> in 1L water.
3. Trypsin solution was prepared by dissolving 3.0 mg of trypsin (Sigma –Aldrich T9201) in 200 mL 1mM HCl. The solution was stored in refrigerator at 4°C, prior to use.
4. Soybean and Jathropha meal were used for assessing efficacy of the method. Untreated meals were obtained by grinding raw beans and passing meals through 100 mesh screen. Treated meals were obtained through wet heat treatment of raw screen meals at 130°C. One gram portions of meals were stirred with 50 mL of 0.01N NaOH for 3 hours at 25°C, pH of the extract was maintained between 8.5 – 10.0. The meal suspensions were transferred to 100 mL volumetric flasks and volume was brought to the mark with HPLC grade water.

5. BAPA solution was in accordance with the standard method AACC 22-40.01.

### **Sample free Enzymatic Hydrolysis of HMTBA [Lys]<sub>2</sub>**

Hydrolysis of the substrate was investigated prior to the initiation of inhibition assay. Five mL aliquots of HMTBA-[Lys]<sub>2</sub> solutions were taken and introduced into 15 mL vials. Three mL of HPLC grade water was added to each vial. Vials were placed in an incubator shaker maintained at 37°C for 30 minutes. After equilibration, 2 mL of trypsin solution, equilibrated at 37°C was added to each vial. Vials were removed from incubator shaker after set equilibration periods (0 – 20 minute) and enzyme was heat deactivated. Aliquots were drawn from the vials and filtered through 0.2 µm filters, 5µL portion of the filtered solution was introduced into ESI-MS. Intensity of protonated ion of HMTBA-[Lys]<sub>2</sub> at m/z 407 was monitored.

### **Substrate Free Enzymatic Reaction Blanks**

Seven mL of HPLC grade water along with one mL of soybean meal extract was added to 15mL vials. Vials were placed in an incubator shaker maintained at 37°C for 30 minutes. After equilibration, 2 mL of trypsin solution was added to each vial. Vials were removed from incubator shaker after 10 minute equilibration periods and enzyme was deactivated. Contents of the vials were allowed to come to 25°C. Aliquots were drawn from the vials and filtered through 0.2 µm filters and 5µL portion of the filtered solution was introduced into ESI-MS.

### **Deactivated Enzyme Samples**

Enzyme blank samples were prepared by adding 2 mL of trypsin solution to 15 mL vials. The enzyme solutions were heated to 85°C for 10 minutes. Solutions were allowed to cool



down to 25°C. Five mL aliquots HMTBA-[Lys]<sub>2</sub> were taken and introduced into vials. Two mL of HPLC grade water along with one mL of soybean meal or Jatropha meal extract was added to the vials. Vials were placed in incubator shaker and equilibrated at 37°C. Contents were allowed to react for 10 minutes. Contents of the vials were cooled down to room temperature and aliquots were filtered through 0.2µm filter, 5µL aliquots of the filtered solutions were introduced into the ESI-MS.

### **Measurement of Trypsin Inhibitor Activity in Soybean and Jatropha Meal Extracts**

Soybean and Jatropha meal extracts were mixed with HPLC grade water in varied ratios, 1mL of the undiluted or the diluted extracts were mixed with 5 mL of the substrate (HMTBA-di lysine) solution and two mL of HPLC grade water. The contents of the vials were mixed, then 2 mL of trypsin solution was added to each vial. Vials were placed in the incubator shaker maintained at 37°C and incubated for 10 minutes. Vials were transferred to a water bath maintained at 85°C to terminate the reaction. Contents of vials were allowed to cool down to 25°C. Aliquots were drawn from vials and filtered through 0.2 µm filters. A 5µL aliquot of the filtered solutions were introduced into ESI-MS.

### **ESI-MS Parameters**

Quantification of HMBTA-di lysine was carried out through direct infusion of 5µL sample aliquots through a fixed volume loop. Samples were carried into the ESI-MS (Varian / Agilent 320 MS/MS system) with acetonitrile:water mixture at 220 µL min<sup>-1</sup>. The ESI-MS was operated in the positive ion mode.

Operation parameters were:

Needle voltage: 4500 V

Shield voltage: 600 V

Capillary voltage: 80 V

Drying gas temperature: 200°C

Nebulizing gas: Nitrogen

Drying gas: Nitrogen

## Results and Discussion

### Characterization of HMTBA-Di lysine

Purity of HMTBA –di-lysine used as the substrate was established through reverse phase liquid chromatography and ESI-MS. Reverse phase chromatogram of the HMTBA-di-lysine preparation showed the presence of one dominant peak which accounted for >95% of the area of the total peak areas in the chromatogram. The ESI-MS spectrum of the HMTBA-di-lysine solution showed a dominant ion at  $m/z$  407, corresponding to  $[\text{HMTBA-dilysine}+\text{H}]^+$ , Figure 3.

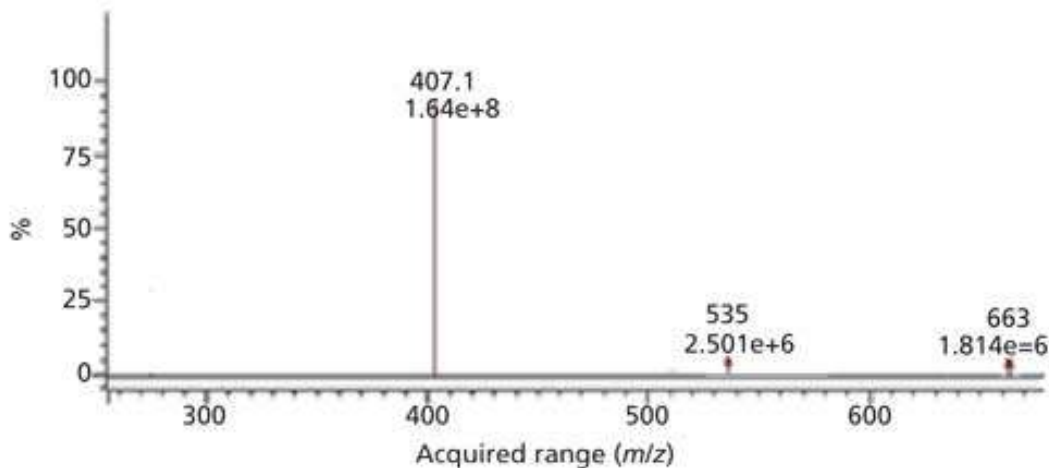


Figure 3: ESI-MS positive ion spectrum of HMTBA-(Lys)<sub>2</sub>

Ions at  $m/z$  535 and 663 correspond to HMTBA – [Lys]<sub>3</sub> and HMTBA-[Lys]<sub>4</sub> were observed, however, their combined concentration was less than five percent. Linearity of ESI-MS response for HMTBA-[Lys]<sub>2</sub> was monitored as intensity of  $m/z$  407 over concentrations ranging from 1 – 203  $\mu\text{g mL}^{-1}$  in aqueous solutions containing soybean or Jathropha meal extracts, response was found to be linear with a correlation co-efficient between 0.998 – 1.002.

### **Trypsin Catalyzed HMTBA-[Lys]<sub>2</sub> Hydrolysis**

Trypsin catalyzed hydrolysis of HMTBA-[Lys]<sub>2</sub> was monitored over incubation periods varying from 0 – 20 minutes. Extent of hydrolysis was monitored by measuring intensity of protonated HMTBA-[Lys]<sub>2</sub> ions at  $m/z$  407 and its hydrolysis product HMTBA-Lys (MW 278) which appears  $m/z$  279 with ESI-MS. Hydrolysis led to appearance of protonated HMTBA- Lys ion at 279  $m/z$ . The appearance of the latter ion can provides an independent means to monitor the extent of trypsin mediated hydrolysis of the substrate, however, it was not examined during this study. Results showed that HMTBA capped di-lysine is rapidly catalyzed by trypsin and therefore a good substrate for trypsin monitoring trypsin catalyzed hydrolysis. The change in HMTBA-[Lys]<sub>2</sub> concentration with the incubation period is shown in Figure 4. As expected the enzymatic hydrolysis of HMTBA – [Lys]<sub>2</sub> occurs at a considerably faster rate than that reported for BAPA, perhaps because of its higher solubility in water and also because it is a more natural substrate for trypsin. Results showed that HMTBA-[Lys]<sub>2</sub> is a suitable substrate for monitoring trypsin catalyzed hydrolysis of the amide bond at the carboxylic end of lysine.

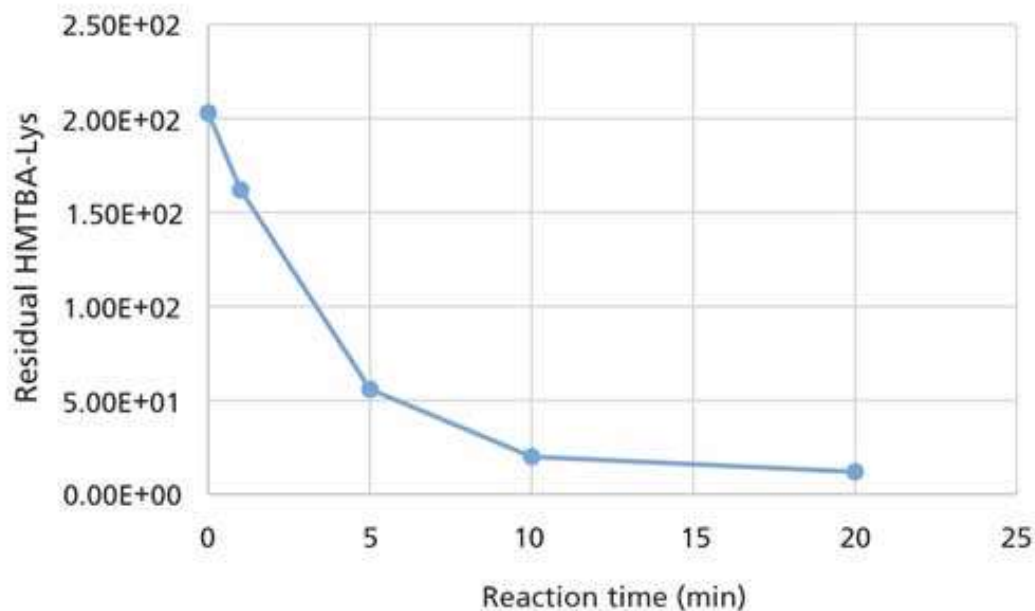


Figure 4: Change in HMTBA-(Lys)<sub>2</sub> concentration with reaction time

### Substrate Free Enzymatic Reactions

Filtered aliquots from reactions carried with soybean and Jathropa meal extracts in the presence of trypsin but without HMTBA-[Lys]<sub>2</sub> were introduced into the ESI-MS. ESI-MS mass spectra for the reaction media aliquots were acquired in the both the scan over  $m/z$  200 – 450 and SIM mode set for monitoring ion  $m/z$  407. The scan mode spectra showed the presence of characteristic background ions observed with CH<sub>3</sub>CN:H<sub>2</sub>O mixtures at very low intensities. Ions at  $m/z$  407 was not observed at all or observed at intensities three orders of magnitude lower than ones observed in the presence of the HMTBA-[Lys]<sub>2</sub>. These results support the contention that HMTBA-[Lys]<sub>2</sub> is a suitable substrate for monitoring trypsin catalyzed hydrolysis.

### Substrate Concentration in Reaction Media with Deactivated Enzyme

Filtered aliquots from reactions media incubated at 37°C for ten minutes with deactivated trypsin containing HMTBA-[Lys]<sub>2</sub> and soybean and Jathropha meal extracts were introduced into the ESI-MS. ESI-MS mass spectra for the reaction media aliquots were acquired in the SIM mode set for ions *m/z* 407 and *m/z* 279. Only ion at *m/z* 407 was observed in the reaction with either the soybean or Jathropha meal extracts. Results showed that HMTBA-[Lys]<sub>2</sub> concentration did not change after incubation with deactivated enzyme. Integration results for *m/z* 407 in reaction media prior to incubation and after incubation are shown in Figure 5.

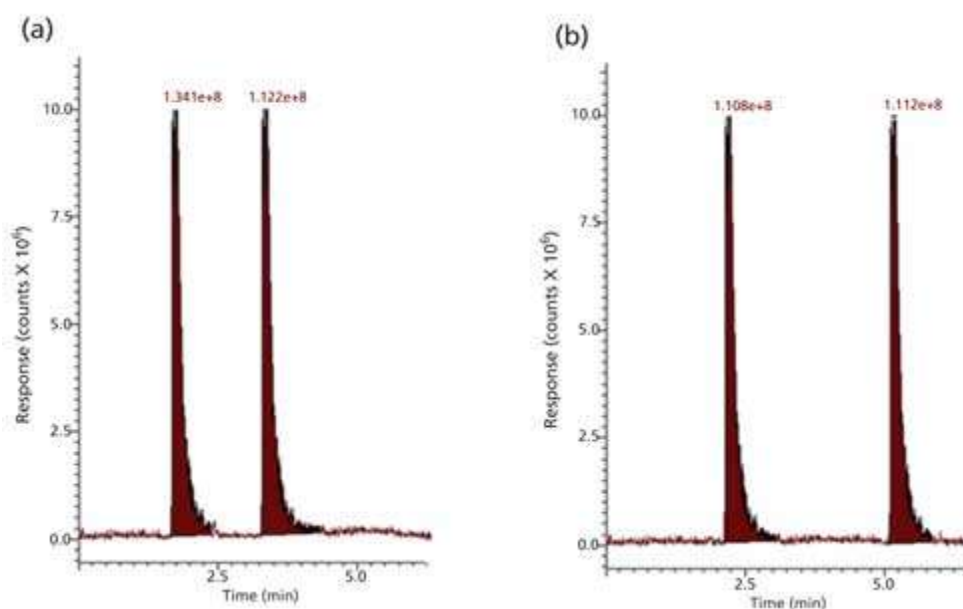


Figure 5: (a) Intensity of HMTBA-(Lys)<sub>2</sub> ion *m/z* 407 in reaction media at the start of incubation with deactivated trypsin. (b) Intensity of HMTBA-(Lys)<sub>2</sub> ion *m/z* 407 in reaction media after a 10-min incubation with deactivated trypsin.

### Measurement of Trypsin Inhibition

Analysis for substrate HMTBA-[Lys]<sub>2</sub>, in reaction media containing the substrate, trypsin with soybean or Jatropha meal extracts showed that the intensity of protonated HMTBA-[Lys]<sub>2</sub> ion at m/z 407 in the reaction media was related to amount of meal extracts introduced, intensity of m/z 407 i.e. the intact (un-hydrolyzed) HMTBA-[Lys]<sub>2</sub> increased indicating a decrease in the extent of HMTBA-[Lys]<sub>2</sub> hydrolysis through trypsin inhibition. The increase in intensity of ion m/z 407 relative to reaction media without the soybean or Jatropha extracts was related to the volumes of meal extracts introduced into the reaction media. It was observed that meal extracts of untreated (raw) soybean and Jatropha meal were more effective in inhibiting the hydrolysis than the extracts of the heated treated meals. Extracted ion chromatograms for m/z 407 obtained for three replicate injections of reaction media aliquots after 10 minute incubation with trypsin in the absence of soybean or Jatropha meal extract are shown in Figure 6. Extracted ion chromatograms for m/z 407 obtained for three replicate injections of reaction media aliquots after 10 minute incubation with trypsin in the presence of diluted Jatropha meal extract (two fold dilution) are shown in Figure 7.

Results show the addition of diluted Jatropha meal reduced HMTBA-[Lys]<sub>2</sub> to half the value obtained in the absence of extract. In the standard BAPA based trypsin inhibitor assay a linear relationship between BAPA hydrolysis over a set time (10 minute) is assumed. If a similar relationship is assumed in the present case this would imply that diluted Jatropha deactivates half of enzyme (~15 µg) present in the reaction media. Thus trypsin inhibitor content of the meal would be 30 µg g<sup>-1</sup>, a value similar to one obtained

with the AACC standard method. Results obtained for untreated and treated soybean and Jatropha meals with the two methods are shown Table-1.

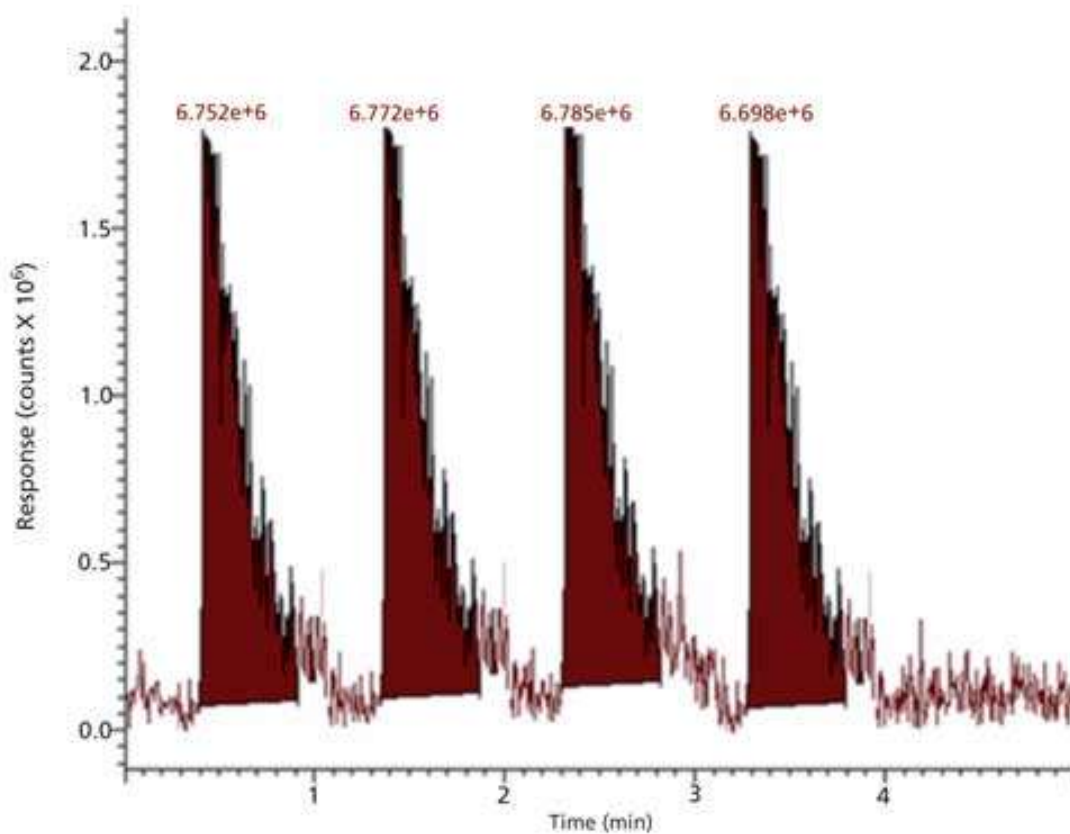


Figure 6: Intensity of HMTBA-(Lys)<sub>2</sub> ion  $m/z$  407 in reaction media after a 10-min incubation with active trypsin

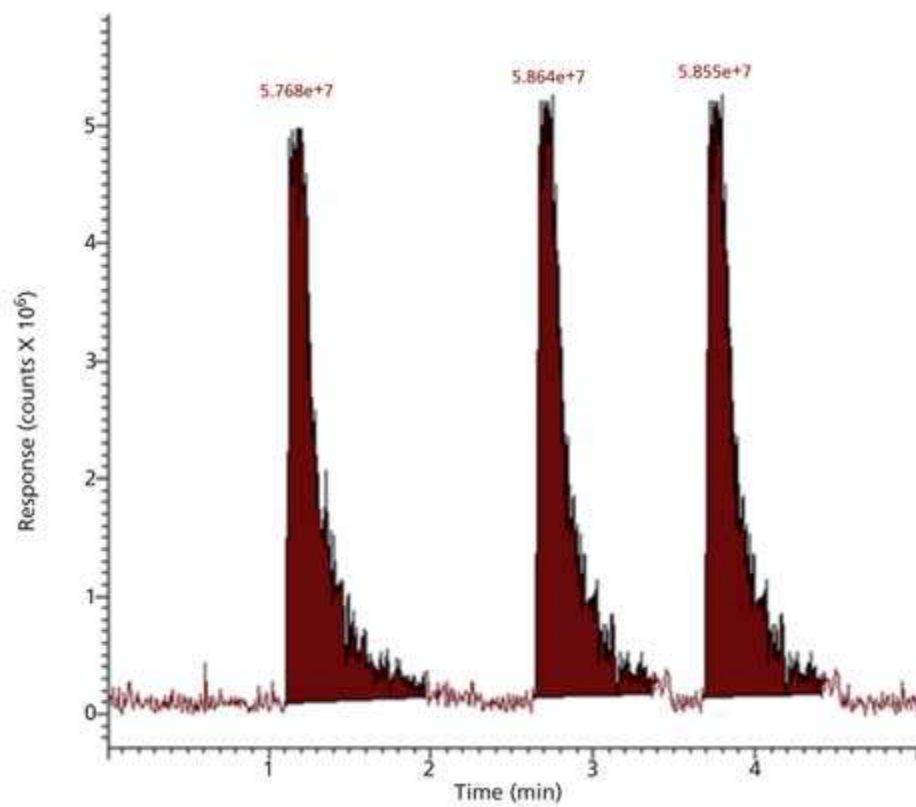


Figure 7: Intensity of HMTBA-(Lys)<sub>2</sub> ion m/z 407 in reaction media after a 10-min incubation with trypsin and Jatropha meal extract.



Table 1:Comparative results for TI

Sample	TI ( $\mu\text{g/g}$ ) AACC 22- 40.01	TI ( $\mu\text{g/g}$ ) Peptide ESI- MS	Rel. percent dev. Six replicate standard method	Rel. percent dev. Six replicate peptide ESI-MS
Defatted Jatropha meal (untreated)	33.8	30.0	6.5	7.2
Defatted soybean (untreated)	34.4	30.0	6.8	6.5
Defatted Jatropha meal(Heat treated)	<2.0	1.4	22.1	12.8
Defatted soy meal(Heat treated)	<2	1.1	27.6	11.4

## Conclusions

The results of this short set of experiments clearly demonstrate that rapid, specific and accurate determination of TI is feasible with ESI-MS, the approach does not suffer from non-linearity issues which mandate careful control of all experimental parameters in absorption based determinations. However, collaborative multi laboratory efforts are required before the ESI-MS based approach can be fully validated.

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## **II. Studies on Oxidation Stability of Natural Esters for Transformers and Similar Electrical Equipment**

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## ABSTRACT

This article describes specifications and test methods for unused natural esters in transformers and similar oil impregnated electrical equipment in which a liquid is required as an insulating and heat transfer medium. These specifications have been adopted in the new IEC standard [IEC 62770]. In the IEC standard, the term “natural esters” applies to insulating fluids for transformers and similar electrical equipment with suitable biodegradability and environmental compatibility. Such natural esters are vegetable oils obtained from seeds and oils obtained from other suitable biological materials. Esters are comprised of triglycerides and as such their chemical composition differ significantly from insulating mineral oils and other insulating fluids such as the synthetic esters or silicones fluids that have high fire points. Because of different chemical composition, these liquids are prone to oxidation and therefore use of natural ester is not recommended for electrical equipment that is open to atmosphere. IEC standard also incorporates a recommended test procedure for establishing oxidation of natural ester liquids for electrical applications. The recommended oxidation stability test was adopted after a series of Round Robins Tests performed by fourteen laboratories around the world.

Index Terms – Natural esters, transformers, oxidation stability.

## INTRODUCTION

Because of their higher fire points and better environmental compatibility relative to petroleum derived insulating mineral oil, the use of vegetable oils and other natural esters is on the rise as insulating and heat transfer fluids in electrical devices such as transformers [1-3]. Due to interest in use of natural esters as insulating liquids, IEC established a new project team (PT-5) under the Technical Committee – 10 (TC-10) to set performance criteria for unused natural esters earmarked for electrical applications. PT-5 efforts over a period of six years resulted in the IEC standard for natural ester, IEC 62770. This standard sets performance criteria for unused natural esters earmarked for electrical applications. However, the standard clearly states that the use of natural esters is recommended only for equipment that is not open to the atmosphere, e.g. sealed transformers and reactors because these fluids are prone to rapid oxidation especially under oxic environments [4-6].

In the standard, the term “natural esters” refers to insulating fluids for transformers and similar electrical equipment with suitable biodegradability and environmental compatibility. Such natural esters are vegetable oils obtained from seeds and oils obtained from other suitable biological materials and delivered to an agreed point, at a set time period. These oils are comprised of triglycerides. Natural esters with additives are within the scope of the standard. Because of different chemical composition, natural esters differ from insulating mineral oils and other insulating fluids that have high fire points, such as synthetic esters or silicone fluids. Natural ester-derived insulating fluids with low viscosity have been introduced but are not covered by this standard. Pertinent properties of such fluids are given in section 5. Development of the standard started with first meeting of PT-5 that was held on May 16, 2007 in Rome, Italy. During the meeting members acknowledge

the existence of ASTM Standard D6871-03: “Specification for Natural (Vegetable Oil) Ester Fluids Used in Electrical Apparatus. A review of D6871-03 showed that the standard did not address two very important aspects related to the use of natural esters i.e. the oxidation stability and biodegradation. It should be noted that ASTM D6871-03 was reapproved in 2008 without any change (7). It was also realized that there were no IEC or other internationally recognized test methods for ascertaining oxidation stability of unused natural esters for use in transformers and similar electrical equipment. However, members were aware of ongoing efforts being made by ASTM and the CIGRE working group D1 aimed at developing oxidation stability test methods for natural esters and synthetic esters, but, no standard protocols to assess oxidation stability of natural esters were available.

As a result, establishing a suitable oxidation stability test method for the unused natural esters became the focus of PT-5 and considerable diligent efforts were put in by members in establishing and validating the oxidation stability test protocol for these liquids. These efforts involved four round robin tests (RRTs) participated by eleven laboratories from seven different countries. PT-5 members decided to establish oxidation stability parameters on the basis of the existing IEC oxidation stability standard for mineral insulating oil i.e. IEC 61125 ED. 1.0 B: 1992 unused hydrocarbon based insulating liquids - Test methods for evaluating the oxidation stability (8). IEC 61125 method C was deemed the most suitable oxidation stability test method for comparing and evaluating different natural esters insulating fluids. This method describes a test for evaluating the oxidation stability of unused hydrocarbon based insulating liquids under accelerated conditions with or without the presence of antioxidant additives.

## 2.EXPERIMENTAL PROCEDURES

### 2.1. Round Robin Test – I

Aliquots of natural ester samples from three suppliers were shipped to the participating laboratories. Laboratories were directed to follow procedure outlined in IEC 61125 method C. Laboratories were requested to determine viscosity, soluble acidity and dielectric dissipation factor ( $\text{Tan } \delta$ ) of unused natural esters prior to oxidation stability test.

For oxidation stability test  $25 \pm 0.5$  gram aliquots of unused natural esters samples were put in suitable borosilicate glass test tubes and coiled clean solid soft copper wire (1-2 mm thickness and surface area of  $28.6 \text{ cm}^2 \pm 0.3 \text{ cm}^2$ ) was inserted in each test tube as the oxidation catalyst. The tubes were transferred to a thermostatically controlled aluminum alloy block maintained at  $120 \pm 0.5$  °C. Clean dry air was made to flow through the samples at  $0.15 \text{ L h}^{-1}$ , air flow rate was checked periodically and adjusted when required. Oxidation under these conditions was allowed to proceed for 164 hours. After the 164 hours' oxidation, natural esters samples were tested for gel formation, viscosity, soluble acidity and dielectric dissipation factor ( $\text{Tan } \delta$ ). One laboratory also evaluated fatty acid composition of the unused natural esters and natural esters after the oxidation stability test. For these analysis, natural esters were trans-esterified with methanol in the presence of a base as the catalyst to yield fatty acid methyl esters which were analyzed with a gas chromatograph interfaced to a mass spectrometer.

### 2.2. Round Robin Test – II

These tests were carried out with the natural ester samples with the same amount of samples, catalyst and air flow rate. However, the oxidation temperature was maintained at  $100 \pm 0.5$  °C. Oxidation was allowed to proceed for 164 hours. After which oils samples



were tested for gel formation, viscosity, soluble acidity and dielectric dissipation factor (Tan  $\delta$ ).

### **2.3. Round Robin Test – III**

These tests were carried out in a manner similar to the one described in section 2.1, however, the duration of test was limited to the induction period that resulted in volatile acidity of 0.1 mg KOH g<sup>-1</sup>. Oxidized natural esters samples were tested for gel formation, viscosity, soluble acidity, and dielectric dissipation factor (Tan  $\delta$ ).

### **2.4. Round Robin Test – IV**

These tests were carried out in a manner similar to the one described in section 2.1, however, the duration of test was limited to 48 hours. After which natural esters samples were tested for gel formation, viscosity, soluble acidity and dielectric dissipation factor (Tan  $\delta$ ).

**2.5. Viscosity determination** Viscosity of unused and oxidized samples were measured at 40 °C in accordance with ISO 3104 [9].

### **2.6. Dielectric dissipation factor (DDF)**

DDF of the unused and oxidized natural ester samples was measured in accordance with IEC 60247 at 90 °C. [10].

### **2.7. Acidity**

Soluble acidity of the unused and oxidized natural esters samples was measured in accordance with IEC 62021-3 [11].

The chemical nomenclature and scientific notations used in the standard and this article are in accordance with the IUPAC Handbook - Quantities, Units and Symbols in Physical Chemistry [12].

### 3. RESULTS AND DISCUSSION

#### 3.1. Salient properties of unused natural esters liquids

##### 3.1.1. Viscosity

A good agreement was obtained in viscosities measured at 40 °C by the participating laboratories for the three unused natural ester samples. Viscosity measurements obtained by the participating laboratories are summarized in Table – I.

Table I: Viscosity of the unused natural esters samples

Natural ester	Natural esters A	Natural esters B	Natural esters C
Aver. Viscosity	37.06	41.21	33.81
Std. deviation	0.14	0.13	0.08
Per. rel. deviation	0.38	0.31	0.24

Measured viscosity values were in good agreement, relative standard deviation was less than 0.5%.

##### 3.1.2. Dielectric dissipation factor (Tan $\delta$ )

Dielectric dissipation factor (Tan  $\delta$ ) of three unused natural ester samples was determined by all participating laboratories at 90 °C, results are summarized in table II.

A good agreement was found in the measured values obtained by participating laboratories, relative percent deviation for the samples ranged between 2.2 – 6.7.

Table II: Tan  $\delta$  of the unused natural esters samples

Natural ester	Natural esters A	Natural esters B	Natural esters C
Ave. Tan $\delta$	0.034	0.010	0.021
Std. Deviation	0.002	0.000	0.000
Per. rel. deviation	6.7	4.5	2.2

### 3.1.3. Soluble Acidity

Soluble acidity of unused natural esters samples was measured by participating laboratories and the results are summarized in Table-III.

Table III: Soluble acidity of the unused natural esters samples

Natural ester	Natural esters A	Natural esters B	Natural esters C
Ave acidity [mg KOH g <sup>-1</sup> liquid]	0.043	0.046	0.037
Std. deviation	0.004	0.003	0.004
Per. rel. deviation	9.49	7.10	10.06

Ideally soluble acidity of insulating liquids should be zero, however, natural esters contain very low concentrations of free fatty acids; presence of free fatty acids can affect acidity of natural esters. A good agreement was found between values reported by different laboratories.

### 3.1.4. Fatty Acid Composition of natural ester samples

One of the participating laboratories determined fatty acid composition of the unused natural ester samples through trans esterification of natural esters with methanol followed by GC-MS characterization of the fatty acid methyl esters. A total ion chromatogram (TIC) obtained for one of the unused natural ester sample is shown in Figure 1. Major fatty acid methyl esters obtained from the sample were identified through retention time matching with fatty acid methyl ester standards and electron ionization spectra of the peaks observed in the sample TIC.

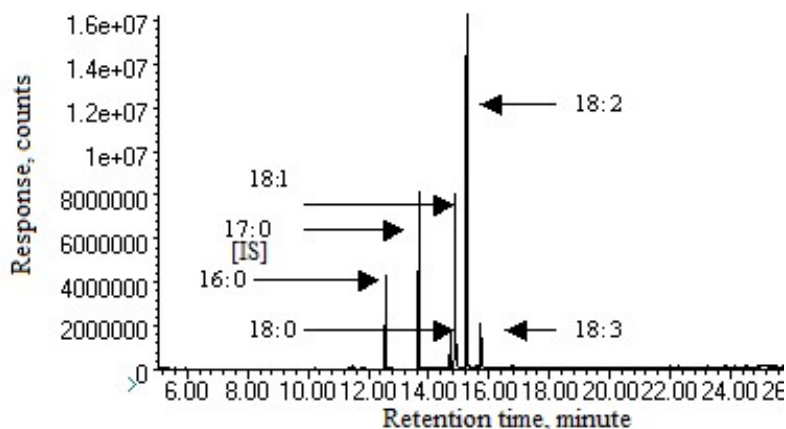


Figure 1: TIC showing the separation of fatty acid methyl esters obtained from natural esters sample C.

All natural esters samples were found to contain palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) i.e. fatty acids with sixteen and eighteen carbon chains with zero to three double bonds. Margoric acid (C-17:0) methyl ester was added as an internal standard. Approximate percent fatty acid compositions of the three natural esters samples is given in Table IV.

### 3.2. Salient properties of natural ester samples after oxidation at 120 °C for 164 hours

Oxidation of natural esters samples at 120 °C for 164 hours brought about a noticeable change in physical appearance of the samples. Color of the samples turned to deep yellow with noticeable gel formation. The gel was found to be soluble in hexane and thus could be classified as sludge as defined in IEC 61125. Specific properties were measured and results presented below.

Table IV: Approximate fatty acid composition of unused natural esters samples

Natural esters	Percent Fatty Acid				
	C16:0	C18:0	C18:1	C18:2	C18:3
A	9.5	5	77.5	8	0
B	8.9	2.0	48.6	26.3	14.2
C	16.3	7.7	20.3	45.5	10.2

### 3.2.1 Viscosity

Viscosity measurements obtained for the oxidized natural esters samples are summarized in Table – V.

Table – V  
Viscosity of the oxidized natural esters samples

Natural esters	Natural esters A	Natural esters B	Natural esters C
Aver. Viscosity	74.4	62.7	92.9
Std. deviation	3.45	28.9	2.8
Per. rel. deviation	4.6	46.1	3.0

Results showed a marked increase in viscosity of all natural esters samples. The increase in viscosity varied between 200 - 300%. Reproducibility (R) of measured values for one of samples was poor with percent relative deviation of 46%. The large percent relative deviation most likely resulted from gel formation which affected sample homogeneity.

### 3.2.2 Dielectric dissipation factor (Tan $\delta$ )

Dissipation factor for three natural esters samples after oxidation was found to be twenty to sixty times higher than the Tan  $\delta$  of the unused natural esters, Table VI.

Table VI-Tan  $\delta$  of the unused natural esters samples

Natural esters	Natural esters A	Natural esters B	Natural esters C
Ave. Tan $\delta$	0.79	0.60	0.69
Std. Deviation	0.075	0.011	0.038
Per. Rel. Deviation	9.5	1.8	5.5

### 3.2.3 Soluble Acidity

Soluble acidity of unused natural ester samples after oxidation was measured by participating laboratories and the results are summarized in Table VII. Results showed that soluble acidity of the oxidized natural esters samples was nearly 40 times higher than the soluble acidity of the unused natural esters sample.

Table VII: Soluble acidity of the oxidized natural esters samples

Natural ester	Natural esters A	Natural esters B	Natural esters C
Ave acidity [mg KOH g <sup>-1</sup> liquid]	2.09	1.37	1.75
Std. Deviation	0.51	0.14	0.21
Per. Rel. Deviation	24.4	10.2	12

The results clearly showed the severity of oxidation of oil at 120 °C for 164 hours. The severity was also observed in the GC-MS data. The TIC of the fatty acid methyl esters obtained from an oxidized natural esters sample showed obvious changes in the fatty acid

composition of the natural esters during oxidation at 120 °C. GC - MS data showed that polyunsaturated fatty acid degraded more rapidly than the monounsaturated and the saturated fatty acids in the natural esters samples, Figure 2.

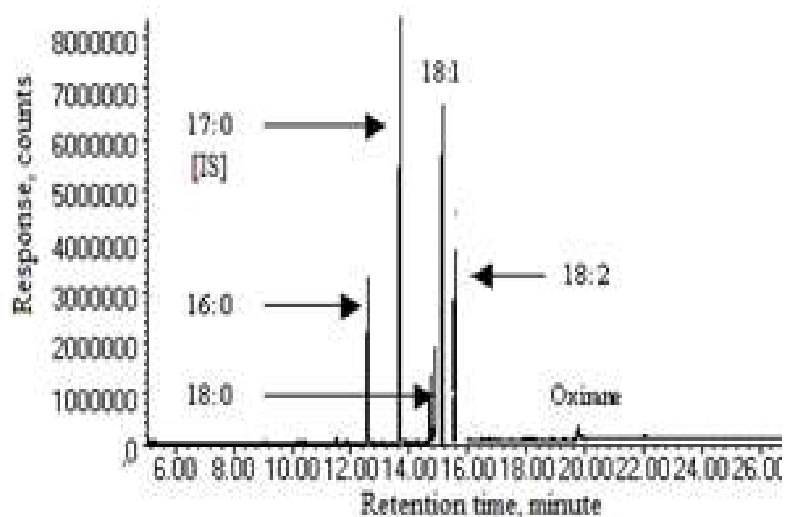


Figure 2: TIC showing the separation of fatty acid methyl esters from oxidized natural ester sample C.

These fatty acids are susceptible to oxidation and yield oxygenated products such as short chain aldehydes, ketones and acids. Decrease in C-18:2 concentration can be clearly seen in the TIC.

Results showed that the oxidation at 120 °C for 164 hours with air as the oxidizer is too harsh for natural esters. Because of their chemical composition, natural esters are more prone to oxidation than the mineral insulating oils. In absence of a standard test method, three alternative oxidation protocols based on IEC 61125 C were proposed and evaluated to determine relative oxidation stability of natural esters.



### 3.3 RRT – II Oxidation at 100 °C for 164 hours

An alternative protocol was used during the second round robin test. For this test the oxidation temperature was set at 100 °C, while the oxidation period and air flow rates were maintained at 164 hours and 0.15L h<sup>-1</sup> respectively. However, despite the lower temperature results obtained with natural ester samples were similar to those obtained after oxidation at 120 °C.

### 3.4 RRT-III oxidation at 120 °C for an induction period required for volatile acidity of 0.1mg KOH g<sup>-1</sup> sample.

Results obtained from the third-round robin test showed a wide variability. The variability in measured properties arose as a result of difficulties encountered in establishing induction period. Variability higher than 40% was observed in reported induction period values, Table VIII.

The variability in induction time determination lead to poor repeatability in measured values for viscosity, soluble acidity and dissipation factor.

Table VIII: Induction period to reach volatile acidity - 0.1mg KOH g<sup>-1</sup>

Natural esters	Natural esters A	Natural esters B	Natural esters C
Ave. induction period (h)	34.4	100.6	42.8
Std. Deviation	10.1	41.7	4.1
Per. Rel. Deviation	29.3	41.5	9.5

### 3.5 RRT – IV Oxidation at 120 °C for 48 hours

During the fourth-round robin test oxidation of natural ester samples was carried out at 120 °C but only for 48 hours. Physical examination of natural esters after oxidation showed that natural esters did not undergo change in color or produce significant amounts of gels. Properties measured after the oxidation were found to be more reproducible and repeatable and summarized below:

#### 3.5.1 Viscosity

Viscosities of the three oxidized natural esters samples measured by the participating laboratories were found to be in good agreement, the percent relative deviation of the measured values was less than 10. The measurements values are summarized in Table IX.

#### 3.5.2 Dielectric dissipation factor (Tan $\delta$ )

Dissipation factor for three natural esters samples measured after oxidation at 120 °C for 48 hours are summarized in Table X.

Table IX: Viscosity of natural esters samples after oxidation at 120 °C for 48 hours

Natural ester	Natural esters A	Natural esters B	Natural esters C
Aver. Viscosity	42.0	42.9	41.08
Std. deviation	1.07	2	2.92
Per. rel. deviation	2.55	4.6	7.1

Table X: Tan  $\delta$  of natural esters samples after oxidation at 120 °C for 48 hours.

Natural ester	Natural esters A	Natural esters B	Natural esters C
Ave. Tan $\delta$	0.058	0.214	0.109
Std. Deviation	0.027	0.044	0.046
Per. rel. deviation	46.5	21	42.2

### 3.5.3 Soluble Acidity

Soluble acidity of unused natural ester samples after oxidation at 120 °C for 48 hours was measured by participating laboratories and the results are summarized in Table -XI.

Table XI: Soluble acidity of the oxidized natural esters samples

Natural ester	Natural esters A	Natural esters B	Natural esters C
Ave acidity [mg KOH g <sup>-1</sup> liquid]	0.1	0.20	0.15
Std. Deviation	0.02	0.03	0.03
Per. Rel. Deviation	20	15	20

The values for oxidized natural esters varied from 0.1 – 0.2 mg KOH g<sup>-1</sup>, relatively large variability was observed in values reported by different laboratories. The variability could be related to gel formation. However, the overall test parameters were considered acceptable for the standard.

#### **3.5.4 Precision**

values obtained during the Round Robin Tests on commercially available natural esters after 48 h oxidation were in general agreement with values reported for mineral insulating oils in IEC 61125.

#### **3.6 Biodegradation**

It was noted that natural esters exhibit better environmental compatibility relative to petroleum-derived insulating mineral oils. However, specific tests need to be undertaken to demonstrate ready biodegradability of these fluids. Tests include OECD 301B, C or F; or US EPA – OPPTS 835.311[31].

It was noted that natural esters can be classified in accordance with IEC 61039, based on biodegradability observed with OECD 301:1992.

#### **3.7 Toxicity**

Unused natural esters are considered non-toxic and suppliers shall supply assays that define the product as non-toxic.

It was noted that toxicity of natural esters can be assessed with test methods such as a modified Ames test or other suitable internationally recognized assays such as OECD 201-203; US EPA 600/4.82.068:1983.

## **4. CLASSIFICATION, IDENTIFICATION, GENERAL DELIVERY REQUIREMENTS AND SAMPLING**

### **4.1 Classification**

For the purpose of the standard, natural esters are classified in a single class – less flammable natural esters.

It was noted that there are other natural ester derived liquids, which may have a different classification. However, these liquids are not covered by the standard, an example of such liquids is described in section 5.

## **5. SPECIFICATIONS OF LOW-VISCOSITY INSULATING FLUIDS DERIVED FROM NATURAL ESTERS**

From a chemical and biochemical point of view, the natural esters (i.e. esters that can be found in biological materials) comprise many other molecules besides the triglycerides to which the definition of natural esters in this standard refers. The triglycerides are the main constituents of vegetable oils (and animal fats) and these fluids are usually characterized by high fire point and flash point, which categorize them in class K according to IEC 61100. Compared to mineral oils, they are less flammable but they have also a higher viscosity and a higher pour point.

NOTE: According to IEC 61100, class K fluids have a fire point  $> 300$  °C (according to ISO 2592 – open cup) and a flash point  $> 250$  °C (according to ISO 2719 – closed cup)

Other insulating fluids, derived from various natural esters, have been developed for use in some electrotechnical applications (e.g. low/medium voltage power transformers with ONAN cooling). Such fluids mainly consist of fatty acid mono-esters that allow lowering the viscosity or mixtures of triglycerides and fatty acid mono-esters.

## **6. CONCLUSIONS**

The new IEC standard, highlights of which are reported in this article, establishes non-subjective criteria for unused natural esters application in transformers and similar electrical equipment. The new standard also sets criteria for oxidative stability of these fluids during laboratory studies. However, authors recognize that more appropriate stability test for these fluids which is more in agreement with the conditions of their use needs to be developed and incorporated into future IEC maintenance guide for these fluids.

## **ACKNOWLEDGMENT**

The authors acknowledge the contributions of the international experts of IEC TC 10 PT-5. This study could not have been completed without their active participation. Suppliers of natural esters are acknowledged for their contribution of the natural esters samples.

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## SECTION

### 2. CONCLUSIONS

Trypsin inhibitor is one of the well-known antinutrient present in most of the plants and their presence in food and feed formulation deteriorate the nutritional aspect of food and feed formulation. So, monitoring of trypsin inhibitor in such food and feed formulation is important especially to food and feed processors who are concerned with providing quality food for animals. In this research work, new approach for evaluation of trypsin inhibitor in food and feed formulation has been described using electrospray ionization mass spectrometry. The study also describes method for the separation of Trypsin inhibitor from feed formulation. The analysis of Trypsin inhibitor was carried out with ESI MS/MS in positive mode by observing prominent ions of unique chemical residue from the reaction mixture. This method is quick and specific having better precision and accuracy. Also, the method is resistant to interferences from presence of any microparticles that come with sample solution during extraction of trypsin inhibitor from feed and feed formulation. It has been assumed that the validation of method relies on multi-laboratory efforts to carry out the analysis and compare the results.

In the second part oxidation evaluation of commercial natural ester oils were evaluated. The natural ester oil is good alternative to mineral oil as dielectric fluids in transformers. The commercial natural ester oils as dielectric fluids have the properties that are comparable with mineral oils. The major problem of natural ester oil is the problem of oxidation. This problem has been partly eliminated by adding antioxidants. The oxidation evaluation of natural ester oil help to evaluate the stability of oil under oxidation. In this work, oxidation of different commercial oil has been evaluated by using method IEC 61125

method c. It has been found that oxidation of natural ester oil depends on the fatty acid composition. The high degree of unsaturation in the fatty acid chain make the oil more prone towards oxidation. Therefore, judicious selectin of oil is very important for transformer as dielectric fluid.

## APPENDIX

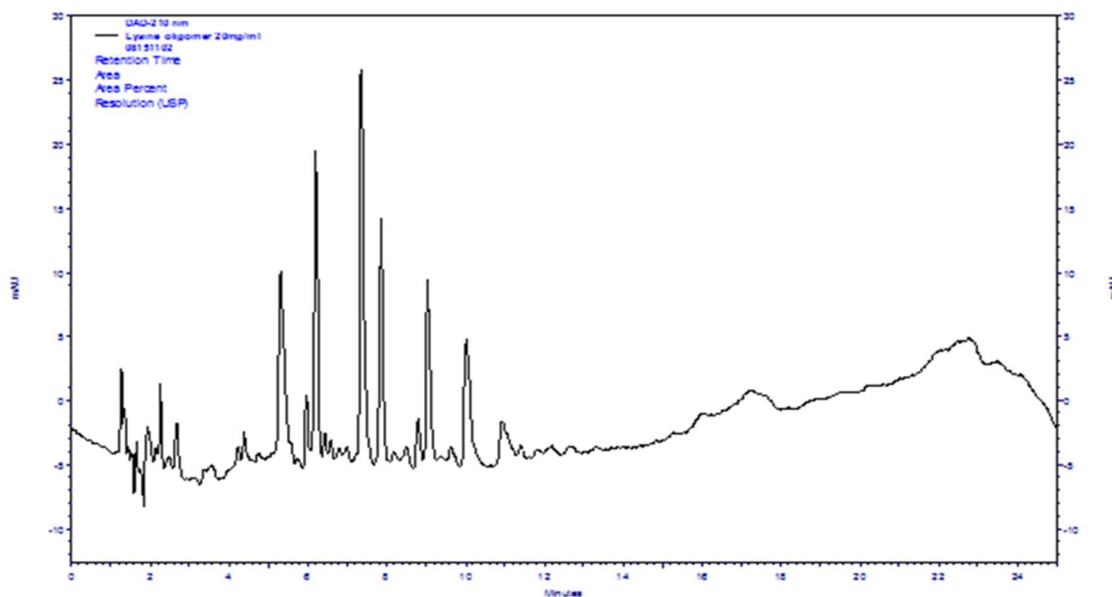


Figure : Ion pair liquid chromatographic separation of oligolysine

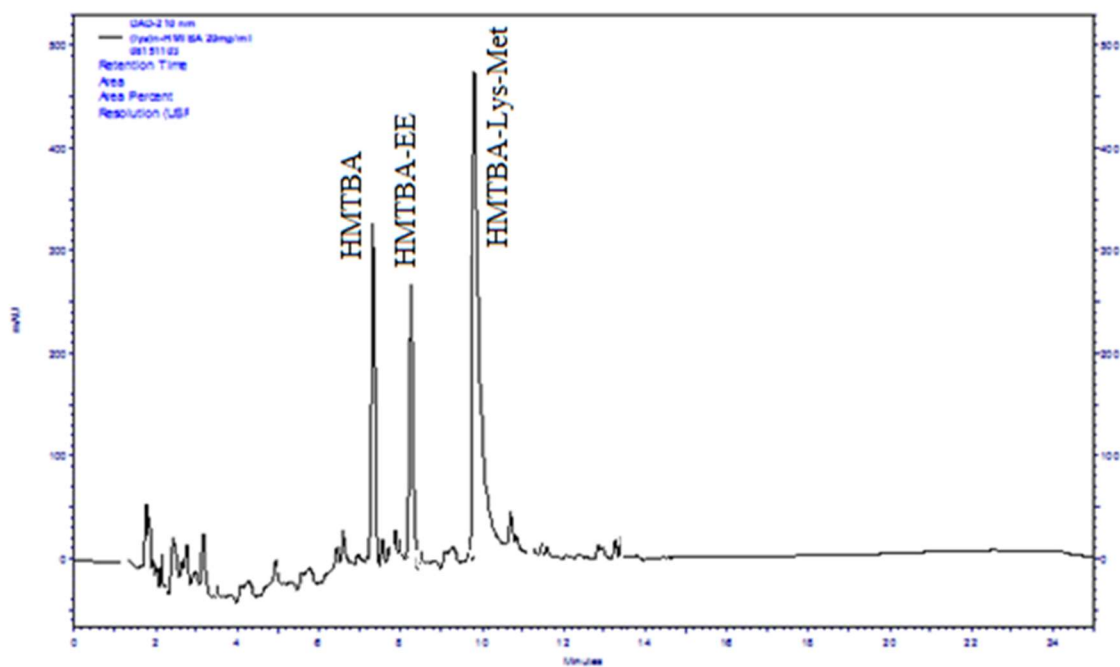


Figure: Liquid chromatographic separation of HMTBA-[Lys-Met]

Table : ESI-MS result of HMTBA oligolsysine

Oligolsysine	MW	Protonated Ion $[M+H]^+$
HMTBA-[Lys] <sub>2</sub>	406	407
HMTBA [Lys] <sub>3</sub>	534	535
HMTBA [Lys] <sub>4</sub>	662	663

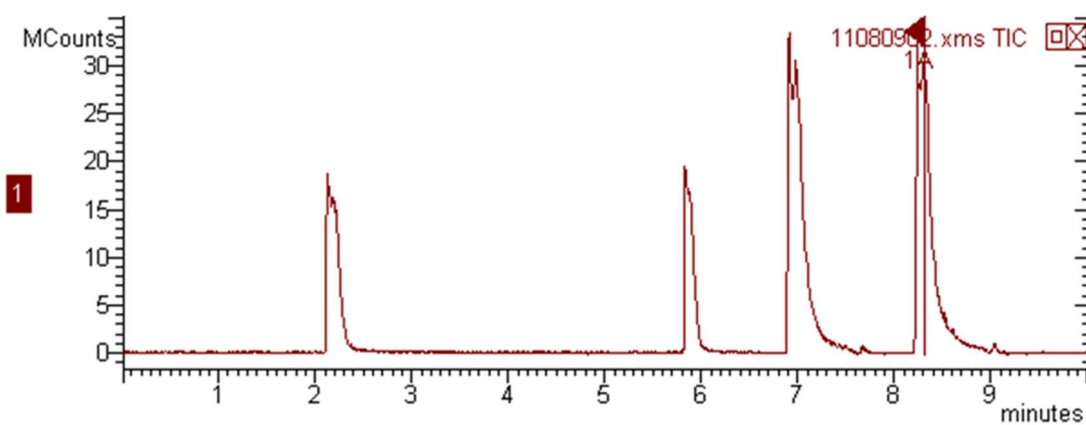
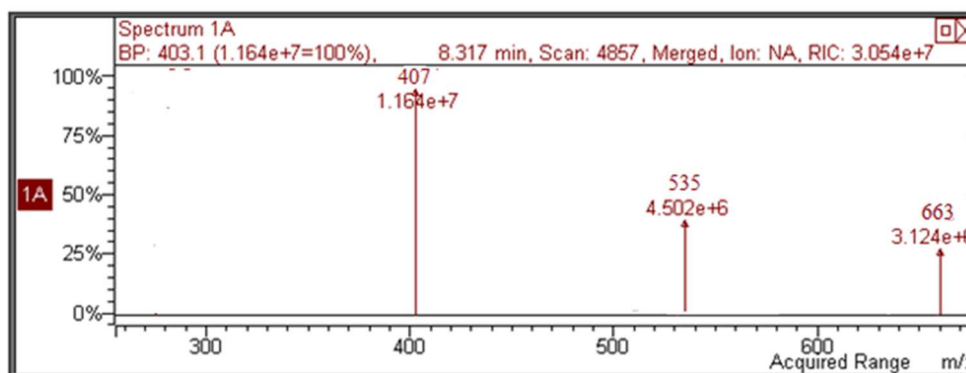
Figure : Total ion chromatogram of  $[Lys]_n$  in the positive ion mode.Figure : ESI-MS Positive Ion Spectrum of  $[Lys]_n$



Figure: oxidation stability study of oil

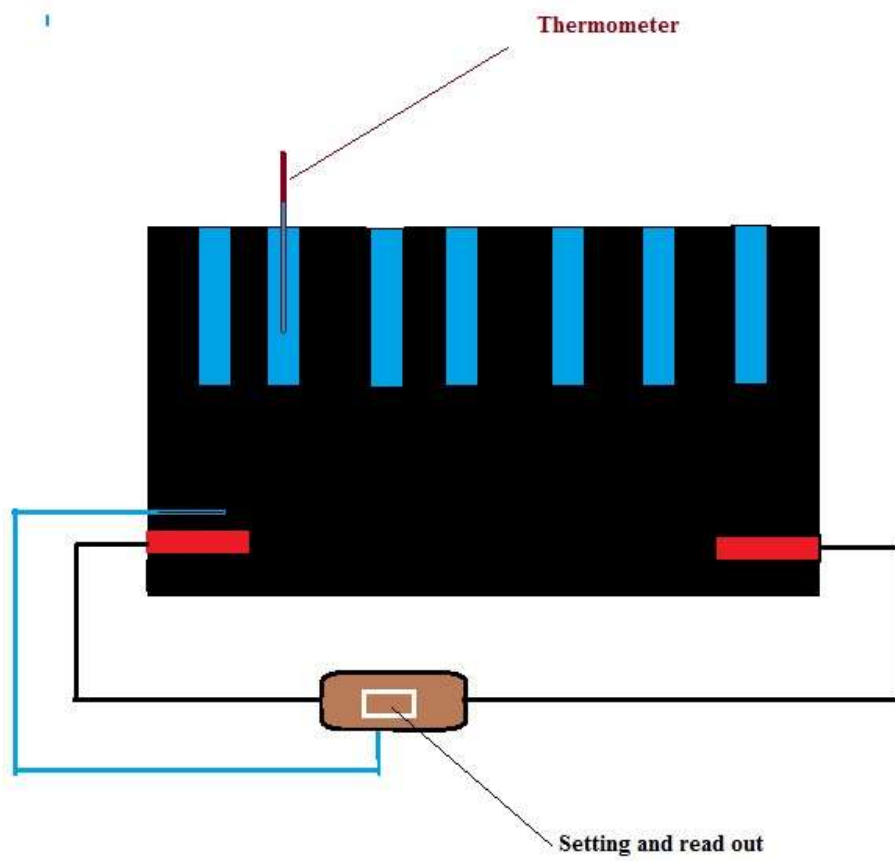


Figure 1.18: Schematic of heating block

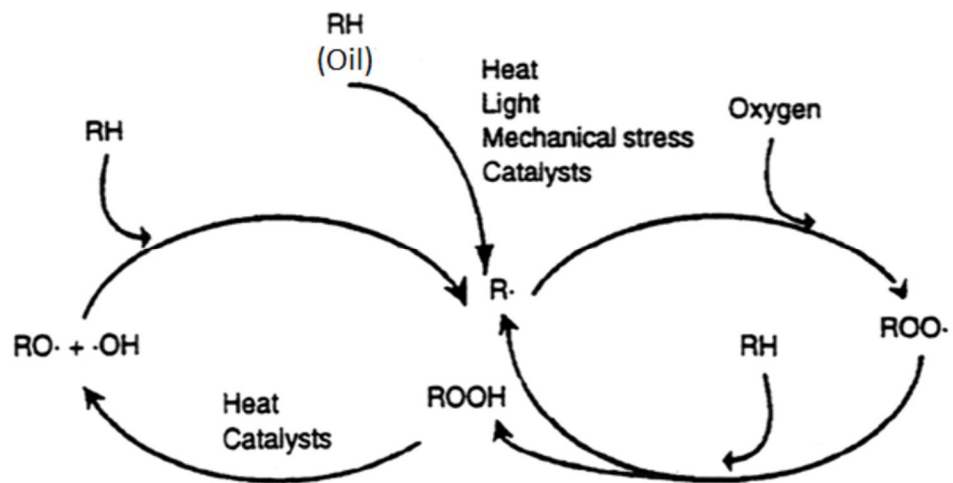


Figure: various reaction paths showing oxidation of oil

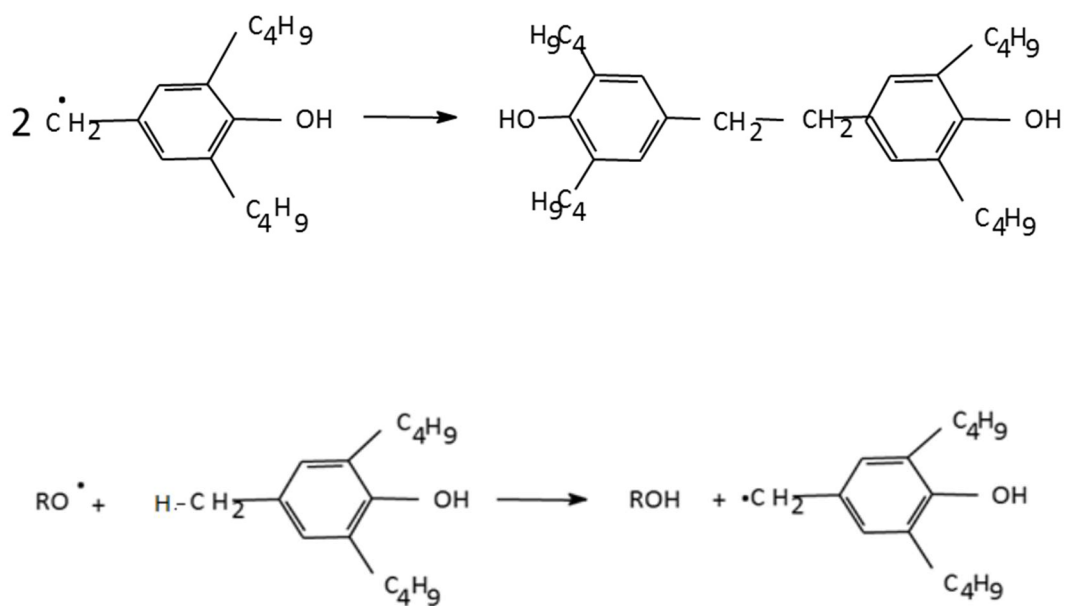


Figure : Antioxidant activity of BHT

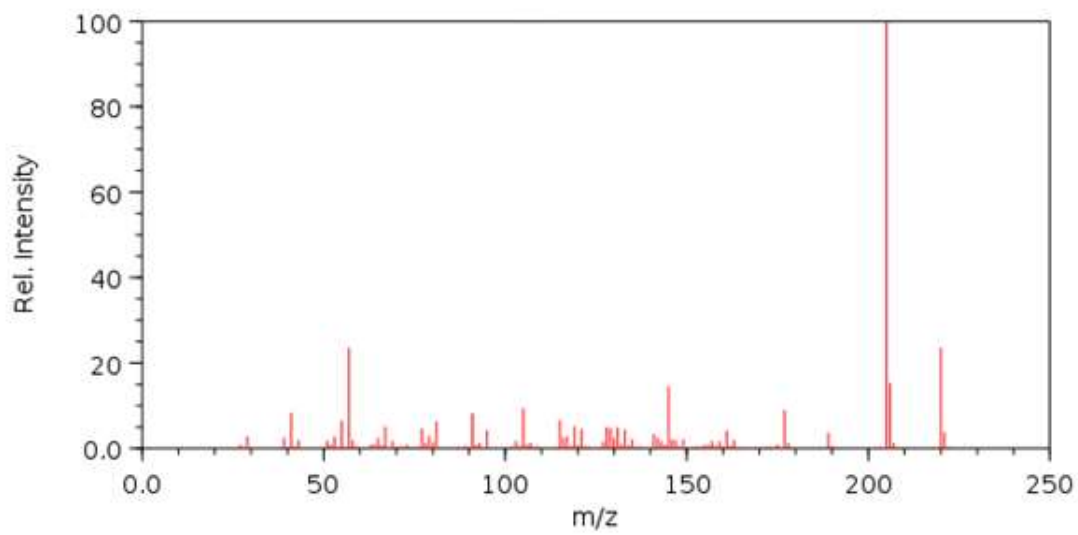


Figure: Mass spectrum of BHT

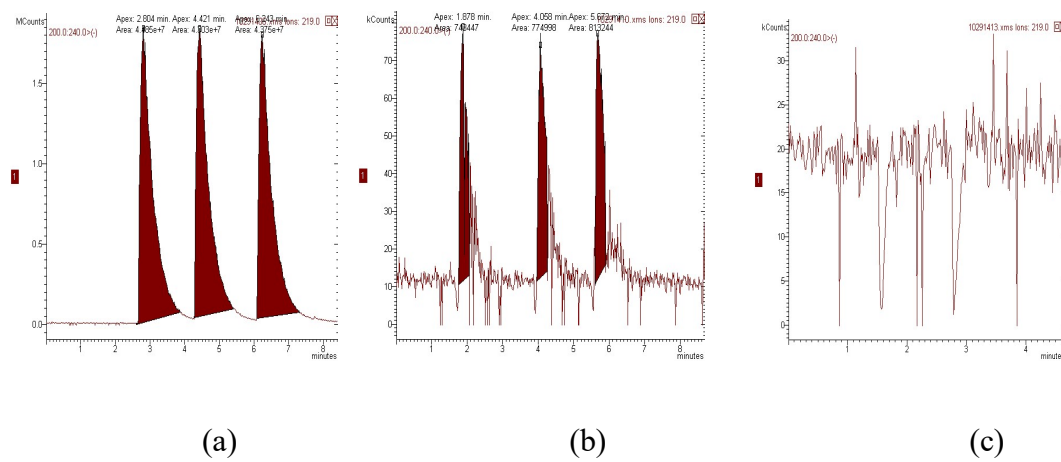


Figure: ESI-MS of (a) Unoxidized oil A BHT – ive m/z= 219, 4.485x10<sup>7</sup>; 4.303x10<sup>7</sup>; 4.375x10<sup>7</sup> (b) oil A 36 hrs oxidation; BHT – ive m/z= 219, 743447; 77998; 813244 (c) oil A 96 hrs oxidation



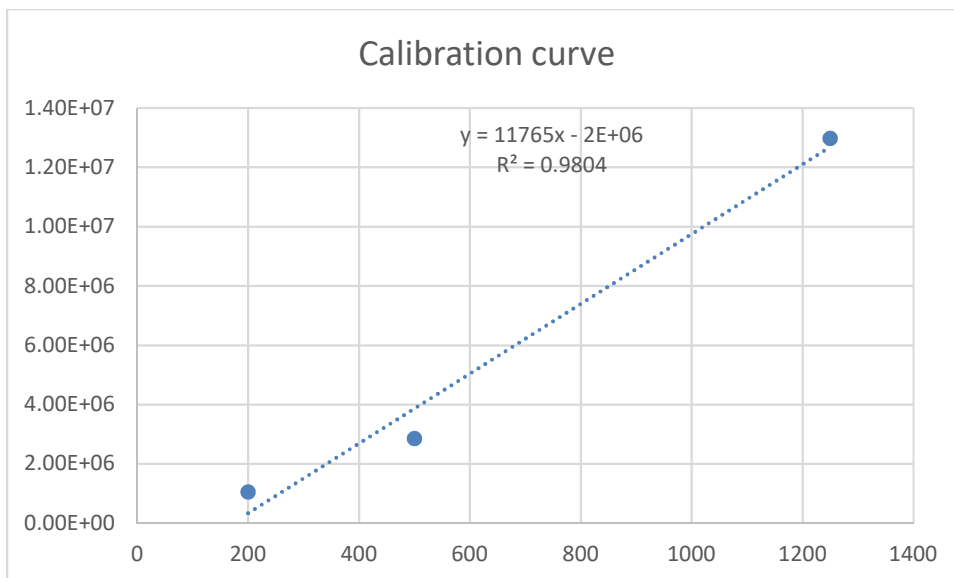
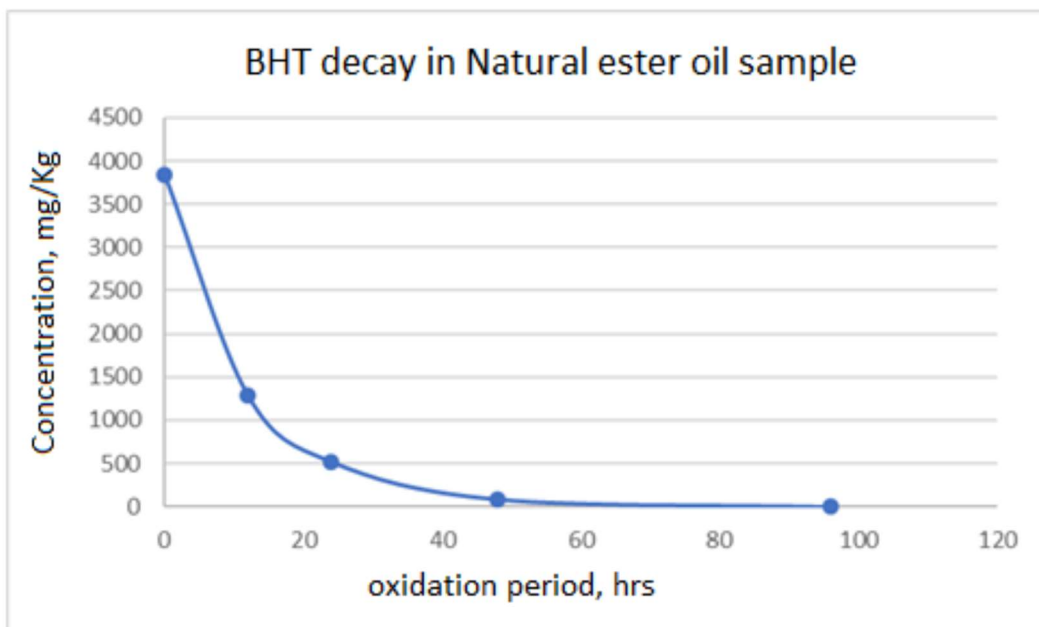


Figure: Calibration curve for antioxidant analysis



(a)

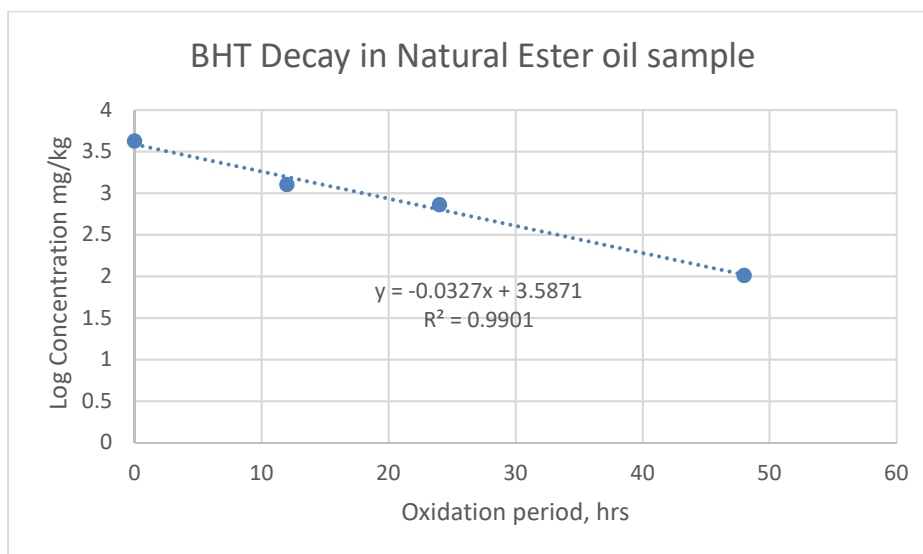


Figure: BHT decay in natural ester oil sample (a) plot of concentration vs oxidation period in hours (b) plot of Log (conc.) vs oxidation period in hours

Table: Percent fatty acid composition of commercial oil A

FAME	0 hr	12 hrs	24 hrs	36 hrs	48 hrs	96 hrs	120 hrs	164 hrs
C16:0	11.98	11.64	11.69	12.15	14.30	13.41	13.87	33.15
C18:0	5.30	5.24	5.35	5.24	5.91	5.54	5.60	15.03
C18:1	25.28	25.05	24.98	26.17	30.04	27.26	28.53	48.5
C18:2	51.11	51.36	51.26	50.15	45.25	48.27	46.87	3.22
C18:3	6.30	6.66	6.69	6.27	4.47	5.50	5.11	-

Table: Percent fatty acid composition of commercial oil B

FAME	0 hr	12 hrs	24 hrs	36 hrs	48 hrs	96 hrs	120 hrs	164 hrs
C16:0	4.24	4.39	4.31	4.41	4.73	4.41	4.50	5.42
C18:0	2.76	2.62	3.79	3.66	3.69	3.76	3.68	3.86
C18:1	87.38	86.99	86.04	86.05	85.92	86.78	87.34	86.54
C18:2	5.61	5.98	5.84	5.86	5.63	5.02	4.46	4.17
C18:3								

Table: Percent fatty acid composition of commercial oil C

FAME	0 hr	12 hrs	24 hrs	36 hrs	48 hrs	96 hrs	120 hrs	164 hrs
C16:0	5.74	6.10	6.54	5.97	6.18	6.68	6.89	9.28
C18:0	1.24	2.10	1.54	2.07	2.41	2.21	2.16	2.86
C18:1	65.96	65.75	66.66	66.28	69.19	69.10	73.98	80.13
C18:2	19.10	18.40	17.88	18.68	16.63	16.56	13.53	7.71
C18:3	7.94	7.59	7.34	6.97	5.56	5.43	3.42	-

## FAME Standard (27-58 ppm)

Chromatogram Plot

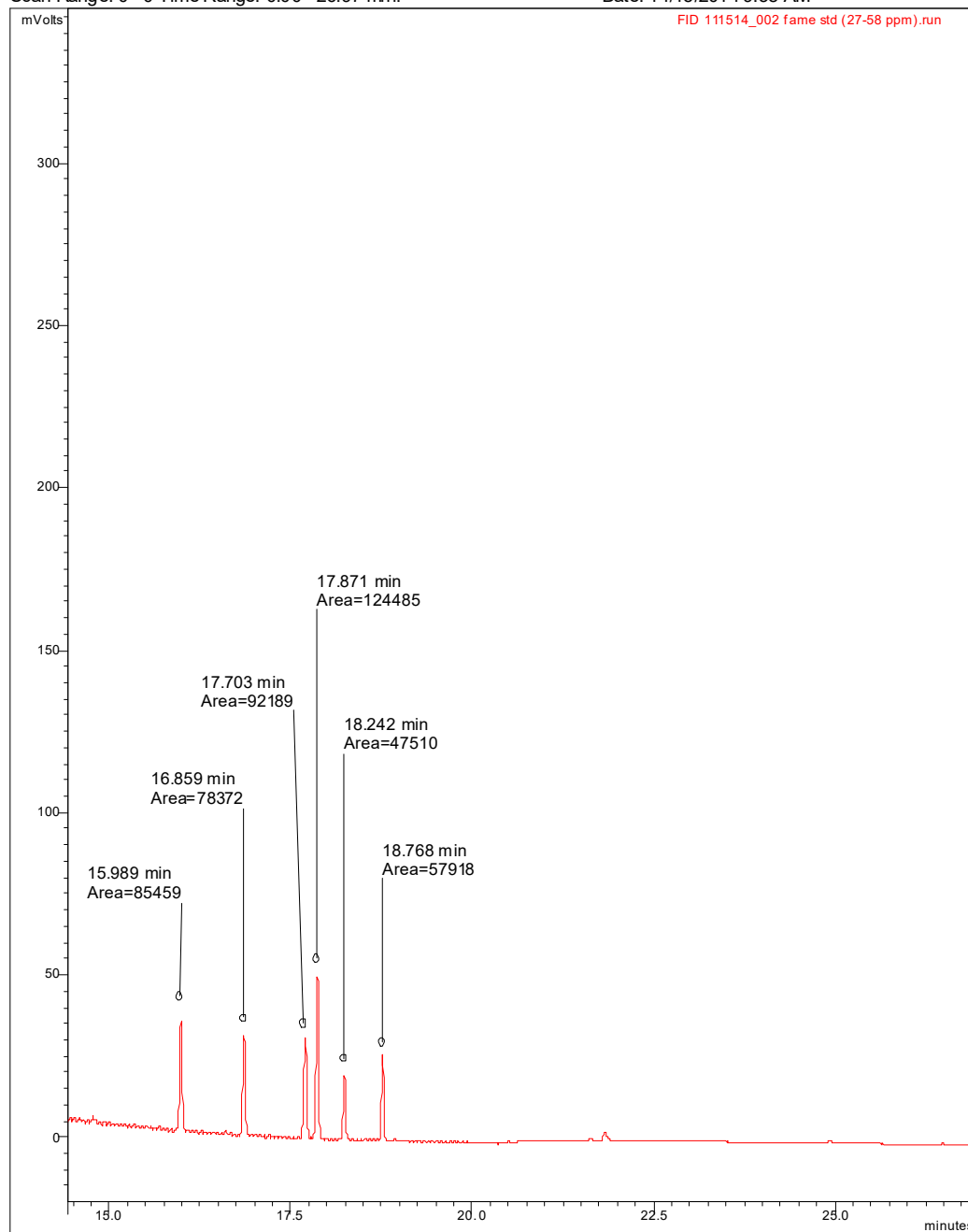
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Sample:

Operator:

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Date: 11/15/2014 9:38 AM



## FAME standard (67-145 ppm)

Chromatogram Plot

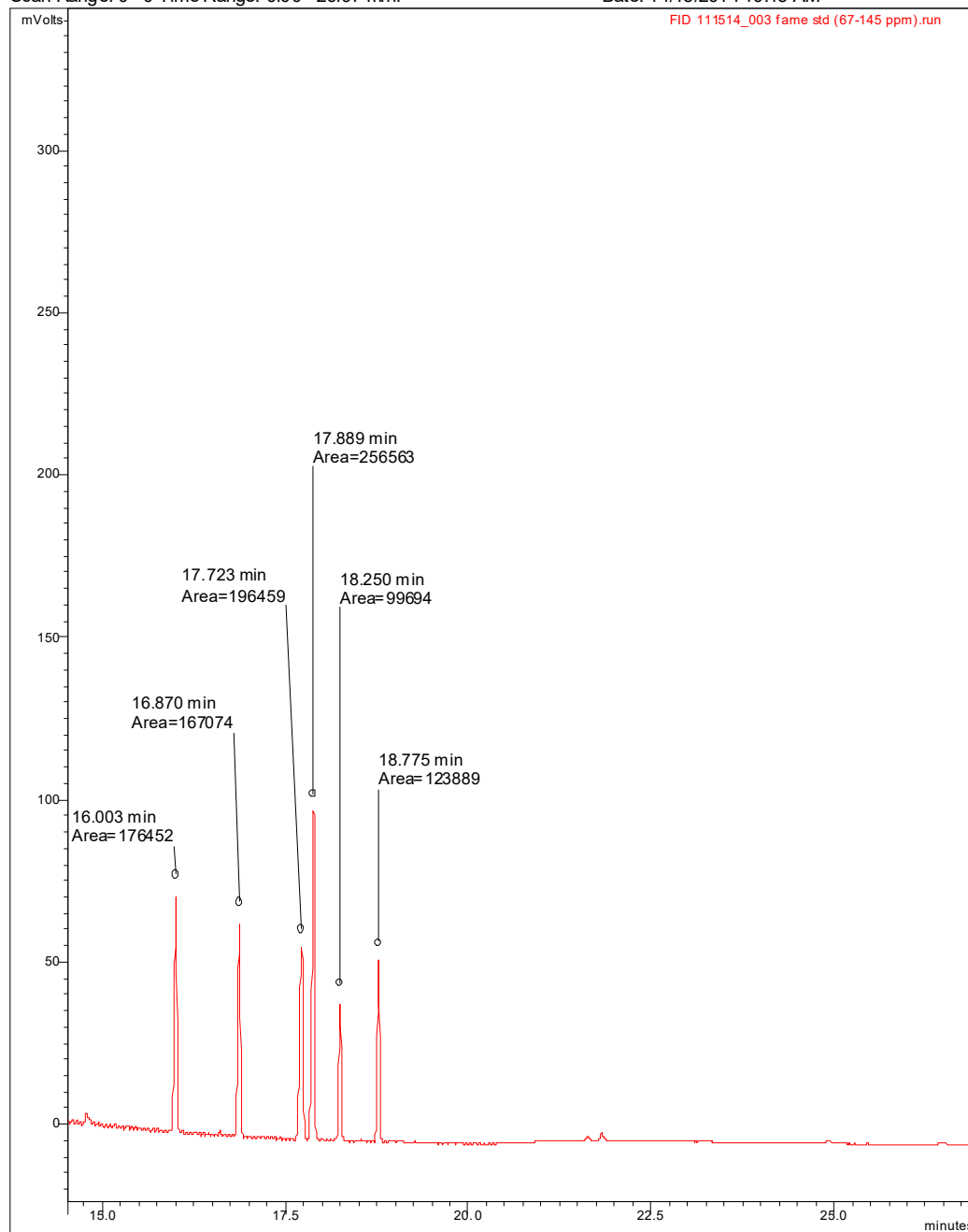
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Sample:

Operator:

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Date: 11/15/2014 10:15 AM



## FAME standard (135-290 ppm)

Chromatogram Plot

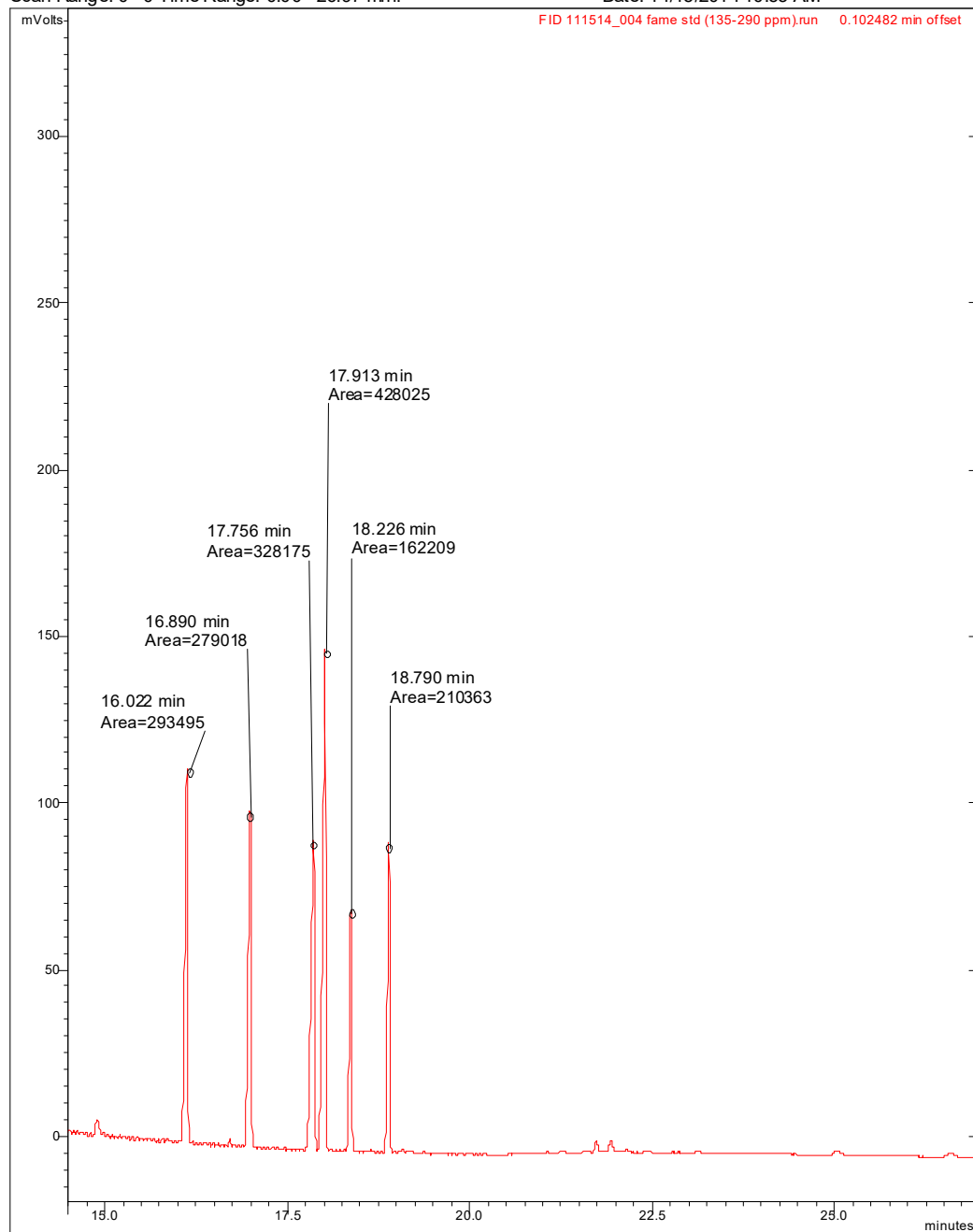
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Sample:

Operator:

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Date: 11/15/2014 10:53 AM



## FAME standard (270-580 ppm)

Chromatogram Plot

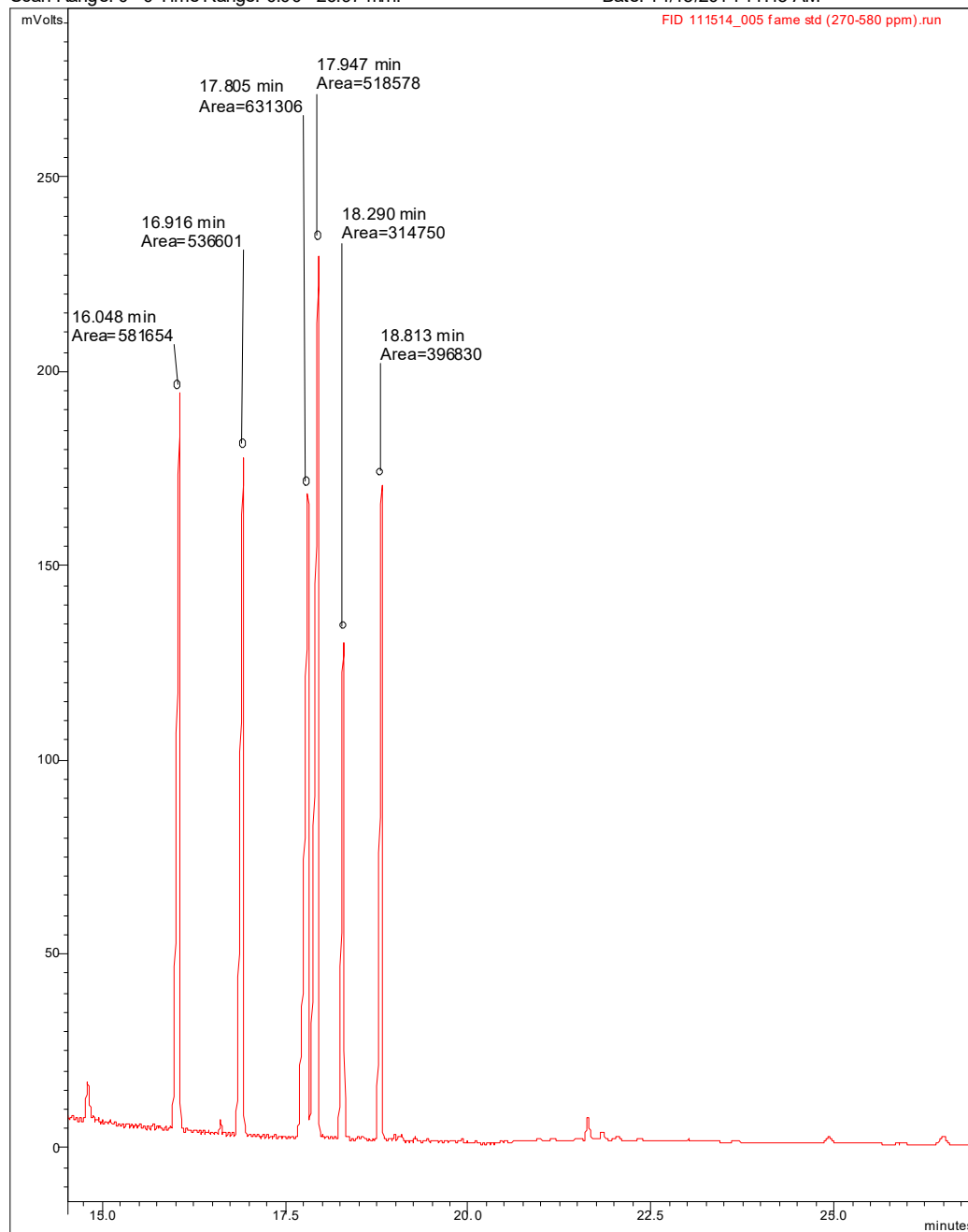
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Sample:

Operator:

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Date: 11/15/2014 11:43 AM



## FAME A (12 hrs oxidation) 1000 ppm

Chromatogram Plot

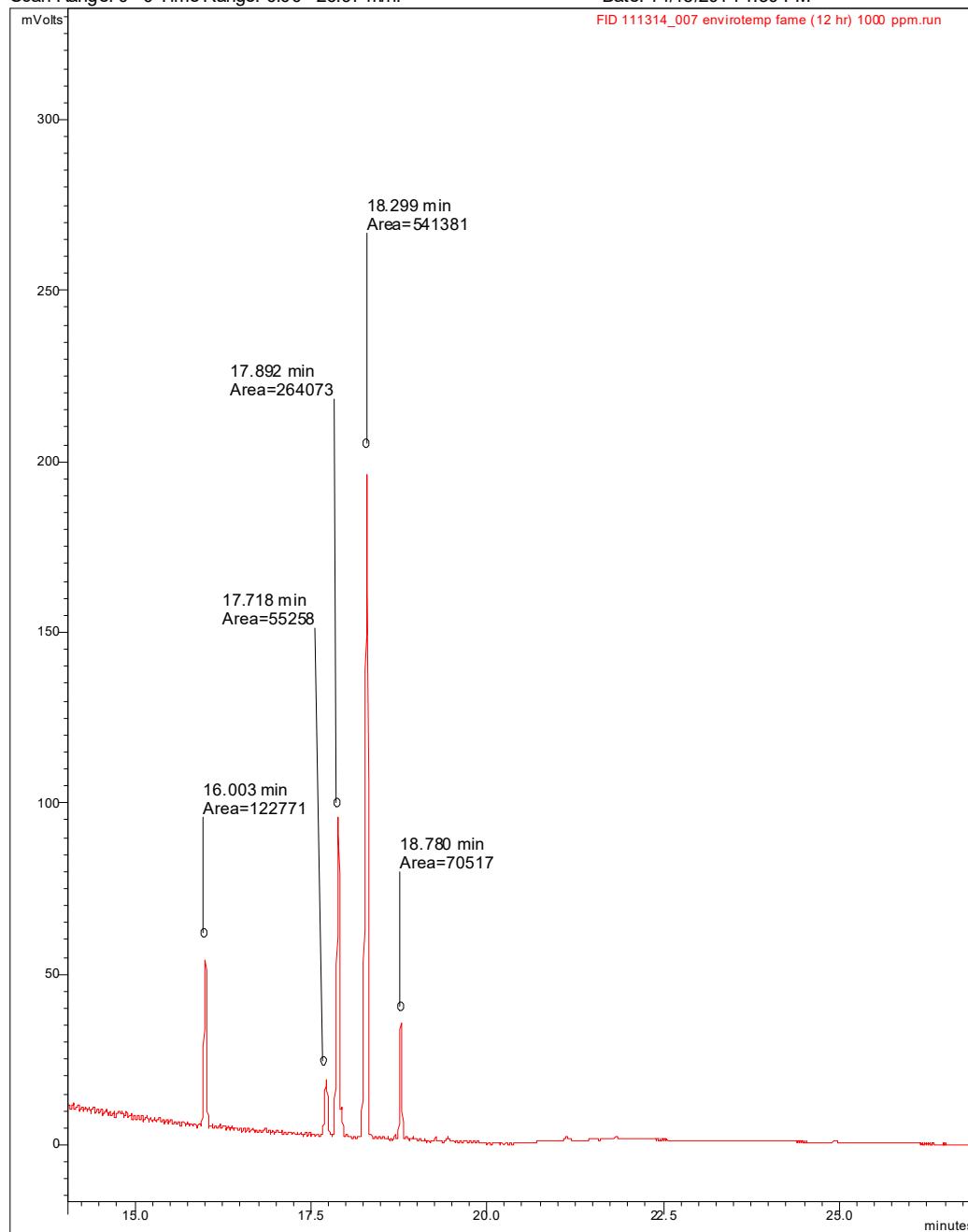
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Operator:

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Date: 11/13/2014 1:30 PM





## FAME A 1000 ppm (24 hrs oxidation)

Chromatogram Plot

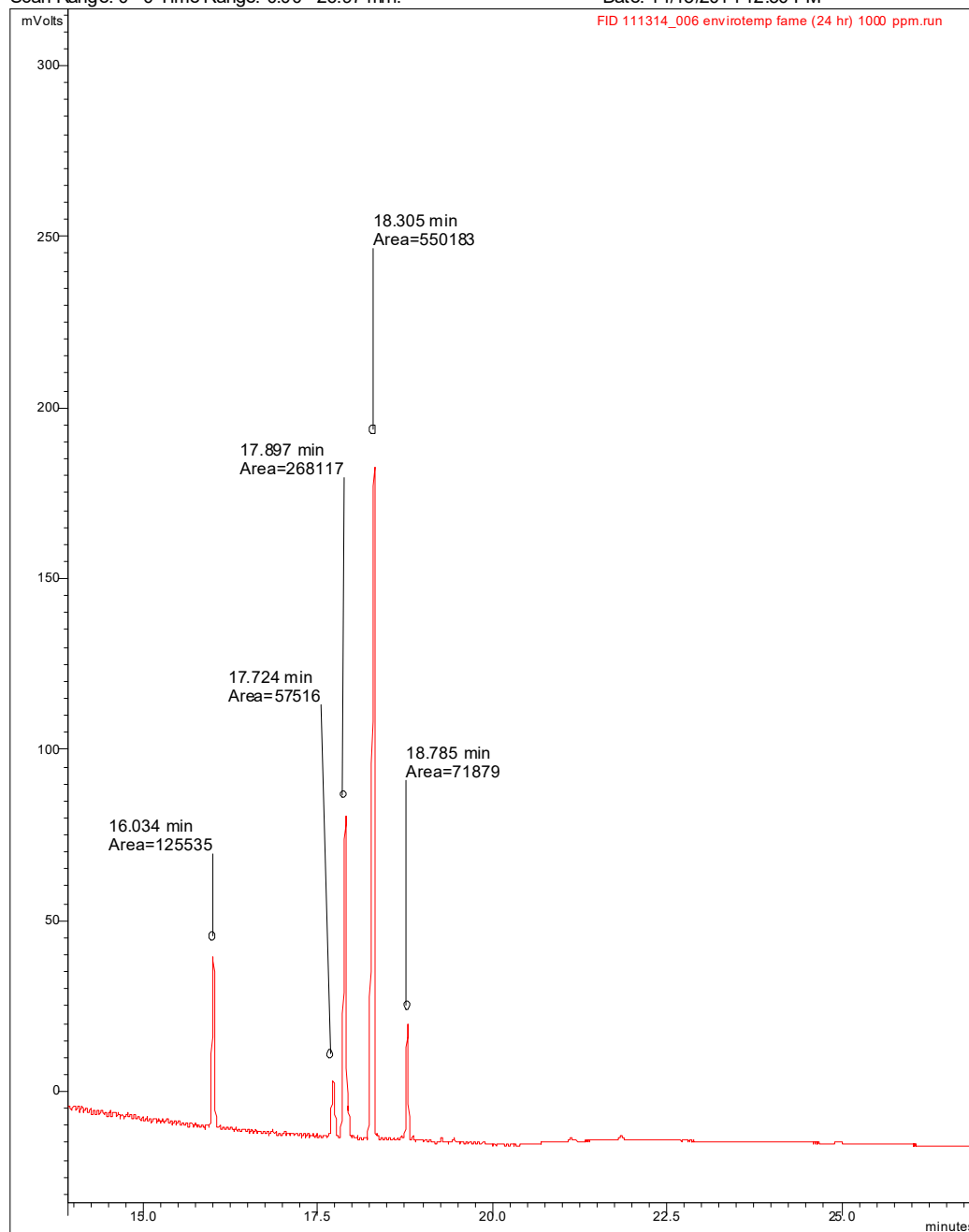
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Sample:

Operator:

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Date: 11/13/2014 12:50 PM



## FAME A 1000 ppm (36 hrs oxidation)

Chromatogram Plot

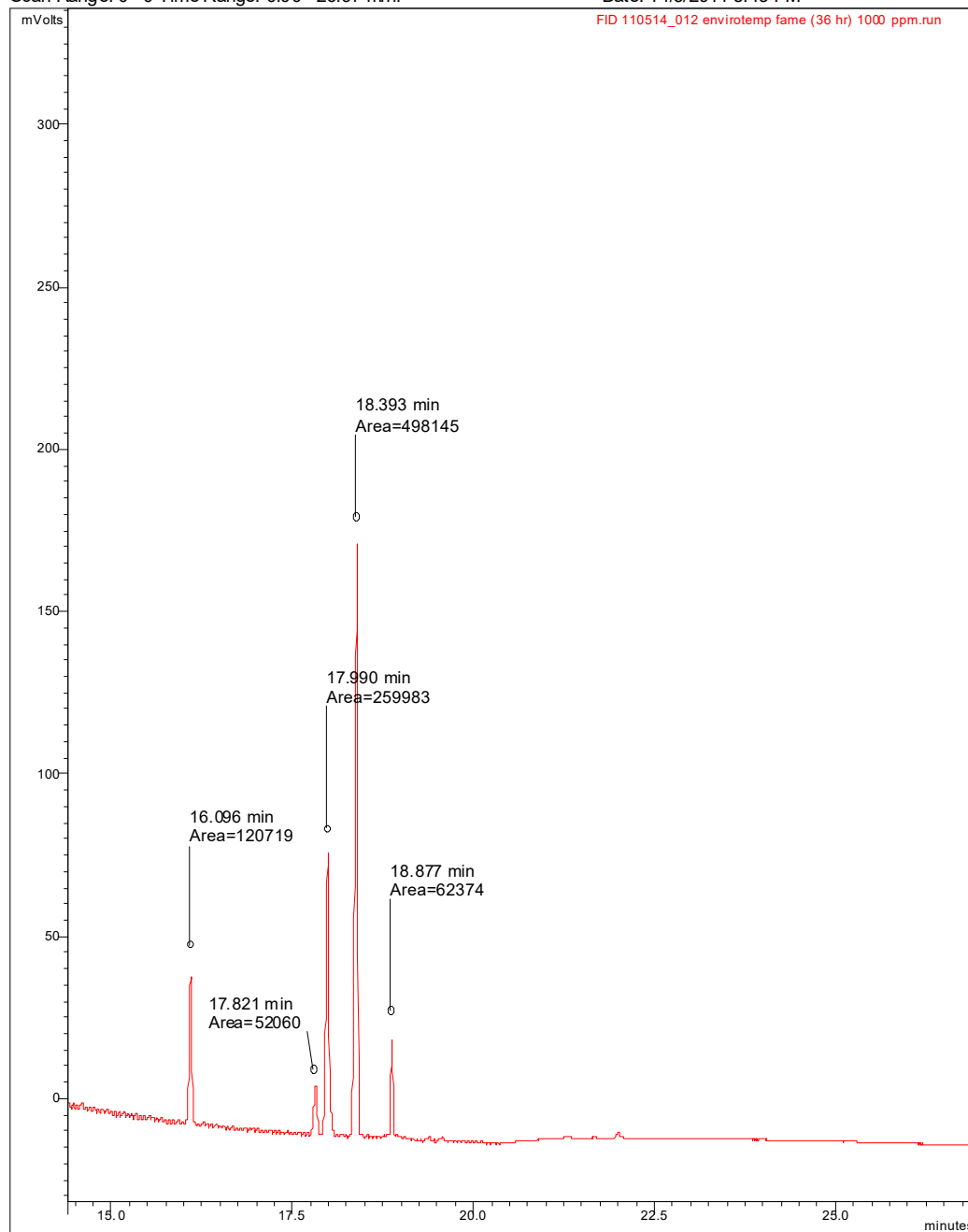
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Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/5/2014 5:43 PM



## FAME A 1000 ppm (48 hrs oxidation)

Chromatogram Plot

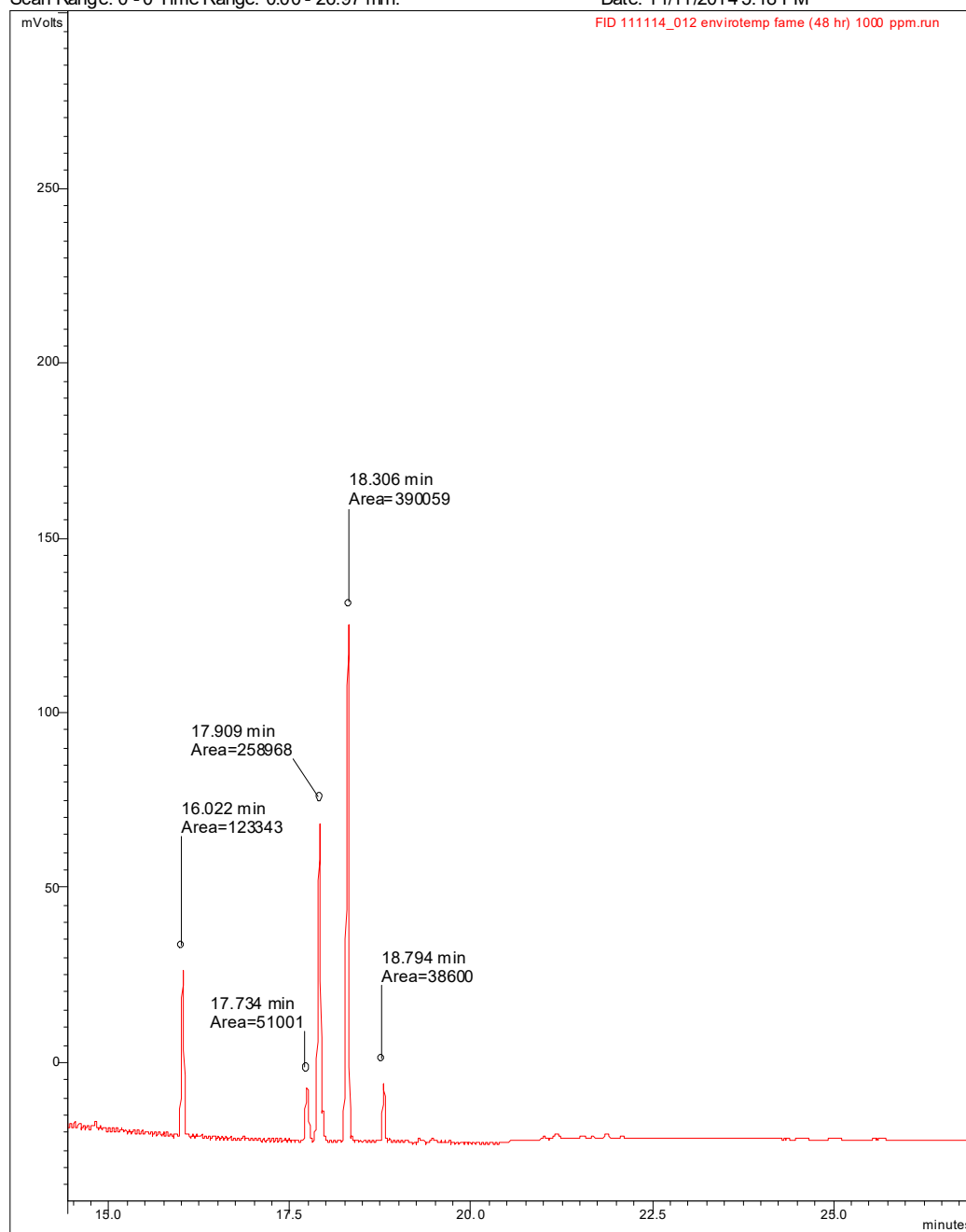
File: ...in isooctane\_032814\111114\_012 envirotemp fame (48 hr) 1000 ppm.run

Sample:

Operator:

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Date: 11/11/2014 5:18 PM



## FAME A 1000 ppm (96 hrs oxidation)

Chromatogram Plot

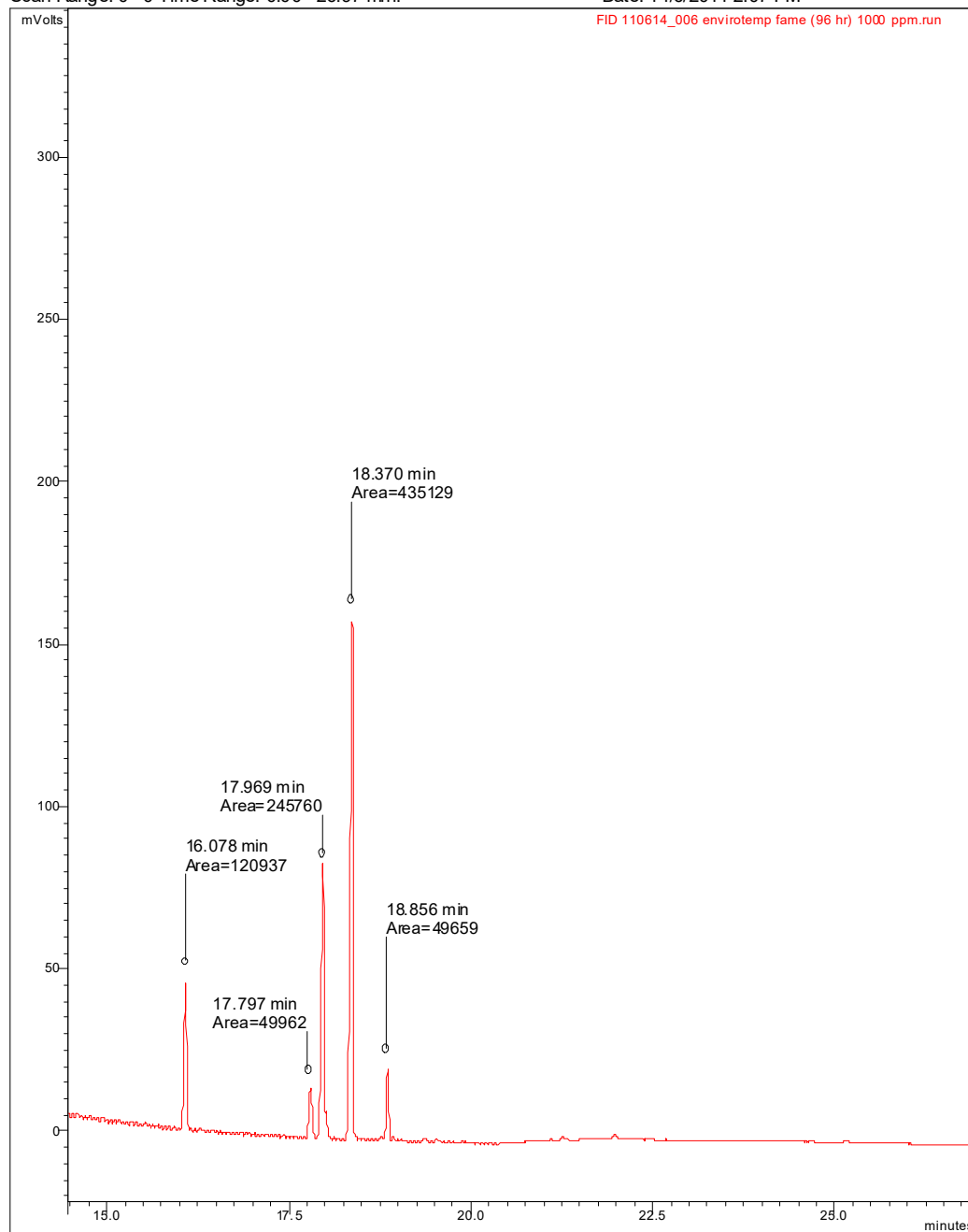
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Sample:

Operator:

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Date: 11/6/2014 2:07 PM



## FAME A 1000 ppm (120 hrs oxidation)

Chromatogram Plot

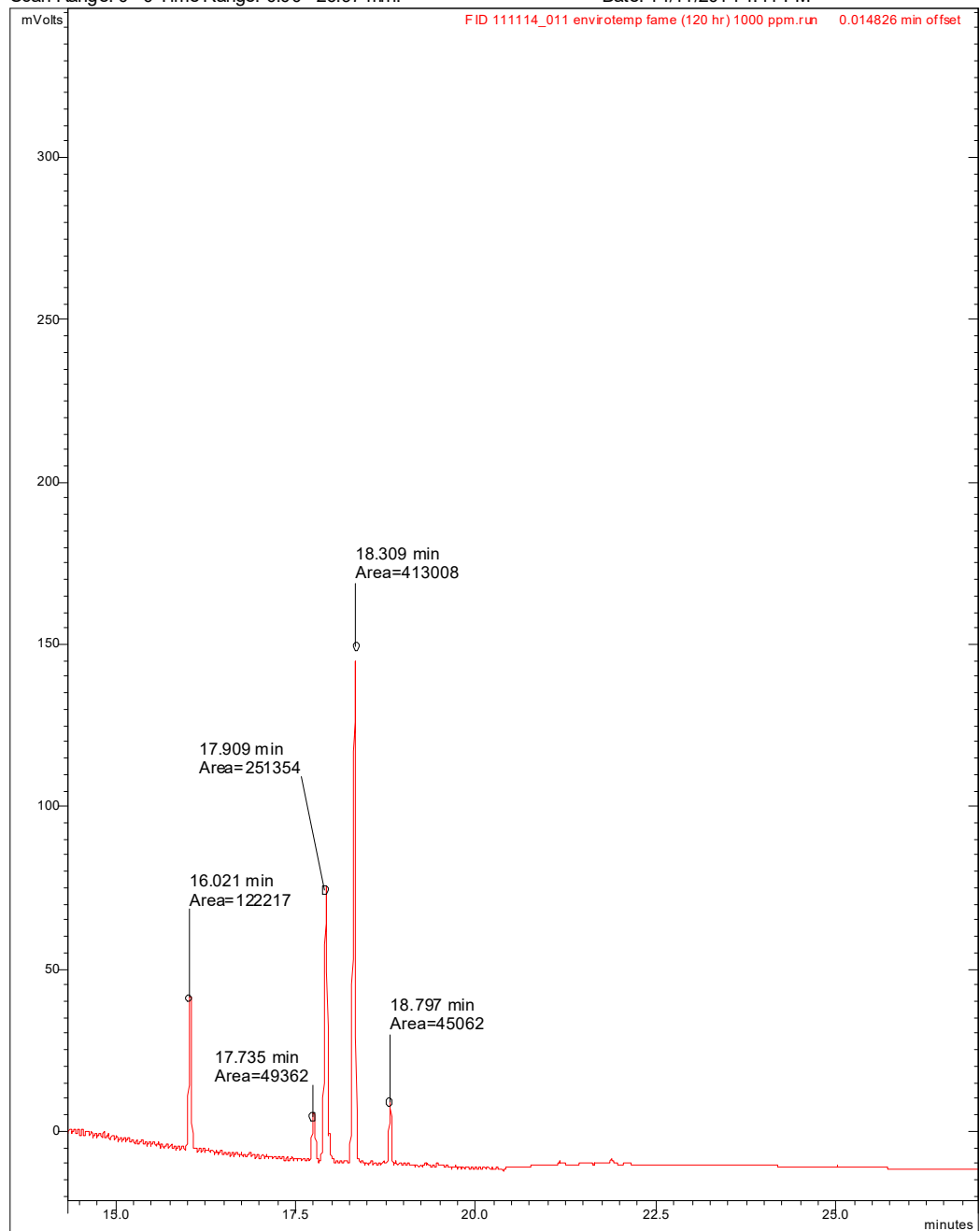
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Sample:

Operator:

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Date: 11/11/2014 4:41 PM



## FAME A 1000 ppm (164 hrs oxidation)

Chromatogram Plot

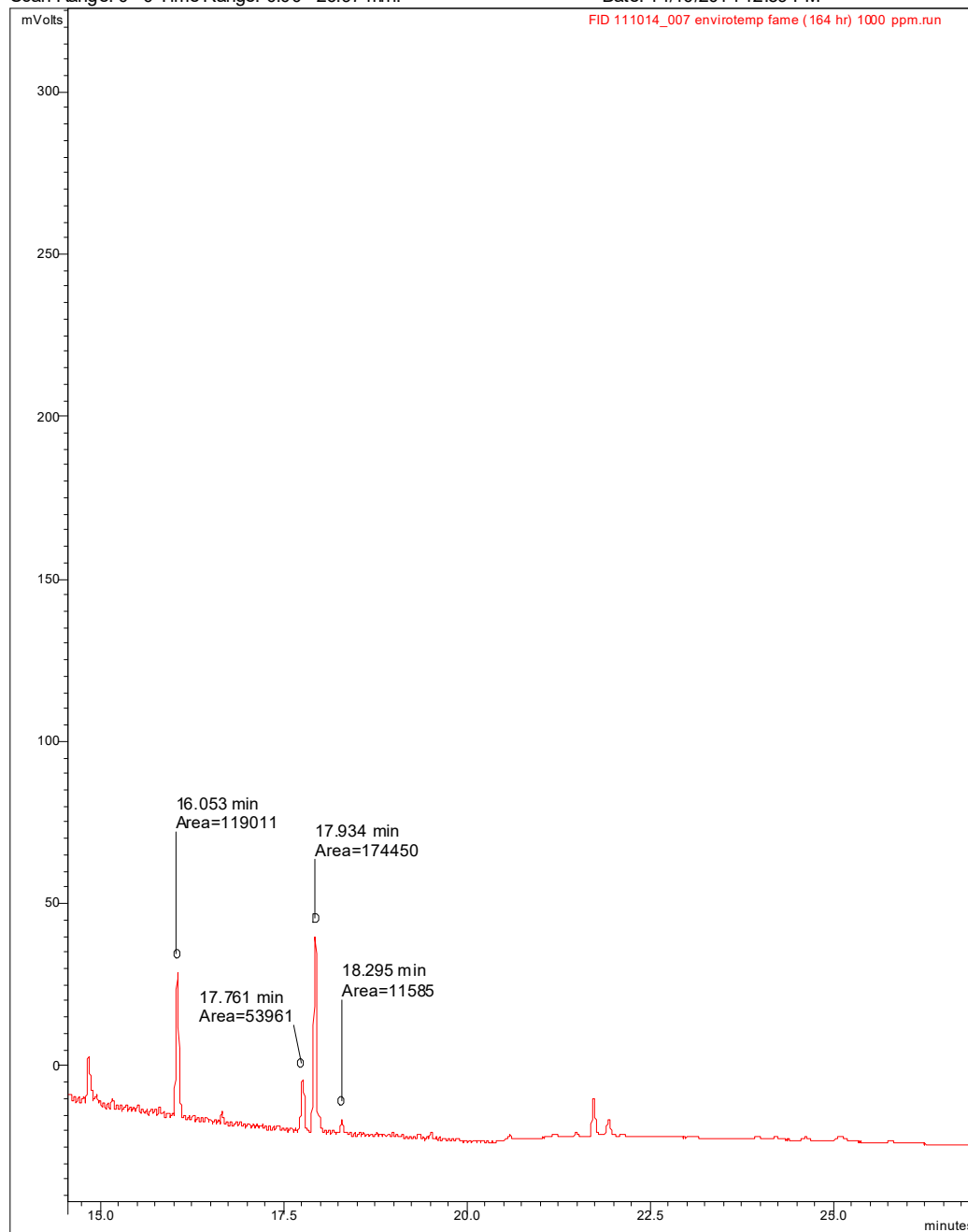
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Sample:

Operator:

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Date: 11/10/2014 12:59 PM



FAME B 1000 ppm (unused oil)

### Chromatogram Plot

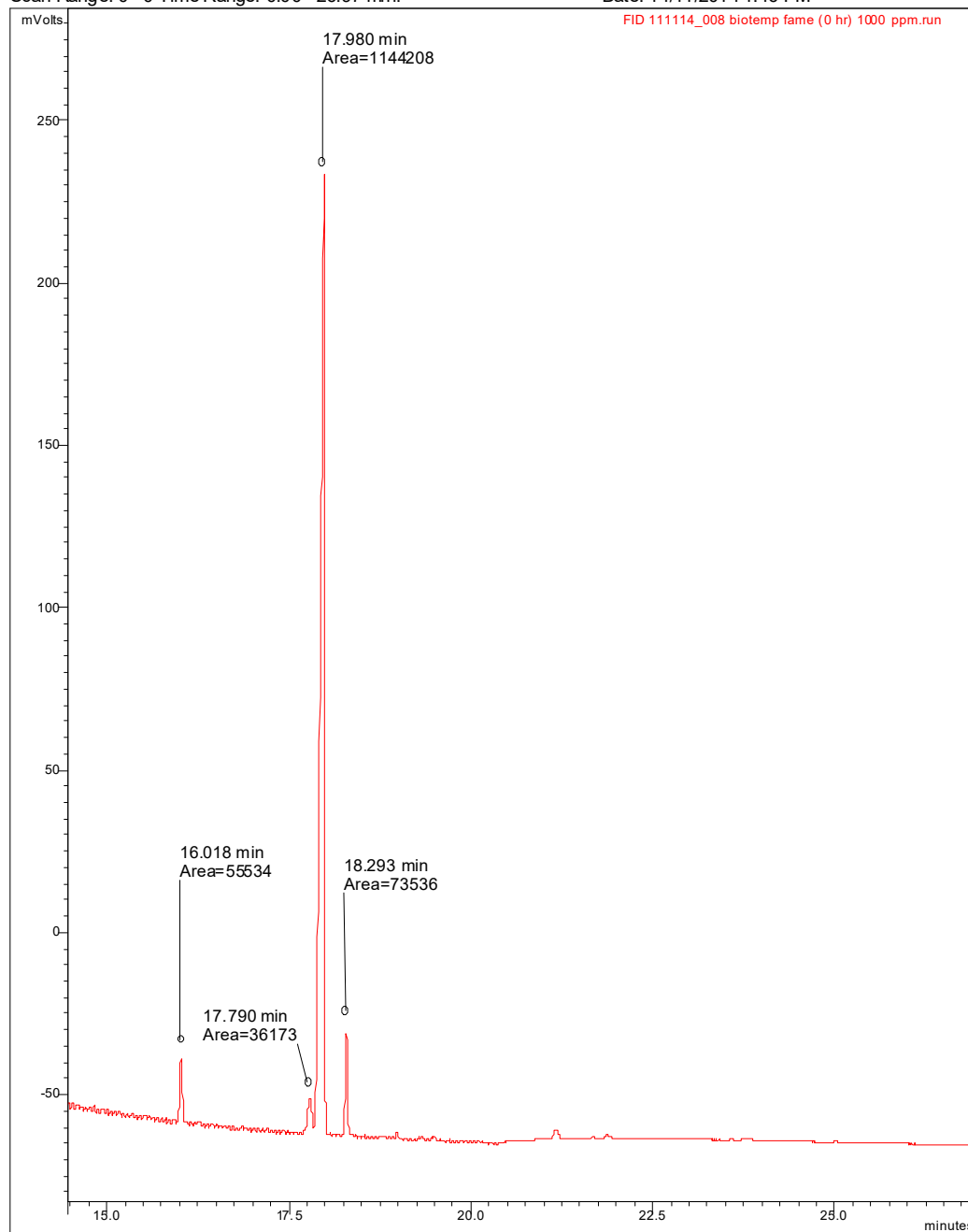
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Sample:

Operator:

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Date: 11/11/2014 1:46 PM



## FAME B 1000 ppm (12 hrs oxidation)

Chromatogram Plot

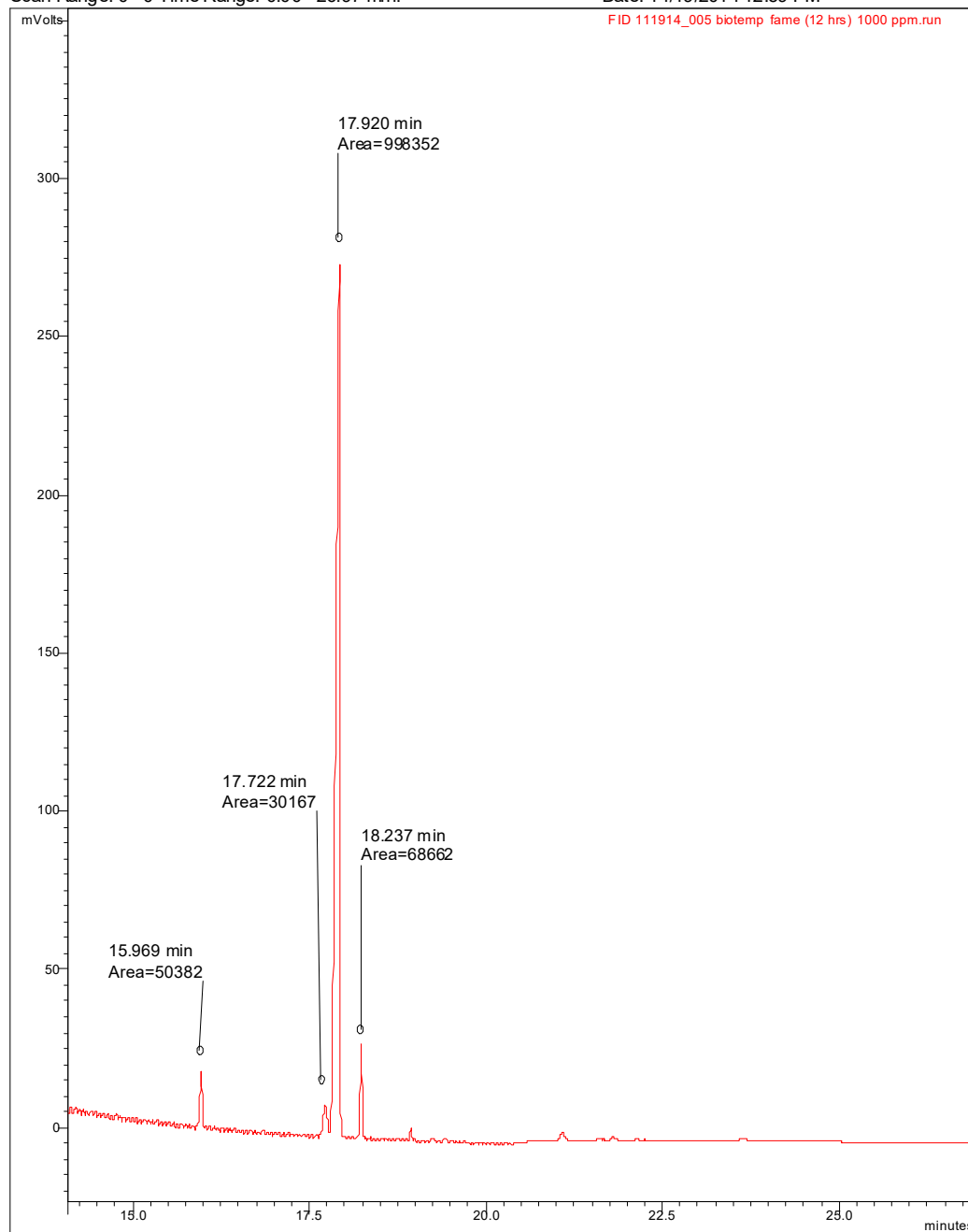
File: ...e in isooctane\_032814\111914\_005 biotemp fame (12 hrs) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/19/2014 12:59 PM





## FAME B 1000 ppm (24 hrs oxidation)

Chromatogram Plot

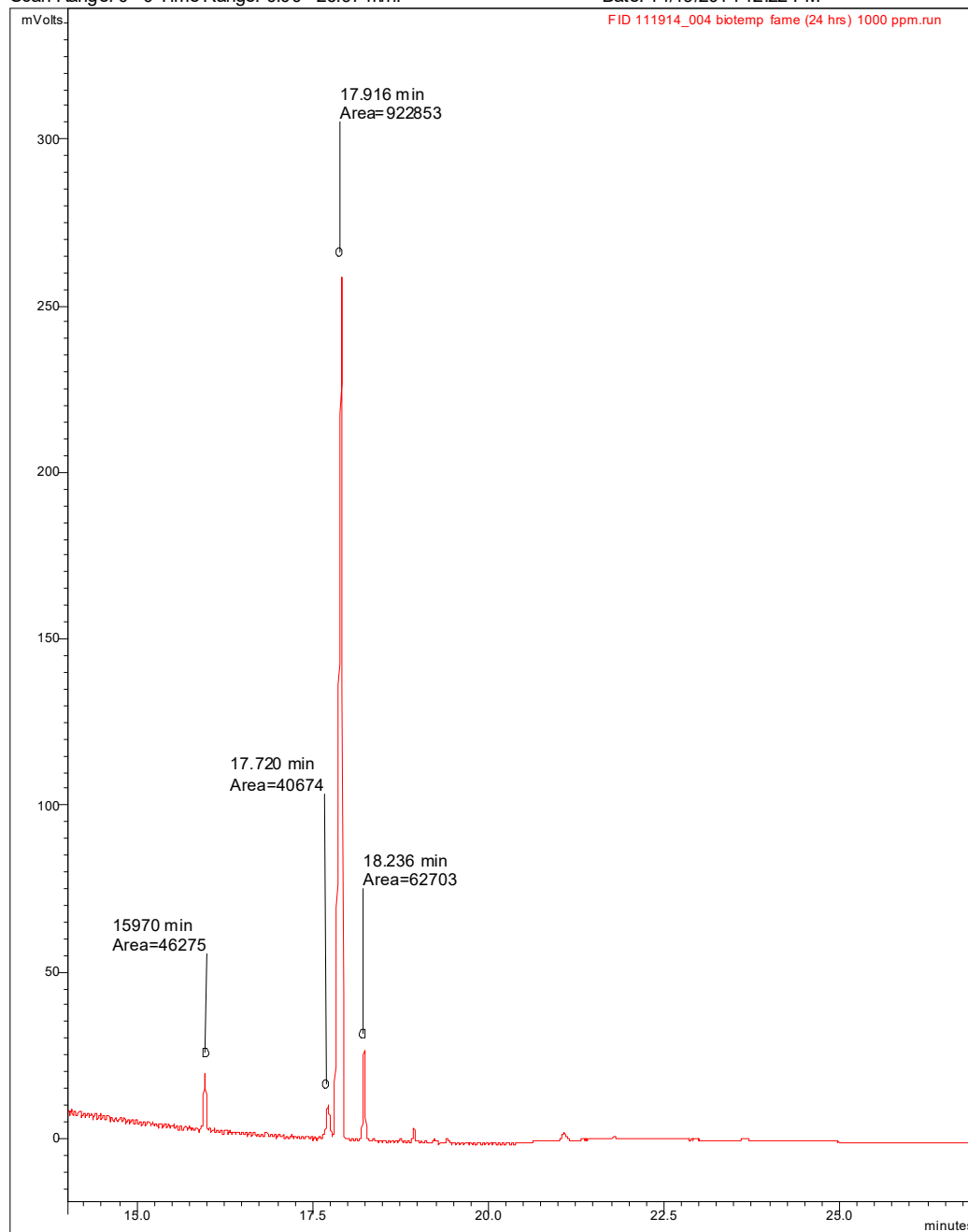
File: ...e in isoctane\_032814\111914\_004 biotemp fame (24 hrs) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/19/2014 12:22 PM



## FAME B 1000 ppm (36 hrs oxidation)

Chromatogram Plot

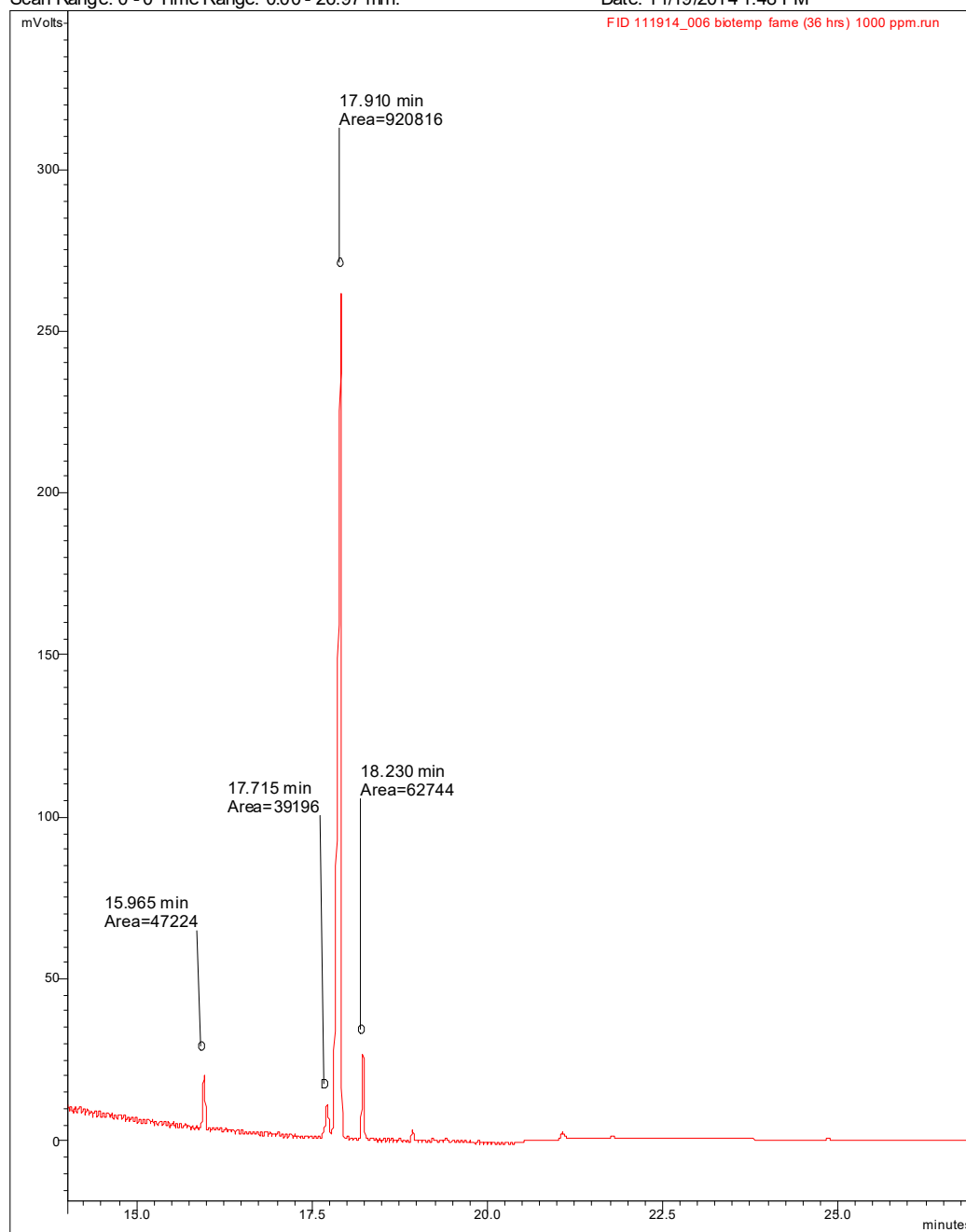
File: ...e in isooctane\_032814\111914\_006 biotemp fame (36 hrs) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/19/2014 1:48 PM



## FAME B 1000 ppm (48 hrs oxidation)

Chromatogram Plot

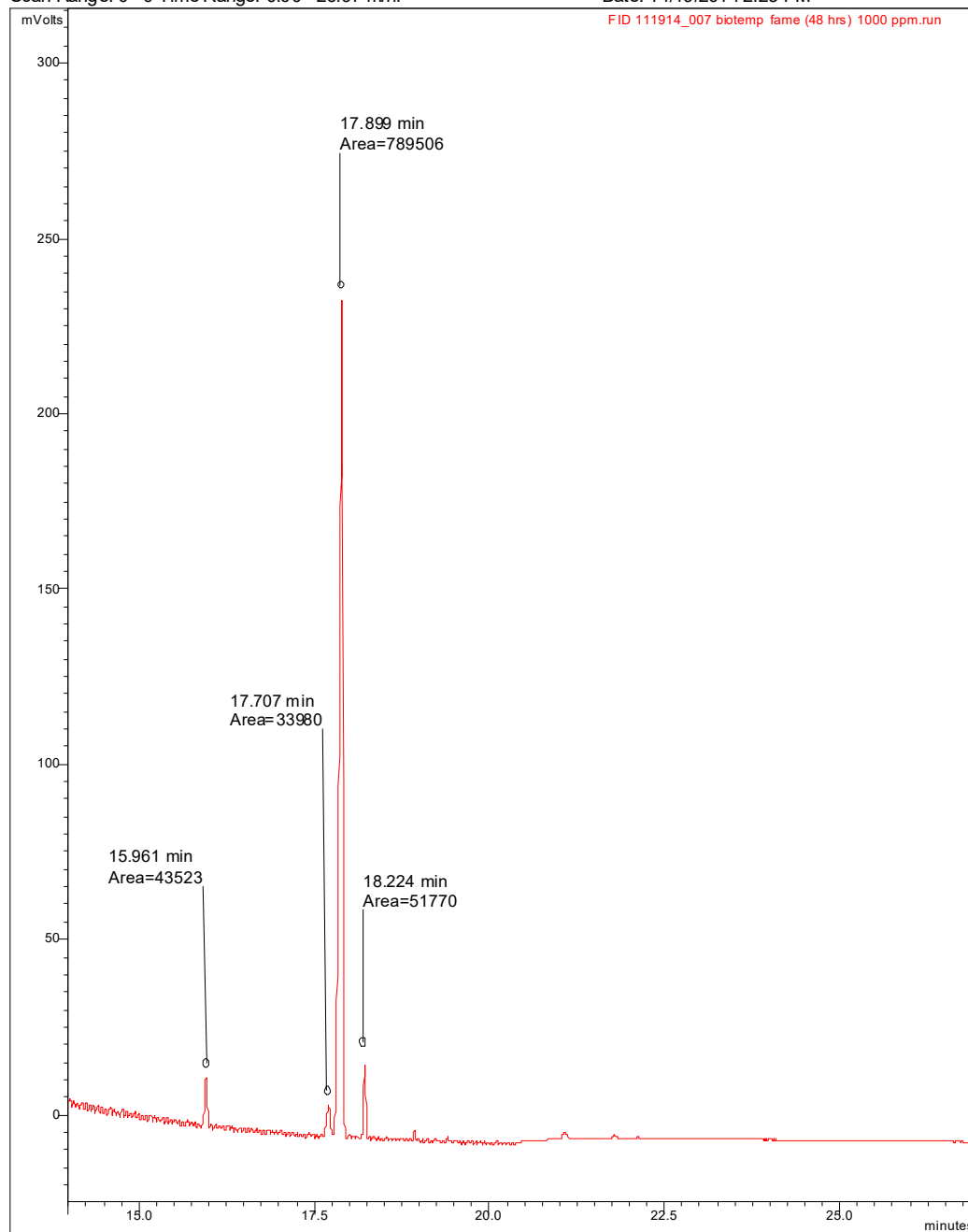
File: ...e in isooctane\_032814\111914\_007 biotemp fame (48 hrs) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/19/2014 2:25 PM



## FAME B 1000 ppm (96 hrs oxidation)

Chromatogram Plot

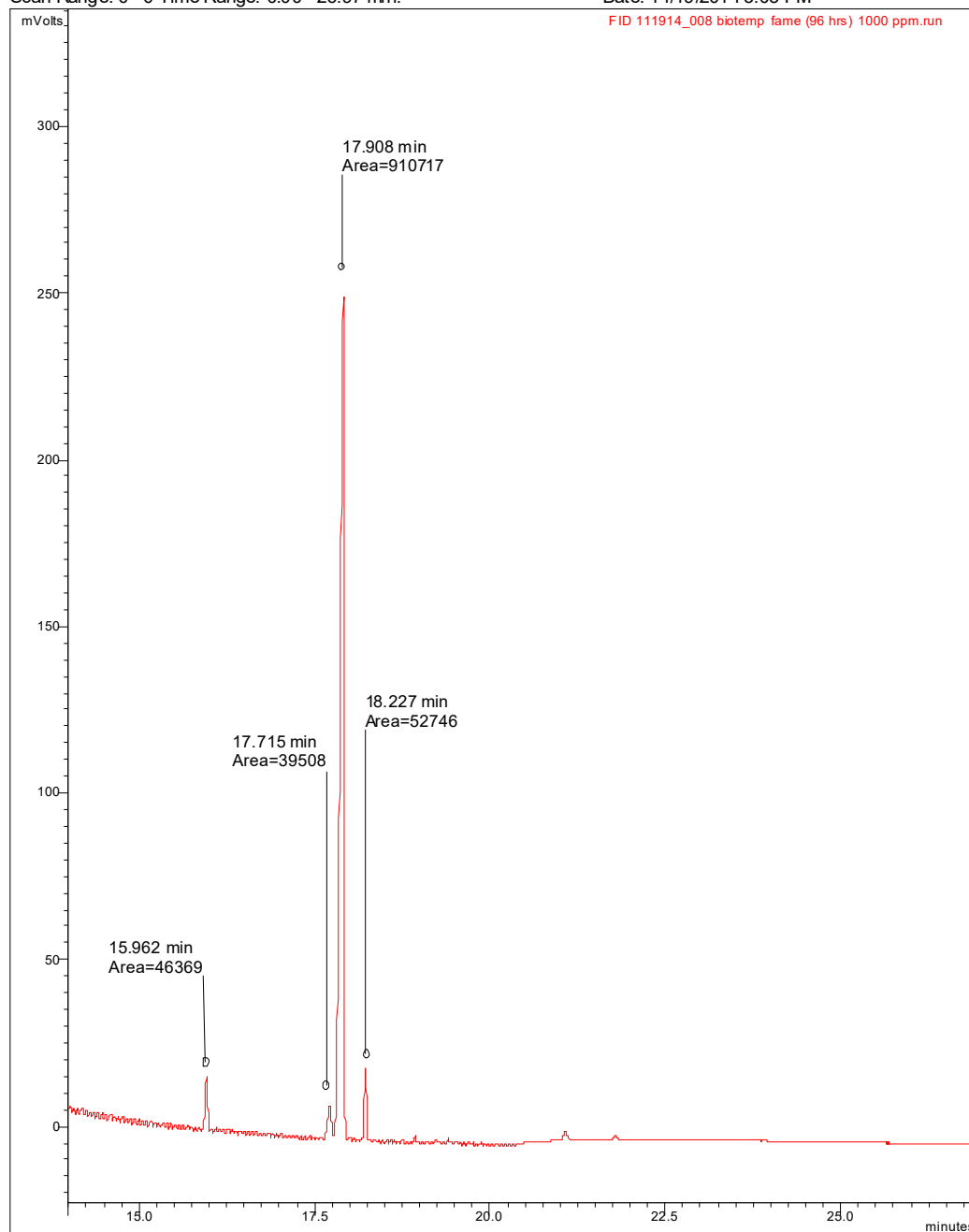
File: ...e in isooctane\_032814\111914\_008 biotemp fame (96 hrs) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/19/2014 3:03 PM



## FAME B 1000 ppm (120 hrs oxidation)

Chromatogram Plot

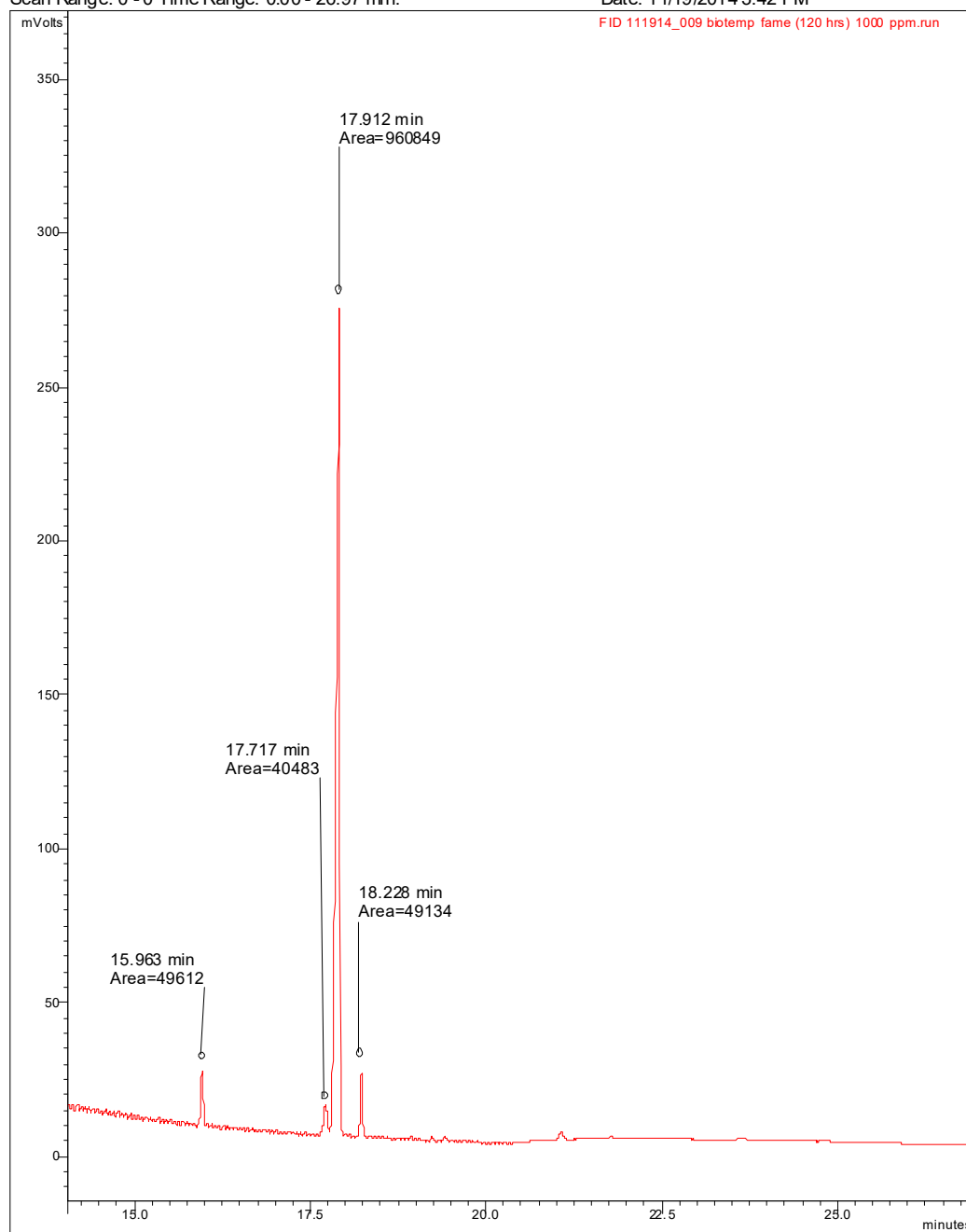
File: ... in isooctane\_032814\111914\_009 biotemp fame (120 hrs) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/19/2014 3:42 PM



## FAME B 1000 ppm (164 hrs oxidation)

Chromatogram Plot

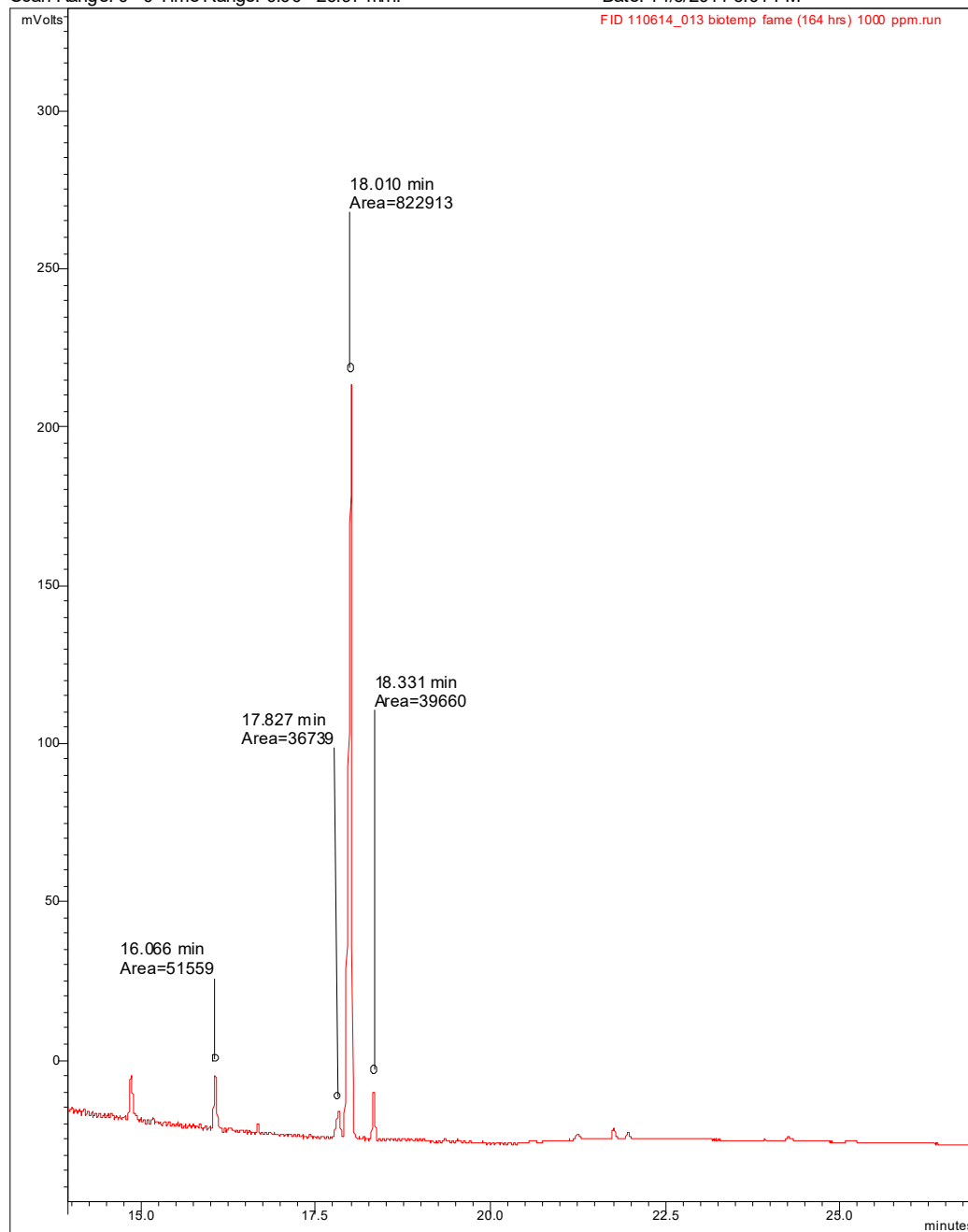
File: ... in isooctane\_032814\110614\_013 biotemp fame (164 hrs) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/6/2014 6:01 PM



## FAME C (unused) 1000 ppm

Chromatogram Plot

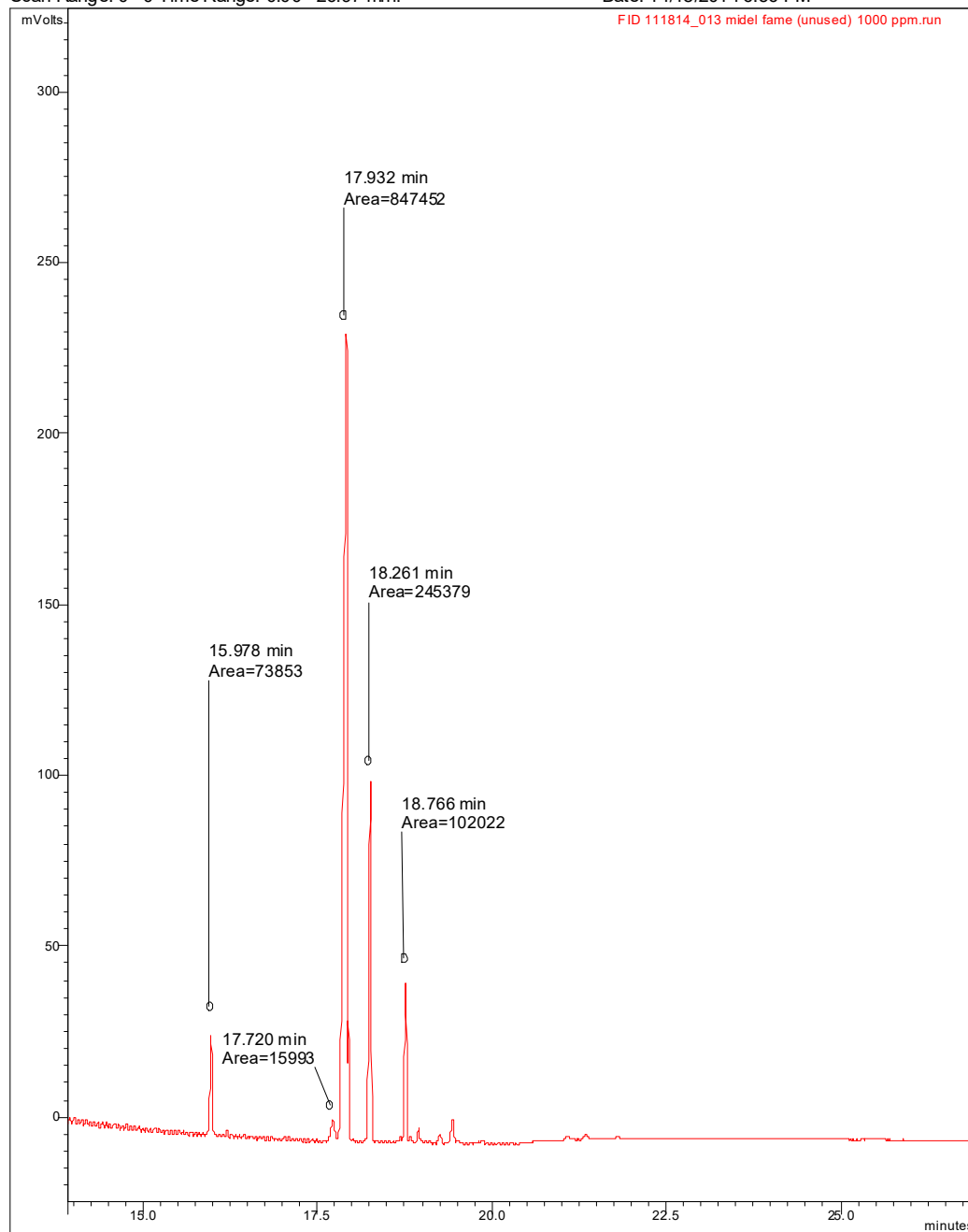
File: ...ame in isooctane\_0328141 11814\_013 midel fame (unused) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/18/2014 6:56 PM



## FAME C (12 hrs oxidation) 1000 ppm

Chromatogram Plot

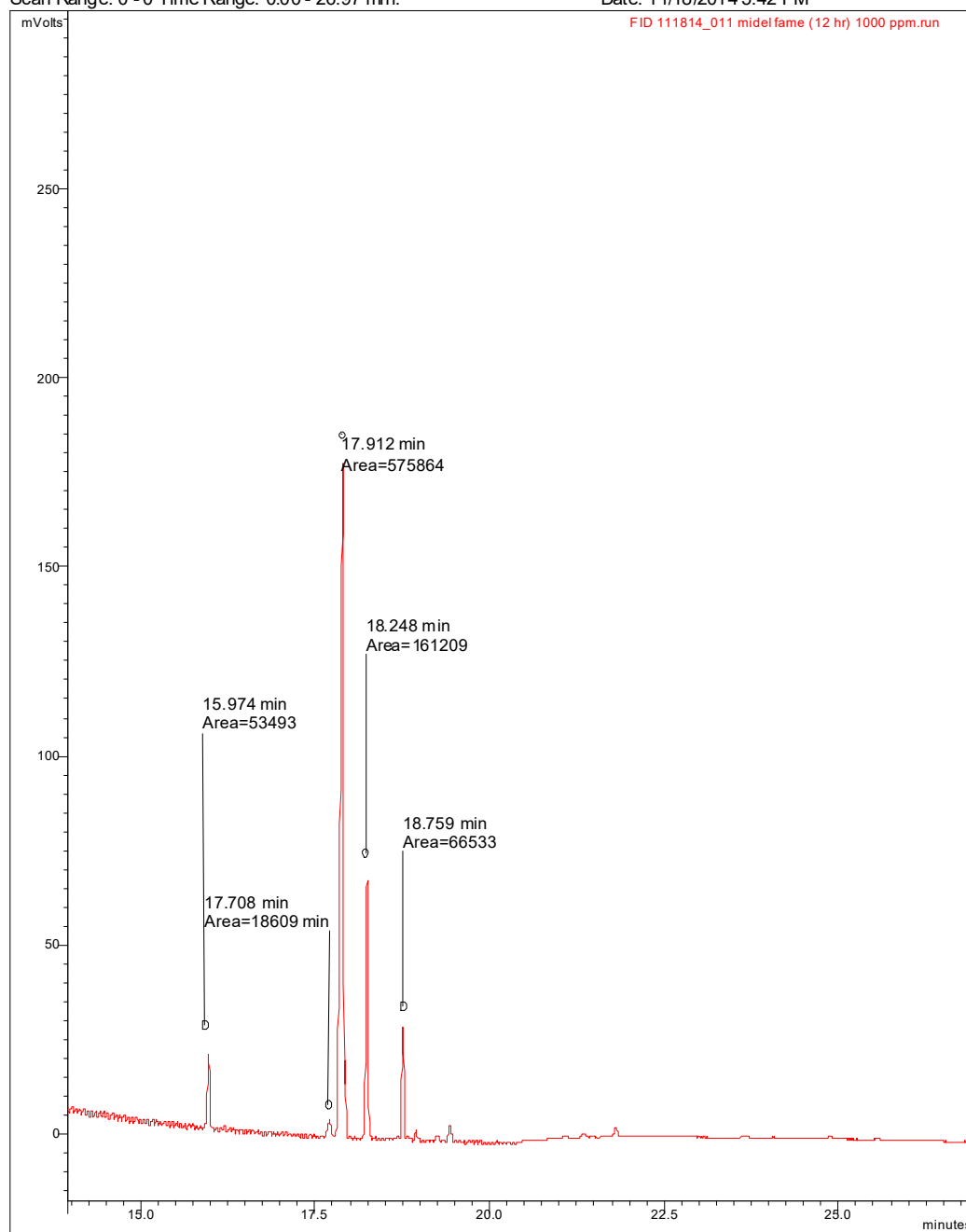
File: ...fame in isooctane\_032814\111814\_011 midel fame (12 hr) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/18/2014 5:42 PM





## FAME C (24 hrs oxidation)

Chromatogram Plot

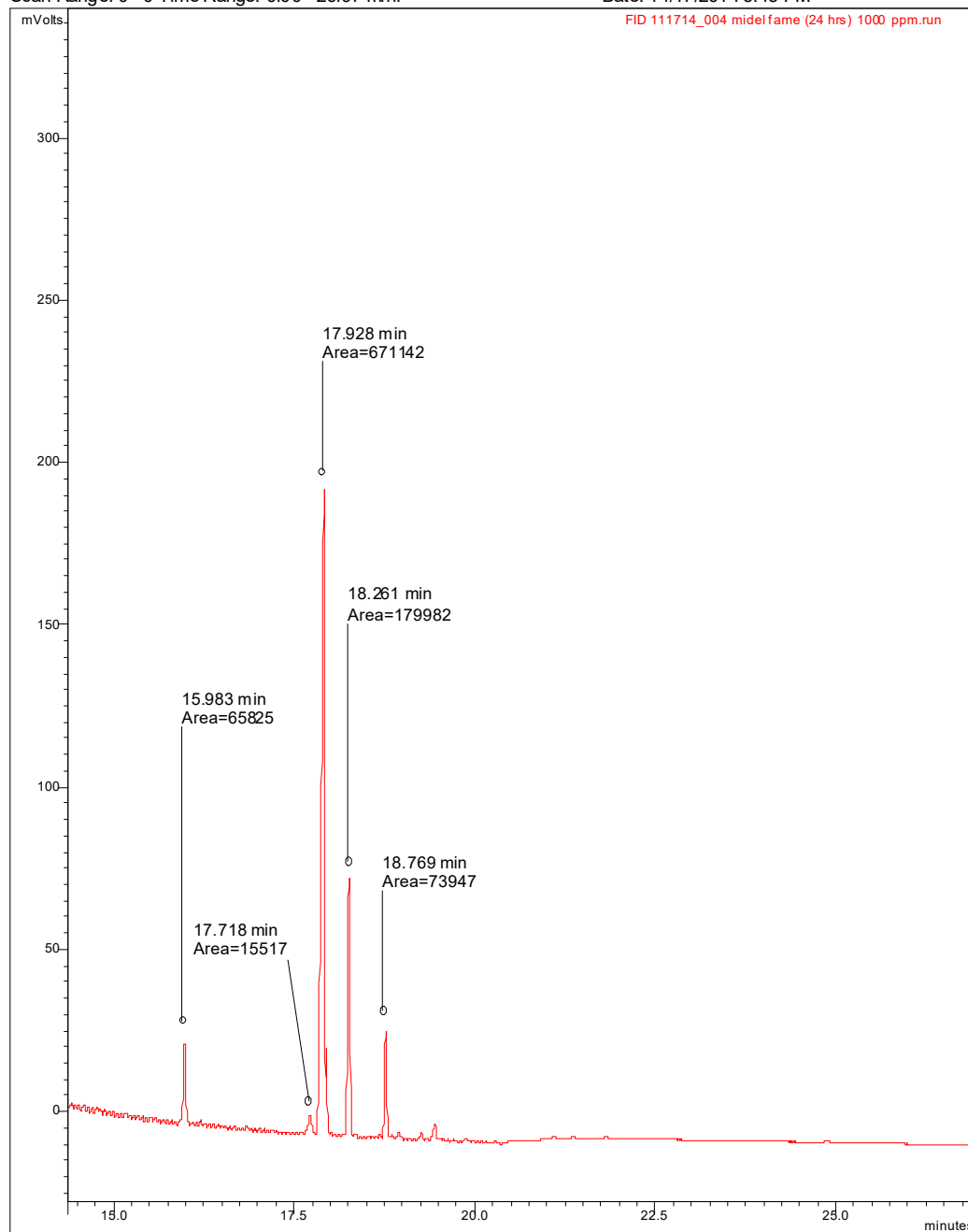
File: ...ame in isoctane\_0328141 11714\_004 midel fame (24 hrs) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/17/2014 3:45 PM



## FAME C (36 hrs oxidation) 1000 ppm

Chromatogram Plot

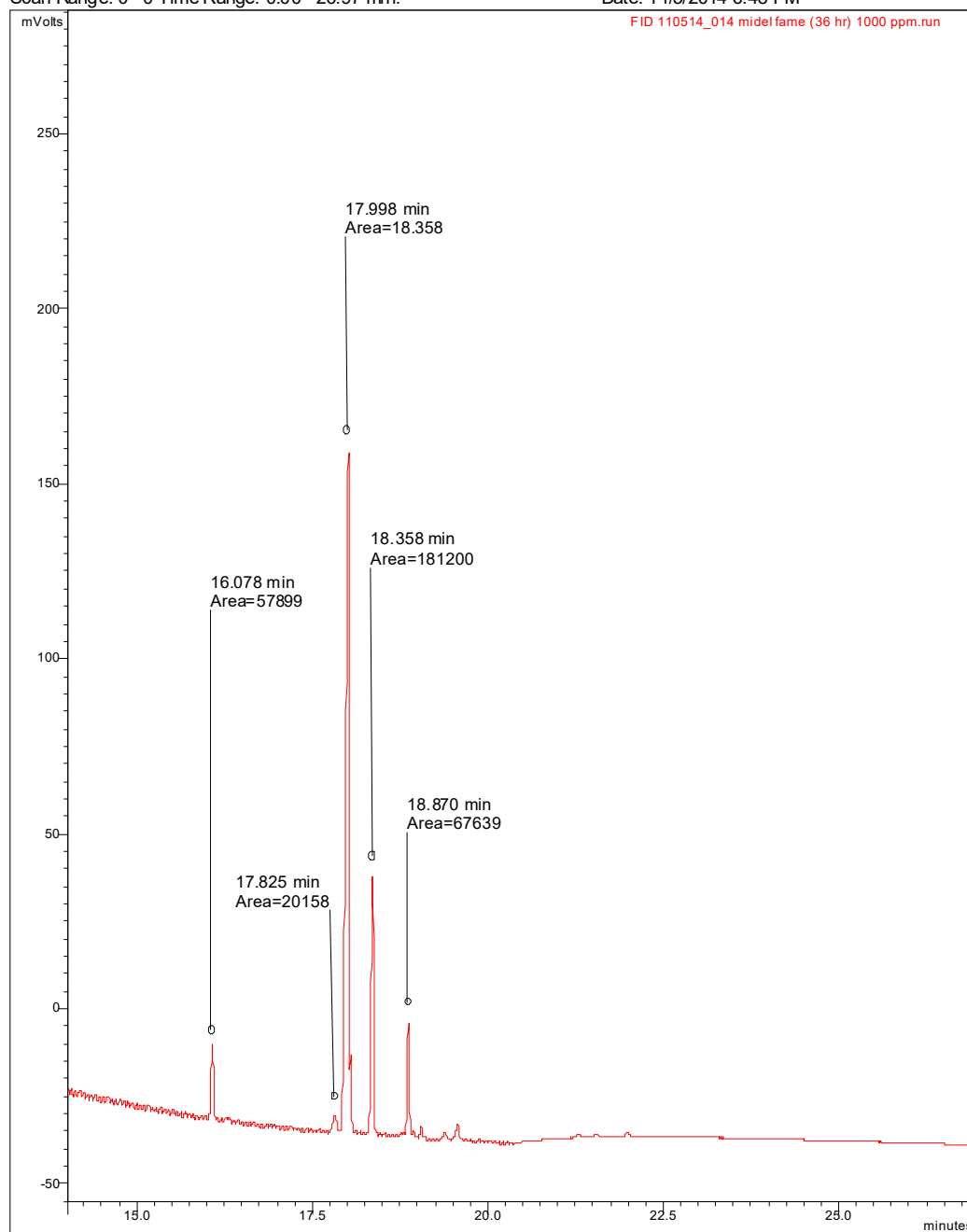
File: ...fame in isooctane\_032814\110514\_014 midel fame (36 hr) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/5/2014 6:48 PM



## FAME C (48 hrs oxidation) 1000 ppm

Chromatogram Plot

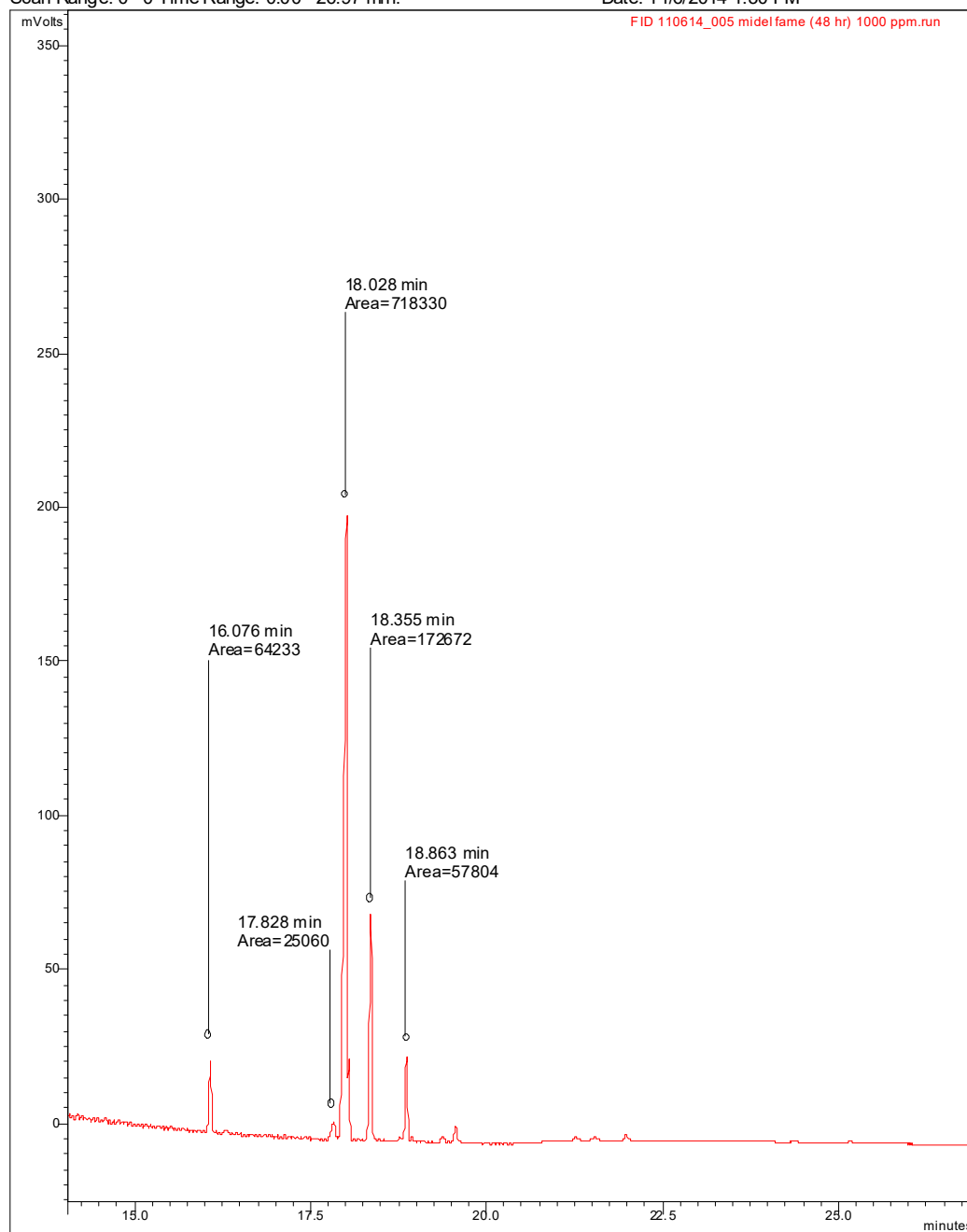
File: ...fame in isooctane\_032814\110614\_005 midel fame (48 hr) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/6/2014 1:30 PM



## FAME C (96 hrs oxidation) 1000 ppm

Chromatogram Plot

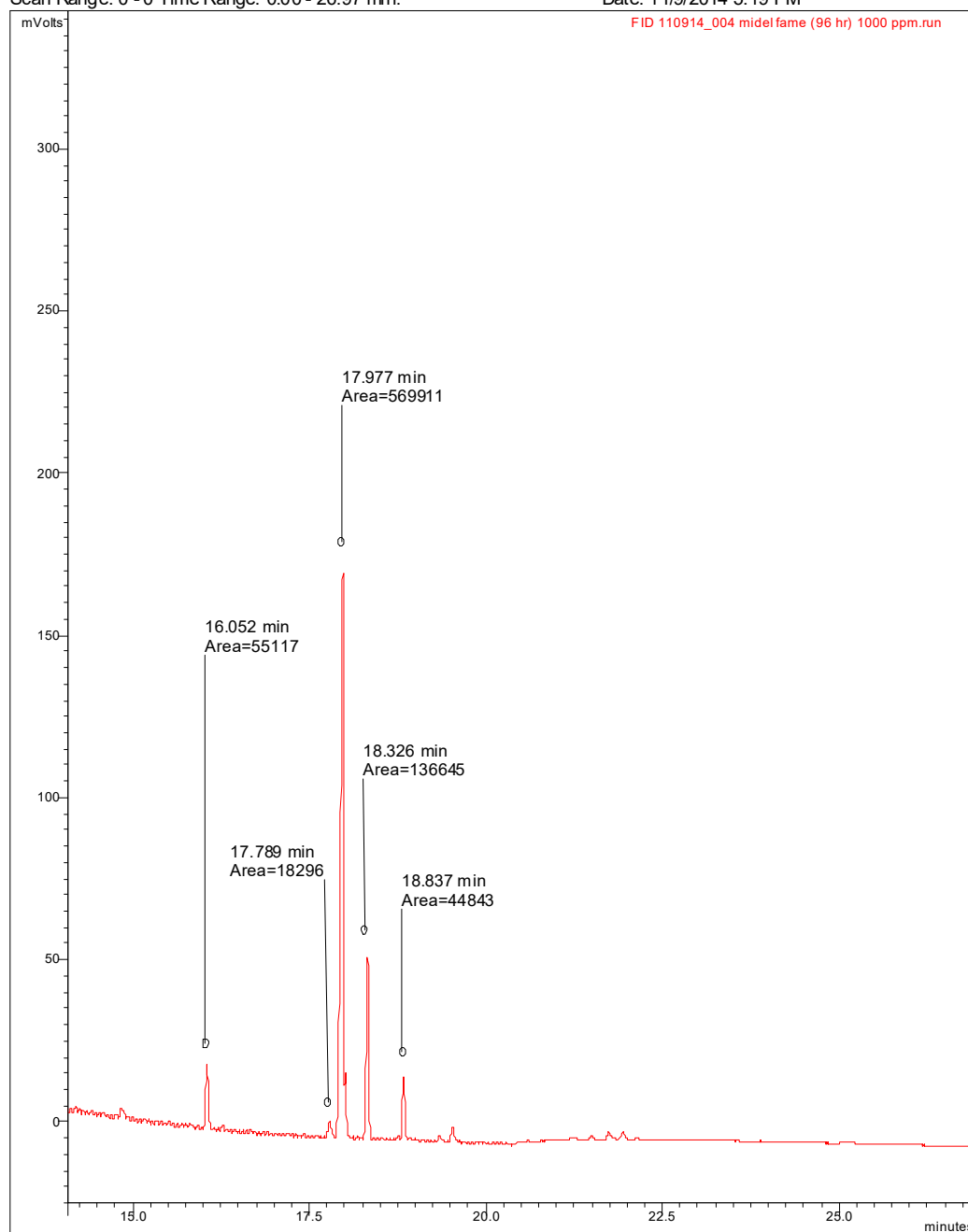
File: ...fame in isooctane\_032814\110914\_004 midel fame (96 hr) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/9/2014 5:19 PM



## FAME C (120 hrs oxidation) 1000 ppm

Chromatogram Plot

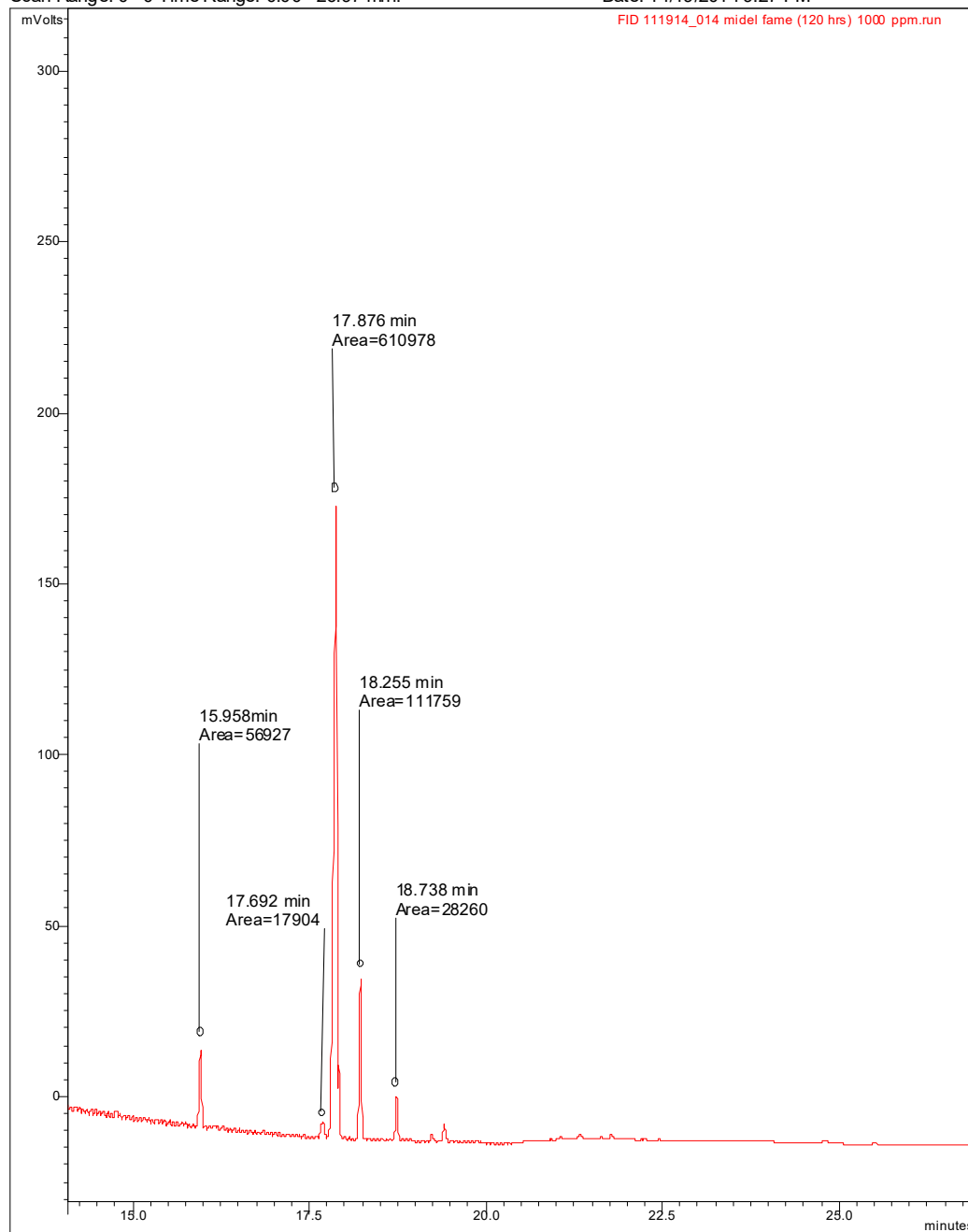
File: ...me in isooctane\_032814\111914\_014 midel fame (120 hrs) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/19/2014 9:27 PM



## FAME C (164 hrs oxidation) 1000 ppm

Chromatogram Plot

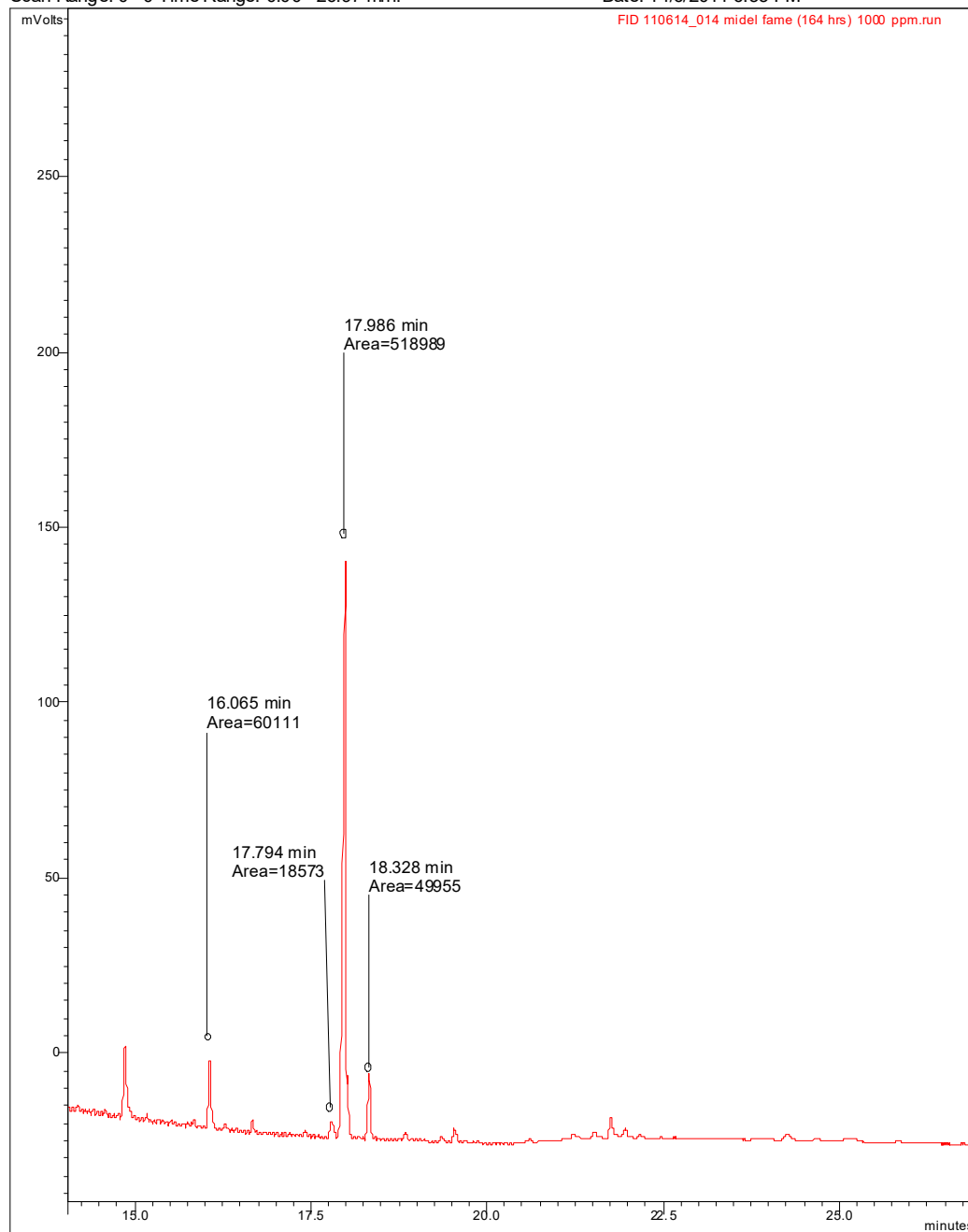
File: ...me in isooctane\_032814\110614\_014 midel fame (164 hrs) 1000 ppm.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 11/6/2014 6:33 PM



Data table for GPC separation of oils.

Oil	Oxidation period (hour)	Wt. of oil taken (g)	Wt. of oil collected (g)
Envirotemp	0	0.200	0.19
Envirotemp	24	0.201	0.200
Envirotemp	36	0.202	0.200
Envirotemp	48	0.206	0.200
Envirotemp	96	0.207	0.200
Envirotemp	120	0.201	0.190
Envirotemp	164	0.221	0.220
Biotemp	0	0.201	0.200
Biotemp	24	0.204	0.200
Biotemp	36	0.201	0.200
Biotemp	48	0.203	0.190
Biotemp	96	0.206	0.200
Biotemp	120	0.202	0.200
Biotemp	164	0.202	0.200
Midel	0	0.202	0.200
Midel	24	0.201	0.190
Midel	36	0.203	0.200
Midel	48	0.202	0.190
Midel	96	0.205	0.200
Midel	120	0.201	0.200
Midel	164	0.205	0.200

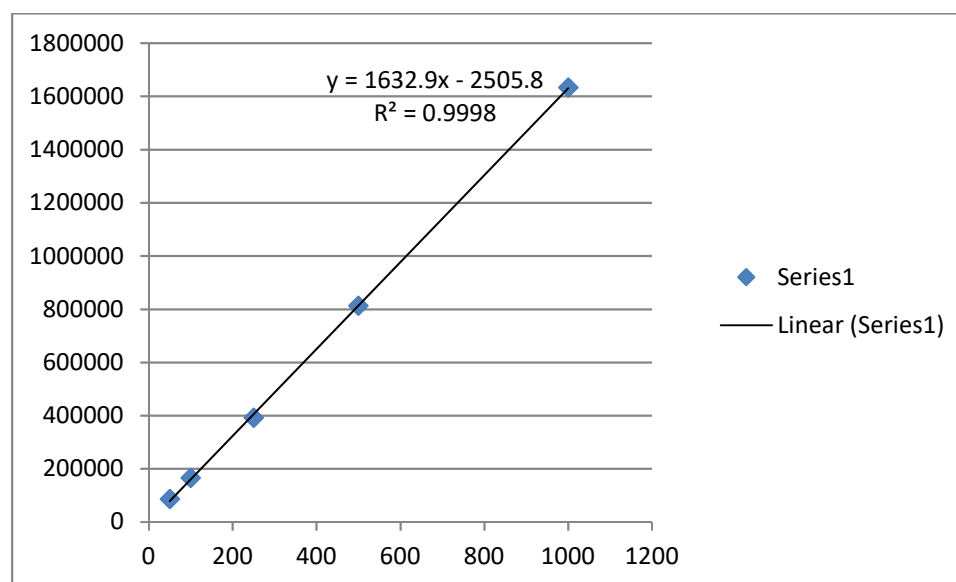


Fig: Calibration curve for BHT standard by GC/FID



## BHT extract from unoxidized envirotemp oil

Chromatogram Plot

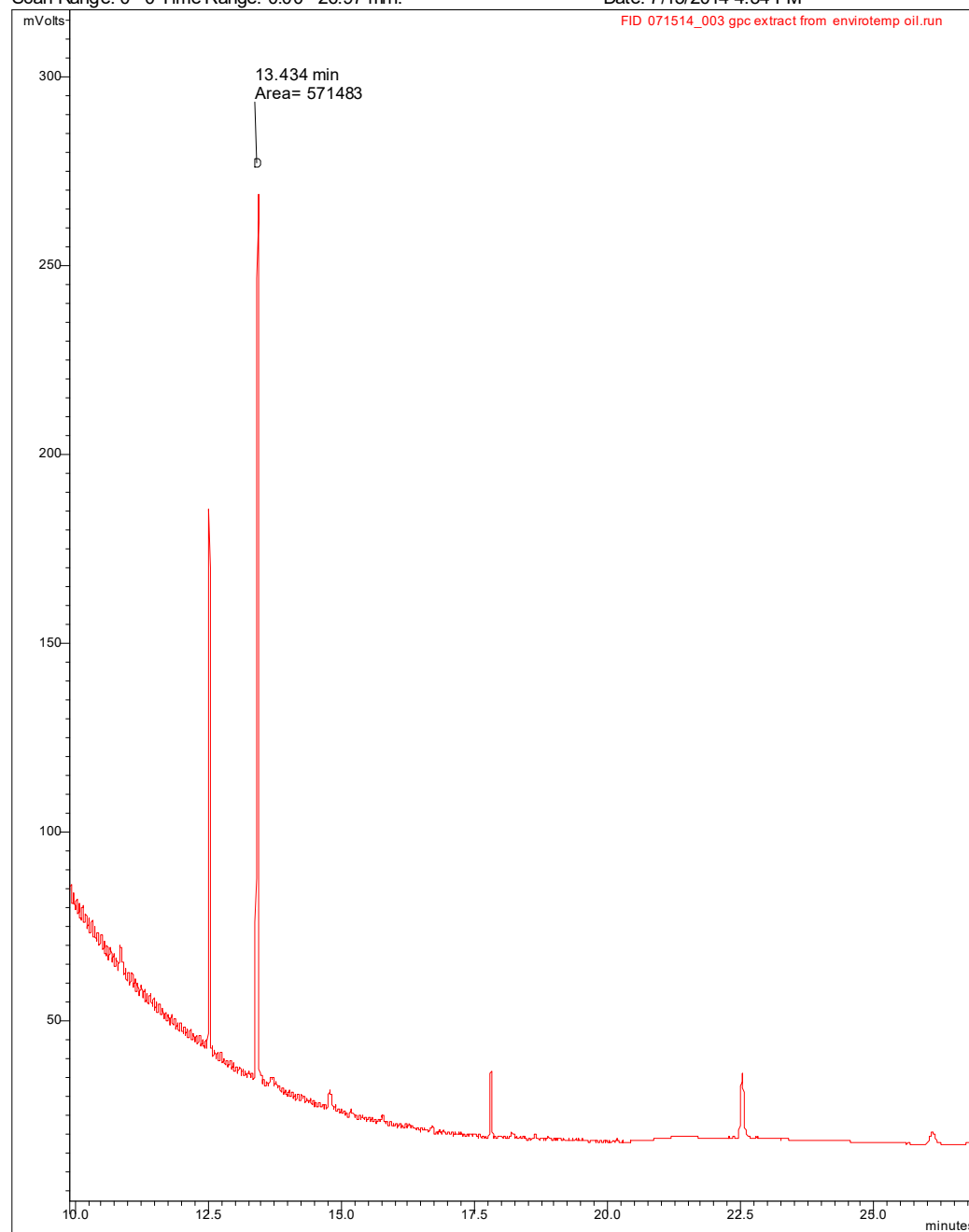
File: ... in isoctane\_032814\071514\_003 gpc extract from envirotemp oil.run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 7/15/2014 4:34 PM



## BHT extract from Envirotemp oil (12 hrs)

Chromatogram Plot

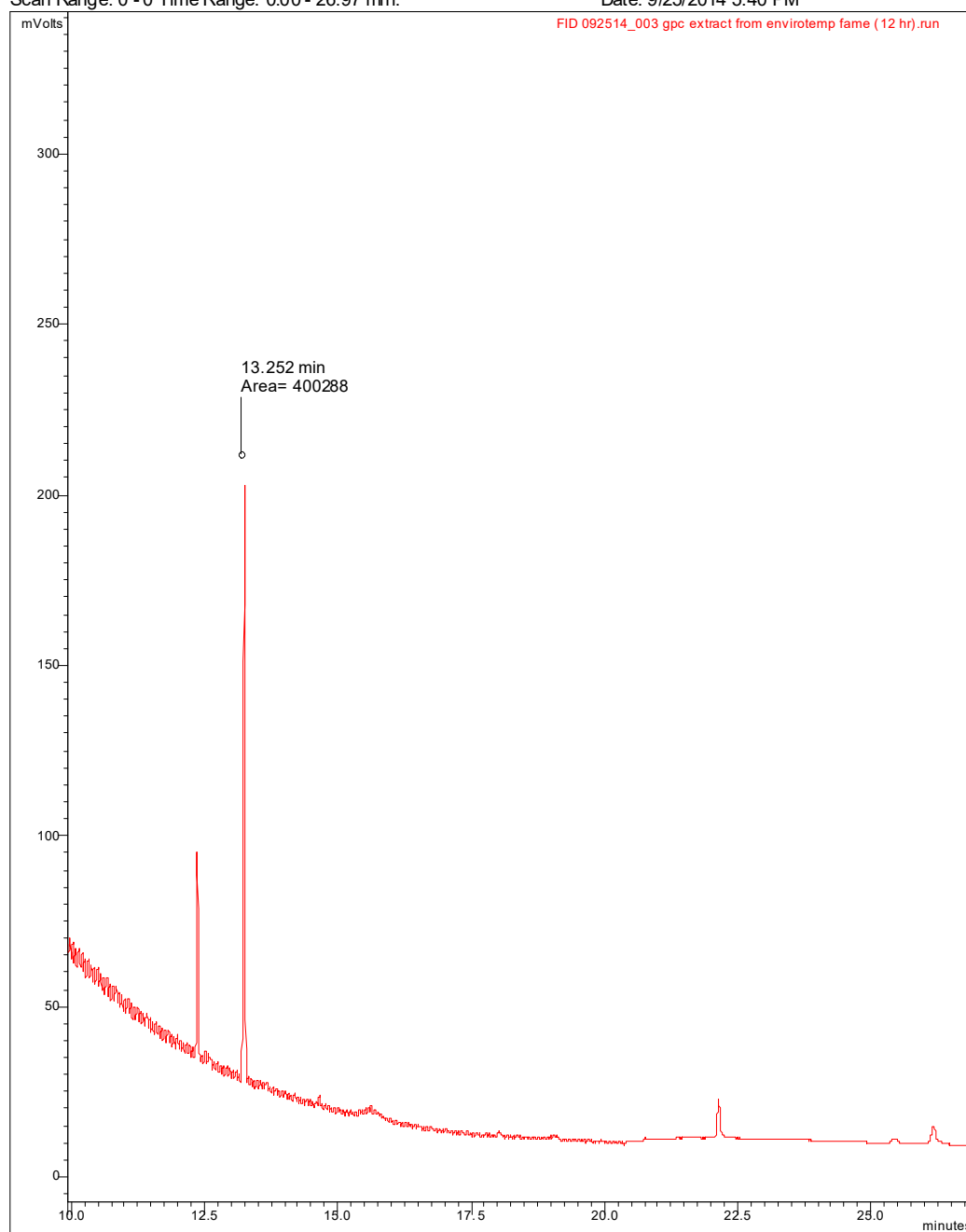
File: ...tane\_032814\092514\_003 gpc extract from envirotemp fame (12 hr).run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 9/25/2014 5:40 PM



## BHT extract from Envirotemp (24 hrs)

## Chromatogram Plot

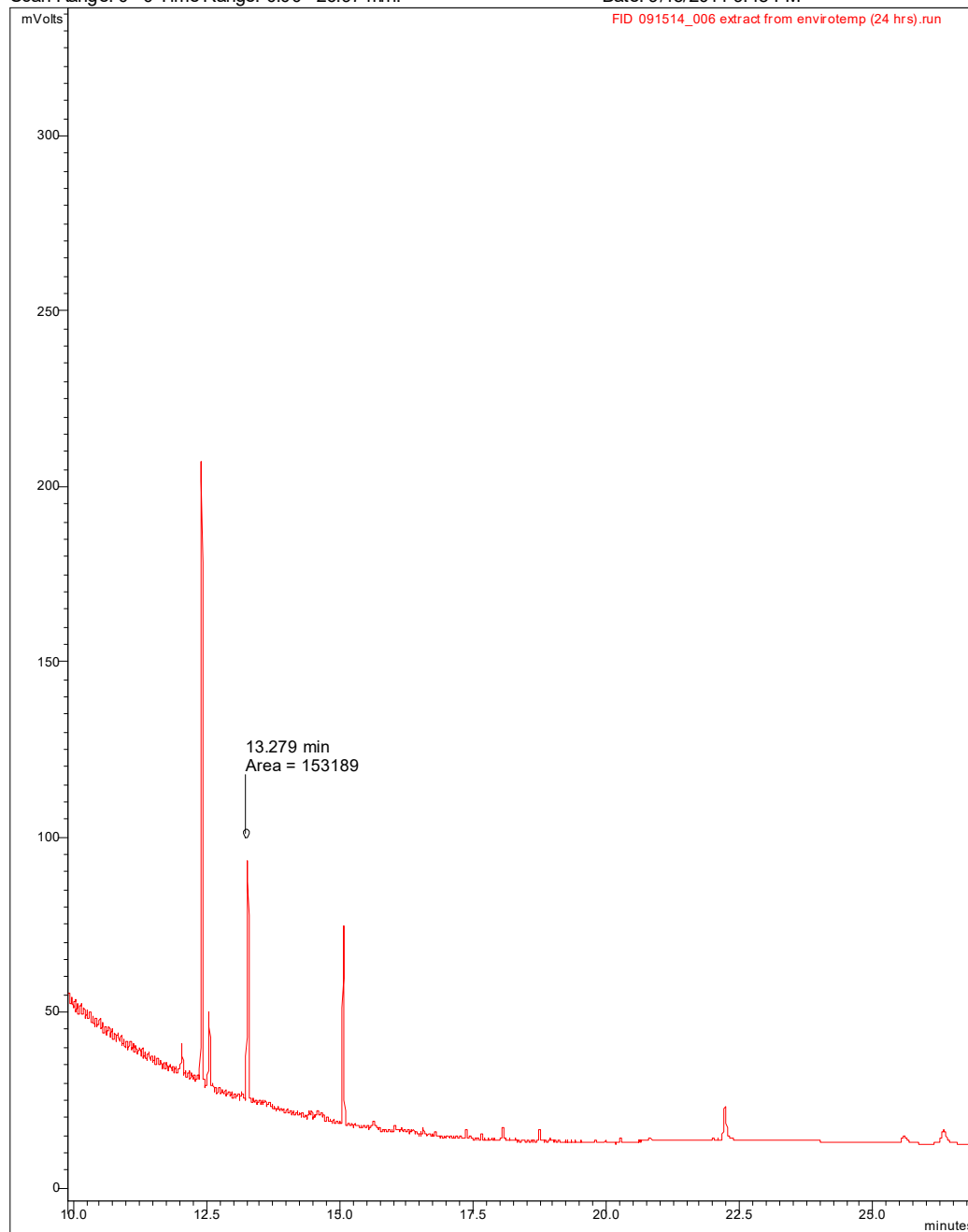
File: ...in isooctane\_032814\091514\_006 extract from envirotamp (24 hrs).run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 9/15/2014 9:48 PM



## BHT extract from Envirotemp oil (36 hrs)

Chromatogram Plot

File: ...fame in isooctane\_032814\092514\_003 envirotemp extract (36 hrs).run

Sample:

Operator:

Scan Range: 0 - 0 Time Range: 0.00 - 26.97 min.

Date: 9/25/2014 12:21 PM

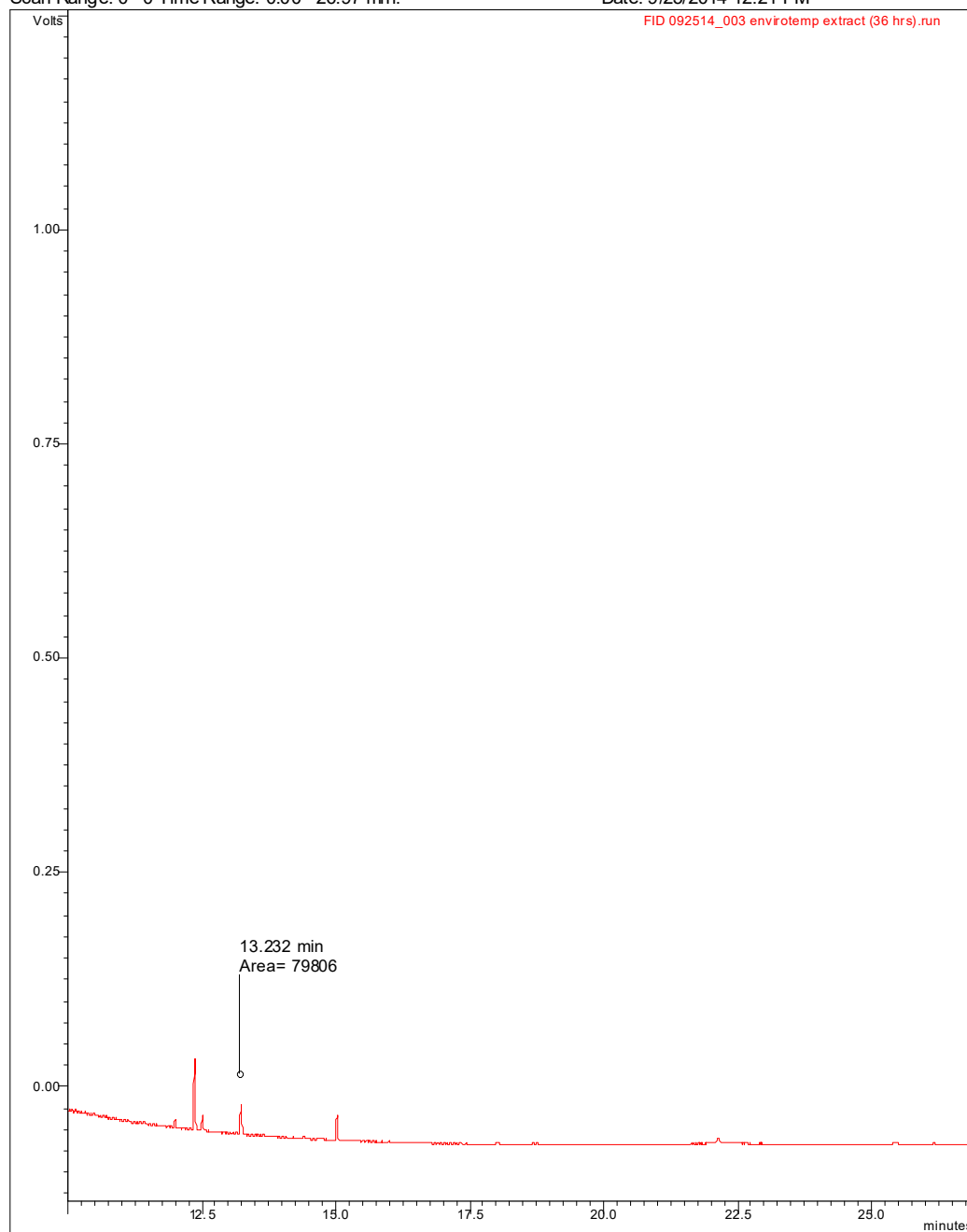
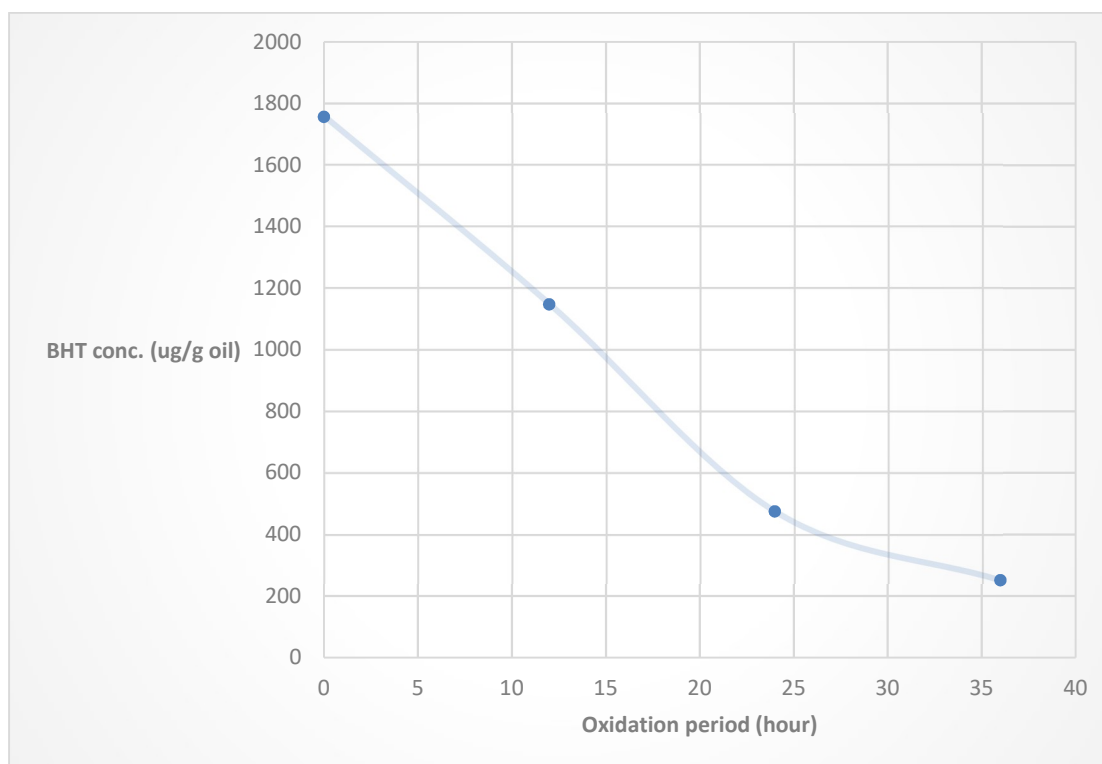


Table: BHT analysis in oil A

Oxidation period	BHT retention time (min)	Area
0	13.434	571483
12	13.252	400288
24	13.279	153189
36	13.232	79806

Antioxidant analysis of commercial oil A



## VITA

Radheshyam Panta was born in Tanahun, Nepal. He did his secondary school from Balkumari secondary school Narayangarh, Nepal. He received his bachelor's degree in Chemistry from Birendra Multiple college, Bharatpur, Nepal. He received his Master's degree in Chemistry from Central Department of Chemistry, Tribhuvan University, Kirtipur, Nepal. After obtaining Master's degree, he started teaching Chemistry for secondary level in Nepal for 4 years. In August 2011, he joined Ph.D. program in Chemistry department at Missouri University of Science and Technology, Rolla, MO. He started working under the guidance of professor Dr. Shubhender Kapila in 2013. He received his PhD degree in May 2017 in Chemistry from Missouri University of Science and Technology.