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The Effect of the Reducing Agent Triphenylphosphine on Arachidonic Acid Metabolism by
Prostaglandin H2 Synthase

By

Lalini Ailaboina

Thesis

to the Department of Chemistry

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In partial fulfillment of the requirements

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ABSTRACT

Prostaglandin H₂ synthase (PGHS) catalyzes the conversion of arachidonic acid to prostaglandin endoperoxide (PGH₂), which is a precursor to a variety of prostaglandins that function as hormones. Gas chromatography combined with mass spectrometry is employed to look for the novel products produced by reaction of triphenylphosphine with arachidonic acid prior to and during the PGHS reaction. Triphenylphosphine (TPP) is known to reduce hydroperoxides to alcohols and is used in the present study to ascertain the extent to which hydroperoxides in arachidonic acid preparations and in PGHS contribute to enzyme activity. Incubation of PGHS with arachidonic acid that was treated with triphenylphosphine resulted in two peaks. One of the peaks was identified as Prostaglandin E₂, the well-characterized metabolite of PGHS activity. PGHS metabolism of arachidonic acid in the presence of TPP resulted in production of a new metabolite that was identified as 12-hydroxyeicosatetraenoic acid.

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Triphenylphosphine (TPP) may be involved in stimulating the production of
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Chapter 1. Introduction

1.1 Research objective

The main research objective was to investigate the role of hydroperoxides contained in arachidonic acid preparations and in PGHS preparations in stimulating cyclooxygenase activity. The project used the reducing agent triphenylphosphine to remove the hydroperoxides during metabolism and in arachidonic acid preparations.

1.2 Arachidonic acid

1.2a. Lipids and fatty acids

The class of biological molecules that are insoluble in aqueous solvents but are soluble in organic solvents are known as lipids (1). Lipids not only comprise the biological membranes that provide a semi-permeable barrier into and out of the cell but are also a storehouse of energy-yielding molecules. The lipids are classified into fatty acids, steroids, triglycerides, phospholipids, and sphingolipids.

1.2b. Structure and classification of fatty acids

The long-chain hydrocarbon molecules with a carboxylic group at one end are known as the fatty acids. Fatty acids vary in the number of carbon atoms and number of double bonds. As shown in Fig. 1, fatty acids that do not possess any double bonds between carbon-carbon atoms are called saturated fatty acids. In contrast, the fatty acids that possess double bonds are known as unsaturated fatty acids. Monounsaturated fatty acids contain a single double bond, whereas polyunsaturated fatty acids contain more than one double bond.

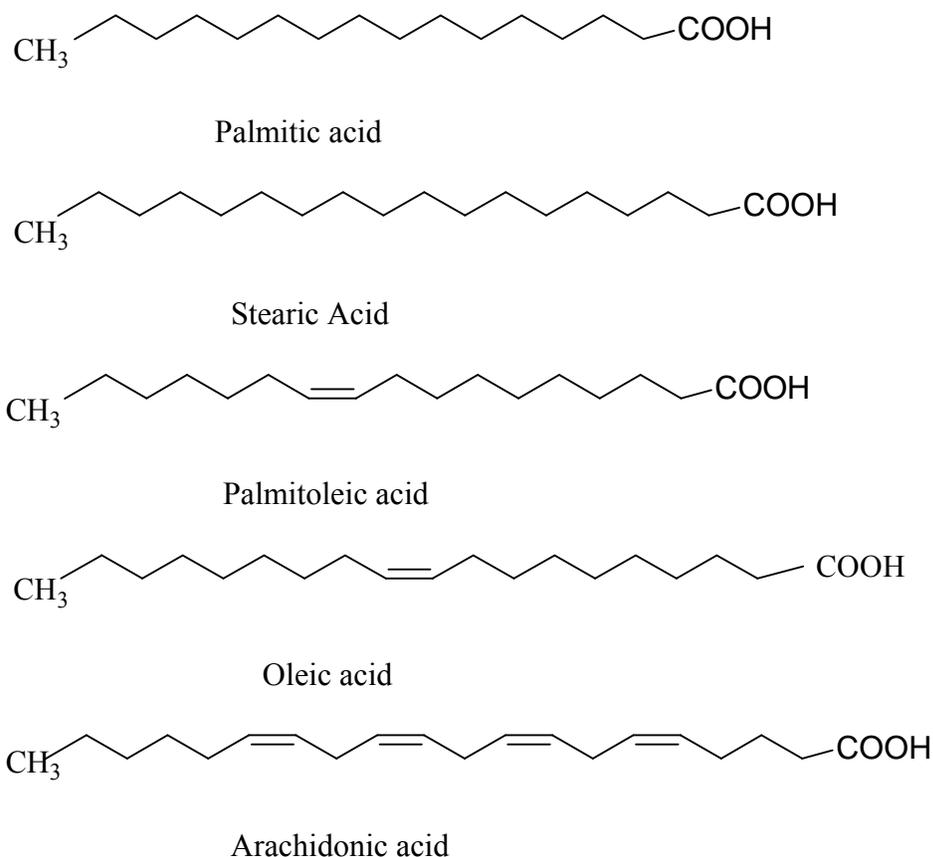


Figure 1. Structures of saturated and unsaturated fatty acids.

These fatty acids occur naturally in plants but not in animals. Some of the fatty acids have to be taken through diet, and they are referred to as essential fatty acids. Examples of essential fatty acids are linoleic, linolenic, and arachidonic acid. The other forms of fatty acids that are produced in the body and thus are not required in the diet are known as nonessential fatty acids.

1.2c. Nomenclature of fatty acids

According to the International Union of Pure and Applied Chemistry system of nomenclature, these fatty acids are named based on number of carbon atoms and the position of the double bond as shown in Table 1. The carboxylic carbon is numbered as one, and all

other carbon atoms are numbered relative to the carboxylic carbon. For example, in 9, 12-octadecanoic acid, the 9, 12 indicates the positions of double bonds at the carbon atoms.

An alternative nomenclature for fatty acids is based on the omega reference, indicating the number of carbon atoms, double bonds, and the position of the double bond relative to the omega carbon, which is the terminal carbon at the methyl end of the fatty acid (2).

Table 1. Nomenclature of fatty acids

Names		Abbreviations	
Trivial	IUPAC	Carboxyl-reference	ω -reference
Palmitic acid	Hexadecanoic acid	16:0	16:0
Stearic acid	Octadecanoic acid	18:0	18:0
Oleic acid	9-octadecanoic acid	18:1; Δ 9	18:1(ω -9)
Linoleic acid	9, 12-octadecanoic acid	18:2; Δ 9, 12	18:2(ω -6)

1.2d. Biological membranes

Fatty acids are the main components of biological membranes as shown in Figure 2. These lipids mostly are in an esterified form, rather than in the free form in the biological membrane (1). The biological membranes are doubled leaflets made up of different types of lipids like glycerophospholipid and sphingolipid.

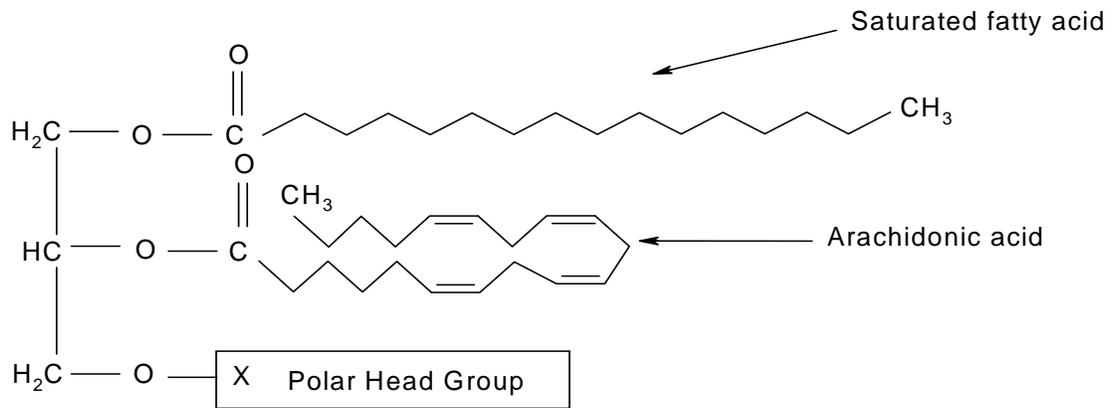


Figure 2. Diagram of membrane phospholipids (1).

In the glycerophospholipid, two different fatty acids are attached to the first carbon atom of SN-1 position and second carbon atom of SN-2 position of glycerol by an ester linkage, and a polar head group is attached to SN-3 position of third carbon by a phosphodiester linkage, as shown in Figure 3.

Arachidonic acid is a polyunsaturated fatty acid present in the cell membrane (3), and it is mainly located at the SN-2 position of phospholipids, where it is liberated by the action of the enzyme phospholipase (1). The phospholipase enzyme is activated by certain growth factors, cytokines, circulating hormones, and calcium (4-5).

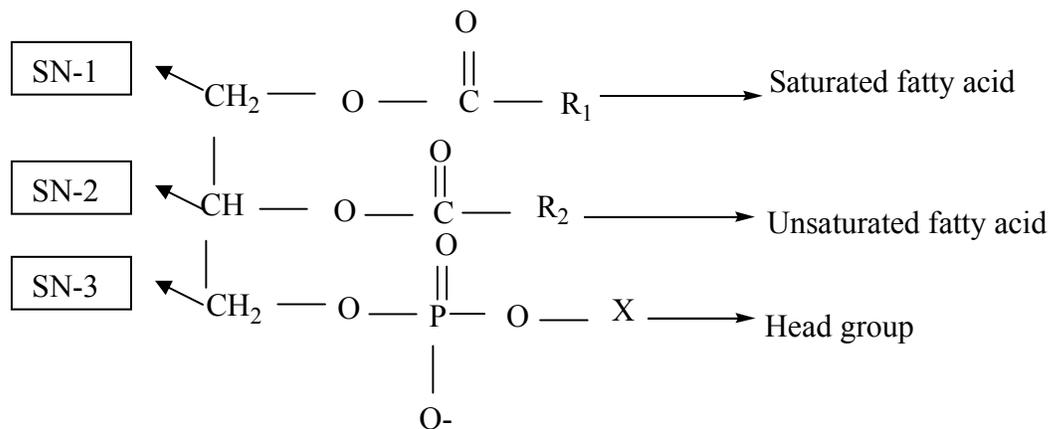


Figure 3. Structure of glycerophospholipid (1).

1.2e. Arachidonic acid metabolism

The arachidonic acid released by phospholipase can be metabolized by different enzymes such as cyclooxygenase, lipoxygenase, and cytochrome P450, which produce a variety of metabolites (6, 7). These metabolic pathways are summarized in Figure 4.

Initial metabolism by the cyclooxygenase yields PGH_2 , which is further metabolized to various prostaglandins and thromboxanes, known as prostanoids. Leukotrienes comprised of epoxyeicosatrienoic acids (EETs) are produced by lipoxygenase and cytochrome P450. All of the metabolites of arachidonic acid are collectively known as eicosanoids, a term that was introduced by Corey in 1980 (8). In all of these reactions, enzymes play a vital role in the metabolism of arachidonic acid. Alternatively, arachidonic acid can also be oxidized in the absence of enzyme. Non-enzymatic lipid oxidation is the pathway by which arachidonic acid can be metabolized in the absence of enzyme to produce a series of compounds known as the isoprostanes (9). Alternatively, the liberated arachidonic acid from phospholipids can be used for phospholipid biosynthesis by gaining reentry or reconstitution into the membrane (1).

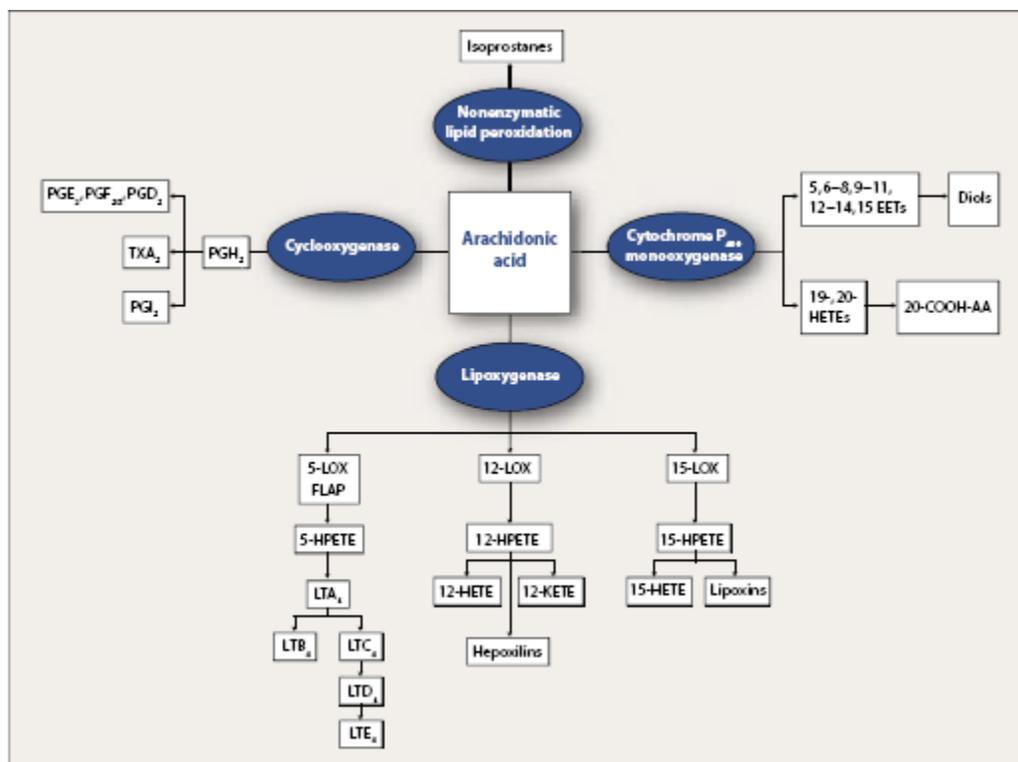


Figure 4. Metabolism of Arachidonic Acid (6).

1.3. Prostaglandins

1.3a. Chemical Structure

All of the metabolic products of arachidonic acid contain 20 carbon atoms and include a variety of compounds known as prostaglandins, leukotrienes, and thromboxanes, some of whose structures are shown in Figure 5. The fundamental unit of all prostaglandins is known as prostanic acid. A Swedish scientist, Von Euler, originally introduced the term *prostaglandin* in 1935 (10). He discovered the prostaglandins in semen in 1930. He thought that the prostaglandins were derived from the prostate gland, but they were originally found in seminal vesicles (11). Extensive research was done to determine the chemical structure of prostaglandins, and the structure of the prostaglandins was revealed in 1960 by Bergstorm

(8). The prostaglandins are characterized by a five-membered ring, and the thromboxanes have a six-membered ring containing two oxygen atoms. The structure of the leukotrienes possesses three conjugated double bonds.

The substituent groups on the cyclopentyl ring are used to categorize the different types of prostaglandins. For example, the five-membered ring of the PGE₂ prostaglandin possesses a keto group and a hydroxyl group, and the remainder of the structure contains two double bonds. In contrast, the PGF_{2α} has two hydroxyl groups in the five-membered ring and a single double bond in the structure as shown in Figure 5.

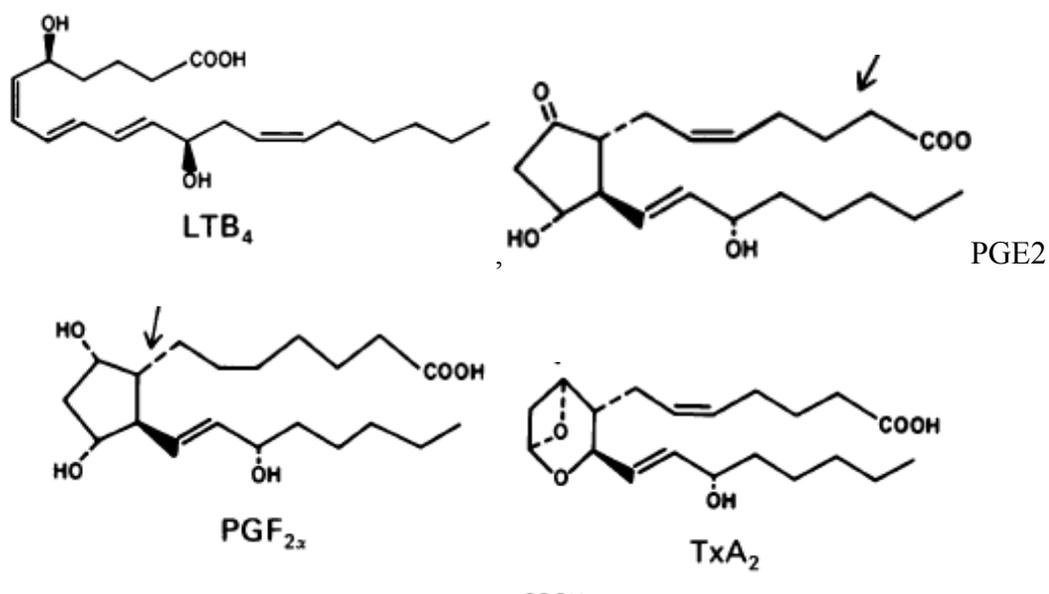


Figure 5. Structures of individual eicosanoids: PGF_{2α} and PGE₂ are prostaglandins, LTB₄ is a leukotriene, TXA₂ is a thromboxane (12).

1.3b. Physiological and pathophysiological role of prostaglandins

Prostaglandins act as chemical mediators in inflammation, which are produced during injury. Prostaglandins are thought to play a significant role in the development of cancer, Alzheimer's disease, arthritis, Type II diabetes, and other diseases. Many non-steroidal anti-inflammatory drugs (NSAIDs) have been developed to reduce inflammation. NSAIDs reduce inflammation by inhibiting the activity of prostaglandin H2 synthase, otherwise known as the cyclooxygenase enzyme, which is responsible for production of the precursor to various prostaglandins. Even though the prostaglandins are responsible for certain pathological disorders, these prostaglandins are also essential for performing certain beneficial physiological functions such as contraction of smooth muscle (uterine, gut, blood vessel). Von Euler and Goldbatt, chemical psychologists, indicated that the contraction of the muscle and lowering of blood pressure via vasodilation is due to the action of certain prostaglandins (13).

Prostanoids are produced differentially in various tissues and sometimes exert opposing effects in the same tissue. For example, PGE₂ and PGF_{2α} are produced by every organ except red blood cells, whereas thromboxane and the prostacyclins are produced by only platelets and endothelials (14, 15). Thromboxane and PGF_{2α} function as vasoconstrictors, whereas prostacyclin and PGE₂ act as vasodilators. Thromboxane promotes aggregation of the platelets, whereas prostacyclin is responsible for prevention of aggregation of platelets; thus they should be in balance to maintain the homeostasis of the body.

1.3c. Substrates

Arachidonic acid acts as a substrate for the cyclooxygenase enzyme that leads to the production of prostaglandins. Alternative substrates for the cyclooxygenase enzyme include dihomolinolenic acid and eicosapentanoic acid, which are converted to the prostaglandins indicated in Figure 6 by the cyclooxygenase enzyme (8). Arachidonic acid, dihomolinolenic acid, and eicosapentanoic acid all possess 20 carbon atoms.

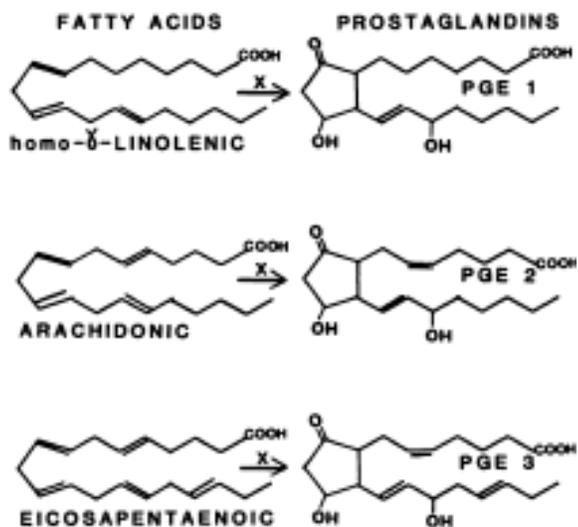


Figure 6. Conversion of 20-carbon polyunsaturated fatty acids to prostaglandins by the cyclooxygenase enzyme (8).

1.4. Cyclooxygenase enzyme or prostaglandin H2 synthase

1.4a. Isoforms of the cyclooxygenase enzyme

The prostaglandin H2 synthase enzyme exists in two isoforms, prostaglandin-H-synthase-I or COX-I, and prostaglandin-H-synthase-II or COX-II. The two isoforms of the enzyme exist in all mammalian cells. Both isoforms of the enzyme are bound to the

membrane (16). COX-I is constitutively expressed (17), whereas COX-II is an inducible form of the enzyme, which is expressed during inflammation through the action of cytokines and growth factors (18-22). The prostaglandins that are produced by COX-I are responsible for protection of the gastric mucosa, whereas the prostaglandins produced by COX II are responsible for the spread of inflammation and the production of pain (23).

COX-I and COX-II are similar in structure as shown in Fig. 7, where the two helices of COX-I and COX-II are perfectly superimposable.

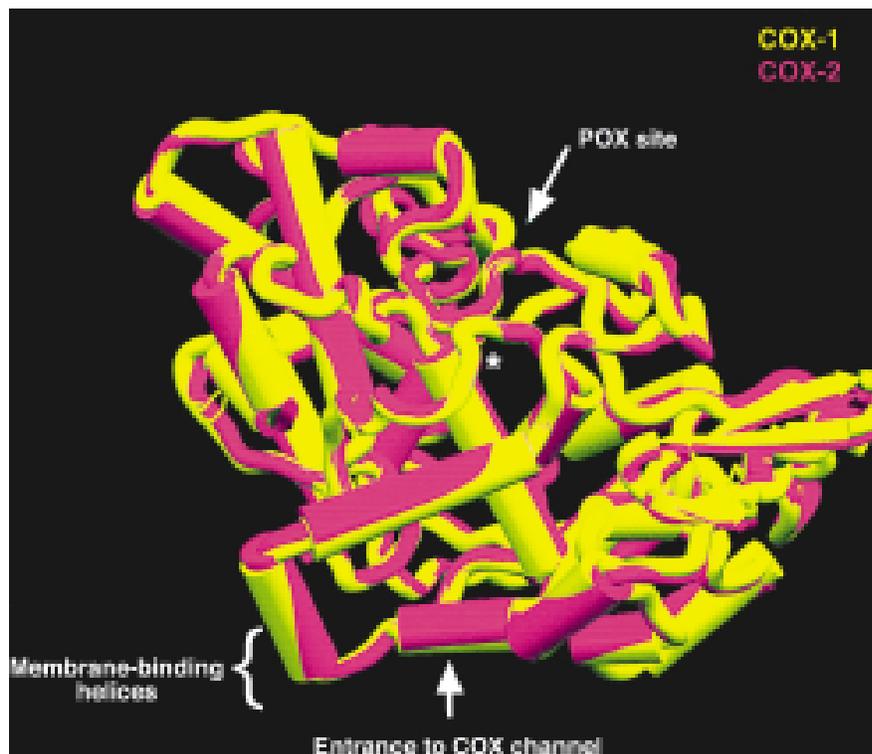


Figure 7. Superimposition of the structures of COX I and COX II (8).

The peroxidase active site lies exactly opposite to the COX active channel. As shown in Figure 7, the asterisk indicates the COX active site, which lies near the center of molecule.

1.4b. Arachidonic acid interaction with the cyclooxygenase enzyme

Crystallographic studies have revealed that the active site of the cyclooxygenase enzyme contains a hydrophobic channel. (25-28). Smith et al. have shown that arachidonic acid makes hydrophobic as well as hydrophilic contacts with the cyclooxygenase enzyme, as shown in Figure 8. It makes a total of 48 hydrophobic contacts and 2 hydrophilic contacts with the residues present in the hydrophobic channel of cyclooxygenase protein. Arachidonic acid bound in the active site of COX is in the form of an extended L shape (28). Several mutational studies have been performed to identify the role of these residues as they interact with the cyclooxygenase enzyme. The residues appear to play an essential role in proper positioning of arachidonic acid to generate the metabolic intermediate, PGG₂, by promoting the abstraction of 13-S- hydrogen (29).

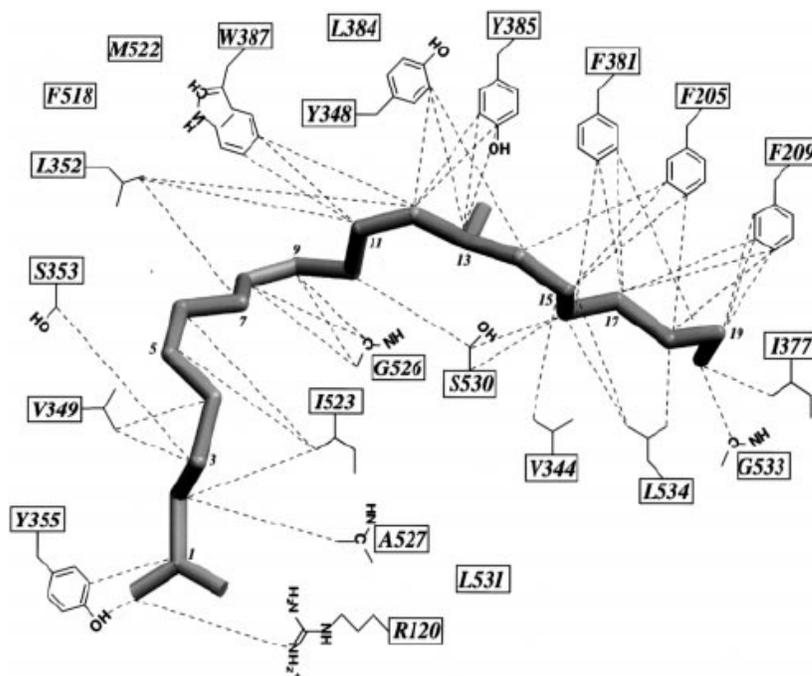


Figure 8. Arachidonic Acid interaction with residues of cyclooxygenase enzyme (24).

1.4c. Activity associated with the cyclooxygenase enzyme

Bergstorm et al. (31) first identified that prostaglandins are produced from arachidonic acid by the cyclooxygenase enzyme. Lands et al. (31) have confirmed the principal enzyme responsible for conversion of arachidonic acid to PGH_2 is the cyclooxygenase enzyme. The cyclooxygenase enzyme is a bifunctional enzyme that possesses two activities, a cyclooxygenase activity and a peroxidase activity (30).

Arachidonic acid is converted to PGG_2 by the cyclooxygenase activity, and by peroxidase activity the hydroperoxyl group at the 15th position of PGG_2 is converted to an alcohol, giving PGH_2 prostaglandin endoperoxide (PGH_2) as shown in Figure 9 (32-34). The generation of PGG_2 involves the abstraction of the 13S-hydrogen from arachidonic acid to yield the arachidonyl radical followed by the addition of oxygen at carbons 11 and 15 of the arachidonyl radical.

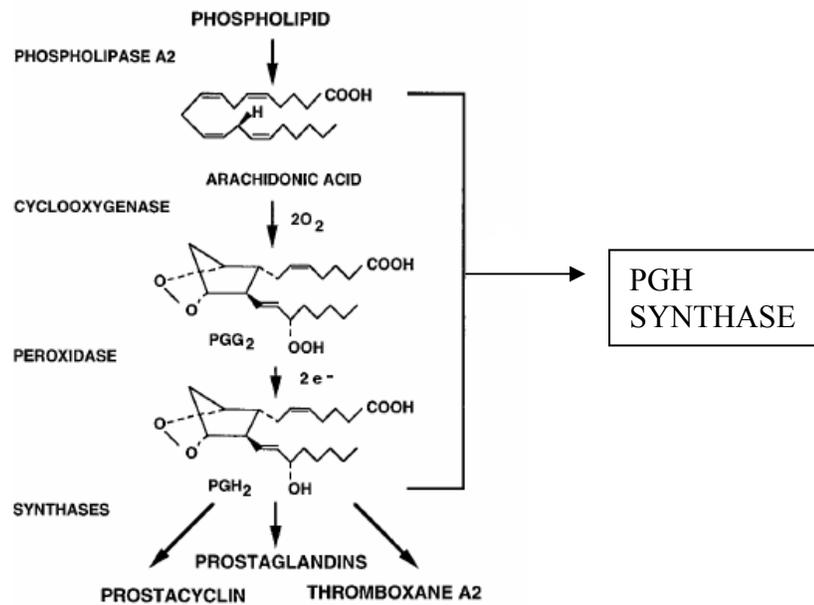


Figure 9. Prostanoid biosynthetic pathway (35).

1.4d. Role of hydroperoxides in cyclooxygenase activity:

Hydroperoxides of arachidonic acid play a significant role in the generation of prostaglandins by stimulating the cyclooxygenase activity. It is unknown as to whether arachidonic acid possesses the hydroperoxide inherently or that the cyclooxygenase enzyme is responsible for generation of hydroperoxides in arachidonic acid.

1.4e. Reducing agents used for cyclooxygenase activity:

There are several reducing agents such as triphenylphosphine and tributylphosphine that are available to reduce the substrate that is arachidonic acid. Among the different reducing agents that are available, the triphenylphosphine is the mild reducing agent that is used for reduction of hydro peroxides of arachidonic acid to alcohol. It is the preferred reducing agent as it does not affect the double bonds and the carbonyl groups present in arachidonic acid.

1.5. Assays for the activity of cyclooxygenase

1.5a Enzyme-linked immunosorbent assay (ELISA)

ELISAs are routinely used for the quantification and detection of proteins, hormones, and antigens produced in a biological process. The proteins or antigens are adsorbed on to an inert solid support. The antibodies that specifically bind to a particular antigen are added. The unbound antibodies are washed off by using a suitable solvent. The antigen bound to an antibody can be detected by incorporation of enzyme directly to the antigen-antibody complex or using a secondary antibody which is specific to the primary antibody that has been added previously, followed by addition of substrate. The enzyme catalyzes the reaction mixture after addition of substrate and develops colour change, which is measured by a spectrophotometer.

Prostaglandins are routinely analyzed by the Enzyme Linked Immunosorbent Assay

(ELISA), which is a simple, sensitive, and selective method (36). The major drawback with the ELISA is that only a particular prostaglandin, PGE₂, can be quantified; PGE₂ is a decomposition product of PGH₂(38). However, preliminary work with COX preparations identified five metabolites of arachidonic acid including PGH₂ and PGG₂ (39). Thus various ELISA assays have to be established for the quantification of all prostaglandins, which is a time consuming process. Kramer and his coworkers mentioned that ELISA technique is unsuitable for measurement of prostaglandins in biological samples. Moreover they also point out that ELISA technique does not differentiate between n-6 and n-3 metabolites (37). The major limitation of this technique is that it would be difficult to analyze the prostaglandin of interest as there may be production of other prostaglandins, which may cross-react with the antibody used in the ELISA assay (36).

1.5b. Oxygen consumption assay (oxygen electrode assay)

The oxygen consumption assay is used to measure the cyclooxygenase activity of the enzyme. The arachidonic acid undergoes oxidation to yield PGG₂, which further undergoes reduction to produce PGH₂. The cyclooxygenase activity of the prostaglandin-H-synthase enzyme is measured based on the consumption of oxygen molecules. The oxygen consumption assay is easy to use and it is a continuous assay. Major limitations of this technique are that it requires a continuous supply of oxygen, yields no information about the products formed, and requires a very high amount of enzyme (41). Several experiments were carried out to study the cyclooxygenase activity by using the oxygen electrode assay (42).

1.5c. High performance liquid chromatography

With the advancement in technology, other assay methods have been developed for the analysis of prostaglandins. Various chromatographic techniques have been developed for

the analysis of prostanoids. Thin layer chromatography detects different prostaglandins based on retention factor, but detection of thromboxanes with thin layer chromatography is difficult (40).

Mikhail Tswett, a Russian scientist in the twentieth century, invented the separation technique known as chromatography (43). Chromatography is a technique that is used to separate the components of mixture into individual components (43). There are two types of chromatography based on the type of mobile phase used: high performance liquid chromatography (HPLC), where the mobile phase is liquid, and gas chromatography, where the mobile phase is a gas.

HPLC is the widely used analytical tool for the separation of various prostaglandins. The analytes are separated based on affinity to the stationary phase. Figure 10 shows the components of the HPLC. The system consists of a mobile phase reservoir to hold the solvent, a pump that pumps the solvent through the column, a data station (computer), which collects the chromatograms and calculates the area under the each peak, a column that contains the stationary phase, an injection valve to deliver the sample to the column, and a detector that monitors the presence of analytes.

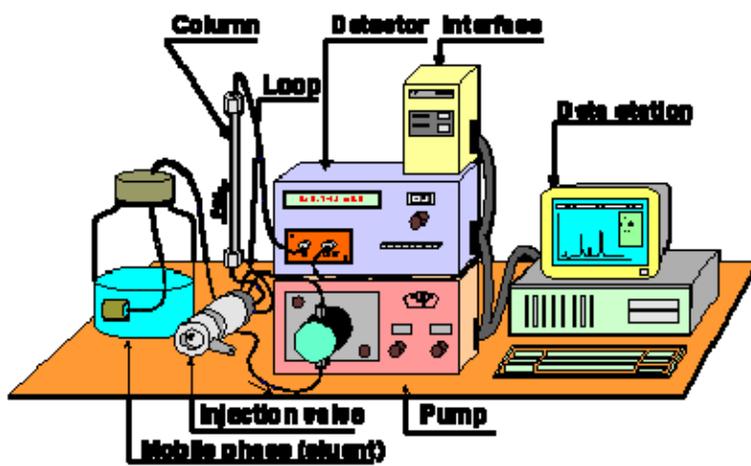


Figure 10. Block diagram of an HPLC (44).

The PGE₂ in the biological matrices can be identified by using HPLC equipped with a UV-Visible detector (45). The HPLC technique is not restricted to biological matrices and has been widely employed for the analysis of prostaglandins in marine organisms by using a fluorometric detector (46). HPLC coupled to electro-spray ionization mass spectrometry has also been used for analysis of prostaglandins in human dermal fibroblasts and in human cancer cells (47).

The HPLC technique offers a major advantage in that it requires no derivatization of the sample, as is needed by the analytical technique, gas chromatography. However, it is more time-consuming than other separation techniques.

1.5d. Gas chromatography-mass spectrometry

Gas chromatography is a widely used analytical separation technique for volatile compounds (48). The components of the gas chromatography include the carrier gas, flow controller, column, injector port, and detector as shown in Figure 11. The carrier gas constitutes the mobile phase, which conveys the sample to the column. Helium, nitrogen, and hydrogen are the most commonly used carrier gases. These gases are inert and do not possess any chemical reactivity towards the sample. The flow controllers are used to regulate the flow of mobile phase through column. The sample is injected through the injector port by using a syringe. The syringe pierces through the rubber septum of injector port and carries the sample to the column. The two types of column that are available are the open tubular column and the packed column (43).

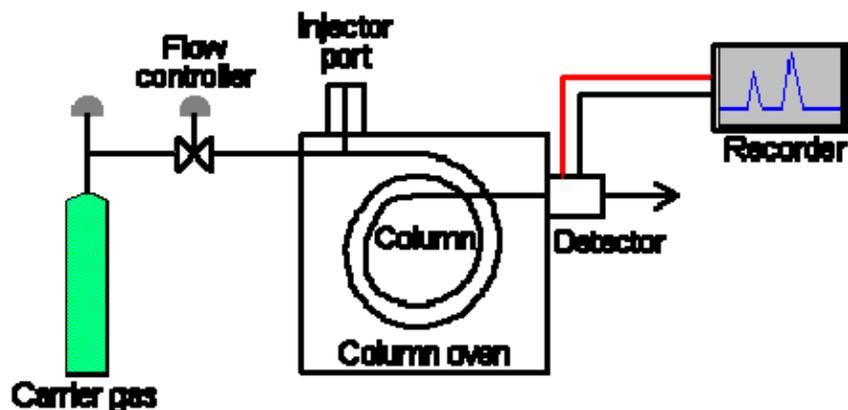


Figure 11. General components of gas chromatography (49).

The components of the mixture are vaporized in the injector port, and they are separated based on their affinity to the stationary phase. The lower the affinity to the stationary phase, the earlier the compounds will elute when compared to the compounds that possess higher affinity to stationary phase. Retention time is the time taken for injected sample to reach the detector. Based on the retention time, the components of the mixture are identified qualitatively.

The mass spectrometer aids in the detection of compounds based on the mass to charge ratio of the molecule. The essential components of all mass spectrometers include the ionizer, the ion analyzer, and the detector. The source of ions for analysis is the ionizer, where the sample is vaporized and subjected to the ionization process. Ionization techniques include electron impact ionization and chemical ionization techniques. Both of these techniques are commonly used for analysis of volatile compounds (50). The electron impact ionization utilizes high-energy electrons to ionize the molecular vapor into ions. The electron impact ionization is also known as a hard ionization technique, because high-energy electrons bombard the molecular vapor.

Electron impact ionization is used in the analysis of samples in the present study. The chemical ionization technique requires less energy than electron impact ionization. Hence it is known as a softer ionization technique. In the chemical ionization technique, chemical reagent gases such as methane or ammonia are commonly used. The ions required for the analysis are produced by interaction of molecular vapor with chemical reagent ions, which are produced by bombardment with electrons. Mass analyzers then analyze the ionized samples. The commonly available mass analyzers are the quadrupole and time-of-flight analyzers. These analyzers analyze the sample based on its mass to charge ratio. The time-of-flight mass spectrometer accelerates the ions produced by the ionizer, and thus all the ions of the sample travel with the same kinetic energy. The fragmented molecules have the same kinetic energy and thus reach the detector based on the mass of the fragmented molecule ions. Thus, the lighter ions reach the detector earlier than the heavier ions, as shown in Figure 12.

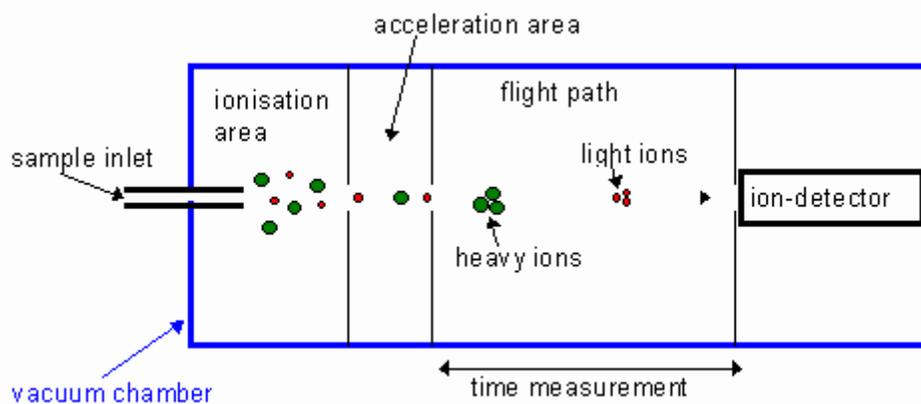


Figure 12. Time of flight spectrometer (51).

The drawback with the gas chromatography technique is that it requires derivatization to promote volatility and heat stability for the samples to be analysed. The GC-MS technique is the favored analytical technique for analysis of a wide range of fatty

acids in various samples. The time required for the analysis of samples is low when compared to all other techniques that have been employed for the analysis of prostaglandins. The derivatized samples for gas chromatography analysis are very stable, which allows for their long-term storage prior to analysis.

Chapter 2. Experimental Procedure

2.1 Preparation of arachidonic acid for cyclooxygenase reaction

An aliquot of 10 μL of arachidonic acid was routinely taken from a stock of 100 mg/mL ethanol in a test tube, which was diluted with 990 μL of ethanol to give a concentration of 1 mg/mL. From this stock 100 μL was arachidonic acid solution transferred to a clean tube, the solvent was evaporated, and the components of the reaction were added to a final volume of 0.5 mL. The final concentration of arachidonic acid used for the cyclooxygenase reaction was therefore 0.2 mg/mL.

2.2 Preparation of reduced form of arachidonic acid

Five milligrams of triphenylphosphine, a reducing agent, was dissolved in 0.2 mg/mL concentration of arachidonic acid and the mixture was allowed to incubate for 30 min at room temperature.

2.3 Treatment of reduced arachidonic acid with cyclooxygenase enzyme

Solvent was then completely removed from the preparation of reduced arachidonic acid by drying under nitrogen, and 142 μL of distilled deionized water was added to the test tube. One hundred microliters of phosphate buffer, pH 7.8, 50 μL of a mixture of 1% Tween-20:1% octylglucopyranoside, 100 μL of glycerol, 100 μL of 2.5 mM phenol, and 8 μL of COX-I enzyme (10kunit, Cayman Chemical) were added to a test tube. The mixture was sonicated for 1 min, and the test tube was then placed in a shaker bath at 37 °C for 2 min. The arachidonic acid preparation was then added to the test tube containing all ingredients to make a total volume of 500 μL . The reaction was allowed to proceed for 15 min, after which it was stopped by transferring the test tubes to an ice bath and by addition of 200 μL methanol and 100 μL 1 M citric acid. The sample was then extracted with 1 mL ethyl ether, vortexed for 1 min, and centrifuged for 1 min from 1200 to 1500 rpm. The extraction process was repeated three times. The extracted organic layer was dried over anhydrous sodium sulfate, and the solvent was completely removed by drying under nitrogen, the dried sample was resuspended in 50 μL of ethyl acetate. The methyl ester, oxime, and silylated derivatives of the sample were prepared according to the procedure provided in the section 2.7.

2.4 Treatment of arachidonic acid with cyclooxygenase enzyme

Instead of the reduced form of arachidonic acid, in this experiment the non-reduced form of arachidonic acid was taken. The prepared concentration of arachidonic acid was

taken in a test tube without the treatment of triphenylphosphine. The remaining procedure for carrying out the reaction was the same as described above.

2.5 Treatment of reduced arachidonic acid with cyclooxygenase enzyme treated with triphenylphosphine

An amount of 0.005 g of triphenylphosphine was added to a test tube containing 8 μL of the cyclooxygenase enzyme-I. They were incubated for half an hour at room temperature. To this test tube 100 μL of phosphate buffer, 50 μL of 1% Tween: 1% octylglucoside, 50% glycerol, and 2.5 mM phenol were added. 100 μL of reduced arachidonic acid was evaporated under nitrogen, and this fatty acid was suspended in 142 μL of distilled deionized water. The remaining procedure was as discussed in the experiment where the reduced arachidonic acid was used with cyclooxygenase enzyme.

2.6 Treatment of denatured cyclooxygenase enzyme with arachidonic acid

Eight microliters of the cyclooxygenase enzyme was denatured by placing the test tube in a hot water bath (turbovap LV evaporator) at 60 $^{\circ}\text{C}$ for 10 min. One hundred microliters of arachidonic acid was evaporated under nitrogen, and fatty acid was suspended in 142 μL of distilled water. The rest of the experiment was carried out according to the procedure for treatment of reduced arachidonic acid with cyclooxygenase enzyme.

2.7 Preparation of derivatives

2.7a Preparation of methyl ester derivative

Six hundred microliters of 20% methanol in acetone was added to a test tube containing 2400 μL of acetone, and the mixture was vortexed for 1 min. A 2.0-M

trimethylsilyldiazomethane solution in hexane was supplied by Aldrich, and 10 μL of this reagent was added to 990 μL of heptane to give 0.02 M TMSD in heptane. The solvent-extracted sample in ethyl acetate was completely dried under nitrogen. To this sample was added 350 μL of 20% methanol in acetone and 100 μL of 0.02 M TMSD in heptane. The sample was vortexed for 1 min, incubated at room temperature for 15 min, vortexed for 1 min, and then dried under nitrogen to prepare the next derivative.

2.7b Preparation of oxime derivative

Prior to the preparation of oxime derivative, the methyl ester derivative was completely dried under nitrogen. Methoximation of carbonyl groups in the sample was accomplished by preparation of the oxime derivative. A 2% w/v methoxylamine-HCl solution in dried pyridine was prepared by addition of 8 mg of methoxylamine-HCl to 400 μL of pyridine. A volume of 50 μL of 2% w/v methoxylamine HCl in dried pyridine was added to the test tube containing the methyl ester derivative sample, which was completely dried under nitrogen. The sample was vortexed for 1 min and incubated at 60 $^{\circ}\text{C}$ for 90 min in a water bath (turbovap LV evaporator). After incubation the sample was prepared for the next derivative by completely drying under nitrogen.

2.7c Preparation of BSTFA derivative

The silylation of the hydroxyl group in the sample was done by preparing the BSTFA derivative. The oxime derivative of the sample was completely dried under nitrogen. The trimethylsilyl derivative was prepared by suspending the sample in 25 μL of pyridine followed by addition of 100 μL BSTFA reagent in 1% trimethylchlorosilane. The sample was capped, vortexed for 1 min, and incubated at 65 $^{\circ}\text{C}$ for 60 min in a water bath. The silyl

derivative of the sample was completely dried under nitrogen, and then suspended in 100 μ L ethyl acetate.

2.7d Purification of prepared derivatives

All the derivatives that were prepared sequentially in a test tube were then subjected to purification by chromatography on a silica column, eluted from the column with ethyl acetate, and the volume of the preparation was reduced to one-half of the original volume prior to GC-MS analysis. The synthetic scheme that shows the preparation of derivatives following oxime formation, methyl ester formation, and silylation of the standard PGE₂ is shown in Figure 13.

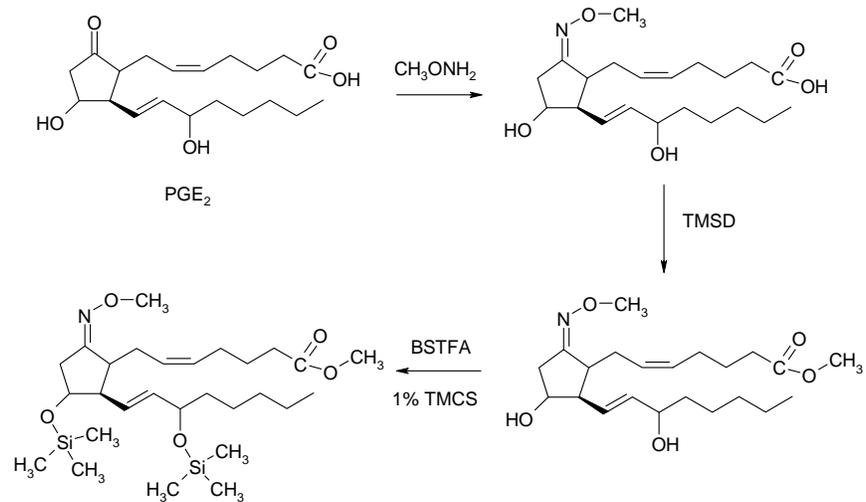


Figure 13. Synthetic scheme for production of the derivative of standard PGE₂ that was subjected to gas chromatography and mass spectral analysis.

2.8 Preparation of different concentrations of standards

All the standards shown in Table 2 were purchased from Cayman chemical. The methyl ester, oxime, and silylated derivatives of the standards were prepared according to the procedure provided in section 2.7. Only derivatives of the standards were prepared. The derivatives of the standards were used for identification of metabolites formed during the cyclooxygenase reaction.

Table 2. Standards used for identification of COX products
and the amount used in analysis.

Standard	Mol. Wt (g/mol)	Amount assayed (μg)
Prostaglandin F _{2α}	354.5	100
Prostaglandin E ₂	352.5	100
12(s)HpETE (hydroperoxy eicosatetraenoic acid)	336.5	10
11,12-DiHETrE(11,12- dihydroxy eicosatrienoic acid)	338.5	12.5
5, 15-DiHETE (dihydroxy eicosatetraenoic acid)	336.5	12.5

Only the methyl ester and silylated derivatives were prepared for the standards 12-HpETE (12-hydroperoxy eicosatetraenoic acid), 11, 12-DiHETrE (11, 12-dihydroxy eicosatrienoic acid), and 5, 15- DiHETE (5, 15-dihydroxy eicosatetraenoic acid) as there were no carbonyl groups in their structures for the preparation of oxime derivatives.

2.9 GC-MS conditions

All of the derivatized samples were analyzed on a model 3800 Saturn Varian gas chromatograph with a model 2200 mass spectrometer. The carrier gas was helium, which is inert, with a fused silica column, which was 30 m long with a diameter of 0.25mm and 0.25 μm film thickness. The mode of ionization of the samples was by electron impact ionization by injecting 1 μL of the sample on the GC column. The column oven temperature conditions for the experiment were in the range of 200-240°C, as shown in Table 3.

Table 3. Temperature programming for gas chromatography

Temperature(°C)	Rate(c/min)	Hold(min)	Total(min)
200	-	2.00	2.00
240	40.0	0.00	3.00
275	8.0	4.50	11.88

Chapter 3. Results

3.1 Treatment of arachidonic acid with triphenylphosphine

Thin layer chromatography has been used to indicate that triphenylphosphine plays a role in the conversion of hydroperoxides to alcohols. Thin layer chromatography was performed using the solvent system in the ratio of 90:10:1 (hexane, ether, acetic acid). Two TLC plates were used separately to run arachidonic acid that had not been treated with triphenylphosphine (TLC plate 1) and the arachidonic acid treated with triphenylphosphine (TLC plate 2) for half an hour. The examination of the TLC plates as shown in Figure 14 revealed that there was only one band for TLC plate 1, whereas TLC plate 2 has showed up two bands. As the reducing agent triphenylphosphine plays a vital role in conversion of hydroperoxides to alcohol, the extra band in the TLC plate is due to conversion of hydroperoxides in arachidonic acid to alcohols.

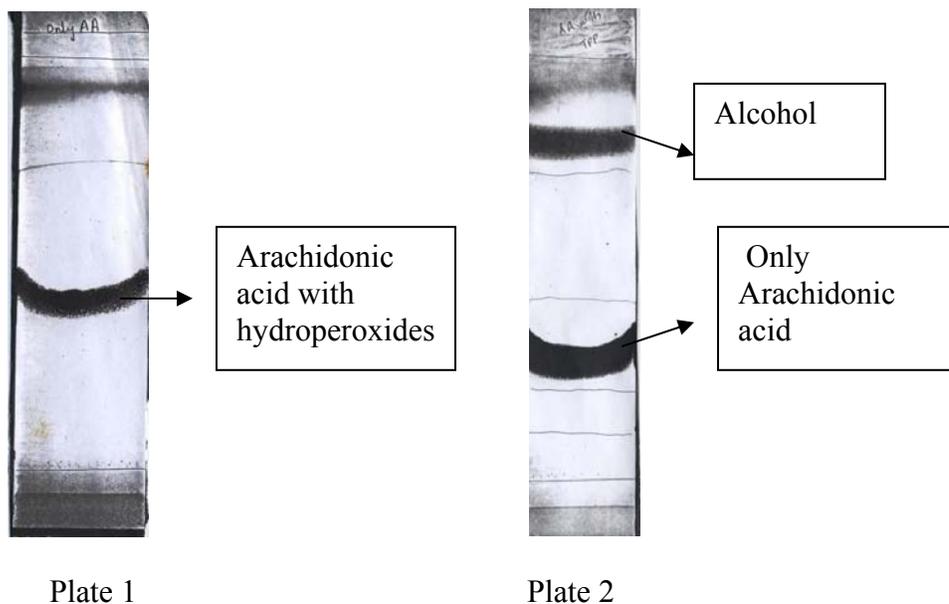


Figure 14. TLC of an arachidonic acid preparation (plate 1) and of arachidonic acid treated with triphenylphosphine (plate 2).

3.2 Treatment of arachidonic acid with cyclooxygenase enzyme

Experiments were first carried out to confirm that PGE₂ is produced by incubation of cyclooxygenase enzyme with arachidonic acid. The reaction with arachidonic acid was carried out at 37°C for 15 min, after which the reaction was quenched. The carboxylic acid moiety was modified to give the methyl ester, the carbonyl groups were modified to form the oxime, and then all hydroxyl groups were derivatized to form the trimethylsilyl moiety. Injection of the treated sample in the GC-MS resulted in the chromatogram that is shown in Figure 15. Two peaks were evident in the chromatogram and are labeled A1 and B1.

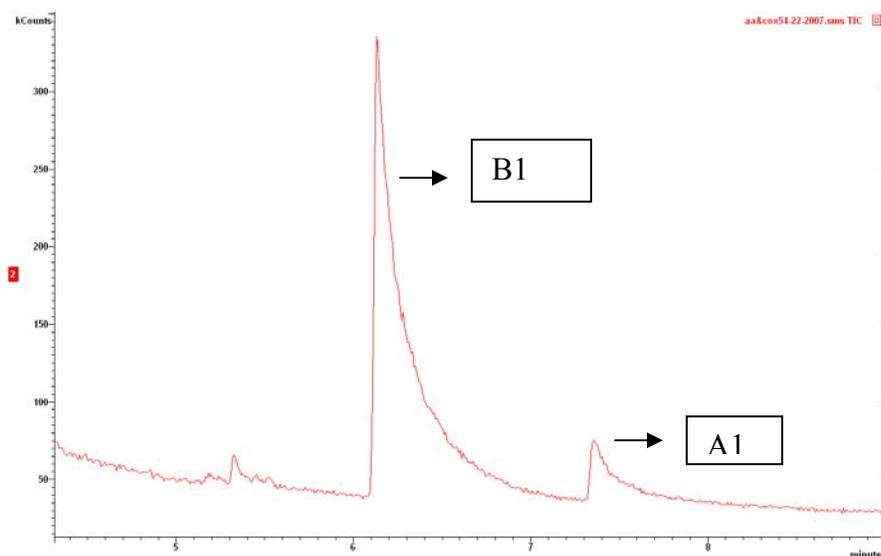


Figure 15. Gas chromatogram of the derivative of the product resulted from reaction of cyclooxygenase enzyme with arachidonic acid.

Peak A1 elutes from 7.311 to 7.505 min. The mass spectrum of peak A1 assayed at the apex is shown in Figure 16, which possesses major ions with mass to charge ratios (m/z) of 512.5, 455.2, 278.9, 168.1 and 149.0.

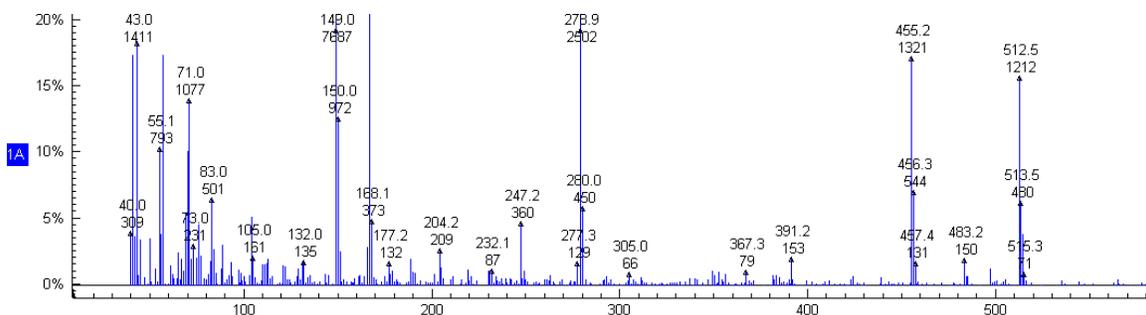


Figure 16. Mass spectrum of peak A1 at 7.3 min.

The peak B1 of the chromatogram shown in Figure 14 eluted from 6.089 min to 6.402 min. The mass spectrum of peak B1 assayed at the apex (6.131 min) of the chromatographic peak is shown in Figure 17, which indicates the presence of major ions with mass to charge ratios (m/z) of 73, 147, 204, 231, and 475.

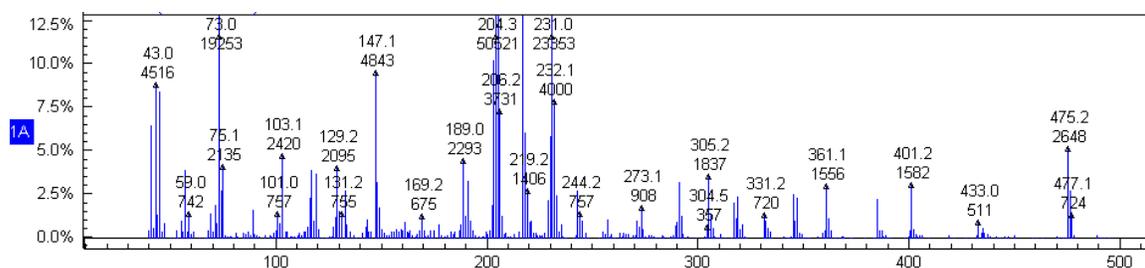


Figure 17. Mass spectrum assayed at the peak B1.

3.3 Incubation of reduced arachidonic acid with cyclooxygenase enzyme

Arachidonic acid preparations were treated with triphenylphosphine to reduce hydroperoxy arachidonic acid to alcohols. Incubation of the treated arachidonic acid with cyclooxygenase enzyme gave the chromatogram shown in Figure 18, which is qualitatively similar to that of the chromatogram obtained when cyclooxygenase enzyme was incubated with arachidonic acid (see Figure 15). The two peaks of the chromatograms in Figure 18 have the same retention times as those in Figure 15. The two chromatograms overlaid are shown in Figure 19.

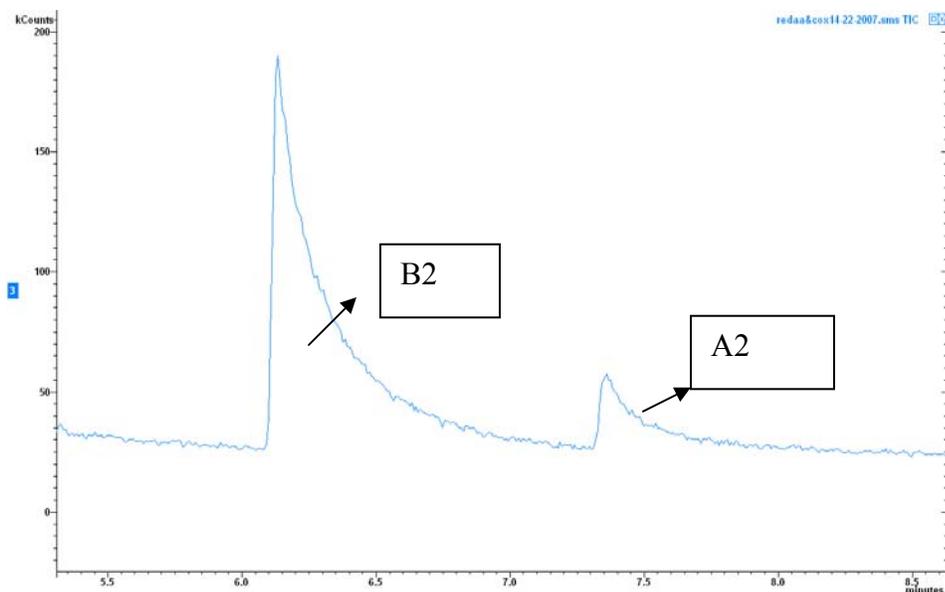


Figure 18. Gas chromatogram of the derivative of product produced by treatment of reduced arachidonic acid with cyclooxygenase enzyme

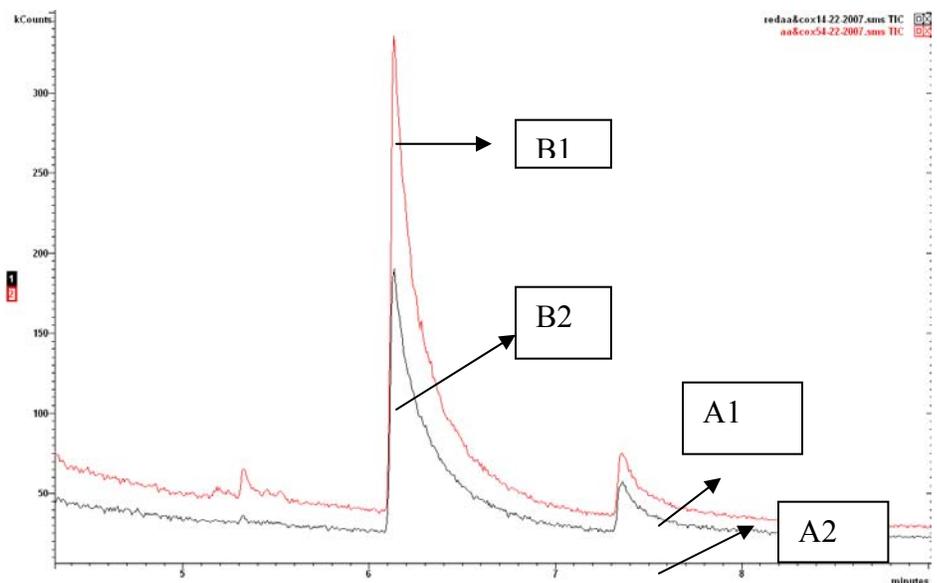


Figure 19. Chromatograms generated by reaction of cyclooxygenase enzyme with arachidonic acid (light line) or with arachidonic acid treated with triphenylphosphine (dark line) prior to incubation with cyclooxygenase enzyme.

The total ion intensity of peak B1 and B2 as shown in Figure 19 is greater than the ion intensity of peak A1 and A2, indicating that the product associated with this peak is most

likely produced to a greater extent than the product that gave rise to peaks A1 and A2. The two very small peaks eluted at 5.3 min, as shown in Figure 19, which are generated by reaction of cyclooxygenase with arachidonic acid and treatment of arachidonic acid with triphenylphosphine prior to incubation with cyclooxygenase enzyme, was due to the ethylacetate solvent background.

The A2 metabolite eluted from 7.303 min to 7.506 min. The mass spectrum of peak A2 has major ions with mass to charge ratios (m/z) of 512.5, 455.3, 391.3, 279.1, 149.1 and 71 as shown in Figure 20.

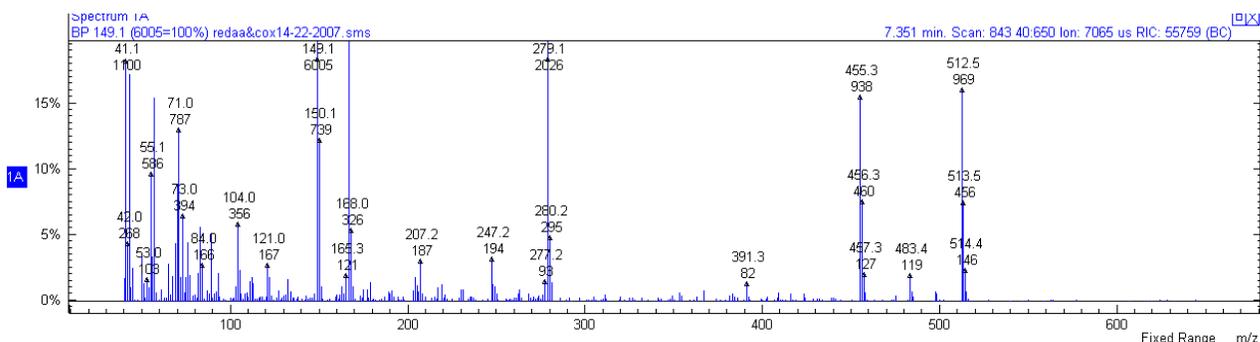


Figure 20. Mass spectrum of peak A2 produced by incubation of cyclooxygenase enzyme with arachidonic acid treated with triphenylphosphine.

The methyl ester, oxime, and silylated derivative of the analyte obtained by reaction of reduced arachidonic acid reaction with cyclooxygenase enzyme was analyzed by gas chromatography and mass spectrometry. One peak in the resultant chromatogram characterized as B2 elutes from 6.093 to 6.409 min. The mass spectrum of the peak B2 assayed at 6.134 min has major ions with mass to charge ratios (m/z) of 73.0, 147.1, 204.3, 231.1, and 475.2, as shown in Figure 21, which are the same ions as are observed for the B1 metabolite presented earlier. Thus, the identity of the B2 metabolite produced by

cyclooxygenase is the same as that of the B1 metabolite produced during incubation of arachidonic acid with cyclooxygenase.

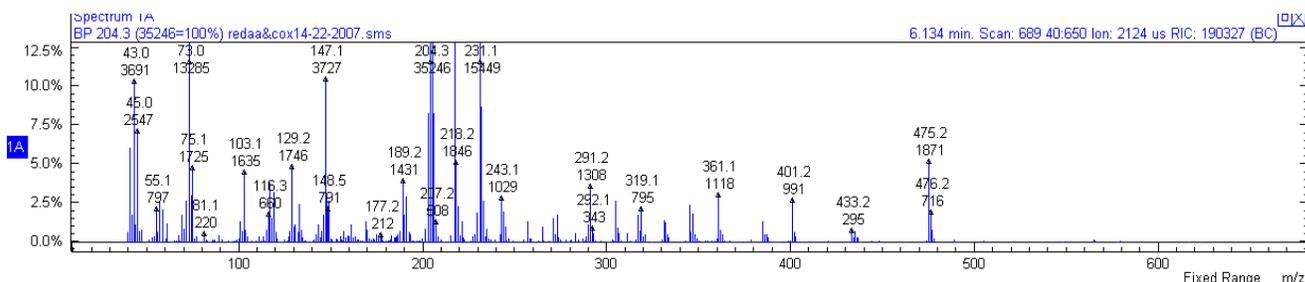


Figure 21. Mass spectrum of peak B2.

3.4 Incubation of reduced arachidonic acid and triphenylphosphine with cyclooxygenase enzyme

The treatment of arachidonic acid with cyclooxygenase enzyme and the incubation of reduced arachidonic acid with cyclooxygenase enzyme have generated similar chromatograms with elution of peaks A1, A2 and B1, B2 at identical retention times. So one more experiment was conducted where the cyclooxygenase enzyme was treated with triphenylphosphine with the assumption that any peroxides present in cyclooxygenase enzyme-I would be reduced with triphenylphosphine.

The prepared derivatives of the product resulting from reaction between cyclooxygenase enzyme treated with triphenylphosphine and reduced arachidonic acid were injected onto the GC and gave three different peaks as shown in Figure 22. This reaction possessed an extra peak C relative to those obtained for the other experiments carried out with cyclooxygenase enzyme. Peak A3 elutes from 7.324 to 7.509 min and the mass

spectrum of peak A3 has major ions with mass to charge ratios (m/z) of 512, 455, 279, 168, 149, and 73 as shown in Figure 23.

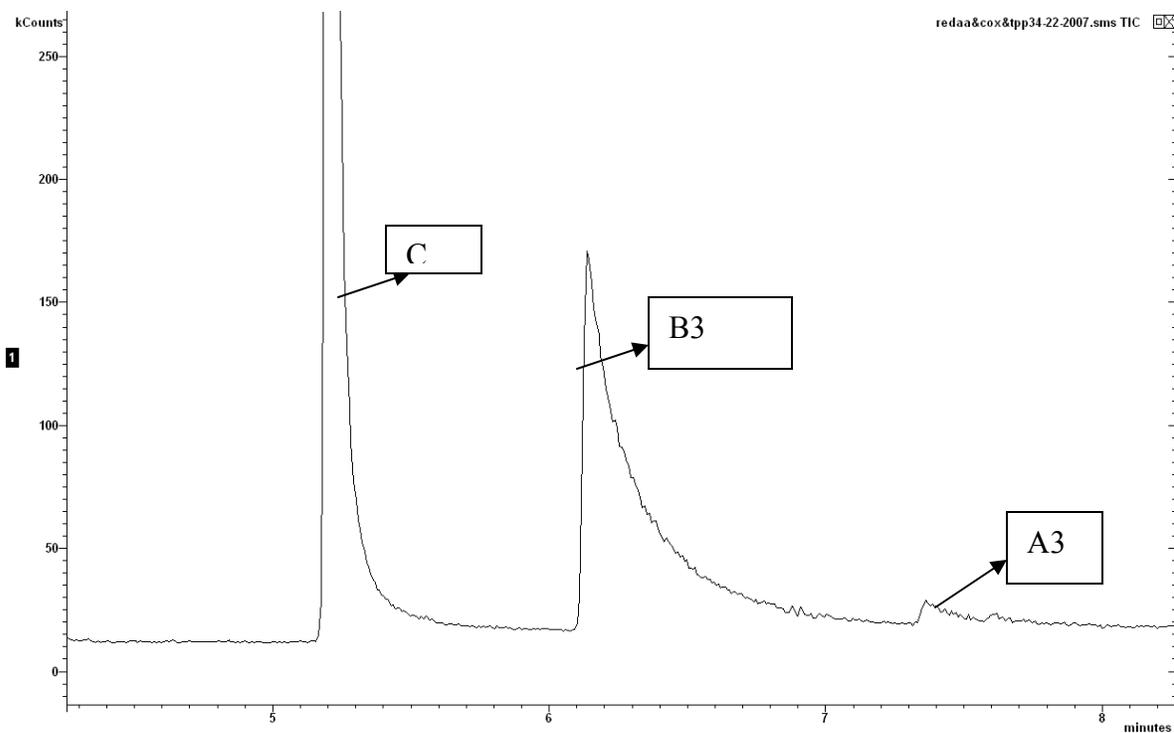


Figure 22. Chromatogram of the derivative of product produced by incubation of reduced arachidonic acid and cyclooxygenase enzyme treated with triphenylphosphine.

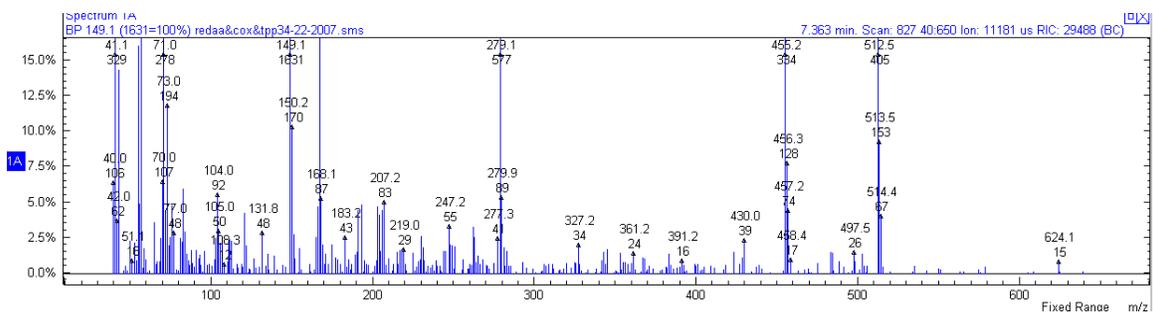


Figure 23. Mass spectrum of peak A3 of the product of the reaction between cyclooxygenase enzyme and triphenylphosphine-treated arachidonic acid and triphenylphosphine.

Peak B3 as shown in Figure 22 elutes on the gas chromatography column from 6.093 to 6.409 min. The mass spectrum of peak B3 is similar to the mass spectrum of peak B2 and peak B1 with the formation of similar ions with mass to charge ratios (m/z) of 73.0, 147, 204, 231, and 475, as shown in Figure 24.

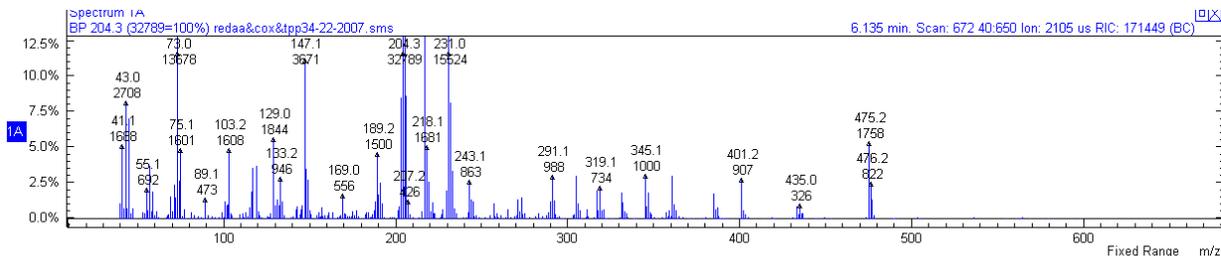


Figure 24. Mass spectrum of peak B3.

Peak C is a new peak that was not observed in all other experiments carried out with cyclooxygenase enzyme. Peak C elutes from 5.166 to 5.138 min and the mass spectrum of peak C shown in Figure 25 contains the major ions with mass to charge ratios (m/z) of 108, 188, 262, 264, 370, and 445.

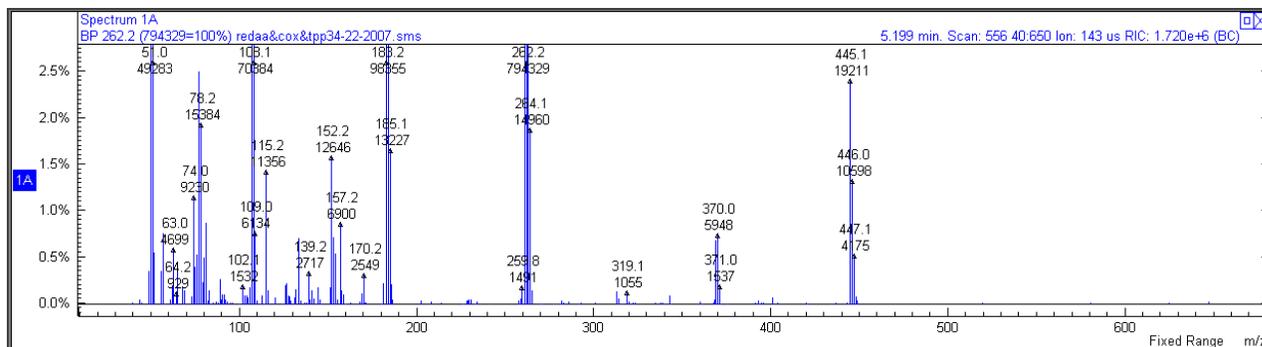


Figure 25. Mass spectrum of peak C generated by incubation of cyclooxygenase enzyme treated with triphenylphosphine and reduced arachidonic acid.

All of the major ions in the mass spectra of peak A1, peak A2, and peak A3 were the same as shown in Tables 4, 5, and 6. The relative abundances of the major ions in the A1 and A2

mass spectra were identical. However, the relative abundance of the ions 279.2, 167.2, and 149.2 for the mass spectrum of A3 were lowered relative to their abundance for A1 and A2.

Table 4. Major ions and their abundances relative to the 512.5 ion in the mass spectrum of the metabolite associated with peak A1.

Major ions in peak A1 mass spectrum	Peak Area Abundance	Abundance relative to the m/z 512.5
512.5	1212	1.00
455.3	1312	1.08
279.2	2502	2.06
167.2	2926	2.41
149.2	7687	6.34

Table 5. Abundances of the major ions relative to that of the m/z 512.5 ion in the A2 mass spectrum.

Major ions in peak A2 mass spectrum	Peak Area Abundance	Abundance relative to the m/z 512.5
512.5	969	1.00
455.3	938	1.00
279.2	2026	2.09
167.2	2986	3.018
149.2	6005	6.19

Table 6. Major ions in peak A3 mass spectrum relative to the abundance of the 512.5 ion.

Major ions in peak A3 mass spectrum	Peak area abundance of major ions in peak A3 mass spectrum	Abundance of major ions relative to the m/z 512.5
512.5	624	1.00
455.3	631	1.01
279.2	312	0.50
167.2	438	0.70
149.2	845	1.36

3.5 Treatment of denatured cyclooxygenase enzyme with arachidonic acid

An experiment was conducted to confirm that cyclooxygenase enzyme is required for production of all peaks generated in the above chromatograms. In this experiment, the enzyme was denatured by heating at 65 °C for three to five min. The methyl ester-oxime-silylated derivative of the reaction with COX and arachidonic acid was then injected into the GC.

The chromatogram in Figure 26 indicated that there was no generation of well-resolved peaks as had been observed in all other chromatograms. Even examination of the mass spectrum of the non-gaussian peak, as shown in the Figure 27, indicated no production of the major ions that were observed in other chromatograms of the cyclooxygenase reaction. Hence, the products A1 and B1 are products of cyclooxygenase metabolism of arachidonic acid.

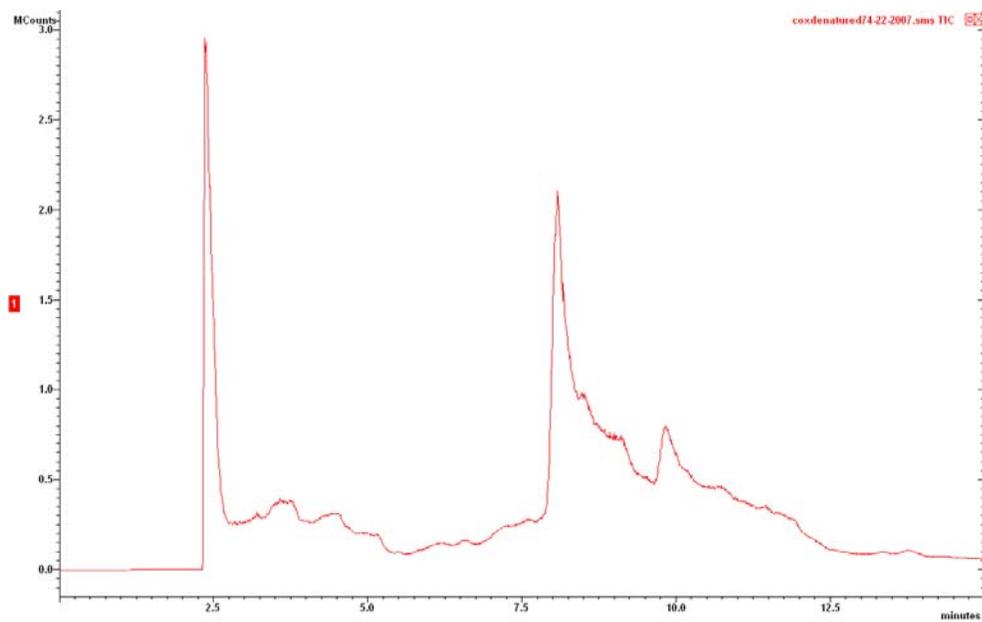


Figure 26. Chromatogram of the derivative product from reaction of denatured cyclooxygenase enzyme with arachidonic acid.

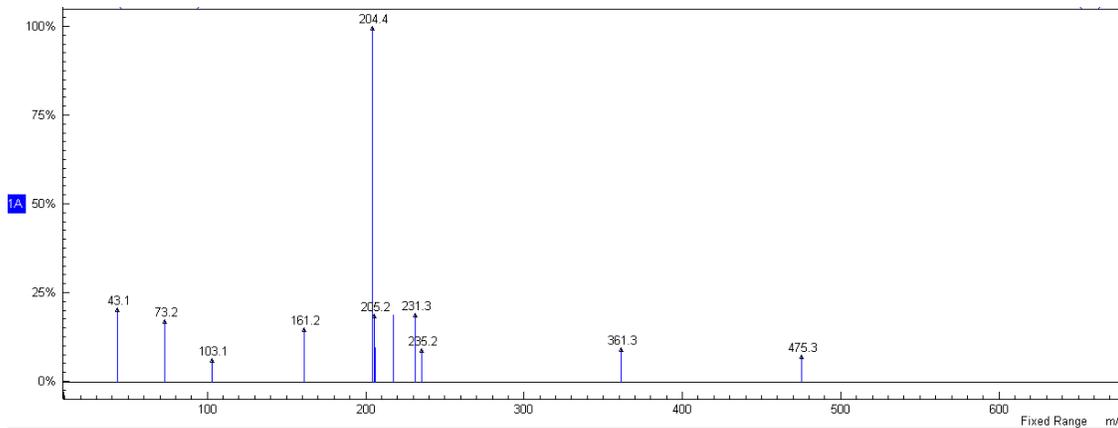


Figure 27. Mass spectrum of the derivative of denatured cyclooxygenase enzyme incubated with arachidonic acid.

3.6 Identification of chromatographic peaks

Attempts were made to identify the peaks produced under various conditions of incubation of arachidonic acid and cyclooxygenase enzyme. Figure 28 is an overlay of all three chromatograms produced by different experiments conducted with the cyclooxygenase enzyme. In all of the experiments, peaks at 7.3 min and 6.2 min were observed. The mass spectrum of peaks A1, A2, and A3 at 7.3 min are qualitatively the same in all of the three cases with major ions of mass to charge ratio of m/z of 512, 455, 391, 279, 73, and all have the same retention time. Even the mass spectrum of peaks B1, B2, and B3 at 6.091 min are identical with the presence of major ions with m/z of 73.0, 147.1, 204.3, 231.0, and 475.2.

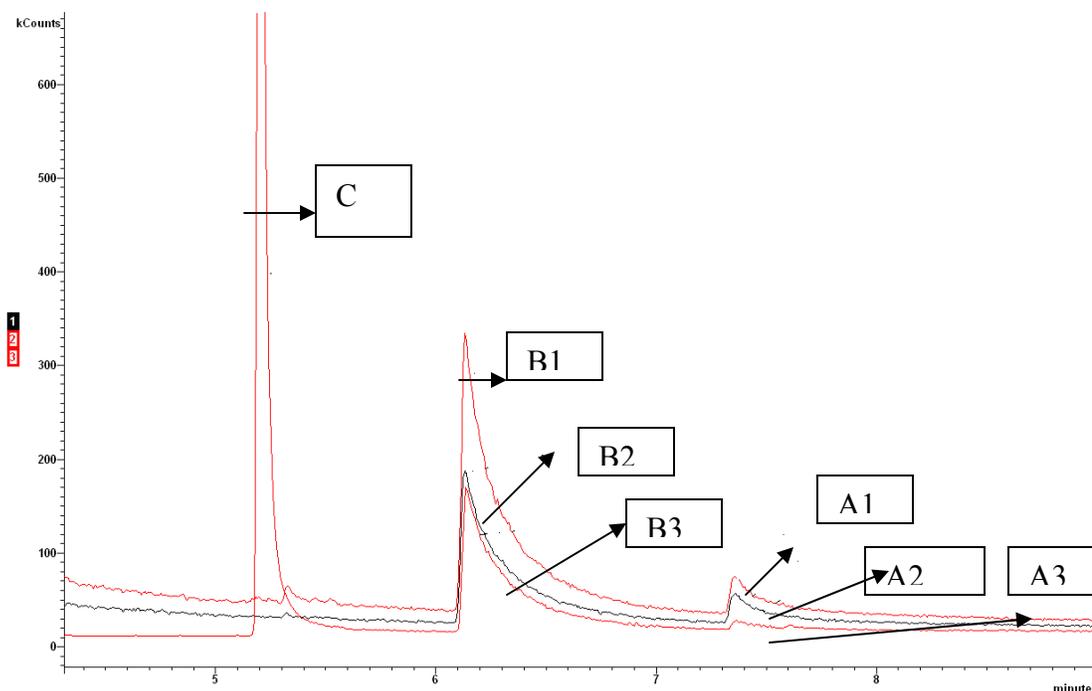


Figure 28. Superimposed chromatograms of derivatives of products resulting from reaction between cyclooxygenase enzyme and arachidonic acid (A1, B1), cyclooxygenase enzyme and triphenylphosphine-treated arachidonic acid (A2,B2), or cyclooxygenase enzyme treated with triphenylphosphine and triphenylphosphine treated with arachidonic acid (A3, B3, C).

Under identical conditions for preparation of the derivative and assay by GC-MS, various standards were run to identify the peaks produced in the cyclooxygenase reactions.

3.6a Identification of peak at 7.3 min (A1, A2, and A3)

The peak at 7.3 min was identified as prostaglandin E₂ (PGE₂), which has similar mass spectrum and retention time similar to that of standard PGE₂. The standard PGE₂ peak eluted from 7.325 to 7.842 min as observed in the Figure 29, and the mass spectrum has been taken with peak apex at 7.394 min. The mass spectrum of standard PGE₂ has shown all the major ions of mass to charge ratios (m/z) of 512.5, 455.5, 391.2, 279.1, 149.1, and 73, as shown in Figure 30, and has qualitatively the same mass spectrum as those for peaks A1, A2, and A3 (see Figures 16, 20, and 23 for comparison).

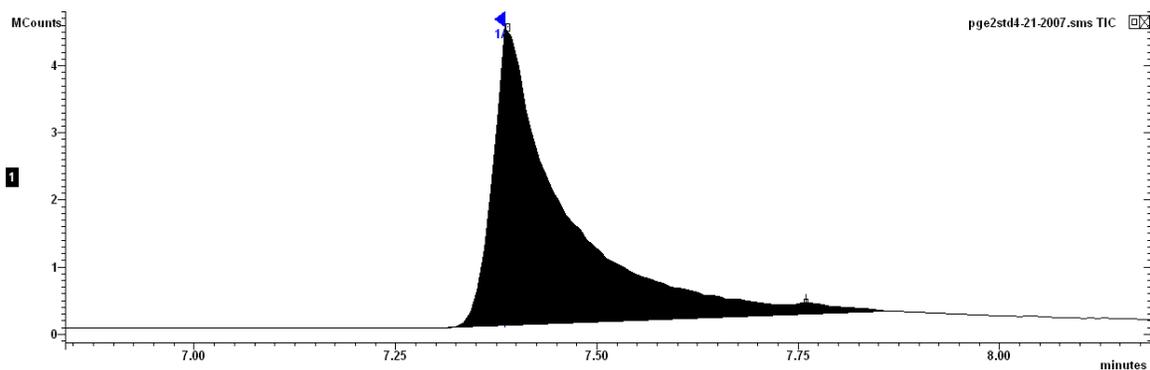


Figure 29. Gas chromatogram of the methyl ester-oxime-silylated derivative of the standard prostaglandin E₂.

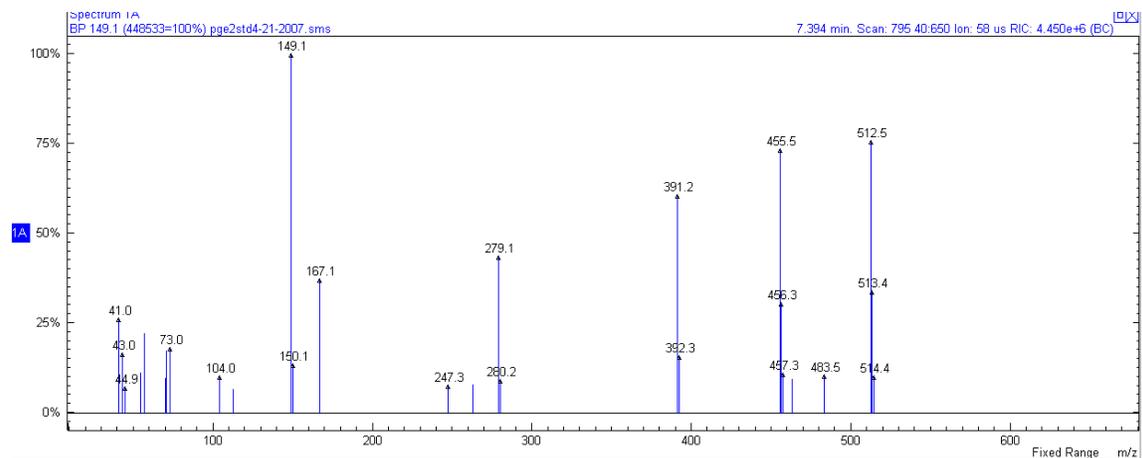


Figure 30. Mass spectrum of standard PGE₂.

3.6b Identification of peak at 5.2 min (Peak C)

The additional peak at 5.2 min was identified as one of the monohydroxy compounds as shown in Figure 31. The standard 12-hydroperoxyeicosatetraenoic acid (HpETE) was treated for 30 min with triphenylphosphine (5mg) dissolved in ether (100μL). The methyl ester and silylated derivative was then prepared according to the procedure described in the experimental section, and the synthetic scheme is shown in Figure 31. As there are no carbonyl groups in the chemical structure of 12-hydroperoxy-eicosatetraenoic acid, the methoximation or oxime derivative was not prepared. The hydroperoxide in the HpETE was reduced to an alcohol by the treatment with the triphenylphosphine. The reduced product of HpETE is 12-hydroxyeicosatetraenoic acid (12-HETE). Peak C elutes at the same time as 12-hydroxyeicosatetraenoic acid at 5.2 min as shown in Figure 32, and the mass spectrum of peak C exactly matches that of 12-HETE shown in Figure 33. Based on retention time and mass spectrum, the peak at 5.2 min (C) has been identified as 12-hydroxyeicosatetraenoic acid.

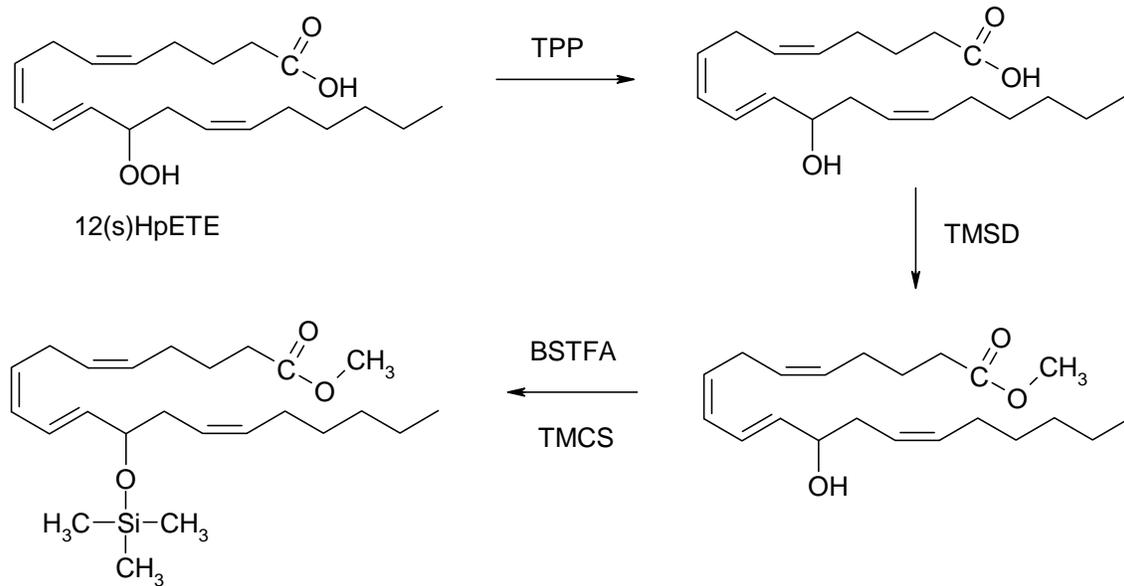


Figure 31. Synthetic scheme for the production of derivative of 12(s) HpETE.

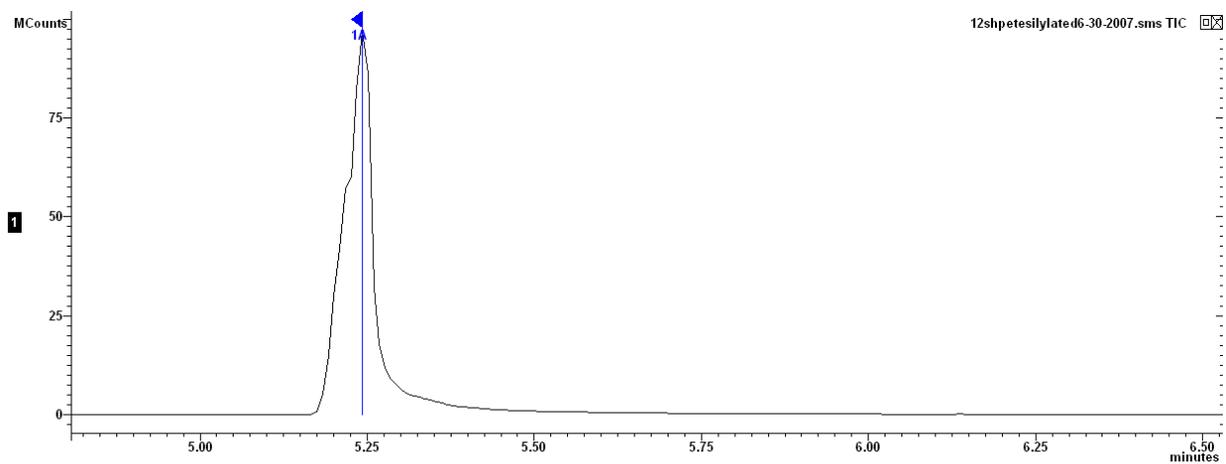


Figure 32. Chromatogram of standard 12-hydroperoxyeicasinotetraenoic acid treated with triphenylphosphine (12-hydroxyeicasinotetraenoic acid).

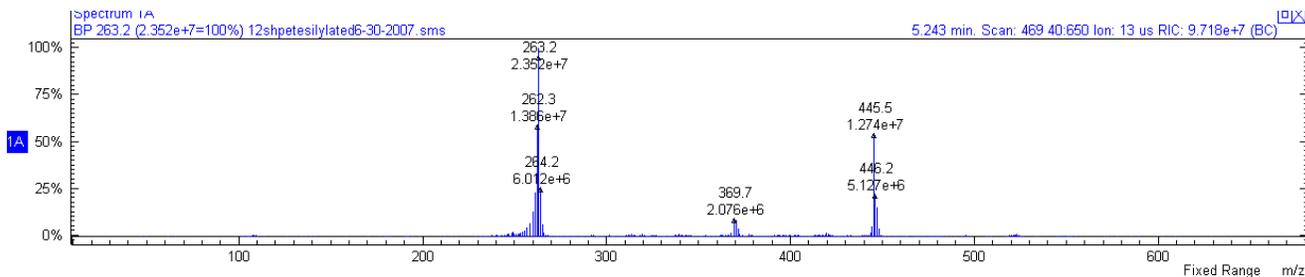


Figure 33. Mass spectrum of the peak presented in Figure 32.

3.6c Attempts to identify the peak at 6.2 min (B1, B2, B3)

Attempts were made to identify the metabolite at 6.2 min, although neither the mass spectra of the standards nor the retention time matched those of this product. As one of the peaks at 5.2 min had been identified as a monohydroxy fatty acid, it was suspected that peaks B1, B2, and B3 were monohydroxy fatty acid as well. The methyl ester-oxime-silyl derivative of the standard 15-hydroxyeicosatetraenoic acid was submitted to evaluation on GC-MS, and the peak eluted from 5.149 to 5.431 min, as shown in Figure 34. The mass spectrum of the 15-hydroxyeicasinotetraenoic acid is shown in Figure 35.

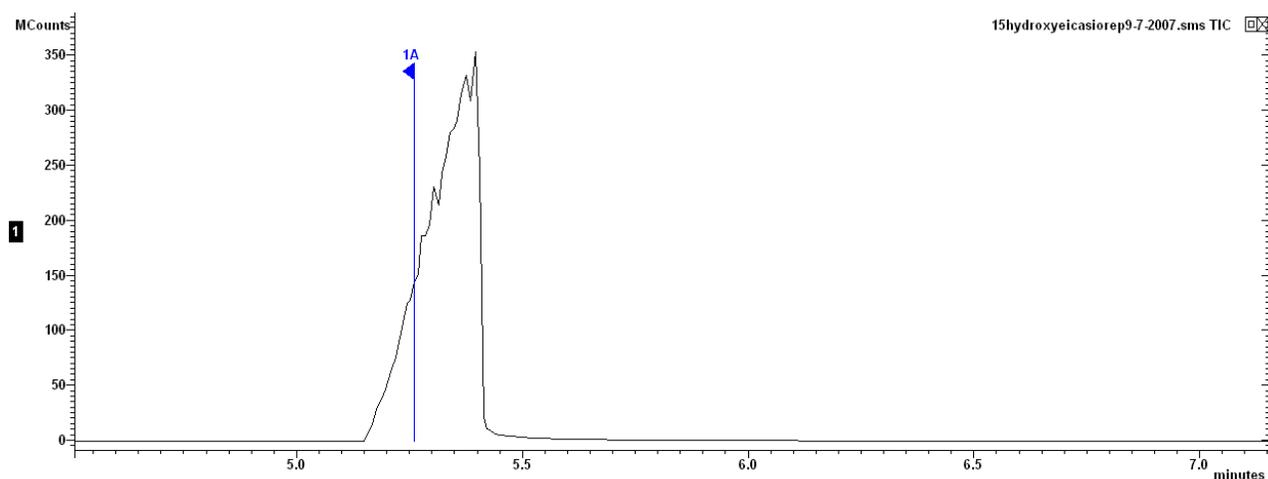


Figure 34. Chromatogram of 15-hydroxyeicosatetraenoic acid.

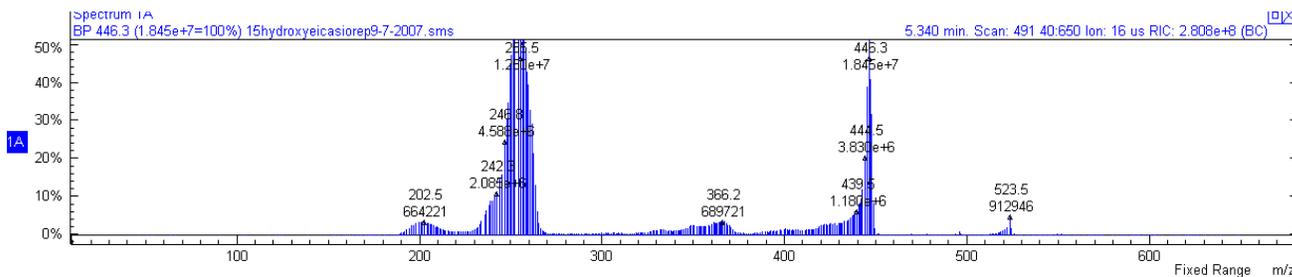


Figure 35. Mass spectrum of 15-hydroxyeicasinotetraenoic acid.

The mass spectrum of 15-hydroxyeicosatetraenoic acid did not match the mass spectrum of the peak at 6.2 min, so more trials were attempted with standard dihydroxycompounds. Similarly derivatized dihydroxy compounds were injected onto the gas chromatograph and analyzed following mass fragmentation. Some of the standards were hydrogenated, which involves the reduction of the double bond, as some of the standard samples did not yield a respectable, gaussian peak. Hydrogenation was performed by addition of 50 μ L glacial acetic acid and addition of a small amount of palladium oxide catalyst, and then the preparation was treated with hydrogen gas for 15 min. After the hydrogenation step, the methyl ester-oxime-silylated derivative was prepared. The standard eluted from 5.280 to 5.468 min, as shown in Figure 36. The mass spectrum is shown in the Figure 37 with a peak apex at 5.323 min.

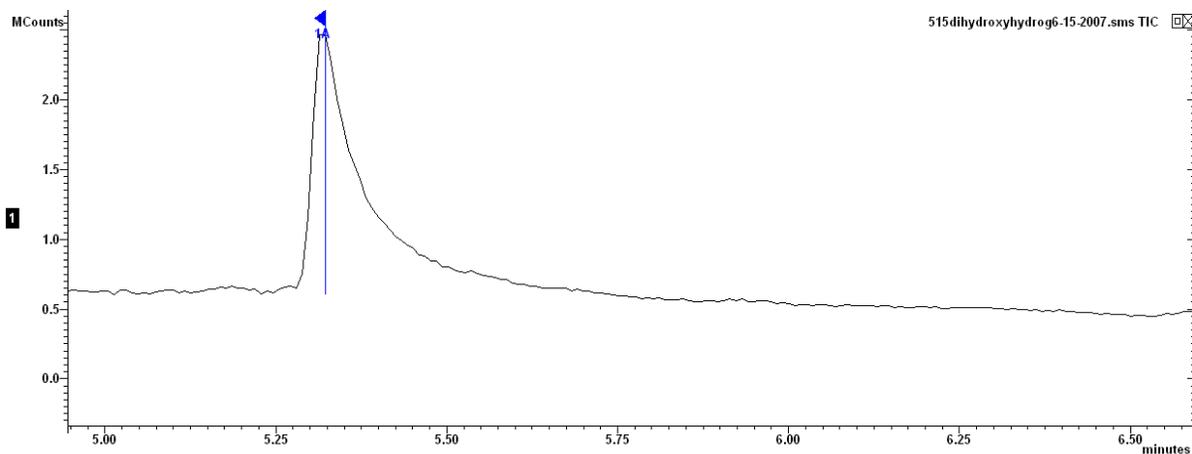


Figure 36. Chromatogram of 5,15-Di-HeTE methyl ester-oxime-silyl derivative following hydrogenation.

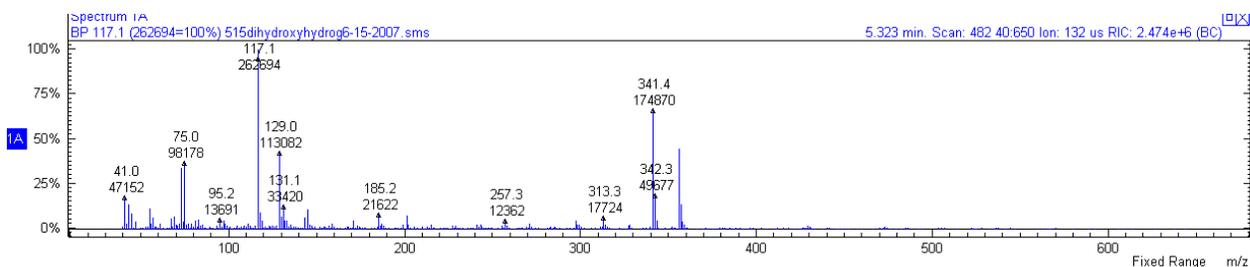


Figure 37. Mass spectrum of 5, 15-Di-HeTE methyl ester-oxime-silyl derivative following hydrogenation.

A second sample of 5,15 Di-HeTE derivative was prepared but without hydrogenation and was injected onto the GC. The retention time of the sample was 5.285 to 5.465 min, as shown in Figure 38. The mass spectrum of the sample is shown in Figure 39 for the peak apex at 5.319min. The mass spectrum was very complex with no identifiable prominent ions.

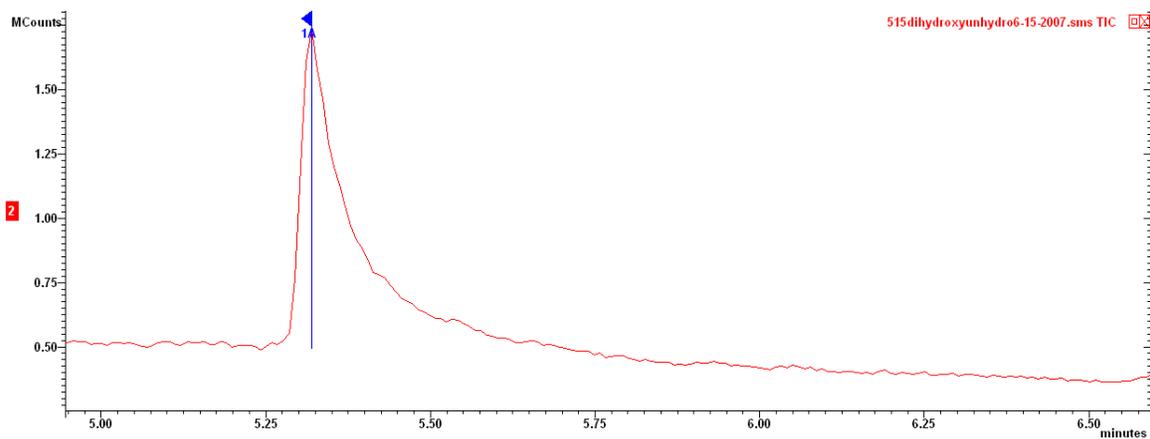


Figure 38. Gas chromatogram of standard 5, 15-DiHeTE.

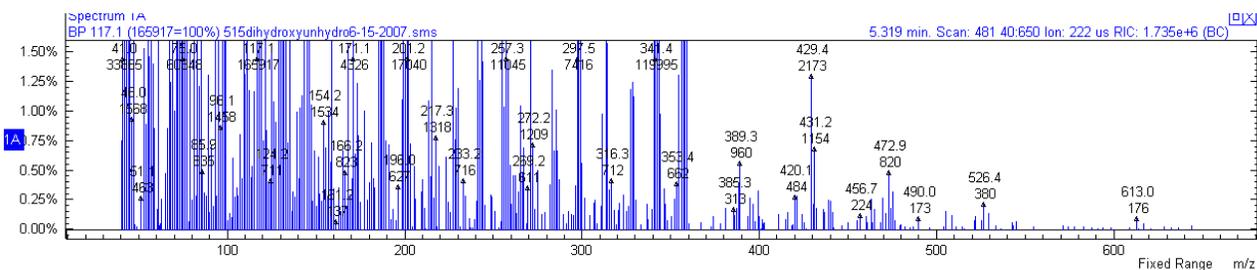


Figure 39. Mass spectrum of 5, 15-DiHeTE derivative sample.

Finally, an 11, 12-DiHETrE sample was hydrogenated and then injected onto the GC, which resulted in a chromatogram as shown in Figure 40 with a retention time ranging from 5.280 to 5.453 min and the mass spectrum as shown in Figure 41.

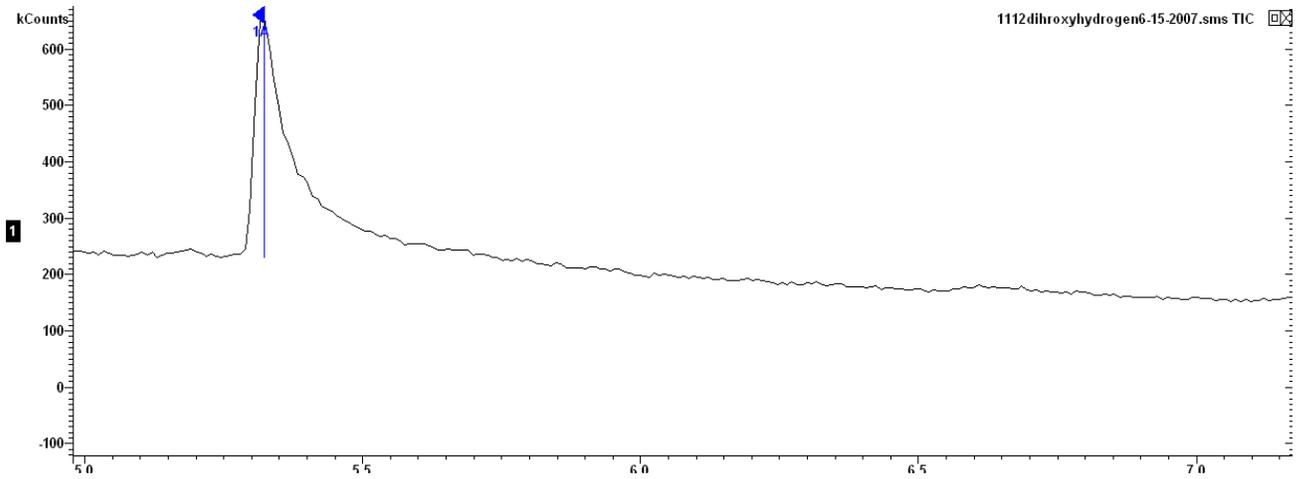


Figure 40. Gas chromatogram of 11, 12-DiHETrE.

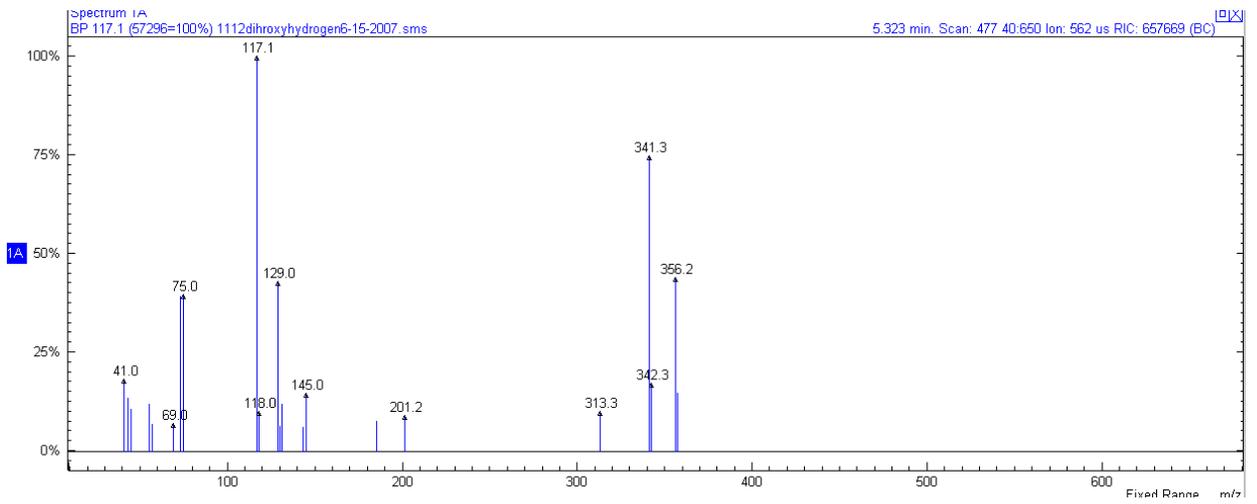


Figure 41. Mass spectrum of 11, 12-DiHETrE.

Chapter 4. Discussion

Many scientists have done extensive research to understand the basis of the enzymatic mechanism of the cyclooxygenase enzyme. In the process of exploring its enzymatic activity, many of the researchers emphasized the importance of characterizing the metabolites produced by the cyclooxygenase enzyme. The principle metabolite from arachidonic acid metabolism by COX, PGE₂, is thought to be mainly responsible for the development of inflammation, which plays a major role in the development of various chronic diseases. In a departure from the usual methods or assays that have been developed for analysis of PGE₂ by cyclooxygenase enzyme, triphenylphosphine, a reducing agent, was incorporated into the reaction mixture along with substrate in the present study. It is known that triphenylphosphine reduces hydroperoxides of arachidonic acid leading to production of alcohols (52). The reducing agent was used to generate a peroxide-free preparation of arachidonic acid that was then used as a substrate in the cyclooxygenase reaction. Peroxide-containing species were found in the preparations of arachidonic acid as judged by thin layer chromatography.

All samples were derivatized according to previously established procedures for the analysis of fatty acids and prostaglandins (53). Under all conditions examined, a peak at 7.3 min and another peak at 6.2 min have been observed in the chromatogram. Identification of the peak at 7.3 min as PGE₂ was accomplished by comparison of the mass spectrum with that of PGE₂ standard. Peaks A1, A2, and A3 are produced in decreasing order, indicating that the amount of production of PGE₂ decreases by the treatment of either arachidonic acid with triphenylphosphine or both arachidonic acid and cyclooxygenase enzyme with triphenylphosphine. The peak at 6.2 min, which has been observed in all experiments, eluted

at the same time and has an identical mass spectrum in all of the chromatograms. The identification of this peak at 6.2 min was not successful despite the preparation and analysis of a number of eicosanoid standards.

A unique peak at 5.2 min has been observed with the reaction of cyclooxygenase enzyme treated with triphenylphosphine and arachidonic acid treated with triphenylphosphine. This peak has been identified as 12-hydroxyeicosatetraenoic acid, as this peak has a similar retention time and a similar mass spectrum pattern as that of the standard 12-hydroxyeicosatetraenoic acid. The 12-hydroxyeicosatetraenoic acid metabolite is usually produced by metabolism of arachidonic acid via the lipoxygenase pathway. It is not certain how the 12-hydroxyeicosatetraenoic acid was produced in this experiment and whether it was cyclooxygenase enzyme or triphenylphosphine that played a role in its production.

Reports in the literature indicate that hydroperoxides are required for cyclooxygenase activity (54, 55). Our results are in agreement with this hypothesis, in that 12-hydroperoxyeicosatetraenoic acid may stimulate the cyclooxygenase activity, thereby facilitating more production of PGG₂, which results in increased amount of PGE₂. Alternatively, it may be stimulating the peroxidase activity of cyclooxygenase enzyme, which results in increased production of prostaglandin endoperoxide (PGH₂) and PGE₂. 12-hydroperoxy eicosatetraenoic acid is reduced to 12-hydroxyeicosatetraenoic acid by the treatment with triphenylphosphine, thereby removing a stimulatory effect on the COX, which leads to a decrease in the level of PGE₂ formed. The hypothetical scheme is shown in Figure 42, which also hypothesizes that TPP directly stimulates the enzyme to generate 12-HETE via formation of 12-HpETE.

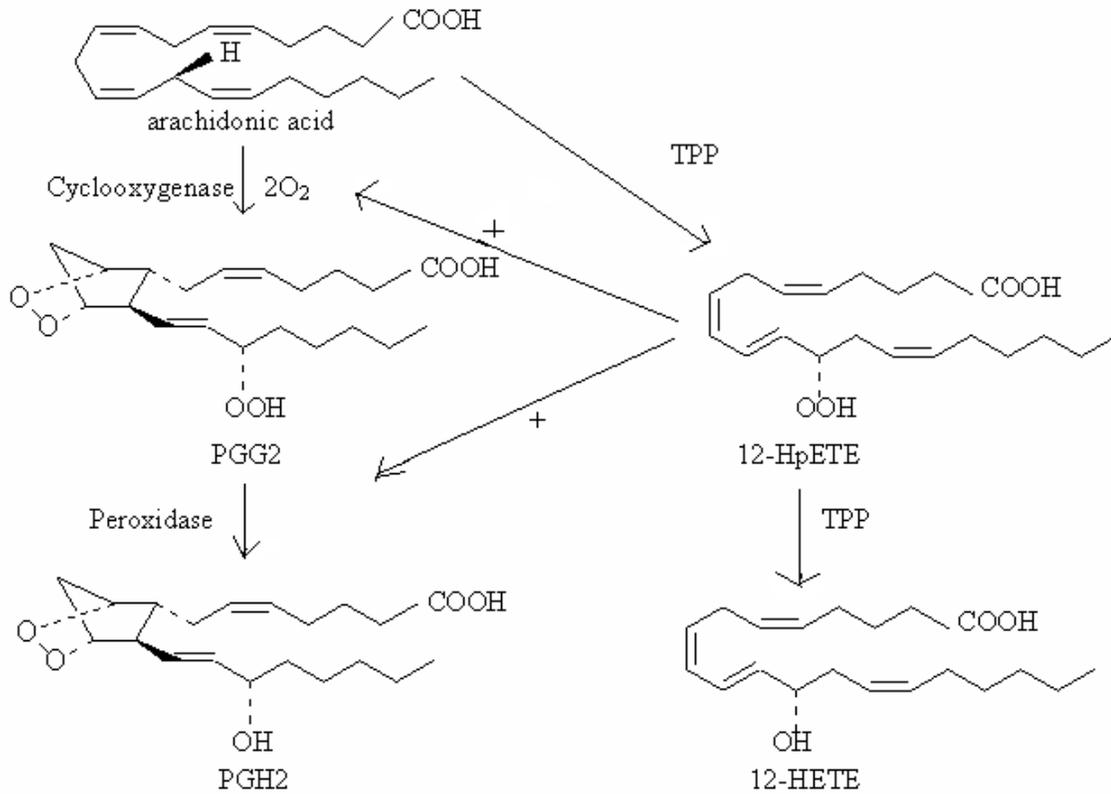


Figure 42. Formation and stimulatory function of 12-HpETE on the cyclooxygenase.

Triphenylphosphine (TPP) may be involved in stimulating the production of 12-HETE, presumably via the 12-HpETE, and the reduction in the level of 12-HpETE lowers the activity of COX and, hence, reduces the PGE₂ produced during metabolism.

One of the future experiments that can be performed is just carrying out the reaction between arachidonic acid and cyclooxygenase enzyme. After addition of a required amount of standard 12-hydroperoxyeicasinoteraenoic acid to the reaction mixture, possible increased production of PGE₂ can be observed in comparison to the amount of PGE₂ produced by reaction between cyclooxygenase enzyme and arachidonic acid. This would reveal or confirm the hypothesis that 12-hydroperoxyeicasinoteraenoic acid is regulating the production of PGE₂. Even further experiments can be carried out to investigate how 12-hydroperoxyeicasinotetraenoic acid is produced.

References

1. Nelson. L. D. *Lehninger Principles Of Biochemistry*. 3rd Edition. Worth Publishers, **2004**, 363-383.
2. Psota. L. T.; Gebauer. K. S.; Etherton. K. P. *Am. J. Cardiol* . **2006**, 98, 3-18.
3. Davidson. F. F.; Dennis. E. A. *J. Mol. Evol.* **1990**, 31, 228-238.
4. Lin. L. L.; Wartmann. M.; Lin. A. Y.; Knopf. J. L.; Seth. A.; Davis. R. J. *Cell*. **1993**, 72, 269-278.
5. Clark. J. D.; Lin. L. L.; Kriz. R. W.; Ramesha. C. S.; Sultzman. L. A.; Lin. A. Y.; Milona. N.; Knopf. J. L. *Cell*. **1991**, 65, 1043-1051.
6. Porter. N. A.; Funk. M. O. *J. Org. Chem.* **1975**, 40, 3614-3615.
7. Pryor. W. A.; Stanley. J. P. *J. Org. Chem.* **1975**, 40, 3615-3617.
8. Samuelson. D. W. *Biol. Bull.* **1987**, 173, 92-109.
9. Jason. D. M.; Hill. K. E.; Burk. F. R.; Nammour. M. T.; Badr. F. K.; Jackson. R. II. L. *Proc. Natl. Acad. Sci.* **1990**, 87, 9383-9387.
10. Von Euler. U. *J. Physiol.* **1936**, 88, 213-34.
11. Eliasson. R. *Acta Physiol Scand.* **1959**, 46, 1-73.
12. Smith. W. L. *Biochem. J.* **1989**, 259, 315-324.
13. Schaafsma. D.; Gosens. R.; Bos. T. S.; Meurs. H.; Zaagsma. *J. Respiratory Research.* **2005**, 85, 1465-9921.
14. Weksler. B.; Marcus. A.; Jaffe. E. *Proc Natl Acad Sci.* **1977**, 74, 3922-6.
15. Macintyre. D.; Pearson. J.; Gordon. *J. Nature.* **1978**, 271, 549-51.
16. Smith. W. L.; Marnett. J. L. *Pharmacol Thera.* **1991**, 49, 153-179.

17. Jones. D. A.; Carlton. D. P.; McIntyre. T. M.; Zimmerman. G. A.; Prescott, S. M. *J. Biol. Chem.* **1993**, *268*, 9049-9054.
18. Evette.G.E.; Xie, W.; Chipman. J. G.; Robertson, D. L.; Simmons. D. L. *Arch. Biochem. Biophys.* **1993**, *306*, 169-177.
19. Herschmann. H R. *Annu. Rev. Biochem.* **1991**, *37*, 281-219.
20. Dewitt. D. L.; Meade. E. A. *Arch. Biochem. Biophys.* **1993**, *306*, 94-102.
21. Kujubu. D. A.; Reddy. S. T.; Fletcher. B. S.; Herschman. H. R. *J. Biol. Chem.* **1993**, *268*, 5425-5430.
22. Pilbeam. C. C.; Kawaguchi. H.; Hakeda. Y.; Vosnesensky. O.; Alander. C. B.; Raisz. L. *G. J. Biol. Chem.* **1993**, *268*, 25643-25649.
23. Brown, R. J.; Dubois. N. R. *J. Clin. Oncol.* **2005**, *23*, 2840-2865.
24. Thuresson. D. E.; Lakkides. M. K.; Rieke. J. C.; Byron. A. Y.; Mulichak. M. A.; Malkowski. G. M.; Garavito. M. R.; Smith. L. W. *J. Biol. Chem.* **2001**, *276*, 10347-10359.
25. Picot, D.; Loll, P.J.; and Garavito, M. *Nature.* **1994**, *367*, 243-249.
26. Kurumbail, R, G.; Steveno, A. M.; Gierce, J. K.; McDonald, J. J.; Stegeman, R, A.; Pak, J. Y.; Gildhause, D.; Miyashiro, J, M.; Penning, T, D.; Seibert, k.; Ieakson, P.C.; and Stallings, W. C. *Nature.* **1996**, *384*, 644-648.
27. Luong, C.; Miler, A., Barnett, J.; Chow, J., Ramesha, C.; and Browner, M. F. *Nat. Struct. Biol.* **1996**, *3*, 927-983.
28. L. Malkowski, M. G.; Ginell, S., Smith, W. L.; Garavito, R. M. *Science.* **2000**, *289*, 1933-1937.

29. Shimokawa. T.; Kulmacz. R. J.; Dewitt. D. L.; Smith. W. L. *J. Biol. Chem.* **1990**, 265, 20073-20076.
30. Smith. W.L.; Marnett, L. J.; Dewitt, D. L. *Pharmacol Ther.* **1991**, 49, 163-179.
31. Simmons, L. D.; Botting, M. R.; Hla, T. *Pharmacol Rev.* **2004**, 56, 387-437.
32. Smith, W. L.; Dewitt, D.L.; Garavito, R. M. *Annu. Rev. Biochem.* **2000**, 69, 149-182.
33. Smith, W.L.; and Dewitt, D.L. *Adv Immunol.* **1996**, 62, 167-215.
34. Marnett, L. J.; Rowlinson, S. W.; Goodwin, D. C., Kalgutkar, A. S.; and Lanzo. *J. Biol. Chem.* **1999**, 274, 22903-22906.
35. Smith. W. L.; Garavito. M. R.; Dewitt. L. D. *J. Biol. Chem.* **1996**, 271, 33157-33160.
36. Margalit. A.; Duffin. L. K.; Isakson. C. P. *Anal. Biochem.* **1996**, 235, 73-81.
37. Kramer. H. J.; Stevens. J.; Seeger. W. *Anal. Biochem.* **1993**, 214, 535-543.
38. Coste, O.; Schimdt, R. *J. Chromatogr.* **2005**, 826, 188-197.
39. Dewitt. L. W.; Garavito. M. R.; Smith. L. W. *J. Biol. Chem.* **1997**, 271, 33157-33160.
40. Pestel. S.; Jungermann. K.; Schieferdecker. L. H. *Prostaglandins Other Lipid Mediat.* **2005**, 75, 123-129.
41. Forghani. F.; Ouellet. M.; Keen. S.; Percival. M. D.; Tagari. P. *Anal. Biochem.* **1998**, 264, 216-221.
42. Kulmacz. J. R.; Wang. L. *J. Biol. Chem.* **1995**, 270, 24019-24023.
43. Skoog. A. D.; Holler. J. F.; Nieman. A. T. *Principles of Instrumental Analysis*. 5th edition, Thomson Publishers, **2005**, 675-678.
44. www.ionsource.com/tutorial/chromatography
45. Buboiss. R. N.; Abramson. S. B.; Crofford. R. A.; Gupta, L. S.; Simon. L. B.A.; Putte. V. *FASEB J.* **1998**, 12, 1063-1073.

46. Alekseev, S. M.; Evstigneeva, R. P.; Isai, S. V.; Karpova, G. V.; Korotchenko, O.D.
Chem. Nat. Compd. **1999**, *35*, 612-615.
47. Mallet, I. A.; Newby, S. C. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1723-1727.
48. Sobotka, P.A.; Brottman, M.D.; Weitz, Z.; Birbaum, A.j.; Skosey, J.L.; Zarling, E.J.
Free Radical Biol. Med. **1993**, *14*, 643-647.
49. <http://teaching.shu.ac.uk/hwb/chemistry/tutorials/chrom/gaschrn.htm>.
50. Moore, K.; Roberts, L. J. *Free. Rad.* **1998**, *28*, 659-671.
51. www.kore.co.uk/graphics/MS-200_tof.gi.
52. Bryant, W. R.; Balley, J. M.; Schewet, T.; Rapoport, M. S. *J. Biol. Chem.* **1982**, *257*,
6050-6055.
53. Maas, L. R.; Turk, J.; Oates, A. J.; Brash, A. R. *J. Biol. Chem.* **1981**, *257*, 7056-7067.
54. Kulmacz, J. R.; Pendleton, B. R.; Lands, E. M. *J. Biol. Chem.* **1994**, *269*, 5527-5536.
55. Kulmacz, J. R.; Wang, L. *J. Biol. Chem.* **1995**, *270*, 24019-24023.