Liquid chromatography tandem mass spectrometry analysis of arachidonic and eicosapentanoic acid metabolites by using experimental design and curve resolution approaches

Phoebe Z. Trio

Thesis for the degree of European Master in Quality in Analytical Laboratories

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Phoebe Z. Trio

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Abstract

Eicosanoids are important lipid mediators in the development of inflammation, progression of cancer and other pain associated diseases. Arachidonic acid (AA) is the precursor molecule for the synthesis of prostaglandin E2 and luekotriene B4 which is considered as pro-inflammatory metabolites. Conversely, eicosapentanoic acid (EPA) is the substrate for the synthesis of anti-inflammatory metabolites such as prostaglandin E3 and luekotriene B5.

In this study, the effects and influence of AA, EPA and inhibitor towards the synthesis of the four metabolites in cell cultures were investigated. AA-derived metabolites showed minimal production when AA is the only substrate present in the cell culture media. Interesting, the levels of AA-derived metabolites increased significantly in the presence of EPA. The results showed that EPA may play a role in the production of the AA-metabolites. An understanding of the effects and interactions between the two molecules is important in establishing these metabolites as inflammatory biomarkers.

Contents

List of Abbreviations

AA	Arachidonic Acid
EPA	Eicosapentanoic acid
LOX	Lipoxygenase
COX	Cyclooxygenase
PGE	Prostaglandin
LTB	Leukotriene
PLA_2	Phospholipase A2
HPETE	Hydroxyeicosapentanoic acid
HETE	Hydroeicosapentanoic acid
LCMS	Liquid chromatography mass spectrometry
GCMS	Gas chromatography mass spectrometry
RIA	Radio-immunoassay
ELISA	Enzyme-linked immunosorbent assay
ITTFA	Iterative target transformation factor analysis
AR	Alternate regression
WFA	Window factor analysis
HELP	Heuristic evolving latent projections
DMEM	Dulbecco’s modified eagle medium

Chapter 1

INTRODUCTION

Fatty acids are organic molecules composed of a long hydrophobic unsaturated or saturated aliphatic chain to which is attached a hydrophilic carboxylic acid group (Fig. 1). The polyunsaturated fatty acids such as arachidonic acid (20:4n-6) and eicosapentanoic acid (20:5n-3) synthesized from linoleic acid (18:2n-6) and alpha-linoleic acid (18:3n-3) respectively, are essential components in human diets and responsible for the production of eicosanoid metabolites (Innis, 1991).

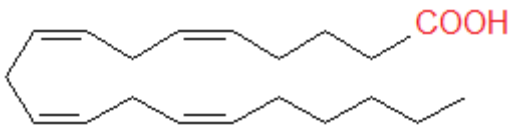

Figure 1. Structure of a fatty acid containing 20 carbon chain (in black) and a carboxylic group (in red).

Eicosanoids are compounds derived from fatty acid precursors generally containing 20-carbon atoms. They are also known as signaling molecules that exert complex control over many human and animal biological systems, mainly in the process of inflammation or immunity, and as messengers in the central nervous system. The networks of controls that depend upon eicosanoids are among the most complex in biological system (Coquette, et. al., 1992, Shapiro, et. al., 1994, Nieves and Moreno, 2006, Miles and Calder, 2007).

The eicosanoid synthesis takes place by means of three main pathways, namely a cyclic pathway characterized by the presence of cyclooxygenase (COX) enzymes, a linear pathway characterized by the lipoxygenase (LOX) enzymes and the cytochrome P450. These pathways can take place regardless of the availability of AA or EPA. The cyclic pathway is catalyzed by the action of the COX-1 and COX-2 which lead to the formation of prostaglandins, prostacyclins and thromboxanes (Fig. 2). The linear pathway is catalyzed by three different lipoxygenases specifically, 5, 12 and 15-lipoxygenases (5-LOX, 12-LOX and 15-LOX respectively) and cytochrome P450 (P450) pathway is catalyzed by P450-dependent monooxygenases through a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent mechanism. These lypoxygenase enzymes along with P450 are responsible for the production of leukotrienes (catalyzed by 5-LOX), hydroperoxyeicosatetraenoic acids (HPETEs) and hence into hydroxyeicosatetraenoic acids (HETEs) respectively and catalyzed by 12-LOX, 15-LOX or P450.

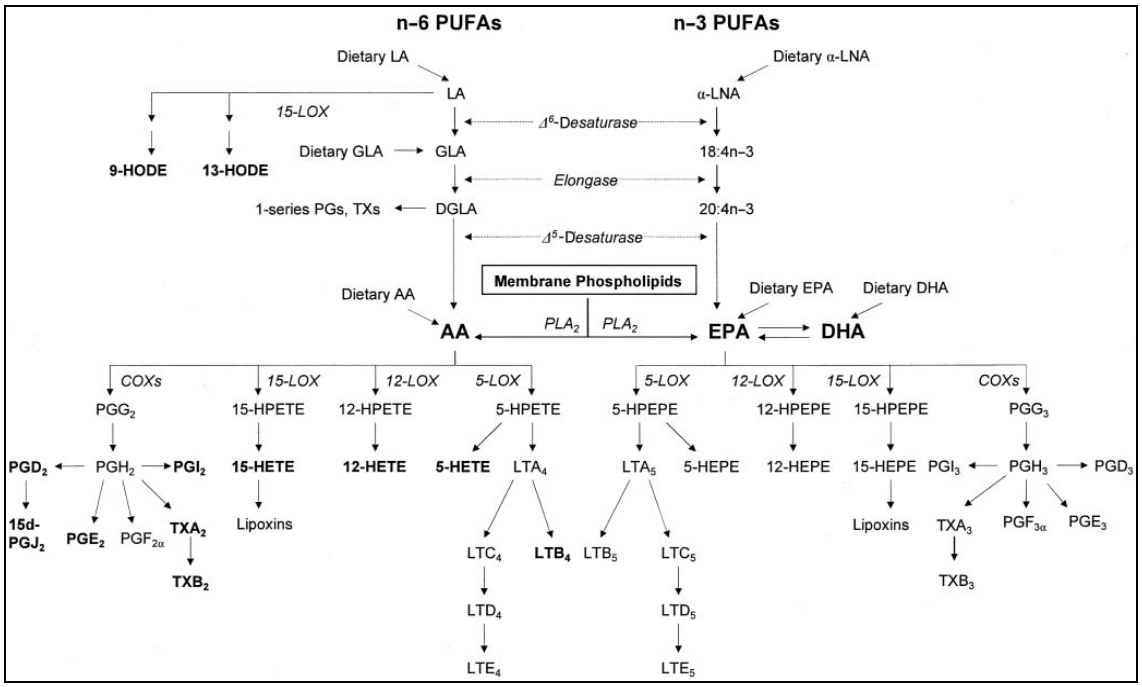

Figure 2. Enzymatic oxygenation pathway of AA and EPA into eicosanoids metabolites (adapted from Arab, 2003).

Prostaglandins, one of the major eicosanoid metabolites generated from the cyclooxygenase mediated pathway of the arachidonic acid and eicosopentanoic acid, are found in every animal tissue. It directs various physiologic and pathologic routes within the cells where they are produced in nearby cells in response to specific stimuli. Several, studies have demonstrated the role of prostaglandin in the cytoprotection of gastric mucosa (Zhang, et. al., 2009), renal physiology (Logan, et. al., 1992), gestation (Urquhart, et. al., 1997) and parturition (Rigby, et. al., 1998) under normal physiologic conditions. However, they are also associated with a number of pathological conditions such as bronchitis (Rigby, et. al., 1998), cancer (Chulada, et. al., 2000) and other kind of pathologies associated with inflammations. For example, depending on the interaction of prostaglandin E2 (PGE_2) with one of the receptor in different cell types can exhibit pro or anti-inflammatory effects (Raud, et. al., 1988).

Other than prostaglandin, leukotrienes, the main products of the 5-LOX pathway, are also effective lipid mediators of inflammation. Research has revealed that overproduction of leukotrienes affects many diseases such as asthma (Wenzel, et. al., 1990), inflammatory bowel movement (Hawkey, et. al. 1992), rheumatoid arthritis (Gürsel, et. al., 1997), autoimmune diseases (Chen, et. al., 2009) and cardiovascular diseases (Freiberg, et. al., 2009). It follows that by inhibiting or controlling the synthesis of leukotrienes might pave the way for the treatment of several diseases.

Fatty acids exhibiting the first double bond at the sixth carbon from the omega (or methyl end) are named omega 6 (ω-6) fatty acid. Arachidonic acid (AA), an ω-6 fatty acid (Fig. 3A), produces 2-series of prostaglandins and 4-series of leukotrienes when metabolized. Conversely, eicosapentanoic acid (EPA), an ω-3 fatty acid (Fig.3B), yields 3-series of prostaglandin and 5-series of leukotrienes as its breakdown products.

Figure 3. Nomenclature of AA (A) and EPA (B) based on the position of the first double bond from the ω-end.

Since the discovery of this group of metabolites, many researchers including pharmaceutical industries still continue to have a great interest in this field of

research due to the role it portrays in pain and inflammation associated diseases. The intricacy of eicosanoids and the lack of powerful instruments for their analysis made the progression of information in this field not similar to the outburst of knowledge of other areas. Recent development in instrumental methods has created a great change in the field of eicosanoid research. Liquid chromatography with tandem mass spectrometry is regarded as a robust technique for quantifying eicosanoid metabolites simultaneously and considered as an information-rich technique as compared to nuclear magnetic resonance (NMR) and gas chromatography mass spectrometry (GC-MS) (Wenk, 2005). The predicaments regarding the elution of many metabolites at a similar retention time and identification of individual peaks were resolved by the aid of mass spectrometry (Katajamaa and Orešič, 2005 & Hu, et. al., 2009). When the coelutants pass the liquid chromatography, through an appropriate interface, they are ionized and introduced into the mass spectrometer. The mass-to-charge ratio is measured and based on this ratio the coelutants can be identified and differentiated. With this instrumentation, changes in the eicosanoid mechanism or the pathway modulation of eicosanoid metabolites due to certain diseases can now be determined in complicated biological matrices. Changes in the metabolic profile can also be correlated with disease.

Epidemiological studies revealed that AA-derived eicosanoids are generally pro-inflammatory lipid mediators whereas EPA-acid derived eicosanoids are referred to as anti-inflammatory (Sastre, et. al., 2008). However, due to the controversies being raised regarding animal testing experiments, the shift to cell and tissue culture techniques become an alternative way for investigating the eicosanoid production (Akthar, et. al., 2008). Cell and tissue culture turn out to be preferable methods due to the feasibility to extensively control the physiochemical environment and physiological conditions and to perform biochemical monitoring and observations (Shaw, 1996). In addition, the cell and tissue culture methods

avoid the ethical and monetary issues like usage and killings of countless animals.

Studies regarding eicosanoid production and synthesis on cell cultures are usually focused on either the LOX or the COX pathway. No studies have been published yet regarding how LOX and COX pathways affect each other towards the production of eicosanoids from both AA and EPA. Thus, it follows that simultaneous quantitative assessment of eicosanoid metabolites from cyclooxygenase and lypoxygenase pathways is vital in evaluating the behavior of the precursor molecules in the cell membrane and how these substrate molecules affects the synthesis of leukotrienes and prostaglandins.

1.2 Significance of the Study

Previous studies indicated that EPA is a poor substrate for cyclooxygenase (COX) but can compete with AA by inhibiting its metabolism thus affecting the production of COX-derived metabolites such as prostaglandin E2 (PGE_2) and prostaglandin E3 (PGE_3). On the contrary, EPA is said to be a good substrate to lypoxygenase enzymes which follows that synthesis of the anti-inflammatory leukotriene B5 (LTB_5) is favorable over the production of pro-inflammatory leukotriene B5 (LTB_4). However, less information is available regarding the effects and interactions of prostaglandins and leukotrienes when both AA and EPA are present in a biological system and the effect of inhibiting the PLA_2 and COX enzymes. The present study would provide preliminary evidence regarding how AA behaves in the cellular membrane in the presence of EPA and which pathway is more favored. Furthermore, this would shed new information on how the precursor molecules affect the production or synthesis of eicosanoids activity in systems where COX and PLA_2 enzymes are blocked simultaneously.

1.3 Aims of the study

The general aim of this research was to evaluate targeted eicosanoids biomarkers derived from arachidonic (AA) and eicosapentanoic (EPA) fatty acids in cell culture experiment by using liquid chromatography tandem mass spectrometry (LCMS-MS) and explore the applicability of some chemometrical strategies that could assist in evaluating the selected metabolites in the cell culture media.

The specific objectives of the research were:

1. To evaluate the influence of AA, EPA and inhibitor on the synthesis of pro and anti-inflammatory COX and LOX associated metabolites, specifically PGE_2, PGE_3, LTB_4 and LTB_5 in cell cultures system by using factorial experimental design and high performance liquid chromatography tandem mass spectrometry.
2. To study the effect of inhibiting phospholipase A_2 (PLA_2) and COX enzymes in cells treated with EPA and/or AA.
3. To study the behavior of EPA and AA towards the COX and LOX enzymes.
4. To study the interaction of EPA, AA and inhibitor in cells towards the synthesis of prostaglandin and leukotrienes.
5. To analyze and resolve the chromatographic and mass spectrum profiles by chemometrics.

Chapter 2

LITERATURE REVIEW

This chapter is divided into three sections. The first section describes important aspects involved in the chemistry and biology of eicosanoids; the second section introduces some analytical techniques generally used for the quantification of eicosanoids and the last section explains some chemometric approaches that could be used in the analysis of eicosanoids.

2.1 Eicosanoids

Eicosanoids were discovered and isolated from human semen by the Swedish Nobel laureate Ulf von Euler in 1930. It has been determined that they exist and are synthesized in every cell of the body and are referred as autocoids chemical messengers that do not move to other sites, but work right within the cells where they are synthesized. Eicosanoids are only active for a short period of time before they are modified into a non-functional form and are subsequently excreted from the body.

2.1.1 Eicosanoids Nomenclature

2.1.1.1 Prostaglandins

The short form for prostaglandin is PG while the ensuing letter indicates either position of the double bond or the constituents of the 5-membered ring. Fig. 4 shows how the positioning of the double bond in the ring system defines the names of PGA, PGB and PGC, while the positioning of the substituents in the ring defines the names of PGD and PGE, for instance.

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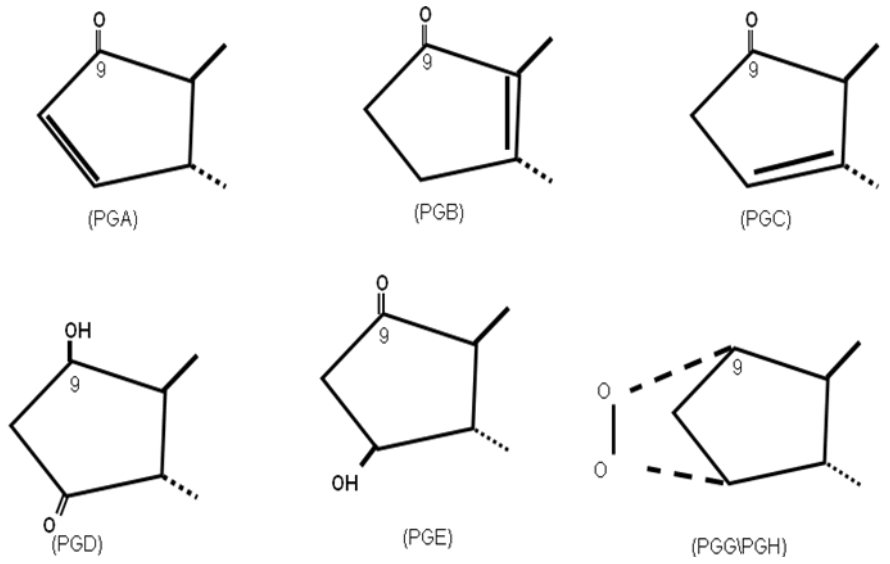

Figure 4. Nomenclature of the prostaglandins based on their ring structures.

The final subscripted number (e.g, PGE_2 or PGE_3) indicates the number of double bonds on the side chains. For example, PGE_2 and PGE_3 have the same substituent in their ring structures and 2 and 3 double bonds on the side chains respectively (Fig. 5).

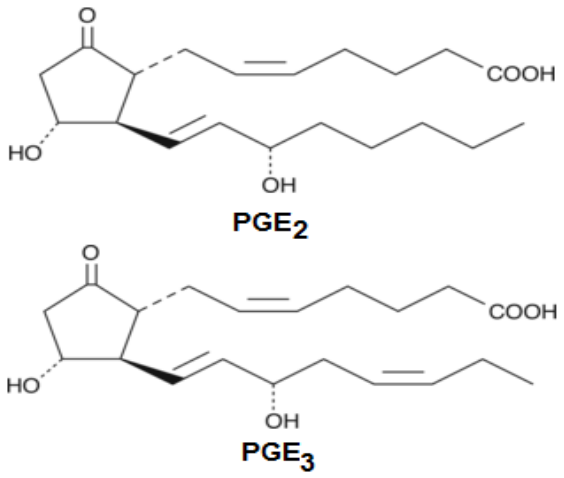

Figure 5. Nomenclature of the prostaglandin based on their side chain structures.

2.1.1.2 Leukotrienes

The short form for leukotrienes is LT, the ensuing letter represents the substituent in the arachidic acid structure and the subscripted number represents the number of double bonds. For instance, LTB_4 and LTB_5 exhibit the same number of hydroxyl substituent (two hydroxyl substituents) and 4 and 5 double bonds along their arachidic structures, respectively (Fig. 6)

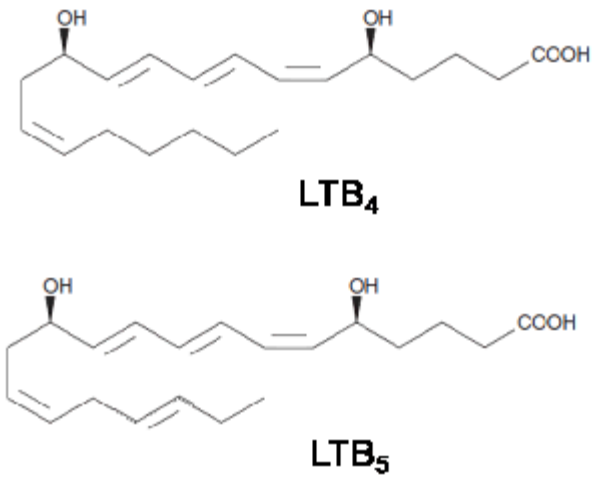

Figure 6. Nomenclature of the leukotrienes.

2.1.2 Eicosanoid Metabolism

Eiocosanoids, such as prostaglandin (PGs), leukotrienes (LTs), thromboxanes (TXs) and lipoxins (LXs), are signaling molecules derived mainly from the enzymatic oxygenation of arachidonic acid (AA) and also from eicosapentanoic

acid (EPA) pathway (Fig. 7) (Wada, et. al., 2007). Since their discovery eight decades ago, countless studies have been conducted to establish the crucial part of eicosanoids in the metabolic pathway. Early investigations recognized the biochemical contribution of eicosanoids to vascular homeostasis, platelet aggregation and gastric mucosa protection (Boughton-Smith and Whittle, 1988). Nowadays, they are also involved in the regulation and control of various biochemical processes related to asthma, chronic pains, cancer (Chulada, et. al., 2000 Larsson, et. al., 2004) and inflammation (Wenzel, et. al., 1990, Chen, et. al., 2009).

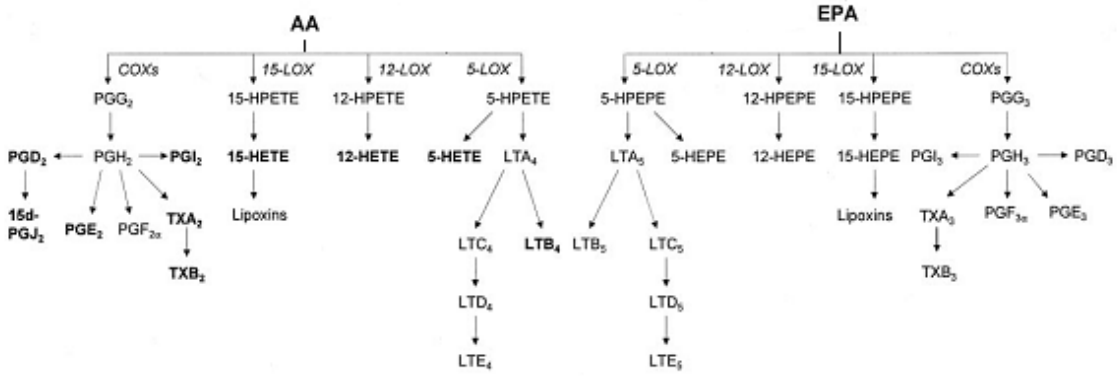

Figure 7. Eicosanoid synthesis via the AA and EPA.

2.1.2.1 Arachidonic Acid Metabolites

The AA cascade (Fig. 8) is the major biosynthetic pathway for the production of the myriad eicosanoid metabolites. Cells exposed to various physiological and pathological stresses, release AA from the phospholipid membrane, catalyzed by the phospholipase A_2 (PLA_2). Free AA then serves as a substrate for eicosanoid synthesis either through the COX or LOX described in Fig. 9 (Harizi, et. al., 2008).

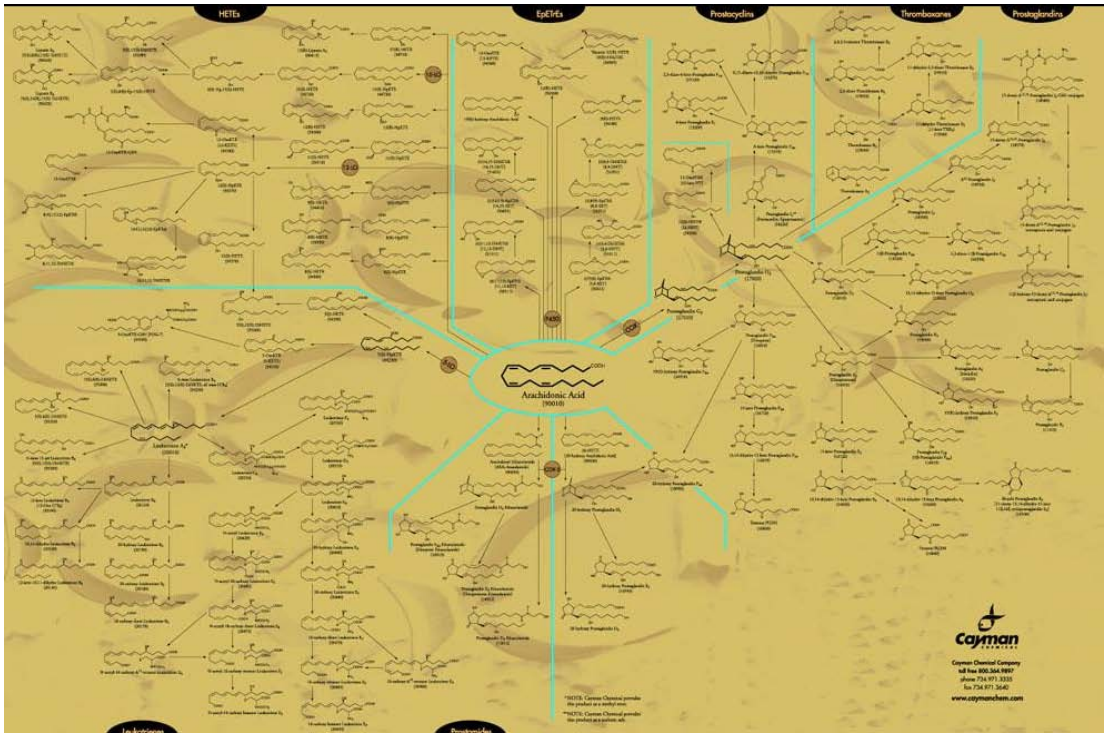

Figure 8. Arachidonic Acid cascade (adapted from Cayman Chemicals, MI, USA)

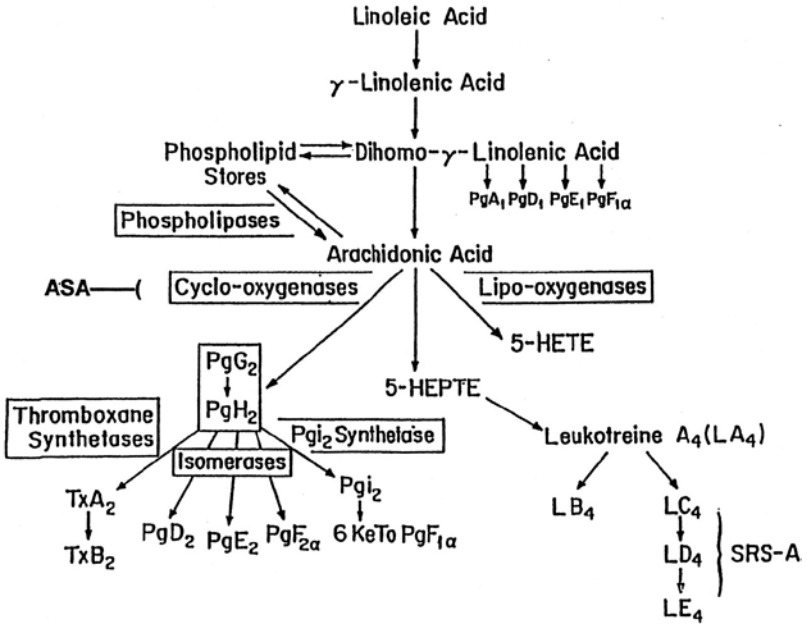

Figure 9. Eicosanoids synthesis of arachidonic acid via COX and LOX pathway.

2.1.2.2 Eicosapentaenoic Acid metabolites

Eicosapentanoic acid (EPA) is also a precursor molecule for the synthesis of eicosanoids. However, only fish are capable of synthesizing EPA whereas human get EPA from the fish they consumed in their diets.

Although EPA and AA are structurally similar (both with 20 carbon atoms), they differ in the number and position of the double bonds. EPA has five double bonds, the first double bond located after the third carbon atom from the methyl end while AA is composed of four double bonds and the first unsaturation found after the sixth carbon from the methyl end. It has been reported that dietary EPA is incorporated into the cells by substituting AA from the cellular membrane (Nieves and Moreno, 2006) and that the same group of enzymes acting on AA can exert similar actions on EPA molecule to produce different eicosanoid metabolites. In this way, PLA_2 releases EPA from the phospholipid bilayer membrane and converts EPA to 3-series of prostaglandins (PGE_3) and 5-series of leukotrienes (LTB_5) by the action of COX and LOX enzymes (Lee, et. al., 1987).

2.1.2.3 Biological Roles of Eicosanoids

Most AA-derived eicosanoids are distinguished for their pro-inflammatory action as indicated by their high proportion during inflammation (Bagga, et. al., 2003). Studies also showed that PGE_2 could be a link in the progression of cancer (Chulada, et. al., 2000). Leukotriene B_4 (LTB_4), another AA-derived product from the LOX pathway, is also associated in tumor cell adhesion leading to the spread of cancer cells in the body (Calder and Grimble, 2002).

Although the majority of the AA-derived eicosanoid metabolites are referred to as pro-inflammatory agents, clinical studies showed that PGE_2 is an exception

(Raud, et. al., 1988). The PGE_2 also exhibits an anti-inflammatory property by inhibiting the 5-LOX and inducing the 15-LOX. The inhibition of 5-LOX results in low production of pro-inflammatory of leukotrienes (4-series species) while induction of 15-LOX enhances the formation of anti-inflammatory lipoxins (Schmitz and Ecker, 2008).

The EPA-generated eicosanoids are regarded as potential anti-inflammatory mediator. However, EPA-derived leukotrienes and prostaglandins are less potent than the AA-derived. Leukotriene metabolites from the EPA via the 5-LOX pathway are less potent than their counterpart product from AA (Terano, et. al., 1984). For instance, LTB_5 is 30 times less bioactive than LTB_4, the counterpart product from AA (Terano, et. al., 1984). Moreover, Hawkey, et. al. (1992) studied the effect of suppression of LTB_4 synthesis by treating the inflammatory bowel disease patients daily with fish oil containing 4.5 g of eicosapentanoic acid for a year. The results showed that LTB_5 concentration increased while those of LTB_4 decreased by approximately 50%. This result indicates that the anti-inflammatory effect of EPA works in three ways. Firstly, the EPA-generated leukotrienes acts as competitive inhibitors because they are less potent as compared from those coming from the AA precursor. Secondly, EPA suppresses LTB_4 production through competition with AA on the lipoxygenase enzymes. Thirdly, EPA and its derivatives hasten inflammatory pathway reactions by inhibiting some steps in the lipoxygenase pathway (Hawkey, et.al., 1992).

2.1.3 Arachidonic Acid in Cells and Tissue

The level of free AA in unstressed cells is generally accepted as “low level”. Reports on the determination of AA are relatively few but usually the level of free AA is quantified using a mass assay. Using a mass per volume calculation, the level of esterified arachidonate in unstressed human platelets (~20 µl volume and

~30 µg arachidonate/109 platelets) is approximately 5 mM (Brash, 2001). The reported amount is the average level for the whole cells but levels in subcellular compartments like the plasma membrane are expected to be higher.

2.1.4 Eicosanoid Synthesis in Liver Cells

Liver is a heterogenous tissue that is made up of parenchymal cells, the hepatocytes (epithelial cells) and at least four major types of non-parenchymal cells (messechymal cells), the Kupffer cells (resident macrophages), the perisinusoidal or stellate cells, the sinusoidal epithelial cells and the large granular lymphocytes (natural killer cells). About 65 % of the cells in the liver are parenchymal cells, which constitute the largest cell number and proportion of liver mass. The non-parenchymal cells are about 35 % which is 10% of the liver mass (Weibel, et. al, 1969 and Malik, et. al., 2002).

The liver plays a vital role in body metabolism especially in eicosanoid synthesis, making it the preferred organ of choice for eicosanoid investigation. Bartolini, et. al. (1978) showed that in parenchymal rat liver cells 61 % of the intracellular fat was accounted for by the phospholipids. Furthermore, it was found that 7 % was free or in neutral lipids and 1 % was transformed into prostaglandins. On the other hand, non parenchymal cells contains less arachidonate in the phospholipid membrane (22 %) but more free fatty acid (23 %) or converted AA into prostaglandins (4.3 %). The synthesis of prostaglandin is found to occur in rat liver homogenates, isolated hepatocytes and in both the parenchymal and the non-parenchymal liver cell fraction (Morita and Murota, 1980).

2.2 Analytical Techniques for Eicosanoids Quantitation

Research studies showed that the eicosanoid synthesis is a web of complicated and interconnected interactions. Therefore, to be able to picture the changes created by blocking one pathway, there is a need to design a sensitive and reliable method that can identify and quantify simultaneously the eicosanoid metabolites.

2.2.1 Immunoassays

Conventionally, antibody based assays such as radio-immuno assays (RIA), have been used in quantifying different eicosanoids (Rocha, et. al., 1997). However, a lot of drawbacks are raised with regards to this technique. Aside from the few available antibodies for eicosanoids, there is also the danger of cross-reaction of isomeric eicosanoids with various antibodies which can result in low selectivity leading to over or under estimation of the amount of metabolite present (Mesaros, et. al., 2009). Metabolite overestimation has been observed by Proudfoot, et. al. (1995) while studying the oxidation products of low density lipids. This study demonstrate that 8-epi PGF_2 cross-reacted with PGE_2 metabolite by 4% which resulted to a much higher amount of PGE_2 observed than the true value. Thus, this makes the use RIA both expensive and inefficient.

A popular technique for the quantitative analysis of eicosanoids is the enzyme-linked immunosorbent assay (ELISA). However, a number of problems are also associated with this technique such as the use of complicated procedures, low sensitivities, poor specificities, cross reactivities and complicated clean-up procedures for eliminating interfering substances in the sample matrix (Hishinuma, et. al., 2007). Il'yasova, et. al. (2004) have compared ELISA and GC-MS methods in determining the levels of PGF_2-isoprostanes and found that

the estimated concentration by ELISA were 30 times higher than by GC-MS. This indicates clearly that ELISA could overestimate the levels of PGF_2 in the sample. In addition, the procedure is limited to only to single product detection at a time. Unpublished studies at NIFES on the determination of PGE_2 in cell culture media observed that the metabolite levels estimated by ELISA were 10 times higher than by LCMS-MS.

2.2.2 Capillary Electrophoresis

Capillary electrophoresis is also an appropriate method in some cases (Jin-Lan, et. al., 1998), but not applicable to the analysis of all types of eicosanoids. Aside from that, the technique has poor concentration sensitivity detection for the majority of analytes tested due to the limited sample-volume capacity of the capillary and the short (optical path length of the capillary when using on-column ultraviolet detection (Guzman, et. al, 2006).

2.2.3 Gas Chromatography

The evolution of gas chromatography-mass spectrometry (GC-MS) paved way for simultaneous analysis of multiple eicosanoids (Ribeiro, et. al., 1992 and Hammes, et. al., 1999)**.** However, this technique requires a very tedious derivatization steps and consequently is quite time- consuming - especially for the analysis of many samples.

2.2.4 Liquid Chromatography

The advent of high performance liquid chromatography tandem mass spectrometry (HPLC-MS), paved the way for the quantification of up to 60 eicosanoids simultaneously as reported by Deems (2007). Based on the eicosanoids chemical and physical characteristics, eicosanoids can be isolated individually through liquid chromatography while collision-induced decomposition can yield distinct precursor or product transition that can be used in multi-reaction MS monitoring mode. Single or tandem LC-MS based methods have become one of the most powerful techniques for determining eicosanoids in cell cultures due to their high sensitivity, high selectivity and simplicity of sample preparation (Deems, 2007). Cao, et. al. (2009) developed a method based on LCMS-MS technique for determining PGE_2 and PGD_2 production in cell culture supernatants and other biological fluids. This study achieved a limit of detection of 20 pg/mL and an inter-day and intra-day coefficient of variation lower than 5%. Furthermore, Araujo, et. al. (2008) devised a much simpler method involving LCMS-MS protocol by replacing the conventional time-consuming sample preparation of cell cultures with direct injection of redissolved cell culture media into the LCMS system and limits of detection and quantification for Williams' medium E and L-15 medium of 0.5 and 1 ng/mL, respectively. In addition, Blewett, et. al. (2008) was able to develop also an LCMS method that can separate and quantitate 23 different eicosanoids from all the three oxidative pathways. The limit of detection ranges from 1.6-174.0 pg/mL for the 23 different metabolites, which is sensitive enough to quantitate endogenous levels of eicosanoids. However, a leukotrienes yeilded very poor chromatographic peak shapes due to the presence of protonated amino acid moieties on the cys-LT (Blewett, et. al., 2008).

2.3 Chemometric Approaches

2.3.1 Design of Experiment

During the past decades the investigative approaches to biological problems have markedly diversified. This diversification has been cause in part by the introduction of methods from mathematics, statistics and chemistry such as factorial design, principal component, factor analysis, etc (Kwak, et. al., 2006, and Benoit, et. al., 2007) . Since the biological processes involved a wide variety of factors, screening approaches are regarded as an important component in experimental design procedures. This is the first stage to efficiently assess the factors influencing an analytical system under investigation.

Different screening approaches for assessing the influence of several factors have been reported (Araujo and Brereton, 1996). The first step is to identify a region of study for each factor under consideration. The chosen region is an experimental sensible region which is referred to as the experimental domain. The results outside this chosen region cannot be classified as valid. The second step is to choose a design. One class of experimental design that can be used to study a biological system is a factorial design. Using this design, the experimenter can vary simultaneously the different factors included in the experiment in a systematic way. Moreover, it can be use to estimate the influence of the factors under study along with the interaction (Deming, 1987). In single factorial design, only one independent factor is involved in which the factor is manipulates at multiple levels and the effect of the factor is based on the results of the analysis of variance. This design can be used when running experiments with less time or material available but it is very limited in scope. However if two or more factors are of equal interest, two-level factorial design is more applicable and a popular choice among experimenters. Morgan (1995) mentioned that this design allows the experimenter to have a glimpse of the effects of the factors involved in a relatively few runs. Aside from that, the results

are easy to evaluate and can provide major trends even though only a small region of the factor space is under investigation. Choosing a response is the third step in screening approach. The chosen response should be easy to quantify such as extraction efficiency, peak area or signal height. Hence, it is important to select an appropriate measurement of response to avoid formulating inaccurate conclusion. The last step is to build a mathematical model relating the response to the factors involved (Araujo & Brereton, 1996). Usually, the raw data are not use, instead coded factors are utilized. Typically, the lower level is denoted by -1 and the higher level by +1.

2.3.2 Multivariate Curve Resolution

Peak resolution and alignment are the most often encountered problems when analyzing LCMS data set (Scott, 1995). Although experimental optimization methods are usually employed, their contribution in improving the chromatographic resolution cannot be regarded as improvement (Massart, et. al., 1988).

The most common approaches available for unbiased preprocessing of information from LC-MS data are peak detection, curve resolution or deconvolution and summing of data to obtain a total mass spectrum for each sample. A peak detection algorithm is used to detect all peaks in the data above the specified noise threshold. Peak area and peak height are then used for quantitatitation while the retention time and m/z are used for identification (Plumb, et. al., 2003). Curve resolution and deconvolution are done to resolve overlapping peaks of the chromatographic and spectral profiles. The areas of chromatographic profiles become the basis for quantitative measure of the compound while the spectral profiles and retention times are used for identification purposes (Idborg-Björkman, et. al., 2003). Summation of data is

done by combining the whole chromatograms or first by dividing the chromatogram into segments and then combining all the segments before subjecting to multivariate analysis (Plumb, et. al., 2002).

Resolution methods can be classified into three kinds: iterative, non-iterative and hybrid methods. Iterative methods begin with guessing or defining an initial set of profiles. Then non-negativity and unimodality constraints are imposed during the iteration process. Iterative target transformation factor analysis (ITTFA) and alternation regression (AR) belongs to this category (Maeder and Neuhold, 2007). They are simple to use but the results of these methods are dependent upon the starting vectors. Better results can be obtained if the separation power of chromatography is used to localize key spectra that are either pure, or whose signal is dominated by one analyte (Shen, et. al., 2001).

The non-iterative methods follow the elution of analytes by rank analysis of evolving matrices (Shen, et. al., 2001). The evolving factor analysis (EFA) (Séquaris and Koglin, 1987), window factor analysis (WFA) (Malinowski, 1992), and heuristic evolving latent projections (HELP) (Liang, et. al., 1992) belong to this cluster. In non-iterative methods, the corresponding concentration and spectral profiles can be estimated when the elution window of each analyte is obtained. However, unresolved background and heterosedastic noise are just a few factors that affect the matrix rank (Liang, et. al., 1993, Wang, et. al., 1996 and Shen, et. al., 2000).

Hybrid procedures are combination of both iterative and non-iterative features. This group includes the automatic window factor analysis (AWFA) (Malinowski, 1996) and *Gentle* procedure (Manne and Grane, 2000). The two methods overcome the drawbacks of iterative and non-iterative procedures.

Gentle procedure, as proposed by Manne and Grande (2000), involves removal of negative region, estimation of elution window, minimization of concentration

profiles outside the elution windows and checking the quality of resolution to estimate the concentration and spectral profile.

Recently, software package based on Gentle principle for multivariate curve resolution become available. It is iterative approach in solving Eq. 1 where ***X*** is the data matrix obtained for a sample, ***C*** is the true chromatographic profile, ***S*** is the spectra for all components and ***E*** is the residuals.

$$\boldsymbol{X} = \boldsymbol{C}\boldsymbol{S}^{\boldsymbol{T}} + \boldsymbol{E} \qquad (1)$$

MS Resolver™ (Pattern Recognition System, Bergen, Norway) is designed to resolve multiple component data from hyphenated instruments such as mass spectroscopy data. Unlike the Xtricator (another curve resolution software from the same company) which depends heavily on interaction with the software user, MS Resolver™ is a fully automated resolution package. The advantages of using MS Resolver™ are the reduction of analysis time, capability to resolve data from samples containing more than 400 components and the possibility to work on more complex data. Spectral similarity and retention times are used by MS Resolver™ to match the resolved components from different samples automatically. The integrated chromatographic areas for each compound in each sample are then placed in a predictor matrix, without any user interaction. This matrix can be exported and used in calibration and regression against an external response variable.

Chapter 3

EXPERIMENTAL

3.1 Materials and Reagents

Dulbecco's modified eagle medium (DMEM), L-15 medium, antibiotic antimycotic solution, flunixin meglumine, N-(2-phenylethyl)indomethacinamide, arachidonic acid (AA), eicosapentanoic acid (EPA), leukotriene B_5 (LTB_5) were from Sigma-Aldrich (St. Louis, MO, USA).

Prostaglandin E_2 (PGE_2), prostanglandin E_3 (PGE_3), leukotriene B_4 (LTB_4) were from Cayman Chemicals (Ann Arbor, MI, USA).

GlutaMax was from Gibco-BRL (Cergy-Pontoise, France).

Penicilline-streptomycine-amphotericine was from BioWittaker (Petit Rechain, Belgium).

Acetonitrile and formic acid were from Merck (Darmstadt, Germany).

De-ionized water was purified in a Milli-Q system (Millipore, Milford, MA).

The 12 well plates used were from Corning (New York, USA).

Non-parenchymal and head kidney cells were isolated and cultured from Wistar rat liver and Atlantic cod and kindly donated by Dr. Zhen-Yu Du and Dr. Elisabeth Holen from the National Institute of Nutrition and Seafood Research (NIFES).

3.2 Culturing Protocol

3.2.1 Non-Parenchymal Cultures

Non-parenchymal cells isolated from Wistar rats and almost free of Kupffer cells were seeded to two 12-well dishes at approximately 5×10^6 cells per unit density. The attached cells were pre-incubated in Dulbecco's modified eagle medium (DMEM) containing 10 % v/v fetal bovine serum and 1.0 % v/v antibiotic antimycotic solution for three hours at 37C under humidified atmosphere (5% CO2, 95% air). After the pre-incubation period, DMEM was removed and the attached cells were washed with DMEM medium alone to remove unattached and viable cells. The incubation period started by adding the specified volume of 50 µM AA (Sigma), 50 µM EPA (Sigma), or inhibitor as indicated in Table 2 and was made up to 1mL total volume by adding DMEM with 10 % v/v bovine serum albumin (BSA) and 1% v/v antibiotic. The cells were then incubated for 48 hours without changing the media.

Table 1. Volumes of the AA, EPA and Inhibitor Added to each Wells

	Volume added in each treatment in mL							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
50 µM Arachidonic Acid	0	0.45	0	0.45	0	0.45	0	0.45
50 µM Eicosapentanoic Acid	0	0	0.45	0.45	0	0	0.45	0.45
Inhibitor (mixture of COX and PLA_2 inhibitors)	0	0	0	0	0.1	0.1	0.1	0.1

3.2.2 Head Kidney Cultures

Head kidney cells isolated from Atlantic cod (800-1000g) were seeded into a 12 well plate with a density of 3×10^6 cells per well. A volume of 2 mL of L-15 medium consisting of 1 % glutaMax, 1 % penicilline-streptomycine-amphotericine and 10% bovine serum was added to each well. The cells were incubated at 9 ºC in a normal atmosphere incubator for 24 hours without changing the medium.

3.3 HPLC-MS Sample Protocol

After incubating non-parenchymal cells and head kidney cells for 48 and 24 h respectively, 1 ml of the DMEM media was centrifuged at 3000 g for 10 min at 4 ºC. An aliquot of 500 µL of supernatant is collected and vacuum dried in a RapidVap vacuum evaporation system (Labconco, Kansas, MO). An aliquot of 500 µL of acetonitrile was added to the dried sample, vortex-mixed for 1 minute and the supernatant collected. The collected supernatant was evaporated to dryness under nitrogen at room temperature and reconstituted in 60 µl of acetonitrile, vortex-mixed for 30 seconds and submitted to LCMS/MS.

The aforementioned method (Araujo, et. al., 2008) permitted the injection of the redissolved cell culture media into HPLC-MS. The selection of this method was based on its several advantages. For instance, there is no need for derivatisation, degradation, solid-phase extraction, liquid-liquid extraction, thin layer chromatography or a combination of all these steps prior to HPLC-MS/MS analysis. In addition, it possesses low limits of detection and quantification in cell culture media (0.5 and 1 ng/mL respectively) and recovery percentages close to 100%.

3.4 External Standards

The quantification of PGE_2, PGE_3, LTB_4 and LTB_5 in the media was carried out by means of external calibration curves (0-500 ng/mL). The standards were mixed in acetonitrile to a final concentration of 700 ng/mL of each metabolite and the calibration curve was prepared as follows. The aliquot of the mixture were collected to yield the nominal concentrations between 0-500 ng/mL and a total volume of 50 µL. The calibration curve standards in acetonitrile solution were prepared in triplicates and evaporated to dryness under a stream of nitrogen at room temperature. Aliquots of 50 µl of fresh culture medium, with no detectable levels of PGE_2, PGE_3, LTB_4 and LTB_5 were added into each set of vials and vortex-mixed for 2 min and submitted to LCMS-MS.

3.5 High Performance Liquid Chromatography tandem Mass Spectrometry

The high performance liquid chromatography system used was an Agilent 1100 series LCMS ion trap detector (SL model) equipped with electrospray interface (ESI), a quaternary pump, degasser, auto-sampler, thermo-stated column compartment, variable-wavelength UV-detector and 25 µl injection volume. The separation was performed using Zorbax Eclipse-C8 reversed phase column (Agilent, Palo Alto, USA) with dimensions of 150 mm × 4.6 mm × 5 µm and maintained at 40 ˚C column oven temperature. An isocratic mode solvent system was employed using acetonitrile with formic acid of 0.1% (v/v) mobile phase, and flow rate and UV wavelength set at 0.2 mL/min and 254 nm, respectively. The sample injection volume was 15 µL. A negative ion mode ESI was used as ionization source and the ion optics responsible for getting the ions in the ion-trap were controlled by using the Smart View option with a resolution of 13000 *m*/*z*/sec (FWHM/*m*/*z* = 0.6-0.7). The nebulizer gas used was nitrogen with a flow of 8 L/min, pressure 350 psi and temperature of 350˚C. The mass profile s were

obtained using multiple reaction monitoring (MRM) with scan mass range of 50-1000 m/z. The total time it required for each sample run was 60 minutes. Data acquisition and pre-processing were done using the Chem Station for LC/MSD version 4.2 from Agilent and converted into CDF files. The transitions monitored are indicated in Table 2.

Table 2. Transition Monitored Among Eicosanoid Metabolites

Eicosanoid Metabolites	Parent Ion Molecular weight, $[M-H]^-$	Product Ion Molecular Weight			
		$[M-H]^- \rightarrow [M-H_2O-H]^-$	$[M-H]^- \rightarrow [M-2H_20-H]^-$	$[M-H]^- \rightarrow [M-2H_2O-CO_2-H]^-$	$[M-H]^- \rightarrow [M-H_2O-CO_2-H]^-$
PGE_2	351	333	315	271	
PGE_3	349	331	313	269	
LTB_4	335	317			273
LTB_5	333	315			271

3.6 Experimental Design

A two-level factorial design involving three variables was used in this study to determine the production of PGE_2, PGE_3, LTB_4 and LTB_5 from non-parenchymal cell cultures. The variables were AA, EPA, and an inhibitor (Inh) that block simultaneously the action of the COX and PLA_2 enzymes. The levels analyzed were absence and presence of the previously mentioned three variables and denoted by a negative (-) or a positive (+) sign. The number of experiments was dictated by the expression 2^3= 8 experiments. The eight experiments allowed determining the effects of AA, EPA and inhibitor along with their interactions. All possible combinations are detailed in Table 3 and were measured in triplicates.

The differences between the variance of treatments were evaluated using single factor ANOVA at a 95 % confidence limit.

The main effects and interaction effects were evaluated using the Minitab® 15.1.30.0 (Minitab® Inc, PA, USA) statistical software package.

Table 3. Treatment Employed to the Non-Parenchymal Cell Cultures

Treatment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
AA	-	+	-	+	-	+	-	+
EPA	-	-	+	+	-	-	+	+
Inhibitor	-	-	-	-	+	+	+	+

3.7 MS Resolver™ and Chromatogram Integration

Data obtained from LC-MS/MS systems are known as two-way data and every sample is characterized by a matrix. The rows and columns of such matrices contain special meanings as illustrated in Fig. 10. A time direction and a mass direction are denoted for a given matrix. Each row signifies the mass spectrum recorded at a specific time. Graphical illustration of the row vector gives a mass spectrum of analytes that eluted at a particular retention time. Similarly, plotting a column vector results in a chromatogram profile of the analytes that has a fragment at that particular mass/charge ratio. In cases of complex samples containing different components, separation is difficult and overlapping is unavoidable. For this kind of problem MS Resolver™ is a great help.

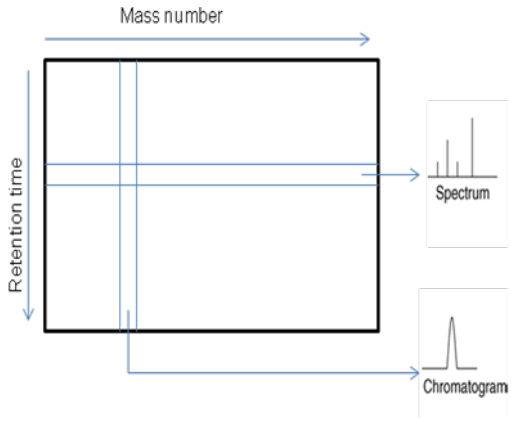

Figure 10. Data matrix for a typical LC/MS data.

MS Resolver™ software (Pattern Recognition System, Bergen, Norway), a fully automated software package, was used for curve resolution process. The CDF files coming from the LC-MS/MS system were imported into the software using a resolution of one mass number. The size of the data sets was reduced by creating a subset and saving parts of the original data set under a different name.

For every metabolite extracted ion chromatogram profiles, data subsets were created by selecting the mass/ratio of a particular metabolite of interest. From the create subset data, all values in the time point rows option were selected while in the mass number option one particular m/z value (PGE_2 = 333, PGE_3 = 331, LTB_4 = 317 and LTB_5 = 315) was chosen as detailed in Fig. 11. The resulting subset yielded a chromatogram plot with only one mass number.

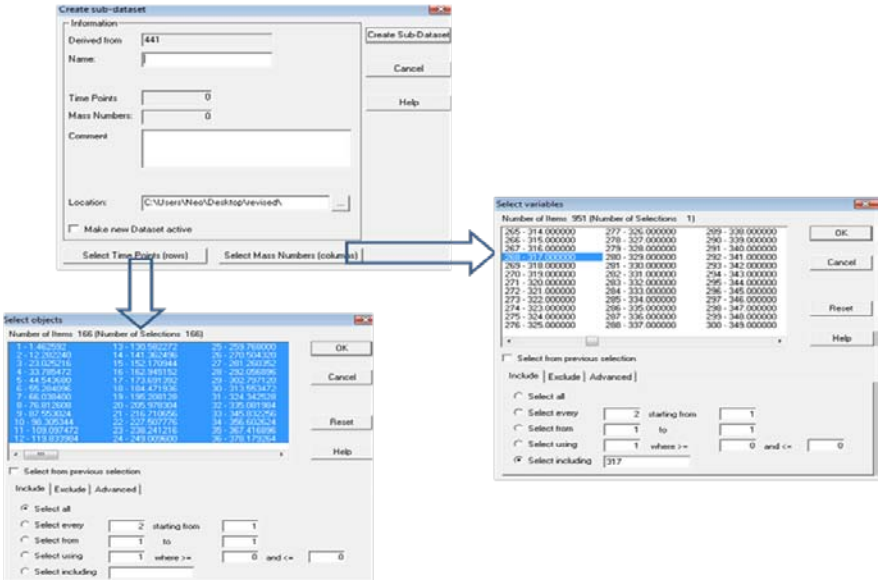

Figure 11. Schematic procedure for creating a subset from a given LCMS data.

Resolved chromatogram profiles were exported and saved as MATLAB files. The area of the chromatogram profiles was further integrated using the trapezoidal method of integration coded in MATLAB 7.9.0.529 (R2009b) (The Mathworks, MA, USA). Microsoft® Excel 2007 SP-2 was used to calculate the mean, the standard error of the mean and the standard deviation. Data were analyzed and displayed using the Data Analysis Tool of Microsoft® Excel 2007 SP-2.

Both information on the m/z direction and retention time direction are taken into consideration by using the MS Resolver™ software.

Chapter 4
RESULTS AND DISCUSSION

The discussion of results is divided into four parts. The first part is a brief discussion of the eicosanoid metabolites LCMS-MS spectra, second part deals with the application of MS Resolver™ in resolving the eicosanoid chromatogram and spectral profiles, third part focuses on the experimental design in determining the effects of AA, EPA and inhibitor on eisosanoid synthesis and the last part discusses the eicosanoid synthesis using different cell to support the data obtained from non-parenchymal cells.

4.1 Identification of the LCMS-MS Profile of Eicosanoid Metabolites

The PGE_2, PGE_3, LTB_4 and LTB_5 external standards (700 ng/mL) prepared individually in acetonitrile were submitted to LCMS-MS to evaluate their elution times and behavior of their mass spectra. The mass spectrometric data were first collected in auto-scan mode from m/z 50-1000 from 0-20 min, in negative ion mode to obtain the full information about the behavior of the mass spectra of the four particular eicosanoids. Inmediately after, the eicosanoids metabolites were monitored using multi-reaction mode so that a high response of the analyte of interest will be obtained. From the chromatogram of a standard mixture (Fig 12), it was found that only one peak exist instead of the expected four different peaks. By selecting the EIC chromatogram of the PGE_2, PGE_3, LTB_4 and LTB_5 metabolites and overlaying the four chromatogram profiles, severe overlapping of chromatogram peaks was observed (Fig. 13). The elution times were 8.5, 8.0, 8.3, and 8.0 min for PGE_2, PGE_3, LTB_4 and LTB_5, respectively..

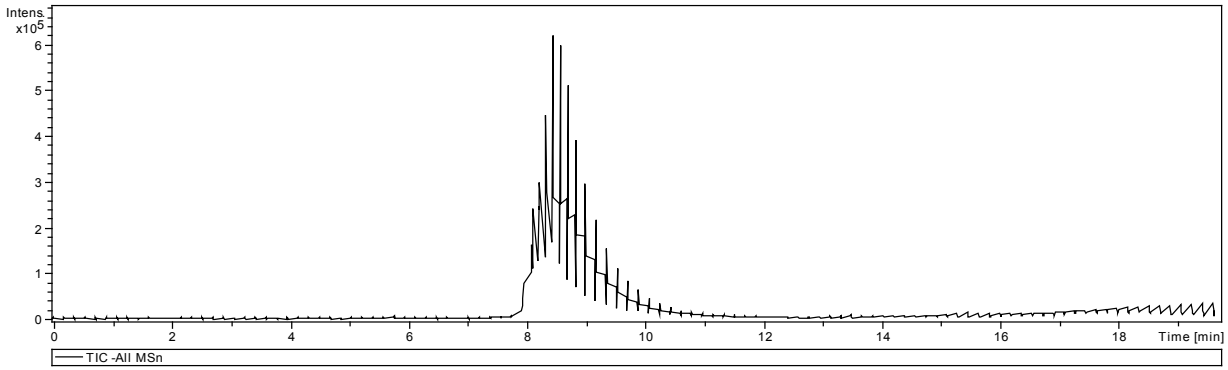

Figure 12. TIC chromatogram of the standard mixture collected in MRM mode.

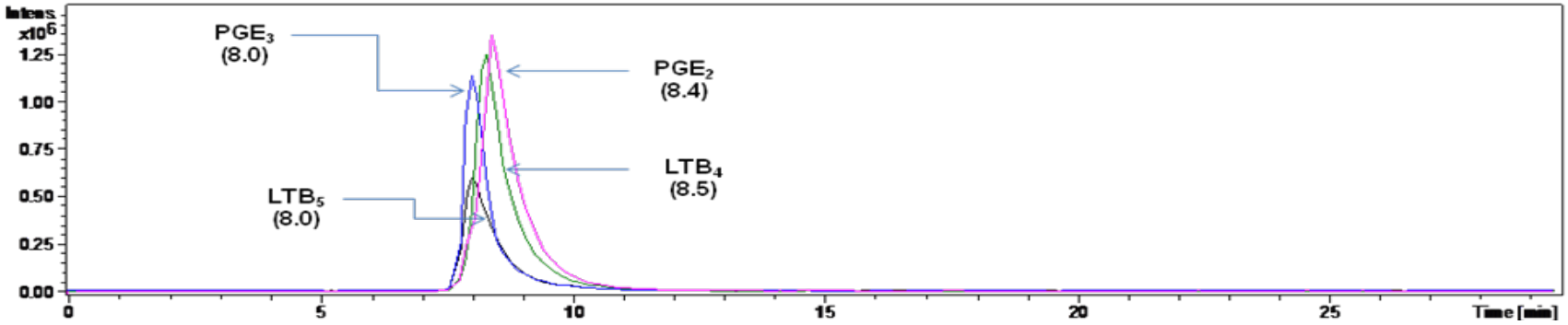

Figure 13. EIC chromatogram of the standard mixture collected in MRM mode.

The tandem mass spectral profiles of the four metabolites are shown in Figs. 14-17. The spectral profile of PGE_2 (Fig. 14) shows the deprotonated precursor ion at m/z = 351 along with its characteristic massfragments at m/z values 333, 315, and 271. The resulting fragments are due to the subsequent loss of two water molecules and a carboxyl group from the deprotonated PGE_2 precursor ion. A similar m/z transitions patterns were also observed from the PGE_3 ion (Fig. 15) giving m/z 331, 313, 269 as the fragment ions. On the other hand, the leukotrines spectra (Figs. 16-17) are characterized in general by the fragments $[M - H_2O - H]^-$ and $[M-H_2O-CO_2-H]^-$ from the precursor ions LTB_4 and LTB_5.

For quantitative evaluation, the first *m/z* transition of every metabolite, corresponding to the loss of a molecule of water (*m/z* 333, 331, 317 and 315 for PGE_2, PGE_3, LTB_4 and LTB_5 respectively) was used because they exhibit the highest intensity and abundance.

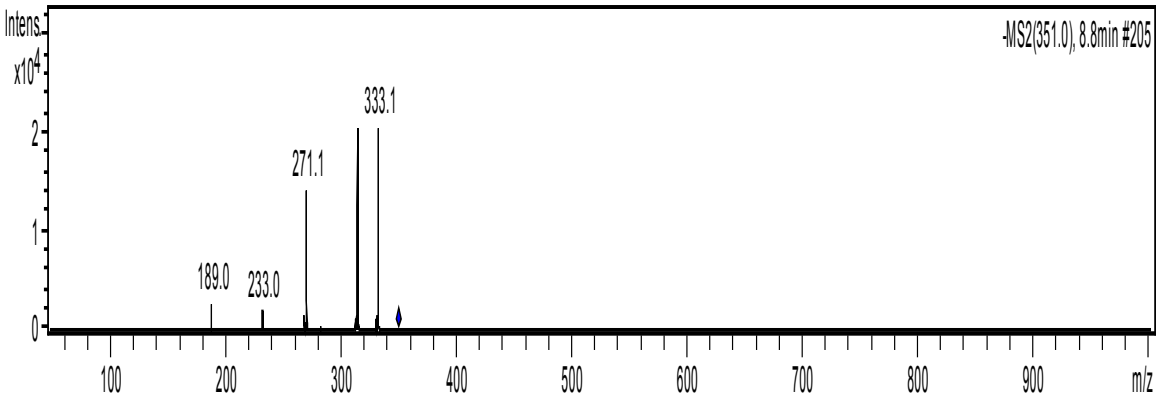

Figure 14. MRM transition of PGE_2 from m/z 351→ 333, 315, 271 m/z.

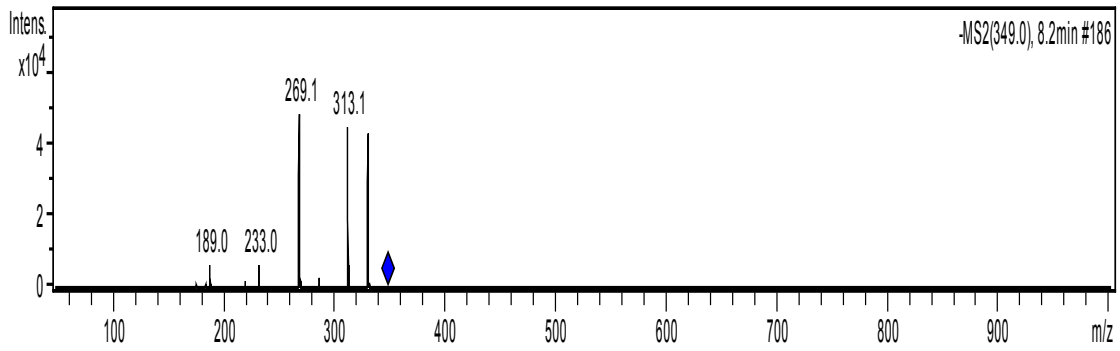

Figure 15. MRM transition of PGE_3 from m/z 349→ 331, 313, 269 m/z.

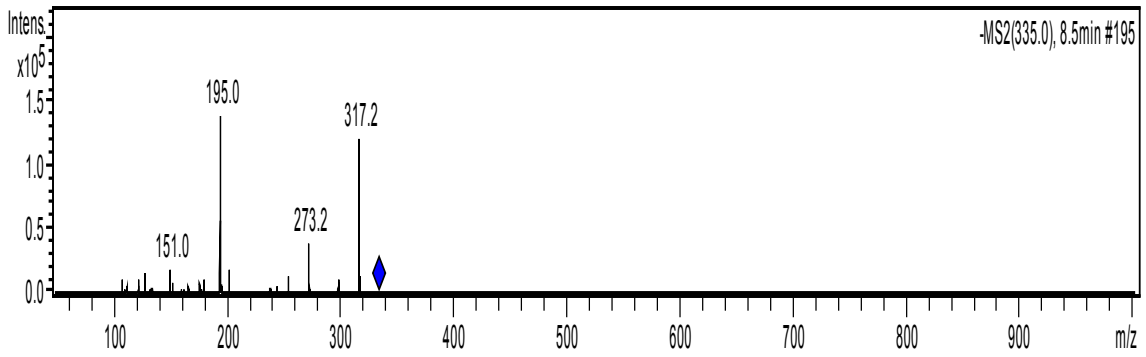

Figure 16. MRM transition of LTB_4 from m/z 335→ 317, 273 m/z.

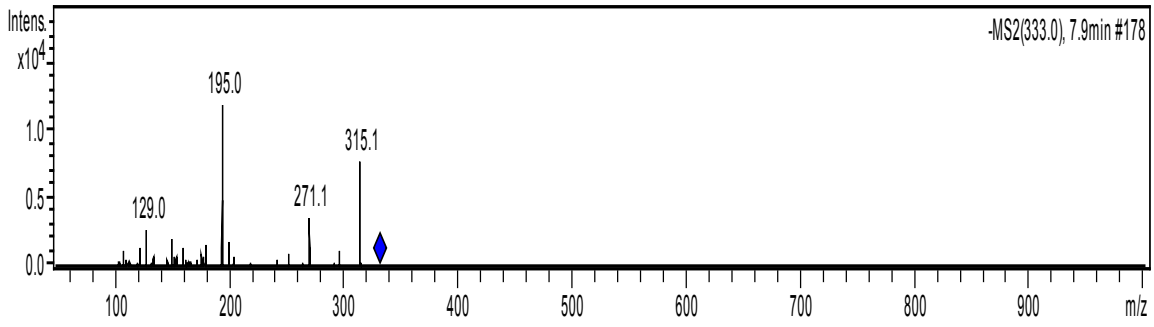

Figure 17. MRM transition of LTB_5 from m/z 333→ 315, 271 m/z

4.2 Application of MS Resolver™ to LCMS-MS Data

The use of hyphenated technique such as LCMS is a now a leading technique in generating metabolite profiles from complex biological samples due to its high sensitivity (Panchuad, et. al., 2005, Kempen, et. al., 2001, Yue, et. al., 2007 and Yan, et. al. 2005). The use of LC-ESI-MS-MS is widely accepted as a reliable technique for characterization and quantification of eicosanoids (Margalit, et. al., 1996, Newman, et. al., 2002 and Schmelzer, et. al., 2005). However, due to the similarity in structure of the various eicosanoids and their chemical characteristics, peak overlapping pose a major problem in acquiring information-rich data. Hence, there is a need for a systematic approach to extract information from LCMS data such as multivariate curve resolution or deconvolution.

In this study the capability of MS Resolver™ (Pattern Recognition System, Bergen, Norway), an automated curve resolution software package, to resolve spectral and chromatographic profiles of AA and EPA derived eicosanoids was explored. The TIC of one of the samples is shown in Fig. 18 where it is evident the overlapping of the mass profiles at different time points. The original chromatogram profiles of the metabolites under study usually contain approximate 170 retention times and 960 mass numbers (Fig. 19). From the plot (Fig. 19), it is also evident that most of the components in the sample elute approximately between the regions of 450-700. Since the chromatograms profiles were monitored via MRM mode, the m/z value of interest is already known. With this, modification in the steps of MS Resolver™ the analysis was done. Instead of dealing with the whole LC MS data set, a subset was created by selecting the m/z value of interest over the given set of time. After creating the subset, a chromatogram profile containing only one m/z value was obtained (Fig. 20). Unlike the previous untreated data, the processed chromatogram exhibits a defined peak of the component which is easy to quantify using the trapezoidal method of integration coded in MATLAB.

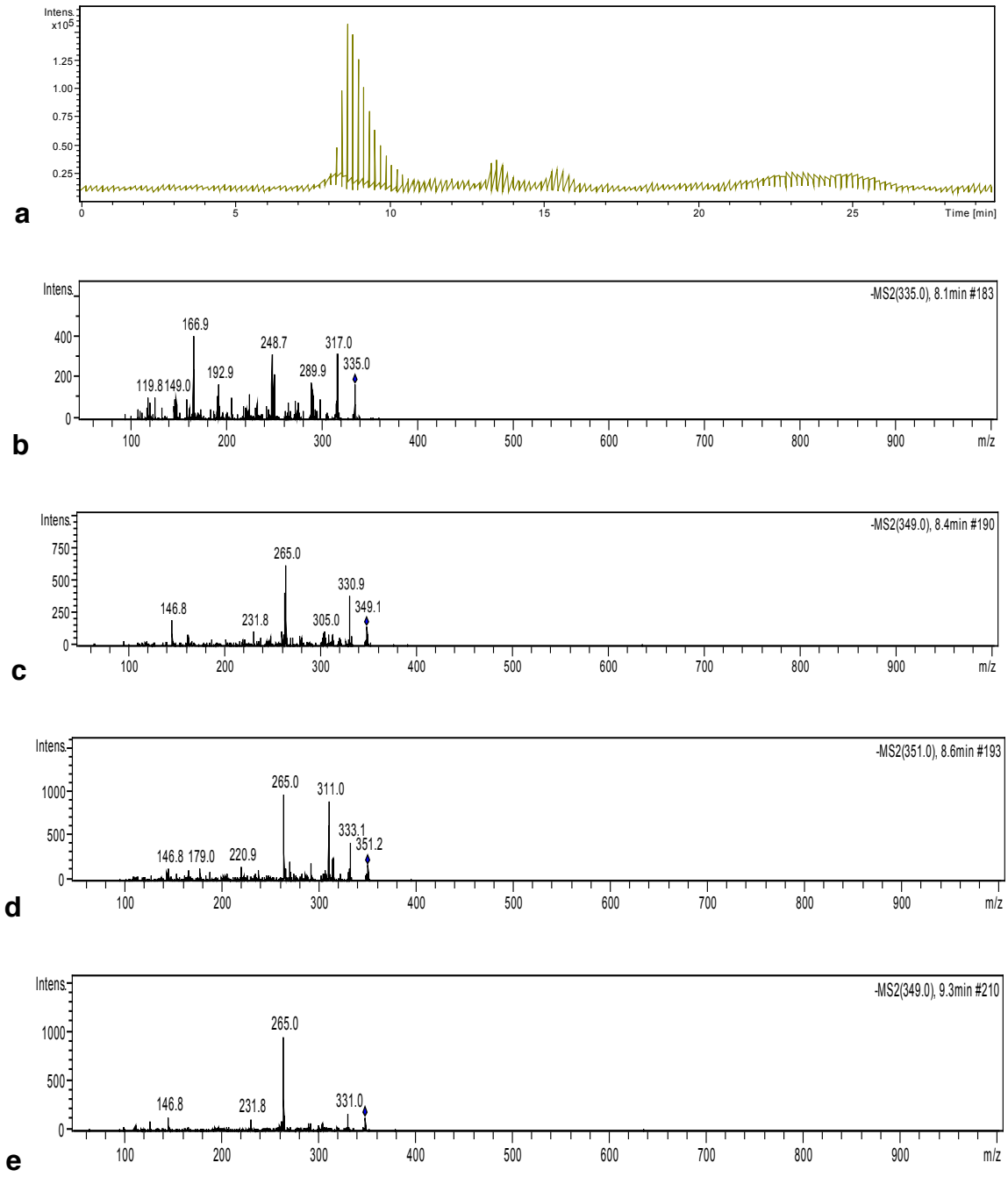

Figure 18. a. TIC chromatogram of a sample depicting the different mass number found at different time points (plots b-e).

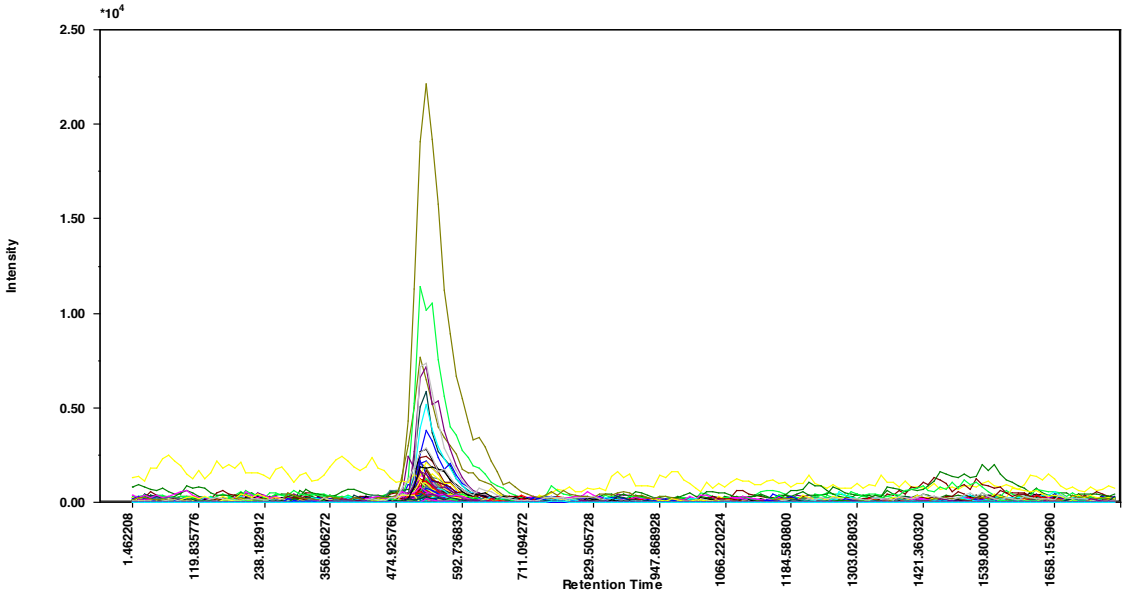

Figure 19. Original chromatogram profile obtained from LCMS data

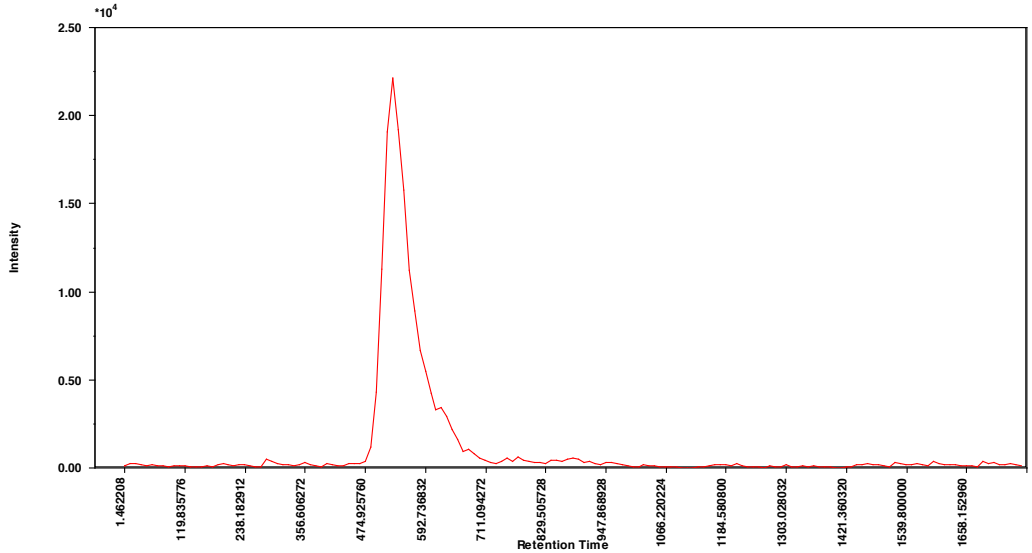

Figure 20. The chromatogram after extracting a specific m/z value using MS Resolver.

4.3 Two-Factorial Experimental Design

The eicosanoid synthesis in vitro or in vivo is dependent not only to the pre-existing AA in the cellular membrane but also to other factors such as added AA and EPA in to the biological system. To explore further the influence of added AA and EPA in non-parenchymal rat liver cells, a 2-level three-factor design (2^3) was carried out. The studied variables were AA, EPA and inhibitor of both cyclooxygenase (COX) and phospholipase A_2 (PLA_2) enzymes. The levels were the presence or absence of the factors. The full 2^3 factorial design allows estimating:

1. The *in vitro* effect of AA and EPA (either individually or in combination) on the production of their COXs (PGE_2 and PGE_3) and LOXs (LTB_4 and LTB_5) metabolites.
2. The *in vitro* effect of COX/PLA_2 inhibitor (either individually or in combination with AA and EPA) on the production of AA and EPA related COXs and LOXs metabolites.

4.3.1 Eicosanoids Responses

The non-parenchymal rat liver cell production of AA and EPA derived eicosanoids, PGE_2/LTB_4 and PGE_3/LTB_5 respectively and released in the medium was determined by using the 2-factorial experimental design and LC-MS-MS. The experimental response selected to assess the effects of the variables and their interactions was the average metabolite concentration expressed in ng/mL and calculated by means of the external standard calibration curve. The estimated concentrations for the various prostaglandins and leukotrienes (PGE_2, PGE_3, LTB_4 and LTB_5) are presented in Table 4. The positive (+) and negative (-) signs denotes the presence and absence of the variables in the treatments, respectively.

Table 4. Eicosanoids Mean Response obtained using 2^3 Factorial Experimental Design.

Treatment	AA	EPA	INH	Response in ng/mL Concentration			
				PGE_2	PGE_3	LTB_4	LTB_5
1	-1	-1	-1	15.2 ± 1.5	7.3 ± 1.0	8.4 ± 0.6	12.1 ± 0.4
2	1	-1	-1	15.4 ± 2.0	11.5 ± 1.0	12.5 ± 3.2	14.0 ± 1.8
3	-1	1	-1	19.7 ± 4.4	19.2 ± 2.7	185.1 ± 27.8	13.1 ± 0.1
4	1	1	-1	22.2 ± 6.1	27.3 ± 3.5	191.8 ± 29.1	16.0 ± 1.8
5	-1	-1	1	18.6 ± 3.2	14.1 ± 3.5	30.8 ± 6.3	5.6 ± 1.0
6	1	-1	1	14.8 ± 0.7	6.3 ± 2.9	23.8 ± 7.1	10.8 ± 0.8
7	-1	1	1	22.4 ± 4.2	33.2 ± 19.6	363.3 ± 98.8	14.9 ± 3.4
8	1	1	1	28.2 ± 15.4	20.8 ± 0.4	333.8 ± 183.7	15.6 ± 8.7

4.3.2 Estimation of Effect

The main effects of the variables under study (AA, EPA, Inh) were computed as follows: firstly, the product of the variable by the response (variable × response) is performed, secondly the average responses at low and high level (-1 and +1) are estimated from the resulting product and thirdly, the algebraic summation at low and high level is used to compute the main effects. For instance, the product of the variable EPA by its LTB_5 response in ng/mL is (-12.1 -14.0 +13.1 +16.0 -5.6 -10.8 +14.9 +15.6), the average at low and high levels are -10.625 and +14.9, and their algebraic summation is 4.275. The interaction effects are calculated in the same fashion by taking firstly the product between variables and the resulting column by the response. For instance the interaction between AA and EPA is calculated by AA × EPA × response.

The main effects and their interactions for the different metabolites considered in the present study are presented in Table 5 and 6.

Table 5. Calculated main effects of AA, EPA and inhibitor towards Eicosanoid production

Metabolites	Main effects		
	AA	EPA	INH
PGE2	1.2	7.1	2.9
PGE3	-5.4	18.8	5.8
LTB4	-6.4	249.6	88.5
LTB5	2.7	4.3	-2.0

Table 6. Calculated interactive effects of AA, EPA and inhibitor towards Eicosanoid production.

Metabolites	Interaction Effects			
	AE	AI	EI	AEI
PGE2	3.0	-0.2	1.5	1.8
PGE3	-3.7	-11.6	5.0	-5.6
LTB4	-4.9	-11.9	71.6	-6.3
LTB5	-0.9	0.3	2.8	-1.3

4.3.3 Main and interaction effects analysis

The analyses of the different treatments (Table 4) were carried out in random order. After conducting the analyses and calculating the analytical concentration of each metabolite, the responses (ng/mL) were entered in the Minitab 15.1.30.0 statistical software (Minitab® Inc, PA, USA).

4.3.3.1 PGE_2 Synthesis

The main effects of the variables on the production of PGE_2 by non-parenchymal rat liver cells are portrayed in Fig. 21. Based on the steepness of the line connecting the PGE_2 mean response without (-) and with (+), it is evident that the presence of EPA or inhibitor favored the synthesis of PGE_2 when compared to the AA or inhibitor. However, it must be said that the observed changes in PGE_2 concentration and consequently the main effect of these variables are not significant at a confidence level of 95 %. The small increment of PGE_2 concentration could be ascribed either to the stress of the cells due to the presence of added EPA or to the release of AA from the cell membrane when EPA is incorporated.

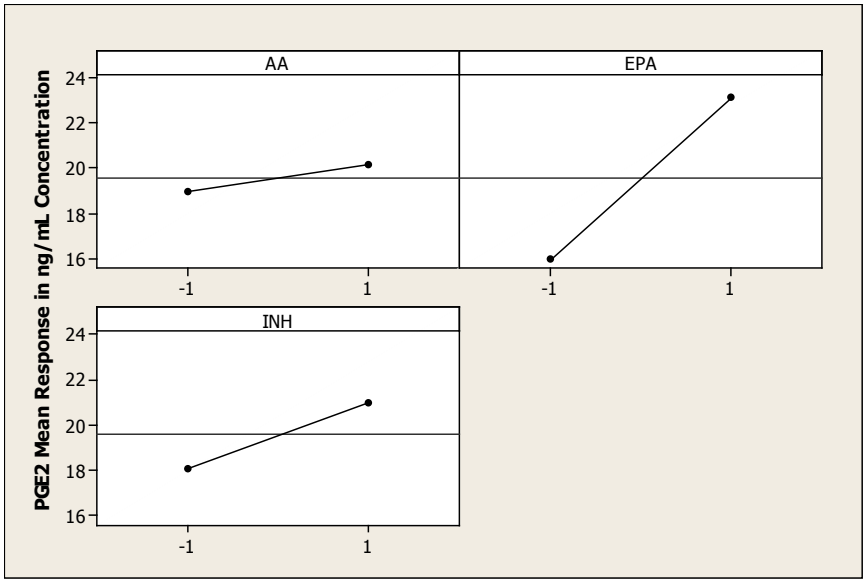

Figure 21. Main Effects Plots of AA, EPA and Inhibitor on PGE_2 Synthesis

The two-factor interaction plots (Fig. 22) define the interaction of AA vs EPA (upper left side plot), AA vs inhibitor (upper right side plot), and EPA vs inhibitor (lower right side plot). The black line indicates the lower level of the variable

present which is equivalent to the absence of the given variable. Conversely, the red line signifies the level of the variable at the higher level (presence of variable). A strong interaction exists when the slopes of the red and black lines are significantly different. A small variation between slopes indicates a minimal interaction while symmetrical slopes indicate no interaction. Based on the interaction plots (Fig.13) it is evident that the degree of interaction in decreasing order of magnitude is EPA-AA > EPA-Inhibitor > AA-Inhibitor. The strong interaction EPA-AA could indicate that EPA causes stress to the membrane resulting in the release of free AA from the phospholipid bilayers and consequently activating the COX enzyme to act on AA to produce PGE_2 as the breakdown product. The minor interaction EPA-inhibitor and AA-inhibitor reflect the lack of activity of the inhibitor towards the blocking of the enzymes responsible for the production of PGE_2.

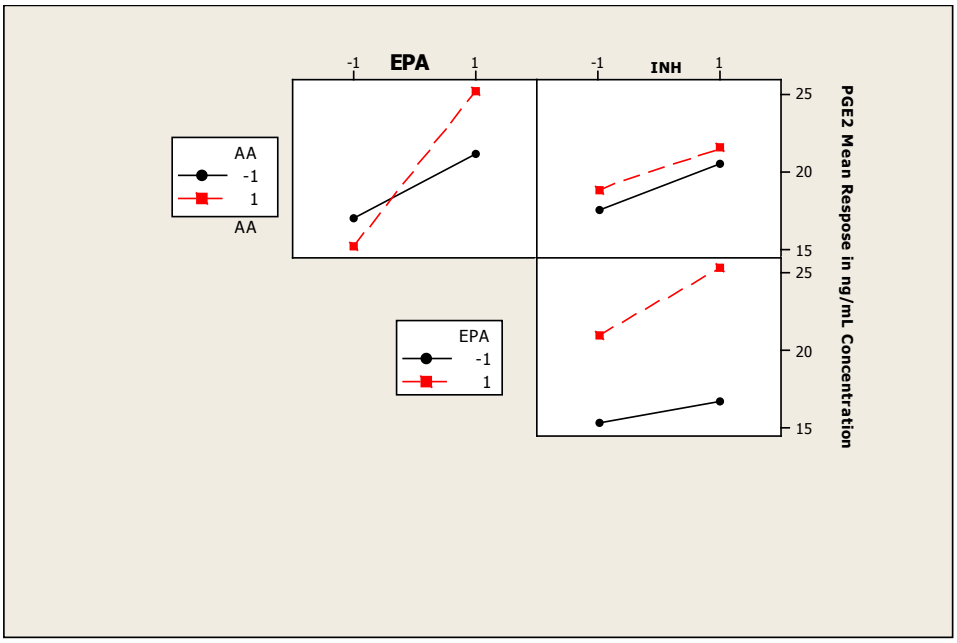

Figure 22. Interaction Effects Plots of AA, EPA and inhibitor on PGE_2 Synthesis

4.3.3.2 PGE_3 Synthesis

Fig. 23 illustrates the main effects of AA, EPA and inhibitor with respect to the production of PGE_3 in the non-parenchymal cell culture. Of the three variables, it is evident that the PGE_3 level increased significantly with the addition of EPA into the culture media (upper right side plot). Thus, it can be inferred that the presence of EPA in the cell culture media might have enhanced the synthesis of PGE_3 via the COX pathway. In the main effect plots of AA (upper left plot) and inhibitor (lower left plot), the AA (-1.9 units) and inhibitor (2.3 units) exhibit almost a similar effect magnitude towards the production of PGE_3 but with different directions. The presence of AA tends to lower the production of PGE_3 while inhibitor tends to increase the level of PGE_3. This implies that the EPA is the precursor molecule primarily responsible in the synthesis of PGE_3. However, the minimal positive effect of the inhibitor on the synthesis of PGE_3 was unexpected. Thus, the interaction plot will further evaluate how inhibitor behaves with respect to EPA and AA in relation to the synthesis of PGE_3 metabolite.

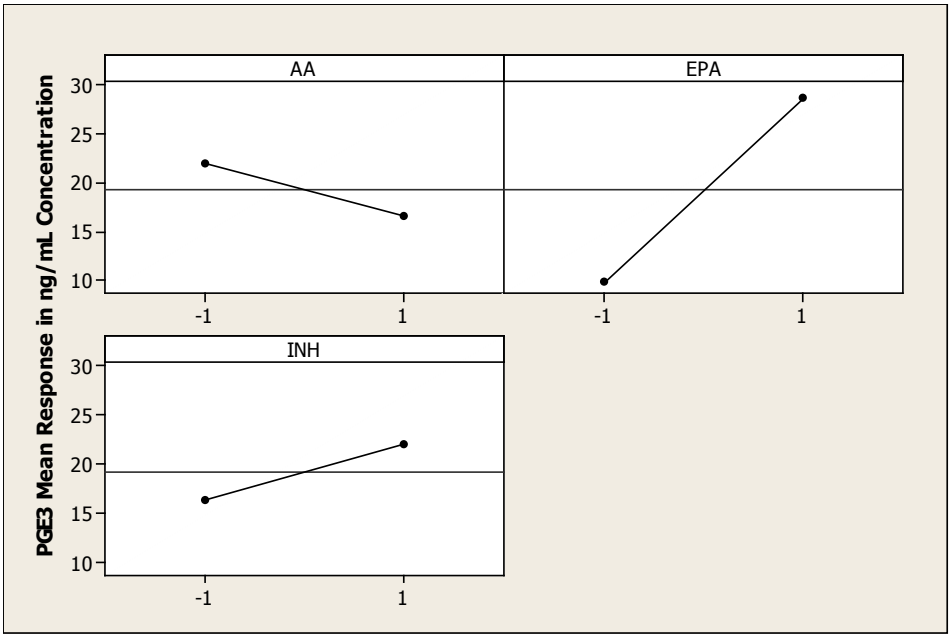

Figure 23. Main Effects Plot of AA, EPA and Inhibitor on PGE_3 Synthesis

Fig. 24 shows the interaction plot of the three variables with respect to the synthesis of PGE_3. The upper left side plot is the interactive effect between AA and EPA, upper right side is the interactive effect of AA and inhibitor and the lower right side plot for the interactive effect between EPA and inhibitor. In the plot, the black line indicates EPA or AA at lower level (absent) while the black line signifies EPA or AA at higher level (present). A significant interaction between AA and inhibitor is indicated by the non-parallelism of the lines in the plot. In the absence of AA in non-parenchymal cell culture, an increase in the mean PGE_3 level is obtained when inhibitor is incorporated to the cell culture system while the opposite happens when inhibitor is added in the presence of AA in the cell cultures. The mean level of PGE_3 tends to decrease in cell culture with AA when the inhibitor is added to the cell culture media. For the interaction between EPA and inhibitor, it can be inferred that there is a minimal interaction, as indicated by the slight difference in the slope of the line for non-parenchymal cell culture media containing EPA and non-parenchymal cell culture without EPA. In the case of AA vs EPA, a lack of interaction between the AA and EPA effects due to the parallelism of the lines in the plot. The level of PGE_3 present in the system is primarily due to the presence of EPA. This indicates that AA does not inhibit EPA in the access of COX enzymes responsible for the production of PGE_3.

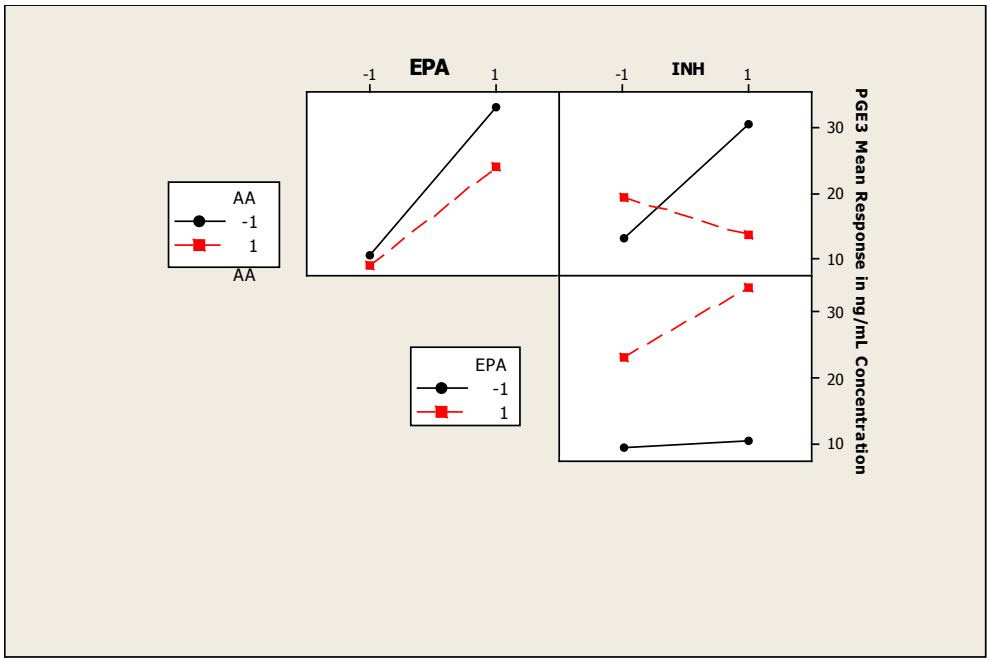

Figure 24. Interaction Effects Plot of AA, EPA and inhibitor on PGE_3 Synthesis

4.3.3.3 LTB_4 Synthesis

Leukotrine B_4 is an arachidonic derived eicosanoid via the lipoxygenase pathway. The production of this metabolite is dependent upon its precursor molecules, AA and EPA, which could compete with each other in the eicosanoid synthesis. The main effects of AA, EPA and inhibitor on LTB_4 production are described in Fig 25.. In the plot of EPA (upper right side) a dramatic increase of LTB_4 is achieved in the presence of EPA. Also, the cell culture media added with EPA produce the highest LTB_4 after incubation. Inhibitor main effect plot (lower right side) suggests also a positive main effect on the LTB_4 synthesis at a minimal level which indicates that perhaps the particular inhibitor used in the present experiments lacks the ability to block effectively the action of COX and PLA_2 enzymes. Unexpectedly, the presence of AA in the cell culture media does

not seem to manifest any effect in the production of LTB_4 metabolites but apparently, the presence of EPA shows to have the greatest influence.

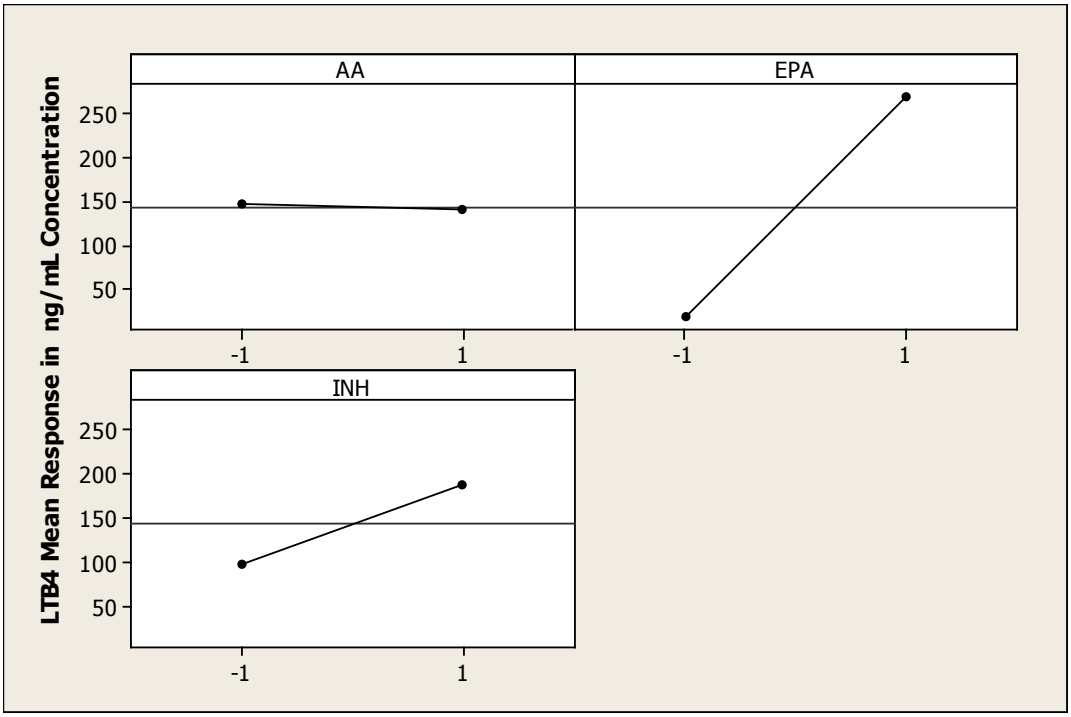

Figure 25. Main Effects Plots of AA, EPA and Inhibitor on LTB_4 Synthesis.

The interaction effects of AA vs EPA, AA vs inhibitor and EPA vs inhibitor with respect to LTB_4 production is portrayed in Fig. 26. The evaluation of the the plots, revealed a strong interaction effect between EPA and inhibitor (lower right side plot). It shows that an apparent increase of LTB_4 production by cells happen when an inhibitor is added to the cell culture media treatment containing EPA. The main features from the three plots (Fig 26) are: 1) EPA increases considerably the LTB_4 production with or without AA. 2) The combined term EPA and inhibitor indicated that the increase of LTB_4 production occurs only when

EPA is present. 3) The combined term AA and inhibitor increased the production of LTB_4. Based on the aforementioned observations, it can be hypothesized that EPA enhances the production of LTB_4 in non-parenchymal rat liver cells due to the replacement of AA for EPA in the phospholipid membrane. The released AA can be metabolized into LTB_4. This explanation is not found in any published report, however experiments with head-kidney cells from Atlantic cod and discussed below (Section 4.3) seem to confirm this hypothesis.

A comparison between the main effects plots for PGE_2 and LTB_4 (Figs 21 & 25) indicate that the three factors have similar behaviors. It follows that in the synthesis of arachidonic acid-derived eicosanoids, EPA has the strongest influence over the level of metabolite produced.

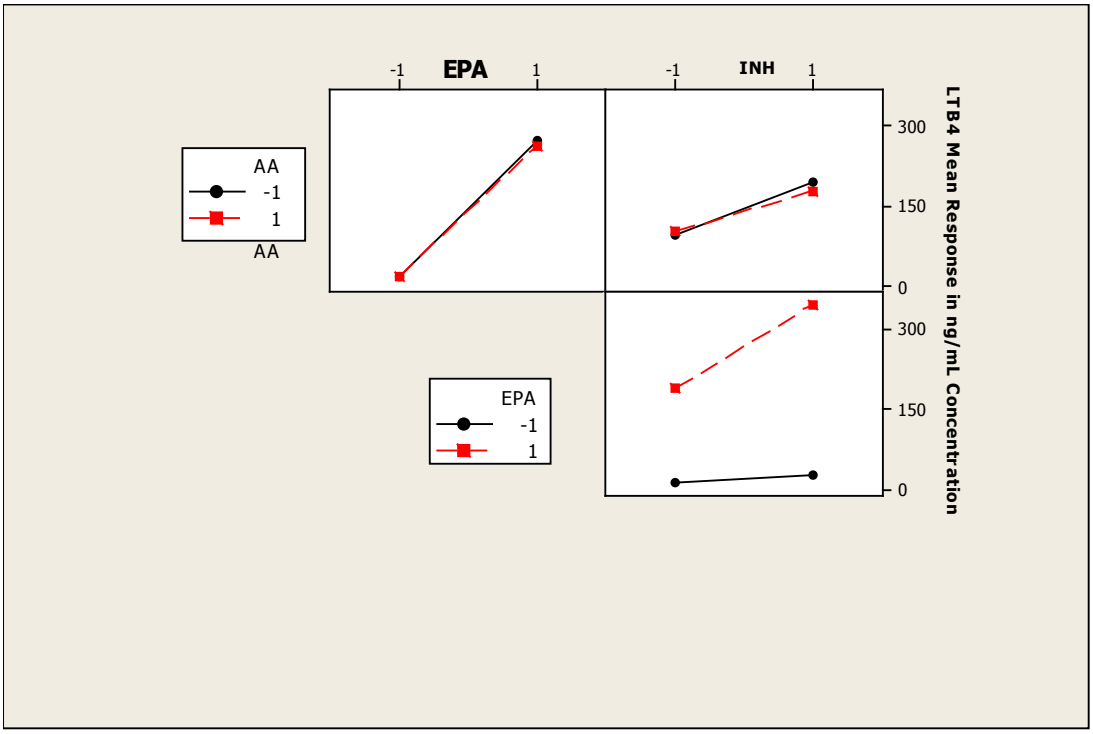

Figure 26. Interaction Effects Plots of AA, EPA and inhibitor on LTB_4 Synthesis.

4.3.3.4 LTB_5 Synthesis

The three factors studied had an influence on the production of LTB_5 (Fig. 27). The effects of AA, EPA and inhibitor were similar. That is, the line containing the mean response for the absence and presence of AA has a slope similar to the slopes of the lines connecting the mean response for the presence and absence of EPA and the mean response for treatments with and without inhibitor. The plot (Fig. 27) also shows that adding EPA or AA to the various treatments bring about an increase in LTB_5 production, while introducing the inhibitor into the cell culture system causes a decreasing in the amount of LTB_5.

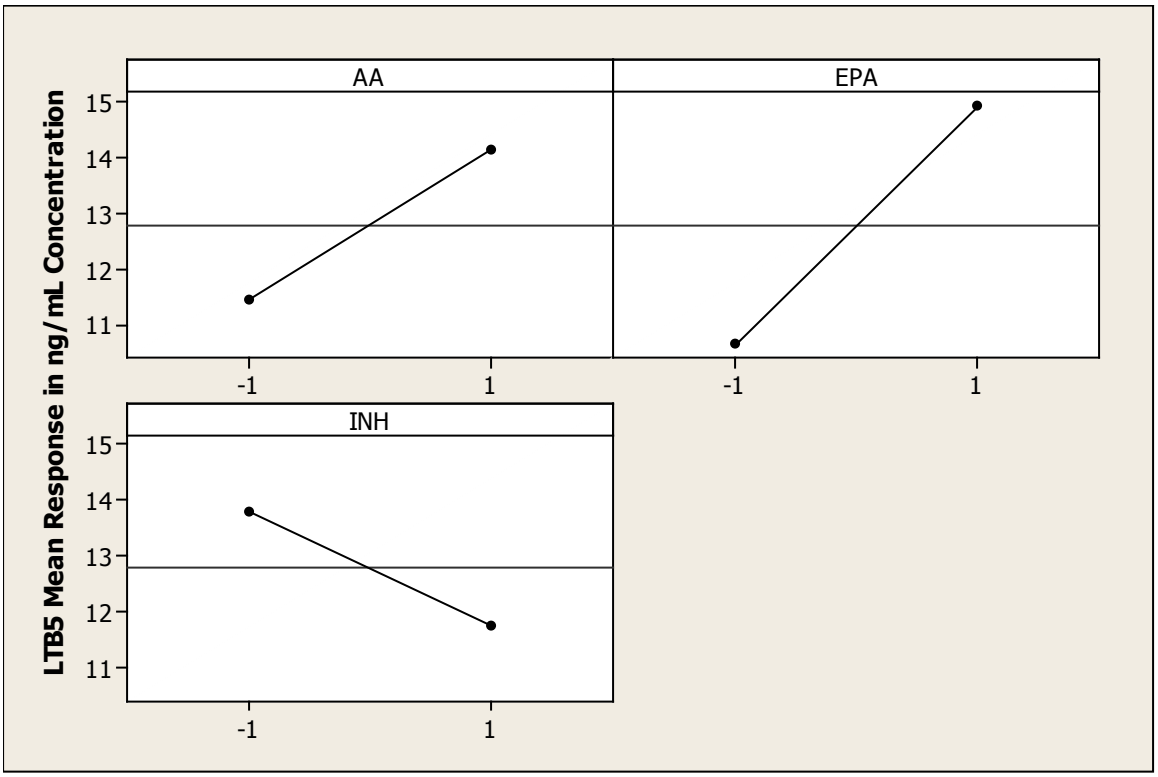

Figure 27. Main Effects Plots of AA, EPA and Inhibitor on LTB_5 Synthesis.

The interaction plot is used to further evaluate the combined effects of the variables. Fig. 28 indicates the interaction between EPA and inhibitor, AA and EPA and AA and inhibitor. The plot of EPA vs inhibitor (lower right) demonstrates that in the synthesis of LTB_5, the inhibitor has a great influence in the decrease of LTB_5 response when the cell culture media is not treated with EPA. In the presence of EPA, the addition of inhibitor elevates the amount of LTB_5 produced. The increasing levels of eicosanoid in the presence of inhibitor could indicate that the inhibitor in question is not appropriate for studying the blockage of COX/PLA_2 enzymes in experiments involving non-parenchymal cells. With respect to AA vs EPA, both treatments enhance the production of LTB_5. But, the presence of both AA and EPA produce a much higher amount of LTB_5 as compared with the treatment without AA. From this interaction effects, it can be deduced that EPA has a strong influence in the synthesis of LTB_5.

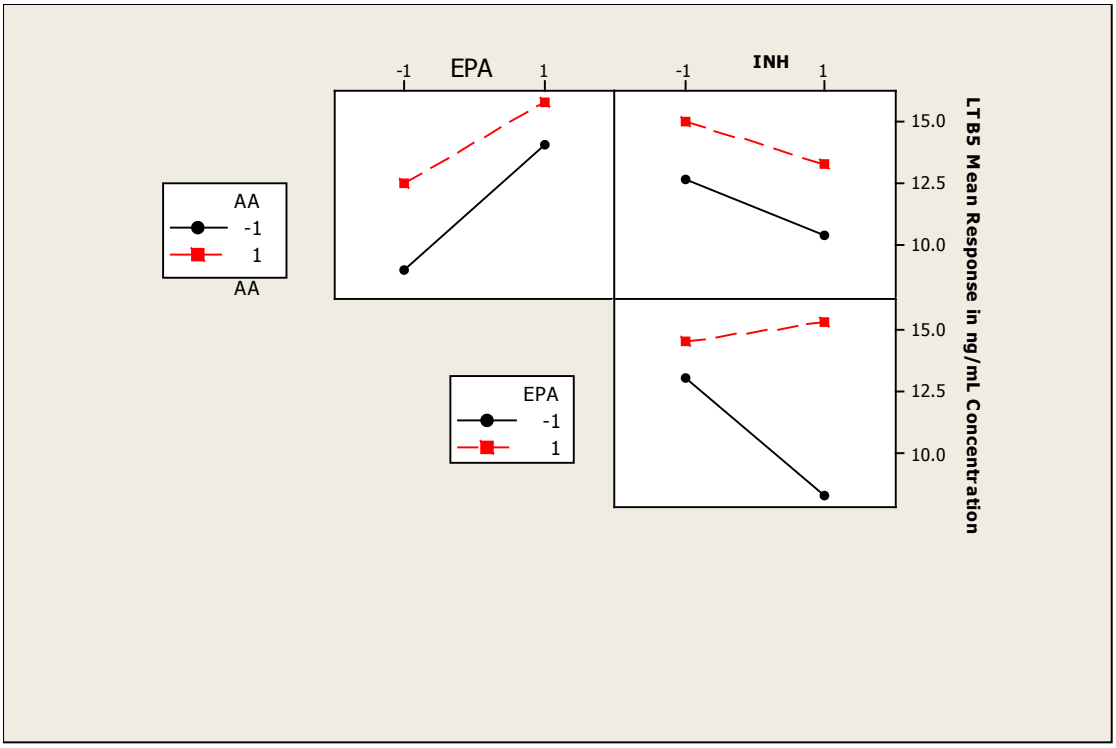

Figure 28. Interaction Effects Plots of AA, EPA and inhibitor on LTB_5 Synthesis

4.4 Eicosanoids Synthesis in Cod Kidney Cells

The unexpected production of AA-derived derived metabolites in the presence of EPA prompted the determination of eicosanoid metabolites using another type of cells to confirm the results. Cod kidney cells treated with EPA, AA and control (plain media) were used to confirm the previously discussed results. Fig. 29-32 show the behavior of eicosanoid metabolites in cell culture media treated with AA and EPA. It was found that there is significant increase in the levels of PGE_2 ($p = 0.0038$) and LTB_4 ($p = 0.0010$) when AA is present in the cell culture media as compared with the cell culture containing AA. This indicates the EPA may have some role in the synthesis of AA-derived metabolites not reported yet in dietary studies. Conversely, in cell media treated with AA no significant increase in EPA-derived metabolites was found. This suggests the AA has no influence over the synthesis of PGE_3 and LTB_5.

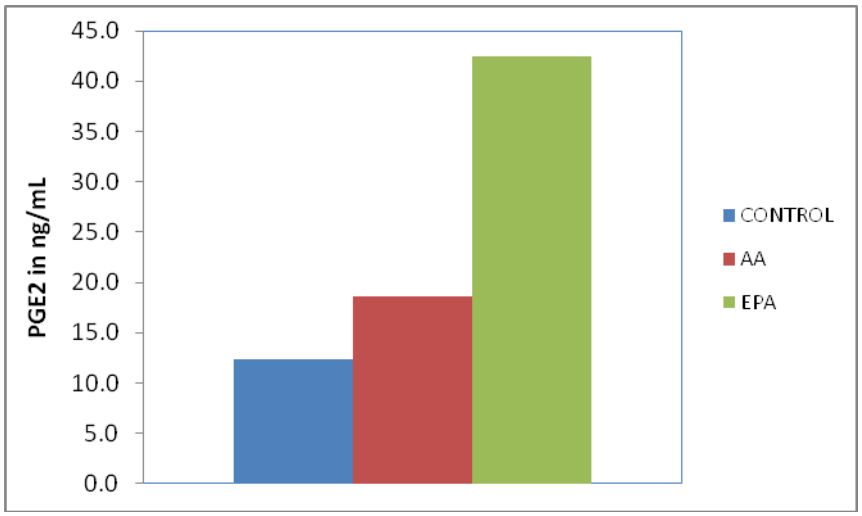

Figure 29. PGE_2 Production from Cod Kidney Cells

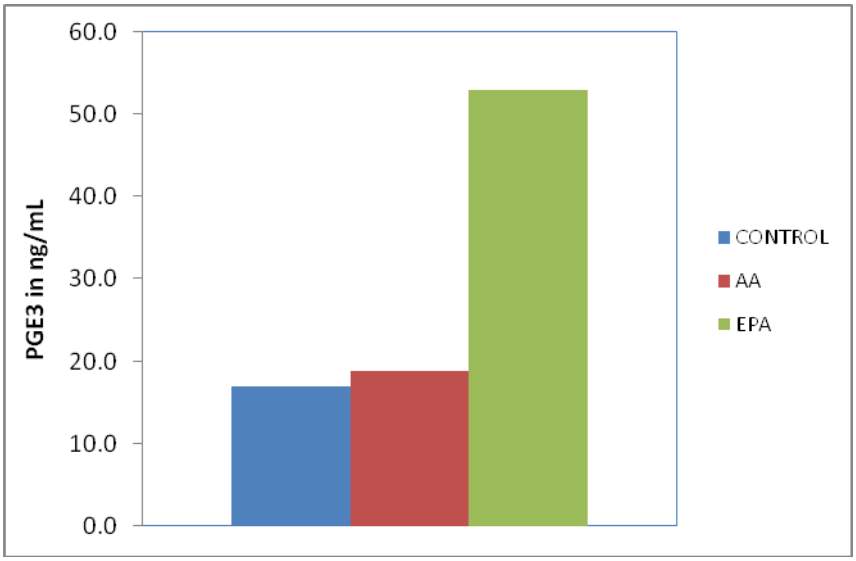

Figure 30. PGE_3 Production from Cod Kidney Cells

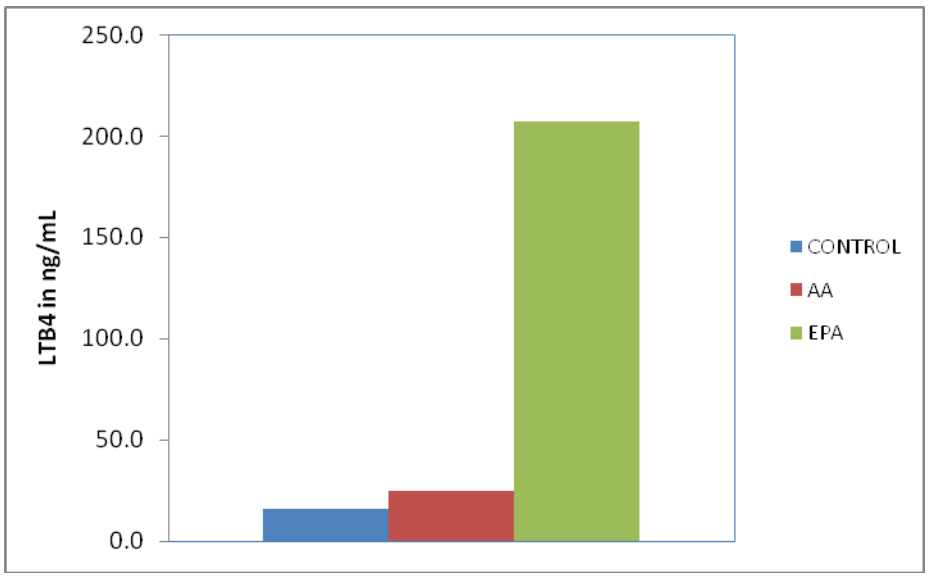

Figure 31. LTB_4 Production from Cod Kidney Cells

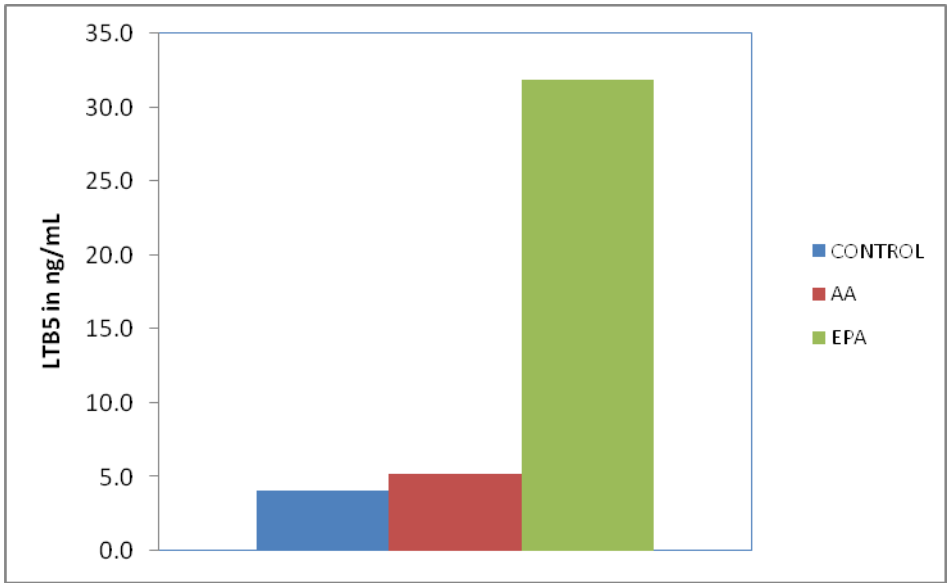

Figure 32. LTB_5 Production from Cod Kidney Cells

Chapter 5

CONCLUDING REMARKS

A significant increase in the production of LTB_4 in the presence of EPA indicates that EPA seems to have a strongest influence in the production of pro-inflammatory LOX associated metabolites. The elevated production of LTB_4 as a result of adding EPA could be rationalized as the replacement of AA for EPA in the phospholipid membrane and subsequently metabolization of the released AA into the proinflammatory LTB_4. It must be said that this is the first ever report that has observed such increase in the production of LTB_4 in the presence of EPA. However, it would be advisable to carry on studying systematically different kinds of cell cultures and AA and EPA concentrations in order to fully clarify the role of these essential fatty acids.

The minimal increase of the production of PGE_2 in the cell culture media indicates that the inhibitor used in the study lacks the ability to block the action of PLA_2 and COX enzymes. It would be advisable to use in a more effective inhibitor in future experiments.

The experimental evidence by using two different kinds of cell cultures (mammalian and fish) showed that the LOX pathway is favoured in *in vitro* experiments involving EPA and AA. Although a similar behavior was observe for the two types of cells tested, It is unclear why the LOX pathway is preferred.

The application of MS Resolver™ in the LCMS data set from AA and EPA derived metabolites demonstrated to be an important tool that can assist in the quantification of overlapping metabolites. The generated chromatograms obtained by MS Resolver™ are easy to quantify.

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