Development of an extraction method for the analysis of pro-inflammatory prostaglandin-E_2 and leukotriene-B_4 in human plasma by liquid chromatography tandem mass spectrometry

By
Zebasil T. Mengesha

Thesis submitted to the fulfilment of the requirement for
European Master in Quality in Analytical Laboratories (EMQAL)

Bergen, Norway
March 2013

Department of Chemistry
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Zebasil T. Mengesha
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Abbreviations

AA	Arachidonic acid (20:4n-6)
COXs	Cyclooxygenases (COX-1 and COX-2)
DHA	Docosahexaenoic acid (22:6n-3)
DGLA	Dihomo-γ-linolenic acid (20:3n-6)
DHET	Dihydroxyecosatrienoic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid (20:5n-3)
GCMS	Gas chromatography mass spectrometry
GLA	γ-linolenic acid (18:3n-6)
HETE	Hydroxyeicosatetraenoic acid
HEPE	Hydroxyeicosapentaenoic acid
HODE	Hydroxyoctadecadienoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HPEPE	Hydroperoxyeicosapentaenoic acid
LA	Linoleic acid (18:2n-6)
α-LNA	α -linolenic acid (18:3n-3)
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOX	Lipoxygenase
LT	Leukotriene
LTB_4	Leukotriene-B_4
LTB_5	Leukotriene- B_5
LTB_4-d_4	Leukotriene B_4 deuterated
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PGE_3	Prostaglandin E_3
PGE_2-d_4	Prostaglandin E_2 deuterated
PLA_2	Phospholipase A_2
PUFA	Polyunsaturated fatty acid
TX	Thromboxane

Abstract

A simple and rapid method for extracting PGE_2 and LTB_4 from human plasma and further determination by LC-MS/MS proposed and validated. Extracting solvents, formic acid (10 µL) and acetonitrile (140 µL) were added subsequently to 50 µL plasma sample. After vortex-mixing and centrifuging the obtained solution, the supernatant was submitted to LC-MS/MS. The quantitative analysis was carried out based on the internal standard method and the chromatographic separation using the LC column gradient mobile phase system. The analytical species were recorded by multiple reaction monitoring in negative mode. The method was validated using blank human plasma in the range of 1-50 ng/mL, and it exhibits good selectivity with LOD and LOQ of 0.4 ng/mL and 1 ng/mL respectively for PGE_2 and LTB_4 analytes. The recovery ranges were from 80.54 - 104.96% for PGE_2 and 72.34 - 105.61% for LTB_4. The method was applied to 40 human plasma samples from patients participating in clinical nutritional intervention studies and suffering from inflammatory disorders.

1. Introduction

Polyunsaturated fatty acids (PUFAs) are biological active fatty acid long carbon chain molecules with a carboxyl group in one end and a methyl group in another. Their long carbon chain and double bonds at different positions make them biologically active. PUFAs have significant involvement in human health. There are two major naturally occurring PUFAs, omega-3 (ω-3 or n-3) and omega-6 (ω-6 or n-6) based on the position of their last double bond with respect to their methyl end. Arachidonic acid (20:4n-6, AA) from the n-6 series and eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) from the n-3 series are important active lipid mediators play vital roles in immune regulation and inflammation [1].

The oxidation metabolites of Arachidonic acid (AA) comprising 20-carbon fatty acid are called eicosanoids according to the IUPAC terminology. The AA derived eicosanoids are the principal mediators and regulators of inflammation. They are often used as biomarkers for diseases and pathological conditions such as cancer, atherosclerosis, arthritis, cardiovascular or other immunological diseases [2-4]. Conversely EPA is among the most important omega-3 fatty acids which suppress inflammation. Accordingly, eicosanoids derived from n-6 PUFA in general are pro-inflammatory while eicosanoids derived from n-3 PUFA are anti-inflammatory [5]. This implies that the optimum levels of n-3 and n-6 PUFA in the body require to be maintained by means of nutritional supplements or diet to relieve the body from inflammatory complications.

The eicosanoid biosynthesis is usually initiated by the activation of the phospholipase A_2 and the release of AA from the bi-layer phospholipid membrane. Subsequently AA is transformed into several bioactive eicosanoids such as prostaglandins (PGs), leukotrienes (LTs) and others bioactive compounds through four different and well defined enzymatic pathways, namely cyclooxygenase (COX), lipoxygenase (LOX), cytochrome P450 (CYP) and non-enzymatic pathways as depicted in Fig. 1 [4,6].

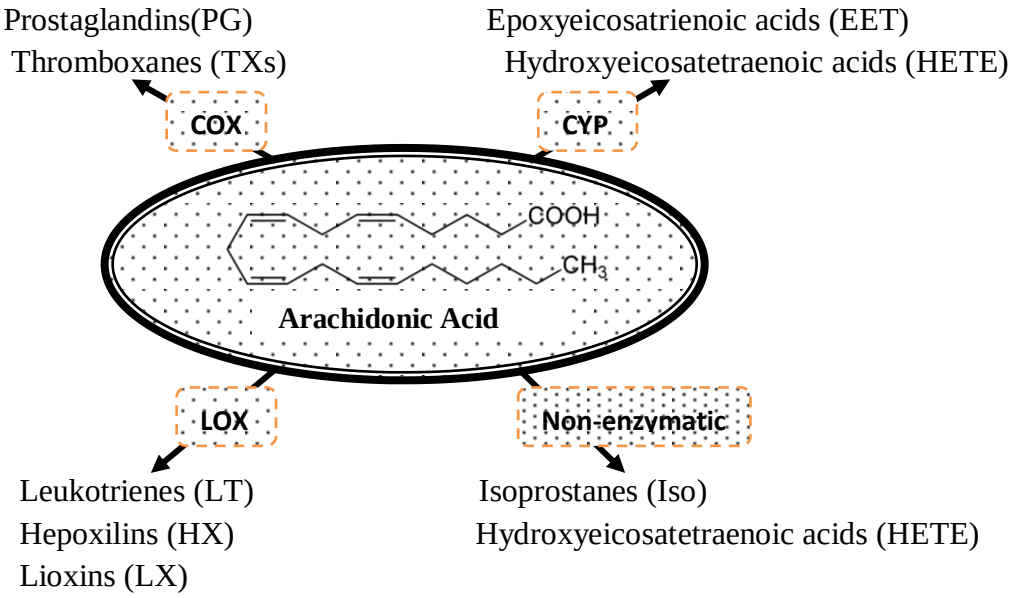


Figure 1. Overview of eicosanoid biosynthesis from AA via enzymatic COX, LOX, CYP P450 and non-enzymatic pathways [4].

AA and its derivative eicosanoids are potent inflammatory mediators; the detection of them provides insight into the development of inflammatory conditions. For instance, the AA related PGs generated through the cyclooxygenase pathway and the AA related LTs generated by the lipoxygenase pathway are among the major groups of mediators which play a key role in sustaining homeostatic functions and mediating pathogenic mechanisms including inflammatory response. During acute inflammation prior to the

recruitment of leukocytes and the intrusion of immune cells, both the level and the profile of those mediators production change dramatically [7]. Consequently, they contribute to the development of cardinal signs like fever, redness, swelling, pain of acute inflammation, which is the good side of inflammation that helps to isolate damaged area and promote healing. Similarly, other eicosanoid precursors also play vital role in the body with slight difference on the physiological sights and effects.

Therefore most researches are focused on the development of analytical methods for the analysis of those inflammation biomarkers. The detection and quantification of them are of great interest as they play vital roles in a range of inflammatory pathologies, and hence it helps to monitor appropriate pharmacological therapy. Several analytical methods have been reported for the analysis of eicosanoids with different detection and quantification principle. Among these methods enzyme immunoassay (EIA) and radioimmunoassay (RIA), capillary electrophoresis (CE), gas chromatography (GC) with flame ionization or mass spectrometry detectors and high performance liquid chromatography (HPLC) with ultraviolet, fluorescent or mass spectrometry detectors have been used [8, 9]. These methods differ in sensitivity and specificity for each individual eicosanoid. Recently, the development and application of liquid chromatography tandem mass spectrometry (LC-MS/MS) for eicosanoids' analysis have been widely recognized due to its sensitivity, specificity, simultaneous analysis of a number of eicosanoids and short time analysis for different kind of samples [10-13].

The main problems found in LC-MS/MS for the analysis of eicosanoids are related to sample treatment. Indeed, the scientific literature revealed that the sample treatment is

the rate determining step for several analytical methods [14]. In addition, prolonged time consumption for sample preparation or instrumental analysis could have a direct impact on the quality of analytical results and often they are not advisable for clinical or routine analysis involving a great number of samples. NIFES has observed that its current method for the extraction of eicosanoids from plasma and further determination by LC-MS/MS does not yield the expected results in the context of its ability to be used as a routine clinical method, due to its multiple extraction steps and lengthy evaporation time under a stream of nitrogen [15]. Based on the above observations, the analysis of eicosanoids in biological fluids predominantly in plasma requires further investigation in order to get rapid and precise quantification of inflammatory signalling eicosanoids during occurrence of inflammation disorders.

1.1 Objective of the study

The aim of the present master thesis is to develop a simple and rapid method for the extraction of the two most potent pro-inflammatory lipid mediators, namely PGE_2 and LTB_4 in human plasma and further quantification by LC-MS/MS.

The main objectives of the intended research are:

- To propose an extraction method for routine analysis of plasma samples collected from patients suffering from inflammatory disorders.
- To optimize the sample extraction and other variables by applying experimental design.
- To validate quantitatively the developed extraction protocol.
- To analyse human plasma samples from clinical nutritional intervention studies.

1.2 Significance of the study

It has been acknowledge that the number of patients suffering from chronic inflammatory disorder is increasing worldwide. A survey conducted in 16 European countries confirms that around 20% of adult Europeans suffer from moderate to severe intensity chronic pain which is seriously affecting the quality of their social and working lives [16]. A recent report of the Norwegian Institute of Public Health (*Nasjonalt folkehelseinstitutt*) has also highlighted that "chronic pain affects about 30% of the adult of Norwegian population and it is the main cause of long term sick leave and disability" [17]. Accordingly health centres are in urgent need of simple and fast sample treatment methods and analytical techniques suitable for routine analysis in

clinical investigations of eicosanoid biomarkers. Moreover, NIFES dietary studies on the effect of omega-3 and omega-6 rich foods and oils, and also clinical intervention studies on the effect of specific fish oils on inflammatory conditions require a rapid and reliable extraction method for determining eicosanoids in plasma samples. LC-MS/MS has been acknowledged in several publications as a better alternative for the analysis of eicosanoids than the traditional enzyme linked immunosorbent assay (ELISA), due to its analytical specificity, absence of cross-reaction and most important accurate quantification provided that an appropriate sample treatment method is used.

Therefore, the present study aims at developing a simple and rapid method for extracting the pro-inflammatory eicosanoids PGE_2 and LTB_4 from human plasma by using LC-MS/MS. The development of such methods will assist the analysis of eicosanoids in research or routine studies and also they could have the potential to become national or international benchmark references. Specifically, it is intended to provide assistance to the various projects of NIFES involving inflammation diagnosis in clinical and nutritional trials. In addition, the developed method will be incorporated in NIFES current series of nationally accredited methods.

2. Theoretical Background

2.1 Eicosanoids

Eicosanoids are oxidation metabolites of essential fatty acids, omega-3 or omega-6 PUFAs. The term Eicosanoids comes from the word "eicosa" derived from the Greek "εικοσι" that means "twenty" to denote the number of carbon atoms in all of them. The eicosanoids are important cell signalling molecules since they have significance impact on many physiological and pathophysiological systems of the body. Eicosanoids are the principal oxidation products of an important large class of biologically active compounds, the arachidonic acid (20:4n-6, AA). There are a number of eicosanoids in the body which are potent inflammatory mediators; although in some cases they also have anti-inflammatory effects [18-20].

2.1.1 Biosynthesis of eicosanoids

Eicosanoids are generated either directly from dietary linoleic acids or from already stored membrane phospholipids through the pathways in Fig 2 [21]. Arachidonic acid (AA; 20:4n-6) is the predominant substrate for eicosanoid biosynthesis. The inflammatory cells typically contain a high proportion of the n-6 PUFA and low proportions of other 20-carbon PUFAs [22]. AA is mobilized by phospholipase enzymes notably phospholipase A_2 (PLA_2) from the bi-layer phospholipid membrane. Although cellular free AA levels are controlled by two competing reactions; firstly the PLA_2 mediated cleavage of the sn-2 position of phospholipids to yield the free AA and secondly the CoA-dependent acyltransferase-modulated acylation reactions that re-incorporate the free AA into phospholipids [23-24]. The eicosanoid precursor AA is

consequently metabolized through several enzymatic pathways. The most common pathways are the cyclooxygenase (COX) pathway that converts AA into the series-2 of eicosanoids principally prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2) and thromboxanes (TXA_2 and TXB_2); and the 5-lipoxygenase (5-LOX) pathway that converts AA into the series-4 of leukotrienes (LTA_4, LTB_4, LTC_4, LTD_4 and LTE_4). Similarly minor eicosanoids are derived from EPA and DHA via COX to produce the series-3 of prostaglandins (PGD_3, PGE_3, $PGF_{3\alpha}$ and PGI_3) and thromboxanes (TXA_3 and TXB_3); and through the 5-LOX pathways these n-3 compunds are metabolized into the series-5 leukotrienes (LTA_5, LTB_5, LTC_5, LTD_5 and LTE_5) and other eicosanoids as shown in Fig 2. It must be mentioned that among the various described eicosanoids, PGE_2 and LTB4 are the most studied and abundant pro-inflamatory eicosanoids.

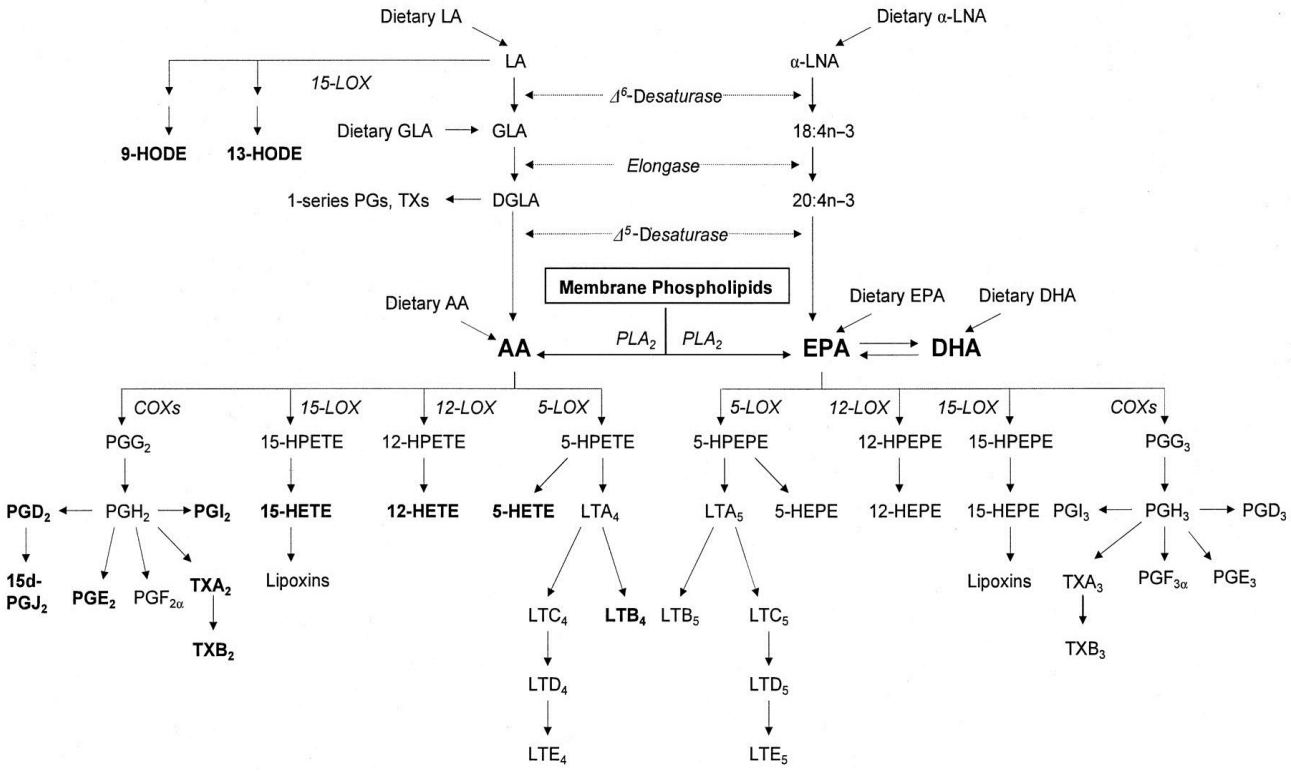


Figure 2. Overview of the metabolism of PUFAs into eicosanoids [21].

2.1.2 Pro- and anti-inflammatory eicosanoids (n-6 versus n-3 eicosanoids)

Eicosanoids exert opposing actions in the human body. Some of them have been labelled as "bad eicosanoids" (those derived from the n-6 family) due to their capacity to promote inflammation, while other have been labelled as "good eicosanoids" (those derived from the n-3 family) due to their healing and capacity to decrease inflammation. A high content of n-6 fatty acid through dietary intake can produce a significant increase in the levels of AA of the cell membrane reservoir and when the immune system is triggered, this AA is released from the membrane and converted into pro-inflammatory eicosanoids including PGE_2 and LTB_4 and as a results of these processes, a pro-inflammatory immune response will occur. On the other hand, high levels of n-3 fatty acids in the cell membrane reservoir will produce anti-inflammatory eicosanoids derived from the release of EPA and consequently an anti-inflammatory immune response will occur [25]. Therefore, a balance of these opposing actions is responsible for good health and wellness whereas an imbalance brings inflammatory disorder.

2.1.3 Historical highlight of prostaglandins and leukotrienes analysis

In 1934, eicosanoids were first observed by Goldblatt and Ulf Von Euler in sheep vesicular gland extracts and human seminal plasma [26-27]. The discovery of the 20 carbon metabolites paved the way for many researchers around the world to continue working on the physiological and pathological roles of these molecules and the development of strategies for their qualitative and quantitative analysis. In 1938, the slow reacting substance (SRS) of unknown eicosanoid compound was introduced by Feldberg and Kellaway for studying smooth muscle contracting factors. In 1960

Bergstrom and Sjovall isolated prostaglandin F (PGF) from sheep prostate glands for the first time and showed as it was a 20-carbon fatty acid [28]. In a similar way, other prostaglandins such as PGE_1 [29], PGE_2 and PGE_3 [30] were isolated from human seminal plasma. Later in 1979 Samuelsson and co-workers demonstrated that the SRS was a mixture of leukotrienes (LTC_4, LTD_4 and LTE_4) [31-32]. In addition, they elucidated their biosynthetic pathway. Following those pioneer reports numerous investigations focusing on various aspects of eicosanoids have been performed in many research centres around the world. Among the various aspects, the quantitative analysis of eicosanoids has been challenging, due to the complex nature of different biological matrices (e.g plasma, urine, etc.) and the very low abundance, similar chemical structures, instability of the eicosanoids and their metabolites.

2.1.4 Eicosanoids in blood plasma

Blood is a very complex sample; it is composed of a cellular component consisting of red and white blood cells and platelets, and a liquid carrier commonly known as plasma. Blood plasma consists of mainly water (above 90% water) and a variety of suspended or dissolved substances including proteins, peptides, nutrients (such as carbohydrates, lipids and amino acids), electrolytes, organic wastes and a variety of other small organic molecules [33]. Fatty acids are among those components of plasma which reflect the dietary fatty acid composition; in particular eicosanoids are components of plasma largely generated from bilayer cell membranes during inflammation and other physiological disorder [34-35]. Consequently, most of recent clinical tests are based on the analysis of blood plasma since tissue inflammations, organ dysfunctions and pathological states can alter the composition of blood plasma.

2.2 Method of extraction and analysis for PGE_2 and LTB_4

Numerous analytical methods of extraction and analyses have been reported for eicosanoids (including PGE_2 and LTB_4) from several biological sample matrices. In general, methods for the analyses of arachidonic acid metabolites in biological fluids require high sensitivity and specificity because of the very low concentrations, similar chemical structures and short half-lives of these metabolites [36].

2.2.1 Extraction methods of eicosanoids

Extraction and sample clean-up of eicosanoids including pro-inflammatory PGE_2 and LTB_4 from biological samples are vital steps for any of the reported eicosanoids' analytical methods. These procedures especially for plasma are relatively complex due to low concentrations and instability and interferences of abundant contaminants in the sample matrix. For that reason, the development of efficient and rapid extraction methods for the analysis of eicosanoids is an on-going process that requires the assistance of very skilled practitioners and the state-of-the-art of instrumentation (e.g. LC-MS/MS) [37-38].

The most common methods of extraction prior to LC-MS/MS analysis are solid phase extraction (SPE), thin layer chromatography (TLC), and repeated liquid-liquid extraction followed by drying and reconstitution of the solvent for pre-concentration, chemical derivatization followed by GC analysis and liquid-liquid extraction followed by clean-up with one or more LC column [39-45]. These procedures are time consuming and they might bring about considerable loss of sample and poor recoveries. Most of the reported methods dealing with biological fluids such as plasma require

relatively large volumes of sample and cautious room temperature pre-concentrating step. In an attempt to develop a method for extracting plasmatic eicosanoids, NIFES observed the total loss of spiked analytes in blank plasma upon pre-concentration of the supernatant with a stream of nitrogen or with vacuum drier at room temperature. Further observations revealed that to avert the loss of the analytes the preconcentration step should be carried out under very low nitrogen flow conditions which in turn demand very long periods of time. NIFES has estimated a total of 8 hours (a working day) to dry 500 µL of supernatant containing eicosanoids for a reliable determination by using LC-MS/MS. Consequently, neither reported methods nor NIFES current operational method [appendix I] for the analysis of eicosanoids extracted from plasma are suitable for research studies or routine analysis. There is a growing interest in developing novel, efficient and rapid extraction methods for eicosanoids in general due to worldwide increase of patients suffering from chronic inflammatory pathologies.

Table 1. Reported extraction methods for eicosanoids from human blood

Method of analysis	**Short description of Method of Extraction**	**Plasma sample Volume used**	**Reference**
GC-MS/MS	Column filtration – derivatization (2 - times) - drying - solvent reconstitution.	200 µL	[39]
LC-MS	LLE washing (2-times) with drying and solvent reconstitution.	1 mL *	[40]
LC-MS/MS	SPE - drying and solvent reconstitution	4.5 mL blood	[41]
LC-MS/MS	On-line SPE - with automatic valve switching and cartridge exchange.	100 µL	[42]
LC-MS/MS	Three solvents LLE - drying and solvent reconstitution.	200 µL	[43]
LC-MS/MS	SPE - drying and solvent reconstitution.	200 µL	[44]
2D- LC-MS/MS	LLE - SPE -drying and solvent reconstitution (4-step extraction).	3 mL	[45]

*Equine blood

2.2.2 Methods of analyses for eicosanoids

The analysis of eicosanoids involved the application of numerous analytical methods. Enzyme immunoassay (EIA) and radioimmunoassay (RIA) methods are among the most commonly applied methods for analysis of eicosanoids. They are based on the principles of the competitive substrate binding assay. The main difference between EIA and RIA is the form of competing antigen which is binding to a specific enzyme in the former and radiolabeled in the latter [46]. These methods are highly sensitive however their main limitations are the detection of a single product at a time and the cross reactivity which can cause variability in sample quantification. Thus it is not suitable to analyse a number of different eicosanoids at a time [47-48].

Gas chromatography with mass spectrometry (GC-MS) can overcome the limitations of immunoassay methods. GC-MS offers increased selectivity for simultaneous detection of multiple eicosanoids. It is important to mention that for GC-MS analysis eicosanoids should be both volatile and thermally stable. For that reason this chromatographic technique requires the use of chemical derivatization for the majority of eicosanoids which makes the procedure tedious and time-consuming [49-50]. However, not all eicosanoids are readily amenable volatile (e.g LTC_4) to be analysed by GC [51].

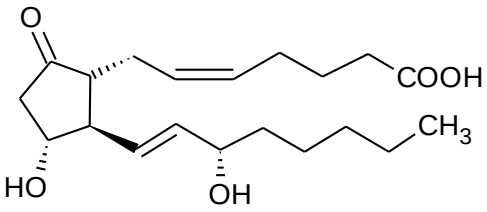
Another technique that has been used for the analysis of eicosanoids is high-performance liquid chromatography (HPLC) coupled with UV detection or fluorescence detection [52-53]. The main disadvantages of the technique are its lack of sensitivity and specificity which are the main drawback for the limited advancement and interest in the scientific community in general for the applicability of HPLC.

The introduction of electrospray ionization (ESI) MS analyzers coupled with HPLC [54] allowed the direct introduction of eicosanoids in aqueous solution without volatilization and with an improvement of sensitivity and specificity. The separation of the analytes in a chromatographic column prior to MS analysis reduces background noise, problems associated with ion suppression from co-eluting compounds, improved detection limits and overall quality of the MS data. Nowadays, LC-MS/MS is becoming the most powerful technique to quantify a large number of eicosanoids simultaneously in various biological matrices [37-38]. In addition, the stable isotope dilution LC-MS/MS is the most specific and sensitive method for eicosanoids and maximal specificity has been achieved using multiple reactions monitoring (MRM) system [2].

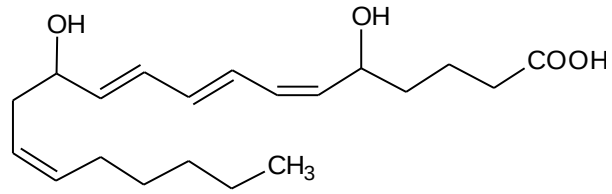
2.3 Chemical structure and mass fragments of PGE_2 and LTB_4

PGE_2 and LTB_4 are among the most studied and abundant pro-inflammatory n-6 carbon-20 metabolites of AA, which are potent mediators of inflammation and other physiopathological systems [55-56]. In LC-MS/MS analysis after the analytes are separated through the LC column they are allowed to pass through the mass analyzer compartment, in which the PGE_2 and LTB_4 molecules are converted to gas phase followed by ion production. The ions or mass fragments are separated in the mass analyzer based on their mass-to-charge ratio (*m/z*). By running the analysis with structural analogues deuterated internal standards to control variation in the responses system and applying the MRM mode for measurement of the target analytes, the quantitative analysis can be carried out successfully [57-58]. The chemical structures of PGE_2 and LTB_4 with their respective deuterated internal standard are shown in Figure 3. PGE_2 and LTB_4 contain two and four double bonds respectively which are indicated on

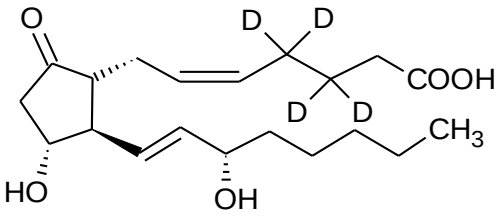
the subscripts of their abbreviated names (PGE_2 and LTB_4). The typical ion fragments for the PGE_2 are $[M-H]^- \rightarrow 351$; $[M-H_2O-H]^- \rightarrow 333$; $[M-2H_2O-H]^- \rightarrow 315$; and $[M-2H_2O-CO_2-H]^- \rightarrow 271$, whereas for the LTB_4 also in negative mode are $[M-H]^- \rightarrow 335$; $[M-H_2O-H]^- \rightarrow 317$; $[M-2H_2O-H]^- \rightarrow 299$; $[M-H_2O-CO_2-H]^- \rightarrow 273$; and $[M-C_9H_{17}O-H]^- \rightarrow 195$ (cleaved at C-11) [59]. The internal standards PGE_2-d_4 (356 Daltons) and LTB_4-d_4 (340 Daltons) are fragmented in a similar fashion.



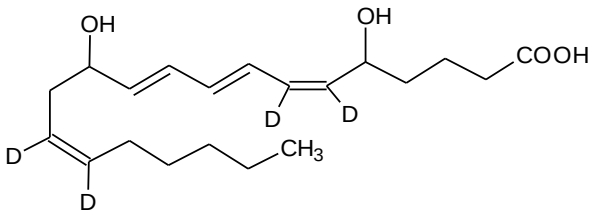
PGE_2



LTB_4



PGE_2-d_4



LTB_4-d_4

Figure 3. Chemical structures of PGE_2, LTB_4, PGE_2-d_4, and LTB_4-d_4

2.4 Doehlert design for optimization of Response Factor

2.4.1 Response factor (RF)

It is common to use internal standards with almost similar chemical structure and property to that of the compound of interest in order to assist the quantification process. It involves preparation of known concentration of analyte [A] spiked with known concentration of internal standard [IS], then determination of their signal ratio (S_A/S_{IS}) and their response factor (RF) computed by the expression [60].

$$RF = \frac{S_A}{S_{IS}} \times \frac{[IS]}{[A]} \quad \text{Eq. (1)}$$

Therefore, once RF is determined at a given known concentration of spiked IS, the unknown concentration of the analyte can be calculated from their response signals by rearranging the expression Eq. 1, assuming that the two factors ([A] and [IS]) exhibited a linear relation towards the detector over studied range of concentrations. The determination of RF helps saving time and resources instead of repeated preparation of calibration curve for each analysis. However, sometimes the linear relation of the analytical species varies over the working range [60-62]. Thus it is necessary to estimate the amount of IS to be spiked for various concentration ranges of analyte. Experimental designs assist to optimization of the concentration level of IS to be spiked on different level of analyte in quantification experiments by modelling the RF. The behaviour of the RF can be modelled, and optimized by using different type of experimental designs. Potential models can be polynomial functions of first (Eq. 2) or second order (Eq. 3 and 4) and their selection could be based on the RF models statistical acceptability and efficiency of prediction [60, 63].

$$\text{RF} = b_o + b_A[A] + b_{IS}[IS] \quad \text{Eq. (2)}$$

$$\text{RF} = b_o + b_A[A] + b_{IS}[IS] + b_{A\times IS}[A] \times [IS] \quad \text{Eq. (3)}$$

$$RF = b_o + b_A[A] + b_{IS}[IS] + b_{A\times IS}[A] \times [IS] + b_A^2\,[A]^2 + b_{IS}^2\,[IS]^2 \quad \text{Eq. (4)}$$

The terms in the above equation represent the intercept (b_o), the linear term coefficients (b_A and b_{IS}), the second order interaction effect coefficient ($b_{A\times IS}$) and the curvature effect coefficients (b_A^2 and b_{IS}^2).

2.4.2 Doehlert design

Doehlert designs proposed by David H. Doehlert (in 1970) can be used to study the RF behaviour as a function of the concentration of analyte [A] and internal standard [IS], and also to establish the optimal concentration of internal standard to be used in a determined quantitative analysis [61, 64]. The experimental points of a Doehlert design are evenly distributed in a hexagon for two variables (Fig. 4A) or a rhombic lattice for three variables (Fig. 4B) or the surface of a hyper-sphere for more than three variables. For a number of *k* factors, the total number of experiments to be carried out is given by $k^2 + k + 1$. Thus the total number of experiments for two factor and three factor designs are 7 and 13, respectively.

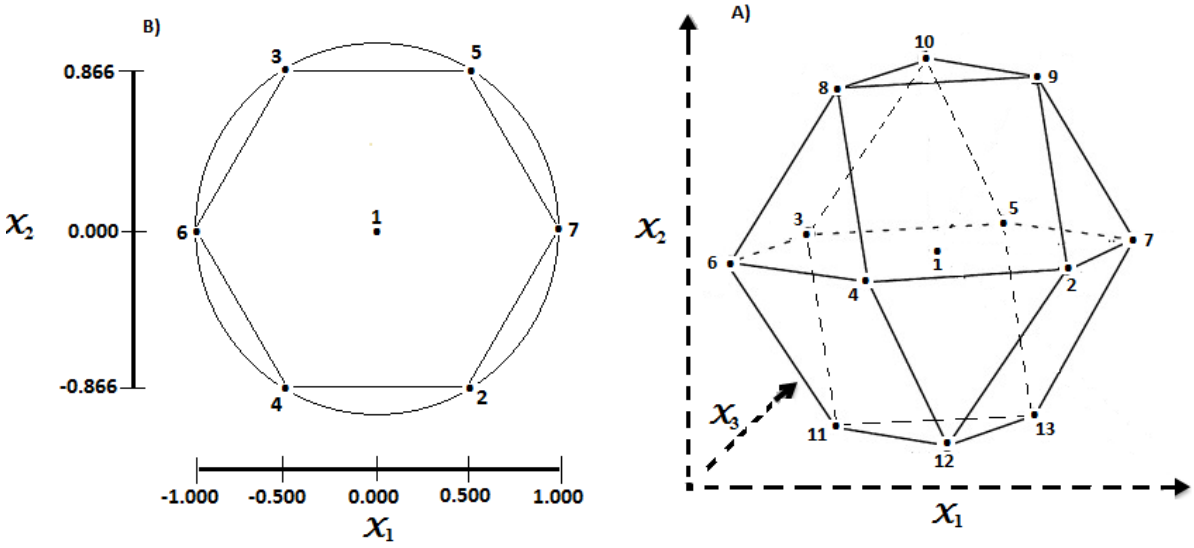


Figure 4. Doehlert design for optimizing two variables (A) and three variables (B).

The two-factor Doehlert design (Fig. 4A), a hexagon with vertices 2 to 6 and centre point 1, define five levels for factor 1 (X_1) and three levels of factor 2 (X_2), that is the Doehlert matrix dimension is 5 × 3. In the design each point has equal distance to the

centre as well to its neighbour experimental points. According to the design the five experimental levels along X_1 dimension have coded value -1.00, -0.50, 0.00, 0.50 and 1.00 respectively. Similarly the three experimental levels along X_2 dimension have coded value -0.866, 0.000 and 0.866 respectively. Accordingly Doehlert design was functional to model the RF behaviour by considering a simultaneous variation of the two variables; concentration of analyte and concentration of internal standard [60, 64].

2.5 Method validation parameters

Method validation, according to the definitions given by the International Standardization (ISO/IEC 17025) and the International Conference on Harmonization (ICH) definition, is a process of proving or finding evidence that an analytical method for particular requirements of specified intended use is fulfilled. Its objective is to demonstrate that the procedure, when correctly applied, produces results that are fit for purpose [65-66]. It is an important feature of any analytical method since it is closely related to the quality of the results [67]. The validation guidelines recommend that an analytical method has to be validated before use. The main parameters recommended to be considered for validation purpose are: selectivity/specificity, limit of detection and quantification (LOD and LOQ), linearity, linear range, precision and accuracy.

2.5.1 Selectivity and Specificity

Selectivity can be defined as the ability of the analytical method to differentiate the analyte(s) or internal standard(s) from endogenous components in the sample matrix. To assess the selectivity of the method several approaches have been used, the most popular approaches are (i) comparison of chromatograms of the blank sample with and

without spiking known analytes; (ii) analysis of certified reference materials and (iii) comparison of the chromatographic response of the sample with and without all the possible interferences [68]. In some cases the term selectivity is used interchangeably with specificity, however they are different terms as specificity refers that 100 % selectivity or without any interference. The International Union of Pure and Applied Chemistry (IUPAC) and other guidelines mention that specificity is the ultimate of selectivity [69].

2.5.2 Precision

Precision is defined as the closeness of agreement between a series of replicate measurements obtained under the prescribed conditions [66]. It is expressed by the standard deviation (SD), variance (SD^2), relative standard variation (RSD), or coefficient of variation (CV) of the replicate analytical measurement results. The estimation of the parameter helps to evaluate the level of random error of the measurements around the mean value.

$$SD = \sqrt{\frac{\sum_{i=0}^{n}(x_i - \bar{x})^2}{n-1}} \quad \text{Eq. (5)}$$

$$\text{CV} = \frac{SD}{\bar{x}} \times 100 \quad \text{Eq. (6)}$$

$$\overline{x} = \frac{\sum_{i=0}^{n} x_i}{n} \quad \text{Eq. (7)}$$

where: SD - standard deviation, CV - coefficient of variation, $\bar{x}$ - mean value, n - number of measurments and $n-1$ degrees of freedom.

2.5.3 Accuracy

Accuracy is the degree of agreement between the experimental value, obtained by replicate measurements, and the accepted reference value. Among the strategies to evaluate the accuracy of the analytical method the commonly used techniques are: i) comparing the measurement result of the analyte in a particular reference material with the certified value; ii) comparing the results of the method under investigation with that of already established reference method; iii) using standard addition method; and iv) calculating percentage of recovery [68]. The percentage recovery, basically for accuracy assessment of the effectiveness of sample preparation can be carried out by spiking a blank sample matrix with a known concentration of analyte. After extraction of the analyte from the matrix, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent [70-72]. Each spiked sample with known concentration (*C*) injected in triplicate and the recovery calculated by the expression:

$$Recovery = \frac{C_{calculated}}{C_{nominal}} \times 100 \qquad \text{Eq. (8)}$$

2.5.4 Limit of detection(LOD) and quantification (LOQ)

The LOD is the lowest concentration or amount of an analyte in a sample that can be detected, but not necessarily quantified whereas LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable level of confidence [72].

Among several methods for estimation of these parameters, commonly applied methods [68, 72-73] are:

- Visual determination, in which the analyte undergo successive dilution up to the lowest concentration level that cannot be detected. The concentration level detected with acceptable level of confidence is considered the LOD.

- Calculating the signal-to-noise ratio (S/N), usually it is applied to analytical methods that exhibit baseline noise in which the peak-to-peak noise around the analyte retention time is measured. Subsequently the concentration of the analyte that would yield a signal equal to certain value of noise to signal ratio is estimated. The noise magnitude can be measured either manually on the chromatogram (Fig. 5) or by auto-integrator of the instrument. The signal-to-noise ratio for LOD and LOQ correspond to 3 and 10 times respectively.

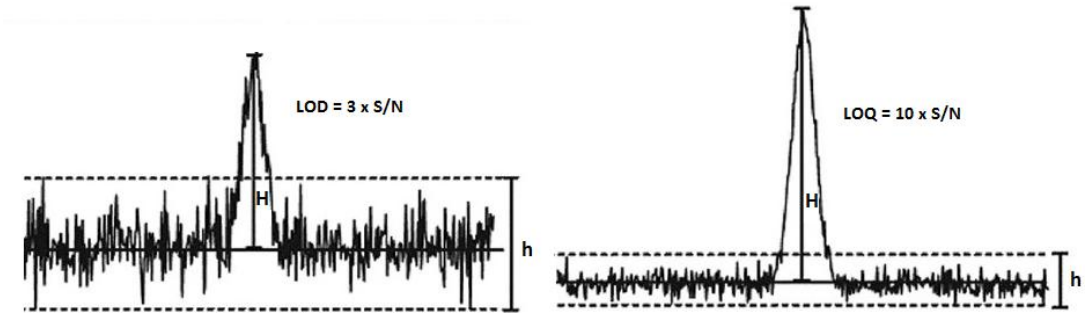


Figure 5. Signal-to-noise examples for LOD and LOQ estimation [70].

- Calculating from the standard deviation of the blank, it is normally used when the blank analysis provide a nonzero standard deviation. By using at least six blank analysis results, the LOD is expressed as the analyte concentration corresponding to the sample blank value plus three standard deviation and LOQ is the analyte concentration corresponding to the sample blank value plus ten standard deviations.

$$LOD = \bar{X} + 3(SD_{blank}) \quad \text{Eq. (9)}$$

$$LOQ = \bar{X} + 10(SD_{blank}) \quad \text{Eq. (10)}$$

where: $\bar{X}$ - concentration (usually zero), SD_{blank} - blank standard deviation of the blank sample.

However, Eq. (10) is usually applied if LOQ is not determined experimentally [74].

- Computing from the calibration line at low concentrations, in which at least three small concentrations near to the estimated LOQ taken and linear regression of the points applied to estimate the LOD and LOQ.

$$LOD = 3.3 \times \frac{SD_{res}}{m} \quad \text{Eq.(11)}$$

$$LOQ = 10 \times \frac{SD_{res}}{m} \quad \text{Eq.(12)}$$

where: SD_{res}- residual standard deviation; and m - slope of the calibration curve.

2.5.5 Linearity and Range

Linearity is the ability of an analytical method to provide an analytical response proportional to the concentration or the amount of analyte within a specified range. Whereas, the range of an analytical method is defined as the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has an acceptable level of precision, accuracy and linearity [66]. Mathematically, Linearity is expressed as:

$$y = m(x) + b \quad \text{Eq. (13)}$$

where: y - analytical response (dependent variable), x - amount of analyte (independent variable), m - slope (sensitivity) and b - intercept (error of y measurement).

The slope (m), the intercept (b) and the coefficient of determination (r) for linear regression (Eq. 13) based on least square method are expressed as:

$$m = \frac{n\sum x_i y_i - \sum x_i \sum y_i}{n\sum(x_i^2) - (\sum x_i)^2} \quad \text{Eq. (14)}$$

$$b = \frac{\sum(x_i^2)\sum y_i - \sum x_i \sum x_i y_i}{n\sum x_i^2 - (\sum x_i)^2} \quad \text{Eq. (15)}$$

$$r = \frac{n\sum x_i y_i - \sum x_i \sum y_i}{\sqrt{[n\sum x_i^2 - (\sum x_i)^2][n\sum y_i^2 - (\sum y_i)^2]}} \quad \text{Eq. (16)}$$

It is common practice explaining linearity by calculating the correlation coefficient (r), and r value close to unit considered as a sufficient evidence for linearity. However, the correlation coefficient close to one does not necessarily imply the linearity of the relation; conversely if the relation is linear, the correlation coefficient should be close to one [68, 76-78]. Furthermore, the literature recommends the Lack-of-fit or Mandel's fitting test as more suitable tests for the validation of a linear calibration model [79].

On the other hand, it is important to realize that the ordinary least square regression assumes that all the responses (y-values) have equal variances (homoscedasticity), but in some cases the variance of the responses rises proportionally to the concentration (heteroscedasticity). Therefore, the calibration data should be assessed for

homoscedasticity; In addition, to avoid the greater influence of the larger concentrations on the fitted regression line, the weighted linear regression is recommended [80].

The weighted linear regression can be obtained by using a weighting factor, w_i to any sum and changing the term n in to $\sum w_i$ on the formula of the slope (m), the intercept (b) and the coefficient of determination (r) (Eqn. 14 - 16) derived from the unweighted linear regression equation. Each data points weighting factor can be calculated by the expression:-

$$w_i = n \frac{\frac{1}{(SD)^2}}{\sum \frac{1}{(SD)^2}} \qquad \text{Eq. (17)}$$

where: $(SD)^2$ is variance of each point and n is the number of measurements.

Therefore, the slope (m_w), intercept (b_w) and correlation coefficient (r_w) of the weighted linear regression can be computed by the following relations respectively.

$$m_w = \frac{\sum w_i \sum w_i x_i y_i - \sum w_i x_i \sum w_i y_i}{\sum w_i \sum w_i x_i^2 - (\sum w_i x_i)^2} \qquad \text{Eq. (18)}$$

$$b_w = \frac{\sum w_i x_i^2 \sum w_i y_i - \sum w_i x_i \sum w_i x_i y_i}{\sum w_i \sum w_i x_i^2 - (\sum w_i x_i)^2} \qquad \text{Eq. (19)}$$

$$r_w = \frac{\sum w_i \sum w_i x_i y_i - \sum w_i x_i \sum w_i y_i}{\sqrt{[\sum w_i \sum w_i x_i^2 - (\sum w_i x_i)^2][\sum w_i \sum w_i y_i^2 - (\sum w_i y_i)^2]}} \qquad \text{Eq. (20)}$$

3. Experimental

3.1 Reagents

Prostaglandin E_2 (PGE_2, 99%), deuterated prostaglandin E_2 (PGE_2-d_4, 99%), leukotriene B_4 (LTB_4, 97%), deuterated leukotriene B_4 (LTB_4-d_4, 99%) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetonitrile (liquid chromatographic grade, 99.8%), formic acid (98%) were obtained from Sigma-Aldrich (St. Louis,MO, USA) and isopropanol (100%) obtained from Kemetyl Norge (Vestby, Norway). Water was produced using a Millipore Milli-Q water system (Millipore, Milford, USA).

3.2 Plasma samples preparation

Blank human plasma sample (approx. 10 mL) from an anonymous donor was used for the analysis carried out in the development of an extraction method. From the blank plasma 3 mL was spiked with 500 ng/mL of each eicosanoid (PGE_2 and LTB_4) and kept at -80 °C until further experiments.

3.3 Selection of the extraction solvents using mixture diagrams

Liquid-liquid extraction was proposed as the simplest and most rapid method of extraction with a selective LC-MS/MS method of analysis. For the development of an extraction method the first task was the selection of suitable solvent(s) and their mixture composition to extract the PGE_2 and LTB_4 from the plasma sample.

The initial selection of potential solvents for extraction was based on published reports. Most frequently applied solvents were acetonitrile, methanol, water, formic acid and their mixtures. To determine the best solvent or solvent mixture composition for

extraction, a simple augmented mixture design (Fig. 6A) and the modified form designs like shown on Fig. 6B and 6C with further volume adjustments were used.

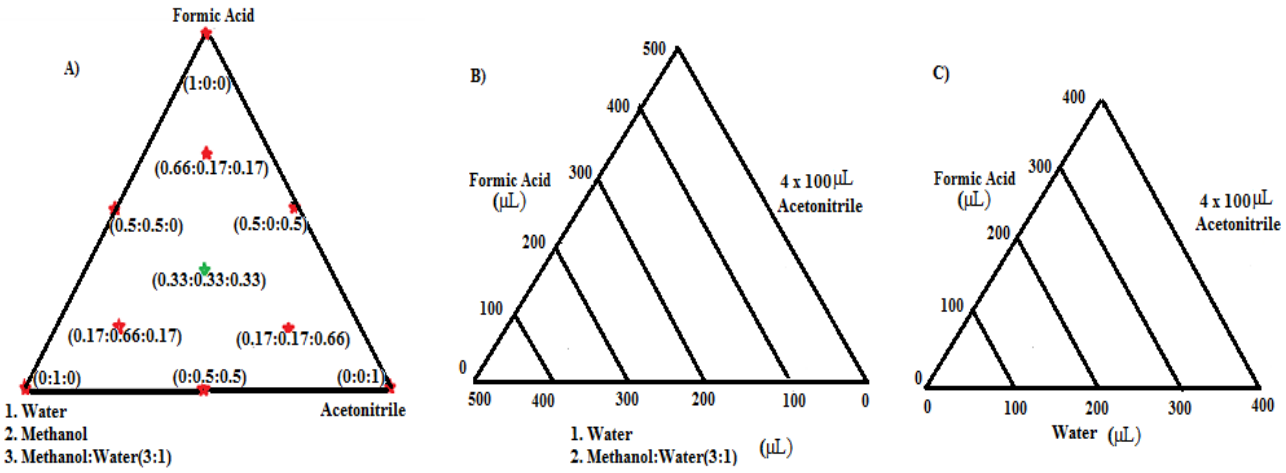


Figure 6. Augmented simplex mixture design (A) and modified mixture designs (B and C)

The preliminary experiments for selection of extraction solvent were carried out using blank plasma samples spiked with equal amounts of PGE_2 and LTB_4 (100 ng/mL). The general procedure applied was as follows: Based on the selected mixture designs at each experimental point triplicate test tubes containing 100 µL plasma samples were treated by extracting solvents acetonitrile, methanol, water, formic acid and their (two or three component) mixtures. At each individual solvent addition the test tubes were vortex mixed for 1 min then centrifuged for 10 min. The obtained solution was visually evaluated for its supernatant clearness. From test tubes that provide clear supernatant, extract was taken and introduced to LC-MS/MS. The remaining solution was dried in a vacuum drier and its residue weighted. During the experiments the concentration and the volume of spiked blank plasma sample were adjusted based on the volume of added extracting solvent.

The experimental responses from the designs applied for selection of suitable extraction solvent and their optimal mixture composition were the visual observation of clearness of supernatant after centrifugation, the weight of the precipitate left after extraction, and/or the strength of signals or highest recovery after introducing to the LC-MS/MS. However, based on the obtained result the mixture of extracting solvent yielding relatively clear supernatant and highest peak areas of extracted ion chromatogram in ion count per seconds (icps) was selected as the optimal system for extracting PGE_2 and LTB_4 from human plasma. Moreover, during the development of an extraction method, to gain maximal signal response and improved detection limit the extracts were exposed to passed through a vacuum drier pre-concentration step.

3.4 Optimization of the amount of internal standards

The initial blank plasma sample containing PGE_2 and LTB_4 (500 ng/mL of each analyte) was diluted with blank plasma to 1.00, 13.50, 25.00, 37.50 and 50.00 ng/mL as described in Fig 7. Moreover; 3.35, 25.00 and 47.50 ng/mL internal standards (PGE_2-d_4 and LTB_4-d_4) in acetonitrile were prepared for optimizing the response factor applying Doehlert uniform shell design.

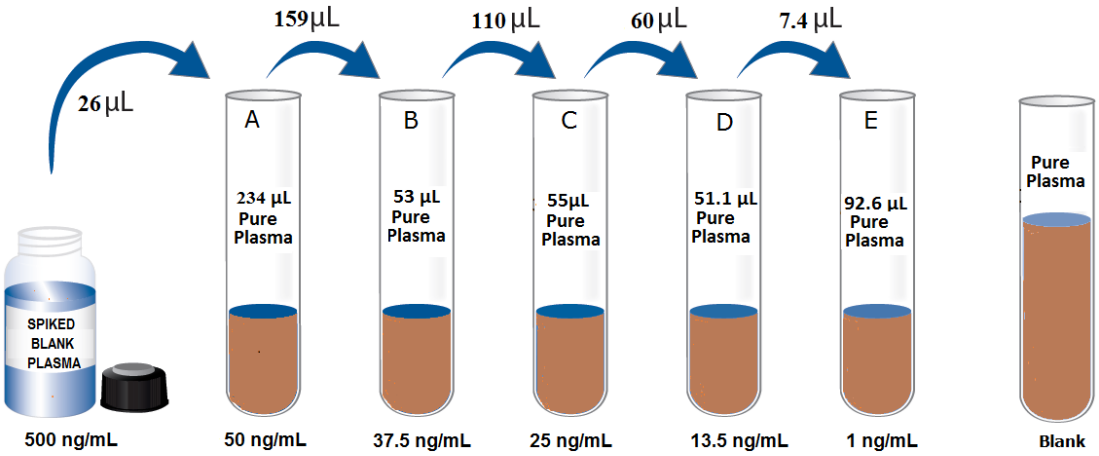


Figure 7. Preparation of different concentrations of PGE_2 and LTB_4 plasma solutions

The response factor (RF) behaviour was studied and modelled by using Doehlert uniform shell design when the concentrations of the PGE_2 and LTB_4 with their respective deuterated internal standards (natural level) were varied simultaneously (Table 2). In the Doehlert design as shown on Fig. 8, the LC-MSMS analysis response data from samples of seven experimental points were used to develop the models.

Table 2: Concentration of PGE_2, LTB_4, PGE_2-d_4 and LTB_4-d_4 in blood plasma at each experimental point of a two-variable Doehlert design.

Experiment no.	Coded level		Natural levels (ng/mL)			
	X_1	X_2	X_1		X_2	
			PGE_2	LTB_4	PGE_2 - d_4	LTB_4 - d_4
1	0.000	0.000	25.00	25.00	25.00	25.00
2	0. 500	-0.866	37.50	37.50	13.50	13.50
3	-0.500	0.866	13.50	13.50	47.50	47.50
4	-0.500	-0.866	13.50	13.50	13.50	13.50
5	0.500	0.866	37.50	37.50	47.50	47.50
6	-1.000	0.000	1.00	1.00	25.00	25.00
7	1.000	0.000	50.00	50.00	25.00	25.00

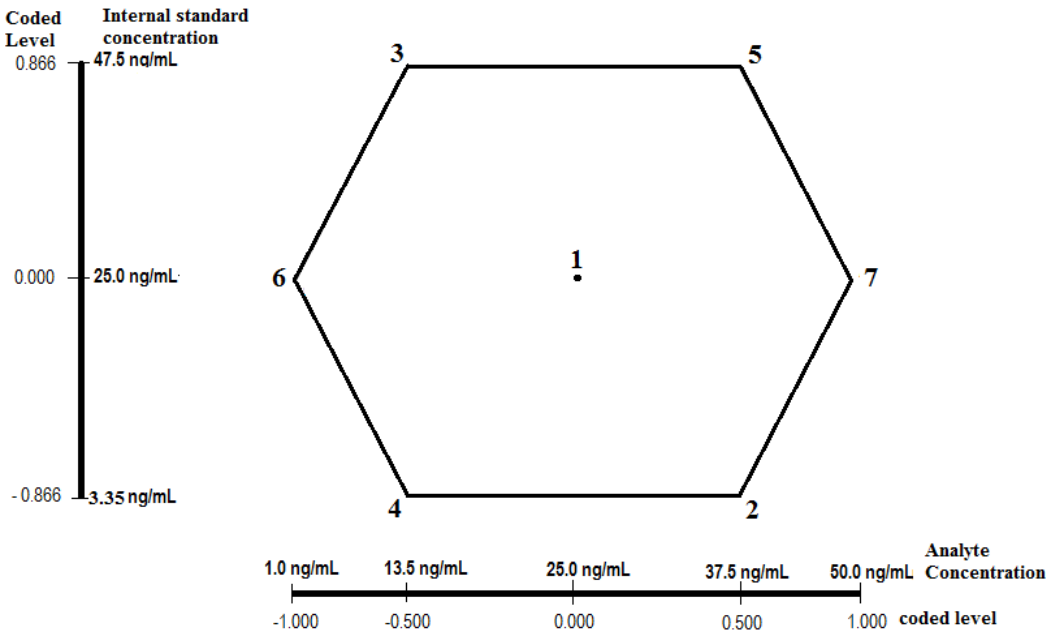


Figure 8. Coded and natural levels of the analytical species over the domain of a two variable Doehlert design.

At those Doehlert design experimental points based on the obtained signal (peak area) of analyte and internal standard, the RF was determined according to the expression shown at equation 1 (Eq. 1). Then the RF is explained or modeled based on the second order polynomial model (Eq. 4). The adequacy of the developed models from the obtained data was evaluated by statistical approach that describes the variation of the obtained values to that of the predicted model. In which the variances of the lack-of-fit and pure error are estimated by dividing their summation with respective degrees of freedom. The variance ratio of the lack-of-fit error to that of pure error known as experimental F-value ($F_{exp.}$) is used to conclude the model fits of the data by comparing with the theoretical F-value ($F_{theo.}$).

3.5 Extraction protocol

After selecting an optimal solvent system from the applied mixture design (section 3.3), the following protocol for the extraction of PGE_2 and LTB_4 from human plasma was proposed: the plasma sample (50 µL) in a micro-vial was treated with 10 µL of formic acid (98%). Subsequently 140 µL of acetonitrile (99.8%) containing the internal standards (PGE_2-d_4 at 15 ng/mL and LTB_4-d_4 at 50 ng/mL) were added to the plasma solution and vortex-mixed for 2 minutes, followed by centrifugation at 3500 rpm for 10 min. The supernatant collected in a conical micro-vial was submitted to LC-MS/MS. It is important to highlight that the mentioned levels of internal standards (15 ng/ml PGE_2-d_4 and 50 ng/mL LTB_4-d_4) were selected after optimizing their amounts by the strategy describe in section 3.4 and discussed in detail in section 4.2 .

3.6 Method validation

The selectivity of the method was evaluated by extracting the ion chromatogram of the analytes and internal standards (PGE_2, LTB_4, PGE_2-d_4 and LTB_4-d_4) in the blank sample spiked with and without those compounds.

The calibration curve using blank plasma samples spiked with PGE_2 and LTB_4 in the concentration range of 1 - 50 ng/mL was studied. Five equally spaced concentration levels (1, 12.5, 25, 37.5 and 50.00 ng/mL) in triplicates (as explained in Fig. 7) were extracted according to the protocol described in section 3.5 using 15 ng/mL of PGE_2-d_4 and 50 ng/mL of LTB_4-d_4. Weighted regression was also performed for both PGE_2 and LTB_4. The LOD was estimated by $3 \times SD_{blank}$ from the standard deviation of six injection results of blank samples as well as visually by using consecutive dilution of the spiked analyte in plasma and the LOQ was estimated by $7 \times SD_{blank}$ experimentally [74]. The recovery was determined by comparison of the nominal (spiked blank plasma) and calculated concentrations based on the constructed calibration curve models. The repeatability of the measurements in the analysis was also evaluated by calculating the coefficient of variance (CV) values of the signal ratio of the analyte to that of internal standards data for calibration curves.

3.7 Liquid chromatography ion-trap mass spectrometry

The LC-MS used was an Agilent 1100 series LC/MSD trap, SL model equipped with an electrospray interface (ESI), a quaternary pump, degasser, autosampler, thermostatted column compartment and a variable-wavelength UV detector. The column, a Zorbax

Eclipse-C_8 RP 150 mm × 4.6 mm, 5 µm (Agilent Technologies, Palo Alto, CA, USA) was kept in the column compartment at 40 ^{o}C. The injection volume was 25 µL with a flow rate of 0.5 mL/min for a 25 min analysis time operated in gradient mode solvent system.

The LC mobile phase system was studied in advance by analyzing the resolution and signal responses (peak areas) of PGE_2 and LTB_4 under isocratic and gradient mode. The mobile phase and their proportions used in these experiments were selected based on reported articles. The highest resolution and signal response was obtained with the following system: Solvent A: acetonitrile/water 63/37 (v/v) containing 0.02% formic acid, and solvent B: acetonitrile/isopropanol 50/50 (v/v), run with 100% of solvent A in between 0 - 5 min; solvent B was increased to 90% from 5 - 13 min and held until 15 min; then dropped to 0% by 20 min then held constant to 25 min. The UV detector was set at 254 nm. Nitrogen was used as nebulizing (50 psi) and drying gas (8 L/min) at 350 ^{o}C. The ESI source was operated in negative ion mode and the ion optics responsible for getting the ions in the ion-trap such as capillary exit, skimmer, lens and octapoles voltages were controlled by using the Smart view option with a resolution of 13,000 *m/z/s* (FWHM/*m/z* = 0.6 - 0.7). Complete system control, data acquisition and processing were done using the ChemStation for LC/MSD trap software 5.3 from Agilent. The monitored fragmentation patterns, recorded in ion counts per second (icps), were *m/z* 351 → 333, 315, 271 for PGE_2, *m/z* 355 → 337, 319, 275 for PGE_2-d_4, *m/z* 335 → 317, 299, 273, 195 for LTB_4 and *m/z* 339 → 321, 303, 277, 197 for LTB_4-d_4.

3.8 Quantification of PGE_2 and LTB_4 in human plasma

Forty plasma samples kindly donated from different projects of NIFES were analysed for their levels of PGE_2 and LTB_4 by the developed method. The first thirty plasma samples were collected in 2009 from a nutritional intervention study involving salmon fish and vitamin D. The remaining plasma samples were part of another project at NIFES involving patients with inflammatory problems. The forty samples were spiked with internal standards and the analytes were extracted by applying the developed extraction procedure. The obtained each sample extract was submitted to LC-MS/MS.

3.9 Statistics

The data is reported as mean and standard deviation. The acceptability of the RF models and linear regressions were performed by testing their Fisher ratio at a 95% confidence levels. The numbers of replicates used throughout the thesis were mostly triplicates, but in some instances it was dictated by the available amount of plasma.

4. Results and Discussion

4.1 Selection of the extraction solvents using mixture diagrams

Several reported methods of extraction were assessed during the development of an extraction method for PGE_2 and LTB_4 in human plasma. The most frequently applied extraction techniques prior to LC-MS/MS analysis are solid phase extraction (SPE) and repeated liquid-liquid extraction and some uses two or more column separation [39-45]. Accordingly, in this work the simple and rapid extraction method that could be applied in routine clinical analysis for a very small blood sample was selected to be liquid-liquid extraction. Its extraction efficiency was maximized by selecting a suitable solvent, the optimal composition of solvent mixture and developing proper extraction procedure.

From previously reported extraction methods [41-45, 82] and from the current NIFES extraction method (Appendix I) the following solvents for the extraction of PGE_2 and LTB_4 from human plasma were selected: acetonitrile, water, methanol, formic acid and their mixtures.

The ideal solvent or solvent mixture composition for best extraction was studied by applying augmented simplex mixture design (Fig. 6A). From the experiments most of the obtained solutions could not provide clear supernatant even after centrifugation, because the plasma components (mainly protein) were precipitated immediately and form colloidal mixture when acetonitrile added. Consequently the precipitation produced before addition of other solvents made further extraction procedures very

difficult. Therefore, by modifying the mixture design as shown in Fig. 6B, 400 µL (4 × 100 µL gradually) acetonitrile was added after adding other solvents (formic acid with water, formic acid with methanol and formic acid with methanol:water (3:1)) to precipitate out those precipitating components of the plasma solution and produce clear supernatant.

Based on the analysis results from mixture designs (Fig. 6A and Fig. 6B) a qualitative result was investigated. That is samples with formic acid, water and acetonitrile provided relatively clear solutions, easily separable supernatant from their residue and relatively better signal after introduced to LC-MS/MS, provided that the acetonitrile was added after adding other solvents. Further studies like as shown in Fig. 6C and other trials led to reduce the volume of extracting solvents (formic acid to 10 µL and acetonitrile to 140 µL) and complete exclusion of water in order to get concentrated solution of analytes from a reduced volume (50 µL) of plasma sample.

4.2 Optimization of the amount of internal standards

When the concentration of analyte and internal standard varied simultaneously, the RF behavior was studied by applying Doehlert uniform shell design. Based on the design five increasing coded levels of PGE_2 and LTB_4 (-1.00, -0.50, 0.00, 0.50, 1.00) along the x-axis and three increasing coded levels of PGE_2-d_4 and LTB_4-d_4 (-0.866, 0.000, 0.866) along the y-axis, a total of seven different concentration but with the same concentration of PGE_2 and LTB_4 as well as PGE_2-d_4 and LTB_4-d_4 solutions were analyzed in a hexagonal design experimental points as shown in Fig. 8.

The behaviour of RF of PGE_2 with PGE_2-d_4 and LTB_4 with LTB_4-d_4 was modelled with the full-second order polynomial function with six coefficients (Eq. 4), but later some models were reduced to lesser coefficients by omitting less contributing coefficients. This was done when the adequacy and prediction capacity of the reduced model was not significantly affected as compared with the unreduced six coefficients model. The adequacy of the developed models was shown by comparing the ratio of experimental lack-of-fit to pure error variance at the determined degrees of freedom (F_{exp}) with F_{crit}, the summery table is shown in table 3.

Table 3. Statistical validation summary for the developed RF models.

Models	Eq.(21)	Eq.(22)	Eq.(23)	Eq.(24)
Residual variance	1.24×10^{-2} (4)	1.60×10^{-1} (4)	1.05×10^{-1} (3)	9.66×10^{-3} (5)
Pure error variance	9.17×10^{-3} (2)	4.43×10^{-2} (2)	2.87×10^{-2} (2)	3.64×10^{-3} (2)
Lack-of-fit variance	3.23×10^{-3} (2)	1.16×10^{-1} (2)	7.60×10^{-2} (1)	6.02×10^{-3} (3)
$F_{experimental}$	0.71	2.62	3.31	1.10
$F_{theoretical}$	18.51	19.00	18.51	19.16

$F_{theoretical}$ is at 95% confidence level and degree of freedom in brackets

4.2.1 Modelling of the RF as a function of PGE_2 and PGE_2-d_4

Experimentally obtained RF data at the various levels of concentrations of PGE_2 and PGE_2-d_4, modelled with six parameters expression (Eq. 4) using regression function. The statistical validity of the full-second order polynomial equation for describing the RF as a function of PGE_2 and PGE_2-d_4 concentrations was evaluated. However, the six parameters can be reduced to five parameters model (Eq. 21) without any significance difference of prediction capacity, its statistical acceptability checked by F-test (table 3).

$$RF = 1.01 - 8.23 \times 10^{-3} [PGE_2] - 9.98 \times 10^{-3} [PGE_2\text{-}d_4] + 2.30 \times 10^{-4} [PGE_2][PGE_2\text{-}d_4] - 1.09 \times 10^{-4} [PGE_2\text{-}d_4]^2 \quad \text{Eq.(21)}$$

The pridiction capacity of Eq. 21 was evaluated statistically. 94.5% of the RF variability was explained by the reduced model while by the full six parameters model was 95.5%. The RF variation as a simultaneous change of concentration (0 - 50 ng/mL) of PGE_2 and PGE_2-d_4 is presented by the graphical representation or contour plot as shown on Fig.9A.

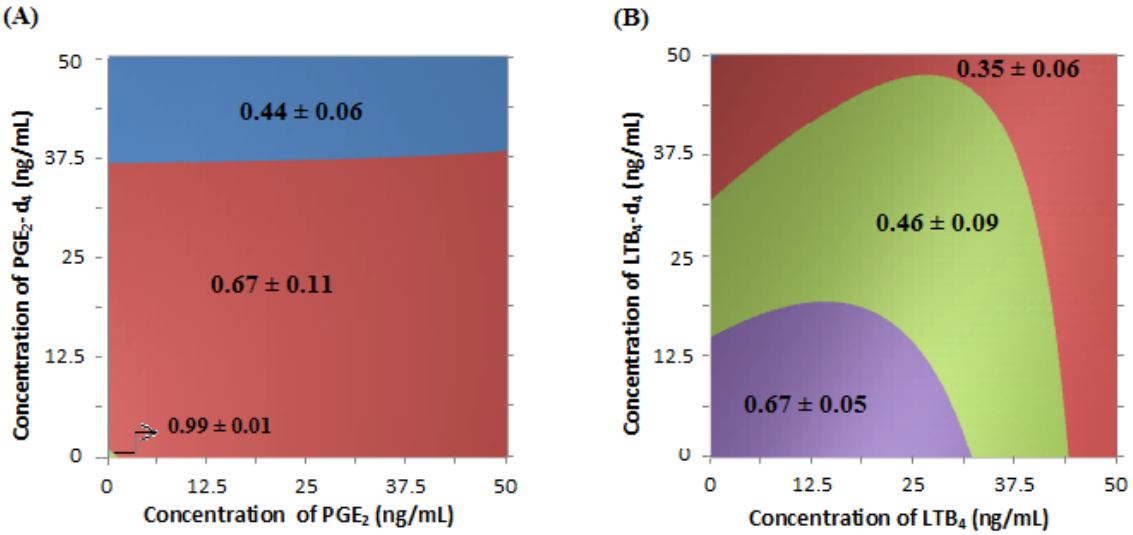


Figure 9 . Contour plot of the response factor (RF) expressed as a function of: A) PGE_2-d_4 vs. PGE_2 (Eq. 21) and B) LTB_4-d_4 vs. LTB_4 (Eq. 22).

As observed from the contour plot almost all of the PGE_2-d_4 concentration regions are suitable to find a constant RF, but it is clearly observed that there is a variation of RF below 2.5 ng/mL and slightly around 37.5 ng/mL of PGE_2-d_4. This constant RF consents to select the optimal concentration of the internal standard to be spiked for quantitative analysis of PGE_2 in plasma. Moreover, from the diagram it is clearly noticed that the RF is decreased as the concentration of PGE_2-d_4 increased, which confirms that the sensitivity of the analysis is also declining as the concentration of spiked PGE_2-d_4.

4.2.2 Modelling of the RF as a function of LTB_4 and LTB_4-d_4

The LTB_4 versus LTB_4-d_4 model evaluation was carried out in the same way as described above for PGE_2 versus PGE_2-d_4. The experimental RF value was described by the reduced five-coefficient model (Eq. 22). The model expresses 69.6% of the experimental variability.

$$RF = 0.78 + 2.93 \times 10^{-3} [LTB_4] - 1.18 \times 10^{-2} [LTB_4\text{-}d_4] + 2.30 \times 10^{-4} [LTB_4][LTB_4\text{-}d_4] - 2.60 \times 10^{-4} [LTB_4]^2 \quad \text{Eq.(22)}$$

The RF variation due to a simultaneous change of concentration (0 - 50 ng/mL) of LTB_4 and LTB_4-d_4 is shown by the contour plot (Fig. 9B). In which it is clearly seen that only the concentration region of LTB_4-d_4 above 46 ng/mL is parallel with the LTB_4 axis in the range of 0 - 50 ng/mL concentration, although the region has relatively less sensitivity of the analysis due to its relatively smaller mean of RF (0.35 ± 0.06) as compared with lower concentration LTB_4-d_4 regions. The observed curvature out of the stated range can be justified due to the quadratic contribution of LTB_4 term.

On the other hand, in the analysis of eicosanoids or other compounds it is usual to use a common internal standard for two or more analytes when there is scarcity of respective deuterated internal standards. Accordingly, it is possible to analyse both PGE_2 and LTB_4 using either of PGE_2-d_4 or LTB_4-d_4 based on the RF modelled below.

4.2.3 Modelling of the RF as PGE_2 vs LTB_4-d_4 or as LTB_4 vs PGE_2-d_4

The following two models were modelled to analyse PGE_2 using LTB_4-d_4 and to analyse LTB_4 using PGE_2-d_4 to assess the possibility to use one deuterated internal standard for two different analytes.

4.2.3.1 Modelling of the RF as a function of PGE_2 and LTB_4-d_4

Different possible polynomial models were evaluated for modelling the RF-behaviour of PGE_2 with LTB_4-d_4, finally the four parameter model showing a clear dependence of concentration of PGE_2, LTB_4-d_4 and their interaction explain RF behaviour adequately.

$$RF = 1.49 - 2.29 \times 10^{-2} [PGE_2] - 2.22 \times 10^{-2} [LTB_4\text{-}d_4] + 4.97 \times 10^{-4} [PGE_2][LTB_4\text{-}d_4] \quad \text{Eq.(23)}$$

The model expresses 97.8% of the experimental variability and the graphical representation (Fig. 10A) of Eq.(23) shows only the concentration region of LTB_4-d_4 45-50 ng/mL is parallel with the PGE_2 axis in the range of 0 - 50 ng/mL concentration. However for samples expected to contain below 40 ng/mL of PGE_2, it is possible to suggest that in the concentration range 22.5 - 50 ng/mL of LTB_4-d_4. The adequacy of the model was evaluated by the F-test (Table 3) and the model expressed by Eq.(23) is well enough to predict the RF over the range of experimental domain of the study.

4.2.3.2 Modelling of the RF as a function of LTB_4 and PGE_2-d_4

Although the RF behaviour of LTB_4 with PGE_2-d_4 was evaluated for reduced second order polynomial models, the full six term second order polynomial model expressed by Eq.(24) was best to describe the RF behaviour of LTB_4 and PGE_2-d_4.

$$RF = 0.54 + 5.84 \times 10^{-3} [LTB_4] - 6.57 \times 10^{-3} [PGE_2\text{-}d_4] + 8.44 \times 10^{-5} [LTB_4][PGE_2\text{-}d_4] - 1.31 \times 10^{-4} [LTB_4]^2 - 3.75 \times 10^{-5} [PGE_2\text{-}d_4]^2 \quad \text{Eq.(24)}$$

The graphical representation (Fig. 10B) of Eq.(24) shows the concentration region of LTB_4-d_4 from 5 - 20 ng/mL (RF = 0.49 ± 0.05) and 37.5 - 42.5 ng/mL (RF = 0.36 ±

0.05) is parallel with the LTB_4 axis in other words RF remain constant in the range of 0 - 50 ng/mL concentration. It is obvious that the observed curvature out of the stated range is due to the quadratic contribution of LTB_4 and PGE_2-d_4.

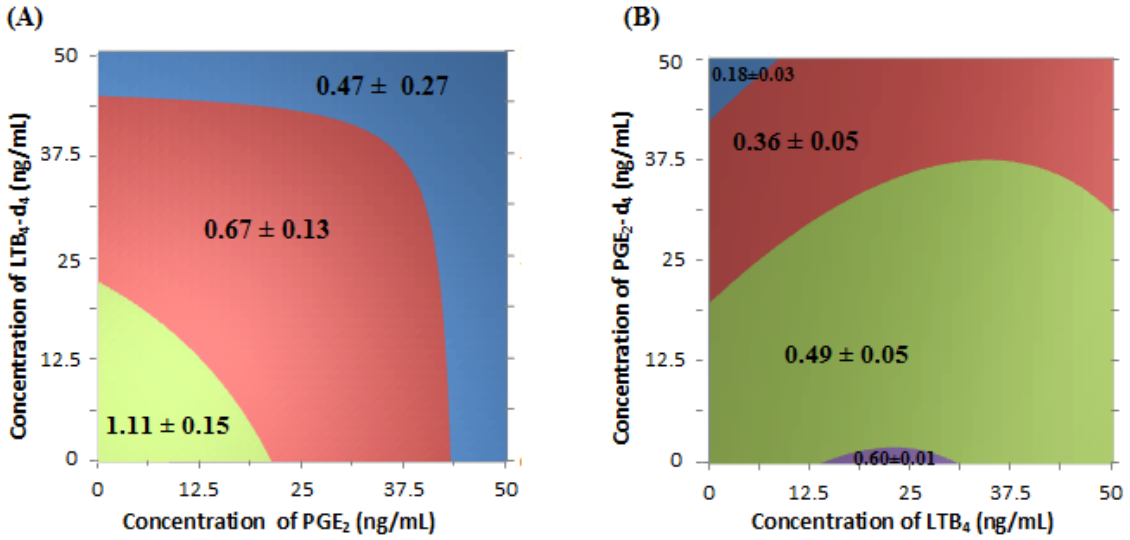


Figure 10. Contour plot of the response factor (RF) expressed as a function of: A) LTB_4-d_4 vs. PGE_2 (Eq. 23) and B) PGE_2-d_4 vs. LTB_4 (Eq. 24).

4.2.4 Optimal amount of internal standards for the analysis

The purpose of optimizing the RF behaviour of PGE_2 and LTB_4 with internal standards in this research was to select the optimal concentration of internal standards to be spiked in the plasma samples, in order to determine the concentration of PGE_2 and LTB_4 in plasma applying Eq.(1) or linear caliberation curve. Thus, based on the above four models the optimal concentrations of internal standards to be used were selected.

The optimal concentrations of PGE_2-d_4 to be used in order to analyse PGE_2 can be from 3 - 30 ng/mL or 38 - 50 ng/mL according to (Fig. 9A), as a result 15 ng/mL is chosen as optimal concentration of PGE_2-d_4 to be used for every analysis. The average RF value at

the region of this level of concentration is 0.67 ± 0.11. Similarly, the optimal concentration of LTB_4-d_4 to be spiked in the plasma sample in order to analyse LTB_4 is in the range of 46 - 50 ng/mL based on Fig.9B. Accordingly the concentration 50 ng/mL of LTB_4-d_4 was decided to be used for analysis of LTB_4. The average RF value at the region of this level of concentration is also 0.35 ± 0.06.

Spiking a plasma sample with 15 ng/mL PGE_2-d_4 can also serve to quantify the LTB_4 simultaneous with PGE_2, since as observed in Fig.10B the RF values change for LTB_4 is not significant around this concentration range. Moreover, 15 ng/mL is relatively small quantity of internal standard as compared with using in the concentration range of 38 - 50 ng/mL, it saves the expense of internal standard. Therefore; for simultaneous analysis of PGE_2 and LTB_4 in a plasma sample, 15 ng/mL PGE_2-d_4 is chosen. At which the average RF-value for LTB_4 is 0.49 ± 0.05. Similarly, the optimal concentration of LTB_4-d_4 to be spiked in the plasma sample in order to analyse PGE_2 is in the range of 44 - 50 ng/mL as shown in Fig.10A. Accordingly the concentration 50 ng/mL of LTB_4-d_4 was decided to be used for simultaneous analysis of both LTB_4 and PGE_2 and the average RF-value of PGE_2 is 0.47 ± 0.27.

4.3 Developed extraction protocol

Based on the previous sections the selected solvents for the quantitative extraction of PGE_2 and LTB_4 from human plasma were formic acid and acetonitrile (section 4.1) and the optimal concentrations of internal standards PGE_2-d_4 and LTB_4-d_4 (section 4.2) were 15 ng/mL and 50 ng/mL respectively. The protocol description was given in detail in the experimental section 3.5. Briefly, formic acid (10 µL) and acetonitrile (140 µL

containing optimal amounts of internal standards) were added to plasma (50 µL), vortex-mixed, centrifuged and supernatant submitted to LC-MS/MS.

According to the analysis procedure, a spiked blank plasma with optimal amount of internal standards using the developed extraction protocol submitted to LC-MS/MS analysis. The extracted ion chromatograms (EICs) were used for quantitative analysis. The EICs of PGE_2 and LTB_4 with respective deuterated internal standards (Fig. 11) provide their respective characteristic fragments in the mass spectra of each compound as shown on Fig. 12. The elution time for PGE_2 and PGE_2-d_4 was around 11.2 min while for LTB_4 and LTB_4-d_4 around 15.3 min by the conditions set, the total analysis time was 25 min.

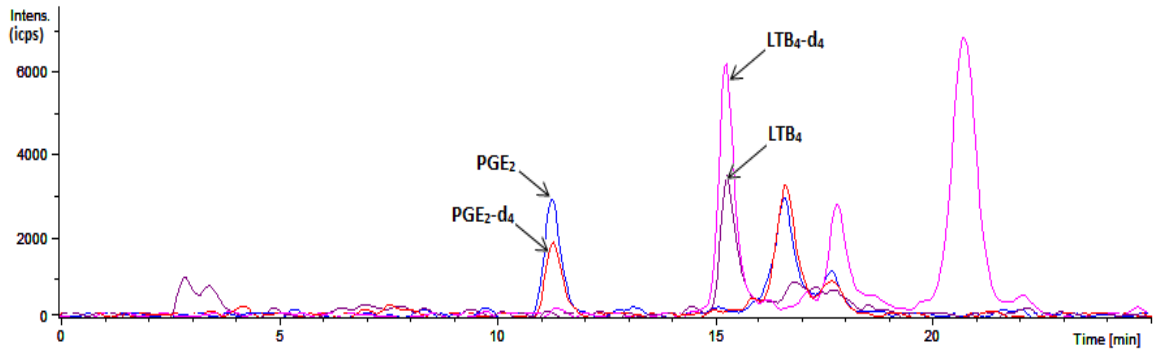


Figure 11. Extracted ion chromatograms (EICs) of PGE_2, PGE_2-d_4, LTB_4 and LTB_4-d_4 extracted from spiked human blank plasma.

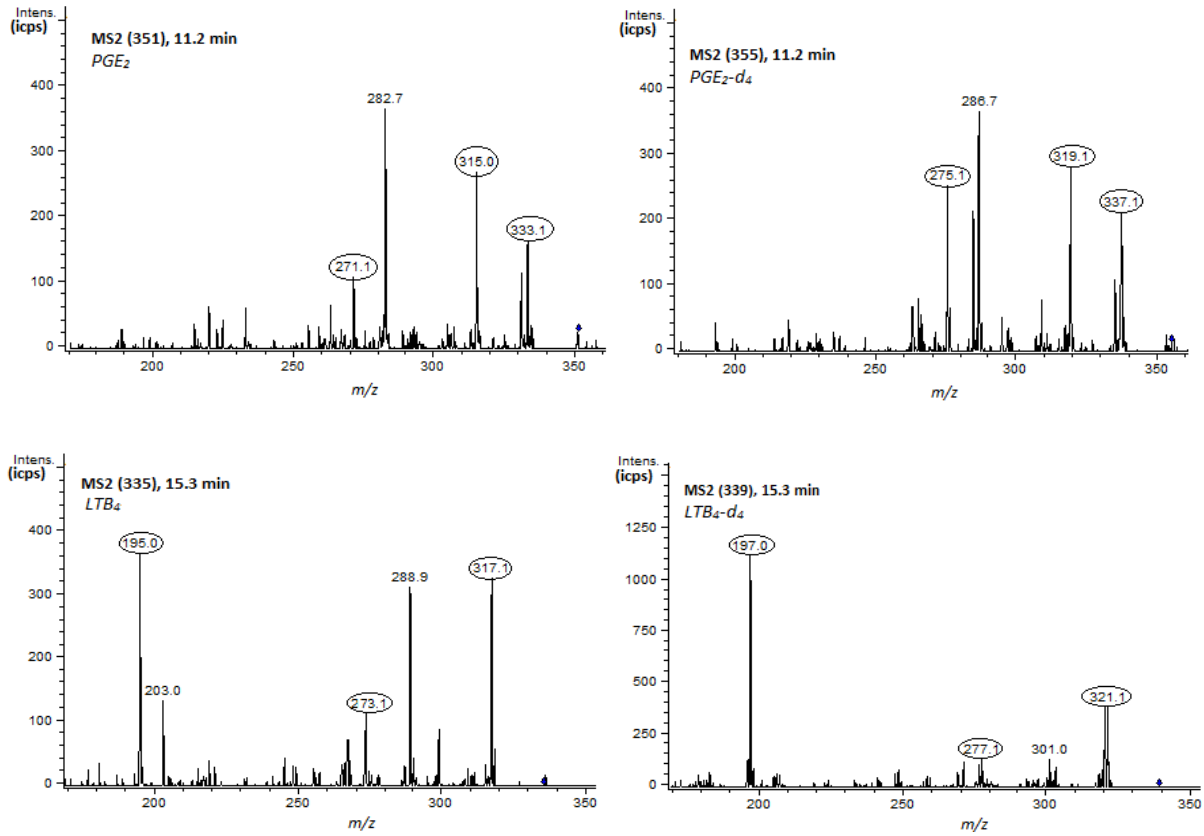


Figure 12. Mass spectra of PGE_2, PGE_2-d_4, LTB_4 and LTB_4-d_4 extracted from spiked human blank plasma.

Furthermore, pre-concentrating with vacuum drier as well nitrogen gas drier at room temperature was carried out in order to enhance the response signal and the detection limit. The result show that signals of eicosanoids has been negligible, even sometimes disappeared. This confirms that pre-concentration of eicosanoids with nitrogen gas or vacuum drier requires great attention. Especially when analytes are in large proportion of acetonitrile the analyte loss was high (Fig. 13), since it is clearly observed in the experiments that when acetonitrile mixed with other solvents the analyte loss was reduced.

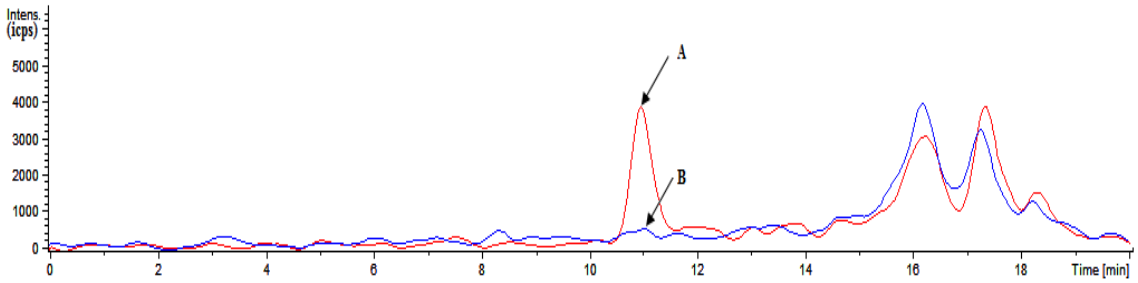


Figure 13. Extracted ion chromatograms (EICs) of PGE_2 in acetonitrile without (A) and with (B) vacuum drying at room temperature.

4.4 Method validation

The selectivity of the method was evaluated by extracting the ion chromatogram of the analytes and internal standards (PGE_2, LTB_4, PGE_2-d_4 and LTB_4-d_4) in the blank sample spiked with and without those analytes. The analysis was highly selective towards those eicosanoids showing well resolved ion chromatogram as observed in Fig. 11. To determine LOD six times blank plasma sample was injected and the standard deviation was estimated by applying Eq.(9), ($LOD = \bar{X} + 3(SD)$) and again it was visually approved by successive dilution of spiked analytes in plasma up to the lowest concentration level that cannot be detected. Finally, the LOD was determined to be 0.4 ng/mL for both PGE_2 and LTB_4. The LOQ also determined experimentally to be 1 ng/mL approximating with modified Eq.(10), almost seven times the standard deviation ($LOQ = \bar{X} + 7(SD)$). However, compared with the reported less than two order of magnitude pico-level (pg/mL) LOD and LOQ reported by other related methods (eg. on-line two-dimensional reversed-phase LC-MS/MS [45, 82]), the obtained LOD and LOQ value for the method is not appreciable to detect concentrations near to the normal endogenous levels. However it can serve for the intended purpose of PGE_2 and LTB_4 routine analysis in human plasma samples from patients suffering from inflammatory

pain, since the level of these pro-inflammatory biomarkers is expected relatively to be high in those patients.

The linearity of the system was already shown by the Doehlert design in the selected range of concentration. The acceptability of the models is also evaluated by statistical F-test as shown in table 3 or the detail in Appendix II-V. Although getting coefficient of determination close to one necessarily do not confirm the linearity of the system, the coefficient of determination was determined by the usual way of simple linear regression curve in the specified concentration range of analyte (1 to 50 ng/mL) against the ratio of signal (peak area) of analyte to that of internal standard (Table 4). That is (signal of $PGE_2/PGE_2\text{-}d_4$) = 0.0257[PGE_2] – 0.0029 for PGE_2 analysis (Fig. 14A) and (signal of $LTB_4/LTB_4\text{-}d_4$) = 0.0094[PGE_2] + 0.0037 for LTB_4 analysis (Fig. 14B), the closed bracket in the expressions represent concentration. Their coefficients of determination (r^2) were 0.9605 and 0.9629, respectively. The linearity of the data was statistically evaluated using F-test (Appendix VI and VII). The $F_{experimental}$ value for PGE_2 and LTB_4 were 0.99 and 0.26 at 95% confidence limit (degree of freedom 3, 10), respectively. While the $F_{critical}$ is 3.71 at stated confidence limit and degree of freedom for both analytes, which confirms that linearity is statistically accepted at 95% confidence limit and 3, 10 degree of freedom.

On the other hand, when the triplicate data points visually observed on the calibration line, their distribution seems like dependent of concentration. Thus the weighted regression based on Eq. 17-20 was determined assuming the data as heteroscedastic. The weighted regression equation were (signal of $PGE_2/PGE_2\text{-}d_4$) = 0.0265[PGE_2] – 0.0087 for PGE_2 and (signal of $LTB_4/LTB_4\text{-}d_4$) = 0.0094[PGE_2] + 0.0012 for LTB_4

analysis which have almost comparable slope and intercept values to that of unweighted curves, and also the coefficient of determination (r^2) was relatively low (around 0.66) and the acceptability of its linearity was not statistically supported at 95% confidence limit for both analytes. Therefore, the linearity was evaluated by unweighted linear regression equation.

Table 4. Experimental data for constructing PGE_2 and LTB_4 calibration curves

Conc. (ng/mL)	Signal of PGE_2/PGE_2-d_4	CV (%)	Signal of LTB_4/LTB_4-d_4	CV (%)
1	0.0178±0.0035	19.66	0.0105±0.0018	17.14
12.5	0.3038±0.0615	20.24	0.1131±0.0236	20.86
25	0.6444±0.1382	21.44	0.2519±0.0496	19.69
37.5	1.0087±0.0673	6.67	0.3589±0.0361	10.05
50	1.2433±0.1674	13.46	0.4632±0.0503	10.85

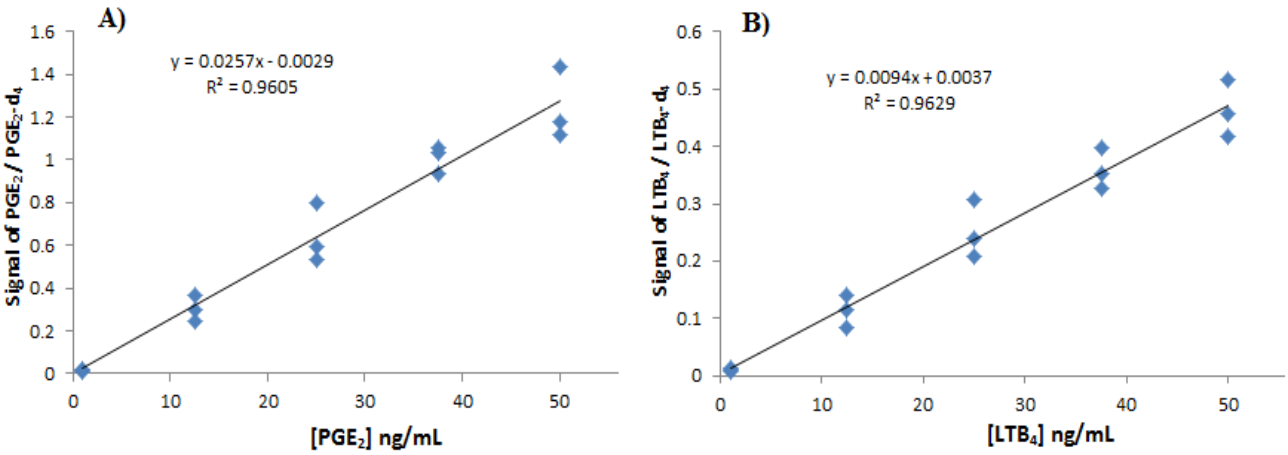


Figure 14. Linear regression graphsfor PGE_2 (A) and LTB_4 (B).

Accuracy was also determined by recalculating each sample based on the calibration curve equation applying the recovery calculation equation, Eq.(12). The obtained result was the recovery of PGE_2 falls within 80.54% - 104.96 %, whereas for LTB_4 72.34% - 105.61%. The other parameter precision that is the repeatability of the method was evaluated by calculating the coefficient of variation (CV) based on Eq.(6) for all

injections at each concentration analysed for calibration curve, thus the CV for both analytes was shown in table 4.

4.5 Method application on real plasma samples

Human plasma samples (40 in number) from different on-going projects at NIFES were examined for their levels of PGE_2 and LTB_4 by the developed extraction method. Based on the quantitative analyses (Table 5) the levels of the PGE_2 and LTB_4 in the majority of the first thirty samples were below the quantification level (LOQ) except three samples for PGE_2 and one sample for LTB_4 were above LOQ.

Table 5. Levels of PGE_2 and LTB_4 in real human plasma samples

Sample		Concentration (ng/mL)		Sample		Concentration (ng/mL)	
No	**Name**	**PGE_2**	**LTB_4**	**No**	**Name**	**PGE_2**	**LTB_4**
1	ID001	*d*	*nd*	**21**	ID030	*nd*	*nd*
2	ID002	*d*	*nd*	**22**	ID032	*d*	*nd*
3	ID003	*d*	*nd*	**23**	ID034	*nd*	*nd*
4	ID004	1.32±0.22	*d*	**24**	ID036	*d*	*nd*
5	ID005	*d*	*nd*	**25**	ID038	*nd*	*nd*
6	ID006	*nd*	*nd*	**26**	ID040	*nd*	*nd*
7	ID007	*nd*	*nd*	**27**	ID041	*nd*	*nd*
8	ID008	*d*	*nd*	**28**	ID043	*d*	*d*
9	ID009	1.21±0.35	*nd*	**29**	ID045	*nd*	*nd*
10	ID010	*d*	*nd*	**30**	ID047	*nd*	*nd*
11	ID012	*nd*	*nd*	**31**	P-13 ^{B}	505.0±56.1	631.6±49.9
12	ID014	*d*	*nd*	**32**	P-21 ^{B}	644.67±5.68	741.8±79.1
13	ID016	*nd*	*nd*	**33**	P-25 ^{B}	562.82±3.86	769.5±41.1
14	ID018	*d*	*d*	**34**	P-28 ^{A}	36.80±6.54	72.35±4.69
15	ID020	*d*	*nd*	**35**	P-33 ^{C}	546.49±6.73	1375.4±96.9
16	ID021	*nd*	*nd*	**36**	P-44 ^{B}	45.74±3.94	958.3±85.7
17	ID023	1.48±0.28	*d*	**37**	P-55 ^{D}	1031±211	2107 ±357
18	ID025	*d*	*nd*	**38**	P-58 ^{B}	333.1±41.1	756.2±29.1
19	ID027	*nd*	1.77±0.41	**39**	P-102 ^{C}	552.1±16.3	939.7±84.2
20	ID029	*nd*	*nd*	**40**	P-312 ^{B}	1132.1±27.7	158.0±13.0

- $nd < LOD \leq d < LOQ$
- *Superscripts on the sample name A, B, C & D are dilution factors used 2, 24, 30 & 50 respectively.*
- *Results are expressed as: mean* $\pm$ $standard\ deviation$.

Several justifications can be forwarded for the low levels of analytes from the expected in the first thirty samples. The main reasons may be the degradation of analytes through time as it is kept for more than three years. For instance PGE_2 may be degraded to a very stable PGA_2 as it is reported in the literature [83-86]. However, the levels of eicosanoids in the last 10 samples were relatively high when compared to reported [15, 82] normal endogenous levels of this eicosanoids (< 12 pg/ml).

Therefore, according to the obtained analyses result the proposed method can effectively serve as a simple and rapid method for extraction and further analysis using LC-MS/MS to estimate the level of PGE_2 and LTB_4 in blood plasma samples of patents suffering from inflammatory pain.

5. Conclusions

The study proposed a simple and most rapid method for the extraction of PGE_2 and LTB_4 from human plasma and subsequent quantification by LC-MS/MS. In the experiment simple mixture design has been applied to assist the selection and evaluation of extracting solvent by changing the proportions of components of a mixture. The design was basic to distinguish the best proportion of extracting solvents. The two-factor Doehlert uniform shell design was also used to assess the effect of four parameters (PGE_2, LTB_4, PGE_2-d_4 and LTB_4-d_4) in modelling of the experimental response and selection of the optimal concentration of internal standards to be applied in further analysis.

The developed method has considerable significance of reducing analysis time. When it is compared with the method of eicosanoids extraction from human plasma and further LC-MS/MS analysis that has been used in NIFES (requires total time of analysis 6-8 hours), the developed method (requires about 50-60 minutes) was many times faster. The method was also validated based on the usual validation parameters and evaluated for the real plasma samples, works appropriately. Therefore, it can serve as a simple and rapid method of extraction in routine clinical analysis to detect and quantify PGE_2 and LTB_4 found in plasma samples collected from patients who are affected by chronic inflammation.

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APPENDICES

I. Extraction procedure for PGE_2 (Eicosanoids) from human plasma for LC-MS/MS analysis used in NIFES [43].

- Plasma sample collected and store at - 80 ^oC until analysis.
- The 200 µL of 30 ng/mL deuterated internal standard (PGE_2-d_4) in acetonitrile dispensed in to test tube and allowed to be dried under a stream of nitrogen at room temperature in a test tube very slowly.
- Aliquot of human plasma (200 µL) is added to the test tubes containing dried internal standard and vortex mixed for 2 min.
- Then 400 µL methanol:water (3:1) solvent mixture added and vortex-mixed for 30 s, again 400 µL acetonitrile is added and vortex-mixed for 30 s.
- The solution centrifuged at 3000 rpm for 10min at room temperature.
- The supernatant is collected and evaporated to dryness under a stream of nitrogen at room temperature.
- Finally the dried sample reconstituted by 30 µL of acetonitrile; sonicated for 30 s and transfered to micro-volume vial for LC-MS/MS analysis

Reported Limitation of the method by the analysts

- When the stream of nitrogen is applied to evaporate the solvent, the analytes often disappear or the amount could be collected is very small, because the solvent evaporation step requires strict follow up and lengthy time of evaporation.
- The overall extraction procedure is relatively time-consuming (not more than 20 samples/day can be analyzed) to be applied in a routine clinical use.

II. Model acceptability for PGE_2 and PGE_2-d_4

$$RF = 1.01 - 8.23 \times 10^{-3} [PGE_2] - 9.98 \times 10^{-3} [PGE_2\text{-}d_4] + 2.30 \times 10^{-4} [PGE_2][PGE_2\text{-}d_4] - 1.09 \times 10^{-4} [PGE_2\text{-}d_4]^2$$

Table 6. Model acceptability for PGE_2 and PGE_2-d_4

			Peak area			Error Decomposition		
Exp. no	$[PGE_2]$	$[PGE_2\text{-}d_4]$	PGE_2	$PGE_2\text{-}d_4$	RFexp.	$(\text{Residual E})^2$	$(\text{Pure E})^2$	$(\text{L.O.F})^2$
1	25.00	25.00	32941	55604	**0.59**	$1.62\text{x}10^{-3}$	$3.31\text{x}10^{-3}$	$3.00\text{x}10^{-4}$
1	25.00	25.00	36796	50784	**0.73**	$8.45\text{x}10^{-3}$	$5.57\text{x}10^{-3}$	$3.00\text{x}10^{-4}$
1	25.00	25.00	26258	41492	**0.63**	$4.83\text{x}10^{-8}$	$2.92\text{x}10^{-4}$	$3.00\text{x}10^{-4}$
2	37.50	3.35	51484	6470	**0.71**	$1.77\text{x}10^{-4}$	0	$1.77\text{x}10^{-4}$
3	13.50	47.50	16001	177931	**0.32**	$1.64\text{x}10^{-4}$	0	$1.64\text{x}10^{-4}$
4	13.50	3.35	23919	6875	**0.86**	$1.77\text{x}10^{-4}$	0	$1.77\text{x}10^{-4}$
5	37.50	47.50	51322	159793	**0.41**	$1.64\text{x}10^{-4}$	0	$1.64\text{x}10^{-4}$
6	1.00	25.00	2193	80814	**0.68**	$1.88\text{x}10^{-4}$	0	$1.88\text{x}10^{-4}$
7	50.00	25.00	51579	48439	**0.53**	$1.46\text{x}10^{-3}$	0	$1.46\text{x}10^{-3}$
					Sum=	**$1.24\text{x}10^{-2}$**	**$9.17\text{x}10^{-3}$**	**$3.23\text{x}10^{-3}$**
					DF=	**4**	**2**	**2**
					Fcrit.=	**18.51**		
					Fexp.=	**0.71**	at 95% Confidence limit and degree of freedom 2, 2.	

III. Model acceptability for LTB_4 and LTB_4-d_4

$$RF = 0.78 + 2.93 \times 10^{-3} [LTB_4] - 1.18 \times 10^{-2} [LTB_4\text{-}d_4] + 2.30 \times 10^{-4} [LTB_4][LTB_4\text{-}d_4] - 2.60 \times 10^{-4} [LTB_4]^2$$

Table 7. Model acceptability for LTB_4 and LTB_4-d_4

			Peak area			Error Decomposition		
Exp. no	$[LTB_4]$	$[LTB_4\text{-}d_4]$	LTB_4	$LTB_4\text{-}d_4$	RFexp.	$(\text{Residual E})^2$	$(\text{Pure E})^2$	$(\text{L.O.F})^2$
1	25.00	25.00	33193	50625	**0.66**	$1.45\text{x}10^{-2}$	$1.97\text{x}10^{-2}$	$3.98\text{x}10^{-4}$
1	25.00	25.00	18177	50586	**0.36**	$3.10\text{x}10^{-2}$	$2.43\text{x}10^{-2}$	$3.98\text{x}10^{-4}$
1	25.00	25.00	19328	36403	**0.53**	$1.87\text{x}10^{-5}$	$2.44\text{x}10^{-2}$	$3.98\text{x}10^{-4}$
2	37.50	3.35	33708	7753	**0.39**	$1.46\text{x}10^{-2}$	0	$1.46\text{x}10^{-2}$
3	13.50	47.50	17940	123677	**0.51**	$2.40\text{x}10^{-2}$	0	$2.40\text{x}10^{-2}$
4	13.50	3.35	18912	5218	**0.89**	$2.59\text{x}10^{-2}$	0	$2.59\text{x}10^{-2}$
5	37.50	47.50	29609	148085	**0.25**	$1.35\text{x}10^{-2}$	0	$1.35\text{x}10^{-2}$
6	1.00	25.00	863	62565	**0.34**	$2.08\text{x}10^{-2}$	0	$2.08\text{x}10^{-2}$
7	50.00	25.00	50755	64930	**0.39**	$1.58\text{x}10^{-2}$	0	$1.58\text{x}10^{-2}$
					Sum=	**$1.60\ \text{x}10^{-1}$**	**$4.43\ \text{x}10^{-2}$**	**$1.16\ \text{x}10^{-1}$**
					DF=	**4**	**2**	**2**
					Fcrit.=	**19.00**		
					Fexp.=	**2.62**	at 95% Confidence limit and degree of freedom 2, 2.	

IV. Model acceptability for LTB_4 and PGE_2-d_4

$$RF = 0.54 + 5.84 \times 10^{-3} [LTB_4] - 6.57 \times 10^{-3} [PGE_2\text{-}d_4] + 8.44 \times 10^{-5} [LTB_4][PGE_2\text{-}d_4] - 1.31 \times 10^{-4} [LTB_4]^2 - 3.75 \times 10^{-5} [PGE_2\text{-}d_4]^2$$

Table 8. Model acceptability for LTB_4 and PGE_2-d_4

			Peak area			Error Decomposition		
Exp. no	$[LTB_4]$	$[PGE_2\text{-}d_4]$	LTB_4	$PGE_2\text{-}d_4$	RFexp.	(Residual E) ^{2}	(Pure E) ^{2}	(L.O.F) ^{2}
1	25.00	25.00	33193	55604	**0.60**	9.99×10^{-3}	1.52×10^{-2}	5.49×10^{-4}
1	25.00	25.00	18177	50784	**0.36**	1.93×10^{-2}	1.34×10^{-2}	5.49×10^{-4}
1	25.00	25.00	19328	41492	**0.47**	9.72×10^{-4}	5.99×10^{-5}	5.49×10^{-4}
2	37.50	3.35	33708	6470	**0.47**	1.04×10^{-2}	0	1.04×10^{-2}
3	13.50	47.50	17940	177931	**0.35**	1.76×10^{-4}	0	1.76×10^{-4}
4	13.50	3.35	18912	6875	**0.68**	1.03×10^{-2}	0	1.03×10^{-2}
5	37.50	47.50	29609	159793	**0.23**	3.33×10^{-2}	0	3.33×10^{-2}
6	1.00	25.00	863	80814	**0.27**	1.47×10^{-2}	0	1.47×10^{-2}
7	50.00	25.00	50755	48439	**0.52**	5.52×10^{-3}	0	5.52×10^{-3}
					Sum=	**1.05×10^{-1}**	**2.87×10^{-2}**	**7.60×10^{-2}**
					DF=	**3**	**2**	**1**
					$F_{crit.}$=	**18.51**		
					$F_{exp.}$=	**3.31**	at 95% Confidence limit and degree of freedom 1, 2.	

V. Model acceptability for PGE_2 and LTB_4-d_4

$$RF = 1.49 - 2.29 \times 10^{-2} [PGE_2] - 2.22 \times 10^{-2} [LTB_4\text{-}d_4] + 4.97 \times 10^{-4} [PGE_2][LTB_4\text{-}d_4]$$

Table 9. Model acceptability for PGE_2 and LTB_4-d_4

			Peak area			Error Decomposition		
Exp. no	$[PGE_2]$	$[LTB_4\text{-}d_4]$	PGE_2	$LTB_4\text{-}d_4$	RFexp.	(Residual E) ^{2}	(Pure E) ^{2}	(L.O.F) ^{2}
1	25.00	25.00	32941	50625	**0.65**	4.42×10^{-4}	0.002412	7.89×10^{-4}
1	25.00	25.00	36796	50586	**0.73**	3.10×10^{-3}	7.62×10^{-4}	7.89×10^{-4}
1	25.00	25.00	26258	36403	**0.72**	2.46×10^{-3}	4.63×10^{-4}	7.89×10^{-4}
2	37.50	3.35	51484	7753	**0.59**	5.51×10^{-4}	0	5.51×10^{-4}
3	13.50	47.50	16001	123677	**0.46**	1.09×10^{-4}	0	1.09×10^{-4}
4	13.50	3.35	23919	5218	**1.14**	1.17×10^{-4}	0	1.17×10^{-4}
5	37.50	47.50	51322	148085	**0.44**	5.11×10^{-4}	0	5.11×10^{-4}
6	1.00	25.00	2193	62565	**0.88**	2.22×10^{-3}	0	2.22×10^{-3}
7	50.00	25.00	51579	64930	**0.40**	1.53×10^{-4}	0	1.53×10^{-4}
					Sum=	**9.66×10^{-3}**	**3.64×10^{-3}**	**6.02×10^{-3}**
					DF=	**5**	**2**	**3**
					$F_{crit.}$=	**19.16**		
					$F_{exp.}$=	**1.10**	at 95% Confidence limit and degree of freedom 3, 2.	

VI. Acceptability of the linear regression model for PGE_2

Table 10. Acceptability of the linear regression model for PGE_2

Concentration (mg/mL)	**$Y_{experimental.}$ (S_A/S_{IS})**	**$Y_{estimated.}$**	**$Y_{average.}$**	**(Resid E)^2**	**(Pure E)^2**	**(L.O.F)^2**
1	0.01949	0.0228	0.01783	1.097E-05	2.75E-06	2.47E-05
1	0.01377	0.0228	0.01783	8.159E-05	1.65E-05	2.47E-05
1	0.02023	0.0228	0.01783	6.585E-06	5.78E-06	2.47E-05
12.5	0.36699	0.3184	0.303772	0.0023662	0.003997	0.000213
12.5	0.30008	0.3184	0.306639	0.0003338	4.3E-05	0.000137
12.5	0.24424	0.3184	0.306639	0.0054918	0.003893	0.000137
25	0.53647	0.6396	0.644423	0.0106363	0.011654	2.33E-05
25	0.75833	0.6396	0.640521	0.0257834	0.025489	8.48E-07
25	0.59663	0.6396	0.640521	0.0018464	0.001926	8.48E-07
37.5	1.05806	0.9609	1.008747	0.0094505	0.002432	0.002294
37.5	0.93206	0.9609	1.002339	0.0008291	0.00494	0.001721
37.5	1.03612	0.9609	1.002339	0.0056655	0.001141	0.001721
50	1.17639	1.2821	1.243336	0.0111748	0.004482	0.001503
50	1.11980	1.2821	1.150776	0.0263416	0.00096	0.017246
50	1.43382	1.2821	1.150776	0.0230189	0.080113	0.017246
			Sum=	**0.1230375**	**0.141096**	**0.042317**
			DF=	13	10	3
			Fexp.=	**0.99**	at 95% Confidence limit and degree of freedom 3, 10.	
			Fcrit.=	**3.71**		

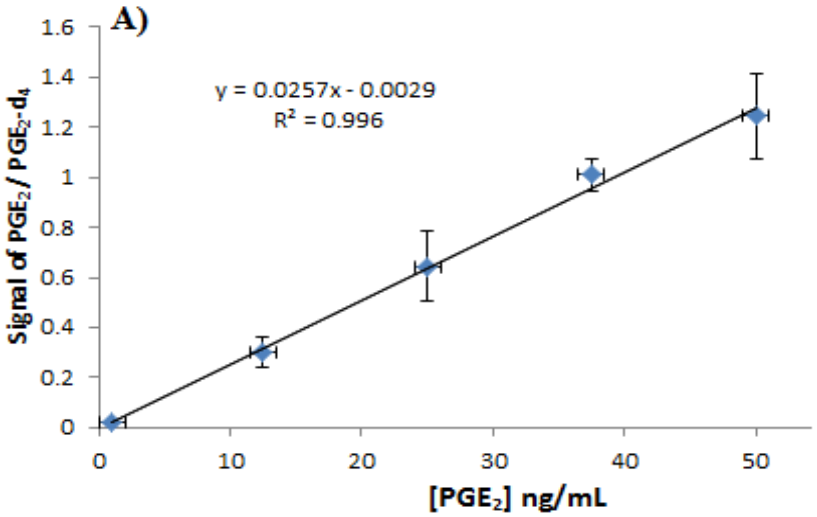

Fig 15. Linear regression graphs for PGE_2 using triplicate mean value and error bar.

VII. Acceptability of the linear regression model for PGE_2

Table 10. Acceptability of the linear regression model for PGE_2

Concentration (mg/mL)	**$Y_{experimental.}$ (S_A/S_{IS})**	**$Y_{estimated.}$**	**$Y_{average.}$**	**(Resid E)^2**	**(Pure E)^2**	**(L.O.F)^2**
1	0.01949	0.00977	0.0131	0.01053	1.11E-05	5.83E-07
1	0.01377	0.01258	0.0131	0.01053	2.73E-07	4.19E-06
1	0.02023	0.00925	0.0131	0.01053	1.49E-05	1.65E-06
12.5	0.36699	0.11505	0.1212	0.113074	3.78E-05	3.9E-06
12.5	0.30008	0.14063	0.1212	0.113074	0.000378	0.000759
12.5	0.24424	0.08354	0.1212	0.113074	0.001418	0.000872
25	0.53647	0.30636	0.2387	0.251926	0.004578	0.002963
25	0.75833	0.2092	0.2387	0.251926	0.000871	0.001826
25	0.59663	0.24022	0.2387	0.251926	2.32E-06	0.000137
37.5	1.05806	0.39816	0.3562	0.358911	0.001761	0.001541
37.5	0.93206	0.35151	0.3562	0.358911	2.2E-05	5.48E-05
37.5	1.03612	0.32706	0.3562	0.358911	0.000849	0.001015
50	1.17639	0.51651	0.4737	0.46316	0.001833	0.002846
50	1.1198	0.41671	0.4737	0.46316	0.003248	0.002158
50	1.43382	0.45627	0.4737	0.46316	0.000304	4.75E-05
			Sum=	**0.015328**	**0.01423**	**0.001098**
			DF=	13	10	3
			Fexp.=	**0.26**	at 95% Confidence limit and degree of freedom 3, 10.	
			Fcrit.=	**3.71**		

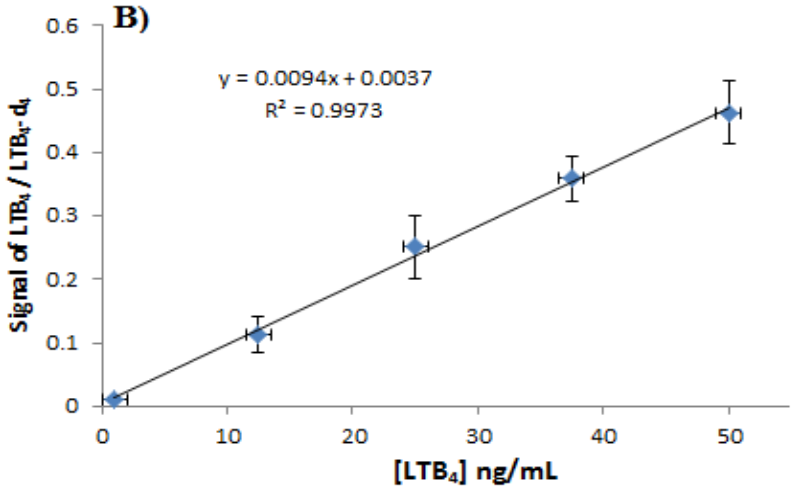

Fig 16. Linear regression graphs for LTB_4 using triplicate mean value and error bar.