Key prostanoid effectors of COX-2 in the embryonic development of the hen

Agenda of this chapter

This chapter aimed at the identification of one or more prostaglandin molecules, which acted during embryonic life, and led to the various defects due to inhibition of COX-2 by etoricoxib administration. This part of the thesis, first of all, describes how important the prostanoids are in the biological systems and the currently known pathologies related to prostanoid level modulation in the introduction. An elaborate idea of methods available to derive error-free quantities of prostanoids is provided as well. Later in this chapter, the methodology of liquid chromatography combined with mass spectroscopy is detailed for four major prostanoids, which are the focus of this particular study. The standardization steps of the LC-MS/MS method for embryonic tissues of chicks are described in-depth. These steps include the preparation of calibration curves for each prostanoid, standardization of sample preparation method for chick embryos aged day-2, recovery analysis of standards in the embryonic matrix, and quantification of prostanoids in the prepared samples. One specific prostanoid, which was found to be actively changing by the addition of etoricoxib, is revealed in the end.

INTRODUCTION

Prostaglandins and their precursor molecule, the 20-carbon fatty acid arachidonic acid (AA), play vital roles in the life functions. AA is usually derived from poultry and animal diet (Taber et al., 1998), or is produced from linoleic acid using a desaturase enzyme. Apart from being a source molecule of all the prostanoids, it serves as a crucial structural component of cell membranes. It allows the membrane to adjust its fluidity in changing temperatures (Tallima and Ridi, 2018). It is known to protect the cells and organelles from the oxidative stress created by Reactive Oxygen Species (ROS) outnumbering the anti-oxidants of the system. AA is much more than just a biological membrane's structural part. In the neuronal transmission, it acts as a calcium-dependent secondary messenger molecule as well (Vijayaraghavan et al., 1995).

AA possesses unique characteristics that facilitate its physiological functions. One such character is the presence of double bonds in its fatty acid chain. The double bond provides the scope of interaction with the free oxygen radicals, which help the resident tissue combating the oxidative stress (Brash, 2001). These four double bonds also enable this molecule to be acted upon by the phospholipases. On account of its amphipathic nature, AA fits in the lipid bilayer hanging its polar group in the aqueous head-region of lipid bilayer, and hydrophobic head (of AA) tucked in the row of other hydrophobic fatty tails of membrane (figure 24) (Beck et al., 2007).

The bound arachidonic acid is freed from such an arrangement by the phospholipase enzyme. The phospholipase enzymes – PLA_2, act on the ester bonds of phospholipids, specifically on sn2-ester bonds (Burke and Denis, 2008). PLA_2 is a superfamily of the enzymes, which derived the name because they all act on the ester bonds of *phospholipids* only. The one involved in prostanoid biosynthesis is cytosolic PLA_2. Freeing of the membrane AA is the rate-limiting step of prostanoid synthesis. The free AA molecules are further acted upon by COX-1, lipooxygenase (LOX), or COX-2. The action of LOX transforms the free AA into hydroperoxieicosatetranoic acid, more commonly known as HPETE (Palumbo, 2017). Multiple types of HPETE function for activation of leukocytes, degranulation of neutrophils, contraction of smooth muscles of the respiratory system, and endothelial muscles of the GI tract (Stanley, 2005). Although, this part of the AA pathway is not the interest of this research. The COX enzymes carry out the bis-oxygenation reaction to derive PGG_2 from AA. The next is the transformation reaction, wherein two electrons are lost from PGG_2 with the help of the peroxidase enzyme. The product of peroxidases, the PGH_2, is extremely short-lived. It survives for 90-100 seconds in the cell, and before it accumulates, it is converted to one or more prostanoids via the action of tissue-specific synthases (Smith et al., 2011).

Prostanoid Synthases

The prostanoids work in autocrine and paracrine fashion rather than endocrine. Their production is, therefore, site-specific. Their short half-lives do not allow them to be functioning for a long time once produced. The tissue-level synthases are the enzymes that mediate their synthesis from relatively long-lived free AA entities. They catalyze the step using up PGH_2 as the substrate, producing the particular prostanoid. The tissue which

requires this catalysis step has structurally unique synthase for a particular PG type (figure 25). PGD_2, for example, is produced in central nervous tissues, lymphocytes, mast cells, gonads, as well as in cardiac tissue during the post-embryonic life of mammals (Rowley et al., 2005).

The tissues of gonads, cardiac regions, and central nervous systems contain the same PGD synthase, which contains identical three-dimensional anti-parallel structure containing eight beta-pleated sheets (Urade and Eguchi, 2002; Flower et al., 1993). Such a structure is known as lipocalin. Therefore, this type of PGDS is named as lipocalin-type PGDS (L-PGDS). Other tissues such as hematopoietic cells, mast cells, as well as most tissue types in vertebrate class, possess the PGDS structurally belonging to glutathione S-transferase (GST) family (Rowley et al., 2005). PGE synthases principally are of two types – cytosolic and membrane-associated forms. Further, the membrane-associated form was later found to occur in two forms, named as mPGES-1 and -2. The stimulators of these PGES are different. Both COX-1 and COX-2 are linked with the activity of mPGES-2, while COX-2 regulators stimulate mPGES-1 along with COX-2 (Murakami et al., 2003). TXA_2 is produced with the help of thromboxane A-synthase, which is a member of the cytochrome P_{450} family (Ullrich et al., 2001). More functional and structural details about prostanoids are described in the following section.

Prostanoids

Prostanoids derive the name from the tissue they were first identified from, i.e., the prostate gland (Euler, 1934). They belong to the oxylipin family of lipids. The prostanoids are short-lived autacoids in the biological systems. They are structurally closely related to each other, and almost all of them are inter-convertible by a one-step reaction. They are released in very small quantities (picograms) when they are required. Their basal level is maintained, however, for housekeeping functions such as the protection of stomach and internal intestinal linings from varied pH of secreted fluids. The two groups of prostanoids are – prostacyclopentanes and thromboxanes. Thromboxanes are named based on their function of clotting – thrombosis.

PGE_2

Initially known as the inflammatory metabolite PGE_2, is now known to mediate numerous physiological processes. It is also found to act anti-inflammatory for the function of neuroprotection in the microglial cells (Riccioti and FitzGerald, 2011). The most important and commonly known function of this metabolite is to protect the internal lining of the intestines and stomach from the acidic pH of the secreted fluids. Externally provided PGE_2 can increase the cell division of hematopoietic stem cells *in-vitro* (Goessling et al., 2011). However, increasing the concentration of PGE_2 in human tendon cells culture can result in dual effects depending upon the added concentration. More than one ng/mL of it could arrest cell division, while lower than that enhanced proliferation of the same cell type (Zhang and Wang, 2014). Cardiomyocytes grow in numbers by the addition of PGE_2 *in-vitro* (Hsueh et al., 2014). In this manner, PGE_2 possesses contrasting roles as per the requirement of its tissue-level niche at a certain timepoint. Its half-life in biological systems is approximately 15-30 seconds (Bygdeman, 2003; Gomez et al., 2013). Some PGs are so labile that their inactive forms or derivatives are quantified as indirect measures of their presence in the cells or tissues. Detection or quantification of PGE_2 is, however, done with the help of ELISA technique or LC-MS/MS, both of which have been used to detect the PGE_2 molecule rather than any of its derivative or inactive forms. Naturally, occurring PGE_2 is also known as dinoprostone and is stable in methanol solution (Srivastava, 1973). Structurally this molecule contains 20 carbons, from which five carbons are involved in the formation of cyclopentane ring. This ring is a characteristic feature of prostacyclopentanes.

TXA_2

Initially described as a platelet-derived factor, TXA_2 is now known to be secreted by several cell types such as neutrophils, macrophages, and endothelial cells. Chemically it resembles PGI_2 more than any other prostaglandin. Functionally, PGI_2, and TXA_2 show opposite physiological effects (Cathcart et al., 2010). It is responsible for platelet aggregation as well as arterial constriction in biological systems (Rucker and Dharmoon, 2020). As the name suggests, its primary function is to stimulate thrombosis via activation of platelets in the case of tissue injury (FitzGerald, 1991).

TXA_2 is now found to be enhancing platelet activation and aggregation in the tumor niche (Mezouar et al., 2016). It is also involved in a cardiorespiratory feedback mechanism,

wherein its role is to cause pulmonary hypertension in response to the reflexes as well as rapid chest breathing leading to hypoventilation. It causes this response via the excitation of autonomic C-fibres, which are associated with the several vagal reflexes (Jang et al., 2020). TXA_2 is also associated with bronchial, intestinal, uterine, and urinary bladder smooth muscles contraction. It can increase contractions via binding to its receptor leading to calcium influx (Rucker and Dhamoon, 2020). Finally, this compound is also known to affect the blood flow in kidneys, thereby it can modulate the concentration of urine (Spurney et al., 1992). Owing to its role in numerous pathophysiological conditions, quantification of TXA_2 is important. However, it is rapidly hydrolyzed to form TXB_2 in tissues and fluids. Therefore, the quantification of TXA_2 is usually done by measuring TXB_2 as an indirect measure of TXA_2 levels (Wang et al., 2001).

PGF2α

$PGF_{2\alpha}$ is known for its involvement in uterine muscle contraction and vasoconstriction. Now it has been found that bone remodeling is not accomplished without the presence of $PGF_{2\alpha}$. It mediates the process of osteoclastogenesis by affecting the phosphorylation of Janus kinase 2 (JAK2) while elevating the synthesis of IL-6 (Visconti et al., 2019). Other than that, $PGF_{2\alpha}$ has been associated with cell proliferation and death pathways. In osteoclasts, it activates the cell survival pathway. On the other hand, it promotes cell death of luteal cells (Visconti et al., 2019; Joncyzk et al., 2020). Pregnancy establishment is not accomplished without the presence of this prostanoid, wherein it functions to induce vascularization on the endometrium. It assists the tissue remodelling near endometrium in the early pregnancy of mammals (Kaczynski et al., 2016).

PGI_2

PGI_2 is known for its contradictory behavior to TXA_2 in tissues. It dilates blood vessels, while TXA_2 constricts them. The balance between these two prostanoids brings about the proper function of coagulation (Haldane, 2015). It is usually produced in smooth muscle cells of blood vessels and endothelium. Other than vasodilation, it affects muscle growth by decreasing their proliferation. It is extremely unstable like TXA_2 and is quantified using its stable metabolite 6-keto-PGF_1 or 2,3-dinor-6-keto-PGF_1 based on the tissue or fluid to look for it (Rosenkranz et al., 1981).

There are numerous other prostanoids, as shown in figure 26, which are not being focussed upon for the current research. The prostanoids are, by definition, the molecules synthesized with the help of specific enzymes, differing from isoprostanes – derived by non-enzymatic methods in cells. The prostanoids show stereospecificity and are chiral at every functional group that they possess (Christie, 2020). Collectively all these prostanoids are named by fundamental structural differences, as shown in figure 26 (derived from Christie, 2020).

Other than prostanoids are the isoprostanes and prostanoid derivatives performing several physiological functions. PGJ_2, for instance, has recently been recognized as a pro-inflammatory derivative of PGD_2 (figure 25; Morgenstern et al., 2018). Isoprostanes, as the name suggests, are the isomers of the PGs. These prostanoids are derived from the described main prostanoids, namely PGE_2, PGD_2, $PGF_{2\alpha}$, and TXA_2, which are therefore called the parent eicosanoids. However, the reverse reaction is also proved by scientists, wherein the isoprostanes mediate the so-called parent prostanoids production via the triple peroxidation pathway independent of COX enzymes (Gao et al., 2003, Musiek et al., 2005). Isoprostanes structurally differ from prostanoids by the possession of a *cis* side chain rather than *trans* of PGs in relation to the prostane ring (Morrow and Roberts, 1997). They are lately suggested as the producers of nitrite in response to lipopolysaccharides (LPS) (Musiek et al., 2005). They are recognized as the indicators of human health as being used to identify various pathological conditions (Cracowski et al., 2002). Some isoprostanes are frequently monitored to understand the state of hepatic injury, renal injury, cardiovascular diseases, persistent gastrointestinal infections, systemic, and inflammatory diseases (Morrow and Roberts, 1997; Cracowski et al., 2002). Some diseases are also known to arise from abnormal concentrations of arachidonic acid, and/or prostanoids.

Prostanoids in pathobiology

The oxidative stress arising as a side-effect of several pathological conditions can stimulate the synthesis of isoprostanes rather than parent prostanoids. Such an effect may, therefore, lead to a decrease in the normal prostanoid levels and loss of prostanoid-led functions. The consecutive diseased conditions arising due to modulation in prostanoid levels are hypothesized since the initial studies of prostanoid functions (Rocha and Carvalho, 2005). The most important and well-known function of prostanoids is inflammation. The

overabundance of prostanoids causes excessive inflammation, which hampers the healing process at the site of injury. In a condition of chronic obstructive pulmonary disease (COPD), surplus PGs contribute to a heightened amount of inflammation and hampers remodeling and regrowth of alveolar tissue post-injury (Wang et al., 2018). Such a condition leads to emphysema. Earlier, PGE_2 and its analogs were being used as bronchodilators for asthmatic patients. However, later it was realized that it produced excessive cough in the patients via case studies. Studies later revealed the mechanism of action behind cough production, which was related to the EP3 receptor of PGE_2, while that of bronchodilation was via the EP4 receptor (Zaslona and Peters-Golden, 2015). The most recently known roles of PGE_2 include its immunosuppressive activity in tumorigenesis. It has been found that it contributes to the risk of GI cancer by providing immunosuppressive niche to the GI cells to promote proliferation (Wang and DuBois, 2018).

Skin diseases like psoriasis and dermatitis are also mediated via the production of TX and PGE_2, respectively (Honda and Kabashima, 2019). The synthesis of PGs was thought to be unique to the vertebrates. However, the process is now being traced in the parasitic protists such as *Entamoeba histolytica* and *Trypanosoma sp.* possess the enzymes for the production of PGs, exactly in the manner that their host mammals synthesize them (Eida, 2015). Some nematode parasites produce PGs via the glutathione S-transferase pathway. Ticks and leeches contain anticoagulant PGs, which also help increasing blood flow and suppressing immune response at the site of feeding on the host (Salzet, 2001; Eida, 2015).

Looking at the research already done, and the results, PGs keep feeding the scientific community with enough curiosity to ponder upon their novel functions. The functional details are limited without digging into the concentrations of the same molecules. Quantification of PGs has created a whole new field of research owing to the similarities and short-lived nature.

Current Techniques for Prostanoid Quantification

On account of their functional importance in a wide variety of homeostatic processes, as well as pathological conditions, prostanoids are required to be quantified often. However, the similarities that the prostanoids share with each other as well as with isoprostanes upsurge the challenges on the way of its accurate quantitation. On top of that, the extremely short half-lives of prostanoids increase the level of challenge for sample preparation steps of any

proposed technique. Immunological assay kits are readily available for the quantification of prostanoids, especially when the focus is only one or a few of the prostanoids and not isoprostanes. These kits notably have several demerits, making it practically futile for the tissue samples. First, isolation of prostanoid from tissue homogenate requires special chromatographic columns and instruments, which is less common in laboratories. The commercially available facilities are expensive for researchers. Secondly, the analysis of outsourced sample results generated by a different group of workers is often complicated to carry out. If all these difficulties are overcome, the kits themselves cost more for each of the prostanoids. Additionally, the amount of sample (small volumes derived from tissue homogenates) is often a limiting factor for assaying more than one prostanoids by different kits (Schmidt et al., 2005).

The kit-based methods work best for serum or urine samples of humans. Measuring prostanoids with LC-MS/MS requires altogether a different approach for the whole methodology starting from sample preparation to the standardization of quantitation method, and the analysis of particular PG from numerous mass peaks arising from tissue homogenate. In this chapter, an attempt was made to standardize the technique for four major prostanoids, namely PGE_2, PGD_2, $PGF_{2\alpha}$, and TXB_2 (as an indirect measure of TXA_2). One or more of these prostanoids were hypothesized to be decreasing in the case of etoricoxib added embryos as compared to the control embryos.

LC-MS/MS technique

Ultraperformance liquid chromatography (UPLC) is one of those methods, which use the mixture of solvents coupled with high pressure pumps to resolve the test compounds along the column. UPLC, coupled with mass spectrometers, allows the synergistic enhancement in the individual capabilities of these techniques for identification and quantification of the chemicals. The mass spectrometry is nothing but an analyzer, used to identify the compound using its molecular weight. Coupling of the LC system with MS was possible only after the electrospray ionization (ESI) was discovered (Fenn et al., 1989). ESI is the ion source that can spray ions even under the continuous flow of liquid and provide the compounds with a charge. The produced ions and the fragments are then identified by the MS based on the mass to charge ratio (*m/z*) unique for each of the chemical compounds. The chemical compound under study in the LC-MS/MS is commonly referred to as an analyte. Figure 27 shows the

coupling of LC with MS instruments, and figure 28 depicts the whole process of LC-MS/MS along with its components.

Usually, the biological mixtures are derived from tissue samples from animals or plants, or the body fluids like serum, plasma, urine, lymph, or blood. The method aims to separate the analyte/s of interest from this kind of mixed solution using the LC method first. The separated components are then identified and quantified using tandem spectrometry for the analyte/s of interest, which requires knowledge of the structure, solubility, and affinity characteristics for each of them before conducting standardization. The described methodology here is constrained to the details for an LC-MS system consisting of Agilent 1290 series UHPLC (Agilent, USA) coupled to a Sciex QTRAP® 5500 mass spectrometer (Sciex, USA), controlled by Analyst® 1.6.2 LC-MS software.

Standardization includes several steps. First, each of the analytes of interest is standardized individually at a higher concentration than usually observed in the biological system. Herein, the individual analyte is characterized for its elution time, ionization mode, and fragmentation pattern under the varied Collision energy (CE) and Declustering potential (DP). The optimum CE and DP for each analyte is saved as a methodology file in the software. Second, that analyte is prepared in a range of concentrations, which are referred to as the calibrants. The range is decided based upon the concentrations commonly observed in biological systems of interest (blood/ urine/ serum/ lymph/ tissue of particular type). The calibrants are mixed in a single solution before adding in the chromatographic column, wherein various parameters are set in separate runs for the same mixture. These parameters include column conditions – its temperature, mobile phase solvent ratios, and solvent gradient profiles. The instrument parameters such as the gas pressure, temperature (TEM), ion spray voltage, entrance potential (EP), and cell exit potential (CXP) are also checked for deriving their optima. Here upon, the parameters yielding required separation of the calibrants are noted, as well as saved in the software as a methodology file, which can be used for the future experiments of similar analyte mixtures and/or samples. The added concentration and calculated concentration derived after the separation in the column, are compared, and the standard graph is generated for each analyte. This standard graph is saved and utilized later, whereas the samples are run using the same methodology derived above, to interpolate the unknown concentrations of analytes in samples. Fourth, some of the samples (raw, freshly isolated) are added with spiking concentrations of the same calibrants before the preparation of samples (homogenate and further procedural steps). This is called a recovery group, which

analyses the manual errors during sample preparation and handling by deriving the percentage recovery with the following formula. Recovery closer to 100% is the measure of optimum handling, purity of reagents, and right procedure.

$$\%\ Recovery = \frac{Calculated\ \ concentration\ \ for\ recovery\ \ group\ - \ calculated\ \ concentration\ \ of\ the\ sample\ \ used\ for\ reovery\ \ group}{Spiked\ concentration}\ x\ 100$$

The majority of analytes present in any biological sample mixtures are separated using this methodology, wherein MS parameters play the main role in identifying the peak of the analyte of interest from hundreds or thousands of peaks of various molecules, usually visible in the LC data. Structurally unique molecules can be easily isolated in chromatography. For instance, the study of uremic toxins focusses on the analytes like dopamine, hydroxyproline, p-cresol, and neopterin. All these are structurally unique and thus get well-resolved in the chromatographic column. The generated peaks post-separation can be identified by their unique *m/z* ratio and fragmentation pattern. An exceptional condition where two or more structurally similar compounds fragment in the same pattern, and generate same *m/z*, can still be identified and quantified from their mixture by modifying the chromatographic parameters to achieve their fine temporal separation. This is sometimes achieved by finding the unique fragments of each of these compounds by modifying the multiple reaction monitoring (MRM) parameters, or by selecting different columns for each of the analytes of the mixture. Due to its faster functioning, LC-MS/MS is often performed for identifying hundreds of analytes from a little volume of sample mix. In such a case, selecting different columns for a different analyte can be time- as well as sample-consuming. The best method thus is to vary the methodology for different analytes until the unique fragmentation is achieved. Another approach is to resolve such analytes on the same chromatographic column with the help of solvent gradient variation.

LC-MS/MS for prostanoids: Challenges and derived solutions.

Quantification of prostanoids has always been a challenge as they act at extremely low concentrations in tissues and fluids in the organisms. It has now been easier to do so with the development in the field of immunetechnology due to the availability of several kit-based assays for quantitation of each of the prostanoids. These kits, apart from being expensive,

give high variances at lower concentration ranges. It is crucial to decrease the variance for their quantities since the type of activity they perform changes with a little change in concentration. Kit-based methods are not apt for tissue samples due to higher noise (background) and loss of prostanoid in the attempt of filtration. Greater cross-reactivity with isoprostanes is also one of the challenges in kit-based assays (Schmidt et al., 2005).

The LC-MS/MS system for prostanoids has different challenges other than the variance of quantitation results. The chromatographic column and mass analyzer work based on the differences in the structures of analytes, whereas the prostanoids are very similar to each other in that manner. PGE_2, PGD_2, $PGF_{2\alpha}$, and TXB_2 – are all 20-carbon long molecules. Structurally, they vary in the position of a double bond, number of hydrogen atoms, and three-dimensional planar arrangement of oxygen. The structural differences can be observed in the table 13. All of these prostanoids absorb electrons and ionize in a negative mode.

Other than the challenges arising from structural similarities of prostanoids, additional hindrances to quantification arise from the isoprostanes. The isoprostanes occur naturally from arachidonic acid via a free radical pathway separate from conventional PG production. Isoprostanes of F series were discovered first in 1990 (Morrow et al., 1990). They show close structural similarities to either of the PGs and are accordingly classified in series. For example, the isoprostanes similar to PGE_2 belong to the E series of isoprostanes, and those similar to PGD_2 belong to the D series. Synthesis of isoprostanes does not require the involvement of enzymes. They are bioactive molecules, continuously present in all the tissue forms at basal levels. However, their levels increase in some pathological conditions (Bauer et al., 2015). For example, the addition of CCl_4 to the rats with hepatic failure causes a hundred-fold increase in the level of isoprostanes in the liver (Morrow et al., 1992). They are found to elevate the disease symptoms in multiple sclerosis as well (Sbardella et al., 2013). Isoprostanes have now been qualified as diagnostic tools for cardiovascular, hepatic, neurodegenerative, and metabolic disorders (Davies et al., 2011; Sbardella et al., 2013; Dhama et al., 2019). Isoprostanes of series F_2 are used the most, as they are metabolically more stable in tissues as well as body fluids (Bauer et al., 2014). Owing to their structural similarities, the isoprostanes fragment in the similar patterns to their respective prostanoid of the same series. One such isoprostane, 8-epi-$PGF_{2\alpha}$, for example, has the same molecular weight (354) as that of $PGF_{2\alpha}$. Both these molecules show similar fragmentation spectra while LC-MS/MS technique is used (Kuligowski et al., 2014). Identification of the exact

quantities of prostanoids becomes tedious because closely isolated and similarly fragmented isoprostanes even migrate at similar rates through the LC column in the effect of the same set of LC parameters. Some of these challenges have generated a new field of research on methodology development for prostanoid LC-MS/MS. This chapter deals with the standardization of LC-MS/MS of the main prostanoids, namely PGD_2, PGE_2, $PGF_{2\alpha}$, and TXB_2. All these prostanoids are short-lived, and co-elutes with their respective isoprostanes. However, earlier studies throw some light on the solvents to be used for chromatography of these. The optimum separation of peaks and omitting co-elution are the major challenges while working with these compounds.

RESULTS

In the current chapter, the standardization of methodology served as results as well. Quantification of prostanoids was performed using several steps of standardization and optimization procedures. First of all, isolation of prostanoids from chick embryos aged day-2 was carried out by trying a number of methods published in the literature already. The optimum solvent concentration, along with temporal parameters of sample preparation, were found for chick embryos. After this, the calibrants were prepared for calibration of each prostanoid. The concentration range for calibration was decided based on the literature survey, as discussed in detail in the following sections. The standard graphs were used to perform recovery analysis by spiking the prostanoids mixed with embryonic homogenate. Finally, the quantification was done using a standardized methodology for the prostanoids mixture.

Standardization of methodology for isolation of prostanoids

Prostanoid isolation from adult rat brain and spinal cord tissue samples is standardized earlier (Gandhi et al., 2007; Brose et al., 2013). Other methods deal with the plasma, cerebrospinal fluid, and urine samples used for PG isolation and quantification of one or two PGs at a time (Griffin et al., 1994; Almer et al., 2002; Kuligowski et al., 2014). However, isolation of prostanoids from the embryonic tissue needs a different approach, as this tissue has a differently composed matrix than the adult brain. The embryos lack hard connective and

interstitial tissues. The majority of early embryonic cells are multipotent in nature, unlike the adult tissues wherein they are unipotent. The early embryonic cells possess more fluid cell membranes than most of the adult cells (Matsuzaki et al., 2018). Therefore, the procedures available for isolation of any macromolecules from the embryonic tissues demands development of new protocols, using different concentrations of solvents and membrane-breaking agents, varying incubation times, and completely different sample handling owing to more labile nature of cells of embryos.

Here, we developed a methodology for the isolation of prostanoids from early chick embryos. These embryos were collected at HH 12/13 (48 hours incubated) stage using the filter ring method (Chapman et al., 2001). The stage selection was based on the activity status and protein level of COX-2 during the overall embryogenesis of chick (Figure 6, 14, and 17 – Chapter-3). COX-2 activity, as well as protein, was highest on day 1, followed by day 2, which showed a decreasing trend later on till day 10. The embryos incubated for 24 hours (HH 6/7) are too tiny to make up for sufficient sample for the assay hence preferred HH 12/13. Thirty embryos per group (control and experimental) were collected in cold methanol solution. The ratio of tissue to methanol was decided after several attempts to obtain the optimum quantity of prostanoids (not too diluted or concentrated, as per the calibrant range) in the sample. Finally, in an mL of methanol per gram of tissue, embryos were homogenized using pre-chilled mortar-pestle. The methanol in each vial was pre-added with internal standard (ISTD) $PGF_{2\alpha}$-d4[#] (10007275, Cayman Chemicals, USA) at an ultimate concentration of 10 ppb. Internal standard (ISTD) $PGF_{2\alpha}$-d4 was purchased as 200 µg/100 µL solution in methanol. Vials were vortexed briefly so that the embryos, methanol, and ISTD get properly mixed in the tube. The samples were then ultrasonicated at room temperature for 5 minutes. Samples were removed from the sonicator and shaken gently. This mixture was centrifuged at 10000 *g* at 4 °C for 15 minutes to yield the prostaglandin-bearing supernatants. The pellet was discarded, and the supernatant was stored at -80 °C for further analysis.

[#]*Significance of using ISTD was to check the extent of loss of prostanoids present in samples, during the sample preparation or LC and MS runs. To understand the kind of degradation the assay compounds (prostanoids in this case) are susceptible to (during sample preparation), we ought to use the ISTD, which has a similar structure to the assay compounds. While assaying more than a single type of compounds in the same solution, like for example, while assaying uremic toxins in urine samples, one needs to add several ISTDs resembling different structural groups of assay compounds, before the sample preparation step itself.* $PGF_{2\alpha}$*-d4 is structurally similar to all the prostanoids used for this assay. Therefore, it was used as a common ISTD for quantification of all the prostanoids here.*

MRM Parameters

Individual solutions of standard pure $PGF_{2\alpha}$, TXB_2, PGD_2, and PGE_2 were added one by one to the LC-MS instrument consisting of Agilent 1290 series UHPLC (Agilent Technologies, USA) coupled to a SCIEX QTRAP® 5500 mass spectrometer (SCIEX, USA). The stock solution of each analytical standard was prepared at a concentration of 1 mg/mL. Since the analytical standards were available as 1 mg vial, they were dissolved in 500 µL methanol in the respective vials, transferred quantitatively into new amber vials, and the difference in weight was calculated to determine the actual amount of each standard present. Weight measurements were done on a CPA225D semi-micro-electronic balance (Sartorius, USA). Finally, excess methanol was added to make up the final concentration to 1 mg/mL. These stock solutions were used to determine unique MRM (Multiple reaction monitoring) parameters for each prostanoid.

The molecular weight of $PGF_{2\alpha}$ is 354 grams. In negative ionization mode, it generated a peak of molecular mass 353 (isotopic peak) and 309 daltons (Da) (table 14). Optimal declustering potential (DP) in this case was -60 mV, while the peak showed its best mass to charge ratio (m/z) when this DP value was combined with -24 collision energy (CE).

TXB_2 has a molecular weight of 370 grams which is different from $PGF_{2\alpha}$, PGD_2, and PGE_2 and in this study it showed and isotopic peak of 369 Da under the negative ionization mode. The fragmentation of this analyte gave the principal peak at 169 Da. Standardized DP and CE were -70 and -22, respectively.

PGD_2 and PGE_2 possess the same molecular weight, i.e., 352 grams/mol. Owing to their remarkably similar structure, both of them showed a fragment of 271 Da, which was used as Q3 for their identification. However, the isotopic peak of PGD_2 varied by 0.1 Da as being 351.1 when compared to 351 of PGE_2. Fragment 271 Da was optimally derived with -60 mV DP, as compared to -70 of PGE_2. The value of optimum CE was -22 in both cases.

The molecular weight of $PGF_{2\alpha}$-d4 is 358, which showed an isotopic peak at 357 and another major peak at 313 Da. Other than prostanoids and ISTD, arachidonic acid (AA) was also checked for its presence and comparative intensity in control and treated samples. AA showed an isotopic peak at 303 (molecular weight 304) and another major peak at 205 Da. PGI_2 is not described in quantitation as it tends to be lost rapidly from the sample. Optimization of PGI_2 was done using its standard and is therefore described in table 14. It did

not appear in the embryos, which could be due to its instability or due to its absence, which cannot be commented upon at this stage.

Overall, all the PGs showed higher ionization efficiency (IE) in negative ionization mode (ESI-) than in positive mode (ESI+), which means all of these prostanoids generate deprotonated analytes easily. All the prostanoid showed quite a close m/z ratio as all of them possess quite close molecular weights, except for TXB_2. For PGD_2 and PGE_2, MRM parameters coincided with each-other. In such a case, identification of these molecules from the mixture (as performed in the next sections) was completely dependent upon LC profiles, thus making optimum separation of peaks crucial.

Chromatographic Separation

Once MRM parameters were determined, the calibrant stock was prepared by mixing all the prostanoids' standard stock solutions. The four standards were combined in equal ratios together to have a working mixture of concentration 250 ppb. An Agilent UHPLC system equipped with a Zorbax Eclipse XDB C18 analytical reverse-phase column (3.0 mm x 100 mm, 3.5 µm particle size, 80 Å pore size), connected to a Phenomenex (Torrance) SecurityGuard C18 pre-column (4.0 mm x 3.0 mm; Phenomenex, USA) was used for the chromatographic separation. The ISTD was added to each calibrant at the concentration of 10 ppb. The mass spectrometric analyses were performed in the negative ionization and MRM modes. Curtain gas pressure was set to 30 psi, whereas collision associated dissociation (CAD) was set to medium and he instrument temperature was adjusted to 500 °C. Other parameters were – ion source gas 1 (GS1): 50, ion source gas 2 (GS2): 50, ion spray voltage -4500 V. The Entrance Potential (EP), and Cell Exit Potential (CXP) were set at -10 V and -15 V respectively. Collision energy (CE) and declustering potential (DP) for each analyte were optimized by manual tuning. The CE and DP for the ISTD were the same as those for $PGF_{2\alpha}$. The compound dependent MS parameters were used, as outlined in table 14.

Reverse-phase chromatography was performed for separation of these prostanoids using 0.1 % acetic acid in water as mobile phase A, along with acetonitrile and water mixture (90:10) as mobile phase B. The gradient profile for the run time of about 11.6 minutes was set as described in table 15, at the same time the flow rate was set at 0.3 mL per minute. Injection volume was 10 µL in a column, which was maintained at 40 °C column

temperature. The instrument parameters and MRM were identical to what is abovementioned under the heading of Calibration curves. The method successfully resolved all the PGs used for this study (figure 29). In the initial runs, even the PGI_2 peak was visible and separated from other PGs in the chromatogram (figure 29B). Since the MRM parameters for PGD_2 and PGE_2 were the same, their peaks looked overlapping in the chromatogram (grey and green peaks in figure 29A). This happened because the software recognized the initially eluting compound as well as a later eluting compound, both as PGD_2 and PGE_2 giving both of them the same color codes. However, to solve the query of which peak to consider for quantification of either of these analytes, the standard solutions of 1 mg/mL PGs were run through LC-MS/MS individually. The peaks obtained are shown in figure 29C, along with the comparison of them with the complete chromatogram when the mixture of analytes was run (figure 29C).

This successful methodology was saved in the instrument connected software and was later used for running the samples after obtaining calibration curves for each prostanoid of analyte mixture.

Calibration Curves

Seven-point calibration curves for each analyte were obtained by serial dilution of the working solution mixture in methanol, resulting in the range 0.3 – 25 ppb. Specifically, the concentrations of the calibrants were 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0.313 ppb. Calibration curves were obtained by plotting the ratio of peak areas of the analyte to that of ISTD response versus the calibrant concentration. Quadratic regression was used to fit the response by using 1/x weighting for all the analytes being quantified.

The standardized parameters of LC and MS worked fine for $PGF_{2\alpha}$ for concentrations between 0.78 and 25 ppb. The calculated concentrations showed close to 100 % accuracy for all calibrants except for the lowest measured calibrant 0.313. The calculated concentration was found to be 0.218, which was about 70 % accurate (table 16A). This concentration was probably too low for quantification using the current method of LC-MS/MS. The notable reduction of areas of peaks can be observed in figure 30A. The quantification was done as compared to the respective ISTD peak for each calibrant. The regression analysis for all the obtained values from 0.3 to 25 ppb yielded 0.9991 r value (figure 31A).

This particular methodology also yielded accurate results for the TXB_2 analyte (figure 30B). Using all the calibrants that were run, the r-value for the regression curve was found to be 0.998 in the quadratic graph (figure 31B). The calibration attained close to 100 % accuracy in the evaluation of the concentration of all the calibrants (table 16B). The less accurate concentrations of the highest and lowest calibrants were supposedly due to the tailing pattern of its LC profile. Attaining a better single peak by modifying LC parameters could eliminate such variations. However, this method gave appropriate results for the range of concentrations a chick embryo possessed, as found out later by quantification.

Calibration of PGD_2 in the mixture of analytes (calibrant solutions) using the standardized method, resulted in 0.9981 r value (table 16C). It was found to be accurate for the range between 0.3 to 25 ppb, as the intensity of the area covered under its peak reduced with its decreasing concentration in calibrants (figure 30C). The area of peaks was selected based on the retention time of this compound in the LC column, which was identified in the LC of its separate solution (figure 29C). The quadratic regression curve was generated by incorporating the values of all the run calibrants (figure 31C).

The current methodology worked well for PGE_2 up to a certain extent. The calibrant, containing 25 ppb PGE_2 did not sync with other parts of data and on addition caused r-value to decrease. Therefore, it was omitted for the result part of this methodology, considering it an outlier for this data. The embryonic samples contained PGE_2 within the range of this calibration curve, as discovered later during quantification. The correct peak of PGE_2 was found to be the one with smaller retention time than PGD_2. This means that PGE_2 eluted earlier than PGD_2 from the column. Therefore, the correct peak was selected during method development from the calibrants showing a decreasing trend with a reduction in added concentration (figure 30D). The quadratic regression curve for this analyte is shown in figure 31D. The exact added versus calculated concentrations are mentioned in table 16D.

It was found that ISTD peaks showed similar areas as the quantity of ISTD was the same in all the calibrants (figure 30E). The same areas of ISTD in all these samples are also a measure of optimum pipetting technique, which helped to eliminate the errors in the added concentration of ISTD in all the samples. This result concludes the absence of erroneous pipetting of all the analytes in the calibrant solutions. In such a case, the accuracy deviation of an analyte in a particular calibrant solution proves the need for further standardization of that concentration of the specific analyte. For example, 0.3 ppb $PGF_{2\alpha}$, as added in calibrant

1, resulted in 70 % accurate quantification, which could be further standardized with protocol modification if one is interested in that much low concentration of $PGF_{2\alpha}$.

Quantification

The analytes in embryonic samples were quantified using the method derived so far and used for the calibrants. The samples were added to the wells in triplicates, while the other wells were filled with the calibrants again. The result was analyzed statistically with the help of Student's t-test (GraphPad PRISM Software Inc., USA).

Quantification revealed that control embryos and experimental embryos had an identical concentration of most PGs such as $PGF_{2\alpha}$, TXB_2, and PGD_2. Furthermore, the concentration of PGE_2 dropped significantly in the experimental embryos than control embryos (table 17, figure 32). The results suggest that the drop in COX-2 activity due to the addition of etoricoxib at day-2 caused PGE_2 reduction among four major prostanoids. Therefore, PGE_2 is construed as the embryonic effector of COX-2 enzyme.

Analytical Method Validation (Recovery Analysis)

Recovery analysis is done to understand the extent to which the loss of analyte that might have occurred in performing the newly developed method. Each analyte under the study has to be added to the samples prior to LC-MS/MS, inside the wells already containing samples in a well plate. These concentrations are also added without the samples (only the analyte solution) in individual wells. The extent to which a spiked concentration of the analyte is recovered from the sample plus analyte well as compared to the analyte well depicts the goodness of the method for the used sample type. That indirectly means that the concentration of unknowns, as derived from the quadratic regression graph generated by new method validation, is accurate for the unknown samples, as it stood accurate for the known spiked concentrations. Practically, the spiked concentration (for example 15 ppb PGE_2) added in a sample containing some concentration of the same analyte already (for example 2 ppb PGE_2), along with the mixture of other analytes (for example $PGF_{2\alpha}$, TXB_2, and PGD_2), should be able to calculate the PGE_2 concentration as a total of spiked and basal value (17 ppb PGE_2) when running through the LC-MS/MS system using the standardized parameters. In short, the recovery analysis proves that the separation of studied analytes is optimum for

the calculation of realistic concentrations, thereby proving the LC parameters are efficient for the set of analytes. It also suggests that the method development (by manual or automatic peak selection mode, whichever was used) using calibrant mixtures and their concentration values is particularly working well (particularly) for the calibration range used for each analyte.

In the current study, 3 and 15 ppb concentrations of each analyte were spiked in two different sample matrices, namely day-2 control and day-2 experimental embryos. The recovery of each analyte in these matrices is shown in table 18. $PGF_{2\alpha}$ could not be recovered properly from the control embryo matrix, leading to over estimation of value. In that case, the value was over-estimated. However, it worked fine for other samples.

PGE_2 ELISA Assay

The LC-MS/MS conclusively pointed out that PGE_2 is the embryonic effector of COX-2, which was confirmed using PGE_2 assay in the chick embryos of day-1 to day-10. The results showed that only except for two stages – day-6 and day-9, PGE_2 levels dropped in treated embryos as compared to control ones (figure 33).

DISCUSSION

Lipids are essential energy producers and energy stores for organisms. In humans, hormonal production depends on lipids (Welte and Gould, 2017). Prostaglandins are small lipid molecules present in all Vertebrates in the lower concentrations of nanograms (Jeffrey and Aspden, 2007). They perform some homeostatic functions like maintenance of gastrointestinal integrity and blood pressure, as well as some inducible functions such as inflammatory response (pain, edema & fever), regeneration, myogenesis, cellular differentiation, etc (Simmons et al., 2004). Lately, these molecules are studied more for their involvement in disease pathology of cancer, neurodegeneration, and osteoarthritis (Sellers et al., 2010). Despite their vast known roles in the adult life of vertebrates, very few studies have focussed on their levels and roles in embryogenesis. Humans, their pets, domestic and captive animals are daily consumers of pain killers of NSAID (non-steroidal anti-inflammatory drugs) class, which suppresses the synthesis of these prostaglandins. Understanding the concentrations and roles of prostaglandins in the embryonic state can

predict a plausible negative outcome of the consumption of NSAIDs in the pregnancy stage by organisms.

Quantification of prostanoids has been a topic of research for numerous analytical chemists across the world. Owing to their striking similarities in structure and fragmentation pattern, the prostanoids are challenging to quantify accurately from the tissue samples supposedly containing several isoprostanes and prostanoid isomers as well. On top of that, they are present in little concentrations in the cells that too, at the time of their activity. Their half-lives are short, and in order to quantify, the homogenates are usually concentrated after isolation steps. The method of PG isolation and sample concentration together increases the loss (mainly degradation) of present PGs, with the time, handling, temperatures, and other assay conditions. Thus, the first step to better quantification was to achieve a more effective and rapid sample preparation method. The earlier methods developed for PG isolation include the acetone extraction, Bligh and Dyer extraction, and methanol extraction. In the acetone extraction technique, the tissue is ground in acetone and saline solutions (Golovko and Murphy, 2008). This homogenate is centrifuged in silanized glass tubes to procure the supernatant containing PGs with other impurities. To this supernatant hexane is added to increase the lipid extraction and the it is acidified using formic acid for obtaining non-ionized forms of PGs, for easy extraction into organic solvents (Puppolo et al., 2014). Finally, chloroform is used here as the organic solvent, which is later cooled at -80 °C for separation and removal of remaining upper phase. Such a multistep liquid-liquid extraction requires enormous volumes of solvents and increases the cost of procedure apart from being time-consuming (Lee et al., 2003). The recovery rate of PGs is low due to the longer procedural times here.

Bligh and Dyer extraction method, on the other hand, utilized a mixture of chloroform and methanol (1:2) in a multistep isolation method (Bligh and Dyer, 1959). This method was based on a salting-out effect. The tissue was ground in these solvents to generate a one-phase system. Homogenate was then centrifuged in salinized glass tubes at low speed for 10 minutes. Equal volumes of chloroform and saline were added to divide this supernatant into two equal phases. After a brief vortex, samples were centrifuged to procure PGs in the chloroform phase. This method uses toxic chemicals, while the researchers nowadays are turning towards the use of greener solvents (Breil et al., 2017). The recovery rate in this method was achieved using several modifications as published by other researchers (Grima et al., 1994; Sheng et al., 2011; Caprioli et al., 2016). Folch and co-workers discovered a

technique to isolate lipids effectively from animal tissues around a similar time when as that of Bligh and Dyer did (Folch et al., 1957). Both the methods of Folch and Bligh-Dyer derived lipids by dividing the homogenates into two phases – the water-rich phase and the organic-rich phase. Both the methods are cited more than fifty thousand times and are the most popular lipid isolation methods even today.

The methods which were considered most effective also could not recover prostanoids more than 70 % from the samples due to their longer sample preparation times. In 2013, Brose and the team, developed a one-step methanol extraction method, especially for the extraction of PGs, to be used for UPLC-MS/MS techniques (Brose et al., 2013). They proved the method worked best so far, even for the isoprostanes. Only methanol was used to homogenate tissue, which was centrifuged after sonication, and the supernatant was transferred to silanized micro inserts. Additional proteins were precipitated by placing the samples in -80 °C for 10 minutes and warming them. They were centrifuged to remove proteins, and the supernatant was dried under liquid nitrogen for concentrating them if required. This methodology was slightly modified in the current work for betterment in recovery. The embryos contained a low amount of hard connective tissues at this stage (Hay, 1973). Embryos were added with methanol at a ratio of 1 mL per gram of tissue, which was nine times less concentration of methanol than what was suggested by Brose and colleagues (Brose et al., 2013). This ratio worked best for the 2-day old embryos. There was no need to concentrate the samples further due to this newly developed LC-MS/MS methodology, which allowed quantification at low concentrations as well. Here, the proteins were not additionally precipitated, but the homogenate of tissue was sonicated for five minutes after brief vortexing. This procedure was rapid as it took less than ten minutes after homogenization to complete the whole procedure. Omitting excessive precipitation, warming, cooling, and nitrogen purging led to better recovery, as discussed in the method validation part.

Efficient chromatographic separation of prostanoids and isoprostanes is a vast area of research. The efforts have been made to resolve the peaks based on a number of parameters related to chromatography. The chromatographic columns are one of those. Luna C18, Lux cellulose 1, Cheralpack – are some of the columns tested so far by scientists to achieve greater resolution (Brose et al., 2011). However, the current method focussed on chromatographic characters regarding solvents, their gradients, and run time rather than types of columns. The earlier studies dealing with prostanoids and isoprostanes used several solvent systems. Some solvent systems contained 0.1 % v/v formic acid (prepared in water)

as solvent A and acetonitrile with 0.1 % v/v formic acid as solvent B (Brose et al., 2011). Acids provide protons in the reverse phase chromatography. Acetic acid was used in the current study to do so. Another solvent A composition is – acetonitrile: water: glacial acetic acid, 45: 55: 0.02 (v/v/v), which was used for separating PGE_2, $PGF_{2\alpha}$, PGD_2, TXB_2, and numerous isoprostanes. This study used acetonitrile: water: glacial acetic acid, 90: 10: 0.02 (v/v/v) as solvent B (Masoodi and Nicolaou, 2007). The run time of this protocol was 30 minutes, as opposed to 11.6 minutes of the current derived method. The protocol described by Masoodi and Nicolaou performed well for higher concentrations of PGs. Here, we used solvent A: 0.1% Acetic acid, Solvent B: 90/10 Acetonitrile/water. Acetonitrile is a medium-polarity solvent, miscible in water, containing a high boiling point. Schmidt and a group of researchers attained successful separation of PGE_2 and PGD_2 in the total run time of 6 minutes (Schmidt et al., 2005). They used acetonitrile/water (40:60, v/v, pH 6.8) as solvent A and methanol as solvent B. Their method could resolve PGE_2 and PGD_2 by 0.25 minutes, which could result in overlapping peaks at higher concentrations. Another study trying to resolve these two molecules used the same MRM parameters for both PGE_2 and PGD_2, as in the case of the current work (Cao et al., 2008). In the current work, an effort was made to evaluate the concentrations of four major prostanoids in the day-2 chick embryos by deriving a new method to attain efficient extraction, proper resolution in LC, and appropriate characterization by tandem MS technique.

PGE_2 quantification using ELISA kit in all the samples showed that PGE_2 level dropped in all the samples except for two – day-6, and day-9. It can not be commented upon whether the levels were rescued by COX-1 at these stages. However, PGE_2 was proved to be the embryonic effector of COX-2, as its level dropped in all the etoricoxib-treated samples.

Finally, the quantification was done using stable isotope dilution against the constant ISTD in the number of calibrants. The LC parameters were able to achieve a resolution of at least 0.3 minutes between the prostanoids under study. TXB_2 was retained in the column for the least time, eluting at 3.9 minutes. It was followed by $PGF_{2\alpha}$, which eluted at 4.4 minutes. The retention time for PGE_2 was 4.7 minutes, while PGD_2 eluted at last at 5.5 minutes. Overall, the derived extraction and LC-MS/MS method, as described in this chapter, proved to be the most rapid extraction, providing considerably good linearity and recovery.

TABLES

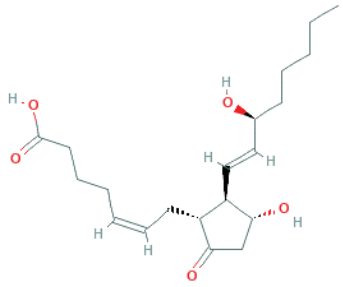
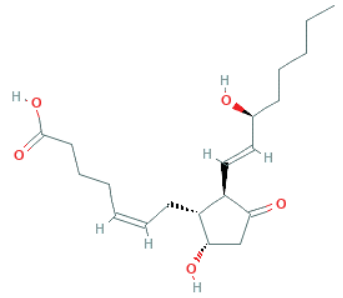
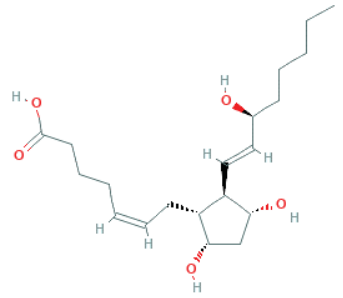
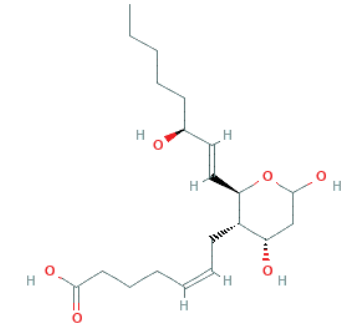
Name of prostanoid	Molecular formula	Structure	Molecular weight	Ionization mode
PGE_2	$C_{20}H_{32}O_5$		352	negative
PGD_2	$C_{20}H_{32}O_5$		352	negative
$PGF_{2\alpha}$	$C_{20}H_{34}O_5$		354	negative
TXB_2	$C_{20}H_{34}O_6$		370	negative

***Table 13**: Structural, molecular, and ionization properties of the major prostanoids*

ID	Q1 Mass (Da)	Q3 Mass (Da)	DP	CE
$PGF_{2\alpha}$	353	309	-60	-24
TXB_2	369	169	-70	-22
PGD_2	351	271	-60	-22
PGE_2	351.1	271	-70	-22
$PGF_{2\alpha}$-d4	357	313	-60	-24
AA	303	205	-60	-20
PGI_2	351	305	-50	-10

***Table 14**: MRM parameters of prostanoids, ISTD, and AA.*

Time (minutes)	% A	% B	Flow rate
0	62	38	0.3
3.5	40	60	0.3
4.25	22.5	77.5	0.3
6.25	5	95	0.3
9	5	95	0.3
9.1	62	38	0.3
11.6	62	38	0.3

***Table 15**: Gradient information for solvents in chromatography.*

Sample name	Analyte retention time (min)	Analyte concentration (ppb)	Calculated concentration (ppb)	Accuracy (%)
Calibrant 7	4.4	25.0	25.20	101.0
Calibrant 6	4.4	12.5	12.10	096.4
Calibrant 5	4.4	06.3	06.16	098.6
Calibrant 4	4.4	03.1	03.26	105.0
Calibrant 3	4.4	01.6	01.76	113.0
Calibrant 2	4.4	00.8	0.909	117.0
Calibrant 1	4.4	00.3	0.218	069.6

***Table 16A**: $PGF_{2\alpha}$ Calibration. The calculated concentration and added analyte concentrations were comparable for all except for one concentration (0.3 ppb).*

Sample name	Analyte retention time (min)	Analyte concentration (ppb)	Calculated concentration (ppb)	Accuracy (%)
Calibrant 7	3.9	25.0	11.5	91.9
Calibrant 6	3.9	12.5	25.3	101
Calibrant 5	3.9	06.3	6.85	110
Calibrant 4	3.9	03.1	3.24	105
Calibrant 3	3.9	01.6	1.69	108
Calibrant 2	3.9	00.8	0.814	104
Calibrant 1	3.9	00.3	0.247	79.1

***Table 16B**: TXB_2 Calibration. The calculated concentration and added analyte concentrations were comparable for all the calibrants.*

Sample name	Analyte retention time (min)	Analyte concentration (ppb)	Calculated concentration (ppb)	Accuracy (%)
Calibrant 7	5.5	25.0	11.5	91.9
Calibrant 6	5.5	12.5	25.3	101
Calibrant 5	5.5	06.3	6.85	110
Calibrant 4	5.5	03.1	3.24	105
Calibrant 3	5.5	01.6	1.69	108
Calibrant 2	5.5	00.8	0.814	104
Calibrant 1	5.5	00.3	0.247	79.1

***Table 16C**: PGD_2 Calibration. The calculated concentration and added analyte concentrations of PGD_2 were comparable for all the calibrants.*

Sample name	Analyte retention time (min)	Analyte concentration (ppb)	Calculated concentration (ppb)	Accuracy (%)
Calibrant 7	4.7	25.0	11.5	91.7
Calibrant 6	4.7	12.5	25.3	101
Calibrant 5	4.7	06.3	6.66	107
Calibrant 4	4.7	03.1	3.42	110
Calibrant 3	4.7	01.6	1.74	112
Calibrant 2	4.7	00.8	0.738	94.6
Calibrant 1	4.7	00.3	0.26	83.1

***Table 16D**: PGE_2 Calibration. The calculated concentration and added analyte concentrations of PGE_2 were comparable for all except for the 25 ppb containing calibrant, which was omitted for generating curve.*

Analyte	Concentrations	
	Control (Mean±SE)	Test (Mean±SE)
$PGF_{2\alpha}$	02.86 ± 0.359	02.49 ± 0.454
TXB_2	08.73 ± 0.222	08.28 ± 1.364
PGD_2	19.15 ± 0.979	14.40 ± 2.959
PGE_2	04.18 ± 0.672	02.05 ± 0.614

***Table 17**: Concentration of prostanoids as derived from the new methodology. n=30*

Analyte	Concentrations spiked									
	3 ppb					15 ppb				
	Sample1	Sample2	Mean (%CV)	STDEV	Std error	Sample1	Sample2	Mean (%CV)	STDEV	Std error
$PGF_{2\alpha}$	138	132	135	4.48	3.17	87	121	104	24.46	17.3
TXB_2	115	116	116	1.18	0.83	78	103	90	18.14	12.83
PGD_2	64	83	74	13.91	9.83	81	81	81	0.47	0.33
PGE_2	85	118	102	23.33	16.5	91	90	91	0.85	0.6

***Table 18**: Method validation result. Recovery percentage for two spiked concentrations for each analyte in two sample matrices, % CV and standard error.*

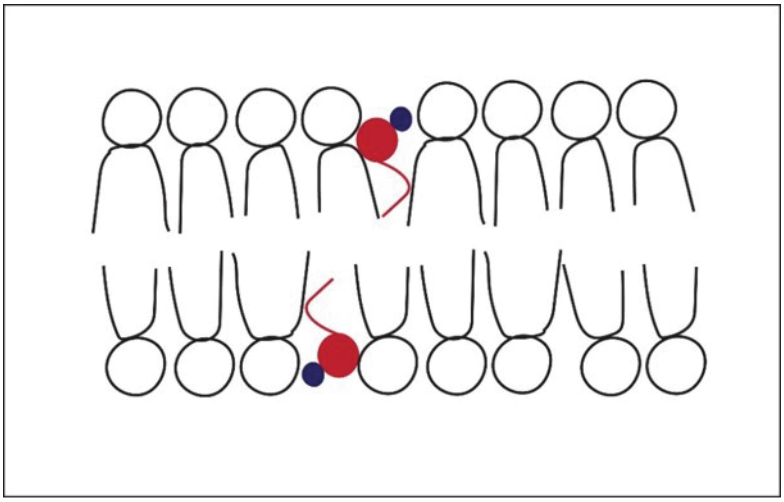


Figure 24: *The arrangement of 20-carbon polyun- saturated arachidonic acid in a lipid bilayer of cell membrane. The red coloured structures represent arachidonic acid simply. The other molecules show the hydrophobic heads and hydrophilic tail regions of other fatty acids forming the membrane.*

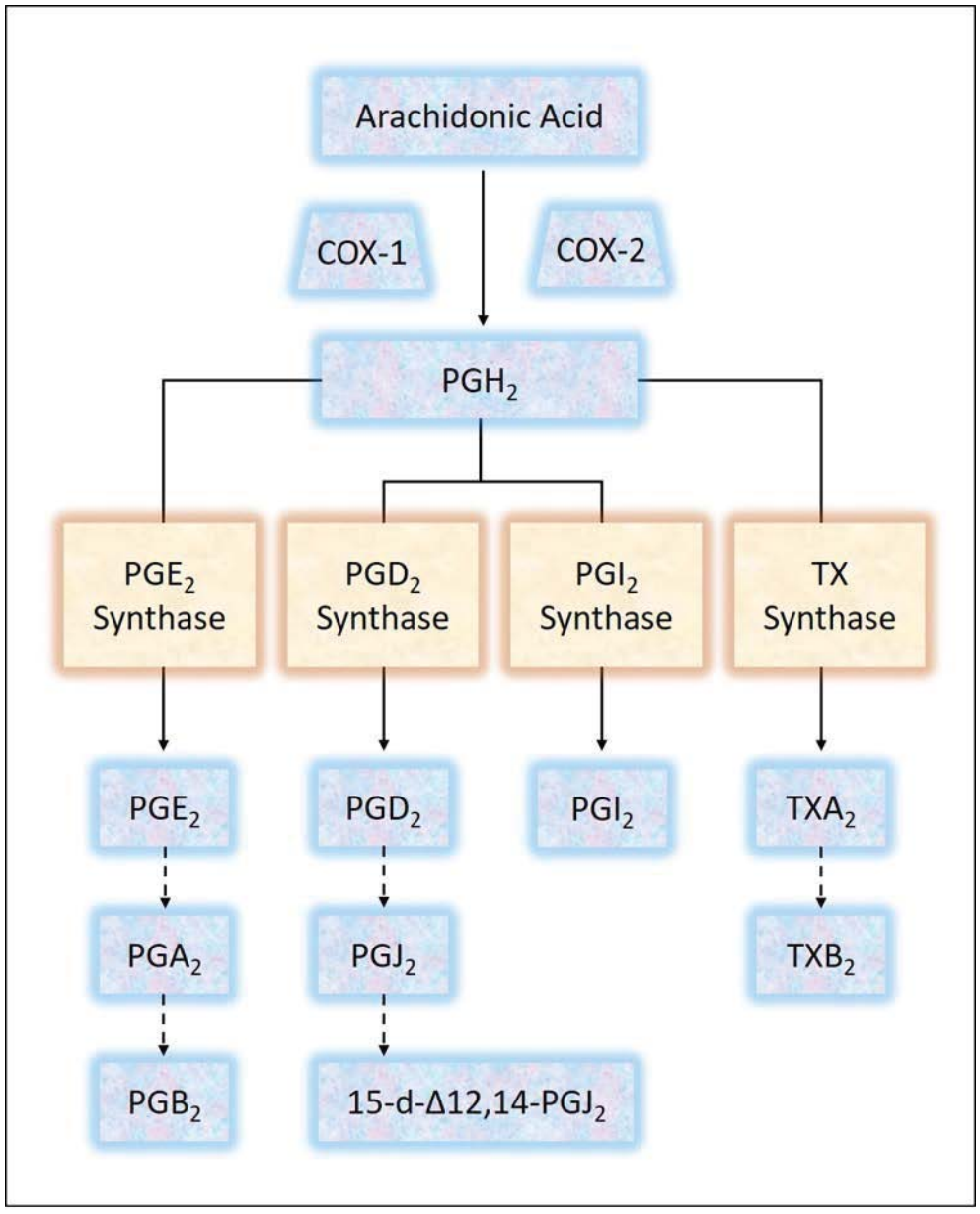


Figure 25: *Tissue-specific Synthases converting AA into various types of PGs biosynthetically. PGE_2 is converted to PGA_2 and PGB_2 by sequential dehydration reactions. PGD_2 gets converted to PGJ_2 by dehydration, which is isomerized to 15-d-Δ12,14-PGJ_2. TXA_2 is rapidly converted to TXB_2.*

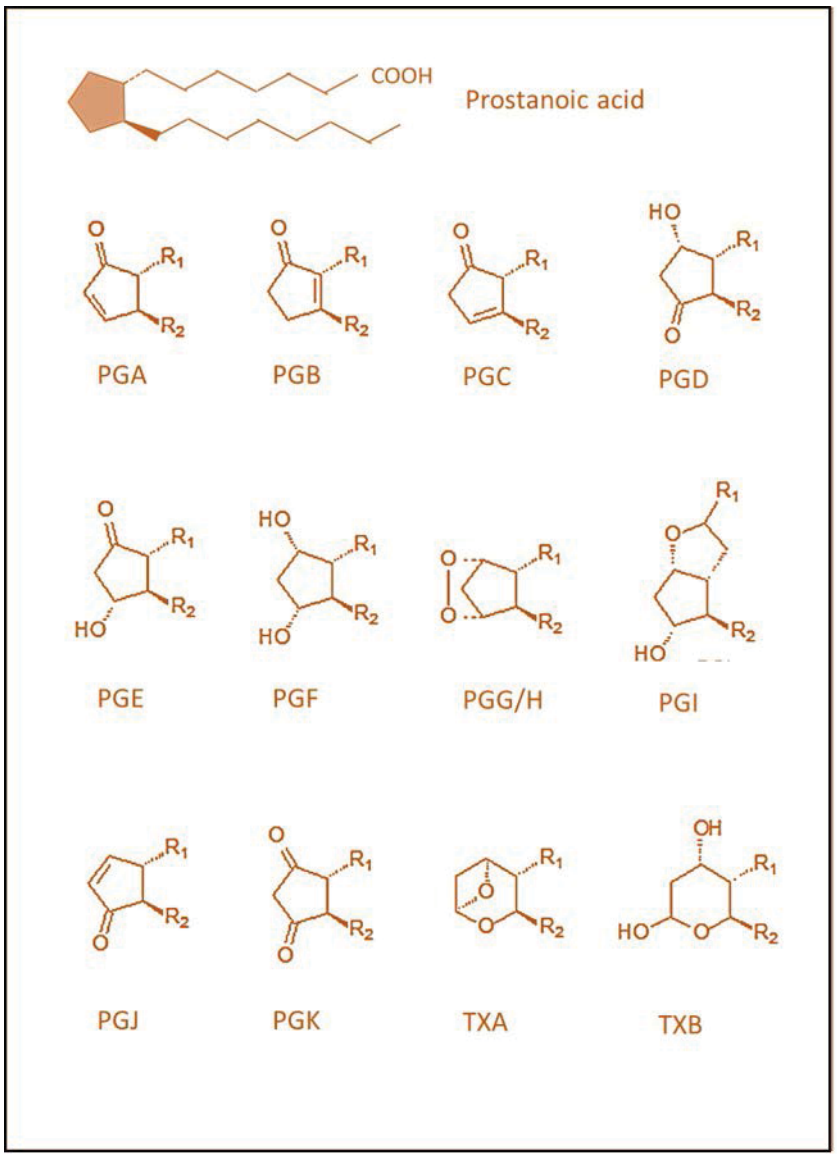


Figure 26: *Nomenclature of PGs from the fundamental arrangement of bonds in the cyclopentane as well as functional groups.*

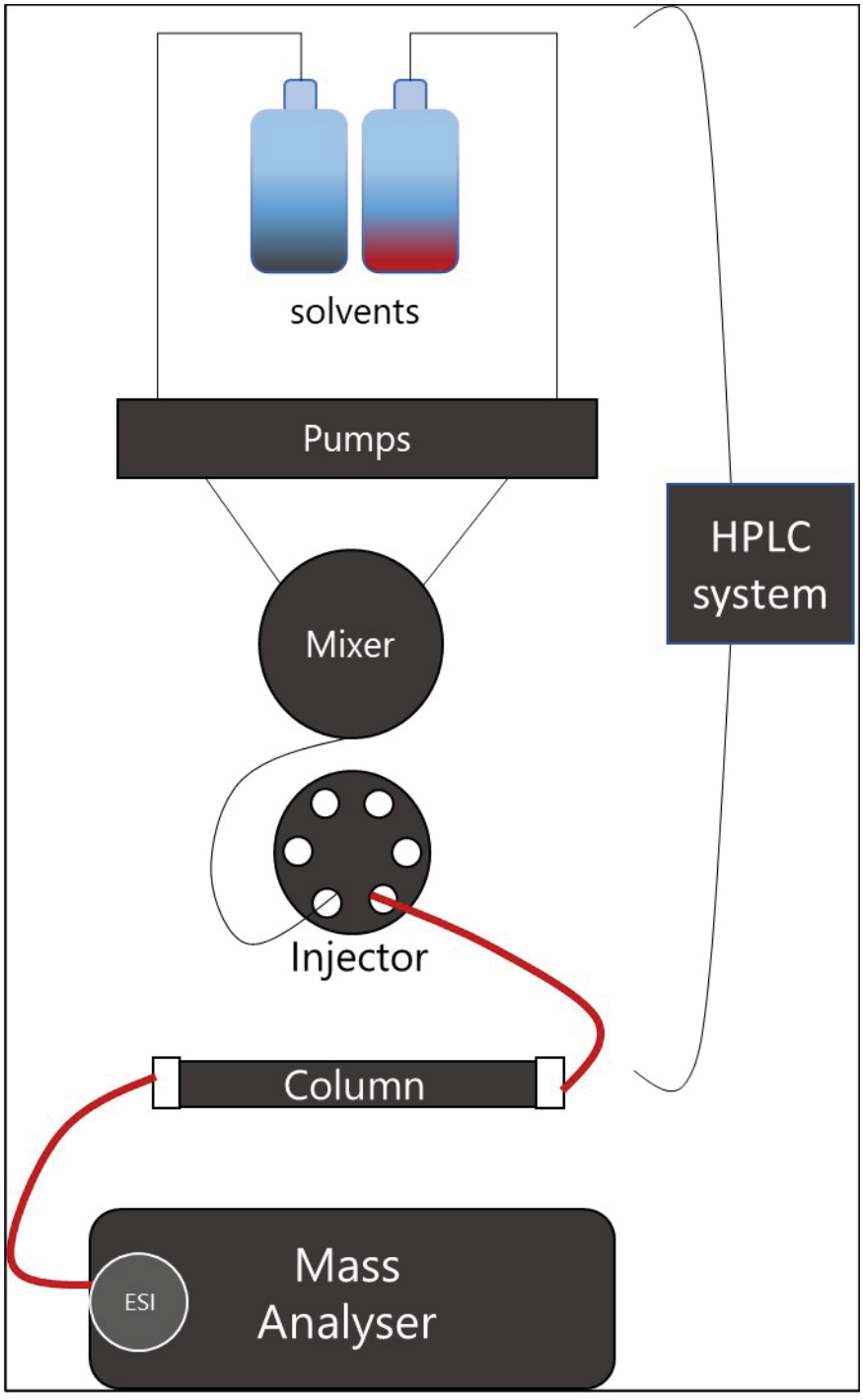


Figure 27: *Coupling LC with MS via ESI*

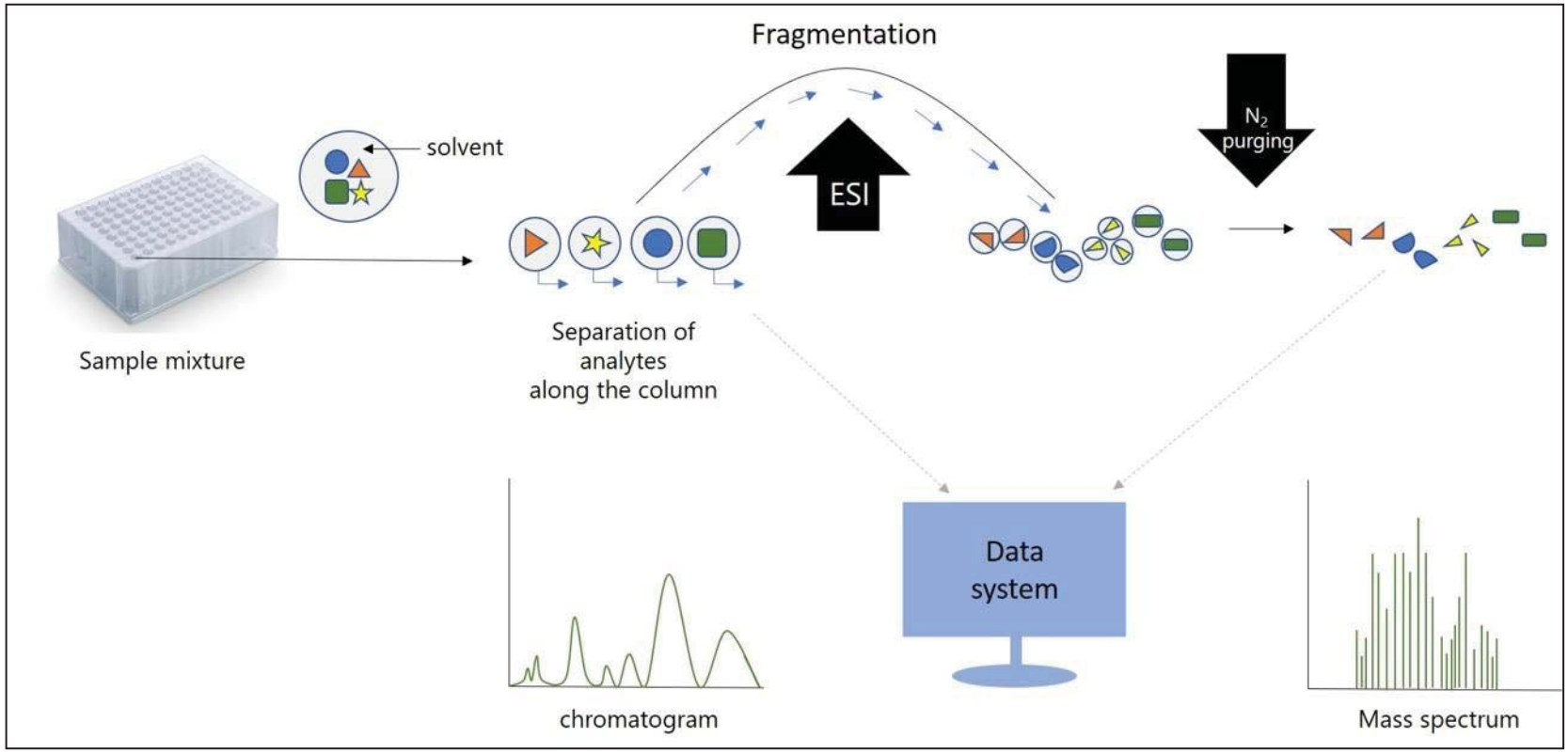


Figure 28: *Graphical representation of LC-MS/MS system*

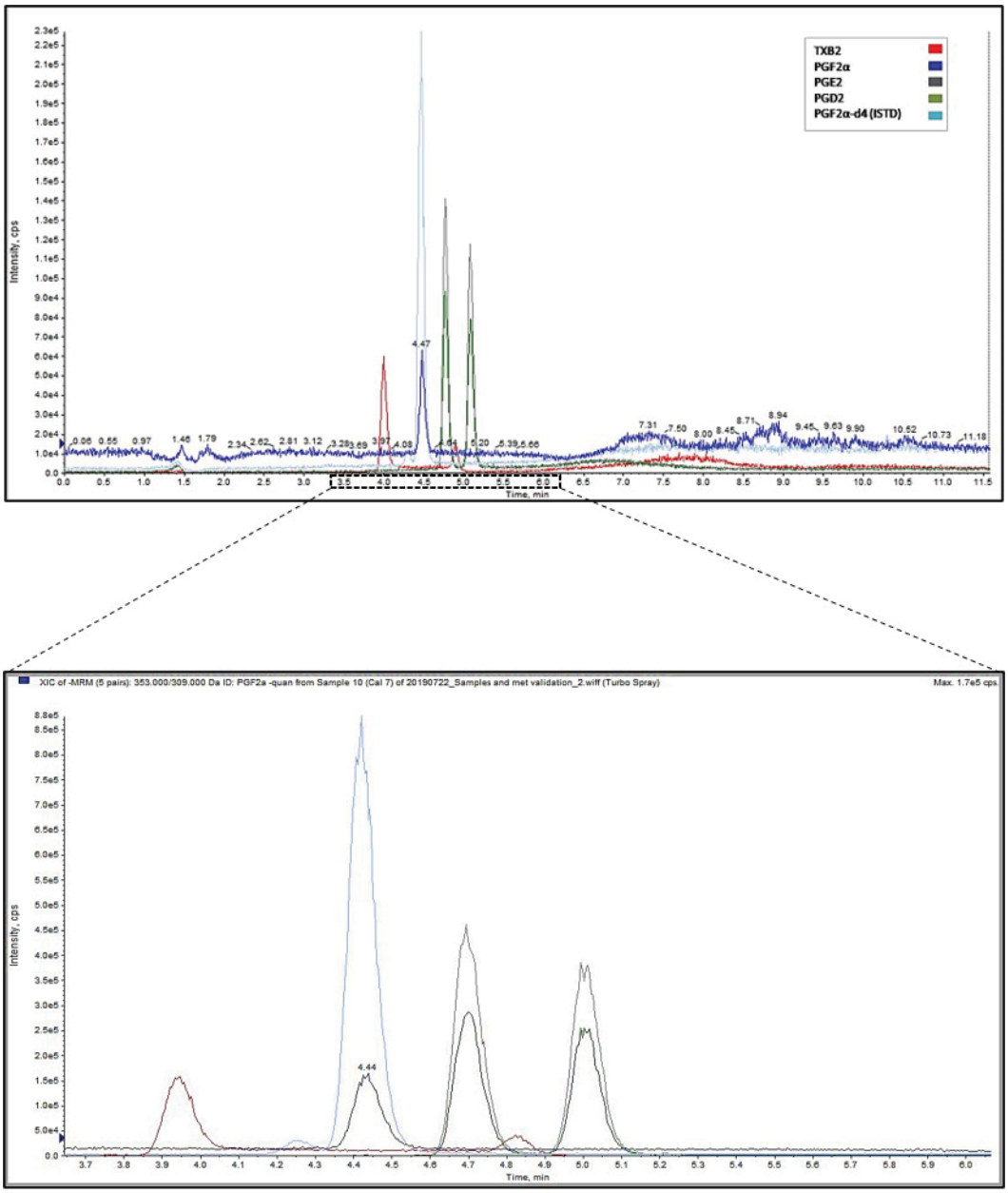


Figure 29A: *Chromatogram of prostanoids as derived from Analyst software. The upper panel shows chromatogram throughout the run-time in LC column. The lower panel is larger view of shorter span between 3.5 to 6 minutes of run. Color scheme remains same for both the panels along with X and Y coordinates. Intensity of peaks is plotted on X-axis, while Time in minutes is plotted on Y-axis.*

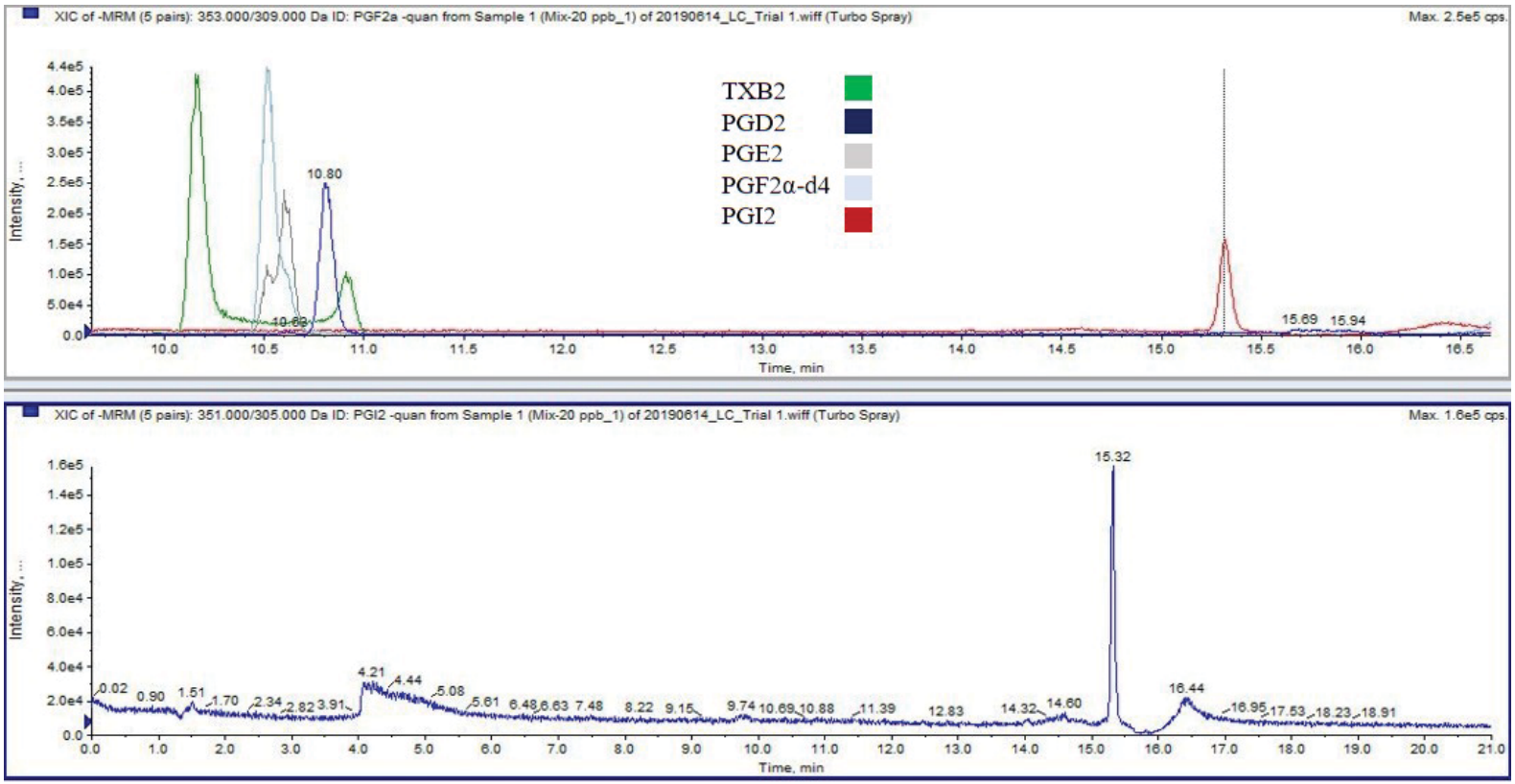


Figure 29B: *Chromatogram of the mixture of prostanoids containing PGI_2 along with $PGF_{2\alpha}$, TXB_2, PGD_2, and PGE_2 in the upper panel. The lower panel is an eluted ion peak for PGI_2 only.*

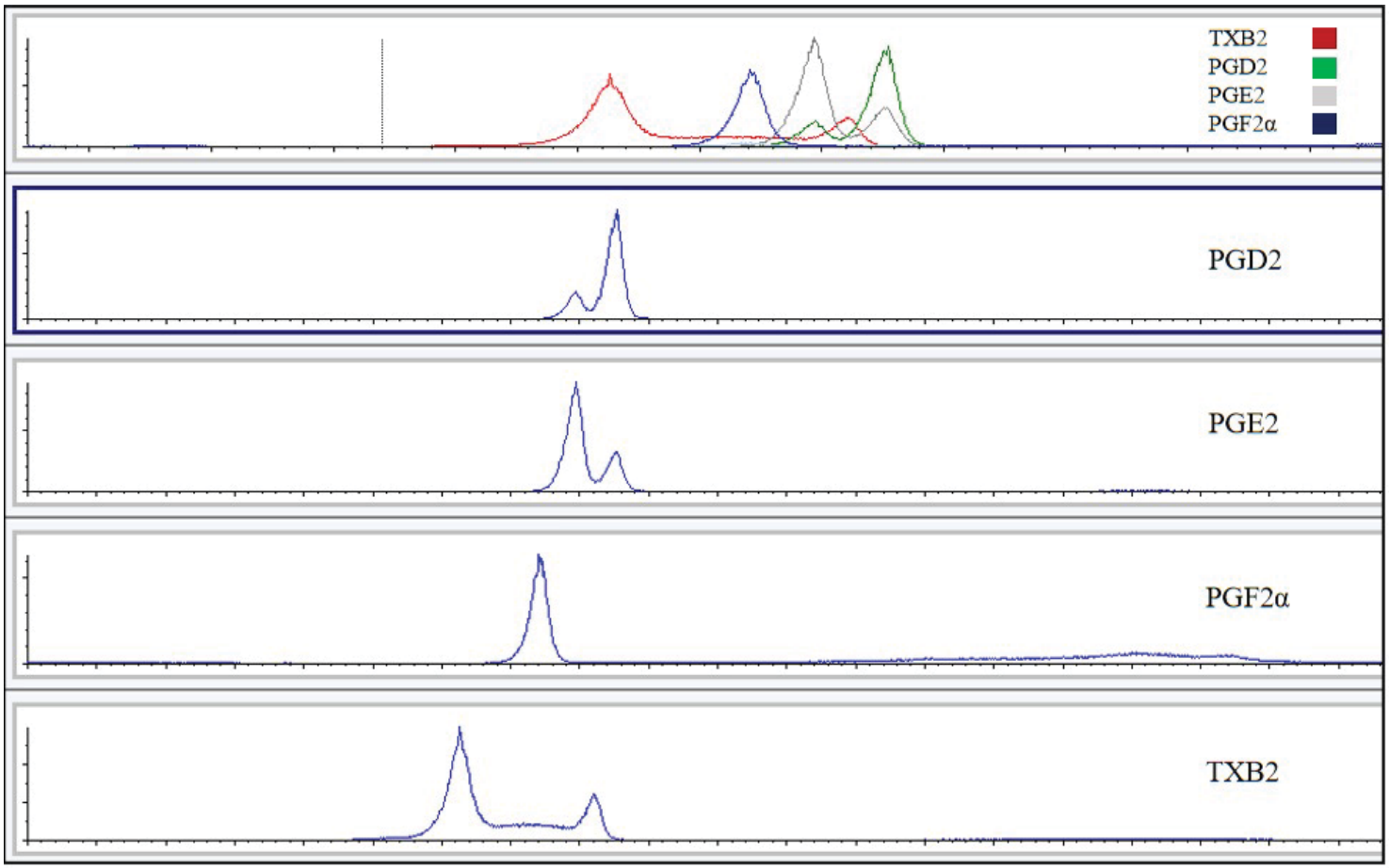


Figure 29C: *Chromatogram of the mixture of prostanoids containing $PGF_{2\alpha}$, TXB_2, PGD_2, and PGE_2 in the first panel. The lower panels are the eluted ion peaks as annotated. Note that the peaks of PGD_2 and PGE_2 eluted at different timepoints as evident from these graphs extracted from chromatographic depictions of Analyst software.*

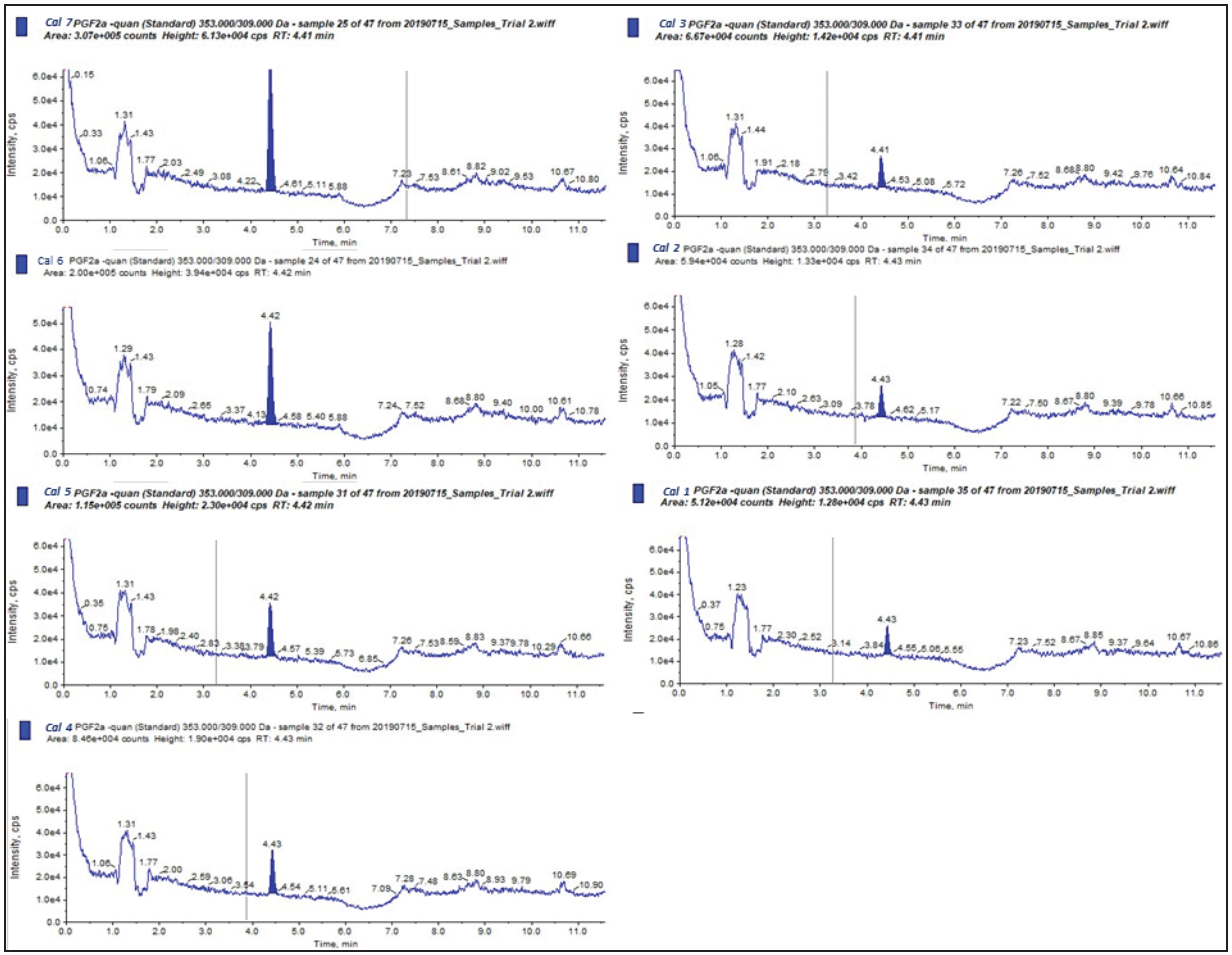


Figure 30A: *Intensity of quantitative mass peaks of $PGF_{2\alpha}$ in calibrants arranged in descending order of concentration from 25 to 0.3 ppb. Note that the area of peaks tends to reduce with the decreasing quantity of analyte concentration, which was further compared with constant ISTD areas.*

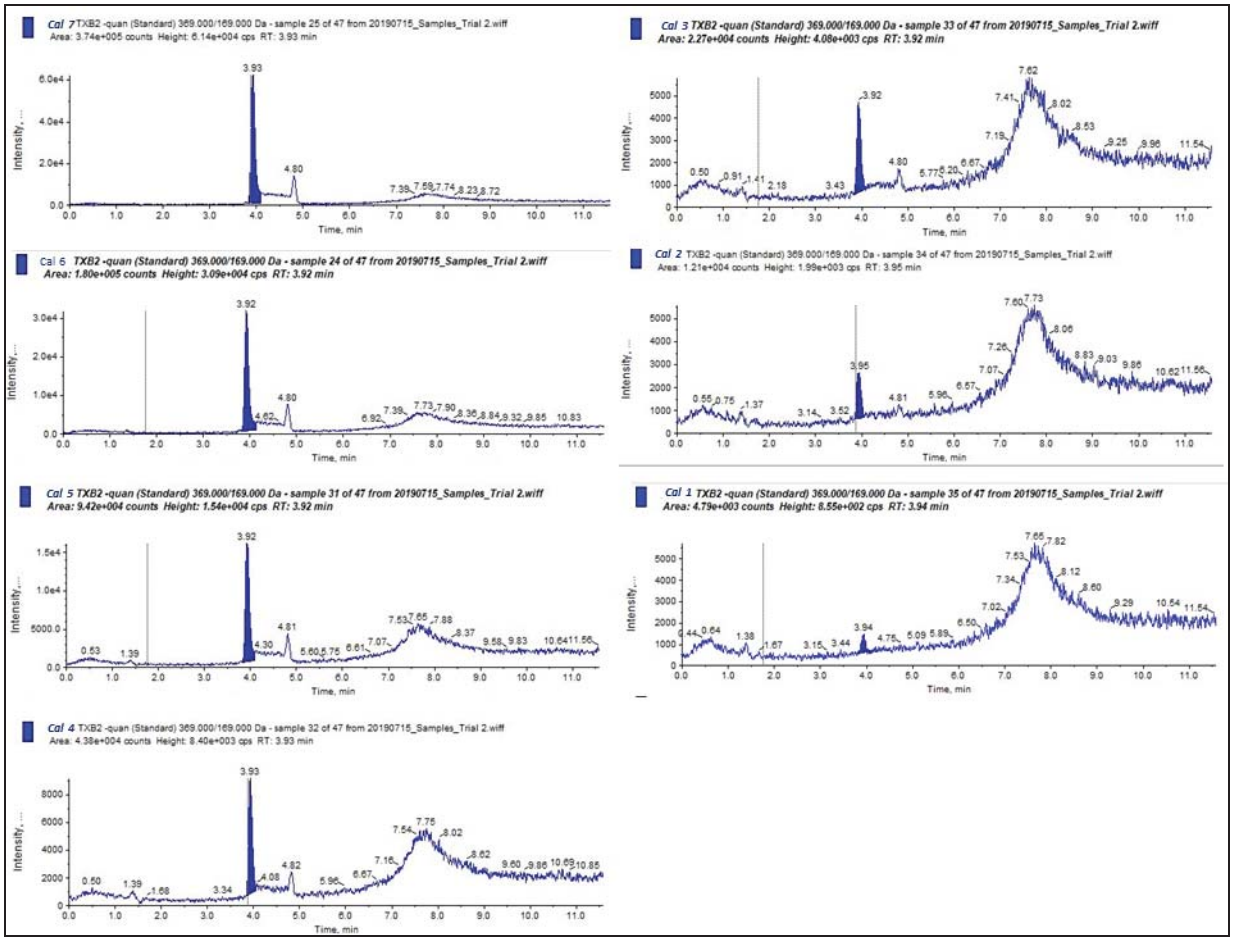


Figure 30B: *Intensity of quantitative mass peaks of TXB_2 in calibrants arranged in descending order of concentration from 25 to 0.3 ppb. Note that the area of peaks tends to reduce with the decreasing quantity of analyte, which was further compared with constant ISTD areas. Intensity in cps unit is plotted on Y-axes.*

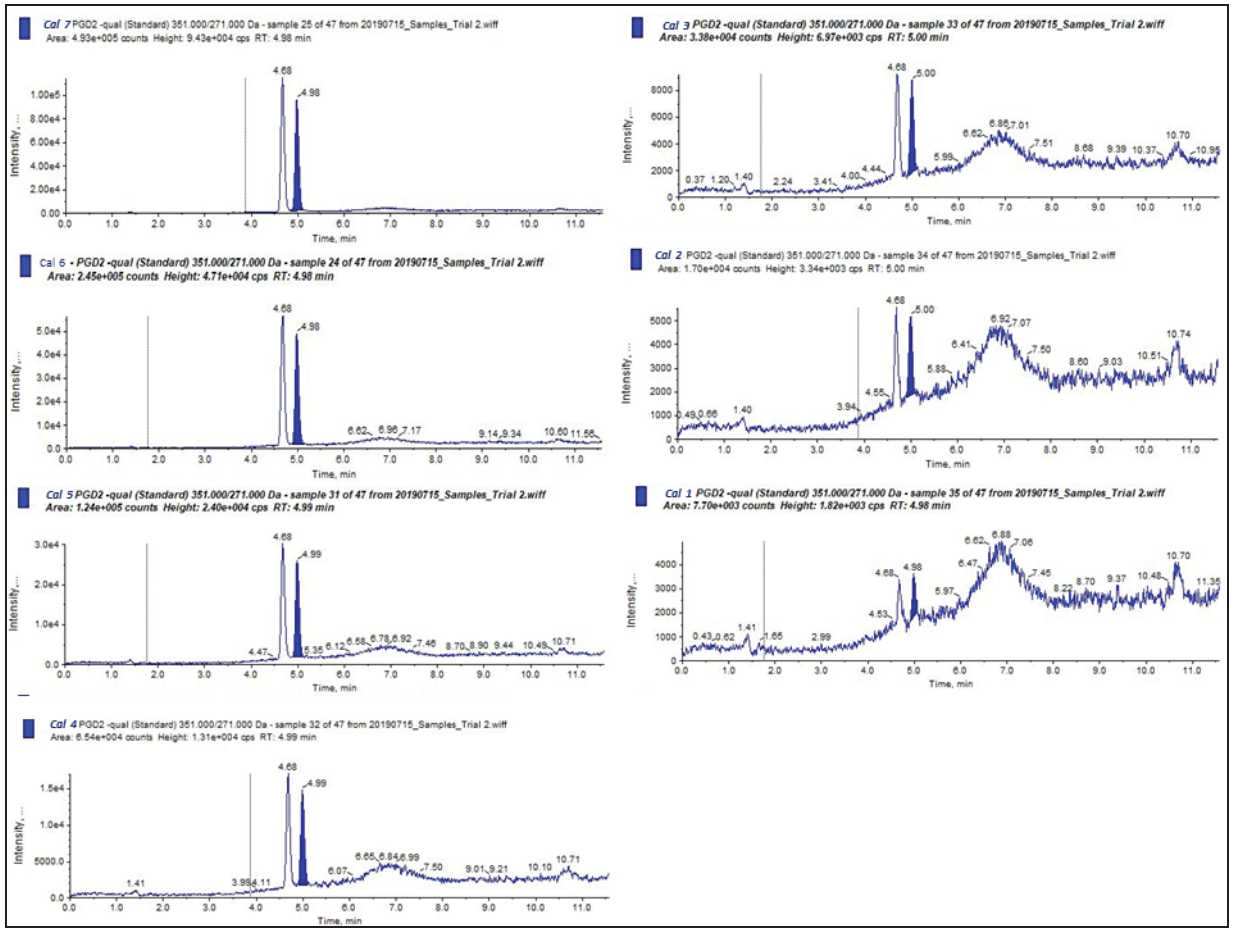


Figure 30C: *Intensity of quantitative mass peaks of PGD_2 in calibrants arranged in descending order of concentration from 25 to 0.3 ppb. Note that the area of peaks tends to reduce with the decreasing quantity of analytes, which was further compared with constant ISTD areas. Intensity in cps unit is plotted on Y-axes. Only the highlighted area (blue) was considered for calculations as the other peak belonged to PGE_2 as identified by individual LC parameters.*

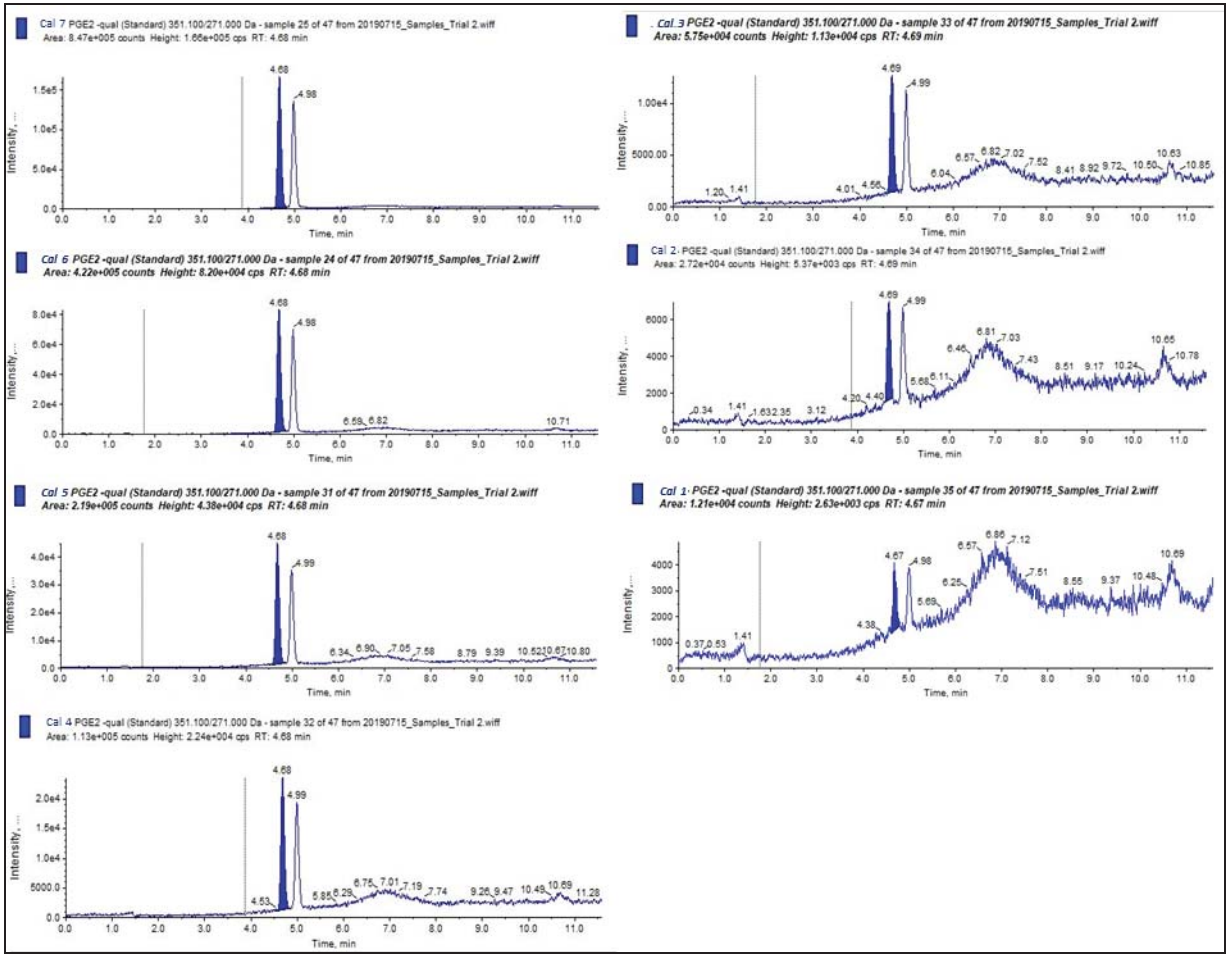


Figure 30D: *Intensity of quantitative mass peaks of PGE_2 in calibrants arranged in descending order of concentration from 25 to 0.3 ppb. Note that the area of peaks tends to reduce with the decreasing quantity of analytes, which was further compared with constant ISTD areas. Intensity in cps unit is plotted on Y-axes. Only the highlighted area (blue) was considered for calculations as the other peak belonged to PGD_2 as identified by individual LC parameters.*

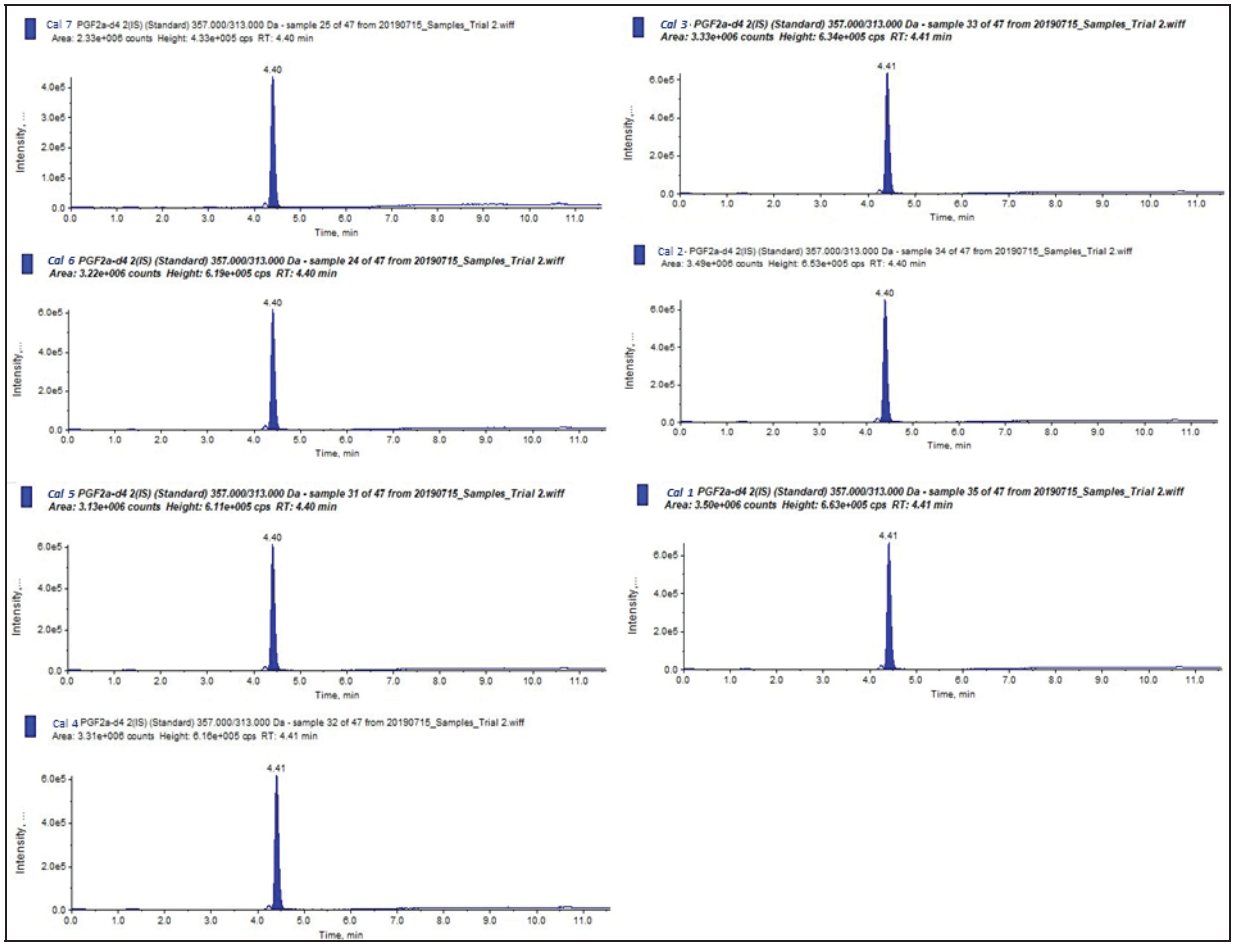


Figure 30E: *Intensity of quantitative mass peaks of $PGF_{2\alpha}$ – d4 in calibrants, arranged in descending order of concentration from 25 to 0.3 ppb. Note that the area of peaks remains similar in all these calibrants. Intensity in cps unit is plotted on Y-axes.*

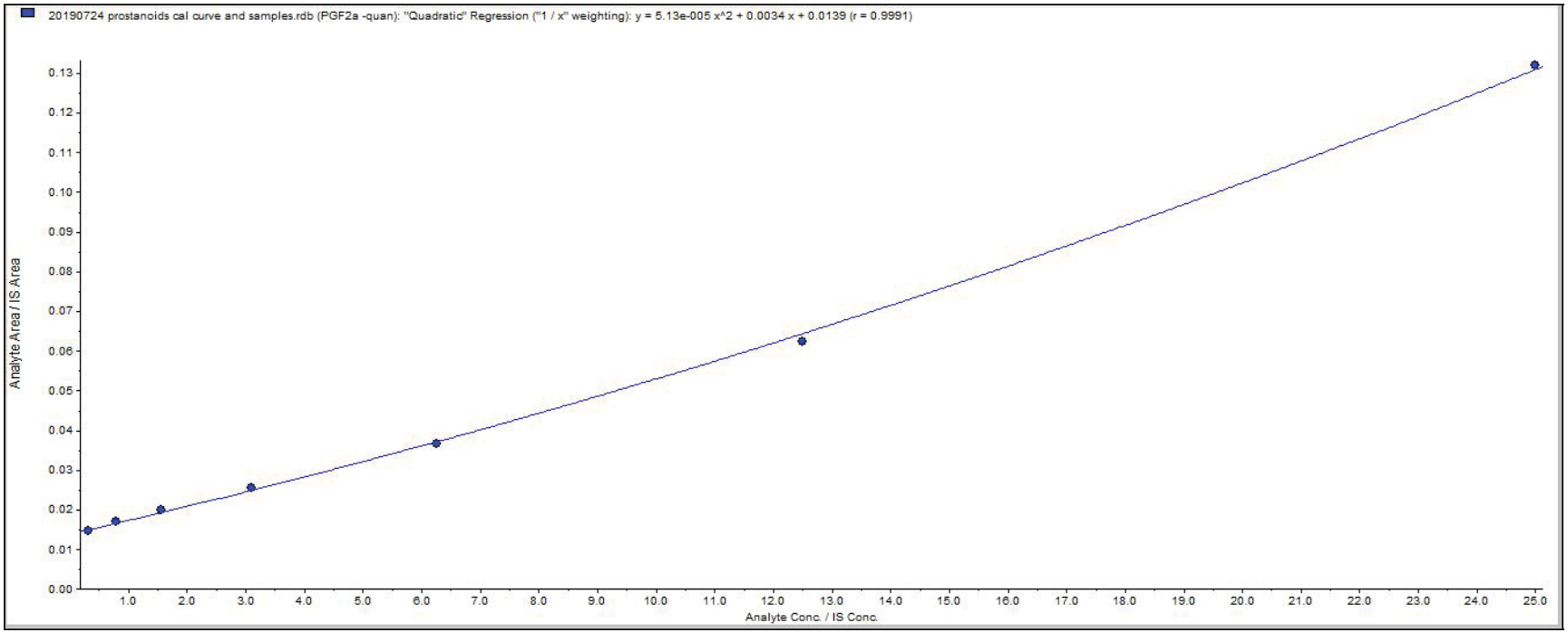


Figure 31A: *Calibration curve of $PGF_{2\alpha}$: range of concentrations 0.3 – 25 ppb, r = 0.9991, regression type quadratic, with "1/x" weighting. Analyte peak area/ISTD peak area is plotted on X-axis, Analyte concentration/ ISTD concentration is plotted on Y-axis.*

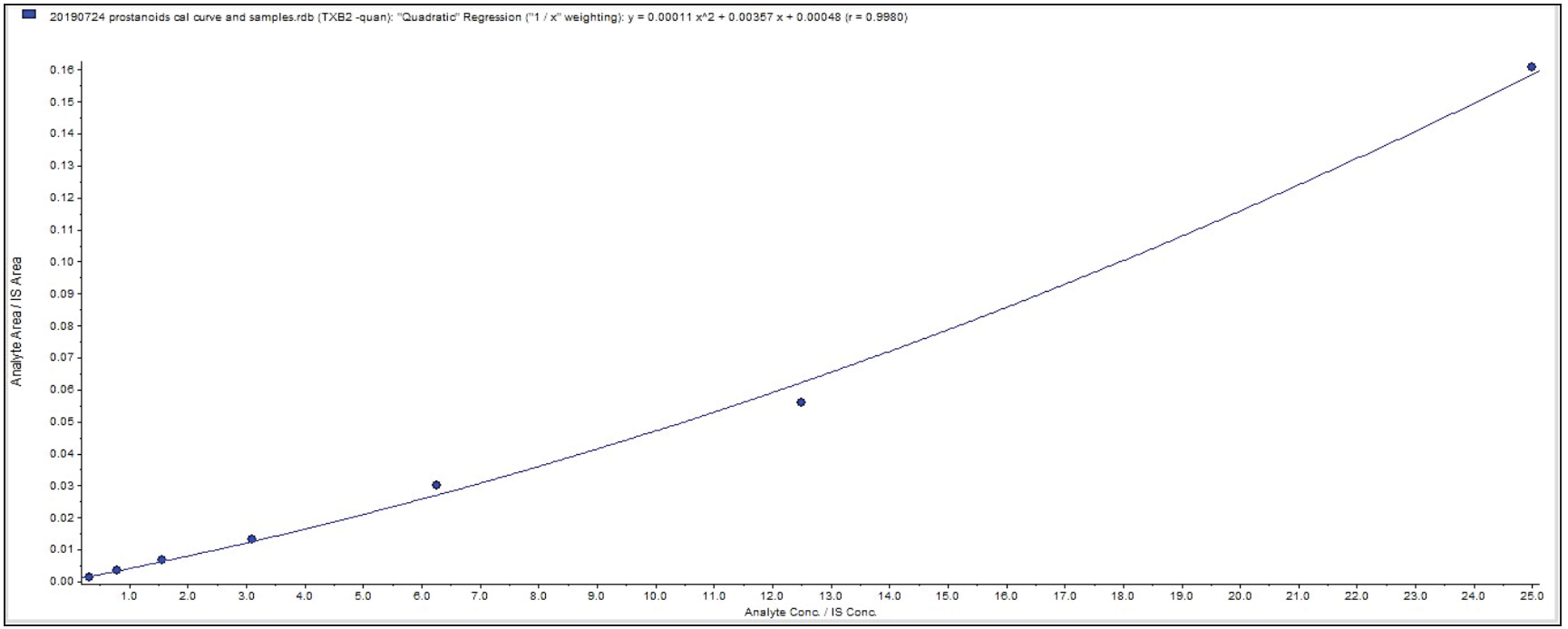


Figure 31B: *Calibration curve of TXB_2: range of concentrations 0.3 – 25 ppb, r = 0.9980, regression type quadratic, with "1/x" weighting. Analyte peak area/ISTD peak area is plotted on X-axis, Analyte concentration/ ISTD concentration is plotted on Y-axis.*

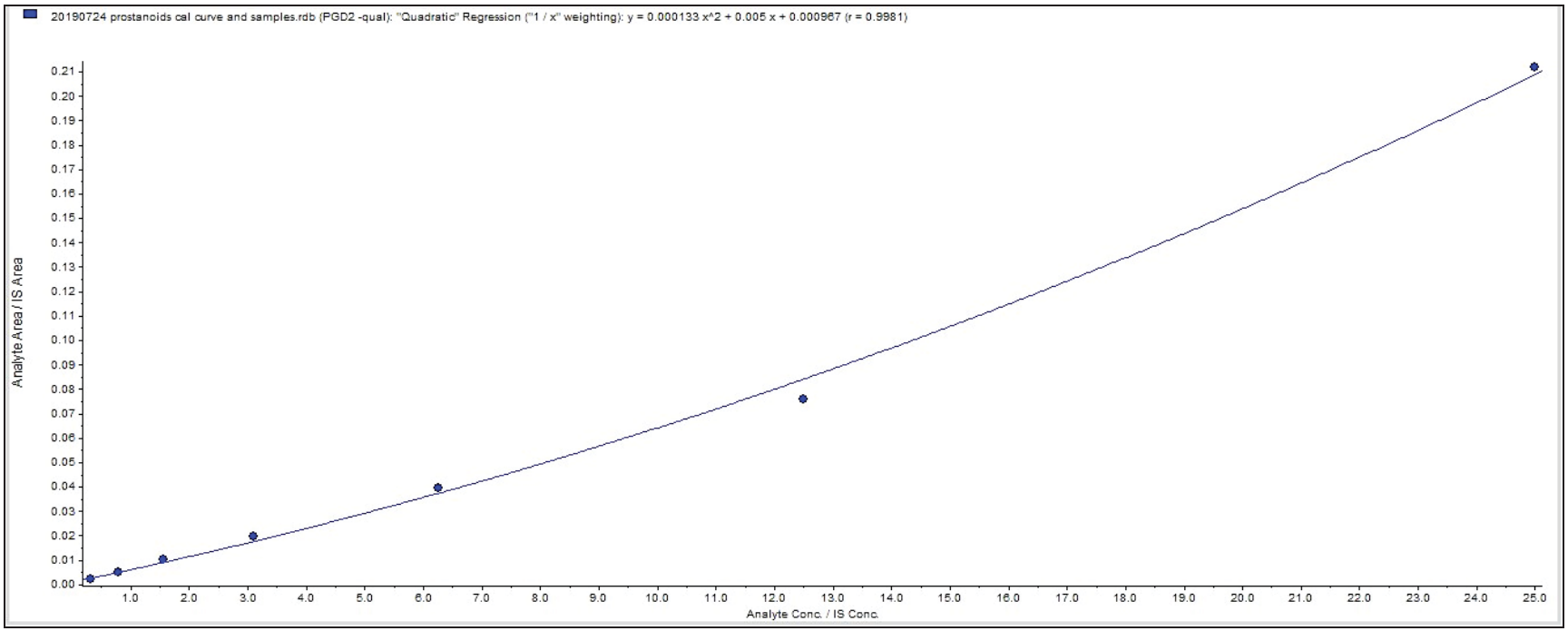


Figure 31C: *Calibration curve of PGD_2: range of concentrations 0.3 – 25 ppb, r = 0.9981, regression type quadratic, with "1/x" weighting. Analyte peak area/ISTD peak area is plotted on X-axis, Analyte concentration/ ISTD concentration is plotted on Y-axis.*

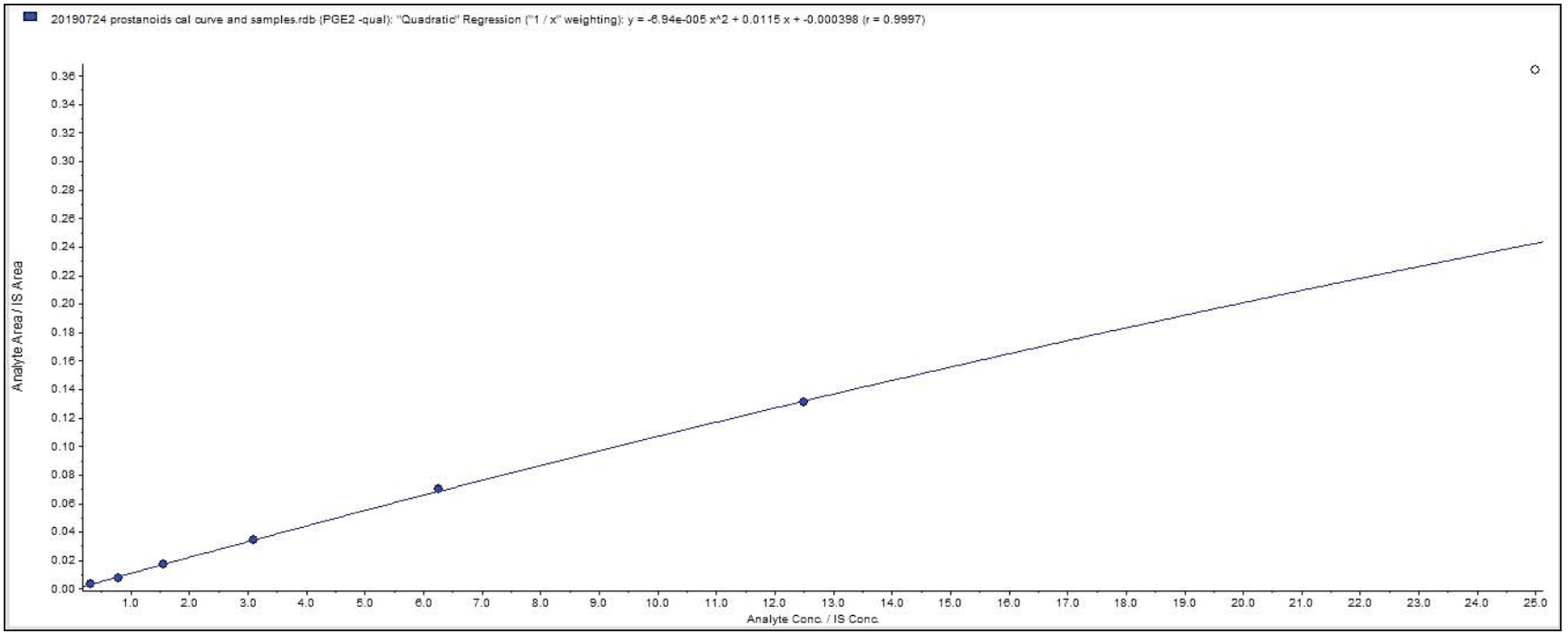


Figure 31D: *Calibration curve of PGE_2: range of concentrations 0.3 – 12.5 ppb, r = 0.9997, regression type quadratic, with "1/x" weighting. Analyte peak area/ISTD peak area is plotted on X-axis, Analyte concentration/ ISTD concentration is plotted on Y-axis.*

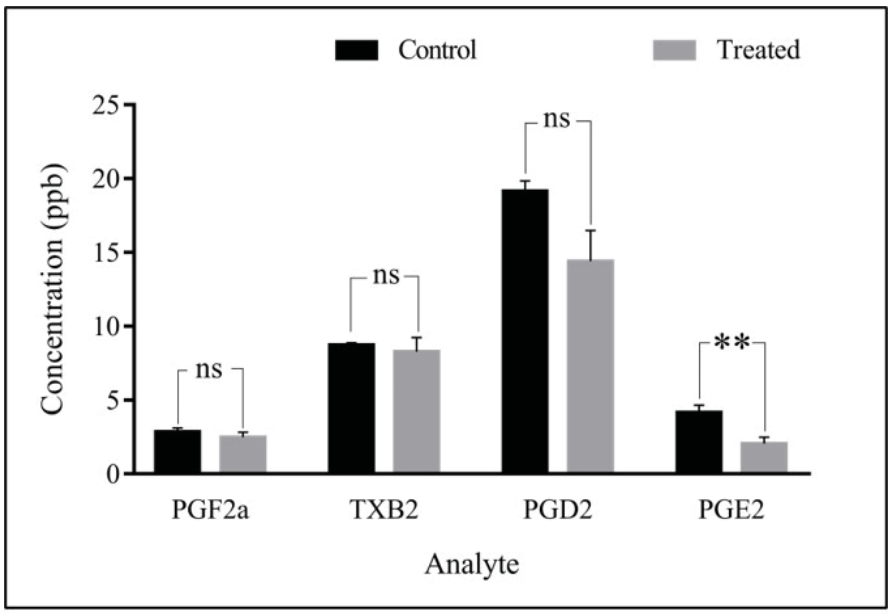


Figure 32: *Concentration of Prostanoids. Level of PGE_2 dropped significantly. Plotted values are Mean ± SEM. **p≤0.01. n=3.*

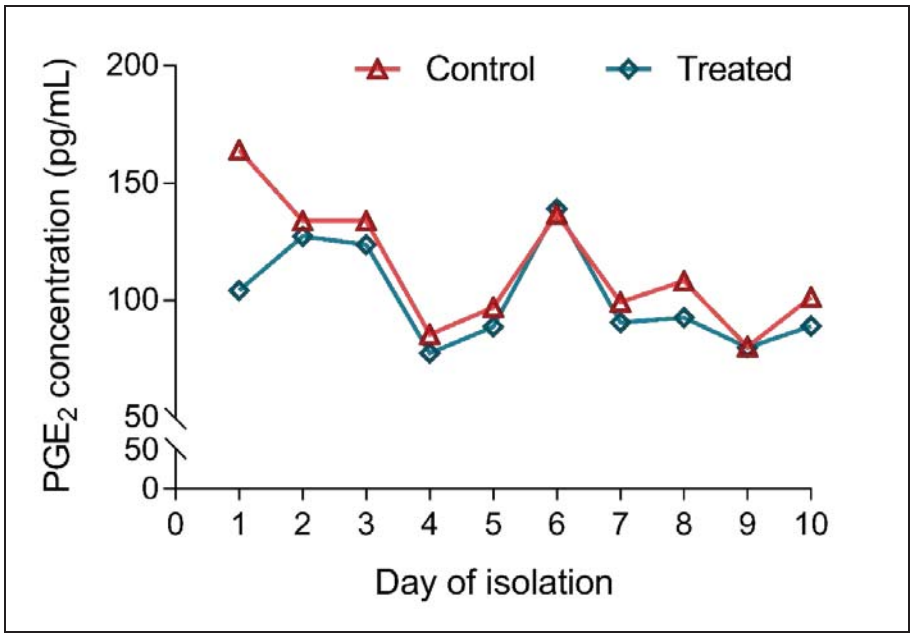


Figure 33: *Concentration of PGE_2. Level of PGE_2 dropped as compared to control in all days except for day-6 and day-9. Plotted values are Mean values of three technical replicates.*