Development of an LC-MS assay for putative biomarkers of anaphylaxis: urinary 11β-prostaglandinF2α and leukotrieneE4

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Abstract

Development of an LC-MS assay for putative biomarkers of anaphylaxis: urinary 11β-prostaglandinF2α and leukotrieneE4

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Anaphylaxis is a hypersensitivity reaction that is rapid in onset and can be fatal. In recent years there has been a concerning increase in anaphylactic cases worldwide. Due to the limitations of currently used biomarkers of anaphylaxis such as narrow time collection window for blood and/or poor applicability to food-induced anaphylaxis, there is a need to assess new biomarkers of anaphylaxis. Urinary 11β-prostaglandinF2α (11β-PGF2α) and leukotrieneE4 (LTE4) were recently proposed as possible new biomarkers of anaphylaxis. However, these studies relied on immunoassay-based methods, which may not be able to distinguish all isomers, and small cohorts, thus requiring more rigorous follow-up studies. The objective of this project was to develop and validate a highly selective ultra-high-performance liquid chromatography-quadrupole time of flight (UHPLC-QTOF) method for the accurate analysis of urinary 11β-PGF2α and LTE4.

C18 reversed-phase chromatography was used for the separation of isomers. Mobile phase A was 0.02% acetic acid in water and mobile phase B was 0.02% acetic acid in acetonitrile/iso-propanol (90:10). The isocratic flow and shallow gradient of mobile phase B gave a baseline separation of PGF isomers and LTE4 isomers, respectively. Both positive and negative electrospray ionization (ESI) were tested for the ionization of LTE4. Negative ESI was selected based on the signal-to-noise ratio, which was 7x higher in negative ESI than positive ESI.

Solid phase extraction (SPE) was selected for sample preparation to achieve sufficient enrichment to enable the measurement of low endogenous levels of these two putative biomarkers. Three different SPE sorbents were tested for recovery of 11β-PGF2α and LTE4: mixed-mode strong anion-exchange, mixed-mode weak anion-exchange and reversed-phase C18. C18 SPE was selected for the sample preparation based on the analyte recovery and minimum extraction of interferences. Separate SPE elution of 11β-PGF2α and LTE4 was chosen as the best strategy to minimize background signal and improve the limits of detection of LTE4. A forced degradation study of LTE4 showed that LTE4 is not stable at a pH below 4 and hence strict control of pH was

required throughout the procedure. The use of 6 µL of 30% glycerol in method (v/v) improved the method precision by reducing analyte losses in non-specific adsorption.

11β-PGF2α is also excreted as glucuronic acid conjugate in urine. Hence, to quantitate total endogenous 11β-PGF2α concentrations in urine, β-glucuronidase enzyme hydrolysis was used.

The method was validated for accuracy, precision, linearity, recovery, matrix effects and stability. The validation was performed using deuterated internal standards as surrogate analytes since both 11β-PGF2α and LTE4 are present endogenously. Intra-day accuracy for ranged from 90-111% and 86-108% for 11β-PGF2α-d4 and LTE4-d5, respectively. The method showed good intra-day precision (% RSD = 4.0-7.4% and % RSD = 6.8-14.9% for 11β-PGF2α-d4 and LTE4-d5 respectively). The method was linear from 9.8-5000 pg/mL concentration of both analytes. Inter-day accuracies were 91-100% (% RSD = 4.2-8.4%) for 11β-PGF2α-d4 and 93-113% (% RSD = 4.9-8.8%) for LTE4-d5. The lower limit of quantitation of both analytes was 15 pg/mL. The recoveries were 92-95% and 77-83% and the matrix effects were 83-88% and 99-115% for 11β-PGF2α-d4 and LTE4-d5, respectively in pooled urine from six different individuals. Recovery and matrix effects were also evaluated in nine individual urine lots. The 11β-PGF2α-d4 recovery ranged from 70-116% and LTE4-d5 recovery ranged from 61-99% across nine urine lots. Matrix effects for 11β-PGF2α-d4 ranged from 69-106% and matrix effects for LTE4-d5 ranged from 87-126% across nine urine lots, suggesting that slight matrix effects were present in individual samples as compared to matrix effects in pooled urine. 11β-PGF2α-d4 was stable for up to three freeze-thaw cycles whereas LTE4 was stable only up to one freeze-thaw cycle indicating that urine samples should be aliquoted and free-thaw cycles should be avoided for LTE4 quantitation. Both analytes were stable in the freezer for up to three-week period.

Overall, this novel validated and highly selective LC-MS method for urinary 11β-PGF2α and LTE4 can be used for anaphylaxis biomarker analysis in clinical studies and addresses some of the key shortcomings in other LC-MS assays reported in literature such as low LTE4 recovery and lack of data on isomer separation.

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This work is dedicated to my late mother Ms. Vijaylakshami Gupta and my grandmother Ms. Basanti Gupta. Thank you for being my role models and setting examples of hard work and dedication. I would also like to thank my family for all the love and encouragement and my husband, Mayank, for his immense support and eternal love throughout this journey.

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Contribution of authors

Chapter 2

This chapter entitled "Development of an LC-MS assay for putative biomarkers of anaphylaxis: urinary 11β-prostaglandinF2α and leukotrieneE4" authored by Ankita Gupta, Natalie Khor, Greg Shand, Moshe Ben-Shoshan and Dajana Vuckovic is the first draft of a manuscript still in preparation to be submitted for publication in the summer of 2019.

A.G. performed all the experimental work and data processing. A.G. and D.V. designed all the experiments and interpreted the results. A.G. wrote the first draft of this manuscript. D.V. supervised all the work. N.K. assisted in ELISA experiment for the measurement of 11β-PGF2α in clinical samples which is not included in the thesis. M. B-S designed the clinical study and G. S. and M. B-S collected all samples for analysis. All authors will review and revise the final version of the manuscript before submission.

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List of abbreviations

11β-PGF2α - 11β-prostaglandinF2α

AA - Acetic acid

ACN - Acetonitrile

AJS - Agilent Jet Stream

AS - Autosampler

C-CARE - Cross Canada Anaphylaxis Registry

CE - Collision energy

CID - Collision-induced dissociation

CNS - Central nervous system

COX - Cyclooxygenase

CPR - Cardiopulmonary resuscitation

CV - Coefficient of variance

CVS - Cardiovascular system

DC - Direct current

EIA - Enzyme immunoassay

EIC - Extracted ion chromatogram

ELISA - Enzyme-linked immunosorbent assay

ESI - Electrospray ionization

FDA - Food and Drug Administration

FZ - Freezer

GI - Gastrointestinal tract

ICR - Ion cyclotron resonance

IgE - Immunoglobulin E

IgG - Immunoglobulin G

IEM - Ion evaporation model

IPA - Isopropanol

IS - Internal standard

LC - Liquid chromatography

LC-MS - Liquid chromatography-mass spectrometry

LLE - Liquid-liquid extraction

LLOQ - Lower limit of quantitation

LOD - Limit of detection

LOX - Lipoxygenase

LT - Leukotriene

LTE4 - Leukotriene E4

MAX - Mixed-mode anion exchange

MCP - Microchannel plate

MeOH - Methanol

MRM - Multiple reaction monitoring

PAF - Platelet activation factor

PMT - Photomultiplier tube

Q-TOF - Quadrupole time-of-flight

QC - Quality control

RF - Radio frequency

RSD - Relative standard deviation

PLA_2 - Phospholipase A_2

ppm - Parts per million

RPLC - Reversed-phase liquid chromatography

SPE - Solid-phase extraction

S/N - Signal-to-noise ratio

SRM - Single reaction monitoring

SRS-A - Slow reacting substances of anaphylaxis

TOF - Time-of-flight

UHPLC - Ultra-high-performance liquid chromatography

ULOQ - Upper limit of quantitation

WAO - World Allergy Organization

WAX - Weak anion exchange

1. Introduction

1.1. Introduction to anaphylaxis

The term anaphylaxis, which refers to a severe allergic reaction, was coined by French physiologists Charles Robert Richet and Paul Portier.[1] Charles Robert Richet received the Nobel Prize in Medicine or Physiology in 1913 for his research on anaphylaxis.[1] According to the World Allergy Organization (WAO), "anaphylaxis is a life-threatening hypersensitivity reaction which is rapid in onset and could be fatal."[2]

1.1.1 Triggers of anaphylaxis

WAO has classified triggers of anaphylaxis into three broad categories based on the mechanism of trigger: IgE-dependent immunologic factors, IgE-independent immunologic factors and non-immunologic factors.[2] IgE-dependent immunologic factors are the most common of the three triggers.[2] IgE-dependent immunologic factors include foods (such as peanuts, tree nuts, shellfish, fish, eggs, soybeans, milk and other dairy products), venoms (such as insect bites), medications (such as β-lactam antibiotics, non-steroidal anti-inflammatory drugs and other biologic agents), natural rubber latex, occupational agents and aeroallergens. IgE-independent immunologic factors include radiocontrast media, certain biologic agents and autoimmune mechanisms. Physical factors like exercise, heat, cold, certain medications like opioids and ethanol are the non-immunologic triggers of anaphylaxis. Certain triggers such as medications, venoms and radiocontrast media can act via more than one mechanism.[3]

1.1.2 Epidemiology

Recent studies have suggested that there has been an increase in hospital admissions of allergy cases in the past few years. In a 2015 survey of National Centre for Health Statistics, a total of 26.5 million people were reported to have allergies (hay fever and food, skin and respiratory allergies) in the US.[4] In another study conducted by collecting data from national databases that recorded hospital admissions of anaphylactic patients in England and Wales from 1992-2012, a 7-fold increase in hospital admissions of anaphylactic patients was found.[5] Worldwide, there has been a 5-7 fold increase in anaphylaxis cases in the past 10-15 years.[6] This conclusion is based on

clinical data of hospitalized patients.[6] The highest increase was seen in children in the age group of 0-9 years with food and drugs acting as the most common triggers.[6] Though anaphylaxis can be fatal, no significant increase in anaphylaxis fatalities has occurred in the past 10-15 years.[5,6] As part of the Cross Canada Anaphylaxis Registry (C-CARE) program, a 4-year study was conducted at the Montreal Children's Hospital from April 2011-April 2015. More than a 2-fold increase in emergency visits of anaphylactic pediatric patients was found over this time period. The most common trigger was food allergies involving mainly nuts and milk.[7] Thus, the increasing number of anaphylaxis cases in children and adolescents is concerning.

1.1.3 Pathophysiology

Anaphylaxis can occur through immunologic and non-immunologic mechanisms.[2] Immunologic anaphylaxis includes IgE-dependent reactions and IgE-independent reactions.

In IgE-dependent pathways, an exposure to allergen activates immune system, resulting in secretion of IgE.[8] Secreted IgE binds to FcεRI (an Fc receptor for IgE) on basophil and mast cell membranes.[8] This IgE-receptor binding and crosslinking of IgE bound FcεRI activates downstream signaling pathways in basophils and mast cells to release intracellularly stored mediators like histamine and certain proteases such as tryptase, chymase and carboxypeptidase A3.[3,8] The signaling pathways also lead to an increase in intracellular Ca^{2+} concentration, by up taking extracellular Ca^{2+} as well as by releasing stored intracellular Ca^{2+} in endoplasmic reticulum, which leads to the activation of phospholipase A_2 (PLA_2) present in cell membranes. PLA_2 causes a release of arachidonic acid, which is further metabolized to release lipid mediators such as leukotrienes (LTs) and prostaglandins.[8] Neutrophils have also been considered as potential effector cells for anaphylaxis along with mast cells and basophils. Upon activation, neutrophils release platelet activation factor (PAF) in addition to histamine release.[9] IgE-independent reactions are mediated by other immunoglobulins such as IgG, but so far this has only been demonstrated in mice models and has yet to be demonstrated in human subjects.[9] Non-immunologic anaphylactic reactions occur due to a direct activation of mast cells, mainly by physical factors like heat, cold and exercise with mechanisms not yet fully understood.[10]

Histamine and tryptase (released during IgE-dependent reactions) have been long considered as main mediators of anaphylaxis. LTs and prostaglandins have also been implicated in physiological

changes during anaphylaxis.[9] These are called slow-reacting substances of anaphylaxis (SRS-A) since they are released later during anaphylactic reaction and participate in the progression of anaphylactic symptoms.[8] Chemical structures of these mediators are shown in Figure 1.1.

The main physiological changes that are caused by mediators via various pathways include smooth muscle contraction, increased vascular permeability, vasodilation, edema, bronchoconstriction, bronchial mucus secretion, leukocyte adhesion and migration, nerve stimulation, proinflammatory responses and changes in myocardial contractility.[11]

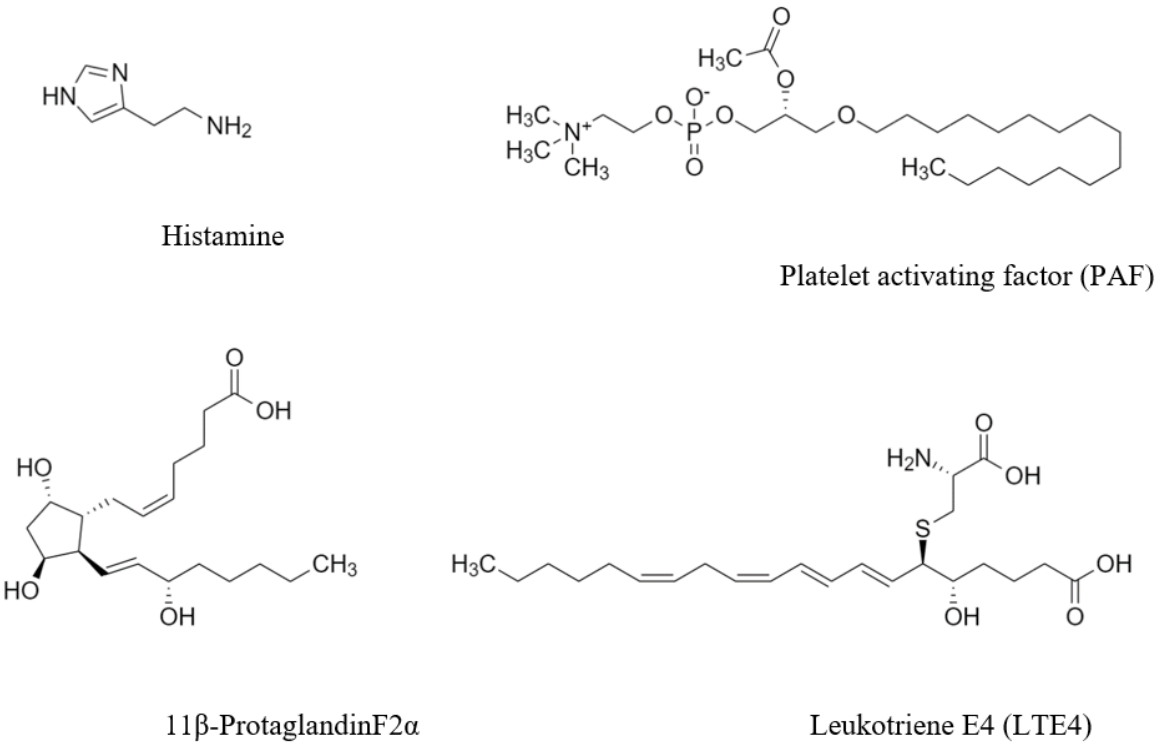

Figure 1.1 Chemical structures of some of the mediators of anaphylaxis

1.1.4 Symptoms and management

The above-mentioned physiological changes caused by mediators result in clinical manifestations involving the target organs: skin, respiratory system, gastrointestinal tract (GI), cardiovascular system (CVS) and central nervous system (CNS).[3] These signs and symptoms are listed in Table 1.1. In 90% of anaphylaxis cases, skin is the target organ and patients show common symptoms

like urticaria (hives), flushing and angioedema (swelling of the mucosal layer of the skin).[13] The respiratory system is the target organ in 70% of cases, followed by GI, CVS and CNS.[3]

Table 1.1 Common signs and symptoms of anaphylaxis. Table created using information from reference 1 and 2.[1,2]

Organ system	Anaphylaxis symptoms
Skin	Hives (urticaria), itching, flushing, angioedema, swelling of lips/tongue
Respiratory	Dyspnea, cough, hoarseness, pain with swallowing cough, stridor, wheeze, nose/throat itching, respiratory arrest
GI	Nausea, vomiting, diarrhea, abdominal pain, dysphagea
CVS	Hypotension, dizziness, incontinence, chest pain, tachycardia, bradycardia (less common), other arrhythmias, palpitations, shock, cardiac arrest
CNS	Headaches, lightheadedness, loss of consciousness, confusion, tunnel vision

For acute treatment, a first assessment of airway, breathing, circulation and orientation and skin evaluation is performed. This is followed by an intramuscular epinephrine injection, which is the first line of treatment for anaphylaxis.[3] The auxiliary treatment, also known as second line of treatment, could include additional medications like antihistamines, vasopressors, β_2-agonists and glucocorticoids and cardiopulmonary resuscitation (CPR), if needed.[2,3] Measures for long-term risk reduction include avoidance of allergens and emergency preparedness (mainly epinephrine autoinjectors).[3]

1.1.5 Diagnosis and currently available laboratory tests

Clinical diagnosis of anaphylaxis first involves a detailed patient history of exposure to potential triggers of anaphylaxis and time taken from the exposure to advancement of symptoms, which can range from minutes to hours. Currently, plasma histamine levels and serum total tryptase levels are used for the clinical diagnosis of anaphylaxis.[14] These are measured using commercially-available enzyme immunoassay (EIA) kits. One example of an enzyme-linked immunosorbent assay (ELISA) kit used clinically to measure serum tryptase is the ImmunoCAP Tryptase kit from Phadia; Uppsala, Sweden. This is a sandwich ELISA kit and utilizes mouse monoclonal anti-

tryptase antibodies.[15] As reported by the kit manufacturer, the average total serum tryptase level in healthy individuals is about 3.4 ng/mL, whereas a value of 10-20 ng/mL represents increased mast cell burden indicating severe anaphylactic reactions.[15] The limit of detection for kit is 1 ng/mL.[15] Another example is the histamine enzyme immunoassay (EIA) kit by Cayman Chemicals.[16] This is a competitive assay that uses free histamine and acetylcholinesterase-linked histamine, which compete for mouse anti-histamine antibody. The limit of detection as reported by the manufacturer is 0.06 ng/mL.[16]

Plasma histamine levels peak as soon as they are within 5 minutes of the onset of the symptoms and decline to baseline in 60 minutes.[14] Therefore, the sample should be collected within 5 to 60 minutes of the onset of the symptoms, which makes it impractical in numerous circumstances. For instance, plasma histamine levels may reach baseline levels by the time the patient reaches a health-care facility.[14] On the other hand, samples to evaluate serum tryptase levels should be collected within 3 hours of the onset of the symptoms. Thus, this test is more widely used than measuring plasma histamine.[14] However, serum tryptase levels are rarely elevated in food-induced anaphylaxis, which is the most common trigger of anaphylaxis.[3] Hence, the absence of elevated serum tryptase alone cannot be used to rule out anaphylaxis. Moreover, the total tryptase level could also be elevated in conditions other than anaphylaxis like mastocytosis, myelocytic leukemia and renal diseases.[14] Hence there is a need to assess other biomarkers of anaphylaxis.

1.2. Why assess urinary 11β-PGF2α and LTE4?

1.2.1 Metabolic pathways for generation of 11β-PGF2α and LTE4

As previously mentioned in Section 1.1.3, in IgE-dependent pathways of hypersensitivity, the interaction of allergens with immune system leads to activation of PLA_2 in immune cell membranes, which causes the release of arachidonic acid from cell membranes.[8] Arachidonic acid is metabolized by oxidation via three different pathways: the cytochrome P-450 pathway, cyclooxygenase (COX) mediated pathway and lipoxygenase (LOX) mediated pathway. The COX pathway leads to the formation of prostaglandins such as prostaglandin H2, prostaglandin I2, prostaglandin G2 and prostaglandin D2. Prostaglandin D2 is further metabolized to 11β-PGF2α. The LOX pathway leads to the formation of leukotrienes, such as leukotriene A4, leukotriene B4, leukotriene C4, leukotriene D4 and leukotriene E4. [17] The sequence of pathways leading to the

production of LTE4 and 11β-PGF2α are represented in Figure 1.2. Arachidonic acid can also be oxidized via non-enzymatic pathways, because of oxidative stress, leading to the formation of prostaglandin isomers, also known as isoprostanes.[17] Arachidonic acid metabolites, also known as eicosanoids, are excreted in urine as free acids as well as conjugates such as glucuronide conjugates to facilitate excretion by making analytes more polar. Mainly, prostaglandins are excreted as glucuronide conjugates.[18]

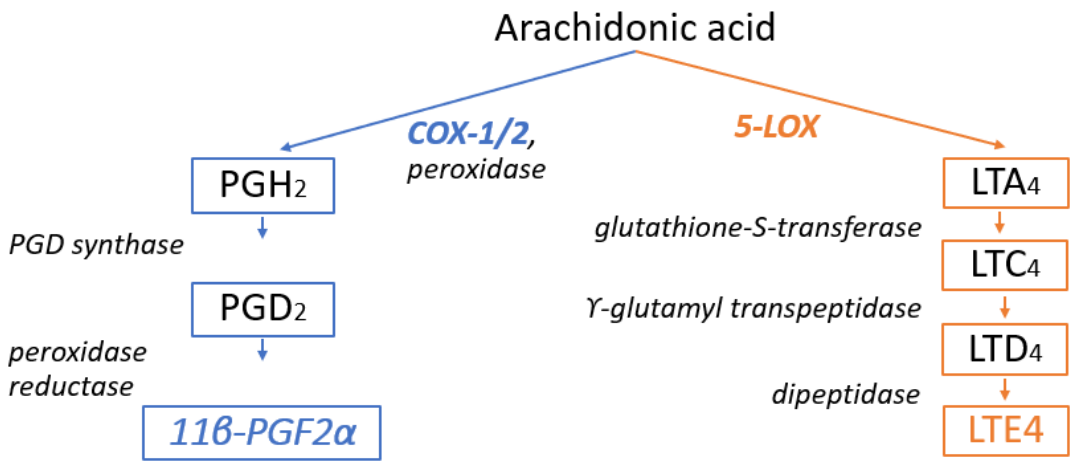

Figure 1.2 Metabolism of arachidonic acid to generate 11β-PGF2α and LTE4. Figure created using information in reference 19.[19]

Leukotrienes and prostaglandins have been implicated as biological mediators of hypersensitivity reactions.[20] Histamine and tryptase are classified as primary mediators since these are pre-formed, stored in mast cells and released upon mast cell activation. In contrast, leukotrienes and prostaglandins are produced by oxidation of arachidonic acid by the enzymatic cascade (shown in Figure 1.2) and hence are secondary mediators. Leukotrienes mediate increased vascular permeability, mucus production and bronchoconstriction whereas prostaglandin D2 has been implicated in causing bronchoconstriction.[8] LTE4 is the most stable and reliable leukotriene to monitor endogenous cysteinyl leukotriene synthesis and 11β-PGF2α is major stable metabolite of prostaglandin D2.[21]

1.2.2 Previous studies with urinary 11β-PGF2α and LTE4 as potential biomarkers

Ono *et al.* investigated the change in urinary concentrations of 11β-PGF2α and LTE4 during anaphylaxis using an enzyme immunoassay kit.[20] This was a hospital-based study that compared urinary levels of 11β-PGF2α and LTE4 in anaphylactic patients (n = 32) to a healthy group (n = 15).[20] 11β-PGF2α was measured after extraction through C18 disk cartridge.[20] It was found that urinary LTE4 concentration was significantly higher (p = 0.006) in the anaphylactic group (median = 772 pg/mg creatinine) than in the healthy group (median = 66 pg/mg creatinine).[20] Similarly, urinary 11β-PGF2α was also significantly higher (p = 0.036) in anaphylactic group (median = 279 pg/mg creatinine) than the healthy group (median = 82 pg/mg creatinine).[20] However, the correlation between concentrations of 11β-PGF2α and LTE4 was poor (r = 0.672) in the anaphylactic group.[20] This suggests that levels of both analytes do not increase at the same rate in the anaphylactic patients.

Higashi *et al.* also assessed urinary 11β-PGF2α and LTE4 as potential biomarkers of anaphylaxis in a hospital-based study.[21] They compared urinary levels of 11β-PGF2α and LTE4 in eight anaphylactic subjects to their baseline levels, using enzyme immunoassay and found that urinary concentrations of LTE4 were 6.5 times higher and urinary concentrations of 11β-PGF2α were 11 times higher in the subjects compared to their baseline concentrations.[21] The reported urinary 11β-PGF2α was 180.4 pg/mg creatinine, which is similar to what was previously reported by Ono *et al.* in the anaphylactic group and reported urinary LTE4 concentration was 1189.5 pg/mg creatinine, which is higher than what was reported by Ono *et al*.[21] They also showed a poor correlation between concentrations of 11β-PGF2α and LTE4 (r = 0.44).[21]

1.2.3 Why assess urinary 11β-PGF2α and LTE4 again?

One of the major shortcomings of previous studies to measure urinary 11β-PGF2α and LTE4 is the cross-reactivity of immunoassays used for quantitative measurement of these two analytes, considering the several structural isomers exist for prostaglandins and two known isomers of LTE4. Some immunoassays may also recognize analyte conjugates such as glucuronide conjugates of PGFs.

Even though immunoassays are very sensitive and easy to use, they suffer from major limitations such as antibody cross-reactivity, poor reproducibility and high overall cost per sample. Cross-

reactivity will be particularly problematic for eicosanoids where many isomers exist. Further the molecules are too small to use a sandwich assay to improve immunoassay selectivity. Batch-to-batch variability of antibodies and variability between antibodies from different manufacturers is also a problem with immunoassays for eicosanoids. The equipment cost is low in immunoassays, but the reagent costs make the overall cost per sample and daily running cost relatively high. Liquid chromatography-mass spectrometry (LC-MS) can overcome some of these limitations of immunoassays. LC separation coupled with modern high-resolution mass spectrometers provides good selectivity. Selectivity is improved both through chromatographic separation, parent mass and characteristic fragment ions. LC-MS also provides the advantage of multiplexing, which means that several biomarkers can be measured in the same run. The day-to-day running cost is low with LC-MS due to low cost of chromatography consumables. Typically, a LC column would be useful for 500-2000 runs before requiring replacement. The solvent requirements are even lower if using ultra-high-performance liquid chromatography (UHPLC) due to shorter run times. The inter-day and intra-day assay reproducibility is high. Thus, many of the disadvantages of immunoassays can be overcome by using LC-MS, after an initial expensive investment in equipment.[22]

Another issue with previous studies is that they were performed in small cohorts. Also, different approaches were used to compare urinary analyte levels in both studies. Eno *et al.* compared the levels between two different groups whereas Higashi *et al*. compared the elevated levels in the same subject group with their baseline levels.[20,21] In order to overcome all these shortcomings, there is a need to further assess urinary 11β-PGF2α and LTE4 in a bigger cohort of anaphylactic patients with an analytical method capable of distinguishing between isomers that will ensure an accurate measurement of the concentrations of the correct analyte.

1.3. Urine sample pretreatment and normalization

Human urine is composed of 90-96% (v/v) water, creatinine, urea, salts, proteins and lipids. Urea, creatinine, hippuric acid and citric acid are the most abundant organic metabolites in urine.[23] One of the advantages of working with urine over other biofluids such as bronchoalveolar lavage fluid and plasma is that urine collection is non-invasive.[23] Other advantages are that urine samples can be available in large quantities if needed and sample preparation is less complex since the protein

and lipid content is lower compared to plasma. On the other hand, many analytes and metabolites can be excreted as conjugated products in urine. For example, with glucuronide conjugates, sulfate conjugates and sulfoglucuronides, the sample preparation may become complex because an extra step of hydrolysis of conjugated analytes needs to be included in the sample preparation in order to measure the total metabolite levels accurately.[23] Another problem with the urine matrix is the variable volume, since this depends on physiological conditions and the water intake of the individual. The urine volume and hence the concentration of metabolites may vary in different individuals and may also vary for the same individual depending upon the hydration levels at the time of urine collection. Therefore, an appropriate normalization step should be included in sample pre-treatment steps, such as creatinine concentration, osmolality or total MS signal normalization.[23] Creatinine normalization is the most common approach used for volume correction and for reporting analyte concentrations in urine.[23] Creatinine can be measured via LC-MS but care should be taken that its concentration is not outside the linear range of the instrument since it is present in high abundance in urine. For this reason, spectrophotometric methods are preferred over LC-MS for creatinine measurement.[23] For example, Clinitek Status®+ Analyzer (Siemens Healthcare Diagnostics) measures creatinine in urine samples spectrophotometrically by reading the absorbance of strips dipped in urine. The color change in the strip is directly proportional to the concentration of creatinine in urine.

Normal human urine pH can range from 5.5-7 and hence the pH adjustment should also be included during sample pre-treatment especially for ionizable analytes, to ensure that small differences in the sample pH do not adversely affect analyte recovery.[23]

1.4. Sample preparation for urine

Sometimes, dilute-and-shoot technique is used to analyze urine samples. In this method, the urine sample is diluted by appropriate buffer or water and injected directly into the LC-MS for analysis without any prior treatment or preparation.[23] This technique is suitable if the target analytes are present in high concentration and there is no interference with other analytes. If the target analytes are present in low abundance, below the detection limit and/or below the lower limit of quantitation (LLOQ), then a sample clean-up and preconcentration method can be used to quantify analytes

after enrichment. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) are commonly used for sample clean-up of urine.[23] LLE is useful for separation of polar and non-polar metabolites but it does not give a high pre-concentration factor, whereas SPE gives a higher preconcentration factor and is very suitable for targeted analysis because of the several types of sorbents available commercially.[23] Hence, SPE gives better selectivity for targeted analysis. After clean-up, the samples are evaporated or lyophilized and then reconstituted in a smaller volume of solvent to get a desired pre-concentration factor.[23] Considering that LTE4 and 11β-PGF2α are present in low levels in urine, SPE was selected to be used in this research and is discussed in more detail below.

1.4.1 Solid phase extraction

1.4.1.1 SPE principle, formats and retention mechanisms

SPE is a sample clean-up and enrichment technique. In SPE, solid particle packing material is used to separate components of a sample. The liquid sample is loaded on a previously-conditioned SPE sorbent. The sorbent is washed to remove unwanted interferences and then analytes are eluted with an appropriate solvent/buffer (Figure 1.3). Various sorbents are commercially available such as normal-phase, reversed-phase, ion-exchange, affinity and mixed-mode sorbents.[24] The sorbent is selected depending upon the retention mechanism of analytes of interest. Sorbents are available in different formats, such as cartridges, 96-well plates and small barrels.[24] The selection of the SPE format depends on the analytical needs. For example, a 96-well plate design is useful for automated high-throughput sample preparation. Small barrel design is useful for low sample loading volumes (in microliters). A cartridge design is the most commonly used format when mL sample loading volumes are needed.

In reversed-phase SPE such as C18, the sorbent is non-polar and non-polar analytes are retained on cartridge. In anion-exchange SPE, the sorbent particles are positively charged to retain anions, whereas in cation-exchange SPE, the sorbent particles are negatively charged to retain cations. Ion-exchange sorbents can be strong ion-exchange or weak ion-exchange sorbents. In strong ion-exchange SPE, sorbents have ionic groups that are always charged such as, for example, sulphonic acids and quaternary amines. These sorbents are used if the analytes to be retained are weakly charged. In weak ion-exchange, the ionic groups are not always charged depending on the pH, for

example, carboxylic acids and secondary amines. Weak ion-exchange sorbents are used for the retention of strongly acidic and strongly basic analytes. In mixed-mode ion-exchange SPE, the reversed-phase sorbent is modified to retain non-polar as well as charged analytes via either strong (mixed-mode anion exchange (MAX)) or weak (mixed-mode weak anion-exchange (WAX)) ion-exchange. Another type of mixed-mode sorbent is hydrophilic lipophilic balanced (HLB) sorbent in which the sorbent has reversed-phase properties as well as functional groups to retain polar analytes. Prostaglandins and leukotrienes are non-polar and have weakly ionizable anionic functional groups such as carboxylic acids. Hence, in this study C18, HLB, MAX and WAX SPE were selected for extraction to exploit non-polar and/or electrostatic interactions.

1.4.1.2 Optimization of SPE steps

SPE steps include sorbent conditioning and equilibration, sample loading, wash, elution and drying and reconstitution (Figure 1.3). Conditioning and equilibration are performed to wet the sorbent pores, which helps analytes to flow in and out of these pores and to ensure good extraction. Usually organic solvents such as acetonitrile (ACN) and methanol (MeOH) are used for conditioning and a weaker solvent is used for equilibration, for example, water in the case of reversed-phase sorbent. Sample loading is the next step in SPE. Sample loading volume is optimized so that loading volume does not exceed the binding capacity of the sorbent. Other loading conditions are also optimized such as the pH. For example, if the analytes are weak anions and MAX sorbent is used, then loading the pH should be at least 2 pH above the lowest pKa in the sample to ensure that the analytes will be fully ionized to be able bind to MAX sorbent for retention. During pH optimization, the working pH range of sorbent is also considered. Loading pH should be within the working pH range of the sorbent for the sorbent chemistry to work properly and bind the analytes of interest. In the wash step, the solvent composition and volume is optimized to remove interferences while retaining the analytes of interest. For example, if polar interferences are to be removed using C18 reversed-phase SPE, then weakly organic wash solvent can be used. If the sample matrix is complex, multiple wash steps with different solvent compositions and pH are used to remove different types of interferences. Stronger solvents are used for the elution step and may include modifiers such as acetic acid and formic acid to change the pH. The elution solvent composition is optimized to elute the retained analytes of interest while leaving the interferences

that might have been more strongly retained on sorbent than analytes of interest. The elution solvent volume is optimized to ensure elution of all the retained analytes. During all of the above steps, a constant flow rate is used for proper retention and elution. For this purpose, sometimes a positive pressure pump or vacuum is used to generate positive pressure for a constant flow rate.[24] Drying and reconstitution is performed after the elution step if a change of solvent composition or pre-concentration of analytes is needed. Usually, high organic percentage is used in SPE elution in order to elute maximum possible analytes. If the subsequent LC analysis is performed using reversed-phase chromatography, then a change of solvent will be required. This is done to ensure good peak shapes since for good chromatography, the injection solvent should be weaker than the mobile phase. For eluate drying, vacuum drying, or flow of nitrogen is usually used. The reconstitution solvent composition and volume is optimized to ensure a complete dissolution of analytes of interest from the container walls. The reconstitution solvent composition should be compatible with the instrument with which sample is to be analyzed and a sufficient volume of solvent should be used to ensure a reproducible recovery of the analytes.

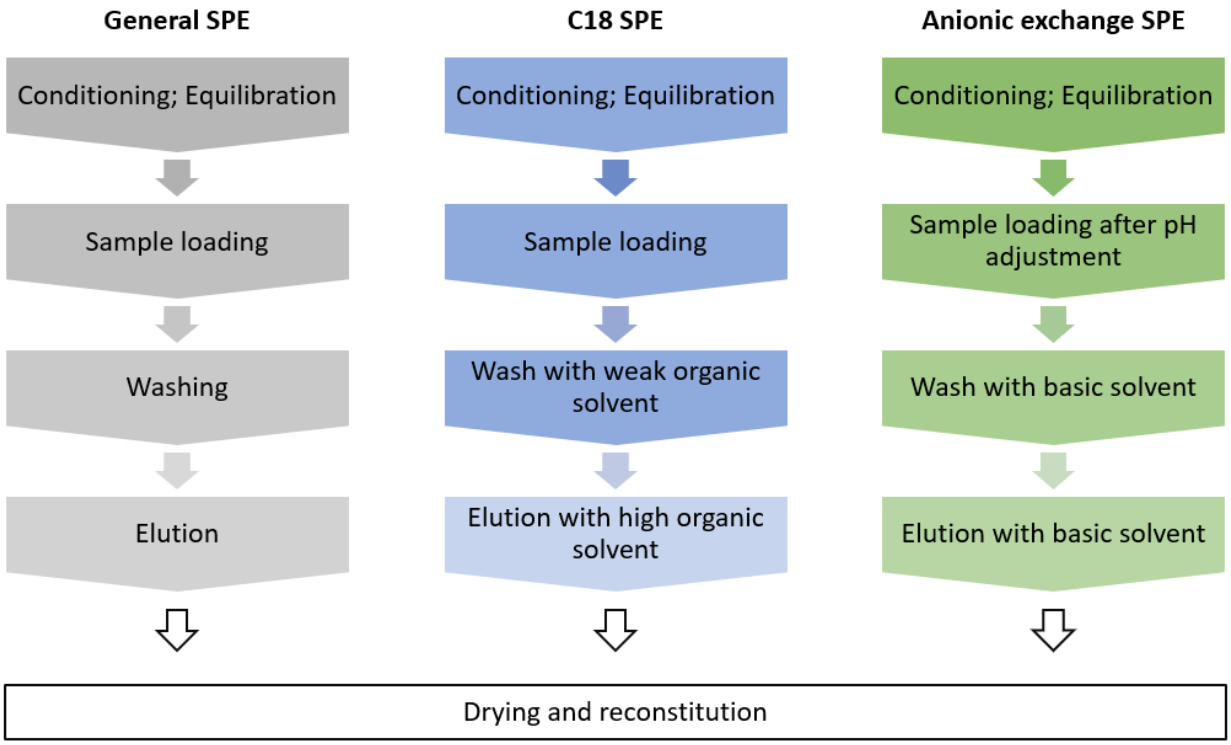

Figure 1.3 Main steps of SPE showing the wash and elution solvent compositions in reversed-phase and anion-exchange SPE.

1.4.1.3 Evaluation of recovery, repeatability and matrix effects

SPE performance is evaluated using the recovery of analyte, matrix effects and repeatability. The recovery is calculated as the percentage of analyte recovered from SPE over the mass of analyte that was loaded on cartridge. A 100% recovery is ideal, meaning that all the analyte that was loaded on the cartridge was recovered and there were no losses along the extraction procedure. To calculate SPE recovery of endogenous analytes accurately, the response due to endogenous concentrations of the analyte must be subtracted from the final response of the spiked samples in order to calculate the amount of spiked analyte recovered from the SPE procedure. In combination with LC-MS analysis, SPE recovery of endogenous analytes is calculated as follows:

$$\%Recovery = \frac{(Average\ response\ in\ pre-spiked\ samples) - (endogenous\ response)}{(Average\ response\ in\ post-spiked\ samples) - (endogenous\ response)}\ x\ 100\%$$

Equation 1.1 % recovery calculation for endogenous analytes.

The above equation is used to eliminate the effect of ionization matrix effects on recovery calculation.

An ionization matrix effect is defined as the enhancement or decrease in the analyte signal than the actual signal (signal in neat solvent) due to the presence of co-eluting species in the matrix. The co-eluting species affect the signal of the analyte by competing with the analyte for a limited amount of charge present in the ionization chamber. These co-eluting species may be present in the sample matrix or may originate from the SPE cartridge material. Matrix effects arising from the cartridge (eg. plasticizers) can be evaluated by comparing a post-spiked blank SPE extraction signal with the analyte signal in the solvent. Ideally, there should not be any matrix effects originating from the cartridge. If there are matrix effects from cartridge, then additional cartridge washing steps should be included during or prior to the sample preparation in order to remove interferences coming from the cartridge. Another solution to this problem is to perform blank water extractions with the cartridge before performing biological sample extraction to get rid of the interferences coming from the cartridge.

For endogenous analytes, matrix effects due to co-eluting interferences are calculated by comparing the analyte response in the spiked matrix (after subtraction of endogenous response) to the analyte response in the neat solvent (Equation 2.3). If % response is more than 100%, then this is called ion enhancement and if it is less than 100% it is called ion suppression. Ideally, there should not be any matrix effects, but in general, the acceptable range for % response is 80-120% for the method to be considered free of significant ionization matrix effects. One of the goals of optimizing SPE washes is to ensure that matrix effects are within acceptable 80-120% range and that there is no significant suppression.

The selectivity of the SPE sorbent and the extent of the SPE enrichment can impact matrix effects. For example, changing the SPE sorbent can help to reduce ion suppression by removing these interferences if ion suppression is an issue. Similarly, a higher SPE enrichment may also impact matrix effects because a higher enrichment will result in an increase in the concentration of analytes as well as an increase in the concentration of co-eluting interferences. This will increase matrix effects because the amount of charge is limited in the ionization chamber.

Usually in LC-MS analysis, internal standards (IS) such as deuterated standards are used to compensate for both recovery and matrix effects. The IS behave the same as the analytes and compensate for the SPE recovery by undergoing similar losses as the analytes during the sample preparation. IS co-elute with analytes during LC-MS analysis and hence undergo a similar ion suppression or ion enhancement as the analyte, thus, compensating for matrix effects. The IS are usually added at the same concentration to all samples at the beginning of the sample preparation so that they correct for as much variability as possible during the sample preparation and LC-MS analysis. During data analysis, in order to cancel the variability in the recovery and matrix effects, the ratio of signal of the analyte to the IS signal is used for quantitation purposes.

1.5. Liquid chromatography-mass spectrometry (LC-MS)

1.5.1. Liquid chromatography (LC)

In LC, a solid stationary phase is used to separate sample components by utilizing the interaction of sample components with solid phase and mobile phases. The separation occurs because of the

different distribution coefficients of the components of mixture in mobile phase and stationary phase. Similar to SPE as described in Section 1.4., different LC columns are available such as normal-phase, reversed-phase, adsorption, size-exclusion and ion-exchange chromatography. Reversed-phase liquid chromatography (RPLC) is most commonly used for the separation of non-polar analytes such as leukotrienes and prostaglandins of interest in this work.[25] In RPLC, the stationary phase is non-polar. The column separation efficiency is inversely proportional to the particle size of sorbent but, as the particle size decreases, column backpressure increases. To achieve a better resolution of prostaglandin and leukotriene isomers, in this study ZORBAX Eclipse Plus C18 with 1.8 μm particle size was used, which works well at high pressures (up to 1200 bars) and is suitable for UHPLC instruments. UHPLC was selected over HPLC because UHPLC gives better peak resolution and decreases analysis time.

Methanol and acetonitrile are commonly used organic solvents for LC-MS mobile phase composition.[26] Volatile solvents and buffers are used in LC-MS mobile phases because the solvent is evaporated at LC-MS interface before ionization.[26] Volatile modifiers/additives are also added in mobile phase or are infused post-column. The purpose of the additives is to improve analyte ionization. Examples of such additives are acetic acid, formic acid, ammonium acetate and ammonium formate. Non-volatile salts and surfactants cannot be used because they would contaminate the ion source.[26]

1.5.2. Electrospray ionization (ESI)

For MS analysis, the analytes must be present as gas-phase ions in order to not disrupt the high vacuum (10^5-10^7 Torr) at which the mass analyzers operate. Different types of interfaces can be used to convert LC eluent into gas phase ions, but electrospray ionization is the most common and widely applicable ionization interface. Dole *et al.* were the first to use electrospray ionization (ESI) with MS in 1968 where they showed that macromolecules in liquid phase could be converted into macroions in the gas phase by electrospraying solution in the nitrogen bath.[26] However, the technique became more widely adopted since the seminal work by John Fenn, which earned him the Nobel Prize in 2002.[27]

ESI is an atmospheric ionization LC-MS interface in which the LC eluent is introduced by a nebulizer that forms small droplets. A Taylor cone is formed in which a jet of charged

microdroplets is emitted from the capillary. These droplets are formed as a result of strong electric potential between the nebulizer needle and counter electrode as well as a high-speed nitrogen gas flow. These small droplets carry positive or negative charges depending upon the state in which the source is operated. For example, in negative ESI, the capillary tip is negatively charged and so are the droplets. As the droplets travel from the nebulizer tip towards the ESI source into the heated capillary, de-solvation occurs due to evaporation of neutral solvent molecules. This results in a decrease of droplet size. As the droplet size further decreases, the Coulomb repulsion between the charges in the droplet increases and when the surface tension is no longer able to stabilize this repulsion, a Coulomb explosion occurs forming smaller droplets.[28] This is also known as Rayleigh fission.[26] The fission process is repeated until final gas-phase ions are formed.

Three different mechanisms have been proposed for a gas-phase ion release in ESI.[26] These are the ion evaporation model (IEM), charged residue model and chain ejection model. In IEM, charged small ions are ejected from the charged droplet. This model is applicable to low molecular analytes such as prostaglandins and leukotrienes of interest in this work.[26]

In the case of small molecules, usually singly charged ions are produced in ESI.[26] The main molecular ions from ESI are either protonated or deprotonated ions. Adducts are also commonly seen in ESI. In positive mode ESI, the most common adducts are ammonium $[M+NH_4]^+$ and sodium adducts $[M+Na]^+$.[26] In negative ESI, the most common adducts are formate $[M+HFa-H]^-$, acetate $[M+HAc-H]^-$, chloride $[M+Cl]^-$ and dehydrated molecular ions $[M-H_2O-H]^-$.[26] In ESI there is a limited amount of charge present and hence it is a competitive process. This means that all the compounds, analytes and interferences, present in ESI at a given time can compete for the charge resulting in possible ionization enhancement or suppression as discussed in Section 1.4.1.3

ESI is a soft ionization technique, meaning that fragmentation is minimal and the parent ion is usually observed. However, in-source fragmentation of very labile bonds in analytes is still possible.[26] In-source fragmentation is also possible in case of conjugates of analytes such as glucuronic acid conjugates.

1.5.3. Mass spectrometry (MS)

A typical MS system consists of an ion source, a mass analyzer, an ion detector and a control computer system. Mass analyzers are operated at high vacuum in order to avoid the collision of

ions with gas molecules and the subsequent loss of their kinetic energy as well as fragmentation.[26] Mass analyzers separate gas-phase ions based on their mass-to-charge ratios (m/z). There are four types of mass analyzers commonly used in analytical laboratories: (i) quadrupole mass analyzer, which uses four oppositely charged electrodes in which direct current and radio-frequency voltages are varied to focus selected m/z v ions from the ion source to the detector, (ii) ion trap mass analyzers, which first trap ions in an electrostatic field generated between electrodes and then release the ions to go to detector by changing radio-frequency, (iii) ion cyclotron resonance mass analyzers, which use a strong magnetic field to trap ions to move in a circular motion with a frequency dependent on their m/z values and measure the image current induced by these oscillations for all ions simultaneously, and (iv) time-of-flight (TOF) mass analyzers, which separate the ions based on the time required to reach the detector after acceleration of all ions to a particular kinetic energy.[26] In this work, the Q-TOF instrument was used, so it will be discussed in more detail below.

1.5.4. Quadrupole-time of flight (Q-TOF)

Quadrupole time of flight (Q-TOF) mass spectrometer consists of a mass filter to separate desired m/z value ions (precursor ions), a collision cell for fragmentation of precursor ions and a TOF mass analyzer for separation of generated fragments (product ions). Depending upon the analytical need, Q-TOF can be operated in MS1 mode where all precursor ions generated in ion source pass through the quadrupole without filtering, and then go directly to the TOF unit for separation. It can also be operated in MS2 mode where the quadrupole is used to select a parent ion of interest followed by the use of the collision cell to generate product ions, which then go to the TOF unit.

For this research, Agilent 6550 iFunnel Q-TOF was used. A flow chart of key components of Agilent 6550 iFunnel Q-TOF is presented in Figure 1.4.

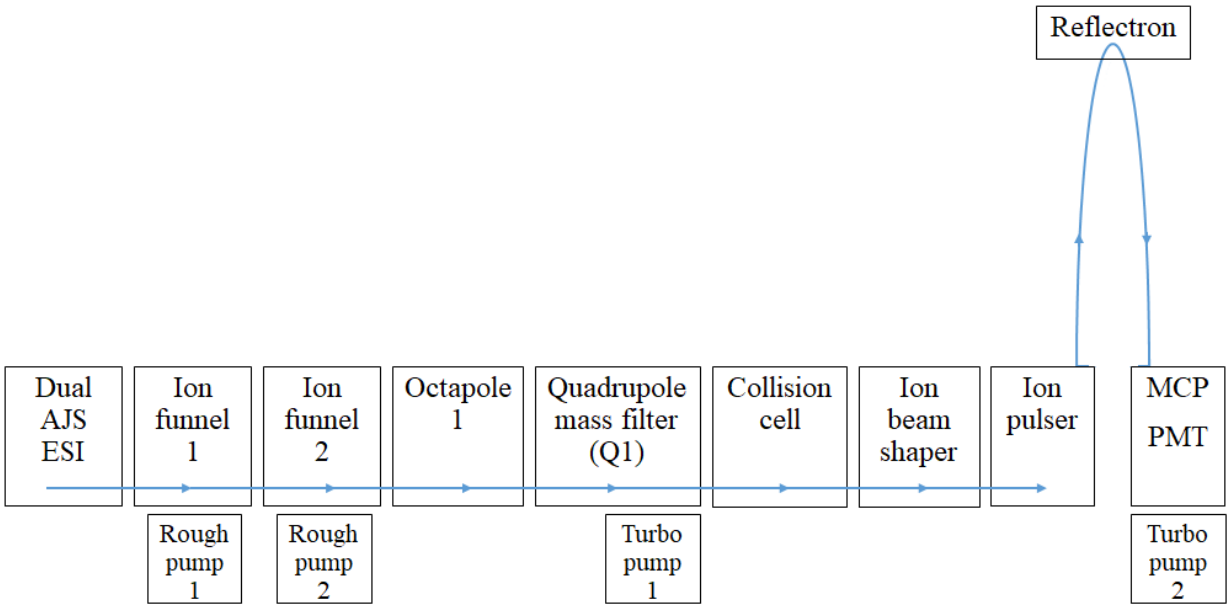

Figure 1.4 Flow chart of main components of Agilent 6550 iFunnel Q-TOF. The blue arrow represents the direction in which ions are transmitted from the ion source all the way to the detector.

This model is equipped with dual Agilent Jet Stream (dual AJS) ESI in which there are two nebulizers: one for a reference spray and one for a LC effluent. For the reference spray, a mixture of calibrant standards is continuously infused for internal mass calibration, which in turn ensures the best mass accuracy. After the ionization in ESI source, the ions enter into a hexabore capillary from where they enter into the dual ion funnel system. The hexabore capillary helps to transfer more ions, formed in ESI, into a mass spectrometer, thus improving the instrument performance for trace analysis. The dual ion funnel system facilitates increased ion transfer and removes neutral noise better, thus improving the instrument sensitivity.[29] In this dual ion funnel system, the front funnel is operated at a high pressure of 7-14 Torr and the second ion funnel is operated at a low pressure of 1-3 Torr.[29] RF voltage is used to focus ions through the funnel and DC voltage is used to accelerate ions to pass through the funnel and enter into the octapole 1. There are two pumps (pump 1 and pump 2) connected to the two ion funnels to remove neutral species. Thus, this setup helps to remove neutral species and gas.[29]

In the MS/MS mode, RF voltage is applied to ions in the octapole 1 that helps to repel a certain range of m/z ions towards the quadrupole mass filter (Q1). This mass filter consists of four hyperbolic rods operating at alternating RF voltages and a constant DC voltage to select and focus

certain m/z ions to pass through the filter. Neutral species and other m/z ions collide with rods and are removed by a turbo pump.[29] The selected ions now enter into collision cell, which is a hexapole operated at a certain potential difference to ensure the ions are transmitted through it. Inert gases such as N_2, are used in collision cell for fragmentation and a set voltage is used to control the energy of collisions.[26] This is known as collision induce dissociation (CID). With the help of a beam shaper, the ions coming out of collision cell are compressed into a denser beam, which then goes into the ion pulser section of the TOF unit.

The ion pulser section of TOF consists of a stack of plates with a hole in the center of each plate. A high voltage pulse is applied to the last plate of the stack, which accelerates the ions orthogonally into the flight tube. The flight tube is about 1 meter in length.[29] The ions travel towards the end of the flight tube where an ion reflectron is present to reflect the ions to make their way towards detector. The purpose of the reflectron is to increase the flight time of ions which improves the resolving power of the mass analyzer and to correct for the difference in kinetic energy of ions with same m/z values. The flight tube is free of an electric field. The potential applied to ions by the ion pulser before flight is converted into kinetic energy of ions. Hence, this potential energy is equal to the kinetic energy of ions. The following equation shows the relationship of m/z of ions with their time of flight:

$$t_{TOF} \quad = \quad \frac{L}{v} \quad = \quad L\sqrt{\frac{m}{2qUa}} \quad \propto \quad \sqrt{\frac{m}{z}}$$

Equation 1.2 m/z of ion is directly proportional to its time of flight, where t_{TOF} is the time of flight of the ion, L is flight distance, v is the velocity of ion after acceleration, q is the charge on the ion, Ua is the accelerating potential difference and m/z is mass to charge ratio of the ion.

Ions coming out of the flight tube then strike the front plate of the detector, which is a microchannel plate (MCP). It consists of many microscopic tubes across the whole length of MCP. When ions hit the front end of the microscopic tubes in MCP with enough energy, electrons are released from the microscopic tube surface. These free electrons further collide with tube surfaces to free up more electrons and this cascade results in a signal amplification up to 10 times.[29] The signal generated from MCP is at very high potential and to convert it into ground potential, a

photomultiplier tube (PMT) is used. For this purpose, after electrons exit from MCP, they hit a scintillator to produce flash light, which is ultimately transmitted onto PMT. The signal from PMT is read by a data system and the output result is generated by the instrument by calculating ions' m/z values from their time of flight.

An external mass calibration is performed by using standards of known masses covering the entire m/z range of the instrument. Internal calibration is performed using known masses by introducing the internal calibrant from the auxiliary nebulizer of dual AJS ESI source. The routine use of internal calibration gives better mass accuracy in long sample batches. Better mass accuracy gives more confidence in analysis.

Mass accuracy and mass resolution: Mass accuracy and mass resolution are two important parameters that are used to evaluate performance of mass analyzers. Mass accuracy (Δm) is defined as the difference of the mass detected by MS from the actual mass of the analyte whereas mass resolution is the smallest difference in masses that can be detected as separate peaks in the spectrum. Mass accuracy is usually expressed in parts per million (ppm). Mass accuracy is calculated by using following equation:

$$mass\ accuracy = \frac{\Delta m}{m_1}\ x\ 10^6$$

Equation 1.3 Mass accuracy calculation, where m_1 is theoretical mass of compound and Δm is the difference observed mass measured by MS and theoretical mass.[26]

For example, if the actual mass of a compound is 354.2406 Da and the observed mass measured by MS is 354.2442 Da then using equation 1.3, mass accuracy is 10.16 ppm.

Mass accuracy is an important parameter because while working with biological matrices, for example, urine, there could be a plethora of interferences with masses close to the analyte of interest and if the mass accuracy and resolving power are higher, it gives more confidence in the accurate analyte detection and measurement. Mass resolving power is calculated using the following equation:

$$Resolving\ power\ = \frac{m}{\Delta m_{FWHM}}$$

Equation 1.4 Mass resolving power calculation, where m is the observed mass and Δm_{FWHM} is mass difference at full width half height.[26]

1.6. Challenges in method development for LC-MS analysis of urinary 11β-PGF2α and LTE4

One of the major challenges of working with eicosanoids is that they are present in trace levels. Reported average urinary LTE4 concentration in normal human subjects is 39.82 pg/mg of creatinine (n = 16),[18] 39.77 pg/mg of creatinine (n = 30),[30] 36.7 pg/mg of creatinine (n = 10)[31] and 66 pg/mg of creatinine (n = 15).[20] Ono *et al.* reported urinary 11β-PGF2α concentrations in healthy subjects (n = 15) as 82 pg/mg of creatinine.[21] Average urinary creatinine in normal human subjects is reported as 1.14 mg/mL,[32] 1.18 mg/mL,[33] and 0.96 mg/mL.[34] Using the average of literature reported values of 11β-PGF2α, LTE4 and creatinine, the expected endogenous level of 11β-PGF2α is ~ 90 pg/mL and of LTE4 is ~ 40 pg/mL. Since these analytes are present in very low concentration in urine, a robust sample preparation method is required to pre-concentrate these analytes in order to analyze them by LC-MS.

Sterz *et al.* reported LTE4 stability in urine at three different concentrations; 0.13 ng/mL, 1.18 ng/mL and 5.02 ng/mL.[30] LTE4 recovery was between 72%-105% at room temperature after 30 hours, 102%-110% after six freeze thaw cycles and 98%-111% at 10 °C after 14 days, at all three concentrations.[30] Armstrong *et al.* also reported acceptable LTE4 recovery in urine after 1, 3 and 5 freeze-thaw cycles (%deviation was -18.3%, -16.33% and -10.79% at 1, 3 and 5 freeze thaw cycles respectively).[31] Thus, from both studies it can be concluded that LTE4 is considerably stable up to 5-6 freeze-thaw cycles. In contrast, not a lot of information is available for the effect of pH on LTE4 stability. Kumlin *et al.* reported that at pH 9, LTE4-d3 recovery in urine was 92%, 92% and 84% after 8, 30 and 60 days, respectively.[35] There is no data regarding LTE4 stability at acidic pH. Similarly, there is very little to no data available in literature for 11β-PGF2α stability. Toewe *et al.* reported autosampler, freeze-thaw and long-term stability of PGF2α isomer in urine.[36] %RSD were reported as 3.3% and 2.5% (at 4.5 ng/l and 16 ng/mL, respectively) for 72 hours autosampler

stability, 1.6% and 2.4% (at 4.5 ng/mL and 16 ng/mL, respectively) after three freeze thaw cycles and 1.9% and 11.9% (at 4.5 ng/mL and 16 ng/mL, respectively) after 90 days storage at -80°C.[36]

Another challenge in the analysis of eicosanoids with LC-MS is the presence of isomers. There are six known isomers of prostaglandinF2: 8-iso-15(R)-PGF2α, 8-iso-PGF2α, 8-iso-PGF2β, 11β-PGF2α, 15(R)-PGF2α and PGF2α and two known isomers of LTE4: LTE4 and 11-trans-LTE4.Their structures are shown in Figure 1.5. Since isomers cannot be distinguished in MS1 and since some isomers may form same fragments in MS2 fragmentation process, it is critical to separate the isomers chromatographically for accurate analysis of a specific isomer such as 11β-PGF2α and LTE4 in our case.

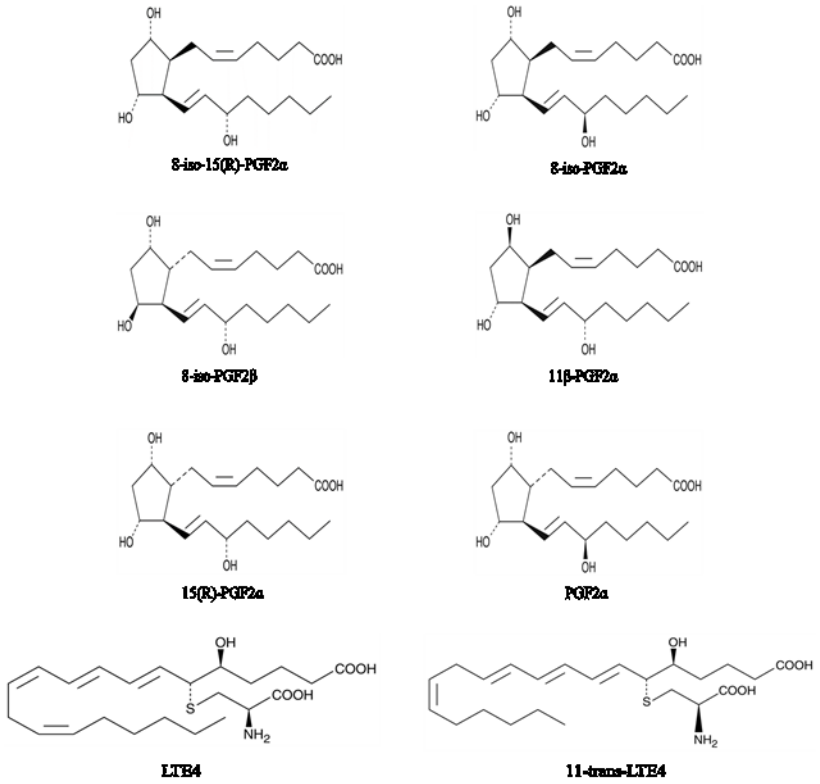

Figure 1.5 Chemical structures of prostaglandinF2 and leukotrieneE4 isomers.[12]

1.7. LC-MS analysis of urinary 11β-PGF2α and LTE4

LC-MS methods and sample preparation methods previously used for the analysis of urinary 11β-PGF2α and LTE4 are summarized in Table 1.2.

Table 1.2 Summary of LC-MS methods used in literature for the analysis of urinary 11β-PGF2α and LTE4.

Citation	Analyte	Sample preparation	LC	MS	Ionization mode	LLOQ
Lueke *et al.* 2016[37]	LTE4	ACN precipitation, filtration, online MAX mixed-mode anion-exchange	C8 Waters Xbridge column (2.1 x 50 mm, 2.5 µm), 60°C Mobile phase: MeOH/NH_4OH H_2O/ NH_4OH	Triple quad (AB Sciex API 5000 MS/MS) <u>MRM transitions:</u> LTE4: 438 → 333, 438 → 235 (CE = 25 V)	Negative	8 pg/mL
Sasaki *et al.* 2015[38]	11β-PGF2α and LTE4	Strata-X-AW weak anion-exchange mixed-mode SPE	Waters Aquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm) Mobile phase: MeOH/ACN/HAc (75:25:0.1) H_2O/HAc (100:0.1)	Triple quad (Xevo TQ-S, Waters) <u>SRM transitions:</u> LTE4: 438 → 333 (CE = 15 eV) 11β-PGF2α: 353 → 193 (CE = 25 V)	Negative	LTE4 was later removed from the panel due to low recovery (36%) 11β-PGF2α: 48 pg/mL

Balgoma *et al.* 2013[18]	11β-PGF2α and LTE4	HLB SPE	Waters Aquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm), 60°C Mobile phase: ACN/IPA/FA (90:10:0.2) H_2O/FA (100:0.2)	Triple quad (Xevo TQ, Waters) SRM transitions: LTE4: 440.3 → 189.2 11β-PGF2α: 353.3 → 291.1	Positive for LTE4 and negative for 11β-PGF2α	LTE4: 263.3 pg/mL 11β-PGF2α: 152.4 pg/mL
Sterz *et al.* 2012[30]	LTE4	LLE (methanol: chloroform), collected chloroform phase	Waters Aquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm), 30°C Mobile phase: ACN:FA (100:0.1) H_2O:FA (100:0.1)	Triple quad (AB Sciex API 5000 MS/MS) <u>MRM transitions:</u> LTE4: 438.0 → 333.1 (CE = 28 V)	Negative	6 pg/mL
Armstrong *et al.* 2009[31]	LTE4	Centrifugation followed by online purification using Agilent Extend-C18 column	Agilent Eclipse C8 (2.1 x 50 mm, 1.8 µm), 40°C Mobile phase: 0.02% AA and 0.007% NH_4OH in ACN 0.02% AA and 0.007% NH_4OH in water	Triple quad (Agilent 6410) <u>MRM transitions:</u> LTE4: 440 → 301, 440 → 189 CE = 8 V	Positive	5 pg/mL

As shown in Table 1.2, both LLE and SPE have been used as sample preparation method in literature, with SPE being used the most commonly. Both negative and positive ionization modes have been in used in literature for LTE4 (Table 1.2). Different methods have shown different LLOQs for both analytes; for LTE4 reported LLOQs vary from 5 pg/mL-263.3 pg/mL and for 11β-PGF2α reported LLOQs vary from 48 pg/mL-152.4 pg/mL (Table 1.2). Some of the LLOQs such as reported by Balgoma *et al.* are higher than normal endogenous levels and hence will not be suitable to measure endogenous levels in our work.[18] Balgoma *et al.* showed LC separation of only two PGF isomers out of six and Sasaki *et al.* showed LC separation of five PGF isomers.[18,38] Sasaki *et al.* later dropped LTE4 from their panel of eicosanoids due to low recovery.[38] Hence both studies, where our two analytes of interest were studied, are not suitable for our work. None of the listed five LC-MS methods in Table 1.2, included 11-trans LTE4 isomer in the panel of eicosanoids. Thus, a highly sensitive LC-MS assay that is fully optimized for recovery of urinary 11β-PGF2α and LTE4 and ensures LC separation of all six isomers of PGFs and two isomers of LTE4 is needed.

1.8. Research objectives

With the increase of the cases of anaphylaxis in the past decade worldwide especially in children and adolescents, there is a need to have tests for anaphylaxis with high selectivity and reproducibility. There is also a need for new reliable biomarkers of anaphylaxis because the currently used biomarkers poorly correlate with food-induced anaphylaxis, such as tryptase; or their blood levels return to baseline level too soon making it difficult to collect the sample before it reaches baseline, such as histamine.[3,14] The aim of the present study is to enable further testing of the recently proposed biomarkers of anaphylaxis: urinary 11β-PGF2α and LTE4, as potential new biomarkers of anaphylaxis.[20,21] The objectives of the research are to optimize LC-MS method for the separation of prostaglandins and LTE4 isomers, develop a solid-phase extraction method to enrich urinary 11β-PGF2α and LTE4 since these are present in pg/mL concentrations and validate the method according to regulatory guidelines.

To develop the method, positive and negative ESI will be tested for LTE4 since LTE4 can ionize in both positive and negative ESI. Reversed-phase, polymeric and anion-exchange SPE will be compared for the recovery of both analytes and matrix effects. This will be done to ensure that the

analytes are not lost during sample preparation and to ensure matrix effects are within 80-120% acceptable range. After the selection of SPE type, SPE steps will be further optimized to achieve the required enrichment factor. In addition, prostaglandins are excreted as glucuronide conjugates in urine and hence, to measure the total endogenous levels of urinary 11β-PGF2α, β-glucuronidase enzyme hydrolysis protocol will also be optimized. The enzyme hydrolysis will be performed before SPE. Since it has been previously reported in literature that LTE4 is not stable at low pH with no detailed data, LTE4 stability will be analyzed at different pH and at higher temperatures for the first time. This will help to select the appropriate pH of SPE loading, washing and elution solvents as well as to decide whether the control of temperature is required to ensure that LTE4 is not lost/degraded during the sample preparation.

The next step after method development will be method validation. The method will be validated as per the United States Food and Drug Administration (FDA) guidelines for bioanalytical method validation. These guidelines are discussed in detail in the following section.

1.9. Food and drug administration guidance for bioanalytical method development

The US FDA provides general recommendations for the validation of bioanalytical methods for quantitative measurements of metabolites and drugs in biological matrices such as plasma, serum, blood or urine. In May 2018, the FDA released a revised guidance for bioanalytical method validation.[39] These guidelines are intended to address if the developed bioanalytical method is sensitive and selective enough to measure intended analytes precisely, accurately and reproducibly in given biological matrix. These guidelines also provide the acceptance criteria for the evaluation of these different parameters.

To ensure the selectivity and specificity of the method FDA requires analyses of blank matrices from at least six different sources and the acceptance criteria is that the blanks should be free of analytes and interferences at the retention time of the analytes and internal standards.[39] The calibration curve must be prepared in the same biological matrix with at least six non-zero points, including LLOQ, blank sample (blank matrix) and zero sample (internal standard + blank matrix) to fit in a regression model of the concentration response curve.[39] For methods that are intended for endogenous compounds or for biomarkers, the FDA directs to use analyte-free biological

matrix or equivalent matrix.[39] Freshly prepared standards should be used for calibration curves.[39] At LLOQ, the response should be at least five times of signal-to-noise ratio. The accuracy at non-zero concentrations is required to be 85-115% for at least 75% of all standards, expect at LLOQ where the accuracy is required to be 80-120%.[39] Similarly for precision, at LLOQ there should not be more than 20% coefficient of variance (CV) and at other non-zero concentrations CV should not exceed 15% for at least 75% of all the standards.[39] The guidelines also direct to prepare quality control (QC) samples in the same biological matrix and the QCs should be used to evaluate the performance of method.[39]

The accuracy, precision and recovery should be evaluated at a minimum of three different concentrations of analytes: low, medium and high concentrations.[39] The FDA does not require the recovery to be 100%, but requires it to be consistent and reproducible within an accepted range of accuracy and precision.[39] The recovery should be evaluated by comparing response in the spiked extracted samples at low, medium and high concentrations with post extraction spiked samples at low, medium and high concentrations respectively as discussed in Section 1.4.1.3.[39] Guidelines direct to evaluate matrix effects by parallelism (serial dilution of sample) and to eliminate any significant interference causing matrix effects for immunoassays. However, there are no clear guidelines provided by the FDA for the evaluation of matrix effects in LC-MS based methods.

The FDA provides guidelines to evaluate the stability of analytes in the given biological matrix throughout the analysis time meaning from the time at which the sample was collected to the time until the sample was analyzed to produce raw data.[39] For this purpose, the FDA requires to perform stability studies at different times and conditions such as autosampler stability, bench-top stability, freeze-thaw stability, stability of processed samples and long term stability.[39] The FDA requires to perform these stability studies with at least three replicates at two levels: low and high concentrations.[39] The acceptance criteria is that the accuracy at nominal concentrations at each level should be ± 15%.[39]

2. Development of an LC-MS assay for putative biomarkers of anaphylaxis: urinary 11β-prostaglandinF2α and leukotrieneE4

2.1. Abstract

Anaphylaxis is a severe and possibly life-threatening allergic reaction characterized by rapid onset. Plasma histamine and serum tryptase measurements are currently used for clinical diagnosis of anaphylaxis. These biomarkers are poorly applicable to food-induced anaphylaxis, have a short measurement window (<60 min) and/or may be elevated in other pathophysiological conditions such as myocardial infraction and renal diseases. Therefore, there is a need to evaluate new biomarkers of anaphylaxis such as recently proposed urinary 11β-prostaglandinF2α (11β-PGF2α) and leukotrieneE4 (LTE4). The objective of this study was to develop an ultra-high-performance liquid chromatography-quadrupole time of flight (UHPLC-Q-TOF) method to measure urinary 11β-PGF2α and LTE4. C18 reversed-phase chromatography and acetonitrile/water/acetic acid mobile phases were used for LC separation of analytes of interest, isomers and LC interferences. Both positive and negative electrospray ionization (ESI) modes were tested for LTE4, and the negative mode was selected based on 7x better signal-to-noise ratios. Mixed-mode anion-exchange solid phase extraction (SPE) and C18 reversed-phase SPE were tested for sample preparation. C18 SPE was selected based on the analyte recovery, matrix effects, enrichment and minimum interferences. A two-step elution protocol was finalized for C18 SPE for separate elution of 11β-PGF2α and LTE4 to improve limits of detection of LTE4 by decreasing background signal and noise. β-Glucuronidase enzyme hydrolysis was used to measure total 11β-PGF2α levels including glucuronides. The method was linear from 9.8 pg/mL to 5000 pg/mL in urine with excellent accuracy and precision. Intra-day accuracies ranged from 90-111% for 11β-PGF2α and 86-108% (% RSD = 4.0-7.4% for 11β-PGF2α and % RSD = 6.8-14.9% for LTE4). Inter-day accuracies were 91-100% (% RSD = 4.2-8.4%) for 11β-PGF2α and 93-113% (% RSD = 4.9-8.8%). LLOQ was 15 pg/mL for both analytes. The method had acceptable recoveries and matrix effects with average recoveries of 92-95% and 77-83% and average matrix effects of 83-88% and 99-115% for 11β-PGF2α-d4 and LTE4-d5, respectively. Both analytes were stable in the freezer for up to three weeks and the prepared samples were stable in an autosampler (6°C) for 48 hours. 11β-PGF2α was stable up to three freeze-thaw cycles. However, LTE4 was stable only up to one freeze-thaw

cycle. In summary, we developed and validated a novel LC-MS method for the analysis of urinary 11β-PGF2α and LTE4, which can be used in future to verify these two analytes as possible biomarkers of anaphylaxis in clinical studies.

2.2. Introduction

Anaphylaxis is a hypersensitivity reaction of human body to antigens.[2] The main triggers of anaphylaxis include certain foods (such as peanuts, shellfish and dairy products), drugs (such as antibiotics and some non-steroidal anti-inflammatory drugs), physical factors (such as exercise and heat/cold temperatures) and other factors (such as insect bites, venoms and radiation). In recent years there has been an increase in emergency visits of anaphylactic patients. For example, the visits of anaphylactic patients to hospitals in England and Wales increased 7-fold from 1992-2012.[5] Globally, in the past 10-15 years there has been a 5-7 fold increase in the number of anaphylactic patients, based on clinical data of hospitalized patients.[6] The most affected age group was children 0-9 years old and the most common causes of anaphylaxis were food and drugs.[6] Another 4-year study that was conducted in the Montreal Children's Hospital from April 2011-April 2015 reported a more than 2-fold increase in emergency visits of anaphylactic children.[7] Food, mainly nuts and milk, were reported as the most common causes for anaphylaxis in this study.[7] Thus, there has been a concerning increase in anaphylaxis cases worldwide in recent years with children and adolescents who are the most affected.

Anaphylactic reaction occurs when antigens bind to IgE antibodies present on the mast cells and basophils causing cross-linking of the antibodies.[8] This cross-linking results in a cascade of downstream reactions, which ultimately results in the release of pre-formed and stored mediators of anaphylaxis such as histamine, and certain proteases such as tryptase, chymase and carboxypeptidase A3.[8] The cross-linking of antibodies also results in the initiation of another pathway in which phospholipase A2 in cell membranes is activated.[8] Phospholipase A2 metabolizes arachidonic acid into prostaglandins (PGFs) and leukotrienes (LTs). PGFs and LTs are called slow-reacting substances of anaphylaxis because they participate in the further progression of anaphylactic symptoms.[8]

Currently for clinical diagnosis of anaphylaxis, plasma histamine and serum tryptase are measured using immunoassays. A major issue with measuring plasma histamine is that its levels drop to a baseline in 60 minutes and hence a plasma sample should be collected within 60 minutes of the onset of anaphylaxis.[14] This is a problem because plasma histamine levels may decline to a baseline by the time the patient reaches a hospital. The issue with measuring serum tryptase is that serum tryptase levels are rarely elevated in food-induced anaphylaxis (which is the most common cause of anaphylaxis) and hence the absence of elevated tryptase in serum cannot be used alone to rule out anaphylaxis.[14] Hence, there is a need to assess new biomarkers of anaphylaxis.

Urinary 11β-prostaglandinF2α (11β-PGF2α) and leukotrieneE4 (LTE4) have been recently proposed as new biomarkers of anaphylaxis.[20,21] Ono *et al.* measured difference in the urinary concentrations of 11β-PGF2α and LTE4 in anaphylactic patients (n = 32) and healthy group (n = 15) using enzyme immunoassays.[20] It was found that urinary 11β-PGF2α was 3.4 times higher ($p = 0.036$) and urinary LTE4 was 11.7 times higher ($p = 0.006$) in anaphylactic group compared to healthy group.[20] Higashi *et al.* also measured urinary 11β-PGF2α and LTE4 as new potential biomarkers of anaphylaxis using enzyme immunoassays and found that urinary 11β-PGF2α was 11 times higher and urinary LTE4 was 6.5 times higher in anaphylactic patients after the onset on anaphylactic reaction compared to the baseline concentrations in the same patients (n = 8).[21] One major issue with both studies is that immunoassays were used for the measurement. The measured levels of analytes may not reflect the actual concentrations because of the possible cross-reactivity to the known six structural isomers of 11β-PGF2α, glucuronide conjugates of 11β-PGF2α and two isomers of LTE4 immunoassays. In addition, both studies were performed in small cohorts, requiring further validation. Hence, the results need to be confirmed in bigger cohorts with an analytical method capable of distinguishing between isomers in order to measure the analyte concentrations accurately. Liquid chromatography-mass spectrometry (LC-MS) is an analytical method of choice in this case since it can give better selectivity than competitive immunoassays. The selectivity in LC-MS is improved by LC separation of isomers and improved confidence in the identification using parent mass and retention time of isomers. In this study, we focused on the development of an LC-MS assay to measure urinary 11β-PGF2α and LTE4 including sample preparation method. Sasaki *et al.* and Balgoma *et al.* used LC-MS methods to measure a panel of urinary eicosanoids including urinary 11β-PGF2α and LTE4.[18,38] However, both studies did not show LC separation of all six isomers of PGFs and neither of the studies included 11-trans

LTE4.[18,38] Sasaki *et al.* also dropped LTE4 from their panel of eicosanoids due to a low recovery (36%).[38] Hence, the development of a new LC-MS method was required to ensure the separation of all six PGF isomers and two LTE4 isomers along with a sample preparation method that can provide acceptable recoveries of both analytes.

The aim of this study was to develop a robust and highly selective LC-MS method for the separation of PGF and LTE4 isomers, optimize β-glucuronidase enzyme hydrolysis protocol for 11β-PGF2α and to develop a SPE method for the enrichment of urinary 11β-PGF2α and LTE4. To the best of our knowledge, for the first time in this research, forced degradation study of LTE4 was performed to investigate the stability of LTE4 at low pH and higher temperatures. The final developed method was validated for selectivity, linearity, accuracy and precision, stability, recovery and matrix effects in human urine.

2.3. Experimental

2.3.1. Materials and reagents

LC-MS grade acetonitrile (ACN), methanol (MeOH), 2-isopropanol (IPA), water, acetic, acid (AA), formic acid (FA), ammonium hydroxide (NH_4OH) and ammonium acetate (NH_4OAc) were purchased from Fisher Chemicals, Ottawa, ON, Canada. 8-iso-15(R)-prostaglandinF2α (8-iso-15(R)-PGF2α), 8-iso-prostaglandinF2α (8-iso-PGF2α), 8-iso-prostaglandinF2β (8-iso-PGF2β), 11β-prostaglandinF2α (11β-PGF2α), 15(R)-prostaglandinF2α (15(R)-PGF2α), prostaglandinF2α (PGF2α), leukotrieneE4 (LTE4), 11β-prostaglandinF2α-d4 (11β-PGF2α-d4), leukotrieneE4-d5 (LTE4-d5) and 11-trans-leukotrieneE4 (11-trans-LTE4) were purchased from Cayman Chemicals, Ann Arbor, MI, USA. β-glucuronidase enzyme from *Helix pomatia* (type HP-2, aqueous solution, ≤ 7,500 units/mL) was purchased from Roche Dignostics, Indianapolis, IN, USA. Oasis MAX and WAX SPE cartridges (60 mg/3 mL) were purchased from Waters, Milford, MA, USA. Strata 200 mg/3 mL reversed-phase C18-E/ODS cartridges were purchased from Phenomenex Inc., Torrance, CA, USA. For creatinine measurement, Clinitek reagent strips were purchased from Siemens, Tarrytown, NY, USA; qUAntify control levels 1 and 2 were purchased from Bio-Rad Laboratories, Irvine, CA, USA.

2.3.2. Preparation of standards

10 µg/mL stock standards of all PGF and LTE4 isomers and deuterated internal standards (IS) were prepared in 50% MeOH and stored as aliquots below -70 °C.

2.3.3. Creatinine analysis

The creatinine was measured in urine samples using Clinitek Status®+ Analyzer (Siemens Healthcare Diagnostics Inc. Deerfield, IL, USA). The analyzer was first calibrated using commercial standards. Creatinine in urine samples was measured by dipping the creatinine measurement strips in urine followed by reading the strip on Clinitek Status+ Urine Analyzer for creatinine concentration. The instrument spectrophotometrically detects the light reflected by strip and the degree of the color change in the strip is directly related to the concentration of creatinine. The creatinine in urine forms metal complexes with strip pads that have peroxidase activity to produce change in color.[40]

2.3.4. Sample preparation

2.3.4.1. Initial sample preparation method development

2.3.4.1.1. *Comparison of analyte recovery using C18, MAX and WAX SPE*

For C18 SPE, the cartridge was first conditioned with 1 mL MeOH, equilibrated with 1 mL 20% ACN, loaded with 100 µL of spiked urine (5 ng/mL), washed with 500 µL of 20% ACN and eluted using 2 mL of 80% ACN containing 4% AA. For MAX and WAX SPE, conditioning, equilibration and loading steps were the same as for C18 SPE. The wash step was performed using 500 µL of 5 mM NH_4OAc and elution was performed using 2 mL of 80% ACN containing 4% AA. For the initial comparison of sorbents, the eluates were injected after 4x dilution with water, whereas in subsequent experiments, the eluates were evaporated to dryness in the speedvac (Savant, SPD111V) and reconstituted in 100 µL of 20% MeOH for enrichment.

2.3.4.1.2. *Sample preparation using β-glucuronidase enzyme hydrolysis*

10 µL of 1ng/mL internal standard mix containing 11β-PGF2α-d4 and LTE4-d5 was added to the volume of urine equivalent to 4 mg of creatinine. β-glucuronidase enzyme hydrolysis was

performed by first adjusting the pH of urine to 5.5 with AA, followed by an addition of 200 µL acetate buffer (1M, pH 5.5) + 10 µL β-glucuronidase/arylsulfatase solution (secondary activity = ≤ 7,500 units/mL) and incubation at 37°C for 16 hours. After letting the samples cool down to room temperature, the pH was adjusted to 4.0 with FA and SPE was performed as in Section 2.3.4.1.1.

2.3.4.1.3. *Sample preparation without β-glucuronidase enzyme hydrolysis*

The pH of urine sample volume equivalent to 4 mg of creatinine (typically this corresponds to 2 mL urine) was first adjusted to 4.0 with AA followed by an addition of 10 µL of 1 ng/mL internal standard mix containing 11β-PGF2α-d4 and LTE4-d5. After this, SPE was performed without performing enzyme hydrolysis.

2.3.4.2. Final sample preparation protocol used for validation

2.3.4.2.1. *Sample preparation using β-glucuronidase enzyme hydrolysis*

To measure total conjugated and unconjugated 11β-PGF2α concentration in urine β-glucuronidase enzyme hydrolysis was performed. 10 µL of 1 ng/mL internal standard mix containing 11β-PGF2α-d4 and LTE4-d5 was added to the volume of urine equivalent to 4 mg of creatinine. Urine pH was then adjusted to 5.5 with AA, followed by an addition of 200 µL acetate buffer (1M, pH 5.5) + 10 µL β-glucuronidase/arylsulfatase solution (secondary activity = ≤ 7,500 units/mL) and incubation at 37°C for 16 hours. Samples were cooled down to room temperature and the pH was adjusted to 4.0 with FA. This was followed by C18 SPE using protocol as in Section 2.3.4.2.3

2.3.4.2.2. *Sample preparation without β-glucuronidase enzyme hydrolysis*

To measure free 11β-PGF2α concentration in urine β-glucuronidase enzyme hydrolysis was not performed. First the pH of urine sample volume equivalent to 4 mg of creatinine was adjusted to 4.0 using AA followed by an addition of 10 µL of 1ng/mL internal standard mix containing 11β-PGF2α-d4 and LTE4-d5. This was followed by C18 SPE using the protocol as in Section 2.3.4.2.3.

2.3.4.2.3. *Final optimized C18 SPE protocol*

C18 SPE was selected and protocol was further optimized to decrease background and to improve the limit of detection of LTE4. The final C18 SPE protocol was as follows:

First conditioning was performed using 3 mL of ACN followed by equilibration with 3 mL of 25% ACN. A urine sample volume equivalent to 200 mg/dL of creatinine was loaded (the pH adjusted to 4 using formic acid). The cartridge was first washed with 2 mL water followed by 3 mL of 25% ACN. After this, elution 1 was performed using 4 mL of 30% ACN (to collect 11β-PGF2α; eluate 1) in polypropylene Eppendorf tubes containing 6 µL of 30% glycerol (v/v) in MeOH. This was followed by a second elution using 1.3 mL of 60% ACN + 0.1% formic acid (to collect LTE4; eluate 2) in a second set of tubes containing 6 µL of 30% glycerol (v/v) in MeOH. Eluate 1 and eluate 2 were evaporated in speedvac and reconstituted in 50 µL of 50% MeOH, separately.

2.3.5. Calibration and quantification

For validation, ten calibration standards were prepared by spiking pooled urine followed by a serial dilution with urine. The ten calibration levels used were: 9.77, 19.5, 39.1, 78.1, 156, 312, 625, 1250, 2500 and 5000 pg/mL. Quality control samples were prepared by spiking pooled urine (from six different individuals; creatinine concentration = 2 mg/mL) pre-extraction with 50 pg/mL of all PGF and LTE4 isomers. All calibration standards and quality control samples were then extracted as per the procedure described in Section 2.3.4.

2.3.6. LC-MS analysis

UHPLC Agilent 1290 Infinity II system (Agilent Technologies, Santa Clara, CA USA) and ZORBAX RRHD Eclipse Plus 95Å C18, 2.1 x 100 mm, 1.8 µm (Agilent Technologies) analytical column was used for chromatographic separation. Autosampler temperature was set at 6°C and the column temperature was maintained at 50°C. Mobile phase A was water with 0.02% acetic acid (v/v) and mobile phase B was composed of ACN/IPA (90:10) with 0.02% acetic acid (v/v).[41] The flow rate was 0.4 mL/min. For initial development experiments, one LC-MS method was used for both analytes in which mobile phase B gradient was increased from initial 5% to 30% from 1 to 1.1 min and 30% isocratic mobile phase B was used until 8.5 min for separation of prostaglandins followed by a 0.5%/min gradient until 24 min for separation of LTE4 isomers and interferences.

This was followed by a column wash with 95% mobile phase B and then equilibration with 5% mobile phase B. Total run time was 34 minutes. The injection volume was 10 µL.
However, to minimize interferences, while ensuring quantitative recovery of both analytes, a separate SPE elution was selected as the best strategy, which in turn required analysis of both analytes by individual LC-MS methods. Two LC methods used for final method validation were; Method A for 11β-PGF2α and method B for LTE4. In method A, mobile phase B gradient was increased from initial 5% to 30% from 1 to 1.1 min and 30% isocratic mobile phase B was used until 8.5 min for separation of prostaglandins followed by column wash with 95% mobile phase B and column equilibration with 5% mobile phase B. The total runtime for method A was 15 min. In method B, the mobile phase gradient was increased from its initial 5% to 34% from 1 to 1.1 min and then a gradient of 0.5%/min was used until 14 min to ensure separation of LTE4 isomers followed by a column wash with 95% mobile phase B and column equilibration with 5% mobile phase B. The total runtime was 20 minutes. The injection volume in both methods was 10 µL.

For all LC methods, MS detection was performed using Agilent 6550 iFunnel quadrupole time-of-flight (Q-TOF) connected to UHPLC Agilent 1290 Infinity II via Dual Agilent Jet Stream (AJS) electrospray ionization (ESI) source interface. ESI was operated in negative mode. The Dual AJS ESI source parameters were set as: drying gas temperature: 250°C, drying gas flow rate: 15 L/min, sheath gas temperature: 275 °C, sheath gas flow: 12 L/min, nebulizer: 30 psig, capillary voltage: 3500 V, nozzle voltage: 500 V. MS scan was collected in the m/z range of 100-1000 with 2 spectra/s. For internal mass calibration, Agilent 1260 isocratic pump was used for continuous infusion of reference standard mixture. Purine (m/z = 119.036320) and HP-0921 acetate adduct (m/z = 980.016375) from Agilent mass reference solution were used as reference standards for internal mass calibration throughout all analyses. Agilent MassHunter data acquisition software version B.06.01 was used for data acquisition.

2.3.7. Data analysis

Agilent MassHunter Analysis software (TOF Qualitative Analysis B.07.00 and TOF Quantitative Analysis B.07.00) was used for qualitative and quantitative data analysis. Monoisotopic masses of deprotonated $[M-H]^-$ ion of both analytes were considered with mass extraction window of ± 5

ppm for 11β-PGF2α a and ± 25 ppm for LTE4. The retention time tolerance was ± 0.15 minutes for both analytes. To make a calibration curve by subtracting endogenous levels of analytes from calibration samples, GraphPad PRISM software version 5.02 was used. The calibration was performed using $1/x^2$ weighted linear regression.

2.3.8. Overview of finalized experimental assay

The main steps for analysis of 11β-PGF2α and LTE4 in human urine samples are shown in Figure 2.1.

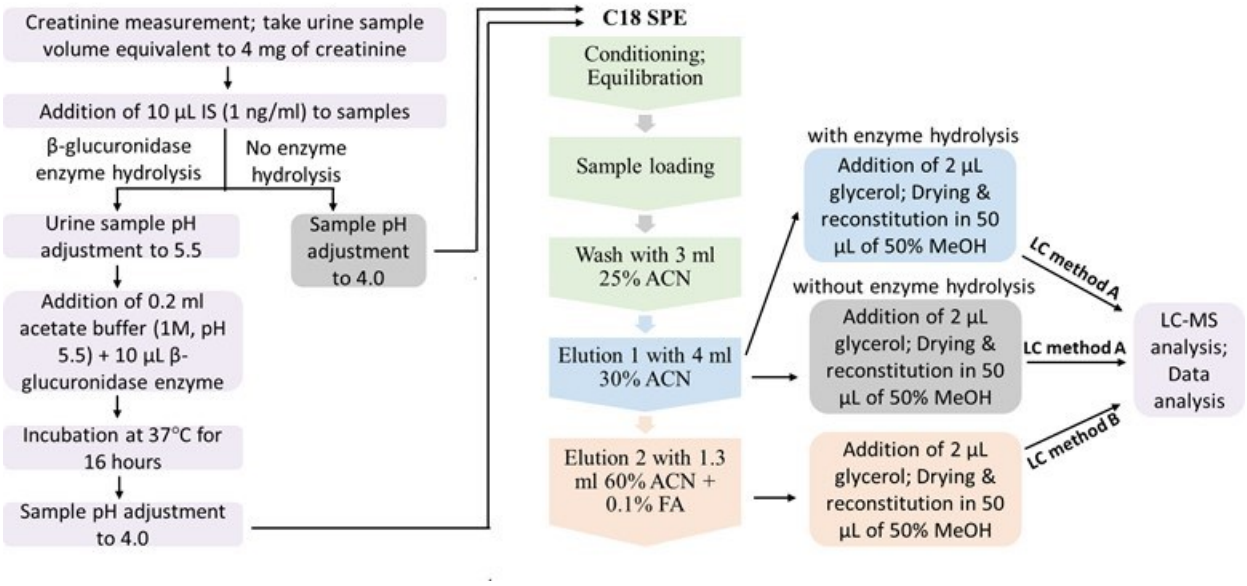

Figure 2.1 Schematic representation of the final method for the measurement of 11β-PGF2α, total 11β-PGF2α and LTE4 in urine.

2.3.9. Method validation

The method validation was performed using deuterated internal standards for both analytes. Pooled urine from six different individuals was used for validation (creatinine concentration = 2 mg/mL)

Selectivity: Since both analytes are present endogenously, it is impossible to get human urine free of endogenous analytes to test for selectivity. The selectivity was ensured by LC separation of all

known isomers of both analytes. Selectivity was further confirmed in nine different lots of urine by MS2 fragmentation of both endogenous analytes to ensure that the mass spectrum of all nine lots gave similar fragmentation pattern and there were no new product ion peaks arising from different urine lots possibly due to co-eluting isobaric interferences.

Intra-day and inter-day accuracy and precision: The intra-day accuracy experiment was performed by spiking pooled urine with 15, 30, 50, 75, 100, 250, 1000 and 4000 pg/mL (n = 6 replicates) with 11β-PGF2α-d4 and LTE4-d5. LLOQ was determined as the concentration tested with an accuracy of 80%-120% and a precision of ≤ 20% RSD. For all other concentrations, the accuracy criterion was 85%-115% and the precision criterion was ≤15% RSD. LODs were determined as the concentration with an analyte signal-to-noise ratio (S/N) of 3.
Inter-day accuracy experiments were performed at four different concentrations: 15, 50, 250 and 4000 pg/mL (n = 3 days). The accuracy and precision passing criterion for LLOQ and the other three concentrations were the same as for the intra-day experiments.

Recovery and matrix effects: The recovery and matrix effects were evaluated in pooled urine. The recovery was evaluated at 50, 250 and 4000 pg/mL (n = 6) by pre-spiking the stated concentrations of deuterated surrogate analytes. Matrix effects were evaluated at 2, 10 and 160 ng/mL post-spiked concentrations (n = 6), which takes into account 40x enrichment.
The recovery and matrix effects were also evaluated at two concentrations; 50 pg/mL and 4000 pg/mL in nine different individual lots of urine (n = 1 for each lot). The samples were spiked pre-extraction at 50 pg/mL and 4000 pg/mL of deuterated analytes. Post-spiked samples were spiked at 2 ng/mL and 160 ng/mL compensating for an enrichment factor of 40x.
The retention time of LTE4 and LTE4-d5 was different by 0.09 min and the retention time of 11β-PGF2α and 11β-PGF2α-d4 was different by 0.05 min. To ensure that deuterated analytes would track and correct for non-deuterated analytes, when used as internal standards during clinical sample analysis, the matrix effect was also evaluated for endogenous analytes by spiking urine post-extraction with 2000 pg/mL of non-deuterated standards and then performing the correction by subtraction of endogenous response. SPE extraction recovery and matrix effects were calculated as follows:

$$\%\ Recovery\ of\ IS = \frac{(IS\ response\ in\ prespiked\ samples)}{(IS\ response\ in\ postspiked\ samples)}\ x\ 100\%$$

Equation 2.1 % recovery calculation for IS.

$$\%\ ME_{IS} = \frac{(IS\ response\ in\ postspiked\ samples)}{(Response\ in\ neat\ solvent)}\ x\ 100\%$$

Equation 2.2 % matrix effects calculation in IS, where $\%ME_{IS}$ represents % matrix effects in deuterated internal standards.

$$\%\ ME_{ndstds} = \frac{(Response\ in\ postspiked\ samples\ ) - \ (endogenous\ response)}{(Response\ in\ neat\ solvent)}\ x\ 100\%$$

Equation 2.3 % matrix effects calculation in 11β-PGF2α and LTE4, where $\%\ ME_{ndstds}$ represents % matrix effects in non-deuterated standards.

Sample stability: The sample stability was assessed at two spiked concentrations of deuterated analytes: 50 pg/mL and 4000 pg/mL (n = 3) After spiking, samples were aliquoted into single-use aliquots and stored below -70°C. Stability of samples was evaluated at one, two and three freeze-thaw cycles, and after 1-week and 3-week freezer storage below -70°C. At appropriate time points, aliquots were thawed, prepared and analyzed. Freshly prepared urine samples at 50 pg/mL and 4000 pg/mL spiked concentration were used as controls. 48-hour autosampler stability of prepared samples was also evaluated at 50 pg/mL and 4000 pg/mL concentrations of deuterated analytes.

2.4. Results and discussion

2.4.1. LC separation of isomers

There are six known isomers of PGFs and two isomers of LTE4. Chromatographic separation of all isomers was ensured, in order to measure one specific isomer of interest accurately. A 30% mobile phase B isocratic separation successfully chromatographically resolved all PGF isomers and a shallow gradient of 0.5%/min gave baseline separation of LTE4 isomers along with ensuring

separation of some of isobaric and high intensity interferences for LTE4 as well as for LTE4-d5. The results of separation with final two LC-MS methods for PGFs and LTE4s in solvent and urine matrix are shown in Figure 2.2.

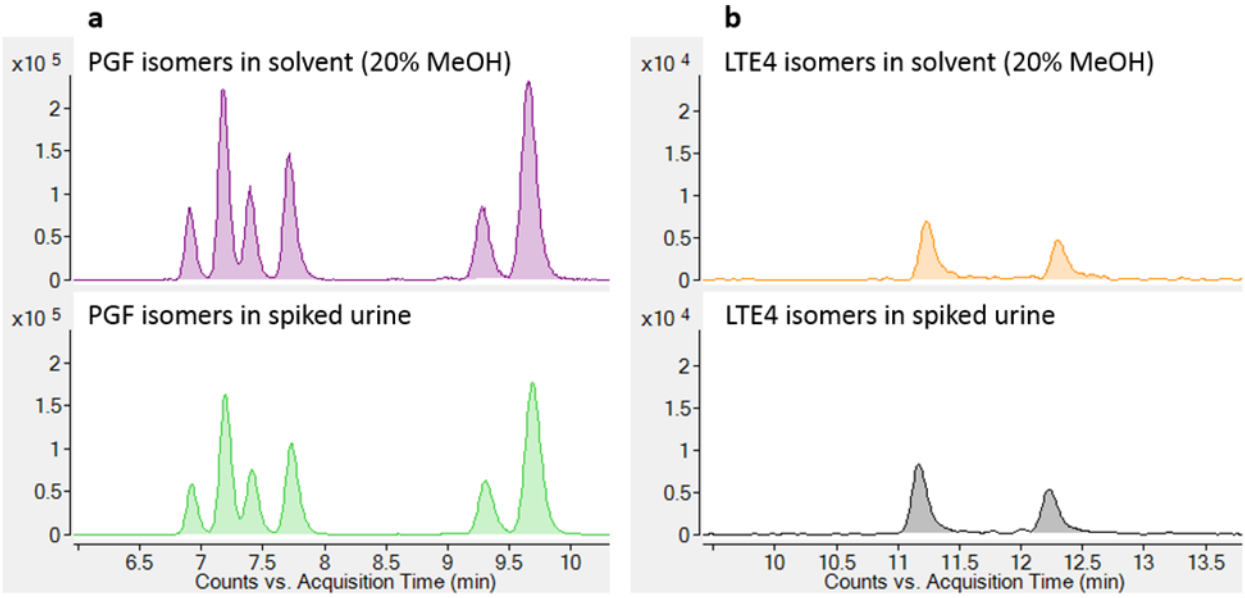

Figure 2.2 Separation of PGF isomers and LTE4 isomers in standard (20% MeOH) and spiked urine at concentration of 5 ng/ml. The PGF isomers (Figure 2.2 a) shown according to their elution order are: 8-iso-15(R)-PGF2α, 8-iso-PGF2α, 8-iso-PGF2β, 11β-PGF2α, 15(R)-PGF2α and PGF2α. LTE4 isomers (Figure 2.2 b) shown according to their elution order are LTE4 and 11-trans-LTE4. For separation of PGF isomers LC method A was used, while for LTE4 separation LC method B was used as described in Section 2.3.6.

MS2 spectra of all six isomers of PGFs and two isomers of LTE4 are very similar (Figure 2.3) and hence they cannot be distinguished with MS2 only. Therefore, an LC separation of isomers is necessary. Sasaki *et al.*[38] used the transition 353 → 193 and Balgoma *et al.*[18] used the transition 353 → 291 for 11β-PGF2α analysis. For LTE4 Lueke *et al.*[37] used the transition 438 → 333 and 438 → 235 and Sasaki *et al.*[38] and Sterz *et al.*[30] used the transition 438 → 333. However, Figure 2.3 shows that all PGF isomers form the 193 and 291 mass fragments and both LTE4 and 11-trans-LTE4 forms the 235 and 333 mass fragments in MS2. The 193 mass fragment is seen in all the isomers of PGFs as well as in both isomers of LTE4. Hence, if co-elution of isomers was present, then it would not have been detected in the previously used methods. Therefore, chromatographic

separation of all isomers is absolutely mandatory to ensure sufficient method accuracy and selectivity.

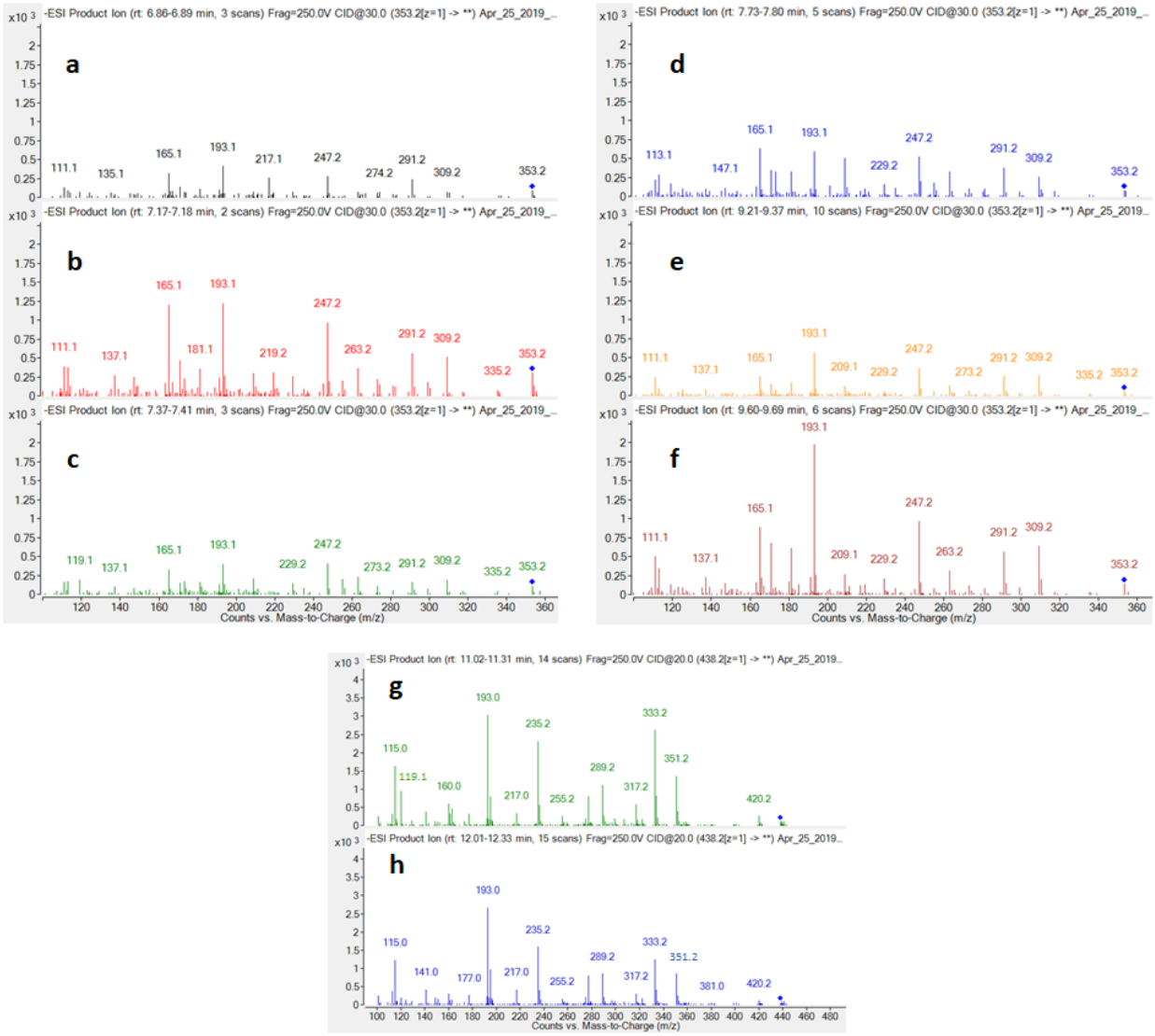

Figure 2.3 MS2 product ion spectra of six PGF isomers and two LTE4 isomers. 10 ng/mL standards in 20% MeOH were prepared for all isomers. 30 V collision energy was used for PGFs and 20 V collision energy was used for LTE4s. 8-iso-15(R)-PGF2α is shown in black (a), 8-iso-PGF2α is shown in red (b), 8-iso-PGF2β is shown in green (c), 11β-PGF2α is shown in blue (d), 15(R)-PGF2α is shown in orange (e), PGF2α is shown in brown (f).; LTE4 is shown in green (g) and 11-trans-LTE4 is shown in blue (h).

2.4.2. Selection of ESI mode for LTE4

Eicosanoids, including 11β-PGF2α and LTE4, are 20-carbon long metabolites derived from oxidation of arachidonic acid. Both metabolites have carboxylic acid groups, which makes them amenable to negative ESI. However, LTE4 has an additional amine group as shown in Figure 2.4, which also enables its efficient ionization in positive ESI. Hence, both positive and negative ESI were tested for LTE4 in terms of signal intensity and S/N. The intensity of LTE4 in negative mode was 5 times higher and its S/N was 7 times higher in negative than in positive ESI as shown in Figure 2.5. Hence, the negative ESI was selected for both analytes. In the literature, both positive[18,31] and negative [30, 37, 38] ESI modes have been used for the ionization of urinary LTE4 showing that possibly ESI source design may impact optimum ionization mode for ESI. In addition, in this work, 0.02% acetic acid (v/v) was used as an additive in the mobile phase since it has previously been shown to improve ionization in lipidomic analysis in negative ESI.[41] This may have also improved ionization of LTE4 in negative ESI and hence the choice of positive v/s negative mode.

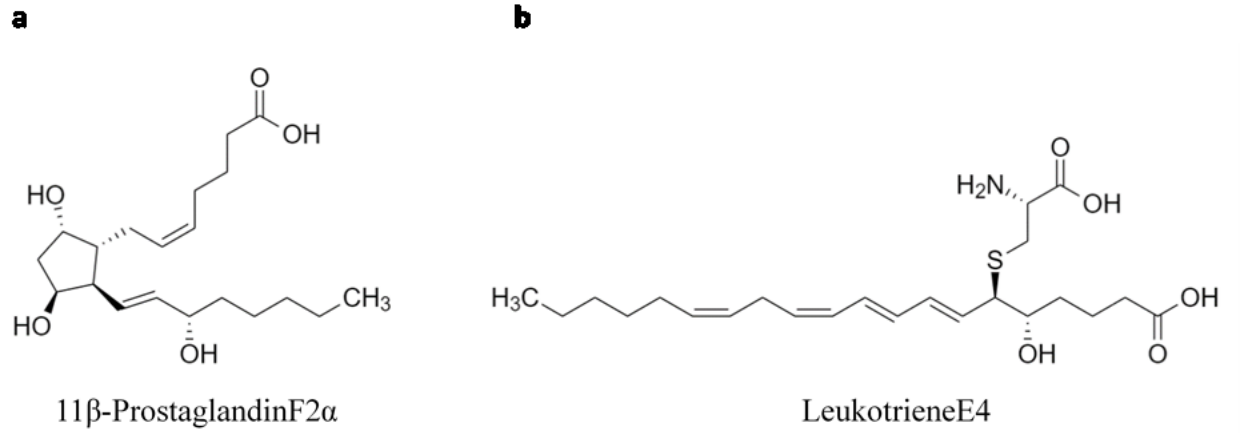

Figure 2.4 Chemical structures of 11β-PGF2α (a) and LTE4 (b).

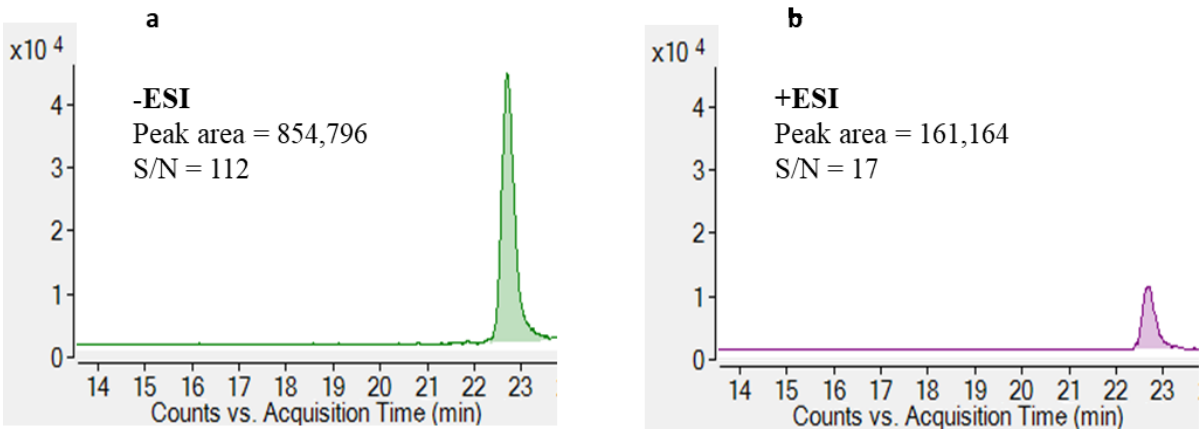

Figure 2.5 EICs of LTE4 in positive ESI (a) and negative ESI (b). LTE4 concentration in solvent (20% MeOH) = 10 ng/mL. 438.23198 m/z was extracted in negative ESI and 440.24653 was extracted in positive ESI. Combined LC-MS method was used for this analysis.

2.4.3. Comparison of different SPE sorbents for analyte recovery

The most common methods used for urine analysis in combination with LC-MS are dilute-and-shoot, liquid-liquid extraction and solid-phase extraction.[23] Dilute-and-shoot is suitable for analytes present in higher concentrations. Both analytes of interest in this work are present in trace levels in urine. The average reported normal 11β-PGF2α concentration in urine is ~ 90 pg/mL and normal LTE4 concentration in urine is ~ 40 pg/mL.[18,20,30,31] In our method, the lowest detectable 11β-PGF2α concentration in solvent was 100 pg/mL and 200 pg/mL for LTE4, which is higher than the reported endogenous levels. Hence, sample preparation method that also provides enrichment was needed. In this study, SPE was used as a sample preparation method since SPE gives a higher pre-concentration factor than LLE and also SPE gives better selectivity than LLE for targeted analysis because of different types of sorbents available commercially.[23] Since 11β-PGF2α and LTE4 are moderately non-polar analytes (logP = 2.61 and 2.02, respectively)[42,43] and weakly anionic (pKa = 4.36 and 2.39, respectively)[42,43], three different SPE sorbents were investigated: C18 reversed-phase, mixed-mode strong anion exchange (MAX) and mixed-mode weak anion exchange (WAX). The final SPE sorbent was selected based on the analyte recovery while minimizing the co-extraction of interferences with the potential to build-up on UHPLC column.

The SPE sorbents were first tested for analyte recovery in solvent (5 ng/mL; n = 3). 11β-PGF2α recovery was quantitative in all three sorbents and LT4 recovery was 71% with C18 SPE and 78% with MAX and WAX SPE. SPE sorbents were also tested for analyte recovery using spiked urine (without enrichment; concentration = 5 ng/mL; n = 3). The recoveries were calculated using post-extraction spiked urine samples. 11β-PGF2α average recovery was quantitative in all three sorbents whereas LTE4 recovery was the highest in C18 SPE (80%) as shown in Figure 2.6. In MAX and WAX SPE, the wash step was performed using 5 mM NH_4OAc to wash cationic species and the elution was performed using 4% AA to ensure that the elution pH was lower than pKa of both analytes of interest so that the analytes of interest would be protonated and disrupt the binding to SPE column.

All three sorbents performed comparably in terms of analyte recovery when no enrichment step was included. In subsequent experiments, when a high enrichment factor was attempted (20x and 40x), MAX and WAX SPE resulted in very high LC back pressure during LC-MS analysis possibly due to the extraction of anionic interferences from urine matrix. Hence, C18 SPE was selected for further sample preparation method development.

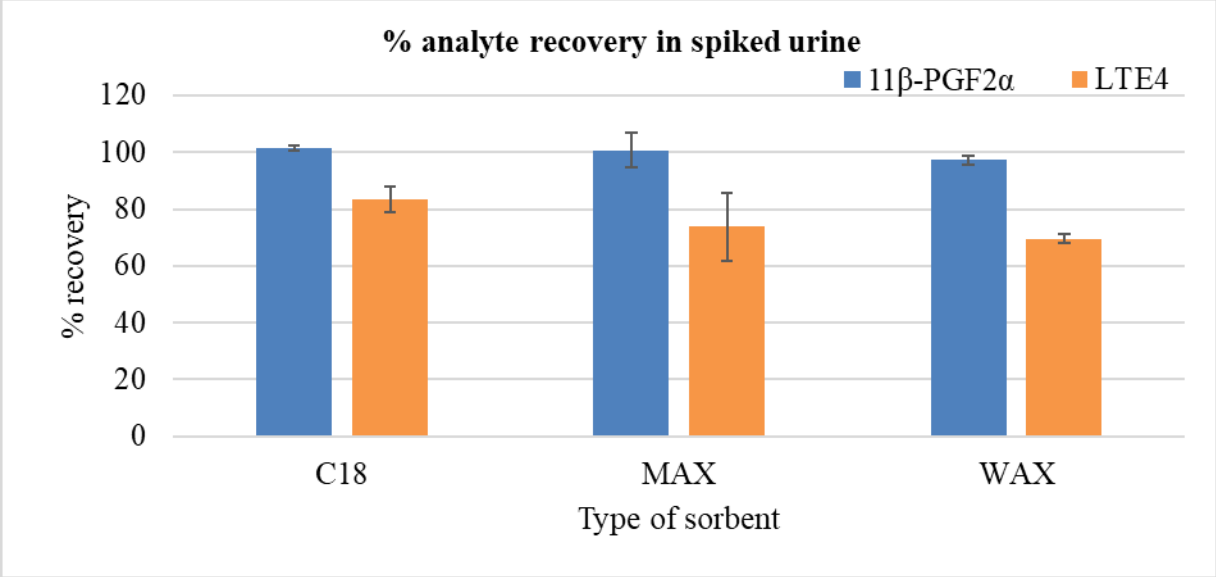

Figure 2.6 % recovery of analytes using different SPE sorbents in urine samples spiked at 5 ng/mL concentration (without enrichment; n = 3).

2.4.4. Addition of evaporation/reconstitution step and impact on analyte recovery

With initial attempts of enrichment (enrichment factor = 5x), a significant drop in the recovery of LTE4 (59%) was seen and poor repeatability was also observed using C18 SPE (Figure 2.7; without glycerol addition). Possible reasons for low recovery could be low SPE recovery, non-specific adsorption losses, poor solubility in reconstitution solvent and/or possible LTE4 degradation during evaporation step. Low SPE recovery was not the likely cause since in the previous section (Section 2.4.3), it was shown that recoveries of LTE4 without enrichment were acceptable. To address the possibility of non-specific adsorption losses during the evaporation step, the addition of 6 µL of 30% glycerol in MeOH (v/v) in evaporation tubes was tested.[18] The addition of glycerol improved precision by reducing % relative standard deviation (% RSD) from 49% to 8% (Figure 2.7; with glycerol addition). In this experiment, 4% AA was used in SPE elution solvent. 4% AA was used because without any acid/modifier in the elution solvent, the recovery of LTE4 was very low as shown and discussed in more detail in the next section (Section 2.4.5). In conclusion, with the addition of glycerol prior to the evaporation step, the method precision for LTE4 improved but its recovery was still low, thus requiring further investigation.

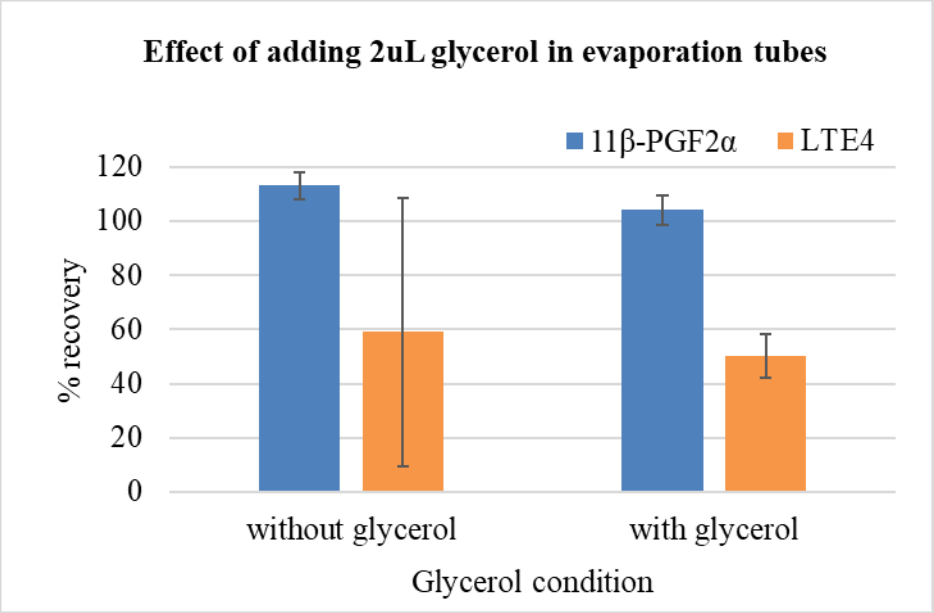

Figure 2.7 Evaluation of recovery of 11β-PGF2α and LTE4 after 5x enrichment and effect of glycerol addition using C18 SPE. Urine was spiked with 5 ng/ml (n = 3). For effect of glycerol addition, 6 µL of 30% glycerol was added in evaporation tubes.

2.4.5. Effect of elution pH on method recovery and selectivity

After improving the precision for LTE4, the next objective was to improve LTE4 SPE recovery. For this purpose, different compositions of SPE elution solvents were tested using different percentages of acids and no acid in elution solvent. Acceptable recoveries were seen for both analytes using 0.2% (v/v) FA and 0.1% (v/v) FA in SPE elution solvent, whereas poor LTE4 recovery was seen when no acid was added in elution solvent, suggesting an acidic pH is required for the complete elution of LTE4 from cartridge or to ensure LTE4 stability/solubilization during sample preparation (Figure 2.8). Increasing the acid concentration to 4% (v/v) resulted in a systematic decrease in LTE4 recovery. 4% FA gave better recovery than 4% AA in elution solvent possibly due to lower pH. The effect of low pH on LTE4 stability was next investigated by forced degradation study as discussed in detail in Section 2.4.6.

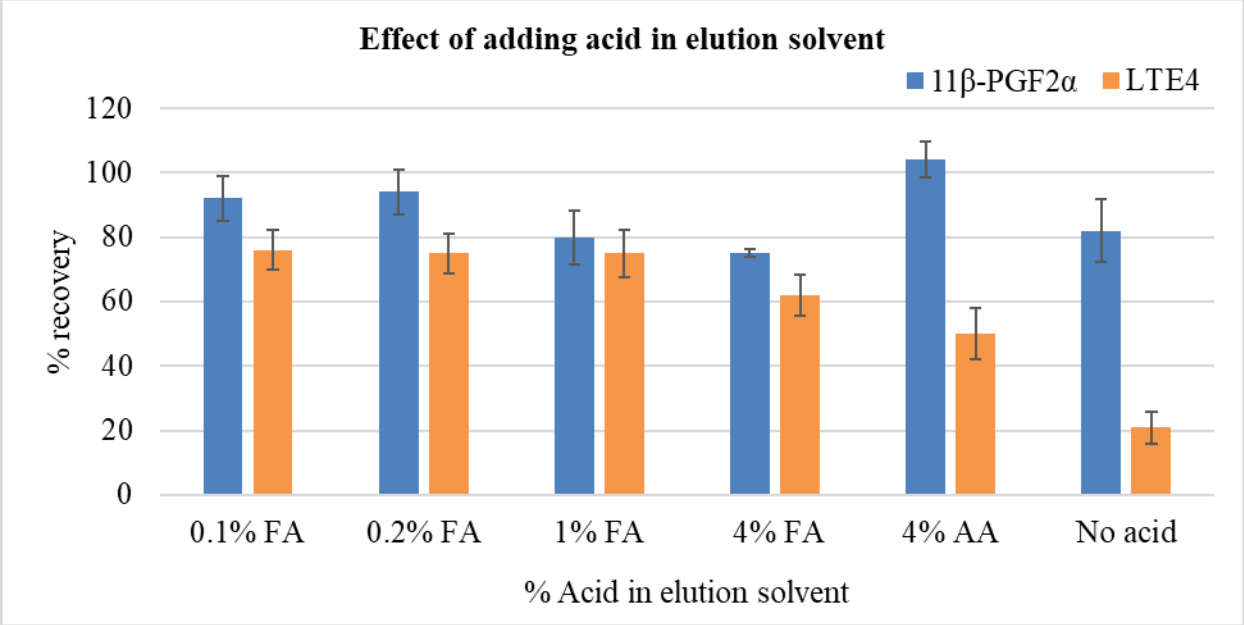

Figure 2.8 The effect of adding acid in elution solvent on analyte recovery from C18 SPE. C18 cartridge was first conditioned with 1 mL MeOH, equilibrated with 1 mL 20% ACN, loaded with 100 µL of spiked urine (5 ng/mL), washed with 500 µL of 20% ACN and eluted using 2 mL of 80% ACN containing 0.1% FA, 0.2% FA, 1% FA, 4% FA, 4% AA or no acid.

In a subsequent experiment to investigate if elution solvent composition affects the extraction of interferences, 0.1% (v/v) FA and 0.2% (v/v) FA in elution solvent were compared in terms of background signal for LTE4. A lower total ion background was seen with 0.1% FA in the elution

solvent, suggesting that a slight difference in pH affected the extraction of interferences (Supplementary Figure S1). Hence, 0.1% FA in elution solvent was selected for LTE4 elution since the recoveries of both analytes were similar to 0.2% FA in elution solvent (Figure 2.8). These results agree with Armstrong *et al.* who used 0.02% AA in mobile phase A and B during online purification of urine samples to extract LTE4 using Extend C18 column for enrichment.[31] The reported average LTE4 recoveries were 90%.[31] In contrast, Balgoma *et al.* used 1 mL MeOH without any acidification for elution of LTE4 from HLB SPE and obtained average LTE4 recoveries of 54%.[18]

Several other parameters of SPE were optimized for optimum recovery of 11β-PGF2α and LTE4 such as sample loading volume and elution volume. It was ensured that the sample loading volume was not exceeding the SPE column binding capacity by evaluating the flow-throughs and washes (Supplementary Figure S2). 1 mL and 2 mL elution volumes were compared, and 2 mL of elution solvent gave higher recoveries of both analytes (Supplementary Figure S3). In further optimization of elution volume, it was found that 1.3 mL elution volume was optimum for complete elution of both analytes (Supplementary Figure S3).

2.4.6. Low stability of LTE4 at low pH and high temperatures

To investigate the effect of pH on LTE4 stability, a short-term (1 week) forced degradation study was performed for LTE4 standard in solvent (20% MeOH) at different solvent pH and different temperatures. For this purpose, LTE4 stability was tested at pH 2, 3, 4, 5 and 6 at 4°C, room temperature and at 37°C. LTE4 was stable at a pH above 4 at 4°C and room temperature for one week, whereas all LTE4 was degraded at 37°C in one week (Figure 2.9). To further understand LTE4 stability at 37°C, a time-course stability test was performed at pH 2, 3, 4, 5 and 6 over 24-hour time period. 37°C temperature was selected for this experiment in order to verify whether LTE4 would remain stable during glucuronidase enzyme hydrolysis of 11β-PGF2α which requires 16 hours of incubation at 37°C. In addition, during the speedvac evaporation, the temperature of the speedvac is not controlled and rises up to 37°C. A typical evaporation time is 4.5-5 hours. The results of this study are shown in Figure 2.10. LTE4 was stable at a pH above 4 at 37 °C for a minimum of 24 hours, suggesting pH 4-6 are optimum working pHs for LTE4. This explains the

results in Section 2.4.5 where decreasing the amount of acid in the elution solvent improved the recovery of LTE4 (Figure 2.8). The pH of 0.2% FA was 3.7 and the pH of 0.1% FA was 3.9, while higher acid percentages in the elution solvent resulted in much lower pH values where LTE4 stability is compromised over time.

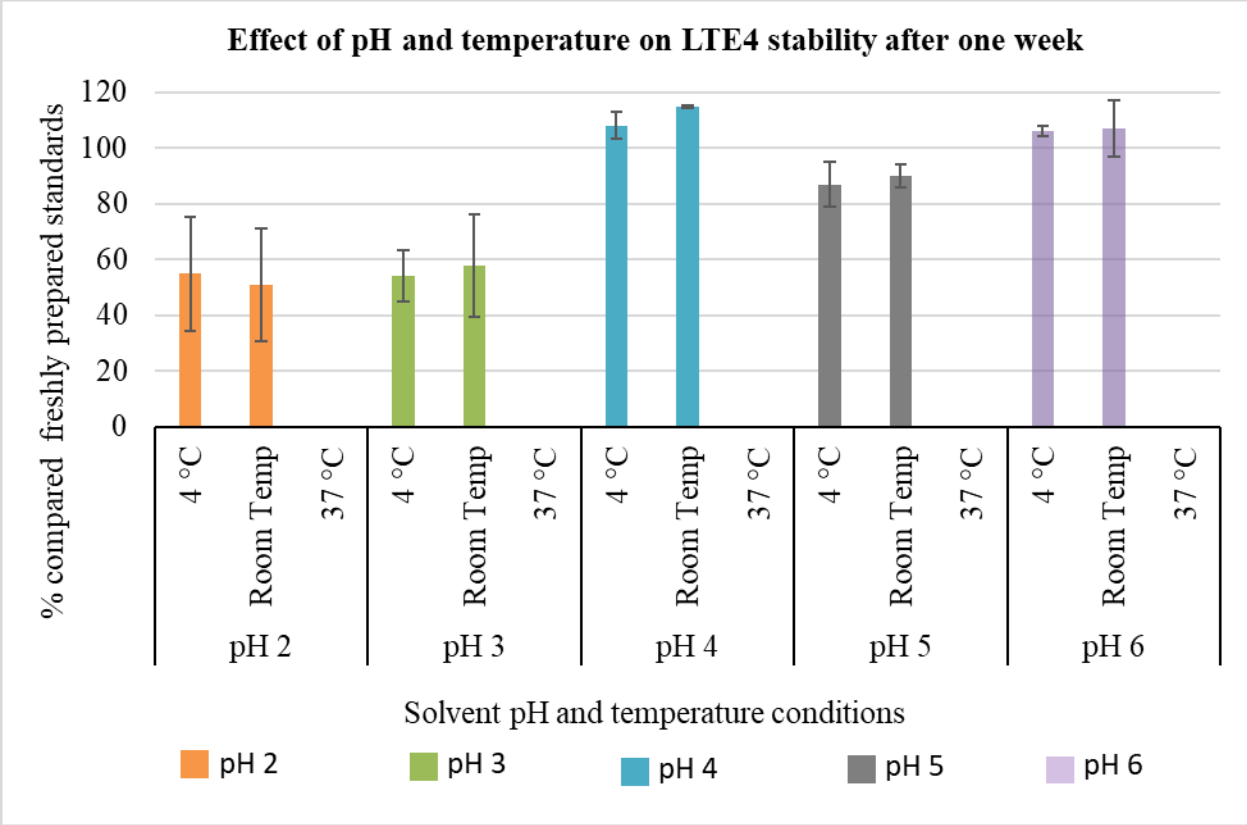

Figure 2.9 Effect of pH and temperature on LTE4 stability over one week. In this experiment, 20 ng/mL LTE4 standard was prepared in 20% MeOH at pH 2, 3, 4, 5 and 6 (n=3). Aliquots were kept at 4°C, room temperature and at 37°C for one week and analyzed. The controls were freshly prepared 20 ng/mL LTE4 standard in 20% MeOH at pH 2, 3, 4, 5 and 6 (n=3).

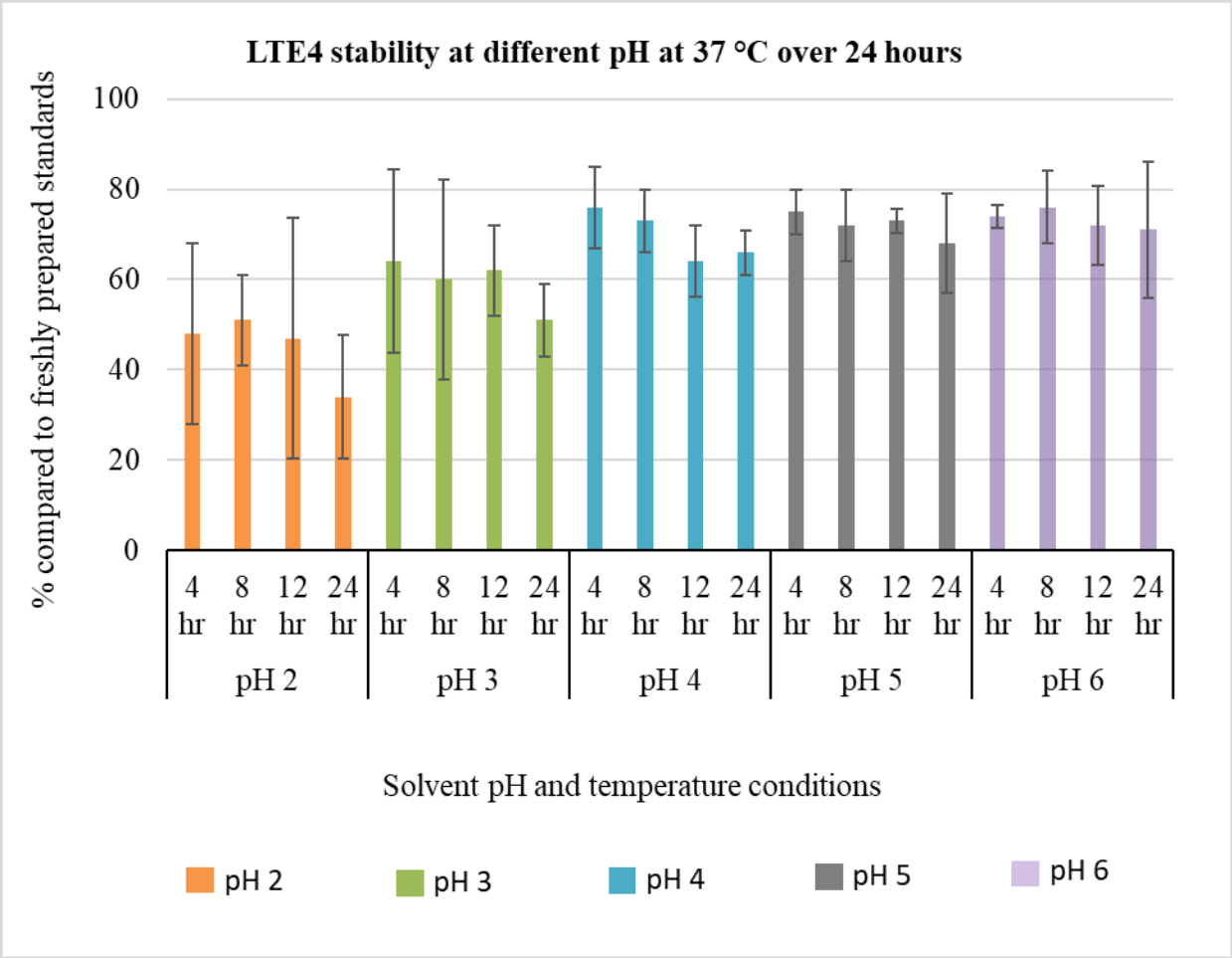

Figure 2.10 The effect of pH and temperature on LTE4 stability over 24 hours. In this experiment, 20 ng/mL LTE4 standard was prepared in 20% MeOH at pH 2, 3, 4, 5 and 6 (n=3). Samples were kept at 37°C for 24 hours and aliquots were analyzed at 4, 8, 12 and 24 hours. The controls were freshly prepared 20 ng/mL LTE4 standard in 20% MeOH at pH 2, 3, 4, 5 and 6 (n=3).

The LC-MS data from the above forced degradation study was then examined to identify the possible degradation products of LTE4. Both oxidation and degradation at S-C bond were considered. It was found that the glycine moiety can break off from LTE4 during degradation studies. The identification of glycine as one of the degradation products was further confirmed using glycine standard (Figure 2.11 (a)). Since Cayman Chemicals[44] reports that at 0°C in about one week, 10% of LTE4 isomerizes into 11-trans-LTE4, the forced degradation samples were checked for isomerization of LTE4 to 11-trans-LTE4. No isomerization was seen in the samples

kept at room temperature and 37°C for one week. However, in the samples that were kept at 4°C for one week, 8.9-10.4% isomerization was seen at pH 4, 5 and 6.

The example EIC of this isomerization is shown in Figure 2.11 (b). This suggests that at low temperatures and pH above 4, LTE4 isomerizes into 11-trans-LTE4 over time.

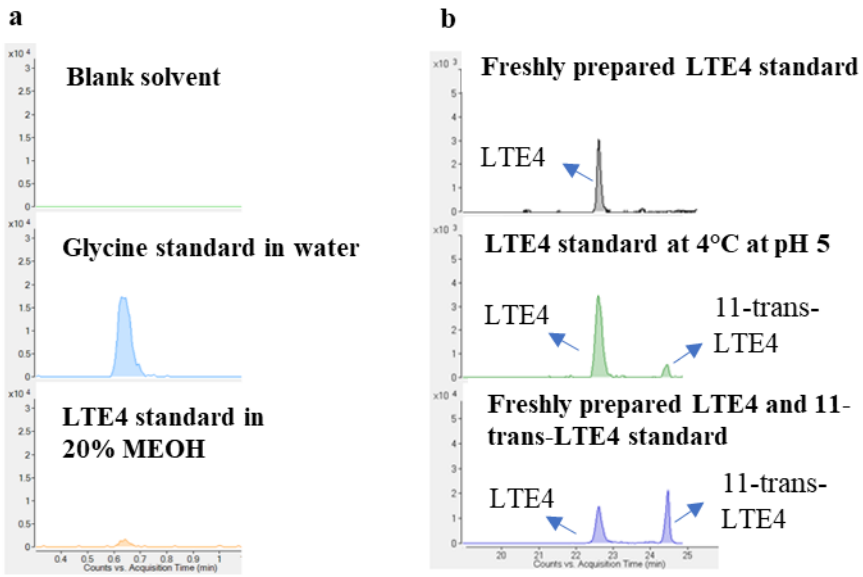

Figure 2.11 Investigation of LTE4 degradation products. (a) EICs of glycine are shown. 200 ng/mL LTE4 standard in 20% MeOH (shown in orange) was kept at 37°C for 4 days and was analyzed on LC-MS. 200 ng/mL glycine standard in water is shown in blue. The blank solvent is shown in green. (b) EICs of LTE4 and 11-trans-LTE4. 20 ng/mL LTE4 standard in 20% MeOH was prepared at pH 5 and kept at 4°C for one week (shown in green) and appearance on 11-trans-LTE4 was compared with freshly prepared LTE4 and 11-trans-LTE4 standard in 20% MeOH. Freshly prepared LTE4 standard is shown in black and freshly prepared LTE4 and 11-trans-LTE4 standard is shown in blue.

2.4.7. Poor LOD for LTE in urine after 40x enrichment

After 40x enrichment of urine using C18 SPE, 20 times higher LOD for LTE4 was observed in urine compared to LOD in solvent (20% MeOH). In solvent LTE4, LOD was 0.2 ng/mL whereas in urine 4 ng/mL LOD was observed. This evaluation was performed by spiking known concentrations of LTE4 post-extraction to determine what is the lowest concentration that can be detected and to give a preliminary indication if matrix effects are present. High noise was observed

in post-extraction spiked urine samples compared to standard in solvent which was the probable cause of high LOD in urine, suggesting that more sample clean-up was required to reduce the noise and improve the LTE4 LOD in urine. The high noise and high LODs are shown in LTE4 EICs in Figure 2.12 (a). Due to such high matrix interference, endogenous levels of LTE4 were not detected. The high background in urine samples was seen compared to solvent (TICs are shown in Figure 2.12 (b)).

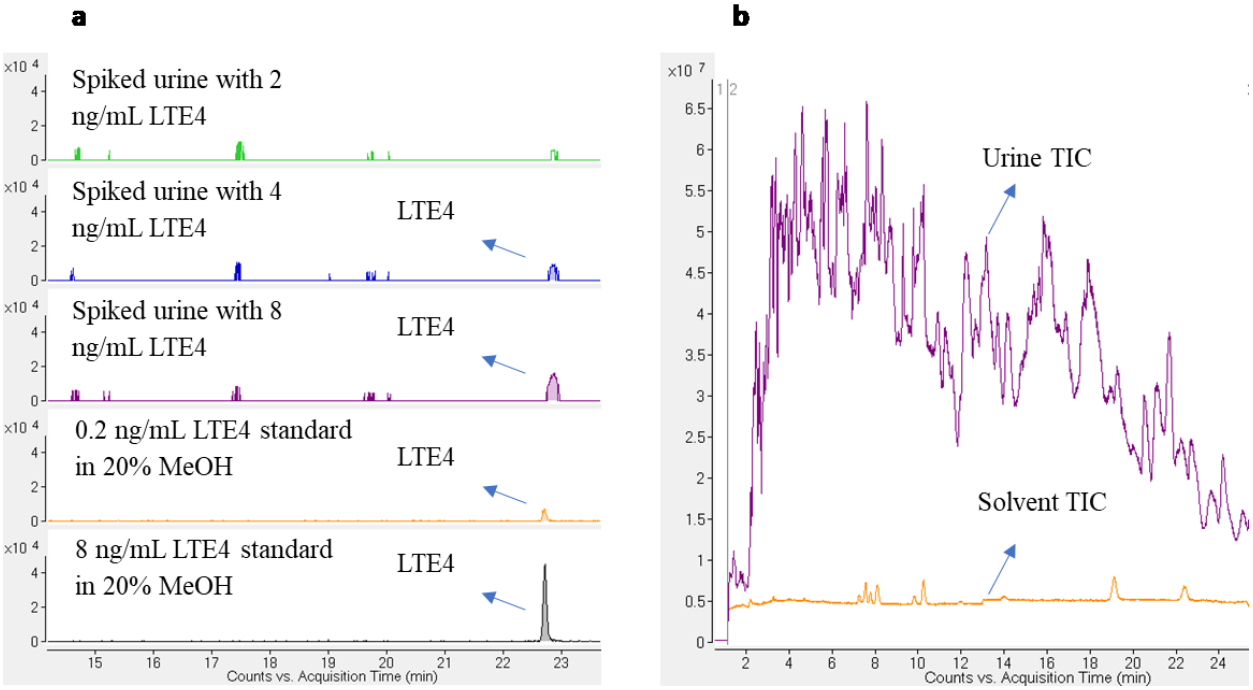

Figure 2.12 (a) LTE4 EICs in post-extraction spiked urine (using 3 mL 20% ACN SPE wash) and in solvent (20% MeOH). After 40x enrichment, urine was post-extraction spiked with 8, 4 and 2 ng/mL. 8 ng/mL is shown in purple color, 4 ng/mL is shown in blue color and 2 ng/mL is shown in green color. LTE4 standard was prepared in 20% MeOH at 0.2 ng/mL (orange) and 8 ng/mL (black). (b) Example TIC of urine (purple) and TIC of standard in solvent (20% MeOH).

2.4.8. Optimization of SPE washes and elution solvents to improve LOD of LTE4

To troubleshoot the inability to detect the endogenous levels of LTE4, different types of wash solvents, wash volumes and elution solvents were tested. The objective of this experiment was to find the best combination of wash and elution solvents that can preserve high analyte recoveries,

while reducing co-extracted interferences and increasing S/N ratio. The parameters tested for different types of washes were: % of organic solvent, type of organic solvent (ACN and MeOH), ionic strength of wash solution and pH of wash solution. The different washes were tested to check whether the interferences were possibly polar, ionic, basic or acidic and to remove them, while maintaining good recovery of LTE4. TICs of some of these washes are shown in Figure 2.13. Figure 2.13(a) shows the change in total background depending on pH and the volume of wash solution used. Their corresponding LTE4 EICs are shown in Figure 2.13 (b) and show the change in noise and LTE4 signal with the change in SPE washes. Comparison of the addition of 1% FA or 1% ammonium hydroxide in wash, showed that LTE4 could not be recovered under basic conditions. A comparison of acidified wash and wash without any acid additives showed lower background for non-acidified wash solutions. Overall, an extensive wash using 7 mL of 30% ACN gave the minimum background (Figure 2.13 a) with lower noise and optimum recovery of LTE4 (Figure 2.13 b) suggesting that a larger wash volume (7 mL of 30% ACN) is required to remove as much of non-polar interferences as possible and to improve the limits of detection for LTE4.

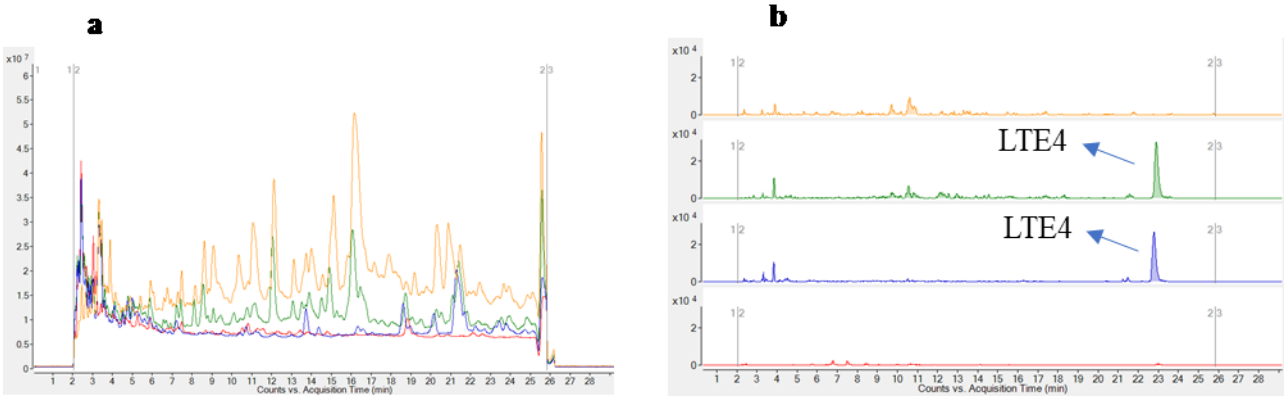

Figure 2.13 The effect of wash solvent volume and composition on method selectivity and LTE4 recovery. (b) EICs of LTE4 with different SPE washes. The different washes tested were - 3 mL 1% FA in 30% ACN wash (orange), 3 mL 1% NH4OH in 30% ACN wash (red), 3 mL 30% ACN wash (green) and 7 mL 30% ACN wash (blue).

In the next experiment, different percentage of organic solvent was also tested during SPE elution step in order to investigate whether it can further minimize background and improve LTE4 signal. Lowering the percentage of organic in the elution solvent helped to reduce the co-extraction of

non-polar interferences and thus reduced background signals. For this purpose, 1.3 mL of 50% ACN + 0.1% FA, 60% ACN + 0.1% FA, 70% ACN + 0.1% FA and 80% ACN + 0.1% FA were tested as elution solvents. 0.1% FA was included in elution solvents because it was previously shown that acidic pH was required to compete the elution of LTE4 from the SPE cartridge (Figure 2.8 and Supplementary Figure S1). 1.3 mL of 50% ACN + 0.1% FA was insufficient to achieve the complete elution of LTE4. 1.3 mL of 60% ACN + 0.1% FA as elution solvent gave maximum recovery of LTE4 while background was slightly improved. TICs and the corresponding LTE4 EICs are shown in Figure 2.14 (a) and Figure 2.14 (b), respectively.

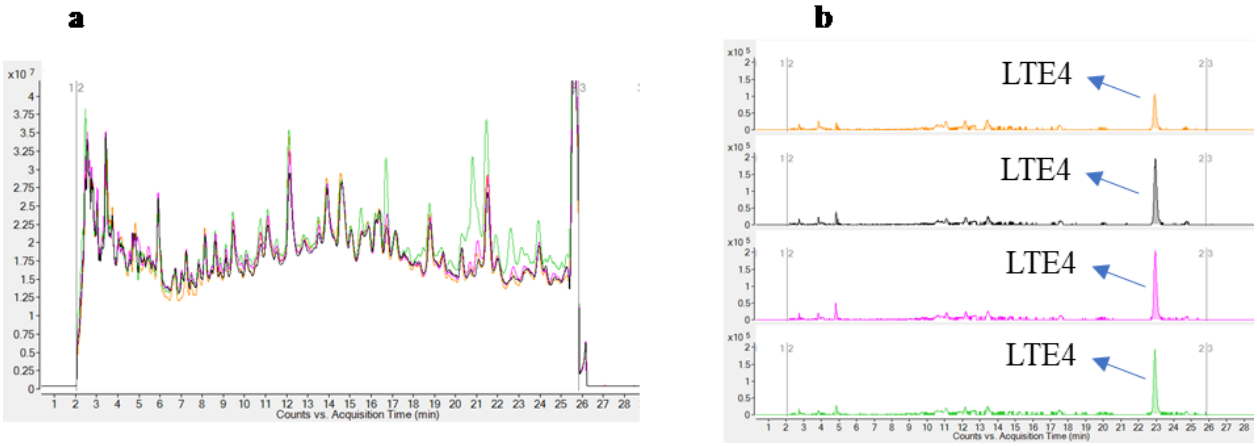

Figure 2.14 The effect of elution solvent composition on (a) TICs (b) EICs of LTE4. Different elution solvents that were tested are: 50% ACN + 0.1% FA (orange), 60% ACN + 0.1% FA (black), 70% ACN + 0.1% FA (pink), 80% ACN + 0.1% FA (green). All elution volumes were 1.3 mL.

The final optimized wash of 7 mL of 30% ACN decreased noise 5 times and improved LTE4 LODs from 8 ng/mL to 2 ng/mL in post-spiked urine when compared to the previous 3 mL 20% ACN wash (Figure 2.12) as shown in Figure 2.15.

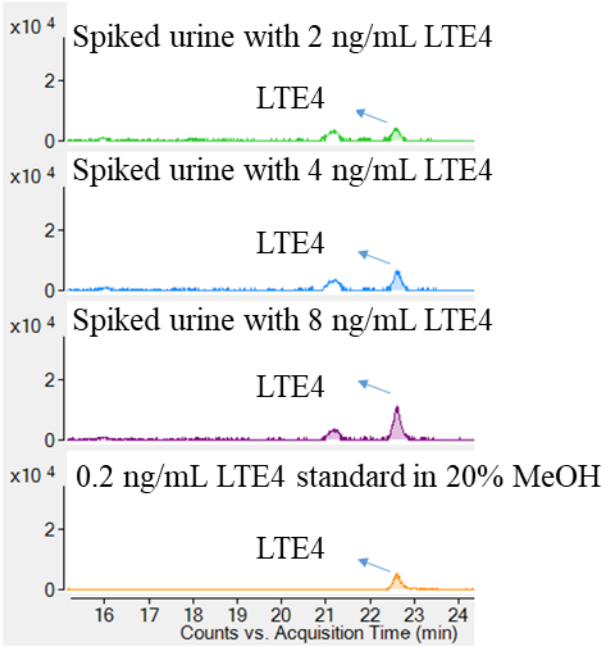

Figure 2.15 LTE4 EICs in post extraction spiked urine (using 7 mL 30% ACN SPE wash) and in solvent (20% MeOH). After 40x enrichment, urine was post-extraction spiked with 8 (purple), 4 (blue) and 2 (green) ng/mL. LTE4 standard was prepared in 20% MeOH at 0.2 ng/mL (orange).

Although the improved wash and elution protocols helped LODs for LTE4, 11β-PGF2α is more polar than LTE4, and it was unfortunately washed away with 7 mL of 30% ACN SPE wash. Thus, to recover both analytes in one SPE protocol, a two-step elution was finalized in which, after loading, the cartridge was first washed with 3 mL 25% ACN, which provided sufficient selectivity to accurately measure 11β-PGF2α. Then elution 1 was performed using 4 mL 30% ACN to collect 11β-PGF2α, followed by elution 2 using 1.3 mL 60% ACN + 0.1% FA to collect LTE4. The TICs and LTE4 EICs with 7 mL 30% of ACN wash and the two-step elution protocol are shown in Supplementary Figure 4, showing that with both protocols the background as well as the LTE4 recovery were comparable.

2.4.9. Deterioration of LTE4 mass accuracy at low concentrations

The final issue that was observed for LTE4 measurement was deterioration of mass accuracy as the concentration of analyte in the sample decreased. For example, mass accuracy tolerances required to integrate 11β-PGF2α and LTE4 peaks at different concentrations in solvent (20%

MeOH) are listed in Table 2.1. Mass accuracy deterioration was seen only for LTE4 and not for 11β-PGF2α (Table 2.1). EICs of LTE4 at different concentrations using different mass extraction windows are shown in Figure 2.16 (b).

Table 2.1 Mass tolerance required to integrate analyte peaks at different concentrations in solvent (20% MeOH).

Concentration (ng/mL)	**Mass accuracy tolerance (Δ ppm)**	
	11β-PGF2α	**LTE4**
15.00	5	5
7.50	5	5
3.75	5	5
1.88	5	10
0.93	5	10
0.47	5	10
0.23	5	20
0.11	5	n.d.*

* n.d. is signal was below LOD and peak integration was not possible.

To further investigate LTE4 mass accuracy deterioration in urine, urine sample was spiked with internal standard, LTE4-d5 post-extraction, at average endogenous level. The average endogenous level was calculated as 1.2 ng/mL at the time of LC-MS injection which is equivalent to 40 pg/mL normal endogenous concentration considering 40x enrichment and 75% SPE recovery. To extract and integrate this low concentration properly, an extraction window of ± 25 ppm was required in the urine matrix (Figure 2.16 a). Hence, to measure and quantitate the endogenous levels of LTE4, a mass extraction window of ± 25 ppm was used.

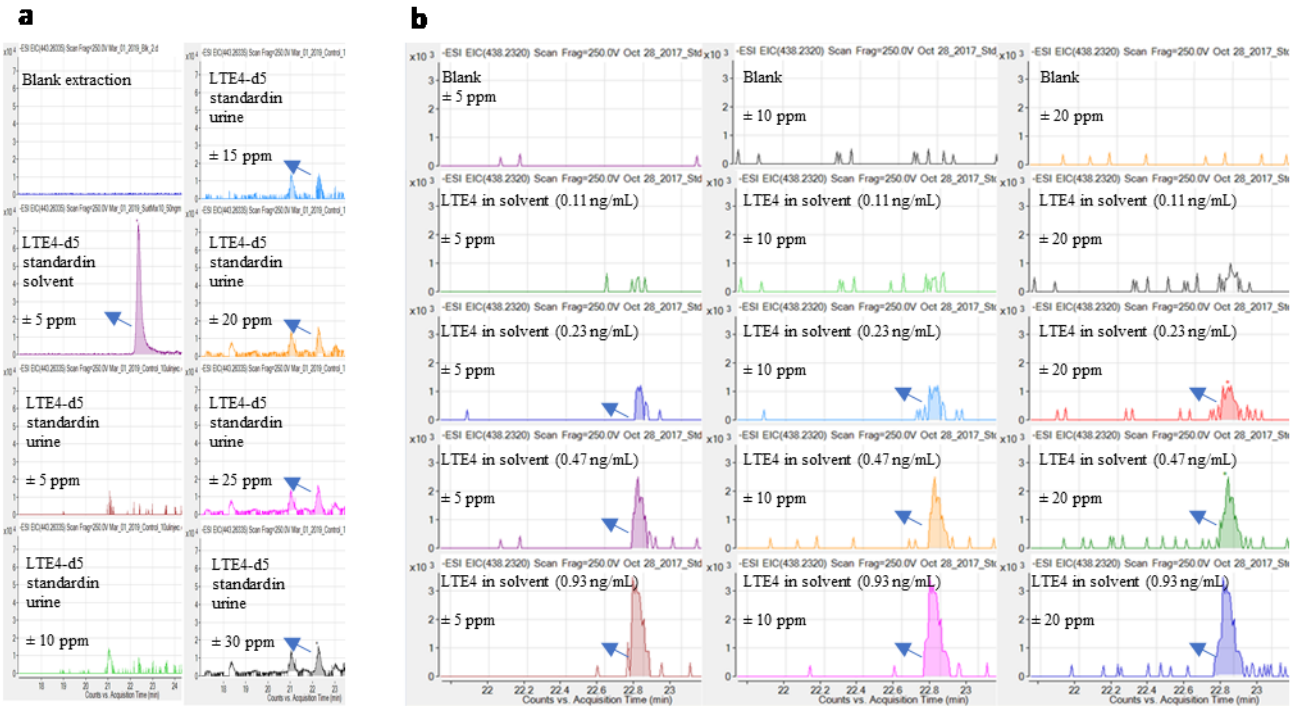

Figure 2.16 (a) EICs of LTE4-d5 are shown in blank, solvent (20% MeOH) and in urine (with different mass extraction window. In urine LTE4-d5 was spiked post-extraction at an average normal endogenous level (1.2 ng/mL). LTE4-d5 was extracted with mass tolerance of ± 5 ppm, ± 10 ppm, ± 15 ppm, ± 20 ppm, ± 25 ppm and ± 30 ppm in urine. (b) EICs of blank (20% MeOH) and LTE4 in solvent (20% MeOH) at 0.11 ng/mL, 0.23 ng/mL, 0.47 ng/mL and 0.93 ng/mL concentration using mass tolerance of ± 5 ppm, ± 10 ppm and ± 20 ppm.

2.4.10. Effectiveness of β-glucuronidase enzyme hydrolysis

B-glucuronidase enzyme hydrolysis was used to measure the total 11β-PGF2α levels. The enzyme volume to be added was optimized by adding different volumes of enzyme and comparing the increase in 11β-PGF2α endogenous signal while maintaining the repeatability. 10 µL of enzyme showed a high 11β-PGF2α endogenous signal with good repeatability (Supplementary Figure 5). The effectiveness of β-glucuronidase enzyme hydrolysis was assessed by analyzing EICs of 11β-PGF2α before and after enzyme hydrolysis (Figure 2.17) which showed large increase in 11β-PGF2α response suggesting that a considerable amount of 11β-PGF2α is excreted in urine as glucuronide conjugate. In literature, enzyme hydrolysis has not been frequently used to measure endogenous PGFs. For example, among the LC-MS methods listed in Table 1.2 for urinary PGF

measurement, only Sasaki *et al.* used β-glucuronidase enzyme hydrolysis to measure endogenous prostaglandins.[38]

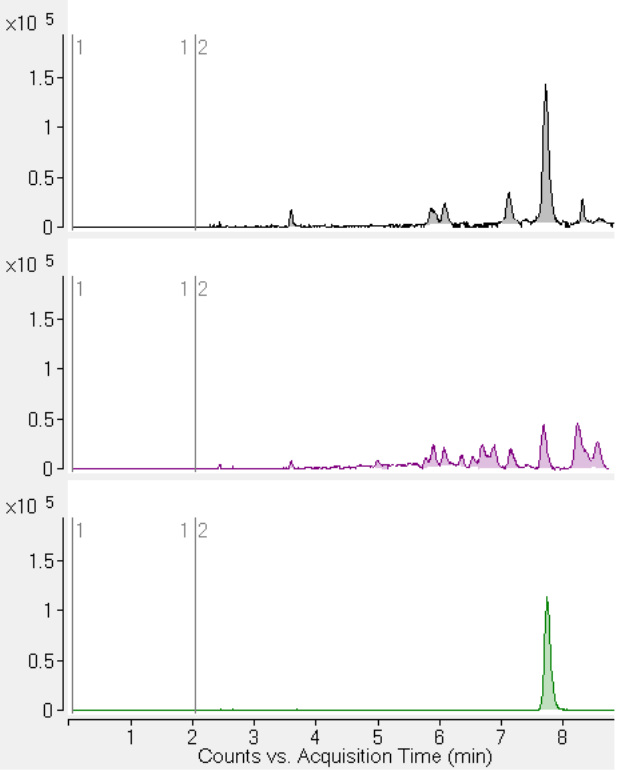

Figure 2.17 An increase in response of endogenous 11β-PGF2α after β-glucuronidase enzyme hydrolysis. The 11β-PGF2α peak area without enzyme hydrolysis (purple) was 36290 counts/sec/sec whereas after enzyme hydrolysis (black) the endogenous 11β-PGF2α peak area was 133400 counts/sec/sec. 10 ng/mL11β-PGF2α standard in solvent (20% MeOH) is shown in green. For β-glucuronidase enzyme hydrolysis experiment, 16-hr hydrolysis was followed by C18 SPE and LC-MS analysis. For experiment performed without enzyme hydrolysis, C18 SPE was performed directly on urine samples followed by LC-MS analysis.

2.4.11. Validation results

Since there is no established guidance for the validation of endogenous biomarkers, we used experiments proposed by the FDA for bioanalytical method validation in order to execute the validation and supplemented them as needed with additional experiments for this application.

Different approaches have been used in the past for the validation of LC-MS methods for endogenous analytes such as the use of surrogate matrix, background subtraction and the use of surrogate analytes.[45] In background subtraction and surrogate analyte methods, the same matrix is used in which the endogenous analytes of interest are present whereas in surrogate matrix approach, other matrices are used such as stripped matrix and artificial matrix.[45] In the surrogate analyte approach, stable isotope labelled analytes are used for validation purposes. In this study, we chose surrogate analyte (deuterated standards) as the most appropriate validation strategy for two reasons. First, both 11β-PGF2α and LTE4 are present in trace levels in urine and hence LLOQ determination was extremely important for this application and routine use of the method. The use of background subtraction validation strategy does not permit very accurate determination of LLOQ as the observed signal intensities are elevated due to the presence of endogenous analytes. Secondly, considerable and variable matrix effects can be expected in urine. The use of artificial matrices, such as artificial or stripped urine would severely underestimate matrix effects and overestimate the resulting accuracy and precision of the method. The isotopically-labelled IS used as surrogate analytes have similar physicochemical properties as the analytes of interest and permitted the use of real urine matrices during the entire validation, thus allowing the evaluation of accuracy and precision under the most stringent and clinically-relevant conditions. The key parameters tested during validation were: selectivity, LOD, LLOQ, linearity, intra-day accuracy and precision, inter-day accuracy and precision, recovery, matrix effects and stability.

2.4.11.1. Selectivity

For exogenous compounds, selectivity is tested by analyzing different lots of matrix and ensuring there is no interfering signals present at the retention time of interest. Since LTE4 and 11β-PGF2α are endogenously present in all urine samples, the selectivity of the method was tested and established using two different experiments. First, the selectivity was ensured by developing an LC method that is capable of separating all known isomers of LTE4 and 11β-PGF2α (Figure 2.2) to ensure no isomeric interferences can impact the analyte measurement. Next, the selectivity of the method was further tested by measuring and examining MS2 product ion spectra of both endogenous analytes in the urine matrix from nine different urine lots. The collected spectra were compared to pure authentic standard spectra. No high intensity product ion peaks were seen other than the expected product ions in standards (Figure 2.18), across all nine lots suggesting that there

were no co-eluting isobaric interferences for both analytes. MS2 spectra of standards are shown in Figure 2.3. Also, no isobaric interferences were detected for deuterated standards for both analytes.

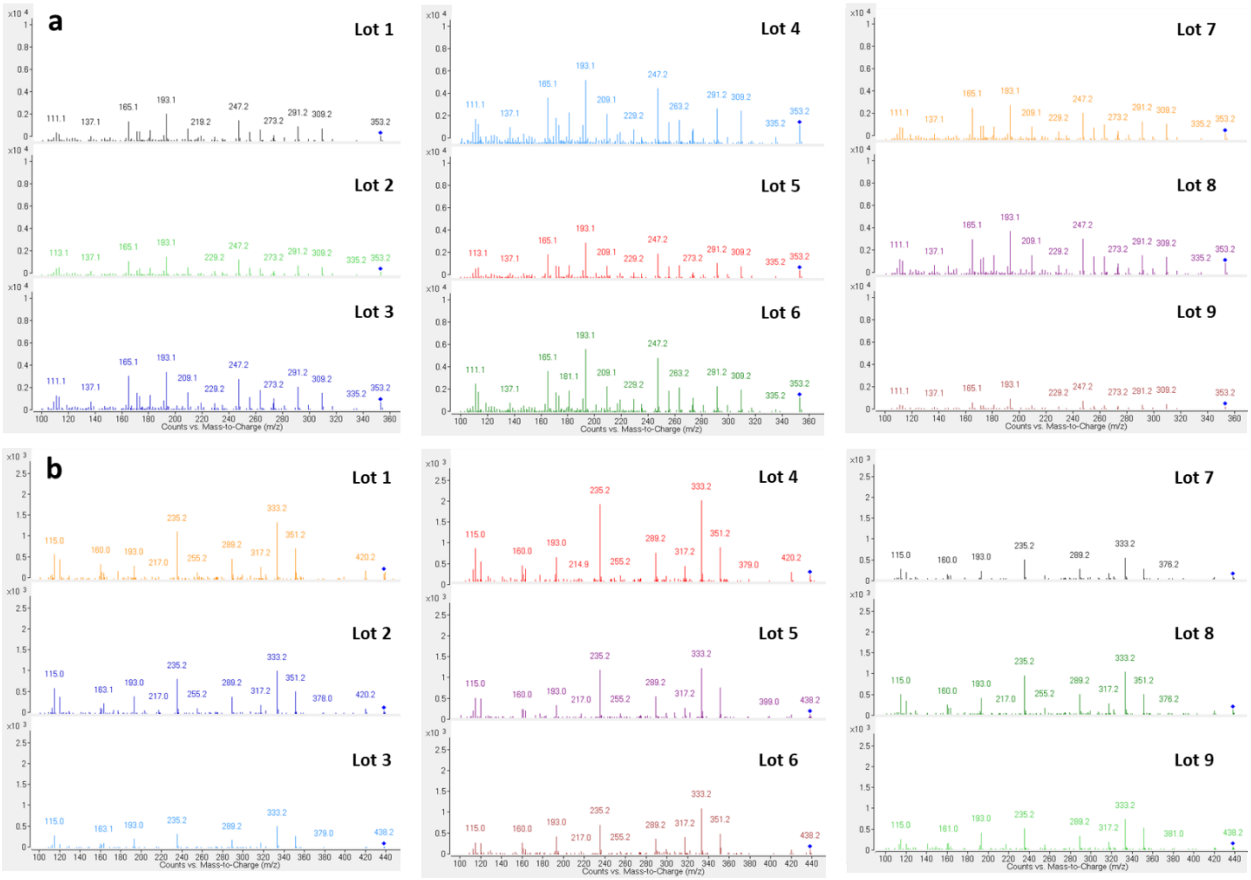

Figure 2.18 Product ion spectra of endogenous 11β-PGF2α (a) and endogenous LTE4 (b) from nine different lots of urine using the final developed SPE protocol and two different LC-MS method for both analytes. 20 V collision energy was used for LTE4 and 30 V collision energy was used for 11β-PGF2α.

Considering the six known PGF isomers, endogenous 8-iso-PGF2α and PGF2α isomers were frequently seen in urine samples whereas 8-iso-15(R)-PGF2α and 15(R)-PGF2α isomers were observed occasionally. The endogenous response of 8-iso-PGF2α, PGF2α, 8-iso-15(R)-PGF2α, 15(R)-PGF2α and endogenous 11β-PGF2α in nine urine lots (that were used for recovery and matrix effects experiment) are shown in Table 2.1. Some of the isomers are present at significantly higher levels than 11β-PGF2α and can interfere with the accurate measurement of 11β-PGF2α when using methods that are not sufficiently selective. These results further confirm how important

isomer separation is, and that existing immunoassay and LC-MS methods which did not properly establish selectivity may lead to inaccurate quantitation of 11β-PGF2α. 8-iso-PGF2β and 11-trans-LTE4 was not detected in any of the urine samples tested.

Table 2.1 Endogenous response of 8-iso-PGF2α, PGF2α, 8-iso-15(R)-PGF2α, 15(R)-PGF2α and 11β-PGF2α in nine different urine lots that were used for recovery and matrix effects evaluation. Response is represented as peak areas (counts/sec/sec).

Urine lot number	Peak area of endogenous 11β-PGF2α	Peak area of endogenous 8-iso-PGF2α	Peak area of endogenous PGF2α	Peak area of endogenous 8-iso-15(R)-PGF2α	Peak area of endogenous 15(R)-PGF2α
Lot 1	194800	22900	n.d.	n.d.	n.d.
Lot 2	148100	198300	269000	407500	68990
Lot 3	257000	n.d.	n.d.	n.d.	n.d.
Lot 4	226600	16140	354900	n.d.	n.d.
Lot 5	268500	22850	n.d.	n.d.	n.d.
Lot 6	249700	n.d.	n.d.	n.d.	n.d.
Lot 7	244900	108900	1966000	n.d.	44870
Lot 8	240400	n.d.	n.d.	10310	n.d.
Lot 9	137200	226100	2578000	292100	34330

n.d. is not detected

2.4.11.2. LOD, LLOQ and linearity

The method was linear from 9.77 to 5000 pg/mL concentration (r^2 = 0.9952 for 11β-PGF2α-d4 and r^2 = 0.9914 for LTE4-d5) for both analytes with 90% of all concentration levels meeting the criteria of accuracy between 85 - 115% and precision of within 15% RSD. Example calibration curves of both analytes in urine are shown in Supplementary Figure S6. The lowest concentration tested for LLOQ was 15 pg/mL for both analytes and passed all accuracy and precision criteria. This concentration was sufficient for the intended use of the method with the expected normal concentrations of 15-135 pg/mL for LTE4[30] and 20-150 pg/mL[20]. A slightly lower 10 pg/mL concentration was used as the lowest concentration for the calibration curve, and also routinely passed all accuracy and precision criteria for calibration. In literature LTE4 LLOQs (using LC-MS/MS; triple quadrupole) have been reported to be 5 pg/mL[31], 6 pg/mL[30], 8 pg/mL[37] and 263.3

pg/mL[18], whereas 11β-PGF2α LLOQs have been reported to be 48 pg/mL[38] and 152.4 pg/mL.[18] Thus, LTE4 LLOQs with our method are similar to the average LLOQs in literature, whereas 11β-PGF2α LLOQs are better than previous methods. LOD for 11β-PGF2α was 2.5 pg/mL and LOD for LTE4 was 4.9 pg/mL.

2.4.11.3. Intra-day and inter-day accuracy and precision

The FDA guidelines require intra-day and inter-day accuracy and precision experiments at a minimum of three concentrations: low, medium and high concentrations in addition to LLOQ. Total of eight concentrations were tested in intra-day experiments whereas the inter-day experiments were performed at four concentrations to cover the range from LLOQ to ULOQ; 15, 50, 250 and 4000 pg/mL. The results for intra-day and inter-day accuracy and precision for both analytes are shown in Figure 2.19. The method meets the all the FDA acceptance criteria for intra-day and inter-day accuracy and precision for all the concentrations tested.

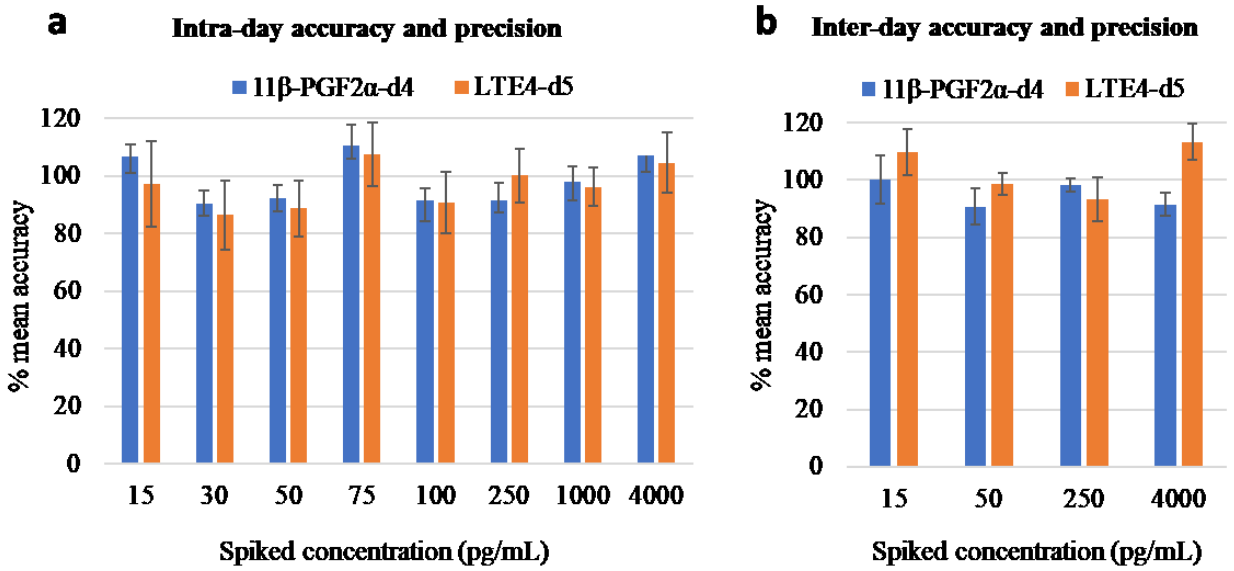

Figure 2.19 Intra-day (a) and inter-day (b) accuracy and precision. For intra-day accuracy and precision pooled urine was spiked with deuterated internal standards of both analytes at 15, 30, 50, 75, 100, 250, 1000 and 4000 pg/mL (n = 6). For inter-day accuracy and precision pooled urine was spiked with deuterated internal standards of both analytes at 15, 50, 250 and 4000 pg/mL (n= 5 days). Calculations were performed using calibration curves prepared using spiked pooled urine

at eleven concentrations (9.8-5000 pg/mL), prepared freshly on each day of analysis alongside the validation samples.

Calibration curve equations and r^2 for intra-day and inter-day calibration curves are shown in Table 2.2. The method showed good linearity with r^2 values from 0.9906 - 0.9952. The %RSD of slopes was 10.8% and 15.0% for 11β-PGF2α-d4 and LTE4-d5, respectively. This variability is acceptable considering no IS correction used.

Table 2.2 Calibration curve equations and r^2 for intra-day and inter-day experiments.

Experiment day	11β-PGF2α-d4		LTE4-d5	
	Equation	r^2	Equation	r^2
Intra-day	Y = 20330x + 9632	0.9952	Y = 1953x + 9498	0.9914
Inter-day 1	Y = 15610x - 41160	0.9914	Y = 1495x - 2735	0.9928
Inter-day 2	Y = 21410x + 25810	0.9918	Y = 2008x - 9120	0.9924
Inter-day 3	Y = 19780x - 43470	0.9916	Y = 2229x - 551.2	0.9936
Inter-day 4	Y = 21280x - 20660	0.9924	Y = 2385x - 126.4	0.9934
Inter-day 5	Y = 19650x - 43570	0.9906	Y = 2074x - 1328	0.9907

Endogenous levels of 11β-PGF2α and LTE4 were seen consistently in all the experiments which further supports the applicability of this method to measure endogenous urinary 11β-PGF2α and LTE4. The endogenous response of both analytes in pooled urine (from six different individuals; creatinine concentration = 2 mg/mL) during intra-day experiment is shown in Figure 2.20. The endogenous LTE4 response was consistent in all 48 replicates with a % RSD of 5.4%. The endogenous 11β-PGF2α response in 48 replicates was acceptable but showed poorer precision (% RSD = 14.8%). In particular, four samples seemed to be outliers on the higher range (Figure 2.20). Possible reason for this could be sample preparation error and/or instrument drift. The sample preparation does not seem to be the likely cause since the LTE4 precision was acceptable (5.4% RSD). The QC run immediately after these samples shows elevated signal intensity, thus instrument drift might have contributed towards these outliers. During clinical sample analysis, if

there were some effects due to matrix interferences or instrument drift then such effects would be corrected by the use of labelled internal standards during method application to clinical samples.

The Clinical Laboratory Improvement Amendments (CLIA) guidelines from The Center of Disease Control and Prevention requires a minimum of 20 replicates for intra-day accuracy and the imprecision requirement is ≤ 20%.[46] In this experiment, even without internal standard correction, 48 replicates had % RSD of 14.8% and 5.4% for 11β-PGF2α and for LTE4, respectively. Thus, our results meet the CLIA guidelines.

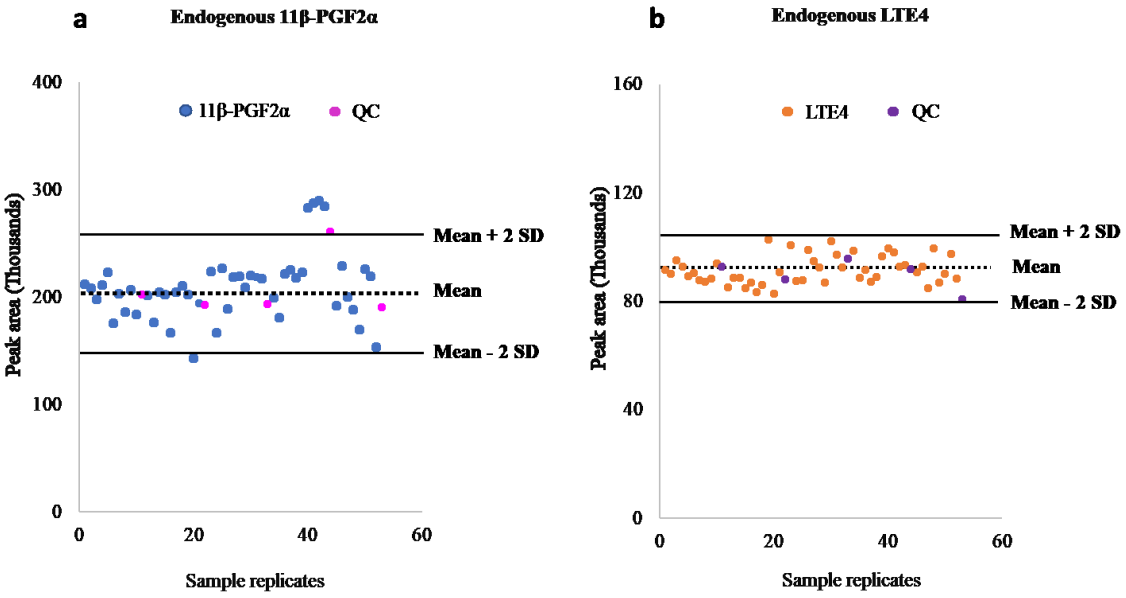

Figure 2.20 Peak areas of endogenous 11β-PGF2α (a) and LTE4 (b) in 48 replicates of pooled urine. Samples are shown according to their run order during intra-day accuracy validation experiment. For 11β-PGF2α (blue) run, QCs are shown in pink and for LTE4 (orange) run, QCs are shown in purple.

2.4.11.4. Recovery and matrix effects

The recovery and matrix effects were first evaluated in pooled urine as per regulatory guidelines. The recovery was evaluated by spiking pooled urine samples at 50, 250 and 4000 pg/mL (n = 6) with deuterated analytes prior to extraction. Matrix effects were evaluated at 2, 10 and 160 ng/mL post-extraction spiked concentrations (n = 6) to account for 40x enrichment prior to LC-MS analysis. 11β-PGF2α-d4 average recovery was 92-95% and LTE4-d5 average recovery was 77-83% at all three concentrations (Figure 2.21 a). 11β-PGF2α-d4 average matrix effects were 83-88% and LTE4-d5 average matrix effects were 99-115% (Figure 2.21 b) suggesting that no significant matrix effects were observed for both analytes in the pooled urine at the three concentrations tested.

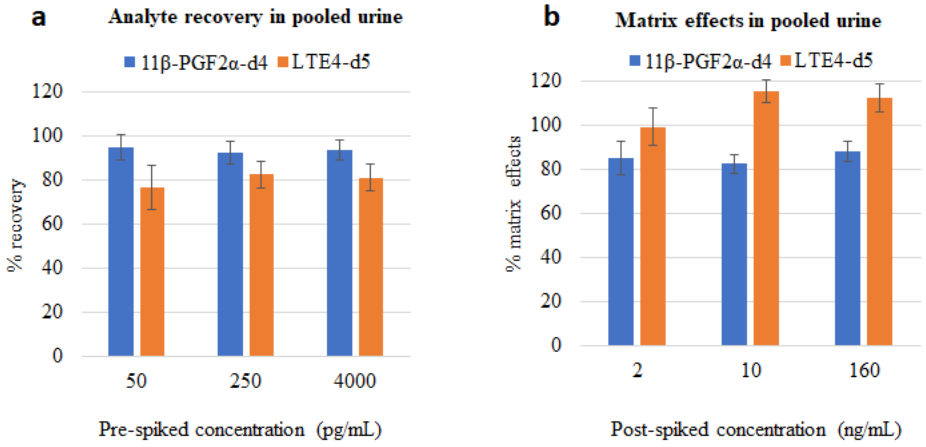

Figure 2.21 11β-PGF2α-d4 and LTE4 recovery (a) and matrix effects (b) in pooled urine. For recovery calculations, urine samples were pre-spiked at 20, 250 and 4000 pg/mL (n = 6) and post-extraction spiked with 2, 10 and 160 ng/mL (n = 6). For matrix effects calculations, urine was post-extraction spiked with 2, 10 and 160 ng/mL (n = 6) and standards in 50% MeOH were prepared at same concentrations.

Urine composition can vary widely from individual to individual even after creatinine normalization and may thus result in different matrix effects as well as recovery. Hence, the recovery and matrix effects were also further evaluated in nine different lots of urine. The results obtained are shown in Figure 2.22. The creatinine level in nine urine lots is listed in Supplementary

Table S1. The 11β-PGF2α-d4 recovery ranged from 70-116% (average = 100%) and LTE4-d5 recovery ranged from 61-99% (average = 79%) across nine urine lots. Similarly, matrix effects for 11β-PGF2α-d4 ranged from 69-106% (average = 86%) and matrix effects for LTE4-d5 ranged from 87-126% (average = 108%) across nine urine lots. This suggests that even after creatinine normalization, the sample variability of urine matrix across varies from individual to individual, and that slight matrix effects are observable in some lots of urine as 80-120% acceptance criteria is occasionally exceeded. This small variability in recovery and matrix effects will be compensated using the labelled internal standards during clinical sample analysis.

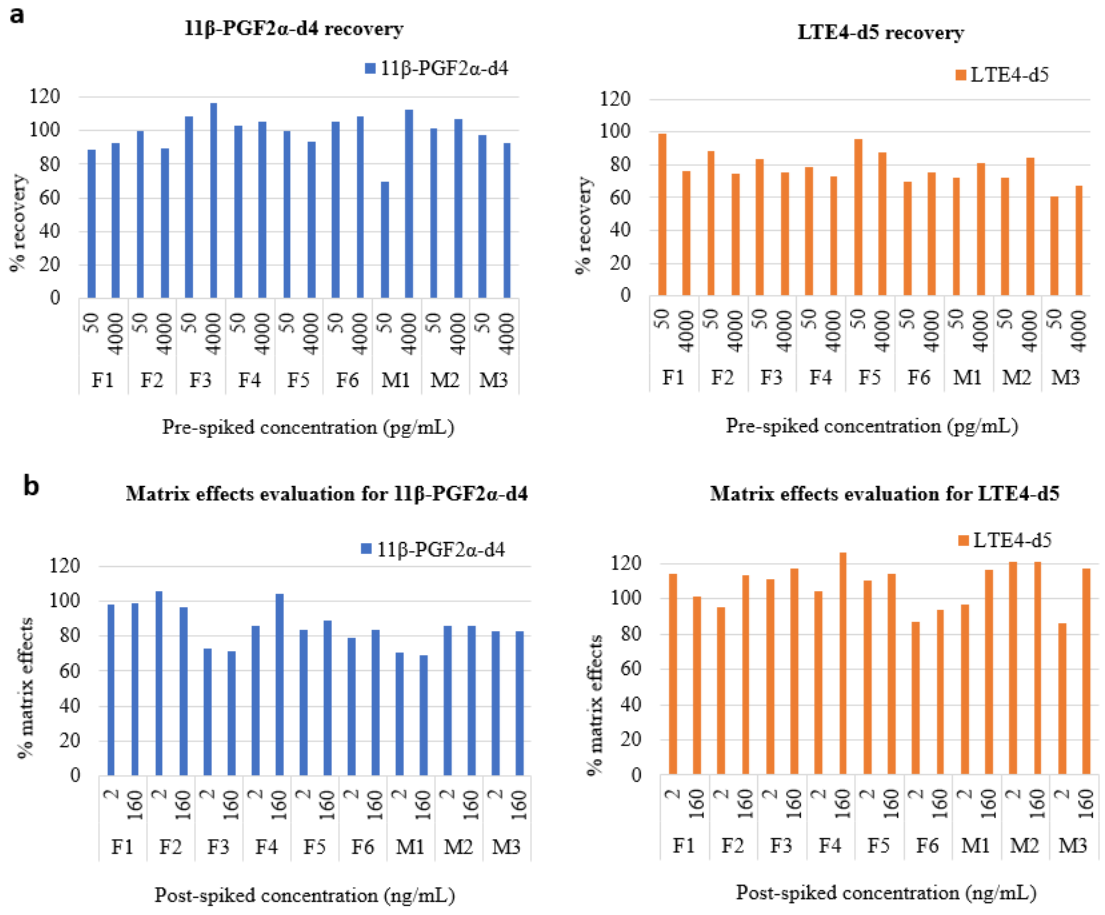

Figure 2.22 11β-PGF2α-d4 and LTE4-d5 recovery (a) and matrix effects (b) in urine samples from six females and 3 males. Recovery was evaluated by comparing pre-extraction spiked samples (spiked at 50 and 4000 pg/mL; n = 1 for each lot) with post-extraction spiked samples (spiked at 2 and 160 ng/mL to account for 40x enrichment during the procedure). Matrix effects were

calculated by comparing post-extraction spiked urine samples (spiked at 2 and 160 ng/mL; n = 1 for each lot) with standards at 2 and 160 ng/mL in solvent (50% MeOH).

Finally, since the retention time of LTE4 and LTE4-d5 was different by 0.09 min and the retention time of 11β-PGF2α and 11β-PGF2α-d4 was different by 0.05 min, matrix effects observed for deuterated and non-deuterated standards were compared to ensure that the deuterated internal standards will be able to compensate for any matrix effects observed during clinical sample analysis. The results are shown in Figure 2.23. The ratio of signal intensities obtained for non-deuterated and deuterated standards for both analytes was between 0.90 and 1.1 suggesting that the use of deuterated internal standards will compensate well for absolute matrix effects in clinical samples despite small difference in retention times between the analytes and their isotopically-labelled analogues.

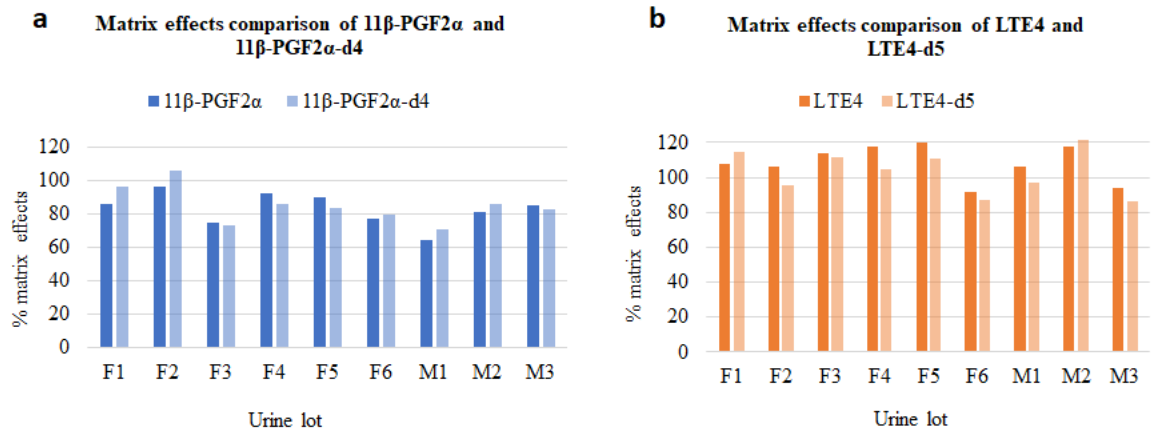

Figure 2.23 Matrix effect comparison of 11β-PGF2α and 11β-PGF2α-d4 (a) and LTE4 and LTE4-d5 (b). Urine from nine different individuals (six females and three males) was post-extraction spiked with 2000 pg/mL (n = 1) of deuterated and non-deuterated standards of both analytes. Matrix effects were calculated by comparing the response in urine with the response in solvent (50% MeOH). For non-deuterated standards the appropriate correction was performed by first subtracting the endogenous response in the non-spiked samples.

2.4.11.5. Sample stability

One, two and three freeze-thaw (F/T) cycle stability was evaluated to ensure that if samples are stored in a freezer they would be stable for at least one F/T cycle in order to be able to perform the analysis. 11β-PGF2α-d4 was stable for up to three F/T cycles (Figure 2.24). After two F/T cycles LTE4 recovery was outside the 80-120% range (recoveries were between 94-122%), suggesting that low levels of LTE4 are not stable for more than one F/T cycle. As such, samples should be aliquoted as needed immediately after collection and freeze-thaw cycles should be avoided. Both analytes were stable in freezer after 1-week and 3-weeks of storage with the recovery of both analytes ranging from 93-114% at both concentrations tested for both time points. A 48-hour autosampler (6°C) stability was also evaluated to ensure that both analytes were stable in the prepared extracts during long batches. LTE4-d5 recovery was 94% and 97% at 50 pg/mL and 4000 pg/mL concentration respectively whereas 11β-PGF2α-d4 stability was 95% and 107% at 50 pg/mL and 4000 pg/mL concentration respectively, suggesting that both analytes are stable in autosampler for up to 48 hours.

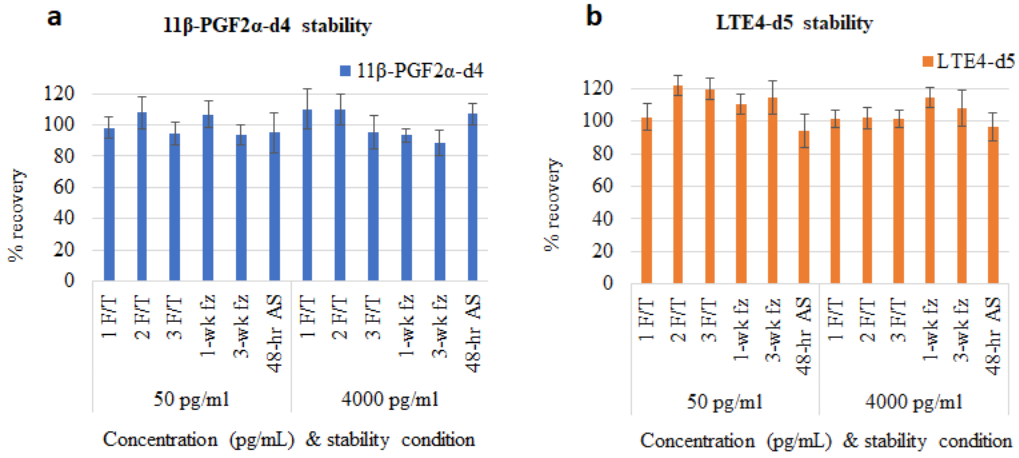

Figure 2.24 Stability of 11β-PGF2α-d4 (a) and LTE4-d5 (b). F/T = freeze-thaw cycle; 1-wk fz = 1-week freezer; 3-wk fz = 3-week freezer; 48-hr AS = 48-hours autosampler. For F/T cycles and freezer stability urine samples were pre-spiked with 50 and 4000 pg/mL (n = 3) followed by sample preparation and LC-MS analysis. For 48-hours autosampler stability prepared samples were kept in autosampler for 48-hours followed by LC-MS analysis. Recovery was compared with freshly prepared samples.

2.5. Conclusions

In this study we developed and validated a highly selective and accurate UHPLC-Q-TOF method for the analysis of urinary 11β-PGF2α and LTE4. A chromatographic separation of all known isomers of both analytes was achieved. The negative ESI gave the best sensitivity for both analytes. After optimization of SPE steps, C18 SPE gave an optimum recovery of both analytes, but required two separate elution steps to achieved adequate LODs. The addition of glycerol in evaporation tubes improved precision by reducing analyte losses by non-specific adsorption. Forced degradation studies of LTE4 revealed that it is not stable under low pH and high temperatures. In contrast, pH values >5 and low temperatures promoted isomerization over time. The method has very good selectivity, linearity, accuracy and precision. Even with the use of creatinine normalization, the results show that the recovery and matrix effects for urinary 11β-PGF2α and LTE4 can vary slightly depending upon the matrix and its composition. These effects will be well-corrected by stable-isotope dilution during clinical sample analysis Overall, this method provides a new analytical tool for the quantification of urinary 11β-PGF2α and LTE4 which can be applied to biomarker identification in clinical studies.

3. Conclusions and future work

3.1. Conclusions

The quantitative analysis of eicosanoids such as urinary 11β-PGF2α and LTE4 is challenging because they are present in trace levels in urine and because of the existence of several isomers which may interfere with the accurate measurement of the selected analyte of interest. Although antibody-based methods can achieve adequate limits of detection, they may not be able to distinguish well among all possible isomers and phase II metabolites, such as glucuronides. LC-MS can help improve the selectivity of this analysis and ensure appropriate separation of all known interreferences. However, achieving adequate limits of detection by LC-MS and good recovery of both analytes during the enrichment step in a variable biofluid such as urine is also challenging. In this work we developed and validated a UHPLC-QTOF method for accurate and highly selective quantitation of urinary 11β-PGF2α and LTE4. Since all isomers generate similar MS2 product ion spectra, LC separation of all isomers was required and was achieved using a C18 stationary phase and an acetonitrile/isopropanol/acetic acid mobile phase. The chromatographic separation of all PGF2 isomers was ensured using a 30% isocratic mobile phase B and separation of LTE4 isomers was ensured using a 0.5%/min mobile phase B gradient. 11β-PGF2α can ionize in negative ESI whereas LTE4 can ionize in both positive and negative ESI and hence, both ionization modes were tested for LTE4. The negative ESI was selected because it gave 7 times higher S/N ratio in negative than in positive ESI for LET4.

11β-PGF2α and LTE4 are moderately non-polar and weak anionic metabolites. Hence, mixed-mode anion-exchange and reversed-phase C18 SPE were tested for sample preparation. In spiked urine, 11β-PGF2α recovery was quantitative in all sorbents whereas LTE4 recovery was the highest (80%) using C18 SPE. Considering normal average endogenous levels of 11β-PGF2α are 90 pg/mL and normal average endogenous levels of LTE4 are 40 pg/mL, enrichment was needed. However, when 20x and 40x enrichment was attempted, high LC back pressure was observed with MAX and WAX SPE possibly due to co-extraction of anionic interferences. Therefore, C18 SPE was selected for further sample preparation method development.

The addition of the evaporation/reconstitution step resulted in low recovery and poor precision for LTE4. Poor precision was attributed to non-specific adsorption and/or solubilization and could be addressed by the addition of glycerol to the evaporation tubes. To troubleshoot low recovery of LTE4, the elution pH was varied. LTE4 recovery was very low (22%) without any acid in elution solvent, but on the other hand increasing the percentage of acid in elution solvent beyond 0.2% (v/v) also caused a systematic decrease in LTE4 recovery. This suggests that a slightly acidic pH is required for acceptable recovery (75%) of LTE4 but increasing acid content beyond this results in too low pH where LTE 4 is unstable, as shown by forced degradation studies.

To further investigate the effect of pH and temperature on LTE4 stability, a forced degradation study was performed at pH 2, 3, 4, 5 and 6 and at 4°C, room temperature and 37°C. LTE4 was stable at pH $\geq$ 4 at 4°C and room temperature but not at 37°C. One of the forced degradation products of LTE4 was identified as glycine using glycine standard suggesting that the S-C bond in LTE4 structure breaks off releasing the glycine moiety. 8.9-10.4% isomerization of LTE4 to 11-trans-LTE4 was observed at 4°C at pH above 4, which suggested that LTE4 slowly isomerizes to 11-trans-LTE4 at low temperature and pH above 4 over time.

For C18 SPE, several parameters were optimized such as the loading volume, wash volume, wash solvent composition, elution volume and type of elution solvent (ACN and MeOH). For the loading volume, 500 µL, 1 mL and 2 mL urine loading volumes were tested and flow-throughs and washes were analyzed to ensure that loading volume does not exceed column binding capacity. The different washes were tested to check whether the nature of interferences were possibly polar, ionic, basic or acidic and to remove them. 7 mL of 30% ACN wash helped to decrease the background significantly while maintaining the LTE4 recovery to 75%. It also helped to improve the LOD of LTE4 in urine 4 times. Since 11β-PGF2α is more polar than LTE4, it was washed away with 7 mL of 30% ACN SPE wash. Hence, in order to recover both analytes in one SPE protocol, a two-step elution was selected as the best strategy in which after washing the SPE cartridge with 3 mL of 25% ACN, elution 1 was performed using 4 mL 30% ACN to collect 11β-PGF2α, followed by elution 2 using 1.3 mL 60% ACN + 0.1% FA to collect LTE4. In turn, this necessitated the use of two shorter LC methods, rather than a single method for the measurement of the two analytes of interest.

B-glucuronidase enzyme hydrolysis was used to measure total endogenous 11β-PGF2α. Enzyme hydrolysis showed 4 times increase in 11β-PGF2α signal suggesting that a considerable amount of endogenous 11β-PGF2α is excreted in urine as glucuronide conjugate.

Since both analytes are present endogenously, method was validated using deuterated surrogate analytes using β-glucuronide enzyme hydrolysis. The method was selective and showed good intra-day accuracy (90-111% for 11β-PGF2α and 86-108% for LTE4) and precision (% RSD = 4.0%-7.4% for 11β-PGF2α and % RSD = 6.8%-14.9% for LTE4). Method was linear from 9.8-5000 pg/mL for both analytes (r^2 = 0.9952 for 11β-PGF2α-d4 and r^2 = 0.9914 for LTE4-d5) and LLOQs for both analytes was 15 pg/mL. The average 11β-PGF2α-d4 recoveries was 92-95% and average LTE4-d5 recoveries ranged from 77-83% in pooled urine. Average absolute matrix effects for 11β-PGF2α-d4 ranged from 83-88% and average absolute matrix effects for LTE4-d5 ranged from 99-115% in pooled urine. Thus, method had acceptable recoveries of both analytes without significant matrix effects in pooled urine. Recovery and matrix effects were also evaluated in nine different urine lots. The 11β-PGF2α-d4 recovery ranged from 70-116% (average = 100%) and LTE4-d5 recovery ranged from 61-99% (average = 79%) across nine urine lots. Matrix effects for 11β-PGF2α-d4 ranged from 69-106% (average = 86%) and matrix effects for LTE4-d5 ranged from 87-126% (average = 108%) across nine urine lots, suggesting that slight matrix effects were present in individual samples as compared to matrix effects in pooled urine.

None of the previously used LC-MS/MS methods for PGFs and LTE4s have shown separation of all six isomers of PGFs and two isomers of LTE4. Balgoma *et al.*[18] showed LC separation of only two PGF isomers out of six and Sasaki *et al.*[38] showed LC separation of five PGF isomers. However, considering the similar fragmentation of isomers in MS2, it is absolutely necessary to separate isomers chromatographically for correct identification of one specific isomer. Our method gives a baseline separation of all PGF and LTE4 isomers. Endogenous 8-iso-PGF2α and endogenous PGF2α isomers were frequently seen in different urine samples from different individuals, while 8-iso-15(R)-PGF2α and 15(R)-PGF2α were seen in three out of nine individual urine samples tested. Previous LC-MS/MS methods had LTE4 LLOQs as 5 pg/mL[31], 6 pg/mL[30], 8 pg/mL[37] and 263.3 pg/mL[18], whereas 11β-PGF2α LLOQs were 48 pg/mL[38] and 152.4 pg/mL.[18] Thus, LTE4 LLOQs with our method are similar to the average LLOQs in literature, whereas 11β-PGF2α LLOQs are better than previous methods. The LLOQs for 11β-PGF2α and LTE4 reported

in commercially available immunoassay kits is 1.6 pg/mL and 7.8 pg/mL, respectively. [47,49] Thus, our method has similar LLOQs but better precision at such low concentrations as compared to immunoassays.

Overall, we developed and validated a UHPLC-QTOF method for quantitation of urinary 11β-PGF2α and LTE4. The method meets general validation criteria provided by the FDA for bioanalytical method validation with good accuracy, linearity, precision and limits of quantitation.

3.2. Future work

To date, the presented method was validated with the inclusion of β-glucuronidase enzyme hydrolysis, which measures the total 11β-PGF2α. Hence, to measure the free 11β-PGF2α concentration in urine, the method should also be validated without enzyme hydrolysis prior to its application to clinical samples, in case the ratio of free and total 11β-PGF2α levels in urine varied between anaphylactic individuals and healthy individuals. The cross-reactivity of existing immunoassays to PGF glucuronides is also not known, so it is not clear if the reported values in anaphylaxis correspond to total or unconjugated 11β-PGF2α.

One weakness of the proposed method is that the elution of 11β-PGF2α with 4 mL of 30% ACN takes a long time to evaporate. In the future, a higher percentage of organic with low volumes can be tested for 11β-PGF2α recovery and evaporation time in a speedvac in order to shorten the sample preparation time if only 11β-PGF2α is the analyte of interest, or if sufficient urine sample is available for two SPE extractions.

A long-term stability study should be performed before application to clinical samples to ensure that the clinical samples are stable during long term storage.

Two previous studies that proposed urinary 11β-PGF2α and LTE4 as possible biomarkers of anaphylaxis used immunoassays and were performed in small cohorts.[20,21] Hence these results need to be validated in a bigger population. In addition, immunoassays have a major problem of cross-reactivity. For example, reported cross-reactivity of 11β-PGF2α immunoassay kit by Cayman Chemicals is 0.1% for 2,3 dinor 11β-PGF2α isomer and 0.01% for PGF2α. However, the endogenous levels of 2,3 dinor 11β-PGF2α isomer in urine is 3.5 times more than 11β-PGF2α and

the endogenous levels of PGF2α is about 20 times more than 11β-PGF2α in urine.[38,47] Similarly, the LTE4 immunoassay kit by Cayman Chemicals has a cross reactivity of 0.1% for LTB4 and the endogenous levels of LTB4 are 3.5 times higher than LTE4 in urine.[48,49] The bigger issue with the immunoassay cross reactivity is that for most of the PGF isomers and for the glucuronide conjugates, the cross-reactivity is not reported by the kit manufacturers which is even bigger potential source of error.

Hence, the urinary 11β-PGF2α and LTE4 needs to be tested as possible biomarkers of anaphylaxis in a large population size with a highly selective LC-MS method in order to avoid incorrect identification of isomers which may have been the case with previous studies where immunoassays were used.

We have developed a very selective LC-MS assay for quantitation of urinary 11β-PGF2α and LTE4 which can be used to further validate these two analytes as potential biomarkers of anaphylaxis in clinical studies.

The clinical samples were provided by Cross Canada anaphylaxis REgistry (C-CARE) project. C-CARE is a project of the Allergy Genes and Environment Network (AllerGen). AllerGen is a federally-funded network in which experts in various disciplines work together to address allergy, asthma, anaphylaxis and related immune diseases in order to reduce the mortality and socio-economic impact of these diseases by creating preventive strategies, diagnostic tests, medical treatments, public policies and patient education. AllerGen launched C-CARE project in 2011. Dr. Moshe Ben-Shoshan, a pediatrician at Montreal Children's Hospital, is the head investigator of C-CARE and is our collaborator in this project. C-CARE aims to register information about the anaphylactic patients brought to the emergency departments by paramedics, patients and allergists and gather data on possible causes of anaphylaxis and measures to take for management of anaphylaxis. The urine samples for the present study were provided by the Montreal Children's Hospital that were collected from anaphylactic patients after the onset of anaphylaxis. Urine samples were also collected from the same patients after anaphylaxis symptoms had subsided following the treatment, to serve as control samples.

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2.5. Appendix A: Supplementary information

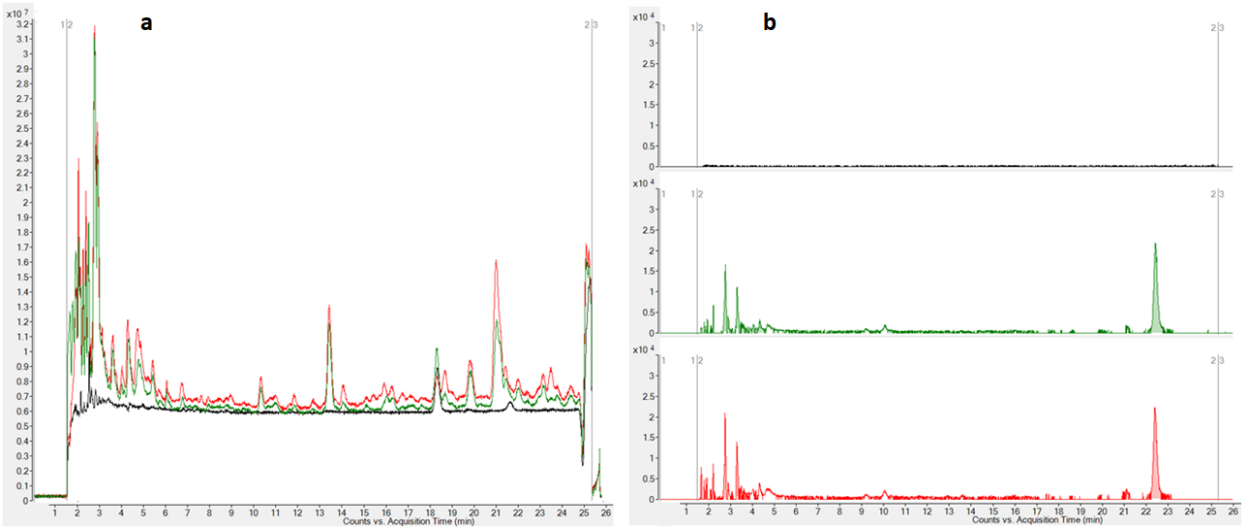

Figure S1. Effect of 0.1% FA and 0.2% FA in SPE elution solvent on LTE4 background. In this experiment, two SPE elution solvents were used: 0.2% FA in 60% ACN (shown in red color) and 0.1% FA in 60% ACN (shown in green color). The blank water extraction is shown in black color. (a) TICs with different percentage of FA in elution solvent and blank extraction. (b) EICs of LTE4 with different percentage of FA in elution solvent and blank extraction.

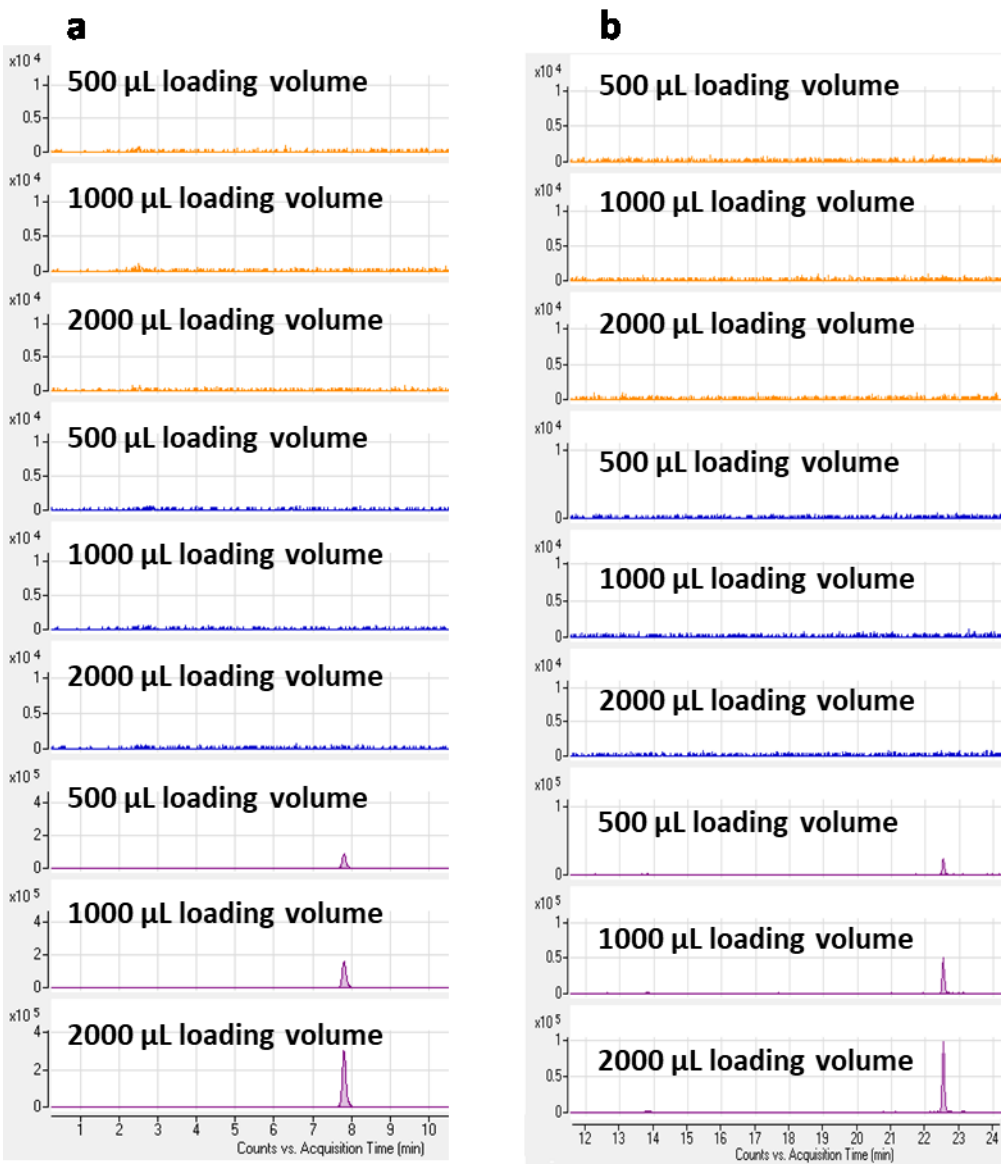

Figure S2. Optimization of urine loading volume for SPE. (a) 11β-PGF2α EICs and (b) LTE4 EICs. After conditioning and equilibration of C18 cartridge, 500 µL, 1000 µL and 2000 µL of urine was loaded. Cartridge was washed with 3 mL 20% ACN and elution was performed with 1.3 mL 80% ACN with 0.1% FA. Analysis of flow throughs are shown in orange, washes are shown in blue and elutes are shown in purple.

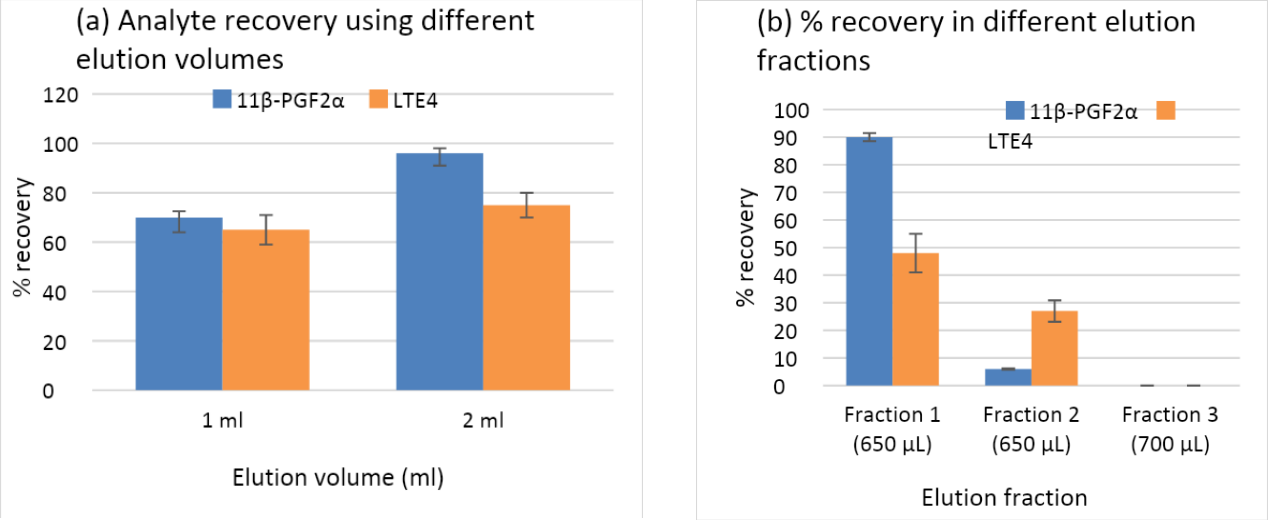

Figure S3. Optimization of SPE elution volume. (a) After condition, equilibration and loading on C18 SPE, the analytes were eluted either with 1 mL 80% ACN + 0.2% FA or with 2 mL 80% ACN + 0.2% FA (n = 3). (b) After condition, equilibration and loading on C18 SPE, the analytes were recovered using 80% + 0.2% FA in three separate fractions of 650 µL, 650 µL and 700 µL. The fractions were evaporated and reconstituted separately and analyzed.

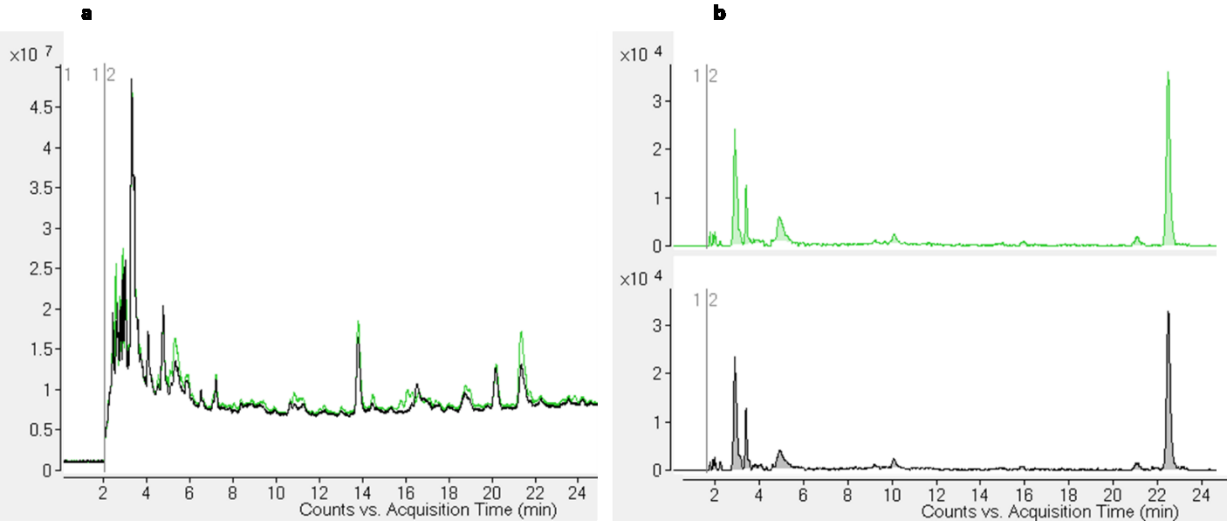

Figure S4. (a) TICs and (b) LTE4 EICs. After conditioning, equilibration and spiked urine sample loading, the cartridge was washed with 3 mL of 25% ACN, elute 1 (for PGF) was collected using 4 mL 30% ACN (shown in green) and then elute 2 (for LTE4) was collected using 1.3 mL of 60% ACN + 0.1% FA. After conditioning, equilibration and spiked urine sample loading, the cartridge was washed with 7 mL of 30% ACN and LTE4 was eluted with 1.3 mL of 60% ACN + 0.1% FA.

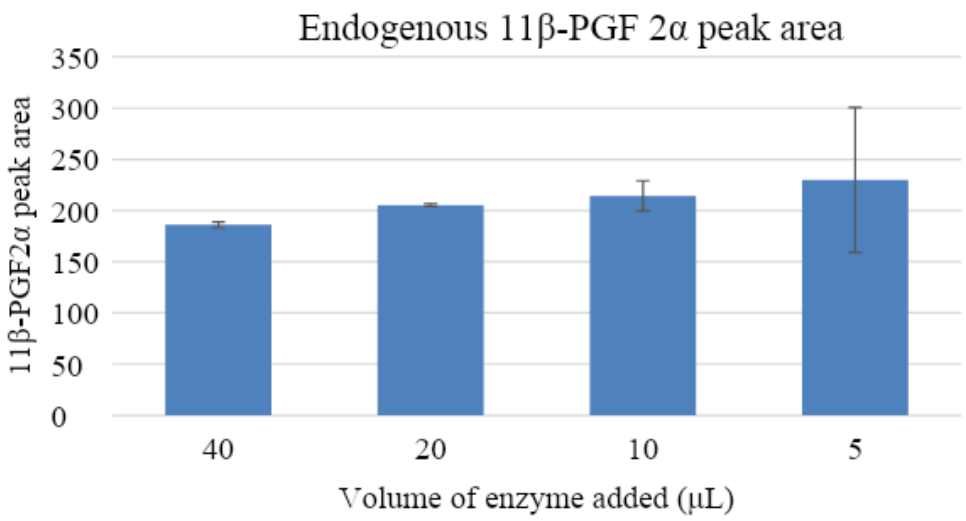

Figure S5. Optimization of volume of enzyme to be added for β-glucuronidase enzyme hydrolysis. pH of 2 mL urine was adjusted to 5.5 with AA and 200 µL of acetate buffer (1M, pH 5.5) was added. This was followed by addition of either 5, 10, 20 or 40 µL of β-glucuronidase/arylsulfatase solution (n = 3 for each).

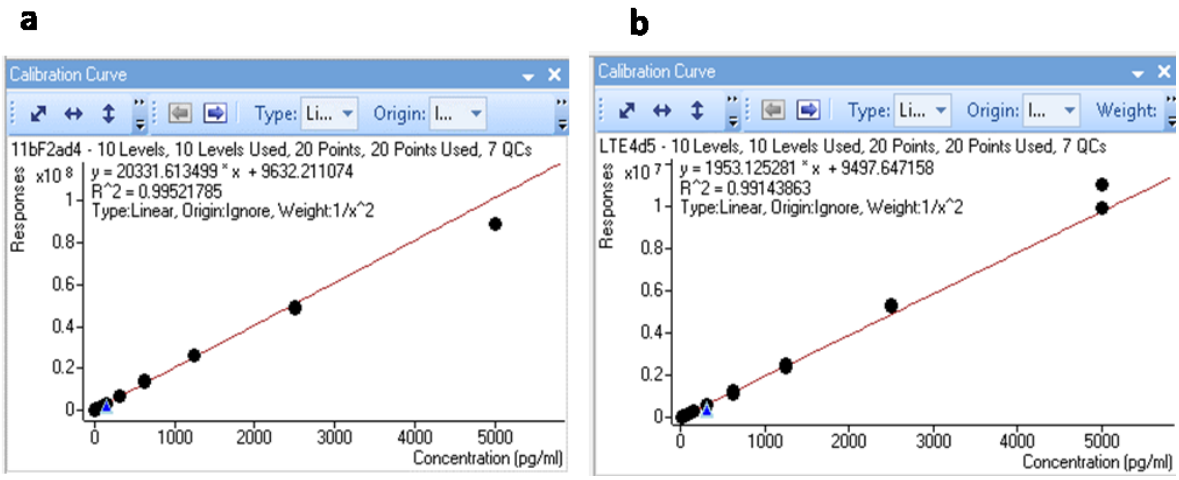

Figure S6. Calibration curves of 11β-PGF2α-d4 (a) and LTE4-d5 (b) in spiked urine. Average of two calibration curves is shown for both analytes. The urine was pre-spiked at 9.77, 19.53, 39.06, 78.12, 156.25, 312.5, 625, 1250, 2500 and 5000 pg/mL. Quality control samples (shown as blue triangles) were prepared by spiking pooled urine pre-extraction with 50 pg/mL of all PGF and LTE4 isomers.

Supplementary Table S1. Creatinine concentration (mg/mL) in the nine different urine lots from different individuals.

Urine lot	Creatinine concentration (mg/mL)
Lot 1	0.5
Lot 2	1
Lot 3	2
Lot 4	1
Lot 5	1
Lot 6	3
Lot 7	3
Lot 8	2
Lot 9	3