Accepted Manuscript

Title: Determination of potential childhood asthma biomarkers using a powerful methodology based on microextraction by packed sorbent combined with ultra-high pressure liquid chromatography. Eicosanoids as case study

Authors: Pedro H. Berenguer, Irene C. Camacho, Rita Câmara, Susana Oliveira, José S. Câmara

PII: S0021-9673(18)31441-9
DOI: https://doi.org/10.1016/j.chroma.2018.11.041
Reference: CHROMA 359820

To appear in: *Journal of Chromatography A*

Received date: 16 April 2018
Revised date: 17 November 2018
Accepted date: 20 November 2018

Please cite this article as: Berenguer PH, Camacho IC, Câmara R, Oliveira S, Câmara JS, Determination of potential childhood asthma biomarkers using a powerful methodology based on microextraction by packed sorbent combined with ultra-high pressure liquid chromatography. Eicosanoids as case study, *Journal of Chromatography A* (2018), https://doi.org/10.1016/j.chroma.2018.11.041

Determination of potential childhood asthma biomarkers using a powerful methodology based on microextraction by packed sorbent combined with ultra-high pressure liquid chromatography. Eicosanoids as case study

Pedro H. Berenguer[a], Irene C. Camacho[b], Rita Câmara[c], Susana Oliveira[c], José S. Câmara[a,d,*]

[a] CQM – Centro de Química da Madeira, Universidade da Madeira, Campus Universitário da Penteada, 9020-105 Funchal, Portugal

[b] Faculdade de Ciências da Vida, Universidade da Madeira, Campus Universitário da Penteada, 9020-105, Funchal, Portugal

[c] Unidade de Imunoalergologia, Hospital Dr. Nélio Mendonça, SESARAM, E.P.E., 9004-514 Funchal, Portugal

[d]Departamento de Química, Faculdade de Ciências Exatas e da Engenharia, Universidade da Madeira, Campus Universitário da Penteada, 9020-105, 9004-514 Funchal, Portugal

* **Corresponding author**

Prof. José S. Câmara, E-mail: jsc@uma.pt; Tel: +351 291705112; Fax: +351 291705149

Faculdade das Ciências Exactas e da Engenharia, Universidade da Madeira, Campus Universitário da Penteada, 9020-105 Funchal, Portugal

Highlights

- Validation of a MEPS/UHPLC-PDA methodology for quantification urinary eicosanoids
- Semi-automatic straightforward sample preparation and extraction was optimized
- Satisfactory figures of merit of method were obtained ensuring robust results
- Eicosanoids levels from asthmatic patients are highest than healthy individuals

ABSTRACT

Leukotrienes and prostaglandins are arachidonic acid bioactive derived eicosanoids and key mediators of bronchial inflammation and response modulation in the airways contributing to the pathophysiology of asthma.

An easy-to-use ultra-high pressure liquid chromatography (UHPLC)-based strategy was developed to characterize biomarkers of lipid peroxidation: leukotrienes E_4 (LTE_4) and B_4 (LTB_4) and 11β-prostaglandin $F_{2\alpha}$ ($11\beta PGF_{2\alpha}$), present in urine of asthmatic patients (N = 27) and healthy individuals (N = 17). A semi-automatic eVol®-microextraction by packed sorbent (MEPS) was used to isolate the target analytes. Several experimental parameters with influence on the extraction efficiency and on the chromatographic resolution, were evaluated and optimized. The method was fully validated under optimal extraction (R-AX sorbent, 3 conditioning-equilibration cycles with 250 µL of ACN-water at 0.1% FA, 10 extract-discard cycles of 250 µL of sample at a pH of 5.1, elution with 2 times 50 µL of MeOH and concentration of the eluate until half of its volume) and chromatographic conditions (14-min analysis at a flow rate of 300 µL min-1 in an UHPLC-PDA equipped with a BEH C18 column), according to IUPAC guidelines. The findings indicated good recoveries (>95%) in addition to excellent extraction efficiency (>95%) at three concentration levels (low mid and high) with precision (RSDs) less than

11%. The lack-of-fit test, goodness-of-fit test and Mandel's fitting test, revealed good linearity within the concentration range. Good selectivity and sensitivity were achieved with a limits of detection ranging from 0.04 μg L^{-1} for LTB4 to 1.12 μg L^{-1} for 11βPGF2α, and limits of quantification from 0.10 μg L^{-1} for the LTB4 to 2.11 μg L^{-1} for 11βPGF2α. The successful application of the fully validated method shows that, on average, the asthmatic patients had significantly higher concentrations of $11\beta PGF_{2\alpha}$ (112.96 μg L^{-1} *vs* 62.56 μg L^{-1} in normal controls), LTE_4 (1.27 μg L^{-1} *vs* 0.89 μg L^{-1} in normal controls), and LTB_4 (1.39 μg L^{-1} *vs* 0.76 μg L^{-1} in normal controls). The results suggest the potential of the target eicosanoids on asthma diagnosis, however, a larger and more extensive study will be necessary to confirm the data obtained and to guarantee a greater robustness to the approach.

Keywords: Asthma; Biomarkers; Eicosanoids; MEPS; UHPLC.

1 Introduction

Asthma is a noncommunicable chronic disease usually characterized by chronic airway inflammation, bronchial hyperresponsiveness and recurrent episodes of reversible airway obstruction [1]. It affects about 300 millions people all over the world [2] with a special prevalence in children from high income countries [3]. This disease is particularly problematic given that it consists of many different phenotypes, each with its own etiologic and pathophysiologic factors, being considered a multifactorial syndrome with a challenging diagnosis [4].

In order to improve asthma diagnosis and treatment, considerable efforts have been made to study potential biomarkers able to discriminate different phenotypes of this disease [5].

Currently, several asthma biomarkers have been found in different biological fluids. In airway remodeling, elevated levels of Th2 cytokines and eosinophilia were observed in the bronchoalveolar lavage (BAL) and endobronchial biopsies of asthmatic patients. The presence of different types of granulocytes, (neutrophils, eosinophilsa and exosomes), and signaling proteins obtained in the induced sputum of asthmatics, helps to identify the phenotype. Also, the fraction of exhaled nitric oxide (FeNO) has been measured routinely by means of commercially available analyzers [6]. Even though bronchial biopsies, BAL and induced sputum are highly relevant to assess the airway physiology and integrity, they are particularly invasive to obtain and generally require trained personnel to make the collection, being relatively inaccessible to obtain. Blood is less invasive to obtain and contains endogenous analytes that provide a good overview of the metabolic status of the patient and potentially of its airway inflammation (e.g. eosinophilia, chemokines and cytokines) but still requires trained personnel to collect with some degree of evasiveness [6-8].

Therefore, there is a need of biosamples that can be easily collected, through non-invasive sampling strategies. Totally non-invasive sampling procedures, such as urine collection, are especially appealing for the child population . It is easily obtained across the full age spectrum, and possesses an array of molecular biomarkers related to airway inflammation [9], including the arachidonic acid end products [10]. Enzymatic peroxidation of the arachidonic acid is a fundamental process that leads to the formation of bioactive eicosanoids such as prostaglandins and leukotrienes, key mediators of airway inflammation and response modulation in airways [11] with a strong potential as asthma biomarkers, in assessing its progression and follow of the therapy efficiency [5, 9].

To date, several analytical platforms have been developed in order to study urinary levels of eicosanoids in asthmatic patients including the traditional sample preparation

techniques such as liquid-liquid extraction (LLE) [12] and SPE [12, 13], usually hyphenated to mass spectrometry; and enzyme immunoassays [14, 15], an alternative to chromatography. Even though these techniques are widely used among researchers, they are not easily hyphenated to an analytical platform, expensive, time consuming, unfeasible sampling procedure, need considerable amounts of harmful organic solvents with strong environmental implications, especially in the case of LLE, and requires substantial amounts of sample [16].

Microextraction techniques emerged in the 90's with SPME [16, 17] and up to date, a noteworthy number of different microextraction techniques are continuously being developed including needle-based sampling techniques such as MEPS [18], a miniaturized SPE technique consisting of a removable cartridge containing a sorbent, and a syringe-like sampler device, which can be manual, semi-automatic or fully automatic [19]. This technique has been recently applied in the analysis of urine samples for the characterization of, among other, environmental/occupational derived metabolites [20, 21], psychoactive metabolites [22, 23] and disease potential biomarkers related to asthma [24]. The miniaturization of the sample treatment techniques and their improvement in terms of sensitivity has allowed the use of absorbance detection techniques [19], hyphenated to liquid chromatography, such as photodiode array (PDA) and UV at a relative reduce cost compared to mass spectrometry.

Taking into consideration the most recent trends in liquid chromatography, UHPLC has been widely spread due to its increased sensitivity, resolution and time of analysis. Therefore, the present work aimed to develop a highly sensitive MEPS/UHPLC-based approach, to identify and quantify lipid peroxidation biomarkers related with asthma – LTE_4, LTB_4 and $11\beta PGF_{2\alpha}$ – present in urine of asthmatic patients and healthy individuals. The most extraction efficiency-influencing experimental parameters,

including the sorbent material, the number and mode (extract-discard and extract-eject) of sample and eluent loading cycles, the pH, the volume of elution solvent and the conditioning solvent, were evaluated and optimized by means of a univariate experimental design. In addition, important instrumental parameters with influence on the chromatographic resolution, such as composition of the mobile phase, the nature of the stationary phase of capillary column and the flow rate of mobile phase, were also optimized. The stability of eicosanoids extracts was checked over a period of 24 hours with sampling throughout a single day. The proposed methodology was applied to the analysis of children urine with allergic asthma and healthy children, used as control group.

2 Materials and methods

2.1 Reagents and materials

All standards and reagents were of analytical grade with a purity higher than 98%. acetonitrile (ACN; 99.9%) and methanol (MeOH; 99.9%) were obtained from Fisher Scientific (Leics, UK). Formic acid (FA; 98%), ethanol (EtOH; 99.5%), calcium chloride-2-hydrate, sodium sulphate anhydrous, sodium carbonate anhydrous, potassium di-hydrogen phosphate and sodium hydroxide (NaOH) were acquired from Panreac (Barcelona, Spain). Magnesium chloride-6-hydrate and potassium chloride were purchased from Honeywell Riedel-de-Haën AG (Seelze, Germany). Tri-Sodium citrate-2-hydrate and ammonium chloride were acquired from Merck (Darmstadt, Germany) and sodium chloride from Sigma-Aldrich (Buchs, Switzerland). Ultrapure water (18 MΩ cm at 23 °C) was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). Kaempferol, used as internal standard (IS), was acquired from Fluka Biochemica AG (Buchs, Switzerland). LTB_4, LTE_4 and $11\beta PGF_{2\alpha}$ standards were acquired from Cayman Chemical Co. (MI, USA).

The eVol® XR hand-held automated analytical syringe (500 µl) and the BINs: C18, C8, C2, SIL, M1, HLB-DVB, R-AX and R-CX, containing the sorbent used in microextraction procedure were purchased from SGE Analytical Science Europe, Ltd. (Milton Keynes, United Kingdom). The polymeric sorbents PEP, VAX and PGC were from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

The capillary columns and pre-columns: Acquity UPLC® BEH C18 (2.1 × 50 mm) protected with an Acquity UPLC® BEH C18 VanGuard™ Pre-column (2.1 × 5 mm), Acquity UPLC® HSS T3 (2.1 × 100 mm) protected with an Acquity UPLC® HSS T3 VanGuard™ Pre-column (2.1 × 5 mm), Acquity UPLC® CSH C18 (2.1 × 150 mm) protected with an Acquity UPLC® CSH C18 VanGuard™ Pre-column (2.1 × 5 mm) and CORTECS UPLC® C18 (2.1 × 100 mm) protected with a CORTECS UPLC® C18 VanGuard™ Pre-column (2.1 × 5 mm), were acquired from Waters Portugal (Lisboa, Portugal). The Kinetex® HILIC (2.1 × 100 mm) protected with SecurityGuard™ ULTRA cartridge for HILIC UHPLC (2.1 × 2 mm) was supplied by Tecnocroma – Técnicas analíticas, LDA (Caldas da Rainha, Portugal).

2.2 Standard solutions and synthetic urine

Individual stock solutions of all standards were prepared in EtOH at 10 mg L^{-1} for the LTB_4 and LTE_4, and 1 g L^{-1} for the $11\beta PGF_{2\alpha}$ and the IS. Stock solutions of the LTB_4 and LTE_4 were used as working solutions, whereas for $11\beta PGF_{2\alpha}$ and IS the working solutions were diluted in EtOH at concentrations of 100 mg L^{-1} and 1 mg L^{-1}, respectively. All stocks and working solutions were aliquoted in 2 mL amber glass vials and stored at -80 ℃.

A synthetic urine (SU) solution was prepared according to Wilsenach et al. [25] with slight modifications (Table 1SM, Supplementary Material). Prior to its use, the pH of

synthetic urine was adjusted to 5.1 (Mettler Toledo™ EL20 Benchtop pH Meter for Teaching and Learning, Mettler Toledo AG, Switzerland) with 10% FA and 1M NaOH, and filtered through a 0.2 µm membrane PTFE filter (Merck Millipore, Milford, MA, USA). The pH of real urine samples including an unspiked real urine (USRU), were adjusted to 5.1, centrifuged at 5000 rpm (2739 × g; Sigma 3K30, Sigma Laborzentrifugen GmbH, Germany) for 15 minutes at 5 °C, and the supernatants filtered through 0.22 µm PTFE syringe filters (BGB Analytik, VA, USA).

For method development, optimization and validation, the assays were carried out using fortified SU with LTB_4, LTE_4 and $11\beta PGF_{2\alpha}$ at different concentrations according to the sensitivity of the UHPLC-PDA and the amount of eicosanoids present in urine.

The stability of the extracts was evaluated in extracts of spiked SU at low level (LL; 25 µg L^{-1} for the $11\beta PGF_{2\alpha}$, and 2.5 µg L^{-1} for the LTB_4 and LTE_4) and medium level (ML; 100 µg L^{-1} for the $11\beta PGF_{2\alpha}$, and 10 µg L^{-1} for the LTB_4 and LTE_4) of concentrations. A set of 4 replicates were taken and analyzed at 0, 8, 16 and 24 hours after extraction. This parameter, evaluated by comparing the concentration at each time compared to the concentration at time 0, was calculated as follows:

$$Stability\ (\%) = (A_{ti}/A_{t0}) \times 100$$

where A_{ti} represents the analyte concentration measured at the specific time and A_{t0} represents analyte concentration measured immediately after MEPS procedure (0 hour), time point zero. This parameter allowed us to know how long the eluates can be in the sample manager chamber before analysis without compromising the integrity of the target analytes.

2.3 Sample collection and preparation

Asthmatic children (N = 27, age = 8.8 ± 4.6Y) were recruited from the emergency and allergology service at Hospital Dr. Nélio Mendonça (Funchal Hospital) and diagnosed with uncontrolled asthma symptoms, while urine samples from healthy children (N = 17, age = 7.8 ± 1.4Y) were collected at two schools in Funchal, Portugal: EB1/PE Tanque – Monte and EB1/PE Visconde Cagongo. Relevant information for the study including gender, age and other information such as familiar diseases, home characteristics, type of disease including clinical data (controlled or not controlled disease, and clinical symptoms), therapies, among others, were obtained at sample collection (Table1).

< Table 1 near here >

The study was performed in accordance with the principles contained in the Declaration of Helsinki and approved by the Ethical Committee for Health of Funchal Hospital (SESARAM, EPE) and by Regional Direction of Education (DRE). All tutors of the individuals signed an informed consent to participate in the study after being informed about the purpose of the study.

No restrictions were applied to the individuals. Each individual, healthy or asthmatic patient, provided a urine sample in a 100-mL urine beaker with integrated transfer device (Greiner Bio-One GmbH, Austria), transported to the laboratory in a portable cooling box, suitable for this kind of biological samples, where were aliquoted in 8-mL glass vials (in order to avoid freezing and thawing cycles) and immediately frozen at -80 ℃ in order to quench any rapid degradation activity such as oxidation of labile metabolites as well as various enzymatic reactions. Compared with other biofluids, urine offers several advantages since it is obtained through an easy and totally non-invasive sampling procedure, may be collected across all ages even in severe patients, is a rich source of metabolites and its composition reflects the imbalances of all biochemical pathways within the body.

2.4 Isolation of targeted analytes by MEPS

The targeted analytes, LTB_4, LTE_4 and $11\beta PGF_{2\alpha}$, were isolated from urine using a semi-automatic miniaturization of solid phase extraction, the MEPS. This off-line procedure combines a digitally controlled and programmable semi-automatic device (eVol® XR) that controls the aspiration/dispensing volume and flow rate with minimal user interference allowing greater precision and accuracy and consequently greater reproducibility; a gas-tight XCHANGE® analytical syringe (500 µL); and a removable MEPS cartridge, known as 'barrel insert and needle' (BIN), containing 1-4 mg of a solid packed sorbent bed. This experimental procedure includes: (*i*) an activation process of the sorbent with water and methanol, (*ii*) the conditioning of the sorbent usually performed with the elution solvent, (*iii*) the sample loading where the sample is forced to pass through the sorbent bed several times, in order to efficiently retain the analytes of interest, (*iv*) a washing step to remove unwanted analytes not retained in the sorbent; and finally (*v*) the elution where the analytes were eluted using an appropriate solvent [19]. A SU sample spiked at 40 µg L^{-1} for the LTB_4 and LTE_4, and 160 µg L^{-1} for the $11\beta PGF_{2\alpha}$, was used in the optimization of the experimental parameters with influence on the MEPS efficiency. The chemical nature of the sorbent material, the number and mode (extract-discard and extract-eject) of sample loading cycles, the pH, the volume of elution solvent and the conditioning solvent, were evaluated and optimized by means of a univariate experimental design.

The extraction efficiency of 11 commercially available MEPS sorbents including, C18 (octadecyl-silica), C8 (octyl-silica), C2 (ethyl-silica), SIL (silica), M1 (80% C8 + 20% SCX – strong cation exchange), PEP (polar enhanced polymer), VAX (verify AX; C8 + SAX – strong anion exchange), HLB-DVB (hydrophilic-lipophilic-balanced reversed-

phase sorbent, divinylbenzene), PGC (porous graphitic carbon), R-AX (retain anion exchange) and R-CX (retain cation exchange), was evaluated and optimized in order to select the sorbent that offers the best performance for isolation of targeted eicosanoids. The main characteristics of the MEPS sorbents used in this study are represented in Table 2SM.

The number of loading cycles was optimized by drawing up 250 µL of sample, 3, 5 and 10 times, while the mode of the loading cycle was evaluated by drawing up the sample and ejecting in the same vial (extract-eject) and by drawing up the sample and discarding as waste (extract-discard).

The influence of pH was evaluated by testing the extraction efficiency at three different pH values (2.8, 5.1 and 8.5), adjusted with NaOH at 1M or FA at 10%, in two opposite ion exchange MEPS sorbents: R-AX and R-CX.

In order to investigate the best elution conditions, the impact of different elution solvents was investigated with different solvent solutions based on pure ACN, pure MeOH, acidified ACN and acidified MeOH, and different proportions of ACN and MeOH (50:50, 75:25 and 25:75). An aqueous solution of FA at 0.1% was also used to investigate if any of our target analytes were washed away in the washing step. After solvent selection, the elution volume (50 µL and 100 µL) and the number of cycles (1, 2 and 3 times) was also investigated.

Eluates were eluted directly into 100-µL micro-inserts (VWR International - Material de Laboratório, Lda., Carnaxide, Portugal), proper for 2-ml vials. In addition, the total dryness of the eluate and its resuspension in 50 µL and 100 µL of mobile phase (95% water at 0.1% FA and 5% ACN at 0.1% FA) and the concentration of the eluate until half of its volume (without dryness) were also investigated.

An important step in MEPS is the conditioning of the sorbent since it can influence the performance of the extraction. It consists on the clean up to avoid carry-over, and regeneration of the active sites in the sorbent for its reuse. According to Pereira et al. [26], different organic solvents can be used to perform this step. In this regard, the effect of the conditioning solvent was performed by testing two common organic solvents, 250 µL of ACN and 250 µL of MeOH, followed by an equilibration with 250 µL of FA at 0.1%. The number of conditioning-equilibration cycles (1, 2 and 3 times) were tested in order to suppress the carry-over effect.

In all steps of the MEPS procedure the aspiration and dispensing flow rate was limited to minimum (20 µL s^{-1}) in order to increase the interaction time of the analytes with the sorbent and to minimize the occurrence of cavitation. All extractions were made in duplicate (N = 2).

2.5 Chromatographic conditions. Optimization

The analysis of the target analytes was carried out on a Waters Ultra Performance Liquid Chromatographic Acquity system (UPLC Acquity H-Class, Waters Corporation, Milford, MA, USA) equipped with a 2996 PDA detector, a Waters Acquity quaternary solvent manager (QSM), an Acquity sample manager, a column heater and a degassing system. The whole configuration was driven by Empower software v2.0 (Waters Corporation). In order to achieve the best performance, important instrumental parameters with influence on the chromatographic resolution: composition of mobile phase, and nature of the stationary phase of capillary column, were optimized. Several types of columns with different dimensions including Acquity UPLC® BEH C18 protected with an Acquity UPLC® BEH C18 VanGuard™ Pre-column, Acquity UPLC® HSS T3 protected with an Acquity UPLC® HSS T3 VanGuard™ Pre-column, Acquity UPLC® CSH C18 protected

with an Acquity UPLC® CSH C18 VanGuard™ Pre-column, CORTECS UPLC® C18 protected with a CORTECS UPLC® C18 VanGuard™ Pre-column, and Kinetex® HILIC protected with SecurityGuard™ ULTRA cartridge for HILIC UHPLC, were evaluated in terms of peak resolution, shape and measurable areas of the target analytes (Table 2). These parameters were developed with spiked SU and real urine at the same concentration as described in a previous section. The optimized gradient was as follow: (A) water at 0.1% FA and (B) ACN at 0.1% FA, filtered through a 0.2 µm PTFE membrane: 95% A (0 min), 47% A (9.5 min), 24% A (11 min), 0% A (11.1 – 12.1 min) and 95% A (14 min) followed by a re-equilibration time of 2 minutes. The vials containing the extracts were kept inside the sample manager at a temperature of 15 °C. The flow rate was 300 µL min^{-1}, the injection volume was set at 5 µL and the capillary columns thermostatted at 30 °C. For quantification purposes, the detector was set to 4 distinct channels corresponding to the maximum absorbance wavelength of each target analyte and IS (*see* Table 3). The identification of the target analytes was made by comparison of the retention time and UV-Vis spectral characteristics with those of pure standards and confirmed by the standard addition to the eluates. All UPLC injections were carried out in triplicate (N = 3).

2.6 Method validation

MEPS/UHPLC-PDA method was validated according to the International Union of Pure and Applied Chemistry (IUPAC) guidelines [27], being assessed the following parameters: selectivity, linearity, method limits, precision, accuracy, extraction efficiency and matrix effect.

The method selectivity was assessed by analyzing the absence of interfering components at the retention time of the eicosanoids.

Linearity was evaluated by spiking SU at different concentrations. A seven-point calibration curve for LTE_4 and $11\beta PGF_{2\alpha}$, and nine concentration levels for the LTB_4 were constructed from the least square linear regression model with a weighing factor of $1/\times^2$ by plotting the peak area ratio between each eicosanoid and the IS ($Area_{analyte}/Area_{IS}$) against the eicosanoid concentration. Since the evaluation of the coefficient of determination (r^2) as a measure of linearity may be misleading, the proportional ratio using chromatographic signal and eicosanoid concentration was confirmed by means of the *F*-test through the lack-of-fit, goodness-of-fit and Mandel's fitting tests.

The method limits were expressed in terms of LOD, defined as the lowest concentration of analyte which can be reliably detectable and identified with an acceptable precision and accuracy (commonly within 20% RSD and within 20% bias), and LOQ, defined as the lowest concentration of analyte which can be identified and quantified with a satisfactory precision and trueness [28]. These parameters were calculated from the least squares linear regression analysis being LOD defined as: $a + 3.3S_{a/b}$ and LOQ as: $a + 10S_{a/b}$, where *a* represents the origin ordinate, *Sa* represents the standard deviation of the experimental value closest to the intercept, and *b* the slope of the calibration curve.

The matrix effect was calculated by the ratio between the slope of the standards in USRU, obtained in the least squares linear regression analysis, and the slope of the standards in SU:

$$Matrix\ effect\ (\%) = (slope_{USRU}/slope_{SU}) \times 100$$

where $slope_{USRU}$ and $slope_{SU}$ are the slope obtained from the analytical curve of the USRU and SU respectively.

In order to determine precision, accuracy and recovery percentage, the SU was spiked at three concentration levels corresponding to the LL (25 µg L^{-1} for the $11\beta PGF_{2\alpha}$, and 2.5 µg L^{-1} for the LTB_4 and LTE_4), ML (100 µg L^{-1} for the $11\beta PGF_{2\alpha}$, and 10 µg L^{-1} for the

LTB_4 and LTE_4) and high level (HL; 200 µg L^{-1} for the $11\beta PGF_{2\alpha}$, and 20 µg L^{-1} for the LTB_4 and LTE_4), covering the entire calibration range. Precision (expressed as percentage of relative standard deviation, % RSD) was evaluated in terms of intraday repeatability obtained from the analyzes of 4 replicates of spiked SU performed at the same day, and analyzed in the same analytical run, assayed in three different runs (N = 12) over a period of three days (reproducibility; N = 27).

Accuracy refers to how close the concentration values obtained experimentally are to the nominal values and is calculated as follow:

$$Accuracy\ (\%) = (x_i/x) \times 100$$

where x_i represents the experimental concentration values and x the nominal concentration values of the analyte. This parameter was calculated with the data obtained in the least squares linear regression analysis.

The recovery study allowed the evaluation of the extraction efficiency carried out in duplicate (N = 2) for each level of concentration. Briefly, one set of aliquots of SU was spiked with the analytes before the MEPS procedure and another set of unspiked SU went through the process and the respective eluates were spiked with the different concentration levels of the target analytes. This parameter is calculated by means of the following formula:

$$Recovery\ (\%) = (S_{SU}/S_{Eluate}) \times 100$$

where S_{SU} represents the obtained concentration of the analytes in the spiked SU when added before the MEPS procedure, and S_{Eluate} represents the concentration obtained from the analysis of the spiked eluates.

2.7 Statistical analysis

Further statistical data analysis was performed by means of the IBM SPSS Statistics Version 22 (IBM Corp., Armonk, NY, USA). The independent samples *t*-test is used in situations with two experimental conditions with different participants in each condition [29]. Regarding this, the *t*-test was performed on the target analytes values obtained from the samples in order to characterize the significant differences among both groups under study: the asthmatic patients and normal controls, in terms of mean values.

3 Results and discussion

With this work, it was intended to develop an easy and quick analytical method able to detect and quantify simultaneously three eicosanoids present in urine that are key biomarkers of the inflammatory process and asthma metabolomics *per se*.

3.1 MEPS optimization

A SU sample spiked at 40 μg L^{-1} for the LTB_4 and LTE_4, and 160 μg L^{-1} 253 for the $11\beta PGF_{2\alpha}$, was used in the optimization of the extraction parameters. Each sorbent was activated with 1 time 250 μL of ACN and 0.1% FA. 3 loading cycles were used by drawing up 250 μL of sample (pH = 5.1) and discarding as waste. 100 μL of 0.1% FA to wash the sorbent and the elution of the sample was made with 100 μL of acidified ACN (0.1% FA).

The efficiency of eleven commercial sorbents suitable for eVol®-MEPS format (Fig. 1A) was evaluated. Each sorbent has specific chemical properties that make them suitable for the extraction of a particular class of compounds (Table 2SM).

As shown in Fig. 1A, R-AX was the most favorable for the isolation of all targeted eicosanoids and thus, was selected as the extraction sorbent for the following experiments. Although R-CX and VAX sorbents, show best efficiency for LTB_4, the

difference is not significant when compared to the overall of the R-AX that had a better extraction efficiency for the LTB_4. Furthermore, the target analytes, $11\beta PGF_{2\alpha}$, LTE_4 and LTB_4, have an acidic nature due to the presence of a carboxylic group making them suitable to be extracted by the R-AX. This sorbent is chemically functionalized with quaternary amine groups that gives to it a balanced retention capacity between acidic and non-polar compounds [19].

< Fig. 1 near here >

The number of loading cycles is determinant when developing a MEPS procedure. This parameter was optimized by drawing up 250 µL of sample 3, 5 and 10 times (Fig. 1B). The results showed good extraction efficiencies for the LTs, in addition to detection of the $11\beta PGF_{2\alpha}$ when the sample is drawn up and down 10 times through the sorbent. It has not been tested more cycles because of sorbent saturation that was reflected on cavitation which started to become more prominent with the increase of loading cycles from 5 cycles. Consequently, more time was needed for each loading cycle and thus a reduction on the sorbent lifetime was expected. Additionally, the mode of the loading cycles was also tested. The extract-discard mode consists on the drawn up of the sample from an aliquot and the waste is discarded, the extract-eject mode involves the drawn-up of the sample from an aliquot and the waste is not discarded, returning to the aliquot. The extract-discard mode was selected because the obtained performance was slightly better (Fig. 1C) for all target analytes.

The quaternary amine groups present on the surface of the R-AX are strong bases with a great acid dissociation constant ($pK_a > 14$), positively-charged at all pHs that, as the name indicates, exchange or attracts anionic compounds in solution. R-CX is another ion exchange sorbent, functionalized with sulfonic acid groups that have very low pK_a (< 1), which make it negatively-charged at all pHs [30]. The targeted eicosanoids have low

strongest acidic pK_a ($11\beta PGF_{2\alpha}$ = 4.36, LTE_4 = 2.39 and LTB_4 = 4.65 [31]. For this reason, at a pH higher than their pK_as, most of the analytes would be deprotonated, being able to be adsorbed through the surface of a basic sorbent. Although R-CX had the best results toward $11\beta PGF_{2\alpha}$ and in terms of total area, the extraction of LTE_4 was not so efficient. Hence, R-AX at pH = 5.1 was chosen as the best pH for subsequent experiments (Fig. 1D).

Other important steps in MEPS procedure are washing and elution. The washing step allows us to remove undesired matrix interferences that are weakly retained on the sorbent while the elution step consists on the use of the best solvent and the least volume possible that is able to elute the target analytes retained on the surface of the sorbent [19, 32]. In this study, the best elution solvent was MeOH followed by ACN (Fig. 2A). The elution volume was also considered and no substantial differences were found between eluting 2 or 3 times with 50 µL of MeOH (Fig. 2B), therefore, in subsequent experiments, the elution was carried out using 2×50 µL of MeOH. The washing solvent, 0.1% FA, was also investigated and none of the target analytes were eluted with this solvent making it suitable for this step (Fig. 2A).

The influence of the eluate concentration was investigated. Comparing the obtained areas from 50% evaporation of the eluates (until 50 µL) with the areas obtained from complete dryness and resuspension in mobile phase (50 and 100 µL), it can be understood that complete dryness of the eluates is not appropriate for the target analytes once there are losses when resuspended. Evaporation of the eluates until half of its volume was the best option not only in terms of areas but also in terms of peak resolution and analyte detection, and therefore were used for further experiments.

< Fig. 2 near here >

An important and final/beginning step of the MEPS procedure is the conditioning/regeneration of the sorbent. Each MEPS procedure starts with the conditioning of the sorbent with an organic solvent followed by its equilibration before the loading of the sample and ends with the regeneration of the sorbent that match with conditioning step if further extractions are made [26]. The influence of the conditioning solvent on the performance of the extractions made with the R-AX sorbent, was assayed with two organic solvents: (*i*) 250 µL of ACN and (*ii*) 250 µL of MeOH. Each conditioning solvent was followed by the previous optimized parameters: equilibration with 0.1% FA, 10 sample (pH = 5.1) loading cycles discarded as waste, 100 µL of 0.1% FA as washing solvent and elution with 2 × 50 µL of MeOH. The eluates obtained with each conditioning solvent were analyzed and the obtained data can be seen on Fig. 2C. Even though the best elution solvent was MeOH, it was remarkable to find out that ACN is a better surfactant, allowing a greater conditioning of the sorbent. ACN is less viscous than MeOH (0.37 centipoise, cp *versus* 0.55 cp at 20 ℃ (Physical Properties of Solvents – https://www.sigmaaldrich.com on November 2017) which can be a determinant factor that allows it to be more efficient involving the sorbent micro-particles reaching all spaces between them and consequently increasing the number of functionalized groups that are conditioned, improving the number of analytes bound to the surface of the sorbent. To the best of our knowledge, no previous report was made regarding the optimization of the conditioning/regeneration step with different solvents. To avoid carry-over, the number of conditioning-equilibration cycles were optimized. The best results, which are the smaller number of cycles enough to remove the carry-over, was 3×250 µL of ACN - 250 µL of 0.1% FA.

3.2 Optimization of chromatographic conditions

The gradient of the mobile phase, the flow rate and several types of columns with different dimensions and particle sizes were tested and optimized.

The influence of five commercial columns suitable for UPLC® analysis of eicosanoids were evaluated (Fig. 1A-SM and Fig. 2-SM). Each column has specific chemical and physical properties that makes them suitable for the separation of a wide range of compounds at diverse conditions (Table 2).

< Table 2 near here >

The BEH C18 column was selected for its narrow and sharp peaks, reproducible retention times and good chromatographic resolution of the target analytes (Fig. 1A-SM) and by the areas obtained (Fig. 3A). This column was previously used by Perestrelo et al. [24] and Chappell et al. [33] for the analysis of leukotrienes in urine and sputum samples.

< Fig. 3 near here >

In terms of gradient and flow rate, the initial conditions selected for the optimization of the chromatographic conditions (also used for the optimization of the MEPS procedure) were a modification of Perestrelo el al. [24] and were tested with spiked SU. It consisted of a BEH C18 column and by a gradient profile composed by (A) water at 0.1% FA and (B) ACN at 0.1% FA, at a flow rate of 500 µL min^{-1}: 95% A (0 min), 47% A (9.5 min), 24% A (11 min), 0% A (11.1 – 12.1 min) and 95% A (14 min) followed by a re-equilibration time of 2 minutes. The chromatogram obtained is shown in Fig. 1A-SM. The best gradient with spiked SU was obtained with a flow rate of 300 µL min^{-1} as follows: 95% A (0 min), 85% A (2 min), 60% A (2.5 min), 45% A (5 min); 95% A (6 – 7 min) (Fig. 1B-SM) followed by a re-equilibration time of 2 min.

The best conditions obtained with SU were tested using spiked real urine. As shown in Fig. 1C-SM, these conditions were not effective once the analytes were co-eluted with urine interferents. A modification of the initial conditions in terms of flow rate (300 µL

min^{-1}) were successfully tested with spiked real urine (Fig. 1D-SM) and spiked SU (Fig. 4). With this optimization, even though we were not able to reduce the run time of analysis, a considerable decrease of 40% on the amount of solvent was achieved which is important regarding environmental issues and cost of analysis. Regarding the gradient of the mobile phase and the flow rate, they were optimized for the best results within a total run time of 16 min, including column equilibration. A representative chromatogram obtained with the final optimized conditions in SU is shown in Fig. 4.

< Fig. 4 near here >

Considering the obtained data, the best chromatographic conditions were obtained using a gradient elution mode, with a flow rate of 300 µL min^{-1}, as follows: 95% A (0 min), 47% A (9.5 min), 24% A (11 min), 0% A (11.1 – 12.1 min) and 95% A (14 min) followed by a re-equilibration time of 2 min using an UHPLC-PDA equipped with a BEH C18 capillary column.

3.3 Method validation

In order to demonstrate the practicability of the developed analytical strategy and its ability to quantify the target analytes in urine samples, the method was fully validated in terms of selectivity, linearity, method limits, precision, accuracy, extraction efficiency and matrix effect for each eicosanoid. The analytical method figures of merit are described in Table 3 and Table 4.

< Table 3 and Table 4 near here >

Selectivity was demonstrated by the absence of interfering peaks at the retention times of the target analytes in blank SU. Figure 4 clearly demonstrates the absence of interfering peaks at the retention times of the target analytes, demonstrating the ability of the analytical method to unequivocally identify and quantify the analytes of interest.

The linearity was assayed using different calibration curves of each analyte obtained by least squares linear regression analysis as described in section *2.6 Method validation*. All of three regression equations of the target analytes showed a coefficient of determination higher than 0.9980 but these values alone, do not guarantee the linearity of the signal. Therefore, linearity was assessed by means of the *F*-test at different approaches: lack-of-fit test, based on the analysis of the residual variance; goodness-of-fit test, based on the ratio between the mean sum of squares of the factors and the residuals; and Mandel's fitting test, based on the difference between the residual standard deviation of the obtained first-order calibration model and a potential second-order calibration model, as described by Kruve et al. [34]. The signal is considered linear when the calculated *F*, by means of the experimental data, is lower than the respective tabulated *F*. As show in Table 3, all signal linearities calculated (Lack-of-fit, Godness-of-fit and Mandel's tests) by the previous mentioned approaches, are confirmed ($F_{calculated} < F_{tabulated}$) with a 95% confidence level.

The method limits can be described as the lowest value of concentration at which the method is able to detect (LOD) and quantify (LOQ) the target analytes (Table 3) with satisfactory accuracy and precision. As previously reported, the developed method showed low values of LOD which ranged between 0.04 µg L^{-1} for LTB_4 and 1.12 µg L^{-1} for $11\beta PGF_{2\alpha}$ while LOQ values ranged between 0.10 µg L^{-1} for the LTB_4 and 2.11 µg L^{-1} for $11\beta PGF_{2\alpha}$.

Matrix effect was also evaluated in order to determine which calibration curve should be used during the real sample analysis. The values obtained with this test is represented in Table 3. All the target analytes have revealed considerable matrix effects with 73% for the $11\beta PGF_{2\alpha}$, 76% for the LTE_4 and 81% for the LTB_4. Consequently, the regression equation obtained with real urine would be used for further real sample analyzes.

An important parameter when validating a method is precision. This parameter can be defined as repeatability at which the % RSD is calculated in order to assess the error associated between replicates performed in the same day, and as reproducibility at which the % RSD is calculated in order to assess the error associated between replicates performed over a certain period of days. The developed method proved to have repeatability with % RSD values that ranged from 3.11% for LTE_4 at HL and 6.63% at LL for the same analyte. As for reproducibility, % RSD values ranged from 1.29% for LTE_4 at HL and 10.43% at LL for the same compound being LTE_4 on the extremes of both precision approaches (Table 4).

The performance of the method can also be assayed as accuracy. In general, all experimental values have shown to be accurate (approximately 100%) during the study of this parameter with less than 7% deviation from the tabulated value as shown in Table 4. The least accurate was $11\beta PGF_{2\alpha}$ at LL with 93.62% and LTB_4 the most accurate with 100.04%.

The extraction efficiency was evaluated in order to know how much of the target analytes is lost during extractions. The obtained recovery percentage for $11\beta PGF_{2\alpha}$ ranged between 95.52% at a LL and 99.71 at a ML; LTE_4 ranged between 74.99% and 91.13% at HL and LL respectively; and LTB_4 ranged from 84.53% at LL and 92.25% at ML (Table 4).

The analytical performance of MEPS/UHPLC-PDA analysis for the quantification of urinary eicosanoids was compared with several other methodologies reported in the literature in the last years for the same group of compounds using other extraction procedures, as described in Table 5. Our developed technique involves lower elution volumes, sample amounts and it is much faster (time of extraction falling few minutes), than the described. In addition, the MEPS procedure was carried out in a semi-automatic

way which is a guarantee of a high precision. Regarding the analytical performance, MEPS/UHPLC-PDA allows equal or even better LODs and LOQs than other published studies with excellent recoveries and extraction efficiency.

< Table 5 near here >

3.4 Stability of the extracts

The stability of the extracts was tested with target analytes at different concentrations (LL and ML), over a 24-hour period with analyzes running each 8 hours.

It was found out that eluates were stable for 8 hours, with less than 6% lost during the tested time, but after that time, the leukotrienes stability decrease, being the 11β$PGF_{2\alpha}$ the most stable with less than 7% lost during the assay as observed in Fig. 3B. All the analysis were performed taking into account the stability of the extracts.

3.5 Application of the developed methodology to real samples

*3.5.*1 *Individuals characterization*

The main characteristics of the subjects of this study are shown in Table 1 (*see* Section 2.3).

Family history is strongly related to asthma. Most of the asthmatic patients (81.0%) that took part of this study, had a family member with asthma being the majority (66.7%), maternal-related. This trend has been reported by Valerio et al. [39] where children with relatives with asthma are 2 to 4 times more likely to have an asthma diagnosis than children with no asthmatic relatives. Even though family history is strongly related to the asthmatic diagnosis in children, many of our asthmatic patients did not have any relative with asthma (19%) and many of the normal controls (59%) had a relative with an asthmatic diagnosis during their lifetime but no asthma or any allergen sensitization was reported by this group. In the present work, it was interesting to find that most of the

asthmatic patients were boys (59% of total population of asthmatic patients and 74% of asthmatic patients until 13 years old) being in accordance with the pattern of other countries [40]. Although the prevalence of asthma is higher in boys at this age (< 13 years old), the risk of developing asthma increases rapidly in women after puberty [41]. It is believed that these changes might have a relationship to the endocrine system, specifically to leptin, an hormone produced by adipocytes, related to inflammation [42] and asthma in children [43].

The main comorbidity found throughout this study was allergic rhinitis with 93% of the asthmatic patients with allergic asthma having also rhinitis. Although higher, this value is in agreement with the literature [44] which stated that allergic rhinitis coexists in 60 to 80% of allergic asthmatic children probably because of their similar pathophysiology. This comorbidity has also been found not only in children but also in general asthmatic population in lower values (52.3%) [45]. The most common allergen sensitization, among the allergic population under study, was sensitivity to house dust mites (70% of the allergic population) followed by animal epithelium and pollens, with 56% and 37% respectively. A similar trend was previously reported by Boulet et al. [46].

3.5.2 Quantification of urinary eicosanoids in asthmatic patients and healthy individuals

In order to evaluate the applicability of the method to urine samples, R-AX sorbent and the developed method proposed in this study were applied to detect the three eicosanoids – 11β$PGF_{2\alpha}$, LTE_4 and LTB_4, in urine of 27 asthmatic and 17 healthy children. The concentration values obtained for the urine of each asthmatic patient and healthy individual (control) is represented in Table 3SM. Fig. 5 represents a chromatogram of an asthmatic patient and another from a normal control. Even though these eicosanoids are

at very low concentration, in a complex matrix, it is clear the difference on peaks intensity between controls and asthmatic groups.

< Fig. 5 near here >

A third part of the normal controls had values bellow the limit of quantification or detection, except for the 11βPGF$_{2\alpha}$ that was not detected in 2 asthmatic patients. In both groups, the major analyte was 11βPGF$_{2\alpha}$ with greater values when compared to the two LTs of this study (Fig. 6). 11βPGF$_{2\alpha}$ values varied from bellow the LOD (< LOD) until 310.32 ± 9.59 μg L^{-1} for the asthmatic patients group, and from 32.16 ± 1.00 until 167.29 ± 17.75 μg L^{-1} for the normal controls. LTE$_4$ values varied from < LOD until 4.94 ± 0.22 μg L^{-1} for the asthmatic group, and from 0.47 ± 0.06 until 2.53 ± 0.41 μg L^{-1} for the normal controls. The tendency for the LTB$_4$ was the same with values between < LOD and 2.64 ± 0.05 μg L^{-1} for asthmatics and < LOD and 1.74 ± 0.05 μg L^{-1} for normal controls.

< Fig. 6 near here >

Before running the *t*-test some specific assumptions required to be evaluated including the type of the variables, independence of observations, normal distribution, outliers and homogeneity of variances [29]. The results have shown that there are significant differences between groups. For this procedure, the logarithmic values of concentrations were used in order to obtain closer values to the normal pattern. For the 11βPGF$_{2\alpha}$, the *t*-test value was 7.415 with 289.6 degrees of freedom (df; $p < 0.001$); the LTE$_4$ had a *t*-test value of 3.653 (df = 217, $p < 0.001$) and LTB$_4$ had 5.437 (df = 198, $p < 0.001$). The mean values calculated by means of the *t*-test procedure of each target analyte and investigated group, are shown in Table 3SM.

Prostaglandin D$_2$ is a major cyclooxygenase metabolite produced during an inflammatory response by mast cells and leukocytes as dendritic cells and type 2 T helper cells with a

pro-inflammatory role. This unstable eicosanoid is rapidly transformed into more stable products, prostaglandins of the J series and $11\beta PGF_{2\alpha}$ [47]. As prostaglandin D_2, $11\beta PGF_{2\alpha}$ also has biological activity as bronchoconstrictor and contraction of coronary arteries [48], and can be found in increased values in the urine of asthmatic patients after allergen-induced bronchoconstriction [49] and after exercise challenge [50]. On average, the asthmatic patients had higher concentrations of $11\beta PGF_{2\alpha}$ (112.96 µg L^{-1}) than normal controls (62.56 µg L^{-1}) with a mean difference of about 50.40 µg L^{-1} between groups. This significant difference ($p < 0.001$) represent a medium-sized effect ($r = 0.40$). The difference among the total population was between 35.97 and 64.83 µg L^{-1} for this eicosanoid, with a confidence level of 95%.

Cysteinyl leukotrienes, powerful bronchoconstrictors, 100 to 1000 times more powerful than histamine [51], play an important role in the pathophysiology of asthma. They also have a role in the vascular permeability allowing the exudation of inflammatory cells from plasma to the airway mucosa, stimulate mucus secretion and inhibit mucociliar clearance – characteristics of the pathophysiology of asthma [51]. Since LTC_4 and LTD_4 are biologically converted into LTE_4, they are not excreted in measurable concentrations in urine. Therefore, LTE_4 is considered a biomarker of the total of cysteinyl leukotrienes levels in urine [52]. Studies suggest that LTE_4 is dependent on allergen dose inhaled during allergen challenge in asthmatic patients [53]. Having this into account, in the evaluation of the groups present in this study, it was found increased mean levels of LTE_4 in the urine of asthmatic patients with 1.27 µg L^{-1} against 0.89 µg L^{-1} in normal controls with a mean difference of 0.38 µg L^{-1}. This difference was significant ($p < 0.001$) and represented a small-sized effect ($r = 0.24$). Regarding the total population, the difference was of 0.11 – 0.64 µg L^{-1} with a confidence level of 95%.

LTB_4 is produced by a diversity of cells including neutrophils and macrophages and it is a powerful pro-inflammatory chemoattractant whose main targets are neutrophils promoting their activation, adhesion to the endothelium and chemotaxis. This LT also activates neutrophils degranulation of additional mediators and it is also involved in inflammatory pain by neutrophils dependent processes [51]. LTB_4 tend to be in higher concentrations in diverse matrices like exhaled breath condensate [54], sputum [55] and urine [24]. The asthmatic patients of our investigations had a tendency for higher values of LTB_4 with a mean concentration of 1.39 μg L^{-1} in the urine of asthmatics patients against 0.76 μg L^{-1} in the normal controls (mean difference = 0.63 μg L^{-1}). This difference was significant ($p < 0.001$) and represented a medium-sized effect ($r = 0.36$). In terms of total population, the difference varied between 0.47 and 0.78 μg L^{-1} with a confidence level of 95%.

4 Conclusions

Throughout this work, it was developed a powerful methodology to simultaneously detect and quantify urinary eicosanoids in asthmatic patients and healthy individuals as powerful strategy to identify potential asthma biomarkers.

The developed MEPS/UHPLC-PDA approach have proven to be an ultra-fast, accurate, precise and easy analytical strategy. The extraction procedure in not time-consuming with a total analysis time of 31 min. Furthermore, The MEPS/UHPLC-PDA procedure revealed to be environmentally friendly due to the reduce levels of harmful organic solvents consumed during both extraction and analytical procedures. The validation of the method revealed compatible values for each parameter including selectivity, linearity, method limits, precision, accuracy, extraction efficiency, matrix effect and extracts stability for each eicosanoid. Therefore, it was possible to successfully apply the method

to the samples and discriminate between asthmatics and healthy individuals. On average, the urine of asthmatic patients presented significantly higher concentrations of all eicosanoids. The obtained values for the 11βPGF$_{2\alpha}$ and LTB$_4$ were about 1.8 times higher, and for the LTE$_4$ were about 1.4 times higher in asthmatic patients than in healthy individuals suggesting the potential of these eicosanoids on asthma diagnosis. Consequently, MEPS/UHPLC-PDA reveled to be a promising method regarding the study of these and other eicosanoids present in other biological matrices as plasma, sputum and EBC of patients from inflammatory illnesses other than asthma.

Acknowledgments

This work was supported by FCT – Fundação para a Ciência e a Tecnologia (project PEst-OE/QUI/UI0674/2013, CQM, Portuguese Government funds), and through Madeira 14-20 Program, project PROEQUIPRAM – Reforço do Investimento em Equipamentos e Infraestruturas Científicas na RAM (M1420-01-0145-FEDER-000008) and by ARDITI – Agência Regional para o Desenvolvimento da Investigação Tecnologia e Inovação, through the project M1420-01-0145-FEDER-000005 – Centro de Química da Madeira – CQM$^+$ (Madeira 14-20).

Compliance with ethical standards

All protocols and procedures were adhered to institutional ethical standards and/or research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by ethics committee of Hospital do Funchal, Madeira, Portugal. Additionally, prior informed consent was obtained from all the participants in the study.

Conflict of interest

Authors declare no conflicts of interest.

References

[1] Global Initiative for Asthma, Global strategy for asthma management and prevention, http://ginasthma.org/, 2017 (accessed 15 October 2017).
[2] Global Asthma Network, The Global Asthma Report 2014, www.globalasthmanetwork.org, 2014 (accessed 15 October 2017).
[3] C.K.W. Lai, R. Beasley, J. Crane, S. Foliaki, J. Shah, S. Weiland, the ISAAC Phase Three Study Group, Global variation in the prevalence and severity of asthma symptoms: Phase Three of the International Study of Asthma and Allergies in Childhood (ISAAC), Thorax 64 (2009) 476-483.
[4] Z. Kuang, J.J. Wilson, S. Luo, S.W. Zhu, R.P. Huang, Deciphering asthma biomarkers with protein profiling technology, Int. J. Inflam. 2015 (2015) 1-13.
[5] A. James, G. Hedlin, Biomarkers for the phenotyping and monitoring of asthma in children, Curr. Treat. Options Allergy 3 (2016) 439-452.
[6] S.J. Vijverberg, B. Hilvering, J.A. Raaijmakers, J.W. Lammers, A.H. Maitland-van der Zee, L. Koenderman, Clinical utility of asthma biomarkers: from bench to bedside, Biologics 7 (2013) 199-210.
[7] K.N. Turi, L. Romick-Rosendale, K.K. Ryckman, T.V. Hartert, A review of metabolomics approaches and their application in identifying causal pathways of childhood asthma, J. Allergy Clin. Immunol. 141 (2018) 1191-1201.
[8] A. Zhang, H. Sun, P. Wang, Y. Han, X. Wang, Recent and potential developments of biofluid analyses in metabolomics, J. Proteomics 75 (2012) 1079-1088.
[9] A.M. Fitzpatrick, Biomarkers of asthma and allergic airway diseases, Ann. Allergy Asthma Immunol. 115 (2015) 335-340.
[10] D. Balgoma, J. Larsson, J. Rokach, J.A. Lawson, K. Daham, B. Dahlén, S.E. Dahlén, C.E. Wheelock, Quantification of lipid mediator metabolites in human urine from asthma patients by electrospray ionization mass spectrometry: controlling matrix effects, Anal. Chem. 85 (2013) 7866-7874.
[11] I. Matacuta, Is there an ideal biomarker for pediatric asthma?, Int. J. Recent Sci. Res. 6 (2015) 5561-5568.
[12] K.N. Cahill, J.C. Bensko, J.A. Boyce, T.M. Laidlaw, Prostaglandin D2: a dominant mediator of aspirin exacerbated respiratory disease, J. Allergy Clin. Immunol. 135 (2015) 245-252.
[13] M. Swierczynska-Krepa, M. Sanak, G. Bochenek, P. Strek, A. Cmiel, A. Gielicz, H. Plutecka, A. Szczeklik, E. Nizankowska-Mogilnicka, Aspirin desensitization in patients with aspirin-induced and aspirin-tolerant asthma: a double-blind study, J. Allergy Clin. Immunol. 134 (2014) 883-890.
[14] H.S. Baek, J.H. Choi, J.W. Oh, H.B. Lee, Leptin and urinary leukotriene E4 and 9α,11β-prostaglandin F2 release after exercise challenge, Ann. Allergy Asthma Immunol. 111 (2013) 112-117.
[15] J.J. Eastman, K.J. Cavagnero, A.S. Deconde, A.S. Kim, M.R. Karta, D.H. Broide, B.L. Zuraw, A.A. White, S.C. Christiansen, T.A. Doherty, Group 2 innate lymphoid cells are recruited to the nasal mucosa in patients with aspirin-exacerbated respiratory disease, J. Allergy Clin. Immunol. 140 (2017) 101-108.e3.
[16] S. Risticevic, D. Vuckovic, H.L. Lord, J. Pawliszyn, Solid-phase microextraction, in J. Pawliszyn (Ed.), Comprehensive Sampling and Sample Preparation, Academic Press, Oxford, 2012, pp. 419-460.
[17] C.L. Arthur, J. Pawliszyn, Solid phase microextraction with thermal desorption using fused silica optical fibers, Anal. Chem. 62 (1990) 2145-2148.

[18] J.A. Ocaña-González, R. Fernández-Torres, M.Á. Bello-López, M. Ramos-Payán, New developments in microextraction techniques in bioanalysis. A review, Anal. Chim. Acta 905 (2016) 8-23.
[19] J. Pereira, J. Gonçalves, V. Alves, J.S. Câmara, Microextraction using packed sorbent as an effective and high-throughput sample extraction technique: recent applications and future trends, Sample Prep. 1 (2013) 38-53.
[20] E. Soleimani, A. Bahrami, A. Afkhami, F.G. Shahna, Determination of urinary trans,trans-muconic acid using molecularly imprinted polymer in microextraction by packed sorbent followed by liquid chromatography with ultraviolet detection, J. Chromatogr. B 1061-1062 (2017) 65-71.
[21] E. Soleimani, A. Bahrami, A. Afkhami, F.G. Shahna, Selective determination of mandelic acid in urine using molecularly imprinted polymer in microextraction by packed sorbent, Arch. Toxicol. 92 (2018) 213-222.
[22] J.L. Gonçalves, V.L. Alves, C.J.F. Conceição, H.M. Teixeira, J.S. Câmara, Development of MEPS–UHPLC/PDA methodology for the quantification of clozapine, risperidone and their major active metabolites in human urine, Microchem. J. 123 (2015) 90-98.
[23] V. Alves, J. Gonçalves, C. Conceição, H.M. Teixeira, J.S. Câmara, An improved analytical strategy combining microextraction by packed sorbent combined with ultra high pressure liquid chromatography for the determination of fluoxetine, clomipramine and their active metabolites in human urine, J. Chromatogr. A 1408 (2015) 30-40.
[24] R. Perestrelo, C.L. Silva, J.S. Câmara, Determination of urinary levels of leukotriene B4 using a highly specific and sensitive methodology based on automatic MEPS combined with UHPLC-PDA analysis, Talanta 144 (2015) 382-389.
[25] J.A. Wilsenach, C.A.H. Schuurbiers, M.C.M. van Loosdrecht, Phosphate and potassium recovery from source separated urine through struvite precipitation, Water Res. 41 (2007) 458-466.
[26] J. Pereira, C.L. Silva, R. Perestrelo, J. Goncalves, V. Alves, J.S. Camara, Re-exploring the high-throughput potential of microextraction techniques, SPME and MEPS, as powerful strategies for medical diagnostic purposes. Innovative approaches, recent applications and future trends, Anal. Bioanal. Chem. 406 (2014) 2101-2122.
[27] M. Thompson, S.L. Ellison, R. Wood, Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC technical report), Pure Appl. Chem. 74 (2002) 835-855.
[28] B. Magnusson, U. Örnemark (Eds.), Eurachem guide: the fitness for purpose of analytical methods – a laboratory guide to method validation and related topics (second edition 2014), www.eurachem.org, 2014 (accessed 15 October 2017).
[29] A. Field, The independent t-test, in: A. Field (Ed.), Discovering statistics using IBM SPSS statistics, SAGE Publications Ltd., London, 2009, pp. 334-341.
[30] Supelco, Guide to solid phase extraction, https://www.sigmaaldrich.com/, 1996 (accessed 15 November 2017).
[31] D.S. Wishart, D. Tzur, C. Knox, R. Eisner, A.C. Guo, N. Young, D. Cheng, K. Jewell, D. Arndt, S. Sawhney, C. Fung, L. Nikolai, M. Lewis, M.A. Coutouly, I. Forsythe, P. Tang, S. Shrivastava, K. Jeroncic, P. Stothard, G. Amegbey, D. Block, D.D. Hau, J. Wagner, J. Miniaci, M. Clements, M. Gebremedhin, N. Guo, Y. Zhang, G.E. Duggan, G.D. Macinnis, A.M. Weljie, R. Dowlatabadi, F. Bamforth, D. Clive, R. Greiner, L. Li, T. Marrie, B.D. Sykes, H.J. Vogel, L. Querengesser, HMDB: the Human Metabolome Database, Nucleic Acids Res. 35 (2007) D521-526.

[32] M. Abdel-Rehim, Recent advances in microextraction by packed sorbent for bioanalysis, J. Chromatogr. A 1217 (2010) 2569-2580.
[33] G.P. Chappell, X. Xiao, A. Pica-Mendez, T. Varnell, S. Green, W.K. Tanaka, O. Laterza, Quantitative measurement of cysteinyl leukotrienes and leukotriene B4 in human sputum using ultra high pressure liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 879 (2011) 277-284.
[34] A. Kruve, R. Rebane, K. Kipper, M.L. Oldekop, H. Evard, K. Herodes, P. Ravio, I. Leito, Tutorial review on validation of liquid chromatography–mass spectrometry methods: part II, Anal. Chim. Acta 870 (2015) 8-28.
[35] H.Y. Lee, Y.M. Ye, S.H. Kim, G.Y. Ban, S.C. Kim, J.H. Kim, Y.S. Shin, H.S. Park, Identification of phenotypic clusters of nonsteroidal anti-inflammatory drugs exacerbated respiratory disease, Allergy, 72 (2017) 616-626.
[36] J.K. Prasain, A. Arabshahi, P.R. Taub, S. Sweeney, R. Moore, J.D. Sharer, S. Barnes, Simultaneous quantification of F2-isoprostanes and prostaglandins in human urine by liquid chromatography tandem-mass spectrometry, J. Chromatogr. B 913-914 (2013) 161-168.
[37] K. Sterz, G. Scherer, J. Ecker, A simple and robust UPLC-SRM/MS method to quantify urinary eicosanoids, J. Lipid Res. 53 (2012) 1026-1036.
[38] B.S. Levison, R. Zhang, Z. Wang, X. Fu, J.A. DiDonato, S.L. Hazen, Quantification of fatty acid oxidation products using online high-performance liquid chromatography tandem mass spectrometry, Free Radic. Biol. Med. 59 (2013) 2-13.
[39] M.A. Valerio, P.M. Andreski, R.F. Schoeni, K.A. McGonagle, Examining the association between childhood asthma and parent and grandparent asthma status: implications for practice, Clin. Pediatr. (Phila.) 49 (2010) 535-541.
[40] L.B. Bacharier, A. Boner, K.H. Carlsen, P.A. Eigenmann, T. Frischer, M. Gotz, P.J. Helms, J. Hunt, A. Liu, N. Papadopoulos, T. Platts-Mills, P. Pohunek, F.E. Simons, E. Valovirta, U. Wahn, J. Wildhaber, Diagnosis and treatment of asthma in childhood: a PRACTALL consensus report, Allergy 63 (2008) 5-34.
[41] R. de Marco, F. Locatelli, J. Sunyer, P. Burney, Differences in incidence of reported asthma related to age in men and women, Am. J. Respir. Crit. Care Med. 162 (2000) 68-74.
[42] P. Fernández-Riejos, S. Najib, J. Santos-Alvarez, C. Martín-Romero, A. Pérez-Pérez, C. González-Yanes, V. Sánchez-Margalet, Role of leptin in the activation of immune cells, Mediators Inflamm. 2010 (2010) 568343.
[43] N. Guler, E. Kirerleri, U. Ones, Z. Tamay, N. Salmayenli, F. Darendeliler, Leptin: does it have any role in childhood asthma?, J. Allergy Clin. Immunol. 114 (2004) 254-259.
[44] E.P. de Groot, E.J. Duiverman, P.L. Brand, Comorbidities of asthma during childhood: possibly important, yet poorly studied, Eur. Respir. J. 36 (2010) 671-678.
[45] A. Sa-Sousa, M. Morais-Almeida, L.F. Azevedo, R. Carvalho, T. Jacinto, A. Todo-Bom, C. Loureiro, A. Bugalho-Almeida, J. Bousquet, J.A. Fonseca, Prevalence of asthma in Portugal - the portuguese national asthma survey, Clin. Trans. Allergy 2 (2012) 15.
[46] L.P. Boulet, H. Turcotte, C. Laprise, C. Lavertu, P.M. Bedard, A. Lavoie, J. Hebert, Comparative degree and type of sensitization to common indoor and outdoor allergens in subjects with allergic rhinitis and/or asthma, Clin. Exp. Allergy 27 (1997) 52-59.

[47] E. Ricciotti, G.A. FitzGerald, Prostaglandins and inflammation, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 986-1000.
[48] S.E. Dahlen, M. Kumlin, Monitoring mast cell activation by prostaglandin D2 in vivo, Thorax 59 (2004) 453-455.
[49] S. O'Sullivan, B. Dahlen, S.E. Dahlen, M. Kumlin, Increased urinary excretion of the prostaglandin D2 metabolite 9 alpha, 11 beta-prostaglandin F2 after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction, J. Allergy Clin. Immunol. 98 (1996) 421-432.
[50] H.S. Baek, J.H. Choi, J.W. Oh, H.B. Lee, Leptin and urinary leukotriene E4 and 9α,11β-prostaglandin F2 release after exercise challenge, Ann. Allergy, Asthma Immunol. 111 (2013) 112-117.
[51] J.N. Sharma, L.A. Mohammed, The role of leukotrienes in the pathophysiology of inflammatory disorders: is there a case for revisiting leukotrienes as therapeutic targets?, Inflammopharmacology, 14 (2006) 10-16.
[52] N. Rabinovitch, Urinary leukotriene E4 as a biomarker of exposure, susceptibility and risk in asthma, Immunol. Allergy Clin. North Am. 32 (2012) 433-445.
[53] L. Bancalari, I. Conti, D. Giannessi, G. Lazzerini, F.L. Dente, R. De Caterina, P.L. Paggiaro, Early increase in urinary leukotriene E4 (LTE4) is dependent on allergen dose inhaled during bronchial challenge in asthmatic subjects, Allergy 54 (1999) 1278-1285.
[54] J. Trischler, C.M. Muller, S. Konitzer, E. Prell, I. Korten, S. Unverzagt, C. Lex, Elevated exhaled leukotriene B(4) in the small airway compartment in children with asthma, Ann. Allergy, Asthma Immunol. 114 (2015) 111-116.
[55] A. Higham, P. Cadden, T. Southworth, M. Rossall, U. Kolsum, S. Lea, R. Knowles, D. Singh, Leukotriene B4 levels in sputum from asthma patients, ERJ Open Res. 2 (2016) 00088-02015.

Figure captions

Fig. 1 (A) Comparison of the efficiency of eleven commercial MEPS sorbents; (B) number and (C) mode of loading cycles of the sample; and (D) influence of the pH of the sample in two ion exchange sorbents: R-AX and R-CX. Values are expressed as mean ± standard deviation (N = 6).

Fig. 2 (A) Influence of the elution solvent and (B) volume on the extractions; (C) conditioning step solvent. Values are expressed as mean ± standard deviation (N = 6).

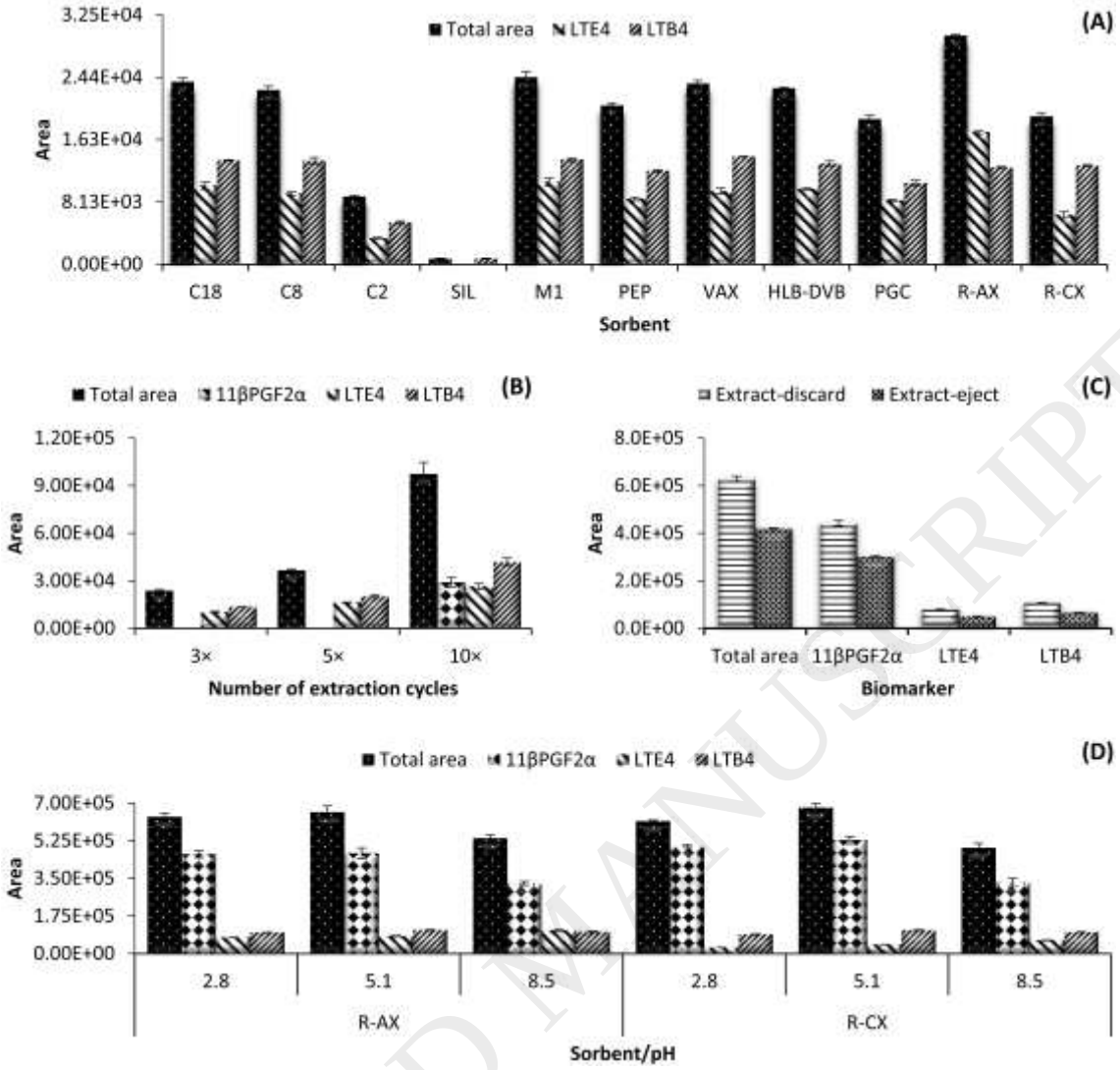
Fig. 3 (A) Influence of five commercial columns suitable for UPLC® analyzes. Values are expressed as mean ± standard deviation (N = 3). (B) Stability of extracts at 0, 8, 16 and 24 hours after extraction. Target analytes were at low level (LL; 25 µg L^{-1} for the

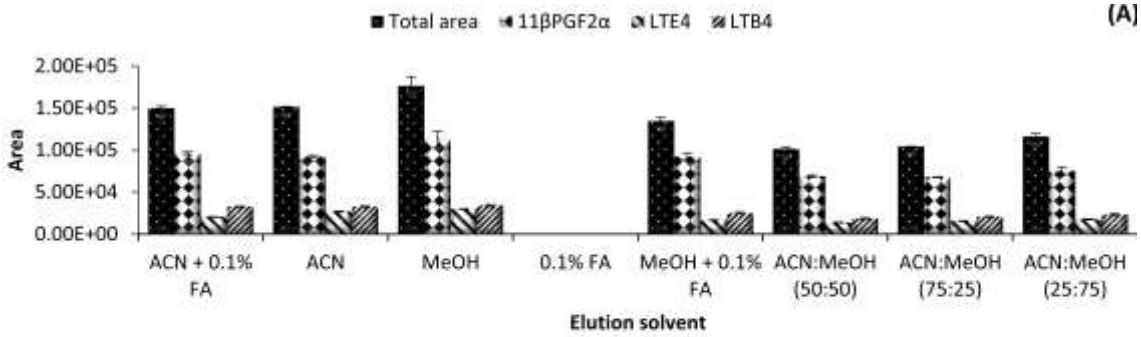
11βPGF$_{2\alpha}$, and 2.5 µg L^{-1} for the LTB$_4$ and LTE$_4$) and medium level (ML; 100 µg L^{-1} for the 11βPGF$_{2\alpha}$, and 10 µg L^{-1} for the LTB$_4$ and LTE$_4$) of concentration.

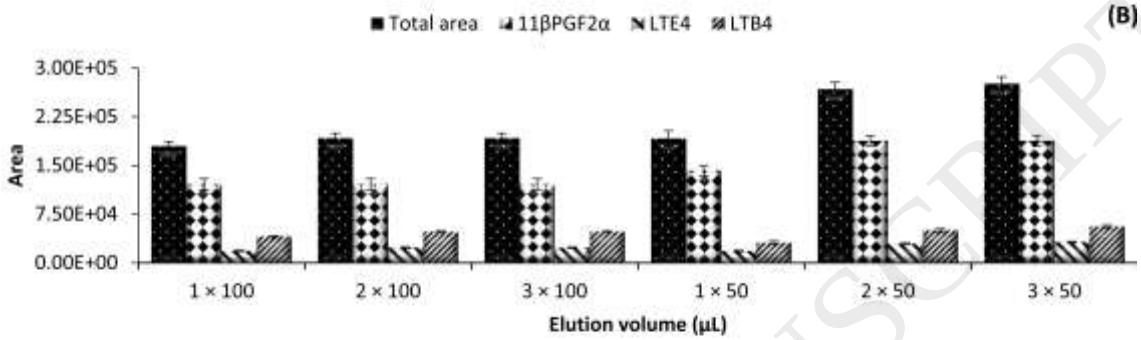
Fig. 4 Representative chromatograms of a blank synthetic urine and synthetic urine spiked with the investigated standards: (1) 11β-prostaglandin F$_{2\alpha}$ (300 µg L^{-1}), (2) leukotriene E$_4$ (30 µg L^{-1}) and (3) leukotriene B$_4$ (30 µg L^{-1}) – at the best optimization parameters obtained at (A) 192 nm and (B) 281 nm. Chemical structures of the target analytes are attached on the side of the respective peak.

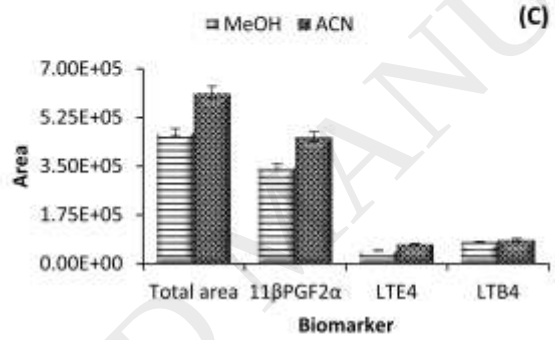
Fig. 5 Representative chromatograms of urine from a normal control (CTRL) and urine from an asthmatic patient (ASMT) at (A) 192, (B) 281, and (C) 270 nm. Numbers represent the target analytes: (1) 11β-prostaglandin F$_{2\alpha}$, (2) leukotriene E$_4$ and (3) leukotriene B$_4$. Boxes on the up-right corner of each chromatogram represent the magnification of the target analyte peak.

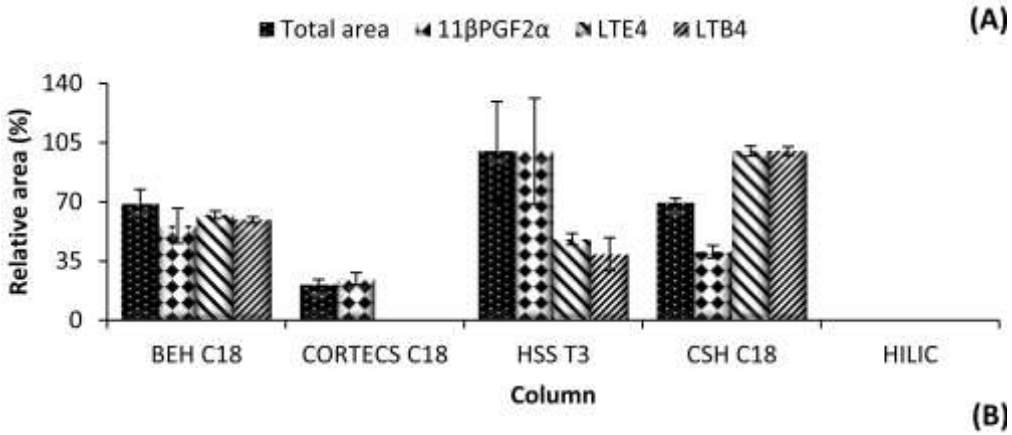
Fig. 6 Average concentrations of 11β-prostaglandin F$_{2\alpha}$ (11βPGF$_{2\alpha}$), leukotriene E$_4$ (LTE$_4$) and leukotriene B$_4$ (LTB$_4$) obtained in the urine of asthmatic patients and healthy individuals.

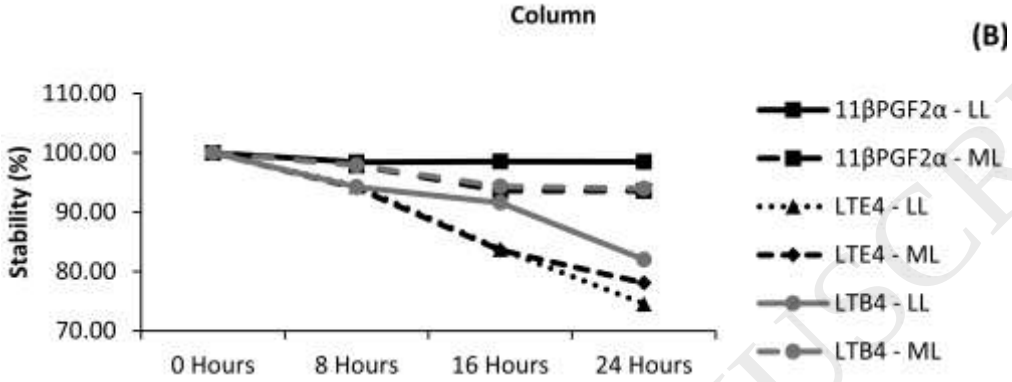


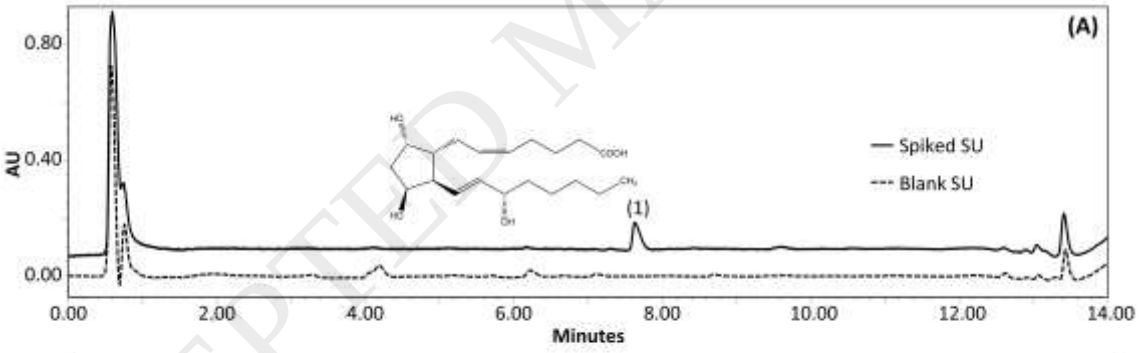


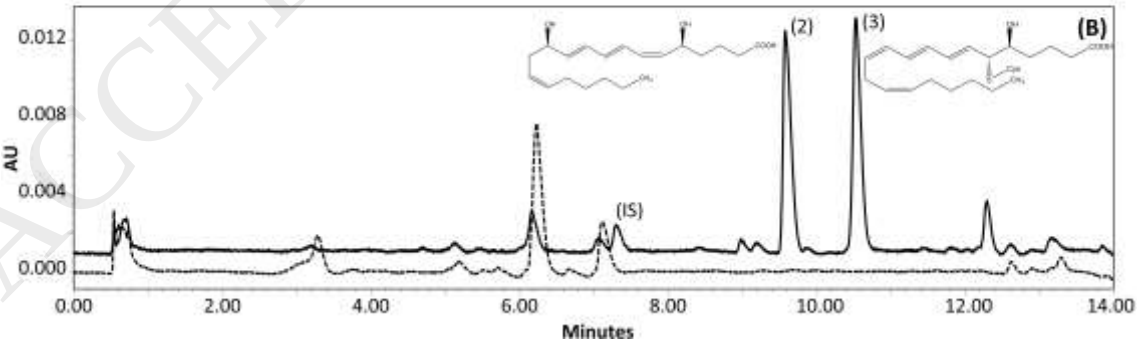


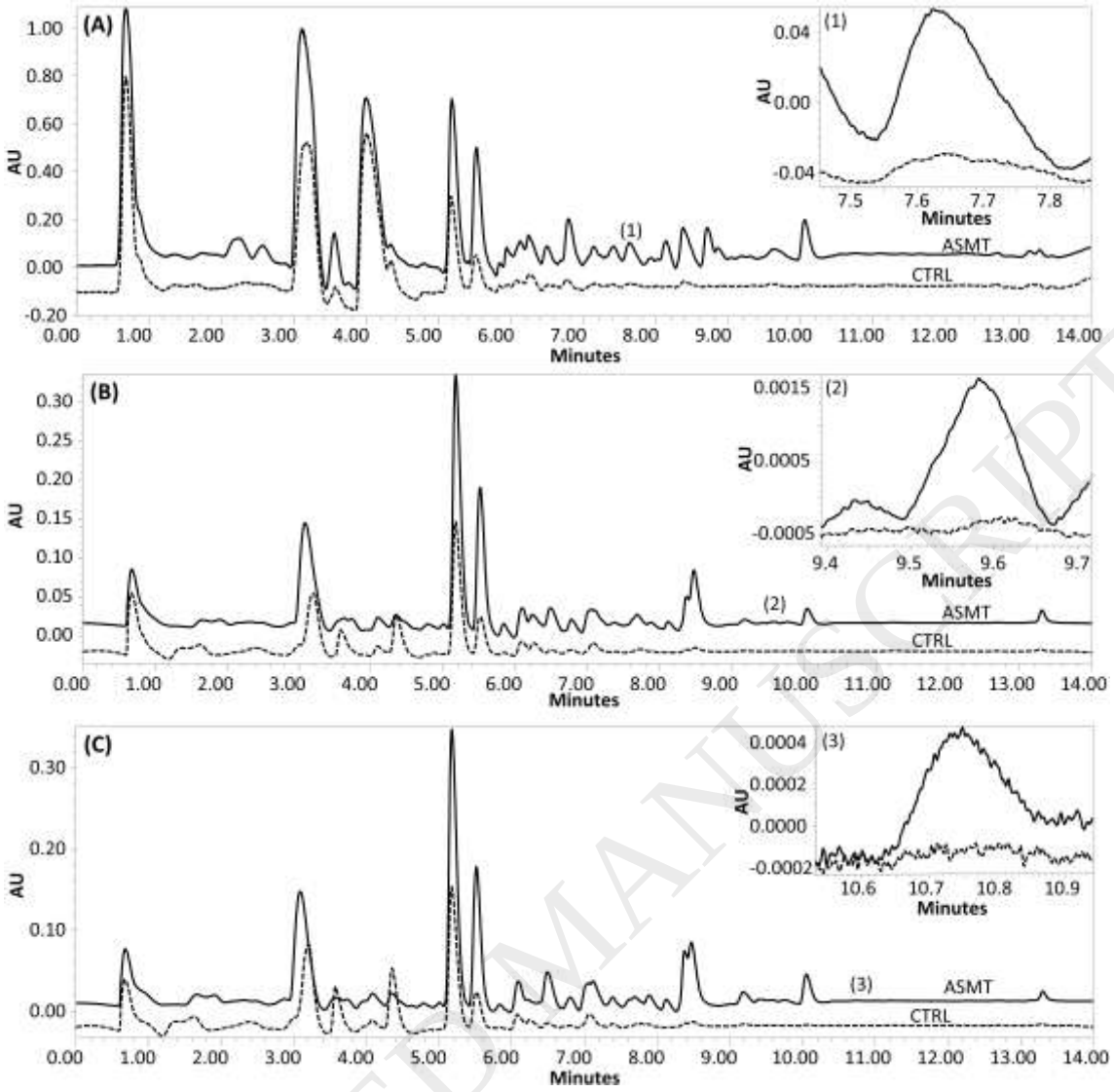












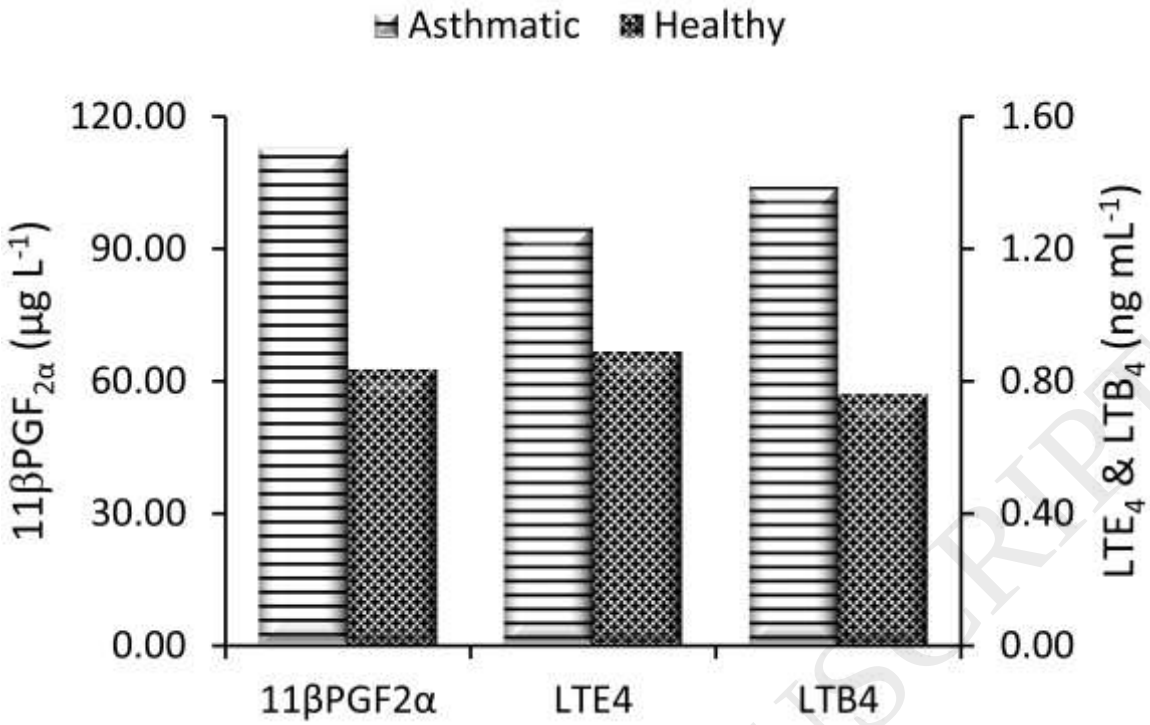


Table 1

Characteristics of the asthmatic patients and healthy individuals (control group).

Subjects	Asthmatic patients	Healthy individuals
n	27	17
Age (years; mean ± std)	8.8 ± 4.6	7.8 ± 1.4
Gender (male:female)	16:11	10:07
Pathology		
Allergic asthma	26 (96%)	0
Allergic asthma + allergic rhinitis	25 (93%)	0
Allergic asthma + sinusitis	2 (7%)	0
Allergic asthma + conjunctivitis	7 (26%)	0
Allergic asthma + eczema	14 (52%)	0
Allergens		
House dust mites	19 (70%)	0
Pollens	10 (37%)	0
Animal epithelium	15 (56%)	0
Moulds	3 (11%)	0
Cockroaches	5 (19%)	0
Initial diagnose (intermittent:mild:moderate:severe:n/a[a])	6:3:12:0:4	-
Uncontrolled asthma	27 (100%)	0
Exacerbation in last 12 months	23 (85%)	0
Missed school in last 12 months because of illness[b]	23 (85%)	8 (47%)
Parent/Tutor missed work in last 12 months because of the child[b]	14 (52%)	7 (41%)
Smoker	3 (11%)	0
Have family members with asthma	22 (81%)	10 (59%)
Relatives with asthma (only paternal:only maternal)	4:13	0:4
Both relatives with asthma	5 (19%)	6 (35%)
Without relatives with asthma	5 (19%)	7 (41%)

[a] n/a – no answer.

[b] Because of asthma, in the case of the asthmatic patients and because of any illness, in the case of normal controls.

Table 2

Properties of the UPLC columns used in this study.

Chromatographic mode	**Name**	**Chemistry**	**Particle technology**	**Particle size (µm)**	**surface area (m^2/g)**	**pH range**	**Pressure Stability (psi)**	**Temperature limits (°C)**		**Properties and purpose**
								Low pH	High pH	
Reversed phase	Acquity UPLC® BEH C18	C18	Fully porous	1.7	185	1 - 12	18000	80	60	Ethylene Bridged hybrid particle technology
										Universal column
										Wide variety of compounds
										Separations at high temperatures and extremes of pH
	Acquity UPLC® HSS T3	Tri-functionally bonded C18 ligand	Fully porous	1.8	230	2 - 8	18000	45	45	High strength silica particle technology
										High retention of polar organic analytes
										Balanced retention of hydrophilic and hydrophobic analytes
	Acquity UPLC® CSH C18	C18	Fully porous	1.7	185	1 - 11	18000	60	45	Charged surface hybrid particle technology
										Separation of basic analytes at low pHs
										Fast pH transitions and equilibration
										Fast method development
	CORTECS UPLC® C18	C18	Solid core	1.6	100	2 - 8	18000	45	20	Solid-core particle technology
										High efficiency and increased sensitivity
Normal phase	Kinetex ® HILIC	Unbonded silica (silanol groups)	Solid core with a porous shell	1.7	200	2 - 7.5	14500	60	< 60	Core-shell particle technology: 1.25 µm solid core with a 0.23 µm porous coating
										Hydrophilic interaction chromatography
										High selectivity and increased retention of highly polar analytes

Table 3

Analytical performance of the optimized MEPS/UHPLC-PDA methodology.

Analytes	**IS**	**11βPGF$_{2\alpha}$**	**LTE$_4$**	**LTB$_4$**
Peak number	-	1	2	3
RT[a] (min)	7.43	7.65	9.61	10.75
λ_{max}[b] (nm)	366	192	281	270
LDR[c] (µg L^{-1})	-	5 - 300	0.5 - 30	0.1 - 30
Calibration in solvent				
Regression equation	-	y = 0.1304x + 0.6418	y = 0.1225x + 0.0627	y = 0.1708x + 0.0105
r^{2}[d]	-	0.9988	0.9989	0.9997
r[e]	-	0.9994	0.9995	0.9998
Calibration in matrix				
Regression equation	-	y = 0.0951x - 2.6893	y = 0.0932x + 0.0224	y = 0.1385x - 0.0640
r^{2}[d]	-	0.9841	0.9884	0.9989
r[e]	-	0.9920	0.9942	0.9994
Matrix effect (%)	-	73	76	81
Method limits				
LOD[f] (µg L^{-1})	-	1.12	0.16	0.04
LOQ[g] (µg L^{-1})	-	2.11	0.35	0.10
Lack-of-fit test				
$F_{calculated}$:$F_{tabulated}$[h]	-	1.48:2.49[h]	0.67:2.49[h]	0.32:2.22[i]
Goodness-of-fit test				
$F_{calculated}$:$F_{tabulated}$	-	0.00:2.49[h]	0.06:2.49[h]	0.11:2.22[i]
Mandel's fitting test				
$F_{calculated}$:$F_{tabulated}$	-	2.50:7.71[j]	0.12:7.71[j]	0.08:5.99[k]

[a] RT – retention time.
[b] λ_{max} – maximum absorbance values obtained in the PDA system detection.
[c] LDR – linear dynamic range.
[d] r^2 – coefficient of determination.
[e] r – coefficient of correlation (Person's r).
[f] LOD – limit of detection.
[g] LOQ – limit of quantification.
[h] $F_{tabulated}$ – numerator degrees of freedom (df1) = 5; denominator degrees of freedom (df2) = 35; p = 95%.
[i] $F_{tabulated}$ – df1 = 7; df2 = 45; p = 95%.
[j] $F_{tabulated}$ – df1 = 1; df2 = 4; p = 95%.
[k] $F_{tabulated}$ – df1 = 1; df2 = 6; p = 95%.

Table 4

Precision, accuracy and extraction efficiency obtained for the 11β-prostaglandin $F_{2\alpha}$ (11βPGF$_{2\alpha}$), leukotriene E_4 (LTE$_4$) and leukotriene B_4 (LTB$_4$) at different levels of concentration.

Analytes	**Precision (% RSD)**

	Level of concentration[a] (µg L^{-1})		Intraday repeatability (*N* = 12)	Intermediate precision (*N* = 9)	Accuracy (%)	Extraction efficiency
11βPGF$_{2\alpha}$	LL	25	3.17	4.81	93.62	95.52
	ML	100	3.17	3.89	100.50	99.71
	HL	200	3.73	5.40	103.02	98.87
LTE_4	LL	2.5	6.63	10.43	96.73	91.13
	ML	10	4.70	2.86	95.85	83.86
	HL	20	3.11	1.29	103.51	74.99
LTB_4	LL	2.5	6.17	6.54	101.08	84.53
	ML	10	4.82	3.57	96.32	92.25
	HL	20	4.58	3.68	100.04	88.14

[a] Levels of concentration in the validation studies: LL – low level, ML – medium level and HL – high level

Table 5

Comparison the analytical performance of different methodologies reported in the literature for the analysis of urinary eicosanoids.

Eicosanoids	Extraction platform	Extraction solvents (µL)	Sample volume (µL)	Analytical platform	Separation solvents	Flow rate (µL min^{-1})	Time of analysis (min)	Method limits (µg L-1)		r^2	Precision (% RSD)		Accuracy (%)	Extraction efficiency (%)	Ref.
								LOD	LOQ		Intraday repeatability	Intermediate precision			
LTB_4	MEPS	350 (0.1% FA) and 350 (ACN)	2500	UHPLC-PDA	0.1% FA and ACN at 0.1% FA	500	10	0.37	1.22	0.995	3.69 - 20.19	3.78 - 20.37	85.2 - 104.3	Not specified	[24]
LTB_4, LTE_4 and $11\beta PGF_{2\alpha}$	MEPS	850 (0.1% FA), 750 (ACN) and 100 (MeOH)	2500	UHPLC-PDA	0.1% FA and ACN at 0.1% FA	300	14	0.04 - 1.12	0.1 - 2.11	0.9988 - 0.9997	3.11 - 6.63	1.29 - 10.43	93.62 - 103.51	74.99 - 99.71	This study
LTE_4	Not used	Not used	Not specified	UHPLC-Q-TOF/MS	0.1% FA, 20 mM ammonium formate and MeOH at 0.1% FA	400	21	Not specified	Not specified	0.9801	Not specified	Not specified	Not specified	Not specified	[35]

PG metabolites, TXs, IsoPs and LTs	SPE	PGs, IsoPs and TXs: 3000 (MeOH), 6000 (0.1% acetic acid), 3000 (ACN) LTs: 4000 (MeOH), 6000 (0.1% acetic acid), 1000 (50% MeOH)	2000 - 2700 (diluted urine)	UHPLC-MS/MS	PGs, IsoPs and TXs: 0.1 % acetic acid and ACN:isopropanol (9:1, v/v) LTs: 0.2% FA and ACN/isopropanol (90:10)	325 - 450	4.25 - 24	0.55 fmol (2,3-dinor-8-isoPGF2α) to 15.4 fmol (tetranorPGEM)	Not specified	linear from > 27-fold concentration range	1.30 - 34.8	Not specified	48.7 - 118.2	Not specified	[10]
PGs and IsoPs	SPE	Equilibration with MeOH and water, elution with 80% MeOH (1% acetic acid). Volumes not specified.	200	UHPLC-MS/MS	Water and MeOH:ACN (1:1, v/v)	200	20	Not specified	All analytes = 0.05	0.99	0.92 - 16.59	0.90 - 9.12	87.98 - 119.46	Not specified	[36]
tetranor-PG, IsoPs; Txs, LTE_4, 12-HETE	LLE	11250 (MeOH:chloroform, 2:1, v/v) and 3750 (chloroform)	3000	HPLC-MS/MS	0.1% FA and ACN at 0.1% FA	600	14	0.002 - 0.06	0.006 - 0.18	0.996 - 0.999	1.08 - 11.20	2.70 - 11.5	94.9 - 112.9	25.00 - 100.00	[37]

HETEs, EETs, oxoETEs, HODEs, PGs, oxoODE	LLE	1000 (10% acetic acid:2-propanol:hexane, 2:20:30, v/v/v) 6000 (hexane),	200	HPLC-MS/MS	0.2% acetic acid and MeOH	200	32	2.6 - 64 pg	0.09 - 2.2	Not specified	3.40 - 14.00	5.3 - 10.9	100.02 - 109.60	10 - >85	[38]