



Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb



Liquid chromatography–mass spectrometry measurement of leukotrienes in asthma and other respiratory diseases[☆]

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ARTICLE INFO

Article history:

Received 21 November 2013

Accepted 17 February 2014

Available online xxx

Keywords:

Airway inflammation

Asthma

Biomarkers

Exhaled breath condensate

Leukotrienes

Liquid chromatography–mass spectrometry

Respiratory medicine

ABSTRACT

Leukotrienes (LTs), including cysteinyl-LTs (LTC₄, LTD₄ and LTE₄) and LTB₄, are potent inflammatory lipid mediators which have been involved in the pathophysiology of respiratory diseases. LC–MS/MS techniques for measuring LT concentrations in sputum supernatants, serum, urine and exhaled breath condensate (EBC) have been developed. In asthmatic adults, reported LTB₄ and LTE₄ concentrations in sputum range from 79 to 7220 pg/ml and from 11.9 to 891 pg/ml, respectively. Data on sputum LT concentrations in healthy subjects are not available. In EBC, reported LTE₄ concentrations range from 38 to 126 pg/ml (95% CI) in adult asthma patients and from 34 to 48 pg/ml in healthy subjects. LTB₄ concentrations in EBC range from 175 to 315 pg/ml (interquartile range) in asthmatic children, and from 25 to 245 pg/ml in healthy children. Enabling an accurate quantitative assessment of LTs in biological fluids, LC–MS/MS techniques provide a valuable tool for exploring the pathophysiological role of LTs in respiratory disease and might be useful for assessing the effects of therapeutic intervention. This review presents the analytical aspects of the LC–MS/MS techniques for measuring LT concentrations in biological fluids and discusses their potential utility for the assessment of airway inflammation and monitoring of pharmacological treatment in patients with asthma phenotypes and other respiratory diseases.

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1. Introduction

Leukotrienes (LTs) include LTB₄ and cysteinyl LTs (CysLTs) (LTC₄, LTD₄, and LTE₄). These potent lipid mediators derive from arachidonic acid through the 5-lipoxygenase (5-LO) activity [1–6] (Fig. 1). The pathway for the complete synthesis of CysLTs is expressed in several inflammatory cell types and becomes activated during atopic airway inflammation [3,5]. Although they lack the complete synthetic pathway, platelets and endothelial cells can produce CysLTs from the chemically reactive intermediate LTA₄ via mechanisms of intercellular transfer [5]. LTs have a pivotal

pathophysiological role in asthma [1–4,7], as demonstrated by the efficacy of CysLT receptor antagonists [8], but their importance may vary among patients with asthma.

CysLTs induce pathophysiological changes that contribute to airway obstruction in patients with asthma [1–3]. LTC₄, LTD₄, and LTE₄ are the most potent endogenous bronchoconstrictors. They have similar contractile activity on human airway smooth muscle in vitro. This effect has been confirmed by bronchoprovocation studies in healthy control subjects [3], whereas asthma patients are hyper-responsive to inhalation of LTC₄, LTD₄, and LTE₄ [3]. CysLTs are elevated in adults and children with exercise-induced bronchoconstriction [9,10]. CysLTs increase lung microvascular permeability in experimental animals and increase mucus secretion in isolated human and animal airways [3]. Inhalation of CysLTs in asthmatic patients causes recruitment of eosinophils into airway mucosa and increases sputum eosinophil counts [11]. In addition to their local effects in the airways, CysLTs have several systemic effects that contribute to the inflammatory process that characterizes asthma [4,12]. The reduced Th2 cell-dependent inflammatory

[☆] This paper is part of the special issues ACIDS edited by Alexander A. Zoerner and Dimitrios Tsikas.

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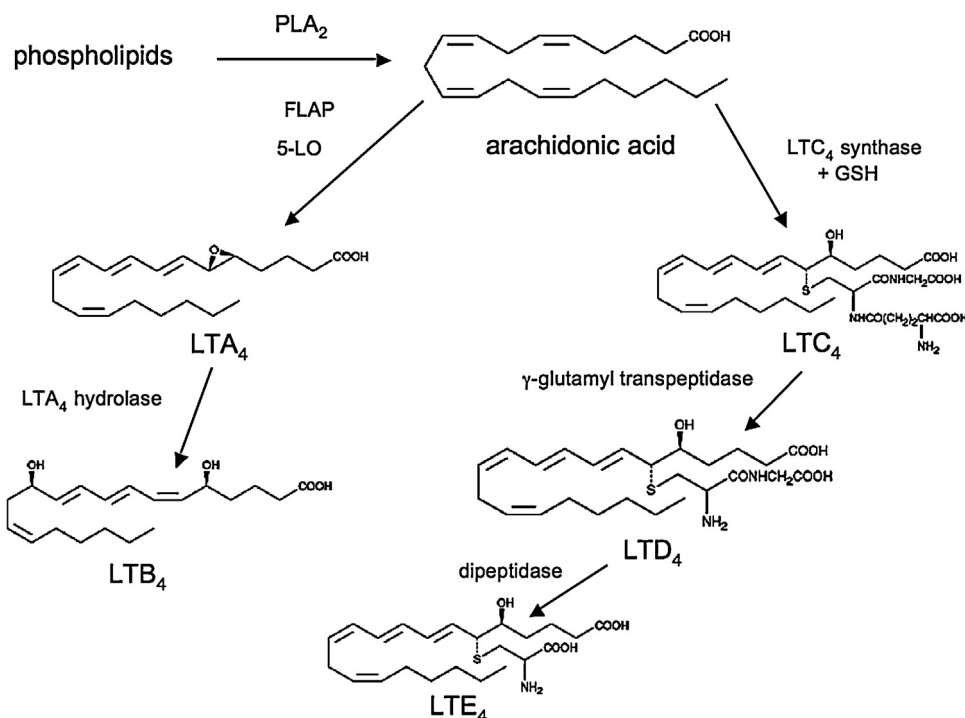


Fig. 1. Biosynthetic pathway of leukotrienes. FLAP, five-lipoxygenase-activating protein; GSH, glutathione; 5-LO, 5-lipoxygenase; LT, leukotriene; PLA₂, phospholipase A₂.

response in LTC₄ synthase null mice demonstrates a central role for CysLTs in airway inflammation induced by allergen challenge [13]. Due to their biological effects, CysLTs might be actively involved in airway remodelling which includes eosinophilic inflammation, mucus gland hyperplasia and mucus hypersecretion, collagen deposition beneath the epithelial layer and in the lung interstitium at sites of leucocyte infiltration, and airway smooth muscle cell hyperplasia [14].

LTB₄ may contribute to airway narrowing by producing local oedema and increasing mucus secretion, although it has no bronchoconstrictor effect in healthy subjects and patients with asthma [2,3]. Due to its potent chemoattractant activity for neutrophils, LTB₄ might be functionally involved in the neutrophilic phenotype of asthma that characterizes patients with severe asthma [15] or asthma exacerbations. LTA₄ hydrolase inhibition attenuates allergic airway inflammation and airway hyper-responsiveness (AHR) in a mast cell-dependent murine model of allergic airway inflammation [16]. Persistently elevated plasma LTB₄ concentrations in children 1 month after an asthma exacerbation [17], and elevated LTB₄ concentrations in exhaled breath condensate (EBC) in adults with mild asthma [18] and children with mild-to-moderate persistent asthma [19], could indicate a pathophysiological role of LTB₄ in persistent asthma of lesser severity [20]. However, the pathophysiological role of LTB₄ in asthma is not completely defined and requires further studies.

In this review, we present the analytical aspects of the LC–MS/MS techniques for measuring LT concentrations in biological fluids and discuss their potential utility for the assessment of airway inflammation and monitoring of pharmacological treatment in patients with asthma phenotypes and other respiratory diseases.

2. Mass spectrometry measurement of LTs in biological fluids: analytical aspects

MS techniques for measuring LTs concentrations in sputum supernatants, EBC, bronchoalveolar lavage (BAL) fluid, serum,

plasma, and urine in healthy subjects and patients with respiratory disease, including asthma, have been developed. Unlike measurement of LT concentrations in serum, plasma and urine which reflects systemic LT production, measurement of LTs in sputum supernatants, EBC, and BAL likely reflects LT production within the respiratory system and is, therefore, more suitable for assessing airway inflammation. However, BAL is invasive and not indicated in patients with asthma who are at higher risk for bronchospasm during this procedure due to their AHR.

2.1. Sputum supernatants

A sensitive and specific solid-phase extraction (SPE) ultra high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for simultaneous measurement of LTB₄ and CysLTs in sputum supernatants has been developed and validated [21]. Stable-isotope dilution method was used for measuring sputum LTB₄ and LTE₄ concentrations, but it is not known whether deuterated internal standards were also used for measuring LTC₄ and LTD₄, making the results related to these LTs less convincing. However, this limitation applies to similar studies aiming at quantifying LT concentrations in biological studies by LC–MS/MS as discussed further on in the text. After ultracentrifugation, samples were stabilized by protease inhibitors, spiked with stable-isotope labelled internal standards, and subjected to SPE and UHPLC analysis on Acquity UHPLC (Waters, USA) with a reversed-phase C-18 column (BEH C18, 2.1 mm × 50 mm, 1.7 μm particle size, Waters, USA) [21]. Column oven temperature was 55 °C. Sample injection volume was 10 μl. Mobile phase A consisted of water with 0.1% formic acid. Following 0.5 min equilibration phase with 18% mobile phase B (acetonitrile with 0.1% formic acid), prostaglandin E₂ was eluted by 4.5 min isocratic elution with 27% phase B; LTB₄ and CysLTs were eluted by a 35–36% gradient of mobile phase B in the next 4.5 min [21].

LT concentrations were measured with a triple quadrupole mass spectrometer (API 5000, Applied Biosystem/MDS SCIEX, Foster City, USA) coupled with an electrospray ionization (ESI) source

Table 1

SRM conditions for LTs and deuterated internal standards.

LTs	SRM transition	Ion polarity	RT (min)	IS (V)	DP (V)	CE (V)	EP (V)	CXP (V)
LTD ₄	497.5/189.3	Positive	6.84	5500	100	24	10	11
LTC ₄	626.4/189.3	Positive	7.11	5500	130	35	10	15
LTE ₄	440.4/189.4	Positive	7.18	5500	70	24	10	15
LTE ₄ -d ₅	445.5/194.4	Positive	7.14	5500	70	23	10	10
LTB ₄	335.2/195.0	Negative	8.22	−4500	−140	−23	−10	−15
LTB ₄ -d ₄	339.2/197.0	Negative	8.17	−4500	−150	−22	−10	−10

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Abbreviations: CE (V): collision energy; CXP (V): exit potential; DP (V): declustering potential; EP (V): entrance potential; IS (V): ion spray voltage; LT: leukotriene; SRM: selected reaction monitoring.

operated in selected-reaction monitoring (SRM) mode and switched between positive and negative modes [21]. SRM conditions for LTs and deuterated internal standards are shown in Table 1.

2.1.1. LTB₄

The limit of detection (LOD) for LTB₄ was 19.5 pg/ml, whereas the lower limit of quantification (LOQ) was 39.0 pg/ml [21]. Standard curve of LTB₄ was linear in the range of 19.5–10000 pg/ml, with average correlation coefficients of 0.9991. The coefficient of variation (CV) for the correlation coefficients for LTB₄ calibration curves was <0.1%. Precision was determined by calculating CV of repeat measurements. CV ranged from 3.4% to 6.3% for intra-day precision and from 1.9% to 9.6% for inter-day precision. Accuracy, determined by spike recovery experiments, ranged from 105% to 111% with CV less than 6.5% [21]. In vivo production of two LTB₄ stereoisomers, 6-*trans*-LTB₄ and 6-*trans*-12-*epi*-LTB₄, due to non-enzymatic hydrolysis of LTA₄, has been described [22]. Conversion of LTB₄ into 6-*trans*-LTB₄ and 6-*trans*-12-*epi*-LTB₄ also occurs in vitro at low pH and high temperature, although its mechanism and the principal products of LTB₄ under these experimental conditions are unknown [21]. By contrast, measurement of LTB₄ was not sensitive to matrix effects. For pre-treated sputum supernatant samples, storage at −80 °C for 75 days, at 4 °C for 4 h and at room temperature for 4 h, and up to 3 freeze/thaw cycles had minimal effects on LTB₄ concentrations (−1% to 6% change) [21].

An SRM transition specific to LTB₄ and its isomers was only obtained in the negative ion mode with a deprotonated precursor ion at *m/z* 335.2 and a product ion at *m/z* 195.0 [21]. Similar fragmentation was observed when LTB₄ was measured in EBC [23]. The LTB₄ isomers 6-*trans*-LTB₄ and 6-*trans*-12-*epi*-LTB₄ had identical SRM transition to LTB₄. However, under optimized HPLC gradient conditions, these LTB₄ isomers, which eluted earlier, were clearly separated from LTB₄ [21].

2.1.2. CysLTs

Regarding the analytical features of this UHPLC–MS/MS method for measuring sputum CysLT concentrations, LOD for LTE₄ was 9.8 pg/ml, whereas LOQ was 19.5 pg/ml [21]. Calibration curve for the LTE₄ assay was linear from 9.8 to 5000 pg/ml. The intra-day CV ranged from 3.2% to 6.4%; the inter-day CV ranged from 3.0% to 8.9%. Spike recovery ranged from 105% to 111% with CV less than 6.5% [21]. LTC₄ and LTD₄ were highly unstable in biological matrices and were converted into LTE₄ in vitro. LTE₄ seems to be a surrogate for total CysLTs in sputum [21]. Likewise, LTE₄, the end-metabolite of CysLTs in humans, has been reported to be the only CysLT sufficiently stable to be measurable in biological fluids and its measurement is used for assessing total CysLT synthesis in vivo [24,25]. However, whether the CysLT biosynthetic pathway in vivo is the same as in vitro is unknown. Under storage conditions tested in this study, changes in LTE₄ concentrations in pre-treated sputum supernatants samples ranged from −9% to 13% [21].

More intensive SRM signals were observed in the positive ion modes with 0.1% formic acid. SRM transitions for CysLTs were as

following: LTC₄, *m/z* 626.4 to *m/z* 189.3; LTD₄, *m/z* 497.5 to *m/z* 189.3; LTE₄, *m/z* 440.4 to *m/z* 189.4 [21].

2.2. EBC

2.2.1. LTB₄

We demonstrated that LC–MS and LC–MS/MS could be used to measure LTB₄ in EBC as a potentially useful approach for studying the pathophysiology of respiratory disease and monitoring respiratory inflammation and pharmacological treatment [19,23]. We studied the MS and MS/MS behaviour of LTB₄ and deuterated LTB₄ (i.e., LTB₄-d₄) used as internal standard, by ESI and atmospheric pressure chemical ionization (APCI) in both positive and negative ion polarity mode [23]. EBC was collected using a commercially available condenser (Ecoscreen, Jaeger, Hoechst, Germany) as described previously [26]. Briefly, subjects were asked to breathe tidally through a mouthpiece connected to the condenser for 15 min. Exhaled air entered and left the chamber through one-way valves at the inlet and outlet, thus keeping the chamber closed. An average of 1.5 ml EBC per subject was collected [23].

EBC samples were stored at −80 °C and analyzed within 2 weeks from collection. To check for possible salivary contamination, α-amylase was measured by an in vitro colorimetric method (Roche Diagnostics, Basel, Switzerland). α-Amylase activity was undetectable in all EBC samples, thus excluding significant salivary contamination [23].

As most of EBC (>99%) consists of water and only less than 1%, consisting of salts and other non-volatile or semi-volatile components, could be responsible for matrix effects [27], we assumed a very limited or lack of matrix effects. A subsequent study has demonstrated the validity of our hypothesis [27]. For this reason, sample pre-treatment was not performed. EBC samples (1 ml) were spiked with LTB₄-d₄ (1.5 ng/ml), concentrated 40-fold under a gentle nitrogen stream, and directly injected into the sample loop. Injection volume was 20 μl [23].

We used a Spectra System P4000 pump coupled with a LCQ Advantage ion trap mass spectrometer (ThermoFinnigan Corp., San José, USA). Chromatography was performed at room temperature using a Lichrospher 100 RP-8 column (125 mm × 4 mm, 5 μm) (Merck, Darmstadt, Germany) [23]. A linear gradient, starting with acetonitrile–water–acetic acid (40:60:0.05, v/v, at pH 5–6) and changing to 100% acetonitrile over 15 min, was used. Flow rate was 0.7 ml/min. The column effluent was introduced into the mass spectrometer through a fused-silica capillary (1 mm i.d. × 0.190 mm o.d.). A sample-loop of 20 μl was used [23].

The mass spectra of standard LTB₄ and LTB₄-d₄ were acquired in full scan mode by infusion of a reference solution using ESI and APCI in positive and negative ion polarity modes. The best analytical conditions were obtained using ESI [23]. In positive ion polarity mode, base peaks at *m/z* 301 for LTB₄ and *m/z* 305 for LTB₄-d₄, corresponding to loss of two molecules of water from the molecular ions ([M+H]⁺) were observed in the ESI mass spectra. The negative ion mass spectra of standard LTB₄ and LTB₄-d₄ revealed a base peak

corresponding to molecular anions ($[M-H]^-$) at m/z 335 for LTB₄ and m/z 339 for LTB₄-d₄ [23]. To confirm its presence in EBC, LTB₄ was also detected in MS² mode which has a higher specificity. In the MS² spectrum of the m/z 301 $[M+H-2H_2O]^+$ precursor ion, the most abundant fragment ions for LTB₄ were observed at m/z 241, 265 and 283 [23]. In the product ion mass spectrum of the carboxylate $[M-H]^-$ ion of LTB₄, m/z 317 (water loss) and m/z 195 were the most abundant fragments [23]. Collisional activation of the carboxylate anion $[M-H]^-$ of LTB₄ causes double bond rearrangement with subsequent carbon-carbon bond cleavage [28]. The proposed pathway for LTB₄ fragmentation involves initial cyclization of the conjugate triene to a cyclic diene [28] followed by abstraction of the carbon-5 proton by a carbon-12 alkoxide anion or the carboxylate anion, and formation of anionic site at carbon-11 which could undergo a proton shift to form the more stable oxygen-centred anion [28] with structural characteristics required for a charge-driven α -hydroxy- β -ene fragmentation often observed for eicosanoids [28,29].

Compared with ESI in positive ion polarity mode, ESI in negative MS mode had a higher sensitivity for both LTB₄ and LTB₄-d₄. This is in line with the fact that ESI is the most readily ionization technique used for the eicosanoid analysis and most of its applications to this purpose have been in the negative ion polarity mode [28].

For quantitative analysis of LTB₄ concentrations in EBC, we used ESI in negative MS¹ polarity mode as the sensitivity of the MS² mode was insufficient [23]. $[M-H]^-$ ions m/z 335 and m/z 339 (internal standard) were used to monitor endogenous LTB₄. LOQ for ion trap analysis of LTB₄ concentrations in MS¹ mode was 100 pg/ml with acceptable signal-to-noise (S/N) ratios [23].

The LTB₄ calibration curve, obtained by plotting the peak area ratios for LTB₄/LTB₄-d₄ versus LTB₄ concentrations, ranged from 100 to 1000 pg/ml. Good linearity was observed over the examined range ($y = 0.0717x - 0.0033$, $r^2 = 0.99$). The CV was 16% ($n = 7$) at the lowest calibration point (100 pg/ml) [23].

In another study, we used LC-ESI-MS/MS for measuring LTB₄ concentrations in EBC in steroid-naïve atopic children with asthma, atopic children with asthma who were being treated with inhaled fluticasone, a glucocorticoid, atopic non-asthmatic children and healthy non-atopic control children [19]. A Finnigan Surveyor® LC System pump coupled with a TSQ Quantum Ultra™ triple quadrupole mass spectrometer, using ESI with negative ion polarity mode with electrospray source, was used (Thermo Electron Corporation, San José, USA) [19]. The LC-MS/MS experimental conditions were similar to those described previously [23]. However, the use of a triple quadrupole mass spectrometer made it possible to increase the analytical sensitivity of the technique by 10-fold (10 pg/ml versus 100 pg/ml) with a LOQ of 50 pg/ml [19]. LTB₄ calibration curve ranged from 10 to 500 pg/ml with good linearity ($r^2 = 0.9965$). Mean accuracy, expressed as percent relative error and calculated over a range of LTB₄ concentrations of 100–1000 pg/ml, was <10%. The intra-class correlation coefficient, expressing between-day variations in EBC LTB₄ measurements, was 0.89 [19]. To increase specificity, quantitative analysis of LTB₄ in EBC was performed in MS² mode. After fragmentation of the parent ions m/z 335 (endogenous LTB₄) and m/z 339 (LTB₄-d₄), the transition m/z 335 $\rightarrow$ m/z 195 and m/z 339 $\rightarrow$ m/z 197, respectively, was monitored to measure LTB₄ concentrations in EBC [19].

2.2.2. CysLTs

A rapid method, which combines the pre-concentration and highly specific separation of CysLTs using immunoaffinity with LC-ESI-MS/MS, was developed for the simultaneous measurement of CysLT concentrations in EBC [27]. CysLT concentrations in EBC were measured using the stable-isotope dilution method [27]. Stable-isotope labelled internal standards (each 250 pg of LTC₄-d₅, LTD₄-d₅, and LTE₄-d₅) were added to 1 ml of EBC immediately after collection. Samples were then stored at -80°C and assayed within 1

month from collection [27]. Potential salivary contamination in EBC samples was assessed by measuring hydrolytic α -amylase activity determined by measuring the amount of reducing sugars generated from starch. Maximal α -amylase activity in all samples did not exceed 0.1% of the saliva activity, thus excluding significant salivary contamination of EBC [27].

Three different pre-treatment techniques for separating and concentrating CysLTs in EBC were compared: immunoaffinity separation, SPE, and lyophilization [27]. In the immunoaffinity separation, the optimal immunoseparation time of CysLTs from EBC was determined as 60 min and sorbent volume enabling the most efficient immunoseparation was determined as 10 μl . CysLTs elution from antibody-antigen complex was performed with methanol. In the SPE extraction, column was activated with methanol and 0.05 M formic acid before EBC sample (1 ml) exposure. Column was then washed with water and CysLTs were eluted with methanol [27]. In the lyophilization, pre-frozen EBC samples (-80°C , 1 ml) were lyophilized for 24 h. With all pre-treatment techniques samples were concentrated 20 fold (1 ml of collected fractions were dried and reconstituted in 50 μl of mobile phase) before LC-ESI-MS/MS measurement. Immunoaffinity separation in combination with LC-ESI-MS/MS was the best performing method in this study in terms of validation parameters (precision, accuracy and recovery), LOD and LOQ [27]. However, SPE and lyophilization can be considered as acceptable alternatives. Results of this study discussed further on in the text are related to immunoaffinity separation.

HPLC was performed using a ProStar HPLC system equipped with a ProStar 210 dual pump, degasser and Varian 410 autosampler (Varian, USA) with a Hypercarb Thermo 100 mm $\times$ 2.1 mm, 5 μm column connected to Hypercarb pre-column (Thermo Electron Corporation, USA) [27]. Isocratic elution was performed at a flow rate of 250 $\mu\text{l}/\text{min}$ using a mobile phase consisting of acetonitrile–water (70:30, v/v) mixture of pH 11. Sample injection volume was 20 μl [27].

Selected-reaction monitoring of CysLTs was performed in the negative ion mode in a highly selective SRM mode using a Varian 1200 L triple quadrupole (Varian, USA) equipped with an electrospray ion source [27]. Full scan mass spectra were acquired. SRM transitions used for analysis were m/z 624.1 $\rightarrow$ m/z 351.2 for LTC₄, m/z 495.2 $\rightarrow$ m/z 477.3 for LTD₄, m/z 438.2 $\rightarrow$ m/z 333.1 for LTE₄ (deprotonated molecular ion $[M-H]^-$ used as a precursor ion $\rightarrow$ most abundant product ion after collision-induced dissociation) (Fig. 2) [27].

LOD and LOQ values were calculated as the mean value of the CysLTs-free EBC matrix signals plus 3 or 10 times the standard deviation, respectively. LOD and LOQ were as follows: LTC₄, 2 pg/ml and 10 pg/ml, respectively; LTD₄, 1 pg/ml and 6 pg/ml, respectively; LTE₄, 1 pg/ml and 5 pg/ml, respectively [27]. Precision, accuracy, and recoveries were assessed by analyzing four concentrations (25, 50, 100 and 250 pg/ml) of LTC₄, LTD₄ and LTE₄ into 5 EBC CysLTs-free matrix. Across the concentrations tested, precision, expressed as relative standard deviation (RSD), ranged from 5.4% to 9.7% for LTC₄, from 4.1% to 8.7% for LTD₄, and from 3.2% to 8.1% for LTE₄ [27]. Accuracy was expressed as relative error calculated as the difference between the standard concentrations in aqueous solutions measured by LC-ESI-MS/MS and the same CysLT concentrations in CysLTs-free EBC samples subjected to immunoaffinity separation and then analyzed by LC-ESI-MS/MS. Accuracy relative error ranged from -7.7% to -9.6% for LTC₄, from -6.9% to -8.0% for LTD₄, and from -6.3% to -7.0% . Recovery values were above 90% for all CysLTs [27].

The Pearson's regression correlation coefficients (r^2) were higher than 0.9955 (RSD, 5.95%) for LTC₄, 0.9983 (RSD, 4.05%) for LTD₄ and 0.9947 (RSD, 3.11%) for LTE₄ calibration curves in CysLTs-free EBC matrix [27]. CysLTs calibration curves in EBC matrix

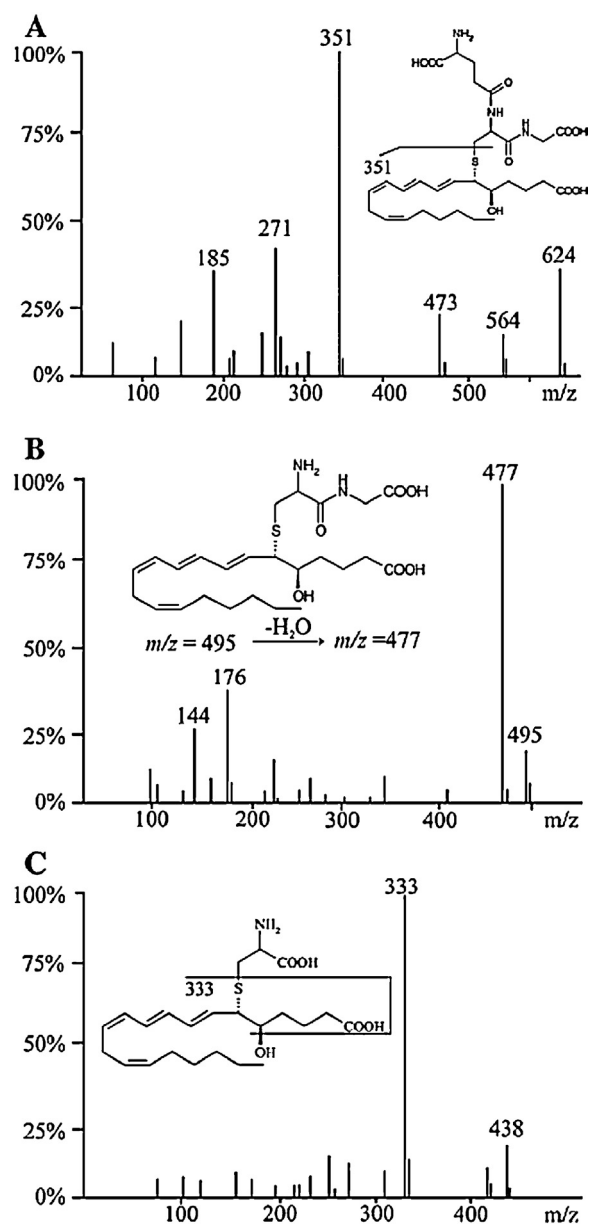


Fig. 2. Negative-ion ESI collision mass spectra of LTC₄ (A), LTD₄ (B) and LTE₄ (C). Precursor ions which were subjected to collision-induced dissociation were m/z 624 for LTC₄, m/z 495 for LTD₄, and m/z 438 for LTE₄.

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and mobile phase were similar, thus excluding significant matrix effects. Moreover, S/N values in LT-free EBC samples spiked with a fixed amount of each single CysLT were high: 346 for LTC₄, 351 for LTD₄, and 376 for LTE₄ [27]. These data suggest that matrix effect in EBC, if any, is of limited extent. In this study, effects of temperature (processing temperature 25 °C and storing temperature –80 °C) and several freeze-thaw cycles on stability of externally added CysLTs in CysLTs-free EBC matrix, water and mobile phase were assessed [27]. Temperature and the number of freeze-thaw cycles were the most important factors affecting CysLTs concentrations in EBC. By contrast, EBC pH and light do not seem to affect EBC CysLTs. Ambient temperature rapidly decreases EBC CysLTs concentrations likely due to the limited stability of these compounds at room temperature [27]. CysLTs enzymatic inter-conversion (i.e., LTC₄ → LTD₄ → LTE₄) could explain the lower LTC₄ and LTD₄ concentrations in EBC as compared with LTE₄ concentrations [27]. However, there is no demonstration of the presence of γ -glutamyl

transpeptidase and dipeptidase in EBC as these enzymatic activities were not measured in this and previous studies. After 12 h at 25 °C, relative CysLTs concentrations in EBC were as follows: 16% for LTC₄; 19% for LTD₄; 30% for LTE₄ [27]. An alternative explanation for the higher LTE₄ recovery at room temperature might be the higher chemical stability of this CysLT. At –80 °C, CysLTs in EBC are stable for up to 3 months [27]. Sample storage at 4 °C is not adequate as CysLTs degradation in EBC and mobile phase also occurs at this temperature, although slower than at room temperature. Each freeze-thaw cycle (from –80 °C to room temperature) reduced the total amount of CysLTs in EBC by 8–9% [27]. Intra-day and inter-day variation of EBC CysLTs was studied in 10 healthy subjects. CysLTs concentrations in EBC were higher by an average of 15.4% during the day and returned to baseline values during the night. Maximal variation in total CysLTs concentrations in EBC was 6.6% for each subject over 5 consecutive days [27].

An HPLC–MS/MS technique was developed for measuring a panel of eicosanoids, including LTs, in EBC [30]. The stable-isotope dilution method was used. EBC was collected from 17 healthy smokers and 41 healthy non-smokers using a commercially available condenser (Ecoscreen, Jaeger, Hoechberg, Germany) [30]. HPLC was performed by injecting 10 μ l of EBC extract on reverse phase column (Zorbax Eclipse XDB C-18, Agilent Technologies, Inc. Santa Clara, USA) stabilized thermally at 37 °C using a Shimadzu Sil-2-AC HPLC (Shimadzu Scientific Instruments, Inc. Columbia, USA) [30]. A gradient consisting of mobile phase A acetonitrile–water–acetic acid (20:80:0.0001, v/v) and phase B acetonitrile–isopropanol–acetic acid (55:45:0.0001, v/v) was used to elute eicosanoids. Flow rate was 0.11 ml/min. The mobile phase binary linear gradient was 1 min 8% B, 9.5 min 8–95% B, 0.5 min 95% B, 0.5 min 95–100% B, 2 min 100% B [30].

LTs were measured using SRM tandem mass spectrometry (QTRAP 4000, Applied Biosystems, Foster City, USA) equipped with electrospray ion source. LT_{B4}, LT_{E4} and their respective internal standards (LT_{B4}-d₄ and LT_{E4}-d₃) were detected in ESI negative ion polarity mode, whereas LTC₄, LTD₄ and their internal standard (LTD₄-d₅) were detected in positive ion polarity mode [30]. Deprotonated molecular ion [M–H][–] was m/z 335 for LT_{B4} and m/z 438 for LT_{E4}. Protonated molecular ion [M+H]⁺ was m/z 626 for LTC₄ and m/z 497 for LTD₄. The product ions monitored were m/z 195 for LT_{B4}, m/z 333 for LT_{E4}, m/z 308 for LTC₄ and m/z 189 for LTD₄. The corresponding ion pairs for internal standards were m/z 339–197 for LT_{B4}-d₄, m/z 441–336 for LT_{E4}-d₃, and m/z 502–194 for LTD₄-d₅ [30].

As the concentrations of volatile or semi-volatile compounds in EBC can be affected by the variable dilution of aerosol particles with condensed water vapor, palmitic acid was measured as a surrogate factor for EBC dilution [30]. Free palmitic acid was chosen because it is a non-volatile diffusible molecule from the vascular compartment into the airways compartment of the lung. Moreover, free palmitic acid concentrations in EBC (average 2.31 μ M) do not depend on gender, age, or tobacco smoking [30]. EBC concentrations of LTs, expressed as medians [interquartile ranges] and measured by HPLC–MS/MS, were 6.54 [3.82–9.87] pg/ml for LT_{B4}, 1.91 [0.89–3.37] pg/ml for LTC₄, 1.98 [1.05–2.80] pg/ml for LTD₄, and 1.16 [0.77–1.75] pg/ml for LT_{E4} [30]. EBC LTs concentrations recalculated as ppm of palmitic acid were as follows: 12.53 [9.27–16.84], 2.92 [1.40–5.46], 3.87 [1.89–5.46], and 2.25 [1.38–3.34] [30]. There was no difference in EBC LTs concentrations between healthy smokers and healthy non-smokers. No information on calibration curve parameters, LOD, LOQ, and accuracy of the technique was provided.

The same analytical technique was used for measuring concentrations of eicosanoids, including LTs, in EBC in 115 adults with asthma (62 with aspirin-intolerant asthma) and 38 healthy subjects [31].

2.3. BAL fluid

2.3.1. LTB₄

A LC–ESI–MS/MS method was used for detecting 39 oxylipins, including LTB₄, in BAL fluid and serum in mice after intraperitoneal (i.p.) or intra-nasal (i.n.) lipopolysaccharide (LPS) injection, a murine model of sepsis [32]. Oxylipins are a group of cyclooxygenase, lipoxygenase and cytochrome P450 oxidized metabolites of polyunsaturated fatty acids (PUFAs). Off-line SPE was performed using Water Oasis HBL 60 mg cartridge columns (Milford, USA) washed with ethyl acetate, methanol and water–methanol–acetic acid (95:5:0.1, v/v) prior to extraction. After BAL sample loading, cartridges were eluted with methanol and ethyl acetate into tubes containing 30% glycerol in methanol as a trap solution. Volatile solvents were then removed and samples were reconstituted in methanol. An Agilent separation module (Santa Clara, USA) coupled to ABI 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems Instrument Corporation, Foster City, USA) equipped with an electrospray source was used for analyses [32]. Separation was performed on a 2.0 mm × 150 mm Pursuit Plus C18 column with a 5 μm particle size (Varian Inc., Palo Alto, USA). Mobile phase A was water with 0.1% acetic acid, whereas mobile phase B consisted of acetonitrile–methanol (84:16, v/v) with 0.1% acetic acid. Flow rate was 400 μl/min. Oxylipins were quantified using stable-isotope labelled internal standards [32]. LC–ESI–MS/MS was performed in negative SRM mode. Transition m/z 335 → m/z 195 with collision energy of 23 eV was monitored for quantifying LTB₄. LOQ, defined as the amount of sample required to produce an S/N of 10 or greater, was 0.2 pg equivalent to 0.06 nM in a 250 μl sample analyzed at a final volume of 100 μl [32].

The linearity of the method was determined by the calibration curves constructed for each analyte. Linearity of LTB₄ was greater than 0.999 over the calibration range (0.06–1000 nM). Accuracy was expressed as the percent difference between the mean quality control (QC) value and the expected value, whereas precision was expressed as the RSD of the measurements. For LTB₄, intra-day accuracy ranged from not defined (the concentration that was below of LOQ at the lowest QC sample) to 12.78% over the concentrations tested (0.40, 2, 20, 200 nM). Inter-day accuracy was between not defined and 11.48%. Intra-day precision ranged from not defined to 12.61%, whereas inter-day precision was between not defined to 13%. Recovery in spiked QC samples ranged from 80% to 103%, except at the lowest QC concentration (0.20 nM) for which recovery could not be defined [32]. Compared with control group, mice which were administered i.n. LPS had an 86-fold increase in LTB₄ concentrations in BAL fluid, whereas it was undetectable in serum [32]. Values of LTB₄ concentrations in BAL fluid were not reported.

The same method was used in a cross-sectional clinical study in which a total of 87 oxylipins, including LTB₄ and 6-*trans*-LTB₄, were screened through LC–ESI–MS/MS following SPE extraction of BAL fluid [33]. The only difference in the analytical procedures was that separation was performed with a different type of column, a 2.1 mm × 150 mm Eclipse plus C18 column with a 1.8 μm particle size (Agilent, Santa Clara, USA) [33]. Samples were obtained from birch pollen steroid-naïve atopic patients with mild asthma and non-atopic healthy control subjects [33].

2.4. Serum

An LC–ESI–MS/MS method for measuring oxylipins, including LTB₄, in a murine model of sepsis was developed by Yang et al. as described above [32].

Lipid mediator profiles of human serum were identified by a LC–MS-based targeted lipidomic analysis [34]. This approach was used for investigating lipid mediator responses to resistance

exercise and treatment with the non-steroidal anti-inflammatory drug ibuprofen in 16 healthy male subjects [34], but has not been applied to patients with respiratory diseases. A total of 87 lipid mediators, including LTB₄, were reliably detected in human serum [34]. Serum samples (0.5 ml) were spiked with 10 ng each of octadeutero-15(S)-hydroxy-eicosatetraenoic acid (HETE)-d₈, 11-dehydro-thromboxane B₂ (TXB₂)-d₄, and TXB₂-d₄ as internal standards for analyte recovery and quantitation. Samples were then extracted for fatty acyl lipid metabolites using C₁₈ extraction columns. The internal standard spiked samples were applied to conditioned C₁₈ cartridges, washed with water followed by hexane, and dried under vacuum. The cartridges were then eluted with 0.5 ml methanol, the eluate was dried under a gentle stream of nitrogen, and the residue was dissolved in 50 μl methanol–25 mM aqueous ammonium acetate (1:1, v/v) and subjected to LC–MS analysis [34].

HPLC was performed on a Prominence XR system (Shimadzu) using Luna C₁₈ (2.1 mm × 150 mm, 3 μm) column. The mobile phase consisted of a gradient between A: methanol–water–acetonitrile (10:85:5, v/v) and B: methanol–water–acetonitrile (90:5:5, v/v), both containing 0.1% ammonium acetate. The gradient programme regarding the composition of B was as follows: 0–1 min, 50%; 1–8 min, 50–80%; 8–15 min, 80–95%; and 15–17 min, 95%. Flow rate was 0.2 ml/min. HPLC eluate was directly introduced to ESI source QTRAP 5500 mass analyzer (ABSCIEX) in the negative ion mode. The eluate was monitored by SRM to detect unique precursor ion-product ion pairs for each of 125 transitions (to monitor a total of 144 lipid mediators and three internal standards). The data were collected using Analyst 1.5.2 software, and the MRM transition chromatograms were quantitated by MultiQuant software (both from ABSCIEX) [34].

There are no published studies on LC–MS methods used for assessing serum LT concentrations in patients with asthma.

2.5. Urine

An LC–MS/MS method for measuring urinary LTE₄ was developed and compared with an enzyme immunoassay (EIA). For LC–MS/MS analysis, 800 μl of supernatant from centrifuged urine was spiked with LTE₄-d₃ as internal standard and loaded directly on to the LC–MS/MS system [35]. A column switching/enrichment strategy was employed resulting in the analyte concentration on the column and removal of contaminants that could interfere with ionization [36]. LTE₄ was measured using an Agilent 6410 triple quadrupole mass spectrometer coupled to a positive ESI source operated in SRM mode [35]. The mass transitions of m/z 440.2 → m/z 301.2 for LTE₄ and m/z 443.2 → m/z 304.2 for LTE₄-d₃ were measured. In some experiments, the transitions of m/z 440.2 → m/z 189.2 for LTE₄ and m/z 443.2 → m/z 192.2 for LTE₄-d₃ were monitored as qualifiers. A typical standard curve was linear in the range 5–500 pg/ml with $r^2 = 0.9985$ for 7 calibration levels. Intra- and inter-day analyses, conducted using both calibration standards and spiked, pooled urine from children with asthma, showed average CV of 5.76% and 7.20%, respectively. Recoveries for the intra-day experiments were 95.3–120.9%; recoveries for the inter-day experiments were 94.2–104.9%. Regarding sample stability, following up to 7 days at 4 °C there was a reduction of urinary LTE₄ concentrations of 11%, whereas a reduction of 26% was observed after 24 h at 30 °C. Single and multiple freeze/thaw cycles had an equivalent effect on sample stability, with reduction in urinary LTE₄ levels ranging from 18.3 to 10.8%. Addition of protease inhibitors did not modify sample stability [35].

Measurement of urinary LTE₄ estimates systemic CysLTs production [24]. HPLC–MS was used for measuring urinary LTE₄ concentrations in adults with asthma and healthy subjects [37]. Urinary samples (25 ml) were pre-treated with affinity purification

and added $\text{LTE}_4\text{-d}_4$ as internal standard. HPLC–MS was performed using a LCQ Advantage mass spectrometer (Thermo Finnigan, San José, USA). Calibration curve of LTE_4 was linear in the range from 0 to 200 pg/ml with $r^2 = 0.999$ [37].

In a complex biological matrix such as urine, the quality of the LC–MS/MS quantification of eicosanoids can be reduced by strong matrix effects as sample background depends on fluid intake and water–salt balance [38]. A platform consisting of 4 ultra performance (UP)–LC methods coupled to ESI–MS/MS that quantify 26 lipid mediators and their metabolites in urine was developed to address these limitations [38]. To compensate for the inter- and intra-individual sample matrix effects, extracted urinary sample volume was normalized by the ratio of absorbance at 300 nm to an optimized reference material [38]. LC–MS/MS separation and quantification of LTs was performed on an UPLC Acquity–Xevo TQ mass spectrometer system (Waters, Milford, USA). SPE was performed on 3 cm^3 /60 mg HLB Oasis SPE cartridges (Waters) conditioned with methanol and water containing 0.1% acetic acid [38]. Urine was diluted with water containing 1% acetic acid, spiked with internal standard mix solution and loaded onto the SPE cartridge. Samples were washed with methanol–water (50:50, v/v) and water with 0.1% of acetic acid. Cartridges were dried under vacuum-induced air stream and LTs were eluted with methanol [38]. Eluates were then evaporated and reconstituted in methanol–water 85:15 (v/v). Model urinary samples (high, medium and low concentration of solutes) were extracted with internal standards added before and after extraction to calculate the matrix effect, the extraction recovery, and the overall process efficiency. The matrix effect of LT internal standards ranged from 82.8% to 175% and was more prominent in highly concentrated urinary samples [38]. Extraction recoveries in model urinary samples ranged from 33% to 83.3% and were less dependent on the sample background [38].

LC–ESI–MS/MS was performed in negative ion polarity mode to measure LTB_4 and its internal standard and in positive ion polarity mode to measure LTC_4 , LTD_4 and LTE_4 and their respective internal standards [38]. The following SRM transitions were used for quantifying urinary LTs: m/z 626.4 $\rightarrow$ m/z 308.2 for LTC_4 ; m/z 497.3 $\rightarrow$ m/z 189.1 for LTD_4 ; m/z 440.3 $\rightarrow$ m/z 189.2 for LTE_4 ; m/z 335.3 $\rightarrow$ m/z 195 for LTB_4 ; m/z 335.3 $\rightarrow$ m/z 195 for 6-*trans*- LTB_4 [38]. LOD values for CysLTs and LTB_4 were lower than 4.60 fmol. Linear range was as follows: 13.5–3380 fmol for LTC_4 ; 4.59–3445 fmol for LTD_4 ; 4.50–1125 fmol for LTE_4 ; 13.4–3343 fmol for LTB_4 [38].

To validate the method, model urinary samples were spiked with external standards at a concentration equivalent to the calibration curve. Accuracy, expressed as percentage of difference between the external standard method and the standard addition method, was lower than –5% for endogenous urinary LTE_4 . Precision, expressed as the RSD of the concentration calculated with the calibration curve, ranged from 8% to 34.8% with lowest precision in highly concentrated urinary samples [38].

2.6. EBC, plasma, urine

2.6.1. CysLTs

A method based on immunomagnetic molecular separation with UHPLC–ESI–MS/MS for measuring CysLTs in EBC, plasma and urine was developed [39]. EBC (1 ml), plasma (0.5 ml) and urine (0.2 ml) samples were immediately spiked with 100 pg of each deuterium-labelled internal standard ($\text{LTC}_4\text{-d}_5$, $\text{LTD}_4\text{-d}_5$ and $\text{LTE}_4\text{-d}_5$), stored at -80°C and analyzed within 1 month. Salivary contamination of EBC was excluded by measuring salivary α -amylase which did not exceed 0.1% of the saliva activity in all samples [39]. Immunomagnetic molecular separation of CysLTs in EBC, plasma and urine was performed by resuspending anti-CysLT coated magnetic beads, previously washed twice with sodium phosphate buffer (0.1 M, pH 7.4), in 1 ml of sample. The suspension

was incubate for 1 h, the supernatant was removed and the beads were washed 5 times with phosphate buffer saline (PBS) containing 0.1% BSA at pH 7.4. After removing supernatant, bound CysLTs were eluted from complex with antibody by putting beads into a 50 μl of glycine buffer (100 nM at pH 2.5) for 1 min. Supernatant was removed immediately and analyzed by UHPLC–ESI–MS/MS [39]. The beads were regenerated by washing 5 times in PBS buffer containing 0.1% BSA [39]. UHPLC analysis was performed using a quaternary pump with an Accela 1250 degasser (Thermo Scientific, San José, USA) with a Kinetex C18 50 mm $\times$ 2.1 mm, 2.7 μm column connected to a Kinetex C18 pre-column (Phenomenex, USA) [39]. A mobile phase consisting of acetonitrile–water–formic acid (70:30:0.1, v/v) was used for isocratic elution. Flow rate was 400 $\mu\text{l}/\text{min}$. Injection volume was 10 μl . MS/MS analysis was performed using a triple quadrupole mass spectrometer TSQ Vantage (Thermo Scientific, San José, USA) operating with ESI in negative polarity mode [39]. The following SRM transitions were monitored: m/z 624.1 $\rightarrow$ m/z 351.2 for LTC_4 ; m/z 629.1 $\rightarrow$ m/z 356.2 for $\text{LTC}_4\text{-d}_5$; m/z 495.2 $\rightarrow$ m/z 477.3 for LTD_4 ; m/z 500.2 $\rightarrow$ m/z 482.3 for $\text{LTD}_4\text{-d}_5$; m/z 438.2 $\rightarrow$ m/z 333.1 for LTE_4 ; m/z 443.2 $\rightarrow$ m/z 338.1 for LTE-d_5 [39].

LOD and LOQ values, intra-day and inter-day precision (expressed as RSD), accuracy (expressed as RE), and extraction recovery for measurement of CysLTs in EBC, plasma and urine are shown in Table 2 [39].

Calibration curves, obtained by adding increasing amounts of LTC_4 , LTD_4 and LTE_4 to a LT-free volume of EBC, plasma or urine containing 100 pg of a particular internal standard, were linear within the range starting at the LOQ up to 5000 pg with $r^2 > 0.997$ ($\text{SD} < 2.8$) and $\text{RSD} < 2.7\%$ for all CysLTs. Free matrix of biological fluids was obtained by repeat separation of CysLTs from EBC, plasma or urine by immunoextraction with immunoparticles [39].

3. Mass spectrometry measurement of leukotrienes in biological fluids: clinical aspects

In the beginning, quantitation of LTs in biological fluids was performed by gas chromatography–mass spectrometry (GC–MS) and gas chromatography–mass spectrometry/mass spectrometry (GC–MS/MS) [40]. Afterwards, LC–MS and LC–MS/MS techniques were developed for the same purposes. LC–MS/MS and GC–MS and/or GC–MS/MS provide similar concentrations of plasma LTB_4 [41,42] and urinary LTE_4 in humans [38,40].

Most of the analytical techniques described above have been used for comparing LT concentrations in sputum supernatants, EBC, BAL, plasma and urine in patients with different asthma phenotypes and/or pharmacological treatment and healthy control subjects. LC–MS/MS has also been used for quantifying LT concentrations in EBC in patients with pneumoconiosis (Tables 3 and 4).

3.1. LC–MS measurement of LTs in patients with adult asthma

3.1.1. Sputum supernatants

In the study by Chappell et al., LTB_4 and CysLT concentrations were measured in 14 spontaneous sputum supernatant samples purchased commercially [21]. Patients' information was not available. In addition, 8 induced sputum samples which had been collected during a clinical trial from 4 adult patients with asthma on two separate visits over a 10-day period were analyzed [21]. Two asthma patients were steroid-naïve, one patient was on a dose of inhaled fluticasone of 88 $\mu\text{g}/\text{day}$ and one patient was on a daily dose of 100/500 μg of inhaled salmeterol/fluticasone [21].

LTB_4 was detected in all samples with concentrations ranging from 79 to 7220 pg/ml. LTE_4 was detected in 20 out of 22 sputum supernatants samples analyzed [21]. LTE_4 concentrations ranged

Table 2
Validation parameters for measurement of CysLTs in EBC, plasma and urine by a method combining immunomagnetic molecular probe separation with UHPLC–MS/MS.

LT	Matrix	LLOD (pg/ml)	LLOQ (pg/ml)	Precision, %RSD ^a		Accuracy, %RE ^b	Recovery, %
				Intra-day	Inter-day		
LTC ₄	EBC ^c	3	6	8.1–9.5	8.8–9.8	–8.6 to 10.5	95.6–96.1
LTD ₄	EBC ^c	2	5	7.4–9.2	7.8–9.5	–8.3 to 10.4	95.8–97.1
LTE ₄	EBC ^c	2	4	7.2–8.5	7.5–8.9	–8.4 to 10.3	95.5–96.8
LTC ₄	Plasma ^d	5	7	8.9–10.1	9.4–10.4	–12.6 to 15.1	95.2–96.2
LTD ₄	Plasma ^d	4	6	8.7–10.3	9.3–10.7	–12.5 to 17.7	93.6–94.8
LTE ₄	Plasma ^d	4	6	8.3–10.0	8.8–10.6	–11.4 to 17.9	93.1–95.6
LTC ₄	Urine ^e	6	8	10.6–13.6	11.2–14.5	–15.2 to 18.8	93.2–95.5
LTD ₄	Urine ^e	5	7	10.2–12.5	10.8–13.1	–13.2 to 16.2	93.4–95.4
LTE ₄	Urine ^e	5	7	9.8–11.7	10.3–12.5	–12.3 to 15.5	93.5–95.7

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Abbreviations: CysLT: cysteinyl-leukotriene; EBC: exhaled breath condensate; LLOD: lower limit of detection; LLOQ: lower limit of quantification; LT: leukotriene; RE: relative error; RSD: relative standard deviation; UHPLC–MS/MS: ultra high performance liquid chromatography–tandem mass spectrometry.

^a Precision was expressed as relative standard deviation (see text).

^b Accuracy was expressed as relative error (see text).

^c Precision and accuracy for CysLTs in EBC was tested at values of LLOD, 50 pg/ml and 100 pg/ml.

^d Precision and accuracy for CysLTs in plasma was tested at values of LLOD, 25 pg/0.5 ml and 50 pg/0.5 ml.

^e Precision and accuracy for CysLTs in urine was tested at values of LLOD, 25 pg/mg creatinine and 50 pg/mg creatinine.

from 11.9 to 891 pg/ml, whereas LTC₄ and LTD₄ concentrations were below LOD in most of sputum samples [21]. However, the lack of a control group makes impossible a comparison of LT levels between asthma patients and healthy subjects.

3.1.2. EBC

In the first study aimed at investigating whether a LC–MS technique could be used for detecting and measuring LTB₄ in EBC, samples were collected from 8 patients with asthma and 2 healthy control subjects [23]. Four patients with asthma were without any treatment, 3 patients were treated with oral montelukast, a CysLT receptor antagonist, at a dose of 10 mg once a day, 1 patient was treated with a combination of oral montelukast and inhaled fluticasone at a daily dose of 500 µg [23].

LTB₄ concentrations in EBC were detected in those patients with asthma who were not on anti-inflammatory drugs for asthma, whereas were below the LOQ of the technique (100 pg/ml) in healthy subjects and in those patients with asthma who were on montelukast or montelukast and fluticasone [23]. However, the small sample size, justified by the fact that the principal aim of this study was to establish whether a LC–MS technique could be applied to LT analysis of EBC, precluded conclusions on between group comparison.

EBC samples obtained from 20 patients with occupational asthma and 20 healthy control subjects were analyzed with a method combining immunoaffinity separation of CysLTs with LC–ESI–MS/MS [27]. EBC was collected by asking subjects to breathe tidally through a mouthpiece connected to an Ecoscreen condenser (Jaeger, Hoechberg, Germany) for 15 min. Compared with those in healthy subjects (LTC₄: 57.4 ± 8.6 pg/ml, 95% CI; LTE₄: 25.3 ± 5.1 pg/ml), CysLT concentrations in EBC were higher in patients with occupational asthma (LTC₄: 80.1 ± 6.2 pg/ml; LTE₄: 35.7 ± 4.8 pg/ml), although *P* values were not reported [27]. There was no difference in LTD₄ concentrations in EBC between patients with asthma and healthy subjects (19.9 ± 4.3 pg/ml versus 13.8 ± 5.2 pg/ml, respectively). Between-group difference was greater in the sum values of CysLTs (patients with asthma, 133.2 ± 20.1 pg/ml; healthy subjects, 77.8 ± 13.8 pg/ml) [27].

HPLC–MS/MS was used for measuring concentrations of CysLTs and LTB₄ in EBC in 62 adults with aspirin-intolerant asthma, 53 patients with aspirin-tolerant asthma and 38 healthy subjects [31]. LT values were expressed as pg/ml or recalculated as ppm of palmitic acid as a dilution factor. The whole asthma group had higher baseline median LTC₄ [4.28 (2.68–8.13) ppm of palmitic acid versus 2.28 (0.81–5.43) ppm of palmitic acid, *P* < 0.01], LTD₄ [2.69

(1.32–5.42) ppm of palmitic acid versus 1.80 (0.80–4.09) ppm of palmitic acid, *P* < 0.05], and LTE₄ [3.40 (1.99–5.49) ppm of palmitic acid versus 2.03 (1.39–4.05) ppm of palmitic acid, *P* < 0.05], but not LTB₄, concentrations in EBC than healthy subjects [31]. LTD₄ and LTE₄ levels were lower in patients with aspirin-intolerant asthma [LTD₄: 1.93 (0.96–4.08) ppm of palmitic acid, *P* < 0.01; LTE₄: 2.54 (1.37–4.66) ppm of palmitic acid, *P* < 0.001] than in those with aspirin-tolerant asthma [LTD₄: 3.77 (2.09–6.94) ppm of palmitic acid; LTE₄: 4.52 (2.96–6.87) ppm of palmitic acid], which might reflect an effect of corticosteroids as most of the patients with aspirin-intolerant asthma were on chronic treatment with these drugs [31]. However, asthma groups did not differ in terms of daily dose of inhaled or oral corticosteroids. Results, expressed as pg/ml or ppm of palmitic acid, were similar [31].

HPLC–MS/MS was used to study the effects of aspirin on EBC levels of eicosanoids in patients with asthma [43]. Twenty-one patients with aspirin-intolerant asthma and 23 patients with aspirin tolerant asthma were included in the study [43]. LT values were recalculated as ppm of palmitic acid as a dilution factor or expressed as absolute concentrations in pg/ml. CysLTs and LTB₄ concentrations in EBC were measured at baseline and after challenge with increasing doses of inhaled lysine-aspirin [43]. Following 15–20 min of tidal breathing, 1–2 ml of EBC was collected using an Ecoscreen® condenser. Patients with aspirin-intolerant and aspirin-tolerant asthma had similar baseline EBC concentrations of LTC₄ (*P* = 0.43), LTD₄ (*P* = 0.22), and LTE₄ (*P* = 0.79) [43]. EBC LTE₄ concentrations, but not total CysLTs (*P* = 0.33), were increased over baseline values after aspirin challenge in both asthma groups (*P* = 0.03) [43]. However, although statistically significant, mean difference in pre- and post-challenge LTE₄ concentrations was 1.19 pg/ml in patients with aspirin-intolerant asthma and 1.66 pg/ml in patients with aspirin-tolerant asthma. In patients with aspirin-intolerant asthma, but not in those with aspirin-tolerant asthma, LTB₄ values were decreased after aspirin-challenge (*P* < 0.05) [43], a finding which is difficult to explain. LT variations in EBC were independent of the dose of inhaled corticosteroids (ICS) used by study patients and their forced expiratory volume in 1 s (FEV₁) values. Study results expressed as pg/ml or normalized for EBC concentrations of palmitic acid were similar [43].

3.1.3. BAL fluid

In a cross-sectional clinical study, 87 oxylipins, including LTB₄ and 6-*trans*-LTB₄, were measured in BAL fluid samples from 8 birch pollen steroid-naïve atopic patients with mild asthma and

Table 3Concentrations of CysLTs and LTB₄ measured in EBC and sputum supernatants in patients with different asthma phenotypes and pneumoconiosis using LC–MS techniques.

LT	Matrix	Subject group	n	Technique	Concentration	Ref.
Adult asthma						
LTB ₄	Sputum	Not defined/asthma	18	SPE-UHPLC–MS/MS	79–7220 pg/ml ^a	[21]
LTC ₄	Sputum	Not defined/asthma	18	SPE-UHPLC–MS/MS	Mostly not detected	[21]
LTD ₄	Sputum	Not defined/asthma	18	SPE-UHPLC–MS/MS	Mostly not detected	[21]
LTE ₄	Sputum	Not defined/asthma	18	SPE-UHPLC–MS/MS	11.9–891 pg/ml ^a	[21]
LTB ₄	EBC	Asthma not treated	4	LC–MS	>100 pg/ml	[23]
LTB ₄	EBC	Asthma treated	4	LC–MS	Not detected	[23]
LTB ₄	EBC	Healthy control	2	LC–MS	Not detected	[23]
LTC ₄	EBC	Occupational asthma	20	i.a.s.–LC–MS/MS	80.1 ± 6.2 pg/ml ^{b,c}	[27]
LTC ₄	EBC	Healthy control	20	i.a.s.–LC–MS/MS	57.4 ± 8.6 pg/ml ^b	[27]
LTD ₄	EBC	Occupational asthma	20	i.a.s.–LC–MS/MS	19.9 ± 4.3 pg/ml ^{b,d}	[27]
LTD ₄	EBC	Healthy control	20	i.a.s.–LC–MS/MS	13.8 ± 5.2 pg/ml ^b	[27]
LTE ₄	EBC	Occupational asthma	20	i.a.s.–LC–MS/MS	35.7 ± 4.8 pg/ml ^{b,c}	[27]
LTE ₄	EBC	Healthy control	20	i.a.s.–LC–MS/MS	25.3 ± 5.1 pg/ml ^b	[27]
Total Cys-LTs	EBC	Occupational asthma	20	i.a.s.–LC–MS/MS	133.2 ± 20.1 pg/ml ^{b,c}	[27]
Total Cys-LTs	EBC	Healthy control	20	i.a.s.–LC–MS/MS	77.8 ± 13.8 pg/ml ^b	[27]
LTC ₄	EBC	Occupational asthma	30	i.m.s.–UHPLC–MS/MS	62–101 pg/ml ^{b,e}	[39]
LTC ₄	EBC	Steroid-resistant asthma	32	i.m.s.–UHPLC–MS/MS	54–78 pg/ml ^b	[39]
LTC ₄	EBC	s.n.-moderate asthma	25	i.m.s.–UHPLC–MS/MS	45–83 pg/ml ^{b,f}	[39]
LTC ₄	EBC	s.t.-moderate asthma	28	i.m.s.–UHPLC–MS/MS	32–58 pg/ml ^b	[39]
LTC ₄	EBC	Healthy control	35	i.m.s.–UHPLC–MS/MS	23–37 pg/ml ^b	[39]
LTD ₄	EBC	Occupational asthma	30	i.m.s.–UHPLC–MS/MS	14–45 pg/ml ^{b,e}	[39]
LTD ₄	EBC	Steroid-resistant asthma	32	i.m.s.–UHPLC–MS/MS	18–43 pg/ml ^b	[39]
LTD ₄	EBC	s.n.-moderate asthma	25	i.m.s.–UHPLC–MS/MS	22–29 pg/ml ^b	[39]
LTD ₄	EBC	s.t.-moderate asthma	28	i.m.s.–UHPLC–MS/MS	18–25 pg/ml ^b	[39]
LTD ₄	EBC	Healthy control	35	i.m.s.–UHPLC–MS/MS	13–19 pg/ml ^b	[39]
LTE ₄	EBC	Occupational asthma	30	i.m.s.–UHPLC–MS/MS	43–126 pg/ml ^{b,e}	[39]
LTE ₄	EBC	Steroid-resistant asthma	32	i.m.s.–UHPLC–MS/MS	48–87 pg/ml ^{b,e}	[39]
LTE ₄	EBC	s.n.-moderate asthma	25	i.m.s.–UHPLC–MS/MS	54–93 pg/ml ^{b,e}	[39]
LTE ₄	EBC	s.t.-moderate asthma	28	i.m.s.–UHPLC–MS/MS	38–72 pg/ml ^b	[39]
LTE ₄	EBC	Healthy control	35	i.m.s.–UHPLC–MS/MS	34–48 pg/ml ^b	[39]
LTC ₄	EBC	AIA	62	HPLC–MS/MS	3.69 (2.39–7.03) ppm/PA ^{g,h}	[31]
LTC ₄	EBC	ATA	53	HPLC–MS/MS	5.59 (3.01–9.56) ppm/PA ^g	[31]
LTC ₄	EBC	Healthy control	38	HPLC–MS/MS	2.28 (0.81–5.43) ppm/PA ^g	[31]
LTD ₄	EBC	AIA	62	HPLC–MS/MS	1.93 (0.96–4.08) ppm/PA ^{g,i}	[31]
LTD ₄	EBC	ATA	53	HPLC–MS/MS	3.77 (2.09–6.94) ppm/PA ^g	[31]
LTD ₄	EBC	Healthy control	38	HPLC–MS/MS	1.80 (0.80–4.09) ppm/PA ^g	[31]
LTE ₄	EBC	AIA	62	HPLC–MS/MS	2.54 (1.37–4.66) ppm/PA ^{g,j}	[31]
LTE ₄	EBC	ATA	53	HPLC–MS/MS	4.52 (2.96–6.87) ppm/PA ^g	[31]
LTE ₄	EBC	Healthy control	38	HPLC–MS/MS	2.03 (1.39–4.05) ppm/PA ^g	[31]
LTC ₄	EBC	AIA pre-challenge	21	HPLC–MS/MS	9.22 (2.18–22.31) ppm/PA ^g	[43]
LTC ₄	EBC	AIA post-challenge	21	HPLC–MS/MS	6.61 (2.08–11.50) ppm/PA ^{g,k}	[43]
LTC ₄	EBC	ATA pre-challenge	23	HPLC–MS/MS	5.40 (2.79–10.84) ppm/PA ^g	[43]
LTC ₄	EBC	ATA post-challenge	23	HPLC–MS/MS	3.24 (1.65–5.84) ppm/PA ^{g,k}	[43]
LTE ₄	EBC	AIA pre-challenge	21	HPLC–MS/MS	5.19 (3.23–8.50) ppm/PA ^g	[43]
LTE ₄	EBC	AIA post-challenge	21	HPLC–MS/MS	6.38 (4.95–14.57) ppm/PA ^{g,l}	[43]
LTE ₄	EBC	ATA pre-challenge	23	HPLC–MS/MS	4.88 (3.10–7.76) ppm/PA ^g	[43]
LTE ₄	EBC	ATA post-challenge	23	HPLC–MS/MS	6.54 (4.42–7.59) ppm/PA ^{g,l}	[43]
LTB ₄	BAL	Atopic asthma pre-challenge	8	LC–ESI–MS/MS	72 pM (59%) ^m	[33]
LTB ₄	BAL	Atopic asthma post-challenge	8	LC–ESI–MS/MS	81 pM (53%) ^m	[33]
LTB ₄	BAL	Healthy control	10	LC–ESI–MS/MS	53 pM (71%) ^m	[33]
Childhood asthma						
LTB ₄	EBC	s.n.a.-asthma	25	LC–MS/MS	255.1 (175.0–314.7) pg ^{n,o}	[19]
LTB ₄	EBC	s.t.a.-asthma	22	LC–MS/MS	125.0 (25.0–245.0) pg ^{n,p}	[19]
LTB ₄	EBC	Atopic nonasthmatic	20	LC–MS/MS	96.5 (87.3–102.5) pg ⁿ	[19]
LTB ₄	EBC	Non-atopic control	15	LC–MS/MS	87.5 (82.5–102.5) pg ⁿ	[19]
LTC ₄	EBC	Severe asthma	27	HPLC–MS/MS	1.95 × 10 ^{−6} (1.32–2.87 × 10 ^{−6})/PA ^{q,r}	[44]
LTC ₄	EBC	s.n.-mild-to-moderate asthma	31	HPLC–MS/MS	1.61 × 10 ^{−6} (1.14–2.26 × 10 ^{−6})/PA ^{q,q}	[44]
LTC ₄	EBC	s.t.-mild-to-moderate asthma	49	HPLC–MS/MS	1.78 × 10 ^{−6} (1.37–2.31 × 10 ^{−6})/PA ^{q,r}	[44]
LTC ₄	EBC	Healthy control	24	HPLC–MS/MS	0.82 × 10 ^{−6} (0.54–1.24 × 10 ^{−6})/PA ^q	[44]
Pneumoconiosis						
LTD ₄	EBC	Silicosis	60	SPE–LC–MS/MS	21.1 ± 2.7 pg/ml ^{f,s}	[45]
LTD ₄	EBC	Healthy control	25	SPE–LC–MS/MS	14.7 ± 2.7 pg/ml ^s	[45]
LTD ₄	EBC	Asbestosis	45	SPE–LC–MS/MS	31.9 ± 1.8 pg/ml ^{f,t}	[42]
LTD ₄	EBC	Silicosis	37	SPE–LC–MS/MS	30.8 ± 1.8 pg/ml ^{f,t}	[42]
LTD ₄	EBC	Healthy control	27	SPE–LC–MS/MS	23.0 ± 1.8 pg/ml ^t	[42]
LTE ₄	EBC	Asbestosis	45	SPE–LC–MS/MS	72.6 ± 8.2 pg/ml ^{f,t}	[42]
LTE ₄	EBC	Silicosis	37	SPE–LC–MS/MS	69.8 ± 7.6 pg/ml ^{f,u}	[42]
LTE ₄	EBC	Healthy control	27	SPE–LC–MS/MS	54.1 ± 8.3 pg/ml ^t	[42]

Table 3 (Continued)

LT	Matrix	Subject group	n	Technique	Concentration	Ref.
Total CysLTs	EBC	Asbestosis	45	SPE-LC-MS/MS	192.4 ± 30.2 pg/ml ^{f,t}	[42]
Total CysLTs	EBC	Silicosis	37	SPE-LC-MS/MS	170.7 ± 9.5 pg/ml ^{e,t}	[42]
Total CysLTs	EBC	Healthy control	27	SPE-LC-MS/MS	143.3 ± 14.4 pg/ml ^t	[42]

Abbreviations: AIA: aspirin-insensitive asthma; ATA: aspirin-tolerant asthma; CysLT: cysteinyl-leukotriene; EBC: exhaled breath condensate; ESI: electrospray ionization; HPLC: high performance liquid chromatography; HPLC-MS/MS: high performance liquid chromatography-tandem mass spectrometry; i.a.s.: immunoaffinity separation; i.m.s.: immunomagnetic separation; LC-ESI-MS/MS: liquid chromatography-electrospray ionization-tandem mass spectrometry; LC-MS: liquid chromatography-mass spectrometry; LC-MS/MS: liquid chromatography-tandem mass spectrometry; LT, leukotriene; MS: mass spectrometry; PA: palmitic acid; SPE: solid phase extraction; s.n.: steroid naive; s.t.: steroid-treated; s.n.a.: steroid-naive atopic; s.t.a.: steroid-treated atopic; UHPLC: ultra high performance liquid chromatography; UHPLC-MS/MS: ultra high performance liquid chromatography-tandem mass spectrometry.

^a Values are expressed as range.

^b Values are expressed as 95% confidence interval.

^c Elevated compared with healthy control subjects. *P* values not provided.

^d Not significant compared with healthy control subjects. *P* values not provided.

^e *P* < 0.05 compared with healthy control subjects.

^f *P* < 0.001 compared with healthy control subjects.

^g Values are expressed as medians and 25th–75th interquartile range.

^h No significant difference compared with ATA asthma patients.

ⁱ *P* < 0.01 compared with ATA asthma patients.

^j *P* < 0.001 compared with ATA asthma patients.

^k Not significant compared with pre-challenge values.

^l *P* < 0.05 compared with pre-challenge values.

^m Values are expressed as medians and CV%.

ⁿ Values are expressed as medians and interquartile range expressed as the total amount (in pg) of eicosanoid exhaled in the 15-min breath test.

^o *P* < 0.001 compared with healthy non-atopic and atopic non-asthmatic children.

^p *P* < 0.01 compared with s.n.a. children with asthma.

^q Values are geometric means with 95% confidence intervals expressed as eicosanoid/PA ratios.

^r *P* < 0.01 compared with healthy control children.

^s Values are expressed as means ± uncertainty of experimentally determined value.

^t Values are expressed as arithmetic mean ± confidence interval.

^u *P* = 0.002 compared with healthy control subjects.

10 non-atopic healthy control subjects by LC-ESI-MS/MS [33]. BAL sampling was performed on all subjects at unprovoked baseline levels outside the birch pollen season and on patients with asthma following bronchoprovocation with birch pollen extract, corresponding to a final drop in FEV₁ ≥ 20% from the baseline value [33]. BAL concentrations of LTB₄ in healthy subjects [53 pM (71%), median and CV; healthy control group versus asthmatic control group, *P* = 0.35; healthy control group versus asthmatic following provocation group, *P* = 0.17], patients with asthma before provocation [72 pM (59%); asthmatic control group versus asthmatic following provocation group, *P* = 0.44], and patients with asthma following provocation [81 pM (53%)] were similar [33]. BAL 6-*trans*-LTB₄ concentrations in healthy subjects [22 pM (56%), median and CV] as compared with patients with asthma before provocation [32 pM (58%), *P* = 0.18] and in patients with asthma before provocation as compared with patients with asthma following provocation [39 pM (45%), *P* = 0.051] were similar. Patients with asthma following provocation had higher BAL 6-*trans*-LTB₄ concentrations than healthy subjects (*P* = 0.030) [33]. A significantly elevated trend in the three groups was observed for both LTB₄ (*P* = 0.02) and 6-*trans*-LTB₄ (*P* = 0.03) [33].

3.1.4. Urine

An HPLC-MS method was used for measuring LTE₄ concentrations in morning urinary samples from 50 adult healthy subjects [21 males, mean age 31 (range 21–60) years] and 124 patients with asthma [46 males, mean age 44 (21–66) years] [37]. Forty-one patients (18 males) had aspirin-intolerant asthma confirmed by the aspirin challenge test. Urinary LTE₄ values were expressed as pg/mg of creatinine. There was a gender difference in urinary LTE₄ excretion, as the latter was twice in females [42.4 (95% CI) 31.1–53.8 pg/mg creatinine] than in males [23.7 (18.5–28.9) pg/mg creatinine, *P* = 0.01] [37]. Compared with healthy subjects (value for the whole healthy control group not provided), urinary LTE₄ was elevated in the whole group of patients with asthma [345.0 (210–498) pg/mg creatinine] and, to a greater extent, in the subgroup of patients

with aspirin-intolerant asthma [808.2 (415.5–1201) pg/mg creatinine] [37]. At the threshold of 48.6 pg/mg creatinine, classification rate between asthma and healthy subjects was 81.9% (sensitivity: 80.6%; specificity: 63.2%), whereas discriminating capacity between aspirin-intolerant and aspirin-tolerant asthma patients was 82.6% (threshold: 71 pg/mg creatinine; sensitivity: 91.3%; specificity: 44.3%) [37].

A LC-MS/MS technique was used for measuring urinary LTE₄ levels in 10 children with asthma and 39 healthy control children and compared with EIA [35]. Following normalization to creatinine, EIA LTE₄ values in urinary samples from 10 asthma patients were up to two orders of magnitude higher [639–5685 pg/mg creatinine with CV ranging from 2.60% to 26.42% (average 14.34%)] than those measured by LC-MS/MS [29–143 pg/mg creatinine with CV ranging from 0.44% to 7.36% (average 2.99%)] [35]. This discrepancy might be partially explained by the fact that affinity purification before urinary LTE₄ measurement by EIA was not performed. However, a wide range of CV (4.2–75%) and percent recoveries (2.2–182.7%) is observed even when various purification methods are used before EIA [35]. No significant correlation was observed between LC-MS/MS versus EIA used for analyzing fresh and frozen urinary samples (−0.1906 and −0.1182 respectively). In 10 healthy control adults, urinary LTE₄ values measured by LC-MS/MS ranged from <17.2 to 63.0 pg/mg creatinine and averaged 36.6 pg/mg creatinine with an average CV of 9.20%. In 39 healthy control children, urinary LTE₄ values measured by LC-MS/MS ranged from 9.0 to 115.1 pg/mg creatinine and averaged 50.7 pg/mg creatinine with an average CV of 5.1%. Urinary LTE₄ values in adults and children were similar (*P* < 0.12) [35].

A platform composed of four LC-MS/MS methods was developed to quantify 26 lipid mediators, including CysLTs and LTB₄, in urinary samples from 16 atopic patients with asthma under allergen challenge. After allergen challenge, urinary excretion of LTE₄ (14.0 ± 11.4 ng/mmol creatinine, mean ± SD; *P* < 0.05), which reflects total CysLTs production, increased over baseline values (4.50 ± 3.37 ng/mmol creatinine) in patients with atopic asthma [38].

Table 4Concentrations of CysLTs and LT_{B4} measured in plasma and urine in patients with different asthma phenotypes and pneumoconiosis using LC–MS techniques.

LT	Matrix	Subject group	n	Technique	Concentration	Ref.
LTC ₄	Plasma	Occupational asthma	30	i.m.s.-UHPLC–MS/MS	53–108 pg/ml ^{a,d}	[39]
LTC ₄	Plasma	Steroid-resistant asthma	32	i.m.s.-UHPLC–MS/MS	54–84 pg/ml ^{a,b}	[39]
LTC ₄	Plasma	s.n.-moderate asthma	25	i.m.s.-UHPLC–MS/MS	54–86 pg/ml ^{a,c}	[39]
LTC ₄	Plasma	s.t.-moderate asthma	28	i.m.s.-UHPLC–MS/MS	52–77 pg/ml ^{a,d}	[39]
LTC ₄	Plasma	Healthy control	35	i.m.s.-UHPLC–MS/MS	49–63 pg/ml ^a	[39]
LTD ₄	Plasma	Occupational asthma	30	i.m.s.-UHPLC–MS/MS	21–38 pg/ml ^{a,b}	[39]
LTD ₄	Plasma	Steroid-resistant asthma	32	i.m.s.-UHPLC–MS/MS	20–33 pg/ml ^{a,c}	[39]
LTD ₄	Plasma	s.n.-moderate asthma	25	i.m.s.-UHPLC–MS/MS	25–32 pg/ml ^{a,d}	[39]
LTD ₄	Plasma	s.t.-moderate asthma	28	i.m.s.-UHPLC–MS/MS	24–28 pg/ml ^{a,d}	[39]
LTD ₄	Plasma	Healthy control	35	i.m.s.-UHPLC–MS/MS	23–27 pg/ml ^a	[39]
LTE ₄	Plasma	Occupational asthma	30	i.m.s.-UHPLC–MS/MS	42–58 pg/ml ^{a,d}	[39]
LTE ₄	Plasma	Steroid-resistant asthma	32	i.m.s.-UHPLC–MS/MS	41–55 pg/ml ^{a,d}	[39]
LTE ₄	Plasma	s.n.-moderate asthma	25	i.m.s.-UHPLC–MS/MS	42–52 pg/ml ^{a,d}	[39]
LTE ₄	Plasma	s.t.-moderate asthma	28	i.m.s.-UHPLC–MS/MS	41–47 pg/ml ^{a,d}	[39]
LTE ₄	Plasma	Healthy control	35	i.m.s.-UHPLC–MS/MS	37–45 pg/ml ^a	[39]
LTB ₄	Plasma	Asbestosis	45	SPE-LC–MS/MS	36.6 ± 3.8 pg/ml ^{f,e}	[42]
LTB ₄	Plasma	Silicosis	37	SPE-LC–MS/MS	33.5 ± 3.7 pg/ml ^{f,b}	[42]
LTB ₄	Plasma	Healthy control	27	SPE-LC–MS/MS	26.9 ± 3.3 pg/ml ^f	[42]
LTE ₄	Plasma	Asbestosis	45	SPE-LC–MS/MS	47.1 ± 4.8 pg/ml ^{f,b}	[42]
LTE ₄	Plasma	Silicosis	37	SPE-LC–MS/MS	44.3 ± 4.4 pg/ml ^f	[42]
LTE ₄	Plasma	Healthy control	27	SPE-LC–MS/MS	37.7 ± 3.3 pg/ml ^f	[42]
LTE ₄	Urine	Atopic asthma pre-challenge	16	LC–MS/MS	14.0 ± 11.4 ng/mmol creatinine ^{c,g}	[38]
LTE ₄	Urine	Atopic asthma post-challenge	16	LC–MS/MS	4.50 ± 3.37 ng/mmol creatinine ^g	[38]
LTC ₄	Urine	Occupational asthma	30	i.m.s.-UHPLC–MS/MS	11–19 pg/mg creatinine ^{a,d}	[39]
LTC ₄	Urine	Steroid-resistant asthma	32	i.m.s.-UHPLC–MS/MS	13–21 pg/mg creatinine ^{a,d}	[39]
LTC ₄	Urine	s.n.-moderate asthma	25	i.m.s.-UHPLC–MS/MS	11–16 pg/mg creatinine ^{a,d}	[39]
LTC ₄	Urine	s.t.-moderate asthma	28	i.m.s.-UHPLC–MS/MS	12–15 pg/mg creatinine ^{a,d}	[39]
LTC ₄	Urine	Healthy control	35	i.m.s.-UHPLC–MS/MS	9–13 pg/mg creatinine ^a	[39]
LTD ₄	Urine	Occupational asthma	30	i.m.s.-UHPLC–MS/MS	9–17 pg/mg creatinine ^{a,d}	[39]
LTD ₄	Urine	Steroid-resistant asthma	32	i.m.s.-UHPLC–MS/MS	10–17 pg/mg creatinine ^{a,d}	[39]
LTD ₄	Urine	s.n.-moderate asthma	25	i.m.s.-UHPLC–MS/MS	11–15 pg/mg creatinine ^{a,d}	[39]
LTD ₄	Urine	s.t.-moderate asthma	28	i.m.s.-UHPLC–MS/MS	10–14 pg/mg creatinine ^{a,d}	[39]
LTD ₄	Urine	Healthy control	35	i.m.s.-UHPLC–MS/MS	9–13 pg/mg creatinine ^a	[39]
LTE ₄	Urine	Occupational asthma	30	i.m.s.-UHPLC–MS/MS	25–44 pg/mg creatinine ^{a,d}	[39]
LTE ₄	Urine	Steroid-resistant asthma	32	i.m.s.-UHPLC–MS/MS	23–48 pg/mg creatinine ^{a,d}	[39]
LTE ₄	Urine	s.n.-moderate asthma	25	i.m.s.-UHPLC–MS/MS	26–39 pg/mg creatinine ^{a,d}	[39]
LTE ₄	Urine	s.t.-moderate asthma	28	i.m.s.-UHPLC–MS/MS	25–34 pg/mg creatinine ^{a,d}	[39]
LTE ₄	Urine	Healthy control	35	i.m.s.-UHPLC–MS/MS	22–31 pg/mg creatinine ^a	[39]
LTD ₄	Urine	Asbestosis	45	SPE-LC–MS/MS	18.9 ± 3.0 pg/mmol creatinine ^{f,h}	[42]
LTD ₄	Urine	Silicosis	37	SPE-LC–MS/MS	16.3 ± 3.4 pg/mmol creatinine ^f	[42]
LTD ₄	Urine	Healthy control	27	SPE-LC–MS/MS	14.2 ± 4.4 pg/mmol creatinine ^f	[42]

Abbreviations: CysLT: cysteinyl-leukotriene; HPLC: high performance liquid chromatography; i.a.s.: immunoaffinity separation; i.m.s.: immunomagnetic separation; LC-MS: liquid chromatography-mass spectrometry; LC-MS/MS: liquid chromatography-tandem mass spectrometry; LT: leukotriene; MS: mass spectrometry; s.n.: steroid naïve; SPE: solid phase extraction; s.t.: steroid-treated; UHPLC: ultra high performance liquid chromatography; UHPLC-MS/MS: ultra high performance liquid chromatography-tandem mass spectrometry.

^a Values are expressed as 95% confidence interval.

^b $P < 0.01$ compared with healthy control subjects.

^c $P < 0.05$ compared with healthy control subjects.

^d Not significantly different from healthy control subjects.

^e $P < 0.001$ compared with healthy control subjects.

^f Values are expressed as arithmetic mean ± 95% confidence interval.

^g Values are expressed as mean ± SD.

^h $P < 0.03$ compared with healthy control subjects.

3.1.5. EBC, plasma, urine

A method combining immunomagnetic molecular separation with UHPLC–ESI-MS/MS was applied to CysLT measurement in EBC, plasma, and urine [39]. Samples were obtained from 30 patients with mild to severe occupational asthma, 32 patients with steroid-resistant or difficult asthma, 25 steroid-naïve patients with moderate asthma, 28 patients with moderate asthma who were being treated with corticosteroids, and 35 healthy non-smokers [39]. EBC samples were collected with a commercially available condenser (Ecoscreen, Jaeger, Hoechberg, Germany) as described previously in the text. Compared with healthy subjects [23–37 pg/ml (95% CI)], LTC₄ concentrations in EBC were increased in steroid-naïve patients with moderate asthma (45–83 pg/ml, $P < 0.001$) and in patients with occupational asthma (62–101 pg/ml, $P < 0.05$) [39]. LTE₄ was elevated in patients with occupational asthma (43–126 pg/ml, $P < 0.05$), steroid-resistant asthma (48–87 pg/ml, $P < 0.05$), and moderate asthma who were not being

treated with ICS (54–93 pg/ml, $P < 0.05$) as compared with healthy subjects [39]. Compared with healthy subjects (13–19 pg/ml), EBC LTD₄ concentrations were elevated patients with steroid-resistant asthma (18–43 pg/ml, $P < 0.05$). Compared with healthy subjects (LTC₄: 49–63 pg/ml; LTD₄: 23–27 pg/ml), plasma LTC₄ concentrations were higher in patients with steroid-resistant asthma (54–84 pg/ml, $P < 0.01$) and steroid-naïve patients with moderate asthma (54–86 pg/ml, $P < 0.05$), LTD₄ was elevated in patients with occupational asthma (21–38 pg/ml, $P < 0.01$) and steroid-resistant asthma (20–33 pg/ml, $P < 0.05$), whereas plasma LTE₄ and total CysLTs concentrations were similar in the different study groups [39]. There was no difference in urinary LTC₄, LTD₄, and total CysLTs concentrations between asthma phenotypes and healthy control group [39]. The observed statistically significant differences in EBC and plasma CysLT concentrations between asthma patients and healthy subjects were relatively small [39]. The greatest differences in CysLT concentrations between asthma phenotypes and

healthy subjects were observed in EBC, particularly for sums of CysLTs [39].

3.2. Measurement of LTs in children with asthma

3.2.1. EBC

In a cross-sectional study, LC–MS/MS was used to measure LTB₄ in EBC in 25 steroid-naïve atopic children with asthma, 22 atopic children with asthma who were being treated with inhaled fluticasone (10 at a dose of 100 µg/day, 12 at a dose of 200 µg/day), 20 atopic non-asthmatic children and 15 healthy non-atopic control children [19]. LTB₄ in EBC was detected in all study samples, but in 7 atopic asthmatic children who were being treated with high daily dose (200 µg) of inhaled fluticasone [19].

Compared with healthy children [87.5 (82.5–102.5) pg, median and interquartile range], LTB₄ values, expressed as the total amount (in pg) of eicosanoid exhaled in the 15-min breath test, were increased in steroid-naïve atopic asthmatic [255.1 (175.0–314.7) pg, $P < 0.001$], but not in atopic nonasthmatic children [96.5 (87.3–102.5) pg, $P = 0.59$] [19]. Asthmatic children who were receiving ICS had lower concentrations of LTB₄ in EBC [125.0 (25.0–245.0) pg, $P < 0.01$] than steroid-naïve asthmatics [255.1 (175.0–314.7) pg] [19]. This study shows that LTB₄ in EBC is selectively elevated in steroid-naïve atopic children with asthma, but not in atopic non-asthmatic children. Among children who were being treated with ICS, those who were on a lower daily dose of fluticasone (100 µg) ($n = 10$) had higher exhaled LTB₄ values than those who were on a higher daily dose of fluticasone (200 µg) ($n = 12$, $P < 0.002$) [19]. Although placebo control studies are required, treatment with ICS seems to reduce exhaled LTB₄ in asthmatic children. LC–MS/MS analysis of LTB₄ in EBC might provide a non-invasive, sensitive, and quantitative method for assessment of airway inflammation and response to pharmacological treatment in children with asthma.

Using an LC–MS/MS technique as described above [30], EBC LTB₄, LTC₄, LTD₄, and LTE₄ were measured in 27 school-aged children with severe asthma, 80 children with mild-to-moderate asthma, and 24 healthy children [44]. LT values were expressed as both concentrations in pg/ml and eicosanoid/palmitic acid ratios to adjust for EBC dilution. Compared with healthy children (0.82 ppm [95% CI, 0.54–1.24 ppm]), children with asthma had higher EBC LTC₄/palmitic acid ratio (1.76 ppm [95% CI, 1.48–2.11 ppm], $P < 0.001$) [44]. LTC₄/palmitic acid ratio was higher in children with severe asthma (1.95 ppm [95% CI, 1.32–2.87 ppm], $P = 0.008$) and in children with mild-to-moderate asthma with (1.78 ppm [95% CI, 1.37–2.31 ppm], $P = 0.007$) and without (1.61 ppm [95% CI, 1.14–2.26 ppm], $P = 0.048$) regular ICS treatment than in healthy children (0.82 ppm [95% CI, 0.54–1.24 ppm]). Similar LTC₄ values were observed in children with different severity of asthma [38]. EBC LTE₄ concentrations ($P = 0.01$) and LTE₄/palmitic acid ratio ($P = 0.046$) were elevated in children with AHR as compared with those without AHR [44].

3.3. Measurement of LTs in pneumoconiosis

A method combining immunoseparation with LC–ESI-MS/MS was used for measuring CysLTs and LTB₄ concentrations in EBC in 60 patients with silicosis (mean exposure to silica 23.6 ± 2.5 years) and 25 healthy control subjects [45]. EBC was collected with an Ecoscreen condenser. Compared with healthy subjects (14.7 ± 2.7 pg/ml, means $\pm$ uncertainty of experimentally determined value), exhaled LTD₄ concentrations were increased in patients with silicosis (21.1 ± 2.7 pg/ml, $P < 0.001$), whereas there was no between-group difference in LTC₄ ($P = 0.416$), LTE₄ ($P = 0.145$) and LTB₄ ($P = 0.529$) concentrations [44].

Another study was performed by the same group to establish which biological matrix (EBC, plasma and urine) could be

more suitable for assessing respiratory inflammation in patients with pneumoconiosis [42]. Forty-five patients with asbestosis, 37 patients with silicosis and 27 healthy control subjects were studied. CysLTs and LTB₄ concentrations were measured in EBC, plasma and urine using LC–ESI-MS/MS after SPE [42].

Compared with healthy subjects (LTD₄: 23.0 ± 1.8 pg/ml, mean $\pm$ 95% CI; LTE₄: 54.1 ± 8.3 pg/ml; total CysLTs: 147.3 ± 14.4 pg/ml), EBC concentrations of LTD₄, LTE₄ and total CysLTs were elevated in patients with asbestosis (LTD₄: 31.9 ± 1.8 pg/ml, $P < 0.001$; LTE₄: 72.6 ± 8.2 pg/ml, $P < 0.001$; total CysLTs: 192.4 ± 30.2 pg/ml, $P < 0.001$) and silicosis (LTD₄: 30.8 ± 1.8 pg/ml, $P < 0.001$; LTE₄: 69.8 ± 7.6 pg/ml, $P = 0.002$; total CysLTs: 170.7 ± 9.5 pg/ml, $P = 0.036$), whereas EBC LTC₄ concentrations were similar in the three study groups [42]. Patients with asbestosis and silicosis had similar values of exhaled LTD₄ and LTE₄ [42]. Patients with asbestosis had higher exhaled LTB₄ concentrations (26.5 ± 4.6 pg/ml) than patients with silicosis (19.0 ± 1.8 pg/ml, $P < 0.001$) and healthy subjects (20.3 ± 2.0 pg/ml, $P < 0.001$). Patients with more severe signs of asbestosis and reduction in pulmonary function had higher total CysLT and LTC₄ concentrations in EBC than patients with mild asbestosis ($P < 0.05$). In patients with asbestosis, total CysLTs and LTC₄ concentrations in EBC were negatively correlated with total lung capacity (total CysLTs: -0.313 , $P < 0.05$; LTC₄: -0.358 , $P < 0.05$) [42].

Plasma LTB₄ concentrations were increased in patients with asbestosis (36.6 ± 3.8 pg/ml, mean $\pm$ 95% CI, $P < 0.001$) and silicosis (33.5 ± 3.7 pg/ml, $P = 0.008$) compared with healthy subjects (26.9 ± 3.3 pg/ml). Compared with healthy subjects (37.7 ± 3.3 pg/ml), plasma LTE₄ concentrations were elevated in patients with asbestosis (47.1 ± 4.8 pg/ml, $P = 0.009$), whereas plasma LTC₄, LTD₄ and total CysLTs concentrations were similar in the three study groups [42].

Urinary LTD₄, but not LTB₄, LTC₄, LTE₄ and total CysLT concentrations, were elevated in patients with asbestosis (18.9 ± 3.0 pg/mmol creatinine, $P = 0.023$) compared with healthy subjects (14.2 ± 4.4 pg/mmol creatinine) [42]. Based on these finding, EBC seems to be the biological matrix of choice for LT analysis in patients with pneumoconiosis.

4. Limitations and shortcomings

Measurement of LTs in biological fluids is an attractive approach for exploring the pathophysiological role of these important inflammatory mediators in respiratory diseases, but has to be carefully addressed from the analytical standpoint. First of all, the choice of the analytical technique is central. There is a huge discrepancy between immunoassay- and MS-based methodologies for the quantitative analysis of LTs in biological fluids as about 80% of the studies used immunoassay techniques. These can overestimate LT concentrations as reported in a study comparing EIA and LC–MS/MS methods which showed up to two order of magnitude higher concentrations of urinary LTE₄ when EIA is used [35].

LC–MS/MS is considered the gold standard technique for measuring LT concentrations in biological fluids. However, this technique has several potential limitations. Analysis of LTs in biological fluids by LC–selected ion monitoring/low resolution MS is unlikely to be of any practical value due to the potential for the co-elution on LC of interfering substances.

For some, but not all, compounds under study in various papers cited here, commercially available deuterium stable-isotope labelled internal standards were used. The use of stable-isotope labelled internal standard is critical and absolutely necessary to eliminate the “relative” matrix effect in complex biological systems. However, in some of these papers only selected analytes were paired with their respective stable-isotope labelled internal standards. In

addition, the position of the deuterium labels in deuterated internal standards is very important for the methods validity. Deuterium atoms need to be placed preferably in a non-exchangeable position in molecules under study. In a number of papers, the apparent lack of compensation for the “relative” matrix effect, in spite of the use of deuterated internal standard, was due to the exchange of the deuterium atom(s) during sample preparation. Thus, the position of deuterium atoms in the labelled molecules should be clearly specified to assess the potential for the exchange.

To demonstrate the selectivity of methods where a number of analytes are determined simultaneously including several internal standards, “cross-talk” between all these analytes has to be excluded by injecting separately each analyte at the upper LOQ and monitoring the MS/MS response in all other analyte channels at the lower LOQ (see for example Ref. [32] where 39 analytes, including LTB₄, were determined simultaneously).

The stability and chemical integrity of all analytes, including internal standards, during sample preparation and analyses has to be confirmed by processing each individual compound and monitoring all mass transitions for all other analytes. This is especially critical in view of the chemical instability of some LTs. Yet, this is not done in the majority of methods for multiple analytes.

For the overall assessment of assay validity, it is important to define how the extraction recovery for the individual analytes in various studies was determined. Assay recoveries (if determined) are often determined erroneously by comparing the MS/MS response of samples spiked before extraction into biological matrix with the same response of standards injected directly instead of standards spiked into matrix extracts.

In order to demonstrate the absence of matrix effect for all analytes, it is recommended to determine slopes of the standard lines in at least five different sources of a biological matrix and confirm that they are not different than 3–4% from each other [46]. Such assessment was not done in most of the studies cited in this review.

The selectivity of all methods in the presence of metabolites has to be established. As extensively demonstrated in the literature, the selectivity of the HPLC–MS and HPLC–MS/MS methods in the presence of metabolites is not guaranteed as metabolites may affect the selectivity of the MS/MS approach. Due to in-source fragmentation of metabolites in the MS interface, metabolites may interfere with the quantification of a parent molecule and/or its internal standard. The evaluation of metabolite interference may be done, for example, by analyzing post-dose samples under more selective chromatographic conditions without the presence of internal standard and other analytes, and by monitoring mass transitions for the LT and the corresponding internal standard. The matrix effect, also due to endogenous metabolites, may be especially severe when a limited sample clean-up is utilized, a relatively poor retention of analytes on HPLC column is provided, an electrospray interface is used, and homologues, i.e., non-stable isotope labelled compounds, are used as internal standards. All these for negatively contributing factors were present in the majority of the methods reviewed in this article.

5. Conclusions

Several internally validated LC–MS and LC–MS/MS techniques for measuring LTs concentrations in different biological matrices have been developed over the past years. Most of these techniques involve non-invasive sampling which is suitable for repeated measures as required in longitudinal studies. Studies aiming at external validation of LC–MS/MS methods for measuring LTs in patients with respiratory disease are warranted. Establishing common analytical protocols for LTs measurement would facilitate between-laboratory data comparison and interpretation of

results of future studies. Exhaled breath analysis using electronic noses [47,48], which are chemical sensor arrays for the detection of volatile molecules, and nitric oxide (NO) analysers [49] along with measurement of semi-volatile compounds in EBC using nuclear magnetic resonance (NMR) spectroscopy [50,51] and/or GC–MS or GC–MS/MS is required for a more comprehensive breathomics approach to non-invasive assessment of respiratory inflammation.

Measurement of LTs in biological fluids using MS techniques, including analytical platforms for detecting several lipid mediators at the same time, might provide a powerful tool for exploring the role of these potent inflammatory mediators in the pathophysiology of asthma and other respiratory diseases, and for assessing airway inflammation. This approach might also be useful for monitoring the anti-inflammatory effects of drugs and help identify phenotypes of asthma patients who are most likely to respond to LT receptor antagonists. More generally, LC–MS/MS measurement of LTs in biological fluids might provide a molecular basis for the implementation of personalized pharmacotherapeutic strategies in patients with respiratory disease.

Nomenclature

AHR	airway hyper-responsiveness
APCI	atmospheric pressure chemical ionization
BAL	bronchoalveolar lavage
CI	confidence interval
CV	coefficient of variation
CysLTs	cysteinyl-leukotrienes
EBC	exhaled breath condensate
EIA	enzyme immunoassay
ESI	electrospray ionization
FEV ₁	forced expiratory volume in 1 s
GC–MS	gas chromatography–mass spectrometry
GC–MS/MS	gas chromatography–tandem mass spectrometry
15(S)-HETE-d ₈	deuterated 15(S)-hydroxy-eicosatetraenoic acid
HPLC	high performance liquid chromatography
HPLC–MS	high performance liquid chromatography–mass spectrometry
HPLC–MS/MS	high performance liquid chromatography–tandem mass spectrometry
ICS	inhaled corticosteroids
i.n.	intra-nasal
i.p.	intra-peritoneal
LC	liquid chromatography
LC–ESI–MS/MS	liquid chromatography–electrospray ionization–tandem mass spectrometry
LC–MS	liquid chromatography–mass spectrometry
LC–MS/MS	liquid chromatography–tandem mass spectrometry
5-LO	5-lipoxygenase
LOD	limit of detection
LOQ	limit of quantification
LPS	lipopolysaccharide
LT	leukotriene
LTA ₄	leukotriene A ₄
LTB ₄	leukotriene B ₄
LTB ₄ -d ₄	tetradeutero-leukotriene B ₄
LTC ₄	leukotriene C ₄
LTC ₄ -d ₅	pentadeutero-leukotriene C ₄
LTD ₄	leukotriene D ₄
LTD ₄ -d ₅	pentadeutero-leukotriene D ₄
LTE ₄	leukotriene E ₄
LTE ₄ -d ₃	trideutero-leukotriene E ₄
MS	mass spectrometry
MS/MS	tandem mass spectrometry
PBS	phosphate buffered saline

QC quality control
RSD relative standard deviation
SD standard deviation
S/N signal-to-noise ratio
SRM selected-reaction monitoring
SPE solid-phase extraction
11-dehydro-TXB₂-d₄ tetradeutero-11-dehydro-thromboxane B₂
TXB₂-d₄ tetradeutero-thromboxane B₂
UHPLC–ESI-MS/MS ultra high performance liquid chromatography–electrospray ionization-tandem mass spectrometry
UHPLC–MS/MS ultra high performance liquid chromatography–tandem mass spectrometry
UP-LC ultra performance-liquid chromatography

Acknowledgement

This study was supported by Catholic University of the Sacred Heart, Fondi di Ateneo 2010–2013.

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