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Determination of cysteinyl leukotrienes in exhaled breath condensate: Method combining immunoseparation with LC–ESI-MS/MS

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ABSTRACT

A rapid and precise method for the identification and quantification of cysteinyl leukotrienes (leukotriene C_4, leukotriene D_4 and leukotriene E_4), essential markers of bronchial asthma, in exhaled breath condensate was developed. The protocol consists of immunoaffinity separation and a detection step, liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). In particular, the selected reaction monitoring mode was used for its extremely high degree of selectivity and the stable-isotope-dilution assay for its high precision of quantification. The developed method was characterized with a high precision (≤7.7%, determined as RSD), an acceptable accuracy (90.4–93.7%, determined as recovery), a low limit of detection (≤2 pg/ml EBC) and a low limit of quantification (≤10 pg/ml EBC). It was compared to other simple, clinically appropriate combinations of pre-treatment methods (solid phase extraction and lyophilization) with LC/MS. Finally, the method (a combination of immunoaffinity separation with LC–MS) was successfully tested in a clinical study where a significant difference was found in the concentration levels of cysteinyl leukotrienes between patients with occupational bronchial asthma and healthy subjects.

1. Introduction

The analysis of exhaled breath condensate (EBC) is a relatively novel method with a good potential to become the preferred and completely non-invasive alternative to the currently practiced invasive (open lung biopsy [1–4], bronchoalveolar lavage [3–5]) and *semi*-invasive (method of induced sputum [6–8]) diagnostic methods for bronchial asthma. Early diagnosis of this life-threatening disease is essential to allow a physician to initiate an effective therapy and minimize harm to the patient. EBC is a liquid matrix that reflects the particular composition of the bronchoalveolar extracellular lung liquid, which replicates the conditions proceeding directly in the lungs and airways. The substances are contained in the exhaled breath in both gaseous and liquid phases (aerosol form). The aerosol particles and the substances present in the gaseous phase could be condensed by breathing over a condenser, which is readily available at specialized clinical facilities. In the obtained liquid, typically known as EBC, more than 1000 compounds have been identified so far, out of which a substantial number are considered to represent sensitive biomarkers of lung diseases (leukotriene B_4 – LTB_4, cysteinyl leukotrienes C_4, D_4 and E_4 – LTC_4, LTD_4, LTE_4, 8-isoprostane, malondialdehyde, 4-hydroxyhexenal, 4-hydroxynonenal, 8–hydroxyguanine, 8-hydroxyguanosine, 8-hydroxy-2′-deoxyguanosine, hydroxymethyluracil, *o*-tyrosine, nitrotyrosine and others) [9–11]. Based on the determination of their content in EBC, the type of ongoing pathological process, the severity of the disorder and the efficiency of a therapeutic procedure etc. can be assessed. In the case of bronchial asthma, cysteinyl leukotrienes (cys LTs) represent a specific group of biomarkers, whose concentration level is significantly elevated in airways and lungs as a result of an ongoing allergic reaction (e.g. aspirin-induced asthma) [12,13]. Leukotrienes are metabolites of arachidonic acid, which is present as phospholipid in cellular membranes [9,14,15]. The enzyme 5-lipooxygenase transforms arachidonic acid into an unstable epoxide, leukotriene A_4 (LTA_4), which can be further transformed by one of two possible enzymatic pathways (Fig. 1). During inflammation, the levels of LTB_4 are elevated by the action of LTA_4 hydrolase, while the second pathway is dominant during allergic reactions [16]. The first member of the family of cys LTs, LTC_4, is produced by LTC_4 synthetase. The next members of the series of cys LTs (LTD_4 and LTE_4) are formed by a gradual transformation occurring sequentially from $LTC_4 \rightarrow LTD_4 \rightarrow LTE_4$ by a consecutive action of the enzymes γ-glutamyltranspeptidase ($LTC_4 \rightarrow LTD_4$)

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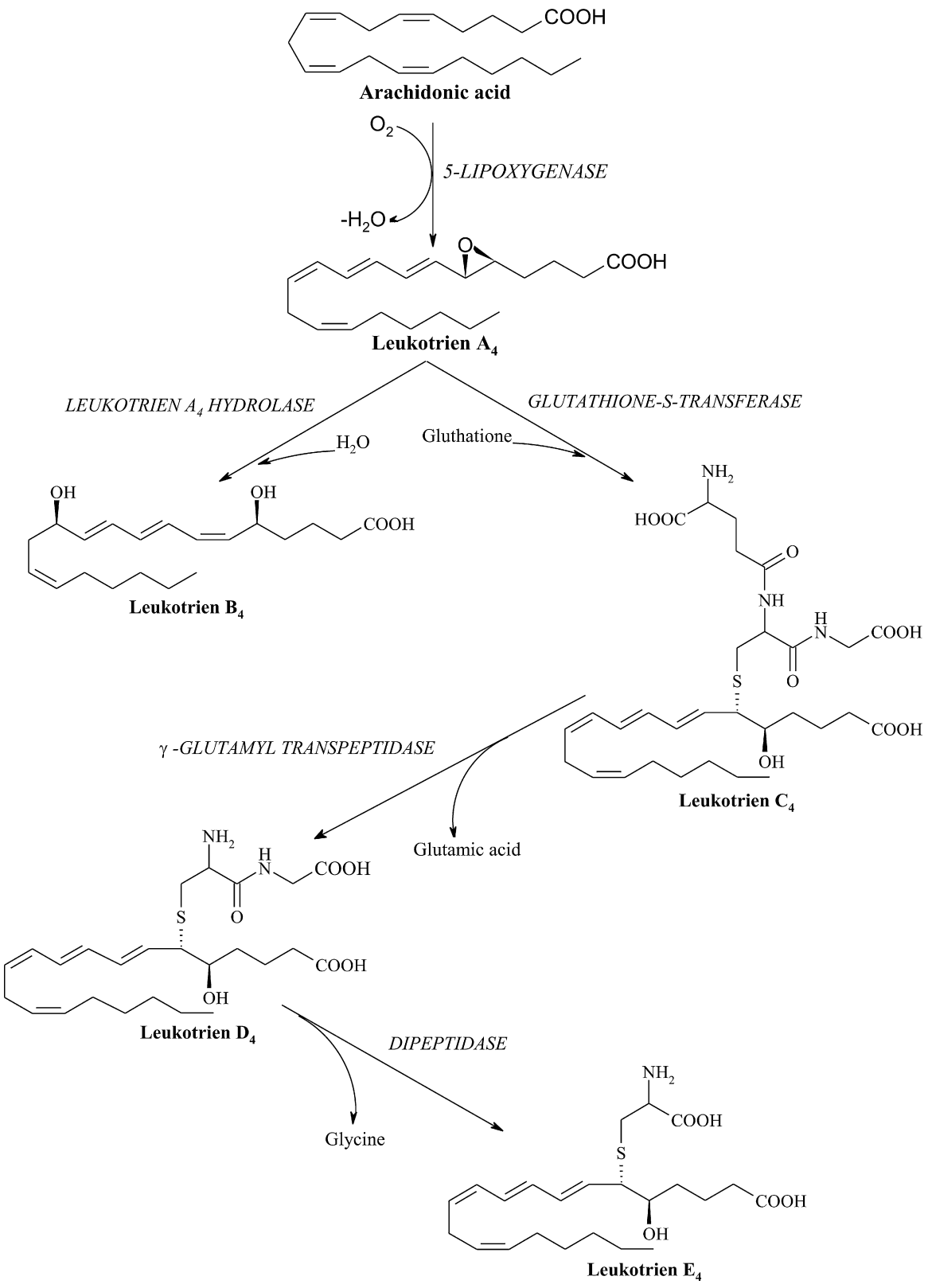

Fig. 1. Scheme of biosynthesis of leukotrienes *in vivo*.

and dipeptidase ($LTD_4 \rightarrow LTE_4$) [15,17]. The produced cys LTs interact with cys LTs receptors that are mainly found on the cells of smooth muscles, eosinophils and various other cells in the organism. Binding of cys LTs to cys LTs-1 receptors, localized in lungs and airways, cause bronchial and bronchiolar constriction and hyperaemia followed by tissue oedema and an excessive secretion of viscous mucus resulting in repeated episodic states of expiratory dyspnoea (breathlessness).

Several publications describe the determination of cys LTs in different body fluids (blood plasma [18,19], urine [20–22] and

EBC [10,16,23–25]) using various analytical methods including RIA (Radioimmunoassay) [20], EIA (Enzyme Immunoassay) [8,21–24], ELISA (Enzyme Linked Immunosorbent Assay) [25,26], GC–MS [10,27] or LC–MS [10,14,27–29]. In general, biochemical methods have demonstrated a lower-order detection limit than those commonly achieved by current mass-spectrometric methods (GC–MS, LC–MS). However, the LOD of the latter instrumental methods have dropped to pico-, femto- or even attomolar levels during the last decade, sufficient for the detection of most biologically active substances in humans. Physiological concentrations of cys LTs in EBC range between 10 and 200 pg/ml of EBC. In comparison with biochemical methods, connecting liquid or gas chromatography to mass spectrometry not only offers precise quantitative data but also structural information (qualitative information). Employing the MS^n techniques offers the development of highly specific methods, inherently eliminating false positive/negative results. This is in contrast to immunochemical or enzymatic biochemical methods, whose design in principle allows cross reactivity leading to an occurrence of these errors.The present work deals with the development of a highly selective and precise analytical method for the simultaneous determination of cys LTs (LTC_4, LTD_4, LTE_4) present in EBC. The method combines the pre-concentration and highly specific separation of cys LTs using immunoaffinity separation (IAS) with a highly selective detection method (LC–ESI-MS/MS). During the method development, three different pre-treatments were tested (IAS, solid phase extraction (SPE), lyophilization (LYO)) and compared. These pre-treatment methods were chosen with regard to their delicacy that respects the limited cys LTs stability which has also been extensively tested. Also other crucial parameters influencing the cys LTs determination, from EBC withdrawing over sample handling and preparation to LC–MS concentration value determination were evaluated and their impact on the quality of the results obtained was expressed. It was confirmed that, when cys LTs are used as biomarkers of bronchial asthma and are determined by the described method, they have the potential to enable the differential diagnosis of the given disease, offering an advantageous alternative to existing diagnostic methods.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents were of commercial origin and sourced as follows: mixture of cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4), [19,19,20,20,20 $^2H_5$] leukotriene C_4 (LTC_4-d_5), [19,19,20,20,20 $^2H_5$] leukotriene D_4 (LTD_4-d_5), [19,19,20,20,20 $^2H_5$] leukotriene E_4 (LTE_4-d_5), cysteinyl leukotriene affinity sorbent (Cayman Chemicals, USA), acetonitrile, water, methanol (LC/MS grade), formic acid, triethylamine (99.5%), ammonium hydroxide (28% NH_3 solution in water), 3,5-dinitrosalicylic acid (>99.0%) (Aldrich, USA), starch (Zulkowsky grade, Merck, Germany), SPE column BOND ELUT C18 sorbent mass 100 mg, particle size 40 μm, volume 1 ml (Varian, USA).

2.2. EBC sample collection

The commercially available EcoScreen condenser equipped with saliva trap (Jaeger, Germany) was used for the EBC sample collection. The subjects (patients diagnosed with occupational bronchial asthma and a control group of healthy subjects) were encouraged to perform tidal breath through a mouthpiece connected to the condenser (−20 °C) for 15 min, while wearing a nose-clip. The acquired EBC volumes of samples ranged from 1 to 2 ml. Immediately after the sample collection, 250 pg of each deuterium labelled internal standard (LTC_4-d_5, LTD_4-d_5 and LTE_4-d_5) was added to 1 ml of EBC and the sample was subsequently frozen to a temperature of −80 °C (stored for a period not exceeding 1 month). All clinical samples were collected between 8 and 12 a.m. A detailed description of the EBC collection is available in a separate publication [9].

2.3. Amylase assay

To exclude contamination of EBC by saliva during its collection, the concentration of α-amylase was monitored using the following procedure: The hydrolytic activity of α-amylase was determined by measuring the amount of reducing sugars generated from starch. Enzyme reactions were carried out by mixing EBC with 1% (w/w) starch in a 1:2 ratio at 37 °C. After 40 min, 3,5-dinitrosalicylic acid was added and the reaction was terminated by heating (90 °C, 5 min). The concentration of reducing sugars was determined by measuring the absorbance of the reaction product (3-amino-5-nitrosalicylic acid) at 530 nm (Rainbow reader, SLT, Austria). Maximal α-amylase activity in all samples did not exceed 0.1% of the saliva activity and thus the absence of the saliva contamination in EBC samples was sufficiently proven.

2.4. Separation of cysteinyl leukotrienes

2.4.1. Immunoaffinity separation

The IAS was carried out by the following procedure: cysteinyl leukotriene affinity sorbent (10 μl) was added to EBC (1 ml, labelled by internal standards (ISs)). The suspension was stirred (60 min, 480 rpm) on a laboratory shaker (IKA KS 130 basic, IKA-Werke GmbH & Co. KG, Germany). The sorbent was then separated by centrifugation (500 × *g*, i.e. 3000 rpm). The supernatant was removed and the sorbent was washed with water (2 × 1 ml). After washing, cold methanol was added (2 × 0.5 ml) to elute cys LTs from the antibody-substrate complex. After the elution, collected methanol fractions were dried by flushing with nitrogen. The resulting solid residue was dissolved in a mobile phase (50 μl) and analyzed by LC–ESI-MS/MS.

2.4.2. Solid phase extraction

Prior to injection of the EBC sample (labelled by ISs), the SPE column was activated with methanol (5 × 1 ml) and 0.05 M formic acid (3 × 1 ml). The EBC sample (1 ml) was injected to the pre-conditioned column, and washed with water (3 × 1 ml). The entrapped substances were eluted with methanol (1 ml). The acquired fraction of methanol was stripped-off to dryness in a flow of nitrogen. The resulting solid residue was dissolved in the mobile phase (50 μl) prior to analysis by LC–ESI-MS/MS.

2.4.3. Lyophilization

Pre-frozen EBC sample (−80 °C; 1 ml; labelled by ISs) was introduced into a lyophilizer (Labconco Free Zone, USA) for 24 h. The spiral of the lyophilizer was cooled to −47 °C and the pressure stabilised within 1 h at 9 kPa. The lyophilized residue was dissolved in the mobile phase (50 μl) and immediately analyzed by LC–ESI-MS/MS.

2.5. LC–ESI-MS/MS analysis – apparatus and conditions

A ProStar HPLC system equipped with a ProStar 210 dual pump, degasser and Varian 410 autosampler (Varian, USA) with a Hypercarb Thermo 100 × 2.1 mm × 5 μm column connected to Hypercarb pre-column (Thermo Electron Corporation, USA) was used. A mobile phase consisting of a 70:30 (v/v) acetonitrile:water mixture adjusted to pH 11 with triethylamine was used for isocratic elution at a flow rate of 250 μl/min. The sample injection volume was 20 μl. The LC system was directly coupled to a Varian 1200L triple quadrupole mass spectrometer (Varian, USA) equipped with

an electrospray ion source operated in the negative ion mode (ESI^-). Full scan mass spectra were acquired using a continual infusion of the standard solutions (concentration 1 ng/ml, flow 50 μl/min). For the measurement of cys LTs and deuterated ISs, the selected reaction monitoring (SRM) mode was used. The scan monitoring reactions used for analyses were: 624.1 → 351.2 for LTC_4 (collision energy 27 eV), 495.2 → 477.3 for LTD_4 (21 eV), 438.2 → 333.1 for LTE_4 (19 eV), 629.1 → 356.2 for LTC_4-d_5 (27 eV), 500.2 → 482.3 for LTD_4-d_5 (21 eV) and 443.2 → 338.1 for LTE_4-d_5 (19 eV). The conditions on the mass spectrometer were optimized to the following values: capillary voltage −70 V, needle voltage −4500 V, pressure of the argon - collision gas (Siad, Czech Republic) 2.2 mTorr. The temperature of the drying gas - nitrogen (Siad, Czech Republic) was 300 °C (pressure 19 psi). Air (Siad, Czech Republic) was used as the nebulising gas (pressure 50 psi, temperature 50 °C). The data were acquired and processed using the software Varian MS Workstation, version 6.52 (Varian, USA).

2.6. Validation of assay procedure

2.6.1. Standard preparation and calibration procedure

The cys LTs stock solution (20 ng/ml) was prepared by diluting cys LTs in the mobile phase (water:acetonitrile – 30:70, pH 11). It was used further for the preparation of a calibration sample with specific leukotriene concentrations of 5, 10, 25, 50, 100, 250 and 500 pg/50 μl (each calibration sample was spiked with 250 pg of each ISs (LTC_4-d_5, LTD_4-d_5 and LTE_4-d_5)). The peak area of particular cys LT with the assigned IS was used as the function to quantify concentration.

2.6.2. Accuracy and precision

Validations of the methods were carried out for each combination of the pre-treatment procedures (IAS, SPE, LYO) and the quantification by LC–ESI-MS/MS. First, LTs-free EBC matrix was prepared as follows: the pooled clinical EBC samples were subjected to repeated IAS to remove all the cys LTs; using LC–ESI-MS/MS, the residual amounts of particular cys LTs were determined below the limit of detection.

The accuracy and precision of the methods were determined by the analysis of four concentration levels of the individual cys LTs (25, 50, 100 and 250 pg) into EBC LTs-free matrix (1 ml). Each concentration was replicated five times ($n = 5$). The samples were submitted to the pre-treatment procedure (IAS, SPE or LYO) followed by LC–ESI-MS/MS. Subsequently, the mean of each set of concentrations, the standard deviation (SD) and the relative standard deviation (RSD) were calculated and the value of the precision parameter was determined. The accuracy (determined as relative error and recovery parameter) of the method was calculated as the difference between the standards aqueous solutions (corresponding concentration level of a particular substrate was dissolved in water and analyzed by LC–ESI-MS/MS), and the samples prepared in the LTs-free EBC matrix and analyzed by the developed method (including IAS, SPE or LYO). The relative error (RE) and the recovery were calculated on this basis.

2.6.3. Limit of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were determined using the assay of the "EBC blank matrix" ($n = 5$). The values of LOD and LOQ were calculated as the mean value of the blank matrix signals plus three times the standard deviation (LOD) and ten times the standard deviation (LOQ), respectively.

2.7. Stability of cys LTs

Experiments on the stability of cys LTs in EBC were assessed by the identical analysis (IAS combined with LC–ESI-MS/MS) of cys LTs-free EBC with externally added cys LTs (100 pg of each cys LTs). Artificially prepared samples of cys LTs in water as well as in mobile phase (acetonitrile:water 70:30; pH 11) were evaluated in parallel. Temperature parameters (processing temperature 25 °C and storing temperature −80 °C) and several thaw–freeze cycles (0–7 cycles, one cycle consisted of a temperature change from −80 °C to 25 °C, which was accompanied by the change in state, i.e. from the solid to the liquid phase) were examined. Every experiment was performed five times.

2.8. Clinical study

EBC samples collected during the clinical study were worked-up by the IAS. At the beginning of the study it was essential to map the behaviour of the involved biological systems with respect to the production of cys LTs during the circadian biorhythm. The intra-day physiological variations were determined by analyzing duplicate EBC samples (2 × 1 ml) collected from ten volunteers (age 18 ± 1, non-smokers, without the diagnosis of bronchial asthma). EBC samples were obtained at different times throughout the day (6, 12, 18 and 24 h). The inter-day physiological variations of the method were assessed by analyzing duplicate samples (10 healthy volunteers) obtained from each subject over five consecutive days.

The clinical study comprised the differential diagnosis of occupational bronchial asthma, which was performed in a group of 20 subjects with a given diagnosis (56–64 years old, male, non-smokers) and with the equal number in the control group (52–66 years old, male, non-smokers).

3. Results and discussion

3.1. Stability of cys LTs in EBC

The biomarkers of bronchial asthma in EBC, cys LTs, are capable of inter-conversion in the presence of the necessary enzymes (Fig. 1). Moreover, these substances are not stable enough to be handled and stored without any particular precautions. The primary effort in the development of the method for the determination of these substances in EBC was to gain deeper insight into their behaviour in the given biological matrix and to find an appropriate procedure for the preparation of a sample suitable for a mass-spectrometric analysis.

The collection of EBC is carried out in a specialized workplace adapted accordingly (see Section 2.2). During the collection it is necessary to employ a standardized procedure compatible with the low stability of the substances in the matrix. During the collection, the patient breathes through their mouth over a condenser for the exhaled air. Therefore, it is advisable to carry out a test for contamination of the sample by saliva, which could occur as a result of faulty collection of EBC. This could potentially lead to false results [30,31]. Contamination can be checked by the determination of α-amylase in the sample (Section 2.3).

With regard to the sensitivity of the biomarkers to several factors, it is essential to label the sample immediately after the collection with an isotope-labelled internal standard (addition of 250 pg of LTC_4-d_5, LTD_4-d_5 and LTE_4-d_5 to 1 ml of EBC). This allows a highly precise quantification together with monitoring the changes in the sample composition, which occur during its processing ("stable-isotope-dilution assay"). Although the EBC (matrix with water as the major component) has a pH in the range of 7.2–7.7 immediately after collection, it rapidly decreased (pH 6.0–6.5) due to absorption of CO_2 from the ambient air, which could have been one of the causes for the consecutive changes occurring in this matrix after its collection [32,33]. It was demonstrated that storing EBC under an inert atmosphere (argon) did not cause a change

Table 1
Stability of cys LTs in EBC and in mobile phase (acetonitrile:water (70:30); pH 11; t = 25 °C).

Time (h)	Matrix: EBC			Matrix: acetonitrile:water solution (70:30)		
	LTC_4 (rel. %)	LTD_4 (rel. %)	LTE_4 (rel. %)	LTC_4 (rel. %)	LTD_4 (rel. %)	LTE_4 (rel. %)
0	100	100	100	100	100	100
0.25	95	104	111	94	95	93
0.5	88	99	106	87	90	86
1	76	89	95	75	78	72
1.5	63	73	86	62	65	58
2	55	60	74	55	54	48
3	44	47	58	42	35	32
6	32	38	46	30	24	21
9	24	26	39	21	15	12
12	16	19	30	14	5	3

in pH; nevertheless, this does not appear to be the main parameter with regard to the determination of cys LTs concentration levels. Additionally, no effect of light on the stability of cys LTs was observed. The most significant parameter affecting the composition of EBC with regard to cys LTs was temperature, as it was demonstrated that it rapidly changed at ambient temperature as a result of (1) their enzymatic inter-conversion and (2) their limited temperature stability. Table 1 depicts the behaviour of cys LTs in the EBC and organic solvents (mobile phase utilized during LC analysis), showing the effect of temperature (25 °C) on the composition of individual cys LTs. In the matrix of EBC, besides the degradation of individual substances (very apparent in the mobile phase), inter-conversions were observed in the series of $LTC_4 \rightarrow LTD_4 \rightarrow LTE_4$, evidently catalyzed by the enzymatic systems present in the EBC. Furthermore, at a temperature of −80 °C, EBC samples could be stored without any detectable change in the cys LTs content for a period of 3 months (similarly, the temperature of −20 °C seemed to be acceptable). On the other hand, a temperature of 4 °C was not sufficient for storage of the EBC sample. Although the inter-conversions as well as the degradations were slowed at this temperature, the degradations of all the biomarkers studied still ranged in a period of hours in both experimentally studied matrices (EBC, mobile phase used).

The number of thaw–freeze cycles was another important parameter (thaw–freeze cycle was performed between −80 °C and laboratory temperature). Apparently, the critical temperature for the stability of substances was the transformation from their solid to their liquid state (temperature of 0 °C). Each cycle leads to an 8–9% decrease of the total amount of cys LT in EBC.

3.2. LC–ESI-MS/MS analysis

LC–MS has become a method of choice for the analysis of picogram levels of cys LTs in EBC with the potential of a future employment in a routine practice. Therefore the LC–MS conditions were optimized first.

LC conditions were optimized with respect to the limitations of the ionization method (electrospray ionization – ESI) and also with the emphasis on high detector sensitivity (Hypercarb column; mobile phase acetonitrile:water 70:30 (v/v) with pH adjustment to the value 11 by triethylamine; isocratic elution at the flow 250 μl/min). Using lower pH values (pH 7–10; adjusted by formic acid, acetic acid or ammonium acetate) the detector sensitivity was depressed as follows: 9% (pH 10), 18% (pH 9), 26% (pH 8), 38% (pH 7). Using higher pH values (pH > 11) resulted in lower analytes stability. LC served not only the purpose of separating the individual cys LTs and other prostanoids but also separated the substances from the analysis front, i.e. preventing the mass detector signal being suppressed ("signal suppression effect") by a potential salt co-elution leading to a negative ionization effect. The optimized LC method met all these demands and allowed the separation of individual cys LTs (Fig. 2) as well as other biologically active eicosanoids detected in the EBC matrix with short retention times (LTC_4: t_R = 1.4 min, LTD_4: t_R = 1.8 min and LTE_4: t_R = 2.0 min; leukotriene B_4: t_R = 2.8 min; 8-isoprostane $F_{2\alpha}$: t_R = 2.2 min; dead time of column: t_R = 0.8 min) [30].

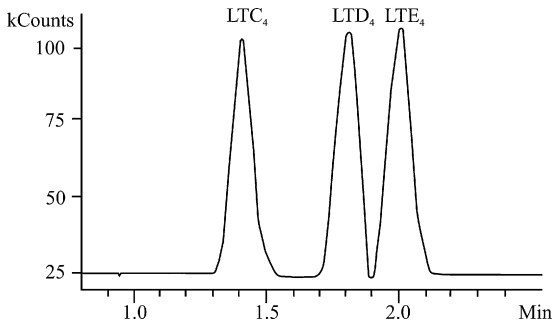

Fig. 2. Chromatogram of cys LTs (retention time of LTC_4 = 1.4 min, LTD_4 = 1.8 min, LTE_4 = 2.0 min).

Mass-spectrometric detection was enabled using negative electrospray ionization (ESI^-) in a highly selective SRM mode. On the first quadrupole Q1, the quasi-molecular ion (deprotonated molecular ion) $[M-H]^-$ of the corresponding cys LT was isolated and used as the precursor ion for the consecutive collision-induced dissociation (CID). In the collision cell (quadrupole Q2), a selective molecular decomposition occurred, producing product spectra (Fig. 3). These were the source of a specific product ion with the highest abundance that was consecutively isolated on the third quadrupole Q3. The collision energy as well as other parameters related to the mass spectrometer was optimized to acquire maximal yield of the dissociation and thereby high sensitivity of the developed method (see Section 2.4).

3.3. Separation of cysteinyl leukotrienes – immunoaffinity separation

Since the concentration of cys LTs was very low in EBC, the separation of the monitored substances and their pre-concentration prior to LC–ESI-MS/MS analysis was highly desirable. The IAS was performed with a commercial cys LTs affinity sorbent (binding capacity 100 μl of the immunoaffinity sorbent/10 ng of cys LTs). The immunoaffinity extraction step was optimized to obtain conditions exhibiting high reproducibility and precision. The experiments were performed with externally added cys LTs into cys LTs-free EBC and 10 μl of cys LTs affinity sorbent. This amount was selected based on the binding capacity and expected levels of cys LTs in EBC (quantity of cys LTs in EBC clinical samples ranged in the interval of 20–250 pg/ml of EBC). The time dependence of the levels of immunoseparated cys LTs at a ratio of immunoaffinity sorbent:cys LTs of 10 μl:100 pg in 1 ml of EBC is depicted in Fig. 4. Apparently,

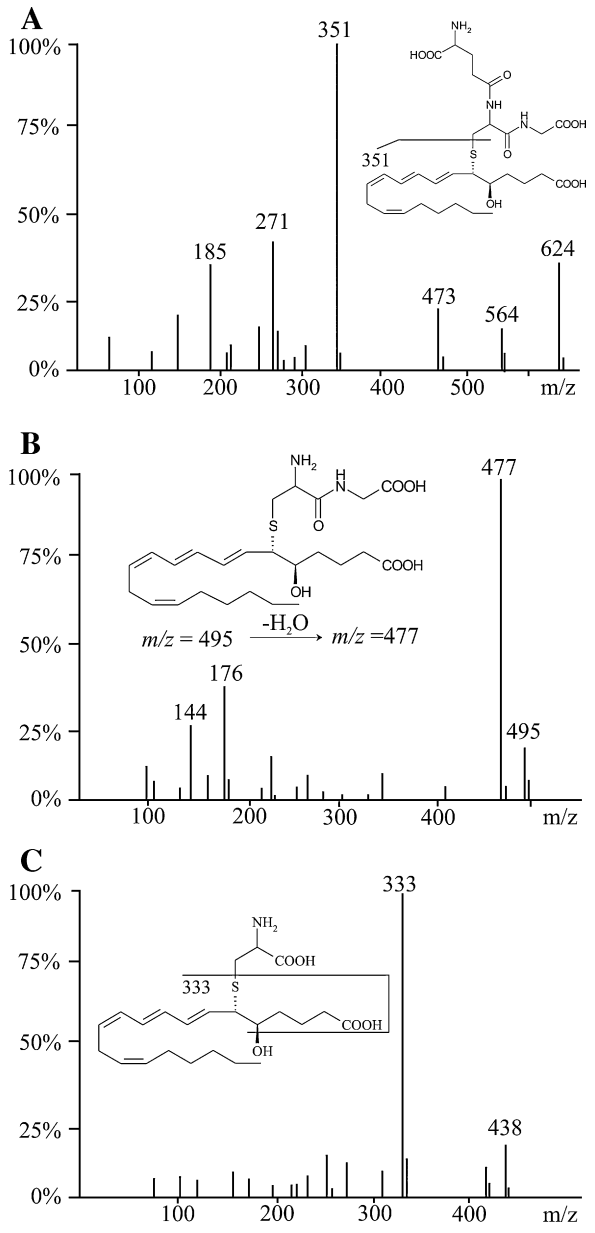

Fig. 3. ESI⁻ collision spectra of cys LTs – LTC_4 (A), LTD_4 (B) and LTE_4 (C).

with prolongation of the immunoseparation time, the separated amount of cys LTs from EBC increased, however decomposition of the substances occurred because of a negative temperature effect on their stability (temperature of immunoseparation = 25 °C). After

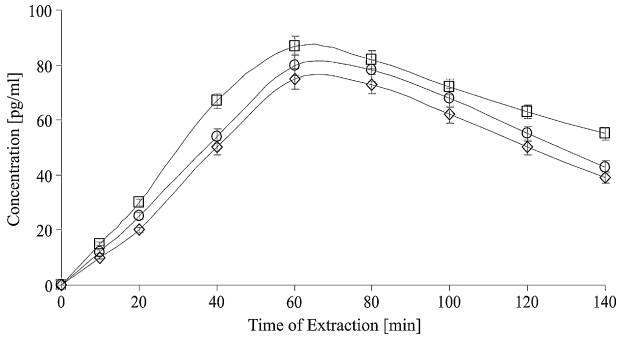

Fig. 4. Time dependence of LTC_4 (◊), LTD_4 (○) and LTE_4 (□) concentrations in immunoaffinity separation.

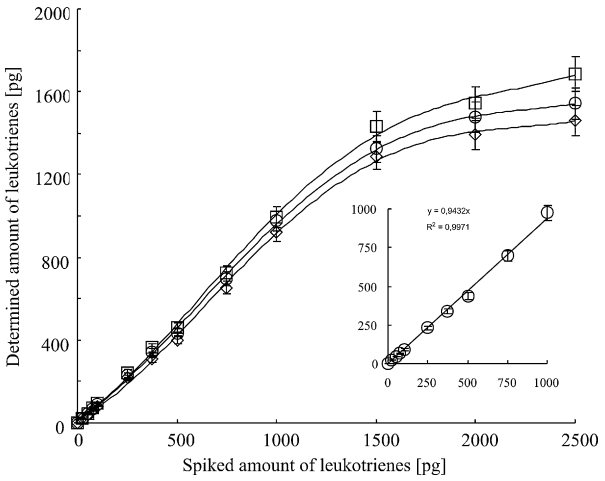

Fig. 5. Efficiency of cys LTs immunoaffinity separation LTC_4 (◊), LTD_4 (○) and LTE_4 (□).

achieving the maximum separated amount (60 min), a degradation process started to prevail, therefore the optimal period for the immunoseparation of LTs from EBC was determined as 60 min. An equally important parameter was the ratio of the quantities of immunoaffinity sorbent to cys LTs. Fig. 5 shows the dependence of the levels of immunoseparated cys LTs on their original levels in the EBC, which conspicuously suggest that the dependencies had the character of an adsorption isotherm, where two different areas could be described separately. In the first area with the linear dependence, the quantity of the antigen–antibody complex produced grew with an increasing amount of biomarkers in the matrix (Fig. 5 – enlarged working area), whereas in the second non-linear area, there were not enough free antibodies on the sorbent surface to bind the excess quantity of cys LTs. With regard to the accuracy of the method, 10 μl of the sorbent was used during the clinical analyses, which corresponded to the immunoseparation proceeding in the linear dependence area. After the immunoseparation, the separated biomarkers (cys LTs) were eluted from the complex substrate–antibody by methanol, which did not denaturize the protein antibody and allowed the regeneration of the sorbent with anchored antibodies. Using the described procedure, it was possible to separate more than 90% of the cys LTs originally contained in the clinical sample (recovery parameter). The ratio of non-specific reactions with the immunoaffinity sorbent was negligible.

3.4. Method validation

Calibration graphs were constructed as the dependence of the ratio of a particular substrate peak area to the deuterium-labelled IS peak area (*x*-axis) *versus* the substrate concentration (*y*-axis). A least squares regression analysis was applied. The Pearson's regression correlation coefficients (R^2) were higher than 0.9993 for LTC_4, 0.9996 for LTD_4 and 0.9991 for LTE_4 calibration curves (the acceptable value of R^2 for each calibration curve was $R^2 \geq 0.99$). The back-calculated values of the calibration points were in a good agreement with the theoretical concentrations with RSD between 3.3 and 6.6% of the nominal concentrations. Table 2 depicts variations in the calibration curves prepared in the course of the study (inter-day variance), characterized by the standard deviation and the RSD ($n = 10$). The calibration graphs were constructed for the cys LTs-free EBC matrix as well. The R^2 were higher than 0.9955 for LTC_4, 0.9983 for LTD_4 and 0.9947 for LTE_4 calibration curves in EBC matrix. The calibrations for particular substances in mobile phase compared to EBC matrix exhibited no substantial differences in the values of correlation coefficients, slopes and

Table 2
Linear regression analysis of calibration curve of cys LTs ($n = 10$).

Analyte	Slope	Intercept	SD	RSD (%)
LTC_4				
Matrix: acetonitrile:water (70:30)	352.18	−28.43	8.93	2.67
Matrix: pooled EBC (residual LTC_4 < LOD)	336.54	−10.87	19.33	5.95
LTD_4				
Matrix: acetonitrile:water solution (70:30)	453.98	−23.45	7.98	1.84
Matrix: pooled EBC (residual LTD_4 < LOD)	444.73	−11.56	16.97	4.05
LTE_4				
Matrix: acetonitrile:water solution (70:30)	571.53	−33.91	6.73	1.23
Matrix: pooled EBC (residual LTE_4 < LOD)	558.09	−39.23	14.12	3.11

intercepts of the calibration curves (Table 2). Based on the results obtained in EBC matrix and mobile phase any matrix effect was excluded.

The accuracy and precision parameters were assessed by analyzing four different concentration levels of the particular substances (LTC_4, LTD_4 and LTE_4). The precision (RSD) was within the interval of 5.4–9.7% for LTC_4, 4.1–8.7% for LTD_4 and 3.2–8.1% for LTE_4. The accuracy (RE) varied from −7.7 to −9.6% for LTC_4, −6.9 to −8.0% for LTD_4 and −6.3 to −7.0% for LTE_4. The developed method enabled the use of the following criteria: an acceptable value of RSD was lower than 10%; an acceptable value of RE lay within the interval ±10%. The values of the precision and accuracy parameters for each particular substrate were summarized in Table 3.

3.5. Clinical study

The intra-day physiological variation of cys LTs in EBC was studied in a group of 10 volunteers (age 18 ± 1, non-smokers not diagnosed with bronchial asthma, clinical samples were collected in the same day-time at 6, 12, 18 and 24 h, subjects were denied sleep during the experimental period). An identical trend was demonstrated in the concentration profiles of cys LTs in all the subjects, which increased during the day and returned to its original level during the night. The level of cys LTs increased by an average of 15.4%. Regarding the inter-day physiological variations in a group of 10 healthy people, the total level of cys LTs did not differ by more than 6.6% for each individual over a period of 5 consecutive days.

The clinical study (all the samples were collected between 8 and 12 a.m.), where the main goal was to verify the possibility of differential diagnosis of bronchial asthma, was carried out using 20 subjects with a previous diagnosis of occupational bronchial asthma and an identically numbered control group (Fig. 6). The results were statistically evaluated. A significant difference was found between the control group and the group with the diagnosis

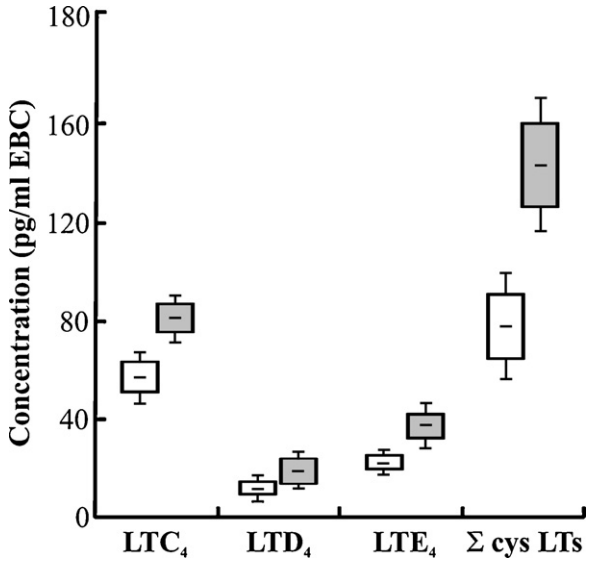

Fig. 6. Clinical study – concentration of cys LTs in EBC bronchial asthma (grey), healthy subjects (white).

in the values of concentration of LTC_4 (57.4 ± 8.6 pg/ml of EBC in the control group and 80.1 ± 6.2 pg/ml of EBC in those diagnosed with bronchial asthma) and LTE_4 (25.3 ± 5.1 pg/ml in the control group *versus* 35.7 ± 4.8 pg/ml in the bronchial asthma group). The difference in the levels was highlighted in the sum values of cys LTs, where the control group reached values of 77.8 ± 13.8 pg/ml of EBC and the asthmatic group 133.2 ± 20.1 pg/ml of EBC. Although a difference in the levels of LTD_4 was observed in its average value as well, differing from 19.9 ± 4.3 pg/ml of EBC in positively diagnosed subjects to 13.8 ± 5.2 pg/ml of EBC in the healthy subjects, the significance of this biomarker for the differential diagnostics of

Table 3
Recovery values of immunoaffinity separation from repeated analysis ($n = 5$).

Added amount (pg/ml)	Found amount (mean) (pg/ml)	SD (pg/ml)	Precision RSD (%)	Accuracy RE (%)	Recovery (%)
LTC_4					
25	22.6	2.2	9.7	−9.6	90.4
50	45.5	4.1	9.0	−9.0	90.8
100	91.6	7.5	8.2	−8.6	91.6
250	230.8	12.5	5.4	−7.7	92.3
LTD_4					
25	23.0	2.0	8.7	−8.0	92.0
50	46.2	3.5	7.6	−7.6	92.4
100	92.9	6.3	6.8	−7.1	92.9
250	232.8	9.5	4.1	−6.9	93.1
LTE_4					
25	23.3	1.9	8.1	−6.8	93.2
50	46.8	3.8	8.1	−6.4	93.6
100	93.7	6.2	6.6	−6.3	93.7
250	232.6	7.5	3.2	−7.0	93.0

Table 4
Validation parameters for various pre-treatment methods (imunoaffinity separation, solid phase extraction and lyophilization) ($n = 5$).

Analyte	LOD (pg/ml EBC)	LOQ (pg/ml EBC)	Precision RSD (%)	Accuracy RE (%)	Recovery (%)
Imunoaffinity separation					
LTC_4	2	10	9.7	−9.6	90.4–92.3
LTD_4	1	6	8.7	−8.0	92.0–93.1
LTE_4	1	5	8.1	−7.0	93.0–93.7
Solid phase extraction					
LTC_4	3	14	10.1	−10.4	89.6–91.8
LTD_4	2	9	9.2	−8.5	91.5–92.9
LTE_4	2	10	8.7	−8.2	91.8–92.4
Lyophilization					
LTC_4	4	21	11.2	−14.2	85.8–87.1
LTD_4	4	19	9.9	−11.5	88.5–90.0
LTE_4	3	13	9.7	−9.3	90.7–92.3

bronchial asthma was not entirely proven as the difference in the confidence intervals was statistically insignificant.

3.6. Comparison of pre-treatment methods

The presented method encompasses a pre-treatment IAS, allowing highly selective and effective concentration of cys LTs from EBC, combined with a highly selective and sensitive detection by LC–ESI-MS/MS. The developed method was compared with other pre-treatment methods (combined with LC–ESI-MS/MS detection) respecting low cys LTs stability and potentially suitable for clinical practice. The other methods used in combination with mass spectrometry detection were the pre-concentration and the pre-separation method, SPE performed on non-polar C18 tips and LYO used for pre-concentrating the sample. The comparison of the methods is shown in Table 4 where the values of precision, accuracy, recovery, LOD and LOQ parameters are presented.

The pre-treatment methods connected with LC–ESI-MS/MS detection produced slightly different values for the validation parameters (precision, accuracy and recovery). The LYO method demonstrated a lower recovery, precision and accuracy compared to the other pre-treatment methods. SPE had almost identical recovery, precision and accuracy values to IAS but differed significantly in terms of LOD and LOQ parameters. A comparison of the pre-treatment methods from the matrix effect point of view is demonstrated in Fig. 7 where cys LTs-free EBC matrix with artificially added cys LTs (125 pg of each particular cys LT) was prepared for an LC–ESI-MS/MS analysis. The signal/noise (S/N) values were calculated (Fig. 7). It was found that with decreasing selectivity of the pre-treatment method (IAS > SPE > LYO) the S/N decreased due to the presence of other matrix components. Based on the results, it is clear that the matrix effect, if any, is present only to a small extent which is caused by the EBC matrix composition. EBC consists of water (>99%) and only a small amount of salts and other components generally responsible for the matrix effect in LC–MS analysis. The comparison of the three pre-treatment methods may lead to a conclusion that IAS in the combination with LC–ESI-MS/MS exhibited the finest values of all the validation parameters, nevertheless LYO and SPE could be seen as acceptable alternatives.

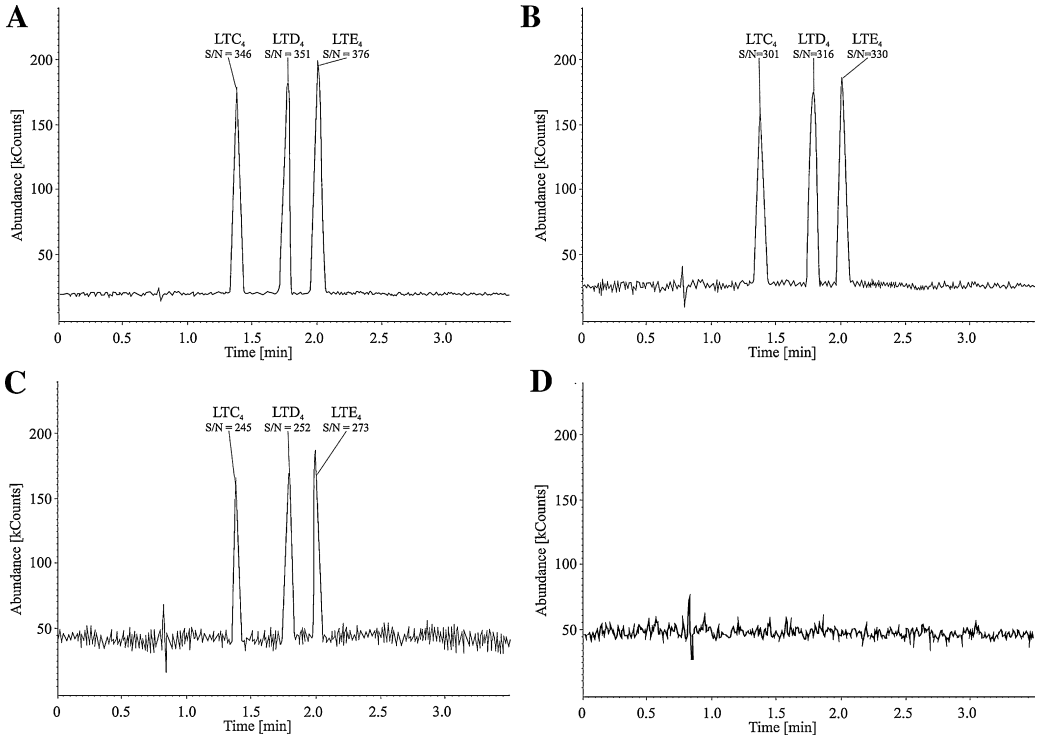

Fig. 7. LC–ESI-MS/MS chromatograms of cysteinyl leukotrienes (125 pg of each cysteinyl leukotriene/ml EBC) for different pre-treatment methods: (A) immunoaffinity separation; (B) solid phase extraction; (C) lyohilization and (D) blank (cysteinyl leukotrienes-free EBC) – immunoaffinity separation.

4. Conclusions

A method for the detection and quantification of cys LTs as potential biomarkers of bronchial asthma in EBC has been developed. The determination of cys LTs in this matrix allows monitoring of the processes occurring in the lungs and airways independently of the processes taking place in the entire organism, commonly monitored by determining biomarkers in blood plasma or urine. Although several methods have recently been developed for the determination of cys LTs in EBC, no standardized procedure for the differential diagnosis of bronchial asthma and the determination of their concentration levels has been verified until now. Moreover, the results published from various sources display a rather noteworthy disunity. This work aims to identify potential sources of the reported inconsistencies and apply the conclusions to the development of a feasible analytical method facilitated by the description of an all-inclusive methodology from the sample collection to measurement, yielding uncompromisingly valid data. These data were obtained in the form of concentrations of individual cys LTs in the given matrix together with their correlations to physiological/pathological processes occurring in the lungs or the airways. The developed method combined extraction techniques with mass spectrometric detection. The extraction techniques demonstrated a selective separation in the case of IAS, but non-selective isolation of the studied biomarkers in SPE and mere concentration of substances when LYO was used. The mass spectrometric detection accommodated all the limitations incurred as a result of complexity of the matrix and the low stability of cys LTs and its performance was characterized by an effective accuracy and high reproducibility.

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