



Non-volatile compounds in exhaled breath condensate: review of methodological aspects

Elaheh Rahimpour¹ · Maryam Khoubnasabjafari² · Vahid Jouyban-Gharamaleki³ · Abolghasem Jouyban^{1,4}

Received: 23 June 2018 / Accepted: 10 July 2018 / Published online: 25 July 2018
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Abstract

In contrast to bronchial and nasal lavages, the analysis of exhaled breath condensate (EBC) is a promising, simple, non-invasive, repeatable, and diagnostic method for studying the composition of airway lining fluid with the potential to assess lung inflammation, exacerbations, and disease severity, and to monitor the effectiveness of treatment regimens. Recent investigations have revealed the potential applications of EBC analysis in systemic diseases. In this review, we highlight the analytical studies conducted on non-volatile compounds/biomarkers in EBC. In contrast to other related articles, this review is classified on the basis of analytical techniques and includes almost all the applied methods and their methodological limitations for quantification of non-volatile compounds in EBC samples, providing a guideline for further researches. The studies were identified by searching the SCOPUS database with the keywords “biomarkers,” “non-volatile compounds,” “determination method,” and “EBC.”

Keywords Exhaled breath condensate · Non-volatile compounds · Biomarkers · Analytical methods

Introduction

The conventional methods for the diagnosis of lung diseases (bronchoscopy or biopsy) are invasive or semi-invasive (e.g., nasal swabs, sputum induction), but are currently used in clinical follow-ups [1]. In contrast, non-invasive methods, such as the collection of exhaled breath condensate (EBC) with analysis of diverse biomarkers, which have been used since the 1980s [2], are easy, repeatable for some biomarkers, and not associated with side effects or any appreciable discomfort or risk to the sample donors [3]. This method also reduces the number of interfering substances compared to more complicated matrices, such as blood, plasma, sputum, and urine [4]. Moreover, the collection of

EBC can be repeated as often as needed without altering the fluids present on the lung surfaces [5].

EBC is the water vapor contained in breath and very small droplets of lung lining fluid, which are condensed typically by using a cooling or condensing collection device (commonly at 4 °C or sub-zero temperatures) [6]. EBC is a highly diluted and low-protein aqueous matrix [7]. It is a unique matrix for the analysis of inflammatory and oxidative stress biomarkers and enables the study of the early effects of different diseases or exposures on the lung and/or upper airways [8]. The analysis of drugs is also another promising procedure that has attracted increasing attention in recent years [9–15].

The number of publications on EBC analysis has increased in recent years, and so has the interest at international conferences. Clinicians and clinical chemists are awaiting the sufficient maturation of EBC-based methods to help them with their daily needs in the clinic. Therefore, it is important to summarize the current understanding of the available methods as well as identify the knowledge gaps in the hope of finding suitable applications for this sampling method in both research and clinical practice.

This paper aims to summarize and review the exhaled breath non-volatile compound-related detection and analytical methods, especially the recent developments relating to EBC biomarker detection systems. It is hoped that although similar articles have been published in the past, this article will attract

✉ Abolghasem Jouyban
ajouyban@hotmail.com

¹ Pharmaceutical Analysis Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

² Tuberculosis and Lung Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

³ Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

⁴ Kimia Idea Pardaz Azarbayjan (KIPA) Science Based Company, Tabriz University of Medical Sciences, Tabriz, Iran

the attention of many research groups as well. The main purpose of this review is to update a previous literature review [16] on the same subject, and to present a broad range of compounds (biomarkers and drugs) used in various diseases; hence, leading researchers from various areas of study are included in this article. In contrast to other related articles, this review is classified on the basis of analytical methods and includes almost all the applied methods and their methodological limitations for quantification of non-volatile compounds in EBC samples, thus providing guidelines for further investigations.

Literature search method

The articles included in this review were identified through literature searches of the SCOPUS database by using the search terms of “biomarkers,” “non-volatile compounds,” “determination method,” and “EBC.” A total of 224,175 studies were identified for the keyword “biomarkers,” out of which 3183 studies pertained to “non-volatile compounds” and 1662 cases related to studies conducted on EBC. Since this study is limited to the determination methods of non-volatile compounds/biomarkers in EBC, the literature search was restricted to the methodological aspects only. It should be noted that published reviews, abstracts, and bibliographies were also examined to identify additional studies.

EBC sampling devices

Various collection and sampling systems have been described by different research groups (Fig. 1). However, the common principle for all of them includes the rapid cooling of exhaled air with consequent condensation of water vapor, as well as the sedimentation of aerosol particles onto a cold surface. Three commercial instruments are available for EBC sampling; the most common according to the literature are EcoScreen (Erich Jaeger, Hoechberg, Germany), which is a stationary system, and RTube™ (Respiratory Research, Charlottesville, VA, USA), which is a portable device and is available in two subtypes. The first subtype is composed of a metal cylindrical tube that is pre-cooled in a freezer at a temperature of less than $-20\text{ }^{\circ}\text{C}$, then it is placed in the RTube chamber, and the exhaled breath is blown into the tube. Finally, owing to the low temperature of the tube, the exhaled breath is condensed into the chamber [17, 18]. In the second subtype of RTube, instead of cooling by using a freezer or dry ice, liquid nitrogen or a low-temperature fluid is used to reduce the temperature inside the RTube chamber [19, 20]. Turbo-DECCS (transportable unit for research on biomarkers obtained from disposable exhaled condensate collection systems; ItalChill, Parma, Italy) is another commercially

available device for EBC collection. In addition, various homemade devices have been described in the literature [21]. One of these homemade EBC collecting devices with the ability to set the temperature from 0 to $-25\text{ }^{\circ}\text{C}$ was also developed by our research group. Variable sampling temperature enables the collection of gaseous substances with boiling points varying from 0 to $-25\text{ }^{\circ}\text{C}$. The sampling time in this device is between 5 to 15 min, depending on the operating temperature [22]. Ahmadzai et al. [23] compared biomarkers measured in collected EBC by using different devices. The design and inner coating materials used in the collecting device as well as the temperature fluctuations during the process of condensate collection influence the biomarkers' concentrations [24]. Uneven condensing temperatures may be present not only between different EBC collecting systems but also in cases when the same condenser is used with different airflows or with different cooling temperatures. Moreover, the variability in EBC pH between various collection systems due to the differences in the concentrations of absorbed volatile salivary contaminants during collection (e.g., CO_2 , ammonia, and acetic acid) can influence the concentrations of biomarkers or non-volatile compounds differently [21]. Finally, to provide consistent data, the EBC collection system needs to be standardized with respect to the collection temperature, material, and flow design, as well as other parameters, such as ventilatory patterns, tidal volumes, breathing frequencies, and exhaled particles.

Non-volatile compounds in EBC

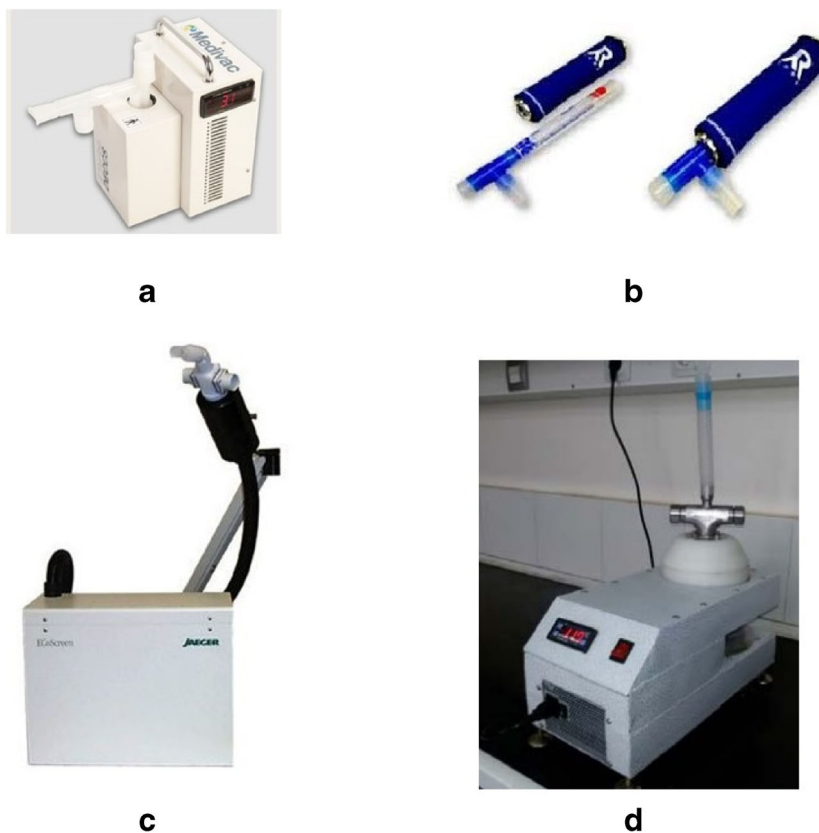
Non-volatile compounds normally present in EBC

The non-volatile compounds in EBC vary from small inorganic ions (anions, cations), larger organic molecules (urea, organic acids, amino acids, and their derivatives) to peptides, proteins, surfactants, and macromolecules. EBC contains many biomarkers of oxidative and nitrative stresses (8-isoprostane, 3-nitrotyrosine (3-NT), S-nitrosothiols, etc.) as well as inflammation mediators (LTB_4 , cysteinyl leukotrienes (Cys-LTs): LTC_4 , LTD_4 , LTE_4 , prostaglandins, histamine, adenosine, interleukins (IL), etc.). Some of these compounds are listed in Table 1 [25–62].

Non-volatile compounds generated in EBC with the appearance of disease signs

In addition to the mentioned compounds, EBC contains some other substances that do not exist in a healthy state and they are only detectable along with the appearance of disease signs. Some of these compounds include:

Fig. 1 EBC sampling devices. **a** Turbo-DECCS, **b** RTube, **c** EcoScreen system, and **d** A lab-made cooling trap



Pepsin

Pepsin is a gastric aspartic proteinase enzyme that degrades food proteins into peptides and initiates digestion. Pepsin is produced only in the stomach; hence, evidence of its presence in other organs indicates that it has reached them as a refluxate. The enzyme has recently been detected within the laryngeal epithelium, in the saliva of patients with suspected reflux laryngitis, and in the EBC of individuals with airways and lung diseases, where reflux has played a part. The advantage of pepsin analysis is that in the normal state, it is not found in the tracheobronchial tree, and thus it may be a specific biomarker for reflux-related lung diseases [63].

Carcinoembryonic antigen

Carcinoembryonic antigen (CEA) is a glycoprotein involved in cell adhesion and is normally produced in gastrointestinal tissue during fetal development, but its production stops before birth. It is usually present only at very low levels in the blood of healthy adults. However, the serum levels of CEA are raised in some types of cancer [64, 65]. Nevertheless, CEA may also exist in EBC and it has been reported in some studies that CEA detection in EBC may diagnose lung cancer earlier than the detection of this biomarker in serum [66]. Therefore,

CEA can be used as a possible and promising tumor biomarker in clinical tests.

N^ε-(Carboxymethyl)lysine

N^ε-(Carboxymethyl)lysine (CML) is one of the best-characterized analytical indicators of advanced glycation end-products (AGEs) and could be formed by a non-enzymatic reaction of reducing carbohydrate with an amino group of protein or other macromolecules followed by a cleavage and/or intramolecular rearrangement [67]. AGE-modified proteins have been found to stimulate monocytes/macrophages to synthesize and release TNF, IL-1, and some growth factors—platelet-derived growth factor (PDGF) and insulin-like growth factor I (IGF-I)—by the AGE receptor system. These cytokines and growth factors secreted by lung macrophages are related to a possible pathogenesis in pulmonary fibrosis [68].

Trimethylamine

Trimethylamine (TMA) is formed in biosynthetic pathways from amino acids via the methylation of monomethylamine, which is metabolically derived from sarcosine or glycine. TMA is also an intermediate product in the metabolism of choline. During the demethylation process, it is decomposed into dimethylamine [68].

Table 1 Biomarkers/non-volatile compounds present in EBC

Biomarkers/non-volatile compounds	Role	Remarks	Concentration in EBC of normal subjects	Variation in patient subjects	Ref.
Nitrite/Nitrate	–	Nitrogen redox forms present in the epithelial lining fluid of the human respiratory tract	0.33–3.3 $\mu\text{mol L}^{-1}$	Increase in case of any pulmonary disease	[25–28]
3-Nitrotyrosine (3-NT)	Marker of oxidant injury	Formed by (1) reaction of peroxynitrite, a reactive form of NO in vivo, with tyrosine (Tyr) residues of proteins; (2) myeloperoxidase related pathways; (3) eosinophil-specific peroxidase related pathways	6.3 ng mL ⁻¹	Increase especially in asthmatic and allergic patients	[29–36]
S-Nitrosothiols	–	Formed by the interaction of NO or NO ₂ ⁻ with glutathione or other compounds containing the –SH group in the airways	0.05 and 0.8 $\mu\text{mol L}^{-1}$	–	[37, 38]
Prostanoids	Lipid mediators	Generated by the action of cyclooxygenase on arachidonic acid	–	–	[39, 40]
- prostaglandins (PG, for instance, PGE ₂ , PGE ₂ , and PGF _{2α})	- Mediators of inflammatory and anaphylactic reactions	- Produced by airway epithelium and smooth muscle	–	–	–
- thromboxanes (TXA ₂ , TXB ₂)	- Mediators of vasoconstriction	–	–	–	–
- prostacyclins	–	–	–	–	–
Isoprostanes	Biomarkers of inflammation	Formed by non-enzymatic direct peroxidation of arachidonic acid on the cell surface during oxidative stress	–	Increase in case of any pulmonary disease	[8, 41–44]
- F ₂ -isoprostanes	- Indices of endogenous oxidative activities and oxidative stress	–	–	–	–
Leukotrienes	- Pro-inflammatory mediators	Released from inflammatory cells of the airways, particularly mast cells and eosinophils	–	Increase in of asthma, COPD and CF	[45–49]
- Cys-LTs (LTC ₄ , LTD ₄ , LTE ₄)	- Pro-fibrogenic mediators	–	–	–	–
- LTB ₄	Regulators of host responses to infection, immune responses, inflammation, and trauma	Released from inflammatory cells and structural cells of the pulmonary system	A few ng L ⁻¹	–	[50–52]
Cytokines	- IL-1, tumor necrosis factor (TNF) and interferon (IFN)- γ	–	–	–	–
- IL-4, IL-10, and IL-13	- Anti-inflammatory cytokines	–	–	–	–
Glutathione (GSH)	Biomarker of oxidant-induced damage	Endogenous antioxidant	–	–	[53, 54]
Adenosine 5'-triphosphate (ATP)	Key mediator in allergic airway inflammation	Released from airway epithelial cells	At the low picomolar range	–	[55, 56]
Arginine (Arg)	An important substrate for enzymes such as the nitric oxide synthases (NOS) and arginases	–	–	–	[57, 58]
Surfactants	Reduce surface tension at the air-liquid interface throughout the lung	Excreted from alveolar type II cells that line the inner surface of the respiratory system	–	Abnormalities in various lung diseases	[59–61]
85–90% Lipids (phospholipids that mainly consist of phosphatidylcholines (PCs), 10% proteins, 2% carbohydrates. Other compounds	–	–	–	Variation in the concentration profile	[62]

Table 1 (continued)

Biomarkers/non-volatile compounds	Role	Remarks	Concentration in EBC of normal subjects	Variation in patient subjects	Ref.
DNA, hepatocyte growth factor, vitronectin, endothelin-1, lactate, glucose, and small ions and molecules, such as chloride, sodium, potassium, urea, and small organic acids					

Studies have proved that the presence of TMA in breath is typical only for patients with renal disorders. In healthy subjects, the amines are normally removed from the body by renal excretion, whereas the amines accumulate in the case of patients with kidney damage [69].

Exogenous compounds

All the above-mentioned compounds are endogenous and are found in different concentrations according to the health status of the individuals. However, exogenous factors can also have an impact on the exhaled composition and require consideration as confounding factors that impede the reliable identification of breath biomarkers. Compounds with exogenous origin can also be excreted via the lungs and detected in EBC after previous uptake from the environment through inhalation or ingestion, and distribution within the body [70]. However, exogenous compounds are beyond the scope of this article and we just focus on drugs as a sub-set of these compounds.

Drugs

Previous studies on breath have confirmed that many drugs or metabolites are exhaled and can be potentially used as diagnostic markers [71–74]. Many factors, such as body mass index, health status, polymedication, addiction, and genetics, influence the quantity of therapeutic dose needed. Therefore, therapeutic drug monitoring (TDM) and knowing the exact body drug concentration are essential to maintain the efficacy of many immunosuppressant drugs. TDM allows the physician to immediately adjust the required drug dose before the patient faces any inconvenience. Analyzing EBC as a very convenient and rapid sampling method enables an access to immediate and accurate information about the pharmacokinetics of drugs.

Reported analytical methods for determination of non-volatile biomarkers/compounds in EBC

The application of EBC is dependent on the development of highly sensitive and reproducible assays. Till date, a lot of methods have been reported for the determination of various non-volatile biomarkers/compounds in EBC samples. Depending on the biomarker panel required for diagnosing or monitoring of a specific disease, different methods or their combinations could be applied. The methods for the analysis of breath condensate are usually based on spectrophotometric or fluorometric assays or high-pressure liquid chromatography (HPLC) in combination with different detection methods, such as UV detection, fluorescence detection (FD),

electrochemical detection (ECD) or mass spectrometry (MS), gas chromatography (GC) in combination with MS, capillary electrophoresis (CE), antibody-based assays, and some other new methods (Fig. 2). The following sections present the typical instrumentation platforms and their utility for EBC analysis in healthy subjects and patients. In general, the analytical methods reported for EBC analysis may be classified into several categories, including:

Optical methods

A number of spectroscopic- and spectrometric-based devices have been developed for use in clinical applications for the detection and monitoring of non-volatile biomarkers/compounds in EBC (Table 2). A range of optical detectors could be used, including absorbance, fluorescence, and luminescence.

Absorbance

The most commonly used detection methods are UV detectors, which rely on the presence of a chromophore in the molecules. The determination of $\text{NO}_2^-/\text{NO}_3^-$ in EBC samples is mostly based on the Griess diazotization reaction, which involves the reaction of NO_2^- with N-1-naphthylethylenediamine in the presence of sulfonic acid and subsequent absorbance measured at 540–550 nm [75–78, 113]. To measure NO_3^- content, the NO_3^- needs to be reduced to NO_2^- enzymatically by NO_3^- reductase, followed by the

measurement of nitrite. In addition to the Griess diazotization reaction, other colorimetric assays could be used as well. Sepehr et al. [9] determined the NO_2^- content in EBC samples by using the Berthelot method. For this purpose, NO_2^- is first reduced to ammonium by adding Zn powder and H_2SO_4 , followed by the derivatization of ammonium with Berthelot's reagent (i.e. phenol and hypochlorite in alkaline solution) to form a blue product that can be measured at 655 nm. The reagents used in these experiments may be prepared as a kit. For example, the enzyme-linked immunosorbent assay (ELISA) kits which have become very popular recently owing to their relatively low cost and ease of application. Some commercial ELISA kits are available for the determination of 3-NT with absorbance measured at 450 nm [79, 80], S-nitrosothiols with absorbance measured at 540–550 nm (as NO_2^- after the cleavage of the RS–NO bond with HgCl_2 or CuCl /cysteine solution) [81, 114], 8-isoprostane [80, 82, 83, 115, 116], LTB_4 [84–88], prostanoids [89, 90], cytokines [26, 91–96, 117–120], GSH [97], TxB_2 [98], TGF- β 1 [99], and other molecules, such as proteins [121] and keratin [100] in the EBC of patients with various respiratory diseases. In a systematic study, Carpagnano et al. used an enzyme immunoassay (EIA) kit for determining a protein hormone called leptin in patients with obstructive sleep apnea syndrome (OSAS) [122] and non-small-cell lung carcinoma (NSCLC) [123]. Leptin controls body weight by influencing appetite and energy expenditure. They reported higher concentrations of exhaled leptin in obese patients with OSAS and in NSCLC patients with relation to tumor progression. The developed

Fig. 2 Applied analytical methods for determination of non-volatile biomarkers/compounds in EBC. *PCR* polymerase chain reaction, *FD* fluorescence detection, *ECD* electrochemical detection, *MS* mass spectrometry, *DAD* diode array detection, *C^dD* contactless conductivity detection, *LIF* laser-induced fluorescence, *PCR* polymerase chain reaction, *NMR* nuclear magnetic resonance

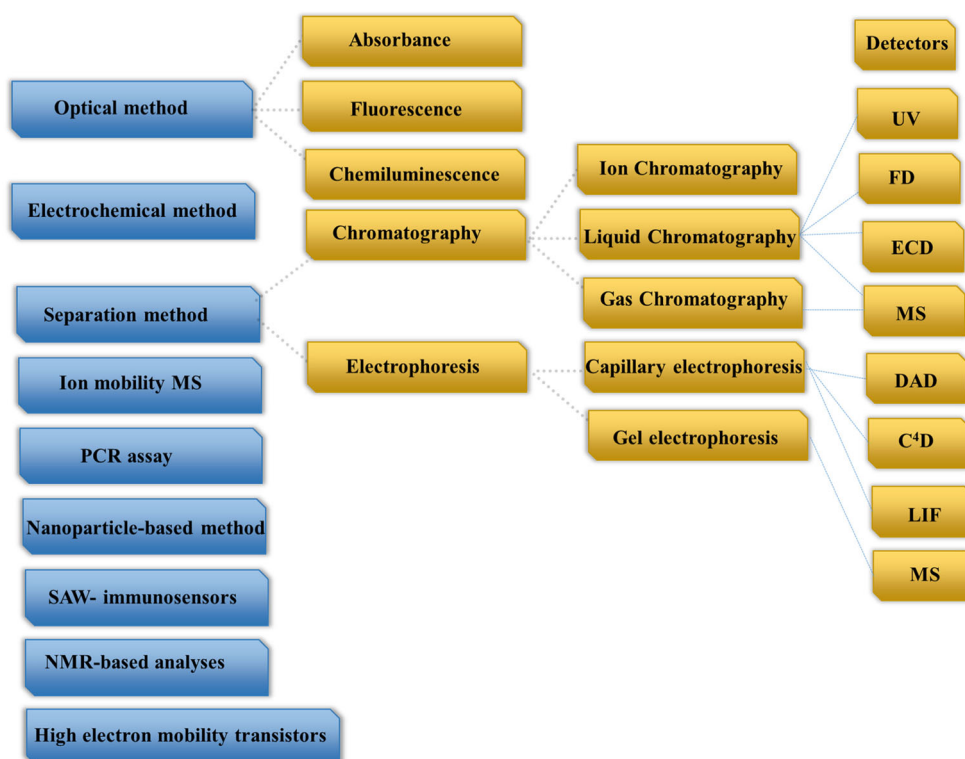


Table 2 Characteristics of included studies on exhaled non-volatile biomarkers/compounds in various diseases by optical methods

Method	Targeted biomarkers	Breath collection device	Disease/Disorder	Linear range	LOD	Detection range	Number of real samples	Ref.
Absorbance	Nitrite/Nitrate	Ecoscreen condenser	OSA	–	2 $\mu\text{mol L}^{-1}$	–	50	[75]
Absorbance	Nitrite/Nitrate	EcoScreen condenser	Asthma	0.5–12.5 $\mu\text{mol L}^{-1}$	0.44 $\mu\text{mol L}^{-1}$	2.24–3.55 $\mu\text{mol L}^{-1}$ 4.4–8.8 $\mu\text{mol L}^{-1}$	14	[76]
Absorbance	Nitrite	EcoScreen condenser	COPD	0.5–12.5 $\mu\text{mol L}^{-1}$	0.44 $\mu\text{mol L}^{-1}$	3.76–4.48 $\mu\text{mol L}^{-1}$	13	[77]
Absorbance	Nitrite Nitrate	EcoScreen condenser	Suspected work-related asthma	–	1.0 $\mu\text{mol L}^{-1}$ 2.5 $\mu\text{mol L}^{-1}$	–	125	[78]
Absorbance	Nitrite	Lab made setup	Healthy subjects	16–620 ng mL ⁻¹	1.5 ng mL ⁻¹	24.2–31.3 ng mL ⁻¹	15	[9]
Absorbance	3-NT	EcoScreen condenser	COPD	–	–	3.31–7.77 n mol L ⁻¹	90	[79]
Absorbance	8-Isoprostane IL-6	EcoScreen condenser	Helicobacter pylori infection	–	–	6.13–8.82 pg mL ⁻¹ 1.48–2.91 pg mL ⁻¹ 1.71–1.87 pg mL ⁻¹	68	[80]
Absorbance	3-NT	Glass-condensing device suspended in an ice bath	Asthma, CF, COPD	–	0.025 $\mu\text{mol L}^{-1}$	–	51	[81]
Absorbance	Nitrosolols	Double-jacketed glass tube cooled by air	COPD	–	4 pg mL ⁻¹	7–34 pg mL ⁻¹	30	[82]
Absorbance	8-Isoprostane	EcoScreen condenser	COPD	–	–	4.2–40 pg mL ⁻¹	50	[83]
Absorbance	LTB ₄	EcoScreen condenser	Asthma	–	4 pg mL ⁻¹	2–23.02 pg mL ⁻¹	68	[84]
Absorbance	LTB ₄	RTube	Asthma	–	13 pg mL ⁻¹	2.30–89.23 pg mL ⁻¹	81	[85]
Absorbance	LTB ₄	Turbo-DECCS	Atopic dermatitis	–	–	3.60–12.85 pg mL ⁻¹	56	[86]
Absorbance	LTB ₄	EcoScreen condenser	Asthma	–	4.4 pg mL ⁻¹	47.9–131.9 pg mL ⁻¹	48	[87]
Absorbance	LTB ₄	EcoScreen condenser	Acute exacerbation of COPD	–	–	20.8–22.6 pg mL ⁻¹ 0.4–0.6 pg mL ⁻¹	20	[88]
Absorbance	IL-6	EcoScreen condenser	Asthma	–	30 pg mL ⁻¹	37–54.1 pg mL ⁻¹	27	[89]
Absorbance	PGE ₂	Tube covered with dry ice	COPD	–	8.0 pg mL ⁻¹	7.7–12.2 pg mL ⁻¹	27	[90]
Absorbance	PGF _{2α}	EcoScreen condenser	Asthma	–	10 pg mL ⁻¹	7.8–10.4 pg mL ⁻¹	49	[91]
Absorbance	TXA ₂	EcoScreen condenser	Asthma	–	13 pg mL ⁻¹	20–41.1 pg mL ⁻¹	49	[91]
Absorbance	PGE ₂	EcoScreen condenser	Asthma	–	0.1 pg mL ⁻¹	–	84	[92]
Absorbance	IL-6	EcoScreen condenser	Inflammatory bowel diseases	–	0.16–0.39 pg mL ⁻¹	3.45–3.74 pg mL ⁻¹ 0.0–0.97 pg mL ⁻¹	84	[92]
Absorbance	TNF- α	EcoScreen condenser	IPF	–	–	2.7–49.9 pg mL ⁻¹	14	[93]
Absorbance	CysLT	EcoScreen condenser	NSCLC	0.0–32 pg mL ⁻¹	0.2 pg mL ⁻¹	0.51–1.02 pg mL ⁻¹	44	[94]
Absorbance	TNF- α	Glass collection device cooled with wet ice	Community-acquired pneumonia	–	–	0.0–3.82 pg mL ⁻¹	74	[95]
Absorbance	Cytokines	RTube	Healthy subjects	–	–	0.3–2.3 pg mL ⁻¹	36	[96]
Absorbance	IL-6	Lab made setup	Prolonged hypoxia	–	–	3.32–6.08 $\mu\text{mol/g}$ Hb	15	[97]
Absorbance	GSH	Homemade setup (Face mask attached to heated tube (40 °C) and a glass collector placed in ice at –5 °C.)	Asthma	–	13.6 pg mL ⁻¹	8–12.5 pg mL ⁻¹	52	[98]
Absorbance	TxB ₂	EcoScreen condenser	Asthma	–	–	–	–	[98]

Table 2 (continued)

Method	Targeted biomarkers	Breath collection device	Disease/Disorder	Linear range	LOD	Detection range	Number of real samples	Ref.
Absorbance	TGF- β 1	EcoScreen condenser	NSCLC	–	–	9.6–20.8 ng mL ⁻¹	269	[99]
Absorbance	Protein	EcoScreen condenser	Lung transplant patients	–	4.0 μ g mL ⁻¹	8–125 μ g mL ⁻¹	49	[100]
Absorbance	Pepsin	EcoScreen condenser	GERD	–	0.26 ng mL ⁻¹	0.0–17 ng mL ⁻¹	62	[101]
Absorbance	Pepsin	EcoScreen condenser	GERD	–	–	–	47	[102]
Fluorescence	Nitrite	EcoScreen condenser	Asthma	–	–	0.42–11.8 μ mol L ⁻¹	36	[103]
Fluorescence	Nitrate	EcoScreen condenser	Asthma	–	–	0.72–47.2 μ mol L ⁻¹	20	[103]
Fluorescence	Cytokines	EcoScreen condenser	COPD	–	–	–	130	[104]
Fluorescence	Cytokines	EcoScreen condenser	ALI/ARDS	0.5–150 pg mL ⁻¹	5 pg mL ⁻¹	–	44	[105]
Fluorescence	Cytokines	Double-jacketed glass tube cooled by ice water	Asthma and CF	–	20 pg mL ⁻¹	0.0–7.2 pg mL ⁻¹	80	[106]
Fluorescence	Cytokines	Tedlar gas sample bag	Asthma	–	1.0–2.9 pg mL ⁻¹	1.7–44.8 pg mL ⁻¹	70	[106]
Fluorescence	EPO	EcoScreen condenser	COPD, OSA	–	0.24 mIU mL ⁻¹	–	22	[107]
CL	TNF- α	EcoScreen condenser	Asthma	–	–	–	18	[108]
CL	Nitrite	EcoScreen condenser	Asthma	–	–	2.18 μ mol L ⁻¹	16	[109]
CL	Nitrite	RTube	Asthma	–	–	2.50 μ mol L ⁻¹	16	[109]
CL	Nitrite/Nitrate	U-shaped borosilicate Glass tubes cooled by ice water	Undergoing heart valve surgery	–	–	–	32	[110]
CL	Cytokines	EcoScreen condenser	Asthma	–	1.0–50,000 pg mL ⁻¹	2.6–27.7%	26	[111]
CL	ATP	RTube	COPD	–	25 pmol L ⁻¹	10–1556 pmol L ⁻¹	56	[56]
CL	CEA	EcoScreen condenser	NSCLC	–	–	0.82–3.59 mg L ⁻¹	262	[112]

kits are portable, rapid, and highly specific, as well as being simple to use.

Furthermore, Timms et al. [101] reported an in-house quantitative ELISA based on a monospecific antibody test for the determination of pepsin in the EBC of patients with obstructive lung disease with gastroesophageal reflux disease (GERD) in comparison to patients with obstructive lung disease without concurrent GERD. A significantly higher mean pepsin concentration was found in the patients with obstructive lung disease with GERD compared to those without GERD and healthy controls. Soyer et al. [102] also determined pepsin in the EBC of pediatric asthmatic patients with GERD with a homemade indirect ELISA kit. They reported a negative correlation between NO_x levels and the severity of gastroesophageal reflux, suggesting decreased levels of NO_x in patients with GERD and increased oxidative stress in the airways of these patients. Lee et al. [124] studied the relationship between pepsin concentration and gastroesophageal reflux in bronchiectasis and COPD by spectrophotometry methods (locally developed ELISA).

The disadvantage of these commercial or homemade kits is that they are for single use only. Additionally, competitive ELISA suffers from a limited detection range due to the narrow sensitivity of the antibodies.

Fluorescence

Till date, a number of fluorescence-based methods for sensing non-volatile biomarkers/compounds in EBC have been presented. For example, the determination of NO_2^- concentration in the EBC of asthma patients performed fluorometrically after derivatization with 2,3-diaminonaphthalene (DAN) by Chladkova et al., a reaction that takes place intramolecularly, because the DAN molecule contains two amino groups in the ortho position. This reaction yields 2,3-naphthotriazole (NAT), a stable fluorescent compound with emission at around 430 nm. NO_3^- was assayed after reduction to NO_2^- with enzymatic reductase [103]. Commercial fluorescence-based kits are also available. Balint et al. reported the determination of 3-NT in the EBC of CF patients by fluorescence-based EIA; they found no significant differences in the levels of NO_2^- and NO_3^- in CF patients and normal subjects [114].

Another fluorescence-based method is flow cytometry. The original name for fluorescence-based flow cytometry technology was “pulse cytophotometry.” Modern flow cytometers are able to analyze several thousand particles per second, in real time, and can actively separate and isolate particles with specified properties. Gessner et al. [104] and Robroeks et al. [125] developed a flow cytometry method for the determination of cytokines in COPD patients and in children with asthma and CF, respectively. They observed a significant increase

in the concentration of cytokines in acute exacerbation of COPD, asthma, and CF patients compared to healthy volunteers. However, they concluded that cytokine concentration did not differ significantly in children with asthma vs. CF. Similar studies have been performed by Sack et al. [105] and Rosias et al. [106] for the determination of cytokines in acute lung injury (ALI) cases and in asthma patients, respectively. Sack et al. reported that in patients with ALI, the cytokine levels for all the investigated cytokines were higher in comparison to those in healthy subjects, and Rosias et al. found a significant decrease in $\text{TNF-}\alpha$ in the EBC of atopic asthmatic children compared to children with viral wheeze and healthy controls. Schumman et al. [107] also reported the detection of erythropoietin (EPO) in the EBC of patients with COPD, OSAS, and OSA by the cytophotometry method. However, they found no significant difference in the EPO levels of patients with COPD compared to non-COPD (OSAS, OSA, and healthy) subjects. Their team also detected $\text{TNF-}\alpha$ simultaneously; $\text{TNF-}\alpha$ was at significantly higher concentrations in COPD patients.

Chemiluminescence

Chemiluminescence (CL) is one of the most attractive methods with a number of clear advantages, such as its high sensitivity, wide linear range, the simplicity of the instrumentation used, and the lack of interference from background scattered light [126]. Pasha et al. [108] determined $\text{NO}_2^-/\text{NO}_3^-$ levels in the EBC of mild persistent asthmatic patients treated with a low dose of fluticasone propionate/salmeterol (FSC) by initial conversion of $\text{NO}_2^-/\text{NO}_3^-$ to NO and then NO was measured by a gas-phase chemiluminescence reaction between NO and ozone. They reported a rapid reduction in airway inflammation and airway hyper-responsiveness in asthmatic patients following a week of low-dose combination therapy with FSC. Furthermore, Hussain et al. [109] used the NO analyzer for the determination of $\text{NO}_2^-/\text{NO}_3^-$ levels in subjects with asthma. A similar study for $\text{NO}_2^-/\text{NO}_3^-$ determination was performed by Arcêncio et al. [110] for the EBC of patients admitted for heart valve surgery. They reported that in patients with postoperative respiratory complications, the postoperative levels of NO_x were significantly higher in EBC from the fourth postoperative hour as compared to those who experienced uneventful postoperative periods. Matsunaga et al. [111] used a CL-based membrane protein array for a simultaneous analysis of cytokine expression in asthmatic subjects. The results showed that IL-4, IL-8, IL-17, $\text{TNF-}\alpha$, RANTES, IFN- γ -inducible protein 10, $\text{TGF-}\beta$, macrophage inflammatory protein (MIP) 1 α , and MIP-1 β exhibited significantly up-regulated expression in asthmatic airways compared to those of healthy subjects. Lázár et al. determined ATP in

the EBC of COPD [56] and asthma [127] patients by using the luciferin–luciferase assay. Luciferase is an enzyme that oxidizes luciferin in the presence of ATP. During the reaction, light is produced and it can be detected by using a luminometer. They reported that the ATP concentration was similar in COPD, asthma, and non-smoking and smoking healthy individuals. Chen et al. [112] used a CL microparticle immunoassay for the determination of CEA in EBC samples collected from patients with NSCLC and healthy individuals. They demonstrated that the CEA levels in the EBC of the NSCLC group were higher as compared to those of the healthy group.

Electrochemical methods

Electrochemical sensors take a biological input and convert it to an electrical signal. In the case of electrochemical sensors for breath analysis, the electrical current produced on the sensor electrode is directly proportional to the concentration of the chemical in the sample. Some reported studies that used electrochemistry methods in EBC are summarized in Table 3, and the following text provides a brief explanation for each applied method:

Effros [128] and Zacharasiewicz [129] used ion-selective electrodes (ISE) for the determination of sodium, potassium, and chloride ions in the EBC of subjects with OSAS, asthma, and CF. Electrochemical sensors for other small molecules in the breath, such as lactate, have also been developed. Karyakina et al. [130] reported a flow-injection (FI) electrochemical system coupled with an ion-exchange pre-concentration column (filled with Strata SAX sorbent-silica gel with quaternary ammonium substituents) for lactate sensing in the EBC of pulmonary patients. The lactate concentration was at the level of $(1.5\text{--}3) \times 10^{-4} \text{ mol L}^{-1}$ for both healthy subjects and pulmonary patients.

These types of sensors offer the possibility of miniature or hand-held sensing capabilities; but so far, they have been limited to the detection of one or a few compounds of interest in biological samples. Melker et al. [133] developed electrochemical- or coulometric-based sensing or monitoring devices to analyze EBC for quantifying glucose concentrations in patients with severe respiratory distress. They found that the concentrations of glucose in the EBC were correlated to the glucose concentrations in the individual subject's body, in particular in blood, and suggested that the developed devices could be applied to improve prognosis and/or reduce the adverse side effects associated with a disease state or condition in a subject. Karyakin et al. [131] also used an electrochemical sensor for monitoring the glucose level in the EBC of diabetic patients. They reported that breath condensate glucose levels correlated positively with blood glucose levels. Carlsen et al. [134]

Table 3 Characteristics of included studies on exhaled non-volatile biomarkers/compounds in various diseases by electrochemical methods

Method	Targeted markers	Breath collection device	Disease/Disorder	Linear range	LOD	Detection range	Number of real samples	Ref.
Electrochemistry	Sodium chloride potassium	Glass condenser cooled by ice water	OSA	–	–	91 mmol L ⁻¹ 102 mmol L ⁻¹	23	[128]
Electrochemistry	Sodium chloride	EcoScreen condenser	Asthma and CF	–	1 mmol L ⁻¹	60 mmol L ⁻¹ 1.6–5.9 mg L ⁻¹ 3.7–5.4 mg L ⁻¹	24	[129]
Electrochemistry	Lactate	EcoScreen condenser	Pulmonary patients	0.1–20 μmol L ⁻¹	0.02 μmol L ⁻¹	150–300 mol L ⁻¹	120	[130]
Electrochemistry	Glucose	EcoScreen condenser	Diabetes	–	0.01 mmol L ⁻¹	11.5 μmol L ⁻¹	30	[131]
Electrochemistry	pH	RTube	Asthma	–	–	7.80–8.40	38	[132]

and Bikov et al. [132] investigated the effect of physical exercise on EBC pH during the development of exercise-induced bronchoconstriction (EIB) in asthmatic individuals. They reported that the decrease in EBC pH was related to the development and severity of EIB, thereby supporting the causal role of airway pH in EIB.

Separation methods

The detection and quantification of extraordinarily low concentrations of non-volatile biomarkers/compounds in EBC requires highly sensitive analytical methods. During the last decade, separation methods have become the method of choice for the trace analysis of various compounds contained in complex biological matrices. In order to achieve such a relatively low detection limit, it is often necessary to incorporate pre-concentration steps (solid-phase extraction, lyophilization, etc.). A number of separation methods developed for the analysis of non-volatile biomarkers/compounds in the EBC of pulmonary patients have been extracted from the literature, and they are classified and explained in brief (Table 4).

Chromatography

Ion chromatography

Ion chromatography (IC) is a sensitive and well-tested separation method that allows the determination of multiple anions or cations in a time scale of minutes, and no sample preparation is needed for making condensate samples. A great advantage of IC is that cations or anions can be separated and quantified simultaneously. An IC method with UV detection was reported for the determination of $\text{NO}_2^-/\text{NO}_3^-$ [103] in well-controlled asthma patients by Chladkova et al.; they reported that the mean level of $\text{NO}_2^-/\text{NO}_3^-$ in the EBC of healthy subjects and patients did not differ. An IC method with conductivity detection was reported for the determination of sodium and potassium [135] in the EBC of healthy volunteers by Svensson et al.; they found that the level of sodium decreased during the day, while the levels of potassium fluctuated.

Greenwald et al. developed an IC method for the simultaneous measurement of EBC's ionic composition, including inorganic anions, cations, and organic acids [172], before and after exercise in adolescent athletes. They reported that exercise resulted in a decrease and an increase in the concentration of propionate and urea, respectively. The source of this variation is gas-phase diffusion from epithelial and oral surface liquids.

In addition, it is possible to determine ammonium by using IC [172], which may be valuable in the determination of other biomarkers in EBC, for example, pH. Normally, the pH of EBC is within the range of 7–8, typical of that for human

blood. Any decreases in pH that are observed in some lung disorders largely reflect the effects of these diseases on the production of NH_3 in the mouth and the efficiency with which NH_3 is collected in the condenser. NH_3 is derived principally from the catalytic degradation of urea in the mouth and it is delivered as ammonia gas in the air exhaled to the condenser. NH_3 is trapped as NH_4^+ in the condenser. It is therefore unlikely that the EBC pH will provide a reliable index of the change in the lung pH [135].

Liquid chromatography

Liquid chromatography (LC) with various applicable detectors is an analytical method used to separate, identify, and quantify each component in a mixture. The simplest LC method uses isocratic reversed-phase high-pressure liquid chromatography (RP-HPLC) and UV absorbance detection. Montuschi et al. used RP-HPLC-UV for the determination of LTB_4 in the EBC of subjects with different lung diseases to qualitatively validate an EIA to measure LTB_4 [136]. Folesani et al. [137] reported a HPLC-UV method for the determination of urea as a normalization factor for non-volatile biomarkers/compounds in EBC. They measured urea EBC concentrations in different clinical conditions and reported that the concentrations were unaffected by the three chronic airway diseases examined—COPD, asthma, and CF. Csoma et al. [138] determined adenosine in the EBC of both asthmatic and control subjects by using a HPLC-UV method. They indicated that physical exercise resulted in bronchoconstriction along with a significant increase of adenosine levels in the EBC of asthmatic patients, but not in that of the healthy subjects. Lázár et al. determined ATP in the EBC of COPD patients [56] to qualitatively validate a luciferin–luciferase fluorometric assay.

However, the main drawback of HPLC-UV methods is the lack of adequate selectivity and sensitivity. For compensation, usually a sample preparation step is applied prior to the injection of the biological sample into the system. Khoubnasabjafari et al. [10] used a liquid–liquid extraction (LLE) procedure to extract/pre-concentrate methadone from the EBC of patients receiving methadone maintenance treatment (MMT) prior to sample injection of HPLC-UV. In a similar study [11], they used two amplified sample preparation procedures, namely dispersive liquid–liquid microextraction (DLLME) and ultrasonic liquid–liquid microextraction (ULLME). They reported a good correlation between the methadone concentration measured in patients and the daily intake of methadone ($R = 0.997$) by using the developed method.

Compared to a UV detector, FD may be applied as a selective detector for HPLC methods. Rihák et al. [139] measured the levels of biomarkers of nitrosative stress (i.e., NO_2^- and NO_3^-) in the EBC of adult patients of asthma, COPD, and idiopathic pulmonary fibrosis (IPF) by using HPLC-FD.

Table 4 Characteristics of included studies on exhaled non-volatile biomarkers/compounds in various diseases by separation methods

Method	Targeted markers	Breath collection device	Disease/Disorder	Linear range	LOD	Detection range	Number of real samples	Ref.
IC	Nitrite	EcoScreen condenser	Asthma	0.4–20 $\mu\text{mol L}^{-1}$	–	0.42–11.8 $\mu\text{mol L}^{-1}$	36	[103]
IC	Nitrate	EcoScreen condenser	Healthy subjects	0.5–50 $\mu\text{mol L}^{-1}$	–	0.72–47.2 $\mu\text{mol L}^{-1}$	20	[135]
	Sodium	EcoScreen condenser		0.02–0.14 mmol L^{-1}	0.002 mg L^{-1}	0.02–0.14 mmol L^{-1}	10	
RP-HPLC-UV	Ammonium Potassium	EcoScreen condenser	Different lung diseases	0.06–0.22 mmol L^{-1}	0.001 mg L^{-1}	0.06–0.22 mmol L^{-1}	22	[136]
	LTB ₄	EcoScreen condenser		0.12–0.46 mmol L^{-1}	0.003 mg L^{-1}	0.12–0.46 mmol L^{-1}	22	
HPLC-UV	Urea	Turbo-DECCS	COPD, asthma, and CF	10–250 pg mL^{-1}	10 pg mL^{-1}	248–1018 pg mL^{-1}	–	[137]
HPLC-UV	Methadone	Lab made cooling trap system	MMT	0.25–1 $\mu\text{mol L}^{-1}$	37.5 pmol L^{-1}	0.7–4 $\mu\text{mol L}^{-1}$	53	[10]
HPLC-UV	Adenosine	EcoScreen condenser	Asthma	0.5–500 $\mu\text{g mL}^{-1}$	–	229–839 $\mu\text{g L}^{-1}$	21	[138]
HPLC-FD	Nitrite	EcoScreen condenser	Asthma, COPD, and IPF	3–48 nmol L^{-1}	–	4–33 nmol L^{-1}	106	[139]
LC-MS/MS	3-NT	Glass chambers cooled with ice	Asthma	–	0.2 nmol L^{-1}	–	38	[32]
SPE-LC-MS/MS	3-NT	EcoScreen condenser	Healthy non-smokers and smokers	3.0–200 nmol L^{-1}	–	< 3.9–184 pg mL^{-1}	20	[33]
LC-MS/MS	LTB ₄	EcoScreen condenser	Asthma	10–500 pg mL^{-1}	100 pg mL^{-1}	255.1–586.7 pg mL^{-1}	10	[140]
LC-MS/MS	LTB ₄	EcoScreen condenser	Asthma	10–500 pg mL^{-1}	–	87.3–314.7 pg	67	[141]
HPLC-MS	8-Isoprostane LTs	EcoScreen condenser	Silicosis	–	1 pg mL^{-1}	43–73.6 pg mL^{-1}	85	[142]
LC-MS/MS	Eicosanoids	RTube	Asthma and COPD	–	1–2 pg mL^{-1}	14.7–69 pg mL^{-1}	87	[143]
LC-MS/MS	Eicosanoids	EcoScreen condenser	Healthy subjects	10–500 pg mL^{-1}	0.05–1 pg mL^{-1}	0.0–7.4 pg mL^{-1}	–	[144]
LC-MS/MS	ATP	RTube	CF	0.1–100 nmol L^{-1}	1 fmol	–	14	[145]
LC-MS/MS	AMP	EcoScreen condenser	COPD and pneumonia	0.1–100 nmol L^{-1}	1 fmol	–	21	[67]
	Urea	EcoScreen condenser		0.1–100 $\mu\text{mol L}^{-1}$	5 pmol	35–110 pg mL^{-1}	44	
LC-MS/MS	CML	EcoScreen condenser	Asbestosis	10–300 pg mL^{-1}	5 pg mL^{-1}	15.9–101.3 pg mL^{-1}	20	[146]
LC-MS/MS	8-Isoprostane	EcoScreen condenser	Asbestosis and silicosis	0–250 pg mL^{-1}	1 pg mL^{-1}	43–66.5 pg mL^{-1}	–	[146]
LC-MS/MS	O-Tyr-hydroxy-2-deoxy-guanosine	EcoScreen condenser	Healthy subjects	25–250 pg mL^{-1}	7.0 pg mL^{-1}	14–51.5 pg mL^{-1}	–	[147]
LC-MS/MS	Lactate	EcoScreen condenser	Healthy subjects	0.5–100 $\mu\text{mol L}^{-1}$	–	–	–	[148]
LC-MS/MS	Amino acids	Turbo-DECCS condenser	Pediatric leukemia patients	0.05–400 ng mL^{-1}	0.05–0.5 ng mL^{-1}	0.15–173.8 ng mL^{-1}	Not mentioned	[148]

Table 4 (continued)

Method	Targeted markers	Breath collection device	Disease/Disorder	Linear range	LOD	Detection range	Number of real samples	Ref.
LC-MS/MS	Fatty acid metabolites	RTube	Asthma	–	–	15–180 pmol L ⁻¹	11	[149]
LC-MS/MS	Proteolytic peptides	RTube	Asthma	–	–	–	70	[150]
LC-MS/MS	Methadone	EcoScreen condenser	MMT	100–2000 pg mL ⁻¹	–	23.6–275 pg min ⁻¹	14	[151]
Nano HPLC/HRMS	Proteomic and metabolomic studies	Cooling trap	Congenital pneumonia, and left-sided congenital diaphragmatic hernia	–	–	–	24	[152]
HILIC/MS/MS	3-NT Tyr Trans-hydroxyproline proline	EcoScreen condenser	Healthy subjects	0.01–1 µg L ⁻¹ 1.0–100 µg L ⁻¹ 0.01–1 µg L ⁻¹ 1.0–100 µg L ⁻¹	5 ng L ⁻¹ 0.5 µg L ⁻¹ 5 ng L ⁻¹ 0.5 µg L ⁻¹	<5–1686.5 µg L ⁻¹ 2.4–99.1 µg L ⁻¹ <5–516.5 µg L ⁻¹ 2.8–51.9 µg L ⁻¹	27	[153]
HILIC/MS/MS	CML Lysine Glucose	EcoScreen condenser RTube	Dialysis patients CFRD	10–1000 pg mL ⁻¹ 1.0–100 ng mL ⁻¹ 0.09–50 µmol L ⁻¹ 0.2–50 µmol L ⁻¹ 0.4–80 µmol L ⁻¹	– – 0.07 µmol L ⁻¹ 0.03 µmol L ⁻¹ 0.1 µmol L ⁻¹	13–205 pg mL ⁻¹ 3.6–46.4 ng mL ⁻¹ –	20 6	[154] [155]
UHPLC/TOF-MS UHPLC/QqQ-MS/MS TWIMS-TOF-MS UPLC-MS/MS UPLC-HRMS	Arginine metabolom Metabolic patterns	Turbo-Deccs EcoScreen condenser	Asthma Patients of ICU	– –	– –	0.2–133 nmol L ⁻¹ –	33 26	[58] [156]
GC-TOF/MS	Metabolomic analysis	EcoScreen condenser	Healthy subjects	–	–	–	50	[157]
GC-NICI-MS	Eicosanoids	EcoScreen condenser	Healthy subject	–	–	0.13–250.4 pg mL ⁻¹	58	[158]
GC/MS/MS	3-NT	EcoScreen condenser	Asthma	0–5 nmol L ⁻¹	0.56 pmol L ⁻¹	14–212 pmol L ⁻¹	18	[34]
GC-NICI-MS	3-NT	EcoScreen condenser	Asthma CF	0–9 nmol L ⁻¹	0.22 nmol L ⁻¹	0.54–6.8 nmol L ⁻¹	42	[36]
GC/NICI-MS GC/MS	8-Isoprostane 8-Isoprostane	Turbo-DECCS Collection tubing cooled with ice bath	Asthma ARDS/ALI	– –	– –	32.8–68 pg mL ⁻¹ 8–78 pg mL ⁻¹	20 22	[159] [109]
GC/MS	LTD ₄ LTE ₄ LTB ₄	EcoScreen condenser	Asthma	–	1.0 pg mL ⁻¹	3–52 pg mL ⁻¹ 51–192 pg mL ⁻¹ 65–369 pg mL ⁻¹	161	[49]
GC/MS	Fatty acids	EcoScreen condenser	Tuberculosis patients	–	–	–	–	[160]
CE-DAD	Methadone	Lab made cooling trap system	MMT	0.3–5 µg mL ⁻¹	–	0.6–2 µg mL ⁻¹	4	[14]

Table 4 (continued)

Method	Targeted markers	Breath collection device	Disease/Disorder	Linear range	LOD	Detection range	Number of real samples	Ref.				
CE-DAD	Methadone	Lab made cooling trap system	MMT	0.15–5 µg mL ⁻¹	–	0.16–1.06 µg mL ⁻¹	7	[15]				
CE-C ₄ D	Chloride	Lab made setup (aluminum cooling cylinder)	Asthma	5–200 µmol L ⁻¹	0.8 µmol L ⁻¹	–	15	[161]				
	Nitrite			2.5–100 µmol L ⁻¹	1.4 µmol L ⁻¹	–						
	Nitrate			2.5–100 µmol L ⁻¹	1.3 µmol L ⁻¹	–						
	Sulfate			2.5–100 µmol L ⁻¹	0.8 µmol L ⁻¹	–						
	Acetate			12.5–500 µmol L ⁻¹	2.7 µmol L ⁻¹	–						
	Lactate			5–200 µmol L ⁻¹	2.9 µmol L ⁻¹	–						
	Propionate			12.5–500 µmol L ⁻¹	2.6 µmol L ⁻¹	–						
	Butyrate			5–200 µmol L ⁻¹	3.7 µmol L ⁻¹	–						
	Ammonium			2–200 µmol L ⁻¹	0.5 µmol L ⁻¹	–						
	Potassium			1–100 µmol L ⁻¹	1.3 µmol L ⁻¹	–						
	Calcium			1–100 µmol L ⁻¹	0.6 µmol L ⁻¹	–						
	Sodium			1–100 µmol L ⁻¹	0.6 µmol L ⁻¹	–						
	Magnesium			1–100 µmol L ⁻¹	0.8 µmol L ⁻¹	–						
	CE-C ₄ D			Chloride	Lab made setup (aluminum cooling cylinder)	CF			5–100 µmol L ⁻¹	separation 10 s/30 s	–	20
CE-C ₄ D	Nitrite	Lab made setup (aluminum cooling cylinder)	CF	1.25–25 µmol L ⁻¹	0.126/0.080 µmol L ⁻¹	–	20	[162]				
	Nitrate			1.25–25 µmol L ⁻¹	0.153/0.095 µmol L ⁻¹	–						
	Sulphate			2.5–50 µmol L ⁻¹	0.106/0.062 µmol L ⁻¹	–						
	Acetate			10–200 µmol L ⁻¹	0.068/0.044 µmol L ⁻¹	–						
	Lactate			10–200 µmol L ⁻¹	0.571/0.371 µmol L ⁻¹	–						
	Propionate			10–200 µmol L ⁻¹	0.392/0.246 µmol L ⁻¹	–						
	Butyrate			10–200 µmol L ⁻¹	0.525/0.321 µmol L ⁻¹	–						
	Formate			10–200 µmol L ⁻¹	0.592/0.363 µmol L ⁻¹	–						
	Oxalate			0–100 µmol L ⁻¹	0.319 µmol L ⁻¹	–			–	–	–	–
	Formate			0–100 µmol L ⁻¹	0.4 µmol L ⁻¹	–			–	–	–	–
	Glycolate			0–100 µmol L ⁻¹	0.6 µmol L ⁻¹	–			–	–	–	–
	Oxalate			0–100 µmol L ⁻¹	1.3 µmol L ⁻¹	–			–	–	–	–
	Formate			0–100 µmol L ⁻¹	0.8 µmol L ⁻¹	–			–	–	–	–
	DOI-CE-C ₄ D			Chloride	Lab made setup (aluminum cooling cylinder)	COPD, asthma, pulmonary fibrosis, sarcoidosis, and CF			0–50 µmol L ⁻¹	1.4 µmol L ⁻¹	0.68–67.75 µmol L ⁻¹	60
Nitrite	0–50 µmol L ⁻¹	1.3 µmol L ⁻¹	0.3–11.34 µmol L ⁻¹									
Nitrate	0–50 µmol L ⁻¹	0.8 µmol L ⁻¹	0.33–17.72 µmol L ⁻¹									
Sulfate	0–50 µmol L ⁻¹	0.8 µmol L ⁻¹	0.16–2.56 µmol L ⁻¹									
Acetate	5–200 µmol L ⁻¹	2.7 µmol L ⁻¹	16.13–176 µmol L ⁻¹									
Lactate	0–150 µmol L ⁻¹	2.9 µmol L ⁻¹	0–112.09 µmol L ⁻¹									
Propionate	0–100 µmol L ⁻¹	2.6 µmol L ⁻¹	4–78.88 µmol L ⁻¹									
Butyrate	0–50 µmol L ⁻¹	3.7 µmol L ⁻¹	0–12.60 µmol L ⁻¹									
Ammonium	10–1000 µmol L ⁻¹	0.5 µmol L ⁻¹	128.7–663 µmol L ⁻¹									
Potassium	0–100 µmol L ⁻¹	1.3 µmol L ⁻¹	1.03–88.69 µmol L ⁻¹									
Calcium	0–100 µmol L ⁻¹	0.6 µmol L ⁻¹	2.07–31.31 µmol L ⁻¹									
Sodium	0–150 µmol L ⁻¹	0.6 µmol L ⁻¹	1.31–29.80 µmol L ⁻¹									
Magnesium	0–50 µmol L ⁻¹	0.8 µmol L ⁻¹	0.33–1.65 µmol L ⁻¹									

Table 4 (continued)

Method	Targeted markers	Breath collection device	Disease/Disorder	Linear range	LOD	Detection range	Number of real samples	Ref.
DOI-CE-C ₄ D	Ammonium	Lab made setup (cooled by ice)	COPD	1–1000 µmol L ⁻¹	0.54 µmol L ⁻¹	0–1000 µmol L ⁻¹	75	[165]
	Potassium			1–250 µmol L ⁻¹	0.33 µmol L ⁻¹	1–150 µmol L ⁻¹		
	Sodium			1–250 µmol L ⁻¹	0.55 µmol L ⁻¹	9–200 µmol L ⁻¹		
	Calcium			1–250 µmol L ⁻¹	0.75 µmol L ⁻¹	20–300 µmol L ⁻¹		
	Magnesium			1–250 µmol L ⁻¹	0.47 µmol L ⁻¹	0–10 µmol L ⁻¹		
	Chloride			1–250 µmol L ⁻¹	0.34 µmol L ⁻¹	0–200 µmol L ⁻¹		
	Nitrite			1–25 µmol L ⁻¹	0.63 µmol L ⁻¹	0–10 µmol L ⁻¹		
	Nitrate			1–25 µmol L ⁻¹	0.56 µmol L ⁻¹	0–10 µmol L ⁻¹		
	Sulfate			1–25 µmol L ⁻¹	0.38 µmol L ⁻¹	0–10 µmol L ⁻¹		
	Thiocyanate			1–250 µmol L ⁻¹	1.25 µmol L ⁻¹	–		
	Acetate			1–250 µmol L ⁻¹	1.25 µmol L ⁻¹	5–200 µmol L ⁻¹		
	Lactate			1–250 µmol L ⁻¹	1.07 µmol L ⁻¹	5–250 µmol L ⁻¹		
	Phosphate			1–250 µmol L ⁻¹	1.15 µmol L ⁻¹	1–150 µmol L ⁻¹		
	GSH			1–250 µmol L ⁻¹	0.18 nmol L ⁻¹	0–1.7 nmol L ⁻¹		
CE-LIF		Lab made setup	Healthy subject				5	[166]
2-DE	Protein pattern	Lab made setup	Healthy subject	–	–	–	5	[167]
1-DE/2-DE/µ-HPLC/MS	Protein profile analysis	RTube	Pulmonary emphysema associated with AATD	–	–	–	20	[17]
Gel electrophoresis coupled with MS/MS	Keratin	EcoScreen condenser	Healthy smokers	–	–	–	46	[168]
		RTube	Healthy subjects	–	–	–	20	[169]
Gel electrophoresis–MS	Protein profile analysis	EcoScreen condenser	ALI/ARDS	–	–	8.3–9.3 pg mL ⁻¹ 11.3 pg mL ⁻¹	40	[170]
	IL 6 IL 8	RTube	Healthy smokers and COPD AATD	–	–	6.8–21.7 µg mL ⁻¹	83	[171]
SDS-PAGE-WB	Cytokines							
SELDI-MS								

They reported that NO_2^- concentration increased in adult corticosteroid-naïve patients with asthma, active IPF, and exacerbation of COPD, whereas in stable COPD, its concentration was comparable to that in healthy subjects.

Electrochemical detection (ECD) with HPLC has the potential to be more selective than the HPLC-UV or HPLC-FD methods. Celio et al. [36] used a HPLC-ECD method for the determination of 3-NT in the EBC of children with asthma, CF, and healthy controls. However, they reported that this method was not suitable for the analysis of free 3-NT in EBC because many of the samples showed concentration levels close to or below the detection limit.

As already described, due to low selectivity of the above-mentioned HPLC detectors, these methods are unable to detect concentrations of non-volatile biomarkers/compounds of interest and require complicated and time-consuming pre-treatment procedures to eliminate interfering substances from the samples. One of the analytical challenges of the HPLC analysis of biological samples is to obtain the necessary selectivity. One straightforward way to achieve this is to couple HPLC with a MS method. This combines the flexibility of LC separation with the robust and unambiguous identification of the MS instrument, especially when used in the MS/MS mode [173]. The sensitivity, selectivity, and superior ability to separate and detect low concentrations, i.e., parts per billion (ppb) and parts per trillion (ppt), of the chemicals offered by MS are likely to be instrumental in the future development of diagnostic biomarker methods for EBC.

Furthermore, LC-MS offers an ideal method for the simultaneous detection of hundreds or even thousands of compounds that differ greatly in size and polarity in the same EBC sample. Other methods of detection of breath compounds, such as some immunoassays and sensors, are very specific for the detection of only one compound. Therefore, LC-MS is considered to be the gold standard, especially for determining low-molecular-weight non-volatile compounds in human breath [174], since they are highly specific and generally sufficiently sensitive. For example, for the analysis of 3-NT in EBC, the highest sensitivity was obtained by using tandem MS. Baraldi et al. [32] measured 3-NT by LC-MS/MS in isotopic dilution in the EBC of asthmatic and healthy children. They reported that 3-NT formation was high in asthmatic children, indicating that nitrosative processes were more active in the lungs of asthma patients.

Sometimes, the limit of detection (LOD) and the limit of quantitation (LOQ) are inadequate, even by the LC-MS methods, to allow a quantitative determination of biomarkers in most biological samples. In order to improve sensitivity, solid-phase extraction (SPE) needs to be used for sample clean-up and pre-concentration prior to analysis. Gönen et al. [33] used a clean-up and pre-concentration step before LC-MS/MS for 3-NT measurement. SPE was performed using C18 POLAR RP cartridges with reverse-phase material.

Montuschi et al. [140] also used two coupled methods, i.e., ion trap LC-MS and LC-MS/MS, for measuring LTB_4 in the EBC of asthmatic patients on anti-inflammatory treatment and asthmatic patients who were not on anti-inflammatory drugs with $\text{LTB}_4\text{-d}_4$ as the internal standard. They reported that exhaled LTB_4 was detected only in the asthmatic patients who were not on anti-inflammatory therapy. Other studies performed by LC-MS methods include the determination of LTB_4 [141], 8-isoprostane [142, 175, 176], the profile of eicosanoid lipid mediators [143, 144], adenylyl purines (ATP, AMP) [145], CML [67], O-Tyr [146], lactate [147], amino acids [148], fatty acid metabolites (9,12,13-trihydroxyoctadecenoic acid (9,12,13-TriHOME), 9,10,13-TriHOME, 12,13-dihydroxyoctadecenoic acid (12,13-DiHOME), 12-hydroxyeicosatetraenoic acid (12-HETE), 12(13)-epoxy octadecanoic acid (12(13)-EpOME)) [149], proteins [150], methadone [151], and proteomic and metabolomic studies [152], which along with some of their analytical details are listed in Table 4.

Hydrophilic interaction liquid chromatography (HILIC) as a variant of normal-phase liquid chromatography provides an alternative approach to effectively separate small polar compounds at polar stationary phases [177]. Like normal-phase LC, HILIC employs traditional polar stationary phases [178], but the mobile phase used is similar to those employed in the reverse-phase LC mode [179]. HILIC also allows the analysis of charged substances, as in IC. Some of the reported studies on EBC for the determination of non-volatile biomarkers/compounds with this method are the simultaneous determination of 3-NT and its precursor Tyr and also trans-hydroxyproline and its precursor proline in EBC as markers of nitrosative/inflammatory stress as well as fibrotic alterations in the lung in the EBC of smokers and healthy subjects [153], and the simultaneous quantifications of CML and lysine as a precursor of CML in the EBC of patients undergoing dialysis [154].

Most HPLC instrument and column manufacturers have introduced ultra-high-pressure liquid chromatography (UHPLC) in recent years [180]. UHPLC substantially enhances performance over conventional HPLC and it is particularly attractive in method-development situations, which demand quick run time and rapid responses to changes in column/mobile-phase conditions [181]. Some measurements for biomarkers in EBC have been performed using this method, including glucose quantification in the EBC of cystic fibrosis-related diabetes (CFRD) patients [155] with three MS-based methods: UHPLC coupled to either time-of-flight (TOF) MS or triple quadrupole (QqQ) tandem MS (MS/MS) and traveling wave ion mobility spectrometry (TWIMS) with TOF-MS detection, Arg metabolome with a UHPLC-MS/MS method enriched online in a trap column [58] in the EBC of asthmatic adolescents, and metabolic patterns with UHPLC coupled with high-resolution mass spectrometry (HRMS)

[156] in the EBC of patients suffering from shock with acute respiratory failure in a pilot study.

Metabolomics is a powerful method that provides metabolic patterns from a combination of small molecules. The analytical methods detect metabolites across a large spectrum of concentrations, polarities, and masses [182–184]. The metabolome may reflect the evolution of physiological states in response to environmental variations or aggressions, such as microbial infections. These approaches enable the semi-quantification of metabolites up to 1500 Da, including organic acids, lipids, amino acids, carbohydrates, peptides, vitamins, steroids, xenobiotics, and many others.

Gas chromatography

Gas chromatography (GC) is a common type of chromatography used for separating and analyzing compounds that can be vaporized without decomposition. GC coupled with MS is very useful for analyzing small molecules with molecular masses of up to about 1000 Da. The GC front end allows for pre-separation of chemicals prior to MS identification. The signal-to-noise ratio (S/N) can be increased by several times by using fluorine-containing derivatization agents and ionization conditions such as negative ion electron capture (NIEC) [173].

However, one challenge associated with the GC-MS analysis of breath is the removal of water from the EBC samples, as its presence could damage the capillary column of GC. So, human breath traditionally requires the isolation and separation of biomarker chemicals from the background. By taking advantage of the polarity of the extractant, LLE can be used to separate compounds from the aqueous matrix before GC-MS characterization.

Alternatively, SPE can be used to concentrate the EBC components and remove matrix interfering components [185]. Peralbo-Molina and his co-workers reported a method for an untargeted metabolomics analysis of EBC in healthy subjects based on GC-TOF-MS profiling in a high-resolution mode. Two different sample preparation methods, i.e., LLE and SPE, were compared for analyzing this biofluid. They found that LLE provided more information on EBC composition [157]. Sanak et al. [158] used GC-negative ion chemical ionization (NICI)-MS for a targeted lipidomics analysis of EBC in healthy subjects. They measured 20 different eicosanoid compounds, representing major arachidonic acid lipoxygenation and cyclooxygenation pathways, by this method. Since these mediators are non-volatile, the EBC extracts were prepared by a three-step derivatization to pentafluorobenzyl ester, trimethylsilyl esters, and methoxyoxime, which modified the carboxyl, hydroxyl, and keto groups of eicosanoids.

Other biomarkers measured by GC-MS include 3-NT [34–36], 8-isoprostane [159, 186], LTB₄, Cys-LTs [49], TMA [187], and fatty acids [160] (see Table 4).

Electrophoresis

Capillary electrophoresis

Capillary electrophoresis (CE) is a family of electrokinetic separation methods performed with submillimeter diameter capillaries and in micro- and nano-fluidic channels. CE surpasses other analytical methods in terms of separation efficiency, short analysis time, high resolution, low sensitivity to sample matrix, and low consumption of sample (about 10–20 nL), solvents, and energy [188].

In the context of CE, different detectors have been reported for determining non-volatile biomarkers/compounds in EBC. Some of the used detectors are reviewed here:

- CE system with diode array detection (DAD): Due to a high rate of data collection, DAD is superior over single-wavelength detection. It is proven that DAD detection can help in developing separation methods, identifying peaks according to their UV-visible spectra, and checking peak purity [189]. Hamidi et al. [14] used CE-DAD in stacking mode for the determination of methadone in the EBC of patients receiving MMT by direct injection of EBC samples. In another study [15], they also used an electro-driven separation method for the quantification of methadone enantiomers in patients receiving racemic methadone. They found no significant differences in the concentrations of *R*- and *S*-methadone in the investigated EBC samples, thereby revealing no stereo-selective penetration of methadone from systemic blood flow to the lung lining fluid. In this study, the inter-correlation of methadone concentrations between EBC and serum or urine was also investigated and a relatively poor correlation was reported between the methadone levels in EBC and those in the serum ($R = 0.45$) or urine ($R = 0.44$) samples.
- CE system with contactless conductivity detection (C⁴D): C⁴D is one of the best detection modes for the analysis of compounds with low molecular weight. Greguš et al. [161] used a CE-C⁴D system for an ionic profile analysis of EBC from a single breath. They reported that the breathing pattern had a significant impact on the concentration of analytes in the collected EBC. As the ventilatory pattern can be easily controlled during a single exhalation, conducting the analysis for a single breath provides a method with applicability in point-of-care diagnostics. In another study, this research group developed a portable CE-C⁴D system for an ionic profile (of nitrosative stress markers, including NO₂⁻ and NO₃⁻) analysis in EBC from a single exhalation [162]. A miniature EBC sampler connected online was used to collect EBC, which was analyzed immediately after collection. The instrument was lightweight (< 5 kg), and all the

necessary parts were placed in a plastic brief-case, allowing for the hydrodynamic injection of small sample volumes (10 μL), and could continuously operate for at least 10 h, with peak area repeatability below 5%. This method presents a fast screening tool that can be applicable in clinical practice. Kubáň et al. [163] used a CE- C^4D for the separation of oxalate, formate, and glycolate as the toxic metabolites after methanol and ethylene glycol intoxication in the EBC of people with suspected intoxication. They found that the EBC of healthy individuals contained small amounts of the analytes, all within physiological ranges. However, in all the samples of the patients intoxicated with methanol, elevated concentrations of formate (8.6–16.6 mmol L^{-1}) were found.

The significant advantage of CE is its ability to analyze anions and cations simultaneously in a single run by applying dual-opposite injection capillary electrophoresis (DOI-CE) [190]. DOI-CE is a separation method that utilizes both ends of the capillary for sample introduction. For example, Greguš et al. [164] reported an analysis of 14 ions (inorganic anions, cations, and organic acids) with a DOI-CE- C^4D method in EBC samples obtained from patients with various respiratory diseases (COPD, asthma, pulmonary fibrosis, sarcoidosis, and CF) in less than 5 min. Kubáň et al. [165] also reported the analysis of ionic contents of EBC in less than 2 min with a DOI-CE- C^4D method. It has been shown that changes in ion concentrations could be observed in acute inflammation of the upper airways and in patients diagnosed with mild COPD. Furthermore, they suggested that lactate concentrations could also be monitored and an approximately fourfold increase of lactate concentration in EBC was determined following an exhausting cycling exercise.

It is worth mentioning that small molecules such as chloride, sodium, potassium, and small organic acids are not considered as biomarkers of pulmonary diseases, but contribute, for instance, to the neutrality of the EBC, pH, and dilution standardization [16]. However, their determination of EBC is important.

- CE system with laser-induced fluorescence (LIF) detection: LIF is another CE detector used for EBC analysis. LIF is much more widespread among CE methods because it can achieve very low LODs. Hodáková et al. [166] have described the use of a 515 nm green laser module and CE-LIF for the quantification of GSH in EBC. Eosin-5-maleimide (EMA) is used as a fluorescent tag for GSH derivatization. They reported that this method could quantify GSH in as little as 20 μL of EBC in less than 3 min.

Gel electrophoresis

Gel electrophoresis is a method for the separation and analysis of macromolecules (DNA, RNA, and proteins) and their

fragments based on their size and charge. The gel is typically made of agar or polyacrylamide [191]. Gel electrophoresis is used for analytical purposes, often after the amplification of DNA via polymerase chain reaction (PCR), but may also be employed as a preparative method prior to the use of other methods, such as MS, restriction fragment length polymorphism (RFLP), PCR, cloning, and DNA sequencing. Griese et al. [167] reported a two-dimensional gel electrophoresis (2-DE) method for a protein pattern analysis of the EBC of healthy subjects. 2-DE is a form of gel electrophoresis commonly used to analyze proteins. 2-DE begins with electrophoresis in the first dimension and then separates the molecules perpendicularly to the first dimension to create an electropherogram in the second dimension. Comparing the spot pattern observed in EBC to the proteome observed in bronchoalveolar lavage fluid, it can be said that the proteins in EBC mirror those present in ELF.

Gel electrophoresis may be coupled with other separation methods; this combination of sensitive methods enables the identification of potential biomarkers in biological samples. For example, a combination of 1-DE/2-DE/ μ -HPLC/MS methods for protein profile analysis of EBC from patients with pulmonary emphysema associated with α 1-antitrypsin deficiency (AATD) [17], gel electrophoresis coupled with MS/MS for keratin content detection in the EBC of healthy smokers [168], gel electrophoresis coupled with MS for proteomic characterization of healthy subjects [169], gel electrophoresis coupled with MALDI/TOF-MS for cytokeratin analysis in patients with ALI/ARDS [170], and gel electrophoresis (SDS-PAGE) followed by Western blot (WB) and surface-enhanced laser desorption ionization (SELDI)-MS for the detection and validation of several inflammatory cytokines (IL-1 α , IL-1 β , IL-2; IL-12, α , and β subunits, IL-15, interferon α and γ , TNF α), type I and II cytokeratins, two SP-A isoforms, calgranulin A and B, and α 1-antitrypsin in the EBC of non-smokers and healthy smokers, COPD patients without emphysema, and subjects with pulmonary emphysema associated with AATD [171].

Other methods

Ion mobility mass spectrometry

Mass spectrometry (MS) is an analytical method that ionizes chemical species and sorts the ions on the basis of their mass-to-charge ratio. In addition to its application coupled with other analytical methods, it can also be used as an independent method, known as ion mobility mass spectrometry, for the analysis of non-volatile biomarkers/compounds in EBC. Zang et al. [192] reported transmission-mode direct analysis in real time (TM-DART)-TWIMS-TOF-MS as a feasible approach and a high-throughput alternative to the conventional direct infusion (DI), ESI, and atmospheric pressure chemical

ionization (APCI) methods for metabolomics studies of EBC samples. They found that multivariate analyses of the resulting TM-DART-TWIMS-TOF-MS datasets could successfully differentiate between EBC samples from CF patients and healthy controls.

Polymerase chain reaction-based assays

Polymerase chain reaction (PCR) is a relatively simple and inexpensive tool used in molecular biology to amplify a single copy or a few copies of a DNA segment across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. PCR is used every day to diagnose diseases, identify bacteria and viruses, match criminals to crime scenes, and in many other ways [193]. A PCR-based assay may also be used for diagnostic purposes pertaining to EBC samples. Some of these studies include the detection of human DNA in the EBC of patients with NSCLC and healthy volunteers with PCR followed by Gel Doc System (Bio-Rad) analysis [194], PCR followed by laser fluorescence in patients with NSCLC as per Carpagnano [195], IS6110 repetitive DNA element of *Mycobacterium tuberculosis* in patients with newly diagnosed active pulmonary tuberculosis [196], fungal DNAC in lung transplant recipients and lung microbiota in patients with ventilator-associated pneumonia (VAP) [197, 198], human papillomavirus (HPV) in patients with lung cancer [199], miR-570-3p as a potential regulator of asthmatic inflammation in asthmatic patients [200], Let-7a in patients with COPD [201], and mitochondrial DNA mutations in patients with lung cancer [202]; the merits of the methods applied for different analytes of interest are tabulated in Table 5.

Nanoparticle-based methods

The application of nanotechnology has enormous potential to greatly influence our world and improve the quality of human life. Plasmonic and paramagnetic nanoparticles, quantum dots, nanoshells, and others are some of the nanoparticles used as contrast agents, fluorescent materials, or molecular research tools for different medical aspects, such as diagnostics and therapeutics, owing to their unique optical, electrical, and mechanical properties [213]. Some studies have reported the detection of biomarkers in EBC with nanotechnology-based methods. For example, Melker et al. [214] used nanoparticle-based assembly systems for the diagnosis of different physical conditions or disease/disorder states. A nanoparticle-based assembly comprises a nanotube, a surrogate marker, and the means for detecting a specific chemical entity (SCE) consisting of an antibody, a protein, and an aptamer. The identification of an SCE by this novel way results in the release of the surrogate marker from the nanoparticle. Since the surrogate marker is released from the nanoparticle only in the presence of an SCE, the detection of the

surrogate marker indicates that the SCE is present in the EBC sample and, consequently, enables the diagnosis of the specific condition, disorder, or disease. The SCE can be found in a broad range of analytes including steroids, viruses, α -fetoprotein, isoprene α II-spectrin breakdown products, and isoprostanes in various pulmonary diseases. In another work [215], this research group used the same system for the determination of a nucleic acid, a protein, an illicit drug, an explosive, a toxin, a pharmaceutical, a carcinogen, a poison, an allergen, and an infectious agent in body fluids including blood and EBC samples. As we know, the most popular molecular recognition agent is an aptamer, which is a highly specific nucleic acid-based ligand that is highly selective for a target analyte/biomarker. Thus, the proposed systems include aptamers in combination with nanotubes attached to signaling agents to provide the means for detecting, signaling, and/or quantifying virtually any compound of interest in EBC.

Moreover, nanoparticle-based methods can be used for monitoring drugs in EBC samples. Recently, our research group, with the aim of conducting a systematic study on drug analysis in EBC samples, used amidosulfonic acid-capped silver nanoparticles (ASA-AgNPs) for the determination of lamotrigine (LTG) in the EBC of epileptic patients undergoing LTG treatment [12]. The method is based on the visual color changes of ASA-AgNPs from yellow to red to violet due to LTG-induced aggregation of nanoparticles. We have also developed an AgNPs-enhanced terbium fluorescence method for quantifying deferiprone (DEF) in the EBC of patients undergoing DEF treatment [13]. Since AgNPs significantly improve the fluorescence intensity of the terbium-DEF system, it allows for the quantification of DEF at very low levels ($60 \mu\text{g L}^{-1}$).

Surface acoustic wave immunosensors

Surface acoustic wave (SAW) sensors are a class of microelectromechanical systems (MEMS) that rely on the modulation of surface acoustic waves to sense a physical phenomenon. SAW as a piezoelectric sensor is a well-performing mass sensing device for label-free immunoassay due to its attractive advantages, including real-time monitoring and simplicity of use [216]. Zhang et al. [64] reported a SAW immunosensor for the detection of CEA in the EBC of cancer patients. A conventional sandwich immunoassay using AuNPs-antibody conjugates and subsequent mass enhancement with gold staining solutions was utilized. In the developed SAW immunosensor, CEA mixed with gold nanoparticles (AuNPs)-antibody conjugates was first recognized by the capture antibody immobilized on the sensor surface; then the sensitivity was enhanced by injecting the gold staining solution. The response of the immunosensor was proportional to the CEA concentration. In another study, this research group [203] reported a miniaturized SAW immunosensor with great

Table 5 Characteristics of included studies on exhaled non-volatile biomarkers/compounds in various diseases by some new methods

Method	Targeted markers	Breath collection device	Disease/Disorder	Linear range	LOD	Detection range	Number of real samples	Ref.
PCR flowed by Gel Doc System	Human DNA	EcoScreen condenser	NSCLC	–	0.05 pg DNA	–	36	[194]
PCR followed by laser fluorescence	Microsatellite marker	EcoScreen condenser	NSCLC	–	–	–	30	[195]
PCR followed by UV	IS6110 repetitive DNA element of M. tuberculosis	Cooling-trap	Newly diagnosed active pulmonary tuberculosis	–	–	–	10	[196]
Luminex multiplex xTAG fungal ASR assay	Fungal DNAC	Not mentioned	Lung transplant recipients	–	–	–	202	[197]
PCR followed by UV	HPV	EcoScreen condenser	Lung cancer	–	–	–	157	[199]
	MiR-570-3p	EcoScreen condenser	Asthma	–	–	–	20	[200]
PCR sequencing approach	Mitochondrial DNA mutations	Lab made glass condenser	Lung cancer	–	–	–	25	[202]
NPs-based sensor	LTG	Lab made cooling trap system	Epileptic patients' receiving LTG	0.02–0.5 µg mL ⁻¹	5 ng mL ⁻¹	0.592–0.771 µg mL ⁻¹	3	[12]
NPs-based assay	DEF	Lab made cooling trap system	Patients' receiving DEF	0.06–1.5 mg L ⁻¹	–	0.06–0.17 mg L ⁻¹	16	[13]
SAW immunosensor	CEA	Not mentioned	Lung cancer	1–16 ng mL ⁻¹	1 ng mL ⁻¹	1–10 ng mL ⁻¹	28	[64]
SAW immunosensor	CEA	Not mentioned	Lung cancer	–	1.25 ng mL ⁻¹	1.0–12 ng mL ⁻¹	30	[203]
SAW immunosensor	CEA	EcoScreen condenser	Lung cancer	–	0.5 ng mL ⁻¹	1.9–4.8 ng mL ⁻¹	161	[66]
	SCC				0.1 ng mL ⁻¹	0.47–1.12 ng mL ⁻¹		
	NSE				0.5 ng mL ⁻¹	4.2–10.5 ng mL ⁻¹		
AlGaIn/GaN HEMTs sensor	Glucose	Lab made setup	Diabetes	–	<0.5 nmol L ⁻¹	–	–	[204]
	Chloride				0.01 µmol L ⁻¹	–	–	
AlGaIn/GaN HEMTs sensor	Glucose	Lab made setup	Healthy subjects	–	<0.5 nmol L ⁻¹	–	–	[205]
NMR	Metabolomic analysis	Tygon tube cooled with ice	Asthma	–	–	–	44	[206]
NMR	Metabolomic analysis	EcoScreen condenser	Laryngectomised patients and COPD	–	–	–	36	[207]
NMR	Metabolomic analysis	EcoScreen condenser	Obese and lean subjects	–	–	–	35	[208]
NMR	Metabolomic analysis	EcoScreen condenser	Asthmatic	–	–	–	70	[209]
NMR	Metabolomic analysis	Turbo-Deccs	CF	–	–	–	84	[210]
NMR	Metabolomic analysis	EcoScreen condenser	AATD	–	–	–	22	[211]
NMR	Metabolomic analysis	RTube	Lung cancer	–	–	–	20	[212]

ability for automatic and online detection of CEA in EBC as compared to the previous version. The detection approach was the same and was based on a sandwich immunoassay using an antibody labeled with AuNPs and, subsequently, mass enhancement with the use of gold staining solutions was integrated onto the SAW sensor surface. The reported cut-off value for the CEA concentration in EBC to distinguish between lung cancer patients and healthy subjects is 1.85 ng mL^{-1} [66]. Since the detection limit of the presented method is less than the cut-off value, this immunosensor could be successfully used for CEA detection in EBC.

High electron mobility transistors

High electron mobility transistors (HEMT), which is also known as a heterostructure field-effect transistor (HFET) or modulation-doped FET (MODFET), is a field-effect transistor that incorporates a junction between two materials with different band gaps (i.e., a heterojunction) as the channel instead of a doped region. Aluminum gallium nitride (AlGaIn)/GaN HEMT is an extremely stable system chemically and includes a high electron sheet carrier concentration channel induced by the piezoelectric polarization of the strained AlGaIn layer and spontaneous polarization. Positive countercharges at the HEMT surface layer are induced by the two-dimensional electron gas cloud located at the AlGaIn/GaN interface. Any slight change in ambient conditions can affect the HEMT's surface charge, thus changing the electron concentration in the channel at the AlGaIn/GaN interface [204]. HEMT sensors have response times of seconds and are probably the cheapest sensing technologies available today. Chu et al. [205] used an AlGaIn/GaN HEMT sensor for measuring pH and glucose in EBC for an investigation related to airway pathology. They designed the sensor as a portable, wireless package for remote monitoring applications. Data from the sensor is sent wirelessly and can be recorded by any device that has an appropriate receiver. Chu et al. [204] and Ren et al. [217] also reported a ZnO nanorod-gated HEMT sensor with immobilized glucose oxidase for detecting glucose in EBC. Furthermore, Chu et al. [204] demonstrated that depending on the immobilized material, HEMT-based sensors can be used for sensing different materials. They used a HEMT sensor immobilized on a thin film of AgCl for the determination of chloride ion and a Sc_2O_3 -gated AlGaIn/GaN HEMT sensor for measuring pH in the EBC of diabetic patients. This technology offers the possibility of biomarker detection in EBC without the need for clinical visits.

Nuclear magnetic resonance-based metabolomic analyses

Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful methods to provide an overall biochemical profile of low-molecular-weight endogenous

metabolites in biological fluids [206], including amino acids, polar/nonpolar metabolites, sugars, and large metabolites. This method enables the characterization of the most represented proton-containing compounds, both known and unknown metabolites as well as novel biomarkers, and their representation in a spectrum, thereby providing a metabolic fingerprint of the analyzed sample that may provide insights into disease mechanisms. Multivariate statistical methods can be applied to extract information from these complex NMR spectra [218]. NMR-based metabolomics has several advantages over other methods such as MS. NMR requires little pretreatment of samples; it is also rapid (10–15 min), non-invasive, nondestructive, and provides highly reproducible results [219–221].

A wide range of NMR-based metabolomics studies have been performed on EBC samples and all of them have shown a clear discrimination between the NMR spectra of patients and healthy individuals. Some of these studies are listed in Table 5, including the metabolomic analysis for discriminating between children with asthma and healthy children by Carraro et al. [206]; discrimination of healthy subjects, laryngectomized, and COPD patients in three separate groups by de Laurentiis et al. [207]; discrimination of obese asthmatic (OA) patients and patients independently affected by asthma or obesity by Maniscalco et al. [222]; discrimination of COPD and OSAS by Ząbek's research group [223]; discrimination of obese and lean subjects by Paris' research group [208]; asthmatic patients by Motta et al. [209]; and CF subjects compared to healthy subjects by Montuschi et al. [210]. Some pilot studies were also undertaken by Airolidi et al. [211] with the aim to differentiate AATD patients, who were diagnosed with moderate to severe emphysema, from healthy individuals and by Ahmed [212] for the identification of metabolic biomarkers of lung cancer with NMR-based metabolomic analysis.

Critical points for EBC analysis methods

The main advantages of EBC as an alternative biological sample were reviewed in a short work recently [224]. The simpler matrix of EBC as compared to other biological sample provides more facilities for an analyst to apply various pre-concentration methods that are required for most analytes due to their very low concentrations in EBC.

All the quantification methods used for EBC analysis have their particular strengths and weaknesses. Although the optical and electrochemical methods are simple, low cost, and portable (in most cases), the main limitations of these methods include low sensitivity, low specificity, and sample throughput for screening applications in routine laboratories. Immunochemical methods (fluoroimmunoassay, ELISA, and

EIA kits) as semiquantitative methods are also subject to inherent uncertainties in selectivity. Lack of selectivity and poor reproducibility/repeatability of electrochemical methods should be considered during their clinical applications. Moreover, most of the above-mentioned methods require a large sample volume depending on the selected analytical method and can only analyze one analyte at a time. Sequential assay processes could be conducted by highly specific analytical methods such as HPLC, GC, and CE instruments with different mentioned detectors for high-throughput applications. The spectrophotometric, spectrofluorimetric, or electrochemical methods cannot compete with the separation methods. However, HPLC and GC methods are more time-consuming and need an extra step for sample preparation prior to injecting the sample into the system. The possibility of directly injecting EBC and the ability to analyze minute sample volumes (< 10 nL) into a CE system is an important characteristic for this analytical method. Online pre-concentration of analytes in CE provides further advantages that could cover for the low sensitivity of CE systems. However, as a separation method, CE has provided less successful results compared to HPLC methods and is not as robust as HPLC. In contrast to HPLC, CE methods can be made portable and can thus be used for point-of-care analysis. MS is a key tool for the selective and sensitive detection of biomarkers at trace levels in biofluids and MS is preferred as the detection section for most methods because of its unambiguity in identification. This hyphenated analytical method has been proved to be highly selective, reproducible, and free from artifacts. GC/MS provides excellent separation and detection, but its applications are relatively few, because most of the investigated compounds are non-volatile; so, a derivatization step is needed that prolongs analysis time. As an alternative to LC, post-ionization gas-phase separation via ion mobility mass spectrometry has shown great promise for analysis due to its ability to separate isobars. High electron mobility transistors-based sensors, which were introduced in the last decades, have high precision, compact size, fast response time, and sensitive to small amounts of biological material. These have potential for point-of-care applications or on-field measurements in order to reduce medical costs and emergency room visits.

Nanoparticle-based method, NMR-based analysis, and SAW-immunosensors could be promising developments for the future of EBC analysis. Recent research has focused on developing innovative nanomaterials and to control their properties to meet the needs of specific applications. The power and scope of such nanomaterials can be improved by coupling with other analytical methods, such as electrical or optical readout protocols. NMR-based analysis being a very new and expensive method is still applied as a diagnostic approach (e.g., for the discrimination of patients from healthy controls) because of poor understanding of disease processes at the metabolic level.

Conclusion

The analysis of EBC that well reflects the composition of the airway surface liquid may provide information about inflammation, oxidative stress, and other conditions (e.g., metabolic disorders, bacterial and viral infections) in the lungs. The detection and quantification of non-volatile biomarkers related to various diseases could help in classifying the severity and type of disease and could be also used for the diagnosis as well as the monitoring of therapy of these diseases. Since many different types of methods are available for breath analysis, the selection of an instrumentation platform for a given application will be easy if the respective merits and limitations of each of the methods are considered carefully. The goal of this review was to provide an overview of the various methods used for the determination of non-volatile biomarkers/compounds in the EBC of patients.

Acknowledgments The authors would like to thank Dr. Ali Shayanfar for his assistance in this study.

Patent Jouyban A, Khoubnasabjafari M, Ansarin K, Jouyban-Gharamaleki V. Breath sampling setup. Iranian Patent, 81363, 2013.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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