

T.R.
EGE UNIVERSITY
Graduate School of Applied and Natural
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**APPLICATIONS OF CHROMATOGRAPHIC
METHODS FOR BREATH ANALYSIS IN
DIAGNOSIS OF HUMAN HEALTH**

Korcan KORBA

Supervisor: Prof. Dr. Levent PELİT
Co-Supervisor: Prof. Dr. Tuncay GÖKSEL

Department of Chemistry
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EÜ Lisansüstü Eğitim ve Öğretim Yönetmeliğinin ilgili hükümleri uyarınca Doktora Tezi olarak sunduğum “**Applications of Chromatographic Methods for Breath Analysis in Diagnosis of Human Health (Kromatografik Yöntemlerin İnsan Nefesinde Tanı Amaçlı Uygulamaları)**” başlıklı bu tezin kendi çalışmam olduğunu, sunduğum tüm sonuç, doküman, bilgi ve belgeleri bizzat ve bu tez çalışması kapsamında elde ettiğimi, bu tez çalışmasıyla elde edilmeyen bütün bilgi ve yorumlara atıf yaptığımı ve bunları kaynaklar listesinde usulüne uygun olarak verdiğimi, tez çalışması ve yazımı sırasında patent ve telif haklarını ihlal edici bir davranışımın olmadığını, bu tezin herhangi bir bölümünü bu üniversite veya diğer bir üniversitede başka bir tez çalışması içinde sunmadığımı, bu tezin planlanmasından yazımına kadar bütün safhalarda bilimsel etik kurallarına uygun olarak davrandığımı ve aksinin ortaya çıkması durumunda her türlü yasal sonucu kabul edeceğimi beyan ederim.

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İmzası

Korcan KORBA

ÖZET

KROMATOĞRAFİK YÖNTEMLERİN İNSAN NEFESİNDE TANI AMAÇLI UYGULAMALARI

KORBA, Korcan

Doktora Tezi, Kimya Anabilim Dalı

Tez Danışmanı: Prof. Dr. Levent PELİT

Eş Danışman: Prof. Dr. Tuncay GÖKSEL

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Nefesteki biyobelirteçlerin analizi ile insanların güncel sağlık durumları hakkında bilgi sahibi olma fikri temelleri antik çağlara kadar dayanan güncel bir konudur. Nefesteki biyobelirteçlerin analizi, normal metabolik yollara invazif olmayan bir bakış açısı sunarak hastalıkların teşhisi ve takibini yapabileceği ortaya konulmuştur. Soluk havasında uçucu ve uçucu olmayan binlerce bileşik bulunmaktadır. Bu bileşikler farklı örneklemme teknikleri ve analiz metodları kullanılarak analizlenebilirler. Yüksek teknoloji analiz teknikleri ve hastalık durumunun hızlı bir şekilde değerlendirilmesini sağlayan gelişmiş veri içeren hastalıklı ilgili bilgiler ortaya çıkarılabilir.

Bu tez kapsamında özellikle çağımızın en yaygın ve düşük tedavi yanıtları bilinen akciğer kanserinin teşhisi üzerine çalışmalar yapılmıştır. Bu amaçla soluk havası örnekleri laboratuvar yapımı örnek toplama cihazları ile yoğunlaştırılarak, soluk havası kondensat (EBC) örnekleri toplanmıştır. EBC örneklerinde bulunan bazı uçucu organik bileşikler Gaz Kromatografisi Kütle Spektroskopisi (GC-MS) ve Sıvı Kromatografisi Uçuş Zamanlı Kuadropol Kütle Spektroskopisi (LC-QTOF) teknikleri ile karşılaştırmalı olarak gerçekleştirilmiştir. GC-MS analiz metodu ile 1-butanol, isopropanol, 3-hidroksi-2-butanon, izopren, dimetilsülfür, karbon

disülfür, benzen, toluen, etilbenzen, 2,2-Dimetildekan, tetradekan, 2,2,4,6,6-pentametilheptan, 2,3,4-trimetildekan, nonan, 3,4,5,6-tetrametiloktan, hekzadekan ile aldehit grubundan; malondialdehit, propanal, bütanal, pentanal, hekzanal, heptanal, oktanal ve nonanal bileşikleri analizlenmiştir. LC-QTOF analiz metodunda ise 2,4-Dinitro fenil hidrazin bileşiği ile türevlendirildikten sonra malondialdehit, 3-hidroksi-2-butanon, propanal, bütanal, pentanal, hekzanal, heptanal, oktanal ve nonanal bileşikleri analiz edilmiştir. Çalışmanın son kısmında ise, EBC örneklerindeki bazı aminoasit ve organik asitlerin stabiliteleri üzerine saklama ve analiz koşullarının etkisi incelenmiş ve sağlıklı ve akciğer kanserli olgulardaki aminoasit ve organik asitlerin miktarları ölçülmüştür.

Anahtar sözcükler: Soluk Havası Kondensatı, Katı Faza Mikroözütleme, Akciğer Kanseri, Uçucu Organik Bileşik, Aminoasit, Organik Asit, Sıvı Kromatografisi Uçuş Zamanlı Kuadropol Kütle Spektroskopisi, Gaz Kromatografisi Kütle Spektroskopisi

ABSTRACT**APPLICATIONS OF CHROMATOGRAPHIC METHODS FOR
BREATH ANALYSIS IN DIAGNOSIS OF HUMAN HEALTH**

KORBA, Korcan

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Supervisor: Prof. Dr. Levent PELİT

Co-Supervisor: Prof. Dr. Tuncay GÖKSEL

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The idea of analyzing biomarkers in breath to provide information about people's current health status is a current topic that dates back to ancient times. Analysis of breath biomarkers has been shown to provide a non-invasive window into normal metabolic pathways, enabling the diagnosis and monitoring of diseases. Exhaled breath contains thousands of volatile and non-volatile compounds. These compounds can be analyzed using different sampling techniques and analysis methods. The disease related knowledge can be revealed from breath which contains an enormous amount of data by the use of high-tech analysis techniques together with advanced data evaluation methods that provide a rapid assessment of the disease status.

In this thesis, experiments have been carried out especially on the diagnosis of lung cancer, which is the most common disease of our time with known low treatment responses. For this purpose, exhaled breath samples were condensed with laboratory-made sample collection devices and exhaled breath condensate (EBC) samples were collected. Some volatile organic compounds in EBC samples were comparatively analyzed by Gas Chromatography Mass Spectroscopy (GC-MS) and Liquid Chromatography Quadrupole Time-of-Flight Mass Spectroscopy (LC-QTOF) techniques. In GC-MS analysis method, 1-butanol, isopropanol,

3-hydroxy-2-butanone, isoprene, dimethylsulfide, carbon disulfide, benzene, toluene, ethylbenzene, 2,2-Dimethyldecane, tetradecane, 2,2,2,4,6,6-pentamethylheptane, 2,3,4-trimethyldecane, nonane, 3,4,5,6-tetramethyloctane, hexadecane and aldehyde group; malondialdehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal and nonanal compounds were analyzed. In LC-QTOF analysis method, malondialdehyde, 3-hydroxy-2-butanone, propanal, butanal, pentanal, hexanal, heptanal, octanal and nonanal compounds were analyzed after derivatization with 2,4-Dinitro phenyl hydrazine compound. In the last part of the thesis, the effects of storage and analysis conditions on the stability of some amino acids and organic acids in EBC samples were examined and the amounts of amino acids and organic acids in healthy and lung cancer patients were measured.

Keywords: Exhaled Breath Condensate, Solid Phase Microextraction, Lung Cancer, Volatile Organic Compound, Amino acid, Organic Acid, Liquid Chromatography Quadrupole Time of Flight Mass Spectroscopy, Gas Chromatography Mass Spectroscopy

ÖNSÖZ

Kanser vakalarında erken teşhis ya da hastalığın seyrinin izlenmesi önemli bir aşamadır. Nefes analizleri antik çağlardan beri hastalık teşhisinde kullanılan yöntemlerden biridir. Nefeste bulunan biyobelirteçler metabolik aktiviteler sonucu üretilir ve insan sağlığı hakkında geniş bilgiler sunar.

Soluk havası, kan ve idrar numunelerine nazaran oldukça temiz bir matrikstir ve gelişen teknoloji ile içindeki binlerce madde tek bir analiz yöntemi ile kolayca saptanabilir. Elde edilen bu büyük veri modern istatistiksel yöntemler ile analizlenip içerisindeki değerli bilgiler ortaya çıkarılabilir. İçerdiği bu büyük potansiyel nedeni ile nefes analizleri büyük önem taşımaktadır. İçerdiği bu büyük potansiyele rağmen standart bir nefes analiz metodunun henüz Kabul görüp uygulamaya girmemiş olması bu analizlerin yaygınlaşmasında karşımıza çıkan en büyük engeldir.

Bu tez kapsamında EBC örneklerindeki bazı uçucu ve uçucu olmayan bileşiklerin analizlerinde karşılaşılan güçlükler ve bunlar için önerilen çözüm yolları sunulmuştur. Bu alanda çalışanlara bu tezin ışık tutmasını diliyoruz.

İZMİR

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Korcan KORBA



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SYMBOLS AND ABBREVIATIONS

<u>Symbols</u>	<u>Description</u>
%	Percantage
>	Grater than sign
°C	Centigrade degree
±	Plus or Minus

Abbreviations

VOC	Volatile Organic Compound
EB	Exhaled Breath
EBA	Exhaled Breath Aerosol
EBC	Exhaled Breath Condensate
COPD	Chronic obstructive pulmonary disease
LC	Lung Cancer
ppb	parts per billion
ppm	parts per million
ppt	parts per trillion
µm	micrometer
µL	microliter

Symbols and Abbreviations (Continue)

SPA	Surfactant Protein A
SIFT	Selected Ion Flow Tube
MS	Mass Spectrometry
PTR	Protein Transfer Reaction
EESI	Extractive Electrosprey Ionization
SESI	Secondary Electrosprey Ionization
PDMS	Polydimethyl Siloxan
DVB	Divinyl Benzene
PA	Polyacrylate
CAR	Carboxen
cm	Centimeter
min	minute
cm ²	Square Centimeters
mL	mililiter
mg	miligram
GC-MS	Gas Chromatography Mass Spectrometry
LC-QTOF	Liquid Chromatography Quadropole Time of Flight

Symbols and Abbreviations (Continue)

PFBHA O-(2,3,4,5,6-Pentaflorobenzyl) hydroxylamine

2,4- DNPH 2,4- Dinitrophenylhydrazine

mM Milimolar

ng Nanogram

LOD Limit of Detection

LOQ Limit of Quantification

1. INTRODUCTION

In modern medicine, various combinations and invasive or risky techniques such as biopsies, radiology and endoscopy are employed for diagnosis diseases (Chen et al., 2021) . However, development of non-invasive diagnosis techniques is very popular issue in the medical science. The smell of different types of body fluids such as breath, urine and sweat have been utilized in medical history for over three thousand years (Buszewski et al., 2007; Dospinescu et al., 2020; Kulkarni et al., 2020; A. Sharma et al., 2023). Among body fluids, breath takes a special place as humans breathe approximately 17,000 to 29,000 times a day and can be sampled non-invasively and limitlessly. Volatile compounds in the blood pass into exhaled breath (EB) during respiration and EB contains thousands of metabolic or non-metabolic compounds (Das & Pal, 2020). So far, various types of volatile and non-volatile compounds have been detected in EB, including volatile organic compounds (VOCs), amino acids or viruses (Ghumra et al., 2023; Liu et al., 2014). The portion of breath containing all components, including liquid droplets, is termed as exhaled breath (EB). The liquid sample obtained by separating liquid droplets from breath is defined as exhaled breath aerosol (EBA) (Johnson & Morawska, 2009). Furthermore, when EB is cooled to the lower temperatures, both moisture and EBA condense, resulting in exhaled breath condensate (EBC) (Mutlu et al., 2001). While volatile compounds are generally analyzed in breath, volatile and non-volatile components such as fatty acids, cytokines, proteins, viruses and bacteria can be analyzed in EBC and EBA. There is a significant amount of the research in the literature focused on the analysis of the types of substances mentioned above in these three forms of exhaled breath.

Analysis of biomarkers in EB is a highly popular research area in medicine for diagnosing certain diseases (Buszewski & Baranowska, 2022; Campanella et al., 2019; Haick, 2022). Breath analysis provides a non-invasive window into normal metabolic pathways and also demonstrates how these pathways change by the disease states (Pereira et al., 2015; Subali et al., 2022). Hence, EB analysis can provide rich information about an individual's current health status. Therefore, it

can be used not only for diagnosing diseases but also for monitoring responses to treatments for these diseases (Konstantinidi et al., 2015).

Recently, numerous studies have investigated volatile organic compounds (VOCs), such as hydrogen, carbon monoxide, methane as potential biomarkers for the diagnosis of various diseases such as lung cancer, asthma and diabetes. However, standardizing the validation and application steps of the methods used in breath analysis is crucial. In the pioneering modern study conducted by Pauling and colleagues in 1971, about 250 VOCs were detected in exhaled breath using a flame ionization detector (A. Sharma et al., 2023). Nowadays, with modern devices, more than 3500 compounds have been detected in human exhaled breath (Chang et al., 2023). Analyzing volatile and non-volatile compounds in exhaled breath is currently being intensively studied, especially for the diagnosis and treatment of respiratory diseases.

Breath analysis, as a non-invasive methodology, allows for continuous monitoring, enabling the observation of instant dynamic changes in patients' conditions. Moreover, compared to methods like biopsies that cannot be repeated frequently, it is a powerful tool not only for disease diagnosis but also for monitoring responses to treatment. Huge amount of information present in EB and using with high-tech analytical techniques together with data evaluation methods provide rapid assessment of patients' conditions.

Generally, chromatographic methods coupled with mass detectors are used to determine compounds in EB. Most of these methods measure in parts per million (ppm). In contrast, the levels of analytes used as biomarkers in EB are typically in parts per billion (ppb) or parts per trillion (ppt). This situation require the use of preconcentration techniques like solid phase microextraction to be extensively employed. These techniques supply significant attention due to their eco-friendliness, cost-effectiveness, speed and ease of use compared to other methods.

This thesis covers the development of analytical methods for the analysis of volatile and non-volatile compounds in exhaled breath and their application for lung cancer diagnosis..

2. GENERAL INFORMATION

2.1 Respiratory Mechanism

Understanding the working principle of the respiratory system is essential before handling breath analysis. Respiration refers to the action of inhaling and exhaling air to facilitate the exchange of oxygen and carbon dioxide in our lungs. Furthermore, the respiratory system performs other functions such as filtering, moisturizing and warming incoming air, as well as aiding in vocalization and coughing (Morawska et al., 2009). During rest, an individual typically breathes approximately 10 to 12 times per minute. Air enters the respiratory system through the nose or mouth and progresses toward the pharynx (windpipe) through the trachea. The windpipe divides into two bronchi, each leading to one lung. Within the lungs, the bronchi further divide into smaller airways called bronchioles. These bronchioles eventually branch into tiny air sacs known as alveoli, which are the primary sites for gas exchange in the respiratory system. Oxygen-rich air is transferred to the alveoli and moves across their thin walls into surrounding capillaries, small blood vessels. Subsequently, oxygen is taken up by red blood cells and transported to the rest of the body. Simultaneously, carbon dioxide and other waste products of metabolism move from the capillaries back into the alveoli. Finally, these metabolic waste products are expelled from the body during exhalation (Cobanoglu, 2014).

Tidal volume represents the volume of air exchanged during a single breath and varies among individuals depending on factors such as age, gender, height and physical activity. The average tidal volume for both male and female individuals is approximately 500 mL per breath (Hallett et al., 2018). A general representation of the respiratory process is shown in Figure 2.1. Only a small portion of the tidal volume reaches the alveoli for gas exchange at inhalation process. The rest of the breath is trapped in the pulmonary pathways comprising the trachea, bronchi and bronchioles. Dead space refers to the volume of air in the respiratory pathways where gas exchange does not occur and is commonly referred to as “dead volume” (Figure 2.1). The dead volume is approximately 150 mL for both males and females.

During exhalation, the concentration of carbon dioxide in the breath increases to around 4% (Buszewski et al., 2007).

A single respiratory cycle can be divided into three different phases: one inhalation (Phase 0) and two exhalations (Phase I and Phase II), which can be detected by observing the CO₂ transferred from the blood to the alveoli during breathing (Figure 2.1). The respiratory cycle, involving inhalation, exhalation and brief pause, can be represented in a capnogram obtained by monitoring the change between time and volume in CO₂ concentration. This capnogram separates the two inhalation phases (Phase 0 and Phase III) and one exhalation phase (divided into Phase I and Phase II). The remaining CO₂ level from the previous exhalation swiftly decreases during Phase 0 after inhalation and then remains constant. Phase I begins at the end of exhalation when CO₂ is at its minimum. The CO₂ concentration in the expired breath affectingly rises, reaching a maximum by the end of Phase I and remains almost constant. Dead volume happens between the start of Phase I and the point where CO₂ begins to rise during exhalation. During Phase I, CO₂ from the alveoli mixes with the anatomical dead volume in the respiratory tract. In phase II, only air from the alveoli is present. There is usually a slight increase in CO₂ levels during this phase. Sometimes, the CO₂ level can plateau and remain constant. The peak of CO₂ is reached at the end of the exhalation phase in Phase II (Cobanoglu, 2014). Tidal volume represents the tidal volume between the start of Phase I and the end of Phase II during exhalation. Finally, the second respiratory cycle begins again in Phase III, and the CO₂ concentration decreases sharply once more.

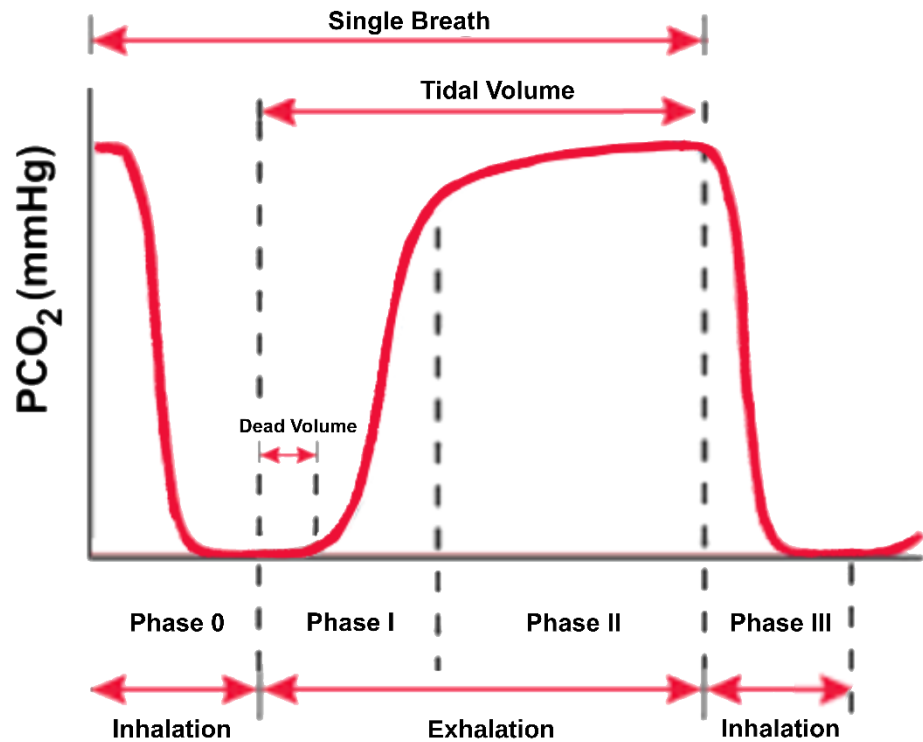


Figure 2.1 The change in CO₂ concentration during the respiratory process (Capnogram)

2.2 Breath

The compounds present in exhaled breath hold significant importance in the field of medicine as they can provide valuable information about an individual's physiological condition, exposure to environmental factors and even the presence of certain diseases or conditions (Drabińska et al., 2021). These aspects are further explained in detail below.

- **Disease diagnosis and monitoring:** Changes in the composition of exhaled breath can serve as biomarkers for various diseases and health conditions. Healthcare professionals have demonstrated the potential to detect diseases such as lung cancer, diabetes, asthma and infectious diseases at an early stage by analyzing specific volatile organic compounds (VOCs) or gases in breath (Kaloumenou et al., 2022).

- **Non-invasive Screening:** Breath analysis offers a non-invasive and painless method for gathering diagnostic information. It makes it more comfortable

and less risky for patients by eliminating the need for invasive procedures like blood draws or tissue biopsies (Guzman, 2021).

- **Personalized Medicine:** Understanding an individual's unique breath profile can help in personalized medicine. By personalizing treatments and medications according to a person's metabolic and physiological characteristics, healthcare providers can optimize therapeutic outcomes and minimize side effects (Amann et al., 2014).

- **Environmental Exposure Assessment:** The composition of exhaled breath can provide information about an individual's exposure to environmental pollutants and toxins. This is crucial for assessing public health risks and informing environmental policies. For instance, monitoring breath for exposure to air pollutants can aid in identifying sources of pollution and reducing their impact on communities (Dixit et al., 2021).

- **Metabolic Information:** Level of specific gases and VOCs in breath reflect metabolic processes occurring in the body. Changes in these markers can indicate metabolic disorders or imbalances and can guide dietary and lifestyle interventions (Popov, 2011).

- **Research and Drug Development:** Breath analysis is a valuable tool in scientific research and drug development. Researchers can use breath samples in order to study metabolic pathways, assess the effectiveness and safety of new drugs and gain a better understanding of human physiology (Haworth et al., 2022).

- **Health and Wellness Tracking:** Breath analysis can be utilized not only for diagnosing diseases but also for general health and wellness tracking. For instance, athletes can use breath analysis to assess their metabolic fitness and individuals can monitor breath acetone level as an indicator of fat metabolism during weight loss efforts (Wong et al., 2019).

- **Point-of-Care Testing:** Advances in breath analysis technology have enabled the development of portable and point-of-care devices for swift screening

and monitoring of health conditions. This could improve access to healthcare services in remote or underserved areas (Guzman, 2021).

In summary, the composition of breath can serve as a rich source of information about an individual's health and exposure history. Healthcare professionals and researchers can make more informed decisions regarding disease diagnosis, treatment and public health interventions by analyzing gases, VOCs and non-volatile compounds in EB. As technology continues to advance, it is likely that breath analysis will play an increasingly important role in healthcare and environmental monitoring.

Many biomarkers found in blood, urine and saliva have also been reported to be detected in Exhaled Breath Condensate (EBC) (Hunt, 2007). EBC contains a wide variety of compounds, both volatile and non-volatile, with different physical and chemical stability and activity (Hunt, 2007). Among the non-volatile compounds in EBC, the most significant ones include polypeptides, proteins, nucleic acids, lipid mediators, inorganic substances, cytokine, chemokines and viruses such as COVID (Khoubnasabjafari et al., 2020; Rahimpour et al., 2018). Various studies have focused on analyzing different biomarkers present in the EBC of respiratory disease like asthma, COPD, among others (Fuschillo et al., 2021; Połomska et al., 2021). Moreover, there has been considerable research interest in recent years regarding the early diagnosis of lung cancer (LC) by means of biomarkers found in EBC (Ratiu et al., 2020; Zakharova et al., 2021). Some studies have shown a high potential not only in the early diagnosis but also in monitoring the treatment of LC (López-Sánchez et al., 2017). The real-time molecular-level monitoring of biomarkers in EBC would be a crucial step forwards achieving treatment success. Additionally, personalized treatment pathways could be established, increasing the possibility of treatment success. In this context, certain VOCs in EBC have been suggested as potential biomarkers for early diagnosis and short-term monitoring for treatment (Broza et al., 2013).

Breath called as exhaled breath (EB), or the whole breath consists of volatile compounds released from the alveoli, along with moisture and aerosols, originating

from primary atmospheric gases. Depending on the sampling method, the phases of breath EB, EBC and EBA, presented in Figure 2.2. EB comprises a mixture of gases and aerosol particles (Amann et al., 2014). On the other hand, EBA is obtained by filtering the liquid droplet part of EB (Johnson & Morawska, 2009). After cooling EB to a low temperature and condensing both low-boiling-point compounds and moisture, and which is called as Exhaled Breath Condensate (EBC) (Tetik Vardarli et al., 2023). The composition of breath parts EB, EBA and EBC is described detailly below.

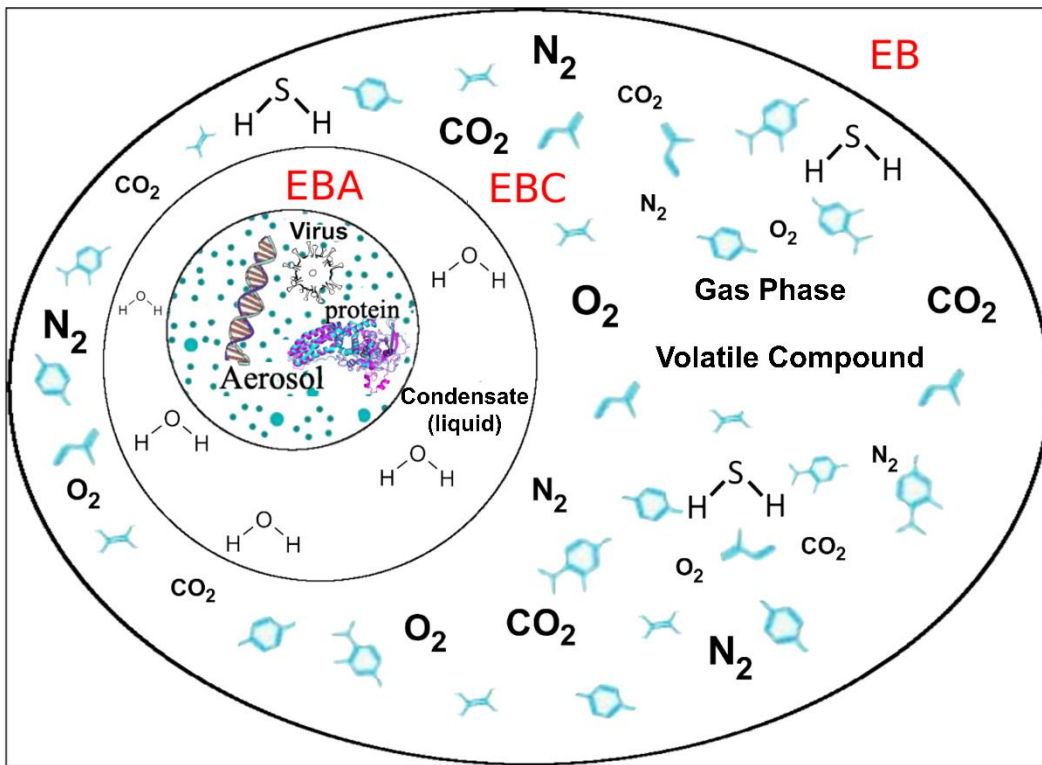


Figure 2.2 Phase diagram of Exhaled Breath

2.2.1. Exhaled Breath

The whole breath, also known as EB, is a mixture of gases and aerosol particle reflecting physiological and biochemical processes occurring in the human body (Amann et al., 2014; Bajtarevic et al., 2009; Gilio et al., 2020). In this section, only the gas composition of EB will be discussed. A comprehensive description of EBA and EBC is presented in separate sections below.

Nitrogen constitutes the primary gas component of exhaled breath, making up approximately 78% of it. It is followed by oxygen at around 16%, carbon dioxide at 4% and water vapor at 1% (Dent et al., 2013; Pleil et al., 2021). In addition to these main compounds, trace amounts of numerous volatile organic compounds (VOCs) and inorganic gases are present in EB. Some volatile compounds in EB possess distinctive odors (Pandey & Kim, 2011). The smell of these compounds in EB is already being applied for diagnosis of disease (A. Sharma et al., 2023). For instance, in individuals with severe diabetes, it has been reported that the breath odor is ketonic due to compounds like acetone, acetoacetate and β -hydroxybutyrate (Rooth & Ostenson, 1966). Simenhoff and colleagues suggested that the presence of ammonia or urea-like smell in breath, originating from dimethylamine and trimethylamine, could be related with the of renal failure (Simenhoff et al., 1977). Several researchers have reported a distinct breath odor resembling rotten eggs in patients with liver cirrhosis, linking it to the presence of hydrogen, sulfur, limonene, aliphatic acids, methyl mercaptan, dimethyl sulfide and ethanethiol (Friedman et al., 1994; Kaji et al., 1978). Leopold and colleagues demonstrated that trimethylaminuria, an inborn metabolic disorder resulting in Fish Odor Syndrome, causes a fish-like odor in EB (Leopold et al., 1990). Some studies have suggested that disturbances in fat digestion might lead to the production of triglycerides, fatty acids, 2-butanone, and styrene, contributing to unpleasant odors in EB (Elliott-Martin et al., 1997; Manson et al., 1999; Scully et al., 1997; Withey, 1978).

VOC analysis in breath by modern instrument supplies useful insights into an individual's health status. Over 3000 volatile organic compounds have been detected in EB to date (Chang et al., 2023). Detailed information about these identified organic and inorganic volatile compounds is summarized in Table 2.1. The levels of these compounds are generally ppb or sub-ppb range. Therefore, sampling step so critical for accurate analysis (Sun et al., 2016). The lack of standardization in the analysis of EB, including Exhaled Breath Aerosol (EBA) and Exhaled Breath Condensate (EBC), is the most significant limitation of the widespread usage of these technique. This situation complicates the comparison of inter-laboratory studies.

Table 2.1 Exhaled breath biomarkers and their disease applications

<u>Class of biomarkers</u>	<u>Biomarker</u>	<u>Disease / Applications</u>	<u>Reference</u>
Alkanes	Nonane, Undecane, Pentadecane, Cyclopropane, Nanodecane, Eicosane, 4-methyl octane, 2,6,10-trimethyldodecane, 3,7- dimethyldodecane	Breast Cancer, Lung Cancer, Alzheimer's, Parkinson's	(Phillips, 1992; Phillips et al., 2010; Tisch et al., 2012; Y. Wang et al., 2012)
Alkenes	1,4- Pentadiene, 1,3 Butadiene, D-Limonene, Cyclohexene, Styrene	Breast Cancer, Liver Cancer, Prostate Cancer	(Peng et al., 2010; Phillips et al., 2010; Qin et al., 2010)
Esters	N-Octadecyl ester, Methyl p-anisate, Ethyl acetate	Lung Cancer, Tuberculosis	(Buszewski et al., 2012; Fend et al., 2006; Y. Wang et al., 2012)
Aldehydes	Nonal, Decanal, Acetaldehyde, Formaldehyde	Colon cancer, Lung Cancer	(Altomare et al., 2013; Fernandes et al., 2015)
Ketones	Acetone	Diabetes	(Guo et al., 2010; Yu et al., 2005)
Sulfites	Carbonyl sulfide, Dimethyl sulfide	Liver	(Sehnert et al., 2002)
Nitrogen oxides	Nitric monoxide	COPD, Asthma	(Zakharkina et al., 2011)
Enzymatic, Vitamins	8-isoprostane, Nitrotyrosine, Cytokines, Methyl nicotinate	COPD, Tuberculosis	(Fend et al., 2006; Zakharkina et al., 2011)

EB analysis offers a fundamental advantage over traditional blood and urine tests: while the latter are limited in tracking a few parameters, EB analysis can simultaneously detect more than thousands of compounds in a single analysis. This allows various parameters related to an individual's health to emerge simultaneously in a single EB analysis, making it a robust method for diagnosing and monitoring diseases. Compared to blood and urine, EB serves as a pure matrix;

however, the challenge lies in sampling low-level biomarkers within it. If the biomarker analysis in EB becomes a standardized method similar to blood or urine analysis, it could enable detection of changes in people's health status before the onset of diseases. As a result, it holds the potential to become one of the most significant tools for preventive medicine.

2.2.2. Exhaled Breath Aerosol

EBA, as mentioned in the previous section, constitutes the liquid particle portion in breath, consisting of aerosol droplets (Duguid, 1946; Marzorati et al., 2019). Representing a small fraction (<0.1%) of Exhaled Breath Condensate (EBC), EBA comprises larger organic compounds, with particle diameters typically exceeding 5 μm (Hayes et al., 2016). Low volatile and more polar compounds tend to remain within aerosols (Pleil, 2016; Ratiu et al., 2020). The compound in breath aerosol can be sampled by using a suitable filter and subsequently analyzed using an appropriate method.

The transfer mechanism of aerosols from the airway of the lung to the breath are still not revealed yet (Horváth, 2003). Alveoli, including points in the respiratory system such as the trachea or mouth can release these respiratory fluid droplets. Some studies claimed that turbulence in the airways leads to the formation of aerosols in the breath (Fairchild & Stampfer, 1987; Papineni & Rosenthal, 2009; Paredi et al., 2002). However, Gebhart and colleagues suggested that aerosols in breath are predominantly released from the lungs. They found a significant decrease in the amount of aerosols in patients' EB following breath-holding, suggesting it as evidence supporting the origin of these aerosols from the depths of the lungs. A schematic representation of alveolar fluid particles within the bronchiole is shown in Figure 2.3. During the respiration process, foamy liquid accumulates in the bronchial tubes due to gas exchange. However, in some bronchioles, the remaining thin channel of air collapses to an extent that the liquid film can completely block the airways. Then, the droplets accumulated in the opening bronchioles during exhalation expand until a small connection remains between the two surfaces. At

the end of this period, the foamy liquid between the two wall of channel breaks apart on the intermediate surface to form aerosols, which are then expelled.

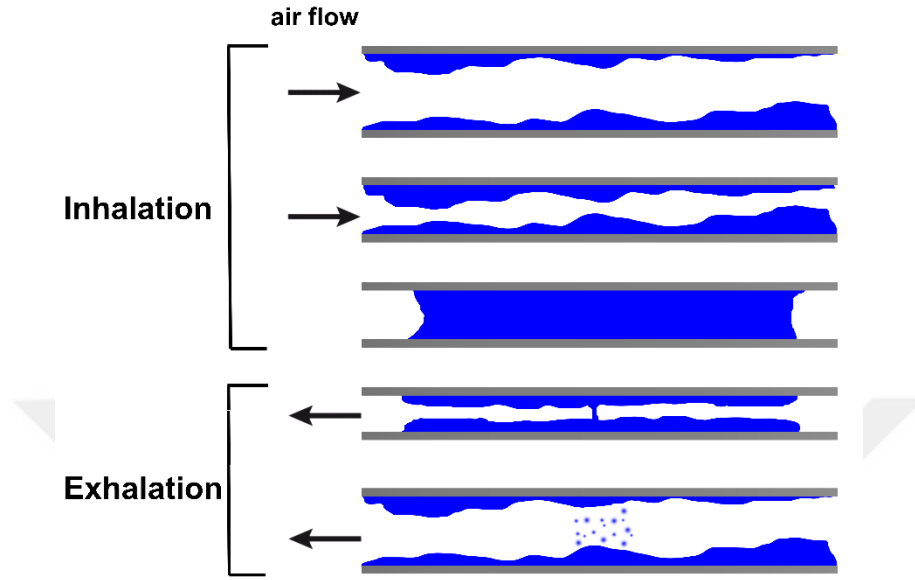


Figure 2.3 The formation mechanism of respiratory aerosol

EBA represents a fraction of the total EBC and includes larger molecules such as fatty acids, cytokine, cellular fractions, proteins, viruses and bacteria (Beck et al., 2016; W. Li et al., 2016; Mitchell et al., 2016; Pleil, 2016; Tinglev et al., 2016). Additionally, non-volatile organic and inorganic compounds and also dissolved volatile compounds in aerosols are found. Sampling of exhaled breath aerosol is already being applied to develop to drop tests (Wallace & Pleil, 2018). A list of substances found in EBA is summarized in Table 2.2.

The number of studies conducted using EBA is relatively low compared to EB and EBC, primarily due to being a relatively new technique (Wallace & Pleil, 2018). Some main advantages of EBA include not requiring sample freezing and being collected simply by wearing standard hospital-type medical masks during sampling. Therefore, sampling EBA is considered simpler compared to EB and EBC studies (W. Li et al., 2016; Pleil, 2016).

Table 2.2 List of biomarkers identified in EBA and their disease applications

<u>Biomarker Class</u>	<u>Biomarker</u>	<u>Disease / Applications</u>	<u>Reference</u>
Drugs	Codeine, Morphine, Buprenorphine, Diazepam, Oxazepam, Methadone	Exposure	(Beck et al., 2016; Beck, Sandqvist, Eriksen, et al., 2011; Ljungkvist et al., 2017)
Proteins	Surfactant Protein A (SPA), Albumine	Asthma	(Soares et al., 2018)
Bacteria	H. Influenzae, P. Influenzae, P. aeruginosa, E. coli, S. aureus, Methicillin-resistant Staphylococcus aureus	Pathogen	(Zheng et al., 2018)
DNA	Mycobacterium tuberculosis (Mtb) cell, Mtb (DNA)	Tuberculosis	(Morozov et al., 2018)
VOCs	Formaldehyde, CO ₂	COVID-19	(Ito et al., 2023)

2.2.3. Exhaled Breath Condensate

The liquid phase obtained by condensing EB is called Exhaled Breath Condensate (EBC) (Tetik Vardarli et al., 2020). EBC originates from aerosolized respiratory fluid droplets released from both water vapor and respiratory epithelial lining fluid (Hunt, 2007). EBC consists predominantly of condensed water vapor (>99%) and aerosolized respiratory tract lining fluid droplets (Koc et al., 2019), in addition to volatile and non-volatile compounds (Kubán & Foret, 2013; Rahimpour et al., 2018).

In the upper part of the respiratory tract mucous membranes containing goblet cells produce mucous (Gustafsson & Johansson, 2022). This part humidifies the inhaled air during the inhalation. The normal range for exhaled breath pH typically

varies between 6.8 and 7.4 (Hunt, 2007). These levels are influenced by a range of factors, including metabolic and respiratory processes occurring within the body.

Various semi-volatile, volatile or non-volatile inorganic or organic compounds, including hydrogen peroxide, proteins, isoprene, ammonia, adenosine, leukotrienes, isoprostanes, nitric oxides, peptides, and cytokines, can be given as example detected in EBC (Baldwin et al., 1986; Cavaleiro Rufo et al., 2019; Horváth et al., 2005; Hunt, 2002; Jöbsis et al., 2001; Kubán & Foret, 2013; Mutlu et al., 2001). A summary of some important compounds detected in EBC is provided in Table 2.3. These compounds are used for the diagnosis of various diseases and for monitoring treatment progress. (Kubán & Foret, 2013).

EBC requires additional equipment such as condensers and storage containers, which can increase the risk of sample contamination. Therefore, EBC is not as widely used as EB for the diagnosis purpose.

Table 2.3 Exhaled breath condensate biomarkers and disease applications

<u>Biomarker Class</u>	<u>Biomarker</u>	<u>Disease / Applications</u>	<u>Reference</u>
Inorganic	pH, inorganic salt	Asthma, COPD	(Hunt, 2002; Loukides et al., 2008; Wheatley et al., 2010)
Nitrogen Compounds	Nitrate, Nitrite, Nitric Oxide	Cystic Fibrosis, Asthma, COPD	(Cunningham et al., 2000; Gessner et al., 2007; Jöbsis et al., 2000)
Enzymatic	Leukotriene, 8-isoprostane, cytokine	Asthma, COPD	(Becher et al., 1997; Gessner et al., 2005; Hanazawa et al., 2012; Kharitonov & Barnes, 2001; Montuschi et al., 2012)
Gene	TP53, EGFR, p16 gene mutation	Lung Cancer	(Gessner et al., 2004; Peralbo-Molina et al., 2016; Zhang et al., 2011)
Protein	proteome	Lung Cancer	(Fedorchenko et al., 2016; Muccilli et al., 2015)
Virus	Human Papillomavirus (HPV)	Lung Cancer	(Carpagnano et al., 2011)

2.3. Breath Sampling

2.3.1. Points to take into consideration

Although the sampling step of breath is considered the most crucial stage, procedures applied before and after sampling have a direct impact on the analysis results. Therefore, procedures to be applied before, during, and after the sampling step should be considered as a whole. In this section, pre-sampling procedures for EB (Exhaled Breath), EBA (Exhaled Breath Analysis), and EBC (Exhaled Breath Condensate) sampling methods, along with post-sampling processes, are discussed in detail. Before sampling for the purpose of breath analysis, it is important to consider specific factors that contribute to the validity of the study, ensuring the accuracy and reliability of the results. Key factors responsible for accurate measurement, such as selection of study groups, food intake, physical activity, etc., which need attention during the sampling step, are summarized below.

Insufficient sample size and dissimilarities between patients and controls (e.g., age, gender, smoking history) can lead to incorrect results. Therefore, the sample size should be taken in account before starting sampling step and the two compared groups should be selected as homogeneously as possible (Lourenço & Turner, 2014).

Food and beverages directly impact the volatile composition of EB, hence imposing certain restrictions on dietary habits is important to ensure the accuracy of breath sampling. Numerous studies address the influence of food and beverages on breath analysis, advising participants to avoid specific foods and drinks (e.g., garlic, onions, and alcohol) before collecting breath samples. Because these substances may potentially contain volatile compounds that could interfere with the analysis. For instance, when alcohol is metabolized in the liver, it produces metabolites like acetaldehyde and acetic acid, which can also be detected in EB (Španěl et al., 2017; C. Turner et al., 2006). In another study, Čalušić and colleagues demonstrated changes in adenosine levels in EB following the consumption of caffeinated beverages (Čalušić et al., 2011). Some research also indicates that

metabolic products such as ammonia, ethanol, glucose, acetaldehyde, and lactic acid after the consumption of staple foods and beverages may elevate the pH of EB from 6.5 to 6.9 (Alonso & Sanchez, 2013; Bikov et al., 2014; Kullmann et al., 2008; Wallace & Pleil, 2018). In other studies are shown that H₂O₂ or increased amounts of oxidants containing food and beverages products may affect the levels of reactive oxygen compounds in EB (Gajdoci et al., 2011). Additionally, a study found that nitrate/nitrite-rich food sources like spinach and beetroot juice might potentially impact the concentrations of markers associated with NO in EB (Martens et al., 2005). Additionally, as in the Hydrogen/Methane test some food and beverages are subject to strict restrictions because they alter breath composition (Rezaie et al., 2017). Smoking can also affect the results of breath composition. Pollutants can enter the body while smoking and subsequently influence the results of EB analysis (Buszewski et al., 2009). For instance, real-time breath analyzer results have shown significant changes in the concentrations of certain analytes in the EB of smokers after smoking (Alonso et al., 2009). Another study indicated the presence of certain compounds in the EB of non-smokers after exposure to secondhand smoke (Dragonieri et al., 2022; Gordon et al., 2002). Due to such reasons, it's recommended that participants abstain from food, drink, smoking, tobacco products, and alcohol for at least eight hours before providing a breath sample (Ćalušić et al., 2011). The primary reasons for this eight-hour avoidance period before collecting a breath sample are to stabilize baseline levels, minimize external effect and daily changes, standardize research and clinical protocols, and ensure accurate decision-making (Henderson et al., 2021). Low water intake is another critical factor to consider when performing breath analysis. Dehydration can be observed due to low water intake, leading to changes in breath composition (Biagini et al., 2022). Maintaining breath sample consistency through proper hydration. Therefore, ensuring adequate hydration is essential to preserve the stability of EB samples (van Nieuwenhoven et al., 2000).

Another significant factor that can affect breath composition is physical activity. Physical effort is also changes the composition of breath due to increased metabolic activity (Miekisch et al., 2004). For instance, it has been shown that the concentration of adenosine in EBC increases with exercise (Csoma et al., 2005).

Therefore, it is recommended that participants rest for at least one hour before the sampling step (Horváth et al., 2005).

Some VOCs can be produced by the bacterial activity or oxidation because of retained food residues in patients' mouth. These compounds might impact the results of breath analysis. To minimize interference, participants are asked to rinse their mouths with distilled water or isotonic saline solution before analysis (Štanfel et al., 2022). This procedure can help reduce significant interference effects of certain compounds on the results of breath analysis.

There are several important points to consider during sample collection. These factors have the potential to affect the analysis results, and they are summarized below. Breath analysis may be affected by the presence of other interfering compounds in the sampling environment. The environment in which the sample is taken has low air quality, particularly due to high levels of particulate matter or volatile compounds, can lead to the mixing of pollutants or foreign substances into breath samples, potentially impacting the results of breath analysis (Huang et al., 2012). Exhaust fumes or solvents such as ethanol, acetone, or isopropyl alcohol are among the most common environmental pollutants in hospital for breath sampling (Sanchez, 2019; Wallace et al., 2019). The levels of non-respiratory compounds in the environment are reduced by effective ventilation, which can reduce the likelihood of cross-contamination and improve the quality of air. Additionally, humidified clean air from a gas cylinder can be delivered to the patient through a mask during breath sampling to prevent external pollutants (Tetik Vardarlı et al., 2023). Therefore, obtaining a contamination-free environment before the sampling stage is crucial. Patients can be isolated from ambient air using appropriate equipment. This can be accomplished by sampling in isolated clean rooms or by allowing individuals to breathe clean, uncontaminated air (Pleil et al., 2013; Tetik Vardarlı et al., 2023). Environmental temperature and humidity can also impact EBC moisture levels. Changes in temperature and humidity during breath sampling can cause condensation or evaporation of moisture generated during exhalation, affecting the concentrations of biomarkers in breath samples (Mansour et al., 2020). Therefore, maintaining a constant temperature and humidity

during sampling is important to obtain more accurate results. The composition of the EBC can also have an effect on the psychological state of patient (Tonacci et al., 2019). To minimize stress level is important to explain the process management and ethical guidelines regarding breath sample collection to patients. Additionally, patient comfort can also impact sampling outcomes. Högman and colleagues demonstrated that changes in a patient's respiratory cycle during sampling could affect nitric oxide (NO) levels in EB (Högman, 2012). Turner and colleagues suggested an increase in concentrations of indole and 2-methylpentadecane in EBC under stress conditions (Turner et al., 2013). It is important to ensure that participants are comfortable during breath sampling to prevent discomfort or anxiety from affecting breathing pattern. Another significant aspect is the method of breath collection, whether it's through the nose or mouth, which can affect the concentration of biomarkers presented in EBC (Vass et al., 2003). Studies have shown that obtaining a higher volume of EBC is possible when individuals breathe through the nose. During the sampling step, it's important to ensure consistency in the breathing style for all cases.

Sampling temperature is another critical parameter that can affect the composition of collected breath samples (Zamuruyev et al., 2018). Therefore, both room temperature and sample collection temperature should be carefully controlled. The duration of breath sample collection is also critical for specific breath tests. In some cases, samples need to be collected at specific intervals or for a predetermined duration (Doran et al., 2018). This is because the concentrations of compounds differ between tidal and alveolar air. Particularly when sampling within a single breath, ensuring that alveolar breath is sampled is essential; otherwise, inaccurate results may be obtained.

Taking safety measures during the sampling stage is also important particularly when working with highly contagious infectious diseases like COVID-19 (Myers et al., 2022). In such situations, appropriate safety protocols, including the use of personal protective equipment, should be implemented to protect both participants and researchers. Additionally, the proper calibration and validation of

the analytical instrument used to analyze breath samples, including flow meters, temperature probes, and humidity meters, are necessary to obtain accurate results.

The sensitivity of breath sampling depends on choosing an appropriate method (Herbig & Beauchamp, 2014). For this purpose, different sampling methods can be applied for EB, EBA, and EBC sampling. Each method is detailed in the following sections.

2.3.2 Exhaled Breath Sampling

In exhaled breath (EB) sampling, volatile substances can be sampled and analyzed in two methods: "real-time" methods, where sampling and analysis of exhaled breath occur simultaneously, and "offline" methods, where samples are collected and later analyzed using a specific device. Real-time measurement methods allow for rapid and accurate analysis of samples taken from patients, eliminating the need for sample preservation. Offline analysis methods require proper sample collection, storage, transportation to the analytical device, and each step needs to be carried out separately.

Real-time measurements utilize high-sensitivity techniques based on mass measurement, such as Selected Ion Flow Tube (SIFT) Mass Spectrometry (MS), Proton Transfer Reaction (PTR)-MS, optical methods relying on light-matter interactions like infrared, or electronic noses based on generating electrical signals. Below is a brief summary of the available techniques in the following table.

Table 2.4 Real time breath analysis techniques

<u>Method</u>	<u>Reference</u>
Selected Ion Flow Tube (SIFT) Mass Spectrometry (MS)	(X. Zhang et al., 2023)
Proton Transfer Reaction (PTR)-MS	(Su et al., 2023)
Extractive Electrospray Ionization (EESI-MS)	(Wu et al., 2021)
Secondary Electrospray Ionization (SESI-MS)	(Blanco & Vidal-de-Miguel, 2023)
<u>Infrared-Based Methods</u> Nondispersive Infrared Spectroscopy, Hollow Waveguide Adsorption Spectroscopy	(Haisch et al., 1994; Zhou et al., 2020)
<u>Laser-Based Methods</u> External Cavity Quantum Lasers, Hollow Ring Spectroscopy Off-Axis Hollow Integrated Spectroscopic Systems Hollow Leak Spectroscopy Optically Feedbacked Hollow Absorption Spectroscopy	(Dahnke et al., 2001; Gohle et al., 2007; Kamat et al., 2007; Larracy et al., 2022; Z. Li et al., 2023; Mitscherling et al., 2009; Panda et al., 2022; Parameswaran et al., 2008; Sahay et al., 2013)
<u>UV-Based Methods</u> UV-Visible Spectroscopy	(Iwata et al., 2016)

Table 2.4 (Continue)

<u>Photoacoustic-Based Methods</u> Quartz-Enhanced Photoacoustic Spectroscopy Console-Enhanced Photoacoustic Spectroscopy	(Cristescu et al., 2014; Maurin et al., 2020; Narasimhan et al., 2001; Schmidt et al., 2011; Spacek et al., 2015; Spagnolo et al., 2019; Wacławek et al., 2014)
<u>Raman-Based Methods</u> Raman Spectroscopy	(Hanf et al., 2014, 2015)
<u>Chemiluminescence-Based Methods</u> Chemiluminescence	(Lv et al., 2022; Z. Zhang et al., 2023)
Fourier Transform Ion Cyclotron Resonance Mass Spectrometry	(Fu et al., 2014)
Semiconductor Chemical Electronic Nose	(Kononov et al., 2020)
Polymer-Based Electronic Nose	(Anzivino et al., 2022)
Piezoelectric-Based Electronic Nose	(Shao et al., 2010)
Electro-chemical-Based Electronic Nose	(Saraoglu et al., 2013)
Surface Acoustic Wave (SAW) Sensor-based Electronic Nose	(D. Wang et al., 2011)
Optical Sensor-Based Electronic Nose	(Eambaipreuk et al., 2011)

In methods where breath is sampled for analysis, the sampling step is employed for the storage or pre-treatment of breath to either store it or prepare it for further analysis. Gas containers such as gas sampling bags, gas bulb and/or adsorption devices (like fibers, membranes, strips, tubes) are used for breath storage. Among the sampling bags commonly used for the analysis and storage of breath samples are polyethylene terephthalate-based Nalophan, polyvinyl fluoride-based Tedlar, and polyvinyl fluoride-based Mylar (BoPET) bags, as well as aluminum, SamplePRO Flexlm and polyvinylidene difluoride-based Kynar bags. However, there is a risk of changes in the concentration of analytes when breath is stored in these bags (Mochalski et al., 2013). Therefore, it is important to optimize parameters such as storage time and temperature in the bags before analysis.

Systems made of gas-impermeable syringes or gas sampling chambers, apart from bags, constructed from glass or polymer, are suitable options for EB sampling (Kwak et al., 2014; Lindstrom & Pleil, 2002; Speiser et al., 2012).

Pre-treatment Methods: Various techniques are used for the pre-treatment of exhaled breath samples. Solid-phase adsorption is one of the most common pre-treatment techniques used in EB sampling. Additionally, alternative sampling methods are limited to chemical or cryoprecipitation technologies (Ochiai et al., 2001). In adsorptive trapping, volatile compounds are adsorbed using materials such as organic polymers like Tenax TA or substances like activated carbon (Mueller et al., 1998; Tangerman et al., 1985). These adsorbents are typically tightly packed into a tube and analyzed after the adsorption step through thermal desorption.

Microextraction-Based Techniques: Solid-phase microextraction (SPME) is the general name for microextraction techniques in general and there are many different types of methods, such as fiber SPME, needle-trap SPME, in-tube SPME etc. (Yuan et al., 2022). The use of solid phase microextraction (SPME) techniques for the analysis of VOCs in breath has become an increasingly interesting area in recent years. The key advantages of these techniques lie in their simplicity, cost-effectiveness, speed and the absence of expensive equipment requirements. A

summary of microextraction-based techniques used in breath analysis is provided in Table 2.5.

Table 2.5 The microextraction techniques used in breath analysis

<u>No</u>	<u>Technique</u>	<u>Reference</u>
1	Solid Phase Microextraction	(Grote & Pawliszyn, 1997)
2	Needle Trap Solid Phase Microextraction	(Mieth et al., 2009)
3	SnifProbe (Aviv Analytical Ltd., Hod Haharon, Israel)	(Gordin & Amirav, 2000)
4	MEPS (SGE, Trajan Scientific Australia Pty Ltd., Australia)	(Revelsky et al., 2022)
5	In-tube extraction solid-phase dynamic extraction	(Manousi et al., 2020)

Solid Phase Microextraction Fiber Technique: The SPME fiber technique is one of the most commonly used microextraction techniques in breath analysis (Lawal et al., 2017). Developed by Pawliszyn and colleagues in 1990s, the SPME fiber technique is a pre-treatment method (Grote & Pawliszyn, 1997). An overview of the SPME fiber is illustrated in Figure 2.4.

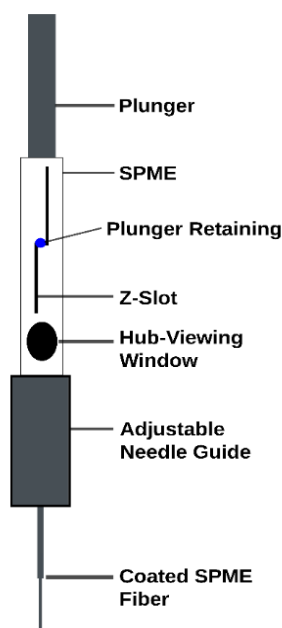


Figure 2.4 General part of SPME fiber

The SPME fiber technique is simple, rapid and solvent-free. Both commercial and custom made SPME fibers have been used for sampling analytes and EB. In the EB sampling, the fiber can be directly placed inside the bag where the sample is contained, under equilibrium or flow condition (Musteata & Pawliszyn, 2007; Queiroz et al., 2022), or it can be applied to the headspace or immersed in a vial after breath condensation for sampling (Figure 2.5) (Zeinali & Pawliszyn, 2022).

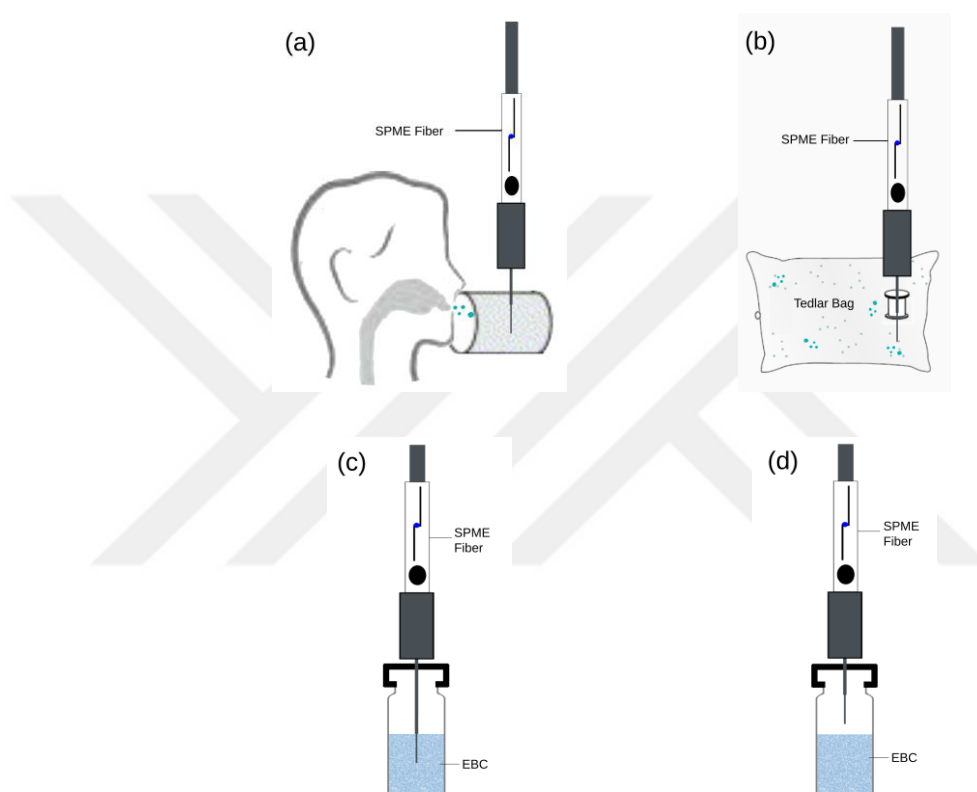


Figure 2.5 Breath sampling methods with SPME (a) Flow sampling (b) Tedlar bag sampling (c) Direct immerse sampling (d) Headspace sampling

The adsorbed analytes are typically analyzed by gas chromatography using thermal desorption, usually without significant loss. The thermal stability of adsorbent materials is crucial; hence, the desorption temperature should be selected at least 10°C below the decomposition temperature. This ensures that the advantages of the SPME fiber listed below make it suitable for clinical applications. It is prepared by coating polydimethylsiloxane (PDMS) or a different material onto a support such as fused silica or stainless-steel wire. The choice of SPME fiber

material should be based on the type of analyte targeted. Both commercial and custom-made SPME fibers are available. Some types of SPME fibers used in breath analysis, depending on the type of analyte, are summarized in Table 2.6.



Table 2.6 Examples of commercial and custom-made SPME fibers used in breath analysis.

No	<u>Coating Material</u>	<u>Analyzed Analyte</u>	<u>Reference</u>
1	PDMS/DVB	Amines, nitro-aromatics, acrolein, aldehyde, acetone, isoprene, menthol, pentane, propanol	(Dias et al., 2017; Fuchs et al., 2010; Roszkowska et al., 2019; Ulanowska et al., 2012)
2	PA	Methadone	(Beck et al. , 2011)
3	DVB/CAR/PDMS	Nonane, 2,2,4,6,6-pentamethylheptane, decane, dodecane, tetradecane, nonanal, decanal, dodecanal, hexane, 3-ethyl, 3-methylheptane, 2,4-dimethylheptane, undecane, propyl cyclohexane, butanal, octanal, benzaldehyde	(Caldeira et al., 2012; Couto et al., 2017)
4	PDMS	Hexadecenal, eicosane, 2,6,11-trimethyldodecane, 8-hexylpentadecane (26VOCs), aromatic hydrocarbons	(Wallace & Pleil, 2018; Wang et al., 2012)
5	Hybrid ZIF-8-90	alkanes ethanol, acetone, hexanal, hexanol, isoprene, heptane, decane	(Ma et al., 2021)
6	CAR/PDMS	Isoprene, menthol, pivalic acid, acetone, tetradecane, 2-methylpentane, 3-methylpentane, hexane, 2,3-dimethylpentane, 2-methylhexane, 3-methylhexane, dodecane, acetone, ethanol, 1-propanol, 2-propanol, 1-butanol, acetic acid, propionic acid, butyric acid, valeric acid, isovaleric acid, 2-methylbutyric acid, hexanoic acid, phenol, pentane, hexane, heptane, benzene, ethylbenzene, xylene, propanal, n-butanol, trans-2-hexanal, trans-2-heptanal	(Corradi et al., 2015; Ma et al., 2014)
7	PDMS/DVB	4,6-dimethyldodecane, 2,2-dimethylpropionic acid, 5-methyl-3-hexanone, 2,2-dimethyldecane, limonene.	(Hakim et al., 2011; Peng et al., 2010)

2.3.3 EBA Sampling

EBA can be sampled by having the subject wear a mask for a specific duration. The main advantage of the technique is the sampling of only dried, non-volatile compounds. Therefore, equipment such as bottles, waste bags, gas cylinder, syringes, pipettes, solvents, refrigerated shipping containers, etc., is not required for transportation. It can be sampled in a manner similar to the EBC system.

Sampling can be performed using filter in different forms at room temperature (Hu, 2022; Pleil et al., 2018). After taking a deep breath, samples can be collected either into a system containing a filter or directly onto a simple medical mask by exhaling into it. If individuals hard to exhale due to the diameter of the filter, a pump can be used to pull exhaled breath in the direction of the filter, allowing individuals to breathe comfortably. There are both commercial and custom made types available. A summary of these is provided in Table 2.7.

Table 2.7 Exhaled Breath Aerosol collection systems

No	EBA Sampling System	Filter Types	Reference
1	Commercial	Gesundheit II, ExABreath	(Kintz et al., 2016; Lai et al., 2022)
2	Impactors	Silicone plate	(Almstrand et al., 2009)
3	Filters	Electret	(Tinglev et al., 2016)
4	Nebulizers	Jet nebulizer; breath-actuated nebulizer; hand-triggered nebulizer; breath powdered nebulizer;	(Lin et al., 2018)
5	Electrostatic precipitators	Polycarbonate plastic	(Pardon et al., 2015)
6	Sampling Chambers	Medical Mask	(Lindsley et al., 2021)
7	Real-time aerosol analyzers	Polyacrylonitrile	(Zeinali et al., 2022)

2.3.4 EBC Sampling

Sampling of EBC is a moderately simple process that involves passing the exhaled breath through a cooled tube in a condenser. During this process, water vapor and EBA, as well as dissolved compounds, condense on the walls of the cooled tube. The collected EBC is then transferred to a suitable vessel for storage or analysis.

EB can be transferred to the condenser unit through different breathing methods. The general approach involves inhaling air through the nose and exhaling it into the condenser unit via the mouth (Grob et al., 2008). In some studies, to prevent external contamination, individuals breathed moistened pure air through a nasal mask and exhaled into the condenser through their mouths (Tetik Vardarlı et al., 2023). Additionally, there are EBC sampling studies in certain cases using a full-face mask for critically ill patients unable to breathe on their own (Pierucci et al., 2021).

Table 2.8 Some of the EBC collection systems and their characteristics

No	Sample Collection System	Material	Cooling Temperature	Reference
1	Laboratory-made	Glass	-20°C	(Tetik Vardarlı et al., 2023)
2	EcoScreen2, EcoScreen Turbo	Aluminum coated with PFTE	-10°C	(Konstantinidi et al., 2015)
3	Rtube, RTubeVOC, Rtube Vent	Polypropylene	Cooling Sleeve (-20°C)	(Połomska et al., 2021)
4	TurboDECCS	Polypropylene	-10°C	(Leese et al., 2016)
5	ANACON		-10°C	(Romero et al., 2006)

As mentioned above, although the collection of EBC is relatively simple, there is no standardized sampling protocol. There exist various commercial and custom-made EBC sampling devices (Konstantinidi et al., 2015). Custom-made devices can be produced in a wide array of materials and designs targeting the analyte. Different types of commercially available devices include EcoScreen2, Rtube, RtubeVOC, Rtube Vent, TurboDECCS, ANACON, EcoScreen Turbo, and Airway Lining Fluid Analyzer (Kubáň & Foret, 2013). A summary of studies conducted with EBC is provided in Table 2.8

Both commercially available and custom-made devices consist of three main parts: the EB sample introduction part, the condenser component, and the EBC sample transfer component. The general section of the EBC sampler is illustrated in Figure 2.6.

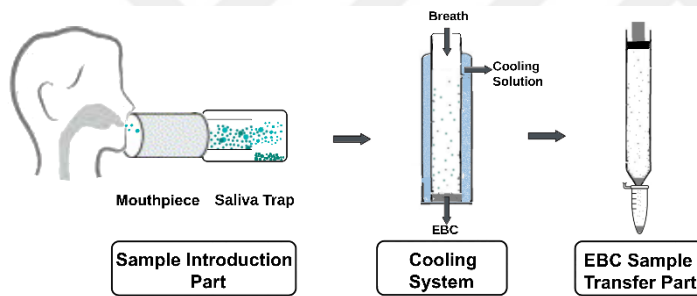


Figure 2.6 General presentation of the EBC sampling device

The Sample Introduction Part typically comprise both a mouthpiece and a saliva trap. The EB is directed into a sample collection device with a cooled tube through a mouthpiece. For the transfer of EB through the condenser, a mouthpiece usually made of silicone rubber-based material is commonly used. If EB is exhaled through the mouth, saliva can enter the cooling tube along with the EB, leading to saliva contamination of EBC. Detection of amylase enzyme in EBC indicates sample contamination with saliva (Zamuruyev et al., 2017). Saliva trap can be placed in front of the cooling tubes to prevent contamination (Figure 2.6). Various saliva traps like swan-neck or U-shaped traps have been used previously (Horváth et al., 2005; Koc et al., 2019; Rosias et al., 2004). In the U-shaped saliva trap used by Koc et al., the trap was heated up to 40°C to prevent condensation of EB (Koc

et al., 2019). EBC contamination can be minimized by gargling with 4.5% sodium bicarbonate solution before sampling or periodically swallowing saliva during sampling procedures (Horváth et al., 2005; Rosias et al., 2004).

The Cooling System is an essential component of the condenser device. EBC samplers can be designed as open-ended or fully isolated systems where the EBC sample is in contact with ambient air (Koc et al., 2019; Rosias et al., 2008). Various cells have been designed for the condensation of EB, including pipes, tubes, or containers made of different lengths, diameters, or dual-walled materials (Horváth et al., 2005; Kietzmann et al., 1993; Schleiss et al., 2000; Sznajder et al., 1989; Szunerits et al., 2023). The choice of construction materials, geometry, and operating conditions for the collection tube is crucial in determining the effectiveness of the EBC condenser. Tubes can be made from various materials such as glass, polypropylene, polyethylene, silicone, PTFE, tygon, aluminum, or inert polymer-coated metal (Mutlu et al., 2001). Cooling systems using materials like ice (0°C), wet ice ($\cong -2^{\circ}\text{C}$), dry ice (-78°C), liquid nitrogen (-196°C), or cooling gels are employed to condense EB. Additionally, an electric cooling system can be used. Electric cooling is utilized in both commercial products like EcoScreen2 and TurboDECCS, as well as in custom-made devices (Ghelli et al., 2022; Tetik Vardarli et al., 2023).

Several important factors in the cooling cell affect the efficiency of EBC collection. When an open-ended design is used, the uncondensed portion of the breath escapes into the environment, resulting in lower sampling efficiency for these systems (Kubán & Foret, 2013). Thus, an isolated system can achieve higher sampling efficiency. Another parameter to consider is the surface area of the condenser tubes, generally increasing surface area improve condensing efficiency (Zamuruyev et al., 2017). In a study, the optimal collection efficiency was found to be 1046 cm², so the length of the cooling tube in a regular system can be chosen as 90 cm (Rosias, 2012). The interaction of cooling tube materials with target analytes in EBC sampling is another crucial issue, and the materials used can directly affect the concentration of some analytes in EBC. To illustrate, under the same collection conditions, when compared to Polytetrafluoroethylene (PTFE), polypropylene, and

aluminum, silicone and glass tubes have shown higher collection efficiency for albumin (Rosias et al., 2006). Thus, the cooling tube should be selected from a proper material that does not interact with the target analytes. Another factor is the EBC sampling temperature, which not only affects condensation efficiency is affected as well as the concentration of analytes in the EBC (Goldoni et al., 2005). The collection temperature of EBC can be set to the desired value using an appropriate system, and sampling efficiency can be increased with decreasing temperature. Nonetheless, the minimum temperature resistance of the condensation unit should be considered during sampling (Vyas et al., 2012). The EBC collection efficiency increases with decreasing sampling temperature (Czebe et al., 2008). Additionally, the increased collection efficiency of EBC can lead to the dilution of compounds in EBC (Zamuruyev et al., 2018). On the other hand, if EBC sampling is performed at a high temperature, the concentration of some unstable compounds may decrease (Davis et al., 2012). Thus, it is important to optimize the sampling temperature before collecting the target analytes in EB. Sampling time is a critical factor that varies with condensation temperature, and is typically continued until the required amount of EBC, often 1-2 mL, is collected (Soyer et al., 2006). While there are some studies in the literature that use very short times, such as 3 minutes, or very long times, like 60 minutes, the total sampling time commonly used in most studies ranges between 10-30 minutes (Scheideler et al., 1993; Vaughan et al., 2003). Some literature suggests that with longer sampling times, dilution problem may be observed for certain analytes but not for others (Kazeminasab et al., 2020). Additionally, it has been shown that some unstable compounds, such as leukotrienes and purines, can degrade when longer sampling durations are applied (P. Rosias, 2012).

EBC device sample transfer compartment is used to transfer the sample into a storage container, and this process can be accomplished using various techniques. In the first method, EBC samples are dripped into a suitable container by the force of gravity (Zamuruyev et al., 2017). Another method involves using a special inert piston to sweep EBC from the condenser wall into dedicated storage bottles. This method maximizes the collected EBC amount. In another study, Vardarli and colleagues developed a needle-type sample transfer line (Figure 2.7). In this system,

after the EBC droplet is swept to the bottom of the cooling tube, the collected sample is pierced with just a needle without any risk of contamination, and then transferred to a vacuum medical tube (Figure 2.7). To achieve more reliable results, the selection of the collection material and storage conditions are also crucial (Kazeminasab et al., 2020). The type and surface character of the sample collection device, saliva traps, the volume of collected EBC, storage duration, and conditions all influence the quality of the biomarkers under investigation. Therefore, the working conditions in EBC sampling studies should be optimized according to the biomarker being studied.

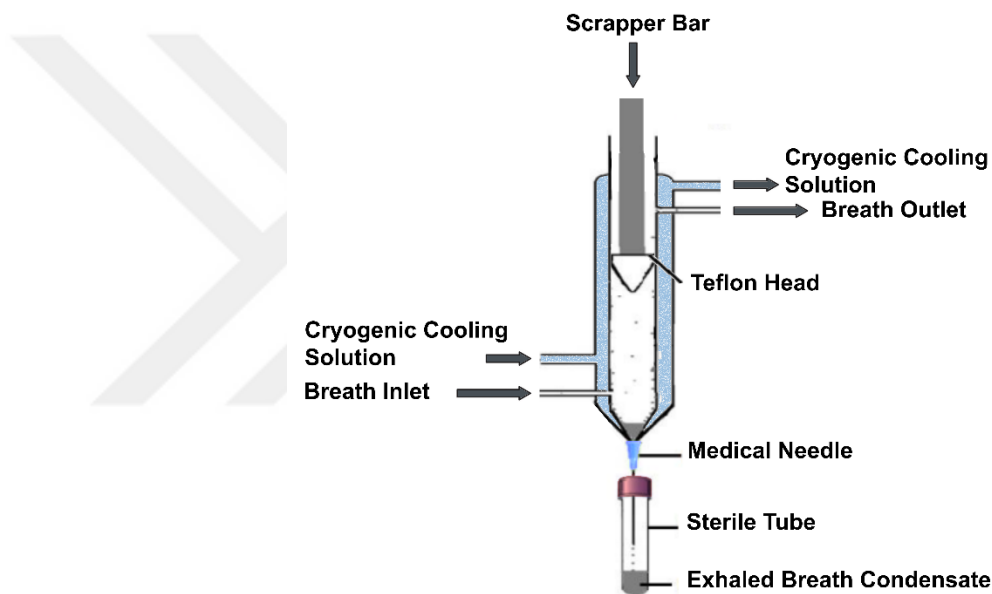


Figure 2.7 Needle type EBC collection system (Tetik Vardarlı et al., 2023)

There are also some considerations to take into account during EBC sampling. As mentioned earlier, the risk of contamination is a serious concern in EBC analysis. Some indoor air pollutants may be present in EBC and react with certain biomarkers, causing changes in their concentrations (Belo et al., 2019). It has been demonstrated that when ambient air is not filtered during the sampling stage, there is an increase in keratins and H_2O_2 concentration (Hoffmann et al., 2008; Knobloch et al., 2008). In another study, only ambient air passed through the EBC sampler, and some cfRNAs were detected in the obtained EBC (Koc et al., 2019). These substances can be present indoors due to the emission of aerosols from human

exhaled breath. Therefore, the using of a fully isolated EBC sampling system is suggested as more suitable for obtaining reliable results without contamination, than the using of a non-isolated system.

The cleaning procedures of the EBC sampling device can also affect the concentrations of biomarkers in EBC (Horváth et al., 2005). Therefore, it is important to carefully follow a cleaning protocol. It is recommended to leave the EBC device in a 1% aqueous sodium hypochlorite solution, for at least 1 hour to ensure effective disinfection. After sterilization, thoroughly rinse the EBC collection system with water to remove any remaining antiseptic solution and then dry it. However, some cleaning materials remaining during the cleaning stage can release compounds that may cause carryover effects (Hakim et al., 2011). Moreover, target analytes may be interfered with in another sampling step by residual disinfectant from the sampling systems (Ahmadzai et al., 2013). While using disposable sampling units in EBC devices eliminates such issues, it also increases the analysis costs.

2.4. Biomarkers Analysis Methods

Some techniques used in the analysis of biomarkers in EBC are provided in Table 2.9.

Table 2.9 List of EBC analysis techniques

<u>No</u>	<u>Analysis technique</u>	<u>Reference</u>
1	Spectroscopic Techniques	(Vlašić et al., 2009)
2	Chromatographic Techniques	(Su et al., 2023)
3	Nanoparticle-Based Techniques	(Jouyban et al., 2017)
4	Immunosensor Techniques	(Zhang et al., 2015)
5	Nuclear Magnetic Resonance Technique	(Maniscalco et al., 2018)
6	Electrophoresis Technique	(Hoffmann et al., 2008)

The detailed descriptions of the chromatographic techniques GC-MS and LC-Q-TOF, which are used in experiments among these methods, are provided below.

2.4.1 Gas Chromatography – Mass Spectrometry (GC-MS)

The gas chromatography technique coupled with different detectors is one of the most commonly used methods for the analysis of volatile organic compounds. It consists of an inlet where analytes are injected and vaporized, an analytical column where analytes are separated, and a detector where analytes are converted into signals (Figure 2.8). In gas chromatography, analytes are generally carried through the column by an inert gas such as helium. Separation of substances is typically achieved in a capillary column with an inner wall coated with a stationary phase. Analytes are separated from the column based on the magnitude of their interactions with the column packing material. A temperature program is often applied for the separation of substances. Analytes leaving the column are commonly detected with a mass spectrometer (MS), which emerged in the 1860s, especially for the analysis of complex matrices (Sharma, 2013). The MS detector provides both qualitative and quantitative information. Generally, the signal intensity or peak area is linearly correlated, allowing the calculation of analyte concentration.

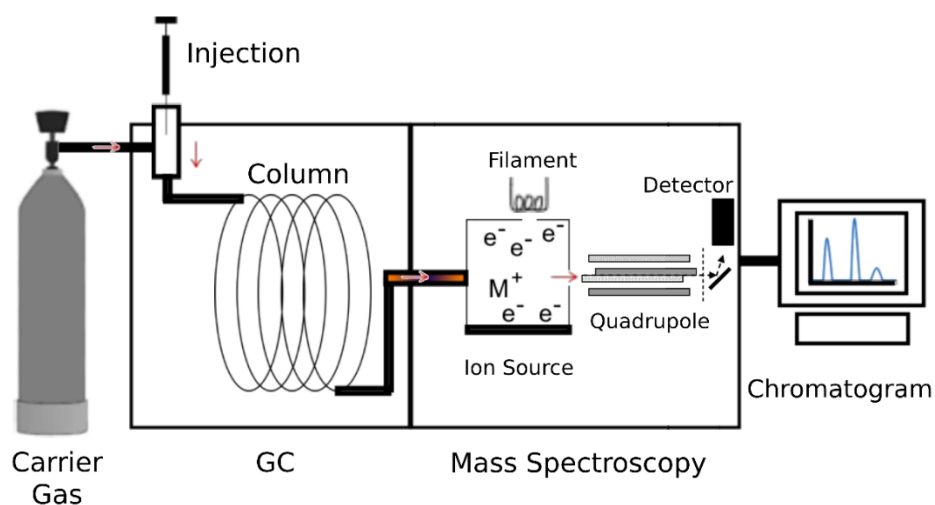


Figure 2.8 General purpose of GC-MS

In some cases, the analysis of certain analytes that cannot be directly analyzed can be performed after a derivatization step. Following the derivatization process, the analysis of non-volatile substances can be facilitated by converting them into volatile forms. The most commonly used derivatization methods for GC-MS are oximation and silylation. Derivatization reactions contribute to the isolation and detection of metabolites (Halket et al., 2005). With derivatization, GC-MS can be used not only for volatile and non-polar compounds but also for the analysis of polar compounds such as fatty acids, amino acids, amines, sterols, and sugars.

2.4.2 Liquid Chromatography Quadrupole Time-of-Flight (LC-QTOF)

Liquid chromatography is a technique that enables the effective separation and analysis of thermally unstable and high-boiling-point compounds. Similar to GC, this system is based on the principle of separating analytes in a moving liquid phase within a column containing a stationary phase. After the analytes are separated by liquid chromatography, charged ions are generated by ionizing them using an ion source. Ionization techniques such as electrospray ionization or atmospheric pressure chemical ionization are employed. Subsequently, ions are filtered or selected based on their different m/z values using the first quadrupole (serving as an ion channel). The ionized analytes are then detected with the powerful component identification capability of mass spectrometry (MS). Detector systems have made significant progress in recent years, especially with time-of-flight mass spectrometry (TOF-MS) systems, allowing for the analysis of analytes with high mass separation power. Therefore, LC-QTOF systems are effective tools for separating and analyzing analytes in biological matrices such as EBC.

After entering the second quadrupole collision cell, where ions can obtain fragment ions through collision, ions with different m/z values move toward the electric field. Under the acceleration of the electric field, ions with the same kinetic energy are obtained. Finally, due to different flight times resulting from the same kinetic energy and different m/z values, mixed ions are separated in the ion drift tube, and the relevant mass spectra are obtained with data analysis software for

qualitative and quantitative analysis. The definition of liquid chromatography, and the general form of LC-QTOF, is shown in Figure 2.9.

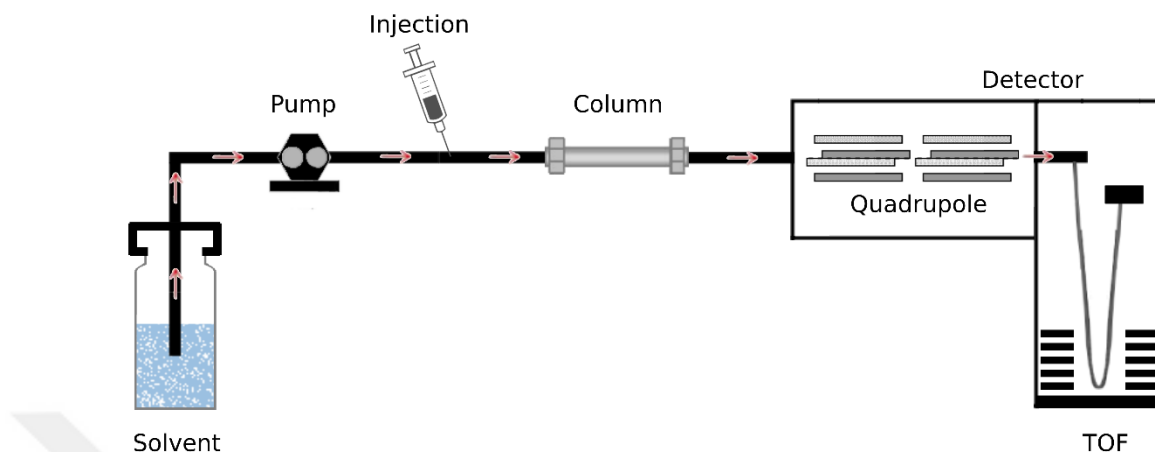


Figure 2.9 Schematic representation of the LC-QTOF device

This technique is mainly used for the analysis of non-volatile compounds. Due to the high complexity of certain samples (e.g., wastewater and biological samples), high-resolution techniques are required to provide additional structural information.

However, with LC/MS/MS, the challenge of identifying an unknown peak only from the mass spectrum still exists. Currently, aside from user-generated libraries, there are no widely available libraries for unknown compounds. Both TOF/MS and sector instruments are typically used in conjunction with LC/MS/MS to address these challenges in accurately determining mass and identifying unknowns.

Recently, LC/TOF/MS has been used for the accurate mass measurement of protonated molecules to confirm the compound in complex matrices (Ferrer & Thurman, 2003). Accurate mass measurements of fragment ions have become especially crucial for elucidating the structure of unknowns. In this regard, Q-TOF MS/MS is unique in its ability to provide accurate mass measurements (1-2 mmu) of fragment ions emerging from the collision cell. This accuracy ensures not only

the precise identification of unknowns but also a high level of confidence in the empirical formula of fragment ions.

Sometimes, a single-stage TOF/MS provides this information by transferring enough energy to the $[M+H]^+$ ions in the source region to cause fragmentation. TOF mass analysis allows for increased resolution of signals along with the m/z axis compared to other quadrupole mass spectrometers. Furthermore, this enhanced resolution power is beneficial for analyses involving complex matrices, as it helps separate isobaric interferences from the signals of interest. The improved resolution power also facilitates the measurement of accurate masses within the accepted 5 ppm for the verification of elemental compositions. The elemental compositions of pollutants and their fragment ions can be clearly established with a higher degree of resolution than provided by nominal mass measurements.

TOF/MS device is a powerful tool for the identification of non-targeted compounds in complex matrices due to three key features:

1. The ability to collect data across a wide mass range without a loss in sensitivity allows for obtaining comprehensive spectral sensitivity;
2. The ability to resolve interference from distant signals with high resolution power; and
3. Obtaining sufficient mass measurement accuracy for the estimation of the elemental composition.

Q-TOF MS combines the simplicity of a quadrupole MS with the ultra-high efficiency of a TOF mass analyzer. In a Q-TOF instrument, the sample passes through the interface, and ions are focused onto the quadrupole MS using a hexapole ion bridge. Here, the precursor ion is then selected with approximately a 3 mass unit-wide mass window for fragmentation and analysis; this is a typical window size to preserve isotopic envelopes in product ion spectra. Ions are launched into the hexapole collision cell, where argon is used for fragmentation. From this point, ions are collected in the TOF region of MS/MS. The entry of ions

is such that the flight path of ions changes 90° , known as orthogonal TOF. The purpose of the change in direction is to optically focus the ions' kinetic energies to be as similar as possible. The ions are then accelerated by the pusher and move down towards the reflector in the flight tube about 1 m below. The purpose of the reflection is to decelerate ions with equal mass but higher kinetic energy and then focus this beam of ions to the detector, allowing ions with the same exact mass but slightly different energies to reach the detector at precisely the same time. This process results in the mass accuracy of Q-TOF MS/MS and is considered a high-resolution device with a resolving power of 10,000 FWHM.

2.5. Lung Cancer

The most common and leading cause of cancer-related deaths worldwide is lung cancer (LC). The incidence of lung cancer has been steadily increasing, primarily due to the rise in tobacco use and exposure to other environmental factors. According to GLOBOCAN 2020 data, lung cancer ranks first among cancers in men (36.5 incidence per 100,000) (Figure 2.10) and third in women (19.9 incidence per 100,000) globally (Figure 2.11) (Sung et al., 2021). Lung cancer is the most prevalent cause of cancer-related deaths worldwide and in Türkiye. According to the World Health Organization (WHO) 2020 death statistics, it ranks 5th in males and 12th in females as a cause of death (Table 2.10) (Global health estimates: Leading causes of death).

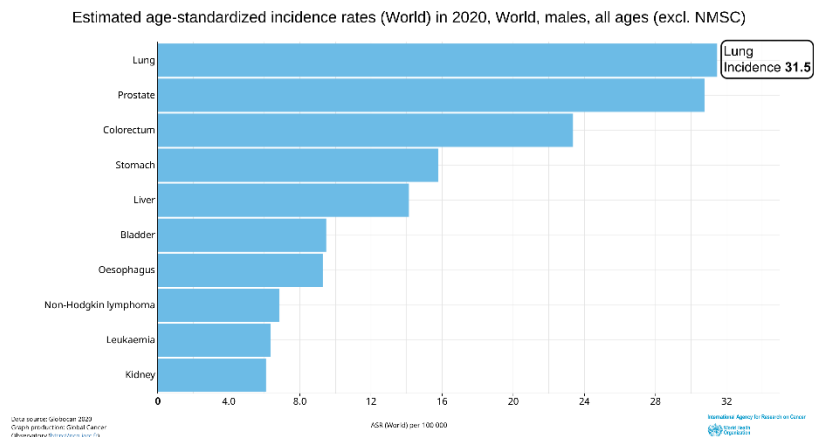


Figure 2.10 Estimated crude incidence rates in 2020, World, males

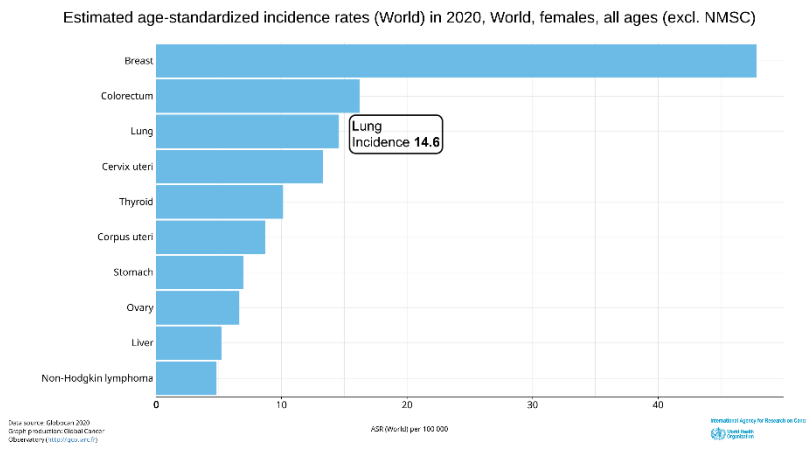


Figure 2.11 Estimated crude incidence rates in 2020, World, females

Table 2.10 Estimated crude incidence and mortality rates in 2020, World, males

Population	Incidence	Mortality Rates
Türkiye	74.8	67.5
Serbia	68.0	59.6
Hungary	66.6	58.6
Bosnia-Herzegovina	64.7	55.8
Montenegro	61.2	49.3
France, New Caledonia	59.9	44.9
Armenia	56.8	52.4
Greece	56.3	46.8
French Polynesia	55.3	48.5
Slovakia	54.3	36.0

Each cancer case accumulates individual-specific mutations in the tumor tissue. While tumors may have similar histological appearances, the fingerprint of cancer creates differences from person to person in tumor behaviors and response to treatment. In lung cancer (LC), revealing the real-time molecular structure of the disease and monitoring changes are essential for determining the most appropriate treatment option.

The non-invasive method under investigation for the real-time and repeatable detection of lung cancer's (LC) molecular structure is the analysis of breath or exhaled breath condensate (EBC), which has become one of the current research topics in recent years (Davis et al., 2012).

It is thought that the diagnosis of lung cancer (LC) at a very early stage and the effective monitoring of patients undergoing treatment can be achieved through the analysis of LC biomarkers in EBC samples. Besides, personalized treatment options can be explored, potentially increasing the success rate of treatment. In this context, it has been suggested that certain VOCs in EBC samples could serve as biomarkers for early diagnosis and short-term monitoring of treatment success (Broza et al., 2013). In a study by Broza and colleagues, they found a decrease in the levels of 2-methyl-1-pentene, 2-hexanone, 3-heptanone, styrene, and 2,2,4-trimethyl-hexane in breath samples of patients before and immediately after surgery (Broza et al., 2013). In another study, Peled et al. demonstrated that α -phellandrene, styrene, and dodecane 4-methyl compounds could serve as biomarkers for monitoring treatment success (Nardi-Agmon & Peled, 2017).

Fuchs and colleagues proposed an increase in the levels of pentanal, hexanal, and octanal in cases of lung cancer (Fuchs et al., 2010). Ulanowska and colleagues demonstrated the association of ethanol, acetone, butane, dimethyl sulfide, isoprene, propanal, 1-propanol, 2-pentanone, furan, o-xylene, ethylbenzene, pentanal, hexanal, and nonane compounds with lung cancer (Ulanowska et al., 2012). In another study, higher levels of isopropyl alcohol were found in cases of lung cancer (Koureas et al., 2020). Ciebiada and colleagues suggested that the levels of 8-isoprostane are associated with lung cancer (Ciebiada et al., 2012). In the same study, it was proposed that the levels of prostaglandin E2, a metabolite of prostaglandin cyclooxygenase-2, and 8-isoprostane are increased in the EB of lung cancer patients and are related to tumor development (Ciebiada et al., 2012). Some studies on the diagnosis of lung cancer in EBC samples are provided in Table 2.11

Table 2.11 Lung cancer biomarkers in EBC

<u>No</u>	<u>Biomarker</u>	<u>Reference</u>
1	Genes and Transcriptome	(Gessner et al., 2004)
2	Metabolomic	(Peralbo-Molina et al., 2016)
3	Protein	(Muccilli et al., 2015)
4	Microbiota	(Carpagnano et al., 2011)
5	VOCs	(Pleil et al., 2008)

3. MATERIALS AND METHODS

3.1 Chemicals

The compounds used, such as 1-butanol, isopropanol, 3-hydroxy-2-butanone, isoprene, dimethyl sulfide, carbon disulfide, benzene, toluene, ethylbenzene, 2,2-dimethyldecane, tetradecane, 2,2,4,6,6-pentamethylheptane, 2,3,4-trimethyldecane, nonane, 3,4,5,6-tetramethylheptane, hexadecane, along with aldehydes including malondialdehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, are used as VOC standards with analytical purity (Sigma Aldrich). The standards for these compounds are prepared by dissolving an appropriate amount of each in ultra pure water and methanol.

The standards of amino acids and organic acids used in the experiments, including lysine, arginine, proline, L-valine, methionine, tyrosine, leucine, phenylalanine, nicotinamide, creatine, trigonelline, pipecolic acid, betaine, tryptophan, L-carnitine, alanine, histidine, glycine, serine, threonine, aspartic acid, glutamic acid, hypoxanthine, hippuric acid, taurine, succinic acid, glycolic acid, vanillin, and taurocholic acid, were obtained from Sigma Aldrich with analytical purity.

Acetonitrile (>99.9%) and methanol (>99.9%) are from Sigma Aldrich. Water used in the preparation of the mobile phase and standards is of LC/MS purity were obtained from Carlo Erba. Formic acid (99%) was obtained from Fluka.

3.2 Device and Equipment

VOC analyses were performed by using an Agilent 7890A gas chromatography instrument coupled with an Agilent 7200B Series MS detector. For GC analyses of VOCs in EBC samples, sampling with Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) solid-phase microextraction (SPME) fibers with a length of 2 cm, a diameter of 23 gauge, and a film thickness of 30/50 μm from Supelco was carried out. The chromatographic separation of analytes was achieved using a Restek DB-624 UI column

(60.0m×1.40μm×0.25 mm). The SPME sampling process utilized Restek 20 mL headspace (HS) vials. Teflon-coated caps from Restek were employed to seal the HS vials. The temperatures of the HS-SPME vials were controlled using an Ika RCT Basic magnetic heating stirrer.

The liquid chromatography analyses of VOCs and amino acids were conducted using the Shimadzu 9030 Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF) instrument. In the separation of analytes, Inertsil ODS-4 (3μm 2.1x 50mm) and Intersil SIL 100A (5μm 4.6x250mm) columns were used.

The weighing of chemical substances was performed using the 5-digit Mettler Toledo MT5 balance. The derivatization reagent and the mixing of samples were carried out using the Boeco V-1 plus vortex mixer, branded as Vortex.

3.3 EBC Sample Collection

The collection of EBC samples was conducted using the specially designed EBC collection device as described in Figure 3.1 (Tetik Vardarlı et al., 2023). A Huber CC805 cryostat was employed to cool the EBC sampling system.

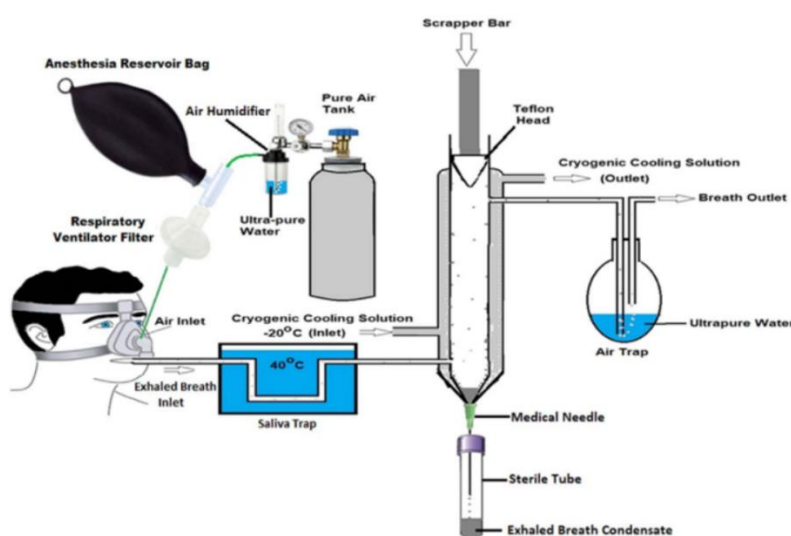


Figure 3.1 Exhaled Breath Condensate collection system (Tetik Vardarlı et al., 2023)

The body of the EBC condenser is made of borosilicate glass tubing, measuring 1.0 m in length and having a 3.0 cm inner diameter. The exhaled breath of patients was condensed at -20°C using a cryostat (Huber CC805). In our previous study (Koc et al., 2019), it was demonstrated that EBC could be contaminated when exposed to the external atmospheric environment. To prevent contamination when exposing EBC to the external environment, patients were ventilated with humidified pure air (99.99%) using a nasal mask (KNGMED Medical, Turquoise nasal CPAP Mask). Patients placed their mouths on the blowing mouthpiece, keeping it in their mouths without separating during the sampling stage. Additionally, an air trap was placed at the system's outlet to complete the isolation of the EBC condenser from the external environment. Pure nitrogen gas (99.99%) was passed through the inside of the EBC condenser to remove potential volatile organic compound residues and contaminants that could come from the system. Both ends of the EBC collection device were sealed with parafilm to isolate it from the external atmosphere until the EBC sampling step.

The EBC samples from the subjects were collected in the morning on an empty stomach, and they were instructed not to smoke or consume any food or drink at least 8 hours before sampling. Patients were asked to rinse their mouths with water before the sampling step. For 5 minutes before the sampling, humidified dry air was introduced to the patients, followed by the continuation of ventilation with pure air until the end of the sampling. Some portion of the pure air was stored in a reusable anesthesia bag to meet the excessive air demand of patients providing EBC samples. The humidified air was then passed through a single-use respiratory ventilator filter during the inhalation process to prevent any contamination. Participants were instructed to inhale pure air through their noses and deliver it to a mouthpiece connected to a U-shaped thermostatic glass saliva trap (+40°C) only through the mouth. This trap was used to prevent the contamination of EBC with saliva by blocking the passage of saliva into the condenser. Participants were instructed to swallow if they felt saliva in their mouths. The process of collecting EBC samples continued for 15 minutes. At the end of the sampling step, EBC on the inner surface of the glass wall of the condenser was scraped from top to bottom with a Teflon scraping rod into a sterile 15 mL sampling tube, collecting

approximately 2–2.5 mL of EBC sample. Subsequently, the samples were stored in -80°C freezers until analysis. The used EBC condenser was cleaned according to the literature before the next EBC sample collection process, sealed at both ends with parafilm, and stored until the next analysis after passing pure nitrogen through it.

3.4 Gas Chromatography Analysis Methods

The analyses of the target VOCs identified in EBC were conducted using gas chromatography techniques. Aldehyde experiments have been presented in the GC-MS section. Due to the EBC matrix containing close to 100% water, VOC experiments were conducted in an ultra-pure water. Standard solutions of the target analytes in appropriate amounts were prepared in ultra-pure water, and experiments were carried out. Each analysis method is provided below.

3.4.1 VOC Analysis with GC-MS

GC-MS analyses were conducted for 1-butanol, isopropanol, 3-hydroxy-2-butanone, isoprene, dimethyl sulfide, carbon disulfide, benzene, toluene, ethylbenzene, 2,2-Dimethyldecane, tetradecane, 2,2,4,6,6-pentamethylheptane, 2,3,4-trimethyldecane, nonane, 3,4,5,6-tetramethyloctane, hexadecane, and aldehydes including malondialdehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal, and nonanal from the aldehyde group. In the experiments, aldehydes and ketones were analyzed after derivatization with O-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine (PFBHA). Firstly, a solution of PFBHA was prepared. The stock solution was stored at +4 °C in the refrigerator. 17 mg of PFBHA was weighed and acidified with 200 µL of formic acid. Then, it was completed to 10 mL with acetonitrile to prepare a PFBHA derivatization solution with a concentration of 1.7 mg/mL. Subsequently, 1 mL of the PFBHA solution was placed in a 20 mL HS vial, tightly sealed, and allowed to equilibrate at 35°C for 5 minutes. Then, a DVB/CAR/PDMS SPME fiber was placed in the headspace of the vial, kept at the same temperature for 10 minutes, allowing the adsorption of the derivatization reagent onto the fiber.

In the next step, the sampling of analytes on the PFBHA-loaded SPME fiber was performed. After that, 0.5 mL of pure water standards containing target VOCs was transferred to another HS vial, tightly sealed. Equilibration was expected to occur at the same temperature for 5 minutes. Then, the fiber loaded with PFBHA was placed in the headspace of the vial containing the sample, allowing the adsorption of analytes onto the fiber for 10 minutes. Meanwhile, aldehydes reacted with the adsorbed aldehydes on the fiber, leading to their adsorption onto the fiber surface, as illustrated in Figure 3.2.

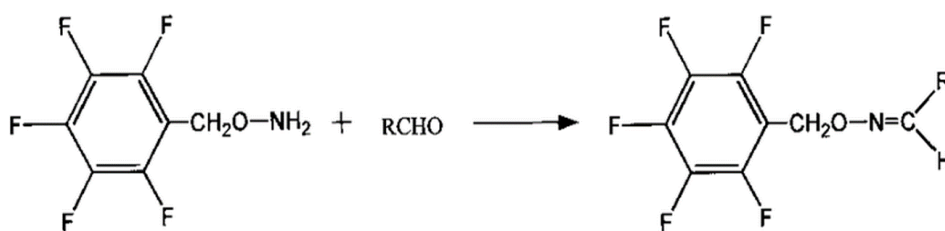


Figure 3.2 The reaction of aldehydes with PFBHA (Bao et al., 2014)

After the sampling step, GC-MS analysis was performed. The GC-MS analysis parameters included the following: electron ionization mode for ionizing VOCs, sample collection in the Extended Dynamic Range mode at 5 scans/s, mass scanning range from 29 to 400 m/z, transfer line and ion source temperatures set at 280 and 230°C, respectively, and ionization utilizing a 70 eV electron bombardment with a 40 μA current.

GC-MS device parameters are as follows: mass spectrometry measurements in full scan mode with a mass range of 29-400 m/z, ion source temperature set to 230°C, injection temperature at 250°C, and a helium flow rate of 2.19 mL/minute (99.99%, Linde). The injection of SPME fibers was carried out in Splitless mode. The column oven temperature program started at 40°C and was held at this temperature for 4 minutes. Subsequently, the column temperature was increased to 200 degrees at a rate of 15°C/minute and held there for 3 minutes. Then, it was further increased to 250°C at a rate of 100°C/minute, and after staying at 250°C for 12 minutes, the analysis was completed. The total analysis time for GC-MS is 30 minutes.

Table 3.1 Analysis parametres of GC-MS

Parameters			
Column	DB-624 UI (60mx0.25mmx1.40 μ m)	Ionization Mode	Electron Ionization
Injection Mode	Splitless	Ion Source Temperature	250°C
Inlet Temperature	250°C	MS Transfer Temperature	280°C
Oven Temperature	250°C	Flow Rate	2.19 mL/min
Initial Temperature	40°C	Scanning Mode	Total ion mode (m/z: 29-400)

Following the sampling step, the fiber was placed into the gas chromatography injection block, and after desorption of the target analytes from the fiber in the inlet block, their analyses were analyzed using gas chromatography. The desorption of analytes from the fiber was carried out by holding the analytes for 5 minutes at 250°C in the inlet block. The desorbed analytes were separated in the gas chromatography column and then detected with the MS detector. The masses and retention times monitored in the GC-MS analysis are provided in Table 3.2. GC-MS analyses for each analyte were conducted using the m/z masses listed in Table 3.2.

Table 3.2 Retention times and m/z ratio of the analytes in GC-MS analysis

Analyte	Retention Time (min)	Formula	Derivatized Formula	m/z Values (Qualitative/Quantative)
Isoprene	1.57	C ₅ H ₈	-	67/53
Benzene	2.46	C ₆ H ₆	-	78/51
Toluene	4.28	C ₇ H ₈	-	91/39
Styrene	6.86	C ₈ H ₈	-	104/78
Nonane	7.00	C ₉ H ₂₀	-	43/57
Propanal	10.73	C ₃ H ₆ O	C ₉ H ₁₀ N ₄ O ₄	238/79
Butanal	11.59-11.65	C ₄ H ₈ O	C ₁₀ H ₁₂ N ₄ O ₄	252/152
Pentanal	12.43-12.48	C ₅ H ₁₀ O	C ₁₁ H ₁₄ N ₄ O ₄	206/266
Tetradecane	12.74	C ₁₄ H ₃₀	-	57/43
Hexanal	13.25-13.29	C ₆ H ₁₂ O	C ₁₂ H ₁₆ N ₄ O ₄	83/206
Heptanal	14.04	C ₇ H ₁₄ O	C ₁₃ H ₁₈ N ₄ O ₄	97/55
Hexadecane	14.40	C ₁₆ H ₃₄	-	57/71
Octanal	14.82	C ₈ H ₁₆ O	C ₁₄ H ₂₀ N ₄ O ₄	69/55
Nonanal	15.64	C ₉ H ₁₈ O	C ₁₅ H ₂₂ N ₄ O ₄	96/206

3.5 LC-QTOF Analysis

The analysis of some volatile organic compounds, amino acids, and organic acids in EBC was carried out using the LC-Q-TOF methods.

3.5.1 Analysis of Volatile Organic Compounds by LC-QTOF

Malondialdehyde, 3-hydroxy-2-butanone, propanal, butanal, pentanal, hexanal, heptanal, octanal, and nonanal target compounds were analyzed using the LC-QTOF method. Suitable calibration curves were obtained for malondialdehyde,

propanal, butanal, pentanal, hexanal, heptanal, octanal, and nonanal using the LC-QTOF instrument, and the analysis of these compounds in EBC samples was performed. To assess the system's stability, a quality control (QC) standard was introduced into the instrument before starting the analysis to verify its stability.

Chromatographic analyses were conducted with a gradient mobile phase program. The mobile phase consisted of (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid, utilizing a gradient program. The gradient conditions of the mobile phase at a flow rate of 0.3 mL/min were as follows: 0-0.1 minutes, 50% B; 0.1-15 minutes, 98% B; 15-20 minutes, 50% B; 20-22 minutes, 50% B for column equilibration. The injection volume of sample was 10 μ L. The column oven temperature was set at 30°C, and the total analysis time was 22 minutes. Chromatographic analysis conditions used in the LC-QTOF analysis are summarized in Table 3.3.

Table 3.3 VOC analysis parameters of LC-QTOF

LC Part					
Mobile Phase A	0.1% formic acid water	Flow Rate		0.3 mL/min	
Mobile Phase B	0.1% formic acid acetonitrile	Injection Volume		10 μL	
Column Temperature	30°C	Column		Inertsil ODS-4 (3μmx 2.1x 50mm)	
Q-TOF Part					
Interface		Ion Source		TOF Parameters	
Ionization Mode	DUIS	Drying Gas Flow	10.0 L/min	Event Time	0.1
Nebulizer Gas Flow	2.0 L/min	DL Temperature	250°C	Interface Voltage	4.00 kV
Heater Gas Flow	10.0 L/min	Heater Block Temperature	400°C	Corona Needle Voltage	4.50 kV
Interface Temperature	160°C			m/z Range	30.000-700.000

LC-QTOF measurement mode was performed in Data-Dependent Analysis (DDA) mode. DUIS (Dual Electrospray Ionization) ionization source was used for ionization. Mass scanning was carried out in positive scanning mode with a mass scanning range of 30.000-700.000 m/z. Collision energy was set at 35 $\pm$ 17, and the mobile phase flow rate was 0.3 mL/min. The device parameters used in LC-QTOF analysis are provided in Table 3.3

The target analytes were derivatized with 2,4-Dinitrophenylhydrazine (2,4-DNPH) and then analyzed using the LC-QTOF device. For this purpose, 25 mg of 2,4-DNPH was weighed, and 200 μ L of formic acid was added, followed by completion with acetonitrile up to 10 mL. This mixture was dissolved in an

ultrasonic bath and vortexed. The stock solution was stored at +4 °C in the refrigerator. From this solution, 100 μ L was taken and placed into a 250 μ L glass insert vial, and 100 μ L of condensate sample was added. The derivatization process for the analytes in EBC with the derivatization reagent was expected to be completed in 4 hours. The derivatization mechanism is provided in Figure 3.3.

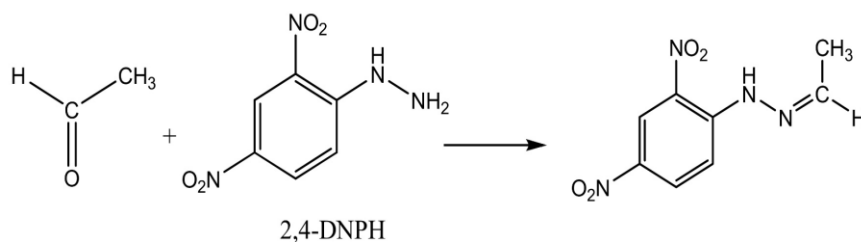


Figure 3.3 The reaction of aldehydes with 2,4-DNPH (Uchiyama et al., 2011)

The EBC samples, subjected to the derivatization process, were analyzed using LC-QTOF by injecting 10 μ L of the samples placed in the autosampler rack at 4°C. The masses and retention times observed in the LC-QTOF analysis are provided in Table 3.4.

Table 3.4 Retention times and m/z ratio of VOC analytes in LC-QTOF

Retention Time (min)	Analyte	Formula	Derivatized Formula	Target Analyte Mass [M ⁺ H] ⁺ m/z
1.493	3-hydroxy 2-butanone	C ₄ H ₈ O ₂	C ₁₀ H ₁₂ N ₄ O ₅	269.0881
1.528	Malondialdehyde	C ₃ H ₄ O ₂	C ₉ H ₆ N ₄ O ₄	235.0462
3.856	Butanal	C ₄ H ₈ O	C ₁₀ H ₁₂ N ₄ O ₄	253.0931
4.8	Pentanal	C ₅ H ₁₀ O	C ₁₁ H ₁₄ N ₄ O ₄	267.1088
6.049	Hexanal	C ₆ H ₁₂ O	C ₁₂ H ₁₆ N ₄ O ₄	281.1244
6.985	Heptanal	C ₇ H ₁₄ O	C ₁₃ H ₁₈ N ₄ O ₄	295.1401
9.416	Nonanal	C ₉ H ₁₈ O	C ₁₅ H ₂₂ N ₄ O ₄	323.1714

3.5.2 Analysis of Amino Acids and Organic Acids in EBC Samples

The LC-QTOF method was used for the analysis of amino acids and organic acids, including Lysine, Arginine, Proline, L-Valine, Methionine, Tyrosine, Leucine, Phenylalanine, Nicotinamide, Creatine, Trigonelline, Pipecolic acid, Betaine, Tryptophan, L-Carnitine, Alanine, Histidine, Glycine, Serine, Threonine, Aspartic acid, Glutamic acid, Hypoxanthine, Hippuric acid, Taurine, Succinic acid, Glycolic acid, Vanillin, and Taurocholic acid. For internal standardization, Sarcosine was utilized in the LC-QTOF device. Suitable calibration curves for the mentioned compounds were generated on the LC-QTOF device, and their analyses in EBC samples were conducted. To ensure the system's stability, a quality control (QC) standard was introduced to the device before initiating the analysis to verify its stability.

Chromatographic analyses were conducted using a gradient mobile phase program. The mobile phase consisted of (A) 5 mM Ammonium Formate, 5% Acetonitrile, and 0.5% Formic acid in water, and (B) acetonitrile. The gradient program for the mobile phase had the following conditions at a flow rate of 0.6 mL/min: 0-21.3 minutes, 58% B. The injection volume was 10 μ L. The column oven temperature was set to 40°C, and the total analysis time was 21.3 minutes. The chromatographic analysis conditions used in the LC-QTOF analysis are summarized in Table 3.5

Table 3.5 LC-QTOF aminoacid analysis parametres

LC Part					
Mobile Phase A	5mM Ammonium Formate, 5% Acetonitrile, 0.5% Formic Acid	Flow Rate		0.6 mL/min	
Mobile Phase B	Acetonitrile	Injection Volume		10 µL	
Column Temperature	40°C	Column		Intersil SIL 100A (5µmx 4.6x250mm)	
Q-TOF Part					
Interface		Ion Source		TOF Parameters	
Ionization Mode	DUIS	Drying Gas Flow	10.0 L/min	Event Time	0.1
Nebulizer Gas Flow	2.0 L/min	DL Temperature	250°C	Interface Voltage	4.00 kV
Heater Gas Flow	10.0 L/min	Heater Block Temperature	400°C	Corona Needle Voltage	4.50 kV
Interface Temperature	160°C			m/z Range	30.000-700.000

The TOF measurement mode was performed in the Data-Dependent Analysis (DDA) mode. The DUIS (Dual Ionization Source) ionization source, including Simultaneous Electron Spray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI), was used. Mass scanning was conducted in the scan mode with a mass scanning range of 30-700 m/z. The collision energy was set at 35±17, and the flow rate of the mobile phase was 0.6 mL/min. The device analysis parameters used in the LC-Q-TOF analysis are provided in Table 3.6.

EBC samples were analyzed by injecting 10 μL of the samples placed in the autosampler rack at 4°C in the LC-QTOF system. The masses and retention times observed in the LC-QTOF analysis are provided in Table 3.6.

Table 3.6 Retention times and m/z ratio of aminoacid analytes in LC-QTOF analysis

<u>Retention Time (min)</u>	<u>Analyte</u>	<u>Formula</u>	<u>Measurement Mode</u>	<u>Target Analyte Mass $[\text{M}^+\text{H}]^+$ m/z</u>
12.141	Lysine	$\text{C}_6\text{H}_{14}\text{N}_2\text{O}_2$	Positive	147.1128
11.502	Arginine	$\text{C}_6\text{H}_{14}\text{N}_4\text{O}_2$	Positive	175.1189
9.992	Proline	$\text{C}_5\text{H}_9\text{NO}_2$	Positive	116.0706
8.300	L-valine	$\text{C}_5\text{H}_{11}\text{NO}_2$	Positive	118.0863
7.700	Methionine	$\text{C}_5\text{H}_{11}\text{NO}_2\text{S}$	Positive	150.0583
7.100	Tyrosine	$\text{C}_9\text{H}_{11}\text{NO}_3$	Positive	182.0812
7.700	Leucine	$\text{C}_6\text{H}_{13}\text{NO}_2$	Positive	132.1019
7.319	Phenylalanine	$\text{C}_9\text{H}_{11}\text{NO}_2$	Positive	166.0863
7.080	Nicotinamide	$\text{C}_6\text{H}_6\text{N}_2\text{O}$	Positive	123.0553
9.060	Creatine	$\text{C}_4\text{H}_7\text{N}_3\text{O}$	Positive	132.0768
12.346	Trigonelline	$\text{C}_7\text{H}_7\text{NO}_2$	Positive	138.0549
9.785	Pipecolic acid	$\text{C}_6\text{H}_{11}\text{NO}_2$	Positive	130.0863
12.891	Betaine	$\text{C}_5\text{H}_{11}\text{NO}_2$	Positive	118.0863
6.900	Tryptophane	$\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_2$	Positive	205.0971
10.298	L-carnitine	$\text{C}_7\text{H}_{15}\text{NO}_3$	Positive	162.1124
8.435	Alanine	$\text{C}_3\text{H}_7\text{NO}_2$	Positive	90.055
11.453	Histidine	$\text{C}_6\text{H}_9\text{N}_3\text{O}_2$	Positive	156.0767

Tablo 3.6. (Continue)

<u>Retention Time (min)</u>	<u>Analyte</u>	<u>Formula</u>	<u>Measurement Mode</u>	Target Analyte Mass [M⁺H]⁺ m/z
8.303	Glycine	C ₂ H ₅ NO ₂	Negative	74.0247
8.106	Serine	C ₃ H ₇ NO ₃	Negative	104.0353
8.184	Threonine	C ₄ H ₉ NO ₃	Negative	118.051
7.356	Aspartic Acid	C ₄ H ₇ NO ₄	Negative	132.0302
7.466	Glutamic acid	C ₅ H ₉ NO ₄	Negative	146.0459
6.745	Hypoxanthine	C ₅ H ₄ N ₄ O	Negative	135.0312
5.632	Hippuric acid	C ₉ H ₉ NO ₃	Negative	178.051
6.708	Taurine	C ₂ H ₇ NO ₃ S	Negative	124.0074
5.669	Succinic acid	C ₄ H ₆ O ₄	Negative	117.0193
5.93	Glycolic acid	C ₂ H ₄ O ₃	Negative	75.0088
5.6	Vanilline	C ₅ H ₁₁ NO ₂	Negative	151.0401
5.305	Taurocholic acid	C ₂₆ H ₄₅ NO ₇ S	Negative	514.2839
9.288	Sarcosine	C ₃ H ₇ NO ₂	Internal Standard	90.050

Statistical analysis of this study was conducted using IBM SPSS V25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp). The assumption of normality for continuous variables was tested with a Shapiro-Wilk Test. Normally distributed continuous variables were presented with mean and standard deviation, while non-normally distributed variables were presented with median, minimum, and maximum values. Mann-Whitney U test was employed to compare the medians of two independent groups (cases and controls). AUC-ROC analysis was employed to categorize subjects as

either diseased or non-diseased. A two-sided p-value of ≤ 0.05 was considered the criterion for statistical significance.

These experiment studied with lung cancer patients and healthy group based on ethical committee decision number 19-11T/49.





4. RESULTS

4.1. GC–MS Analysis Results of VOCs

The GC-MS analysis parameters were optimized for the compounds such as isoprene, benzene, toluene, styrene, propanal, butanal, pentanal, nonane, tetradecane, hexanal, heptanal, hexadecane, octanal, and nonanal after the derivatization step. The sample GC-MS chromatogram obtained under the conditions given in Table 3.1 is presented in Figure 4.1. It can be observed that each analyte is successfully separate in GC-MS analysis.

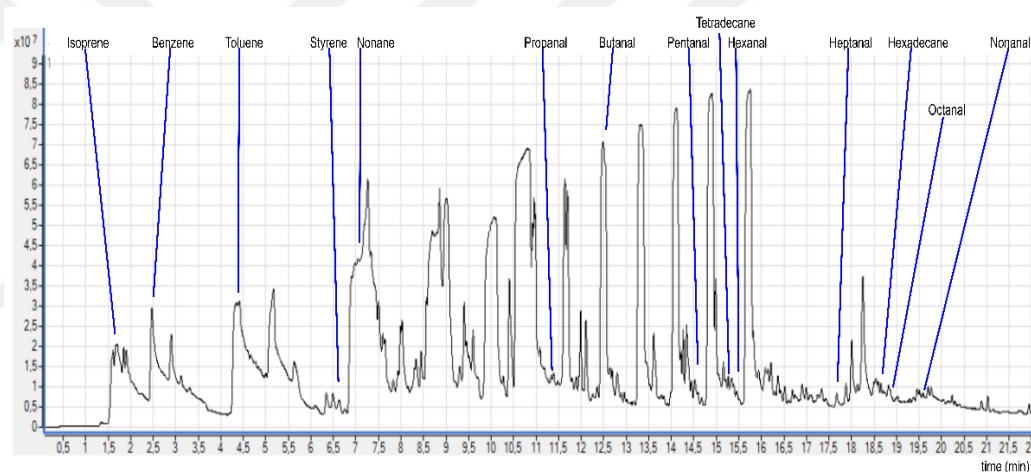


Figure 4.1 Sample chromatogram of optimized SPME GC-MS analysis

Calibration graphs were then performed for each analyte (Figure 4.2-Figure 4.15). As can be seen from the figures most of the targeted compounds have a good chromatogram except Benzene, Propanal, Pentanal and Hexanal. This may be attributed to unsuitable analysis conditions for the analytes mentioned. However, linear calibration curves could also be obtained for the mentioned compounds.

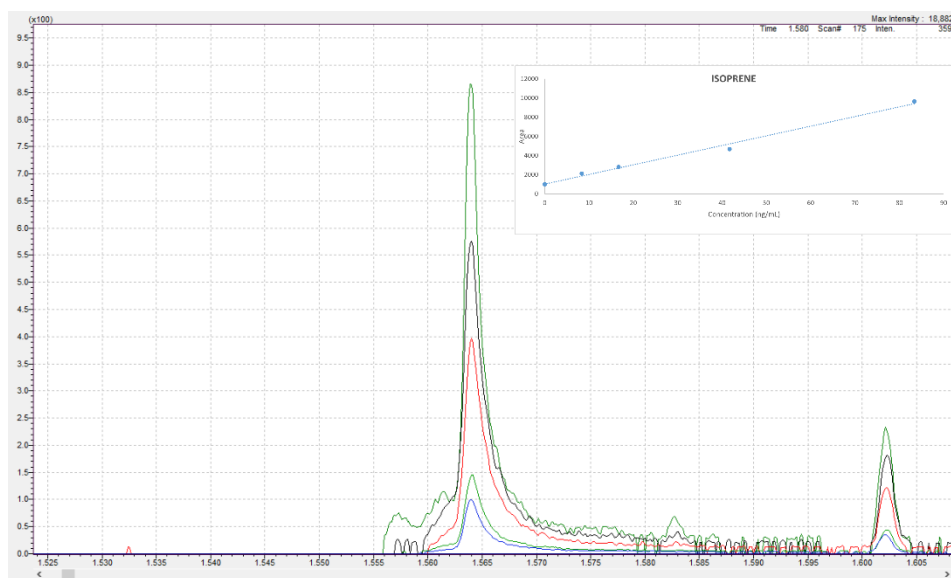


Figure 4.2 Calibration curve and chromatogram of Isoprene in GC-MS

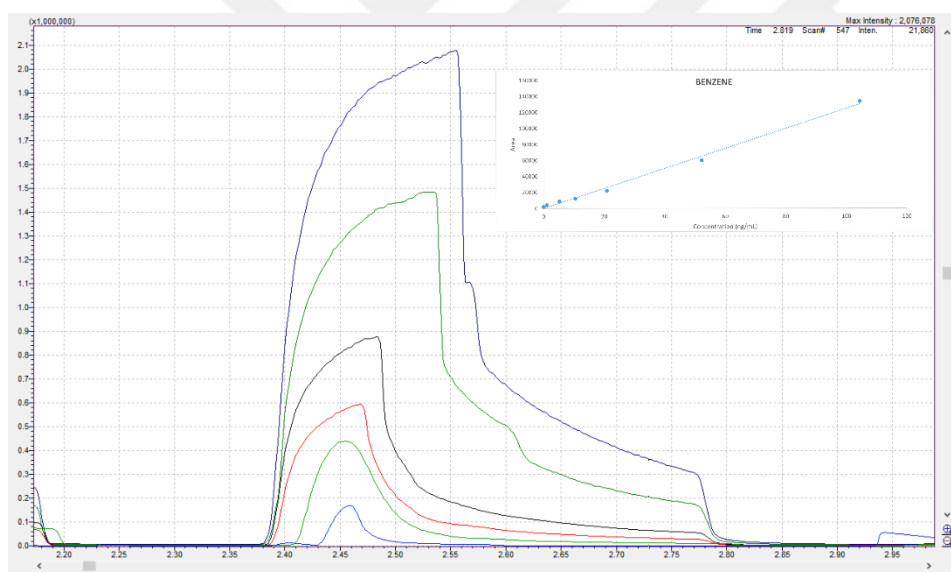


Figure 4.3 Calibration curve and chromatogram of Benzene in GC-MS

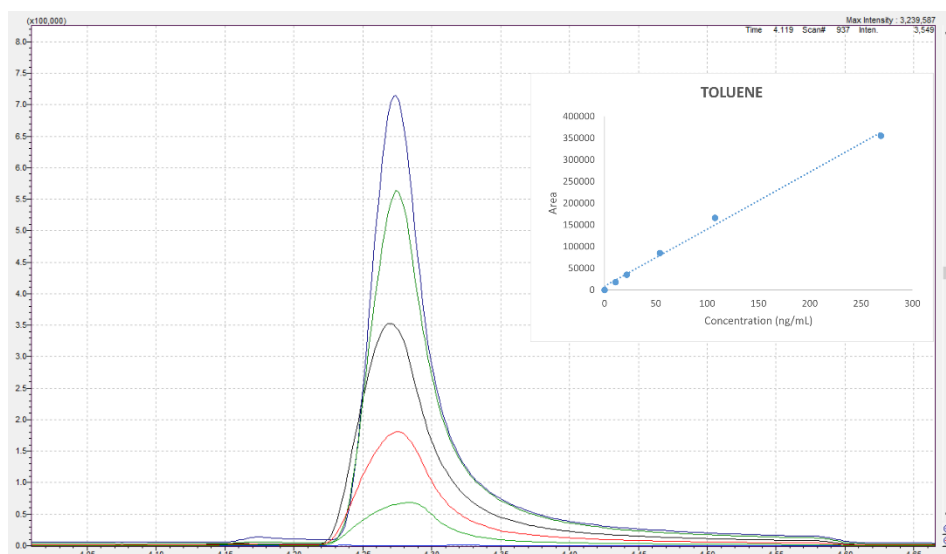


Figure 4.4 Calibration curve and chromatogram of Toluene in GC-MS

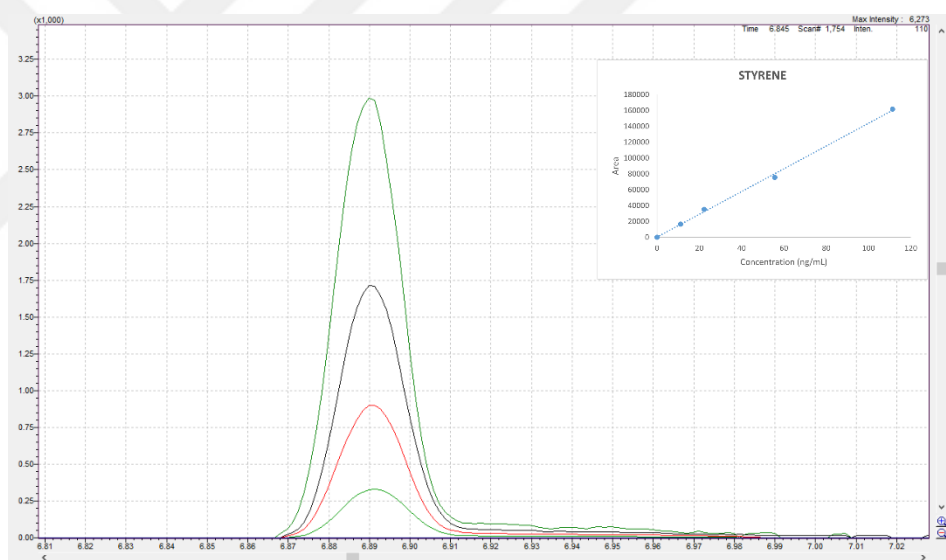


Figure 4.5 Calibration curve and chromatogram of Styrene in GC-MS

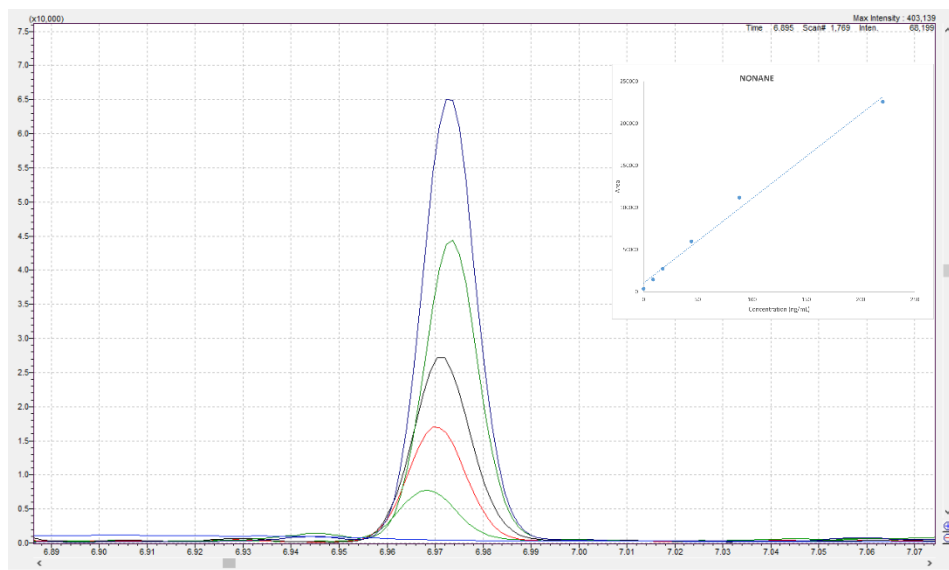


Figure 4.6 Calibration curve and chromatogram of Nonane in GC-MS

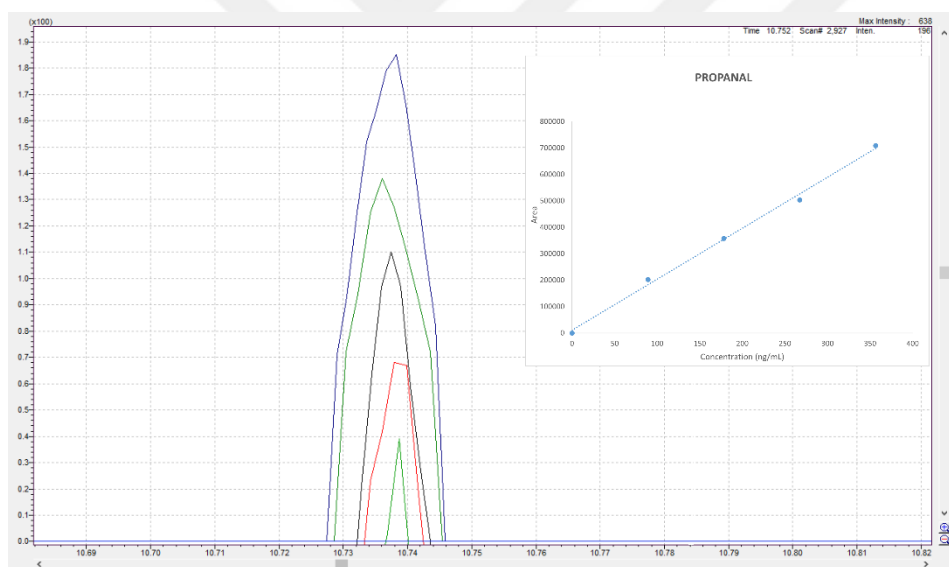


Figure 4.7 Calibration curve and chromatogram of Propanal in GC-MS

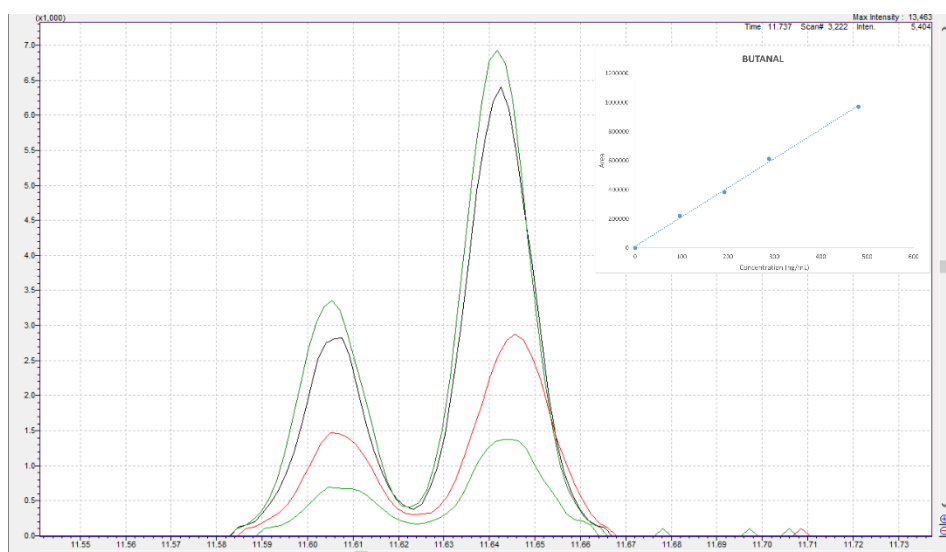


Figure 4.8 Calibration curve and chromatogram of Butanal in GC-MS

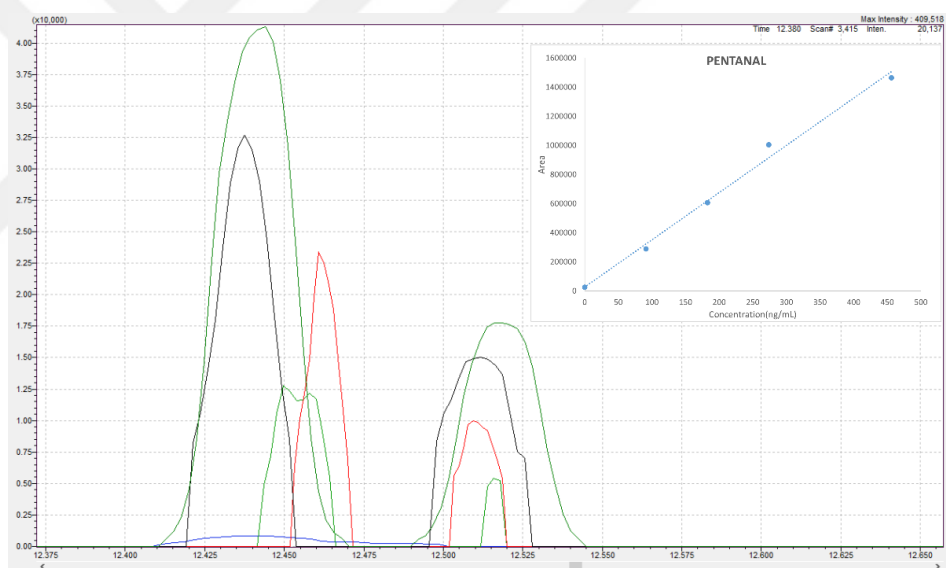


Figure 4.9 Calibration curve and chromatogram of Pentanal in GC-MS

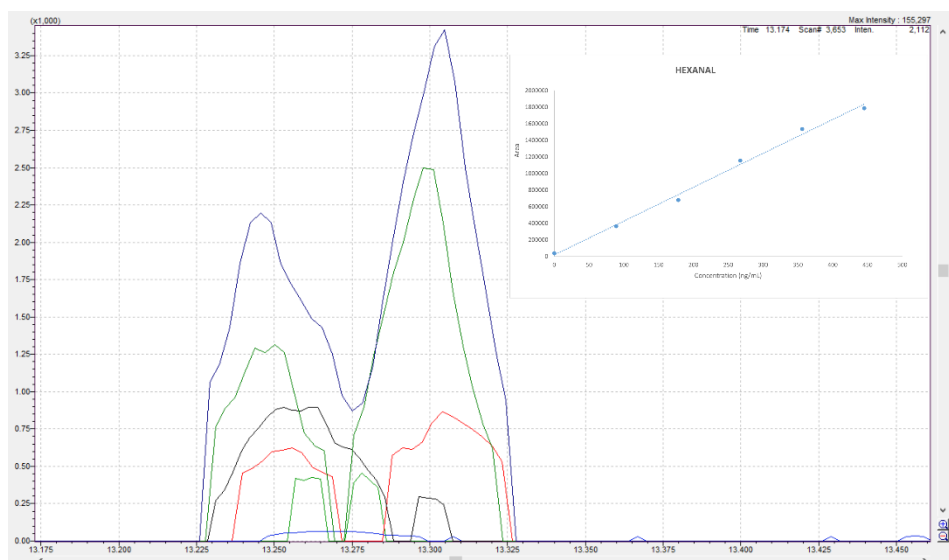


Figure 4.10 Calibration curve and chromatogram of Hexanal in GC-MS

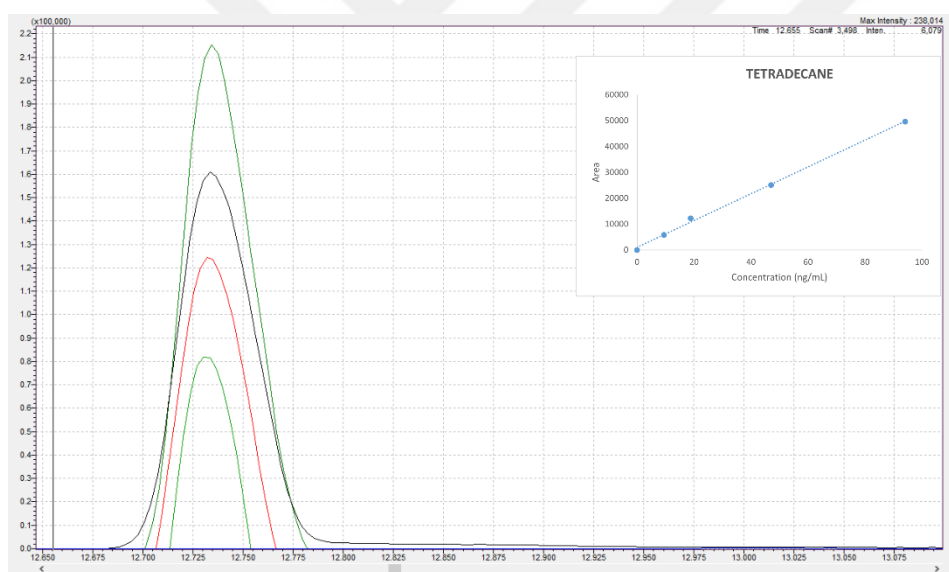


Figure 4.11 Calibration curve and chromatogram of Tetradeane in GC-MS

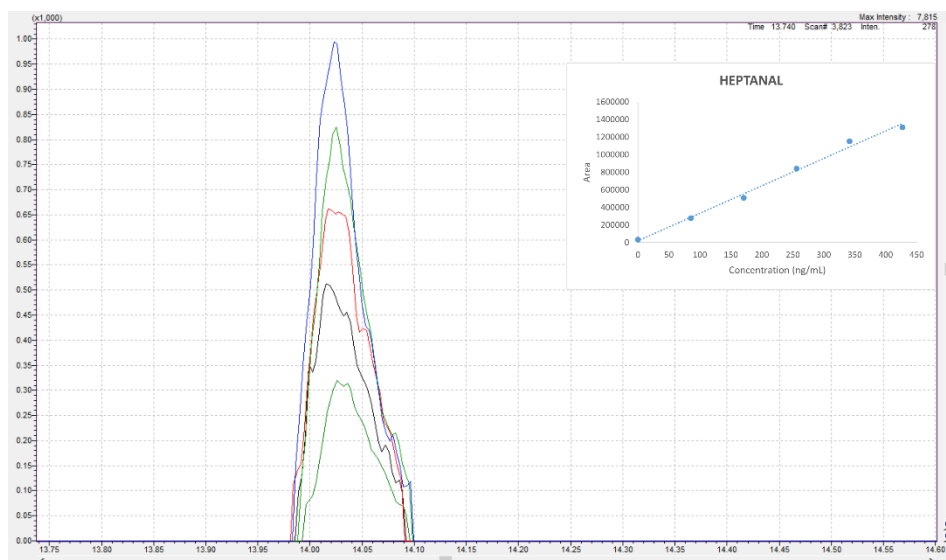


Figure 4.12 Calibration curve and chromatogram of Heptanal in GC-MS

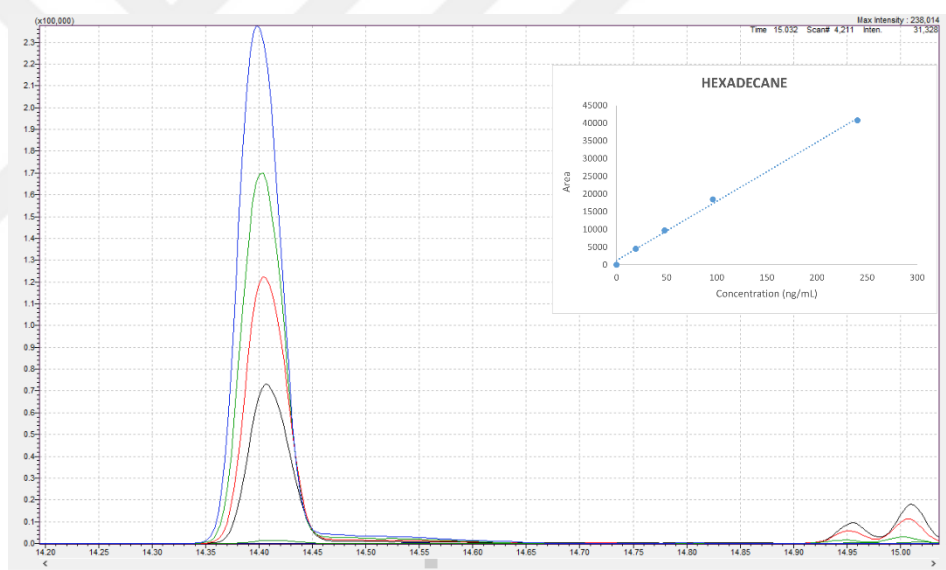


Figure 4.13 Calibration curve and chromatogram of Hexadecane in GC-MS

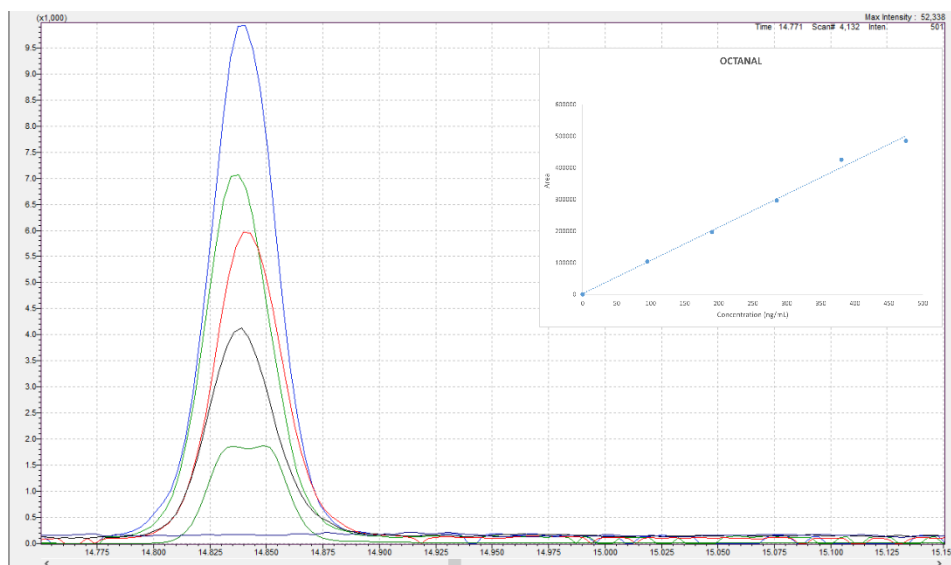


Figure 4.14 Calibration curve and chromatogram of Octanal in GC-MS

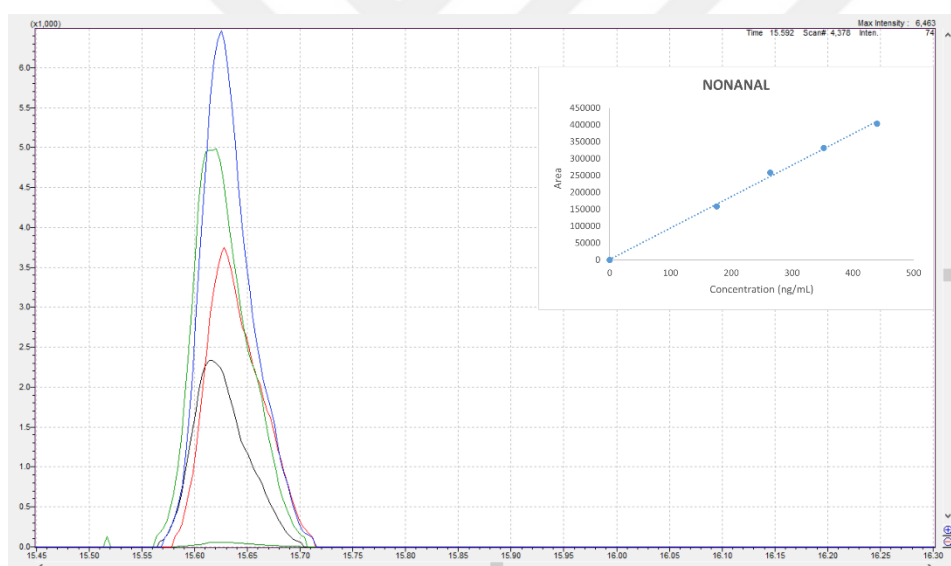


Figure 4.15 Calibration curve and chromatogram of Nonanal in GC-MS

The analytical parameters for each compound are given in Table 4.1. The limit of detection (LOD) in GC-MS analysis was calculated at $S/N = 3$, and the limit of quantification (LOQ) was calculated at $S/N = 10$.

Table 4.1 The analytical parameters of the characteristics VOC SPME-GC-MS analysis method

Analyte	Calibration Curve Equation	R ²	Linear Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Isoprene	$y = 100.61x + 1027.3$	0.9905	4.51-100	1.35	4.51
Benzene	$y = 1245.3x + 225.19$	0.9946	3.01-200	0.9	3.01
Toluene	$y = 1316.3x + 8451$	0.9952	0.33-100	0.1	0.33
Styrene	$y = 1435.2x + 363.53$	0.9981	0.36-100	0.11	0.36
Nonane	$y = 1002.5x + 10528$	0.9914	3.14-200	0.95	3.14
Propanal	$y = 1929.1x + 10683$	0.9960	2.30-200	0.69	2.30
Butanal	$y = 2013.1x + 11179$	0.9979	0.5-100	0.15	0.49
Pentanal	$y = 3245.7x + 26733$	0.9915	0.93-200	0.28	0.93
Hexanal	$y = 4081.4x + 18325$	0.9942	0.86-200	0.26	0.86
Tetradecane	$y = 518.49x + 989.48$	0.9979	10.89-250	3.30	10.89
Heptanal	$y = 3123.7x + 21077$	0.9931	0.79-100	0.24	0.79
Hexadecane	$y = 167.15x + 1212.8$	0.9966	3.77-250	1.14	3.77
Octanal	$y = 1049.5x + 2190.9$	0.9948	0.61-100	0.24	0.61
Nonanal	$y = 929.49x + 1458$	0.9976	0.55-100	0.24	0.55

4.1.1. VOC Analysis by GC–MS Method in Real EBC Samples

After the optimization step, the analysis of EBC samples was carried out. The demographic characteristics of the subjects analyzed with the GC-MS method in the experiments are presented in Table 4.2.

Table 4.2 Demographic properties of the lung cancer patients

Demographic Feature	Number
Male	33
Female	17
Age Range	45-65
Active Smoker	29
Ex-Smoker	12
Passive Smoker	1
Total	50

A sample chromatogram related to the analysis of targeted VOCs in EBC samples using SPME-GC-MS is provided in Figure 4.16.



Figure 4.16 The sample chromatogram of SPME-GC-MS analysis of EBC for lung cancer patient

In this section, the measurement of the levels of target analytes in the breath samples of lung cancer patients was studied. The analysis results obtained from the EBC samples of each patient are presented in Appendix-1 (Page 171). The number of individuals identified in the EBC samples of lung cancer patients, along with their mean values and standard deviations, are provided in Table 4.3.

Table 4.3 The SPME-GC-MS analysis results in EBC samples of lung cancer patients

Compound	Number of Detected Individuals	Average $\pm$ SD (ng/mL)
Isoprene	5	7.17 $\pm$ 2.81
Benzene	45	269.61 $\pm$ 94.62
Toluene	48	406.08 $\pm$ 89.21
Styrene	49	51.56 $\pm$ 10.85
Nonane	6	53.26 $\pm$ 5.88
Propanal	45	29.83 $\pm$ 7.24
Butanal	48	46.60 $\pm$ 12.56
Pentanal	44	12.91 $\pm$ 4.94
Hexanal	45	17.79 $\pm$ 4.22
Tetradecane	48	79.54 $\pm$ 20.39
Heptanal	43	184.48 $\pm$ 18.30
Hexadecane	44	354.62 $\pm$ 114.61
Octanal	42	27.20 $\pm$ 4.80
Nonanal	37	16.83 $\pm$ 6.00

4.2. LC-QTOF Analysis Results

4.2.1 VOC Analysis by LC-QTOF

After determining the appropriate separation conditions for the mobile phase using the liquid chromatography device, the focus shifted to identifying concentration ranges where the signal of each analyte linearly changes and determining the lower limit of detection concentrations. For this purpose, analyses were conducted using an LC-QTOF device for 3-Hydroxy-2-Butanone, Malondialdehyde, Butanal, Pentanal, Hexanal, Heptanal, and Nonanal compounds, and the sample chromatogram is provided in Figure 4.17.

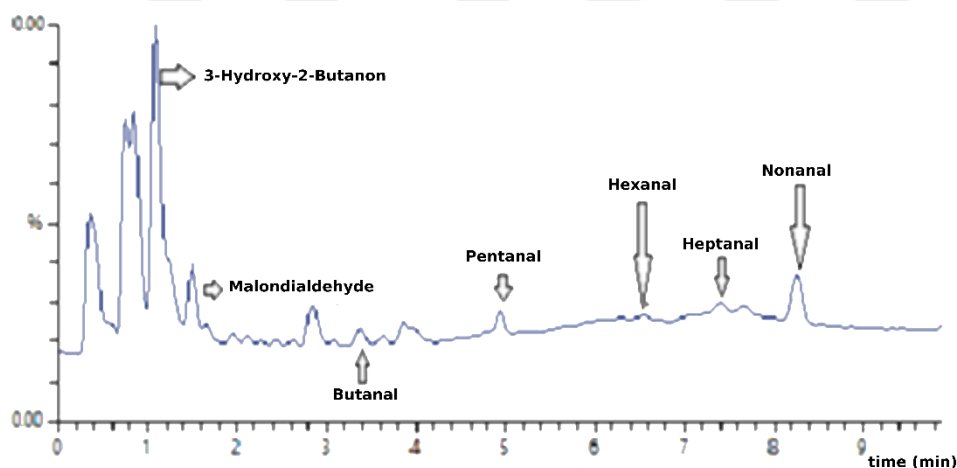


Figure 4.17 The sample LC-QTOF chromatogram of the targeted analytes

Subsequently, calibration curves were generated for each analyte under optimum conditions (Figure 4.18-Figure 4.24). As can be seen from the figures well shape peaks can not be obtained for Heptanal, Hexanal and Nonanal. This can be observed because of insufficient derivatization of these analytes. Nevertheless, linear calibration curves could also be obtained for the mentioned compounds. On the other hand, it was observed that, 3-hydroxy-2-butanone and malondialdehyde, had a low background noise in the chromatogram. Therefore, a lower detection limit can be obtained for 3-hydroxy-2-butanone and malondialdehyde compounds.

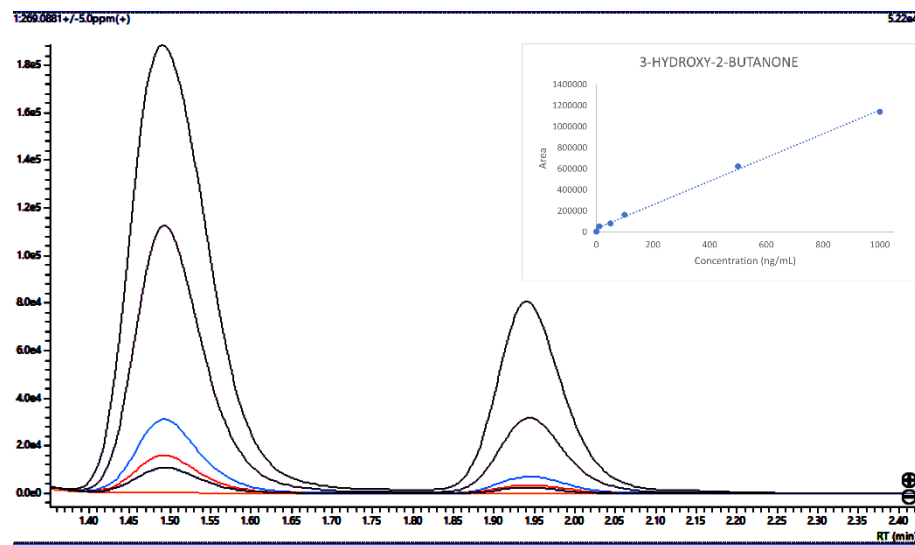


Figure 4.18 Calibration curve and chromatogram of 3-hidroxy, 2-butanone in LC-QTOF

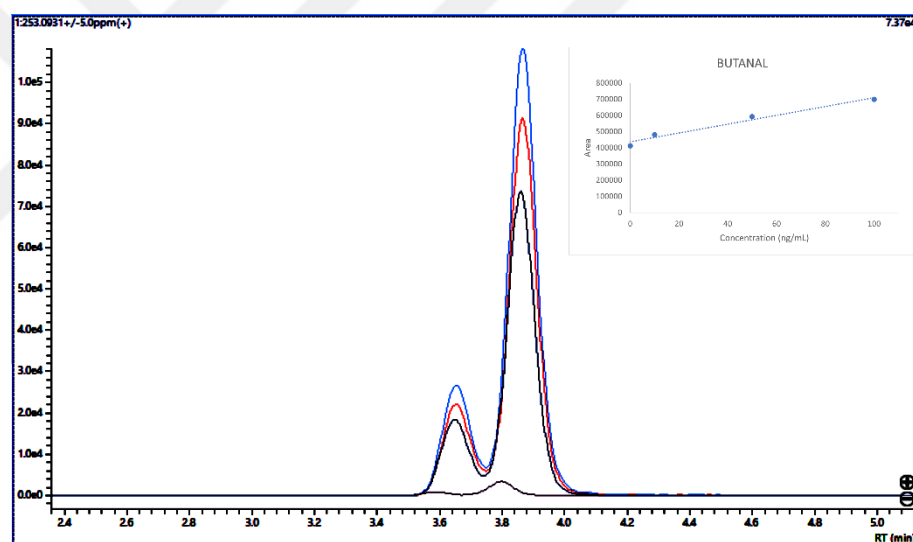


Figure 4.19 Calibration curve and chromatogram of Butanal in LC-QTOF

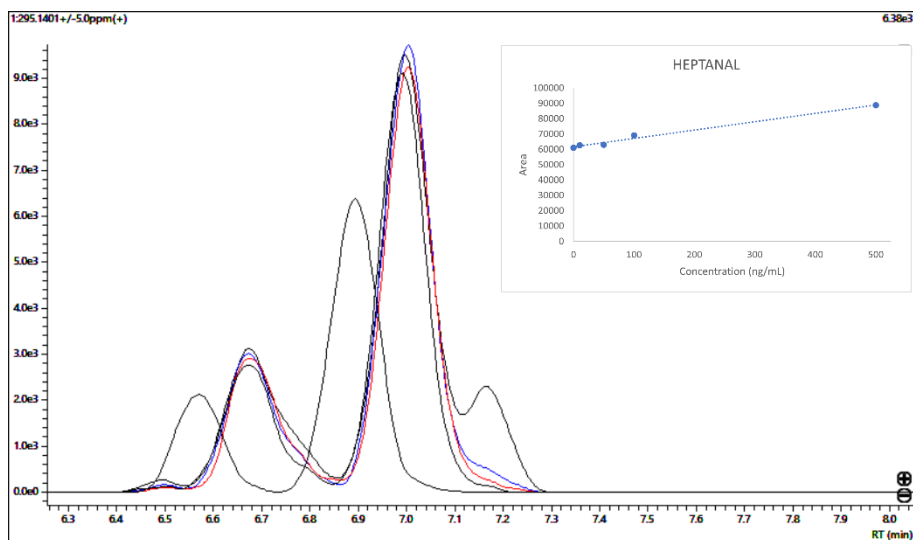


Figure 4.20 Calibration curve and chromatogram of Heptanal in LC-QTOF

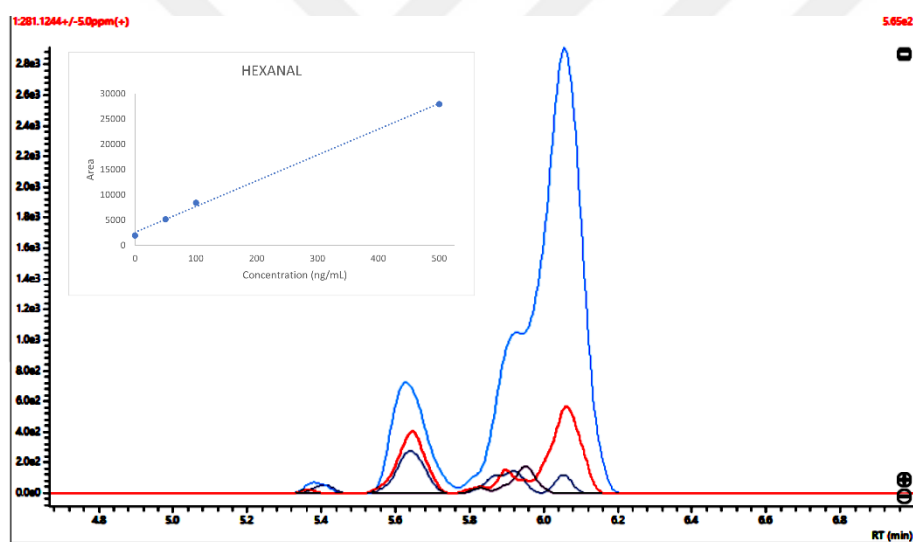


Figure 4.21 Calibration curve and chromatogram of Hexanal in LC-QTOF

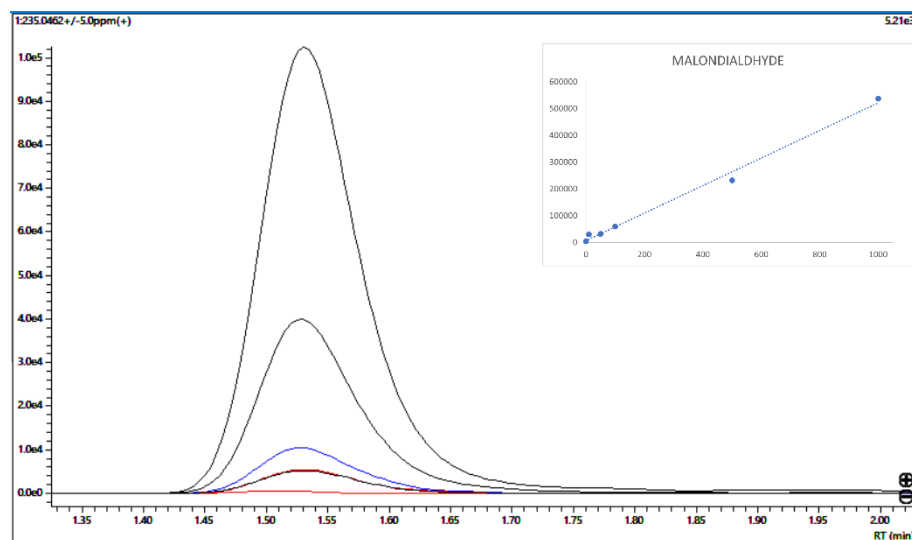


Figure 4.22 Calibration curve and chromatogram of Malondialdehyde in LC-QTOF

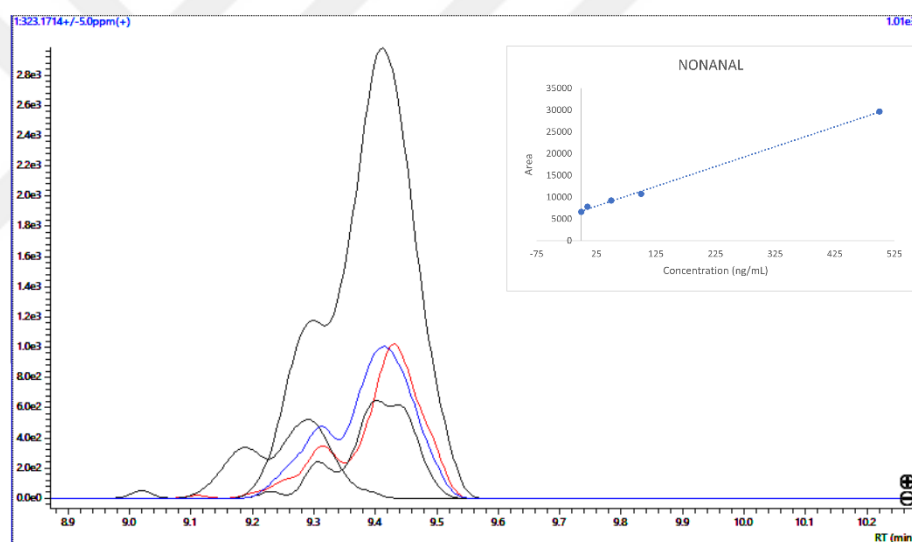


Figure 4.23 Calibration curve and chromatogram of Nonanal in LC-QTOF

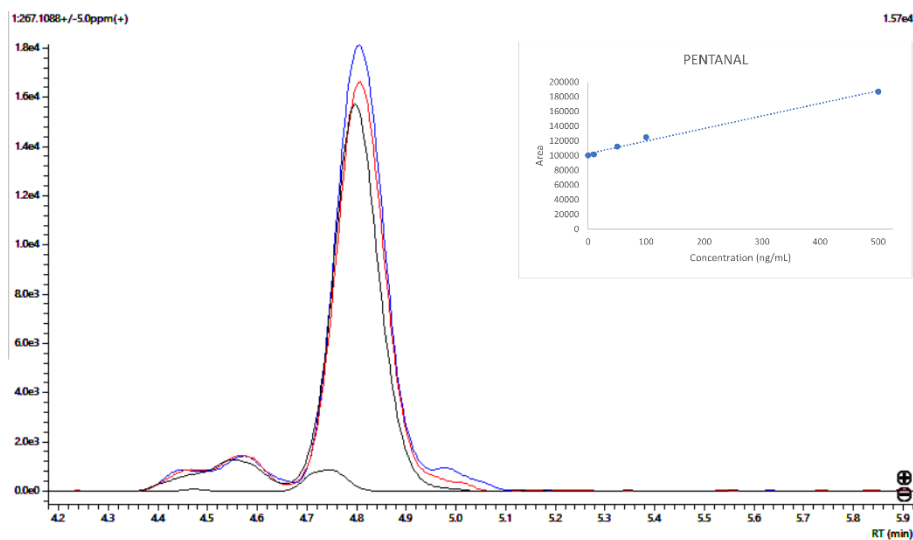


Figure 4.24 Calibration curve and chromatogram of Pentanal in LC-QTOF

After, the analytical parameters of the method were calculated, and the results are presented in Table 4.4. As can be seen, the high background noise level in the analyses carried out by LC-QTOF prevents the determination of healthy aldehyde content. This was attributed to the impurities in the ambient air.

Table 4.4 The analytical parameters of the LC-QTOF VOC analysis method

Analyte	Calibration Curve Equation	R ²	Linear Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Butanal	$y = 2734.3x + 436545$	0.9714	33.0-100	10	33.0
Pentanal	$y = 170.78x + 102786$	0.9916	24.82-500	7.52	24.82
Hexanal	$y = 50.97x + 2600.7$	0.9976	28.65-500	8.68	28.65
Heptanal	$y = 54.519x + 61740$	0.9871	141.92-500	43.0	141.92
Nonanal	$y = 45531x + 6824.9$	0.998	20.14-500	6.10	20.14
3-Hydroxy, 2-Butanone	$y = 1125x + 33418$	0.9976	0.89-1000	0.27	0.89
Malondialdehyde	$y = 514.64x + 7191.2$	0.9924	1.07-1000	0.32	1.07

As shown in the calibration curves in Table 4.4, linearity has been achieved with the method developed using liquid chromatographic separation. Calculations for the limit of detection (LOD) and limit of quantification (LOQ) of the analytical separation method have also been performed. The limit of detection (LOD) of the method is determined based on the $S/N = 3$ principle, and the detection limit (LOQ) is calculated as ($LOQ = 3.3 LOD$). The results indicate that the method can measure the target analytes at the ng/mL level.

4.2.1.1 VOC Analyses in Real EBC Samples in the LC-QTOF Method

In this section, analyses were conducted on EBC samples under optimum conditions, and the results are provided in Appendix-2 (Page 182). The analyses of the same subjects that underwent GC-MS analysis were also carried out using LC-QTOF. An example chromatogram of the EBC sample analysis with LC-QTOF is shown in Figure 4.25.

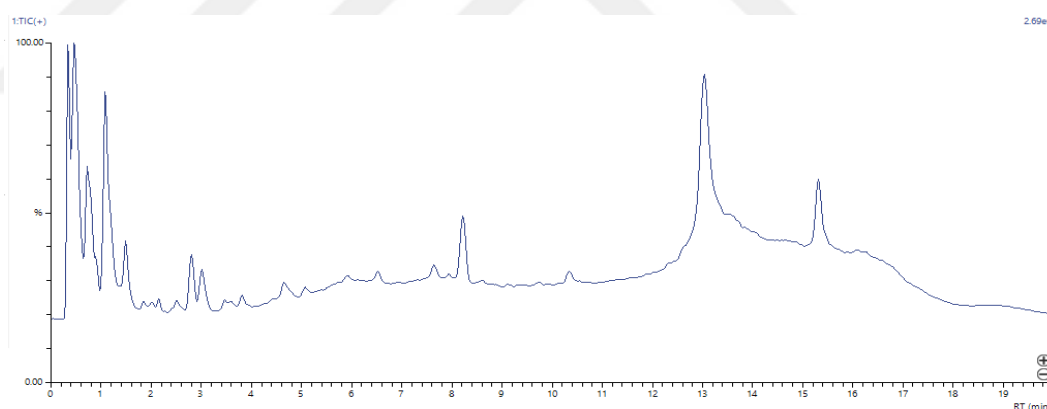


Figure 4.25 The sample LC-QTOF chromatogram of target VOCs in a EBC sample of lung cancer patient

The average values, standard deviations, and the number of detections in subjects obtained from the analysis of target compounds in EBC samples using the LC-QTOF method are provided in Table 4.5.

Table 4.5 LC-QTOF analysis results of targeted VOCs in the EBC of lung cancer patients

Compound	Numbers of Detected Individual	Average $\pm$SD (ng/mL)
Butanal	12	88.67 $\pm$ 42.14
Pentanal	26	470.97 $\pm$ 192.20
Hexanal	2	39.35 $\pm$ 10.11
Heptanal	19	399.36 $\pm$ 141.24
Nonanal	19	152.04 $\pm$ 50.34
3-Hydroxy 2- Butanone	38	44.79 $\pm$ 13.05
Malondialdehyde	11	5.64 $\pm$ 1.94

The average values of compounds detected in the EBC samples of the same subjects using both GC-MS and LC-QTOF are comparatively presented in Table 4.6. As observed, some aldehydes have lower average values in the GC-MS device. Aldehydes such as 3-Hydroxy-2-Butanone and Malondialdehyde can only be analyzed using the LC-QTOF device.

Table 4.6 Comparison of LC-QTOF and GC-MS results

Compound	Average Concentration Values (ng/mL)	
	LC-QTOF	GC-MS
Butanal	88.67	46.60
Pentanal	470.97	12.91
Hexanal	39.35	17.79
Heptanal	399.36	184.48
Nonanal	152.04	16.83
3-Hydroxy 2-Butanone	44.79	Not Detected
Malondialdehyde	5.64	Not Detected

4.2.2. LC-QTOF Amino Acids and Organic Acids Analysis Results

After determining the appropriate separation conditions for the mobile phase using the liquid chromatography device, each analyte's signal was investigated by preparing them in pure water for the analysis of amino acids and organic acids with the LC-QTOF device. For this purpose, the analyses of target compounds such as Lysine, Arginine, Proline, L-valine, Methionine, Tyrosine, Leucine, Phenylalanine, Nicotinamide, Creatine, Trigonelline, Pipelic acid, Betaine, Tryptophan, L-carnitine, Alanine, Histidine, Glycine, Serine, Threonine, Aspartic acid, Glutamic acid, Hypoxanthine, Hippuric acid, Taurine, Succinic acid, Glycolic acid, Vanillin, and Taurocholic acid were performed in the presence of Sarcosine internal standard. The target analytes were analyzed in both positive and negative scan modes for detection. Calibration curves and chromatograms under appropriate conditions are provided between Figure 4.26 and Figure 4.53.

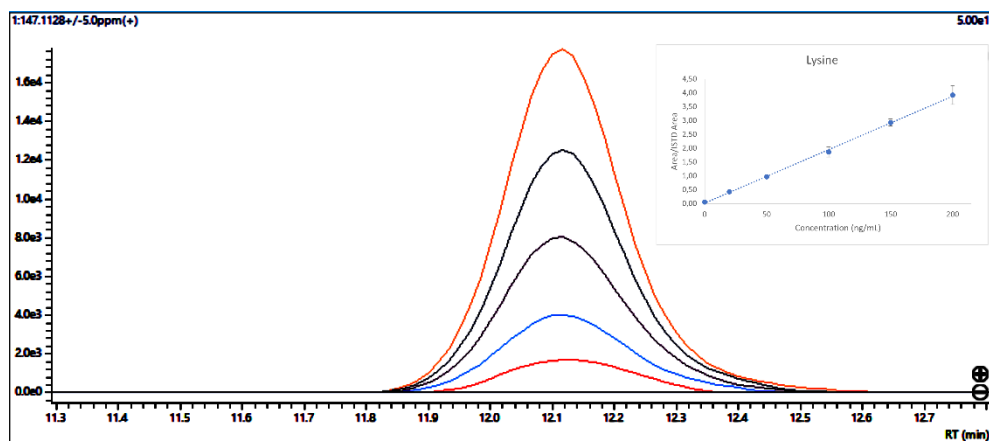


Figure 4.26 Calibration curve and chromatogram of Lysine

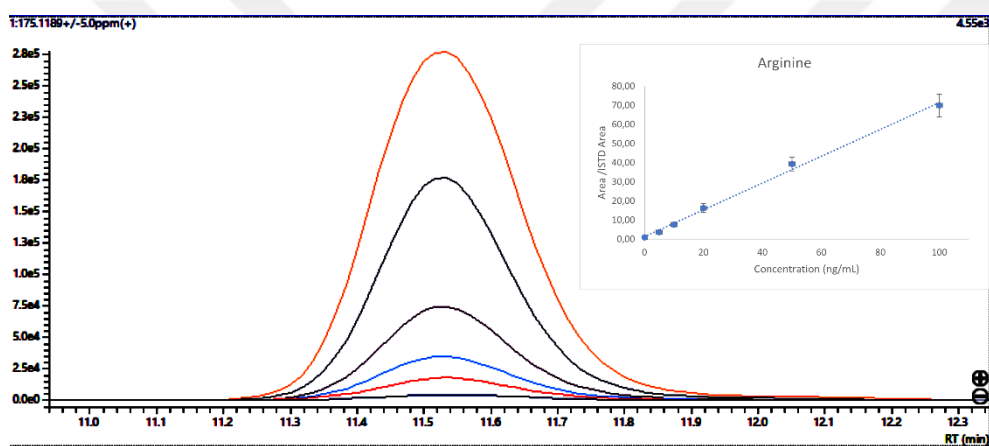


Figure 4.27 Calibration curve and chromatogram of Arginine

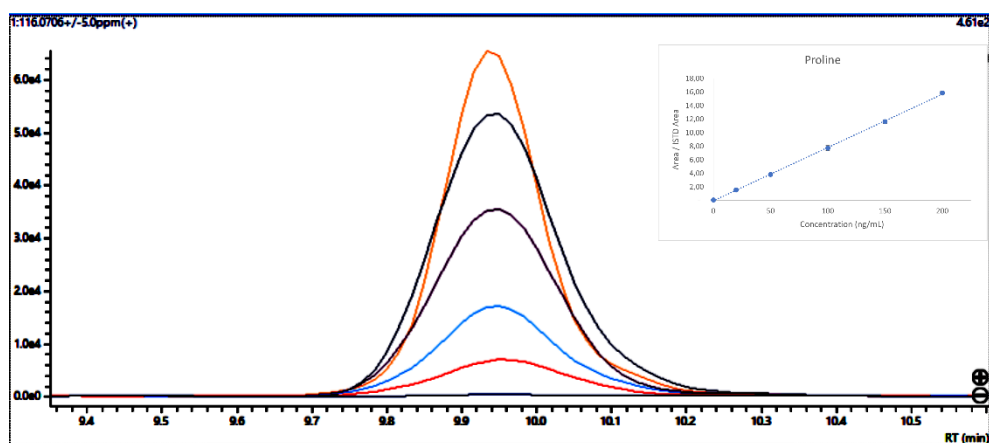


Figure 4.28 Calibration curve and chromatogram of Proline

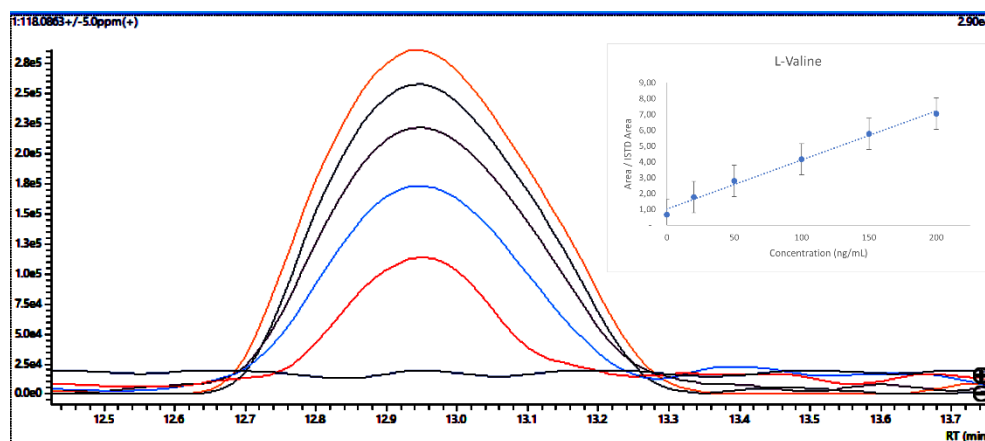


Figure 4.29 Calibration curve and chromatogram of L-Valine

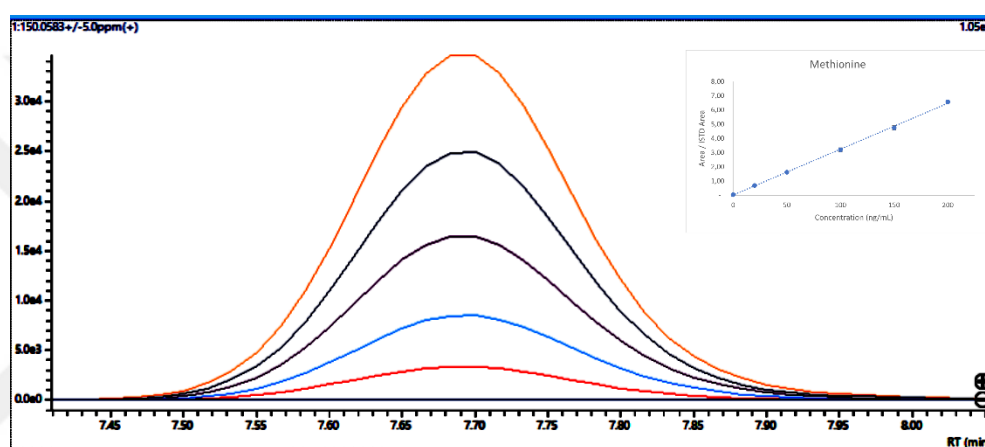


Figure 4.30 Calibration curve and chromatogram of Methionine

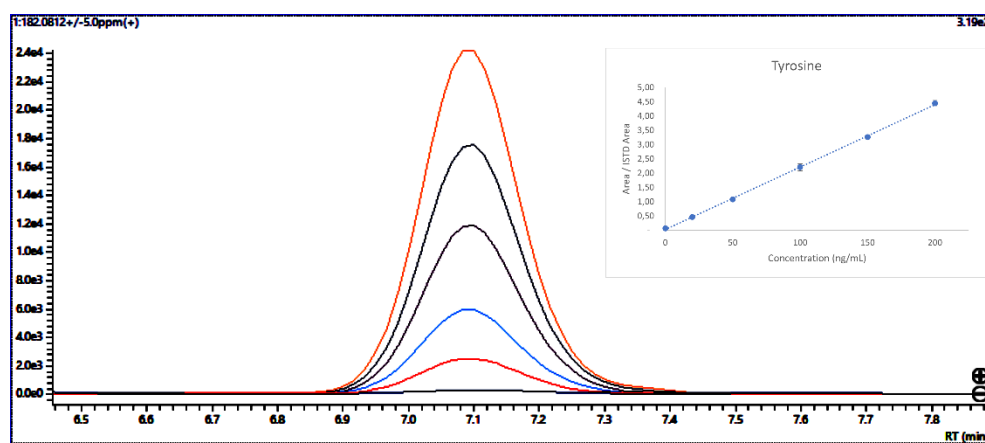


Figure 4.31 Calibration curve and chromatogram of Tyrosine

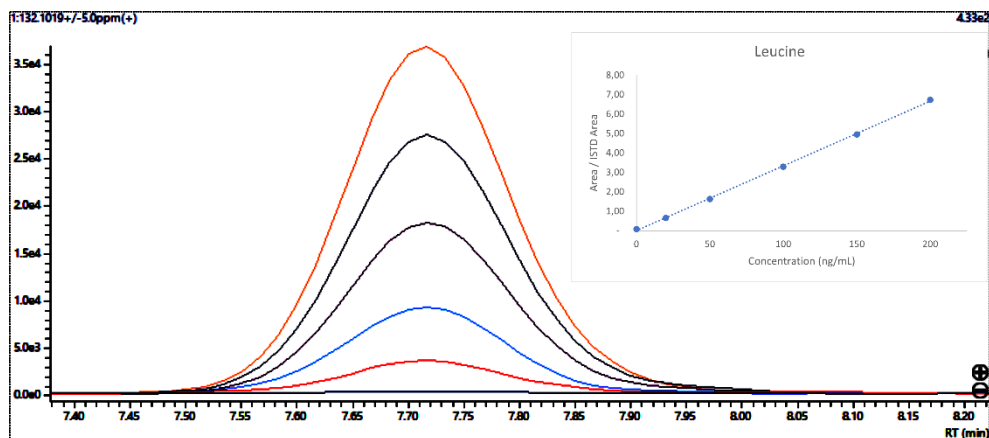


Figure 4.32 Calibration curve and chromatogram of Leucine

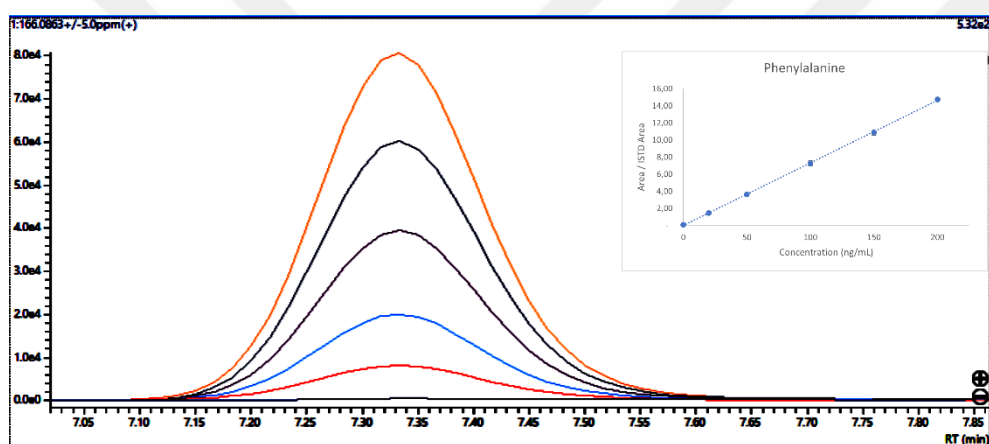


Figure 4.33 Calibration curve and chromatogram of Phenylalanine

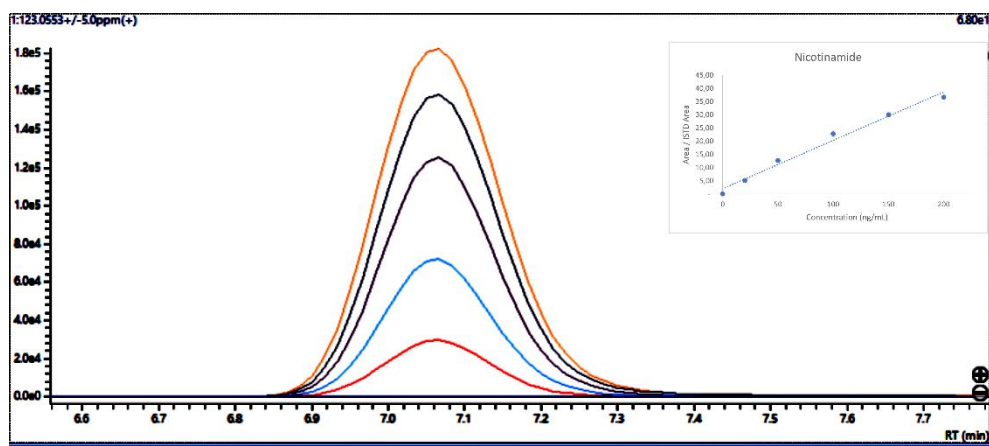


Figure 4.34 Calibration curve and chromatogram of Nicotinamide

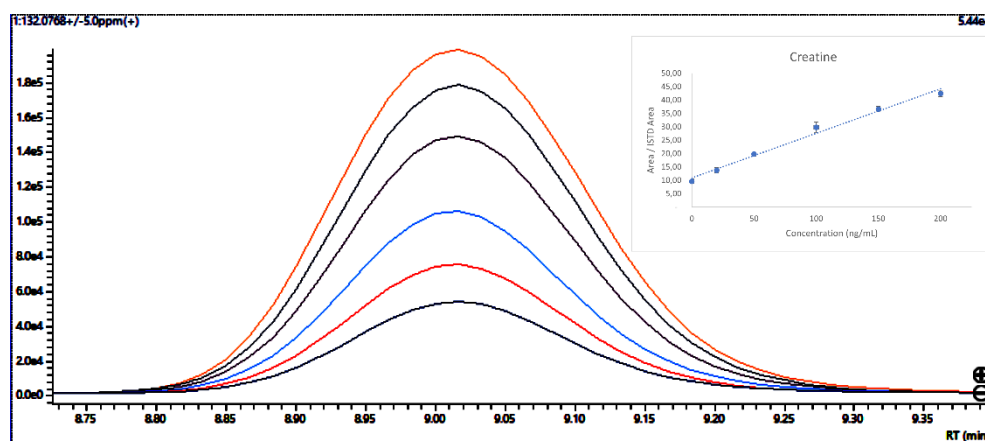


Figure 4.35 Calibration curve and chromatogram of Creatine

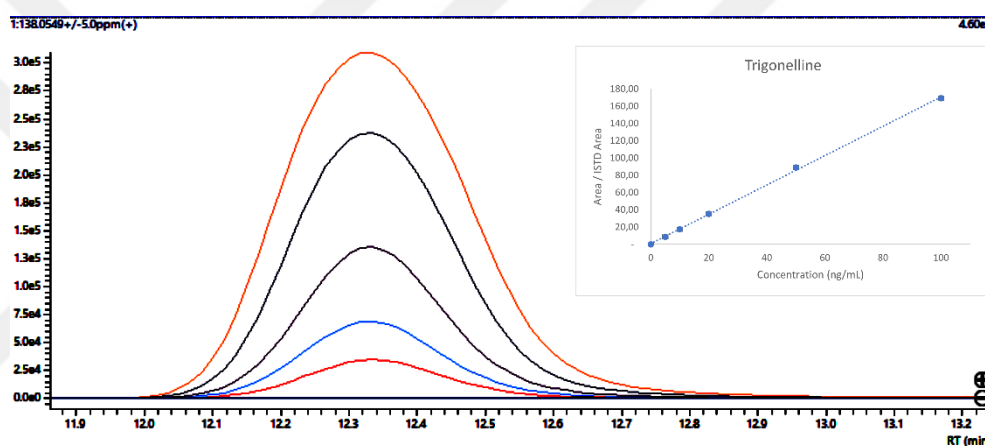


Figure 4.36 Calibration curve and chromatogram of Trigonelline

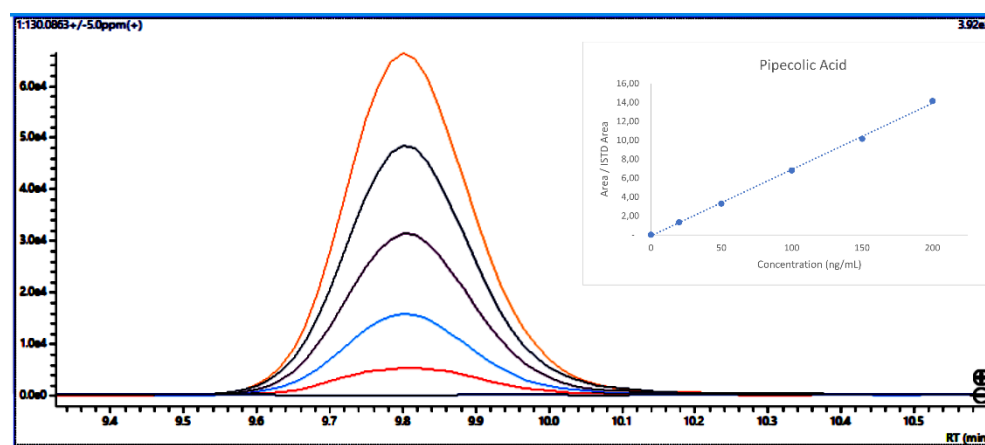


Figure 4.37 Calibration curve and chromatogram of Pipecolic acid

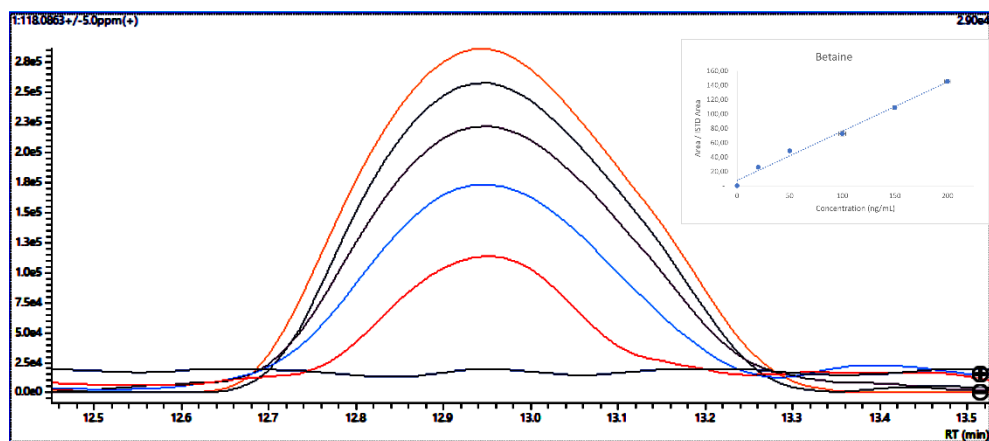


Figure 4.38 Calibration curve and chromatogram of Betaine

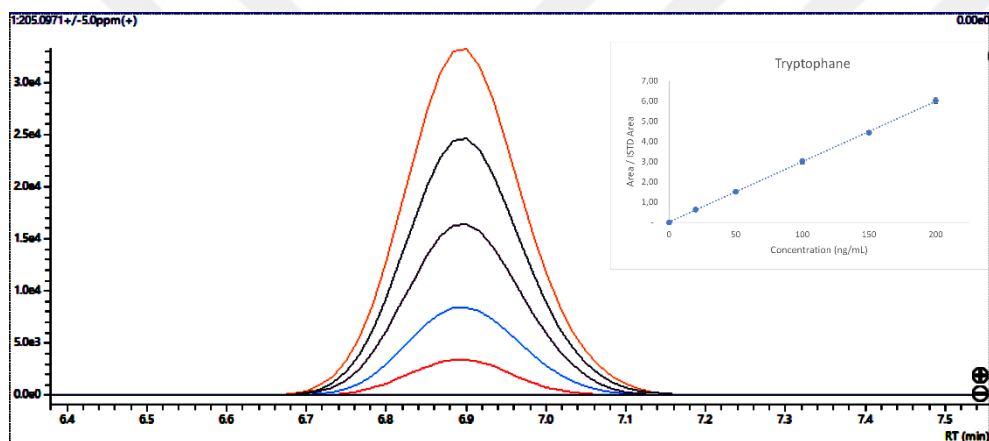


Figure 4.39 Calibration curve and chromatogram of Tryptophane

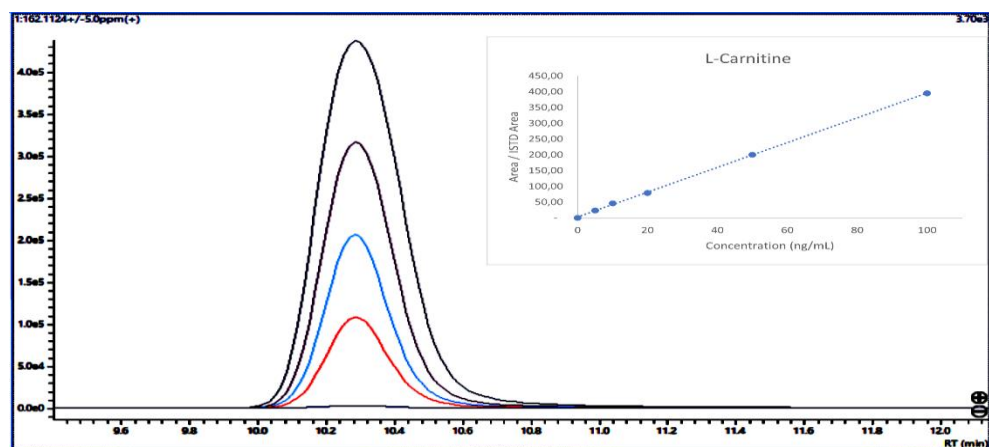


Figure 4.40 Calibration curve and chromatogram of L-Carnitine

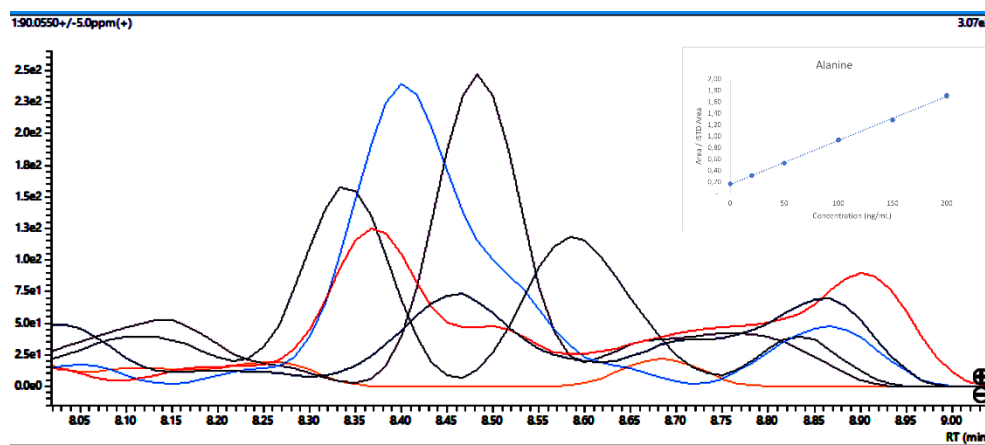


Figure 4.41 Calibration curve and chromatogram of Alanine

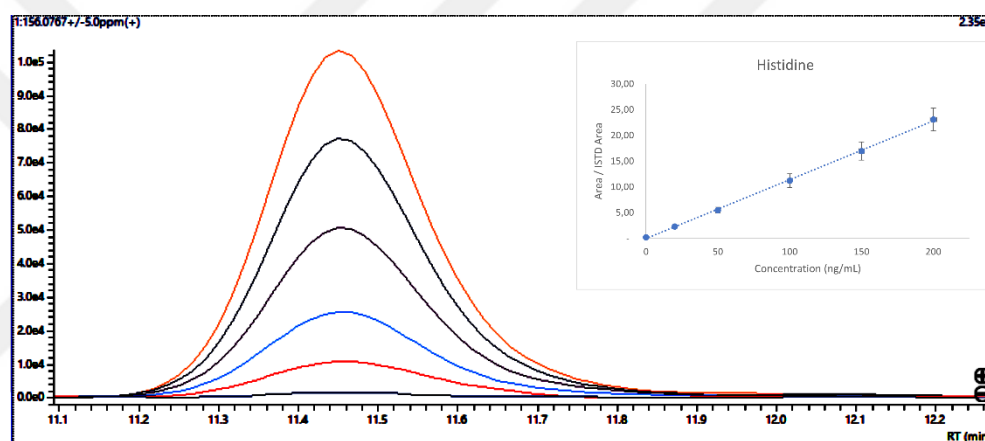


Figure 4.42 Calibration curve and chromatogram of Histidine

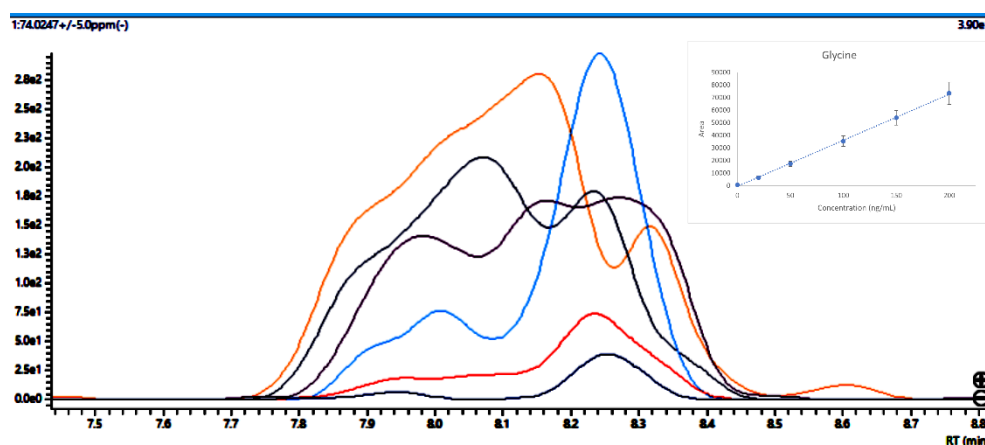


Figure 4.43 Calibration curve and chromatogram of Glycine

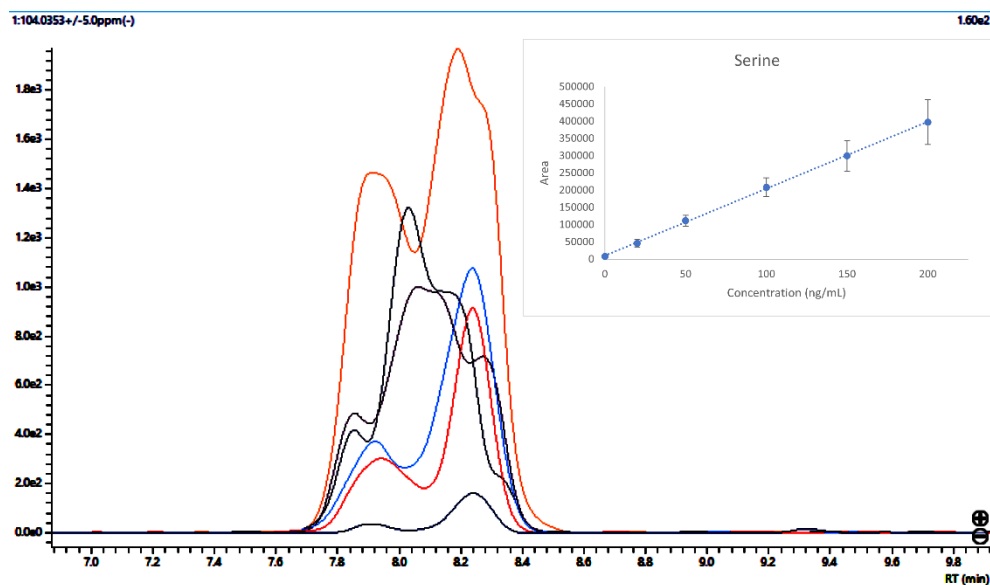


Figure 4.44 Calibration curve and chromatogram of Serine

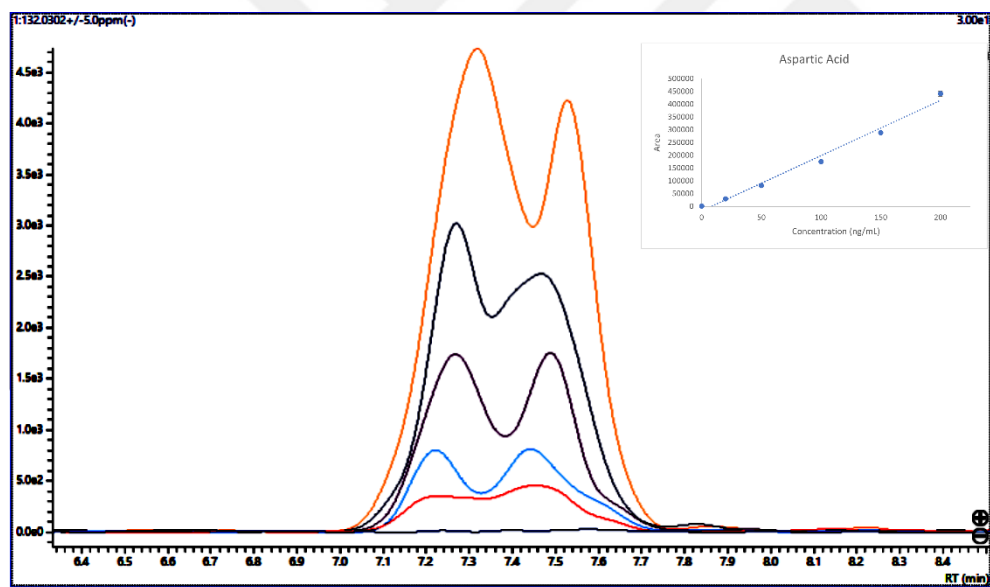


Figure 4.45 Calibration curve and chromatogram of Aspartic acid

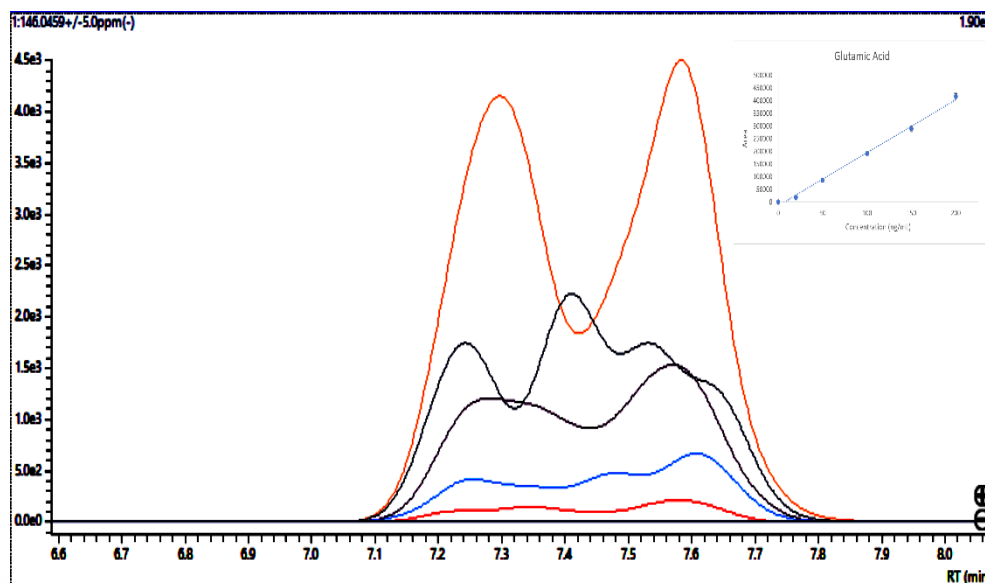


Figure 4.46 Calibration curve and chromatogram of Glutamic acid

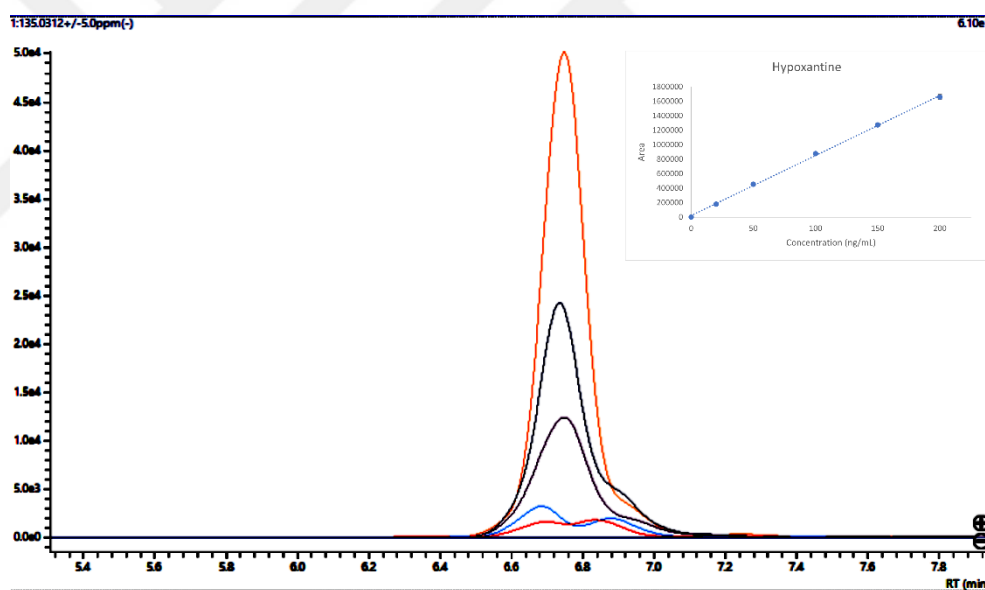


Figure 4.47 Calibration curve and chromatogram of Hypoxanthine

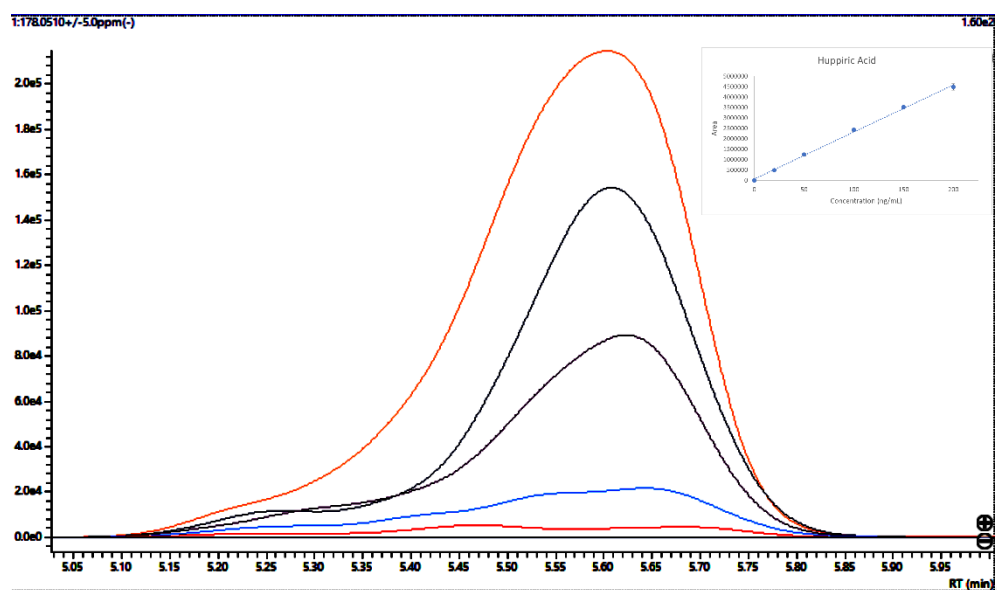


Figure 4.48 Calibration curve and chromatogram of Hippuric acid

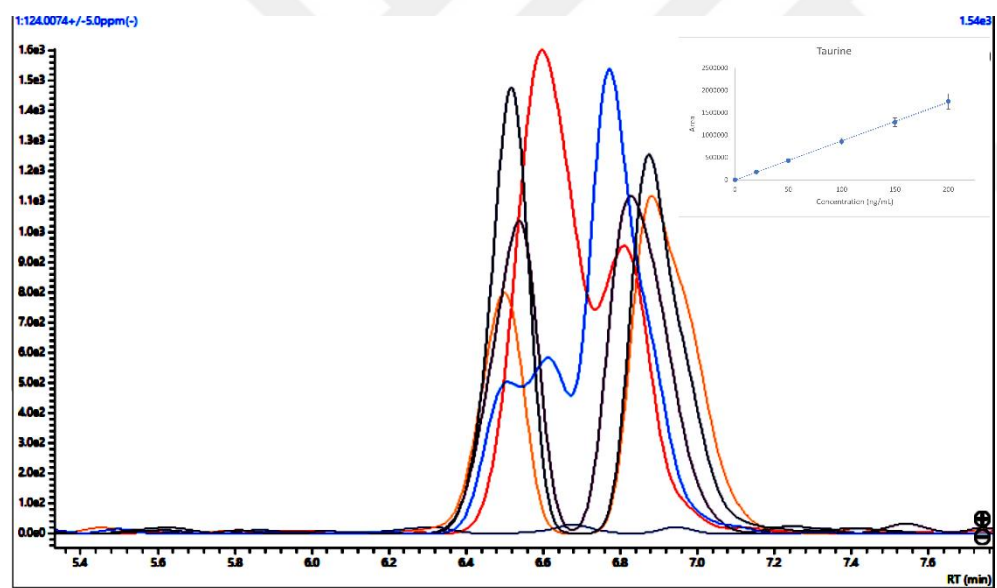


Figure 4.49 Calibration curve and chromatogram of Taurine

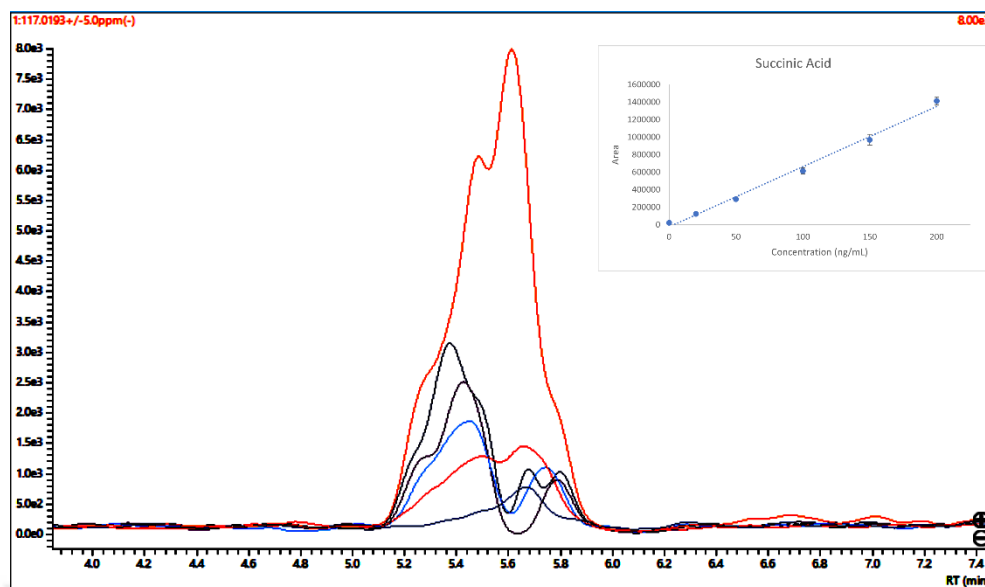


Figure 4.50 Calibration curve and chromatogram of Succinic acid

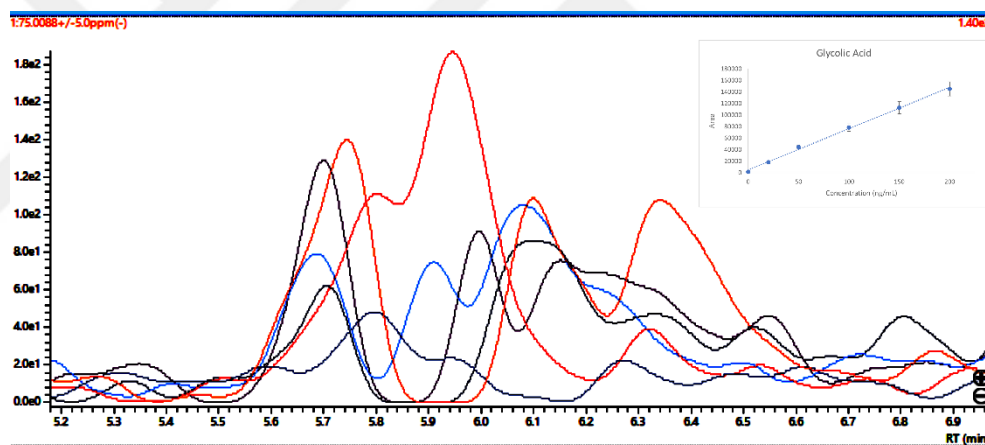


Figure 4.51 Calibration curve and chromatogram of Glycolic acid

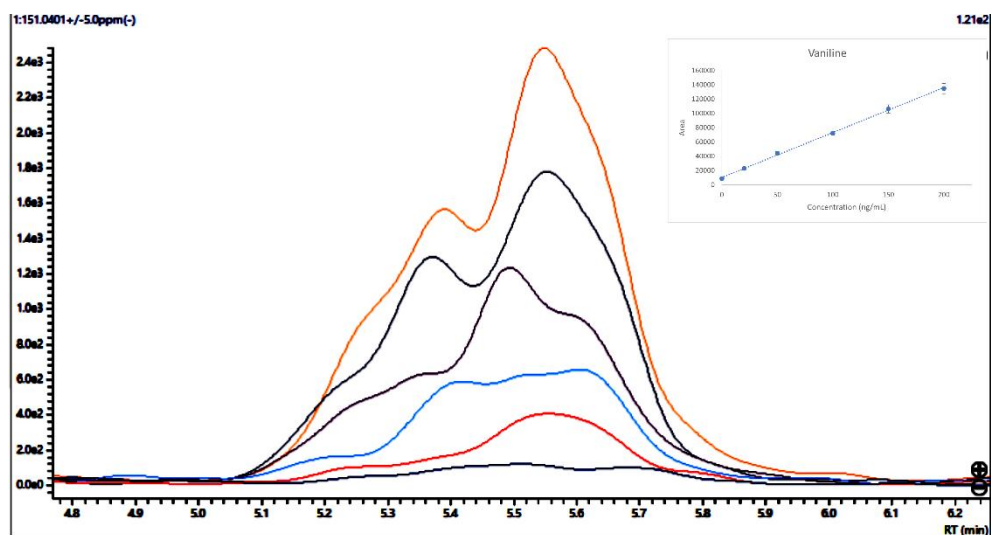


Figure 4.52 Calibration curve and chromatogram of Vaniline

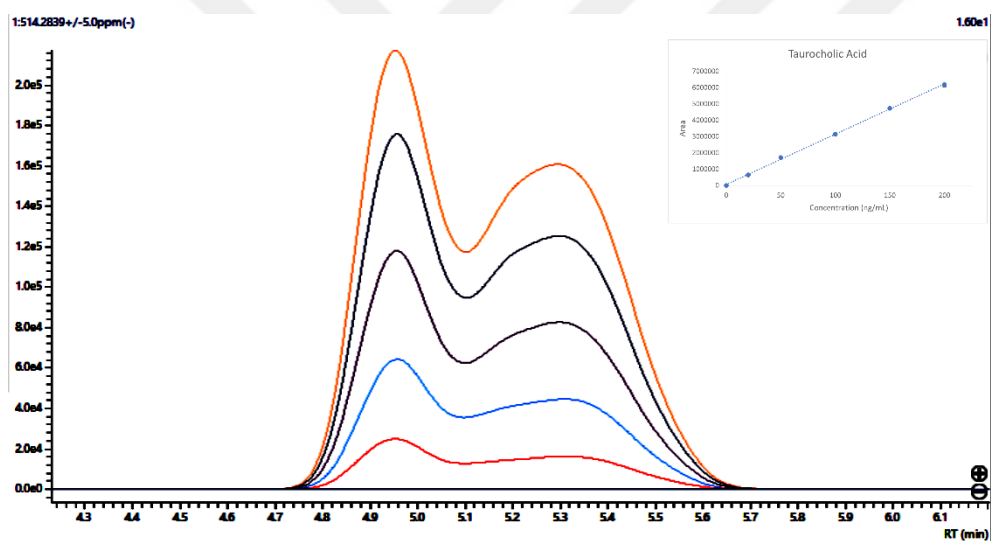


Figure 4.53 Calibration curve and chromatogram of Taurocholic acid

Table 4.7 Analytical parameters of aminoacid analysis for LC-QTOF analysis method

Compound	Calibration Curve Equation	R²	Linear Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Lysine	$y = 0.0193x + 0.0202$	0.9991	3.71-200	1.12	3.71
Arginine	$y = 0.1534x + 0.6211$	0.995	0.34-100	0.10	0.34
Proline	$y = 0.0787x - 0.0433$	0.9996	2.67-200	0.81	2.67
L-Valine	$y = 0.031x + 1.017$	0.9919	90.1-200	27.30	90.10
Methionine	$y = 0.0323x + 0.009$	0.9991	0.12-200	0.04	0.12
Tyrosine	$y = 0.0219x + 0.0265$	0.9995	4.72-200	1.43	4.72
Leucine	$y = 0.0333x + 0.0093$	0.9995	4.52-200	1.37	4.52
Phenylalanine	$y = 0.0732x + 0.0098$	0.9998	1.76-200	0.53	1.76
Nicotinamide	$y = 0.1832x + 2.0124$	0.984	0.24-200	0.07	0.24
Creatine	$y = 0.1667x + 10.906$	0.9874	1.15-200	0.35	1.15
Trigonelline	$y = 1.6997x + 0.8498$	0.9993	0.03-100	0.01	0.03
Pipecolic acid	$y = 0.0702x - 0.1214$	0.999	1.92-200	0.58	1.92
Betaine	$y = 0.6851x + 7.6447$	0.9902	1.78-200	0.54	1.78
Tryptophane	$y = 0.0299x + 0.0151$	0.9998	0.07-200	0.02	0.07
L-Carnitine	$y = 3.9247x + 3.2197$	0.9997	0.06-100	0.02	0.06
Alanine	$y = 0.0077x + 0.1571$	0.9992	1.82-200	0.55	1.82
Histidine	$y = 0.1143x - 0.0261$	0.9994	2.76-200	0.84	2.76
Glycine	$y = 365.75x - 503.24$	0.9992	3.3-200	1.00	3.3
Serine	$y = 1943.9x + 9810.1$	0.9995	0.56-200	0.17	0.56
Theronine	$y = 2414.7x + 3658.7$	0.9999	0.35-200	0.11	0.35
Aspartic acid	$y = 2161.1x - 17633$	0.9858	4.43-200	1.34	4.43

Table 4.7 (Continue)

Compound	Calibration Curve Equation	R ²	Linear Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Glutamic acid	$y = 2098x - 14566$	0.9959	2.11-200	0.64	2.11
Hypoxanthine	$y = 8289.9x + 23115$	0.999	0.75-200	0.23	0.75
Huppirc acid	$y = 22589x + 72419$	0.998	0.54-200	0.16	0.54
Taurine	$y = 8708.9x - 3129.9$	0.9997	3.31-200	1.00	3.31
Succinic acid	$y = 6879x - 23619$	0.9926	5.56-200	1.68	5.56
Glycolic acid	$y = 714.48x + 4871.7$	0.9976	6.35-200	1.92	6.35
Vaniline	$y = 628.91x + 10297$	0.9989	12.03-200	3.65	12.03
Taurocholic acid	$y = 30847x + 59916$	0.9993	0.09-200	0.03	0.09

4.2.2.1. The Effect of Storage Conditions on EBC Amino Acid and Organic Acid Concentrations

Some of the target analytes can be unstable necessitates the investigation of the effect of storage conditions on the concentrations of amino acids and organic acids, especially during and before the analysis. For this purpose, target analytes and internal standard were added to EBC samples at appropriate concentrations for the analysis and also internal standard of positive ions, with a concentration of 100 ng/mL. Then, the standard of analytes were added into the EBC sample. After that was divided into three portion and one of them was place on the rack of the device at +4°C and they were analyzed separately in positive and negative modes for 24 hours. One of the other two parts was stored -20°C and the other at -80°C, they were thawed daily just before analysis and placed on the rack at +4°C for analysis. Then the samples placed back into the freezer at the appropriate temperature. Additionally, parallel experiments were conducted using standards prepared only in pure water. In this way, the stability of analytes at this temperature and the

changes in amino acids and organic acids in EBC during the freezing and thawing process were examined. The results for each analyte are presented between Figure 4.54 and Figure 4.81.

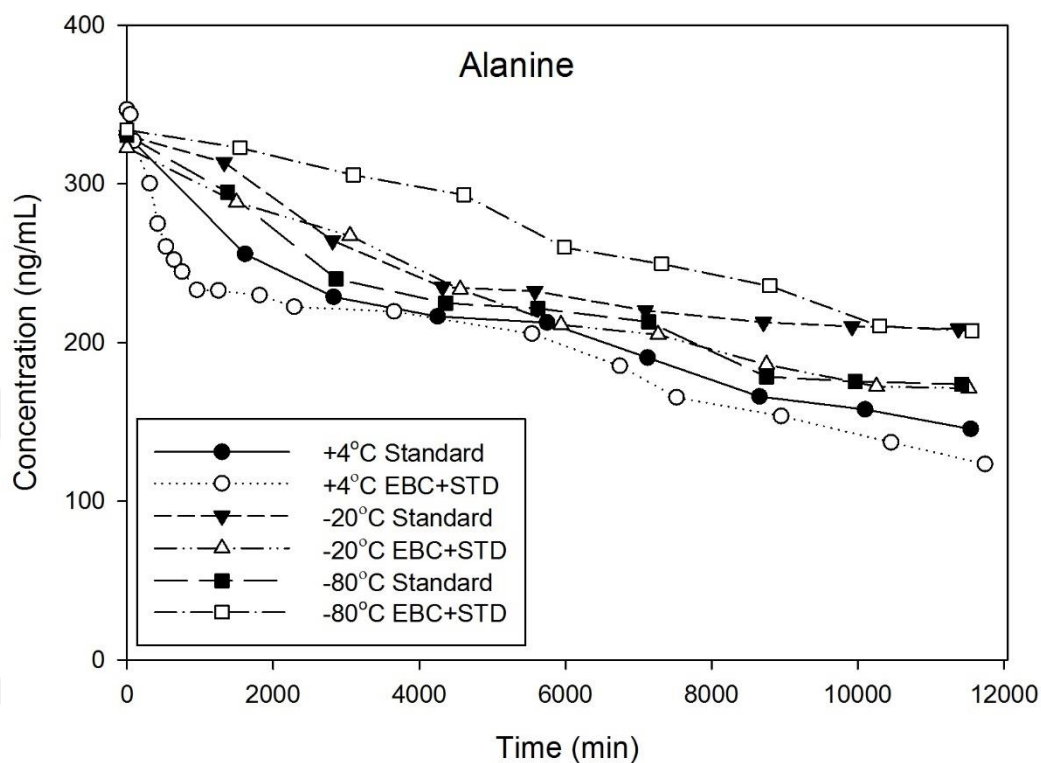


Figure 4.54 The effect of storage conditions on Alanine stability

Figure 4.54 reveals that the concentration of Alanine in EBC at +4°C rapidly decreases within the first 24 hours. Although the Alanine standard prepared in a pure water at +4°C shows a much slower decreasing trend, it is also evident that its concentration decreases in the pure water. When compared with the -20°C conditions, a parallel change is observed between the pure water and the EBC medium. In the EBC medium at -80°C, degradation shows a similar trend to the EBC sample stored at -20°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -80°C. This indicates that the concentration of Alanine standard in the EBC medium decreases more slowly.

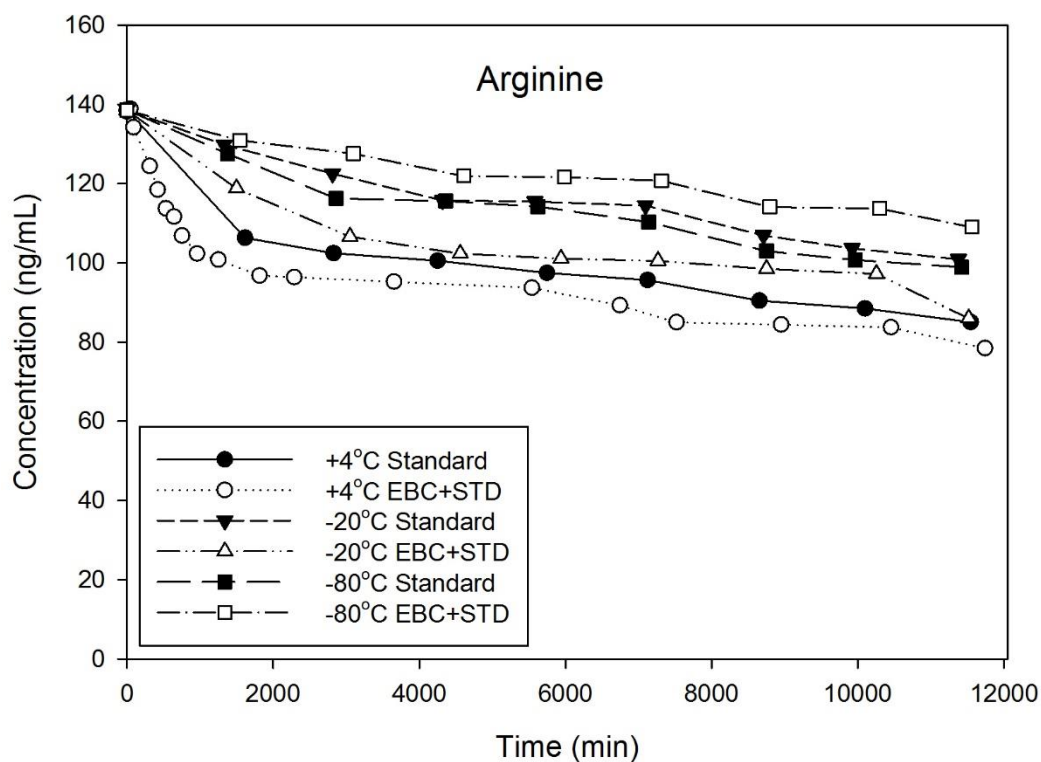


Figure 4.55 The effect of storage conditions on Arginine stability

Figure 4.55 reveals that the concentration of Arginine EBC at +4 °C decreases rapidly within the first 24 hours. In spite of the fact that the Arginine standard prepared in a pure water at +4°C shows a much slower decreasing trend, it is also evident that its concentration decreases in the pure water. When compared with -20 °C conditions, a parallel change is observed between the pure water and the EBC medium. In the EBC medium at -80 °C, degradation shows a similar trend to the EBC sampled stored at -20 °C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -80°C. This indicates that the concentration of the Arginine standard in the EBC medium decreases more slowly.

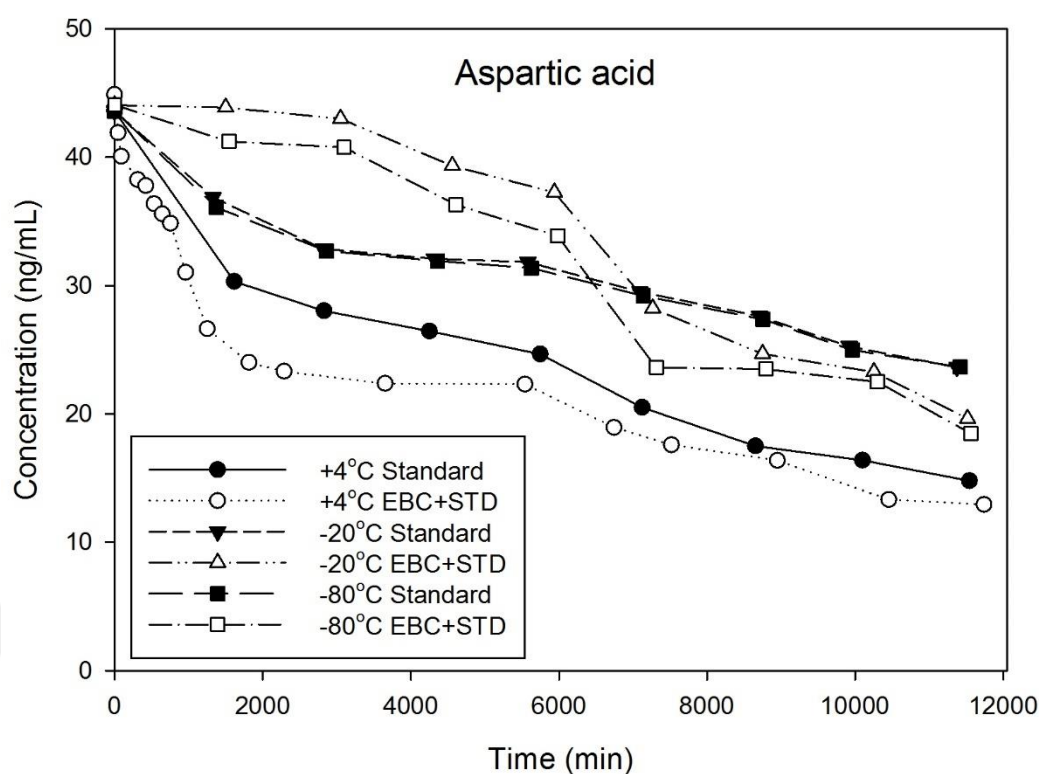


Figure 4.56 The effect of storage conditions on Aspartic acid stability

Figure 4.56 reveals that the concentration of Aspartic acid in EBC at +4°C rapidly decreases within the first 24 hours. Although the Aspartic acid standard prepared in a pure water at +4°C shows a much slower decreasing graph, it is also evident that its concentration decreases in the pure water. When comparing the concentrations of aspartic acid in the pure water at -20°C and -80°C, a parallel change is observed. In the EBC medium at -20°C, degradation shows a similar trend to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C. This indicates that the concentration of Aspartic acid standard decreases more slowly in the EBC medium and remains more stable in the EBC medium.

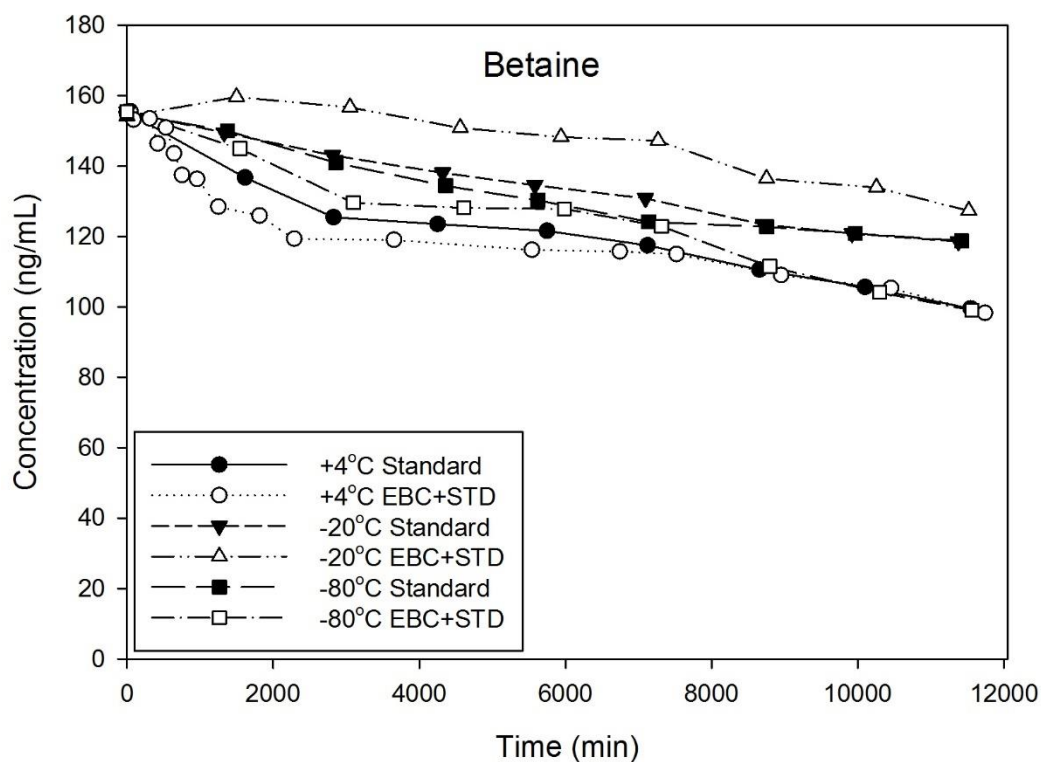


Figure 4.57 The effect of storage conditions on Betaine stability

Figure 4.57 reveals that the concentration of Betaine in EBC at +4°C rapidly decreases within the first 24 hours. Although the Betaine standard prepared in a pure water at +4 °C shows much slower decreasing graph, it is also seen that its concentration decreases in the pure water. When comparing the concentrations of Betaine in the pure water at -20°C and -80°C, a parallel change is observed. In the EBC medium at -20°C, degradation shows a similar trend to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C. This indicates that the concentration of Betaine standard decreases more slowly in the EBC medium and remains more stable in the EBC medium.

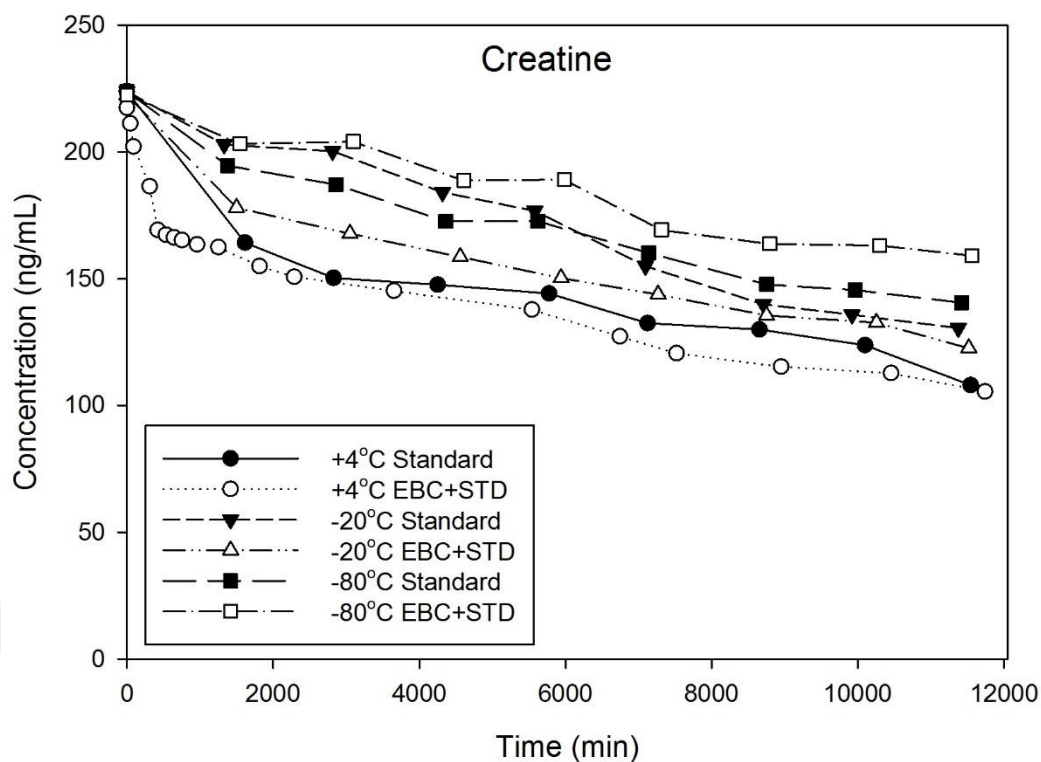


Figure 4.58 The effect of storage conditions on Creatine stability

Figure 4.58 reveals that the concentration of Creatine in EBC at +4°C rapidly decreases within the first 24 hours. Although the Creatine standard prepared in a pure water at +4°C shows a much slower decreasing trend, it is also evident that its concentration decreases in the pure water. When comparing the concentrations of Creatine in the pure water at -20°C and -80°C, a parallel change is observed. In the EBC medium at -20°C, degradation shows a similar trend to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -80°C. This indicates that the concentration of Creatine standard decreases more slowly in the EBC medium and remains more stable in the EBC medium.

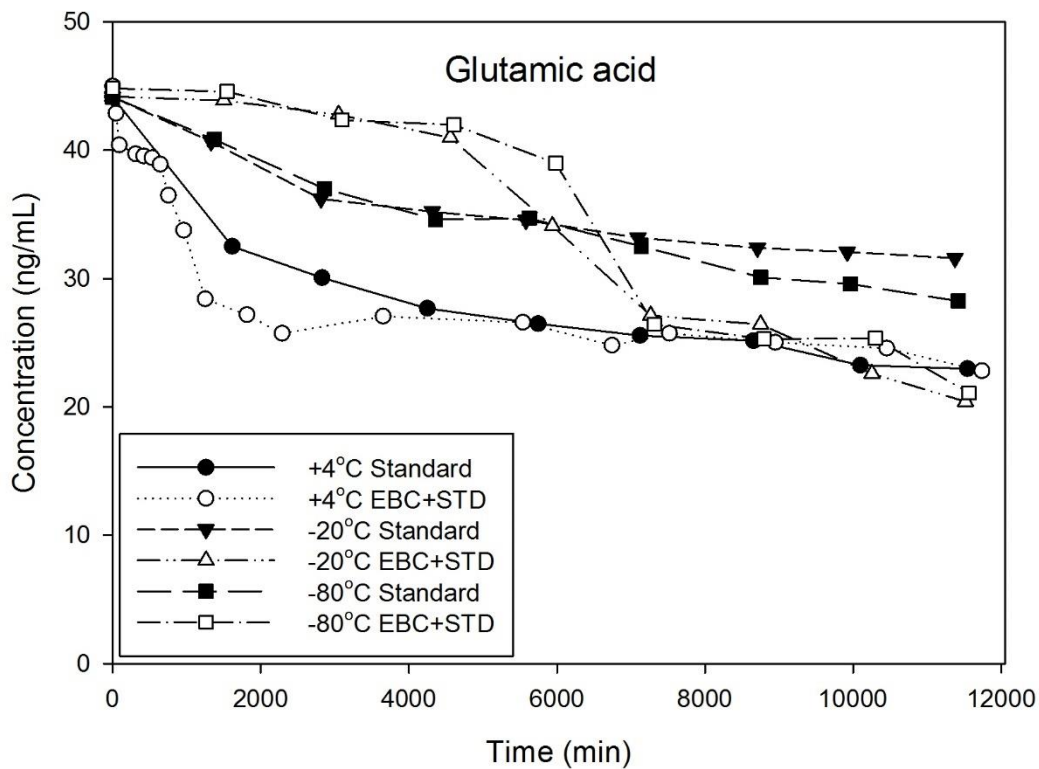


Figure 4.59 The effect of storage conditions on Glutamic acid stability

Figure 4.59 reveals that the concentration of Glutamic acid in EBC at +4°C rapidly decreases within the first 24 hours. Although the Glutamic acid standard prepared in a pure water at +4°C shows a much slower decreasing graph, it is also evident that its concentration decreases in the pure water. When comparing the concentrations of Glutamic acid in the pure water at -20°C and -80°C, a parallel change is observed. In the EBC medium at -20°C, degradation shows a similar trend to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -80°C. This indicates that the concentration of Glutamic acid standard decreases more slowly in the EBC medium and remains more stable in the EBC medium.

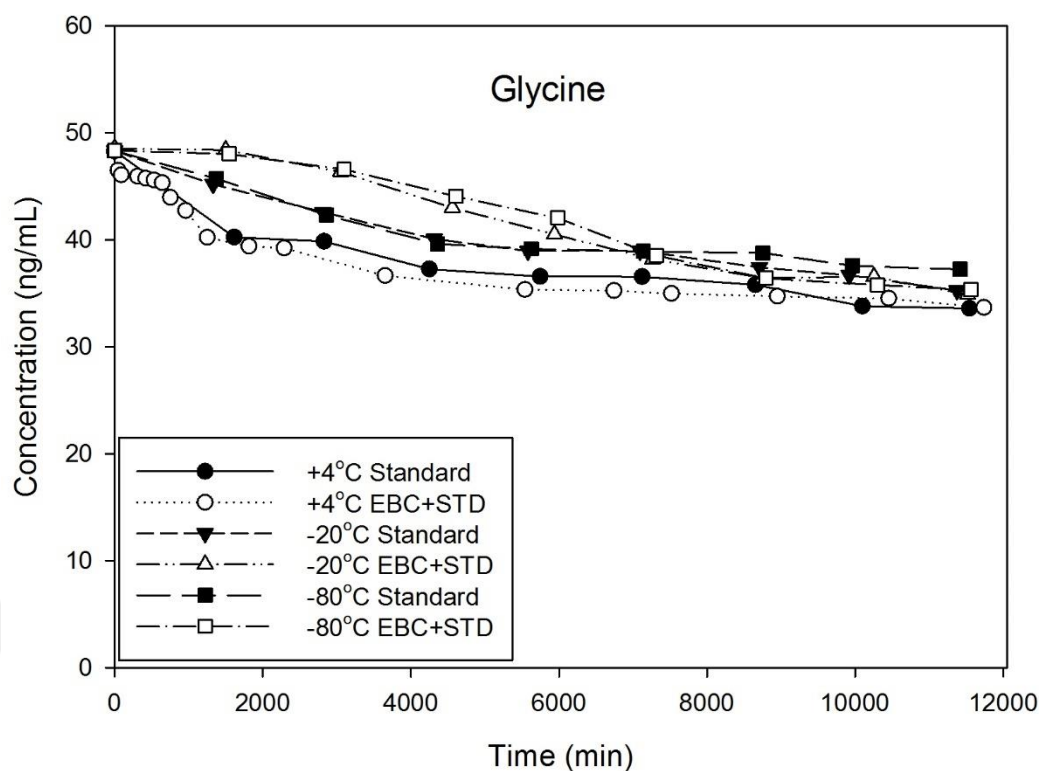


Figure 4.60 The effect of storage conditions on Glycine stability

Figure 4.60 reveals that the concentration of Glycine in EBC at +4°C decreases very rapidly within the first 24 hours. Although the Glycine standard prepared in the pure water at +4°C shows a much slower decrease graph, it is also observed that its concentration tends to decrease in the pure water. When the concentrations of Glycine in the pure water at -20°C and -80°C are compared, it is seen that they exhibit a parallel change. The change of the concentration in the EBC medium at -20°C also shows a similar pattern of change as the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -80°C and -20°C. This indicates that the concentration of Glycine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

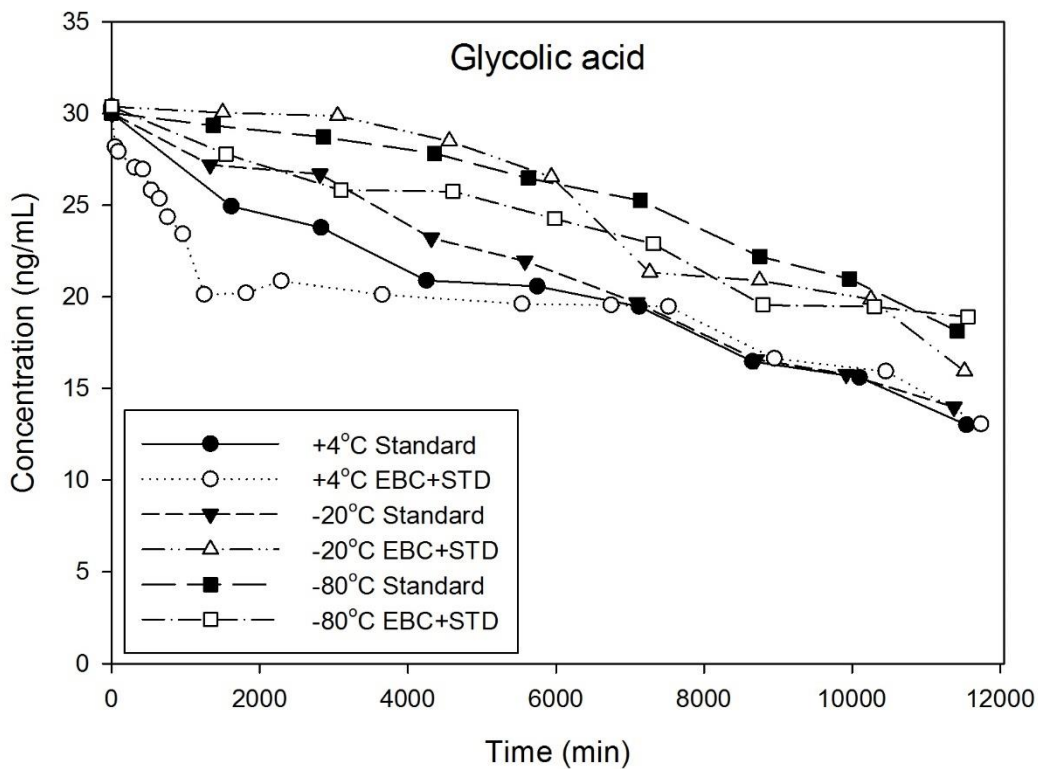


Figure 4.61 The effect of storage conditions on Glycolic acid stability

Figure 4.61 reveals that the concentration of Glycolic acid in EBC at +4°C decreases very rapidly within the first 24 hours. Even though the Glycolic acid standard prepared in the pure water at +4°C shows a much slower decrease graph, it is also observed that its concentration graph to decrease in the pure water. When the concentrations of Glycolic acid in the pure water at -20°C and -80°C are compared, it is seen that they demonstrate a parallel change. The change of the concentration in the EBC medium at -20°C also shows a similar pattern of change as the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C. This indicates that the concentration of the Glycolic acid standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

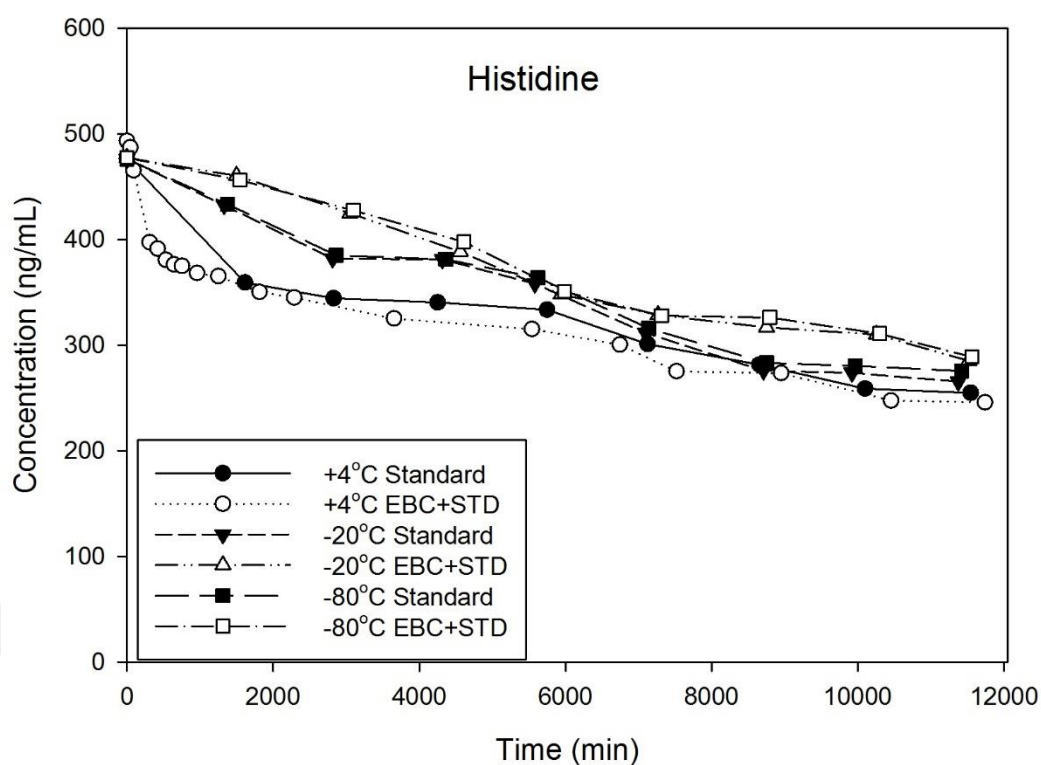


Figure 4.62 The effect of storage conditions on Histidine stability

Figure 4.62 reveals that the concentration of Histidine in +4°C EBC rapidly decreases within the first 24 hours. Although the Histidine standard prepared in a pure water at +4°C shows a much slower decrease, its concentration also tends to decrease in the pure water. When the concentrations of Histidine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change similar to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Histidine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

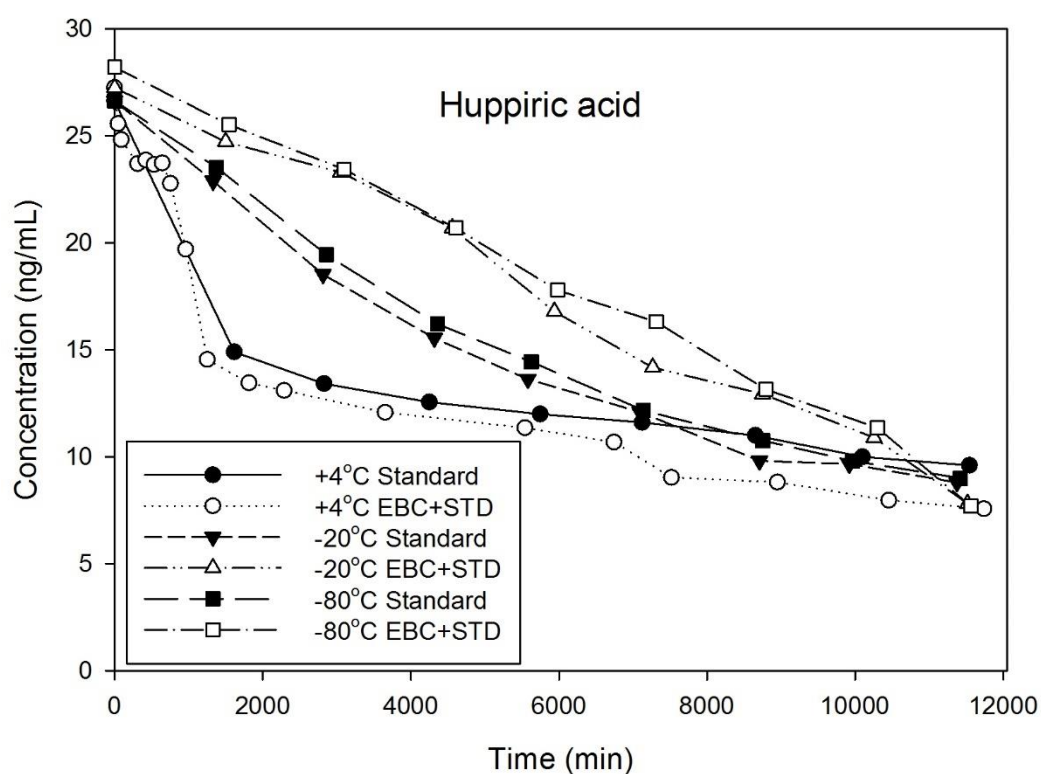


Figure 4.63 The effect of storage conditions on Huppiric acid stability

Figure 4.63 reveals that the concentration of Huppiric acid in +4°C EBC rapidly decreases within the first 24 hours. Although the Huppiric acid standard prepared in a pure water at +4°C shows a much slower decrease, its concentration also tends to decrease in the pure water. When the concentrations of Huppiric acid in pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change similar to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Huppiric acid standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

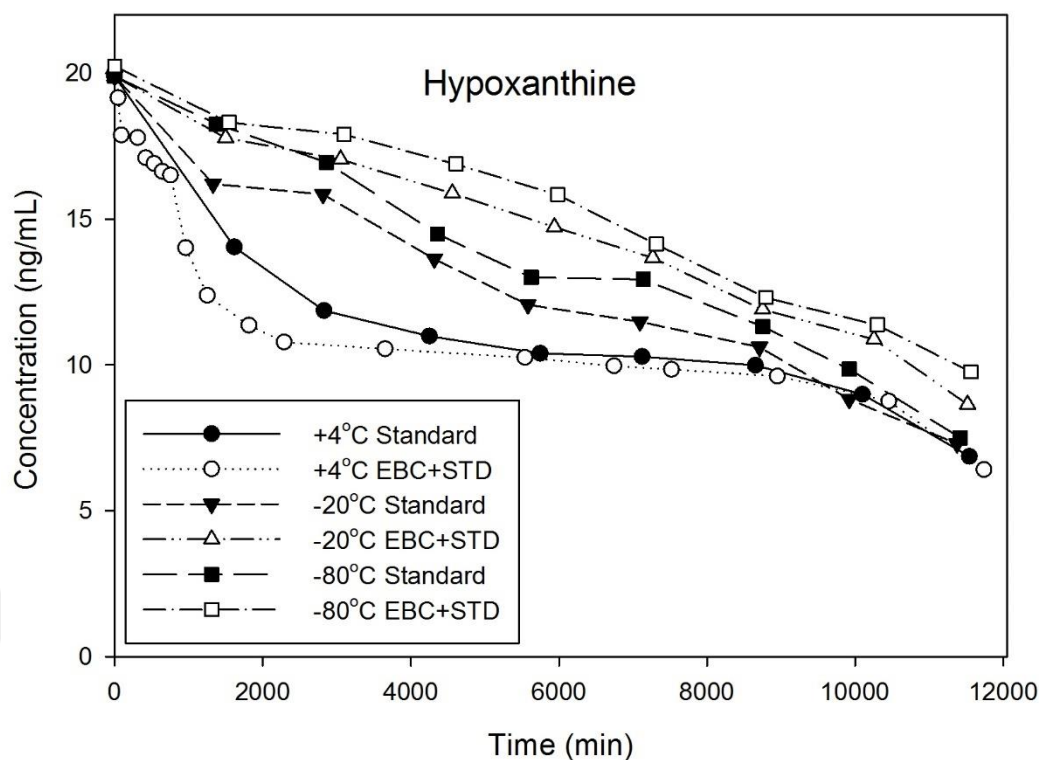


Figure 4.64 The effect of storage conditions on Hypoxanthine stability

Figure 4.64 reveals that the concentration of Hypoxanthine in +4°C EBC rapidly decreases within the first 24 hours. Although the Hypoxanthine standard prepared in a pure water at +4°C shows a much slower decrease, its concentration also tends to decrease in the pure water. When the concentrations of Hypoxanthine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -80°C. This indicates that the concentration of Hypoxanthine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

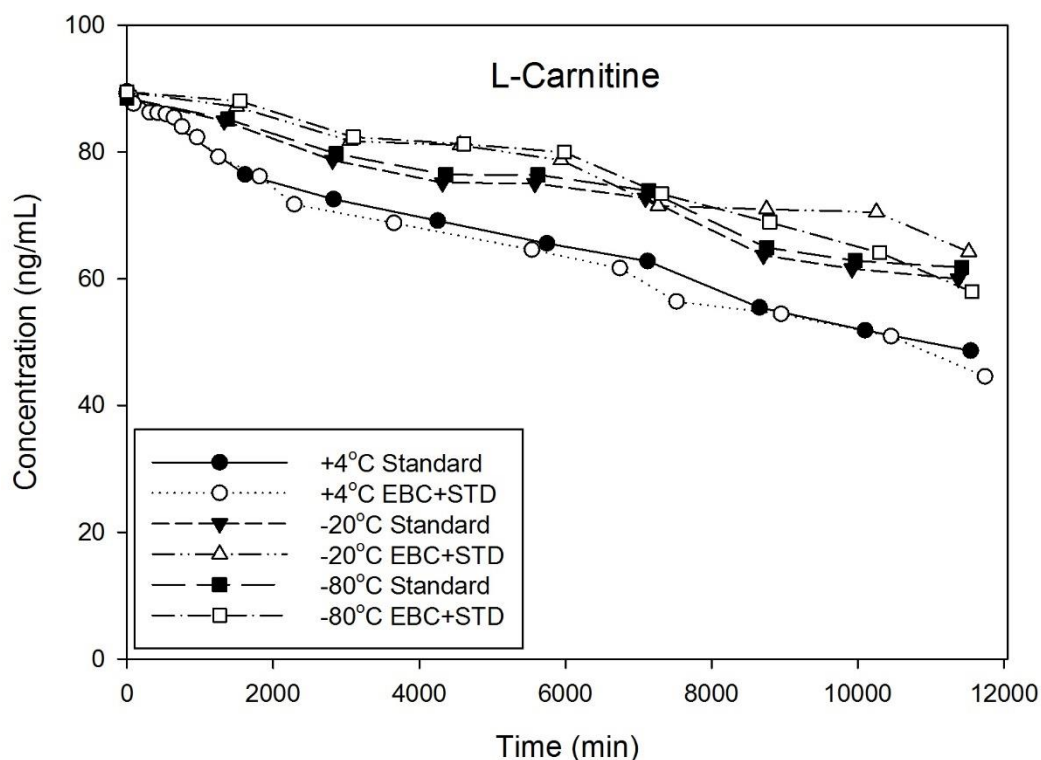


Figure 4.65 The effect of storage conditions on L-Carnitine stability

Figure 4.65 reveals that the concentration of L-Carnitine in +4°C EBC rapidly decreases within the first 24 hours. The L-Carnitine standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of L-Carnitine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of L-Carnitine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

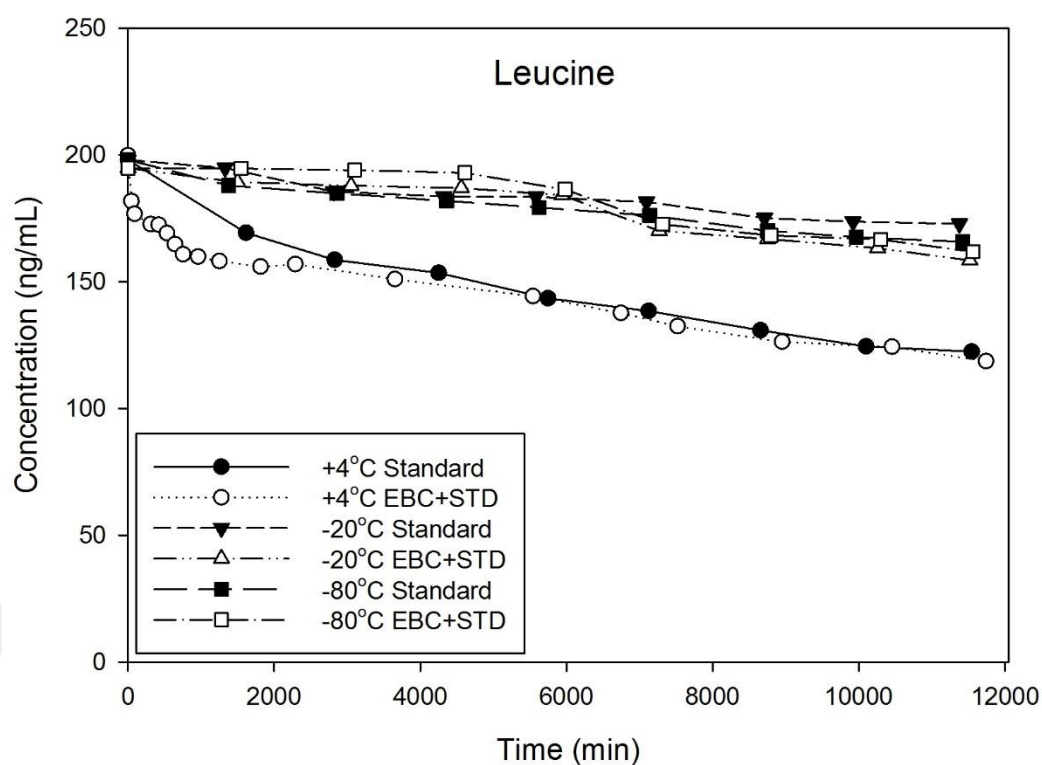


Figure 4.66 The effect of storage conditions on Leucine stability

Figure 4.66 reveals that the concentration of Leucine in +4°C EBC rapidly decreases within the first 24 hours. The Leucine standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Leucine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Leucine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

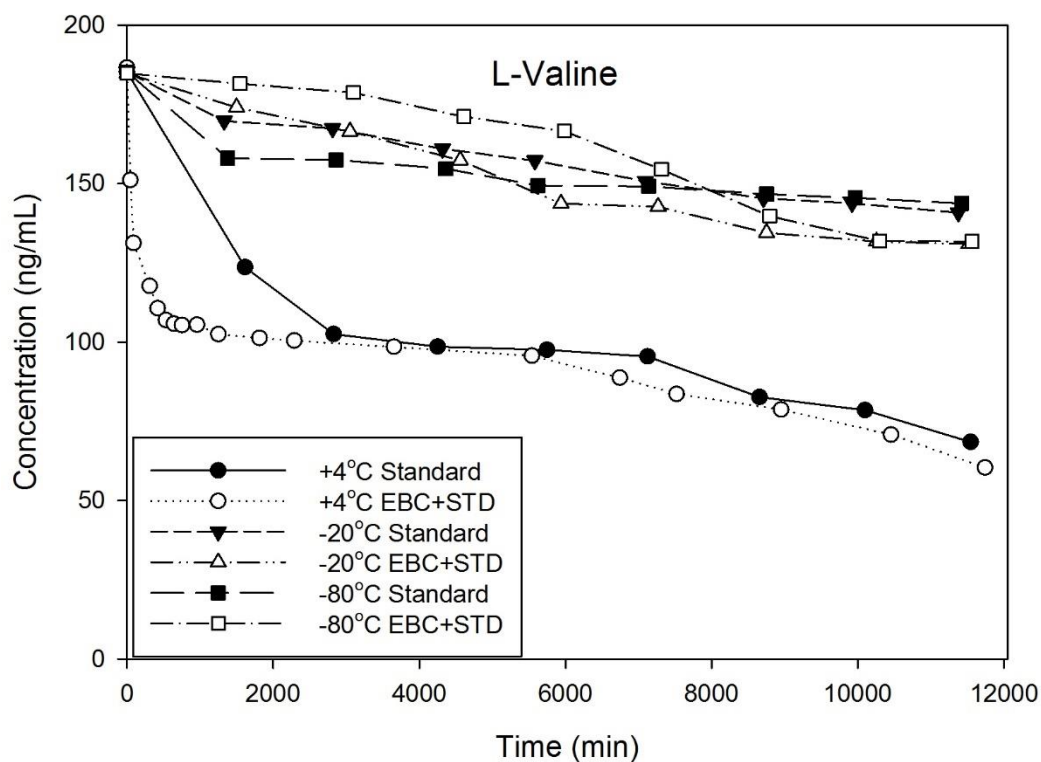


Figure 4.67 The effect of storage conditions on L-Valine stability

Figure 4.67 reveals that the concentration of L-Valine in +4°C EBC rapidly decreases within the first 24 hours. The L-Valine standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of L-Valine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of L-Valine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

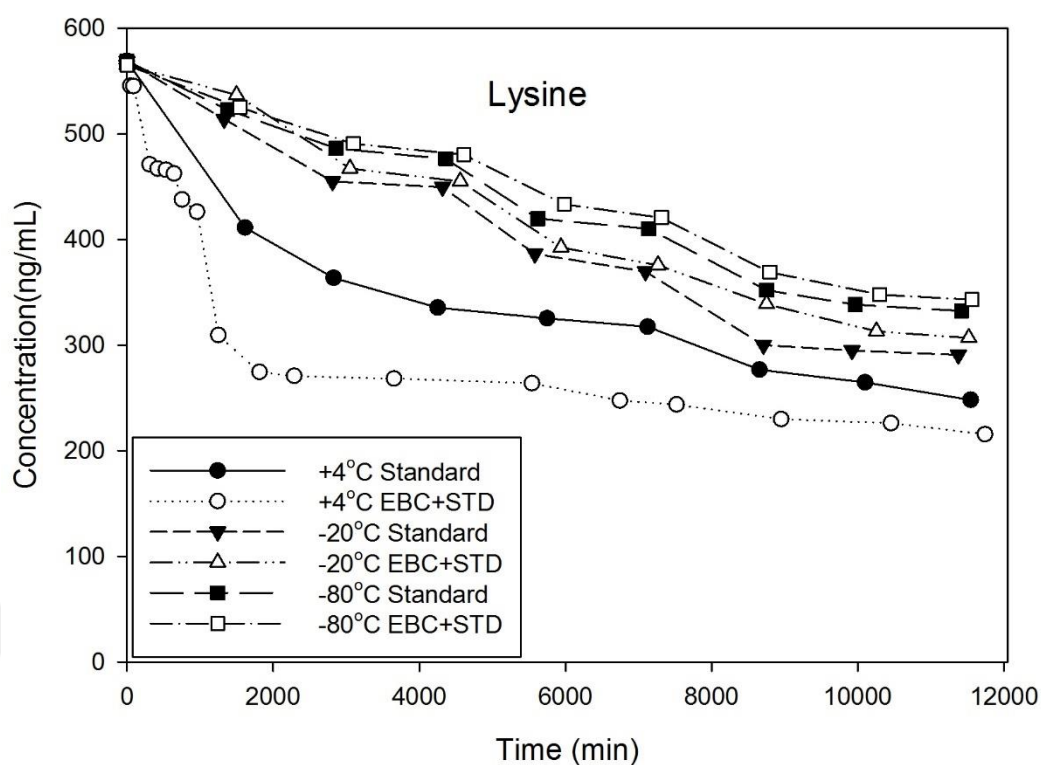


Figure 4.68 The effect of storage conditions on Lysine stability

Figure 4.68 reveals that the concentration of Lysine in +4°C EBC rapidly decreases within the first 24 hours. The Lysine standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Lysine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Lysine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

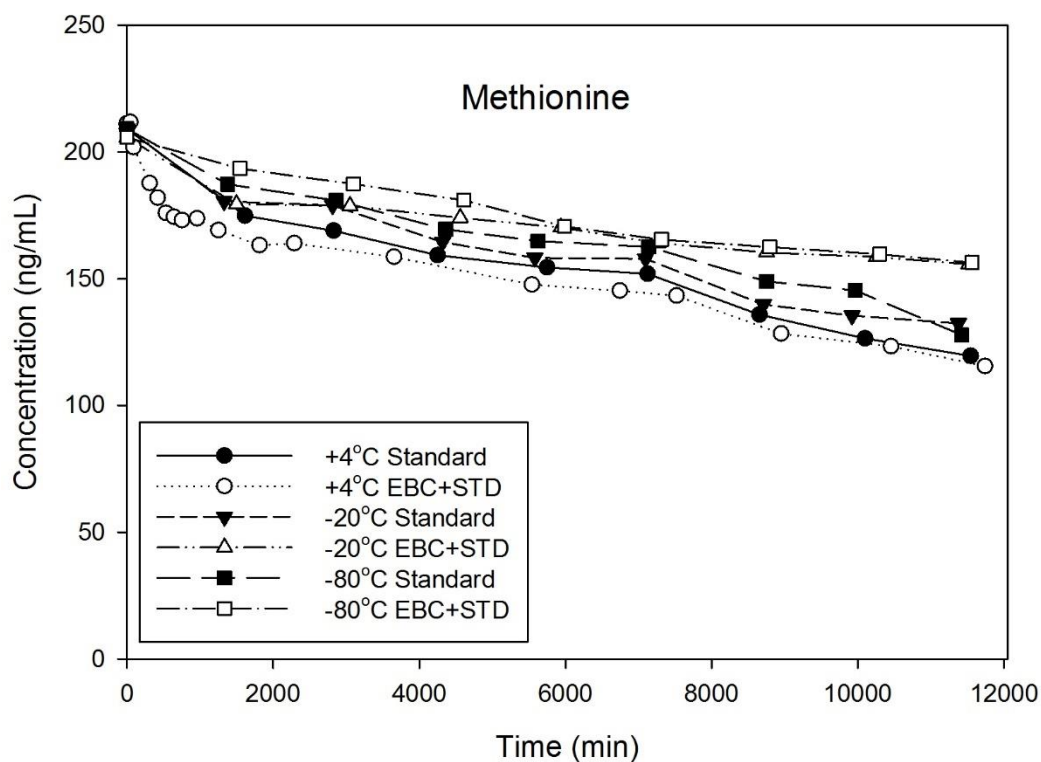


Figure 4.69 The effect of storage conditions on Methionine stability

Figure 4.69 reveals that the concentration of Methionine in +4°C EBC rapidly decreases within the first 24 hours. The Methionine standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Methionine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Methionine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

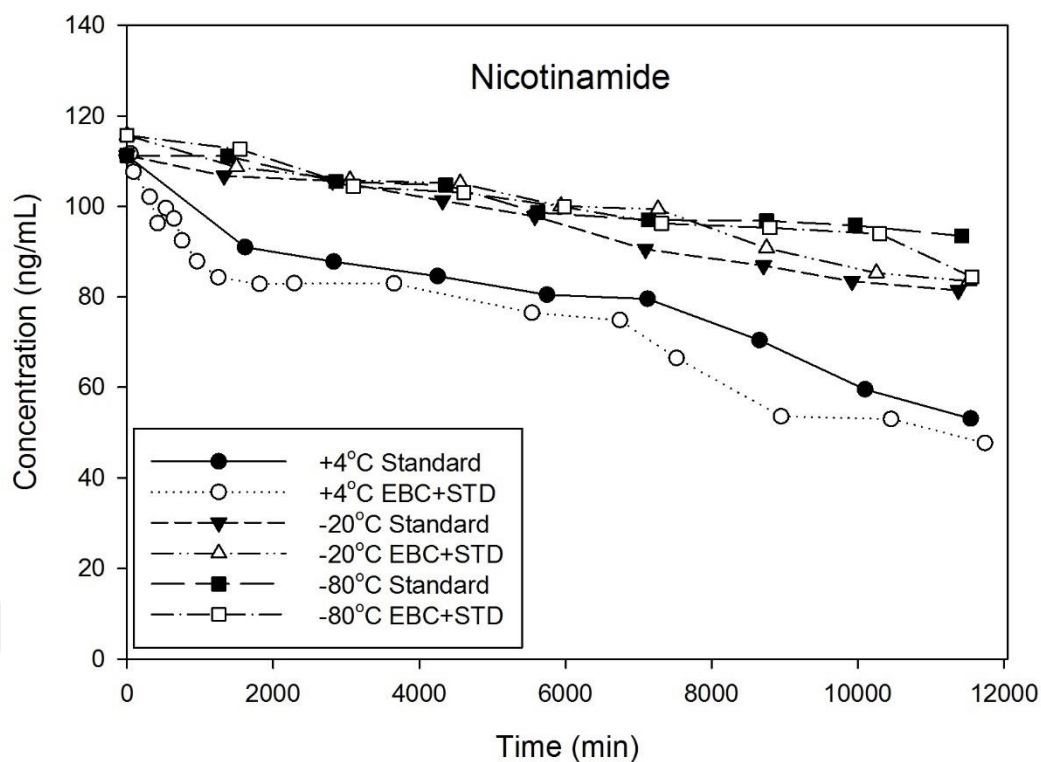


Figure 4.70 The effect of storage conditions on Nicotinamide stability

Figure 4.70 reveals that the concentration of Nicotinamide in +4°C EBC rapidly decreases within the first 24 hours. The Nicotinamide standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Nicotinamide in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Nicotinamide standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

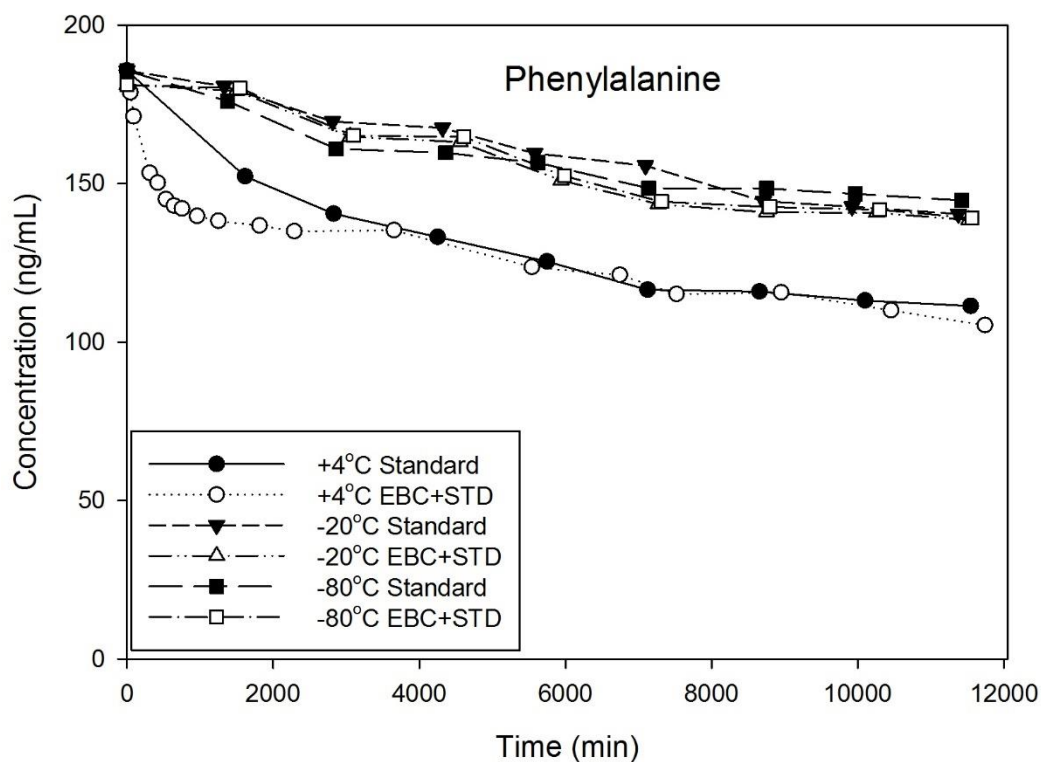


Figure 4.71 The effect of storage conditions on Phenylalanine stability

Figure 4.71 reveals that the concentration of Phenylalanine in +4°C EBC rapidly decreases within the first 24 hours. The Phenylalanine standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Phenylalanine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Phenylalanine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

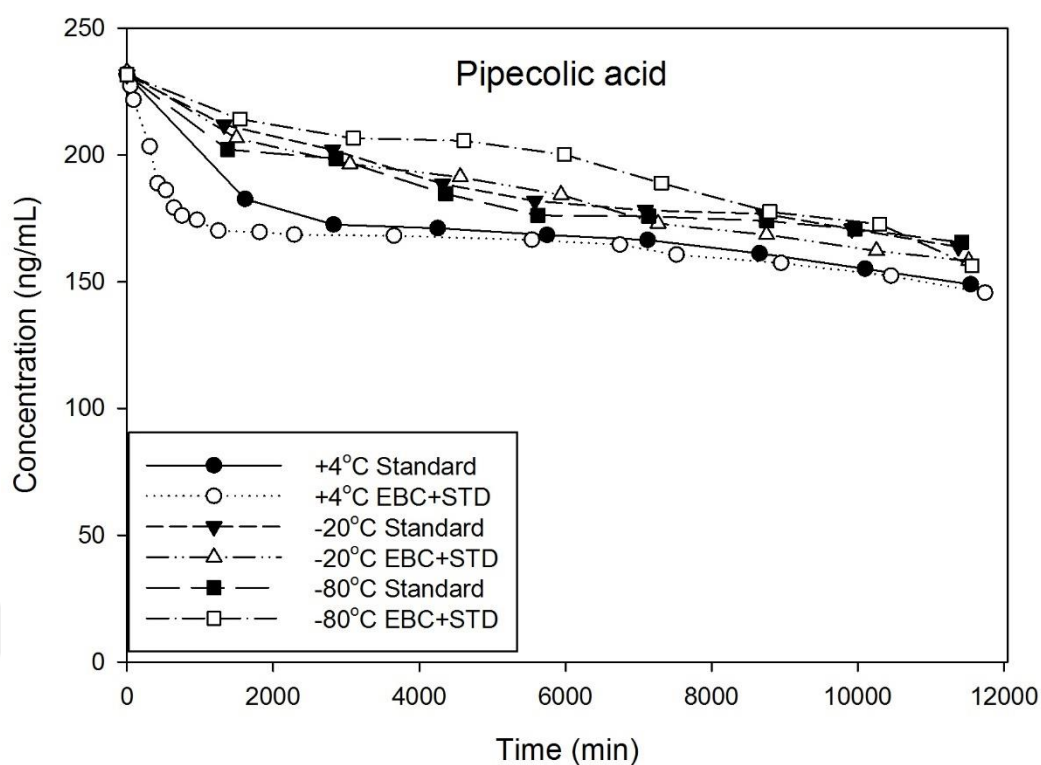


Figure 4.72 The effect of storage conditions on Pipelicolic acid stability

Figure 4.72 reveals that the concentration of Pipelicolic acid in +4°C EBC rapidly decreases within the first 24 hours. The Pipelicolic acid standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Pipelicolic acid in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Pipelicolic acid standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

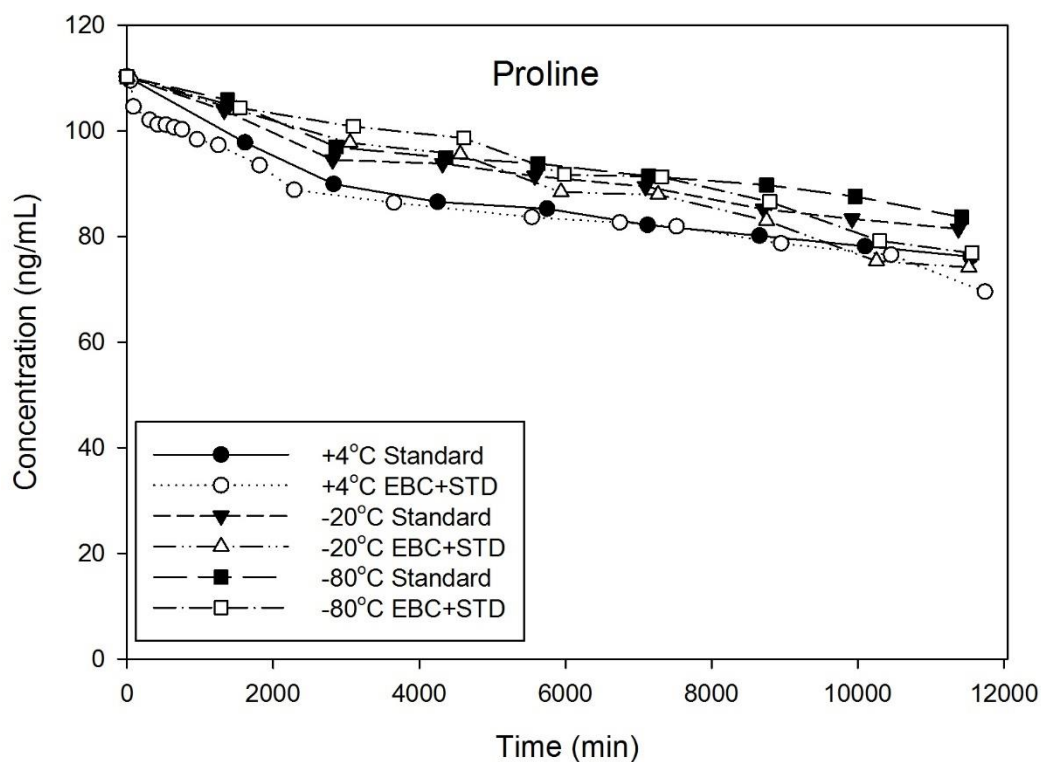


Figure 4.73 The effect of storage conditions on Proline stability

Figure 4.73 reveals that the concentration of Proline in +4°C EBC rapidly decreases within the first 24 hours. The Proline standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Proline in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Proline standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

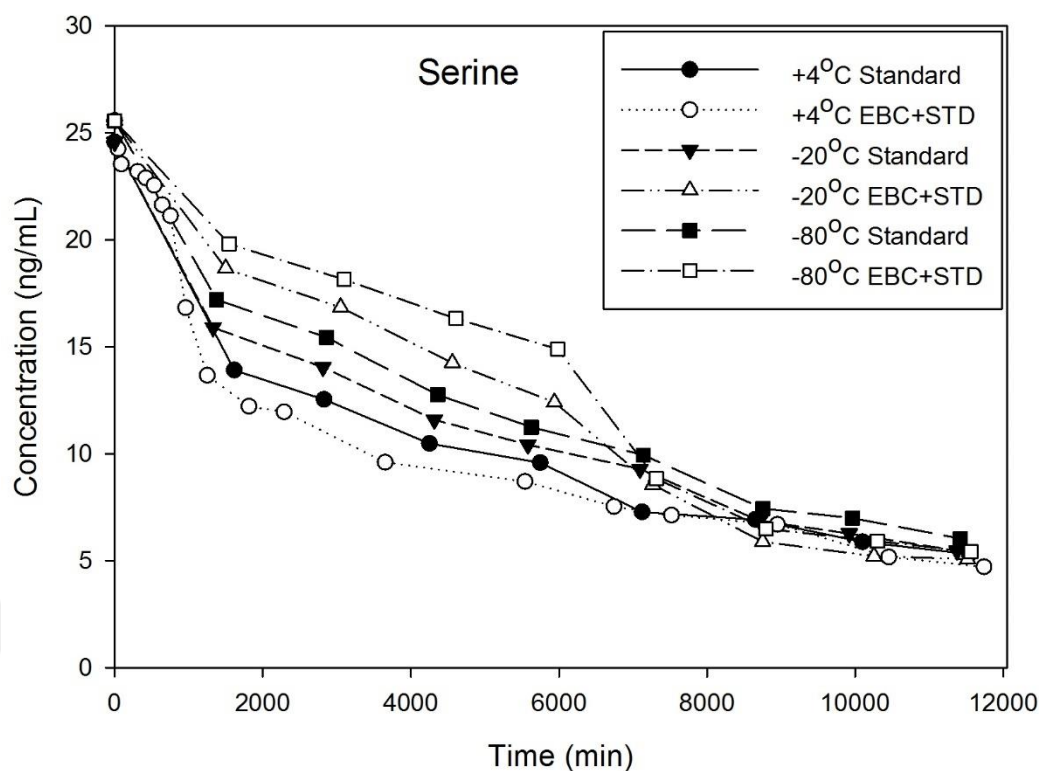


Figure 4.74 The effect of storage conditions on Serine stability

Figure 4.74 reveals that the concentration of Serine in +4°C EBC rapidly decreases within the first 24 hours. The Serine standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Serine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Serine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

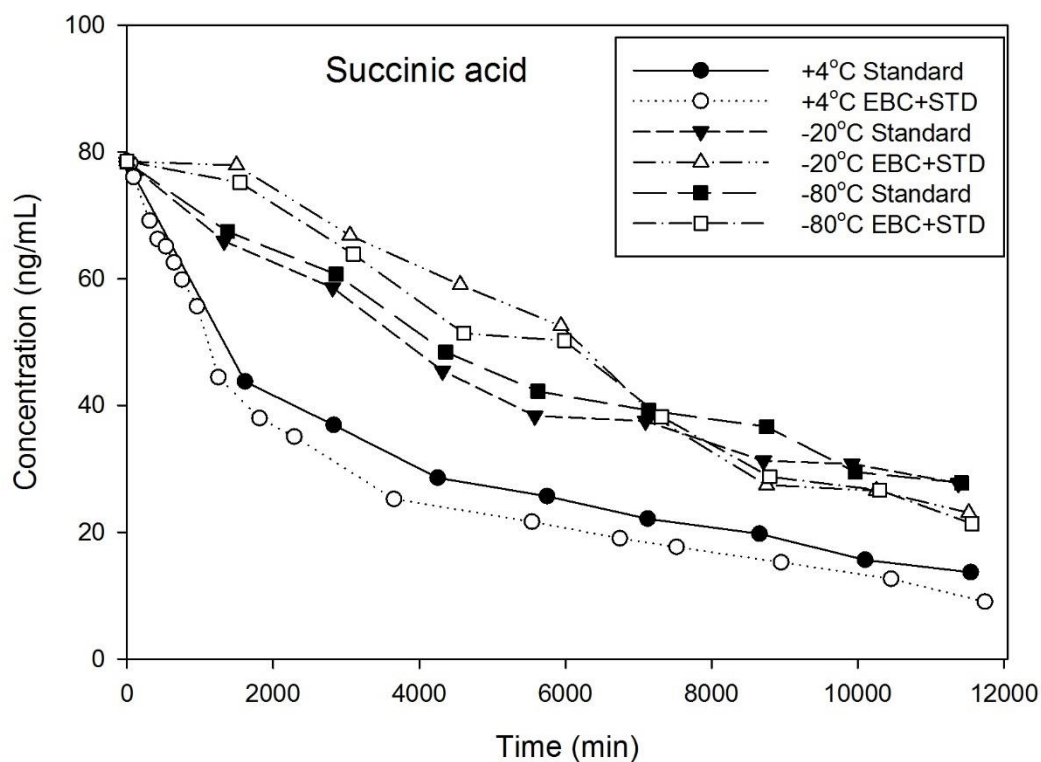


Figure 4.75 The effect of storage conditions on Succinic acid stability

Figure 4.75 reveals that the concentration of Succinic acid in +4°C EBC rapidly decreases within the first 24 hours. The Succinic acid standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Succinic acid in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Succinic acid standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

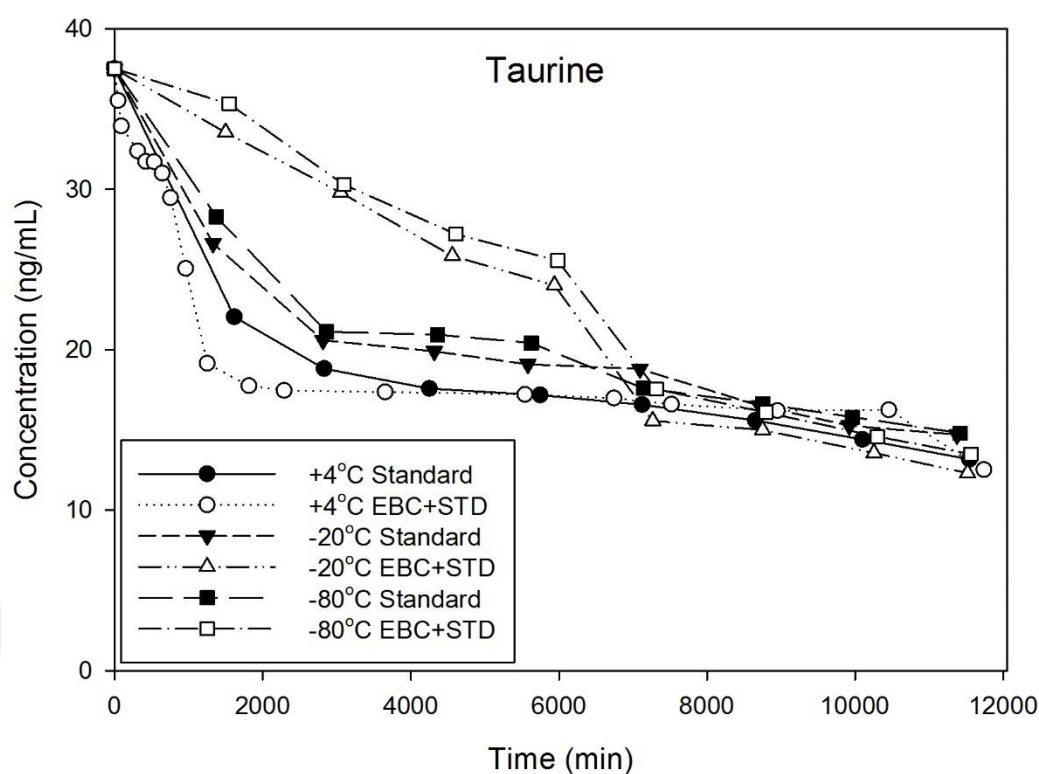


Figure 4.76 The effect of storage conditions on Taurine stability

Figure 4.76 reveals that the concentration of Taurine in +4°C EBC rapidly decreases within the first 24 hours. The Taurine standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Taurine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Taurine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

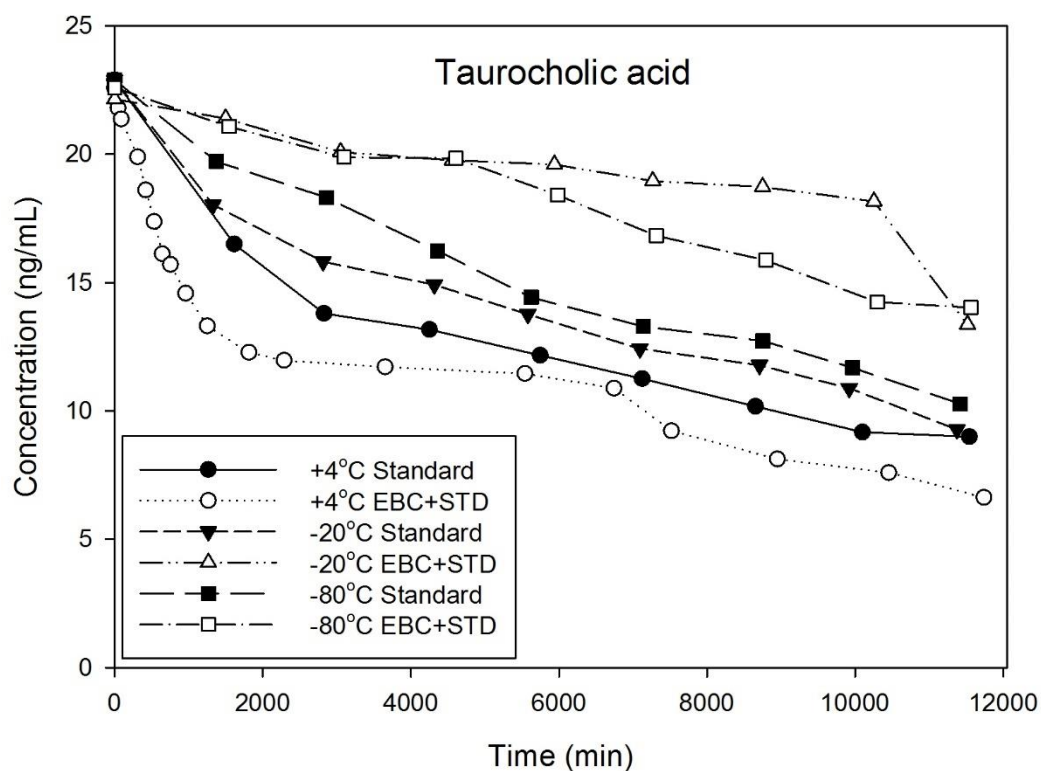


Figure 4.77 The effect of storage conditions on Taurocholic acid stability

Figure 4.77 reveals that the concentration of Taurocholic acid in +4°C EBC rapidly decreases within the first 24 hours. The Taurocholic acid standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Taurocholic acid in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Taurocholic acid standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

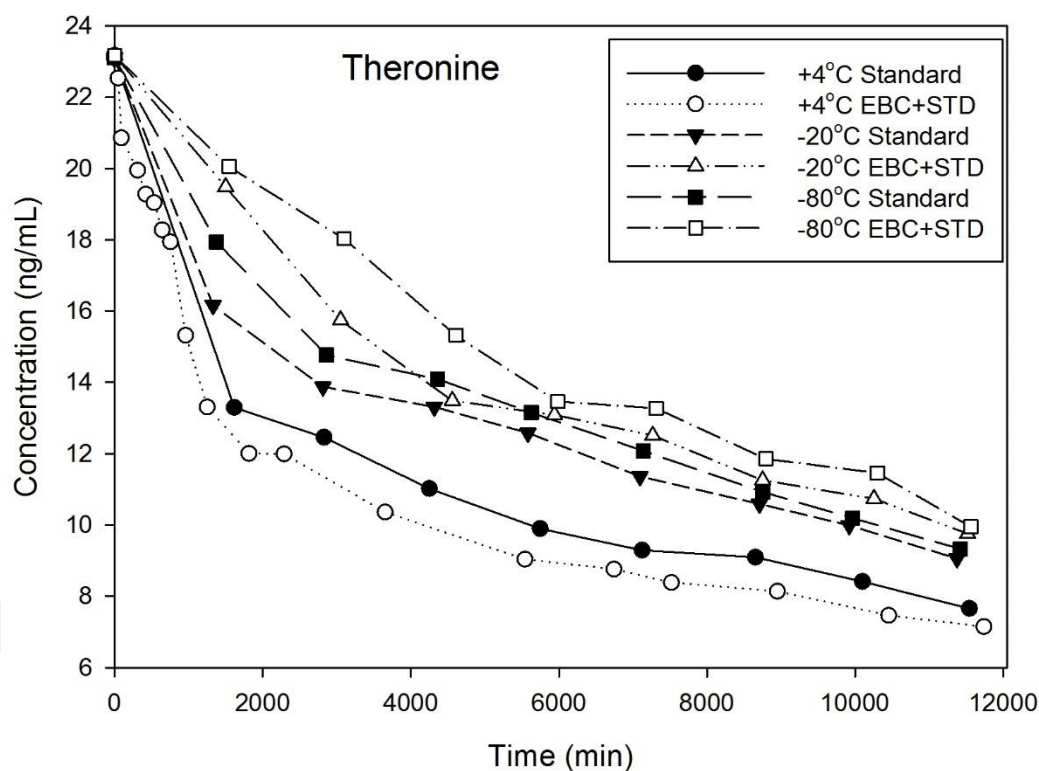


Figure 4.78 The effect of storage conditions on Theronine stability

Figure 4.78 reveals that the concentration of Threonine in +4°C EBC rapidly decreases within the first 24 hours. The Threonine standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Threonine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Threonine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

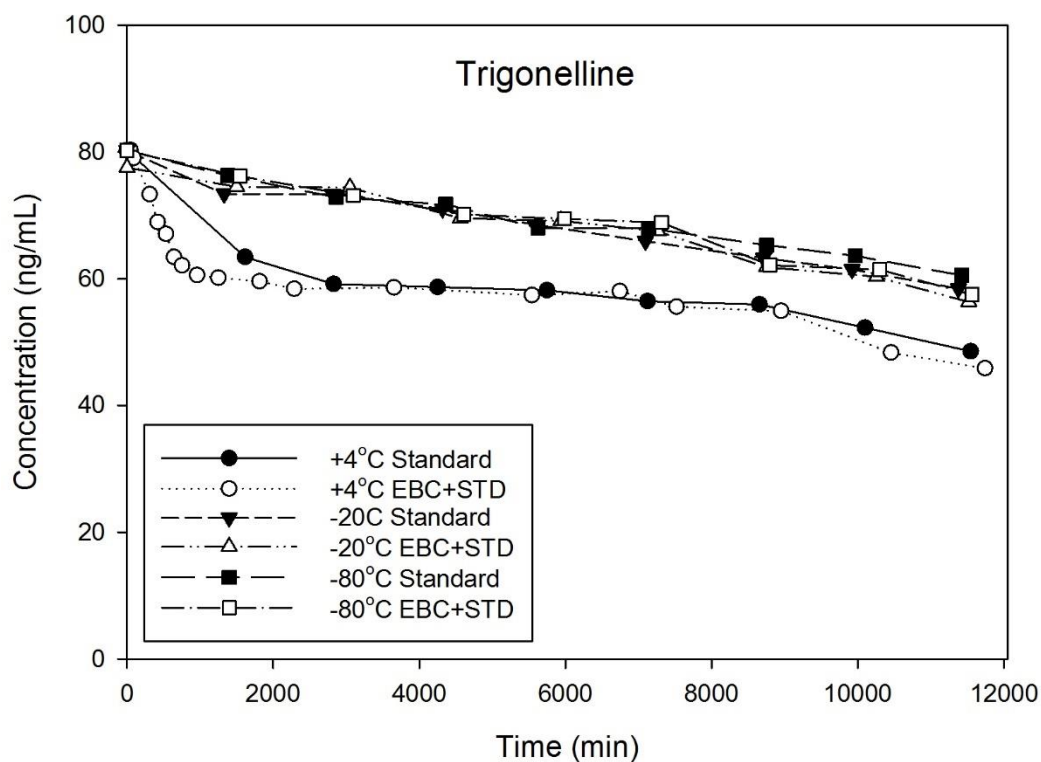


Figure 4.79 The effect of storage conditions on Trigonelline stability

Figure 4.79 reveals that the concentration of Trigonelline in +4°C EBC rapidly decreases within the first 24 hours. The Trigonelline standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Trigonelline in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Trigonelline standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

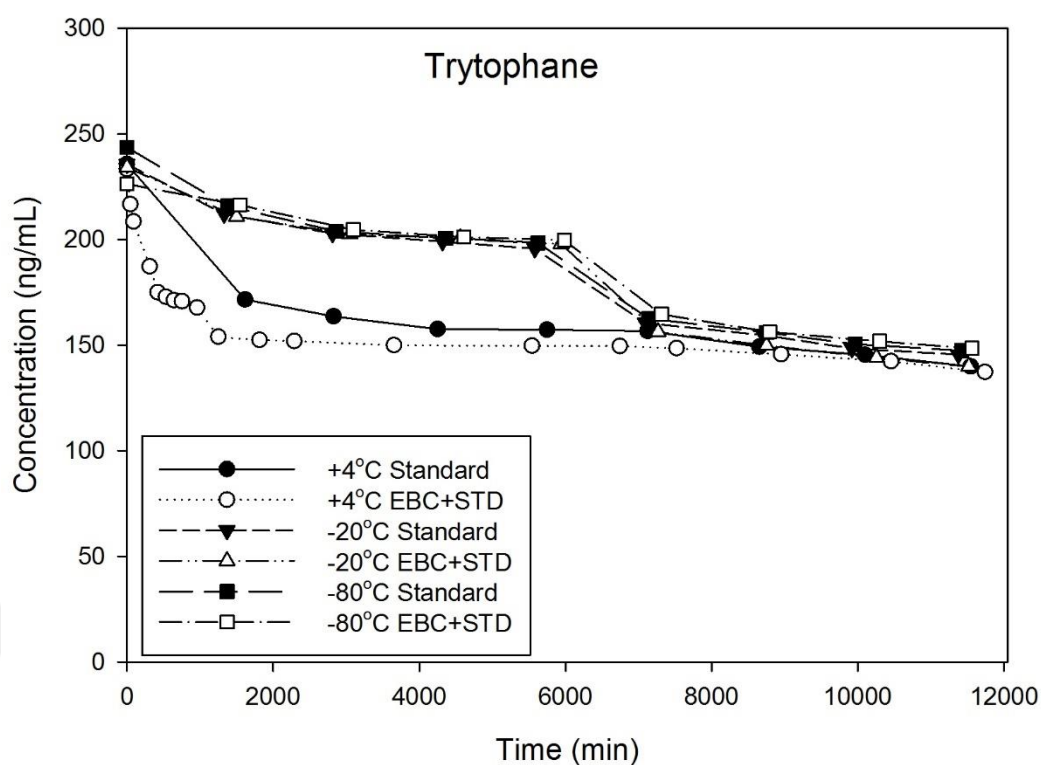


Figure 4.80 The effect of storage conditions on Tryptophane stability

Figure 4.80 reveals that the concentration of Tryptophane in +4°C EBC rapidly decreases within the first 24 hours. The Tryptophane standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Tryptophane in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC and pure waters at -20°C and -80°C. This indicates that the concentration of Tryptophane standard in both the EBC and pure water decreases more slowly and remains more stable in the EBC medium.

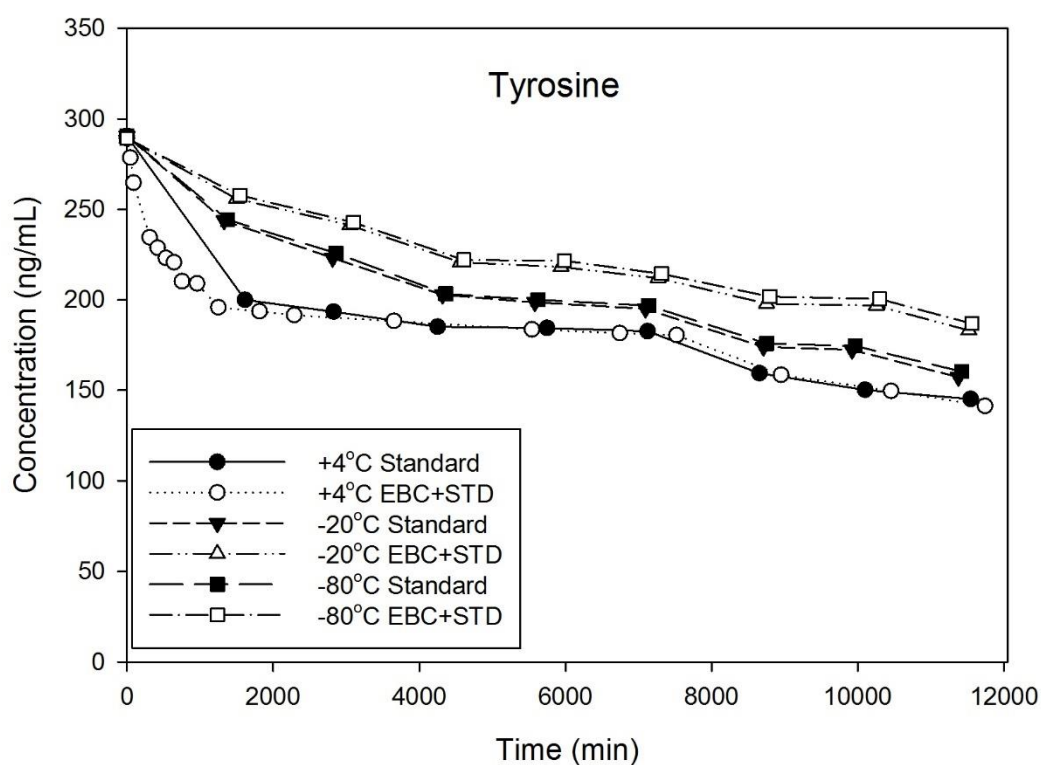


Figure 4.81 The effect of storage conditions on Tyrosine stability

Figure 4.81 reveals that the concentration of Tyrosine in +4°C EBC rapidly decreases within the first 24 hours. The Tyrosine standard prepared in a pure water at +4°C also shows a decrease at the same rate. When the concentrations of Tyrosine in the pure water at -20°C and -80°C are compared, a similar change is observed. In the -20°C EBC medium, the decomposition shows a concentration change parallel to the EBC sample stored at -80°C. The lowest concentration decrease is observed in the standard prepared in the EBC medium at -20°C and -80°C. This indicates that the concentration of Tyrosine standard in the EBC medium decreases more slowly and remains more stable in the EBC medium.

4.2.3. Application to Real Samples

EBC samples of the healthy and LC patients were directly injected into the LC-QTOF system at injection volumes of 5 μL and 10 μL , and the obtained chromatograms are presented in Figure 4.82, The peak area detected amino acids in patient's EBC were summarized in Table 4.8.

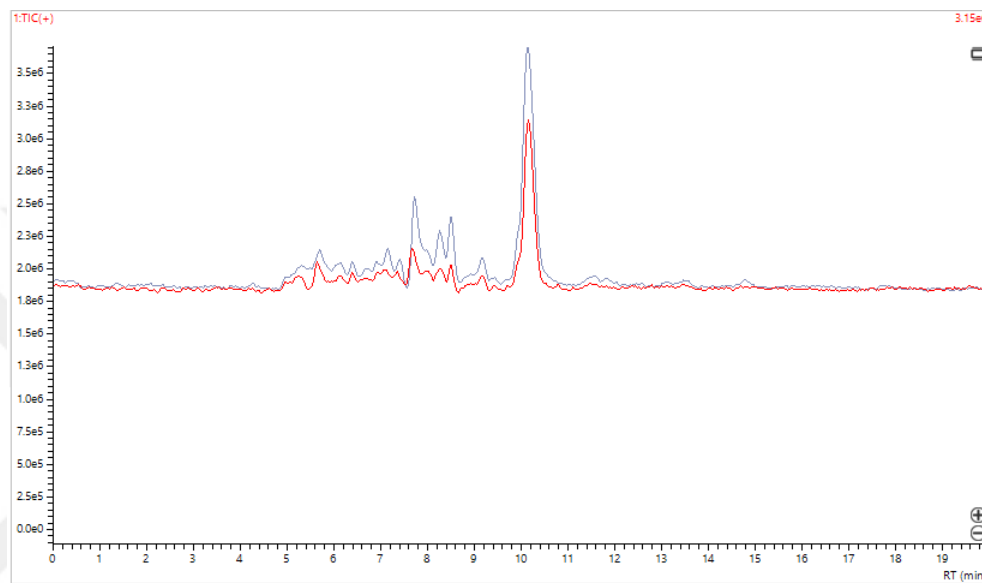


Figure 4.82 The sample LC-QTOF chromatogram of aminoacids and organic acids analysis obtained for EBC sample injection volume of 5 μL and 10 μL

Table 4.8 The effect of sample injection volume for LC-QTOF analysis of EBC

Analytes	5 μL Injection	10 μL Injection	Analytes	5 μL Injection	10 μL Injection
Lysine	4501	14407	L-carnitine	2929	10769
Arginine	15178	312684	Alanine	2632	3574
Proline	5439	13973	Histidine	7299	98015
L-valine	62557	135080	Glycine	18	120
Methionine	140	1661	Serine	262	1503
Tyrosine	2652	15210	Theronine	69	464
Leucine	7685	29750	Aspartic acid	1640	1927
Phenylalanine	3654	22450	Glutamic acid	36	84
Nicotinamide	354	1652	Hypoxantine	1921	740
Creatine	2371	12616	Huppirc acid	1240	576
Trigonelline	2806	7592	Taurine	663	366
Pipecolic acid	896	3312	Succinic acid	14415	13936
Betaine	4750	15784	Glycolic acid	4969	3644
Tryptophane	196	1295	Vaniline	1878	1798
Sarcosine	87362	176662	Taurocholic acid	296	123

When 5 μ L of EBC sample injected signal intensity of the most analytes were below the detection limit. To increase the accuracies of the analysis results 10 μ L sample injection volume was selected for further experiment. Higher injection volumes were also not preferred to prevent system contamination. In the experiments, amino acid and organic acid composition of 19 healthy subjects and 30 lung cancer patients were analyzed. The demographic features of studied group were summarized in Table 4.9.

Table 4.9 Demographic properties of studied groups

<u>Features</u>	<u>Cancer Patients</u>	<u>Healthy Subjects</u>
Gender	23 Male	8 Male
	7 Female	11 Female
Age	45-81	25-65
Smoker	13 Smoker	3 Smoker
	12 Ex-smoker	3 Ex-smoker
	3-Non-smoker	13 Non-smoker
	2 Passive Smoker	---
<u>Total</u>	30	19

The identified analytes and their concentration values from the analysis of the subjects are presented in Appendix-3 (Page 189). The statistical analysis of the results was performed by Mann-Whitney U test and the results were presented in Table 4.10. According to the results, no statistically significant difference can be detected within the 95% confidence interval between the amino acid and organic acid concentration medians detected in EBC ($p > 0.05$). Based on the results of the AUC-ROC analysis, none of the measured amino acids and organic acids were identified as a biomarker distinguishing between the patient and healthy groups (AUC-ROC p values > 0.05). The results were also presented in Table 4.11.

Table 4.10 The statistical summary of Mann- Whitney U test

Compound	Healthy Subjects		Lung Cancer Patients		Test statistics; p-value
	$\bar{x} \pm SD$	$\tilde{x} [min - max]$	$\bar{x} \pm SD$	$\tilde{x} [min - max]$	
Lysine	8.1767±8.5642	5.7188 [0-28.0313]	7.4198±9.6711	4.2034 [0-43.9832]	U=266.5 p=0.704
Arginine	1.6133±2.6568	0.4325 [0-9.493]	0.8459±1.4354	0.1244 [0-6.0086]	U=253 p=0.494
Proline	1.5923±1.4106	1.0168 [0-4.8997]	1.5192±1.4986	0.9433 [0-7.6714]	U=280.5 p=0.926
L-valine	0.0637±0.2779	0 [0-1.2112]	0.0784±0.4295	0 [0-2.3523]	U=280 p=0.765
Methionine	0.148±0.5051	0 [0-2.1323]	0.0634±0.2242	0 [0-1.0229]	U=282.5 p=0.922
Tyrosine	4.5621±4.3783	4.486 [0-15.3739]	4.7032±5.8226	2.0209 [0-26.797]	U=277 p=0.868
Leucine	7.7973±6.6255	7.799 [0.1383-21.4526]	7.8753±8.2017	4.8112 [0.2288-39.0167]	U=284 p=0.984
Phenylalanine	2.0275±1.865	1.6741 [0-5.8552]	2.8186±4.5753	1.2439 [0-23.6331]	U=277.5 p=0.878
Trigonelline	0.1188±0.0411	0.1173 [0.0663-0.2257]	0.1356±0.0725	0.1131 [0.0738-0.3749]	U=271 p=0.774
Pipecolic acid	1.3724±0.0747	1.3495 [1.3009-1.6065]	1.4781±0.2678	1.3824 [1.2903-2.5954]	U=210 p=0.124
Tryptophane	0.0119±0.0363	0 [0-0.1344]	0±0	0 [0-0]	U=255 p=0.073
Histidine	9.4305±11.0977	4.4411 [0.1451-36.1115]	6.731±8.2575	2.4518 [0.08-27.8134]	U=250 p=0.473
Glycine	1.6415±1.4315	1.5125 [0-4.7551]	2.5114±2.2469	1.8707 [0-10.0716]	U=208.5 p=0.156
Serine	0.2313±1.0046	0 [0-4.3798]	1.2691±3.3486	0 [0-13.7665]	U=255 p=0.339
Theronine	0±0	0 [0-0]	0.1233±0.4539	0 [0-2.0224]	U=256.5 p=0.159
Aspartic acid	9.4399±1.3299	8.9506 [8.1756-13.1707]	10.0831±2.3486	9.0865 [8.17-15.6897]	U=263 p=0.652
Glutamic acid	2.6117±3.5148	0 [0-7.3951]	2.7881±3.791	0 [0-10.0696]	U=280 p=0.906
Taurine	0.4869±0.1162	0.468 [0.3654-0.8575]	0.5205±0.235	0.4551 [0.3674-1.6126]	U=275 p=0.837
Succinic acid	5.9906±5.0887	4.4903 [3.7885-26.4678]	6.8536±5.0105	5.1943 [3.8129-28.2803]	U=217 p=0.163

Table 4.11 The statistical summary of AU-ROC evaluations

Compound	Area	Std. Error ^a	p-value	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Lysine	0.468	0.086	0.704	0.300	0.636
Arginine	0.444	0.088	0.511	0.271	0.616
Proline	0.492	0.091	0.926	0.314	0.670
L-valine	0.491	0.086	0.918	0.323	0.659
Methionine	0.496	0.086	0.959	0.328	0.664
Tyrosine	0.486	0.086	0.870	0.318	0.654
Leucine	0.498	0.089	0.984	0.324	0.673
Phenylalanine	0.513	0.088	0.878	0.341	0.685
Trigonelline	0.525	0.088	0.774	0.352	0.697
Pipecolic acid	0.632	0.080	0.124	0.475	0.788
Tryptophane	0.447	0.087	0.538	0.277	0.617
Histidine	0.439	0.088	0.473	0.267	0.610
Glycine	0.622	0.084	0.158	0.457	0.786
Serine	0.553	0.083	0.538	0.389	0.716
Theronine	0.550	0.083	0.559	0.387	0.713
Aspartic acid	0.539	0.084	0.652	0.373	0.704
Glutamic acid	0.509	0.085	0.918	0.342	0.675
Taurine	0.482	0.085	0.837	0.317	0.648
Succinic acid	0.619	0.083	0.163	0.457	0.781
Glycolic acid	0.491	0.086	0.918	0.323	0.659



5. DISCUSSION

Within the scope of this thesis, the analysis of some volatile and non-volatile substances in EBC samples has been conducted by using different methods. The collection of EBC samples was carried out by using a laboratory-made EBC collection device. For this purpose, subjects were instructed to breathe fresh air and after 15 minutes of sampling, approximately ~ 2.5 mL of EBC samples were collected. The collected condensate samples were stored at -80°C deep freezer until they are analyzed.

Some volatile compounds in EBC samples were analyzed separately using LC-QTOF and GC-MS methods. Compounds containing carbonyl groups could be detected in higher numbers with the LC-QTOF device. However, due to high noise, consistency could not be achieved between the GC-MS and LC-QTOF analysis results.

Studies have been carried out on some amino acids and organic acids found in EBC. The analysis conditions of these compounds were investigated, and it was determined that most amino acids decomposed rapidly at 4°C in the EBC medium. Although there is a decrease in concentration in some analytes, it has been observed that most compounds are stable under -80°C. For this reason, it was decided that these compounds should be stored at least at -80°C. For some compounds, which still showed a tendency to decompose at -80°C, storing them at lower temperatures, such as -150°C, is considered to eliminate this deterioration.

Although breath analysis remains a promising non-invasive technique, there is still a long way to go. Instead of focusing on the analysis of different compounds with any method, the emphasis is on factors affecting the analysis of analytes in real breath samples. This focus will make breath analysis one of the most important instruments used in the future for disease diagnosis and treatment.



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05/02/2024

Signature

CV-CURRICULUM VITAE

Name: Korcan

Surname: KORBA

Nationality: Republic of Türkiye

Birthplace:

Date of Birth:

Address: Ege University, Faculty of Engineering, Department of Chemical
Engineering, 35040, Bornova, Izmir/TÜRKİYE

Education: Bachelor's, Ege University, Chemistry, 2009.

Master's degree, Ege University, Analytical Chemistry, 2011.



APPENDIX-1 GC-MS VOC ANALYSIS RESULTS

Patient Code	Isoprene	Benzene	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Toluene	Nonane	Styrene	Tetradecane	Hexadecane
C001	<LOD	142.13	0.56	1.49	<LOD	12.90	<LOD	23.08	<LOD	151.06	0.46	6.78	52.93	696.37
C002	<LOD	364.99	7.80	4.72	10.78	9.83	25.41	64.96	5.31	483.92	0.72	73.13	48.97	526.32
C003	<LOD	446.29	7.37	4.05	<LOD	9.42	4.42	29.37	10.63	539.59	<LOD	52.98	65.27	608.89
C004	<LOD	519.86	3.28	14.75	23.31	13.19	44.40	40.97	9.20	573.72	0.69	38.86	34.12	494.00
C005	<LOD	592.03	11.02	7.39	19.27	22.32	84.58	47.64	55.56	499.84	0.31	56.06	130.43	1520.58

APPENDIX-1 (Continue)

Patient Code	Isoprene	Benzene	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Toluene	Nonane	Styrene	Tetradecane	Hexadecane
C008	<LOD	286.88	5.28	6.15	16.69	12.66	5.54	25.05	17.94	237.79	<LOD	11.50	37.48	443.63
C010	<LOD	195.93	3.93	2.30	<LOD	1.05	<LOD	19.85	<LOD	169.42	0.24	7.60	51.78	620.64
C011	<LOD	1032.43	2.72	7.73	2.32	12.88	18.48	23.75	18.42	1088.65	<LOD	36.69	52.26	535.30
C012	<LOD	213.73	59.72	<LOD	17.56	1.59	<LOD	3.51	3.18	116.51	<LOD	6.88	51.38	644.06
C013	<LOD	68.47	4.83	4.61	<LOD	14.89	1.75	19.80	3.18	61.34	<LOD	14.41	41.45	412.14

APPENDIX-1 (Continue)

Patient Code	Isoprene	Benzene	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Toluene	Nonane	Styrene	Tetradecane	Hexadecane
C014	<LOD	151.25	17.31	17.52	2.93	14.05	<LOD	42.11	<LOD	158.63	1.07	8.78	26.26	266.08
C015	<LOD	115.74	12.21	4.76	15.05	11.06	<LOD	25.31	15.09	81.03	<LOD	8.02	82.47	1017.40
C016	<LOD	838.64	20.70	9.57	7.35	19.62	294.67	42.42	3.60	1021.53	1.13	70.23	82.47	611.95
C017	10.50	32.17	5.20	9.57	4.70	8.14	4.16	1.08	<LOD	12.88	<LOD	2.23	6.45	7.44
C018	1.80	4492.46	10.70	13.22	22.50	12.38	17.77	55.69	<LOD	3338.57	0.30	106.68	81.46	80.60

APPENDIX-1 (Continue)

Patient Code	Isoprene	Benzene	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Toluene	Nonane	Styrene	Tetradecane	Hexadecane
C019	<LOD	7272.18	10.50	17.40	4.45	24.22	162.33	15.75	20.87	7736.49	2.64	145.73	74.03	132.02
C020	8.50	47.00	6.99	8.75	13.38	<LOD	<LOD	<LOD	<LOD	8.35	<LOD	2.12	23.30	24.00
C022	2.71	868.01	14.43	11.55	10.71	15.29	21.57	<LOD	6.06	806.98	1.28	41.53	67.12	70.45
C023	0.77	6882.90	7.25	11.07	1.35	21.80	30.08	7.15	17.88	2919.55	2.55	23.52	69.07	117.69
C024	2.04	3630.53	4.15	16.60	0.97	10.82	206.00	4.51	29.38	4618.51	1.87	90.58	61.78	58.68

APPENDIX-1 (Continue)

Patient Code	Isoprene	Benzene	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Toluene	Nonane	Styrene	Tetradecane	Hexadecane
C025	0.89	14.91	3.63	5.77	7.83	7.64	7.63	<LOD	<LOD	13.08	<LOD	3.23	16.07	19.03
C026	2.84	5897.65	17.10	251.26	13.65	36.79	945.47	6.94	<LOD	6848.37	<LOD	0.46	40.06	431.47
C027	4.07	8337.56	3.77	28.49	1.30	16.78	42.63	1.59	14.81	5594.79	1.60	153.98	37.95	38.48
C030	<LOD	11952.10	60.32	493.57	17.76	55.14	2376.23	61.21	28.85	1229.41	2.34	44.62	77.60	1116.38
C031	<LOD	14122.70	11.57	65.16	5.18	22.52	275.58	<LOD	6.71	18113.68	0.41	1149.79	120.07	51.55

APPENDIX-1 (Continue)

Patient Code	Isoprene	Benzene	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Toluene	Nonane	Styrene	Tetradecane	Hexadecane
C032	<LOD	3432.72	<LOD	2.07	42.21	11.31	1706.80	24.51	<LOD	15.37	<LOD	3.97	54.13	687.46
C033	1.05	<LOD	3.71	5.90	11.45	5.12	3.08	<LOD	<LOD	361.65	2.12	8.04	<LOD	14.40
C034	<LOD	425.60	22.42	35.70	12.28	24.15	908.51	167.56	23.61	1150.85	<LOD	1.80	96.99	754.13
C035	0.53	8.99	1.26	0.37	0.29	0.85	<LOD	<LOD	<LOD	7.53	<LOD	2.82	<LOD	<LOD
C036	<LOD	13358.36	46.86	383.52	11.92	36.62	1364.18	42.77	4.03	15787.52	<LOD	0.25	48.21	371.14

APPENDIX-1 (Continue)

Patient Code	Isoprene	Benzene	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Toluene	Nonane	Styrene	Tetradecane	Hexadecane
C037	<LOD	3.96	11.10	22.78	2.20	3.13	7.90	<LOD	<LOD	9.52	<LOD	0.81	4.91	2.71
C039	<LOD	906.75	5.40	5.41	3.90	17.09	23.93	22.50	26.76	1173.48	2.17	55.37	75.21	179.04
C040	1.86	722.40	14.92	9.69	9.06	18.60	25.90	25.14	10.25	939.79	3.59	28.06	57.42	61.05
C041	3.91	889.81	11.44	8.64	0.45	11.14	16.15	3.85	21.56	801.07	2.52	20.96	57.00	62.04
C042	<LOD	320.45	N. D.	10.39	5.68	24.64	33.80	4.37	6.69	265.35	2.71	8.36	56.10	<LOD

APPENDIX-1 (Continue)

Patient Code	Isoprene	Benzene	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Toluene	Nonane	Styrene	Tetradecane	Hexadecane
C043	<LOD	738.45	33.12	14.78	8.83	25.53	34.96	50.93	37.85	989.91	16.49	53.47	76.40	137.75
C044	3.13	635.20	17.17	28.54	7.61	24.10	33.09	6.64	23.04	354.69	5.67	9.10	58.23	50.13
C045	1.90	375.48	11.92	8.08	5.01	20.90	<LOD	<LOD	28.51	320.68	0.58	15.18	148.46	65.04
C046	2.31	802.80	10.05	7.61	2.97	15.78	<LOD	<LOD	12.03	726.33	0.53	31.39	133.89	13.91
C047	1.52	3628.15	13.66	19.77	3.40	13.79	8.47	<LOD	18.11	4753.62	0.75	97.43	141.15	6.80

APPENDIX-1 (Continue)

Patient Code	Isoprene	Benzene	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Toluene	Nonane	Styrene	Tetradecane	Hexadecane
C048	1.94	224.45	0.28	3.59	<LOD	10.68	15.56	8.38	5.15	424.79	235.22	50.35	180.68	169.64
C049	<LOD	608.51	7.27	6.34	3.45	19.69	27.33	12.31	22.72	836.27	2.07	42.74	66.17	137.56
C050	1.49	170.89	8.20	5.67	<LOD	11.95	17.21	13.43	40.27	339.33	0.51	40.50	198.72	216.87
C051	<LOD	333.63	N. D.	21.79	4.65	19.46	27.03	9.44	<LOD	697.91	0.93	36.83	155.98	81.21
C052	5.00	259.75	N. D.	12.77	4.75	17.47	24.43	32.76	2.98	404.89	5.37	34.33	146.85	53.02

APPENDIX-1 (Continue)

Patient Code	Isoprene	Benzene	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Toluene	Nonane	Styrene	Tetradecane	Hexadecane
C053	0.98	3358.03	24.41	55.56	7.39	22.39	61.42	35.21	17.31	9371.17	1.04	868.51	92.56	101.07
C054	1.84	351.28	20.48	11.65	2.72	14.37	20.38	23.98	17.02	837.14	<LOD	96.04	74.08	924.84
C055	2.75	18.93	691.34	585.93	120.07	59.73	79.65	21.36	<LOD	61.90	1.33	6.49	99.92	151.55
C056	4.69	11.83	27.30	9.07	62.17	20.33	28.17	23.04	21.56	26.97	0.31	4.87	116.60	640.66
C058	3.53	10.22	14.00	14.65	5.71	18.82	26.19	21.50	9.87	74.83	1.78	7.35	110.07	286.63

APPENDIX-1 (Continue)

Patient Code	Isoprene	Benzene	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal	Toluene	Nonane	Styrene	Tetradecane	Hexadecane
C060	4.52	13.69	16.56	7.61	4.36	19.34	26.87	18.07	6.83	29.17	3.53	4.76	85.81	1002.54
C061	0.76	11.98	19.61	15.14	7.27	16.23	22.80	13.05	<LOD	29.41	1.48	6.07	61.35	662.56

***<LOD: Below limit of detection**

APPENDIX-2 (Continue)

Patient Code	Butanal	Pentanal	Hexanal	Heptanal	Nonanal	3-Hidroxy-2-Butanon	Malondialdehyde
C011	134.5	425	< LOD	410	26	10.3	2
C012	51.9	428.5	< LOD	< LOD	28.7	241	0.8
C013	< LOD	< LOD	< LOD	< LOD	< LOD	16.9	< LOD
C014	< LOD	< LOD	< LOD	< LOD	< LOD	7.9	2.8
C018	< LOD	431	8.9	201	76.1	39.9	1.4
C019	32.2	515	32.2	500	505	6.1	7.3

APPENDIX-2 (Continue)

Patient Code	Butanal	Pentanal	Hexanal	Heptanal	Nonanal	3-Hidroxy-2-Butanon	Malondialdehyde
C022	13.9	493	13.9	403.3	132.5	29.3	1.7
C023	16.7	498	16.7	502	132.9	6	3.7
C026	112.1	533	< LOD	387	62.6	8.6	< LOD
C030	175	529	< LOD	513.8	76.9	8.1	1
C031	46.5	594	46.5	524	154.1	5.6	4
C032	119.1	556	< LOD	400	30.2	199.2	< LOD

APPENDIX-2 (Continue)

Patient Code	Butanal	Pentanal	Hexanal	Heptanal	Nonanal	3-Hidroxy-2-Butanon	Malondialdehyde
C033	66.5	306.3	< LOD	129.5	< LOD	10.9	< LOD
C034	103.9	472	< LOD	368	20.3	< LOD	0.9
C035	103.1	552	< LOD	269	< LOD	5.2	< LOD
C036	45.9	531	< LOD	172.9	11.5	10.5	< LOD
C039	< LOD	154.1	< LOD	286.5	16.9	17.5	1.6
C040	< LOD	31.9	< LOD	< LOD	8.9	51.2	< LOD

APPENDIX-2 (Continue)

Patient Code	Butanal	Pentanal	Hexanal	Heptanal	Nonanal	3-Hidroxy-2-Butanon	Malondialdehyde
C041	< LOD	13.8	< LOD	146.8	98.9	40.7	1.2
C042	< LOD	< LOD	< LOD	< LOD	< LOD	30.9	< LOD
C043	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	34.1
C044	< LOD	80.5	< LOD	447.4	192.1	6	0.9
C045	< LOD	< LOD	< LOD	526	398.1	118.9	< LOD
C046	< LOD	< LOD	< LOD	< LOD	279.5	46.2	< LOD

APPENDIX-2 (Continue)

Patient Code	Butanal	Pentanal	Hexanal	Heptanal	Nonanal	3-Hidroxy-2-Butanon	Malondialdehyde
C053	< LOD	2056.1	< LOD	< LOD	< LOD	46.8	< LOD
C054	< LOD	75.1	< LOD	< LOD	15.2	50.8	< LOD
C055	< LOD	104.4	8.7	< LOD	< LOD	52.6	2.2
C060	< LOD	15.5	< LOD	670.3	225.6	73	< LOD
C061	< LOD	< LOD	< LOD	< LOD	108.9	51.9	< LOD

***<LOD: Below limit of detection**

APPENDIX-3 LC-QTOF AMINOACIDS and ORGANIC ACIDS ANALYSIS RESULTS

Patient Code	C001	C002	C003	C004
Taurocholicacid	N.D.	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.	N.D.
Glycolicacid	N.D.	N.D.	N.D.	N.D.
Succinicacid	4.7951	6.8232	4.4521	5.4469
Taurine	0.578	0.4431	0.3674	0.4498
Hupiricacid	N.D.	N.D.	N.D.	N.D.
Hypoxantine	N.D.	N.D.	N.D.	N.D.
Glutamicacid	N.D.	N.D.	6.9647	6.9799
Asparticacid	8.8283	8.8479	8.2376	8.3042
Therinine	N.D.	N.D.	N.D.	N.D.
Serine	N.D.	N.D.	N.D.	N.D.
Glycine	1.5152	2.1851	N.D.	1.6109
Histidine	4.0095	1.1645	0.198	1.5079
Alanine	N.D.	N.D.	N.D.	N.D.
Lcarnitine	N.D.	N.D.	N.D.	N.D.
Tryptophane	N.D.	N.D.	N.D.	N.D.
Betaine	N.D.	N.D.	N.D.	N.D.
Pipecolicacid	1.3749	1.3549	1.5944	1.3775
Trigonelline	0.1298	0.1041	0.0972	0.1724
Creatine	N.D.	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.	N.D.
Phenylalanine	0.6148	0.3412	N.D.	1.4022
Leucine	3.9191	1.7674	0.3569	7.0624
Tyrosine	1.077	N.D.	N.D.	1.9051
Methionine	N.D.	N.D.	N.D.	N.D.
L-valine	N.D.	N.D.	N.D.	N.D.
Proline	1.0581	0.9034	0.4749	1.7888
Arginine	N.D.	N.D.	N.D.	N.D.
Lysine	4.6376	0.3647	N.D.	2.2972

APPENDIX-3 (Continue)

Patient Code	C005	C006	C007	C008
Taurocholicacid	N.D.	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.	N.D.
Glycolicacid	N.D.	0.848	N.D.	N.D.
Succinicacid	3.8455	3.9211	5.5432	5.0115
Taurine	0.3855	0.4576	0.3831	0.3833
Hippuricacid	N.D.	N.D.	N.D.	N.D.
Hypoxanthine	N.D.	N.D.	N.D.	N.D.
Glutamicacid	N.D.	7.0109	6.9685	N.D.
Asparticacid	8.2212	9.4576	8.1939	8.4777
Theronine	N.D.	N.D.	N.D.	N.D.
Serine	N.D.	N.D.	N.D.	N.D.
Glycine	1.384	2.1003	1.5344	1.4195
Histidine	0.5689	2.4632	1.4397	0.7881
Alanine	N.D.	N.D.	N.D.	N.D.
Lcarnitine	N.D.	N.D.	N.D.	N.D.
Tryptophane	N.D.	N.D.	N.D.	N.D.
Betaine	N.D.	N.D.	N.D.	N.D.
Pipecolicacid	1.3943	1.3252	1.3316	1.321
Trigonelline	0.0899	0.1079	0.0954	0.1443
Creatine	N.D.	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.	N.D.
Phenylalanine	0.3131	1.0517	0.3429	0.2992
Leucine	1.9858	4.3621	1.7468	1.6314
Tyrosine	N.D.	2.8616	N.D.	N.D.
Methionine	N.D.	N.D.	N.D.	N.D.
L-valine	N.D.	N.D.	N.D.	N.D.
Proline	0.7443	1.232	0.3531	0.8486
Arginine	N.D.	N.D.	N.D.	N.D.
Lysine	N.D.	3.8907	0.7089	N.D.

APPENDIX-3 (Continue)

Patient Code	C009	C010	C011	C012
Taurocholicacid	N.D.	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.	N.D.
Glycolicacid	N.D.	N.D.	N.D.	N.D.
Succinicacid	11.7808	4.4686	5.0166	4.4617
Taurine	0.3836	0.3929	0.4184	0.4559
Hippuricacid	N.D.	N.D.	N.D.	N.D.
Hypoxanthine	N.D.	N.D.	N.D.	N.D.
Glutamicacid	N.D.	N.D.	N.D.	7.5629
Asparticacid	8.5235	8.2367	8.17	10.4989
Theronine	N.D.	N.D.	N.D.	N.D.
Serine	N.D.	N.D.	N.D.	N.D.
Glycine	1.5125	N.D.	N.D.	1.9691
Histidine	1.4707	0.1092	0.08	17.6506
Alanine	N.D.	N.D.	N.D.	N.D.
Lcarnitine	N.D.	N.D.	N.D.	N.D.
Tryptophane	N.D.	N.D.	N.D.	N.D.
Betaine	N.D.	N.D.	N.D.	N.D.
Pipecolicacid	1.4075	1.3399	1.4657	1.778
Trigonelline	0.1308	0.0738	0.1256	0.1114
Creatine	N.D.	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.	N.D.
Phenylalanine	0.8176	N.D.	N.D.	5.1699
Leucine	1.1128	0.374	0.2288	14.3735
Tyrosine	N.D.	N.D.	N.D.	7.4882
Methionine	N.D.	N.D.	N.D.	N.D.
L-valine	N.D.	N.D.	N.D.	N.D.
Proline	0.7829	0.6449	0.6781	0.8837
Arginine	N.D.	N.D.	N.D.	1.9702
Lysine	0.8191	N.D.	N.D.	13.5525

APPENDIX-3 (Continue)

Patient Code	C013	C014	C015	C016
Taurocholicacid	N.D.	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.	N.D.
Glycolicacid	N.D.	N.D.	N.D.	N.D.
Succinicacid	4.5987	5.781	7.283	28.2803
Taurine	0.652	0.3774	0.4567	0.7192
Hippuricacid	N.D.	N.D.	N.D.	N.D.
Hypoxanthine	N.D.	N.D.	N.D.	N.D.
Glutamicacid	N.D.	N.D.	N.D.	N.D.
Asparticacid	12.1999	8.5541	8.7646	15.6897
Theronine	N.D.	N.D.	N.D.	1.5217
Serine	3.549	N.D.	N.D.	11.661
Glycine	3.872	1.5057	1.6287	8.1372
Histidine	24.624	1.6802	2.3191	10.4952
Alanine	N.D.	N.D.	N.D.	N.D.
Lcarnitine	N.D.	N.D.	N.D.	N.D.
Tryptophane	N.D.	N.D.	N.D.	N.D.
Betaine	N.D.	N.D.	N.D.	N.D.
Pipecolicacid	1.3278	1.4503	1.5039	1.3872
Trigonelline	0.3749	0.117	0.0828	0.1274
Creatine	N.D.	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.	N.D.
Phenylalanine	4.3633	0.7441	0.6903	5.6684
Leucine	16.4043	3.2688	2.4761	18.8434
Tyrosine	8.7604	1.5326	1.8037	12.4057
Methionine	0.1707	N.D.	N.D.	N.D.
L-valine	N.D.	N.D.	N.D.	N.D.
Proline	2.6735	0.8036	1.0871	3.3654
Arginine	3.3465	N.D.	0.1531	1.0245
Lysine	20.6232	1.5845	1.3529	12.8086

APPENDIX-3 (Continue)

Patient Code	C017	C018	C019	C020
Taurocholicacid	N.D.	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.	N.D.
Glycolicacid	N.D.	N.D.	N.D.	N.D.
Succinicacid	7.5364	17.3894	12.1377	3.8343
Taurine	0.4151	1.6126	0.6947	0.528
Hippuricacid	N.D.	N.D.	N.D.	N.D.
Hypoxanthine	N.D.	N.D.	N.D.	N.D.
Glutamicacid	N.D.	9.8623	N.D.	N.D.
Asparticacid	8.251	15.4695	14.4283	9.6517
Theronine	N.D.	2.0224	0.1534	N.D.
Serine	N.D.	13.767	5.2389	N.D.
Glycine	N.D.	10.072	5.4059	2.1126
Histidine	1.0491	18.644	27.8134	4.5949
Alanine	N.D.	N.D.	N.D.	N.D.
Lcarnitine	N.D.	N.D.	N.D.	N.D.
Tryptophane	N.D.	N.D.	N.D.	N.D.
Betaine	N.D.	N.D.	N.D.	N.D.
Pipecolicacid	1.2951	1.519	1.3156	1.4307
Trigonelline	0.0912	0.2969	0.2825	0.1651
Creatine	N.D.	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.	N.D.
Phenylalanine	0.596	5.4612	10.7204	2.6656
Leucine	2.4952	18.1662	39.0167	10.9504
Tyrosine	0.6167	9.6593	26.797	5.3113
Methionine	N.D.	N.D.	N.D.	N.D.
L-valine	N.D.	N.D.	N.D.	N.D.
Proline	0.8433	3.4788	7.6714	1.8041
Arginine	N.D.	4.2941	1.677	1.021
Lysine	0.0139	16.4769	43.9832	6.2555

APPENDIX-3 (Continue)

Patient Code	C021	C022	C023	C024
Taurocholicacid	N.D.	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.	N.D.
Glycolicacid	N.D.	N.D.	N.D.	N.D.
Succinicacid	7.0523	6.006	4.3895	4.1563
Taurine	0.3757	0.523	0.3728	0.5927
Hippuricacid	N.D.	N.D.	N.D.	N.D.
Hypoxanthine	N.D.	N.D.	N.D.	N.D.
Glutamicacid	N.D.	7.0481	6.9694	N.D.
Asparticacid	9.3588	9.9126	8.47	8.7278
Theronine	N.D.	N.D.	N.D.	N.D.
Serine	N.D.	N.D.	N.D.	N.D.
Glycine	1.8707	2.1044	1.7012	N.D.
Histidine	2.4403	6.1449	2.192	3.273
Alanine	N.D.	N.D.	N.D.	N.D.
Lcarnitine	N.D.	N.D.	N.D.	N.D.
Tryptophane	N.D.	N.D.	N.D.	N.D.
Betaine	N.D.	N.D.	N.D.	N.D.
Pipecolicacid	1.6891	1.3345	1.4524	1.2903
Trigonelline	0.1241	0.097	0.0835	0.0899
Creatine	N.D.	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.	N.D.
Phenylalanine	1.3613	1.7678	1.058	1.1264
Leucine	5.0694	6.6354	4.0158	4.553
Tyrosine	1.7703	4.1276	2.1366	1.0407
Methionine	N.D.	N.D.	N.D.	N.D.
L-valine	N.D.	N.D.	N.D.	N.D.
Proline	0.4582	1.3415	0.9832	0.5992
Arginine	0.0956	1.2345	0.0072	0.404
Lysine	1.862	7.144	2.4255	4.5161

APPENDIX-3 (Continue)

Patient Code	C025	C026	C027	C028
Taurocholicacid	N.D.	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.	N.D.
Glycolicacid	N.D.	N.D.	N.D.	N.D.
Succinicacid	3.8129	6.3011	5.372	7.6473
Taurine	0.4542	0.4854	0.5764	0.3834
Hippuricacid	N.D.	N.D.	N.D.	N.D.
Hypoxanthine	N.D.	N.D.	N.D.	N.D.
Glutamicacid	7.2378	10.0696	N.D.	N.D.
Asparticacid	10.1403	10.8233	15.1298	10.8788
Theronine	N.D.	N.D.	N.D.	N.D.
Serine	N.D.	N.D.	2.4435	N.D.
Glycine	4.1099	3.1147	3.8748	2.7866
Histidine	12.4209	25.9201	8.1016	2.2227
Alanine	N.D.	N.D.	N.D.	N.D.
Lcarnitine	N.D.	N.D.	N.D.	N.D.
Tryptophane	N.D.	N.D.	N.D.	N.D.
Betaine	N.D.	N.D.	N.D.	N.D.
Pipecolicacid	2.0445	2.5954	1.6094	1.3105
Trigonelline	0.2544	0.1203	0.1067	0.0753
Creatine	N.D.	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.	N.D.
Phenylalanine	4.1483	23.6331	3.0554	2.0321
Leucine	17.1316	10.3371	13.05	7.529
Tyrosine	11.5956	11.4542	8.722	6.377
Methionine	0.7089	1.0229	N.D.	N.D.
L-valine	N.D.	2.3523	N.D.	N.D.
Proline	3.0581	0.4769	3.0827	N.D.
Arginine	1.8565	6.0086	0.5361	0.7653
Lysine	12.3028	24.7679	14.7263	5.3697

APPENDIX-3 (Continue)

Patient Code	C029	C030
Taurocholicacid	N.D.	N.D.
Vaniline	N.D.	N.D.
Glycolicacid	N.D.	N.D.
Succinicacid	4.2401	4.2241
Taurine	0.5243	0.7728
Hippuricacid	N.D.	N.D.
Hypoxanthine	N.D.	N.D.
Glutamicacid	6.9685	N.D.
Asparticacid	9.325	12.7211
Theronine	N.D.	N.D.
Serine	N.D.	1.4144
Glycine	1.5945	3.708
Histidine	4.2004	12.3441
Alanine	N.D.	N.D.
Lcarnitine	N.D.	N.D.
Tryptophane	N.D.	N.D.
Betaine	N.D.	N.D.
Pipecolicacid	1.3718	1.3491
Trigonelline	0.1148	0.081
Creatine	N.D.	N.D.
Nicotinamide	N.D.	N.D.
Phenylalanine	2.3451	2.769
Leucine	7.8209	9.5739
Tyrosine	7.0176	6.6363
Methionine	N.D.	N.D.
L-valine	N.D.	N.D.
Proline	1.4052	2.0502
Arginine	0.199	0.785
Lysine	8.5177	11.5911

*N.D.: Not Detected

APPENDIX-3 (Continue)
HEALTHY

Subject Code	K001	K002	K003	K004
Taurocholicacid	N.D.	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.	N.D.
Glycolicacid	N.D.	N.D.	N.D.	N.D.
Succinicacid	6.6148	26.4678	4.9701	4.6896
Taurine	0.5102	0.8575	0.5578	0.4737
Huppuricacid	N.D.	N.D.	N.D.	N.D.
Hypoxanthine	N.D.	N.D.	N.D.	N.D.
Glutamicacid	7.0567	7.3951	N.D.	6.9918
Asparticacid	10.287	11.1303	8.4747	8.9506
Theronine	N.D.	N.D.	N.D.	N.D.
Serine	N.D.	0.0144	N.D.	N.D.
Glycine	3.2076	3.1037	1.8187	1.4387
Histidine	12.5511	36.1115	29.3125	3.6857
Alanine	N.D.	N.D.	N.D.	N.D.
L-carnitine	N.D.	N.D.	N.D.	N.D.
Tryptophane	N.D.	0.0911	0.1344	N.D.
Betaine	N.D.	N.D.	N.D.	N.D.
Pipecolicacid	1.375	1.416	1.4449	1.3128
Trigonelline	0.1483	0.1524	0.0961	0.0725
Creatine	N.D.	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.	N.D.
Phenylalanine	3.341	4.8246	2.8923	0.8132
Leucine	11.4303	17.2187	9.3682	2.9288
Tyrosine	9.3672	7.9612	8.2043	2.3354
Methionine	N.D.	2.1323	0.6801	N.D.
L-valine	N.D.	N.D.	1.2112	N.D.
Proline	2.7673	3.169	0.3631	0.7731
Arginine	2.6113	9.493	7.547	N.D.
Lysine	11.1374	26.8886	14.3989	4.3911

APPENDIX-3 (Continue)
HEALTHY

Subject Code	K005	K006	K007	K008
Taurocholicacid	N.D.	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.	N.D.
Glycolicacid	0.1642	N.D.	N.D.	N.D.
Succinicacid	4.0066	4.075	4.5367	4.5615
Taurine	0.5645	0.434	0.3846	0.5437
Huppuricacid	N.D.	N.D.	N.D.	N.D.
Hypoxanthine	N.D.	N.D.	N.D.	N.D.
Glutamicacid	N.D.	6.959	N.D.	N.D.
Asparticacid	10.2074	9.555	8.1756	8.7586
Theronine	N.D.	N.D.	N.D.	N.D.
Serine	N.D.	N.D.	N.D.	N.D.
Glycine	3.626	1.8707	N.D.	1.8816
Histidine	11.2361	5.9301	0.1451	8.0452
Alanine	N.D.	N.D.	N.D.	N.D.
L-carnitine	N.D.	N.D.	N.D.	N.D.
Tryptophane	N.D.	N.D.	N.D.	N.D.
Betaine	N.D.	N.D.	N.D.	N.D.
Pipecolicacid	1.3171	1.3495	1.6065	1.3137
Trigonelline	0.1727	0.1231	0.0663	0.2257
Creatine	N.D.	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.	N.D.
Phenylalanine	4.764	1.6421	0.065	2.2442
Leucine	16.9949	6.7112	0.1383	9.0033
Tyrosine	10.2238	3.8079	N.D.	5.6454
Methionine	N.D.	N.D.	N.D.	N.D.
L-valine	N.D.	N.D.	N.D.	N.D.
Proline	3.5043	0.8842	N.D.	1.0168
Arginine	2.0922	0.4325	N.D.	0.8881
Lysine	15.3316	5.7191	N.D.	11.2236

APPENDIX-3 (Continue)
HEALTHY

Subject Code	K009	K010	K011	K012
Taurocholicacid	N.D.	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.	N.D.
Glycolicacid	N.D.	N.D.	N.D.	N.D.
Succinicacid	3.7885	4.0948	4.4476	4.8857
Taurine	0.468	0.58	0.4052	0.4114
Huppuricacid	N.D.	N.D.	N.D.	N.D.
Hypoxanthine	N.D.	N.D.	N.D.	N.D.
Glutamicacid	N.D.	6.9504	N.D.	N.D.
Asparticacid	8.3607	9.9529	8.3732	8.2515
Theronine	N.D.	N.D.	N.D.	N.D.
Serine	N.D.	N.D.	N.D.	N.D.
Glycine	1.4195	1.5125	N.D.	N.D.
Histidine	0.3038	11.1129	1.1495	2.7113
Alanine	N.D.	N.D.	N.D.	N.D.
L-carnitine	N.D.	N.D.	N.D.	N.D.
Tryptophane	N.D.	N.D.	N.D.	N.D.
Betaine	N.D.	N.D.	N.D.	N.D.
Pipecolicacid	1.3126	1.3254	1.4205	1.3359
Trigonelline	0.0842	0.1478	0.1173	0.1089
Creatine	N.D.	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.	N.D.
Phenylalanine	N.D.	3.7011	0.207	0.6886
Leucine	0.2059	12.4044	1.5915	2.9628
Tyrosine	N.D.	5.7216	N.D.	1.0484
Methionine	N.D.	N.D.	N.D.	N.D.
L-valine	N.D.	N.D.	N.D.	N.D.
Proline	1.17	2.4599	0.4764	N.D.
Arginine	N.D.	3.2296	N.D.	0.1369
Lysine	N.D.	12.2427	0.4878	1.0588

APPENDIX-3 (Continue)
HEALTHY

Subject Code	K013	K014	K015	K016
Taurocholicacid	N.D.	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.	N.D.
Glycolicacid	N.D.	N.D.	N.D.	N.D.
Succinicacid	4.3718	8.1548	6.9396	3.85
Taurine	0.3986	0.3654	0.3705	0.3949
Huppuricacid	N.D.	N.D.	N.D.	N.D.
Hypoxanthine	N.D.	N.D.	N.D.	N.D.
Glutamicacid	7.2864	6.9833	N.D.	N.D.
Asparticacid	8.1774	8.6124	8.2325	13.1707
Theronine	N.D.	N.D.	N.D.	N.D.
Serine	N.D.	N.D.	N.D.	4.3798
Glycine	N.D.	N.D.	N.D.	4.7551
Histidine	0.7479	1.6701	0.1523	28.9752
Alanine	N.D.	N.D.	N.D.	N.D.
L-carnitine	N.D.	N.D.	N.D.	N.D.
Tryptophane	N.D.	N.D.	N.D.	N.D.
Betaine	N.D.	N.D.	N.D.	N.D.
Pipecolicacid	1.4092	1.3061	1.3571	1.3009
Trigonelline	0.1034	0.0906	0.0678	0.1387
Creatine	N.D.	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.	N.D.
Phenylalanine	0.1383	0.2082	0.0156	5.8552
Leucine	0.9963	1.8035	0.3141	21.4526
Tyrosine	N.D.	0.5573	N.D.	15.3739
Methionine	N.D.	N.D.	N.D.	N.D.
L-valine	N.D.	N.D.	N.D.	N.D.
Proline	0.5154	0.8658	N.D.	4.8997
Arginine	N.D.	N.D.	N.D.	0.7303
Lysine	0.2679	3.2479	N.D.	28.0313

APPENDIX-3 (Continue)
HEALTHY

Subject Code	K017	K018	K019
Taurocholicacid	N.D.	N.D.	N.D.
Vaniline	N.D.	N.D.	N.D.
Glycolicacid	N.D.	N.D.	N.D.
Succinicacid	4.4109	4.4646	4.4903
Taurine	0.5903	0.476	0.4653
Huppuricacid	N.D.	N.D.	N.D.
Hypoxanthine	N.D.	N.D.	N.D.
Glutamicacid	N.D.	N.D.	N.D.
Asparticacid	10.8367	9.4967	10.3536
Theronine	N.D.	N.D.	N.D.
Serine	N.D.	N.D.	N.D.
Glycine	3.0737	2.0374	1.4428
Histidine	18.5967	4.4411	2.3011
Alanine	N.D.	N.D.	N.D.
L-carnitine	N.D.	N.D.	N.D.
Tryptophane	N.D.	N.D.	N.D.
Betaine	N.D.	N.D.	N.D.
Pipecolicacid	1.3314	1.3961	1.4441
Trigonelline	0.1442	0.1212	0.0756
Creatine	N.D.	N.D.	N.D.
Nicotinamide	N.D.	N.D.	N.D.
Phenylalanine	3.2769	2.1708	1.6741
Leucine	14.4313	10.3944	7.799
Tyrosine	7.3351	4.6126	4.486
Methionine	N.D.	N.D.	N.D.
L-valine	N.D.	N.D.	N.D.
Proline	2.8408	2.7118	1.8367
Arginine	2.0495	1.4425	N.D.
Lysine	11.2901	5.7188	3.9226

***N.D.:** Not Detected