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(MSc THESIS)

GAS CHROMATOGRAPHIC DETERMINATION OF SOME BIOLOGICALLY IMPORTANT COMPOUNDS IN CLINICAL SAMPLES

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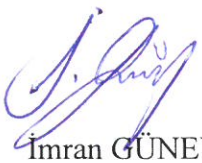

İmran GÜNEY

ÖZET

KLİNİK ÖRNEKLERDE KİMİ BİYOLOJİK ÖNEMİ OLAN BİLEŞİKLERİN GAZ KROMATOGRAFİK TAYİNİ

GÜNEY, İmran

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Eser düzeyde bulunan bu organik bileşiklerin tanımlanması ve saptanması biyokimya, tıp ve çevre analizlerinde büyük önem taşımaktadır. Günümüzde biyolojik matrikslerde eser düzeyde aseton düzeyleri şeker hastalığının biyomarkeri olduğundan, bu uçucu bileşiğin tayinine ilişkin basit, ucuz ve hızlı analitik yöntemlerin geliştirilmesi büyük önem taşımaktadır. Bu tez çalışmasında ileride nefes ve idrar örneklerinde kullanım potansiyelini araştırmak üzere gaz ve sentetik idrar örneklerinde aseton tayinine ilişkin yöntem geliştirilmesi üzerinde durulmuştur.

Asetonun biyolojik ortamda genellikle eser düzeyde bulunduğu göz önüne alındığında bir analit zenginleştirme işlemine gereksinim vardır. Bu çalışmada katı faza mikro ekstraksiyon (SPME) fiberleri örnek hazırlama ve zenginleştirmede kullanılmıştır. Ardından kütle veya alev iyonlaşama dedektörü ile eşleşmiş gaz kromatografi cihazı ile analiz gerçekleştirilmiştir. Denel parametreler her seferinde bir parametre değiştirilerek veya denel tasarım kullanılarak optimize edilmiştir. GC-FID sistemi için sentetik idrar örneklerinde optimum koşullar pH 2.14 ortamında 49.8^{o}C'de 19.6 dak adsorpsiyon olarak tespit edilmiştir. Bu koşullarda ulaşılan LOD değeri 6.0 ng/mL olup, gerçek örneklere uygulanabilir düzeyde bulunmuştur.

Anahtar sözcükler: Gaz kromatografisi, tepe boşluğu, katı faza mikro özütleme, aseton.

ABSTRACT

GAS CHROMATOGRAPHIC DETERMINATION OF SOME BIOLOGICALLY IMPORTANT COMPOUNDS IN CLINICAL SAMPLES

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Identification and detection of organic compounds at trace levels is an important analytical problem in biochemistry, medicine and environmental control. There is an increasing demand for simple, inexpensive and rapid analytical tests for the determination of trace concentrations of acetone in biological matrixes as a biomarker for diabetes mellitus. In this thesis, it was aimed to develop a chromatographic method for sensitive and selective acetone determination in gaseous and in synthetic urine for searching any potential to be applied to breath and real urine samples.

Considering that acetone in biological matrixes is in trace level, an enrichment technique is necessary. In this study, solid phase micro extraction (SPME) fibers were used as the effective tools for sample pretreatment and enrichment. Gas chromatography systems equipped with a mass and flame ionization detectors were utilized for the quantification. Experimental parameters were optimized by either one-variable at time or factorial design. According the GC-FID results, adsorption time of 19.6 min, adsorption temperature of 49.8^{o}C and pH of 2.14 was found as the optimal conditions. The LOD level calculated was found as 6.0 ng/mL and this method can be applied for a real sample.

Keywords: Gas chromatography, head space analysis, solid phase micro extraction, acetone.

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İmran GÜNEY

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SYMBOLS AND ABBREVIATIONS

Abbreviations:		Explanations
ANOVA	:	Analysis of Variance or Regression Analysis.
CAR	:	Carboxen
CW	:	Carbowax
DM	:	Diabetes Mellitus (DM)
DVB	:	Divinilbenzen
ECD	:	Electron Capture Detector
FID	:	Flame Ionization Detector
FTICR	:	Fourier Transform-Ion Cyclotron Resonance
GC	:	Gas Chromatography
HS	:	Headspace
MMt	:	Montmorillonite
MS	:	Mass Detector
NTD	:	Needle Trap Devices
PA	:	Polyacrylate
PANI	:	Polyaniline
PDMS	:	Polydimethyl Siloxane
PTh	:	Polythiophene
PTR-MS	:	Proton Transfer Mass Spectrometry
QCM	:	Quartz Chemical Balance
SDME	:	Head Space Single Drop Microextraction
SPME	:	Solid Phase Micro Extraction
VOC	:	Volatile organic compounds
HT	:	Hadamard Transform
UV–IMS	:	Ion mobility ultraviolet spectrometry

SYMBOLS AND ABBREVIATIONS (Continue)

Abbreviations:	Explanations
HPLC-FLD :	High Performance Liquid Chromatography Fluorescence Detector

1. INTRODUCTION

1.1 Analytical Methods Developed for Clinically Important Compounds

Biologically and clinically important compounds include a variety of substances such as amino acids, peptides, drugs, vitamins, pharmaceuticals and pesticides. Nowadays, there is an increasing demand for simple, rapid and low cost analytical tests for their determination in many matrixes (Sethi, 2013). The main difficulty in the analysis is usually resulted from their amount which is typically in ppb levels. Therefore, their detection, identification and determination constitute an important analytical problem in medicine, biochemistry and environmental control studies (Beninghoven et al., 1978).

A new analytical methodology called metabolomics was emerged in last two decades for the analysis of all low molecular weight compounds in a cell. (Dunn et al., 2005). Analytical methodology includes a number of steps namely; sampling, storage, preparation and analysis. The storage of samples is important for the final result as the analyte stability is limited. Sample preparation steps with such matrixes are rather cumbersome due to the presence of possible interferents in the matrixes (Braun et al., 2012).

Undeniably, modern chromatographic systems coupled to mass detector and NMR spectroscopy helped to enable separation, detection and quantification of such metabolites (German et al., 2005). Biological samples namely blood, urine and feces have been used for a long time and recently it was found that breath odors also contain information about the metabolism. Considering that non-invasive, painless and simple screening methods are being searched for early detection of diseases, breath analyses are emerging as a new diagnostic method.

Table 1.1 shows the comparison of biological matrixes and techniques for diagnostic tests (Braun et al., 2012). Various compounds present in human breath have been associated with pathological states. Sensitive and selective measurement of exhaled volatile organic compounds (VOCs) and aerosolized particles at very low concentrations was made possible by advances in analytical methodologies.

Table 1.1 Comparison of the biological matrixes and techniques for diagnostic tests.

	Type	Invasiveness	Other	Repeatability
Biological Matrix	Blood	Invasive	Painful	Non-repeatable
	Biopsy	Invasive	Painful	Non-repeatable
	Endoscopy	Invasive	Painful	Non-repeatable
	Urine	Non-invasive	Not Painful	Non-repeatable
	Breath	Non-invasive	Not Painful	Repeatable
Imaging Technique	X-ray/CT scan	Invasive	Radiation Exposure	Non-repeatable
	MRI	Invasive	Injected dyes	Non-repeatable
	Sonography	Non-invasive	-	Non-repeatable

Breath analysis provides a simple alternative to traditional biological matrixes in clinical laboratory diagnosis (Zhou et al., 2012). Since it is in contact human blood, breath samples contain important knowledge about metabolism and bacterial populations of the biological samples. Therefore, they are different from common VOCs and from the normal odor which refers to biological, physical and psychological effects caused by the interaction between aromas and fragrances and human olfactive system (Zhang et al., 2012). Greek physicians used to diagnose pathological states by using certain breath odors such as fishy smell indicated renal failure and fruity smell is associated with diabetes. Instrumental basis to these analyses was first established by Pauling and 250 types of gaseous compounds in human breath were detected by Gas Chromatography (GC) (Pauling et al, 1971). Since then, a vast number of researches have been devoted to the analysis and identification of breath biomarkers. Recently, more than 3000 different VOCs and other aerosolized particles in the breath can be detected (Ratray et al., 2014).

Breath-based diagnosis of alcohol intoxication, asthma, carbon monoxide (CO) poisoning, and some other medical conditions have been approved by US Food and Drug Administration (FDA). Non-invasive tests for diagnostic and monitoring purposes are also found very useful for Diabetes and its related states. First study on this subject has started in the 19th century by Nebelthau who found acetone in the breath of diabetics (Minh et al., 2012). Acetone has been regarded as an important disease marker of diabetes and ketoacidosis.

Next chapter includes the relation with acetone levels of urine, blood and breath samples and diabetes which is a chronic disease in which the body does not produce or properly use insulin.

1.2. Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic diseases in which blood sugar level are high over a prolonged period (WHO, 2014). Frequent urination, increased thirst and hunger are the well-known symptoms. Long-term complications include stroke, chronic kidney failure, cardiovascular disease, foot ulcers, and damage to the eyes.

There are three main types of diabetes mellitus; Type 1 results from the pancreas failure to produce enough insulin. Type 2 begins with insulin resistance, a condition in which cells fail to respond to insulin properly (Figure 1.1). As the disease progresses a lack of insulin may also develop. Type 3 is the gestational diabetes and occurs when pregnant women without a previous history of diabetes develop a high blood sugar level.

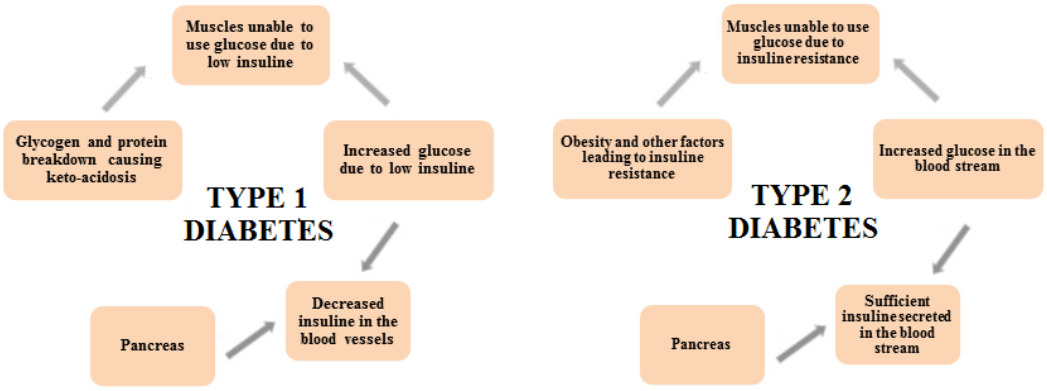

Figure 1.1 Types of diabetes mellitus.

Generally, diabetes is diagnosed by measuring the glucose concentration in blood higher or lower than the normal range of 80-120 mg dL^{-1} by an amperometric enzyme electrode (Wang, 2008). Glucose biosensors account for about 85% of the entire biosensor market since they provide a simple, user friendly, reliable and tight glycemic control for millions of diabetics. Since it was first introduced by Clark and Lyons in 1962, glucose enzyme electrodes have been through an enormous change toward the development of inexpensive and reliable devices for diabetes control. After more than 50 years and vast number of publications, there are still challenges in development of continuous glycemic sensing and even coupled with optimal insulin delivery system.

Considering that Type 1 and Type 2 diabetes affect nearly 450 million people worldwide, diagnosis and management of this disorder on blood tests may be expensive and unpractical since it is invasive and painful.

In addition, precise measurement of plasma insulin is clinically useful as an indicator of impaired glucose metabolism but, tests for insulin concentrations are very laborious. Therefore, a non-invasive monitoring would certainly improve diagnosis and treatment of diabetes.

Breath analysis has recently emerged as a noninvasive method. Breath-based devices have several advantages since it is acceptable by patients and provides a good glycemic control. Sample collection is easy and can even be obtained during surgery.

1.3. Acetone as a Biomarker for Diabetes Mellitus

In diabetic patients, the breakdown of excess Acetyl CoA from fatty acid metabolism result an increase in the level of blood acetone. This acetone is excreted through urine and exhaled through the lungs (Miekisch, 2004).

In addition to starvation, ketone bodies in the blood are also increased in patients with uncontrolled diabetes (Deng et al., 2004a). In mammals, acetone can be produced by either the decarboxylation of acetoacetate or the dehydrogenation of isopropanol (Figure 1.2). Acetoacetate is the major source and arises from either lipolysis or amino acid degradation. Next headings are dealing with the acetone levels and their determination in different biological matrixes.

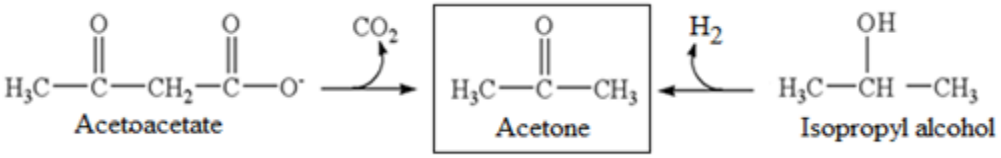

Figure 1.2 Acetone formation mechanism in human body.

1.3.1. Acetone in Breath

Exhaled breath is a mixture of alveolar and ambient air retained in the respiratory dead space. Alveolar air is in contact with blood inside alveoli. Dead space air does not enter the gas exchange region of the lung, and includes: mouth, nose, pharynx, trachea and bronchi (Figure 1.3).

For healthy humans, breath acetone concentrations range from 0.3 to 0.9 ppm but for Type 2 patients, this concentration can rise to more than 1.8 ppm (Nasution et al., 2013). However, there are difficulties in the sampling and analysis of exhaled breath.

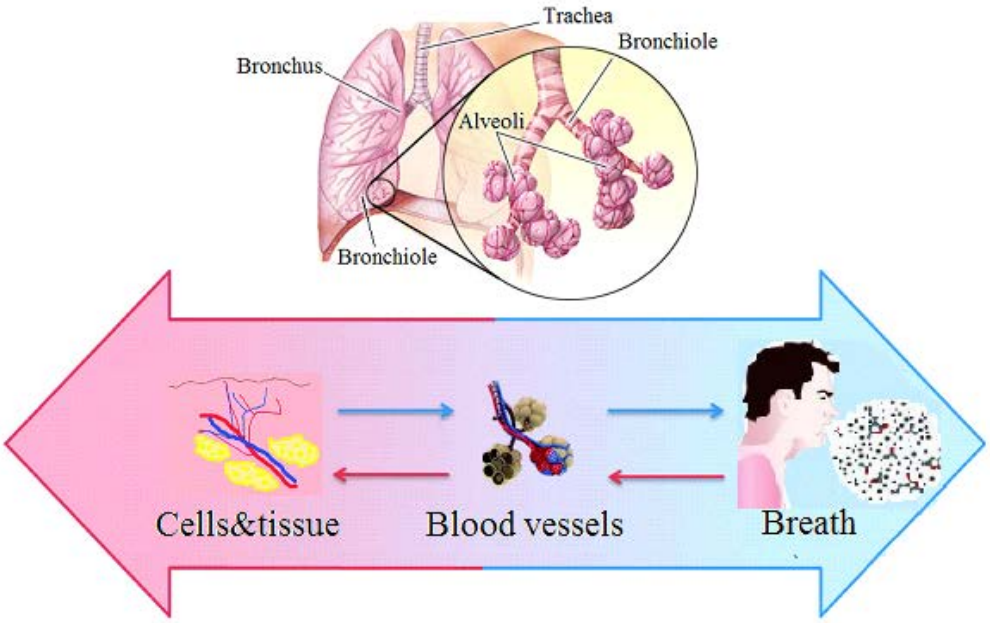

Figure 1.3 Alveolar air and its interaction with cells and tissue through blood vessels.

During sample collection dead space air enters the sampling system diluting the alveolar breath. The representativeness of the sample can be controlled by measuring the partial pressure of CO_2 in breath. A potential error from exogenous contaminants can be corrected by subtracting their concentration from the exhaled breath concentrations. For this purpose, parallel sampling of breath and environmental air is required. Another approach is lung washout by breathing ultrapure air for a while before sampling.

Breath samples are usually collected into sampling canisters, bags, syringes and sorbent tubes (Buszewsky, 2007). Canisters are made from stainless steel to have a 1-3 L volume. Their inner surface is treated to minimize the loss of gaseous compounds by adsorption.

Tedlar bags are chemically inert and made from polyvinyl fluoride (PVF) which is resistant to gas permeation and adsorption of analytes. Yet, permeation of water should be bear in mind. Prior to reuse, the bags must be evacuated and cleaned with nitrogen gas.

In Proton Transfer Mass Spectrometric (PTR-MS) system, the breath can be analyzed directly without any sample preparation. However, considering sub ppb level of the VOCs, it is clear that a sample enrichment technique is required prior to the analysis. The major enrichment technique used for breath analysis is adsorption of the volatile analytes on a suitable support in a collection trap and then, their thermal desorption to the GC/MS system (Miekisch et al., 2004).

Several materials are utilized for this purpose including organic polymers, activated charcoal and graphitized carbon. These adsorbents are chosen on a basis of their hydrophobic high surface area and thus they are less affected by water contents. However, it is difficult to find a material that allows complete adsorption as well as easy desorption of all volatile components present in exhaled air. Mixed bed sorbent traps are also used to solve this problem.

There are two main methodologies for this purpose: adsorption on solid phase micro extraction (SPME) fibers and on solid sorbents in tubes and needle trap devices (NTD). SPME is an efficient and solventless technique and particularly suited to the head space analysis of volatile compounds (Pawliszyn, 1997). The small volume of the stationary phase, i.e. polymeric coating on a fused silica or a steel rod, is an advantage of SPME when sample sizes are not large. LODs are commonly in the sub ppb range but, the technique is prone to water content in breath samples. When Tedlar bag is used for breath sampling, the SPME is usually coupled with headspace (HS) technique (Deng et al., 2004a).

Sorbent traps can be prepared on a microscale and coupled on-line with a GC system. NTDs allow direct thermal desorption inside a GC injector like an SPME and have displayed similar performance. However, their compact structure limits the maximum flow rate that can be used during sample collection. Therefore, alternative approaches are being searched.

Table 1.2 summarizes the methods used for sampling and detection of acetone in breath samples. As can be followed from the Table, gas chromatography coupled with FID or MS detector is the method of choice. However, Quartz Chemical Balance (QCM) or other electrochemical methods are also employed for sensitive acetone detection in breath. Saraoğlu et al. has described a method for the acetone detection in breath with QCM sensors containing zeolite absorbent (Saraoğlu et al., 2010). The sensor data obtained from breath is compared with blood glucose value.

Table 1.2 Details of the methods used for acetone detection in breath samples.

Sample collection	Enrichment Technique	Detection	LOD	Reference
40 L plexiglass chamber	A charcoal or silica sorbent in a GC syringe needle	GC-FID	1-0.1 nmol/L	Qin et al., 1997
Tedlar bag	HS-SPME (PDMS-DVB)	GC–MS	0.049 ppbv	Deng et al., 2004a
Sampling bag	Electrode modified with Ag^{+}-ZSM-5 zeolite	QCM	< 1ppm	Huang et al., 2004
5 L Mylar balloon	Derivatization with alkaline salicylaldehyde	LED-based photometry	14 ppbv	Teshima et al., 2005
Prototype hand-held breath acetone analyzer.		GC	500 nmol/L	Veloso et al. 2006
1 L Tedlar bag	NTD (methacrylic acid and ethylene glycol dimethacrylate copolymer)	GC/MS	5.0×10^{-4} ppmv	Ueta et al., 2009
Real time		SIFT-MS PTR-MS	-	Smith et al., 2011
Latex balloon	Au np on an array of functionalized alkyl thiols	Chemiresistor	1 ppmv	Luo et al., 2012
Portable gas sensor	Si-doped WO_3 np films on an Al_2O_3 substrates	Chemiresistor	20 ppb	Righettoni et al., 2012
Teflon bag	Resorcinol containing Nafion membranes	UV/vis LED		Worrall et al., 2013
Real-time	p-type Co_3O_4 nanofiber with Ir nanoparticles and graphene oxide	Chemiresistor	120 ppb	Choi et al., 2014
Tedlar bag	HS-SPME (Car/PDMS)	HT-GC/MS	0.1 ppmv	Fan et al., 2014
Sampling bag	NTD (graphite C Carbopack X and a C molecular sieve)	GC–MS	1 ng/L	Ueta et al., 2014
Portable breath Analyzer	Mixed metal oxide sensor	Chemiresistor and SIFT-MS	0–10 ppmv	Walton et al., 2014
Real-time	Layered double oxide of Mg–Al–CO_3	Cata-luminescence	0.02 mM	Zhang et al., 2014
Real-time	CNT–SnO_2 nanocomposite sensor	Chemiresistor	0.5-5ppm	Salehi et al., 2014

FTICR-MS: Fourier transform-ion cyclotron resonance mass spectrometry, HT: Hadamard Transform

1.3.2. Acetone Determination in Blood and Urine

The mechanism of lipolysis is generally beta-oxidation to acetyl-CoA. Acetyl-CoA can be converted to acetone and two other ketones via ketogenesis which generally occurs in the liver (Talbert, 2007).

In the blood, acetone concentrations around 1 mg/dL are considered normal. In diabetic patients this concentration may elevate by at least two degrees of magnitude. Acetone levels can also be higher than average for people on ketogenic diets that are low in carbohydrates so fat is the primary source of energy (Kalapos, 2007). Such diets elevate the level of acetone in the brain.

Chromatography is the method of choice for detection of the VOCs in biological fluids and the static headspace technique is extensively used for this purpose. Gas chromatographic methods combined with sample enrichment techniques were also developed for direct determination of acetone in plasma samples. Considering its nature of high activity and volatility, derivatization of acetone was required prior to GC analysis. After derivatization with PFBHA (O-2,3,4,5,6-(pentafluorobenzyl) hydroxylamine hydrochloride) acetone oxime can be sensitively determined by GC-MS method (Deng, 2004b). By using SPME fibers, it has been established that acetone level in diabetes plasma is higher than 1.8 mM while in normal plasma this level is lower than 0.017 mM.

Single drop microextraction methodology was also utilized in enrichment of oxime derivative prior to detection by GC-MS (Li, 2005, Dong, 2006). Similarly, SPME and head space methodologies were used for acetone detection in urine samples. Table 1.3 summarizes the methods used in acetone determination in blood and urine samples, respectively.

Table 1.3 Details of the methodology used in acetone detection in blood and urine samples.

	Enrichment Technique	**Detection**	**LOD**	**Reference**
Blood	HS-SPME	GC-FID	0.005 mg/L	Zuba, 2002
	SPME Acetone + PFBHA → Acetone oxime	GC–MS	0.0672 µM.	Deng, 2004b
	HS-SDME Acetone + PFBHA → Acetone oxime	GC-MS	0.24- 0.62 nM	Li, 2005
	HS-SDME Acetone + PFBHA → Acetone oxime	GC-MS	2.0 nM	Dong, 2006
Urine	SPME	GC-FID	0.30 mg/L	Matin et al., 2007,
	Headspace	GC-FID	0.1 mg/L	Oliveira, 2007
		UV–IMS	3 mg/L	Delgado, 2009
		HPLC-FLD	0.032 µmol/mL	Fujii, 2014

HS-SDME: Head Space Single drop microextraction, UV–IMS: Ion mobility ultraviolet spectrometry, HPLC-FLD: High Performance Liquid Chromatography Flourescence Detector

1.4. The Aim of the Thesis

There is an increasing demand for simple, inexpensive and rapid analytical tests for the determination of trace concentrations of acetone in biological matrixes as a biomarker for diabetes mellitus. In this thesis, it was aimed to develop a chromatographic method for sensitive and selective acetone determination. The results of this study will be evaluated in terms of their potential to be used for breath and urine samples.

However, acetone is usually in trace levels and therefore, recently developed sample preparation techniques such as solid phase micro extraction (SPME) fibers developed were used as the effective tools for analyte enrichment. Experimental parameters were optimized by either one-variable at time or factorial design. Next section gives theoretical information about the methods used for the analysis.

1.5. Theoretical Basis of the Analytical Methods

1.5.1. Gas Chromatography

In GC system, the sample is injected into the inlet where it is volatilized and by the aid of a carrier gas, a small portion of a sample is carried onto the column. The sample components are separated according to their interaction with the stationary phase and then, eluted from the column into the detector where a signal proportional to their amount is recorded. The chromatogram contains various peaks at different retention times and peak areas giving the qualitative and quantitative information, respectively.

Wide range of detectors are available each having different operating characteristics. Electron capture detector (ECD), flame ionization detector (FID) and mass detector (MS) are the widely used for the detection of volatile substances. In FID, the effluent passes through a narrow tube where a microburner ionizes the compounds in the flame and these ions increases the current flow in the external circuit.

MS detectors, on the other hand, operated on the basis of ionizing molecules and then sorting and identifying these ions according to their mass-to-charge (m/z) ratios. Two main components of the instrument are the ion source and the mass analyzer (Figure 1.4).

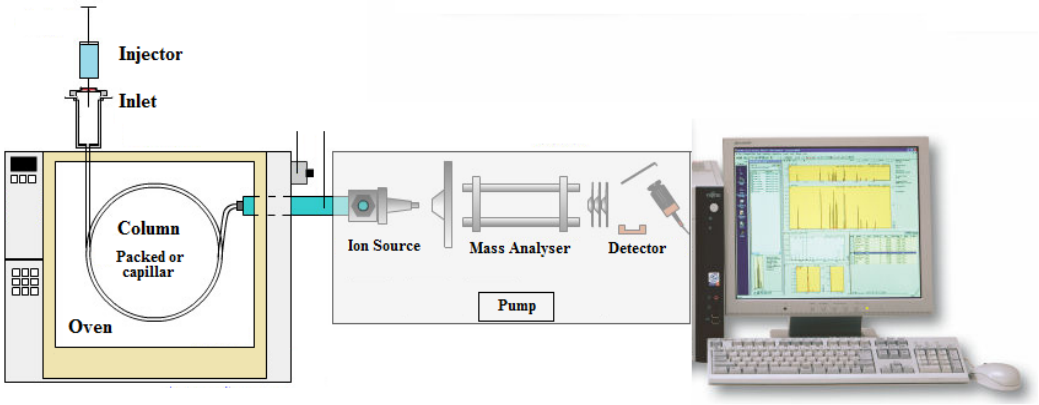

Figure 1.4 Schematic representation of a GC-MS System.

1.5.2. Solid Phase Microextraction (SPME)

SPME is a simple, fast, inexpensive extraction technique that can achieve very low levels of detection of trace organic compounds (Pawlisyzn et al., 1990). Analytes are concentrated on one cm length of fused silica fiber, coated with a polymer, and then are rapidly delivered to a capillary GC column. The fiber is either placed directly into the sample matrix or the headspace for volatile analytes. The equilibrium is established among the analyte in the sample or above the sample, and in the polymer coating on the fiber. For high accuracy and precision, the sampling parameters should be kept constant at their optimal values.

The sorbents used in SPME vary in a range of materials including polydimethyl siloxane (PDMS), poliacrylate (PA) and their blended types with divinilbenzen (DVB), and Carboxen particles or Carbowax (CW). The selection of the fiber type is dependent on the fact that the polarity of the sorbent coating should match the polarity of the analyte. Coating should also be resistant to high temperature conditions and extremes in pH, salts and other additives (Pawlisyzn et al., 1994).

1.5.3. Experimental Design

In analytical chemistry a vast number of data is produced during method development and a statistical analysis is mandatory to sort out the necessary information. Chemometrics has emerged as a special branch of chemistry to provide maximum chemical information by analyzing these data by using mathematical and statistical methods. Method optimization and experimental design can be achieved by this means (Brereton, 2007).

Screening experiments help to sort out which factors have important effect on the signal such as temperature, pH, stirring rate, etc. Traditional screening method for optimization is one-variable-at a time (OVAT) method where all other variables are kept constant while the effect of one variable is examined. Multivariate method, on the other hand, includes a series of experiments which many parameters are changed at a time. Results are evaluated by using Analysis of Variance (ANOVA) or regression analysis.

An ***Experimental Design*** consists of a series of experiments made under different conditions. Experimental data can be described as a mathematical relationship between the independent variables. ***Factorial Designs*** are often used for screening or when there are a large number of possible factors. In ***Full Factorial Design***, the number of experiments is large and therefore, two level fractional factorial design is used to reduce the number of experiments. High and low levels were selected for each factor and a standard design is used where the value of each factor is usually coded. Then, the data obtained are analyzed by setting up a design matrix based on the model. The components of a typical equation for three factors are given below.

$$\hat{y} : b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_{12} + b_{22}x_{22} + b_{33}x_{32} + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$$

$\hat{y}$: Estimated response

b_0 : an intercept

$b_1x_1 + b_2x_2 + b_3x_3$: linear terms depending on each of the three factors

$b_{11}x_{12} + b_{22}x_{22} + b_{33}x_{32}$: quadratic terms depending on each of the three factors

$b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$: interaction terms between the factors

Plackett and Burman proposed a number of two level factorial designs, where the number of experiments is a multiple of four. The number of experiments exceeds the number of factors, k, by one (k+1). Pareto charts display the relative importance of any parameter and $\bar{y}_+ - \bar{y}_-$ differences for each factor are calculated and divided by estimated standard error ($\sqrt{V_{effect}}$).

The chart includes a vertical line at the critical t-value for p = 0.05 and regardless being negative or positive, the factors those exceed the t value are taken into account for further optimization. By using the replicates, standard deviation (s) of the responses and then related average variance of individual runs (s_i^2) can be estimated where n is the number of replication.

$$s_i^2 = s^2 / n \quad (1.1)$$

Response surface methodology uses the graphs that are very useful to interpret the effect on the response of each pair of factors graphically and enable us to evaluate their interactions. In ***Box-Behnken Design***, exploration of quadratic response surface and construction of a second-order polynomial model is performed where y is the response variable (relative area); b_0 is an intercept; b_i, b_{ii}, and b_{ij} are constant regression coefficients of the model; and xi, xj (i=1–4; j=1–4 and i≠j) represent the independent variables.

$$y = b_0 + \Sigma\, b_i xi + \Sigma\, b_{ii} xi^2 + \Sigma\, b_i b_j x_i x_j \quad (1.2)$$

The number of experimental points (N) is defined by the expression where *K* is the number of variables and C_0 is the number of center points;

$$N = 2\,K\,(K\text{-}1) + C_0 \quad (1.3)$$

2. EXPERIMENTAL

2.1. Instrumentation

Acetone determination was maintained by using an Agilent 7820A gas chromatograph equipped with a flame ionization detector (GC-FID) and a Thermo Scientific Trace 1300 ISQ GC-MS system. The details of both GC systems are given in Table 2.1 and Table 2.2, respectively.

Table 2.1 Details of GC-FID system used for acetone determination.

Parameter	FID	Parameter	FID
Column	DB1 (30mx0.32mmx0.25 µm)	Injection	Splitless
		Detector temp.	250ºC
Inlet temp.	250ºC	Flow rate	0.5 mL/min
Oven temp.	250ºC	Hydrogen flow	40 mL/min
Initial temp.	40ºC	Air rate	450 mL/min

Table 2.2 Details of GC-MS system used for acetone in synthetic urine.

Parameter	MS	Parameter	MS
Column	Carbowax (30mx0.25mmx0.25 µm)	Ionization mode	Electron Impact
Injection	PTV Splitless	Ion source temp.	200ºC
Inlet temp.	220ºC	MS transfer temp.	220ºC
Oven temp.	210ºC	Flow rate	1 mL/min
Initial temp.	50ºC	Screening mode/masses	SIM/43,58

The temperature programming in GC-FID system is as follows; 10 min at 40ºC and then, the temperature is risen at a rate of 50ºC/min to 250ºC and then hold for 3 min. The total run is 17.2 min.

The temperature programming in GC-MS system is as follows; 1 min at 50ºC and then, the temperature is elevated first to 95ºC at a rate of 5ºC/min and then 210ºC a rate of 50ºC/min and hold for 2 min. The total run is 14.5 min.

SPME fiber coating was carried out using a Metrohm Autolab PGStat 101 voltammetric analyzer with a three-electrode system including stainless steel wire (316 type, i.d. 0.3 mm) as the working electrode, a platinum wire as the auxiliary electrode and a Ag/AgCl electrode as the reference electrode.

The SPME holder for manual sampling was obtained from Supelco (Bellefonte, PA, USA). Commercial SPME fibers PDMS, PEG, PA, PDMS/DVB, CAR/PDMS and DVB/CAR/PDMS were also supplied from Supelco. The fibers were conditioned before use. Fiber conditioning was accomplished by using Heraeus RT500. Transsonic 460/H was used for ultrasonic treatments. A heater/magnetic stirrer for head space studies was purchased from IKA-RCT (Germany). Nüve BM 402 cryostat was used to maintain fixed temperature for head space during adsorption.

2.2 Reagents

All the reagents were of analytical reagent grade. Aqueous solutions were prepared with ultrapure water (18.2 MΩ/cm) from a MilliPore Milli-Q Gradient water purification system. Sodium chloride and other salt reagents were purchased from Merck. Acetone (99.8%) was obtained from Lab-Scan Analytical Sciences.

Synthetic urine samples were prepared according to the procedure given elsewhere (Deroco et al., 2014). In a 250 ml volumetric flask, 0.73 g of NaCl, 0.40 g of KCl, 0.28 g of $CaCl_2.2H_2O$, 0.56 g of Na_2SO_4, 0.35 g of KH_2PO_4, 0.25 g of NH_4Cl, and 6.25 g of urea was simply mixed and completed with ultrapure water. The pH was adjusted to 6.0 by dropwise addition of NaOH solution. In experimental design studies, the pH was set at two levels being 2.0 and 5.5.

2.3. Procedures

2.3.1. Fiber coating

Prior to the electropolymerization, the stainless steel wire was cleaned ultrasonically in a test tube containing acetone for 15 min and rinsed with distilled water. The procedure applied for different polymeric coatings varied in the solution composition and the potential applied depending on the nature of the monomer.

Polyaniline (PANI) coated fiber was prepared from an aqueous solution of 0.5 M H_2SO_4 containing 0.025 M aniline. The potential was cycled for 30 times between -0.2 - 1.0 V at a rate of 30 mV/s. Then, the fiber was washed with methanol, acetone, and water in sequence respectively, and it was heated in an oven at 100^{o}Cfor 20 min. After it was connected to an SPME holder, the fiber was conditioned in a GC injection port set at 200^{o}C for an hour till a clear blank sample was obtained.

Polythiophene (PTh) fiber was prepared by electropolymerization from a 0.1 M NaClO4 solution containing 1.25 M thiophene in acetonitrile (Pelit and Dizdaş, 2013). After deoxygenation of the solution with nitrogen for 5 min, the electropolymerization procedure was initiated by cycling the potential in a range of −0.2 to 2.2 V at a rate of 50 mV/s for two times. The oxidation signal of thiophene at 1.3 V increased on successive cycling indicating the polymerization. The rest of the procedure was quite similar to PANI fiber development. The procedure for Polyimidazole (PIm) coating was similar to PTh conditions.

The PTh coating was also modified with a Montmorillonite (MMt) clay to improve the adsorption characteristics of the fiber. For this purpose, the monomer solution given above was added clay material and the same procedure was applied (Dizdaş et al., 2014). A further modification was made by inserting ionic liquid into the monomer solution given above. The ionic liquid used for the synthesis of composite fiber was 1-methyl-3-undecyl-imidazolium bromide [C12mimBr] and it was prepared as given elsewhere (Pelit et al., 2015).

2.3.2. Head Space Solid Phase Microextraction Procedure

The performance of the lab-made fibers along with the commercial fibers ones was tested by exposing the SPME fibers to the head space (HS) of acetone standards. For this purpose, 1 µL of pure concentrated acetone was injected into 20.0 mL HS vial with a PTFE-silicon septum and the temperature was set at 25^{o}C with an aid of cryostat. For calibration studies, 40^{o}C was selected as it is close to the body temperature. After the temperature equilibrium was established, the fiber was immersed in HS vial at a fixed height and exposed to the adsorption of acetone for 5 minutes. Then, the fiber was placed into injection block of the GC-FID allowing the adsorbed acetone sent to the column with carrier gas by thermal desorption.

In the studies with synthetic urine samples, 10 mL of aqueous solution was placed in a 20.0 mL glass vial with a PTFE-silicon septum. The vial was tightly sealed with an aluminum cap to prevent sample loss due to the evaporation and placed on a hot plate. The temperature was set at 40-70^{o}C for 5 min by using a cryostat. The fiber was immersed in HS vial at a fixed height and exposed to the adsorption of acetone for 2-20 minutes. Then, the fiber was withdrawn with the needle and immediately introduced into the GC injection port and analyzed according to the procedure.

3. RESULTS AND DISCUSSION

In this thesis it was aimed to develop a sensitive and selective method for acetone determination in different biological matrixes. The studies were conducted in two means; acetone in gaseous samples and synthetic urine for searching any potential to be applied to breath and real urine samples.

3.1. Acetone in gaseous samples

From the literature it was clear that enrichment technique is required for selective and sensitive determination of volatile substances. Therefore, HS-SPME technique was chosen for acetone determination in gaseous samples. The SPME fibers fabricated in the lab namely; PANI, PIm, PTh, and PTh coating modified with clay and clay-ionic liquid composite have been utilized for HS enrichment procedure.

As described in Experimental Section, 1 µL of acetone was injected into a 20.0 mL vial and thermally equilibrated at 25ºC for 5 min. Then, the fibers were exposed to the evaporated acetone in the vial for 5 min. After adsorption process was completed, the fibers were withdrawn into the needle and injected into the port of GC-FID system. The chromatographic conditions on Table 2.1 have been applied. The acetone peak area at around 6th min was plotted in the graph.

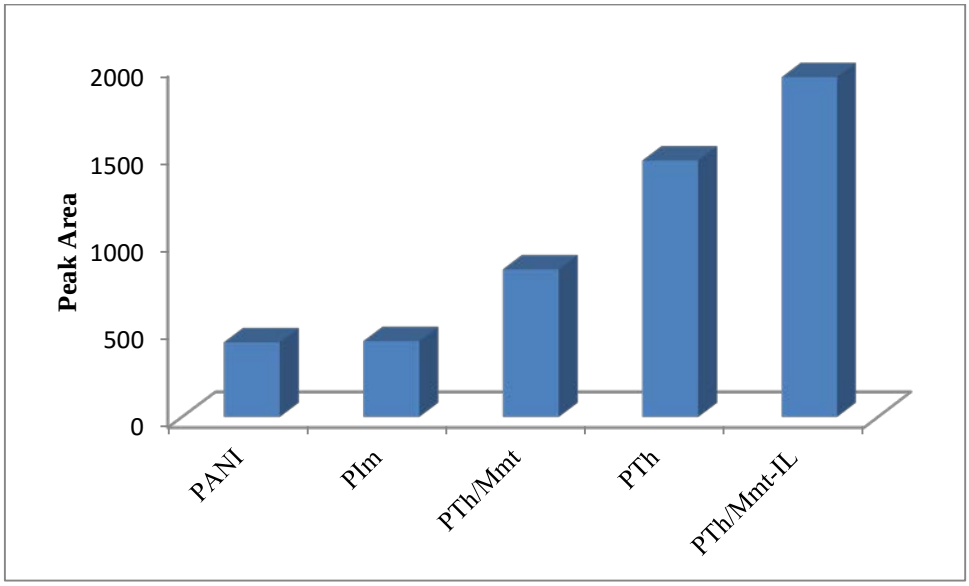

Figure 3.1 Chromatographic peak area values obtained with different lab-made fibers for 1 µL of acetone injected in a 20.0 mL of vial (39 mg/L).

As shown in Figure 3.1 chemical structure of the fiber has a strong effect on the response. The best peak area was obtained with the PTh-clay-IL composite fiber which can be attributed to the enhanced surface capacity with clay nanoparticles and bipolar characteristics of IL.

Commercial fibers with single adsorbent (PDMS, PA, PEG) and blended (PDMS/DVB, CAR/PDMS and DVB/CAR/PDMS) types of fibers were also tested to compare their performances. Similar procedure was applied and resulting chromatograms have revealed that acetone peak around 6th min has given the best results at a CAR/PDMS fiber. It was concluded that porous structure of Carboxen might have increased the adsorption capacity while decreasing the desorption chance of acetone in inner layers. Therefore, further studies have been conducted with this fiber.

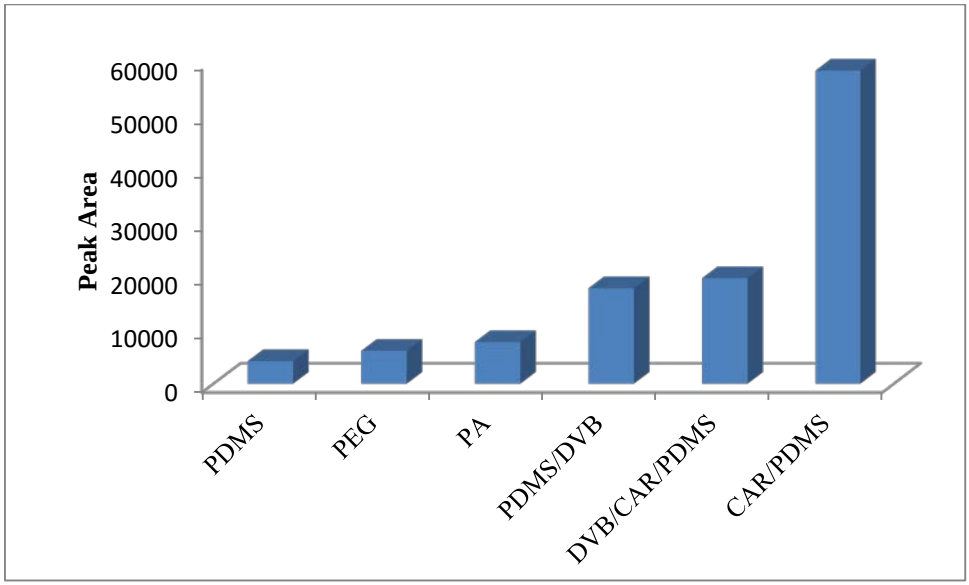

Figure 3.2 Chromatographic peak area values obtained with different commercial fibers for 1 µL of acetone injected into a 20.0 mL of vial (39 mg/L).

In the calibration studies, the difficult part is to prepare the gaseous standards. For this purpose, 1 µL acetone was injected into the 20.0 mL of vial and then, the cap was tightened. A series of calibration standards were prepared by drawing 2, 5, 10, 25, 50 and 75 µL and injecting into 6 set of vials with 20.0 mL of volume. The CAR/PDMS fiber was inserted into each vial and equilibrated for 5 min at 40^{o}C. Then, it was withdrawn and injected into the port of GC-FID system. This procedure was repeated for the rest of the standards and parallel studies were carried out.

Resulting chromatogram can be seen in Figure 3.3. The peak areas were plotted against the acetone concentration in the gaseous mixture.

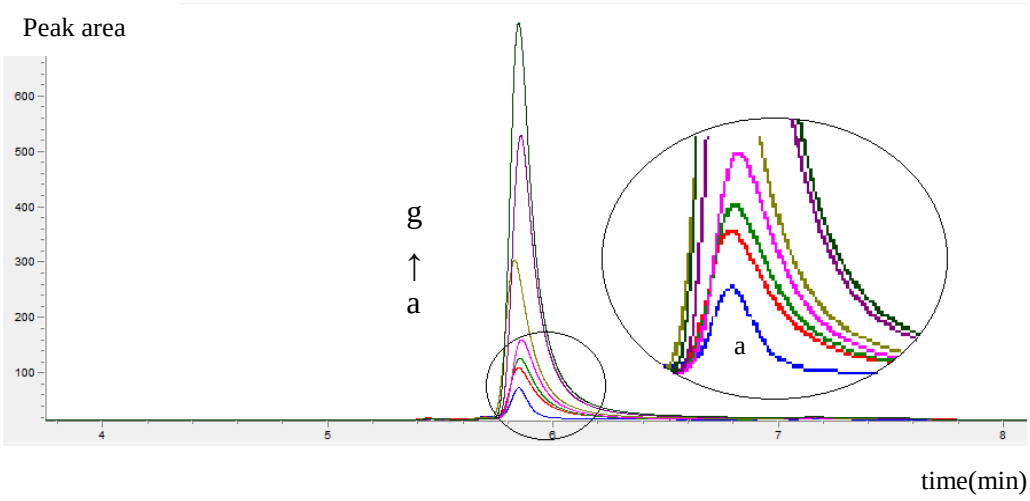

Figure 3.3 HS-SPME-GC-FID Chromatograms obtained for a) blank, b) 0,0039 mg/L, c) 0,00975 mg/L, d) 0,0195 mg/L, e) 0,04875 mg/L, f) 0,0975 mg/L, g) 0.14625 mg/L acetone.

Figure 3.4 shows the calibration graph obtained for the acetone in gaseous mixtures.

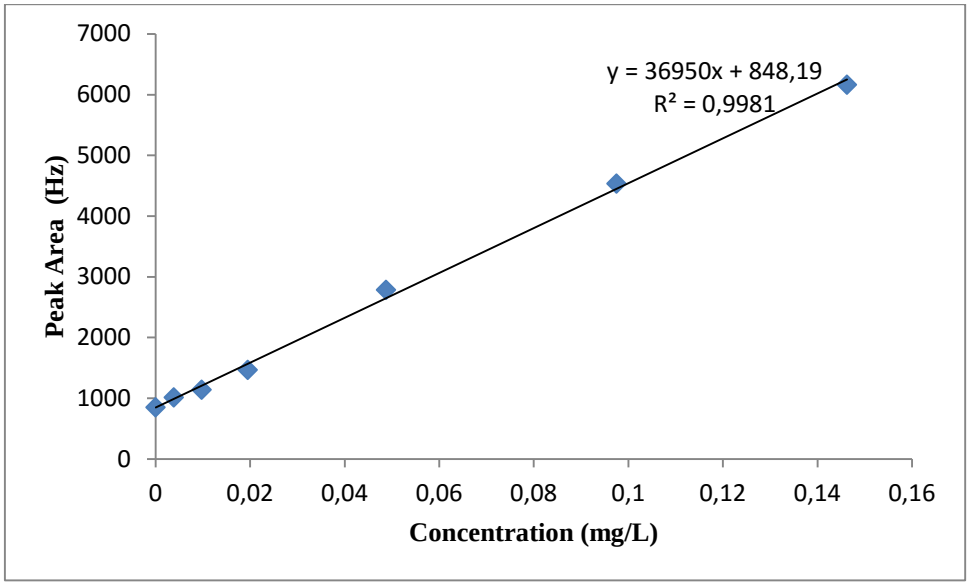

Figure 3.4 Calibration graph for acetone constructed by HS/SPME/GC/FID.

The calibration graph was found linear in a concentration range of 0.010 – 0.15 mg/L with a correlation coefficient of 0.9981. The limit of detection (LOD) was calculated as 0.0126 mg/L from the calibration graph. This difference is resulted from the fluctuation in the blank signal.

Here, it should be bear in mind that the volume studied is very low simply being 20 mL. Considering that average volume of breath is about 500 mL and subsequent breathing at a certain rate is required for previously developed methods, this level can easily be taken down to more sensitive levels enough to determine the acetone. In normal patients, acetone level in breath samples which is usually in a range of 0.3-0.9 ppm. In diabetes patients, this level is higher than 1.8 ppm.

3.2. The Studies with Synthetic Urine

The studies were carried out with GC-MS and GC-FID systems. Overall results were compared to evaluate the performance of the methods.

3.2.1. The Studies with GC-MS system

In the studies with mass detector, experimental parameters such as adsorption temperature and time, sample volume and salt effect were optimized by using OVAT method. However, during these studies CAR/PDMS (75 µm) fiber was not available and therefore, GC-MS studies were conducted with polyacrylate (PA, 85 µm) fiber to reveal the effect of experimental parameters. For this purpose, 50 mL of synthetic urine solution was spiked with 1.0 µl acetone and mixed well. From this solution, 2 mL aliquot was transferred to a 20.0 mL headspace vial and sealed with an aluminum cap. The temperature of the vial was set at 30ºC by using a cryostat. After equilibrium was reached, the PA SPME fiber was inserted into the vial at a fixed height and exposed to the acetone in head space for 10 min.

After this step was completed, the fiber was withdrawn into the needle and injected into the port to thermal desorption for 5 min. This procedure was repeated for 40, 60, 70 and 80ºC and peak area values obtained were plotted against the adsorption temperature. Figure 3.5 shows the effect of adsorption temperature on the peak area of acetone.

As can be seen from the figure, adsorption temperature has a strong effect on the response since headspace concentration is dependent on the temperature. Even though higher values were obtained for 80ºC, high temperature has a risk for escaping of volatile substances. Therefore, further studies were conducted at 70ºC.

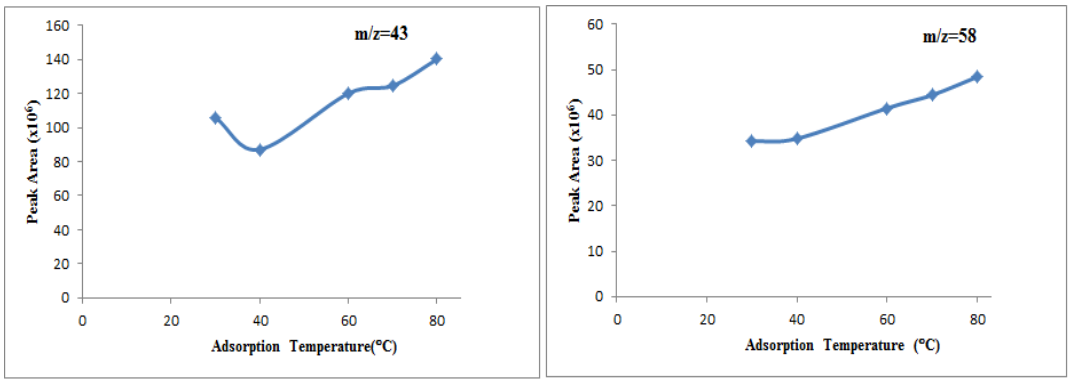

Figure 3.5 Influence of adsorption temperature on the peak area values of 2.0 mL of synthetic urine containing 15.6 mg/L acetone injected into a 20.0 mL of vial.

Sample volume determines the headspace volume and therefore, the extraction efficiency is dependent on the volume change. The volume transferred from the stock solution was changed in a range of 1 -10 mL and then, the procedure was repeated. Figure 3.6 shows that peak area increases with the sample volume as expected. Further studies were carried out with 10 mL of sample solution.

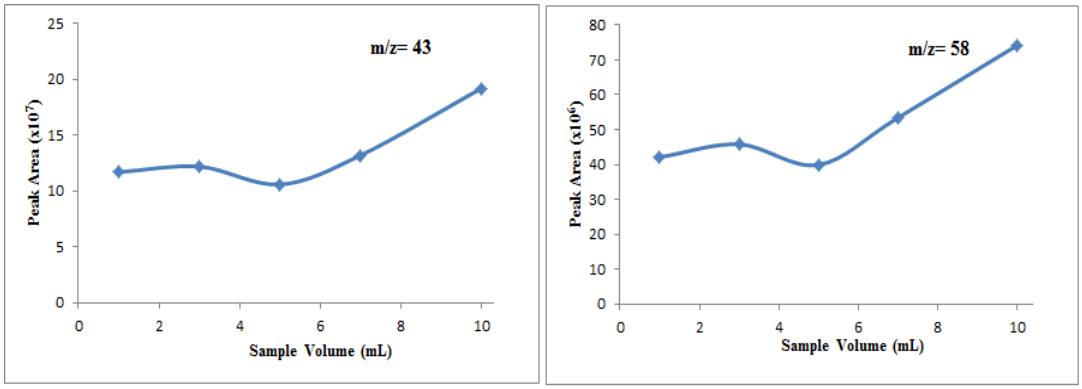

Figure 3.6 Effect of synthetic urine volume in arrange of 1.0-10.0 mL containing 15.6 mg/L acetone injected into a 20.0 mL of vial.

Ionic strength is another parameter to be optimized since dissolved ions increases the passage of volatile compounds to the head space. However, considering that synthetic urine sample has already includes several ions to maintain the necessary ionic strength, this salting out effect can be omitted. Indeed, the addition of salt from 0.6 M NaCl solution into the sample has resulted a slight decrease in the signal.

Adsorption time is another important parameter and determines the amount of analyte adsorbed on the fiber. Longer times, on the other hand, might result desorption since high temperature conditions are use. Therefore, the exposure time of the fiber must be justified. As can be consisted from the Figure 3.7, 5min adsorption time is optimal for the acetone determination.

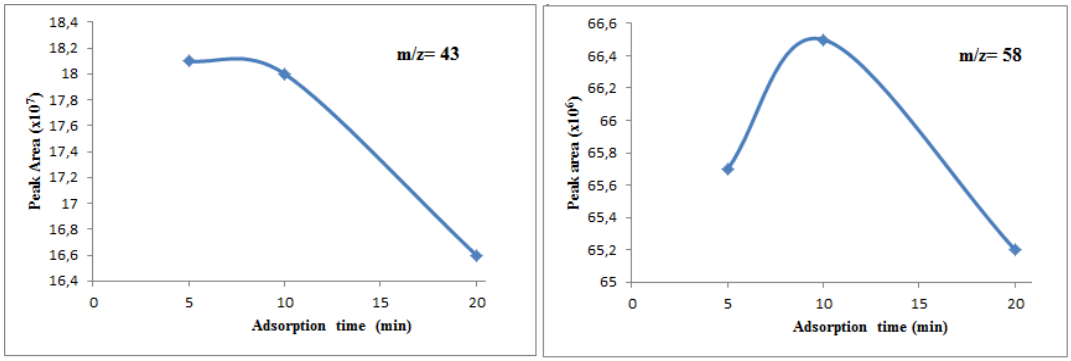

Figure 3.7 Effect of adsorption time on the response of 10.0 mL synthetic urine sample containing 15.6 mg/L acetone injected into a 20.0 mL of vial.

Overall results have shown that optimal conditions for HS-SPME-GC-MS determination of acetone are as follows; 10 mL sample at 70ºC for 5 min. The calibration graph constructed under these conditions was given in Figure 3.8.

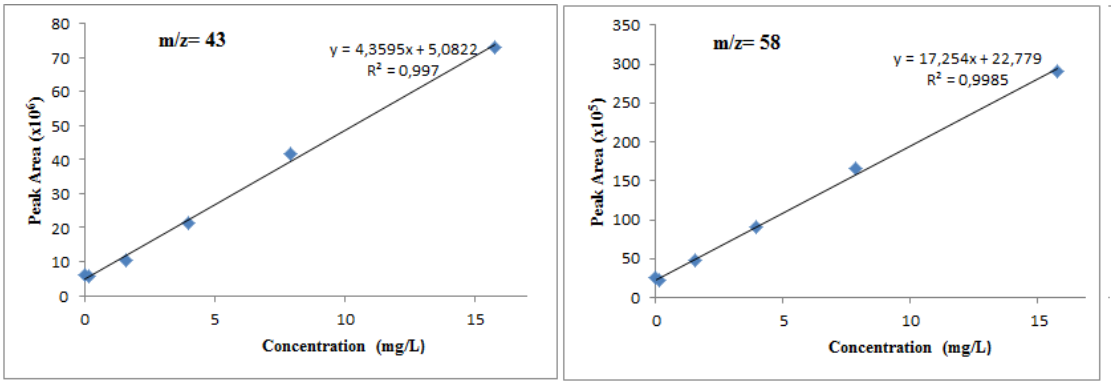

Figure 3.8 Calibration curve for acetone obtained with GC-MS system for two mass to charge ratios. The HS-SPME was performed by using 10.0 mL of synthetic urine sample in a 20.0 mL of vial set at 70ºC.

As can be seen from Figure 3.8, the linear range was between 2.0 -15 mg/L acetone. Better correlation coefficient and slope were obtained for 58 mass to charge ratio. Analytical characteristics are summarized in Table 3.1.

Table 3.1 Analytical characteristics of the HS-SPME- GC-MS method (m/z = 58).

Analytical Parameter	Value
LOD (mg/L)	1.45
LOQ (mg/L)	4.69
R^2	0.9944
Linear Range (mg/L)	4.69-17.4

The sensitivity of the method was not satisfactory considering the trace amount of acetone in the biological matrixes. One of the reasons is that commercial SPME fiber is PA not the CAR/PDMS fiber. Therefore, an alternative method for comparing the performances was searched. It is known that GC-FID system is commonly encountered instrument and very convenient for acetone determination. Therefore, another method development was carried out with this instrument using CAR/PDMS as given in next chapter.

3.2.2. The Studies with GC-FID System

During the studies with GC-FID, CAR/PDMS fiber was utilized. The procedure given in Experimental Section was used and 10 mL of synthetic urine sample was spiked with acetone to be 0.3 mg/L. The 20.0 mL of vial set at 50ºC and CAR/PDMS fiber was exposed to the acetone in headspace for 20 min. Then, the fiber was withdrawn into the needle and injected into the GC-FID system. The chromatogram obtained can be seen in Figure 3.9. The red lines indicate the blank signal which shows a fluctuation probably due to the acetone contamination of the lab atmosphere. The acetone peak was observed at around 5.80 min.

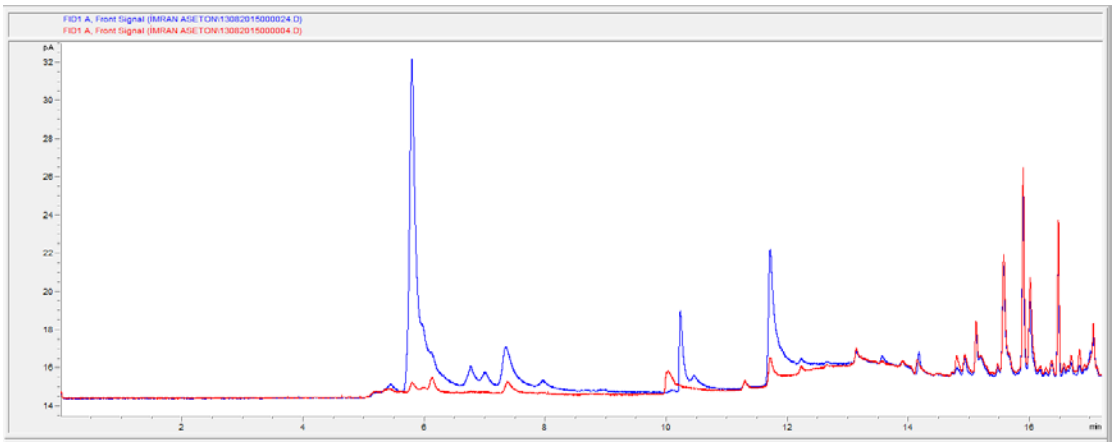

Figure 3.9 Typical HS-SPME-GC-FID chromatogram for blank and 0.3 mg/L acetone.

Considering the number of parameters those are likely affecting the signal, an experimental design was found necessary. ***Plackett Burmann Design*** is very useful in preliminary studies in order to reveal the most affecting parameters within the large number of factors. Then, ***Box-Behnken Design*** was constructed to optimize response surface points correctly. The procedure given in Experimental Section was applied and a synthetic urine solution containing $5x10^{-6}$ M acetone was used for these studies. The factors and their levels were given in Table 3.2 and the design model for this Plackett Burman Design was shown in Table 3.3. Selected factors were assessed at two levels coded as + (high), - (low).

Table 3.2 Selected factors and their levels for Plackett Burman Design.

Factor Code	**Explanation**	**(+) Value**	**(-) Value**
A	Adsorption time (min)	20	2
B	Adsorption temperature (°C)	70	40
C	Desorption time (min)	5	2
D	Desorption temperature(°C)	250	200
E	Sample volume (mL)	10	2
F	Salt effect (g)	0.1	0
G	Stirring rate (rmp)	600	0
H	pH	5.5	2

Table 3.3 Plackett Burman Design model applied for model solutions.

	A	B	C	D	E	F	G	H	J	K	L	Response
1	20	70	2	250	10	0.1	0	2	-1	1	-1	146
2	2	70	5	200	10	0.1	600	2	-1	-1	1	9.8
3	20	40	5	250	2	0.1	600	5.5	-1	-1	-1	69
4	2	70	2	250	10	0	600	5.5	1	-1	-1	9
5	2	40	5	200	10	0.1	0	5.5	1	1	-1	25.3
6	2	40	2	250	2	0.1	600	2	1	1	1	17.9
7	20	40	2	200	10	0	600	5.5	-1	1	1	71
8	20	70	2	200	2	0.1	0	5.5	1	-1	1	44.5
9	20	70	5	200	2	0	600	2	1	1	-1	143.9
10	2	70	5	250	2	0	0	5.5	-1	1	1	17
11	20	40	5	250	10	0	0	2	1	-1	1	63.4
12	2	40	2	200	2	0	0	2	-1	-1	-1	9.8

The main effects of the factors were calculated in relation with the parameter of peak areas obtained from the chromatograms. Twelve experiments were performed and standard effects of the factors for acetone were given in Table 3.4.

Table 3.4 Standard Effects of the factors for acetone.

Term	**Acetone**
A- Adsorption time	74.8
B- Adsorption temperature	19
C- Desorption time	5.0
D- Desorption temperature	3
E- Sample volume	3.7
F- Salt effect	-0.3
G Stirring rate	2.4
H- pH	-25.8
J-Dummy-3	-3.1
K-Dummy-1	35.9
L-Dummy-2	29.9

Resulting parameters were evaluated by using Pareto charts and they were found to be agreement with the resulting values obtained from the regression line plotted through Excel program. For each analyte values of "Probability > F" less than 0.0500 indicate model terms and the model are significant.

As can be seen from the Pareto charts figure (Figure 3.10) three parameters have significant effect on the peak area of the chromatographic analysis namely; adsorption time, adsorption temperature and pH. These three parameters were exploited to construct Box-Behnken for response surface optimization of acetone. The other parameters kept constant as desorption time: 5 min, desorption temperature: 250°C, sample volume: 10 mL, stirring rate: 600 rpm. The addition of salt was omitted as discussed earlier.

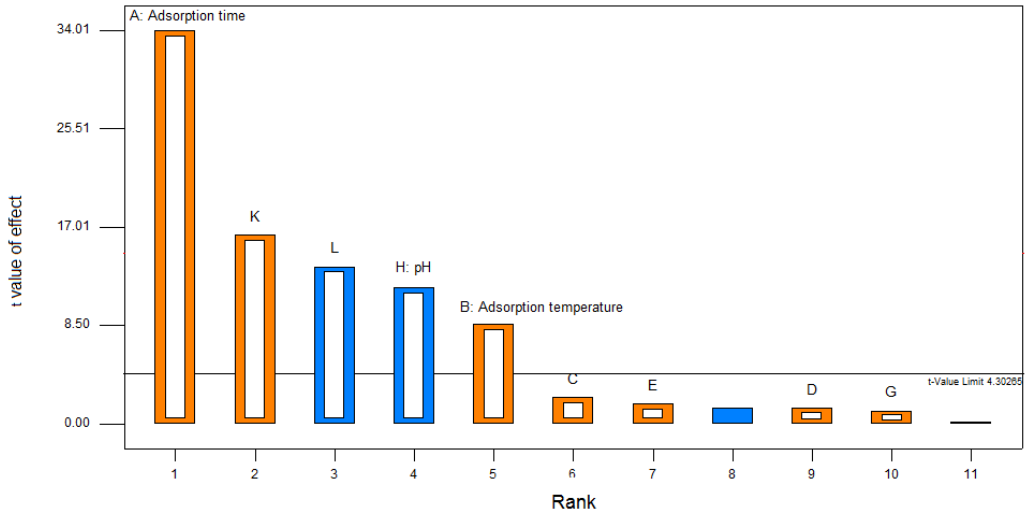

Figure 3.10 Pareto chart for acetone determination with GC-FID system.

In this part of the study, these three factors were selected to be applied for Box-Behnken Design. The examined levels of the factors are given in Table 3.5 where A is the adsorption time, B is the adsorption temperature, C is the pH. The number of experimental points (N) is defined by the Eq. 1.3 given in Introduction Part ($N = 2\ K\ (K\text{-}1) + C_0$) where, K is the number of variables and C_0 is the number of center points. In this study, K and C_0 were set at 3 and 2, respectively, which meant 13 experiments had to be performed. The obtained data were evaluated by Analysis of variance (ANOVA) test for acetone. The ANOVA results of acetone can be seen in Table 3.6.

According to these results, The Model's F-value of 3204.5 implies that the model is significant and there is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC, A^2, B^2, A^2B, A^2C, AB^2 are significant model terms. Values greater than 0.1 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model.

The experimental data for acetone showed a good accordance with the second order polynomial equations. The coefficient of determination R^2 (1.0000) were more than 0.95 for the areas; all were statistically acceptable. Adjusted and predicted R^2 were calculated as 0.9997 and 0.9904, respectively. The "Pred R^2" of 0.9904 is in reasonable agreement with the "Adj R^2" of 0.9997. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable.

Table 3.5 Experimental variables and levels of Box-Behnken Design.

Run	A (min)	B (°C)	C (pH)	Peak Area
1	2	40	3.75	13.9
2	20	40	3.75	43.9
3	2	70	3.75	20.1
4	20	70	3.75	37.1
5	2	55	2	19.5
6	20	55	2	57.7
7	2	55	5.5	16.8
8	20	55	5.5	50.5
9	11	40	2	46.9
10	11	70	2	24.3
11	11	40	5.5	29.2
12	11	55	3.75	42.6
13	11	55	3.75	41.2

Table 3.6 Analysis of variance table for Response Surface Reduced Cubic Model for Acetone.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	2467.46	11	224.31	3204.50	0.0138
A-Adsorption time	1296.00	1	1296.00	18514.29	0.0047
B-Adsorption temperature	24.01	1	24.01	343.00	0.0343
C-pH	0.000	1	0.000	0.000	1.0000
AB	42.25	1	42.25	603.57	0.0259
AC	4.84	1	4.84	69.14	0.0762
BC	309.76	1	309.76	4425.14	0.0096
A^2	127.57	1	127.57	1822.50	0.0149
B^2	149.21	1	149.21	2131.60	0.0138
ABC	0.000	0			
A^2B	10.13	1	10.13	144.64	0.0528
A^2C	12.50	1	12.50	178.57	0.0476
AB^2	78.13	1	78.13	1116.07	0.0191
Residual	0.070	1	0.070		
Cor total	2467.53	12			

According to our results a ratio of 171.915 indicates an adequate signal and this model can be used to navigate the design space. Final equation in terms of coded factors for acetone was calculated as:

$$Response = 42.8 + 18A - 2.45B + C - 3.25AB - 1.10AC + 8.8BC - 6.75A^2 - 7.3B^2 + 2.25A^2B - 2.5A^2C - 6.25AB^2$$

Typical response surface for extraction was shown in Figure 3.11 for acetone. According the results, adsorption time of 19.6 min, adsorption temperature of 49.8^{o}C and pH of 2.14 was calculated as optimum experimental conditions.

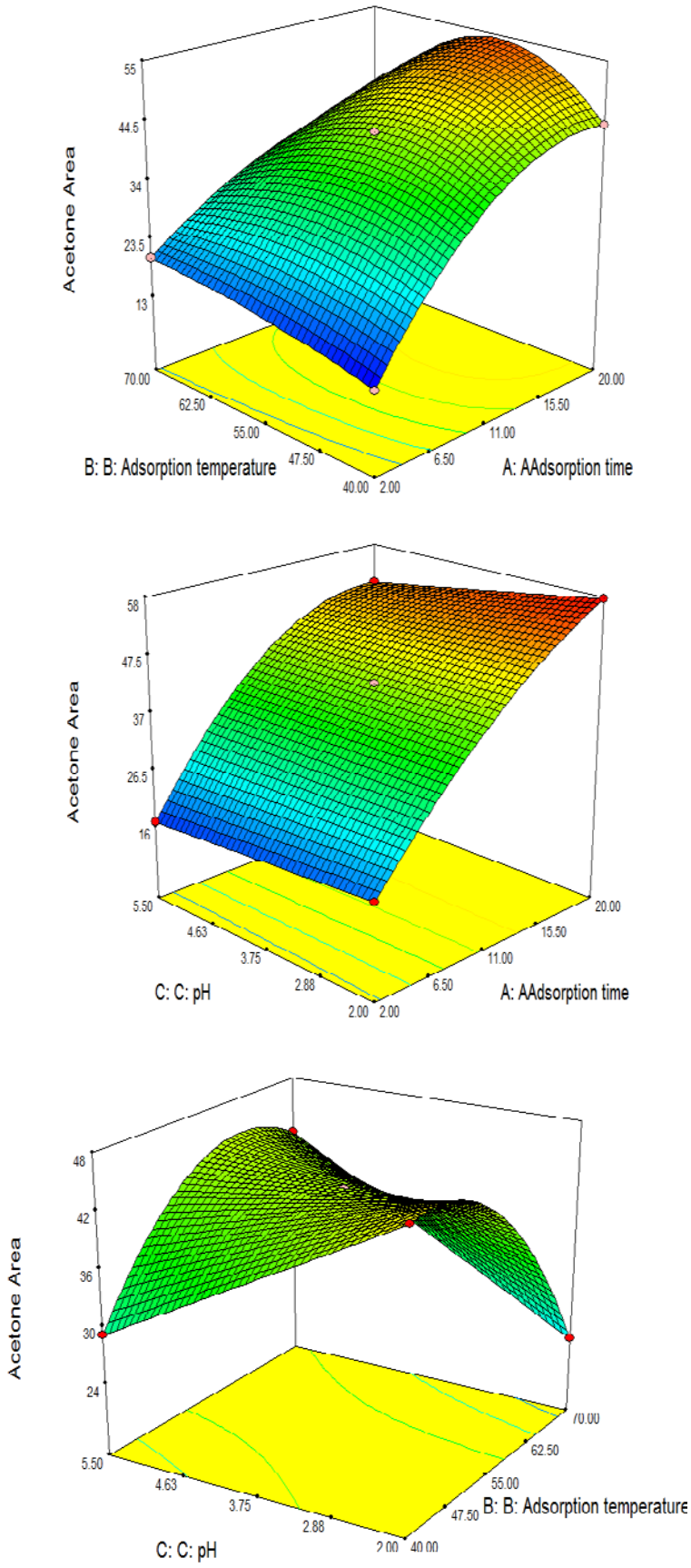

Figure 3.11 A typical response surfaces and related contours for acetone determination.

Calibration studies were conducted under these conditions and obtained graphs were given in Figure 3.12. As can be seen from the figure, the calibration curve was found linear ver a wide range being 0.004- 0.4 mg/L concentration range. The correlation coefficient was calculated as 0.9982 indicating a good correlation.

Analytical charcteristics of the method developed were given in Table 3.7. The reproducibility of the method was calculated as 4.7% As can be concluded from the figures, the LOD level was calculated as 0.006 mg/L which is higher than the starting point of linear range. This can be explained as the fluctuations in the baseline signal. The acetone level in the lab atmosphere daily changes and this change is reflected in the response.

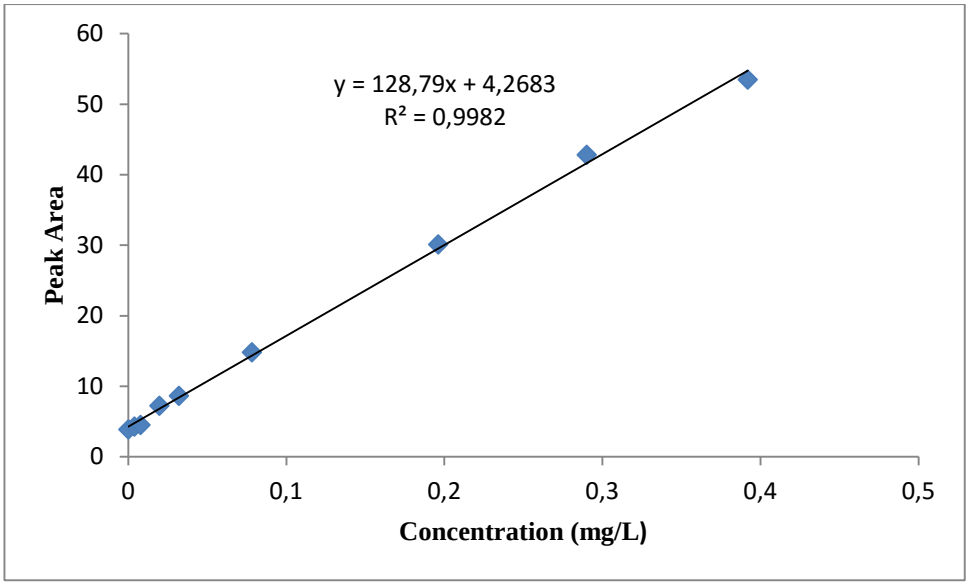

Figure 3.12 Calibration graph for acetone determination by HS-SPME-GC-FID system.

Table 3.7 Analytical characteristics of the HS-SPME-GC-FID method for acetone determination.

Analytical Parameter	Value
LOD (mg/L)	0.006
LOQ (mg/L)	0.020
%RSD	4.7
R^2	0.9982
Linear Range (mg/L)	0.02-0.4

4. CONCLUSION

In this thesis, it was aimed to develop a chromatographic method for sensitive and selective acetone determination in gaseous and in synthetic urine for searching any potential to be applied to breath and real urine samples.

Considering that acetone in biological matrixes is in trace level, an enrichment technique was used. For this purpose, lab made and commercial solid phase micro extraction (SPME) fibers were utilized and among them, best results were obtained with CAR/PDMS (75 µm) fiber. Gas chromatography systems equipped with a mass and flame ionization detectors were utilized for the quantification.

For acetone determination in gaseous sample, the standards were prepared by injecting acetone in a sealed glass vessels and diluting with air. The temperature of the samples was kept 40^{o}C close to human breath temperature. The calibration graph was constructed with a correlation coefficient of 0.9981 and LOD was calculated as 0.0126 mg/L from the calibration graph.

Considering the volume studied is very low simply being 20 mL, this method has a potential to be used for breath sample since each breath has an average volume of 500 mL and subsequent breathing at a certain rate will help to pre-concentrate the acetone content to more sensitive levels.

In synthetic urine samples, two instrumental approaches were tested; the GC-MS system with a PA fiber (85 µm) was utilized for the head space analysis. Experimental parameters namely; sample volume, adsorption time and temperature and salt amount were optimized by using one-variable at time method. The sensitivity of the method was not satisfactory considering the trace amount of acetone in the biological matrixes. Therefore, GC-FID method development was carried out with this instrument using CAR/PDMS.

Experimental parameters were optimized by factorial design. Among the parameters studied, three parameters had a significant effect on the peak area of the chromatographic analysis namely; adsorption time, adsorption temperature and the pH. These parameters were exploited to construct Box-Behnken for response surface optimization of acetone.

According the GC-FID results, adsorption time of 19.6 min, adsorption temperature of 49.8^{o}C and pH of 2.14 was found as the optimal conditions. From the calibration curve the LOD was calculated 6.0 ng/mL and the method was found sensitive enough to determine the acetone level in urine samples.

Overall results have demonstrated that HS-SPME methods developed for acetone in gaseous and aqueous samples have a great potential for application. Future studies will be dealing with applications of the methods for real samples.

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