**TC**
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**(Ph.D. THESIS)**

# CHEMOMETRIC EVALUATION AND OPTIMIZATION OF BLOOD ALCOHOL DETERMINATION BY USING GAS CHROMATOGRAPHY

**Pembe Öztürk**

**Chemistry Department**
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**Supervisors :**
**Prof. Dr. F. Nil Ertaş**
**Öğr. Gör. Dr. Hasan Ertaş**

**Bornova, İZMİR**

# ONAY SAYFASI

Sayın **Pembe Öztürk** tarafından **Doktora** Tezi olarak sunulan "***Chemometric Evaluation and Optimization of Blood Alcohol Determination by Using Gas Chromatography*"**adlı bu çalışma "Lisansüstü Eğitim ve Öğretim Yönetmeliği'nin ve Enstitüsü yönergesinin ilgili hükümleri dikkate alınarak

**Jüri Başkanı :.**Prof. Dr. F. Nil Ertaş.....

**Raportör Üye :** Doç. Dr. Durmuş Özdemir.....

**Üye:** Prof. Dr. Emür Henden.....

**Üye:** Prof. Dr. Çetin Güler…….....

**Üye:** Doç. Dr. Kenan Dost.....

tarafından değerlendirilmiş olup, yapılan tez savunma sınavında aday oybirliği/oyçokluğu ile başarılı bulunmuştur.

# ÖZET

## Kan Alkol Düzeylerinin Gaz Kromatografisi ile Tayininde Kemometrik Optimizasyon ve Değerlendirme

**Öztürk, Pembe**
1. Danışman : Prof.Dr. F. Nil Ertaş
2. Danışman : Öğr. Gör. Dr. Hasan Ertaş
165 sayfa

Kronik veya akut alkol zehirlenmelerinin birçok ciddi patolojik sorunla ve çoğu suça yönelik davranışlarla ilişkili olduğu bilinmektedir. Kandaki uçucu bileşiklerin bir arada tayini adli tıp, klinik ve acil durumlarda gereklidir. Bu amaçla kanda alkol düzeyleri için geliştirilen yöntemler arasında çoğunlukla gaz kromatografisi yeğlenir.

Bu tez kapsamında, metanol, etanol, isopropanol, aseton, n-propanol ve n-butanol standartlarının bir arada tayini statik üst boşluk ve üst boşluk-katı faza mikro özütleme teknikleri iki farklı kolon kullanılarak denenmiştir. Örnek enjeksiyonu ve kromatografik koşulların taranması ve optimizasyonu her iki teknik için faktöriyel tasarım, Plackett-Burman tasarımı ve Merkezi Kompozit Tasarım uygulanmıştır. İncelenen faktörlerin bağıl etkilerinin yansıtılması ve koşulların optimizasyonu amacıyla Pareto ve yanıt yüzeyli grafikler kullanılmıştır.

Statik üst boşluk tekniği için HP 20M kolonu kullanılarak, optimize edilen koşullarda yöntem validasyonu gerçekleştirilmiştir. Yöntemin metanol, etanol ve aseton için geniş bir derişim aralığında doğrusal yanıt verdiği, tekrarlanabilirlik gösterdiği ve düşük belirtme ve saptama sınırlarına sahip olduğu deneysel bulgularla saptanmıştır.

Tüm verilerin ışığında Rtx BAC 1 kolonunun HP 20M kolonuna göre alkoller ve aseton için daha seçimli ve duyar olduğu ve daha kısa sürede analizin tamamlandığı görülmüştür. Öte yandan üst boşluk katı faza mikro özütleme tekniğinin statik üst boşluk tekniğine göre daha duyar ve seçimli yanıtlar verdiği saptanmıştır.



**Anahtar sözcükler:** kan, etanol, metanol, gaz kromatografisi, üstboşluk

## ABSTRACT

### Chemometric Evaluation and Optimization of Blood Alcohol Determination by Using Gas Chromatography

**By Öztürk, Pembe**
Supervisor : Prof.Dr. F. Nil Ertaş
Co-supervisor : Lecturer Dr. Hasan Ertaş
165 pages

Chronic or acute alcohol intoxication has been demonstrated to be connected with many serious pathologies and most criminal offences. Simultaneous determination of volatile compounds in blood is required in clinical, medico-legal and emergency cases. Among the methods developed for determination of blood alcohol levels; gas chromatography is the method of choice.

In this thesis, the simultaneous determination of methanol, ethanol, isopropanol, acetone, n-propanol and n-buthanol was achieved by using static headspace and headspace-sold phase micro extraction techniques in connection with two different gas chromatographic columns. Screening and optimization of sample injection and chromatographic conditions were performed by the application of experimental designs for both injection techniques. Factorial Design, Plackett-Burman Design and Central Composite Design were used for this purpose. Pareto charts and surface plots were used in order to optimize the conditions and reflect the relative effects of the factors investigated.

Method validation using HP 20M column for static headspace technique was performed at optimum conditions obtained from the experimental designs. The method demonstrated good precision, wide working ranges for methanol, ethanol and acetone, good linearity, low detection and quantification limits.

Evaluation of overall results revealed that Rtx BAC 1 column is more selective and sensitive to the alcohols and acetone with less run time in comparison to HP 20M column. On the other hand, HS-SPME technique was found more sensitive and also selective than static headspace technique.



**Keywords :** blood, ethanol, methanol, gas chromatography, headspace

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## LIST OF SYMBOLS

| | |
|---|---|
| $A$: | Peak area |
| $a$: | İntercept |
| $\alpha$: | Levels of star points in central composite design |
| $\boldsymbol{b}$ : | Vector of estimated parameters |
| $b_s$: | Slope of a calibration curve |
| $\beta$: | Phase ratio in headspace |
| $\hat{\boldsymbol{c}}$: | Estimated concentrations |
| $C_0$: | Initial concentration of a given analyte in the sample. |
| $C_G$: | Concentration of analyte in gas phase |
| $C_L^0$ : | Initial concentration of the analyte in liquid phase |
| $C_L$: | concentration of analyte in liquid phase |
| $C_s$ : | Concentration of alcohol standard solution |
| $F_{table}$: | Tabulated F value |
| $H_{IS}$: | Peak height of IS from the "unknown" blood |
| $H'_{IS}$: | Peak height of IS from known ethanol solution |
| $H_x$: | Peak height of ethanol from "unknown" blood |
| $H'_x$: | Peak height of ethanol from known ethanol solution. |
| $K$: | Distribution coefficient |
| $K_{fs}$: | Fiber coating/sample matrix distribution constant |
| $M_o$: | Original weight of analyte in condensed phase |
| $m$: | Number of replication in calibration |
| $N$: | Total number of runs in Plackett-Burmann design with replicates |
| $N_f$: | Number of experiments in full factorial design |
| $n_{cal}$ | *N*umber of points in calibration plot. |
| $n_{ex}$: | Number of moles extracted by the coating |
| $n$: | Number of replication |
| $p$: | Probability level |
| $\hat{r}$ . | Estimated response |
| $\boldsymbol{r}$: | Vector of residual values |
| $R_t$: | Retention times of an analyte |
| $R_s$: | Peak height ratio of an alcohol standard to that of IS |
| $R_u$: | Peak height ratio of the specific alcohol in the sample to that of internal standard |
| $s$: | Standard deviation |
| $s^2$ : | Variance |

$s_i^2$: Average variance of individual run
$\boldsymbol{S}$: Matrix of pure standards
$SS_{residual}$: Sum of squares of residuals
$SS_{replicates}$: Sum of squares of replicates
$s^2_{LOF}$: Variance of lack of fit
$s^2_{residual}$: Variance of residual
$s^2_{parameters}$: Variance of each parameter
$s_{xo}$: Estimated standard deviation of $x_o$
$t_{experimental}$: Experimental student t values.
$V$: Fixed volume of the container
$V_{effect}$: Variance of each effect
$V_f$ : Fiber coating volume
$V_L$: Volume of the solution
$V_G$: Volume of the gas phase
$V_s$ : Sample volume
$\boldsymbol{X}$ : Design matrix
$x_o$ concentration value interpolated from calibration graph
$Y_{replicates}$ Response of replicates
$\overline{Y}$ The mean of response of replicates.
$\boldsymbol{y}$ : Vector of responses for validation mixtures
$\overline{y}_+$ : Mean of positive effects
$\overline{y}_-$ : Mean of negative effects
$\hat{y}$ : Estimated response
$\hat{\boldsymbol{y}}$ : Vector of estimated responses
$y_{o:}$ Experimental value of y

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| AcH: | Acetaldehyde |
| ADH: | Alcohol dehydrogenase |
| AlDH: | Aldehyde dehydrogenase |
| ANOVA: | Analysis of Variance |
| BTEX: | Benzene, toluene, ethylbenzene, xylenes |
| CE: | Capillary Electrophoresis |
| COT-GC: | Cryogenic oven-trapping gas chromatography |
| CV: | Coefficient of variability |
| DCLS: | Direct Classical Least Squares |
| df: | Degrees of freedom |
| DVB: | Divinylbenzene |
| EtOH: | Ethanol |
| FID: | Flame Ionization Detector |
| GC: | Gas Chromatography |
| GC-MS: | Gas chromatography-mass spectrometry |
| HPLC: | High Performance Liquid Chromatography |
| HS: | Headspace |
| HS-LPME: | Headspace-Liquid Phase Micro Extraction Method |
| HS-SPME: | Headspace–Solid Phase Micro Extraction Method |
| IprOH: | Isopropanol |
| IS: | Internal standard |
| LOD: | Limit of Detection |
| LOF: | Lack of fit |
| LOQ: | Limit of Quantitation |
| MeOH: | Methanol |
| MLR: | Multiple linear regression |
| OVAT : | One-variable-at a time method for screening |
| PDMS: | Polydimethylsiloxane |
| RMSE: | Root mean square error |
| RMSEP: | Root mean square error of prediction |
| RSD: | Relative Standard Deviation |
| SEC: | Standard error of calibration |
| SEP : | Standard error of prediction |
| S/N: | Signal/noise ratio |
| TCA : | Trichloroanisole |
| TCD: | Thermal Conductivity Detector |

# 1. INTRODUCTION

The use of alcoholic beverages is probably the most ancient and widespread social habit in western culture. Chronic and/or acute alcohol intoxication has been demonstrated to be connected with many serious pathologies, suicides, homicides, fatal road and industrial accidents and most criminal offences (Tagliaro, 1992; Rosano, 2000). As the analytical chain includes the choice of the sample, its collection and storage and the choice of the analytical approaches, these important aspects which are strictly connected with the analytical strategy must be considered.

Ethanol (EtOH) is almost the only alcohol occuring in the human diet. It is found in beers, ales (3-6%, v/v) and wines (10-12%, v/v), by natural fermentation; higher concentrations (35-50%) can be achieved only by distillation, because fermentation is inhibited by alcohol concentrations exceeding 14-16% (Tagliaro et al., 1992).

Methanol (MeOH) is mainly used as an industrial solvent. It is found in small percentages in wines (up to 0.5% of EtOH) and in some distillates (up to 1% of EtOH). However, it has been used illegally as a cheap substituent for EtOH to adulterate alcoholic beverages, with deleterious and sometimes fatal consequences (Yaycı et al 2003). Occasionally, chronic alcoholics, unaware of its toxicity, consume products containing MeOH (Liu et al., 1999).

Isopropanol (IprOH) is a commonly used antiseptic. It does not have the toxicity of methanol but produces greater central nervous system depression than EtOH (Levine, 1999; Dimaio and Dimaio, 2001).

### 1.1. Metabolism of Alcohol

Alcohol is rapidly absorbed in the stomach and intestine after oral ingestion. About 75% of a dose of ethanol is absorbed in the small intestine, because the surface area available for contact with blood is far greater in the small intestine than in other parts of the gastrointestinal tract (Levine, 1999). After absorbtion, alcohol, lacking any binding to proteins, distributes fairly evenly throughout all tissues and fluids of the body, in relation to the water content. More than 90% of an alcohol dose is metabolized in liver, while the reminder is excreted unchanged with breath, urine, sweat and faeces (Tagliaro, 1992; Levine, 1999). It is reported that, peak ethanol concentrations can be reached within 15-90 minutes. Blood half-life is 2-14 hours with zero order kinetics (Rosano, 2000). In nonhabituated individual, blood alcohol concentration can be expected to fall at the rate of approximately 15 mg/dL/hr. Chronic drinkers may metabolize EtOH at a much greater rate, owing to induction of alcohol dehydrogenase in the liver (Ellis et al., 1992).

Alcohol is metabolized to acetaldehyde by a NAD-dependent enzyme, alcohol dehydrogenase (ADH). Acetaldehyde is further metabolized to acetic acid; this conversion is faciliated by the enzyme aldehyde dehydrogenase (AlDH) (Figure 1.1). A genetic polimorphism of both ADH and AlDH, with phenotypes differing in catalytic features has been reported, which could explain the marked variability of

individual and inter-racial alcohol tolerance (Tagliaro et al., 1992; Levine, 1999).

1. ethanol + NAD → acetaldehyde + NADH

2. acetaldehyde + NAD → acetate + NADH

**Figure 1.1** Primary pathway for ethanol metabolism.

The absorbtion and distribution of MeOH in the body are similar to those of EtOH; the same enzymes oxidise MeOH to formaldehyde and subsequently to formic acid. Formic acid can be used as the marker of MeOH intoxication. The main differences consist in a lower affinity, about 1/100 in oxidation rate of ADH for MeOH, in comparison to EtOH (Tagliaro et al., 1992). The peak concentration is similar with ETOH. Blood half-life is 2-24 hours with zero order kinetics (Rosano, 2000).

IprOH, is slowly converted, probably by ADH, into its major metabolite, acetone. This metabolite is a depressant of the central nervous system like IprOH itself and other alcohols. Blood peak concentration is also like EtOH, blood half-life is 3-6.5 hours with first-order kinetics (Tagliaro et al., 1992; Rosano, 2000).

### 1.2 Toxicology of Alcohol

From a pharmacological point of view, alcohol is a primary depressant of the central nervous system; the apparent stimulation, which occurs at moderate blood alcohol levels, results from the impairment of inhibitory mechanisms operating in the brain. At higher concentrations, the depressive action of alcohol is generally observed with a progressive reduction of alertness, typical drunkenness and, finally, a state of pharmacological anaesthesia.

Differently from normal anesthetics, the window between anesthetic and lethal alcohol levels is fairly narrow. In acute intoxications, death is caused by irreversible depression of respiration with hypoglycaemia, caused by depression of neoglucogenesis. Among the psychiatric and neurological consequences of chronic alcohol intoxication, sleep disturbances, mental deteriation, psychoses, polyneuritis can be given. On the other hand, hepatitis, cirrhosis, mouth, larynx and eausephagus cancers, cardiomyopathy, arrithmia, fatal alcohol syndrome etc. are some of the pathologies caused by alcohol. EtOH interferes with the metabolism of many drugs by competition for the same metabolic pathways and through other mechanisms (Tagliaro et al., 1992; Rosano, 2000, Zuba et al., 2002a).

Acute methanol intoxication is associated with less severe narcotic effects, with a typical symptom latency of 6-36 hours. After this, headache, vertigo, dyspnea and vomiting appear. The most relevant damage concerns sight; inflammation followed by atrophy of the retina with blindness. Coma, respiration failure, pancreas necrosis, acidosis due to formic acid formation and death may occur. Blood MeOH levels of about 0.4 g/L are generally considered as life threatening. Simultaneous administration of EtOH can delay and reduce the MeOH intoxication syndrome, because of competetion of both alcohols for ADH (Tagliaro et al., 1992). In humans EtOH is used for in the treatment of methanol intoxication, since EtOH can inhibit the oxidation of methanol to its toxic metabolite. The blood concentration of EtOH is maintained at ≈ 0.1% to ensure optimal competetion with MeOH for AlDH.

Faci et al. (1998), have also indicated that chloral hydrate enhances EtOH-induced inhibition of MeOH oxidation in mice. The symptomatology of IprOH intoxication resembles that of EtOH, but lasts longer and is characterized by severe gastritis and vomiting with ketoacidosis (Tagliaro et al., 1992). Poisoning by toxic volatile alcohols is relatively uncommon, but early detection and quantification are essential to effective treatment (Smith, 1984; Levine, 1999).

### 1.3 Interpretation of Alcohol Levels

#### 1.3.1 Concentration units of EtOH used in liquid samples

Clinical toxicology; mg/dL,

Forensic toxicology; percent gram (w/v)

Literature; mmol/L

(100 mg/dL = 0.10 % (w/v) = 21.7 mmol/L) (Rosano, 2000).

#### 1.3.2 Blood alcohol levels

0.5 g/L is considered to be the threshold of the symptomatology

0.8-1.0 g/L the limit of severe impairment of body reflexes (and the level of most statutory limits).

-The statutory limit of blood-alcohol concentration as evidence of impairement of driving is 0.5 g/L in Turkey (Demiralp, 1997).

1.5 g/L the concentration above which intoxication is evident

4-6 g/L corresponds to the threshold of fatal concentrations.

The distribution of alcohol in the body fluids and tissues, regulated

essentially by distribution law, related with their water contents. Serum water content is 12-20% higher than whole blood. Plasma/whole blood ratio of alcohol concentrations averages 1.12-1.18, whereas the plasma/serum ratio averages 1.00. Statututory limits generally refer to "blood level", which is almost universally interpreted as whole blood. The alcohol content of whole blood is a weighted average of the alcohol concentrations in plasma, erythrocytes, leukocytes, and platelets (Tagliaro et al., 1992; Rainey, 1993; Williams, 1998, Levine, 1999).

Differences in alcohol concentrations between arterial blood and venous blood are observed depending on the absorbtion status of the individual. During the absorbtion period, alcohol is more concentrated in the arterial and capillary blood than in the venous blood. During this time, the blood from an arm vein will not reflect truly the concentration of EtOH supplied to the brain, where its effects are produced. On the other hand, in post-absorbtive state, the concentration gradients between capillaries and tissues are reversed, with a consequent higher alcohol level in the venous blood (Jones, 1997; Levine, 1999).

In living persons alternative biological samples for alcohol analysis are breath, urine and saliva. The ratio of alcohol concentrations; urine/whole blood is 1.3. This ratio is obtained in the elimination period. Urine EtOH concentrations does not represent the true level, so they are not used for medicolegal purposes. Urine can be used for monitoring alcohol abuse in the workplace and used in rehabilitation programs (Tagliaro et al., 1992; Correa and Pedroso, 1997; Williams, 1998; Levine, 1999).

Identifying the presence of a drug or poison in two or more different biological fluids is a well proven strategy; by contrast, collecting more than one biological specimen for analytical toxicology is uncommon (Jones and Helander, 1998). The ratio of ethanol concentrations in a large variety of fluids and tissues to the blood ethanol concentration has been widely investigated as mentioned above. Table 1.1 provides a guide to the average specimen to blood ratios.

**Table 1.1** Average ratios for specimen to blood ethanol concentrations (g/dL) (Levine, 1999).

| Specimen | Ratio |
|---|---|
| Saliva | 1.1 |
| Vitreous humor | 1.2 |
| Bile | 1.0 |
| Spinal fluid | 1.1 |
| Liver | 0.6 |
| Kidney | 0.7 |
| Brain | 0.8 |

The determination of EtOH in breath is the main alternative to the direct analysis of blood. Breath test devices can use chemical, electrochemical, infrared absorbtion and thermochemical methods. Commercially available evidential breath testing devices are based on the relationship that the amount of EtOH in 2100 mL of breath equals the amount of EtOH in 1 mL of blood at 34°C which is the temperature of expired air. This 2100:1 ratio was based on early scientific studies; more recent works indicates that 2300:1 is a better representation of the actual breath to blood ratio (Levine, 1999).

Although the constancy of this ratio and the practical usefullness of breathe analysis to control blood alcohol concentrations is still debated among forensic toxicologists (Tagliaro et al., 1992).

MeOH level; low serum or plasma concentration of methanol (<5 mg/dL) may result from EtOH abuse or from consumption of denatured EtOH or congeners in alcoholic beverages. MeOH concentrations >25 mg/dL are generally considered toxic, but the degree of toxicity is better related to the formate concentrations in blood (Rosano, 2000).

### 1.4. Determination of Blood Alcohol

#### 1.4.1 Blood sampling and stroge

Important issues in blood sampling and storage are discussed;

-The skin should not be cleaned with EtOH or IprOH before venipuncture.

-For prevention of EtOH loss, tightly closed (sealed) containers must be used.

-To avoid from clotted or semiclotted samples, blood must thoroughly mixed with the anticogulant (EDTA, potassium oxalate) in the tube.

-The main factors affecting alcohol determination in stored blood are the duration and temperature of storage.

-During the storage, some microorganisms which use EtOH may be produced. A preservative (0.5% or 1% w/v NaF) is added to prevent this formation.

-If the containers sealed properly, blood can be kept at room temperature (22-29 °C) or in refrigerator (0-3 °C) for 14 days without being an effective EtOH loss. The common toxic volatile alcohols are also sampled and stored similarly, because of their high volatility.

-According to Somogyl et al. (1986), blood containing 0.5% of sodium flouride maintained constant EtOH levels of ≥2 g/L for 3 months of storage at 4 °C, even if containers opened several times; levels ≤1 g/L decreased rectilinearly with time. In the absence of preservative, opening of the containers caused substantial reduction in the sample stability (Tagliaro et al., 1992).

-EtOH loss due to elevated temperatures is the consequence of oxidation to acetaldehyde (Tagliaro, 1992; Williams, 1998; Levine, 1999).

### 1.4.2 Analysis Methods

Many methods have been developed for alcohol determination in various matrixes. Among these methods chromatographic techniques are the method of choice. Therefore, these methods can be grouped as chromatographic and non-chromatographic methods.

#### 1.4.2.1 Non-chromatographic methods

***Chemical methods:*** Due to its volatility, alcohol can be easily seperated from a biological matrix by distillation or microdiffusion. Microdiffusion is performed on fluids and tissues with a Conway cell, which is porcelain or glass dish containing two concentric wells. To the outer well is added the specimen and to the inner well, a color reagent, combination of potassium dichromate and sulfuric acid.

The cell is covered and allowed to wait at room temperature. EtOH and other volatile substances will diffuse out of the specimen into the center well where the chemical reaction occurs. In the presence of EtOH or any other volatile reducing substance, dichromate is converted to the chromic ion, with a change of color from yellow to green. This color test can be observed visually, making this a simple screening method or alternatively, can be made semiquantitative by prepearing ethanol standards and treating them in a similar fashion to the case specimen. Spectrophotometric measurements are made at 450 nm. This method is nonspecific for EtOH, aldehydes and ketones are the interferring substances (Levine, 1999).

***Enzymatic methods:*** In clinical laboratories, mostly alcohol dehydrogenase (ADH) is used for EtOH analysis. This method uses the enzymes involved in alcohol metabolism and absorbance of NADH produced is measured at 340 nm. This is directly related with the EtOH content of the sample, so quantitave results are possible. EtOH levels of serum, urine, fresh blood, and postmortem blood specimens can be obtained by automated analysers working with this principle (Levine, 1999).

***Breath test analyzers:*** Breath-testing device was developed by Robert Brokenstein in 1954. Some selected types of these analyzers are given below.

***Infrared (IR) Spectrophotometry:*** Absorbtion of IR energy between 800 and 4000 nm by some of the bonds caused IR technology to be used in evidential breath-test devices.

Ethanol has several types of bonds: C-H, C-C, C-O, and O-H bonds. Selecting a wavelength where these bonds specifically absorb energy, allows identification and quantification of ethanol in breath. Since many compounds have these type of chemical bonds, interference with EtOH analysis is possible. Several evidential breath-testing devices use IR technology, the theory of the different instrumentation is the same.

An alveolar air sample enters a cell where it is irradiated with IR energy. If EtOH is present in the sample, it will absorb some of the IR energy in relation to the amount of ethanol which is in the sample. This means that less energy reaches the detector in comparison to an air sample that does not contain EtOH. By calibrating the instrument with simulator solutions of known EtOH concentration, the EtOH concentration in the subject's breath can be obtained (Levine, 1999).

***Electrochemical oxidation:*** The oxidation of EtOH to acetic acid can be used to measure the alcohol concentration in breath samples. This oxidation is performed by a fuel cell, which consists of two platinum-coated conduction electrolyte layers. The conversion of EtOH present in breath to acetic acid produces a current. The current flow generated is directly proportional to the amount of EtOH present in the sample.

The fuel cell will react with other alcohols such as MeOH and IprOH but does not respond to any great extent of acetone. Fuel cell technology has been used for many years in breath-testing devices (Levine, 1999).

### 1.4.2.2 Chromatographic Methods

These methods include liquid and gas chromatographic techniques.

***High Performance Liquid Chromatography (HPLC):*** While the separation of aliphatic alcohols is easily accomplished by LC under reversed phase conditions, the lack of an adequately sensitive Detector has hindered the application of HPLC to blood alcohol determination (Tagliaro et al., 1992).

***Column Liquid Chromatography with Biosensor Detection (LC-Biosensor):*** An enzyme based amperometric biosensor used as a selective and sensitive detection unit in column liquid chromatography for the determination of EtOH and MeOH in biological fluids such as plasma and urine (Liden et al., 1998).

***Gas Chromatography (GC):*** For over 40 years gas chromatography has been used for blood EtOH determination and is still considered to be the reference method. GC has encountered only limited favour in clinical chemistry and emergency toxicology, but has gained widespread acceptance in forensic toxicology. The reason is mainly related to the need for specific expertise of the personnel and the assumption that the purchase and maintenance of sophisticated instrumentation dedicated only to a single class of analyte is not cost effective. In addition, the ruggedness of chromatographic methods and instrumentation is often inferior to that of modern fully automated analysers for clinical chemistry. Details of GC determination of alcohol in biological fluids are given below.

The unique possibility of simultaneous monitoring of other alcohols (and volatiles) of toxicological interest, such as MeOH and IprOH, which otherwise would be ignored, strongly supports the adoption of chromatographic alcohol analysis for clinical purposes (Tagliaro et al., 1992).

GC is an excellent technique for determining volatile molecules, such as alcohols and related compounds. Almost all GC methods for EtOH determination allow the simultaneous determination of a wide range of volatile analytes (alcohols, aldehydes, ketones, glycols, etc.). The determination of low-molecular-mass compounds like acetaldehyde, EtOH, MeOH, acetone, 1-propanol and IprOH in biological fluids may be expected to be a great importance for forensic and clinical purposes, so the procedures of EtOH determination must be sensitive enough to quantify other volatile compounds, whether on physiological levels or typical after consumption of alcoholic beverages. The concentrations of the volatile compounds, which are of interest of forensic toxicologists, range from 10 μg/L to 10 mg/L (Zuba et al., 2002b).

Methods requiring solvent extraction (e.g. with n-propyl acetate**,** n-butanol or dioxane) or distillation, are time and sample consuming and scarcely susceptible for automation. Direct injection and headspace (HS) GC are the only techniques in general use that can be fully and easily automated (Tagliaro et al., 1992).

***The interfering substances of GC alcohol methods:*** One source of error has shown to occur if the specimen has not properly prepared for gas chromatography using HS analysis.

A false-positive methanol can occur from serum (red top) tubes containing glycerin as a result of co-elution of glycerin with methanol. It is recommended that red top tıbes containing glycerin should be openned at least 24 hours prior to HS analysis. The compound, 2,3-butanediol has been shown to co-elute with ethylene glycol with some gas chromatographic techniques (Williams, 1998).

***Chromatographic determination of alcohol related compounds:***

Most GC methods for alcohols allow the simultaneous determination of acetaldehyde (AcH), but problems arise when AcH levels (roughly 1/1000 of EtOH concentrations) are to be measured in blood. It has been reported that an artificial formation of AcH during storage or due to the use of deproteinizing agents and/or elevated temperatures, unless special precautions are adopted (e.g. addition of sodium azide, sodium nitrite or thiourea, deproteinization with perchloric or sulphosalysilic acid, derivatization with semicarbazide, control of of equilibrium temperature in HS-GC. Because of the large differences in relative concentrations, a slight conversion of EtOH, which would be significant for its accurate determination, can alter completely the measurement of the true AcH levels in blood (Tagliaro et al., 1992).

## 1.5. The Techniques Used in GC Determination

### 1.5.1 Direct injection

Direct injection of blood or serum has been applied since the first applications of GC to alcohol analysis. However, there are some drawbacks of this technique.

Direct injection of the sample may spoil the column and injector; give interfering peaks and/or plugg up the syringe (Tagliaro et al., 1992; Klee and Meng, 1998; Levine, 1999). Precautions which must be taken include; diluting 1:1 the blood with an aqueous solution containing internal standard, injecting small volumes (0.5 μL) and throughly washing the syringe immediately after each injection. Then a sensitivity of 10 mg/L was reported (Jain, 1971). According to Manno and Manno (1978), additional protection from contamination by the injected dense material was obtained with a silanized precolumn glass insert filled with a silanized glass-wool plug (Tagliaro et al., 1992).

Direct injection of serum samples generally required previous protein precipitation and protection of the capillary column (methylsilicone coating) with a short glass column containing 3% OV-1 on Gas ChromQ. When sensitivity was not crucial, it was found to be convenient to use higher dilution ratios of blood in order to reduce the amount of sample and the overall amount of biological matrix loaded in the injection port. This technique also allowes direct analysis of brain homogenates. Alternatively, Triton X-100 was reported to improve the performance of the direct injection of serum, even at low sample dilutions (1:1), by acting as a protein dispersing agent (Tagliaro, 1992).

Protein precipitation, by mixing the sample with the internal standard (IS), has been proposed as a simple means of overcoming the feared problems related to the injection of whole blood top into the chromatograph. This approach has also been adopted with excellent results by workers using capillary chromatography as an alternative to high sample dilution (1:50) (Tagliaro et al., 1992).

Calculation of alcohol concentration in direct injection is given below (Jain, 1971).

$$\text{mg of alcohol/100 mL of unknown} = C_s \frac{H_x H'_{is}}{H_{is} H'_x} \qquad (1.1)$$

$C_s$ = Concentration of alcohol standard solution (mg/100 mL).

$H_x$ = the peak height of ethanol from "unknown" blood.

$H_{is}$ = the peak height of IS from the "unknown" blood.

$H'_x$ = the peak heigt of ethanol from known ethanol solution.

$H'_{is}$ = the peak heigt of IS from known ethanol solution.

### 1.5.2 Headspace Analysis (HS)

HS-GC has emerged as the method of choice for ethanol as well as other low molecular weight volatiles in body fluids for research and medicolegal purposes (Jones and Schuberth, 1989). The popularity of this analytical technique stems from the high precision, accuracy and speed of analysis. This is particularly important because results are often challenged in court (Solansky and Wylie, 1993).

Jeszenszky et al., have stated that besides the high concentration of blood alcohol, the so-called biochemical markers of chronic alcoholism are taken into account, one of them is the increased methanol level of the blood. Blood tests of alcoholics revealed that there is a correlation between congeners in alcoholic beverages and biochemical markers, specifically methanol, 1-propanol and 2-propanol elimination (Jeszenszky et al., 2000).

Headspace technique is based on the equilibrium between sample and headspace in a closed container. According to gas laws, when a volatile liquid in a dilute solution is placed in a closed container, at a given temperature, the amount of volatile in the air space above the liquid (headspace) is proportional to the concentration of the volatile liquid in the solution. Therefore, the sampling of the heated specimens and similarly treated EtOH calibrators allows calculation of the EtOH concentration in the specimen. If the specimen is diluted five to ten fold, aqueous calibrators can be used (Levine, 1999).

The amount of an analyte in the air space depends on its concentration in the original sample, its solubility (the less solubility, the more the concentration in the headspace), the relative volumes of the sample and the headspace (the more sample, the more headspace concentration), and the partition coefficient of the analyte. The partition coefficient varies with the nature of the analyte and the sample, the pressure in the container, and the temperature.

Sample and container volume are easily controlled, leaving the temperature as the external variable. For the highest reproducibility, the temperature must be closely controlled as well. However the impact of the temperature variation on analytical results may be minimal in some cases: when;

-the partition coefficient is only weakly dependent on temperature,

-the temperature is insignificant compared to the required accuracy,

-the ambient (laboratory) temperature is well controlled, and

-the temperature effect can be reduced using a relative measurement such as using an internal standard.

Internal standard helps to compensate the temperature, matrix, and sample volume variations (Klee and Meng, 1998).

HS analysis inherently prevents any contamination of the column and injector and, consequently, has always been prefered in laboratories dealing with heavy routine workloads, often coupled with aotomated data handling systems.The reproducibility is often better than in direct injection (within-run and between-run CV's are less than 1.5% and 2.5%, respectively) (Tagliaro et al., 1992).

HS techniques require larger volumes of blood sample than direct injection, but it has been observed that the dependence of the peak height on the sample volume is modest. Consequently, according to Wilkinson et al. (1975) and Cingolani et al. (1991), as little as 20-50 μL of blood has been used which is compatible with capillary blood sampling (Tagliaro et al., 1992). Advantages of HS sampling over direct injection:

-the injectors and columns do not contaminate by nonvolatiles,

-the amount of solvent is reduced, which may obscure early peaks,

-the sensitivity of volatile analytes is increased and

-analysis of volatiles in solids is possible as well as liquids (Klee and Meng, 1998).

***Drawing and collection of biological samples for HS-GC:***

Vacutainers and other securely capped containers are suggested. The use of alcohol containing cleansers should be avoided before venipuncture, because some cleansers could contain alcohols used as IS in HS-GC.

Urine, serum, plasma, and saliva can be suitably stored at 4°C for 7 days and at -20°C for many months (up to 2 years) without any loss of EtOH. Centrifuging saliva before analysis is recommended to obtain a homogenous and useful analytical sample. Whole blood samples should be assayed as soon as possible because the composition is altered by freezing and inaccuracy could result. This problem can be reduced if samples are put in headspace vials before freezing (Macchia et al., 1995).

Semiclotted or completely clotted blood samples should be avoided, even though the EtOH content can also be determined in a clotted blood sample (Senkowski and Thompson, 1990). The stability of EtOH in human whole blood, were investigated by Dubowski et al. (1997), with the addition of sodium azide as antioxidant, sodium flouride as preservative, sodium heparin and EDTA as anticoagulant. No alcohol losses during the 1-year period in blood samples containing sodium azide were observed. Samples containing 1.0% (w/v) sodium flouride and anticoagulants, but not azide, had small decrease in total alcohol content, less than 5% for one year. Since sodium azide is toxic and can accumulate highly explosive compounds, as lead azide or copper azide, requires special procedures. Consequently, the addition of sodium azide to blood control samples was found unnecessary.

Storage conditions before analysis and the headspace equilibrium temperature during analysis were important for the determination of blood ethanol concentrations. The decrease in blood ethanol concentration due to chemical oxidation resulted in a nearly equivalent increase in acetaldehyde concentration (Kristoffersen et al., 2006).

***Selection of equilibrium temperature:*** According to Chiarotti and Giovanni (1982) at temperatures above 40°C, EtOH converts to acetaldehyde. On the other hand, high temperatures increase the air-blood partition coefficient, so the sensitivity. It has been reported that the addition of sodium nitrite or sodium dithionite inhibits the conversion of EtOH to AcH at 60°C (Tagliaro et al., 1992).

Ambient headspace sampling is used for many purposes in blood alcohol analysis. This technique needs more sophisticated autosampler. Without any heating device, IS helps to compensate for temperature, matrix, and sample volume variations (Klee and Meng, 1998).

***Salting-out:*** The use of anticoagulants in HS analysis is still a matter of debate. Plain heparin would be the ideal anticoagulant because it does not ionize in solution and does not affect alcohol vapor concentration. The effect of more commonly used lithium-heparin is negligible because the amount of lithium-heparin added is small and standardized (Macchia et al., 1995).

The addition of salting-out agents such as $(NH_4)_2SO_4$ and/or NaCl to the sample solutions improved the extraction efficiency for some drugs and poisons in biological samples. The peak areas of methanol in whole blood and urine samples were 3-4 times better than those without the salts (Lee et al., 1999; Zuba et al., 2002a).

The salting-out effect increases with compound polarity. Salting out can be used not only to lower the detection limits, but also to buffer random salt concentration in body fluids (Zuba et al., 2002a).

***Selection of Internal Standard (IS):*** Internal standard is a compound of known concentration that is added to every sample including the alcohol standard. The purpose of the use of IS is to account for any variability in chromatographic conditions, such as temperature, gas flow rate, column length, that could affect the retention time of the specific alcohol of interest. The concentration of alcohol in the sample ($C_u$) is given below (Williams, 1998)

$$C_u = \frac{R_u}{R_s} C_s \qquad (1.2)$$

$R_u$ = The peak height ratio of the specific alcohol in the sample to peak height of IS

$R_s$ = The peak height ratio of an alcohol standard to that of IS

$C_s$ = The concentration of alcohol standard

Additionally to the basic requirement of high chemical similarity, the standard should elute in the chromatogram without overlap with sample signals, its retention time, however, should not differ too much from those of the analytes to be determined. Volatility and the concentration of the reference compound should be of the same order of magnitude compared to the sample components of interest.

In practice, alcohols like tert-butanol, n-propanol and IprOH can potentially meet those requirements and have been considered as the internal standards for the analysis of EtOH in protein containing solutions.

IprOH and tert-butanol were less suited from the chromatographic standpoint, since either resolution or total time needed for analysis was beyond the desired limits under the given experimental conditions. Consequently n-propanol was chosen for all further studies as IS (Strassnig and Lankmayr, 1999).

In postmortem specimens, not only EtOH is produced by microbial activity, but other volatile substances such as AcH, acetone and n-propanol are also produced (Levine, 1999).

### 1.5.3. Headspace Methods

***Ambient headspace analysis:*** This method has several advantages over fully automated and temperature controlled headspace sampling; it has few experimental variables that need to be optimized, it can be accomplished with little incremental ivestment in instrument, it can be performed with a standard GC system.

Disadvantages of this method are; results are sometimes less reproducible than with fully automated and temperature controlled headspace sampling; sensitivity is often lower due to smaller injection volume and lower sample temperature.

Ambient headspace analysis can be quite useful in qualitative analysis such as, alcohols in water/blood, residual solvents in pharmaceuticals, trichloroanisole (TCA) in wine and wine cork, BTEX (benzene, toluene, ethylbenzene, xylenes) in water and industrial solvents in water (Klee and Meng, 1998).

***Static headspace sampling:*** Static headspace sampling is a single

stage gas extraction of a volatile component from a solution. The container is held at constant temperature until the volatile analytes have equilibrated between the liquid and gas phases. The phase ratio ($\beta$) is given below;

$$\beta = \frac{V_G}{V_L} \tag{1.3}$$

$V_L$ = volume of the solution

$C_L^0$ = initial concentration of the analyte

$V$ = fixed volume of the container

$V_G$ = volume of the gas phase

The quantity of the substance extracted from the solution ($C_0$), which is expressed as peak area $A$, in the gas phase depends on the relationship of $\beta$ and the value of the distribution coefficient $K$.

$$K = \frac{C_L}{C_G} \tag{1.4}$$

$C_L$ = concentration of analyte in liquid phase

$C_G$ = concentration of analyte in gas phase

For optimal sensitivity, $K$ should be as small as possible, majority of the analyte is in gas phase, this is the principle of static headspace. The peak area is given in terms of concentration of the substance extracted from the solution ($C_0$).

$$A \cong C_G = \frac{C_0}{K + \beta} \tag{1.5}$$

With the proper selection of equilibrium conditions, primarily the parameters of equilibrium temperature and thermostating time, response of volatile components can be enhanced to determine trace concentrations in the sample. Sensitivity can also be increased by adjusting the pH, salting-out or raising the thermostating temperature (Natishan, 1998). Literature survey revealed that LOD and LOQ values found for static-headspace ethanol analyses for blood and urine samples were as given in Table 1.2.

**Table 1.2** LOD and LOQ values for ethanol analysis in biological matrix

| LOD | LOQ | Literature |
|---|---|---|
| 1.0 mg/100 mL | | Macchia et.al.., 1995; |
| 0.8 mg/100mL | 1.0 mg/100mL | Correa and Pedroso, 1997 |
| 0.37 mg/100mL | | Strassning and Lankmayr, 1999 |
| | 0.01 mg/g | Jones and Andersson, 2003 |
| 0.02 mg/100mL | 0.2 mg/100mL | Wasfi et al., 2004 |

***Elimination of matrix effects for static headspace analysis:***

***(Full Evaporation Technique)***

The criterion of full evaporation technique is described by the nearly complete transfer of analytes, not the sample itself, from the condensed matrix into the vapor phase. This criterion can be met by using high equilibration temperature and an adequate small sample size. Evaporation temperature does not reach a level which results in sample decomposition or a formation of artefacts.

In the case of full evaporation of the volatiles they will be

quantitatively transferred into the gas phase, which means, that the distribution coefficient K goes to zero. Furthermore, the volume of the residual condensed phase becomes neglectible compared to the gas phase volume in the head space vial. The sensitivity equation for static headspace analysis may be reduced to equation given below.

$$Cg = \frac{M_O}{V_g} \qquad (1.6)$$

$C_g$ = concentration of analyte in gas phase

$M_o$ = original weight of analyte in condensed phase

$V_g$ = volume of the gas phase

Consequently, the concentration of the analyte is only determined by the sample amount in the headspace vial. The sample amount which can be processed for a single analysis depends on the size of the headspace vials and is restricted by the maximum volume that allows total vaporization of the volatile sample constituents. The optimized parameters were found to be 20 µL sample volume, 5 min equilibration at 100°C, for 22.5 mL vial. These conditions allow for a quantitative transfer of the analyte into the gas phase without noticeable sample decomposition.

The application of this technique eliminates effectively the matrix effect and allows calibrating even in plain aqueous solution. The limit of detection calculated from the calibration graph was 3.7 mg ethanol/L. This value is about 10-times higher than those obtained with conventional headspace techniques (Strassnig and Lankmayr, 1999).

Conventional headspace analysis: 2 mL sample, 10 min, 60°C

Full evaporation technique: 20 μL, 5 min, 100°C

The comparison of the techniques develeoped for head space analysis is summarized in Table-1.3 (Natishan and Wu, 1998).

**Table 1.3** Comparison of headspace techniques.

| **Static headspace** | **Multiple headspace** | **Dynamic headspace (Purge and trap)** |
|---|---|---|
| Method development is simple | Extensive, lengthy method development | Extensive, lengthy method development |
| Easily automated for the analysis of large number of samples | | More sensitivive, but are not easily automated, require repeated cleaning of fragile glassware |
| Wide use with a liquid matrix containing dissolved solid sample | More applicable for undissolved solid samples | |

***Headspace–Solid Phase Micro Extraction Method (HS-SPME):***

SPME is a relatively new solventless sample preperation technique that allows simultaneous sampling, extraction, pre-concentration, and introduction of analytes from a matrix in a single procedure. This method was developed in early 1990's by Pawliszyn.

SPME uses a fused silica fibre that is coated on the outside with an appropriate stationary phase. The analytes in the sample are directly extracted to the fiber coating. The most important feature determining the analytical performance of SPME is the type and thickness of the coating material.

Direct immersion or headspace (HS-SPME) techniques are used for this extraction method. Extraction is based on a similar principle to chromatography, based on gas-liquid or liquid-liquid partitioning.

Equilibrium is attained more rapidly in HS-SPME than in immersion SPME, because the analytes can diffuse more rapidly to the coating on the fiber. In addition, HS-SPME is ideal for minimizing interferences with an analysis, and can prolong the lifetime of the fiber (Supelco, 1998).

The sampling fibres can be used for multiple times, hundreds of analyses in the case of HS analysis and dozens of times in the case of immersion analysis (Vas and Vekey, 2004).

The SPME technique can be routinely used in combination with GC, HPLC, and Capillarry Electrophoresis (CE). Volatile and semivolatile analytes extracted on the fiber are analysed by GC and GC/MS succesfully. For weakly volatile or thermally labile compounds, SPME is used in combination with HPLC and HPLC/MS (Vas and Vekey, 2004; Sigma Aldrich, 1998).

***Principles of SPME***

In SPME, a small amount of extracting phase associated with a solid support is placed in contact with the sample matrix for a predetermined amount of time (Wells, 2003). If the time is long enough, concentration equilibrium is established between the sample matrixes and the extraction phase. When equilibrium conditions are reached, then exposing the fiber for a longer time does not accumulate more analytes.

The equilibrium conditions can be described as;

$$n_{ex} = \frac{K_{f_s} V_f V_s C_0}{K_{fs} V_f + V_s} \qquad (1.7)$$

$n_{ex}$ is the number of moles extracted by the coating

$K_{fs}$ is a fiber coating/sample matrix distribution constant

$V_f$ is the fiber coating volume

$V_s$ is the sample volume

$C_0$ is the initial concentration of a given analyte in the sample.

This principle is shown in Figure 1.2.

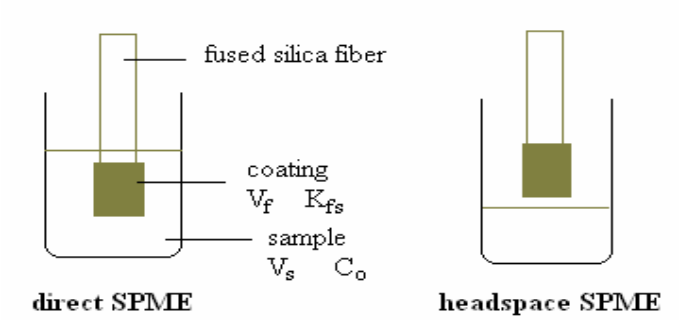


**Figure 1.2** Microextractions with SPME.

Once equilibrium has been reached, there is a direct proportional relationship between sample concentration and the amount of analyte extracted. This constitutes the basic principle for analyte quantification.

The factors that effect the extraction of analytes on the fiber in HS-SPME are; the type and thickness of coating material, the time necessary for reaching the equilibrium between headspace and sample, sample agitation, extraction time and salting out effects (Lord and Pawlisyn, 2000).

On the other hand the factors that affect the desorbtion of analytes from the fiber are stated as; the boiling point of the analyte, the thickness of the fiber coating and the inlet temperature of GC (Lord and Pawliszyn, 2000; Vas and Vekey, 2004).

Lee et al. have determined the whole blood and urine methanol and the metabolite, formic acid levels by HS-SPME/GC (1999). The septum piercing needle of the SPME device was passed through the septum. The pretreated fiber was pushed out from the needle and exposed to the headspace of the vial at 60°C for 10 min to allow adsorbtion of the compounds. The fiber was withdrawn into the needle and pulled out from the vial. It was immediately injected into the GC port and exposed in the injection port for 1.5 min for complete desorbtion of the compounds (Lee et al., 1999).

In another study, the alcohol markers of chronic alcohol users were analysed by SPME. An equilibrium distribution of the analytes is established between the sample matrix, the headspace above the sample and a polymer-coated fused silica fiber. Then the analytes are desorbed from the fiber to a capillary GC. The column detection limits are improved while the resolution is maintained (Zuba et al., 2002a).

An increase in extraction temperature leads to a growth in analyte concentration in the headspace and therefore increases the extraction rate. On the other hand, the partition coefficient of an analyte between fiber and headspace gives a decrease with the temperature. The fibers used for this purpose are polydimethylsiloxane (PDMS), PDMS/DVB, carbowax/DVB and polyacrilate coated fibers.

This method was found to be sensitive for the concentration range of 0.01-10.0 mg/L. Simultaneous determination of volatile compounds in blood may be possible concerning the type and amount of alcoholic drink consumed. The results may appear helpful also in diagnosis of the alcohol abuse. Because the SPME is a rapid, simple and solvent-free technique of sample preparation, the method is highly recommended for forensic, clinical and emergency laboratories (Zuba et al., 2002a).

HS-SPME method is the most effective and suitable method for the detection ethanol in postmortem blood, urine and vitrous humor (Martinis and Martin, 2002). Headspace GC technique needs a relatively expensive hardware for automated analysis and also can cause sample carryover in syringes. Therefore, more precise and rapid methods for the analysis of ethanol in biological fluids are needed (Martinis et al., 2004).

To date, there are limited references from indexed literature on ethanol analysis by HS-SPME in blood and urine specimens.

***Headspace-Liquid Phase (Single Drop) Micro Extraction Method (HS-LPME):***

By using this technique, the related compound (EtOH, MeOH, 1-propanol, 2-propanol, 1-butanol, 1-pentanol, and 2-pentanol) can be selectively extracted and analysed with packed colomn on GC. With this approach, other compounds can also be analysed in combination with other analysis techniques, such as HPLC (Tankeviciute, 2001).

***Cryogenic oven-trapping gas chromatography (COT-GC)***

Chloroform, dichloromethane, trichloroethylene, diethyl ether, the compounds of solvent thinner (ethyl acetate, benzene, n-butanol, toluene, and others), xylene isomers, cyanide, EtOH, hexanes, general anesthetics, and styrene in human body fluids can be analysed by COT with capillary GC. For all these compounds, the sensitivity achieved by using COT-GC was more than 10-times higher compared to that of HS-SPME-GC technique. This method is recommended for widespread use in forensic and environmental toxicology, because it is simple, requires no special GC operations, and yet enables high sensitivity and high resolution (Watanabe-Suzuki et al., 2002).

## 1.6. Instrumentation of Gas Chromatography

As mentioned before, the method of choice is the GC. This technique will be used in this thesis and then the detailed instrumentation of this technique is given below.

### 1.6.1 Columns

GC methods use succesfully either adsorbtion or partition stationary phases with a wide range of polarity. Simultaneous or subsequent GC analyses of volatiles on columns with different elution patterns (e.g., Carbowax 1500 on Carbopack C and polyethyleneglycol on Celite; DB-1 and DB-wax) was suggested as a powerfull and rapid means for confirmation of results.

According to Anthony et al (1980), Carbopack B coated with Carbowax 20M proved superior to Carbopack C coated with Carbowax

1500 for the determination of AcH and MeOH, which co-eluted with each other and with water on the latter stationary phase (Tagliaro et al., 1992). For these purposes, partition chromatography using a Carbowax 20M coating was also proved superior to adsorbtion chromatography on Porapak Q and Chromosorb. These stationary phases, additionally, are temperature resistant and have been used succesfully.

Capillary chromatography (Carbowax 20M coating), which allows a higher separation performance and easier coupling with mass spectrometry, was preffered for the determination of lower volatile alcohols in very complex mixtures such as human stool.

In order to reduce the amount of water loaded on to the column, workers using capillary chromatography generally preferred headspace injection (Penton, 1987). The use of capillary columns has widespread in GC due to clear advantages in efficiency and resolution (Correa and Pedroso, 1997).

Non-polar coating, such as methylsilicone, failed to resolve AcH and MeOH whereas the polar poly-ethylene glycol did succeed. Poly-ethylene glycol was also proved superior in handling direct injections of aqueous samples but showed a lower temperature limit (250 vs. 300°C) in comparison with methylsilicone columns, which renders non-polar columns.

Fused-silica open tubular columns offer several advantages over packed columns:

- they can easily be installed in any GC with use of an inexpensive adaptor,
- they generally contain liquid phases cross-linked and bonded to the column wall,
- they are very durable,
- peak widths tend to be narrow as compared with those seen with porous polymer column, resulting in enhanced sensitivity and shorter run time (Penton, 1987; Tagliaro et al., 1992).

Dual FID/megabore capillary columns are used in confirmation blood alcohol analysis and their metabolites. BAC1 and BAC2 columns are prepared for this purpose. These columns show excellent resolution for all analytes (Rankin, 1999).

In conclusion, alcohols can be efficiently seperated with different GC columns and often the choice is based only on practical considerations such as total analysis time, cost, column life and the possibility of using the same column for different analysis.

### 1.6.2. Temperature

Separation is generally carried out under constant-temperature conditions; temperature programming has been used for the simultaneous determination of less volatile compounds (e.g., ethylene glycol).

### 1.6.3. Detectors

Since the sensitivity and versatility is low, Thermal Conductivity

Detector (TCD) has not been used in alcohol determination. Detection is universally carried out by Flame Ionization Detector (FID) (Figure 1.3). This is proved to be the best compromise in terms of sensitivity, ruggedness and cost (Willet, 1993).

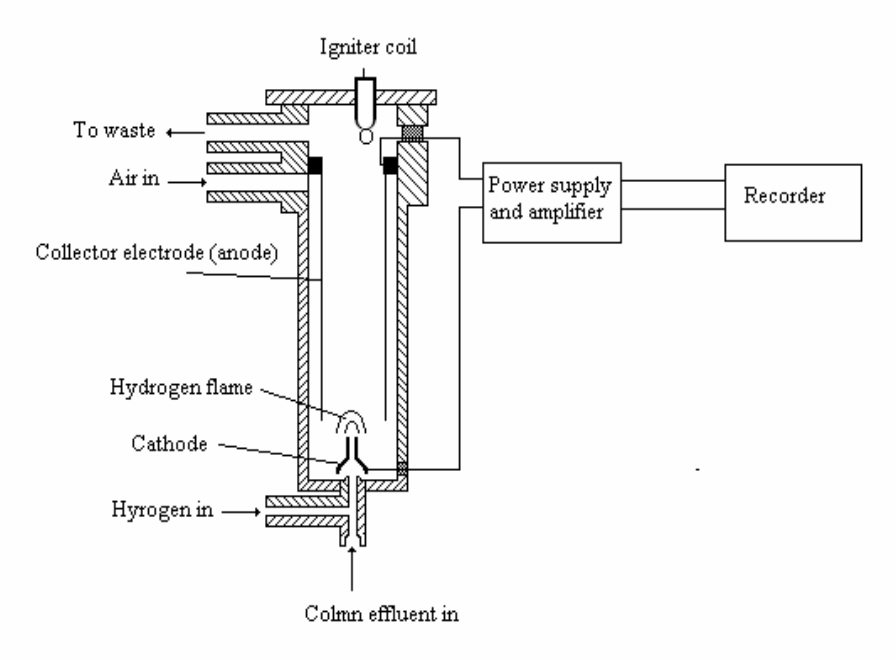


**Figure 1.3** A flame ionisation detector.

In FID, the column effluent passes through a narrow tube which is part of an electrical circuit and is also the base of a microburner which supports a hydrogen-air flame. Above the flame jet is a cylindrical electrode which bears a charge opposite to that of the effluent tube.

Separated compounds emerging from the column are ionized in the flame. The mass of ions produced reduces the resistance across the electrode gap. This decreased resistance alters the current flow in the

external circuit, which is directly proportional to the mass of the carbon atoms in the organic compound, a change which is amplified the electrometer and recorded by the pen trace on the chart. Thus, each emerging component causes a peak to be generated in the chromatogram. Non-ionized compounds do not give response (Williams, 1998).

Gas chromatography-mass spectrometry (GC-MS) has found little application for routine alcohol determinations, but found application only; when, very low level of endogenous higher molecular mass alcohols were to be investigated in serum and urine, and when the sample was extremely complex such as human stool (Tagliaro et al., 1992).

### 1.7. Chemometrics: Experimental Design

Chemometrics is the chemical discipline that uses mathematical and statistical methods to design or select optimal procedures and experiments, and to provide maximum chemical information by analyzing chemical data (Brereton, 2004). The experimental designs are used for screening, optimization, saving time and quantitative modeling.

#### 1.7.1. Screening Methods and Optimization

Screening is used to determine the effects of a number of factors on a response and to eliminate those that are not significant. There are two common screening methods for optimization.

- One-variable-at a time (OVAT) is the method that other variables are kept constant while the effect of one variable is examined.
- Multivariate method includes a series of experiments which

many parameters are changed at a time (Figure 1.4). Results are evaluated by using Analysis of Variance (ANOVA) or regression analysis.

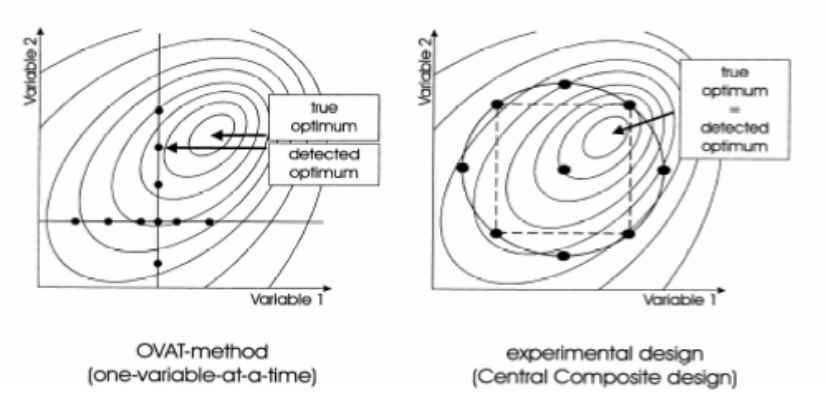


**Figure 1.4** The comparison of screening methods

**1.7.2. Design Matrices and Modelling**

An experimental design may consist of a series of experiments performed under different conditions. Experimental data can be described as a mathematical relationship between the factors or independent variables. A typical equation for three factors might be of the following form including the term; $\hat{y}$ since the equation estimates its value, and is unlikely to give an exact value that agrees experimentally due to the error.

| | |
|---|---|
| $\hat{y}$ = | Estimated response |
| $b_0$ | an intercept or average |
| $b_1x_1 + b_2x_2 + b_3x_3$ | 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 |

The intercept is an average in certain circumstances. It is an important term because the average response is not normally achieved when the factors are at their average values. This term can be ignored if it is known that there are no baseline problems or interferents.

The linear terms allow for a direct relationship between the response and a given factor. For some experimental data, there are only linear terms. On the other hand, quadratic terms are important in many situations. This allows curvature, and is one way of obtaining a maximum or minimum. Interaction terms arise because the influence of two factors on the response is rarely independent. In advance of experimentation it is often hard to predict which factors are important. Many chemometricians find it convenient to work using matrices.

The design matrix is simply one in which the rows refer to experiments and the columns refer to individual parameters in the mathematical model or equation linking the response to the values of the individual factors. There are two considerations required when computing a design matrix, namely; the number and arrangement of the experiments, including replication and the mathematical model to be tested. The relationship between the response, the coefficients and the experimental conditions can be expressed in matrix form given below.

$$\hat{\boldsymbol{y}} = \boldsymbol{Db} \qquad (1.8)$$

It is easy to calculate $b$ (or the coefficients in the model) knowing $D$ and $y$ using MLR (multiple linear regression).

If $\boldsymbol{D}$ is a square matrix, then there is exactly the same number of experiments as coefficients in the model. If $\boldsymbol{D}$ is not a square matrix pseudo-inverse form is used with an easy calculation with all matrix based software. Once $\boldsymbol{b}$ has been determined, it is then possible to predict $\boldsymbol{y}$ and so calculate the sums of squares and other statistics. The statistical

significance can be obtained from a two-tailed *t*-distribution and alternatively with *F*-test.

The size of the coefficients reflects their significance; however, the physical scale for each variable is different. It is useful to put each variable on a comparable scale in order to have a better idea of the significance. Coding the experimental data is commonly preferred for this purpose. Each variable is placed on a common scale, often with the highest coded value of each variable equal to +1 and the lowest to −1.

### 1.7.3. Factorial Designs

Factorial designs are often used for screening or when there are a large number of possible factors. Full factorial designs at two levels are mainly used for screening and are also called as saturated designs. The first step is to choose a high and low level for each factor. Then a standard design is used where the value of each factor is usually coded. Following the experiments the data obtained are analysed by setting up a design matrix. Here an interaction term must be taken into account, and a design matrix based on the below model is set up.

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 \qquad (1.9)$$

This can be expressed either as a function of the true or coded concentrations. It should also be noted that each of the columns in the set up matrix is different. This is an important and crucial property and allows each of the possible terms to be distinguished uniquely from one another, and is called ***orthogonality***. Final next step is to calculate and then interpret the coefficients.

Major drawback of full factorial design is that large number of experiments must be performed. Two level fractional factorial designs are used to reduce the number of experiments by ½, ¼, ⅛ and so on. Such fractional factorial designs only exist when the number of experiments equals a power of 2. Where the number of factors is fairly large, this situation can be rather restrictive. Figure-1.5 shows the difference between two kinds of factorial designs (Brereton, 2004).

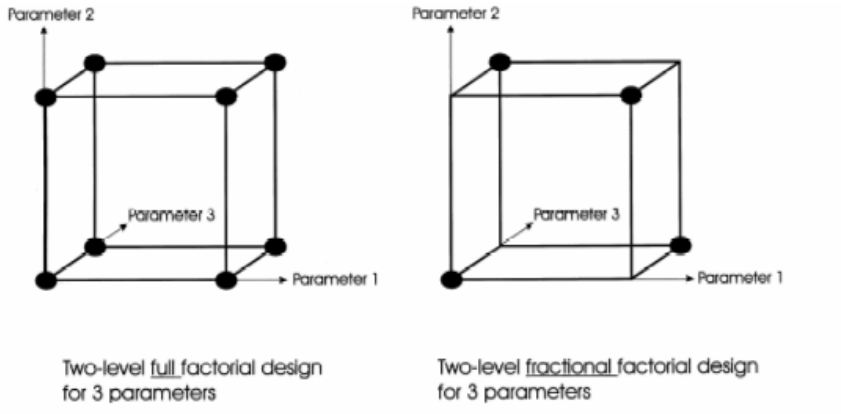


**Figure 1.5** Schematic representation of two level full and fractional factorial designs

### 1.7.4. Plackett and Burman Design and Pareto Charts

Plackett and Burman proposed a number of two level factorial designs, where the number of experiments is a multiple of four. Hence design exist for 4, 8, 12, 16, 20, 24, etc. experiments. The number of experiments exceeds the number of factors, $k$, by one ($k+1$). Generators for Plackett-Burman designs are given in Table 1.4 (Brereton, 2004).

**Table 1.4** Generators used in Plackett-Burman design

| Factors | Generator |
|---|---|
| 7 | + + + - + - - |
| 11 | + + - + + + - - - + - |
| 15 | + + + + - + - + + - - + - - - |
| 19 | + + - + + + + - + - + - - - - + + - |
| 23 | + + + + + - + - + + - - + + - - + - + - - - - |

***Pareto charts*** are special form of a bar graph and are used to display the relative importance of problem or conditions. A pareto chart, is used for focusing on critical issues by ranking them of importance and frequency; prioritizing problem so causes to efficiently initiate problem solving; analyzing problem for causes by different groupings of data, and analyzing the before and after impact of changes made in process (http://www.hanford.gov).

In constructing Pareto charts, $\overline{y}_+ - \overline{y}_-$ differences for each factor are calculated and divided by estimated standard error ($\sqrt{V_{effect}}$) as given below. These values were used to plot bar graphics to show the relative effects of all the factors on the results in increasing trend.

These effects can be negative or positive and those exceed the t value are taken into account for optimization. The chart includes a vertical line at the critical t-value for p = 0.05.

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.

$$s_i^2 = s^2 / n \tag{1.10}$$

Pooled estimate of variance for replicates can be calculated as;

Pooled estimate = Total estimated variance / number of runs

Since each main effect and interaction is a statistic of the form ( $\bar{y}_+ - \bar{y}_-$ ) where each effect contains certain number of observations, the variance ($V_{effect}$) of each effect is given by below equation.

$$V_{effect} = V(\bar{y}_+ - \bar{y}_-) \tag{1.11}$$

To generalize, if *N* total number of runs with replicates is used in conducting a two level replicated factorial design, the formula given below is used (Morgan, 1997).

$$V_{effect} = \frac{4}{N} s^2 \tag{1.12}$$

**1.7.5. Central Composite Design**

There are two reasons for constructing such a design. The first is for optimization, the second is to produce a detailed quantitative model. The main effects and interaction terms were included in this design. A central composite design is composed of a full factorial design, a star design and replicates (Figure 1.6) (Brereton, 2004).

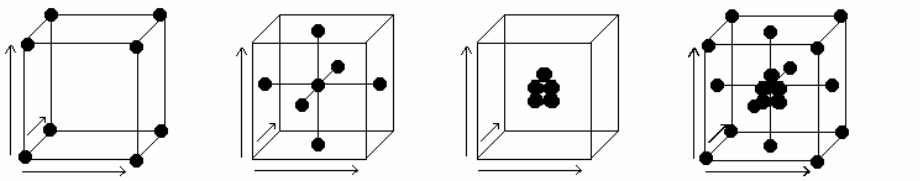


**Figure 1.6** Elements of a central composite design.

Number of experiments is calculated from ($2^k + 2k + 1$) where the *k* is number of factors. $2^k$ is the number of experiments in full factorial

design ($N_f$) in the model and all interaction terms are included. The levels of factors are expressed as +1 and -1. *2k + 1* term represents the number of experiments in star design of this model. The levels are expressed as ±α 1 level, 0 being in the center (Figure-1.6).

α levels take different values for rotational and orthogonal designs. For rotational design; N being the total number of experiments;

$$\alpha = \pm\sqrt[4]{2^k} \tag{1.13}$$

For orthogonal design

$$\alpha = \pm\sqrt{\frac{\sqrt{NN_f} - N_f}{2}} \tag{1.14}$$

Replicates in central composite design can be used to provide a mean response and an estimate of pure experimental uncertainty. These runs are generally at center level and at least five replicates are proposed (Brereton, 2004).

±α values for the factors were calculated as follows,

$$\pm\alpha = \frac{X_1 - Med.value}{Max.value - Med.value} \text{ or } \pm\alpha = \frac{X_1 - Med.value}{Min.value - Med.value} \tag{1.15}$$

Using the model design matrix (***X***) estimated b parameters can be calculated as;

$$\boldsymbol{b} = (\boldsymbol{X}^T \boldsymbol{X})^{-1} \boldsymbol{X}^T \boldsymbol{y} \tag{1.16}$$

Superscripts T and -1 denote the transpose and inverse of a matrix respectively. Estimated responses are calculated as

$$\hat{\boldsymbol{y}} = \boldsymbol{X}\,\boldsymbol{b} \tag{1.17}$$

After calculation of residual values (***r***), sum of squares of residuals ($SS_{residual}$) can be obtained

$$\boldsymbol{r} = \boldsymbol{y} - \hat{\boldsymbol{y}} \tag{1.18}$$

$$SS_{residual} = \Sigma(\boldsymbol{y} - \hat{\boldsymbol{y}})^2 \tag{1.19}$$

The mean response of replicates and the difference between mean and experimental responses are calculated. Then sum of squares of experimental uncertainity ($SS_{replicates}$) can be obtained as given below where $Y_{replicates}$ represents the response of replicates and $\overline{Y}$ denotes the mean value.

$$SS_{replicates} = \sum (Y_{replicates} - \overline{Y})^2 \tag{1.20}$$

The sum of squares due to lack of fit (*LOFSS*) can then be calculated.

$$LOFSS = SS_{residual} - SS_{replicates} \tag{1.21}$$

Calculated $SS_{residual}$, $SS_{replicates}$ and *LOFSS* values are divided by their degrees of freedom and variances ($s^2$) can be obtained. The ratio of variances for *F* value are calculated *F* ($s^2_{LOF}/s^2_{residual}$) and compared with tabulated *F* values ($F_{table}$) at a probability level p = 0.05.

Variances ($s^2_{parameters}$) for each parameter are also calculated for the t test as, $(\boldsymbol{X}^{\boldsymbol{T}}\,\boldsymbol{X})^{-1}$. Main diagonal elements are multiplied with $SS_{residual}$ and square roots are taken and then *b* coefficients are divided by the calculated values to obtain $t_{experimental}$ values.

$$t_{experimental} = \frac{b}{\sqrt{SS_{residuals}\ S^2}} \quad (1.22)$$

Eventually, to test how well the model is fitted, root mean square error in percentage (*%RMSE*) can be calculated. The smaller is the percentage, the better of fitting the model.

$$RMSE = (SS_{residuals}/d.f.)^{½} \quad (1.23)$$

$$\%RMSE = (S_{mean\ residuals} / \bar{Y}) \times 100 \quad (1.24)$$

***Response surface methodology***: The nature of response surface system (maximum, minimum, or saddle point) depends on the signs and magnitudes of the coefficients in the model. The second order coefficients (interaction and pure quadratic terms) play a vital role. These coefficients are estimates of the *b* coefficients of the model equation (Myers and Montgomery, 2002). These graphs are very useful for interpreting graphically the effect on the response of each pair of independent variables (Pellati et al., 2005).

### 1.8. Method Validation

Method validation is the process of providing that an analytical method via acceptible for its intended purpose (Green, 1996, Hubert et al., 1999). The parameters used in validation of chromatographic methods are described below;

***Selectivity*** is considered the ability of a method to differentiate and to quantify an analyte in the presence of other components in a sample.

***Linearity*** is defined as the ability of a method to produce results

directly proportional to the concentration of the analytes within a range of concentrations.

***Precision*** is a measure of random error and defined as the agreement between the experimental results when the method is applied repeatedly to different aliquots taken from a homogenous sample. Precision is evaluated by the means of the coefficient of variability (CV) or Relative Standard Deviation (RSD).

$$\%RSD = (\text{standard deviation/mean}) \times 100 \qquad (1.25)$$

Precision may be considered in three levels: repeatability, intermediate precision and reproducibility. ***Repeatability*** expresses the precision under the same operating conditions over a short interval or time and termed as within-run, within day or intraassay precision. ***Intermediate precision*** expresses within-laboratories variations: different days, different analytes, different equipment etc. and termed as between-run, between-day or inter-assay precision.

Between-day precision is evaluated by the means of ANOVA. ***Reproducibility*** expresses the precision between laboratories (Peters and Maurer, 2002; Causon, 1997).

***Limit of Detection (LOD)*** corresponds to the lowest concentration of an anlyte in a sample that can be detected and calculated as three times of signal/noise (*S/N*) ratio, (*S/N*x3).

***Limit of Quantitation (LOQ)*** is the minimal quantifiable concentration that can be determined with acceptable precision and calculated as *S/N*x10 (Green, 1996, Hubert et al., 1999).

***Recovery*** is related with the accuracy which is the difference between the theoretical concentration and the value determined experimentally. It is expressed as the % recovery.

***Stability*** is the influence of time, storage and the sample containers on the concentration.

The computer rarely fails to produce a prediction result given a model of an unknown. It is therefore important to validate even appearently reasonable results. Being able to validate prediction results is the greatest advantages of multivariate techniques (Beebe et al., 1998).

### 1.9. Linear Regression and Calibration

Linear regression is one of the most frequently used statistical methods in calibration (Burke, 2001). Linearity is defined as the ability of a method to produce results directly proportional to the concentration of the analytes within a range of concentrations ($y = a + b_s x$).

The random error in the values for the slope ($b_s$) and intercept ($a$) are important and was calculated by the following equation;

$$s_{y/x} = \left\{ \frac{\sum_i (y_i - \hat{y}_i)}{n_{cal} - 2} \right\}^{1/2} \qquad (1.26)$$

The $\hat{y}_i$ values are the points on the calculated regression line corresponding to the individual $x$ values. In a linear regression calculation the number of degrees of freedom (d.f.) is $n_{cal}$-2. Here $n_{cal}$ denotes the number of points in calibration plot.

Once these values were calculated, determination of the error in *x*-value corresponding to any measured *y*-value could be possible by using the following formula;

$$s_{xo} = \frac{s_{y/x}}{b_s}\left\{\frac{1}{m}+\frac{1}{n_{cal}}+\frac{(y_i-\bar{y})^2}{b_s^{\,2}\sum_i (x_i-\bar{x})^2}\right\}^{1/2} \qquad (1.27)$$

Here, $y_o$ is the experimental value of y from which the concentration value $x_o$ is to be determined. $s_{xo}$ is the estimated standard deviation of $x_o$ and $m$ is the number of replicates in calibration plot (Miller and Miller, 1993).

### 1.10. Classical Multivariate Calibration

Multivariate calibration is the process of relating multiple responses from an instrument to a property of a sample. By this means a multicomponent sample can be analyzed simultaneously.

***Direct Classical Least Squares (DCLS)*** can be used when the system under investigation obeys a linear relationship between the measurement vectors and concentration. Measurement vectors can contain spectra, chromatograms, electrochemical data, etc. Linear additivity is used in CLS models. The response of an instrument to a mixture of analytes is equal to the sum of the instrument responses to the pure component responses (Olivieri et.al., 2006; Danzer et. al., 2004; Geladi; 2002; Beebe et. al. ,1998).

Multivariate calibration methos may produce valid predictions from highly unselective data as well. Therefore, in case of overlapped

peaks in a chromatogram of a mixture, multivariate analysis is preferred. For this purpose, a certain number of original measurement points (100 is recommended) for pure standards (***S*** matrix) and for the calibration and validation mixtures (***y***) are selected for each chromatogram and consisted the variable range. Concentrations in the calibration and validation samples can be estimated by using the coefficients of the linear combination.

Estimated concentrations ($\hat{\boldsymbol{c}}$) for the mixtures can be calculated as;

$$\boldsymbol{y} = \boldsymbol{c}\ \boldsymbol{S} \tag{1.28}$$

$$\boldsymbol{y}\ \boldsymbol{S}^{\boldsymbol{T}}(\boldsymbol{S}\ \boldsymbol{S}^{\boldsymbol{T}})^{-1} = \hat{\boldsymbol{c}} \tag{1.29}$$

Estimated measurement variables were calculated as;

$$\hat{\boldsymbol{y}} = \hat{\boldsymbol{c}}\ \boldsymbol{S} \tag{1.30}$$

Then residuals were calculated as;

$$Residual = \boldsymbol{y} - \hat{\boldsymbol{y}} \tag{1.31}$$

Since the predicted concentrations are the regression coefficients of the linear regression, uncertainities in the concentrations can be predicted. These are defined as standard error of calibration and prediction (*SEC* and *SEP* respectively) and can be calculated by using the following formulas.

$$SEC = \sqrt{\frac{\sum_{i=1}^{n_{cal}} (\hat{c} - c_i)^2}{n_{cal} - 2}} \tag{1.32}$$

$$SEP = \sqrt{\frac{\sum_{i=1}^{n_{val}} (\hat{c} - c_i)^2}{n_{val}}} \qquad (1.33)$$

However, in literature it is plausible to encounter Root Mean Square Error of Prediction (*RMSEP)* instead of *SEP* for examining errors in the prediction concentrations and judging the performance of a multivariate calibration model (Faber, 1999; Beebe et al.; 1998).

### 1.11. The Aim of the Thesis

In this thesis, a method development for ethanol and methanol analysis in blood employing chemometric assesment and comparison of the methods was aimed. To date, there are limited references from indexed literature on ethanol analysis by HS-SPME in blood and urine specimens

. In this thesis, blood alcohol levels, which have an importance in Forensic Medicine, were determined by headspace-GC methods (static headspace and headspace solid phase microextraction techniques) using FID. The results were evaluated by using chemometric methods.

The chromatographic separation of alcohols and their metabolites by GC-Headspace are known to be affected by several factors. So far a very limited number of studies have been encountered in the literature for determination of blood alcohol levels including chemometric approach. A recent study deals with the determination of aceataldehyde, the main metabolite of ethanol, during storage conditions and includes a factorial design (Kristoffersen et al., 2006).

The main objective of the present thesis is to examine the effect of factors related with sample injection and GC conditions and to optimize separation of alcohols (ethanol, methanol, isopropanol, n-propanol and n-buthanol) and their metabolite (acetone). Chemometric approaches constitute the original part of the thesis and provide a fully understanding of the main and interactive effects underlined the analytical method chosen for blood alcohol levels. For this purpose, experimental designs were used for screening and optimization of the method parameters.

Pareto charts and surface plots were utilized for reflecting the results of screening and optimization methods for focusing on critical issues by ranking them of importance and frequency. Multivariate calibration methods were employed for evaluation of the overlapped peaks. By this means, it was possible to quantify individual peaks correctly within a certain degree of confidence. Method validation was performed in order to provide analytical method acceptable for alcohol analysis in blood.

## 2. EXPERIMENTAL

In the context of the thesis, the analysis of alcohol standards was carried out by a gas chromatography connected to a flame ionization detector (GC-FID). Direct injection, static headspace and head space solid phase micro extraction (HS-SPME) techniques have been used. General instrumentation of each part was described and further explanation was also given under related headings.

### 2.1 Instrumentation

Initial studies included the direct injection of standard solutions to a GC connected to FID. Headspace analysis was carried out with two columns in connection with Finnigan Trace GC Ultra/FID system. The carrier gas was helium.

(I): HP 20M (25 m, 0.32 mm id., 0.3 µm film thickness), Agilent.

(II): Rtx BAC1 (30 m; 0.32 mm id., 1.8 µm film thickness), Restek.

For the first column, chemical composition of the stationary phase was given as polyethylene glycol ($C_{2n}H_{4n+2}O_{n+1}$). The name of the second column stands for the initials of Blood Alcohol Column and stationary phase was not declared.

HS-SPME analysis was carried out with both columns. Extraction procedure was performed under controlled temperatures with 65 µm Carbowax/DVB fiber (Supelco) with the aid of manual fiber holder (Supelco). Inlet liner with 0.8 mm id. (Supelco) was used in inlet block of GC during SPME analysis.

Chemometric calculations were performed at Microsoft Office Excel program. Surface plots and optimization for central composite designs were performed by Matlab 7.0 package program.

### 2.2. Chemical Reagents and Samples

Standards: HPLC grade acetone, methanol, isopropanol, n-propanol and n-buthanol were obtained from Lab Scan, absolute ethanol was obtained from J.T.Baker. De-ionized water, obtained from Dialysis Center of Ege University Hospital, was used throughout the study. Whole blood samples used for method validation were obtained from Blood Center of Ege University Hospital. Medico-legal blood samples were obtained from Emergency Medicine Service of the same hospital and were kept at -20 ºC until analysis. These samples were chosen from the samples exceeded the period for legal purposes.

In validation studies, stock solutions (ethanol 2.5 g/100mL, acetone 1.0 g/100mL and methanol 1.0 g/100mL) were prepared in blank blood. Dilutions were made by addition of blank blood to stock solutions. Each blood sample contained equal amount of n-propanol as the internal standard (10.0 mg/100mL). 1.0 mL of blood and 1.0 mL of IS placed into the vials and capped immediately. Blood samples were prepared daily and kept in refrigerator until analysis.

In the studies carried out with Column II stock blood solutions spiked with standard alcohol solutions (Ethanol 2.5 g/100mL, acetone 1.0 g/100mL, methanol 1.0 g/100mL, isopropanol 1.0 g/100mL and n-butanol 0.05 g/100mL) and dilutions were made with blank blood solutions.

Every blood sample contained equal amount of internal standard (n-propanol 5 mg/100mL). 1 mL blood and 0.5 mL of IS placed into the vials and capped immediately

**2.3. Procedure**

***Direct Injection Technique*** was performed by simply injecting 0.1 µL of pure standards or their mixture onto GC column.

***Static Headspace Technique*** was performed as described below. 2.0 mL of aqueous standard solutions were placed in headspace vials (Agilent, US) and capped with aluminum caps and PTFE septa (Hewlett-Packard, US) by using vial crimper (Agilent, US). These vials were kept at chosen temperature. 1.0 mL headspace sample was pulled by a gas tight syringe (5MAX-HASV, Supelco SGE) and injected to the GC. Carrier gas flow rate was 1.0 mL /min.

***Headspace Solid Phase Micro Extraction Technique*** was performed under controlled temperatures with 65 µm Carbowax/DVB fiber with the aid of manual fiber holder Fiber was pulled out from the vials and injected to the GC and hold for certain desorption period. The SPME fiber was conditioned at 220 ºC for 30 min before every set of experiments.

# 3. RESULTS AND DISCUSSION PART I

## EXPERIMENTS WITH COLUMN I

### 3.1 Preliminary Studies with Direct Injection

Initial studies included the direct injection of mixture of alcohol standards (ethanol, methanol, isopropanol, acetone, n-propanol and n-buthanol) at equal volumes onto a GC column (HP-20M) connected to a flame ionization detector (FID). Resulting chromatograms were evaluated to establish their retention times ($R_t$) as given in Table-3.1.

**Table 3.1** Data related with direct injection of alcohol/metabolite standards

| **standard** | Acetone | Methanol | Ethanol + isopropanol | n-propanol | n-buthanol |
|---|---|---|---|---|---|
| ***$R_t$*, (min)** | 1.38 | 1.60 | 1.73 | 2.40 | 3.95 |

The effect of the main parameters namely the temperatures of detector, inlet block, and column were investigated by OVAT technique and optimal conditions were found as 200, 150 and 60 $^{o}$C, respectively.

### 3.2 Studies with Static Headspace GC-FID

#### 3.2.1. Initial Studies

In the light of the direct injection studies, headspace measurements were performed. Preliminary studies have revealed that the split mode is appropriate for headspace analysis as a capillary column is used. Aqueous ethanol solutions at different concentrations were analyzed at various split ratios and split ratio of 1:50 results in a smaller and better shaped peaks.

### *3.2.2 Factorial Design for Headspace Ethanol Analysis and Chemometric Evaluation*

In the chromatographic separation of alcohols and their metabolites by GC-Headspace, several factors are known to influence their separation. The aim of this part of the study is to screen the temperature factors related with GC and to optimize the separation of alcohols and their metabolites. For this purpose, aqueous ethanol standard solutions (0.1 % (v/v)) were prepared and analyzed by headspace technique.

Three factors were selected at two levels for this experimental design. These factors were injection (I), column (oven) (C) and detector (D) temperatures. The levels were chosen as 10% below and above of the temperatures (±10%) stated above (150, 60 and 200$^{o}$C respectively).

The main and interactive effects of the factors were calculated in relation with the parameters such as peak area, resolution, asymmetry and the number of theoretical plates obtained from the chromatograms. The treatments were considered to be in two halves with the factors at their lower levels in one half and the higher levels in the other. By means of this division, the responses obtained were averaged and the average at the lower level subtracted from that of higher level. Interactions were considered in the same way. Main and interactive effects were calculated by using the following formula (Morgan, 1997).

$$IC = \frac{y_1 + y_4 + y_5 + y_8}{4} - \frac{y_2 + y_3 + y_6 + y_7}{4} \qquad (3.1)$$

In order to estimate the effects of I, C and D temperature, a $2^3$ factorial design was constructed (Table 3.2). Headspace samples were

injected in triplicate throughout the 8 method (run) of experimental design. (–) and (+) sign represents the factor at its lower and higher level, respectively. Applied levels for three factors were given in Table 3.3.

**Table 3.2** Factorial design for 3 factors and two levels

| Run no | I | C | D | IC | ID | CD | ICD |
|---|---|---|---|---|---|---|---|
| 1 | - | - | - | + | + | + | - |
| 2 | + | - | - | - | - | + | + |
| 3 | - | + | - | - | + | - | + |
| 4 | + | + | - | + | - | - | - |
| 5 | - | - | + | + | - | - | + |
| 6 | + | - | + | - | + | - | - |
| 7 | - | + | + | - | - | + | - |
| 8 | + | + | + | + | + | + | + |

**Table 3.3** Selected factors and levels for factorial design by static HS-GC-FID system with HP20M column

| Run no | Temperature (°C) | | |
|---|---|---|---|
| | I | C | D |
| 1 | 135 | 54 | 180 |
| 2 | 165 | 54 | 180 |
| 3 | 135 | 66 | 180 |
| 4 | 165 | 66 | 180 |
| 5 | 135 | 54 | 220 |
| 6 | 165 | 54 | 220 |
| 7 | 135 | 66 | 220 |
| 8 | 165 | 66 | 220 |

Data related with ethanol peak obtained from $2^3$ factorial design model were given in Table 3.4 and chemometrically estimated effects for each parameter from the chromatogram are shown in Table 3.5.

**Table 3.4** Data related with ethanol peak obtained from $2^3$ factorial design model

| Method | I | C | D | IC | ID | CD | ICD | Mean area | Asymmetry (%10) | Width | Theoretical plates |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | - | - | + | + | + | - | 1513825 | 1.231 | 0.29 | 23016 |
| 2 | + | - | - | - | - | + | + | 1407400 | 1.196 | 0.34 | 22934 |
| 3 | - | + | - | - | + | - | + | 1572450 | 1.116 | 0.29 | 21051 |
| 4 | + | + | - | + | - | - | - | 1465383 | 1.143 | 0.28 | 22793 |
| 5 | - | - | + | + | - | - | + | 1524258 | 1.242 | 0.32 | 24317 |
| 6 | + | - | + | - | + | - | - | 1447812 | 1.229 | 0.35 | 24617 |
| 7 | - | + | + | - | - | + | - | 1581475 | 1.102 | 0.32 | 21331 |
| 8 | + | + | + | + | + | + | + | 1583168 | 1.115 | 0.27 | 22236 |

**Table 3.5** Chemometrically estimated effects for each parameter from the chromatogram

| Effect | Estimate for Area | Estimate for Asymmetry | Estimate for Width | Estimate for theoretical plates |
|---|---|---|---|---|
| I | -72062 | -0.011 | 0.00 | 717 |
| C | 77296 | -0.099 | -0.04 | -1869 |
| D | 44414 | -0.192 | 0.02 | -677 |
| IC | 19374 | 0.030 | -0.04 | -2092 |
| ID | 34684 | 0.002 | -0.02 | -113 |
| CD | 18992 | -0.021 | -0.01 | -815 |
| ICD | 19696 | -0.009 | -0.01 | -305 |

Evaluation of the effects from Table 3.5 revealed that high inlet temperature has negative effect (-72062) while the column (77296) and detector temperature (44414) have positive effect on ethanol peak area. Interactive effects (IC, ID, CD and IDC) were observed to have positive but rather lower effect on the peak area.

### 3.2.3 Factorial Design for Headspace Alcohol Analysis and Chemometric Evaluation

In order to see the effects of the temperature parameters, aqueous mixture of 6 standards, each of concentration 0.1 % (v/v), were prepared and factorial design model were applied as described in Table 3.2. Chromatogram of the standard mixture related with the low column temperature (54ºC) was shown in Figure 3.1.

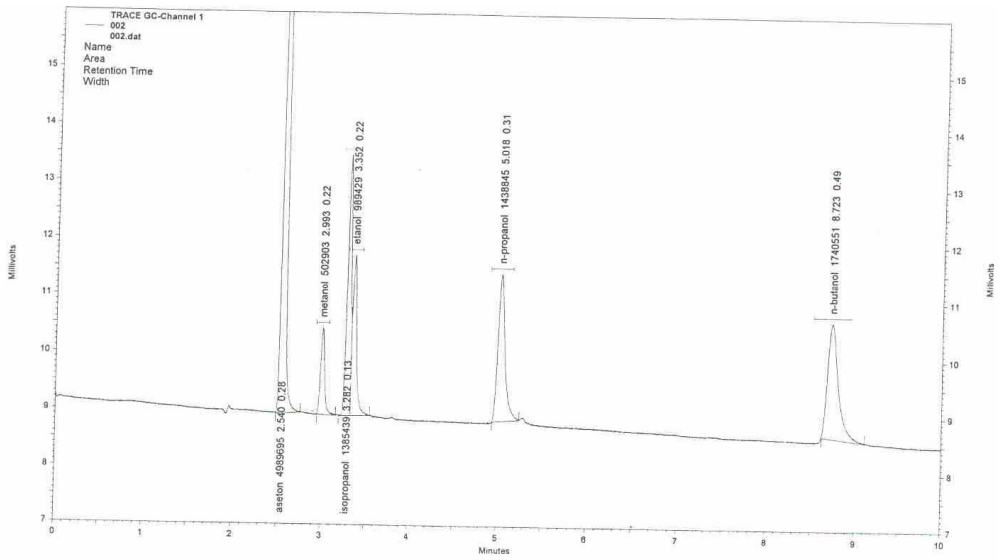


**Figure 3.1** Chromatogram of the standard mixture recorded with HP 20M at the column temperature of 54 ºC. The peaks are in order of acetone, methanol, isopropanol, ethanol, n-propanol and n-buthanol.

Chromatographic peaks obtained with aqueous standard mixture were analyzed according to peak area and width, retention time, resolution, asymmetry and number of theoretical plates. Data related with each standard peak and chemometric calculations were given in Table 3.6 and 3.7 respectively.

The overall evaluation of the results obtained from the experimental design revealed that high column temperature was not appropriate especially for the chromatographic separation of isopropanol and ethanol. Although high column temperature showed positive effect on acetone, methanol, n-propanol and n-buthanol peak areas, the aim of the study was to achieve the best separation for the six standards. High inlet and detector temperatures mostly showed negative effect on the peak areas. The first and second methods of design in Table 3.2 were the preferred methods in terms of good separation.

As followed from Table 3.6, method 4 (where I: +, C: + and D: -) displays poor repeatability as the highest standard deviation (s) was obtained. The lowest s values were observed for method 2 (I:+, C: -, D: -) and method 6 (I: +, C: -, D: +). In relation with peak areas, the first method was the most appropriate method for separation of the standards.

A series of headspace analysis were performed at lower oven temperatures but these studies resulted in smaller peaks areas. On the other hand, as the oven temperature increased, poorer resolution was obtained for isopropanol and ethanol.

**Table 3.6** Data related with analyte peaks according to the $2^3$ factorial designs obtained by static HC-GC technique with HP 20M column.

| Analyte | Method | Mean area (±s) | Resolution | Asymmetry at 10% | Width | Theoretical plates |
|---|---|---|---|---|---|---|
| Acetone | 1 | 5120566 ±123283 | | 1.100 | 0.28 | 22869 |
| | 2 | 5124476 ± 43034 | | 1.0661 | 0.28 | 24425 |
| | 3 | 5254582 ± 133545 | | 0.977 | 0.23 | 18888 |
| | 4 | 5277042 ± 319350 | | 1.001 | 0.23 | 22316 |
| | 5 | 5006858 ± 193360 | | 0.992 | 0.29 | 23778 |
| | 6 | 4843392 ± 46941 | | 1.023 | 0.27 | 24364 |
| | 7 | 5144760 ± 28493 | | 0.942 | 0.27 | 20422 |
| | 8 | 5357912 ± 217323 | | 0.987 | 0.25 | 21005 |
| Methanol | 1 | 521873 ± 16617 | 6.004 | 1.192 | 0.22 | 20013 |
| | 2 | 512611 ± 2480 | 6.161 | 1.197 | 0.24 | 21039 |
| | 3 | 543784 ± 23158 | 3.708 | 1.046 | 0.16 | 17888 |
| | 4 | 527752 ± 43498 | 3.978 | 1.079 | 0.15 | 20278 |
| | 5 | 506099 ± 21659 | 6.102 | 1.137 | 0.24 | 20671 |
| | 6 | 483771 ± 3423 | 6.209 | 1.131 | 0.22 | 21312 |
| | 7 | 526210 ± 14575 | 3.796 | 1.029 | 0.16 | 18470 |
| | 8 | 543185 ± 25176 | 3.861 | 1.081 | 0.17 | 10229 |
| Iso-propanol | 1 | 1422693 ± 35420 | 3.430 | 0.000 | 0.19 | 24598 |
| | 2 | 1391259 ± 16792 | 3.499 | 0.000 | 0.11 | 26304 |
| | 3 | 2603707 ± 98545 | 1.758 | 1.578 | 0.30 | 14012 |
| | 4 | 2541178 ± 153479 | 1.986 | 1.719 | 0.33 | 15319 |
| | 5 | 1410886 ± 60511 | 3.456 | 0.000 | 0.11 | 28453 |
| | 6 | 1353610 ± 17077 | 3.538 | 0.000 | 0.11 | 26919 |
| | 7 | 2530728 ± 43150 | 1.904 | 1.540 | 0.32 | 13302 |
| | 8 | 2602265 ± 120035 | 1.858 | 1.626 | 0.31 | 15647 |
| n-propanol | 1 | 1485254 ± 42113 | 13.298 | 1.366 | 0.31 | 20587 |
| | 2 | 1461970 ± 1897 | 13.556 | 1.276 | 0.31 | 20928 |
| | 3 | 1580455 ± 51634 | 9.192 | 1.193 | 0.23 | 17784 |
| | 4 | 1568968 ± 85566 | 10.209 | 1.238 | 0.23 | 18855 |
| | 5 | 1452391 ± 57185 | 13.829 | 1.384 | 0.31 | 21381 |
| | 6 | 1386200 ± 8692 | 14.037 | 1.317 | 0.30 | 21921 |
| | 7 | 1563895 ± 34753 | 9.949 | 1.3254 | 0.23 | 19343 |
| | 8 | 1616756 ± 67660 | 9.777 | 1.215 | 0.23 | 20034 |

**Table 3.7** Data related with methanol peak and chemometrically estimated effects

| Analyte | Effect | Estimate for Area | Estimate for Resolution | Estimate for Asymmetry | Estimate for Width | Estimate for Theoretical plates |
|---|---|---|---|---|---|---|
| **Acetone** | I | 19014 | | 0.016 | -0.01 | 1538 |
| | C | 234751 | | -0.069 | -0.04 | -3202 |
| | D | -10594 | | -0.050 | 0.02 | 4443 |
| | IC | 98792 | | 0.018 | 0.00 | 468 |
| | ID | 5829 | | 0.018 | -0.01 | -954 |
| | CD | 91459 | | 0.026 | 0.02 | -156 |
| | ICD | 891517 | | -0.012 | 0.00 | -468 |
| **Methanol** | I | -7662 | 0.149 | 0.021 | 0.05 | 1204 |
| | C | 29144 | -2.283 | -0.106 | -0.07 | -1792 |
| | D | -11689 | 0.029 | -0.034 | 0.01 | 116 |
| | IC | 8133 | 0.018 | 0.022 | 0.00 | 370 |
| | ID | 4985 | -0.064 | 0.002 | 0.00 | -504 |
| | CD | 10618 | -0.044 | 0.026 | 0.01 | -350 |
| | ICD | 11518 | -0.039 | -0.018 | 0.02 | -312 |
| **Isopropanol** | I | 297524 | 0.084 | | 0.07 | -1948 |
| | C | 19925 | -1.604 | | 0.19 | -11498 |
| | D | -15337 | -0.008 | | -0.02 | 522 |
| | IC | 24429 | 0.258 | | 0.03 | 370 |
| | ID | 27138 | -0.065 | | 0.01 | -50 |
| | CD | 9391 | -0.012 | | 0.02 | -713 |
| | ICD | 39977 | -0.072 | | -0.03 | 570 |

Table 3.7 Continue

| Analyte | Effect | Estimate for Area | Estimate for Resolution | Estimate for Asymmetry | Estimate for Width | Estimate for Theoretical plates |
|---|---|---|---|---|---|---|
| **n-propanol** | I | -12025 | 0.327 | -0.038 | -0.002 | 661 |
| | C | 136063 | -3.897 | -0.108 | -0.700 | -2200 |
| | D | -19351 | 0.335 | 0.024 | -0.002 | 1131 |
| | IC | 32712 | 0.096 | 0.041 | 0.003 | 219 |
| | ID | 5385 | -0.309 | -0.015 | -0.003 | 741 |
| | CD | 34965 | -0.172 | -0.005 | 0.003 | 47 |
| | ICD | 26814 | -0.286 | -0.027 | 0.003 | -145 |
| **n-buthanol** | I | 55292 | 0.308 | -0.001 | -0.05 | 584 |
| | C | 147369 | -4.618 | -0.092 | -0.07 | 1834 |
| | D | -114587 | 0.511 | -0.039 | -0.01 | 1673 |
| | IC | 119264 | 0.002 | -0.001 | -0.02 | 312 |
| | ID | 80436 | 0.045 | 0.006 | 0.01 | -159 |
| | CD | -36841 | -0.132 | 0.014 | 0.03 | -180 |
| | ICD | 114398 | 0.008 | 0.012 | -0.01 | -135 |

### 3.2.4 The Enhancement of Qualitative Determination of Alcohols by Their Relative Retention Time

The purpose of the use of internal standard (IS) is to account for any variability in chromatographic conditions, such as temperature, gas flow rate, column length, that could effect the retention time of the specific alcohol of interest. Relative retention times (RRT) are the ratio of the retention times of the analyte to that of internal standard and very useful tool for the enhancement of the qualitative determination. The RRT values of the standards studied were calculated for n-propanol as the internal standard and given in Table 3.8.

**Table 3.8** Relative retention times (RRT) of the analyte peaks obtained with HS-GC-FID system.

| Method | RRT(Mean) ± s | | | | |
|---|---|---|---|---|---|
| | **acetone** | **MeOH** | **IprOH** | **EtOH** | **n-but** |
| 1 | 50.621 ± 0.006 | 59.671 ± 0.023 | 65.412 ± 0.014 | 66.795 ± 0.008 | 173.832 ± 0.017 |
| 2 | 50.622 ± 0.024 | 59.688 ± 0.059 | 65.419 ± 0.036 | 66.774 ± 0.017 | 173.807 ± 0.097 |
| 3 | 61.612 ± 0.003 | 68.786 ± 0.076 | 73.051 ± 0.054 | | 154.245 ± 0.042 |
| 4 | 61.672 ± 0.067 | 68.771 ± 0.018 | 72.931 ± 0.143 | | 154.268 ± 0.045 |
| 5 | 50.565 ± 0.019 | 59.631 ± 0.030 | 65.312 ± 0.059 | 66.721 ± 0.055 | 173.911 ± 0.104 |
| 6 | 50.528 ± 0.005 | 59.614 ± 0.025 | 65.325 ± 0.019 | 66.699 ± 0.023 | 173.949 ± 0.022 |
| 7 | 61.652 ± 0.020 | 68.762 ± 0.031 | 73.085 ± 0.021 | | 154.175 ± 0.056 |
| 8 | 61.618 ± 0.012 | 68.734 ± 0.009 | 73.048 ± 0.025 | | 154.285 ± 0.052 |

Here, the RRT values of the 6 standards revealed that the methods applied in factorial design provides better selectivity with improved

precision in qualitative detection of these standards within 95% confidence limits (n=3, $t_{table}$. 4.30).

### 3.2.5 Evaluation of the main effects of the factors by Plackett-Burman Design

In order to find the major effects on the simultaneous determination of alcohol mixture by HS-GC-FID, 14 factors were selected and completed to 15 by using a dummy factor. Then, Plackett-Burman Design was constructed for two levels. The factors and their levels were given in Table 3.9 and the design model is shown in Table 3.10.

**Table 3.9** Selected factors and their levels for for Plackett-Burman design by static HS-GC-FID system with HP20M column

| Factor No | Explanation | (+) level | (-) level |
|---|---|---|---|
| $F_1$ | Detector $H_2$ gas flow rate (mL/min) | 35 | 34 |
| $F_2$ | Sample concentration (v/v %) | 100 | 50 |
| $F_3$ | Injected headspace volume (mL) | 1.0 | 0.5 |
| $F_4$ | Carrier gas flow rate (mL/min) | 1.0 | 0.8 |
| $F_5$ | Inlet temperature (ºC) | 165 | 135 |
| $F_6$ | Split flow (mL/min) | 20* | 30 |
| $F_7$ | Sample volume in vial (mL) | 4.0 | 2.0 |
| $F_8$ | Dummy factor | | |
| $F_9$ | Equilibrium temperature (ºC) | 60 | 40 |
| $F_{10}$ | Equilibrium time(min) | 20 | 10 |
| $F_{11}$ | Oven temperature (ºC) | 65 | 55 |
| $F_{12}$ | Detector temperature (ºC) | 220 | 180 |
| $F_{13}$ | Salting out (2 g NaCl) | Yes | No |
| $F_{14}$ | Air flow rate (mL/min) | 350 | 340 |
| $F_{15}$ | Make-up gas | Yes | No |

*Split flow of 20 mL/min was chosen as the high level since more analyte is allowed to enter the column with lower split flow rate.

**Table 3.10** Plackett-Burman design model

| Method | $F_1$ | $F_2$ | $F_3$ | $F_4$ | $F_5$ | $F_6$ | $F_7$ | $F_8$ | $F_9$ | $F_{10}$ | $F_{11}$ | $F_{12}$ | $F_{13}$ | $F_{14}$ | $F_{15}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 2 | + | + | + | + | - | + | - | + | + | - | - | + | - | - | - |
| 3 | - | + | + | + | + | - | + | - | + | + | - | - | + | - | - |
| 4 | - | - | + | + | + | + | - | + | - | + | + | - | - | + | - |
| 5 | - | - | - | + | + | + | + | - | + | - | + | + | - | - | + |
| 6 | + | - | - | - | + | + | + | + | - | + | - | + | + | - | - |
| 7 | - | + | - | - | - | + | + | + | + | - | + | - | + | + | - |
| 8 | - | - | + | - | - | - | + | + | + | + | - | + | - | + | + |
| 9 | + | - | - | + | - | - | - | + | + | + | + | - | + | - | + |
| 10 | + | + | - | - | + | - | - | - | + | + | + | + | - | + | - |
| 11 | - | + | + | - | - | + | - | - | - | + | + | + | + | - | + |
| 12 | + | - | + | + | - | - | + | - | - | - | + | + | + | + | - |
| 13 | - | + | - | + | + | - | - | + | - | - | - | + | + | + | + |
| 14 | + | - | + | - | + | + | - | - | + | - | - | - | + | + | + |
| 15 | + | + | - | + | - | + | + | - | - | + | - | - | - | + | + |
| 16 | + | + | + | - | + | - | + | + | - | - | + | - | - | - | + |

Aqueous alcohol standard mixture containing 0.1% v/v each was analyzed by HS-GC technique. The main effects of the factors were calculated in relation with the parameters such as peak area, resolution, asymmetry and the number of theoretical plates obtained from the chromatograms. Table 3.11 gives a list of data related with the acetone and their estimated values are given in Table 3.12. Similarly, Table 3.13 and 3.14 also show the results obtained for methanol standard.

The treatments were considered to be in two halves with the factors at their lower levels in one half and the higher levels in the other. By means of this division, the responses obtained for these treatments were averaged and the average at the lower level subtracted from that of higher level ( $\overline{y}_+ - \overline{y}_-$ ).

**Table 3.11** Data related with acetone peak obtained by HS-GC-FID

| Method | Peak area (± s) | Asymmetry (%10) (± s) | Width (± s) | Theoretical plates (± s) |
|---|---|---|---|---|
| 1 | 3104722 ± 274989 | 1.233 ± 0.023 | 0.310 ± 0.025 | 14907 ± 2690 |
| 2 | 27129143 ± 336775 | 0.978 ± 0.045 | 0.390 ± 0.000 | 10873 ± 466 |
| 3 | 91241068 ± 463119 | 1.085 ± 0.048 | 0.300 ± 0.170 | 8118 ± 865 |
| 4 | 7495549 ± 279876 | 1.047 ± 0.095 | 0.250 ± 0.005 | 10408 ± 739 |
| 5 | 8529174 ± 352057 | 1.268 ± 0.062 | 0.250 ± 0.005 | 14834 ± 313 |
| 6 | 4727304 ± 423343 | 1.375 ± 0.361 | 0.290 ± 0.020 | 24684 ± 3692 |
| 7 | 20391750 ± 1516491 | 1.163 ± 0.032 | 0.310 ± 0.001 | 20463 ± 2139 |
| 8 | 20960247 ± 1409471 | 1.043 ± 0.036 | 0.420 ± 0.070 | 9455 ± 344 |
| 9 | 25475356± 5101846 | 1.398 ± 0.282 | 0.260 ± 0.000 | 8497 ± 140 |
| 10 | 16917353 ± 1314134 | 1.148 ± 0.091 | 0.310 ± 0.005 | 15134 ± 2552 |
| 11 | 48421730 ± 1491548 | 1.049 ± 0.043 | 0.330 ± 0.020 | 15678 ± 2793 |
| 12 | 17237179 ± 1344304 | 0.904 ± 0.261 | 0.250 ± 0.000 | 6099 ± 1120 |
| 13 | 31193489 ± 1683433 | 1.217 ± 0.094 | 0.410 ± 0.020 | 12928 ± 942 |
| 14 | 31577797 ± 1619678 | 1.417 ± 0.039 | 0.470± 0.005 | 15518 ± 116 |
| 15 | 9682554 ± 459085 | 1.143 ± 0.092 | 0.260 ± 0.005 | 18763 ± 4041 |
| 16 | 18678627 ± 298076 | 1.026 ± 0.051 | 0.310 ± 0.005 | 9535 ± 601 |

**Table 3.12** Estimated values related with acetone peak from Table 3.11

| Factor | Estimated effects for | | | |
|---|---|---|---|---|
| | Peak area | Asymmetry | Width | Theo. plates |
| $F_1$ | -9739050 | 0.037 | -0.02 | 289 |
| $F_2$ | 18348548 | -0.073 | 0.02 | 886 |
| $F_3$ | 18089955 | -0.099 | 0.06 | -5566 |
| $F_4$ | 6400498 | 0.00 | -0.04 | -4356 |
| $F_5$ | 4494710 | 0.083 | 0.004 | 803 |
| $F_6$ | -8606630 | 0.047 | -0.02 | 5818 |
| $F_7$ | -233405 | -0.062 | -0.05 | 1001 |
| $F_8$ | | | | |
| $F_9$ | 12460092 | 0.063 | 0.05 | -1264 |
| $F_{10}$ | 8634910 | 0.011 | -0.04 | 698 |
| $F_{11}$ | -7308701 | -0.063 | -0.06 | -1824 |
| $F_{12}$ | -9763925 | 0.058 | 0.02 | 434 |
| $F_{13}$ | 19971038 | 0.096 | 0.004 | 1010 |
| $F_{14}$ | -8731403 | -0.041 | 0.04 | 264 |
| $F_{15}$ | 534363 | 0.078 | 0.05 | -684 |

**Table 3.13** Data related with methanol peak obtained by HS-GC-FID

| Method | Area (± s) | Resolution (± s) | Asymmetry (%10) (± s) | Width(±s) | Theoretical plates (± s) |
|---|---|---|---|---|---|
| 1 | 308556 ± 40607 | 4.68811± 0.316 | 1.414 ± 0.055 | 0.21± 0.03 | 13209 ± 1578 |
| 2 | 3031146 ± 13071 | 4.10239 ± 0.084 | 1.109 ± 0.042 | 0.240 ± 0.005 | 10774 ± 362 |
| 3 | 7241685 ± 301235 | 3.53630± 0.177 | 1.444 ± 0.476 | 0.20 ± 0.07 | 7916 ± 713 |
| 4 | 773515 ± 29803 | 2.82828 ± 0.062 | 1.138 ± 0.062 | 0.150 ± 0.005 | 10015 ± 176 |
| 5 | 692498 ± 75207 | 3.38321± 0.055 | 1.250 ± 0.037 | 0.15 ± 0.01 | 13662 ± 199 |
| 6 | 3876880 ± 32435 | 5.51882 ± 0.272 | 1.254 ± 0.035 | 0.25 ± 0.03 | 18116 ± 1399 |
| 7 | 1616565 ± 19075 | 3.89013± 0.119 | 1.197 ± 0.047 | 0.180 ± 0.005 | 17272 ± 866 |
| 8 | 2153301 ± 265350 | 3.78876 ± 0.118 | 1.167 ± 0.009 | 0.280 ± 0.005 | 9014 ± 595 |
| 9 | 1804364 ± 1560881 | 2.63892 ± 0.026 | 1.305 ± 0.015 | 0.15 ± 0.00 | 8520 ± 184 |
| 10 | 1839773 ± 225032 | 3.23600 ± 0.278 | 1.197 ± 0.070 | 0.180 ± 0.005 | 16810 ± 3690 |
| 11 | 3264272 ± 118435 | 3.43091 ± 0.272 | 1.126 ± 0.015 | 0.180 ± 0.005 | 14481± 1556 |
| 12 | 1543515 ± 93567 | 2.14043 ± 0.176 | 0.000 | 0.15 ± 0.00 | 5623 ± 809 |
| 13 | 2082997 ± 76403 | 4.40719± 0.145 | 1.328 ± 0.058 | 0.230 ± 0.005 | 12065 ± 691 |
| 14 | 2909113 ± 253541 | 4.83657± 0.0178 | 1.215 ± 0.017 | 0.290 ± 0.005 | 14209 ± 91 |
| 15 | 850512 ± 45882 | 5.24743 ± 0.416 | 1.266 ± 0.110 | 0.230 ± 0.005 | 16456 ± 1784 |
| 16 | 1611628 ± 76045 | 2.70298 ± 0.096 | 1.076 ± 0.013 | 0.180 ± 0.005 | 9143 ± 648 |

**Table 3.14** Estimated values related with methanol peak from Table 3.13

| | Estimated effects for | | | |
|---|---|---|---|---|
| **Factor** | **Peak area** | **Resolution** | **Width** | **Theo. plates** |
| $F_1$ | -519432 | 0.071 | 0.011 | 252 |
| $F_2$ | 1370703 | 0.079 | -0.001 | 1568 |
| $F_3$ | 1618128 | -0.718 | 0.011 | -4367 |
| $F_4$ | 491143 | -0.488 | -0.005 | -3403 |
| $F_5$ | 383364 | 0.078 | 0.001 | 824 |
| $F_6$ | -632540 | 0.774 | 0.011 | 4116 |
| $F_7$ | 10481 | 0.018 | -0.007 | -360 |
| $F_8$ | | | | |
| $F_9$ | 1308196 | -0.206 | 0.011 | -1463 |
| $F_{10}$ | 564910 | 0.022 | -0.007 | 672 |
| $F_{11}$ | -727382 | -1.496 | -0.098 | -779 |
| $F_{12}$ | -265070 | -0.032 | 0.009 | 476 |
| $F_{13}$ | 1198682 | 0.065 | 0.001 | -110 |
| $F_{14}$ | -571592 | 0.034 | 0.016 | 706 |
| $F_{15}$ | -171744 | 0.049 | 0.016 | -273 |

As can be followed from above tables, sixteen experiments in triplicates were performed. By using the replicates, standard deviation (s) of the responses and then standard deviation of effects was estimated (see Eq. 1.10). Pooled estimate of variance for replicates was calculated for 16 experiments as;

Pooled estimate = Total estimated variance / 16

Then the variance ($V_{effect}$) of each effect was calculated for N = 36 (see Eq.1.12). Then, Pareto charts were constructed for each alcohol component of aqueous mixture where the red line indicates the critical t-values for p = 0.05. In addition pink bars represent the positive effect while blue bars represent negative effect on data.

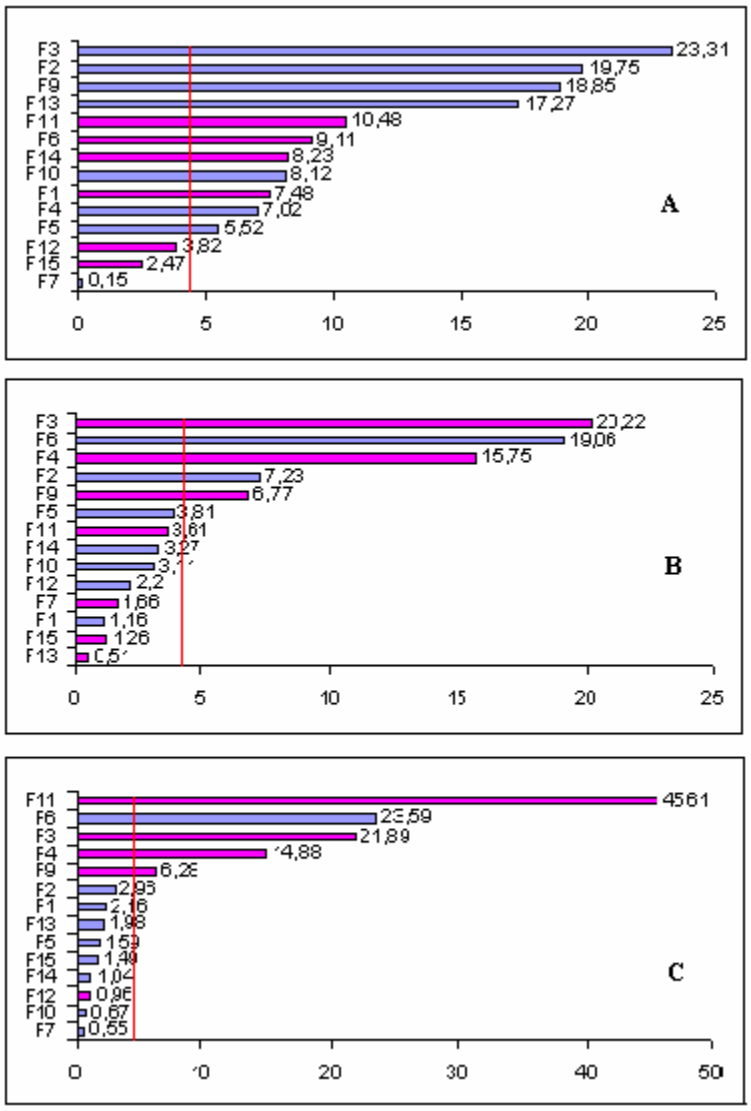


**Figure 3.2** Standardized effects of the factors on A) methanol peak area, B) number of theoretical plates, C) methanol peak resolution.

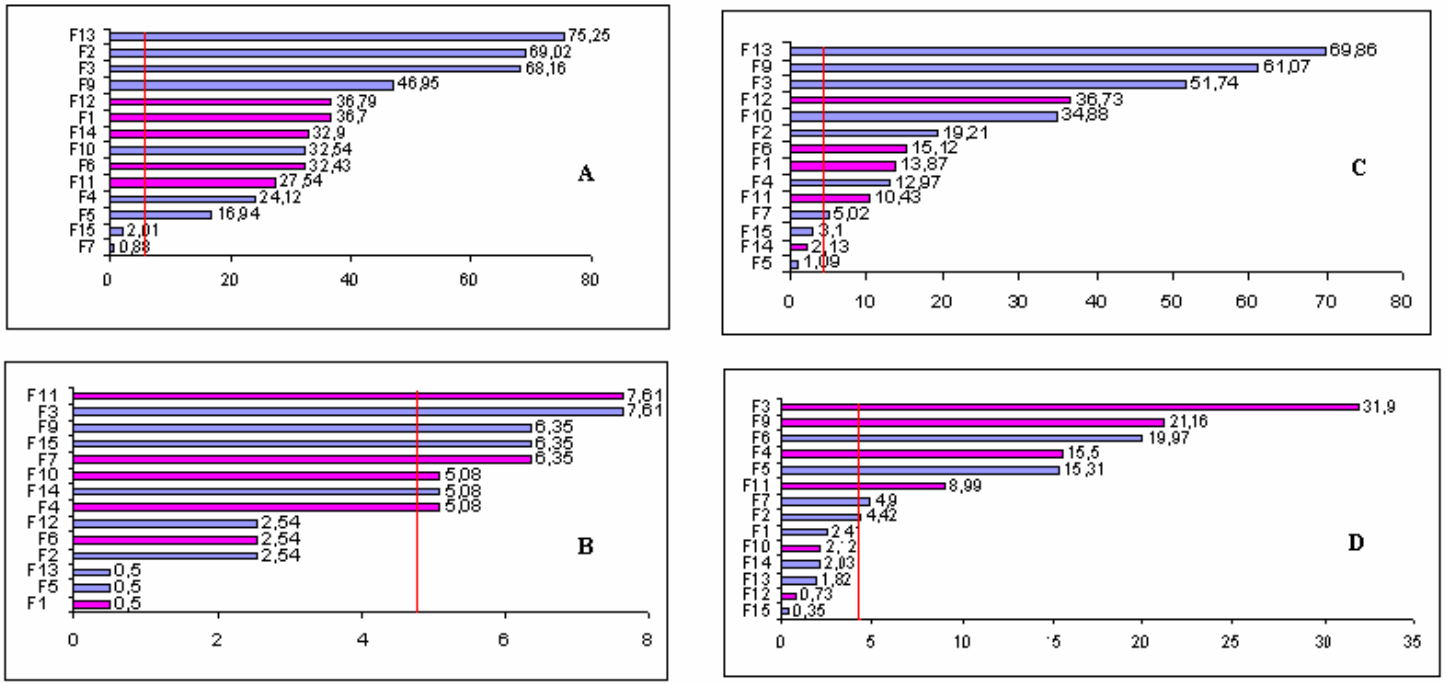


**Figure 3.3** Standardized effects of the factors on A) acetone peak area, B) acetone peak width, C) n-propanol peak area and D) number of theoretical plates calculated for n-propanol.

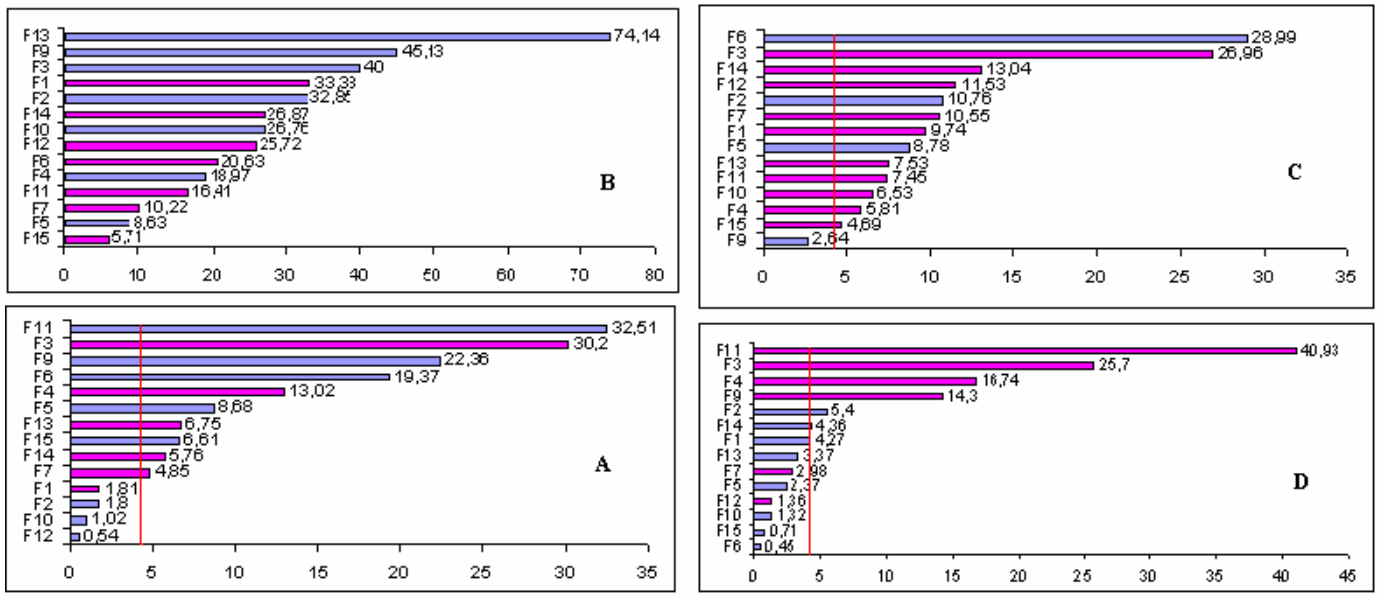


Figure 3.4 Standardized effects of the factors on A) n-buthanol peak area, B) n-buthanol peak resolution, C) number of theoretical plates calculated for n-buthanol and D) .n-propanol peak resolution.

As can be followed from the Pareto charts, a few parameters are mostly affected on the peak area and resolution of the chromatographic separation namely; salting out ($F_{13}$), equilibrium temperature ($F_9$), injected headspace volume ($F_3$), sample concentration ($F_2$), equilibrium time ($F_{10}$) and carrier gas flow rate ($F_4$).

It is well known that addition of salt to the sample increases the polarity and therefore improves the extraction efficiency for a number of analytes in biological samples. Equilibrium temperature and time had a significant positive effect on the peak area as expected.

On the other hand, relative effects of detector and inlet temperature ($F_{12}$ and $F_5$) exceeded the t value from time to time and their effect was mostly in negative direction.

### 3.2.6 Optimization of Static-HS Injection and GC conditions

***Central Composite Design (1)***

A Central Composite Design was constructed with four factors ($k = 4$) affecting the headspace alcohol analysis. Number of experiments can be calculated as $(2^k + 2k + 1) = 16 + 8 + 1 = 25$.

Number of replicates at center point= 11 (for orthogonal and rotational design) Total number of experiments ($N$) = 25 + 11 = 36.

The values of axial points for star design were calculated (see Eq.1.13).±α values for the factors were calculated by using Eq.1.15. The coded values in the Table 3.15 were calculated for each factor by using above equations. Four factors central composite design model was given in Table 3.16. Determined factors and levels are given in Table 3.17

**Table 3.15** Factors and coded values for central composite design by static HS-GC-FID system with HP20M column

| Factor | -2.000 (α) | -1 | 0 | 1 | 2.000 (α) |
|---|---|---|---|---|---|
| $X_1$ : Salting out (NaCl, g) | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 |
| $X_2$ : Eq. temp. of the sample(˚C) | 45 | 50 | 55 | 60 | 65 |
| $X_3$ : Eq. time of the sample (min) | 5 | 10 | 15 | 20 | 25 |
| $X_4$ : Oven temperature: (˚C) | 50 | 55 | 60 | 65 | 70 |

Eq. temp: Equilibrium temperature, Eq. time: Equilibrium time

Model equation for this design is seen below;

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4 + b_{123}x_1x_2x_3 + b_{124}x_1x_2x_4 + b_{134}x_1x_3x_4 + b_{234}x_2x_3x_4 + b_{1234}x_1x_2x_3x_4$$

Table 3.10 showed the determined factors and their levels applied in the design model. Using the model design matrix (***X***) estimated ***b*** parameters were calculated by using Eq.1.16. Estimated responses (see Eq.1.17), residual values (***r***) (Eq.1.18) and sum of squares of residuals ($SS_{residual}$) (Eq.1.19) were calculated.

Degree of freedom (d.f.) for design model, replicates and Lack of fit (*LOF*) were calculated as given below;

Total number of experiments: 36

Number of parameters in the model: 20

d.f. for residuals: 36 – 20 = 16

d.f. for pure experimental uncertainty: 11

d.f. for the LOF (lack of fit of the model): 36 – 20 – 11 = 5

**Table 3.16** Four factors Central Composite Design applied for HS-GC-FID determination of alcohol standards.

| Run no | $X_0$ | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_{11}$ | $X_{22}$ | $X_{33}$ | $X_{44}$ | $X_{12}$ | $X_{13}$ | $X_{14}$ | $X_{23}$ | $X_{24}$ | $X_{34}$ | $X_{123}$ | $X_{124}$ | $X_{134}$ | $X_{234}$ | $X_{1234}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 1 | -1 | -1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 |
| **2** | 1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 |
| **3** | 1 | -1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | -1 |
| **4** | 1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | -1 | 1 | 1 | 1 |
| **5** | 1 | -1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | -1 |
| **6** | 1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | -1 | 1 | -1 | -1 | 1 | -1 | 1 | 1 |
| **7** | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | -1 | -1 | 1 | 1 | -1 | 1 |
| **8** | 1 | 1 | 1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | -1 | 1 | -1 | -1 | -1 | -1 |
| **9** | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 |
| **10** | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | -1 | 1 | -1 | -1 | 1 | 1 |
| **11** | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | -1 | 1 |
| **12** | 1 | 1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | -1 | 1 | -1 | -1 | -1 |
| **13** | 1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | -1 | 1 |
| **14** | 1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | -1 | -1 | 1 | -1 | -1 |
| **15** | 1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 |
| **16** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **17** | 1 | +2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **18** | 1 | -2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **19** | 1 | 0 | +2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **20** | 1 | 0 | -2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **21** | 1 | 0 | 0 | +2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **22** | 1 | 0 | 0 | -2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **23** | 1 | 0 | 0 | 0 | +2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **24** | 1 | 0 | 0 | 0 | -2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **25** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **26** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **27** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **28** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **29** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **30** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **31** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **32** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **33** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **34** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **35** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **36** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 3.17** Determined factors and levels for central composite design by static HS-GC-FID system with HP20M column

| Run no | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Run no | $X_1$ | $X_2$ | $X_3$ | $X_4$ |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 1.5 | 50 | 10 | 55 | **19** | 2 | 65 | 15 | 60 |
| **2** | 2.5 | 50 | 10 | 55 | **20** | 2 | 45 | 15 | 60 |
| **3** | 1.5 | 60 | 10 | 55 | **21** | 2 | 55 | 25 | 60 |
| **4** | 2.5 | 60 | 10 | 55 | **22** | 2 | 55 | 5 | 60 |
| **5** | 1.5 | 50 | 20 | 55 | **23** | 2 | 55 | 15 | 70 |
| **6** | 2.5 | 50 | 20 | 55 | **24** | 2 | 55 | 15 | 50 |
| **7** | 1.5 | 60 | 20 | 55 | **25** | 2 | 55 | 15 | 60 |
| **8** | 2.5 | 60 | 20 | 55 | **26** | 2 | 55 | 15 | 60 |
| **9** | 1.5 | 50 | 10 | 65 | **27** | 2 | 55 | 15 | 60 |
| **10** | 2.5 | 50 | 10 | 65 | **28** | 2 | 55 | 15 | 60 |
| **11** | 1.5 | 60 | 10 | 65 | **29** | 2 | 55 | 15 | 60 |
| **12** | 2.5 | 60 | 10 | 65 | **30** | 2 | 55 | 15 | 60 |
| **13** | 1.5 | 50 | 20 | 65 | **31** | 2 | 55 | 15 | 60 |
| **14** | 2.5 | 50 | 20 | 65 | **32** | 2 | 55 | 15 | 60 |
| **15** | 1.5 | 60 | 20 | 65 | **33** | 2 | 55 | 15 | 60 |
| **16** | 2.5 | 60 | 20 | 65 | **34** | 2 | 55 | 15 | 60 |
| **17** | 3 | 55 | 15 | 60 | **35** | 2 | 55 | 15 | 60 |
| **18** | 1 | 55 | 15 | 60 | **36** | 2 | 55 | 15 | 60 |

The mean response of replicates was calculated. The difference between mean and experimental responses was calculated and then sum of squares of experimental uncertainty was obtained by using Eq.1.20.

The sum of squares due to lack of fit were calculated from Eq.1.21.

Calculated $SS_{residual}$, $SS_{replicates}$ and *LOFSS* values were divided by their degrees of freedom and variances ($s^2$) were obtained.

The ratio of variances for *F* value were calculated ($s^2_{LOF}/s^2_{residual}$) and compared with tabulated *F* value ($F_{table}$) at a probability level p= 0.05 (for $F_{5,11}$). Lower $F_{experimental}$ values than $F_{table}$ values proved that the experimental model was adequate.

Variances ($s^2_{parameters}$) for each parameter were calculated for the t test as, $(X^TX)^{-1}$. $t_{experimental}$ was calculate by using Eq.1.22 and compared with $t_{table}$ value at probability level p = 0.025. A new design model was constructed with the intercept point and significant parameters those exceeds the $t_{table}$ value.

Estimated response values, residuals and sum of squares due to residuals were calculated as described above. Eventually to test how well the model is fitted, *%RMSE* was calculated (see Eq.1.23 and 1.24). The smaller is the percentage, the better fitting the model.

The adequacy of the constructed central composite design model was evaluated according to the parameters obtained from the chromatograms. Peak areas were selected as the response factors. Experimental F values obtained for the applied design model were listed in Table 3.11. $F_{table}$ ($F_{5,11}$) value for p = 0.05 was given as 3.20.

It can be followed from the table that the experimental model is adequate for acetone, methanol and n-propanol, but not for n-buthanol.

**Table 3.18** F test results for the applied model

| **Peak name** | $\mathbf{F_{experimental}}$ |
|---|---|
| Acetone | 1.36 |
| Methanol | 1.38 |
| n-propanol | 1.06 |
| n-butanol | 4.67 |

t test was performed for testing the significance of selected parameters. $t_{experimental}$ values for each parameters were calculated. The values obtained were compared with the $t_{table}$ values for f.d. 5 ($t_{table}$ =2.57, p = 0.05) in Table 3.19. Those exceed the tabulated value were given in bold character.

**Table 3.19** *b* coefficients associated with peak area of acetone, methanol and n-propanol and their $t_{experimental}$ values

| **b** | **Acetone** | **Methanol** | **n-Propanol** |
|---|---|---|---|
| $b_0$ | **17.36** | **23.14** | **34.67** |
| $b_1$ | 0.05 | 0.03 | 0.05 |
| $b_2$ | **3.61** | **7.22** | **10.83** |
| $b_3$ | 2.35 | **2.90** | **5.52** |
| $b_4$ | 0.24 | 0.63 | 0.54 |
| $b_{11}$ | 0.37 | 0.16 | 0.34 |
| $b_{22}$ | 0.68 | 2.06 | 2.46 |
| $b_{33}$ | 0.71 | 1.03 | 1.67 |
| $b_{44}$ | 0.03 | 0.62 | 0.50 |
| $b_{12}$ | 0.15 | 0.02 | 0.26 |
| $b_{13}$ | 0.13 | 0.23 | 0.44 |
| $b_{14}$ | 0.17 | 0.28 | 0.35 |
| $b_{23}$ | 0.05 | 0.24 | 0.58 |
| $b_{24}$ | 0.36 | 0.22 | 0.42 |
| $b_{34}$ | 0.20 | 0.08 | 0.37 |
| $b_{123}$ | 0.30 | 0.44 | 0.73 |
| $b_{124}$ | 0.04 | 0.43 | 0.25 |
| $b_{134}$ | 0.02 | 0.05 | 0.11 |
| $b_{234}$ | 0.14 | 0.21 | 0.31 |
| $b_{1234}$ | 0.02 | 0.04 | 0.06 |

The evaluation of the results revealed that most significant parameter for the constructed central composite design was the equilibrium temperature of the sample and this was followed by equilibrium time. These two parameters had a positive effect on the peak areas. Other parameters namely salting out and column temperature were found to affect the results not in significant degree.

Considering the t test values, a new design was constructed including significant parameters alone. The percentage of mean squares due to residuals *% RMSE* values for acetone, methanol and n-propanol were calculated (see Eq.1.23 and 1.24) and given in Table 3.20. These values were proved to be well fitted by F test.

**Table 3.20** %RMSE values for applied model

| **Peak name** | ***% RMSE*** |
|---|---|
| Acetone | 6.39 |
| Methanol | 4,85 |
| n-propanol | 4.87 |

Partial derivatives for each significant parameter in the model equation revealed that the optimum values were the center points for equilibrium temperature and time.

Ethanol and isopropanol peaks were not resolved chromatographically and therefore, calculations related to these standards were not performed. Biological matrixes like blood decompose at higher temperatures (Zuba; 2002b) and ethanol is oxidized to acetaldehyde (Tagliaro et al.; 1992). Therefore, equilibrium temperatures exceeding 60˚C were avoided in this study.

***Central Composite Design (2)***

Since isopropanol and ethanol were not resolved well under the optimized conditions given above, another central composite design was performed with the previously selected parameters. Here, isopropanol was excluded due to the interference of ethanol peak.

Four factors Central Composite Design model (see Table 3.9) was constructed for the optimization of static headspace injection and chromatographic conditions. Number of runs, replicates at the center and total number of runs were calculated as described earlier. Axial points were calculated for four factors (see Eq.1.15). Coded and applied levels determined for the factors were the same as in Table 3.8 and Table 3.10 respectively.

Chemometric calculations were performed as described above. The adequacy of the constructed central composite design model was evaluated according to the parameters obtained from the chromatograms. Peak areas were selected as the response factors. Experimental F values obtained for the applied design model were given in Table 3.21.

**Table 3.21** F test results for the applied model

| **Peak name** | $\mathbf{F_{experimental}}$ |
|---|---|
| Acetone | 2.25 |
| Methanol | 2.99 |
| Ethanol | 1.75 |
| n-propanol | 1.75 |
| n-butanol | 1.99 |

On comparing the $F_{experimental}$ values with $F_{table}$ ($F_{5,11}$) for p = 0.05 it can be concluded that experimental model is adequate for all the analytes since they all are exceeded by the tabulated value (3.20).

On the other hand t test was performed for evaluation of the significance of selected parameters. $t_{experimental}$ values for each parameter were calculated by using Eq 1.22 (Table 3.22).The values obtained were compared with the $t_{table}$ values for d.f. 5 ($t_{table}$ = 2.57 for p = 0.05).

**Table 3.22** $t_{experimental}$ value**s** for b coefficients calculated in relation with the peak areas of the analytes

| **b** | **Acetone** | **Methanol** | **Ethanol** | **n-propanol** | **n-butanol** |
|---|---|---|---|---|---|
| $b_0$ | **20.63** | **19.83** | **21.28** | **24.01** | **27.52** |
| $b_1$ | 0.31 | 0.16 | 0.36 | 0.47 | 0.67 |
| $b_2$ | **3.46** | **5.16** | **6.04** | **6.45** | **7.33** |
| $b_3$ | **2.51** | 2.11 | **2.72** | **3.37** | **4.47** |
| $b_4$ | 0.40 | 0.17 | 0.24 | 0.52 | 0.69 |
| $b_{11}$ | 0.02 | 0.74 | 0.66 | 0.48 | 0.66 |
| $b_{22}$ | 0.06 | 1.18 | 1.28 | 0.84 | 0.84 |
| $b_{33}$ | 1.18 | 0.57 | 0.84 | 1.17 | 1.10 |
| $b_{44}$ | 0.01 | 0.36 | 0.30 | 0.36 | 0.31 |
| $b_{12}$ | 0.20 | 0.14 | 0.24 | 0.25 | 0.37 |
| $b_{13}$ | 0.08 | 0.39 | 0.42 | 0.31 | 0.22 |
| $b_{14}$ | 0.12 | 0.20 | 0.18 | 0.21 | 0.21 |
| $b_{23}$ | 0.41 | 0.48 | 0.67 | 0.74 | 0.92 |
| $b_{24}$ | 0.16 | 0.02 | 0.10 | 0.20 | 0.28 |
| $b_{34}$ | 0.32 | 0.05 | 0.07 | 0.18 | 0.39 |
| $b_{123}$ | 0.02 | 0.21 | 0.24 | 0.16 | 0.09 |
| $b_{124}$ | 0.03 | 0.22 | 0.22 | 0.12 | 0.10 |
| $b_{134}$ | 0.04 | 0.28 | 0.24 | 0.1 | 0.04 |
| $b_{234}$ | 0.07 | 0.17 | 0.18 | 0.22 | 0.20 |
| $b_{1234}$ | 0.22 | 0.09 | 0.14 | 0.24 | 0.52 |

The evaluation of the results revealed that intercept ($b_0$) has relatively high effect on the signal indicating the baseline of volatile constituents. It can be deducted that $b_2$ and $b_3$ related with the equilibrium temperature and time significantly affect the linear relation of response with the concentration of analytes in positive direction.

Since all the samples were saturated with salt, the effects of salting out and also column temperature were not found significant on the results. On the other hand interaction terms and quadratic terms were not found significant statistically.

According to t test results new designs were constructed with significant parameters and *%RMSE* values were calculated and given in Table 3.23.

**Table 3.23** The %RMSE values of the applied model.

| **Peak name** | ***% RMSE*** |
|---|---|
| Acetone | 6.72 |
| Methanol | 7.38 |
| Ethanol | 7.54 |
| n-propanol | 6.62 |
| n-butanol | 6.30 |

As illustrated in the former model, partial derivatives for each significant parameter in the model equation revealed that the optimum values for equilibrium temperature and time were the center points. Therefore, a validation study was conducted under optimized conditions.

### 3.2.7 Static- HS-GC Method Validation in Blood Samples

Validation studies were carried out with HP 20 M column. Stock solutions were prepared as given in Experimental Section. Each blood sample contained equal amount of n-propanol as the internal standard (10.0 mg/100mL). 1.0 mL of blood and 1.0 mL of IS solution were placed into the vials and capped immediately. Then the analytical procedure was initiated. Experimental conditions as optimized before for static HS injection and GC operation were given in Table 3.24.

**Table 3.24** Optimum parameters for determination of alcohol standards by static HS-GC-FID method with HP 20M column.

| **Parameters for sample and GC** | **Conditions** |
|---|---|
| Inlet temperature | 165 °C |
| Oven temperature | 60° C |
| Detector temperature | 180 °C |
| Air flow rate | 350 mL/min |
| $H_2$ gas flow rate | 35 mL/min |
| Split flow rate | 30 mL/min |
| Carrier gas flow rate | 1.0 mL/min |
| Equilibrium temperature | 55 °C |
| Equilibrium time | 15 min |
| Injected headspace volume | 1.0 mL |
| Salting out (NaCl) | 2.0 g |
| Sample volume in vial | 2.0 mL |

The method was found selective to differentiate and to quantify alcohol standards as the R>1 was observed except isopropanol which gave overlapped peaks with that of ethanol. Therefore this component was excluded in the validation studies. The actual chromatogram can be seen in Figure 3.5.

**Figure 3.5** Static HS-GC-FID chromatogram of the standards with HP 20M column. (1: Acetone, 2: Methanol, 3: Ethanol, 4: n-propanol)

The peak area of each standard displayed results proportional to the concentration of the analyte and calibration graphs for the standards were obtained in the concentration ranges given in Table 3.25. Here, it should be noted that these concentration ranges were selected in typical blood alcohol levels to suit the actual purpose.

Figure 3.6 shows the calibration graphs constructed with five points and three replicates. Table 3.26 summarizes the characteristics of these calibration graphs.

**Table 3.25** Concentration ranges of the analytes used in calibration graph.

| **Standard** | **Concentration (mg/100mL)** |
|---|---|
| Acetone | 0.1, 1.0, 10, 50, 100 |
| Methanol | 1.0, 10, 100, 150, 200 |
| Ethanol | 0.1, 1.0, 10 100, 300 |

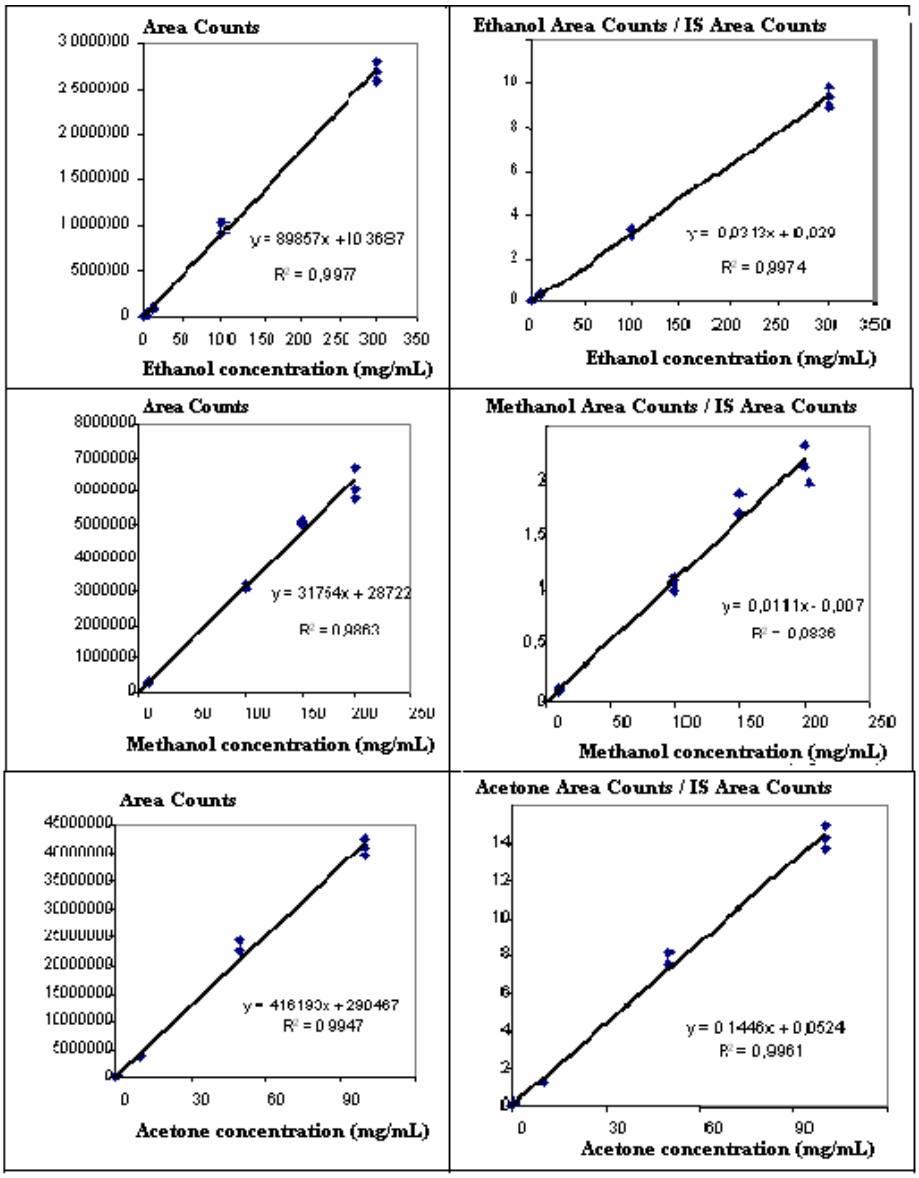


**Figure 3.6** Calibration graphs for ethanol, methanol and acetone solutions obtained by static HS-GC-FID system with HP 20M column.

**Table 3.26** Characteristics of the calibration graphs for acetone, methanol and ethanol

| Standard | y (according to peak area) | $R^2$ | y (peak area/IS area) | $R^2$ |
|---|---|---|---|---|
| Acetone | y = 416193x + 290467 | 0.9947 | y = 0.1446x + 0.0524 | 0.9961 |
| Methanol | y = 31754x + 28722 | 0.9863 | y = 0.0111x + 0.00 | 0.9836 |
| Ethanol | y = 89857x + 103687 | 0.9977 | y = 0.0313x + 0.029 | 0.9974 |

Precision was evaluated by the means of RSD values for each standard. Blood samples containing standard concentrations at four levels were analyzed in five replicates for five consecutive days. RSD values detected for within-day repeatability were under the proposed limits for low, middle and high concentrations. Within-day precision in RSD is shown in Table 3.27.

As proposed earlier, RSD% limit acceptable for low concentrations was ± 20 % and for middle and high concentrations was ± 15 % (Peters and Maurer, 2002; Causon, 1997). Within-day repeatability of the present study was found in acceptable range for different concentration levels.

Between-day repeatability was evaluated by the means of ANOVA and the results were given in Table 3.28. The results were compared with the tabulated F value (2.87) for the d.f. of 4. Corresponding p values were also given in the table.

**Table 3.27** RSD values at four concentration levels with five replicates in consecutive days for each standard.

| Concentration (mg/100mL) | Standard | RSD values for days (%) | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 0.2 | Acetone | 4.28 | 3.94 | 12.32 | 6.45 | 11.44 |
| 2.0 | Methanol | 7.07 | 6.4 | 13.13 | 13.08 | 14.53 |
| 0.2 | Ethanol | 19.76 | 18.80 | 15.88 | - | 18.95 |
| | n-propanol | 4.70 | 3.28 | 3.02 | 2.71 | 4.55 |
| 1.0 | Acetone | 6.58 | 2.45 | 3.56 | 6.63 | 2.49 |
| 10 | Methanol | 6.93 | 17.01 | 4.09 | 6.37 | 3.16 |
| 1.0 | Ethanol | 15.61 | 1.18 | 3.50 | 9.37 | 8.35 |
| | n-propanol | 4.26 | 3.93 | 2.69 | 6.08 | 3.32 |
| 10 | Acetone | 6.17 | 5.15 | 4.22 | 1.22 | 4.32 |
| 100 | Methanol | 9.40 | 5.52 | 6.37 | 12.48 | 3.78 |
| 10 | Ethanol | 8.77 | 4.72 | 5.37 | 3.89 | 5.11 |
| | n-propanol | 6.89 | 2.76 | 4.13 | 4.08 | 6.37 |
| 100 | Acetone | 4.82 | 1.11 | 5.71 | 3.72 | 4.26 |
| 200 | Methanol | 10.26 | 10.99 | 3.94 | 3.79 | 4.62 |
| 300 | Ethanol | 3.187 | 1.00 | 6.69 | 3.07 | 3.27 |
| | n-propanol | 4.74 | 2.55 | 3.81 | 2.64 | 2.07 |

**Table 3.28** ANOVA results for between-day repeatability.

| Standard | Concentration (mg/100mL) | $F_{experimental}$ | p |
|---|---|---|---|
| Acetone | 0.2 | 7.36 | 8.0E-05 |
| | 1.0 | 5.89 | 2.7E-03 |
| | 10 | 4.57 | 8.7E-03 |
| | 100 | 14.35 | 1.1E-05 |
| Methanol | 2.0 | 38.50 | 3.9E-09 |
| | 10 | 29.71 | 3.7E-08 |
| | 100 | 7.64 | 6.6E-04 |
| | 200 | 15.57 | 6.2E-06 |
| Ethanol | 0.2 | 2.72 | 6.9E-02 |
| | 1.0 | 10.92 | 7.4E-05 |
| | 10 | 7.40 | 7.9E-04 |
| | 300 | 13.71 | 1.6E-05 |

As can be followed from the table, the ANOVA results between-day repeatability were not found satisfactory for five consecutive days. This can be attributed to the nature of the batch analysis employed.

The sensitivity of the method was tested upon calculating the LOD and LOQ values given in Table 3.29. Analyses were carried out for spiked blood samples obtained from three different people for the lowest concentration level in the calibration range and LOQ were detected within the confidence interval of 95% ($t$ = 4.30, $n$ = 3). RSD values for each standard were determined.

**Table 3.29** LOD and LOQ values of the standards in blood.

| **Parameter** | **Acetone** | **Methanol** | **Ethanol** | **n-propanol** |
|---|---|---|---|---|
| LOD ±RSD (mg/100mL) | 0.021 ± 0.021 | 0.913 ± 0.618 | 0.236 ± 0.089 | 0.161 ± 0.039 |
| LOQ ±RSD (mg/100mL) | 0.069 ± 0.076 | 3.040 ± 2.060 | 0.787 ± 0.300 | 0.537 ± 0.134 |

LOD and LOQ values found for static-headspace ethanol analyses for blood samples were found in agreement with those values found for static-headspace ethanol analyses for blood and urine samples in the literature (Table 1.2).

In order to determine the accuracy of the method, both spiked aqueous and blood samples were prepared at low, middle and high concentration levels. First, ratios of peak areas of standards for blood and aqueous samples were evaluated. Then, for each standard peak areas/IS areas were taken into consideration for the calculation of percent recovery. Recoveries obtained for the standards in low, middle and high concentration levels were between 70- 104% (Table 3.30).

**Table 3.30** Percent recovery values for each standard at high, middle and low concentrations in aqueous and blood samples.

| Conc. level | Standard | Concentration (mg/100mL) | %Recovery based on | |
|---|---|---|---|---|
| | | | Peak area | Peak area/IS area |
| High | Acetone | 100 | 75.20 | 75.27 |
| | Methanol | 200 | 84.36 | 84.45 |
| | Ethanol | 300 | 95.63 | 95.72 |
| | n-propanol | 10.0 | 99.89 | |
| Middle | Acetone | 20.0 | 75.10 | 71.90 |
| | Methanol | 50.0 | 102.57 | 98.20 |
| | Ethanol | 50.0 | 97.77 | 93.61 |
| | n-propanol | 10.0 | 104.44 | |
| Low | Acetone | 10.0 | 89.35 | 101.4 |
| | Methanol | 2.50 | 68.34 | 77.50 |
| | Ethanol | 2.50 | 84.56 | 95.91 |
| | n-propanol | 10.0 | 88.16 | |

On the other hand, three point calibration graphs were also constructed for the analyzed blood and aqueous standard samples and the ratios of the slopes of the linear equations were used in recovery calculations. Calibration graph data and calculated recoveries obtained for each standard in two matrixes were given in Table 3.31.

**Table 3.31** Recovery percentages of the standards related with slopes of the calibration curves.

| **Standard** | **Matrix** | | **Recovery (%)** |
|---|---|---|---|
| | **Blood** | **Deionized water** | |
| **Acetone** | y = 430850x + 93487 | y = 573706x + 58690 | 75.09 |
| | $R^2 = 1$ | $R^2 = 1$ | |
| **Methanol** | y = 36100x + 205090 | y = 43477x + 32177 | 83.03 |
| | $R^2 = 0.9954$ | $R^2 = 1$ | |
| **Ethanol** | y = 106311x + 70315 | y= 111245x + 35525 | 95.56 |
| | $R^2 = 1$ | $R^2 = 1$ | |

Stability of the method was tested upon analyzing the blood samples in consecutive days. For this purpose blood samples were spiked with standards at high, middle and low concentration levels and then analyzed freshly, after 1, 2, 3 and 6 days. Samples were stored in refrigerator until the analyses. The effect of freeze and thaw was also investigated for the three concentration levels. Stability was evaluated by the means of ANOVA as shown in Table 3.32.

**Table 3.32** ANOVA results for stability

| Concentration | $F_{experimental}$ | P |
|---|---|---|
| High | 0.032 | 0.9994 |
| Middle | 0.02 | 0.9998 |
| Low | 0.01 | 0.9999 |

Following the table above, it is seen that detected *F* values were far below the $F_{table}$ value (2.77 for d.f. 5). It can be concluded that concentration of samples stored in refrigerator and deep freeze conditions at certain period, were not significantly changed during that time.

### 3.2.8 Blood alcohol analysis by HS-GC/FID

Blood samples were analyzed by the present validated method. During the analysis, freshly prepared quality control samples for three levels were also analyzed and concentrations of alcohols in blood samples were calculated by means of the daily constructed calibration graphs. Data related with the calibration graphs were given in Table 3.33.

Due to the slopes obtained from calibration graphs it was seen that there was variations between-days. Daily variations of the calibration curve can influence the bias. Therefore, during blood analysis daily obtained calibration graphs were constructed in order to avoid the bias.

**Table 3.33** Calibration data related with the quality control samples during the blood analyses

| Day | Standard | y (peak area based) | $R^2$ | y (peak area/IS area) | $R^2$ |
|---|---|---|---|---|---|
| 1 | Acetone | y = 514999x - 292573 | 0.9997 | y = 0.1756x – 0.0187 | 0.9999 |
| | Methanol | y = 36274x + 110650 | 0.9987 | y = 0.0.0124x + 0.0546 | 0.9976 |
| | Ethanol | y = 74497x + 951896 | 0.9935 | y = 0.0.0254x + 0.3735 | 0.9915 |
| 2 | Acetone | y = 537745x - 267727 | 0.9998 | y = 0.2068x - 0.3162 | 0.9986 |
| | Methanol | y = 40375x + 81369 | 0.9991 | y = 0.0156x - 0.0156 | 1.000 |
| | Ethanol | y = 35516x + 62256 | 0.9997 | y = 0.0137x - 0.018 | 1.000 |
| 3 | Acetone | y = 447672x - 245350 | 0.9988 | y = 0.1664x - 0.1442 | 0.9971 |
| | Methanol | y = 34127x + 62942 | 0.9995 | y = 0.0127x + 0.0121 | 0.9994 |
| | Ethanol | y = 29479x + 33297 | 0.9997 | y = 0.011x + 0.0025 | 0.9987 |

### 3.3 Headspace- Solid Phase Micro Extraction Studies

In this part of the study, a rather novel technique, headspace-solid phase micro extraction (HS-SPME) was used for simultaneous determination of alcohol standards with GC-FID. This technique is known to allow simultaneous sampling, extraction, pre-concentration, and introduction of analytes from a matrix in a single procedure.

Preliminary studies with HS-SPME technique were carried out with aqueous alcohol standard solutions and very first chromatograms have displayed rather broad peaks with poor resolution. It was anticipated that such peaks were due to the GC inlet liner which was assumed as inappropriate for SPME. Literature survey revealed that for sharper peaks inlet liners with internal diameter, 0.75 mm is proposed for HS-SPME analysis (Sigma-Aldrich, 1998, Mills and Walker, 2000). Alcohol standards were well separated by this means. Chromatogram of the standards was given in Figure 3.7.

**Figure 3.7** HS-SPME-GC-FID chromatogram of 1) acetone 2) methanol, 3) isopropanol, 4) ethanol, 5) n-propanol and 6) n-buthanol.

The main parameters affecting the results were evaluated chemometrically and compared with those obtained with static headspace analysis. For this purpose, a Plackett-Burman design was constructed in order to investigate the effects of the 15 parameters related with the HS-SPME-GC-FID technique. The treatments were considered to be in two halves with the factors at their lower levels in one half and the higher levels in the other. The number of treatments for the 15 factors design is 16. Experiments were performed in three replicates. The factors and their levels for this experimental design were given in Table 3.34. A design model was constructed for 15 factors described earlier (Table 3.7).

**Table 3.34** Factors and levels used for experimental design with HS-SPME-GC-FID system with HP 20M column.

| **Factor** | **Detail** | **(+) level** | **(-) level** |
|---|---|---|---|
| $F_1$ | Inlet temperature (ºC) | 240 | 210 |
| $F_2$ | Oven temperature (ºC) | 60 | 55 |
| $F_3$ | Detector temperature (ºC) | 250 | 180 |
| $F_4$ | Carrier gas flow rate (mL/min) | 1.3 | 1 |
| $F_5$ | Split flow rate (mL/min) | 30 | 50 |
| $F_6$ | $H_2$ gas flow rate (mL/min) | 35 | 40 |
| $F_7$ | Depth gauge of the needle on fiber holder | 3.2 | 2.8 |
| $F_8$ | Sample volume in vial (mL) | 2.0 | 1.0 |
| $F_9$ | Sample concentration (mL/100mL) | 0.05 | 0.03 |
| $F_{10}$ | Equilibrium temperature (ºC) | 60 | 55 |
| $F_{11}$ | Equilibrium time (min) | 10 | 5 |
| $F_{12}$ | Extraction (adsorption) time (min) | 5 | 2 |
| $F_{13}$ | Desorption time (min) | 1.5 | 0.5 |
| $F_{14}$ | Salting out (1 g NaCl) | Yes | No |
| $F_{15}$ | Make-up gas | Yes | No |

Matrix of the designed model (***D***) was used in the calculation of estimated ***b*** coefficients.

$$\boldsymbol{b} = \boldsymbol{D}^{-1}\,\boldsymbol{y} \qquad (3.2)$$

$$\boldsymbol{D}^{-1} = (1/N)\,\boldsymbol{D}^{T} \qquad (3.3)$$

Matrix of the designed model (***D***) was used in the calculation of estimated ***b*** coefficients where N = 16. Chemometric calculations were performed for each alcohol standards and given in Table 3.35. Pareto charts were constructed for each alcohol component of aqueous standard mixture. Figure 3.8 and 3.9 show the relative effects of these factors on peak areas. Figure 3.10 displays the pareto charts drawn for peak resolution.

**Table 3.35** Estimated (Est.) and standardized (Stand.) effects calculated from peak areas obtained from Plackett-Burman design by HS-SPME-GC-FID system with HP 20M column.

| Factor no. | Acetone | | Methanol | | Isopropanol | |
|---|---|---|---|---|---|---|
| | **Est.** | **Stand.** | **Est.** | **Stand.** | **Est.** | **Stand.** |
| $F_1$ | -2455140 | -6.74 | -40252 | -0.35 | -4795685 | -2.73 |
| $F_2$ | -1272012 | -3.49 | -275397 | -2.38 | -1452189 | -0.83 |
| $F_3$ | 134455 | 0.37 | -134475 | -1.16 | -681290 | -0.39 |
| $F_4$ | 885667 | 2.43 | 476502 | 4.12 | 1808686 | 1.03 |
| $F_5$ | -119356 | -0.33 | -47617 | -0.41 | 1854033 | 1.06 |
| $F_6$ | -883442 | -2.43 | 203617 | 1.76 | 1307315 | 0.74 |
| $F_7$ | 275683 | 0.76 | 87406 | 0.76 | -357352 | -0.20 |
| $F_8$ | 1401507 | 3.85 | -415154 | -3.59 | 2332072 | 1.33 |
| $F_9$ | 3026379 | 8.31 | 721278 | 6.24 | 1650847 | 0.94 |
| $F_{10}$ | -977548 | -2.68 | -59622 | -0.52 | -3826449 | -2.18 |
| $F_{11}$ | -741071 | -2.03 | 110042 | 0.95 | 334781 | 0.19 |
| $F_{12}$ | 565250 | 1.55 | -66868 | -0.58 | -201513 | -0.12 |
| $F_{13}$ | 830841 | 2.28 | 83668 | 0.72 | 2539471 | 1.45 |
| $F_{14}$ | 5750048 | 15.79 | 721718 | 6.25 | 12028034 | 6.85 |
| $F_{15}$ | 2512459 | 6.90 | 677210 | 5.86 | 2712881 | 1.55 |

**Table 3.35** continue

| | Ethanol | | n-propanol | | Isopropanol | |
|---|---|---|---|---|---|---|
| **Factor no.** | **Est.** | **Stand.** | **Est.** | **Stand.** | **Est.** | **Stand.** |
| $F_1$ | 111673 | 0.15 | -7933644 | -4.90 | -16192773 | -5.41 |
| $F_2$ | -1164395 | -1.55 | -2256707 | -1.39 | -4271684 | -1.43 |
| $F_3$ | -286488 | -0.38 | -389806 | -0.24 | 654445 | 0.22 |
| $F_4$ | 1638482 | 2.18 | -1551335 | -0.96 | -22101031 | -7.39 |
| $F_5$ | -312619 | -0.42 | 200023 | 0.12 | -6646464 | -2.22 |
| $F_6$ | 144724 | 0.19 | -601212 | -0.37 | -1112335 | -0.37 |
| $F_7$ | 662091 | 0.88 | 2110363 | 1.30 | 6805235 | 2.27 |
| $F_8$ | -1530569 | -2.04 | 3340817 | 2.06 | 12814084 | 4.28 |
| $F_9$ | 2016412 | 2.69 | 8680841 | 5.37 | 41536794 | 13.88 |
| $F_{10}$ | -628960 | -0.84 | -2832554 | -1.75 | -5374608 | -1.80 |
| $F_{11}$ | -1188550 | -1.58 | -2366429 | -1.46 | -645686 | -0.22 |
| $F_{12}$ | -983126 | -1.31 | -309264 | -0.19 | -2891175 | -0.97 |
| $F_{13}$ | 1534489 | 2.04 | 2065728 | 1.28 | 6627139 | 2.22 |
| $F_{14}$ | 11544795 | 15.38 | 38694160 | 23.91 | 78995200 | 26.40 |
| $F_{15}$ | 3557883 | 4.74 | 7574877 | 4.68 | 13854975 | 4.63 |

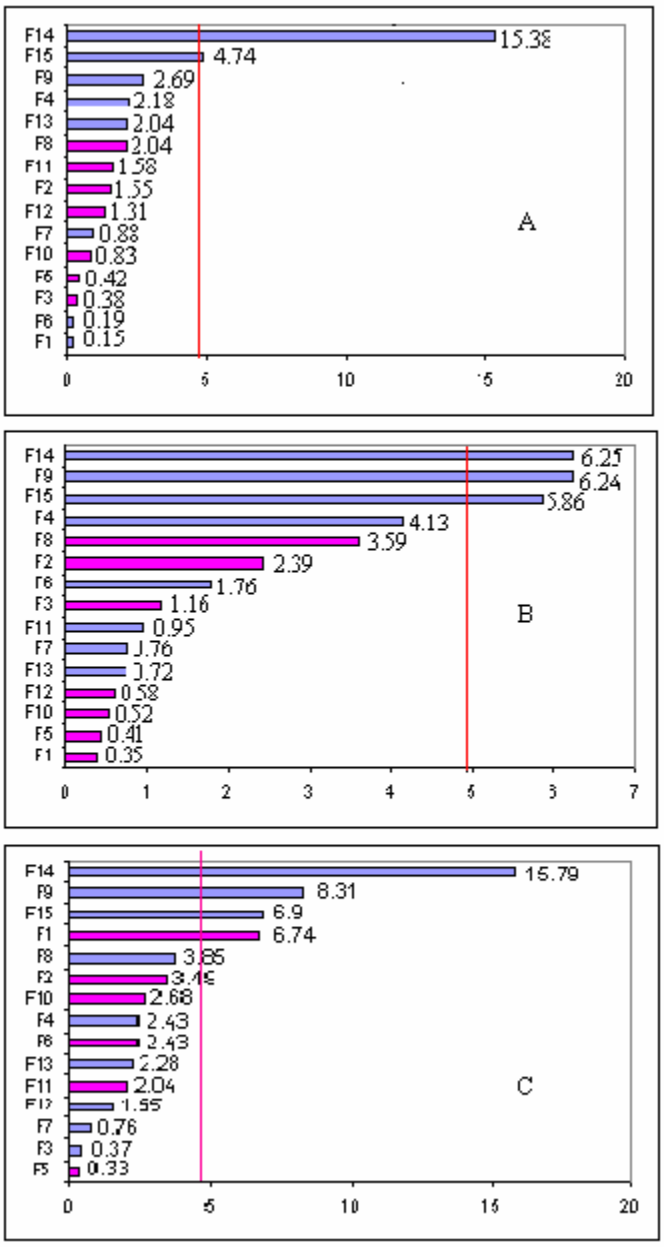


**Figure 3.8** Pareto charts for standardized effects on peak area of A) metanol, B)ethanol and C) acetone standards analyzed by HS-SPME-GC-FID system with HP 20M column.

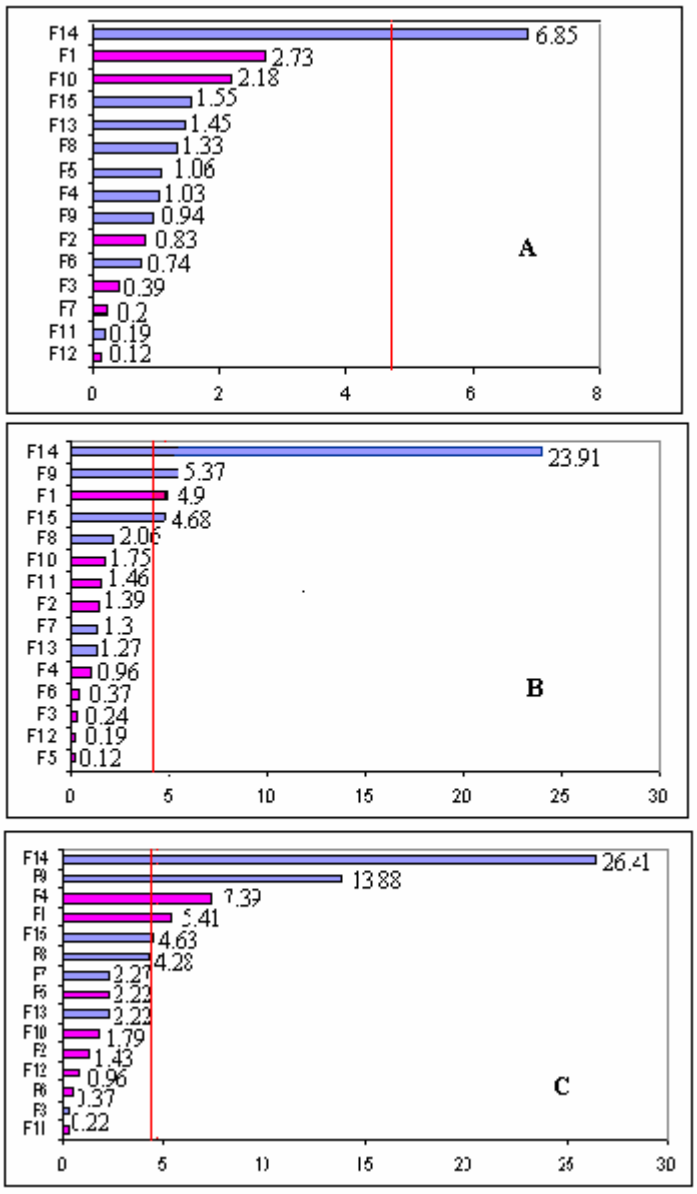


**Figure 3.9** Pareto charts for standardized effects on peak area of A)isopropanol, B) n-propanol and C) n-buthanol standards analyzed by HS-SPME-GC-FID system with HP 20M column.

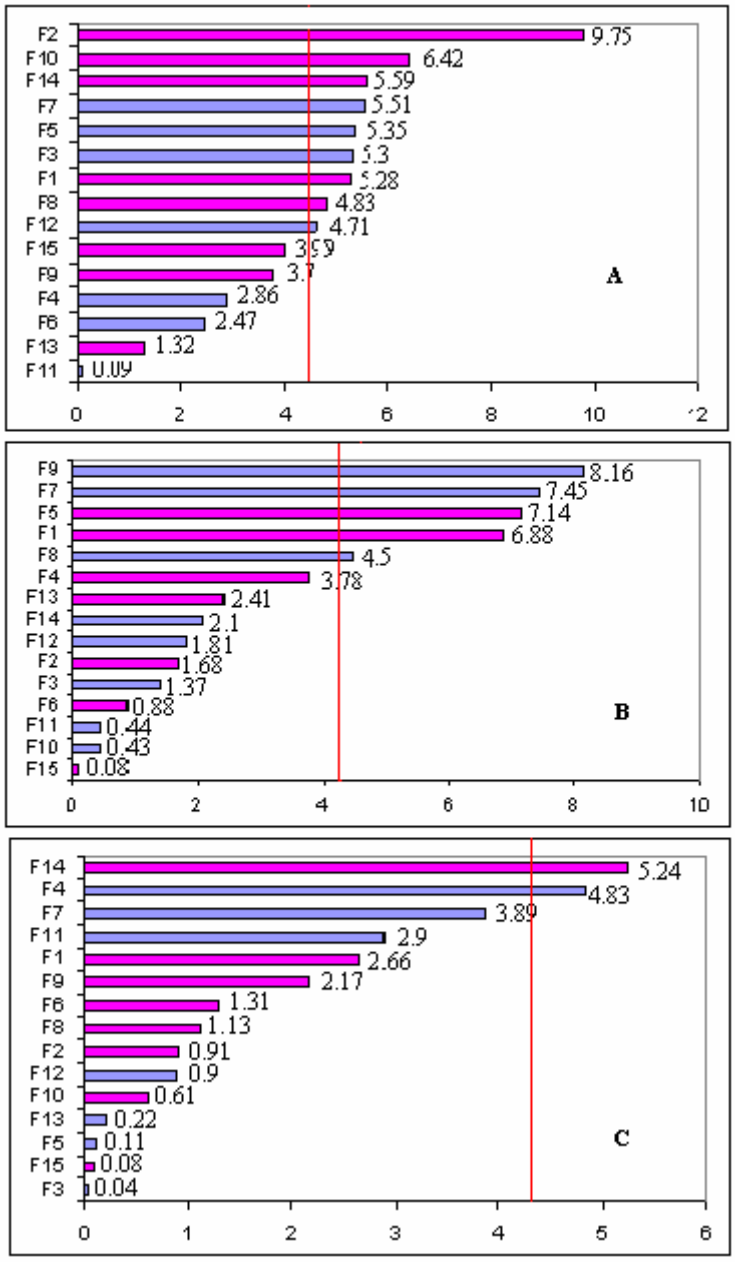


**Figure 3.10** Pareto charts for standardized effects on peak resolution of A)methanol, B) ethanol and C) isopropanol standards analyzed by HS-SPME-GC-FID system with HP 20M column.

As can be followed from these pareto charts, only few parameters among 15 factors chosen had a significant effect on the peak area and resolution of the chromatographic peaks. Primary factors were found as salting out, sample concentration and make-up gas ($F_{14}$, $F_9$ and $F_{15}$). Carrier gas flow rate ($F_4$) and inlet temperature ($F_1$) affected the peak areas negatively for acetone, n-propanol and n-buthanol.

# 4. RESULTS AND DISCUSSION PART II

# EXPERIMENTS WITH COLUMN II

The second part of the thesis includes the studies with another capillary column (Rtx BAC 1) which can provide better resolution for six standards was used in conjunction with static headspace analysis.

## 4.1 Static Headspace Studies

Initial chromatograms recorded by using BAC1 column revealed that peaks of the standards were in the order of methanol, ethanol, isopropanol, acetone, n-propanol and n-buthanol on the chromatogram according to their retention times (Figure-4.1). As can be clearly seen better resolution was obtained for ethanol and isopropanol peaks.

**Figure 4.1** HS-GC-FID chromatogram of 1) methanol, 2) ethanol, 3) isopropanol, 4) acetone, 5). n-propanol and 6) n-buthanol.

### 4.1.1. Evaluation of the Main Effects of the Factors by Plackett-Burman Design for Static Headspace Analysis

In order to find the major effects of 11 factors on the separation of alcohols with static-HS technique a Plackett-Burman Design was constructed for two levels by using the related generator (see Table 1.4).

Aqueous five alcohol standards and acetone mixture each at 0.1% (v/v) concentration were analyzed by HS-GC technique. To increase the polarity of the sample and therefore the extraction efficiency of the analytes, NaCl was added to each vial in 1.0 g proportions.

The factors and their levels were given in Table 4.1 and the design model for 12 run is shown in Table 4.2.

**Table 4.1** Factors and levels used for Plackett-Burman design with static HS-GC-FID system with BAC1 column

| Factor No | Explanation | (+) level | (-) level |
|---|---|---|---|
| $F_1$ | Inlet temperature (ºC) | 200 | 150 |
| $F_2$ | Oven temperature (ºC) | 60 | 40 |
| $F_3$ | Detector temperature (ºC) | 250 | 200 |
| $F_4$ | Carrier gas flow rate (mL/min) | 2 | 1 |
| $F_5$ | $H_2$ gas flow rate (mL/min) | 35 | 30 |
| $F_6$ | Air flow rate (mL/min) | 350 | 300 |
| $F_7$ | Split flow rate (mL/min) | 30* | 50 |
| $F_8$ | Injected headspace volume (mL) | 1.0 | 0.5 |
| $F_9$ | Solution volume in vial (mL) | 2.0 | 1.0 |
| $F_{10}$ | Equilibrium temperature (ºC) | 60 | 40 |
| $F_{11}$ | Equilibrium time (min) | 20 | 10 |

*split flow rate of 30 mL/min was chosen as the (+) level as more analyte is allowed to enter the column at this rate.

**Table 4.2** Plackett-Burman design model

| **Method** | **$F_1$** | **$F_2$** | **$F_3$** | **$F_4$** | **$F_5$** | **$F_6$** | **$F_7$** | **$F_8$** | **$F_9$** | **$F_{10}$** | **$F_{11}$** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| 2 | +1 | +1 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | +1 | -1 |
| 3 | -1 | +1 | +1 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | +1 |
| 4 | +1 | -1 | +1 | +1 | -1 | +1 | +1 | +1 | -1 | -1 | -1 |
| 5 | -1 | +1 | -1 | +1 | +1 | -1 | +1 | +1 | +1 | -1 | -1 |
| 6 | -1 | -1 | +1 | -1 | +1 | +1 | -1 | +1 | +1 | +1 | -1 |
| 7 | -1 | -1 | -1 | +1 | -1 | +1 | +1 | -1 | +1 | +1 | +1 |
| 8 | +1 | -1 | -1 | -1 | +1 | -1 | +1 | +1 | -1 | +1 | +1 |
| 9 | +1 | +1 | -1 | -1 | -1 | +1 | -1 | +1 | +1 | -1 | +1 |
| 10 | +1 | +1 | +1 | -1 | -1 | -1 | +1 | -1 | +1 | +1 | -1 |
| 11 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | +1 | -1 | +1 | +1 |
| 12 | +1 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | +1 | -1 | +1 |

Matrix of the designed model (***D***) was used in the calculation of estimated ***b*** coefficient where N = 12 as given Eq. 3.2 and 3.3. Twelve experiments in triplicates were performed. By using the replicates, standard deviation (s) of the responses and then standard deviation of effects were estimated from Eq.1.10. Pooled estimate of variance for replicates was calculated for 12 runs. Then, the variance of each effect ($V_{effect}$) was calculated from Eq.1.12 for total number of runs.

Chemometric calculations for peak areas and peak heights of each standard were made Table 4.3 and Table 4.4. Pareto charts were shown (in Figure 4.2, 4.3 and 4.4). From the aspect of quantitative analysis, the factors those have a significant effect on peak areas were determined.

**Table 4.3** Estimated (Est.) and standardized (Stand.) effects calculated from peak areas obtained from Plackett-Burman design performed with HS-GC-FID system with BAC1 column.

| Factor no. | Ethanol | | Methanol | | Isopropanol | | Acetone | | n-propanol | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Est.** | **Stand.** | **Est.** | **Stand.** | **Est.** | **Stand.** | **Est.** | **Stand.** | **Est.** | **Stand.** |
| $\mathbf{F_1}$ | -12044.8 | -0.32 | 5713 | 0.45 | -101223 | -0.99 | -170977 | -1.423 | -95691 | -1.05 |
| $\mathbf{F_2}$ | -449485 | -11.83 | -126730 | -9.96 | -1231524 | -12.12 | -1263922 | -10.54 | -1136734 | -12.50 |
| $\mathbf{F_3}$ | -242898 | -6.39 | -63320 | -4.98 | -753591 | -7.42 | -940731 | -7.85 | -666844 | -7.34 |
| $\mathbf{F_4}$ | 391112 | 10.29 | 140752 | 11.06 | 1243993 | 12.24 | 1941033 | 16.19 | 991561 | 10.91 |
| $\mathbf{F_5}$ | -202056 | -5.32 | -53904 | -4.24 | -779027 | -7.67 | -1093426 | -9.12 | -575761 | -6.33 |
| $\mathbf{F_6}$ | -746202 | -19.63 | -196725 | -15.46 | -1809092 | -17.80 | -1593328 | -13.29 | -1860452 | -20.46 |
| $\mathbf{F_7}$ | 1229603 | 32.35 | 385199 | 30.27 | 3729948 | 36.70 | 5312872 | 44.31 | 3129456 | 34.42 |
| $\mathbf{F_8}$ | 2390746 | 62.90 | 738787 | 58.05 | 6828593 | 67.19 | 9166944 | 76.45 | 6023652 | 66.26 |
| $\mathbf{F_9}$ | -853470 | -22.46 | -243673 | -19.15 | -1975627 | -19.44 | -1435734 | -11.97 | -2003953 | -22.04 |
| $\mathbf{F_{10}}$ | 2251125 | 59.23 | 579678 | 45.55 | 5387502 | 53.01 | 4648011 | 38.77 | 5591928 | 61.51 |
| $\mathbf{F_{11}}$ | 1003643 | 26.41 | 232596 | 18.28 | 2687375 | 26.44 | 2233151 | 18.63 | 2647332 | 29.12 |

**Table 4.4** Estimated (Est.) and standardized (Stand.) effects calculated from peak heights obtained from Plackett-Burman design performed with HS-SPME-GC-FID system with BAC1 column.

| Factor no. | Ethanol | | Methanol | | Isopropanol | | Acetone | | n-propanol | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Est.** | **Stand.** | **Est.** | **Stand.** | **Est.** | **Stand.** | **Est.** | **Stand.** | **Est.** | **Stand.** |
| $\mathbf{F_1}$ | -455882 | -22.37 | -76886 | -10.43 | -744646 | -17.64 | -875562 | -19.87 | -654702 | -20.62 |
| $\mathbf{F_2}$ | 87898 | 4.31 | 55950 | 7.59 | 823136.8 | 19.50 | 1158438 | 26.29 | 809637 | 25.50 |
| $\mathbf{F_3}$ | 269572 | 13.23 | 38570 | 5.23 | 200698.2 | 4.754 | 113042.2 | 2.57 | 173495 | 5.46 |
| $\mathbf{F_4}$ | 761499 | 37.37 | 313466 | 42.52 | 2218113 | 52.54 | 2806156 | 63.68 | 1698715 | 53.51 |
| $\mathbf{F_5}$ | -594743 | -29.18 | -127293 | -17.27 | -1043047 | -24.71 | -1092784 | -24.80 | -796031 | -25.07 |
| $\mathbf{F_6}$ | -410891 | -20.16 | -54896 | -7.45 | -671453 | -15.90 | -706519 | -16.03 | -656629 | -20.68 |
| $\mathbf{F_7}$ | 247245 | 12.13 | 26349 | 3.57 | 399091.7 | 9.45 | 817652.8 | 18.56 | 218455 | 6.88 |
| $\mathbf{F_8}$ | 983273 | 48.25 | 262397 | 35.59 | 2159157 | 51.14 | 2711879 | 61.54 | 1650792 | 51.99 |
| $\mathbf{F_9}$ | -302483 | -14.84 | -143008 | -19.40 | -947594 | -22.44 | -701918 | -15.93 | -795495 | -25.06 |
| $\mathbf{F_{10}}$ | 774886 | 38.02 | 2725101 | 36.96 | 1891258 | 44.80 | 1374980 | 31.20 | 1605277 | 50.57 |
| $\mathbf{F_{11}}$ | 581375 | 28.53 | 109623 | 14.87 | 923898 | 21.88 | 625056 | 14.19 | 743886 | 23.43 |

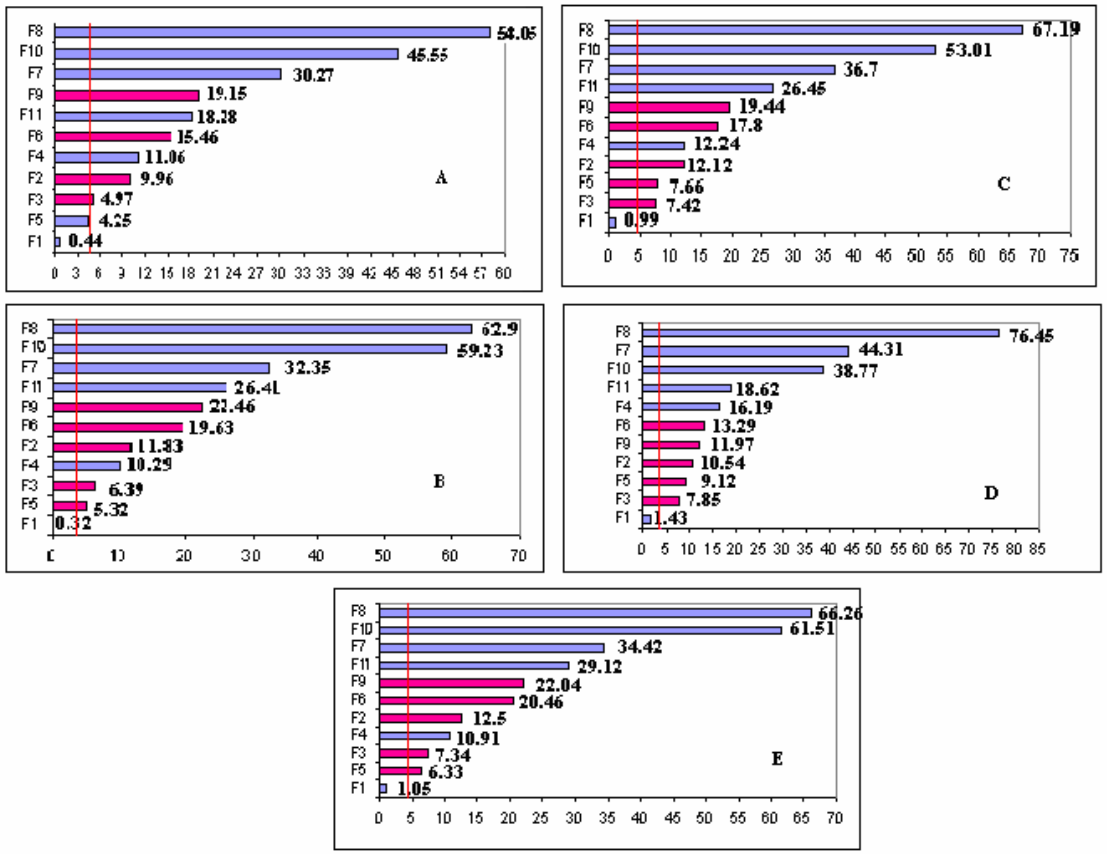


**Figure 4.2** Standardized effects on peak areas of A) methanol, B) ethanol, C) isopropanol, D) acetone and E) n-propanol standards analyzed by HS-GC-FID system with BAC1 column

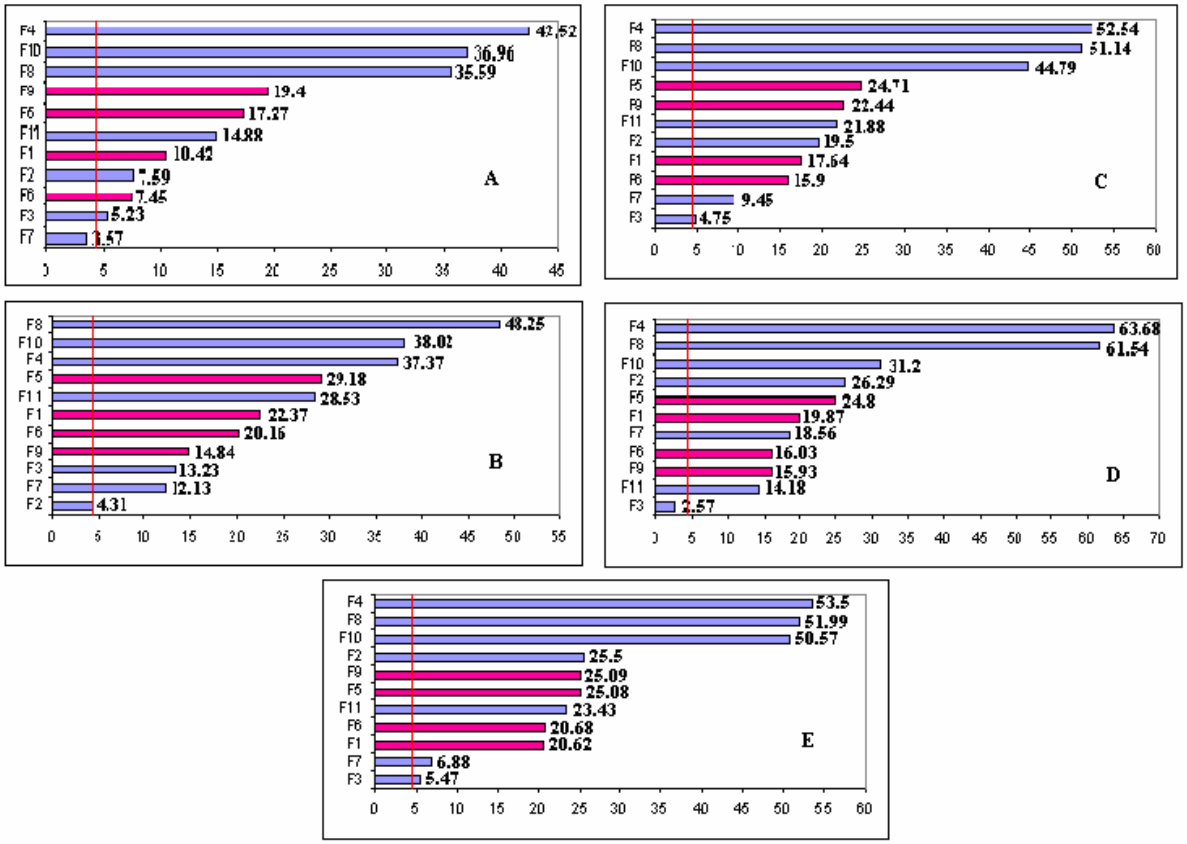


**Figure 4.3** Standardized effects on peak heights of A) methanol, B) ethanol, C) isopropanol, D) acetone and E) n-propanol standards analyzed by HS-GC-FID system with BAC1 column

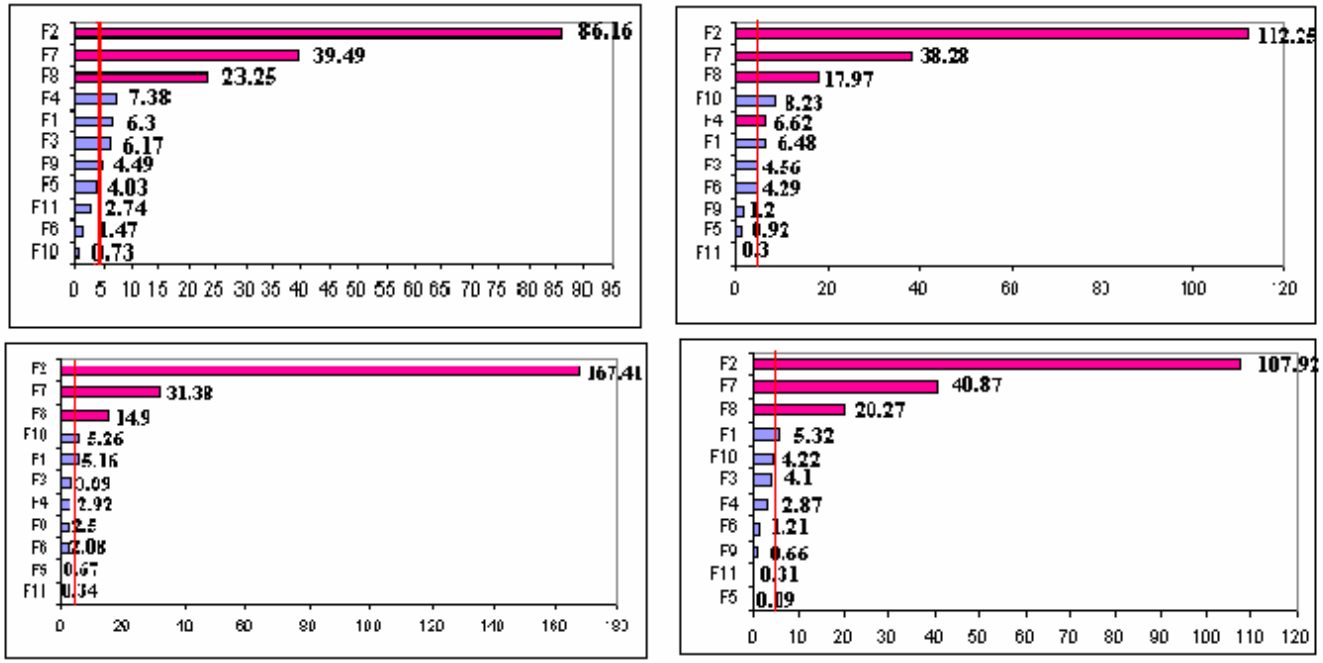


**Figure 4.4** Standardized effects on peak resolutions of A) ethanol, B) n-propanol C) acetone and D) Isopropanol standards analyzed by HS-GC-FID system with BAC1 column.

Evaluation of the results shown in Figure 4.2 revealed that most of the factors except inlet temperature ($F_1$) have exceeded the critical t value and had a significant effect on the peak areas. Among these factors, most significant effect on peak area of standards was injected headspace volume ($F_8$). Other affects are equilibrium temperature ($F_{10}$), split flow rate ($F_7$), equilibrium time ($F_{11}$) and carrier gas flow rate ($F_4$). These factors have displayed positive effect on the result.

Conversely, solution volume in vial ($F_9$) had a relatively high but negative effect on the peak areas. It was followed by air flow rate, oven temperature, $H_2$ flow rate and detector temperature ($F_6$, $F_5$, $F_2$ and $F_3$).

From the aspect of sensitivity, the factors those have a significant effect on peak heights were taken into consideration (Figure 4.3). Main positive effect was carrier gas flow rate ($F_4$). This can be explained in a way that higher flow rates increases the desorption rate of the analytes from stationary phase. This was followed by equilibrium temperature ($F_{10}$), injected sample volume ($F_8$), equilibrium time ($F_{11}$), oven temperature ($F_2$), split gas flow rate ($F_7$) and detector temperature ($F_3$).

On the other hand, $H_2$ flow rate, inlet temperature, solution volume in vial and air flow rate ($F_5$, $F_1$, $F_9$, $F_6$) have displayed negative effects on the peak heights.

In terms of selectivity, resolution of the peaks was taken into consideration (Figure 4.4). Major effect was determined as oven temperature ($F_2$) in negative direction. This result agrees well with the well known fact that column temperature deteriorates the resolution.

Although high temperature usually leads the enhancement of the column performance by increasing theoretical plate heights, closer peak formation is observed. Other most significant factors were split flow rate ($F_7$) and injected volume ($F_8$). Factors those exceeded the critical value and had positive effect were noted as equilibrium, inlet and detector temperature ($F_{10}$, $F_7$, $F_3$) respectively.

### 4.1.2 Optimization of Static HS-Injection and GC Conditions

A Central Composite Design was constructed with four factors affecting the headspace alcohol analysis. Static headspace analysis for a mixture containing 5 aqueous standards was performed by using BAC 1 column. k = 4 (number of factors)

Number of experiments = $2^k + 2k + 1 = 16 + 8 + 1 = 25$

Number of replicates at center point= 5

Total no. of experiments (N) = 25 + 5 = 30

The coded values of axial points for star design and the applied levels of the factors were calculated as previously described (Eq. 1.13 and Eq.1.15). Factors and their levels were given in Table 4.5. Four factor central composite design model was given in Table 4.6. For the design model equation see the equation for the model given in Table 3.9.

**Table 4.5** Factors and coded levels used for central composite design with static HS-GC-FID system with BAC1 column

| **Factor** | **-2.000 (α)** | **-1** | **0** | **1** | **2.000 (α)** |
|---|---|---|---|---|---|
| $X_1$ : Eq.temperature (˚C) | 45 | 50 | 55 | 60 | 65 |
| $X_2$ : Eq. time (min) | 5 | 10 | 15 | 20 | 25 |
| $X_3$ : Oven temperature (˚C) | 45 | 50 | 55 | 60 | 65 |
| $X_4$ : Carrier gas flow rate (mL/min) | 1.6 | 1.9 | 2.2 | 2.5 | 2.8 |

**Table 4.6** Four factor central composite design

| Run no | $X_0$ | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_{11}$ | $X_{22}$ | $X_{33}$ | $X_{44}$ | $X_{12}$ | $X_{13}$ | $X_{14}$ | $X_{23}$ | $X_{24}$ | $X_{34}$ | $X_{12}$ | $X_{124}$ | $X_{134}$ | $X_{234}$ | $X_{1234}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 |
| 2 | 1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 |
| 3 | 1 | -1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | -1 |
| 4 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | -1 | 1 | 1 | 1 |
| 5 | 1 | -1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | -1 |
| 6 | 1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | -1 | 1 | -1 | -1 | 1 | -1 | 1 | 1 |
| 7 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | -1 | -1 | 1 | 1 | -1 | 1 |
| 8 | 1 | 1 | 1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | -1 | 1 | -1 | -1 | -1 | -1 |
| 9 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 |
| 10 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | -1 | 1 | -1 | -1 | 1 | 1 |
| 11 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | -1 | 1 |
| 12 | 1 | 1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | -1 | 1 | -1 | -1 | -1 |
| 13 | 1 | -1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | -1 | 1 |
| 14 | 1 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | -1 | -1 | 1 | -1 | -1 |
| 15 | 1 | -1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 |
| 16 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 17 | 1 | +2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | 1 | -2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | 1 | 0 | +2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20 | 1 | 0 | -2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 21 | 1 | 0 | 0 | +2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | 1 | 0 | 0 | -2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | 1 | 0 | 0 | 0 | +2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | 1 | 0 | 0 | 0 | -2 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 27 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 29 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table 4.7 showed the determined factors and their levels applied in this design model.

**Table 4.7** Factors and levels used for central composite design with static HS-GC-FID system with BAC1 column

| Run no | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Run no | $X_1$ | $X_2$ | $X_3$ | $X_4$ |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 50 | 10 | 50 | 1.9 | **16** | 60 | 20 | 60 | 2.5 |
| **2** | 60 | 10 | 50 | 1.9 | **17** | 65 | 15 | 55 | 2.2 |
| **3** | 50 | 20 | 50 | 1.9 | **18** | 45 | 15 | 55 | 2.2 |
| **4** | 60 | 20 | 50 | 1.9 | **19** | 55 | 25 | 55 | 2.2 |
| **5** | 50 | 10 | 60 | 1.9 | **20** | 55 | 5 | 55 | 2.2 |
| **6** | 60 | 10 | 60 | 1.9 | **21** | 55 | 15 | 65 | 2.2 |
| **7** | 50 | 20 | 60 | 1.9 | **22** | 55 | 15 | 45 | 2.2 |
| **8** | 60 | 20 | 60 | 1.9 | **23** | 55 | 15 | 55 | 2.8 |
| **9** | 50 | 10 | 50 | 2.5 | **24** | 55 | 15 | 55 | 1.6 |
| **10** | 60 | 10 | 50 | 2.5 | **25** | 55 | 15 | 55 | 2.2 |
| **11** | 50 | 20 | 50 | 2.5 | **26** | 55 | 15 | 55 | 2.2 |
| **12** | 60 | 20 | 50 | 2.5 | **27** | 55 | 15 | 55 | 2.2 |
| **13** | 50 | 10 | 60 | 2.5 | **28** | 55 | 15 | 55 | 2.2 |
| **14** | 60 | 10 | 60 | 2.5 | **29** | 55 | 15 | 55 | 2.2 |
| **15** | 50 | 20 | 60 | 2.5 | **30** | 55 | 15 | 55 | 2.2 |

Chemometric calculations were performed as described in Section 1.7.5. The adequacy of the constructed central composite design model was evaluated according to the parameters obtained from the chromatograms. Peak areas and peak heights were selected as the response factors. F values obtained for the applied design model were given in Table 4.8. These values were compare with the tabulated value.

**Table 4.8** F test results according to peak areas for the applied model

| Peak name | $F_{experimental}$ based on | |
|---|---|---|
| | Peak area | Peak height |
| Methanol | 199.75 | 1.75 |
| Ethanol | 469.21 | 4.28 |
| Isopropanol | 48.89 | 5.86 |
| Acetone | 80.82 | 20.40 |
| n-propanol | 304.25 | 88.67 |

As it can be seen in Table 4.8 some of the $F_{experimental}$ values obtained exceeded tabulated value ($F_{5,5}$: 5.05). This high difference can be concluded as the selected factors have very significant effects on the responses. In addition, if the factor levels are chosen too apart, the true response surface might be quite different from the estimated response surface therefore, F values are higher than the tabulated values (Massart, 1988; Morgan, 1999).

t test was performed for testing the significance of selected parameters. $t_{experimental}$ values for each parameters were calculated (see Eq.1.22). The values obtained for peak areas and peak heights for methanol, ethanol, isopropanol, acetone and n-propanol were compared with the $t_{table}$ values (for d.f.. 5, $t_{table}$ =2,57, p=0.05) on Table 4.9 and Table 4.10 respectively .The values exceeding the $t_{table}$ value were shown in bold character.

**Table 4.9** Experimentally found $t_{experimental}$ values for b coefficients calculated in relation with the peak areas of alcohol standards and acetone.

| | **Methanol** | **Ethanol** | **Isopropanol** | **Acetone** | **n-Propanol** |
|---|---|---|---|---|---|
| $\mathbf{b_0}$ | **64.54** | **99.64** | **30.95** | **39.18** | **79.99** |
| $\mathbf{b_1}$ | **17.57** | **27.50** | **7.23** | **5.45** | **20.84** |
| $\mathbf{b_2}$ | **7.45** | **14.44** | **5.11** | **5.66** | **13.34** |
| $\mathbf{b_3}$ | 0.19 | 0.40 | 0.16 | 0.74 | 0.42 |
| $\mathbf{b_4}$ | 0.67 | 1.21 | 0.60 | 1.15 | 0.67 |
| $\mathbf{b_{11}}$ | **3.85** | **5.44** | 1.17 | 0.88 | **3.74** |
| $\mathbf{b_{22}}$ | 1.97 | **3.60** | 0.95 | 0.95 | **2.93** |
| $\mathbf{b_{33}}$ | 1.40 | **2.59** | 0.91 | 0.95 | 2.42 |
| $\mathbf{b_{44}}$ | 1.84 | **3.08** | 0.91 | 1.35 | **2.64** |
| $\mathbf{b_{12}}$ | 1.83 | **3.12** | 0.93 | 1.37 | 2.54 |
| $\mathbf{b_{13}}$ | 0.31 | 0.80 | 0.32 | 0.98 | 0.74 |
| $\mathbf{b_{14}}$ | 1.43 | 2.12 | 0.41 | 0.99 | 1.32 |
| $\mathbf{b_{23}}$ | 0.40 | 0.61 | 0.14 | 0.80 | 0.39 |
| $\mathbf{b_{24}}$ | 0.10 | 0.35 | 0.21 | 0.47 | 0.51 |
| $\mathbf{b_{34}}$ | 0.48 | 0.46 | 0.05 | 0.70 | 0.21 |
| $\mathbf{b_{123}}$ | 0.05 | 0.24 | 0.02 | 0.69 | 0.05 |
| $\mathbf{b_{124}}$ | 0.08 | 0.11 | 0.05 | 0.64 | 0.13 |
| $\mathbf{b_{134}}$ | 0.11 | 0.46 | 0.16 | 0.54 | 0.37 |
| $\mathbf{b_{234}}$ | 1.21 | 1.68 | 0.48 | 0.23 | 1.33 |
| $\mathbf{b_{1234}}$ | 0.51 | 0.99 | 0.26 | 0.50 | 0.73 |

Here, it is worth to note that even the linear terms, $b_3$ and $b_4$, corresponding to the oven temperature and carrier gas flow rate were found lower than the critical *t* value; their quadratic effects ($b_{33}$ and $b_{44}$) exceeded this value for ethanol and $b_{44}$ for n-propanol.

**Table 4.10 $t_{experimental}$ values for *b* coefficients calculated in relation with the peak heights of alcohol standards and acetone.**

| | **Methanol** | **Ethanol** | **Isopropanol** | **Acetone** | **n-Propanol** |
|---|---|---|---|---|---|
| $\mathbf{b_0}$ | **20.95** | **26.50** | **22.38** | **41.78** | **76.74** |
| $\mathbf{b_1}$ | **6.44** | **7.76** | **5.35** | **6.52** | **19.89** |
| $\mathbf{b_2}$ | 2.39 | **3.75** | **3.63** | **5.31** | **12.66** |
| $\mathbf{b_3}$ | 0.54 | 0.01 | 0.72 | 1.95 | **4.53** |
| $\mathbf{b_4}$ | **3.71** | **4.35** | **3.35** | **6.31** | **11.50** |
| $\mathbf{b_{11}}$ | 0.37 | 0.51 | 0.29 | 0.24 | 2.38 |
| $\mathbf{b_{22}}$ | 0.62 | 1.03 | 0.86 | 1.13 | **3.41** |
| $\mathbf{b_{33}}$ | 0.18 | 0.10 | 0.04 | 0.07 | 0.39 |
| $\mathbf{b_{44}}$ | 0.02 | 0.19 | 0.35 | 0.84 | 1.76 |
| $\mathbf{b_{12}}$ | 0.86 | 1.15 | 0.88 | 1.05 | **3.01** |
| $\mathbf{b_{13}}$ | 0.15 | 0.42 | 0.51 | 0.73 | 1.70 |
| $\mathbf{b_{14}}$ | 1.05 | 0.94 | 0.54 | 0.71 | 1.38 |
| $\mathbf{b_{23}}$ | 0.02 | 0.03 | 0.08 | 0.22 | 0.44 |
| $\mathbf{b_{24}}$ | 0.03 | 0.08 | 0.12 | 0.10 | 0.62 |
| $\mathbf{b_{34}}$ | 0.28 | 0.16 | 0.09 | 0.37 | 0.63 |
| $\mathbf{b_{123}}$ | 0.35 | 0.39 | 0.31 | 0.39 | 0.73 |
| $\mathbf{b_{124}}$ | 0.03 | 0.03 | 0.03 | 0.03 | 0.15 |
| $\mathbf{b_{134}}$ | 0.33 | 0.27 | 0.11 | 0.08 | 0.02 |
| $\mathbf{b_{234}}$ | 0.46 | 0.45 | 0.32 | 0.41 | 1.14 |
| $\mathbf{b_{1234}}$ | 0.20 | 0.10 | 0.03 | 0.06 | 0.52 |

The evaluation of the results revealed that the most significant parameter for the constructed central composite design was the equilibrium temperature of the sample and this was followed by equilibrium time. Positive effects of these two parameters were observed. Only one interaction term ($b_{12}$) has exceeded the critical value for ethanol. The effect of the column temperature was found insignificant on the results. When peak areas are taken into consideration, quadratic terms were also significant for ethanol and n-propanol as stated above.

On the other hand, t values calculated according to the peak heights have revealed that linear terms of equilibrium temperature and carrier gas flow rate were significant for methanol, equilibrium temperature, equilibrium time and carrier gas flow rate were significant for ethanol, isopropanol and acetone and four factors together were significant for n-propanol.

Taking into consideration the t test results, new designs were constructed with significant parameters. Under these conditions *%RMSE* values were calculated for constructed models (see Eq.1.23 and 1.24). Table 4.11 shows the %RMSE values indicating the adequacy of the model for peak areas and peak heights.

**Table 4.11** The %RMSE values indicating the adequacy of the model for peak area and peak heights.

| **Peak name** | ***% RMSE* for** | |
|---|---|---|
| | **peak area** | **peak height** |
| Methanol | 15.48 | 11.03 |
| Ethanol | 16.16 | 12.47 |
| Isopropanol | 16.03 | 9.93 |
| Acetone | 16.53 | 9.28 |
| n-propanol | 15.91 | 9.88 |

Considering the %RMSE values in Table 4.11 revealed that for the applied design model evaluation of peak heights was more appropriate, since the %RMSE values were small compared to those obtained related with peak areas.

Partial derivatives were taken for each significant parameter in the model equation and optimum values for the factors were calculated from the coded values. Besides, in the limits of α value as -2 and 2 coded optimum values for the four factors were also iterated by Microsoft Excel Solver. These two optimization results were given in Table 4.12 and Table 4.13 for peak areas and peak heights respectively.

**Table 4.12** Calculated coded optimum values considering peak areas

| **Standard** | **Factor** | **Coded values** | |
|---|---|---|---|
| | | **By partial derivatives** | **With iteration** |
| Methanol | $X_1$ | -2.63 | 2 |
| | $X_2$ | 0 | 2 |
| | $X_3$ | 0 | -2 |
| | $X_4$ | 0 | -2 |
| Ethanol | $X_1$ | -2.92 | 2 |
| | $X_2$ | 2.31 | 2 |
| | $X_3$ | 0 | 2 |
| | $X_4$ | 0 | 1.27 |
| Isopropanol | $X_1$ | 0 | 2 |
| | $X_2$ | 0 | 2 |
| | $X_3$ | 0 | 2 |
| | $X_4$ | 0 | 1.07 |
| Acetone | $X_1$ | 0 | 2 |
| | $X_2$ | 0 | 2 |
| | $X_3$ | 0 | -2 |
| | $X_4$ | 0 | 1.21 |
| n-propanol | $X_1$ | -3.21 | 2 |
| | $X_2$ | 2.62 | 2 |
| | $X_3$ | 0 | 2 |
| | $X_4$ | 0 | 1.21 |

**Table 4.13** Calculated coded optimum values considering peak heights

| Standard | Factor | Coded values | |
|---|---|---|---|
| | | By partial derivatives | With iteration |
| Methanol | $X_1$ | -1.17 | 2 |
| | $X_2$ | 0 | 2 |
| | $X_3$ | 0 | -1.86 |
| | $X_4$ | 0 | 2 |
| Ethanol | $X_1$ | 0 | 2 |
| | $X_2$ | 0 | 2 |
| | $X_3$ | 0 | 2 |
| | $X_4$ | 0 | 2 |
| Isopropanol | $X_1$ | 0 | 2 |
| | $X_2$ | 0 | 2 |
| | $X_3$ | 0 | 2 |
| | $X_4$ | 0 | 2 |
| Acetone | $X_1$ | 0 | 2 |
| | $X_2$ | 0 | 2 |
| | $X_3$ | 0 | 2 |
| | $X_4$ | 0 | 2 |
| n-propanol | $X_1$ | 0 | 2 |
| | $X_2$ | 2.14 | 2 |
| | $X_3$ | 0 | 2 |
| | $X_4$ | 0 | 2 |

Response surfaces for each standard were constructed and effect on the peak areas of each pair of factors were evaluated graphically (Figure 4.5 - 4.11). Besides the surface plots, optimization of four factors for each standard was performed by Matlab Version 7.0. Optimum coded values of four factors for methanol, ethanol, isopropanol, acetone and n-propanol related with peak areas were given in Figure 4.12.

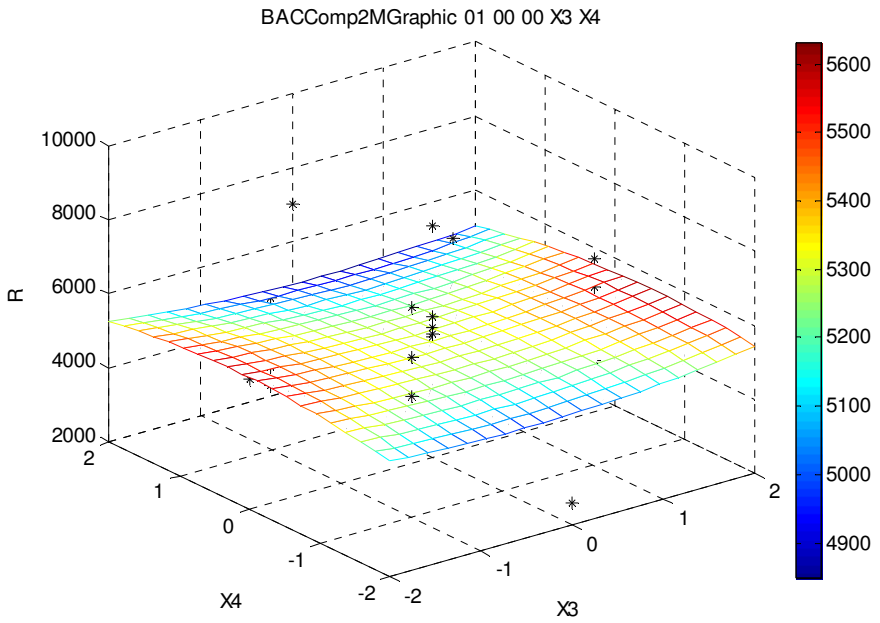


**Figure 4.5** Response surface for methanol peak area (x1000) versus oven temperature (X3) and carrier gas flow rate (X4).

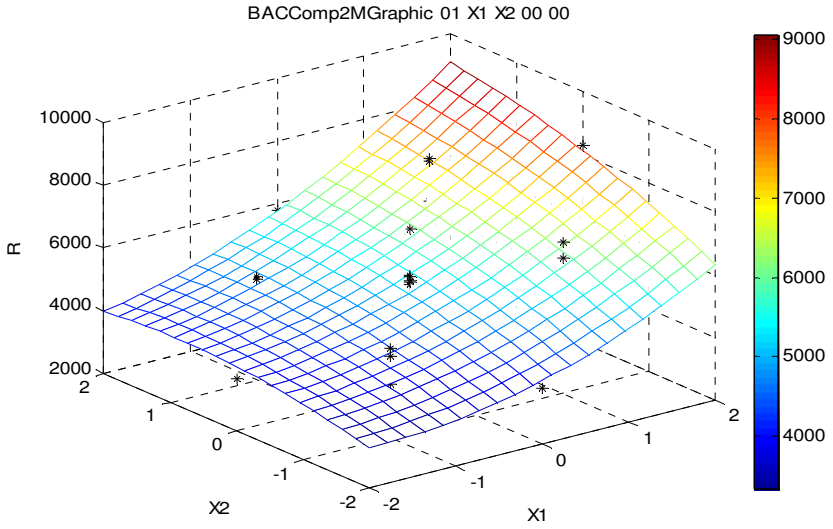


**Figure 4.6** Response surface for methanol peak area (x1000) versus equilibrium temperature (X1) and equilibrium time (X2).

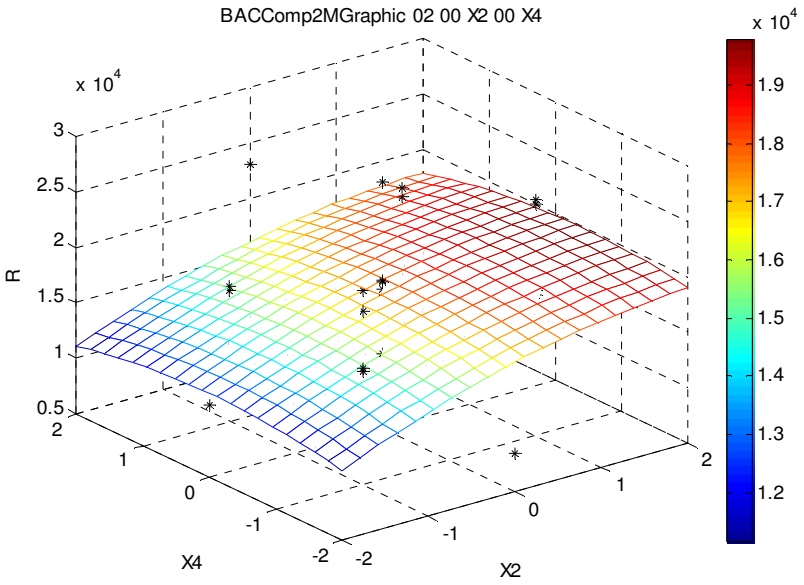


**Figure 4.7** Response surface for ethanol peak area (x1000) versus carrier gas flow rate (X4) and equilibrium time (X2).

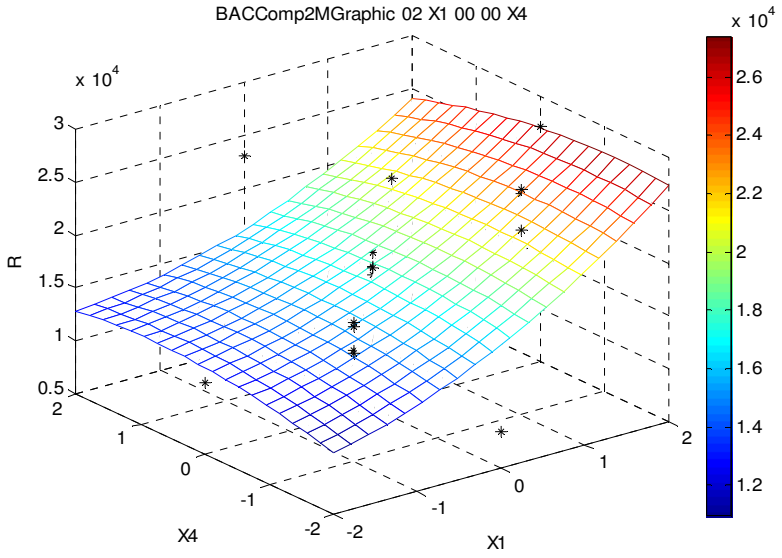


**Figure 4.8** Response surface for ethanol peak area (x1000) versus equilibrium temperature (X1) carrier gas flow rate (X4).

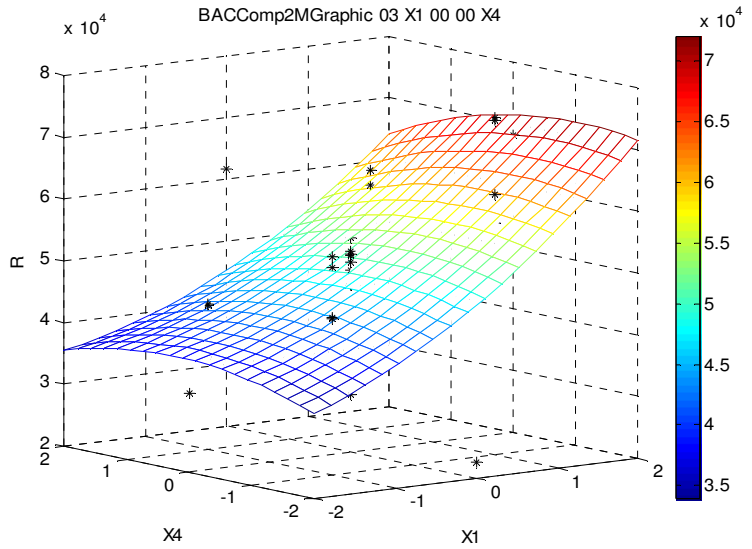


**Figure 4.9** Response surface for ispropanol peak area (x1000) versus equilibrium temperature (X1) carrier gas flow rate (X4).

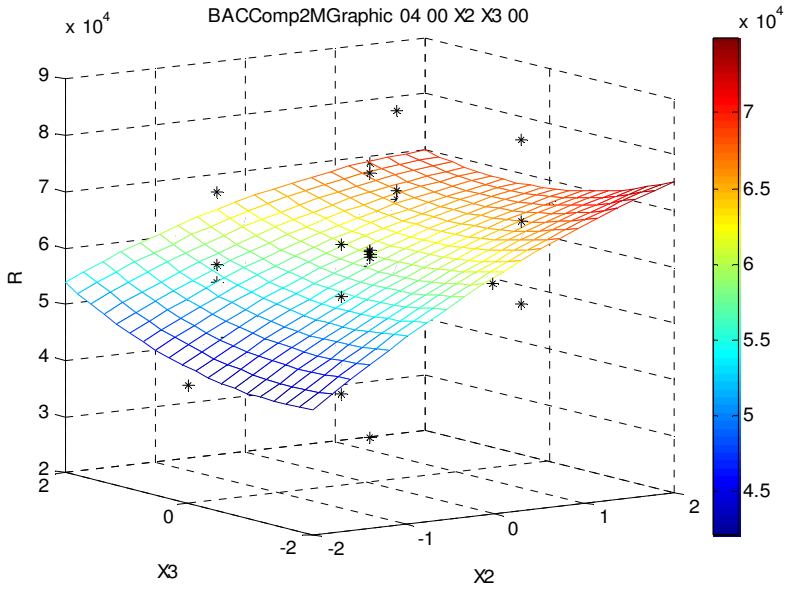


**Figure 4.10** Response surface for acetone peak area (x1000) versus equilibrium time (X2) oven temperature (X3).

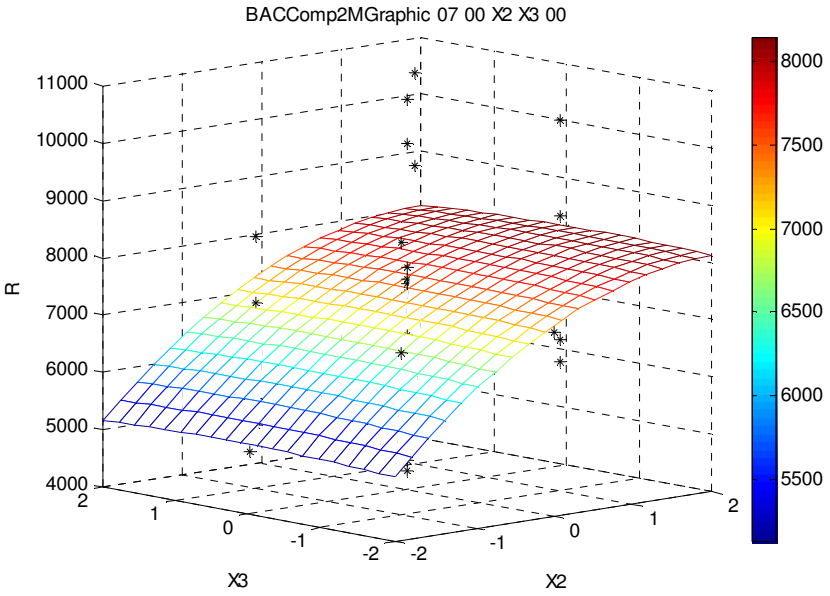


**Figure 4.11** Response surface for ethanol peak height (x1000) versus equilibrium time (X2) oven temperature (X3).

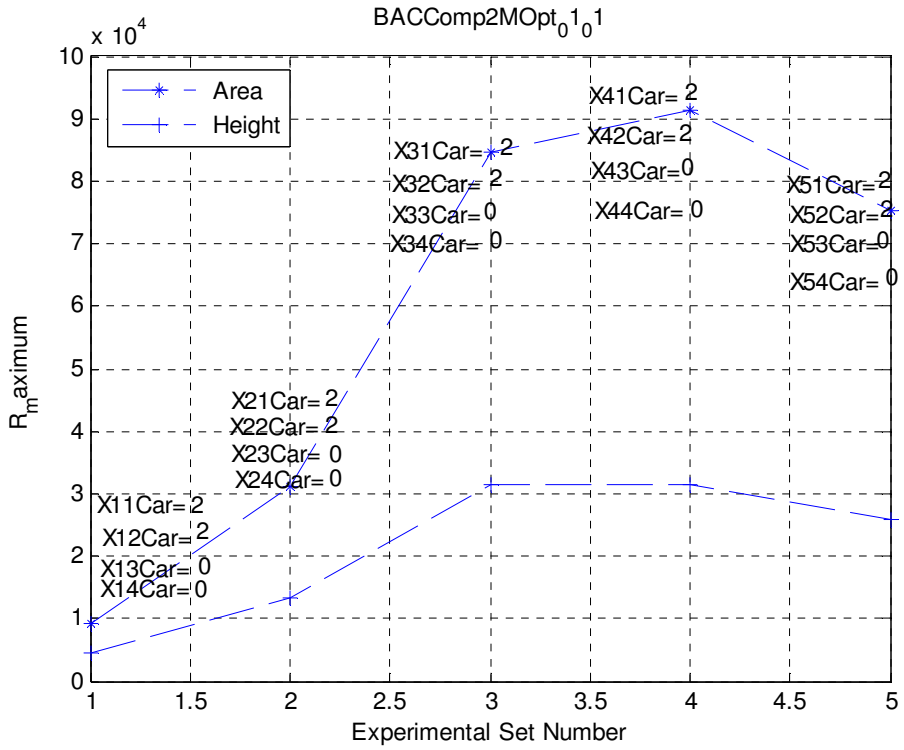


**Figure 4.12** Optimum coded levels of the four factors for methanol, ethanol, isopropanol, acetone and n-propanol related with peak areas.

**Table 4.14** Coded optimum values of the factors established for alcohol standards and acetone.

| Standard | Factor | Coded value |
|---|---|---|
| Methanol | $X_1$ | 2 |
| | $X_2$ | 2 |
| | $X_3$ | 0 |
| | $X_4$ | 0 |
| Ethanol | $X_1$ | 2 |
| | $X_2$ | 2 |
| | $X_3$ | 0 |
| | $X_4$ | 0 |
| Isopropanol | $X_1$ | 2 |
| | $X_2$ | 2 |
| | $X_3$ | 0 |
| | $X_4$ | 0 |
| Acetone | $X_1$ | 2 |
| | $X_2$ | 2 |
| | $X_3$ | 0 |
| | $X_4$ | 0 |
| n-propanol | $X_1$ | 2 |
| | $X_2$ | 2 |
| | $X_3$ | 0 |
| | $X_4$ | 0 |

These results were in agreement with t test values. According to t-test the most significant parameter for the constructed central composite design was the equilibrium temperature of the sample ($X_1$) and this was followed by equilibrium time ($X_2$). These two factors have positive effects on the peak areas. Considering the development of headspace concentration, equilibrium parameters like temperature and time are expected to have a main effect on the results as established.

### 4.2. Studies with Headspace-Solid Phase Micro Extraction

Preliminary studies of HS-SPME analysis of alcohol standards, carried out with column II (Rtx BAC 1)**,** revealed that injection step of SPME holder into GC for analyte desorption was crucial especially for the first and second peaks. Even though the injector was hold in inlet block for a predetermined time and hence desorption time was assumed to be controlled by this means, inserting time of the fiber into the inlet liner was found to be critical for reproducible peak development. It was observed that rapid injection of the fiber caused narrower peaks or vice versa. Chromatogram of the standards was given in Figure 4.13.

**Figure 4.13** HS-SPME-GC-FID chromatogram recorded with BAC1 column for 1) methanol, 2) ethanol, 3) isopropanol, 4) acetone, 5) n-propanol and 6) n-buthanol standards

### 4.2.1 Evaluation of the Main Effects of the Factors, Plackett-Burman Design

Aqueous five alcohol standards and acetone mixture were analyzed by HS-SPME technique. Among the factors those considered to be effective on the separation of alcohols, the major effects were searched by means of screening 11 factors with SPME technique.

A Plackett-Burman Design was constructed for two levels by using the related generator (see Table 1.4). The factors and their levels were given in Table 4.15 and the design model for 12 run was shown previously (see Table 4.2).

**Table 4.15** Factors and levels used for Plackett-Burman design with static HS-SPME-GC-FID system with BAC1 column

| Factor No | Explanation | (+) level | (-) level |
|---|---|---|---|
| $F_1$ | Inlet temperature (ºC) | 245 | 205 |
| $F_2$ | Oven temperature (ºC) | 80 | 50 |
| $F_3$ | Detector temperature (ºC) | 250 | 200 |
| $F_4$ | Carrier gas flow rate (mL/min) | 2.8 | 1.8 |
| $F_5$ | Split flow rate (mL/min) | 30 | 50 |
| $F_6$ | $H_2$ gas flow rate (mL/min) | 40 | 35 |
| $F_7$ | Solution volume in vial (mL) | 2.0 | 1.0 |
| *$F_8$ | Sample concentration (mL/100mL) | 0.05 | 0.03 |
| $F_9$ | Equilibrium temperature (ºC) | 60 | 50 |
| $F_{10}$ | Salting out (NaCl, g) | 1.0 | 0.1 |
| $F_{11}$ | Extraction time (min) | 4 | 2 |

*Concentration only for n-butanol in this mixture was 0.03 and 0.01mL/100mL

Chemometric calculations were performed by using the related equations described in Section 1.7.4. Table 4.17 and 4.18 summarize the

results obtained for estimated and standardized effects calculated from peak areas and peak heights obtained from Plackett-Burman design.

**Table 4.16** Estimated (Est.) and standardized (Stand.) effects calculated from peak areas obtained from Plackett-Burman design performed with HS-SPME-GC-FID system with BAC1 column.

| Factor | Ethanol | | Methanol | | Isopropanol | |
|---|---|---|---|---|---|---|
| no. | Est. | Stand. | Est. | Stand. | Est. | Stand. |
| $F_1$ | -2267899 | -19.15 | -93515 | -3.37 | -3822945 | -9.47 |
| $F_2$ | 983943 | 8.31 | -304262 | -10.93 | 1010825 | 2.50 |
| $F_3$ | 240918 | 2.03 | -86592 | -3.11 | 1811818 | 4.49 |
| $F_4$ | 945712 | 7.98 | 310291 | 11.14 | 2711348 | 6.72 |
| $F_5$ | 52833 | 0.45 | -88963 | -3.19 | -1621218 | -4.02 |
| $F_6$ | -1041767 | -8.80 | 86839 | 3.12 | -1505883 | -3.73 |
| $F_7$ | -1578086 | -13.32 | 85977 | 3.09 | -3414585 | -8.46 |
| $F_8$ | 1841473 | 15.55 | -298452 | -10.72 | 5360740 | 13.28 |
| $F_9$ | -277993 | -2.35 | 305022 | 10.95 | -2567066 | -6.36 |
| $F_{10}$ | 4194864 | 35.42 | 93992 | 3.37 | 12035965 | 29.81 |
| $F_{11}$ | 984949 | 8.32 | 306198 | 10.99 | 3184250 | 7.89 |
| **Factor** | **Acetone** | | **n-propanol** | | **n-buthanol** | |
| **no.** | **Est.** | **Stand.** | **Est.** | **Stand.** | **Est.** | **Stand.** |
| $F_1$ | -1937198 | -14.91 | -6848300 | -13.02 | -19047535 | -7.84 |
| $F_2$ | 203734 | 1.57 | 3174959 | 6.04 | -210591 | -0.09 |
| $F_3$ | 296936 | 2.29 | 2949580 | 5.61 | 13081416 | 5.38 |
| $F_4$ | 1964457 | 15.12 | 7349091 | 13.97 | 5601170 | 2.31 |
| $F_5$ | -692482 | -5.33 | -2652620 | -5.04 | -3919169 | -1.61 |
| $F_6$ | -1074731 | -8.27 | -3361732 | -6.39 | -15032606 | -6.19 |
| $F_7$ | -452093 | -3.48 | -6739720 | -12.82 | -13322599 | -5.48 |
| $F_8$ | 2818544 | 21.70 | 8895042 | 16.91 | 39112632 | 16.10 |
| $F_9$ | -1330892 | -10.25 | -3778386 | -7.18 | -11917302 | -4.91 |
| $F_{10}$ | 4876236 | 37.54 | 26123762 | 49.67 | 50013398 | 20.58 |
| $F_{11}$ | 1447598 | 11.14 | 4697921 | 8.93 | 16514320 | 6.80 |

**Table 4.17** Estimated (Est.) and standardized (Stand.) effects calculated from peak heights obtained from Plackett-Burman design performed with HS-SPME-GC-FID system with BAC1 column.

| Factor no. | Ethanol | | Methanol | | Isopropanol | |
|---|---|---|---|---|---|---|
| | **Est.** | **Stand.** | **Est.** | **Stand.** | **Est.** | **Stand.** |
| $F_1$ | -686335 | -20.41 | -82864 | -0.68 | -1688360 | -18.33 |
| $F_2$ | 444113 | 13.21 | -7146 | -0.06 | 1115063 | 12.11 |
| $F_3$ | 290496 | 8.64 | -23805 | -0.19 | 699282 | 7.59 |
| $F_4$ | 90446 | 2.69 | 169660 | 1.39 | 2091696 | 22.71 |
| $F_5$ | -226893 | -6.75 | 60121 | 0.49 | -692217 | -7.52 |
| $F_6$ | -242432 | -7.21 | -173074 | -1.42 | -680532 | -7.39 |
| $F_7$ | -513950 | -15.29 | 29863 | 0.24 | -1343970 | -14.59 |
| $F_8$ | 620707 | 18.46 | 311873 | 2.55 | 1460664 | 15.86 |
| $F_9$ | -426648 | -12.69 | -206354 | -1.69 | -1167768 | -12.68 |
| $F_{10}$ | 856767 | 25.48 | 163560 | 0.71 | 2777950 | 30.16 |
| $F_{11}$ | 174099 | 5.18 | 86272 | 0.71 | 777068 | 8.44 |
| **Factor no.** | **Acetone** | | **n-propanol** | | **n-buthanol** | |
| | **Est.** | **Stand.** | **Est.** | **Stand.** | **Est.** | **Stand.** |
| $F_1$ | -819140 | -15.47 | -2497142 | -12.78 | -4258429 | -17.75 |
| $F_2$ | 715850 | 13.52 | 1935973 | 9.91 | 2500280 | 10.42 |
| $F_3$ | 827584 | 15.63 | 868345 | 4.45 | 2772378 | 11.55 |
| $F_4$ | 593954 | 11.22 | 3722829 | 19.06 | 4079428 | 17.00 |
| $F_5$ | -957226 | -18.08 | -1088646 | -5.57 | -1837116 | -7.66 |
| $F_6$ | -1038486 | -19.61 | -701578 | -3.59 | -2807480 | -11.70 |
| $F_7$ | 308284 | 5.82 | -2375104 | -12.16 | -3700470 | -15.42 |
| $F_8$ | 682198 | 12.88 | 1756091 | 8.99 | 6201261 | 25.85 |
| $F_9$ | 85888 | 1.62 | -2047481 | -10.48 | -3583204 | -14.93 |
| $F_{10}$ | 1114528 | 21.05 | 5700692 | 29.18 | 8934880 | 37.24 |
| $F_{11}$ | 163639 | 3.09 | 1120609 | 5.74 | 2862713 | 11.93 |

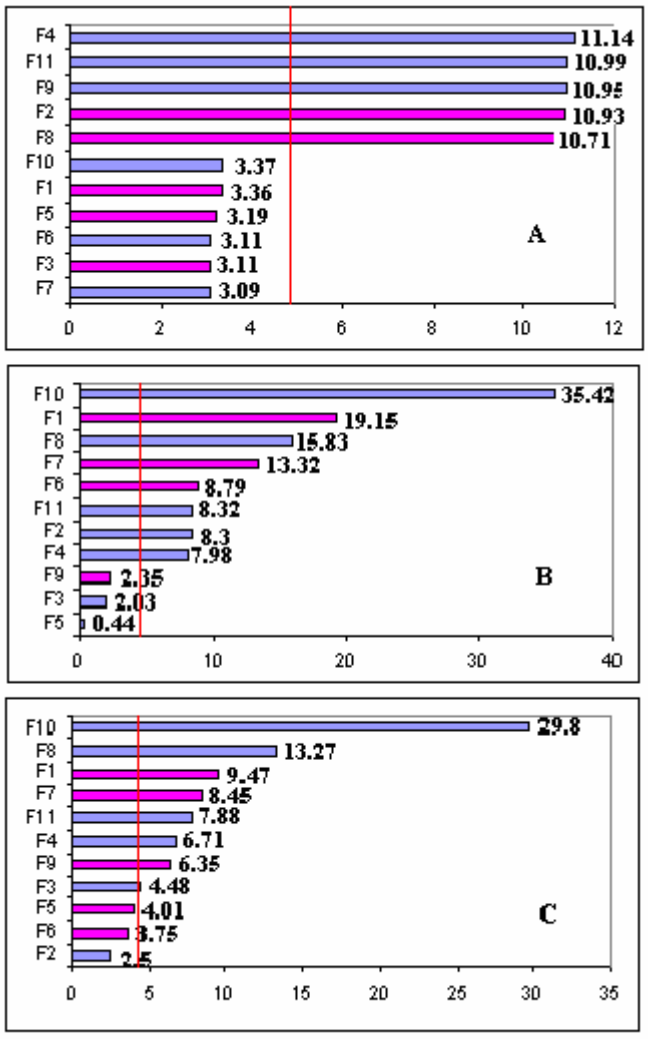


**Figure 4.14** Standardized effects of the factors on peak area of A) methanol, B) ethanol and C) isopropanol standards analyzed by HS-SPME-GC-FID system with BAC1 column.

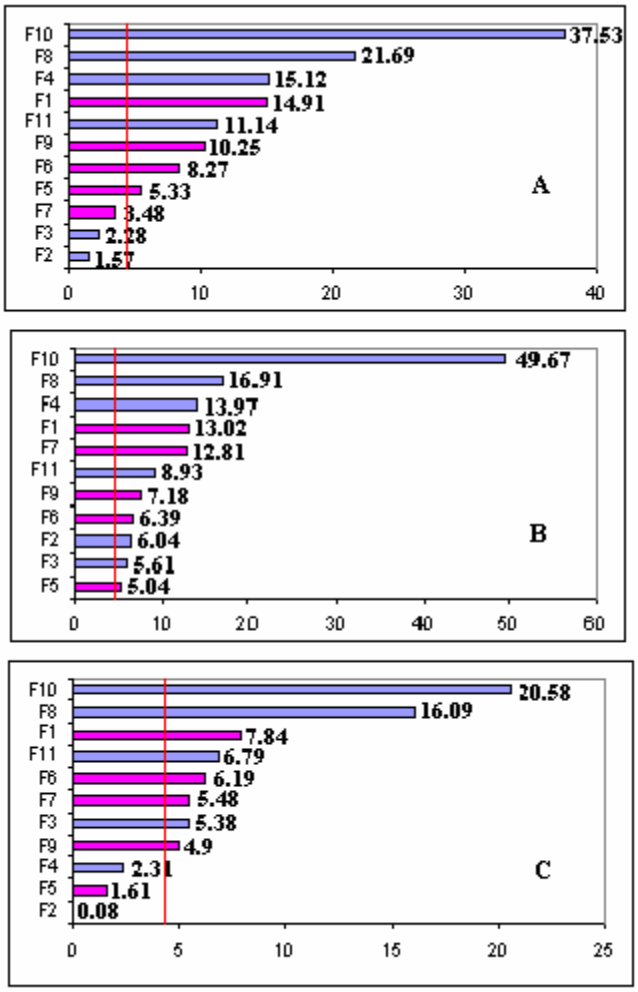


**Figure 4.15** Standardized effects of the factors on peak area of A) acetone, B) n-propanol and C) n-buthanol standards analyzed by HS-SPME-GC-FID system with BAC1 column.

It was concluded that among eleven factors, only a few parameters significantly effect on the peak areas. These factors were found as salting out, sample concentration, inlet temperature, carrier gas flow rate and solution volume in vial mainly ($F_{10}$, $F_8$, $F_1$, $F_4$ and $F_7$).

The effect of these factors on peak areas were mostly in positive direction, on the other hand, high inlet temperature affected the peak areas negatively. In all pareto charts a positive effect of extraction time ($F_{11}$) was observed. Since oven temperature showed positive effect on ethanol response, low column temperature was preferred for better resolution in the following optimization study.

Chemometric calculations for peak heights revealed that almost all the factors have affected on peak heights of each standard except methanol. The most effective factor was salting out ($F_{10}$) for peak heights. Inlet temperature ($F_1$) has effected on the peak heights in negative direction.

Since the calculated standardized effects for peak heights exceeded the t values, pareto charts were not constructed to display the relative importance of the effects on peak heights.

### 4.2.2 Optimization of HS-SPME Injection and GC Conditions

**Central Composite Design**

Considering all the chemometric results obtained, a Central Composite Design was constructed with four factors affecting the HS-SPME alcohol analysis. This design was performed with 6 aqueous standard mixture using BAC 1 column.

k = 4 (number of factors)

Number of experiments = 25

Number of replicates at center point= 5

Total no. of experiments (N) = 25 + 5 = 30

The coded values of axial points for star design and the applied levels of the factors were calculated as previously described (see Eq. 1.13 and 1.15). Coded factors were given in Table 4.18.

**Table 4.18** Factors and coded levels used for central composite design with static HS-SPME-GC-FID system with BAC1 column

| Factor | -2.000 (α) | -1 | 0 | 1 | 2.000 (α) |
|---|---|---|---|---|---|
| $X_1$ : Inlet temperature (˚C) | 205 | 215 | 225 | 235 | 245 |
| $X_2$ : Carrier gas flow rat (mL/min) | 2.2 | 2.4 | 2.6 | 2.8 | 3.0 |
| $X_3$ : Sample volume in vial (mL) | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 |
| $X_4$ : Extraction time (min) | 2 | 3 | 4 | 5 | 6 |

Four factor central composite design model was given previously (see Table 4.4). Table 4.19 shows the determined factors and their levels applied in this design model.

**Table 4.19** Factors and levels used for central composite design with static HS-SPME-GC-FID system with BAC1 column

| Run no | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Run no | $X_1$ | $X_2$ | $X_3$ | $X_4$ |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 215 | 2.4 | 1 | 3 | **16** | 235 | 2.8 | 2 | 5 |
| **2** | 235 | 2.4 | 1 | 3 | **17** | 245 | 2.6 | 1.5 | 4 |
| **3** | 215 | 2.8 | 1 | 3 | **18** | 205 | 2.6 | 1.5 | 4 |
| **4** | 235 | 2.8 | 1 | 3 | **19** | 225 | 3.0 | 1.5 | 4 |
| **5** | 215 | 2.4 | 2 | 3 | **20** | 225 | 2.2 | 1.5 | 4 |
| **6** | 235 | 2.4 | 2 | 3 | **21** | 225 | 2.6 | 2.5 | 4 |
| **7** | 215 | 2.8 | 2 | 3 | **22** | 225 | 2.6 | 0.5 | 4 |
| **8** | 235 | 2.8 | 2 | 3 | **23** | 225 | 2.6 | 1.5 | 6 |
| **9** | 215 | 2.4 | 1 | 5 | **24** | 225 | .6 | 1.5 | 2 |
| **10** | 235 | 2.4 | 1 | 5 | **25** | 225 | 2.6 | 1.5 | 4 |
| **11** | 215 | 2.8 | 1 | 5 | **26** | 225 | 2.6 | 1.5 | 4 |
| **12** | 235 | 2.8 | 1 | 5 | **27** | 225 | 2.6 | 1.5 | 4 |
| **13** | 215 | 2.4 | 2 | 5 | **28** | 225 | 2.6 | 1.5 | 4 |
| **14** | 235 | 2.4 | 2 | 5 | **29** | 225 | 2.6 | 1.5 | 4 |
| **15** | 215 | 2.8 | 2 | 5 | **30** | 225 | 2.6 | 1.5 | 4 |

Chemometric calculations were performed as described in Section 4.1.2. The adequacy of the constructed central composite design model was evaluated according to the parameters obtained from the chromatograms. Peak areas were selected as the response factor. F values obtained for the applied design model was given in Table 4.20.

**Table 4.20** F test results according to peak areas calculated for the applied model

| **Peak name** | $\mathbf{F_{experimental}}$ |
|---|---|
| Methanol | 0.69 |
| Ethanol | 1.99 |
| Isopropanol | 3.65 |
| Acetone | 4.65 |
| n-propanol | 3.44 |
| n-butanol | 9.51 |

As can be seen from the table above, experimental model is adequate for methanol, ethanol, isopropanol, acetone and n-propanol but not for n-buthanol since its $F$ value exceeds $F_{table}$ ($F_{5,11}$: 5.05 for p=0.05).

For testing the significance of selected parameters, t test was performed and $t_{experimental}$ values for each parameter were calculated (see Eq 1.22). The values obtained for peak areas and peak heights for methanol, ethanol, isopropanol, acetone n-propanol and n-butanol were compared with the $t_{table}$ values (for d.f. 5, $t_{table}$ =2,57, p=0.05) on Table 4.21 and Table 4.22. The values exceeding the $t_{table}$ value were shown in bold character.

**Table 4.21** $t_{experimental}$ value**s** for b coefficients calculated in relation with the peak areas of alcohol standards and acetone.

| | **Methanol** | **Ethanol** | **Isopropanol** | **Acetone** | **n-Propanol** | **n-buthanol** |
|---|---|---|---|---|---|---|
| **$b_0$** | **16.27** | **23.04** | **29.02** | **28.28** | **27.91** | **35.57** |
| **b1** | 2.19 | **3.29** | **3.77** | **3.85** | **4.81** | **5.71** |
| **b2** | 0.35 | 0.70 | 1.03 | 1.15 | 2.17 | 1.57 |
| **b3** | 0.94 | 1.84 | 0.59 | 2.54 | 2.50 | 0.87 |
| **b4** | 0.08 | 0.20 | 0.72 | 1.22 | 1.67 | 0.28 |
| **b11** | 0.42 | 1.68 | 1.55 | 1.85 | 0.80 | 2.18 |
| **b22** | 0.77 | 0.65 | 0.57 | 0.39 | 0.15 | 0.21 |
| **b33** | 0.39 | 0.60 | 1.37 | 2.15 | 1.49 | **2.60** |
| **b44** | 0.24 | 0.61 | 0.54 | 0.28 | 0.07 | 0.35 |
| **b12** | 0.37 | 0.53 | 0.53 | 1.05 | 1.88 | 0.61 |
| **b13** | 0.63 | 0.09 | 0.62 | 0.59 | 2.13 | 0.67 |
| **b14** | 0.46 | 0.21 | 1.18 | 2.01 | 0.65 | 0.81 |
| **b23** | 0.34 | 0.64 | 0.89 | 0.27 | 2.17 | 0.58 |
| **b24** | 0.57 | 0.89 | 1.11 | 0.85 | 2.52 | 1.56 |
| **b34** | 0.01 | 0.57 | 0.39 | 0.69 | 1.39 | 0.15 |
| **b123** | 0.04 | 0.01 | 0.34 | 1.04 | 1.18 | 1.06 |
| **b124** | 0.25 | 0.29 | 0.40 | 0.10 | 1.85 | 0.80 |
| **b134** | 0.74 | 0.31 | 0.69 | 0.31 | 2.21 | 1.02 |
| **b234** | 0.09 | 0.60 | 0.94 | 0.65 | 0.89 | 0.17 |
| **b1234** | 0.35 | 0.16 | 0.17 | 0.22 | 1.59 | 0.58 |

**Table 4.22** $t_{experimental}$ values for b coefficients calculated in relation with the peak heights of alcohol standards and acetone.

| | **Methanol** | **Ethanol** | **Isopropanol** | **Acetone** | **n-Propanol** | **n-butanol** |
|---|---|---|---|---|---|---|
| $b_0$ | **26.73** | **24.83** | **29.56** | **27.73** | **34.12** | **45.34** |
| $b_1$ | **4.97** | **4.45** | **6.46** | **5.10** | **5.52** | **6.40** |
| $b_2$ | 0.63 | 1.43 | **3.47** | 2.16 | **2.73** | **4.54** |
| $b_3$ | **3.04** | **2.95** | 0.32 | 2.02 | 2.33 | 1.50 |
| $b_4$ | 0.38 | 0.04 | 0.34 | 1.15 | 0.87 | 0.90 |
| $b_{11}$ | 2.54 | **2.55** | 2.30 | **2.59** | 2.25 | 1.32 |
| $b_{22}$ | 1.92 | 1.45 | 0.95 | 1.04 | 1.23 | 0.61 |
| $b_{33}$ | 0.86 | 0.09 | 1.38 | 1.58 | 0.92 | 2.54 |
| $b_{44}$ | 1.87 | 1.51 | 0.98 | 0.51 | 1.42 | 1.22 |
| $b_{12}$ | 0.71 | 0.21 | 2.02 | 1.19 | 0.32 | 0.40 |
| $b_{13}$ | **2.82** | 1.90 | 0.64 | 1.79 | **2.56** | 2.06 |
| $b_{14}$ | 1.96 | 1.20 | **3.24** | **2.60** | 0.95 | 0.63 |
| $b_{23}$ | 0.01 | 0.16 | 1.49 | 0.96 | 0.40 | 0.59 |
| $b_{24}$ | **3.04** | **3.12** | 1.99 | **2.60** | **4.01** | **4.12** |
| $b_{34}$ | 1.14 | 1.26 | 2.24 | 0.37 | 1.02 | 0.92 |
| $b_{123}$ | 0.18 | 0.61 | **2.59** | 1.56 | 1.15 | 2.16 |
| $b_{124}$ | 1.40 | 1.79 | 0.69 | 1.39 | **2.65** | **2.75** |
| $b_{134}$ | **3.51** | 2.35 | **3.93** | 1.89 | **2.71** | 2.41 |
| $b_{234}$ | 0.86 | 1.11 | 0.11 | 0.73 | 1.51 | 1.35 |
| $b_{1234}$ | 1.32 | 0.29 | 1.33 | 0.13 | 0.09 | 0.02 |

t test was performed for testing the significance of selected parameters. The evaluation of the results revealed that the most significant linear term for the constructed central composite design was related with inlet temperature. Negative effect of this factor was observed on peak areas and peak heights.

When peak heights were taken into consideration, quadratic terms related with inlet temperature were significant for ethanol and acetone. Some of the interaction terms ($b_{13}$ for methanol and n-propanol, $b_{14}$ for isopropanol and acetone, $b_{24}$ for all the analytes except isopropanol) were also significant for all the standards.

On the other hand, triple interaction term $b_{123}$ was found to be significant for isopropanol, $b_{124}$ for n-propanol, n-buthanol and $b_{134}$ for methanol, isopropanol and n-propanol.

Consequently, it can be seen from the *b* values calculated from the design matrix as linear relation, quadratic effects and interaction terms between the parameters have been compiled in a table to give some insight to experimenter. This result underlines the importance of experimental design for exploiting optimization of many parameters.

According to t test results new designs were constructed with significant parameters and under these condition the constructed model were calculated as *%RMSE* (see Eq. 1.23 and 1.24).

Since new designs could not be constructed for methanol, ethanol, isopropanol, n-propanol according to the peak areas related *%RMSE* values were not calculated., though, *%RMSE* values for acetone and n-butanol were calculated (Table 4.23) and given together with *%RMSE* values according to peak heights for all the analytes.

**Table 4.23** The %RMSE values indicating the adequacy of the model for peak area and height.

| Peak | *% RMSE for* | |
|---|---|---|
| | **Peak areas** | **Peak heights** |
| Methanol | - | 17.06 |
| Ethanol | - | 16.54 |
| Isopropanol | - | 14.32 |
| Acetone | 11.68 | 15.96 |
| n-propanol | - | 13.17 |
| n-butanol | 9.31 | 9.56 |

Considering the %RMSE values in Table 13.7 revealed that for the applied design model evaluation of peak areas as the response factor was more appropriate for optimization.

Partial derivatives were taken for each significant parameter in the model equation and optimum values for the factors were calculated from the coded values. According to peak heights, optimization for inlet temperature is needed for methanol, ethanol and acetone and the calculated coded values were -1.13, -1.01 and -1.13 respectively. For the other factors, levels for center points were appropriate.

Response surfaces for each standard were constructed and effect on the peak areas and peak heights of each pair of factors were evaluated graphically. Besides the surface plots, optimization of four factors for each standard peak areas was performed by Matlab Version 7.0.

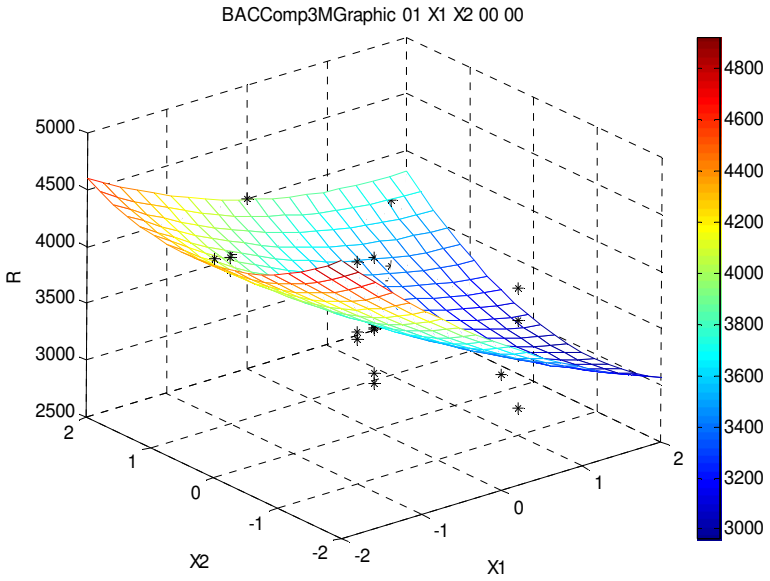


**Figure 4.16** Response surface for methanol peak area (x1000) versus inlet temperature (X1) and carrier gas flow rate (X2).

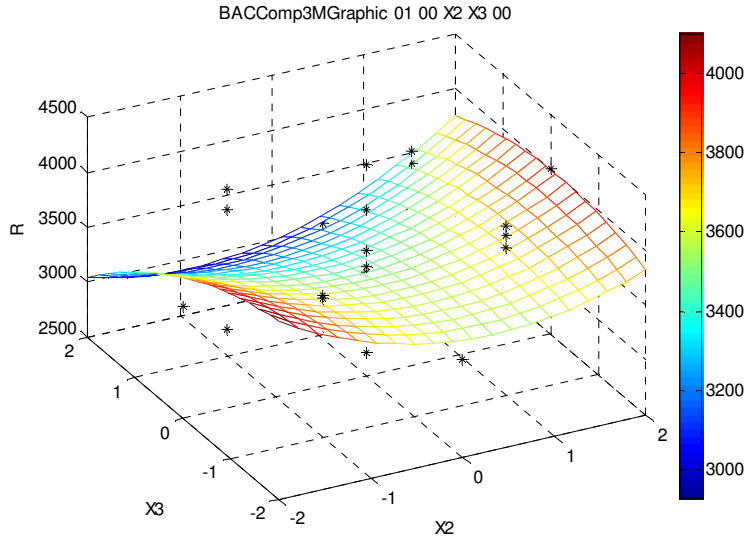


**Figure 4.17** Response surface for methanol peak area (x1000) versus carrier gas flow rate (X2) and sample volume in vial (X3).

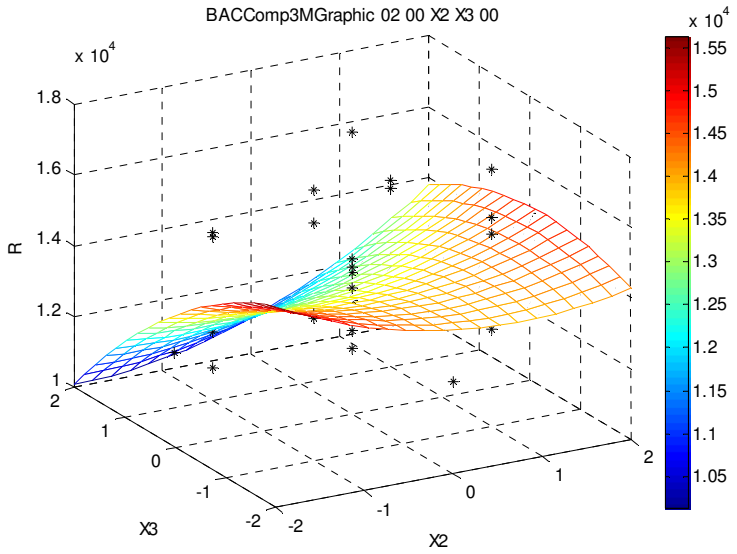


**Figure 4.18** Response surface for ethanol peak area (x1000) versus carrier gas flow rate (X2) and sample volume in vial (X3).

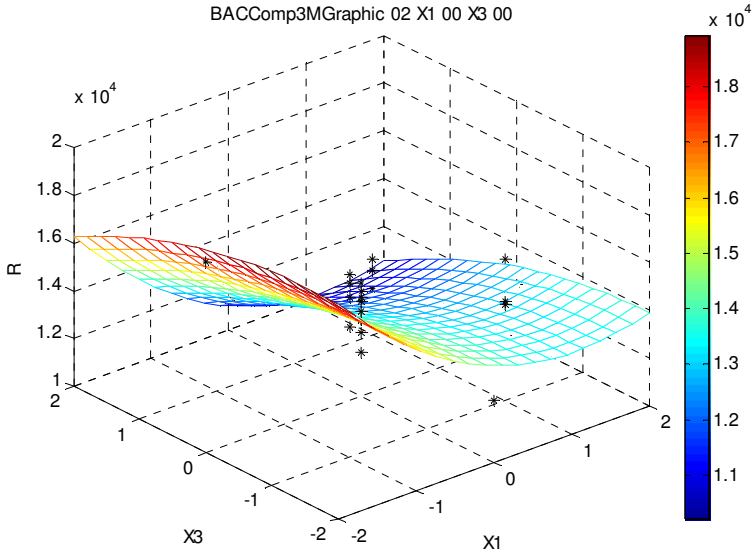


**Figure 4.19** Response surface for ethanol peak area (x1000) versus inlet temperature (X1) and sample volume in vial (X3).

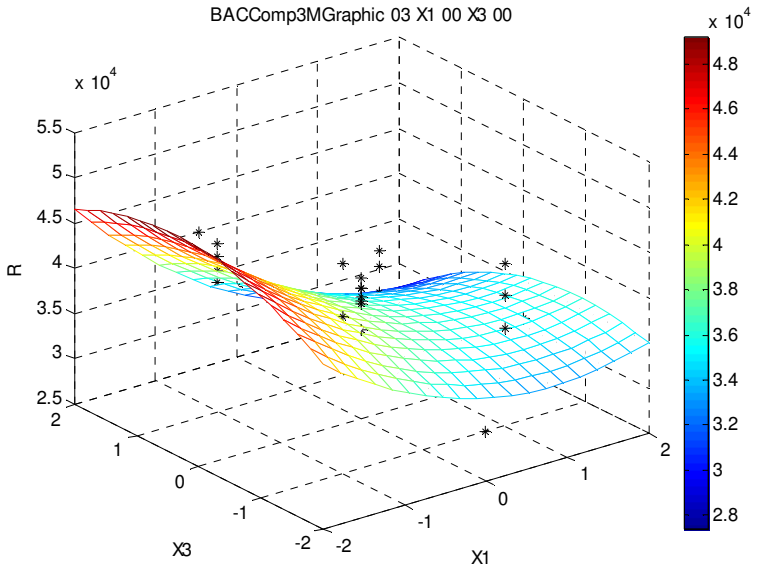


**Figure 4.20** Response surface for isopropanol peak area (x1000) versus inlet temperature (X1) and sample volume in vial (X3).

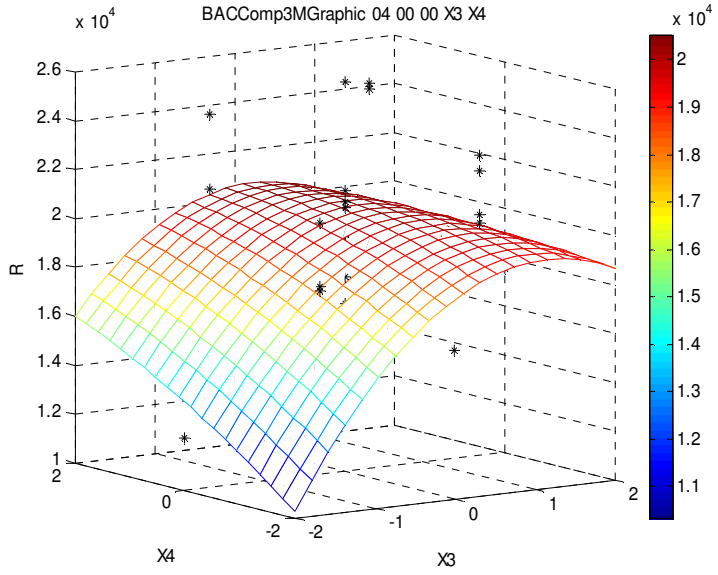


**Figure 4.21** Response surface for acetone peak area (x1000) versus sample volume in vial (X3) and extraction time (X4).

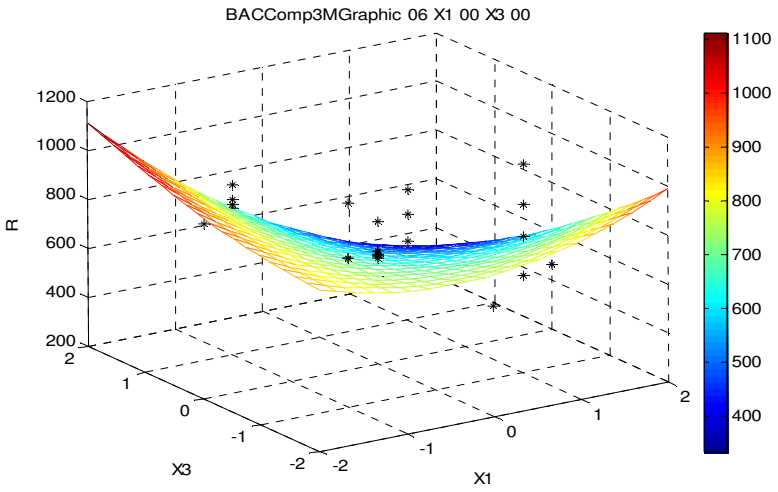


**Figure 4.22** Response surface for methanol peak height (x1000) versus inlet temperature (X1) and sample volume in vial (X3).

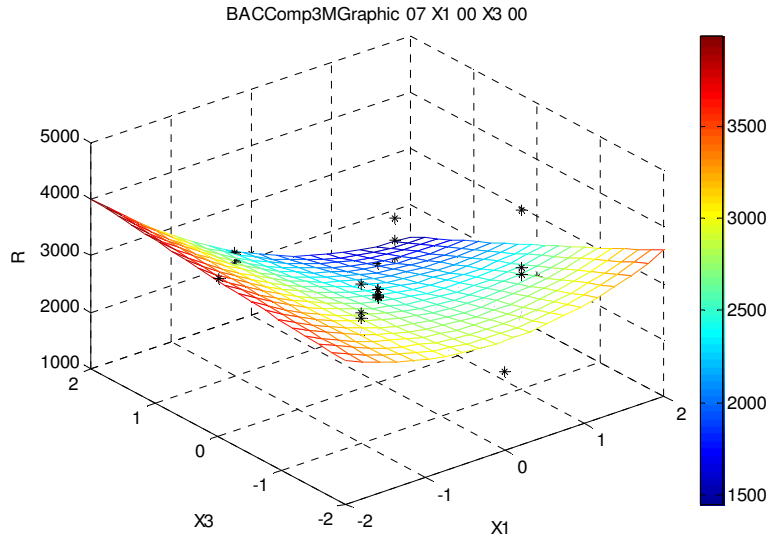


**Figure 4.23** Response surface for ethanol peak height (x1000) versus inlet temperature (X1) and sample volume in vial (X3).

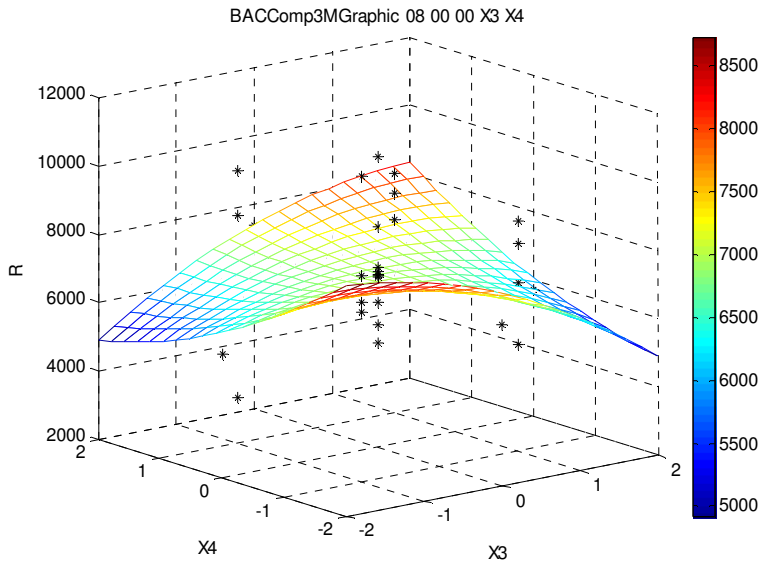


**Figure 4.24** Response surface for isopropanol peak height (x1000) versus sample volume in vial (X3) and extraction time(X4).

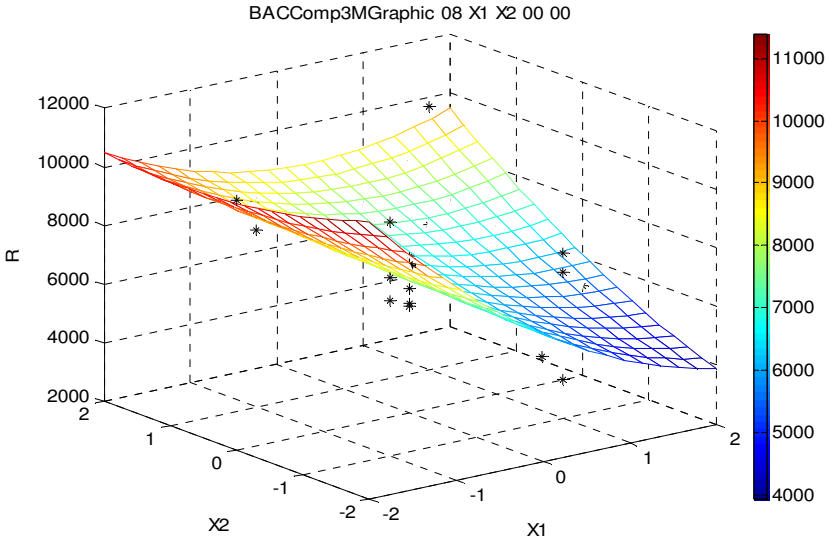


**Figure 4.25** Response surface for isopropanol peak height (x1000) versus inlet temperature(X1) and carrier gas flow rate(X2).

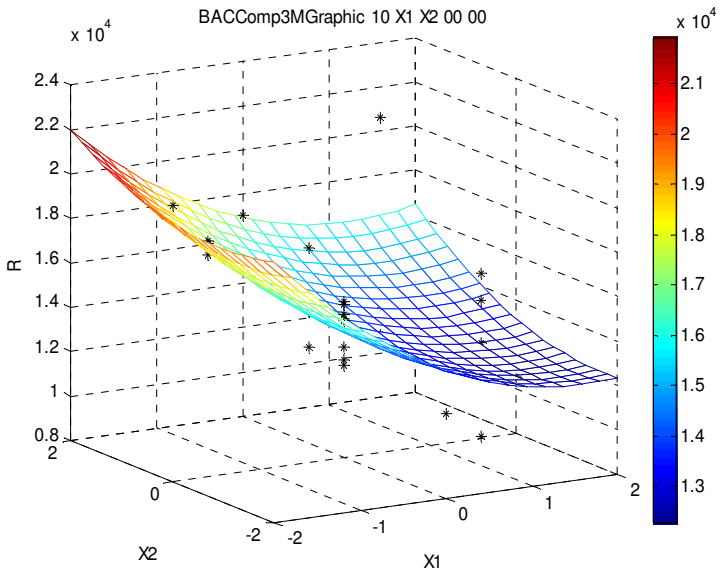


**Figure 4.26** Response surface for n-propanol peak height (x1000) versus inlet temperature(X1) and carrier gas flow rate(X2).

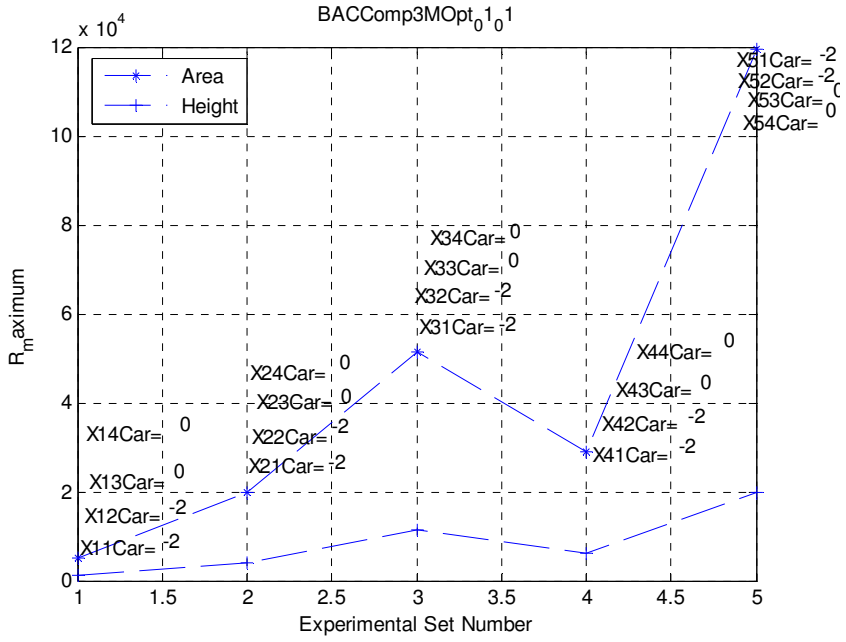


**Figure 4.27** Optimum coded levels of the four factors for methanol, ethanol, isopropanol, acetone and n-propanol related with peak areas.

Optimum coded values for peak areas and peak heights of four factors for each standard were given in Table 4.24.

**Table 4.24** Coded optimum values of the factors for peak area and height.

| Standard | For peak area | | For peak height | |
|---|---|---|---|---|
| | **Factor** | **Coded value** | **Factor** | **Coded value** |
| Methanol | $X_1$ | -2 | $X_1$ | 1.80 |
| | $X_2$ | -2 | $X_2$ | 1.77 |
| | $X_3$ | 0 | $X_3$ | 1.80 |
| | $X_4$ | 0 | $X_4$ | 1.77 |
| Ethanol | $X_1$ | -2 | $X_1$ | 1.93 |
| | $X_2$ | -2 | $X_2$ | 1.05 |
| | $X_3$ | 0 | $X_3$ | 2 |
| | $X_4$ | 0 | $X_4$ | 1.19 |
| Isopropanol | $X_1$ | -2 | $X_1$ | 1.32 |
| | $X_2$ | -2 | $X_2$ | 0.51 |
| | $X_3$ | 0 | $X_3$ | 1.34 |
| | $X_4$ | 0 | $X_4$ | 2 |
| Acetone | $X_1$ | -2 | $X_1$ | 1.59 |
| | $X_2$ | -2 | $X_2$ | 0.05 |
| | $X_3$ | 0 | $X_3$ | 1.31 |
| | $X_4$ | 0 | $X_4$ | 2 |
| n-propanol | $X_1$ | -2 | $X_1$ | 1.85 |
| | $X_2$ | -2 | $X_2$ | 0.35 |
| | $X_3$ | 0 | $X_3$ | 2 |
| | $X_4$ | 0 | $X_4$ | 1.32 |

These results were found in agreement with t test values. According to t-test, the most significant parameter for the constructed central composite design was the inlet temperature of the sample ($X_1$) and this was followed by carrier gas flow rate ($X_2$). The effect of the former factor was in negative direction for peak areas and peak heights. On the other hand the effect of carrier gas flow rate on peak heights was in positive direction.

It is interesting to note that the most significant coded parameters shown in the Table 4.24 have revealed that the optimum coded values for X1 and X2 were found to be minus 2, while they were obtained as "zero" for X3 and X4 considering only the peak areas.

On the other hand, interaction terms for peak heights in Table 4.24 indicate that optimum coded values for the four factors were mostly different from the highest and the lowest values used (2 and -2) and they were positively signed. This as well constitutes the advantage of experimental designs in order to optimize the experimental conditions for obtaining different data (i.e. peak area or height).

### 4.2.3 Application of HS-SPME-GC-FID Method for Blood Alcohol Analysis

Stock blood solutions spiked with standard alcohol solutions as describe in Section 2.2. As given in the Procedure Section, 1.0 mL of blood and 0.5 mL of IS placed into the vials and capped immediately. HS-SPME injection and GC operation conditions used in calibration studies were given in Table 4.25.

The calibration graphs were prepared using five concentration levels of each analyte in the ranges given in Table 4.26. The peak areas served as a response, since the ratio of peak areas of the analyte and IS did not show consistency. The $R^2$ values exceeded 0.941 (average of 0.979).

**Table 4.25** Optimum parameters for HS-SPME injection and GC instrumentation

| Parameters for sample and GC | Conditions |
|---|---|
| Inlet temperature | 213°C |
| Oven temperature | 50°C |
| Detector temperature | 250°C |
| Air flow rate | 350 mL/min |
| $H_2$ gas flow rate | 35 mL/min |
| Split flow rate | 30 mL/min |
| Carrier gas flow rate | 2.6 mL/min |
| Equilibrium temperature | 55°C |
| Equilibrium time | 10 min |
| Extraction time | 4 min |
| Desorption time | 1.0 min |
| Salting out (NaCl) | 1.0 g |
| Sample volume in vial | 1.5 mL |
| Depth gauge of the fiber holder | 3.2 |

**Table 4.26** Concentration ranges (mg/100mL) and calibration graph characteristics related with alcohol standards and acetone.

| Standard | Conc. Range | Equation | $R^2$ |
|---|---|---|---|
| Methanol | 2.0 - 200 | y=5783x+53646 | 0,9877 |
| Ethanol | 0.2 - 300 | y=302552x-5E+06 | 0.9407 |
| Isopropanol | 0.2 - 100 | y=404658x-34688 | 0.9724 |
| Acetone | 0.2 - 100 | y=353157x+282437 | 0.9978 |
| n-buthanol | 0.02 - 10 | y=2E+07x+2E+06 | 0.9937 |

HS-SPME-GC/FID method application to six original ethanol positive medico-legal blood samples was performed and the ethanol levels of these samples were determined. The ethanol concentration of the samples were found in the range of 34.0 -276.4 mg/100 mL.

# 5. RESULTS AND DISCUSSION PART III

## COMPARISON OF THE CALIBRATION TECHNIQUES FOR STATIC HEADSPACE STUDIES

The poor resolution of ethanol and isopropanol with column I (HP 20M) has motivated us to compare the calibration techniques for static headspace analysis of the alcohol standards with both column. Further studies include the linear regression and calibration studies with column II to compare the results obtained with column I in terms of uncertainty of the measurement

### 5.1. Linear Regression and Calibration

Calibration studies with static headspace technique were carried out with column II (Rtx BAC1) studies. Aqueous calibration solutions were prepared for ethanol and isopropanol standards. Table 5.1 summarizes the experimental parameters used for sample and the instrument.

**Table 5.1** Optimum parameters for static HS injection and GC instrumentation

| **Parameters for sample and GC** | **Conditions** |
|---|---|
| Inlet temperature | 150 °C |
| Oven temperature | 55° C |
| Detector temperature | 250 °C |
| Air flow rate | 300 mL/min |
| $H_2$ gas flow rate | 30 mL/min |
| Split ratio | 30 |
| Carrier gas flow rate | 2.2 mL/min |
| Equilibrium temperature | 55 °C |
| Equilibrium time | 15 min |
| Injected headspace volume | 1.0 mL |
| Salting out (NaCl) | 1.0 g |
| Sample volume in vial | 1.0 mL |

Calibration graphs (Figure 5.1) for the standards were obtained in

the concentration ranges for five points and three replicates (0.005, 0.010, 0.050, 0.150, and 0.400 v/v %). Calibration graph characteristics were revealed in Table 5.2.

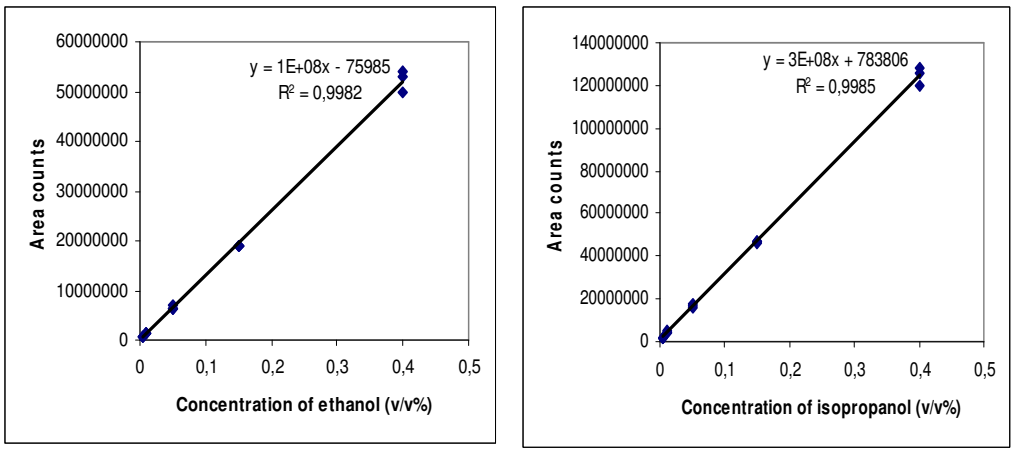


**Figure 5.1** Calibration graphs for ethanol and isopropanol

**Table 5.2** Data related with the calibration graphs for ethanol and isopropanol

| **Standard** | **y** | **$R^2$** |
|---|---|---|
| Ethanol | y = 1E+08x-75985 | 0.9982 |
| Isopropanol | y = 3E+08x + 783806722 | 0.9985 |

The random error in the values for the slope and intercept are important and were calculated from the Eq. 1.26. Then, determination of the error in *x*-value corresponding to any measured *y*-value could be possible by using the Eq. 1.27. Data related with the calculation of $s_{x0}$ values for ethanol and isopropanol were given in Table 5.3 and Table 5.6. respectively.

**Table 5.3** Data for ethanol obtained with linear regression

| $x_i$ | $y_i$ | $\hat{y}$ | $(y_i - \hat{y})$ | $(x_i - \bar{x})$ | $(y_i - \bar{y})$ |
|---|---|---|---|---|---|
| 0.005 | 657261 | 575556 | 81705 | -0.118 | -15294689 |
| 0.005 | 638252 | 575556 | 62696 | -0.118 | -15313698 |
| 0.005 | 632137 | 575556 | 56581 | -0.118 | -15319813 |
| 0.010 | 1482259 | 1227098 | 255161 | -0.113 | -14469691 |
| 0.010 | 1542928 | 1227098 | 315830 | -0.113 | -14409022 |
| 0.010 | 1457852 | 1227098 | 230754 | -0.113 | -14494098 |
| 0.050 | 6308068 | 6439435 | -131367 | -0.073 | -9643882 |
| 0.050 | 6255550 | 6439435 | -183885 | -0.073 | -9696400 |
| 0.050 | 6862173 | 6439435 | 422738 | -0.073 | -9089777 |
| 0.150 | 18986121 | 19470277 | -484156 | 0.027 | 3034171 |
| 0.150 | 18834986 | 19470277 | -635291 | 0.027 | 2883036 |
| 0.150 | 18870982 | 19470277 | -599295 | 0.027 | 2919032 |
| 0.400 | 52951933 | 52047382 | 904551 | 0.277 | 36999983 |
| 0.400 | 49908652 | 52047382 | -2138730 | 0.277 | 33956702 |
| 0.400 | 53890101 | 52047382 | 1842719 | 0.277 | 37938151 |

**Table 5.4** Characteristics of linear regression for ethanol

| $(y_i - \hat{y}_i)^2$ | $(x_i - \bar{x})^2$ | $(y_i - \bar{y})^2$ | $\mathbf{s_{y/x}}$ |
|---|---|---|---|
| 1.0247E+13 | 0.32844 | 5.5873E+15 | 887817 |

**Table 5.5** Data for isopropanol obtained with linear regression

| $x_i$ | $y_i$ | $\hat{y}$ | $(y_i - \hat{y})$ | $(x_i - \bar{x})$ | $(y_i - \bar{y})^2$ |
|---|---|---|---|---|---|
| 0.005 | 1542725 | 2328806 | -786081 | -0.118 | -37306701 |
| 0.005 | 1510049 | 2328806 | -818757 | -0.118 | -37339377 |
| 0.005 | 1573884 | 2328806 | -754922 | -0.118 | -37275542 |
| 0.010 | 4387068 | 3873806 | 513262 | -0.113 | -34462358 |
| 0.010 | 4732227 | 3873806 | 858421 | -0.113 | -34117199 |
| 0.010 | 4461384 | 3873806 | 587578 | -0.113 | -34388042 |
| 0.050 | 16182105 | 16233806 | -51701 | -0.073 | -22667321 |
| 0.050 | 16444881 | 16233806 | 211075 | -0.073 | -22404545 |
| 0.050 | 17475393 | 16233806 | 1241587 | -0.073 | -21374033 |
| 0.150 | 47237281 | 47133806 | 103475 | 0.027 | 8387855 |
| 0.150 | 46829846 | 47133806 | -303960 | 0.027 | 7980420 |
| 0.150 | 46393063 | 47133806 | -740743 | 0.027 | 7543637 |
| 0.400 | 125541603 | 124383806 | 1157797 | 0.277 | 86692177 |
| 0.400 | 119751551 | 124383806 | -4632255 | 0.277 | 80902125 |
| 0.400 | 128678327 | 124383806 | 4294521 | 0.277 | 89828901 |

**Table 5.6** Characteristics of linear regression for isopropanol

| $(y_i - \hat{y}_i)^2$ | $(x_i - \bar{x})^2$ | $(y_i - \bar{y})^2$ | $s_{y/x}$ |
|---|---|---|---|
| 4.6686E+13 | 0.32844 | 3.15031E+16 | 1895045 |

Estimated Standard deviations for each concentration were calculated and given in Table 5.7.

**Table 5.7** Calculated estimated standard deviation values for ethanol and isopropanol at various concentrations (v/v%).

| Concentration | $s_{xo}$ (Ethanol) | $s_{xo}$ (Isopropanol) |
|---|---|---|
| 0.005 | 0.0057 | 0.0040 |
| 0.010 | 0.0057 | 0.0040 |
| 0.050 | 0.0056 | 0.0040 |
| 0.150 | 0.0056 | 0.0040 |
| 0.400 | 0.0059 | 0.0041 |

The confidence limits of $x$ can be calculated as $x_0 \pm t\, s_{xo}$. As can be followed from the tables above, quite similar estimated standard deviations were calculated for ethanol and isopropanol as well. This indicates the homoscedastic relation of data for both alcohols.

### 5.2. Classical Multivariate Calibration

The unique possibility of simultaneous monitoring of ethanol and other volatile alcohols of toxicological interest, such as MeOH and IprOH, strongly supports the adoption of chromatographic alcohol analysis for clinical purposes. However, the overlapping of isopropanol and ethanol peaks on the chromatogram recorded with HP 20M column threatens the selectivity which is considered the ability of a method to differentiate an analyte in the presence of other components.

Therefore, multivariate calibration study was carried out with Column I and the results were compared with those obtained with Column II.

To begin with, chromatograms of aqueous isopropanol and ethanol

standard solutions were recorded. Then, 25 aqueous calibration and validation samples were prepared containing the two standards randomly varying in concentration (Figure 5.2). Chromatograms of pure standards and validation samples were obtained under the optimized conditions. 100 original measurement points for pure standards (***S*** matrix) and for the validation mixtures (***y*** vector) were selected for each chromatogram and these points constitute the variable range studied.

Concentrations (***ĉ***) in the calibration and validation samples can be estimated by using the coefficients of the linear combination. Estimated concentrations for the mixtures can be calculated by using Eq. 1.28 and 1.29. Estimated measurement variables and residuals were calculated from Eq. 1.30 and 1.31 respectively.

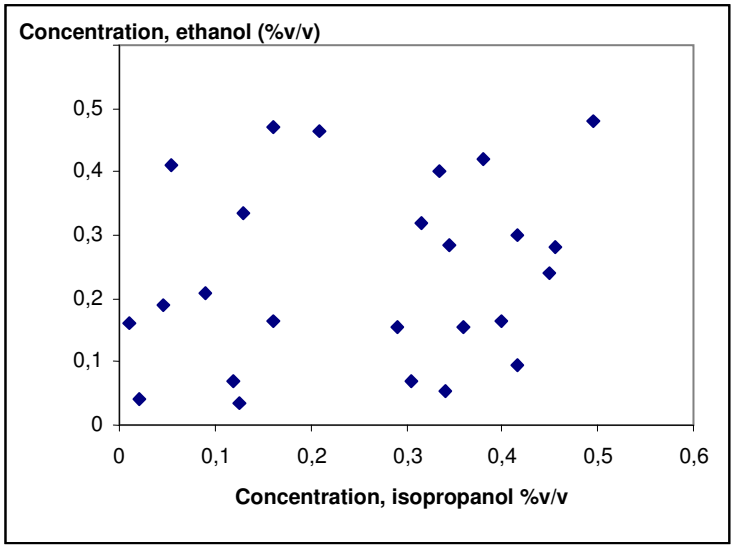


**Figure 5.2** Concentration of ethanol versus the concentration of isopropanol for the calibration and validation samples.

The plot of predicted concentration versus known concentration for isopropanol and ethanol is shown as Figure.5.3 and 5.4 respectively.

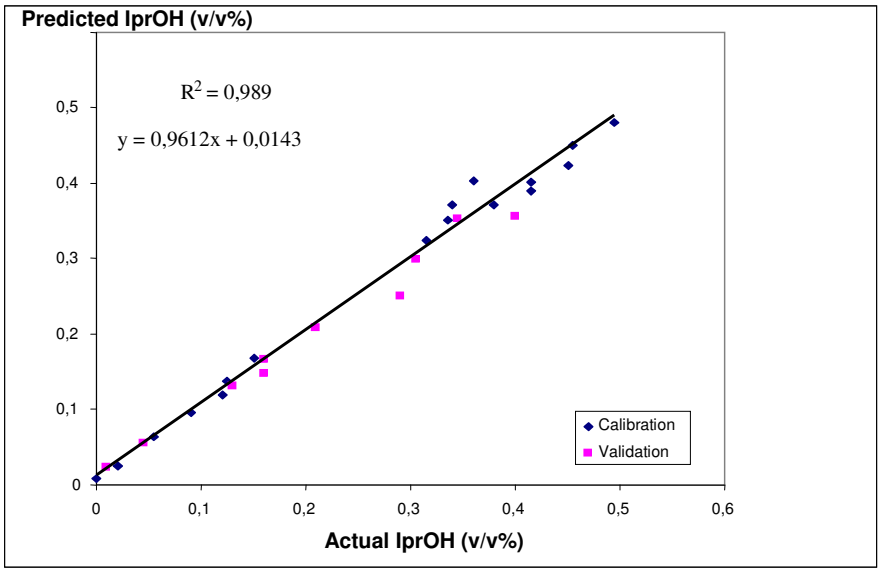


**Figure 5.3** The plot of predicted concentration versus actual concentration of isopropanol

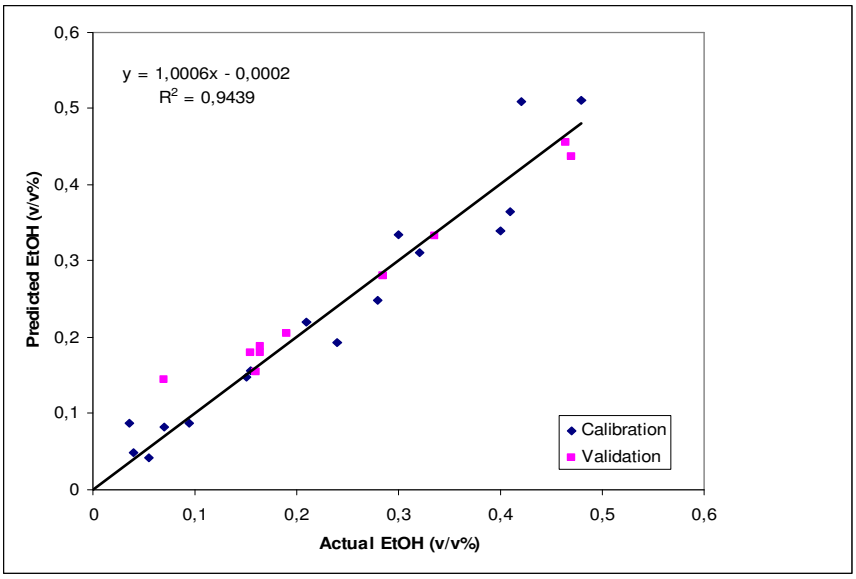


**Figure.5.4** The plot of predicted concentration versus actual concentration of ethanol

The random distribution of the data throughout the ideal line clearly indicates the adequacy of the model. Predicted concentrations versus known concentrations for isopropanol and ethanol gave a linear relationship with $R^2 = 0.989$ and $R^2 = 0.9439$, respectively.

Since the predicted concentrations are the regression coefficients of the linear regression, uncertainties in the concentrations can be predicted. These are defined as standard error of calibration and prediction and can be calculated by using Eq. 1.32 and Eq. 1.33. The standard error of prediction (stated as Root Mean Square Error of Prediction (RMSEP) in some references) is given in concentration units and represents the uncertainty in the predicted concentrations due to the deviations from the model assumptions, measurement noise, and degree of overlap of the pure chromatogram.

As the system deviates from the underlying assumptions of CLS, the $\sum_{i=1}^{n}\left(c_i - \hat{c}_i\right)^2$ increases. If the measurement noise is high, this value will also increase (Beebe, 1998).

The standard error of calibration and prediction were calculated as 0.0195 and 0.0201 for isopropanol and 0.0391 and 0.0291 for ethanol, respectively.

Concentration residuals ($\boldsymbol{c} - \hat{\boldsymbol{c}}$) were calculated and graphs were constructed with the predicted versus residual concentrations for both calibration and validation standards (Figure 5.5 and Figure 5.6, and Figure 5.7 and Figure 5.8) for each alcohol.

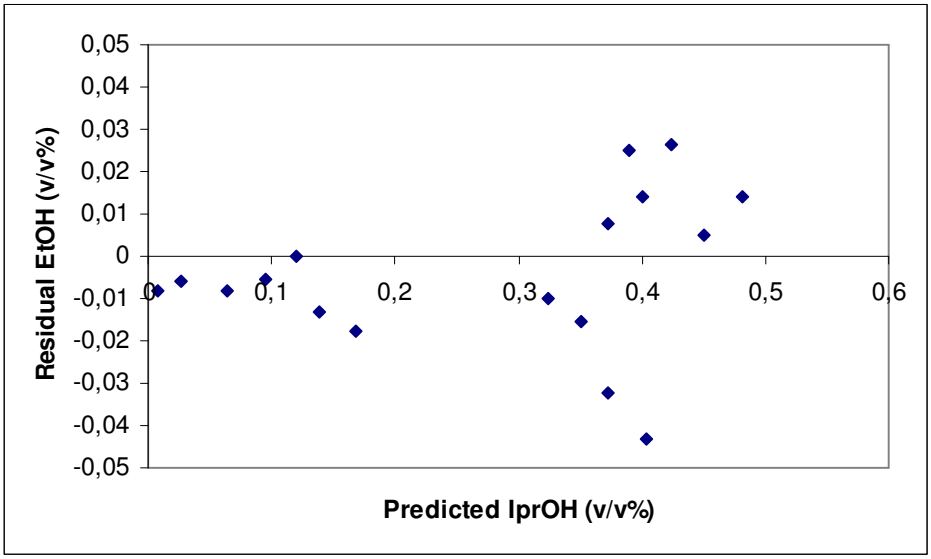


**Figure.5.5** Concentration residuals of calibration samples versus predicted concentration for isopropanol

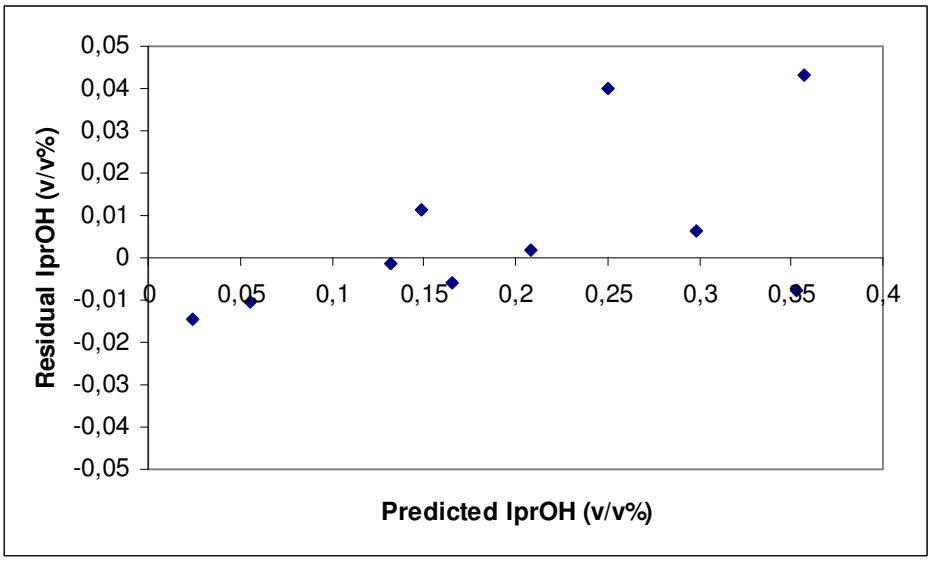


**Figure.5.6** Concentration residuals of validation samples versus predicted concentration for isopropanol

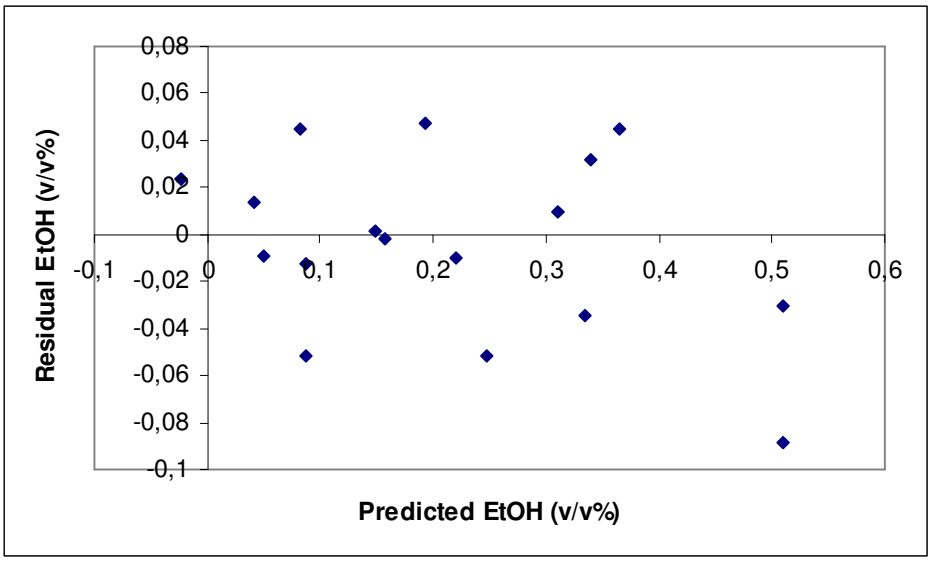


**Figure 5.7** Concentration residuals of calibration samples versus predicted concentration for ethanol

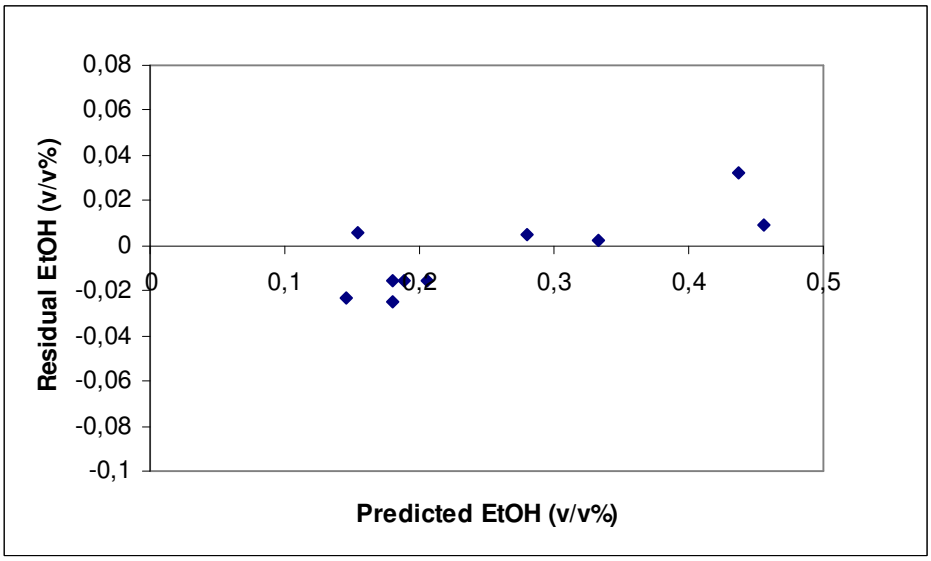


**Figure.5.8** Concentration residuals of validation samples versus predicted concentration for ethanol

Considering the calibration and validation results given above it can be concluded that, CLS calibration model can be easily used for calculation of the concentrations of overlapped peaks obtained from a sample with unknown concentration.

In other words, multivariate methods may allow one to extract truly quantitative information from highly unselective data (Olivieri et al., 2006).

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