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Gas chromatographic analysis of alcohols in blood with a chemometric approach

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The objective of the present study is to examine the main and interactive effects of different factors related with headspace-solid phase extraction (HS-SPME) and gas chromatography (GC) conditions for the simultaneous determination of alcohols (ethanol, methanol, isopropanol, *n*-propanol) and acetone in blood samples. In order to evaluate the simultaneous effects of the significant variables, a chemometric approach was utilized to reveal the important parameters. The factors affecting the headspace extraction of alcohols on the SPME fiber was first deduced in a screening study by applying a Plackett–Burman design. According to the screening results, the factors that possess a positive effect on peak areas are sample concentration, inlet temperature, flow rate for the carrier gas and volume of the solution. Based on the results of the screening study, to continue optimization, six insignificant values were fixed at the appropriate amounts (oven temperature: 50 °C; detector temperature: 250 °C; H_2 gas flow rate: 35 mL min^{-1}; split flow rate: 30 mL min^{-1}; equilibrium temperature: 55 °C, extraction time: 4 min). Subsequently, a central composite design (CCD) was constructed for optimization and response surface methodology (RSM) was used to interpret the effect of each pair of independent variables on the response. The optimized method for alcohol and acetone analysis *via* HS-SPME-GC was validated through spike recovery tests.

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1 Introduction

The simultaneous determination of low molecular weight alcohols and aldehydes in biological fluids is of great importance for forensic and clinical purposes. Such volatile compounds, namely acetaldehyde, ethanol, methanol, acetone, 1-propanol, and 2-propanol, are formed in the course of physiological processes and their concentrations increase after the consumption of alcoholic beverages. Almost every alcoholic drink contains acetone, methanol, and other alcohols as impurities and alcohol addiction leads to their accumulation in the body and therefore, their simultaneous determination in blood receives great attention.[1,2] Jeszenszky *et al.* have stated a correlation between the congeners in alcoholic beverages and biochemical markers (specifically methanol, 1-propanol and 2-propanol) in the blood tests of alcoholics.[3] The methods for alcohol determination must, therefore, be sensitive enough to quantify other volatile compounds, especially after the consumption of alcoholic beverages.[4]

Gas chromatography (GC) is a precise and reliable method for alcohol determination in blood and other biological fluids, and has become a reference method in forensic toxicology.[5] Direct injection of blood or serum has been applied since the first applications of GC for alcohol analysis.[6] Headspace technique coupled with GC (HS-GC) has emerged as the method of choice for ethanol and other low molecular weight volatiles for research and medico-legal purposes in body fluids.[7–9]

Solid Phase Micro Extraction (SPME) is a relatively new solvent free sample preparation technique, developed in the early 1990's by Pawliszyn, that allows simultaneous sampling, extraction, pre-concentration, and introduction of analytes from a matrix in a single procedure.[10] SPME uses a fused silica fiber that is coated on the outside with an appropriate stationary phase. This fiber is placed in contact with the sample matrix for a predetermined time and then, rapidly delivered to a GC column. The method can also be combined with headspace technique for the determination of volatile organic compounds in complex matrices, including blood, to eliminate the interferences.[10–14]

A number of studies were presented in the literature on ethanol and analysis of volatile compounds by HS-SPME in blood and urine specimens.[15–18] In the optimization studies, generally the effect of one variable at a time (OVAT) is evaluated while other variables are held constant. These types of experiments reveal the effect of the chosen variable under the set

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conditions assuming that the variables are independent. Such studies do not give any information about the effects if the other variables are also changed at the same time.[19,20] In an experimental design study, on the other hand, a series of experiments can be performed under different conditions and the simultaneous effects of each variable can be estimated simultaneously.

Response surface methodology (RSM), which consists of mathematical and statistical techniques, is useful in the modeling and analysis of the processes.[21,22] So far a very limited number of studies including a chemometric approach have been encountered for the determination of blood alcohol levels in the literature.[4,23] A recent study has been performed by Kristoffersen *et al.* in which a factorial design was applied to study the effects of blood storage and headspace conditions on ethanol stability and acetaldehyde formation in whole blood and plasma.[23]

The main objective of the present study is to examine the main and interactive effects of different factors related with HS-SPME and GC conditions for determination of alcohols (ethanol, methanol, isopropanol, *n*-propanol, *n*-butanol) and acetone in blood samples, simultaneously. A number of factors affecting the headspace extraction of alcohols on the SPME fiber were first deduced in a screening study by applying a Plackett–Burman design. This design is a powerful tool to search for the key variables in a multivariable system rapidly.[24,25] Pareto charts were utilized to reflect the results of this screening study.

Furthermore, a central composite design (CCD) was constructed for optimization and a detailed quantitative model is proposed. Subsequently, RSM was used to interpret the effect on the response of each pair of independent variables graphically.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals and reagents were at least analytical grade. Deionized water (18.2 MΩ) was used throughout the study. Acetone, methanol, isopropanol, *n*-propanol and *n*-butanol (HPLC grade) were obtained from Lab Scan (Dublin, Ireland) and absolute ethanol (GC grade) was obtained from J. T. Baker (Deventer, Holland). Whole blood samples (used for the validation of the method) were obtained from the Blood Center of Ege University Hospital, İzmir, Turkey. The medico-legal blood samples (containing EDTA as an anticoagulant) were obtained from the Emergency Medicine Service of the same hospital and were kept at −20 °C until analysis.

2.2 Samples

Aqueous standard solutions of alcohol and acetone were prepared in whole blood solutions and used in the screening and optimization studies of the proposed method. The standard stock whole blood solutions were prepared by spiking with mixed alcohol solutions (ethanol 2.5 mg 100 mL^{-1}, acetone 1.0 mg 100 mL^{-1}, methanol 1.0 mg 100 mL^{-1}, isopropanol 1.0 mg 100 mL^{-1}, *n*-butanol 0.05 mg 100 mL^{-1}) and diluting to an appropriate volume with the blank blood. Each blood sample contained the same amount of *n*-propanol as the internal standard (IS) (10.0 mg 100 mL^{-1}). The blood samples were prepared daily, sealed and kept in a refrigerator prior to analysis.

2.3 Headspace-solid phase micro extraction (HS-SPME)

HS-SPME was used to extract the volatile compounds of the materials. The extraction procedure was performed under controlled temperatures with 65 μm Carbowax/DVB (CW-DVB, 65 μm) fiber (Supelco, Bellefonte, PA, USA) with the aid of a manual fiber holder (Supelco, Bellefonte, PA, USA). Prior to the HS-SPME procedure, the fiber was thermally cleaned and conditioned in the GC injection port at 250 °C for 30 minutes to remove the fiber contaminants that were likely to exist.

The samples were put in glass headspace vials (20.0 mL), sealed with a Teflon-lined silicone septum and an aluminum cap, then placed in a thermostatic oven. The vial content was allowed to stand for a while in a thermostatic oven at an optimum temperature, which depended on the experimental design. Subsequently, the SPME device was inserted into the sealed vial manually by penetrating the septum and the fiber was exposed to the headspace of the sample for a predetermined time. After the sampling, desorption of the analytes from the fiber coating was performed thermally in the GC injection port at 250 °C during 1.0 min in splitless mode. Prior to sampling, each fiber was reconditioned for 5 minutes in the GC injection port at 250 °C.

2.4 Gas chromatographic conditions

The analyses were carried out with a Thermo Finnigan Trace GC Ultra GC coupled with a flame ionization detector (FID). The gas chromatograph was equipped with a capillary column Rtx-BAC1 (30 m × 0.32 mm i.d. × 1.8 μm film thickness) and helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. An inlet liner (0.8 mm i.d.) purchased from Supelco (Bellefonte, PA, USA) was used in the GC inlet block during SPME analysis to prevent significant peak broadening of the early peaks. The responses based on the peak areas of the GC-FID analysis were obtained after sample preparation with HS-SPME.

2.5 Experimental designs and statistical analysis

Plackett–Burman design and central composite design were exploited to measure the effects of changes in the operating variables. The mutual interactions on HS-SPME and GC analysis through several experiments were investigated.

2.5.1 Plackett–Burman design. Plackett–Burman (P–B) experimental design was used to evaluate the major factors for HS-SPME and GC conditions. This design considers that there are no interactions between the different factors. The main effect of such a design may simply be calculated as the difference between the average of measurements made at high (+1) and low levels (−1).[26]

Twelve experiments were performed for the eleven factors selected, namely inlet, oven and detector temperature (°C), carrier gas and split flow rate (mL min^{-1}), H_2 gas flow rate at the detector (mL min^{-1}), solution volume (mL), sample concentration (mg 100 min^{-1}), equilibrium temperature (°C), salt

amount (NaCl, g) and extraction time (min) at two levels. The values for the upper (+) and lower (−) levels of each variable were selected based on the preliminary analysis performed. The data were processed using Microsoft Excel and Pareto charts were utilized for reflecting the results of the screening methods by ranking them for their importance and frequency.

A Pareto chart is a bar graph in which the lengths of the bars are proportional to the absolute value of the estimated effects, divided by the standard error. The bars are arranged in size order of the effects to visually depict the more significant parameters. It also includes a vertical line at the critical *t*-value ($p = 0.05$) where a bar that exceeds this line is considered to be significant.[27]

2.5.2 Central composite design. Based on the results obtained from Plackett–Burman design, a central composite design (CCD) was performed for four variables (inlet temperature, carrier gas flow rate, solution volume and extraction time), with a 2^4 factorial design (eight axial points and five replicates in the center). Each factor in the design was studied at five different levels (−2, −1, 0, 1, 2). The factor levels, experimental conditions and the design matrix corresponding to the CCD are shown in Table 1. Statistical calculations were performed by using Microsoft Excel and the results generated from the design matrices were evaluated by Matlab 7.0.

3 Results and discussion

3.1 Screening design

The major effects were screened for eleven factors on the separation of alcohols and acetone with HS-SPME. Twelve experiments of the experimental design were performed randomly in three replicates. Pareto charts were used to identify the most influential factors on the peak areas.

The Pareto charts constructed have revealed that among the eleven factors only a few have a significant effect on the peak areas, namely salt amount, sample concentration, inlet temperature, and carrier gas flow rate and sample volume. Among them only high inlet temperature negatively affected the peak areas in accordance with a previous study.[2] Extraction time displayed a positive effect in all the charts as expected. In addition, since oven temperature showed a positive effect on the ethanol response, low column temperature was preferred for better resolution in the following optimization study. Based on the results of the screening study, the six parameters other than the significant ones were fixed at appropriate amounts (oven temperature: 50 °C; detector temperature: 250 °C; H_2 gas flow rate: 35 mL min^{-1}; split flow rate: 30 mL min^{-1}; equilibrium temperature: 55 °C, extraction time: 4 min).

Table 1 Real values of variables selected for the central composite design

	−2	−1	0	1	2
X_1: inlet temperature (°C)	205	215	225	235	245
X_2: carrier gas flow rate (mL min^{-1})	2.2	2.4	2.6	2.8	3.0
X_3: solution volume (mL)	0.5	1.0	1.5	2.0	2.5
X_4: extraction time (min)	2.0	3.0	4.0	5.0	6.0

3.2 Optimization design

To optimize the variables that have a significant influence on HS-SPME, a response surface methodology coupled with a central composite was applied in this study. Considering all the screening results obtained, a central composite design (CCD) with five replicates of the center point was constructed to give a total of 30 experiments. From the screening studies performed, the most significant factors affecting the HS-SPME alcohol analysis (salt amount, inlet temperature, carrier gas flow rate, and sample volume and concentration) were used for the subsequent studies in the experimental design. Such an experimental design allows the estimation of the coefficients of the following postulated model in which 20 coefficients are estimated. The design was performed with six aqueous standard mixtures each 0.05 mL in 100 mL solution, except for butanol being 0.03 mL in 100 mL.

Apart from the linear effects of the parameters on HS-SPME, an insight into the quadratic and interaction effects of the parameters by means of a Fisher's *F*-test and a Student's *t*-test was provided. The Student's *t*-test was used to determine the significance of the regression coefficients of the parameters. The *p*-values were used as a tool to check the significance of each of the interactions among the variables. The *F*-test tables given by Draper and Smith[28] were used to determine the *F* values of the compounds to be analyzed. The absolute experimental *F* value (F_{ex}) should be greater than the critical *F* value (F_{cr}) obtained from the standard *F*-distribution. The experimental model is adequate at a 95% confidence level for all of the compounds (methanol, ethanol, isopropanol, acetone and *n*-propanol) except *n*-butanol since its *F* value exceeds F_{cr} ($F_{5,11}$: 5.05 for $p = 0.05$).

As mentioned above, the significance of the selected parameters was also evaluated by means of a *t*-test. Experimental (t_{ex}) values obtained for the peak areas for methanol, ethanol, isopropanol, acetone, *n*-propanol and *n*-butanol were compared with the critical (t_{cr}) values. The evaluation of the results revealed that the most significant linear term for the

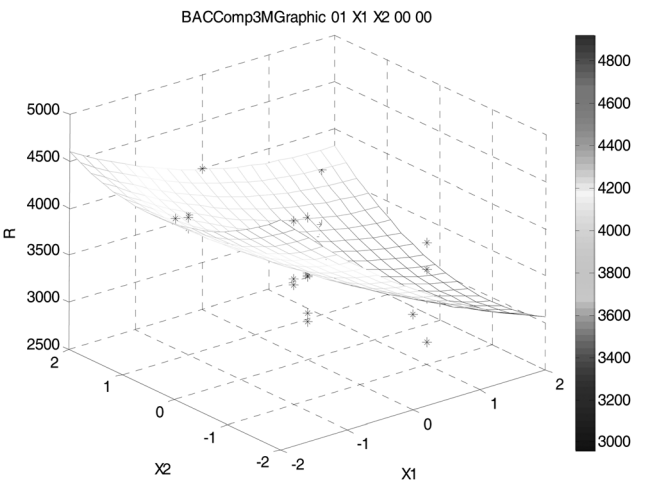

Fig. 1 Response surface of methanol peak area (×1000) for inlet temperature (X_1) *vs.* carrier gas flow rate (X_2).

constructed CCD was related to inlet temperature, in which a negative effect of this factor was observed. Consequently, the linear relation, quadratic effects and interaction terms between the parameters can be seen from the b values calculated from the design matrix and this gives an insight to the experimenter for the HS-SPME conditions of analysis. These results underline the importance of experimental design for exploiting the optimization of many parameters.

The t-test was also performed for testing the significance of selected parameters. t_{ex} values for each parameter were calculated. The values obtained for the peak areas for methanol, ethanol, isopropanol, acetone and n-propanol were compared with the t_{table} values (d.f. 5, $t_{table} = 2, 57$, $p = 0.05$) in Table 2. The values exceeding the t_{table} value were shown in bold character. Here, it is worth noting that even the linear terms, b_3 and b_4, corresponding to the oven temperature and carrier gas flow rate were found to be lower than the critical t value; their quadratic effects (b_{33} and b_{44}) exceeded this value for ethanol, as did b_{44} for n-propanol.

Table 2 Experimentally found t_{ex} values for the b coefficients calculated in relation to the peak areas of methanol, ethanol, isopropanol, acetone and n-propanol. Values exceeding the t_{table} value are shown in bold character

	Methanol	Ethanol	Isopropanol	Acetone	n-Propanol
b_0	**64.54**	**99.64**	**30.95**	**39.18**	**80.00**
b_1	**17.57**	**27.50**	**7.23**	**5.45**	**20.84**
b_2	**7.45**	**14.44**	**5.11**	**5.66**	**13.34**
b_3	0.19	0.40	0.16	0.74	0.42
b_4	0.67	1.21	0.60	1.15	0.67
b_{11}	**3.85**	**5.44**	1.17	0.88	**3.74**
b_{22}	1.97	**3.60**	0.95	0.95	**2.93**
b_{33}	1.40	**2.59**	0.90	0.95	2.42
b_{44}	1.84	**3.08**	0.91	1.35	**2.64**
b_{12}	1.83	**3.12**	0.93	1.37	2.54
b_{13}	0.31	0.80	0.32	0.98	0.74
b_{14}	1.43	2.12	0.41	0.99	1.32
b_{23}	0.40	0.61	0.14	0.81	0.39
b_{24}	0.10	0.35	0.21	0.47	0.51
b_{34}	0.48	0.46	0.05	0.70	0.21
b_{123}	0.05	0.24	0.02	0.69	0.05
b_{124}	0.08	0.11	0.05	0.64	0.13
b_{134}	0.11	0.46	0.16	0.54	0.36
b_{234}	1.21	1.68	0.48	0.23	1.33
b_{1234}	0.51	0.99	0.26	0.50	0.73

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}) exceeded the critical value for ethanol. The effect of column temperature on the results was found to be insignificant. When the peak areas are taken into consideration, quadratic terms were also significant for ethanol and n-propanol.

Moreover, the response surfaces for each standard were constructed and the effects on the peak areas of each pair of factors were evaluated graphically. The significant independent variables (salt amount, inlet temperature, carrier gas flow rate, and sample volume and concentration) were used to determine the optimum levels of these parameters by using RSM based on the CCD based on the above results. For the graphical interpretation of the interactions, the use of three-dimensional surface (3D) plots for the regression model was applied, which is highly recommended.[29] These 3D surfaces can provide useful information about the behavior of the system within the experimental design, facilitate an examination of the effects of the experimental factors on the responses and contour plots between the factors.[30,31] The circular nature of the contours signifies that the interactive effects between the variables are not significant and the optimum values of the test variables cannot be easily obtained.

Fig. 1 shows the interaction effect of inlet temperature (X_1) and carrier gas flow rate (X_2) on the peak area. The response surface and the contour plot indicated that the interaction between these two parameters produced a minimum response at higher flow rates. In addition, the response was increased when X_2 was increased with further decrement in X_1.

Higher peak area values for methanol and ethanol were obtained for the medium values of sample volume in the vial (data not shown). In addition, an increment was observed in the peak area for the higher values of carrier gas flow rate. On the other hand, higher peak areas were obtained for a decrease in inlet temperature while sample volume is increased. The interaction of flow rate and sample volume displayed a parabolic change upwards for methanol and revealed the same maximum values near the central point of the experimental design.

The optimum coded values for the peak areas and peak heights of the four factors were found to be in agreement with t-test values. According to the t-test, the most significant parameter for the CCD was the inlet temperature of the sample (X_1) which was followed by carrier gas flow rate (X_2). The effect of the former factor was in the negative direction for peak areas. It is interesting to note that the most significant coded parameters revealed that the optimum coded values for X_1 and X_2 were found to be minus 2, while they were obtained as "zero" for X_3 and X_4, considering only peak areas.

The previous studies generally focused on one variable at time (OVAT) for the optimization of the analysis conditions. The present study, on the other hand, has revealed the impact of two or more factors on the HS-SPME and GC conditions and the interaction of these terms are also taken into consideration. Equilibrium time, for instance, was optimized as 7.5 minutes on the basis of OVAT while in our work, it was revealed that 4 minutes of equilibrium time is the optimum. Since adding an inorganic salt improves the headspace extraction efficiency of volatile compounds in biological fluids, the effect of salt amount was also investigated in the present study. A significant effect was evaluated on the extraction efficiency of the compounds which is in accordance with a previous study.[4]

In another study, factorial design was used to study the effects of blood storage and headspace conditions on ethanol stability. The study focused on four parameters, namely HS temperature, equilibrium time, analyte concentration and

storage time. According to the work, HS temperature was of importance while equilibrium time was less critical. Although an increase in extraction temperature may enhance the peak height/area of the relevant compound in the headspace, enhancement in the peak size was not found to be significant in our study due to the partition coefficient of the analytes between the fiber and the HS.[23]

3.3 Analytical characteristics of the method

Method validation is the process of proving that an analytical method is acceptable for its intended purpose.[32] Therefore, a validation study was conducted under optimized conditions.

3.3.1 Selectivity. The method was found to be selective enough to differentiate and to quantify alcohol standards, as an $R \geq 1.5$ was observed on BAC1 column.

3.3.2 Linearity. The peak area of each standard displayed results proportional to the concentration of the analyte. The calibration graphs for the standards and the data related to these calibration graphs are given in Table 3. Here, it should be noted that the concentration ranges selected were within typical blood alcohol levels to suit the actual purpose.

3.3.3 Precision. Precision was evaluated by the means of relative standard deviation (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 4.

As proposed earlier, the RSD% limit acceptable for low concentrations was ±20% and for middle and high concentrations was ±15%.[33,34] Within-day repeatability of the present study was found to be in the acceptable range for different concentration levels. Between-day repeatability was evaluated by the means of ANOVA and the results are given in Table 5. The results were compared with the tabulated F value (2.87) for the d.f. of 4. Corresponding p values are also given in the table. As can be followed from the table, the ANOVA results for between-day repeatability were not found to be satisfactory for five consecutive days. This can be attributed to the nature of the batch analysis employed.

3.3.4 Sensitivity. The sensitivity of the method was tested upon calculating the limit of detection (LOD) and limit of quantitation (LOQ) values given in Table 6. 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. LOD and LOQ values were found.

Table 4 RSD values for four concentration levels for five replicates for five consecutive days for each standard

Standard	Concentration (mg 100 mL^{-1})	RSD values for days				
		1 day	2 day	3 day	4 day	5 day
Acetone	0.2	4.28	3.94	12.32	6.45	11.44
Methanol	2	7.07	6.64	13.13	13.08	14.53
Ethanol	0.2	19.76	18.80	15.88	—	18.95
n-Propanol	10	4.70	3.28	3.02	2.71	4.55
Acetone	1	6.58	2.45	3.56	6.63	2.49
Methanol	10	6.93	17.01	4.09	6.37	3.16
Ethanol	1	15.61	1.18	3.50	9.37	8.35
n-Propanol	10	4.26	3.93	2.69	6.08	3.32
Acetone	10	6.17	5.15	4.22	1.22	4.32
Methanol	100	9.40	5.52	6.37	12.48	3.78
Ethanol	10	8.77	4.72	5.37	3.89	5.11
n-Propanol	10	6.89	2.76	4.13	4.08	6.37
Acetone	100	4.82	1.11	5.71	3.72	4.26
Methanol	200	10.26	10.99	3.94	3.79	4.62
Ethanol	300	3.187	1.00	6.69	3.07	3.27
n-Propanol	10	4.74	2.55	3.81	2.64	2.07

Table 5 ANOVA results for between-day repeatability

Standard	Conc. (mg 100 mL^{-1})	F_{ex}	p
Acetone	0.2	7.36	8.2×10^{-5}
	1	5.89	2.7×10^{-3}
	10	4.57	8.7×10^{-3}
	100	14.35	1.1×10^{-5}
Methanol	2	38.50	4.0×10^{-9}
	10	29.71	3.7×10^{-8}
	100	7.64	6.6×10^{-4}
	200	15.57	6.2×10^{-6}
Ethanol	0.2	2.72	6.9×10^{-2}
	1	10.92	7.3×10^{-5}
	10	7.39	8.0×10^{-4}
	300	13.71	1.6×10^{-5}

3.3.5 Stability. The stability of the method was tested upon analyzing the blood samples on consecutive days. For this purpose blood samples were spiked with standards at high, middle and low concentration levels and then analyzed freshly, and after 1, 2, 3 and 6 days. Samples were stored in a refrigerator until the analyses. The effect of freeze and thaw was also investigated for the three concentration levels. Freeze and thaw stability was evaluated by the means of ANOVA as shown in Table 7. Following the table, it is seen that the detected F values were far below the F_{table} value (2.77 for d.f. 5). It can be concluded that the concentration of samples stored in a

Table 3 Data related to the calibration graphs for acetone, methanol and ethanol

Standard	Concentration (mg 100 mL^{-1})	y (according to peak area)	R^2	y (peak area/IS area)	R^2
Acetone	0.1, 1, 10, 50, 100	$y = 416\,193x + 290\,467$	0.9947	$y = 0.1446x + 0.0524$	0.9961
Methanol	1, 10, 100, 150, 200	$y = 31\,754x + 28\,722$	0.9863	$y = 0.0111x + 0.00$	0.9836
Ethanol	0.1, 1, 10 100, 300	$y = 89\,857x + 103\,687$	0.9977	$y = 0.0313x + 0.029$	0.9974

Table 6 LOD and LOQ values

Parameter	Acetone	Methanol	Ethanol	*n*-Propanol
LOD ± RSD (mg 100 mL^{-1})	0.021 ± 0.022	0.913 ± 0.618	0.236 ± 0.089	0.161 ± 0.039
LOQ ± RSD (mg 100 mL^{-1})	0.069 ± 0.076	3.040 ± 2.060	0.787 ± 0.300	0.537 ± 0.134

Table 7 ANOVA results for freeze and thaw stability

Concentration	F_{ex}	F_{table}	p
High	0.031668	2.772853	0.9994
Middle	0.020905	2.772853	0.999782
Low	0.010464	2.772853	0.99996

refrigerator or deep freeze conditions for a certain period were not significantly changed during that time.

3.4 Application of real sample analysis by HS-SPME-GC

HS-SPME is a very convenient technique for the analysis of ethanol, methanol, isopropanol, *n*-propanol, *n*-butanol and acetone in blood as it is simple, fast and solvent-free. The chromatographic conditions were optimized with the aim of obtaining a good separation of adjacent peaks within a short analysis time. Fig. 2 shows the chromatogram of the HS-SPME of the alcohols and acetone using the optimum conditions found in this study.

The optimized method for alcohol and acetone analysis by HS-SPME-GC was validated through spike recovery tests. Stock blood solutions were spiked with standard alcohol solutions as described in Section 2.2. Each blood sample contained the same amount of *n*-propanol as the internal standard (10.0 mg in 100 mL). 1.0 mL of blood and 0.5 mL of internal standard were placed into the vials and capped immediately. The percent recovery results of acetone, ethanol, methanol, *n*-propanol, isopropanol and *n*-butanol in the blood samples were found to be 89.4, 95.6, 97.8, 95.9, 90.6 and 92.1 mg 100 mL^{-1}, respectively.

Five levels of concentration of each analyte were prepared to plot the standard calibration curves. The HS-SPME procedure showed a good linear behavior in the concentration ranges studied. All correlation coefficient (r) values exceeded 0.970 with an average of 0.989.

The HS-SPME-GC method was applied to six original ethanol positive medico-legal blood samples and the ethanol levels of these samples were determined. The ethanol concentrations of the samples were found to be in the range of 34.0–276.4 mg in 100 mL.

4 Conclusions

The present study describes a method for simultaneous determination of volatile compounds in blood by HS-SPME coupled with a gas chromatograph. The method provides a rapid, simple and convenient way to extract methanol, ethanol, isopropanol, *n*-butanol and acetone from a complicated matrix such as blood. Considering the effect of a number of parameters on the analytical signal and their interaction as well, an experimental design was utilized for the optimization of the HS-SPME and GC conditions. Screening and optimization of sample injection and chromatographic conditions were performed by the application

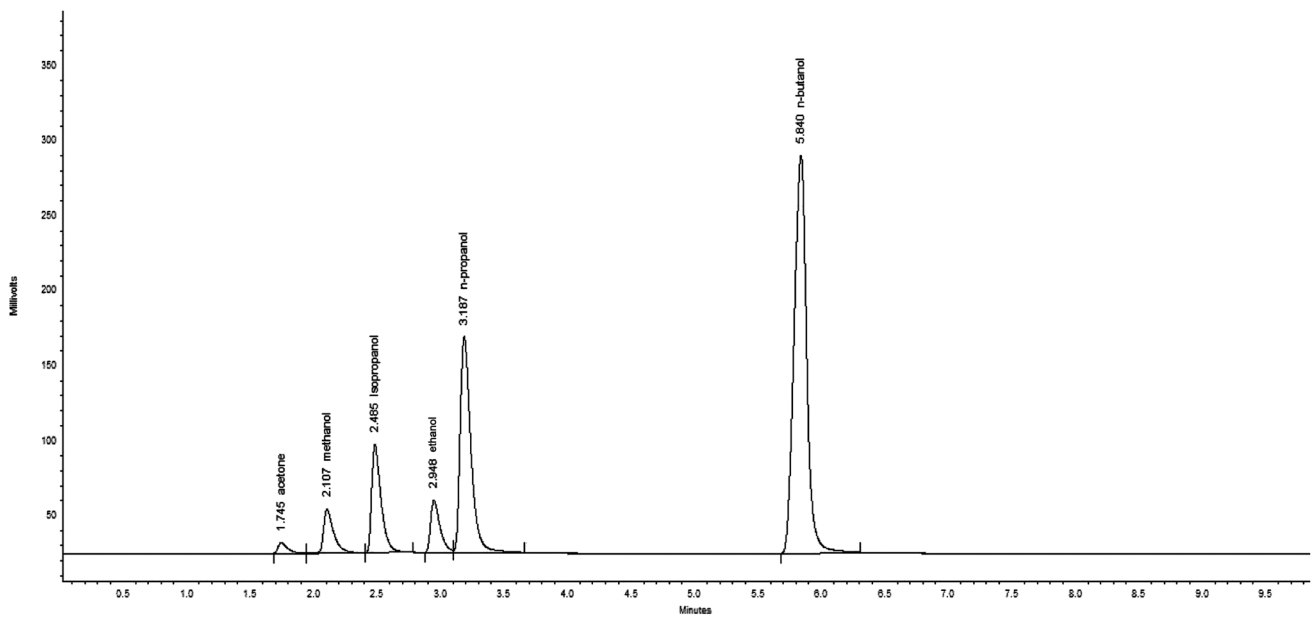

Fig. 2 The chromatogram of the HS-SPME of the alcohols (ethanol, methanol, isopropanol, *n*-propanol, *n*-butanol) and acetone in blood.

of experimental design. The results indicated that the most significant factors affecting the HS-SPME alcohol and acetone analysis were amount of salt, inlet temperature, carrier gas flow rate, and sample volume and concentration in the vial.

In addition to the linear terms, quadratic and some of the interaction terms were also found to be significant for the analytes. Therefore, it can be seen from the *b* values calculated from the design matrix that the linear relation, quadratic effects and interaction terms between the parameters have been compiled to give some insight to the experimenter. This result underlines the importance of experimental design for exploiting the optimization of many parameters.

Method validation 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, and low detection and quantification limits. By considering the results obtained in this study, the proposed conditions were found to be appropriate for the determination of ethanol, methanol, isopropanol, *n*-propanol and *n*-butanol in real blood samples.

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