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Optimization of solid-phase microextraction conditions for gas chromatographic determination of ethanol and other volatile compounds in blood

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This paper is dedicated to Professor Dr. Klaus Danzer on the occasion of his 65th birthday.

Abstract

A procedure for the determination of acetaldehyde, acetone, methanol, ethanol, 1-propanol and 2-propanol in blood was developed. Separation of analytes was carried out on DB-wax capillary column (l=30 m, I.D.=0.32 mm, dF=0.5 μm) at 40 °C, hydrogen was used as a carrier gas (at 30 kPa) and FID as a detector. Quantification was performed with the use of 2-butanol as an internal standard. Headspace solid-phase microextraction was applied as the sample preparation technique. The usefulness of most commercially available fiber coatings was checked and 65 μm Carbowax/DVB proved most effective. Microextraction was carried out from the headspace at 60 °C for 10 min. The sample was stirred at 750 rpm. In order to improve the extraction efficiency of analytes, salting-out agents were also applied. Potassium carbonate turned out to be the most efficient. A 1.0-g amount of this salt and 0.1 ml of I.S. were added to 0.5 ml of sample. Validation of the worked-out method was performed. For each analyte, the limits of detection and quantification, linearity, working range, accuracy and precision were determined or tested.

Keywords: Ethanol; Volatiles

1. Introduction

The determination of ethanol in biological specimens is the most common analytical procedure requested for legal purposes. Over 100 000 such analyses are performed in Polish forensic laboratories each year [1]. Numerous gas chromatographic (GC) methods have been described [2,3] for the determination of alcohols in blood. Headspace GC technique is preferred in the laboratories dealing with heavy routine workloads, often coupled with an automated data handling system. GC methods are suitable for the separation of other volatile substances in addition to ethanol. However, most of the existing methods are applied only to the quantification of ethanol in drunk drivers' blood or to the determination of some higher-molecular-mass alcohols from the exogenous sources in conjunction with intoxication.

The determination of low-molecular-mass compounds like acetaldehyde, acetone, ethanol, methanol, 1-propanol and 2-propanol in biological fluids

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may be expected to be of great importance for forensic and clinical purposes. Knowledge of the content of these compounds can help in the assessment of sobriety, especially when blood is taken a few hours after an accident [4]. Methanol, acetone and 2-propanol can also be used to screen people with alcoholic problems [5].

The present procedures of ethanol determination are not 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 [6]. Some modification of the headspace procedures like adding of inorganic salt to a sample were proposed in order to increase sensitivity [7]. The purpose of our research was to examine the possibility of applying solid-phase microextraction (SPME) to GC determination of acetaldehyde, acetone, ethanol, methanol, 1-propanol and 2-propanol in blood.

SPME is the fast, solventless alternative to the conventional sample extraction procedures. In SPME, an equilibrium distribution of the analytes is established between the sample matrix, the headspace above the sample (in a headspace mode) and a polymer-coated fused-silica fiber. Then the analytes are desorbed from the fiber to a capillary GC or HPLC column. Because the analytes are concentrated on the fiber and are rapidly delivered to the column, detection limits are improved while the resolution is maintained [8].

2. Experimental

2.1. Chemicals

Acetaldehyde, acetone, ethanol, methanol, 1-propanol, 2-propanol and 2-butanol, the latter used as internal standard, were purchased from Merck (Darmstadt, Germany).

Thirteen inorganic salts (NaCl, KCl, NH_4Cl, $MgCl_2$, $NaNO_3$, KNO_3, NH_4NO_3, Na_2SO_4, K_2SO_4, $(NH_4)_2SO_4$, $MgSO_4$, Na_2CO_3, K_2CO_3), all of analytical purity grade (Merck), were used to assess their salting-out abilities.

2.2. Standard solutions

Stock standard solutions of analytes each of concentration 2.5 mg/ml were prepared by the gravimetric–volumetric method. A 20-ml volumetric flask was partly filled with ultra-pure water, closed and weighed. Approximately 65 μl of pure analyte (the volume corresponding to ~50 mg of the analyte, according to its density at room temperature) was then measured with a 100-μl syringe (Hamilton, Reno, NV, USA) and added to the flask. The flask was subsequently closed again and reweighed to determine the exact mass of analyte added. Finally, the flask was filled with water to the mark. The above procedure was repeated for each analyte. Working standard solutions used in the calibration were prepared shortly before the analysis from the stock solutions and ultra-pure water by spiking appropriate volumes of stock solutions into the 100-ml volumetric flasks filled with water.

2.3. Sample solutions

The sample solutions used in optimization and validation of the method proposed were prepared in the same way as the working standard solutions (see above).

2.4. Fibers

The SPME devices and their fiber assemblies, polydimethylsiloxane (PDMS) with coating thickness of 7, 30 and 100 μm, 65 μm PDMS/divinylbenzene (DVB), 65 μm Carbowax/DVB and 85 μm polyacrylate fibers were purchased from Supelco (Bellefonte, PA, USA).

2.5. GC conditions

GC analyses were carried out on a Perkin-Elmer Autosystem gas chromatograph equipped with a flame ionisation detector (FID). A DB-WAX capillary column (l=30 m, I.D.=0.32 mm, dF=0.5 μm) was applied. The isothermal separation of analytes was reached in 8.5 min at 40 °C. Hydrogen at 30 kPa was used as a carrier gas. The injector was heated to 200 °C and the FID to 300 °C. The samples were

injected in the splitless mode, and the splitter was opened after 1 min.

3. Results and discussion

The first step of the method development was to optimize absorption of the analytes from the sample to the SPME fiber. The main factors influencing equilibrium were taken into account.

3.1. Extraction mode

Acetaldehyde, acetone, ethanol, methanol, 1-propanol and 2-propanol are volatile compounds, therefore headspace extraction was the technique of choice. For volatiles the equilibrium time is shorter in this mode as compared to direct extraction from the liquid phase. There are other advantages of the headspace mode: prevention of fiber coating contamination or decomposition (because it does not make contact with the sample) and reduction of matrix influences [9].

3.1.1. Fiber coating

The six commercially available fiber coatings mentioned above were tested for their extraction efficiency of the analytes. The examination was performed using a model solution which contained 10 mg/l of each analyte. Selection was based on the following criterion function:

$$F_j = \frac{1}{n}\sum_{i=1}^{n}\frac{H_{ij}}{\frac{1}{k}\sum_{j=1}^{k}H_{ij}}$$

where H_{ij} is the peak height of i analyte with use of j fiber coating. The values of the F_j function are shown in Table 1.

The first two fiber coatings, which are porous polymers, showed much higher extraction efficiency than the absorption-type ones like polyacrylate or PDMS. The choice between PDMS/DVB and Carbowax/DVB was based on comparison of extraction efficiency for particular analytes at the concentration levels corresponding to their content in the body fluids. In further studies Carbowax/DVB was applied.

Table 1
Values of the criterion function F_j calculated for different fiber coatings

Fiber coating	F_j
65 μm CW/DVB	1.79
60 μm PDMS/DVB	1.63
85 μm PA	0.97
7 μm PDMS	0.52
30 μm PDMS	0.40
100 μm PDMS	0.69

3.2. Extraction conditions

An increase in extraction temperature leads to a growth of analytes' concentration in the headspace and increases the extraction rate. On the other hand, the partition coefficient of an analyte between fiber and headspace decreases with temperature [10]. The dependence of chromatographic peak area on temperature is shown in Fig. 1, for all tested analytes. The linear increase of the response is observed up to 50 °C for each analyte. Over this temperature the increased rate becomes smaller for acetaldehyde, ethanol and 1-propanol. The response for acetone and I.S. seems to be constant over 50 °C and for methanol over 60 °C. However, at high temperature, biological matrices like blood or urine can decompose. Therefore, the measurements were carried out at 60 °C. In Fig. 2 the time profiles, obtained at 60 °C, are presented. Equilibrium was reached after 7.5 min for all analytes. We selected 10 min as the extraction time in our study. During this time each analyte reached equilibrium and the responses were stable. In order to shorten the time required to reach equilibrium, the samples were stirred at 750 rpm. Before extraction each sample was heated for 5 min to reach ~60 °C.

3.3. Salting out

Adding an inorganic salt to a sample solution improves extraction efficiency for volatile compounds in water as well as in biological fluids. The salting 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 [11]. The effect of 13 inorganic salts on the extraction efficiency of ana-

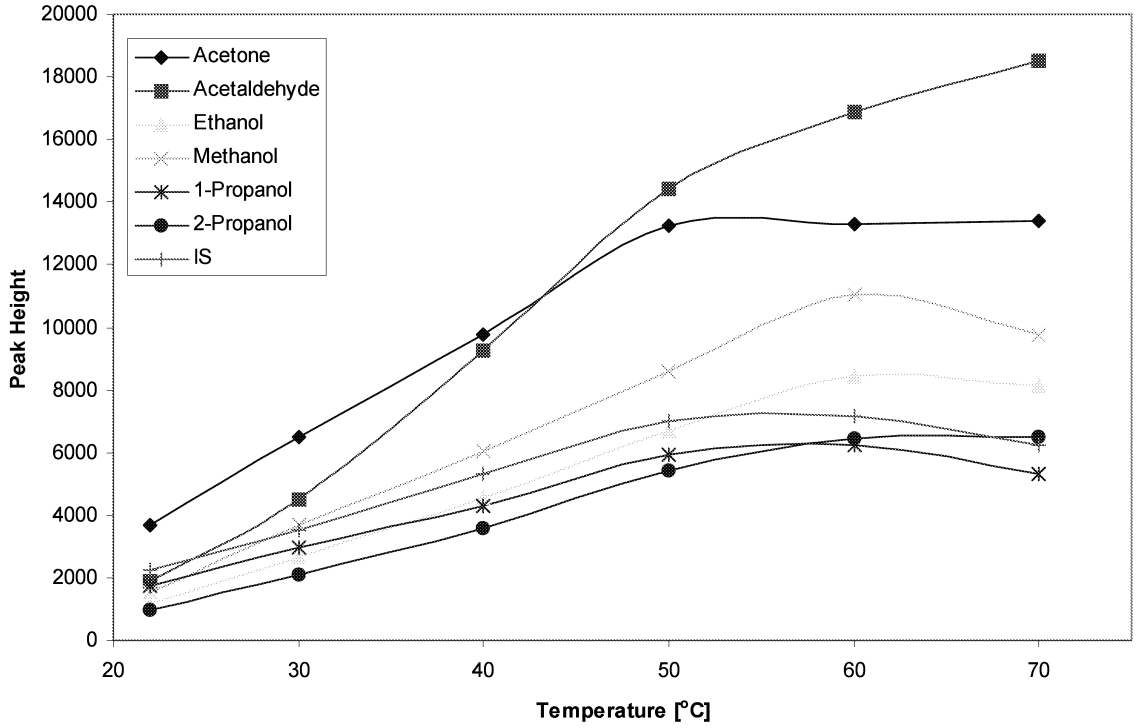

Fig. 1. Temperature profiles for the analytes.

lytes was tested. The amount of salt was first optimized. Addition of solid salts as well as their saturated solutions was tested. Analyte concentration in the headspace increased exponentially with salt concentration in a sample. In Fig. 3 the dependence of analytes signals vs. salt concentration (Na_2CO_3) is shown. The highest peaks were obtained when a salt was added as a solid in an amount that caused sample saturation at the extraction temperature.

In Table 2 the amplifications of analytes signals after adding of different salts are shown; K_2CO_3 appeared to be the most effective. It significantly improved the sensitivity of the determination method: from 2.0 times for methanol up to 220 times for 2-propanol.

In consequence, the following procedure was used for the preparation of solutions for the headspace analysis: 1.0 g of potassium carbonate and 100 μl of I.S. were added to 500 μl of sample (see Sample solutions).

3.4. Validation of the method

3.4.1. Resolution

The proposed method was validated according to the standard procedures [12,13]. The GC separation of the analytes (acetaldehyde, acetone, ethanol, methanol, 1-propanol and 2-propanol and 2-butanol) assures resolution $R > 1$ for all pairs of chromatographic peaks.

3.4.2. Linearity

In Fig. 4 the relationship between logarithm of analytes signals (relative peak heights) and logarithm of their concentrations in standard solutions were shown. Analytes concentration was expressed in mg/l. The responses were linear over the whole range: 0.1–20.0 mg/l for acetone, 1-propanol and 2-propanol and 0.4–100 mg/l for acetaldehyde, methanol and ethanol. In practice, however, we used only part

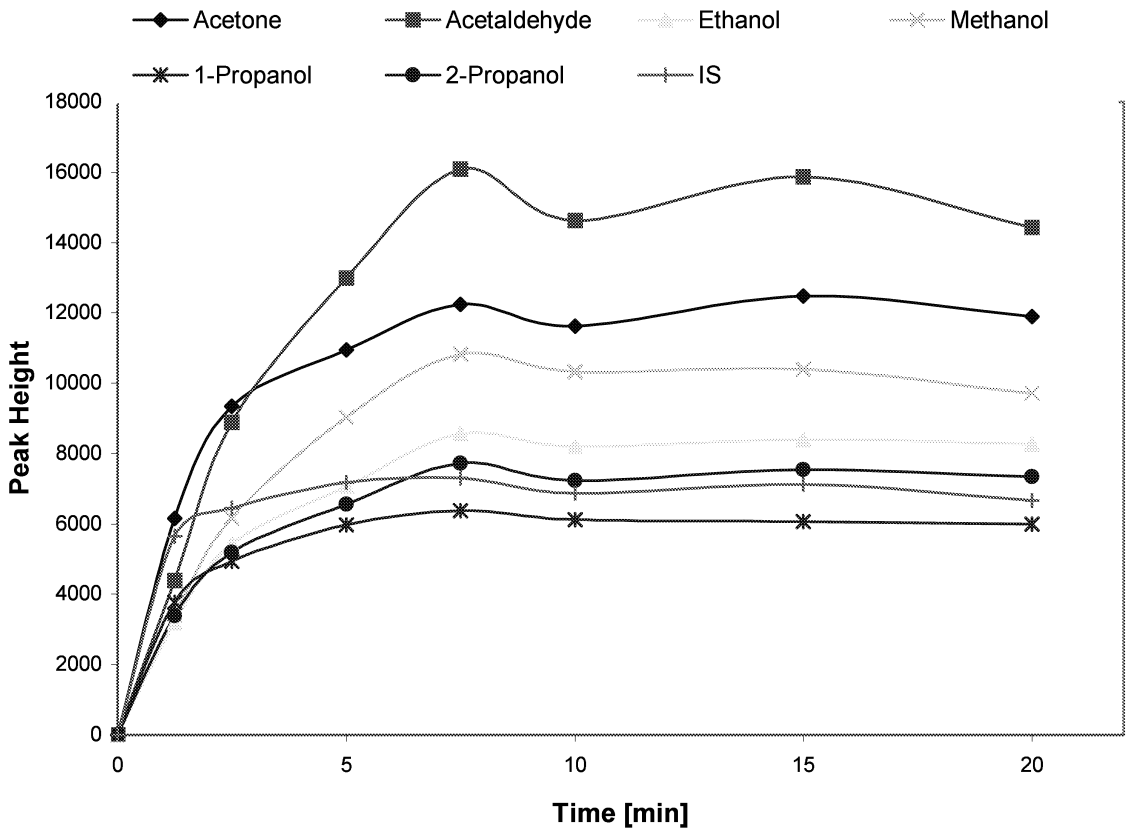

Fig. 2. Time profiles for the analytes.

of the above intervals of linearity which are presented in Table 3 as working ranges. The calibration graphs were prepared using six concentration levels of each analyte which covered the working ranges. The ratio of peaks areas of the analyte and I.S., respectively, served as a response. The correlation coefficients exceeded 0.980 for all analytes.

3.4.3. LOD and LOQ

In order to determine the limit of detection (LOD) and limit of quantification (LOQ) for an element the calibration graph was prepared that covered low analyte concentrations, at the lower range of the working range. The procedure according to the German Standard DIN 32 645 was applied in calculation of LODs and LOQs. The standard uses the statistics of linear calibration graph. The significance level $\alpha = 0.05$ was assumed. The LODs and LOQs for all analytes tested are presented in Table 3.

3.4.4. Precision

For each analyte the relative standard deviation (RSD) was calculated from the residuals in calibration graph and it corresponds to the mean analyte concentration in the standards. The RSD values did not exceed 8%.

3.4.5. Accuracy

The recovery values, calculated for particular analytes, ranged from 99.0 to 101.4%. This shows that accuracy of the method was very good.

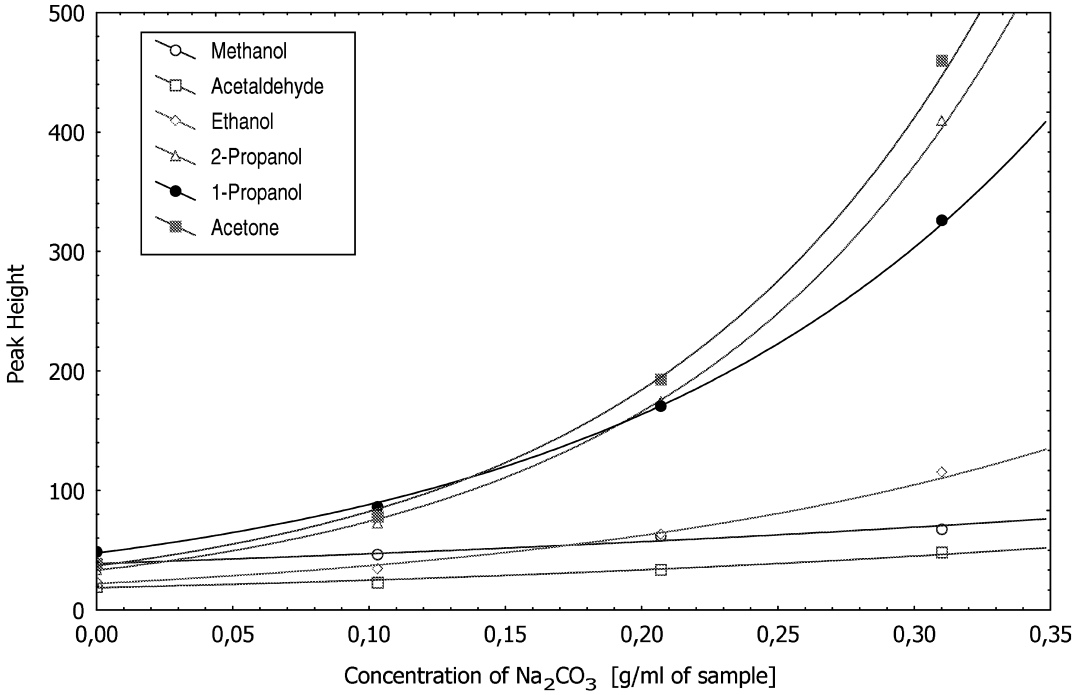

Fig. 3. Dependence of analytes' peak heights vs. salt concentration.

4. Conclusions

The proposed method allows simultaneous determination of acetaldehyde, acetone, methanol, ethanol, 1- and 2-propanol in blood. The method demonstrates good precision, wide working ranges for all analytes, good linearity, low detection and quantification limits.

Table 2
Amplification of analytes signals after adding of inorganic salt

Salt	Signal amplification			
	Mean	Median	Minimum	Maximum
K_2CO_3	86.4	76.5	2.0	220
Na_2CO_3	7.9	8.05	2.1	15
K_2SO_4	1.5	1.5	1.2	1.9
Na_2SO_4	3.5	3.4	1.8	5.5
$(NH_4)_2SO_4$	9.5	9.0	1.5	22
$MgSO_4$	7.0	6.55	1.8	14
KNO_3	1.5	1.35	1.1	2.1
$NaNO_3$	3.4	2.55	0.9	8.0
NH_4NO_3	1.3	1.2	0.2	2.5
KCl	2.2	1.8	1.0	4.0
NaCl	3.0	2.6	0.8	6.8
NH_4Cl	1.4	1.25	0.3	2.4
$MgCl_2$	1.1	1.1	0.1	2.1

Both the physiological concentration levels of the volatiles tested as well as their concentrations in blood resulting from alcohol consumption, are very low (apart from ethanol after it has been consumed), usually 0.01–10 mg/l. It is why the sensitivity of the method was the primary optimization parameter taken into account.

The result of simultaneous determination of volatile compounds in blood may be used in verification of testifying concerning the type and amount of alcoholic drink consumed. The result may appear helpful also in diagnosis of the alcohol abuse. The determination of volatile compounds in blood after ethanol has been eliminated is very important. Ethanol stops the metabolism of other short-chained alcohols. The corresponding enzyme, alcohol dehydrogenase (ADH) demonstrates much stronger affinity to ethanol than to other alcohols. Only when the ethanol concentration drops below 0.2 g/l level (i.e. it has been practically eliminated from the body)

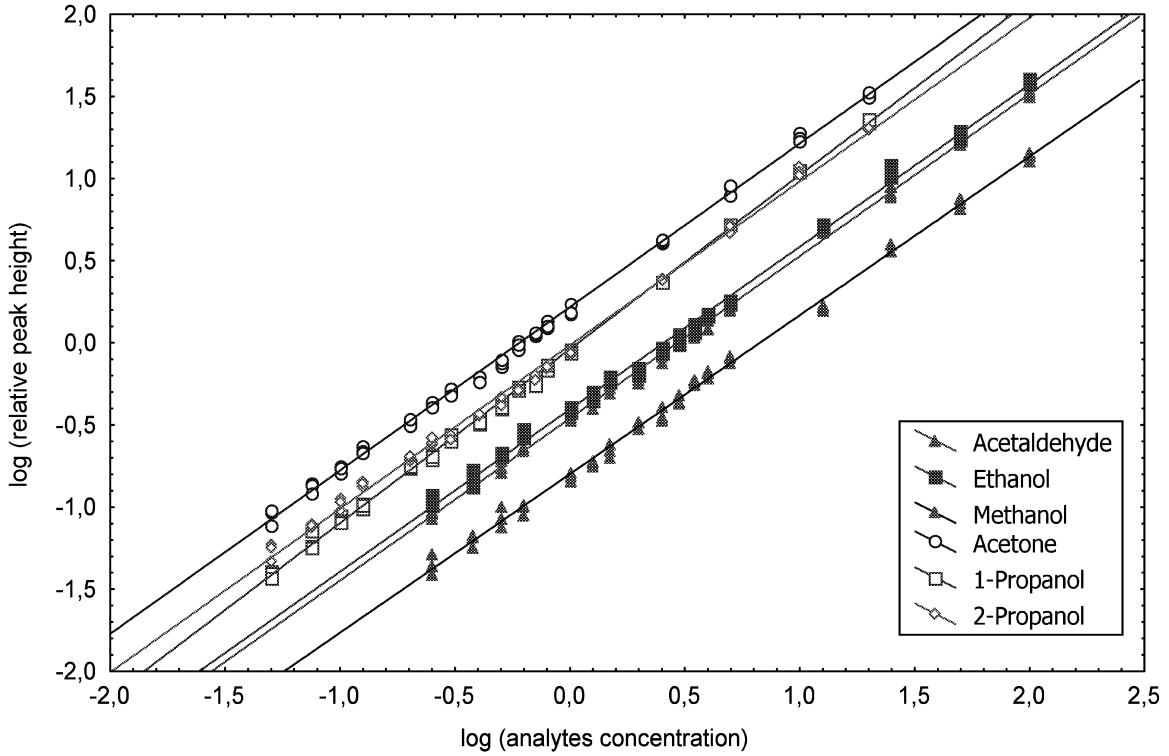

Fig. 4. Relationship between logarithm of analytes signals and logarithm of analytes concentrations in standard solutions.

does the metabolism of other alcohols start. The period of time a person has been under the influence of alcohol and the kind of alcoholic beverage consumed determines the level of methanol and other low-molecular-mass alcohols. It is why the determination of these alcohols and some of their metabolites may be useful in cases concerning drunk driving suspects who run away from an accident scene and are examined after he/she has sobered in a hidden place. An elevated concentration of methanol and other low-molecular-mass alcohols may indicate one has just sobered.

Because the headspace SPME is a rapid, simple and solvent-free technique of sample preparation, the method can be recommended for forensic, clinical and emergency laboratories. Actually, it proves efficient in co-operative research on the determination of the alcohol abuse markers.

Table 3
Analytical parameters of the worked-out method

Analyte	LOD (mg/l)	LOQ (mg/l)	Working range (mg/l)	RSD (%)	Recovery (%)
Acetaldehyde	0.040	0.120	0.150–5.0	7.5	99.0
Acetone	0.005	0.015	0.050–1.0	7.7	99.0
Ethanol	0.025	0.080	0.150–5.0	6.0	99.2
Methanol	0.045	0.125	0.150–5.0	7.4	99.2
1-Propanol	0.005	0.015	0.050–1.0	6.3	101.4
2-Propanol	0.005	0.015	0.050–1.0	5.1	100.5

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