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A NEW DIRECT EXTRACTION BY GAS-CHROMATOGRAPHY WITH FLAME IONIZATION DETECTOR COUPLED TO HEAD SPACE METHOD FOR THE DETERMINATION OF ALCOHOL CONTENT OF HIGH MATRIX WINE PRODUCTS

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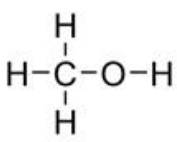
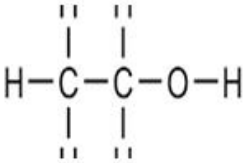
Detection, identification, and quantitation of alcohol in any matrix rich medium represent a common practice although sample preparation is inevitable and time consuming. A sensitive, precise and ultimately wide range method for detection, identification and quantification of main content/residual/impurity alcohols without any matrix interference that can be used for production phase quality control, pharmaceutical and/or bio-technological refinement or toxicological evaluation and for forensics is always needed. Even for quality control also for toxicological considerations, ethanol (EtOH) and the very similar compound methanol have to be detected and identified. However, with the fermented products, the matrix becomes a challenging process, makes the methods inefficient or more extraction methods have to be implanted. Here we propose a new simple and reliable direct extraction method that has been developed for the determination of alcohol content of high matrix wine products using the gas-chromatography with flame ionization detector coupled to head space. The method was developed with a rich and complex component mixture of fermented alcoholic beverages (wine) with very high matrix effects. Isopropanol (IPA) was preferred as an internal standard, and Triton X-100 (TX-100) was used as diluting solution in this method. The amount of TX-100, extraction temperature, and the total volume of solution in head space vial (20 mL) were optimized. 2.5% TX-100, 80°C extraction temperature, and 2.0 ml of total volume were used as optimum condition. Stationary phase was the fused silica, Agilent J&W DB-624 column (30 m x 320 μm x 1.8 μm), and Helium was used as a mobile phase. GC oven temperature programme was 40°C (5 min), 5°C/min ramp to 60°C (0 min) and 30°C/min to 150°C (4 min). Performance of the method was assessed by evaluating the recovery, accuracy, precision, linearity, limits of detection (LOD) and limit of quantification (LOQ). Calibration curve was drawn between the concentration of 2.5% to 15.0% EtOH ($y = 1.572x - 0.702$, $R^2 = 0.9960$, y; the ratio of peak area of EtOH to IPA, x: EtOH%). The slopes of standard addition and external calibration curve were statistically the same. Recovery of the method was 97.5 ± 3.5 for three different concentrations and the precision was %5.8 (n= 11). LOD and LOQ were calculated as 0.80% and 2.5%, respectively. The proposed method has a potential for application in industry and academia with determination of the alcohol content/residual/impurity and also check the quality and content of the fermented medium without the effect of matrix.







INTRODUCTION

Although they are primarily used because of their solvent properties to help solubilize many drugs, ethanol and isopropanol are found as an active ingredient in oral, parenteral, and topical (including inhalational) prescription and nonprescription drug products as they also keep

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several concentration-dependent pharmacological actions, including sedative, carminative, cooling, antipyretic, rubefacient, cleansing, and antiseptic properties.[1] Thus, biotechnological and pharmaceutical applications spark new tools and new value-added compounds from new sources with high commercial importance for the food, nutraceutical, and pharmaceutical industries.[2] While most of the applications need solvent extraction, growing awareness of environmental and health risks and legislative restrictions on the presence of possible toxic solvents[3] have led to the search for safe, food-grade, and environmentally safe solvents and extraction procedures. Methanol for high yield, and also ethanol as a non-toxic, food-grade, and environmentally friendly solvent found a ground in this industry.[4]

The food and alcoholic fermentations represent another well-known industry using yeast ever since the first ages while yeasts have become very important experimental models in both microbiological and genetic research, as well as the main characters in many fermentative production processes.[5] No matter home-made or industrial, an obvious example is wine – an alcoholic drink typically made from fermented grapes: yeast consumes the sugar in the grapes and converts it to ethanol, carbon dioxide and heat. Quantitative analysis of ethanol is also important for the control of fermentation and certification of alcoholic drinks.

Several methods are currently used in the analysis of ethanol content in all kinds of matrixes and also alcoholic beverages. Colorimetric method, gas chromatography flame ionization detector and mass spectrometry detector by using solid phase micro extraction,[6] spectrophotometric detection based on enzymatic reaction,[7-10] flow injection analysis with an amperometry detector using an oxidized nickel wire,[11] optical alcohol meter based on nanostructured silicon,[12] capillary electrophoresis,[13] high performance liquid chromatography (HPLC),[14] NIR spectroscopy method,[15] head space – gas chromatography – mass spectrometry (HS-GC-MS),[16-18] and head-space single-drop microextraction in potassium dichromate in sulfuric acid medium,[19] capillary gas chromatography[20] were used to determine alcohol in wine.

In head space sampling, the sample is placed in a closed vial and heated to a certain temperature for an interval, the more volatile compounds in the vial pass into the gas phase above the sample, while the less volatile or non-GC friendly components that make up the bulk of the sample are kept in the liquid phase, thereby partially reducing the matrix effect.[21]

We developed a head space gas chromatography (HS-GC) method to analyze EtOH in wine. The proposed simple, rapid, reliable, and without any sample pretreatment direct extraction method has been developed for the determination of alcohol content of fermented high matrix wine products using the gas-chromatography with flame ionization detector coupled to head space. Isopropanol (IPA) was preferred as an internal standard, and Triton X-100 (TX-100) was used as diluting solution in this method. The main goal of this study was to evaluate the direct analysis method of the ethanol content of wine. Glycerol, sugars, acids, and tannins and the other compounds did not interfere with this analysis. This method offers the speed and direct analysis of sample.

MATERIALS AND METHOD

Experimental

Reagents and samples: All chemicals were gas chromatographic quality. Absolute ethanol (EtOH), isopropanol (IPA), and Triton X-100 were obtained from Merck, Darmstadt, Germany. High purity helium, hydrogen, and dried air gases (for gas chromatography, ≥99.999%) were purchased from HatGrup, Ltd. Ankara, Turkey. Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Instrumentation and chromatographic conditions: Gas Chromatography-Flame Ionization Detector (GC-FID) (7890A GC System) equipped with Head space Sampler (7697A), 20 mL head space vial and head space Al crimp cap, PTFE/Si septum, and DB-624 (6% cyanopropyl phenyl and 94% dimethylpolysiloxane copolymer) capillary column (30 m x 0.32 mm x 1.8 μm) were Agilent Technologies Inc., Santa Clara, USA. Helium was used as a mobile phase. Operating parameters applied in the method are presented in Table 1.

Table 1

Gas chromatographic conditions

Parameter	GC-FID Condition
Injector temperature	200°C
Detector temperature	230°C
Helium (carrier gas) flow rate	1.0 mL/min, constant flow mode
Hydrogen	30 mL/min
Dried air	400 mL/min
Split ratio	1:200
Oven program	40°C (5.0 min), 5°C/min ramp to 60°C (0.0 min), 30°C/min ramp to 150°C (4.0 min). Post run was 210°C (1 min)
Run time	18.00 min
Sample injection	Head space
Head space sampler parameters	
Oven temperature	80°C
Loop temperature	85°C
Transfer line temperature	90°C
Injection volume	1 mL
GC cycle time	18.00 min
HS vial equilibration time	2.00 min
Injection duration	0.06 min

Sample preparation

Wine contains phenolic compounds (tannin, resveratrol), anthocyanins, mineral elements (copper, zinc, iron etc), alcohol, glycerol, tartaric acid, and shikimic acid.[22-24] Polyphenols are the main antioxidant constituents in grapes and wine.[25] Red wine gains its color and flavor from tannins and anthocyanins present in the grape skin, by allowing the grapes to soak in the extracted juice.[24] The tartaric acid concentration in grapes plays a vital role in wine making. The relative proportion of water and ethanol present in alcoholic beverages can significantly influence the perception of wine sensory attributes.[17,26] This makes wine a perfect choice for developing this method as the fermented end product here, which is wine, should contain all of the mentioned compounds, while the alcohol content should be determined preciously without any matrix effect on the method.

Seven different wines (rose, red, white) produced in İzmir/Turkey were evaluated in this study. All wines were purchased from their producers. Wine bottles were stored in the refrigerator (4 °C) before testing began. The amount of TX-100, extraction temperature, and the total volume of solution in head space vial were optimized. 1.0 mL of the wine, 10 µL of concentrated IPA (as an internal standard), and 2.5% (v/v) TX-100 were added to the head space vial up to total volume of 2.0 mL, then the vial immediately sealed with a head space Al crimp cap, PTFE/Si septum. Ethanol was analyzed with Head Space-Gas Chromatography-Flame Ionization Detector (HS-GC-FID).

Standard solutions preparation

In this method IPA was preferred as an internal standard, and Triton X-100 (TX-100) was used as diluting solution. 50, 100, 150, 200, and 300 µL EtOH, and 10 µL IPA were added, then the volumes were completed to 2.0 mL with 2.5% (v/v) TX-100. The vial immediately sealed with a Head space Al crimp cap, PTFE/Si septum. Ethanol was analyzed with Head Space-Gas Chromatography-Flame Ionization Detector (HS-GC-FID).

Method validation

Performance of the method was assessed by evaluating the recovery, accuracy, precision, linearity, limits of detection (LOD), and limit of quantification (LOQ).

Linearity: The slopes of standard addition and external linear calibration curve were statistically compared, and EtOH was quantified by using an external calibration curve. Linear least squares regression calibration curves were constructed by plotting the ratio of GC peak area (EtOH/IPA) versus the percentage of EtOH (2.5, 5.0, 7.5, 10.0 and 15.0%, v/v) at five concentrations.

Precision: Relative standard deviation (RSD%) was calculated to determine the precision of the

method. The precision was performed with eleven replicates of sample #n01.

Recovery: The percentage recoveries were determined by spiking three known quantities of the standards (EtOH) and 10 µL internal standard (IPA) to the wine sample and analyzed with HS-GC-FID.

LOQ and LOD: The limit of the detection (LOD) and limit of the quantification (LOQ) were evaluated from the lowest measurable concentration which could be determined. The limit of detection (LOD) is defined as the detectable concentration yielding as S/N of 3 and the limit of quantification (LOQ) is defined as the detectable concentration yielding as S/N of 10.

Selectivity: Identification of the EtOH, and IPA were performed based on the comparisons of their retention times (t_R) with pure standard under the same chromatographic conditions of the samples. Methanol (MeOH) standard was spiked on the wine sample, and a methanol peak was observed at a different retention time of the other alcohols. It is known that wine products contain phenolic compounds (tannin, resveratrol), anthocyanins, mineral elements (copper, zinc, iron etc.), alcohol, glycerol, tartaric acid, and shikimic acid while this has not interfered with chromatograms. The slopes of standard addition and external calibration curve were statistically same.

RESULTS

Optimization of head space-gas chromatographic condition

Factors that can affect the concentration of an analyte in the head space phase are extraction solvent (TX-100), extraction temperature, sample volume, and head space volume in the vial.

TritonX-100% (v/v) optimization: The migration of compounds into the head space phase depends on the volatility of the components and their affinity for the original sample phase. If the sample is left in the vial long enough, the relative concentrations of the compound between the liquid and gas phase reach a constant value, *i.e.* equilibrium.[27,28] The presence of each compound in the liquid phase and gas phase depends on partition coeffition (K) of the analyte between the two phases.

$$K = C_{liquid} / C_{gas} \qquad \text{Equation 1}$$

C_{liquid} is the concentration of analyte in the sample or liquid phase,

C_{gas} is the concentration of that analyte in the gas or head space phase

Compounds with high "K" value prefer the liquid phase, while compounds with low "K" value prefer the gas (head space) phase. In head space analysis, K values for analytes should be much lower than for undesirable components in the sample matrix. The "K" value depends on both the compound and the sample matrix and is also strongly influenced by temperature. K value of a compound in the sample is related to the inverse its vapor pressure when pure. Vapor pressure increases with temperature and so K value is decrease and more of the analyte pass into the head space phase. When K is low, there is only minor change in the head space concentration as the temperature is raised. With a very low K value, a small change in the sample volume makes a big difference in head space concentration. In these instances, analytical detection limits are greatly enhanced by an increase in sample volume.[27]

The high "K" value of ethanol in aqueous media means that the volumetric ethanol concentration in the liquid is higher than in the gas phase. It can be thought that this is due to the hydrogen bonding between the hydroxyl groups of alcohol and water.[27]

For this reason, in our study, a solvent medium that does not have a significant hydrogen bond with ethanol was chosen and it was aimed to increase the concentration of EtOH passing into the gas phase.[27] Thus, TX-100 was chosen as the solvent due to its low partition coefficient and high boiling temperature, and an optimization study was carried out between 1% and 10% (v/v) TX-100 concentration range (Fig. 1). TX-100 concentration of 2.5% (v/v) at which the highest peak area obtained was accepted as the optimum value.

Extraction temperature optimization: The more volatile compounds in the vial tend to pass into the head space phase. The more volatile the analytes, the more concentrated they will be in the head space. In contrast, the less volatile (or non-GC friendly) components that make up the bulk of the sample tend to remain in the liquid phase. In our study, extraction temperature optimization was performed at temperatures of 80, 85, 90°C (EtOH boiling point: 78.23°C) (Fig. 2). When we lower the dispersion coefficient (K) by raising the vial temperature, more compounds will pass into the head space. However, in this case, there is a risk

that a more sample matrix will pass into the upper cavity, increasing the pressure inside the vial, which will affect the sampling process and in extreme cases may cause leakage or breakage.

Although the peak areas do not change much in the temperature range of 80 to 90°C, 80°C was chosen as the head space oven temperature in order to prevent matrix entry into the GC column.

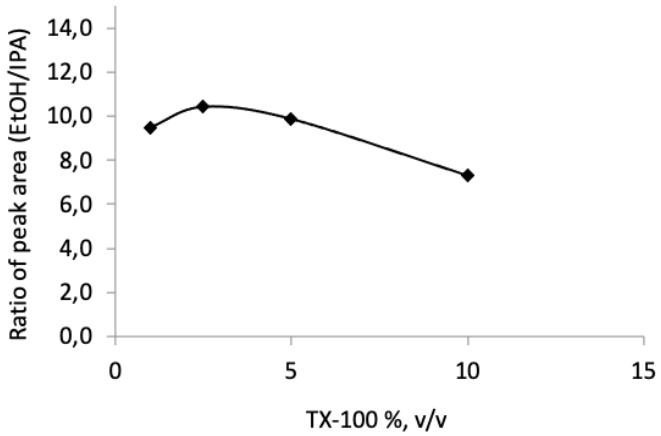

Fig. 1 – The effect of TX-100% (v/v) to the ratio of EtOH/IPA peak areas (1.0 mL sample volume, 80°C extraction temperature, 10 µL IPA, 2.0 mL total volume of solution).

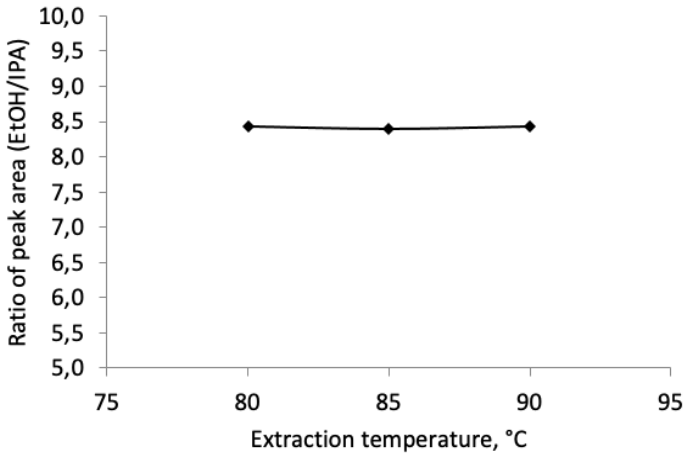

Fig. 2 – The effect of extraction temperature to the ratio of EtOH/IPA peak areas (1.0 mL sample volume, 2.5% (v/v) TX-100, 10 µL IPA, 2.0 mL total volume of solution).

Total volume optimization: Another factor that can affect the concentration of an analyte in the gas phase is the head space volume in the closed vial. In our study, the effect of total solution volume on the peak area was investigated by using 1.0 mL of wine sample, 10 µL of IPA and increasing volumes of TX-100. As the head space volume in the vial decreases with the increase of the solution amount in the lower phase, it is expected that the concentration of all compounds in the head space phase will increase. However, since the concentration of the internal standard we used in our study in the upper cavity increased at the same rate, no change was observed in the peak ratios of EtOH/IPA. When the effect of 2.0, 2.5, 3.5 and 4.0 mL total solution volume on the ratio of GC peak areas (EtOH/IPA) was examined (Fig. 3). Optimum total solution volume was chosen as 2 mL.

Sample volume optimization: As the amount of analyte in the solution increases, the amount of analyte that passes into the head space phase is predictable to increase as well. As expected, linear increase was observed when EtOH/IPA peak ratios were plotted in 0.1 to 1.0 mL sample volume by keeping the internal standard amount (10 µL) and total volume (2.0 mL) constant in all solutions (Fig. 4). Because of the low K value, a small change in the sample volume makes a big difference in head space concentration. It was preferred to use 1.0 mL sample volume in ongoing studies.

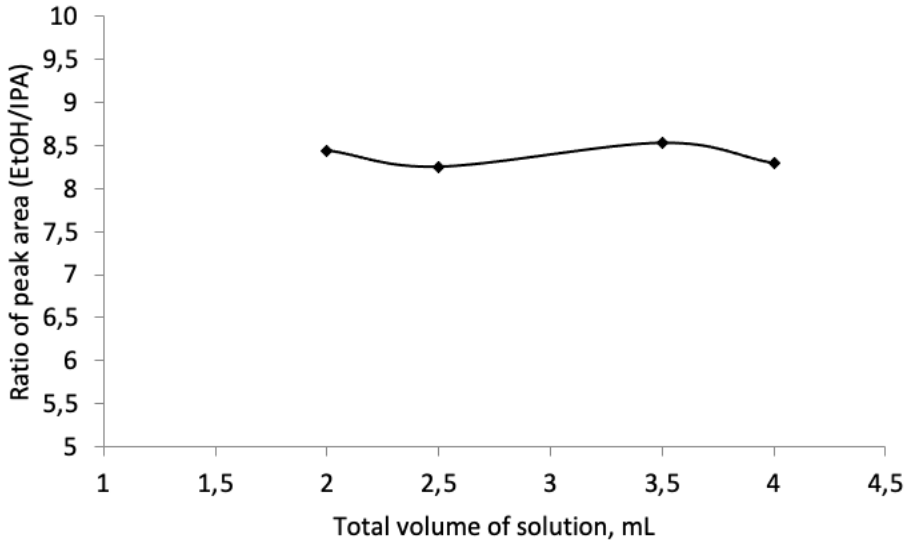

Fig. 3 – The effect of total volume of solution to the ratio of EtOH/IPA peak areas (1.0 mL sample volume, 80 °C extraction temperature, 10 µL IPA, 2.5% (v/v) TX-100).

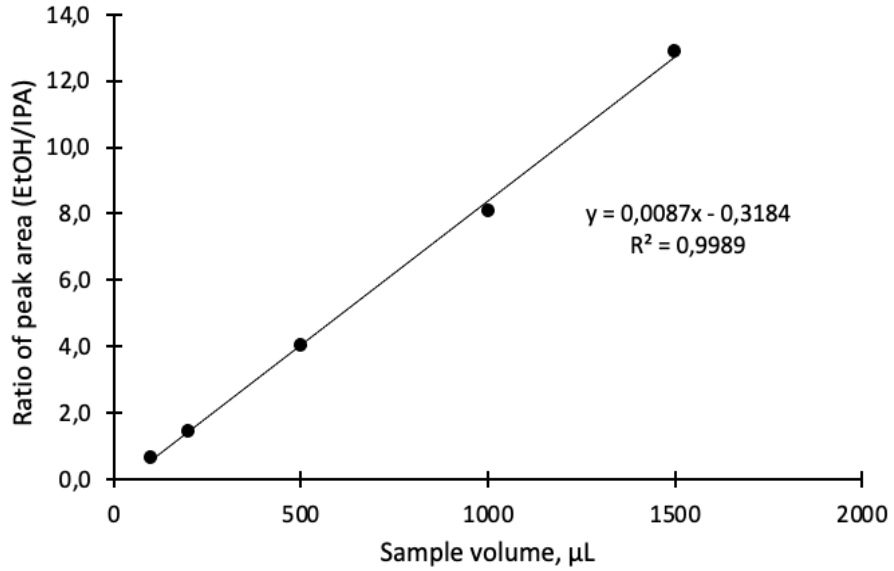

Fig. 4 – The effect of sample volume to the ratio of EtOH/IPA peak areas (80 °C extraction temperature, 10 µL IPA, 2.5% (v/v) TX-100, total volume 2.0 mL).

Qualitative and quantitative analysis

Identification of the EtOH and IPA were performed based on comparison of their retention times (t_R) with a pure standard of EtOH (t_R= 5.639 min) and IPA (t_R= 6.849 min) (Fig. 5a) under the Head space-Gas Chromatography–Flame Ionization Detector (HS–GC–FID) analysis program (Table 1). As shown in Figs. 5b and 5c, EtOH in the sample were detected in the presence of components without any need of purification process; MeOH, IPA and EtOH can effortlessly detected within the samples with no interference.

The quantitative characteristics of EtOH (linearity range, precision, LOD, LOQ, and recovery) are listed in Table 2. Linear least square regression calibration curves were constructed by plotting the ratio of GC peak area (EtOH/IPA) versus the percentage of EtOH. The slopes of standard addition (y= 1.5738x + 8.1676, R^2= 0.9782) and external linear calibration curve (y = 1.572x – 0.702, R^2= 0,9960) were statistically compared, and EtOH in seven different wines were quantified by using an external calibration curve. High determination coefficient (R^2= 0.9960) value showed good linearity.

LOD and LOQ of EtOH were calculated as 0.80% and 2.5%, respectively (Fig. 6 a, b).

The amount of EtOH was found as 12.18% ± 1.05 in seven different wines. The proposed method has a potential for application into the industry which want to check the quality and content of the fermented alcohol without the effect of matrix.

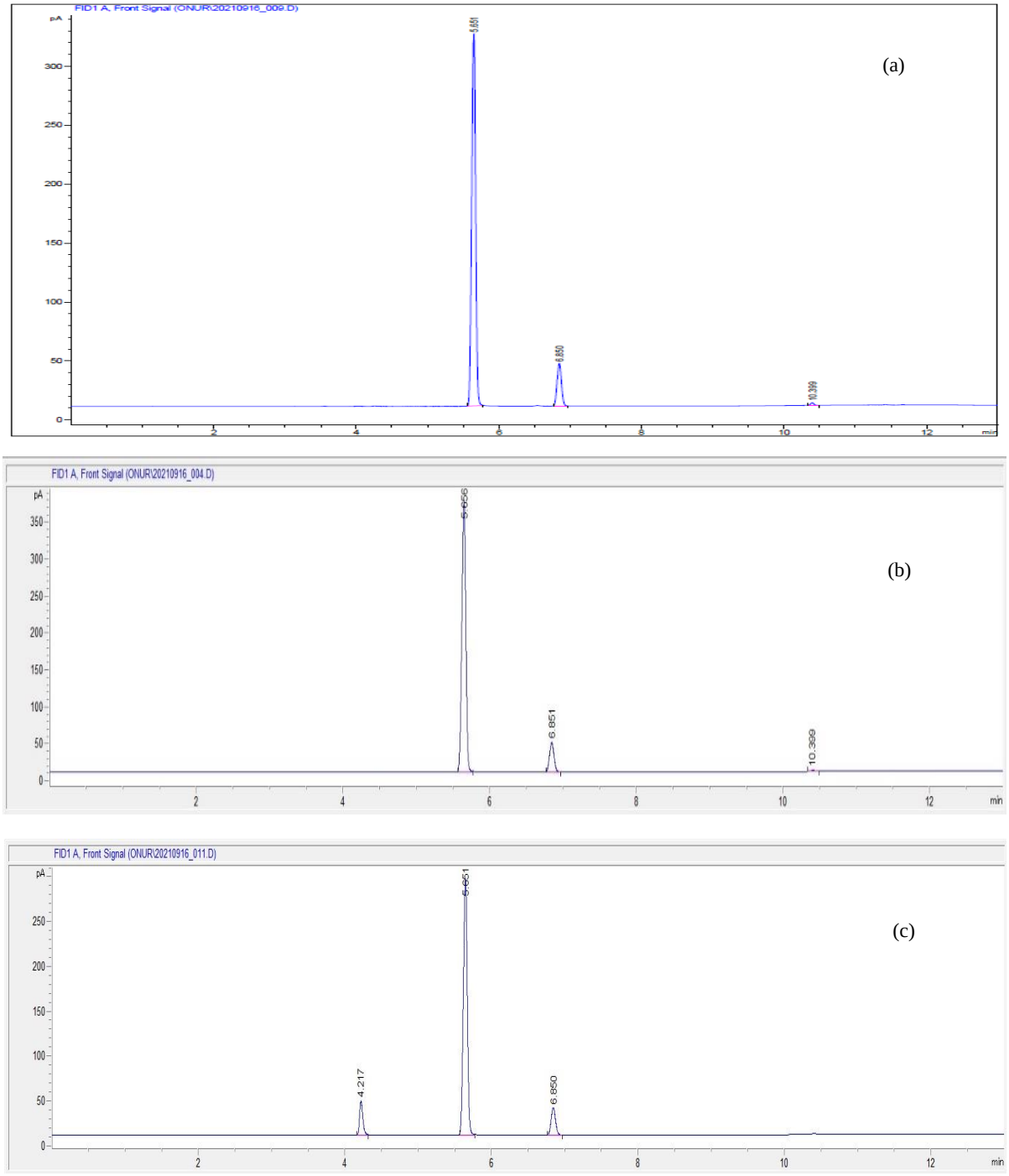

Fig. 5 – Chromatogram of (a) EtOH and IPA (t_REtOH= 5.656 min, t_R IPA= 6.851 min) (b) IPA spiked sample #n01, (c) IPA and MeOH spikes #n01 (t_RMetOH= 4.217 min).

Table 2

Quantitative characteristics of EtOH

Component	EtOH
RSD% (n= 11)	5.8
LOD, %	0.80
LOQ, %	2.5
Range, %	2.5 – 15.0
Linear regression equation*	y = 1.572x – 0.702 (n= 5 point)
Determination coefficient (R^2)	0,9960
Recovery%	97.5 ± 3.5

*y is the ratio of peak area (EtOH/IPA), x is the % EtOH concentration

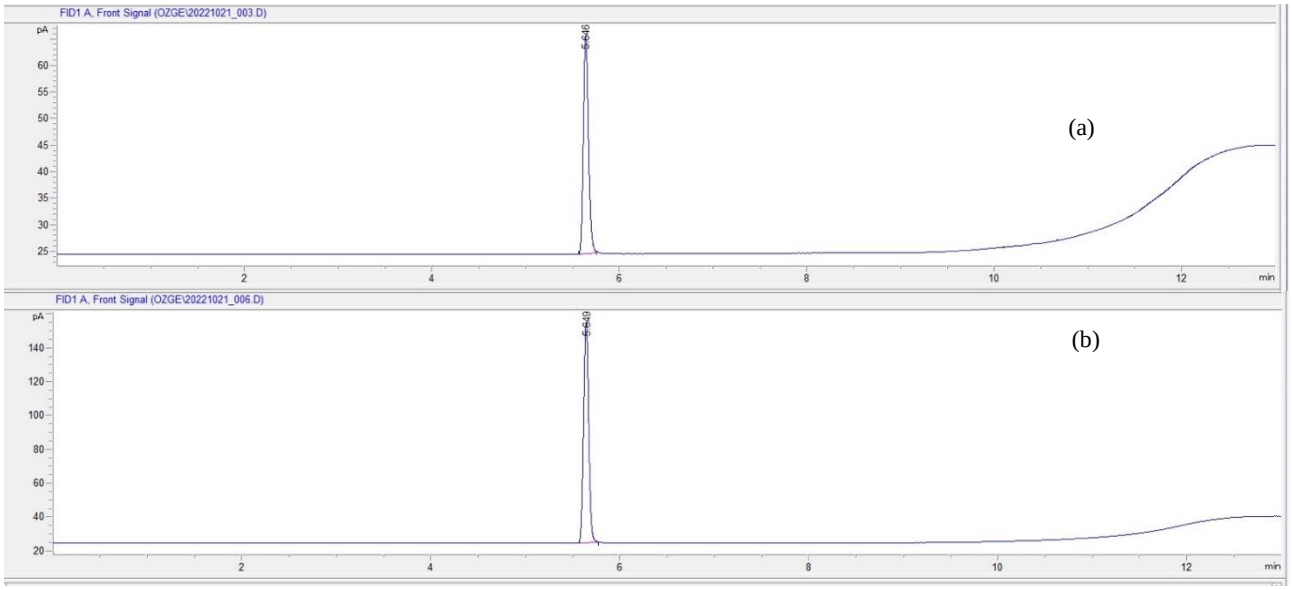

Fig. 6 – Chromatograms of (a) LOD level of 0.8% (v/v) EtOH standard and, (b) LOQ level of 2.5% (v/v) EtOH standard (t_REtOH= 5.656 min).

DISCUSSION

Methanol selectivity

Methanol is highly toxic to human beings following oral, pulmonary and/or skin exposures.[29] Serious systemic toxicity including deaths may arise with intentional, non-intentional or occupational or non-occupational exposures.[30-35] It naturally exists in some beverages while having access to an easy and cheap method for its determination is of great importance to increase the safety of use of these beverages.[36] In acute poisoning, typically 12–24 h after exposure the main toxicity of methanol manifest as its metabolite formic acid has accumulated to toxic levels[37] and unless timely antidotal therapy is given,[38] coma, seizures, death, permanent blindness, and permanent damage to the central nervous system have been reported with substantial exposures.[30] Our method can easily detect methanol in wine samples with a distinctive extra retention time peak while without any extra effort or program change. The illicit drinks made from "industrial methylated spirits" [5% (v/v) methanol: 95% (v/v) ethanol] can cause severe and even fatal illness,[39] the proposed method is also toxicologically important and feasible solution for investigational manners.

CONCLUSION

In chromatographic methods, while we have to spend time separating the compounds we are not interested in, we may also encounter problems

such as the compounds that are not suitable for most gas chromatography, gradually contaminating the system or reacting with the stationary phase in the column. With head space sampling, volatile analytes are easily extracted from the sample matrix and less volatile or column-damaging components that make up the bulk of the sample remain in the liquid phase, giving a clean, easy and faster sample to the column.

The head space gas chromatography (HS-GC-FID) method for the determination of EtOH in wines has been evaluated and fully validated. TX-100 is an appropriate solvent for the extraction of EtOH from wine matrix.

The fact that the slopes of standard addition and external calibration curve were statistically the same means there is no physical interference in extraction method. The method is suitable for working even with a sample amount as low as 0.1 mL. Even the method can be used quite selectively for methanol analysis in ilicit/homemade beverages (moonshine) control for toxicological considerations. The described technique is sensitive and precise and may have found other application grounds with a sufficiently wide range; therefore, it can be applied for quality control of wine industry.

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