VALIDATION OF HEADSPACE SOLID-PHASE MICROEXTRACTION WITH GAS CHROMATOGRAPHY-FLAME IONISATION DETECTOR METHOD FOR ALCOHOL ANALYSIS ON GASTRIC FLUID

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Abstract

Identifying the concentration of alcohol compounds in postmortem analysis of biological fluids can help the investigation, just as postmortem analysis of gastric juices can reveal the concentrations of alcohol consumed. However, an efficient and effective combination of extraction and measurement methods is required when looking at complex postmortem samples. Therefore, a headspace-solid phase microextraction (HS-SPME) method was developed using gas chromatography with a flame ionization detector (GC-FID) to identify postmortem alcohol concentrations. This present study optimizes and validates an effective and efficient method for postmortem alcohol extraction and quantification in gastric fluid. The optimal conditions for HS-SPME extraction using 65μm Polydimethy lsiloxane Divinyl benzene (PDMS/DVB) fiber for analyte isolation were 15 minutes. at 60°C and an exposure time of 1 minute. The validation investigation shows that the suggested approach satisfies the criteria for linearity, precision, accuracy, LOD, and LOQ for postmortem measurement of alcohol in gastric fluid.

***Keywords**: alcohol, gastric fluid, GC-FID, HS-SPME*

Introduction

The World Health Organisation had 3.3 million deaths from metabolic acidosis following consumption of illegal alcoholic beverages with high concentrations of ethanol and methanol (overdose) in 2020 (WHO 2020). The role of alcohol in individual deaths in medicolegal sources depends on proper analysis of the alcohol content in the victim's body. Alcohol testing by forensic toxicologists and/or pathologists in criminal cases resulting from alcohol overdose is essential to determine whether or not alcohol causes death, subject to physiology and subject to antemortem.

Quantitative analysis of alcohol in the blood (Mihretu *et al*., 2020), in urine, saliva, and breath specimens (Matsumura *et al*., 2010), on brain tissue (Hao JC *et al*., 2016); on the glass humor (Szeremeta *et al*., 2018) has been carried out, but this quantitative analysis has the disadvantage that the postmortem alcohol content does not always reflect the concentration at death due to compound instability and redistribution phenomena. In addition, the phenomenon of post-mortem decay makes it difficult to obtain specimens of these biological fluids. (Hao JC *et al*., 2016). Gastric fluid can be an alternative to alcohol analysis if other fluids, such as blood and urine, cannot be found or have been damaged by putrefaction. Research conducted by Wibowo (2018) states that gastric secretions in albino rats (*Rattus norvegicus*) are still positive for alcohol up to day 5. Alcohol analysis in biological

samples is quickly extracted from a complex mixture, so several procedures must be performed before analysis. to consider at the preconcentration stage.

Preconcentration techniques that have been developed for the analysis of volatile compounds or alcohols include Liquid-Liquid Extraction (LLE) by Ivanova *et al*., (2012), which has the disadvantage of relatively low reproducibility and possible harm with solvents; besides that, the Liquid-Liquid Microextraction (LME) technique by Hamidi and Ghorbani (2017) and Solid Phase Extraction by Abukhalaf *et al*., (2001) although fast and cheap, require a concentration step (evaporative dissolution) so that loss of volatile analytes can occur. When compared with the three preconcentration techniques, due to its high specificity for samples with complex matrices, integration of solvent-free sampling, and one-step preconcentration of analytes without sample preparation, the Solid Phase Microextraction (SPME) technique exhibits some ideal properties in the preconcentration technique for the analysis of volatile compounds or alcohols (Gherghel *et al*., 2018).

The development of the HS-SPME method includes several important considerations in selecting sampling methods, fiber types, holders, extraction conditions (equilibrium), and appropriate calibration procedures (Schmidt *et a*l., 2015). Solid Phase Microextraction (SPME) is sensitive to experimental conditions that affect the coefficient distribution and rate of absorption; it will also affect the amount of analyte adsorbed on the SPME fiber, so it is necessary to develop the SPME method for alcohol analysis, which then separates alcohol compounds in the form of ethanol and methanol using a Gas Chromatography Flame Ionization Detector (GC-FID) (Hao et al., 2016).

The GC-FID instrument is a chromatography commonly used to separate and analyze volatile compounds. The FID detector is very sensitive to hydrocarbons and sample components with alkyl groups, making it more efficient in alcohol analysis. Good separation between alcohol compounds and their derivatives will be obtained if gas chromatography is optimized, where these conditions include carrier gas, oven temperature, split ratio, detector temperature, and gas flow rate (Maleki et al., 2005). After obtaining optimal HS-SPME conditions with GC-FID, the analytical method can only be applied if validation has been carried out.

To provide results that are close to the truth, the validation analysis approach attempts to reduce the likelihood that the data obtained will depart from the actual situation. (Maleki et al., 2005). The United States Pharmacopoeia (USP 40) (2017) presents validation parameters for biological fluid specimens that are linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy (USP, 2017).

The main goal of this study was to explore the potential of the SPME technique for quantifying alcohol postmortem in gastric fluid matrices. This research began with several stages, optimization of GC-FID conditions, optimization of HS-SPME extraction and validation methods, including standard curve linearity, accuracy, precision, detection, and quantification limits. The type of research used was true experimental laboratory research, whose purpose was to obtain the optimal conditions of the GC-FID instrument and HS-SPME extraction and the reliability of the HS-SPME and GC-FID methods for alcohol analysis.

Research Methods

Reagents and standards

All reagents (ethanol, methanol, iso-propanol as an internal standard, dichloromethane, and ammonium sulfate $(NH_4)_2SO_4$) from Merck, Darmstadt, Germany. Stock solutions (2%) for

ethanol and methanol were prepared in dichloromethane. Working analytical standard solutions ranging from 0.2 to 1% were created by diluting the stock solution of ethanol and methanol with dichloromethane. All standard solutions and internal standard (2% isopropanol in dichloromethane) were made every day before the study; negative control gastric fluid samples for spiking with ethanol and methanol were obtained from Albino Rats (*Rattus norvegicus*).

Sample preparation

Adult male albino rats (*Rattus norvegicus*) were purchased from the Faculty of Veterinary Medicine, Airlangga University. The body weight used was 200-250 g and the age used was 12 weeks. The animals were adapted to relatively similar environmental conditions for one week. During the adaptation process, the animals were given a standard feed of 10% body weight and were allowed to drink water ad libitum. The animals were fasted for 12 hours, then decapitated and autopsied for gastric fluid. The gastric fluid samples of albino rats (*Rattus norvegicus*) were stored at 5°C.

SPME equipment and instruments

The SPME fiber and a holder for manual sampling are from Supelco 595 Nort Harrison Road, Bellefonte, PA 16823-0048, USA. Fiber with 65µm Polydimethy lsiloxane Divinyl benzene (PDMS/DVB) 57310-U for manual holder blue was used. Before use, this fiber was heated in a gas chromatographic injection port for five minutes at 230°C. During extraction, samples were mixed utilizing a hot plate or stirrer with 1cm stir bars and a 20mm vial closure with a white PTFE or blue silicone (3mm) thick aluminum cap with a septum. This is typical for 10 mL chromatography vials.

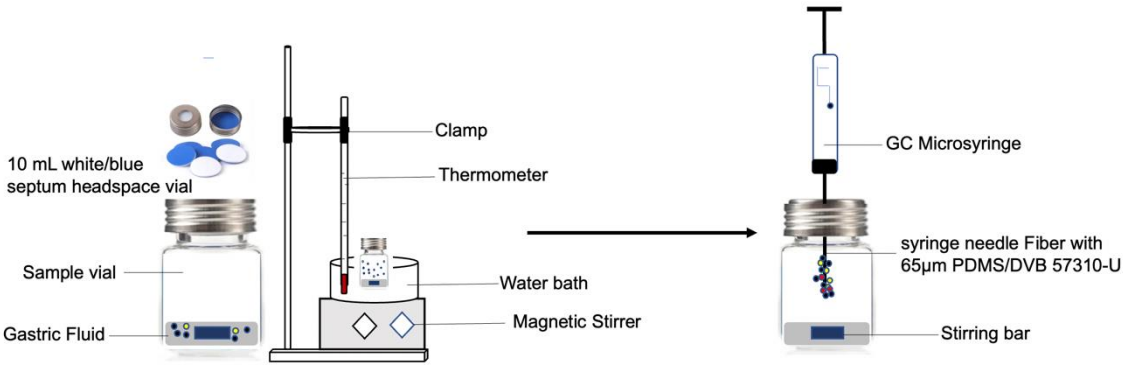

Figure 1. Schematic diagram of HS-SPME Sampling

GC-FID conditions

The Gas Chromatography (GC) instrument used was an Agilent 6890 series with HP-5 column capillary (5 phenyl 95% methyl siloxane (J&W)) (30.0 m x 320 µm x 0.25 µm), with a detector used being a Flame Ionisation Detector (FID).

The optimal condition was an inlet temperature of 230°C, and as the carrier gas, helium with a discharge rate of 1.0 mL/min was utilized. The inlet temperature used was 230°C; The programmed oven temperature was 40°C, which was maintained for 0.30 minutes. The temperature was then increased at 10°C/minute to 60°C and maintained for 1 minute. The temperature was then increased at 30°C/min to 120°C and held for 1 minute. The split ratio of the optimal GC-FID condition in this research was 1:50. All data were obtained by collecting the full-scan analyte chromatogram (ethanol, methanol, isopropanol, and dichloromethane), which was extracted

for the quantitative determination of ethanol and methanol, respectively.

Headspace-SPME sampling

Solid Phase Microextraction (SPME) extraction was performed with Polydimethylsiloxane Divinylbenzene (PDMS/DVB) fiber. The fiber was conditioned at 230°C for 5 min prior to use. Blank desorption was periodically carried out before the study. Periodically, the fiber samples were tested to ensure the absence of contaminants and carryover effects.

The SPME procedure was as follows: 1 g of gastric fluid sample, 0.5 g of $(NH_4)_2SO_4$) 500µl internal standard solution (2% isopropanol in dichloromethane), and a stir bar were placed in a 10 ml headspace-vial and quickly sealed with a septa white PTFE/Blue silicone (3 mm) thick aluminum cap. The vial was heated on the hotplate while stirring at 1000 rpm. Before HS-SPME analysis, the sample vial was agitated and conditioned for 15 minutes at 60°C in a thermostatic water bath. The extraction fiber was then exposed for one minute to the environment of the vial. After removing the syringe from the vial, it was inserted into the injection port of the GC-FID system. The fiber was exposed in the injection port (230°C) for one minute, after which the analytes were desorbed into the GC column.

Headspace-SPME optimization

To optimize the extraction with 65m Polydimethylsiloxane-Divinylbenzene (PDMS/DVB) fiber, a fractional factorial design with 3x3x3 experiments was carried out, resulting in 27 HS-SPME conditions. Variables that have a greater influence on the extraction, such as extraction time (5, 10, and 15 min), heating temperature (50, 60, and 70°C), and adsorption time fiber (1, 3, and 5 min), were examined using ANOVA.

To determine the optimal extraction conditions, a 1 g sample of drug-free gastric fluid was spiked with a 500 µm solution of (0.6% ethanol: 0.6% methanol, 2% I.S) and 0.5 g $(NH_4)_2SO_4$) before being placed in a 10 ml headspace vial and rapidly sealed with a septa aluminum cap. The vial was heated on the hotplate while being stirred at 1000 revolutions per minute. Using PDMS/DVB fiber with a diameter of 65 µl, additional work was conducted on optimizing the heating temperature, extraction time, and adsorption times (the fiber was subjected to the headspace of the vial) with three replicates (n = 3). The fibre was exposed in the injection port (230°C) for one minute, after which the analytes were desorbed into the GC column. Peak areas of ethanol-isopropanol and methanol-isopropanol were used to determine the ideal conditions.

Validation procedure

All statistical data (Linearity, LOD, LOQ, precision (RSD), recovery, and selectivity) were evaluated according to the USP 40 NF-35 procedure (USP, 2017). Linearity was verified by analyzing spiked gastric fluid samples mixture in the concentration ratio of ethanol to methanol (0.2:1.0; 0.4:0.8; 0.6:0.6; 0.8:0.4; 1.0:0.2 %) in the SPME. Determining linearity uses at least five concentration variations ranging from 50-120% of the estimated analyte content (USP 40 NF-35, 2017).

Process with three replicates using isopropanol as an internal standard at a concentration of 2% in dichloromethane. The linear regression equation and correlation coefficient (r^2) were calculated using the least squares method. Using previously reported methodologies, the limit of detection (LOD) and limit of quantitation (LOQ) were determined. Specifically, the LOD was determined to be the concentration at which the signal-to-noise ratio exceeded 3, and the LOQ was determined to be the concentration at

which the signal-to-noise ratio exceeded 10. Within-day (n = 6) precision was evaluated at the same concentrations. Precision was expressed as a relative standard deviation (RSD%). Recovery was determined by analyzing three replicates of analytical standard samples at a concentration ratio of ethanol to methanol (0.4:0.8; 0.6:0.6; 1.0:0.2 %) and isopropanol at 2% I.S.

Results and Discussion

Headspace-SPME optimization

To ascertain the optimal extraction conditions, a 3x3x3 fractional factorial experiment with 27 HS-SPME conditions with fiber 65 µm PDMS/DVB and salt ($(NH_4)_2SO_4$) for the salting-out effect was conducted. This effect reduces the solubility of the solute molecules, resulting in separation or precipitate; however, the matrix effect can be mitigated by the addition of salt. Three replicates (n = 3) were used to evaluate the effects of varying extraction parameters, including extraction time (5, 10, and 15 min), heating temperature (50, 60, and 70°C), and adsorption time fiber (1, 3, and 5 min).

Sampling for optimization of SPME extraction conditions with GC-FID was carried out in a headspace (HS) manner because it is suitable for the extraction of volatile components in the sample used, namely gastric juice, which has a complex matrix, thus avoiding analytes from non-volatile compounds in the sample. The SPME fiber used is 65 µm PDMS-DVB in extracting ethanol and methanol analytes. The type of fiber coating polymer affects the absorption of components based on the level of polarity (Supelco, 2018), and the polarity of the fiber affects the selectivity of the fiber based on the principle of polarity similarity. Polar components are easier to extract using polar-type fibers. However, not all non-polar substances are easier to extract using non-polar fiber types (Wasylka *et al.*, 2017). The 65 µm PDMS-DVB fiber coating belongs to the bipolar type, which is very suitable for the analysis of polar or near-polar volatile components such as ethanol and methanol analytes so that it will absorb the analytes maximally in gastric fluid samples.

In this study, ammonium sulfate salt [$(NH_4)_2SO_4$] was employed to achieve a desalinization effect. It is well known that introducing salts decreases the water solubility of organic compounds. This effect reduces the solubility of solute molecules, resulting in their separation or precipitate; however, the matrix effect can be diminished by the addition of salt. According to Maleki (2006), ammonium sulfate salt [(NH4)2SO4] is the most effective method for analyzing ethanol and methanol in biological fluids.

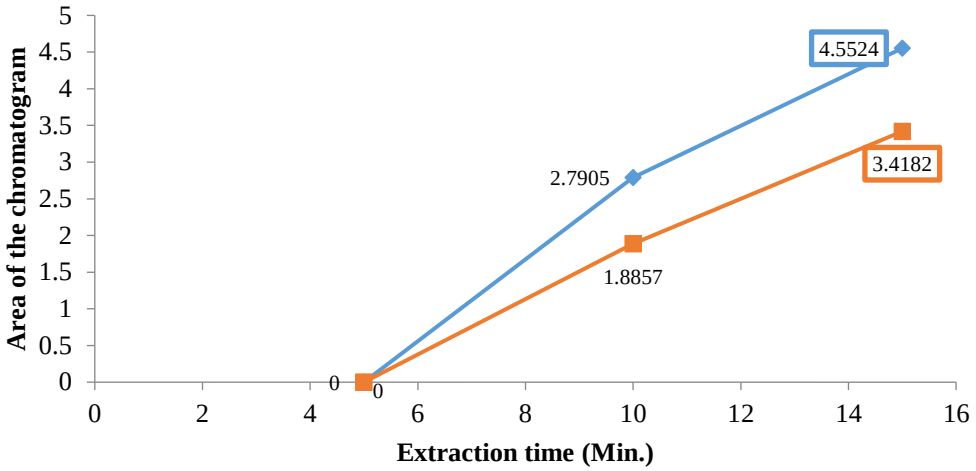

Figure. 1. Effect of extraction time on desorption for ethanol:methanol:isoptopanol (0.6 : 0.6 : 2.0%) under the following conditions: PDMS/DVB fiber, 60°C extraction temperature, $(NH_4)_2SO_4$) and speed of 1000 rpm.

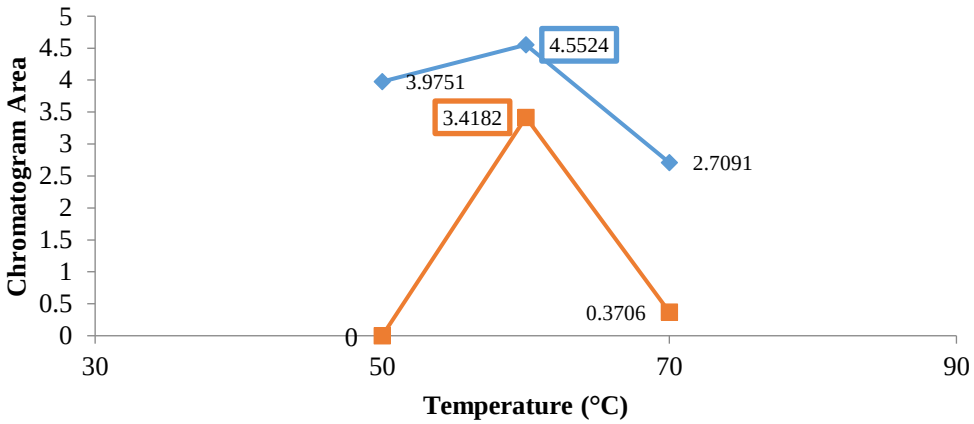

Figure 2. Effect of temperature on desorption for ethanol:methanol:Isoptopanol (0.6 : 0.6 : 2.0%) under the following conditions: PDMS/DVB fiber,15 min extraction time, $(NH_4)_2SO_4$) and speed of 1000 rpm.

Figures 1 and 2 depict the effect of heating temperature and extraction time on the peak areas of ethanol-isopropanol and methanol-isopropanol; they disclose that the extracted quantities increased with time up to 15 minutes when the temperature reached 60°C. According to our knowledge, the heating temperature influenced the extraction the most. Temperature can act in two distinct ways: by increasing the analyte diffusion and, consequently, the extraction yield or by decreasing the analyte partition coefficient between the fiber and the matrix, thereby reducing the extraction yield (Fernandes *et al*., 2007). In these experiments, diffusion effects predominated, so an increase in temperature facilitated extraction. However, minimal volatility may slow mass transfer from the matrix to the headspace, requiring a higher temperature for detectable analyte concentrations. Due to the high boiling point and molecular weight of ethanol and methanol, the extraction time was lengthened (Sha *et al.*, 2005).

Furthermore, the duration of the extraction had a greater positive effect on the extraction. The extracted quantity

increased as time passed. Once SPME is based on the partition between two phases, sufficient time is required to attain equilibrium, according to the literature (Fernandes *et al*., 2007). Positive interaction between time and heating temperature parameters was observed. It indicates that extraction increases as temperature and time increase.

Using PDMS/DVB fiber, the effect of sampling temperature on ethanol and methanol extraction yield was studied between 50 and 70°C. Temperature variation is restricted to 70°C to prevent matrix or other sample components besides ethanol and methanol from evaporating due to excessive heating by the SPME fiber. A maximum was observed at 60°C (Figure 1). At temperatures between 50°C and 60°C, increasing concentrations of ethanol and methanol were observed in the atmosphere. At temperatures above 60 degrees Celsius, the equilibrium between fiber and plenum becomes increasingly shifted towards the headspace. Previous research observed the same trend for HS-SPME-GC-MS (Jessica L. *et al*., 2013). However, after the extraction temperature exceeded 60°C, the peak areas of the ethanol and methanol compounds decreased, which could be explained by the competitive effect during adsorption onto the fibers. Due to the fact that our targets are alcohol compounds with a higher affinity for fiber, some of our targets can be desorbed from fiber due to competition (Xiao *et al*., 2020). Therefore, an extraction temperature of 60°C was utilized as the incubation temperature throughout the development of the HS-SPME method, as the optimum ethanol and methanol responses were observed at this temperature. The time required to attain equilibrium was 15 minutes; therefore, this value was deemed optimal.

Method validation

1) Specificity

The method's selectivity was demonstrated by the effective separation of the compounds in the absence of interfering peaks on the chromatogram. No peak eluted in the same retention time as ethanol, methanol, isopropanol as an internal standard, and dichloromethane solvent. Fig. 3 shows chromatograms of analytes (ethanol and methanol) detected after desorption in gastric fluid samples of albino rats (Rattus norvegicus). In the study, the resulting peak chromatograms were methanol, ethanol, isopropanol, and dichloromethane, respectively. This is based on the polarity of the column and the boiling point of each analyte. The degree of separation (Rs) of methanol-ethanol is 2.42; methanol-isopropanol is 5.05; methanol-dichloromethane is 6.79; ethanol-isopropanol is 2.49; ethanol-dichloromethane is 4.45; and isopropanol-dichloromethane is 3.97. The degree of separation (Rs) of all analytes meets the requirements for validity resolution values, where specifications are categorized as good if separation occurs on the chromatogram with a value of $Rs \geq 2$ (Astuti *et al.*, 2018) so that each analyte peak internal standard, and dissolution are well separated.

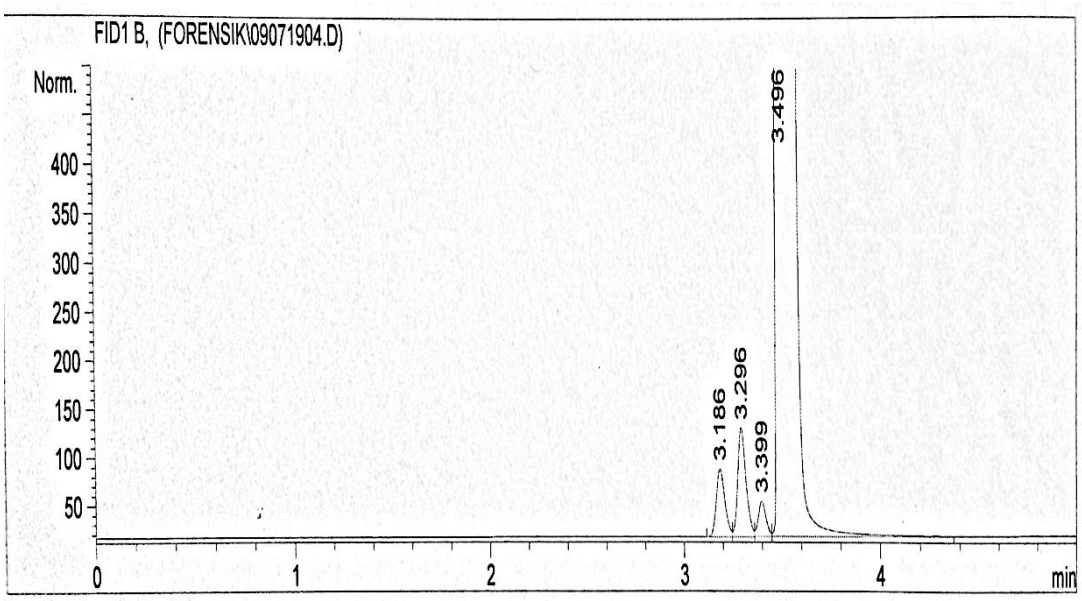

Figure. 3. Typical chromatogram of methanol (3.186), ethanol (3.296), Isopropanol (3.399), and dichloromethane (3.496) in a gastric fluid albino rat (Rattus norvegicus), with resolution value (Rs) between analytes > 2.

2) Linearity
The range of ethanol and methanol was evaluated from 0.2% to 1.0% (n = 3). Peak area data for ethanol-isopropanol and methanol-isopropanol obtained were normally distributed, with correlation coefficients higher than 0.95 ($R^2 \geq 0.95$), 0.987, and 0.998 respectively (Table 1). Correlation coefficients indicate a linear relationship between the concentration and the observed response. The obtained linear regression equations for ethanol and methanol were y = 4.896x + 0.986 and y = 5.111x - 0.044, respectively. The shape of the curve obtained follows the Lambert-Beer law, that is, with increasing concentration, the area produced is higher.

Table 1. Quantitative characteristics of the proposed method (HS-SPME-GC-FID)

Compound	Regression curve equation	R^2	LOD	LOQ	RSD %
Ethanol	y = 4.896x + 0.986	0.987	0.13%	0.42%	6.0
Methanol	y = 5.111x – 0.044	0.998	0.04%	0.15%	7.7

3) Limit of detection and quantification
The limit of detection (LOD) is the lowest analyte concentration that can be detected under the intended analytical conditions. The LOD was 0.13% for ethanol and 0.04% for methanol (Table 1). The limit of quantification (LOQ) is the smallest concentration that can be determined with sufficient accuracy and precision under the analysis conditions. The LOQ was 0.42% for ethanol and 0.15% for methanol. The high correlation coefficients and low detection limits are advantages of the proposed method.

4) Precision
The RSD obtained in the evaluation within a day was between 1% and 12% (Supelco, 2018), and reproducibility experiments conducted on three distinct fibers indicate that the fiber-to-fiber RSD% for all compounds is less than 15% (Maleki *et al.*, 2005) in the same concentration of 0.6%:0.6% (n = 6). The results (Table 1) demonstrate

that the developed method has adequate precision. These results indicate that the % RSD for ethanol and methanol is less than ≤ 7.7%, indicating that the proposed method is repeatable (Maleki, 2006). In addition, Supelco (1998) states that % RSD for the SPME technique is in the range of <1–12%. So that the % RSD ethanol and methanol data obtained met the requirements for repeatability. A high RSD value indicates the level of accuracy of the analyst; the smaller the % RSD produced, the higher the level of accuracy.

5) Recovery

The Extraction recovery was calculated by comparing the peak areas obtained from gastric fluid samples after analysis with HS-SPME and GC-FID with aqueous solutions of the same concentration. The recovery of ethanol and methanol extraction in gastric fluid from albino rats (Rattus norvegicus) was almost 100% (Table 2). In biological samples, recovery should be ±10% (Yuwono et al., 2015). The recovery percentage smaller than the accepted value indicates the inaccuracy of determining 2% ethanol concentration using the HS-SPME method. From the accuracy measurement results obtained, the % recovery of ethanol was 102.20%, while the % recovery of methanol was 101.45%. The % recovery requirement for acceptable accuracy corresponds to the concentration level, namely the analyte concentration (≥ 0.1%), which is in the range of 95–105%. This is based on the AOAC % recovery acceptance requirement (AOAC, 2019).

Table 2. Accuracy/recovery for the analysis of alcohol in gastric fluid albino rats

Concentration (%)	Recovery (%)	
n = 3	Ethanol	Methanol
0.2	85.28	92.76
0.4	99.95	102.43
0.6	109.74	102.54
0.8	100.40	109.06
1.0	96.90	99.43

The devised method was applied to determine ethanol and methanol in gastric fluid. To evaluate the efficacy of the proposed method on actual samples, albino rats were treated orally until they were overdosed. The experiments effectively applied a method to assay ethanol and methanol in the gastric fluid of albino rats (*Rattus norvegicus*); however, no analyte or matrix peak eluted at the same retention time. Due to the complex matrix components of gastric fluid, its analysis is directly problematic; therefore, headspace-SPME is optimal for sampling analytes from biological specimens (gastric fluid).

Conclusions

In this study, a combination of HS-SPME and GC-FID methods was used to quantitatively determine postmortem alcohol in gastric fluid. Optimization was carried out at the extraction stage, obtaining HS-SPME extraction conditions at a temperature of 60°C, 15 minutes of extraction time, and 1 minute of exposure time. The optimal result for GC-FID conditions is an inlet temperature of 230°C; the oven temperature program is 40°C, held for 0.30 minutes, then the temperature is increased to 10°C/min. until it reaches 60°C and is held for 1 minute, then increased at 30°C/min. until it reaches 120°C and is held for 1 minute. The optimal ratio for GC-FID conditions

is 1:50. The validation study showed that the proposed method meets the requirements of linearity, precision, accuracy, LOD, and LOQ for gastric fluid postmortem alcohol measurement.

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Conflict of Interest

All authors declare no conflicts of interest and concur with the manuscript's contents.

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