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The Formation of Ethanol in Postmortem Tissues

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16. Abstract <p>During the investigation of aviation accidents, postmortem samples obtained from fatal accident victims are submitted to the FAA's Civil Aerospace Medical Institute for toxicological analysis. During toxicological evaluations, ethanol analysis is performed on all cases. Many species of bacteria, yeast and fungi have the ability to produce ethanol and other volatile organic compounds in postmortem specimens. The potential for postmortem ethanol formation complicates the interpretation of ethanol-positive results from accident victims. Therefore, the prevention of ethanol formation at all steps following specimen collection is a priority. Sodium fluoride is the most commonly used preservative for postmortem specimens. Several studies have been published detailing the effectiveness of sodium fluoride for the prevention of ethanol formation in blood and urine specimens; however, our laboratory receives blood or urine in approximately 70% of cases. Thus, we frequently rely on tissue specimens for ethanol analysis. The postmortem tissue specimens received by our laboratory have generally been subjected to severe trauma and may have been exposed to numerous microbial species capable of ethanol production. With this in mind, we designed an experiment utilizing unadulterated tissue specimens obtained from aviation accident victims to determine the effectiveness of sodium fluoride at various storage temperatures for the prevention of microbial ethanol formation. We found that without preservative, specimens stored at 4°C for 96 h showed an average increase in ethanol concentration of 1470%. At 25°C, these same specimens showed an average ethanol increase of 1432% after 48 h. With the addition of 1.00% sodium fluoride, there was no significant increase in ethanol concentration at either temperature.</p>					
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THE FORMATION OF ETHANOL IN POSTMORTEM TISSUES

INTRODUCTION

The Federal Aviation Administration's (FAA) Civil Aerospace Medical Institute (CAMI) is responsible under Department of Transportation (DOT) orders 8020.11A and 1100.2C, to "conduct toxicologic analysis on specimens from ... aircraft accident fatalities" and "investigate ... general aviation and air carrier accidents and search for biomedical and clinical causes of the accidents, including evidence of ... chemical (use)." Therefore, following an aviation accident, samples are collected at autopsy and sent to CAMI's Bioaeronautical Sciences Research Laboratory, Forensic Toxicology Research Team where toxicological analysis is conducted on various postmortem fluids and tissues.

Ethanol analysis is one of the most common tests performed on forensic specimens. The presence of ethanol in aircraft accident victims constitutes an important part of both aircraft accident investigations and litigation. Using modern analytical techniques, there is little doubt as to the quantitative amount of ethanol present in a postmortem specimen. The origin of the ethanol detected, however, is an important variable that must be considered when interpreting ethanol results in postmortem specimens.

The microbial formation of ethanol in postmortem specimens is the most significant problem encountered when evaluating ethanol results. The first report dealing with postmortem ethanol production in corpses appeared in 1936 (1). At that time, a postmortem increase in ethanol concentration was occasionally observed, but the source of the increase was unknown. The first studies to elucidate the mechanism of postmortem ethanol formation occurred in the early 1970s (2, 3). From these initial studies it became clear that microbes are responsible for postmortem formation of ethanol. Many species of bacteria, yeast and fungi produce ethanol and other volatile organic compounds as a byproduct of their metabolism (4-12). *Candida albicans* has been identified as the microbe most often responsible for postmortem production of ethanol in humans (9, 13). This species of yeast is commonly found in humans *in vivo*. However, microbes not normally found *in vivo*, such as the *Mucor* species of mold, are commonly found in decaying organic matter and soil (14); they may be introduced from the environment if the circumstances surrounding death are violent, as is often seen in aviation accidents. After death, endogenous and/or exogenous microbes begin rapidly consuming glucose and other nutrients present in the

body and produce ethanol and/or other organic volatiles as metabolic byproducts (12, 15-17). Under optimal conditions, substantial concentrations of ethanol may be formed within hours of death (18). The relatively short time required for microbes to begin producing ethanol complicates the interpretation of a positive ethanol result in postmortem specimens. The potential for postmortem ethanol formation is disconcerting when considering that a positive ethanol result may help discern the cause of an aviation accident. Therefore, the preservation of biological specimens to minimize postmortem ethanol formation has been a priority for nearly 30 years.

The most commonly utilized substance for the preservation of postmortem specimens is sodium fluoride. The fluoride ion prevents the formation of polysaccharides by the microbe and, in turn, prevents microbial growth (19). Storage temperature and duration play an important role in the microbial formation of ethanol. Numerous studies have examined the amount of time required for the microbial production of ethanol and the optimum storage temperature for the prevention of postmortem ethanol in blood and urine specimens (18-20). Additionally, these studies have demonstrated the effectiveness of sodium fluoride for the prevention of microbial ethanol formation. There are, however, at least two published reports concluding that sodium fluoride may be ineffective for the prevention of ethanol formation in blood samples containing sufficiently high concentrations of *C. albicans* (2, 9).

The vast majority of research publications involving microbial ethanol formation investigate blood and/or urine spiked with glucose and *C. albicans* or other microbes. Blood is submitted to our laboratory in approximately 70% of all cases, urine even less frequently. Thus, various tissues are often examined to evaluate ethanol concentration in aviation accident victims. Furthermore, the tissue specimens received by our laboratory typically have been subjected to trauma as a result of the violent nature of aviation accidents. As a result, the tissues may be contaminated with endogenous and/or exogenous microbes capable of producing significant amounts of ethanol. In a previous study using rDNA amplification, which examined 45 blood and tissue specimens obtained from fatal aviation accident victims, 95% were contaminated with enteric bacteria capable of ethanol production (21). Therefore, we examined the formation of ethanol in unadulterated

tissue specimens obtained from fatal aviation accident victims, which were prepared both with and without sodium fluoride as a preservative and were stored for up to 96 h at either 4°C or 25°C.

MATERIALS AND METHODS

Chemicals and Solutions

All aqueous solutions were prepared using double deionized water (DDW), which was obtained using a Milli-QT^{plus} Ultra-Pure Reagent Water System (Millipore®, Continental Water Systems, El Paso, TX). Sodium fluoride, t-butanol, acetaldehyde, methanol, 2-propanol, acetone, n-propanol, isobutanol, n-butanol, sec-butanol and ethanol were purchased from Sigma Chemical Company (St. Louis, MO) in high purity and used without further purification. Purchased ethanol controls were obtained as 25, 50, 100, 150 and 300 mg/dL ethanol standards from Restek Corporation (Restek Corp., Bellefonte, PA).

A volatiles calibration solution was prepared in a matrix of certified-negative human blood at a final concentration of 157 mg/dL ethanol, 33 mg/dL acetaldehyde, 158 mg/dL methanol, 157 mg/dL 2-propanol, 31 mg/dL acetone, 241 mg/dL n-propanol, 32 mg/dL isobutanol, 80 mg/dL n-butanol and 80 mg/dL sec-butanol. Two mL portions of the calibrator solution were transferred to 16 x 100 mm screw-cap culture tubes and stored at -20°C for up to 6 months.

The internal standard solution was prepared as 39 mg/dL t-butanol and 0.40% sodium fluoride. Sodium fluoride was added to the internal standard solution to increase the ionic strength of this solution. The increased ionic strength of the aqueous phase causes a larger portion of the volatile organic compounds to move into the headspace of the vial. This technique is commonly used in volatile analysis and is known as "salting out" (22). The internal standard solution was stored at 4°C for up to 6 months. The sodium fluoride solution used in the homogenization procedure below was prepared weekly at a final concentration of 1.00% (w/v) and stored in the refrigerator at 4°C.

Instrumentation

Analyte separation was achieved using a Varian CP-3800 gas chromatograph (GC) (Varian, INC., Palo Alto, CA) equipped with dual fused silica capillary columns obtained from Restek Corporation (Restek Corp., Bellefonte, PA). The columns were a Rtx-BAC1 (0.53 mm i.d. x 3.0 µm x 30 m) and a Rtx-BAC2 (0.53 mm i.d. x 2.0 µm x 30 m). The GC was fitted with a Varian

CombiPAL® (Varian, INC., Palo Alto, CA) headspace GC injection system that allowed for automated sample pretreatment and injection. The CombiPAL® was utilized in conjunction with a 1 mL SGE gas tight syringe (400 psi max pressure) obtained from SGE (SGE Chromatography Products, Austin, TX). The sample volume was set to 500 µL. The specimens were incubated at 50°C for 13.30 min prior to injection, and the syringe was heated to 50°C. The syringe fill speed was set to 100 µL/sec, and the injection speed was 150 µL/sec. Following sample injection, the syringe was flushed for 30 sec with carrier gas. Identification and quantitation of the analytes of interest was accomplished using a flame ionization detector (FID). Control of the GC system, integration of chromatographic peaks and communication with the GC system were achieved using a Dell™ Optiplex GX1 (Dell™, Round Rock, TX) personal computer system equipped with Varian Star® Chromatography Workstation software version 5.3.1 (Varian, INC., Palo Alto, CA).

GC Method

The GC injector and detector temperatures were set at 150°C. The GC oven was held isothermally at 40°C for the duration of the 10-min run. Helium was used as the GC carrier gas at a flow rate of 20 mL/min. The gases required to operate the FID were hydrogen and compressed air and had flow rates of 30 and 300 mL/min, respectively.

The Varian Star® Chromatography Workstation software allowed for automated identification and integration of all obtained peaks. The software integrated all peaks in a chromatogram with a S/N of 5 or greater. Peak area was used for the quantitation of each analyte of interest from a chromatogram. The retention times for ethanol and other volatiles were established on both columns following the analysis of a prepared blood calibrator.

Analytical Parameters

Both the limit of detection (LOD) and the limit of quantitation (LOQ) were determined for the detection of ethanol using this technique. The LOD, as defined by our laboratory, is the lowest analyte concentration detected with a peak S/N of at least 5 and a retention time within ± 2% of a known standard. The LOD for this method was determined to be 0.10 mg/dL with respect to ethanol. The LOQ was the lowest concentration detected that met the parameters described above and had a S/N of at least 10. The LOQ for this method was determined to be 1.00 mg/dL with respect to ethanol. A representative chromatogram for the analysis of ethanol and other volatiles using this method can be seen in Figure 1.

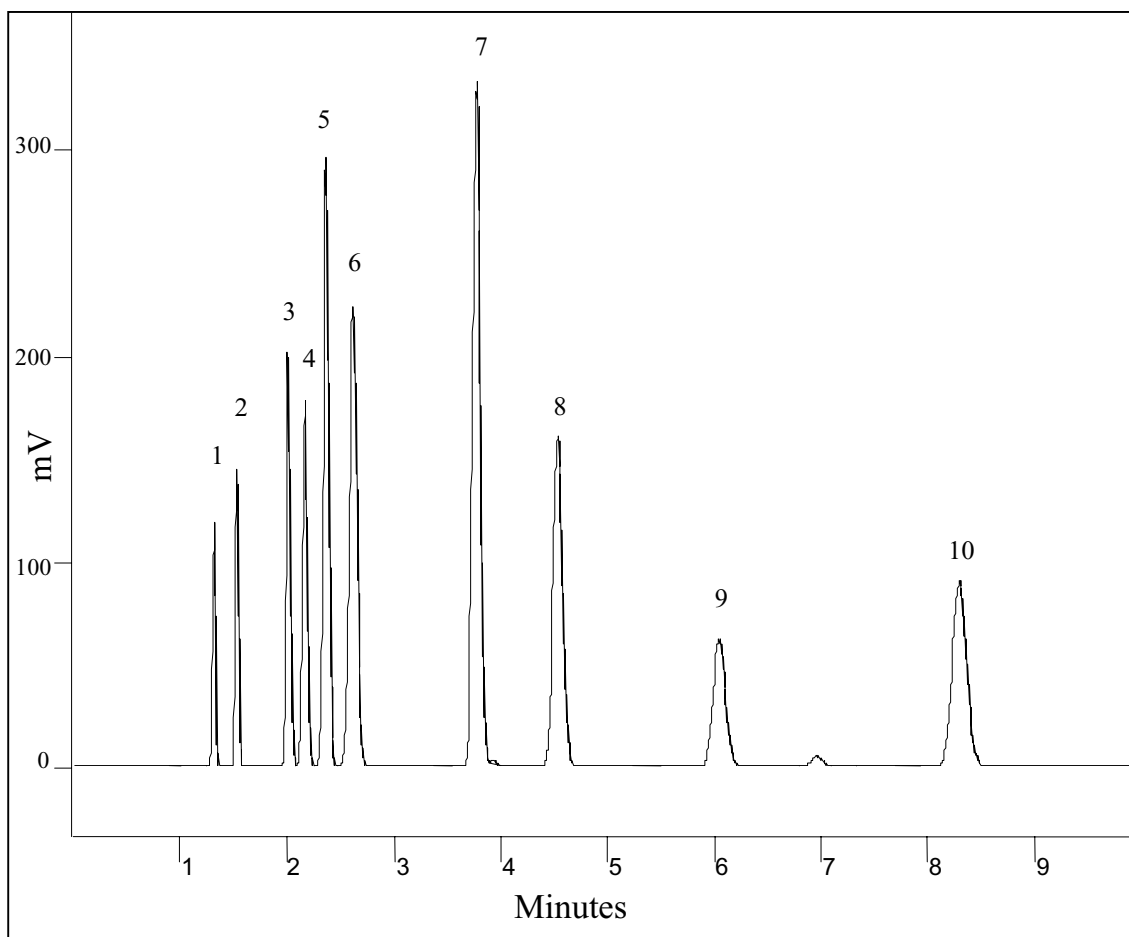


Figure 1. A representative headspace GC chromatogram for the analysis of volatiles in a postmortem specimen. Peaks of interest are: 1. acetaldehyde, 2. methanol, 3. ethanol, 4. acetone, 5. isopropanol, 6. t-butanol, 7. n-propanol, 8. sec-butanol, 9. isobutanol and 10. n-butanol.

Specimen Selection and Preparation

Biological specimens obtained from fatal aircraft accident victims are stored at -20°C at CAMI for up to 5 years following their initial analysis. Using our laboratory database, specimens were selected from cases where microbial contamination was suspected.

Tissue specimens were thawed, divided into two approximately equal parts and weighed. Specimens were diluted with 2 parts of either DDW or 1.00% sodium fluoride. The tissue samples were then homogenized using a PRO250 Post Mounted Homogenizer (PRO Scientific, Oxford, CT) employing a 10.0 mm saw-toothed generator set at 22,000 RPM. To prevent sample-to-sample contamination, the generator was thoroughly cleaned between tissue specimens. Following homogenization, both the water and 1.00% sodium fluoride tissue homogenates were divided again into two parts by transferring approximately half of the volume to clean 16 x 100 mm screw-capped culture tubes. All four samples from the

original tissue specimen were labeled appropriately and placed on ice awaiting an initial volatile analysis.

Volatile Analysis

The instrument was calibrated using the previously described calibration solution. The calibration solution was run as a test mix each day prior to analysis to evaluate the operation of the instrument. Ethanol controls were run before, during and after sample analysis. Specimens were analyzed immediately after homogenization to establish a baseline concentration of volatiles. Following the initial analysis, each specimen was capped and placed either in a refrigerator at 4°C or on a shelf at 25°C . The homogenates remained either in the refrigerator or on the shelf for the remainder of the experiment. At various times following initial homogenization, a portion of each specimen was aliquoted into a headspace vial for volatile analysis.

RESULTS

The specimens selected for this study had indications of microbial activity in the original volatiles analysis, as will be discussed in the next section. Identification of the microbial species was not attempted and is beyond the scope of this paper. Table 1 outlines the different specimen types chosen. Each specimen was periodically uncovered for sampling and, therefore, potentially exposed to the microorganisms ubiquitously present in the environment. However, specimens analyzed in this study were examined without the intentional addition of microbes or substrate.

The 9 tissue specimens selected for this study were each divided into 2 equal parts and homogenized after the addition of either water or 1.00% sodium fluoride. Volatile analysis was immediately performed on these samples. Following specimen homogenization, both water and sodium fluoride homogenates were again split, and the specimens were then stored at either 4°C or 25°C. The samples stored at 4°C were re-examined for ethanol and other volatiles at $t = 24, 48, 72$ and 96 h. The samples stored at 25°C were re-examined for ethanol and other volatiles at $t = 24$ and 48 h.

An initial comparison of the 9 specimens homogenized in water and 1.00% sodium fluoride yielded the following results. The range and average initial ethanol concentration ($t = 0$) for these specimens was the same for both groups and was 1 - 28 mg/hg and 11 mg/hg, respectively. Table 1 shows the individual initial ethanol concentrations present in these samples.

The samples homogenized in 1.00% sodium fluoride and stored at 4°C and 25°C demonstrated no significant difference in ethanol concentrations between the two storage temperatures throughout the duration of the experiment. The largest individual increase in ethanol concentration observed for the sodium fluoride group occurred in a kidney specimen (#7) stored at 4°C. This specimen had an initial ethanol concentration of 28 mg/hg and at 96 h contained 33 mg/hg ethanol. This slight increase, however, was likely due to experimental and/or instrumental error. The initial and final ethanol concentrations for all sodium fluoride preserved specimens can be seen in Table 2.

The 9 tissue specimens homogenized without preservative and subsequently stored at 4°C demonstrated significant ethanol formation over the course of the experiment. The average ethanol increase for these 9 specimens stored at 4°C was 1470% after 96 h, with increases ranging from 107% to 7500%. The largest individual increase in ethanol concentration observed occurred in a kidney specimen (#8). While it initially contained 1 mg/hg ethanol, at 96 h this specimen was found to contain 76 mg/hg ethanol. This specimen had a net ethanol concentration increase of 75 mg/hg over the course of the experiment, an increase of 7500% over its initial ethanol concentration. The initial and final ethanol concentrations for the specimens investigated without preservative and stored at 4°C are shown in Table 3.

The non-preserved specimens were also examined at 25°C after 24 and 48 h. As expected, all 9 showed a dramatic increase in ethanol concentration. The average

Table 1. Specimen types and initial ethanol concentrations.

Specimen Number	Specimen Type	Ethanol Concentration (mg/hg)
1	Muscle	6
2	Kidney	18
3	Muscle	4
4	Kidney	4
5	Muscle	23
6	Kidney	5
7	Kidney	28
8	Kidney	1
9	Muscle	2

Table 2. The effect of temperature on ethanol production in specimens homogenized in 1.00% sodium fluoride.

Specimen #	Preservative	Temperature	Initial Ethanol (mg/hg)	Final Ethanol* (mg/hg)
1	NaF	4°C	6	6
		25°C		6
2	NaF	4°C	18	20
		25°C		16
3	NaF	4°C	4	3
		25°C		3
4	NaF	4°C	4	2
		25°C		4
5	NaF	4°C	23	23
		25°C		22
6	NaF	4°C	5	5
		25°C		6
7	NaF	4°C	28	33
		25°C		29
8	NaF	4°C	1	1
		25°C		1
9	NaF	4°C	2	1
		25°C		1

* Final ethanol concentrations were recorded after an incubation time of 48 h at 25°C and 96 h at 4°C

increase in ethanol concentration at 25°C for these 9 specimens was 1432% at 48 h, with increases ranging from 109% to 6100%. The largest individual increase in ethanol concentration observed occurred in a kidney specimen (#8), with an initial ethanol of 1 mg/hg and an ethanol of 62 mg/hg at 48 h. The individual ethanol concentrations, at t = 0 and 48 h, for the specimens stored without preservative at 25°C are shown in Table 3.

DISCUSSION

The main objective of this study was to compare postmortem ethanol formation in unadulterated tissue specimens homogenized in either water or 1.00% sodium fluoride and stored at both 4°C and 25°C. We examined a single tissue specimen from each of 9 separate aviation fatalities for the formation of postmortem ethanol. All cases selected were chosen because they were suspected of microbial contamination. This suspicion arose due to either an abnormal distribution of ethanol in various tissues and fluids initially examined or abnormally high concentrations of various volatile organic compounds not

normally present *in vivo*. As has been well documented, under normal circumstances following ingestion, ethanol distributes throughout the body according to the water content of various tissues and fluids (23-28). Therefore, when abnormal ethanol distribution is observed between two or more tissues and/or fluids from one victim, post-mortem ethanol formation may be suspected. Another commonly used criteria to evaluate postmortem ethanol formation is the presence of other volatiles in a specimen. Volatiles such as acetaldehyde, n-propanol, sec-butanol, isopropanol, acetone and iso-butanol, when discovered during routine ethanol analysis, suggest the possibility of microbial formation of ethanol (4, 10, 13, 18, 29-32).

One of the initial goals of this study was to make a direct comparison between the increase in ethanol concentration in specimens homogenized in water and 1.00% sodium fluoride. The differences observed between these two groups were substantial. Out of the 36 individual experiments performed, i.e., 18 in water at 4°C or 25°C and 18 in 1.00% sodium fluoride at 4°C or 25°C, all 18 of the non-preserved samples showed a significant increase in ethanol concentration between the

Table 3. The effect of temperature on ethanol production in specimens homogenized in water.

Specimen #	Preservative	Temperature	Initial Ethanol (mg/hg)	Final Ethanol* (mg/hg)	% Increase
1	None	4°C	6	28	367
		25°C		25	317
2	None	4°C	18	45	150
		25°C		40	122
3	None	4°C	4	46	1050
		25°C		30	650
4	None	4°C	4	40	900
		25°C		88	2100
5	None	4°C	23	80	252
		25°C		48	109
6	None	4°C	5	49	880
		25°C		71	1320
7	None	4°C	28	58	107
		25°C		90	221
8	None	4°C	1	76	7500
		25°C		62	6100
9	None	4°C	2	43	2050
		25°C		41	1950

*Final ethanol concentrations were recorded after an incubation time of 48 h at 25°C and 96 h at 4°C.

initial and the final measurement. While both the 4°C and 25°C groups without preservative showed dramatic increases in ethanol concentration, the increase occurred at a slower rate in the refrigerated specimens. Conversely, the 18 specimens preserved with 1.00% sodium fluoride demonstrated insignificant increases in ethanol concentration. These data are best represented graphically (Fig. 2 - 10), where ethanol concentration is plotted versus time for all 9 specimens examined. Each Figure displays the results of the four experiments performed on each specimen. After completion of this cursory comparison, it was clear that sodium fluoride was extremely effective at inhibiting microbial ethanol formation. This agrees well with other sodium fluoride-preservative publications (18-20).

In a majority of the specimens investigated, the overall increase in ethanol concentration after 96 h at 4°C was similar to the increase observed after 48 h at 25°C. This comparable concentration of ethanol formation was expected and may be explained by examining three factors. First, at lower temperatures, one would expect the metabolic rate of microbes responsible for ethanol formation to be slower than those at room temperature. Second, the formation of acids from the fermentation of

carbohydrates, amino acids and alcohols may result in a pH that is harmful to pH-sensitive organisms. Lastly, there is a finite amount of substrate available in these specimens for the microbes to consume, and after the substrate supply has been depleted, no further ethanol can be produced. Therefore, the time required to consume all available substrate and/or create an unsuitable environment should take longer at lower temperatures, but the amount of ethanol produced as a byproduct of this metabolism should be similar.

Six of the specimens investigated were initially reported negative for ethanol, as defined by our laboratory's criteria (< 0.010%, < 10 mg/hg, < 10 mg/dL). After 96 h of storage at 4°C with no preservative, all 6 specimens had ethanol concentrations above our laboratory's cutoff. Furthermore, 5 of the 6 were above 0.040% (40 mg/hg, 40 mg/dL), which is the FAA's legal cutoff for ethanol (14CFR91.17). At 25°C, all 6 previously reported negative specimens had values above 0.010% after 48 h, with 4 of the 6 above 0.040%.

The results of this study reveal several trends. First, at both temperatures, ethanol was formed in all of the specimens when sodium fluoride was absent. Second, ethanol formation rate was slower at reduced temperatures, but

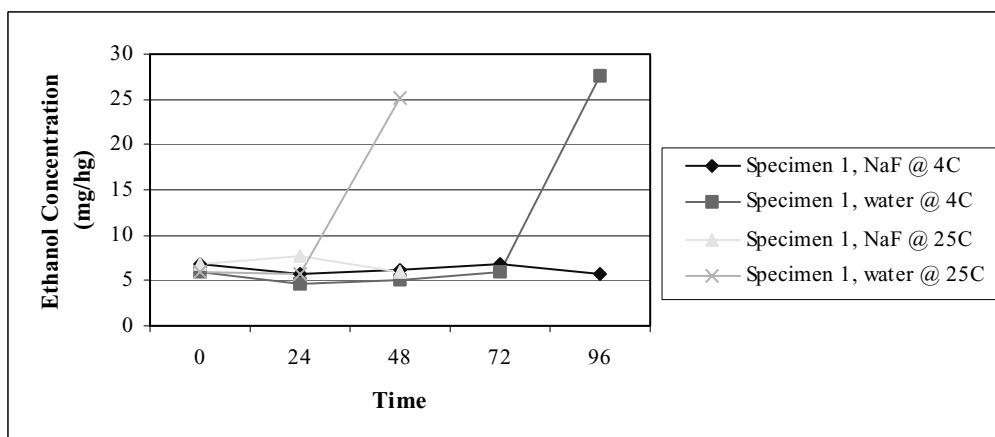


Figure 2. Ethanol concentration vs. time for specimen # 1.

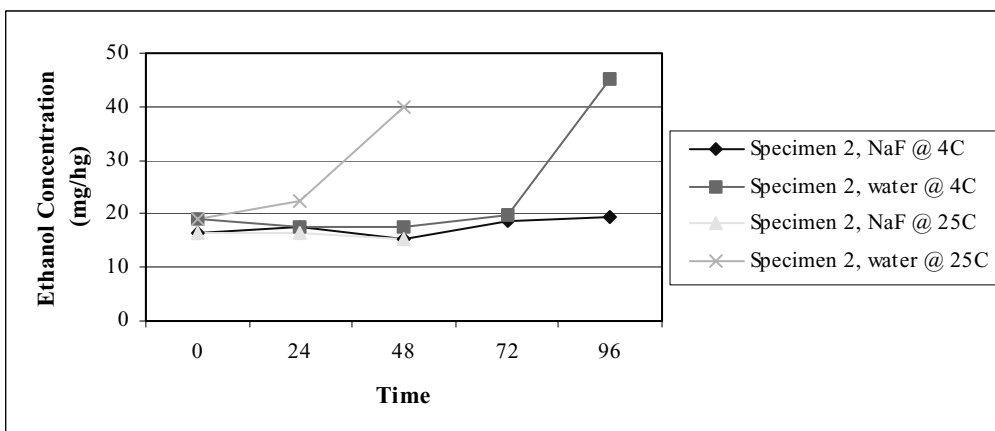


Figure 3. Ethanol concentration vs. time for specimen # 2.

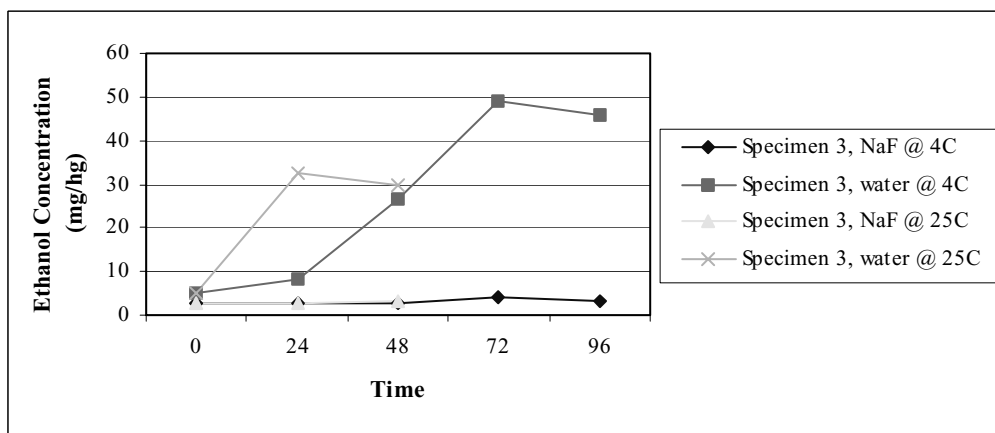


Figure 4. Ethanol concentration vs. time for specimen # 3.

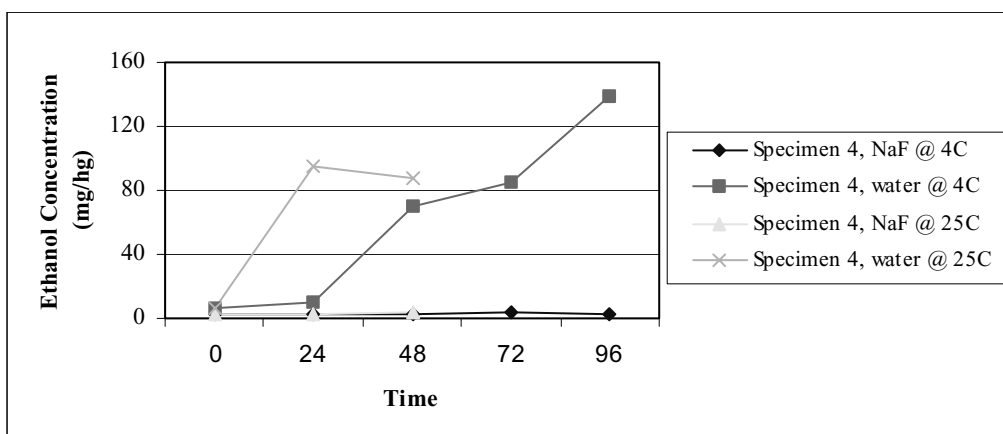


Figure 5. Ethanol concentration vs. time for specimen # 4.

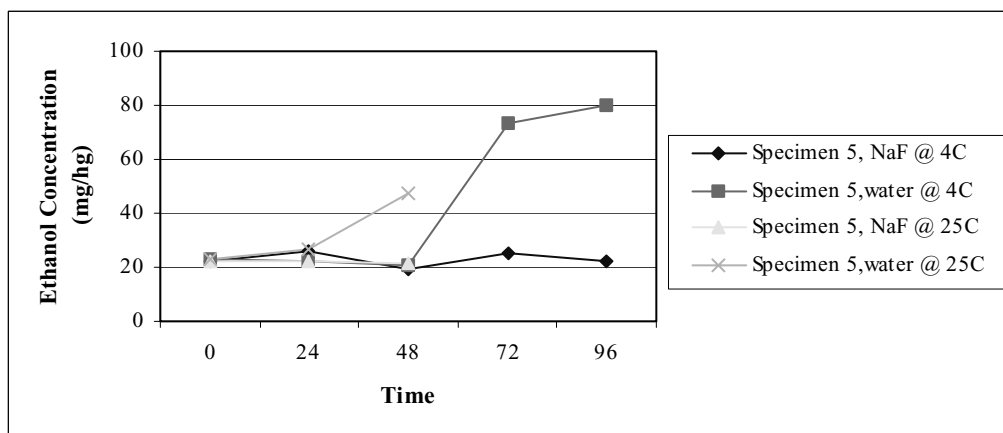


Figure 6. Ethanol concentration vs. time for specimen # 5.

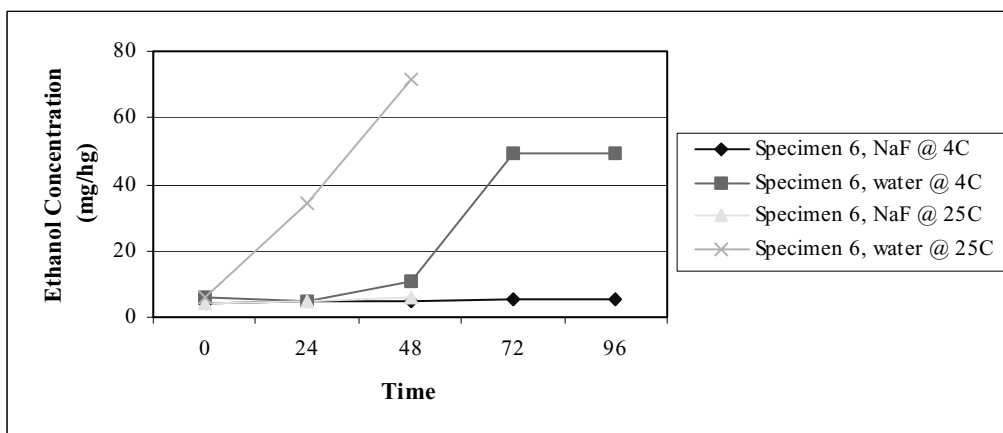


Figure 7. Ethanol concentration vs. time for specimen # 6.

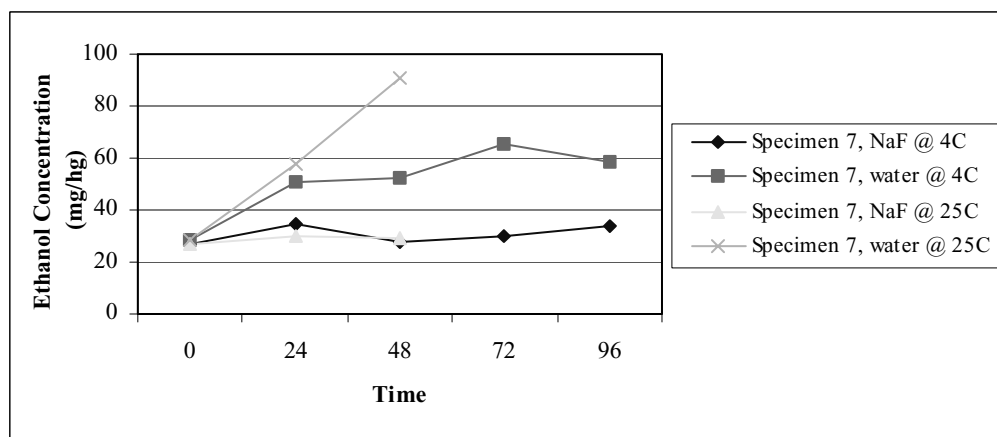


Figure 8. Ethanol concentration vs. time for specimen # 7.

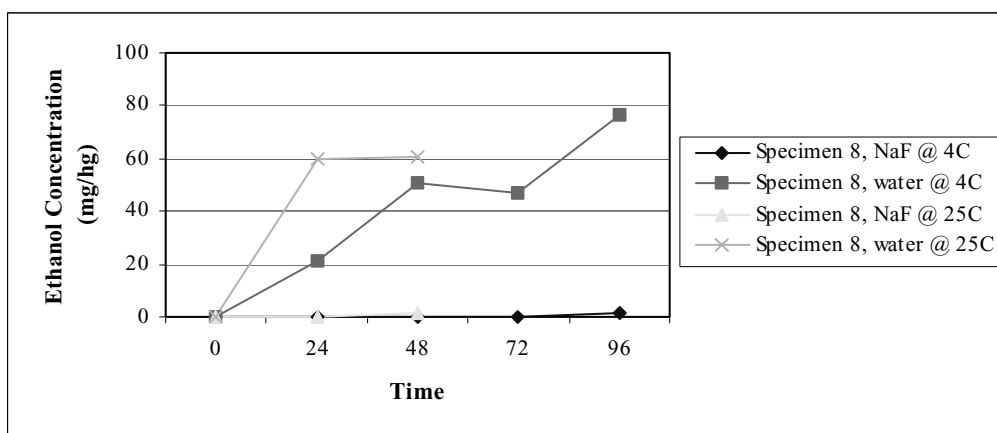


Figure 9. Ethanol concentration vs. time for specimen # 8.

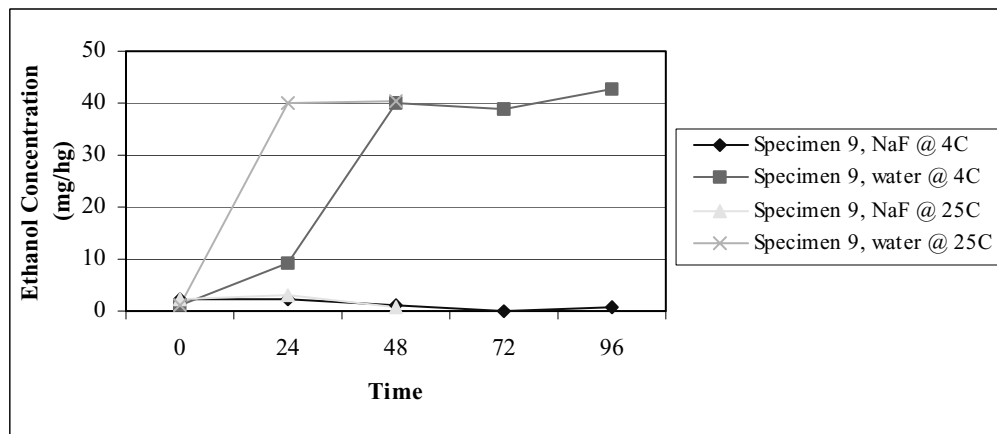


Figure 10. Ethanol concentration vs. time for specimen # 9.

with time, the refrigerated specimens produced ethanol concentrations similar to those at room temperature. Third, ethanol formation was virtually eliminated when specimens were homogenized in 1.00% sodium fluoride and stored at either 4°C or 25°C.

During the course of our experiments, we observed various volatile organic compounds being formed in these samples. We also observed volatiles, which were initially present, decrease in concentration over time. Some examples of volatiles that were either present initially or formed with time are acetaldehyde, n-propanol, sec-butanol, isopropanol, acetone and iso-butanol. These compounds, if present, were quantitated for each specimen at each analysis time, but after collection of the data, and subsequent data analysis, no discernable pattern could be established.

CONCLUSIONS

It is clear from these experiments that all 9 unadulterated specimens from actual aviation accident victims contained microbes capable of ethanol production. We have demonstrated that even at 4°C, significant amounts of postmortem ethanol can form in the absence of a preservative. Additionally, we have demonstrated that the addition of sodium fluoride to postmortem tissue specimens during the homogenization process prevents the formation of ethanol at storage temperatures of 4°C and 25°C. Therefore, we believe that sodium fluoride should be added as a precaution to all postmortem specimens. However, caution must always be used when interpreting ethanol results from postmortem samples since, even with these precautions, we cannot rule out the phenomenon of ethanol production in a small percentage of cases where larger numbers of microbes are present when the sample is received.

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