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TECHNICAL NOTE

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Improper Sealing Caused by the Styrofoam Integrity Seals in Leakproof Plastic Bottles Lead to Significant Loss of Ethanol in Frozen Evidentiary Urine Samples*

ABSTRACT: Evidentiary urine samples ($n = 345$) stored frozen at −20°C in their original containers (leakproof 100 mL plastic bottles) upon re-testing for ethanol resulted in concentrations that were significantly lower (average loss = ~30%) than those prior to their storage at −20°C ($p \leq 0.0001$). The observed loss of ethanol was independent of the method of thawing or the concentration of ethanol in the samples, but was dependent on the sample volume in the container, i.e., the larger the volume of sample the larger the magnitude of ethanol loss. The loss of ethanol was determined to be due to improper sealing by a Styrofoam integrity seal attached to the mouth of the container. Accordingly, adopting leakproof plastic containers that do not contain Styrofoam integrity seals, but rather an outside and across the cap tape integrity seal for evidence collection and long-term storage, will prevent loss of ethanol due to evaporation.

KEYWORDS: forensic science, ethanol, urine, head-space gas chromatography

Social use of ethanol and its abuse continues to be important from the viewpoint of clinical and forensic interest (1). Accordingly, almost every state in the United States and many foreign countries around the world have per se laws governing the measurement of ethanol concentrations for forensic purposes (1). The goal of measuring ethanol concentrations is to establish the degree of intoxication either at the time of sample collection or another time. For this purpose, one of three principal media, viz., blood, breath and/or urine, are used to measure ethanol concentrations. Often the sample choice is single and ethanol concentrations in the other samples may need to be derived. If the sample of choice were urine, then the interpretation of the degree of intoxication would be difficult but not impossible, because urine is not a dynamic body fluid like blood. Accordingly, the accuracy of measurement of ethanol concentrations in evidentiary samples and their interpretation, especially in the case of urine, are extremely important.

The accuracy of measurement and interpretation of ethanol results may be compromised for many reasons. One such scenario is the presence of high concentration of sugar, contamination of such samples by ethanol producing/metabolizing bacteria and/or yeast, e.g., *E. coli* and *C. albicans*, and storage of contaminated samples at room temperature that promotes in vitro ethanol production/consumption (2–8). To correct such a situation NaF (10 mg/mL) is added to samples at the time of collection and the samples are stored at 0–4°C prior to analysis (9–11). The latter corrective step is made possible by collection of samples in containers having a fixed amount of sodium fluoride. This in turn will lead to the presence of elevated levels of NaF in samples because small volumes of samples are collected in bottles containing a fixed concentration of NaF, which has been shown to result in salting out of ethanol during storage and analysis (9,10,13).

Improper storage methods and procedures may also result in compromising the accuracy of measurement and interpretation of ethanol results. Described herein is one such scenario, which we have discovered and corrected. We, like many other laboratories, routinely treat urine samples with NaF (10 mg/mL) and store them at 4°C prior to any toxicological analysis. After the completion of ethanol analysis, the urine samples are treated in one or more of the following ways. First, urine samples are stored frozen at −20°C until they are tested for drugs of abuse (a preventive measure to preserve drugs of abuse and their metabolites). Second, urine samples are stored frozen at −20°C until they are returned to the agencies that collected them for safekeeping and/or to use them as items of physical evidence in court proceedings. Third, urine samples are stored frozen at −20°C until they are transferred to defense counsel for re-testing of ethanol and drugs of abuse when the state laboratory results are disputed. Finally, urine samples are stored frozen at −20°C until they are destroyed when they are no longer required. Although this practice has been discontinued lately, the

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past practice was that the urine samples being transferred to the defense counsel were re-tested for ethanol. This re-testing process led to the observation that the ethanol concentrations determined in previously frozen urine samples were significantly lower than the values obtained prior to their storage at −20°C. Previous studies have carefully evaluated storage (short-term and long-term) methods of blood and urine samples under various conditions (14,15) including the conditions described above, and using leak-proof plastic bottles similar to the ones used in the present study. Contrary to our experience, the urine samples frozen and thawed in leak proof plastic bottles lost little or no ethanol in these studies (14,15). Accordingly this study was initiated to establish the basis for lowering of ethanol concentrations in post-frozen urine samples and suggest corrective measures to prevent it.

Materials and Methods

Ethanol and Internal Standards

Aqueous solutions of ethanol prepared in distilled water were used as ethanol standards and they were purchased from College of American Pathologists (Northfield, IL), New England Reagent Laboratory (Providence, RI), and/or Radian International (Austin, TX). Absolute ethanol was purchased from National Institute of Standards and Technology (Gaithersburg, MD). An aqueous solution of *n*-propanol (5 mg/mL) was used as internal standard.

Urine Storage

Leak proof plastic evidentiary urine collection bottles (100 mL) containing 1 g sodium fluoride were used for urine storage and processing. Most of these bottles contained a Styrofoam integrity seal attached to the rim of the bottle mouth. Some bottles did not contain Styrofoam integrity seal. Urine samples were also stored in Vacutainer blood collection tubes. All of these containers were either purchased from or donated by Tri-Tech, Inc., Greensboro, NC.

Urine Samples, Source and Their Processing

This study was approved and performed in accordance with the ethical standards established by the Institutional Review Board and the urine samples used in this study belonged to 3 groups.

1. Urine samples spiked with known concentrations of ethanol ($n = 100$) and stored frozen for up to 1 year. Two healthy male volunteers, age 34 and 36 years, donated these samples.
2. Actual case samples designated for disposal and stored for up to 2 years ($n = 345$). The age of these subjects ranged from 21–65 years.
3. Urine samples collected from 38 healthy volunteers (Caucasians; 34 male, 3 female and 1 male of Southeast Asian origin) participating in controlled drinking studies ($n = 38$) and stored frozen for up to 1 year. All of the subjects of this subset were peace officers in the age group of 24–45 years.

Samples from Group 1 were prepared by spiking urine with different concentrations of ethanol (0.05–0.40 g/67 mL). These samples were then fractionated into leak proof 100 mL plastic bottles containing 1g NaF. The plastic bottles used for this purpose were same as the ones used for actual evidentiary urine sample collection.

The frozen urine samples described above were either allowed to thaw slowly at 4°C in a walk-in cooler or allowed to thaw quickly at room temperature (RT) in a chemical hood.

Sample Dilution

Sample dilutions were performed with the aid of a Hamilton Microlab 500 dispenser/diluter. Aliquots (0.5 mL) of aqueous ethanol standards, positive controls, negative controls and urine samples were diluted (1:5) with aqueous *n*-propanol (5 mg/mL) internal standard (with 30 mg/mL sodium fluoride), and dispensed into 20 mL headspace vials.

Headspace Gas Chromatography Analysis of Ethanol

Quantitative analysis of ethanol was by headspace gas chromatography (16,17) using a Perkin-Elmer AutoSystem XL gas chromatograph equipped with a Perkin-Elmer HS-40 headspace analyzer.

Alcohol Concentrations

Since 1978 The State of Minnesota has had a per se statute that defines "Alcohol Concentration" as grams of alcohol per 100 mL of blood, or per 210 L of breath or per 67 mL of urine. The protocol for collecting the urine sample was to collect a grab sample. Accordingly, the per 67 mL of urine insures that in cases where the subjects were in the post-absorptive phase (virtually all suspected DWI situations), the ethanol concentration determined will underrepresent the corresponding ethanol concentration determined in a blood sample collected at the same time as the urine sample. We have evaluated simultaneously collected blood and urine samples, both in a controlled laboratory setting and in a field study, and have found that in all cases the ethanol concentration in the urine samples to be less than or equal to the ethanol concentration in the corresponding blood samples (18,19).

Data Analysis

The Macintosh-based STATView II (Brainpower, Inc., Calabas, CA) computer program was used to generate means, standard deviations, compare means (two-tailed, paired, Student's *t*-test), linear regression lines and *p*-values.

Results and Discussion

The alcohol concentrations in urine samples (0.104 ± 0.05 mg/67 mL) stored frozen in evidentiary urine collections bottles were found to be significantly lower (average loss = ~30%, $p \leq 0.0001$) as compared with the values obtained prior to their long-term storage at −20°C (0.151 ± 0.048 mg/67 mL), Table 1. The method used to thaw the samples did influence the magnitude of loss of ethanol from these urine samples, Fig. 1 and Table 1. Among the large number of samples tested, very few samples retained their original ethanol concentrations and it is clearly supported by the poor correlation demonstrated in Fig. 1. On the other hand, urine samples stored in identical storage containers but at 4°C (without ever being frozen) for up to 1.5 years did not show significant change in ethanol concentrations, Table 2. Further, the concentration of ethanol in the urine samples had little or no effect on the magnitude of ethanol lost during the thawing process, Table 3. However, a close examination revealed that the decreases in ethanol concentrations observed were dependent on the sample volumes, Table 4. Additional investigations revealed that the ethanol concentrations in urine samples were unaltered when they were frozen and thawed (at RT) in airtight Vacutainer tubes irrespective the volume of urine samples in them, Table 5. A closer examination of the design of evidentiary urine collection bottles re-

vealed that the leakproof 100 mL plastic bottles contain a Styrofoam integrity seal attached to their mouths. This seal is intended to prevent accidental loss of NaF from the bottle and provide a tamper proof for evidence collection. This observation led to our working hypothesis that improper sealing may occur between the rim of plastic bottle and the inner lining of its cap due to incomplete/improper removal of the Styrofoam seal during long-term sample

TABLE 1—*Alcohol concentrations (AC) in urine samples before freezing and after thawing: effect of thawing method.**

Samples thawed at room temperature AC (Mean ± SD), g/67 mL (n = 255)		
Before Freezing	After Thawing	% Diff
0.151 ± 0.048	0.104 ± 0.05	31%
Samples thawed at 4°C AC (Mean ± SD), g/67 mL (n = 90)		
Before Freezing	After Thawing	% Diff
0.149 ± 0.048	0.100 ± 0.058	33%

* Urine samples used in this study were evidentiary samples designated for disposal. They were stored in their original containers (leak proof plastic 100 mL bottles) in which the evidence was collected. They were stored at 4°C before analysis, at −20°C during long-term storage (~2 years) at 4°C after thawing at room temperature or 4°C. The ethanol concentrations determined before freezing and after thawing were significantly different (p = <0.0001).

TABLE 2—*Alcohol concentrations (AC) in urine samples stored at 4°C: effect of long-term storage.**

Time of Storage at 4°C	AC (Mean ± SD), g/67 mL (n = 38)
Zero	0.071 ± 0.014
1 month	0.071 ± 0.013
3 months	0.072 ± 0.012
6 months	0.070 ± 0.016
1.5 years	0.066 ± 0.017

* Urine samples used in this study were samples collected from a controlled drinking study. They were stored in the evidentiary leak proof plastic 100 mL bottles containing 1 g NaF. The sample volumes in the containers varied from 10–100 mL. They were stored at 4°C for the duration of the study (~1.5 years). The alcohol concentrations shown above are not significantly different from each other.

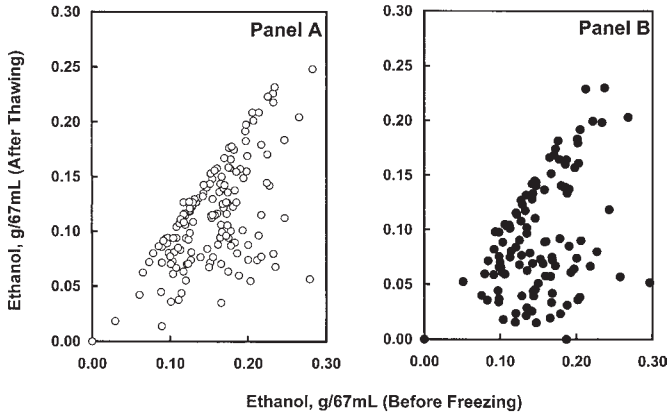

FIG. 1—*Alcohol concentrations in urine samples before freezing and after thawing: effect of thawing method. Urine samples used in this study were evidentiary samples designated for disposal. They were stored in their original containers (leak proof plastic 100 mL bottles). These samples were stored at 4°C prior to their analysis, at −20°C during long-term storage (up to 2 years) and at 4°C after thawing at room temperature (○) or 4°C (●). Panel A: urine samples (*n *= 255) were subjected to thawing at room temperature on the bench-top. Panel B: urine samples (*n *= 90) were subjected to thawing at 4°C in a walk-in cooler. The ethanol concentrations were determined as described in Materials and Methods. The data shown in this figure are summarized in Table 1.*

TABLE 4—*Ethanol concentrations in urine samples: sample volume dependent loss.**

Urine, mL	Before Freezing	After Thawing	Avg. % Change
1–10	0.157 ± 0.03 (7)	0.144 ± 0.03 (7)	7.9
11–20	0.185 ± 0.04 (9)	0.163 ± 0.04 (9)	11
21–30	0.152 ± 0.05 (17)	0.140 ± 0.05 (17)	8.3
31–40	0.140 ± 0.04 (11)	0.124 ± 0.04 (11)	9.8
41–50	0.130 ± 0.04 (17)	0.122 ± 0.04 (17)	7.2
51–60	0.154 ± 0.04 (22)	0.123 ± 0.04 (22)	18
61–70	0.126 ± 0.05 (19)	0.094 ± 0.04 (19)	23
71–80	0.155 ± 0.05 (29)	0.115 ± 0.05 (29)	26
81–90	0.156 ± 0.04 (37)	0.104 ± 0.04 (37)	32
91–100	0.160 ± 0.05 (87)	0.080 ± 0.04 (87)	47

* Urine samples used in this study were evidentiary samples designated for disposal. They were stored in their original containers (leak proof plastic 100 mL bottles in which the evidence was collected). Storage was at 4°C before analysis, at −20°C during long-term storage (up to 2 years) and at 4°C after thawing. The samples were thawed at RT.

TABLE 3—*Effect of alcohol concentration (AC) on the magnitude of its loss due to freezing and thawing at constant volume.**

	AC (Mean ± SD), g/67 mL			
Original Concentration	After Thawing at RT (n)	Avg. % Change	After Thawing at 4°C (n)	Avg. % Change
0.050	0.039 ± 0.008 (10)	22	0.038 ± 0.008 (10)	24
0.100	0.081 ± 0.007 (10)	19	0.080 ± 0.008 (10)	20
0.151	0.110 ± 0.011 (10)	27	0.109 ± 0.015 (10)	28
0.302	0.237 ± 0.025 (10)	22	0.240 ± 0.030 (10)	21
0.402	0.321 ± 0.03 (10)	20	0.311 ± 0.04 (10)	23

* Urine samples (n = 100) used in this study were urine samples spiked with known but different concentrations of ethanol (0.05–0.4 g/67 mL). They were then fractionated (60 mL each) and stored frozen in evidentiary leak proof plastic 100 mL bottles for 1 month. At the end of this storage period, they were thawed at RT or 4°C and ethanol concentrations were determined. The ethanol concentrations determined after thawing (irrespective of thawing method) were significantly different from those of the original values in each case.

TABLE 5—*Ethanol concentration (AC) in urine samples stored in Vacutainer blood tubes, leakproof 100 mL plastic bottles with and without Styrofoam integrity seal.**

Urine Set #	Storage Container	AC (Mean ± SD), g/67 mL (*n*; volume)		Avg % Change
		AC Before Freezing	AC After Thawing	
1	Air-tight Vacutainer blood tubes	0.069 ± 0.014 (38, 6 mL)	0.071 ± 0.015 (38; 6 mL)	3.0
1	Leakproof plastic bottles with Styrofoam seal	0.069 ± 0.014 (38, 60 mL)	0.048 ± 0.011 (38; 60 mL)	30.0
1	Stored in plastic bottles without Styrofoam seal	0.069 ± 0.014 (38, 60 mL)	0.067 ± 0.015 (38, 60 mL)	3.0
2	Air-tight Vacutainer blood tubes	0.111 ± 0.065 (19; 3–8 mL)	0.113 ± 0.062 (19; 3–8 mL)	2.0
2	Leakproof plastic bottles with Styrofoam seal	0.111 ± 0.065 (19; 10–80 mL)	0.079 ± 0.044 (19; 10–80 mL)	29.0
2	Stored in plastic bottles without Styrofoam seal	0.111 ± 0.065 (19; 10–80 mL)	0.110 ± 0.060 (19; 10–80 mL)	1.0

* Urine samples used in this study were either collected from a controlled drinking study ($n = 38$, Set 1) or evidentiary urine samples designated for disposal ($n = 19$, Set 2). They were stored frozen in three different ways. 1. Samples was stored in Vacutainer tubes with soft-rubber plug cap at a constant volume of 6 mL/tube (set 1) or variable volume of 3–8 mL (set 2). 2. The samples were stored in leak proof plastic bottles with Styrofoam seal at a constant volume of 60 mL/bottle (set 1) or variable volume of 10–80 mL (set 2). 3. The samples were stored in leakproof plastic bottles without Styrofoam seal at a constant volume of 60 mL/bottle (set 1) or variable volume of 10–80 mL (set 2). Storage was 4°C before analysis, at −20°C during long-term storage (up to 1 year) and at 4°C after thawing. The samples were thawed at RT.

storage at −20°C and thus leading to loss of ethanol during its storage or thawing process. In fact, we routinely observe incomplete/improper removal of the Styrofoam seal from the mouth of sample collection bottles. Accordingly, during storage or in the process of thawing ethanol may evaporate and escape from the bottle through the small gaps/pores created due to improper sealing, and thus leading to significant decreases in the ethanol concentrations. Further, the pronounced loss of ethanol from the bottles containing larger volumes of urine samples is most likely due to volume expansion during freezing, which further leads to disruption of Styrofoam seal and increased evaporation of ethanol from smaller head space volume. Yet another possibility, the unfrozen ethanol (ethanol does not freeze at −20°C) is absorbed into the Styrofoam seal during urine sample storage. These notions were tested in three different ways. One, Styrofoam integrity seal was first scraped off from the mouths of leakproof plastic bottles and then tested their ability to prevent ethanol loss as before. These experiments prevented the loss of ethanol from urine samples significantly but not completely (data not shown). Two, the ethanol concentrations were identical when the storage containers were leak proof 100 mL plastic bottles except that they did not have inner Styrofoam integrity seal (Table 5 and Fig. 2) and it is supported by a strong correlation demonstrated in Fig. 2. The actual ethanol concentrations in these urine samples ($n = 19$, sample volume 10–80 mL) before freezing were 0.11 ± 0.065, and after freezing (up to 3 months) and thawing were 0.11 ± 0.061 g/67 mL. Three, the Styrofoam seals from the urine sample bottles that contained just thawed urine samples or frozen urine samples were analyzed for ethanol and found to contain only trace amounts of ethanol, negating the possibility of absorption of ethanol into Styrofoam seals (data not shown). Clearly, the use of leak proof 100 mL plastic bottles without Styrofoam integrity seal results in prevention of loss of ethanol from these bottles during storage and/or thawing process, and the poor sealing of the containers was responsible for the observed loss of ethanol concentrations when the contents were frozen. Thus, paying close attention to, and experimental evaluation of loss of ethanol, in any particular system used by a laboratory can prevent the loss of ethanol.

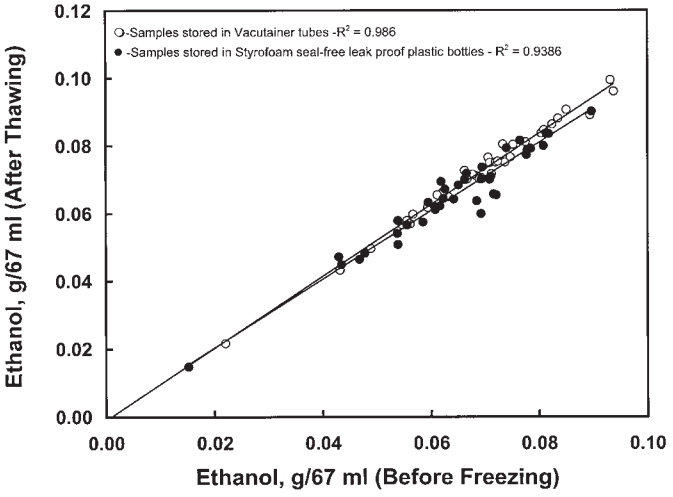

FIG. 2—*A comparison of ethanol concentrations in urine samples stored in Vacutainer blood tubes, and leak proof 100 mL plastic bottles without Styrofoam integrity seal before freezing and after thawing. Urine samples used in this study were evidentiary urine samples designated for disposal (*n = 36*). They were stored frozen in two different ways: 1. Aliquots of samples (3–8 mL) were stored in Vacutainer tubes with soft-rubber plug cap (○). 2. Aliquots of samples (10–80 mL) were stored in leak proof plastic bottles without Styrofoam seal (●). All of the samples were stored at 4°C prior to their analysis, at −20°C during long-term storage (up to 1 year) and at 4°C after thawing at room temperature. The ethanol concentrations were determined as described in Materials and Methods.*

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