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Improved Recovery and Stability of Ethanol in Automated Headspace Analysis

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ABSTRACT: Solutions are reported here for two problems in automated headspace gas chromatographic analysis for ethanol. The first is the catalytic oxidation of ethanol by oxyhemoglobin, a process that is limited only by the amount of oxygen in the sealed vessel. This reaction is prevented by the addition of sodium dithionite. The other problem, the lack of a suitable salting-out agent, led to the selection of ammonium sulfate, which improved recovery of ethanol as well as enhancing method precision. A procedure incorporating these improvements is described.

KEYWORDS: toxicology, alcohol, chromatographic analysis

The method of choice for alcohol analysis in the clinical and forensic science setting is gas chromatography (GC) [*1*]. Among the most specific and sensitive approaches is headspace gas chromatography, in which ethanol and an internal standard, usually another alcohol, are volatilized from a liquid specimen in a sealed container by a combination of heat and an inorganic salt.

The automated headspace gas chromatographic procedure in use in our laboratory until recently (a modification of Ref *2*) employed solid sodium chloride as a salting-out agent and equilibration at 60°C prior to chromatographic analysis. The assay was suboptimal in two important respects: first, the coefficient of variation of 3 to 5% was larger than expected for automated analysis and, secondly, in large sample runs, ethanol concentrations showed a progressive decline.

A similar decomposition process was observed in stored blood samples by Brown and coworkers [*3*]. They found that the loss of ethanol was a result of a reaction catalyzed by hemoglobin and was proportional to the amount of headspace in the storage tube, that is, the amount of available oxygen. Among the agents that prevented the process was sodium dithionite, which converts oxyhemoglobin to reduced hemoglobin. We have coupled the use of this material (to inhibit oxidative loss) with a more effective salting-out agent (ammonium sulfate) to produce a headspace GC method for ethanol exhibiting greatly improved accuracy, sensitivity, and precision.

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Equipment, Materials, and Methods

Equipment

A Perkin-Elmer (Norwalk, CT) Model F-45 Automated Headspace Analyzer was used. The instrument was equipped with a 1.8-m (6-ft) stainless steel column (2-mm inside diameter) packed with 5% Carbowax 20M on Carbopak B (Supelco, Inc., Bellefonte, PA). The carrier gas (nitrogen) flow rate was 30 ± 2 mL/min. Flame ionization detector air and hydrogen flow rates were 420 and 42 mL/min, respectively. Temperature controlled zones were maintained as follows: heating block, 60°C; injection needle, 100°C; oven, 65°C; and detector, 150°C. Timed intervals were: injection valve, 5 s; vial pressurization, 15 s; and analysis time, 5 min. The detector amplifier settings were: range, 10; attentuation, 8. A Hamilton (Hamilton Co., Reno, NV) Model 100004 Diluter/Dispenser was used to pipet sample diluted with diluent.

Sample vials, butyl rubber caps, and seals (Perkin-Elmer) were used.

Materials

Absolute alcohol (ethanol) meeting American Chemical Society (ACS) specifications was purchased from United States Industrial (Louisville, KY); 1-propanol (*n*-propanol), 99+% from Aldrich Chemical Co. (Milwaukee, WI); and ammonium sulfate, ACS reagent grade and sodium dithionite, purified technical grade from Matheson, Coleman and Bell (Cincinnati, OH). All other reagents were of analytical reagent grade from Mallinckrodt (St. Louis, MO).

Aqueous ethanol standards were prepared at a concentration of 0.200% (g/100 mL). Blood, serum, urine, and aqueous controls were prepared with concentrations of 0.115, 0.150, 0.200, 0.300, 0.400, and 0.500% each containing 10 mg of sodium fluoride and 2 mg of sodium oxalate per millilitre. These solutions were aliquotted and stored in airtight containers at 2 to 8°C.

The diluent, developed as described below, was an aqueous solution of 1.0*M* ammonium sulfate and 0.1*M* sodium dithionite containing the internal standard, 1-propanol, at a concentration of 0.03%; this was stored in an airtight container at ambient temperature for up to 90 days.

Methods

General Analytical Procedure—With the diluter/dispenser, aspirate 150-μL sample (standard, control, or patient specimen) and dispense with 1.0-mL diluent into glass autosampler vials. Seal vials with butyl rubber caps and aluminum crimp seals.

Place vials into heating block of the F-45 and incubate for 30 min before initiating analysis. For determination of ethanol, equal portions of the headspace are injected into the gas chromatograph.

For the experiments described under Results, the composition of the diluents were as indicated there. The composition of the final diluent is given above. All other conditions remained the same. The procedure in use before these studies (the "original" procedure) used normal saline containing 0.03% 1-propanol as a diluent and the vials into which the diluted samples were dispensed contained about 0.2 g of solid sodium chloride.

Results

Characterization of Original Procedure

Ethanol controls (0.150%) in distilled water, urine, serum, and oxalated-fluoridated whole blood were analyzed by the original procedure (modified from Ref *2*). For these experiments, 30 sealed vials for each matrix were loaded into the gas chromatograph and analyzed sequen-

tially allowing only 15 min for equilibration. Accuracy, precision, and trends in the data were noted.

The results for the whole blood sample are shown in Fig. 1. The initial rapid rise in peak area ratio for ethanol (open circles) demonstrates that during the distribution equilibration phase, ethanol enters the headspace more slowly than the larger internal standard alcohol, 1-propanol. After equilibration, a progressive decline in ethanol was seen. Coincident with this process, there was the growth of another early GC peak, represented in Fig. 1 by the closed circles. This material was identified by retention time as acetaldehyde, an oxidation product of ethanol [3]. Chromatograms are shown in Fig. 2 for the aqueous control (A), a serum control sample (B), and a whole blood control (C). Only the whole blood specimen demonstrates the presence of the early peak. The ethanol decay process was not seen with water, serum, or urine samples.

Stabilization of Ethanol

For this purpose, sodium dithionite at a concentration of 0.1*M* was added to the diluent containing the internal standard, and its effects were evaluated as described above. With dithionite present, loss of ethanol was not observed (data not shown), so this material was included in later studies, described below.

Optimization of Salting-out Agent

The effects of several salts on recovery of ethanol and on analytical precision were studied. Initially, crystalline ammonium sulfate was substituted for sodium chloride as a salting-out agent in the vial. Subsequently, several salts at 1.0*M* concentration were added individually to the stabilized diluent/internal standard solution, which now contained sodium dithionite and 1-propanol. Six vials of the blood control were analyzed using each diluent solution, following a 30-min incubation period in the instrument. Absolute recoveries of ethanol and 1-propanol (by GC peak area) were compared to those obtained if the diluent solution contained only water. Precision was expressed in the usual way, as coefficient of variation (CV).

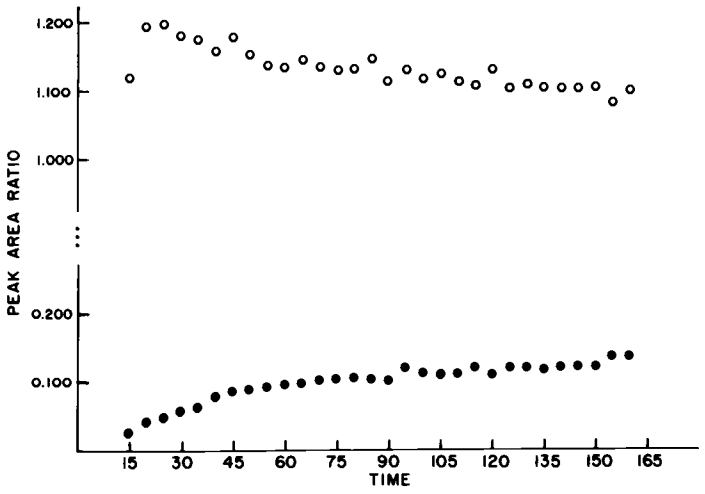

FIG. 1—*Peak area ratios for whole blood control as a function of incubation time at 60°C, using the original procedure (no stabilizer added). The open circles represent ethanol and the closed circles, acetaldehyde.*

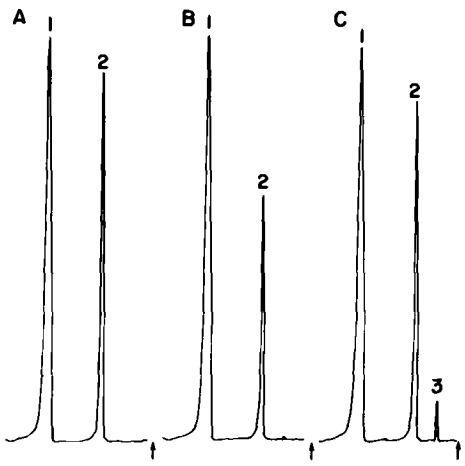

FIG. 2—*Chromatograms for an aqueous control (0.150%, A), serum control (0.110%, B), and whole blood control (0.150%, C) after incubation, using the original procedure (no stabilizer added). The peaks are l-propanol* (1), *ethanol* (2), *and acetaldehyde* (3). *The arrows indicate the point of injection.*

Recoveries observed with the various salt solutions are shown in Fig. 3. The addition of 0.1*M* sodium dithionite improved recovery for the two alcohols about 10%. Recoveries for the other salts increased in the order ammonium chloride, sodium chloride, calcium chloride, magnesium sulfate, ammonium sulfate, and sodium sulfate.

Precision did not follow exactly the same pattern. The following CVs were observed for the various salts in decreasing order: calcium chloride (1.41%), sodium sulfate (1.33%), sodium chloride (1.31%), no added salts (1.30%), magnesium sulfate (1.07%), ammonium sulfate

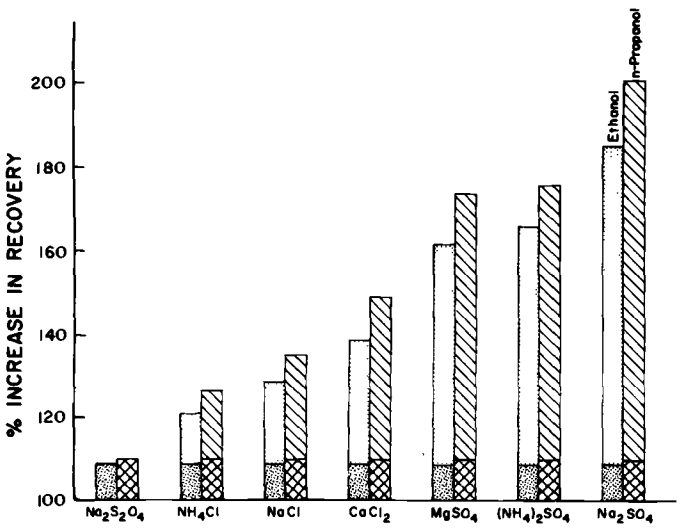

FIG. 3—*Increase in headspace recovery with various salting-out agents, as a percentage of that observed with distilled deionized water containing no additives. Within each pair of bars, the left bar represents ethanol and the right one, l-propanol. The contribution to recovery of sodium dithionite (left-most pair of bars) was assumed to be additive in the other mixtures and is carried across in the figure. See text for further details.*

(0.93%), ammonium chloride (0.81%), and sodium dithionite alone (0.52%). The salt with the best combination of recovery and precision was ammonium sulfate.

Characteristics of the Optimized Assay

The procedure given in the Methods section incorporates the improvements described above. This procedure was applied to controls spiked to three alcohol concentrations (0.115, 0.200, and 0.300%) in two matrixes, blood and urine. The results are shown in Table 1. CVs were consistently below 1%. Achieved mean values for a commercially available serum control and an aqueous ethanol standard from the College of American Pathologists were within 1% of the target values (see Table 2). Linearity was documented (using blood, serum, urine, and water) from the detection limit of 0.001 to at least 0.500% for each matrix. No difference was seen in the slopes for the various matrixes, indicating equivalent relative recovery for each. Typical GC retention times measured for ethanol and other common volatile compounds are shown in Table 3. There were no interferences with either ethanol or the internal standard 1-propanol, and thus other substances could be determined by the same procedure.

Discussion

In 1973 Brown and co-workers described three processes that can result in loss of ethanol from stored blood samples [*3*]. The first was loss from the growth of contaminating microorganisms, which can be prevented in all cases (other than yeasts) by the usual sodium fluoride concentrations of 5 mg/mL or greater [*4*]. Secondly, alcohol can be lost by evaporation if the storage container is not tightly capped. Lastly, loss can occur through chemical oxidation quite apart from the presence or absence of microorganisms. This process was shown to require hemoglobin; it did not occur in serum or plasma samples. Furthermore, the extent of loss was directly proportional to the amount of vapor space over the sample in the storage tube. When the amount of oxygen present in this zone was exhausted, the process came to an end. Smalldon and Brown suggested that oxidation of ethanol to acetaldehyde was coupled to conversion of oxyhemoglobin to reduced hemoglobin through an unknown intermediate [*4*]. If all of the oxyhemoglobin could be converted to the reduced form initially, that should block the process. They described a number of agents that were able to this.

We have discovered that the decomposition process which occurs in stored samples over long periods is accelerated at the elevated temperatures commonly used for headspace analysis. We have successfully prevented it by employing one of the substances described by Smalldon and Brown—sodium dithionite.

Salting-out agents have been used in headspace alcohol analysis almost since the inception of the technique. The mechanism of the salting-out effect is understood only on a very rudimentary basis. Certainly, the agents act to reduce the solubility of alcohol in the liquid phase, causing more to enter the headspace, the vapor phase zone above the sample.

It is likely that the salting-out effect bears some relationship to the fact that water is an *asso-*

TABLE 1—*Method precision.*

Ethanol Concentration, %	Matrix	%CV ($N = 40$)
0.110	blood	0.89
	urine	0.96
0.200	blood	0.69
	urine	0.74
0.300	blood	0.57
	urine	0.59

TABLE 2—*Achieved accuracy for commercial materials.*

Specimen	Target Concentration, %	*N*	Achieved Mean ± Standard Deviation
Serum control	0.150	12	0.149 ± 0.001
CAP standard	0.200	12	0.202 ± 0.001

ciated liquid [5], that is, each water molecule engages in hydrogen bonding interactions with numerous others. Because water is both a hydrogen bond donor and acceptor, association results in continuous lattices. This is what accounts for such bulk properties of water as its high freezing and boiling temperatures [5]. On a molecular scale, it has been suggested that even in liquid water there are "icebergs" of associated water, continually gaining and losing hydrogen bonding partners [5].

Alcohol molecules are analogous to water molecules except that one proton is replaced with an alkyl group. This group cannot be a hydrogen bond donor or acceptor and, since it is nonpolar, forces water to organize around it, which has an unfavorable positive free energy associated with it. Notwithstanding these facts, alcohols are very soluble in liquid water, presumably because they can fit in the interstices of the lattices and in the less organized areas of the microsolution. When water freezes, however, alcohol is largely excluded from the crystal structure because of these thermodynamic handicaps.

In solutions of an ionic salt, each ion is surrounded by a tight shell of water molecules, as was directly demonstrated recently by Narten and Hahn [6]. Thus, salting-out agents presumably act by making water more "ice-like" because of the formation of hydration shells around the component ions. This is especially important at the elevated temperatures which are used to enhance the rate of equilibration of alcohol between the headspace and the liquid solution.

By far the most common salting out agent is sodium chloride, often used in the solid state to produce a saturated solution. In our hands, this approach leads to excessive imprecision. Automated headspace analysis is intrinsically capable of excellent precision, accuracy, and sensitivity. In an attempt to improve the precision, and, if possible, the recovery of ethanol, we switched to salt solutions, which can be dispensed more reproducibly. We compared six salts, at a concentration of 1.0*M*. These salts spanned the range of large cations (ammonium and potassium) versus small (sodium); uni-univalent salts (sodium chloride and ammonium chloride) versus uni-divalent (sodium sulfate), and di-divalent (magnesium sulfate).

Recovery was enhanced if a salt released multiple ions. Secondly, various ions differed in their effectiveness as salting-out agents, presumably because of their size, charge density, and resultant effects on water structure. Sulfate was a more effective anion than chloride, and sodium a more effective cation than ammonium. The latter is not unreasonable when one remembers that ammonium ion is structurally analogous to water, and could fit in a water lattice

TABLE 3—*Retention times of various volatiles.*

Compound	Retention Time, min	Relative Retention Time
Acetaldehyde	0.58	0.13
Methanol	0.91	0.20
Acetone	1.39	0.31
Ethanol	1.75	0.39
2-Propanol	2.78	0.68
1-Propanol[a]	4.47	1.00

[a] Internal standard.

with considerably less deformation of the lattice than sodium. Overall, anions were more important than cations in producing the salting-out effect.

In spite of the fact that sodium sulfate gave the highest recovery (see Fig. 3), precision of the calculated result was best with ammonium salts. The salt with the best combination of recovery and precision was ammonium sulfate. The procedure that resulted from all of these improvements is given in the Methods section and its performance is set forth in Tables 1 through 3. In addition to providing excellent accuracy, precision, and sensitivity, the method resolves acetaldehyde and methanol because it uses a column of Carbowax 20M on Carbopak B, in preference to the commonly used Carbowax 1500 on Carbopak C [*2*].

In conclusion, we have developed a headspace GC method for determination of alcohol in blood in which the analyte is stable under the analytical conditions for at least 3 h; the method is accurate, linear, and extremely precise and sensitive.

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