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MANUAL FOR ANALYSIS OF ETHANOL IN BIOLOGICAL LIQUIDS

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FINAL REPORT

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16. Abstract

This manual covers selected aspects of the analysis of ethanol in biological liquids and the interpretation of the results of such analyses. Recommendations are made concerning the selection, collection, identification, and preservation of suitable biological liquid specimens from living and dead subjects for traffic law enforcement and related purposes. Procedural details are given for analysis of ethanol in such biological liquids by both automated and manual versions of gas chromatography of headspace vapors, with and without internal standards; and the analytical performance characteristics of the method are set forth. Brief consideration is also given to the interpretation of the results of alcohol analysis in blood and other biological liquids.

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PREFACE

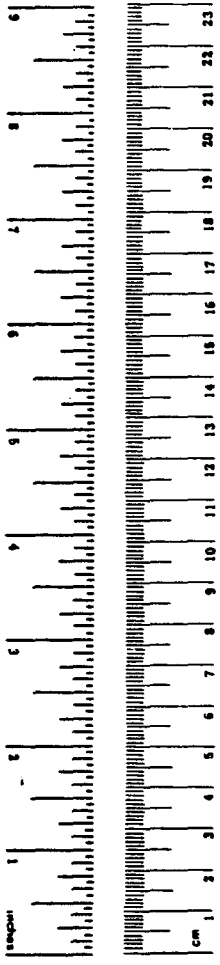
This manual was prepared for the U. S. Department of Transportation as a contribution to efforts by the Department and practicing forensic scientists toward improvement of the state of alcohol analysis for traffic law enforcement and related purposes. Accordingly, it covers pertinent aspects of the selection, procurement, and retention of biological liquid specimens, of their analysis for alcohol, and of the interpretation of the findings. Details are given for a highly reliable and practical method of gas chromatographic headspace analysis for alcohol, the performance characteristics of which make it suitable for use as a reference or referee method as well as for routine applications.

It is hoped that this information will assist forensic scientists everywhere in their important functions of performing valid and reliable analyses for alcohol in biological liquids and interpreting the results of such analyses for traffic law enforcement purposes.

METRIC CONVERSION FACTORS

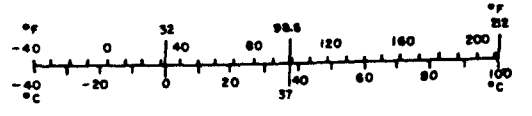
Approximate Conversions to Metric Measures

Symbol	When You Know	Multiply by	To Find	Symbol
		LENGTH		
in	inches	2.5	centimeters	cm
ft	feet	30	centimeters	cm
yd	yards	0.9	meters	m
mi	miles	1.6	kilometers	km
		AREA		
in^2	square inches	6.5	square centimeters	cm^2
ft^2	square feet	0.09	square meters	m^2
yd^2	square yards	0.8	square meters	m^2
mi^2	square miles	2.6	square kilometers	km^2
	acres	0.4	hectares	ha
		MASS (weight)		
oz	ounces	28	grams	g
lb	pounds	0.45	kilograms	kg
	short tons (2000 lb)	0.9	tonnes	t
		VOLUME		
tsp	teaspoons	5	milliliters	ml
Tbsp	tablespoons	15	milliliters	ml
fl oz	fluid ounces	30	milliliters	ml
c	cups	0.24	liters	l
pt	pints	0.47	liters	l
qt	quarts	0.95	liters	l
gal	gallons	3.8	liters	l
ft^3	cubic feet	0.03	cubic meters	m^3
yd^3	cubic yards	0.76	cubic meters	m^3
		TEMPERATURE (exact)		
°F	Fahrenheit temperature	5/9 (after subtracting 32)	Celsius temperature	°C



Approximate Conversions from Metric Measures

Symbol	When You Know	Multiply by	To Find	Symbol
		LENGTH		
mm	millimeters	0.04	inches	in
cm	centimeters	0.4	inches	in
m	meters	3.3	feet	ft
m	meters	1.1	yards	yd
km	kilometers	0.6	miles	mi
		AREA		
cm^2	square centimeters	0.16	square inches	in^2
m^2	square meters	1.2	square yards	yd^2
km^2	square kilometers	0.4	square miles	mi^2
ha	hectares (10,000 m^2)	2.5	acres	
		MASS (weight)		
g	grams	0.035	ounces	oz
kg	kilograms	2.2	pounds	lb
t	tonnes (1000 kg)	1.1	short tons	
		VOLUME		
ml	milliliters	0.03	fluid ounces	fl oz
l	liters	2.1	pints	pt
l	liters	1.06	quarts	qt
l	liters	0.26	gallons	gal
m^3	cubic meters	35	cubic feet	ft^3
m^3	cubic meters	1.3	cubic yards	yd^3
		TEMPERATURE (exact)		
°C	Celsius temperature	9/5 (then add 32)	Fahrenheit temperature	°F



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1. INTRODUCTION

Analysis of biological specimens for alcohol[1] is the most commonly performed forensic science procedure and has occupied that position for many decades. More than a dozen analytical principles have been applied to this task, and hundreds of methodological modifications have been developed and published during the past one hundred years for the analysis of alcohol in blood and other biological liquids.

As can be anticipated, many of the published procedures vary greatly from each other in their significant analytical characteristics, practicability features, and consequent applicability, acceptability, and utility for forensic-scientific purposes. This diversity has undoubtedly played a prominent role, together with other factors, in the undesirably and unacceptably wide range of reported results of alcohol analyses upon supposedly identical specimens which has been repeatedly demonstrated by participating laboratories in proficiency surveys (1). The elucidation and elimination of the causes of such inter-laboratory variations in the analysis of biological liquids for alcohol, which presumably also affect the routine workload of many laboratories, would be much facilitated by the availability and use of an analytical method for alcohol in biological specimens whose procedural details and performance characteristics were precisely and comprehensively established, widely known and appreciated, and capable of being commonly if not universally employed as a referee or reference method.

Preparation of a manual containing the details of such a method for alcohol analysis, together with other relevent information, was among the recommendations made be a widely representative group which met in Washington on May 10, 1973, under the auspices of the U. S. Department of Transportation, to inquire into various aspects of the standardization and improvement of

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[1] In this report, the unmodified term *alcohol* refers to ethanol; *BAC* refers to blood-alcohol concentration

forensic alcohol analysis (2).

The author* accepted responsibility for preparation of the manual, and this report constitutes the resultant manual. Its contents are intended and should be considered to be recommendations. No inference is intended, and none should be drawn, that other methods, techniques, and procedures are necessarily inferior to those described herein.

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2. SAMPLES AND SAMPLING FOR ALCOHOL ANALYSIS

2.1 SELECTION OF SPECIMENS[2]

Various factors enter into the decision concerning the selection of appropriate biological specimens for alcohol analysis, either as a matter of general policy or in a given instance. In most jurisdictions, statutory provisions govern specimen procurement from living subjects, in relation to alcohol-related traffic offenses. Leaving aside consideration of breath,[3] some jurisdictions provide that only blood may be collected for analysis for alcohol intended to be introduced into evidence at the trial of alcohol-related traffic offenses, others vest choice of specimen in the subject to be tested, and many impose specific requirements for or limitations upon persons authorized to obtain specified specimen materials such as blood under implied consent statutes or other applicable laws. The respective statutory provisions, when they exist, of course govern the situation in living subjects.

Specimen selection in dead persons who are the subjects of official investigations or inquiries into the death is generally placed, statutorily or by regulation of comparable import, within the discretion of the investigating medicolegal authority, e.g., the medical examiner or coroner system.

Because of these and other practical considerations, it is convenient to consider samples and sampling separately for living and dead persons, and the following subject matter is so arranged.

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[2] In this report, the term *specimen* is generally intended to apply to the entire quantity of a biological liquid obtained for analysis, while the term *sample* generally applies to that portion of a specimen which is subjected to analysis.

[3] Although breath is a suitable specimen for alcohol analysis in law enforcement, the subject of breath-alcohol analysis is not treated in this report.

2.1.1 Living Subjects

Blood. Blood has been the traditional and most commonly obtained biological liquid for forensic alcohol analysis. It has many actual and potential limitations and shortcomings for that purpose (3,4), especially when venous whole blood is the specimen collected, as is almost universally the case. In most jurisdictions, however, the evidential import of the body burden of alcohol, with respect to alcohol-related traffic offenses such as operation of a motor vehicle under the influence of alcohol, is stipulated exclusively in terms of concentration of alcohol in the blood. All existing laws provide for admissibility into evidence of information concerning the alleged blood-alcohol concentration of the accused at the material time.[4]

It is usually appropriate, therefore, to select *blood* as the specimen for analysis in order to obviate the often difficult problem of translating the results of alcohol analyses of other body fluids into corresponding BAC's. When a choice is possible, the physiologically and parmacokinetically most logical and useful "blood" specimen for alcohol analysis is *capillary plasma,* obtained by centrifugation of whole blood collected, from a suitably deep skin puncture, into an anticoagulant-treated micro blood-collection tube. Plasma obtained from anticoagulant-treated whole venous blood is the next choice for a specimen, followed by serum promptly separated from untreated whole venous blood after coagulation and anticoagulant-treated venous whole blood, in that order. *As a practical matter, however, leaving theoretical considerations aside, venous whole blood is generally the most appropriate blood specimen to be collected for alcohol analysis in law-enforcement practice at the time of preparation of this report.*

The usual site of collection for venous blood specimens is an antecubital

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[4] The *material time* is not necessarily the time at which the specimen was obtained and frequently is not; the controlling factors may be established by statute and/or case law and are often highly complex (see Section 4.1).

vein unless special considerations (e.g., injuries) dictate otherwise. Other suitable sites are equally acceptable, it being understood that the concentration of alcohol in a blood specimen obtained from *any* given site is not necessarily identical to that of blood in the contemporaneous peripheral circulation at another site, nor necessarily fully representative of the concentration of alcohol in the blood circulation as a whole.

Cerebrospinal fluid. This is not a specimen material of practical value for alcohol analysis for law enforcement purposes. Its only applicability would presumably arise in the unique circumstance of its sole availability (as a medically-obtained specimen for clinical diagnostic purposes) to the exclusion of other biological liquids.

Saliva. Because of various practical constraints such as the substantial subject cooperation required for its collection, saliva has not been employed as a specimen material for alcohol analysis in law-enforcement practice recently. Parotid saliva is generally considered to be in alcohol equilibrium with the peripheral blood circulation, at the time of secretion. It could thus, in theory, be used to estimate the coexisting blood-alcohol concentration, and it possesses certain practicability advantages such as the relatively non-invasive nature of the feasible collection techniques. However, its suitability for that purpose under current conditions has not been adequately evaluated, nor have possible differences between parotid, sublingual, submaxillary, and pooled saliva been adequately investigated.

In view of the lack of documented suitability and of other information needed for proper interpretation, saliva is not at present considered a suitable specimen for law-enforcement applications of alcohol analysis.

Urine. The only urine specimen which could, in theory, be confidently employed for alcohol analysis for the purpose of estimating the coexisting BAC is *ureteral* urine - clearly an impracticable specimen for law-enforcement

purposes. The hypothetically-acceptable expedient of collecting vesical urine after a specified time interval following voluntary emptying of the bladder by micturition often does not achieve the desired purpose of eliminating the effects of pooled bladder urine upon the alcohol content of the specimen. The various, numerous difficulties in collecting appropriate urine specimens for alcohol analysis and in validly interpreting the results of such analyses for medicolegal purposes - including, especially, estimation of the coexisting BAC - have been extensively documented (5). The 1968 declaration (6) of an ad hoc committee of the National Safety Council's Committee on Alcohol and Drugs, in this respect, subsequently approved by the Committee on Alcohol and Drugs, is still pertinent and applicable:

> "Because of various problems in the interpretation of the results of analyses of urine specimens for alcohol which cannot readily be overcome in law enforcement practice, analysis of urine for the purpose of determining blood alcohol concentration is to be discouraged except under the strictly controlled conditions employed in determining renal solute clearances."

Additional practical constraints upon urine specimens or their collection further limit their suitability for alcohol analysis for law-enforcement purposes, such as the invasion of privacy required to eliminate subsequent legal questions regarding the unequivocal origin of such specimens and the absence of tampering (e.g., dilution with water) with them.

Occurrence of fermentable carbohydrates, especially glucose, in urine is very common, as is contamination with yeast spores and other microorganisms. These and other adventitious factors can lead to alcohol neoformation in voided urine specimens. Unless statutorily mandated, therefore, use should not be made of urine specimens for alcohol analysis for law-enforcement purposes, except in the unique circumstance of sole availability of such a specimen to the exclusion of more suitable specimens; in any event, it should be used with full realization of the inherent, unavoidable strictures and

limitations of urine as a specimen material for forensic alcohol determination and the attendant uncertainties necessarily engendered in the valid interpretation of the significance of the alcohol analysis results. Urine is *not* a recommended specimen material.

In summary, in the living human subject, only blood, saliva, and urine are potentially usable specimens in law-enforcement practice; of these only *blood* is sufficiently free of forensically-disabling disadvantages and contraindications to be a practical specimen material for routine applications. (While *breath* is also a suitable specimen, it is not considered here.)

2.1.2 Cadavers

Although a variety of biological specimens (including tissues and body fluids) can be obtained postmortem for alcohol analysis, examination of *tissues* is a technique generally limited to specialized forensic toxicology laboratories and thus is not further considered here. In dead subjects, particularly those subjected to autopsy, the functional restraints upon invasion in living subjects are, of course, absent and do not limit selection of specimen materials. However, other and often more complex and binding considerations are always present. Admixture of various body liquids and both internal and external contamination are factors which must be minimized; it is often difficult or impossible to ascertain whether and to what extent postmortem biological specimens have been diluted, contaminated, or otherwise altered from their immediate postmortem or agonal state. In persons whose death was preceded by even brief hospitalization or by emergency medical treatment at the scene of injury or during subsequent transportation from the scene, the composition of body fluids may be substantially altered by routine trauma care such as intravenous fluid therapy, blood transfusions, etc., as well as by significant blood loss. The postmortem composition of even uncontaminated, carefully collected specimens, therefore, may not be even remotely representative

of the pretrauma subject state. Alcohol metabolism and excretion during any substantial period of survival after a subsequently fatal injury is also a commonly encountered factor, even when uncomplicated by medical therapy or clinical factors such as shock which can further complicate it.

These considerations, among others, make it highly desirable, if not imperative, to select two or more different specimen materials for alcohol analysis in many cases and/or to collect such specimens from two or more completely dissociated sites, whenever possible.

Blood. Blood is the postmortem specimen of first choice in undecomposed bodies when available and properly selected (with regard to collection site) and collected. Large blood vessels in the extremities (e.g., femoral artery or vein) are the recommended sampling site in unautopsied bodies, in order to obviate such problems as internal specimen contamination through unrecognized organ rupture or by needle penetration into the gastrointestinal tract during attempted blood withdrawal from the heart or great vessels in the thorax. A substantial literature has developed on the alleged contamination of blood in the heart and thoracic great vessels by alcohol diffusion from gastrointestinal contents and on related matters (7-17). Many of these publications are further complicated by confusion between the effects of biological factors (e.g., inter-tissue or inter-organ diffusion, or decomposition), artefactual difficulties created by the collection process or technique (e.g., needle penetration of adjacent hollow organs or external microbial contamination), and the characteristics and limitations of analytical methods (e.g., nonspecificity of chemical oxidation-reduction procedures resulting in unrecognized effects of nonethanol substances).

Postmortem liquid specimens submitted as "blood" frequently bear little resemblance to the circulating whole blood of living subjects. They commonly display all manner and degree of alteration from the in-vivo state in such respects as presence of clots and lipids, hemolysis varying from slight to

complete, dilution with water or biological liquids, and many, often extreme biochemical changes. Several of these factors result from collection of "blood" from pooled liquids in the pericardial or pleural cavities or even from liquid pools in the thoracic or abdominal cavities at autopsy. The contents of these cavities should not be collected and submitted as "blood." When collection of suitable quantities of blood from large peripheral vessels is infeasible, blood properly taken from the intact chambers of the heart is usually an acceptable specimen.

Cerebrospinal Fluid. Because of its distant location from the gastrointestinal tract and its relatively protected position with respect to external contamination, as well as its intimate association during life with the brain, CSF is a most suitable postmortem specimen for forensic alcohol determination (but not for estimation of the BAC).

Urine. The limitations outlined above for urine as a specimen material in living subjects apply fully in the postmortem situation, recognizing that only pooled bladder urine present, if any, can constitute the specimen. When collected with due care to avoid contamination with blood, water, or other substances it may be a functional specimen for analyses of admittedly limited significance or validity. (A common reason for collection of postmortem urine is to examine it for drugs other than alcohol or their metabolites.) It has been reported that alcohol diffusion through the bladder mucosa can occur both antemortem and postmortem (18).

Vitreous Humor. Because of its specially protected location, normal sterility during life, low protein contents, and other useful characteristics, vitreous fluid (or "humor") is a postmortem specimen of choice for alcohol analysis. As with other specimens, due caution is required in the interpretation of the results of alcohol determinations on this material, especially in respect to estimation of the coexisting BAC or antemortem intoxication

states. In decomposing or decomposed cadavers, it will often be significantly less affected than other available specimen materials and is usually accessible without dissection, even in bodies of persons who have died in aviation crashes or which have been partially incinerated. A number of recent studies have described use of vitreous humor as a postmortem specimen for alcohol analysis (or other chemical studies) and the interpretation of the results (19-26).

2.2 COLLECTION OF SPECIMENS

The collection of specimens for forensic alcohol analysis is subject to a combination of either pertinent and relevant or controlling factors of medical, legal, scientific, and technical nature or combination thereof. Several are discussed below, but others of equal pertinence cannot be considered here.

Ideally, the collection procedure and its ancillary processes should accomplish the following:

a. Provide any and all necessary and desirable specimens, in ample quantities and of suitable quality
b. Maintain and secure the *integrity* of each specimen material - i.e., its original unique composition at the time of collection - by avoiding any adventitious change resulting from the collection technique or other factors and by assuring the continued maintenance of that integrity and original composition without significant alteration(s) from biological processes or handling/storage effects or attempted interference, as by tampering
c. Establish and maintain the *identity* of the person from whom the specimen(s) were collected, as well as that of the specimen(s)

themselves with respect to their nature, origin, and significant time factors (place, date, time of collection)

d. Establish and maintain a readily traceable, demonstrably unbroken chain of custody and possession of the specimen(s), with appropriate indication of the identity of all persons associated with each specimen in any capacity from original collector to the last analyst and of the location, date, and time of any transfer(s).

Some individual features of recommended collection procedures and safeguards are set forth by specimen material category below.

Blood. In living subjects, various legal considerations arising from local statutory provisions or applicable case law apply to collection of blood specimens in the law-enforcement context. Two decisions by the U. S. Supreme Court include relevant elements.

In Breithaupt vs. Abram (27), the U. S. Supreme Court sanctioned the withdrawal of blood from an unconscious subject, without his consent, and the subsequent admission of evidence concerning the results of analysis of that blood specimen for alcohol, over the defendant's timely objection at his trial on a charge of motor vehicle homicide. Relevant excerpts from the majority opinion are:

> "...there is nothing 'brutal' or 'offensive' in *taking of a sample of blood when done, as in this case, under the protective eye of a physician...*" (Emphasis added.)
>
> "...the individual's right to immunity from *such invasion of the body as is involved in a properly safeguarded blood test* is far outweighed by the value of its deterrent effect..." (Emphasis added.)

In Schmerber vs. California (28), the U. S. Supreme Court held that the collection of blood for subsequent evidential alcohol analysis may be carried out upon a lawfully arrested subject without warrant and without his consent, under stipulated conditions set forth in the following relevant excerpt from the majority opinion:

> "...Finally, the record shows that the test was performed in a reasonable manner. Petitioner's *blood was taken by a physician in a hospital environment according to accepted medical practices.* We are not thus presented with the *serious questions which would arise if a search involving use of medical technique, even of the most rudimentary sort, were made by other than medical personnel or in other than a medical environment* - for example, if it were administered by police in the privacy of the stationhouse. To tolerate searches under these conditions might be to invite an unjustified element of personal risk of infection and pain..." (Emphasis added.)

The Supreme Court, in Schmerber, also reaffirmed the decision in Breithaupt that it is not a violation of the fourteenth amendment due process for the state to order withdrawal of a blood specimen from an unconscious, nonconsenting person "*by a physician in a simple, medically acceptable manner in a hospital environment.*" (29) (Emphasis added.)

It should be recognized that collection of blood from living subjects, whether by phlebotomy, skin puncture, or otherwise is a professional activity within the scope of clinical laboratory technology, clinical laboratory sciences, and the appropriate healing arts. For full discussion of the several relevant procedural aspects, standard texts and appropriate manuals should be consulted; the recommendations and comments relating to this subject here are not intended as a substitute for such references.

Medical considerations include avoidance of infection of the puncture site, the

necessity for completely aseptic technique, and various other factors beyond the scope of this report. Skin preparation and disinfection before penetration by needle or lancet should be accomplished by means of a medically acceptable and clinically effective *nonalcoholic* (and preferably *nonvolatile*) preparation, in order to obviate later fruitless arguments over the possible contribution of an alcoholic skin disinfectant to any alcohol concentration found in the blood. Aqueous solutions of benzalkonium chloride and of thimerosal, among others, have been successfully used for this purpose, as has an aqueous solution of povidone-iodine. Unduly long application of tourniquets should be avoided during venipuncture both on medical grounds and because the induced stasis can induce hemoconcentration and thus alter the original physiological alcohol distribution between blood at the sampling site and the surrounding tissues as well as between the liquid and formed element portions of the intravascular blood. The blood collection technique should conform, in all significant respects, to currently accepted clinical standards and good practice.

All instruments employed in skin penetration or blood withdrawal must be sterile and pyrogen free at time of use, preferably through prior heat sterilization. The blood containers should likewise be pyrogen-free, sterile, and dry. Employment of individual-use, disposable equipment and supplies of reputable manufacture, accompanied by appropriate manufacturers' affidavits, is to be greatly preferred over use of locally prepared expedients.

Containers for blood specimens and any closure device should be chemically inert, chemically clean, dry, and of suitable durability and dimensions. It follows that glass containers of proper construction are to be preferred over most other materials. Closure devices such as screw caps or stoppers should have only chemically clean and inert surfaces exposed to the blood specimen and should be so designed or employed as to have the required mechanical security against displacement and specimen loss or accessibility. Suitable seals for securing specimen containers such as vials or tubes and outer con-

tainers such as mailing tubes and cartons are readily available commercially. They should have non-recurring serial identification numbers, whether factory or locally affixed.

When venous blood is collected from living subjects, the collected volume should be 5 or 10 ml for convenience in collection and handling to allow replicate analyses or partial sharing of the specimen if necessary at a later time. It is difficult to obtain more than 200 to 500 microliters of blood from a single skin puncture, and that quantity is adequate for modern analysis methods. In cadavers it is possible and desirable to collect larger quantities of blood (e.g., 30 ml) since additional laboratory procedures may be required to validate the blood-alcohol analysis results or to prepare a suitable sample aliquot for analysis. (These recommendations pertain solely to analysis for alcohol and do not comtemplate use of the specimen for other examinations.)

In collection of blood from cadavers, rigorous precautions should be exercised against the various contaminations referred to in Section 2.1 above. In particular, the specimen should be obtained with due regard for the separation of blood components which occurs postmortem by sedimentation and clotting; appropriate mixing or withdrawal of all portions of a specimen pool are desirable. Obviously, blood specimens should be withdrawn *prior to embalming*, whether by intravascular or cavity injection (30), and in particular, blood should not be obtained by displacement drainage or aspiration during vascular injection of embalming fluid. Embalming fluids and their components, expecially formaldehyde, are ubiquitous in mortuaries, cadaver preparation rooms, and autopsy suites. As a necessary precaution against specimen contamination with these materials, blood (and other specimens) should be collected exclusively with clean, individually packaged, initially sealed instruments and containers of commercial or comparable local manufacture.

It is good practice to minimize trauma to and alterations in composition of blood specimens (e.g., hemolysis) during the collection process by use of the largest gauge needles compatible with the medical situation and by removing needles from syringes prior to ejection of the blood into storage containers. In collection of blood by skin puncture, the puncture site should be aseptically dried after disinfection to permit "beading" of the blood and to avoid its dilution. The puncture must be sufficiently deep to allow ready and adequate blood flow. Undue stasis during collection (e.g., "milking" of a digit) should be avoided. The usual skin puncture site in adults is a digit; when the extremities or ear lobes are collection sites, it is good practice to warm the site prior to skin penetration to promote "arteriolization" of the capillary blood.

Cerebrospinal Fluid. The specimen must be collected from cadavers by an appropriately professionally qualified person, normally a pathologist. Precautions should be observed against specimen dilution by water or contamination with blood or other substances. A specimen volume of 10-30 ml is convenient and desirable.

Urine. Since use of this specimen material is not recommended in living subjects, no recommendations are given with respect to collection techniques. If urine is, however, collected, the above precautionary notes should be observed with respect to unequivocal identification and positive establishment of absence of dilution with water or other tampering. Urine from cadavers should be collected with due regard for avoidance of dilution or contamination. A specimen volume of 10-30 ml is convenient and desirable.

Vitreous Humor. Collection should be carried out from cadavers by appropriately professionally qualified persons, with due precautions against specimen dilution or contamination. Withdrawal of all available vitreous humor (typically 2-3.5 ml) is recommended. If vitreous humor is collected from both

eyes, the specimens should be collected and kept separately and identified as to their respective origin.

Commercially-available Collection "Kits" and Containers. Availability of such items is subject to demand, marketing, and other economic considerations, and there has consequently been considerable fluctuation in both availability and cost of commercial materials for blood collection for forensic alcohol analysis in assembled "kit" form in recent years. At the time of this writing (June 1976) the following items intended for collection and forwarding of blood specimens for forensic alcohol analysis were understood by the author to be commercially available:

a. Blood Alcohol Specimen Kit
   Reorder No. KT-101AK
   Manufacturer: Kimble-Terumo, Inc., P. O. Box 605, Elkton, Maryland 21921.

b. Vacutainer® Brand Blood Collection Kit for Blood Alcohol Determinations
   Order No. 4990
   Manufacturer: Protective Products Division of Becton, Dickinson and Co., P. O. Box 291, Grand Prairie,Texas 75050.

c. Vacutainer® Brand Blood Collection Kit for Post Mortem Blood Alcohol Determinations
   Order No. 4991
   Manufacturer: Protective Products Division of Becton, Dickinson and Co., P. O. Box 291, Grand Prairie, Texas 75050.

The specifications of these items, including that of the components and the "kit" contents, were not necessarily in agreement with the recommendations given here. While these specimen collection "kits" were intended for use in the collection of blood specimens for forensic alcohol analysis, they may also be suitable for use with other specimen materials.

Individual components, such as blood collection tubes, syringes, needles, lancets, nonalcoholic antiseptic pads, etc., for local assembly of collection sets are widely available through the clinical laboratory and healing arts supply industry. In many instances, such items as blood collection tubes can be purchased from commercial sources prepared according to local proprietary formulation (e.g., with respect to anticoagulant and preservative formulation, closure, or labeling).

It is necessary to assure absence of tampering with specimen containers and associated collection paraphernalia both before and after they have been employed in collecting specimen materials. Original sealed commercial items serve that purpose where provided with separate, distinctive re-sealing devices. When specimen containers, syringe holders, etc., are locally prepared, it is recommended that the respective glass, plastic, or other containers be sealed, after preparation, with shrink-fit cellulose sealing bands, or caps, as is common practice in the alcoholic beverage, pharmaceutical, and cosmetic industries.[5] When quantities used are sufficiently great, these can be obtained with appropriate proprietary legends, logos, or other designs.

2.3 IDENTIFICATION OF SPECIMENS

Information should be obtained and recorded, appropriately, at the time any specimen is obtained with respect to at least the following items:

a. Full identity of the subject from whom specimen was obtained (complete name, address if known, age, sex, race, whether subject was living or dead when specimen was obtained)
b. Nature of specimen (e.g., blood, vitreous humor, etc.) and anatomical location from which obtained

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[5] These bands are available through the pharmaceutical and cosmetics industry. For commercial sources for this and comparable items, consult the current edition of THOMAS REGISTER (31)

c. Nature and quantity of any additive, preservative, or adjuvant added to the specimen or initially present in the specimen container

d. Geographical location, date, and exact time the specimen was obtained

e. Name and other identifying data (e.g., profession, title, and affiliation) of the person(s) who collected the specimen and established its identity[6] (the simple principle recommended here is the same as underlies the ritual attest every notary public provides with each notarization to establish the identity and authority of the official performing the act)

f. Names and affiliation of any witnesses to the specimen procurement

g. Full identity and other particulars needed (e.g., mailing address) of the official or agency submitting the specimen and/or ordering its collection.

Many of these information items can be provided readily and conveniently by means of a suitable form. Figure 1 illustrates a form which has satisfactorily served this purpose in connection with the submission to the author's laboratory of blood specimens for forensic alcohol analysis. A unique, non-replicating numbering system for specimen containers should be established, and all information forms used should bear the same number as the corresponding specimen container. Reliance on color-coding or other pre-use identification for nature of specimens (e.g., blood) should be scrupulously avoided since ample experience has established that the intended guidelines for use of such containers are frequently disregarded.

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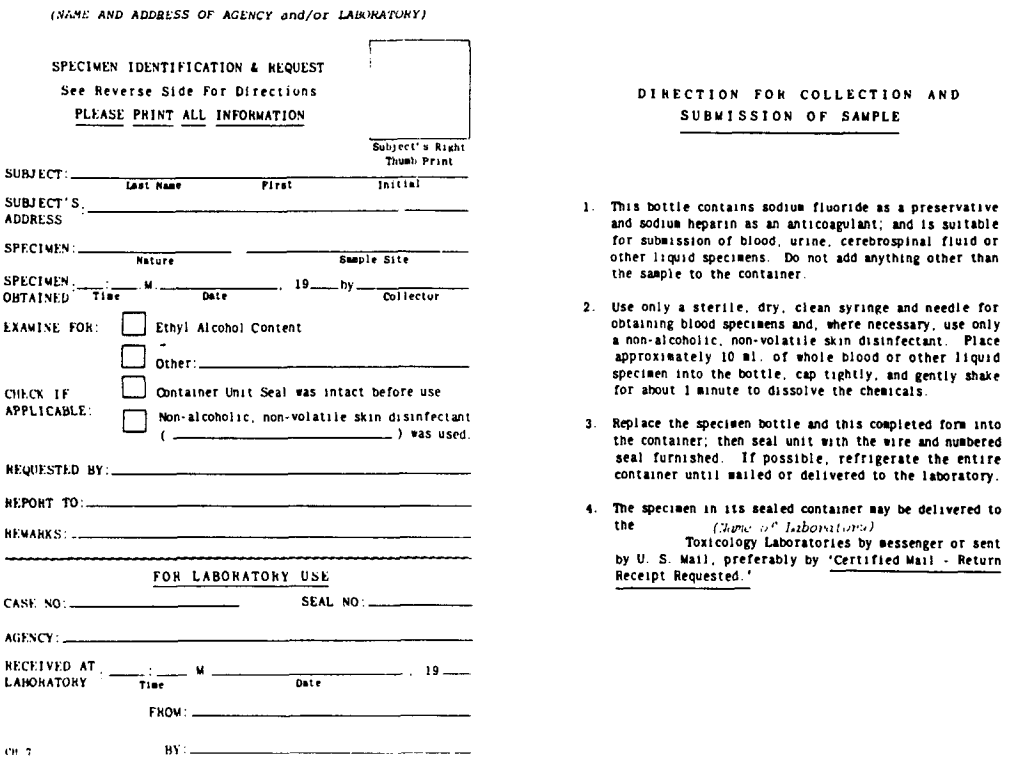
[6] Failure or inability to provide this information (e.g., affirmatively to establish that a person who withdrew a blood specimen from a lawfully arrested subject for subsequent forensic alcohol analysis met the statutory qualifications for such an act) by acceptable means may result at any subsequent trial in suppression of the evidence or reversal of a conviction relying thereon (32).



(NAME AND ADDRESS OF AGENCY and/or LABORATORY)

SPECIMEN IDENTIFICATION & REQUEST
See Reverse Side For Directions
PLEASE PRINT ALL INFORMATION

Subject's Right Thumb Print

SUBJECT: _____ (Last Name) (First) (Initial)

SUBJECT'S ADDRESS: _____

SPECIMEN: _____ (Nature) _____ (Sample Site)

SPECIMEN OBTAINED: ___:___ M. _____ (Time / Date), 19___ by _____ (Collector)

EXAMINE FOR: ☐ Ethyl Alcohol Content
☐ Other: _____

CHECK IF APPLICABLE: ☐ Container Unit Seal was intact before use
☐ Non-alcoholic, non-volatile skin disinfectant (_____) was used.

REQUESTED BY: _____

REPORT TO: _____

REMARKS: _____

FOR LABORATORY USE

CASE NO: _____ SEAL NO: _____

AGENCY: _____

RECEIVED AT LABORATORY: ___:___ M _____ (Time / Date), 19___

FROM: _____

BY: _____

DIRECTION FOR COLLECTION AND SUBMISSION OF SAMPLE

1. This bottle contains sodium fluoride as a preservative and sodium heparin as an anticoagulant; and is suitable for submission of blood, urine, cerebrospinal fluid or other liquid specimens. Do not add anything other than the sample to the container.

2. Use only a sterile, dry, clean syringe and needle for obtaining blood specimens and, where necessary, use only a non-alcoholic, non-volatile skin disinfectant. Place approximately 10 ml. of whole blood or other liquid specimen into the bottle, cap tightly, and gently shake for about 1 minute to dissolve the chemicals.

3. Replace the specimen bottle and this completed form into the container; then seal unit with the wire and numbered seal furnished. If possible, refrigerate the entire container until mailed or delivered to the laboratory.

4. The specimen in its sealed container may be delivered to the (Name of Laboratory) Toxicology Laboratories by messenger or sent by U. S. Mail, preferably by 'Certified Mail - Return Receipt Requested.'

FIGURE 1. SPECIMEN IDENTIFICATION AND EXAMINATION REQUEST (OBVERSE AND REVERSE SIDES SHOWN)

Provision must be made for establishment and documentation of the requisite physical security of all specimens from the moment of procurement and for the customary chain of custody and possession as the specimen changes location and/or hands. Discussion of further details is beyond the scope of this report.

2.4 PRESERVATION AND STORAGE OF SPECIMENS

The purpose and function of preservatives or other additives used to treat biological specimens is basically to prevent or retard normal biological changes (such as coagulation of blood), to prevent or retard decomposition of the specimens, and to assist in maintaining the original chemical composition of such specimens by preventing loss of any component or constituent initially present and generation of any neoformed substances or compounds. Toward these goals, use of additives is often, desirably, combined with appropriate physical measures such as hermetic closure of containers and low-temperature storage.

No single set of treatments or conditions has been found equally suitable and efficacious for all possible uses, and the recommendations made here are addressed specifically only to the task of facilitating maintenance of the integrity and initial composition of biological liquids intended for forensic alcohol analysis. Ideally, no additives should be employed, and sole reliance should be placed on physical measures such as storage at sufficiently low temperatures. However, freshly shed blood from living subjects will promptly coagulate, and chemical treatment will be required to prevent such coagulation since unclotted whole blood specimens are easier to analyze. Further, specimens are often collected remote from the site of analysis, and transportation over long distances and times under often extreme conditions of heat is a common requirement.

Accordingly, chemical treatment of biological liquids by means of additives is a common, if not universal practice. Although the following procedures have been found satisfactory, no implication is intended that others are not equally effective and useful.

Blood. Freshly drawn blood from living subjects requires immediate treatment with anticoagulants to prevent coagulation under the routine conditions here contemplated, and such anticoagulant treatment can be efficiently combined with use of preservatives to retard specimen decomposition and alcohol loss or neoformation. While most postmortem blood specimens will not further coagulate in vitro, it is more convenient to treat blood from both living and dead subjects alike.

Suitable anticoagulants are citrate, edetate, oxalate salts, and heparin. Fluoride salts have both anticoagulant and preservative action, and various mercury salts and azide salts are also suitable preservatives. A combination of sodium citrate or potassium oxalate (5 mg/ml blood) with sodium fluoride (1 mg/ml blood) has been commonly employed.

The author has for some years successfully employed the combination of 0.5 mg heparin sodium and 1.5 mg sodium fluoride per ml blood as an anticoagulant-preservative mixture for freshly-drawn sterile blood from living subjects. It preserves the original alcohol contents of such specimens without detectible changes for at least 18 days at ordinary room temperature, for more than 1 month when the blood is refrigerated at 5°C., and apparently indefinitely when the specimen is stored at -20°C. (33). The mixture is conveniently dispensed, in liquid form, into specimen containers in quantities which will yield at least the stated concentrations when the containers are filled to capacity, and the additives are then deposited in film form on the container walls by low-heat (below 90°C) evaporation of the water. Disodium edetate (1 mg/ml blood) can be substituted for the heparin.

Postmortem blood specimens, with their inherent risk of microbial contamination

before sampling require higher concentrations of preservatives to prevent bacterial alcohol production and significant enzymatic changes. Use of sodium fluoride in a concentration of 1% w/v (10 mg per ml blood) was found effective by Blackmore (17), and by Glendening and Waugh (34) who found no significant changes from the original alcohol contents of blood specimens, thus treated, after 2 months storage at room temperature or after 10 months storage at 1°C. Plueckhahn (35) found that a sodium fluoride concentration of 0.2% w/v did not consistently prevent alcohol neoformation in autopsy blood specimens contaminated with microbial inoculates but that 1% w/v of sodium fluoride was an adequate preservative for autopsy blood specimens. Likewise, Blume and Lakatua (36) reported that addition of 10 mg sodium fluoride per ml of blood (i.e., 1% w/v) prevented production of ethanol in bank blood after 24 hours of incubation at 37°C despite initial deliberate microbial contamination, except for specimens inoculated with C. albicans. Bradford (37) has proposed use of 5 mg sodium citrate and 0.1 mg mercuric chloride per ml of blood as the anticoagulant-preservative mixture of choice, reporting that blood specimens containing alcohol and treated with mercuric chloride (1:10,000) were stable at room temperature for periods of six months and longer. Christopoulos et al. (15) found that when autopsy blood specimens were treated with 1% w/v of sodium fluoride, no alcohol was produced after refrigerated storage of up to 45 days in originally alcohol-free specimens, while those with initially elevated concentrations of alcohol or alcohol and glucose demonstrated only slight changes (predominantly insignificant alcohol decreases) after refrigerated storage of up to 45 days.

The stability of ethanol in stored blood specimens was extensively investigated by Smalldon, Brown, and associates (38-40), who found that human erythrocytes could oxidize ethanol to acetaldehyde in such specimens, that the mechanism for alcohol loss which was linked with the oxyhemoglobin-methemoglobin redox system was strongly temperature-dependent, and that these

losses were *not* inhibited by 1% w/v sodium fluoride. Sodium azide, dithionite, and sodium nitrite were found to inhibit alcohol oxidation significantly during storage of blood specimens for 16 weeks at 20°C. The combination of at least 1% w/v sodium fluoride and 0.5% w/v of sodium nitrite added to blood was recommended to inhibit both alcohol neoformation and alcohol loss. Marbach et al. (41) investigated glycolysis in specimens of whole blood and serum, and found that sodium iodoacetate (0.05 to 0.2% w/v) and sodium fluoride (0.25% w/v) both satisfactorily inhibited glycolysis in such specimens at room temperature for periods of up to 24 hours.

High concentrations of both inorganic anticoagulants such as sodium oxalate and preservatives such as sodium fluoride lead to hemolysis of the blood specimen as well as to alteration of the concentrations of plasma components through erythrocyte shrinkage and water displacement. These effects can be disregarded when whole blood is the sample subsequently analyzed for its alcohol content, the specimen is well mixed before withdrawal of the analyzed aliquot, and the latter is completely hemolyzed prior to analysis. However, these effects cannot be tolerated if the desired, retained specimen is blood plasma; in that case, heparin sodium or disodium edetate or dipotassium edetate should be employed as the anticoagulant, followed by prompt separation of the supernatant plasma from the formed blood elements by centrifugation, and further preservation of the plasma with low concentrations of sodium or potassium fluoride (i.e., 0.5-1 mg per ml of blood). High concentrations of additives, especially inorganic salts, also alter the density and solids content of whole blood substantially, a factor that must be taken into consideration in analysis methods which involve the weighing of samples or the use of headspace gases above untreated samples. (The analysis methods described herein are not affected adversely by this factor.)

The several combined anticoagulant-preservatives mixtures can be placed into specimen containers in powder form, as well as deposited as dried films therein. In the former situation, a warning should accompany the container against

discarding the additive as a presumed contaminant, and in the latter a warning against rinsing of the container prior to collection of the specimen is appropriate. When blood is collected with capillary micro-collection tubes, the anticoagulant-preservative mixture must be deposited on the walls as dried films. Thorough mixing of the specimen with the additives is required.

In addition to chemical treatment, as outlined above, the blood specimen should be stored at reduced temperature until after analysis. When whole blood is to be analyzed, such storage should preferably be at -20°C or lower temperature, which permits long-term storage without significant changes in original alcohol contents of the specimen. The hemolytic effect of freezing is an automatic preparation step for subsequent analysis, but care must be exercised upon removal from low-temperature storage properly to reconstitute the specimen by effective mixing after thawing. Storage at ordinary refrigerator temperature (about 3-5°C) will suffice for short-term storage (up to 4 weeks) of blood specimens which have been properly chemically preserved, as outlined above, or when immediate separation of supernatant plasma or serum from cellular components of blood[7] cannot be accomplished. Refrigeration is now widely available in transportation facilities, and blood specimens for alcohol analysis should not be left at room or higher temperatures for periods of hours or longer when avoidable.

Storage in glass containers with adequate mechanical closures (screw-caps secured with tape) is recommended since many polymeric substances (plastics) are not impervious to gaseous or vapor state substances, including ethanol, over prolonged periods, and since leaching of plasticizers from such container materials into the specimens is an undesirable event.

Although alcohol loss from liquid specimens into the airspace above them in

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[7] It is noted, parenthetically, that centrifugal separation of blood components should be carried out in sealed containers since the substantial air movements thus generated will cause loss of alcohol and water from open specimen containers, thus substantially altering the original specimen composition.

properly-sealed containers is negligible at room or lower temperatures because of the favorable partition ratio, container volume should be suitably proportional to anticipated specimen volume.

Cerebrospinal Fluid. For all practical purposes in preservation and storage, CSF specimens can be treated like whole blood. Since they will generally be of autopsy origin, they should be considered microbially contaminated, and low temperature storage, preferably at -20°C., should be routinely employed. In other respects, the recommendations and comments given under *Blood*, supra, apply.

Saliva. Saliva specimens can be treated like blood with respect to preservation and storage, although less intensive chemical treatment will suffice to assure stability of their alcohol content because of the absence of many of the blood components. Addition of picric acid (0.1-0.5% w/v) has been recommended (42). Friedemann et al. (43) employed sodium fluoride (2 mg per ml of saliva) for preservation, combined with refrigeration, and Nanikawa and Kotoku (44) treated saliva with zinc sulfate (2 mg per ml of saliva), or cupric sulfate (20 mg per ml of saliva), or mercuric chloride and hydrochloric acid (0.4 mg/ml and 1 ml of N/10 per ml of saliva, respectively). Low-temperature storage of saliva specimens is indicated.

Urine. These will generally be specimens obtained at autopsy and must be assumed to be microbially contaminated. The author has effectively employed preservation with 15 mg of sodium fluoride per ml of urine (in dried film form pre-deposited on container walls with allowance for the maximum contents) combined with low-temperature storage; saturation of the urine specimen with excess benzoic acid (approx. 5 mg per ml of urine) was equally effective (33). Phenylmercuric nitrate has been commonly employed as a urine preservative, usually added to specimen containers in tablet or powder form to provide sufficient preservative for a saturated solution (about 1:1000). Christopoulos et al. (15) reported slight decreases from original alcohol

contents of urine specimens obtained at autopsy and stored for periods up to 30 days at room temperature after treatment with phenylmercuric nitrate; none demonstrated any significant neoformation of alcohol in originally alcohol-free urine specimens. Refrigeration, with and without preservative, of autopsy-derived urine specimens yielded comparable results Reduction of the pH of urine specimens will delay and minimize decomposition with formation of ammonia when combined with refrigeration; hydrochloric acid is a satisfactory additive for this purpose to yield a pH of 1-3. In clinical practice, numerous volatile organic compounds or substances have been employed, alone or in combination, to retard decomposition of urine (camphor, formalin, thymol, etc.); all of these should be avoided in preserving urine specimens for alcohol analysis. Urine specimens can generally be treated like blood specimens with respect to chemical preservation with due allowance for the specimen volume concerned.

Urine specimens should be stored at low temperature, before and after analysis. Freezing of such specimens at -20°C. or lower temperature is most satisfactory; care is indicated in subsequent thawing to mix the specimen well since layering occurs during freezing. The specimens should be stored in glass containers; transfer from any plastic or cardboard containers used in collection or clinical examinations should be carried out as soon as possible.

Vitreous Humor. For all practical purposes, these specimens can be treated like blood, with respect to preservation and storage. Being of autopsy origin, they should be considered to be microbially contaminated, and accordingly should be stored at low temperature until analyzed.

2.5 PREPARATION FOR ANALYSIS

Biological specimens tend to be nonhomogeneous systems in which various changes occur with time, including biochemical, chemical, and physical alterations. Some of these are of spontaneous, natural origin, while others are storage-induced artefacts such as the hemolysis of blood specimens induced by freezing. Good laboratory practice therefore requires examination of biological specimens prior to analysis, especially when they have been subjected to prior storage and after reconditioning or rehabilitation of such specimens prior to removal of aliquot samples, and appropriate preparation to counteract any such effects.

Preliminary Observations and Measurements. Visual observations should be made and recorded of such specimen characteristics as appearance, clarity, color, apparent homogeneity or inhomogeneity, and viscosity, as appropriate. In blood specimens, color, evidence of hemolysis, presence of clots, and odor should be especially noted; in urine specimens, the foregoing observations and preliminary pH measurement are useful. Measurement or estimation of initial specimen volume or quantity is useful. Occasionally, repeating the same observations after mixing or other preliminary specimen treatment is desirable.

Reconditioning or Rehabilitation of Specimens. All biological liquids should be vigorously mixed, in their original containers, to restore the maximum feasible specimen homogeneity. Thereafter, depending on nature of the specimen, it may be desirable to employ homogenization technique, or alternatively, to employ separational procedures such as centrifugation or filtration to obtain the most suitable sample for analysis. If a blood fraction, such as plasma or serum is the desired specimen, centrifugation in closed vessels to obtain a supernatant liquid free of formed blood elements, cellular debris, and other particulate matter is the initial step, followed by prompt and

careful removal of the supernatant phase. When whole blood is to be analyzed, thorough mixing to restore the original liquid/cell distribution is indicated; if clots are present, it is desirable to homogenize the entire specimen by mechanical or ultrasonic techniques.[8] The incidental cell disruption and hemolysis is a helpful side effect of effective homogenization. For other well-mixed liquids, such as cerebrospinal fluid, urine, and vitreous humor in which alcohol distribution can be safely assumed to be uniform, the aim is to eliminate precipitated proteins, debris, and other particulate and solid matter from the aliquot analyzed. Centrifugation, filtration, or occasionally simple sedimentation can be advantageously employed for this purpose.

Recording of Particulars. In addition to and apart from the routine but careful noting and recording of identification data concerning the specimen and its container(s) as well as the usual data concerning location, date and time of the examination, apparent absence of tampering with the specimen (if applicable), and identification of the examiner, an initial contemporaneous, permanent record should be made of all steps of the examination including significant omissions. If a prescribed, written protocol for preparation and analysis of specimens is available and used, a notation to that effect suffices, giving sufficient details to identify the protocol and any discretionary actions. In the absence of such complete protocol, all observations, manipulations, procedural steps, computations, and other component parts of the analysis should be contemporaneously entered into a permanent notebook and authenticated by the analyst.

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[8] For occasional use or small specimen volumes, tissue grinders with Teflon pestles are convenient and efficient; e.g., Cat. No. 3431-E04 through 3431-E25, Arthur H. Thomas Co., P. O. Box 779, Philadelphia PA 19105

3. ANALYSIS OF BIOLOGICAL LIQUIDS FOR ETHANOL

This section contains the operational and procedural details of the method for alcohol analysis in biological liquids which has been selected for presentation in this report, together with information concerning the performance characteristics of the method.

The basic method presented here is analysis of alcohol in the equilibrated headspace above liquid specimens by means of gas chromatography. Four versions of the basic method are described:

1) Automated gas chromatographic analysis of headspace vapor *without* use of internal standards
2) Automated gas chromatographic analysis of headspace vapor *with* use of internal standards
3) Manual gas chromatographic analysis of headspace vapor *without* use of internal standards
4) Manual gas chromatographic analysis of headspace vapor *with* use of internal standards.

The method described is based on that of Machata (45-47). For convenience in reference, the automated and manual procedures are described separately, although they are in fact modifications of the same method.

3.1 PRINCIPLES

From the general gas law PV = nRT and Dalton's law of partial pressures, it follows that the partial pressure of a gas or conforming vapor, for dilute solutions, is directly related to the mole fraction (or concentration) of that component in the mixture and to the absolute temperature of the system. Further, Raoult's law states that the partial vapor pressure of a constituent is proportional to its mole fraction in the liquid at all compositions; Henry's law can be expressed

$$\frac{\text{Concentration of gas or vapor in gaseous phase}}{\text{Concentration of gas or vapor in liquid phase}} = \text{constant}$$

at definite temperature, independent of pressure (48). At isothermal equilibrium of a dilute solution of volatile substances and the adjacent gas phase in a closed system, therefore, the concentration in the liquid phase of the component of interest can be determined from experimental measurement of the concentration of that component in the gas phase. In the methods considered here, the "headspace" above the sample constitutes such a gas phase. The functional relations can be expressed as follows:

$$P_{alc} = k_{alc} \times c_{alc}$$

$$P = \Sigma P_i$$

$$P = P_{alc} + P_{H_2O} + P_x + \ldots$$

where P_{alc} = Partial Pressure of Ethanol

k_{alc} = Ostwald Partition Coefficient for Ethanol

c_{alc} = Concentration of Ethanol in the solution (or liquid mixture)

P = Total Pressure

P_i = Partial Pressure of any Compound Present.

Further, gases and vapors are generally less soluble in aqueous solutions of electrolytes than in pure water, a phenomenon known as the *salting-out effect* (49).

The basic method thus utilizes the principle of isothermal equilibrium partition of ethanol and other volatile substances between liquid samples and the "headspace" gas in a closed system, and employs gas chromatographic

analysis to separate the components of the headspace vapor, allowing individual quantitation of their respective concentrations by means of a flame ionization detector.[9] Addition of sodium chloride to the liquid sample in sufficient quantity to assure saturation eliminates differences between blood and other biological liquids and aqueous alcohol solutions in their respective ethanol partition into air, and thus allows use of aqueous reference standards. Quantitation of the detector signal response is accomplished by manual measurement of the resultant trace peak heights on a potentiometric recorder chart, or by mechanical or electronic integration of the peak areas, or by other measurement of the electrical detector signal as a function of time. These data are translated into corresponding concentrations of ethanol and other substances of interest in the sample by comparison with suitable contemporaneous calibration standards. Identification of ethanol and other components of interest is established by comparison of their relative retention times with those of contemporaneous reference samples.

The several physico-chemical relationships are fixed. For consistent, reproducible conditions the functional constants for a given system can be experimentally determined by suitable calibration techniques and then applied to the experimental determination of the liquid phase composition.

The ratio of ethanol concentration in the gas and liquid phases (also termed partition coefficient or partition ratio) is temperature dependent. The thermodynamic theory for the mixtures of interest indicates that the logarithms of the partition coefficients should vary linearly with the reciprocal of the absolute temperature; for narrow ranges the linear relation should also hold for absolute temperature or system temperature expressed as ^{o}C. This relation is illustrated in Figure 2 for the data of Harger et al. (50). The higher partition ratio, at a given temperature, for the

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[9] Presumably, other detectors can be used, provided they have sufficient sensitivity and linearity for the volatiles involved and low response to water.

air/blood system compared to the air/water system is presumably the result of the difference in water content and the salting-out effect. These differences can be abolished by saturation of the liquid phase with soluble inorganic salts, which also substantially increases the absolute value of the partition ratios.

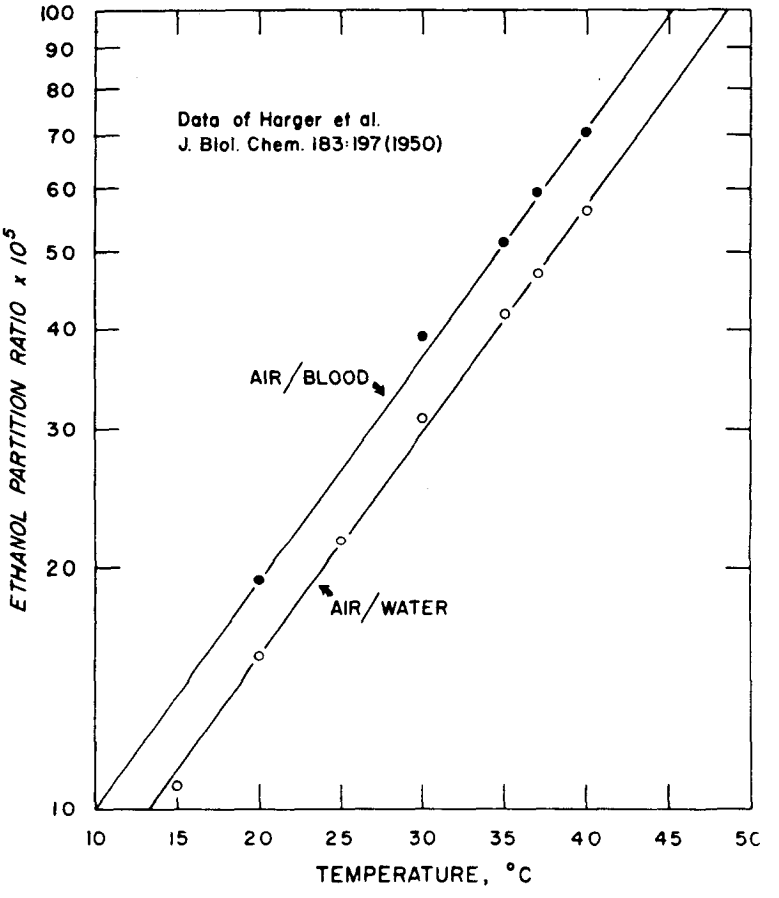

FIGURE 2. IN-VITRO ETHANOL PARTITION RATIOS FOR AIR/BLOOD AND AIR/WATER SYSTEMS (DATA OF HARGER ET AL. (50))

3.2 AUTOMATED METHOD

3.2.1 Materials: Apparatus, Reagents, Supplies

Apparatus

1. Automated Gas Chromatographic Headspace Analyzer (Model F-40 Multifract,[10] Perkin-Elmer Corp., Norwalk, Conn. 06856)
2. Potentiometric Strip-Chart Recorder, 250 mm. (Model 194; Honeywell, Inc., Ft. Washington, Pa. 19034); equipped with Mechanical Trace Integrator (Model 252-A, Disc Instruments Inc., Santa Ana, Calif. 92705) and with Automatic Printing Integrator (Model 610, Disc Instruments, Inc., Santa Ana, Calif. 92705)
3. Automatic Diluter (Cat. No. 2112, York Instrument Corp., Berkeley, Calif. 95710)
4. Hydrogen Generator, Elhygen (Model ER-150, Milton Roy Co., St. Petersburg, Fla. 33733).

Reagents

1. Calibration Reference Materials (Calibrators)
   a. Ethanol, 0.10, 0.20, 0.40% w/v (1.00, 2.00, 4.00 g/Liter) Aqueous Solutions
   b. Mixed Calibrator, "Low" (Acetone 1.00 g/Liter; Ethanol 2.00 g/Liter; Isopropanol 1.00 g/Liter; Methanol 1.00 g/Liter Aqueous Solution)
   c. Mixed Calibrator, "High" (Acetone 1.00 g/Liter; Ethanol 4.00 g/Liter, Isopropanol 1.00 g/Liter; Methanol 1.00 g/Liter Aqueous Solution)

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[10] The Model F-42 instrument can also be used.

2. Internal Standard Solution

   Acetonitrile, A.R., Nanograde, 0.15% v/v

   (1.50 ml. per Liter Aqueous Solution)

3. Sodium Chloride, A.R., granular[11]

Supplies

Glass Vials, 24 ml., with polymer septum stoppers and aluminum seals (Cat. No. 105-0118, Perkin-Elmer Corp., Norwalk, Conn. 06856)

3.2.2 Summary of Operation

The liquid sample (10 microliters to 1 ml) is placed into a glass septum vial (after dilution in fixed proportion with the internal standard solution when that procedural modification is used) together with sufficient sodium chloride to assure saturation. Reference samples are similarly treated, and all vials are sealed with a polymeric septum stopper and a crimped aluminum cap and inserted into the thermostated instrument turntable, which has a capacity of 30 vials. After a 45-minute equilibration period, the septum vials are sequentially presented to the chromatograph in accordance with a preset instrument control program. A dosing capillary penetrates the septum cap of the vial, and pressurization of the vial with N_2 occurs. A highly reproducible quantity of the headspace vapor is then pneumatically transferred to the separating column, and the analysis proceeds for an adjustable, preselected analysis time. The response of the flame ionization detector is recorded, as a function of time, on a potentiometric stripchart recorder and/or monitored at a separate detector signal integrator output. Each sample is identified on the recorder chart by the relative length of an identification trace which precedes the respective chromatogram.

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[11] The author has successfully substituted food-grade salt (Kraft Salt, ~1 g. packet, Kraft Foods Div., Kraftco Corp., Chicago, Ill. 60611) for the reagent material.

The sequence of these steps in the automated headspace analysis is illustrated in Figure 3.

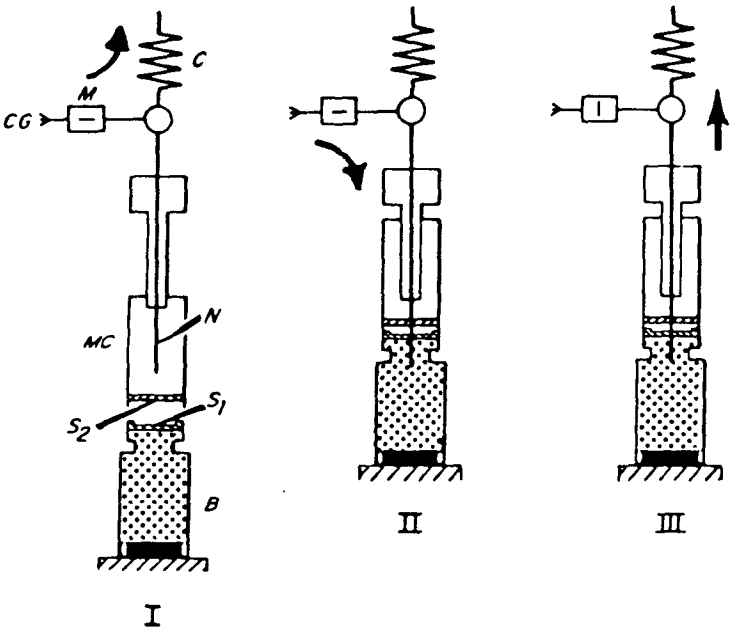

FIGURE 3. PRINCIPLE OF HEADSPACE SAMPLING WITH THE MULTIFRACT F-40 AUTOMATED GAS CHROMATOGRAPH (STEPS SHOWN ARE INITIAL NORMAL STATE (I), PRESSURIZATION (II), AND HEADSPACE SAMPLING (III). B = SAMPLE VIAL; C = CG COLUMN; CG = CARRIER GAS; M = SOLENOID VALVE; MC = MOVABLE DOSING HEAD CYLINDER; N = DOSING NEEDLE; S_1 = SAMPLE VIAL SEPTUM; S_2 = MOVABLE CYLINDER SEPTUM)

3.2.3 Instrument and Analysis Conditions

The following are typical instrument and analysis conditions which have been found satisfactory in the author's laboratory. Numerous modifications of these conditions are also acceptable, or occasionally preferable in special circumstances.

Column:	Carbowax 1500 (0.4%) on 60/80 mesh Carbopack A (51, 53) 1.8 M x 3.2 mm O.D. (6 ft x 1/8 in) stainless steel column	
Carrier Gas:	Nitrogen; inlet pressure	20 psig
	manometer	1.1 kp/cm^2 (117.7 kPa)
	flow rate	35 ml/min.
Temperatures:	Heating bath (turntable)	65°C ± 0.03°C
	Dosing line	175°C
	Injection block	150°C
	Column oven	80°C
FID:	Hydrogen; inlet pressure	20 psig
	manometer	1.1 kp/cm^2 (107.9 kPa)
	flow rate	35 ml/min.
	Air; inlet pressure	28 psig
	manometer	1.2 kp/cm^2 (117.7 kPa)
	flow rate	300 ml/min.
Program:	Pressurization	60 seconds
	Injection	3 seconds
	Analysis	300 seconds
	Flushing (Purge)	12 seconds
	Stabilization	12 seconds
	Analyses/vial	1
Electrometer:	Range	10
	Attenuation	x 128
Baseline Correction:	Automatic	
Recorder:	Input Range	1 mv
	Chart Speed	5 mm/minute

3.2.4 Procedure - Direct Analysis

1. A well-mixed aliquot of the blood specimen,[12] 1 ml in volume,[13] is delivered into each of *two* clean, dry, numbered septum vials which contain approximately 1 gram of crystalline sodium chloride. The vials are closed with a polymeric stopper and sealed with a crimped aluminum cap, and the contents are thoroughly mixed by horizontal rotation.

2. Ethanol or mixed-volatiles reference solutions (normally the "Low" and "High" mixed Calibrators) are similarly treated, and all sealed vials are inserted into the preheated turntable water bath at 65°C and allowed to equilibrate for at least 45 minutes. Calibrators are placed at the beginning, middle, and end of a run.

3. Automatic gas chromatographic analysis of headspace vapor from each vial is initiated after the minimum equilibration period, and the potentiometric strip-chart recorder and peak-area integrator-printer (and/or other signal conditioner and recorder) are activated.

4. The analysis record is appropriately identified (we routinely employ a date/time stamp imprint also naming the laboratory), and a stamp with the chief particulars of the analysis conditions (Figure 4) is affixed.

Operator	Date
Column	Detector
Length	Range
Dia.	Atten
Liquid Phase	Flow Rates, ml/min
Wt. %	Hydrogen Air
Support	Scavenge
Mesh	Split
Carrier Gas	Temperature, °C
Rotameter	Det. Inj.
Inlet Press psig	Column Initial
Rate ml/min	Final
CHART SPEED	Rate
SAMPLE	Solvent
Size	Concn.

FIGURE 4. STAMP FOR RECORDING PARTICULARS OF GAS CHROMATOGRAPHIC ANALYSIS

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[12] For simplicity and brevity the procedural directions were limited to blood. Other biological or nonbiological liquids are treated identically except for possible preparation (e.g., dilution of alcoholic beverages).

[13] In this modification of the method, the sample volume is not a critical variable, and use of pipettes calibrated "to contain" is not required; however, see Section 3.4 and Figure 7 below.

5. Results[14] are computed as follows:

a. By *Peak Height* Measurements

$$BAC_{unknown} = \frac{PH_{unknown}}{PH_{calibrator}} \times Conc._{EtOH,\ calibrator}$$

where PH = Peak Height of the Ethanol Detector Response Recording.

b. By *Peak Area* Measurements

$$BAC_{unknown} = \frac{PA_{unknown}}{PA_{calibrator}} \times Conc._{EtOH,\ calibrator}$$

where PA = Peak Area of the Ethanol Detector Response Recording.

6. Ethanol and other volatile substances in the sample, if present, are identified by their relative retention time(s) in comparison with those of the *contemporaneous* reference solutions.

7. *Micro-Sample Modification:* If indicated because of restricted specimen volume, the above procedure is modified as follows, without other changes in preparation or analysis of samples or computation of results: A well-mixed 100 µl specimen aliquot is delivered into each of two clean, dry numbered septum vials which contain 0.1 gram of crystalline sodium chloride. Calibrators are treated identically.

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[14] Concentration of ethanol in the unknown and reference samples can be expressed in any desired units, arbitrary or otherwise. In recognition of common current practices, it is recommended that ethanol concentration of blood and other biological specimens be reported in *percent w/v* units (% w/v) to two decimal places, truncated (i.e., with the third or additional digits dropped *without* rounding), or in *grams per liter* (g/liter) to two decimal places, truncated.

8. *Ultra-Micro Sample Modification*:[15] An aliquot of precisely 40.0 µl of well-mixed specimen is diluted with fully quantitative technique (using pipettes calibrated *"to contain"*) with 0.9% w/v aqueous sodium chloride to a total volume of 200 µl. A 100 µl aliquot of that diluted sample is then placed into each of two clean, dry numbered septum vials which contain 0.1 gram of crystalline sodium chloride. Calibrators are treated identically. Appropriate change in electrometer range and/or attenuation settings may be needed to achieve suitably large recorder response.

3.2.5 Procedure - Internal Standard Analysis

1. A well-mixed aliquot[16] of the blood specimen is mixed with an identical volume of the acetonitrile internal standard solution, using fully quantitative technique (including pipettes calibrated *"to contain"* when an automatic diluter is not used). Normally, the respective volumes are both 500 µl. The sample measurement and internal standard solution measurement and mixing are carried out with an automatic diluter (appropriately preset, with calibration verified by weighing of sampled and dispensed quantities), and the sample-internal standard mixture is delivered directly into one clean, dry numbered septum vial containing 1 gram of crystalline sodium chloride.

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[15] For the analytical system and procedure described, the gas chromatograph electrometer is normally operated at 1/1280 or 1/640 of maximum sensitivity. It is thus feasible to detect and quantitate ethanol in sample volumes varying from the routine sample volume by at least these factors. Additional sensitivity can be gained by increases in equilibration temperature and/or use of inorganic salts other than sodium chloride

[16] Provided a simple fixed ratio of blood/internal standard solution is maintained for both unknown specimens and calibrators within a given analytical run, the exact volumes and proportion are not critical variables. However, for reproducibility of detector response, etc., between runs, it is recommended that the fixed volumes and proportion given be uniformly employed.

2. Ethanol or mixed-volatiles reference solutions (normally the "Low" and "High" mixed Calibrators) are treated *identically*.

3. The remainder of the procedure for analysis is identical with that described in Section 3.2.4. It may be desirable to employ an electrometer attenuation setting 0.5x that normally used with the Direct Analysis procedure, in order to achieve sutably large recorder responses to the detector signal.

4. Results are computed as follows:

a. By *Peak Height* Measurements

$$BAC_{unknown} = \frac{R_1}{R_2} \times Conc._{EtOH,\ calibrator}$$

where PH = Peak Height of the Indicated Detector Response Recording (e.g., PH_{is} = Peak Height of the Acetonitrile Internal Standard Response)

$$R_1 = \frac{PH_{EtOH}}{PH_{is}}$$ for the unknown sample-internal standard mixture

$$R_2 = \frac{PH_{EtOH}}{PH_{is}}$$ for the calibrator-internal std. mixture.

b. By *Peak Area* Measurements

$$BAC_{unknown} = \frac{R_1}{R_2} \times Conc._{EtOH,\ calibrator}$$

where PA = Peak Area of the Indicated Detector Response Recording (e.g., PA_{is} = Peak Area of the Acetonitrile Internal Standard Response)

$$R_1 = \frac{PA_{EtOH}}{PA_{is}}$$ for the unknown sample-internal standard mixture

$$R_2 = \frac{PA_{EtOH}}{PA_{is}}$$ for the calibrator-internal std. mixture.

5. *Micro-Sample Modification*: When a suitable mechanical diluter is available, exactly 50.0 µl of well-mixed specimen are diluted with exactly 50.0 µl of the acetonitrile internal standard solution, with fully quantitative technique, and the mixture is delivered into a single dry, clean numbered septum vial which contains 0.1 gram of crystalline sodium chloride. Alternately, larger *identical* volumes of the specimen and of the internal standard solution are premixed with fully quantitative technique, and approximately 100 µl of the resultant mixture is employed as the sample for analysis. Calibrators are treated *identically*.

6. *Ultra-Micro Sample Modification*: Using a suitable mechanical diluter, exactly 20.0 µl of well-mixed specimen are diluted with exactly 80.0 µl of a *modified* 0.09375% v/v acetonitrile internal standard solution (containing 0.9375 ml of acetonitrile per liter of aqueous solution) and delivered directly into a single, dry, clean, numbered septum vial which contains approximately 0.1 gram of crystalline sodium chloride. Calibrators are treated *identically*. Appropriate change in electrometer range and/or attenuation setting may be needed to achieve suitably large recorder response.

3.3 MANUAL METHOD

3.3.1 Materials: Apparatus, Reagents, Supplies

Apparatus

1. Gas Chromatograph with Flame Ionization Detector (Model 1860-3, Varian Instruments Div., Palo Alto, Calif. 94303).
2. Potentiometric Strip-Chart Recorder, 250 mm. (Model 194, Honeywell, Inc., Ft. Washington, Pa. 19034); equipped with Mechanical Trace Integrator (Model 252-A, Disc Instruments, Inc., Santa Ana, Calif. 92705) and with Automatic Printing Integrator (Model 610, Disc Instruments, Inc., Santa Ana, Calif 92705).

3. Hydrogen Generator, Elhygen (Model ER-150, Milton Roy Co., St. Petersburg, Fla. 33733)

4. Constant-Temperature Water Bath, 1000 Watts, Temp. Regulation ±0.02°C (Lauda Model K-4, Brinkmann Instruments, Inc., Westbury, N.Y. 11590)

5. Automatic Diluter (Cat. No. 2112, York Instruments Corp., Berkeley, Calif. 94710)

6. Gas-Tight Syringe, 1 ml., with Chaney Adaptor (Model 1001LLCH, Hamilton Co., Reno, Nev. 89510 *or* Plastipak Disposable Tuberculin Syringe, Cat. No. 5602, Becton-Dickinson, Rutherford, N.J. 07070 with Chaney Adapter, Model 14725, Hamilton Co., Reno, Nev. 89510); and Disposable Hypodermic Needle, 25 gauge 1.5 inch (38 mm) (Cat. No. 5028, Becton-Dickinson, Rutherford, N.J. 07070)

7. Culture Incubator (Model 1A6825E, Thermolyne Corp., Dubuque, Ia. 52001).

Reagents

1. Calibration Reference Materials (Calibrators)
   a. Ethanol, 0.10, 0.20, 0.40% w/v (1.00, 2.00, 4.00 g/Liter) Aqueous Solutions
   b. Mixed Calibrator, "Low" (Acetone 1.00 g/Liter; Ethanol 2.00 g/Liter; Isopropanol 1.00 g/Liter; Methanol 1.00 g/Liter Aqueous Solution)
   c. Mixed Calibrator, "High" (Acetone 1.00 g/Liter; Ethanol, 4.00 g/Liter; Isopropanol 1.00 g/Liter; Methanol 1.00 g/Liter Aqueous Solution)
2. Internal Standard Solution
   Acetonitrile, A.R., Nanograde, 0.15% v/v
   (1.50 ml. per Liter Aqueous Solution)
3. Sodium Chloride, A.R., Granular.

Supplies

Glass Septum Vials, Screw-capped, Erlenmeyer, 10 ml or 25 ml. (Products No. 13310 and 13320, Pierce Chemical Co., Rockford, Ill. 61105); *or* Cylindrical Glass Septum Vials, Screw-capped, 14 ml. (Product No. 13043, Pierce Chemical Co., Rockford, Ill. 61105) with polymer septa; *or* Hypo-Vials, 15 ml. (Stock No. 12911, Pierce Chemical Co., Rockford, Ill. 61105) with elastomer septum stoppers and aluminum seals.

3.3.2 Summary of Operations

The liquid sample (20 microliters to 1 ml) is placed into a glass septum vial, together with sufficient sodium chloride to assure saturation of the liquid (and after dilution in fixed proportion with the internal standard solution when that procedural modification is used). Reference samples are treated identically, and all sealed vials are inserted into a thermostated water bath. After a 45-minute equilibration period, a fixed quantity of the headspace vapor of each septum vial is sequentially sampled with a gas-tight syringe and injected into the preset gas chromatograph. The analysis proceeds for a fixed time interval, and the response of the flame ionization detector is recorded, as a function of time, on a potentiometric strip-chart recorder.

3.3.3 Instrument and Analysis Conditions

The following are typical instrument and analysis conditions which have been found satisfactory in the author's laboratory. Numerous modifications of these conditions are also acceptable, or occasionally preferable in special circumstances.

Column: Carbowax 1500 (0.4%) on 60/80 mesh Carbopack A (51, 53)
1.8 M x 3.2 mm O.D. (6 ft x 1/8 in) stainless steel column

Carrier Gas:	Helium[17];	inlet pressure	55 psig
		rotameter	85-90% of maximum scale
		flow rate	45 ml/min.
Temperatures:	Column Oven		80°C
	Injection Port		175°C
	Detector		225°C
FID:	Hydrogen;	inlet pressure	20 psig
		flow rate	45 ml/min.
	Air:	inlet pressure	15 psig
		flow rate	330 ml/min.
Temperature Program:	Isothermal		
Electrometer:	Range		10^{-11} amps/mv
	Attenuation		x 32
Recorder:	Input Range		1 mv
	Chart Speed		5 mm/minute.

3.3.4 Procedure - Direct Analysis

1. A well-mixed aliquot of the blood specimen approximately 1 ml in volume is delivered into a clean, dry numbered septum vial which contains 1 gram of crystalline sodium chloride. The vials are closed with a polymeric stopper or septum and a screw cap or crimped aluminum cap, as appropriate, after which the contents are thoroughly mixed by horizontal rotation.

2. Ethanol or mixed-volatiles reference solutions (normally the "Low" and "High" mixed calibrators) are similarly treated, and all sealed vials are inserted into the preheated water bath, at 38±0.05°C, being immersed to just below the closures, and allowed to equilibrate for at least 45 minutes. Sufficient calibrators are equilibrated to allow their analysis at the beginning, middle, and end of a run.

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[17]Nitrogen can also be employed as the carrier gas, at slightly greater sensitivity

3. After the minimum equilibration period, a fixed, identical volume of the headspace vapor is withdrawn, using the gas-tight syringe adjusted to fixed volume (usually 250 microliters) with the Chaney Adaptor, the syringe having been preheated to 38°C in the incubator or similar device. Repeated rapid "pumping" of the syringe is employed to obtain a fully-representative aliquot of the headspace vapor, which is then immediately injected into the gas chromatograph. Immediately following the headspace sample withdrawal, each septum vial is vented with a 25 gauge needle to reestablish atmospheric pressure. The syringe and needle are thoroughly purged by rapid, repetitive intake and expulsion of room air, and the syringe is returned to the incubator until its next use.

4. The analysis is allowed to proceed for approximately 5 minutes (or 3 minutes if ethanol is the only compound of concern in the sample).

5. The same gas-tight syringe, with volume calibration intact, is used for all unknown and reference samples. Each specimen is analyzed by this procedure in at least independent duplicate, as is each calibrator, after allowing each septum vial to re-equilibrate for at least 20 minutes after the prior headspace sample withdrawal.

6. The analysis record is appropriately identified (we routinely employ a date/time stamp imprint also naming the laboratory) and a stamp with the chief particulars of the analytical conditions (Figure 4) is affixed.

7. Results are computed as follows:

a. By *Peak Height* Measurements

$$BAC_{unknown} = \frac{PH_{unknown}}{PH_{calibrator}} \times Conc._{EtOH,\ calibrator}$$

where PH = Peak Height of the Ethanol Detector Response Recording.

b. By *Peak Area* Measurements

$$BAC_{unknown} = \frac{PA_{unknown}}{PA_{calibrator}} \times Conc._{EtOH,\ calibrator}$$

where PA = Peak Area of the Ethanol Detector Response Recording.

8. Ethanol and other volatile substances in the sample, if present, are identified by their relative retention time(s) in comparison with those of *contemporaneous* reference solutions.

9. *Micro-Sample Modification:* If indicated because of restricted specimen volume, the above procedure is modified as follows, without other changes in preparation or analysis of samples or computation of results: A well-mixed 100 µl aliquot of the specimen is delivered into a single clean, dry numbered septum vial which contains 0.1 gram of crystalline sodium chloride. Calibrators are treated identically.

10. *Ultra-Micro Sample Modification:* An aliquot of exactly 20.0 µl of well-mixed specimen is diluted with fully quantitative technique (using pipettes calibrated *"to contain"*) with 0.90% w/v aqueous sodium chloride to a total volume of 100 µl and that diluted sample is then delivered into a single clean, dry numbered septum vial which contains 0.1 gram of crystalline sodium chloride. Appropriate change in electrometer range or attenuation settings may be needed to achieve suitably large recorder response. Calibrators are treated identically.

3.3.5 Procedure - Internal Standard Analysis

1. A well-mixed aliquot of the blood specimen is mixed with an identical volume of the acetonitrile internal standard solution, using fully quantitative technique (including pipettes calibrated *"to contain"* when an automatic diluter is not used). Normally, the respective volumes are both 500 µl. The sample measurement and internal standard solution measurement and mixing are carried out with an automatic diluter (appropriately preset with calibration verified by weighing of sampled and dispensed quantities), and the sample-internal standard mixture is delivered directly into one clean, dry numbered septum vial containing 1 gram of crystalline sodium chloride.

2. Ethanol or mixed volatiles reference solutions (usually the "Low" and "High" mixed Calibrators) are treated *identically*.

3. The remainder of the procedure for analysis is identical with that described in Section 3.3.4 above. It may be desirable to employ an attenuation setting 0.5x that normally used with the Direct Analysis procedure, in order to achieve suitably large recorder responses to the detector signal.

4. Results are computed as follows:

a. By *Peak Height* Measurements

$$BAC_{unknown} = \frac{R_1}{R_2} \times Conc._{EtOH,\ calibrator}$$

where PH = Peak Height of the Indicated Detector Response Recording (e.g., PH_{is} = Peak Height of the Acetonitrile Internal Standard Response)

$$R_1 = \frac{PH_{EtOH}}{PH_{is}}$$ for the unknown sample-internal standard mixture

$$R_2 = \frac{PH_{EtOH}}{PH_{is}}$$ for the calibrator-internal std. mixture.

b. By *Peak Area* Measurements

$$BAC_{unknown} = \frac{R_1}{R_2} \times Conc._{EtOH,\ calibrator}$$

where PA = Peak Area of the Indicated Detector Response Recording (e.g., PA_{is} = Peak Area of the Acetonitrile Internal Standard Response)

$$R_1 = \frac{PA_{EtOH}}{PA_{is}}$$ for the unknown sample-internal standard mixture

$$R_2 = \frac{PA_{EtOH}}{PA_{is}}$$ for the calibrator-internal std. mixture.

5. *Micro-Sample Modification:* When a suitable mechanical diluter is available, exactly 50.0 µl of well-mixed specimen are diluted with exactly 50.0 µl of the acetonitrile internal standard solution, with fully quantitative technique, and the mixture is delivered directly into a single dry, clean numbered septum vial which contains 0.1 gram of crystalline sodium chloride. Alternately, larger *identical* volumes of the specimen and of the internal standard solution are premixed with fully quantitative technique, and approximately 100 µl of the resultant mixture is employed as the sample for analysis. Calibrators are treated *identically*.

6. *Ultra-Micro Sample Modification:* Using a suitable mechanical diluter, exactly 20.0 µl of well-mixed specimen are diluted with exactly 80.0 µl of a modified 0.09375% v/v acetonitrile internal standard solution (containing 0.9375 ml of acetonitrile per liter of aqueous solution) and delivered directly into a clean, dry numbered septum vial which contains 0.1 grams of crystalline sodium chloride. Calibrators are treated *identically*. Appropriate change in electrometer range and/or attenuation setting may be needed to achieve suitably large recorder response.

3.4 PROCEDURAL NOTES AND COMMENTS

Innumerable modifications of certain details of the above procedures are feasible with respect to techniques, instrument operating parameters, data treatment, etc. Some of the more significant factors are discussed below.

Gas Chromatographic Columns. Many column packings have been used successfully for alcohol analysis in biological specimens, and several of these have proven useful in the author's laboratory. Table 1 reflects, in part, this laboratory's experience with several commonly-employed GC column packings with respect to absolute ethanol retention time under the stated conditions, using a manual gas chromatograph as the comparison device. It should be recognized that other significant factors may also vary, such as capability or ease of separation of certain sample components of interest (e.g., acetaldehyde, formaldehyde, or methanol).

The column specified in the procedural section, 0.4% Carbowax 1500 on 60/80 mesh Carbopack A, was introduced about 1974 but is no longer commercially available. A replacement, Carbopack C, has been marketed for Carbopack A as the support material, and the liquid phase loading is 0.5x that previously used on Carbopack A (53). Among the voluminous literature on column packings are two reports which specifically deal with graphitized carbon and open-pore polyurethane as column packings for the gas chromatographic analysis of ethanol in blood (54-55). In general, increasing the column oven temperature decreases absolute retention times for ethanol and related compounds of interest, shortening total elapsed time per analysis; but column temperature increases also reduce the efficiency of separation of closely adjacent sample components, sometimes to the point of inadequate resolution. In practice, therefore, the optimum conditions, for a given column packing, of column geometry, oven temperature, and carrier gas pressure and flow rate represent compromises to attain the currently most important goal, whether speed of analysis or best resolution.

TABLE 1.

GAS CHROMATOGRAPHIC COLUMN CHARACTERISTICS FOR ETHANOL
UNDER THE STATED GC CONDITIONS (52)

Column	Packing	Column Temperature, °C	Absolute Retention Time for Ethanol (min)
1	0.5% Carbowax K-600 + 3.8% Hallcomid 18 on Teflon 6 HC, 40/60 mesh	100	0.24
2	0.5% Carbowax K-600 + 3.8% Hallcomid 18 on Teflon 6 HC, 40/60 mesh	50	1.80
3	0.4% Carbowax 1500 on Carbo-pack A, 60/80 mesh	80	1.32
4	Porapak Q, 80/100 mesh	155	1.60
5	Chromosorb 102, 100/120 mesh	155	2.16
6	15% Carbowax 1500 on Celite 525, 80/100 mesh	80	2.58
7	Porapak S, 80/100 mesh	155	2.51
8	30% Carbowax 20M* on Chromosorb W, AW, 60/80 mesh	130	2.12

*1.8M x 6.4mm o.d. column, rather than the 1.8M x 3.2mm size used for the other supports

The several column packings listed above also differ significantly in the absolute retention times for volatile compounds other that ethanol, and consequently in their ability to achieve satisfactory and practical separations of the components of interest in biological liquids. Table 2 lists the retention times relative to ethanol of several commonly encountered volatile substances on a Porapak S column and a Carbowax 1500 on Carbopack A column.

TABLE 2.

TYPICAL RELATIVE RETENTION TIMES OF VOLATILE SUBSTANCES ON PORAPAK S AND CARBOWAX 1500 ON CARBOPACK A COLUMNS

Compound	Relative Retention Time[a]	
	Porapak S	Carbowax 1500
Acetone	1.44	1.35
Acetonitrile	1.23	0.87
Ethanol	1.00	1.00
Isopropanol	1.80	1.98
Methanol	0.48	0.52

[a]Relative to the retention time of ethanol

Machata (47) has reported absolute retention times for various volatile compounds on three commonly employed columns, as shown in Table 3.

It is evident that greatly increased certainty of identification of ethanol and other volatile substances from their retention times can be obtained by the simultaneous or sequential GC analysis of headspace vapors on two different columns, and that procedure is recommended.

TABLE 3.

TYPICAL ABSOLUTE RETENTION TIMES OF VARIOUS SUBSTANCES OBSERVED ON THREE COLUMNS (47)

	Carbowax 1500	Hallcomid 18	Porapak
Column Temperature, °C	90	90	175
Compound	Absolute Retention Time, Minutes		
Acetaldehyde	1.1	1.7	1.5
Acetone	1.5	2.1	3.2
Chloroform	2.8	2.4	7.2
Diethyl Ether	0.8	1.3	4.2
Ethanol	2.6	4.0	2.1
Isopropanol	2.5	4.8	3.4
Methanol	2.1	2.6	1.1
Methyl Ethyl Ketone	2.1	4.2	6.9
n-Butanol	7.2	19.3	10.2
n-Propanol	4.2	8.7	4.5
Paraldehyde	4.6	12.7	4.3
tert. Butanol	2.2	5.4	5.4

Gas Chromatographic Instrument Parameters. The several GC instrument temperatures specified in the procedural section have proven suitable in the author's laboratory; others are feasible. Since the headspace samples always contain water vapor in addition to any volatile substances present, the entire analysis train inclusive of the sample inlet must be maintained at a sufficiently high temperature to prevent condensation of any sample component. Analysis time per sample is partly determined by the foregoing column considerations and partly by the desired information. When ethanol or other volatiles eluting earlier (e.g., acetaldehyde) are the only components of interest, chromatography can be terminated after the complete ethanol peak has been recorded. Temperature programming can be employed to accelerate elution of any later-eluting sample components, or timed injections of succeeding samples can be made while the preceding sample is being chromatographed. Acetonitrile has the advantage, as an internal standard in this system, of eluting before ethanol and thus not prolonging the analysis.

It is usually desirable to record (or otherwise measure) GC detector response at 75-80% of maximum full-scale response for the highest expected sample component concentration, where feasible. The interaction of sample quantity chromatographed, electrometer sensitivity setting (e.g., range and attenuation), and recorder full-scale span is suitably adjusted toward this goal, with due attention to short-term baseline stability and long-term baseline drift. In the automated method, length of injection time governs the headspace sample quantity chromatographed; in the manual procedure it is determined by the headspace volume transferred to the gas chromatograph. For large specimen numbers, fully automatic result calculation and reporting accessories for the automated gas chromatograph are available (46, 56).

Equilibration Considerations. Isothermal equilibration is a complex phenomenon which is affected by such factors as temperature, time, volume, and geometry of the equilibration vessel, and presence and concentration of inorganic salts in the liquid phase. In general, the headspace volume should be kept as small as is compatible with the sample volume required by the sensitivity

of the gas chromatograph employed and by the number of replicate headspace vapor samplings contemplated; 10-25 ml are appropriate volumes for the recommended scheme. While the cylindrical equilibration vessel form is dictated by the turntable requirements of the F-40 instrument, larger interface areas accelerate equilibration, and the Erlenmeyer flask shape is thus more efficient. Close temperature regulation is required and the equilibration vessel must be immersed in the water bath to cap level; spring retainers or weighting with suitable washers or lead doughnuts[18] are used as required. The vapor pressure of volatile compounds increases as a function of the temperature, but the vapor pressure curves of ethanol and many other volatile compounds potentially useful as internal standards are not parallel over the temperature range of interest. Hence, calibration factors and curves established at one temperature cannot be used at other temperatures. While the concentration of ethanol in headspace vapor can be increased by equilibration at higher temperatures, other factors (such as the greater differential between equilibration and room temperatures) may offset any advantage gained through condensation effects, etc. Hence, the manual procedure, which involves manual headspace transfer, employs a compromise equilibration temperature of 38°C. In theory, equilibration could be accomplished at room temperature if the latter were sufficiently stable over time. In practice, the requisite identity of equilibration temperatures for unknowns and reference specimens can only be assured by use of high-precision thermostating.

Time to reach complete equilibrium of volatile components of the sample between the liquid and gaseous/vapor phases in the equilibration vessel is a key factor which is often disregarded. Incomplete equilibration will yield variable results, and failure to achieve identical equilibration of reference and unknown samples (e.g., because of analysis of insufficiently-equilibrated reference samples at the beginning of a run) will yield erroneous results. Figures 5 and 6 illustrate this phenomenon.

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[18] Instruments for Research and Industry, Cheltenham, Pa. 19012.

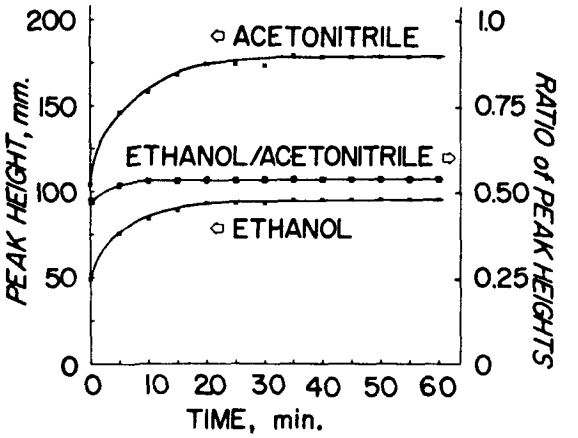

FIGURE 5. TIME COURSE OF GC DETECTOR RESPONSES TO ETHANOL AND ACETONITRILE AND THEIR RATIO, SHOWING TIME REQUIRED FOR EQUILIBRATION OF HEADSPACE VAPOR AT 65°C. DATA POINTS SHOWN ARE SINGLE OBSERVATIONS (100 MG/DL AQUEOUS ETHANOL SAMPLE IN THE PRESENCE OF SODIUM CHLORIDE)

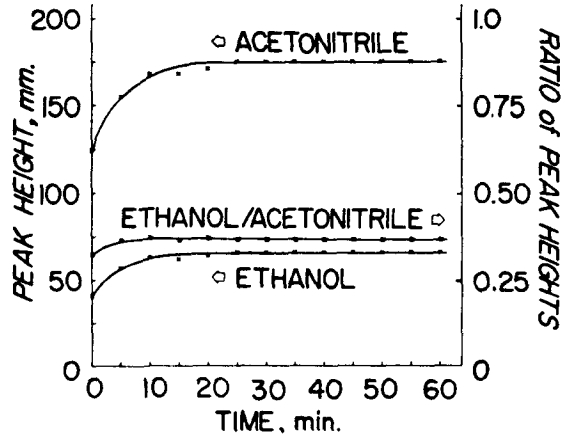

FIGURE 6. TIME COURSE OF GC DETECTOR RESPONSES TO ETHANOL AND ACETONITRILE, AND THEIR RATIO, SHOWING TIME REQUIRED FOR EQUILIBRATION OF HEADSPACE VAPOR AT 38°C. DATA POINTS SHOWN ARE SINGLE OBSERVATIONS. (100 MG/DL AQUEOUS ETHANOL SAMPLE IN THE PRESENCE OF SODIUM CHLORIDE)

Addition of inorganic salts decreases the vapor pressure of water and increases that of organic substances with proportionate effects upon their relative partial pressures in the headspace gas. Saturation of the liquid phase with sodium chloride yields an approximate 3-fold increase in alcohol concentration of the vapor phase compared with the same system in the absence of added sodium chloride; e.g., at 40°C, the GC peak height ratio for ethanol in the headspace gas above NaCl-saturated liquid vs. that above untreated liquid specimens is 3.2. Machata (47) has reported the following "vapor pressure increases" for ethanol at 60°C by addition of various salts to an aqueous ethanol solution (the respective salt concentrations not being specified):

Anhydrous Salt Added	Vapor Pressure Increase
Ammonium chloride	2 x
Ammonium sulfate	5 x
Potassium carbonate	8 x
Sodium chloride	3 x
Sodium citrate	5 x

Unionized dissolved solids in the specimen exert much smaller effects on the relative fugacity of volatile compounds; e.g., addition of 1 gram of sucrose to 1 ml of a 100 mg/dl aqueous ethanol specimen increases the gas chromatographic ethanol peak height at 35°C to 1.3x that yielded by the untreated specimen. The observations are in accord with theories regarding decreased mutual solubility of solvents or complete or partial removal of one solvent in such systems via preferential solvation processes.

In the manual method, equilibration should preferably occur at atmospheric pressure. Partial loss of a headspace sample can occur during its transfer with a syringe whose needle is open to the atmosphere when equilibration

has been carried out above atmospheric pressure, e.g., at elevated temperature in an unvented vessel; or dilution of a headspace sample obtained below atmospheric pressure can occur from air influx. Atmospheric pressure is, therefore, established by hypodermic needle puncture through the equilibration vessel septum stopper prior to headspace sampling, and again thereafter if sampling is to be repeated.

Sample Measurement. In the manual method *direct* procedure, measurement of the liquid specimens placed into equilibration vessels need only be approximate. In both the manual and automated methods, the *internal standard* technique requires exact, reproducible measurement of unknown or reference specimen and internal standard reagent quantities. Further, in the automated method direct procedure, the headspace vapor quantity actually transferred pneumatically into the chromatograph is significantly affected by the total liquid volume in the septum vial. Figure 7 illustrates the relation between total liquid volume in the 24 ml septum vial and the peak height of the GC ethanol trace for a 200 mg/dl aqueous ethanol specimen with added sodium chloride in the usual (1 g NaCl/1 ml sample) proportions. The experimental curve corresponds to a power equation ($y=ax^b$), but apparent best-fit regression of the data is with the parabolic or quadratic curve equation $y = 0.400 + 0.748x - 0.201x^2$ ($r = 0.93$).

Since the sample is not diluted in the direct procedure, wash-out ("to contain") pipettes cannot be employed for delivery of the sample. Whole blood can be measured with Ostwald-Folin pipettes calibrated "to deliver," allowing drainage to proceed slowly enough to avoid blood film residues. If necessary, treatment of pipettes with a suitable silicone coating compound[19] will reduce wettability of pipette walls and avoid adhesion of blood. When the volume of analyses is not large enough to warrant use of automated mechanical pipetting or pipetting-diluting machines, manually-operated repetitive

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[19] SILICLAD water soluble silicone concentrate (Cat. No. 1950, Clay Adams Div., Becton-Dickinson & Co., Parsippany, N. J. 07054) has been found suitable.

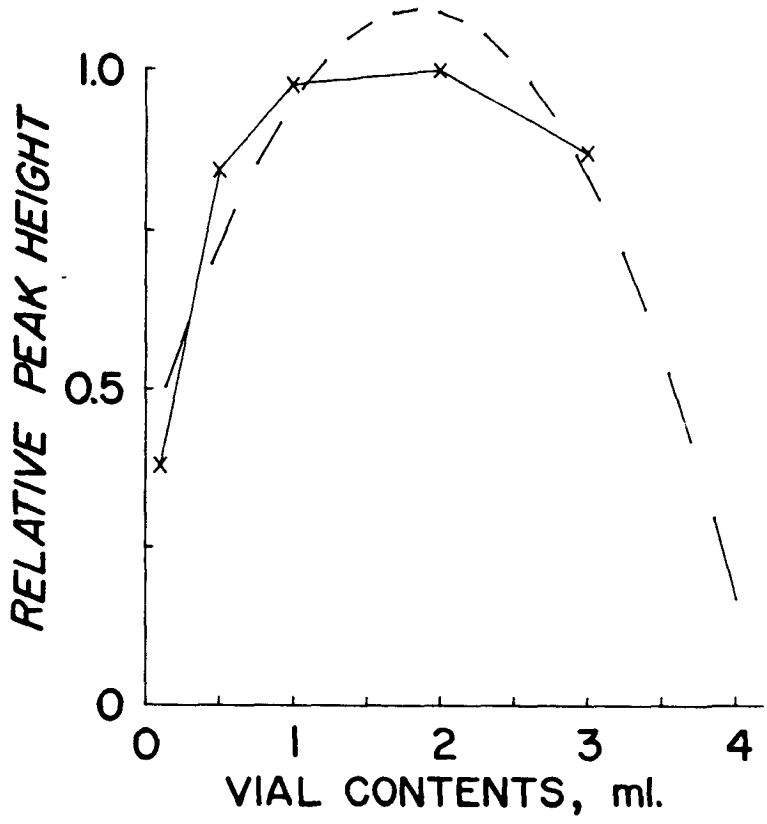

FIGURE 7. EFFECT OF SAMPLE VOLUME IN 24-ML SEPTUM VIAL ON DETECTOR RESPONSE TO ETHANOL WITH MULTIFRACT F-40 AUTOMATED GAS CHROMATOGRAPH. EACH DATA POINT IS MEAN OF FOUR OBSERVATIONS. DASHED CURVE REPRESENTS BEST-FIT PARABOLIC REGRESSION EQUATION $Y = 0.400 + 0.748X - 0.201X^2$ (R=0.93) (200 MG/DL AQUEOUS ETHANOL SAMPLE IN PRESENCE OF SODIUM CHLORIDE)

pipetters or pipetter-diluters are convenient; the syringe-based types[20] have proven suitable for the dilutions involving internal standard reagent, while hand-held automatic pipets of the Eppendorf type with disposable polypropylene tips have proven suitable for measurement and delivery of blood and other liquid samples.

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[20] Mini-Repipet Diluter (Cat. No. Mini-80501, Labindustries, Berkeley, CA 94710).

The high concentration of sodium chloride, incidentally, aids in hemolysis of the whole blood sample, a desirable effect since the ethanol concentration of the sample is thus equalized. Mixing an aqueous internal standard with the whole blood specimen, of course, further effectively promotes rupture of the walls of the cellular components.

Headspace Sample Measurement and Transfer. In these procedures, a headspace vapor aliquot is chromatographed; hence, measurement of that aliquot is critical. In the automated method, measurement and introduction of the headspace vapor sample occurs automatically through suitably heated connections; but in the manual method manipulation by the analyst is required. Although many devices and schemes can be used for measuring and transferring the headspace vapor aliquot, we have found use of a fixed-volume syringe to be the most convenient suitable procedure. The plastic tuberculin-pattern syringe is readily heated to a temperature slightly above that of the septum vial and retains the heat adequately during the brief headspace aspiration-and-transfer operation, when handheld. A specialized gas-tight glass syringe with heating mantle can be similarly employed. Reproducible-volume sampling is assured by means of a Chaney adaptor or similar volume-limiting device. The entire headspace aliquot removed by syringe is then immediately injected into the gas chromatograph.

Alternately, a heated gas-sample valve, maintained at a fixed temperature slightly above that of the septum vial, can be used to measure and inject the headspace vapor aliquot, after a suitably larger roughly-measured volume of headspace gas has been transferred from the septum vial to the gas-sample loop. Identity of volumes of unknown and reference sample headspace vapors is assured by using the same syringe, after repeated air-purging between samples. Absence of carry-over or sample-memory effects can be documented, if necessary, by chromatographing such a blank sample.

When an internal standard is used, the ratio of GC detector signals for ethanol and the internal standard (e.g., acetonitrile) is identical for any

appropriate sample volume, and hence minor sample losses through leaks, etc., do not affect the results. In the direct procedures, however, the chromatographed sample quantity is a critical variable, and steps are required to exclude unrecognized losses of the headspace vapor aliquot. The simplest routine procedure toward that end is that employed here; namely, independent replicate (i.e., *at least* duplicate) sampling of the headspace, from two or more separately but identically equilibrated specimen aliquots, in order to avoid the effects of rapidly repeated withdrawals of headspace aliquots upon the analysis results. Coincidence of these replicate results within the precision limits for the procedure then excludes unrecognized sample losses or alterations. If the volume of analyses to be accomplished militates against the replicate analysis practice, internal standard use becomes the mandatory alternative. When time permits, replicate sampling from a single vial suffices.

Of the various volatile compounds proposed as candidate internal standards, many have limitations of one sort or another. Acetonitrile was found in this laboratory to be acceptable on general practical grounds and to yield the same GC detector response ratio (e.g., peak heights) for ethanol/internal standard in both aqueous reference solutions of ethanol and biological fluids of the same ethanol concentration when both are saturated with sodium chloride. (This limitation does not apply to use of internal standards as biological fluid diluents for direct *liquid* sample injection in gas chromatography.)

Quantitation of GC Detector Response. Many of the typical detector-response quantitation problems encountered in gas chromatography of injected biological liquid samples are obviated by use of the equilibrated headspace technique. Asymmetry and tailing of peaks, significant baseline offset or drift, and other problems resulting from flash-vaporization effects or column changes induced by liquid samples are absent. As illustrated in Figure 8, under routine operating conditions the headspace analysis method described herein yields highly symmetrical component peaks for all substances of interest and good return to baseline. Under these conditions, quantitation of detector

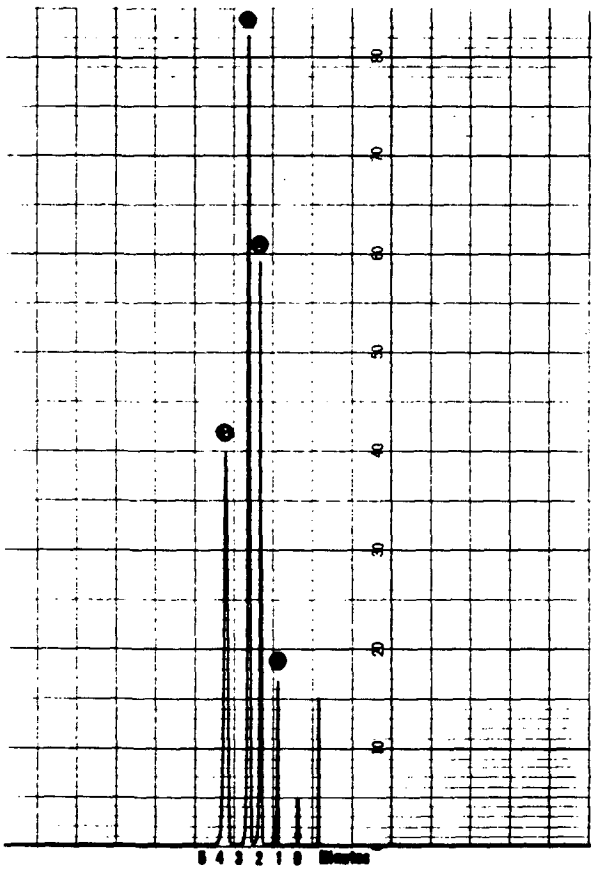

FIGURE 8. TYPICAL HEADSPACE CHROMATOGRAM OBTAINED WITH MULTIFRACT F-40 AUTOMATED GAS CHROMATOGRAPH (DIRECT PROCEDURE), UNDER CONDITIONS DESCRIBED IN TEXT. (HITACHI PERKIN-ELMER MODEL 159 RECORDER WAS USED.) SAMPLE COMPONENTS SHOWN ARE: 1 = METHANOL, 2 = ETHANOL, 3 = ACETONE, 4 = ISOPROPANOL

response can be carried out equally satisfactorily by measurement of peak height or peak area. In the internal standard mode, the peak height (and area) of the internal standard should ideally be identical to that of the peak of interest, ethanol; in practice, similarity is, of course, all that can be attained when the ethanol concentrations of unknown specimens vary. The peak height for the internal standard should therefore be adjusted to match that of the most commonly occurring unknown value according to prior experience (i.e., the modal concentration).

The various methods of recording, measuring, and computing GC detector response as a function of sample mass all have characteristic inherent limitations as well as advantages. For the gas chromatographic system described herein, the basic detector signal output is a d.c. voltage as a function of time. For a narrow Gaussian peak, the peak height at maximum is directly proportional to the total peak area; therefore the d.c. voltage as a function of time (i.e., mv x min) is also proportional to the peak area and hence to the mass of analyte present in the carrier gas at the relevant peak time. The several common result computation methods all utilize the two key variables, d.c. voltage and time, of the relevant chromatographic peak, either directly or indirectly. Direct quantitation from the potentiometric strip-chart recorder tracing (whether by manual peak height measurements or manual peak area estimates or by accessory mechanical integrator reflecting *recorder* response over time) necessarily entails the detector signal degradation and variability attributable to the signal conditioning and display by a separate device, especially one of limited quantitative discriminating capacity such as a recorder with 200-250 mm (or possible lesser) chart width. Ideally, therefore, an electronic quantitation device of precision and accuracy at least equal to the detector signal output should be employed, such as a suitable electronic integrator capable of accepting the electrometer output signal directly.

The printing (Disc) integrator employed in this laboratory is an accessory for the (Disc) mechanical integrator, itself an accessory to a potentiometric strip-chart recorder. It has proven satisfactory for the application described and has yielded results equivalent to those obtainable with more sophisticated direct digital voltage measurements, direct electronic integrators,[21] or the fully automatic data processing system.

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[21] The Multifract F-40 instrument has a direct electrometer output signal for integrators (100, 250, or 1000 mv f.s.d.)

For large-scale forensic alcohol analysis, use of an electronic integrator directly coupled to the electrometer output signal and preferably equipped with hard-copy reporting (e.g., by means of a digital printer or teletype-writer) is desirable and recommended because of the substantial savings in personnel time and the probable reduction in errors in computing and transcribing results which can be achieved.

When instrumental conditions are optimal, quantitation of detector response by either peak height or peak area measurements is acceptable and appropriate. It may, however, be more conservative to base quantitation of ethanol in the headspace sample on the peak areas ratio for ethanol and an appropriate internal standard.

Several common quantitation methods employing either peak height or peak area measurements assume direct proportionality between the peak height or peak area and the concentration of the analyte. Over a narrow concentration range this is normally a tenable assumption when gas chromatgraphic analysis conditions are optimized. It is, however, good practice to use at least three reference samples of different concentrations over the full range of interest to assure that adequate linearity of detector response is present.

Specificity Considerations. In routine gas chromatographic analysis, putative identification of the unknown is universally based on comparison of its retention time with that of contemporaneously chromatographed reference compounds. In the absence of sophisticated instrumentation with inherently high specificity for qualitative identification of unknowns (e.g., by combined gas chromatography-mass spectrometry (57) or mass fragmentometry (58)) several simple expedients can significantly increase the certainty of identification of ethanol and other sample components based on their relative retention time. The first, and simplest, involves employing two or more different GC columns, so selected that the expected analyte (e.g., ethanol) elutes at substantially different absolute times after injection and prefer-

ably in altered sequence relative to a reference compound such as the internal standard. (Cf. Tables 1-3) Coincidence of these significant variables for the unknown and for a contemporaneous authentic ethanol-internal standard reference, with two or more different columns, normally increases the confidence in the identification to the extent required.

The alternative approach of Blume et al. (59), a recent variant of a classic analytic technique, is to demonstrate disappearance of the putative ethanol peak of a gas chromatogram following treatment of the unknown specimen with alcohol dehydrogenase. Essentially complete disappearance of the supposed ethanol peak is the requisite identity-confirmation criterion, since alcohol dehydrogenase also partially oxidizes higher alcohols (e.g., isopropanol, n-propanol, and n-butanol) under certain conditions.

3.5 ANALYTICAL AND PERFORMANCE CHARACTERISTICS OF THE METHOD

The following information illustrates some key performance characteristics of the above method for gas chromatographic analysis for ethanol of headspace above biological liquids. These data are not intended to constitute a full method evaluation; rather, they are typical of a much larger body of data obtained in the author's laboratory in the process of developing, modifying, and validating the basic method and its procedural subsets presented herein. This presentation is limited to several of the major characteristics of the method, including accuracy, precision, sensitivity, specificity, and to typical examples of its performance in the analysis for alcohol[22] of reference specimens, of proficiency survey specimens, and of routine case specimens submitted for alcohol analysis in connection with traffic law enforcement.

Equilibration Time. Time to achieve presumably complete equilibration of the volatile components of aqueous reference specimens, as reflected by peak heights of the relevant gas chromatographic responses, is illustrated in Figure 5 for the 65°C equilibration temperature employed in the automated analysis and in Figure 6 for the 38°C equilibration temperature employed in the manual method, for a nominal sample ethanol concentration of 100 mg/dl.

It is evident that at least 35 minutes are required to reach steady state; hence 45 minute pre-analysis equilibration times are specified in the procedural sections above, to afford an appropriate safety margin. The peak heights ratio of ethanol and acetonitrile internal standard becomes asymptotic (or nearly so) more rapidly than the ethanol response per se. How-

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[22] In order better to illustrate the characteristics of the method and to facilitate statistical comparisons, the ethanol concentrations in this section are generally reported to three significant figures. However, it deserves reemphasis that the results of alcohol analyses of biological specimens, when intended for traffic law enforcement or similar forensic applications, should be reported in percent w/v to two decimal places, truncated; i.e., to *two* significant figures only.

ever, since the time course of the alcohol and the internal standard responses are not entirely parallel, sampling of the headspace vapor should be avoided until complete equilibration has occurred. These effects are also concentration related, and the illustrated time courses may not be applicable to specimens with substantially different compositions.

Chromatographic Separations and Specificity. A typical gas chromatogram for the automatic headspace analysis method, internal standard mode, is shown in Figure 9. Certain specificity considerations, such as order of elution of various volatile substances, absolute and relative retention times, are given in Tables 1 to 3 for several commonly employed columns.

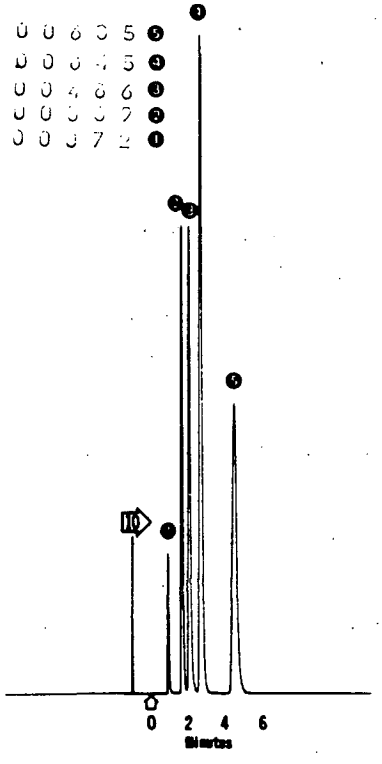

FIGURE 9. TYPICAL HEADSPACE CHROMATOGRAM OBTAINED WITH MULTIFRACT F-40 AUTOMATED GAS CHROMATOGRAPH (INTERNAL STANDARD PROCEDURE), UNDER CONDITIONS DESCRIBED IN TEXT. SAMPLE COMPONENTS SHOWN ARE: 1 = METHANOL, 2 = ACETONITRILE, 3 = ETHANOL, 4 = ACETONE, 5 = ISOPROPANOL; MARKER "10" IS SAMPLE IDENTITY INDICATION. CORRESPONDING INTEGRATOR VALUES ALSO SHOWN.

When alcohol is the only analyte of immediate concern, the chromatography time can be reduced to 3 min. or less per sample since the internal standard, acetonitrile, elutes before ethanol. Alterations in the gas chromatographic instrument parameters (e.g., column packing and temperature), can, of course, substantially alter the chromatogram.

Figure 10 is a chromatogram of a blood specimen from a human subject intoxicated with isopropanol, followed by the chromatogram of the "Low" mixed Calibrator; analysis was performed by the manual headspace analysis method, direct (non-internal standard) mode. By coincidence, the acetone peak height and peak areas of unknown reference samples are virtually identical; the concentration of blood acetone (100 mg/dl) and of blood isopropanol (62 mg/dl) are typical of moderate isopropanol intoxication after substantial metabolic conversion of the latter to the former has occurred.

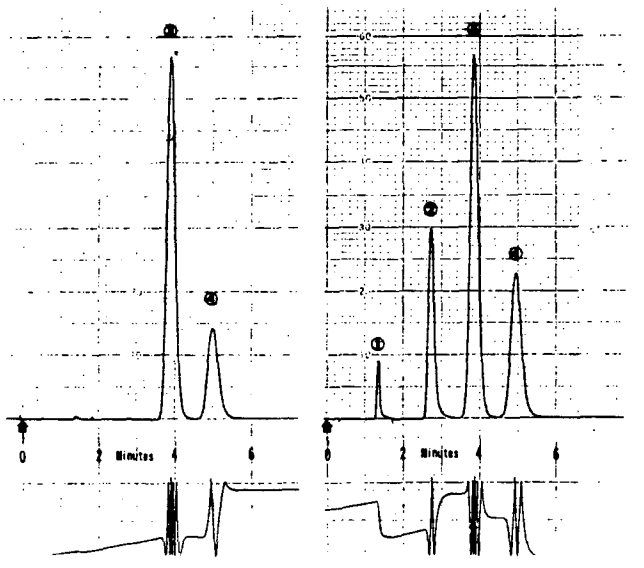

FIGURE 10. HEADSPACE CHROMATOGRAMS OBTAINED WITH MANUAL METHOD (DIRECT PROCEDURE), UNDER CONDITIONS DESCRIBED IN TEXT. LEFT = HUMAN BLOOD FROM SUBJECT INTOXICATED WITH ISOPROPANOL, RIGHT = CONTEMPORANEOUS MIXED "LOW" CALIBRATOR RUN. SAMPLE COMPONENTS SHOWN ARE: 1 = METHANOL, 2 = ETHANOL, 3 = ACETONE, 4 = ISOPROPANOL

Calibration Curves and Linearity. Our typical calibration curves for the automated headspace analysis method, in both the direct and internal standard modes, are illustrated in Figure 11. The curves all demonstrate high

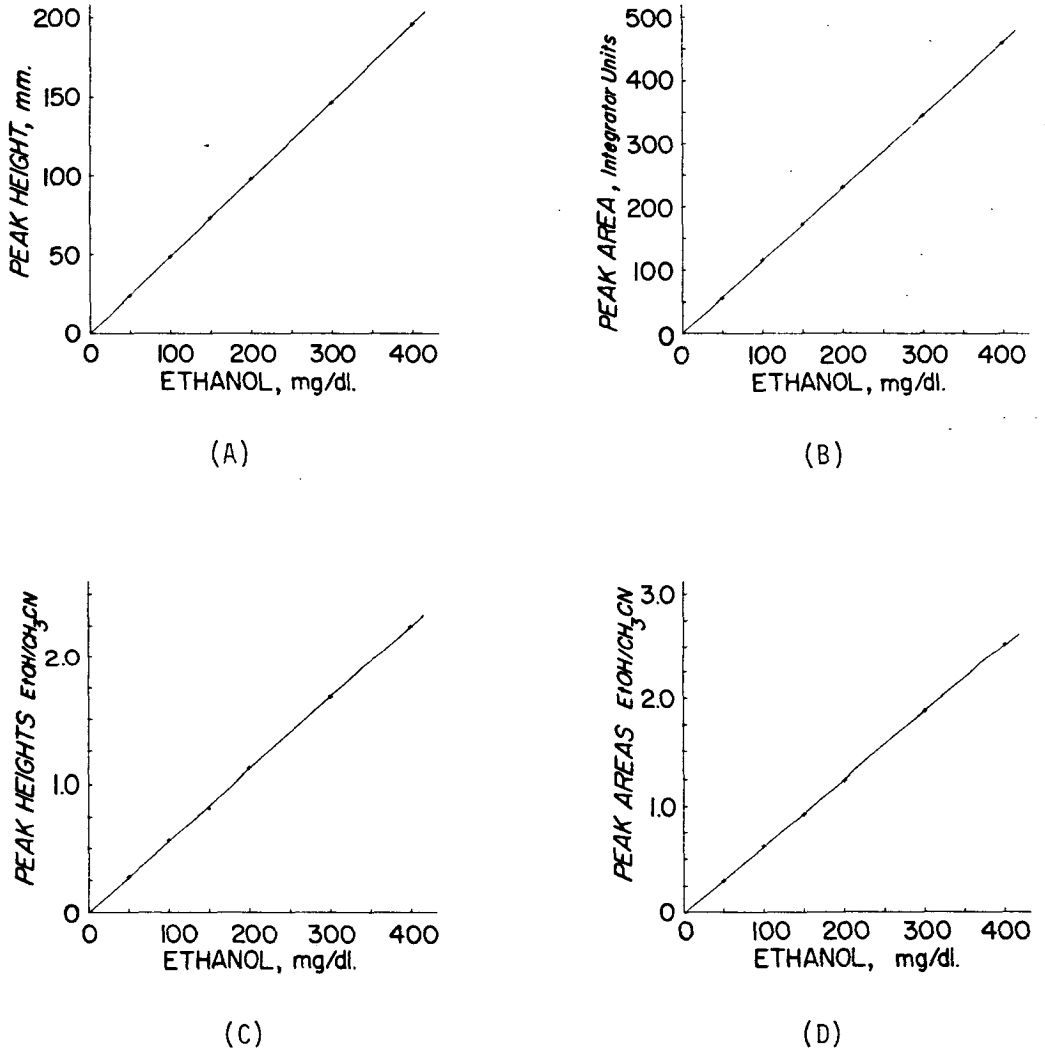

FIGURE 11. TYPICAL CALIBRATION CURVES FOR ETHANOL WITH AUTOMATED GAS CHROMATOGRAPHIC HEADSPACE METHOD, UNDER CONDITIONS DESCRIBED IN TEXT. EACH DATA POINT IS MEAN OF FIVE OBSERVATIONS.
A. PEAK HEIGHT, DIRECT PROCEDURE
B. PEAK AREA, DIRECT PROCEDURE
C. RATIO OF PEAK HEIGHTS, INTERNAL STANDARD PROCEDURE
D. RATIO OF PEAK AREAS, INTERNAL STANDARD PROCEDURE

reproducibility and linearity of calibration of the respective analysis modes and means of quantitating the ethanol content of the reference specimens. Hence, each of these four quantitation schemes is potentially acceptable and satisfactory for determination of ethanol in liquid specimens. Linearity of response over the immediate range of interest in any given analysis run is, of course, an important consideration. Typical direct experimental verification of such linearity over the immediate range of interest for ethanol concentrations in blood is illustrated in Figure 12.

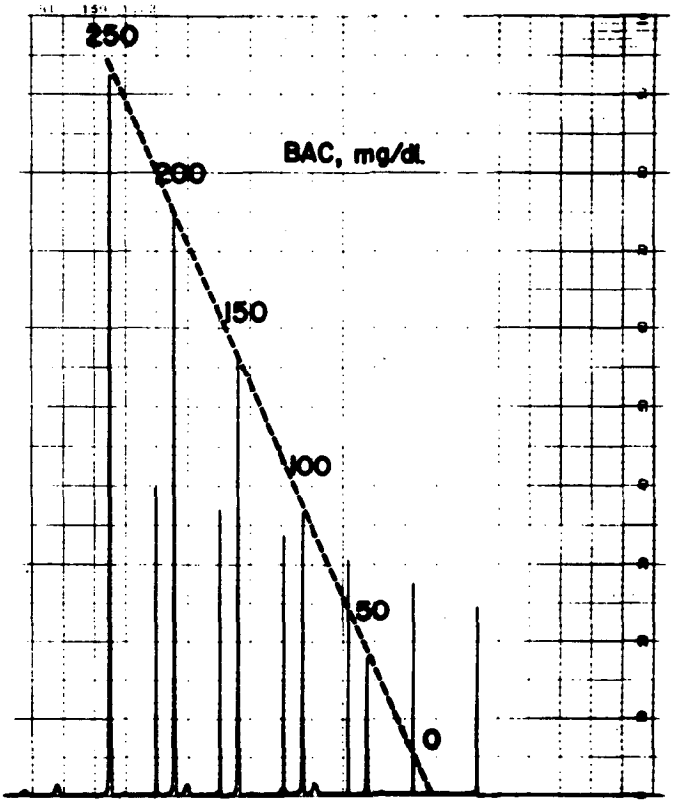

FIGURE 12. LINEARITY OF DETECTOR RESPONSE TO EHTANOL WITH AUTOMATED GAS CHROMATOGRAPHIC HEADSPACE METHOD (DIRECT PROCEDURE). EACH ETHANOL PEAK IS PRECEDED BY A SAMPLE IDENTITY MARKER

It can be estimated from the gas chromatographic instrument parameters and the typical calibration results that the automated method described, conservatively, has a functional sensitivity of 10 ng of ethanol per ml of specimen and a functional absolute detection limit for ethanol of approximately 1 nanogram.

Precision. Reproducibility of the automated headspace analysis method is shown in Tables 4 and 5, respectively, for the direct and internal standard modes.

TABLE 4.

PRECISION OF AUTOMATED GC HEADSPACE ANALYSIS FOR ETHANOL WITH MULTIFRACT F-40 INSTRUMENT IN REPLICATE[a] DETERMINATIONS BY DIRECT PROCEDURE

at	50 mg./dl.	Mean = 50.0 mg./dl.	S.D. = 0.27 mg./dl.	C.V. = 0.55%
	100 mg./dl.	100.0 mg./dl.	1.05 mg./dl.	1.05%
	150 mg./dl.	150.0 mg./dl.	0.95 mg./dl.	0.63%
	200 mg./dl.	200.0 mg./dl.	1.77 mg./dl.	0.89%

[a] (N=29, 28, 27, 24)

TABLE 5.

PRECISION OF AUTOMATED GC HEADSPACE ANALYSIS FOR ETHANOL WITH MULTIFRACT F-40 INSTRUMENT IN REPLICATE[a] DETERMINATIONS BY INTERNAL STANDARD PROCEDURE

at	100 mg./dl.	Mean = 100.0 mg./dl.	S.D. = 0.62 mg./dl.	C.V. = 0.62%
	200 mg./dl.	200.0 mg./dl.	0.55 mg./dl.	0.28%
	300 mg./dl.	300.0 mg./dl.	1.65 mg./dl.	0.55%
	400 mg./dl.	400.0 mg./dl.	2.01 mg./dl.	0.50%

[a] (N=14, 15, 15, 15)

Figures 13 and 14 depict the typical precision of replicate analyses for alcohol with the automated method in the direct and internal standard modes, respectively.

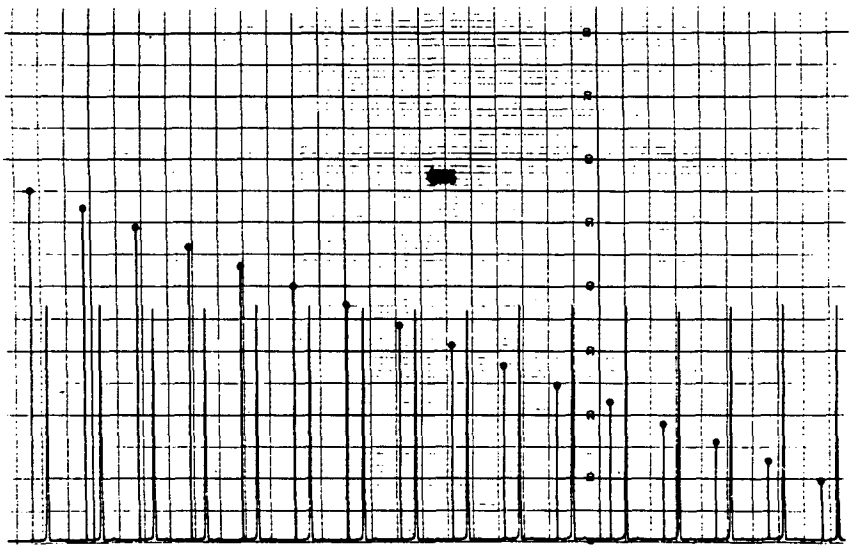

FIGURE 13. CHROMATOGRAM ILLUSTRATING TYPICAL PRECISION OF REPLICATE ANALYSES FOR ETHANOL IN BLOOD WITH AUTOMATED GAS CHROMATOGRAPHIC HEADSPACE METHOD (DIRECT PROCEDURE), UNDER CONDITIONS DESCRIBED IN TEXT. SIXTEEN CONSECUTIVE ANALYSES ARE SHOWN (SAMPLE VOLUME = 100 MICROLITERS; BAC = 200 MG/DL). EACH ETHANOL PEAK IS PRECEDED BY A DOTTED SAMPLE IDENTITY MARKER

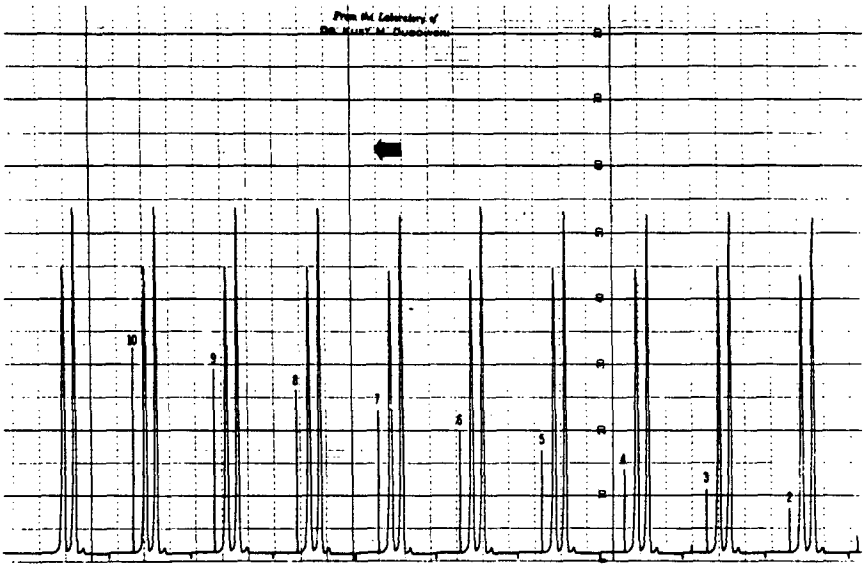

FIGURE 14. CHROMATOGRAM ILLUSTRATING TYPICAL PRECISION OF REPLICATE ANALYSES FOR ETHANOL IN BLOOD WITH AUTOMATED GAS CHROMATOGRAPHIC HEADSPACE METHOD (INTERNAL STANDARD PROCEDURE), UNDER CONDITIONS DESCRIBED IN TEXT. TEN CONSECUTIVE ANALYSES ARE SHOWN (SAMPLE VOLUME = ½ ML, BAC = 167 MG/DL). EACH ETHANOL PEAK IS PRECEDED BY A NUMBERED SAMPLE IDENTITY MARKER

The precision illustrated for the automated method is substantially better than can be achieved with manual methods, even with careful manipulations. Typical precision for the manual headspace method (direct non-internal standard mode) using peak area measurements, at a specimen concentration of 100 mg /dl , is: Standard deviation of replicate analyses = ±2.80 mg /dl ; coefficient of variation = 2.8%.

Accuracy. The term "accuracy" is generally understood, in scientific disciplines, to mean concordance or exact agreement between an experimentally determined result and the true value of the item under consideration. Although the "true" value for the alcohol content of any unknown or reference specimen is, of course, unknown, several experimental approaches can yield adequate information regarding the accuracy of a candidate method for alcohol analysis. Findings for two of these are documented here.

Table 6 reflects the results of independent *routine* analyses for alcohol on a series of whole blood specimens submitted for forensic blood-alcohol analysis by the automated headspace analysis method in the direct non-internal standard mode, employed independently by two geographically separted laboratories. The agreement of the results is remarkably good, especially when it is considered that individual calibrations were carried out independently in the two laboratories and that all of the specimens were stored at 4°C for intervals up to 60 days between separate analyses. The mean difference between the independent BAC results, 0.0012% w/v, is 0.53% of the mean BAC of the 25 specimens (0.226% w/v), and the high correlation coefficient (r=0.998) confirms the excellent agreement of these independently obtained results. Statistical correlation analysis of these results reveals a negligibly slight bias between the two series of results, reflecting the independent calibrations.

When a large number of laboratories carry out independent analyses upon aliquots of the same specimen pool, using several different analytical methods, the mean, mode, and median of the combined results closely reflect the "true" value, provided suitable precautions have been taken against specimen alter-

TABLE 6.

RESULTS OF INDEPENDENT ROUTINE ANALYSES FOR ETHANOL ON IDENTICAL BLOOD SPECIMENS, IN TWO SEPARATE LABORATORIES, BY AUTOMATED GC HEADSPACE ANALYSIS (DIRECT PROCEDURE)

Specimen	BAC Found, % w/v[a]		A-B Difference[b] % w/v
	Laboratory A	Laboratory B	
1	0.16	0.16	0
2	0.33	0.33	0
3	0.18	0.17	0.01
4	0.17	0.17	0
5	0.25	0.25	0
6	0.40	0.40	0
7	0.17	0.16	0.01
8	0.10	0.09	0.01
9	0.27	0.27	0
10	0.17	0.18	-0.01
11	0.37	0.36	0.01
12	0.12	0.12	0
13	0.19	0.19	0
14	0.32	0.32	0
15	0.37	0.37	0
16	0.04	0.05	-0.01
17	0.12	0.13	-0.01
18	0.27	0.27	0
19	0.22	0.22	0
20	0.19	0.19	0
21	0.24	0.24	0
22	0.31	0.30	0.01
23	0.26	0.25	0.01
24	0.15	0.15	0
25	0.29	0.29	0
Mean			0.0012
S.D.			±0.0060

[a] Analysis in Laboratory A preceded those in Laboratory B by intervals of up to 60 days, with intervening specimen storage at 4°C. Both laboratories employed the automated GC headspace analysis method.

[b] Correlation Coefficient: r = 0.998 for Laboratory A Results vs. Laboratory B Results

ation during aliquoting and against decomposition in transit. These conditions exist in "double-blind" proficiency surveys of alcohol analysis in aqueous and biological specimens. Table 7 illustrates performance of the automated method (direct non-internal standard mode) in a monthly state alcohol-testing proficiency survey, vis-à-vis the participant median results for 31 laboratories and the mean results for the 3 reference laboratories. Very high correlation was found for the automated method results with the reference laboratory mean results as well as with the participant laboratory median results. The "blood" specimens in this survey are whole blood hemolysates with significant concentrations of fluoride.

The same high degree of correlation is shown in Table 8 between the automated method (direct, non-internal standard mode) results and the mean values of the results of all 85 participant laboratories. The differences probably relate, at least in part, to alterations of the whole blood specimen pool during aliquoting as revealed by differences in the respective hematocrit values in the preparing and this laboratory, and to the substantially different receipt dates for these specimens in different laboratories. Some bias resulting from different calibrations is also probable.

These comparisons adequately establish the accuracy of the method described herein for alcohol analysis.[23]

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[23] "...Analytical procedure(s) for determining alcohol in blood should meet the following performance requirements: a. The accuracy and sensitivity of the procedure should be such as consistently to attain results within ±0.01% w/v of the known value over the range of 0 to 0.30% w/v in analyses of appropriate reference materials of known ethyl alcohol concentration. b. The precision of the procedure should be such as consistently to attain a standard deviation not greater than 0.003% w/v in replicate analyses. c. The blank values yielded by the procedure in analyses of alcohol-free blood specimens consistently should be not greater than 0.01% w/v. d. The specificity of the procedure should be adequate and appropriate for the analysis of biological specimens for the determination of the blood alcohol concentration in traffic law enforcement and highway crash investigations." (71)

TABLE 7.

TYPICAL PERFORMANCE OF THE AUTOMATED GC HEADSPACE ANALYSIS (DIRECT PROCEDURE) IN "DOUBLE-BLIND" ANALYSES FOR ETHANOL IN A STATE PROFICIENCY SURVEY[a]

Specimen[b]	Nature[c]	Reported Ethanol Concentrations, mg/dl[d]		
		F-40 Result	Ref. Labs. Mean (N=3)	Participant Labs. Median (N=31)
1	U	196	209	201
2	A	323	325	320
3	B	80	82	83
4	A	232	233	232
5	B	157	159	162
6	U	281	283	286
7	U	197	196	198
8	A	325	326	320
9	B	155	162	160
10	A	320	324	312
11	B	295	298	286
12	U	236	239	239
13	U	160	160	162
14	A	72	71	73
15	B	296	300	290
16	A	323	320	319
17	B	0	2	0
18	U	240	249	240
19	B	250	256	250
20	U	158	167	162
21	B	325	336	316
22	U	201	206	204
23	U	86	90	90
24	A	296	297	293
25	B	254	259	252
26	A	157	161	159

[a] Wisconsin State Laboratory of Hygiene Alcohol Testing Evaluation Program (60)

[b] Consecutive paired monthly specimens (e.g., 1 + 2, 3 + 4, etc.)

[c] B = Blood; U = Urine; A = Aqueous

[d] Correlation Coefficients: r=0.999 for Reference Labs. Mean vs. F-40 Result; r=0.978 for Participant Labs. Median vs. F-40 Result

TABLE 8.

TYPICAL PERFORMANCE OF THE AUTOMATED GC HEADSPACE ANALYSIS (DIRECT PROCEDURE) IN "DOUBLE-BLIND" ANALYSES FOR ETHANOL IN A NATIONAL PROFICIENCY SURVEY[a]

Specimen[b]	Nature[c]	Reported Ethanol Concentrations, mg/dl[d]	
		F-40 Result	Participant Labs. Mean (N=85)
1	B	127	123
2	B	76	76
3	A	191	185
4	A	48	49
5	B	216	225
6	B	165	167
7	A	76	76
8	A	178	179
9	B	193	194
10	B	99	99
11	A	196	198
12	A	96	99
13	B	213	216
14	B	121	125
15	A	82	83
16	A	148	149
17	B	91	94
18	B	145	149
19	B	92	97
20	B	195	193
21	B	268	272
22	B	53	61
23	B	155	164
24	B	85	91
25	B	214	229
26	B	110	114
27	B	68	71
28	B	158	161

[a] U. S. Department of Transportation, Transportation Systems Center Blood-Alcohol Proficiency Testing Program (61)

[b] Consecutive Sets of 4 quadrimonthly specimens (e.g., 1-4, 5-8, etc.)

[c] B = Blood; A = Aqueous

[d] Correlation Coefficient: r = 0.998 for Participant Labs. Mean vs. F-40 Result

4. QUALITY ASSURANCE: SELECTED ASPECTS

In modern laboratory practice, quality assurance is generally understood to encompass those plans and activities which are intended to provide certainty that the laboratory operations are proper and suitable to the assigned tasks, and that the reported laboratory results are correct, reliable, and valid. It is evident that quality assurance is a very large subject, only a few selected aspects of which can be considered here.

4.1 REFERENCE MATERIALS AND THEIR VALIDATION

Gas chromatographic methods are basically comparison procedures in which qualitative and quantitative results are obtained by comparison of the instrument response generated by an unknown sample with those generated by samples representing reference materials or calibrators, commonly (but improperly) referred to as "standards." The selection, validation and use of reference materials and calibrators is thus critically important to the quality and validity of the gas chromatographic analysis data.

Reference Materials. Pending the development and marketing by the National Bureau of Standards of an ethanol SRM (=Standard Reference Material) to which working ethanol reference specimens can be traced, alternate means of validating the latter must be employed.

Ethanol reference specimens (often referred to as "calibrators") can be purchased from commercial sources or prepared locally from reagent-grade ethanol. Typical aqueous calibrators understood by the author to be available commercially at the time of this writing (June 1976) are:

Alcohol Stock Solutions (Aqueous)

Cat. No. 8988/0001 Ampoules, 0.5 mg in 1 ml
Cat. No. 8991/0001 Ampoules, 1.0 mg in 1 ml
Cat. No. 9003/0001 Ampoules, 2.0 mg in 1 ml
Cat. No. 9008/0001 Ampoules, 4.0 mg in 1 ml
Cat. No. 8985.0001 Large Series (0.8-1.9 mg in 1 ml in Steps of 0.1 and 2.0-3.0 mg in 1 ml in Steps of 0.2)
Manufacturer: E. Merck, Darmstadt, Germany (E. M. Laboratories, Inc., 500 Executive Blvd., Elmsford, N. Y. 10523)

Ethanol Standard Solution, 0.08% w/v

Product No. 330-20
Manufacturer: Sigma Chemical Co., P. O. Box 14508, St. Louis, Mo. 63178.

Ethanol Calibrators are readily prepared from reagent-grade ethanol, commercially available at 190 Proof (~95% v/v) or 200 Proof (~100% v/v). Typical sources understood by the author to be available at the time of this writing (June 1976) are[24]:

Pure Anhydrous Ethyl Alcohol

Manufacturer: Commercial Solvents Corporation
245 Park Ave., New York, N.Y. 10017

Absolute Pure Ethyl Alcohol

Manufacturer: U. S. Industrial Chemicals Co., 99 Park Ave., New York, N.Y. 10016.

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[24] Purchase and use of "pure" (i.e., undenatured) ethyl alcohol is subject to Federal regulations (26 CRF), whether tax-paid or tax-free use is involved. Details of these regulations are available from the Bureau of Alcohol, Tobacco and Firearms, U. S. Department of the Treasury.

Specifications for reagent-grade Ethyl Alcohol (190 Proof) and Ethyl Alcohol, Absolute are given in the AMERICAN CHEMICAL SOCIETY SPECIFICATIONS for reagent chemicals (62). Since anhydrous ethanol is highly hygroscopic, it should be obtained, for this use, in the smallest unit size available. Absolute ethanol in original, tightly sealed commercial containers is generally water-free prior to opening. If necessary, traces of water can be removed with calcium oxide or molecular sieve 3A. Because it is much less hygroscopic, 190-Proof ethanol is much easier to use as a stock reagent, but does require the additional step of determining its exact initial ethanol concentration by suitable quantitative analysis. Dilute ethanol solutions, such as those prepared as calibrators, are stable when properly stored and protected from chemical and microbial contamination. Stored in screw-capped borosilicate glass containers at 0-5°C, such solutions are stable for at least one year; in individual 5-10 ml portions in flame-sealed borosilicate glass ampoules, they appear to be stable indefinitely (63, 64).

Aqueous calibrators of desired alcohol concentrations are readily prepared by critically careful dilution of appropriate stock reference ethanol solutions, with fully quantitative technique. Stock reference ethanol solutions, in turn, are best prepared by *weighing* the required quantity of absolute ethanol into a conveniently-sized, tared flask; diluting with approximately 50 ml of reagent-grade water and quantitatively transferring the mixture to a 1 liter volumetric flask; and finally, diluting to volume with reagent-grade water, with due attention to the flask calibration temperature, normally 20°C for Grade A volumetric ware. Alternately (and less preferably), the desired calibrator concentration is established by *volumetric measurement* of the required ethanol quantity, calculated from the desired mass of anhydrous ethanol and its density at the existing laboratory temperature (e.g., 0.78522 g/cm^3 at 25°C), obtained from various handbooks.

Aqueous calibrators yield results identical to those yielded by calibrators based on biological liquids such as whole blood or whole blood hemolysates

in the methods detailed here. That is not necessarily true for other methods of analysis; in fact, it is generally *not* true for many alcohol analysis methods. On purely theoretical grounds, calibrators should be identical to unknown specimens in terms of biological matrix composition or properties and should cover the entire range of alcohol concentrations of interest. Further, at least three calibrators of different alcohol content should be used in any given calibration to provide appropriate information about the linearity of calibration in the specific instance. In practice, complete identity of composition and other relevant properties for calibrators and unknowns is often both unachievable or unnecessary (as in the methods described here). Surrogate calibrators are often employed effectively; these are specimens which possess the critical properties of the unknowns (e.g., viscosity, protein content, nonhomogeneity) to an extent sufficient to duplicate or simulate the behavior of the unknowns in the various steps of the analysis. Considerable sophistication is required to make and implement appropriate choices of surrogate materials, and specialized references on this topic should be consulted. Human whole blood, for example, differs substantially from that of some animal species in respect to erythrocyte fragility (and hence extent of hemolysis of samples), concentration and nature of plasma proteins, cellular composition, sedimentation rate, etc. Whole blood of animal origin, especially that obtained incident to slaughter, is usually not a suitable substitute for preparation of calibrators or control specimens which are to duplicate the properties of human blood. In turn, human whole blood obtained in the form of outdated blood-bank materials is not identical in properties or composition to freshly shed whole human blood, often being substantially diluted with liquid anticoagulants and of greatly different carbohydrate and/or electrolytes composition. Additional safety aspects are also relevant such as the need to guard against hepatitis transmitted by contact with human blood or blood components. For these and other cogent reasons, calibrators and control specimens should be prepared with commercially available products of human origin which include proper safeguards, if possible.

Validation of Reference Specimens and Calibrators. The exact ethanol concentration of stock reference solutions and dilute calibrators, prepared as described above, should be validated by independent analysis. The usual progression is to employ three linked sets of reference materials: 1) A *Primary Standard* such as the NBS potassium dichromate SRM, of known and validated chemical characteristics and established quantitative chemical reaction relationship to ethanol; 2) a *Secondary Standard* such as an aqueous solution of ethanol whose concentration has been validly established by quantitative analysis using or traceable to a Primary Standard (e.g., by oxidimetry with NBS potassium dichromate) and which serves as a stock solution; 3) a *Tertiary Standard*, such as a ready-to-use, dilute aqueous ethanol solution prepared by dilution from the Secondary Standard, to be employed directly as a calibrator or for the preparation of other reference samples or of control specimens of known alcohol content.

A variety of chemical and physical methods can be used for the establishment and/or verification of the ethanol concentration of reference materials and calibrators. Suitable physical methods of analysis include pycnometry and refractometry, the experimental results being converted to the corresponding ethanol concentration from handbook tables (e.g., 65, 66). Osmometry has been advocated by Redetzki (63) for analysis and control of aqueous alcohol reference solutions; only osmometers operating on the freezing-point depression principle are suitable for this application. The author has found this technique useful for the rapid, simple pre-use checking of reference solutions to exclude gross dilution errors or unsuspected deterioration.

Chemical analysis by oxidimetry using a potassium dichromate reagent is a suitable method for initial and periodic verification of the alcohol content of stock and dilute alcohol reference solutions and calibrators, since

the results are traceable directly to a true Primary Standard;[25] it is the method of choice recommended here. The reaction, for the oxidation of ethanol utilizing potassium dichromate in the presence of sulfuric acid, is

$$2Cr_2O_7^{=} + 3C_2H_5OH + 16H^+ \longrightarrow 4Cr^{+++} + 3CH_3COOH + 11H_2O.$$

Under suitably moderate oxidation conditions (acid concentration below 18N, etc.) the reaction does not proceed beyond acetic acid. It follows that under such conditions 4.257 mg of $K_2Cr_2O_7$ are equivalent to 1.000 mg of C_2H_5OH. Most commonly, a measured aliquot of the ethanol sample is reacted with excess standard dichromate solution and the unreacted dichromate determined spectrophotometrically or titrimetrically. The Dubowski spectrophotometric microdetermination method with final measurement at 350 nanometers (67) is well-suited to this oxidimetric analysis, since it requires only a single stable reagent and is rapid, simple, and sensitive. When an aqueous alcohol reference solution is to be analyzed, the distillation step of the procedure can be omitted, and the reaction is carried out directly on a 1:5 dilution of the aqueous specimen. *Oxidimetry with potassium dichromate* is also the procedure for determination of the ethanol concentration of standard stock solutions stipulated in the DOT Performance Standard for Calibrating Units for Breath Alcohol Testers (68).

Enzymatic oxidation of ethanol in the presence of NAD oxidoreductase, EC 1.1.1.1 (=alcohol dehydrogenase) can also be used to quantitate ethanol in reference solutions and calibrators, the reaction being:

$$C_2H_5OH + NAD^+ \overset{ADH}{\rightleftharpoons} CH_3CHO + NADH + H^+.$$

The ultraviolet spectrophotometric measurement method based on this reaction

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[25] Potassium dichromate, oxidimetric, SRM 136c, Office of Standard Reference Materials, Rm. B311, Chemistry Building, National Bureau of Standards, Washington, D. C. 20234.

is very sensitive and rapid; it can also be simplified by eliminating the primary separation step (e.g., distillation) when analyzing suitably-diluted aqueous alcohol solutions. Under ideal analysis conditions, the alcohol concentration of aqueous samples can be determined solely from the difference between the measured absorbances at 340 nanometers of the unknown and blank samples and the established molar absorptivity of NADH; in practice, an ethanol calibrator is always included in the analysis series (69, 70).

Whatever scheme is employed to establish the actual concentration of ethanol in stock and dilute reference solutions and calibrators, it should be emphasized that an experimental confirmation of the ethanol concentration independent of the preparation procedure should be made initially (after preparation) and at appropriate intervals thereafter if the materials are stored.

4.2 PERFORMANCE MONITORING

Both internal and external performance monitoring are accepted quality assurance practices. Of the many variants, those considered here are use of within-run and between-run controls, use of internally-prepared "unknown" specimens, and participation in internal and external performance monitoring (often referred to as "proficiency testing").

Use of Within-run and Between-run Controls. Individual aliquots of a suitably large biological specimen pool, so treated and stored as to maintain stability of composition, are included in each series of analyses. The results should lie within acceptable limits[26] of the previously-estab-

[26] "Procedures for blood analysis should include the following controls in conjunction with each batch of samples analyzed: ...b. Analysis of a suitable reference or control blood sample of known alcohol content within the range 0.10 to 0.30% w/v; the result of which analysis must coincide with the known blood alcohol value of the reference specimen within ±0.01% w/v if validity is to be assigned to the results for the batch analyzed." (72)

lished concentration. To avoid contamination of the specimen pool by repeated entry to withdraw aliquots, as well as the necessity to thaw or bring it to room temperature on each occasion of use, it is good practice to divide the homogeneous or well-mixed specimen pool into single-use aliquots (e.g., 1 ml portions) to be stored preferably in flame-sealed ampoules.

Whole-blood alcohol control specimens are commercially available. At the time of this writing (June 1976), the following such item was understood by the author to be marketed:

Product No. 2930-12, LederEth® Whole Blood Alcohol Control
Manufacturer: Lederle Diagnostics, American Cyanamid Co.,
Pearl River, N. Y. 10965.

These materials consist of human whole blood containing ethanol (typically at a BAC of 0.15% w/v) and are furnished with analysis results applicable to the specific lot of control material. Commercial or laboratory-prepared controls (i.e., surrogate specimens identical (if possible) or appropriately similar to the unknown specimens in overall composition and expected alcohol content) should be included in every analysis series ("run"). Only if the experimental result obtained for the control specimen in that run coincides acceptably with the established target value should the results for unknowns in that analysis series be cleared for release. Conversely, should the control specimen result not be acceptable, search for the cause of the discrepancy should be instituted and any indicated remedial measures completed before analysis of unknown specimens is resumed. It should be emphasized that the control specimen(s) are to be included in each run *in addition to and apart from* the calibrators and should not be aliquots of any calibrator used in that run.

Internal and External Proficiency Testing. Performance evaluation with survey or proficiency testing specimens has become an indispensable part of laboratory quality assurance practice and of laboratory regulatory programs. Internal and external proficiency testing have some similarities and several significant differences, especially in objective. Internal proficiency testing is a useful and objective but limited technique for detecting and documenting presence or absence of acceptable laboratory performance (in the tested area) according to predetermined criteria and perhaps pinpointing one or more commonly encountered sources of error or indicating gradual changes in laboratory performance. In this respect, it serves in part as a control function.

External proficiency testing has the advantage of a larger data base from (often many) participating laboratories and, usually, from a separate group or "reference" or "referee" laboratories.[27] It thus permits relative assessment of the laboratory's performance in comparison with a peer group and often compared with different analytical methodologies which may not be in use locally. Since the external proficiency test specimens are identified as such upon receipt, whether delivered by common carrier or hand-carried by inspectors, they clearly often receive special treatment not accorded routinely submitted specimens. To that extent, the results of participation in external proficiency testing or surveys constitute a measure of best laboratory capability rather than an indication of standard or routine performance. This limitation also exists to some degree in internal proficiency testing but can be more easily overcome, in part at least, by use of split-specimens, masked submissions by supervisors, and similar techniques. External proficiency testing periodically documents elements of laboratory performance,

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[27] As commonly understood, "reference" laboratories determine the true identity or composition of a specimen, or characterize it; "referee" laboratories establish the level of acceptable performance for participant laboratories, usually for quantitative or semiquantitative results.(74)

and continued satisfactory performance according to predetermined criteria thus established is often a requirement for laboratory licensure in the tested category of laboratory activity. The techniques of proficiency testing and its multiple aspects are a highly specialized field and the applicable literature should be consulted. (73-78).

Use of internal proficiency testing is recommended as a good management practice for surveillance of laboratory performance. If "blind" specimen handling is possible (i.e., use of proficiency test specimens sufficiently similar to those routinely analyzed and submitted like the latter with respect to containers, laboratory request forms, putative identification, use of the mails, etc.) it adds an element of credibility, but analysts quickly become adept in identifying such "blind" submissions unless extraordinary care is taken in their camouflage.

External proficiency testing in the health laboratory field is widely carried out by Federal agencies, units of many state governments, and professional organizations in the private sector. Although occasional probes and periodic surveys of laboratory performance in analyses of aqueous or biological specimens for alcohol have been carried out by several organizations for varying intervals in the past (e.g., by the American Academy of Forensic Sciences Toxicology Section, California Association of Criminalists, Center for Disease Control), the sole current *national* program of proficiency testing in alcohol analysis known to the writer is the Blood-Alcohol Proficiency Testing Program of the National Highway Traffic Safety Administration, U. S. Department of Transportation (operated by Transportation Systems Center, U. S. Department of Transportation, Cambridge, Mass. 02142). Many states, however, operate individual statewide proficiency testing programs in alcohol analysis.

4.3 OTHER QUALITY ASSURANCE PRACTICES

There is at present no universally recognized equivalent, in forensic or health laboratory practice, to the overall generally-accepted operational

standards encompassed under the term "GMP" (Good Manufacturing Practices) in, for example, the pharmaceutical industry. In their absence, each laboratory must strive individually to identify and implement operational practices which the consensus of informed practitioners would identify as "Good Laboratory Practices," and to eliminate any local practices inconsistent therewith.

Certain desirable practices are obvious, and many are common to all forensic laboratories (and often health laboratories as well). These include the full, contemporaneous, unique labeling and identification of every incoming specimen and its associated items (laboratory request forms, shipping containers, etc.) and of every laboratory-generated hard-copy data form. Raw data should be entered into permanent bound data log books if handwritten or assembled into permanent data log books if computer-terminal generated, etc. There is no substitute for dating (and timing when indicated, as when multiple analytical runs are made in a given day) and identifying with the name of the writer all record entries. The usual bookkeeping rules should be followed; all entries are made in permanent reproducing ink and are thereafter never obliterated or eradicated; errors or deletions are treated by ~~non-obliterating-through-lining-of-the-item(s)-concerned~~, associated ibidem with any corrections or superseding information. Liberal use should be made of time stamps, numbering machines, etc. In addition to raw data compilations associated with each specimen analyzed, all computations entering into the final reported result should be shown in full in the permanent record log, including any unique factors (special dilutions, individual sample quantities other than routinely used, etc.). Data files should be maintained for an *adequately* long period to meet all legal and operational requirements; when necessary, they can be microfilmed.

A separate permanent, complete, chronological file should be maintained for methods. This includes complete documentation of all procedural steps for a given analysis, and literature reference(s), reagent preparation directions,

sample calculation, instruction for reporting of results, instrumental parameter listings, etc. The initial method description should bear the date of preparation and full name of the preparer, and all subsequent changes should consist of dated, signed *additions* (perhaps on differently colored paper stock), but never of physical deletions. Separate bench reminder cards can be used to summarize salient steps in the procedure for time/point-of-use availability.

Reports of examination of blood and other biological liquids for alcohol should be complete, correct, and fully self-contained. Hence, they should always include the following data:

a. Laboratory case identification number
b. Complete subject and specimen identification (Cf. Section 1.3)
c. Unequivocal statement of the examination(s) or analyses performed, together with brief identification of the analysis method employed
d. Unequivocal report of the findings, always inclusive of concentration units employed
e. Interpretation of the findings, if required
f. Significant date/time information relating to specimen collection, specimen receipt in the laboratory, analysis, and reporting
g. Legible name, title, signature of the reporting analyst(s) and official(s)
h. Authentication device(s).

The last item deserves further comment. In this era of efficient, ubiquitous copying machines, it seems obvious that original authentic reports should be readily distinguishable from unofficial copies and forgeries. Report forms can be printed on security paper which is uniquely identifiable and reveals attempted alterations; versions are available which resist copying by xerographic methods. It is, additionally, recommended good practice to authenticate the completed report and any authorized copies with an embossed, ser-

rated, or penetrating seal or legend, such as those used for commercial checkwriters, passport photograph attachment, bank cancellation of checks, etc. The embossed authentication used for reports originating from the writer's laboratory is illustrated in Figure 15. Distribution of official copies should be shown on the original of the report.

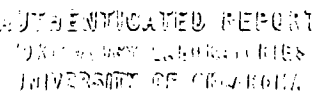

FIGURE 15. EMBOSSED AUTHENTICATION FOR TOXICOLOGY LABORATORY REPORTS, EMPLOYED IN AUTHOR'S LABORATORY

It has a salutary effect on the credibility of the laboratory and of its reports, as well as on the conscientiousness of laboratory personnel in recording information and data, if the laboratory observes a universal "open book" policy. By this is meant that original raw data entries, chromatograms, calculations, proficiency testing information, etc., are available for inspection in the laboratory, at reasonable times, by any appropriately interested party (e.g., attorneys representing a tested subject) without further legal process. The advantages of such a policy far outweigh any potential disadvantages, and it should be pursued whenever it is statutorily permissible.

5. INTERPRETATION OF RESULTS

In forensic and clinical laboratory practice, proper interpretation of results depends in substantial measure on the use to be made of those laboratory results. Further, many jurisdictions have statute law governing the interpretation of the results of alcohol analyses in relation to the alleged offense of operating a (motor) vehicle under the influence of alcohol, which remains among the most common applications of breath-alcohol and blood-alcohol analyses. Virtually all jurisdictions also have applicable case law relating to the interpretation of traffic law-related alcohol analyses. The material which follows is not intended to supersede or replace such applicable statute or case law, but rather to supplement them.

An understanding of the somewhat unique position of ethanol in the broad spectrum of interpretative toxicology may be useful at this point. With most other drugs (e.g., the barbiturates), the usual practice is to define expectable therapeutic (where applicable), toxic, and lethal concentrations in a specified medium (e.g., blood plasma). In this context, therapeutic concentration is generally considered that accompanying effective therapeutic action of the drug and/or its metabolites in humans, and toxic concentration is often regarded as that associated with serious toxic symptoms in humans (79). With alcohol, interpretation of concentrations found in whole blood (for example, and limited to that medium for the moment to simplify the discussion) is typically required for the following purposes:

1) To establish whether or not alcohol is a significant factor in a patient's clinical condition, or whether a patient is intoxicated

2) To establish whether or not the alcohol-related element of a motor vehicle or other traffic offense is present.

Thus, in the former, the BAC becomes an element in decisions concerning diagnosis, prognosis, or treatment, while in the latter it becomes an element in the investigation and prosecution of an alleged traffic offense. It is easier to consider these two applications separately since they involve significant differences. In general, however, and in contrast to the usual practice with respect to most other drugs subject to abuse, four special features apply: (1) There is no accepted therapeutic concentration in the usual sense; (2) the demonstrable effects of alcohol are of substantial legal (and clinical) significance at other than the toxic and lethal concentrations in blood, and hence lesser concentrations are critically important; (3) in many instances existence of a BAC less than, or equal to or greater than a statutory-specified value is per se the conclusive decisive criterion (or more commonly is a presumptive evidential factor); and (4) unequivocal demonstration of the presence of alcohol is the sole decisive criterion in certain special situations.

5.1 BLOOD-ALCOHOL CONCENTRATIONS

While artificial in a sense, it is convenient to consider interpretation of BACs separately with respect to clinical and law enforcement applications. Figure 16 is a schematic flow-chart illustrative of the interpretation of BACs (or other alcohol findings) in typical civil and criminal justice proceedings.

Clinical Applications. Disregarding its social-and-recreational-drug use aspects, the acute effects (if any) of alcohol intake on a person can only be properly evaluated in the light of the total individual situation and of the entirety of his or her environment at the time concerned. Most often, the acute effects of alcohol of interest are those related to alcoholic intoxication, usually understood to mean the demonstrable, significantly deleterious acute effects of alcohol attributable to its direct pharmacological actions.

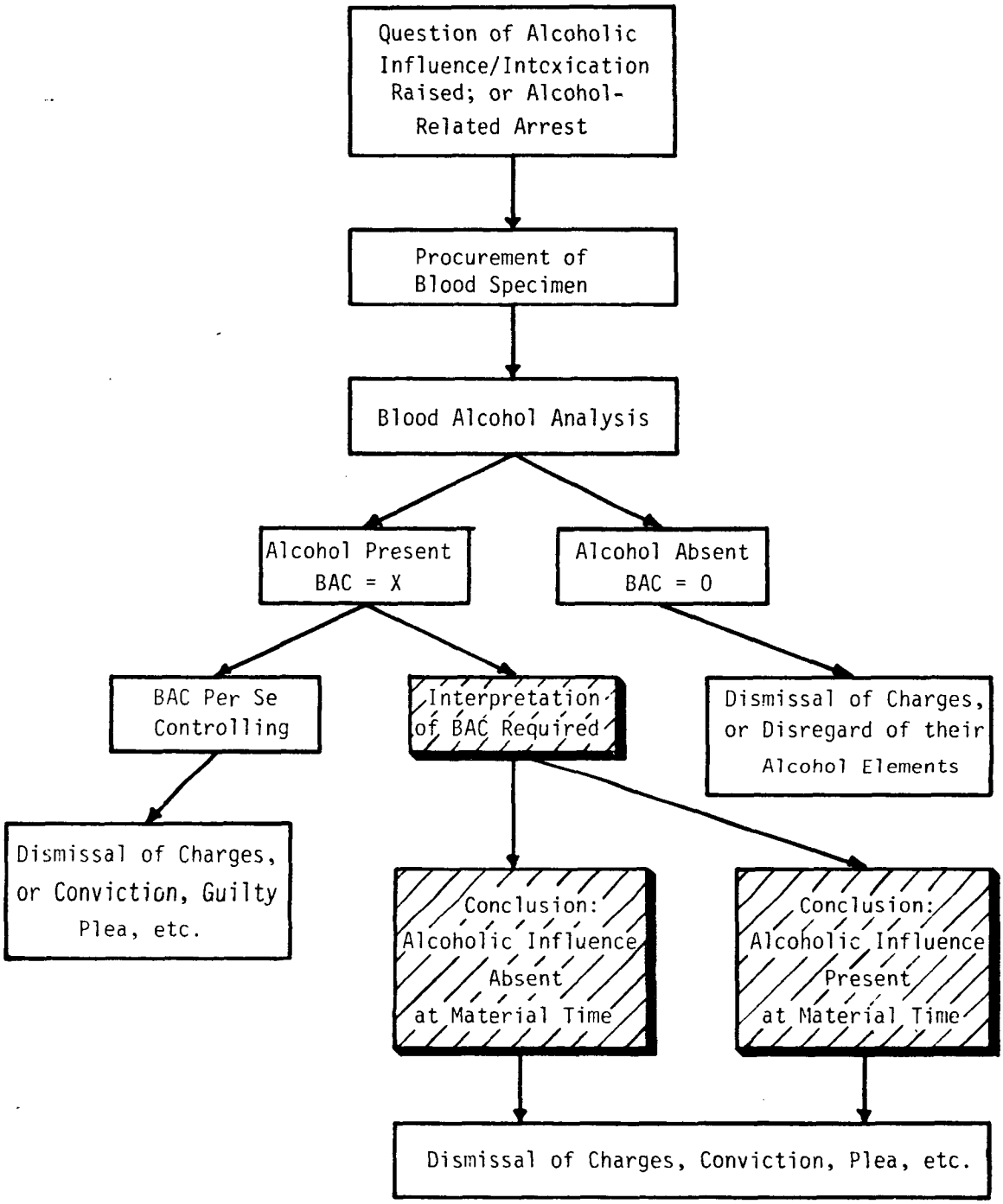

FIGURE 16. SCHEMATIC FLOWCHART ILLUSTRATING ROLE OF INTERPRETATION OF BLOOD ALCOHOL FINDINGS IN TYPICAL CRIMINAL AND CIVIL JUSTICE PROCEEDINGS (INTERPRETATION FUNCTIONS CROSSHATCHED)

Against the background that these acute effects of alcohol can range continuously from subclinical to lethal, it can be said that (acute) alcoholic intoxication constitutes

1) a state of unfitness, attributable to the acute effects of alcohol upon the human body and mind and their several processes and functions, safely to undertake the task(s) attempted; or
2) a state of significant danger to the life of the individual concerned, resulting from the acute effects of alcohol upon the human body and mind and their several processes and functions.

The second situation is analagous to any life-threatening drug overdose.

The continuum of the acute effects of alcohol upon the human body and mind and their function and processes is reflected in Table 9, developed by Dubowski (80). It should be noted that there is deliberate overlap between the designated stages of alcoholic influence and cross-over between these stages and the adjacent clinical signs and symptoms,[28] as well as between both of these categories and the BAC ranges shown. While the tabulation is, in the author's experience, typical, exceptions to the categorized information are encountered.

Space does not permit further consideration herein of the nature and extent of individual fluctuations in consumption tolerance and constitutional tolerance to alcohol. It is also recognized that interpretations analagous to those required for clinical applications are frequently called for in connec-

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[28] In common clinical usage, signs denote *objective* indications or manifestations of the existence of health, disorder, or disease; symptoms refer to *subjective* functional evidence or manifestations of a patient's condition. The former are thus observable, but the latter require reporting by the subject.

TABLE 9. STAGES OF ACUTE ALCOHOLIC INFLUENCE/INTOXICATION (80)

BLOOD-ALCOHOL CONCENTRATION, %W/V	STAGE OF ALCOHOLIC INFLUENCE	CLINICAL SIGNS/SYMPTOMS
0.01-0.05	Sobriety	No apparent influence Behavior nearly normal by ordinary observation Slight changes detectable by special tests
0.03-0.12	Euphoria	Mild euphoria, sociability, talkativeness Increased self-confidence; decreased inhibitions Diminution of attention, judgment, and control Loss of efficiency in finer performance tests
0.09-0.25	Excitement	Emotional instability; decreased inhibitions Loss of critical judgment Impairment of memory and comprehension Decreased sensitory response; increased reaction time Some muscular incoordination
0.18-0.30	Confusion	Disorientation, mental confusion; dizziness Exaggerated emotional states (fear, anger, grief, etc.) Disturbance of sensation (diplopia, etc.) and of perception of color, form, motion, dimensions Decreased pain sense Impaired balance; muscular incoordination; staggering gait, slurred speech
0.27-0.40	Stupor	Apathy; general inertia, approaching paralysis Markedly decreased response to stimuli Marked muscular incoordination; inability to stand or walk Vomiting; incontinence of urine and feces Impaired consciousness; sleep or stupor
0.35-0.50	Coma	Complete unconsciousness; coma; anesthesia Depressed or abolished reflexes Subnormal temperature Incontinence of urine and feces Embarrassment of circulation and respiration Possible death
0.45+	Death	Death from respiratory paralysis

tion with judicial and quasi-judicial proceedings. Common problems in these categories involve questions regarding the existence and extent of alcoholic intoxication as a partial or complete defense against workmen's compensation claims or as an issue in other types of civil litigation; questions regarding the defendant's capacity to form intent for (or execute) an alleged criminal act; and questions concerning a subject's ability adequately to understand his rights in an investigative or custodial situation and his or her ability intelligently, knowingly, and understandingly to waive *Miranda*-derived rights.

Applications to Traffic Law Enforcement.[29] These fall into two principal categories:

1) To establish whether or not the alcohol element of the alleged offense is present in a jurisdiction which prohibits operation or actual physical control of a (motor) vehicle by a person whose BAC is equal to or greater than a specified value

2) To establish whether or not the alcoholic influence or alcoholic impairment element of the alleged offense is present in a jurisdiction which prohibits operation or actual physical control of a (motor) vehicle by a person while under the influence of alcohol (etc.) or while such person's ability to operate a (motor) vehicle is impaired by the presence of alcohol in the blood.

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[29] UVC §11-902: "Driving while under influence of alcohol or drugs. (a) A person shall not drive or be in actual physical control of any vehicle while: 1. There is 0.10 percent or more by weight of alcohol in his blood; 2. Under the influence of alcohol... 4. Under the combined influence of alcohol and any drug to a degree which renders him incapable of safely driving..." (81)

In the first instance, the reported BAC result becomes a controlling element in the adjudication of this so-called "per se" offense which makes driving illegal at or in excess of a stipulated blood-alcohol concentration. In the second instance, the statutory provisions merely create a factual, rebuttable presumption of being under the influence of alcohol, based upon the same evidence (i.e., BAC $\geq$ a specified value). Suitably expert interpretation is therefore required in the latter situation on the issue of whether the BAC in evidence is indicative of existence of alcoholic intoxication, influence, or impairment at the material time, to the requisite standard of proof - customarily, in criminal litigation, beyond a reasonable doubt. In reaching that opinion, the expert witness may, depending upon the jurisdiction involved, need to be cognizant of applicable case law defining such relevant terms as "intoxicated," "under the influence of alcohol (or intoxicating liquor)," "impaired," etc. There is massive case law on this subject (82); the following classical examples show the diversity of these interpretations:

a. Deskin v. State (Oklahoma, 1951): "Not drunk is he who from the floor can arise and drink once more, but is he drunk who prostrate lies and can neither drink nor rise." (83)

b. State v. Stout (Iowa, 1956): "...if by reason of the use of alcoholic liquors...he has lost control in any manner or to any extent of his reason or faculties, or the control of motion of his person or body, then he is in an intoxicated condition within the meaning of the law..." (84)

c. Steffani v. State (Ariz., 1935): "The expression 'under the influence of intoxicating liquor' covers not only all the well known and easily recognized conditions and degrees of intoxication, but any abnormal mental or physical condition which is the result of indulging in any degree in intoxicating liquors, and which tends to deprive him of that clearness of intellect and control of himself which he would otherwise possess. If the ability of the driver of an automobile has been lessened in the slightest degree by the use of intoxicating liquors, then

the driver is deemed to be under the influence of intoxicating liquor..." (85)

Other jurisdictions have appellate decisions which promulgate as the standard for establishment of alcoholic influence or intoxication that the subject lack to "an appreciable degree the ability to function properly..." or that he be "so affected by the ingestion of... alcohol as to impair his ability to drive in an appreciable degree..." (86). It is altogether esoteric and treacherous territory, and the interpretation of alcohol concentrations in blood (or in other specimens) in relation to alleged traffic law violations or other offenses is distinctly no task for a tyro, but requires an experienced, seasoned toxicologist with directly applicable first-hand experience in this field.

There is a further complication. In traffic law enforcement, the time of blood sampling is commonly not the material time of evidential interest; hence, retrograde extrapolation of the BAC found to an earlier time (e.g., that of a vehicular crash or an alleged traffic law violation) is not infrequently requested. Unfortunately, that material time is virtually never[30] identified statutorily as the time of collection of the specimen.

This is a most complex matter involving biological and other factors, which has been covered in detail elsewhere (3, 4, 87). For the present purpose it suffices to note that various assumptions of uncertain validity would

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[30] What, never? Well, hardly ever: Illinois Vehicle Code 1972: "§11-501. Persons under the influence of intoxicating liquor or narcotic drugs... c) evidence of the amount of alcohol in the person's blood *at the time of the act alleged* as shown by a chemical analysis of his breath, blood, urine, saliva or other bodily substances, is admissible...and the result of any such analysis shall give rise to the following presumptions: 1. If there was *at the time of such analysis* 0.05 percent or less by weight of alcohol in the person's blood, it shall be presumed...2. If there was *at the time of such analysis* in excess of 0.05 percent but less than 0.10 percent by weight of alcohol in the person's blood, such fact shall not give rise to any presumption...3. If there was *at the time of such analysis* 0.10 percent or more by weight of alcohol in the person's blood, it shall be presumed..." (emphasis added) (It seems probable that the legislature intended to equate "time of analysis" with "time of test" and "time of specimen collection.").

generally be required for such an exercise in a given real-world case; hence, in the view of the writer, such speculative retrograde extrapolation of the BAC found is unwarranted and should not be undertaken. Recent case law supports the view that courts are becoming alerted to these problems and will not require inappropriate speculative retrograde extrapolation. To quote the Idaho Supreme Court in State v. Sutliff (1976):

> "...The primary question for resolution by this Court is whether the results of a properly administered blood alcohol test must be related back to the time of the alcohol related offense as a foundational prerequisite to admissability...
> *...We hold that this statute does not require extrapolation back but establishes that the percentage of blood alcohol as shown by chemical analysis relates back to the time of the alleged offense for purposes of applying the statutory presumption. This holding is in accord with those of other jurisdictions who have considered the question...*
> A contrary result could defeat the statute entirely since an extrapolation, particularly to a period prior to the defendant's 'peak' period, would often be based solely on the defendant's own testimony as to the amount of alcohol ingested, the period of time over which it was ingested and the time of the last consumption of alcohol..." (88) (Emphasis added.)

Similar considerations apply to the attempt to state unequivocally what dose of alcohol or quantity of alcoholic beverage(s) was ingested by a subject, solely on the basis of the BAC and such general factors as the subject's body weight, sex, and the supposed time of alcohol intake.

A synopsis of information bearing on the interpretation of BACs for legal purposes has been published (89).

5.2 ALCOHOL CONCENTRATIONS IN OTHER SPECIMEN MATERIALS

In autopsy toxicology, studies of alcohol distribution in various body liquids and tissues may be warranted in special circumstances, e.g., to estimate the state of alcohol distribution in the subject at the time of death, or for statistical purposes. The uses and limitations for alcohol analysis of specimens in living subjects other than blood and breath have been covered previously (Section 2.1.1). It is clear from that discussion that interpretation of alcohol findings in most other specimens is fraught with pitfalls for most purposes.

In the absence of blood, such findings can establish presence or absence of alcohol in the body, which (infrequently) may be useful to rebut, say, alleged lifelong abstention from alcohol. Brief consideration of other specimens follows and documents the hazards of estimating BACs from other materials.

Cerebrospinal Fluid. This has not been extensively utilized for alcohol determination in recent years. At alcohol distribution equilibrium (which is always an uncertain factor in routine cases), a mean alcohol quotient for human lumbar CSF/blood of 1.14 in 46 samples was reported by Harger et al. (90). Gettler and Freireich reported a mean CSF/blood quotient of 1.08 for 15 subjects (91). Other tabulations give a mean ethanol content value for CSF of 1.27 relative to that in blood (92). It is relevant that alcohol in CSF lags behind that in blood during uptake as well as during elimination. The reported data indicate that fluctuation in experimentally determined alcohol ratios for CSF/blood are so wide as to make attempts to estimate the coexisting BAC from CSF-alcohol analysis futile.

Saliva. Alcohol apparently reaches saliva by passive diffusion and should, therefore, partition between it and plasma according to their relative water content. The weighted average quotient of saliva to venous blood-alcohol was reported by Coldwell and Smith (93) as 1.12 for 244 sample pairs; Friede-

man et al. (43) had earlier reported a mean saliva/venous blood quotient of 1.10. With renewed interest in saliva as a practical medium for drug determinations of clinical relevance and improved routine collection techniques for secreted parotid saliva now available, it is likely that the prediction of BACs from saliva-alcohol analysis can be improved to the point of practical utility by modern standards.

Urine. The voluminous literature on the relationship of alcohol concentrations of alcohol concentrations in blood and urine (5) has been referred to above. It supports virtually any ratio anyone would wish to use for the estimation of BACs from urine-alcohol values, a practice to be deprecated for the reasons stated above (Section 2.1.1). Under special, experimentally-controlled conditions the relationship may be reasonably consistent (e.g., Coldwell and Smith (93) found a urine/venous blood alcohol quotient of 1.24 at the half-time period of urine accumulation in the bladder); however, in routine applications to case material that consistency is notably absent. There is no assurance that any of the many suggested formulae will yield BAC estimates from urine-alcohol values sufficiently valid for forensic or clinical use in a given instance.

To quote Kaye and Cardona (94): "Our data clearly confirm what other investigators have claimed: that the relationship (ratio-range) between the concentrations of alcohol in urine and in blood may vary widely. This renders it unreliable to use an average conversion factor in medicolegal cases."

Vitreous Humor. Comparative studies of alcohol and other drugs in vitreous humor and blood are of relatively recent origin. For Sturner and Coumbis' series of paired blood and vitreous humor specimens (19), a mean vitreous humor/venous blood alcohol ratio of 1.10:1 (s.d. ±0.74:1) can be calculated for 38 specimen pairs. For the series of 30 paired vitreous humor and heart/

great vessel blood specimens of Leahy et al. (20) a mean quotient of 1.07 ±0.09 can be calculated. Felby and Olsen (21) postulated that the relative alcohol concentrations in vitreous humor and blood, at diffusion equilibrium, should conform to their respective water content and proposed the theoretical quotient 1.27 for vitreous humor/blood alcohol. However, their data for 27 pairs of specimens yields a mean quotient of 1.38 ±0.17. In Coe and Sherman's 174 cases a vitreous humor/blood alcohol quotient of 1.12 was indicated (22). Heumann and Pribilla's series of 36 paired vitreous and serum specimens (23) yields a mean vitreous/serum alcohol quotient of 1.04 ±0.26. Norheim (25) analyzed the distribution of alcohol between vitreous humor and blood in 73 cases and found a mean quotient of 1.19 with a range of 0.90-1.89.

Finally, Scott et al. (26) reported 38 cases in which blood obtained prior to embalming was compared for alcohol content with vitreous humor obtained after embalming (and with pre-embalming vitreous humor in 9 of these cases). The data yielded a mean post-embalming vitreous humor/blood alcohol quotient of 1.03 ±0.33 for the 27 pairs in which alcohol was found in both specimens, and a mean pre-embalming vitreous humor/blood alcohol quotient of 0.90 ±0.16 for 8 such pairs.

The use of vitreous humor has been increasing as a postmortem specimen in situations in which blood is unavailable, and it is a useful material in that circumstance. As with other relatively isolated body components, the vitreous humor lags behind blood in alcohol uptake and elimination. Hence, although congruence between the alcohol content of vitreous humor and blood at alcohol-distribution equilibration has been experimentally demonstrated in animal models, it often cannot be established with the requisite certainty for vitreous humor → blood alcohol conversions whether a real-world subject was in the postabsorptive state. In the circumstances, only guarded interpretations can validly be made, such as ruling out significant body alcohol presence at the time of death when the vitreous humor is alcohol-free.

5.3 DECOMPOSED SPECIMENS

This is a large, tedious, and controversial subject which continues to generate literature. Because it has been convincingly demonstrated that neoformation of ethanol can take place in body fluids and tissues during decomposition under both aerobic and anaerobic conditions, interpretation of the ethanol content demonstrated in such specimens is a treacherous, hazardour, and generally unrewarding task. It complicates matters that decomposition and alcohol neoformation can occur in postmortem specimens both before and after their removal from the body; but specimens from living subjects are also subject to decomposition after procurement when specimen preservation and/or storage conditions are inappropriate or improper. The simplest response is to forgo alcohol analysis on decomposed specimens.

Examination of Decomposed Specimens. The interpretation of findings in decomposed specimens (if their analysis is undertaken at all) involves three separate but related tasks:

1) Chemically valid establishment of whether ethyl alcohol is conclusively present, and if so, in what concentration

2) Chemically valid establishment of the absence, or presence and concentration, of certain other compounds of interest in relation to assessing the presence and extent of putrefaction and the likelihood of alcohol neoformation

3) Assessment of the significance of these findings.

The common initial step is to identify and document whether consequential decomposition (especially to the point of putrefaction) has occurred in the specimen under consideration. In the event extensive putrefaction has occurred, the indicia are usually obvious (using blood as an exemplar): The distinctive odor representing inorganic sulfur compounds and mercaptans, abnormal color reflecting the changes in hemoglobins, complete hemolysis, abnormal

physical state of the specimen with respect to viscosity and liquefaction, etc. Less extreme putrefaction is more subtle to detect and more difficult to document. Gas chromatographic evidence of the presence of methane and other decompositional products is usually present; Osterhaus (11, 16), for example, demonstrated presence of acetone, isopropanol, n-propanol, n-amyl alcohol, and tert-butanol in trace quantities in some (but not all) decomposed blood specimens after long-term storage.

It is, of course, necessary to establish conclusively that a volatile substance present in a decomposed specimen and tentatively identified as ethanol on the basis of its GC retention time is, in fact ethanol, and this matter was covered previously. Conclusive identification of ethanol does not per se exclude the possibility of neoformation. Some authors have suggested elaborate schemes for the attempted differentiation between neoformed alcohol resulting from putrefactive processes and that initially present in the specimen. Smith et al. (96), for example, routinely culture postportem specimens derived in connection with aircraft accident investigations. They assess the ability of contaminating microorganisms to produce alcohol by 24-hour incubation in a brain-heart infusion medium containing 2% glucose, subtracting the alcohol value thus found from the total in the original specimen. When such subtraction leaves an alcohol remainder equal to or greater than 0.05% they consider "it is highly probable that alcohol has been ingested by the subject," although they have documented occasional instances of positive, alcohol-producing bacterial or fungal cultures yielding alcohol values corresponding to 0.067 and 0.075%. (Presumably, blood is the specimen material to which these findings pertain.) Others have reported substantially higher blood alcohol concentrations attributed to putrefactive neoformation.

Assessment of the Significance of Findings. As already noted, the literature pertaining to decomposed specimens, postmortem changes of drug concentrations, and postmortem generation of alcohol is substantial and still increasing. Two recent summaries are pertinent (97, 98).

Perhaps the most conservative approach to interpretation of the results of alcohol analyses on specimens from decomposed cadavers is the 1963 position of Dubowski: "No valid conclusions are routinely possible from alcohol determinations performed on blood specimens obtained from decomposed bodies or those subjected to prolonged submersion" (7). The same view can be held with respect to the significance of findings on specimens which have decomposed subsequent to their procurement from living or dead subjects. For the reasons discussed, the intervening widescale use of gas chromatography and the consequent increased specificity of alcohol analyses in biological specimens does not add a proportionately greater element of validity to the interpretation of the findings. It is evident that both alcohol loss and neoformation can occur in postmortem specimens before and after their removal from the body but that only the latter can occur in specimens from living subjects. The resulting combinations and permutations of conditions and effects can yield various final results which are capable of ready differentiation, the principal ones being:

a. A specimen can become alcohol-free if its initial concentration was low

b. A moderate initial alcohol concentration can be reduced to an insignificant one

c. An initially alcohol-free specimen can gain a low or moderate alcohol concentration

d. An initially high alcohol concentration can be reduced to a moderate concentration

e. An initially moderate alcohol concentration can be increased to a high one

f. The initial alcohol concentration can be accidentally reproduced by offsetting losses and neoformation.

If three time factors (before/after/before and after removal from body) and three directional factors (alcohol increase/decrease/no change) are considered, there are nine possible combinations for postmortem specimens. Rarely, if ever, will the ancillary information be available to decide which of these possibilities led to the observed result. If any assessment of the results is to be made, it should probably be limited to the opinion that complete absence of alcohol negates, with high probability, the initial existence of a highly elevated BAC and that a very high BAC (in or near the lethal concentration range) is unlikely to be attributable only to neoformation.

APPENDIX - REPORT OF INVENTIONS

After a diligent review of the work performed under this contract, no discovery, improvement, or invention deemed patentable was found. Details are given for a highly reliable and practical method of analysis of alcohol. This information should assist forensic scientists in performing valid and reliable analysis for alcohol in biological liquids and interpreting the results of such analysis for traffic law enforcement purposes.

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