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Review

Chromatographic methods for blood alcohol determination

F. Tagliaro and G. Lubli

Institute of Forensic Medicine, University of Verona, Policlinic, 37134 Verona (Italy)

S. Ghielmi

Institute of Chemistry, School of Medicine, University of Brescia, 25100 Brescia (Italy)

D. Franchi

Glaxo Research Laboratories, Verona (Italy)

M. Marigo

Institute of Forensic Medicine, University of Verona, Policlinic, 37134 Verona (Italy)

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ABSTRACT

This review is focused on the different chromatographic strategies for blood alcohol determination which can be adopted for clinical and/or forensic purposes. Particular attention is paid to gas chromatography and to high-performance liquid chromatography. However, other analytical techniques in common use, such as chemical and enzymic methods, are also briefly presented, together with some, at present unusual or experimental, approaches, such as enzymic reactors and catalytic electrodes, which are suitable for application in column liquid chromatography. Finally, mention is made of the methods for the determination of acetaldehyde, the major ethanol metabolite, and of some proposed markers of chronic alcohol abuse, such as acetaldehyde–protein adducts and carbohydrate-deficient transferrin. In order to give the background of knowledge for the rational choice of an analytical strategy, an updated outline of ethanol metabolism and toxicology is presented, together with basic information for the interpretation of the results. Problems concerning blood sampling and storage are also discussed.

Correspondence to: Dr. F. Tagliaro, Institute of Forensic Medicine, University of Verona, Policlinic, 37134 Verona, Italy.

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CONTENTS

LIST OF ABBREVIATIONS

AcH	Acetaldehyde
ADH	Alcohol dehydrogenase (E.C. 1.1.1.1)
AlDH	Aldehyde dehydrogenase (E.C. 1.2.1.5)
AO	Alcohol oxidase (E.C. 1.1.3.13)
βIMD	β-Ionization micro detector
C.V.	Coefficient of variation
EtOH	Ethanol
FIA	Flow-injection analysis
FID	Flame ionization detection
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IMER	Immobilized enzyme reactor
IprOH	Isopropyl alcohol (2-propanol)
ISFET	Ion-sensitive field effect transistor
MEK	Methyl ethyl ketone
MeOH	Methanol
NAD(P), NAD(P)H	The oxidized and reduced forms, respectively, of the coenzyme nicotinamide adenine dinucleotide (phosphate)
TCD	Thermal conductivity detection
THF	Tetrahydrofuran

1. INTRODUCTION

The use of alcoholic beverages is probably the most ancient and still widespread social habit in western culture. This has generated, since biblical times (see Noah's drunkenness), severe problems of abuse and has prompted a great deal of efforts by the various competent authorities to limit it. In fact, chronic and/or acute alcohol intoxication has been demonstrated to be connected with

many serious pathologies, suicides, homicides, fatal road and industrial accidents and most criminal offences [1].

Great attention has been paid in the biomedical environment to alcohol [a,b] related problems, as witnessed by the extensive literature, including a number of journals specifically devoted to this subject (*e.g., Alcohol, Alcoholism, Alcohol and Alcoholism, Blutalkohol, Drug and Alcohol Dependence and Journal of Studies on Alcohol*).

This is the reason why no review on alcohol, even if limited to a selected topic such as chromatographic alcohol determination, can claim to be adequate. On the other hand, strictly restraining the scope of the paper to the mere review of the chromatographic procedures would have limited its practical usefulness.

As the analytical chain includes the choice of the sample, its collection and storage and the choice of the analytical approach(es), we decided to consider these important aspects which are preliminary, but strictly connected with the analytical strategy.

Ethanol (EtOH) is almost the only alcohol occurring in the human diet, and therefore this paper is focused on this compound. It is present in beers and ales (3–6%, v/v) and wines (10–12%, v/v), by natural fermentation; higher concentrations (35–50%), such as in spirits, can be achieved only by distillation, because fermentation is inhibited by alcohol concentrations exceeding 14–16%.

Methanol (MeOH) is mainly an industrial solvent and is used to denature "tax free" EtOH. In addition, it is contained in small percentages in wines (up to 0.5% of EtOH) and in some distillates (up to 1% of EtOH), but has been used illegally as a cheap substitute for EtOH to adulterate alcoholic beverages, with deleterious and sometimes fatal consequences.

2-Propanol (IprOH) is a common industrial and pharmaceutical solvent, which has occasionally been the cause of poisoning in humans.

1.1. Metabolism of alcohol

Alcohol is rapidly absorbed in the stomach and intestine, but an important route of absorption can be via the lung in cases of exposure to alcohol vapours [2]. The main factor affecting absorption is the presence of food in the stomach [3], which delays the intestinal phase of absorption.

After absorption, alcohol, lacking any binding to proteins, distributes fairly evenly throughout all tissues and fluids of the body, in relation to the water content. Between 90 and 98% of an alcohol dose is metabolized, while the remainder is excreted unchanged with breath, urine, sweat and faeces. Larger amounts (as high as 10%) escape oxidation, after high doses of alcohol [4]. EtOH metabolism consists of oxidation to acetaldehyde (AcH), which is further oxidized to acetate [5]. Oxidation to AcH is mainly catalysed by alcohol dehydrogenase (ADH), an NAD-dependent enzyme. It is located chiefly in the liver, but gastric ADH, responsible for a significant first-pass metabolism of EtOH, has been reported as a factor affecting blood alcohol concentrations after alcohol ingestion [6–8]. In particular, gastric ADH activity is lower in women than in men (either non-alcoholic or alcoholic), thus accounting for enhanced vulnerability of women to acute and chronic complications of alcohol abuse [9]. A cytochrome P-450 enzyme, highly specific for EtOH, is induced by chronic alcohol intake. AcH is pharmacologically active, but its role in the actions of EtOH is still controversial [10]. Oxidation of AcH is catalysed by aldehyde dehydrogenase AlDH in the liver and other tissues.

A genetic polymorphism of both alcohol and aldehyde dehydrogenases, with phenotypes differing in catalytic features, has been reported [11], which could explain the marked variability of individual and inter-racial alcohol tolerance.

[a] For the sake of convenience, unless specified differently, the term alcohol refers to ethanol.

[b] In the literature, alcohol concentration has been expressed in different units, often in mg/dl, but in this present paper, according to Ruz *et al.* [62], g/l has been adopted. As the SI recommended expression (mmol/l) is rarely used, here we give the conversion factors: from g/l into mmol/l = 21.7 and from mg/dl into mmol/l = 0.217.

The absorption and distribution of MeOH in the body are similar to those of EtOH; the same enzymes oxidize MeOH to formaldehyde and subsequently to formic acid, which can be a marker of MeOH intoxication [12]. The main differences consist in a lower affinity (about 1/100) and oxidation rate of ADH for MeOH in comparison with EtOH. IprOH is slowly converted, probably by ADH, into acetone, its major metabolite, which is a depressant of the central nervous system like IprOH itself and other alcohols.

It is generally assumed that the kinetics of alcohol elimination from the body are independent of the dose (zero order), because of saturation of the NAD-dependent ADH system [13]. In the adult, alcohol is oxidized at an average rate of 120 mg/kg · h [4]. However, first-order kinetics of disappearance have been demonstrated at either very high or very low concentrations [13–15]. Zero-order kinetics also characterize the elimination of MeOH [16], while IprOH is metabolized with first-order kinetics [17].

1.2. Toxicology of alcohol

From a pharmacological point of view, alcohol is a primary depressant of the central nervous system; the apparent stimulation, which occurs at moderate blood alcohol levels, results from the impairment of inhibitory mechanisms operating in the brain. At higher concentrations, the depressive action of alcohol is general with a progressive reduction of alertness, typical drunkenness and, finally, a state of pharmacological anaesthesia. Differently from modern anaesthetics, the window between anaesthetic and lethal alcohol levels is fairly narrow. In acute intoxications, death is caused by irreversible depression of respiration with hypoglycaemia, caused by depression of neoglucogenesis. Psychiatric and neurological consequences of chronic alcohol intoxication include sleep disturbances, mental deterioration, psychoses, polyneuritis and Korsakoff's syndrome.

In addition, a host of effects of alcohol have been ascertained on the cardiovascular system (vasodilatation, cardiomyopathy), liver function (hepatitis and cirrhosis), plasma lipoproteins (increase of high-density lipoproteins HDL3), triglycerides (increase), gastric secretion (increase), blood (megaloblastic and sideroblastic anaemias), diuresis (increase), sexual function (impotence, sterility) and body temperature (heat loss).

EtOH interferes with the metabolism of many drugs [18] by induction of enzymic systems, by competition for the same metabolic pathways (*e.g.*, microsomal oxidases) and through other mechanisms.

Acute MeOH intoxication is associated with less severe narcotic effects, with a typical symptom latency of 6–36 h. After this, headache, vertigo, dyspnea and vomiting appear; however, the most relevant damage concerns sight (inflammation followed by atrophy of the retina with blindness), alertness (coma), respiration (failure), pancreas (necrosis) and pH homeostasis (acidosis). They seem to be related to the formation of formic acid and to acidosis. Blood MeOH levels of about 0.4 g/l are generally considered life threatening. The presence of MeOH in the blood in addition to EtOH could indicate long-standing EtOH intoxication [19]. Simultaneous administration of EtOH can delay and reduce (or mask) the MeOH intoxication syndrome, because of competition of both alcohols for ADH.

IprOH is another depressant of the central nervous system. The symptomatology of intoxication resembles that of EtOH, but lasts longer and is characterized by severe gastritis and vomiting with ketoacidosis.

1.3. Interpretation of alcohol levels

EtOH is physiologically present at trace levels (mg/l) in the blood and urine of man [20,21], but this has never generated difficulties in the interpretation of the analytical data on EtOH intoxication.

In general, acute alcohol effects on the brain function are correlated, although with great variability, with the concentrations in the blood [22]. Roughly, 0.5 g/l is considered to be the threshold of the symptomatology, 0.8–1 g/l the limit of severe impairment of body reflexes (and the level of

most statutory limits) and 1.5 g/l the concentration above which intoxication is evident. A blood alcohol level of 4–6 g/l corresponds to the threshold of fatal concentrations [23,24], but some authors have reported patients surviving much higher levels up to 11.2 g/l [25,26].

On the other hand, the distribution of alcohol in the body fluids and tissues, although regulated essentially by the diffusion law, still poses problems of interpretation. It has been calculated that the plasma/whole blood ratio of alcohol concentrations averages 1.12–1.18, whereas the plasma/serum ratio averages 1.00 [27,28]. This point should be taken into consideration as statutory limits generally refer to "blood level", which is almost universally interpreted as whole blood. In constrast, plasma is the blood fraction which is more intimately in equilibrium with the tissues, where alcohol exerts its action. Thus, in our opinion, plasma should be the sample of choice, whenever possible, for alcohol assays aimed at correlating concentrations with physiological effects (see also ref. 22).

After ingestion of a dose of alcohol, the compound is rapidly absorbed and distributed throughout the body with the blood. At the capillary level, a diffusion-regulated distribution into the different organs takes place. During this period, alcohol is more concentrated in the arterial and capillary blood than in the venous blood, in relation to the rapidity of absorption. During this time, the blood from an arm vein will not reflect truly the concentration of EtOH supplied to the brain, where its effects are produced [29].

In the post-absorptive state, the concentration gradients between capillaries and tissues are reversed, with a consequent higher alcohol level in the venous blood. The cross-over for capillary and venous blood alcohol concentration has been reported about 40 min after the end of drinking (drinking time 30 min) [30]. As the post-absorptive state has slower kinetics than the absorption phase, it is associated with smaller capillary–venous blood differences in EtOH concentrations [30].

Small differences in EtOH concentrations between the left and right arms have been reported, especially during the absorption phase [30]. This could be accounted for considering the existence of differences in the vascular anatomy of the two sides of the body. On this basis , multi-site sampling seems advisable in order to compensate for sampling variations when blood alcohol is to be determined for legal purposes.

Some workers have proposed methods for calculating the blood alcohol level at the time of an event on the basis of the alcohol concentration measured in a sample taken some hours later. Simple and reportedly accurate equations have been proposed by some authors among whom Zink and Reinhardt [31]. However in agreement with Dubowski [22], we believe that the mentioned variability in alcohol metabolism and distribution limit very much, in practice, the claimed accuracy of such a theoretical approach.

In addition to this, differences in alcohol concentrations have been reported between heart blood and other vascular sites [32]. Several factors related to agonal or post-mortem events have been suggested to account for these differences [33]. A traditionally accepted factor is diffusion of EtOH from the stomach into the heart blood but, recently, the agonal aspiration of vomitus containing at least 8 g/l of alcohol has been reported as a factor associated with an increase in aortic EtOH [34].

The possible formation of EtOH in post-mortem blood after ante-mortem therapeutic administration of the sugar alcohol mannitol has been reported [35].

Another extensively investigated point is the correlation of alcohol concentrations in post-mortem fluids and tissues. The calculation of reliable fluid–tissue/blood ratios could, theoretically, be useful in forensic cases to infer the blood alcohol concentration when blood is not available or contaminated. Considerable efforts have been made [36], but the results have allowed only estimates with a wide range of variability [37–40]. Closer relationships were reported between blood alcohol levels and alcohol concentrations in bone marrow (corrected for the lipid fraction) [41], thigh muscles [42] and vitreous humour [43].

The water content of each tissue or fluid [23]

and the distribution phase (absorption, post-absorption) during which death had occurred [36] were reported as the most relevant factors influencing fluid-tissue/blood ratios.

The use of alternative biological fluids to blood in order to estimate the blood alcohol level can be extremely important not only in the investigation of autopsy cases, but also in living persons to control drunk-driving and to investigate alcohol ingestion in emergency-service patients with signs of central nervous system depression. For these purposes, breath, urine and saliva have been received widespread consideration [44].

Saliva alcohol levels, when corrected for the water content, have been shown to parallel blood levels in the elimination phase [45], more closely referring to capillary blood than to venous blood [46]; however, there is not complete agreement on this point [47]. Other aliphatic alcohols (*e.g.*, propanol and isobutanol) were identified in saliva, although with different saliva/blood ratios [48].

Urine has been taken into consideration by some workers as an alternative to blood, but the correlation between the two fluids is generally considered to be unsatisfactory [44]. Urine alcohol levels are lower than blood concentrations until the haematic peak is reached; during the declining phase, in contrast, alcohol is slightly more concentrated in urine than in blood. In general, alcohol concentration in a urine sample is considered to reflect blood levels over a certain period of time; therefore, it has been proposed as a tool for monitoring alcohol abuse in the workplace and during alcohol rehabilitation programmes [49].

The determination of EtOH in breath is the main alternative to the direct analysis of blood. It is especially adopted for the roadside enforcement of drink-driving legislation, but also for the bedside diagnosis of acute intoxication [50]. Much has been published on this subject, which is substantially beyond the limits of this review. In fact, it commonly involves determinations using dedicated non-chromatographic instrumentation based on chemical, electrochemical, infrared absorption or thermochemical methods [51] (see also ref. 44); only a brief mention of the applications of chromatography will be made in the chapter devoted to gas chromatography.

Blood–air and plasma–air partition coefficients were thoroughly investigated by Jones [52]. In practice, alcohol in the blood diffuses through the alveolar system of the lung and, during the elimination phase, the blood alcohol/breath alcohol ratio averages 2100 (*i.e.*, the mass of EtOH contained in 1 ml of blood is contained in 2100 ml of breath). Although the constancy of this ratio and the practical usefulness of breath analysis to control blood alcohol concentrations is still debated among forensic toxicologists [44,53–59], breath analysis is adopted worldwide by different authorities. This is a particularly delicate question in those countries where drink-driving legislation defines statutory blood alcohol limits, but breath analysis is adopted in the field for law enforcement purposes.

2. DETERMINATION OF BLOOD ALCOHOL

The determination of alcohol has already been the subject of excellent reviews by Jain and Cravey [60,61] and by Ruz *et al.* [44,62]. For this reason, we have decided to follow, as far as possible, their approach and to avoid mentioning work already covered by their papers, unless important for our purposes.

2.1. Blood sampling and storage

Issues concerning the comparability of blood samples taken from different vascular points in living persons and bodies have already been discussed above.

A recent study has shown that, differently from a consolidated opinion, the interference in alcohol analysis induced by cleansing of the skin with EtOH or IprOH before venepuncture is negligible, not only for clinical purposes but also in forensic situations [63].

The stability of EtOH during storage is another problem investigated by several workers. It it a crucial issue when the results of determinations carried out on the same sample at different times have to be compared, as is usual in a forensic

environment. In two fundamental papers by Brown *et al.* [64] and Smalldon and Brown [65], it was demonstrated that the main factors affecting alcohol determination in stored blood are the duration and temperature of storage, with negligible losses in the frozen state, and the presence of a preservative. Three mechanisms accounting for these changes were suggested: oxidation to AcH (highly temperature dependent, utilizing oxygen from oxyhaemoglobin), growth of microorganisms metabolizing ethanol (inhibited by sodium fluoride at $\geq 0.5\%$, w/v) and diffusion from containers owing to closure failure.

More recent studies have shown that alcohol levels in sterilely collected blood from living subjects are stable for at least 14 days, even in the absence of any preservatives and at room temperature [66]. Similar data were published by Manno and Manno [67], who reported a micro-sampling procedure using plain capillary tubes similar to those for micro-haematocrit.

According to Somogyi *et al.* [68], blood containing 0.5% of sodium fluoride maintained constant EtOH levels of ≥ 2 g/l for 3 months of storage at 4°C, even if the containers were opened several times; levels ≤ 1 g/l decreased rectilinearly with time. In the absence of a preservative, opening of the containers caused substantial reduction in sample stability.

Long-term stability of blood alcohol levels was examined by Chang *et al.* [69] in specimens containing 0.36% (w/v) of sodium fluoride after 3.0–6.75 years of storage at room temperature. All samples showed a decline in EtOH levels independent of the original concentration, averaging 0.19 (range 0.03–0.37) and 0.33 (range 0.03 0.72) g/l, respectively, in never opened tubes. Reopening the tubes during storage increased losses to an average of 0.61 g/l.

EtOH production in post-mortem tissues by bacteria and yeasts has been domonstrated [13], which, reportedly, can account for levels up to 0.5 g/l [70]. This represents a further potentially interfering factor, especially in autopsy cases, even if the cadaver has been kept refrigerated [23]. Freezing seems to be an idoneous precaution in order to maintain the original alcohol levels.

2.2. Chromatographic methods

For over 30 years (gas) chromatography has been used for blood EtOH determination and is still considered to be the reference method. Surprisingly, although this view has gained widespread acceptance in forensic toxicology, GC has encountered only limited favour in clinical chemistry and emergency toxicology. The reasons are mainly related to the need for specific expertise of the personnel [71] and the assumption that the purchase and maintenance of sophisticated instrumentation dedicated only to a single class of analytes is not cost effective. In addition, the ruggedness of chromatographic methods and instrumentation is often inferior to that of modern fully automated analysers for clinical chemistry.

Notwithstanding this, the unique possibility of simultaneous monitoring of other alcohols (and volatiles) of toxicological interest, such as MeOH and IprOH, which otherwise would be ignored, strongly supports the adoption of chromatographic alcohol analysis for clinical purposes [72].

Besides, it must be emphasized that blood alcohol determinations carried out in the hospital laboratory for diagnostic purposes can subsequently assume forensic importance as evidence of intoxication (*e.g.*, if the patient is charged with driving while under the influence of alcohol). Also for this reason, the adoption of GC, which is widely accepted in the forensic environment, seems particularly advisable.

So far, HPLC has found little application because of the lack of adequate detectors but, taking advantage of the active research in the field of enzymic and electrocatalytic sensors, this situation could change in the near future.

2.2.1. Gas chromatography

GC is *par excellence* the all-pupose technique for determining volatile molecules, such as alcohols and related compounds. Almost all GC methods for EtOH determination allow the simultaneous determination of a wide range of volatile analytes (alcohols, aldehydes, ketones, glycols, etc.), but, for the sake of convenience, the

TABLE 1

DIRECT INJECTION GAS CHROMATOGRAPHY

Abbreviations: B = blood; S = serum; P = plasma; U = urine; V = vitreous humour; W = water; Int. standard solution = aqueous solution of the internal standard used at a suitable concentration; a.w. = acid-washed.

Specimen (ml or g)	Diluent (ml)	Column (m × mm I.D.)	Packing (mesh)
B (0.05)	Int. standard solution (0.05) 5% zinc sulphate (0.05) 0.15 *M* barium hydroxide (0.05)	1.8 × 6	Polypak-2 (80–120)
B (0.5)	Int. standard solution (0.5)	1.8 × 6	30% Carbowax 20 M on Chromosorb W (60–80)
W	–	3.0 × 2	20% Carbowax
B/S (0.5)	Int. standard solution (0.2)	–	15% PEG 1500 on Celite 545 (60–100)
U (0.002)	–		10% Carbowax 1500 on Teflon and 20% Ucon LB-550-X on Chromosorb
B (1)	Int. standard solution (1.0)	–	40% Castorwax on a.w. Chromosorb W (60–80)
B,P,S (2)	Int. standard solution (0.2)	–	20% Hallcomid on a.w. Diatoport W (60–80)
B (0.1)	Int. standard solution (0.010)	–	10% PEG 400 on Celite (100–120)
P, S, U (0.5 μl)	–	–	5% Hallcomid M-18 and 0.5% Carbowax 600 on Teflon 6 (40–60)
B (0.2)	Int. standard solution (3.0) 10% sodium tungstate (0.15) 0.33 *M* sulphuric acid (0.15)	1.5 × –	Porapak Q
B, U (0.2)	Int. standard solution (0.09)	1.8 × 3	Porapak Q (80–100)
B (0.01)	Int. standard solution (0.1)	1.5 × 4.8	10% Carbowax 400 on Chromosorb W (80–100)
B, U, S, P (0.5 μl)	Int. standard solution (0.5 μl)	2 × 3	(1) 0.2% Carbowax 1500 on Carbopack C (80–100) (2) 30% Carbowax 20 M on Chromosorb W HP (60–80)

Oven temperature (°C)	Carrier gas (ml/min)	Detection	Internal standard	Ref.
110	Helium (96)	FID	*tert.*-Butanol	Roach and Creaven [203]
100	Nitrogen (35)	FID	Isobutanol	Jain [78]
170	Helium	MS	–	Bonnichsen and Ryhage [108]
100	–	FID	Acetone	Machata, 1962 (in ref. 61)
100	–	FID	–	Bonnichsen, 1962 (in ref. 61)
120	Nitrogen (13.6)	FID	Ethyl acetate	Parker, 1962 (in ref. 61)
70	Helium (60–80)	FID	Dioxane	Mather, 1965 (in ref. 61)
85	–	FID	*n*-Propanol	Curry, 1966 (in ref. 61)
50	–	FID	–	Finkle, 1971 (in ref. 61)
170	– (50)	FID	*n*-Propanol	Cooper, 1971 (in ref. 61)
170	Helium (50)	MS	[$^2H_5$]Ethanol	Pereira *et al.* [109]
75	Nitrogen (75)	FID	*n*-Propanol	Jones [80]
120	– (20)	FID	*n*-Propanol	Manno and Manno [67]
100			Isobutanol	

(Continued on p. 170)

TABLE 1 (*continued*)

Specimen (ml or g)	Diluent (ml)	Column (m × mm I.D.)	Packing (mesh)
B	Int. standard solution + Triton X-100	1.8 × 3	Porapak S (80–100)
P, B (0.1)	Int. standard solution (0.1)	1.8 × 3	0.2% Carbowax 1500 on Carbopack C (60–80)
S (0.1)	Int. standard solution + Triton X-100 (0.1)	3.0 × 3.2	Porapak Q (80–100)
S, U (0.5)	Int. standard solution (2 μl)	100 × 0.5	Emulphor ON-870
S (0.2)	Int. standard solution (0.2) Sodium tungstate 0.2 *M* (0.2) Copper(II) sulphate 0.2 *M* (0.2)	30 × 0.25	Methylsilicone-bonded phase (0.25 μm)
B	Water (tenfold sample vol.)	2 × 2	Porapak S (80–100)
B, U (0.05)	Int. standard solution (0.5)	2 × 2	0.3% Carbowax 20 M on Carbopack C (80–100)
P, S (0.5)	Int. standard solution (0.1)	1.8 × 4	Porapak Q (50–80)
B	Water (fiftyfold sample vol.)	15 × 0.53	Polyethylene glycol (1.0 μm)
B, P, S, U, V, W	Int. standard solution (twofold)	1.8 × 2	Porapak S (80–100)
B (0.1–0.3)	Sodium tungstate 12.5% (0.2) Sulphuric acid 0.33 *M* (0.2)	2 × 3	Porapak Q (80–100)
B (0.2)	Int. standard solution (0.8)	1.2 × 4	5% Carbowax 20 M on Supelcoport (100–120)

scope of this review is restricted to EtOH, giving only a glimpse of some applications to MeOH, IprOH and AcH.

Already at the beginning of 1960s, a number of GC methods for blood alcohol determination had been developed (*e.g.*, refs. 73–77). Almost all the analytical strategies still used were presented in the review by Jain and Cravey [61] in 1972 and a further, updated, review was published by Ruz *et al.* in 1986 [44,62].

Let us consider what is new in GC alcohol determination. In order to simplify the discussion, the analytical conditions of the most interesting methods (some mainly historical) are summarized in Tables 1 and 2.

2.2.1.1. Sample pretreatment and injection. Methods requiring solvent extraction (*e.g.*, with *n*-propyl acetate, *n*-butanol or dioxane) or distillation [61], notwithstanding excellent cleanness of the resulting chromatograms, should be considered obsolete, mainly because they are time and sample consuming and scarcely susceptible of automation. Direct injection and headspace GC are the only techniques in general use that can be fully and easily automated.

2.2.1.1.1. Direct injection. The direct injection

Oven temperature (°C)	Carrier gas (ml/min)	Detection	Internal standard	Ref.
165	Helium (45)	FID	Acetonitrile	Dubowski [204]
125	Nitrogen (17)	FID	MEK	Baselt [205]
155	Nitrogen (18)	FID	Acetonitrile	Valentine *et al.* [79]
65	Helium (4)	MS	Diethyl ether	Liebich *et al.* [20]
35	Helium	FID	*n*-Propanol	Smith [84]
180	Nitrogen (30)	FID	*n*-Propanol	Penton [96]
120	Nitrogen (30)	FID	*n*-Propanol	Widdop [206]
100 210	Nitrogen (30)	FID	*n*-Propanol	Cheung and Lin [107]
40	Helium (25)	FID	–	Penton [106]
165	Nitrogen (45)	FID	Acetonitrile	Watts and McDonald [99]
180	Nitrogen (30)	FID	Isopropanol	Beljean-Leymarie and Le Henaff [83]
100	Helium (30)	FID	*n*-Butanol	Burstein and Greenblatt [81]

of blood or serum has been applied since the first applications of GC to alcohol analysis [76,77]. Despite the concern of many workers that this rough practice would have spoiled the column and the injector, given interfering peaks and/or plugged up the syringe, Jain [78] in 1971 demonstrated its feasibility with the only precautions of diluting 1:1 the blood with an aqueous solution containing the internal standard, injecting small volumes (0.5 μl) and thoroughly washing the syringe immediately after each injection. A sensitivity of 10 mg/l was reported. Under these conditions, the column reportedly could be used for years. Once in a while, "it was found advisable to disconnect the column at the injection port and remove all blood residues from the port with a pipe cleaner".

Additional protection from contamination by the injected ballast was obtained with a glass sleeve inserted in the injection port [79] or with a precolumn glass insert filled with a silanized glass-wool plug [67]. Substantially the same approach was adopted by Manno and Manno [67], who published an ultra-micro method using as little as 0.5 μl of blood, plasma, serum or urine, showing a reproducibility comparable to a refer-

TABLE 2

HEADSPACE GAS CHROMATOGRAPHY

Abbreviations: B = blood; S = serum; P = plasma; U = urine; W = water; BS = biological samples.

Specimen (ml or g)	Incubation		Packing (mesh)
	Temperature (°C)	Time (min)	
B, W	60	15–20	–
	25	–	Flexol 8N8–diisodecyl phthalate–PEG 600 (15:10:3) on firebrick (42–60)
B, U, W (0.2)	30	5	Flexol 8N8–diisodecyl phthalate–polyethylene glycol 600 (1.5:1.5:1.5) on Chromosorb P (100–120)
B, S, U (0.5)	60	–	15% PEG 1500 on Celite (60–100)
B, S, U (0.5)	60	–	15% PEG 1540 on silanized Chromosorb W (80–100)
B (1.0)	85	5	Flexol 8N8–diisodecyl phthalate–PEG 600 (15:10:3) on firebrick (42–60)
B, U (1.0)	–	15	Porapak S (80–100)
B	–	–	5% Carbowax 1500 on Haloport 60F (30–60)
B (0.02)	60	3	Porapak Q (80–100)
B, S, U (0.5)	60	–	15% polyethylene glycol 1500
S (0.25)	–	–	Carbowax 1500 on Chromosorb W (80–100)
B (1.0)	38	45	Porapak S (80–100)
BS (0.5)	60	30	5% Carbowax 20M on Carbopack B (60–80)
B (0.2)	60	20	(1) 0.2% Carbowax 1540 on Carbopack C (60–80) (2) 15% Polyethylene glycol on Celite (60–100)
B (0.04)	40	–	Porapak Q
B, P, U (0.2)	60	30	5% Carbowax 20M on Carbopack B (60–80)
B, P, W	20–40	30	Porapak Q

Oven temperature (°C)	Carrier gas (ml/min)	Detection	Internal standard	Reference
–	–	–	*tert.*-Butanol	Hauck and Terfloth [91]
95	–	βIMD	–	Golbaum, 1964 (in ref. 61)
75	Nitrogen (19)	FID	–	Wallace, 1966 (in ref. 61)
100	–	FID	Acetone	Machata, 1964 (in ref. 61)
100	–	FID	MEK	Machata, 1964 (in ref. 61)
100	Helium (40)	TCD	1,4-Dioxane	Reed, 1972 (in ref. 61)
132	Helium (50)	TCD	–	Luckey, 1971 (in ref. 61)
75–100	Nitrogen (30)	TCD	–	Goldwell, 1971 (in ref. 61)
150	Nitrogen (30)	FID	*n*-Propanol	Wilkinson *et al.* [92]
65 72	–	FID	THF	Kisser [207]
80–100	–	–	*tert.*-Butanol	Mebs *et al.* [86]
165	Helium (45)	FID	–	Dubowski [204]
65–110	Nitrogen (30)	–	*n*-Propanol	Anthony *et al.* [89]
85–100	–	FID	*tert.*-Butanol	Klug and Schmidt [102]
160	Nitrogen (30)	FID	*n*-Propanol	Chiarotti and De Giovanni [94]
65–110	Nitrogen (30)	FID	*n*-Propanol	Suitheimer *et al.* [208]
100	–	FID	–	Jones [52]

(Continued on p. 174)

TABLE 2 (*continued*)

Specimen (ml or g)	Incubation		Packing (mesh)
	Temperature (°C)	Time (min)	
B	70	20	Carbowax 20M (capillary column)
B (0.1)	60	15	5% Carbowax 20M on Haloport F (30–60)
B (0.2)	20–40	30	0.2% Carbowax 1500 on Carbopack C (80–100)
B	45	150	Polyoxyethylene glycol on Celite C-22
B	20	–	Carbowax on Chromosorb
B, W	60	15	Porapak Q
B, W	60	15	5% Carbowax 20M on Carbopack B (60–80)
B, W	60	15	0.2% Carbowax 1500 on Carbopack C (80–100)
B	20–40	30	Methylsilicone
B, W	37	45	Porapak S (80–100)
B (0.5)	55	12	(1) Methylsilicone (megabore column) (2) DB-wax (megabore column)
B, U (0.1)	40	18	0.2% Carbowax 1500 on Carbopack C (80–100) 5% Carbowax 20M on Carbopack B (60–80) 15% Carbowax 20M on Chromosorb W
P	25	–	Porapak S (80–100)

ence macro-method (typical between-run C.V.s of 2.8% and 2.5%, respectively).

When sensitivity was not crucial, it was found convenient to use higher dilution ratios of blood in order to reduce the amount of sample and the overall amount of biological matrix loaded in the injection port [19,80,81]. This also allowed the direct analysis of brain homogenates [81] (Fig. 1), but careful control of the dilution error is strongly needed. Alternatively, the detergent Triton X-100 was reported to improve the performance of the direct injection of serum, even at low sample dilutions (1:1), by acting as a protein dispersing agent [79].

Protein precipitation, which could easily be carried out in conjunction with the addition of the sample with the internal standard, has been proposed as a simple means of overcoming the feared problems related to the injection of whole blood into the chromatograph [82,83]. This approach has also been adopted with excellent results by workers using capillary chromatography [84,85] (Fig. 2) as an alternative to high sample dilution (1:50) [106], with obvious advantages in sensitivity (a few mg/l) and with acceptable reproducibility (typical within-run C.V.s <2.5–3%).

2.2.1.1.2. Headspace analysis. Headspace analysis inherently prevents any contamination of the column and injector and, consequently, has al-

Oven temperature (°C)	Carrier gas (ml/min)	Detection	Internal standard	Reference
20–90	–	FID/MS	Isoamyl alcohol	Doizaki and Levitt [97]
90	Nitrogen (30)	FID	*n*-Propanol	Steenaart *et al.* [105]
125	Nitrogen (20)	FID	*n*-Propanol	Penton [96]
80	–	FID	–	Gambaro, 1975 (in ref. 62)
110	–	FID	–	Martin, 1981 (in ref. 62)
160	–	FID	*tert.*-Butanol	Chiarotti *et al.* [100]
95–120	–	FID	Isopropanol–*n*-Propanol	
70–150	–	FID	*n*-Propanol	Chiarotti *et al.* [100]
35–40	Helium (25)	FID	*n*-Propanol	Penton [106]
165	Nitrogen (45)	FID		Watts and McDonald [99]
45	Helium (7.5)	FID	*n*-Propanol	Brown and Long [90]
100	Nitrogen (20)	FID	*n*-Propanol	Jones and Schuberth [104]
165	Nitrogen (45)	FID	–	Watts and McDonald [98]

ways been preferred in laboratories dealing with heavy routine workloads, often coupled with automated data handling systems [86,87]. Also, the reproducibility is often better than in direct injection (typical within-run and between-run C.V.s $<1.5\%$ and $<2.5\%$, respectively). In 1975, headspace GC for blood alcohol was the subject of a short review by Machata [88], who made many contributions to the development of this technique.

Headspace techniques *per se* require larger volumes of blood sample than direct injection [89,90], but is has been observed that the dependence of the peak height on the sample volume is modest [91]. Consequently, as little as 20–50 μl of blood has been used [92,93], which is compatible with capillary blood sampling.

Additional problems concern the choice of the temperature at which the sample is equilibrated. In fact, as previously mentioned, oxidation of EtOH to AcH takes place at temperatures exceeding 40°C [94], but, on the other hand, high temperatures increase the air–blood partition coefficient and, consequently, the sensitivity [91].

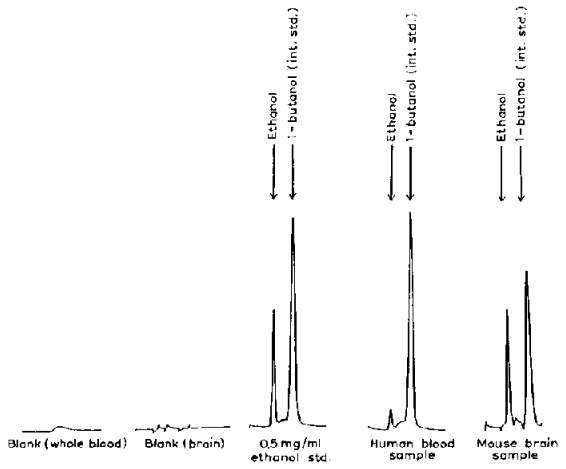

Fig. 1. GC using direct injection of (left to right): blank sample of human whole blood; blank homogenate of mouse brain; calibration standard containing 0.49 g/l of EtOH; actual human blood sample following ingestion of EtOH; and mouse brain sample after a 2 g/kg injection of EtOH. Retention times: EtOH, 2.1 min; 1-butanol, 3.9 min (reprinted from ref. 81 with permission).

EtOH to AcH conversion at 60°C was reportedly inhibited by the addition of sodium nitrite [92] or sodium dithionite [95] (see also Section 2.2.3). On the other hand, equilibration at room temperature has been found suitable for headspace analysis of blood alcohol for most purposes, allowing a slightly modified autosampler for liquid injection to serve as an automatic headspace sampler [96]. In this instance, lacking any thermostating device, the internal standard itself (*n*-propanol) reportedly corrected for temperature fluctuations, allowing C.V.s of $\leqslant 1.1\%$ even under conditions of changing temperature.

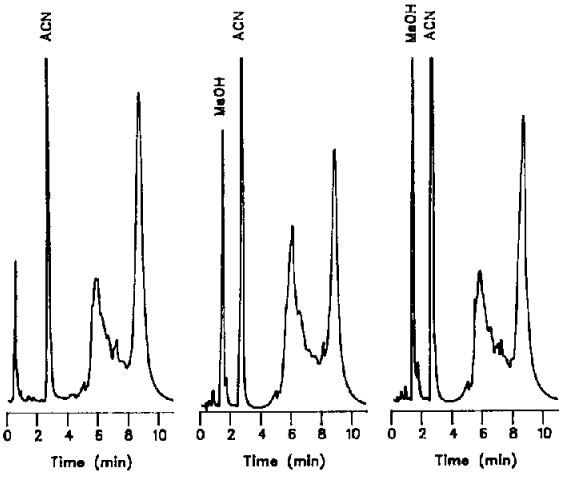

Fig. 2. Representative gas chromatograms for (left) whole blood obtained from a naive rat (*i.e.* not treated with MeOH), (centre) whole rat blood with MeOH (0.010 g/l) and acetonitrile (ACN) added *in vitro* and (right) whole blood obtained from a rat 3 h following intravenous injection of 250 mg/kg of MeOH (calculated MeOH concentration = 0.049 g/l) (reprinted from ref. 85 with permission).

Increased sensitivity, due to a salting-out effect of EtOH from blood solutions, was obtained by using sodium chloride [93,96], sodium nitrite [92], potassium carbonate [19,97], sodium fluoride [52] and ammonium sulphate [95].

However, the vapour pressures of volatile components at a fixed temperature have been reported to depend on the water content of the sample [88]. It has been demonstrated that saturation with sodium chloride produces solutions with different sodium molarities in water and in plasma, which causes little change in the partitioning of EtOH, but severe differences in partitioning of other alcohols frequently used as internal standards, such as *n*-propanol and *tert.*-butanol. The presence of proteins in the biological samples was found to account for a large part of this phenomenon [98]. Sample dilution (at least 1:5) has been suggested to minimize these errors [99].

An additional advantage of headspace techniques is the almost complete elimination of matrix-related effects, which prompted its use for the analysis of tissues [38] and stool samples [97]. Also, it has been used successfully for the chromatographic validation of breath alcohol analyses, after breath alcohol adsorption on silica cartridges [100], and direct injection GC has also proved useful for this purpose [101]. This subject was extensively reviewed by Ruz *et al.* [44].

2.2.1.2. GC separation and detection. As far as chromatographic conditions are concerned, modern GC methods use successfully either adsorption or partition stationary phases with a wide range of polarity [88]. Taking advantage of this, simultaneous or subsequent GC analyses of volatiles on columns with different elution patterns (*e.g.*, Carbowax 1500 on Carbopack C and

polyethyleneglycol on Celite [102]; DB-1 and DB-wax [90]) was suggested as a powerful and rapid means for confirmation of results [103, 104].

Carbopack B coated with Carbowax 20M proved superior to Carbopack C coated with Carbowax 1500 for the determination of AcH and MeOH, which co-eluted with each other and with water on the latter stationary phase [89]. For these purposes, partition chromatography using a Carbowax 20M coating also proved superior to adsorption chromatography on Porapak Q and Chromosorb 102 [105]. These stationary phases, additionally, are temperature resistant and have been used successfully by several other workers (see tables).

Capillary chromatography (Carbowax 20M coating), which allows a higher separation performance and easier coupling with mass spectrometry, was preferred for the determination of lower volatile alcohols in very complex mixtures such as human stool [97]. In order to reduce the amount of water loaded on to the column, workers using capillary chromatography generally preferred headspace injection [90,106]. Direct injection of serum samples generally required previous protein precipitation and protection of the capillary column (methylsilicone coating) with a short glass column containing 3% OV-1 on Gas-Chrom Q [84].

Non-polar coatings, such as methylsilicone, failed to resolve AcH and MeOH, whereas the polar polyethylene glycol did succeed [106]. Polyethylene glycol also proved superior in handling direct injections of aqueous samples but showed a lower temperature limit (250 *vs.* 300°C) in comparison with methylsilicone columns, which renders non-polar columns preferable in terms of versatility [106].

In conclusion, alcohols can be efficiently separated with different GC columns and often the choice is based only on practical considerations such as total analysis time, cost, column life and the possibility of using the same column for different analyses.

Separation is generally carried out under constant-temperature conditions; temperature programming has been used for the simultaneous determination of less volatile compounds (*e.g.*, ethylene glycol [107].

Nowadays, detection is universally carried out by FID, which proved to be the best compromise in terms of sensitivity, ruggedness and cost. The use of TCD in alcohol determination, because of the lower sensitivity and versatility, has long since been dropped.

2.2.1.2.1. Gas chromatography–mass spectrometry. Gas chromatography–mass spectrometry (GC–MS) has found little application for routine alcohol determinations, but already in the early 1970s it was reported as a powerful tool to fulfil the strictest requirements on specificity and accuracy.

In a paper by Bonnichsen and Ryhage [108], after separation on a packed column, determination of EtOH was based on the ions (generated under electron impact conditions at 70 eV) at *m/z* 45 and 46 and identification was by the retention time and by monitoring the ion currents due to ions at *m/z* 31 ($-CH_2OH$), 45 ($M-1$) and 46. The method was later computerized and slightly modified, using the molecular ion $M^{+\cdot}$ (*m/z* 46) for quantification and the intensity ratio of ions at *m/z* 31, 45 and 46 for identification. AcH was identified by peaks at *m/z* 29 and 44 and MeOH by peaks at *m/z* 31 and 32.

Mass fragmentography of EtOH in blood and urine, after GC on a Porapak Q column, was carried out by Pereira *et al.* [109] with a quadrupole mass spectrometer, using ions at *m/z* 31 and 45 for EtOH and 33 and 49 for $[^2H_5]$EtOH. As little as 5 ng of alcohol could be detected.

In reality, the specificity and accuracy of GC with FID for blood alcohol determination have never been seriously questioned; hence GC–MS has found application only in selected cases, when, for instance, very low levels of endogenous higher molecular mass alcohols were to be investigated in serum and urine [20], and when the sample was extremely complex such as human stool [97]. In both these recent applications, following the modern trends, capillary GC was used in tandem with MS.

2.2.2. High-performance liquid chromatography

While the separation of aliphatic alcohols is easily accomplished by LC under reversed-phase conditions, the lack of an adequately sensitive detector has hindered the application of HPLC to blood alcohol determination.

As aliphatic alcohols have no chromophore or fluorophore groups and exhibit very poor electroactivity (because of rapid fouling of the electrode surface by oxidation products), until recently almost only refractive index detection has been used, with inherent limits in sensitivity and selectivity. However, this approach allowed the determination of EtOH in alcoholic beverages simply after sample filtration [110,111]. The authors used reversed-phase separation with a Varian MCH 5N column (150 mm × 4.4 mm I.D.) and distilled water as mobile phase (1 ml/min) with refractometric detection; only sample filtration was needed. Simultaneous determination of MeOH was possible and no interferences were found from other compounds present in alcoholic beverages. The repeatability was satisfactory, but the application range was limited to 1–550 g/l of EtOH, *i.e.*, the alcohol concentration range in beverages.

Another strategy to detect a UV-transparent compound, such as alcohol, with a UV detector is indirect detection, which was successfully applied in an HPLC method for EtOH determination in alcoholic beverages [112]. After reversed-phase chromatography on a C_{18} column, EtOH was detected using 0.2–0.3% MEK as the UV background-forming compound at a wavelength of 280 nm. The minimum detectable concentration was 1 g/l, with good reproducibility, but the selectivity of the technique seems inherently too low for application to the assay of complex biological matrices.

Another detection technique used in HPLC, so far applicable only to the analysis of beverages, is pulsed amperometric detection. As is well known, it overcomes the problems of electrode surface fouling by combining amperometric detection with alternating anodic and cathodic polarizations of the electrode. Pulsed amperometric detection, not effective with carbon electrodes, proved suitable for aliphatic organic compounds using platinum or gold electrodes.

LaCourse *et al.* [113] successfully used pulsed amperometric detection at platinum or gold electrodes to determine as low as 0.02 g/l of EtOH and MeOH. Isocratic separation was obtained employing a polymeric ion-exclusion Ion Pac ICE-AS-1 column (Dionex, Sunnyvale, CA, USA), but working in the reversed-phase mode. The mobile phase was 50 mmol/l $HClO_4$, which enhanced EtOH retention (because of a larger stationary phase volume/pore size ratio) but required a platinum electrode. Under these conditions, EtOH detection in beverages was easily accomplished. The same authors also envisaged a gradient separation (acetonitrile–water) on an OmniPac PCX-500 column (Dionex) with post-column addition of 0.3 mol/l of NaOH, allowing pulsed amperometric detection of alcohol at a gold electrode. It showed a dramatic reduction in sensitivity (3.7 g/l), attributed to the presence of acetonitrile, blocking the surface sites necessary for anodic detection of alcohols. Any practical usefulness of the approach with regard to our aims therefore seems precluded.

Indirect electrochemical detection of aliphatic alcohols (including IprOH but not EtOH or MeOH) was also envisaged, using a reversed-phase C_8 column and a mobile phase composed of a mixture of MeOH and 0.12 mol/l phosphate–0.083 mol/l KNO_3 (40:60) buffer (pH 3.0) containing *p*-hydroquinone as the background electroactive compound [114]. Detection was accomplished in an amperometric mode with a glassy carbon electrode set at potentials within the range +0.26–0.38 V (*vs.* Ag/AgCl).

The use of enzymic reactors with in-solution or immobilized enzymes greatly improved the sensitivity of alcohol detection and allowed the application of HPLC to blood alcohol determination. In fact, only very few applications have so far been reported (employing either ADH or AO) and therefore this approach, although promising, should still be considered preliminary (for details on alcohol-coverting enzymes, see Section 2.3.2).

To the best of our knowledge, the first application of HPLC to blood analysis came from Kis-

singer's group, who used HPLC with electrochemical detection to determine precolumn-generated NADH from ADH-catalysed EtOH oxidation [115]. In this method, of course, the use of HPLC improved the alcohol enzymic assay only indirectly, by improving the NADH measurement step, but leaving unchanged the inherent selectivity limits of the enzymic assays.

On the other hand, the methods using postcolumn reactors take advatage of the coupling of the chromatographic and enzymic selectivities. As a postcolumn ADH reactor would have required the addition of NAD to the mobile phase and would not have converted MeOH, AO appeared a more promising enzyme. The use of an AO reactor in an HPLC system dates back to 1988 and was aimed at wine analysis [116]. The commercial reactor used contained Sepharose-supported alcohol and glucose oxidases (Biometra, Göttingen, Germany) and was placed downstream of a reversed-phase column and upstream of an amperometric detector, monitoring the enzymically produced H_2O_2. This set-up allowed the simultaneous assay of EtOH and glucose in wine, requiring only sample dilution (1:1000). MeOH was also enzymically converted, but the peak co-eluted with glucose.

The development of immobilized AO reactors in HPLC systems for blood alcohol determination was investigated by Tagliaro and co-workers [117,118], who demonstrated the advantages of chemical binding over ion-exchange pseudo-immobilization of the enzyme to the support packed in the reactor cartridge. Polymeric hydrophilic supports with a particle size of 10 μm or less were preferred in order to maintain the efficiency of the HPLC separation and to avoid the pH limitations of silica, considering the slightly alkaline eluent needed by the enzyme. In a further paper [119] (Fig. 3), the same workers reported a method for the direct injection HPLC determination of EtOH and MeOH in plasma and serum using a postcolumn reactor consisting of AO from *Candida boidinii* chemically immobilized on a highly biocompatible methacrylate support (HEMA-BIO 1000 VS-L, 10 μm; Tessek, Prague, Czechoslovakia), packed in a 30 mm × 3 mm I.D. glass cartridge. The analytical column

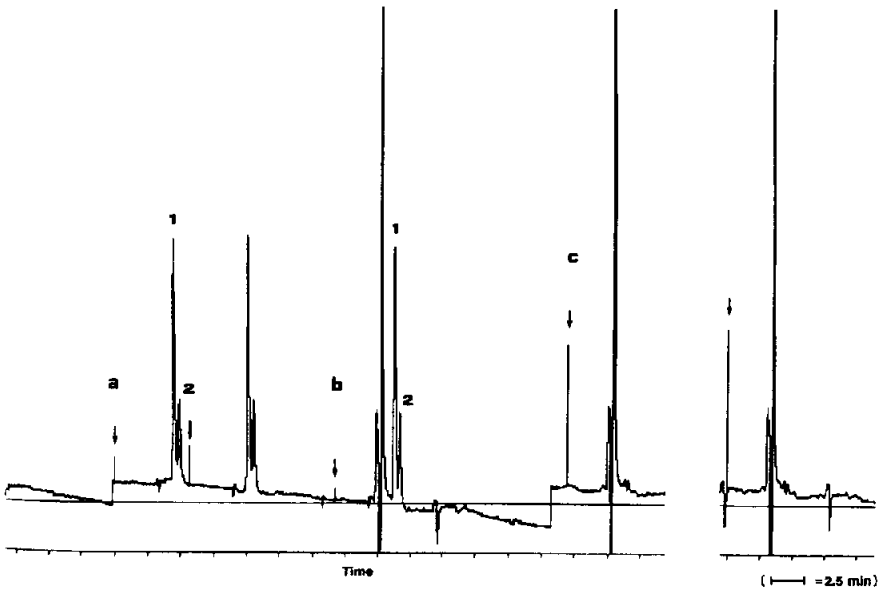

Fig. 3. Direct injection HPLC with electrochemical detection of (from left to right) (a) standard solution of MeOH (0.050 g/l) and EtOH (0.100 g/l) (two injections); (b) plasma spiked with MeOH and EtOH at 0.5 and 1.0 g/l, respectively, diluted tenfold with the mobile phase and injected; and (c) blank plasma diluted as before and injected (two injections) (reprinted from ref. 199 with permission).

consisted of two glass cartridges packed with an underivatized hydrophilic methacrylate polymer (HEMA-S 1000; Tessek) and the mobile phase was 0.01 mol/l phosphate (pH 7.5). Monitoring of the H_2O_2 produced was accomplished amperometrically at a platinum electrode set at +0.5 V (*vs.* Ag/AgCl) and shielded with a Nafion 117 membrane (Aldrich, Steinheim, Germany). Under these conditions, MeOH and EtOH were well resolved from each other and from the front of the chromatogram, the sensitivity was good (about 0.05 g/l for EtOH and 0.01 g/l for MeOH in plasma), as was the reproducibility. The response was linear for EtOH up to 2 g/l, but not for MeOH above 0.25 g/l, in agreement with the different Michaelis constants of AO (9.0 *vs.* 0.7 mmol/l, respectively (see also Section 2.3.2.1). A final problem concerned the enzymic reactor stability, which proved limited when the enzyme source was *Pichia pastoris*, while AO from *Candida boidinii* allowed a reactor life of about 1 month. The loss of activity of the reactor was tentatively ascribed to non-specific production of H_2O_2 by AO which, accumulating inside the reactor during storage, would damage the enzyme itself. Accordingly, the *C. boidinii* enzyme, which is retro-inhibited by H_2O_2, proved much more stable than the *P. pastoris* enzyme, which is not.

Unfortunately, AO is not active on IprOH and, consequently, the described method did not allow the determination of this potentially toxic alcohol.

2.2.3. Chromatographic determination of alcohol-related compounds

While the direct determination of alcohols in blood provides precise information on a state of acute intoxication, there are few and often unreliable biochemical markers of chronic alcoholism (*e.g.*, mean corpuscular volume, γ-glutamyltransferase and aspartate aminotransferase activities). Although this would need much wider and more detailed discussion, it must be mentioned that AcH, the major product of EtOH metabolism, has been reported to form adducts with haemoglobin, via formation of Schiff's base bonds with primary amine residues of amino acids [120]. Thus, acetylated haemoglobin (or more generally, proteins) could be, at least theoretically, a marker of alcoholism, even if not absolutely specific [121,122] and questioned by some workers [123].

Most GC methods for alcohols allow the simultaneous determination of AcH [105], but problems arise when AcH levels (roughly 1/1000 of EtOH concentrations) are to be measured in blood. In fact, artifactual formation of AcH has been reported during storage or due to the use of deproteinizing reagents and/or elevated temperatures [65,92,124], unless special precautions are adopted (*e.g.*, addition of sodium azide, sodium nitrite or thiourea, deproteinization with perchloric of sulphosalicylic acid, derivatization with semicarbazide, control of equilibration temperature in headspace GC) [94,105,125–129]. Because of the large difference in relative concentrations, a slight conversion of EtOH, which would be insignificant for its accurate determination, can alter completely the measurement of the true AcH levels in blood.

A sensitive HPLC method with fluoresence detection for AcH determination in blood has recently been reported [130]. It was based on the reaction of AcH with 1,3-cyclohexanedione and ammonium ion to form a fluorogenic adduct, which was separated by reversed-phase HPLC and determined fluorimetrically; propionaldehyde was used as the intenal standard. The sensitivity was about 0.1 μmol/l and the precision was acceptable. Only 50 μl of blood sample were required. Under these analytical conditions, the amount of AcH present in the samples as an artifact deriving from EtOH was negligible. However, traces of endogenous components chromatographically identical with the AcH derivative were found in the blood of monkeys and humans not consuming EtOH.

Using the described analytical approach, Peterson and Polizzi [131] investigated plasma AcH and haemoglobin-associated AcH in a group of alcoholics, both of which were found to be elevated, in comparison with a control group of teetotalers.

Alternatively, acetylated haemoglobin was di-

rectly determined with an HPLC method using cation-exchange separation and absorption detection at 415 nm, after previous removal of glycated haemoglobin with affinity columns [132]. This allowed the accurate determination of acetylated haemoglobin also in presence of glycated haemoglobin, which could not be separated by ion-exchange chromatography. This haemoglobin adduct was higher in alcoholics than in controls, but in some pregnant women proportions of acetylated haemoglobin as high as in alcoholic subjects were found.

2.3. Non-chromatographic methods

Although chromatography has become the favourite analytical approach in forensic toxicology, in clinical toxicology and emergency laboratories it has often been disregarded as being a technique full of difficulties and requiring specifically and highly trained personnel. This opinion is now slowly changing in favour of chromatography, but chemical and enzymic methods are still considered convenient tools, at least for a first screening [71,133].

2.3.1. Chemical assays

Determination of alcohol by means of chemical methods, utilizing its reducing or ester-formation capabilities in titrimetric or photometric assays, has been extensively investigated in the past.

The most popular approach was based on the quantitative oxidation of EtOH by potassium dichromate or other oxidizing agents in a strongly acid medium:

$$2\ K_2Cr_2O_7 + 8\ H_2SO_4 + 3\ CH_3CH_2OH \rightleftharpoons$$
$$2\ Cr_2(SO_4)_3 + 2\ K_2SO_4 + 3\ CH_3COOH + 11\ H_2O$$

The oxidation products could be, as alternatives to acetic acid, AcH or CO_2, depending on the reaction conditions. Because of the low selectivity of the oxidation process, chemical methods require an initial separation from the biological matrix, accomplished by distillation, diffusion, aeration or extraction, taking advantage of the volatility of alcohol [60,62]. Most of these methods are now considered obsolete because of their poor specificity, tediousness and poor susceptibility to automation.

Although rarely mentioned in the recent literature, Widmark's method [134], based on distillation and quantitative oxidation of EtOH by dichromate followed by back-titration, is still widely used; in fact, it is very cheap and simple, and possesses good precision [135] and accuracy (in the absence of other reducing distillable volatiles), provided that non-putrefied samples are analysed [23].

A recent commercial microdiffusion method [136] is based on substantially the same principles. Reduction of chromic acid reagent to blue chromic oxide is accomplished within 5 min by alcohol diffusing into a sheet of glass microfibre paper, containing the reactant, placed above the samples heated at 80–120°C. The assay can be carried out directly on whole blood, saliva or urine and, because of its simplicity, it is adopted in several laboratories for screening samples before more expensive and sophisticated confirmatory methods. It has recently been validated in comparison with GC [137].

2.3.2. Enzymic assays

Enzymic methods have found favour since the early 1950s, when they were proposed, almost simultaneously, by Bucher and Redetzki [138] and Bonnichsen and Theorell [139].

ADH (E.C. 1.1.1.1) (common sources: equine liver, Bakers' yeast), which catalyses the reaction

$$CH_3CH_2OH + NAD^+ \rightleftharpoons CH_3CHO + NADH + H^+$$

has been proposed in a variety of analytical schemes, but AO (E.C. 1.1.3.13) (common sources: *Candida boidinii*, *Pichia pastoris*), catalysing the reaction

$$CH_3CH_2OH + O_2 \rightleftharpoons CH_3CHO + H_2O_2$$

has also been used by some workers. As the equilibrium lies toward EtOH formation, in order to

promote completion of reaction the process is carried out in the presence of a trapping agent, or coupled to a second enzymic reaction; direct or indirect monitoring by photometry, fluorimetry or electrochemical methods has been adopted.

Although not absolutely specific for EtOH, enzymic methods satisfied strict accuracy, precision and reliability requirements, allowing alcohol assay to be widely adopted in clinical chemistry laboratories [140]. In addition, enzymic methods, supported by the wide availability of reagents, fit modern clinical chemistry instrumentation very well and often require minimal or no sample pretreatment. Correlation with more sophisticated GC methods has proved good also with problematic samples, such as post-mortem blood [141].

On the other hand, the high specificity of ADH for EtOH, which excludes MeOH and, to a lesser extent, IprOH from detection, may be the source of misleading results in cases of intoxication from MeOH or IprOH (pure or mixed with EtOH [142]. Comparison of the results with those from less selective assays (chemical or osmometric) could be helpful in revealing the presence of enzymically unidentified alcohols.

Ruz *et al.* [62] described and discussed in their review most of enzymic methods published up to 1985. Therefore, in order to avoid tedious repetitions, we have limited our attention to more recent papers and new trends.

2.3.2.1. Photometric and colorimetric methods. Recent research in photometric–enzymic methods for blood alcohol determination has been focused on the development or improvement of ready-to-use reagents for use in emergency-room cases [143–146] and of procedures for automated clinical chemistry instrumentation (*e.g.*, [49,147–149]). Both approaches, in general, use "in solution" ADH with absorption photometry monitoring of the reduced cofactor NADH in the 340–360 nm wavelength region [62]. Acknowledged advantages of automation, in comparison with manual methods, are shorter analysis times, smaller volumes of sample and reagent (often very expensive) and higher precision. Although important for routine work, these papers are peripheral to the focus of this review. Theoretically more interesting with respect to our aims are some improvements made to the classical ADH reaction scheme.

Chang and McCroskey [150] and Young and Rafter-Tadgell [151] proposed the addition of a competitive inhibitor of ADH (pyrazole) to kinetic enzymic (ADH) methods (manual and automated, respectively), in order to extend the linearity of the assay, by increasing the apparent K_m of the enzyme. In fact, kinetic methods require that the substrate concentration be kept much lower than the K_m (by using large dilutions or very small volumes of sample) in order to ensure first-order reaction conditions. These limitations have hampered the use of kinetic approaches, which are otherwise much more rapid than the equilibrium methods.

As monitoring ADH-catalysed alcohol oxidation by measurement of NADH at 340 nm suffers from poor selectivity, Gibitz and Fenninger [152] proposed to carry out a preliminary isothermal distillation (for 100 min at 20°C) of volatile substances from 5-μl serum samples, adsorbed in filter-paper placed 12 mm above the reaction mixture in a closed flask.

In order to allow the monitoring of enzymic alcohol oxidation at a higher and more selective wavelength than 340 nm, Kovar *et al.* [153] recently reported a method based on the simultaneous oxidation of EtOH and irreversible reduction of nitrosodimethylaniline to a quinonediimine derivative in the presence of small amounts of NAD and horse liver ADH. Quinonediimine reacted with a coupler (salicylamide), resulting in a blue indaniline dye which could be monitored at 680 nm in the visible region.

For enzymic alcohol determination, instead of the cytosolic NAD-linked ADH, the membrane-bound form (source: *Acetobacter aceti*), having a 300-fold greater activity and catalysing an irreversible reaction, has also been used [154,155]. No NAD or NADP was required; the reaction involved the reduction of an indicator tetrazolium dye monitored at 570 nm.

AO obtained from *Pichia pastoris* also shows interesting features, such as easy monitoring at 600 nm by a peroxidase-coupled chromogen sys-

tem (4-aminophenazone–chromotropic acid), no inhibition by H_2O_2 (differently from AO from *Candida* cultures) and extended linearity up to 4 g/l [156].

It should be pointed out that AO is not specific for EtOH: it converts all lower alcohols into the respective aldehydes. AO oxidizes MeOH to an even greater extent than EtOH (100:75) and, consequently, AO-based assays can reveal also dangerous intoxications from MeOH, which elude the ADH-based assays.

Coupling the non-specific MeOH oxidation by AO with the specific transformation of the formaldehyde produced into formic acid by NAD-dependent AlDH (with monitoring of NADH at 340 nm), it was possible to develop a specific enzymic assay for MeOH [157,158].

Some test strips for alcohol determination in saliva are based on AO with a coupled chromogenic reaction [159,160].

2.3.2.2. Radiative energy attenuation methods. The so-called radiative energy attenuation (REA) technique has recently been developed for implementing alcohol assay in a commercial microprocessor-controlled automated fluorimeter, commonly used for fluorescence polarization immunoassays (FPIA) of therapeutic and illicit drugs [161]. In this coupled reaction scheme, NADH produced by the ADH-catalysed oxidation of EtOH is re-oxidized in presence of diaphorase (E.C. 1.6.4.3) with reduction of iodonitrotetrazolium violet (INT) to formazon-INT. This product has an absorbance peak at 492 nm, overlapping the excitation and emission spectra of fluorescein, which is added to the reaction mixture. Hence the fluoresence intensity decreases with increasing amounts of EtOH, because of an increasing inner filter effect of formazan-INT. This technique showed good correlations with accepted reference methods and was able to deal with whole blood, even from post-mortem cases, with only a small positive bias [161,162]. The orginal reagents were later reformulated with replacement of INT with an alternative tetrazolium dye [thiazolyl blye (MTT), absorbance peak at 565 nm], resulting in a decrease in the above-mentioned bias in post-mortem cases [163,164].

2.3.2.3. Electroanalytical methods. Electroanalytical enzymic methods for alcohol determination have attracted the interest of many researchers since the mid-1970s because of the high sensitivity, simplicity, low costs and susceptibility to automation [62].

The early methods were based on the direct electrochemical determination (at about +0.8 V *vs.* Ag/AgCl) of NADH produced in the ADH-catalysed oxidation of EtOH, which, especially in biological matrices, was problematic [165]. Thereafter alternative ways were tried. Coupled enzymic reactions between NADH and an oxidant [*e.g.*, hexacyanoferrate(III) or 2,6-dichlorophenolindophenol in the presence of diaphorase], allowing more favourable detection (in general, amperometric) conditions than NADH itself, have been extensively investigated [166,167] (see also ref. 62). Recently, the reduction of hexacyanoferrate(III) to hexacyanoferrate(II), reflecting the EtOH concentration through ADH-generated NADH, was detected with a redox potentiometric electrode [168].

As the enzymic oxidations of EtOH and AcH, catalysed by ADH and AlDH, respectively, liberate H^+, they could be monitored by measuring the changes in the pH of the medium with a special pH meter [169], as recently reported [170]. A conductimetric technique, based on the conductance changes due to the formation of acetate ions (in presence of ADH and AlDH) has also been reported [171,172]

Several attempts have been made to develop enzyme bioelectrodes [173–175]. An amperometric alcohol sensor was developed, adsorbing on a nitrocellulose filter cell membranes of acetic acid-producing bacteria (*Gluconobacter suboxydans* IFO 12528) which was placed in contact with an oxygen electrode and covered with a gas-permeable membrane. The device, in the presence of alcohol, registered a decrease in the current due to oxygen consumption [176].

More recently, the same group developed an alcohol-FET sensor based on an ISFET on the gate of which the cell membranes of *Gluconobacter suboxydans*, consisting of membrane-bound ADH, AlDH and an electron-transfer system,

were immobilized [177]. The difference in signal between this enzyme ISFET and a reference ISFET was recorded.

2.3.3. Flow-injection (and continuous-flow) analysis

Flow-injection analysis (FIA) is one of the most interesting techniques for application in clinical chemistry, particularly owing to its rapidity, simplicity, low costs and easy automation. The merging zones and stopped-flow modes and also enzyme immobilization have been adopted by different workers in order to reduce reagent and sample consumption. Beyond the importance of FIA and other flow analysis techniques *per se*, they are worthy of particular attention because of their suitability for use in LC systems.

Worsfold *et al.* [178] reported a stopped-flow FIA method with photometric detection, which was applied in both blood and beverage assays. Higher sensitivity and smaller blood and reagent consumption was achieved by Fernandez Gomez *et al.* [179], who used the merging zones mode (with and without stopped flow) and fluorimetric detection. Although this approach, involving the direct monitoring of NADH (λ_{ex} = 340 nm, λ_{em} = 460 nm), had rarely been used in the past [180,181], it showed higher sensitivity and selectivity (with absence of a blank signal) than absorption photometry.

Two automated FIA methods were reported by the same group using electrochemical determination of NADH [182]. The reduced coenzyme formed was measured either directly (at +0.8 V *vs.* Ag/AgCl) or indirectly (at +0.07 V) via a coupled reaction involving 2,6-dichlorophenolindophenol reduction, catalysed by diaphorase. The coupling method was more sensitive and selective than the direct method.

An alternative detection approach, applied in a continuous-flow system, is bioluminescence. NAD(P)H:FMN oxidoreductase and bacterial luciferase were co-immobilized in a nylon coil (0.5–1 m), which was inserted directly into a luminometer and placed downstream of another coil (1 m) with immobilized ADH [183]. The system produced light in the presence of oxygen, a long-chain aldehyde and NAD(P)H, which was the substrate limiting the reaction (*i.e.* the reaction was controlled by the concentration of NADPH, depending on the alcohol level). Thus light intensity was proportional to alcohol concentration in the sample. High sensitivity (50 μg/l), extended linearity of response, low background signal and high stability of reactors (up to 900 samples) were the main features of the method, which seems promising for use also in other flow analysis schemes (FIA, HPLC).

ADH immobilized in a nylon tube was used also by Attiyat and Christian [184] in an unsegmented flow system in which the sample, the enzyme catalysing the coupled reaction (diaphorase), NAD and hexacyanoferrate(III) merged at a point close to the ADH reactor; biamperometric detection of hexacyanoferrate(II) assured the indirect measurement of EtOH in the sample.

Continuous improvement in enzyme immobilization techniques has provided flow analysis techniques with excellent tools for saving very expensive reagents, such as enzymes, and increasing their stability (this point, in our opinion, has sometimes been over-estimated). Many workers preferred packed reactors to coils (as reported above), because of the larger amount of enzyme that could be immobilized.

Ruz *et al.* [185] published several FIA methods using controlled-pore glass packed reactors with photometric, fluorimetric and amperometric (pulsed and conventional) detection of the ADH-catalysed NADH formation from alcohol oxidation [186]. Amperometry has also been used to monitor a coupled reaction (2,6-dichlorophenolindophenol reduction) catalysed by diaphorase, immobilized in a downstream reactor [186]. Although any of the proposed methods was suitable for the determination of EtOH in blood, the fluorimetric determination (especially in the stopped-flow mode) and the amperometric method with coupled reaction were the most promising, because of their intrinsically high selectivity and sensitivity. Kubiak and Wang [187] reported an amperometric bioelectrode based on the incorporation of yeasts with ADH activity into a carbon paste matrix, with the addition of hexacyanofer-

rate(III) as the redox mediator. It was used in a FIA system with a detection limit of 9 ng of EtOH and applied to the analysis of alcoholic beverages.

In order to allow the direct assay of whole blood (without pretreatment) in a FIA system using an ADH IMER with photometric detection of NADH, Maeder *et al.* [188] inserted an on-line dialyser, containing a dialysis membrane with a relative molecular mass cut-off of $15 \cdot 10^3$, between the injector and the reactor. This device allowed the complete removal of any interferences related to the biological matrix, even at the rather non-specific wavelength of 340 nm.

Yao *et al.* [189] developed a FIA method with immobilized AO, in which the H_2O_2 produced converted hexacyanoferrate(II) into hexacyanoferrate(III), measured with a peroxidase electrode at 0.0 V (*vs.* Ag/AgCl).

2.3.4. Osmolal gap assay

The presence of alcohol, as might be expected, increases plasma osmolality. Owing to its great theoretical and clinical interest, several workers in the past proposed equations that, on the basis of commonly investigated blood parameters (sodium, urea, glucose and, sometimes, potassium and calcium concentrations), allow the calculation of plasma osmolality, without its direct measurement [190]. Notwithstanding some discrepancies among the different equations, the most recent of them [*e.g.*, osmolality = 1.86 (Na + K) + glucose + urea + 10] yield fairly accurate results [191].

Obviously, in the presence of alcohol there will be a gap between the measured (by freezing point depression) and calculated osmolality values, which already in the early 1970s was proposed for the evaluation of alcohol intoxication [192] (see also ref. 62). This osmolal gap was correlated with alcohol concentration but, even using the most sophisticated equations for calculating the expected osmolality, an overestimation of plasma alcohol concentration has been observed (11–30% in the concentration range 0.4–2.4 g/l) [193]. This discrepancy has tentatively been ascribed to a non-ideal osmotic behaviour of EtOH, which could alter the degree of dissociation of solutes [193]. On the other hand, the osmolal gap evaluation could be helpful in the differential diagnosis of acutely intoxicated patients in emergency situations. Besides, being non-selective for EtOH, it could reveal the presence of alcohols other than EtOH if, compared with an enzymic alcohol assay, a discrepancy of results (osmolal gap $>>$ enzymic assay) was observed [142]. For this purpose, a computerized calculation was reported, checking also for internal consistency of data and alerting the operator in the event of disparity [194].

2.3.5. Other methods

As direct electrochemical determinations of alcohols at carbon electrodes require a large overvoltage for their electro-oxidation, research has been focused on developing alternative electrode materials (*e.g.*, platinum and gold), which, however, require a pulsed potential waveform to maintain a stable response.

Ruthenium dioxide-containing carbon paste electrodes showed interesting electrocatalytic features, particularly long-term stability, which allowed the development of a FIA method for simple alcohols at a constant potential of +0.4 V (*vs.* Ag/AgCl) [195]. Unfortunately, the electrode response was dependent on the number (and position) of hydroxyl groups, thus limiting the sensitivity for EtOH to 3 μg (in comparison with 30 ng for glycerol) in 20-μl injection volumes.

Another theoretically interesting approach to FIA with electrochemical detection, although not directly applied to blood analysis, employed the reaction of EtOH with a component of a redox couple [Os(VIII) Os(VI)] in contact with a porous platinum electrode (held at +0.3 V *vs.* the standard calomel electrode), placed on the electrochemical cell side of a gas-permeable membrane electrode [196].

The direct electrochemical determination of EtOH represents one of the main ways for breath alcohol determination [44], but has encountered some interest also for blood alcohol determination. The adaptation of commercial breath-testing instrumentation, based on an electrochemical

(fuel cell) sensor, for the assay of biological fluids was easily carried out by means of headspace analysis [197]. Another method used isothermal distillation of alcohol from the sample (deposited on filter-paper) and simultaneous electrochemical oxidation of EtOH to acetic acid at a platinum–gold PVC bipolar electrode [198]. A very simple and inexpensive instrument, but relatively precise and accurate, used a pair of gas sensors (semiconductors composed of tin dioxide deposited on a ceramic tubular former), one of which was the measuring unit and the other the reference [199].

3. PERSPECTIVES

GC blood alcohol determination appears to be a well established and widely accepted technique susceptible only to a few refinements, particularly in the use of capillary columns and in microsample injection techniques. At present, HPLC is not to be considered as a mature alternative to GC; however, being in principle more compatible than GC with automated clinical chemistry instrumentation, it could find application for diagnostic purposes. For this purpose, postcolumn enzymic reactors seem the most practicable approach.

On the other hand, much research is still needed to investigate the biochemical changes occurring in chronic alcoholism. AcH and acetylated proteins seem to be a promising field of investigation. Although sample storage is problematic, GC and HPLC have proved suitable for accurate AcH determination if strict precautions to avoid analytical artifacts are adopted. Direct determination of acetylated haemoglobin or proteins by HPLC (or by means of modern electrophoretic techniques) could become an interesting alternative worthy of further research.

An alternative, although indirect, marker of chronic alcohol use can be carbohydrate-deficient transferrin, consisting of isoforms of transferrin that are deficient in their terminal carbohydrates. From a methodological point of view, isoelectric focusing, chromatofocusing and anion-exchange chromatography have been used for separation and immunometric techniques for quantification. The subject has recently been reviewed by Stibler [200].

Recent promising results on the molecular mass determination of intact proteins have been achieved by mass spectrometry using two new desorption–ionization methods, which could usefully be applied to study protein adducts in support of chromatography and electrophoresis. The first, called electrospray, is based on the interaction of the molecule under study with high electric fields, resulting in the deposition of n positive charges on the molecule. Thus, the molecular ion will be detected at an m/z value corresponding to M/n [201]. The second technique, called MALDI (matrix-assisted laser desorption–ionization), consists in the interaction of a laser beam with the compound under study, mixed with a matrix absorbing at the laser wavelength. The $[M + H]^+$ ion of the protein is generated, and for mass analysis a time-of-flight instrument is generally employed [202].

Finally, endogenous formation of alcohol and other volatile compounds is, in our opinion, a subject susceptible *par excellence* to the application of GC–MS. Again, the need to control any possible spurious formation of these substances under storage or analysis conditions must be stressed.

4. CONCLUSION

After prolonged research and extended field application, GC with FID has found general acceptance for blood alcohol determination, especially for forensic purposes. Headspace analysis has regularly been adopted for large routines, while direct injection is preferred for emergency toxicology applications and, generally, for moderate workloads. FID is by far the most favourite detection technique, also for the most demanding purposes. Although the specificity and accuracy of GC with FID for alcohol determination have seldom been questioned, the use of simultaneous or subsequent GC analyses of volatiles on columns with different elution patterns seems advisable for validation of the results. GC–MS has

so far been applied mainly to solve particular problems, depending on the complexity of the biological matrix or on the width of the range of alcohols under study. Capillary GC, which has not yet proved clearly superior to packed column GC for routine applications, can find a specific role owing to its much easier coupling with MS.

Surprisingly, GC has found only limited application in clinical and emergency toxicology, notwithstanding clear advantages in terms of specificity and analytical range (including EtOH, MeOH, IprOH and other volatiles) over enzymic and chemical assays. The main reasons seem to be related to the peculiarity of GC, which hardly fits the philosophy of the clinical chemistry laboratory, generally oriented towards "push and forget" technologies. For this aim, HPLC with enzymic reactors, because of a closer similarity to consolidated techniques in clinical chemistry, could find wider application in the near future, provided that fully automated and rugged instrumentation becomes commercially available. HPLC seems very promising also for the determination of blood AcH and, particularly, AcH–protein adducts, which could become markers of chronic alcoholism, a pathology of high social relevance still lacking specific diagnostic means. For this purpose, modern electrophoretic techniques such as high-performance capillary electrophoresis could become powerful additional tools of analysis.

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