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TITOLO DELLA TESI DI DOTTORATO

NEW APPROACHES TO THE OBJECTIVE DIAGNOSIS OF ALCOHOL ABUSES

S.S.D. ***MED/43***
(indicare il settore scientifico disciplinare di riferimento della tesi dato obbligatorio)*

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1. Introduction and purpose of the study

Since the detection of ethanol in the body is possible only for a relatively short time after intake, the assessment of a condition of alcohol chronic intoxication/addiction has been and still is an issue in clinical and forensic toxicology. In the recent years, however, the diagnosis of alcohol abuses has been substantially improved in terms of sensitivity and relibility by the introduction of new specific biomarkers, among which EtG is one of the most interesting.

Ethyl glucuronide (EtG) (Figure 1), a minor alcohol metabolite formed by conjugation of ethanol with activated glucuronic acid, has been proposed as a stable marker to detect and quantify alcohol consumption. It can be detected in matrices such as hair, urine and blood, with different diagnostic windows.

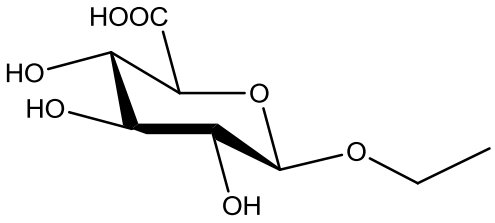

Figure 1. Ethylglucuronide.

The aim of this study has been the evaluation of the possible use of blood EtG in the study of the association of EtG with the occurrence of alcohol-related road accidents and to establish if the ethanol ingestion occurred close to the road accident or hours before. To the best of our knowledge, such association has not been reported to date. On a different note, a similar evaluation has been studied in details with regards to THC abuse in cases of accident investigations and clinical evaluations [1]. It is, in fact, reported that knowing the time cannabis was last used could be important for determining impairment in driving. The well known predictive models, derived by these studies, provide an objective and validated method for assessing the contribution of cannabis to accidents from a forensic point of view [2,3].

2. Alcohol abuse biomarkers

Ethanol is one of the most common psychoactive compound used worldwide. Abuse of ethanol in the Western societies and even more in the developing countries is responsible for various effects on health and above all on safety [4]. It is rapidly absorbed after ingestion and *per se* it can be considered the marker of alcohol acute intoxication. Measurements of ethanol in blood, breath or urine samples are usually carried out in cases of suspected ethanol intoxication. Moreover, clinical tests may prove useful, in combination with detected excessive consumtion of alcohol, to reach conclusions on long-term drinking habits[5].

Most ethanol is primarily (90–95%) metabolized by the human liver in an enzimatically catalysed oxidation process. It is first converted to acetaldehyde by alcohol dehydrogenase and then further metabolized to acetate by aldehyde dehydrogenase. Also, a small amount is excreted unchanged by kidneys, lungs, skin and in the form of water and carbonic gas (Figure 2) [6].

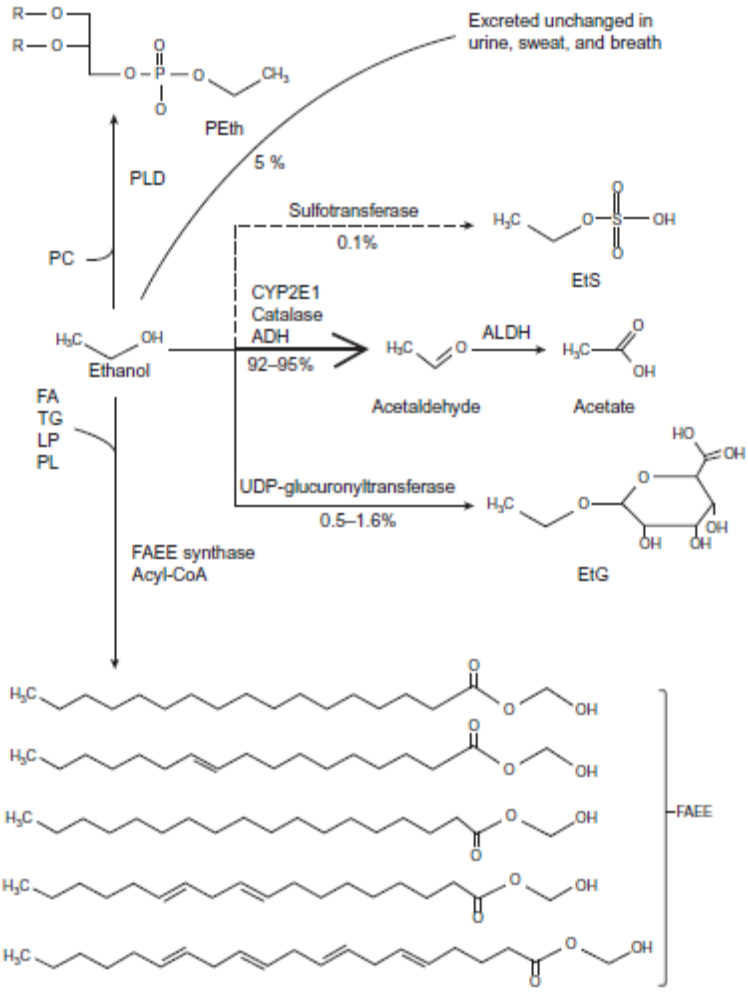

Figure 2. Alcohol metabolism and formation of non-oxidative metabolites [7].

However, other ethanol metabolites are formed after alcohol consumption through minor pathways of EtOH elimination. They have different time frames of detection and can be determined in different matrices (Table 1).

In fact, they reflect the spectrum between short term and long term ingestion of different amounts of alcohol [8].

Detection of ethanol in the body is possible only for a relatively short time [9]. For this reason, in the recent years, besides traditional clinical biochemistry parameters like GGT (γ-glutamyl transferase), ALT (alanine aminotransferase), AST (aspartate aminotransferase), MCV (mean corpuscular volume), medical history information and clinical examination, new specific biomarkers have been

introduced. These new biomarkers (Table 1) have revolutionised the objective diagnosis of alcohol abuse.

Table 1. Biomarkers [10].

Marker type	Marker name	Biochemical changes	Detection window	Diagnostic specificity	Diagnostic sensitivity
Non-oxidative metabolites of ethanol	EtG	Coniugation of ethanol with glucuronic acid by UDP-glucuronyltransferase	In blood and urine: hours-days; in hair: months	High	High
	EtS	Coniugation of ethanol with sulfate by sulfo-transferase	Hours-days	High	High
	FAEEs	Esterification of free fatty acids with ethanol by FAEEs esterases	In blood: hours-days; in hair: months	High	High
	PEth	Transphosphstidylation from phosphatidylchiline in presence of ethanol catalyzed by phospholipase D	2-3 weeks	High	High
Acetaldehyde products	Acetaldehyde protein adducts	Non-enzymatic reaction of acetaldehyde with serum proteins via Shiff's base formation	Depends on half-life of serum protein	Low moderate	Moderate high
Markers of alcohol-related metabolic changes	CDT	Group of minor glycoforms of transferring including asialo, monosialo and disialo-Tf (serum concentration increases after sustained alcohol intake)	2-3 weeks	Moderate high	High
Markers of alcohol related organ damage	AST ALT	Liver enzymes (serum concentration increase after liver damage)	Weeks	Low-moderate	Low-moderate
	GGT	Liver enzymes (serum concentration increase after liver damage)	Weeks	Low moderate	Moderate high
	MCV	Average size of red blood cells (increases after heavy drinking)	Months	Low moderate	Low-moderate

Biological markers of alcohol can be divided into two groups: direct and indirect markers. The alcohol biomarkers GGT, AST, ALT, MCV and CDT (carbohydrate-deficient transferrin) can be classified as "indirect markers" because they change as a consequence of alcohol abuse, but are not directly related to alcohol metabolism. In fact, they are influenced by a steady and significant alcohol intake. Alcohol acetaldehyde adducts, PEth (Phosphatidylethanol), FAEEs (fatty acids ethyl esters), EtG and EtS (ethyl sulphate) can be classified as "direct markers", because they directly track the intake of alcohol [11,12].

2.1 Gamma-glutamyltransferase (GGT)

GGT is a serum enzyme having a glycoprotein structure, mainly produced by the liver. It catalyses the transfer of glutathione to various peptide acceptors; as a consequence, it is involved in the synthesis and in the degradation of glutathione, drug and xenobiotic detoxification. GGT is the parameter most traditionally used for the diagnosis of chronic alcohol abuse. A long term excessive and repeated alcohol consumption (more than 60 g/day for several weeks) increases the serum GGT concentration. GGT serum levels return within the normal limits after 20-25 days from the drinking suspension.

GGT is not considered a specific marker because the serum levels are affected by gender, age, pregnancy and tobacco smoking but also, by non-alcoholic liver disease like hepatitis C. Therefore, GGT has been proposed as a disease marker in several medical fields [10,12].

2.2 Aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

AST and ALT are liver enzyme involved in amino acid metabolism. It is possible to find them in several bodily tissues and red cells. They are usually used as markers of liver health. The serum concentration of AST and ALT increases significantly in heavy drinkers versus moderate drinkers and teetotallers. Moreover, the presence of high concentrations of these enzymes can be observed in abstinent alcoholics with a chronic liver disease. Both enzymes could provide useful information about the interpretation of liver disease. The ratio of AST to ALT over two suggests an alcohol related liver damage, otherwise, a ratio less than one suggests a non-alcohol-related liver damage. However, similarly to GGT, these biomarkers are not specific [5,10,12].

2.3 Mean corpuscular volume (MCV)

MCV refers to the average size of erythrocytes (red blood cells). It is often used in screening tests for the detection of alcohol abuse. MCV level increases with a continuous and prolonged alcohol consumption (4-8 weeks). The increase depends on the toxic action of alcohol on the membrane viscosity of erythrocytes, on the toxicity of acetaldehyde on the red cell precursors in bone marrow and on the interference of ethanol on the intestinal absorption and metabolism of vitamin B_{12} and folic acid. Thanks to a dose-response correlation between alcohol consumption and MCV, it is believed a good marker to monitor a subject who drinks for a long period. Although, MCV demonstrates to have a poor sensitivity and a low specificity because of the presence of non-alcohol-related diseases which may give false-positive results. MCV level returns within the normal limits after 2-4 months from the drinking suspension [5,10,12].

2.4 Carbohydrate-deficient transferrin (CDT)

Among the new markers, CDT has gained a neat prevalence in the diagnostics of chronic alcohol abuse. CDT represents a group of minor glycoforms of human transferrin (Tf), the major serum iron transporting protein. Transferrin can bind a maximum of two Fe^{3+} ions. It is made of a polypeptide chain of 679 amino acids with two N-linked complex oligosaccharide chains. The major glycoform of Tf contains two biantennary *N*-glycans with a total number of four sialic acid residues (tetrasialo-Tf, p*I* 5.4), but other isoforms with two (disialo-Tf, p*I* 5.7), three (trisialo-Tf, p*I* 5.6), five (pentasialo-Tf, p*I* 5.2) and six (hexasialo-Tf, p*I* 5.0) sialic residues (Figure 3) have been identified in normal human serum. In addition, traces of monosialo-Tf can also be determined in pathological conditions. Finally, asialo-Tf is commonly detected when disialo-Tf is elevated due to excessive alcohol consumtion.

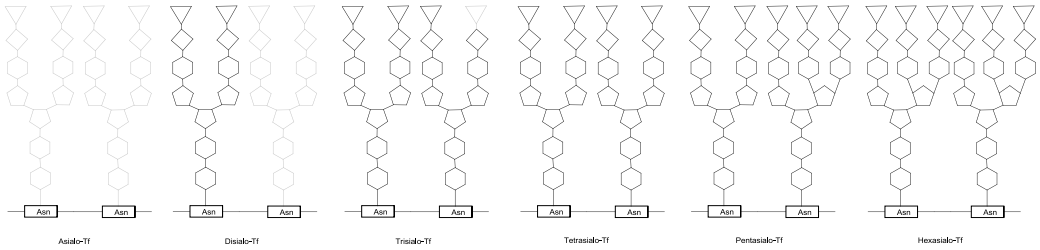

Figure 3. Schematic representation of the clinically relevant Tf isoforms. Asn, asparagine residue; hexagon, N-acetylglucosamine; pentagon, mannose; diamond galactose; and triangle, sialic acid. Trisialo-Tf contains one biantennary di-sialylated N-glycan and one biantennary mono-sialylated N-glycan [13].

Normal human serum typically contains a predominant transferrin glycoform, the tetrasialo-Tf isoform (75–80%). Two additional minor glycoforms are also normally observed: pentasialo-Tf represents about 15% of overall transferrin and trisialo-Tf, which would derive from the pentasialo-Tf isoform by loss of the biantennary chain and represents about 5% of total transferrin [11].

The formation of hyposialylated transferrin isoforms due to chronic alcohol exposure was first described by Stibler et al. in 1978 [14], however, still, the biochemical mechanisms of the alcohol-related CDT increase are not well elucidated.

CDT is the biomarker mostly used to study chronic alcohol abuse and the main reason is its high specificity. However, it cannot identify occasional abuses or binge drinking behaviour.

The two hyposialylated isoforms produced as a consequence of sustained alcohol intake are disialo-Tf and asialo-Tf. These two isoforms are referred to as CDT. CDT concentration (%CDT) is expressed as a ratio of the sum of the hyposialylated isoforms on the total transferrin. However, it is well known that asialo-Tf is typically identified in the sera of alcohol abusers showing high increases of disialo-Tf [10,11,15]. To support a diagnosis of alcohol abuse, since disialo-Tf is present also in normal subjects, it is necessary to establish a "cut-off" (usually about 1.8%) above which subjects are considered alcohol abusers.

The plasma half-life of CDT, about 2 weeks, is longer than that of normally glycosylated transferrin (7 days). After 2 weeks of alcohol abstinence (maximum, 3 weeks), the concentration of CDT returns to physiological levels.

CDT is a very sensitive marker to detect relapse in alcohol-dependent subjects and to monitor abstinence during detoxification treatments. Besides, this marker is used to evaluate the physical fitness of subjects either to apply for or to regain the driving license, after its confiscation for drunk driving, or to hold safety-sensitive jobs [10,15].

2.5 Fatty acid ethyl esters (FAEEs)

Fatty acid ethyl esters are minor direct metabolites of ethanol that are formed after alcohol consumption due to interaction of ethanol with free fatty acids as well as triglycerides, lipoproteins, and phospholipids. The reaction is an enzyme-mediated esterification of fatty acid or fatty acetyl-CoA by ethanol. The formation of fatty acid ethyl esters can take place through the hydrolysis of a fatty acid from a phospholipid or a triglyceride molecule in the presence of ethanol. FAEEs are present in blood, hair and several organs which are known to be prone to alcohol-related damages [7,10].

They are formed primarily in the liver and pancreas and then are released into the circulation. These metabolites are detectable in serum up to 24 hours after alcohol consumption in the case of acute intoxication while, up to 100 hours in chronic alcohol abusers. Thanks to these different detection windows in serum, it is possible to use these metabolites to differentiate chronic abusers from binge drinkers [10]. These compounds are also incorporated into hair follicles through sebum and they can be detected up to three months in hair [7,8,12].

2.6 Phosphatidylethanol (PEth)

Phosphatidylethanol is an abnormal group of phospholipids formed from ethanol and phosphatidylcholine in cell membranes via the transphosphatidylation

reaction catalysed by the enzyme phospholipase D. PEth comprises a group of phospholipids with a common phosphoethanol head onto which two fatty acids of variable carbon chain length and degree of saturation are attached [10]. Various homologues of PEth can be detected but, the most common in human blood are 16:0/18:1 and 16:0/18:2 [8]. After a sustained intake of ethanol for two weeks, PEth is accumulated in the red blood cells and may remain in circulation for more than 2 weeks. Thus, PEth is determined in whole blood and represents a marker for long-term alcohol abuse [5,16]. It can be detected up to 14 – 21 days after abstinence in heavy alcohol users [7,12]. Unfortunately, PEth is not commonly analyzed in routine analysis due to the complexity of its determination, notwithstanding a high specificity [10].

2.7 Acetaldehyde adducts

The first metabolite of ethanol, acetaldehyde which plays an important role in the pathogenesis of tissue damage in alcoholics, by formation of protein adducts. Chemical modification of native proteins has been found to occur in the liver of alcoholic patients. Acetaldehyde-protein adducts have been measured from erythrocytes and plasma proteins being detected up to 3 weeks after the last alcohol intake. Acetaldehyde direct adducts, markers of alcohol consumption, are not routinely used in clinical practice [5].

2.8 Ethylsulphate (EtS)

Ethanol metabolism leads mainly to the formation of acetaldehyde, but, in minor proportion, also of ethylsulfate. EtS is formed via sulphonation of ethanol in presence of the enzyme sulphotransferases. Less than 0.1% of the ingested ethanol is excreted in urine as EtS. This metabolite is non-volatile and water-soluble, and has a longer half-life than ethanol and acetaldehyde. It can be routinely detected by LC-MS/MS in urine for up to 32 hours after alcohol intake occurred [17]. However, it can also be detected in blood and hair matrices [8].

EtS indicates recent alcohol consumption with high specificity and selectivity, thus covering a clinically and forensically important time window between short-term markers, such as ethanol itself, and long-term markers, such as CDT [5,12,16].

3. Ethylglucuronide (EtG)

EtG is a minor metabolite of ethanol and a specific marker of alcohol intake. It is a non-volatile and water-soluble molecule. Moreover, it has a low molecular weight (222 g/mol) and is stable upon storage. This direct metabolite of ethanol can be detected in body fluids such as blood and urine but, also, in hair for an extended time period after the complete elimination of alcohol from the body.

The existence of EtG has been known for over a century. In 1901, Neubauer reported qualitative detection of an ethanol conjugate in the urine of dogs and rabbits [18]. Fifty years later, Kamil and co-workers isolated the conjugate of ethanol as the triacetylmethyl ester derivative from the urine of rabbits orally dosed with ethanol [19]. In 1967 the first identification of EtG as a minor ethanol metabolite in humans was performed [9,20]. In 1994 EtG was first synthesized by Schmitt and co-workers[21]. Moreover, EtG determination was carried out not only in human serum and urine [21] but also in hair [22].

EtG is formed in the liver by conjugation of ethanol with UDP-glucuronic acid. This reaction is catalysed by the action of UDP-glucuronosyl transferase (UDP-GT) (Figure 4).

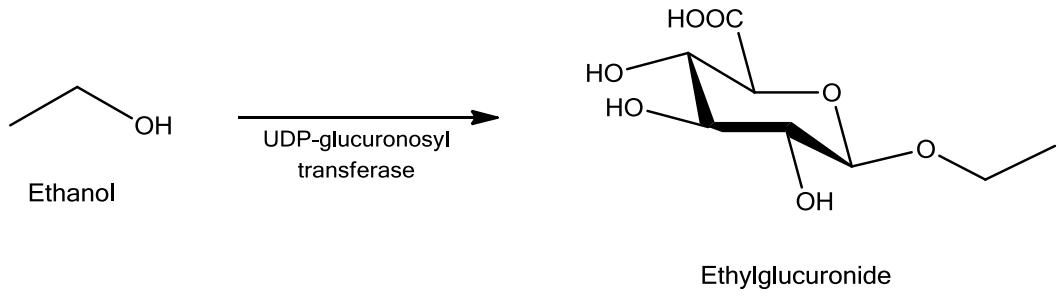

Figure 4. Formation of ethylglucuronide.

Whereas the elimination of EtOH via glucuronidation is a minor pathway of alcohol metabolism (less than 0.1%), EtG is a valuable biomarker of ethanol

intake. Depending on the amount of consumed alcohol, EtG is still detectable in the body long after completion of alcohol elimination [8,23].

Since EtG is a direct ethanol metabolite, a positive finding is regarded as a very reliable indicator of drinking. Infact, the major advantage of EtG is to extend the detection window for ethanol. EtG can be determined in different matrices like blood, hair and urine, each of those are typified by a characteristic detection window.

In literature a few works on EtG kinetics in blood and urine are present.

Urinary EtG is a useful tool for objective identification of present drinking and relapse detection. The excretion of EtG is markedly delayed and it remains detectable in urine from several hours (13-20 hours) up to some days (3.5 days) [24] after ethanol has been eliminated, the time-lag largely being dose-dependent.

The blood-EtG concentration peaks about 3 hours later than ethanol; this metabolite can be found in the blood about 8 hours after ethanol has been disasppeared [11]. The kinetics of EtG in blood is less known and only one study reported on kinetic data of EtG in serum.

This study indicated that, after variable ingested doses of ethanol, EtG first became detectable in serum with a lag time of up to 45 minutes as compared to ethanol. The maximum EtG concentration showed significant inter individual variations and was reached 3.5-5.5 hours after alcohol intake, which was 2.0-3.5 hours later than for ethanol. Unfortunately, this study only collected blood samples for 8 hours, but theoretical calculations indicated that EtG would have remained detectable in blood for up to 17 hours [25,26].

The pharmacokinetics of EtG in blood and urine was also studied by Høiseth et al. using 10 male volunteers who consumed alcohol at a fixed dosage of 0.5 g/kg of body weight in a fasting stage. The blood was collected for 14 hours and urine samples for 45-50 hours after consumption of alcohol. The observed median time for maximum blood EtG concentration was 4 hours (range 3.5-5 hours), whereas peak alcohol concentration was observed at between 0.5 and 2.0 hours (median, 1.0 hour). The half-life of elimination of ethylglucuronide from blood was 2.2 hours (range, 1.7-3.1 hours). Blood alcohol was detected up to 5.0-7.0 hours after consumption of alcohol, whereas ethyl glucuronide in blood was detected up to

10-14 hours after consumption (Figure 5). The urinary concentration of ethyl glucuronide was much higher than the serum concentration. Detection time for EtG in urine is broader than blood (ranging from 14 to 24 hours) which makes urinary EtG the most sensitive biomarker of recent drinking [27].

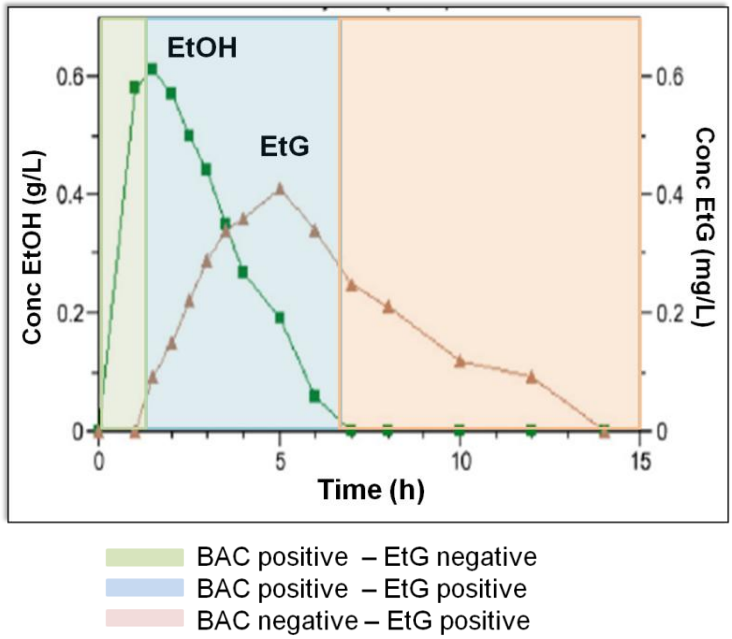

Figure 5. Example of pharmacokinetics of ethanol and EtG in blood [27].

Halter and co-workers studied serum and urinary excretion of EtG and EtS after consumption of a moderate amount of alcohol by 13 volunteers. The peak EtG concentration was observed 4 hours after initiation of drinking. The mean time difference between reaching maximum blood alcohol level and blood EtG level was 2.3 hours. Maximum serum EtG showed wide inter-individual variations and did not correlate with serum ethanol level. Mean time for peak urinary EtG concentration after initiation of drinking was 6.2 hours [28].

In a study of 32 alcohol-dependent patients who had a blood alcohol level of 100-340 mg/dL on admission to the hospital, Helander and co-workers observed that detection time of EtG in urine was weakly correlated with initial alcohol concentration. EtG in urine was detected up to 130 hours (median window of

detection, 78 hours; range, 40-130 hours). The authors concluded that during alcohol detoxification, EtG remained detectable in urine for several days, and the detection window also showed wide inter-individual variations [29]. Wurst et al. reported that the detection of EtG in urine, after consumption of ethanol, could vary. The variations in the detection window reported by different investigators may be related to the amount of alcohol consumed by subjects as well as the wide inter-individual variation in metabolism of alcohol through minor metabolic pathways producing EtG. Moreover, renal impairment may prolong detection time of EtG in patients with decreased renal function [30].

Analysis of drugs of abuse in hair sample is used to identify chronic misuse over a period of weeks to months before sample collection. EtG in hair has relatively recently begun to be used as an alcohol biomarker. Only a small amount is incorporated into hair (pg/mg). The major analytical challenge is the use of a sensitive analytical method to detect these small quantities [31]. On the other side, the advantage of analysis of ethyl glucuronide in hair is that this biomarker can be detected also months after alcohol consumption [6,32,33].
Summarising, the EtG analysed in different matrices presents different detection windows (Figure 6), applications and limitations:

- hair EtG: detectable for up to three months [7]; the application field is the evaluation of chronic alcohol abuse; limits: variability of concentrations found in literature in respect to the quantity of alcohol ingested and the impossibility to use pubic hair;
- urine EtG: detectable for up to three days [7]; the main application field is monitoring the abstinence during detoxification therapeutic programs and the suitability of driving licence; limits: positivity reported in case of accidental alcohol contact and the possible formation post collection;
- blood EtG: detectable for up to ten hours [7]; the application field is still minimal; limits: relatively restricted diagnostic window.

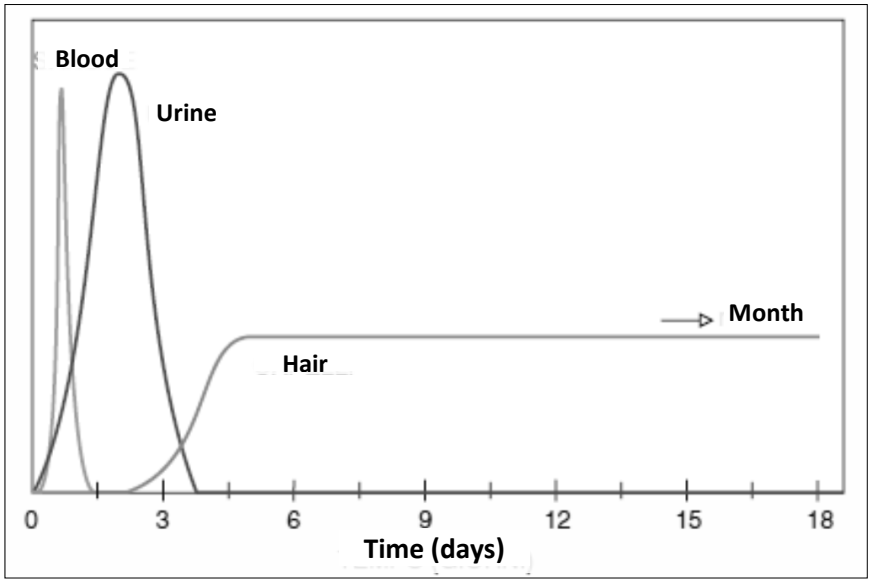

Figure 6. The EtG detection window of blood, hair and urine [34].

4. Driving under the influence of alcohol

Driving under the influence (DUI), is the act of driving or operating a motor vehicle while impaired by alcohol or other drugs (including drugs of abuse and drugs prescribed by physicians, for example, benzodiazepines), to a level that makes the driver incapable of driving or operating safely. DUI is one of the prominent social problems troughout the world. In Italy and in the majority of countries, driving under the influence of alcohol means having a blood alcohol concentration, BAC, over the legal limit. In order to verify the drunk driver's level of intoxication, breath measurements and blood alcohol analysis are usually performed. The former can be directly carried out by the Police officers. However, there is usually a time-lag between the arrest of a drunk driver and the time of traffic accident and the time of blood sample colletion. This time-lag is crucial because leads to a decrease of alcohol concentration in blood because of the rapid alcohol metabolism [35].

In most countries, if the driver is positive to alcohol tests, he can be punished by driver's licence suspension, fines and even prison sentences for DUI offenses.

In Italy, driving under the influence of alcohol is regulated by the law 186 CdS D.Lgs 285/1992. If a driver is found with a BAC between 0.5 and 0.8 g/L, they have to face a fine and three to six months licence suspension. From 0.8 to 1.5 g/L, they have to face a fine, six to twelve months licence suspension and up to six months imprisonment in extreme circumstances. Over 1.5 g/L they have to face a fine, one to two years licence suspension, six to twelve months imprisonment, and vehicle seizure. The BAC limit is zero for newly qualified drivers (those who have hold their license for less than three years).

On March 25th, 2016, a new law was enacted known as “traffic homicide” (law 41/2016). Under this law, a driver under influence of alcohol, who causes severe injuries or the death of a person, is imprisoned from five up to ten years if the BAC is between 0.8 to 1.5 g/L while, the penalty is from eight to twelve years of imprisonment with a BAC above 1.5 g/L.

5. BAC and increase of traffic accident risk

It is widely agreed that drunk-driving is one of the most important risk factor of road accidents and it is one of the main causes of mortality and injuries on roads. According to the Focal Point of WHO (World Health Organisation) on alcohol-related problems, in Europe 25% of traffic accidents is associated with alcohol intoxication.

Alcohol is a CNS (Central Nervous System) depressant: this means that normal brain function is impaired, particularly in the person's information-processing skills, cognitive skills and hand-eye coordination, also referred to as psychomotor skills. When alcohol is consumed, many other skills that safe driving requires, such as judgment, concentration, comprehension, coordination, visual acuity, and reaction time, become impaired. Consuming alcohol prior to driving greatly increases the risk of accidents. The greater the amount of alcohol consumed, the more likely a person can be involved in an accident.

However, if there is a widespread consensus on the correlation of the degree of impairment of the driving ability with the blood alcohol concentration (BAC), a much weaker evidence can be found in the current literature on the correlation between the chronic abuse of alcohol and an increased risk of causing traffic accidents (Figure 7).

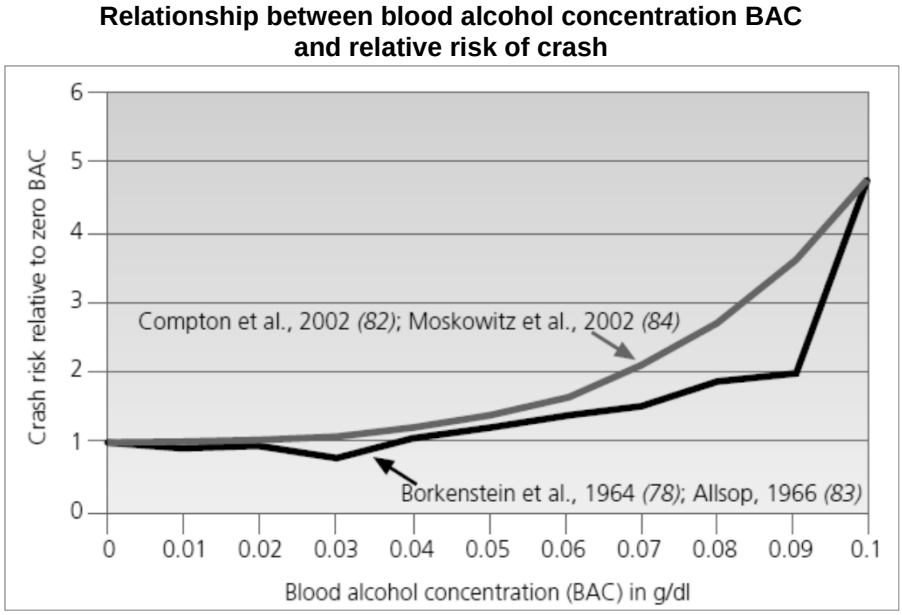

Figure 7. Relative risk of driver involvement in police-reported crashes [36].

The correlation between BAC over 0.5 g/L and the increase of traffic accident risk has been demonstrated in the 1960s. In fact, in 1964 a case-control study, known as Grand Rapids Study, showed that drivers who had consumed alcohol had a higher risk of involvement in accidents than those with a zero BAC and this risk increased rapidly with BAC [37].

In 1994 the study was re-analysed concluding that the risk of accident is present also with lower BAC levels [38]. This correlation has been confirmed by a recent analysis of 554 articles highlighting an increase of traffic accident risk with a BAC over 0.4 g/L [39].

Although, since in a relevant percentage of cases, the lapse of time between when the traffic accident takes place and when the blood sample is collected consists in some hours. The present work aims at studying the possible evaluation of blood EtG to unravel the problem of interpretation of BAC values in presence of delayed blood sample collecion.

6. Materials and methods

6.1. Ethanol analysis

Ethanol analysis for forensic purposes is performed with Head Space GC (HS-GC), either with flame ionization (FID) or with MS detection.

Chemicals and reagents

Calibration standards: 100 and 300 mg/dL ethyl alcohol in tris buffer with sodium azide as a preservative (Microgenics corporation, Fremont, CA, USA); tert-butanol analytical grade (Sigma-Aldrich, Steinheim, Germany). Water for dilution was purified by filtering deionised water on a Millipore Ultra-Pure Water system (Millipore, Bedford, MA, USA).

Instrumentation

A headspace gaschromatograph Acme 6100 (Kunash Instruments, Mumbai, India) with FID detector (Young Lin, Anyang, South Korea) was used to perform alcohol analysis of blood samples. The column was a Metablood 2 (30 m x 0.53 mm x 2 µm) (Teknokroma, Barcelona, Spain) and the oven temperature was kept at 40 °C. The carrier gas was helium with a rate flow of 18 mL/min. Syringe temperature was 80 °C and 1,250 µL of sample was injected from the head space. The detector temperature was set at 250 °C.
The calibration curve used was from 0.1 up to 3.0 g/L. The detection limit and the the quantitation limit are 0.02 g/L (S/N=3) and 0.1 g/L respectively.

Biosamples

Blank blood samples were obtained from the hospital of Verona. Real samples from persons involved in traffic accidents were obtained from the emergency room and from autopsy cases.

All samples where psychoactive drugs had been detected were excluded from the study.

Sample pre-treatment

Fifty microlitres of blood were added to 200 µL of IS (tert-butyl alcohol 0.8 g/L) in a headspace crimp vial. The sample was heated at 80 °C for 10 minutes before the injection.

6.2. EtG analysis

The most commonly used and recommended analytical methods for EtG analysis is chromatography hyphenated with mass spectrometry. The GC-MS or LC-MS methods are considered the gold standard in the forensic context, particularly for the analysis of drugs and small organic molecules. In partucular, GC-MS and LC-MS, having high sensitivity, offer the possibility of determining EtG in hair, where it is present in very low concentrations (pg/mg).

In the context of the present study, fully validated methods for EtG determination were developed. In particular, blood and urine analysis for EtG were performed by LC-MS/MS with electrospray ionization in negative mode. Because of the selectivity of this technique, the sample pre-treatment is simple, since it is not necessary a derivatisation step. Instead, EtG analysis in hair were performed by GC-MS/MS with negative chemical ionization [40,41].

6.3. Blood EtG analysis

Chemicals and reagents

Ethylglucuronide and internal standard pentadeuterated–EtG (EtG-D_5) were obtained from Medichem Diagnostica GmbH.

The two standards were reconstituted with methanol obtaining a final concentration of 2 mg/mL.
The water used was purified by filtering deionised water on a Millipore Ultra-Pure Water system. HPLC-grade acetonitrile (ACN), methanol and formic acid (HCOOH) were obtained from Sigma Aldrich (Steinheim, Germany).

Instrumentation

Analysis were performed by using an LC-ESI-QqQ MS, Agilent 1290 Infinity – 6460 MS (Agilent, Milford, MA, USA), with electrospray ionization in negative mode. The mass spectrometer was operated with a drying gas flow of 8 L/h, nebulizer pressure of 50 psi, drying gas temperature of 350 °C, capillary voltage of 3.75(+) 4.5(–) kV, and fragmentation voltage of 140 eV at negative polarity.
Solvent A was 0.1% HCOOH in water and solvent B: 0.1% HCOOH in ACN/H_2O 9:1.
EtG was separated with a flow-rate of 0.5 mL/min using the following gradient: 5% solvent B for 3 minutes; 10% solvent B for 2 minutes; 90% solvent B for 3 minutes; re-equilibration with 5% solvent B for 2.5 minutes.
The column used was Hypercarb (100 mm x 2.1 mm, 5 µm particle size) (Thermo Fisher Scientific, Waltham, MA,USA) and kept at 50 °C during the analysis.
The product ion transitions monitored are: EtG *m/z* 221→113, *m/z* 221→85, *m/z* 221→75; EtG-d_5 *m/z* 226→75, *m/z* 226→85 CE 12 eV.

Biosamples

Blank blood and serum samples were obtained from the hospital of Verona. Real samples from live and dead people involved in traffic accidents were obtained from the emergency room. The exclusion criteria applied to the samples was the presence of psychoactive drugs.

Sample pre-treatment

Serum and blood samples were prepared according to an existent method [35]. Briefly: samples (200 µL) were deprotonised by addition of 1 mL of cold methanol in the presence of 50 µL of pentadeuterated–EtG solution (12 µg/mL in methanol). The samples were vortexed for 60 seconds and centrifuged at 13000 rpm for 5 minutes. Then, 750 µL of supernatant was evaporated at 40 °C under air flow. The sample was reconstituted with 100 µL of milli-Q water and centrifuged at 4500 rpm for 10 minutes. Ten microlitres of the processed samples were injected in the LC–ESI–MS/MS system.

6.3.1. Method validation

Stock solutions, calibration standards and control samples

The eight-point calibration curves (three replicates of each standard) were based on peak-area ratios of the analyte relative to the internal standard using linear line and excluding the origin.

Calibration curves were prepared (0.45 - 90 µmol/L; 20 - 0.1 µg/mL) from ethylglucuronide stock solution 2 mg/mL.
The detection limit (LOD) and the the quantitation limit (LOQ) were 0.03 µg/mL (S/N=3) and 0.1 µg/mL respectively.

Selectivity

Five blank serum samples from different sources were analysed for possible interfering peaks during the validation phase.

Linearity of calibration

To evaluate the linearity of calibration, five replicates for each calibration level (0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20 µg/mL; 0.45, 1.13, 2.25, 4.5, 11.3, 22.5, 45, 90

μmol/L) were prepared and analysed on different days. The calibration curve was estimated by least-squares regression procedure. The results are reported in Table 2.

Table 2. Calibration curves.

Day	m	q	R^2
1	0.23858	0.09393	0.99213
2	0.30760	-0.04213	0.99847
3	0.34366	-0.09054	0.99859
4	0.26513	0.06492	0.99668

The mean value of the slope was 0.287 with a standard deviation of 0.046 while the slope intercept was 0.006 with a standard deviation of 0.087. The coefficient of regression was always higher than 0.990.

Precision and accuracy

Intra-day precision, calculated as the relative standard deviation (RSD), was determined analysing the quality control (QC) samples (0.5, 2.5 and 10 μg/mL; 2.25, 11.3 and 22.5 μmol/L) in six replicates, using the procedure described above, while inter-day precision was established analysing the QC samples on four different days within a week. The concentration of the analytes in the QC samples was calculated on the basis of the calibration curves of the day. Accuracy (exspressed as relative error, RE) was calculated as the percentage of the ratio between the average value obtained (n = 6; absolute value) and the corresponding nominal value (Table 3, 4 and 5) .

Table 3. Quality control 0.5 µg/mL.

Day	QC 0.5 µg/mL						Mean value	Median	σ	σ_{rel}	$\%_{accuracy}$
1	0.4280	0.5684	0.4494	0.4369	0.5356	0.5413	0.493	0.493	0.062	0.125	98.7
2	0.5478	0.5593	0.5225	0.5094	0.5049	0.5042	0.525	0.516	0.024	0.045	104.9
3	0.6165	0.6098	0.6199	0.6092	0.6363	0.6350	0.621	0.618	0.012	0.019	124.2
4	0.4957	0.5132	0.4486	0.4846	0.5438	0.4769	0.494	0.490	0.033	0.066	98.8

Table 4. Quality control 2.5 µg/mL.

Day	QC 2.5 µg/mL						Mean value	Median	σ	σ_{rel}	$\%_{accuracy}$
1	2.4529	2.4812	2.1331	2.7281	2.6624	2.9784	2.573	2.572	0.287	0.112	102.9
2	2.4659	2.3337	2.3898	2.2948	2.4637	2.4073	2.393	2.399	0.069	0.029	95.7
3	2.1788	2.2154	2.1809	2.2675	2.2362	2.1818	2.210	2.199	0.036	0.017	88.4
4	2.2406	2.2430	2.3743	2.4323	2.3644	2.4275	2.347	2.369	0.086	0.037	93.9

Table 5. Quality control 10 µg/mL.

Day	QC 10 µg/mL						Mean value	Median	σ	σ_{rel}	$\%_{accuracy}$
1	10.4328	9.9756	11.2667	10.4759	11.4322	9.8509	10.572	10.454	0.652	0.062	105.7
2	10.3709	10.2191	10.6131	10.2535	10.3661	10.1472	10.328	10.310	0.164	0.016	103.3
3	9.6436	9.8206	9.6779	9.5348	9.4357	9.6881	9.633	9.661	0.133	0.014	96.3
4	11.1023	10.1975	10.081	10.6226	11.3036	10.7162	10.671	10.669	0.482	0.045	106.7

Matrix effect and recovery

The matrix effect (ME) corrected with internal standard was evaluated using the post extraction approach [42] at two different QC concentrations levels (2.5 and 10 µg/mL). Sample preparation was based on two sets. Set A consists of three extracts of the blank matrices with compounds of interest, added post extraction and compared to set B which consisted of three replicates with neat solutions containing equivalent amounts of compounds of interest prepared in the solution used for reconstitution. ME was calculated by comparison of peak area of sample

spiked before extraction whit those of the neat standard prepared in water, according to the following formula .

$$ME\% = \frac{Area\ spiked\ after\ extraction}{Area\ spiked\ water} \cdot 100$$

Where ME > 100 indicates ion enhancement and ME < 100 ion suppression.
As summarized in Table 6, the ME% where always in the acceptable range (± 25%). Quantification with the use of internal standard yielded better results and the internal standard shown to be appropriate for a correct quantification in the assay.

Recovery was evaluated at two different QC levels (2.5 and 10 µg/mL). Recovery was calculated by comparison of peak spiked before and after extraction, according to the formula:

$$RE\% = \frac{Area\ spiked\ before\ extraction}{Area\ spiked\ after\ extraction} \cdot 100$$

Satisfactory recovery was obtained as shown in Table 6.

Table 6. Recovery and matrix effect.

	2.5 µg/mL			**10 µg/mL**		
	Spiked before extraction	**Spiked after extraction**	**Spiked water**	**Spiked before extraction**	**Spiked after extraction**	**Spiked water**
Area	135329	215641	678001	567544	1048881	3590941
Area IS	205591	361844	957649	175227	280999	1142549
RE%	110			87		
ME%	84			119		

7. Results and discussion

7.1. Blood EtG

The aim of this study has been to determine the correlation between ethanol and EtG in order to infer if the ethanol ingestion occurred close to the traffic accident or hours before.
The investigated blood samples were from drivers involved in fatal and non-fatal traffic accidents.

Before starting the study, the validated method was tested on blood samples of eight healthy volunteers from the laboratory. The volunteers were asked to consume an amount of alcohol (two glasses of red wine) while fasting, which would lead to a blood ethanol concentration of about 0.5 g/L. Forty minutes after the completion of drinking, the blood samples were collected and then analysed for both ethanol and EtG. The results are shown in Table 7. Samples collected were from 3 males and 5 females. The BAC mean value was 0.59 g/L (range 0.43-0.77 g/L) and median 0.55 g/L. The EtG mean value was 0.31 µg/mL (range 0.18-0.75 µg/mL) and median 0.25 µg/mL.

Table 7. Blood EtG samples from eight volunteers.

N. sample	Gender	BAC g/L	EtG µg/mL
1	F	0.77	0.38
2	F	0.56	0.18
3	M	0.54	0.17
4	F	0.70	0.26
5	M	0.54	0.21
6	F	0.48	0.31
7	F	0.70	0.75
8	M	0.43	0.23

7.1.1. Ratio serum/blood forEtG

Studies on serum/blood (S/B) concentration ratio for ethylglucuronide are almost missing in the literature. To the best of our knowledge, there is only one article published by Høiseth et *al.* [43]. They evaluated the serum/whole blood ratio in 13 patients for EtG and EtS. The median concentration in blood was 2.69 mg/L (range 0.13-5.53 mg/L) and in serum 4.59 mg/L (range 0.25-9.81 mg/L). The median value of S/B ratio for EtG is 1.69 (range 1.33-1.90). The ratio obtained are higher than those previously reported for ethanol (1.12-1.14). The EtG and EtS are water-soluble molecules, much larger than ethanol. Because of this reason and considering the minimal hydrophobicity of EtG, the penetration into the red blood cells could be hampered. There are no reports on EtG or EtS binding to plasma proteins, indicating this type of mechanism is not responsible for the skewed distribution of these ethanol metabolites.

The aim of the study was to verify the values obtained by Høiseth and co-workers but, on a greater number of samples. In fact, fifty samples of patients from the emergency room, have been analysed for BAC in blood and for EtG in blood and serum. The sample pre-treatment and the method used are reported in paragraph *6.5*.
The obtained data are shown in *Appendix* Table 1.

The S/B ratio values obtained confirmed the different concentration of EtG in serum and whole blood as already showed by Høiset et *al.*.
Ethanol range is from 0.10 g/L to 3.17 g/L while, the mean value and the median are 1.55 g/L and 1.58 g/L respectively. The EtG range was 0.22-6.84 µg/mL for blood and 0.37-18.80 µg/mL for serum. The mean value of blood was 2.15 µg/mL and the median was 1.52 µg/mL. The mean value of serum was 4.41 µg/mL and the median was 3.01 µg/mL. S/B ratio range was from 1.09 to 3.91 while, the mean value and median were 2.02 and 1.90 respectively (Table 8).

Table 8. Values of EtG in Blood and Serum and S/B ratio in 50 subjects.

	Mean value	**Median**
Ethanol (g/L)	1.55	1.58
EtG serum (µg/mL)	4.41	3.01
EtG blood (µg/mL)	2.15	1.52
S/B ratio	2.02	1.90

7.1.2. Fatal accidents

The aim of the present study was the evaluation of a possible use of blood EtG in the study of alcohol-related traffic accident. A possible correlation between BAC and EtG has been studied to determine if the ethanol ingestion occurred close to the traffic accident or hours before. Preliminary results are reported below.

The most important difference between EtG and BAC is the kinetic that is delayed of an average of about one hour.

The study has been carried out on 25 subjects (M/F: 92% M, 8% F; range 19-79 years), who died in traffic accidents and underwent post-mortem examination in the years 2014-2016 in the Verona district.

The samples have been evaluated on the basis of the EtG and the BAC concentration (Table 9).

Table 9. Data of 25 subjects analysed.

N. sample	BAC g/L	EtG µg/mL
1	1.27	1.41
2	1.68	0.10
3	2.42	3.92
4	1.08	1.20
5	0.38	< 0.10
6	< 0.10	< 0.10
7	2.12	2.09
8	< 0.10	< 0.10
9	1.78	0.43
10	0.99	< 0.10
11	< 0.10	< 0.10
12	0.20	1.24
13	1.95	1.54
14	2.43	1.40
15	< 0.10	< 0.10
16	0.18	0.16
17	2.75	4.80
18	< 0.10	< 0.10
19	< 0.10	< 0.10
20	< 0.10	< 0.10
21	< 0.10	< 0.10
22	< 0.10	< 0.10
23	< 0.10	< 0.10
24	< 0.10	< 0.10
25	0.28	< 0.10

The results of BAC showed 14 positive cases of which 10 (40%) are above the limit 0.5 g/L**.** The range was from 0.18 to 2.75 g/L (mean value 1.39 g/L, median 1.48 g/L).

Regarding the EtG results, 11 cases (44%) resulted positive (above the LOQ 0.10 µg/mL). The range was from 0.1 to 4.8 µg/mL (mean value 1.41 µg/mL; median 1.40 µg/mL).

On the grounds of the obtained results, four groups have been identified (Table 10):

a. samples positive for BAC and negative for EtG (3 cases);
b. samples positive for both BAC and EtG (11 cases);
c. samples negative for BAC and positive for EtG (none);
d. samples negative for both BAC and EtG (11 cases).

Table 10. BAC and EtG results.

BAC (g/L)	**EtG (µg/mL)**	**N. samples**	**%**
< 0.10	< 0.10	11	44
< 0.10	> 0.10	0	0
> 0.10	< 0.10	3	12
> 0.10	> 0.10	11	44
Total		**25**	100

a. Samples positive for BAC and negative for EtG

Three subjects (range 21 – 47 years) were a part of this group. The obtained BAC values were the following:

- range 0.28 – 0.99 g/L,
- mean value 0.55 g/L,
- median 0.38 g/L.

Two cases out of three had a BAC below the 0.5 g/L limit.

b. Samples positive for both BAC and EtG

Eleven subjects (range 19 – 75 years) were part of this group (Table 11). The BAC values were:

- range 0.18 – 2.75 g/L,
- mean value 1.62 g/L,
- median 1.78 g/L.

The EtG values were:

- range 0.10 – 4.80 µg/mL,
- mean value 1.66 µg/mL,
- median 1.40 µg/mL.

Table 11. Samples positive for both BAC and EtG.

BAC (g/L)	**EtG (µg/mL)**
0.18	0.16
0.20	1.24
1.08	1.20
1.27	1.41
1.68	***0.10***
1.78	0.43
1.95	1.54
2.12	2.09
2.42	3.92
2.43	1.40
2.75	4.80

The bold type highlight a singular case in which BAC was above the limit 1.5 g/L. In fact, an elevated BAC value was associated with an EtG concentration equal to the LOQ value (0.10 µg/mL).

c. Samples negative for BAC and positive for EtG

Unfortunately, none of the samples analysed resulted negative for BAC and positive for EtG.

d. Samples negative for both BAC and EtG

Among the samples analysed, 11 subjects resulted negative for both BAC and EtG. For this reason, these subjects were considered not involved in alcohol-related traffic accident.

These preliminary data suggested that the blood EtG analysis could provide indications on the time elapsed between the alcohol ingestion and the collection of blood.

The limited number of dead cases precluded a more detailed investigation on a possible application of blood EtG in case of non fatal alcohol-related traffic accidents.

It will be necessary to increase the number of blood samples from crash victims.

7.1.3. Non-fatal accidents

Samples were collected from the emergency room from subjects involved in non-fatal traffic accidents in the years 2016-2017 in Verona and Vicenza districts. The exclusion criteria applied was the presence of psychoactive drugs The samples analysed were 697 but, among these, 19 samples were discarded because of the lack of information about the traffic accident time.
The samples investigated were 678 (517 males, 161 females; range 14-87 years mean value 41 years) (refer to *Appendix* Table 2).

Regarding BAC, 572 samples resulted negative (below the LOQ 0.1 g/L) while, 106 samples were positive (above the LOQ) of which, 14 samples with BAC < 0.5 g/L and 92 with BAC > 0.5 g/L. Among the 92 cases, 9 samples resulted in the range 0.5-0.8 g/L, 36 samples in the range 0.8-1.5 g/L and 47 above 1.5 g/L (Table 12).

Table 12. BAC cases.

BAC (g/L)				
< 0.1	**< 0.5**	**0.5 - 0.8**	**0.8 - 1.5**	**> 1.5**
572	14	9	36	47

Evaluating the samples resulted positive for BAC (n = 106) (Table 13), it was noticed that BAC mean value was almost coincident with the median value, 1.54 g/L and 1.45 g/L respectively. Instead, for the EtG concentration the mean value was 3.08 µg/mL while the median value 2.49 µg/mL.

Table 13. Samples resulted positive for BAC.

	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
Mean value	1.54	3.08	1:36
Median	1.45	2.49	1:25
Range	0.10 - 4.25	0.21 - 12.44	0:20 - 7:10

As far as EtG is concerned, 487 samples resulted negative (below the LOQ 0.10 µg/mL) and 191 samples positive (above the LOQ). The positive cases were investigated on the basis of ethanol concentration (Table 14).

Table 14. Samples resulted positive for EtG.

BAC (g/L)	N. samples	%	BAC mean value (g/L)	BAC range (g/L)	EtG mean value (µg/mL)	EtG range (µg/mL)	Δt mean value (hh:mm)
< 0.10	85	44.5	-	-	0.31	0.10 - 1.6	2:03
< 0.5	14	7.3	0.18	0.10 - 0.46	1.09	0.24 - 2.38	1:51
0.5 - 0.8	9	4.7	0.70	0.55 - 0.77	1.78	0.84 - 3.04	1:39
0.8 - 1.5	36	18.9	1.16	0.82 - 1.47	2.41	0.37 - 10.76	1:52
> 1.5	47	24.6	2.37	1.53 - 4.25	4.48	0.21 - 12.44	1:25

Regarding the time between traffic accident and the blood collection the range was from 20 minutes to 9 hours 40 minutes. The mean value was 2 hours 2 minutes while the median 1 hour 46 minutes.

The samples were divided into groups on the basis of this time (Table 15). It has been noticed that in most cases, more than 50% (381 cases of which 125 cases less than one hour), the time difference resulted below two hours. A percentage of 26 (171 cases) was in the range from 2 to 3 hours while 10.8% (73 cases) was in the range from 3 to 4 hours. The rest of cases, about 7%, were above four hours

and, unfortunately, a small percentage (3.5%, 24 cases) was even beyond five hours.

Following this analysis, it could be hypothesised that EtG level could provide valuable information on the amount of alcohol consumed before the accident in cases where the interval between the accident and blood collection is high.

Table 15. Time between traffic accident and blood collection.

$\Delta t_{accident\text{-}blood\ collection}$ (h)	N. sample	%
< 1	125	18.4
1 – 2	256	37.8
2 – 3	176	26.0
3 – 4	73	10.8
4 – 5	24	3.5
> 5	24	3.5

Among the analysed samples, cases with a BAC approximately the legal limits (0.5, 0.8 and 1.5 g/L) were chosen and described on the basis of BAC, serum EtG and the ratio between BAC and EtG (Table 16, 17 and 18).

The ratio of blood ethanol (g/L) and blood EtG (mg/L) may be used to approximate the ingestion time. When the ratio is higher than one, it suggests ethanol ingestion within 3.5 hours before sample collection. A ratio less than one suggests ethanol was ingested less than 2.5 hours before collection. If only one blood sample is available, the ratio higher than one will indicate a recent drinking; on the contrary, a ratio lower than one will exclude a recent dringing [27].

Subjects with BAC ~ 0.5 g/L

In all subjects, the ratio, between BAC and EtG, is less than 1 so, the recent drinking could be excluded.

Table 16. Cases investigated with BAC < 0.5 g/L.

Subject	BAC (g/L)	EtG (µg/mL)	Time (hh:mm)	BAC/EtG ratio
1	0.55	0.97	01:06	0.57
2	0.45	0.58	01:15	0.78
3	0.64	2.99	00:50	0.21
4	0.64	2.56	01:55	0.25
5	0.46	1.30	03:30	0.35

Subjects with BAC ~ 0.8 g/L

In all subjects but one, the ratio between BAC and EtG, is less than 1 so, the recent drinking could be excluded. In subject 8, the ratio (2.51) is higher than one, indicating a recent drinking.

Table 17. Cases investigated with BAC ~ 0.8 g/L.

Subject	BAC (g/L)	EtG (µg/mL)	Time (hh:mm)	BAC/EtG ratio
1	0.76	1.67	01:30	0.46
2	0.73	3.04	02:22	0.24
3	0.76	0.84	01:30	0.90
4	0.71	1.25	02:04	0.57
5	0.77	1.56	02:32	0.49
6	0.93	3.82	02:05	0.24
7	0.93	1.23	01:40	0.76
8	0.93	0.37	00:54	**2.51**
9	0.84	1.66	01:18	0.51

Subjects with BAC ~ 1.5 g/L

These subjects are the most interesting because, according to the italian law, drivers who caused an road accident whith BAC higher than 1.5 g/L can be punished with eight to twelve years of imprisonment. Besides, the BAC mean value and median (1.54 g/L and 1.45 g/L respectively) of all the samples analysed is about 1.5 g/L.

In most of the subjects, the ratio is less than 1 thus the recent drinking could be excluded. In samples 5 and 8, the ratio is 1.17 and 1.56 respectively thus confirming a recent drinking. In subject 3, the ratio is about 1 and it could hypothesize that BAC value was higher. In subject 10, the ratio 3.33 may indicate a recent drinking but it is not possible to hypothesize.

Table 18. Cases investigated with BAC ~ 1.5 g/L.

Subject	**BAC (g/L)**	**EtG (µg/mL)**	**Time (hh:mm)**	**BAC/EtG ratio**
1	1.45	2.94	02:08	0.49
2	1.45	3.69	02:20	0.39
3	1.46	1.47	01:00	**0.99**
4	1.44	1.94	02:55	0.74
5	1.47	1.26	03:21	**1.17**
6	1.45	5.00	03:45	0.29
7	1.56	4.09	01:35	0.38
8	1.54	0.99	01:00	**1.56**
9	1.54	3.90	01:20	0.39
10	1.53	0.46	01:45	**3.33**

8. Conclusions

In the present study, a sensitive HPLC-MS/MS method for the determination of EtG in serum has been developed and fully validated. The procedure was found to be sufficiently sensitive and specific to be applicable in monitoring recent alcohol use even in case ethanol has been completely eliminated from blood. The assay was successfully applied on 678 samples of subjets involved in road traffic accident.

Moreover, the possible use of serum EtG to assist the interpretation of blood alcohol concentration in road traffic accidents was evaluated, particularly in case of delayed sample collection. Indeed, among the analysed samples, the 18% of the cases, the time between blood collection and the traffic accident was more than three hours. Many subjects with similar BAC and different serum EtG were identified. A specific study was performed on these subjects by evaluating BAC, serum EtG and time fromblood collection in order to identify the phase of the ethanol curve when the sample was collected. The preliminary results show that an BAC(g/L)/EtG(µg/mL) higher than one is consistent with a sample collection in the phase of ethanol elimination. More study is still needed to clarify these points, taking also in consideration that the distribution of EtG between serum and whole blood has a ratio about two, and therefore the meaning of this ratio can vary according to the biological matrix used. However, since now EtG looks particurlarly useful for the interpretation of cases when BAC is close to concentration limits established by law (e.g. 0.5 g/L, 0.8 g/L, 1.5 g/L) whose exceeding may lead to important consequences for the person.

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9. References

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Appendix 1

Table 1. S/B concentration ratio on 50 subjects.

ID sample	Ethanol (g/L)	EtG serum (µg/mL)	EtG blood (µg/mL)	S/B ratio
1	1.77	0.93	0.45	2.07
2	0.93	0.49	0.22	2.23
3	2.57	3.52	2.69	1.31
4	1.81	2.49	1.86	1.34
5	1.71	1.11	0.69	1.61
6	1.93	1.31	0.64	2.05
7	0.64	0.37	0.26	1.42
8	1.25	1.76	1.48	1.19
9	0.83	1.56	0.88	1.77
10	1.69	2.76	1.40	1.97
11	0.94	1.33	1.12	1.19
12	1.16	1.15	0.83	1.39
13	0.94	0.70	0.40	1.75
14	1.10	1.92	0.98	1.96
15	2.54	3.29	1.35	2.44
16	2.03	3.31	1.03	3.21
17	2.52	9.36	5.93	1.58
18	0.92	1.29	0.87	1.48
19	0.64	2.99	1.74	1.72
20	1.47	1.26	0.74	1.70
21	0.84	1.66	0.69	2.41
22	2.65	10.16	3.57	2.85
23	1.40	2.87	2.04	1.41
24	1.85	5.62	2.62	2.15
25	1.08	3.02	1.63	1.85
26	2.05	3.03	1.70	1.78
27	0.91	3.92	1.17	3.35
28	2.70	15.70	4.10	3.83
29	0.64	2.56	2.34	1.09
30	2.42	5.34	2.69	1.99
31	2.94	6.70	4.60	1.46
32	3.17	9.40	3.91	2.40
33	2.34	6.30	3.83	1.64
34	2.35	9.50	5.10	1.86
35	2.58	8.45	4.20	2.01
36	1.87	3.51	1.56	2.25

ID sample	Ethanol (g/L)	EtG serum (µg/mL)	EtG blood (µg/mL)	S/B ratio
37	0.76	1.67	0.98	1.70
38	2.00	10.60	3.30	3.21
39	2.10	7.10	5.01	1.42
40	2.23	8.00	5.21	1.54
41	0.28	0.83	0.43	1.93
42	0.05	0.64	0.37	1.73
43	0.11	1.57	0.78	2.01
44	1.43	2.31	1.69	1.37
45	1.96	13.98	6.45	2.17
46	1.19	4.46	1.14	3.91
47	1.70	4.90	2.19	2.24
48	0.46	1.30	0.62	2.10
49	0.71	3.64	1.08	3.37
50	1.12	18.80	6.84	2.75

Table 2. Samples of non-fatal traffic accidents.

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
1	1.93	3.29	1:25
2	< 0.10	< 0.10	5:30
3	< 0.10	< 0.10	1:45
4	1.71	3.03	1:10
5	2.27	4.90	3:37
6	3.80	4.60	2:22
7	< 0.10	< 0.10	2:15
8	< 0.10	< 0.10	2:28
9	< 0.10	< 0.10	1:50
10	1.00	0.90	0:45
11	< 0.10	< 0.10	1:55
12	2.24	3.65	1:15
13	< 0.10	< 0.10	1:45
14	< 0.10	< 0.10	3:35
15	< 0.10	< 0.10	1:10
16	< 0.10	< 0.10	0:43
17	2.64	6.19	0:20
18	< 0.10	< 0.10	0:37
19	< 0.10	< 0.10	0:20
20	< 0.10	< 0.10	1:45
21	< 0.10	< 0.10	1:23
22	< 0.10	< 0.10	1:10
23	< 0.10	< 0.10	1:10
24	< 0.10	< 0.10	3:05
25	< 0.10	< 0.10	1:21
26	< 0.10	< 0.10	2:00
27	2.97	4.47	2:25
28	< 0.10	< 0.10	0:20
29	< 0.10	0.14	1:45
30	< 0.10	< 0.10	2:05
31	1.47	1.66	2:15
32	< 0.10	0.22	0:32
33	< 0.10	< 0.10	1:10
34	< 0.10	0.11	3:10
35	< 0.10	< 0.10	1:00
36	< 0.10	< 0.10	7:20
37	< 0.10	< 0.10	0:50
38	< 0.10	< 0.10	1:25
39	0.76	0.84	1:30
40	2.98	7.03	0:55
41	< 0.10	0.27	1:20
42	< 0.10	< 0.10	5:00
43	< 0.10	0.22	0:22
44	< 0.10	0.41	1:50
45	1.15	3.54	1:27
46	< 0.10	0.10	1:45
47	< 0.10	< 0.10	0:44
48	1.08	1.87	2:14
49	1.81	2.65	0:20
50	< 0.10	< 0.10	1:30

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
51	< 0.10	< 0.10	1:00
52	< 0.10	< 0.10	0:55
53	1.26	1.02	0:20
54	< 0.10	< 0.10	0:21
55	< 0.10	< 0.10	1:25
56	< 0.10	< 0.10	2:35
57	< 0.10	< 0.10	1:02
58	< 0.10	< 0.10	0:25
59	< 0.10	< 0.10	1:00
60	< 0.10	< 0.10	1:30
61	< 0.10	< 0.10	0:30
62	< 0.10	< 0.10	2:23
63	0.72	1.17	1:02
64	1.54	3.90	1:20
65	< 0.10	< 0.10	2:05
66	< 0.10	< 0.10	2:55
67	< 0.10	< 0.10	2:45
68	< 0.10	< 0.10	1:25
69	< 0.10	< 0.10	1:30
70	< 0.10	0.12	2:39
71	0.97	1.11	1:25
72	< 0.10	< 0.10	1:40
73	< 0.10	< 0.10	1:00
74	< 0.10	< 0.10	2:55
75	< 0.10	< 0.10	5:20
76	< 0.10	< 0.10	0:47
77	< 0.10	< 0.10	0:55
78	< 0.10	0.11	2:25
79	< 0.10	< 0.10	2:00
80	2.02	6.79	0:36
81	0.55	0.97	1:06
82	< 0.10	< 0.10	1:30
83	< 0.10	< 0.10	1:20
84	< 0.10	< 0.10	1:00
85	< 0.10	< 0.10	6:06
86	0.23	1.05	1:18
87	< 0.10	0.28	0:31
88	< 0.10	0.38	2:00
89	0.45	0.58	1:15
90	< 0.10	< 0.10	1:00
91	1.87	2.76	0:45
92	< 0.10	< 0.10	1:24
93	< 0.10	0.10	2:35
94	< 0.10	< 0.10	2:00
95	< 0.10	< 0.10	3:25
96	< 0.10	< 0.10	1:30
97	< 0.10	< 0.10	2:05
98	< 0.10	0.11	4:39
99	< 0.10	< 0.10	1:46
100	2.35	4.23	1:30

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
101	< 0.10	0.38	1:55
102	< 0.10	< 0.10	1:11
103	< 0.10	< 0.10	1:05
104	< 0.10	0.46	1:55
105	< 0.10	< 0.10	2:12
106	< 0.10	0.39	2:00
107	< 0.10	0.41	0:45
108	1.70	2.41	0:54
109	< 0.10	< 0.10	0:20
110	< 0.10	< 0.10	1:10
111	< 0.10	0.48	0:59
112	< 0.10	0.10	1:25
113	< 0.10	< 0.10	1:15
114	< 0.10	< 0.10	3:30
115	< 0.10	< 0.10	2:30
116	< 0.10	1.6	5:04
117	< 0.10	< 0.10	3:55
118	< 0.10	< 0.10	3:30
119	< 0.10	< 0.10	2:00
120	< 0.10	< 0.10	0:20
121	< 0.10	< 0.10	2:00
122	2.00	5.5	1:10
123	< 0.10	< 0.10	1:44
124	< 0.10	< 0.10	2:28
125	< 0.10	0.38	0:40
126	< 0.10	< 0.10	5:00
127	< 0.10	0.17	1:19
128	< 0.10	< 0.10	1:30
129	< 0.10	0.68	2:00
130	< 0.10	< 0.10	2:00
131	< 0.10	< 0.10	1:58
132	< 0.10	< 0.10	3:00
133	< 0.10	0.21	1:40
134	< 0.10	< 0.10	2:37
135	< 0.10	1.53	0:30
136	< 0.10	0.26	1:47
137	< 0.10	0.25	2:52
138	< 0.10	0.22	0:39
139	< 0.10	< 0.10	0:40
140	1.53	0.46	1:45
141	< 0.10	< 0.10	1:33
142	< 0.10	< 0.10	2:00
143	< 0.10	< 0.10	6:58
144	< 0.10	0.11	2:15
145	< 0.10	0.23	1:50
146	< 0.10	0.12	2:00
147	< 0.10	< 0.10	2:48
148	< 0.10	0.46	2:15
149	0.27	1.16	1:06
150	< 0.10	< 0.10	1:40
151	< 0.10	0.52	0:20
152	< 0.10	< 0.10	2:30

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
153	< 0.10	< 0.10	1:40
154	< 0.10	< 0.10	1:10
155	< 0.10	0.92	0:31
156	< 0.10	< 0.10	1:30
157	< 0.10	0.11	0:50
158	< 0.10	< 0.10	2:56
159	< 0.10	0.22	4:17
160	< 0.10	< 0.10	3:11
161	< 0.10	0.15	1:02
162	< 0.10	< 0.10	2:40
163	< 0.10	< 0.10	2:06
164	< 0.10	0.10	2:10
165	< 0.10	0.14	0:43
166	< 0.10	< 0.10	6:33
167	1.01	1.52	1:21
168	< 0.10	< 0.10	1:15
169	< 0.10	< 0.10	2:40
170	< 0.10	< 0.10	1:10
171	< 0.10	0.12	1:34
172	< 0.10	< 0.10	2:38
173	< 0.10	0.11	2:25
174	< 0.10	0.30	1:45
175	< 0.10	0.13	3:00
176	< 0.10	< 0.10	4:55
177	< 0.10	< 0.10	0:22
178	< 0.10	< 0.10	2:25
179	< 0.10	< 0.10	3:45
180	< 0.10	< 0.10	2:04
181	1.87	4.45	1:45
182	< 0.10	0.11	3:40
183	< 0.10	0.10	2:35
184	< 0.10	< 0.10	2:16
185	0.73	3.04	2:22
186	< 0.10	< 0.10	3:40
187	< 0.10	0.16	4:00
188	< 0.10	0.10	1:36
189	2.94	0.63	3:27
190	< 0.10	0.12	1:43
191	1.46	3.82	3:12
192	< 0.10	0.16	1:40
193	< 0.10	0.15	1:35
194	< 0.10	< 0.10	2:05
195	< 0.10	< 0.10	1:16
196	< 0.10	< 0.10	1:33
197	0.93	3.06	2:05
198	< 0.10	< 0.10	3:45
199	0.91	2.09	1:35
200	3.75	8.04	7:10
201	2.20	4.85	1:40
202	< 0.10	< 0.10	2:02
203	< 0.10	1.37	4:03
204	< 0.10	< 0.10	2:10

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
205	< 0.10	0.23	2:30
206	< 0.10	< 0.10	1:05
207	< 0.10	0.28	3:24
208	< 0.10	0.10	1:19
209	< 0.10	< 0.10	3:00
210	< 0.10	< 0.10	3:15
211	< 0.10	< 0.10	3:00
212	0.16	2.38	1:35
213	< 0.10	< 0.10	2:06
214	< 0.10	< 0.10	5:35
215	< 0.10	< 0.10	2:05
216	< 0.10	< 0.10	1:40
217	< 0.10	< 0.10	2:14
218	< 0.10	1.08	3:40
219	< 0.10	< 0.10	3:30
220	< 0.10	< 0.10	1:30
221	1.29	2.00	1:57
222	< 0.10	< 0.10	0:28
223	< 0.10	0.13	1:40
224	< 0.10	0.24	0:55
225	< 0.10	< 0.10	0:20
226	< 0.10	0.16	1:20
227	< 0.10	< 0.10	0:54
228	< 0.10	< 0.10	2:20
229	< 0.10	< 0.10	0:59
230	< 0.10	< 0.10	2:30
231	< 0.10	< 0.10	3:40
232	< 0.10	< 0.10	3:25
233	< 0.10	< 0.10	2:00
234	< 0.10	< 0.10	2:35
235	< 0.10	< 0.10	1:00
236	< 0.10	< 0.10	2:50
237	< 0.10	< 0.10	4:53
238	0.93	1.23	1:40
239	< 0.10	< 0.10	0:47
240	< 0.10	< 0.10	0:43
241	< 0.10	< 0.10	1:45
242	< 0.10	< 0.10	1:41
243	< 0.10	< 0.10	1:50
244	< 0.10	< 0.10	1:08
245	< 0.10	< 0.10	1:54
246	< 0.10	0.25	0:35
247	< 0.10	< 0.10	1:45
248	< 0.10	0.11	2:35
249	< 0.10	< 0.10	3:20
250	< 0.10	< 0.10	3:40
251	< 0.10	< 0.10	9:40
252	< 0.10	0.12	2:08
253	0.82	1.05	1:45
254	< 0.10	0.17	2:19
255	< 0.10	< 0.10	3:55
256	< 0.10	< 0.10	2:35

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
257	< 0.10	< 0.10	2:10
258	< 0.10	0.25	2:00
259	< 0.10	0.17	0:36
260	< 0.10	< 0.10	1:58
261	< 0.10	< 0.10	3:15
262	< 0.10	< 0.10	2:51
263	< 0.10	< 0.10	1:40
264	< 0.10	< 0.10	1:30
265	< 0.10	< 0.10	4:36
266	< 0.10	< 0.10	1:57
267	< 0.10	< 0.10	2:10
268	< 0.10	< 0.10	1:45
269	< 0.10	0.32	1:30
270	1.38	1.64	1:45
271	< 0.10	< 0.10	6:34
272	< 0.10	< 0.10	0:45
273	< 0.10	0.18	2:10
274	2.36	4.33	1:00
275	< 0.10	< 0.10	0:55
276	< 0.10	< 0.10	2:45
277	< 0.10	0.14	1:15
278	< 0.10	< 0.10	3:30
279	< 0.10	< 0.10	0:26
280	< 0.10	< 0.10	1:10
281	< 0.10	< 0.10	1:58
282	< 0.10	< 0.10	1:20
283	< 0.10	< 0.10	2:28
284	< 0.10	< 0.10	3:40
285	< 0.10	< 0.10	4:00
286	< 0.10	< 0.10	2:15
287	< 0.10	< 0.10	3:04
288	< 0.10	< 0.10	0:24
289	< 0.10	< 0.10	2:05
290	< 0.10	< 0.10	0:47
291	1.45	2.94	2:08
292	< 0.10	< 0.10	1:10
293	2.37	1.97	0:40
294	< 0.10	< 0.10	3:36
295	< 0.10	< 0.10	2:29
296	< 0.10	< 0.10	0:20
297	1.15	1.06	1:44
298	< 0.10	< 0.10	3:00
299	1.07	0.46	6:07
300	< 0.10	< 0.10	2:05
301	< 0.10	< 0.10	1:04
302	< 0.10	< 0.10	1:15
303	< 0.10	< 0.10	2:55
304	1.45	3.69	2:20
305	< 0.10	< 0.10	2:09
306	< 0.10	< 0.10	1:10
307	2.81	6.59	1:50
308	< 0.10	< 0.10	1:33

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
309	< 0.10	< 0.10	0:30
310	< 0.10	< 0.10	1:05
311	< 0.10	< 0.10	2:40
312	< 0.10	< 0.10	1:25
313	< 0.10	< 0.10	1:35
314	< 0.10	< 0.10	2:05
315	< 0.10	0.55	5:10
316	< 0.10	< 0.10	1:30
317	< 0.10	0.10	0:30
318	< 0.10	0.21	1:53
319	< 0.10	< 0.10	0:55
320	1.54	0.99	1:05
321	< 0.10	< 0.10	1:11
322	< 0.10	0.35	2:50
323	< 0.10	< 0.10	2:00
324	< 0.10	< 0.10	1:45
325	< 0.10	< 0.10	2:27
326	< 0.10	< 0.10	3:30
327	< 0.10	< 0.10	0:56
328	< 0.10	< 0.10	1:20
329	< 0.10	< 0.10	7:13
330	< 0.10	< 0.10	1:33
331	< 0.10	< 0.10	2:43
332	< 0.10	< 0.10	4:00
333	2.19	3.71	0:29
334	< 0.10	< 0.10	2:58
335	< 0.10	< 0.10	1:15
336	< 0.10	< 0.10	6:01
337	4.25	12.44	0:25
338	1.12	1.63	2:30
339	2.34	3.95	3:00
340	< 0.10	0.50	2:20
341	< 0.10	< 0.10	1:30
342	3.67	0.21	1:35
343	< 0.10	0.17	1:53
344	< 0.10	< 0.10	4:05
345	2.99	5.12	0:50
346	< 0.10	< 0.10	0:47
347	< 0.10	< 0.10	4:44
348	< 0.10	< 0.10	2:05
349	< 0.10	< 0.10	0:30
350	< 0.10	< 0.10	1:15
351	< 0.10	< 0.10	1:30
352	< 0.10	< 0.10	1:30
353	1.92	0.80	0:20
354	< 0.10	< 0.10	1:18
355	< 0.10	< 0.10	1:50
356	< 0.10	< 0.10	1:54
357	1.99	6.26	2:45
358	3.07	3.79	0:23
359	< 0.10	< 0.10	3:00
360	< 0.10	< 0.10	1:55

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
361	< 0.10	< 0.10	0:28
362	< 0.10	< 0.10	0:20
363	< 0.10	< 0.10	3:23
364	< 0.10	< 0.10	1:39
365	< 0.10	< 0.10	3:12
366	< 0.10	< 0.10	3:24
367	< 0.10	< 0.10	4:15
368	< 0.10	< 0.10	0:50
369	< 0.10	< 0.10	1:32
370	3.16	7.27	0:28
371	< 0.10	< 0.10	2:25
372	1.24	2.97	0:30
373	< 0.10	< 0.10	2:05
374	< 0.10	< 0.10	1:21
375	1.46	1.47	1:00
376	< 0.10	< 0.10	2:20
377	< 0.10	< 0.10	2:54
378	< 0.10	0.16	2:20
379	< 0.10	< 0.10	1:35
380	< 0.10	< 0.10	3:10
381	< 0.10	< 0.10	1:08
382	0.21	0.38	2:05
383	< 0.10	< 0.10	3:05
384	< 0.10	< 0.10	1:55
385	1.01	1.3	0:47
386	< 0.10	< 0.10	1:27
387	< 0.10	< 0.10	1:30
388	< 0.10	< 0.10	2:10
389	0.71	1.25	2:04
390	< 0.10	0.33	2:15
391	< 0.10	< 0.10	3:19
392	< 0.10	< 0.10	1:20
393	< 0.10	< 0.10	0:41
394	< 0.10	< 0.10	6:25
395	< 0.10	< 0.10	2:25
396	< 0.10	< 0.10	2:16
397	< 0.10	< 0.10	2:10
398	< 0.10	< 0.10	3:25
399	< 0.10	< 0.10	2:13
400	< 0.10	< 0.10	2:35
401	< 0.10	< 0.10	2:05
402	< 0.10	< 0.10	1:45
403	< 0.10	< 0.10	2:15
404	< 0.10	< 0.10	1:33
405	< 0.10	< 0.10	3:39
406	< 0.10	< 0.10	1:26
407	< 0.10	0.20	1:24
408	< 0.10	< 0.10	2:10
409	< 0.10	< 0.10	1:25
410	< 0.10	< 0.10	0:54
411	< 0.10	< 0.10	3:00
412	< 0.10	< 0.10	3:50

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
413	< 0.10	< 0.10	2:22
414	< 0.10	< 0.10	2:00
415	< 0.10	< 0.10	2:55
416	1.44	1.94	2:55
417	1.71	0.42	0:25
418	< 0.10	< 0.10	3:30
419	< 0.10	< 0.10	1:02
420	< 0.10	< 0.10	0:40
421	< 0.10	< 0.10	1:34
422	< 0.10	< 0.10	1:26
423	< 0.10	< 0.10	1:25
424	< 0.10	< 0.10	2:15
425	< 0.10	< 0.10	2:30
426	< 0.10	< 0.10	2:03
427	1.25	1.39	1:00
428	< 0.10	< 0.10	1:55
429	0.93	0.37	0:54
430	2.57	1.76	0:40
431	< 0.10	< 0.10	1:54
432	< 0.10	< 0.10	2:37
433	< 0.10	< 0.10	1:35
434	< 0.10	< 0.10	1:13
435	< 0.10	< 0.10	1:34
436	< 0.10	< 0.10	4:13
437	< 0.10	< 0.10	4:35
438	< 0.10	< 0.10	0:31
439	< 0.10	< 0.10	4:37
440	< 0.10	< 0.10	1:00
441	< 0.10	< 0.10	5:40
442	< 0.10	< 0.10	2:40
443	< 0.10	< 0.10	1:43
444	< 0.10	< 0.10	1:20
445	< 0.10	< 0.10	0:55
446	< 0.10	< 0.10	3:10
447	< 0.10	< 0.10	1:20
448	0.77	1.56	2:32
449	< 0.10	< 0.10	0:46
450	< 0.10	< 0.10	1:10
451	< 0.10	< 0.10	1:10
452	< 0.10	0.13	1:05
453	< 0.10	< 0.10	0:24
454	< 0.10	< 0.10	1:11
455	< 0.10	< 0.10	2:02
456	< 0.10	< 0.10	3:02
457	< 0.10	< 0.10	1:35
458	< 0.10	< 0.10	1:15
459	0.11	0.24	1:20
460	3.00	4.11	0:35
461	< 0.10	< 0.10	1:30
462	< 0.10	< 0.10	2:26
463	< 0.10	< 0.10	1:29
464	< 0.10	< 0.10	1:55

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
465	< 0.10	< 0.10	1:25
466	< 0.10	< 0.10	0:52
467	1.10	2.64	0:47
468	2.54	3.97	1:07
469	< 0.10	< 0.10	1:45
470	< 0.10	0.37	2:55
471	2.03	3.72	1:20
472	< 0.10	0.40	4:15
473	< 0.10	< 0.10	1:35
474	< 0.10	< 0.10	1:40
475	< 0.10	< 0.10	1:40
476	< 0.10	< 0.10	3:00
477	< 0.10	< 0.10	1:30
478	< 0.10	< 0.10	1:30
479	< 0.10	0.22	4:35
480	< 0.10	< 0.10	0:53
481	< 0.10	< 0.10	0:21
482	< 0.10	< 0.10	2:10
483	< 0.10	< 0.10	0:40
484	< 0.10	< 0.10	1:04
485	< 0.10	< 0.10	0:20
486	< 0.10	< 0.10	2:57
487	< 0.10	< 0.10	1:50
488	< 0.10	< 0.10	1:20
489	< 0.10	< 0.10	1:37
490	< 0.10	< 0.10	2:54
491	1.69	2.76	1:00
492	< 0.10	< 0.10	1:35
493	< 0.10	< 0.10	2:16
494	1.16	1.15	1:45
495	< 0.10	< 0.10	0:26
496	< 0.10	< 0.10	3:20
497	< 0.10	< 0.10	2:05
498	< 0.10	< 0.10	2:45
499	< 0.10	< 0.10	3:50
500	< 0.10	< 0.10	1:50
501	< 0.10	< 0.10	1:53
502	< 0.10	< 0.10	1:10
503	< 0.10	< 0.10	0:45
504	< 0.10	< 0.10	1:41
505	< 0.10	0.17	2:05
506	< 0.10	< 0.10	4:50
507	< 0.10	< 0.10	1:25
508	< 0.10	< 0.10	2:52
509	< 0.10	< 0.10	0:41
510	< 0.10	< 0.10	1:05
511	< 0.10	< 0.10	0:42
512	< 0.10	< 0.10	2:13
513	< 0.10	< 0.10	1:22
514	< 0.10	< 0.10	2:07
515	< 0.10	< 0.10	1:27
516	< 0.10	< 0.10	2:01

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
517	0.64	2.99	0:50
518	< 0.10	< 0.10	1:15
519	1.47	1.26	3:21
520	< 0.10	< 0.10	1:15
521	< 0.10	< 0.10	0:57
522	< 0.10	< 0.10	2:35
523	< 0.10	< 0.10	3:45
524	0.84	1.66	1:18
525	< 0.10	< 0.10	2:15
526	< 0.10	< 0.10	1:15
527	< 0.10	< 0.10	2:00
528	< 0.10	< 0.10	2:10
529	< 0.10	< 0.10	2:45
530	< 0.10	< 0.10	2:17
531	< 0.10	< 0.10	1:15
532	< 0.10	< 0.10	1:15
533	< 0.10	< 0.10	2:25
534	< 0.10	< 0.10	2:25
535	< 0.10	< 0.10	1:50
536	< 0.10	< 0.10	1:35
537	< 0.10	< 0.10	1:20
538	< 0.10	< 0.10	0:20
539	< 0.10	< 0.10	1:48
540	< 0.10	< 0.10	1:50
541	< 0.10	< 0.10	1:19
542	< 0.10	< 0.10	1:33
543	< 0.10	< 0.10	3:45
544	< 0.10	0.11	3:50
545	< 0.10	< 0.10	2:05
546	< 0.10	< 0.10	2:45
547	< 0.10	< 0.10	1:57
548	< 0.10	< 0.10	1:57
549	< 0.10	< 0.10	1:00
550	< 0.10	< 0.10	2:12
551	< 0.10	< 0.10	5:15
552	< 0.10	< 0.10	1:30
553	< 0.10	< 0.10	2:10
554	< 0.10	< 0.10	0:50
555	< 0.10	< 0.10	2:20
556	< 0.10	< 0.10	2:02
557	< 0.10	< 0.10	0:20
558	< 0.10	< 0.10	2:30
559	< 0.10	0.53	1:50
560	< 0.10	< 0.10	3:20
561	< 0.10	< 0.10	3:00
562	0.91	3.92	0:21
563	< 0.10	< 0.10	4:00
564	< 0.10	< 0.10	1:12
565	< 0.10	< 0.10	0:30
566	< 0.10	< 0.10	2:15
567	< 0.10	< 0.10	2:20
568	< 0.10	< 0.10	4:50

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
569	< 0.10	< 0.10	4:20
570	< 0.10	< 0.10	0:50
571	< 0.10	1.46	3:42
572	< 0.10	< 0.10	3:00
573	< 0.10	< 0.10	1:50
574	< 0.10	< 0.10	1:30
575	< 0.10	< 0.10	1:40
576	< 0.10	< 0.10	1:50
577	0.64	2.56	1:55
578	< 0.10	< 0.10	1:50
579	< 0.10	< 0.10	1:40
580	< 0.10	< 0.10	1:10
581	< 0.10	< 0.10	0:20
582	< 0.10	< 0.10	4:08
583	< 0.10	< 0.10	4:25
584	< 0.10	< 0.10	0:48
585	< 0.10	< 0.10	0:58
586	2.94	6.70	1:35
587	2.34	6.30	1:22
588	< 0.10	< 0.10	3:00
589	< 0.10	< 0.10	1:15
590	< 0.10	< 0.10	2:21
591	< 0.10	< 0.10	3:42
592	< 0.10	< 0.10	2:00
593	< 0.10	< 0.10	8:22
594	< 0.10	< 0.10	2:25
595	< 0.10	< 0.10	3:35
596	< 0.10	< 0.10	2:30
597	< 0.10	< 0.10	3:05
598	< 0.10	< 0.10	2:55
599	< 0.10	< 0.10	1:36
600	< 0.10	< 0.10	1:22
601	< 0.10	< 0.10	1:16
602	1.87	3.51	1:30
603	0.76	1.67	1:30
604	< 0.10	< 0.10	0:40
605	< 0.10	< 0.10	2:41
606	< 0.10	0.24	0:40
607	< 0.10	< 0.10	3:40
608	2.00	10.6	0:50
609	< 0.10	< 0.10	2:20
610	< 0.10	< 0.10	9:10
611	< 0.10	< 0.10	1:35
612	< 0.10	< 0.10	2:25
613	< 0.10	< 0.10	1:30
614	< 0.10	< 0.10	2:19
615	< 0.10	< 0.10	2:20
616	< 0.10	< 0.10	0:21
617	< 0.10	< 0.10	5:03
618	< 0.10	< 0.10	2:35
619	2.23	8.00	2:00
620	< 0.10	< 0.10	1:16

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
621	< 0.10	< 0.10	1:57
622	< 0.10	< 0.10	0:26
623	< 0.10	< 0.10	3:45
624	< 0.10	< 0.10	0:24
625	< 0.10	< 0.10	0:50
626	< 0.10	< 0.10	5:20
627	< 0.10	< 0.10	2:25
628	< 0.10	< 0.10	2:35
629	< 0.10	< 0.10	2:00
630	0.28	0.83	0:30
631	< 0.10	< 0.10	1:45
632	< 0.10	< 0.10	2:15
633	< 0.10	< 0.10	2:05
634	< 0.10	< 0.10	1:50
635	< 0.10	< 0.10	3:15
636	< 0.10	< 0.10	1:40
637	< 0.10	< 0.10	1:33
638	< 0.10	< 0.10	2:00
639	0.10	0.64	1:46
640	< 0.10	< 0.10	2:40
641	< 0.10	< 0.10	0:51
642	0.11	1.57	2:10
643	< 0.10	< 0.10	3:10
644	< 0.10	< 0.10	1:28
645	< 0.10	< 0.10	1:44
646	< 0.10	< 0.10	1:15
647	< 0.10	< 0.10	1:28
648	< 0.10	< 0.10	2:10
649	< 0.10	< 0.10	0:21

N. sample	BAC (g/L)	EtG (µg/mL)	$\Delta t_{accident\text{-}blood\ collection}$ (hh:mm)
650	1.35	10.76	0:53
651	2.54	11.30	1:19
652	< 0.10	< 0.10	0:39
653	< 0.10	< 0.10	2:50
654	< 0.10	< 0.10	2:19
655	< 0.10	< 0.10	2:30
656	< 0.10	< 0.10	1:57
657	< 0.10	< 0.10	1:40
658	< 0.10	< 0.10	1:40
659	< 0.10	< 0.10	0:29
660	1.19	4.46	0:55
661	< 0.10	< 0.10	1:40
662	1.61	1.83	0:50
663	< 0.10	< 0.10	0:50
664	< 0.10	< 0.10	0:48
665	0.46	1.30	3:30
666	< 0.10	< 0.10	1:20
667	< 0.10	< 0.10	4:27
668	< 0.10	< 0.10	1:47
669	< 0.10	< 0.10	2:45
670	0.98	2.93	3:40
671	1.18	3.49	1:10
672	1.19	2.05	0:29
672	1.19	2.05	0:29
673	0.35	1.10	1:47
674	0.20	1.30	1:40
675	0.18	1.82	3:26
676	0.25	0.82	2:00
677	1.45	5.00	3:45
678	1.56	4.09	1:35

Appendix 2

In the present appendix the results of a research study of EtG in hair are summarised.

The aim of this applicative study has been to evaluate the association of hEtG (hair EtG) with the occurrence of alcohol-related fatal traffic accidents.

Materials and method

Chemicals and reagents

Ethylglucuronide and internal standard pentadeuterated–EtG (EtG-D_5) were obtained from Medichem Diagnostica GmbH (Steinenbronn, Germany). The two standards were reconstituted with water obtaining a final concentration of 2 mg/mL. The water used was purified by filtering deionised water on a Millipore Ultra-Pure Water system. HPLC-grade methanol, dichloromethane, ethyl acetate, acetone and the derivatising agent, pentafluoropropionic acid anhydride (PFPA), were obtained from Sigma Aldrich (Steinheim, Germany) while, ammonia 30% from Carlo Erba (Milano, Italy). Nitrogen, methane and helium were from Air Liquide (Paris, France). The Oasis MAX SPE polymeric columns were purchased from Waters, (Milford, MA, USA)

Instrumentation

Analysis were performed with GC-MS/MS in negative chemical ionisation by GCsampler 120 - GC7890A – 7000 QqQ from Agilent (Santa Clara, CA, USA) with methane as reagent gas. The column used was ZB-5MS (30 m x 0.25 mm x 0.25 µm) (Phenomenex, Torrance, CA, USA).
The product ion transitions monitored are: EtG *347→163* CE 5 eV, *347→119* CE 10 eV; EtG-d_5 *352→163* CE 5 eV.
The method had been previously validated showing a linearity in the range 3-200 pg/mg; LOD 2 pg/mg; LOQ 4 pg/mg. A cut-off at 7-30 pg/mg was set according to the Society of Hair Testing (SoHT) [44].

Biosamples

Blank hair samples used for calibration were obtained from abstinent volunteers from this laboratory. Real samples were from dead people involved in traffic accidents and who had undergone post-mortem examination. The main exclusion criteria applied to the samples was the presence of psychoactive drugs.

Sample pre-treatment

The traditional extraction procedure from the hair matrix was based on a simple aqueous elution; in fact, after the sonication in water, the surnatant were dried under air flow and then derivatised. The temperature should not be above 40 °C, because of the thermolability of the analyte. This procedure is time consuming because of the drying step and, many impurities, present in the samples, remained in the final extract. The presence of these interferents influenced the quantitation of the analyte since the retention times of interferences were very close and in some cases co-eluted with EtG; hence, the quantitation was not possible. Later, a wash procedure of hair was introduced, using water and acetone and maintaining the water incubation procedure but, it proved not effective. Therefore, solid phase extraction (SPE) was introduced for sample extract clean up [31,45]. The SPE procedure was based on the use of polymeric Oasis MAX SPE columns that allowed to successfully remove the interferents, present in the samples (Figure 1), and to make the procedure less time consuming.

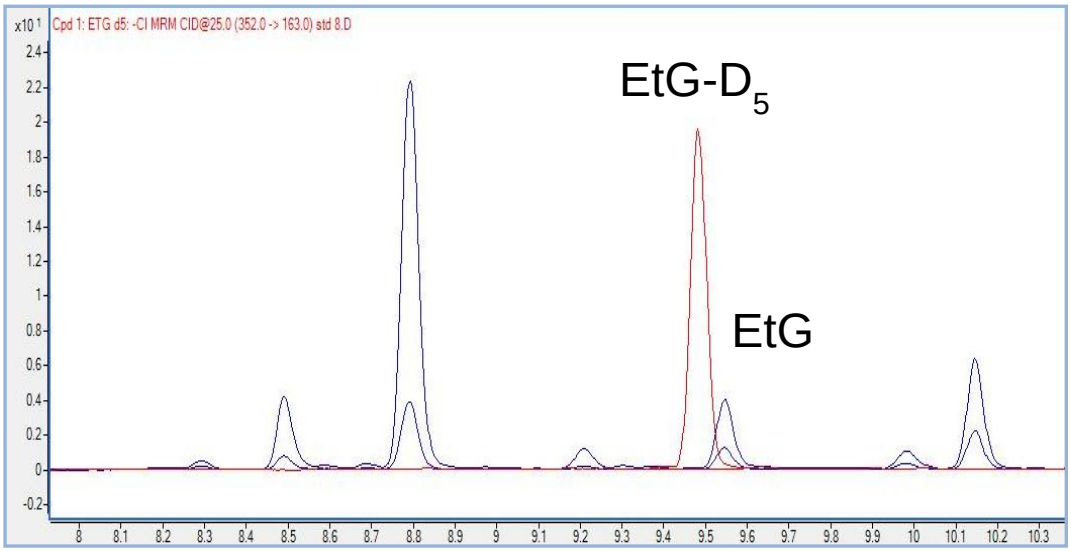

Figure 1. Chromatogram of a sample after SPE purification.

The definitive procedure is reported below.

Hair samples were washed with CH_2Cl_2 and methanol and cut into small pieces. The IS (20 µL of EtG-d_5 0.4 µg/mL) and 700 µL of milli-Q water were added to 50 mg of the hair sample. The aqueous extraction was performed using an ultrasonic incubation for 2 hours. After centrifugation (3500 rpm for 10 minutes), the supernatant was applied to a polymeric SPE cartridge (Oasis MAX) with anion-exchange groups, conditioned with 2 mL of methanol and 2 mL of deionised water. The cartridge was washed with 1 mL of water/ammonia solution (95:5, *v/v*) and 2 mL of methanol and then dried for 10 minutes. EtG was eluted from the column using 2 mL of a methanol/formic acid solution (98:2, *v/v*). The eluate was dried under air flow at 40 °C. The obtained residue was derivatised with 100 µL of PFPA at 80 °C for 30 minutes. Then, it was dried under air flow, reconstituted in 50 µL of ethyl acetate and then 2 µL were injected in GC-QqQ system.

Analytical conditions

The method was also improved, from an instrumental regulation point of view, refining the collision energies (CE) (EtG *m/z 347→163* CE 5 eV, *m/z 347→119* CE 10 eV; EtG-d_5 *m/z 352→163* CE 5 eV). The method was then validated in

terms of selectivity (ten blank samples were analysed to verify the absence of interfering substances that could co-elute with the interested analytes), sensitivity (LOD 2 pg/mg, LOQ 4 pg/mg) and linearity (range 3-200 pg/mg; 0.0078 – 0.5 µg/mL).

Results and discussion

Since 2009 SoHT has proposed the determination of EtG in hair to investigate past excessive/chronic alcohol consumption. On this basis, EtG in hair has been used to verify the suitability for the re-issuing of the driving license after its confiscation for drunk driving [46].

The study has been carried out on 90 subjects (M/F: 79% M, 21% F; age range 17-87 years), who died in traffic accidents and underwent post-mortem examination in the years 2012-2015 in the Verona district.
The hETG cut-off values proposed by SoHT [44] and adopted for the interpretation of the results, are illustrated in Table 1.

Table 1. Hair EtG cut-off values.

EtG < 7 pg/mg	Abstinence
7 pg/mg < EtG < 30 pg/mg	Social alcohol consumption
EtG > 30 pg/mg	Chronic/excessive alcohol consumption

In order to have a homogeneous group of samples some exclusion criteria were adopted.

The exclusion criteria were:

- presence of psychoactive drugs (n = 20) (the accident could have been caused by psychoactive drugs and not by alcohol assumption);
- passengers and pedestrians (n = 4) (they didn't cause the accident);

- pubic hair samples (n = 9) (it is a type of sample of difficult interpretation);
- subjects showing cosmetic hair treatment (n = 3) (in literature there are some articles that explain EtG decrease after hair treatments).

With these conditions the number of samples was reduced from 90 to 54.

The samples tested were divided into two groups based on BAC:

- group A (n = 31; age: mean value 40 years, range 17-87 years; M/F: 65% M, 35% F) with BAC ≤ 0.5 g/L (the legal limit);
- group B (n = 23; age: mean value 36 years, range 17-73 years; M/F: 78% M, 22% F; BAC range: 0.58-2.58 g/L) with BAC > 0.5 g/L.

The obtained results for the two groups were identified in two ranges: 4-183 pg/mg (mean value 16 pg/mg) for the former and 4-339 pg/mg (mean value 94 pg/mg) for the latter. The results are summarised in Table 2.

Table 2. Data of group A and B.

Group A (n = 31)	**Group B (n = 23)**
BAC ≤ 0.5 g/L	**BAC > 0.5 g/L**
Age: mean value 40 years range 17 – 87 years M/F: 65% M, 35% F	Age: mean value 36 years range 17 – 73 years M/F: 78% M, 22% F BAC range: 0.58 – 2.58 g/L
3 < hEtG < 183 pg/mg Mean value 16 pg/mg	4 < hEtG < 399 pg/mg Mean value 94 pg/mg

On the interpretation side, the obtained results were evaluated using the cut-off values proposed by SoHT.

By using a cut-off of 7 pg/mg, among the subjects having BAC ≤ 0.5 g/L, 8 resulted positive (EtG > 7 pg/mg) and 23 negative (EtG ≤ 7 pg/mg). Instead, in the group with BAC > 0.5 g/L, 16 resulted positive and 7 negative (Table 3).

Table 3. Data analysed with the cut-off of 7 pg/mg.

Subjects	**BAC ≤ 0.5 g/L**	**BAC > 0.5 g/L**	**Total**
EtG ≤ 7 pg/mg	23	7	30
EtG > 7 pg/mg	8	16	24
Total	31	23	54

A non-parametric statistical evaluation using the χ^2 resulted 8.544 with $p < 0.003$. Therefore, the data were statistically significant and the odds ratio was 6.6 with p-value < 0.002 (Fisher's exact test) (Table 4).

Table 4. Odds ratio (cut-off 7 pg/mg).

BAC ≤ 0.5 g/L	*Odds = 8/23= 0.35*	Odds ratio 6.6 *p-value* : 0.002 (Fisher's exact test)
BAC > 0.5 g/L	*Odds =16/7 = 2.29*	

Likewise, the tests were repeated by using a cut-off of 30 pg/mg. Among the subjects having BAC ≤ 0.5 g/L, only 4 samples resulted positive (EtG > 30 pg/mg) and 27 negative (EtG ≤ 30 pg/mg). Instead, in the group with BAC > 0.5 g/L, 12 resulted positive and 11 negative (Table 5).

Table 5. Data analysed with the cut-off of 30 pg/mg.

Subjects	**BAC ≤ 0.5 g/L**	**BAC > 0.5 g/L**	**Total**
EtG ≤ 30 pg/mg	27	11	38
EtG > 30 pg/mg	4	12	16
Total	31	23	54

The value of χ^2 was 7.973 with $p < 0.005$. Therefore, the data were statistically significant and the odds ratio was 7.4 with p-value < 0.003 (Fisher's exact test) (Table 6).

Table 6. Odds ratio (cut-off 30 pg/mg).

BAC ≤ 0.5 g/L	*Odds = 4/27 = 0.15*	Odds ratio 7.4 *p-value* : 0.003 (Fisher's exact test)
BAC > 0.5 g/L	*Odds =12/11 = 1.05*	

The obtained data confirm a significant statistical association between elevated hEtG and alcohol-related fatal traffic accidents.

Conclusions and future directions

These findings support strongly the general hypothesis that chronic alcohol abuse is an important risk factor in the occurrence of alcohol-related traffic accidents.

The limited number of cases however precluded a more detailed investigation on a possible correlation between concentration of hEtG and the frequency of alcohol-related traffic deaths.

Abbreviations

EtG Ethylglucuronide

THC tetrahydrocannabinol

EtOH Ethanol

GGT γ-glutamyl transferase

ALT alanine aminotransferase

AST aspartate aminotransferase

MCV mean corpuscular volume

CDT carbohydrate-deficient transferring

PEth phosphatidylethanol

FAEEs fatty acid ethyl esters

EtS ethylsulfate

Tf transferrin

LC-MS/MS liquid chromatography coupled to mass spectrometry

UDP-GT UDP-glucuronyl transferase

DUI driving under influence

BAC blood alcohol concentration

WHO World Health Organisation

CNS Central Nervous System

HS-GC head space gascromatography

FID flame ionization detection

S/N signal to noise

IS internal standard

GC-MS/MS gas chromatography coupled to mass spectrometry

GC-NCI-MS/MS gas chromatography coupled to mass spectrometry with negative chemical ionisation

EtG-D_5 pentadeuterated-ethylglucuronide

SoHT Society of Hair Testing

QqQ triple quadrupole

LOD limit of detection

LOQ limit of quantification

CE collision energy

SPE solid phase extraction

CH_2Cl_2 dichloromethane

ACN acetonitrile

PFPA pentafluoropropionic acid

HCOOH formic acid

LC-ESI-QqQ MS liquid chromatography coupled to mass spectrometry (triple quadrupole) with electrospary ionisation

QC quality control

RE relative error

hEtG hair EtG

$\chi 2$ chi value

p value Fisher's exact test

S/B serum/blood concentration ratio

$\Delta t_{accident\text{-}blood\ collection}$ time between traffic accident and blood collection