UNIVERSITA' DEGLI STUDI DI VERONA

DEPARTMENT OF

Diagnostics and Public Health

DOCTORAL PROGRAM IN

Nanoscience and Advanced Technologies

WITH THE FINANCIAL CONTRIBUTION OF

University of Verona

Cycle 38 - 2022/2023

TITLE OF THE DOCTORAL THESIS

"Study on Blood Ethanol Stability in the Context of Driving under influence of Alcohol Investigations"

S.S.D. MED/43

Doctoral Student: Laura Al Chaer

Coordinator: Prof. Adolfo Speghini

Tutor: Prof. Rossella Gottardo

UNIONE EUROPEA
Fondo Sociale Europeo
Ministero dell'Università e della Ricerca
PON RICERCA E INNOVAZIONE 2014 - 2020
REACT EU

La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020, risorse FSE REACT-EU
Azione IV.4 “Dottorati e contratti di ricerca su tematiche dell’innovazione”
e Azione IV.5 “Dottorati su tematiche Green”

ACKNOWLEDGMENTS

This research was conducted during three years of hard work. In the end of this cycle, I am deeply grateful for the opportunity that was presented to me in order to complete this work.

Special thanks to my professors for accompanying me on this journey and for proofreading this manuscript.

Last but not the least, I want to thank all the committee members at the University of Verona for taking the time to read this thesis and for making this work happen.

ABSTRACT

Driving under the influence of alcohol is a major public health and legal issue, as a substantial proportion of traffic accidents are associated with alcohol consumption. In Italy, Articles 186 and 186-bis of the Highway Code establish legal thresholds for blood alcohol concentration (BAC) and impose sanctions according to specific limits (0 g/L, 0.5 g/L, 0.8 g/L, and 1.5 g/L) based on driver categories. Following ethanol determination in such cases, blood samples are typically stored at −20 °C for at least one year to allow potential counter-analysis requested by judicial authorities or the individuals involved. However, because ethanol is volatile and susceptible to microbial activity after collection, its concentration may change over time, potentially affecting forensic interpretations—especially when initial results are near legal thresholds.

This study evaluated the long-term stability of ethanol in blood samples collected under Articles 186 and 186-bis, stored at −20 °C for up to 30 months, and re-analyzed to verify analyte preservation and analytical reliability. A total of 780 blood samples collected between 2022 and 2023, each initially testing positive for ethanol (BAC > 0.05 g/L), were re-analyzed in 2024 using a validated Headspace Gas Chromatography–Flame Ionization Detection (HS-GC-FID) method. The limits of detection and quantification were 0.01 g/L and 0.05 g/L, respectively, and a ±10% analytical tolerance was applied. Results showed that approximately 60% of samples remained within this tolerance range, while 24% exhibited moderate decreases and 16% showed increases, mostly within the 10–20% range. A two-factor ANOVA indicated a significant effect of storage duration ($p<0.05$) but no significant influence of vacutainer type ($p>0.05$), demonstrating comparable performance between sodium fluoride and EDTA-based tubes. Overall, these findings confirm that ethanol concentrations remain stable for up to 30 months when samples are properly sealed and stored at −20°C, ensuring analytical reliability and legal defensibility in forensic counter-analyses.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

1. **ADH:** Alcohol Dehydrogenase.
2. **ALDH:** Aldehyde Dehydrogenase.
3. **ANOVA**: Analysis of variance.
4. **AUC:** Area Under Concentration.
5. **BAC:** Blood Alcohol Concentration.
6. **CNS:** Central Nervous System.
7. **DUI**: Driving Under the Influence.
8. **EDTA:** Ethylene-Diamine Tetra-acetic Acid.
9. **EtG**: ethyl glucuronide.
10. **EtOH:** Ethanol.
11. **FID:** Flame Ionization Detector.
12. **HS-GC:** Headspace Gas Chromatography.
13. **IS:** Internal Standard.
14. **GI:** Gastrointestinal tract.
15. **LOQ:** Limit of Quantification.
16. **LOD:** Limit of Detection.
17. **MSB:** Mean Square Between Groups.
18. **MSW:** Mean Square Within Groups.
19. **RT**: Retention Time.
20. **Vd:** Volume of distribution.
21. **TBW:** Total Body Water

1. Introduction

Alcohol consumption is deeply embedded in social life and cultural practices, yet it remains a major contributor to preventable morbidity and mortality. Ethanol, the psychoactive substance responsible for alcohol's effects, depresses the central nervous system and alters perception, coordination, and reaction time. These impairments have important consequences for road safety: alcohol use is consistently associated with elevated crash risk, severe injuries, and fatalities. As a result, driving under the influence (DUI) has long been recognised as both a public health challenge and a legal issue requiring clear regulation and reliable enforcement.

In Italy, legal responsibility for impaired driving is determined by blood alcohol concentration (BAC) thresholds established in the Highway Code. These thresholds differentiate administrative from criminal offences and vary according to driver category. Since even small differences in measured BAC can alter legal outcomes, forensic analyses must be precise, reproducible, and defensible in court. Beyond the initial determination, archived samples are often re-analysed months or years later at the request of judicial authorities, which places additional emphasis on long-term sample stability.

Despite the widespread use of preservatives and freezing, ethanol is a volatile compound that can undergo evaporation, oxidation, or microbial transformation after collection. Such processes may modify BAC values over time, particularly when concentrations lie near legal cut-offs. Ensuring that stored blood samples reliably reflect ethanol levels at the time of collection is therefore essential to guarantee fairness and accuracy in legal proceedings.

Forensic toxicology has consequently devoted significant attention to the preservation and analysis of ethanol in biological matrices. Headspace gas chromatography with flame

ionisation detection (HS-GC-FID) remains the analytical gold standard, offering both sensitivity and robustness. However, real-world laboratory practices—including differences in storage duration, container type, and handling procedures—may introduce variability that does not necessarily emerge under controlled experimental conditions. Understanding how these factors influence ethanol stability over time is crucial for interpreting BAC results in retrospective analyses.

The structure of this thesis reflects a logical progression from fundamental biochemical concepts to applied forensic investigation and interpretation. The first two chapters introduce the scientific basis of the research by describing ethanol's molecular characteristics, pharmacokinetics, and the clinical manifestations associated with acute intoxication, thereby contextualising its effects on human performance and driving ability. The third chapter shifts toward a forensic perspective, examining the role of ethanol testing in medico-legal practice, the biological matrices used for analysis, and the analytical and regulatory frameworks that govern DUI investigations. Building on this foundation, the fourth chapter reviews current knowledge regarding ethanol stability in stored samples and highlights the practical challenges posed by sample preservation, providing the rationale for the experimental investigation. Subsequent sections detail the materials and methods employed in analysing a large cohort of blood samples collected during DUI investigations, followed by the presentation of results concerning the effects of storage duration and vacutainer type on ethanol stability. The thesis concludes with a critical discussion of these findings in relation to forensic reliability and legal interpretation, and it closes by identifying potential avenues for improving analytical practice and guiding future research.

2. Ethyl Alcohol

2.1 Molecular structure and Pharmacokinetics

Ethanol—also known as ethyl alcohol (EtOH), grain alcohol, or drinking alcohol—is an organic compound with the molecular formula C_2H_6O (Figure 1). It is a volatile, flammable, and colorless liquid characterized by a distinctive pungent odor and taste [1]. As a psychoactive central nervous system (CNS) depressant, ethanol is the primary active ingredient in alcoholic beverages and is the second most widely consumed psychoactive substance worldwide, following caffeine [2]. It remains one of the most historically and culturally pervasive mind-altering compounds.

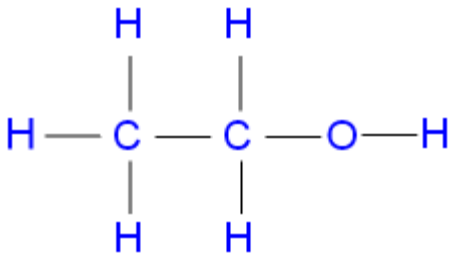

Figure 1: Molecular structure of ethyl alcohol.

Ethanol occurs naturally through the fermentation of sugars by yeast and can also be produced industrially by hydrating ethylene [3]. Historically, ethanol was used as a general anesthetic and continues to serve as an antiseptic, disinfectant, solvent, and antidote for methanol and ethylene glycol poisoning [4]. Beyond its medical uses, ethanol functions as a versatile industrial solvent, a precursor for organic synthesis, and a renewable fuel for lamps, stoves, and internal-combustion engines. It may also be dehydrated to yield ethylene, an important industrial feedstock [5].

The term ethanol derives from the ethyl radical first described in 1834 and was formally adopted in 1892 [6]. The word alcohol, now applied to a class of organic compounds containing a hydroxyl group, originally referred to a fine cosmetic powder before evolving to denote ethanol specifically [6]. Ethanol is naturally present in trace amounts as a by-product of yeast metabolism in fruits, palm blossoms, and germinating plants under anaerobic conditions, and it has even been detected in interstellar space [7].

Because of its long history of use, ethanol has been independently discovered and distilled by numerous civilizations. Its chemical identification and synthetic production in the nineteenth century marked the beginning of its modern scientific and industrial importance [8].

Ethanol can enter the body through oral ingestion, inhalation of vapor, or transdermal absorption from topical formulations (Figure 2) [9]. After oral intake, ethanol passes through the stomach and small intestine and is absorbed primarily by passive diffusion across the gastrointestinal (GI) mucosa [10]. Roughly 80% of absorption occurs in the small intestine and 20 percent in the stomach [11]. Under normal conditions, absorption is complete within 60–90 minutes after ingestion. The rate depends largely on gastric emptying, which governs transfer of stomach contents to the small intestine through the pyloric sphincter [12]. Factors such as ethanol concentration, gastric content, body composition, age, and metabolic rate also influence absorption [13].

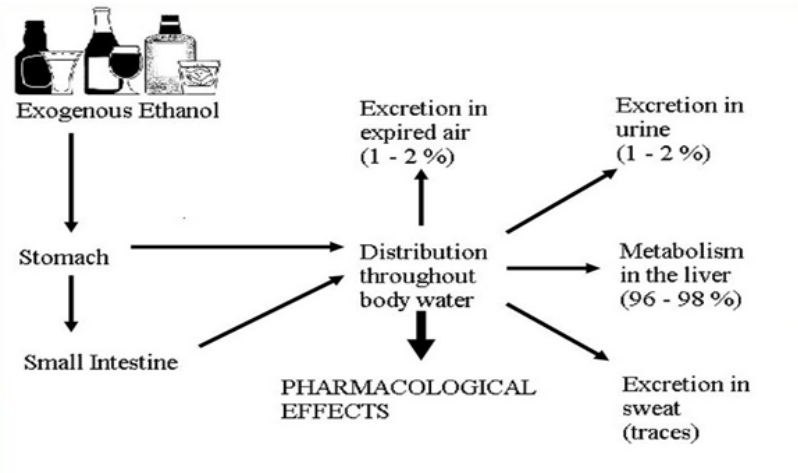

Figure 2: Scheme representing the route of alcohol in the body after intake [9].

After entering the portal circulation, ethanol undergoes first-pass metabolism in the liver before reaching systemic circulation. Up to 20% of ingested ethanol may be metabolized during this process [11]. Alcohol dehydrogenase (ADH) activity in the gastric mucosa suggests that part of this metabolism occurs in the stomach itself [11].

Once in systemic circulation, ethanol distributes throughout total body water and diffuses into most tissues, including the brain and skeletal muscle. Because it is a small hydrophilic molecule, its volume of distribution (Vd) correlates with total body water (TBW) [11]. Average Vd values are approximately 0.7 L/kg in men and 0.6 L/kg in women [14].

Ethanol is eliminated primarily by hepatic oxidation (90–98 percent of total clearance) [15]. Minor pathways include conjugation with glucuronic acid or sulfate and excretion of unchanged ethanol in urine, breath, and sweat [16]. The main enzyme system, alcohol dehydrogenase (ADH), located in hepatocyte cytosol, metabolizes roughly 85 percent of ethanol [17]. A secondary system, cytochrome P450 2E1 (CYP2E1), is inducible with chronic alcohol use and contributes to metabolic tolerance. Reported elimination rates range between 0.10 and 0.25 g/L per hour [18].

Gastric emptying is the most critical determinant of ethanol absorption from the GI tract into systemic circulation [19]. Although its exact mechanism remains under investigation, two primary hypotheses have been proposed. One suggests that gastric ADH metabolizes a fraction of ethanol prior to absorption into the portal system [19]. The other posits that delayed gastric emptying slows the delivery of ethanol to the liver, thereby prolonging exposure to first-pass metabolism. Due to the saturable kinetics of ADH, slower gastric emptying enhances the fraction of ethanol metabolized before systemic absorption [20].

The presence of food in the stomach significantly reduces the rate and extent of ethanol absorption. Co-ingestion of alcohol with food slows gastric emptying, thereby lowering the peak blood ethanol concentration (C-max), delaying the time to peak concentration (Tmax), and reducing the area under the concentration–time curve (AUC) [21,22].

Recent studies have examined the impact of low-calorie mixers on ethanol pharmacokinetics. Consumption of alcoholic beverages prepared with non-caloric sodas results in higher blood ethanol concentrations compared to those mixed with sucrose-sweetened beverages, an effect attributed to faster gastric emptying [23]. Notably, existing studies have primarily evaluated artificial sweeteners such as cyclamate and aspartame; to date, no studies have investigated natural low-calorie sweeteners such as “steviol glycosides” in this context.

Because the liver is the principal site of ethanol elimination, hepatic blood flow directly affects clearance. Ingestion of a caloric meal can increase portal venous blood flow by 52–107%, thereby enhancing hepatic metabolism and reducing blood ethanol concentrations when alcohol is consumed with food [24].

Ethanol metabolism occurs primarily in the liver via sequential oxidation reactions. In the first step, ADH catalyzes the conversion of ethanol to acetaldehyde, which is subsequently oxidized to acetic acid by aldehyde dehydrogenase (ALDH). Acetic acid then enters the Krebs cycle and is ultimately metabolized to carbon dioxide and water [13]. The average rate of decrease in blood alcohol concentration (BAC) is approximately 0.15 g/L per hour, though individual variability is substantial (Figure 3).

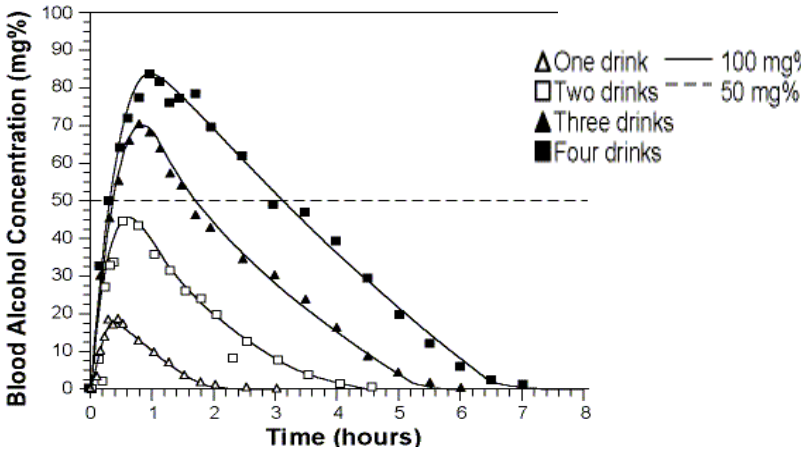

Figure 3: Widmark's curve used to determine the elimination rate of alcohol from blood by fitting a straight line to selected concentration-time points on the declining portions of the blood alcohol concentration (BAC) curve and then extrapolating back to the time of starting to drink [25].

At higher BAC levels, ethanol elimination follows zero-order kinetics, meaning the rate of metabolism remains constant and independent of concentration—a process governed by the saturation of enzymatic capacity, analogous to the Michaelis–Menten model. A small fraction of ethanol is excreted unchanged via breath (≈0.7%), urine (≈0.3%), and sweat (≈0.1%) [26]. With chronic or frequent consumption, the CYP2E1 pathway becomes increasingly active, enhancing ethanol clearance and contributing to metabolic tolerance [27].

2.2 Acute and chronic alcohol intoxication: clinical and diagnostic aspects

Despite its widespread consumption, ethanol is consistently associated with a range of adverse health and social outcomes, including increased risks of homicide, suicide, occupational injuries, motor vehicle collisions, drowning, and other forms of violent or accidental death [28].

As a central nervous system (CNS) depressant, ethanol exerts diverse behavioral and physiological effects that depend on both dose and individual susceptibility [29].

At low concentrations, it typically produces euphoria, relaxation, and mild stimulation; however, higher concentrations result in progressive CNS depression, manifesting as sedation, slurred speech, disinhibited behavior, memory impairment, aggression, and deficits in sensory and motor coordination [30].

Acute ethanol intoxication arises when alcohol intake exceeds the rate of metabolic clearance, leading to a spectrum of clinical manifestations ranging from mild euphoria to life-threatening complications such as coma and death.

Symptoms are dose-dependent and correlate with increasing blood alcohol concentration (BAC) (a term used to describe a forensic analysis performed to quantify the amount of ethanol present in the blood of a subject), including confusion, impaired coordination, vomiting, respiratory depression, hypothermia, and reduced levels of consciousness [31], as detailed below:

- <0.5 g/L: often no symptoms or mild euphoria and relaxation;
- 0.5-1 g/L: euphoria, sedation, prolonged reaction time, and mild psychomotor impairment;
- 1–2 g/L: impaired motor coordination, slurred speech, and gait instability;

- 2–3 g/L: nausea, vomiting, hypothermia, and stupor;
- >4 g/L: respiratory depression, coma, and potentially fatal outcomes.

Additional signs may include ataxia, confusion, seizures, pallor or cyanosis, hypothermia, and reduced responsiveness [31].

Diagnosis is primarily clinical and supported by the measurement of BAC through blood or breath analysis.

Management is largely supportive, as no specific antidote exists for ethanol toxicity. Treatment focuses on maintaining airway patency and cardiorespiratory stability, correcting dehydration and electrolyte imbalances with intravenous fluids, managing hypoglycemia with glucose administration, and addressing hypothermia through active rewarming. In cases of severe respiratory depression, airway protection via endotracheal intubation may be required [31].

Chronic or excessive ethanol exposure contributes to significant organ damage, particularly hepatic cirrhosis, and is associated with an elevated risk of malignancy. It may also cause irritation of the skin, eyes, and respiratory tract. At toxic concentrations, ethanol intoxication can progress to confusion, stupor, coma, and ultimately, death [32].

Ethanol analysis plays a pivotal role in forensic investigations, particularly because of its involvement in criminal acts, traffic or occupational accidents, and suicides [33].

As a potent central nervous system (CNS) depressant, ethanol produces psychoactive effects including impaired motor coordination, delayed reaction time, reduced judgment, and behavioral disinhibition. These effects often contribute to uncontrolled or hazardous behaviors that may culminate in accidents, injuries, or fatalities.

In cases involving death or serious injury, the interpretation of toxicological findings—notably blood alcohol concentration (BAC)—is essential. Such interpretation carries significant legal and financial implications, as the presence of ethanol may influence determinations of liability, insurance indemnification, and criminal responsibility. For example, in vehicle accidents, BAC results may distinguish between accidental and culpable homicide or determine the extent of compensation under insurance policies. In some jurisdictions, the identification of alcohol intoxication may reduce or nullify insurance claims, underscoring the medico-legal importance of accurate ethanol quantification [34].

Therefore, forensic autopsies must be complemented by comprehensive toxicological analyses of biological specimens, particularly those aimed at detecting ethanol and other psychoactive substances. The assessment of ethanol levels aids in reconstructing the victim's psychological and psychomotor state prior to the accident, providing critical context for forensic interpretation and judicial decision-making.

As mentioned earlier, Blood Alcohol Concentration (BAC), is the percentage of alcohol in a person's bloodstream, commonly used to measure the level of intoxication. It serves as the principal quantitative indicator of ethanol intoxication and remains one of the most frequently performed tests in forensic toxicology laboratories [35].

Applications of BAC analysis include:

- Human performance toxicology: Measurement of ethanol in blood, breath, or urine to assess impairment at the time of an incident, such as in cases of driving under the influence (DUI) or drug-facilitated crimes [36].

- Postmortem toxicology: Evaluation of ethanol in biological fluids and tissues after death to determine ante-mortem consumption and to differentiate it from postmortem ethanol production [36].

Ethanol distributes uniformly throughout total body water, permitting its detection in nearly all body fluids and tissues.

The most commonly analyzed biological matrices include:

1. Blood: the primary specimen for ethanol quantification, reflecting concentrations that influence CNS activity.
2. Breath: breath analysis, based on infrared spectrometry or electrochemical oxidation, is widely employed for roadside and workplace testing of suspected intoxication [36].
3. Urine: useful in both ante- and postmortem cases, this biological sample provides insight into prior alcohol exposure and is less susceptible to microbial contamination.
4. Vitreous humor: a metabolically stable ocular fluid valuable in postmortem analysis, offering reliable evidence of ante-mortem alcohol intake.
5. Hair: it provides a retrospective index of chronic alcohol consumption, typically via detection of ethyl glucuronide (EtG), a direct ethanol metabolite.

Historically, BAC was estimated using chemical oxidation techniques, which have largely been replaced by enzymatic reactions measure via spectrophotometry and chromatographic methods.

Chemical oxidation methods were common in the late 19th century but were non-specific, yielding false results in the presence of other substances like acetone. Enzymatic methods became more common in the mid-20th century and are still used in clinical settings [37].

Currently, headspace gas chromatography (HS-GC) represents the gold standard for quantitative determination of ethanol and other volatile compounds in blood, owing to its high precision, sensitivity, and reproducibility [38].

This methodology remains the preferred analytical approach in both forensic and clinical toxicology laboratories.

Headspace gas chromatography (HS-GC) is an analytical technique used for the analysis of the concentrations of many organic volatile compounds, one of them being ethanol.

The popularity of this technique has grown and has gained worldwide acceptance for analyses of alcohols in the blood [39].

HS-GC relies on the headspace principle where only an aliquot of the vapor in the headspace above a water solution containing alcohol, will be delivered to the GC system. The sample will be introduced into a hermetically sealed vial and then heated in an oven at a given temperature and time, till it reaches an equilibrium between ethanol in solution and ethanol in the vapor phase (figure 4). Once the equilibrium is reached, an aliquot will be taken from the headspace using a gas-tight syringe and the sample will then be introduced into the GC for analysis [40].

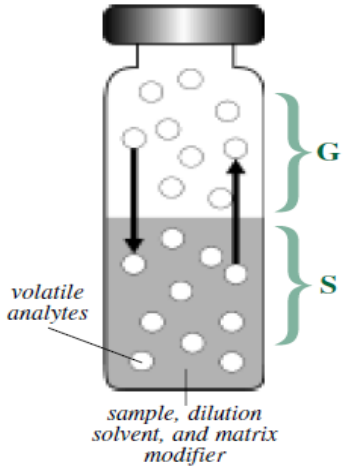

Figure 4: The distinct phases present in a headspace vial. The gaseous phase (G) represents the headspace region above the condensed sample, whereas the sample phase (S) contains the target analytes, generally in liquid or solid form and frequently mixed with a dilution solvent or matrix modifier. After the sample is introduced and the vial is hermetically sealed, volatile compounds undergo partitioning between the two phases. Equilibrium is achieved when the concentration of volatile components in the gaseous and condensed phases remains constant, as illustrated by the bidirectional arrows. Sampling for analysis is performed from the gaseous headspace phase in vapor form [41].

The chromatographic separation used for ethanol analysis is capillary GC which uses a carrier gas (nitrogen, hydrogen or helium), which acts as the mobile phase. The sample will enter the GC system through an injector and then transported by the mobile phase through the separation column towards the detector.

The chromatographic column is a long thin spiral capillary tube, in which a stationary liquid phase is present as a thin film on an absorbent inert material (the capillary wall), thus providing a large stationary phase.

The choice of the stationary phase is a key decision to ensure that the volatile components in the sample are separated from each other within the column.

The GC column is heated in an oven which can be programmed to a certain temperature. According to the physical and chemical properties of the substances, such as the boiling point, the solubility in water with respect to lipids and the polarity, they are divided between

the stationary phase and the mobile phase. Some substances are retained in the column for shorter or longer times, then elute in a defined timed order from when injected, known as retention time (RT). The RT of the analyte is the parameter used for the qualitative determination, assuming that it is different from the RT's of the other substances present in the injection mixture (figure 5).

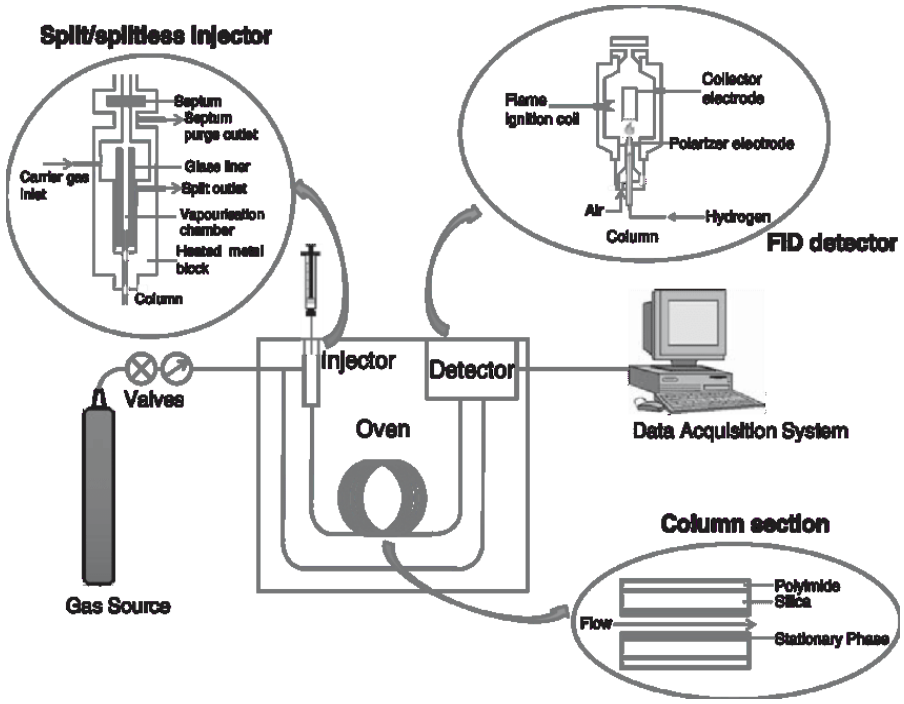

Figure 5: HS-GC-FID instrumentation scheme. After sampling from the headspace of the sample vial, the sample will enter the injector and will then be carried through the column located in the oven by a carrier gas and finally burnt ad analyzed by the FID detector [42]

In practice, under proper chromatographic conditions, complete or partial separation of volatile components occurs within the column before they reach the flame ionization detector (FID) detector, which is fitted with a burning air-hydrogen flame.

The combustion produced ions and electrons. In particular, the ion CHO^+, which is produced in the combustion process involving the analyte, hydrogen and oxygen, interacts with a plate to which is applied a negative voltage (a positive voltage is applied to the flame nozzle) and generates an electric signal which is amplified and recorded in function of the

time elapsed from the start of analysis as a series of “peaks” appearing on the chromatogram.

The FID is almost ideal for analyzing carbon-based molecules having also the great advantage to not be affect by the content of water in the sample. Also, it has excellent linearity over a wide concentration range and signal intensity is directly proportional to the amount of substance in the sample.

Applied to ethanol determination, this method provides a fast and clean analysis. The advantages of using the vapor in the headspace (avoiding the injection of a sample as a liquid) is that only the compounds that are volatile (such as ethanol and other small non-ionic molecules) will be analyzed while all other potential interferences will remain in the sample phase.

Coupling gas phase injection, gas chromatographic separation and a flame ionization detector, this method has proved adequate sensitivity and specificity for detecting many organic volatile compounds in biofluids [43].

In terms of quantification of the analytes, the modern approach is based on the use of an “internal standard” (IS), a compound chemically similar to the analyte, which is added in a fixed amount to the liquid sample. The relative response of the detector to a series of known ethanol concentrations compared to that of the IS (standard samples) is recorded and then used to construct a calibration curve. This curve (ethanol peak/IS peak areas vs. ethanol concentration in the sample) is then used for calculation by interpolation of the ethanol concentration in the unknown samples [44].

3. Experimental part

3.1 Introduction and aim

Driving under the influence of alcohol (DUI) constitutes a major public health and legal concern, as alcohol consumption is a leading contributor to traffic accidents, injuries, and fatalities.

As said before, ethanol impairs critical psychomotor and cognitive functions—including attention, coordination, and reaction time—thereby compromising driving ability and increasing the risk of accidents [45].

In Italy, DUI is regulated under Articles 186 and 186-bis of the "Codice della Strada" (Highway Code), which define legal blood alcohol concentration (BAC) limits and establish a graduated system of administrative and criminal sanctions according to measured ethanol levels and driver categories (e.g., novice, professional, or public service drivers) [46]. For standard drivers, Article 186 sets a BAC limit of 0.5 g/L.

Exceeding this threshold triggers escalating penalties: BAC 0.5–0.8 g/L incurs administrative fines and driving license suspension (3–6 months); BAC 0.8–1.5 g/L constitutes a criminal offense with arrest up to six months, higher fines, and license suspension of six months to one year; BAC above 1.5 g/L carries more severe criminal sanctions, including arrest up to one year, higher fines, long-term license suspension or revocation, and potential vehicle confiscation.

Article 186-bis imposes stricter rules for high-responsibility drivers—such as newly licensed, professional, or public transport drivers—by enforcing a zero-tolerance limit (0.0 g/L) [47]. In these cases, any detectable ethanol constitutes an infraction, highlighting the heightened duty of care associated with their role.

Given these legal thresholds, the accuracy and stability of BAC determination are critical [48]. Minor deviations in measured ethanol concentration—arising from analytical uncertainty, sample degradation, or post-collection ethanol formation—can materially alter judicial outcomes. For instance, a measured value of 0.78 g/L versus 0.82 g/L may determine whether an incident is treated as an administrative violation or a criminal offense, with corresponding differences in penalties, criminal records, and insurance or employment consequences.

Maintaining the integrity of blood samples is therefore essential.

Standard forensic protocols recommend preserving post-analysis specimens at −20 °C for at least one year, allowing counter-analysis (contro-analisi), if requested, thereby ensuring procedural fairness and verifiable results.

However, ethanol is volatile, and its concentration may change over time due to evaporation, oxidation, or microbial fermentation, particularly if storage conditions are suboptimal. Both decreases and artificial increases in ethanol can occur, introducing interpretative challenges when results approach legal thresholds. The stability of ethanol in blood has been extensively investigated [49-51].

Early chromatographic studies demonstrated gradual ethanol decline in stored specimens, with loss magnitude depending primarily on temperature, storage duration, and preservative use.

Room-temperature storage, even with preservatives, accelerates ethanol loss, whereas refrigeration or freezing markedly slows it.

Sodium fluoride (NaF), typically combined with an anticoagulant, inhibits glycolysis and microbial activity, enhancing short- to medium-term stability [52].

Modern ante-mortem studies indicate that refrigerated or frozen samples remain stable for weeks to months, with losses becoming appreciable only over prolonged storage at 4 °C or

ambient conditions. Postmortem specimens are further complicated by microbial ethanol production during decomposition, making matrix selection (e.g., vitreous humor) and adjunct assays important for accurate interpretation [45].

As mentioned earlier, headspace gas chromatography (HS-GC), with flame ionization detection (FID) is still the preferred method for forensic investigations, minimizing loss and interference.

Forensic guidelines emphasize prompt analysis, use of preserved specimens, careful documentation of storage history (temperature, time, tube openings), and parallel testing of alternative matrices when postmortem production is a concern [52].

Based on the literature, best practices include [52]:

1. Collecting blood into tubes with a metabolic inhibitor (e.g., NaF) and anticoagulant.
2. Refrigerating or freezing specimens if analysis is delayed.
3. Analyzing samples promptly and documenting all handling.
4. Interpreting isolated ethanol results cautiously when decomposition or prolonged ambient storage is involved.

Although previous studies have explored the stability of ethanol in stored blood samples, findings remain highly dependent on storage conditions, preservation methods, duration of storage, and analytical variability. Earlier investigations suggested that ethanol concentrations remain relatively stable when samples are properly preserved and refrigerated, whereas more recent studies have highlighted the potential for minor variability over prolonged storage periods, particularly in the absence of preservatives. Furthermore, research on post-mortem samples has emphasized the possibility of endogenous ethanol formation due to microbial activity, complicating forensic interpretation.

Despite these contributions, limited data are available evaluating ethanol stability over extended storage periods under routine forensic laboratory conditions using contemporary analytical techniques and real-world workflows.

Building on these considerations, the present study, conducted at the Unit of Forensic Medicine, Hospital G.B. Rossi, Verona, Italy, aimed to evaluate long-term ethanol stability in blood samples analyzed for BAC in 2022 and 2023 and subsequently stored at −20 °C for one to two years. The primary goal was to determine whether storage time and preservative type could significantly impact ethanol stability in blood samples and to assess whether analytical results remain robust and legally defensible under real forensic conditions, including the possibility of re-analysis well after the initial determination.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

All chemicals were of analytical reagent grade. Tert-butanol alcohol, used as internal standard, was purchased from Carlo Erba Reagents. Six standard solution for the calibration curve “ethanol control in whole blood” were purchased from ACG Science GmbH.

3.2.2 Sample collection and sample preparation

The study was not submitted for approval to an ethics committee, as such approval was not required. The samples had been collected with informed consent from the individuals for ethanol analysis and were subsequently anonymized prior to their use in this study.
Blood samples from subjects involved in traffic accidents who underwent mandatory BAC determination under Articles 186 and 186-bis of the Highway Code, that were tested positive for ethanol (BAC > 0.05 g/L), in the year 2022 and 2023 and had been in storage under -20°C were retrieved, thawed and re-analyzed.
A total of 780 samples were collected, 394 in the year 2022 and 386 in the year 2023.
The interval between the first and second analysis ranged from 12 to 30 months.

3.2.3 Method

Alcohol measurement was carried out using a Head-Space–Gas Chromatography–flame ionisation detector (HS-GC-FID) method using a Shimadzu GC2030 Head Space GC analyser with a flame ionisation detector (Shimadzu, Kyoto, Japan) equipped with a fused

silica capillary column “TEKNOKROMA MetaBLOOD2” with the following characteristics:

- Length: 30 meters — T max: 350˚C
- Internal Diameter (ID): 0,53 mm — T min: -60˚C
- Film thickness: 2 µm

Samples were prepared by adding 50µL of the sample in a 20 mL Heaspace glass vial (purchased from Agilent Technologies).

Each sample was spiked with 200µL of a prepared solution of the internal standard (IS) Tert-butanol (0,0975 g/L).

The samples were then sealed hermetically with a 20mm chrome isolation cap (purchased from Agilent Technologies) (figure 6) and analyzed under the following conditions:

- Split Temperature: 200˚C — Split Pressure: 22.4.4 kPa
- Flow rate: 0.2 mL/min — Split Ratio: 100
- Column Temperature: 60˚C — Carrier gas: Helium
- Run time: 5 minutes

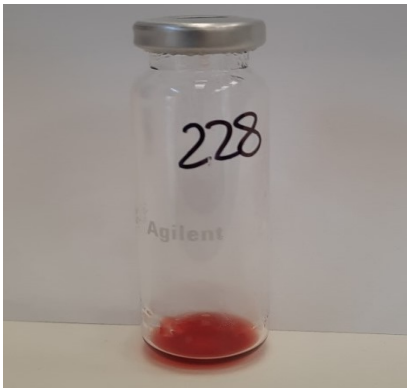

Figure 6: Headspace analysis glass vial containing the solution to be analyzed (sample + IS).

The analytical method employed for the re-analysis was identical to that used in the primary and routine analyses. This method had been previously validated and demonstrated to be suitable for its intended purpose in accordance with established validation parameters.

The calibration range was maintained consistent with that of the initial analysis, which also corresponds to the range routinely applied in standard analytical procedures.

The calibration curve was constructed using six standard concentrations prepared in the same biological matrix as the study samples, specifically: 0.2 g/L, 0.3 g/L, 0.5 g/L, 0.8 g/L, 2.0 g/L, and 3.0 g/L.

Standard calibration curve of ethanol obtained through routine analysis (figure 7) with a correlation coefficients (R2) of: 0.9998997.

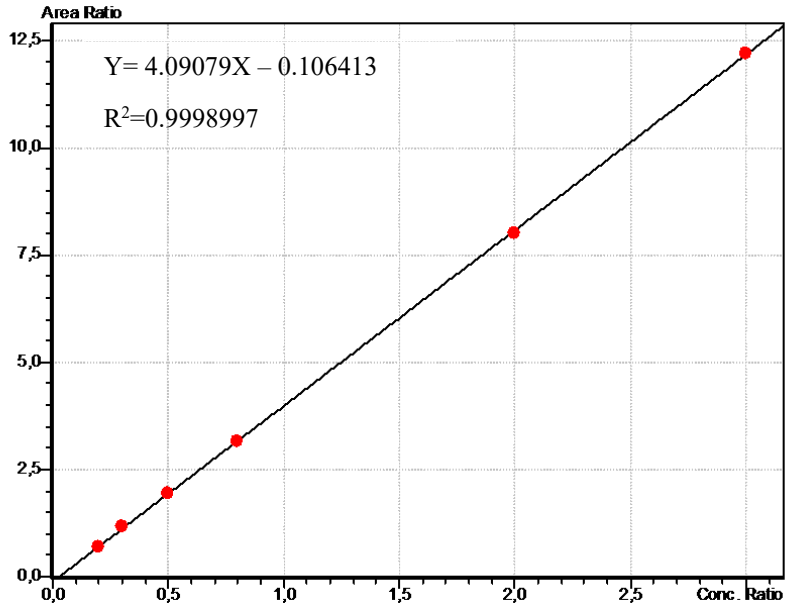

Figure 7: Graph of the constructed calibration curve of ethanol obtained from the pre-prepared six standards in increasing concentrations: 0.2 g/L, 0.3 g/L, 0.5 g/L, 0.8 g/L, 2.0 g/L, and 3.0 g/L.

The method's limit of detection (LOD) and limit of quantification (LOQ) were established at 0.01 g/L and 0.05 g/L, respectively, as determined during the initial method validation. Stability was evaluated by monitoring blood alcohol concentration (BAC) values to the

second decimal place, applying a tolerance range that accounted for an analytical variability of ±10%.

3.3 Results

The analysis by HS-GC-FID of the positive samples collected produced a chromatogram, as shown in Figure 8, typically containing two peaks, one of ethanol and the other one of Tert-Butanol which was used as internal standard as mentioned earlier.

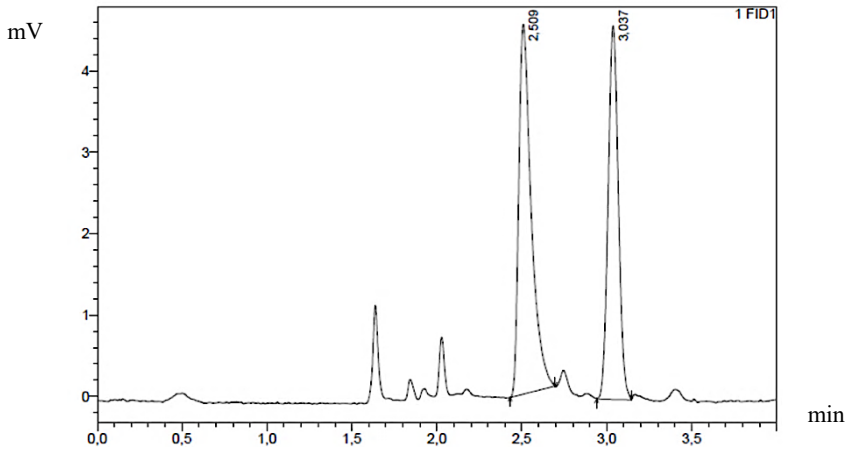

Figure 8: Chromatogram of a positive sample containing ethanol at2.509 min and IS at 3.037 min.

The data from the second determination of BAC were divided into three different groups according to the type of variation they underwent:

- **Group 1**: Samples in which **any variation** in BAC was within the tolerance margin of ±10%.
- **Group 2**: Samples that showed a **reduction** in the initial BAC value, within the range of 10% to 40%.
- **Group 3**: Samples that showed an **increase** in BAC values, within the range of 10% to 40%.

These data were then analyzed considering two factors:

1. **The time interval** between the first and second analysis: samples from the year 2022 were re-analyzed after a time interval that ranged from 30 to 19 months from

the first analysis date, while samples of the year 2023 were analyzed after 18 to 11 months after their first analysis.

2. **The type of vacutainer** in which the blood sample was stored.

Blood specimens are typically collected using evacuated collection tubes, commonly referred to as Vacutainers. These tubes are manufactured with various additives or preservatives designed to stabilize specific analytes and thereby ensure the integrity of subsequent laboratory analyses. Each Vacutainer type is distinguished by the color of its stopper, which denotes the nature of the additive it contains, as summarized in Table 1.

Color	Red (plain)	Red (speckled)	Gold	Light blue	Green (dark)	Green (light)	Lavender	Pink	Gray	Royal blue
Additive	None	Clot activator (silica particles)	Clot activator (silica particles) & Gel separator	Sodium citrate 3.2%	Sodium heparin	Lithium heparin (with or without gel)	Potassium EDTA (K2EDTA)	Potassium EDTA (K2EDTA)	Sodium Fluoride & Potassium Oxalate / Sodium Oxalate	Potassium EDTA (K2EDTA)
Uses	Chemistry panels (after serum separation)	Chemistry panels (after serum separation)	Chemistry panels (after serum separation)	Coagulation studies (PT, PTT, INR)	Chemistry panels (especially "stat" tests), Blood gas analysis	Chemistry panels (especially "stat" tests), Blood gas analysis	CBC, Blood bank testing	CBC, Blood bank testing	Blood glucose	Trace element and heavy metal testing (more common)

Table 1: Summary of the standardized color coding of Vacutainer stoppers, outlining the corresponding additives and the principal analytical applications associated with each tube type.

In our study, we observed six different types of vacutainers (figure 9):

- Grey vacutainer that contained Sodium fluoride + anticoagulant (EDTA K3, potassium oxalate…),
- Violet vacutainer that contained anticoagulant EDTA K2 or EDTA K3.
- Blue vacutainer that contained sodium heparin.
- Pink vacutainer that contained EDTA K3 with aprotinin.

- Light blue (Turquoise) vacutainer that contained a buffered trisodium citrate solution.
- Green vacutainer that contained NH sodium heparin,

Figure 9: representation of the six different vacutainers that were present during the study.

a) Results considering the time factor:

Upon re-analysis, the 2022 samples exhibited consistent behavior across all months, as illustrated in Figure 10.

Overall, 234 of BACs (59%) remained within the margin of tolerance, 95 samples (24.1%) showed a decrease in blood alcohol concentration (BAC), and 65 samples (16.5%) showed an increase. Samples whose second BAC measurements fell within the tolerance margin (Group 1) constituted the majority in each month, as expected. A notable rise in samples exhibiting an increased second BAC (Group 3) was observed during the 20th and 30th months, accounting for approximately 50% and 60% of samples, respectively. Conversely, decreases in second BAC results (Group 2) were observed throughout the study period, which is consistent with the volatility of ethanol.

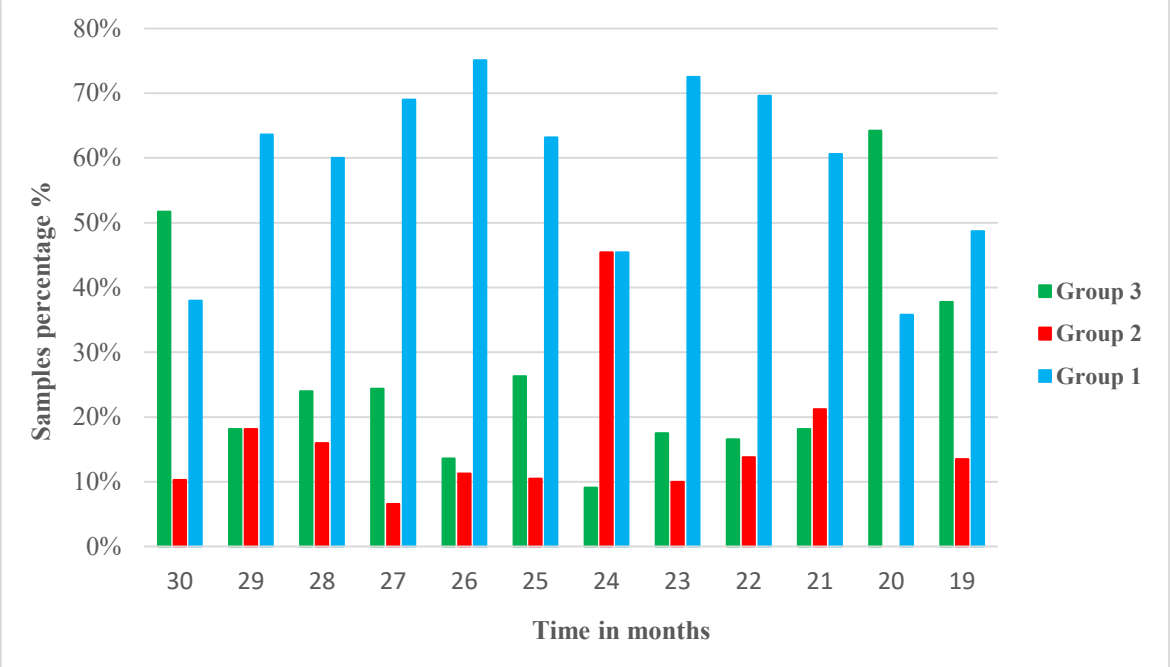

Figure 10: Chart depicting the temporal distribution of newly determined blood alcohol concentrations (BACs) from samples collected in 2022. The data are stratified into three groups based on variation trends: Group 3 (increased values), Group 2 (decreased values), and Group 1 (values within the established margin of tolerance).

In contrast to the 2022 findings, the analysis of samples collected in 2023 revealed a distinct pattern, with the majority of BAC values exhibiting a monthly decrease throughout the year. Only 146 BACs (~38%) remained within the margin of tolerance, while 227 samples (~59%) showed a reduction in BAC. Conversely, samples displaying an increase in their second BAC measurement were rare, totaling only nine (~2.3%), with no increases observed during five months (months 20, 17, 16, 14, and 12), as shown in Figure 11.

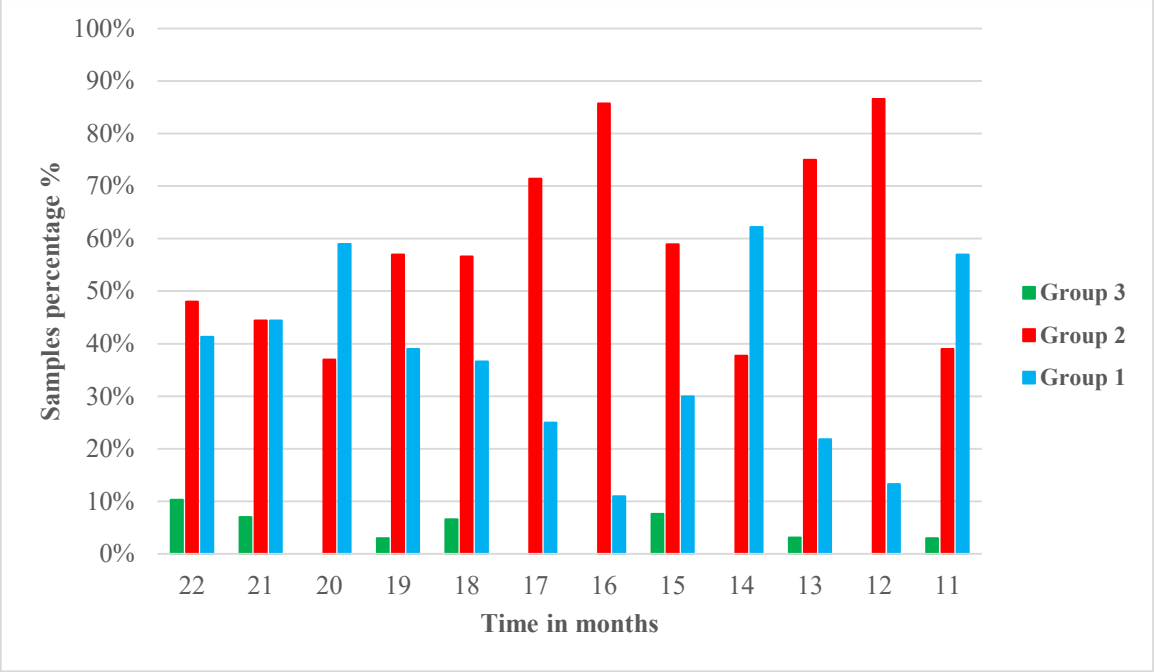

Figure 11: Chart depicting the temporal distribution of newly determined blood alcohol concentrations (BACs) from samples collected in 2023. The data are stratified into three groups based on variation trends: Group 3 (increased values), Group 2 (decreased values), and Group 1 (values within the established margin of tolerance).

Following the second determination of BAC, the samples were categorized into two primary groups: **Group 2**, representing a **decrease** in BAC, and **Group 3**, representing an **increase**. Each group was subsequently stratified according to the percentage variation observed between the two determinations. Three variation ranges were established to facilitate this classification: 10–20%, 20–30%, and 30–40% changes in BAC levels.

For the 2022 samples, most variations in second BAC measurements were moderate in magnitude, as illustrated in Figure 12. Among the samples with decreased BAC values (n = 95), approximately 59% showed a 10–20% reduction, while 22 samples (~23%) exhibited a decrease between 20–30%, and only about 7% showed a decrease greater than 30%. Similarly, among the samples with increased BAC values (n=67), around 68% demonstrated a 10–20% rise, approximately 27% showed an increase of 20–30%, and only 4.4% exhibited an increase exceeding 30% as shown in table 2.

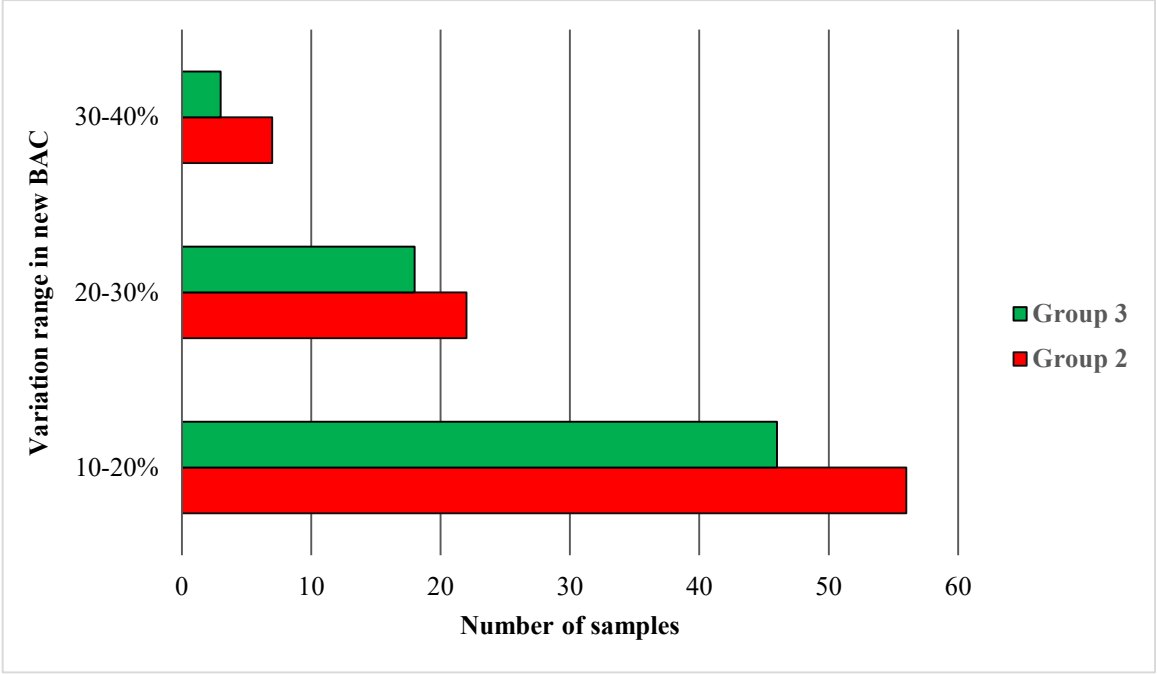

Figure 12: Classification of BAC changes of the samples of the year 2022 by direction and magnitude of variation

	Decreased BACs	Increased BACs
10-20%	56	46
20-30%	22	18
30-40%	7	3

Table 2: Summary of 2022 BAC variation by direction and percentage range.

For the 2023 samples, changes in second BAC values were also predominantly moderate, similar to the 2022 data. Approximately 82% of samples with decreased BAC exhibited a 10–20% reduction, while 77% of the samples with increased BAC showed a comparable 10–20% rise, as illustrated in Figure 13. Notably, the number of samples exhibiting an increase in BAC (Group 3) was very low, accounting for only ~2.1% of the total dataset, highlighting a strong bias toward decreases in BAC during this year as shown in table 3.

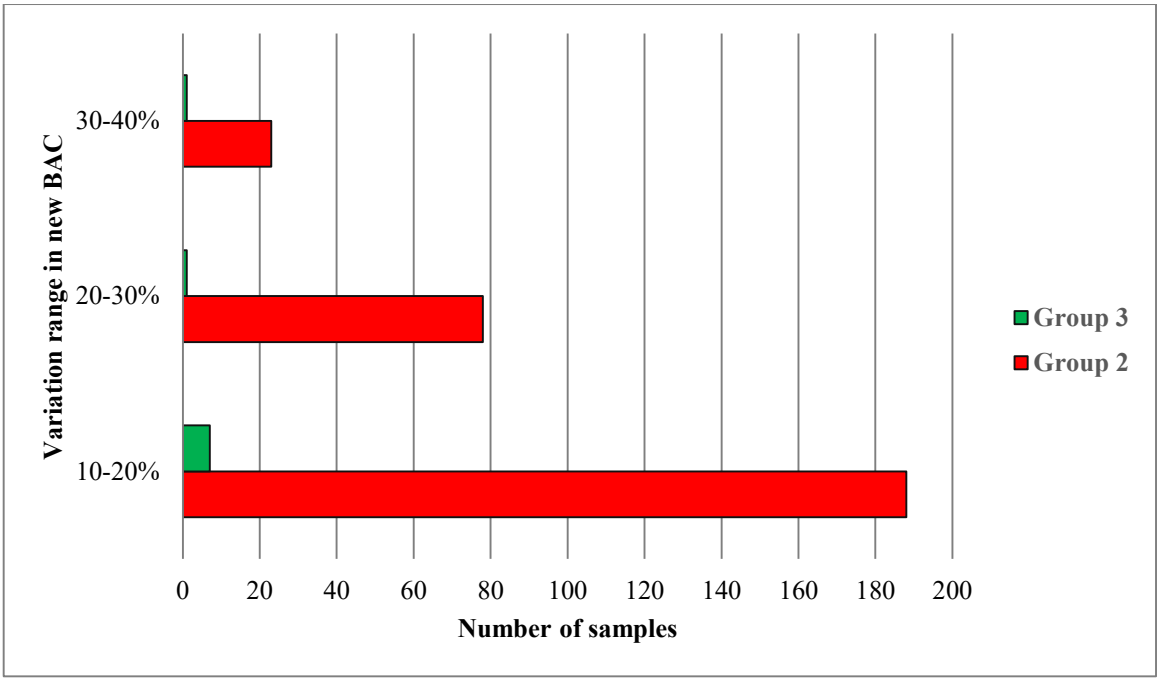

Figure 13: Classification of BAC changes of the samples of the year 2023 by direction and magnitude of variation

	Decreased BACs	Increased BACs
10-20%	188	7
20-30%	78	1
30-40%	23	1

Table 3: Summary of 2023 BAC variation by direction and percentage range.

b) Results of the obtained data considering the vacutainer type:

→ Samples of the year 2022:

Vacutainer type	Total number of samples
Grey	269
Violet	90
Pink	27
Blue	6
Light blue	2
Total	**394**

Table 4: Summary of blood samples distribution of the year 2022 by vacutainer type

Considering the type of vacutainer used, most Group 1 samples—those with results within the margin of tolerance (39.09%)—were stored in grey vacutainers containing sodium fluoride as a preservative. The second largest portion of Group 1 samples (16%) was stored in violet vacutainers containing EDTA.

As illustrated in Figure 14, the three most prevalent preservative types—sodium fluoride, EDTA, and EDTA combined with aprotinin—showed the highest proportion of second BAC results within the tolerance margin (57.6%, 68.8%, and 51.8%, respectively). Approximately 26% of samples stored in grey and pink vacutainers exhibited a decrease in BAC, while only about 12% of those stored in violet vacutainers showed a reduction. Notably, pink vacutainers were the only type in which the proportion of samples with increased BAC exceeded those with decreased values, indicating a distinct pattern compared to the other vacutainer types.

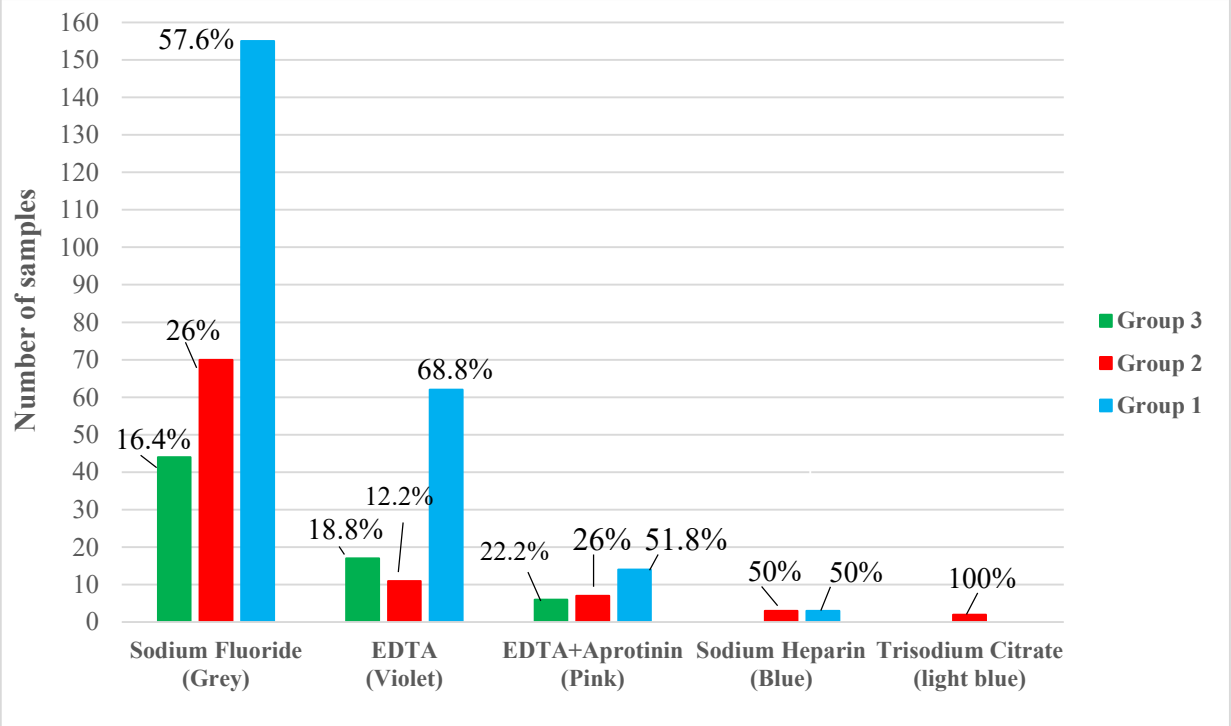

Figure 14: Chart presenting the distribution of newly determined blood alcohol concentrations (BACs) from 2022 samples, organized by the type of collection tube in which the blood was preserved. The data are further classified based on variation trends: Group 3 (increased values), Group 2 (decreased values), and Group 1 (values within the established margin of tolerance).

→ Samples of the year 2023:

Vacutainer type	Total number of samples
Grey	242
Violet	97
Pink	40
Blue	5
Green	2
Total	**386**

Table 5: Summary of blood samples distribution of the year 2023 by vacutainer type

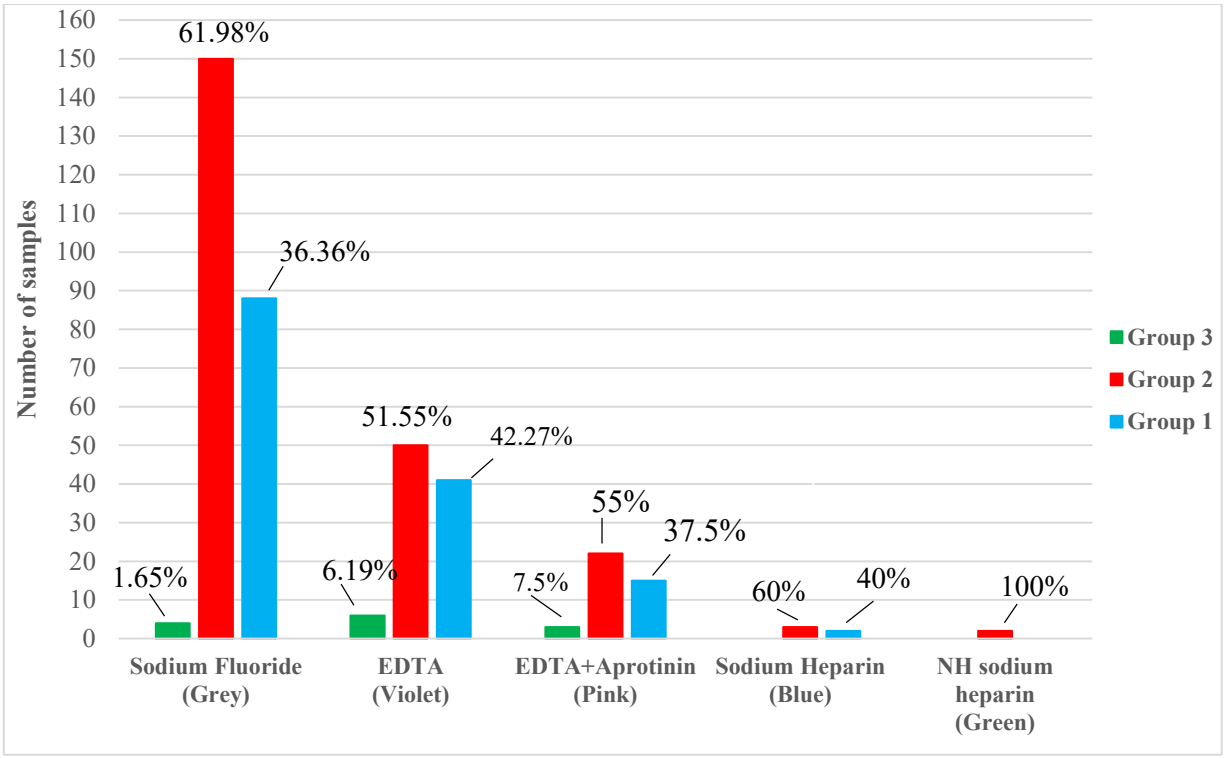

Figure 15: Chart presenting the distribution of newly determined blood alcohol concentrations (BACs) from 2023 samples, organized by the type of collection tube in which the blood was preserved. The data are further classified based on variation trends: Group 3 (increased values), Group 2 (decreased values), and Group 1 (values within the established margin of tolerance).

Although the majority of the 2023 samples were preserved in grey vacutainers (~63%), a distinct pattern emerged compared to the previous year.

In this dataset, samples showing a decrease in BAC constituted the majority across all vacutainer types—approximately 62% in grey, 52% in violet, and 55% in pink vacutainers, as illustrated in Figure 15. Conversely, only a small proportion of samples, not exceeding 10% within any vacutainer type, exhibited an increase in BAC.

Further analysis was conducted by merging the data obtained previously “based on the type of vacutainer in which each sample was preserved” and “the magnitude of change witnessed in the second measurement of BAC”. This allowed for assessment of the influence of storage medium on BAC variability.

As shown in Figure 16, the 2022 data reveal that most changes within each vacutainer type were moderate, predominantly falling within the 10–20% range for both increases and decreases. For instance, among the 46 BAC values that increased in grey vacutainers, approximately 77% exhibited a 10–20% rise, while ~69% of decreased BACs in the same vacutainer type showed a 10–20% reduction. Larger changes were rare: decreases exceeding 30% were observed only in grey vacutainers (~9%) and were absent in all other vacutainer types, while increases greater than 30% occurred solely in grey and violet vacutainers (4.6% and 5.5%, respectively). These results indicate that substantial deviations were uncommon, and the majority of BAC variations were moderate and not of significant analytical concern.

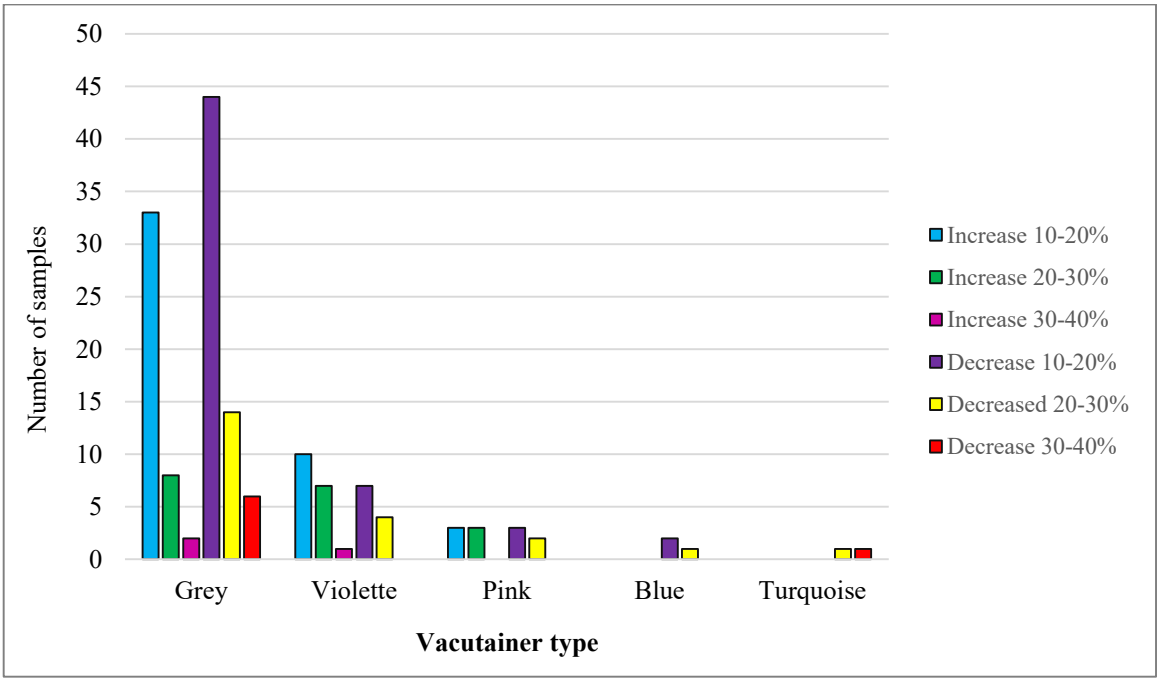

Figure 16: Distribution of new 2022 BAC variation by vacutainer type. Each bar represents the total number of samples for a given tube type, subdivided by the direction (increase or decrease) and magnitude (10–20%, 20–30%, or 30–40%) of BAC variation.

In contrast to the 2022 data, which exhibited a clear variation in both the direction and magnitude of BAC changes, the 2023 samples displayed a more uniform pattern, with the majority of results falling into a single category: decreases (Group 2).

As shown in Figure 17, decreases dominated across all vacutainer types and were primarily stratified into two ranges: 10–20% and 20–30% (reaching 67% and 24% respectively in grey vacutainers). The same can be said for the violet vacutainers where 62% witnessed a decrease from 10-20% while 32% witnessed a decrease from 20-30%). Similar to the 2022 data, most decreased BAC values across all vacutainer types were confined to the 10–20% range. Only a small proportion of samples exhibited an increase in BAC, and all increases were limited to the 10–20% category, further highlighting the strong bias toward BAC reduction in 2023.

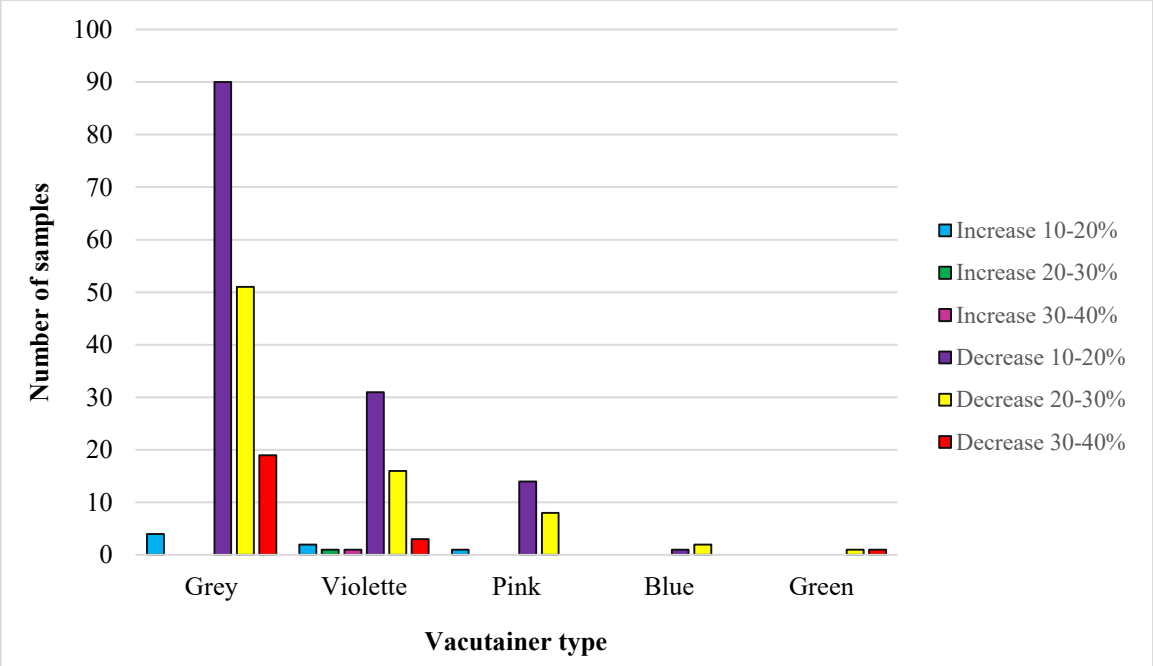

Figure 17: Distribution of new 2023 BAC variation by vacutainer type. Each bar represents the total number of samples for a given tube type, subdivided by the direction (increase or decrease) and magnitude (10–20%, 20–30%, or 30–40%) of BAC variation.

This stratified approach provided a more nuanced understanding of the relationship between vacutainer type and the degree of BAC fluctuation, highlighting the potential impact of preservative agents and storage conditions on analytical outcomes.

Furthermore, to evaluate whether, in combination, storage duration and vacutainer type influence the stability of blood alcohol concentration (BAC) over time, a series of one-way analyses of variance (ANOVA) " two factors without replication" were conducted on the data obtained from the second determination of BAC of the samples collected in 2022 and 2023. The analysis aimed to determine whether the year of collection, reflecting the time elapsed between sampling and re-analysis, and the preservation system used in each tube (conservative type) had a measurable effect on BAC stability over time.

Three types of vacutainer tubes were included in this statistical analysis: Grey (sodium fluoride/potassium oxalate), Violet (EDTA), Pink (EDTA variant).

The other three types: Blue (Sodium Heparin/citrate), Turquoise (trisodium citrate solution) and Green (NH sodium heparin) were excluded from this analysis due to small sample size (11 blue samples and two green and turquoise samples).

As mentioned earlier, for each vacutainer type and year, BAC results from the initial and re-analysis measurements were compared, and each sample was classified into one of three categories: Group 1, 2 and 3.

Firstly, the sample proportion for each group and for each vacutainer type was calculated for both years (Tables 6, 7, and 8). The sample proportion was defined as the ratio between the number of samples meeting the specified condition and the total number of samples within the corresponding group:

$$\boldsymbol{Sample\ proportion} = \frac{\text{Number of samples satisfying a condition}}{\text{Total number of the samples}}$$

For example, the sample proportion of the Grey samples of the year 2022 (Total number of samples) that witnessed any variation in their BAC within the tolerance margin (condition) would be equal to 0.57249 since 154/269 = 0.57249.

The calculation of sample proportions is essential to evaluate the effect of both vacutainer type and year on the stability of BAC in the analyzed samples:

Group 1: Samples in which any variation in BAC was within the tolerance margin of ±10%

	Grey	Violet	Pink	Blue
2022	0.57037	0.696629	0.518519	0.5
2023	0.363636	0.42268	0.375	0.4

Table 6: Summary of sample proportion for each vacutainer type for the year 2022 and 2023

Group 2: Samples that showed a reduction in the initial BAC value, within the range of 10% to 40%.

	Grey	Violet	Pink	Blue
2022	0.27037	0.123596	0.259259	0.5
2023	0.619835	0.515464	0.55	0.6

Table 7: Group 1 sample proportion for each vacutainer type for the year 2022 and 2023

Group 3: Samples that showed an increase in BAC values, within the range of 10% to 40%.

	Grey	Violet	Pink	Blue
2022	0.159259	0.179775	0.222222	0
2023	0.016529	0.061856	0.075	0

Table 8: Summary of sample proportion for each vacutainer type for the year 2022 and 2023

This step was critical for the following reasons:

- ✓ To create a dependent variable for ANOVA, a standard ANOVA requires a continuous dependent variable. However, it's a common practice to transform or model proportional data in a way that allows it to be analyzed using methods that are related to ANOVA. This calculation provides the core data point (for example: the proportion of samples that decreased, increased, or was within the tollerance margin) that is being analyzed.
- ✓ To assess the effect of factors: The ANOVA is used to determine if the mean values of a dependent variable are statistically different across the levels of one or more independent variables (factors). In our case, the independent variables are the "vacutainer type" and the "time," while the dependent variable is the proportion of samples with a decreased, increased or “equal” (withing margin of tollerance) BAC.

The calculation provides the numbers needed to see if the average proportion of decreases, increases or equals is significantly different between the different vacutainer types and years.

By calculating this proportion for each combination of vacutainer type and year, we can then compare these proportions. The “ANOVA two factor without replication” test will then determine if any observed differences are statistically significant or likely due to random chance. For example, the test might show that one type of vacutainer has a significantly lower proportion of samples with decreased BAC compared to another, indicating it is better for preserving the samples.

Subsequently, descriptive statistics (count, mean, sum, and variance) were computed for each group, and these values were used to perform a separate ANOVA test for each dependent variable (“increase,” “decrease,” and “equal/in-margin”) as shown in tables 9, 10 &11.

1. **Within-year variance estimation (MSB):** Variance values within each vacutainer/year combination were calculated to represent the variability of BAC differences for that group. These were used to compute the "within-group" mean square error (MSE).
2. **Between-group mean comparison:** The mean BAC difference (or proportion of samples in each category) (MSB) for each tube type was compared across years using the standard ANOVA F-ratio: $$F=\frac{\text{Mean Square Between Groups (MSB)}}{Mean\ Square\ Within\ Groups\ (MSW)}$$

   The resulting F-statistics for each dependent variable test whether the observed differences among Vacutainer types and years are greater than expected by random variation alone.

   The F-statistic and associated p-value were calculated for each model to determine whether differences between years or tube types were statistically significant at a significance level of $\alpha = 0.05$. The F-test at the heart of ANOVA compares the variability between the group means to the variability within the groups. This is used to determine if the differences between group means are likely due to a real effect rather than random chance. The sample sizes determine the degrees of freedom used to calculate the F-statistic.

   Each ANOVA output includes an F-statistic and associated p-value. The significance threshold was set at $\boldsymbol{\alpha = 0.05}$. If $p < 0.05$, a difference among tube types or between years would be considered statistically significant.

   The averages and variances were visually compared between 2022 and 2023 to identify patterns consistent across the three dependent variables (e.g., whether one vacutainer type consistently showed higher or lower BAC).

   This analytical design allowed for a direct evaluation of whether temporal storage effects (2022 vs. 2023) or conservative effects (vacutainer type) contributed to measurable differences in BAC stability upon re-analysis:

Group 1: Samples in which any variation in BAC was within the tolerance margin of ±10%

ANOVA: Two-Factor Without Replication						
SUMMARY	Count	Sum	Average	Variance		
2022	4	2.285518	0.57138	0.007859		
2023	4	1.417503	0.354376	0.002103		
Grey	2	0.907535	0.453768	0.027192		
Violet	2	1.11931	0.559655	0.037524		
Pink	2	0.842843	0.421421	0.018856		
ANOVA						
Source of Variation	SS	*df*	MS	F	P-value	F-crit
Rows	0.094181	1	0.094181	86.15015	0.002647	10.12796
Columns	0.026606	3	0.008869	8.112336	0.059643	9.276628
Error	0.00328	3	0.001093			
Total	0.124067	7				

Table 9: Results of the ANOVA two factor without replication statistical analysis for the samples of Group 1, were ROWS represent "time" and COLUMNS represent "Vacutainer type". (SS= Sum of Squares, *df*=Degrees of Freedom, MS= Mean Square, F= F-statistics and F-crit= critical value of F).

The ANOVA results for group 1, showed a P-value for "Rows" (Years) of **0.002647** and a P-value for "Columns" (Vacutainer Types) of **0.059643.**

A P-value of 0.002647 for rows is less than the conventional significance level of 0.05 which means that the year (time) has a statistically significant effect on the proportion of samples where BAC remained equal. **The difference between the average proportions in 2022 and 2023 is not due to random chance.**

However, regarding the P-value for columns (0.059643) which is just slightly above the conventional significance of 0.05 demonstrates that vacutainer type does not have a statistically significant effect to some extent on the proportion of samples where BAC remained equal. The differences observed between the "Grey," "Violet," and "Pink," vacutainers are likely due to random chance and not to a real effect of the vacutainer type itself.

Group 2: Samples that showed a reduction in the initial BAC value, within the range of 10% to 40%.

ANOVA: Two-Factor Without Replication						
SUMMARY	Count	Sum	Average	Variance		
2022	4	1.153225	0.288306	0.02437		
2023	4	2.416572	0.604143	0.00438		
Grey	2	0.910217	0.455109	0.068256		
Violet	2	0.639059	0.31953	0.07678		
Pink	2	0.853854	0.426927	0.056225		
ANOVA						
Source of Variation	SS	*df*	MS	F	P-value	F-crit
Rows	0.199506	1	0.199506	38.2562	0.008511	10.12796
Columns	0.070603	3	0.023534	4.512854	0.123768	9.276628
Error	0.015645	3	0.005215			
Total	0.285754	7				

Table 10: Results of the ANOVA two factor without replication statistical analysis for the samples of Group 2, were ROWS represent “time” and COLUMNS represent “Vacutainer type”. (SS= Sum of Squares, *df*=Degrees of Freedom, MS= Mean Square, F= F-statistics and F-crit= critical value of F).

The ANOVA results for group 2, showed a P-value for “Rows” (Years) of **0.0085110** and a P-value for “Columns” (Vacutainer Types) of **0.123768**.

A P-value of 0.0085110 for rows is again, less than the conventional significance level of 0.05 which means that the year (time) does have a statistically significant effect on the proportion of samples where BAC decreased. However, regarding the P-value for columns 0.123768, which is above the conventional significance of 0.05 demonstrates that vacutainer type does not have a statistically significant effect to some extent on the proportion of samples where BAC decreased. The differences observed between the "Grey," "Violet," and "Pink," vacutainers are likely due to random chance and not to a real effect of the vacutainer type itself.

Group 3: Samples that showed an increase in BAC values, within the range of 10% to 40%.

ANOVA: Two-Factor Without Replication						
SUMMARY	Count	Sum	Average	Variance		
2022	4	0.561257	0.140314	0.009438		
2023	4	0.165925	0.041481	0.001349		
Grey	2	0.182248	0.091124	0.009285		
Violet	2	0.241631	0.120815	0.006953		
Pink	2	0.303303	0.151652	0.00996		
ANOVA						
Source of Variation	SS	*df*	MS	F	P-value	F-crit
Rows	0.019536	1	0.019536	8.797417	0.059256	10.12796
Columns	0.025697	3	0.008566	3.857318	0.148395	9.276628
Error	0.006662	3	0.002221			
Total	0.051895	7				

Table 11: Results of the ANOVA two factor without replication statistical analysis for the samples of Group 3, were ROWS represent "time" and COLUMNS represent "Vacutainer type". (SS= Sum of Squares, *df*=Degrees of Freedom, MS= Mean Square, F= F-statistics and F-crit= critical value of F).

The ANOVA results for group 3, showed a P-value for "Rows" (Years) of 0.059256 and a P-value for "Columns" (Vacutainer Types) of 0.148395.

A P-value of 0.059256 for rows is slightly more than the conventional significance level of 0.05 which means that the year (time) does have a statistically significant effect to some extent on the proportion of samples where BAC remained equal. However, regarding the P-value for columns 0.148395, which is above the conventional significance of 0.05

demonstrates that vacutainer type does not have a statistically significant effect to some extent on the proportion of samples where BAC increased. The differences observed between the "Grey," "Violet," and "Pink," vacutainers are likely due to random chance and not to a real effect of the vacutainer type itself.

3.4 Discussion

The present study investigated the long-term stability of blood ethanol concentration (BAC) in forensic samples stored under controlled conditions, with particular attention to the influence of storage duration (samples collected in 2022 and 2023, both re-analysed in 2024) and vacutainer type (conservatives such as sodium fluoride, EDTA, etc…) on ethanol persistence.

Through an extensive dataset comprising 780 samples and a two-factor ANOVA analysis without replication, the research sought to quantify whether these variables exerted a measurable effect on the accuracy and reproducibility of delayed BAC determinations.

1. The 2022 data demonstrate that analytical performance and sample stability were generally quite well maintained throughout the year. The majority of samples remained within the margin of tolerance, indicating reliable reproducibility of BAC determinations under the applied storage conditions. Most observed changes in second BAC measurements were moderate (10–20%), suggesting that deviations largely reflect expected experimental variability rather than systematic analytical error. Larger changes were limited to a small proportion of samples and likely resulted from ethanol volatility, minor handling differences, or storage conditions. The relatively balanced distribution of moderate increases and decreases further supports the conclusion that, despite minor fluctuations, the majority of samples remained sufficiently stable to provide reliable BAC determinations.

   Vacutainer type had a clear influence on BAC stability.

   Samples stored in grey vacutainers containing sodium fluoride exhibited the highest stability, consistent with sodium fluoride's inhibitory effect on glycolytic enzymes and its established use as a preservative in forensic toxicology.

EDTA-containing vacutainers also demonstrated strong preservation efficiency, likely due to their ability to chelate metal ions that catalyze ethanol oxidation.

Samples preserved with EDTA combined with aprotinin showed slightly lower stability, potentially reflecting differences in formulation or chemical interactions affecting ethanol retention.

Pink vacutainers displayed a distinct pattern, with increases in BAC more prevalent than decreases, possibly due to matrix effects, analytical interferences, or sample heterogeneity.

Overall, the predominance of moderate changes across all vacutainer types indicates that preservative selection effectively maintained BAC stability in 2022. Rare occurrences of larger deviations were exceptional and likely attributable to minor handling or storage differences rather than methodological shortcomings. These findings highlight the robustness of the analytical method while emphasizing the importance of careful sample preservation, proper storage, and timely analysis to minimize ethanol loss or fluctuation in repeated measurements.

2. The 2023 results reveal a marked shift from the pattern observed in 2022, with a predominant decrease in BAC values across nearly all months.

   This trend suggests that sample degradation or ethanol loss was more pronounced during this period, potentially due to differences in storage duration, container integrity, or environmental conditions such as temperature and humidity. The minimal proportion of samples exhibiting increased BAC values (~2.1%) indicates reduced analytical variability compared to 2022 but also highlights a systematic bias toward ethanol volatilization over time.

   An overall evaluation of sample storage procedure showed that in 2023, in comparison to that occurred in 2022, there was an increase of the time interval in which the blood samples were kept at+4°C after the analysis before to be definitively stored at -20°C.

Indeed, after BAC determination, because of laboratory organizational aspects, the blood samples are kept at +4°C for a few days before being transferred at -20°C. This time interval in 2022 was less than 1 week, while in 2023 it was up to two weeks.

Despite this downward trend, most BAC changes remained within the 10–20% range, indicating that deviations were moderate and likely attributable to predictable physicochemical processes rather than analytical error.

Vacutainer type had limited influence on this pattern, as decreases dominated across all preservation media. Even with stabilizing agents such as sodium fluoride and EDTA, which are known to inhibit enzymatic degradation and microbial activity, BAC reductions were consistently observed. This uniformity suggests that preservative efficacy alone may be insufficient to prevent ethanol loss under certain storage or handling conditions. Systematic factors—such as prolonged storage, suboptimal temperature control, or increased exposure to air—likely contributed to the observed decreases. Notably, when changes occurred, they were predominantly moderate (10–20%), regardless of vacutainer type, indicating that extreme deviations were rare.

Compared to 2022, where increases and decreases were more balanced and distributed across multiple magnitude categories, the 2023 dataset demonstrates a clear downward bias in BAC values. The restriction of most decreases to the 10–20% range further emphasizes that ethanol loss was consistent but moderate. These observations underscore the critical importance of standardized post-collection protocols, including proper storage duration, temperature control, and timely analysis, to maintain reliable and reproducible BAC measurements over time.

The results revealed that, across all analytical groups, time of storage had a more consistent impact on BAC variation than the type of preservative tube, although the magnitude of this effect was modest and largely within analytical uncertainty limits. Specifically, the ANOVA for Group 1 (samples within ±10% variation) showed a statistically significant year effect ($p = 0.0026$), suggesting a slightly higher proportion of stable samples in 2022 compared to 2023. Similarly, Group 2 (samples showing a decrease in BAC) demonstrated a significant year effect ($p = 0.0085$), indicating a greater frequency of ethanol loss among samples stored for shorter intervals.

In contrast, for all three groups, vacutainer type (Grey, Violet, Pink) exhibited no statistically significant effect on BAC stability ($p > 0.05$ in all cases).

This lack of inter-vacutainer variability implies that the conservative systems tested—whether sodium fluoride/potassium oxalate or EDTA-based—offer comparable ethanol stability over periods extending up to 30 months. These findings reinforce the view that modern vacutainers, when stored under proper refrigeration or freezing conditions, effectively maintain analyte integrity regardless of additive composition.

The stability of ethanol in stored blood samples has been examined in previous studies, although results have varied depending on storage conditions, preservatives, and analytical methods employed. Early work by Winek and Murphy (1984) suggested that ethanol concentrations remain relatively stable under appropriate preservation conditions, while Jones (2007) emphasized that most observed changes during storage are more likely attributable to analytical variability than true biochemical degradation.

More recent investigations have reinforced the general stability of ethanol under refrigerated conditions. Kosecki and Raines (2022) demonstrated that antemortem blood samples stored for up to seven years exhibited minimal clinically significant changes in

ethanol concentration. Similarly, Kosecki et al. (2023) reported that ethanol remained largely stable even in unpreserved refrigerated samples, although minor variability was observed over time.

However, literature has also highlighted confounding factors that may influence ethanol measurements during storage. Corry (1978) and Boumba (2022) discussed the potential for endogenous ethanol formation in post-mortem samples due to microbial activity, emphasizing the need to distinguish between true degradation and biological production.

In this context, the present study extends previous findings by evaluating ethanol stability under conditions representative of routine forensic laboratory practice rather than strictly controlled experimental settings. By examining real-world storage timelines and contemporary analytical workflows, this work provides further support for the reliability of delayed ethanol analysis in antemortem samples.

It's important to say that the observed patterns are consistent with previous investigations reporting minimal ethanol degradation in preserved frozen blood samples over extended storage. Studies by Kosecki et al. (2022, 2023) demonstrated ethanol recoveries above 95% even after seven years of refrigerated storage, while Thorburn Burns et al. (2019) similarly noted that ethanol concentration changes rarely exceeded the analytical imprecision threshold of ±5–10% under controlled conditions. In contrast**, significant ethanol loss has been documented only when storage occurred at ambient temperature or in unpreserved blood,** highlighting the critical role of sodium fluoride and related preservatives in suppressing microbial glycolysis and oxidative loss [53,54].

Although most samples exhibited stable or a decreased blood alcohol concentrations (BAC) upon re-analysis, a subset also demonstrated notable increases, in some cases exceeding

20% relative to the original measurement. While these cases represent a minority of the total dataset, their magnitude exceeds typical analytical imprecision and therefore warrants careful interpretation. Several mechanisms may account for such apparent increases:

1. Analytical and procedural variability:

   Even under tightly controlled conditions, ethanol quantification by HS-GC-FID is subject to small systematic differences between analytical runs performed years apart. Variations in calibration standard preparation, instrument sensitivity, column aging, or auto-sampler headspace equilibrium can collectively contribute to measured deviations. However, such instrumental factors alone are unlikely to explain increases as large as 20%, suggesting additional influences.

2. Post-collection chemical changes:

   In rare cases, endogenous ethanol formation can occur during storage, typically through microbial fermentation of residual glucose or other substrates present in the blood [56]. This process requires both microbial contamination and the absence or depletion of effective preservatives. Although unlikely in properly sealed and frozen tubes, even minute contamination before freezing could, over prolonged storage, generate small but measurable ethanol quantities. The sporadic nature of the ≥ 20% increases observed here—limited to isolated samples rather than a consistent tube or year pattern—supports the interpretation of localized microbial or sample-handling artefacts rather than a systematic preservative failure.

3. Matrix concentration effects:

   Repeated freeze–thaw cycles or partial evaporation of water from the headspace over long-term storage could lead to concentration of solutes, including ethanol. Even minor fluid loss through micro-leakage or changes in vapor-liquid partitioning may elevate the apparent

ethanol concentration during reanalysis. Such effects are consistent with increases that occur sporadically rather than across entire tube groups.

Taken together, these considerations suggest that the observed ≥20% increases reflect isolated artefacts—likely due to a combination of minor microbial activity, matrix concentration effects, and analytical variation—rather than true ethanol synthesis or preservative breakdown. Importantly, these cases were rare and did not cluster by tube type or year of collection, as confirmed by the ANOVA results showing no significant main effect or interaction for the "increase" category ($p > 0.05$).

Thus, the present findings confirm that even under realistic forensic storage conditions—samples collected during routine investigations, stored for 12–30 months, and re-analysed using the same validated HS-GC-FID method—ethanol remains pretty stable.

Importantly, the data show no evidence of post-storage ethanol formation, as would be expected if microbial fermentation or contamination had occurred. Instead, variations were balanced between slight increases and decreases, supporting the conclusion that random analytical variability and sample handling differences account for most deviations.

From a forensic toxicology standpoint, these results carry significant implications for both case interpretation and judicial reliability. First, they provide empirical support for the long-term validity of archived blood samples, affirming that ethanol quantification remains trustworthy even when re-analysis is performed one to two years after initial testing. This is crucial in legal contexts where re-analysis is requested months or years after the original measurement, such as in driving under the influence (DUI) cases governed by Articles 186 and 186-bis of the Italian Highway Code.

Second, the demonstration that vacutainer type does not significantly affect BAC stability enhances flexibility in forensic sampling practices. While sodium fluoride tubes remain the

gold standard, the findings suggest that EDTA-based tubes, when properly refrigerated or frozen, are also capable of preserving ethanol levels reliably. This is particularly relevant for institutions where diverse vacutainer types are used depending on availability or concurrent clinical testing requirements.

Ethanol loss in stored samples typically results from evaporation through imperfect seals, chemical oxidation to acetaldehyde, or microbial metabolism. Conversely, ethanol formation can occur post-collection via endogenous fermentation if microbial contamination is present and preservative concentrations are insufficient. The absence of significant changes across tube types in this study indicates that preservative efficacy remained adequate to inhibit both processes. The slight statistical difference across years (2022 vs. 2023) may reflect subtle differences in storage time (average 30 vs. 18 months), temperature fluctuations, or sample handling frequency, rather than intrinsic instability.

Furthermore, the close alignment of results between vacutainer types containing fluoride/oxalate and those with EDTA suggests that while sodium fluoride provides additional enzymatic inhibition, EDTA's chelating effect may also indirectly stabilize ethanol by limiting metal-catalyzed oxidation pathways. Thus, multiple mechanisms may contribute to ethanol preservation beyond simple enzymatic inhibition.

Third, the study underscores the robustness of headspace gas chromatography (HS-GC-FID) as an analytical platform for ethanol quantification. Its high reproducibility and tolerance to matrix effects likely contributed to the minimal intergroup variance observed, reinforcing its status as the method of choice for forensic alcohol analysis.

Nevertheless, several limitations should be considered when interpreting the present findings:

- First, the retrospective nature of the study did not allow strict control over all pre-analytical variables, including the precise interval between blood collection and initial analysis, possible short-term temperature variations prior to long-term storage, and individual differences in sample handling. Although samples were stored at −20 °C in accordance with routine forensic protocols, minor fluctuations in storage conditions over time cannot be entirely excluded. Additionally, the study was conducted within a single forensic laboratory setting, and therefore the results may reflect local procedural practices, instrumentation, and storage infrastructure, potentially limiting the generalizability of the findings to other institutions with different analytical workflows or environmental conditions.
- Another limitation concerns the storage conditions evaluated. Only samples stored at −20°C were examined, and no direct comparison was performed with alternative temperatures such as refrigerated (4°C) or ultra-low temperature stroage (−80°C). Consequently, while the study provides valuable information regarding ethanol stability under commonly used forensic storage conditions, it does not allow conclusions about the relative superiority of different preservation strategies. Furthermore, the storage period assessed (one to two years) represents a relevant but finite timeframe, and extrapolation beyond this duration should be approached cautiously.
- From an analytical perspective, although headspace gas chromatography with flame ionization detection is considered a robust and reliable method for ethanol quantification, minor variations in measured concentrations over time may

reflect inherent analytical imprecision, calibration variability, or instrumental drift rather than true biochemical degradation. The absence of complementary microbiological testing also prevents definitive exclusion of rare cases of endogenous ethanol formation, particularly in samples with extended storage histories.

Nonetheless, it is important to emphasize that these limitations mirror real-world forensic conditions rather than strictly controlled experimental settings. As such, the study provides ecologically valid and practically relevant insight into the long-term stability of ethanol in routinely stored forensic blood samples, supporting the reliability of delayed reanalysis in medico-legal contexts.

3.5 Conclusions

This study provides strong evidence that blood alcohol concentrations (BAC) remain stable for at least two to three years when samples are properly sealed, preserved, and stored at −20 °C. Across all analyses, storage duration exerted a modest but measurable influence on mean BAC change, while vacutainer type showed no statistically significant effect on ethanol stability, confirming that both fluoride/oxalate and EDTA-based tubes are suitable for forensic alcohol testing.

A portion of samples exhibited modest decreases in BAC upon reanalysis. These reductions were generally minor—predominantly within the 10–20% range—and are most plausibly attributed to routine analytical variability, micro-evaporation, or slow oxidative processes during frozen storage. The decreases were consistent across vacutainer types and did not reach statistical significance, indicating that preservatives effectively prevented microbial activity and chemical degradation. From a forensic perspective, these small declines fall within the accepted analytical uncertainty of HS-GC-FID quantification and do not alter the evidential interpretation of alcohol levels relevant to legal thresholds. Accordingly, measured BAC values remain a trustworthy reflection of the original concentration at the time of sampling.

Conversely, a limited number of samples displayed increases in BAC, occasionally exceeding 20% of the original measurement. These occurrences were sporadic, lacked consistent association with tube type or year, and are best explained by isolated artefacts, such as minor matrix concentration effects from freeze–thaw cycles, localized microbial activity, or minor handling variability. Importantly, no systematic trends or statistical significance were observed, confirming that these increases do not reflect true post-collection ethanol formation. From a forensic standpoint, such rare upward deviations do not compromise evidential reliability but underscore the importance of replicate analysis

and careful inspection of unexpected results. In practical terms, this means that even when minor decreases occur after long-term storage, the measured BAC remains a trustworthy reflection of the original concentration at the time of sampling. Such findings further reinforce the scientific reliability and judicial defensibility of re-analysed blood samples in forensic casework.

The study also demonstrates that vacutainer type has minimal impact on long-term BAC stability, reinforcing the practical flexibility of using either sodium fluoride/oxalate or EDTA-based tubes without compromising analytical integrity. This is particularly relevant for laboratories managing diverse sample collection protocols while maintaining forensic reliability.

Overall, the findings confirm that ethanol is chemically resilient and analytically consistent under appropriate preservation and frozen storage conditions. HS-GC-FID quantification remains robust and reliable for long-term BAC determination, supporting both scientific understanding and judicial defensibility in forensic toxicology.

Despite the robustness of the findings, certain limitations warrant consideration certain limitations should be acknowledged:

- Unequal group sizes: Grey and violet tubes predominated, limiting generalizability to less common tube types.
- Aggregate-level analysis: ANOVA was performed on proportional data, potentially obscuring subtle within-sample variability.
- Storage condition records: Minor variations in handling or temperature exposure could not be fully controlled.
- Single-site study: All analyses were conducted in one forensic laboratory, which may limit extrapolation to other facilities.

Ultimately, this research strengthens the scientific and legal foundations of forensic toxicology practice, affirming that blood alcohol measurements remain stable, reliable, and defensible over extended storage periods, thus ensuring the continued integrity of analytical evidence in judicial processes.

4. Future Perspectives

Although the present study confirms the long-term stability of blood alcohol concentration under frozen storage, several avenues for future investigation remain open.

Extending the monitoring period beyond two to three years would help define the upper temporal limits of ethanol stability and determine whether minimal changes might accumulate over longer durations. Future research should also aim to include multi-centric datasets involving multiple forensic laboratories, storage environments, and analytical platforms to enhance generalizability and inter-laboratory comparability.

In addition, microbiological and physicochemical analyses could be incorporated to explore the underlying mechanisms responsible for occasional increases or decreases in BAC, such as subtle preservative depletion, tube permeability, or microbial resilience under freezing conditions. Investigating alternative biological matrices—including vitreous humor, urine, and tissue homogenates—would further clarify whether similar stability patterns apply beyond whole blood.

Finally, establishing standardized international guidelines for long-term sample preservation, re-analysis, and uncertainty evaluation would ensure consistent evidential reliability worldwide. Such research directions would not only consolidate the scientific foundation of ethanol stability but also strengthen the judicial robustness and transparency of forensic toxicology practices in alcohol-related casework.

REFERENCES

1. Mathew, F., & Goyal, A. (2024). Ethanol (StatPearls). StatPearls Publishing. Retrieved February 22, 2026, from https://www.ncbi.nlm.nih.gov/books/NBK556147/
2. Barceloux, D. G. (2012). Medical toxicology of drug abuse: Synthesized chemicals and psychoactive plants (pp. 394–480). John Wiley & Sons.
3. Mohsenzadeh, A., Zamani, A., & Taherzadeh, M. J. (2017). Bioethylene production from ethanol: A review and techno-economical evaluation. ChemBioEng Reviews, 4(2), 75–91.
4. McDonnell, G., & Russell, A. D. (1999). Antiseptics and disinfectants: Activity, action, and resistance. Clinical Microbiology Reviews, 12(1), 147–179.
5. Renewable Fuels Association. (2008). 2008 world fuel ethanol production. Ellisville, MO, USA. Retrieved February 22, 2026, from [publisher website]
6. Oxford English Dictionary. (1913). The Oxford English Dictionary: Being a corrected re-issue with an introduction, supplement and bibliography of a new English dictionary on historical principles (Vol. 1, A–B, p. 209). Clarendon Press.
7. Dashko, S., Zhou, N., Compagno, C., & Piškur, J. (2014). Why, when, and how did yeast evolve alcoholic fermentation? FEMS Yeast Research, 14(6), 826–832. https://doi.org/10.1111/1567-1364.12161
8. Powell, M. A. (1998). Wine and the vine in ancient Mesopotamia: The cuneiform evidence. In P. E. McGovern, S. J. Fleming, & S. H. Katz (Eds.), The origins and ancient history of wine (Vol. 11, pp. 96–124). Food and Nutrition in History and Anthropology.
9. Paton, A. (2005). Alcohol in the body. BMJ, 85–87.
10. Jones, A. W., Wigmore, J. G., & House, C. J. (2006). The course of the blood-alcohol curve after consumption of large amounts of alcohol under realistic conditions. Canadian Society of Forensic Science Journal, 125–140.

11. Zekan, P. (2023). Pharmacokinetic analysis of ethanol in a human study. Pharmacology & Therapeutics. Advance online publication. https://doi.org/10.1016/j.pharmthera.2023.108500
12. Holford, N. H. G. (1987). Clinical pharmacokinetics of ethanol. Clinical Pharmacokinetics, 13(5), 273–292.
13. Cederbaum, A. I. (2012). Alcohol metabolism. Clinics in Liver Disease, 16(4), 667–685. https://doi.org/10.1016/j.cld.2012.08.002
14. Contreras-Zentella, M. L., Villalobos-García, D., & Hernández-Muñoz, R. (2022). Ethanol metabolism in the liver, the induction of oxidant stress, and the antioxidant defense system. Antioxidants, 11(7), 1258. https://doi.org/10.3390/antiox11071258
15. Jones, A. W. (2019). Alcohol, its absorption, distribution, metabolism and excretion in the body and pharmacokinetic calculations. Wiley Interdisciplinary Reviews: Forensic Science, 1(6), e1340. https://doi.org/10.1002/wfs2.1340
16. Heier, C., Xie, H., & Zimmermann, R. (2016). Nonoxidative ethanol metabolism in humans—from biomarkers to bioactive lipids. IUBMB Life, 68(12), 916–923. https://doi.org/10.1002/iub.1560
17. Jiang, Y., Zhang, T., Kusumanchi, P., Han, S., Yang, Z., & Liangpunsakul, S. (2020). Alcohol metabolizing enzymes in alcohol-associated liver disease. Biomedicines, 8(3), 50. https://doi.org/10.3390/biomedicines8030050
18. Ronis, M. J., Huang, J., Crouch, J., Mercado, C., Irby, D., Valentine, C. R., Lumpkin, C. K., Ingelman-Sundberg, M., & Badger, T. M. (1993). Cytochrome P450 2E1 induction during chronic alcohol exposure occurs by a two-step mechanism associated with blood alcohol concentrations in rats. Journal of Pharmacology and Experimental Therapeutics, 264(2), 944–950.

19. Seitz, H. K., & Pöschl, G. (1997). The role of gastrointestinal factors in alcohol metabolism. Alcohol and Alcoholism, 32(5), 543–549. https://doi.org/10.1093/oxfordjournals.alcalc.a008293
20. Holt, S. (1981). Observations on the relation between alcohol absorption and the rate of gastric emptying. Canadian Medical Association Journal, 124(3), 267–277.
21. Goldman, M. R., Molina-Castro, M., Etkins, J. C., Koide, T. L., Ramchandani, V. A., Plawecki, M. H., Mennella, J. A., & Pepino, M. Y. (2025). Recent advances in alcohol metabolism: From the gut to the brain. Physiological Reviews, 105(4), 2501–2535. https://doi.org/10.1152/physrev.000XX.2024
22. Kechagias, S., Jönsson, K. A., & Jones, A. W. (1999). Impact of gastric emptying on the pharmacokinetics of ethanol as influenced by cisapride. British Journal of Clinical Pharmacology, 48(5), 728–732. https://doi.org/10.1046/j.1365-2125.1999.00092.x
23. Stamates, A. L., Maloney, S. F., & Marczinski, C. A. (2015). Effects of artificial sweeteners on breath alcohol concentrations in male and female social drinkers. Drug and Alcohol Dependence, 157, 197–199. https://doi.org/10.1016/j.drugalcdep.2015.10.006
24. Morgan & Claypool. (2009). Hepatic circulation and toxicology (Chapter 12). Morgan & Claypool Life Sciences.
25. Winek, C. L., & Murphy, K. L. (1984). The rate and kinetic order of ethanol elimination. Forensic Science International, 25(3), 159–166. https://doi.org/10.1016/0379-0738(84)90044-2
26. Djordjević, D., Nikolić, J., & Stefanović, V. (1998). Ethanol interactions with other cytochrome P450 substrates including drugs, xenobiotics, and carcinogens. Pathologie Biologie, 46(10), 760–770.
27. Christophersen, A. S., Morland, J., Stewart, K., & Gjerde, H. (2016). International trends in alcohol and drug use among motor vehicle drivers. Forensic Science Review, 37–66.

28. Pervin, Z. (2021). Effect of alcohol on the central nervous system to develop alcohol use disorder. Frontiers in Pharmacology, 12, 674623. https://doi.org/10.3389/fphar.2021.674623
29. Steele, C. M., & Southwick, L. (1985). Alcohol and social behavior I: The psychology of drunken excess. Journal of Personality and Social Psychology, 48(1), 18–34.
30. LaHood, A. J., & Kok, S. J. (2023). Ethanol toxicity. NIH. Retrieved February 22, 2026, from https://www.ncbi.nlm.nih.gov/books/NBK554518/
31. Cartmell, M. T., Schulz, H. U., O'Reilly, D. A., Yang, B. M., Kielstein, V., Dunlop, S. P., Halangk, W., Demaine, A. G., & Kingsnorth, A. N. (2005). Cytochrome P450 2E1 high activity polymorphism in alcohol abuse and end-organ disease. World Journal of Gastroenterology, 11(41), 6445–6449. https://doi.org/10.3748/wjg.v11.i41.6445\
32. Jones, A. W. (2019). Alcohol, its analysis in blood and breath for forensic purposes, impairment effects and acute toxicity. WIREs Forensic Science, 1(6), e1340. https://doi.org/10.1002/wfs2.1340
33. National Academies of Sciences, Engineering, and Medicine; Health and Medicine Division; Board on Population Health and Public Health Practice; Committee on Accelerating Progress to Reduce Alcohol-Impaired Driving Fatalities; Negussie, Y., Geller, A., & Teutsch, S. M. (Eds.). (2018). Getting to zero alcohol-impaired driving fatalities: A comprehensive approach to a persistent problem. Washington, DC: National Academies Press. https://doi.org/10.17226/24928
34. Kelly, A. T., & Mozayani, A. (2012). An overview of alcohol testing and interpretation in the 21st century. SAGE Journals, 25(1), 1–12. https://doi.org/10.1177/109158181202500101
35. Levine, B. S., Caplan, Y. H., & Jones, A. W. (2020). Alcohol. In Principles of forensic toxicology (pp. 287–316).

36. Savini, F., Tartaglia, A., Coccia, L., Palestini, D., D'Ovidio, C., de Grazia, U., Merone, G. M., Bassotti, E., & Locatelli, M. (2020). Ethanol determination in post-mortem samples: Correlation between blood and vitreous humor concentration. Molecules, 25(12), 2724. https://doi.org/10.3390/molecules25122724

37. Ialongo, C. (2024). Blood alcohol concentration in the clinical laboratory: A narrative review of the preanalytical phase in diagnostic and forensic testing. Biochemia Medica (Zagreb), 34(1), 010501. https://doi.org/10.11613/BM.2024.010501

38. Sithersingh, M. J., & Snow, N. H. (2012). Chapter 9: Headspace-gas chromatography. In C. Poole (Ed.), Gas chromatography (pp. 221–234). Elsevier. https://doi.org/10.1016/B978-0-12-391920-0.00009-7

39. Ozturk, A., Temel, I., Yalcindag, A., Ucar, F., & Yay, F. (2022). Verification of enzymatic ethanol analysis method and method comparison with headspace gas chromatography. Turkish Journal of Biochemistry. https://doi.org/10.1515/tjb-2022-0001

40. Xiao, H., He, L., Tong, R., Yu, J., Chen, L., Zou, J., Li, J., Bian, Y., & Zhang, Y. (2014). Rapid and sensitive headspace gas chromatography-mass spectrometry method for the analysis of ethanol in the whole blood. Journal of Clinical Laboratory Analysis, 28(5), 386–390. https://doi.org/10.1002/jcla.21754

41. Restek Corp. (2000). A technical guide for static headspace analysis using GC. Restek Corporation. Retrieved February 22, 2026, from https://www.restek.com/technical-resources

42. Mihajlović, F., Andrić, I., Slović, Ž., Vujović, M., & Piskulić, K. (2025). Development and validation of headspace gas chromatography with a flame ionization detector method for the determination of ethanol in the vitreous humor. Open Medicine, 20(1), 1–9. https://doi.org/10.1515/med-2025-0001

43. Joseph, J. A., Akkermans, S., & Van Impe, J. F. M. (2022). Processing method for the quantification of methanol and ethanol from bioreactor samples using gas chromatography−flame ionization detection. ACS Omega, 7(28), 24121–24133. https://doi.org/10.1021/acsomega.2c03456
44. Parlamento Italiano. (n.d.). Articles 186 e 186-bis del Codice della Strada ("Art. 186 C.d.S." e "Art. 186-bis C.d.S."). Retrieved February 22, 2026, from https://www.normattiva.it
45. Parlamento Italiano. (n.d.). Art. 186 C.d.S., comma 2, lett. a, b, c. Retrieved February 22, 2026, from https://www.normattiva.it
46. Kosecki, P. A., Canonico, E., & Abbott, L. (2023). Ethanol stability in unpreserved refrigerated antemortem blood. Journal of Forensic Sciences, 68(2), 682–687. https://doi.org/10.1111/1556-4029.15102
47. Kosecki, P. A., & Raines, M. E. (2022). Testing antemortem blood samples for ethanol after four to seven years of refrigerated storage. *Journal of Forensic Sciences, 67*(3), 1250–1257. https://doi.org/10.1111/1556-4029.15018
48. Jones, A. W. (2007). Are changes in blood-ethanol concentration during storage analytically significant? Importance of method imprecision. Clinical Chemistry and Laboratory Medicine, 45(10), 1299–1304. https://doi.org/10.1515/CCLM.2007.241
49. Burnsa, D. T., Breena, C., & Walkerb, M. J. (2019). The stability of ethanol in forensic blood samples with storage conditions. Journal of the Association of Public Analysts, 47, 036–045.
50. Boumba, V. A. (2022). Modeling postmortem ethanol production/insights into the origin of higher alcohols. Molecules, 27(3), 700. https://doi.org/10.3390/molecules27030700
51. Mihajlović, F., Andrić, I., Slović, Ž., Vujović, M., Piskulić, K., & Đorđević, S. (2025). Development and validation of headspace gas chromatography with a flame ionization

detector method for the determination of ethanol in the vitreous humor. *Open Medicine (Warsaw), 20*(1), 20241123. https://doi.org/10.1515/med-2025-1123

52. González-Domínguez, R., González-Domínguez, Á., Sayago, A., & Fernández-Recamales, Á. (2020). Recommendations and best practices for standardizing the pre-analytical processing of blood and urine samples in metabolomics. Metabolites, 10(6), 229. https://doi.org/10.3390/metabo10060229
53. Corry, J. E. L. (1978). Possible sources of ethanol ante- and post-mortem: Its relationship to the biochemistry and microbiology of decomposition. Journal of Applied Bacteriology, 44(1), 1–56. https://doi.org/10.1111/j.1365-2672.1978.tb02497.x
54. Wongchanapai, W., Dokpuang, D., Sasithonrojanachai, S., & Tamtakerngkit, S. (2008). Stability of postmortem blood ethanol under experimental conditions. Siriraj Medical Journal, 60, 547–552