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Quantitative Determination of Ethyl Glucuronide and Ethyl Sulfate in Postmortem Blood Using Hybrid-SPE-PPT and UHPLC-MS/MS

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ABSTRACT

Background: Alcohol misuse is associated with significant morbidity and mortality. Ethyl glucuronide (EtG) and Ethyl sulfate (EtS) are direct ethanol metabolites and powerful biomarkers for previous alcohol consumption. EtG and EtS can be determined in blood for an extended period after complete elimination of ethanol itself. Their presence is therefore indicative of recent alcohol consumption in case of delayed sampling after an event (e.g., car crash)

Methods: An ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method for the determination of EtG and EtS in postmortem blood was developed and validated. Postmortem blood samples (100 µL) were prepared using protein precipitation technique (PPT) with acetonitrile (ACN). Filtration of the supernatants through Hybrid SPE-PPT appeared to be necessary to reduce matrix effect (ME) caused by co-elution of phospholipids with EtG and EtS. Identification of compounds was performed using multiple reaction monitoring (MRM) in the positive mode. Deuterated analogous of EtG and EtS was used as internal standard. The compounds were separated using reversed phase chromatography C_{18} column (2.1 mm x 100 mm, 1.8 µm). The flow rate of 0.4 mL /min was used, 0.5 µL injected and gradient elution with 0.1% formic acid (FA) and methanol (MeOH) as organic modifier.

Results: Excellent linearity for EtG from (0.4 - 100 µmol/L) and EtS from (0.2 -50 µmol/L) was achieved ($R^2 \geq 0{,}999$). Limit of detection (LOD) and limit of quantification (LOQ) were 0.04 and 0.3 µmol/L for EtG and 0.02 and 0.15 µmol/L for EtS, respectively. Accuracy (bias) and precision (relative standard deviation) were studied at four different levels of quality control (QC) samples, and were always better than ±10%. The use of the internal standard appears to be appropriate for the EtG and EtS assay and the matrix effects were found to be negligible. The method was applied on 26 DUI-cases and postmortem cases, good agreements of quantified concentrations were found for the concentrations examined.

Conclusion: A sensitive and specific method for quantitative determination of EtG and EtS in postmortem blood was developed despite a simple, fast and robust with high throughput sample preparation procedure.

ACRONYMS AND ABBREVIATION

EtG	Ethyl glucuronide
EtS	Ethyl sulfate
MeOH	Methanol
DUI	Driving under the influence
PM	Postmortem
HS-GC	Headspace gas chromatography
FID	Flame ionization detector
BAC	Blood alcohol concentration
NIPH	Norwegian Institute of Public Health
ALDH	Aldehyde dehydrogenase
ADH	Aldehyde hydrogenase
FAEEs	Fatty acid ethyl esters
UHPLC	Ultra High Performance Liquid Chromatography
HPLC	High performance liquid chromatography
MP	Mobile phase
MA	Mobile phase A
MB	Mobile phase B
LC	Liquid chromatography
GC	Gas chromatography
CE	Capillary electrophoresis
MS/MS	Tandem mass spectrometry
RP	Reversed phase
ACN	Acetonitrile
FA	Formic acid
QQQ	Triple quadrupole
ESI	Electrospray ionization
MS	Mass spectrometry
MRM	Multiple reaction monitoring
SRM	Selected reaction monitoring
CID	Collision induced dissociation

TIC	Total ion chromatogram
SPE	Solid phase extraction
PPT	Protein precipitation technique
LLE	Liquid –liquid extraction
ME	Matrix effect
RSD	Relative standard deviation
SIL-ISTD	Stable isotopically labeled internal standards
LM	Low mass
HM	High mass
GHB	Gamma-hydroxybutyrate
LLOD	Lower limit of detection
LLOQ	Lower limit of quanitificatiz

CONTENTS

1. INTRODUCTION AND BACKGROUND

1.1 Forensic toxicology

Forensic toxicology is the study of adverse effects of drugs and chemicals on biological systems. In forensic toxicology, information is shared with law enforcement, death investigators, crime scene investigators, clinicians, other forensic scientists or forensic pathologists, and legal professionals.

The field of forensic toxicology includes three main disciplines: postmortem forensic toxicology, human performance toxicology, and forensic drug testing. These specialized fields offer a variety of exciting career paths [1].

In forensic toxicology a wide range of analytical methods are used, which produce a wealth of data. One of the main purpose of this data is to help to determine the cause of deaths resulting from accidents, suicides and crime of violence [1].

Division of Forensic Sciences at Norwegian Institute of Public Health (NIPH) routinely screened and confirmed drugs in blood, urine and different body fluids. Analytical methods such as chromatography and spectrometry may be used for screening and further confirmations of drugs. Forensic pathologist determines the cause and manner of death. Interpretation of postmortem toxicology is based on several confounding factors and toxicological results must always be interpreted with caution. The abuse of alcohol still causes more deaths than any other substance [2]. Alcohol is also known to interact with other drugs and it is often found to have played a major part in causing death.

1.1.2 Importance of ethanol in forensic toxicology

Driving under the influence (DUI) of alcohol is a common social problem throughout the world. Alcohol consumption reduces driving ability and increases crash risk [3].

Ingestion of large doses of ethanol can result in intoxication and ethanol causes more deaths than any other substance due to trauma, such as road traffic incidents and falls, drowning, burns and carbon monoxide poisoning. In such cases of death, analyzes of several different samples for the parent drug and its metabolite(s) may go some way towards distinguishing acute from chronic use of drugs and may assist the pathologist results [2, 4, 5].

Ethanol has been known as one of the most important substance within the field of forensic toxicology [2]. Endogenous ethanol can be produced via different metabolic processes in the

body. Endogenous ethanol blood concentrations in living humans are too low to be detected by modern analytical equipment (~ 0.001 g/kg) [5, 6]. Ethanol can also rise as a putrefactive product formed by a wide range of species of microorganisms. The formation may occur as a part of postmortem degenerative processes. Ethanol can also rise pronounced after severe traumas. The substrate used in this reaction is often glucose [7]. The level of glucose fermentation depends on microorganisms and it might rise considerably after death, causing endogenous ethanol levels that are difficult to distinguish from those caused by ingestion [5]. The temperature on the location of the body can affect the probability of postmortem alcohol production [8, 9].

The concentration of ethanol measured in postmortem blood needs to be interpreted in relation to whether the detected ethanol is from postmortem production or ingestion prior to death [5].

Interpretation of postmortem ethanol has become relatively simple, accurate and precise. Specific result can be obtained by measuring the direct metabolites of ethanol ethyl glucuronide (EtG) and ethyl sulfate (EtS) [9]. They are measurable in body fluids for a sufficiently extended time after the complete elimination of ethanol from the body. EtG and EtS have are sensitive and specific ethanol markers and are superior compared to other ethanol markers [7, 10].

EtG and EtS are even detectable in living subjects after intake of small amounts of alcohol. The period for the detectability of the EtG and EtS depends on the quantity of the consumed ethanol. EtG could be detected in serum up to 10 h and in urine up to about 100 h. For EtS, a similar detection window was determined in urine samples [11].

1.2 Ethanol metabolism

Ethyl alcohol (CH_3CH_2OH) also known as ethanol is metabolized in the body through several pathways. The pathways can be divided into oxidative and non-oxidative.

Ethanol is completely soluble in aqueous environments. The molecular structure as illustrated in Figure 1 consists of ion pairs, which make it reactive with a variety of reagents. It can be absorbed through the stomach and pass rapidly into the bloodstream. The rapid circulation of the blood carries ethanol to all parts of the body, especially into organs with large bloodstream, such as the brain. This phenomenon continues until the concentration of

ethanol in the tissues reach equilibrium in the body. The concentration of ethanol in the breath can be related to the level in blood. Hence, determination of breath ethanol concentration can be used to determine the BAC of a driver. The major limitation of breath and blood ethanol determination is the short detection window (typically < 6 h) [12-15].

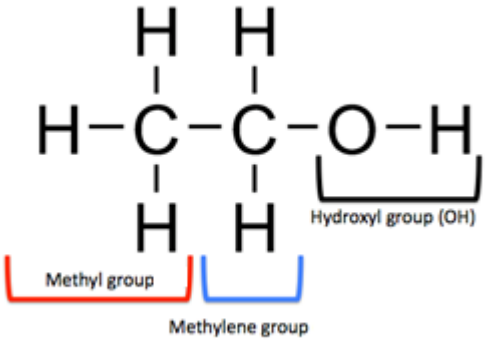


Figure 1: Molecular structure of Ethanol structure and chemical formula.

1.2.1 Oxidative pathway

About 92–95% of the ingested ethanol is eliminated primarily in the liver cells via a 2-step oxidative pathway as illustrated in Figure 2 [16]. The primary pathway involves an oxidation of alcohol to acetaldehyde (CH_3CHO). NAD^+ is used as oxidizing agent. This step in the reaction is catalyzed by alcohol dehydrogenase (ADH) enzymes [17]. Acetaldehyde is toxic and contribute to damage cells and tissues in various way and rapid oxidation is important in the second step of this reaction giving acetate as the final degradation product [18]. A small fraction of ethanol is oxidized in liver cells in the cytosol by aldehyde dehydrogenase ALDH1, where the principal fraction is oxidized in the mitochondria by ALDH2 [19]. The rest of the alcohol (non-metabolized) is eliminated in small quantities by the kidneys (0.5–2%), lungs (1.6–6%) and the skin (< 0.5%) [16]. The alcohol elimination rate varies widely among individuals. It is influenced by factors such as chronic alcohol consumption, diet, age and smoking [19]. Ethanol can reach its maximum concentration in the blood 20-60 min after ingestion on an empty stomach whereas absorption can take about 2 hours in combination with food [16, 20].

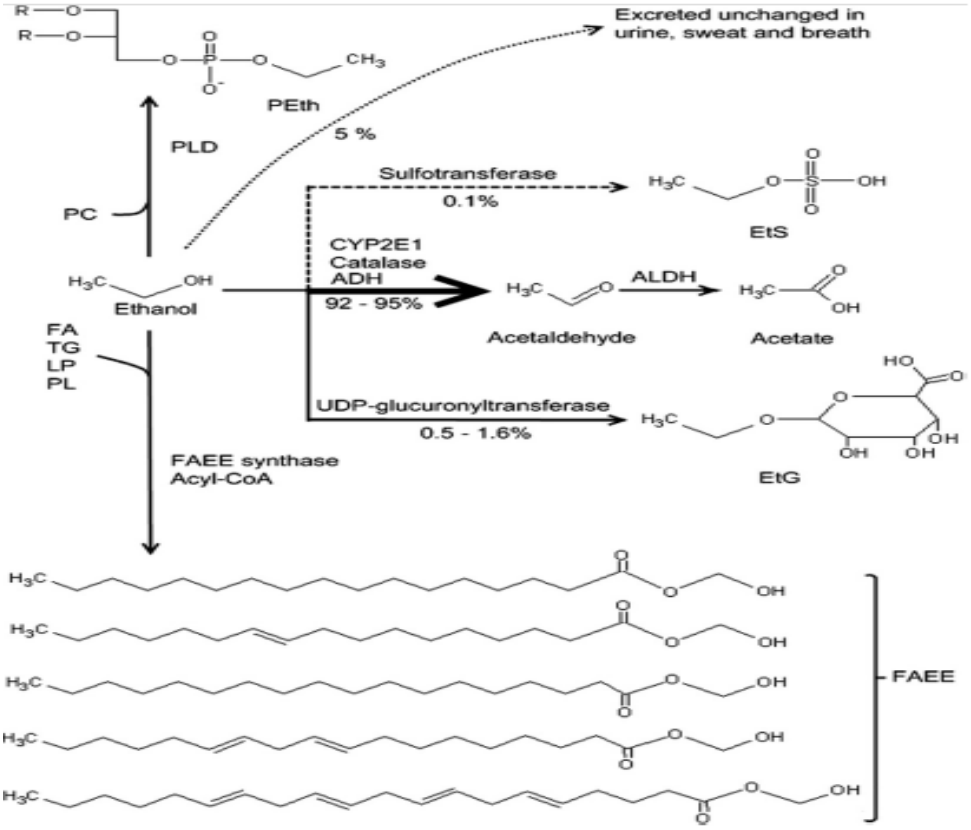


Figure 2: Ethanol metabolism and formation of EtG and EtS [16].

1.2.2 Non-oxidative pathway

*S*mall percentage (0.6–1.5%) of the ethanol consumptions undergoes non-oxidative metabolism yielding EtG and EtS [16, 21, 22], Figure 2. This step is minimal and fatty acid ethyl esters (FAEE) are the main products of the non-oxidative metabolism of ethanol. Phosphatidylethanol is formed in the cellular membrane in the presence of ethanol by transphosphatidylation of phosphatidylcholine with the participation of phospholipase [16]. Formation of EtG is a result of the reaction between ethanol and glucuronic acid. UDP glucuronosyltransferase enzymes catalyzed this step in the reaction, which is located in the endoplasmic reticulum in the liver. EtS is formed after conjugation with sulfate (3´-phosfoadenosine 5´-phosfosulphate) and catalyzed by the cytosolic sulphotransferase in the liver [23].

About (0.02-0.03%) of the consumed ethanol is excreted as EtG, and (0.01 – 0.02%) as EtS [16]. EtG and EtS are detectable in several body fluids and post mortem tissues and are useful markers for alcohol ingestion. Both of these metabolites remain in the body longer than ethanol itself [24, 25]. EtG is eliminated with a half time of ~2.5 h. Ethanol doses

between ~ 0.25-0.5 g/ kg are detectable for ~ 24-48h after the start of ingestion. About 95-99.5% of EtG and EtS are eliminated during the first 24 h. About 79-92% of the EtG and EtS being excreted in the first 10 to 12 h after alcohol consumption [24].EtG and EtS close the gap between short term markers (e.g., ethanol) and long term markers (e.g., carbohydrate deficient transferrin in serum) [24, 26, 27].

1.3 Ethyl glucuronide and ethyl sulfate

EtG and EtS are non-volatile, acidic and water soluble phase II metabolites of alcohol, which are widely used for clinical, forensic and traffic cases. EtG and EtS has gained popularity due to their use as physiological indicators for screening previous alcohol consumption and to differentiate between ingested ethanol prior to death and post mortem formation of ethanol [4, 11, 26].

The existence of EtG has been known for over a century. EtG was first described in 1901 and verified in 1950 by Dr. Friedrich Wurst and Dr. Gregory Skipper [28], whereas EtS was first described in 1960 [16, 29]. The clinical use of the EtG as an alcohol marker started in 2001 [28]. Until 2004 EtS was only demonstrated as an ethanol metabolite in an animal model [22].

Consumption of EtS containing beverages like alcohol free wine or grape juice contributes to false positive results of EtS. EtG concentration above 0.1 mg/L can be found in urine after consumption of food or non-alcoholic beer and yeast in combination with sugar and after the use of alcohol containing mouthwash [24]. In this assay, a cut-off limit of 0.088mg/L for EtG and 0.024 mg/L for EtS in blood was chosen to distinguish between intentional and unintentional alcohol consumption.

Table 1:Chemical properties of EtG and EtS.

Compound	Ethyl glucuronide	Ethyl sulfate
Molecular formula	$C_8H_{14}O$	$C_2H_6O_4S$
Exact mass	222.07 g/mol	125.99 g/mol
pKa [30]	~ 2.84	~ -3.14
Log p[31]	1.51	0.62

1.3.1 Ethyl glucuronide and ethyl sulfate in postmortem specimens

A more reliable indication for excluding postmortem ethanol formation is the detections of the non-oxidative metabolites of ethanol. For the correct interpretation of EtG and EtS concentrations, knowledge of the stability and degradation or possible formation of these biomarkers is needed.

The presence of EtG in autopsy samples is not a unique indicator of recent drinking, but might originate from post collection synthesis if specimens are E. *coli* or C. *sordellii* infected [12]. After sampling EtG can be formed or destroyed in urine by bacteria, especially in the case of insufficiently cooled urine samples.

A wide range of bacteria and yeast can produce ethanol from glucose present in the blood, especially in the case of insulin depended diabetics. The level of ethanol may increase by bacterial postmortem production after death [30]. Tissues and fluids with high glucose concentration such as urine are more affected to postmortem production of ethanol. Postmortem blood and urine results must be careful interpreted in cases with heavy putrefaction [7]. As part of a large stability study, Hoiseth assayed blood from 39 autopsy cases containing EtG. In these cases ethanol was present as a result of postmortem production. EtG was present at a median concentration of 1.6 mg/l in 19 cases, whereas it was not detected in blood from the remaining cases. The absence of EtG might actually represent a false negative due to postmortem degradation of EtG. Therefore, negative results of EtG must be interpreted with caution, especially in heavily putrefied bodies. Isolated tissues such as brain and vitreous humour are less susceptible to [8]. Ethyl sulfate is biodegradable only under conditions of high bacterial density. Consequently, EtS seems to be more reliable marker for monitoring alcohol abstinence [24, 32].

1.4 Current methods

Annually NIPH performs alcohol screening of about 1800 post mortem whole blood samples by using Headspace gas chromatography-flame ionization detector (HS-GC-FID) [33]. The samples screened positive for alcohol are further analyzed to confirm the presence of EtG and EtS [34].

The most commonly applied technique for quantification of EtG and EtS is liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) due to high selectivity and sensitivity. This technology has been used to determine EtG and EtS in urine

[35-40], serum/whole blood [41-43], dried blood spots [44] and meconium [45] in combination with simple dilution or protein precipitation as sample preparation.
The already existing sample preparation method for EtG and EtS at NIPH is limited to protein precipitation with methanol and consists of many steps [34]. The method demonstrates good performance for routine determination of EtG and EtS in ante mortem cases. Many autopsy samples are of poor quality and generally they are quite different from ante mortem blood. However, post mortem samples obtained by PPT contain high amount of endogenous matrix compounds. As a consequence of this, unpredictable mass spectrometry results were obtained, deviation in ion ratio, occasionally split peaks were observed, and variation in internal standard response were relative frequently observed in the current method.

1.5 Aim of the study

The aim of this study was twofold: to explore sample preparation procedures which could be simple and fast with a minimal number of steps. The procedure should also provide clean extract and eliminate interferences. Secondly, to develop and validate a high throughput, specific and sensitive LC-MS/MS method for the quantitative determination of EtG and EtS in postmortem blood.

2. THEORY

2.1 Quantification of drugs in biological samples

Chromatographic separations of biological samples can be obtained by different techniques. The most commonly used separation techniques are gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE). The use of LC in combination with sensitive and specific tandem mass spectrometry (MS/MS) can be considered as being one of the most important techniques in forensic toxicology [46].

LC-MS/MS play an important role in forensic analysis due to their possibility for identification based on the mass to charge ratio (*m/z*) and to use isotope labelled compounds as internal standards [47].

Most separations of drug or organic compounds can be achieved in reversed phase (RP)-LC. RP chromatography employs a non-polar stationary phase and polar solvent. Typically, an acidic or basic solution is used as mobile phase and acetonitrile (ACN) or methanol (MeOH) as organic modifier. Stationary phase consist of micro porous silica with chemical bonded groups where C_{18} is widely used. Retention time can be manipulated by changing mobile phase compositions, pH, column temperature and/or non-polar stationary phase characteristics [48]. UPLC HSS-T3 alkyl phase are RP C_{18} columns.

2.1.1 Ultra High Performance Liquid Chromatography

Standard HPLC typically use column particles with size about (3 – 5 µm) and pressures around 400 bar. Whereas Ultra High Performance Liquid Chromatography (UHPLC) use specially designed columns with very small particle size (1.2 µm). In UHPLC high pressure, up to 1000 bar is observed when the mobile phase is forced through the column. High-resolution separations are obtained in UHPLC. The three main issues in the UHPLC field are sensitivity, selectivity and speed. These factors can contribute to rapid and high quality levels of results. UHPLC systems are faster, smaller volumes of organic solvents are utilized compared to standard HPLC. Newer technology of UHPLC is being developed to use column particles with 1µm size, and pressure potentials up to 6,800 bar [49].

2.1.2 Particle size and chromatographic efficiency

By using smaller particles, speed and peak capacity can be extended to new limits. Van Deemter's equation (plate height equation) for LC separation illustrate the importance of reducing the particle size to increase the chromatographic efficiency, equation 1 [50]

A-term B-term C-term

Eddy diffusion Longitudinal diffusion Resistance to mass transfer

$$H = 2\lambda . d_p + \frac{2\gamma . Dm}{u} + \frac{f\,(k).dp^2\ .u}{D_m} \qquad \text{Eq 1}$$

Substituting eq. 1, the reduced van Deemter eq.2 becomes

A-term B-term C-term

$$H \approx A . d_p + \frac{B}{u} + C . d_p^2 . u \qquad \text{Eq 2}$$

Where:

H: plate height and it's proportional to the variance of a chromatographic band

λ: packing factor

d_p: particle size

γ: obstruction factor

f(k): function of the retentions factor

u: mobile phase linear flow rate

D_m: diffusion coefficient of the solute in the mobile phase

The reduction of the particle size can affect both eddy diffusion (A-term) and resistance to the mass transfer (C-term) as described by eq.1 and eq.2. The use of small particles size result in high plate number (N) as well as faster separations and it is one of the best solutions to improve chromatographic performances. Figure 3 illustrate the relationship

between particle size, flow rate (linear velocity) and plate height for columns packed with various particle sizes.

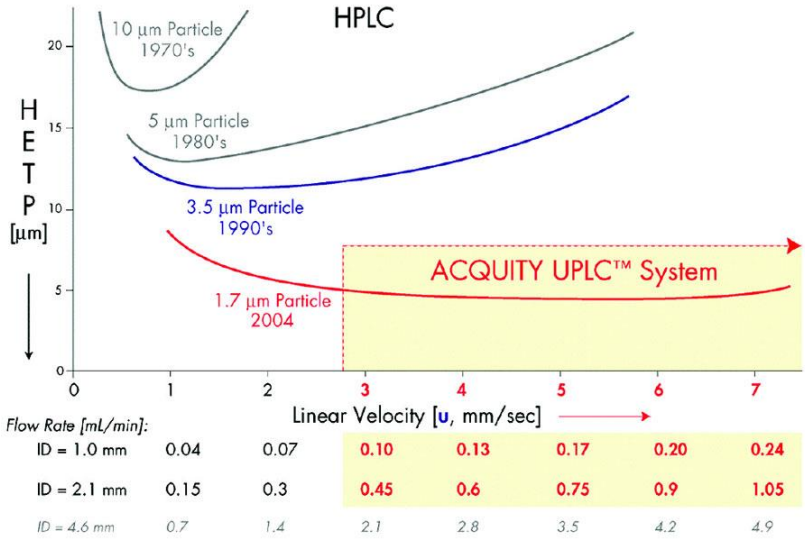


Figure 3: Plate height as a function of flow rate for stationary phases with different particle diameters [49].

As presented in figure 3 the curve is less affected by higher column flow rates and it becomes flatter with smaller particle size. By using smaller particles, speed and peak capacity can be extended, less peak dispersion and also more uniform flow through the column is obtained. The analyte spends less time inside the particle where peak diffusion can occur [50]

2.3 Mass spectrometry

A mass spectrometer consists of the following elements, as illustrated in figure 4. A sample inlet to introduce the compound that is analyzed and to produce ions from the sample. The ions are separated according to their mass to ratio by one or several mass analyzers. Ions from the last analyzer are detected in the detector.

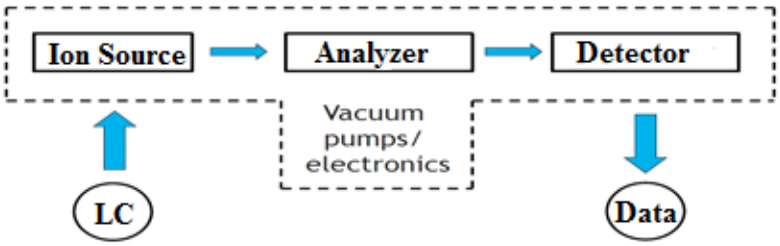


Figure 4:Scheme of Mass Spectrometry.

2.3.1 Electrospray ionization mass spectrometry

Electrospray ionization (ESI) is a soft ionization technique and provides little fragmentation. The analyte is ionized at atmospheric pressure in the ion source, forming protonated [M+1] or de-protonated [M-1] respectively for positive or negative mode. Negative mode is less sensitive. An exception is where the nature of the analyte leads to the formation of stable anions, such as carboxylic acids [51].

The electrospray process is based on the production of a fine mist of ionized droplets of sample solution by applying a strong electric field (Figure 5). The spray passes through the outlet of a small tube (capillary) with a weak flux (normally 1-10 µL min $^{-1}$), under atmospheric pressure in the presence of a warm nitrogen flow. The electric field is obtained by applying a potential difference of 3-6 kV between the capillary and the counter electrode. Typically, nitrogen gas is used in nebulization process. Nitrogen is also used as an assist to evaporate the solvent from the droplets. The droplets become smaller and the charge density increase, then the droplets breaks down due to the instability. This process produces de-solvated ions and allows them to enter the mass analyzer [52, 53].

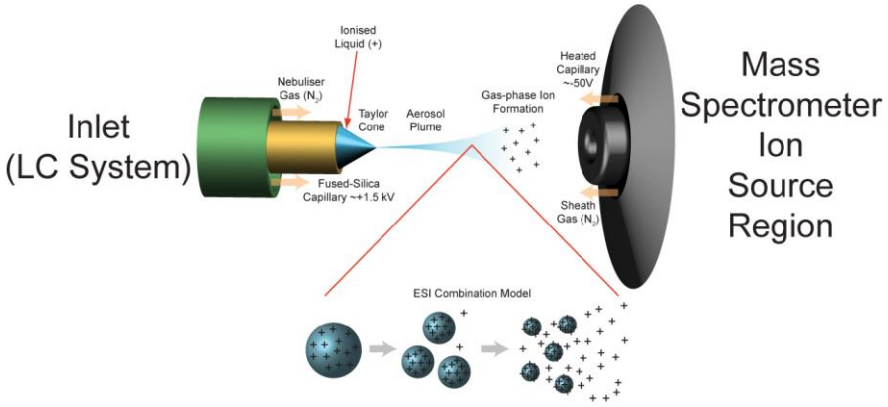


Figure 5: Schematic of the positive elektrospray ionization (ESI) [54]

2.3.2 Triple quadrupole instruments

The triple quadrupole (QqQ) system consists of a linear combination of a linear combination of three quadrupoles mass analyzers. Q1 and Q3 are working as mass filters to separate a specific precursor ion of interest. Q2 is reaction region acting as collision cell in RF- only mode, which transmits all incoming ions from Q1 to Q3. Q2 is placed in a partially closed area filled with collision gas, where a noble gas is used. Triple quadrupole instruments provide high sensitivity and selectivity for quantitative analysis.

2.3.3 Tandem mass spectrometry with multiple reaction monitoring

Multiple reactions monitoring (MRM) is the most common mode for using a triple quadrupole. A triple quadrupole can monitor a large number of transitions simultaneously [48]. By analyzing only a few selected ions, the dwell time for each ion is increased, improving the single to noise ratio [55]. The forensic field uses multiple MRM transitions per analyte in order to confirm the presence of the desired compound, typically two transitions per analyte, but in some cases three. As illustrated in Figure 6 the first quadrupole (Q1) selects a specific precursor ion of a given *m/z*. The second quadrupole act as a collision cell and is called Collision Induced Dissociation (CID). The resulting product ions are mass analyzed by using the third quadrupole (Q3) where only a specific *m/z* is allowed to pass. All other product ions are filtered out in Q3. This detection mode is powerful, especially with complex matrices such as biological samples, where sensitivity and selectivity are particularly important.

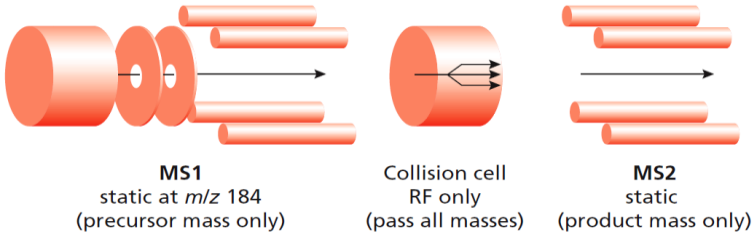


Figure 6: Single/ multiple reaction monitoring [56].

2.3.4 Matrix effect in LC-MS/MS methods

The phenomenon of ion suppression and ion enhancement is one of the most challenging issues in LC-MS/MS analysis, known as matrix effect [57]. Matrix effect is also compound dependent. Earlier studies has shown that the most polar compounds are most effected by ion suppression [58].

Phospholipids have been found to cause significant matrix effects [57]. Phospholipids are a major component of all cell membranes. The total concentration of all phospholipids in human plasma is about 1.6 -3.0 mg/mL[59]. The molecular structures of phospholipids consist of two major functional groups. A polar head group substituent, which includes an ionizable organic phosphate and one/two long chain fatty acids ester groups, which create hydrophobicity in the molecule. Characteristic fragment ions are formed by the high energy ion-source at *m/z* 184. These ions can be monitored by different analytical methods in order to evaluate matrix effect. Due to the high ionic nature of phospholipids, they are responsible

for influencing the ionization in electrospray MS sources. Thus, elimination of coeluting substances is necessary to obtain a robust HPLC-ESI-MS/MS method [60].

2.4 Sample preparation

The choice of the sample preparation is the most critical step in the whole analysis. An ideal routine sample preparation is simple, fast with a minimal number of steps. Good and successful sample preparation needs to be carefully designed. Strategies in this step should lead to extraction of the target analytes from the biological matrix with high recovery and produce clean samples containing only low levels of interfering substances from the matrix.

2.4.1 Pre-treatment of postmortem blood

In forensic post mortem blood analysis, only whole blood is obtained. Whole blood is a complex matrix and it should be handled such as plasma and serum. The only difference is presence of red blood cells. Postmortem blood collected at autopsy is notably different from ante mortem blood. Postmortem blood may contain 60-90% water, and is subject to different degrees of haemolysis. Postmortem blood are more viscous, contain small clots or sediment cells. The pH value in postmortem blood can be as low as 5,5 due to protein degradation [61]. In addition, limited sample volume is received in some cases. Hence specific protocols for sample preparation are mandatory.

2.4.2 Protein precipitation technique

Presence of endogenous compounds such as proteins, phospholipids and other interfering compounds in the sample can cause significant problems in glucuronide analysis [60]. The EtG molecule is approximately 50% protein bound, which may negatively affect the absolute method recovery [62]. Analyte binding to proteins can be disrupted by organic solvent, acid, inorganic salt or metal ion treatment [60]. In routine analysis there is a tendency to minimize the sample preparation steps. Protein precipitation technique (PPT) is one of such fast, easy to automate and quite popular especially for plasma preparation. The solubility of proteins is considered by four variables, pH, ionic strength, temperature and protein concentration. PPT is utilized by modifying one or more of these parameters. Precipitation with an organic solvent such as acetonitrile (ACN), methanol (MeOH) or ACN-MeOH mixtures is most commonly employed [62].

To obtain an efficient protein removal, at least an addition of an organic solvent in 3:1 (v/v) ratio to the sample is required. Precipitation with ACN can remove > 97% proteins, while

methanol removes ~ 94%. Unfortunately, sample obtained by PPT may contain higher amount of matrix compounds compared to SPE and LLE procedures. Matrix compounds that remain in the final extract leads to increased variability between samples and can cause matrix effects [41, 42]. PPT removes large degree of proteins, but phospholipids are remaining in the final extract.

2.4.3 Hybrid-SPE-PPT

Hybrid SPE-PPT is the combination of the protein precipitation technique (PPT) and solid phase extraction (SPE). This technique provides phospholipids free extracts. The advantages of Hybrid SPE-PPT are simplicity, easy automation and fast in the cleanup process. The proteins in the biological samples are precipitated with an organic solvent, and the supernatant is passed through the filter plate cartridges (SPE). The bed sorbent contains a low porosity hydrophobic filters which removes both proteins and phospholipids from the sample [63].

The mechanism underlying the phospholipids removal by using filter plates is based on Lewis acid-base interactions between the zirconium (Zr) coated silica particles and the phosphate group of phospholipids (Figure 7). When the sample is passed through the packed sorbent of the filter 96-well plate which contains Zr atoms, the phospholipids are removed efficiently from the sample through strong interactions between the phosphate groups (Lewis base) and Zr atoms (Lewis acid) bonded to the silica surface [63].

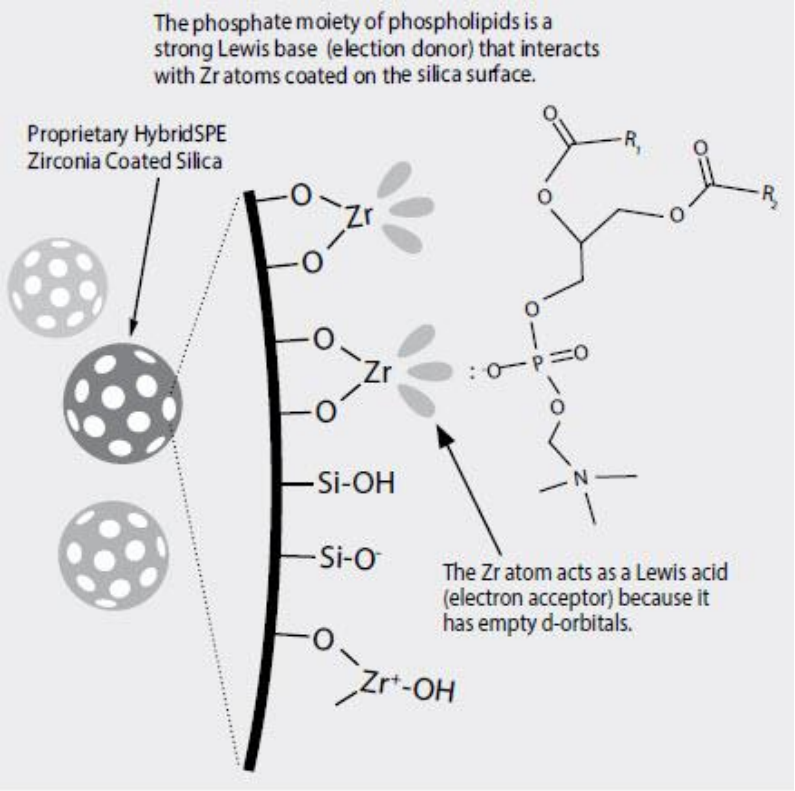


Figure 7: Mechanism of HybridSPE [63]

2.4.4 Other extraction techniques

In addition to simple PPT approaches, there is a recent increase in the development of more selective strategies based on solid phase extraction (SPE) and liquid –liquid extraction (LLE).The mechanism of SPE extraction is based on an analyte binding with a great affinity to the sorbent. SPE require several steps, conditioning, loading of the sample, washing and elution of analytes. The analytes in the sample solution bind with greater affinity to the solid surface than the matrix compounds, washing the sorbent to remove various interference that are less strongly bound to the sorbent than the analyte of interest. The analyte will be eluted from the sorbent by using a stronger elution solvent (organic solvent) and collect them for further analysis. The main goal with SPE method is to obtain high reproducibility, satisfactory recovery and clean extract.

Sometimes low recovery of hydrophilic compounds and instability of analyte in elution solvent is observed. Precipitation and high viscosity of samples can leads to column blockage and variability in column packaging [62].

LLE is an extraction of an analyte from one liquid phase to another liquid phase (a two-phase distribution of a solute), usually water and an organic solvent. The mechanism underlying LLE is based on transferring the analyte from matrix compounds to an appropriate liquid phase.

2.4.5 Stable isotopic labeled internal standards

The use of internal standard is recommended in quantitative MS analysis and isotope labeled compounds are recommended/most often used [52].

Stable-isotope-labeled internal standard (SIL-ISTD) is routinely used in forensic toxicology to correct for matrix effects during LC-MS/MS analysis. SIL-ISTD internal standards are compounds in which several atoms in the analyte are replaced by their stable isotopes. For instance, hydrogen (^{1}H) is exchanged with deuterium (^{2}H), carbon molecule (^{12}C) is exchanged with ^{13}C. During labeling at least three atoms should be exchanged. When the difference is less than three mass units the isotope peaks of the analyte may interfere with the signal of the internal standard. Furthermore, the SIL-ISTDs should behave nearly the same way as the analyte of interest, with identical retention times and similar behavior in the ion source. The use of SIL-ISTDs can compensate for loss of analyte in the sample preparation steps and for possible matrix effects in the MS source. It is important to add the ISTD as early as possible in the procedure, in order to obtain maximum precision. To avoid ion suppression obtained from ISTD, the concentration of ISTD must be appropriate and it should be tested within the calibration curve concentration range [64, 65]. Molecular structures of EtG and EtS and their labeled internal standards used in this study are shown in Figure 8.

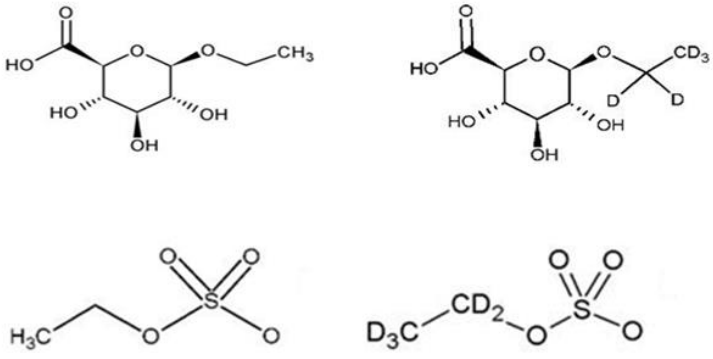


Figure 8: Molecular structurs of EtG and EtS and their deuterium labelled internal standard [66].

3. EXPERIMENTAL

3.1 Chemicals and materials

The reference substances for calibrators and QC samples were for both EtG and EtS obtained from Lipomed (Arlesheim, Zwitzerland) and Cerillant (Round Rock, TX, land). EtG-d5 was obtained from Cerillant and EtS-d5 from Lipomed as 1 mg/mL solutions. Ammonium acetate, 32% ammonia and acetic acid were provided by Merck, and ammonium format from Prolab (Briare, France). HPLC grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany) Heptane and formic acid were purchased from BHD Prolab (Briare, France). Water was purified by filtering deionized water on a Milli-Q filtration system (Millipore, Bedford, MA, USA) and Laboratory pH meter, Lab 870 from SCHOTT instruments GmbH (Mainz, Germany) was used for pH adjustments.

3.2 Preparation of solutions

3.2.1 Stock solutions, calibrators and quality control samples

The stock solution for EtG and EtS were prepared by dissolving a suitable quantity of the standard compound in pure methanol. Stock solutions were further diluted to prepare working solutions in methanol. Calibrator and quality control (QC) samples were prepared by diluting suitable amounts of each working solution with Milli-Q water. Working solutions for calibrator and QC samples were further diluted to the concentrations provided in Table 2 respectively for calibrators and QC samples. All stock and working solutions were stored at -20°C. Calibrators and QC samples were stored at 4°C until empty.

Table 2:Analyte concentrations in calibration solutions and control samples.

	Ethyl glucuronide(µM)	Ethyl sulfate (µM)
Calibrator 1	0.40	0.20
Calibrator 2	1.00	0.50
Calibrator 3	5.00	2.50
Calibrator 4	35.00	17.50
Calibrator 5	100.0	50.00
QC_{low}	0.6	0.3
QC_{heigh}	80	40

3.2.2 Internal standard

Internal standard (IS) stock solution of EtG and EtS (1mg/mL) were prepared in MeOH for each compound. From stock solutions, IS solution was prepared with concentrations presented in Table 3 in Milli-Q water and stored at 4 °C until empty. Internal standard stock solutions were stored at -20°C.

Table 3: Internal standard concentrations

	Ethyl glucuronide d5 (µM)	Ethyl sulfate d5 (µM)
Internal standard	8.5	4.5

3.2.3 Buffers and mobile phases

Ammonium acetate buffer

5 mM ammonium acetate solution was prepared by dissolving 3.85 g of ammonium acetate in 1000 mL Milli-Q water. The pH was adjusted to 5 by adding acetic acid.

Ammonium format buffer

5mM Ammonium format buffer solution of 5 mM was prepared by dissolving 0.50 g of ammonium format in 1000 mL ultra-grade water. The pH was adjusted to 3.1 with 32% ammonia.

0.1% formic acid and 0.2 % formic acid

The solution was prepared by adding 1 mL formic acid (FA) to 1000 mL ultra-grade water for 0.1% and 2 mL FA in 1000 ml ultra-grade water for 0.2% FA. The pH was measured to be 2.6 and 2.5, respectively. FA solutions stored at room temperature for 5 weeks gave no change in EtG and EtS during analysis.

3.3 Instrumentation

3.3.1 LC and MS/MS conditions

An Acquity UPLC module (Waters Corp., Milford, MA, USA) was used for separation with either a Quattro Premier XE detector or Xevo TQS. Electrospray (ESI) ionization source in negative mode was employed. Chromatographic separation was carried out using an ACQUITY UPLC HSS T3 C_{18} Column (2.1 mm X 100 mm, 1.8 µm particular size) coupled to a guard column (2.1 mm X 50 mm) with a flow rate of 0.4 ml/min and column temperature of 65 °C. The injection volume was 0.5 µL. Instrument parameters are presented in Table 4.

Table 4: Instrument parameters

Injector	Mode	Partial loop with needle overfil
	Injected amount	0.5 µL
	Loop volume	1.0 µL
Solutions	Weak wash	5% MeOH
	Strong wash	90% MeOH
	Seal wash	20% ACN
	Washing solvent	30% MeOH
Sample	Solvent	0.1% ACN + 0.1% FA in ultra-grade water
	volum	150 µl
Mobile phase	Buffer	0.1% Formic acid
	Organic modifier	MeOH
	Gradient program:	Initial 99% A
		2:00 80% A
		2:01 10% A
		3:00 10% A
		4:00 99% A
	Flow	0.4 ml/min
Column	Stationary phase	HSS T3 C_{18}
	Length	100 mm
	Inner diameter	2.1 mm
	Particle size	1.8 µm
	Temperature	65 °C
Guard column	Stationary phase	C_{18}
	Length	50 mm
	Inner diameter	2.1 mm
	Particle size	1.8 µm

3.3.2 Tuning and MS parameters

Intellistart software version 4.1 from Waters was used for tuning and optimization of mass spectrometer parameters. The system was tuned using a continuous 5 µL per minute infusion of EtG and EtS solution according to the tuning procedure. Infusion into the mass spectrometer was performed as follows. The mobile phase in combination with standard solution (calibrator 2) of EtG and EtS coming from the built in syringe pump at a flow rate 10 µl/min was mixed with mobile phase (200 µl/min) through a T-piece. Mobile phase with 0.1 %FA and MeOH were used for all analytes. Intellistart automated the optimization of

parameters for mass spectrometric detection and quantification. Negative ESI was employed to tune EtG and EtS. Desolvation gas flow rate, source temperature and collision energy were also optimized and displayed in Table 5. Other parameters such as MRM transitions, capillary voltage, and cone voltage are given in Table 6.

Table 5: MS/MS parameters

Mass analyzer	LM 1 resolution	2.87
	HM 1 resolution	15.1
	Ion energy 1	0.7
	Collision energy ()	MRM specific
	Collision gas flow (ml/min)	0.13
	Ion energy 2 (V)	0.9
	LM 2 resolution	2.75
	HM 2 resolution	15.0
Detector	Multiplier voltage (V)	560
MS- Source	Capillary voltage	1
	Capillary angle button adjustment	5.5
	Desolvation gas temperature (°C)	500
	Desolvation gas flow (l/h)	900
	Cone voltage	MRM Specific
	Cone gas flow (ml/min)	60
	Source temperature (°C)	150

Table 6: MRM transitions, cone voltages, collision energies, dwell times for EtG, EtS and their deuterated analogues.

Compound	Precursor ion *(m/z)*	Product ion *(m/z)*	Cone voltage (V)	Collision energy (V)	Dwell time (s)
EtG(Quantifier)	220.96	74.95	14	12	0.01
EtG (Qualifier)	220.96	84.96	14	12	0.01
EtG d_5	225.97	84.90	14	12	0.01
EtS (Quantifier)	124.90	80.10	16	14	0.01
EtS (Qualifier)	124.90	96.88	16	14	0.01
EtS d_5	129.97	97.85	16	14	0.01

3.3.3 Investigated UHPLC columns

To investigate the selectivity of the separation, different columns and mobile phases were tested. Different LC columns from different suppliers were tested with various mobile phases and organic solvents in order to determine the ideal mobile phase for the analysis of EtG and EtS. Three different RP UHPLC columns were tested (Table7).

Table 7: UHPLC column spesifications

	Waters Acquity UPLC High strength silica HSS T3	Waters Acquity UPLC Cortecs	Fortis Technologies Ltd
Chemistries	C_{18}	C_{18}	C_{18}
pH range	2-8	1-8	1-12
Particle size	1.8 µm	1.6 µm	1.7 µm

3.4 Method development

3.4.1 Blood samples

Blood samples containing 2 g sodium fluoride, 6 mL heparin and 10 mL water per 450 mL blood used for development and validation of the method, and were purchased from the blood bank at Oslo University Hospital (Oslo, Norway). Confirmation analysis of EtG and EtS in whole blood samples at NIPH are utilized in DUI cases and forensic autopsy cases. The DUI samples were received in 5 mL BD Vacutainer Plus Plastic Blood Collection Tubes (BD Vacutainer Systems, Frankling Lake,NJ, USA) containing 10 mg sodium fluoride and 8 mg potassiumoxalate. The forensic autopsy cases were received in 25 mL Sterilin tubes (Sterilin, Caerphilly, U.K.) containing 200 mg potassium fluoride.

Before spiking, blood samples were checked to be negative with respect to EtG and EtS using the same method used for the measurement of the real samples. Calibrators and QC samples were made by adding EtG and EtS working solutions at five different concentrations and two different concentrations (Table2).

3.4.3 Reversed phase SPE on Bond Elute Plexa

Bond Elute Plexa cartridge C_{18} is a polymer SPE material with a hydrophobic core. The following sample preparation was utilized on the Bond Elute Plexa cartridges C_{18}. To an aliquot of 200 µL blood was added 50 µL internal standard and mixed with 800 µL ACN. The

sample was vortexed at 1 min and centrifuged at 4500 rpm for 5 min. Then, 800 µL supernatant was tranfered to glass tubes and mixed with 25 µL formic acid. The SPE cartridge was conditioned with 1 mL 1% formic acid. The sample was decanted onto the SPE column and there after washed with 1 mL water. EtG and EtS were eluted by adding 1 mL 1% formic acid in methanol. The residue was evaporated to dryness under a stream of nitrogen at 50 °C. The extract was reconcentrated with 50 µL water/ACN/FA (99/0.1/0.1, v/v) solution and shaken for 1 min before injection of 3 µL into LC-MS/MS Quattro Premier XE detector or 0.5 µL to Waters Acquity Xevo TQ-S detector.

3.4.4 Ion- exchange SPE on Oasis MAX

The following sample preparation was utilized on Oasis MAX (Mixed- Mode Anion-eXchange) 96-well plate. To an aliquot of 200 µL blood was added 50 µL internal standard and mixed with 800 µL ACN. The sample was vortexed at 1 min and centrifuged at 4500 rpm for 5 min. Then, 800 µL supernatant was transferred to the glass tubes and mixed with 250 µL acetate buffer (pH 4). The MAX plate cartridge was conditioned with 1 mL MeOH followed by 1 mL water. The samples were decanted onto the SPE column and washed by 1 mL water. EtG and EtS were eluted by adding 1 mL 2% formic acid in methanol. The residue was evaporated to dryness under a stream of nitrogen at 50 °C. The extract was re-concentrated with 50 µL water/ACN/FA (99/0.1/0.1, v/v) solution and shaken for 1 min before injection of 3 µL into LC-MS/MS Quattro Premier XE detector or 0.5 µL to Waters Acquity Xevo TQ-S detector.

3.4.5 Deprotonation Procedure

Standard protein precipitation procedure was utilized by using different types of organic solvents and adjusting the pH levels of precipitating agents. One part of spiked blood sample was deprotonated by addition of different parts of organic solvents (Table 8) in the presence of 50 µL internal standard. Different volumes of organic solvent were evaluated during method development. A mixture of 1% formic acid in acetonitrile and other organic solvents with various mixture ratio were also evaluated. The samples were vortexed at high speed for approximately 1 min to mix the samples and to precipitate the proteins. Then, centrifugation at 4500 rpm for 5 min was applied. After centrifugation the supernatant was transferred to a 96-well Hybrid SPE filter plate for further extraction.

Table 8: Reagents and volumes used in PPT experiment.

Organic solvent	Blood volume	Organic solvent volume
Methnol	One volume	5 volumes
Methanol:ACN(50:50, v:v)	One volume	5 volumes
Methanol:ACN (85:15, v:v)	One volume	5 volumes
ACN	One volume	4 volumes
ACN:Heptane (85:15, v:v)	One volume	4 volumes
1% FA in ACN	One volume	3 volumes

3.4.6 Hybrid-SPE-PPT

Different Hybrid-SPE phospholipids and protein removal 96-well plates were tested to remove endogenous compounds from blood samples. Different 96-well filter plates were examined with various organic precipitation solvents (Table 8 and 9). 96-filtration plates were used without prior conditioning. The supernatants obtained from simple PPT were filtered through 96-well hybrid-SPE-PPT plates by using vacuum manifold. Then, the extracts were evaporated to dryness under a gentle stream of nitrogen at 50°C for methanol, 65 °C for ACN and 60 °C for other mixtures. The residues were reconstituted with 50- 250 µL of a mixture of water/ACN/FA (99/0.1/0.1, v/v), shaken for 1 min before LC-MS/MS injection.

Table 9: Filter plates and equipments used for hybrid-SPE

	Product	supplier
Hybrid-SPE cartridges	Captiva ND Lipids	Agilent
	Supelco Hybrid-SPE-Phospholipid-Technology	Sigma
	Ostro Protein Precipitation & Phospholipid Removal	Waters
	Phree Phospholipid Removal	Phenomenex
	ISOLUTE® PLD+ Protein and Phospholipid Removal	Biotage
Whirlmixer	Reax control / MSI	Heidolph
Nitrogen vaporizer	Turbo Vap LV	Zymark

3.5 Validated Hybrid SPE-PPT sample preparation method

Human blood of 100 µL were spiked with 100 µL calibrators /QC solution in the presence of 50 µL of IS solution. The samples were mixed for 1 min, after which 400 µL of ACN was added to precipitate the proteins. Following centrifugation, the supernatants were filtered through a 96-well hybrid-SPE-PPT Phree plate from Phenomenex (Torrance, CA, USA). The filtered samples were evaporated to dryness under a stream of nitrogen at 65 °C. The residue was dissolved in 150 µL water/ACN/FA (99/0.1/0.1, v/v) solution and shaken for 1 min before injection of 0.5 µL into the UHPLC-MS/MS.

3.6 Validation

The following parameters were considered for method validation: Linearity, limit of detection and limit of quantification, intra-day and inter-day accuracy and precision, specificity, matrix effect, recovery, carry over, robustness of the method and processed sample and freeze/thaw stability. In addition a method comparison was carried out comparing the developed Hybrid SPE-PPT method with the current PPT UHPLC-MS/MS method used at NIPH [34]. All validation parameters were evaluated using spiked blood samples.

3.7 Data analysis

Masslynx software version 4.1 obtained from Waters was used for instrument control, Data acquisition and processing.

4. RESULTS AND DISCUSSIONS

To our knowledge only a few LC-MS/MS methods for determination of EtG and EtS in biological samples have been published. With the exception of the method reported by Høiseth et al., in whole blood [34], there are no earlier publications on blood we have been able to find. The analysis in serum [43], dried blood spots [44], meconium [45], and hair [67]. Most research on EtG and EtS has been performed in urine [26, 35, 37, 39, 40].

In the following section the development of a UHPLC-MS/MS method for determination of EtG and EtS is presented and discussed.

4.1 Method development

The development of the method was performed using Waters Acquity UPLC with two different MS/MS instruments equipped with negative electrospray ionization (ESI) in the MRM mode. Various detection alternatives were investigated during the method development. The preliminary experiments were employed on Waters Acquity Quattro Premier XE detector. Forensic analysis by MS/MS in MRM mode requires the detection of minimum two transitions of each compound. Sometimes it is difficult to find the second transition for EtS by using LC-ESI-MS/MS. Due to the low intensity of the second transition of EtS, high sensitivity was necessary for quantification. Based on these reasons mentioned above, the final experiments and the method validation were performed on Waters Acquity Xevo TQ-S detector for improving detection selectivity and sensitivity. The second transition of EtS is about 10-fold increased using Xevo TQ-S. The system is equipped with Step-Wave ion optics, which increase the sensitivity and enhances both the signal and signal to noise when using negative ESI [49].

The chromatographic method was developed parallel to the development of the sample preparation procedure. Different RP-stationary phase and mobile phases were also tested.

4.1.1 Stationary phase consideration

UHPLC columns with small particles are expected to give better resolution and narrow peaks are obtained, which should translate to better sensitivity. In order to enhance the peak intensity and retention of EtG and EtS, three different RF C18 columns were evaluated using various chromatographic conditions (Table 7).

EtG and EtS are poorly retained in conventional RP chromatography. To overcome this issue, a polar end-capped 1.6 µm CORTECS C_{18} column was tested using different buffers and

different organic modifiers in order to enhance the retention and the peak shape. Satisfactory retention of EtS was obtained by using 0.1% formic acid in water as mobile phase A (MA) and MeOH as mobile phase B (MB) at a flow rate of 0.4 ml/min, but most importantly peak intensity for EtS was decreased when compared to the HSS T3 column. Based on this observation the selection of CORTECS column was discarded and Fortis C_{18} was evaluated.

The Fortis C_{18} column was examined with 0.1% and 0.2% FA as MA and MeOH or ACN as MB. Good retention was obtained on this column under chromatographic conditions used for method validation (0.1% FA and MeOH). In addition, symmetrical peak shape was obtained. The peak intensity of EtS, specifically, the second transition of EtS was 2-fold decreased when compared to the HSS T3 column. Thus, the selection of Fortis column was unsatisfactory. The reason for the change in the peak intensity of EtS is not known. Compared to the Cortecs and Fortis C18 columns, a better separation was obtained by using the HSS T3 C_{18} column (Figure 9). The current method for determination of EtG and EtS is performed on HSS T_3 C_{18} column [34]. Highly aqueous conditions are used for an adequate retention and an organic modifier is used to achieve adequate MS sensitivity. EtG and EtS were baseline separated within 2 minutes by using 0.1% formic acid in water as MA and MeOH as MB with a flow rate of 0.400 ml/min. The second transition for EtS using HSS is much higher compared to FORTIS and CORTEX columns (results not shown). Based on this observation, HSS T3 C_{18} column was chosen for the method validation and sample analysis of EtG and EtS.

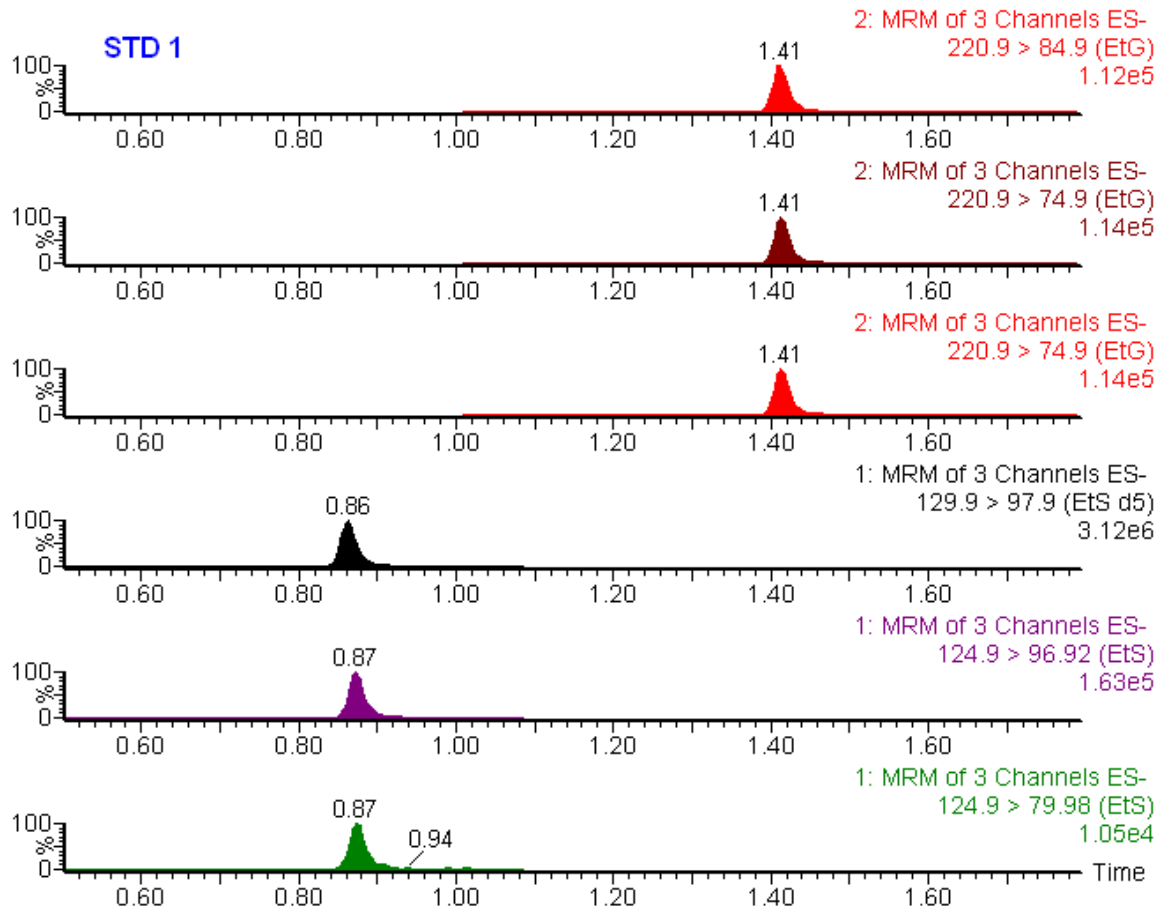
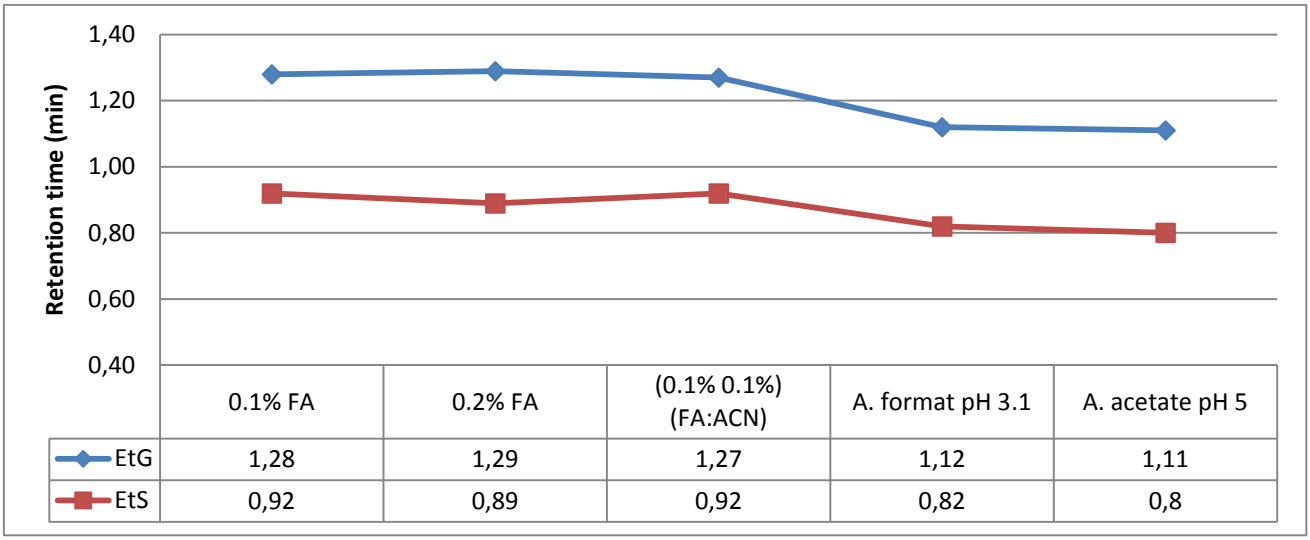


Figure 9: Ion chomatogram of EtG and EtS for the lowest calibrator separated on HSS T3 C_{18} column.

4.1.2 Acidic mobile phases with different pH values

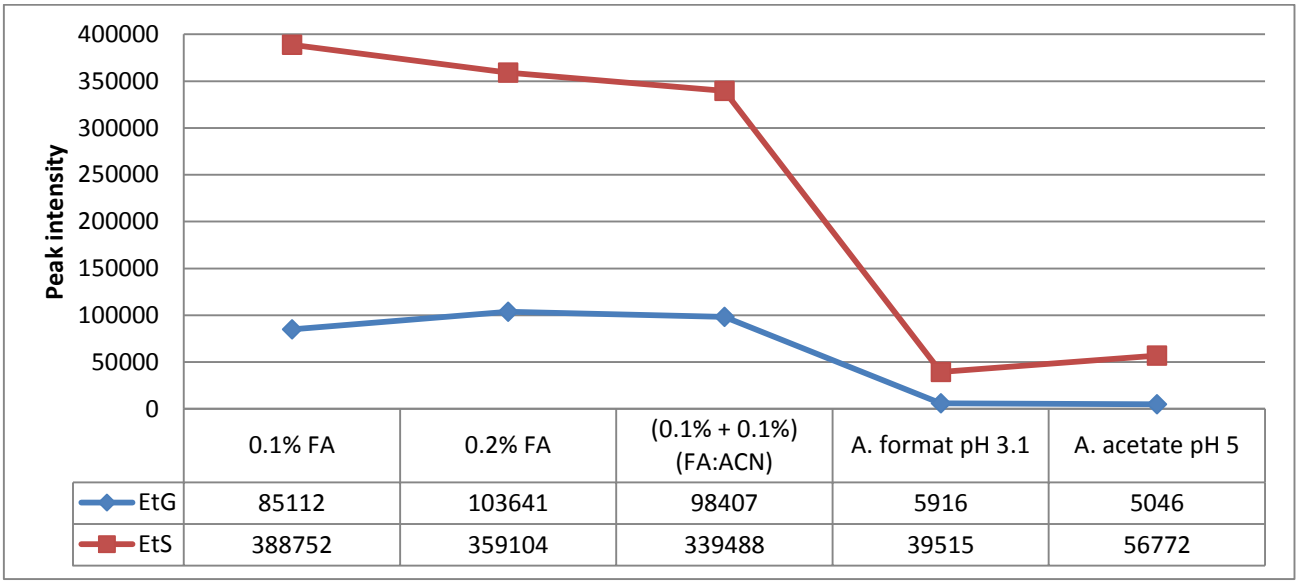
Separation of EtG and EtS on HSS T3 C_{18} column was examined with different mobile phases. EtG with reported pka value ~ 3 and EtS with pka value around (-3) do not interact strongly with C_{18} reversed phase. Thus, it is necessary to add formic acid to mobile phase to increase the retention on the column (Figure 10).The selection of pH values and organic modifier content in the mobile phases were based on the retention, peak shape and response of the EtG and EtS. The aqueous eluents investigated are shown in Figure 10. The selected mobile phases were tested for the separation of the analyzes from the blood under gradient elution. No major changes in the peak shape or retention time were observed using FA mobile phases in combination with MeOH. Small alterations in retention time were observed using ammonium acetate and ammonium formate buffer compared to FA mobile phases.



	0.1% FA	0.2% FA	(0.1% 0.1%) (FA:ACN)	A. format pH 3.1	A. acetate pH 5
EtG	1,28	1,29	1,27	1,12	1,11
EtS	0,92	0,89	0,92	0,82	0,8

Figure 10: Retention time of EtG and EtS using various acidic mobile phases and MeOH as organic solvent.

As shown in the Figure 11, the peaks intensity of EtG and EtS were significantly reduced using ammonium format and ammonium acetate buffer compared to formic acid. The reason for the dramatic reduction in the peak intensity was not further investigated. Based on this observation a mobile phase containing 0.1% FA and MeOH was chosen.



	0.1% FA	0.2% FA	(0.1% + 0.1%) (FA:ACN)	A. format pH 3.1	A. acetate pH 5
EtG	85112	103641	98407	5916	5046
EtS	388752	359104	339488	39515	56772

Figure 11: Peak intensity of EtG and EtS using different acidic mobile phases in combination with MeOH as organic mobile phase.

4.1.3 Optimization with organic solvents

MeOH and ACN were investigated as organic mobile phases (MB) in combination with 0.1% formic acid (MA). The comparison between ACN and MeOH as organic modifiers (MB), were performed on the HSS T3 C_{18} column. ACN has been reported to provide a better peak shape of glucuronides, higher MS response and better resolution among the structural glucuronide isomers [62]. ACN is a stronger eluent than MeOH in RP-LC [55]. Minor differences in the separation were observed using ACN, the peak shape of EtG was more symmetrical, and small alternation in the retention time was observed when compared to MeOH. Based on this observation, the selection of ACN as organic modifier was discarded. MeOH was chosen due to its low toxicity and enhanced retention of EtG and EtS.

4.1.4 Column temperature

Column temperature above ambient can contribute to faster separations, improved resolution and peak shape. Furthermore, higher column temperature contributes to decreased mobile phase viscosity and decreased column backpressure. In order to improve the retention time and the peak shape of EtG and EtS, different column temperatures were investigated. No major difference in the peak shape was observed. As expected, the retention time decreased with increased column temperature (Figure 12). A temperature of 45°C was the maximum column temperature recommended by the manufacturer, but long-term practical experience at NIPH has shown that the column can withstand 65°C [68, 69]. Based on satisfactory peak shape, resolution and retention of EtG and EtS, a column temperature of 65°C was utilized for method validation and further analysis of EtG and EtS.

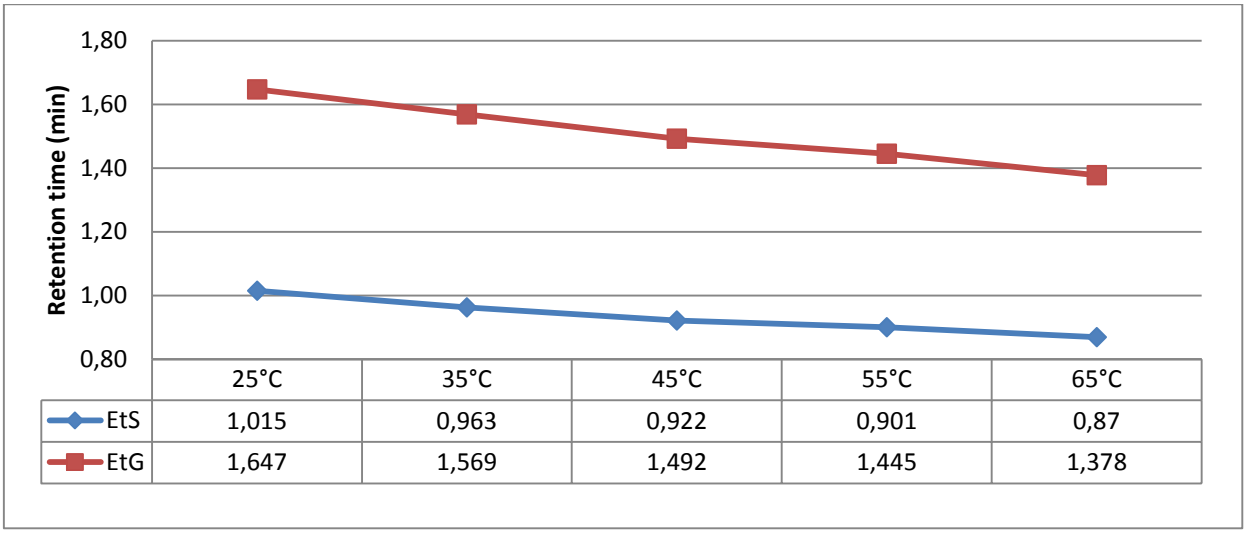


Figure 12: Retention time of EtG and EtS as function of different column temperatures.

4.1.5 Optimization of reversed phase conditions

The use of reversed phase conditions for the determination of EtG and EtS was quite successful. The retention of polar acidic compounds such as EtG (pKa ~ 2.84) and EtS (pKa ~ -3.14) was achived under highly aqueous conditions. Organic content (MeOH) in the mobile phase was reduced to 1%. The use of the mobile phase containing less organic solvent is undesirable due to the analyte ionization efficiency and hence, assay sensitivity will adversely be affected (data not shown). Furthermore, it was necessary to add formic acid to the mobile phase in order to increase the molecular ionization of EtG and EtS and hence improving the sensitivity.

The solvent strength of the injected sample is known to affect the peak shape. The organic content in the processed sample should be equal to or lower than that of the mobile phase [70, 71]. To evaluate the influence of sample solvent strength on analyte peak shape, two processed blood samples were prepared and assayed with two different diluting methods. ACN content in the prepared sample was about 60% after adding 400 µl ACN to precipitate the proteins in the blood. In the first prepared sample 40 µl of the filtered supernatant was diluted with 60 µl water/ FA (99/0.1, v/v). To further decrease the ACN content, the filtered supernatant was evaporated to dryness under a stream of nitrogen at 65 °C in the second prepared sample. The extract was redissolved with 150 µl water/ACN/FA (99/0.1/0.1, v/v) solution, and shaken for 1 min before injection of 0,5 µl into LC-MS/MS.

The peak shapes for the sample which was evaporated and redissolved was symmetrical and sharp. However, the peak shapes for the sample containing diluted filtered supernatant was not consistent, split and double peaks of EtG internal standard were observed. Based on these observations, it was concluded that less organic solvent in the injection solvent led to less variable, and hence more rugged chromatographic resultes. Based on this observation, the evaporation of the supernatant and redissolution with a solution with low content of organic solvent was mandatory for the method validation and further sample analysis of EtG and EtS.

4.2 Sample preparation

Blood/ postmortem blood is a complex matrix containing many compounds that may co-elute with analytes of interest when using traditional sample preparation procedures. Different PPT solvent agents (Table 8) in combination with Hybrid SPE 96-well plates (Table 9) were evaluated in the method development. The aim was to achieve sufficient recovery and minimize matrix effect. Furthermore, the sample preparation for determination of EtG and EtS in blood by UHPLC-MS/MS should be simple, fast and time saving with high throughput. To obtain a robust, selective and sensitive UHPLC-MS/MS method a sample preparation technique with high recoveries of the extracts and high ability to remove matrix compounds from the blood was desired.

4.2.1 Reversed phase SPE on Bond Elute Plexa

A pilot experiment of polymer SPE was performed on Bond Elute Plexa cartridges using the sample extraction procedure described in section 3.4.1. The extraction of EtG and EtS was difficult and no peaks were detected (data not shown). The observed results may be explained by the high percentage of the organic solvent in the supernatant and EtG and EtS may be washed into waste when the samples were decanted onto the SPE columns. Due to the time limitation, no further experiments of SPE were explored.

4.2.2 Ion- exchange SPE on Oasis MAX

In order to achieve minimum matrix effect and maximum recovery, another pilot experiment was explored on Oasis MAX weak anion exchange cartridges as described in 3.4.2. EtG and EtS have very hydrophilic natures with a pKa ~ 2.84 for EtG and pKa ~ -3.14 for EtS. Very low recovery from both analyzes were obtained (data not shown). This may be explained by

several parameters, EtG and EtS may be washed into waste when the samples were decanted onto the SPE columns, due to the high precentage of the organic solvent in the supernatant, or may they were very sensitive to the elution solvent. The elution with 1 mL of 2% FA in MeOH may not disrupt all the interaction mechanism between anion exchange group (N^+) and EtG and EtS. According to another article for determination of EtG and EtS in human urine, 3 mL of 6% formic acid in ACN was used to elute EtS and 2 mL of 2% formic acid in MeOH was used to elute EtG. Due to the lack of the time no further experiments were performed on Oasis MAX cartridges or on other types of commonly used anion exchange sorbents.

4.2.3 Deproteinization Procedure; Effect of the organic solvents

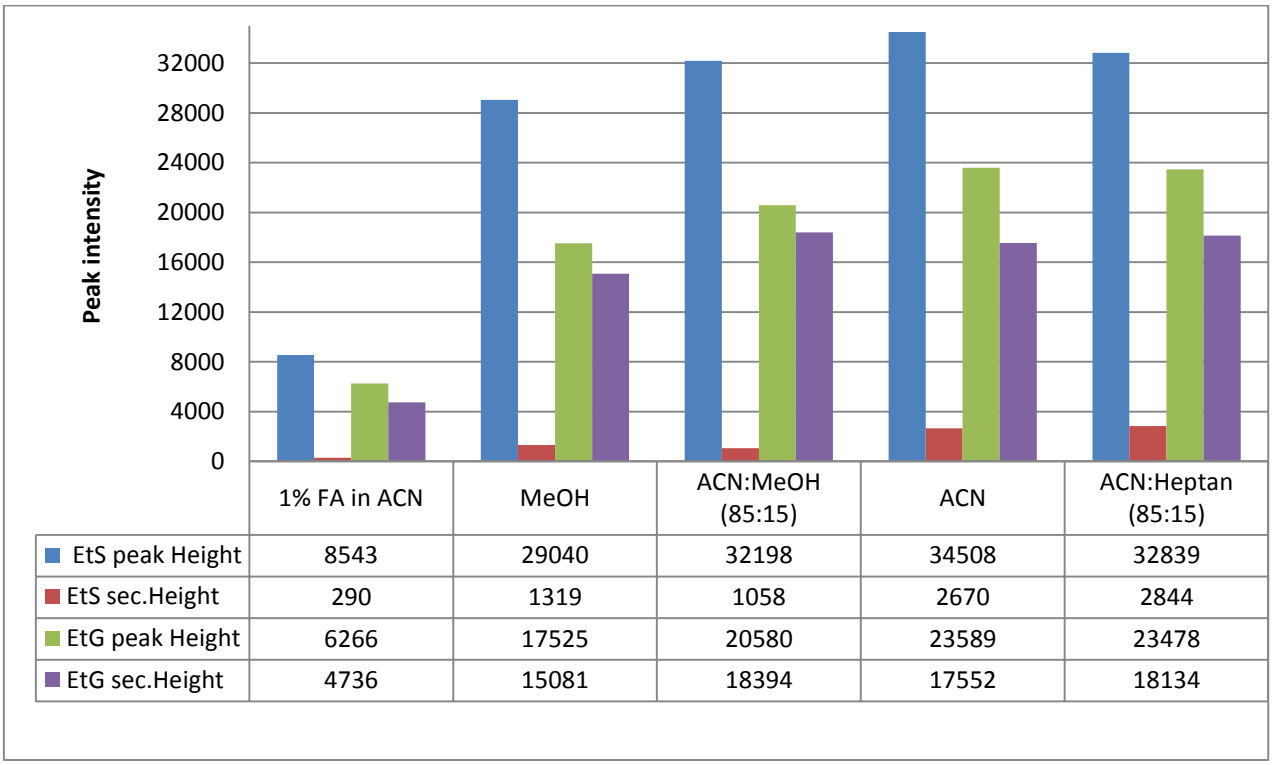
Protein precipitation does not result in a very clean final extract and none of the employed precipitation agents provides complete protein removal. Remaining proteins in the final extract are depended on the solvent agent and ratios evaluated [72]. In order to eliminate proteins and phospholipids and in addition to obtain satisfactory recovery, different PPT solvents have been utilized and compared. MeOH and ACN are the most commonly used organic solvents [72]. Different precipitating agents listed in Table 8 were examined. The precipitation procedures were carried out by using increasing volume of ACN and MeOH or mixtures, from 300 to 1000 µl. The sample volume, 100 µl, was kept constant in all the experiments. As expected less ACN was needed for efficient precipitation when compared to MeOH [72]. At least 5 volumes of MeOH were required for one volume of blood compared to 3 volumes of ACN.

Acidification of blood samples with 1% FA in ACN is typically used in combination with hybrid SPE sample procedure. As displayed in Figure 13, the presence of the formic acid causes low peak intensity of EtG and EtS. The second transition of EtS was adversely affected using 1% FA as precipitation agent. Using 1% FA in ACN as precipitation agent required a higher ratio of solvent (5:1, v/v) compared to pure ACN (Table 8).

PPT with MeOH or a mixture containing MeOH: ACN indicated lower performance/efficiency. The peak intensity of EtS observed from these MeOH containing solvents was decreased compared to ACN. EtS peak intensity was very sensitive to the amount of organic solvent and increased with decreased amount of MeOH.

PPT with ACN or a mixture containing ACN and heptan as precipitation agents were most efficient and satisfactory and were more promising than other mixtures examined. EtS peak intensity, especially the second transition was about three fold higher compared to MeOH precipitation.

The effect of the organic solvent on EtG and EtS peak intensity may be explained by several variables such as the concentration and hydrophobicity of the organic solvent may contributes to this effect. Based on this observation, ACN was selected for method validation and further analysis of EtG and EtS.



	1% FA in ACN	MeOH	ACN:MeOH (85:15)	ACN	ACN:Heptan (85:15)
EtS peak Height	8543	29040	32198	34508	32839
EtS sec.Height	290	1319	1058	2670	2844
EtG peak Height	6266	17525	20580	23589	23478
EtG sec.Height	4736	15081	18394	17552	18134

Figure 13: EtG and EtS peak height using only protein precipitation technique with different organic solvents as precipitation agent.

4.2.4 Phospholipids monitoring

In order to evaluate the presence of phospholipids and to overcome the contamination of the ion source, ionization suppression and protein build-up, that affects the accuracy and quality of the results. A pilot experiment was carried out after PPT only or PPT combined with different phospholipids removal plates (Hybrid SPE). All phospholipids and lysophospholipids generate an intense fragment ion at m/z 184 for the

trimetylammoniumethyl phosphate in the source, due to the collision induced dissociation (CID). Total phospholipids in blood samples were monitored using this characteristic MRM transition.the obtained results indicated that, there was a cluster of compounds that co-eluted with EtG and EtS (figure 14 A2).

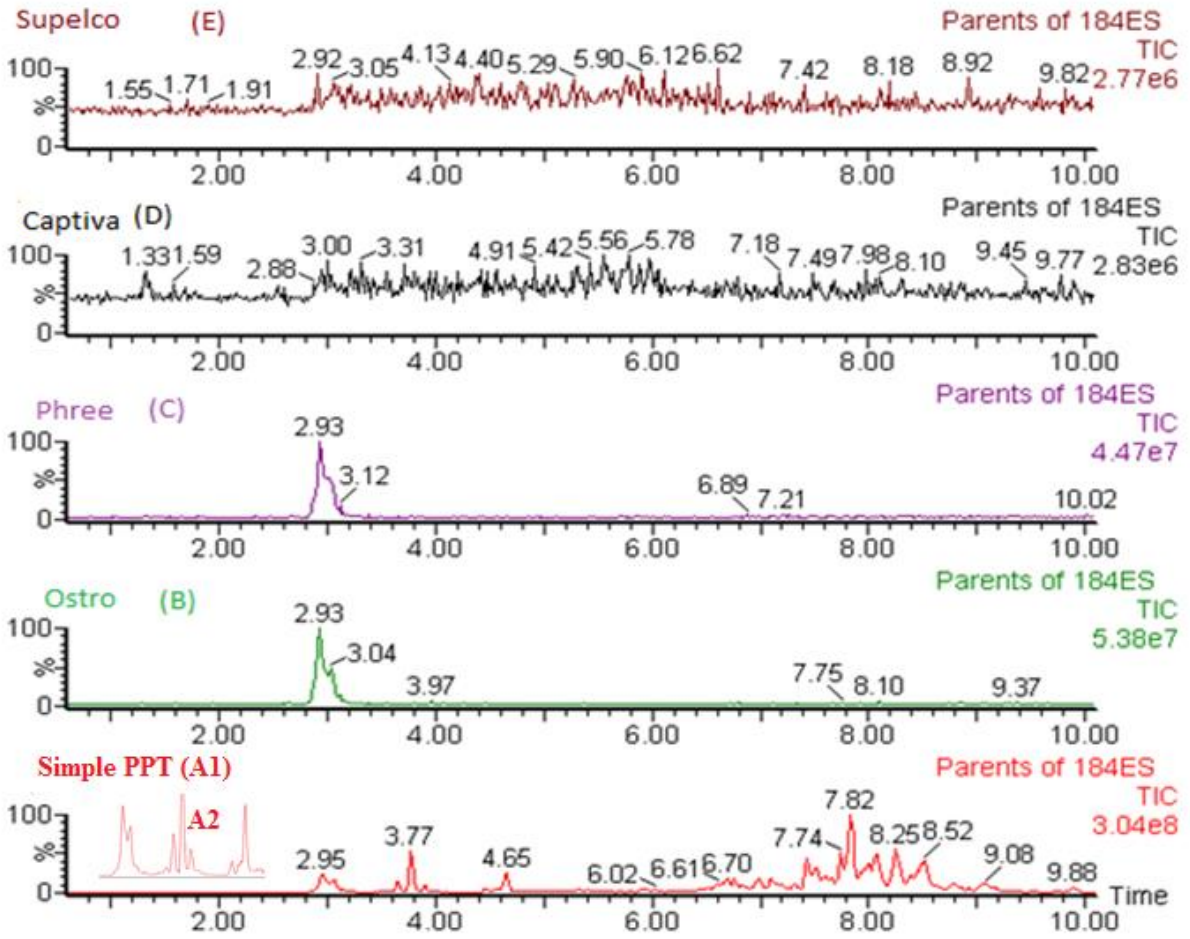


Figure 14:Positive ESI Total ion chromatograms of *m/z* 184 for protein precipitated blood sample before (A) and after (B) filtered through hybrid-SPE-PPT Ostro plate, (C) Phree plate, (D) Captiva plate, (E) Supelco plate. Isolute plate was not examined for phospholipids due to late delivery.

As displyed in the figur 14, the blank blood sample prepared by using simple protein precipitation demonstrates a significant amount of phospholipids and hence, further sample cleanup was needed. Proteins and phospholipids were removed using Supelco and Captiva, whereas Ostro and Phree filter plates provided also a significant elimination of phospholipids but with remaining one peak, which eluted after EtG and EtS. Isolute plate was not included in phospholipids monitoring experiments due to late delivery.

4.2.5 Hybrid-SPE-PPT: Removal of phospholipids during sample extraction

Further exploration of the sample clean up procedure was needed to develop a selective an d sensitive UHPLC-MS/MS assay for the determination of EtG and EtS in postmortem blood samples. In order to eliminate proteins and phospholipids, different Hybrid-SPE in the 96-well plates format were evaluated. Both in well and off line protein precipitation can be used in combination with Hybrid-SPE 96 well plate. Due to the complexity of the postmortem blood, off line protein precipitation was used in this assay. Sample procedure is shown in Figure 15.

Different phospholipids removal plates were evaluated in combination with different precipitation solvents. The results revealed that the recovery was dependent of the filter plate used. This may be explained by different interactions between analyte and filter plate sorbent. As presented in the Figure 16, Captiva provide a significant elimination of phospholipids. The results obtained with Captiva show low peak intensity of the second transition of EtS. EtG peak intensity was satisfactory. A possible explanation for the observed result may be strong interactions between the compounds and the sorbent.

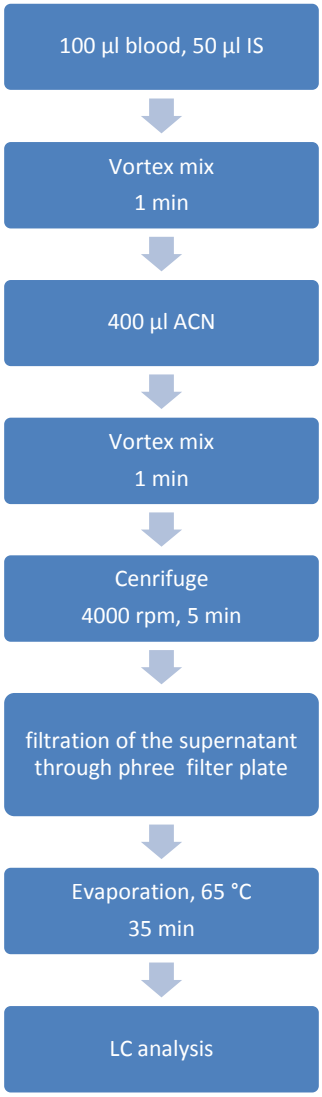
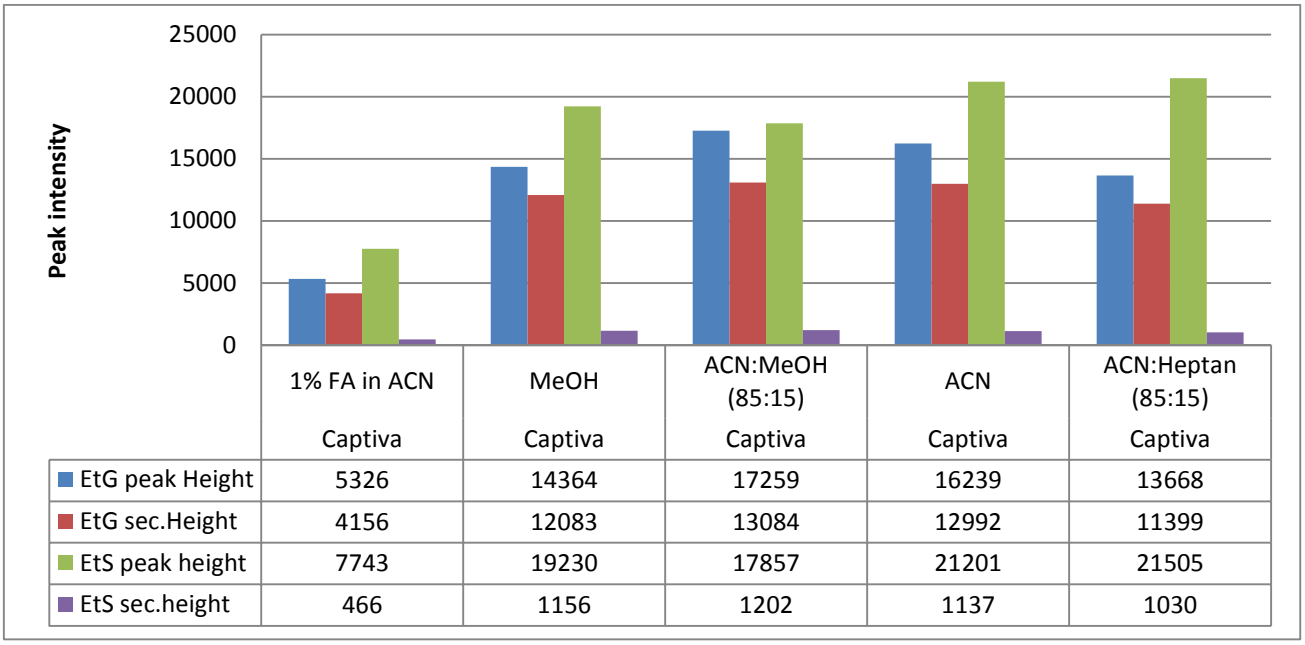


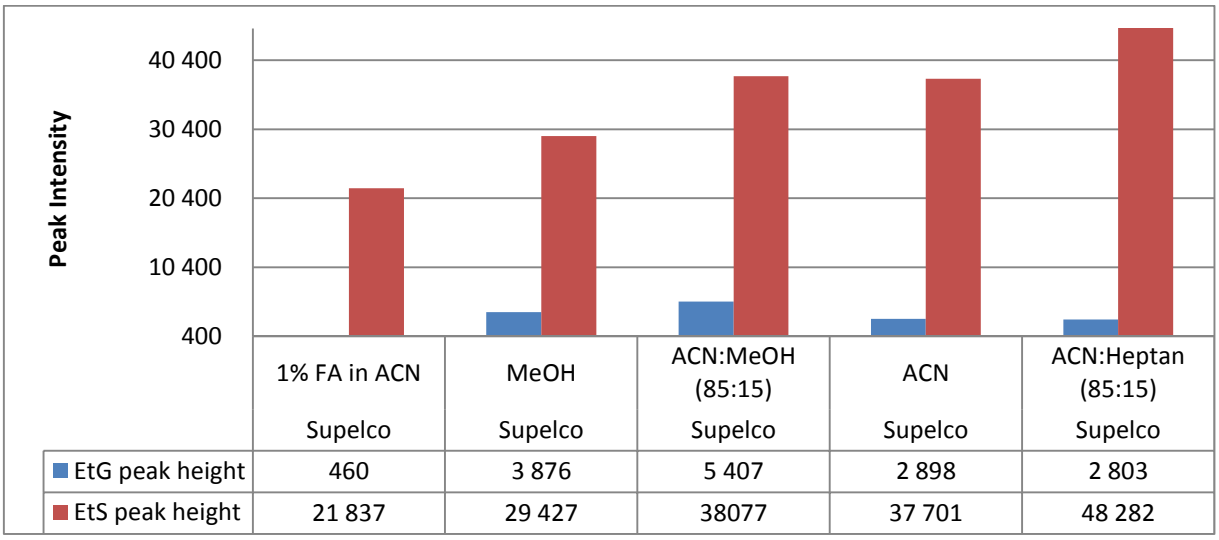
Figure 15: Depiction of basic sample preparation by using HybridSPE.



	1% FA in ACN Captiva	MeOH Captiva	ACN:MeOH (85:15) Captiva	ACN Captiva	ACN:Heptan (85:15) Captiva
EtG peak Height	5326	14364	17259	16239	13668
EtG sec.Height	4156	12083	13084	12992	11399
EtS peak height	7743	19230	17857	21201	21505
EtS sec.height	466	1156	1202	1137	1030

Figure 16: EtG and EtS peak height using hybrid SPE by Captiva plate in combination with protein precipitation technique with different organic solvents as precipitation agent.

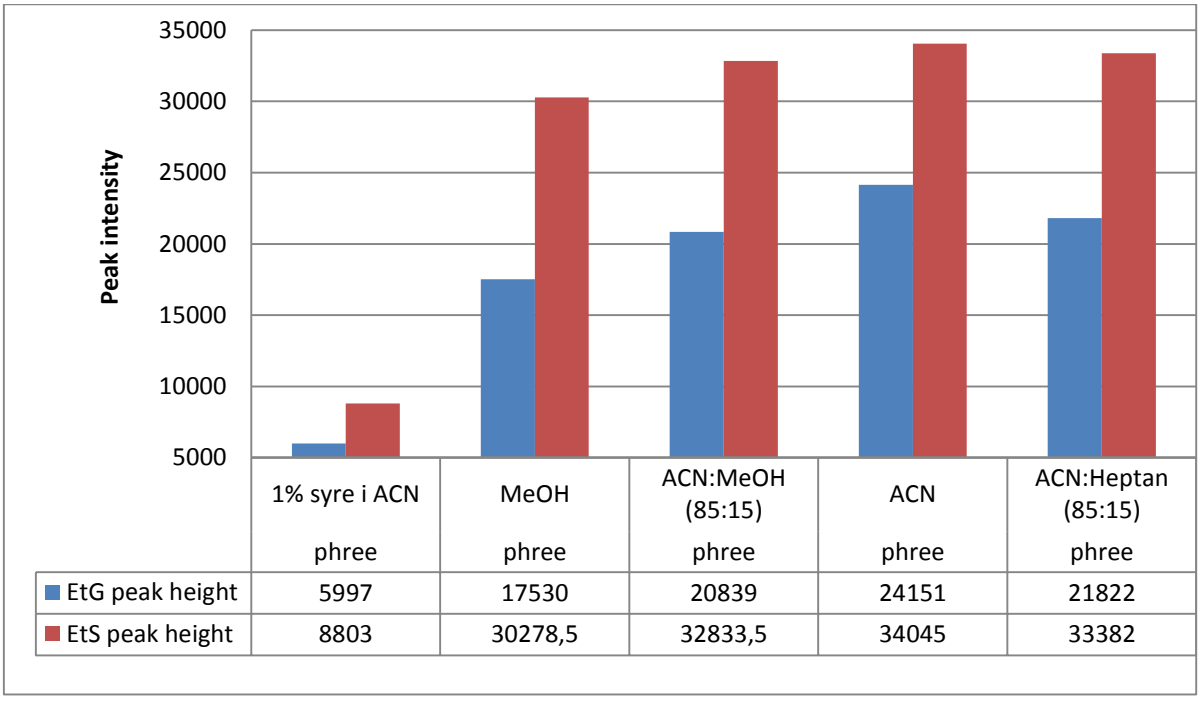
The obtained results on Supelco filter 96-well plate were unsatisfactory. EtG was strongly retained on the Supelco filter plate and very low recovery was obtained. This can be explained by several parameters. EtG are more than 50 % protein bound and the Supelco packed filter acts as a depth filter resulting in concurrent removal of both proteins and phospholipids. As displayed in Figure 17, EtG molecule was strongly interacted on Supelco bed sorbent and contributed to low recovery, whereas EtS peak intensity was satisfactory.



	1% FA in ACN Supelco	MeOH Supelco	ACN:MeOH (85:15) Supelco	ACN Supelco	ACN:Heptan (85:15) Supelco
EtG peak height	460	3 876	5 407	2 898	2 803
EtS peak height	21 837	29 427	38077	37 701	48 282

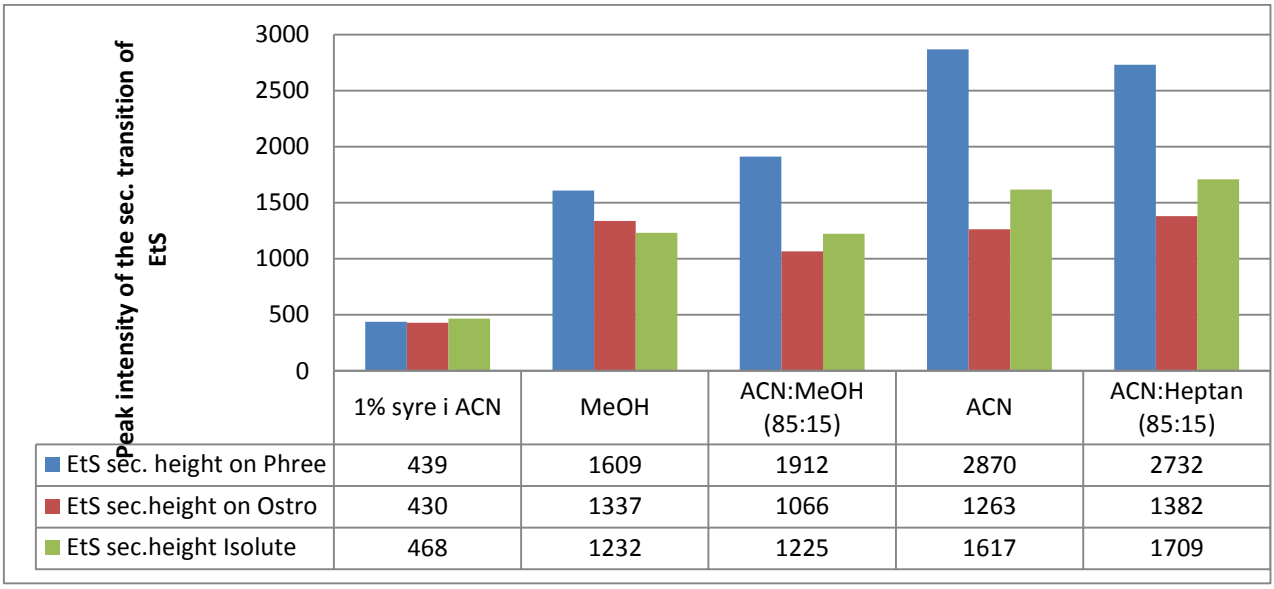
Figure 17: EtG and EtS peak height using hybrid SPE by Supelco plate in combination with protein precipitation technique with different organic solvents as precipitation agent.

Furthermore, sufficient sensitivity was obtained by using Phree, Ostro and Isolute 96-well plates (Figure18 and 19). Satisfactory sensitivity and phospholipids removal were achieved by using off-line PPT with ACN as precipitation reagent in combination with Phree, Ostro and Isolute Hybrid SPE 96-well plates. Phree filter plate in combination with ACN as precipitation agent was selected for method validation and further EtG and EtS analysis. The selection of Phree was based on higher recovery of EtS when compared to Ostro and Isolute (figure 19).



	1% syre i ACN phree	MeOH phree	ACN:MeOH (85:15) phree	ACN phree	ACN:Heptan (85:15) phree
EtG peak height	5997	17530	20839	24151	21822
EtS peak height	8803	30278,5	32833,5	34045	33382

Figure 18: EtG and EtS peak height using Phree hybrid-SPE in combination with protein precipitation technique with different organic solvents as precipitation agent.



	1% syre i ACN	MeOH	ACN:MeOH (85:15)	ACN	ACN:Heptan (85:15)
EtS sec. height on Phree	439	1609	1912	2870	2732
EtS sec.height on Ostro	430	1337	1066	1263	1382
EtS sec.height Isolute	468	1232	1225	1617	1709

Figure 19: Peak intensity of the secound transition of EtS using hybrid SPE by Phree, Ostro and Isolute plate in combination with protein precipitation technique with different organic solvents as precipitation agent.

4.2.6 Chromatographic separation of the analyte from phospholipids

Blood sample obtained from phree HybridSPE demonstrated a significant reduction of matrix effect when compared to PPT (Figure 20). However, it was observed significant phospholipids around the retention time of three. EtG and EtS were baseline eluted within < 2 min. The major peak around the retention time of EtG and EtS was about two fold decreased with filtration through Phree filter plate and no peak at the same retention times of EtG and EtS were observed in the chromatogram. Based on this observation, significant elimination in phospholipids by using HybridSPE was obtained. Hence, the HybridSPE-PPT procedure provided significantly cleaner extract with an efficient reduction of matrix effect due to the removal of phospholipids for polar analytes such as EtG and EtS.

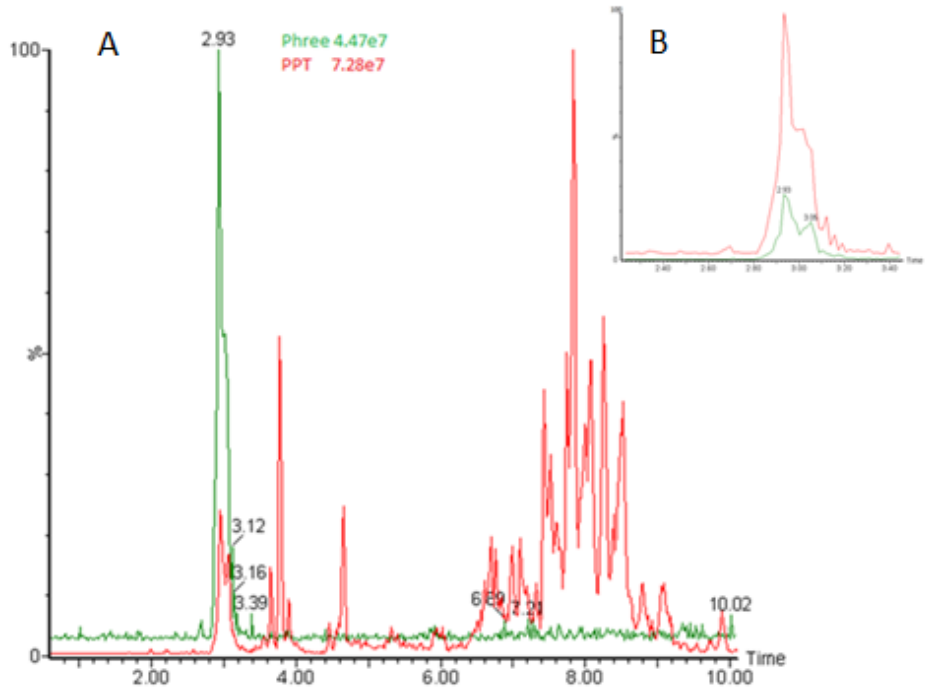


Figure 20: Total ion chromatogram (TIC *m/z* 184) of blank blood sample prepared by PPT compared to the same blood sample prepared using Phree HybridSPE (A). Significant phospholipids (Figure B) around the retention time of EtS 0.9 min and EtG 1.4 min (<3 min).

4.3 Mass spectrometry

4.3.1 Cone voltage and collision energy

Yen et al. has reported that a low cone voltage value contributes to higher sensitivity in glucuronide analysis [73]. Hence, the effect of the cone voltage was evaluated Cone voltages and collision energies for EtG and EtS were optimized by post column infusion as described in section 3.3.2. In addition increasing cone voltage values (12V – 40V) were examined and compared. No obvious differences in the peak intensity were observed (data not shown). The reason for this observation may be explained by different structures of glucuronides (O,N or ß) that behave different in the ion source. A cone voltage of 14 V was chosen for the method validation.

4.3.2 Desolvation gas flow

In order to increase the peak intensity of both compounds, a gas flow of 900-1000 ml/h was evaluated. The desolvation temperature was 500 °C. No differences in the peak intensity of EtG and EtS were observed at different desolvation gas flow.

4.3.3 Electrospray ionization in negative mode

Formic acid in various concentrations was added to the mobile phase to increase the molecular ionization of the compounds and improve the detection. Optimal ionization of EtG and EtS was achieved using 0.1% formic acid-water (MA) and in combination with MeOH as organic modifier at a flow rate of 0.4 ml/min. Lowering the content of the formic acid in the aqueous mobile phase resulted in decreasing ionization of EtG and EtS.

5. METHOD VALIDATION

The LC-MS/MS method was validated according to the international guidelines [74].All validation parameters were evaluated using spiked blood samples and calculations were performed using Excel software.

5.1 Linearity

A total of nine calibrators were used during method validation, but on routine base five calibrators are used. The lowest calibrators were prepared to 1/10 of the concentration level (cut-off) above which EtG and EtS, respectively, are reported as positive to the customers of NIPH (Table 2). The calibration curves were evaluated based on one assay with six replicates of each of the nine calibrators. The goodness of fit was established as the difference between the calculated calibrator value and its nominal value. The residuals should be < 10%. The linear ranges were based on the measurement of the peak area of the compounds versus the peak area of the corresponding deuterated IS analogue. Linear calibration curves with R^2 values ≥0,999 (Appendix) were achieved using a weighting factor (1/x) for both compounds and including the origin point for the concentration range (0.4-100 µM for EtG and 0.2-50 µM for EtS). Validation standards were analyzed to investigate the whole linear range from LOD to the maximum concentration. Increased residuals were observed at the lowest concentration range (below cut-off). The concentration range using five calibrators of each compound was able to cover the concentration range required for forensic toxicology.

5.2 Limit of detection and limit of quantification

The LLOQ (lower limit of quantification) was calculated as the QC concentration where RSD was ≤ 20% and bias was within ± 20% and with signal to noise higher than 10 for both transitions.

The LLOD (lower limit of detection) was determined by extracting dilutions of a low calibrator (figure 21). Blood samples were treated as ordinary spiked in five different lots of human blood. The calculation was based on signal to noise better than three for both transitions. The concentrations level of 0.3 µmol/L for EtG and 0.15 µmol/L for EtS were chosen as LLOQ. RSD and bias er summarized in Table 11. The LOD was set at 0.04 µmol/L for EtG and 0.02 µmol/L for EtS. In order to enhance LOD or to achive lower LOQ it is poosible to increase the injection volume.

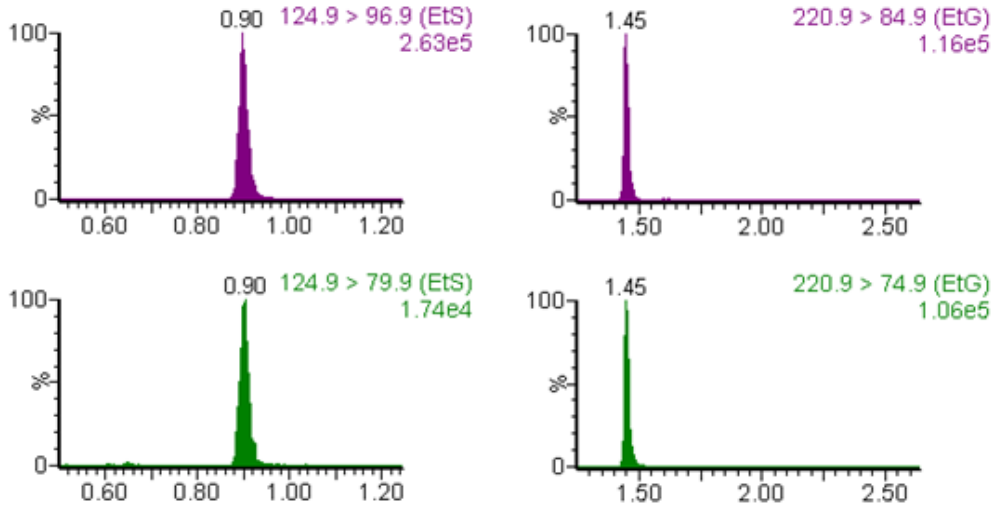


Figure 21: Representative MRM chromatograms of EtS and EtG of the lowest point of the calibration curve.

5.3 Intra-day accuracy and precision

The precision of the method was expressed as relative standard deviation (RSD). Validation samples were prepared in four different concentrations in blood samples Low, middle and high QC. Replicates (n=10) at each of the concentration levels were prepared in one assay. Accuracy given as bias was calculated as the percent deviation of the measured mean of the QC samples from the nominal concentration. Satisfactory accuracy and precision were obtained and summarized in Table 10.

Table 10: Intera-day accuracy and precision of EtG and EtS at different concentration levels.

	EtG				EtS			
Nominal concentration (µM)	0.3	0.6	40	80	0.15	0.3	20	40
Replicats (n)	10	10	10	10	10	10	10	10
Average concentration (µM)	0.31	0.64	34.2	77.7	0.15	0.30	17.9	36.2
Bias%	-3	6	-4	-3	0	-0.3	-10	-9
CV%	2.7	2.4	1.9	1.6	1.6	2.6	1.5	1.9

5.4 Inter-day accuracy and precision

The inter-day precision of the method was assessed by analyzing validation QC samples at four different concentration levels spanning from low to high concentrations. Eight replicates (n=8) at each concentration from eight independed assays were prepared by three different persons. Accuracy expressed as bias in percent and precision expressed as (relative

standard deviation) for QC samples. Satisfactory accuracy and precision were obtained and displyed in Table 11.

Table 11: Inter-day accuracy and precision of EtG and EtS at different concentration levels.

	EtG				EtS			
Nominal concentration (µM)	0.3	0.6	40	80	0.15	0.3	20	40
Replicats (n)	8	8	8	8	8	8	8	8
Average concentration (µM)	0.32	0.64	40.9	80.9	0,15	0.30	18.0	36.6
Bias%	6	6	2	2	0	0	-10	-8
CV%	6.5	3.7	1.3	1.7	2.4	4.3	8.6	1.9

5.5 Specificity

The specificity of the method was investigated by analysis of blank whole blood in five different lots. In addition the method was tested using frequently found drugs (n=102). The chromatograms were evaluated for interfering peaks at the same retention time as the compounds. Only GHB-(Appendix) eluted at the same retention time as EtS. However GHB (*m/z* 105 —> 87.05) and EtS (*m/z* 124.9 —> 79.9) have different moleculare masses and MRM transitions and GHB did not interfere with the EtS determination. No interfering peaks from the 140 investigated compounds were observed at or close to the retention time of EtG and EtS.

5.6 Matrix effect

The matrix effect (ME) corrected with internal standard was evaluated using the post extraction approach at three different QC concentrations levels. Blank blood from(n=6) different lots of human blood obtained from local blood bank (Oslo university Hospital) were used.

Sample preparation was based on two sets. Set A consists of six extracts of the blank matrices with compounds of interest, added post extraction and compared to set B which consisted of four replicates with neat solutions containing equivalent amounts of compounds of interest prepared in the solution used for reconstitution. Internal standard (IS) was added after extraction and prior to evaporation. ME was calculated by comparison of peak area of sample spiked before (set A) and after (set B) extraction.

$$ME = \frac{[A]}{[B]} \times 100 \qquad \text{eq 3}$$

ME > 100 indicates ion enhancement and ME< 100 ion suppression.

As it is demonstrated in Table 12, low ME levels were observed without internal standard. Quantification with the use of internal standard yielded better results and the internal standard shown to be appropriate for a correct quantification in the assay.

5.7 Recovery

Recovery was evalueted at three different QC levels. The peak area of six blood blank samples, spiked with EtG and EtS were compared with the peak areas obtained for six blood samples where an appropriate EtG and EtS amount was added at the end of the extraction procedure. Satisfactory recovery for both compounds was obtained (Table 12).

Table 12: Matrix effect and extraction recovery

Compound	Conc. (µM)	Recovery (%)	ME (%)	RSD (%)	Recovery (%)	ME (%)	RSD (%)
EtG		Corrected with internal standard			No internal standard		
QC LOW	0.6	62	99	3	86	62	11
QC MIDDEL	40	62	100	3	70	76	7
QC HIGH	80	61	100	4	57	74	12
EtS							
QC LOW	0,3	77	103	3	94	62	10
QC MIDDEL	20	78	100	3	76	70	7
QC HIGH	40	79	100	2	66	70	13

5.8 Carry over

The carry over for the method was evaluated by preparing a calibrator with a concentration of three fold of the highest calibrator followed by injecting of two extracted matrix blanks run consecutively after the calibrator. The carry- over was calculated by measuring concentration of the blank sample versus concentration of the standard solution with three fold calibrator concentration. The carry-over was found to be < 0.001% for EtS and EtG. No false positive result due to carry over has been observed in the use of the method.

5.9 Stability

In order to determine a potential decrease of EtG/EtS concentrations, stability of the EtG and EtS in stored blood samples were examined. Calibrators and QC samples were analyzed repeatedly by the UPLC-MS/MS method when the samples were extracted. Then, the samples were kept for three days in the auto-sampler at 10°C as well as one week in a freezer at -20 °C and then reanalyzed. The concentrations from the LC-MS/MS methods were compared and found to be within ± 5% for both compounds. Due to the lack of the time, long term stability over a 4-week period of storage at -20 °C will later be investigated. During this study, the stability of 0.1 % FA solution stored at room temperature for 5 weeks was also examined. There was no evidence for peak boarding/decreased peak intensity and gave no change in EtG/EtS concentrations.

5.10 Robustness of the method

The robustness of the method was evaluated (Figure 10) by analysing the influence of pH level on the retention. Stored 0.1% FA at room temperature was also examined. Different re-solutions of FA in combination with ACN were also investigated. No major differences in the retention time were observed, but the peak shape with 0.2% ACN as resolution was more symmetrical.

5.11 Method comparison

Forensic samples (n=26) consisting of only autopsy cases that were simultaneously analyzed for EtG and EtS using the existing method [34], and the developed Hybrid-SPE-PPT UHPLC-MS/MS method for comparison of the analyte concentrations. No false positive or negative were found for the UHPLC-MS/MS method. In general good correlation and good agreements of quantified concentrations were found for the concentrations examined, but it also showed a negative analytical bias in the EtG results, as shown in the figure 22. A possible explanation may be; in the current method, occasionally split peaks of the compounds and their deuterated IS analogue is observed in postmortem samples (Figure 23) and the comparison of findings was difficult. Furthermore, the measurement of the concentrations in current method was based on peak height where as in the new developed method peak area was used and the comparison of split peaks findings between to methods

was not straight forward. The introduced analytical bias will be investigated in future work by the analysis of an independent reference material.

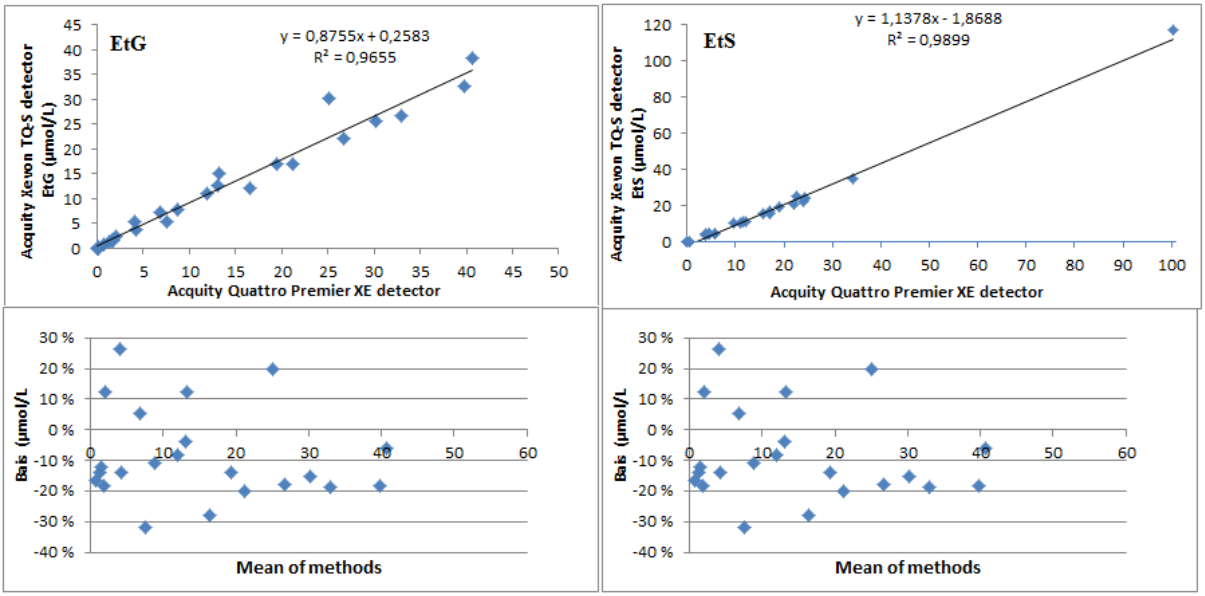


Figure 22: Comparison between EtG and EtS concentrations determined by the current method and the new developed method.

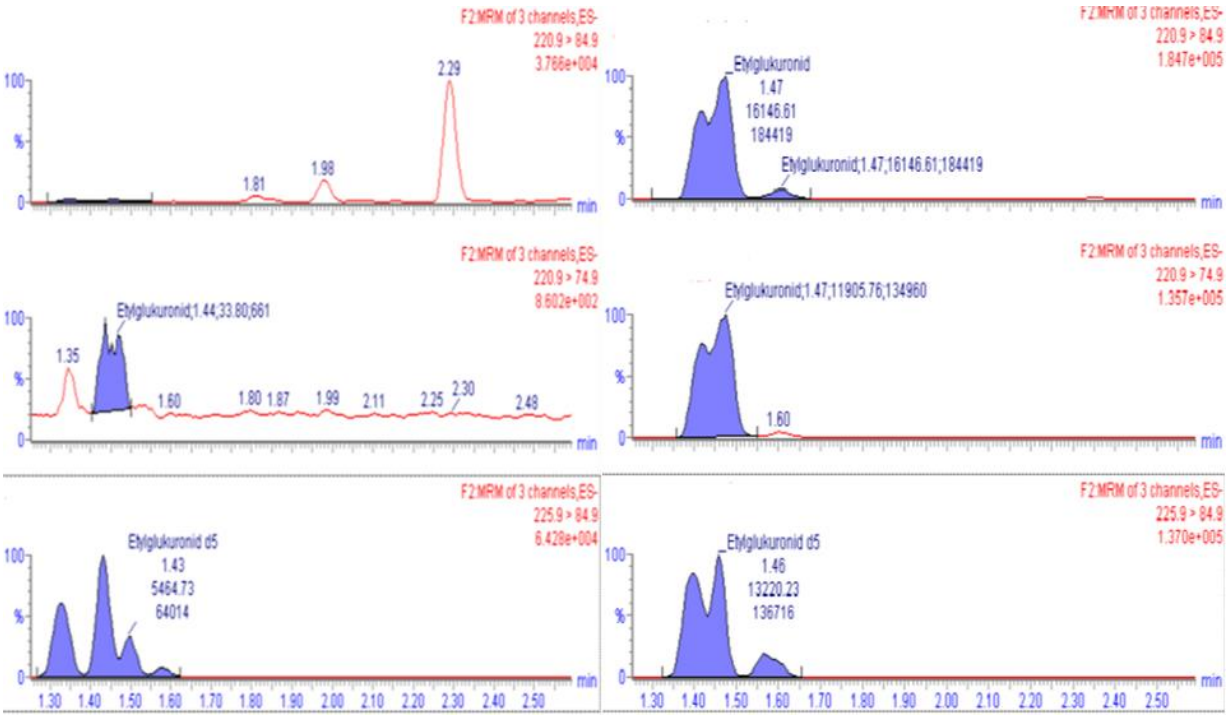


Figure 23: Some instances from current method of EtG chromatograms and internal standard showing split peaks in postmortem cases.

5.12 Method application

The UHPLC-MS/MS method has been in use for quantitative determination and confirmation for EtG and EtS in some DUI-cases and postmortem cases since April 2015. Altogether, about 26 whole blood samples have been analyzed on the method.

5.14 Further work

While this thesis has demonstrated a new developed method for quantitative determination of EtG and EtS, many opportunities for extending the scope of this thesis remain. The sample preparation is not automated yet. Therefore, further work will focus on implementation and documentation of the assay on the routine, automated sample preparation procedure, long term stability of the compounds stored at -20 °C and most importantly investigation of the analytical bias.

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6. CONCLUSION

In the present study, a simple, sensitive and robust UHPLC-MS/MS method based on hybrid-SPE-PPT with off-line sample clean up, using only 100 µl postmortem blood and acidic mobile phase is developed and fully validated. Both compounds use deuterated analogues as IS. This improves the robustness of the quantitative determination with respect to variation in experimental conditions and reduces possible matrix effect. The procedure was found to be sufficiently sensitive and specific to be applicable in monitoring recent alcohol use when ethanol has been completely eliminated. The assay was successfully applied on authentic postmortem cases, demonstrating high specificity without any suspicious results. Good correlation and good agreements of quantified results were found by the new developed method when compared to the current method.

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8. APPENDIX

Compound name: Etylglukuronid
Correlation coefficient: r = 0.999707, r^2 = 0.999413
Calibration curve: 0.0714674 * x + 0.00138083
Response type: Internal Std (Ref 4), Height * (IS Conc. / IS Height)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

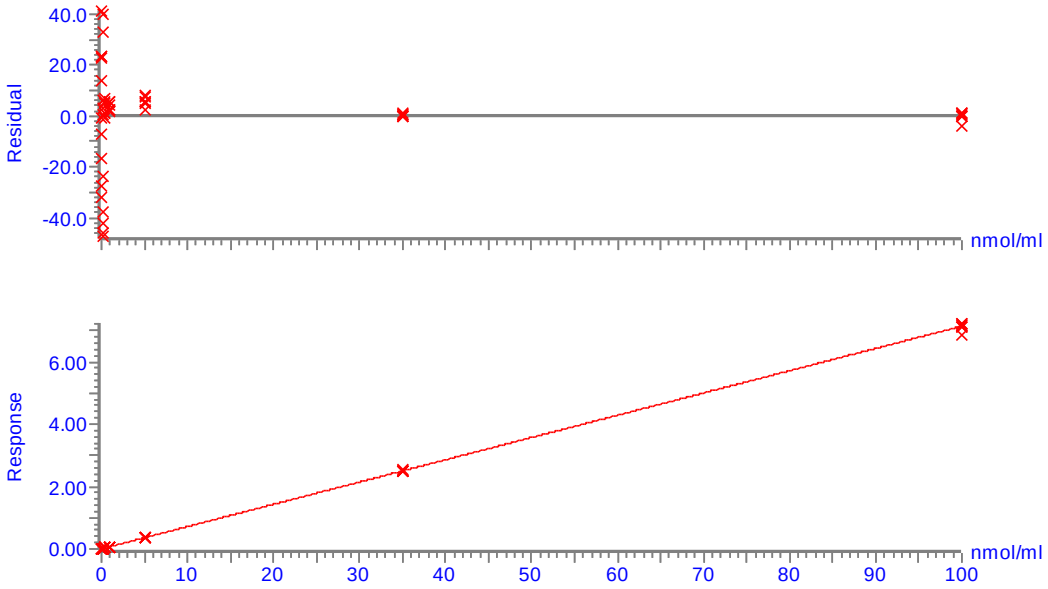


Figure 24: calibration curve of EtG.

Compound name: Etylsulfat
Correlation coefficient: r = 0.999811, r^2 = 0.999623
Calibration curve: 0.101411 * x + 0.00123585
Response type: Internal Std (Ref 3), Height * (IS Conc. / IS Height)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

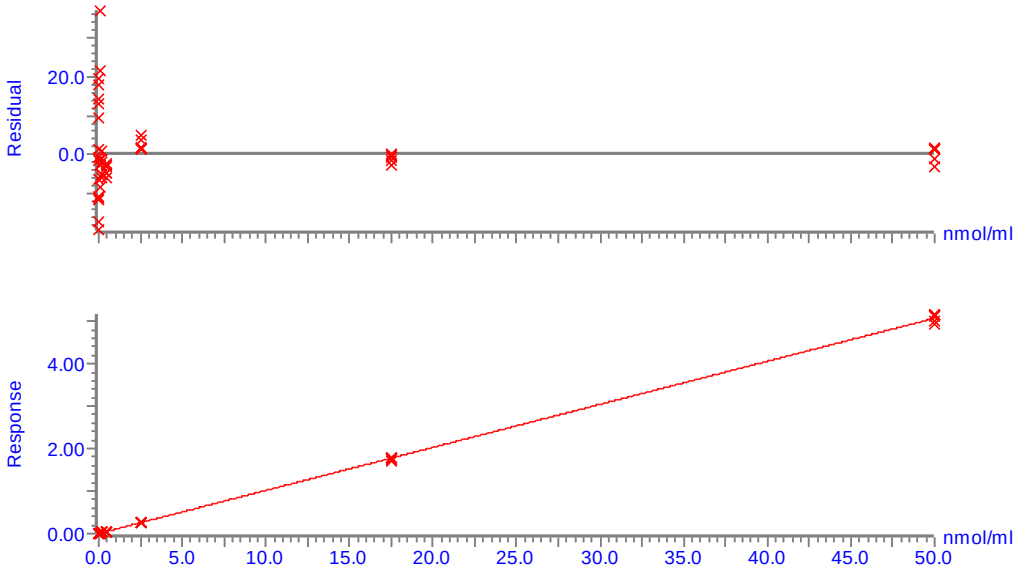
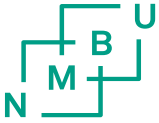


Figure 2: Calibration curve of EtS.

Table 13: Retention times and molecular weight for legal and illegal compounds.

Stoff	RT	MW
EtG	1,4	222,2
EtS	0,9	126,1
Pregabalin	7,78	159,2
GHB	0,91	104.5
Sotalol	6,78	272,4
Paracetamol	6,94	151,2
Teofyllin	0,65	180,2
Gabapentin	7,78	171,2
Amisulprid	8,54	369,5
Ketobemidon	8,54	247,3
Salisylsyre M-	9,95	138,1
Olanzapin	8,03	312,4
Moklobemid	8,79	268,7
Lamotrigin	9,13	256,1
Metoprolol	9,21	267,4
Mirtazapin	9,46	265,4
Tramadol	9,21	263,4
Petidin	9,63	247,3
Risperidon	9,88	410,5
Risperidon	3,78	410,5
Venlafaxin	9,88	277,4
Propranolol	10,13	259,3
Diltiazem	10,55	414,5
Quetiapin	10,38	383,5
Haloperidol	10,63	375,9
Klozapin	10,3	326,8
Reboxetin	10,55	313,4
Citalopram	10,47	324,4
Prometazin	10,72	284,4
Perfenazin	11,31	404
Flupentixol	11,64	434,5
Sertralin	11,31	306,2
Klorprotixen	11,39	315,9
Nortriptylin	10,97	263,4
Amitriptylin	11,05	277,4
Trimipramin	11,14	294,4
Alimemazin	10,97	298,4
Fenytoin M-	11,29	252,3
Mianserin	10,47	264,4
Verapamil	10,89	454,6
Fluoksetin	11,22	309,3
Paroxetin	10,8	329,3
Fluvoksamin	10,97	318,3
Levomepromazin	11,05	282,5
Karbamazepin	11,22	236,3
Morfin	4,67	285,4
Amfetamin	7,87	135,2
Buprenorfin gluk.	9,63	643,8
Etylmorfin glukuronid	7,96	
Alprazolam	11,64	308,8
Fenazepam	12,31	349,6
Fentanyl	10,3	336,5
Flunitrazepam	2,81	313,3
Zopiklon	2,62	388,8
Zolpidem	9,71	307,4
Oksykodon-d6	2,88	321,3
Karisoprodol	3,18	260,3
Nitrazepam	2,59	281,3
Ketamin	8,71	237,7
Klonazepam	2,65	315,7
Kodein	2,46	299,4
Midazolam	10,55	325,8
Metylfenidat	9,46	233,3
Metamfetamin	2,74	149,2
Kokain	9,54	303,4

Stoff	RT	MW
MDMA	8,29	193,2
Metadon	11,05	309,4
Meprobamat	10,22	218,3
Losartan	2,52	422,9
Fenytoin (M-)	1,5	252,3
Topiramat	10,86	339,4
Etosuksimid	9,13	141,2
Klobazam	3,05	300,7
Pregabalin	0,93	159,2
Hydromorfon	1,58	285,3
Flecainid	10,55	414,3
Amlodipin (M-)	3,82	408,9
Amiodaron		645,3
N-desm.diazepam	3,58	270,7



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