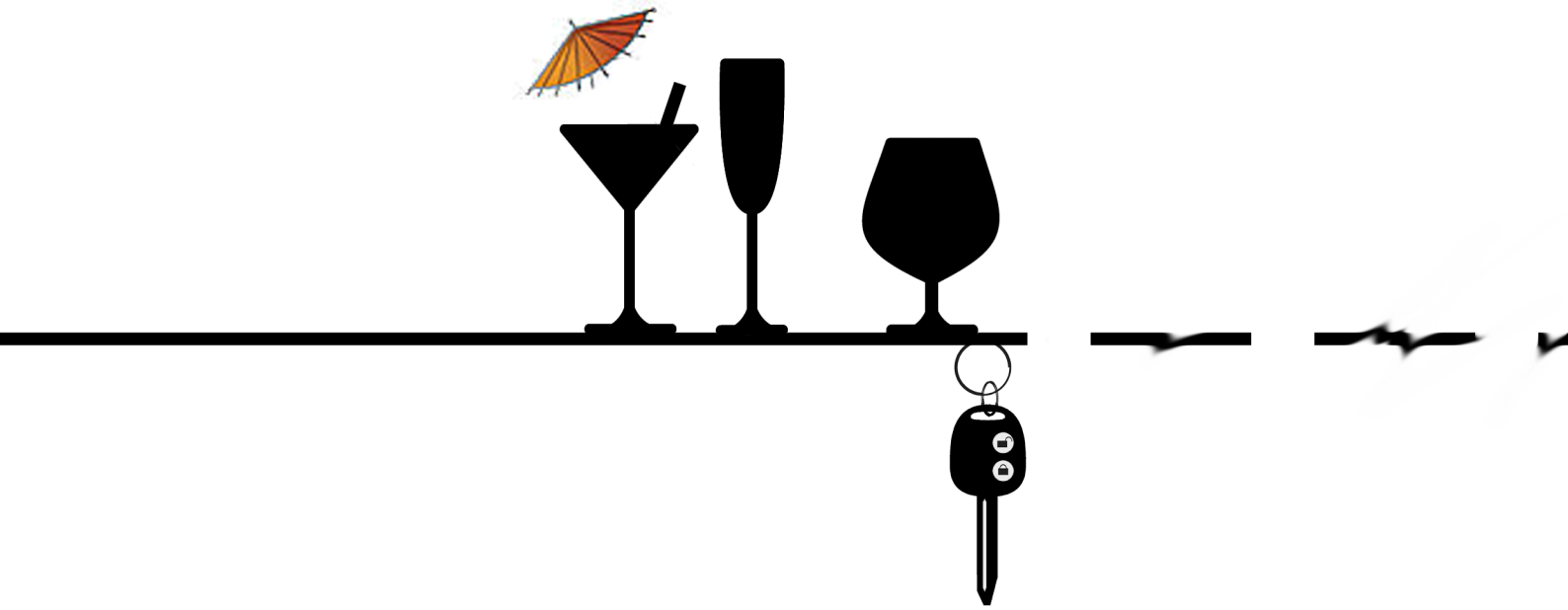
Alternative sampling strategies to monitor alcohol consumption in case of driver's licence regranting

Natalie Kummer

2016



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Ghent University
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ALTERNATIVE SAMPLING STRATEGIES TO MONITOR ALCOHOL CONSUMPTION IN CASE OF DRIVER'S LICENCE REGRANTING

Thesis submitted to obtain the degree of Doctor in Pharmaceutical Sciences

Natalie Kummer

2016

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This work was conducted at the National Institute of Criminalistics and Criminology, in Brussels, Belgium.

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A José

“Que la force me soit donnée de supporter ce qui ne peut être changé, et le courage de changer ce qui peut l'être mais aussi la sagesse de distinguer l'un de l'autre.”

Marc Aurèle

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The promoter,

Prof. Dr. Christophe Stove

The author,

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The co-promoters,

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List of abbreviations

AAbuffer	25 mM ammonium acetate buffer (pH=6)
ABV	alcohol by volume
ACN	acetonitrile
ADH	alcohol dehydrogenase
AEAT	acyl-CoA: ethanol O-acyltransferase
ALDH	aldehyde dehydrogenase
ALT	alanine aminotransferase
APCI	atmospheric pressure chemical ionisation
AST	aspartate aminotransferase
AUDIT	alcohol use disorders identification test
AUROC	area under the curve
BAC	blood alcohol concentration
BrAC	breath alcohol concentration
C	conditioning
Cal-Stock	stock solution for calibration
Cal-WS	calibration working solution
C-DBS(s)	capillary dried blood spot(s)
CDT	carbohydrate deficient transferrin
CE	capillary electrophoresis
D	drying
DAST	Drug Abuse Screening Test
DBS(s)	dried blood spot(s)
DRUID	Driving Under the Influence of Drugs, Alcohol and Medicines
DUI	driving under the influence
DUS(s)	dried urine spot(s)
E	elution
EE	extraction efficiency
ELSD	evaporative light scattering detector
ESI	electrospray ionisation
EtG	ethyl glucuronide
EtG_{100}	EtG concentration normalised to a creatinine concentration of 100 mg/dL
Ethyl myristate	E14:0
Ethyl oleate	E18:1
Ethyl palmitate	E16:0
Ethyl stearate	E18:0
EtOH	Ethanol
EtS	ethyl sulfate
EtS_{100}	EtS concentration normalised to a creatinine concentration of 100 mg/dL

FA	formic acid
FAEEs	fatty acid ethyl esters
FIPI	5-Fluoro-2-indolyl des-chlorohalopemide
GC-MS	gas chromatography coupled to mass spectrometry
GGT	gamma-glutamyltransferase
GTFCh	German Society of Toxicological and Forensic Chemistry
H	high
H_2O	water
Hct	hematocrit
HPLC	high performance liquid chromatography
IBSR/BIVV	Belgian Road Safety Institute
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IS	internal standard
IS-Stock	stock solution for internal standard
L	low
LC-MS	liquid chromatography coupled to mass spectrometry
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LFT	liver function test
LLE	liquid-liquid extraction
LLOQ	lower limit of quantification
LOD	limit of detection
LOQ	limit of quantification
M	medium
MAST	Michigan Alcohol Screening Test
MCV	mean corpuscular volume
ME	matrix effect
ME_{IS}	matrix effect corrected by the internal standard
MeOH	methanol
MRM	multiple reaction monitoring
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
ND-APCI	no-discharge atmospheric pressure chemical ionisation
NH_3	Ammonia
NICC	National Institute of Criminalistics and Criminology

NOPT	*N*-[2-(4-oxo-1-phenyl-1,3,8-triazaspiro[4,5]dec-8-yl)ethyl]-2-naphthalenecarboxamide
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PEth	phosphatidylethanol
PEths	phosphatidylethanol species
PLD	phospholipase D
QC	quality control
QC-Stock	stock solution for quality control
QC-WS	quality control working solution
RMS_{bias}	uncertainty of the inaccuracy of measurement
ROC	Receiver operating characteristic
RP	reversed-phase
RSD	relative standard deviation
RSD_r	Repeatability
RSD_t	intermediate precision
SAX	strong anion exchange
SCRAM	Secure Continuous Remote Alcohol Monitor
SoHT	Society of Hair Testing
SPE	solid-phase extraction
TAC	transdermal alcohol measurement
Tf	Transferrin
U	measurement uncertainty
$u(C_{ref})$	uncertainty of the certified value
UDPGA	uridine 5'-diphospho-β-glucuronic acid
UHPLC	ultra high performance liquid chromatography
UPLC	ultra performance liquid chromatography
UV	Ultraviolet
V-DBS(s)	venous dried blood spot(s)
W	washing
WAX	weak anion exchange
WHO	World Health Organization

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Chapter 1

Introduction

Based on

N. Kummer, W.E. Lambert, N. Samyn, C.P. Stove, Alternative sampling strategies for the assessment of alcohol intake of living persons, Clinical Biochemistry. In Press (2016).

N. Kummer, S.M.R. Wille, A. Poll, W.E. Lambert, N. Samyn, C.P. Stove, Quantification of EtG in hair, EtG and EtS in urine and PEth species in capillary dried blood spots to assess the alcohol consumption in driver's licence regranting cases, Drug and Alcohol Dependence. 165 (2016) 191–197.

Abstract

Alcohol is a legal psychoactive substance that has been widely used in many cultures for centuries. General background, definitions of alcohol and alcoholic beverages as well as the different patterns of alcohol consumption are given in the first Section of this Chapter (Section 1.1), while the metabolism of ethanol is depicted in Section 1.2. The first part of Section 1.3 (1.3.1) reviews the analytical methods to monitor alcohol consumption via the so-called 'classical analyses', i.e. breath, blood and urine for the determination of ethanol itself and blood and urine for the detection of direct and indirect markers of ethanol that are widely used to estimate the alcohol intake. In the second part of Section 1.3 (1.3.2) so-called 'alternative sampling strategies' are discussed. These cover the collection of classical blood/urine samples from a living person in an alternative way (i.e. dried blood spots (DBSs) and dried urine spots (DUSs)), as well as the collection of 'alternative' samples (i.e. different from blood, plasma, serum or urine). Amongst the alternative sampling strategies covered are the sampling of DBSs (CDT%, EtG/EtS, PEths), DUSs (EtG/EtS), sweat and skin surface lipids (ethanol, EtG, FAEEs), oral fluid (ethanol, EtG), exhaled breath (PEths), hair (EtG, FAEEs), nail (EtG). Post-mortem matrices (e.g. bone, muscle, bone marrow, adipose tissue, and vitreous humour) and samples specific to newborns (e.g. meconium, umbilical cord and placenta) will not be discussed here. Section 1.4 presents the legal issues regarding the driving under the influence of alcohol legislation and the driver's licence regranting process currently in force in Belgium.

1.1 General background and definitions about alcohol

Alcohol is the name commonly used to define the ethanol present in alcoholic beverages after the fermentation of sugar by yeast. Ethanol, which is one of the oldest psychoactive drugs used by humans, is a depressant of the central nervous system. Small doses typically create a feeling of euphoria, while higher doses affect the consumer's performance and behaviour (impaired coordination, lack of good judgment, sedation, ataxia, incoherent speech, loss of motor control). Finally, at a very high dose (leading to a blood alcohol concentration (BAC) > 4 g/L) alcohol consumption can lead to unconsciousness and death [1].

Alcohol is a volatile, flammable and colorless liquid also used as a solvent, antiseptic and fuel. The ethanol content of commercially available alcoholic beverages is indicated on the packaging and is expressed as alcohol by volume (ABV, or alc. % vol) and corresponds to the number of milliliters of pure ethanol present in 100 mL of the solution at 20° C. Alcoholic beverages are typically divided into three categories, based upon their ethanol content; beers (alc. 4-5 % vol.), wines (alc. 11-16 % vol.) and spirits (alc. 40 % vol.) [2,3]. To standardise the amount of ethanol ingested by an individual, regarding the beverages consumed (type and amount), publications often refer to the standard drink/unit. The World Health Organization (WHO) has defined a standard drink as a volume of a specific alcoholic beverage (e.g. a glass of wine or a can of beer) that contains approximatively the same amount of ethanol regardless the type of beverage [4]. The amount of ethanol used to define a standard drink changes from one country to another (Table 1.1).

Country	Standard drink definition
Canada	13.60 g
UK	8.00 g
USA	12.00-14.00 g
New Zeeland and Australia	10.00 g
Japan	19.75 g

Table 1.1 Standard drink definition for Canada, UK, USA, New Zeeland, Australia and Japan reported by the WHO [5,6].

Knowing that 1 mL of ethanol equals to 0.79 g of ethanol (volumetric mass density) and taking into account the ethanol content estimation per type of alcoholic beverage, a chart of common standard drinks containing 10 g of ethanol is presented in Figure 1.1.

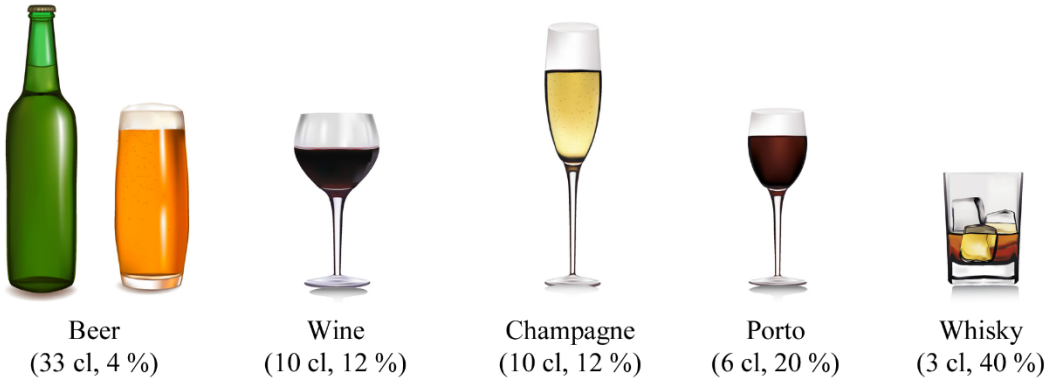

Figure 1.1 Illustration of standard drink containing approximately 10 g ethanol.

In literature, variable amounts of ethanol per day (sometimes expressed as number of standard drink) are used to classify volunteers based on their drinking pattern. Briefly, "abstainers" or "teetotallers" are generally defined as those with a total abstinence for at least some months [7,8]. A person who is not strictly abstinent, but who drinks alcohol only at rare occasions and then in low doses, (for instance one glass champagne to clink glasses on a birthday party) was defined as "low moderate drinker [7]". Within "social drinkers" (< 60 g ethanol per day [9]), one refers to "low-risk drinkers" (1-21 units alcohol per week [8] or < 30 (< 20 for women) g ethanol per day [10]), "increasing-risk drinkers" (22-50 units per week [8]) or "high-risk drinkers" (20-60 g ethanol per day for women and 30-60 g ethanol per day for men [10]). "Heavy drinkers" (≥ 50 g ethanol per day [11] or ≥ 60 g ethanol per day [10]) or "high-risk drinkers" (≥ 50 units per week [8]) have been defined more in detail as "risky drinkers" (60-120 g ethanol per day [9]) or "excessive drinkers" (> 120 g ethanol per day [9]). The WHO defined a "low-risk drinker" as a person with an alcohol consumption of less than 20 g of alcohol per day, less than 5 days a week (recommending 2 non-drinking days) [6]. The WHO has published in 2014 a lexicon of alcohol and drug terms which provides definitions (Table 1.2) for different drinking patterns [4].

Drinking pattern name	Definition by the WHO
Abstinence	Refraining from drinking alcoholic beverages, whether as a matter of principle or for other reasons. Those who practise abstinence from alcohol are termed “abstainers” or “teetotallers”.
Moderate drinking	An inexact term for a pattern of drinking that is by implication contrasted with heavy drinking. It denotes drinking that is moderate in amount and does not cause problems.
Social drinking	The use of alcoholic beverages in compliance with social custom, primarily in the company of others, and then only for socially acceptable reasons and in social acceptable ways. Often used loosely to mean a drinking pattern that is not problem drinking.
Heavy drinking	A pattern of drinking that exceeds some standard of moderate drinking or more equivocally social drinking. It is often defined in terms of exceeding a certain daily volume or quantity per occasion.
Excessive drinking	Currently a non-preferred term for a pattern of drinking considered to exceed some standard of moderate drinking of acceptability.
Binge drinking	A pattern of heavy drinking that occurs in an extended period set aside for purpose.
Hazardous drinking	A pattern of drinking that increases the risk of harmful consequences for the user. Consequences can be physical, mental, or even social.
Harmful drinking	A pattern of drinking that is causing damage to health. The damage may be physical or mental (e.g. episodes of depressive disorder secondary to heavy consumption of alcohol).

Table 1.2 Drinking patterns defined by the WHO [4].

1.2 Metabolism of ethanol

After alcohol consumption, ethanol is readily absorbed from the stomach and from the small intestine (duodenum and jejunum) into the blood stream [12]. The speed of absorption is influenced by many factors, such as the quantity consumed, the rate of drinking, the type of beverage (beer, wine, spirit) and the consumption of food before and/or during the ingestion of alcohol [12]. Ethanol is a small size (molecular weight (MW) = 46 g/mol) weak acid (pKa 15.9 at 25°C), which can easily penetrate biological membranes by passive diffusion through aqueous channels. Ethanol is distributed into all body fluids and tissues, in proportion to their water content [13]. Between 2 to 5 % of an ingested dose is excreted unchanged in the urine, breath and sweat. The ingested ethanol is mainly (about 95 %) removed from the body by oxidative metabolism (phase I) and partially (< 0.1 %) by non-oxidative metabolism (phase II), i.e. via conjugation reactions. The oxidative metabolism of ethanol takes places via three pathways, namely alcohol dehydrogenase (ADH), catalase and cytochrome. The alcohol dehydrogenase metabolism is the principal reaction. Via a two-stage oxidation process in the liver, it transforms ethanol into acetic acid, via acetaldehyde (Figure 1.2). The reaction is catalysed by the alcohol dehydrogenase (ADH) and the aldehyde dehydrogenase (ALDH) and requires involvement of the coenzyme oxidised nicotinamide adenine dinucleotide (NAD+), which is converted into its reduced form (NADH).

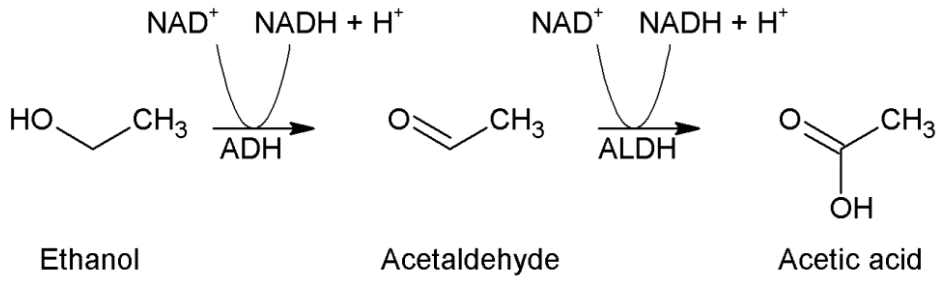

Figure 1.2 Ethanol metabolised to acetic acid, via acetaldehyde. ADH: alcohol dehydrogenase, ALDH: aldehyde dehydrogenase, NAD+: oxidised nicotinamide adenine dinucleotide, NADH: reduced nicotinamide adenine dinucleotide.

1.2.1 Direct biomarkers

The non-oxidative metabolism of ethanol results in the formation of ethyl glucuronide (EtG), ethyl sulfate (EtS), phosphatidylethanol species (PEths) and fatty acid ethyl esters (FAEEs) (Figure 1.3). These are discussed more into detail in Section 1.3.1.3, where the 'classical' analyses of direct biomarkers in urine and blood are outlined.

1.2.2 Indirect biomarkers

After excessive and chronic alcohol consumption, ethanol can induce indirect effects on the body via its interference with glycosylation (increased carbohydrate deficient transferrin (CDT%), with liver function (increased gamma-glutamyltransferase (GGT), aspartate aminotransferase/alanine aminotransferase (AST/ALT)) and via its effect on the size of red blood cells (increased mean corpuscular volume (MCV)). Also these are discussed more into detail in Section 1.3.1.2, where the 'classical' analyses of indirect biomarkers in blood are outlined.

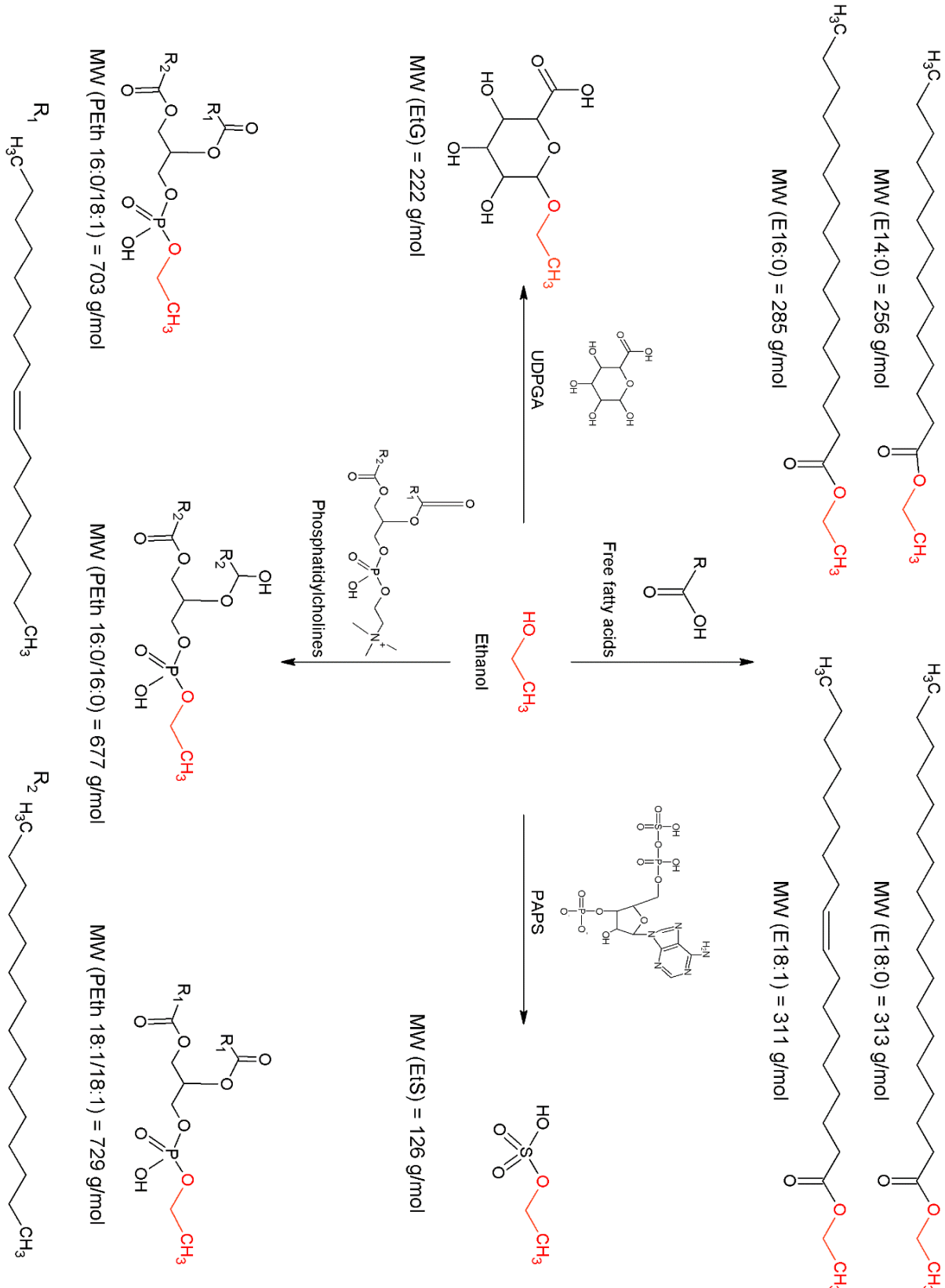

Figure 1.3 Non-oxidative phase II metabolism of ethanol into EtG, EtS, PEths (PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0) and FAEEs (ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl stearate (E18:0) and ethyl oleate (E18:1)), with indication of the molecular weight (MW). PAPS: 3'-phosphoadenosine 5'-phosphosulfate, UDPGA: uridine 5'-diphospho-β-glucuronic acid.

1.3 Analyses of interest

Monitoring of alcohol consumption is most often performed in breath, blood and urine. The blood alcohol concentration (BAC), obtained by quantification of ethanol in blood, is of particular interest to monitor alcohol consumption, due to its correlation with the effect of alcohol. To avoid sampling by venepuncture and to allow the evaluation of alcohol consumption in traffic situations, breath sampling has since long been introduced to detect persons under the influence of alcohol. Then, application of a factor, based upon the blood-breath concentration ratio, allows the conversion of the ethanol concentration in breath (BrAC) to the BAC [1]. Analysis of ethanol, EtG and EtS in urine, as well as the determination of EtG, EtS, FAEEs and PEths in blood, allows a longer detection window, but does not lead to information concerning the status of the person e.g. at the time of an accident. Indirect biomarkers of alcohol measured in whole blood (MCV) or serum (CDT%, GGT, ALT, AST) are traditionally used to detect chronic and excessive alcohol consumption (as seen in alcohol dependent individuals), for instance in case of fitness to drive decision. These 'classical analyses' are presented more in detail in Section 1.3.1.

The alcohol consumption of a living person can also be monitored via so-called 'alternative sampling strategies', which include 'classical' samples obtained from a living person via an alternative way (i.e. dried blood spots (DBSs) and dried urine spots (DUSs)), as well as 'alternative' samples (i.e. different from blood, plasma, serum or urine). Amongst the alternative sampling strategies covered in Section 1.3.2, are the sampling of DBSs, DUSs, sweat/skin surface lipids, oral fluid, exhaled breath, hair and nail. Post-mortem matrices (e.g. bone, muscle, bone marrow, adipose tissue, and vitreous humour) [14–17] and samples specific to newborns (meconium, umbilical cord and placenta) will not be discussed here. Key results, issues and considerations specific to each matrix are reported. For details about sample preparation and analytical methods, the interested reader is referred to the original articles or to recent reviews on this topic [9,18–30].

1.3.1 'Classical' analyses

1.3.1.1 Ethanol

The concentration-time profiles of ethanol in blood, breath and saliva (Figure 1.4, upper) follow the same overall shape [1].

The peak concentrations are reached after about 30 minutes for breath and saliva and 1 hour for blood. The peak concentrations of ethanol in blood and saliva are similar and approximatively 2100 times higher than in breath. The peak concentration of ethanol in urine is the highest and is reached later (after about 2h). The peak concentration of ethanol in sweat (Figure 1.4, lower) is lower than in blood, urine and saliva and is reached later than in other matrices (~4.5 h).

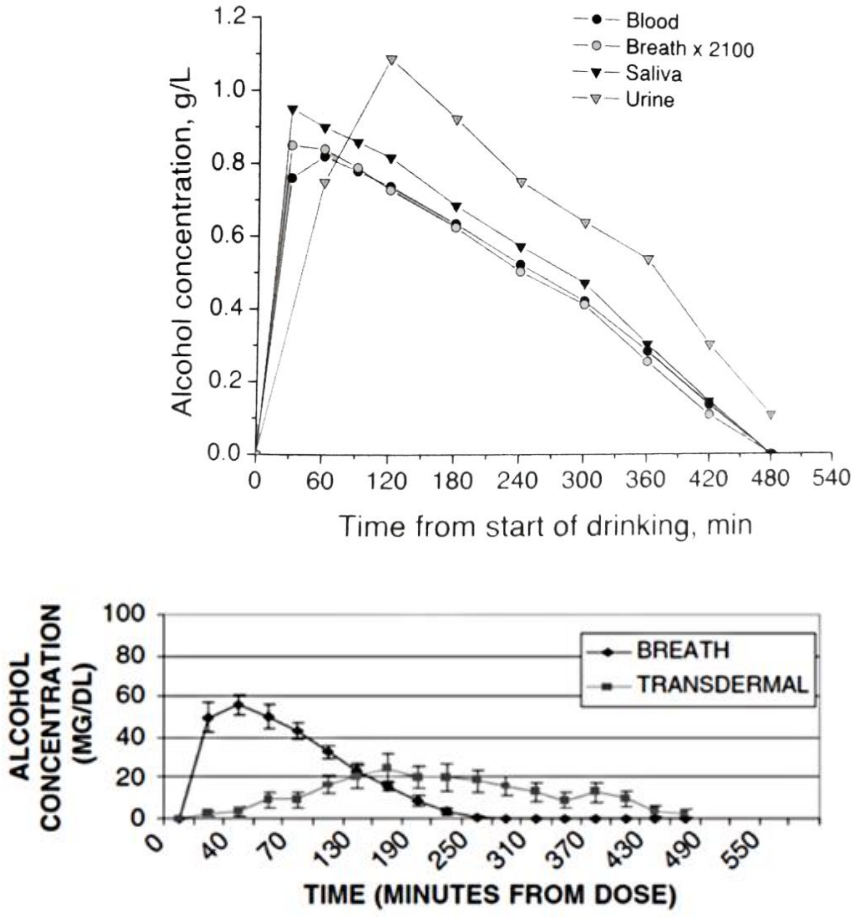

Figure 1.4 (Upper) Ethanol mean (N=21) concentration-time profile in blood, urine, breath and saliva after a consumption of 0.68 g ethanol/kg body weight [1]. (Lower) Ethanol mean (N=8) concentration-time profile of ethanol in sweat and breath after the consumption of 0.56 g/kg body weight [31].

1.3.1.1.1 Ethanol in blood

In literature, the alcohol concentration in blood is expressed using different units (i.e. g/kg, g/L (‰), mol/L). Use of the mean density of whole blood (1.055 g/mL) and/or the molecular weight (MW) of ethanol (46 g/mol) allows to convert results from one unit to another [1].

After alcohol consumption between 0.50 and 0.78 g ethanol/kg body weight, peak concentrations in blood were reached between 1.3 and 2.1 hours [32] after the start of

drinking (N=13). Ethanol was detectable up to 8.6 hours after the start of drinking. The concentration-time profile of ethanol in blood after the consumption of 0.68 g/kg body weight is presented in Figure 1.4 (upper). The rate of disappearance of ethanol in blood was estimated at 0.12 g/L/h [33]. The elimination rate is up to 1.5 times higher for heavy drinkers [34]. Ethanol concentrations up to 3.7 g/L have been measured in blood of alcoholic patients in withdrawal [35].

Ethanol levels exceeding 1.5 g/L without sign of intoxication or exceeding 3.0 g/L at any time are a sign of abnormal ethanol tolerance and suggest alcohol misuse [34].

1.3.1.1.2 Ethanol in urine

A good correlation (r=0.96) has been observed between urine and blood concentrations and the urine-blood concentration ratio was estimated at 1.3:1 [1]. In urine, the mean peak ethanol concentration (17.0 mmol/L = 0.782 g/L (MW = 46 g/mol)) after consumption of 0.50 g ethanol/kg body weight, was reached 1.5 h after the start of drinking [15]. Ethanol was detectable up to 6.5 hours after the consumption of alcohol. The concentration-time profile of ethanol in urine after the consumption of 0.68 g/kg body weight is presented in Figure 1.4 (upper). Ethanol concentrations up to 4.76 g/L were measured in urine from patients when admitted to a detoxification unit [13].

1.3.1.1.3 Ethanol in breath

Breath alcohol concentration (BrAC) measurement is performed with a Breath Analyser System and gives a direct result. BrAC measurement is, in contrast to blood sampling, non-invasive and is therefore often used in road traffic control.

A good correlation (r=0.98) between the concentration of ethanol in breath and blood has been reported [1]. Most publications expressed the breath ethanol concentration not as the mg ethanol per liter of breath measured, but as the corresponding calculated blood alcohol concentration in g/L or ‰. This inference requires the use of a blood-breath concentration ratio (conversion factor), which differs from one country to another (2300:1 for Great Britain, The Netherlands and Belgium, 2000:1 for most European countries and 2100:1 for USA and Canada).

After the consumption of 1.07 g ethanol/kg body weight, BrAC values between 0.245 and 0.730 mg/L were measured 33 min after the termination of drinking. Ethanol was still

detectable in breath after 5 h. The concentration-time profile of ethanol in breath after the consumption of 0.68 g/kg body weight is presented in Figure 1.4 (upper). The mean elimination rate of ethanol in breath was estimated at 0.082 mg/L/h (0.078-0.086, 95 % CI)[70], which corresponds to 0.16 g/kg/h (0.15-0.17, 95 % CI) when expressed as BAC (blood-breath concentration ratio 2000:1). Ethanol concentrations in breath up to 2.2 mg/L (reported in the article as 4.7 g/L in blood (conversion factor 2100:1)) have been measured in breath of alcoholic patients on arrival in a detoxification unit [64].

1.3.1.2 Indirect biomarkers

1.3.1.2.1 Carbohydrate deficient transferrin (CDT)

Transferrin (Tf) is a group of glycoproteins synthesised and secreted by the liver, that transport iron through the bloodstream. These are present in the body under different isoforms (Figure 1.5) all composed of a polypeptide chain with two binding sites and two carbohydrate chains. These polysaccharide chains are branched with sialic acid residues.

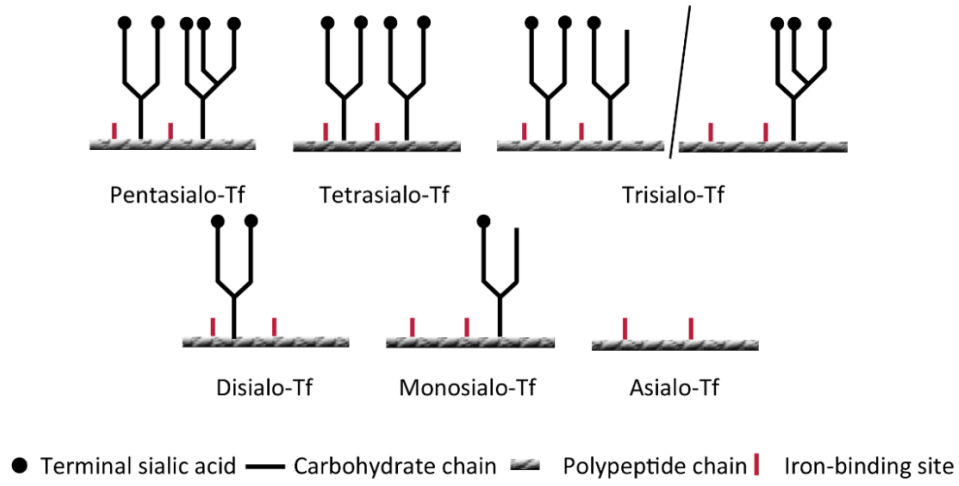

Figure 1.5 Representation of the different isoforms of transferrin present in the human body.

Percentages of most important isoforms in the serum of healthy persons are between 64 and 80 % for tetrasialo-Tf, 12 and 18 % for pentasialo-Tf, 4.5 and 9 % for trisialo-Tf and less than 2.5 % for disialo-Tf. Asialo-Tf and monosialo-Tf are not present, or in a very small amount (< 1 %) in healthy persons [36]. Alcohol consumption induces an increase of isoforms with less carbohydrate chains and less sialic acid groups, named carbohydrate-deficient transferrin (CDT). This has been explained by an underglycosylation of Tf (most likely due to the effect of ethanol and or acetaldehyde on the N-glycan chain synthesis) [37]

or by an enhanced activity (induced by ethanol or acetaldehyde) of the sialidase enzyme that removes the carbohydrate groups from Tf [34].

Different methods, which consider different transferrin isoforms as CDT, have been developed. This involves that cut-off values used for the interpretation of results are method-dependent. Arndt has published in 2001 an interesting review on this issue [36].

In its original definition, CDT encompassed the sum of transferrin glycoforms with either zero (asialo-Tf)), one (monosialo-Tf) or two (disialo-Tf) sialic acid residues on the carbohydrate side chain of the transferrin molecule [36]. Several studies demonstrated that trisialo-Tf was not correlated with alcohol consumption [38,39]. Subsequent investigations have recognised disialo-Tf and asialo-Tf as being the main alcohol-related glycoforms [40,41]. In 2007, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) workgroup on CDT [41] proposed to exclude the monosialo-Tf from this definition because it is linked to trisialo-Tf and not to alcohol consumption [42,43]. Some authors [44,45] proposed to used asialo-Tf instead of a total CDT as test diagnostic of alcohol abuse. CDT results are generally expressed as a % of total transferrin (CDT%).

It is observed that asialo-Tf is usually not detected in serum of healthy persons. Disialo-Tf is normally detected in serum of healthy volunteers but the concentration increased after high alcohol consumption. A daily consumption between 50 and 80 g during 1 to 2 weeks is required to rise the CDT level. In this way, short periods of high alcohol consumption are not detected. Abnormal CDT level is detected in alcoholic patients up to 2 weeks after the cessation of drinking. The mean half-life of CDT in blood is about 7 to 10 days [34]. CDT (asialo-Tf, disialo-Tf and monosialo-Tf) values up to 6.6 % have been measured in blood of alcoholics patient in withdrawal [46].

Amongst the indirect markers, CDT% is the most reliable marker to detect chronic alcohol consumption (specificity between 80 and 95 % [22]). Nevertheless, liver diseases can lead to false positive results [47–49]. The sensitivity for CDT has been reported between 20 and 80 % [22]. Possibilities, limitations, outcomes and pitfalls concerning the use of CDT in the context of driver's licence regranting programs have been discussed in detail in a recent publication [50].

1.3.1.2.2 Gamma-glutamyltranspeptidase (GGT)

Gamma-glutamyltranspeptidase (gamma-glutamyltransferase, GGT) is an enzyme located in the cell surface membrane of many tissues (i.e. liver, kidneys, bile duct, pancreas, gall bladder, spleen, heart, brain, and seminal vesicles), which catalyses the transfer of a gamma-glutamyl moiety of the glutathione to amino acids, peptides or water to form glutamate [51].

This reaction is possibly involved in protection against oxidative stress (induced from the metabolism of alcohol), via the regulation of the intracellular glutathione (body's anti-oxidant) levels [52,53]. Although present in many tissues, only the isoform of GGT from the liver is detected in serum [19].

GGT activity, which is measured in serum, is not increased after the consumption of a single dose of alcohol [53], and a consumption between 80 and 200 g of ethanol per day for a period of several weeks is required to increase the activity of GGT in serum [49]. A 15 % increase has been observed after daily alcohol consumption of 60 g ethanol for 3 weeks, with increases up to 50 % after 5 weeks of high alcohol consumption [53]. GGT mean activity measured in blood of heavy drinkers was 177 U/L (N=133) [54]. The half-life of GGT is between 14 and 26 days [34,55]. Normal GGT values are detected within 2 to 5 weeks after the start of an alcohol withdrawal [12,49]. GGT activity up to 945 U/L has been measured in blood of alcoholic patients in withdrawal [56].

Because cholestasis, a liver injury which stops the bile flow from the liver to the duodenum, induces an increased synthesis of GGT [57], GGT has been widely used for the assessment of liver damage. In addition, GGT can increase due to biliary, heart, pancreas or kidney damage, obesity, type 2 diabetes, the use of drugs like barbiturates, anticonvulsants and alcohol [25,48,49,55]. Many different values have been reported regarding the sensitivity and specificity of GGT to detect excessive and chronic drinkers. These variations are explained by different study protocols (including variable subjects), using variable cut-off values and taking into account variable parameters (gender, age, medical conditions). As a result, sensitivity values between 30 and 60 % have been reported [22]. GGT is rarely elevated in subjects under 30 years [53,55], even when they have alcohol dependence [58]. Specificities between 65 and 95 % have been reported [22]. The IFCC has published a procedure for the measurement of GGT, which recommends an upper reference limit at 38 U/L for females and 55 U/L for males [59].

1.3.1.2.3 Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT)

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are two transaminase enzymes used in liver function tests (LFT), to detect liver injury. AST and ALT catalyse the reversible transfer of an α-amino group from aspartate (AST) or alanine (ALT) to α-ketoglutarate to create oxaloacetate (AST) or pyruvate (ALT) and glutamate (Figure 1.6). AST is present predominantly in the liver, but also in heart, muscle, kidneys, brain, pancreas, lung and red and white blood cells [25]. ALT is mainly present in hepatic tissue [25].

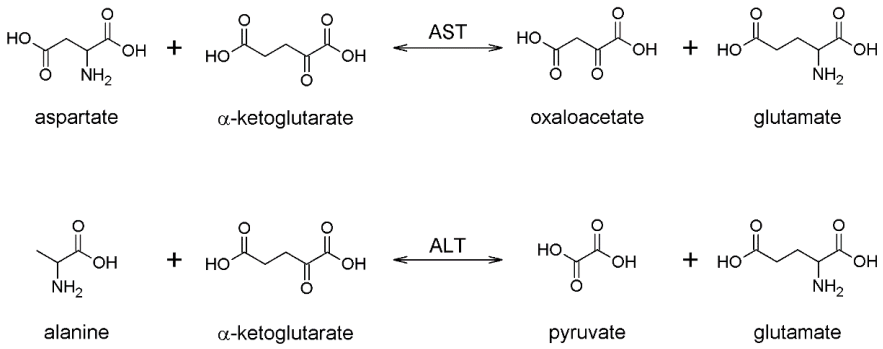

Figure 1.6 Typical reaction between aspartate or alanine and α-ketoglutarate catalysed by aspartate aminotransferase (upper) or alanine aminotransferase (lower).

AST and ALT activity are determined in serum. The half-life of these enzymes is about 13 (AST) and 16 days (ALT) [53], and normal values are reached in alcoholic patients between 2 and 3 weeks after the cessation of alcohol consumption [34,48]. Concentrations up to 422 U/L for AST and 225 U/L for ALT have been measured in blood from patients in alcohol withdrawal [46]. The sensitivity of the method to detect alcohol misuse was reported between 23 and 50 % [34,53,60]. Both AST and ALT levels have been reported to be less likely elevated in those aged less than 30, and possibly in the elderly, and to be elevated in case of obesity, liver and biliary diseases [53]. A study has demonstrated [53], that abnormal ALT activity could be attributed in 22 % of the cases to obesity and in between 17 to 20 % of the cases to hepatitis C. In addition, strenuous exercise, muscle disorders and many drugs have been shown to possibly increase AST levels [25,48]. Despite this, abnormal ALT and AST levels were observed in only 4 % of "moderate drinkers" (N=1504) [54] and so the specificity to distinguish between heavy drinkers and reference individuals has been calculated at 87 % for ALT and 95 % for AST [60]. This may be explained by a low prevalence of factors that may

elevate ALT and AST activity (e.g. obesity, liver and biliary diseases) in the reference populations involved.

The IFCC has published procedures for the measurement of AST and ALT, which recommended an upper reference limit for AST at 31 U/L for females and 35 U/L for males and for ALT at 34 U/L for females and 45 U/L for males [59]. In addition, an AST/ALT ratio over 2 has been proposed to suggest alcohol induced liver damage in 90-95 % of the cases (specificity) but this ratio is not necessary elevated for all alcohol dependent patients (sensitivity below 40 %) [68, 84, 92].

1.3.1.2.4 Mean corpuscular volume (MCV)

The mean corpuscular volume (mean volume of erythrocytes) is the average volume of erythrocytes (red blood cells). The value results from the hematocrit level (volume (%) of erythrocytes in total volume blood) divided by the number of erythrocytes. The result is expressed in femtoliters (fL, or 10^{-15}L).

The normal range of MCV is between 86 and 98 fL [62]. The cut-off value used to detect dependence of alcohol is between 93 and 96 fL [25]. A chronic and excessive alcohol consumption period is known to increase the size of red blood cells (macrocytosis). Values up to 109 fL have been measured for patients in alcohol withdrawal [56]. With a lifespan of the red blood cells of 120 days, MCV values remain abnormal for between 3 and 4 months after cessation of heavy drinking [12,34,63].

MCV is also influenced by folic acid or vitamin B12 deficiency, bleeding, hematological diseases, bone marrow disorders, liver diseases, hypothyroidism, hyperglycaemia and smoking [34,48]. MCV is less likely to be elevated in those aged less than 30, and possibly in the elderly [53]. MCV has shown a specificity between 75 and 95 % [22], and a sensitivity below 50 % [22,25,34,60].

1.3.1.3 Direct biomarkers

1.3.1.3.1 Ethyl glucuronide (EtG) and ethyl sulfate (EtS)

Glucuronidation of ethanol is a phase II conjugation reaction with UDPGA (uridine 5'-diphospho-β-glucuronic acid) through the action of endoplasmic reticulum UDP-glucuronosyltransferase enzyme (Figure 1.3) [64]. About 0.02 % of consumed ethanol is

excreted in urine as EtG [65,66]. Sulfation of ethanol is a phase II conjugation reaction with PAPS (3'-phosphoadenosine 5'-phosphosulfate) through the action of cytosolic sulfotransferase (Figure 1.3) [67]. Only 0.01-0.02 % of the consumed ethanol is excreted in urine as EtS on a molar basis [68,69].

EtG and EtS are two small, polar and acidic metabolites of ethanol [70]. The pKa of EtG was estimated between 2.84 and 3.21 [70–72] and at -3.14 for EtS [70].

1.3.1.3.1.1 EtG and EtS in blood

After the consumption of between 0.50 and 0.78 g ethanol/kg body weight, the peak concentrations (N=13) in serum for EtG were between 0.3 and 1.1 mg/L (reported as 1.2-4.9 µmol/L in the publication (MW = 222 g/mol)) and were reached between 2.3 and 5 hours after the start of drinking (drinking time was about 30 min) [32]. For EtS the peak concentrations were between 0.1 and 0.8 mg/L (1.0-6.4 µmol/L, MW = 126 g/mol) and were observed between 2.1 and 3.9 hours. EtG was still detected 10 hours after the start of drinking in 7 out of 13 volunteers. EtS concentrations return to zero more rapidly (between 4 and up to more than 10 h) [32]. The concentration-time profiles of ethanol, EtG and EtS in serum are presented in Figure 1.7. The half-life of EtG and EtS in blood from alcohol dependent patients was calculated at 3.3 h (range 2.6-4.3) for EtG and at 3.6 h (range 2.7-5.4) for EtS [35]. EtG and EtS were detectable in blood for about a two times longer period than ethanol [32].

In blood of patients in alcohol withdrawal concentrations up to 17.9 mg/L for EtG and up to 5.9 mg/L for EtS [35] were reported. EtG and EtS were detectable for up to 40 hours in blood of alcohol dependent patients after the cessation of drinking [35].

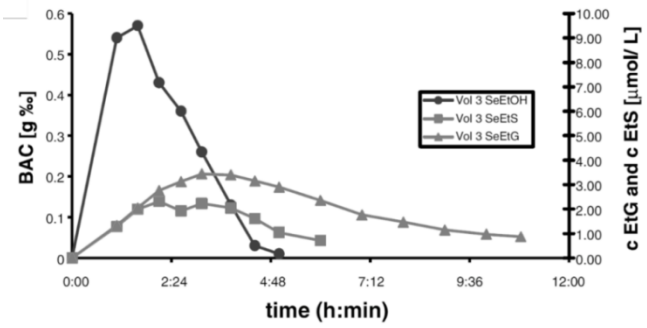

Figure 1.7 Serum ethanol, EtG and EtS concentration-time profiles in one volunteer after consumption of 0.5 g ethanol/kg body weight [32]. 1 µmol/L is equal to 0.22 mg/L for EtG (MW = 222 g/mol) and to 0.13 mg/L for EtS (MW = 126 g/mol).

1.3.1.3.1.2 EtG and EtS in urine

In a study evaluating the kinetics of EtG and EtS in urine (Figure 1.8), peak EtG concentrations (N=13) were reached between 5.0 and 7.5 hours and were between 23 and 179 mg/L (104-805 µmol/L, MW = 222 g/mol) [32]. For EtS, peak concentrations between 6 and 67 mg/L (46-533 µmol/L, MW = 126 g/mol) were observed between 3.1 and 7.4 hours. Four out of 13 volunteers had positive EtG urinary levels after 44 h. In urine, EtG was detectable between 26.6 and more than 44 h after the start of drinking, while EtS was detectable between 22.8 and more than 47 h after consumption of alcohol quantities between 0.5 and 0.78 g /kg body weight [32]. EtG was detected for about 10 times longer than ethanol, whereas EtS was detectable for about 3-8 times longer [32]. The EtG concentration in urine decreased with a half-life of approximately 2.5 h [64,66]. Urine samples from alcohol-dependent patients during detoxification can have EtG concentrations up to 1240 mg/L [64] and EtS concentrations up to 264 mg/L [73] remaining detectable for up to 5 days [74].

Due to the possibility of finding EtG and EtS concentrations in urine without consumption of alcoholic beverages, e.g. via the intake of certain food or beverages and the use of certain cosmetics (mouthwash and hand sanitizers) or e-cigarettes [75–82], low concentration results have to be interpreted with caution. No cut-offs are fixed yet by international guidelines and values currently used to detect intentional alcohol consumption vary between 0.05 and 1.1 mg/L [83,84].

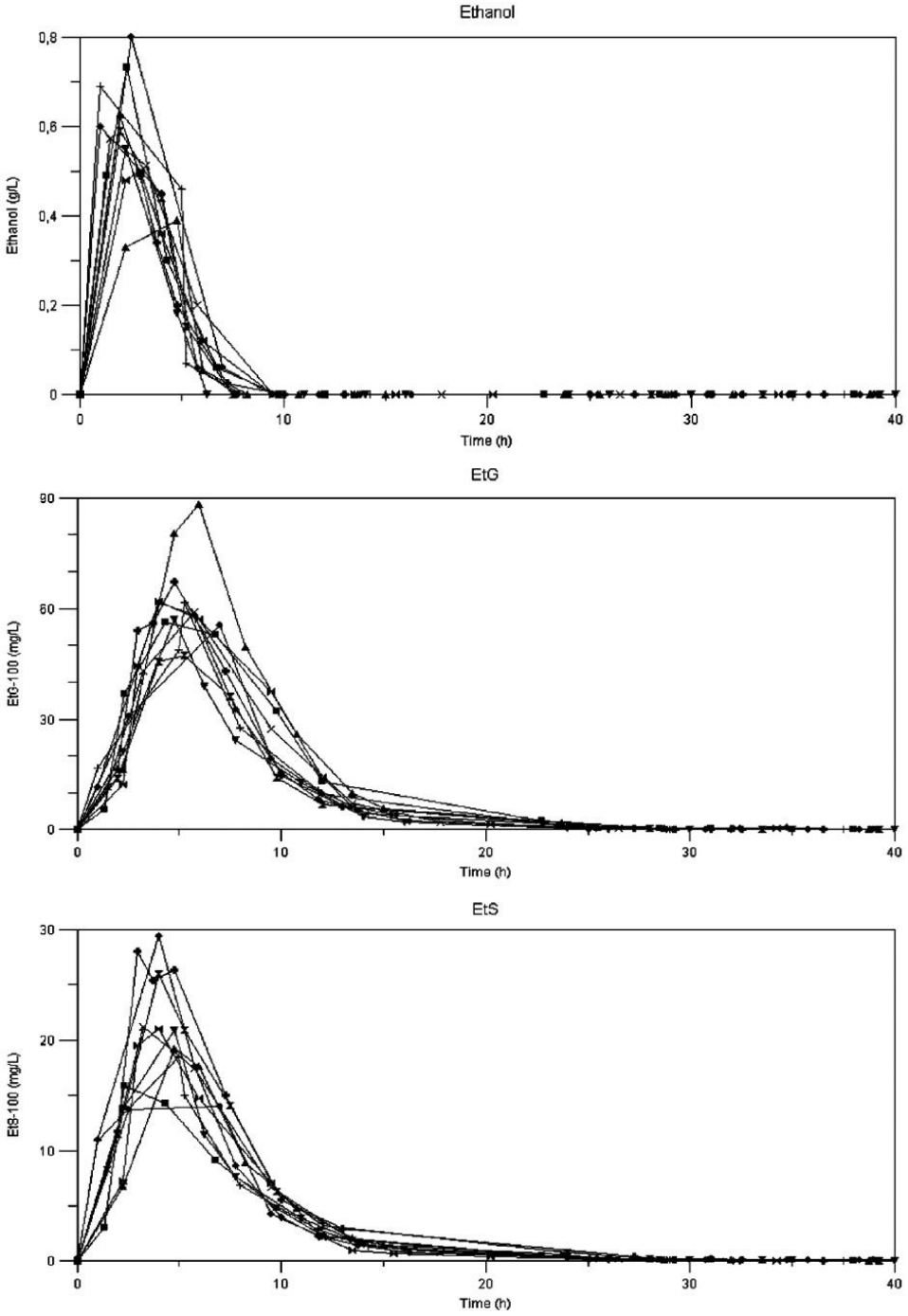

Figure 1.8 Urinary ethanol, EtG and EtS concentration-time profiles after consumption of 0.5 g ethanol/kg body weight [68].

1.3.1.3.2 Fatty acid ethyl esters (FAEEs)

FAEEs are a group of more than 20 substances formed by enzymatic esterification of ethanol and free fatty acids (Figure 1.3). Ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl stearate (E18:0) and ethyl oleate (E18:1) are the most studied FAEEs. Different enzymes (i.e. FAEE synthase, acyl-CoA:ethanol O-acyltransferase (AEAT), lipoprotein lipase, cholesterol esterase, carboxylesterase and carboxylester lipase) catalyse the esterification of ethanol to free fatty acids. More information about the biochemistry of FAEEs can be found in an article published in 2003 [85]. Politi *et al*. in 2007 and Cabarcos *et al.* in 2015 have published interesting reviews about the detection of FAEEs in biological samples [18,19].

FAEEs are present in blood of alcohol drinkers and abstainers. In abstainers, FAEEs concentrations in serum between 24 and 87 nmol/L have been suggested as reference range [86].

Blood ethanol and serum FAEEs concentrations follow similar kinetics (Figure 1.9), except that FAEEs remain longer detectable in blood (up to 99 h) than ethanol [86,87].

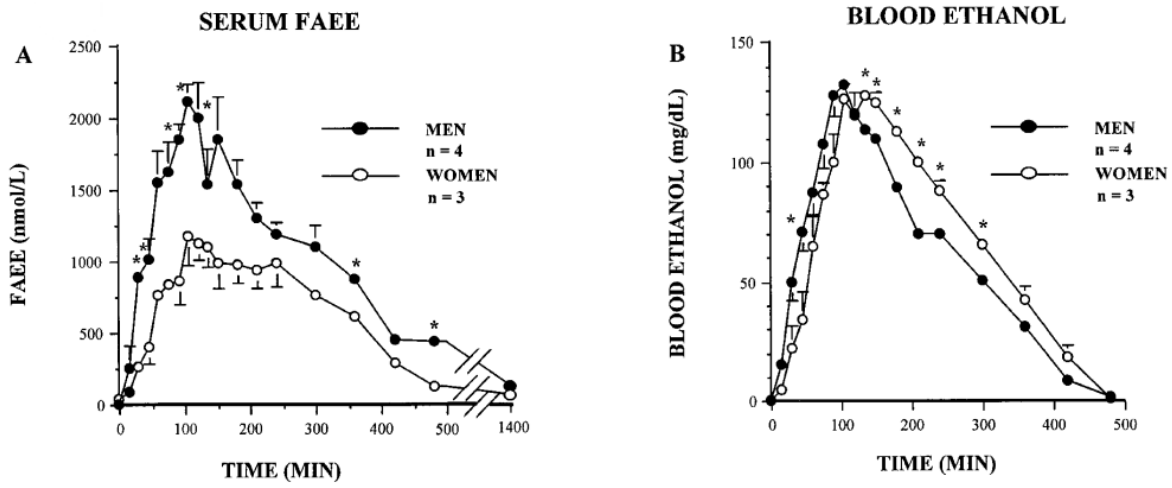

Figure 1.9 Serum FAEEs and blood ethanol concentration-time profiles in subjects after the consumption of 0.43 g ethanol/kg body weight [88].

After the consumption of 0.43 g ethanol/kg body weight, a mean peak serum concentration at ~2200 nmol/L (N=4) for men and at ~1200 nmol/L for women (N=3) has been observed (Figure 1.9, left) [88]. During the first 17.7 hours after alcohol consumption, 95 % of the FAEEs detected in serum were eliminated. In serum from a hospitalised inpatient with

known history of chronic alcohol abuse, a FAEEs concentration up to 42.1 mg/L (E16:0 = 7.2 mg/L, E18:0 = 5.2 mg/L, E18:1 = 23.9 mg/L, E18:2 = 4.4 mg/L and E20:4 1.4 mg/L) was reported [89].

1.3.1.3.3 Phosphatidylethanol species (PEths)

PEths are a group of abnormal phospholipids formed in the presence of ethanol (Figure 1.3), via the action of phospholipase D (PLD), which normally hydrolyses phosphatidylcholine into phosphatidic acid and choline in cell membranes (Figure 1.10) [21].

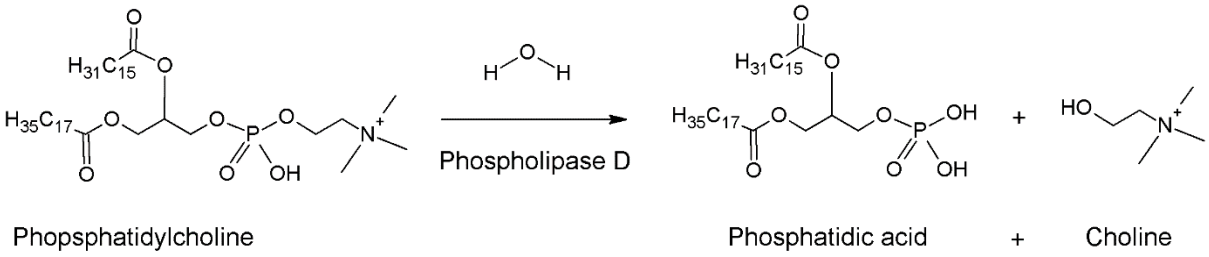

Figure 1.10 Normal hydrolysis of phosphatidylcholine into phosphatidic acid and choline in cell membranes.

In blood, PEths peak concentrations appear between the 3th and 6th day after five days of consecutive high alcohol consumption (between 50 and 109 g ethanol daily) and were detected up to more than 16 days [90]. Up to 48 different PEths have been detected in blood collected during autopsy of heavy drinkers [91]. All species have a common phosphoethanol head onto which two fatty acids of variable chain length and degree of saturation are attached. Although analysis of blood from heavy drinkers shows a huge interindividual variation of the distribution of the different PEths [92], PEth 16:0/18:1 and PEth 16:0/18:2 are the two predominant PEths detected [91–94]. Preliminary studies suggest that PEth 16:0/18:1, PEth 16:0/18:2, PEth 18:1/18:1, PEth 16:0/20:4 and PEth 18:1/18:2 could constitute together more than 80 % of total PEths, whereas PEth 16:0/16:0 alone could represent about 1-5 % [24]. The distribution of PEths species from different publications is presented in Figure 1.11.

While some methods (e.g. high performance liquid chromatography (HPLC) coupled to evaporative light scattering detector (ELSD) [95–98] and non-aqueous capillary electrophoresis coupled to UV detection [99]) measure the total amount of PEths, other methods (such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)) are able to identify and quantify individual molecular species [92–94,100–103].

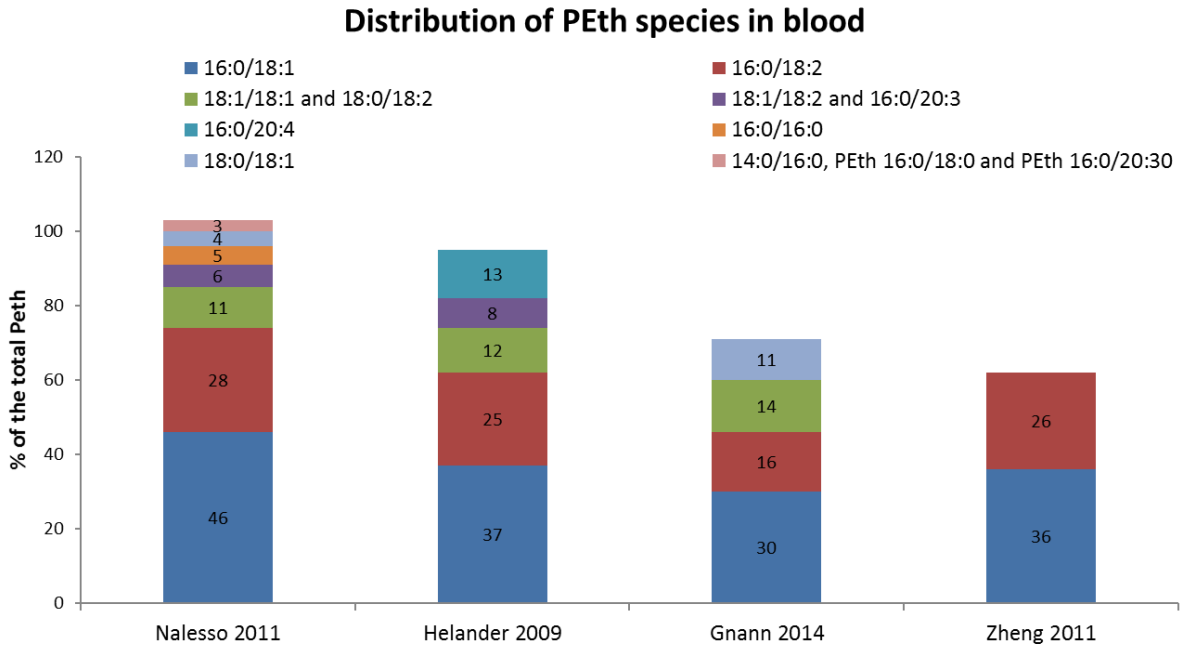

Figure 1.11 Distribution of PEths in human blood according to 4 studies [92–94,104].

1.3.2 'Alternative' sampling strategies

1.3.2.1 Dried blood spot (DBS)

A DBS is obtained by depositing a blood sample onto a filter paper, followed by drying. These samples are known to improve the stability of many analytes and to facilitate storage and transportation issues [105]. While so-called venous DBS (V-DBS) can be generated from venous blood, obtained by classical venepuncture, capillary DBS (C-DBS) are typically generated by direct collection of blood drops appearing after a finger or heel prick. The sampling can be performed either in a volumetric (using a precision microcapillary) or a non-volumetric way (direct application from the finger/heel). Compared to venepuncture, the sampling of C-DBS offers the advantage of being less invasive and, as long as no accurate handling is required, does not require a nurse or physician [106].

DBS collected in a non-volumetric way are mostly processed by excision of fixed-size punches (typically 3-6 mm diameter) from the global spot. This partial-spot approach requires the assessment of the impact of variables such as hematocrit, punch localisation and spot volume on the quantitative result [107,108]. To cope with a possible bias imposed by deviating hematocrit values, different strategies have been proposed [106]. These include

volumetric deposition using special devices followed by full-spot analysis, the use of special filter paper, the normalisation following hematocrit prediction [106] or the collection of a fixed volume of blood from a non-volumetrically deposited sample (e.g. using a microfluidic device or volumetric absorptive microsampling) [109]. Another issue in DBS analysis is how to apply an internal standard (IS) to a dried matrix spot [110]. Whilst in most DBS-based procedures, the IS is added to the extraction solvent, it can also be spiked to or sprayed onto the DBS prior to extraction. Having the IS in the DBS prior to the extraction offers the advantage that any variability during the extraction process is corrected for [110].

Below, we provide an elaborated update on the use of DBS for detecting a subset of ethanol markers, which was briefly covered in an overview by Sadones *et al.* on the use of DBS for detecting (markers of) abused substances [29].

1.3.2.1.1 CDT% in DBS

In a report dating from 1996, a good agreement (as suggested by a correlation coefficient of 0.94) was found between CDT% in serum and DBS [111]. CDT% values in DBS were demonstrated to be stable for up to 2-3 days at room temperature, and 2 weeks at 4°C or frozen (-20°C). In 2016, Bertaso *et al.* [112] reported on the development of a capillary electrophoresis method for straightforward quantification of CDT% in DBS.

Further studies about the stability of CDT in DBS for longer periods of time, as well as the evaluation of the influence of hematocrit, punch localisation and spot volume are needed. In addition, and to ensure equivalence of venous and capillary samples, concentrations obtained from venous blood and C-DBS samples should be statistically compared. An advantage of CDT% is that it is a relative measure (expressed as a % of total transferrin). Hence, while the absolute amount of CDT and transferrin may differ, depending on several factors, the CDT% is likely to remain the same. Such an observation was readily made by De Kesel *et al.*, albeit in the context of CYP1A2 phenotyping, where the use of ratios (paraxanthine:caffeine in that case) compensated for effects of volume and hematocrit, as well as for capillary-venous differences [106].

1.3.2.1.2 EtG and EtS in DBS

While multiple methods for the quantification of EtG and EtS in blood have been published [35,65,71,78,113–119], there is currently only one report on their quantification in DBS

[120]. That study utilised full-spot extraction of 10-µL V-DBS, the IS being added to the extraction solvent. EtG and EtS were demonstrated to be stable in DBS for at least 3 weeks when stored at room temperature. Blood and DBS concentrations detected in blood samples from traffic offense cases (N=76) were compared using the matched-paired t test, Wilcoxon test, Bland-Altman analysis and Mountain plot of the percentage differences [120]. This study concluded that EtG and EtS measurement in DBS is a simple and cost-effective method that allows to shorten the time gap between a possible offense and blood sampling [120].

In addition, the stability of EtG has been examined in dried blood stains. Evaluation of blood, spotted and dried onto different surfaces (glass, carpet, wall paper, car seat, calf leather and cotton swab), revealed that the EtG concentration remains relatively constant for 24 hours after the deposit [121]. Even though there may be differences in absolute concentration between varying samples, Kaufmann and Alt proposed that 3 ng EtG/mg dried blood samples at a crime scene could be used as a cut-off value to suggest “a forensic relevant degree of alcoholisation” [122].

Further studies about stability in dried blood stains and DBS for longer storage periods are warranted. Before routine implementation of EtG quantification via DBS, evaluation of the equivalence of V- and C-DBS concentrations, as well as of the influence of hematocrit, punch localisation and spot volume on the analytical result is needed.

1.3.2.1.3 PEth species in DBS

PEths have been widely analysed in blood [90–94,99,101,102,104,123–130], and since 2011, publications have reported on the quantification of PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 in V-DBS samples [100,101,123] and in C-DBS [105,123]. Both whole-spot [101,127] and partial-spot approaches [100,105,123,131] have been described. In all methods the IS was added to the extraction solvent. The extraction efficiency was reported at 55 and 78 % for V-DBS and C-DBS, respectively [123]. Stability was demonstrated for up to 6 months for DBS stored in zip-closure plastic bags containing a desiccant packet at room temperature [123]. One-way ANOVA tests did not reveal a significant influence ($p>0.05$) of the hematocrit (range 0.39-0.57), punch localisation and spot volume on quantification of the evaluated PEths [123]. Comparison between concentrations measured in blood and in DBS has been performed using Wilcoxon signed rank test analyses [123], Bland-Altman analyses [100,101,123], linear regression [100] and Passing-Bablok regressions [123]. All

studies concluded that determination of PEths in DBS is a useful tool to monitor alcohol misuse. Especially in the context of driver's license regranting programs, where in many instances the indirect marker CDT% is used, the use of C-DBS for PEths monitoring seems promising [132]. Samples can be conveniently collected from a patient and the result allows to demonstrate (in)compatibility with ethanol abstinence and/or to make a distinction between teetotallers, social drinkers and heavy drinkers.

Further improvement will be possible with the commercialisation of other species (especially PEth 16:0/18:2) and with the use of deuterated IS, which have recently become commercially available [132,133]. In addition, independent, large-scale, prospective studies are warranted to confirm the promising results we obtained in an exploratory study that demonstrated the potential of PEth monitoring for improving driver's license regranting programs [132].

1.3.2.2 Dried urine spot (DUS)

Similar to DBS, a dried urine spot (DUS) can be generated by depositing an amount of urine onto filter paper, followed by drying. As for DBS, DUS can be interesting to improve the stability of analytes and to facilitate storage and transportation of the samples [105].

1.3.2.2.1 EtG and EtS in DUS

Though widely analysed in urine [64–66,68,70,71,74–80,83,84,115,118,119,135–154], the quantification of EtG and EtS in DUSs (generated by applying 30 µL of urine on filter paper stripes) has only been reported by one research group [155]. While EtG in urine may be subject to degradation [156,157] or post-collection formation [158], these phenomena have not been observed for EtS [157–159]. The analysis of EtG from DUS was demonstrated to avoid the bacterial degradation of EtG in contaminated urine. EtG and EtS were stable up to 7 days in DUS stored at room temperature. Creatinine was also measured in the DUS to compensate for possible dilution of the sample. The IS was added to the extraction solvent. The recovery for EtG and EtS from DUS was higher than 32 and 38 %, respectively. The reproducibility of the extraction efficiency from DUS is difficult to estimate because no %RSD values were published. Lower limits of quantification (LLOQs) were reported at 0.175 µg/mL for EtG and 0.340 µg/mL for EtS, values that lie somewhat above the ones typically reported for EtG/EtS in urine at 0.1 µg/mL (with reports down to 0.02 µg/mL) [160]. Additional

research on the extraction efficiency from DUS could be useful to decrease the LLOQ (by increasing the recovery) and to assess the reproducibility. Further evaluation of the stability for a longer period of time could be useful as well. In addition, to overcome the need for accurate pipetting of a urine sample, future applications could make use of novel technologies that allow volumetric collection of urine. Technologies originally developed for DBS sampling (volumetric absorptive microsampling and microfluidics) could turn out useful for this [109].

1.3.2.3 Sweat and skin surface lipids

The human skin (Figure 1.12) is an organ made up of multiple layers of tissue, which guards the underlying muscles, bones, ligaments and internal organs. The skin is composed of three primary layers, i.e. the epidermis, the dermis and the hypodermis. Glands present in the skin produce sweat (sweat gland) and sebum (sebaceous glandes).

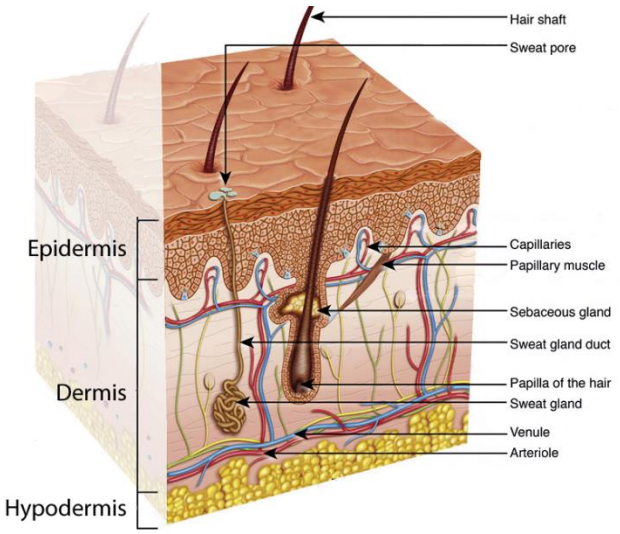

Figure 1.12 Schematic structure of the skin [50].

Sweat (perspiration) is a biological fluid, mainly composed of water (99 %), secreted by the body through the skin to maintain a constant core body temperature [27]. The number and type of sweat glands are not constant and vary from one area of the body to another (i.e. hands count most sweat glands). Eccrine sweat glands are located in the dermal layer of most skin surfaces, while apocrine sweat glands are only present in specific areas, such as axilla, pubic and nipples zone. Approximately 50 % of sweat originates from the trunk, 25 %

from the legs and 25 % from the head and upper extremities. The sweat production rate, which depends upon environmental temperature, emotional state and activities, was estimated between 300 and 700 mL per day and can be up to 2-4 L/h in case of extensive exercise [161]. The pH of the sweat ranges between 4 and 6.8 and increases with the flow rate. More information is presented in a review published in 2013 by De Giovanni and Fucci [27].

Skin surface lipids consist of a mixture of epidermal and sebaceous lipids. The ratio between these two components depends on the body region. In regions with a high density of sebaceous glands (i.e. forehead, scalp, thorax and the upper part of the trunk) the skin surface lipids originate mainly (96-97 %) from sebum [162]. Sebum, secreted by the sebaceous gland in humans, is primarily composed of triglycerides (~41 %), wax esters (~ 6 %), squalene (~12 %), and free fatty acids (~16 %) [163]. A transition time of about 1 week has been reported between the sebum production and its appearance at the skin surface [164].

The excretion of drugs through the skin is not fully understood, but seems to be possible through passive diffusion from blood into sweat glands and via transdermal migration across the skin. The excretion into sweat is dependent on the physicochemical properties of the compound (i.e. mass, pKa, protein binding and lipophilicity). Generally, parent drugs are expected to be detected at higher concentrations than the more polar metabolites. Sweat samples are a mixture of sweat and skin surface lipids present on the skin (especially on the face and scalp).

The sampling of sweat and skin surface lipids was first performed using patches, worn for a variable period of time (from some hours up to some days), with the accumulation of drugs into these absorbent pads reflecting the total consumption during the period the patch was worn. Sebum has also been collected by wiping a wetted cotton bud on the skin [164]. When the total amount of sweat/sebum is not known, results are expressed semi-quantitatively and represent the total amount of drugs accumulated per patch/wipe. Quantitative results can be obtained when the amount of sweat/sebum accumulated on patches/wipes is also measured [16]. This can be performed by using pre-weighed patches/wipes or by measuring the sodium content in the sweat [165] or the squalene (or total lipid) content in the sebum [164,166,167]. For the detection of volatile compounds, electrochemical methods that

convert vapours into an electrical signal proportional to the concentration have been proposed [168,169]. Depending on the device, monitoring can be based on successive or continuous measurements. These electrochemical devices are strapped on the forearm, wrist or ankle (Figure 1.13).

1.3.2.3.1 Ethanol in sweat

In the eighties, studies (described in a review published in 2006 [28]) were performed to develop methods to monitor alcohol consumption via transdermal alcohol measurement (TAC). While some studies were based on the analysis of sweat accumulated in a sweat patch [170–173], other studies quantified ethanol in vapours formed above the skin (insensible perspiration) [174–178]. In the nineties, electrochemical devices such as the Secure Continuous Remote Alcohol Monitor (SCRAM) bracelet Device [31,179,180] and the WrisTAS device (Figure 1.13) [179,181,182] were developed, originally meant to monitor alcohol consumption but subsequently also tested as an alternative to breath analysers which are widely used to provide a quantitative BAC. More details about the specification of these devices can be found in publications by Leffingwell *et al.* and Marques and McKnight [168,179].

Figure 1.13 Secure Continuous Remote Alcohol Monitor (SCRAM) bracelet Device and WrisTAS device [168].

Research to date has shown that transdermal alcohol sensors allow the continuous remote monitoring of (absence of) alcohol consumption, without an intrusive daily contact [168] such as required by BrAC or BAC measurements. Variable correlations -from poor to good- have been reported; between TAC and BrAC/BAC peak concentrations [31,183], between the area under the curve (AUROC) for TAC and BrAC/BAC [31,183], between self-reported

alcohol consumption and TAC AUROC [184] and between self-reported alcohol consumption and peak TAC [184]. A shift (delay estimated between 30 and 180 minutes) and a lower magnitude of the TAC curve (a difference which was demonstrated to be gender dependent [179,185]) when compared with BAC and/or BrAC curves have been observed [12,124,126,128,129]. Overall, these studies have pointed out a lack of reliability to record and retrieve data for the WrisTAS [179] and a water accumulation issue for the SCRAM [179]. While neither the WrisTAS nor the SCRAM suffered from false positive results, both were somehow limited by false negative results (sensitivity estimated at 43 % for the WrisTAS and between 57 and 87 % for the SCRAM) [179,187]. The good specificity was confirmed in a study published in 2009 [180], which showed that abnormally high consumption of a non-alcoholic energy drink (containing up to 0.23 % ethanol) did not induce positive results (< 0.02 % w/v). Studies based on later-generation devices (i.e. WrisTAS 7 [188] and SCRAM II [180,186,189,190]), as well as other biosensing devices [169], have not reported problems to record and retrieve data. When combined with sophisticated mathematical models, these new devices are able to semi-quantitatively predict BrAC or BAC from TAC [169,181,185,186,188]. These studies have provided very promising results but should be tested and validated using larger datasets.

In 2014, the SCRAM II was able to detect 38 % (8/21) of the drinking episodes when one alcoholic drink was consumed and all drinking episodes (N=83) when two or more alcoholic drinks were consumed. To date, TAC seems better suited to distinguish between the consumption of 1-2 beers *vs.* more than two beers (cut-off value proposed at 0.024 g/dL [186]), rather than to really monitor an alcohol abstinence period [185]. More recently, the SCRAMx (third generation of SCRAM devices) became commercially available.

1.3.2.3.2 EtG in sweat

In 2008, Schummer *et al.* reported on the quantification of EtG in sweat [16]. The amount of sweat accumulated on patches was determined by measuring the sodium content in the extract. In this study, 14 volunteers wore a sweat patch during the time they had planned to consume alcohol. They wore the patch for 3 to 12 hours and reported alcohol consumption varying from 38 to 155 g of pure ethanol. Four teetotallers were also involved in the protocol. EtG could be measured (1.7-103 µg/L) in patches from all subjects that had consumed alcohol, in concentrations that were about 100 times lower than those in blood.

We are not aware of other reports that have pursued the determination of EtG or determined EtS in sweat. While such methods should be fully validated, the interpretation of a quantitative result (and/or the use of a cut-off value) will likely remain challenging, given the anticipated inter-individual variability. Hence, while technically possible, we consider it likely that EtG determination in sweat will have to give way to other alternatives mentioned in this overview.

1.3.2.3.3 FAEEs in skin surface lipids

Concentrations of FAEEs in skin surface lipids (sebum) of teetotallers, social drinkers and alcoholics were estimated using patches [164,166,167] or wipe tests [164]. The endogenous concentration of FAEEs was reported up to 13.85 pg/mg sebum [167] or 1.12 ng/µg squalene [164]. After the consumption of a single high dose ethanol (92 and 112 g) by two volunteer abstainers, the highest increase of the concentration was observed between 8-12 days after the drinking event. This time delay corresponds to the transition period required by the sebum to reach the skin surface. Total FAEEs concentrations between 11.10 and 86.55 pg/mg sebum (N=11) were measured in patches worn for 45 min by alcohol drinkers without dependence [167]. Using a wipe test, concentrations between 0.08 and 1.56 ng/µg squalene were reported for alcohol drinkers without dependence (N=16) [164] and with a self-reported alcohol consumption between 9 and 261 g ethanol (~1 and 26 drinks per week) the week prior to the sampling. In alcoholics, FAEEs concentrations up to 23.33 ng/µg squalene using the wipe-test [164] and up to 1243.40 pg/mg sebum using patches have been reported [167]. Differences in results can be attributed to a variety of factors, amongst which the timing of the sampling, the use of different sampling approaches (wipe-test *vs.* patches) and the means for normalising the data (squalene *vs.* sebum). Further studies are needed to ensure the accuracy of the quantification and to provide information regarding interpretative issues. In addition, the same limitations hold true as those mentioned for EtG in sweat.

1.3.2.4 Oral fluid

Oral fluid consists of saliva (the aqueous secretion produced by the three pairs of major salivary glands (Figure 1.14)), other secretes, as well as other (solid) constituents. It is composed of mainly water (99 %), with 0.3 % proteins (mostly enzymes) and 0.3 % electrolytes (i.e. sodium, potassium, chloride, bicarbonate), besides bacteria, epithelial cells and food debris. The composition and the volume of saliva produced are variable within individuals and are influenced by the moment of the day and the type of stimulus. The production of saliva is estimated between 0-6 mL/min (500-1500 mL per day). More information about the physiology of saliva/oral fluid and the incorporation of drugs into it are available in reviews published in 1998 by Kidwell *et al.* [161] and in 2005 by Aps and Martens [191]. The pH of unstimulated saliva ranges from 2.6 to 7 and -in case of stimulation- increases up to 8 [161]. The collection of oral fluid can be performed by spitting, expectoration, or absorption into a swab.

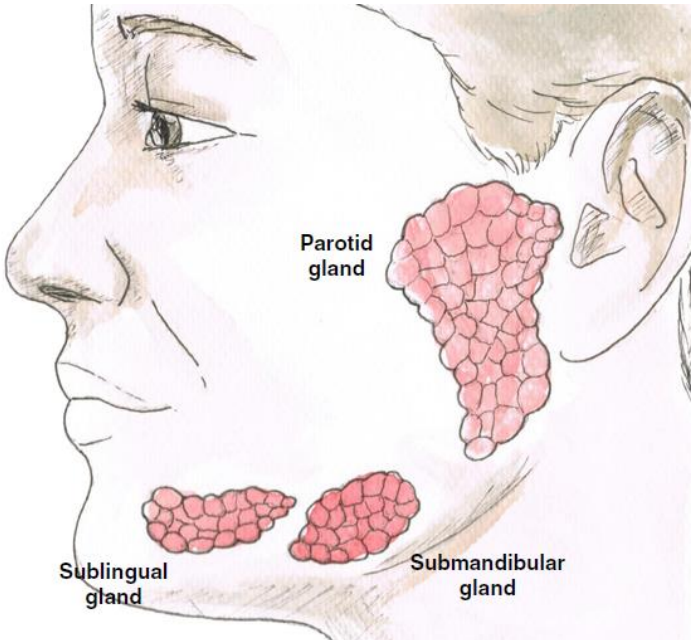

Figure 1.14 Representation of the three pairs of salivary glands [55].

1.3.2.4.1 Ethanol in oral fluid

The detection of ethanol in oral fluid has been widely performed using enzymatic screening [192–198,13], while the quantification using chromatographic methods [199–201] is not commonly used. A good correlation (r=0.98) between the concentration of ethanol in blood and oral fluid has been observed [1]. Concentration-time profiles of ethanol in oral fluid, blood and breath are similar (Figure 1.4, upper) [199,201], with a mean oral fluid-blood ratio estimated at 1.09:1 (range 0.96:1-1.23:1) [71]. After the consumption of 0.68 g/kg body weight of ethanol, mean (N=21) peak concentrations (1.9 g/L) were reached between 10 to 100 minutes from the end of drinking. The mean disappearance rate of ethanol in oral fluid was estimated at 0.13 g/L/h [33]. Ethanol concentrations up to 4.8 g/L were measured in oral fluid from alcohol abusers [13]. Oral fluid concentrations can be influenced by the presence of residual unabsorbed alcohol within the oral cavity due to recent ingestion or regurgitation. To avoid a bias due to these possible ethanol residuals, a delay of at least 10 minutes has been proposed before the sampling [192]. Several on-the-spot enzymatic devices (alcohol test stick) for the analysis of ethanol in oral fluid (i.e. QED kits or AlcoScreen) have been used in emergency rooms and ambulances [192–198] to obtain rapid results, even from unconscious persons. Given the ease of and expertise with breath sampling and the fact that blood will remain the gold standard to verify whether or not someone is under the influence of alcohol, we feel that the main (and possibly only) use of ethanol determination in oral fluid indeed lies in screening, in cases where a person is unconscious or for another reason cannot perform a breath test.

1.3.2.4.2 EtG in oral fluid

The quantification of EtG in oral fluid has been reported in a few publications [71,78,202] and is based on a method published in 2009 by Hegstad *et al.* [203]. An oral fluid collector (Statsure Saliva Sampler), consisting of a collection pad, a stabilising solution and a transport tube, was used. The amount of collected oral fluid was determined by weighing the collector. The peak concentration of EtG in oral fluid (between 0.008 and 0.014 mg/L) after consumption of 0.5 g ethanol/kg body weight was observed 3.5 h after the start of drinking [71]. After consumption of 1.0 g ethanol/kg body weight (Figure 1.15), peak concentrations between 0.013 and 0.059 mg/L were measured after 3.5 to 5.5 hours. EtG was detected in oral fluid up to 11.5 hours after the end of drinking. EtG concentrations in oral fluid and

blood were similar. No EtG was detected in oral fluid after the use of mouthwash containing ethanol (22 %) or after the consumption of one bottle (7.5 dL) of non-alcoholic wine (which contains 3 mg/L EtG) [78]. Results from a small population study (N=3) have shown that the detection of EtG in oral fluid indicates an alcohol consumption of 6 or more drinks the night before the sampling [203].

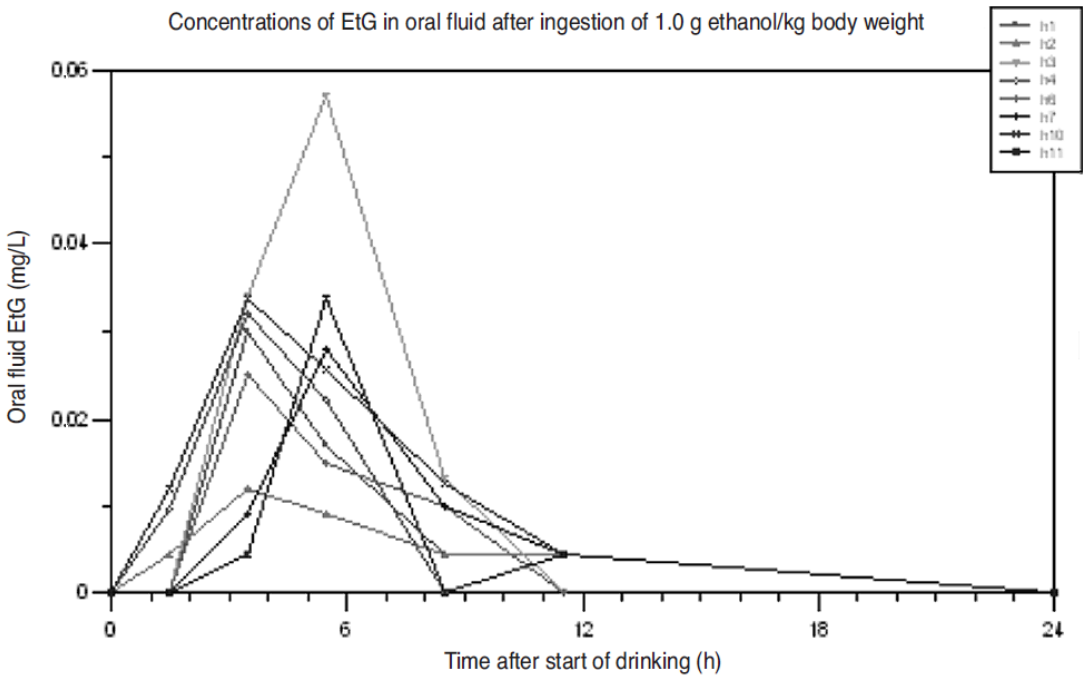

Figure 1.15 Oral fluid EtG concentration-time profile after the consumption of 1.0 g ethanol/kg body [71].

Even though some publications have used the quantification of EtG in oral fluid [71,78,202,203], only one has reported on the validation [203]. To confirm the utility of EtG in oral fluid, more data are needed, including more data on its stability in oral fluid and possible cut-off values for interpretation. In addition, the data should be corroborated by others. Potential may lie in the rapid and non-invasive sample collection for determination of EtG in oral fluid from drivers that were apprehended after a hit and run case and had a negative alcohol test at the time of testing. In these cases, the wider window of detection offered by EtG monitoring in oral fluid may still allow to detect a recent drinking episode. It should be considered that an even wider detection window is offered by EtG monitoring in urine and also ethanol determination in urine may allow to pick up recent drinking in cases in which ethanol is no longer measurable in breath or blood. To our knowledge, the detection or quantification of EtS in oral fluid has not yet been reported.

1.3.2.5 Exhaled breath

Exhaled breath (mainly composed of water vapour and inhaled air that has not reached alveoli) contains volatile as well as non-volatile compounds. As already mentioned, the best known application of exhaled breath testing is monitoring of the volatile ethanol (Section 1.3.1.1.3). However, recently, also the determination of non-volatile compounds in exhaled breath has gained interest. Different sampling approaches have been proposed and were presented in a recent review written by Beck *et al.* [204]. The principle comes down to the fact that virtually any compound, via its deposition in small particles that are exhaled, is present in exhaled breath. Via a simple and disposable sampling device, these exhaled breath particles can be trapped and processed for analysis on the presence of drugs or markers of e.g. alcohol use.

1.3.2.5.1 PEth species in exhaled breath

The quantification of PEth 16:0/18:1 and PEth 16:0/16:0 in exhaled breath has recently been presented as a non-invasive method to detect moderate to heavy drinking [134]. Breath samples were collected using a commercial disposable device (SensAbues), which traps aerosol microparticles that mainly originate from the airway lining fluid (surfactant). PEth 16:0/18:1 could be measured (range 20-77 pg/filter, median 45.5) in all self-reported heavy drinking volunteers (N=12), while PEth 16:0/16:0 was not detectable (LLOQ = 5 pg/filter). The concentration of PEth in breath and BAC was not significantly correlated (ρ=0.660, Spearman). The breath samples of all (N=12) control volunteers (self-reported alcohol abstinence or regular low drinking but no alcohol intake in the previous 2 days) were negative. Further research is needed to establish if and how breath PEth results correlate with blood PEth data. Interesting to note is that for drugs of abuse, there is not a consistent quantitative correlation between breath and blood data; in the sense that data should primarily be considered as qualitative (a drug is present or not). However, because of the nature of PEth -being a modification of an endogenous phospholipid- exhaled breath actually contains an intrinsic control, being the non-modified phospholipids, which may serve to normalise the breath PEth data. If this approach turns out successful, studies could be set up to establish cut-off values, as has been done for blood. Obviously, the non-invasive nature of breath testing offers a promising and major advantage, and may possibly open a new field of research to provide an alternative approach of monitoring alcohol consumption.

1.3.2.6 Hair

Hair as an alternative matrix is especially useful to provide long term information about consumption/ingestion of drugs, after their elimination from the body [26]. Briefly, the retrospective investigation of past consumption is possible because compounds are incorporated into hair. This is possible by passive diffusion from blood capillaries into growing cells, during the hair shaft formation via surrounding tissues from deep skin and by diffusion from sweat and sebum along the completed hair shaft (Figure 1.16).

The melanin content of hair and the chemical properties of the compound (lipophilicity and basicity) are the main factors influencing the incorporation process. External contamination of hair -via for example dust, smoke, dirty hands, sweat or sebum- is possible and must be taken into account, especially when working with compounds which are not metabolites. In the same way, drugs and metabolites can be removed from hair, by decomposition (e.g. bleaching, UV radiation) or by extraction (e.g. shampooing and hair cosmetic treatments).

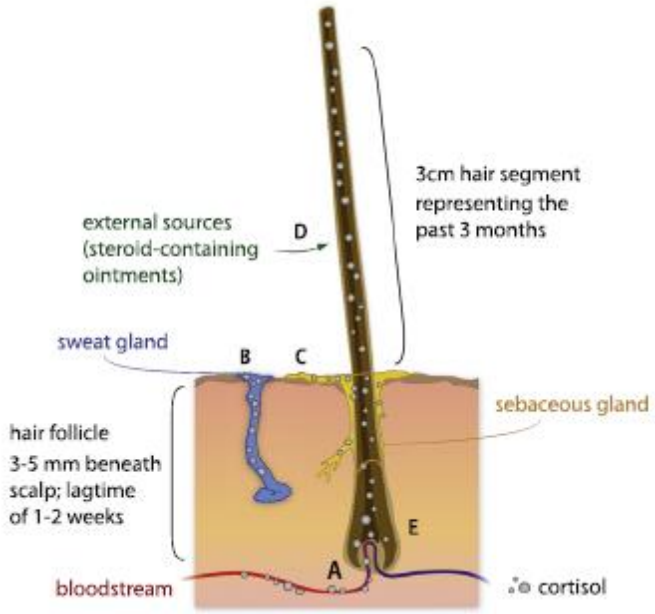

Figure 1.16 Illustration of cortisol incorporation into hair [205].

Hair grows in a cycle composed of 3 stages (Figure 1.17), starting with an active growing (anagen) period (4-6 months), followed by a transition period of a few weeks (catagen), which ends with a resting phase (telogen). The growth rate range of scalp hair is estimated between 0.6 and 1.4 cm per month. The Society of Hair Testing (SoHT) guidelines for drug

testing in hair propose an average scalp hair growth of 1 cm/month [206]. The preferred sampling site is the vertex posterior part of the scalp, because it contains less telogen hair and a relatively uniform growth rate. Typically, a hair strand with a diameter of 3-4 mm is fixed with a string attached as close as possible to the scalp and hairs are cut at the skin surface. The string marks the proximal end of the sample. If no head hair is available, the SoHT states that other body hair can be collected, but the different physiology of non-head hair has to be considered during the interpretation [207].

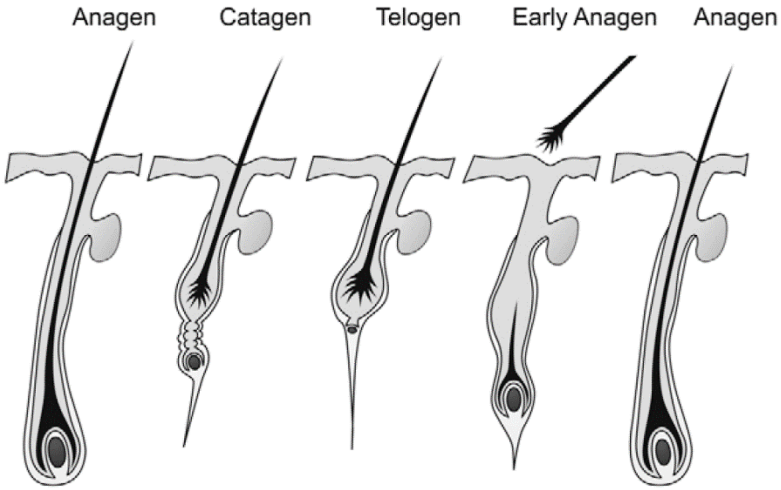

Figure 1.17 Illustration of the growing cycle of hair [59].

Pre-analytical steps prior to analysis include washing the hair strand, segmenting the hair (optional), cutting the hair into small pieces or grinding it. A washing step is necessary to remove residues of hair products, sweat, sebum and dust and to remove target drugs that may originate from external contamination (e.g. in individuals involved with illegal drugs, smoking), although this may not be completely feasible and might even lead to incorporation of some compounds. Segmental analysis can provide information concerning the evolution of the consumption with respect to time course. When taking into account a mean scalp hair growth of 1 cm per month, analysis of 1-cm segments allows the evaluation of the consumption per month during the period before sampling. To avoid that hair shifts within the hair strand during the segmentation, a tuft of hair could be aligned into folded graph paper and cut to the appropriate length with a razor blade. Nevertheless, the 2014 SoHT consensus guideline for the use of alcohol markers in hair recommends not to segment

hair but to analyse 0-3 up to 0-6 cm proximal scalp hair [207]. Compounds trapped into the hair shaft must be extracted by solubilisation or digestion. In case of solubilisation, hair is typically first cut into 1-3 mm pieces or pulverised. The 2014 SoHT consensus for the use of alcohol markers in hair advises to grind the hair prior to analysis or, if not, to demonstrate a comparable recovery [207]. The analysis of ethanol in hair and nail, which would offer a longer detection window, is not usable for evaluation of alcohol consumption due to the instability of ethanol in these solid matrices. Therefore, the focus has been on the detection of direct ethanol markers [207].

1.3.2.6.1 EtG in hair

The quantification of EtG in hair has been reported in numerous publications [7,9,11,144,160,208–220] and has been reviewed in 2008 by Pragst and Yegles [221] and in 2014 by Crunelle *et al.* [22]. The incorporation mechanism of EtG in hair has not been totally explained yet, but due to its acidic and extremely hydrophilic properties this seems to occur mainly by diffusion from blood and deposition from sweat (Figure 1.16) [26,63,160]. Quantification of EtG in hair is widely used to monitor chronic alcohol consumption and to establish abstinence (or not) in cases where chronic excessive drinking is suspected.

The concentration of EtG in the first 3-cm proximal hair segment from 3 month-abstainers ranged up to 4.5 pg/mg. After a regular ethanol consumption of 100 g ethanol per week for 3 months, the concentration of EtG was between 2.0 and 9.8 pg/mg hair (median = 5.6 pg/mg, N=10). A regular ethanol consumption of 150 g ethanol per week for 3 months led to an EtG concentration between 7.7 and 38.9 pg/mg (median 11.3 pg/mg, N=10) [220]. EtG was detected in hair of patients in alcohol withdrawal studies in concentrations up to 528 pg/mg hair [46]. A significant correlation between the EtG concentration in hair (measured up to 261 pg/mg hair) and the alcohol consumption (estimated using self-report questionnaires and medical records) has been reported by several authors [56,222,223], while others have reported no correlation at all [224–226]. These differences may be explained first by the difficulty to obtain sufficiently reliable information about the alcohol consumption, especially when a long period of time is involved, second by cosmetic treatments that have been shown to have an impact on the concentration of EtG in hair and third by a variation in the correlation, depending on the concentration range. Further studies are still required to resolves these issues.

The SoHT has published guidelines [207] concerning the use of EtG in hair for the detection of chronic/excessive alcohol consumption. A cut-off value at 30 pg EtG/mg hair, measured in the 0-3 up to 0-6 cm proximal segment, has been proposed to strongly suggest excessive/chronic alcohol consumption. An EtG concentration ≥ 7 pg/mg (but below 30 pg/mg) in the 0-3 up to 0-6 cm proximal scalp hair segment strongly suggests repeated alcohol consumption and an EtG concentration < 7 pg/mg does not contradict self-reported abstinence of a person during the corresponding time period before sampling.

Hair melanin content does not influence the concentration of EtG [227]. Bleaching, perming, straightening and dyeing of hair are known to decrease the concentration of EtG in hair, whereas other cosmetic hair treatments (use of cosmetics containing ethanol, hair spray, gel, wax, oil or grease) were reported to have no effect [207,228–230], except in one case report where EtG was detected in a hair sample due to the use of a hair care product containing EtG (alcoholic plant extract) [231]. Extraction experiments show that the time required to extract EtG from hair is decreased when the matrix is pulverised [209] and that the concentration determined in pulverised hair samples was higher than in cut hair samples [208,210,218,232]. The 2014 SoHT guideline states that “powdering hair prior to the extraction of EtG is preferred. Laboratories utilising other sample preparation procedures should demonstrate comparable recovery of EtG” [207]. An interesting publication has demonstrated that the washing and the nature of extraction solvents influences the quantification of EtG in hair [233]. Additional guidelines to streamline washing and extraction procedures may be useful to decrease the variations observed between reported EtG concentrations from different laboratories [218]. Moreover, labs aiming at routine implementation of EtG monitoring in hair should use external QCs and/or participate to proficiency testing schemes, to ensure an accurate quantification and/or comparability of results.

1.3.2.6.2 FAEEs in hair

Numerous studies about the quantification of FAEEs in hair have been published [7,9,221,233,226,234–245]. Because of their lipophilic character, most authors explain the incorporation of FAEEs into the hair mainly through sebum [19]. FAEEs concentrations increase from the proximal region to the distal [221] and -according to recent publications- decrease after 5-10 cm in length [240]. This phenomenon has been explained by the contact

of hair with the sebum from the sebaceous gland or by a more intense hair wash near the scalp. An interesting review has been published in 2008 by Pragst and Yegles [221].

Hair melanin content does not influence the concentration of FAEEs [227]. Bleaching and perming hair may influence the concentration of FAEEs in hair [207], while dyeing has been shown to decrease the FAEEs concentration in hair [237].

The concentration of FAEEs in teetotallers was between 0.06 and 0.37 ng/mg [240]. The reason for this baseline presence remains unclear and different possible causes have been put forward, such as endogenous formation, incorporation via capillary products containing ethanol, external contamination, diet or medication [19]. The SoHT recommends not to use the analysis of FAEEs alone to assess abstinence and suggests a cut-off value to detect alcohol consumption at 0.2 ng/mg when measured in the 0-3 cm proximal segment (0.4 ng/mg when measured in the 0-6 cm proximal segment) [207]. False positive results, due to an external contamination via cosmetic products containing ethanol and/or FAEEs, which have been detected in all of 49 frequently used hair products analysed, have been reported [237,242].

After “moderate” alcohol consumption, the total FAEEs (E14:0, E16:0, E18:0, E18:1) concentrations measured in the 0-6 cm proximal segment were between 0.20 and 0.85 ng/mg (mean = 0.41 ng/mg, N=13) [240]. FAEEs (E14:0, E16:0, E18:0, E18:1) concentrations up to 11.6 ng/mg hair were measured in hair of patients in alcohol withdrawal studies [240]. The SoHT proposed a cut-off value to strongly suggest chronic and excessive alcohol consumption at 0.5 ng/mg FAEEs when measured in the 0-3 cm proximal segment (1.0 ng/mg when measured in the 0-6 cm proximal segment) [207].

1.3.2.7 Nail

Nail is a solid keratinised layer covering the tips of the fingers and toes in humans, which - like hair- accumulates drugs and allows a retrospective investigation. A review about nail analysis for the detection of drugs of abuse and pharmaceuticals has been published by Cappelle *et al*. in 2014 [23]. The nail consists of the nail plate (hard part of the nail), the nail bed (skin beneath the nail plate) and the nail matrix (part of the nail bed which contains nerves, lymph and blood vessels). The incorporation of compounds into nails (Figure 1.18) occurs mainly via the nail bed (along the nail plate) and via the nail matrix (at the root of the nail), in two directions (vertically and horizontally) [23]. The growth rate of nails is constant

and was estimated at about 3 mm/month for fingernails and 1.1 mm/month for toenails. Three to five months (8-16 for toenails) for fingernails are required to grow from the germinal matrix to the free edge. Sampling is mainly performed by clipping, but can also be performed via scraping. As for hair analysis, nail samples have to be washed, cut or grinded and drugs have to be extracted (via solubilisation or digestion).

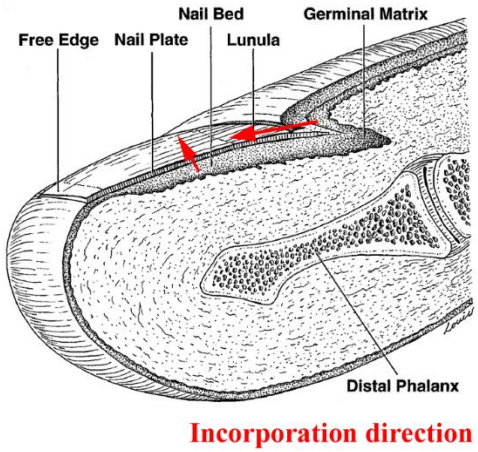

Figure 1.18 Schematic structure of the nail [63].The incorporation directions are indicated in red.

1.3.2.7.1 EtG in nail

Since 2012, quantitative methods for EtG in nails have been reported [14,246–250], as reviewed by Cappelle *et al.* [23]. The EtG concentrations in nails from 5 alcohol abstainers were all below 10 pg/mg (LLOQ = 2 pg/mg). After a self-reported mean alcohol consumption between 10 and 60 g of ethanol per day, the concentrations measured in nail were between 12.3 and 84.3 pg/mg [247]. The EtG concentration in nails from alcohol abusers ranges between 40 and 91 pg/mg [14]. In 529 students, 203 nail samples had quantifiable (≥ 8 pg/mg) EtG concentrations [246], up to 397 pg/mg (mean 29 pg/mg). Further studies are required to better understand the concentration of EtG in nails. In addition, the stability of EtG in nails, the equivalence between EtG levels in finger and toe nails, as well as the influence of external parameters (e.g. influence of the use of nail polish) should be studied. Undoubtedly, when a statement is to be made on the timing of ethanol consumption, this will be even more challenging for nails than for hair.

1.4 Driving under the influence of alcohol and regranting of a driver's licence

The Belgian legislation for driving under the influence (DUI) of alcohol (Art. 35-37/1) fixed the limit concentration and the sentences [251]. The ethanol concentration in blood (BAC) is limited to 0.5 gram per litre of blood (BrAC ≥ 0.22 mg/L) and drivers above this limit are condemned to a fine, depending on the level of intoxication (Table 1.3). In case of alcohol-impaired driving (Art. 35), the law provides a fine between 200 and 2000 Euro and the confiscation of the driver's licence for 1 month up to lifelong. Imprisonment for 1 month to 2 years, a fine between 400 and 5000 Euro and/or the confiscation of the driver's licence for 3 months up to lifelong can be pronounced in case of recidivism (Art. 36).

	Art. 34 § 1	Art. 34 § 2	Art. 35	Art. 36
Breath concentration (mg/L)	0.22-0.35	≥ 0.35	*	**
Blood concentration (g/L)	0.50-0.80	≥ 0.80		
Fine (Euro)	25-500	200-2000	200-2000	400-5000
Deprivation to drive (time)			1 m -5 y (lifelong)	3 m - 5 y (lifelong)
Imprisonment (time)				1 m - 2 y

Table 1.3 Definitions and sentences for DUI offences (*state of drunkenness (observable signs of impairment); **recidivism to Art. 34 § 2 or Art. 35 (within 3 years) after a conviction for Art. 34 § 2 or Art. 35. m (month), y (years).

The annex 14 of the driver's licence regulation [252], which defines the medical/psychological norms to assess the fitness to drive, states that "all the resources offered by medicine can be used" and adds that the medical doctor can make this decision dependent on a blood analysis for DUI of alcohol offences and on a hair analysis in case of DUI of drugs offences. In Belgium, if the medical assessment by the physician deciding about the fitness to drive includes a blood analysis, the sampling is not performed directly by himself. The volunteer is asked to visit his family doctor, who then performs the venepuncture and sends the blood sample to an authorised laboratory for analysis. Hence, the current process implies an invasive sampling and there may be a long time period between the blood analysis request and the final decision. In addition, the chain of custody is not ensured during the whole process.

The medical norm of the annex 6 of the driver's licence regulation [253] and the Directive 2006/126/EC of the European Parliament and of the Council of December 20, 2006 on driver's licences [254] declare that alcohol dependent persons or persons who cannot stay abstinent while driving are not fit to drive. The Belgian driver's licence regranting legislation requires in case of alcohol dependence a 6-month period of proven abstinence [253]. Administrative documents (i.e. attestation of alcohol withdrawal, letter from a psychologist) are currently used to monitor the abstinence period.

In Belgium, as in many European countries, analyses of indirect biomarkers such as CDT%, GGT, AST/ALT and MCV, are the current analytical methods used by physicians to monitor the alcohol consumption in case of a driver's licence regranting process [50,209,255–258]. These markers reflect the indirect effects of ethanol on the body, via its interference with glycosylation (increased CDT%) and with liver function (increased GGT, ALT, AST) and its effect on the size of red blood cells (increased MCV). Because of a lack of sensitivity and specificity to detect alcohol consumption (illustrated in Figure 1.19), these analyses are unable to detect all cases of alcohol dependence and are not adapted to evaluate strict alcohol abstinence periods either.

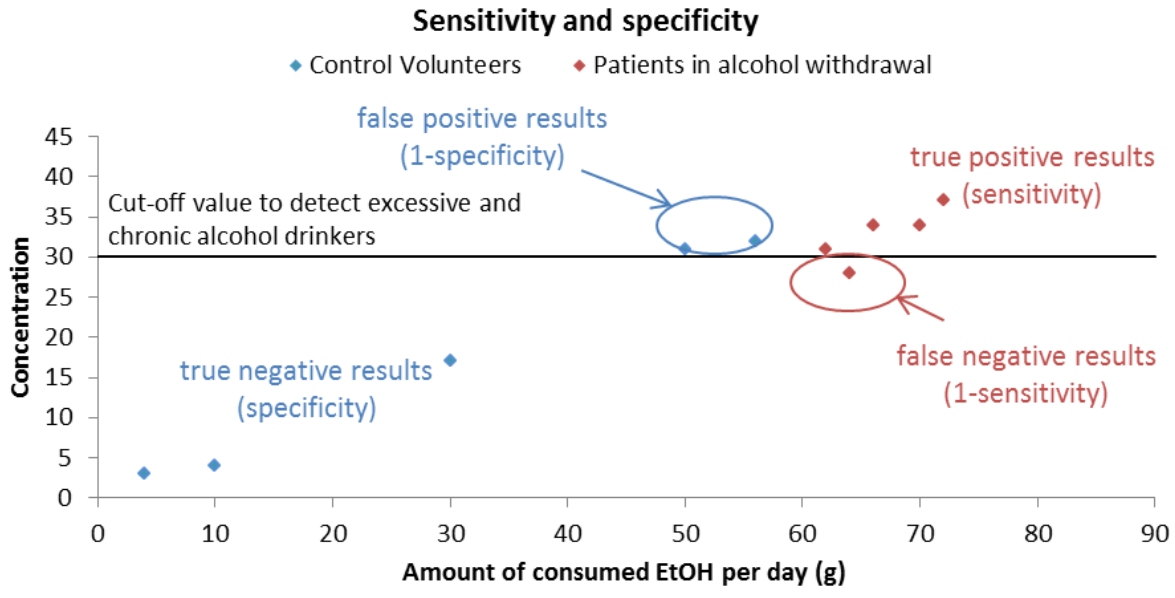

Figure 1.19 Illustration of sensitivity and specificity of an analytical method used to detect excessive and chronic alcohol consumers, using a cut-off value defined between patients in alcohol withdrawal and control volunteers.

In several countries (e.g. Italy, Germany, Switzerland and Sweden) the quantitative determination of ethanol metabolites, such as ethylglucuronide (EtG) and ethylsulfate (EtS) in urine and/or hair is used to monitor an alcohol abstinence period. According to the Italian driver's licence regranting program, abstinence periods are monitored via urinary EtG and EtS, determined in three to five unannounced collections, over a period between 2 and 4 weeks [83]. The Swedish [209] and Swiss [257] driver's licence regranting programs have introduced the quantification of EtG in hair as a complementary tool to the analysis of indirect biomarkers. The German driver's licence regranting guidelines to monitor abstinence periods require the quantification of EtG in six random urine or four hair samples [75,160,259]. Recently, Schröck *et al.* recommended to include the quantification of PEth 16:0/18:1 in whole blood as a routine analysis for the detection of prolonged excessive alcohol consumption (currently based on a BAC above 1.6 ‰) in "driving under the influence" cases [260]. In the United States of America, where alcohol ignition interlock devices are used to prevent recidivism, the ability of direct biomarkers (blood total PEths, hair EtG and FAEEs and urine EtG and EtS) and indirect biomarkers (CDT%, ALT, AST, GGT) to predict recidivism has been tested [261]. From that study it appears that, except for FAEEs in hair, all alcohol biomarkers were significantly related to the interlock BAC test profiles and that PEths in blood was a remarkably strong, general alcohol risk indicator.

The European Integrated Project DRUID (Driving under the Influence of Drugs, Alcohol and Medicines) has published in 2008 a report on state of the art regarding the rehabilitation in the European Union [262].

1.5 References

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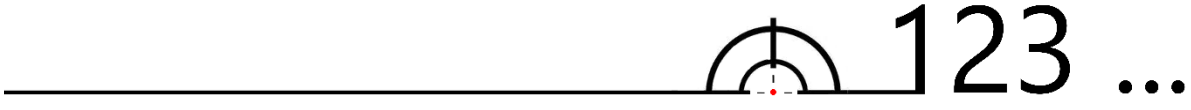
Chapter 2

Current situation, objectives and structure



The Belgian driver's licence regranting regulation states two important facts regarding alcohol consumption and driving. First, an alcohol dependent person or a person who cannot stay abstinent while driving is not fit to drive; second, a person with a deprivation to the right to drive, who has been declared alcohol dependent, has to prove an abstinence period of minimum 6 months to regrant his driver's licence.

To date, the abstinence period is monitored using administrative documents (e.g. attestation of alcohol withdrawal, letter from a psychologist) and the procedure to assess the fitness to drive is based on a psychological and medical assessment, which can -but doesn't have to- include a blood analysis. In Belgium, if a blood analysis is included in the medical assessment by the physician deciding about the fitness to drive, the blood sampling itself is not performed by this physician. Instead, the volunteer is asked to visit a sampling centre or his family doctor to perform the venepuncture. Blood samples are then sent to an authorised laboratory for analysis. Hence, the current process implies an invasive sampling and may result in a long time period between the blood analysis request and the final decision. In addition, the chain of custody is not guaranteed during the whole process. Another drawback of the process currently used in Belgium, is the compounds targeted. As in many European countries, indirect biomarkers of ethanol consumption are used to monitor (cessation of) alcohol abuse in case of a driver's licence regranting process. These include carbohydrate deficient transferrin (CDT%), gamma-glutamyltransferase (GGT), aspartate aminotransferase/alanine aminotransferase (AST/ALT) and mean corpuscular volume (MCV) [1–6]. These markers reflect the indirect effects of ethanol on the body, via its interference with glycosylation (increased CDT%), with liver function (increased GGT, ALT, AST) and its effect on the size of red blood cells (increased MCV). Because of a lack of sensitivity and specificity [7], these analyses are unable to detect all cases of chronic and excessive alcohol consumption (as seen in persons with alcohol dependence) and are not adapted to evaluate strict alcohol abstinence periods either.

For all these reasons, the aim of this thesis was to select, develop and test alternative methods for quantification of *direct biomarkers* of ethanol consumption, allowing the detection of excessive and chronic alcohol consumption and the monitoring of abstinence periods, thereby using samples that can be obtained via non- or minimally invasive sampling.

For those purposes, a literature search has been performed (**Chapter 1**) and has shown that apart from the 'classical' methods currently used to monitor (excessive) alcohol consumption (e.g. ethanol in blood, breath and urine; EtG, EtS, FAEEs and PEths in blood and/or urine; and CDT%, GGT, ALT, AST and MCV in blood or serum), 'alternative' sampling strategies (e.g. dried blood spots; dried urine spots; sweat and skin surface lipids; oral fluid; exhaled breath; hair and nail) were available.

Methods based on dried urine spots (**DUSs**) are rare and have been developed to improve the stability of compounds (e.g. EtG and EtS) and to simplify the storage and transfer of samples. The analysis of compounds in dried blood spots (**DBSs**) could offer the same advantages of DUSs regarding the transfer/storage of samples and stability of compounds (e.g. CDT%, EtG, EtS and PEths), and in case of capillary DBS (C-DBS), would offer in addition a minimally invasive sampling approach that may be performed by minimally trained staff members. The same cut-off values that have been established for the interpretation of urine and blood results can be used for the interpretation of DUS and DBS results if a good agreement between results from the two methods has been demonstrated. To ensure the reliability of C-DBS methods, it is needed to include specific parameters into the validation process, such as hematocrit, punch localisation and volume effects. The measurement of ethanol with an electrochemical device, worn during a defined period of time, and of ethanol, EtG or FAEEs in **sweat/skin surface lipids** accumulated on a patch, offers an interesting approach to monitor an alcohol abstinence period. The similar pharmacokinetics of ethanol in oral fluid and blood would allow **oral fluid** to be an alternative matrix to blood to detect subjects under the influence of alcohol. An important limitation remains the short detection time of ethanol and EtG in oral fluid. The quantification of PEths in **exhaled breath** has been proposed as a promising non-invasive method to detect moderate to heavy drinking and is to date an interesting research field that is only starting to be explored. Quantification of EtG and FAEEs in **hair** provides long-term information about alcohol consumption prior to the sampling. The possible segmental analysis of hair samples -though not routinely implemented- might offer interesting insights into a drinking pattern. **Nail**, like hair a keratinised matrix, also offers the advantage of accumulating compounds (e.g. EtG).

Within the framework of this thesis, we have decided to work with hair samples instead of nails or sweat/skin surface lipids samples (which also offer the advantage to store compounds) for different reasons. First there is ample literature about the quantification of EtG and FAEEs in hair, whereas there is only a limited number of reports about EtG in nails or ethanol and EtG in sweat or FAEEs in skin surface lipids. Although scientific literature provides plenty of reliable information about analytical methods, sample preparation and interpretation issues for hair analysis, several issues (e.g. segmental analysis, impact of the decontamination and pulverisation, impact of nutritional components, drugs, genetic polymorphisms, and diseases on the metabolism) remain and should be studied further. In addition, sweat/skin surface lipids accumulation in patches or measurement with an electrochemical device requires a patch/device to be worn for several days, whereas nails and hair naturally accumulate compounds and so only require one sampling. In addition, because the incorporation of compounds into nails occurs horizontally (nail matrix) and vertically (nail bed), the relation between the detected concentration and the alcohol consumption pattern is challenging. Last but not least, the possible segmental analysis of hair samples can be very interesting to infer a drinking pattern. EtG and FAEEs are the two possible analytes of interest that can be analysed in hair to monitor alcohol consumption. In this study, we have chosen for the analysis of EtG for three main reasons. First, the SoHT states that “EtG should be the first choice in abstinence assessment” [8]. Second, EtG has a higher sensitivity and specificity to detect excessive and chronic alcohol consumption than FAEEs when measured in hair [9]. Third, FAEEs are subject to post-collection synthesis after exposure to ethanol vapour [10] and to false positive results when using popular ethanol/FAEEs containing cosmetic products [11].

Amongst the other methods based on the analysis of direct biomarkers of ethanol (EtG, EtS, PEths and FAEEs), we selected those with long detection windows in body fluids. Knowing that in persons with chronic and excessive alcohol consumption in detoxification, the detection time is up to 28 days for PEths in blood, up to 5 days for EtG/EtS in urine, up to 4 days for FAEEs in blood, up to 40 hours for EtG/EtS in blood, and up to 11.5 hours for EtG in saliva, we decided to develop methods for the quantification of PEths in blood (and DBSs) and EtG/EtS in urine.

The first method described in this work is the **quantification of EtG and EtS in urine**. This method offers the advantage, compared to the other methods selected, that it is able to detect the consumption of a single dose of alcohol. This is highly relevant in case of monitoring alcohol abstinence. **Chapter 3** presents the development and validation of this method and its application to a small population study.

The second method described in this work is the **quantification of EtG in hair**. Special attention was paid to optimise the sample preparation (solid-phase extraction). The influence of the grinding process on the final quantitative result was studied. The development, validation and application of this method are presented in **Chapter 4**.

A third series of methods described in this work relate to the **quantification of PEth species in blood and DBS (venous and capillary)**. The development and the validation of these methods are presented in **Chapter 5**. In addition, the agreement between the quantitative results from the analysis of whole blood, V-DBS and C-DBS was tested via a population study. Based upon this, a cut-off value to distinguish between inpatients on alcohol withdrawal and control volunteers was suggested.

To evaluate the potential added-value of our three-tiered approach to monitor the abstinence period, to detect the presence of chronic and excessive alcohol consumption and/or to infer information about the evolution of alcohol consumption, these three validated methods were applied to 50 volunteers, for whom fitness to drive had to be assessed. Results obtained from the quantification of EtG and EtS in urine, EtG in hair and PEths in C-DBS were compared with currently used indirect biomarkers (CDT%, GGT, ALT, AST and MCV), with a psychological test (AUDIT) and with the final decision concerning the fitness to drive. This **population study** is presented in **Chapter 6**.

The **broader international context, the relevance and the future perspectives of this research** are presented in **Chapter 7**. While Chapters 3, 4 and 5 are primarily analytically oriented, **Chapter 7** also discusses limitations and advantages related to the compounds and matrices chosen. A general **conclusion** is formulated in **Chapter 8**.

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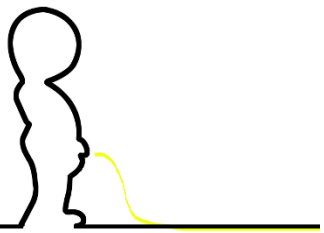
Chapter 3

Quantification of EtG and EtS in urine



Based on

N. Kummer, S. Wille, V. Di Fazio, W. Lambert, N. Samyn, A fully validated method for the quantification of ethyl glucuronide and ethyl sulphate in urine by UPLC–ESI-MS/MS applied in a prospective alcohol self-monitoring study, Journal of Chromatography B. 929 (2013) 149–154.

Abstract

A method for the quantification of ethyl glucuronide (EtG) and ethyl sulfate (EtS) in human urine was developed and fully validated according to international guidelines. Protein precipitation with methanol was chosen as sample preparation and EtG-d_5 and EtS-d_5 were used as internal standards. The method was developed and validated on an Acquity UPLC® coupled to a Xevo TQ MS tandem mass spectrometer using a CSH C18 column. The method was linear (1/x for EtG and $1/x^2$ for EtS) from 100 (LLOQ) to 10000 ng/mL for both analytes. Acceptable accuracy and precision were demonstrated (%bias < 13 %, $\%RSD_r$ < 7, $\%RSD_t$ < 10 %)). A matrix effect (expressed as % recovery) between 76 and 84 % was observed for EtG and no significant matrix effect (< 12 %) was measured for EtS. The extraction efficiency was between 76 and 81 % (%RSD < 14 %). EtG and EtS were stable up to 11 days in the original sampling device (4°C) and after three freeze/thaw cycles and up to 2 months when stored in Greiner bio-one tubes (-20°C and 4°C). Moreover, EtG and EtS extracts were stable in the autosampler during 72 h (4°C). EtG_{100} and EtS_{100} concentrations were calculated by normalising the measured EtG and EtS to a creatinine concentration of 100 mg/dL. The developed and fully validated method was transferred to another UPLC®-MS/MS system (composed of a Xevo TQ S tandem mass spectrometer) and partially revalidated (%bias < 12 %, $\%RSD_r$ < 5 %, $\%RSD_t$ < 9 %). The reproducibility on both systems has been evaluated by successful (Z-score < 2) participation in proficiency tests.

The measurement uncertainties ($\%U=2.12*\%RSD_t$) at the LLOQ (%U = 21 % for EtG and %U = 8 % for EtS) were used to interpret quantitative results close to the LLOQ. Concentrations above or equal to 121 ng/mL for EtG_{100} and 108 ng/mL for EtS_{100} (LLOQ + %U) were used to suggest alcohol intake the days prior to the sampling and to disprove strict abstinence. These decision limits were tested by analysing urine samples obtained from twenty-seven volunteers whose alcohol consumption was monitored during the 5 days before sampling.

3.1 Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are two specific metabolites of ethanol, respectively generated by conjugation with UDP-glucuronic acid [1] and 3'-phosphoadenosine 5'-phosphosulfate [2].

Quantification of EtG and EtS in urine is used to detect recent alcohol consumption. These biomarkers extend the detection window relative to blood ethanol measurement and, compared to long term biomarkers, allow the detection of drinking of small quantities. This permits to monitor alcohol consumption during withdrawal treatment [3,4] or for workplace testing [5,6]. As presented in Section 1.4, several countries -such as Italy [7] and Germany [8,9]- have integrated the quantification of EtG and EtS in urine into their driver's licence regranting program to monitor the abstinence period. In post-mortem cases, the detection of EtG and EtS in urine is useful to distinguish between ante-mortem alcohol intake and post-mortem formation of ethanol [10–12].

EtG and EtS are detectable in urine up to 24 h after intake of 0.25 g/kg ethanol and up to 48 h after intake of 0.50 g/kg ethanol [2,13–18]. After alcohol intoxication, they can be detected in urine during a few days. EtG is eliminated with a half-life of 2.5 h [1,13]. After consumption of alcohol and depending on the amount of consumed alcohol, urinary concentrations of EtG and EtS can vary from some µg/mL [11,18–21] to hundreds of µg/mL [13,15,17,21–23]. Urine samples from alcohol-dependent patients during detoxification can reach EtG concentrations up to 1240 µg/mL [1,2,4,24] and EtS concentrations up to 264 µg/mL [2].

Due to the possibility of finding EtG and EtS in urine even without consumption of alcoholic beverages (Table 3.1) [18,19,25–30], a cut-off limit is generally used to avoid false positive results. Cut-offs are not fixed yet in international guidelines and the ones currently used vary between 50 and 1100 ng/mL [7,9]. Urine analysis of teetotallers (which are persons who practise abstinence from alcohol) shows no EtG [1,31] and no EtS [32] above 100 ng/mL.

Compounds	Ethanol content	Max. concentration (ng/mL)		Ref.
	(Amount consumed/used)	EtG	EtG	
Non-alcoholic beer	3.6 g/L (2-3 L)	512	169	[18]
Non-alcoholic beer	3.1-3.2 g/kg (2.5 L)	870	70	[19]
Non-alcoholic wine*	0.2% (7.5 dL)	<LOD[1]	2150	[26]
Sauerkraut	2 g/kg (0.8-1.3 kg)	200	55	[18]
Appel juice	0.1-0.4 g/L (1.1-2 L)	<LLOQ[2]	<LLOQ[2]	[18]
Grape juice**	0.3-1.8 g/L (1.5-2 L)	<LLOQ[2]	648	[18]
Bananas (peeled)	5 g/kg (670-690 g)	120	55	[18]
Yeast/sugar	21-42 g / 50 g	670	1410	[28]
Mouthwash	21.6% (120 mL over 5 min)	<LOD[1]	<LOD[3]	[26]
Mouthwash	12% (118 mL over 15 min)	341	Not tested	[27]
Hand sanitiser	62% (every 15 min)[4]	62	Not tested	[25]
Hand antisepsis	96% (32 times, 3-4 mL)[5]	958	Not tested	[30]
E-cigarettes	23.5% (puffing)	371[6]	Not tested	[29]

Table 3.1 Maximum EtG and EtS concentrations, which are not due to ethanol consumption, detected in urine. [1] LLOQ = 170 ng/mL, [2] LLOQ = 19 ng/mL., [3] LLOQ = 60 ng/mL, [4] hand sanitiser applied every 15 min throughout a workday, [5] hand antisepsis procedure performed by health care workers during 8-hour clinical work, [6] mean concentration (N=3), * containing 3.0 mg/L EtG and 1.5 mg/L EtS, ** containing EtS.

The most commonly applied technique for quantification of EtG and EtS in urine is liquid chromatography coupled to mass spectrometry (LC-MS) [4,13] or coupled with tandem mass spectrometry (LC-MS/MS) [1,7,11,12,17,26,32–35] in combination with simple dilution or protein precipitation as sample preparation. A few methods have been published using gas

chromatography coupled to mass spectrometry [3,31,36,37] or capillary zone electrophoresis [38–41] for the analysis of EtG and EtS in urine or serum.

To decrease matrix effects, especially for EtS, sample preparation should be adapted. Dilution of urine is the easiest 'sample preparation' method, however, high matrix effects and higher instrument maintenance can be problematic in routine analysis. Even with a 1/20 dilution, relevant matrix effects were observed at low concentrations [32]. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are conventional sample preparation techniques for non-volatile compounds. Due to the highly polar and acidic character of EtG and EtS in combination with a different acidic strength, the development of LLE and SPE is, however, not straightforward. Protein precipitation can be an alternative clean-up method for this type of analytes [12,17–19], if the matrix effects are carefully monitored. No matrix effects were reported after protein precipitation [12] using an LC system coupled with ion trap MS.

Reversed-phase (RP) chromatography used with negative electrospray ionisation mode (ESI-) is the most commonly used approach [1,4,11,13,17,23,26,33,35,42]. The retention of very polar acidic compounds, such as EtG (pKa estimated between 2.84 and 3.21 [11,43,44]) and EtS (pKa estimated at -3.14 [11]), is achieved in RP only under highly aqueous conditions. As highly aqueous conditions might not be optimal for ESI ionisation, post-column addition of an organic modifier is used to enhance the ionisation of compounds, to improve sensitivity. A chromatographic possibility to improve the retention is to use a normal-phase column [12] or another specific column with particular retention behaviour [11]. Nevertheless, normal-phase chromatography is known to provide variable retention times [45]. The use of no-discharge atmospheric pressure chemical ionisation (ND-APCI) [7] or APCI [32] represents another solution to increase the ionisation and so to improve the limit of quantification.

According to international guidelines, forensic analysis by MS/MS in multiple reaction monitoring (MRM) mode requires the detection of minimum two transitions for each compound; one for identification and one for quantification [35,46]. When LC-MS is used, three characteristic ions are required. Sometimes it is difficult to find a second transition for EtS using LC-ESI-MS/MS [12,33], because of the low intensity of the second transition and the presence of interfering compounds in urine.

Low limits of quantification have been reported using an LC-MS/MS system coupled with ion trap MS [7,11,12,47]. Using LC-ESI-MS/MS systems equipped with a triple quadrupole, only one published method [19] has reported an LLOQ at 100 ng/mL for EtG and EtS. Unfortunately, no details of the method validation are given in that publication.

While several LC-MS(/MS) methods have been described for the quantification of EtG and EtS in urine, most of these are not fully validated (following all criteria for chromatographic assays), especially regarding the measurement of accuracy and precision with external and certified quality controls. Moreover, to our knowledge, to date, only two published reports [47,48] have evaluated the reproducibility of the method by participation to interlaboratory tests.

This chapter describes and discusses the development of a method for the quantification of EtG and EtS in urine and presents the results of the validation using two UPLC®-ESI-MS/MS systems, with as tandem mass spectrometer either a Xevo TQ MS or a Xevo TQ S. A prospective study, based on 27 volunteers declaring their daily alcohol consumption, was performed to evaluate the sensitivity and specificity of the method to detect alcohol intake.

3.2 Experimental

3.2.1 Chemicals

Ethyl glucuronide (EtG), ethyl sulfate (EtS) and their pentadeuterated analogues (EtG-d_5 and EtS-d_5) were obtained from Sigma-Aldrich (Steinheim, Germany) as a methanolic 1 mg/mL solution. ULC/MS grade acetonitrile, methanol and 0.1% formic acid in water were purchased from Biosolve (Valkenswaard, The Netherlands). Blank urine was purchased from Bio-Rad Laboratories (Nazareth Eke, Belgium).

3.2.2 Standard Solutions, Calibrators and Quality Control Samples (QC)

Two stock solutions, one for calibration (Cal-Stock) and one for the internal quality controls (QC-Stock), with EtG and EtS each at a concentration of 20 µg/mL were prepared in methanol. The stock solution with internal standards (IS-Stock) each at a concentration of 4 µg/mL was prepared in methanol. All solutions were stored at -18°C.

Daily calibration working solutions (Cal-WS) with concentrations of EtG and EtS each at 100, 5000 and 10000 ng/mL were prepared by diluting the Cal-Stock solution. Calibrators were prepared by spiking 30 µL of the IS-Stock solution to 50 µL of commercial blank urine, an

adequate amount of Cal-WS solution, and methanol until a total volume of 280 µL was reached.

Daily quality control working solutions (QC-WS) with concentrations of 0.5 and 5 µg/mL were prepared by diluting the QC-Stock solution. Internal quality controls (300, 4000 and 7500 ng/mL) were prepared by spiking 30 µL of IS-Stock solution to 50 µL of commercial blank urine, an adequate amount of QC-WS solution and methanol until a total volume of 280 µL.

External quality controls Medidrug ETG 1/10-B, Medidrug ETG 2/09-B, Medidrug ETG 3/10-B, Medidrug ETG 2/12-B were purchased from Medichem (Steinenbronn, Germany). Proficiency tests for EtG and EtS in urine organised by the German Society of Toxicological and Forensic Chemistry (GTFCh), were performed between 2011 and 2015.

3.2.3 Sample preparation

Methanol (250 µL) and 30 µL of the IS-Stock solution (4 µg/mL) were added to 50 µL of urine. After precipitation, the sample was centrifuged at 14000 rpm (20800 g) during 10 min at 4°C. 250 µL of the supernatant was transferred to a total recovery glass vial (Waters, Zellik, Belgium), which is a vial -containing an interior sharp bottom- specifically designed to allow analysis of all volume available, and evaporated to dryness under a stream of nitrogen using a heated metal block at 38°C. The residue was reconstituted in 300 µL of 0.1 % formic acid in water.

To ensure a reliable quantification of samples having a concentration of EtG or EtS above the upper calibrators, an additional 1/1000 dilution was systematically performed for each authentic sample.

3.2.4 Liquid chromatography and mass spectrometry conditions

Separation was performed on an Acquity UPLC® system (Waters, Manchester, UK) equipped with an electrospray ionisation (ESI) source operated in negative mode. Gradient elution was performed on an Acquity UPLC® CSH C18 (2.1 x 100 mm, 1.8 µm) column (Waters, Milford, MA, USA) with 0.1 % formic acid in water (A) and acetonitrile (B) at a flow rate of 300 µL/min. The gradient elution started with 99.3 % of solution A for 2.4 min, decreasing to 40 % of solution A at 3.0 min, and to 20 % of solution A at 4.4 min. The washing step contained only 2 % of solution A and was held from 4.41 to 5.40 min. The initial condition was applied from 5.41 min to 7 min. The column temperature was set at 55°C. The injection

volume was 5 µL using full-loop mode. Post-column addition of acetonitrile was performed at 400 µL/min.

ESI source parameters specific to each mass spectrometer were used. For the Xevo TQ MS mass spectrometer, cone gas flow (nitrogen) was 40 L/h, desolvation gas flow (nitrogen) was 900 L/h at 650°C, collision gas flow (argon) was 0.35 mL/min and capillary voltage was 1 KV. For the Xevo TQ S mass spectrometer, cone gas flow (nitrogen) was 150 L/h, desolvation gas flow (nitrogen) was 1000 L/h at 650°C, collision gas flow (argon) was 0.15 mL/min and capillary voltage was 0.8 kV.

Detection was performed in the MRM mode using the appropriate parameters for each compound and each mass spectrometer (Table 3.2). Two product ions were monitored for EtG and EtS; a quantifier ion for the quantification and a qualifier to confirm the identification. For the ISs, only one ion was used. The chemical structure of the precursor ion and the two product ions for EtG and EtS are represented in Figure 3.1.

		Xevo TQ MS / Xevo TQ S		
	Precursor/product ion (m/z)	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
EtG (Quantifier)	221/75	0.11/0.11	30/40	22/15
EtG (Qualifier)	221/85	0.11/0.11	30/40	24/15
EtG-d_5	226/85	0.19/0.11	28/30	30/15
EtS (Quantifier)	125/80	0.11/0.11	26/50	26/20
EtS (Qualifier)	125/97	0.11/0.11	26/50	18/13
EtS-d_5	130/98	0.11/0.11	28/45	18/15

Table 3.2 MRM transitions and conditions for EtG, EtS and their deuterated analogues.

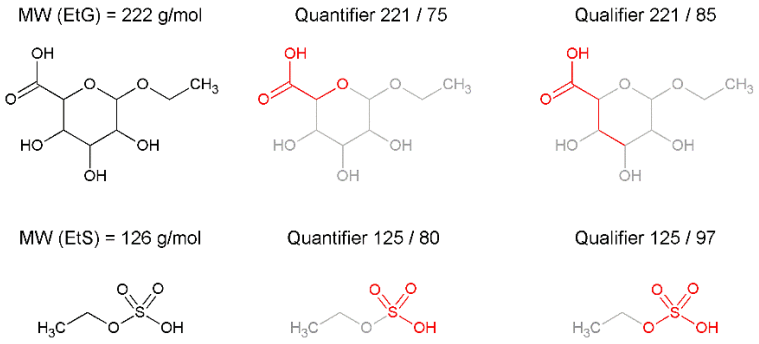


Figure 3.1 Chemical structure of EtG (upper) and EtS (lower) and of their two product ions (red), with the transition (precursor ion / product ion) used. MW: molecular weight.

EtG and EtS mass spectra, extracted from the total ion current plot obtained after the analysis of a urine sample using a Xevo TQ MS mass spectrometer operating in the scan mode, are presented in Figure 3.2.

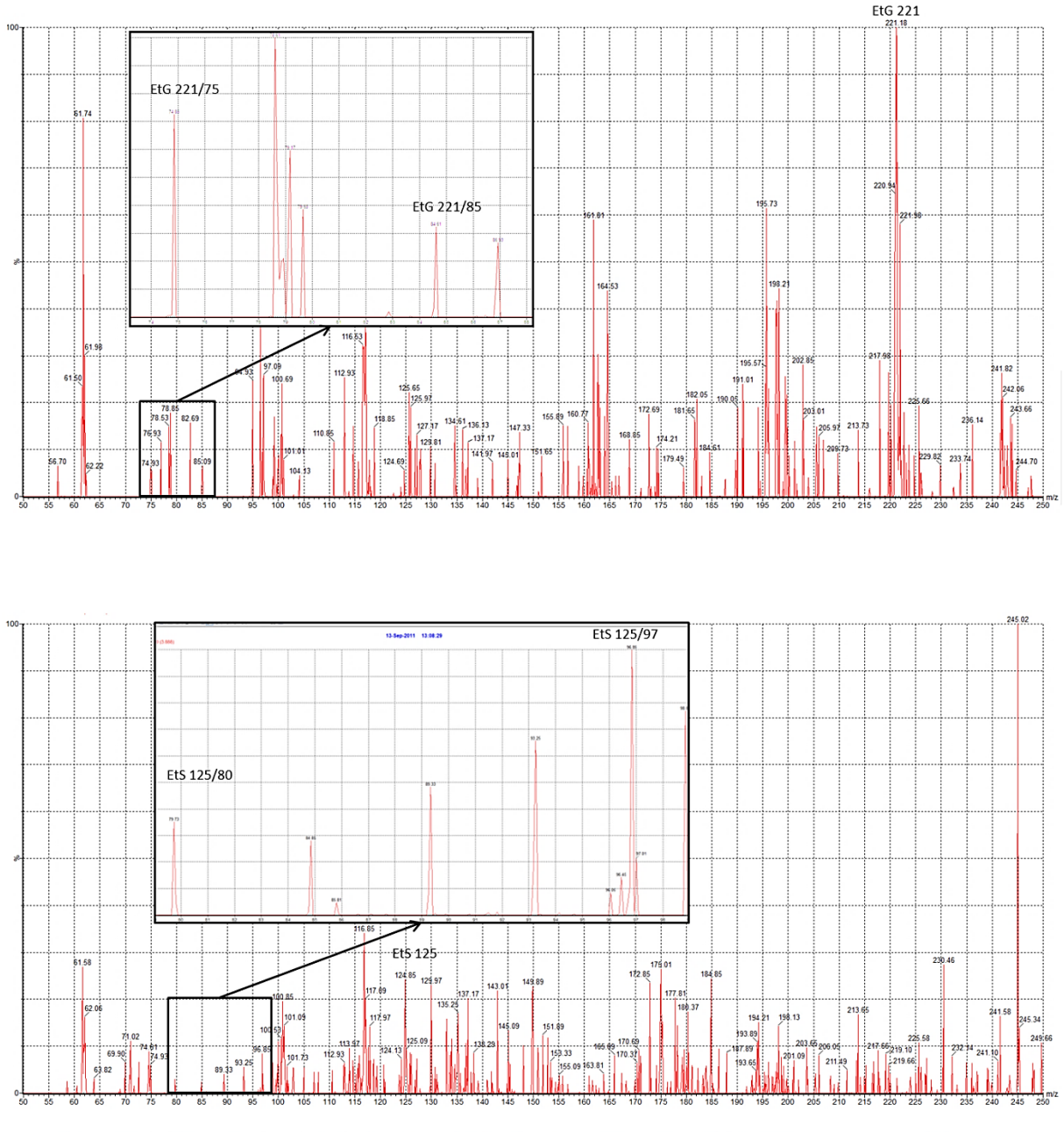


Figure 3.2 Mass spectrum extracted at 2.4 min (upper) and 3.8 min (lower) from total ion current plot obtained after the analysis of a urine sample spiked with EtG and EtS at 7500 ng/mL. Analysis was performed using a Xevo TQ MS mass spectrometer operating in the scan mode. Precursor and product ions (inside the enlarged area) are indicated.

3.2.5 Method validation

Selectivity, sensitivity, matrix effects, extraction efficiency, limit of detection (LOD), lower limit of quantification (LLOQ), linearity, accuracy, precision and stability were evaluated according to international guidelines [49].

To evaluate possible endogenous interferences, six blank urine samples from different individuals were analysed. To verify that there were no isotope exchange reactions with non-labelled compounds, two zero samples (blank urine spiked with internal standard) were analysed. According to the EMA guideline [49], interferences are acceptable in our type of method as long as the response of the interfering peak remains lower than 20 % of the response at the LLOQ.

Matrix effects expressed as % recovery (%ME) were quantified and evaluated using the post-extraction addition technique [50]. To this end, six blank urine samples from different persons were spiked after sample preparation and compared with compounds spiked at the same theoretical concentration in the mobile phase. Extraction efficiency (%EE) was evaluated by comparing responses of six blank urine samples spiked before sample preparation with responses of six blank urine samples spiked after sample preparation. These experiments were done at low (300 ng/mL), medium (4000 ng/mL) and high (7500 ng/mL) concentration.

The limit of detection (LOD) was determined by analysing decreasing concentrations of the analytes (40, 60 and 80 ng/mL, respectively). The LOD was defined as the lowest concentration of the analyte for which the signal-to-noise ratio of both transitions was at least 3/1.

The LLOQ is the lowest concentration of an analyte with a signal-to-noise ratio greater than 10/1 for both transitions and for which the accuracy (%bias) and precision (%RSD) were less than 20 %. Other identification criteria, such as a stable ion ratio (%RSD < 20 %) between the quantifier and the qualifier also had to be reached.

The calibration model (N=6) was tested over the range 100 (LLOQ) to 10000 ng/mL. Calibration model and weighting factor were evaluated for each compound. The goodness of fit was established as the difference between the calculated calibrator value and its nominal value. The %RSD should be lower than 15 % except for the LLOQ (< 20 %).

Accuracy (%bias) and precision (repeatability (%RSD_r) and intermediate precision (%RSD_t) were measured analysing QCs. Three internal quality controls (QCs) at low (300 ng/mL), medium (4000 ng/mL) and high (7500 ng/mL) concentrations and two external QCs were analysed in replicates on 8 different days. One-way ANOVA test with significance level (α) of 0.05 allows to calculate bias, repeatability and intermediate precision with these data (see the Appendix 2 for more explanation). The results were acceptable when they were less than 15 % (20 % for the LLOQ). The reproducibility was evaluated by participation in proficiency tests organised by GTFCh. The measurement uncertainty was also calculated (%$U=2.12*\%RSD_t$) and used to interpret quantitative results close to the LLOQ.

Freeze/thaw stability (3 cycles), processed samples stability (42 and 72 h in the autosampler, 4°C) and long term storage stability (2 months at 4°C and -20°C) were evaluated at low (300 ng/mL) and high (7500 ng/mL) concentrations. Freeze/thaw stability and long term storage stability were evaluated with samples kept in 4-mL Greiner bio-one tubes (Frickengrasen, Germany). Stability of compounds in the original sampling device stored at 4°C for 5 and 11 days was also tested. The mean of the stability samples (N=6) should be within 90 – 110 % of the mean of the control samples (N=6) and the 90 % confidence interval of the stability sample results should be within ± 20 % of the control samples (see the Appendix 2 for more explanation).

Creatinine concentration was measured using a Cobas Integra analyser (Roche Diagnostics Limited, Switzerland).

3.2.6 Population study

A prospective alcohol self-monitoring study was performed by asking 27 volunteers to declare their exact alcohol consumption per day during the 5 days preceding the sampling. Urine samples were collected in 100-mL urine containers from Sarstedt (Nümbrecht, Germany), transferred to 4-mL Greiner bio-one tubes and stored at 4°C until analysis. Samples were analysed within 5 days after collection.

EtG_{100} and EtS_{100} concentrations were calculated by normalising the measured EtG and EtS to a creatinine concentration of 100 mg/dL [2]. The measurement uncertainties (see the Appendix 2 for more explanation) at the LLOQ (%U = 21 % for EtG and %U = 8 % for EtS) were used to interpret quantitative results close to the LLOQ. Concentrations above or equal

to 121 ng/mL for EtG_{100} and 108 ng/mL for EtS_{100} (LLOQ + %U) were used to suggest alcohol intake the days prior to the sampling and to disprove strict abstinence.

3.3 Results and discussion

3.3.1 Method validations

3.3.1.1 Xevo TQ MS mass spectrometer

The method was validated for selectivity, sensitivity, matrix effects, extraction efficiency, limits, linearity, accuracy, precision and stability. Identification of compounds was based on retention time and on the presence of a stable ratio between the two MRM transitions (< 20 %). It is well known that EtG and EtS can be present in small amounts in urine even without voluntary consumption of alcohol [18,19,25–27].

In one of the blank urine samples (N=8), EtG could be detected, but the calculated concentration (approximately 40 ng/mL) was below the LLOQ (100 ng/mL). By a combination of retention time and stable ratio between the qualifier and quantifier, no interfering signal for EtS was detected in blank urine samples. Results of matrix effect and extraction efficiency are presented in Table 3.3.

A matrix effect expressed as % recovery (%ME) between 76 and 84 % was observed for EtG (Table 3.3). The use of EtG-d_5 as IS compensated for the matrix effect ($\%ME_{EtG\text{-}d5}$ in Table 3.3). No significant matrix effects were observed for EtS. As comparison, %ME up to 69, 80 and 171 % were reported for EtG and up to 94, 110, and 179 % for EtS in literature when using LC systems and simple dilution of urines as sample preparation [11,32,47]. The influence of co-eluting compounds influencing the ionisation of the target compounds can be minimised by an efficient sample preparation –in our case, protein precipitation instead of a simple dilution- and by a chromatographic separation of the analytes from interfering compounds (e.g. by using an adequate column and an adapted gradient elution). The extraction efficiency (%EE) of EtG and EtS was reproducible, concentration independent and about 80 %.

The LLOQ was 100 ng/mL for EtG and for EtS (Figure 3.3). The LOD was 60 ng/mL for EtG and 80 ng/mL for EtS.

The calibration curve (N=6) was linear over the range 100 (LLOQ), 250, 500, 2500, 5000, 10000 ng/mL for EtG and EtS. A weighting factor of 1/x (for EtG) and $1/x^2$ (for EtS) was

applied. The %bias of the method was lower than 13 %. The repeatability (%RSD_r) and intermediate precision (%RSD_t) were acceptable, with value lower than 10 % (Table 3.4).

	EtG			EtS		
Concentration	L	M	H	L	M	H
Nominal value (ng/mL)	300	4000	7500	300	4000	7500
%ME (%RSD)	84 (12)	80 (15)	76 (8)	106 (9)	95 (8)	88 (3)
$\%ME_{EtG\text{-}d5}$ (%RSD)	110 (11)	102 (4)	102 (3)	108 (7)	96 (7)	113 (6)
%EE (%RSD)	81 (14)	80 (3)	79 (3)	76 (5)	81 (7)	80 (6)

Table 3.3 Matrix effect (%ME) and extraction efficiency (%EE) for EtG and EtS in urine measured at low (L), medium (M) and high (H) concentration.

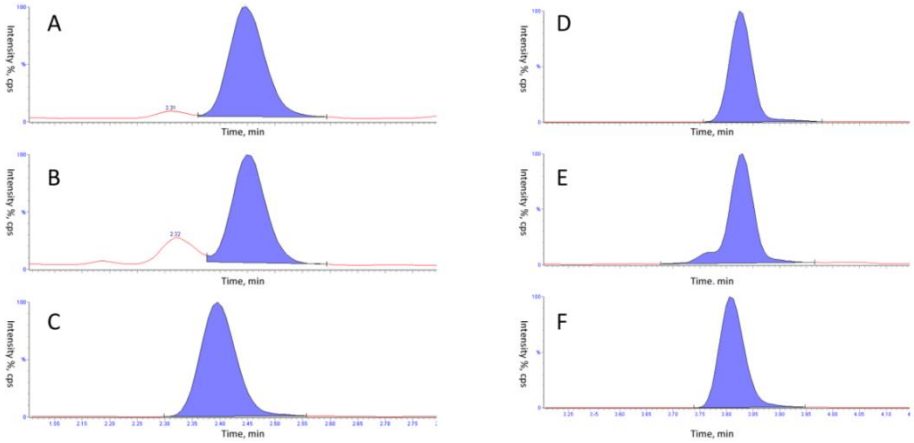


Figure 3.3 MRM Chromatogram for EtG (m/z 221→75 (A), m/z 221→85 (B)) and EtG-d_5 (m/z 226→85 (C)) and EtS (m/z 125→80 (D), m/z 125→97 (E)) and EtS-d_5 (m/z 130→98 (F)) at the LLOQ (100 ng/mL).

	EtG				EtS			
QC	Nominal value (ng/mL)	%RSD_r	%RSD_t	%Bias	Nominal value (ng/mL)	%RSD_r	%RSD_t	%Bias
LLOQ	100	7	10	4	100	4	4	-5
EtG 1/10-B	878	4	3	-4	920	2	4	-13
EtG 2/09-B	3020	4	4	-1	1750	3	6	-4
QC L	300	6	7	-1	300	4	7	6
QC M	4000	2	5	-1	4000	3	4	0
QC H	7500	2	6	0	7500	2	5	-5

Table 3.4 Precision (repeatability (%RSD_r), intermediate precision (%RSD_t)) and accuracy (%bias) for EtG and EtS in urine, measured using the Xevo TQ MS, for the LLOQ and 5 QCs (2 external and 3 internal).

No instability was observed (Figure 3.4) for samples staying in the autosampler during 24 and 72 h (4°C). Moreover, EtG and EtS were stable after three freeze/thaw cycles and after 2 months at -20°C and 4°C when stored in Greiner bio-one tubes. EtG and EtS were also stable after 5 and 11 days when stored in the original sampling device at 4°C. Results of the stability experiments are presented Table 3.5 and in Figure 3.4.

	EtG		EtS	
Concentration	L	H	L	H
Nominal value (ng/mL)	300	7500	300	7500
Processed sample stability (24 hours, 4°C)				
Mean stability %	102	103	109	105
90 % CI of stability samples	62-62	1414-1448	63-64	1232-1268
± 20 % of control samples	49-73	1111-1666	47-70	953-1429
Processed sample stability (72 hours, 4°C)				
Mean stability %	95	102	108	105
90 % CI of stability samples	56-59	1389-1435	62-64	1240-1273
± 20 % of control samples	49-73	1111-1666	47-70	953-1429
Storage stability (5 days, 4°C)				
Mean stability %	98	106	102	108
90 % CI of stability samples	69-72	1637-1659	71-73	1342-1385
± 20 % of control samples	57-86	1242-1864	56-84	1014-1522
Storage stability (11 days, 4°C)				
Mean stability %	100	111	101	110
90 % CI of stability samples	71-73	1342-1385	71-73	1342-1385
± 20 % of control samples	56-84	1014-1522	56-84	1014-1522
Long term storage stability (2 months, 4°C)				
Mean stability %	104	107	102	102
90 % CI of stability samples	64-67	1467-1524	66-70	1227-1291
± 20 % of control samples	51-76	1113-1670	53-80	989-1484
Long term storage stability (2 months, -20°C)				
Mean stability %	101	110	102	107
90 % CI of stability samples	62-66	1514-1537	66-70	1311-1325
± 20 % of control samples	51-76	1113-1670	53-80	989-1484
Freeze/Thaw (3 cycles)				
Mean stability %	101	100	109	101
90 % CI of stability samples	60-63	1370-1414	62-65	1196-1218
± 20 % of control samples	49-73	1111-1666	47-70	953-1429

Table 3.5 Stability results for EtG and EtS in urine measured at two concentrations (Nominal value); Low (L) and high (H). The mean of the stability is expressed in %. The range corresponding to ± 20 % of the mean responses of the control samples and the 90 % confidence interval of the responses for stability samples are presented (values are divided by 100 for readability purpose).

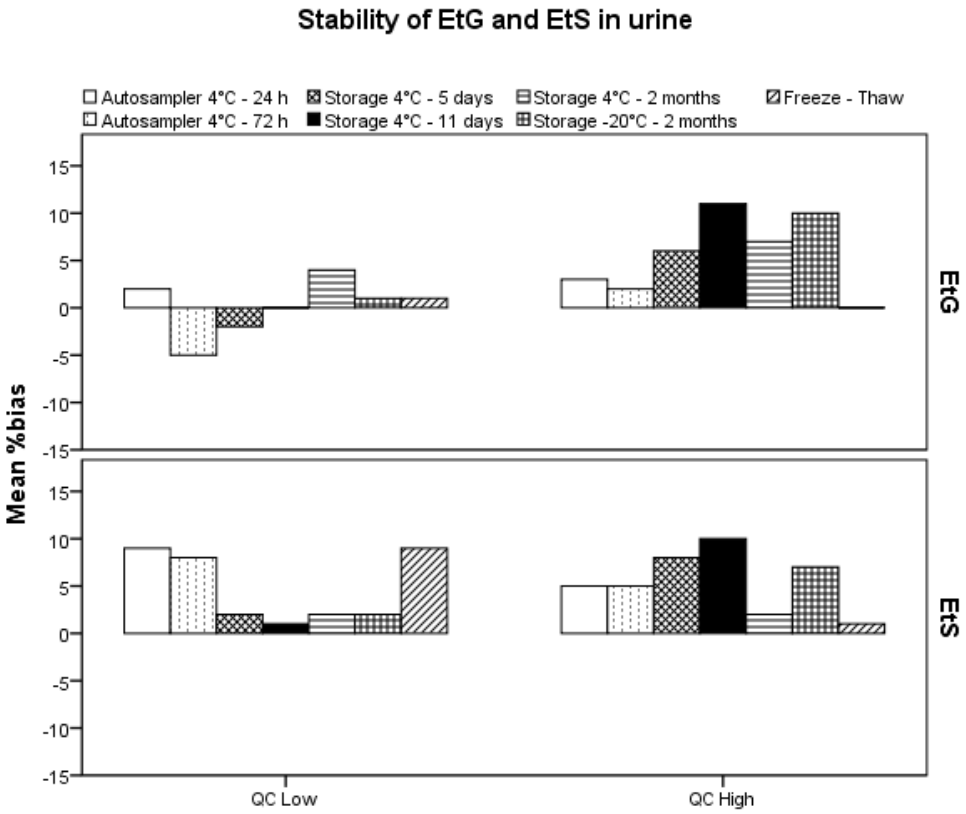


Figure 3.4 Stability of EtG and EtS in low and high urine QC samples after 24 and 72 hours in the autosampler (4°C), after 3 freeze/thaw cycles, after 2 months storage at 4°C and -20°C and after 5 and 11 days storage in the original sampling device (4°C).

The reproducibility of the method was evaluated via analysis of proficiency tests between 2011 and 2014, organised by the German Society of Toxicological and Forensic Chemistry (GTFCh). Results are presented in Table 3.6.

	EtG			EtS		
Proficiency test	Nominal value (ng/mL)	Reported value (ng/mL)	Z-score	Nominal value (ng/mL)	Reported value (ng/mL)	Z-score
EtG 3/11	1450	1400	-0.22	885	818	-0.46
EtG 1/12	800	814	0.57	1130	975	-0.86
EtG 2/12	556	566	0.10	1070	1110	0.23
EtG 3/12	832	872	0.23	924	899	-0.16
EtG 1/13	1100	1100	0.00	1020	964	-0.32
EtG 2/13	505	440	-0.72	587	515	-0.71
EtG 3/13	1740	1760	0.07	1350	1020	-1.57
EtG 1/14	1240	1173	-0.33	790	604	-1.41

Table 3.6 Proficiency test results for EtG and EtS in urine using a Xevo TQ MS tandem mass spectrometer.

3.3.1.2 Xevo TQ S mass spectrometer

The fully validated method using a Xevo TQ MS mass spectrometer was transferred to another system equipped with a Xevo TQ S mass spectrometer. More details about the difference between the two devices are presented in the Appendix 3. Because of the use of another tandem mass spectrometer, linearity, accuracy, precision and reproducibility were validated again. The results for the validation of the method on the Acquity UPLC® coupled to a Xevo TQ S tandem mass spectrometer, are presented in Table 3.7 and Table 3.8. The calibration model (N=8) was linear (1/x) for EtG and linear ($1/x^2$) for EtS over the range 100, 250, 500, 1000, 2500, 5000, 7000 and 10000 ng/mL.

	EtG				EtS			
QC	Nominal value (ng/mL)	$\%RSD_r$	$\%RSD_t$	%Bias	Nominal value (ng/mL)	$\%RSD_r$	$\%RSD_t$	%Bias
EtG 3/10-B	1270	2	2	-3	810	2	2	-8
EtG 2/12-B	556	1	5	1	1250	3	9	-12
QC-L	300	3	4	-8	300	5	8	-5
QC-M	4000	2	4	-6	4000	4	4	-3
QC-H	7500	3	4	-3	7500	5	6	-1

Table 3.7 Precision (repeatability ($\%RSD_r$) and intermediate precision ($\%RSD_t$)) and %bias for EtG and EtS in urine, using a Xevo TQ S mass spectrometer, for 5 QCs (2 external and 3 internal).

The %bias, repeatability ($\%RSD_r$) and intermediate precision ($\%RSD_t$) were calculated for the internal and external QCs and were less than 12 % (Table 3.7). The reproducibility of the method has been monitored by successful (Z-scores < 1.04) participation in four proficiency tests (Table 3.8).

	EtG			EtS		
Proficiency test	Nominal value (ng/mL)	Reported value (ng/mL)	Z-score	Nominal value (ng/mL)	Reported value (ng/mL)	Z-score
EtG 2/14	621	616	-0.04	1230	1230	0
EtG 3/14	1350	1310	-0.19	715	631	-0.69
EtG 1/15	945	999	0.35	796	658	-1.04
EtG 2/15	1150	1100	-0.26	1440	1310	-0.59

Table 3.8 Proficiency test results for EtG and EtS in urine using a Xevo TQ S tandem mass spectrometer.

3.3.1.3 Xevo TQ MS *vs.* Xevo TQ S

The accuracy (%bias) and precision (repeatability ($\%RSD_r$) and intermediate precision ($\%RSD_t$)) were calculated for both devices. No differences were observed regarding the accuracy and precision (Figure 3.5) or Z-scores (between -1.57 and 0.57 (Xevo TQ MS) and between -1.04 and 0.35 (Xevo TQ S)) when using one or another device. This seems to confirm that both systems can be used interchangeably.

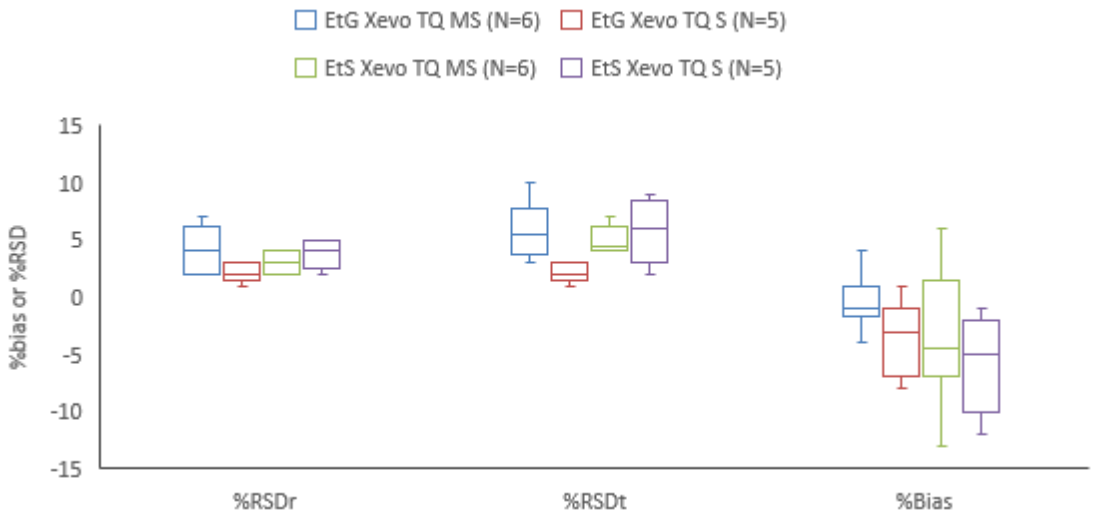


Figure 3.5 Boxplots depicting %bias, repeatability ($\%RSD_r$) and intermediate precision ($\%RSD_t$) obtained using a Xevo TQ MS and a Xevo TQ S (values of Tables 3.4 and 3.7). The boxes represent the values between the lower and upper quartile, the middle line represents the median and the whiskers represent the extremes value.

3.3.2 Population study

Twenty-seven urine samples from volunteers were analysed on the Acquity UPLC® coupled to a Xevo TQ MS tandem mass spectrometer. Urine samples from volunteers (N=14) who did not drink alcoholic beverages the day before the sampling were all negative for EtG and EtS (EtG_{100} < 121 ng/mL and EtS_{100} < 108 ng/mL (LLOQ + %U)).

The chromatograms obtained from one volunteer who declared having drunk 3 alcohol units the day before the sampling, which led to measured EtG_{100} and EtS_{100} concentrations of 496 and 209 ng/mL, respectively, are presented in Figure 3.6.

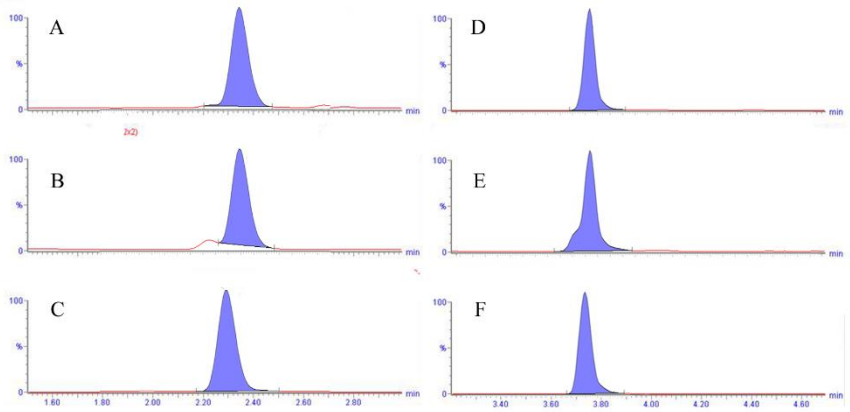


Figure 3.6 EtG (m/z 221→75 (A), m/z 221→85 (B)), EtG-d_5 (m/z 226→85 (C)), EtS (m/z 125→80 (D), m/z 125→97 (E)) and EtS-d_5 (m/z 130→98 (F)) MRM Chromatogram obtained by the analysis of urine sample from one volunteer. Concentrations of 496 (EtG_{100}) and 209 (EtS_{100}) ng/mL were measured.

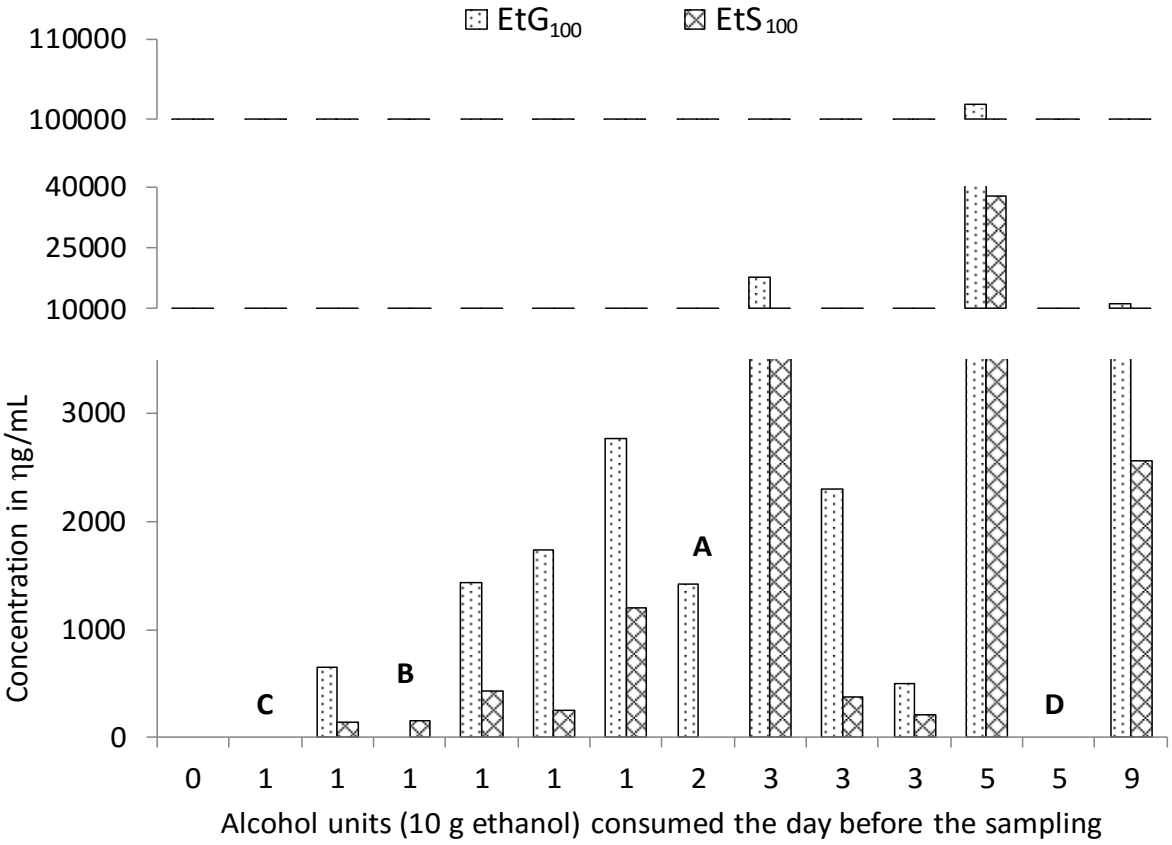


Figure 3.7 EtG_{100} and EtS_{100} concentrations in subjects who declared having been drinking alcohol 24 hours before the sampling.

In 10 samples from volunteers who declared to have consumed alcohol the day before the sampling (N=13) a concentration between 646 and 101917 ng/mL (mean 10908, median

1433) for EtG_{100} and between 145 and 37853 ng/mL (mean 3613, median 256) for EtS_{100} was determined (Figure 3.7).

EtG and EtS were in agreement in 25 out of 27 cases. EtG and EtS concentrations in urine were highly correlated ($R=0.996$, $p<0.001$). A lower correlation between the number of drinks the day before the sampling and the concentration of EtG ($R=0.448$, $p<0.02$) and EtS ($R=0.406$, $p<0.04$) in urine was observed. This result can be explained by an inaccurate self-reported alcohol consumption, as well as by the high inter-individual variation of EtG and EtS concentrations in urine after the consumption of equal amounts of ethanol [17], due to different metabolism and elimination rates varying according to individual characteristics (e.g. age, gender, genetic polymorphisms and diseases) or to the intake of certain substances (e.g. foods and drugs) that may affect the glucuronidation and or sulfonation of ethanol (see Chapter 7).

Twenty-four hours after the ingestion of 1 unit of alcohol, EtG was not detected (EtG_{100} < 121 ng/mL and EtS_{100} = 153 ng/mL) in one volunteer (Figure 3.7, B), while neither EtG nor EtS (EtG_{100} < 121 ng/mL and EtS_{100} < 108 ng/mL) were detected in another volunteer (Figure 3.7, C). A recent study [9] has demonstrated that after the consumption of approximately two units of alcohol (0.2 L of wine or 0.66 L of beer), urinary concentrations were below 100 ng/mL 24 hours after the intake in 1 out of 7 cases for EtG and in 6 out of 7 cases for EtS. Taking these results into account, it is indeed possible to have no EtG or EtS in urine 24 hours after the consumption of only one unit.

In one case (Figure 3.7, A), no EtS (EtS_{100} < 108 ng/mL) was detected after the consumption of two alcohol units, while the EtG concentration was 1422 ng/mL. A mismatch between EtG and EtS results may be explained by the fact that the two compounds are formed via different pathways (i.e. via glucuronidation and sulfonation), with different factors (e.g. compounds or diseases) that may affect one of these pathways (formation of EtS in this case) but not necessarily the other.

In one volunteer (Figure 3.7, D) who declared a consumption of five alcohol units the day before the sampling no EtG and no EtS were detected in urine (EtG_{100} < 121 ng/mL and EtS_{100} < 108 ng/mL). The creatinine concentration measured in that sample was abnormally low (12 mg/dL). In Germany, a urinary creatinine concentration below 20 mg/dL is declared as “not usable” for analysis [51]. This abnormally low urinary creatinine concentration, which

may be induced by diabetes insipidus, potomania or tampering, can explain the negative result for EtG and EtS in this sample [47].

The three subjects who reported a consumption of alcohol (2, 4 and 6 units) 2 days before the sampling and no consumption the day before were all negative for EtG and EtS. Kinetic studies show that EtG and EtS are detectable in urine up to 24 h after intake of about 2 units of alcohol and up to 48 h after intake of about 4 units of alcohol [2,13–18].

The use of the LLOQ increased by the measurement uncertainty of the method (EtG_{100} = 121 ng/mL and EtS_{100} = 108 ng/mL) as cut-off values allows the demonstration of alcohol consumption approximately 24 hours after the intake, without showing any false positive results.

3.4 Conclusion

This report describes a validated method for the quantification of EtG and EtS in urine by UPLC®-ESI-MS/MS using protein precipitation with methanol as clean-up step. The chromatographic run time for one analysis is 7 minutes. The extraction efficiency (EE%) was around 80 % for both compounds and matrix effect (expressed as % recovery) were between 76 and 84 % for EtG and virtually absent for EtS. This method provides good precision ($\%RSD_r$ and $\%RSD_t$ < 10 %) and accuracy (%bias < 15 %) using a Xevo TQ MS or a Xevo TQ S mass spectrometer. The validity of the method was confirmed by successful participation to 12 proficiency tests (Z-scores ≤ 1.41).

The measurement uncertainties ($\%U=2.12*\%RSD_t$) obtained using the Xevo TQ MS at the LLOQ (%U = 21 % for EtG and %U = 8 % for EtS) were used to interpret quantitative results close to the LLOQ. Concentrations above or equal to 121 ng/mL for EtG_{100} and 108 ng/mL for EtS_{100} (LLOQ + %U) were used to suggest alcohol intake the days prior to the sampling and to disprove strict abstinence. Analysing urine samples from 27 volunteers showed that subjects (N=14) who did not drink alcoholic beverages the day before the sampling were all negative for EtG and EtS and that 10 out of the 13 subjects who declared having consumed alcohol the day before the sampling were positive. In these subjects, the determined concentrations (normalised to 100 mg/dL creatinine) lay between 646 and 101917 ng/mL for EtG_{100} and between 145 and 37853 ng/mL for EtS_{100}.

3.5 References

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Chapter 4

Quantification of EtG in hair

Based on

N. Kummer, S.M. Wille, V. Di Fazio, M. del M. Ramírez Fernández, M. Yegles, W.E.E. Lambert, N. Samyn, Impact of the Grinding Process on the Quantification of Ethyl Glucuronide in Hair Using a Validated UPLC-ESI-MS-MS Method, Journal of Analytical Toxicology. 39 (2015) 17–23.

Abstract

A UHPLC-ESI-MS/MS method for the quantification of ethyl glucuronide (EtG) in human hair was developed and fully validated according to international guidelines on a Xevo TQ MS tandem mass spectrometer. The solid-phase extraction (SPE) was optimised (nine different SPE sorbents were tested and different washing and elution solvents were compared regarding the matrix effect and extraction efficiency) and the effect of the pulverisation on the quantification was evaluated. Differences were observed in the EtG concentration obtained depending on the grinding process and special attention was paid to optimise the extraction of EtG from 50 mg of hair with 1.5 mL of water. Using a Bond Elut SAX cartridge, extraction efficiency was higher than 53 % (%RSD < 15 %) and matrix effect (expressed as % recovery) was between 66 and 76 % (%RSD < 24 %) and compensated by the use of EtG-d_5 as internal standard. The method was linear from 10 (LLOQ) to 500 pg EtG/mg hair. A %bias, repeatability (%RSD_r) and intermediate precision (%RSD_t) of less than 16 % were obtained. This method was applied to authentic samples from 6 social drinkers (mean ethanol intake per day between 10 and 32 g) and one teetotaller. A cut-off value at 30 pg/mg hair was used to strongly suggest chronic excessive alcohol consumption. Monitoring periods of alcohol abstinence requires a method with a lower LLOQ -i.e. able to quantify concentrations equal to or below 7 pg/mg hair- which is the cut-off value used to strongly suggest repeated alcohol consumption and disprove a strict abstinence period. Therefore, the method was transferred to another tandem mass spectrometer (Xevo TQ S instead of the Xevo TQ MS) and optimised (the mobile phase A (0.1 % formic acid in water) was changed into 0.01 % formic acid in water), to reach a lower LLOQ (at 2 pg EtG/mg hair). To ensure the accuracy and precision of the modified method, a partial validation was performed (%bias < 9 %, %RSD_r < 8 %, %RSD_t < 12 %). The reproducibility on both systems has been demonstrated by successful (Z-score < 1.8) participation in proficiency tests.

4.1 Introduction

Quantification of ethyl glucuronide (EtG) in hair is used to detect excessive/chronic alcohol consumption and to assess alcohol abstinence. As direct quantification of ethanol in hair is not possible [1], specific non-oxidative metabolites of ethanol [2–4], such as ethyl glucuronide (EtG), ethyl sulfate (EtS) and/or fatty acid ethyl esters (FAEEs) are targeted. Hair is advantageous over traditional matrices such as blood or urine because of its extended detection window. The Society of Hair Testing (SoHT) has published guidelines [5] concerning the use of EtG and FAEEs in hair for the detection of chronic/excessive alcohol consumption and the monitoring of alcohol abstinence periods. Although these markers can be used independently, the consensus is that in some cases determination of both can prove useful. For EtG, two cut-off values have been proposed by the SoHT [6], at 7 and at 30 pg/mg hair, respectively, to strongly suggest repeated alcohol consumption and to disprove a strict abstinence period and to strongly suggest excessive/chronic alcohol consumption (consumption of ≥ 60 g ethanol/day over several months).

Sample preparation steps, such as decontamination and extraction, have been demonstrated to influence the measured EtG concentration [7–14]. It has been shown that a simple decontamination with 1 mL of methanol (30 seconds vortex mixing) was not enough to clean the sample and that a first dichloromethane wash was needed to eliminate lipids [15]. The most common decontamination solvents used are dichloromethane alone [16] or in combination with methanol [10,15,17–21] or water [22,23], methanol [24,25] or methanol and acetone [26,27], water and acetone [8,11,28–31], or water and n-heptane [32,33]. Bossers *et al.* [14] have pointed out that among different washing solvents tested, a wash with dichloromethane followed by methanol was a very efficient washing protocol. Prior to extraction of the analytes, an attempt to improve sample homogeneity is usually made by reducing the hair into smaller pieces, e.g. by cutting or pulverising. Extraction experiments show that the time required to extract EtG from hair is decreased when the matrix is pulverised to a powder [10] and that the determined concentration in pulverised hair samples was higher than in cut hair samples [7,13,34]. In 2014, the SoHT has published guidelines which state that: “powdering hair prior to the extraction of EtG is preferred. Laboratories utilising other sample preparation procedures should demonstrate comparable recovery of EtG” [6].

Direct analysis of the aqueous extract is the easiest 'sample preparation' method [7,11,17,18,27,35–37], however, to clean up the extract, solid-phase extraction (SPE) is the conventional technique applied [8,10,21–23,25,26,28–32,38]. Several validated methods based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [7,10,15–18,21–23,31,34,39–42], or gas chromatography coupled to single or tandem mass spectrometry (GC-MS(/MS)) [8,13,25,28–30,32,37,43] have been published. Most of these make use of internal quality control samples (QCs) that have been prepared using either spiked QCs [7,15–17,22,23,25,28,37,39,40,42] and/or authentic hair [8,10,15,18,31,44], to estimate the accuracy of the method. Whilst spiked QCs are suitable to estimate accuracy and precision, they do not take into account the extraction efficiency of compounds from the hair matrix; QCs based on authentic hair partially overcome this problem but unfortunately they are not suitable to estimate the accuracy, because the real concentration is not known. Thus, to estimate the real capability of methods to correctly quantify authentic samples, QCs with a certified reference value and/or proficiency tests are required [41,43]. Nowadays a number of commercial sources of hair QCs (external QCs) exist; these samples may be supplied either in cut or pulverised form. Furthermore, to assess the reproducibility, proficiency tests are organised by the German Society of Toxicological and Forensic Chemistry (GTFCh) and by the SoHT in collaboration with Medichem. The SoHT provides samples in both cut and pulverised form, while the GTFCh provides pulverised samples. Although several publications report successful participation in these schemes [23,40,42,45], the quantitative results from these proficiency tests show an overall lack of reproducibility for the EtG quantification in hair [41,45].

This chapter describes and discusses first the selection of an SPE cartridge and the optimisation of the SPE process, second the impact of different grinding and extraction conditions on the measured EtG concentrations, third the validation of a quantitative method on a Xevo TQ MS mass spectrometer and its application to a small population study, fourth the transfer (and optimisation) of the developed method to a more sensitive mass spectrometer (Xevo TQ S) and finally the results of past proficiency tests.

4.2 Materials and methods

4.2.1 Chemicals and stock solutions

Certified reference standards for EtG and its pentadeuterated analogue (EtG-d_5) were obtained from Sigma-Aldrich (Steinheim, Germany) and were supplied at 1 mg/mL in methanol. Formic acid (mass spectrometric quality i.e. ~98 %) was obtained from the same supplier. A separate source of EtG was also purchased from Lipomed (Arlesheim, Switzerland) in powder form and was used for the preparation of internal QCs. UPLC/MS grade acetonitrile, methanol and 0.1 % formic acid in water were purchased from Biosolve (Valkenswaard, The Netherlands).

Two stock solutions, one for calibration (Cal-Stock) and one for the preparation of the internal QCs (QC-Stock) with EtG at a concentration of 5000 ng/mL were prepared in methanol, using a different source of reference standard. The stock solution containing the internal standard (IS-Stock) at a concentration of 50 ng/mL was prepared in methanol. All solutions were stored at -18°C.

4.2.2 Blank hair samples, external QCs and proficiency tests

Blank hair samples from children and teetotallers were collected on a voluntary basis and were used for the validation, for the calibrators and for the spiked QC samples.

Hair samples were washed with dichloromethane (vortex 30 seconds, sonication 10 minutes) and methanol (vortex 30 seconds, sonication 2 minutes), and dried overnight at room temperature. The samples were cut into small pieces with scissors and stored at room temperature.

One external QC sample, EGH 2/12-A from ACQ Science GmbH (Rottenburg-Hailfingen, Germany) was used during validation. This QC sample consists of pulverised authentic human hair with an EtG concentration of 25 pg/mg hair.

Between 2011 and 2015 proficiency tests (N=16) for EtG in hair were organised by the GTFCh (GTFCh 3/11, GTFCh 1/12, GTFCh 2/12, GTFCh 3/12, GTFCh 1/13, GTFCh 2/13, GTFCh 3/13, GTFCh 1/14, GTFCh 2/14, GTFCh 3/14, GTFCh 1/15,and GTFCh 2/15) and by the SoHT in collaboration with Medichem (SoHT 2011, SoHT 2012, SoHT 2013 and SoHT 2014).

4.2.3 Xevo TQ MS mass spectrometer method

4.2.3.1 Optimisation of the solid-phase extraction

Nine SPE cartridges, all based on ion exchange mechanisms, were tested: Oasis MAX (Waters, 60 mg, 3 mL), Strata X-AW, Strata SAX and Strata Screen A (100 mg, 3 mL) from Phenomenex (Utrecht, The Netherlands), Isolute SAX and Isolute PE-AX (Biotage, 100 mg, 3 mL) purchased from Sopachem (Eke, Belgium), Varian Bond Elut SAX (100 mg, 3 mL) and Varian Bond Elut NH_2 (50 mg, 1 mL) from Agilent (Diegem, Belgium) and Clean Screen EtG (UCT, 200 mg, 3 mL) purchased from Achrom (Zulte, Belgium). More details concerning these cartridges are provided in the Appendix 1.

Hair from children (up to 5 years old) and from one alcohol abstainer (48 years old) were used as blank samples. Samples were first washed (with water and acetone), then cut into small pieces with scissors before being homogenised. During method development, we used 30-mg samples, which were weighed into a 2-mL Precellys tube containing six 2.8-mm diameter stainless steel beads (Precellys Lysing kit, Hard tissue grinding) and were pulverised (three cycles (6200 rpm) of 90 seconds with a cooling time of 5 seconds in between) using the Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Samples spiked with EtG (500 pg/mg hair) were incubated 2 hours at 40°C with 1.5 mL water and were then loaded onto the SPE cartridges. The extraction protocol for each cartridge (presented in Table 4.1) was chosen based on recommended methods from the manufacturer or using published procedures [8,22,32,46].

Loading, wash and elution solutions were collected separately, and analysed to detect EtG. Wash and/or elution solvents were optimised for cartridges with good retention during the loading and washing steps. Matrix effect and extraction efficiency were calculated for the three cartridges showing the best results, at a concentration of 100 pg/mg hair (N=6).

Oasis MAX (SAX)	
C:	2 mL MeOH - 2 mL H_2O
W:	1 mL NH_3 - 1 mL MeOH
D:	2 min vacuum
E:	1 mL MeOH/FA (98/2)

Bond Elut SAX (SAX)	
C:	2 mL MeOH - 2 mL H_2O
W:	1 mL H_2O - 1 mL ACN
D:	2 min vacuum
E:	1 mL ACN/H_2O/AF (94/3/3)

Strata SAX (SAX)	
C:	2mL MeOH - 2 mL AAbuffer
W:	1.2 mL AAbuffer/MeOH (50/50) - 2 mL MeOH
D:	2 min vacuum
E:	1.2 mL MeOH/FA (85/15)

Isolute PE-AX (SAX)	
C:	1 mL MeOH - 1 mL H_2O
W:	1 mL H_2O/MeOH (50/50)
D:	2 min vacuum
E:	1 mL MeOH/FA (98/2)

Isolute SAX (SAX)	
C:	2 mL MeOH - 2 mL H_2O
W:	2 mL H_2O/MeOH (50/50)
D:	15 min vacuum
E:	2 mL MeOH/FA (98/2)

Strata-X-AW (WAX)	
C:	1 mL MeOH - 1 mL H_2O
W:	2 mL AAbuffer - 2 mL MeOH
D:	2 min vacuum
E:	2 mL MeOH/NH_4OH (95/5)

Screen-A (SAX + apolar)	
C:	2 mL MeOH - 2 mL AAbuffer
W:	1.2 mL AAbuffer/MeOH (50/50)
D:	2 min vacuum
E:	1.2 mL MeOH/FA (85/15)

Clean Screen (WAX + apolar)	
C:	2 mL MeOH/FA (99/1) - 2 mL H_2O/FA(99/1)
W:	2 mL H_2O
D:	10 min vacuum
E:	3 mL MeOH/FA (99/1)

Bond Elut NH_2 (WAX + polar)	
C:	1 mL MeOH - 1 mL H_2O
W:	1 mL ACN - 1 mL *n*-hexane
D:	15 min vacuum
E:	1 mL H_2O/NH_3 (32%) (90/10)

Table 4.1 Classification of SPE cartridges tested for the extraction of EtG from hair. FA: formic acid, AAbuffer: 25 mM ammonium acetate buffer (pH=6), MeOH: methanol, H_2O: water, NH_3: ammonia, ACN: acetonitrile, C: conditioning, W: washing, D: drying, E: elution, SAX: strong anion exchange, WAX: weak anion exchange.

4.2.3.2 Grinding experiments

Experiments on the extraction process (state of hair and extraction condition), in order to fully extract EtG from 50 mg of hair with 1.5 ml water, were performed. Hair from two volunteers (alcohol consumers) and two external QC samples were used. The two volunteers were Caucasian with cosmetically untreated hair; one was a male with brown hair, the other, a female with blond hair. Samples were first washed and cut into small pieces with scissors. The external QCs Medidrug ALCM 1/11-C (12 pg/mg hair) and Medidrug ALCM 12-A (39 pg/mg hair) from Medichem (Steinenbronn, Germany) -both authentic hair reference materials obtained in a cut (non-pulverised) form- were used.

Approximately 50 mg of cut hair were weighed into a 2-mL Precellys tube and were pulverised with the Precellys 24 homogenizer. Pulverisation is performed mechanically by the movement of the six stainless steel beads, that are present in each tube. The same sample was processed according to four different procedures (4 to 6 replicates for each pulverisation procedure). The first three procedures aimed to compare the determined EtG concentration when using cut hair (process n°1), weakly pulverised hair (process n°2) or extensively pulverised hair (process n°3, which is the standard grinding protocol used for the validation studies). Cut hair samples (process n°1) were initially incubated for 16 hours at 40°C and then sonicated for 2 hours (40°C) with 1.5 mL water and 50 µL of IS-Stock solution. Pulverisation was performed using the Precellys 24 homogenizer; one cycle of 30 seconds (at a speed of 6500 rpm) was used for the weakly pulverised samples (process n°2) and three cycles of 60 seconds (with a cooling time of 2 minutes in between) was used for the extensively pulverised samples (process n°3). Pulverised hair samples were sonicated for 2 hours (40°C) with 1.5 mL water and 50 µL of IS-Stock solution. For the extensively pulverised samples, the sonication time was also evaluated (2 hours for process n°3 and 6 hours for process n°4).

The EtG mean calculated concentrations obtained from the four different processes were compared using One-way ANOVA test (α=0.05) to detect statistical differences.

The impact of the different grinding procedures on the hair was studied using a Phenom G2 pro electron microscope (Phenom-World BV, Eindhoven, The Netherlands).

To ensure that the grinding process selected (process n°3) sufficiently pulverised real samples (hair strand up to 6 cm not cut into pieces prior to the pulverisation), additional

analyses of real positive samples (three alcohol consumers) were performed. Hair strands from the volunteers were divided into aliquots, which were pulverised using one, two, three and up to six grinding processes. Results were visually compared.

4.2.3.3 Final sample preparation procedure

Fifty milligrams of hair sample were weighed into a 2-mL Precellys tube and pulverised with the Precellys 24 homogenizer. For the validated method a pulverisation protocol based on three grinding cycles of 60 seconds at 6500 rpm with a cooling time of 2 minutes between each cycle, was used. EtG was subsequently extracted from the pulverised hair samples with 1.5 mL water and 50 µL of IS-Stock solution using 2 hours of sonication (40°C).

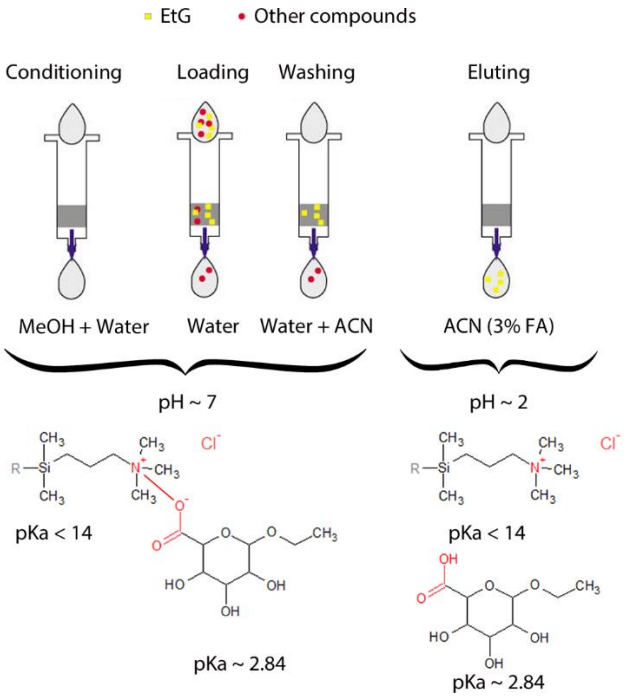

Figure 4.1 Representation of the different solid-phase extraction steps used for the quantification of EtG in hair. Interactions between the cartridge stationary phase and EtG at different pH are presented. FA: formic acid, MeOH: methanol, ACN: acetonitrile.

For clean-up (see Figure 4.1), the sample was centrifuged (20800 g) for 10 minutes at 4°C and the supernatant was applied to a Bond Elut SAX (100 mg, 3 mL) SPE cartridge from Agilent (Diegem, Belgium), conditioned with 2 mL of methanol and 2 mL of water. Special care was paid to ensure the cartridge did not dry out between conditioning steps. The SPE cartridge was washed with 2 mL of water and 2 mL of acetonitrile and was dried under vacuum (-0.3 bar) during 2 minutes. EtG was eluted with 1 mL of a formic acid/acetonitrile

solution (3/97, v/v) in a total recovery glass vial (Waters, Zellik, Belgium), which is a vial -containing an interior sharp bottom- specifically designed to allow analysis of all volume available. The eluate was evaporated to dryness using a vacuum centrifuge at 38°C for 45 minutes (Labconco, Kansas City, Missouri, USA). The residue was reconstituted in 100 µL of 0.1 % formic acid in water. Ten microliters were injected onto the UPLC®-MS/MS system using the full-loop mode.

4.2.3.4 Preparation of calibrators, QCs and proficiency test samples

Daily calibration working solutions (Cal-WS) at 10, 50 and 250 ng/mL were prepared by diluting the Cal-Stock solution in water. Calibrators (10, 15, 20, 50, 100, 250 and 500 pg/mg hair) were prepared with 50 mg of pulverised blank hair spiked with 50 µL of the IS-Stock solution, an adequate amount (50, 75 or 100 µL) of a Cal-WS solution, and water until a total volume of 1.5 mL was reached.

Daily QC working solutions (QC-WS) at 10, 25 and 250 ng/mL were prepared by diluting the QC-Stock solution in water. Spiked quality controls at 16, 30 and 300 pg/mg hair were prepared by adding 50 µL of the IS-Stock solution, an adequate amount (60 or 80 µL) of a QC-WS and water until a total volume of 1.5 mL to 50 mg of pulverised blank hair. EGH 2/12-A QC and proficiency test samples were prepared by spiking 50 µL of IS-Stock solution and 1.45 mL of water to 50 mg of pulverised hair sample.

4.2.3.5 Liquid chromatography and mass spectrometry conditions

Analyses were performed on an Acquity UPLC® coupled to a Xevo TQ MS tandem mass spectrometer (Waters) equipped with an electrospray ionisation source operated in negative mode. Gradient elution was performed on an Acquity UPLC® HSS T3 (2.1 x 100 mm, 1.8 µm) column (Waters) with 0.1 % formic acid in water (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 400 µL/min. The gradient elution started with 99 % of mobile phase A, decreasing to 88 % mobile phase A, at 2.0 min. The washing step consisted of 100 % of mobile phase B held from 2.1 to 2.5 min. The initial condition was applied from 2.6 min to 5 min. The column temperature was set at 60°C.

MS/MS detection was performed in the multiple reaction monitoring mode (MRM) with a dwell time fixed at 0.078 sec using the following precursor/product ion transitions (cone voltage, collision energy): EtG for quantification 221/85 (30 V, 24 eV), EtG for qualification 221/75 (30 V, 22 eV) and EtG-d_5 226/85 (28 V, 30 eV). ESI source parameters used were the same as described in Chapter 3 (3.2.4) [47]. Briefly, nitrogen was applied as cone gas (40 L/h) and as the desolvation gas (900 L/h at 650°C). Argon was used as collision gas (0.35 mL/min). The capillary voltage and the cone voltage were 1 kV.

4.2.3.6 Method validation

Selectivity, matrix effect, extraction efficiency, lower limit of quantification (LLOQ), linearity, accuracy, precision, stability and reproducibility were evaluated according to international guidelines [48].

Identification was based on the following criteria: a stable retention time (%RSD < 5 %) and stable ion ratios for the MRM transitions (within 20 % of expected) [5]. Two MRM transitions were used for EtG and one transition was used for the IS.

Selectivity was determined by the analysis of six blank hair samples from different individuals. To verify that there were no isotope exchange reactions with non-labelled compounds, two samples without hair but with internal standard were analysed. According to the EMA guideline [48], interferences are acceptable in our type of method as long as the response of the interfering peak remains lower than 20 % of the response at the LLOQ.

Matrix effects expressed as % recovery (%ME) were quantified and evaluated using the post-extraction addition technique [49]. Six blank hair samples from different individuals were

spiked after the sample preparation and compared with the analytes spiked at the same theoretical concentration in the mobile phase. Extraction efficiency (%EE) was evaluated by comparing the responses of six blank hair samples spiked before solid-phase extraction with responses of six blank hair samples spiked after solid-phase extraction. These experiments were performed at LLOQ (10 pg EtG/mg hair), and at low (16 pg EtG/mg hair), medium (30 pg EtG/mg hair) and high (300 pg EtG/mg hair) concentration.

The lower limit of quantification (LLOQ) was defined as the lowest concentration of the analyte with a signal-to-noise ratio calculated as root mean square above 10/1 (for both transitions) and for which the %bias and %RSD was below 20 %. Other identification criteria, such as a stable ion ratio between the quantifier and the qualifier had to be met.

The calibration model (N=7) and the weighting factor were tested over the range 10 to 500 pg EtG/mg hair and were evaluated via residual plots [48]. The goodness of fit was established as the difference between the calculated calibrator value and its nominal value. The coefficient of variation should be lower than 15 % except at the LLOQ (%RSD < 20 %).

Accuracy (%bias) and precision (repeatability ($\%RSD_r$) and intermediate precision ($\%RSD_t$)) were determined analysing three internal QCs spiked at low (16 pg EtG/mg hair), medium (30 pg EtG/mg hair) and high (300 pg EtG/mg hair) concentration and one external QC; EGH 2/12-A (25 pg EtG/mg hair). QCs were analysed in replicates on eight different days. One-way ANOVA test with significance level (α) of 0.05 allows calculating %bias, $\%RSD_r$ and $\%RSD_t$ with these data (see the Appendix 2 for more explanation). The results are acceptable when they are below 15 % (20 % at the LLOQ).

The stability of processed samples when stored in the autosampler (72 hours, 4°C) was evaluated at low (16 pg EtG/mg hair) and high (300 pg EtG/mg hair) concentrations and using six blank hair samples spiked with EtG. Controls and stability samples (N=6) were prepared at the same time and processed samples were stored in the autosampler for up to 72 hours prior to the analysis. The mean response of the stability samples should be within 90 – 110 % of the mean response of the control samples and the 90 % confidence interval of the stability sample responses should be within ± 20 % of the control sample responses (see the Appendix 2 for more explanation).

The reproducibility has been evaluated by participation in proficiency tests organised either by the GTFCh or by the SoHT in co-operation with Medichem and the Federal Institute for Materials Research and Testing between 2012 and 2014.

4.2.3.7 Population study

To verify that no false positive results were obtained in the detection of chronic/excessive alcohol consumption applying the recommended cut-off value (30 pg EtG/mg hair) and using the Xevo TQ MS mass spectrometer method developed, a prospective alcohol self-monitoring study was performed. Seven healthy volunteers were asked to declare their alcohol consumption per day during the 4 weeks preceding the sampling. The mean daily alcohol intake was calculated based on the volume and on the % (v/v) alcohol content of the declared consumption. Hair strands were sampled at the end of the 4 weeks and the first 1-cm proximal segments were analysed.

4.2.4 Method transfer (Xevo TQ S mass spectrometer)

Because monitoring of an alcohol abstinence period requires a method with a lower LLOQ, i.e. able to quantify concentrations equal to or below 7 pg/mg hair, which is the cut-off value used to strongly suggest repeated alcohol consumption and disprove a strict abstinence period, the method presented in Section 4.2.3 was transferred to another tandem mass spectrometer (Xevo TQ S instead of the Xevo TQ MS) and optimised. More details about the difference between the two devices are presented in the Appendix 3.

Some minor modifications concerning the preparation of the daily calibration working solutions (Cal-WS) and daily QC working solutions (QC-WS) were necessary to add lower calibrators and QCs. Briefly, 4 Cal-WS solutions (at 2.5, 10, 50 and 250 ng/mL) were prepared by diluting the Cal-Stock solution (5000 ng/mL) in water. Calibrators (2, 5, 10, 20, 50, 250 and 500 pg/mg hair) were prepared with 50 mg of pulverised blank hair spiked with 50 µL of the IS-Stock solution, an adequate amount (40, 50 or 100 µL) of the Cal-WS solution, and water until a total volume of 1.5 mL was reached. Daily QC-WS at 10, 25 and 250 ng/mL were prepared by diluting the QC-Stock solution in water. Spiked quality controls at 6, 30 and 300 pg/mg hair were prepared by adding 50 µL of the IS-Stock solution, an adequate amount (30 or 60 µL) of the QC-WS solution and water until a total volume of 1.5 mL to 50 mg of pulverised blank hair.

The final sample preparation procedure described in Section 4.2.3.3 was used. Analysis was performed according to the developed UHPLC-ESI-MS/MS method described in Section 4.2.3.5, with some modifications, one concerning the mobile phase A used (0.01 instead of 0.1% formic acid in water) and the others concerning some parameters specific to the mass spectrometer used (Table 4.2).

Parameters	Xevo TQ MS	Xevo TQ S
Cone voltage, collision energy		
EtG quantifier (221/85)	30 V, 24 eV	40 V, 15 eV
EtG for qualification (221/75)	30 V, 22 eV	40 V, 15 eV
EtG-d_5 (226/85)	30 V, 24 eV	40 V, 15 eV
Dwell time	0.078 sec	0.047 sec
Cone gas (nitrogen)	40 L/h	40 L/h
Desolvation gas (nitrogen)	900 L/h at 650°C	1000 L/h at 650°C
Collision gas (argon)	0.35 mL/min	0.15 mL/min
Capillary voltage	1 kV	0.8 kV

Table 4.2 Specific MS/MS parameters used for analyses on a Xevo TQ MS and Xevo TQ S mass spectrometers.

Because the method was previously fully validated (see section 4.2.3.6), only a partial validation (LLOQ, linearity, accuracy and reproducibility) was performed to ensure the accuracy of the transferred method.

4.2.5 Proficiency test results analysis

The uncertainty of measurement (%U=2.12*%RSD_t) was calculated using proficiency test results according to the GTFCh guidelines [45,48,50]. The combined uncertainty u(x) was calculated based on the uncertainty of the inaccuracy of measurement (RMS_{bias}), the uncertainty of the certified value ($u(C_{ref})$) and the intermediate precision of the method (%RSD_t) using the equations presented in Figure 4.2.

$$u(x) = \sqrt{(RMS_{bias})^2 + (U(C_{ref}))^2 + (\%RSD_t)^2} \qquad (eq.1)$$

$$\%Bias = \frac{100 * (Reported\ value - Target\ value)}{Target\ Value} \qquad (eq.2)$$

$$u(C_{ref}) = \frac{\sum(100 * \frac{SD_H}{Target\ Value})}{\sqrt{average\ number\ of\ participants}} \qquad (eq.3)$$

$$RMS_{bias} = \sqrt{\frac{\sum((\%Bias)^2)}{N_{PT}}} \qquad (eq.4)$$

Figure 4.2 Equations used to calculate the uncertainty of measurement. SDH: standard deviation, NPT: number of proficiency test samples.

4.3 Results and discussion

4.3.1 Xevo TQ MS mass spectrometer method

4.3.1.1 Optimisation of the solid-phase extraction

Nine cartridges were selected because of their potential interaction with EtG (Figure 4.3). Because EtG is a very polar acidic compound (pKa estimated between 2.84 and 3.21 [44,51,52]), polar and anion exchange interactions seemed to be well suited for the extraction of EtG. Polymer based cartridges were of interest because they are stable within a larger pH range (pH 0 to 14) compared to silica based cartridges (pH 2 to 7).

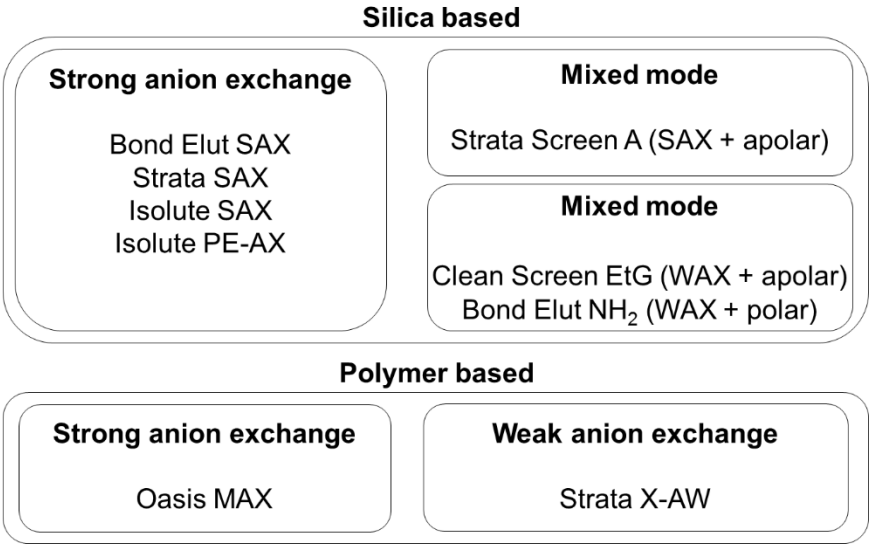

Figure 4.3 Classification of SPE cartridges tested for the extraction of EtG from hair samples according to the type (silica *vs.* polymer based) of sorbent and the interaction involved (strong anion exchange (SAX), weak anion exchange (WAX) or mixed mode).

EtG was clearly detected in loading and/or wash solvents (Figure 4.4) using the Strata SAX, Strata Screen A, Bond Elut NH_2 and Strata X-AW cartridges. No further experiments were done with these cartridges. The five other cartridges (Bond Elut SAX, Isolute PE-AX, Isolute SAX, Clean Screen EtG and Oasis MAX) retain EtG and do not show any loss during loading and washing steps.

Three out of these 5 cartridges (Bond Elut SAX, Isolute SAX, Isolute PE-AX) are very similar, due to a common silica based and strong anion exchange mode (Figure 4.3). Because the Bond Elut SAX cartridge showed the best result for matrix effect for EtG-d_5 (estimated at 71, 64 and 46 % for the Bond Elut SAX, Isolute PE-AX and Isolute SAX, respectively), this cartridge was selected (along with Oasis MAX and Clean Screen EtG) for further experiments.

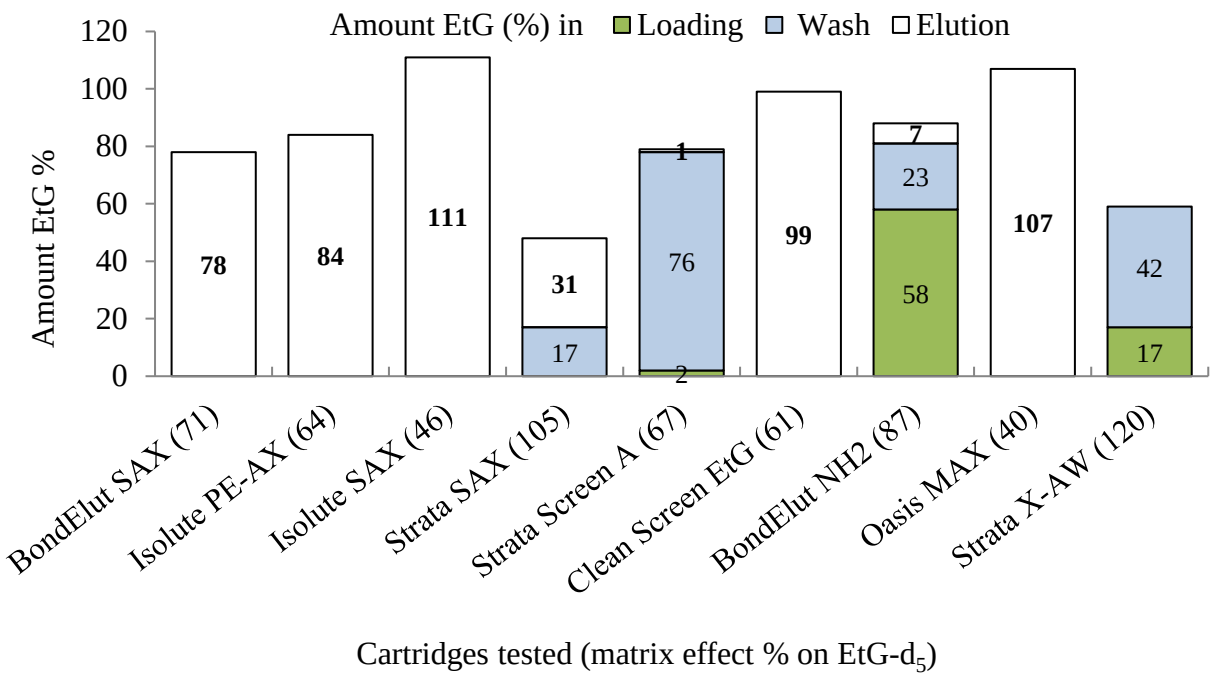

Figure 4.4 Mean percentage (N=6) of EtG measured in loading, wash and elution solvent for all SPE cartridges tested, with the matrix effect (expressed as % recovery) measured on the internal standard.

The matrix effect expressed as % recovery (%ME) and extraction efficiency (%EE) for three cartridges (Bond Elut SAX, Oasis MAX and Clean Screen EtG) were measured and are presented in Table 4.3. The best SPE cartridges with regard to matrix effect and extraction efficiency were the Clean Screen EtG and the Bond Elut SAX. This result is in agreement with earlier observations reported in literature [28]. Because of a comparable efficiency but a considerable price difference between these two products, the Bond Elut SAX cartridge was chosen for further experiments (grinding experiments) and for the method validation.

	%ME (%RSD)	%EE (%RSD)
Clean Screen EtG	73 (13)	83 (5)
Bond Elut SAX	81 (18)	69 (14)
Oasis MAX	71 (20)	53 (21)

Table 4.3 Matrix effect (%ME) and extraction efficiency (%EE) of EtG calculated at a concentration of 100 pg EtG/mg hair (N=6).

4.3.1.2 Grinding experiments

The results of the different analyses of hair samples from two volunteers and data on the two QCs (Medidrug ALCM 1/11-C and Medidrug ALCM 12-A) are shown in Figure 4.5.

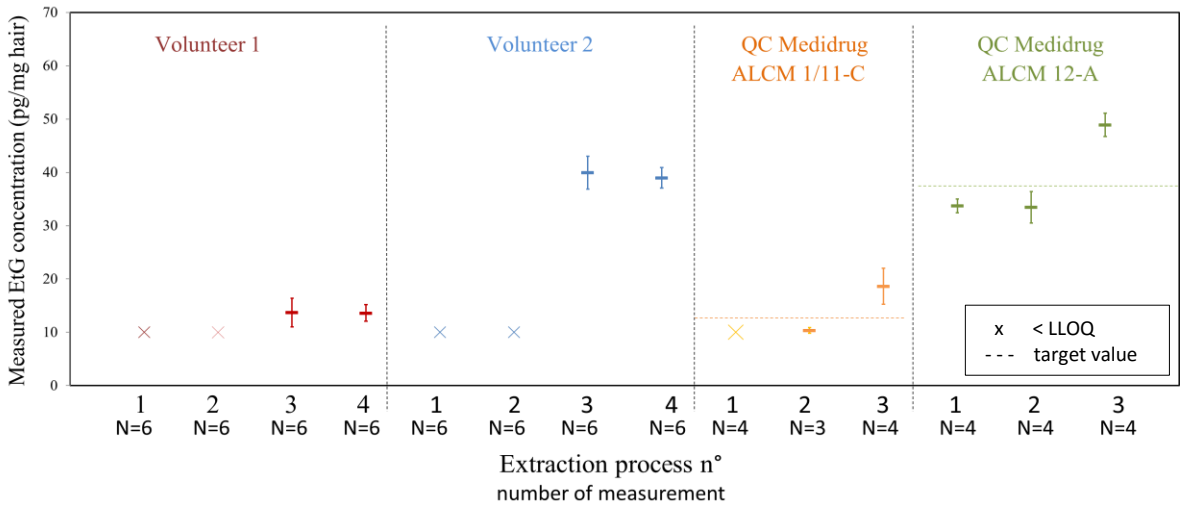

Figure 4.5 Impact of different extraction processes (cut hair (process n°1), weakly pulverised hair (process n°2), extensively pulverised hair (process n°3) and extensively pulverised hair with longer sonication time (process n°4)) on the mean EtG concentration measured (y-axis), with standard deviation. This experiment was performed on 2 authentic positive hair samples (Volunteer 1 and 2) and on two external QC samples (QC Medidrug ALCM 1/11-C and QC Medidrug ALCM 12-A).

A result below LLOQ (at 10 pg EtG/mg hair) was obtained for EtG using cut hair (process n°1) or weakly pulverised hair (process n°2) for volunteer 1 and 2. After extensive pulverisation (process n°3), a mean concentration of 14 pg EtG/mg hair (%RSD = 20 %, N=6) and 40 pg EtG/mg hair (%RSD = 8 %, N=6) were determined in the hair of volunteer 1 and 2, respectively.

For the QCs Medidrug ALCM 1/11-C and Medidrug ALCM 12-A, One-way ANOVA tests demonstrated statistical differences in the determined EtG concentration with regard to the sample preparation. For Medidrug ALCM 1/11-C -with a target value at 12 pg EtG/mg hair- EtG was not quantifiable using cut hair (process n°1) while a mean concentration of 10 pg EtG/mg hair (%RSD = 5 %, N=3) and 19 pg EtG/mg hair (%RSD = 18 %, N=4) were calculated using weakly pulverised hair (process n°2) and extensively pulverised hair (process n°3), respectively. For the Medidrug ALCM 12-A -with a target value at 39 pg EtG/mg hair- a mean EtG concentration of 34 pg/mg hair (%RSD = 4 %, N=4) and 33 pg/mg hair (%RSD = 9 %, N=4) were calculated using cut (process n°1) and weakly pulverised hair (process n°2), respectively. The mean concentration measured (N=4) using extensively pulverised hair (process n°3) was significantly higher (49 pg EtG/mg hair, %RSD = 4 %). An extensive pulverisation of hair samples leads to a significantly higher amount of EtG measured, which exceeded the reference value of the commercially available QCs. Indeed, to reach the

certified values, these hair samples had to be weakly pulverised or even not pulverised at all. This could partially be explained by the fact that certified values were determined with a mean calculated concentration obtained from different laboratories, working either with cut hair samples or with pulverised hair samples. To avoid that bias, we suggest the creation of different QCs with a different certified value for a method based on cut hair and for a method based on pulverised hair.

Furthermore, experiments on the hair from the two volunteers showed that the results obtained after two hours of sonication (process n°3) did not differ statistically from those obtained after six hours of sonication (process n°4).

Electron microscopy was used to illustrate the impact of different grinding procedures on hair. The results of the grinding process are visualised in Figure 4.6. Weak pulverisation (process n°2) damaged the external structure of the hair segment (Figure 4.6-B) while extensive pulverisation (process n°3) destroyed the structure of the hair segment (Figure 4.6-C) and so increases the surface in contact with the extraction solvent. The microscopic structure of the external QC EGH 2/12-A, which consists of pulverised authentic human hair, is presented as a comparison in Figure 4.6-D and is similar to the structure obtained after an extensive pulverisation of hair (process n°3) with the Precellys 24 homogenizer (Figure 4.6-C).

Three real samples (hair strands up to 6 cm not cut into pieces prior to the pulverisation) were used to ensure that one grinding process was efficient enough to pulverise the hair and to allow to extract all EtG (Figure 4.7) from the matrix.

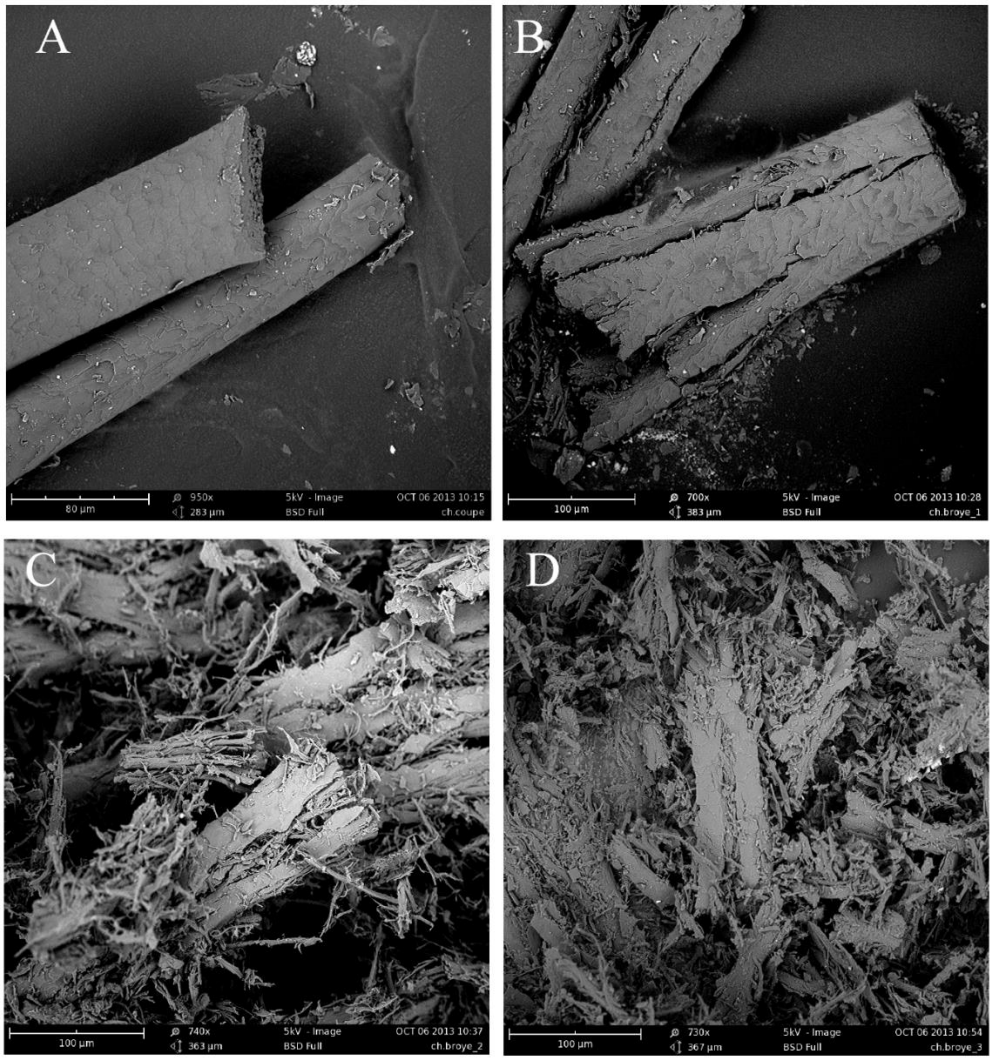

Figure 4.6 Microscopic observation of A: hair cut into small pieces (process n°1), B: hair cut into small pieces and weakly pulverised (process n°2), C: hair cut into small pieces and extensively pulverised (process n°3), D: external QC EGH 2/12-A.

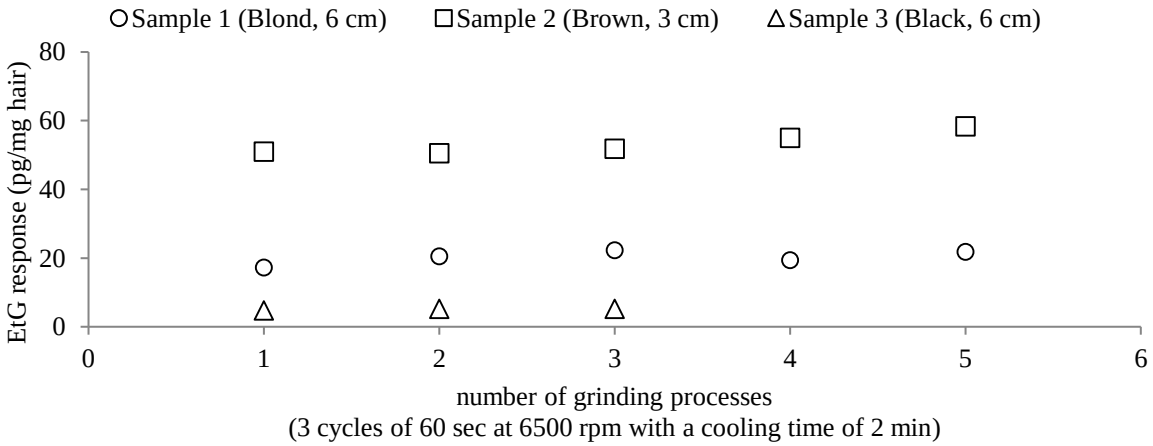

Figure 4.7 Effect of the number of grinding processes on the measured EtG concentration in real samples (N=3), which are not cut into small pieces (as calibrators and QCs). The methods developed and validated are based on one grinding process.

Pulverisation was performed in disposable tubes containing six stainless steel beads, placed on a Precellys 24 homogenizer. The multi-directional motion gives a high energy level to the beads that grind up to a total 24 samples simultaneously. The motion speed, the number of cycles, the time of the cycles and the pause between cycles are variable parameters, which could potentially be optimised to improve the grinding of samples. This system allows a rapid pulverisation of samples without a risk of contamination. As the samples are directly weighed and pulverised in the same vial that is used for the extraction, loss of sample is minimised. Further experimental studies based on more hair samples, from different ethnicities, with different colours and which have been subject to different cosmetic treatment are required to propose an optimal sample preparation procedure.

4.3.1.3 Method validation

The method was validated using a Acquity UPLC® coupled to a Xevo TQ MS mass spectrometer for lower limit of quantification (LLOQ), selectivity, linearity, matrix effects, extraction efficiency, accuracy, precision, stability and reproducibility.

The LLOQ was calculated at 10 pg EtG/mg hair (Figure 4.8). Variation in the ratio between the two transitions (%RSD > 20%) at concentrations below 10 pg EtG/mg hair limited the sensitivity of the presented method. Nevertheless, the cut-off value used to detect abuse and excessive alcohol consumption, defined as 30 pg EtG/mg hair by the SoHT, exceeds by far the LLOQ of 10 pg EtG/mg hair of the presented method. Furthermore, this LLOQ was suitable for the purpose of evaluating the grinding process.

An interfering peak at the same retention time of EtG and with a stable ratio between the two transitions (< 20 %) was detected in some blank hair samples (3/6); the estimated concentrations were around 0.5 pg EtG/mg hair (~20 times lower than the LLOQ). The response obtained for the interfering peak (11290) was 6 % of the response obtained for the LLOQ (168242). The origin of the peak was not determined. Interfering peaks have also been reported in other publications [7,17] and have been separated from EtG using a 100 % porous graphitic carbon column instead of a more conventional silica-based bonded phase column. We opted for an Acquity UPLC® HSS T3 column because of enhanced retention of polar compounds on this column. Other published methods based on liquid chromatography [15–17,22] did not report on interfering peaks. However, these research groups used hair

samples in the cut form and, as already demonstrated in literature [7,13,34], grinding of hair samples increases the extraction efficiency.

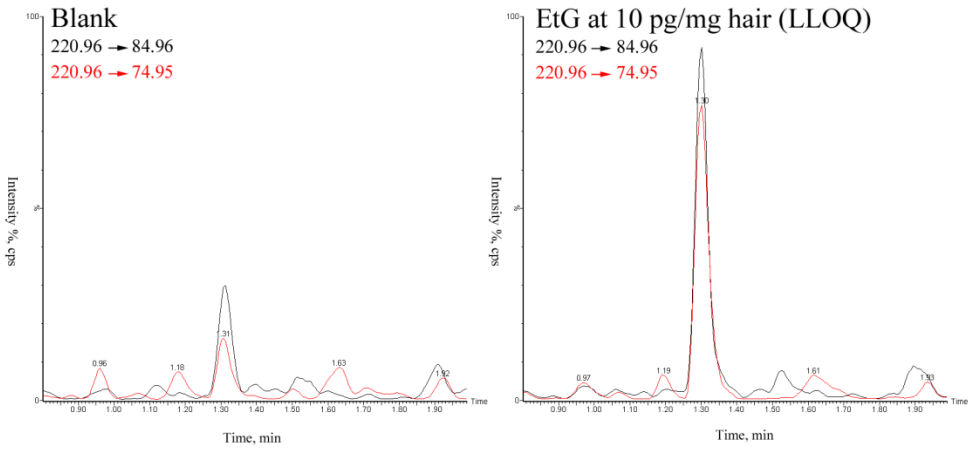

Figure 4.8 MRM Chromatogram for EtG (m/z 221→85 and m/z 221→75) in a 'blank' sample (left) and spiked at the LLOQ (10 pg/mg hair) (right) obtained after the analysis of hair using a Xevo TQ MS mass spectrometer.

The calibration curve (Figure 4.9) was linear over the range 10 (LLOQ), 15, 20, 50, 100, 250 and 500 pg EtG/mg hair with correlation coefficients above 0.99. A weighted 1/x linear regression was applied.

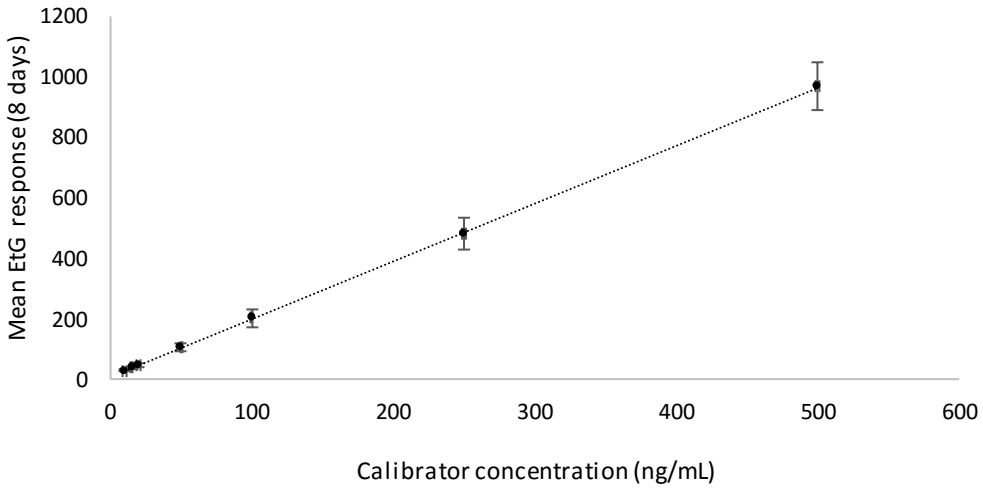

Figure 4.9 Calibration curve representing the mean EtG response (measured on 8 different days), with standard deviations, obtained analysing 7 calibrators.

Data on matrix effect (expressed as % recovery), extraction efficiency and accuracy and precision are reported in Table 4.4. A matrix effect (%ME) between 66 and 76 % (%RSD < 24 %) was observed for EtG. The %ME compensated by the use of EtG-d_5 as IS (%ME_{IS}) was

between 105 and 136 % (%RSD < 13 %). The extraction efficiency (%EE) of EtG was higher than 53 % (%RSD < 15 %).

Concentration	LLOQ	L	M	H	EGH 2/12-A
Nominal value (pg/mg hair)	10	16	30	300	25.4
%ME (%RSD)	76 (6)	66 (24)	69 (16)	68 (21)	
$\%ME_{IS}$ (%RSD)	136 (6)	116 (13)	112 (13)	105 (8)	
%EE (%RSD)	71 (13)	57 (15)	56 (10)	53 (6)	
%Bias	1	0	-1	1	-12
$\%RSD_r$	11	9	7	7	16
$\%RSD_t$	13	12	13	9	15

Table 4.4 Matrix effect expressed as % recovery (%ME), extraction efficiency (%EE), %bias, repeatability ($\%RSD_r$) and intermediate precision ($\%RSD_t$) for EtG in hair, measured using a Xevo TQ MS mass spectrometer for the LLOQ and 4 QCs (3 internal and 1 external QCs).

The %bias of the method was ≤ 1 % using internal QCs and ≤ 12 % using an external QC. The repeatability ($\%RSD_r$) and intermediate precision ($\%RSD_t$) were ≤ 16 % (for both internal and external QCs). Bias, repeatability and intermediate precision obtained from analysis of spiked QC samples (≤ 13 %) are comparable with results from published studies [7,25,28,37,41]. The measured $\%RSD_r$ and $\%RSD_t$ (≤ 16 %) and the calculated %bias (≤ 12 %) for the external QC (EGH 2/12-A) -consisting of authentic hair in pulverised form- demonstrate the accuracy and precision of this method. Comparable precision (< 13 %) has been reported by other groups [8,44] using authentic hair samples. To our knowledge, no reports on accuracy -based on the analysis of external QC samples with a certified reference value- have been published yet. In one published study, cross-validation including several laboratories has been used to determine the accuracy (%bias < 13 %) [8]. The reference value was determined based on the mean of the EtG concentrations obtained from each laboratory. According to our results, commercially available QC samples, in pulverised form, can be integrated in quality control programs to measure the accuracy and precision, but do not take into account variations due to the sample preparation.

No instability was observed for samples staying in the autosampler for 72 h at 4°C. The mean stability was at 109 % for the QC low and at 104 % for the QC high. The 90 % confidence interval of stability sample responses (QC low = 38318-41218, QC high = 520898-549493

were within the ± 20 % of the mean response for control samples (QC low = 29098-43647, QC high = 410152-615228).

The reproducibility of the method was evaluated via successful (Z-score ≤ 1.8) participation to 5 proficiency tests organised by the SoHT and the GTFCh. Results are presented in Table 4.5. Some results from proficiency tests were not reported, because results were not available at the deadline (GTFCh 3/11, GTFCh 3/12, GTFCh 2/12, GTFCh 3/12, SoHT 2011) or because samples were never received (SoHT 2013).

Proficiency test	Sample	Nominal value (pg/mg hair)	Reported value (pg/mg hair)	Z-score
SoHT 2012	A	39	63	1.8
	B	69	90	1.1
GTFCh 1/13	A	42	33	-0.8
	B	60	53	-0.5
GTFCh 2/13	A	47	40	-0.6
	B	38	33	-0.5
GTFCh 3/13	A	22	29	1.3
	B	31	34	0.3
GTFCh 1/14	A	< 7	< 10	-
	B	39	35	-0.4

Table 4.5 Proficiency test results for EtG in hair using a Xevo TQ MS mass spectrometer.

4.3.1.4 Population study

The mean daily alcohol consumption and the EtG concentrations determined in the proximal 1-cm segments -i.e. closest to the skin- from 7 volunteers are described in Table 4.6.

Volunteer	Gender	Mean daily alcohol consumption (g/day)	EtG concentration (pg/mg hair)
1	Male	16	< LLOQ
2	Male	21	< LLOQ
3	Female	0	< LLOQ
4	Male	10	< LLOQ
5	Female	27	< LLOQ
6	Female	32	10.4
7	Female	17	10.1

Table 4.6 Data of the population study, with indication of the gender, the mean daily alcohol consumption in g/day and the EtG concentration measured in the hair strand using a Xevo TQ MS mass spectrometer. LLOQ = 10 pg EtG/mg hair.

With a mean alcohol intake per day between 10 and 32 g for the social drinkers and of 0 g for one teetotaller, EtG concentrations in hair were below LLOQ (10 pg EtG/mg hair) for 5 subjects and at 10.1 and 10.4 pg/mg hair for 2 subjects, the latter having a mean alcohol intake per day at 17 and 32 g, respectively. A recent controlled alcohol-dosing study [53] has shown that a mean daily ethanol consumption of 14 g ethanol for 3 months led to an EtG concentration between 2.0 and 9.8 pg/mg hair (median = 5.6 pg/mg, N=10), while a mean daily ethanol consumption of 21 g ethanol for 3 months led to an EtG concentration between 7.7 and 15.2 pg/mg (median 11.3 pg/mg, N=10), with one volunteer having an especially high EtG concentration at 38.9 pg/mg. Recalling that the LLOQ of our method was at 10 pg/mg and taking into consideration the considerable inter-individual variability observed for EtG in hair after the consumption of a fixed amount of alcohol [53], our results are in agreement with the results of this controlled alcohol-dosing study, even if a higher EtG concentration could have been expected for two volunteers (n°5 and n°6), having declared a mean daily alcohol consumption of 27 and 32 g/day. Cosmetic treatment (e.g. bleaching, perming and thermal hair straightening [31,32,38,54,55]), which may lead to significant decreases of EtG concentration in hair, could explain the somewhat low EtG concentrations observed in these 2 cases. In our study, no information concerning hair treatment was

available. Some drugs or diseases that may affect the glucuronidation of ethanol (see Chapter 7), could also possibly lead to decreased EtG concentrations in hair. Such issues still remain to be studied.

Hair analysis from 6 social drinkers (mean ethanol intake per day ≤ 32 g) and 1 teetotaller did not show an EtG concentration above 30 pg/mg hair. Results of this limited population study showed no false positive results of the method in the diagnosis of excessive/chronic alcohol consumption using the cut-off value fixed by the SoHT. Because of the too small size of the population study, the specificity of the presented method was not estimated. However, a recently published method has reported a specificity at 0.93 using 25 pg/mg hair as cut-off value to distinguish between social and excessive/chronic drinkers [56]. Based on that estimation and working with a higher cut-off value (i.e. 30 pg EtG/mg hair), the risk of a false positive result for our method can be estimated at 7 % or less.

4.3.2 Method transfer (Xevo TQ S mass spectrometer)

The fully validated method using a Xevo TQ MS mass spectrometer (see section 4.2.3) was transferred to another system equipped with a Xevo TQ S mass spectrometer and partially validated (LLOQ, linearity, accuracy, precision and reproducibility).

Using a system equipped with a more sensitive mass spectrometer (a Xevo TQ S instead of Xevo TQ MS) and with 0.01 % formic acid in water (instead of 0.1 %) as mobile phase improved the lower limit of quantification (LLOQ) for EtG in hair from 10 to 2 pg/mg hair (Figure 4.10). This is of major importance to apply the cut-off value at 7 pg/mg hair proposed by the SoHT to detect repeated alcohol consumption and so to disclaim a strict abstinence period. The calibration model (N=8) was linear (1/x) over the range 2 (LLOQ), 5, 10, 20, 50, 250 and 500 pg EtG/mg hair. The %bias, repeatability ($\%RSD_r$) and intermediate precision ($\%RSD_t$) for internal and external QCs were ≤ 12 % (Table 4.7).

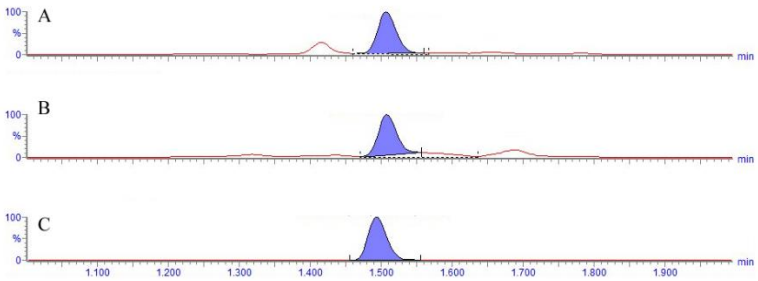

Figure 4.10 MRM Chromatogram for EtG (m/z 221→85 (A) and m/z 221→75 (B)) and EtG-d_5 (m/z 226→85 (C)) spiked at the LLOQ (2 pg/mg hair) obtained after the analysis of hair using a Xevo TQ S mass spectrometer.

Concentration	LLOQ	QC-L	QC-M	QC-H	EGH 2/12-A
Nominal value (pg/mg hair)	2	6	30	300	25.4
%Bias	6	3	8	8	-9
$\%RSD_r$	8	3	2	3	5
$\%RSD_t$	10	9	7	4	12

Table 4.7 Precision (repeatability (%RSDr), intermediate precision ($\%RSD_t$)) and accuracy (%bias) for EtG in hair, measured using a Xevo TQ S mass spectrometer for the LLOQ and 4 QCs (1 external and 3 internal).

The reproducibility of the method was evaluated via successful participation to 3 proficiency tests organised by the SoHT and the GTFCh. Results for two proficiency tests (GTFCh 2/14 and GTFCh 1/15) were not reported, because results were not available at the deadline. Samples from older proficiency tests (between 2011 and 2012) that were still available were analysed. All results are reported in Table 4.8.

Proficiency Test	Sample	Nominal value (pg/mg hair)	Reported value (pg/mg hair)	Z-score	Measured value (pg/mg hair)
GTFCh 3/11	B	72			68
SoHT 2011	B	24			27
	C	16			13
GTFCh 1/12	A	27			23
	B	54			46
GTFCh 2/12	A	25			24
	B	41			50
GTFCh 3/12	A	5			4
SoHT 2014	A	9	8	-0.4	
	B	29	27	-0.4	
GTFCh 3/14	A	11	8	-1.0	
	B	26	18	-1.1	
GTFCh 1/15	A	4			< 2
	B	12			9
GTFCh 2/15	A	23	18	-0.7	
	B	44	35	-0.8	

Table 4.8 Proficiency test results for EtG in hair using a Xevo TQ S mass spectrometer.

4.3.3 Xevo TQ MS *vs.* Xevo TQ S

The accuracy (%bias) and precision (repeatability (%RSD_r) and intermediate precision (%RSD_t)) were calculated for both devices. No differences were observed regarding the accuracy and precision (Figure 4.11) or Z-score (between -0.8 and 1.8 (Xevo TQ MS) and between -1.1 and -0.4 (Xevo TQ S)) when using one or another device. This seems to confirm that both systems can be used interchangeably.

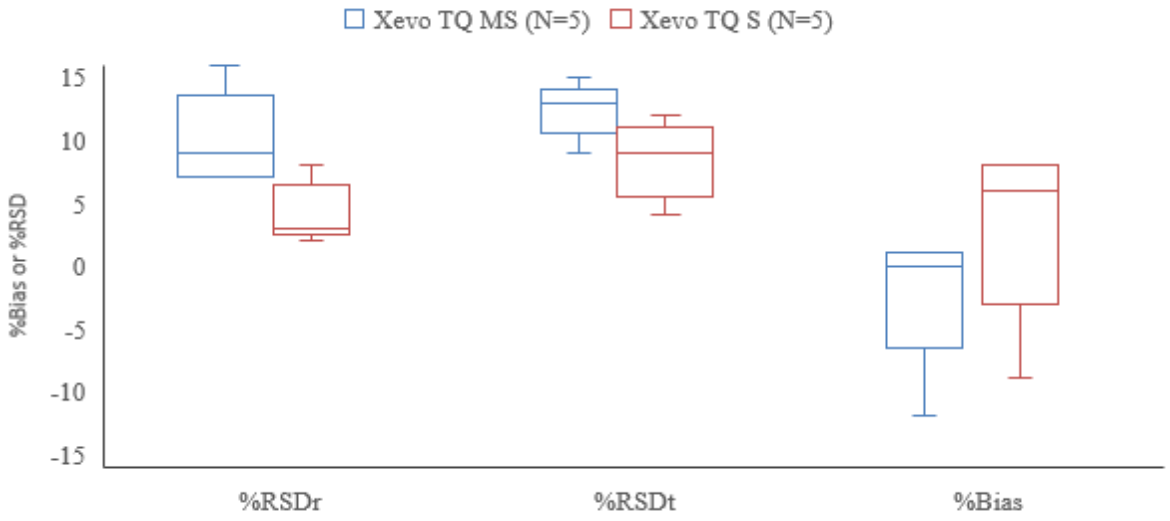

Figure 4.11 Boxplots depicting the accuracy (%bias), repeatability (%RSD_r) and intermediate precision (%RSD_t), obtained using a Xevo TQ MS and a Xevo TQ S mass spectrometer (values of Tables 4.4 and 4.7). The boxes represent the values between the lower and upper quartile, the horizontal line represents the median and the whiskers represent the extreme values.

4.3.4 Proficiency test results between 2011 and 2015

Quantitative results from the proficiency tests, organised either by the GTFCh or by the SoHT, in co-operation with Medichem and the Federal Institute for Materials Research and Testing, are presented in Figure 4.12. The target values, %RSD, accepted ranges, number of laboratories with a successful participation, reported values, obtained Z-score and measured value (but not reported) are reported. The %RSD reported are the standard deviation according to Horwitz (except for the SoHT 2011 and the SoHT 2012 where the %RSD provided by the report are the 'coefficient of variation 'resp. the 'reproducibility %RSD').

The tests, based on authentic hair samples that had been cut into small pieces (organised by the SoHT), show a variation in the quantification between laboratories (%RSD) between 24 and 102 %. The reported variation (%RSD) for the quantification of EtG in pulverised hair from proficiency tests organised by the GTFCh and the SoHT is between 23 and 35 %. The reproducibility of the presented methods was evaluated by means of participation in 5 proficiency tests using the Xevo TQ MS mass spectrometer (SoHT 2012, GTFCh 1/13, GTFCh 2/13, GTFCh 3/13, GTFCh 1/14) and in 3 proficiency tests using the Xevo TQ S mass spectrometer (SoHT 2014, GTFCh 3/14 and GTFCh 2/15). In agreement with data published in 2011 [45], the combined uncertainty (u(x) = 30 %) calculated on results from proficiency tests [48–50] is mainly due to the uncertainty of the inaccuracy of measurement (RMS_{bias} = 26 %, N=15) and partially due to the uncertainty of the certified value ($u(C_{ref})$ = 6 %, N=36) and intermediate precision of the method ($\%RSD_t$ = 13 %). The inaccuracy of measurement (RMS_{bias} = 22 %, N=11) and so the combined uncertainty (u(x) = 26 %) decrease when proficiency tests based on cut hair samples, thus with variable sample preparation, are not taken into account. These results tend to suggest that recommendations or guidelines concerning the sample preparation protocol are to date crucial to lower the observed variation between laboratories in the determination of EtG from cut hair samples.

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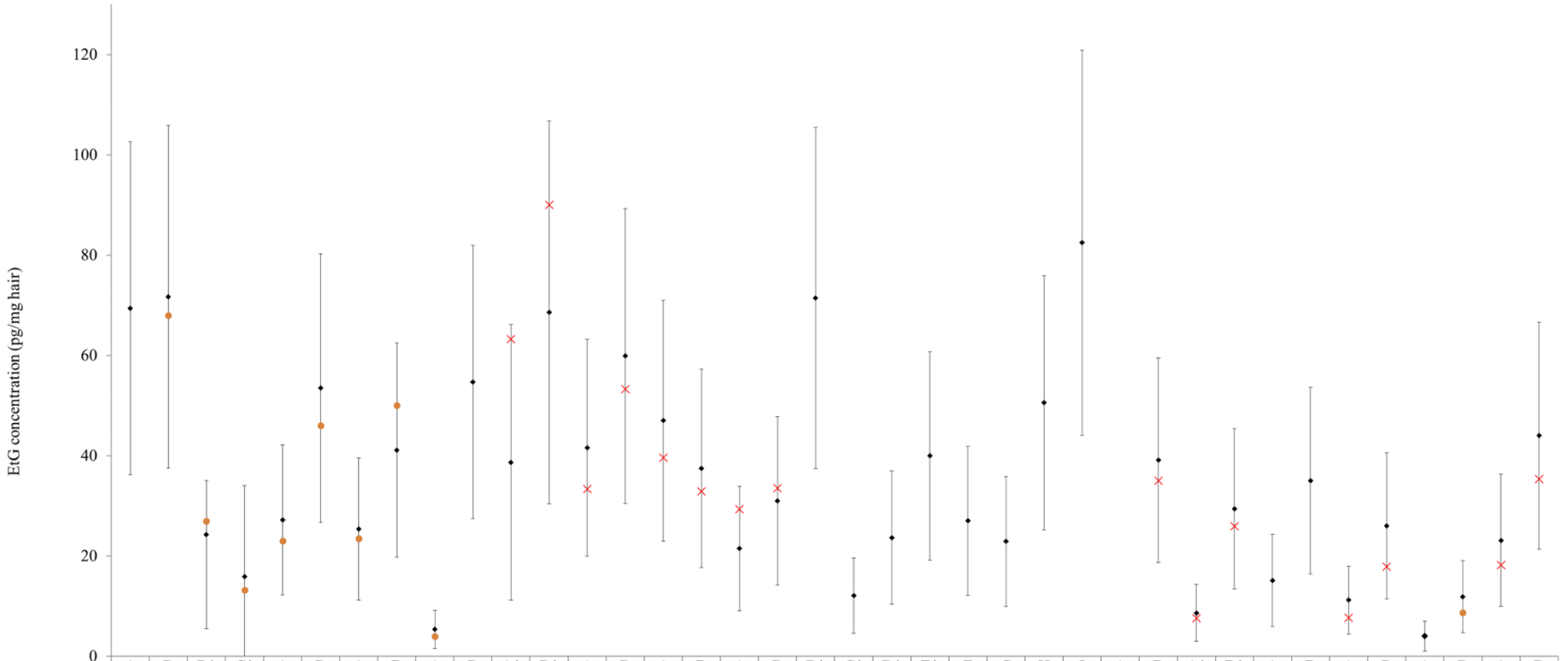

	A	B	B*	C*	A	B	A	B	A	B	A*	B*	A	B	A	B	A	B	B*	C*	D*	E*	F	G	H	I	A	B	A*	B*	A	B	A	B	A	B	A	B
	GTFCh 3/11		SoHT 2011		GTFCh 1/12		GTFCh 2/12		GTFCh 3/12		SoHT 2012		GTFCh 1/13		GTFCh 2/13		GTFCh 3/13		SoHT 2013								GTFCh 1/14		SoHT 2014		GTFCh 2/14		GTFCh 3/14		GTFCh 1/15		GTFCh 2/15	
◆ Target Value	69	72	24	16	27	54	25	41	5	55	39	69	42	60	47	38	22	31	71	12	24	40	27	23	51	82	< 7	39	9	29	15	35	11	26	4	12	23	44
RSD%	24	24	62	102	28	25	28	26	35	25	36	28	26	25	26	26	29	27	24	31	28	26	28	28	25	23		26	33	27	30	27	30	28		30	29	26
No of laboratories	25	23	30	29	26	23	27	26	14	25	28	27	33	33	29	28	29	29	24	21	23	22	23	23	26	26	9	37	24	30	28	31	31	37	33	30	45	46
× Reported value											63	90	33	53	40	33	29	34										35	8	26			8	18			18	35
Z-score											1.8	1.1	-0.8	-0.5	-0.6	-0.5	1.3	0.3									< 10	-0.4	-0.4	-0.4			-1.0	-1.1			-0.7	-0.8
● Measured value		68	27	13	23	46	24	50	4																										< 2	9		

Figure 4.12 Target values, %RSD, accepted ranges, number of laboratories with a successful participation, reported values, obtained Z-score for this result and measured value (but not reported) for proficiency tests organised by the GTFCh and the SoHT between 2011 and 2015 (with permission of the GTFCh and the SoHT). Samples delivered in the cut form are indicated with an asterisk.

4.4 Conclusion

This report describes the development and validation of a method for the quantification of EtG in hair by UHPLC-ESI$^-$-MS/MS using a Xevo TQ MS mass spectrometer. A SPE cartridge (the Bond Elut SAX) was selected among nine tested, to optimise the matrix effect and extraction efficiency. The influence of the preparation of the hair on the quantitative result has been studied. An extensive pulverisation of hair samples leads to a significantly higher amount of EtG measured and is therefore essential to assure a high extraction efficiency of EtG from hair and consequently a correct quantification of authentic samples. In addition, pulverisation of hair saves time and leads to more homogenous samples. The developed method has been fully validated using a Xevo TQ MS mass spectrometer. The accuracy and precision of the method has been demonstrated with spiked QC samples (%bias, $\%RSD_t$ and $\%RSD_r \leq 13$ %) and with one external QC sample (%bias, $\%RSD_t$ and $\%RSD_r \leq 16$ %), consisting of authentic pulverised hair, with a reference concentration value at 25.4 pg EtG/mg hair. With its LLOQ at 10 pg EtG/mg hair this method is suitable to diagnose chronic and excessive alcohol consumption according to the Society of Hair Testing (SoHT) as the proposed cut-off is 30 pg EtG/mg hair. Because monitoring of an alcohol abstinence period requires a method able to quantify concentrations equal to or below 7 pg/mg hair, which is the cut-off value used to strongly suggest repeated alcohol consumption and disprove a strict abstinence period, the method was transferred to another tandem mass spectrometer (Xevo TQ S instead of the Xevo TQ MS), optimised and partially validated (%bias, $\%RSD_t$ and $\%RSD_r \leq 12$ %). The LLOQ for EtG in hair was improved from 10 to 2 pg/mg hair. The reproducibility of both methods was assessed via successful participation (Z-scores ≤ 1.8) to eight proficiency tests. However, recommendations or guidelines concerning the sample preparation protocol are crucial to lower the observed variations between laboratories in the determination of EtG in hair.

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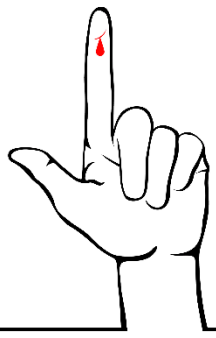
Chapter 5

Quantification of PEths in whole blood, V-DBS and C-DBS



Based on

N. Kummer, A.S. Ingels, S.M. Wille, C. Hanak, P. Verbanck, W.E. Lambert, N. Samyn, C.P. Stove, Quantification of phosphatidylethanol 16:0/18:1, 18:1/18:1, and 16:0/16:0 in venous blood and venous and capillary dried blood spots from patients in alcohol withdrawal and control volunteers, Analytical and Bioanalytical Chemistry. 408 (2016) 825–838.

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Abstract

Phosphatidylethanol species (PEths) are promising biomarkers of alcohol consumption. This chapter reports on the set-up, validation and application of a novel UHPLC-ESI-MS/MS method for the quantification of PEth 16:0/18:1, PEth 18:1/18:1, and PEth 16:0/16:0 in whole blood (30 µL) and in venous (V, 30 µL) or capillary (C, 3 punches (3 mm)) dried blood spots (DBS). The methods were linear from 10 (LLOQ) to 2000 ng/mL for PEth 16:0/18:1, from 10 (LLOQ) to 1940 ng/mL for PEth 18:1/18:1, and from 19 (LLOQ) to 3872 ng/mL for PEth 16:0/16:0. Extraction efficiencies were higher than 55 % (%RSD < 18 %) and matrix effects compensated by IS were between 77 and 125 % (%RSD < 10 %). Accuracy and precision fulfilled acceptance criteria (%bias, $\%RSD_r$ and $\%RSD_t$ below 13 %). Validity of the procedure for determination of PEth 16:0/18:1 in blood was demonstrated by the successful participation to a proficiency test. The quantification of PEths in C-DBS was not significantly influenced by the hematocrit (Hct), punch localisation or spot volume. The stability of PEths in V-DBS stored at room temperature was demonstrated up to 6 months. The method was applied to authentic samples (whole blood, V-DBS and C-DBS) from 50 inpatients in alcohol withdrawal and 50 control volunteers. Applying a cut-off value to detect inpatients at 221 ng/mL for PEth 16:0/18:1 provided no false positive results and a good sensitivity (86 %). Comparison of quantitative results (Bland-Altman plot, Passing-Bablok regression and Wilcoxon signed rank test) revealed that V-DBS and C-DBS are valid alternatives to venous blood for the detection of alcohol consumption.

5.1 Introduction

Phosphatidylethanols (PEths) are a group of abnormal phospholipids formed by the presence of ethanol in cell membranes [1]. They are biomarkers of alcohol consumption [2] present in blood, mainly located in erythrocytes [3], and in different organs [4]. Up to forty-eight different PEths have been detected in blood collected in autopsy cases of heavy drinkers [5]. All PEths have a common phosphoethanol head on which two fatty acid chains of variable length and degree of saturation are attached. Although blood analysis from heavy drinkers shows inter-individual variations of the distribution of the different PEths [6], the predominant species in blood after alcohol consumption are PEth 16:0/18:1 (30-46 %) and PEth 16:0/18:2 (16-28 %) [5–9]. Other PEths detected are PEth 18:1/18:1 and PEth 18:0/18:2 (identical molecular masses), together accounting for about 11-12 % of total PEths [6,7] while PEth 16:0/16:0 accounts for about 5 % [6]. The half-life of PEths in whole blood was calculated to be 4.0 ± 0.7 days [3]. In case of chronic/excessive alcohol consumption, PEths are detectable in blood up to 28 days after sobriety [10]. Moreover, quantification of PEths can be used to detect the degree of alcohol consumption as a significant correlation between the PEths concentrations in blood and the amount of consumed ethanol has been demonstrated [11].

Numerous studies have been published on the quantification of PEths in blood and these have been reviewed in 2012 [10]. The most used extraction technique is a liquid-liquid extraction (LLE) with *n*-hexane [5–7,11–17] (or heptane [8]) after stepwise addition of blood to isopropanol and the internal standard (IS) solution. Some methods added water [6], borate buffer (pH=9) [5] or sodium acetate buffer (pH=5) [15] to dilute the blood. Some publications reported other types of sample preparation, such as protein precipitation with methanol [18] or protein precipitation followed by an online solid-phase extraction [19]. A number of detection methods is based on high performance liquid chromatography (HPLC) with normal-phase columns coupled to evaporative light scattering detection (ELSD); chromatography has been carried out with *n*-hexane and propanol-based gradients containing acetic acid and triethylamine [3,4,11,12]. Quantification limits (LLOQ) obtained with these methods ranged between 100–500 ng/mL [4,11], analysing 250 to 300 µL of whole blood. PEths have also been analysed with non-aqueous capillary electrophoresis (CE) coupled to ultraviolet (UV) detection [13]. Both HPLC-ELSD and CE-UV [13] methods measure

the total amount of PEths. However, LC methods coupled to MS/MS detection allow to obtain much lower LLOQs (between 0.7 and 83 ng/mL, based on the analysis of between 100 and 300 µL of whole blood) and are able to identify and quantify individual molecular species [6–8,15,18–20].

To improve the stability of compounds in whole blood and to facilitate the storage and transportation of samples [21], DBS methods have been developed. Numerous DBS-based methods have been published for a wide variety of applications, including therapeutic drug monitoring and toxicology [22]. Also alcohol markers such as ethyl glucuronide, ethyl sulfate and PEths have been determined, starting from DBS [15,18,21,23–25] (reviewed by Sadones *et al.* [26]). Since 2011, two publications have reported on the quantification of PEth 16:0/18:1 [15,18] and PEth 18:1/18:1 [15] in V-DBS samples, while only one [21] reported on the analysis of C-DBS samples (detection of PEth 16:0/18:1 in newborns to detect prenatal alcohol exposure). V-DBS are prepared by spotting a fixed volume of venous blood onto a filter paper, whereas C-DBS are generated by direct collection of blood drops appearing after a finger or heel prick onto a filter paper. C-DBS offer the advantage compared to venepuncture of being less invasive and not requiring the service of nurses or physicians. Since these are typically collected in a non-volumetric way, these samples are mostly processed by excising punches with a fixed diameter from the global spot. This partial-spot approach requires the assessment of the impact of variables such as hematocrit (Hct), punch localisation and spot volume on the quantitative result [27,28].

This chapter presents the validation of UHPLC-ESI-MS/MS methods for the quantification of three PEths (PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0) in whole blood, V-DBS and C-DBS according to international guidelines [29] and published recommendations [27]. To our knowledge, this is the first report on the rigorous validation of the differences between capillary and venous DBS including the impact of specific parameters such as the influence of hematocrit, punch localisation and spot volume on PEths. In addition, a sensitive method for PEth 16:0/16:0 in DBSs was developed and stability of the three species in V-DBS was evaluated over a period of 6 months. Moreover, successful participation to a proficiency test demonstrated the validity of the method for blood (no proficiency tests for DBS are available). Finally, the developed methods were applied to evaluate the agreement between the quantitative results from the analysis of whole blood, V-DBS and C-DBS obtained from

100 volunteers (50 inpatients in alcohol withdrawal and 50 control volunteers). Receiver operating characteristic (ROC) curves performed on these results allow us to propose a possible cut-off value to detect chronic and excessive alcohol consumption. It was our main objective to investigate whether C-DBS could be a reliable alternative for the detection of PEths in whole blood, as this could lead to a more user friendly and practical approach to detect excessive and chronic alcohol consumption.

5.2 Materials and Methods

5.2.1 Chemical

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanol (sodium salt; PEth 18:1/18:1) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol (sodium salt; PEth 16:0/16:0) were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol (PEth 16:0/18:1) was purchased from Enzo Life Sciences (Antwerp, Belgium). As deuterated analogues had not been commercialised at that time, four different internal standards from Avanti Polar Lipids were evaluated during validation: 1,2-dipalmitoyl-*sn*-glycero-3-phosphomethanol (sodium salt; PMeth 16:0/16:0), 1,2-dioleoyl-*sn*-glycero-3-phosphomethanol (sodium salt; PMeth 18:1/18:1), 1,2-dipalmitoyl-*sn*-glycero-3-phosphopropanol (sodium salt; PProp 16:0/16:0) and 1,2-dioleoyl-*sn*-glycero-3-phosphopropanol (sodium salt; PProp 18:1/18:1).

Isopropanol (ULC/MS), tetrahydrofuran (ULC/MS), ammonium acetate (ULC/MS), water (HPLC) and methanol (ULC/MS) were purchased from Biosolve (Valkenswaard, The Netherlands). Isopropanol and *n*-hexane, gradient grade for liquid chromatography, were purchased from Merck KGaA (Darmstadt, Germany). Formic acid for mass spectrometry (~98 %) was purchased from Sigma-Aldrich (Steinheim, Germany).

5.2.2 Standard solutions, calibrators and quality control (QC) samples

Stock solutions of PEths (PEth 16:0/18:1 (1.000 mg/mL), PEth 18:1/18:1 (0.970 mg/mL) and PEth 16:0/16:0 (0.968 mg/mL)) and stock solutions of the 4 evaluated ISs (PMeth 16:0/16:0 (0.968 mg/mL), PMeth 18:1/18:1 (0.971 mg/mL), PProp 16:0/16:0 (0.969 mg/mL) and PProp 18:1/18:1 (0.971 mg/mL)) were prepared in methanol. For the blood and V-DBS methods, two stock solutions, one for the calibrators (Cal-Stock, 100 µg/mL) and one for the QCs (QC-Stock, 50 µg/mL) -containing PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0- were prepared by diluting stock solutions of each PEth in methanol. An IS stock solution (IS-Stock)

with the 4 ISs each at a concentration of 5 µg/mL was prepared in methanol. For the C-DBS method, a Cal-Stock solution of 250 µg/mL, a QC-Stock solution of 250 µg/mL and an IS-Stock solution of 0.25 µg/mL were prepared in methanol. All working solutions were stored at -18°C.

Daily dilutions of IS-Stock solutions were performed in solution A, consisting of isopropanol, 10 mM ammonium acetate buffer and formic acid (6:4:0.2, v/v), to reach a concentration of 100 ng/mL (IS working solution (IS-WS-1) used for the whole blood and V-DBS method) and 10 ng/mL (IS working solution (IS-WS-2) used for the C-DBS method).

Daily dilutions of Cal-Stock solutions and QC-Stock solutions were performed in water to obtain 8 different concentrations for calibrators and 3 for QCs. A second dilution was performed in EDTA blank whole blood. Final calibrator concentrations in blood were between 10 and 2000 ng/mL for PEth 16:0/18:1, 10 and 1940 ng/mL for PEth 18:1/18:1 and between 19 and 3872 ng/mL for PEth 16:0/16:0. For the two DBS methods, 30 µL of calibrators and QCs in blood were spotted onto Whatman 903 filter paper (GE Healthcare). Spots were dried for minimum 2 hours at room temperature. The complete DBS was used for the V-DBS method and 3 punches (3 mm) were used for the C-DBS method, unless indicated otherwise. Here, we typically used 3 punches from the same DBS, except in the application study, where not from all C-DBS three 3-mm punches could be obtained. The hematocrit of the blood used to prepare the DBS calibrators was 0.48 ± 0.02, as measured using a Sysmex XP-300™ automated hematology analyser (Sysmex America, Inc.). The three methods are presented in Figure 5.1.

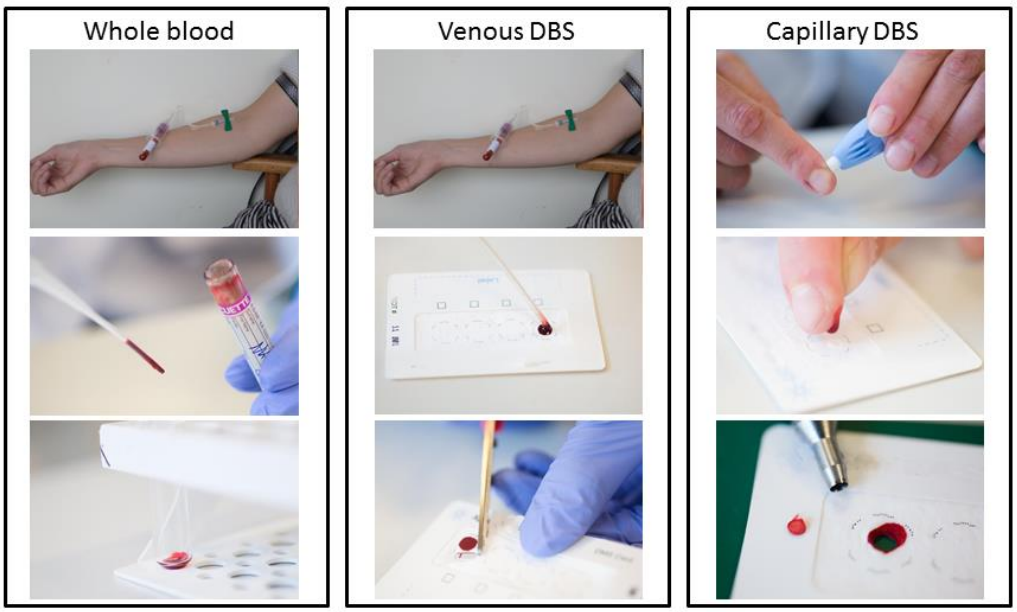

Figure 5.1 Illustration of the whole blood, venous DBS (V-DBS) and capillary DBS (C-DBS) sampling process.

5.2.3 Sample preparation

PEths were extracted by liquid-liquid extraction (LLE) with *n*-hexane. For the whole blood method, 30 µL of the sample was added to a 5 mL disposable glass tube containing 250 µL of solution A (consisting of isopropanol, 10 mM ammonium acetate buffer and formic acid (6:4:0.2, v/v)) and 50 µL of the IS-WS-1 solution (100 ng/mL). After a quick mixing (vortex), 1 mL *n*-hexane was added and the sample was gently mixed for 10 minutes. The tubes were centrifuged (10 min, 14000 rpm (20800 g), 4°C) and the clear supernatant was transferred to a total recovery glass vial (Waters, Zellik, Belgium) and evaporated to dryness during 30 minutes in a rotational vacuum concentrator (RVC 2-33 IR, Martin Christ, Osterode am Harz, Germany). The final dried extract was dissolved in 250 µL of a solution B (50 % of mobile phase A and 50 % of mobile phase B, see below).

For the V-DBS method, the complete DBS (30 µL) was excised and placed in a 5 mL disposable glass tube containing 250 µL of solution A and 50 µL of the IS-WS-1 solution (100 ng/mL). For the C-DBS method, three (or one, where indicated) punches (3 mm) were excised from the DBS and placed in a 5-mL disposable glass tube containing 250 µL of solution A and 50 µL of the IS-WS-2 solution (10 ng/mL). For both DBS methods, the tubes were gently mixed for 1 hour. After adding 1 mL of *n*-hexane, the samples were mixed for another 10 minutes. After centrifugation, the clear supernatant was transferred in total

recovery glass vials and evaporated to dryness. The final dried extract was dissolved in 250 µL of solution B for the V-DBS and in 100 µL of solution B for the C-DBS.

For the whole blood method and the V-DBS method, 5 µL was injected in partial loop with needle overfill mode. For the C-DBS method, 10 µL was injected in full loop mode.

5.2.4 Liquid chromatographic and mass spectrometric conditions

Analyses were performed on an Acquity UPLC® system coupled to a Xevo TQ S tandem mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionisation source operated in negative mode (ESI^-). The compounds were separated on an Acquity UPLC® BEH C8 (2.1 x 50 mm, 1.7 µm) column (Waters) using as mobile phase A 10 mM ammonium acetate buffer with 0.05 % formic acid (pH 2) and as mobile phase B isopropanol with 10 % of tetrahydrofuran at a flow rate of 400 µL/min. The gradient elution started with 40 % of mobile phase A and decreased to 0 % of mobile phase A at 1.5 minutes. The washing step, containing 100 % of solution B, was held for 1 minute and was followed by 1 minute re-equilibration with the starting condition, resulting in a total run time of 3.5 minutes. The column temperature was set at 60°C.

Detection was performed in the multiple reaction monitoring mode (MRM), with a dwell time fixed at 0.017 sec, using the appropriate parameters for each compound (Table 5.1). Two transitions were monitored for the PEths (Figure 5.2), one for quantification (underlined) and one for qualification. For the ISs only one MRM transition was used.

	Precursor/product ion (m/z)	Cone voltage (V)	Collision energy (eV)
PEth 16:0/18:1	702/255	10	35
PEth 16:0/18:1	702/125	10	40
PEth 18:1/18:1	728/281	10	35
PEth 18:1/18:1	728/463	10	25
PEth 16:0/16:0	676/255	10	30
PEth 16:0/16:0	676/125	10	35
PProp 18:1/18:1	742/281	20	35
PProp 16:0/16:0	690/255	20	30
PMeth 18:1/18:1	714/281	20	30
PMeth 16:0/16:0	662/255	10	30

Table 5.1 MRM transitions and conditions for PEths and ISs (PProp 18:1/18:1, PProp 16:0/16:0, PMeth 18:1/18:1, PMeth 16:0/16:0).

For the MS/MS detection, the following parameters were used: temperature of source gas (nitrogen) was 150°C, desolvatation gas (nitrogen) flow was 1000 L/h at 650°C, capillary voltage was 3 KV, cone gas flow at 150 L/h and collision gas (argon) flow was 0.15 mL/min.

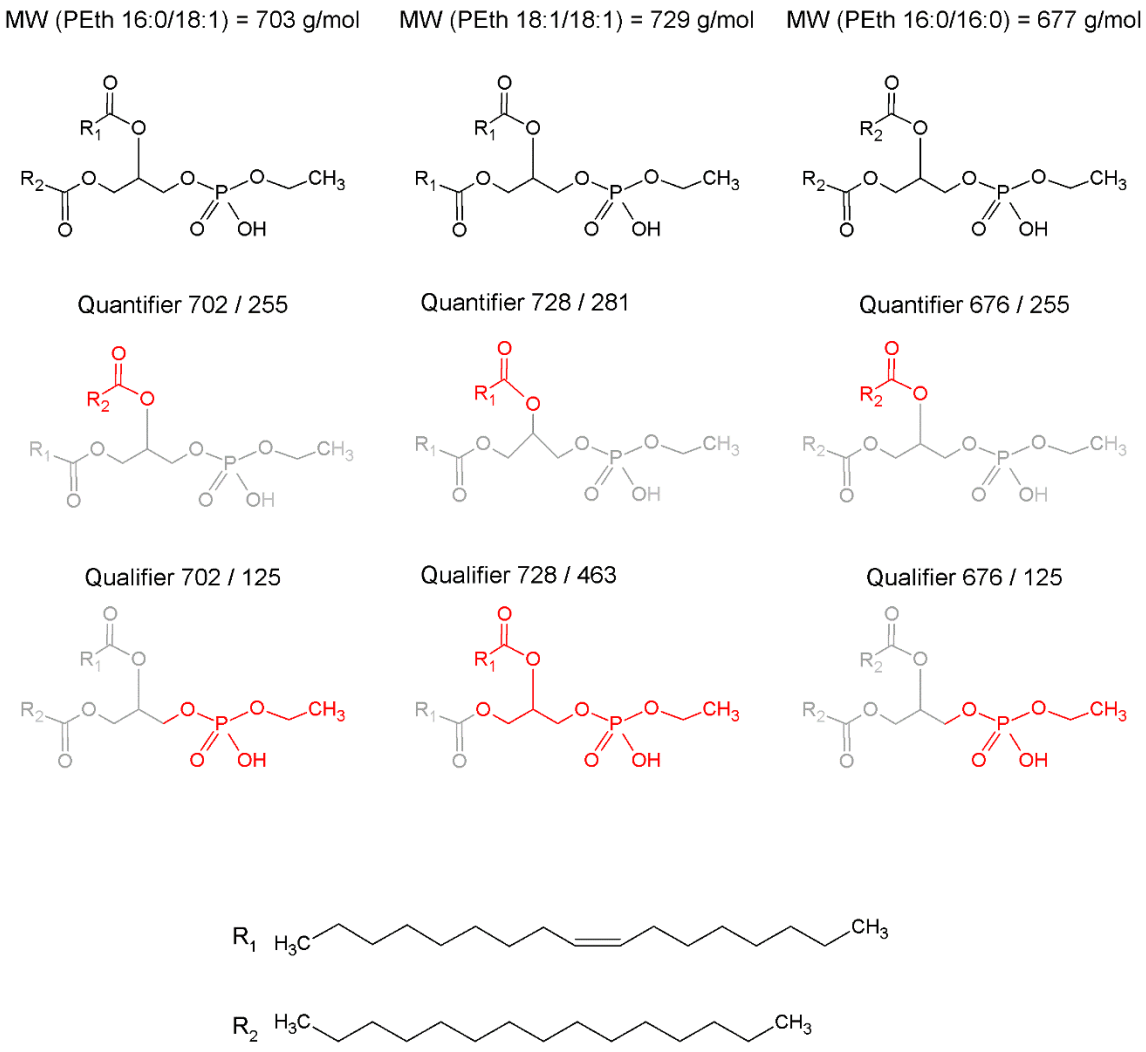

Figure 5.2 Chemical structure of PEth 16:0/18:1 (left), PEth 18:1/18:1(middle) and PEth 16:0/16:0 (right) and of their two product ions (red), with the transition (precursor ion / product ion) used. MW: molecular weight.

5.2.5 Method validation

Selectivity, sensitivity, matrix effect, extraction efficiency, lower limit of quantification (LLOQ), linearity, accuracy, precision (repeatability (%RSD_r) and intermediate precision (%RSD_t)) and stability were evaluated based upon international guidelines [29]. The influence of hematocrit, punch localisation and spot volume were evaluated for the C-DBS method [27,28].

To study endogenous interferences, six blank whole blood samples from different teetotallers were analysed. To verify that IS compounds do not interact with PEths, two zero samples (blank samples spiked with IS-WS solution) were analysed. According to the EMA guideline, in our method interferences are acceptable as long as the signal was lower than 20 % of the response at the LLOQ [30].

Matrix effect expressed as % recovery (%ME) was quantified and evaluated by the post-extraction addition technique using six different blank bloods from teetotallers [31]. Whole blood (30 µL), V-DBS (complete 30 µL DBS) and C-DBS (3 filter paper punches spiked each with 3.5 µL of whole blood) were extracted. The reference standards and IS (diluted in the mobile phase) were added in the total recovery vial before the injection. These samples were compared with control samples spiked at the same theoretical concentration in the mobile phase. Extraction efficiency (%EE) was evaluated by comparing responses of six blank samples spiked before sample preparation with responses of six blank samples, where the reference standards were spiked after the sample preparation in the mobile phase. Matrix effect and extraction efficiency were evaluated at low, medium and high concentrations. For the C-DBS method, blood samples with varying hematocrit levels (measured from 0.31 to 0.58) were used, to study the influence of the hematocrit variation on the extraction efficiency and on the matrix effect.

The LLOQ is the lowest concentration of analyte with a signal-to-noise ratio greater than 10/1 for both transitions and for which the bias and precision deviation is less than 20 %.

Calibration model and weighting factor were evaluated for each compound and each method. The linearity was tested by performing F-Tests (α=0.05). Homoscedasticity was tested visually by plotting residuals *vs.* fitted value. In case of heteroscedasticity, a weighted regression (1/x and $1/x^2$) was applied (slope and intercept). The sum of relative errors (difference between the calculated concentration and its nominal concentration) for each

model was calculated and plotted against the nominal concentrations. The model with a $R^2 \geq 0.99$ with the lowest sum of relative errors was selected. The goodness of fit of the selected model was tested, calculating the relative errors for calibrators and QCs. The relative errors should be lower than 15 % except for the LLOQ (< 20 %) [30].

Three internal QCs spiked at low, medium and high concentration, were analysed in duplicate on 8 different days to assess accuracy (%bias) and precision ($\%RSD_r$ and $\%RSD_t$). A One-way ANOVA test with significance level (α) of 0.05 allows calculating bias, repeatability and intermediate precision with these data (see the Appendix 2 for more explanation), with acceptance criteria of 15 % (20 % for the LLOQ). The measurement uncertainty was also calculated ($\%U=2.12*\%RSD_t$) and used to interpret quantitative results close to the LLOQ or close to the cut-off value.

The validity of the PEth 16:0/18:1 quantification in blood was tested by participation to a proficiency test organised by Equalis (Uppsala, Sweden).

Processed sample stability and long term storage stability were evaluated at low and high concentrations for the whole blood method and for the V-DBS method. The mean response of the stability samples should be within 90 – 110 % of the mean response of the control samples and the 90 % confidence interval of the stability sample responses should be within ± 20 % of the control sample responses data (see the Appendix 2 for more explanation).

The influence of the hematocrit on the response was evaluated for five hematocrit values at low and high concentrations. Blank blood samples with variable hematocrit level were prepared by adding or removing plasma to EDTA blank blood samples. The measured hematocrit values were 0.39, 0.42, 0.48, 0.50 and 0.57. Six spots per concentration and per hematocrit level were prepared and single centrally located punches were analysed. Measured responses were compared with a One-way ANOVA test (α=0.05). To evaluate whether no artefactual results were obtained because spiked samples might behave differently from real samples (where PEth species are presumably located in erythrocytes), we set up an experiment in which blood with different hematocrit was prepared from blood of two inpatients. More specifically, 200 µL of blood of an inpatient was diluted with plasma (between 25 and 200 µL) and erythrocytes (between 0 and 175 µL) of an alcohol abstainer to generate 6 blood samples of 400 µL with a different hematocrit (with measured hematocrits between 0.20 and 0.60) but with the same PEths concentrations (PEths virtually exclusively

being derived from the 200 µL of inpatient blood). This blood was used to generate DBS, which were processed as real samples (see Section 5.2.3). The DBS analysis was performed in quadruplicate at each hematocrit level. Also the blood PEths concentrations were determined and served as a reference.

The influence of the punch localisation (peripherally or centrally) was evaluated at low and high concentrations and at low (0.39), intermediate (0.48) and high (0.57) hematocrit levels. Six spots per concentration and per hematocrit level were prepared and the responses measured in peripherally and centrally located punches (one central and one peripheral punch were analysed per DBS) were compared using a One-way ANOVA test (α=0.05).

Three blood spot volumes (20, 35, 50 µL) were tested at low (0.32), intermediate (0.48) and high (0.67) hematocrit levels and at 2 concentrations; low and high. Six spots per concentration and per hematocrit level were prepared and centrally located 3 mm punches (1/DBS) were analysed. Responses were compared using a One-way ANOVA test (α=0.05) to detect significant differences.

The normality of the distributions and the homogeneity of variances were tested using the Shapiro-Wilk test and the Levene's test prior to One-way ANOVA tests [32].

5.2.6 Application to a comparative study

5.2.6.1 Sample collection

Whole blood and C-DBS from inpatients in alcohol withdrawal were collected at the Brugmann Hospital (Brussels, Belgium) one business day after their admission. Whole blood and C-DBS from control volunteers were collected by the medical staff of the Military Hospital in Brussels (Belgium). The inpatients group was composed of 37 males and 13 females, between 27 and 71 years (mean = 47, median = 47) and with a self-reported number of abstinence days before the sampling between 1 and 21 (mean = 4, median = 2). The control group was composed of 23 males and 27 females, between 22 and 64 years (mean = 40, median = 37) and with a self-reported mean alcohol consumption per week between 0 and 16 units (mean = 5, median = 6). Seven out of the 50 control volunteers were teetotallers.

Venous whole blood samples were collected in a 4-mL EDTA tube and were stored at -80°C until analysis. Five C-DBS were collected onto a Whatman 903 filter paper card after a

fingertip prick with a contact-activated lancet (BD Microtainer®, Becton Dickinson). Five V-DBS were prepared from the EDTA tubes by pipetting 30 µL of venous blood onto a filter paper. C-DBS and V-DBS were left to dry for minimum 2 hours at room temperature and were then stored in zip-closure plastic bags containing a desiccant packet (Sigma-Aldrich) at room temperature until the analysis.

The study protocol was approved by the ethics committee of the Brugmann Hospital (Brussels, Belgium) and informed consent was obtained from each subject before enrolment in the study (B077201420445).

5.2.6.2 Statistical analysis

Bland-Altman plot, Passing-Bablok regression analysis and Wilcoxon signed rank test were used to study the agreement between quantitative results obtained from whole blood, V-DBS and C-DBS samples [33]. A Bland-Altman plot is used to assess the absence of systematic differences between two measurements. The mean of the two measurements is plotted against the difference between these, 95 % of the differences are expected to lie within the limits of agreement (mean ± 1.96 SD). The Passing-Bablok regression analysis is a scatter diagram of the concentrations obtained with two different methods. The regression line and equation are used to detect measurement errors. No proportional differences are observed as long as the 95 % confidence interval of the slope includes 1 and no systematic differences are observed as long as the 95 % confidence interval of the intercept includes the zero value. Wilcoxon signed rank test was performed to detect significant differences (p-value<0.05) between the concentrations obtained from two methods.

ROC curve analyses were performed to determine optimal cut-off values (higher sensitivity with 0 false positive results) to distinguish between inpatients in alcohol withdrawal and control volunteers. The area under the curve (AUROC) was used to quantify the overall ability of the method to discriminate between the two populations. A perfect diagnostic method (0 false positives and 0 false negatives) will have an area of 1, where a method with no diagnostic ability will have an area of 0.5.

5.3 Results

5.3.1 Method Validation

Selectivity, sensitivity, linearity, LLOQ, matrix effects, extraction efficiency, accuracy and precision were assessed for the three methods. Stability was tested for the whole blood and V-DBS methods. For the C-DBS method, the impact of hematocrit, punch localisation and blood spot volume were evaluated.

In summary, no interfering peaks were detected in blank samples and the addition of the IS did not interfere with PEths detection. The linear (1/x) calibration curves ranged from 10 (LLOQ) to 2000 ng/mL for PEth 16:0/18:1, from 10 (LLOQ) to 1940 ng/mL for PEth 18:1/18:1 and from 19 (LLOQ) to 3872 ng/mL for PEth 16:0/16:0.

The non-IS-compensated matrix effect (%ME) was between 68 and 137 % (%RSD < 20 %) for PEth 16:0/18:1, between 73 and 121 % (%RSD < 12 %) for PEth 18:1/18:1 and between 59 and 110 % (%RSD < 20 %) for PEth 16:0/16:0. For the three PEths, PMeth 18:1/18:1 was selected as IS, because it better compensated for matrix effect than PMeth 16:0/16:0 and PProp 18:1/18:1 (Table 5.2) and had a better peak shape compared to PProp 16:0/16:0. The matrix effect compensated by this IS ($\%ME_{IS}$) was between 77 and 125 % (%RSD < 10 %) for all PEths. The extraction efficiency (%EE) was between 66 and 100 % (%RSD < 18 %) for the blood method, between 55 and 63 % (%RSD < 14 %) for the V-DBS method and between 61 and 78 % (%RSD < 15 %) for the C-DBS method. Visual inspection of the results indicated no influence of the hematocrit level on matrix effect and extraction efficiency for the C-DBS method (Figure 5.3).

Table 5.2 Matrix effect (%ME), matrix effect ► compensated with ISs ($\%ME_{IS}$) and extraction efficiency (EE) in whole blood, V-DBS and C-DBS for the three PEths calculated at low (L), medium (M) and high (H) concentrations.

Concentration		L	M	H
PEth 16:0/18:1 (Nominal value ng/mL)		133	2000	6250
Blood	%ME (%RSD)	116 (16)	89 (4)	72 (12)
	$\%ME_{PMeth\ 18:1/18:1}$ (%RSD)	116 (10)	102 (5)	88 (3)
	$\%ME_{PMeth\ 16:0/16:0}$ (%RSD)	81 (11)	67 (8)	57 (10)
	$\%ME_{PProp\ 18:1/18:1}$ (%RSD)	43 (18)	36 (12)	30 (15)
	$\%ME_{PProp\ 16:0/16:0}$ (%RSD)	118 (10)	101 (5)	91 (3)
	%EE (%RSD)	90 (10)	80 (9)	79 (17)
V-DBS	%ME (%RSD)	137 (9)	99 (9)	76 (8)
	$\%ME_{PMeth\ 18:1/18:1}$ (%RSD)	125 (6)	108 (4)	87 (3)
	%EE (%RSD)	59 (14)	61 (5)	58 (11)
C-DBS	%ME (%RSD)	101 (15)	79 (17)	68 (20)
	$\%ME_{PMeth\ 18:1/18:1}$ (%RSD)	108 (6)	89 (4)	89 (3)
	%EE (%RSD)	67 (4)	65 (8)	74 (13)
PEth 18:1/18:1 (Nominal value ng/mL)		129	1940	6063
Blood	%ME (%RSD)	115 (10)	101 (3)	97 (8)
	$\%ME_{PMeth\ 18:1/18:1}$ (%RSD)	115 (6)	117 (6)	118 (5)
	$\%ME_{PMeth\ 16:0/16:0}$ (%RSD)	80 (6)	77 (9)	76 (7)
	$\%ME_{PProp\ 18:1/18:1}$ (%RSD)	43 (12)	41 (8)	41 (11)
	$\%ME_{PProp\ 16:0/16:0}$ (%RSD)	118 (6)	116 (6)	122 (4)
	%EE (%RSD)	96 (7)	85 (9)	81 (18)
V-DBS	%ME (%RSD)	121 (6)	97 (5)	94 (6)
	$\%ME_{PMeth\ 18:1/18:1}$ (%RSD)	110 (5)	107 (4)	108 (2)
	%EE (%RSD)	61 (7)	63 (6)	59 (11)
C-DBS	%ME (%RSD)	106 (11)	89 (12)	73 (12)
	$\%ME_{PMeth\ 18:1/18:1}$ (%RSD)	114 (4)	101 (3)	97 (8)
	%EE (%RSD)	64 (9)	61 (13)	77 (12)
PEth 16:0/16:0 (Nominal value ng/mL)		258	3872	12100
Blood	%ME (%RSD)	103 (8)	88 (3)	83 (6)
	$\%ME_{PMeth\ 18:1/18:1}$ (%RSD)	103 (4)	102 (7)	101 (5)
	$\%ME_{PMeth\ 16:0/16:0}$ (%RSD)	72 (5)	66 (7)	65 (6)
	$\%ME_{PProp\ 18:1/18:1}$ (%RSD)	38 (9)	36 (8)	35 (9)
	$\%ME_{PProp\ 16:0/16:0}$ (%RSD)	105 (4)	100 (7)	104 (3)
	%EE (%RSD)	100 (13)	79 (11)	66 (15)
V-DBS	%ME (%RSD)	110 (9)	81 (11)	84 (6)
	$\%ME_{PMeth\ 18:1/18:1}$ (%RSD)	102 (8)	89 (9)	96 (5)
	%EE (%RSD)	58 (14)	57 (4)	55 (9)
C-DBS	%ME (%RSD)	107 (9)	69 (16)	59 (20)
	$\%ME_{PMeth\ 18:1/18:1}$ (%RSD)	116 (7)	78 (3)	77 (5)
	%EE (%RSD)	78 (13)	66 (7)	74 (15)

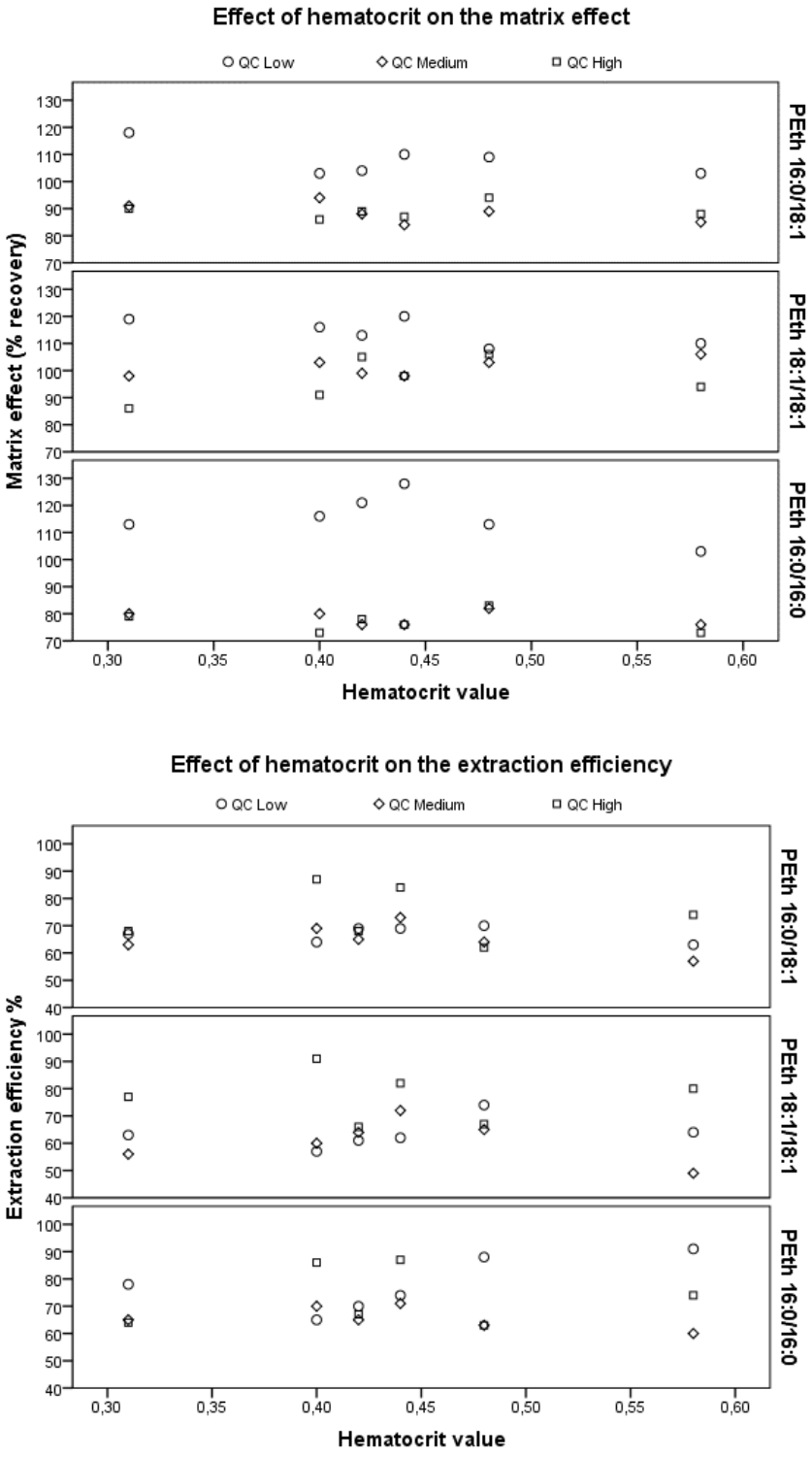

Figure 5.3 (Upper) Matrix effect (% recovery) for PEths from DBS at three concentrations (low, medium and high) and prepared from 6 whole blood samples with varying hematocrit levels. (Lower) Extraction efficiency % for PEths from DBS at three concentrations (low, medium and high) and prepared from 6 whole blood samples with varying hematocrit levels.

The %bias, $\%RSD_r$ and $\%RSD_t$ were ≤ 13 % for the whole blood, V-DBS and C-DBS methods (Table 5.3). The maximal uncertainties of measurement ($\%U=2.12*\%RSD_t$) were 23 %

(blood), 25 % (V-DBS) and 24 % (C-DBS) for PEth 16:0/18:1; 25 % (blood), 22 % (V-DBS) and 23 % (C-DBS) for PEth 18:1/18:1 and 28 % (blood), 21 % (V-DBS) and 22% (C-DBS) for PEth 16:0/16:0.

	PEth 16:0/18:1				PEth 18:1/18:1				PEth 16:0/16:0			
QC	*	L	M	H	*	L	M	H	*	L	M	H
Blood												
%RSD_r	9	9	6	4	7	4	4	4	11	6	3	4
%RSD_t	11	9	6	5	12	7	3	4	13	10	8	7
%Bias	3	5	2	3	-1	3	4	3	-5	-3	1	-1
V-DBS												
%RSD_r	11	7	3	4	6	5	3	4	7	6	4	3
%RSD_t	12	12	6	5	10	8	6	5	10	9	6	6
%Bias	5	3	6	4	2	3	2	1	0	0	4	1
C-DBS												
%RSD_r	11	7	4	3	7	5	3	3	7	4	3	2
%RSD_t	11	11	6	6	11	8	7	5	9	11	6	6
%Bias	0	3	6	1	0	4	3	-1	-2	-2	4	0

Table 5.3 Precision (repeatability (%RSD_r) and intermediate precision (RSD_t) and %bias for PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 in blood, V-DBS and C-DBS. *: LLOQ, L: low, M: medium, H: high.

The validity of the quantification of PEth 16:0/18:1 in blood was demonstrated by the successful participation (Z-score < 1.43) to a proficiency test. The Z-score obtained ((reported value – target value) / SD) was 0.11 for sample B (reported value = 2.52 µmol/L, target value = 2.50 µmol/L, SD = 0.23, N=8) and 1.43 for sample C (reported value = 0.17 µmol/L, target value = 0.16 µmol/L, SD = 0.01, N=8). The samples were used to create V-DBS and Z-scores of 0.38 for sample B (measured value = 2.59 µmol/L) and 0.22 for sample C (measured value = 0.16 µmol/L) were calculated ((measured value – target value) / SD). PEths were not detected in sample A (reported value < LLOQ). Sample A was whole blood from a teetotaller.

Similar mean responses (One-way ANOVA) were obtained from 20, 35, 50 µL V-DBS samples and from peripherally and centrally located punches (Figure 5.4).

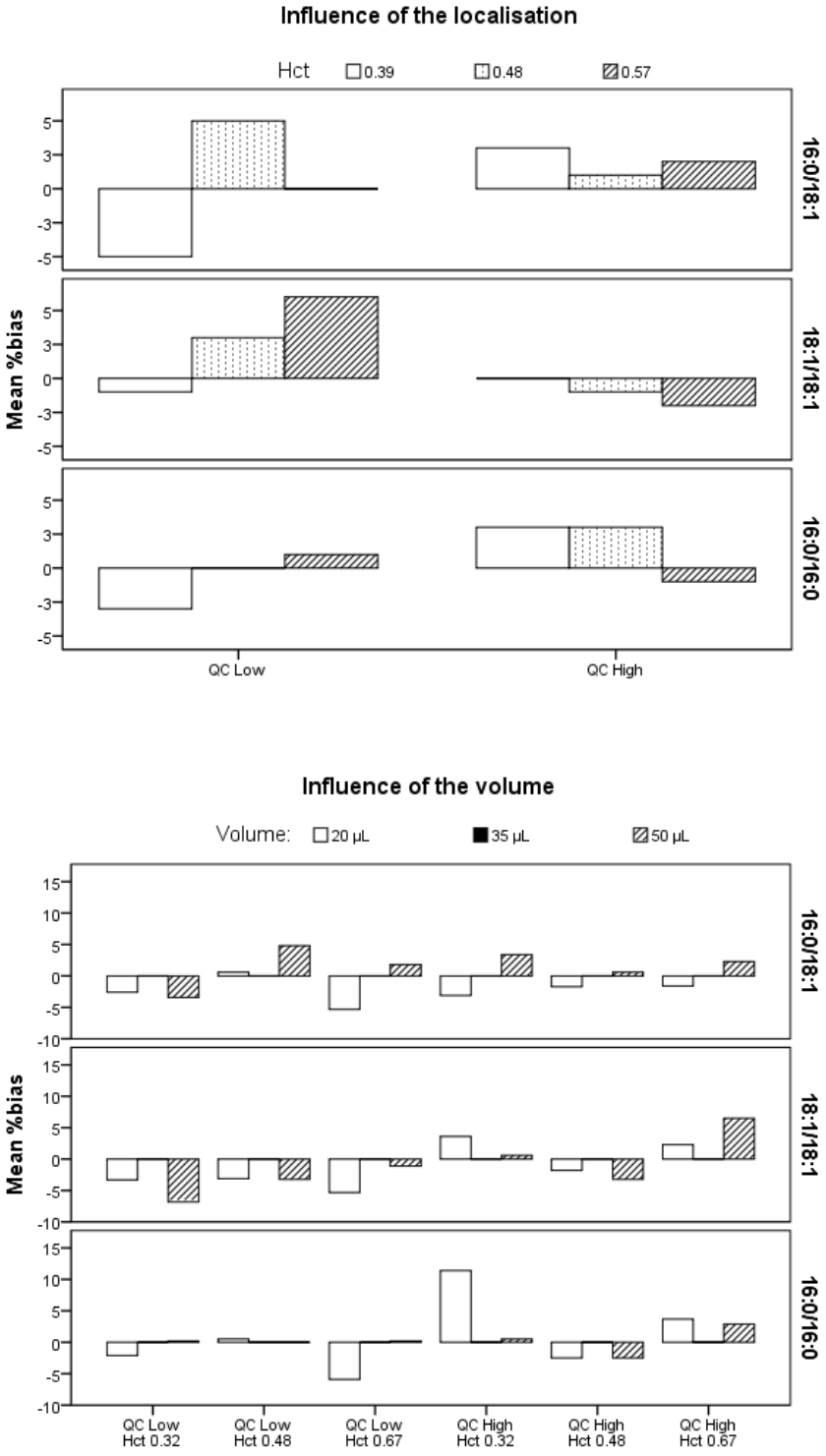

Figure 5.4 Influence of the localisation (upper) and of the volume (lower) on PEths responses measured in low and high QCs at three hematocrit (Hct) levels. Results are presented as a mean %bias compared with the reference value (localisation = centrally and volume = 35 µL).

As presented in Figure 5.5, no significant differences ($p>0.05$) were observed between the mean responses obtained for the analysis of V-DBS samples spiked with PEths reference standard, prepared from blood with hematocrit levels spanning a normal to high range (0.39, 0.42, 0.48, 0.50, 0.57). In addition, varying the hematocrit level (between 0.20 and 0.60) of real inpatient's blood samples (by adding blank plasma and red blood cells) did not adversely affect quantification, as demonstrated in Figure 5.6, which depicts the %bias when comparing results obtained from DBS with those obtained from blood.

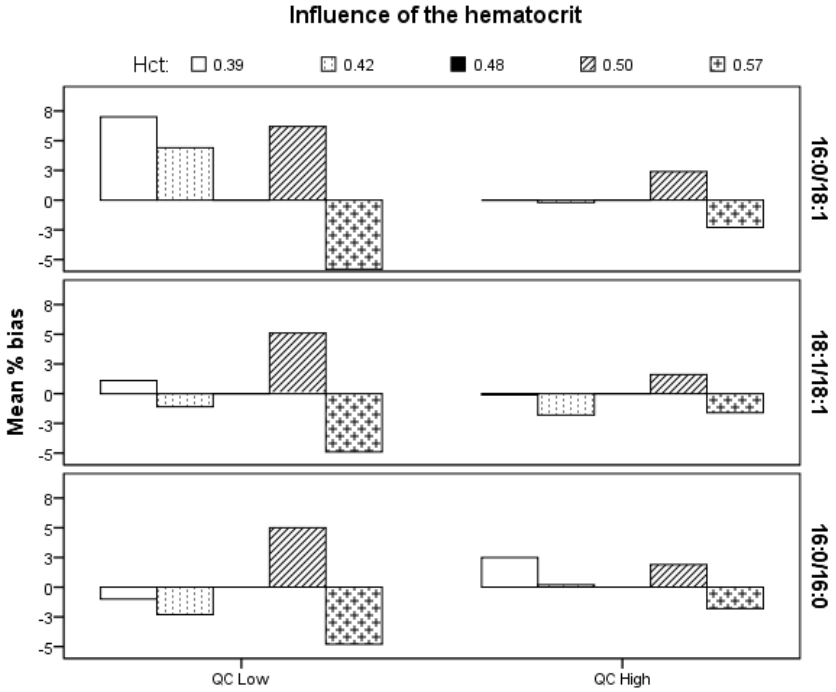

Figure 5.5 Influence of the hematocrit (Hct) on PEths responses measured in low and high QCs. Results are presented as mean %bias compared with the reference value (Hct = 0.48).

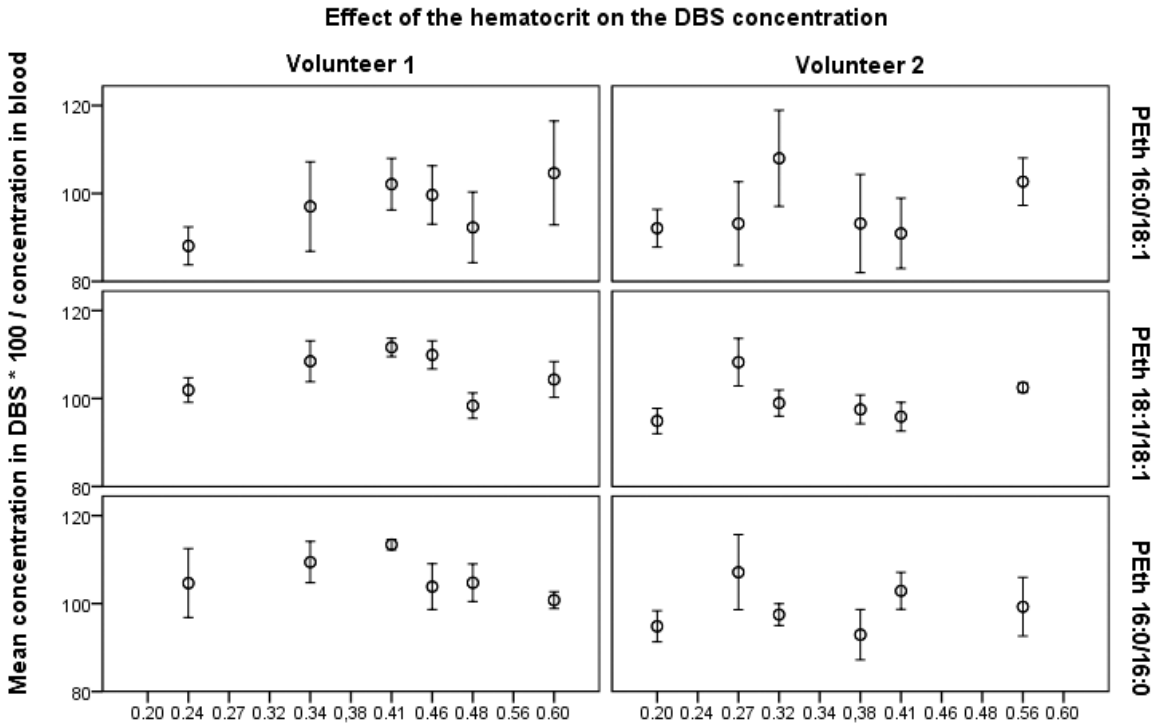

Figure 5.6 Influence of the hematocrit on PEths quantification in real positive samples (3 punches excised from DBS created from the blood of inpatients in alcohol withdrawal). Results, with standard deviation (%RSD), are presented for each hematocrit level as mean %bias (N=4) compared with the reference value measured in whole blood. The mean measured blood concentrations for PEth 16:0:18:1, PEth 18:1/18:1 and PEth 16:0/16:0 were 728, 52 and 89 ng/mL for inpatient 1 and 659, 46 and 100 ng/mL for inpatient 2, respectively.

All samples were stable in the autosampler for 72 h at 4°C. PEths were stable up to 6 months when stored at -80°C in EDTA tubes. PEth 16:0/18:1 and PEth 18:1/18:1 were stable during 6 months in V-DBS samples stored at room temperature in zip-closure plastic bags containing a desiccant packet. The 90 % confidence interval of the stability sample responses for PEth 16:0/16:0 in V-DBS were within ± 20 % of the control sample responses, although the mean response of the high stability samples was 119 % of the mean response of the control samples. Results are presented in Table 5.4.

Table 5.4 Stability results for PEths. The mean ► stability is expressed in %. The range corresponding to ± 20 % of the mean responses of the control samples and the 90 % confidence interval of the responses for stability samples are presented. L: low, M: medium, H: high.

	Blood		V-DBS	
QC	L	H	L	H
PEth 16:0/18:1				
Processed sample stability (72 hours, 4°C)				
Mean stability (%)	92	101	109	110
90 % CI of stability samples	34492-40052	1382828-1554029	38376-45647	1577485-1725941
± 20 % of control samples	32251-48377	1166722-1750083	30975-46462	1198474-1797711
Long term storage stability (6 months)				
Mean stability (%)	97	90	96	107
90 % CI of stability samples	25436-28940	903666-951676	48121-51264	1444751-16027
± 20 % of control samples	22365-33547	820487-1230731	41315-61973	1143333-1714999
PEth 18:1/18:1				
Processed sample stability (72 hours, 4°C)				
Mean stability (%)	108	110	101	106
90 % CI of stability samples	173374-181763	7666345-8489003	167975-187316	7720659-8480599
± 20 % of control samples	131102-196652	5863684-8795525	140435-210653	6141100-9211650
Long term storage stability (6 months)				
Mean stability (%)	98	92	98	93
90 % CI of stability samples	110842-128008	4344047-4819524	172200-181353	5856412-6368162
± 20 % of control samples	97928-146892	3999944-5999916	144788-217183	5269491-7904237
PEth 16:0/16:0				
Processed sample stability (72 hours, 4°C)				
Mean stability (%)	94	90	104	110
90 % CI of stability samples	254950-301409	7499912-8846416	325240-379137	9500878-10624858
± 20 % of control samples	236161-354241	7294071-10941106	271852-407778	7291702-10937552
Long term storage stability (6 months)				
Mean stability (%)	105	91	103	119
90 % CI of stability samples	369239-419978	6264007-7484137	442774-495796	14506830-12771817
± 20 % of control samples	300887-451331	6060575-9090863	366051-549076	9959212-14938818

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5.3.2 Comparative study

Whole blood, V-DBS and C-DBS from inpatients in alcohol withdrawal (N=50) and control volunteers (N=50) were analysed to quantify PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0. For the C-DBS method, the 3 punches analysed were excised either from the same spot (15 % of the cases), from two different spots (30 % of the cases) or from three different spots (55 % of the cases). Concentrations measured in whole blood, V-DBS and C-DBS from all study participants were compared using Bland-Altman plot, Passing-Bablok regression analysis and Wilcoxon signed rank test.

PEth 16:0/18:1 was quantified (> LLOQ) in 50/50 inpatients and in 18/50 control volunteers. Concentrations measured in blood ranged from 16 to more than 2000 ng/mL (mean = 1232, median = 1087) in alcoholics and were between 13 and 220 ng/mL (mean = 59, median = 49) in control volunteers with a quantifiable result. PEth 18:1/18:1, with blood concentrations ranging from 17 to 307 ng/mL (mean = 101, median = 78), was measured in 47/50 inpatients and in 1/50 control volunteers (17 ng/mL). PEth 16:0/16:0 was quantified only in some inpatient samples (34/50) with concentrations varying from 25 to 203 ng/mL (mean = 97, median = 89). An overview is given in Figure 5.7.

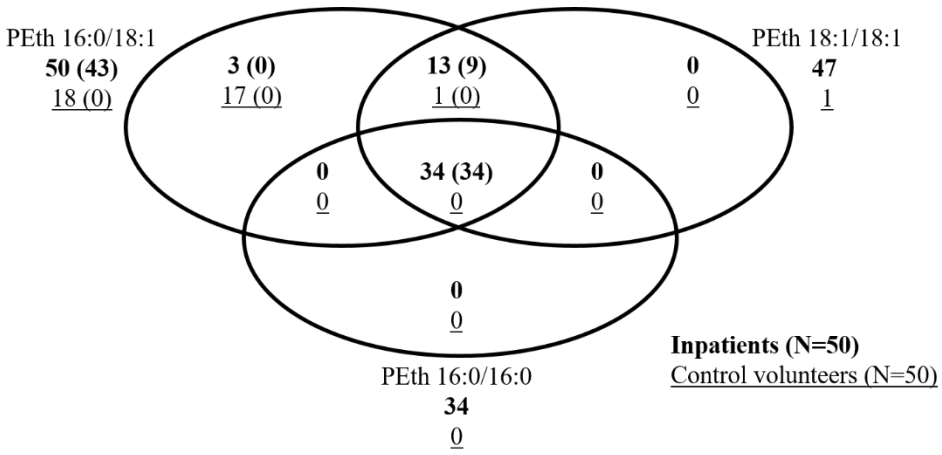

Figure 5.7 Number of blood samples with a measured concentration for PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 above the LLOQ (+ %U). The numbers between brackets indicate the number of samples with a PEth 16:0/18:1 concentration above the cut-off value (+ %U) that suggest an excessive and chronic alcohol consumption.

In the comparison of the results obtained from blood, C-DBS and V-DBS (Table 5.5), correlation coefficients exceeded 0.995 for PEth 16:0/18:1 (N=68), 0.978 for PEth 18:1/18:1 (N=48) and 0.962 for PEth 16:0/16:0 (N=32). As reported in Table 5.5 (presenting the numerical results) and shown in Figure 5.8 (right), the mean % differences in the concentration between whole blood and C-DBS included the 0 value for the three PEths. The 95 % confidence intervals of the slope obtained from the Passing-Bablok regression analysis included or were very close to 1 and the 95 % confidence intervals of the intercept included the 0 value (Figure 5.8 (left) and Table 5.5). No significant differences ($p \geq 0.05$) in the mean measured concentrations were detected using Wilcoxon signed rank test. The same comparisons were performed between blood and V-DBS and between V-DBS and C-DBS for the three compounds (Table 5.5 and Figures 5.9 and 5.10), with essentially the same conclusions. Only in 3 cases with measurable (i.e. above LLOQ) PEth 16:0/16:0 in whole blood, V-DBS and C-DBS, a discrepancy was observed, when taking into account the measurement uncertainty at the LLOQ. The blood, V-DBS and C-DBS concentrations in these 3 cases were respectively 32, 23* and 25 ng/mL (case 1), 22*, 31 and 21* ng/mL (case 2) and 27, 29 and 22* ng/mL (case 3). For 4 quantitative results (indicated with an asterisk) in these 3 cases, the results should actually be considered negative when the measurement uncertainty is taken into account (exemplified in Figure 5.11). These three cases were not taken into account for the statistical analysis.

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Compounds	Correlation	Passing-Bablok regression		Bland-Altman analysis		Wilcoxon
Methods	R (N)	Intercept [95 % CI]	Slope [95 % CI]	Limits of agreement	% Mean diff. [95 % CI]	p
PEth 16:0/18:1						
Blood C-DBS	0.996 (68)	-0.56 [-2.82/5.43]	1.00 [0.97/1.02]	-29.11/27.89	-0.61 [-4.13/2.91]	0.94
Blood V-DBS	0.995 (68)	1.96 [-3.32/6.90]	0.98 [0.96/0.99]	-24.39/24.82	0.22 [-2.82/3.26]	0.07
V-DBS C-DBS	0.996 (68)	-1.25 [-5.64/2.54]	1.02 [1.00/1.05]	-25.19/23.42	-0.89 [-3.89/2.12]	0.18
PEth 18:1/18:1						
Blood C-DBS	0.978 (48)	3.12 [-1.00/6.44]	0.94 [0.89/1.00]	-31.25/33.62	1.19 [-3.62/5.99]	0.19
Blood V-DBS	0.990 (48)	1.20 [-0.41/3.38]	0.95 [0.92/0.98]	-19.72/22.66	1.47 [-1.67/4.61]	0.05
V-DBS C-DBS	0.985 (48)	1.01 [-1.60/3.55]	0.97 [0.93/1.01]	-26.50/25.97	-0.26 [-4.15/3.62]	0.41
PEth 16:0/16:0						
Blood C-DBS	0.971 (32*)	-4.20 [-10.76/2.00]	1.11 [1.00/1.19]	-26.43/22.58	-1.92 [-6.43/2.58]	0.11
Blood V-DBS	0.976 (32*)	-4.68 [-11.14/3.36]	1.09 [1.01/1.18]	-26.90/21.26	-2.82 [-7.25/1.61]	0.05
V-DBS C-DBS	0.962 (32*)	-0.58 [-5.55/6.13]	0.97 [0.89/1.06]	-25.44/27.30	0.93 [-3.92/5.78]	0.41

Table 5.5 Results for the Passing-Bablok analysis, Bland-Altman analysis and Wilcoxon signed rank test performed on PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 to compare Blood *vs.* C-DBS, Blood *vs.* V-DBS and V-DBS *vs.* C-DBS. N: number of positive results (above LLOQ + %U), CI: confidence interval.
* 3 samples were in disagreement concerning the detection or no detection of PEth 16:0/16:0 when analysed in blood, V-DBS and C-DBS. These samples were not used in the statistical analysis.

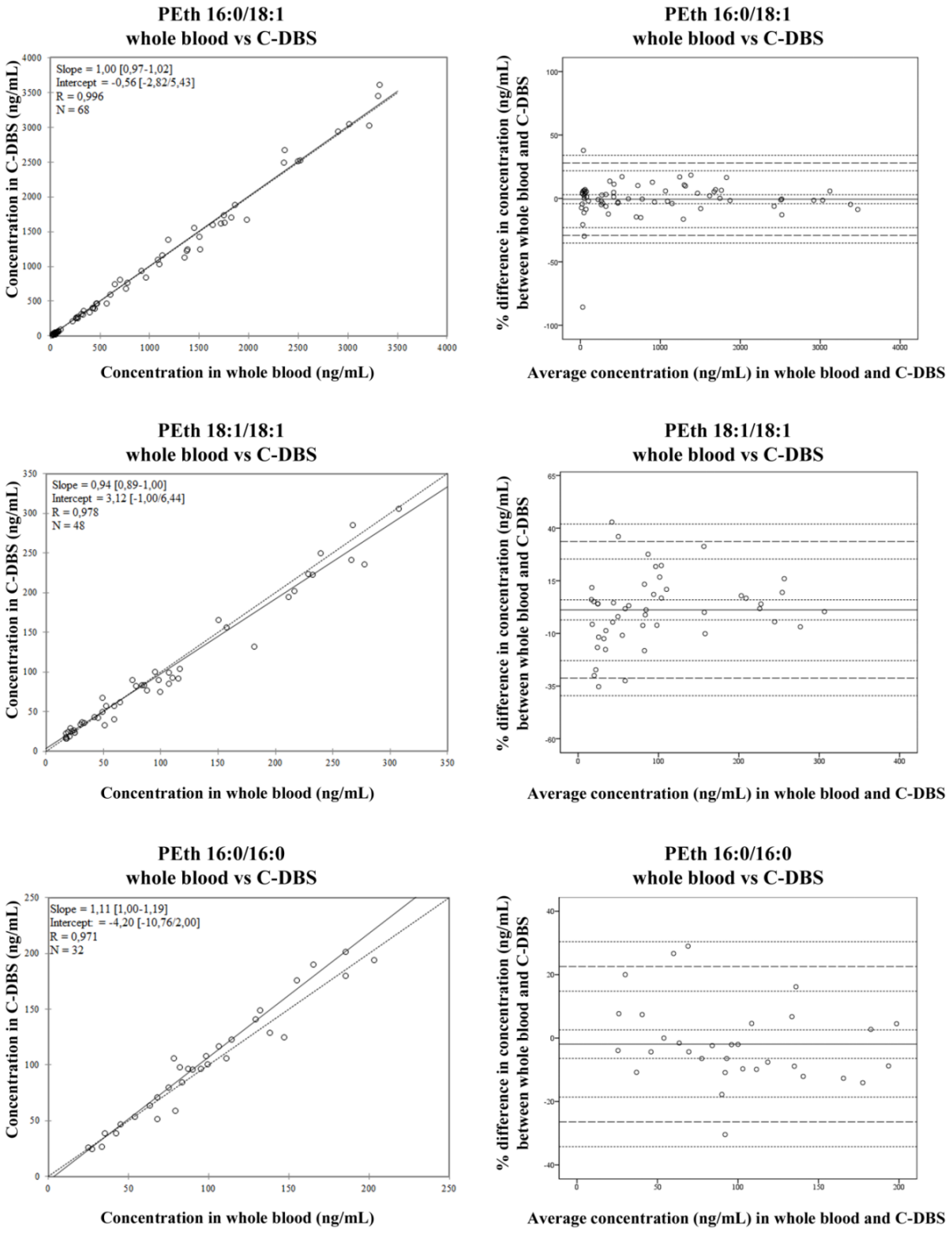

Figure 5.8 (Left) Passing-Bablok regression analyses of PEths concentrations measured in blood and in C-DBS. The identity line is indicated using a dotted line. (Right) Bland-Altman analyses of PEths plotting the % difference between blood and C-DBS concentrations. The average difference is represented by a solid line, the limits of agreement (1.96 SD) by dashed lines. The 95 % confidence intervals for the mean and the limits of agreement are shown with dotted lines.

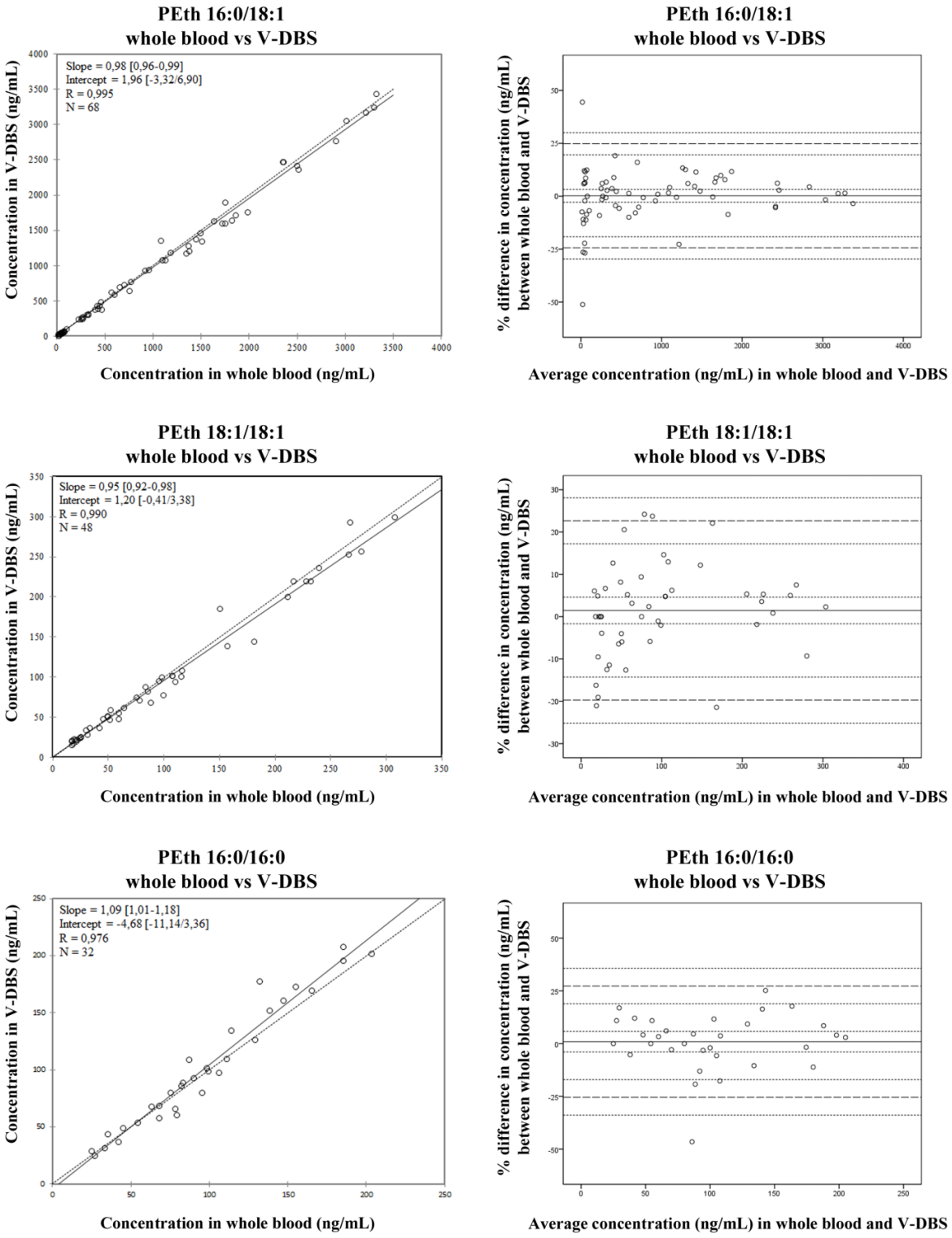

Figure 5.9 (Left) Passing-Bablok regression analyses of PEth species concentrations measured in blood and in V-DBS. The identity line is indicated using a dotted line. (Right) Bland-Altman analyses of PEth species plotting the % difference between blood and V-DBS concentration. The average difference is represented by a solid line, the limits of agreement (1.96 SD) by dashed lines and the 95 % confidence intervals for the mean and the limits of agreement by dotted lines.

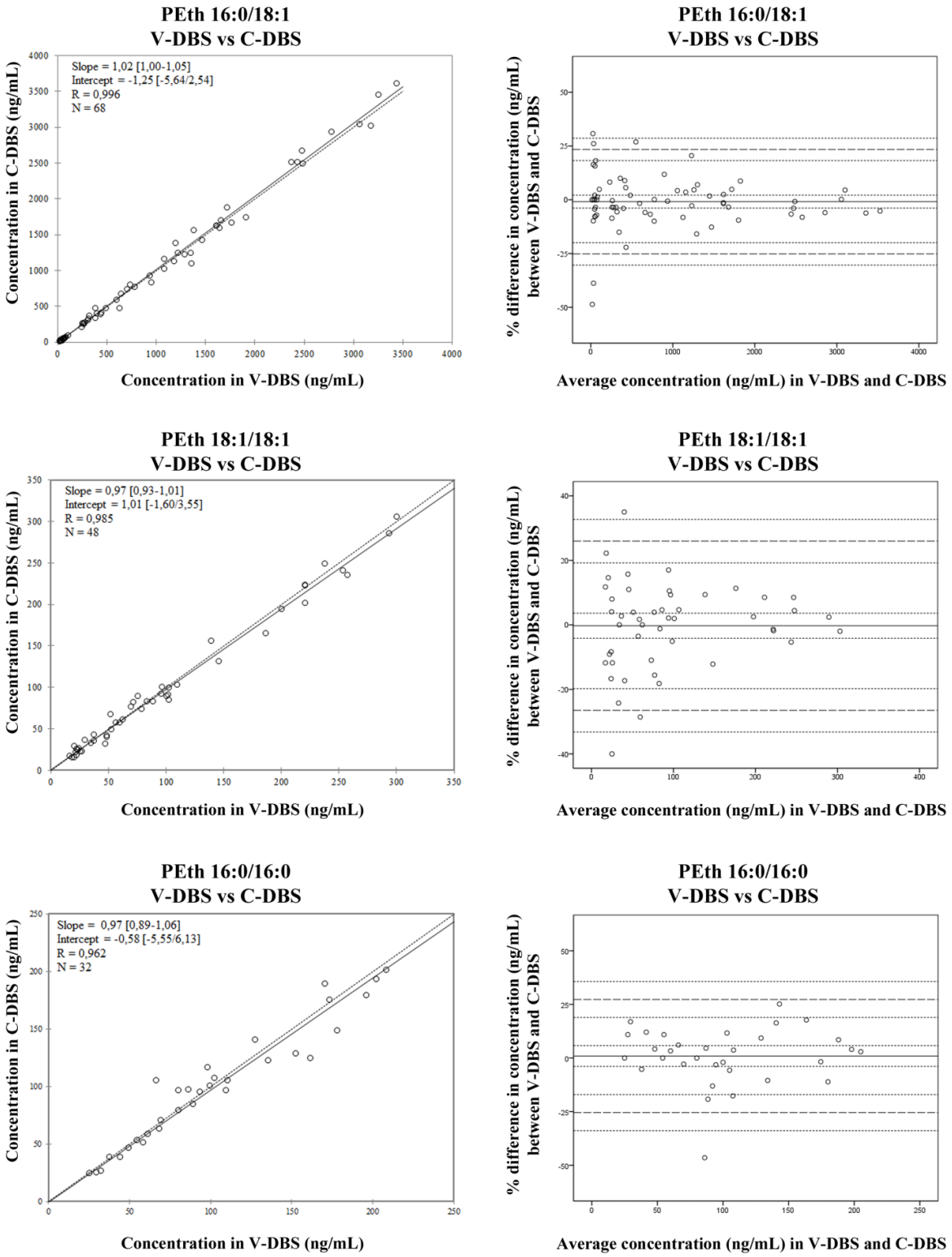

Figure 5.10 (Left) Passing-Bablok regression analyses of PEth species concentrations measured in V-DBS and in C-DBS. The identity line is indicated using a dotted line. (Right) Bland-Altman analyses of PEth species plotting the % difference between V-DBS and C-DBS concentration. The average difference is represented by a solid line, the limits of agreement (1.96 SD) by dashed lines and the 95 % confidence intervals for the mean and the limits of agreement by dotted lines.

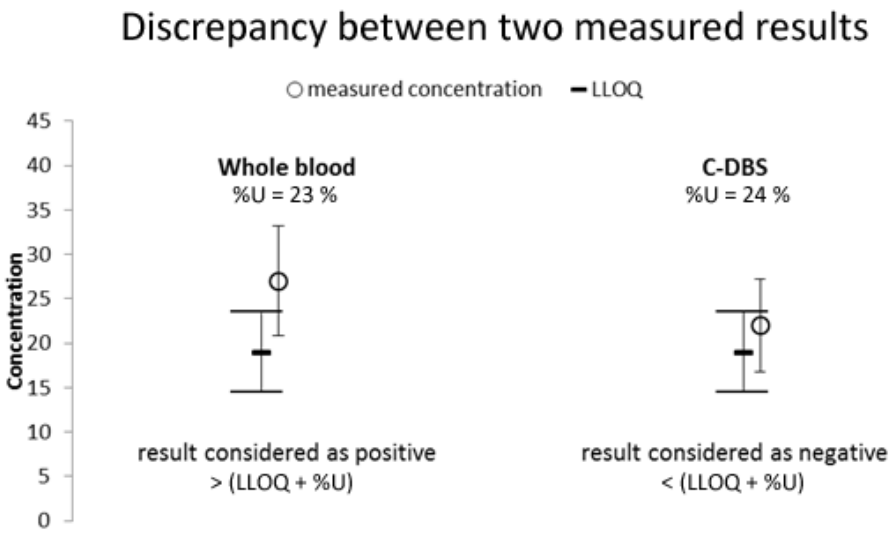

Figure 5.11 Concentrations measured (case 3) and LLOQ in whole blood and C-DBS presented with the measurement uncertainty (%U). Taking into account the measurement uncertainty for the LLOQ, the whole blood result is considered as positive while the C-DBS result is considered as negative.

Distributions of the concentrations of PEth 16:0/18:1 measured in whole blood, V-DBS and C-DBS from inpatients in alcohol withdrawal and control volunteers are presented in Figure 5.12.

ROC analysis was performed to determine a cut-off value to distinguish between control volunteers and inpatients in alcohol withdrawal using the concentration of PEth 16:0/18:1 in blood. A cut-off value at 221 ng/mL (AUROC = 0.947) for PEth 16:0/18:1 provided no false positive results (1-specificity = 0) and a sensitivity of 0.86 (7 out of 50 inpatients were classified as control volunteers). Among these 7 false negative results, 3 can be explained by a declared cessation of alcohol consumption between 16 and 21 days prior to the sampling. Among the 4 other false negative results (with a self-reported cessation of alcohol consumption between 1 and 2 days before the sampling), 2 have a PEth 16:0/18:1 concentration between 271 and 272 ng/mL, which is very close to the cut-off limit. The 2 last false negative results, with abnormally low concentration of PEth 16:0/18:1 (between 16 and 40 ng/mL), could be explained by a decreased transphosphatidylation rate of ethanol due to an altered phosphatidylcholine availability, a changed enzyme activity of PLDs or other, as yet unidentified, variables (see Chapter 7 for more details). Application of this cut-off for C-DBS and V-DBS yielded exactly the same result, lending further support to the validity of the approach of using DBS.

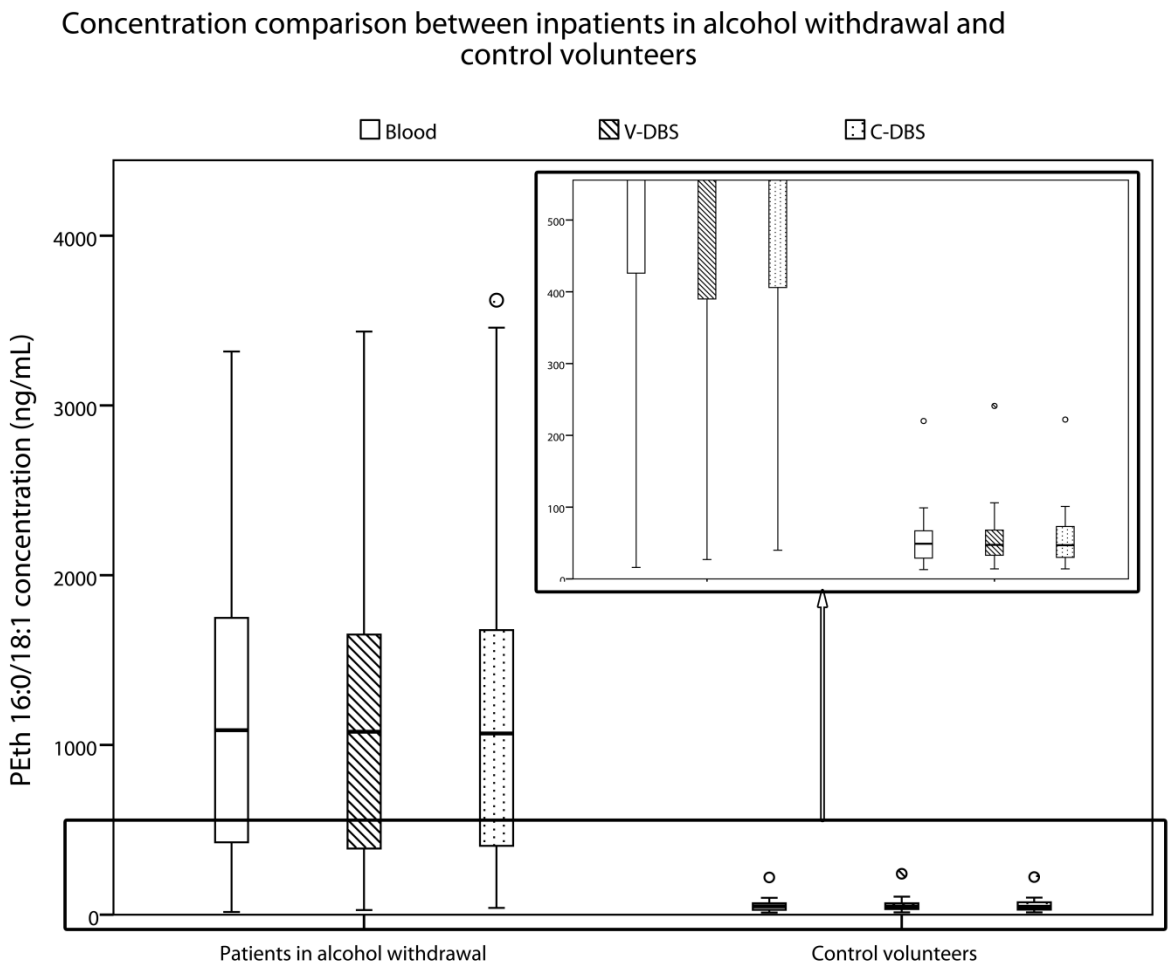

Figure 5.12 Distribution of the PEth 16:0/18:1 concentrations measured in blood, V-DBS and C-DBS of patients in alcohol withdrawal (N=50) and in control volunteers (N=18). The box represents the values between the lower and upper quartile and the middle line represents the median. The whiskers represent the extreme values, excluding outliers (represented by dots). The indicated area (below) is enlarged (right above).

5.4 Discussion

UHPLC-ESI-MS/MS methods for the quantification of PEths in whole blood, V-DBS and C-DBS have been developed and validated using international guidelines [29] and published recommendations [27]. PEth 16:0/18:1 and PEth 16:0/18:2 are the two predominant PEths detected in blood after alcohol consumption. Taking into account the commercial availability of the PEths standards at the moment, the methods presented in this chapter have been developed for PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0. Because PEth 18:1/18:1 and PEth 16:0/16:0 have also been detected in blood of heavy drinkers, these were included in this study, even though these are generally not analysed in other studies (especially PEth 16:0/16:0). As deuterated analogues were not commercially available at that time, four different ISs (PMeth 16:0/16:0, PMeth 18:1/18:1, PProp 16:0/16:0 and PProp 18:1/18:1) were evaluated during validation. In this study, PMeth 18:1/18:1 compensated best for the matrix effect for each compound and was therefore chosen as IS for all 3 methods.

The detection of PEths requires highly sensitive techniques, due to the low amount of a certain PEth present in the sample (e.g. 16:0/16:0) and/or due to a low amount of sample (e.g. C-DBS). Therefore, special attention was paid to decrease possible ion-suppression by optimising both the extraction and the chromatographic separation.

Variable extraction efficiencies -ranging from 33 % (PEth 16:0/16:0 and PEth 16:0/18:1) [20] to 80 % (PEth 16:0/16:0, PEth 18:1/16:0, PEth 18:1/18) [7]- have been reported in past publications using a LLE with a mixture of isopropanol and *n*-hexane (2:3, v/v). In this chapter, the LLE procedure to extract PEths from venous blood has been optimised. Therefore, the pH during extraction was adjusted to 2 by adding 2 % formic acid in a mixture of 10 mM ammonium acetate buffer and isopropanol before extraction with *n*-hexane. This resulted in a mean extraction efficiency from venous blood of 83 % (%RSD = 13 %) for PEth 16:0/18:1, 87 % (%RSD = 13 %) for PEth 18:1/18:1 and 82 % (%RSD = 20 %) for PEth 16:0/16:0. Somewhat lower percentages were observed (between 55 and 78 %) for the V-DBS and the C-DBS methods. Similar percentages, ranging from 68 to 91 % [15] and 56 to 76 % for PEth 16:0/18:1 [18] and from 27 and 43 % for PEth 18:1/18:1) [15], were reported earlier for other DBS-based methods. The basis for this somewhat lower extraction efficiency is not known. Interaction with the filter paper might be a possibility, as recently suggested by Koster *et al.* for immunosuppressants [34].

Reversed-phase LC separation is the method of choice for the identification and quantification of phosphatidylethanol species. The retention is based on the lipophilicity, determined by the length and number of double bonds present in the fatty acid side chains [35]. Because the non-polar part of PEths tends to interact very strongly with the non-polar hydrocarbon phase of a reversed-phase column, the use of a more polar phase (i.e. C8 [14,18,20], C4 [7,15] or phenyl [16]) instead of a C18 phase allows to decrease the retention of PEths [14]. The use of less polar solvents, such as tetrahydrofuran (index polarity = 4.0), isopropanol (index polarity = 3.9) or methanol (index polarity = 5.1) instead of acetonitrile (index polarity = 5.8) also improved the elution of PEths using a reversed-phase column. In our methods, gradient elution based on an ammonium acetate buffer and a mixture of 10 % tetrahydrofuran in isopropanol on a 50-mm C8 column was chosen.

The three methods (blood, V-DBS and C-DBS) developed in this chapter, have a LLOQ of 10 ng/mL for PEth 16:0/18:1 and PEth 18:1/18:1 and of 19 ng/mL for PEth 16:0/16:0. In literature, LC-MS/MS methods for PEths quantification in blood have reported LLOQs between 8 to 83 ng/mL for PEth 16:0/18:1, between 0.7 to 73 ng/mL for PEth 18:1/18:1 and between 0.7 to 68 ng/mL for PEth 16:0/16:0. While it seems at first sight that our method is less sensitive, the most sensitive method published [6] required 300 µL of blood while our methods use only 30 µL of sample. Using a low sample volume, such as 30 µL, was necessary for development of the C-DBS method. Published methods about the validation of PEths in DBS have reported LLOQs of 8 ng/mL (3x3 mm punches from a 30 µL DBS) [18] and 87 ng/mL (100 µL DBS) [15] for PEth 16:0/18:1 and of 23 ng/mL (100 µL DBS) for PEth 18:1/18:1 [15]. The DBS methods presented here provide comparable or lower LLOQs for PEth 16:0/18:1 and 18:1/18:1, and have included PEth 16:0/16:0. Furthermore, to our knowledge, no publication has already evaluated the influence of hematocrit, punch localisation and spot volume on the quantification of PEths in DBS. One-way ANOVA tests did not reveal a significant influence ($p>0.05$) of these parameters on quantification of the evaluated PEths (Figure 5.4 and Figure 5.5). In addition, no influence of the hematocrit on matrix effect and extraction efficiency (Figure 5.3) was observed and quantification was not affected when comparing DBS and blood concentrations in real samples with a wide hematocrit range (Figure 5.6). Hematocrit levels generally vary between 0.20 and 0.50 (except in patients from neonatal care in which hematocrit levels may exceed 0.50) [36]. However, experiments were

performed on a wide range of values to detect possible hematocrit effect in persons with extreme hematocrit values.

An important advantage of DBS compared with venous blood is the improvement of analyte stability, avoiding the degradation of PEths in venous blood not stored at -80°C [24] and the post-collection synthesis of PEths in samples exposed to ethanol [18]. Helander *et al*. have demonstrated that PEths were stable in venous blood, if stored at −80°C, and this up to 14 months [7]. A decrease of the concentration of PEth 18:1/18:1 (18 %) and PEth 16:0/18:1 (25 %) has been described for EDTA whole blood samples stored at -20°C for 30 days [24]. Stability of PEth in DBS (at -20°C and 20°C) has been assessed up to 30 days by Faller *et al.* [24]. Our results confirm the stability of PEths in blood stored at -80°C and, more importantly, demonstrate that PEths were stable in DBS samples stored in zip-closure plastic bags containing a desiccant packet at room temperature for up to 6 months, although a slight %bias (119 %) was observed for PEth16:0/16:0 in the QC high.

Finally, the successful participation (Z-scores < 1.43) to an international proficiency test organised by Equalis (Uppsala, Sweden) proved that the venous blood method for the quantification of PEth 16:0/18:1 is accurate.

Hundred authentic samples (50 inpatients in alcohol withdrawal and 50 control volunteers) were analysed using the 3 developed methods. To ensure C-DBS method validity, the hematocrit level of inpatients in withdrawal therapy (N=48) was measured and ranged between 0.33 and 0.49 (mean = 0.43, median = 0.44), with 83 % (40/48) of the inpatient hematocrit levels lying within the reference range [28,37] (0.41-0.50 for men and 0.36-0.44 for women).

Comparisons of the PEths concentrations measured using the three assays (Table 5.5) have shown limits of agreement of less than 33.62 %, with no significant differences using Wilcoxon signed rank test analyses ($p \geq 0.05$) and Bland-Altman analyses (mean differences < 2.82 %, with the zero value included in the 95 % CI). Passing-Bablok regressions indicated a good overall correlation ($R>0.962$), no systematic differences (95 % CI of the intercept values include the zero value) and no proportional differences, although 1 was just not included in the 95 % CI of the slope in 3 out of 9 comparisons. In literature, agreement between whole blood and V-DBS concentrations has been assessed using Bland-Altman analysis for PEth 18:1/18:1 and PEth 16:0/18:1 [15,18]. One study showed good agreement, with a mean

difference of 95.8 ng/mL (%RSD = 3.0 %) and -4.3 ng/mL (%RSD = 2.9 %) for PEth 16:0/18:1 and PEth 18:1/18:1, respectively [15]. Another study, despite a limit of agreement of more than 50 %, reported no significant bias (mean -4.5 %; %RSD = 33.8 %) for PEth 16:0/18:1 and a good correlation (R=0.94) when comparing 281 results obtained from the analysis of venous blood and of 3 punches excised from V-DBS [18]. Both studies concluded that PEth 16:0/18:1 and 18:1/18:1 in V-DBS were a useful tool to monitor alcohol misuse. Our population study not only confirms these conclusions, but also extends these to PEth 16:0/16:0, and, importantly, demonstrates the agreement between blood and C-DBS. The latter is the most relevant comparison, as in real practice C-DBS will be collected from a fingertip. Thus, the results presented here strongly suggest that C-DBS analysis is a valid alternative to venous blood analysis for the quantification of PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0. In addition, we studied the distribution of PEths within the two groups (inpatients in alcohol withdrawal and control volunteers). In 50, 47 and 34 out of the 50 inpatients in alcohol withdrawal, PEth 16:0/18:1 (from 16 to more than 2000 ng/mL), PEth 18:1/18:1 (17-307 ng/mL) and PEth 16:0/16:0 (25-203 ng/mL), respectively, were quantified. PEth 16:0/18:1 was quantified in 18 out of the 50 control volunteers (13-220 ng/mL), while PEth 18:1/18:1 (17 ng/mL) was quantifiable in only one. PEth 16:0/16:0 was not present above LLOQ in control volunteers. These results suggest that, using the methods presented in this publication, only PEth 16:0/18:1 could be used to distinguish inpatients in alcohol withdrawal from control volunteers. More sensitive methods are required to search for a cut-off value for PEth 18:1/18:1 and PEth 16:0/16:0.

In literature, HPLC-ELSD methods analysing total PEths in blood generally used cut-off values between 0.2 and 1 µmol/L [7,10,11,17,19] to detect alcohol consumption. In Sweden, 0.7 µmol/L of total PEths is used as the clinical threshold [7]. These values were fixed by the LLOQ of the methods used and are limited to the detection of relatively high alcohol consumption (i.e. more than 50 g ethanol per day at an LLOQ of 0.7 µmol/L total PEths [17]). For PEth 16:0/18:1, an upper reference value for blood donors (N=200) of 141 ng/mL (0.2 µmol/L) has been proposed, which provided 5 % false positive results and 17 samples detected as outliers [8]. In addition, cut-off values at 210, 700 and 800 ng/mL, for PEth 16:0/18:1 have been proposed in literature [38–40]. In our case, we have calculated a cut-off value of 221 ng/mL in blood to detect chronic and excessive alcohol consumption (inpatients on alcohol withdrawal), based on the highest sensitivity (86 %) which was associated with

the absence of false positive results (specificity = 100 %). It is of interest to add that 3 out of the 7 inpatients with PEth 16:0/18:1 concentrations lower than the chosen cut-off value declared to have ceased their alcohol consumption 2-3 weeks before the sampling. Importantly, and lending further support to the validity of using C-DBS, is that application of the blood cut-off to the C-DBS and V-DBS data yielded the same sensitivity and specificity.

5.5 Conclusion

This chapter describes the validation of three UHPLC-ESI-MS/MS methods for the quantification of PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 in 30 µL venous blood, 30 µL V-DBS and 3 punches (3 mm) from C-DBS. The calibration curves ranged from 10 (LLOQ) to 2000 ng/mL for PEth 16:0/18:1, from 10 (LLOQ) to 1940 ng/mL for PEth 18:1/18:1 and from 19 (LLOQ) to 3872 ng/mL for PEth 16:0/16:0. Our results have confirmed the stability of PEths in blood stored at -80°C and have demonstrated that PEth 16:0/18:1 and PEth 18:1/18:1 were stable in V-DBS at room temperature for up to 6 months. The quantification of PEths via the C-DBS method was not significantly influenced by the hematocrit, the punch localisation or the spot volume. Statistical comparisons (Bland-Altman plot, Passing-Bablok regression analysis and Wilcoxon signed rank test) of the measured concentrations obtained from venous blood, V-DBS and C-DBS from 100 volunteers (alcoholic inpatients and control volunteers) showed good agreement. Furthermore, application of a cut-off value of 221 ng/mL for PEth 16:0/18:1 to distinguish between inpatients in alcohol withdrawal and control volunteers provided a sensitivity of 86 % and no false positive results (specificity = 100 %). To conclude, the developed method for C-DBS can be of interest to detect high and chronic alcohol consumption, as it offers distinct advantages such as a less invasive blood sample collection, stability during storage and transportation and a relatively simple sample preparation before analysis.

5.6 References

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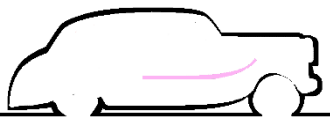
Chapter 6

Hair EtG, urine EtG/EtS and C-DBS PEths in driver's licence regranting cases



Based on

N. Kummer, S.M.R. Wille, A. Poll, W.E.E. Lambert, N. Samyn, C.P. Stove, Quantification of EtG in hair, EtG and EtS in urine and PEth species in capillary dried blood spots to assess the alcohol consumption in driver's licence regranting cases, Drug and Alcohol Dependence. 165 (2016) 191-197.

Abstract

In Belgium, the analysis of indirect biomarkers such as carbohydrate deficient transferrin (CDT%), gamma-glutamyltransferase (GGT), aspartate aminotransferase/alanine aminotransferase (AST/ALT) and mean corpuscular volume (MCV), is currently used to monitor the alcohol consumption in cases of fitness to drive assessment. This chapter aims to evaluate the use of direct ethanol markers for this purpose, exclusively determined in matrices obtained via non- or minimally invasive sampling.

The three quantitative methods developed and validated within the framework of this thesis, i.e. ethylglucuronide and ethylsulfate (EtS) in urine (Chapter 3), EtG in hair (Chapter 4) and phosphatidylethanol species (PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0) in capillary dried blood spots (C-DBS)) (Chapter 5) were used. Fifty volunteers, for whom fitness to drive had to be assessed and for whom a blood analysis for indirect biomarkers was requested, were included in the study. The sampling and analysis of hair, urine and C-DBS were added to the process currently used.

Hair EtG (24/50) and C-DBS PEths (29/50) are more sensitive than the currently used indirect biomarkers (13/50 for CDT%) to detect excessive and chronic alcohol consumption and allow to disprove an abstinence period. Urinary EtG and EtS are useful parameters to determine recent alcohol consumption.

The combined use of the three strategies allows better inference about the evolution of the alcohol consumption prior to the sampling. An inference scheme to integrate the results of hair EtG, C-DBS PEths and urine EtG/EtS into the fitness to drive decision process - complementing the psychological assessment- is proposed Figure 6.4.

Moreover, the exclusive use of non- or minimally invasive sampling (hair, urine and C-DBS) allows this to be performed directly during the fitness to drive assessment by regular staff members. This approach offers the potential to improve the Belgian driver's licence regranting process.

6.1 Introduction

As introduced in Chapter 1 (Section 1.4), the Belgian legislation for driving under the influence (DUI) of alcohol (Art. 35-37/1) states that drivers with a measured ethanol concentration above the limit of 0.5 gram ethanol per liter of blood are condemnable to a fine and in some cases to a confiscation of the driver's licence [1]. The assessment of the fitness to drive can be requested by the judge in case of drunk driving or recidivism. The annex 14 of the driver's licence regulation [2], which defines the medical/psychological norms to assess the fitness to drive, states that "all the resources offered by medicine can be used" and adds that the medical doctor can make this decision dependent on a blood analysis for DUI of alcohol offences and on a hair analysis in case of DUI of drug offences. In Belgium, if the medical assessment by the physician deciding about the fitness to drive includes a blood analysis, the sampling is not performed directly by himself. The volunteer is asked to visit a sampling centre or his family doctor to perform the venepuncture. Blood samples are then sent to an authorised laboratory for analysis. Hence, the current process implies an invasive sampling and there may be a long time period between the blood analysis request and the final decision. In addition, the chain of custody is not ensured during the whole process.

The medical norm of the annex 6 of the driver's licence regulation [3] and the Directive 2006/126/EC of the European Parliament and of the Council of December 20, 2006 on driving licences [4] declare that alcohol dependent persons or persons who cannot stay abstinent while driving are not fit to drive. If an alcohol dependence is detected during the fitness to drive assessment, the person will be declared unfit to drive. The Belgian driver's licence regranting legislation requires a 6-month period of proven abstinence after an unfit to drive decision [3]. Administrative documents (i.e. attestation of alcohol withdrawal, letter from a psychologist) are currently used to monitor the abstinence period.

In Belgium, as in many European countries, analyses of indirect biomarkers such as carbohydrate deficient transferrin (CDT%), gamma-glutamyltransferase (GGT), aspartate aminotransferase/alanine aminotransferase (AST/ALT) and mean corpuscular volume (MCV), are the current analytical methods used by physicians to monitor (cessation of) alcohol abuse in case of a driver's licence regranting process [5–10]. These markers reflect the indirect effects of ethanol on the body, via its interference with glycosylation present in the

body (increased CDT%), with liver function (increased GGT, ALT, AST) and via its effect on the size of red blood cells (increased MCV). Because of a lack of sensitivity and specificity [11], these analyses are unable to detect all cases of chronic and excessive alcohol consumption and are not adapted to evaluate strict alcohol abstinence periods either. To overcome these problems, the quantification of ethanol metabolites (direct biomarkers), such as ethylglucuronide (EtG), ethylsulfate (EtS) and phosphatidylethanol species (PEths) has been advocated [12,13]. Urinary EtG and EtS have been used to detect alcohol consumption up to 5 days after intake [14], allowing a longer detection window than for ethanol itself and providing a tool to evaluate short term abstinence [15–17]. PEths in blood and dried blood spots (DBSs) have been used to detect chronic and excessive alcohol consumption and allow monitoring ethanol (ab)use during the month prior to the sampling [18]. The quantification of EtG in hair has proven to be an efficient method to monitor long term abstinence and to detect alcohol misuse, as outlined in a recent review by Crunelle *et al.* [19]. In several countries (e.g. Italy, Germany, Switzerland and Sweden) the quantitative determination of ethanol metabolites, such as ethylglucuronide (EtG) and ethylsulfate (EtS) in urine and/or hair is used to monitor an alcohol abstinence period [7,8,20–23]. Recently, Schröck *et al.* recommended to include the quantification of PEth 16:0/18:1 in whole blood as a routine analysis for the detection of prolonged excessive alcohol consumption (currently based on a BAC above 1.6 ‰) in 'driving under the influence' cases [24]. In the United States of America, where alcohol ignition interlock devices are used to prevent recidivism, the ability of direct biomarkers (blood PEths, hair EtG and FAEE and urine EtG and EtS) and indirect biomarkers (CDT%, ALT, AST, GGT) to predict recidivism has been tested [25].

Previously, our research group has set up validated approaches to quantify EtG and EtS in urine (Chapter 3), EtG in hair (Chapter 4) and PEths in capillary dried blood spots (C-DBS) (Chapter 5) [26–28]. In this Chapter, we applied these strategies on samples obtained from fifty volunteers, for whom fitness to drive had to be assessed and for whom a blood analysis was requested by the physician. Although the currently used process, based on psychological and medical assessments, remained the basis to decide on the actual fitness to drive, we also evaluated in this context the potential added-value of our three-tiered approach to monitor the abstinence period and/or chronic and excessive alcohol consumption. Importantly, the proposed strategy offers the advantage that it can be performed during the

psychological/medical assessment by a non-physician (and potentially even by non-medical, minimally trained staff), since it involves only non- or minimally invasive sampling.

To our knowledge, we are the first to test the combination of these three methods for both the detection of excessive and chronic alcohol consumption and the monitoring of the abstinence period in case of fitness to drive decisions. In addition, analysis of 50 cases revealed practical information concerning interpretation of the results obtained from the three tested methods. The approach of assessing different analytes in distinct matrices, with each of these matrices covering another time window, should reduce both the false positive and false negative rate of the actual fitness to drive evaluation procedure. Hence, this three-tiered strategy should allow for a better assessment of the fitness to drive.

6.2 Materials and Methods

6.2.1 Population study

This study was conducted between May 2014 and September 2015. Fifty volunteers, for whom fitness to drive had to be assessed, were recruited by medical physicians from the Belgian Road Safety Institute (IBSR/BIVV). Subjects compelled to undergo a blood analysis were asked to participate. The study was approved by the Ethics Committee of Ghent University Hospital (B670201215604) and informed consent was obtained from each subject before enrolment in the study.

All volunteers were asked to provide venous blood (one serum tube and one EDTA-anticoagulated tube), urine, hair and a C-DBS sample. The serum was separated by centrifugation (10 min, 3500 rpm, 4°C) and stored at -20°C for maximum one week until analysis. The EDTA-anticoagulated blood was brought the same day to the Military Hospital in Brussels for determination of the MCV. Urine was stored at -20°C for maximum one week before analysis. Hair samples were stored in aluminium foil until analysis. Five C-DBS were collected onto a Whatman 903 filter paper card (GE Healthcare) after a fingertip prick performed with a contact-activated lancet (BD Microtainer®, Becton Dickinson). C-DBS were left to dry for minimum 2 hours at room temperature and were then stored in a zip-closure plastic bag containing a desiccant packet (Sigma-Aldrich) at room temperature until analysis.

EtG and EtS in urine, EtG in hair and the PEth species (PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0) in C-DBS were analysed at the NICC (National Institute of Criminalistics and Criminology).

The Alcohol Use Disorders Identification Test (AUDIT) developed by the World Health Organization (WHO) was used to detect persons with hazardous and harmful alcohol use and possible alcohol dependence [29]. The total score of the AUDIT test, along with other information about the volunteers, such as gender, age, self-reported liver problems, self-reported hair treatment, self-reported alcohol consumption, the final fitness to drive decision (only based on the blood analysis of indirect biomarkers and the psychological assessment) and the indication of a requested abstinence period were collected by IBSR/BIVV members for each volunteer.

6.2.2 Traditional biomarkers (CDT%, GGT, ALT/AST) in serum

CDT%, GGT, ALT and AST were analysed in serum at the central laboratory of Ghent University Hospital. CDT% (percentage of asialo-transferrin and disialo-transferrin of the total transferrin isoforms) was measured by capillary zone electrophoresis using a Capillarys 2™ system (Sebia, France) [30]. GGT was measured using a kinetic spectrophotometric assay (405 nm) with carboxynitroanilide as a substrate [31]. ALT and AST were measured using a kinetic ultraviolet spectrophotometer [32,33]. Values (females/males) above 31/37 U/L for AST, 31/40 U/L for ALT, 36/61 U/L for GGT were classified as above the reference range [34]. For CDT%, the cut-off value proposed by Maenhout *et al.* [9], which includes the measurement uncertainty, was used to suggest excessive and chronic alcohol consumption (concentrations ≥ 2.4%).

6.2.3 MCV in EDTA whole blood

MCV was measured in EDTA whole blood using a Sysmex XP-300™ automated hematology analyser (Sysmex Belgium, Belgium). MCV values above 96.4 fL were considered as being above the reference range [34].

6.2.4 EtG in hair

Analysis of EtG in hair samples was based upon a previously published fully validated method [27], using EtG-d_5 as internal standard (see Chapter 4). Briefly, hair samples were washed (dichloromethane and methanol) and dried overnight at room temperature. The 0-6 cm proximal scalp hair segment (50 mg) was selected and pulverised as recommended by the Society of Hair Testing (SoHT), which states that a pulverised 0-3 up to 0-6 cm hair segment should be analysed [35]. EtG was extracted with 1.5 mL of water (2 hours of sonication (40°C)). After a solid-phase extraction (BondElut SAX cartridge), EtG was

quantified by UHPLC-ESI-MS/MS after separation on an Acquity UPLC® HSS T3 (2.1 x 100 mm, 1.8 µm) column (Waters). The use of a Xevo TQ S mass spectrometer (Waters, Manchester, UK), as outlined in Chapter 4 (4.2.4 and 4.3.2), allowed an analytical range from 2 (LLOQ) to 500 pg/mg hair. The measurement uncertainty ($2.12*\%RSD_t$) at the LLOQ (U = 25 %) was used to interpret quantitative results close to the LLOQ and cut-off values. The two cut-off values proposed by the SoHT [35], at 7 and at 30 pg/mg hair, respectively, were used to interpret the results. Concentrations above or equal to 9 pg/mg hair (7 + 25 %) were used to strongly suggest repeated alcohol consumption and disprove a strict abstinence period, while concentrations ≥ 38 pg/mg hair (30 + 25 %) were used to strongly suggest chronic excessive alcohol consumption (consumption of ≥ 60 g ethanol/day over several months).

6.2.5 EtG and EtS in urine

The quantification of EtG and EtS in urine was performed after protein precipitation followed by UHPLC-ESI-MS/MS according to a fully validated method previously published [26], using EtG-d_5 and EtS-d_5 as internal standards (see Chapter 3). Samples were analysed either using an Acquity UPLC® coupled to a Xevo TQ MS tandem mass spectrometer (used in the published method) or coupled to a Xevo TQ S tandem mass spectrometer (Waters, Manchester, UK), as outlined in Chapter 3. Briefly, methanol (250 µL) was added to 50 µL of urine. The sample was centrifuged (14000 rpm, 10 min at 4°C) and 250 µL of the supernatant was transferred to a total recovery glass vial (Waters, Zellik, Belgium), evaporated to dryness and reconstituted in 300 µL of 0.1 % formic acid in water. Gradient elution was performed on an Acquity UPLC® CSH C_{18} (2.1 x 100 mm, 1.8 µm) column (Waters, Milford, MA, USA). The analytical range was from 100 (LLOQ) to 10000 ng/mL. EtG_{100} and EtS_{100} concentrations were calculated by normalising the measured EtG and EtS to a creatinine concentration of 100 mg/dL. The measurement uncertainties ($2.12*\%RSD_t$) at the LLOQ (%U = 21 % for EtG and %U = 8 % for EtS) were used to interpret quantitative results close to the LLOQ. Concentrations above or equal to 121 ng/mL for EtG_{100} and 108 ng/mL for EtS_{100} (LLOQ + %U) were used to suggest alcohol intake the days prior to the sampling and to disprove strict abstinence.

6.2.6 PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 in C-DBS

Taking into account the commercial availability of the PEth standards at the time of the development of the method, three PEth species (PEth 16:0/18:1, PEth 18:1/18:1 and PEth

16:0/16:0) were quantified using PMeth 18:1/18:1 as an internal standard in C-DBS using a fully validated method, as described in Chapter 5 [28]. Briefly, three punches (3 mm) were excised from C-DBSs and blood was extracted from the paper using 250 µL of a mixture containing 10 mM ammonium acetate buffer with 0.2 % formic acid and isopropanol. PEths were extracted by liquid-liquid extraction (LLE) with *n*-hexane (1 mL). Analyses were performed on an Acquity UPLC® coupled to a Xevo TQ S tandem mass spectrometer (Waters, Manchester, UK) using an Acquity UPLC®BEH C8 (2.1 x 50 mm, 1.7µm) column (Waters, Milford, MA, USA). The analytical ranges were from 10 (LLOQ) to 2000 ng/mL for PEth 16:0/18:1, from 10 (LLOQ) to 1940 ng/mL for PEth 18:1/18:1 and from 19 (LLOQ) to 3872 ng/mL for PEth 16:0/16:0. The measurement uncertainties ($\%U=2.12*\%RSD_t$) at the LLOQ were used to interpret results close to the LLOQ and close to the cut-off value (for PEth 16:0/18:1) and were 24 % for PEth16:0/18:1, 23 % for PEth 18:1/18:1 and 22 % for PEth 16:0/16:0. A cut-off value at 221 ng/mL for PEth 16:0/18:1, previously proposed (see Section 5.3.2) to distinguish between inpatients on alcohol withdrawal and control volunteers, was used [28]. Concentrations ≥ 274 ng/mL (221 + 24 %) were used to suggest excessive and chronic alcohol consumption. Measured concentrations ≥ 12 ng/mL for PEth 18:1/18:1 and above 23 ng/mL for PEth 16:0/16:0 (LLOQ + %U) were used to confirm excessive and chronic alcohol consumption suggested by other biomarkers.

6.3 Results

Venous blood (CDT%, GGT, ALT, AST and MCV), hair (EtG), C-DBS (PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0) and urine (EtG_{100} and EtS_{100}) from fifty volunteers, for whom fitness to drive had to be assessed, were analysed. The group was composed of 45 males and 5 females, aged between 25 and 69 years (mean = 45, median = 45). Ten out of the 50 volunteers had an AUDIT total score of 8 or more, which is used as an indicator of hazardous and harmful alcohol use and possible alcohol dependence [29]. Based on psychological and medical assessments, including the results of indirect biomarkers (CDT%, GGT, ALT, AST and MCV) measured in venous blood, 18 volunteers were declared fit to drive, 20 fit to drive for one year and 11 unfit to drive (for one volunteer no decision was taken, because some requirements were not fulfilled). Table 6.1 contains the following details for each volunteer: age, gender, liver problems, self-reported alcohol consumption, AUDIT total score, fitness to drive decision, abstinence period required, indirect biomarkers concentrations (CDT%, GGT, ALT, AST and MCV values), hair sample data (treatment, hair length and weight of hair analysed, EtG concentration), C-DBS data (PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 concentrations) and urine data (creatinine, EtG_{100} and EtS_{100} concentrations).

								Indirect biomarkers						Hair			C-DBS			Urine		
N°	Age (years)	Gender	Liver problem	Self-reported alcohol consumption (units/week)	AUDIT	Decision	Abstinence period	CDT (%)	GGT (U/L)	ALT (U/L)	AST (U/L)	MCV (fL)	Treatment	Length (cm)	Weight (mg)	EtG (pg/mg)	PEth 16:0/18:1 (ng/mL)	PEth 18:1/18:1 (ng/mL)	PEth 16:0/16:0 (ng/mL)	Creatinine (mg/dL)	EtG_{100} (ng/mL)	EtS_{100} (ng/mL)
1	45	M	No	0	2	F	Yes	1.4	20	34	33	86	No	2	14.6	<LLOQ	<LLOQ	<LLOQ	<LLOQ	79	155	129
2	55	M	No	0	0-4	F1	No	1	33	16	19	88	No	5	54.3	<LLOQ	<LLOQ	<LLOQ	<LLOQ	38	<LLOQ	*369*
3	49	M	No	0	0-4	F	Yes	0.9	**139**	14	15	90	No	2	45.3	<LLOQ	<LLOQ	<LLOQ	<LLOQ	29	<LLOQ	<LLOQ
4	35	M	No	< 5	6	F	Yes	0.8	13	26	22	78	No	5	35.9	<LLOQ	<LLOQ	<LLOQ	<LLOQ	247	<LLOQ	<LLOQ
5	51	M	No	0	0-4	F	Yes	0.8	20	24	22	82	No	0	6.7	<LLOQ	<LLOQ	<LLOQ	<LLOQ	30	<LLOQ	<LLOQ
6	27	M	No	0	0-4	F1	No	0.7	44	**64**	25	83	No	3	49.3	<LLOQ	<LLOQ	<LLOQ	<LLOQ	91	<LLOQ	<LLOQ
7	49	F	-	< 5	6	F	No	0.5	22	29	30	89	Yes	6	51.9	<LLOQ	<LLOQ	<LLOQ	<LLOQ	31	<LLOQ	<LLOQ
8	38	M	No	0	2	F	Yes	<LLOQ	11	15	19	91	No	0	10.6	<LLOQ	<LLOQ	<LLOQ	<LLOQ	17	<LLOQ	<LLOQ
9	52	M	No	0	0-4	F1	Yes	1.9	42	13	20	88	No	6	55.6	17	<LLOQ	<LLOQ	<LLOQ	203	<LLOQ	<LLOQ
10	45	M	No	5-21	9	F1	No	1.4	25	26	29	89	No	2	49.3	18	212	***14***	<LLOQ	35	3139	439
11	57	M	No	< 5	6	F	No	1.2	37	37	28	83	No	6	58.6	21	98	<LLOQ	<LLOQ	132	<LLOQ	<LLOQ
12	40	F	No	0	0-4	F1	Yes	0.9	8	9	19	88	Yes	25	79	29	46	<LLOQ	<LLOQ	75	<LLOQ	<LLOQ
13	26	M	No	< 5	6	UF (THC)	No	1.1	12	15	20	92	No	6	43.7	30	160	<LLOQ	<LLOQ	76	<LLOQ	<LLOQ
14	46	M	No	< 5	6	F1	No	1.7	14	12	18	90	No	4	38.5	30	96	<LLOQ	<LLOQ	81	243	<LLOQ
15	29	M	-	< 5	0-4	F	Yes	0.9	**64**	**50**	33	96	-	-	-	-	132	<LLOQ	<LLOQ	-	-	-
16	29	M	No	0	0-4	F1	Yes	1.2	20	18	18	93	-	-	-	-	35	<LLOQ	<LLOQ	51	1773	<LLOQ
17	38	M	No	< 5	4	F	No	0.8	28	20	22	80	-	-	-	-	26	<LLOQ	<LLOQ	251	<LLOQ	<LLOQ
18	46	F	No	< 5	4	UF	No	**15.6**	**51**	16	26	**98**	Yes	6	49.7	**154**	**1620**	***66***	***123***	13	>76278	>76278
19	47	M	Yes	5-21	6	UF	No	**10**	**163**	36	**46**	92	No	3	44.2	**203**	**1181**	***115***	***74***	25	21640	8315
20	35	M	No	> 24	19	UF	No	**5.9**	**63**	31	**37**	95	No	6	50.7	**573**	**2085**	***93***	***161***	142	>7027	>7027
21	67	M	No	> 24	16	UF	No	**3.5**	**169**	19	23	93	No	5	67.5	**231**	**1644**	***154***	***97***	181	>5520	>5520
22	32	M	No	5-21	8	UF	No	**3.3**	24	22	30	82	No	6	53.3	**95**	**541**	***24***	***43***	367	2092	1107
23	27	M	No	5-21	4	UF	No	**3**	**99**	**64**	**44**	94	No	6	48.7	**144**	**1671**	***123***	***94***	331	>3021	>3021

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24	62	M	No	5-21	13	UF	-	**2.9**	**1074**	**42**	**78**	96	No	4	44.5	**99**	**1921**	***107***	***221***	247	>4041	>4041
25	53	M	No	5-21	4	F1	No	**2.7**	57	28	34	92	No	6	27.7	**66**	**1035**	***51***	***69***	114	>8768	4242
26	44	M	No	5-21	16	UF	No	**2.7**	**140**	39	**46**	95	No	2	14.1	**249**	**834**	***53***	***72***	80	<LLOQ	<LLOQ
27	44	M	No	21-24	7	UF	No	**3.1**	**124**	27	33	**97**	No	3	15.3	**127**	**1263**	***89***	***89***	37	>27049	<LLOQ
28	34	M	No	< 5	6	UF	No	**3.1**	14	20	31	92	No	2	29.4	**52**	192	<LLOQ	<LLOQ	91	7552	1448
29	52	M	No	5-21	7	UF	No	**2.7**	**82**	27	31	88	No	1	11.1	30	**813**	***23***	***71***	251	<LLOQ	148
30	47	M	No	5-21	7	F	No	**3.3**	**73**	20	26	93	No	3	31.3	15	**311**	***26***	<LLOQ	163	134	<LLOQ
31	51	M	No	21-24	9	-	No	-	-	-	-	91	No	6	32.1	**58**	**1105**	***70***	***93***	189	>5288	<LLOQ
32	41	M	No	< 5	3	F1	Yes	1.6	41	18	21	88	No	9	58.2	**74**	**943**	***73***	***67***	184	197	153
33	25	M	Yes	-	9	F1	No	2.1	27	21	23	92	No	6	51.4	**76**	**377**	***18***	***35***	360	1995	577
34	58	M	No	5-21	4	F1	No	0.7	**74**	23	21	91	No	6	51.4	**214**	**457**	***35***	***37***	69	6850	3232
35	50	F	No	5-21	6	F1	No	1	**59**	23	**45**	94	Yes	6	50.6	**314**	**1648**	***95***	***143***	143	<LLOQ	3333
36	51	M	Yes	5-21	4	F1	No	1.7	54	33	22	88	No	5	48.9	**44**	**493**	***24***	***39***	129	<LLOQ	<LLOQ
37	35	M	No	5-21	6	F1	No	1.3	15	17	25	88	No	6	46.3	**44**	**450**	***20***	***43***	103	<LLOQ	<LLOQ
38	37	M	No	5-21	10	F1	No	0.5	24	24	23	**98**	No	6	36.8	**104**	**293**	***14***	***37***	53	<LLOQ	<LLOQ
39	68	M	No	< 5	5	F	Yes	2	30	27	14	93	No	5	49.3	**141**	**413**	***19***	***38***	50	<LLOQ	<LLOQ
40	48	M	No	0	2	F	Yes	0.9	**166**	**91**	**72**	88	No	2	18.7	**143**	**971**	***84***	***69***	37	<LLOQ	<LLOQ
41	42	M	No	< 5	5	F	No	0.8	56	30	26	91	-	-	-	-	**736**	***42***	<LLOQ	-	-	-
42	56	F	No	5-14	5	F1	No	1.5	**118**	**72**	**104**	77	-	-	-	-	**3689**	***143***	***260***	48	>20661	>20661
43	26	M	No	0	0-4	F1	Yes	1.9	21	16	32	88	-	-	-	-	**365**	***13***	***34***	120	>8348	>8348
44	38	M	No	< 5	1	F	No	<LLOQ	19	22	22	91	-	-	-	-	**363**	***12***	***38***	142	<LLOQ	<LLOQ
45	41	M	No	5-21	2	F	No	1.1	**195**	**57**	**50**	93	No	6	63.1	9	**1056**	***33***	***86***	59	>170678	>170678
46	57	M	-	< 5	3	F1	No	1.8	23	20	22	93	No	6	49.1	32	**381**	***23***	***31***	123	2167	917
47	34	M	No	21-24	6	F	No	0.4	**76**	34	**51**	87	Yes	5	18.9	10	**457**	***31***	<LLOQ	93	6697	2753
48	47	M	No	5-21	4	F	No	0.7	42	25	21	88	No	4	50.9	**130**	68	<LLOQ	<LLOQ	302	<LLOQ	<LLOQ
49	69	M	No	< 5	4	F1	No	1.2	11	12	16	88	No	5	50.4	**250**	110	<LLOQ	<LLOQ	48	<LLOQ	<LLOQ
50	62	M	No	5-21	7	F	No	1	**119**	18	19	**98**	No	2	19	**269**	75	<LLOQ	<LLOQ	58	<LLOQ	<LLOQ

Table 6.1 Age, gender, liver problems, self-reported alcohol consumption, AUDIT score, fitness to drive decision (F: fit, F1: fit 1 year, UF: unfit), abstinence period required, indirect biomarkers concentrations (CDT%, GGT, ALT, AST and MCV values), hair sample data (treatment, hair length and weight of hair analysed, EtG concentration), C-DBS data (PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0 concentrations) and urine data (creatinine, EtG_{100} and EtS_{100} concentrations). Results that suggest an excessive and chronic alcohol consumption are indicated in bold and results that confirm an excessive and chronic alcohol consumption are indicated in bold and italic (criteria are listed in Table 6.2).

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Quantitative results were interpreted using cut-off values and/or LLOQs as presented in Table 6.2. For serum CDT%, hair EtG, C-DBS PEths, and urine EtG_{100}/EtS_{100}, the measurement uncertainty (%U) of the method at the LLOQ was taken into account before considering a concentration above the LLOQ or cut-off values. For the purpose of readability, this will not be repeated further in the text.

	%U	LLOQ	Cut-off	Cut-off/LLOQ + %U
Results suggesting excessive and chronic alcohol consumption				
(Concentrations ≥ cut-off + measurement uncertainty (%U))				
Serum CDT%	-	-	-	2.4%
Hair EtG	25 %		30 pg/mg	38 pg/mg
C-DBS PEth 16:0/18:1	24 %		221 ng/mL	274 ng/mL
Results confirming excessive and chronic alcohol consumption				
(Concentrations ≥ LLOQ + measurement uncertainty (%U))				
C-DBS PEth 18:1/18:1	23 %	10 ng/mL		12 ng/mL
C-DBS PEth 16:0/16:0	22 %	19 ng/mL		23 ng/mL
Results suggesting alcohol intake the days prior to the sampling and disproving strict abstinence				
(Concentrations ≥ LLOQ + measurement uncertainty (%U))				
Urine EtG_{100}	21 %	100 ng/mL		121 ng/mL
Urine EtS_{100}	8 %	100 ng/mL		108 ng/mL
Results suggesting repeated alcohol consumption (disproving strict abstinence)				
(Concentrations ≥ cut-off/LLOQ + measurement uncertainty (%U))				
Hair EtG	25 %		7 pg/mg	9 pg/mg
C-DBS PEth 16:0/18:1	24 %	10 ng/mL		12 ng/mL

Table 6.2 Criteria used to interpret the measured concentration for serum CDT%, hair EtG, C-DBS PEths and urine EtG_{100}/EtS_{100}.

A graphical comparison between the concentrations measured in hair (EtG), serum (CDT%) and C-DBS (PEth 16:0/18:1) is given in Figure 6.1.

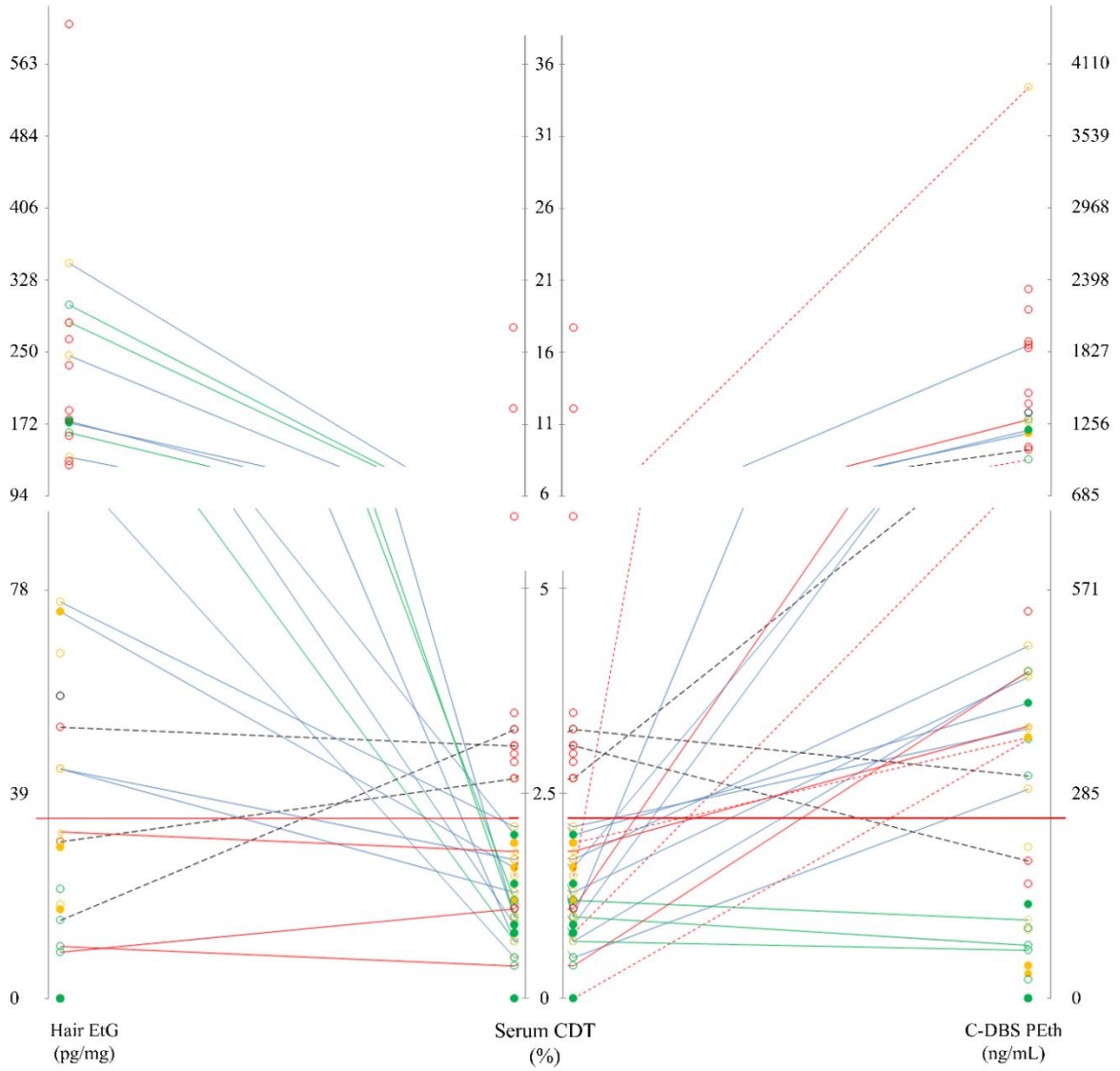

Figure 6.1 Measured concentrations of EtG in hair (left), CDT% in serum (middle) and PEth16:0/18:1 in C-DBS (right) are represented with dots. Hair EtG and C-DBS PEth16:0/18:1 concentrations are normalised to serum CDT% to have a horizontal alignment of the three cut-off values (indicated with a red line) used to detect chronic and excessive alcohol consumption. The final decision regarding the fitness to drive is indicated using different colours for each dot (green = fit, orange = fit for one year, red = unfit). Individuals subjected to an abstinence period are indicated with full dots. In cases where different conclusions were obtained for hair EtG, serum CDT% and/or C-DBS PEth 16:0/18:1 (N=22), lines were used to link the results per volunteer. Results from volunteers with concentrations in hair, serum and C-DBS all above (N=11) or all below (N=17) the cut-off values are not linked using lines. Blue lines indicate 9 volunteers with CDT% results below the cut-off value and EtG in hair and PEth 16:0/18:1 in C-DBS results above the cut-off values. Green lines indicate 3 volunteers with CDT% and PEth 16:0/18:1 in C-DBS results below the cut-off values and EtG in hair results above the cut-off value. Full red lines indicate 3 volunteers with CDT% and EtG in hair results below the cut-off values but PEth 16:0/18:1 in C-DBS results above the cut-off value. The red dashed lines represent 4 cases where a hair sample was not available. Black dotted lines indicate 3 cases with CDT% value above the cut-off but C-DBS PEth 16:0/18:1 or hair EtG below the cut-off values. To improve the readability, the y axes are not linear, the lower part is more expanded than the upper part.

Seventeen volunteers (Table 6.1, n° 1-17) had serum CDT%, hair EtG and C-DBS PEth 16:0/18:1 concentrations that do not suggest excessive or chronic alcohol consumption. All of them, except one with a cannabis addiction, were declared fit to drive (7 for one year). The values obtained for indirect biomarkers were in the reference range in 15 (GGT and ALT), and 17 (AST and MCV) volunteers.

Thirty-three volunteers (Table 6.1, n° 18-50) had at least one result (serum CDT%, hair EtG and/or C-DBS PEth 16:0/18:1) that suggests excessive and chronic alcohol consumption (Figure 6.2). Among them, 13 (Table 6.1, n° 18-30) had a CDT% value that suggests excessive and chronic alcohol consumption, which is confirmed by C-DBS (N=12) and/or hair (N=11) results. Other indirect biomarkers were measured above the upper reference limit in 10 (GGT), 2 (ALT), 5 (AST) and 2 (MCV) out of these 13 cases. For one individual (n° 31), the only indirect biomarker available was MCV. Of note, urine analysis for EtG and EtS indicated recent alcohol consumption in 12 of these cases. The positive urine results are compatible with the alcohol dependence suggested by other biomarkers. Notably, in most cases the urinary concentrations were relatively high (between 2092 and up to more than 10000 ng/mL for EtG_{100} and between 1107 and up to more than 10000 ng/mL for EtS_{100}), when compared with concentrations measured in dependent patients during detoxification (about 10000 ng/mL for EtG_{100} and about 3000 ng/mL for EtS_{100} 20 hours after the cessation of alcohol intake) [14].

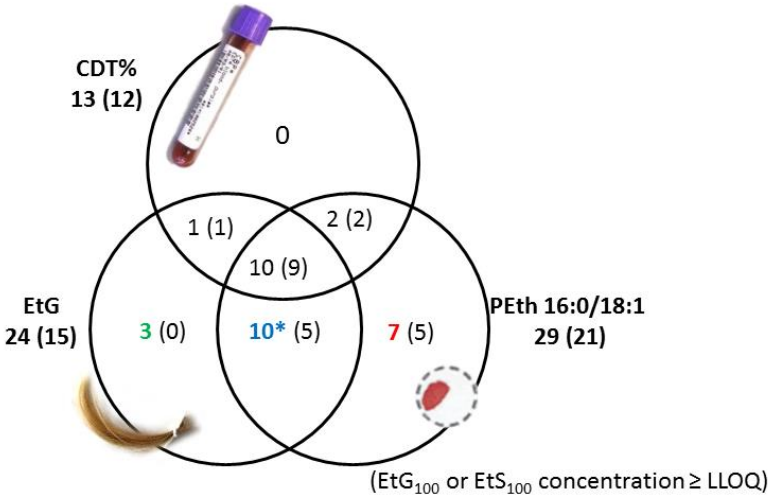

Figure 6.2 Numbers out of 50 volunteers with results above the cut-off values for CDT%, EtG and/or PEth 16:0/18:1, suggesting alcohol dependence. The number of samples with EtG_{100} or EtS_{100} concentrations in urine above or equal to the LLOQ is indicated between brackets. CDT%, hair-EtG, C-DBS-PEth and urine-EtG/EtS data were available for respectively 49, 43, 50 and 48 volunteers. * 9 CDT% values below the cut-off and one with a missing result.

Seventeen volunteers (Table 6.1, n° 31-47) had a PEth 16:0/18:1 concentration in C-DBS that suggests excessive and chronic alcohol consumption and a serum CDT% below the cut-off (or with a missing result). Out of them, 10 (Table 6.1, n° 31-40, represented by blue lines in Figure 6.1 and in blue in Fig. 6.2) had an EtG concentration in hair that also suggests an alcohol misuse, 4 (Table 6.1, n° 41-44, represented by red dashed lines in Figure 6.1) had not provided a hair sample, while 3 (Table 6.1, n° 45-47, represented by red lines in Figure 6.1) had a measured EtG concentration in hair that suggests alcohol intake but with no suggestion of chronic and excessive alcohol consumption. All of the 10 volunteers with C-DBS PEth 16:0/18:1 and hair EtG concentrations that suggest alcohol dependence also had quantifiable PEth 18:1/18:1 and PEth 16:0/16:0 levels. One noteworthy example in this context is the individual (Table 6.1, n° 42) with the highest C-DBS PEth 16:0/18:1 concentration of the whole evaluated cohort (3689 ng/mL). In this individual, CDT% fell below the cut-off and the person was declared fit to drive (albeit for 1 year). Interestingly, also urinary EtG/EtS was positive in this individual (no hair was available for analysis).

Three volunteers out of the 50 (Table 6.1, n° 48-50, Figure 6.2) had an EtG concentration in hair that suggests excessive and chronic alcohol consumption, which was not confirmed, neither by serum CDT% nor by C-DBS PEth 16:0/18:1 results. In these 3 cases, urine analysis for EtG and EtS was negative.

Of the 50 volunteers, 13 had been submitted to an abstinence period (filled dots in Figure 6.1). Out of these 13 cases, recent alcohol consumption was suggested in 4 (EtG_{100}) and 3 (EtS_{100}) cases using urine results, while strict abstinence can be disproved by C-DBS analysis in 7 cases and by hair analysis in 5 cases (3 volunteers did not provide a hair sample). Although in total, only 4 volunteers tested negative for all evaluated direct markers, it should be noted that EtG/EtS concentrations in 1 individual (n° 1) were low, while the positivity in hair of another individual (n° 9) might be owing to residual EtG present in hair, dating from before the abstinence period.

6.4 Discussion

A driver's licence regranting process like the one currently used in Belgium has to manage two main issues. First, medical doctors and psychologists have to detect people with chronic and excessive alcohol consumption. Second, after an unfit to drive decision, an abstinence period of minimum 6 months has to be proven. To date, medical doctors can use the results

from the analysis of indirect biomarkers of alcohol consumption (CDT%, GGT, ALT, AST and MCV) to help in the decision process. These analyses have as a drawback the necessity of a venepuncture by a physician. An interesting step towards a solution to avoid this invasive sampling has recently been presented by Bertaso *et al.* [36], who developed a method for the quantification of CDT% from DBS. However, still, one is confronted with the well-documented lack of sensitivity and specificity of these indirect biomarkers. Three non- or minimally invasive methods analysing direct biomarkers (hair EtG, C-DBS PEths and urine EtG_{100}/EtS_{100}) were applied on 50 real cases, where the fitness to drive had to be assessed using a blood analysis.

6.4.1 Methods and cut-off values

For direct markers in urine and hair, internationally recommended cut-off values could be applied in this study. For PEth 16:0/18:1 in blood, a cut-off value at 274 ng/mL was used to suggest excessive and chronic alcohol consumption in 29 volunteers. Others have proposed a lower (210 ng/mL) or substantially higher (700 or 800 ng/mL) cut-off value [24,37,38]. When applying C-DBS PEth 16:0/18:1 cut-off values of 210, 700 or 800 ng/mL, a suggestion of excessive and chronic alcohol consumption would be made for resp. 30, 17 and 16 volunteers. It remains to be evaluated what C-DBS PEth cut-off value should ideally be used in the context of driver's license regranting. For CDT%, despite an international guideline advocating the measurement of disialo-transferrin as a single analyte, both asialo-transferrin and disialo-transferrin were used in this study, to be in accordance with the driver's licence regranting process currently used in Belgium. For the same reason, cut-off values used (female/male) in this study (36/61 U/L for GGT, 31/40 U/L for ALT and 31/37 U/L for AST) slightly differ from the internationally accepted ones (38/55 U/L for GGT, 34/45 U/L for ALT and 31/35 U/L for AST) [39].

6.4.2 Inference processes

Different information can be obtained from the quantification of hair EtG, C-DBS PEths and urine EtG/EtS. Knowing that the analysis of a proximal hair segment of up to 6-cm length will reflect the alcohol consumption during the period up to 6 months before the sampling (assuming a hair growth rate of 1 cm/month [40]), and recalling that PEth 16:0/18:1 is detected in an alcohol dependent patient up to 1 month after cessation of alcohol consumption [41], the evolution of the drinking pattern can be inferred. As an illustration, an

increase of the mean alcohol consumption at least the month prior to the sampling could explain the apparent mismatch of the results for volunteers n° 45-47 (Table 6.1; data points linked by full red lines in Figure 6.1). In these volunteers, C-DBS PEths results suggest an excessive and chronic alcohol consumption, which is not suggested by the hair EtG results. Hair lengths of these three volunteers were between 5 and 6 cm, which means that the EtG concentration measured in hair represented the mean alcohol consumption the 5-6 months prior to the sampling. In addition, for these 3 volunteers an excessive and chronic alcohol consumption is also confirmed by the concentration of PEth 18:1/18:1 measured in C-DBS. Two of them (Table 6.1, n° 45-46) also have PEth 16:0/16:0 concentrations in C-DBS above the LLOQ. Moreover, urinary EtG_{100} and EtS_{100} concentrations suggest for all 3 cases a recent alcohol consumption. This reflection is illustrated in Figure 6.3, which shows the results of volunteer n° 45. A segmental analysis of hair samples in such cases may provide relevant supplementary information [10,19,42,43], which could confirm or refute such inferences. From these three cases it is readily clear that the combined results can provide possible explanations for certain observations and may give clues about the evolution of a person's alcohol consumption.

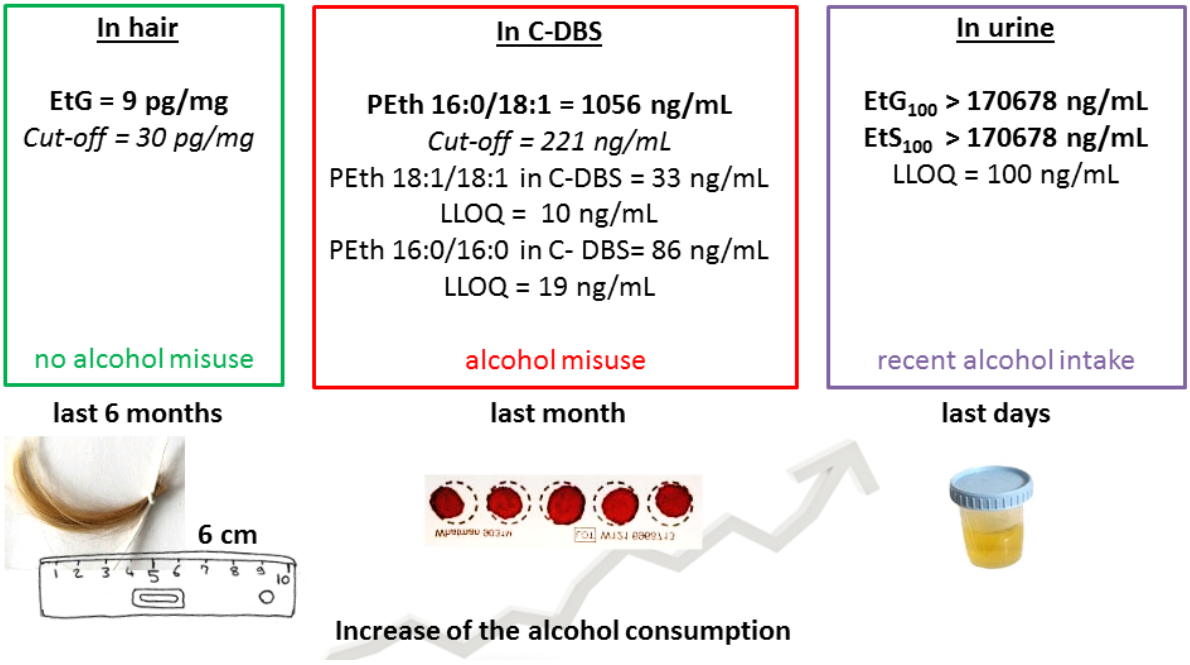

Figure 6.3 Hypothetical drinking pattern of volunteer n° 45 based on the results of EtG in hair and PEths in C-DBS and confirmed by the concentration of EtG_{100} and EtS_{100} in urine.

Using the same reasoning, a mean decrease of alcohol consumption during at least the month prior to sampling could be suggested in volunteers n° 48-50 (Table 6.1; data points linked by green lines in Figure 6.1), where the hair EtG results but not the C-DBS PEth results suggest excessive and chronic alcohol consumption. In addition, EtG_{100} and EtS_{100} concentrations in urine do not suggest an alcohol consumption by these individuals during the few days prior to the sampling.

Among the 13 volunteers (Table 6.1, n° 18-30) with CDT% results that suggest excessive and chronic alcohol consumption, two had C-DBS PEth 16:0/18:1 and one had hair EtG concentrations below the cut-off values used to suggest alcohol dependence, which did not confirm the alcohol dependence suggested by the CDT% result. Among these three exceptions (indicated in Figure 6.1 using black dotted lines), one had a PEth 16:0/18:1 concentration at 192 ng/mL (which is below but already relatively close to the cut-off at 274), another had a EtG concentration at 30 pg/mg hair (which is below but rather close to the cut-off at 38) and the last had an EtG concentration at 15 pg/mg hair. This last result, measured in the 3-cm proximal hair segment, could be explained by an increase of the alcohol consumption at least the last month prior to the sampling. Both volunteers with EtG concentrations below the cut-off reported no cosmetic treatment (bleaching, perming or straightening) of their hair, which could lead to a decreased concentration of EtG in hair [44].

An inference scheme to integrate the results of hair EtG, C-DBS PEths and urine EtG/EtS into the fitness to drive decision process, complementing the psychological assessment is proposed in Figure 6.4.

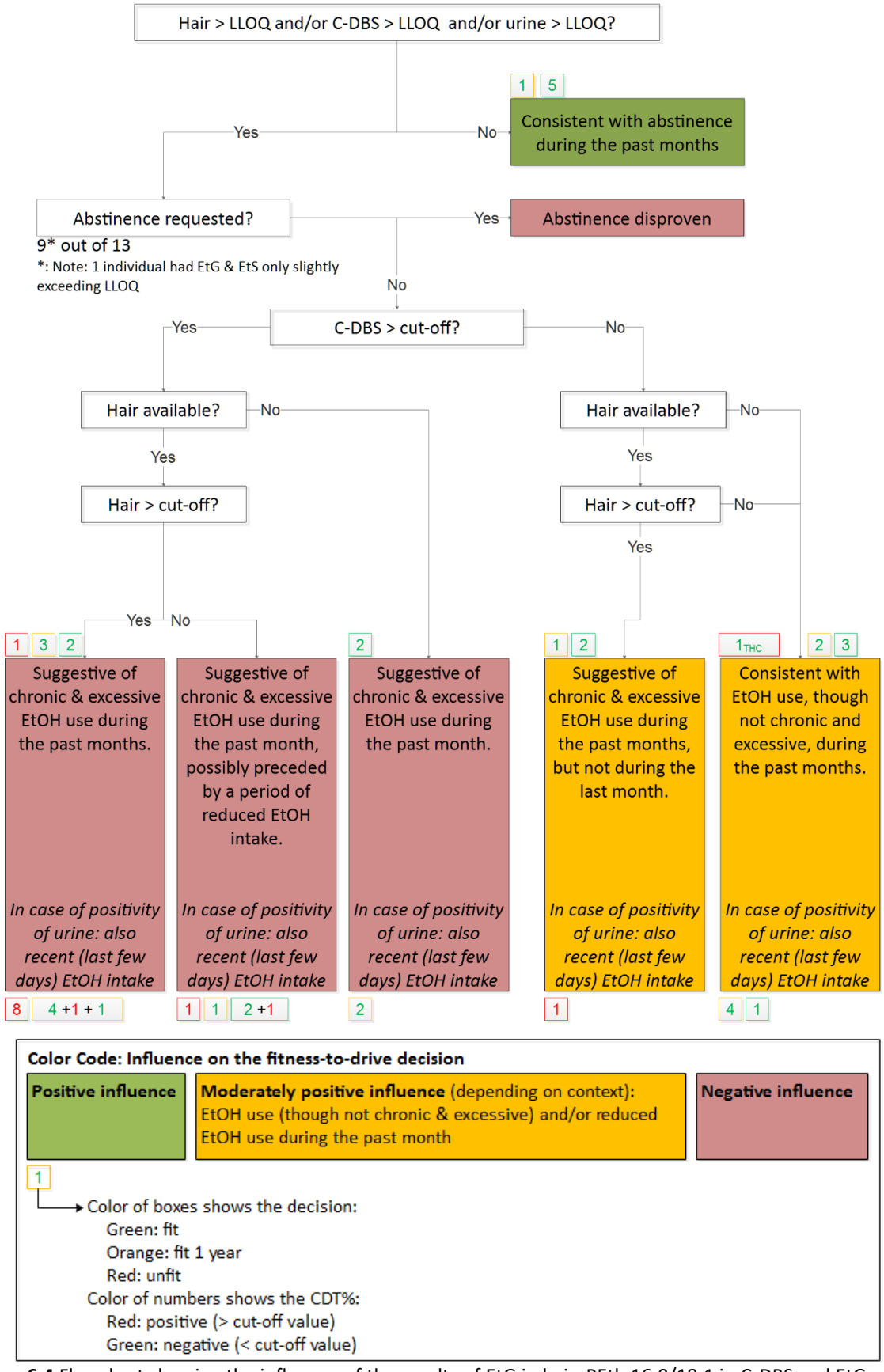

Figure 6.4 Flowchart showing the influence of the results of EtG in hair, PEth 16:0/18:1 in C-DBS and EtG_{100} and EtS_{100} in urine on the fitness to drive decision. The number of cases observed in the 50 volunteers of our study is indicated above (cases with negative urine sample for EtG or EtS) or below each category (cases with positive urine sample for EtG or EtS). EtOH: ethanol.

6.4.3 Sensitivity and specificity

The results of our study confirm the good specificity of hair EtG and C-DBS PEth 16:0/18:1 to detect chronic and excessive alcohol consumption reported by others in literature [11,45,46]. Among the 13 volunteers with CDT% values above the cut-off value observed in our population study, all have C-DBS PEth 16:0/18:1 and/or hair EtG concentrations that confirm the alcohol misuse suggested by the CDT% result.

Our results also confirm i) the superior specificity of CDT% over other indirect biomarkers (especially GGT, ALT and AST) to detect chronic and excessive alcohol consumption [11,45,46] and ii) that hair EtG (24/50) and C-DBS PEths (29/50) are more sensitive to detect excessive and chronic alcohol consumption than the currently used indirect biomarkers (13/50 for CDT%) and are more efficient to disprove an alcohol abstinence period than urinary EtG/EtS [16,20]. Nevertheless, and as mentioned by some authors [16,47], urinary EtG/EtS remains of special importance to disprove recent strict abstinence, because of its ability to detect one single alcohol consumption. In our study, one volunteer submitted to abstinence (volunteer n° 1) had no increased CDT%, a concentration of hair EtG and C-DBS PEths below the LLOQs but concentrations of EtG (155 ng/mL) and EtS (129 ng/mL) in urine above the limits used to suggest alcohol consumption. In that case, strict abstinence could only be disproved by urine analysis. However, due to the possibility of finding EtG and EtS concentrations above 100 ng/mL in urine without consumption of alcoholic beverages [23,48–52] low concentrations -as observed in this case- have to be interpreted with caution. In Germany, participants of abstinence programs are informed about the alcohol content of certain food, beverages and cosmetics (mouthwash and hand sanitisers), whose consumption or use may give rise to results that are in conflict with strict abstinence [15].

Of the thirty-three volunteers that had at least one result (serum CDT%, hair EtG and/or C-DBS PEth 16:0/18:1) that suggests excessive and chronic alcohol consumption (Figure 6.2), only 11 were declared unfit to drive (all having a positive CDT% result). This means that in this study (in which C-DBS and hair results were not used in the fitness-to-drive decision), 2/3 of the cases with evidence of excessive and chronic alcohol consumption were declared fit to drive (albeit in many cases only for 1 year), which is worrisome.

6.4.4 Comparison with results in literature

While many studies have already reported on the comparison of direct alcohol biomarkers with indirect biomarkers, most of these have only made this comparison with one of the 3 direct biomarkers, such as hair EtG [8,11,42,46,53] or blood PEths [25,41,45]. Two studies have compared PEths in blood and EtG/EtS in urine with indirect biomarkers [16,54]. To our knowledge, to date only two publications have included in their population study the analysis of both EtG/EtS in urine, EtG in hair and PEths in blood or DBS [55,56]. Marques *et al.* [55] aimed to estimate the ability of alcohol biomarkers to predict DUI of alcohol recidivism. For that purpose, subjects were classified into three risk-groups, based on the number of attempts to drive a vehicle with an elevated breath alcohol concentration (measured and recorded by the Alcohol Ignition Interlocks Device (IIDs) installed on their vehicle). Mean biomarker concentrations per risk-group were compared and the correlation between biomarkers was evaluated. The interesting results of this study cannot be compared with our results, because both the aim and the procedure differ. The second study [56] compared PEths from venous DBS (100 µL venous blood spotted onto a filter paper) and blood, EtG (from serum, urine and hair), EtS (from serum and urine), CDT%, GGT, ALT and AST concentrations during 12 days (samples at 0, 6 and 12 days), in 81 subjects with an alcohol misuse history. The aim of that study was to determine the agreement and stability of PEths in blood and DBS. Other biomarkers were only used to compare their respective detection times and ability to distinguish between subjects drinking more or less than 85 g ethanol per day. Results were thereby limited to tables containing the mean and range of concentrations observed for each biomarker at days 0, 6 and 12. In addition, no significant differences for the median concentrations were reported between subjects drinking more or less than 85 g ethanol per day, and that for CDT%, EtG/EtS in urine and PEths in blood or DBS.

6.4.5 Hair EtG *vs.* PEth C-DBS

While there is currently more experience with hair EtG analysis in driver's licence regranting programs abroad, C-DBS PEths analysis may have a similar utility in future. Indeed, both approaches have several advantages and disadvantages. A single analysis of a 3-6 cm hair strand is less labour intensive and may be less costly than multiple (e.g. monthly) PEth analyses in case of monitoring long term abstinence. Nevertheless, hair samples are not systematically available and a sufficient amount of hair is not always easy to obtain, especially when volunteers have short hair. While sampling at sites other than the scalp is possible, this poses problems as to the interpretation, when using the cut-off values for scalp hair. Moreover, correct sampling requires some expertise and the collection of a hair strand may be considered somewhat intrusive. In addition, false negative and false positive results due to hair treatments cannot be excluded [35,44,57]. Lastly, hair analysis typically requires a more dedicated sample preparation, often involving an SPE step (as is the case for the EtG analysis performed here). C-DBS, on the other hand, can be collected in any case, always yielding a result. Moreover, the availability of high-throughput-capable fully automated systems (from DBS card to chromatogram without hands-on) may offer a cost-effective alternative in future.

6.5 Conclusion

To evaluate how the Belgian driver's licence regranting process could be improved, we applied three strategies (hair EtG, C-DBS PEths and urine EtG_{100}/EtS_{100}) on 50 real cases, where the fitness to drive had to be assessed using a blood analysis. While currently regular blood sampling is not part of the medico-psychological evaluation (the individual needs to visit a sampling centre or his family doctor to perform a venepuncture), the exclusive use of non- or minimally invasive sampling in this study (hair, urine and C-DBS) allows sampling to be performed directly during the fitness to drive assessment by regular staff members. Each of the strategies applied here provides a different level of information and can be used separately or combined. The quantification of EtG and EtS in urine is a useful method to detect recent alcohol intake and can thus be used to disprove strict abstinence during the days prior to sampling. The determination of PEths in C-DBS is a user-friendly minimally invasive approach that allows to detect chronic and excessive alcohol consumption at least the month prior to the sampling and to disprove an abstinence period. The quantification of EtG in the up to 6-cm proximal hair segment is a non-invasive approach that allows to estimate the mean alcohol consumption for up to 6 months prior to sampling and can be used to strongly suggest repeated alcohol consumption or disprove strict abstinence. Both DBS and hair are easy to transfer and store and are far more sensitive than CDT% to detect chronic and excessive alcohol consumption. In conclusion, in conjunction with the psychological assessments, the approach proposed here allows to obtain a more detailed view on (the evolution of) the alcohol consumption of a subject. This allows a better judgment about the fitness to drive and hence has the potential to improve the driver's licence regranting process.

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Chapter 7

Broader international context, relevance and future perspectives

7.1 Broader international context

In the late 1960s, European countries started to develop driver rehabilitation programs for drinking driving offences, with the aim to avoid recidivism and to re-integrate driving under the influence (DUI) offenders into the traffic system without imposing a risk on other traffic users [1]. Even if differences are observed between countries, the fitness to drive decision in European countries follows the Directive 2006/126/EC of the European Parliament and of the Council of December 20, 2006 on driver's licences [2]. Nine different regranting conditions (i.e. medical assessment, psychological assessment, screening for substance markers in blood/urine/hair, driver rehabilitation, treatment program, theoretical driving lessons, practical driving lessons, theoretical driving test and practical driving test) were detected in a survey performed in the DRUID project [3]. Amongst these, the first and second most common conditions for regranting after an alcohol offence, were, respectively, the medical assessment (used in 22 out of 30 countries either systematically (8/30) or in specific cases (14/30)) and the theoretical driving test (21/30). Medical/psychological examination may include a functional survey (e.g. observation of tremors, smell of the breath, skin changes (tiny red dots from which small blood vessels radiate or skin condition similar to acne)), a medical examination (e.g. blood pressure, heart rate, examination of the belly for an enlarged or tender liver), questionnaires for detecting heavy and problem drinking (e.g. AUDIT, CAGE, DAST, MAST, etc.) and toxicological analyses. In Belgium, as in many European countries, analyses of indirect biomarkers such as carbohydrate deficient transferrin (CDT%), gamma-glutamyltransferase (GGT), aspartate aminotransferase/alanine aminotransferase (AST/ALT) and mean corpuscular volume (MCV), are the current analytical methods used by physicians to help in their assessment of (cessation of) alcohol abuse in case of a driver's licence regranting process [4–9]. Because of a lack of sensitivity and specificity [10], the efficiency of these analyses to detect excessive and chronic alcohol consumption and to assess strict alcohol abstinence periods has become an international issue.

Since 2010, several countries (i.e. Italy, Germany, Switzerland and Sweden) advocate the quantification of ethanol metabolites, such as ethylglucuronide (EtG) and ethylsulfate (EtS) in urine and/or hair to monitor an alcohol abstinence period. According to the Italian driver's licence regranting program, abstinence periods are monitored via urinary EtG and EtS,

determined in three to five unannounced collections, over a period between 2 and 4 weeks [11]. The Swedish [6] and Swiss [7] driver's licence regranting programs have introduced the quantification of EtG in hair as a complementary tool to the analysis of indirect biomarkers. The German driver's licence regranting guidelines to monitor abstinence periods require the quantification of EtG in six random urine or four hair samples [12–14]. In 2016, some authors [15] have strongly recommended to include the quantification of PEth 16:0/18:1 in whole blood in routine analysis for the detection of prolonged excessive alcohol consumption (currently based on a BAC above 1.6 ‰). In the United States of America, where alcohol ignition interlock devices are used to prevent recidivism, the ability of direct biomarkers (blood PEths, hair EtG and FAEE, and urine EtG and EtS) and indirect biomarkers (CDT%, ALT, AST, GGT) to predict recidivism has been tested [16,17]. One-way ANOVA tests have shown that mean concentrations of direct and indirect biomarkers measured in blood and urine were different when measured in subjects with a low rate of attempts to drive with an BAC ≥ 0.4 g/L or in subjects with a high ignition lockout rate. In addition, PEths in blood were depicted as "a remarkably strong, general alcohol risk indicator" correlating with all of the other biomarkers tested.

7.2 Relevance

To our knowledge, the work presented in this thesis is the first to test the combined quantification of EtG/EtS in urine, EtG in hair and PEths in capillary dried blood spots (C-DBS) for both the detection of excessive and chronic alcohol consumption and the monitoring of the abstinence period in case of fitness to drive decisions. These methods are known to be more sensitive and specific than indirect biomarkers currently analysed in blood/serum. Yet, as outlined below, there are still some limitations and challenges that have to be taken into consideration.

7.2.1 Choice of the analytes

The concentration of metabolites in humans strongly depends on the individual metabolism and elimination rate, which are related to factors such as gender and age. Briefly, ethanol metabolism has been described to be on average faster in women (0.17 g/L) than in men (0.15 g/L) and the elimination rate decreases with age [18]. Other factors such as differences in liver volume, enzymes and substrate levels, and pregnancy may affect the metabolism of ethanol [18].

EtG is produced by the transfer of a glucuronic acid moiety from uridine 5'-diphospho-β-glucuronic acid (UDPGA) to an ethanol molecule by multiple UDP-glucuronosyltransferases (i.e. UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15), with UGT1A9, 2B7 (and 1A1 according to one study [19]) contributing most to ethanol glucuronidation.

Genetic polymorphisms, due to variations in the coding regions and/or promoters of UGT isoforms, have been identified for different UGT enzymes [20,21] and may result in variable glucuronidation activity. For example, a common genetic polymorphism in the promoter region of the UGT1A1 gene (*UGT1A1*28*) results in a reduced enzyme expression and is associated with Gilbert's syndrome [21]. Hence, one could hypothesize that EtG formation may be hampered in these individuals and, as a result, EtG may not be a good marker for alcohol consumption in these individuals. A recent study evaluated this hypothesis and found no evidence of impaired EtG formation [22] in individuals suffering from Gilbert's syndrome, having a reduced activity of the UGT1A1 enzyme. This could be explained by the fact that functional differences in one isoform (UGT1A1 in case of Gilbert's syndrome), may be compensated by the activity of other UGT isoforms involved in formation of EtG [19].

UGT activity is not only determined by genetic factors, also a variety of other factors may have an influence on UGT activity. Indeed, inhibition of various UGT isoforms has been observed by a wide set of compounds, including drugs (e.g. tolcapone and entacapone [23] used to treat Parkinson's disease; medroxyprogesterone acetate used as contraceptive, in hormone therapy and to cure advanced breast cancer [24]; and chlormadinone acetate [25]) and dietary substances (e.g. carvacrol from numerous aromatic plants [26]; psoralidin from the psoraleacorylifolia [27]; nor-oleanane triterpenoid saponins from Stauntonia brachyanthera [28]; and ginsenosides from ginseng [29] used in traditional Chinese medicine). More studies are needed to evaluate whether these compounds may affect the glucuronidation of ethanol. A study like this has been performed for polyphenols (i.e. quercetin, kaempferol and resveratrol -naturally present in fruits, vegetables, wines, beers, herbs or spices) and results have shown that the glucuronidation of ethanol was inhibited, via UGT1A1, 1A9 (resveratrol) and UGT1A1, 1A3, 1A6, 1A9, 2B7, 2B10 (quercetin, kaempferol) [30]. However, this inhibition of ethanol glucuronidation was considered as weak to negligible by the authors. As demonstrated by the unaffected formation rate of EtG in subjects suffering from Gilbert's syndrome [22], and knowing that each UGT isoform

exhibits a distinct, but sometimes overlapping, inhibitor selectivity [20], it can be expected that the inhibition of one enzyme will most likely be compensated by the activity of the remaining isoforms. Alternatively, a depletion of UDPGA -as induced by drugs known to be glucuronidated (e.g. valproic acid, chloramphenicol, salicylamide, clofibric acid and galactosamine) [31,32] or after diethyl ether narcosis [32]- could result in a lower glucuronidation of ethanol and should be evaluated. In addition, further research about the effect of drugs (e.g. paracetamol, chloramphenicol, fenofibrate, nicotine, ibuprofen, etc [20,21,33]) known to be conjugated extensively by UGT1A9 or 2B7, which are the two isoforms showing the highest rate of ethanol glucuronidation, and about the effect of polymorphisms on these two isoforms (e.g. UGT1A9*22, UGT1A9*1c, UGT2B7*1a, UGT2B7*2 [33]), may provide valuable insights to better understand the inter- and intra-individual variability of EtG formation and to more efficiently interpret results.

Different sulfotransferase enzymes (i.e. SULT1A1, 1A2, 1A3, 1B1, 1C4, 1E1 and 2A1) are involved in the formation of EtS via the transfer of the sulfo moiety (SO_3^-) of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to ethanol, with SULT1A1 exhibiting the highest EtS formation rate, followed by SULT1A2, 2A1, 1A3, 1B1, 1C4 and 1E1 [30,34,35]. The activity of SULTs may be inhibited when humans are exposed to certain xenobiotics, including drugs (e.g. mefenamic acid, salicylic acid, clomiphene, danazol, nimesulide, meclofenamate, piroxicam, sulindac, aspirin and ibuprofen), dietary chemicals (e.g. ethyl acetate from red wine; catechins from wine and tea; caffeic acid from coffee and tea; quercetin from red wine, green tea and coffee; food colorants; flavonoids and phytoestrogens) and environmental chemicals (e.g. phthalates, hydroxylated polychlorinated biphenyls, hydroxylated polyhalogenated aromatic hydrocarbons, pentachlorophenol, triclosan and bisphenol A) [36–38]. Polyphenols (e.g. resveratrol, quercetin, kaempferol) have been shown to inhibit (in a way considered as moderate to weak by the authors) the sulfonation of ethanol [30]. Sulfotransferase expression and activity has been shown to decrease with liver disease (i.e. steatosis, diabetes, diabetic cirrhosis, alcoholic cirrhosis [39]). Genetic polymorphisms are known for all human SULT isoforms [40,41] and polymorphisms in the SULT1A family have been shown to result in altered enzyme activity [42,43]. We are not aware of studies that systematically investigated the influence of SULTs activity on EtS formation. Similar to glucuronidation, sulfonation is subject to substantial redundancy, where a reduction in activity of one enzyme may be compensated for by other enzymes.

Another important aspect when considering sulfonation is the availability of PAPS, which provides the required sulfonic group, which in turn is dependent on the availability of inorganic sulfate and on the activity of the two enzymes of its synthesis, PAPSS1 and PAPSS2 [44]. Depletions of PAPS have been described in cases of administration of xenobiotics that are eliminated via sulfonation (e.g. salicylamide, phenol and naphthol) and molybdate [45,46]. Further studies are required to determine the impact of PAPS depletion on the sulfonation of ethanol.

Currently available evidence points to the fact that, while nutritional components, drugs, genetic polymorphisms and diseases may affect the activity of UDP and SULT enzymes, the effect on EtG and/or EtS formation effect is modest at most, primarily because of redundancy and the presence of compensatory mechanisms. Yet, more studies are warranted to evaluate under what circumstances there may be a considerable effect on the glucuronidation/sulfonation of ethanol, which might lead to a significant variation of the concentration detected in blood, urine or hair. Depletion of UDPGA or PAPS due to drugs – as reported in a study from the 1990s- could also affect the glucuronidation/sulfonation rate of ethanol and should be further investigated. As a consequence, one should always keep in mind that certain factors may influence to some extent the quantitative results, and in cases of strange results for one subject, one should ask the person more information about drugs, illicit drugs, foods consumed and diseases diagnosed that could affect these results. Importantly, it is unlikely that variations in glucuronidation and/or sulfonation as the ones described above would lead to false positives (i.e. someone being accused of being a chronic drinker and/or not having respected an abstinence period): the influences that have been described mostly lead to a reduction, rather than an increase in enzyme activity. False positive results for EtG and EtS are possible due to items that contain ethanol (e.g. certain food or beverages, medications, cosmetics/sanitizers or e-cigarettes [47,48]). To avoid misleading interpretations two solutions arise. First the use of higher cut-off values, which will, however, decrease the detection window of alcohol consumption, and second, to inform participants about the items which could lead to positive results and to perform the urine sampling within 24 h after giving the advice- as performed in Germany [14].

PEths are a group of abnormal phospholipids formed in cell membranes when ethanol is present, via the action of phospholipase D (PLD), which normally hydrolyses

phosphatidylcholine into phosphatidic acid and choline in cell membranes [49]. Two human PLD isoforms (i.e. PLD1 and PLD2) have been reported [50–52], with cells expressing different splice variants [52]. Because PLD1 and PLD2 have been implicated in human cancer cell progression, compounds that inhibit these enzymes (e.g. FIPI and NOPT [53–56]) are targeted for the treatment of cancer [57] and could possibly affect the transphosphatidylation rate of ethanol. PLD1 and/or PLD2 inhibition is also used in some treatments against Alzheimer's, thrombotic diseases, hypertension, influenza and multiple sclerosis [58–61]. In addition, an altered phosphatidylcholine metabolism (as observed for example in subjects suffering from Alzheimer's disease [62]) may affect the transphosphatidylation rate of ethanol. Different compounds found in food (e.g. resveratrol, honokiol and saponin) have been shown to decrease the activity of PLD enzymes [58]. In addition, trans-diethylstilbestrol, which is a synthetic estrogen used during the 1950s-1970s to prevent miscarriages, has been shown to reduce PEths formation in cells [63].

While also plasma contains phosphatidylcholine (e.g. in lipoproteins) [64], Varga and Alling have demonstrated that PEths were formed *in vivo* within the red blood cells after alcohol consumption [49]. In another study the analysis of cell fractions (erythrocytes, leukocytes and plasma) of blood from 6 alcoholic patients, has shown that PEths were mainly located in erythrocytes and that PEths were not present in plasma [65]. In that study, although no significant difference was observed between whole blood and erythrocyte PEths concentrations, concentrations in erythrocytes tended to be somewhat lower. This could be explained by the presence of a minor part of PEths in platelets (which were not analysed in that study) or by a concentration of PEths in leukocytes or plasma, but at a concentration below the limit of detection of the method used. Although we could not find studies that evaluated whether different plasma phosphatidylcholine concentrations might result in different whole blood phosphatidylethanol levels, it is unlikely that fluctuations in plasma phosphatidylcholine (and, possibly, plasma phosphatidylethanol) levels would impact the whole blood level significantly.

Yet, it may still be worthwhile to evaluate in future studies i) if an altered substrate availability (phosphatidylcholine) or a changed enzyme activity of PLDs would affect the transphosphatidylation rate of ethanol and/or the whole blood PEth level, and ii) which PLD isoforms are involved in the transphosphatidylation of ethanol.

7.2.2 Choice of the matrices

Hair is a very interesting matrix especially due to the wide detection window of compounds trapped into it. However, because hair is an external matrix exposed to environmental conditions, compounds can be washed out to some extent, for example by cosmetic treatments. In addition, the sampling can be considered somewhat intrusive and requires a certain expertise [56]. Hair is not systematically available and a sufficient amount is not always available, e.g. in bald subjects or in subjects having short hair. Three-cm segments may be preferred over 6-cm segments to avoid "dilution" of possibly positive segments by adjacent negative segments. Since every 3-cm segment covers a period of about 3 months, four samplings may be sufficient to cover a whole year. The Swedish [6], Swiss [7] and German [12–14] driver's licence regranting programs have introduced the quantification of EtG in hair as a complementary tool to the analysis of indirect biomarkers. In Switzerland, the 0-5 cm proximal scalp hair segment is analysed and hair from the arms, legs and chest are sometimes used [67]. In that regard, concentration in scalp hair has been shown to be similar to that in beard, chest, arms and legs [18]. In Germany, the analysis of the proximal 3 cm segment of scalp hair is one option for subjects that want to recover their driver's license and have to attest an abstinence period [12,13]. Four separate hair analyses are planned for persons who have to attest an abstinence over a period of one year [12].

The **C-DBS** approach is a minimally invasive sampling procedure that is relatively easy to perform and provides samples that are easy to transfer and store. DBSs are also interesting as they improve the stability of several analytes. This is relevant in the context of PEths, which may be formed *in vitro*, in cases where ethanol is present in the blood and there is a delay in processing the sample. C-DBS also represent a very interesting solution to avoid the whole blood degradation which can affect for example the quantification of CDT% in blood [68]. Sampling of C-DBS has not been used routinely in the context of driving license regranting programs. However, one can envisage a system where follow-up of persons is performed using C-DBS. Since the time window covered is about a month, theoretically, twelve samplings should be performed to have full coverage of a year. However, this is not realistic from both a practical and financial point-of-view. Therefore, similar to the systems where random (unannounced) samplings of urine are performed (e.g. in Italy and Germany [11,12]), one may envisage a system in which persons get a phone call and have to present

within a certain time interval for C-DBS collection. If positive, sampling frequency might be increased. If negative, sampling frequency might be decreased. Important in this system is that persons do not know on beforehand when they will be sampled: e.g. sampling in January may or may not be followed by sampling in February (otherwise some people might have a tendency to start drinking right after a sample was collected). As such, the number of samplings may remain limited - ideally less than 6 per year, which is close to the frequency of hair sampling.

Urine is a non-invasive sampling approach, which, however, requires supervised collection when it is to be used for legal purposes. This is somewhat inconvenient for both the person him/herself, as for the supervising person. Moreover, both a male and female should be available to perform the supervision. Urine allows monitoring of EtG and EtS to detect recent alcohol consumption, also if only low amounts of ethanol have been consumed. Obviously, the time window is much shorter than in the case of hair or C-DBS. Because dilution is known to affect the quantification, creatinine normalisation is required. Urine analysis might actually also be performed on-site, using simple, commercially available tests, such as the DRI® Ethyl Glucuronide Assay (Thermo Fisher Scientific Microgenics) [69–72]. A positive result might readily lead the individual to confirm recent ethanol intake. Obviously, one should always be cautious with such rapid tests as they may generate false positive results [73]. Hence, one may envisage a system in which the individual would be given the opportunity to challenge a positive screening result (provided that he/she would have to pay for the confirmatory chromatographic analysis in case the positive result is confirmed). As with DBS, dried urine spot analysis is also an interesting option: urine may be collected in a traditional way (supervised sampling, using a beaker), but, subsequently, dried urine samples could be generated (e.g. using volumetric absorptive microsampling), which would improve the stability of compounds and also facilitate transfer and storage.

Obviously, a balance should be found between what is feasible and what is preferred from a scientific and clinical perspective. Moreover, the use of one sampling strategy or marker does not exclude the use of another sampling strategy or marker: depending on the case, another strategy or marker may be ideal: e.g. in someone that has to remain abstinent, a simple positive urinary test for EtG may readily lead the person to give in that he/she indeed still uses ethanol. In other cases, very high CDT% levels may readily confirm a suspicion of

chronic excessive ethanol use. Alternatively, in cases where CDT% would not lead to a suspicion of chronic excessive ethanol use, the availability of direct markers in non- or minimally invasive matrices may confirm or refute the absence of (excessive) ethanol consumption.

7.3 Future perspectives

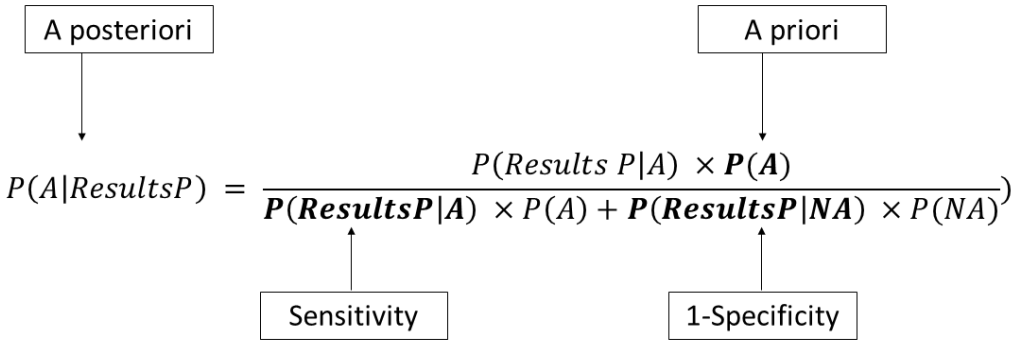
As discussed before, further studies are required to estimate if nutritional components, drugs, genetic polymorphisms and diseases that have been demonstrated to affect the activity of UDP, SULT and PLD enzymes or to deplete necessary substrates (e.g. UDPGA, PAPS, phosphatidylcholine), will have an effect on the glucuronidation, sulfonation or transphosphatidylation of ethanol, which might have some impact on the observed concentrations of the corresponding direct ethanol markers, thereby explaining interindividual differences in concentrations of these markers between individuals that consumed similar amounts of ethanol.

Although the three strategies presented in this research have been fully validated according to international guidelines and published recommendations, additional work seems required to ensure an accurate quantification and an adapted interpretation of the results. As discussed in **Chapter 4**, an extensive pulverisation of hair samples leads to a significantly higher amount of EtG measured. External QC samples with hair in a cut (non-pulverised) form were not used for the validation, because of a bias induced by the pulverisation process applied. To avoid such bias, the creation of different external quality control samples with a different certified value for methods based on cut hair and for methods based on pulverised hair seems necessary. Data analysis of proficiency tests between 2011 and 2015, using cut hair samples, has shown an overall lack of reproducibility (%RSD values from 24 up to 102 %). Less variation was observed between reported EtG concentrations from different laboratories (%RSD from 23 to 35 %) for proficiency tests using hair in powdered form. The observed variations can partially be explained by inhomogeneity within samples, but also illustrate the influence of the grinding process on the quantification of EtG in hair. Recommendations concerning the sample preparation protocol have been published in 2015 by the Society of Hair Testing (SoHT) and in 2016 by Salomone *et al.* [74], and the impact of such guidelines on the observed variation between laboratories in the determination of EtG in proficiency test samples has to be studied. As discussed in **Chapter 6**, different cut-off

values for PEths have been proposed to suggest excessive and chronic alcohol consumption and it remains to be evaluated what PEths cut-off values are the most adapted to the fitness to drive assessment. To evaluate to what extent the determination of hair EtG, C-DBS PEths and urine EtG/EtS may improve the assessment of the fitness to drive, a population study including 50 volunteers was performed. The outcome of this limited study indicated that two thirds of the individuals in which our analyses indicated evidence of excessive and chronic alcohol consumption were declared fit to drive (albeit many for 1 year). This striking observation lends great support to the implementation of direct alcohol markers into the fitness to drive decision process. A decision scheme to integrate direct biomarker results (EtG in hair, PEth in C-DBS and EtG_{100} and EtS_{100} in urine), complementing the psychological assessment, has been presented in **Chapter 6** and could be applied and tested in larger cohorts of individuals in driver's license regranting programs in Belgium, as well as in other countries. In that perspective, the development of quantitative methods for ETG and EtS from dried urine spots and for CDT% from dried blood spots represents a very interesting improvement. This would allow to avoid the currently performed venepuncture and provide samples more easy to transfer and less subject to degradation [68,75]. In addition, monitoring ethanol in sweat via electrochemical devices could be an interesting approach to monitor alcohol abstinence period, while monitoring EtG in nail may be interesting, especially when no hair is available.

Another future perspective inherent to this research field concerns the interpretation of results. A common thread throughout this research is the interpretation of quantitative results using cut-off values (e.g. to detect excessive and chronic alcohol consumption). Cut-off values are defined according to the sensitivity and specificity of the method to distinguish between two populations (e.g. volunteers entering an alcohol withdrawal treatment *vs*. a control group). This approach, which is currently the common way of interpreting results in the toxicological field, is nevertheless not the only possible approach and one promising alternative is to choose a Bayesian probabilistic approach. As illustrated by Taroni *et al.* for the interpretation of THC concentrations in blood using a legal cut-off value to detect DUI of illicit drugs [76], a Bayesian probabilistic approach allows to provide as an output the probability (called posterior probability) for a THC concentration to exceed the legal

threshold, instead of the categorical conclusion (e.g. being below or above the cut-off value). In case of monitoring alcohol consumption (Figure 7.1), this would provide for example the probability of being an excessive and chronic alcohol consumer having an analytical result (e.g. CDT% in serum, EtG in hair, etc.) suggesting this.



A: Being an excessive and chronic alcohol consumer
NA: Not being an excessive and chronic alcohol consumer
Results P: Having a quantitative result that suggests an excessive and chronic alcohol consumption

Figure 7.1 Illustration of the Bayes theorem**.**

In addition, this approach allows to integrate the initial beliefs (prior to looking at analytical results) of the person in charge of taking a decision, as much as the sensitivity and specificity of the analytical method used. The posterior probability indeed combines both personal initial beliefs and analytical results (e.g. THC concentration in blood, CDT% value in serum). Another advantage is the possibility to combine several results (e.g. CDT% in serum, hair EtG, urine EtG/EtS, C-DBS PEths concentrations, AUDIT score, etc.) to calculate a posterior probability (i.e. the probability of being an excessive and chronic alcohol consumer), using a Bayesian network. Such network was developed and tested for the interpretation of post-mortem drug concentrations [77] and in another field, for age estimation of living persons [78] and can serve as a reliable example.

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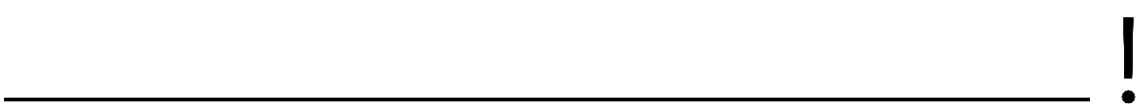
Chapter 8

Conclusion



The objectives of this thesis, presented in **Chapter 2**, were to select, develop and test alternative methods to detect excessive and chronic alcohol consumption and to monitor abstinence periods in individuals within a driver's licence regranting program. Among the non- or minimally invasive sampling strategies for the assessment of alcohol intake of living persons found in literature and presented in **Chapter 1**, three methods were selected, developed and validated, all towards the quantitative determination of direct alcohol markers; ethyl glucuronide (EtG) and ethyl sulfate (EtS) in urine (**Chapter 3**), EtG in hair (**Chapter 4**) and phosphatidylethanol species (PEths) in capillary dried blood spots (C-DBSs) (**Chapter 5**).

To estimate how these three alternative methods could improve the current process, samples (urine, hair and C-DBS) from 50 volunteers for whom fitness to drive had to be assessed and for whom a blood analysis was requested by the physician, were analysed. These three methods, which assess different analytes in distinct matrices, with each of these matrices covering another time window, provide different information about the alcohol consumption of the volunteer. Although there still remain some questions regarding interindividual variability in the levels of direct ethanol markers, our work supports the increasing amount of literature demonstrating the added-value of using these markers. EtG and EtS in urine form a relevant parameter to detect recent alcohol intake (even one single alcohol consumption) during the days (up to 5 days) prior to the sampling and can thus be used to disprove strict abstinence. The determination of PEths in C-DBS allows to detect chronic and excessive alcohol consumption at least the month prior to the sampling and to disprove an abstinence period. Monitoring an even longer abstinence period (e.g. the 6-months abstinence period requested by the Belgian driver's licence regranting legislation), using urine or C-DBS, requires multiple analyses, which can be avoided by hair analysis. The quantification of EtG in an up to 6-cm proximal hair segment allows indeed to estimate the mean alcohol consumption for up to 6 months prior to sampling. Quantitative results can disprove strict abstinence or strongly suggest repeated alcohol consumption. Nevertheless, hair samples are not systematically available and the required amount of hair is not always easy to achieve. In addition, false negative and false positive results due to hair treatments cannot be excluded. When combined together, the results obtained from the urine, hair and C-DBS analyses, allow to infer what the evolution of the alcohol consumption prior to the sampling was, thereby allowing a more detailed view on this consumption. A decision

scheme to integrate the results of these three alternative methods into the fitness to drive decision process, complementing the currently used psychological/medical assessment (based on CDT%, GGT, ALT, AST and MCV), has been proposed and can be used by physicians to monitor the alcohol consumption in case of a driver's licence regranting process. Moreover, the exclusive use of non- or minimally invasive sampling allows this to be performed directly during the fitness to drive assessment by regular staff members. In conclusion, the three approaches that were evaluated in this work offer the potential to improve the Belgian driver's licence regranting process.

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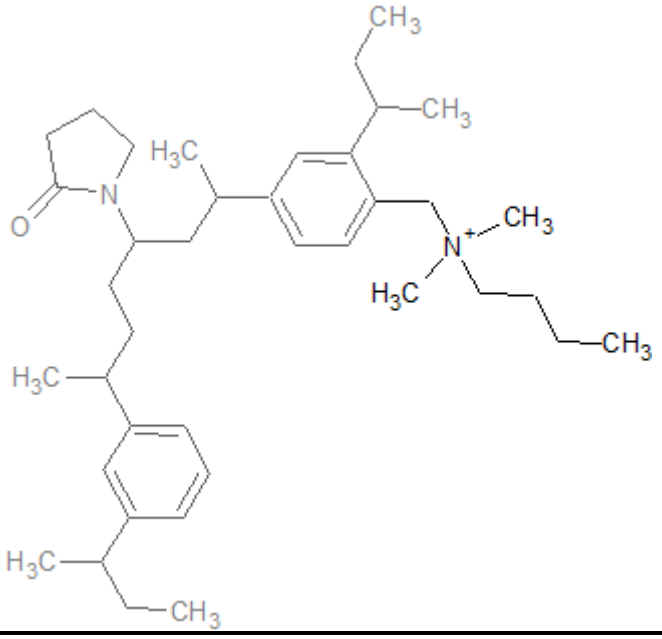
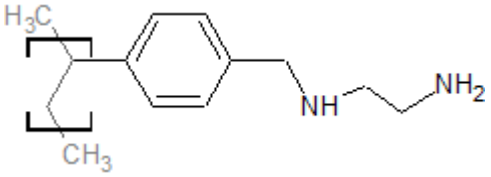
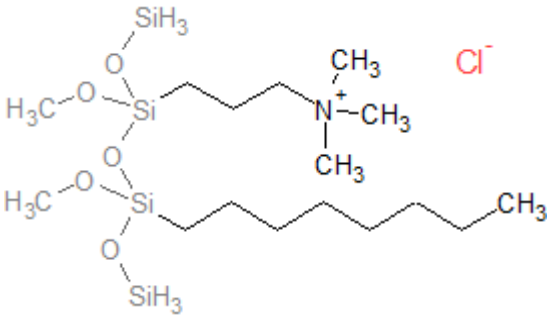
Appendix

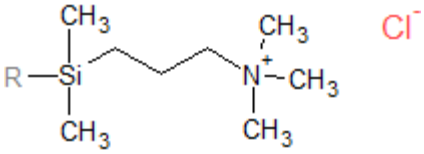
Appendix 1: Solid-phase extraction cartridges

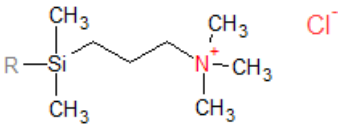
To optimise the extraction efficiency and to decrease the matrix effect for the quantification of EtG in hair, several SPE cartridges, all based on ion exchange mechanisms, were tested. Details about these cartridges, such as the backbones, the functional group, the retention mode, the pka and the chemical representation of the phase are given below.

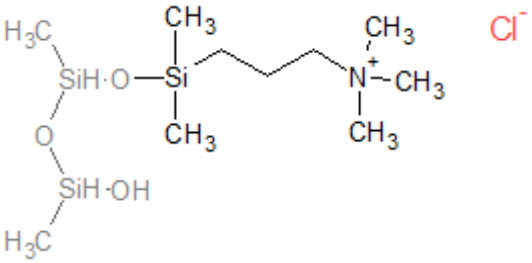
Oasis MAX (Waters Corp., Milford, USA)	
Sorbent weight, barrel size	60 mg, 3cc
Sorbent Substrate	Polymeric
Functional group	Quaternary amide
Retention mode	Reversed-phase and ion exchange
pKa	>18
Chemical representation	

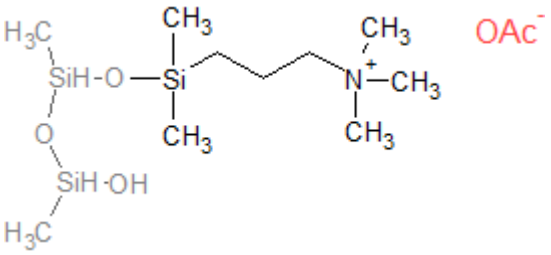
Strata-X-AW (Phenomenex, Utrecht, The Netherlands)	
Sorbent weight, barrel size	100 mg, 3 mL
Sorbent Substrate	Polymeric
Functional group	
Retention mode	Weak anion exchange, π-π Bonding and hydrophobic interaction
pKa	9
Chemical representation	

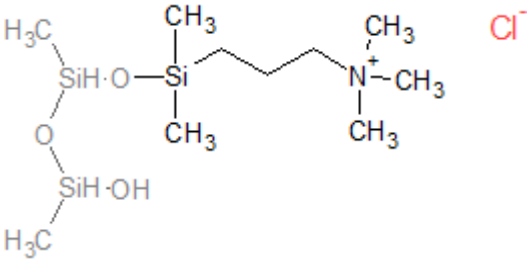
Screen-A (Phenomenex, Utrecht, The Netherlands)	
Sorbent weight, barrel size	100 mg, 1 mL
Sorbent Substrate	Silica
Functional group	C8 + quaternary amine
Retention mode	Hydrophobic selectivity + strong anion exchange
pKa	
Chemical representation	

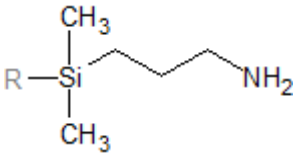
Clean Screen (UCT, Achrom, Zulte, Belgium)	
Sorbent weight, barrel size	200 mg, 3 mL
Sorbent Substrate	Silica
Functional group	
Retention mode	WAX + apolar
pKa	Neutral
Chemical representation	

Bond Elut SAX (Agilent Technologies, Diegem, Belgium)	
Sorbent weight, barrel size	100 mg, 3 mL
Sorbent Substrate	Silica
Functional group	Quaternary amine
Retention mode	Strong anion exchange
pKa	< 14
Chemical representation	

Strata-SAX (Phenomenex, Utrecht, The Netherlands)	
Sorbent weight, barrel size	100 mg, 3 mL
Sorbent Substrate	Silica
Functional group	Quaternary amine
Retention mode	Strong anion exchange
pKa	< 14
Chemical representation	

Isolute PE-AX (Biotage, Sopachem, Eke, Belgium)	
Sorbent weight, barrel size	100 mg, 3 mL
Sorbent Substrate	Silica
Functional group	Quaternary amine
Retention mode	Strong anion exchange
pKa	< 14
Chemical representation	

Isolute SAX (Biotage, Sopachem, Eke, Belgium)	
Sorbent weight, barrel size	100 mg, 3 mL
Sorbent Substrate	Silica
Functional group	Quaternary amine
Retention mode	Strong anion exchange
pKa	< 14
Chemical representation	

Bond Elut NH_2 (50 mg, 1 mL) (Agilent Technologies, Diegem, Belgium)	
Sorbent weight, barrel size	100 mg, 3 mL
Sorbent Substrate	Silica
Functional group	Aminopropyl
Retention mode	Polar and weak anion exchange
pKa	9.8
Chemical representation	

Appendix 2: Statistics

Accuracy, precision and measurement uncertainty

To evaluate the accuracy (%bias) and precision (repeatability (%RSD_r) and intermediate precision (%RSD_t)) of the methods, quality controls were analysed in replicates (N=2) on 8 different days. The bias is a systematic error which induces an overall deviation of the result from the true value (Figure A2.1, right). The imprecision, which is a random error, corresponds to the spreading of the quantitative results when measuring samples from the same concentration (Figure A2.1, middle). Imprecision may be due to a lack of repeatability and/or intermediate precision. Repeatability (%RSD_r) is the precision obtained when samples (replicates in our case) are analysed within short intervals of time (the same day in our case), using the same method, in the same laboratory, by the same operator, using the same equipment. The intermediate precision (%RSD_t) is the precision obtained, when samples (replicates analysed on 8 different days) are analysed within a longer interval of time (the validation periods in our case were from one to a few months). Figure A2.1, left shows the ideal case, in which both imprecision and bias are minimal (in our case there are international guidelines that need to be fulfilled).

Figure A2.1 Illustration of an accurate method (left) and of bias (right) and imprecision (middle).

The measurement uncertainty (%U) is used to interpret quantitative results close to the LLOQ or close to a cut-off value and is calculated using the equation %U = 2.12 * %RSD_t.

Reproducibility

The validity of the quantification (reproducibility) may be demonstrated by the successful participation (Z-score < 2) to proficiency tests. The Z-score is calculated using the reported value, the target value and the standard deviation observed between all results reported (SD) : ((reported value – target value) / SD).

Stability

Stability is evaluated by comparing the response of control samples (CS) and stability samples (SS). Control samples and stability samples were prepared at the same time in replicates (N=6). Control samples are analysed immediately, while stability samples were stored prior to the analysis. The mean response of the stability samples should be within 90 – 110% of the mean response of the control samples and the 90% confidence interval of the stability sample responses should be within ± 20% of the control sample responses.

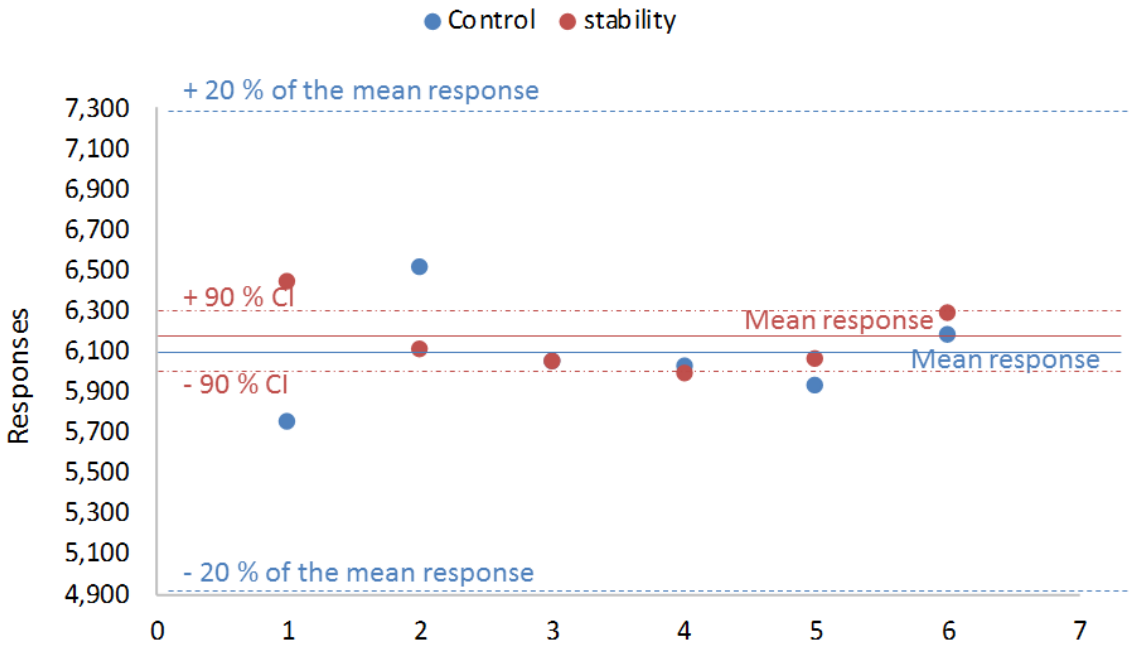


Figure A2.2 Illustration where the 90% confidence interval (CI) of the stability sample responses are within ± 20% of the control sample responses.

Appendix 3: Xevo TQ MS *vs.* Xevo TQ S

The methods for the quantification of EtG/EtS in urine and for the quantification of EtG in hair were both developed and fully validated using a Xevo TQ MS mass spectrometer before being transferred to another system equipped with a Xevo TQ S mass spectrometer.

Both devices are triple quadrupole mass spectrometers. Due to its innovative StepWave ion guide (as second quadrupole), the Xevo TQ S mass spectrometer (Figure A3.1) allows to achieve lower detection limits as compared to the Xevo TQ MS mass spectrometer. The StepWave ion guide (Figure A3.2) is designed to maximise ion transmission from the source to the mass analyser and allows to remove neutral contaminants. This leads to an enhancement of the overall signal to noise ratio.

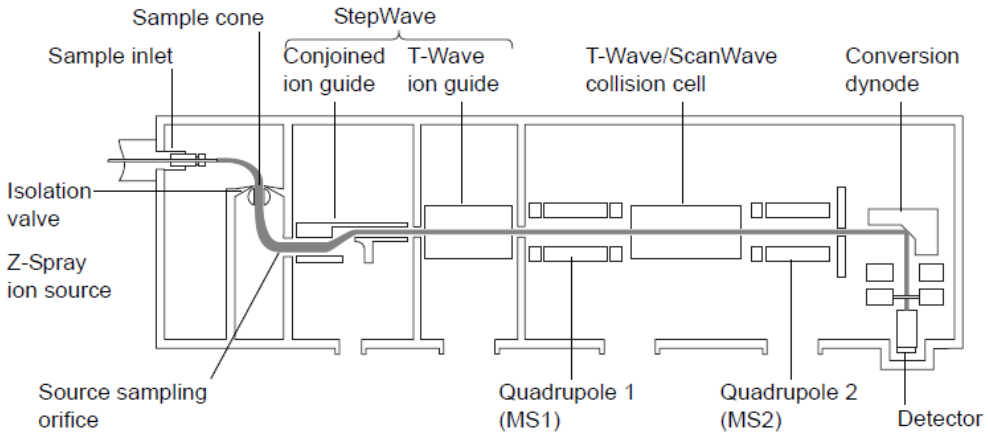


Figure A3.1 Schematic representation of the Xevo TQ S mass spectrometer (Picture from Waters Xevo TQ-S Operator's Overview & Maintenance Guide, Revision A).

Figure A3.2 Illustration of targeted compounds (yellow) transferred from the source to the mass analyser and neutral contaminants (blue) removed by the StepWave ion guide (Picture from http://www.waters.com).

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Summary

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Chapter 1 provides a general background and some definitions related to alcohol and to alcoholic beverages, information about the metabolites of ethanol, analytical methods available to monitor alcohol consumption, the legal issues regarding driving under the influence of alcohol, and the driver's licence regranting process currently in force in Belgium.

The objectives of this thesis, presented in **Chapter 2**, were to select, develop and test alternative methods to detect excessive and chronic alcohol consumption and to monitor abstinence periods in individuals within a driver's licence regranting program.

The first method, described in **Chapter 3**, deals with the quantification of ethyl glucuronide (EtG) and ethyl sulfate (EtS) in urine. The second method, described in **Chapter 4**, allows quantification of EtG in hair. In the latter method, sample preparation (grinding process and solid-phase extraction) was optimised, with special attention for the effect of the grinding process to ensure an accurate quantification from real hair samples. The third method, described in **Chapter 5**, is suited for the quantification of phosphatidylethanol (PEth) species (PEth 16:0/18:1, PEth 18:1/18:1, and PEth 16:0/16:0) in blood, venous (V) and capillary (C) dried blood spots (DBSs). The quantification in C-DBS was not significantly influenced by the hematocrit, spot volumes and punch localisation. In addition, the good agreement of the measured concentrations obtained from venous blood, V-DBS and C-DBS from 100 volunteers (alcoholic inpatients and control volunteers) revealed that V-DBS and C-DBS are valid alternatives to venous blood for the detection of alcohol consumption. Based upon this population study, a cut-off value for PEth 16:0/18:1 to distinguish between inpatients on alcohol withdrawal and control volunteers was suggested.

These three methods were validated regarding selectivity, sensitivity, matrix effects, extraction efficiency, limits of detection/quantification, linearity, accuracy, precision and stability. The validity of the procedures was demonstrated via successful participation to proficiency tests.

The procedures for quantifying EtG/EtS in urine, EtG in hair and PEths in C-DBS were applied on samples obtained from fifty volunteers, for whom fitness to drive had to be assessed and for whom a blood analysis was requested by the physician (**Chapter 6**). The results showed that hair EtG and C-DBS PEths are more sensitive than the currently used indirect biomarkers (CDT%, GGT, ALT, AST and MCV) to detect excessive and chronic alcohol consumption and allow to disprove an abstinence period.

Different information, covering different time windows, can be obtained from the three approaches that are applied.

When combined, these three approaches allow to infer what the evolution of the alcohol consumption prior to the sampling was, thereby allowing a more detailed view on this consumption. A decision scheme to integrate the results of EtG in hair, PEths in C-DBS and EtG/EtS in urine into the fitness to drive decision process, complementing the currently used psychological/medical assessment, has been proposed and can be used by the physicians that are involved in the fitness to drive decision in case of a driver's licence regranting process.

The broader international context, the relevance and the future perspectives related to this research along with factors that may influence the formation and/or elimination of direct ethanol markers, are presented in **Chapter 7**, while a general conclusion is formulated in **Chapter 8**.

Additional information and details about the solid-phase extraction cartridges, the statistics, and the two mass spectrometers used in this project are given in the **Appendix 1, 2 and 3,** respectively.

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Samenvatting

Hoofdstuk 1 bevat de algemene achtergrond en enkele definities met betrekking tot alcohol en alcoholische dranken, informatie betreffende de metabolieten van alcohol, de analytische methodes die beschikbaar zijn om alcoholgebruik te monitoren, wettelijke bepalingen aangaande rijden onder invloed van alcohol, en de huidige procedure voor de teruggave van het rijbewijs in België. De doelstellingen van deze thesis, samengevat in **Hoofdstuk 2**, omvatten het selecteren, ontwikkelen en testen van 'alternatieve methodes' om excessief en chronisch alcoholgebruik aan te tonen en om periodes van abstinentie te monitoren bij deelnemers aan een omkaderingsprogramma voor de teruggave van het rijbewijs.

De eerste methode, uitgewerkt in **Hoofdstuk 3**, betreft de kwantitatieve bepaling van ethylglucuronide (EtG) en ethylsulfaat (EtS) in urine. De tweede methode, beschreven in **Hoofdstuk 4**, focust op de kwantitatieve bepaling van EtG in haar. Hiervoor werd de staalvoorbereiding (verpulveren en vaste fase extractie) geoptimaliseerd, met bijzondere aandacht voor de impact van het verpulveren op het uiteindelijke kwantitatieve resultaat. De derde methode, beschreven in **Hoofdstuk 5**, betreft de kwantitatieve bepaling van drie fosfatidylethanol (PEth) species (PEth 16:0/18:1, PEth 18:1/18:1, and PEth 16:0/16:0) in bloed en droge bloedspots (DBS, veneus en capillair). De concentratie in capillaire droge bloedspots (C-DBS) werd niet significant beïnvloed door hematocriet, spot volume en 'punch' lokalisatie. Bovendien waren de PEth concentraties in veneus bloed, veneuze droge bloed spots (V-DBS) en C-DBS van honderd personen (patiënten in een ontwenningskliniek en een controlegroep) niet significant verschillend, wat erop wijst dat V-DBS en C-DBS valabele alternatieven zijn voor veneus bloed om het alcoholgebruik op te volgen. In deze studie werd een grenswaarde voor PEth 16:0/18:1 gesuggereerd om een onderscheid te maken tussen zware drinkers en personen die geen of matige hoeveelheden alcohol gebruikten.

Deze drie methodes werden gevalideerd met betrekking tot selectiviteit, gevoeligheid, matrix effecten, extractie efficiëntie, detectie- en kwantificatielimieten, lineariteit, accuraatheid en stabiliteit. De succesvolle deelname aan externe kwaliteitscontroleprogramma's bevestigde de validiteit van de gebruikte procedures.

De procedures voor de kwantitatieve bepaling van EtG/EtS in urine, EtG in haar en PEths in C-DBS werden toegepast op vijftig vrijwilligers, voor wie de rijgeschiktheid geëvalueerd moest worden en voor wie een bloedanalyse werd opgelegd door de arts (**Hoofdstuk 6**). De

resultaten toonden aan dat de analyses van EtG in haar en PEths in C-DBS gevoeliger waren dan de huidige gebruikte indirecte biomarkers in bloed (CDT%, GGT, ALT, AST en MCV) om excessief en chronisch alcoholgebruik op te sporen of om abstinentie te weerleggen.

Aan de hand van de resultaten van de drie toegepaste methodes kan uiteenlopende informatie bekomen worden die geassocieerd wordt met verschillende tijdsvensters van gebruik.

De combinatie van de drie toegepaste analysemethodes geeft de evolutie weer van het alcoholgebruik en laat toe om een meer gedetailleerd beeld te krijgen van het gebruik voor de monstername. Een beslissingsschema waarin de resultaten van EtG in haar, PEths in C-DBS en EtG/EtS in urine geïntegreerd worden in de huidige rijgeschiktheidsevaluatie (momenteel bestaand uit een psychologische en medische beoordeling) werd voorgesteld en kan gebruikt worden door de artsen die betrokken zijn bij deze evaluatie in het kader van de teruggave van het rijbewijs.

De bredere internationale context, de relevantie en de toekomstperspectieven van dit onderzoeksproject worden besproken in **Hoofdstuk 7**, alsook de factoren die de productie en eliminatie van directe ethanol markers kunnen beïnvloeden. Een algemeen besluit volgt in **Hoofdstuk 8**.

Bijkomende informatie en details met betrekking tot de vaste fase extractie kolommen, de toegepaste statistiek en de twee massaspectrometers gebruikt binnen dit project, wordt weergegeven in respectievelijk **Appendix 1,2 en 3.**

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Curriculum Vitae

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Natalie KUMMER
Bruynstraat 141 (n°13) - 1120 Brussels
nataliekummer@bluewin.ch
12.02.1983
Swiss nationality

Work experience

2016	**National Institute of Criminalistics and Criminology (NICC), Belgium** Researcher for the Generic Integrated Forensic Toolbox for CBRN incidents
2015 Three months	**Netherlands Forensic Institute (NFI), The Hague, The Netherlands** Researcher in biometric sciences
2010 Five years	**National Institute of Criminalistics and Criminology – NICC / Belgium** Researcher in toxicological sciences
2011 Six years	**University of Brussels (ULB) – Belgium** Teaching assistant for the course "Criminalistics and legal medicine"
2010 Six months	**Federal Criminal Police, Switzerland** Preliminary and criminal investigations in case of organized crime and economic crime
2009 Two months	**Police cantonal of Zurich, Switzerland** Quantification of cocaine and heroin by IR spectroscopy
2007 One month	**Police cantonal of Wallis, Switzerland** Crime Scenes investigation and documentation, examination of evidence, writing report

Education

2009	**MSc in forensic science** - University of Lausanne, Switzerland Dissertation: "Illicit drugs residues in wastewater – Analysis by CE-MS and by GC-MS "
2007	**BSc in forensic science**- University of Lausanne, Switzerland Dissertation: "Utilisation d'un produit détachant pour les faux par enlèvement"

Language

French	Mother tongue
English	Upper intermediate (B2*)
Dutch	Intermediate (B1*)

**in the CEFR, i.e. Common European Framework of Reference for languages levels*

Continuous training

February 2011	**Waters, Zellik, Belgium**
1 day	"Acquity UPLC School for the lab practitioner: Maintenance"
January 2011	**Waters, Zellik, Belgium**
2 days	"Empower software – Fundamentals using the PRO Interface"
December 2010	**Waters, Zellik, Belgium**
2 days	"Acquity UPLC School for the lab practitioner (with lab experiments)"

Publications

Kummer N, Lambert WE, Samyn N, Stove CP. Alternative sampling strategies for the assessment of alcohol intake of living persons. *Clinical Biochemistry*. In Press (2016).
IF (2015): 2.382, Q2 (9/30) of 'Medical laboratory technology', times cited: 0

Kummer N, Wille SMR, Poll A, Lambert WE, Samyn N, Stove CP. Quantification of EtG in hair, EtG and EtS in urine and PEth species in capillary dried blood spots to assess the alcohol consumption in driver's licence regranting cases. *Drug and Alcohol Dependence*. 165 (2016) 191-197.
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Kummer N, Ingels AS, Wille SM, Hanak C, Verbanck P, Lambert WE, *et al.* Quantification of phosphatidylethanol 16:0/18:1, 18:1/18:1, and 16:0/16:0 in venous blood and venous and capillary dried blood spots from patients in alcohol withdrawal and control volunteers. *Analytical and Bioanalytical Chemistry*. 408 (2016) 825–838.
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Kummer N, Wille SM, Di Fazio V, Ramírez Fernández M del M, Yegles M, Lambert WE, *et al.* Impact of the Grinding Process on the Quantification of Ethyl Glucuronide in Hair Using a Validated UPLC-ESI-MS-MS Method. *Journal of Analytical Toxicology*. 39 (2015) 17–23.
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Ramírez Fernández M del M, Wille SM, di Fazio V, **Kummer N**, Hill V, Samyn N. Detection of Benzodiazepines and z-Drugs in Hair Using an UHPLC-MS/MS Validated Method: Application to Workplace Drug Testing. *Therapeutic Drug Monitoring*. 37 (2015) 600–608.
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Ramírez Fernández M del M, Di Fazio V, Wille SMR, **Kummer N**, Samyn N. A quantitative, selective and fast ultra-high performance liquid chromatography tandem mass spectrometry method for the simultaneous analysis of 33 basic drugs in hair. *Journal of Chromatography B*. 965 (2014) 7-18.
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Ramírez Fernández M del M, Van Durme F, Wille SMR, di Fazio V, **Kummer N**, Samyn N. Validation of an Automated Solid-Phase Extraction Method for the Analysis of 23 Opioids, Cocaine, and Metabolites in Urine with Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry. *Journal of Analytical Toxicology*. 38 (2014) 280–288.
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Kummer N, Wille S, Di Fazio V, Lambert WE, Samyn N. A fully validated method for the quantification of ethyl glucuronide and ethyl sulphate in urine by UPLC–ESI-MS/MS applied in a prospective alcohol self-monitoring study. *Journal of Chromatography B*. 929 (2013) 149–154.
IF (2015): 2.687, Q2 (22/75) of 'Chemistry, Analytical', times cited: 6

Wille S, Di Fazio V, Ramírez Fernández M del M, **Kummer N**, Samyn N. Driving under the influence of cannabis: pitfalls, validation, and quality control of a UPLC-MS/MS method for the quantification of tetrahydrocannabinol in oral fluid collected with StatSure, Quantisal, or Certus collector. *Therapeutic Drug Monitoring*. 35 (2013) 101–111.
IF (2015): 2.094, Q3 (148/253) of 'Pharmacology, Pharmacy', times cited: 12

Ramírez Fernández M del M, Wille SMR, **Kummer N**, Di Fazio V, Ruyssinckx E, Samyn N. Quantitative Analysis of 26 Opioids, Cocaine, and Their Metabolites in Human Blood by Ultra Performance Liquid Chromatography–Tandem Mass Spectrometry. *Therapeutic Drug Monitoring*. 35 (2013) 510–521.
IF (2015): 2.094, Q3 (148/253) of 'Pharmacology, Pharmacy', times cited: 14

Presentations

August 2016 4 days	**54th Annual Meeting of the International Association of Forensic Toxicologists (TIAFT), Brisbane, Australia.** Poster presentation: "Quantification of EtG in hair, EtG and EtS in urine, and PEth species in capillary dried blood spots to assess the alcohol consumption in driver's licence regranting cases". *Kummer Natalie, Wille Sarah, Anneleen Poll, Lambert Willy, Samyn Nele, Christophe Stove*.
March 2014 2 days	**Waters European Toxicology Forum, Barcelona, Spain.** Oral presentation: "Monitoring alcohol consumption using UPLC-MS/MS".
October 2013 1 day	**Waters MS Technology Days 2013, Brussels, Belgium**. Oral presentation: "Quantification of EtG in hair to monitor alcohol consumption".
October 2013 3 days	**18th Meeting of the Society of Hair Testing, Geneva, Switzerland.** Poster presentation: "Determination of ethylglucuronide in hair: optimization of the extraction process and validation of an UPLC-ESI--MS/MS procedure". *Kummer Natalie, Wille Sarah, Lambert Willy, Samyn Nele.*

June 2012
3 days

17th Meeting of the Society of Hair Testing, Toronto, Canada.
Poster presentation: "Comparison of solid phase extraction procedures for the segmental analysis of ethyl glucuronide in hair by UPLC MS/M
Kummer Natalie, Beuckelaers Astrid, Lambert Willy, Samyn Nele.