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[a]
Sarah M.R. Wille[a]
Anneleen Poll[c]
Willy E.E. Lambert[b]
Nele Samyn[a]
Christophe Stove[b]

nataliekummer@bluewin.ch

Postprint version of the article published in *Drug and Alcohol Dependence* **2016**, *165*, 191–197. https://doi.org/10.1016/j.drugalcdep.2016.06.012.

Abstract Background: 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. We evaluated the use of direct ethanol markers for this purpose, exclusively determined in matrices obtained via non- or minimally invasive sampling.

Methods: Three validated quantitative methods (ethylglucuronide (EtG) in hair and urine, ethylsulfate (EtS) in urine, 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)) 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.

Results: 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 allow to disprove an abstinence period. Urinary EtG and EtS are useful parameters to determine recent alcohol consumption.

Conclusion: The combined use of the three strategies allows better inference about the evolution of the alcohol consumption prior to the sampling. 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.

Keywords Alcohol monitoring; Fitness to drive; Direct biomarkers; Dependence; Abstinence period; Driver's licence

Contents

1 Introduction

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 litre of blood are condemnable to a fine and in some cases to a confiscation of the driving licence (Belgian Royal Decree, 1968). 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 driving licence regulation (Belgian Royal Decree, 2006), 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 a sampling centre or his family doctor to perform the venepuncture. Blood samples are then sent to an authorized laboratory for analysis. Hence, the current process implies an invasive sampling and 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 driving licence regulation (Belgian Royal Decree, 1998) and the Directive 2006/126/EC of the European Parliament and of the Council of 20 December 2006 on driving licences (European Communities, 2015) 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 driving licence regranting legislation requires a 6-month period of proven abstinence after an unfit to drive decision (Belgian Royal Decree, 1998). 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 driving licence regranting process (Appenzeller et al., 2005; Bortolotti et al., 2002; Kronstrand et al., 2012; Liniger et al., 2010; Maenhout et al., 2012; Wurst et al., 2008). These markers reflect the indirect effects of ethanol on the body, via its interference with glycoproteins present in the body (increased CDT%) and 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 (Pirro et al., 2011), 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. Two interesting reviews published in 2015 can be consulted for more detailed information about EtG and PEths (Cabarcos et al., 2015) and about EtG, EtS, PEths and CDT (Nanau and Neuman, 2015). Urinary EtG and EtS have been used to detect alcohol consumption up to 5 days after intake (Helander et al., 2009), allowing a longer detection window than for ethanol itself and providing a tool to evaluate short-term abstinence (Albermann et al., 2012a; Winkler et al., 2013). PEths in blood and dried blood spots (DBS) have been used to detect chronic and excessive alcohol consumption and allow monitoring ethanol (ab)use during the month prior to the sampling (Schröck et al., 2014). 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. (2014). 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 (Favretto et al., 2010). The Swedish (Kronstrand et al., 2012) and Swiss (Liniger et al., 2010) 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 driving licence regranting guidelines to monitor abstinence periods require the quantification of EtG in six random urine or four hair samples (Agius et al., 2012; Albermann et al., 2010; Musshoff et al., 2010). All these publications focus on abstinence monitoring. 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 (Marques et al., 2011).

Previously, our research group has set up validated approaches to quantify EtG and EtS in urine, EtG in hair and PEths in capillary dried blood spots (C-DBS) (Kummer et al., 2016, 2015, 2013). In this report, 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 alcohol dependence. 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, our manuscript is 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.

2 Materials and Method

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, 3,500 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 (World Health Orgnization, 2015). 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.

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) (Schellenberg et al., 2007). GGT was measured using a kinetic spectrophotometric assay (405 nm) with carboxynitroanilide as a substrate (Schumann et al., 2002a). ALT and AST were measured using a kinetic ultraviolet spectrophotometer (Schumann et al., 2002b, 2002c). Values (males/females) 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 (Maenhout et al., 2014). For CDT%, the cut-off value proposed by Maenhout et al. (2012), which includes the measurement uncertainty, was used to suggest excessive and chronic alcohol consumption (concentrations ≥ 2.4%).

2.3 MCV in EDTA whole blood

MCV was measured in EDTA whole blood using a Sysmex XP-300™ automated haematology analyser (Sysmex Belgium, Belgium). MCV values above 96.4 fL were considered as being above the reference range (Maenhout et al., 2014).

2.4 EtG in hair

Analysis of EtG in hair samples was based upon a previously published fully validated method (Kummer et al., 2015), using EtG-d5 as internal standard. 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 recommends to analyse pulverised 0-3 up to 0-6 cm hair segment (Kintz, 2015). 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). To improve the LLOQ at 10 pg/mg hair of the published method, two modifications have been performed; the first concerning the tandem mass spectrometer used Xevo TQ S instead of the Xevo TQ MS (Waters, Manchester, UK)), the second concerning the mobile phase A used (0.01 instead of 0.1% formic acid in water). To ensure the accuracy of the modified method, a partial validation was performed. The process used for the partial validation and the results are presented in the electronic supplementary material (ESM). Briefly, the analytical range of the modified method was from 2 (LLOQ) to 500 pg/mg hair. Bias and imprecision of less than 12% were obtained (ESM Table S1) and the method was proven to be accurate via successful participation (z-score<1.10) to three proficiency tests (ESM Table S2). The measurement uncertainty (2.12*RSDt) 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 (Kintz, 2015), 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).

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 (Kummer et al., 2013), using EtG-d5 and EtS-d5 as internal standards. 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). To ensure the accuracy of the quantification on the Xevo TQ S, a partial validation was performed. The partial validation process and results are given in the ESM. Bias and imprecision of less than 12% were obtained (ESM Table S3) and the method was proven to be accurate via successful participation (z-score<1.04) to four proficiency tests (ESM Table S4). The published method (extraction, clean-up and chromatography) was not modified. Briefly, methanol (250 µL) was added to 50 µL of urine. The sample was centrifuged (14'000 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 C18 (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 normalizing the measured EtG and EtS to a creatinine concentration of 100 mg/dL. The measurement uncertainties (2.12*RSDt) 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.

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 PEths standards at the moment, 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 (Kummer et al., 2016). 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 0.02% 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, 1.7 µm) 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 (2.12*RSDt) 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 to distinguish between inpatients on alcohol withdrawal and control volunteers, was used (Kummer et al., 2016). 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.

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 (EtG100 and EtS100) 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 (World Health Orgnization, 2015). 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 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, 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).

Seventeen volunteers (Table 1, n° 1-17) had serum CDT%, hair EtG and C-DBS PEth 16:0/18:1 concentrations that do not suggest excessive and chronic alcohol consumption. Sixteen out of these 17 volunteers were declared fit to drive (7 for one year). One was declared unfit to drive because of a cannabis addiction. Among them, the number of individuals with values in the reference range was 12 for GGT, 13 for ALT, 15 for AST and 17 for MCV.

Thirty-three volunteers (Table 1, n° 18-50) have at least one results (serum CDT%, hair EtG and/or C-DBS PEth 16:0/18:1) that suggests excessive and chronic alcohol consumption (Figure 1). Among them, 13 had CDT% concentrations above the cut-off value (Table 1, n° 19-31; these are further referred to as CDT-positives). Other indirect biomarkers were measured above the upper reference limit in 10 (GGT), 5 (ALT), 9 (AST) and 2 (MCV) out of these 13 cases. For one individual (n° 18), the only indirect biomarker available was MCV. For all but three out of the 13 CDT-positives, a chronic and excessive alcohol consumption was also suggested by both the PEth 16:0/18:1 concentration in C-DBS and the EtG concentration in hair. Twenty-nine volunteers (Table 1, n°18-47 (except 29)) have a PEth 16:0/18:1 concentration in C-DBS that suggests excessive and chronic alcohol consumption, which is confirm in 12 cases by CDT results and in 20 cases by hair EtG results.

Table 1: 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, length and weight of hair, 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). A Chronic and excessive alcohol consumption (bold) was suggested when the measured concentration was above 2.4% for serum CDT, 38 pg/mg for hair EtG and 274 ng/mL for C-DBS PEth 16:0/18:1 (cut-off value + U%). Concentrations above 12 ng/ for PEth 18:1/18:1 and 23 ng/mL for PEth 16:0/16:0 mL (LLOQ + U%) in C-DBS were used to confirm an excessive and chronic alcohol consumption (bold and italic) suggested by serum CDT, hair EtG and/or C-DBS PEth 16:0/18:1 concentrations. An EtG concentration in hair between 9 (LLOQ + U%) and 38 pg/mg hair has been used to strongly suggests repeated alcohol consumption and disproves a strict abstinence. Recent alcohol intake (within a few days before the sampling) was suggested (strict abstinence was disproven) when urine EtG_{100} and/or EtS_{100} were measured above 121 and 108 ng/mL (LLOQs + U%), respectively.

								Indirect biomarkers					Hair				C-DBS			Urine		
N°	Age (years)	Gender	Liver problem	Self-reported alcohol consumption	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	Fit	Yes	1.4	20	***34***	***33***	86	No	2.0	14.6	<LLOQ	<LLOQ	<LLOQ	<LLOQ	79	155	129
2	55	M	No	0	0 - 4	Fit 1 year	No	1.0	33	16	19	88	No	5.0	54.3	<LLOQ	<LLOQ	<LLOQ	<LLOQ	38	<LLOQ	*369*
3	49	M	No	0	0 - 4	Fit	Yes	0.9	**139**	14	15	90	No	2.0	45.3	<LLOQ	<LLOQ	<LLOQ	<LLOQ	29	<LLOQ	<LLOQ
4	35	M	No	< 5	6	Fit	Yes	0.8	13	26	22	78	No	5.0	35.9	<LLOQ	<LLOQ	<LLOQ	<LLOQ	247	<LLOQ	<LLOQ
5	51	M	No	0	0 - 4	Fit	Yes	0.8	20	24	22	82	No	0.1	6.7	<LLOQ	<LLOQ	<LLOQ	<LLOQ	30	<LLOQ	<LLOQ
6	27	M	No	0	0 - 4	Fit 1 year	No	0.7	**44**	**64**	25	83	No	2.5	49.3	<LLOQ	<LLOQ	<LLOQ	<LLOQ	91	<LLOQ	<LLOQ
7	49	F	-	< 5	6	Fit	No	0.5	22	29	30	89	Yes	6.0	51.9	<LLOQ	<LLOQ	<LLOQ	<LLOQ	31	<LLOQ	<LLOQ
8	38	M	No	0	2	Fit	Yes	<LLOQ	11	15	19	91	No	0.1	10.6	<LLOQ	<LLOQ	<LLOQ	<LLOQ	17	<LLOQ	<LLOQ
9	52	M	No	0	0 - 4	Fit 1 year	Yes	1.9	**42**	13	20	88	No	6.0	55.6	17	<LLOQ	<LLOQ	<LLOQ	203	<LLOQ	<LLOQ
10	45	M	No	5-21	9	Fit 1 year	No	1.4	25	26	29	89	No	2.0	49.3	18	212	***14***	<LLOQ	35	3139	439
11	57	M	No	< 5	6	Fit	No	1.2	**37**	**37**	28	83	No	5.5	58.6	21	98	<LLOQ	<LLOQ	132	<LLOQ	<LLOQ
12	40	F	No	0	0 - 4	Fit 1 year	Yes	0.9	8	9	19	88	Yes	6.0	79.0	29	46	<LLOQ	<LLOQ	75	<LLOQ	<LLOQ
13	26	M	No	< 5	6	Unfit (THC)	No	1.1	12	15	20	92	No	6.0	43.7	30	160	<LLOQ	<LLOQ	76	<LLOQ	<LLOQ
14	46	M	No	< 5	6	Fit 1 year	No	1.7	14	12	18	90	No	3.5	38.5	30	96	<LLOQ	<LLOQ	81	243	<LLOQ
15	29	M	-	< 5	0 - 4	Fit	Yes	0.9	**64**	**50**	**33**	96	-	-	-	-	132	<LLOQ	<LLOQ	-	-	-
16	29	M	No	0	0 - 4	Fit 1 year	Yes	1.2	20	18	18	93	-	-	-	-	35	<LLOQ	<LLOQ	51	1773	<LLOQ
17	38	M	No	< 5	4	Fit	No	0.8	28	20	22	80	-	-	-	-	26	<LLOQ	<LLOQ	251	<LLOQ	<LLOQ
18	51	M	No	21-24	9	-	No	-	-	-	-	91	No	5.5	32.1	**58**	**1105**	***70***	***93***	189	>5288	<LLOQ
19	46	F	No	< 5	4	Unfit	No	**15.6**	51	16	26	**98**	Yes	6.0	49.7	**154**	**1620**	***66***	***123***	13	>76278	>76278
20	47	M	Yes	5-21	6	Unfit	No	**10.0**	**163**	**36**	**46**	92	No	2.5	44.2	**203**	**1181**	***115***	***74***	25	21640	8315

21	35	M	No	> 24	19	Unfit	No	**5.9**	**63**	**31**	**37**	95	No	6.0	50.7	**573**	**2085**	***93***	***161***	142	>7027	>7027
22	67	M	No	> 24	16	Unfit	No	**3.5**	**169**	19	23	93	No	5.0	67.5	**231**	**1644**	***154***	***97***	181	>5520	>5520
23	32	M	No	5-21	8	Unfit	No	**3.3**	24	22	30	82	No	6.0	53.3	**95**	**541**	***24***	***43***	367	2092	1107
24	27	M	No	5-21	4	Unfit	No	**3.0**	**99**	**64**	**44**	94	No	6.0	48.7	**144**	**1671**	***123***	***94***	331	>3021	>3021
								Indirect biomarkers					**Hair**				**C-DBS**			**Urine**		
N°	**Age (years)**	**Gender**	**Liver problem**	**Self-reported alcohol consumption**	**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)**
25	62	M	No	5-21	13	Unfit	-	**2.9**	**1074**	**42**	**78**	96	No	4.0	44.5	**99**	**1921**	***107***	***221***	247	>4041	>4041
26	53	M	No	5-21	4	Fit 1 year	No	**2.7**	**57**	28	**34**	92	No	6.0	27.7	**66**	**1035**	***51***	***69***	114	>8768	4242
27	44	M	No	5-21	16	Unfit	No	**2.7**	**140**	**39**	**46**	95	No	1.5	14.1	**249**	**834**	***53***	***72***	80	<LLOQ	<LLOQ
28	44	M	No	21-24	7	Unfit	No	**3.1**	**124**	27	**33**	**97**	No	2.5	15.3	**127**	**1263**	***89***	***89***	37	>27049	<LLOQ
29	34	M	No	< 5	6	Unfit	No	**3.1**	14	20	**31**	92	No	1.5	29.4	**52**	192	<LLOQ	<LLOQ	91	7552	1448
30	52	M	No	5-21	7	Unfit	No	**2.7**	**82**	27	**31**	88	No	1.0	11.1	30	**813**	***23***	***71***	251	<LLOQ	148
31	47	M	No	5-21	7	Fit	No	**3.3**	**73**	20	26	93	No	3.0	31.3	15	**311**	***26***	<LLOQ	163	134	<LLOQ
32	41	M	No	< 5	3	Fit 1 year	Yes	1.6	**41**	18	21	88	No	6.0	58.2	**74**	**943**	***73***	***67***	184	197	153
33	25	M	Yes	-	9	Fit 1 year	No	2.1	27	21	23	92	No	6.0	51.4	**76**	**377**	***18***	***35***	360	1995	577
34	58	M	No	5-21	4	Fit 1 year	No	0.7	**74**	23	21	91	No	6.0	51.4	**214**	**457**	***35***	***37***	69	6850	3232
35	50	F	No	5-14	6	Fit 1 year	No	1.0	59	23	**45**	94	Yes	6.0	50.6	**314**	**1648**	***95***	***143***	143	<LLOQ	3333
36	51	M	Yes	5-21	4	Fit 1 year	No	1.7	**54**	**33**	22	88	No	4.5	48.9	**44**	**493**	***24***	***39***	129	<LLOQ	<LLOQ
37	35	M	No	5-21	6	Fit 1 year	No	1.3	15	17	25	88	No	6.0	46.3	**44**	**450**	***20***	***43***	103	<LLOQ	<LLOQ
38	37	M	No	5-21	10	Fit 1 year	No	0.5	24	24	23	**98**	No	6.0	36.8	**104**	**293**	***14***	***37***	53	<LLOQ	<LLOQ
39	68	M	No	< 5	5	Fit	Yes	2.0	30	27	14	93	No	5.0	49.3	**141**	**413**	***19***	***38***	50	<LLOQ	<LLOQ
40	48	M	No	0	2	Fit	Yes	0.9	**166**	**91**	**72**	88	No	2.0	18.7	**143**	**971**	***84***	***69***	37	<LLOQ	<LLOQ
41	42	M	No	< 5	5	Fit	No	0.8	**56**	30	26	91	-	-	-	-	**736**	***42***	<LLOQ	-	-	-
42	56	F	No	5-14	5	Fit 1 year	No	1.5	**118**	**72**	**104**	77	-	-	-	-	**3689**	***143***	***260***	48	>20661	>20661
43	26	M	No	0	0 - 4	Fit 1 year	Yes	1.9	21	16	**32**	88	-	-	-	-	**365**	***13***	***34***	120	>8348	>8348
44	38	M	No	< 5	1	Fit	No	<LLOQ	19	22	22	91	-	-	-	-	**363**	***12***	***38***	142	<LLOQ	<LLOQ
45	41	M	No	5-21	2	Fit	No	1.1	**195**	**57**	**50**	93	No	6.0	63.1	9	**1056**	***33***	***86***	59	>170678	>170678
46	57	M	-	< 5	3	Fit 1 year	No	1.8	23	20	22	93	No	6.0	49.1	32	**381**	***23***	***31***	123	2167	917

47	34	M	No	21-24	6	Fit	No	0.4	**76**	**34**	**51**	87	Yes	5.0	18.9	10	**457**	***31***	<LLOQ	93	6697	2753
48	47	M	No	5-21	4	Fit	No	0.7	**42**	25	21	88	No	4.0	50.9	**130**	68	<LLOQ	<LLOQ	302	<LLOQ	<LLOQ
49	69	M	No	< 5	4	Fit 1 year	No	1.2	11	12	16	88	No	5.0	50.4	**250**	110	<LLOQ	<LLOQ	48	<LLOQ	<LLOQ
50	62	M	No	5-21	7	Fit	No	1.0	**119**	18	19	**98**	No	1.5	19.0	**269**	75	<LLOQ	<LLOQ	58	<LLOQ	<LLOQ

Amongst the volunteers with a serum CDT concentration below the cut-off (or with a missing result (Table 1, n°18)) and a C-DBS PEth 16:0/18:1 concentration that suggests chronic and excessive alcohol consumption (N=17), 10 (Table 1, n° 18, 32-40, Figure 1) have an EtG concentration in hair that also suggests an alcohol misuse, 4 (Table 1, n°41-44) had not provided a hair sample, while 3 (Table 1, n°45-47) have a measured EtG concentration in hair that suggests alcohol intake but with no suggestion of chronic and excessive alcohol consumption. Three volunteers out of the 50 (Table 1, n°48-50, Figure 1) have an EtG concentration in hair that suggests excessive and chronic alcohol consumption, which is not confirm neither by serum CDT nor by C-DBS PEth 16:0/18:1 results.

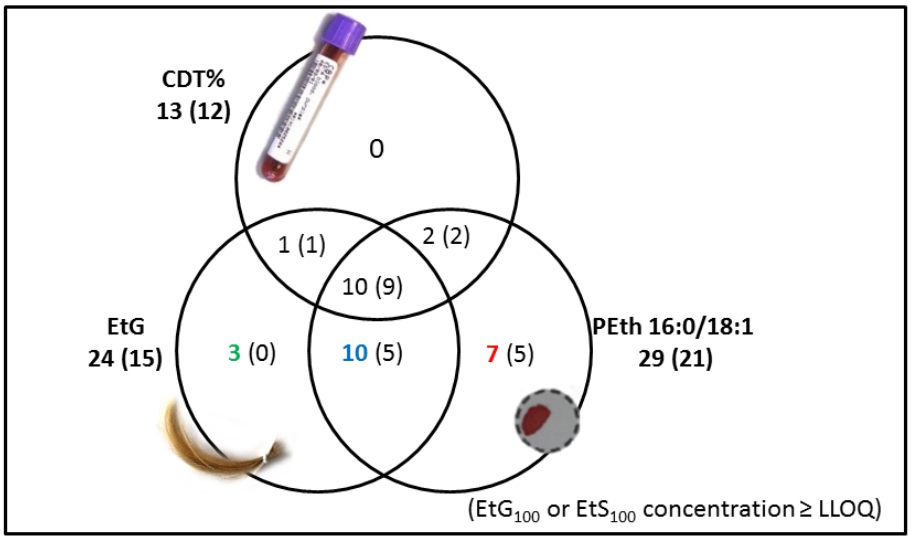

Figure 1: Numbers out of 50 volunteers with results above the cut-off values for CDT% (concentration > 2.4 %), EtG (concentration > 38 pg/mg) and PEth 16:0/18:1 (concentration > 274 ng/mL), suggesting excessive and chronic alcohol consumption. The number of samples with concentrations in urine above or equal to the LLOQ for EtG_{100} (concentration > 121 ng/mL) or EtS_{100} (concentration > 108 ng/mL) is indicated between brackets. CDT%, EtG (hair), PEth (C-DBS) and EtG/EtS (urine) data were available for respectively 49, 43, 50 and 48 volunteers.

Of the 50 volunteers, 13 had been submitted to an abstinence period. Amongst them, EtG_{100} and EtS_{100} exceeded the LLOQ in 4 resp. 3 cases, suggesting a recent alcohol consumption. Out of these 13 cases, strict abstinence can be disproved by a hair EtG concentration above 9 pg/mg and a C-DBS PEth 16:0/18:1 concentration above 12 ng/mL in 5 (3 volunteers did not provide a hair sample) and 7 cases, respectively.

4 Discussion

A cut-off value at 274 ng/mL for PEth 16:0/18:1 was used to suggest excessive and chronic alcohol consumption. Others have proposed a lower (210 ng/mL) or substantially higher (700 or 800 ng/mL) cut-off value (Gnann et al., 2012; Helander and Hansson, 2013; Schrock et al., 2015). On the basis of applying C-DBS PEth 16:0/18:1 cut-offs 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 should be used in those cases. For CDT, despite the guidelines from the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), which advocates the measurement of disialo-transferrin as the single analyte, in this study both asialo-transferrin and disialo-transferrin were used to respect the driving licence regranting process currently used in Belgium. For the same reason, cut-off value used (female/male) in this study (31/37 U/L for AST, 31/40 U/L for ALT and 36/61 U/L for GGT) slightly differs from the internationally accepted ones (31/35 U/L for AST, 34/45 U/L for ALT and 38/55 U/L for GGT) (Schumann and Klauke, 2003).

Some results from the population study performed can be used to illustrate the different information which can be obtained from the quantification of hair EtG, C-DBS PEths and urine EtG/EtS. For the three cases (Table 1, n°45-47) which have C-DBS PEths results that suggest an excessive and chronic alcohol consumption, which is not suggested by the results of EtG in hair, a hypothesis about the evolution of the alcohol consumption pattern can be proposed based on the different information given by these matrices. 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 (Cooper et al., 2012)), and recalling that PEth 16:0/18:1 is detected in an alcohol dependent patient up to 1 month after cessation of alcohol consumption (Wurst et al., 2010), the evolution of the drinking pattern can be inferred. In these 3 cases, an increase of the mean alcohol consumption at least the month prior to the sampling could explain the apparent mismatch of the results provided by C-DBS and hair analysis. 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 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. It seems of interest to added

here that segmental analysis of hair samples in such case would provide relevant supplementary information (Appenzeller et al., 2007; Crunelle et al., 2014; Wurst et al., 2008), 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. 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 the ESM (Figure S1). Using the same reasoning, a mean decrease of alcohol consumption during at least the month prior to sampling could be suggested in the 3 volunteers (Table 1, n°48-50, Figure 1) with hair EtG concentrations that suggest a mean alcohol dependence during the period (between 1.5 and 5 months) prior to the sampling and C-DBS concentrations that do not suggest any alcohol dependence at least the month before the sampling.

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 (Hartmann et al., 2007; Kharbouche et al., 2012; Pirro et al., 2011). Among the 13 CDT-positives results observed in our population study, two (Table 1, n°30-31) had C-DBS PEth 16:0/18:1 and one (Table 1, n°29) had hair EtG concentrations below the cut-off values used to suggest chronic and excessive alcohol consumption, which did not confirm the alcohol misuse suggested by the CDT% result. Among these three exceptions, 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 (Ettlinger et al., 2014).

Our results also confirm the superior specificity of CDT% over other indirect biomarkers (especially GGT, ALT and AST) to detetct chronic and excessive alcohol consumption (Hartmann et al., 2007; Kharbouche et al., 2012; Pirro et al., 2011).

In addition, our study confirms 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 (Agius et al., 2012; Winkler et al., 2013). Nevertheless, and as mentioned by some authors (Albermann et al., 2012b; Winkler et al., 2013), 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 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 (Costantino et al., 2006; Høiseth et al., 2010; Musshoff et al., 2010; Rohrig et al., 2006; Thierauf et al., 2010, 2009) 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 sanitizers), whose consumption or use may give rise to results that are in conflict with strict abstinence (Albermann et al., 2012a).

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 (Appenzeller et al., 2007; Hastedt et al., 2012; Kharbouche et al., 2012; Liniger et al., 2010; Pirro et al., 2011) or blood PEths (Hartmann et al., 2007; Marques et al., 2011; Wurst et al., 2010)). Two studies have compared PEths in blood and EtG/EtS in urine with indirect biomarkers (Helander et al., 2012; Winkler et al., 2013). 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 (Marques et al., 2010; Stadler, 2013). The interesting results of these studies cannot be compared with our results, because both the aim and the procedure differ.

5 Conclusion

To evaluate how the Belgian driver's licence regranting process could be improved, we applied three methods (hair EtG, C-DBS PEths and urine EtG100/EtS100) on 50 real cases, where the fitness to drive had to be assessed using a blood analysis. 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 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 allows to estimate the mean alcohol consumption for up to 6 months prior to sampling. Quantitative results can strongly suggest repeated alcohol consumption or disprove strict abstinence. The three analyses used here provide different levels of information and can be used separately or combined, to obtain a more detailed view on the evolution of the alcohol consumption of a subject. 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. In fine, this approach offers the potential to improve the Belgian driver's licence regranting process.

References

Agius, R., Nadulski, T., Kahl, H.G., Dufaux, B., 2012. Ethyl glucuronide in hair - A highly effective test for the monitoring of alcohol consumption. Forensic Sci. Int. 218, 10–14. doi:10.1016/j.forsciint.2011.10.007

Albermann, M.E., Musshoff, F., Doberentz, E., Heese, P., Banger, M., Madea, B., 2012a. Preliminary investigations on ethyl glucuronide and ethyl sulfate cutoffs for detecting alcohol consumption on the basis of an ingestion experiment and on data from withdrawal treatment. Int. J. Legal Med. 126, 757–764. doi:10.1007/s00414-012-0725-3

Albermann, M.E., Musshoff, F., Madea, B., 2012b. A High-Performance Liquid Chromatographic–Tandem Mass Spectrometric Method for the Determination of Ethyl Glucuronide and Ethyl Sulfate in Urine Validated According to Forensic Guidelines. J. Chromatogr. Sci. 50, 51–56. doi:10.1093/chromsci/bmr012

Albermann, M.E., Musshoff, F., Madea, B., 2010. A fully validated high-performance liquid chromatography-tandem mass spectrometry method for the determination of ethyl glucuronide in hair for the proof of strict alcohol abstinence. Anal. Bioanal. Chem. 396, 2441–2447. doi:10.1007/s00216-009-3388-2

Appenzeller, B.M.R., Agirman, R., Neuberg, P., Yegles, M., Wennig, R., 2007. Segmental determination of ethyl glucuronide in hair: A pilot study. Forensic Sci. Int. 173, 87–92. doi:10.1016/j.forsciint.2007.01.025

Appenzeller, B.M.R., Schneider, S., Maul, A., Wennig, R., 2005. Relationship between blood alcohol concentration and carbohydrate-deficient transferrin among drivers. Drug Alcohol Depend. 79, 261–265. doi:10.1016/j.drugalcdep.2005.01.017

Belgian Royal Decree, 2006. Annexe 14 of the driving licence regulation (2006014056).

Belgian Royal Decree, 1998. Driving licence regulation (1998014078).

Belgian Royal Decree, 1968. Law on police traffic (1968031601).

Bortolotti, F., Tagliaro, F., Cittadini, F., Gottardo, R., Trettene, M., Marigo, M., 2002. Determination of CDT, a marker of chronic alcohol abuse, for driving license issuing: immunoassay versus capillary electrophoresis. Forensic Sci. Int. 128, 53–58. doi:10.1016/S0379-0738(02)00153-6

Cabarcos, P., Álvarez, I., Tabernero, M., Bermejo, A., 2015. Determination of direct alcohol markers: a review. Anal. Bioanal. Chem. 407, 4907–4925. doi:10.1007/s00216-015-8701-7

Cooper, G.A.A., Kronstrand, R., Kintz, P., 2012. Society of Hair Testing guidelines for drug testing in hair. Forensic Sci. Int. 218, 20–24. doi:10.1016/j.forsciint.2011.10.024

Costantino, A., DiGregorio, E.J., Korn, W., Spayd, S., Rieders, F., 2006. The Effect of the Use of Mouthwash on Ethylglucuronide Concentrations in Urine. J. Anal. Toxicol. 30, 659–662. doi:10.1093/jat/30.9.659

Crunelle, C.L., Yegles, M., van Nuijs, A.L.N., Covaci, A., De Doncker, M., Maudens, K.E., Sabbe, B., Dom, G., Lambert, W.E., Michielsen, P., Neels, H., 2014. Hair ethyl glucuronide levels as a marker for alcohol use and abuse: a review of the current state of the art. Drug Alcohol Depend. 134, 1–11. doi:10.1016/j.drugalcdep.2013.10.008

Ettlinger, J., Kirchen, L., Yegles, M., 2014. Influence of thermal hair straightening on ethyl glucuronide content in hair. Drug Test Anal 6 Suppl 1, 74–77. doi:10.1002/dta.1648

European Communities, 2006. Directive 2006/126/EC of the European Parliament and of the Council of 20 December 2006 on driving licences.

Favretto, D., Nalesso, A., Frison, G., Viel, G., Traldi, P., Ferrara, S., 2010. A novel and an effective analytical approach for the LC-MS determination of ethyl glucuronide and ethyl sulfate in urine. Int. J. Legal Med. 124, 161–164. doi:10.1007/s00414-009-0376-1

Gnann, H., 2011. Phosphatidylethanol - Ein Alkoholkonsummarker auf dem Weg in die klinisch-forensische Routinediagnostik. Dissertation, University of Freiburg, Germany. Available from: https://www.freidok.uni-freiburg.de/data/8428. Accessed 06 Janvier 2016.

Gnann, H., Weinmann, W., Thierauf, A., 2012. Formation of Phosphatidylethanol and Its Subsequent Elimination During an Extensive Drinking Experiment Over 5 Days. Alcohol. Clin. Exp. Res. 36, 1507–1511. doi:10.1111/j.1530-0277.2012.01768.x

Hartmann, S., Aradottir, S., Graf, M., Wiesbeck, G., Lesch, O., Ramskogler, K., Wolfersdorf, M., Alling, C., Wurst, F.M., 2007. Phosphatidylethanol as a sensitive and specific biomarker: comparison with gamma-glutamyl transpeptidase, mean corpuscular volume and carbohydrate-deficient transferrin. Addict. Biol. 12, 81–84. doi:10.1111/j.1369-1600.2006.00040.x

Hastedt, M., Herre, S., Pragst, F., Rothe, M., Hartwig, S., 2012. Workplace Alcohol Testing Program by Combined Use of Ethyl Glucuronide and Fatty Acid Ethyl Esters in Hair. Alcohol Alcohol 47, 127–132. doi:10.1093/alcalc/agr148

Helander, A., Böttcher, M., Fehr, C., Dahmen, N., Beck, O., 2009. Detection Times for Urinary Ethyl Glucuronide and Ethyl Sulfate in Heavy Drinkers during Alcohol Detoxification. Alcohol Alcohol 44, 55–61. doi:10.1093/alcalc/agn084

Helander, A., Hansson, T., 2013. National harmonization of the alcohol biomarker PEth. Lakartidningen 110, 1747–1748.

Helander, A., Péter, O., Zheng, Y., 2012. Monitoring of the Alcohol Biomarkers PEth, CDT and EtG/EtS in an Outpatient Treatment Setting. Alcohol Alcohol 47, 552–557. doi:10.1093/alcalc/ags065

Høiseth, G., Yttredal, B., Karinen, R., Gjerde, H., Christophersen, A., 2010. Levels of Ethyl Glucuronide and Ethyl Sulfate in Oral Fluid, Blood, and Urine After Use of Mouthwash and Ingestion of Nonalcoholic Wine. J. Anal. Toxicol. 34, 84–88. doi:10.1093/jat/34.2.84

Kharbouche, H., Faouzi, M., Sanchez, N., Daeppen, J.B., Augsburger, M., Mangin, P., Staub, C., Sporkert, F., 2012. Diagnostic performance of ethyl glucuronide in hair for the investigation of alcohol drinking behavior: a comparison with traditional biomarkers. Int. J. Legal Med. 126, 243–250. doi:10.1007/s00414-011-0619-9

Kintz, P., 2015. 2014 consensus for the use of alcohol markers in hair for assessment of both abstinence and chronic excessive alcohol consumption. Forensic Sci. Int. 249, A1–2. doi:10.1016/j.forsciint.2014.11.001

Kronstrand, R., Brinkhagen, L., Nystrom, F.H., 2012. Ethyl glucuronide in human hair after daily consumption of 16 or 32 g of ethanol for 3 months. Forensic Sci. Int. 215, 51–55. doi:10.1016/j.forsciint.2011.01.044

Kummer, N., Ingels, A.S., Wille, S.M., Hanak, C., Verbanck, P., Lambert, W.E., Samyn, N., Stove, C.P., 2016. 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. Anal. Bioanal. Chem. 408, 825–838. doi:10.1007/s00216-015-9169-1

Kummer, N., Wille, S., Di Fazio, V., Lambert, W.E., Samyn, N., 2013. 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. J. Chromatogr. B 929, 149–154. doi:10.1016/j.jchromb.2013.04.011

Kummer, N., Wille, S.M., Di Fazio, V., Ramírez Fernández, M. del M., Yegles, M., Lambert, W.E., Samyn, N., 2015. Impact of the Grinding Process on the Quantification of Ethyl Glucuronide in Hair Using a Validated UPLC-ESI-MS-MS Method. J Anal Toxicol 39, 17–23. doi:10.1093/jat/bku108

Liniger, B., Nguyen, A., Friedrich-Koch, A., Yegles, M., 2010. Abstinence Monitoring of Suspected Drinking Drivers: Ethyl Glucuronide in Hair Versus CDT. Traffic Inj. Prev. 11, 123–126. doi:10.1080/15389580903518280

Maenhout, T., Baten, G., De Buyzere, M.L., Delanghe, J.R., 2012. Carbohydrate deficient transferrin in a driver's license regranting program. Alcohol Alcohol 47, 253–260. doi:10.1093/alcalc/ags013

Maenhout, T., Poll, A., Vermassen, T., De Buyzere, M.L., Delanghe, J.R., 2014. Usefulness of indirect alcohol biomarkers for predicting recidivism of drunk-driving among previously convicted drunk-driving offenders: results from the Recidivism Of Alcohol-impaired Driving (ROAD) study. Addiction 109, 71–78. doi:10.1111/add.12372

Marques, P., Hansson, T., Isaksson, A., Walther, L., Jones, J., Lewis, D., Jones, M., 2011. Detection of Phosphatidylethanol (PEth) in the Blood of Drivers in an Alcohol Ignition Interlock Program. Traffic Inj. Prev. 12, 136–141. doi:10.1080/15389588.2010.544048

Marques, P., Tippetts, S., Allen, J., Javors, M., Alling, C., Yegles, M., Pragst, F., Wurst, F., 2010. Estimating driver risk using alcohol biomarkers, interlock blood alcohol concentration tests and psychometric assessments: initial descriptives. Addiction 105, 226–239. doi:10.1111/j.1360-0443.2009.02738.x

Musshoff, F., Albermann, M., Madea, B., 2010. Ethyl glucuronide and ethyl sulfate in urine after consumption of various beverages and foods—misleading results? Int. J. Legal Med. 124, 623–630. doi:10.1007/s00414-010-0511-z

Nanau, R., Neuman, M., 2015. Biomolecules and Biomarkers Used in Diagnosis of Alcohol Drinking and in Monitoring Therapeutic Interventions. Biomolecules 5, 1339–1385. doi:10.3390/biom5031339

Pirro, V., Valente, V., Oliveri, P., De Bernardis, A., Salomone, A., Vincenti, M., 2011. Chemometric evaluation of nine alcohol biomarkers in a large population of clinically-classified subjects: pre-eminence of ethyl glucuronide concentration in hair for confirmatory classification. Anal. Bioanal. Chem. 401, 2153–2164. doi:10.1007/s00216-011-5314-7

Rohrig, T.P., Huber, C., Goodson, L., Ross, W., 2006. Detection of ethylglucuronide in urine following the application of Germ-X. J. Anal. Toxicol. 30, 703–704. doi:10.1093/jat/30.9.703

Schellenberg, F., Girre, C., Nalpas, B., Plat, A., Tome, A., Pagès, J.C., 2007. Analytical evaluation of a new capillary electrophoresis method for carbohydrate-deficient transferrin measurement. Clin. Chim. Acta 382, 48–53. doi:10.1016/j.cca.2007.03.020

Schrock, A., Hernandez Redondo, A., Martin Fabritius, M., Konig, S., Weinmann, W., 2015. Phosphatidylethanol (PEth) in blood samples from “driving under the influence” cases as indicator for prolonged excessive alcohol consumption. Int J Leg. Med In Press. doi:10.1007/s00414-015-1300-5

Schröck, A., Thierauf, A., Wurst, F.M., Thon, N., Weinmann, W., 2014. Progress in monitoring alcohol consumption and alcohol abuse by phosphatidylethanol. Bioanalysis 6, 2285–2294. doi:10.4155/bio.14.195

Schumann, G., Bonora, R., Ceriotti, F., Ferard, G., Ferrero, C.A., Franck, P.F., Gella, F.J., Hoelzel, W., Jorgensen, P.J., Kanno, T., Kessner, A., Klauke, R., Kristiansen, N., Lessinger, J.M., Linsinger, T.P., Misaki, H., Panteghini, M., Pauwels, J., Schiele, F., Schimmel, H.G., Weidemann, G., Siekmann, L., 2002a. IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 degrees C. International Federation of Clinical Chemistry and Laboratory Medicine. Part 6. Reference procedure for the measurement of catalytic concentration of gamma-glutamyltransferase. Clin Chem Lab Med 40, 734–738. doi:10.1515/CCLM.2002.126

Schumann, G., Bonora, R., Ceriotti, F., Ferard, G., Ferrero, C.A., Franck, P.F., Gella, F.J., Hoelzel, W., Jorgensen, P.J., Kanno, T., Kessner, A., Klauke, R., Kristiansen, N., Lessinger, J.M., Linsinger, T.P., Misaki, H., Panteghini, M., Pauwels, J., Schiele, F., Schimmel, H.G., Weidemann, G., Siekmann, L., 2002b. IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 degrees C. International Federation of Clinical Chemistry and Laboratory Medicine. Part 4. Reference procedure for the measurement of catalytic concentration of alanine aminotransferase. Clin Chem Lab Med 40, 718–724. doi:10.1515/CCLM.2002.124

Schumann, G., Bonora, R., Ceriotti, F., Ferard, G., Ferrero, C.A., Franck, P.F., Gella, F.J., Hoelzel, W., Jorgensen, P.J., Kanno, T., Kessner, A., Klauke, R., Kristiansen, N., Lessinger, J.M., Linsinger, T.P., Misaki, H., Panteghini, M., Pauwels, J., Schiele, F., Schimmel, H.G., Weidemann, G., Siekmann, L., 2002c. IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 degrees C. International Federation of Clinical Chemistry and Laboratory Medicine. Part 5. Reference procedure for the measurement of catalytic concentration of aspartate aminotransferase. Clin Chem Lab Med 40, 725–733. doi:10.1515/CCLM.2002.125

Schumann, G., Klauke, R., 2003. New IFCC reference procedures for the determination of catalytic activity concentrations of five enzymes in serum: preliminary upper reference limits obtained in hospitalized subjects. Clin Chim Acta 327, 69–79. doi:10.1016/S0009-8981(02)00341-8

Stadler, A., 2013. Phosphatidylethanol compared to established indirect and direct markers of alcohol consumption. Toxichem Krimtech 80, 168–177.

Thierauf, A., Gnann, H., Wohlfarth, A., Auwarter, V., Perdekamp, M.G., Buttler, K.J., Wurst, F.M., Weinmann, W., 2010. Urine tested positive for ethyl glucuronide and ethyl sulphate after the consumption of “non-alcoholic” beer. Forensic Sci. Int. 202, 82–85. doi:10.1016/j.forsciint.2010.04.031

Thierauf, A., Wohlfarth, A., Auwärter, V., Perdekamp, M.G., Wurst, F.M., Weinmann, W., 2009. Urine tested positive for ethyl glucuronide and ethyl

sulfate after the consumption of yeast and sugar. Forensic Sci. Int. 202, e45–47. doi:10.1016/j.forsciint.2010.06.028

Winkler, M., Skopp, G., Alt, A., Miltner, E., Jochum, T., Daenhardt, C., Sporkert, F., Gnann, H., Weinmann, W., Thierauf, A., 2013. Comparison of direct and indirect alcohol markers with PEth in blood and urine in alcohol dependent inpatients during detoxication. Int. J. Legal Med. 127, 761–768. doi:10.1007/s00414-012-0812-5

World Health Organization, 2001. The Alcohol Use Disorders Identification Test (AUDIT): Guidelines for use in primary care (Second Edition).

Wurst, F.M., Thon, N., Aradottir, S., Hartmann, S., Wiesbeck, G.A., Lesch, O., Skala, K., Wolfersdorf, M., Weinmann, W., Alling, C., 2010. Phosphatidylethanol: normalization during detoxification, gender aspects and correlation with other biomarkers and self-reports. Addict. Biol. 15, 88–95. doi:10.1111/j.1369-1600.2009.00185.x

Wurst, F.M., Yegles, M., Alling, C., Aradottir, S., Dierkes, J., Wiesbeck, G., Halter, C., Pragst, F., Auwaerter, V., 2008. Measurement of direct ethanol metabolites in a case of a former driving under the influence (DUI) of alcohol offender, now claiming abstinence. Int. J. Legal Med. 122, 235–239. doi:10.1007/s00414-007-0218-y

Supplementary information

Number of pages: 6

Number of tables: 4

Number of figures: 1

Partial validation procedure

The quantitative methods for EtG and EtS in urine and EtG in hair have been fully validated on an Acquity UPLC® coupled to a Xevo TQ MS tandem mass spectrometer (Waters, Manchester, UK). The validation results have been published (Kummer et al., 2015, 2013). For the present study, samples were also analysed on an Acquity UPLC® coupled to a Xevo TQ S tandem mass spectrometer (Waters, Manchester, UK). Because of the use of another tandem mass spectrometer, the LLOQ, linearity, accuracy and reproducibility were validated (Wille et al., 2011). The calibration model and weighting factor were evaluated. 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 established as the difference between the calculated calibrator value and its nominal value. The relative errors should be lower than 15% except for the LLOQ (< 20%) (Committee for medicinal products for human use (CHMP) and European medicines agency (EMEA), 2011). Accuracy was measured through the determination of bias and precision (repeatability and intermediate precision). Internal spiked and external QCs were analysed in replicates on 8 different days. For EtG and EtS in urine, two external quality controls from Medichem (Steinenbronn, Germany), Medidrug ETG 3/10 and Medidrug ETG 2/12, were used. For EtG in hair, one external QC sample, EGH 2/12 A HA, from ACQ Science GmbH (Rottenburg-Hailfingen, Germany) was used. A single factor ANOVA test with significance level (α) of 0.05 was performed to calculate the bias, repeatability and intermediate precision. These results were acceptable when they were below 15% (20% at the LLOQ). The reproducibility has been evaluated by successful (z-score<2) participation in proficiency tests. For the quantification of EtG and EtS in urine, proficiency tests organised by GTFCh were performed. For EtG in hair, 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, were performed.

Validation results

EtG in hair

Using a more sensitive apparatus (XEVO TQ S) 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. 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, 5, 10, 20, 50, 250 and 500 pg/mg hair. The bias (%), repeatability (%RSD_r) and intermediate precision (%RSD_t) for internal and external QCs were less than 12% (Table S1).

Table S1: Validation results for the quantification of EtG in hair using a Xevo TQ S tandem mass spectrometer.

	Nominal value (pg/mg)	RSDr (%)	RSDt (%)	Bias (%)
LOQ	2.0	8	10	6
Low	6.0	3	9	3
Medium	30.0	2	7	8
High	300.0	3	4	8
EGH 2/12 A HA	25.4	5	12	-9

Results of the proficiency tests for EtG in hair are presented in Table S2.

Table S2: Proficiency test results for EtG and EtS in urine using a Xevo TQ S tandem mass spectrometer.

	Sample A		Sample B	
	z-score	Target value (pg/mg hair)	z-score	Target value (pg/mg hair)
SoHT 2014	-0.38	8.7	-0.44	29.4
GTFCh 3/14	-1.03	11.2	-1.10	26.0
GTFCh 2/15	-0.74	23.1	-0.77	44.0

EtG and EtS in urine

The results for the validation of the method, on the Acquity UPLC® coupled to a Xevo TQ S tandem mass spectrometer, are presented here. 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. The bias (%), repeatability (%RSD_r) and intermediate precision (%RSD_t) were calculated for the internal and external QCs and were less than 12% (Table S3).

Table S3: Validation results for the quantification of EtG and EtS in urine using a Xevo TQ S tandem mass spectrometer.

	Nominal value (ng/mL)	RSD_r (%)	RSD_t (%)	Bias (%)
EtG				
Low	300	3	4	-8
Medium	4000	2	4	-6
High	7500	3	4	-3
EtG3/10	1270	2	2	-3
EtG 2/12	556	1	5	1
EtS				
Low	300	5	8	-5
Medium	4000	4	4	-3
High	7500	5	6	-1
EtG 3/10	810	2	2	-8
EtG 2/12	1270	3	9	-12

Results of the proficiency tests for EtG and EtS in urine are presented in Table S4.

Table S4: Proficiency test results for EtG and EtS in urine using a Xevo TQ S tandem mass spectrometer.

	EtG		EtS	
	z-score	Target value (ng/mL)	z-score	Target value (ng/mL)
GTFCh 2/14	-0.04	621	0	1230
GTFCh 3/14	-0.19	1350	-0.69	715
GTFCh 1/15	0.35	945	-1.04	796
GTFCh 2/15	-0.77	1150	-0.59	1440

Flowchart of direct EtOH markers and CDT% in fitness-to-drive assessment

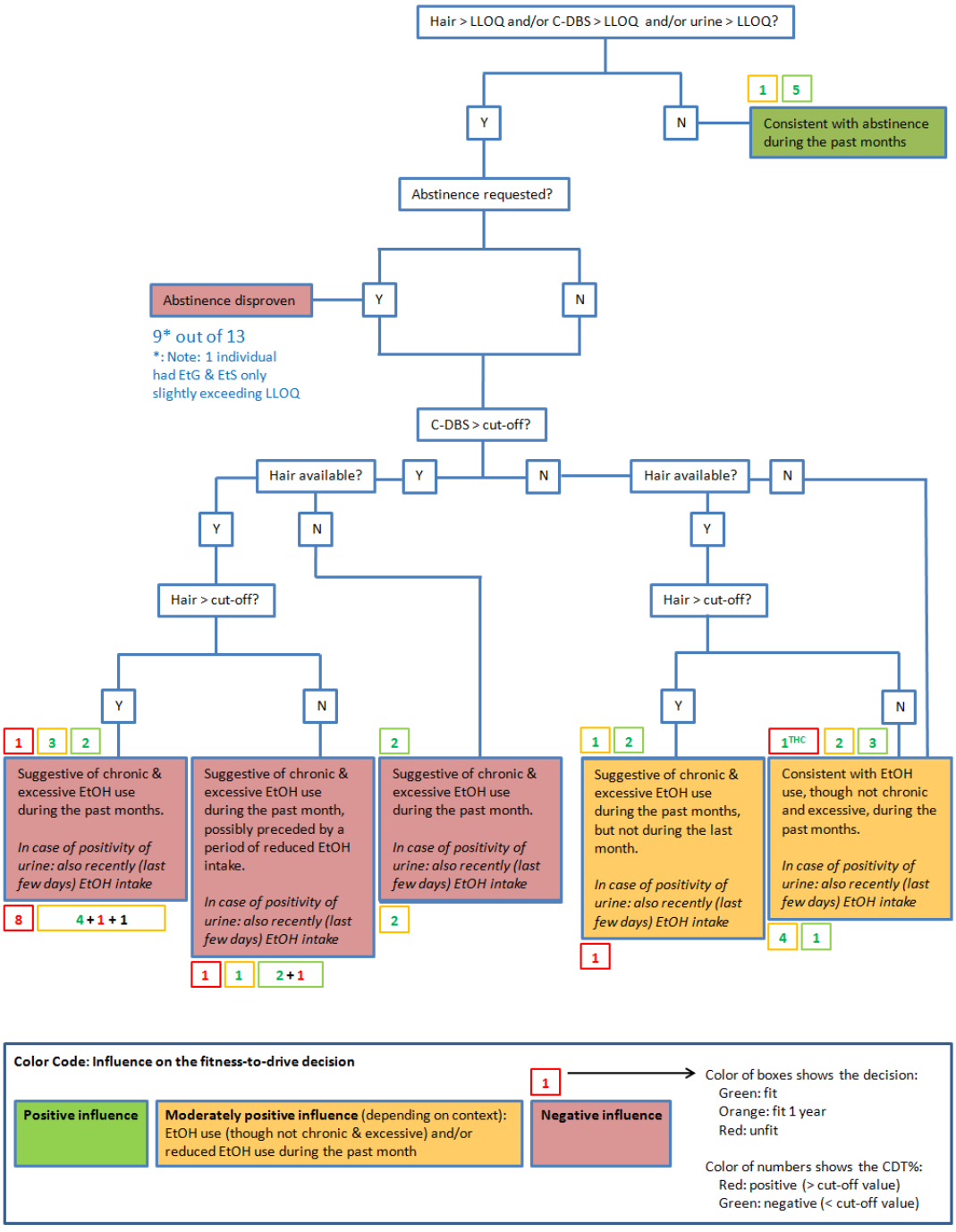

Figure S1: Flowchart showing the influence of the results of EtG in hair, Peth 16:0/18:1 in C-DBS and EtG_{100} and EtS_{100} in urine on the fitness to drive decision. The number of cases observed in the 50 volunteers of our study is indicated above (cases with negative urine sample for EtG or EtS) or below each category (cases with positive urine sample for EtG or EtS).

References

Committee for medicinal products for human use (CHMP), European medicines agency (EMEA), 2011. Guideline on validation of bioanalytical methods (Document 192217/2011). Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf. Accessed 15 June 2015.

Kummer, N., Wille, S., Di Fazio, V., Lambert, W., Samyn, N., 2013. 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. J. Chromatogr. B 929, 149–154. doi:10.1016/j.jchromb.2013.04.011

Kummer, N., Wille, S.M., Di Fazio, V., Ramírez Fernández, M. del M., Yegles, M., Lambert, W.E.E., Samyn, N., 2015. Impact of the Grinding Process on the Quantification of Ethyl Glucuronide in Hair Using a Validated UPLC-ESI-MS-MS Method. J Anal Toxicol 39, 17–23. doi:10.1093/jat/bku108

Wille, S., Peters, F., Di Fazio, V., Samyn, N., 2011. Practical aspects concerning validation and quality control for forensic and clinical bioanalytical quantitative methods. Accreditation Qual. Assur. 16, 279–292. doi:10.1007/s00769-011-0775-0