Alternative sampling strategies for the assessment of alcohol intake of living persons

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Abstract Monitoring of alcohol consumption by living persons takes place in various contexts, amongst which workplace drug testing, driving under the influence of alcohol, driving licence regranting programs, alcohol withdrawal treatment, diagnosis of acute intoxication or fetal alcohol ingestion. The matrices that are mostly used today include blood, breath and urine. The aim of this review is to present alternative sampling strategies that allow monitoring of the alcohol consumption in living subjects. Ethanol itself, indirect (carbohydrate deficient transferrin, CDT%) as well as direct biomarkers (ethyl glucuronide, EtG; ethyl sulphate, EtS; fatty acid ethyl esters, FAEEs and phosphatidylethanol species, PEths) of ethanol consumption will be considered. This review covers dried blood spots (CDT%, EtG/EtS, PEths), dried urine spots (EtG/EtS), sweat and skin surface lipids (ethanol, EtG, FAEEs), oral fluid (ethanol, EtG), exhaled breath (PEths), hair (EtG, FAEEs), nail (EtG), meconium (EtG/EtS, FAEEs), umbilical cord and placenta (EtG/EtS and PEth 16:0/18:1). Main results, issues and considerations specific to each matrix are reported. Details about sample preparation and analytical methods are not within the scope of this review.

Keywords Alcohol; Review; Dried blood spots; Dried urine spots; Sweat; Skin surface lipids; Oral fluid; Exhaled breath; Hair; Nail; Meconium; Umbilical cord; Placenta

Contents

1 Introduction

Alcohol is a legal psychoactive substance that has been widely used in many cultures for centuries. The severity of an alcohol intoxication is related to the volume of alcohol consumed and to the drinking pattern. Alcohol consumption can lead to impairment of physical coordination, consciousness, cognition, perception or behaviour [1] and is therefore not compatible with some professional activities or with driving a vehicle. Prenatal alcohol exposure, induced by alcohol consumption during pregnancy, can engender consequences on the newbo0rn, such as distinctive craniofacial dysmorphology, growth retardation, common cognitive disorders, and social impairment [2]. Chronic misuse of alcohol can cause diseases, while acute intoxications may result in coma and death or be a contributing factor resulting in death. Moreover, alcohol use may induce social and economic problems.

After consumption, ethanol is readily absorbed from the stomach and from the small intestine (duodenum and jejunum) into the blood stream [3]. 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 [4]. 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 non-oxidative metabolism of ethanol results in the formation of ethyl glucuronide (EtG), ethyl sulphate (EtS), phosphatidylethanol species (PEths) and fatty acid ethyl esters (FAEEs) (Figure 1).

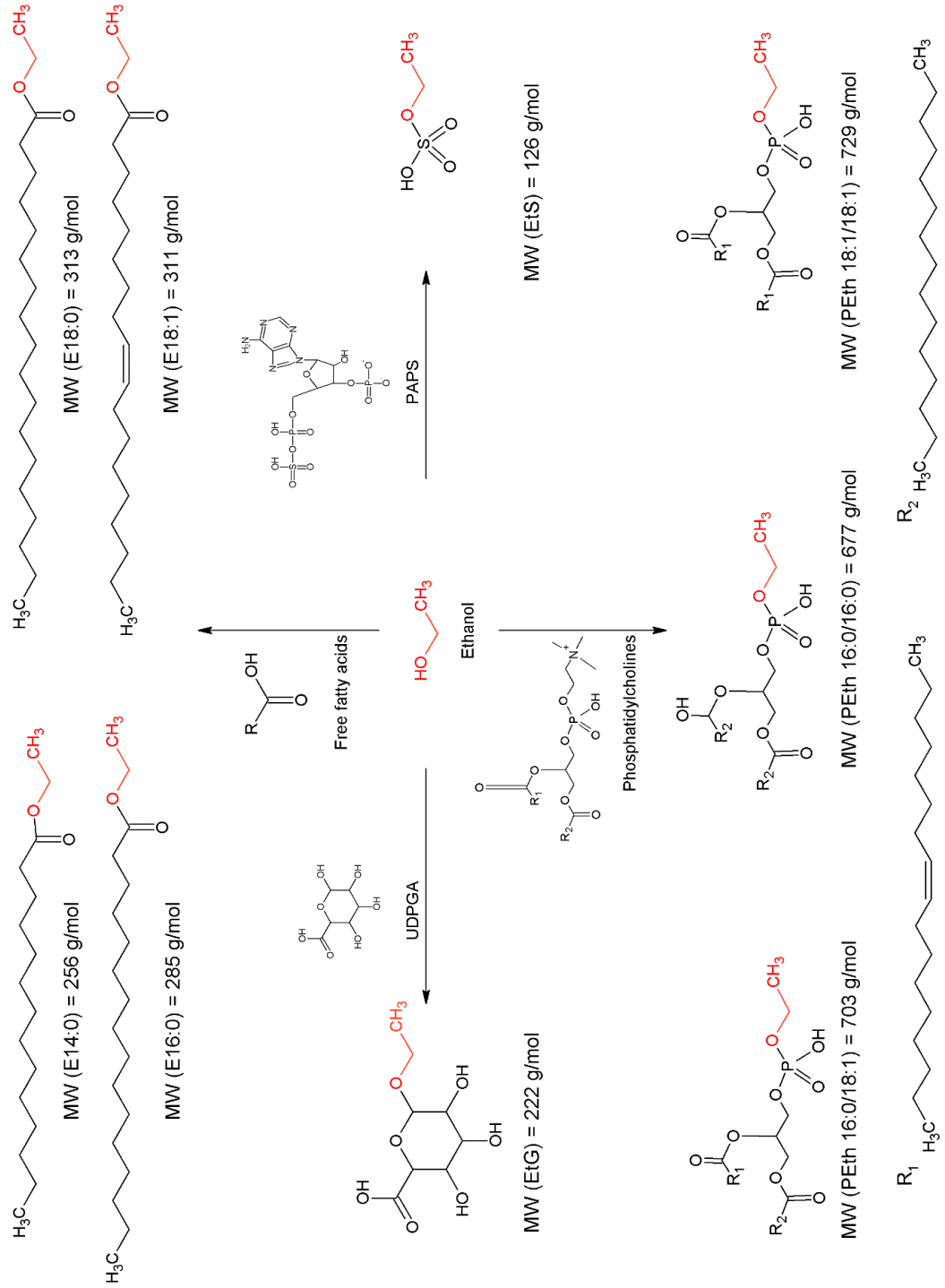

Figure 1: Non-oxidative phase II metabolism of ethanol into EtG, EtS, PEths (PEth 16:0/18:1, PEth 18:1/18:1 and PEth 16:0/16:0) and FAEEs (ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl stearate (E18:0) and ethyl oleate (E18:1)), with indication of the molecular weight (MW).

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 enzymes [5]. About 0.02 % of consumed ethanol is excreted in urine as EtG [6,7]. Sulfation of ethanol is a phase II conjugation reaction with PAPS (3'-phosphoadenosine 5'-phosphosulphate) through the action of cytosolic sulfotransferase [8]. Only 0.01-0.02 % of the consumed ethanol is excreted in urine as EtS on a molar basis [9,10].

EtG and EtS are two small, polar and acidic metabolites of ethanol [11]. The pKa of EtG was estimated between 2.84 and 3.21 [11–13] and of EtS at -3.14 [11].

PEths are a group of abnormal phospholipids formed in the presence of ethanol, via the action of phospholipidase D, which normally hydrolyses phosphatidylcholine into phosphatidylic acid and choline in cell membranes [14]. Up to 48 different PEths have been detected in blood collected during autopsy of heavy drinkers [15]. 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 [16], PEth 16:0/18:1 and PEth 16:0/18:2 are the two predominant PEths detected [15–18]. 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 % [19]. While some methods (e.g. HPLC coupled to light-scattering detection [20–23] and non-aqueous capillary electrophoresis coupled to UV detection) [24] measure the total amount of PEths, other methods (such as LC-MS/MS) are able to identify and quantify individual molecular species [16–18,25–28]. The three commercially available PEths are presented in Figure 1.

FAEEs are a group of more than 20 substances formed by enzymatic esterification of ethanol and free fatty acids. Ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl stearate (E18:0) and ethyl oleate (E18:1) (Figure 1) are the most studied FAEEs.

Different enzymes (i.e. FAEE synthase, acyl-CoA: ethanol O-acyltransferase, 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 [29]. Politi

et al. in 2007 and Cabarcos *et al.* in 2015 have published interesting reviews about the detection of FAEEs in biological samples [2,30].

After excessive and chronic alcohol consumption, ethanol can induce indirect effects on the body via its interference with liver function (increased carbohydrate deficient transferrin (CDT%), gamma-glutamyltransferase (GGT), aspartate aminotransferase/alanine aminotransferase (AST/ALT)) and its effect on the size of red blood cells (increased mean corpuscular volume (MCV)).

Up to now, indirect biomarkers of alcohol measured in whole blood (MCV) or serum (CDT%, GGT, ALT, AST) are traditionally used to detect alcohol dependence. Monitoring of alcohol consumption is most often performed in breath, blood and urine. Blood alcohol concentration (BAC) is of particular interest, due to its correlation with the effect of alcohol. To avoid venepuncture sampling 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 [31]. Analysis of ethanol and/or EtG/EtS in urine 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.

Besides breath, blood and urine for the determination of ethanol itself and blood and urine for the detection of direct and indirect markers of ethanol use, the alcohol consumption of a living person can also be monitored via so-called 'alternative sampling strategies'. These will be the subject of this review, which will discuss 'classical' samples obtained from a living person via an alternative way (e.g. dried blood spots (DBSs)), as well as 'alternative' samples (i.e. different from blood, plasma, serum or urine). Amongst the alternative sampling strategies covered are the sampling of dried blood and urine spots (DUSs), as well as the sampling of sweat/skin surface lipids, oral fluid, exhaled breath, hair, meconium, umbilical cord and placenta. Post-mortem matrices (e.g. bone, muscle, bone marrow, adipose tissue, and vitreous humour) [32–35] 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 [2,14,19,30,36–45].

2 Biologial matrices of interest

2.1 Dried blood spots

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 [46]. 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 [47].

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 localization and spot volume on the quantitative result [48,49]. To cope with a possible bias imposed by deviating hematocrit values, different strategies have been proposed [47]. These include volumetric deposition using special devices followed by full-spot analysis, the use of special filter paper and normalisation following hematocrit prediction [47]. Another issue in DBS analysis is how to apply an internal standard (IS) to a dried matrix spot [50]. 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 [50].

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 [44].

2.1.1 CDT%

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 [51]. CDT% in DBS was demonstrated to be stable for up to 2-3 days at room temperature, and 2 weeks at 4° C or frozen (-20° C). In 2014, Bertaso *et al.* [52] 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 localization 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 [47].

2.1.2 EtG and EtS

After the consumption of 0.50-0.78 g ethanol/kg body weight, EtG and EtS remained detectable in blood for up to more than 10 h (with a detection window twice the one for ethanol) [53]. While multiple methods for the quantification of EtG and EtS in blood have been published [6,12,54–62], there is currently only one report on their quantification in DBS [63]. That study utilized 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 [63]. 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 [63].

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 [64]. 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” [65].

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 localization and spot volume on the analytical result is needed.

2.1.3 PEth species

PEths peak concentrations appear between the 3rd 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 [66]. PEths have been widely analysed in blood [15–18,24,26,27,66–75], 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 [25,26,67] and in C-DBS [46,67]. Both whole-spot [26,71] and partial-spot approaches [25,46,67,76] have been described. In all methods the IS was added to the extraction solvent. The extraction efficiency was reported between 55 and 78 % for V-DBS and C-DBS, respectively [67]. Stability was demonstrated for up to 6 months for DBS stored in zip-closure plastic bags containing a desiccant packet at room temperature [67]. One-way ANOVA tests did not reveal a significant influence ($p>0.05$) of the hematocrit (range 0.39-0.57), punch localization and spot volume on quantification of the evaluated PEths [67]. Comparison between concentrations measured in blood and in DBS has been performed using Wilcoxon signed rank test analyses [67], Bland-Altman analyses [25,26,67], linear regression [25] and Passing-Bablok regressions [67]. All studies concluded that determination of PEths in DBS is a useful tool to monitor alcohol misuse. Especially in the context of driving license regranting programs, where in many instances the indirect marker CDT% is used, the use of C-DBS for PEths monitoring seems promising [77]. 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 [78,79]. 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 driving license regranting programs [77].

2.2 Dried urine spots

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 [46].

2.2.1 EtG and EtS

Though widely analysed in urine [5–7,9,11,12,57,60–62,80–107], 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 [108]. While EtG in urine may be subject to degradation [109,110] or post-collection formation [111], these phenomena have not been observed for EtS [111–113]. 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. The 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) [114]. 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 volumetric DBS sampling (volumetric absorptive microsampling and microfluidics) could turn out useful for this [115].

2.3 Sweat and skin surface lipids

The human skin 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 gland).

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 [42]. 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 [116]. 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 [42].

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 [117]. Sebum, secreted by the sebaceous gland in humans, is primarily composed of triglycerides (~41 %), wax esters (~26 %), squalene (~12 %), and free fatty acids (~16 %) [118]. A transition time of about 1 week has been reported between the sebum production and its appearance at the skin surface [119].

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 [119]. 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 [34]. This can be performed by using pre-weighed patches/wipes or by measuring the sodium content in the sweat [120] or the squalene (or total lipid) content in the sebum [119,121,122]. For the detection of volatile compounds, electrochemical methods that convert vapours into an electrical signal proportional to the concentration have been proposed [123,124]. Depending on the device, monitoring can be based on successive or continuous measurements. These electrochemical devices are strapped on the forearm, wrist or ankle.

2.3.1 Ethanol

In the eighties, studies (described in a review published in 2006 [43]) 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 [125–128], other studies quantified ethanol in vapours formed above the skin (insensible perspiration) [129–133]. In the nineties, electrochemical devices such as the Secure Continuous Remote Alcohol Monitor (SCRAM) bracelet Device [134–136] and the WrisTAS device [134,137,138] 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 [123,134]. Research to date has shown that transdermal alcohol sensors allow the continuous remote monitoring of (absence of) alcohol consumption, without an intrusive daily contact [123] such as required by BrAC or BAC measurements. Variable correlations -from poor to good- have been reported, between TAC and BrAC/BAC peak concentrations [136,139], between the area under the curve (AUC) for TAC and BrAC/BAC [136,139], between self-reported alcohol consumption and TAC AUC [140] and between self-reported alcohol consumption and peak TAC [140]. 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 [134,141]) 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 [134] and a water accumulation issue for the SCRAM [134]. 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) [134,143]. The good specificity was confirmed in a study published in 2009 [135], 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 [144] and SCRAM II [135,142,145,146]), as well as other biosensing devices [124], 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 [124,137,141,142,144]. 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 [142]), rather than to really monitor an alcohol abstinence period [141]. More recently, the SCRAMx (third generation of SCRAM devices) became commercially available.

2.3.2 EtG

In 2008, Schummer *et al*. reported on the quantification of EtG in sweat [34]. 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 review.

2.3.3 FAEEs

Concentrations of FAEEs in skin surface lipids (sebum) of teetotallers, social drinkers and alcoholics were estimated using patches [119,121,122] or wipe tests [119]. The endogenous concentration of FAEEs was reported up to 13.85 pg/mg sebum [122] or 1.12 ng/µg squalene [119]. 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 FAEE 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 [122]. Using a wipe test, concentrations between 0.08 and 1.56 ng/µg squalene were reported for alcohol drinkers without dependence (N=16) [119] 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 [119] and up to 1243.40 pg/mg sebum using patches have been reported [122]. 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.

2.4 Oral fluid

Oral fluid consists of saliva (the aqueous secretion produced by the three pairs of major salivary glands), other secretes, as well as other (solid) constituents. It is composed of mainly water (99 %), with 0.3 % protein (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.* [116] and in 2005 by Aps and Martens [147]. The pH of unstimulated saliva ranges from 2.6 to 7 and -in case of stimulation- increases up to 8 [116]. The collection of oral fluid can be performed by spitting, expectoration, or absorption in a swab.

2.4.1 Ethanol

The detection of ethanol in oral fluid has been widely performed using enzymatic screening [148–154,4], while the quantification using chromatographic methods [155–157] is not commonly used. A good correlation (r=0.98) between the concentration of ethanol in blood and oral fluid has been observed [31]. Concentration-time profiles of ethanol in oral fluid, blood and breath are similar [155,157], with a mean oral fluid-blood ratio estimated at 1.09:1 (range 0.96:1-1.23:1) [12]. 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 [158]. Ethanol concentrations up to 4.8 g/L were measured in oral fluid from alcohol abusers [4]. 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 [148]. 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 [148–154] 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.

2.4.2 EtG

The quantification of EtG in oral fluid has been reported in a few publications [12,62,159] and is based on a method published in 2009 by Hegstad *et al*. [160]. 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 [12]. After consumption of 1.0 g ethanol/kg body weight, 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) [62]. 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 [160].

Even though some publications have used the quantification of EtG in oral fluid [12,62,159,160], only one has reported on the validation [160]. 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.

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. 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.* [161]. 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.

2.5.1 PEth species

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 [79]. 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 (P = 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 normalize 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.

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 [41]. 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.

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 (bleaching, UV radiation) or by extraction (shampooing and hair cosmetic treatments).

Hair grows in a cycle composed of 3 stages, 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 [162]. The preferred sampling site is the vertex posterior part of the scalp, because it contains less telogen hair and a relatively uniform growth rate. 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 [163]. 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.

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 [163]. 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 pulverized. 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 [163]. The detection of ethanol itself in hair is not possible due to its volatility and potential absorption via external contamination. Therefore, the focus has been on the detection of direct ethanol markers [163].

2.6.1 EtG

The quantification of EtG in hair has been reported in numerous publications [37,93,114,164–178] and has been reviewed in 2008 by Pragst and Yegles [179] and in 2014 by Crunelle *et al*. [38]. The incorporation mechanism of EtG in hair has not been totally explained yet, but due to its acidic and extremely hydrophilic properties only a weak incorporation rate occurs in hair via diffusion from blood and deposition from sweat and sebum [41,114,180]. 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) [178]. EtG was detected in hair of patients in alcohol withdrawal studies in concentrations up to 528 pg/mg hair [181].

The SoHT has published guidelines [163] 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 [182]. Bleaching, perming, straightening and dyeing of hair are known to decrease the concentration of EtG in hair, whereas other cosmetic hair treatments (use of hair spray, gel, wax, oil or grease) were reported to have no effect [163,183], 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) [184]. Extraction experiments show that the time required to extract EtG from hair is decreased when the matrix is pulverized [165] and that the concentration determined in pulverized hair samples was higher than in cut hair samples [164,166,176,185]. The 2014 SoHT guideline states that "powdering hair prior to the extraction of EtG is preferred. Laboratories utilizing other sample preparation procedures should demonstrate comparable recovery of EtG" [163]. An interesting publication has demonstrated that the washing and the nature of extraction solvents influences the quantification of EtG in hair [186]. Additional guidelines to streamline washing and extraction procedures may be useful to decrease the variations observed between reported EtG concentrations from different laboratories [176]. 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.

2.6.2 FAEEs

Numerous studies about the quantification of FAEEs in hair have been published [37,171,179,186–199]. Because of their lipophilic character, most authors explain the incorporation of FAEEs into the hair mainly through sebum [2]. FAEE concentrations increase from the proximal region to the distal [179] and - according to recent publications- decrease after 5–10 cm in length [194]. 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. Cosmetics containing ethanol can increase FAEEs concentrations in hair and lead to false positive results. An interesting review has been published in 2008 by Pragst and Yegles [179].

Hair melanin content does not influence the concentration of FAEEs [182]. Bleaching and perming hair may influence the concentration of FAEE in hair [163], while dyeing has been shown to decrease the FAEEs concentration in hair [191].

The concentration of FAEEs in teetotallers was between 0.06 and 0.37 ng/mg [194]. 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

[2]. 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) [163]. 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 [191,196].

After alcohol consumption (self-reported ethanol consumption up to 20 g/day), the mean 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) [194]. FAEE concentrations up to 11.6 ng/mg hair were measured in hair of patients in alcohol withdrawal studies [68]. 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) [163].

2.7 Nail

Nail is a solid keratinized 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 [39]. 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 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) [39]. 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 a fingernail 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).

2.7.1 EtG

Since 2012, quantitative methods for EtG in nails have been reported [32,200–203], as reviewed by Cappelle *et al.* [39]. 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 [201]. The EtG concentration in nails from alcohol abusers ranges between 40 and 91 pg/mg [32]. In 529 students, 203 nail samples had quantifiable (≥ 8 pg/mg) EtG concentrations [200], 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 (i.e. influence of nail polish used) 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.

2.8 Meconium

Meconium is the first fecal matter of a newborn, which starts to accumulate in the fetal bowel from the 12^{th} to 16^{th} week of gestation [204]. It is a complex matrix composed of water, mucopolysaccharides, bile salts, bile acids, epithelial cells and residues of swallowed amniotic fluid. The detection of compounds in meconium reflects the consumption of the mother during the last 20 weeks of gestation. Sampling can be performed between 1 and 5 days after birth by scraping the contents of the soiled diaper. Contamination via urine is an issue that needs to be taken into account for the interpretation. The analysis starts by extracting compounds from meconium by solubilisation. A review about the detection of biomarkers of alcohol exposure in meconium has been published in 2012 [14].

2.8.1 EtG and EtS

In 2008, the first method to quantify EtG and EtS in meconium was published [205] and was rapidly followed by other reports [33,203,206–211]. Transplacental transfer of EtG and EtS has been suggested, meaning that their detection in meconium can be due to transfer from the mother in addition to -or instead of- metabolism of ethanol by the foetus [212]. Based on the analysis of meconium samples (N=110) from newborns not exposed to alcohol during *in utero* development, endogenous values up to 1.87 nmol/g (415 ng/g) for EtG and up to 0.03 nmol/g (3.78 ng/g) for EtS were detected [209]. Different cut-off values to detect prenatal exposure to ethanol have been suggested, at 1.5 or 2 nmol/g (333 or 444 ng/g) [33,207] for EtG and at 0.012 nmol/g (1.512 ng/g) [33] for EtS. Further studies to fix a consensus value are still needed.

2.8.2 FAEEs

Since 1999, when the first methods were published [213,214], quantification of FAEEs in meconium has been studied [203,211,215–223] and reviewed [14,36,216,224]. Important findings were: i) FAEEs do not cross the placenta and

so are only produced by the foetus from the alcohol present in the blood stream of the mother; ii) 75 % of the FAEEs detected in meconium originate from the last 8 weeks of pregnancy; iii) the decision on cut-off values for FAEEs to distinguish prenatal alcohol exposure from exposure to endogenous ethanol or to ethanol present in common food is necessary [14]. Different FAEEs (laurate (E12:0), myristate (E14:0), palmitate (E16:0), palmitoleate (E16:1), stearate (E18:0), oleate (E18:1), linoleate (E18:2), linoleneate (E18:3), arachidonate (E20:4) and docosahexanoate (E22:6) acid ethyl esters) are measured and considered for the cumulative FAEE concentration [211]. Different cut-off values have been proposed and the ones based on cumulative FAEEs [216,217,223] have been said [211] to be more efficient than the ones based on only one FAEE [213,218,215]. Amongst those based on cumulative FAEEs, some are based on 3 FAEEs (E14:0, E16:0, E18:0) at 600 ng/g, [210], 4 FAEEs (E16:0, E18:0, E18:1, E18:2) at 500 ng/g [223] and 2.0 nmol/g (~600 ng/g [211]) [216,219], on 6 FAEEs (E16:0, E16:1, E18:0, E18:1, E18:2, E20:4) at > 10000 ng/g [211], and on 7 FAEEs (E16:0, E16:1, E18:0, E18:1, E18:2, E18:3, E20:4) at 2.0 nmol/g (~600 ng/g) [225,216,226,221,205,227]. This last cut-off value is internationally accepted to differentiate heavy (seven or more drinks per week or five or more drinks per occasion (i.e. “binge” drinking) [216,221,227]) maternal alcohol consumption during pregnancy from occasional use or no use at all [205].

2.9 Umbilical cord and placenta

Following every birth, umbilical cord and placenta are available for diagnostic analysis. One study has developed a method for the quantification of EtG and PEth 16:0/18:1 in umbilical cord [228]. From 308 specimens, 5 contained quantifiable levels of PEth 16:0/18:1 (mean 11.4 ng/g ± 9.4 ng/g), while in 12 EtG could be quantified (mean 127.2 ± 227.7 ng/g). Four specimens contained both EtG and PEth 16:0/18:1. Another study developed a method to detect EtG and EtS in placenta and fetal remains of pregnancies that had been voluntarily terminated in the 12th week [212]. The placenta could be sampled after birth to detect *in utero* exposure to ethanol prior to birth. Further work is required to develop and validate quantitative methods for the analysis of umbilical cord and placenta, and to evaluate how results should be interpreted.

3 Discussion and conclusion

Alcohol consumption is monitored in particular cases, especially in the context of workplace drug testing, driving under the influence of alcohol, driving licence regranting programs, alcohol withdrawal treatment, diagnosis of acute alcohol intoxication or suspected and at-risk pregnancies. The currently used methods are the quantification of ethanol in blood, breath and urine. The detection of ethanol itself offers the advantage of being highly specific to alcohol consumption, but the relatively short detection window in these matrices only allows detection for a short period of time after consumption. In addition, 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. Indirect biomarkers, such as CDT%, GGT, ALT, AST and MCV, measured in blood are also widely used, especially to detect excessive and chronic alcohol consumption and alcohol dependence. As demonstrated by others and ourselves, these indirect markers -with e.g. CDT% being widely used- are known to suffer from a lack of sensitivity and specificity and therefore are not optimal to detect all cases with chronic alcohol consumption and to monitor abstinence periods [77].

In this review, we provided an overview of alternative sampling strategies that can be applied to monitor the alcohol consumption of living subjects. Briefly, quantitative methods to analyse dried blood spots (CDT, EtG/EtS, PEths), dried urine spots (EtG/EtS), sweat and skin surface lipids (ethanol, EtG, FAEEs), oral fluid (ethanol, EtG), exhaled breath (PEths), hair (EtG, FAEEs), nail (EtG), meconium (EtG/EtS, FAEEs), umbilical cord and placenta (EtG/EtS and PEth 16:0/18:1) have been covered here. The determination of FAEEs in DBS has not been published so far and could be an interesting complement to the currently available DBS analyses [229–233].

Methods based on dried urine spots (**DUSs**) are rare and have been developed to improve the stability of compounds 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, and in case of capillary DBS (C-DBS), would offer in addition a non-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 haematocrit, punch localization and volume effects.

The different detection windows of PEths in blood (up to 28 days [19]), CDT in blood (up to 14 days [234]), EtG/EtS in urine (up to 5 days [86]), FAEEs in blood (up to 4 days [230]) and EtG/EtS in blood (40 hours [55]), measured in alcohol-dependent patient samples, indicate that a combined determination of several of these markers allows to derive complementary information. In this context, the higher convenience offered by dried matrix sampling (blood/urine) could provide an interesting approach to monitor an alcohol abstinence period, for example in driving licence regranting programs or alcohol withdrawal treatment, or to detect alcohol intake during a pregnancy-at-risk. In addition, the (repeated) determination of PEths in DBS seems to be a reliable method to detect alcohol dependence, when the quantitative results are interpreted according to published cut-off values. However, while cut-off values for PEths in DBS have been proposed and are in line with some proposed cut-offs in blood, these are not yet supported by international guidelines [67]. In this context, implementation of DBS sampling as a more convenient sampling approach might actually be helpful to set-up large-scale studies supporting the determination of such cut-offs.

The measurement of ethanol with an electrochemical device, worn during a defined period of time, and of ethanol, EtG or FAEEs in **sweat/sebum** accumulated on a patch, offers an interesting approach to monitor an alcohol abstinence period.

The concentration-time profiles of ethanol in **oral fluid** closely mirror those in blood and breath [31] and the concentrations in oral fluid and blood are similar, with a mean oral fluid-blood ratio estimated at 1.09:1 (range 0.96-1.23:1) [31]. 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 [158] and EtG [12] in oral fluid. Given the quality of modern breath devices, we see little added-value in oral fluid monitoring of ethanol. However, for EtG monitoring, oral fluid sampling, which is non-invasive and does not involve privacy issues, might offer opportunities when aiming at evaluating recent alcohol consumption, in the presence of a negative breath test for ethanol. An example might be hit and run cases, with longer timeframes (many hours) before a suspect can be intercepted.

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.

There is ample literature on the quantification of EtG and FAEEs in **hair**, providing information about analytical methods, sample preparation and interpretation issues. Cut-off values published in international guidelines are available to make statements on compatibility with alcohol abstinence and to detect excessive and chronic alcohol consumption. The possible segmental analysis of hair samples - though not routinely implemented- might offer interesting insights into a drinking pattern. EtG and FAEEs are the two possible compounds of interest to monitor alcohol consumption using hair, EtG being the SoHT's first choice for abstinence assessment [163]. FAEEs suffer from several drawbacks compared to EtG when measured in hair: i) a lower sensitivity and specificity to detect excessive and chronic alcohol consumption [37]; ii) a possible post-collection synthesis after exposure to ethanol vapour [191] and iii) false positive results when using popular ethanol containing cosmetic products [196]. Therefore, according to the SOHT, the analysis of FAEEs alone is not recommended but may be used in case of a suspected false negative EtG finding in hair.

Nail, like hair a keratinized matrix, also offers the advantage of accumulating compounds. However, 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. When no hair is available to (dis)prove alcohol abstinence, nails might be an alternative, although it should be taken into account that the covered timeframes will be different.

The detection of EtG, EtS and FAEEs in **meconium** seems to be the gold standard to detect *in utero* exposure to ethanol. Determination of FAEEs in meconium -even with different cut-off values being proposed- seems to become generally accepted by the scientific community. For EtG, further studies are still needed to fix an international cut-off value. EtG and FAEEs in meconium are generally analysed separately using two different aliquots. To ensure the homogeneity of the different aliquots analysed, two solutions have been proposed; first to homogenise the meconium, second to use a combined extraction and analysis for EtG and FAEEs [206]. The *in utero* exposure to ethanol can also be estimated via monitoring of hair or nails from mother and/or newborn (EtG, EtS or FAEE) or DBS from the mother and/or newborn (PEths), although it should be kept in mind that each of these matrices covers another time window. A comparative study has

shown that meconium was more sensitive to detect alcohol consumption during pregnancy, because hair requires higher alcohol consumption (min 30 g per day) to allow the detection of FAEEs or EtG [33].

Umbilical cord and placenta are two alternative samples available at birth to detect *in utero* exposure to ethanol. However, research in this field is currently limited.

Overall, it is clear that alternative sampling strategies offer distinct advantages over traditional sampling of breath, blood or urine, allowing e.g. remote sampling or retrospective analysis to derive information about someone's alcohol consumption. Yet, much research is still required in this field to confirm promising results, establish widely accepted cut-offs, organize proficiency tests using alternative samples, etc. When successful, this may lead some alternative sampling strategies for the assessment of alcohol intake of living persons to become "established" rather than "alternative" sampling strategies.

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