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LC-MS/MS analysis of Phosphatidylethanol for abstinence testing and alcohol consumption monitoring in different cohorts, including comparisons to other biomarkers

Dissertation

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by

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Dedicated to my Mom

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- Aboutara, N; Jungen, H; Szewczyk, A; Sterneck, M; Müller, A; Iwersen-Bergmann, S (2021): Analysis of six different homologues of phosphatidylethanol from dried blood spots using liquid chromatography-tandem mass spectrometry. In *Drug Testing and Analysis*

- Aboutara, N; Szewczyk, A; Jungen, H; Mosebach, A; Rodriguez Lago, M; Vettorazzi, E; Iwersen-Bergmann, S; Müller A; Sterneck, M. (2022): Phosphatidylethanol in patients with liver diseases of different etiologies: Analysis of six homologues and comparison with other alcohol markers. In *Clinica Chimica Acta*

- Aboutara, N; Müller, A; Jungen, H; Szewczyk, A; van Rüth, V; Bertram, F; Püschel, K; Heinrich, F; Iwersen-Bergmann, S. (2022): Investigating the use of PEth, CDT and MCV to evaluate alcohol consumption in a cohort of homeless individuals- A comparison of different alcohol biomarkers. In *Forensic Science International*

- Mosebach, A; Aboutara, N; Lago Rodriguez, M; Müller, A; Lang, M; Fischer, L; Iwersen-Bergmann, S; Sterneck, M (2020): Impaired diagnostic accuracy of hair ethyl glucuronide testing in patients with renal dysfunction. In *Forensic Science International.*

- Szewczyk, A; Hof, C; Müller, A; Kocygit, D; Jungen, H; Aboutara, N; Iwersen-Bergmann, S; Andresen-Streichert, H (2020). Vorkommen und Bedeutung von Pregabalin bei Verkehrsdelikten in Deutschland. In *Blutalkohol*.

- Aboutara, N; Jungen, H; Schaper, A; Sterneck, M; Püschel, K; Iwersen-Bergmann, S (2019) Poisoning with paracetamol: Cases from Hamburg and the GIZ Nord Poison Center with Special respect to Legislation Change in 2009 concerning packsizes. In: *Rechtsmedizin*

II. List of abbreviations

ADH	alcohol dehydrogenase
ALD	alcoholic liver disease
ALDH	acetaldehyde dehydrogenase
ALT	alanine aminotransferase
AST	aspartate transaminase
AUDIT	Alcohol Use Disorders Identification Test
bac	blood alcohol concentration
BMI	body mass index
CRM	charge residue model
CDT	Carbohydrate-deficient transferrin
CYP	Cytochrome P450
DC	direct current
DBS	dried blood spots
DSM	Diagnostic and Statistical Manual of Mental Disorders
EDTA	ethylenediaminetetraacetic acid
ELSD	evaporative light scattering detector
ESI	Electrospray ionization
Etg	ethyl glucuronide
EtOH	ethanol
Ets	ethyl sulfate
GABA	gamma-aminobutyricacid
GGT	gamma-glutamyltransferase
Hct	hematocrit
HPLC	high-performance liquid chromatography
ICD	International Statistical Classification of Diseases and Related Health Problems
IEM	ion evaporation model
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MCV	mean corpuscular volume

MEOS	microsomal ethanol oxidizing system
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NMDA	*N*-methyl-D-aspartate
PC	phosphatidylcholine
PEth	phosphatidylethanol
PLD	phospholipase D
RF	radio frequency
RP	reversed phase
sd	standard deviation
SIM	selected ion monitoring
Tf	transferrin
UDP-GT	uridine 5'-diphospho-glucuronosyltransferase
UPLC	ultra-performance liquid chromatography
WHO	World Health Organization

1. Abstract

The question of the nature and extent of alcohol exposure of individuals is of substantial importance in forensic assessments and clinical diagnostics. The evaluation is usually based on chemical-toxicological test results. Various alcohol biomarkers have been established for routine testing, but none of them has 100% sensitivity and specificity.

Phosphatidylethanol (PEth) has gained increasing recognition as an alcohol consumption marker in recent years. Unlike indirect alcohol markers, which represent biochemical changes in the body (e.g., liver enzyme levels, carbohydrate-deficient transferrin, altered erythrocyte volume), direct alcohol markers are biotransformation products of ethanol, making them highly specific. These include ethyl glucuronide (Etg) and ethyl sulfate (Ets), as well as PEth, an abnormal phospholipid consisting of a glycerol backbone esterified with fatty acid residues at sn-1 and sn-2 positions and with phosphoethanol at sn-3 position. While Etg is measured in urine (detection for about 3 days) and in hair (detection period about 1 to 3 months), PEth is analyzed in blood and provides a detection period of up to several weeks.

After successfully establishing a method to quantify six different homologues of PEth by LC-MS/MS, the applicability and diagnostic value of PEth was assessed in two studies with different cohorts. Primary scope was on PEth's performance in comparison with already established alcohol biomarkers and analysis of the different homologues. In a collective of patients with liver diseases of different etiologies, PEth had a specificity of 100% for alcohol consumption in the last three months, the highest sensitivity (75%) for the detection of consumption in the last week compared to Etg in urine (28%), as well as for consumption in the last three months (53%) compared to Etg in hair (37%). For alcohol uptake in the past four weeks, PEth had a specificity of 98% and a sensitivity of 58%. In a collective of subjects with presumably very high alcohol consumption, PEth and carbohydrate-deficient transferrin (CDT) correlated well ($\rho = 0.77$ ($p < 0.001$) for PEth 16:0/18:1), but overall PEth also performed better than the already established markers.

PEth analyses were performed from dried blood spots (DBS) generated from venous or capillary blood. The stability of all PEth homologues in blood stored at room temperature proved to be very variable in 62 samples in which PEth concentrations were quantified over a period of 30 days at several time points. Thus, it was concluded that blood samples that are planned to be analyzed for PEth should be sent to the analytical laboratory as soon as possible or DBS should be generated at the site of blood sampling to inhibit decrease of concentration.

Overall, results of this work, especially the results of a study with subjects who consumed small amounts of alcohol, led to the conclusion that the currently applied cut-off for PEth should be lowered to improve the sensitivity of this alcohol biomarker.

All in all, the compilation of studies on PEth in this work shows why this alcohol biomarker should have a place in routine analysis for abstinence testing and alcohol consumption monitoring, while short comings, that are intensely discussed in this work, need to be considered.

2. Zusammenfassung

Die Beantwortung der Frage nach Art und Umfang einer Alkoholexposition ist von erheblicher Bedeutung in der forensischen Begutachtung sowie der klinischen Diagnostik. Die Beurteilung stützt sich hierbei in erheblichem Maße auf chemisch-toxikologische Untersuchungsergebnisse. Für Routineuntersuchungen haben sich verschiedene Alkoholbiomarker etabliert, von denen jedoch keiner eine Sensitivität und Spezifität besitzt von 100% besitzt.

Phosphatidylethanol (PEth) hat in den letzten Jahren zunehmend an Interesse als Alkoholkonsummarker gewonnen. Im Gegensatz zu den indirekten Alkoholmarkern, welche biochemische Veränderungen im Körper darstellen (z.B. Leberenzymwerte, Carbohydratdefizientes Transferrin, verändertes Erythrozytenvolumen), sind die direkten Alkoholmarker Biotransformationsprodukte des Ethanols, wodurch sie grundsätzlich sehr spezifisch sind. Hierzu gehören das Ethylglucuronid (Etg) und das Ethylsulfat (Ets), sowie das PEth, ein modifiziertes Phospholipid, bestehend aus einem Glycerolgrundgerüst das an sn-1-und sn-2-Position mit Fettsäureresten und an sn-3-Position mit Phosphoethanol verestert ist. Während Etg im Urin (Nachweis für ca. 3 Tage) und im Haar (Nachweiszeitraum ca. 1 bis 3 Monate) gemessen wird, erfolgt die Bestimmung von PEth im Blut und ermöglicht einen Nachweiszeitraum von bis zu mehreren Wochen.

Nachdem zunächst eine Analysenmethode etabliert wurde, um sechs verschiedene Homologe von PEth mittels LC-MS/MS zu quantifizieren, wurde in zwei Studien mit unterschiedlichen Kohorten die Anwendbarkeit und die diagnostische Leistungsfähigkeit von PEth beurteilt. Dies wurde in erster Linie in Hinblick auf den Vergleich mit anderen, bereits etablierten Alkohol Biomarkern und in Hinsicht auf die Analyse der verschiedenen Homologen genauer betrachtet. In einem Kollektiv von Patienten mit Lebererkrankungen verschiedener Ätiologien, zeigte PEth, neben einer Spezifität von 100% für einen Alkoholkonsum in den letzten drei Monaten, auch die höchste Senstitivität (75%) für den Nachweis eines Konsums in der letzten Woche im Vergleich zu Etg im Urin (28%), sowie eines Konsums in den letzten drei Monaten (53%) im Vergleich zu Etg im Haar (37%). Für einen Konsum in den letzten vier Wochen wies PEth eine Spezifität von 98% und eine Sensitivität von 58% auf. In einem Kollektiv von Probanden mit sehr hohem Alkoholkonsum korrelierten PEth und CDT gut miteinander ($\rho = 0.77$ ($p < 0.001$) für PEth 16:0/18:1), insgesamt zeigte PEth jedoch ebenfalls eine bessere Leistung als die bereits etablierten Marker.

Die PEth Analysen erfolgten aus Dried Blood Spots (DBS), welche zuvor aus Venen- oder Kapillarblut generiert wurden. Die Stabilität von allen PEth Homologen in Blut, welches bei Raumtemperatur gelagert wurde, erwies sich als sehr unterschiedlich in 62 authentischen Proben, in denen über einen Zeitraum von 30 Tagen and mehreren Zeitpunkten PEth quantifiziert wurde. Aus den Ergebnissen lässt sich schließen, dass Blutproben, die auf PEth getestet werden sollen, möglichst schnell in das Analyselabor übersendet oder aber direkt am Ort der Blutabnahme DBS generiert werden sollten, um einen Konzentrationsabfall zu inhibieren.

Insgesamt zeigen die Ergebnisse der Arbeit, dass der aktuell angewendete Cut-off für PEth abgesenkt werden sollte, um die Sensitivität des Alkoholbiomarkers zu verbessern, was in einer zusätzlichen Studie mit Probanden, die nur geringe Mengen an Alkohol konsumierten, besonders offensichtlich wurde.

In der Zusammenschau zeigen die Untersuchungen dieser Arbeit, warum PEth - unter Berücksichtigung der in dieser Arbeit intensiv diskutierten Schwächen - einen Platz als Alkoholbiomarker in der Routineanalyse für Abstinenznachweise und die Überwachung des Alkoholkonsums haben sollte.

3. Introduction

3.1. Alcohol

3.1.1 Numbers and facts

Alcohol is the most present and most accepted drug in our society. The World Health Organization (WHO) presented the Global status report on alcohol and health lastly in 2018 [1]. It reports that the harmful use of alcohol led to approximately three million deaths worldwide in that year (which corresponds to 5.3% of all deaths) and that 5.1% of the global burden of disease and injury could be led back to alcohol. In 2016 it was calculated that 7.2% of all premature deaths worldwide were a consequence of alcohol, while stressing that among 20–39-year old's it was 13.5% [1].

Among people in Germany the average consumption amount of pure alcohol was calculated to be 12.8 L in 2019 [2], which ranks it in the top-ten globally. In the Epidemiologic Substance Abuse Survey [3] 71.6% (36.9 million persons) reported to have drunk alcohol in the past 30 days. Consumption of risky amounts of alcohol were stated by 18.1%. Three million adults (18-64 years) had a diagnosis of an alcohol-related disorder in 2018 in Germany. Data from 2019 calculated that about 1.6 million people in Germany abuse alcohol and about 1.8 million were alcohol dependent [3]. It is known that about 200 diseases and injury conditions can be caused or worsened by alcohol consumption. Liver diseases, cardiovascular diseases, mental health disorders and cancer are just a few examples [1]. Further causal relationships have been found between harmful drinking and incidence of infectious diseases such as tuberculosis [1]. Each year about 74,000 people in Germany die as a direct or indirect consequence of alcohol consumption [4,5].

Not only can alcohol harm the individual consuming it, but there are many ways in which third-party persons can be affected as well. This might be due to accidents caused by driving under the influence of alcohol, increased potential of aggression and violence under the influence of alcohol or when an expectant mother fails to be abstinent from alcohol during pregnancy. It is estimated that about 12,650 children are born with fetal alcohol spectrum disorder and 2,930 with fetal alcohol syndrome each year in Germany [6].

In 2020 it was counted that 31,540 accidents in traffic happened influenced by alcohol in Germany [7]. Kraus et al. reported that in the year 2014 about 45% of all third-party traffic accident fatalities were attributable to alcohol (about 1,214 fatal casualties) [6]. Furthermore, the working group published that 55 out of 368 deaths caused by interpersonal violence were under the influence of alcohol.

3.1.2 Definitions

While the influence of alcohol on individuals differs a lot and it cannot exactly be defined which amounts cause specific consequences, definitions have been established for the risk potential of consumed alcohol. Low risk alcohol consumption is defined as a maximum of 12 g and 24 g pure alcohol per day for women and men, respectively, as described in the recommendations of the scientific board of trustees of the German Central Office for Addiction Issues (Deutsche Hauptstelle für Suchtfragen) [8]. Nevertheless, it was postulated in the Global Burden of Disease Study 2016 that not consuming any alcohol is the safest [9]. Risky amounts are set to 12-40 g for women and 24-60 g for men [8]. Consumption of more than those amounts per day are considered hazardous to one's health. Binge drinking is the consumption of alcohol that results in a blood alcohol concentration (bac) of 0.8 ‰ or higher. For women that averagely means consumption of four and for men of five drinks within 2 hours [10]. While a standard drink in the United States is defined as 14 g of pure alcohol in a beverage in Germany it is 10-12 g [11,12].

The Diagnostic and Statistical Manual of Mental Disorders (DSM) defines an alcohol use disorder as 'an impaired ability to stop or control alcohol use despite adverse social, occupational, or health consequences' [10]. It can be diagnosed as mild, moderate or severe. The commonly used International Statistical Classification of Diseases and Related Health Problems (ICD-10), a classification system published by the WHO, lists mental and behavioral disorders caused by alcohol as a diagnosis category (ICD-code F10) [14]. Under this category further specifications are found, like acute intoxication (F10.0), addiction syndrome (F10.2), withdrawal syndrome (F10.3), psychotic disorder (F10.5) and amnesic syndrome (F10.6). For diagnosis of an alcohol dependence three or more of the following aspects need to be fulfilled regarding the past year [10]:

- a strong desire or compulsion to consume alcohol
- development of tolerance; increasing amounts of alcohol need to be consumed to achieve an effect
- continued consumption of alcohol, despite consequential harms
- difficulties to control start, termination and amounts of consumption
- a physical withdrawal syndrome, when no or less alcohol is consumed
- progressing neglect of other interests in favor of alcohol consumption

3.1.3 Alcohol effects in the central nervous system

After resorption, ethanol impacts several different sites of the body, for example by influencing different neurotransmitter systems. Ethanol activates opioid receptors (μ) which leads to the release of dopamine in the ventricle tegmentum [15,16]. Furthermore, the substance binds to the

glycine binding site of NMDA-receptors, which inhibits the excitatory effects of glutamate binding to the receptor, by stopping the confirmational change of the ion channels resulting from glycine interaction at its binding site [17]. This has for example been shown to cause the perception and memory problems after alcohol consumption. After chronic alcohol presence in the organism NMDA-receptors are upregulated to keep homeostasis up. If suddenly alcohol input is stopped, the disturbance in balance of excitatory and sedative signals leads to symptoms of withdrawal such as shaking, dizziness and seizures [18].

Another system with which alcohol interacts are the GABA-receptors, more specifically the GABA-A-receptors [19]. Gamma-aminobutyricacid is the most important inhibitory neurotransmitter in the central nervous system [18]. As the receptor is ligand-gated, binding of GABA results in the opening of an ion channel for chloride. This influx of negative ions inhibits the neuron, by causing hyperpolarization. Besides the GABA-binding-site the GABA-A-receptors have allosteric binding-sites for other molecules. Ethanol acts as a positive GABA-modulator, meaning its binding to the allosteric binding site leads to a confirmation of the active center that enhances affinity to GABA, thus enhancing the effect of GABA [20]. This explains the relaxing and anxiolytic effects of alcohol.

In the early 90ties research on the effects of alcohol on neurotransmitters in the brain, revealed that after acute alcohol consumption metabolites of serotonin in blood and urine were increased, thus it was concluded that ethanol raises serotonin levels in the CNS [21]. Due to its role as a key regulator of reinforcement learning and behavioral plasticity the serotonergic system of the brain is considered as an important factor in alcohol-related physiological and behavioral adaptations [22]. The most important enforcing neuromodulator in the reward-circuit of the brain is dopamine, the release of which is caused by alcohol, as described above [18].

Therefore, influence on the serotonin and the dopamine systems cause the ‘euphoric’ and ‘pleasurable’ feelings experienced after alcohol uptake [23]. In addition, these two systems play a crucial role in the addictive properties of ethanol and the high relapse probability, because the constant input of alcohol leads to a reduction of base dopamine and serotonin levels and the number of both substances’ receptors, causing psychological effects if alcohol is suddenly no longer present.

3.1.4 Harms to health caused by alcohol

Alcohol is a toxic substance that is distributed in the entire body and can cause harm to any organ [24]. Beside the ‘mental and behavioral disorders caused by alcohol’ under the F10-category in the ICD-10 (see 3.1.2) there are ten more diagnosis in the ICD-10 classified as exclusively alcohol-related diseases. The diseases are listed in the following (ICD-codes included) [14]:

- Alcohol-induced Pseudo-Cushing's syndrome (E24.4)

- Niacin deficiency (Alcohol-induced pellagra) (E52)
- Degeneration of the nervous system caused by alcohol (G31.2)
- Alcoholic polyneuropathy (G62.1)
- Alcoholic myopathy (G72.1)
- Alcoholic cardiomyopathy (I42.6)
- Alcoholic gastritis (K29.2)
- Alcoholic liver disease (K70)
- Alcohol-induced acute/chronic pancreatitis (K85.2/K86.0)
- Alcoholic embryopathy (with dysmorphism) (Q86.0)

In addition, as mentioned in 3.1.1, alcohol can be a contributing factor in development of more than 200 diseases [1]. Both alcohol and acetaldehyde are classified as carcinogenic for humans (group 1) (oral cavity, pharynx, larynx, esophagus, colorectal, liver and female breast) by the International Agency for Research for Cancer [25,26]

Overall, it is calculated that about 5.5% of all cancer cases are a consequence of alcohol consumption [24].

3.1.5 Alcohol resorption and metabolism

When ingesting ethanol, very small portions are already resorbed by the oral mucosa. About 20% of the ethanol is then resorbed in the stomach and the rest in the small intestines, solely by diffusion [27]. Ethanol undergoes a partial first-pass-metabolism in the liver and encounters alcohol dehydrogenase (ADH) in the stomach [28]. This results in a 'resorption deficit' (reduced bioavailability) of 10-30%. Resorption of ethanol can be accelerated or slowed down by several different factors: from higher percent beverages, warm beverages and carbonated beverages ethanol is resorbed faster, some medications such as acetylsalicylic acid also enhance resorption [29]. If the stomach is filled with food after a meal resorption of ethanol is retarded [30].

After reaching the blood circulation ethanol is quickly distributed in the body, reaching the organs best supplied by blood, such as the brain, the liver and the lungs first. Because ethanol is a dipole, due to the hydroxyl group in which the hydrogen atom carries a partial positive charge, it is extremely soluble in water. Therefore, ethanol is quickly distributed in the body water. Women have a lower total portion of body water (51%) than men (65%). This is the reason why women usually have higher bac than men after consumption of the same amount of alcohol, as the alcohol has a smaller distribution volume in women [27]. Older people's distribution volume is smaller than

that of younger people and overweight people's is smaller than of people with normal weight [24]. Peak alcohol concentrations are reached approximately 10-75 min after end of ingestion [27,31]. Approximately 2-5% of the ethanol is excreted unchanged via breath, sweat and urine [30]. Metabolism of ethanol can be split into one major pathway and several minor ones. Mainly ethanol is metabolized by an oxidative pathway in the liver. The first step takes place in the cytosol of hepatocytes. Here ethanol is converted into acetaldehyde, catalyzed by the enzyme ADH [32]. If bac exceeds about 0.15-0.2 ‰ this reaction is characterized by a zero-order kinetic, because the enzyme is saturated with ethanol. That means that independent from the concentration of alcohol a fixed amount is converted by the enzyme [31,33]. If ethanol is below the substrate saturation, elimination follows a first-order kinetic, meaning that a certain proportion of the substrate is converted, depending on the remaining concentration [33].

In the second step the acetaldehyde, a cell toxic substance believed to be related to 'hang-over'-symptoms, such as nausea, vomiting and headaches, is dehydrogenized to acetate, catalyzed by acetaldehyde dehydrogenase (ALDH), mainly in mitochondria. The pathway is illustrated in figure 1. Both reactions need the cofactor nicotinamide adenine dinucleotide (NAD+). NAD+ acts as an electron- and hydrogen-acceptor and is therefore reduced to NADH. This has consequences on other biochemical pathways because it generates a highly reduced surrounding, leading to morphological and functional changes [32]. The resulting acetate is then converted to acetyl-CoA (fig. 1), which is further processed in the citrate cycle or oxidized to carbon dioxide [34].

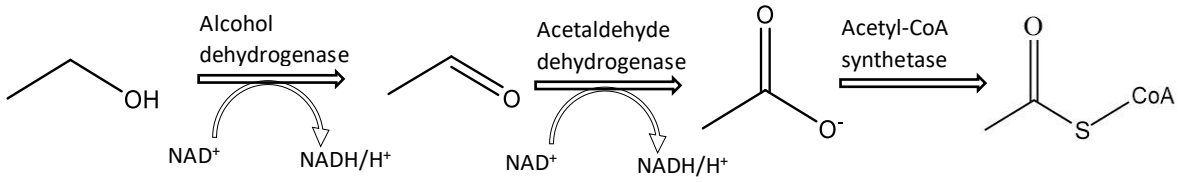

Fig. 1: Metabolic pathway of ethanol: oxidation of ethanol to acetaldehyde catalyzed by ADH, oxidation of acetaldehyde to acetate catalyzed by ALDH, conversion of acetate to acetyl-CoA

A minor pathway for metabolism of ethanol (about 10-20%) is carried out by the microsomal ethanol oxidizing system (MEOS). This involves the enzyme CYP2E1, located in the endoplasmic reticulum. Cofactor here is NADPH, which donates the hydrogen. Thus, while ethanol is oxidized to acetaldehyde with the help of O_2, NADP+ and two water molecules emerge (fig. 2) [35].

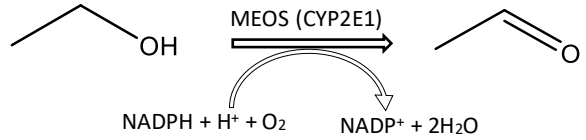

Fig. 1: Metabolic pathway of ethanol: oxidation of ethanol to acetaldehyde catalyzed by the MEOS, e.g. CYP2E1

CYP2E1 is inducible by ethanol, explaining why alcohol metabolism is enhanced in individuals who drink chronically [27]. It has been reported that up to about 0.2‰/h can be eliminated additionally. Furthermore, reactive oxygen species are formed in the redox reaction, which leads to oxidative stress in the cells and lipid peroxidation, which is an important factor in development of alcohol-related damages to the organism [35].

Another oxidative, but very minor pathway of ethanol metabolism is performed by the enzyme catalase, which can be found in peroxisomes of liver cells. In the presence of hydroxy peroxide catalase oxidizes ethanol to acetaldehyde [32].

Besides the oxidative pathways there are also non-oxidative pathways. Although they are a lot less effective for ethanol metabolism and only add up to a minor fraction of overall metabolism (<0.1%), they are certainly interesting and have been groundbreaking for diagnostics and biomarker analysis. Some of the resulting products are used as alcohol biomarkers, such as ethyl glucuronide (Etg), ethyl sulfate (Ets) and Phosphatidylethanol (PEth). Their role in ethanol metabolism will be looked at in detail in 3.2.

3.2 Alcohol biomarkers used in forensic and clinical toxicology

The question of the nature and extent of alcohol exposure is of importance in forensic assessments and clinical diagnostics. Beside the interview of individuals (AUDIT, see 3.3) the assessment is usually based on chemical-toxicological test results. Alcohol biomarker analysis has therefore long been established as a major part in toxicological analysis.

Reasons for evaluating the alcohol consumption of individuals are versatile. In clinical contexts monitoring of alcohol consumption is relevant in patients with an alcohol dependency, whether they are in psychiatric treatment for withdrawal therapy or need to proof their abstinence before being eligible for an organ transplantation (see 5.2). In traffic medicine alcohol and alcohol biomarker analysis is important to judge if a vehicle was handled under the influence of alcohol and for abstinence monitoring in programs to reclaim a revoked driver's license. Courts or authorities

may order alcohol consumption monitoring of individuals as a condition for probation or in questions of awarding custody of children.

While the direct detection of ethanol in the body can simply answer the question of current alcohol intake or influence, the detection of alcohol exposure over a longer period or chronic exposure is more difficult. Alcohol biomarkers are used for this purpose. They can be classified as indirect and direct biomarkers.

3.2.1 Indirect alcohol biomarkers

The indirect markers represent ethanol-related biochemical changes that occur due to alcohol uptake but are not directly related to the presence of alcohol in the body. For example, they can be indicators of organ damage. Gamma-glutamyltransferase (GGT), alanine aminotransferase (ALT), aspartate transaminase (AST), changes in the mean corpuscular volume (MCV) or the increased formation of certain isoforms glycoprotein transferrin [27] are examples for indirect biomarkers that are applied in routine analysis. These markers tend to be nonspecific, as abnormalities can be caused by other reasons but alcohol.

3.2.1.1 Liver enzymes

GGT is a membrane-bound enzyme that transfers glutamyl groups to amino acids or water. The main enzymatic activity is localized in the liver, but it is also found in the kidneys, lungs, intestines, spleen and pancreas [27]. Increased GGT activity in blood serum has traditionally been used as a biomarker of heavy and harmful alcohol use, but it is not a specific biomarker because the GGT value can be increased due to various liver diseases of other etiologies than alcohol abuse. Furthermore, other diseases such as type 2 diabetes and cardiovascular disease or a variation of drugs, e.g., acetaminophen, benzodiazepines or fluoxetine may increase values [27]. As an explanation how alcohol causes increase of serum GGT it is discussed that an enzyme induction may directly be triggered by ethanol and that ethanol exposure harms the hepatocytes and eventually leads to necrosis, resulting in an increased release of GGT into the serum [36]. GGT is considered elevated if exceeding 40 U/L in females and 64 U/L in males [37]. Specificity and sensitivity have been calculated between 72-98% and 54-65%, respectively [38,39]. If caused by alcohol consumption it usually takes GGT values between six to twelve weeks to normalize after reduction of consumption.

Two more enzymes that are traditionally used as alcohol biomarkers are ALT and AST, which both can indicate general liver damage if their activities are elevated in serum. Transaminases transfer amino groups from amino acids to α-keto acids [27]. AST is a mitochondrial enzyme found predominantly in the liver but is also found in the skeletal muscle and almost all organs. Therefore,

non-hepatic causes of elevation are divers, with strenuous exercise, muscle disorders and multiple drugs being the most common. ALT is located in the cytosol, mainly in the liver which makes it slightly more specific for liver damage than AST. Still, elevation of both has low specificity for alcohol induced damage compared to GGT [40]. Wide ranges for sensitivity and specificity are given in different studies, roughly ranging from 10-90% for both [27,41].

3.2.1.2 Mean corpuscular volume

The mean corpuscular volume of erythrocytes is calculated by dividing the hematocrit (hct) by the number of erythrocytes and is defined as normal at volumes of 80-96 fl [37]. Deviations from this norm are possible in both directions. Most common cause for abnormalities is anemia. An increase in MCV > 96 fl is caused by deficiency of vitamins, especially folic acid and vitamin B12. Alcohol consumption also causes macrocytosis [42]. How exactly remains still unclear. It has been proposed that ethanol and acetaldehyde might have a direct hematotoxic effect, by permeating the cell membrane and altering its lipid structure [43]. Additionally, an impaired uptake of vitamin B12 and folic acid due to alcohol abuse have been proposed as a possible reason. In a study carried out in the seventies it was shown that consumption of 60 g of alcohol per day increased MCV above the reference range in most individuals [44]. Other causes of macrocytosis may be hypothyroidism, anorexia nervosa or certain drugs, such as metformin [45,46]. In a review of clinical biomarkers for the detection of alcohol dependence sensitivities of 39-76% and specificities of 75-98% were reported [38].

3.2.1.3 Carbohydrate-deficient Transferrin

Transferrin (Tf) is a glycoprotein responsible for transport of iron, that is synthesized in hepatocytes and secreted into the blood. Transferrin is built up of a polypeptide chain with two glycosylation sites where oligosaccharide chains are attached in a post-translational step. The last moiety of this carbohydrate chains is sialic acid. Different glycoforms of transferrin exist, which are isoforms that are defined by the glycosylation pattern and are named after the number of present sialic acids as terminal sugars in the carbohydrate chains [47] (fig. 3). Normally, the carbohydrate chains are terminated with sialic acid residues at two to six sites by glycosylation reactions. The most represented transferrin is the tetrasialotransferrin, which makes up about 64 to 80% of total Tf molecules [48].

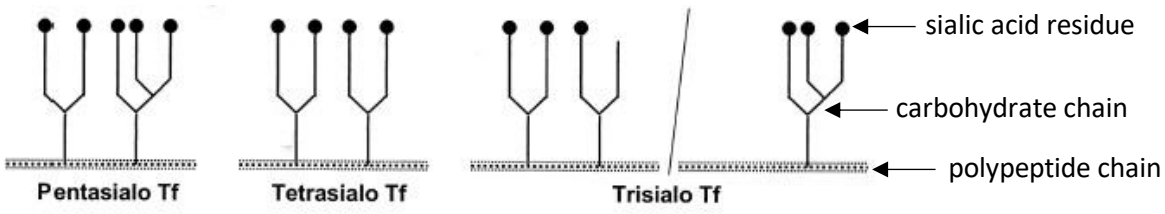

Fig. 3: Different isoforms of the iron transport protein Transferrin (Tf) (modified from [47])

The exact ways in which alcohol influences Tf synthesis are not completely discovered yet. Two major principles have been established though. Ethanol or its metabolite acetaldehyde inhibit the enzymes such as sialyltransferase, galactosyltransferase, and N-acetylglucosamine transferase, in the Golgi-apparatus of hepatocytes, which are responsible for glycosylation of the peptide chain of Tf. This leads to less incorporation of sialic acid residues, thus increasing the isoforms with lower sialylation rates, disialotransferrin, monosialotransferrin and asialotransferrin (fig. 4) [27]. They are collectively named carbohydrate deficient Tf [49].

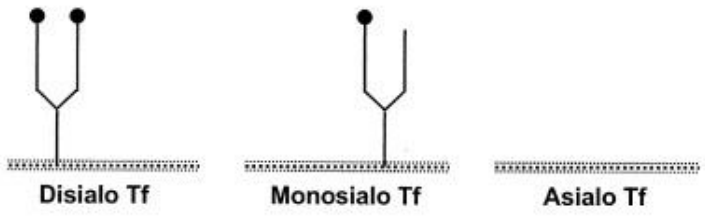

Fig. 4: Different carbohydrate-deficient transferrin isoforms, which increase due to reduced sialyation (modified from [47])

Another theory of how alcohol influences the increase of those isoforms is by enhancement of enzymes like sialidase, which removes the carbohydrate moiety. CDT values are expressed as a percentage of total transferrin (%CDT) [27].

CDT is a biomarker for chronic excessive alcohol consumption, as elevated levels can normally be expected after about 1-2 weeks of consumption of at least 50 g of alcohol per day. Average half-life of CDT is reported as 14 days, with normalization occurring after approximately 10-30 days [50,51]. Several methods for analysis of CDT are available, such as isoelectric focusing, capillary electrophoresis, immunoassays and high-performance liquid chromatography (HPLC). The latter is currently considered the reference and most widely used method for CDT analysis. The isoforms are chromatographically separated and expressed as area percent of total transferrin and is thus independent of fluctuations in the transferrin concentration. Figure 5 shows an example of HPLC chromatograms of a normal finding and a finding after chronic alcohol consumption.

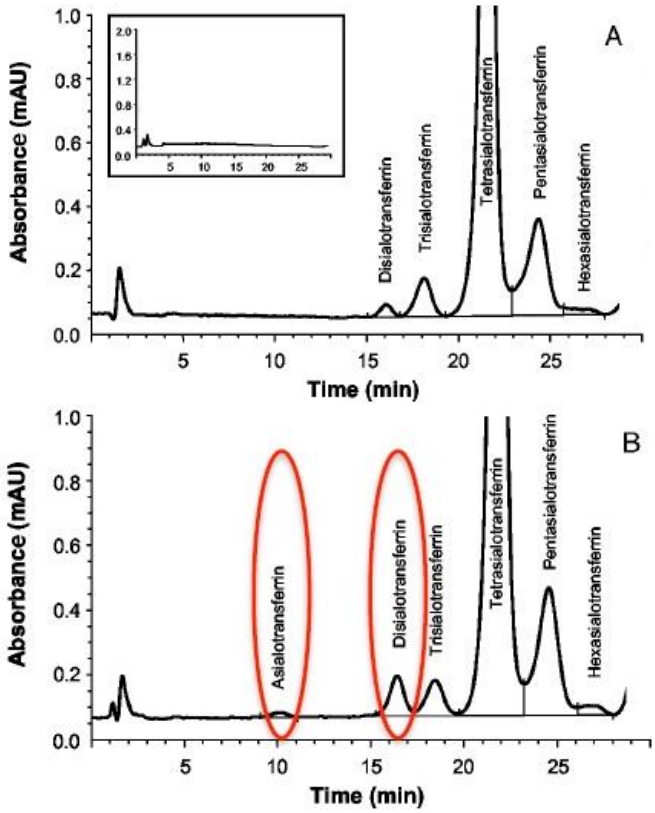

Fig. 5: Chromatograms after HPLC-UV/VIS analysis of a) a normal finding and b) after chronic alcohol consumption (modified from [52])

CDT reference ranges and decision limits are strongly dependent on the analytical methods. For HPLC methods the cut-off us lies between 1.7% and 2.5%. [53].

CDT can be elevated due to other reasons than alcohol as well, such as genetic variation in glycosylation, pregnancy, end-stage liver diseases of different etiologies or anorexia nervosa [27,54,55]. The specificity that has been calculated varies from approximately 60% to 98% [49].

3.2.1.4 Methanol

Methanol is present in the body endogenously as it is formed from pectins that are present in certain foods and drinks and it can be externally taken in from methanol-containing drinks. These sources lead to a physiological methanol concentration of 0.35–3.2 mg/L in blood [56]. Metabolism takes place almost exclusively in the liver by the enzyme ADH (see 3.4.1). As the enzyme has a much higher affinity to ethanol than to methanol, the metabolism of the latter is stalled, leading to an accumulation in the blood at bac of ethanol >0.2-0.5‰ [57]. Continuous ethanol intake can increase the methanol blood concentration by more than tenfold. A methanol concentration of 5 mg/L is interpreted as cut-off to differentiate between patients with and without alcohol dependence, with a specificity of 98% [58]. In scientific drinking experiments it was evaluated that a methanol

concentration of > 10 mg/L can be considered a marker of prior prolonged, continuous alcohol exposure for at least numerous hours [56].

3.2.2 Direct biomarkers

Direct biomarkers are products of ethanol metabolism or of reaction of ethanol with substances in the body. Consequently, ethanol's methylene are part of the molecular structure of direct biomarkers.

3.2.2.1 Ethyl glucuronide and ethyl sulfate

As described in 3.1.4 ethanol metabolism is not solely along the oxidative pathway. Etg and Ets are phase II metabolites of ethanol, produced by non-oxidative pathways. Etg and Ets account for about 0.02%-0.06% and 0.010–0.016% of the ingested alcohol dose, respectively [59]. Etg results from conjugation of ethanol and glucuronic acid from uridine-diphospho-(UDP)-alpha-D-glucuronic acid, catalyzed by uridine 5'-diphospho-glucuronosyltransferases (UGTs) (fig. 6), while Ets yields from conjugation of ethanol with a sulfate group of 3'-phosphoadenosine-5'-phosphosulfate through the action of sulfotransferase (SULT) [27,59].

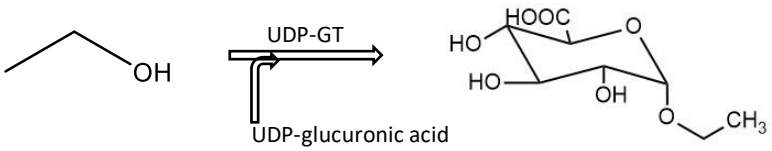

Fig. 6: Non-oxidative metabolism of ethanol: conjugation of UDP-glucuronic acid via UDP-GT, resulting in Etg

They can be detected in blood within 45 min after the beginning of alcohol consumption and are excreted in urine, where urinary Etg (uEtg) can be detected even after the ingestion of very small amounts of ethanol. After small or moderate consumption, the detection window is marked as one to three days, after excessive consumption up to five days. Sensitivity for detecting an alcohol consumption is dependent on alcohol quantity, time interval until sample collection [60-62]. Thus, it is not possible to draw conclusions about the quantity of ethanol that was ingested. Furthermore, very small amounts of alcohol that might be ingested unintentionally may be detectable if a sample is taken shortly after the intake.

For abstinence testing sensitivity has been reported to be 70%-89%. Specificity reached 93-99% in different studies [63]. Since diuresis influences Etg-levels in urine, the intake of high volumes of water leads to a decrease of concentration, which could result in a false-negative result. Therefore, urinary creatinine is analyzed along with Etg, as an indicator for dilution of the sample, which is invalid if creatinine concentration is below 20 mg/dL [64].

Urinary tract infections with *Escherichia coli* can be the reason for false negative uEtg results because the bacteria contain the enzyme beta-glucuronidase that is responsible for hydrolyses of Etg [65,66]. On the other hand, it has been observed that post collection synthesis can occur if urine samples contain ethanol and are infected with *Escherichia coli* [66]. Several studies were carried out to evaluate the influence of alcohol being resorbed from other sources but drinking alcohol. After high frequented use of ethanol-containing disinfection, positive results of Etg have been reported [67,68]. In further studies positive uEtg results were found after consumption of alcohol-free beer, grape juice or yeast and sugar [69]. It should be noted that realistically cases in which this occurs are probably rather rare, but still it should be kept in mind.

Different cut-offs are applied for different settings. While a low cut-off of 0.1 ng/ml is used for driving suitability programs, a cut-off for 0.5 ng/ml is used in abstinence testing for liver transplantation candidates whose need for transplantation is due to chronic alcohol misuse [70].

A small portion of Etg is incorporated into the hair and other keratinized tissues. With analytical methods that are capable to quantify the small concentrations, such as LC-MS/MS, analysis of Etg in hair (hEtg) has become a long-term marker for alcohol consumption. Hair as a matrix allows to screen for alcohol consumption over a period of months. As Etg concentration in hair will decrease due to external influences, such as weather, UV-radiation and washing procedures, hEtg analysis is limited to the proximal 3 cm segment of hair, which approximately represents a time frame of three months [71]. Based on the consensus statement of the Society of Hair Testing (SoHT) from 2019 a hEtG concentration of less than 5 pg/mg in scalp hair is not in contradiction to self-reported abstinence, whereas hEtg above 30 pg/mg in the proximal scalp hair segment is related to chronic excessive drinking [71]. A hEtG concentration between 5 pg/mg and 30 pg/mg on the other hand suggests repeated moderate alcohol consumption and is classified as ‘social consumption’. HEtg analysis has susceptibilities to several influencing factors. While the results are not impacted by age, gender or ethnicity, chemical hair treatment, such as bleaching, dyeing or perming can lead to a strong decrease in the hEtG concentration [72,73,74].

On the other hand, there are Etg containing cosmetic hair products, which have shown to potentially cause false positive results [75,76]. Furthermore, obesity and kidney function can influence hEtg concentrations [77].

3.2.2.2 Phosphatidylethanol

Phosphatidylethanol (PEth) is an abnormal phospholipid consisting of a glycerol backbone which is esterified with fatty acid residues and at sn-3 position with phosphoethanol. In the presence of ethanol, PEth is synthesized from phosphatidylcholine (PC) by phospholipase D (fig. 7), which normally catalyzes the hydrolysis of PC to phosphatidic acid. Due to the higher affinity of short-

chain alcohols to the enzyme compared to water, the presence of ethanol in the blood leads to transphosphorylation, resulting in PEth [27].

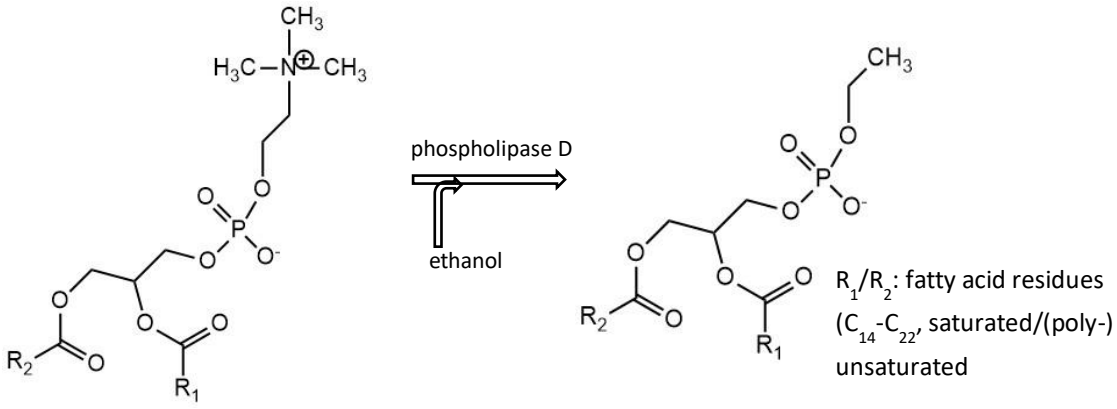

Fig. 7: PEth is synthesized from PC, catalyzed by phospholipase D in the presence of ethanol in the blood

As the composition of fatty residues varies, similar to the precursor molecule, PEth is not a single molecule but a group of molecules. The fatty acid residues differ in number of carbon atoms and double bonds. The most abundant fatty acids in the body are those with a chain length of 14 to 24 carbon atoms, with different saturation levels. Gnann et al. were able to identify 48 different homologues of PEth with individual compositions of the fatty acid chains [78]. The homologues with palmitic and oleic acid (16:0/18:1) [79,80] as well as palmitic and linoleic acid (16:0/18:2) were found to make up the largest proportion [78].

In drinking experiments, PEth was detected in blood after 30 minutes and peaked after 90 to 120 minutes [81]. With frequent alcohol consumption, PEth accumulates in whole blood, mainly due to the lack of degrading enzyme in erythrocytes. Half-life of PEth in blood was shown to be 3–10 days in blood [81,82]. In previous studies sensitivity and specificity of 73–100% and 90–96%, respectively, to determine alcohol consumption in the previous 1–4 weeks, were reported [63]. A correlation between drinking amounts and PEth concentrations in whole blood was demonstrated [83,84]. Earlier measurements of PEth included the whole fraction and were performed using HPLC-ELSD (evaporative light scattering detector). The methodology is rather unspecific and of poor sensitivity [78]. The detection by means of mass spectrometry, which allows differentiation of homologues, improved the analytical outcome significantly [80]. By the more widespread application of LC-MS/MS for PEth analysis, a quantitative evaluation of individual PEth isoforms in context of biomarker research was enabled. While most applications focus on PEth 16:0/18:1 today, discussions about cut-off establishment are still on-going and different suggestions have been made, some of which are widely applied currently. Mostly, the suggestion from a Swedish group is

being followed in europe, which categorizes concentrations <35 ng/ml of PEth 16:0/18:1 as still compatible with abstinence and 210 ng/ml as cut-off for excessive consumption (about 50-60 g ethanol per day) [85].

3.3 AUDIT

The Alcohol Use Disorders Identification Test (AUDIT), a screening instrument for alcohol-related disorders, was developed by the WHO. It is particularly intended to identify individuals with problematic alcohol consumption. It aims at helping patients to reduce or cease alcohol consumption, by offering a framework for intervention and ultimately offering support to avoid the harmful consequences of the patients' drinking behavior. Of the AUDIT's ten items, three relate to alcohol consumption, three to alcohol dependence, and four to alcohol abuse [86]. There is also a short version, the AUDIT-C, only including the three consumption items [87]. Both versions are found in the appendix of this work (9.)

It is explicitly recommended that patients are provided with information on the content and purpose of the application of the AUDIT and that it is explained that accuracy for answers is necessary. Furthermore, the meaning of 'standard drink' needs to be explained to patients, especially as the definition differs between some countries (also see 3.1.2). It is recommended to mention the most common alcoholic beverages that are most likely consumed.

Questions in the AUDIT offer a set of responses to choose from. Each of the chosen answers is scored with points from 0-4 (or 0 and 2-4 for some questions). The total score used for evaluation is calculated by adding up all points and can range from 0-40. The WHO guidelines define a score of 1-7 as low-risk consumption, whereas scores from 8-14 suggest hazardous or harmful alcohol consumption and 15 or more indicate the likelihood of alcohol dependence (moderate-severe alcohol use disorder) [88].

3.4 LC-MS/MS

3.4.1 Mass spectrometry

3.4.1.1 Ionization

The principle of mass spectrometry (MS) is that molecules are converted into ions. This process happens in the ion source of a MS. Different procedures to create the ions are used.

In this work all measurements are carried out with a MS using electrospray ionization (ESI). This technique was first reported in 1984 by Yamashita and Fenn [89]. The electrospray describes the

dispersion of the eluent fluid in which the analytes are dissolved and present in an ionized state. The ions need to be transferred into a gas phase. The fluid is lead through a capillary (about 50 to 100 µm inner diameter) and sprayed into an electric field, that is created by the applied high voltage between the capillary and a counter electrode. This happens at atmospheric pressure surrounding the field [90,91]. A gas (nitrogen), which is applied around the capillary supports the nebulization process, so that complete evaporation is possible even at high flow rates. The electric field between capillary and counter electrode, which has an opening leading to the mass spectrometer, causes an ion separation in the fluid being lead through the capillary. For analyzing negative ions, the capillary needs to be charged negatively, so that the counter electrode is positively charged. The anions move to the surface of the fluid and are being pulled in the direction of the anode. A characteristic cone is formed at the tip of the capillary (Taylor-cone), resulting from equilibrium of the surface tension of the fluid and the electric field. At a certain distance from the cathode the cone disintegrates into droplets. As more and more solvent molecules leave due to evaporation, the charge density continuously increases until the repulsive forces between the equally charged molecules result in an explosion like disintegration into tiny droplets (coulomb explosion), after the so called 'Rayleigh' limit of tension is reached [92]. The whole process is schematically shown in figure 8. In detail, two mechanisms are assumed for the complete process of transferring the ions into gas phase. One is the ion evaporation model (IEM), where the electric field of the surface of highly charged droplets becomes sufficient to release desorbed ions directly from the surface, as the droplets become smaller. According to the charge residue model (CRM) ions finally become de-solvated as solvent molecules exit from the droplet surface. For analysis of positive ions, the voltage must be applied inverted [90-92]. ESI is considered to be a 'soft ionization' technique, because typically only little fragmentation occurs during ionization [93].

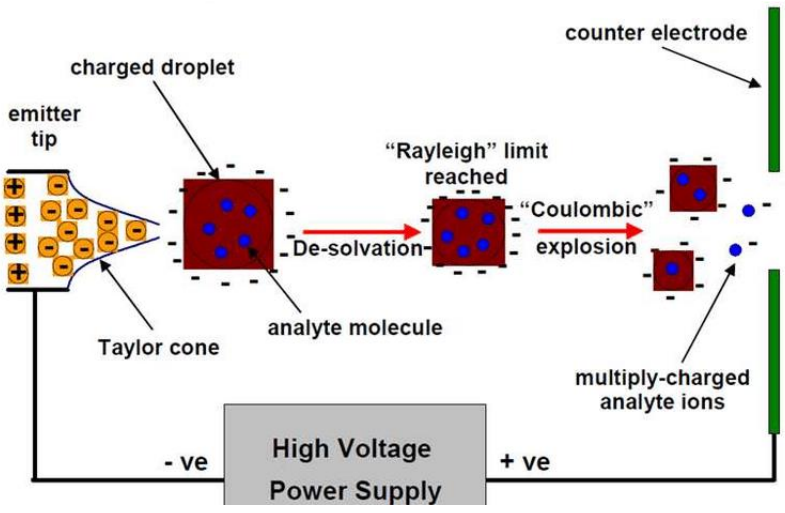

Fig. 8: Schematic representation of electrospray ionization for negatively charged ions [92]

3.4.1.2 Quadrupoles and collision cell

The MS that was used for the analysis in this work was a tandem quadrupole MS. The generated ions are directed into the vacuum region. Here they are lead through a first quadrupole. In a quadrupole four metal rods are ordered around an axis with equal distances. By applying a direct current (DC) and radio frequency (RF) between the rods, where opposite rods have the same voltage, a fluctuating electric field is generated between the rods. The stability of an ion in that electric field is dependent on its mass to charge ratio (m/z). Each m/z has an optimum RF-DC-setting which enables a stable trajectory through the quadrupole. Therefore, the voltages can be set in a way that only ions with defined m/z are stable, ions with other m/z have an unstable flight path and collide with the rods and do not make it through the length of the quadrupole [90,94]. The principle is shown in figure 9.

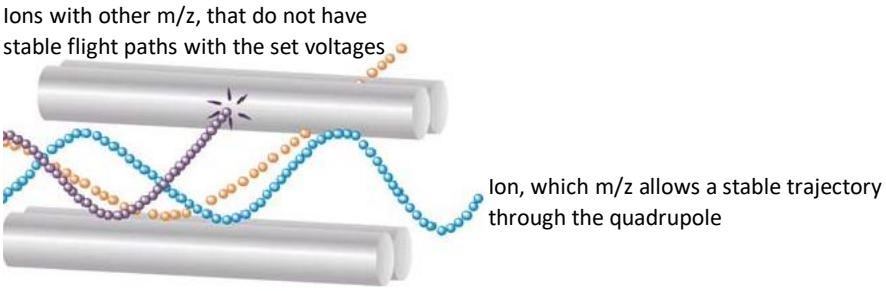

Fig. 9: The electric field set between the rods stabilizes the trajectory of one ion with a specific m/z (modified from [94])

Quadrupoles can be operated in different acquisition modes which are either static or scanning. When set to static mode, the voltages in the quadrupole only let ions with one m/z through and be further transmitted. This allows to look at ions with one specific m/z of interest and therefore yields a very high sensitivity. This is called selected ion monitoring (SIM). The other mode is a scanning one in which the RF-DC-voltages that are applied to the quadrupole are ramped over time [90]. Therefore, each time the voltages are changed an ion with a certain m/z is stabilized in the electric field and can be further transmitted. The scan time reflects the duration the quadrupole spends scanning the set mass-range. This is called full-scan mode and the typical mass spectrums are derived (mass spectrums for PEth homologues are found in 5.1). While this acquisition mode provides information of all ions in the sample, it forfeits sensitivity.

In tandem mass spectrometry two MS are applied after each other. In between a collision cell, sometimes also a quadrupole and hence the term triple quadrupole, is found (fig. 10). The collision cell is where fragmentation takes place. The information gained from fragmenting the ion can be

used to elucidate composition and connectivities of a molecule. Fragmentation also increases the specificity of analysis by enabling to measure the precursor and the fragment masses. Most common and installed in the apparatus used in this work, is the collision-induced dissociation (CID) which uses relatively low energies to yield the fragments. Here basically the molecules are accelerated into the collision cell, that is filled with an inert gas e.g., nitrogen or argon, and when the analyte molecules collide with the gas molecules, energy is transferred, resulting in bonds to break [94]. The site where the break is caused depends on the bond energy, location of bonds and other factors within the analyte molecule. The fragmentation of PEth 16:0/18:1 is shown in 5.1.

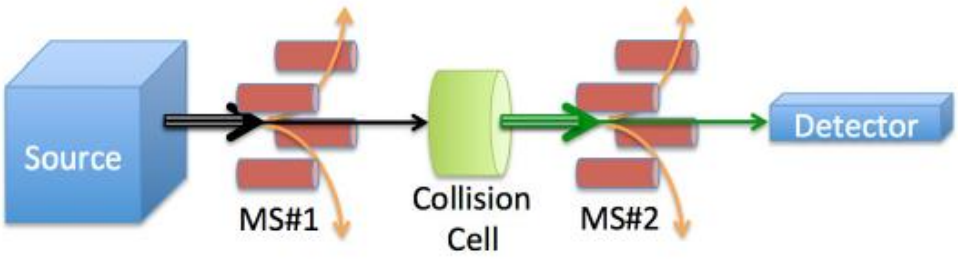

Fig. 10: Schematic set-up of a tandem/triple quadrupole mass spectrometer [96]

With set up of a tandem MS different MS/MS-acquisition modes can be applied, besides only using one quadrupole in full scan or selected ion mode [97]:

- Product ion scan: Quadrupole 1 is set to a static mode and selects a specific ion with a defined m/z and only this ion subsequently enters the collision cell, where the precursor ion is fragmented by the set collision energies. The fragments then enter the second (or third, if the collision cell is counted as quadrupole 2) quadrupole that is operating in a scanning mode, so a defined mass range is scanned and corresponding fragments are continuing to the detector.
- Precursor ion scan: Quadrupole 1 is set to scanning mode, so a certain mass range is scanned and all corresponding ions enter the collision cell. After fragmentation takes place, the fragment ions are going on to quadrupole 2, which is set to static mode. Therefore, only one ion, which m/z matches the applied voltages, can pass through to the detector.
- Neutral loss scan: Both quadrupoles operate in scanning mode. They are set with a defined mass difference, so the quadrupole after the collision cell is offset by the neutral loss of the molecule. Only ions with this mass difference are detected.
- Multiple reaction monitoring (MRM): This mode, in which both quadrupoles are set to static mode, is the most sensitive one for quantitative analysis. The first quadrupole will only pass one precursor molecule, which is then fragmented in the collision cell. The second

quadrupole is set to only pass one specific fragment of the analyte, which is then detected (fig. 11). Multiple of these processes can be combined in one method, which is often done in quantitative analysis and in the method to analyze six homologues of PEth (5.1).

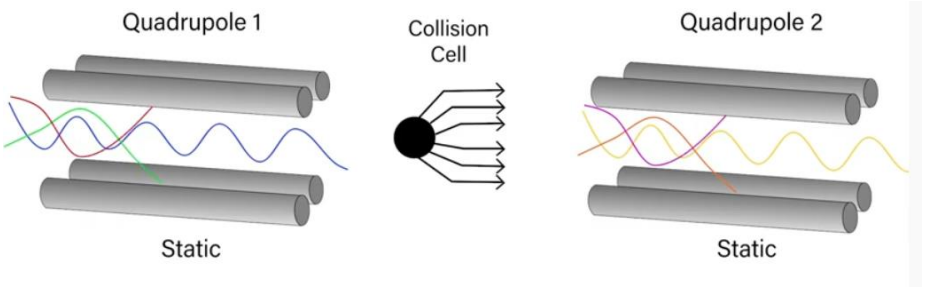

Fig.11: Set-up of an MRM-acquisition-mode, in which both quadrupoles are operated in static mode (modified from [97])

3.4.1.3 Detector

Different detectors can be implemented as the last part in MS-analysis, to convert a current of separated ions into measurable signals. The detection of ions can be carried out by detectors such as Faraday Cup detector, electron multiplier, different array detectors or photomultiplier [90]. The latter is part of the analytical system used for this work. In a photomultiplier the ions hit a dynode which results in electron emission. The electrons in turn strike a phosphorous screen resulting in a release of photons. The photons then enter the multiplier where amplification occurs in a cascade fashion: the photons hit a photocathode which then releases electrons. These released photoelectrons are accelerated in an electric field and interact with further electrodes (dynodes), which shoot out several secondary electrons. With several dynodes following each other, the number of electrons cascades from dynode to dynode. For this to work the dynodes need an increasingly positive potential for the electrons to be accelerating towards them. Finally, the electrons hit an anode and produce a measurable electrical signal [98].

3.4.2 Liquid chromatography

In liquid chromatography (LC) different distribution behaviors of the analytes between the stationary (the column) and mobile phases (the solvents) are used to separate analytes from one another for subsequent detection. The stronger the affinity of the analyte to mobile phase, the faster it moves through the column along with the mobile phase. With stronger affinity to the stationary phase, it moves slower through the column. High- or ultra-performance liquid chromatography (HPLC, UPLC) systems consist of a sample inlet (from the sample manager), a high-pressure pumping unit including various mixers, an oven to control temperature of the separation

column (in the column manager) and a connection to the chosen detector for analysis, besides the stationary and the mobile phase [91].

In the method validated in this work (5.1), reversed phase (RP) chromatography was used. This means that the polar end groups of the silica particles, which is the packing material in the column, are modified by non-polar end groups. In the column used in his work the modification consists of Ethylene Bridged Hybrid (BEH), where an ethyl group bridges across two silicon atoms (fig. 12). The particles become very stable mechanically, which enables a very high packaging rate that still withstands high pressures and supports a wide pH-range [99].

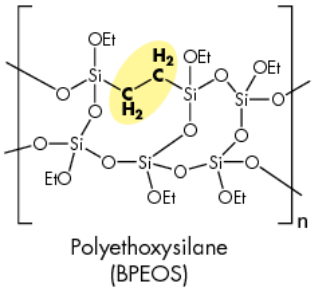

Fig. 12: Structure of BEH material as a stationary phase in a LC-column (modified from [99])

The sample mixture containing the analytes is transferred from a sample vial into a moving fluidic stream of mobile phase, which carries the analytes to the separation column with the help of a high-pressure pump. The mobile phase with the analytes enters, passes through the particle bed in the column and exits the analytes separated by time depending on their chromatographic properties. The elution behavior of the analytes is thus determined by distribution processes between the stationary phase and the selected elution strength of the mobile phase. The retention time of each analyte represents how strongly it is retained on the column and is finally an identification criterion.

4. Objectives of the work

The use of any of the established alcohol markers alone cannot achieve 100% sensitivity and selectivity for the reasons stated in the introduction (3.). It has been shown that diagnostic confidence in the assessment of alcohol exposure is significantly increased when different markers are included in combination. PEth has been discovered as an alcohol biomarker with promising properties: analysis from blood, which leaves no room for falsification of samples, in contrast to urine or hair; detection window of several weeks, which lies between that of Etg in urine and hair; good sensitivity and specificity for alcohol uptake and possible correlation to consumption amounts.

The positive assumption about PEth usage demanded for more research on the alcohol biomarker. First, the aim was to develop and validate a LC-MS/MS method for quantification of PEth. Focus was on enabling measurement from DBS to evade problems of possible sample instability and post-sampling synthesis of PEth in whole blood and with farsightedness to potentially sample capillary blood from the fingertip instead of blood from veins. This bares the advantage of being a less invasive procedure that does not need personnel that is trained in veinous blood sampling. It was important to evaluate different handling techniques of DBS and take a closer look at potential influence of blood characteristics like hct.

Furthermore, it was aimed at establishing a method to quantify several PEth -homologues simultaneously to open the door for research on other homologues but PEth 16:0/18:1 which is the mostly analyzed and best researched homologue. The question was raised whether analysis of multiple homologues, which suspectedly differed in synthesis and elimination rate, is useful, not only for validating alcohol consumption, but to specify further information such as consumption amount or time.

As PEth had been applied in studies for abstinence testing in patients with liver diseases caused by alcohol abuse (ALD), a study was planned that did not only include ALD patients but patients with other etiologies of liver diseases and patients with impaired kidney function to evaluate the applicability of PEth analysis on a broader patient collective and with different drinking behaviors.

The main objective was how well PEth performed analytically (sensitivity and specificity) in the setting especially compared to the routinely used alcohol biomarkers Etg in urine and hair, CDT and MCV.

Further interest was on investigating PEths performance as a marker for high and excessive alcohol consumption behavior, especially the suggested cut-off of 210 ng/ml for that drinking behavior. Particularly the comparison to CDT, specific for consumption of high alcohol amounts for several weeks, was of interest.

In both studies the major limitation, as predicted beforehand, were the potential bias of self-reported alcohol consumption behavior, which was tried to be mitigated by not only handing out questionnaires but also interviewing the patients and participants face to face.
Publications concerning PEth stability in whole blood were first of all rare and second of all non-existent for multiple of the six homologues integrated in our method for PEth analysis. Therefore, a further objective claimed was to thoroughly investigate and evaluate stability of PEth homologues in such detail, that well-founded suggestions for pre-analysis sample handling could be obtained.
At last, another aim was to contribute to the on-going discussion about an optimal cut-off for differentiating between abstainers and consumers of alcohol. Here a study was planned with at least 60 participants who consume small amounts of alcohol on several consecutive days after a phase of abstinence and 20 participants in a control group consuming alcohol-free beer. Previous studies had investigated PEth synthesis after uptake of higher amount of alcohol or single drinking events. Therefore, we predicted a high benefit of such a study to evaluate different cut-offs for PEth.

In the following, the work presents analysis of PEth concerning different settings, different cohorts, different consumption amounts, differences between sexes and benefits of analysis of multiple homologues.

5. Cumulative Part/Synopsis

5.1 LC/MS-MS method for the analysis of six homologues of PEth

The method to quantify six different homologues of PEth was published in *Drug Testing And Analysis* in 2020 (online) [100].

To my knowledge it was the first method that enables the simultaneous measurement of six different PEth-homologues (PEth 16:0/18:1, PEth 16:0/18:2, PEth 16:0/20:4, PEth 18:0/18:1, PEth 18:0/18:2, PEth 18:1/18:1).

Initial experiments started on a tandem quadrupole analyzer (Xevo TQ, with AQCUITY UPLC (TQ-MS) from Waters, Milford, MA, USA). Various parameters for the best possible fragmentation and detection of PEth (capillary voltage, cone voltage, desolvation temperature, source temperature, high mass and low mass resolution, ion energy, collision energy, gas flows) were determined using direct infusion of standard solutions into the mass spectrometer. The detection was performed in an MRM (see 3.4.1.2).

At that time only three homologues were commercially available (PEth 16:0/18:1, PEth 16:0/18:2, PEth 18:1/18:1) and a propyl derivate (Phosphatidylpropanol 18:1/18:1) was used as an internal standard, so the primary method development was based on these substances. The chromatographic settings were initially inspired by a method of Helander et al. [80], with slight differences using a C4 high purity column (50mm) (Thermo Fisher Scientific, Waltham, MA, USA), with a 4:6-water and acetonitrile mix (2 mM ammonium-acetate as additive) and isopropanol as eluents. After all, satisfactory separation of the substances could not be achieved. In addition, the viscosity of isopropanol resulted in a high pressure in the system and problems with other methods running in parallel on the same LC-MS/MS instrument, since a very long rinse had to take place to free the system from the running medium rests. Therefore, various other eluent compositions were tested for their applicability, and a combination of methanol and an ammonium-acetate buffer proved to be particularly suitable regarding chromatography. Once further homologues became commercially available, it was decided to adapt the method to quantify six homologues simultaneously in a single run. Due to the very similar molecular weights of some of the homologues and to ensure accurate analysis it was aimed at chromatographically separating the homologues from each other. This could not be achieved on the short HYPURITY C4 column that was previously used. To enhance interaction with the fatty acid residues of the PEth molecules and retention times a long BEH column (Acquity, 2.1x150 mm, 1.7m (Waters)) (see 3.4.1) was finally used for the method.

As deuterated standards of all homologues (except for PEth 18:1/18:1) became available as well, they were acquired and incorporated into the method, ensuring more reliable quantification of the molecules and to enable correction of matrix effects [101].

Dried blood spots (DBS) were chosen as a matrix carrier, as sample handling is very easy, extraction is simpler than from whole blood, stability of PEth was thought to be inadequate for storage of several days in EDTA tubes [102] and with looking ahead to advantages of an analysis from capillary blood. Stability of PEth will be subject in 6.1 and sampling of capillary blood will be addressed as well in later chapters.

Different circumstances for extraction of the molecules from filter paper (Whatman #903) were tested. Experiments were performed with different extraction agents and their amounts, extraction time, temperature during the extraction process and different shaker frequencies. Extraction with 1000 µL methanol, under room temperature and shaking for one hour at about 190 rpm was satisfactory. Due to the small sample volume of 20 µL (compared to 100 µL of used in methods with whole blood), special care had to be taken to ensure that the sensitivity of the method was sufficient to analyze concentrations as low as those obtained with a whole blood method. For this purpose, the analysis was taken onto a newer UPLC-MS/MS (TQS (Waters)) instrument that offers very high sensitivity and robustness due an enhanced ion guidance system of the apparatus ("StepWave®").

The publication ‘Analysis of six different homologues of PEth from dried blood spots using liquid chromatography–tandem mass spectrometry’ mainly describes the method settings and validation in detail. Furthermore, it contains information about the percentile distribution of the six analyzed homologues in authentic samples of patients of the outpatient liver transplantation center of the University Medical Center Hamburg Eppendorf (UKE). This was firstly done, and interesting findings were made, e.g. that PEth 16:0/18:1 is replaced as most abundant homologue in some patients by 16:0/18:2 [78].

The aim of establishing a method to quantify different homologues was not only to use PEth as an abstinence marker, but to get a further insight into the potential contribution to drinking behavior monitoring by measuring multiple homologues. Javors et al. [103] and Hill-Kapturczak et al. [104] found differences in the synthesis- and elimination kinetics, which led to the idea that measuring multiple homologues could give further insight into the timing of the drinking events. Figure 13 shows the molecular structure of PEth 16:0/18:1 in its ionized form ($[M-H]^-$), in which it is needed for MS-detection. Additionally molecular masses for different parts, especially relevant for MS-detection are given.

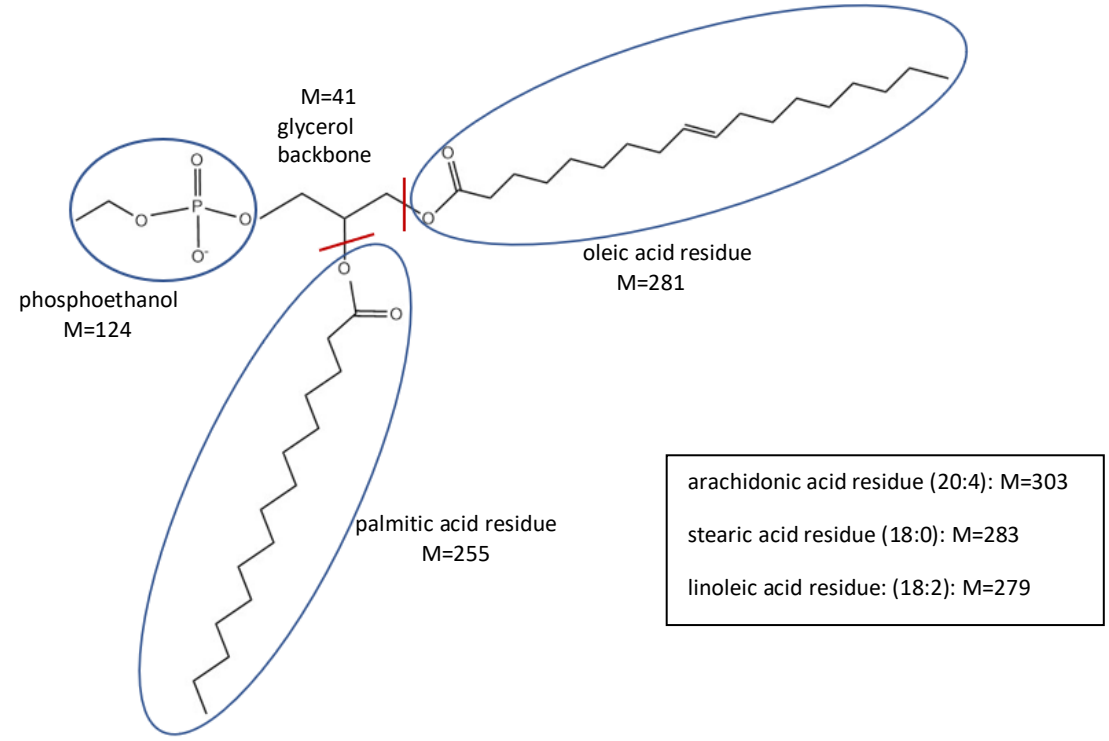

Fig. 13: PEth 16:0/18:1 molecule, marked with the fragmentation sites (red lines) for the analyzed transitions and with the molecular weights (blue)

In Figure 14 a mass spectrum of PEth 16:0/18:1 is shown, where the molecular ion (m/z=701) and daughter ions (m/z=255, m/z=281) can be seen.

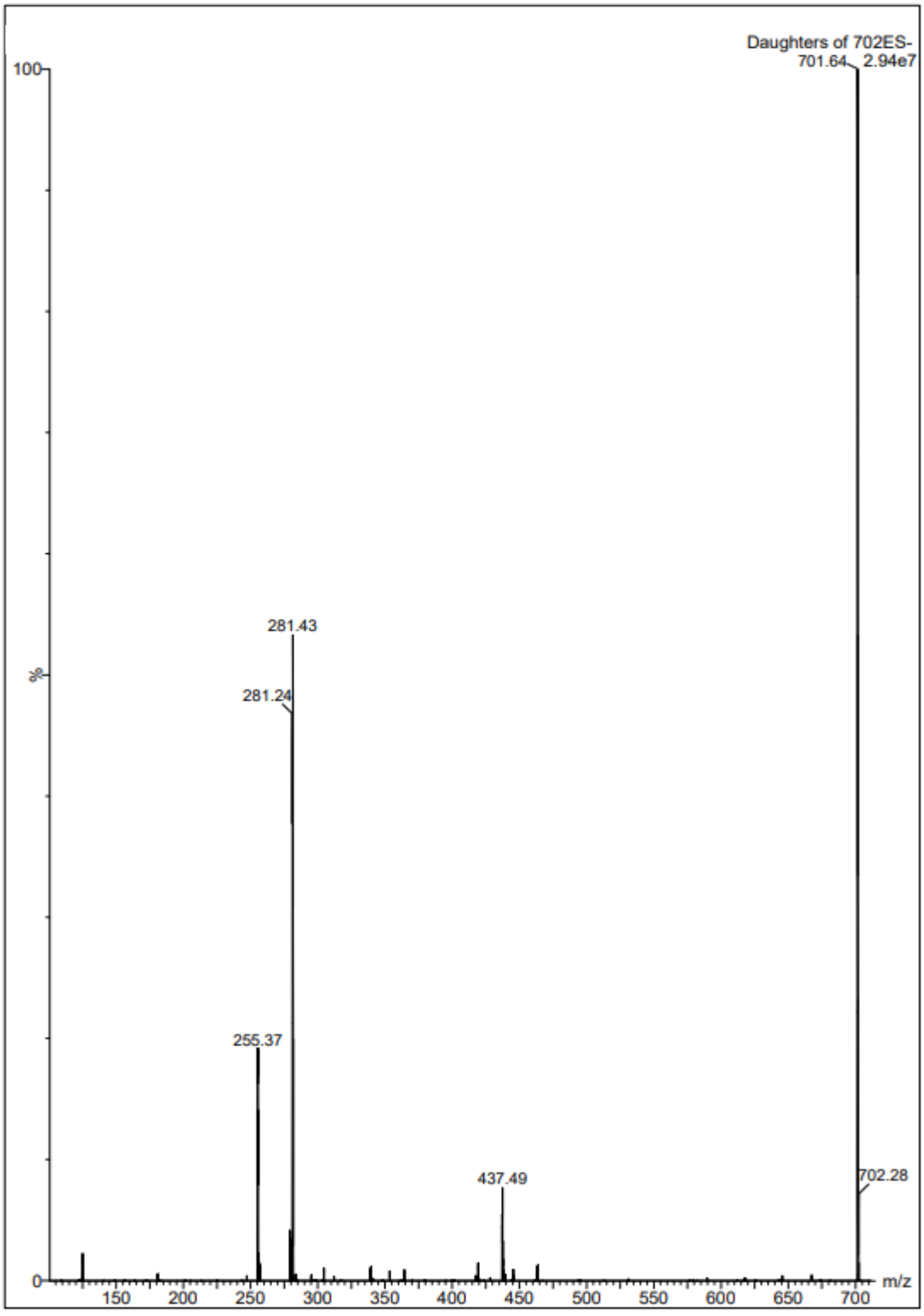

Fig. 14: Mass spectrum of a daughter scan of PEth 16:0/18:1, settings as described in the publication, collision energy 36 eV

Figure 15 displays the mass spectrum of a solution of a native mix of PEth homologues produced with a scan with the MS settings described in the publication.

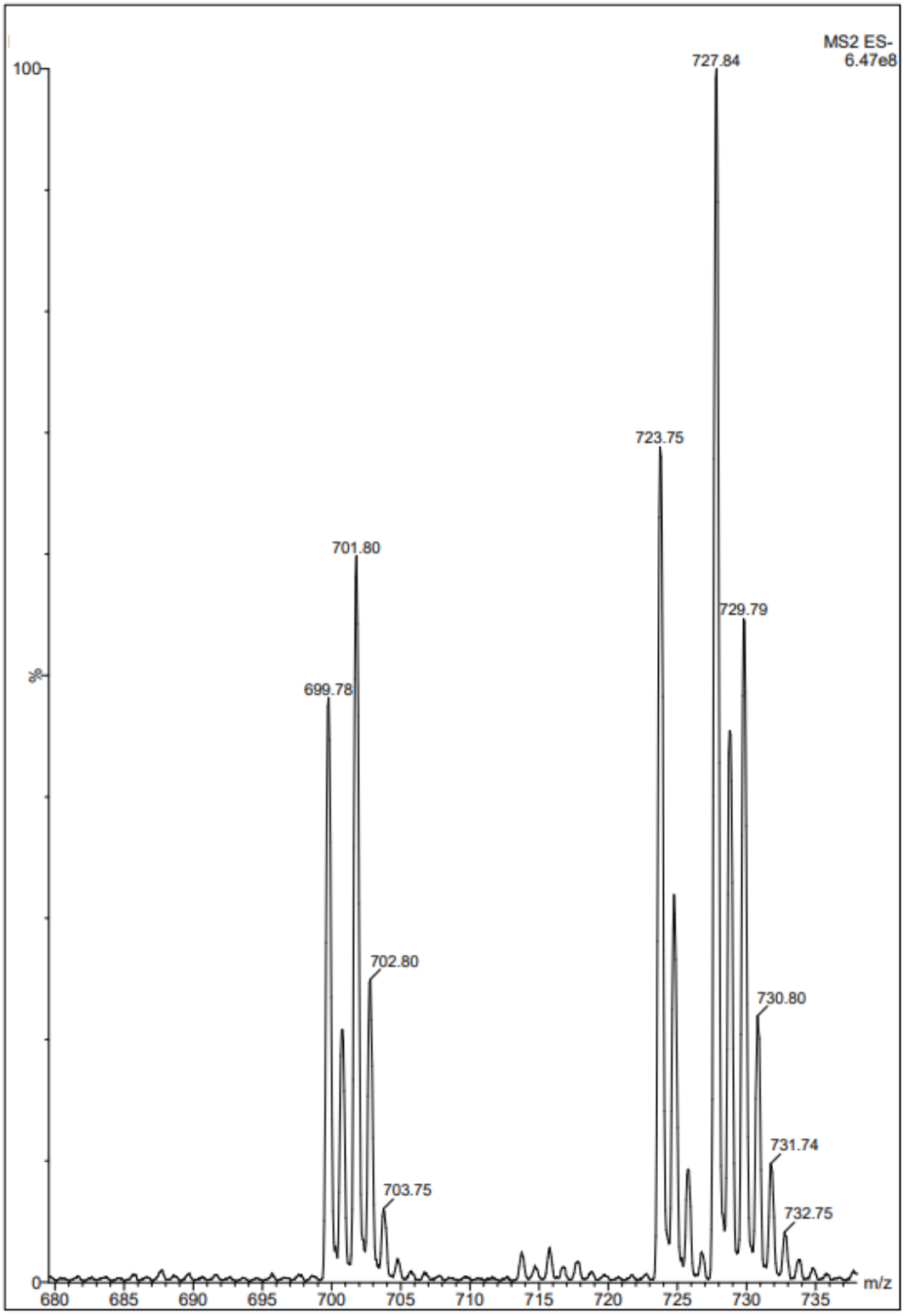

Fig. 15: Mass spectrum of a mix of all native standards (MS1 scan), settings as described in the publication

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RESEARCH ARTICLE

Analysis of six different homologues of phosphatidylethanol from dried blood spots using liquid chromatography–tandem mass spectrometry

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Abstract

Phosphatidylethanol (PEth) is a direct biomarker for alcohol consumption consisting of a fraction of different ethanol-modified, homologue phospholipids. The aim of this study was to validate an ultra-high-performance liquid chromatography–tandem mass spectrometry method to quantitate six different homologues of PEth (16:0/18:1, 16:0/18:2, 16:0/20:4, 18:0/18:1, 18:0/18:2, and 18:1/18:1) from dried blood spots (DBSs). DBSs were prepared volumetrically (20 μL of whole blood) and extracted with 1 mL of methanol (0.02 ng/μL internal standards). PEth homologues were separated on a BEH C18 column (2.1 × 150 mm, 1.7 μm) using methanol and ammonium acetate buffer (25 mM) in a 7 min isocratic run. Multiple reaction monitoring mode was used for the detection of PEth and the internal standards. Calibrators (10–1000 ng/mL) and quality controls (40, 400, and 700 ng/mL) were prepared from spiked whole blood; external control samples were obtained from proficiency testing schemes. After a comprehensive validation of the method, quantitative patterns of the different homologues were investigated in PEth positive samples (n = 57) from patients in a transplant setting. Satisfactory chromatographic separation, sensitive detection, and reliable quantification of the PEth homologues in DBSs can be achieved using the liquid chromatography–tandem mass spectrometry (LC/MS/MS) procedure. Validation results, including accuracy, linearity, recovery, matrix effects, and in-process stability, complied with international standards, and the analytical performance of the procedure was not affected by the hematocrit of the blood samples. Different quantitative patterns of the investigated PEth homologues were observed in authentic samples from liver transplant patients. This method will enable the study of the kinetics of six PEth homologues simultaneously and investigate the meaning of the homologues' distribution in individuals.

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KEYWORDS
alcohol biomarker, dried blood spots, LC/MS/MS, phosphatidylethanol

1 | INTRODUCTION

An impartial assessment of patients' alcohol consumption is of great interest in multiple clinical and forensic settings. Bioanalytical examinations determining the so-called alcohol biomarkers are carried out to evaluate the extent and duration of the preceding alcohol exposure of an individual. This is important in different circumstances, for example, monitoring of alcohol recovery patients, identification of alcohol exposure of newborns during pregnancy, abstinence testing in organ transplant recipients, traffic medicine, and custody cases. A panel of different biomarkers from different tissues (body fluids, keratinous tissues) is available to estimate ethanol exposure of an individual today. There are substantial differences in terms of sensitivity, specificity, detection window, and diagnostic performance of alcohol markers. Phosphatidylethanol (PEth), a fraction of ethanol-modified phospholipids, is a highly specific (formed exclusively in the presence of ethanol) marker in blood, enabling the detection of ethanol exposure up to several weeks. The phospholipids derive from phosphatidylcholine (PC) and are formed enzymatically by the action of phospholipase D in the presence of ethanol.[1] Corresponding to the precursor's diversity, PEth is a group of different homologue compounds, consisting of a phosphoethanol head group on which a variety of fatty acid chains are attached via a glycerol backbone (at sn-1 and sn-2 positions). Gnann et al identified 48 different homologues of PEth.[2] The most abundant and most researched homologues in red blood cells are the ones with palmitic and oleic acid chains (16:0/18:1) and palmitic and linoleic acid chains (16:0/18:2),[2,3] whereas less attention has been paid to the less-prominent compounds. Little is known about the homologues' distribution patterns and the kinetic properties of the homologues. This information might allow one to detect further correlation between the PEth-homologue values and drinking habits.

Due to chemical instability and possible post-sampling formation from ethanol in whole blood, strong freezing (−80°C) is necessary in the context of PEth analysis in whole blood, which is a major limitation.[4] Sampling of dried blood spots (DBSs) was found to be beneficial: a strong improvement of stability (up to 1 year[4]) and the absence of post-sampling formation of PEth in DBSs enable easier handling, storage, and transportation of samples.[5] In addition, the alternative preparation of DBSs directly from capillary blood could be a more convenient way (a prick on the fingertip instead of a puncture of a vein) of sampling in PEth, as it is less invasive and there is no need for a phlebotomist. One of the most challenging analytical issues in the context of DBSs is the hematocrit (Hct) effect: spot formation, drying time, spot homogeneity, and size are primarily influenced by blood Hct (in terms of blood viscosity); in addition, serious impact on analyte recovery, matrix effects (MEs), ruggedness, and reproducibility of the assay can occur.[6] The aim of the present work was to establish a validated liquid chromatography–tandem mass spectrometry (LC/MS/MS) method for monitoring several PEth homologues simultaneously from (volumetrically defined) DBS samples, which is suitable for routine analysis and will further enable the investigation of the kinetics of PEth homologues (eg, formation, elimination, and interindividual differences) related to alcohol consumption in future studies.

2 | EXPERIMENTAL

2.1 | Chemicals

1,2-Dioleoyl-sn-glycero-3-phosphoethanol (PEth 18:1/18:1), 1-palmitoyl-2-lineoyl-sn-glycero-3-phosphoethanol (PEth 16:0/18:2), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol (PEth 16:0/18:1), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoethanol (PEth 16:0/20:4), 1-stearoyl-2-lineoyl-sn-glycero-3-phosphoethanol (PEth 18:0/18:2), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoethanol (PEth 18:0/18:1), PEth 16:0/18:2-d5, PEth 16:0/18:1-d5, PEth 16:0/20:4-d5, PEth 18:0/18:2-d5, and PEth 18:0/18:1-d5 were purchased from Echelon (Salt Lake City, UT, USA). 1,2-Dioleoyl-sn-glycero-3-phosphopropanol (PProP 18:1/18:1) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). All solids were reconstituted in methanol (MeOH) (J. T. Baker, Deventer, the Netherlands) to make a stock solution of 1 mg/mL. For further dilution to yield working solutions, MeOH was used. LC/MS-grade MeOH and LC/MS-grade water were obtained from Honeywell (Seelze, Germany). Ammonium acetate (eluent additive) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 | Sample preparation

Whole blood collected from alcohol-abstinent volunteers, previously tested negative for PEth, was used to prepare calibrator and control samples. Working solutions containing all homologues were prepared to spike the calibrators (10, 50, 100, 200, 500, 1000 ng/mL) and the quality controls (QCs) (40, 400, and 700 ng/mL). To minimize precipitation in the blood caused by the solvent MeOH, the working solutions were pre-diluted, enabling the addition of a single 4 μL aliquot of standard mix to 400 μL of whole blood. After the standard solution was spiked, each sample was vortexed for 30 s and then shaken on a sample mixer for at least 10 min. Blood spots (20 μL) were pipetted onto Whatman #903 filter paper (GE Healthcare, Buckinghamshire, UK) using an Eppendorf pipette. The blood spots were left to dry for at least 3 h at room temperature and then stored in a Ziploc bag with a desiccant bag (Sigma-Aldrich), shielded from direct sunlight, at room temperature, for further sample analysis, not longer than 3 weeks

after preparation. DBSs were cut out as a whole and put into a disposable glass tube. MeOH (1 mL) containing all internal standards (0.02 ng/μL) was added. After the disposable glass tubes were sealed, they were shaken for 60 min at ~190 rpm on an orbital shaker. Then, 800 μL of MeOH was transferred into a V-vial and evaporated at 40°C using a gentle stream of nitrogen. The residue was reconstituted in 200 μL of mobile phase A (90:10 [vol%] MeOH and acetate buffer) and vortexed for 30 s.

3 | APPARATUS

3.1 | LC/MS/MS

The analyses were carried out on a tandem-quadrupole mass spectrometer (XEVO TQ-S) coupled to a UPLC (Acquity System) and a sample manager (Acquity I-class) (all from Waters, Milford, MA, USA). To control system operation parameters and to acquire and process data, the software Masslynx 4.1 (Waters) was used.

3.2 | Chromatography

An Acquity UPLC BEH C18 column (2.1 × 150 mm, 1.7 μm; Waters) operating at 40°C was used for separation. Mobile phase A consisted of 90% MeOH and 10% 25 mM ammonium acetate, and mobile phase B was MeOH. Elution was carried out in a 7 min isocratic run with 40% A/60% B at a flow rate of 0.5 mL/min. The injection volume was 10 μL.

3.3 | Mass spectrometry

For MS/MS detection, electrospray ionization in negative mode was used. The settings were as follows: source temperature, 150°C; capillary voltage, 2.5 V; desolvation temperature, 600°C; and desolvation gas flow rate, 1000 L/h. For all measured PEth homologues, two mass transitions were chosen. The internal standards were each detected by a single transition (Table 1).

4 | METHODS

4.1 | Validation

The method was validated according to the German Society for Toxicology and Forensic Chemistry guidelines,[7] which are based on international guidelines. Calculations were carried out using the software Valistat 2.1.[8]

External QC (only PEth 16:0/18:1) in blood was achieved by participation in eight rounds of a proficiency test offered by Equalis (Uppsala, Sweden).

4.2 | Selectivity

Blank blood from six different persons was measured with and without internal standards. In addition, each PEth homologue was used to spike blank blood exclusively with one of the analytes. It was examined for interferences that could influence quantification.

4.3 | Calibration range

A seven-point calibration curve was prepared as mentioned earlier. Linearity of calibration and variance homogeneity were checked by regression analysis of area ratios (analyte/internal standard) to the calibrator concentration and by performance of a Cochran test and a Mandel-F test.

TABLE 1 *m/z* of monitored precursor, target, and qualifier ions; set cone voltage and collision energies for six PEth homologues and respective internal standards

	Transitions (*m/z*)				Collision energy (eV)	
PEth/prop-homologue	Parent	Target	Qualifier	Cone voltage (V)	Target	Qualifier
16:0/18:2	700.0	279.4	255.4	66	32	36
16:0/18:1	702.0	281.4	255.3	22	34	36
16:0/20:4	723.9	303.4	255.4	8	30	36
18:0/18:1	730.0	283.4	281.4	14	44	34
18:0/18:2	728.0	283.3	279.4	42	32	36
18:1/18:1	728.1	281.4	463.5	94	28	26
16:0/18:2-d5	705.0	279.3		14	32	
16:0/18:1-d5	707.0	281.4		6	30	
16:0/20:4-d5	729.0	303.4		16	28	
18:0/18:1-d5	735.1	281.4		74	34	
18:0/18:2-d5	733.0	279.3		10	34	
PProp18:1/18:1	742.0	281.3		68	44	

4.4 | Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined by the DIN32645 procedure.[7] Seven blood concentrations around the expected limits were spiked from PEth-free blood (3, 5, 7, 10, 12, 15, and 20 ng/mL). The linearity of this curve and the presence of stragglers were tested on a 99% significance level for both qualifier and target transitions.

4.5 | Recovery

The extraction recovery (ER) of PEth from DBSs was determined at concentration levels of QC1 and QC3 and three different Hct levels (0.2, 0.4, and 0.6). After sedimentation of red blood cells, specified volumes of plasma were either added (from the same blood donor) or removed to achieve different Hct values. Hct was measured (hematology analyzer ADVIA 2020i, Siemens, Munich, Germany) after preparation. ER was calculated using the absolute areas from six independent measurements of the extracted DBSs and from spiked extraction solvent from PEth-free blood.

4.6 | Matrix effects

MEs were assessed at two concentration levels corresponding to QC1 and QC3. DBSs of PEth-negative blood of six different alcohol-abstinent volunteers were extracted as described earlier and finally reconstituted with mobile phase A, which was spiked to the corresponding concentrations. The impact of Hct on MEs was investigated at three different Hct levels prepared from the blood of two individuals (see earlier). The resulting areas and responses were compared to mobile phase A that was directly spiked with analytes and injected.

4.7 | Precision and accuracy

To measure the accuracy and between-day and within-day precision, the three QC levels were each analyzed in triplicate on nine different days. Precision was evaluated by the relative standard deviation (RSD, %), accuracy by the bias (%), and the 95% tolerance intervals were determined. Three different Hct levels were investigated in these experiments.

4.8 | Processed sample stability

To evaluate the stability of the analytes during the analytical procedure, six samples of each QC1 and QC3 were prepared. Then the six samples were pooled and aliquoted to six vials. The pooled samples of each concentration were injected regularly over the duration of a complete series of samples, and absolute signal areas were observed. The duration of the series for QC1 and QC3 testing was 6.6 and 9.4 h, respectively, and the temperature in the autosampler was 10°C.

4.9 | Authentic samples

Authentic samples were obtained from patients who participated in a clinical study at the University Medical Center Hamburg-Eppendorf (UKE) to evaluate the value of PEth measurement in addition to other alcohol biomarkers in the transplant setting. All patients were informed about the measurements that were made and about the fact that quantification of the additional alcohol marker would not influence their treatment in the clinic. All patients who participated provided written consent. The ethics committee agreed to the study (PV5068).

Blood was taken by venous puncture into an EDTA tube. The authentic blood samples were immediately stored at 4°C and were volumetrically spotted (20 μL) within a maximum of 4 h thereafter. The DBSs were stored under the same conditions as those for the calibrator samples.

Ethanol was also tested from serum taken at the same time as the whole blood by an enzymatic test (AU 480, Beckmann Coulter, Brea, CA, USA), as *in vitro* PEth formation takes place if EtOH is present in the whole blood sample.[9] Furthermore, the Hct levels of all blood samples were determined. When referring to total PEth concentrations, the sum of the concentration of PEth 16:0/18:1, PEth 16:0/18:2, PEth 16:0/20:4, PEth 18:0/18:1, PEth 18:0/18:2, and PEth 18:1/18:1 is described here. Concentrations below LOQ were excluded from the calculations for the sum of all PEths. The values between LOQ and the lowest calibration point were quantified via extrapolation.

5 | RESULTS

5.1 | Method

An LC/MS/MS method for the separation of PEth 16:0/18:1, PEth 16:0/18:2, PEth 16:0/20:4, PEth 18:0/18:1, PEth 18:0/18:2, and PEth 18:1/18:1 after extraction from DBSs was achieved in a 7 min isocratic run, without interfering peaks (Figure 1). For all homologues, except for PEth 18:1/18:1, deuterated internal standards are commercially available. Due to the relatively high molecular weight of the molecules, the deuteration (d5) of the standards has a minor impact on retention, leading to the almost-simultaneous elution of analyte and corresponding internal standard. This is beneficial for peak detection and for compensation of MEs.

5.2 | Validation

5.2.1 | Selectivity

There was no interference from other components in blood at the retention times of the analytes. Furthermore, the homologues did not influence each other's detection and quantification, and no interferences of the internal standards were observed.

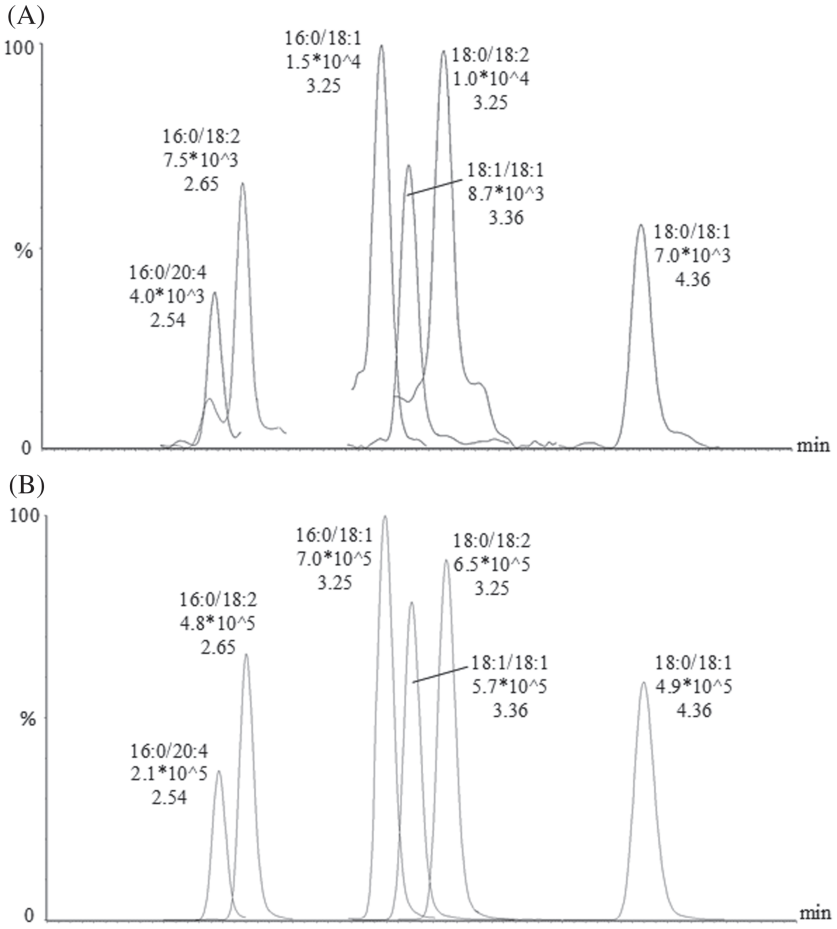

FIGURE 1 Chromatogram of all six PEth-homologues, A) lowest point of calibration (10 ng/ml)) B) QC3 (700 ng/ml) (homologue, retention time, area marked to corresponding peak)

5.2.2 | Calibration range

The calibration was linear from 10 to 1000 ng/mL with $r^2 > 0.995$ and the residuals spreading randomly less than 15% around zero. For all homologues, neither the target nor the qualifier ion presented stragglers at the two significance levels, the Cochran test and the Mandel-F test were passed, and weighing of calibrators was not necessary.

5.3 | LOD and LOQ

The low calibration curves passed the test for linearity, and no stragglers were observed. The values of LOD < 4 ng/mL and LOQ < 9 ng/mL were found for the six homologues, which are presented in Table 2.

TABLE 2 LOD and LOQ for each homologue

Peth	LOD (ng/mL)	LOQ (ng/mL)
16:0/18:1	3.9	8.6
16:0/18:2	3.9	6.0
16:0/20:4	3.4	7.7
18:0/18:1	2.8	6.1
18:0/18:2	2.8	7.5
18:1/18:1	3.2	6.6

Abbreviations: LOD, limit of detection; LOQ, limit of quantification.

5.3.1 | Recovery

The ER rates at different Hct levels are presented in Table 3. The exact Hct levels of the used blood samples were 0.217, 0.399, and 0.597. At all Hct levels, the ER rate was higher than 71% for PEth 16:0/18:1, 58% for PEth 16:0/18:2, 53% for PEth 16:0/20:4, 66% for PEth 18:0/18:1, 65% for PEth 18:0/18:2, and 62% for PEth 18:1/18:1. The maximum coefficient of variation (CV) was 15% for 16:0/18:1 (QC1, Hct of 0.2).

5.3.2 | Matrix effects

MEs were between 93% and 112% (CV < 5%) except for PEth 18:0/18:1: significant (ME: 130%–142%) and reproducible (CV < 3%) signal enhancement was observed for this homologue, but MEs were compensated by internal standard (100%–111%) in these cases.

There was no significant difference in ME noticeable between the different Hct levels.

5.3.3 | Precision and accuracy

All homologues passed the tests for precision and accuracy. The results did not differ at the tested Hct levels. The maximum RSD

TABLE 3 Extraction recovery rates of all PEth homologues at QC1 and QC3 levels at hematocrit values of 0.217, 0.399, and 0.597

	0.217		0.399		0.597	
	QC1 (%)	QC3 (%)	QC1 (%)	QC3 (%)	QC1 (%)	QC3 (%)
16:0/18:1	71	78	81	84	78	71
16:0/18:2	72	81	67	81	63	58
16:0/20:4	61	65	55	66	55	53
18:0/18:1	66	83	83	91	84	85
18:0/18:2	65	77	71	82	70	71
18:1/18:1	62	82	66	69	67	73

Abbreviation: QC, quality control.

TABLE 4 Range of absolute values and ratio in percentage of all six homologues (n = 57)

	Range	
Peth	Absolute (ng/mL)	Percentage
16:0/18:1	11.9–1597.2[a]	19–68
16:0/18:2	6.0[b]–687.9	11–42
16:0/20:4	<LOQ–162.5	0–18
18:0/18:1	<LOQ–300.1	0–25
18:0/18:2	<LOQ–504.6	0–31
18:1/18:1	<LOQ–444.0	0–18

[a]Value > calibration range.
[b]Value < calibration range.

values for within-assay precision, between-precision, and bias at QC1 level were 1%, 6.5%, and −6.1%, respectively (Hct of 0.4); 1.6%, 10.3%, and 5.1% at QC2 level; and 0.2%, 6.5%, and 4.8% at QC3 level; 95% of all measured values (all homologues, all QC levels, all Hct levels) were included in a range of target ±30%.

5.3.4 | Processed sample stability

The maximum differences between the absolute signal areas were found to be 19% (QC1) and 11% (QC3); trend plotting of signal areas of stability samples resulted in no (QC1) or very low (QC3, slope −0.0008) decreasing trends.

5.3.5 | External QC

Quantitative results were satisfactory in the proficiency testing schemes; the mean deviation was found to be +15%, range −18% to 21%.

6 | AUTHENTIC SAMPLES

Quantitative PEth results of the 57 authentic samples from patients with detectable PEth are presented in Table 4 showing the range of values of the collective; 31 patients were males and 26 were females. The mean age of the patients was 54.9 ± 13.4 years (range 18–77 years). None of the samples was positive for ethanol. In three cases the concentration of PEth 16:0/18:1 exceeded the calibration range and was therefore estimated via extrapolation. The total PEth concentration (sum of all measured PEth concentrations) was between 18.6 and 2959.1 ng/mL. Differences were observed in both the absolute concentrations and percentage distribution of PEth homologues. The absolute PEth concentrations of each patient can be found in detail in Supporting Information.

Six of the patients exclusively tested positive (>LOQ) for two homologues (16:0/18:1 and 16:0/18:2), which were the ones with the lowest (<42.5 ng/mL) total PEth concentration. In these patients, the ratio of 16:0/18:1 from the total PEth concentration ranged from 57% to 72%. In six patients, three homologues tested positive, the third one being 16:0/20:4 in four cases and 18:0/18:1 in two cases. When four homologues were positive (seven patients), it was 16:0/18:1, 16:0/18:2, 16:0/20:4, and 18:0/18:1 in six cases. In one case, 18:0/18:2 was positive instead of 16:0/20:4. In all three cases with five positive PEth homologues, 18:1/18:1 was the one less than LOQ. All homologues were positive in 34 patients. The lowest value for total PEth concentration, with all homologues being present, was 83 ng/mL.

In nine cases, 16:0/18:2 was the most prominent homologue. In other cases it was 16:0/18:1, and in one case, the concentrations of 16:0/18:1 and 16:0/18:2 were identical.

The lowest total PEth concentration at which 16:0/20:4 and 18:0/18:1 were quantified was 46.5 ng/mL. For 18:0/18:2, the total PEth concentration was 71.3 ng/mL and for 18:1/18:1, 83.7 ng/mL. The average value for the Hct was 0.393, with the full range of 0.267–0.500. Therefore, none of the participants had to be eliminated due to Hct, as it was demonstrated by validation that quantification is valid for an Hct of 0.2–0.6. The Hct level of all blood samples used for the calibration curves ranged from 0.39 to 0.45.

7 | DISCUSSION

7.1 | Method and validation

An LC/MS/MS method for the determination of six different Peth homologues from DBSs in a single run was validated and applied for

the analysis of authentic blood samples. The assay was successfully tested in a proficiency testing scheme (eight rounds till now), and more than 4000 samples have already been processed demonstrating its practicability for routine application. The calibration range from 10 to 1000 ng/mL was found to be adequate for the majority of the samples and for the main future applications: abstinence testing and investigation of kinetics of the six different homologues after moderate alcohol intake. The suggested cutoff of 210 ng/mL PEth 16:0/18:1 for the detection of chronic alcohol consumption,[10,11] which was proposed by a Swedish group, was covered by the calibration range of the method. Because PEth concentrations may exceed 1000 ng/mL in cases of severe alcoholism,[12] an accurate quantification of PEth in the blood of heavy drinking patients would require an extended calibration range.

Whereas analytical procedures including one,[3,13] two,[14,15] or three[16] homologues of PEth can be already found in literature today, parallel quantification of PEth 16:0/18:1, PEth 16:0/18:2, PEth 16:0/20:4, PEth 18:0/18:1, PEth 18:0/18:2, and PEth 18:1/18:1 has (to our knowledge) not been described before. Currently, reference materials for these six molecules are available; additional homologues could be introduced in the procedure if they become available.

Different columns for the analytical separation of different homologues have been discussed in previous methods. Andreassen et al[13] used a 30 mm long BEH-phenyl column for high-throughput analysis of PEth 16:0/18:1. Other authors used predominantly C4 or C8 columns,[3,15,17] which had also been considered in the present work during method development. However, optimum chromatographic separation was achieved by standard C18 columns using methanolic eluents. Because PEth homologues are strongly retained by C18 columns, it was previously concluded that those columns are not suitable for the separation of PEth homologues.[2,17] This is finally not confirmed by our work.

The influence of Hct in DBS testing has been discussed in detail and is considered as one of the most important issues in this field.[6,18,19] In 2019 the International Association for Therapeutic Drug Monitoring and Clinical Toxicology published a review for the development and validation of DBS-based methods.[20] The present method was validated in accordance with these guidelines. The impact of Hct on analytical performance was evaluated in terms of ER, ME, and precision. In summary, the results obtained from PEth-spiked, negative blood samples were not affected by Hct in our study, which is consistent with the findings of Kummer et al.[16] Beck et al also investigated the influence of Hct effect in the context of PEth biomarker analysis and reported an impact of Hct on PEth-testing results, predominately at higher levels of PEth.[21] However, Hct was changed in PEth-positive blood samples retroactively by the addition or removal of plasma, so dilution or concentration of red blood cells (containing the highest portions of PEth) occurs in this experimental setting. In the present study, we spiked the blood sample after adapting the Hct as described for validation. The entire spot (volumetrically defined to be 20 μL) is used in the present method, so the influence of the DBS size and homogeneity is negligible.[22]

7.2 | Authentic samples

In this study the occurrence of the six different homologues was shown in relation to the total PEth concentration. The distribution of PEth homologues in patient samples was found to be individually different (see "Results"),[23] which might be due to the differing drinking behaviors of the patients and individual occurrence of the PC-precursor molecules. Therefore, the simultaneous quantification of several homologues in specific samples (a) might allow one to elucidate the reason for those differences, (b) could detect further correlations of homologues with alcohol consumption patterns,[24] and (c) can enable a more comprehensive study of PEth kinetics. The results show that for testing of abstinence, the analysis of the two most prominent homologues (16:0/18:1 and 16:0/18:2) is suitable, as they presented positive in all patients, and found to have positive PEth. Other studies that investigated PEth as a marker for alcohol consumption in patients with liver disease measured either only 16:0/18:1 or homologues 16:0/18:1 and 16:0/18:2. To further investigate the use of the markers for the evaluation of the drinking event, such as time and amount of ethanol that was consumed, further studies need to be carried out with controlled consumption of alcohol. Multiple subsequent sample collections need to be carried out, to investigate the formation and degradation of each homologue. This will help to possibly connect the ratio of homologues to the drinking event.

All patients of the study had a liver damage and some additionally an impaired kidney function. Even though studies have shown that this does not have an effect on PEth values, this might be a limitation of the study.[19] Further experiments should be carried out with a healthy collective. All patients in this study had blood taken for routine analysis. In this clinical setting, it was easier to additionally draw blood into an EDTA tube than take an additional sample of capillary blood (fingertip). Generally, using a volumetric device for capillary blood sampling could be an alternative. Capillary blood has been used in different studies in our laboratory and showed no differences to venous blood, which is in accordance with other published studies.[17,21,22]

8 | CONCLUSION

The present method enables the valid quantification of six different homologues of PEth in DBSs. This will be useful for a comprehensive monitoring of PEth homologues in the context of alcohol exposure and biomarker application. Consequently, this will be important in different clinical and forensic settings, such as alcohol recovery programs, identification of *in utero* alcohol exposure of newborns, custody cases, traffic medicine, and transplant medicine.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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5.2 PEth-analysis for abstinence testing and drinking behavior monitoring

The article 'Phosphatidylethanol in patients with liver diseases of different etiologies: Analysis of six homologues and comparison with other alcohol markers' was published in Clinica Chimica Acta in 2021 (online) [104].

After introduction of the analytical method (see 5.1), a study was carried out to evaluate PEth's performance in context of biomarker analysis. Patients answered questions about their alcohol consumption during the three months prior to the doctor's appointment. The information was applied to investigate the question raised in 5.1: can analysis of multiple homologues give insight into drinking events. Patient characteristics such as age, BMI, diseases were included into evaluation. Patients' blood, urine and hair samples and questionnaires, which had been collected by a doctoral PhD-student at the clinical site were sent to our laboratory for analysis.

Patients with different etiologies of liver diseases were included. Patients with alcoholic liver disease (ALD) are obliged by law to stay abstinent for at least six months before they qualify to be listed for liver transplantation [106]. This is very important as continuation of alcohol consumption tremendously endangers the transplanted organ and reduces long term survival immensely. Early detection of relapse is important to offer professional support to cease alcohol consumption [60]. According to the transplantation guideline of the federal medical association ('Bundesärztekammer') proof of abstinence is mandatory by negative uEtg testing [70]. As described in 3.2.2.1 this marker is known to be very sensitive to alcohol intake. That is the reason why a cut-off of 0.5 mg/l is used for this setting, as it must be ruled out that false interpretations occur, potentially caused by incidental alcohol ingestion (e.g., alcohol containing foods), because this could lead to the wrongful denial of a liver transplantation. Furthermore, uEtg's detection time only spans up to 3-4 days [60-62, 107]. As the patients are not spontaneously invited for appointments by the clinic but rather make an appointment for themselves weeks ahead of time, it is quite easy for patients to create a negative uEtg by stopping consumption of alcohol several days before their appointment. Therefore, the use of PEth for abstinence testing was evaluated. Patients with other etiologies of liver disease are not obliged to be abstinent from alcohol but avoidance of alcohol consumption is recommended [108]. Including patients with other liver diseases besides ALD increased data for thorough analysis of PEth (sensitivity calculation), as they are believed to be more honest about their consumption behavior than ALD patients, since they do not have to fear negative consequences concerning their treatment. Furthermore, as PEth allows to differentiate between moderate and excessive drinking behavior, monitoring the non-ALD patients can be useful to reveal potential harmful alcohol consumption and therefore a misdiagnosis. This also opens the possibility to confront the patients and offer support.

The publication focuses on comparing PEth to uEtg and hEtg, sensitivity and specificity of the different alcohol biomarkers, differences between males and females concerning PEth and the application of combining different PEth homologues. Additionally, hEtg was further evaluated in this cohort and a separate paper was published presenting hEtg results in context of impaired kidney function. ([77], see I).

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Phosphatidylethanol in patients with liver diseases of different etiologies: Analysis of six homologues and comparison with other alcohol markers

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ABSTRACT

Background and aims: Phosphatidylethanol (PEth) is a direct alcohol biomarker. Aim of the study was to evaluate the performance of six homologues of PEth in comparison to other alcohol markers in patients with liver diseases.
Methods: The study included 234 patients with liver disease, who gave statements about alcohol consumption during the three months prior to the doctor's appointment. Ethylglucuronide in urine (uEtG) and in hair (hEtG) and carbohydrate-deficient transferrin (CDT) were analyzed in addition to PEth.
Results: Of all patients 47% stated to have drunk alcohol during the past three months. UEtG, hEtG and CDT showed a sensitivity of 29% and a specificity of 92% together for ingestion of at least two standard drinks (24 g) per week. With PEth 16:0/18:1 in addition, sensitivity increased to 59%. For consumption in the last week uEtG's sensitivity and specificity was 28% and 100%, respectively. PEth's was 75% and 93%. When looking at patients who consumed at least two standard drinks per week during the past three months and of which a hair sample could be obtained, hEtG's sensitivity was 37% and specificity 90%. PEth had a sensitivity of 53% and specificity of 100%. Quotients of PEth 16:0/18:1 with 16:0/18:2, 16:0/20:4 and 18:0/18:2 were smaller when alcohol had been consumed more recently.
Conclusion: Despite the rather poor overall sensitivity of alcohol biomarkers in this study, PEth showed best sensitivity for all time periods of alcohol consumption.

1. Introduction

In multiple clinical and forensic settings an objective evaluation of the patients' alcohol consumption is important. In particular, in liver transplant candidates with alcoholic liver disease (ALD) abstinence checks are mandatory and required by law in Germany [1]. In addition, evaluating alcohol consumption behavior plays a role in treatment for patients with various liver pathologies [2].

To investigate the nature, extent and duration of alcohol exposure, alcohol biomarkers are measured from body fluids or keratinous tissue. Besides the traditional indirect biomarkers, which are rather insensitive (carbohydrate-deficient transferrin (CDT)) and non-specific (alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyl transpeptidase (GGT), mean corpuscular volume (MCV)), direct alcohol biomarkers can be measured [3]. These direct alcohol biomarkers are derivatives of ethanol, making them highly specific. For example, ethyl glucuronide (EtG) is synthesized when ethanol is glucuronidated by uridine 5′-diphosphoglucuronosyltransferase in the hepatocytes, the gastro-intestinal-tract and in the kidneys [4]. EtG is usually determined in urine (uEtG) and hair (hEtG). While the maximum detection window of EtG has been reported to be up to 5 days [5] in urine, it accumulates in hair and allows to detect alcohol consumption over the past months [3]. For patients with liver disease, sensitivity and specificity of uEtG have been reported to be 70–89% and 93–99% respectively for any alcohol consumption in the past 3–7 days [6]. Sensitivity and specificity of EtG in a 3 cm hair strand for detecting moderate and excessive alcohol consumption during the past three months were demonstrated to be as high as 85–100% and 97–100%, respectively [6].

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Phosphatidylethanol (PEth) is an abnormal phospholipid consisting of a phosphoethanol headgroup with a variety of fatty acid chains attached to a glycerol backbone. Because PEth production by the enzyme phospholipase D requires the presence of ethanol [7], it can be used as a direct alcohol marker. Previously, it was reported to have a sensitivity and specificity of 73–100% and 90–96%, respectively to determine any alcohol consumption in the previous one to four weeks [6]. The existence of at least 48 different homologues of PEth was described [8]. Simultaneous quantification of six of these homologues via LC/MS/MS has been established [9]. PEth was shown to have a half-life of 3–10 days [10,11]. Helander et al. [12] specified between homologues and reported half-lives of 3.7–10.4 days, 2.7–8.5 days and 2.3–8.4 days for PEth 16:0/18:1, PEth 16:0/18:2 and PEth 16:0/20:4, respectively. Therefore, PEth may have a detection window of several weeks [13]. A linear correlation between PEth concentrations in whole blood and ethanol intake was demonstrated [14,15]. It is generally accepted that it is impossible to reach complete specificity and selectivity for determination of alcohol consumption, but diagnostic certainty is increased by taking the results of different alcohol markers into consideration when evaluating an alcohol exposure [16].

Therefore, the aim of this study was to assess the diagnostic value of PEth homologues in comparison to other alcohol biomarkers regarding different consumption times and amounts.

2. Methods and materials

2.1. Analysis of alcohol biomarkers

For the analysis of uEtG the samples were measured via an enzymatic test (AU 480, Beckmann Coulter, Brea, California, USA). If the immune assay yielded a concentration above 300 ng/l, an aliquot from the same sample was quantitatively measured by LC/MS/MS as described previously [17]. Eventually a cut-off of 500 ng/ml was applied as it is set in the German legal transplant guidelines [1].

Hair samples were taken (if at least 3 cm long) by cutting it directly at the scalp and prepared as previously described and subsequently analyzed for EtG by a validated LC/MS/MS-method [18]. Hair could be sampled and analyzed from 91 patients. Reasons for not sampling or analyzing the hair samples were that the hair was too short/patients were bald (n = 51), the hair was chemically treated (n = 36) or that not enough material was available (strand too thin) (n = 7). Furthermore, patients refused hair sampling (n = 37) and in six cases the reason for missing hair sample analysis is unknown. According to international standards from the society of hair testing (SoHT) a cut-off of 5 pg/mg was used for abstinence [19]. Values >30 pg/mg suggest chronic, excessive alcohol intake. Analysis of 3 cm hair represents consumption of approximately the past three months.

CDT was analyzed by HPLC using a commercially available, fully validated, and IVD-CE-labeled kit (CDT in blood ClinRep© Komplettkit 'CDT im Serum– HPLC', Recipe, München, Germany). If the fraction of disialotransferrin exceeds 2.0% it indicates that alcohol was consumed excessively for two to six weeks [20]. MeOH and EtOH were measured via GC-FID as previously described [17]. EtOH was primarily analyzed to exclude the possibility of post-sampling formation of PEth [21].

PEth was analyzed from dried blood spots (DBS) that were volumetrically generated (20 μl) from EDTA-blood. For analysis one spot was processed as whole. Detailed information about sample preparation, instrument settings and validation results can be found in our previous work [9]. Additional validation for a calibration range up to 2000 ng/ml was performed and passed. PEth-homologues 16:0/18:1, 16:0/18:2, 16:0/20:4, 18:0/18:1, 18:0/18:2 and 18:1/18:1 were simultaneously quantified. Furthermore, the haematocrit (hct) of all blood samples was determined (haematology-analyzer ADVIA 2020i, Siemens, Munich, Germany). During validation of the applied method, matrix effect and recovery were inquired for hcts of 20%, 40% and 60% to exclude major analytical hct effects [9].

2.2. Patients

In the study 234 patients were included who presented to the outpatient liver and kidney clinic of the University Medical Center Hamburg- Eppendorf between October 2017-September 2018.

Of those, 87 had alcoholic liver disease (ALD), 124 had non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steato-hepatitis (NASH) and 23 suffered from cryptogenic or other rare liver diseases (e.g., Wilson Disease).

To evaluate patients' alcohol consumption, a three-page questionnaire with adapted AUDIT elements was given out for self-assessment. Included in the form were questions about alcohol consumption over (I) the last three months, (II) the last four weeks and (III) the last week. All responses were kept anonymous. In parallel, alcohol markers were quantified in blood, urine and hair samples. Possible factors that might interfere with alcohol marker analysis were taken into consideration, such as consumption of alcohol-free beer or alcohol containing foods, the use of EtOH containing hygiene/cosmetic products and chemical treatment of hair. Informed written consent was given by all participating subjects and the study was approved by the local ethics committee (PV5068).

2.3. Clinical parameters

Creatinine, total bilirubin, liver enzyme activity and MCV were analyzed on the appointment day. Body mass index (BMI) was calculated using weight and height measured on the date of study entrance. Glomerular filtration rate (GFR) was calculated using the Modification of Diet in Renal Disease (MDRD) equation.

2.4. Statistical analysis

For statistical analysis, the program SPSS (IBM SPSS Statistics for Windows, Version 27.0) was used. Based on questionnaire responses an average estimated weekly alcohol intake was calculated. Table 2 presents the number of patients who made a statement about their consumption in the different time periods. Diagnostic accuracy was calculated based on questionnaire responses and as such, values were excluded if no response was given for alcohol consumption in the corresponding time-period (Table 2). Data was similarly excluded if at least two direct alcohol markers were positive while complete abstinence was claimed (n = 6). The PEth homologue 16:0/18:1 was used for comparison with other markers, as laboratories use this homologue primarily for analysis.

3. Results

3.1. Patient characteristics and alcohol consumption behavior

Characteristics of all 234 patients are summarized in Table 1. Of all included patients (n = 228), 50% (n = 114) stated that they had consumed alcohol at some point. As mentioned in 2.4 an overview of patient-declared alcohol consumption is given in Table 2. Significantly more patients with NAFLD/NASH admitted consumption of alcohol in the past three months and four weeks ($p < 0.001$) compared to patients with ALD. But the mean amounts of alcohol consumed during the four weeks and the three months prior to the appointment was significantly higher in patients with ALD compared to patients with NASH/NAFLD (factor 2.8, $p = 0.026$). Furthermore, the mean amount of alcohol consumed per week was significantly lower during the last week compared to the four weeks prior to the appointment ($p = 0.02$) (including all diagnosis).

Although there was no significant difference between males and females in terms of the percentage that admitted alcohol consumption during the past three months and four weeks, men stated a significantly higher consumption amount (factor of 2.1, $p = 0.006$). The consumed

Table 1
Patients' characteristics.

Characteristic	Total (n = 234)	ALD (n = 87)	NASH/ NAFLD (n = 124)	Other/ unclear (n = 23)
Sex male (%)	130 (56)	55 (63)	63 (51)	12 (52)
Age (years), median (range)	58 (18–86)	62 (38–77)	56 (23–86)	53 (18–71)
Creatinine (mg/l), median (range)	9.8 (5–97)	12 (5–50)	9.4 (4.6–97)	8.7 (6.3–12)
Bilirubin (mg/l), median (range)	6 (2–77)	7 (2–77)	5 (2–29)	4 (3–39)
Albumin (g/l), median (range)	38 (19–47)	34 (19–45)	39 (24–47)	40 (24–47)
ASAT (U/l), median (range)	29 (4–266)	33 (9–266)	29 (4–230)	28 (18–130)
ALAT(U/l), median (range)	36 (6–259)	28 (9–256)	43 (6–259)	50 (30–239)
GGT (U/l), median (range)	83 (5–1772)	77 (5–1772)	80 (12–906)	126 (31–594)
BMI (kg/m^2), median (range)	27.9 (15.4–48.9)	27.5 (15.4–48.9)	28.4 (16.6–53.5)	28.1 (19.5–42.7)
GFR (ml/min), median (range)	78 (4.1–137)	53 (12–121)	85 (4.1–137)	91 (49–123)
MCV (fl) median (range)	89 (66.2–113)	92.7 (77.8–113)	87.9 (66.2–110)	89 (80–106)
Post-LTX	40	33	7	
Pre-LTX	156	53	103	
Pre-KTX	13	0	13	
Pre-KTX, Post LTX	2	1	1	
Liver cirrhosis	128	81	46	1

KTX = kidney transplantation.

amount did not differ between sexes during the week before the appointment.

3.2. Alcohol biomarkers

Of all 228 included patients, 33% (n = 76) had a positive alcohol biomarker in at least one of the three sample materials. Fig. 1 illustrates the number of cases with positive biomarkers, highlighting those with exclusively one positive marker. Interestingly, 46 (20%) patients admitted to alcohol consumption without having any positive alcohol biomarker. Positive alcohol consumption was defined as an ingestion of >24 g of alcohol per week, which is equivalent to two standard alcoholic drinks. The traditional markers uEtG, hEtG and CDT together showed a sensitivity of only 29% and a specificity of 92% for any alcohol consumption during the preceding three months. With PEth 16:0/18:1 in addition to those markers, sensitivity could be increased to 59%, and specificity remained similar with 93%.

3.2.1. PEth

PEth 16:0/18:1 was positive (≥10 ng/ml) in 63 cases (28%, total n = 228). In 32 cases it was the only positive alcohol marker (compared with hEtG, uEtG, CDT), with PEth concentrations from 12 to 772 ng/ml (mean: 66 ng/ml; median: 27 ng/ml). All 63 patients admitted to alcohol consumption within the last three months, so specificity is 100%. Sensitivity for alcohol consumption during that period was 53% for ≥24 g/week. Sensitivity and specificity of PEth 16:0/18:1 was 58% and 98% respectively when exclusively analyzing the four weeks before the appointment. The two patients who had a positive PEth but denied alcohol consumption in the four weeks prior to the visit, stated to have consumed alcohol in the preceding three months (60 g/week). When taking patients into consideration who drank at least 84 g of alcohol per week, which corresponds to seven standard drinks per week, sensitivity of PEth 16:0/18:1 was 92%, specificity 89%. Table 3 shows that this homologue has the highest sensitivity of the six homologues. Interestingly, PEth detected 50% of patients who claimed to have stopped alcohol consumption four weeks prior to the appointment (n = 8), but consumed alcohol in the months before, which demonstrates the potentially long detection window. In detail: Alcohol amounts the four patients with PEth <10 ng/ml stated to have drunk until four weeks prior to the appointment were 48 g/week, 60 g/week (twice) and 216 g/week. One patient who stated to have consumed 60 g/week had a PEth concentration of 14 ng/ml, another one 24 ng/ml. PEth 16:0/18:1

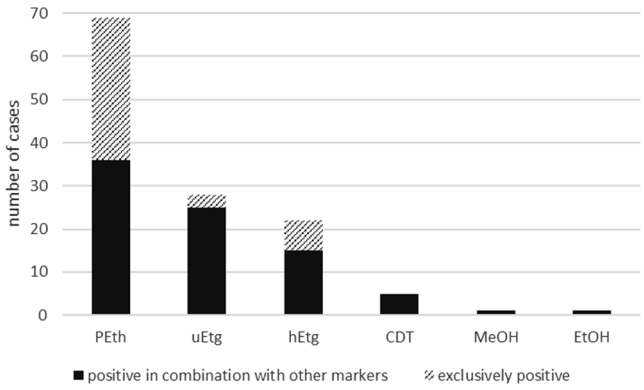

Fig. 1. Number of positive alcohol biomarkers using the applied cut-offs: 10 ng/ml PEth (n = 228), 0.5 mg/l uEtg (n = 228), 5 pg/mg hEtg (n = 91), 1.7% CDT (n = 224), 5 μg/ml MeOH (n = 228), 0.1‰ EtOH (n = 228).

Table 3
Specificity, sensitivity and AUC-ROC of six PEth homologues for different minimum amounts of alcohol consumption during the past four weeks.

	specificity (%)		sensitivity (%)		AUC-ROC	
	≥24 g	≥84 g	≥24 g	≥84 g	≥24 g	≥84 g
PEth 16:0/18:1	98	89	58	92	0.78	0.93
PEth 16:0/18:2	98	88	53	84	0.76	0.89
PEth 16:0/20:4	98	90	44	71	0.71	0.82
PEth 18:0/18:1	99	92	40	68	0.70	0.82
PEth 18:0/18:2	98	93	40	68	0.70	0.82
PEth 18:1/18:1	99	95	33	60	0.66	0.78

Table 2
Alcohol consumption according to patients' statements in the questionnaire.

time period	alcohol consumption	Total	ALD	NAFLD/NASH	unclear/others
Last week	admitted in %	32 (n = 226)	15	41	52
	g/week EtOH mean (range)	65 (12–358)	108 (12–358)	53 (12–317)	66 (12–246)
Last four weeks	admitted in %	39 (n = 223)	22	48	57
	g/week EtOH mean (range)	199 (24–1792)	502 (24–1792)	116 (24–490)	166 (24–336)
Last three months	admitted in %	47 (n = 222)	29	55	70
	g/week EtOH mean (range)	195 (24–1792)	496 (24–1792)	108 (24–490)	159 (24–490)

concentration was 20 ng/ml with a reported consumption of 72 g/week and 39 ng/ml for 336 g/week until four weeks prior to the appointment.

Fig. 2 represents ranges and medians of PEth concentration in three different categories of alcohol amount consumed in the prior four weeks: 24–144 g/week (2–12 standard drinks), 156–336 g/week (13–28 standard drinks) and anything above 336 g/week, which equals the definition of excessive alcohol consumption (50 g/d). Although concentrations of all categories overlap, all PEth concentrations are significantly higher in the highest consumption category than in the others ($p = 0.038$, $U = 59$, $z = -2.1$).

Receiver-operating-characteristics (ROC) curves for all homologues are shown in Fig. 3 for different cut-off levels of alcohol consumption. AUC (area under the curve)-ROCs can be found in Table 3. For the consumption of ≥84 g/week in the previous four weeks, the AUC under the ROC curve for PEth 16:0/18:1 is 0.93. This result indicates PEth 16:0/18:1 is capable of differentiating between those who drink and those who abstain from alcohol or only drink occasionally.

All homologues showed correlation between their concentration and the claimed ethanol intake in the spearman ranks analysis ($p < 0.001$), with a correlation coefficient of 0.73 for PEth 16:0/18:1, 0.70 for 16:0/18:2, 0.61 for 16:0/20:4 and 18:0/18:2, 0.60 for 18:0/18:1, 0.56 for 18:1/18:1.

Despite women stating to have consumed significantly less alcohol (see 3.1), the concentrations of the PEth-homologues did not differ between the sexes ($p = 0.61$ for 16:0/18:1, $p = 0.41$ for 16:0/18:2, $p = 0.84$ for 16:0/20:4, $p = 0.76$ for 18:0/18:1, $p = 0.24$ for 18:0/18:2, $p = 0.34$ for 18:1/18:1). When comparing the ROC curves (alcohol ≥84 g/week), the AUCs of all homologues were closer in value to each other in females than in males (Fig. 4). Although the AUCs and sensitivities were higher in women compared to men, the differences in AUC-ROCs were not statistically significant ($p = 0.70$ for 16:0/18:1, $p = 0.35$ for 16:0/18:2, $p = 0.26$ for 16:0/20:4, $p = 0.07$ for 18:0/18:1, $p = 0.24$ for 18:0/18:2, $p = 0.05$ for 18:1/18:1) (Table 4).

For evaluating applicability of different cut-off values for PEth higher cut-offs were applied: specificity for consumption of ≥24 g/week and ≥84 g/week is 98% and 95% for 20 ng/ml respectively and 99% and 96% for 35 ng/ml. Sensitivity at a 20 ng/ml cut-off is 39% and 74% for ≥24 g/week and ≥84 g/week, respectively and 29% and 53% for 35 ng/ml.

It was evaluated if a combination of different PEth homologues can indicate how recently alcohol was consumed. In the group of patients who consumed ≥24 g of alcohol/week in the past four weeks, the ratios of PEth 16:0/18:1 to the other homologues were calculated. Subsequently, the quotients were compared with the Mann-Whitney-Test between (I) patients who stopped consumption one week before the appointment and (II) patients who drank consistently until the appointment. The median quotients were (II) 1.1, 2.0, 1.7 and (I) 1.6, 10.0, 3.3 for PEth 16:0/18:1 / PEth 16:0/18:2, PEth 16:0/18:1 / PEth 16:0/20:4 and 16:0/18:1 / PEth 18:0/18:2, respectively (Fig. 5). Thus, the quotients were significantly smaller if alcohol was consumed during the week before blood sampling ($p = 0.028$, $p = 0.002$, $p = 0.011$, respectively)

3.2.2. Urine-EtG vs PEth

The diagnostic accuracy of PEth was compared with uEtG regarding consumption during the week prior to the appointment. Altogether uEtG was positive (≥500 ng/ml) in 22 cases (10%, total n = 228). In two of those PEth was negative (<LOQ), although alcohol consumption was admitted by the patients (36 g/week and 24 g/week). Nonetheless, sensitivity of uEtG was very low (28%) for detecting alcohol consumption in the past week while specificity was very high (100%). Combination with PEth increases sensitivity strongly (77%) (Table 5).

3.2.3. Hair-EtG vs PEth

Of the 91 hair samples that were obtained 22 (24%) tested positive for EtG (>5 pg/mg) (range: 9–292 pg/mg; mean: 70 pg/mg; median 54 pg/mg). In seven cases it was the only positive alcohol marker (range: 9–114 pg/mg; mean: 37 pg/mg; median 17 pg/mg). Five of the seven were classified as false-positive, because alcohol consumption was completely denied. Enhanced incorporation into the hair matrix and reduced rate of hair growth could have prolonged the detection window beyond three months. Sensitivity and specificity for detecting alcohol consumption in the three months before the appointment are shown in Table 5. PEth alone had a better sensitivity and specificity than hEtG. Combination of both markers improved sensitivity further. Of the 13 cases with positive PEth and hEtG both markers exceeded cut-off for excessive alcohol consumption (30 pg/mg for hEtG; 210 ng/ml for PEth) in four cases. In six cases hEtG concentrations indicated excessive alcohol consumption, while PEth concentrations did not; and in one case vice versa. Exact concentrations of both markers and the self-reported alcohol consumption can be found in the supplementary data (Table S1).

3.2.4. CDT vs PEth

CDT was positive (≥2.0%) in three (1%) cases (total n = 224). PEth was also positive in all three patients (183–473 ng/ml). Two more patients had CDT values between 1.7 and 2.0% which is suspicious for excessive alcohol consumption, PEth was positive in both (221 and 1141 ng/ml). Corresponding CDT and PEth concentrations are listed in the supplementary data, including the stated consumed alcohol amount (Table S2). Looking at patients with excessive alcohol consumption (at

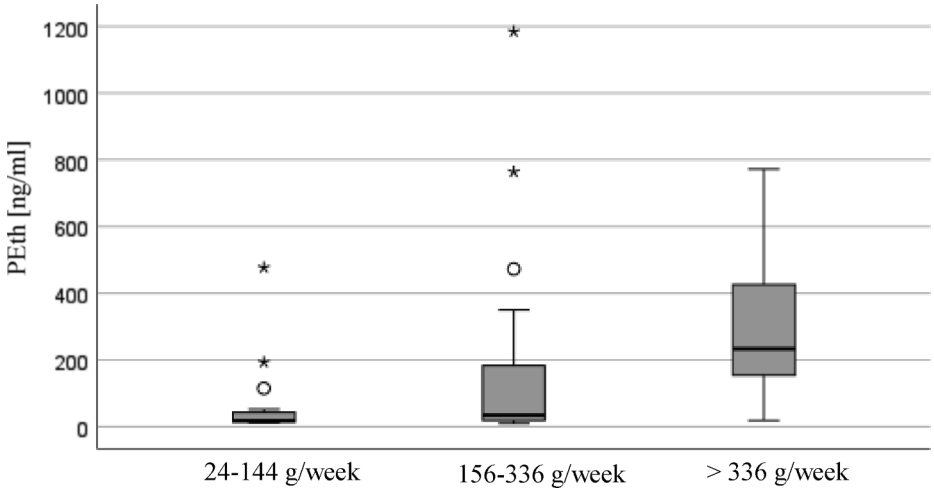

Fig. 2. Boxplots of PEth 16:0/18:1 concentrations corresponding to three different alcohol consumption amount groups.

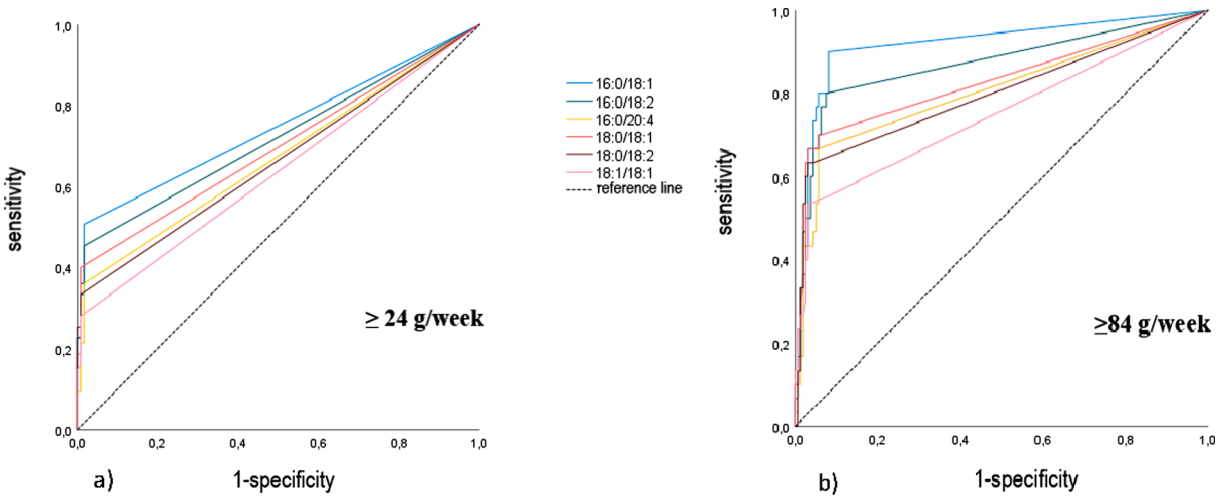

Fig. 3. ROC of PEth-homologues for a) ≥24 g alcohol/week, b) ≥84 g alcohol/week.

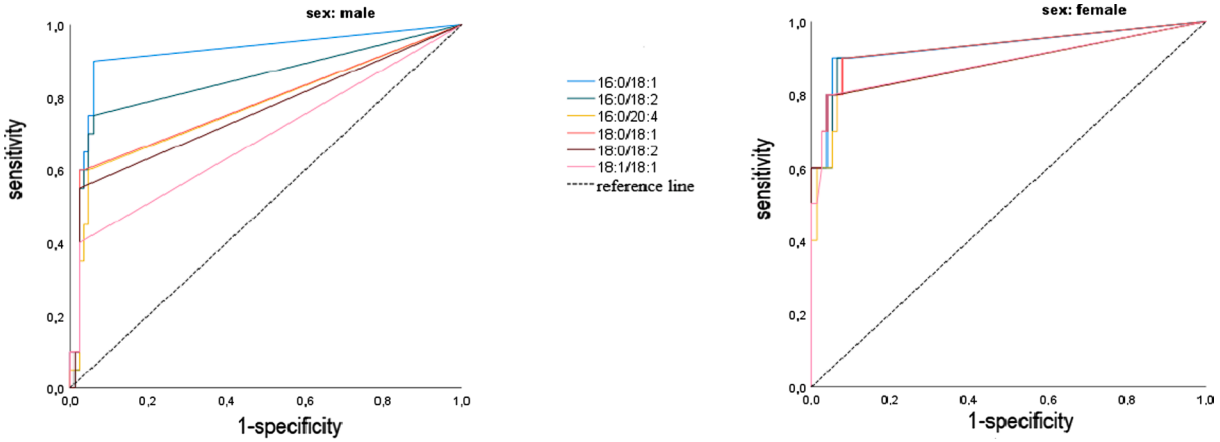

Fig. 4. AUC-ROC for ≥84 g alcohol/week for male and female patients.

Table 4
AUC-ROC and sensitivity of six PEth-homologues for ≥84 g alcohol/week in the past four weeks for male and female patients.

	AUC-ROC		sensitivity (%)	
	male (n = 114)	female (n = 91)	male (n = 38)	female (n = 23)
PEth 16:0/18:1	0.92	0.94	92	92
PEth 16:0/18:2	0.87	0.94	81	92
PEth 16:0/20:4	0.79	0.89	65	83
PEth 18:0/18:1	0.77	0.93	57	90
PEth 18:0/18:2	0.79	0.90	62	83
PEth 18:1/18:1	0.72	0.90	48	83

least 350 g/week, n = 10), PEth 16:0/18:1 and 16:0/18:2 were positive in all cases, whereas CDT was negative in all (<2.0%).

3.2.5. MeOH, EtOH

MeOH was found to be positive (≥5 μg/ml) in one sample with a value of 18 μg/ml. In this case PEth 16:0/18:1 and uEtG and were also positive with high concentrations. One patient was found to have a blood alcohol concentration of 0.2 ‰. Urine of the patient could not be sampled. Both PEth 16:0/18:1 (concentration 426 ng/ml) and hEtG (84 pg/mg) were positive.

4. Discussion

This study evaluated the diagnostic performance of PEth (six of its homologues) in comparison to other alcohol markers based on self-reported alcohol consumption in patients with liver diseases.

4.1. Specificity and sensitivity of PEth

The observed specificity of PEth was 98% for detecting alcohol consumption of >24 g/week in the past four weeks and even 100% when considering three months prior to blood sampling. This is especially important as false-positive results might wrongfully lead to denial of a liver transplant. On the other hand, in this study the observed sensitivity of PEth 16:0/18:1 for consumption of >24 g/week during the past week (75%) and past four weeks (58%) was rather low. This contrasts with a previous study of our group in pre- and post-transplant patients with alcoholic liver disease [22] which revealed a PEth 16:0/18:1 sensitivity of 100% despite of using a higher cut- off level of 20 ng/ml instead of 10 ng/ml. So, many more patients admitting alcohol consumption tested negative for PEth in this study. Due to poor chemical stability of PEth in whole blood, pre-analytical deterioration of the target analyte can reduce analytical outcome in PEth analysis [23] and impact sensitivity.

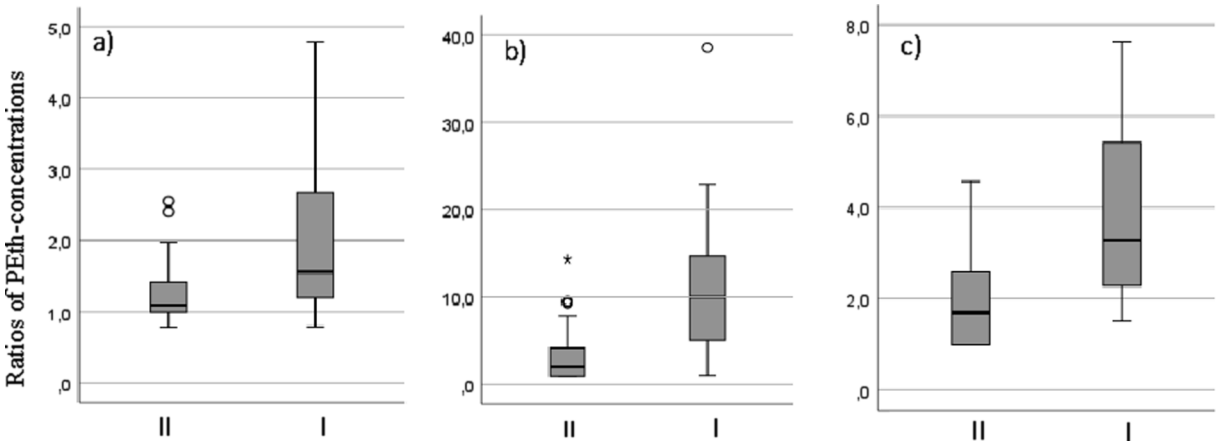

Fig. 5. Boxplots of the ratios of a) 16:0/18:2, b) 16:0/20:4 and c) 18:0/18:2 to 16:0/18:1 comparing I) patients who stopped consumption one week before the appointment and II) patients who drank consistently until the appointment.

Table 5
Specificity and sensitivity of uEtG (500 ng/ml), hEtG (5 pg/mg) and PEth (10 ng/ml).

		specificity (%)		sensitivity (%)	
		≥24 g	≥84 g	≥24 g	≥84 g
Last week	uEtG	100	96	28	41
	uEtG or PEth	93	79	77	88
	PEth	93	80	75	88
Last three months	hEtG	90	89	37	57
	hEtG or PEth	95	87	55	93
	PEth	100	90	53	90

In this study, DBS were generated at the site of sampling within four hours, so influence on sensitivity would be negligible. But there are other explanations for the lower sensitivity in this compared to our previous study. Firstly, it is possible that in the previous investigation the amount of alcohol intake of patients was higher and therefore more likely to be detected. The exact ethanol intake amount was not given in detail, so direct comparison is not possible. The previous study only included patients with ALD and according to the current study ALD patients were generally found to consume more alcohol than NAFLD patients. Secondly, it is possible that in the previous study, which included only patients in the transplant setting, patients were more likely to conceal their alcohol consumption out for fear of negative consequences. Indeed, overall, more patients (50%) admitted to alcohol consumption in this study compared to the previous one (19%). Other authors calculated a sensitivity of 79% for PEth for any drinking in the past four weeks (cut-off 8 ng/ml), with medians of alcohol amount being similar to the presented study (66 g/week and 70 g/week) [13].

Generally, referencing alcohol consumption to self-reports is one of the most critical issues in alcohol biomarker studies. Underreporting of alcohol consumption due to patients' fear of stigmatization is usually assumed. In addition, retrospective questionnaires on alcohol consumption might be difficult to fill out for some patients as estimating the amount of ingested alcohol after several weeks could be a challenge, especially for patients who drink moderately, and do not give special attention to their consumption behavior. This might especially apply to the NAFLD patients in this study. This is a general limitation to the study, which could be avoided by having participants fill out a drinking journal during the questioned time frame. In a study by Walther et al. [24] correlation of PEth was a lot better to alcohol consumption documented in a diary than to the retrospective consumption data, with correlations of 0.56 and 0.23 respectively.

A quantifiable PEth concentration excludes abstinence, but due to its relatively long half-life it might still be detectable after several weeks, depending on the concentration at the onset of abstinence. This was probably the case with patients in this study who claimed abstinence in the four weeks prior to blood sampling. Therefore, a patient's statement of abstinence for four weeks should not immediately be questioned because of detectable PEth.

4.2. PEth-homologues

There was a significant correlation of the amounts of ingested alcohol and all PEth homologue concentrations. This is in accordance with the results of other studies regarding PEth 16:0/18:1 [25,26] and supports its ability to estimate drinking patterns.

Concentration ratios of PEth 16:0/18:1 to the homologues 16:0/18:2, 16:0/20:4 and 18:0/18:2 could be promising in respect of estimating consumption time, since the concentration ratios were found to be markedly lower if alcohol was consumed during the week prior to blood sampling compared to abstinence during that week. This supports the use of PEth homologues in estimating timing of abstinence onset. Our data is in accordance with the observations of Javors et al. [27] and Hill-Kapturczak et al. [28] who studied synthesis and elimination of PEth 16:0/18:1 and 16:0/18:2. Since PEth 16:0/18:2 showed a faster initial synthesis rate and a shorter half-life than PEth 16:0/18:1, the authors concluded that this could be used to specify information about ingestion times.

4.3. Differences between sexes

In this study males consumed significantly more alcohol, but none of the PEth homologue concentrations differed significantly between the sexes. This may be because women's blood alcohol concentrations (bac) are averagely higher after consumption of equal amounts of alcohol, due to a lower distribution volume for ethanol. Higher bac leads to higher PEth concentrations. Sex was reported not to influence the diagnostic performance of PEth 16:0/18:1 in previous studies [6,29]. By comparing sensitivities and specificities between males and females, this was also observed in the current study. There was also no significant difference between the AUC-ROCs of the other homologues (p-value of 18:1/18:1 was 0.05 though). The sensitivities of all homologues, but 16:0/18:1, were higher in females, which means they detected more right positives in females than males. To our knowledge no other study has so far investigated these other homologues concerning sex.

4.4. Cut-off for PEth 16:0/18:1

In 3.2.1 it is shown that specificity was barely increased using 20 ng/ml or even 35 ng/ml as cut-off level when testing for abstinence. This

implies, that the currently recommended cut -off of 35 ng/ml could be lowered to improve sensitivity. Studies that investigate influence of ethanol uptake from alternative sources, like hygiene products or foods are still rare. Reisfield et al. [30] studied the influence of ethanol-containing mouthwash. In one of the 25 participants PEth 16:0/18:1 was 12 ng/ml after using the mouthwash four times per day for 12 days, which is above our suggested cut-off of 10 ng/ml. Several potential reasons for the increase in PEth are described by the authors, though. Nevertheless, as suggested in the study, potential heterogeneity in PEth response to small amounts of extraneous ethanol exposure should be further investigated.

4.5. UEtG vs PEth

Strikingly, sensitivity of uEtG for any consumption during the week prior to sampling was very low at 28%. In previous studies it was much higher at 71% and 86% [17,22]. Because EtG has a detection window in urine of approximately two to three days, the difference could arise from the day on which the alcohol was consumed during the week. Furthermore, the amount of ingested alcohol could have influenced the different outcomes. Bacterial infections of the urinary tract can cause degradation of EtG resulting in false-negative uEtG results [31,32]. Additionally, high urine dilution or medication with diuretics leads to reduced detection of EtG in urine [33]. Because there is no reason for a larger number of false negatives to exist in this study as compared to others, these are weak explanations for the low sensitivity. On the other hand, UEtG was the only positive marker in two patients. The amount of consumed alcohol (24 and 36 g/week) during the four weeks before blood sampling apparently was not enough in these patients for PEth to be quantified > LOQ. On the other hand, the consumption reported during the last week might have taken place in the days before the doctor's appointment, so urine was sampled within the detection window of uEtg. This demonstrates the benefit of uEtG analysis in addition to PEth's.

4.6. HEtG vs PEth

HEtG is a well-established alcohol-consumption marker. Its use however is limited because of sample availability, as a certain quantity and length of hair is required for analysis. Furthermore, hair that has been chemically treated is not suitable for EtG analysis [34,35]. In this study PEth 16:0/18:1 presented better sensitivity, specificity, and AUC-ROC than hEtG for detecting alcohol consumption in the three months prior to the appointment. As hEtG alone detected alcohol consumption in two cases there is value in testing hEtG in addition to PEth. HEtG has been shown to be influenced by kidney function [36]. In a study of Mosebach et al. [18] patients with suboptimal GFR had higher concentrations of hEtG. This is thought to be due to slow elimination secondary to inadequate kidney function, giving it more time to incorporate into hair matrix. This might have been the case for four patients in this study who had GFRs <50 ml/min and who tested positive for hEtG but claimed abstinence during the past three months. Other individual factors have been demonstrated to influence hEtG interpretation, such as obesity, which could have been the case for the other false-positive patient with a BMI of 31 kg/m^2, and a reduced rate of hair growth, which can be a symptom of kidney or liver disease [37]. As they primarily detect consumption in different time frames and are both known to be able to differentiate between excessive and light drinking, hEtG and PEth complement each other well and can be used together to potentially estimate drinking patterns.

4.7. CDT vs PEth

CDT did not have any additional use in detecting alcohol consumption in the context of abstinence testing, as it was never positive without PEth being positive as well. As such, these findings support the presumption of Arnts et al. [6] that PEth will soon gain importance over CDT.

5. Conclusion

All in all, sensitivity of the investigated alcohol consumption markers was lower than expected in this study. Nevertheless, PEth yielded the best sensitivity and specificity for consumption during all time periods prior to blood sampling. Especially the number of cases in which alcohol consumption was solely detected by PEth (n = 33), underlines the benefit of integrating PEth into standard alcohol marker measurement. This is supported by its easy sample handling and costs which align to other biomarker analysis.

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CRediT authorship contribution statement

Nadine Aboutara: Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Anne Szewczyk:** Investigation. **Hilke Jungen:** Investigation. **Amadea Mosebach:** Methodology, Investigation. **Maria Rodriguez Lago:** Methodology, Investigation. **Eik Vettorazzi:** Formal analysis. **Stefanie Iwersen-Bergmann:** Conceptualization, Resources, Writing – review & editing. **Alexander Müller:** Conceptualization, Writing – review & editing, Supervision. **Martina Sterneck:** Conceptualization, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2021.11.013.

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5.3 Use of PEth as a marker for excessive alcohol consumption

The paper with the title 'Investigating the use of PEth, CDT and MCV to evaluate alcohol consumption in a cohort of homeless individuals- a comparison of different alcohol biomarkers' was published in *Forensic Science International* [109].

After evaluating PEth in abstinence testing and consumption monitoring (5.2) in a clinical setting, it was aimed at evaluating PEth in the context of high or excessive alcohol consumption. Especially, the comparison with the long-term marker CDT was in focus. The Department of Legal Medicine carried out a study in which a cohort of homeless people in Hamburg were examined and inquired about different aspects of their lives. Since substantial alcohol consumption was suspected in this population, we participated in this study with alcohol biomarker testing. The study questionnaire, filled out by the participants, included the self-assessment of general alcohol consumption behavior. Blood samples were taken for the analysis of CDT and bac (serum gel) and PEth (EDTA) and analyzed in our laboratory. MCV and hct were analyzed in the laboratory of the Department of Clinical Chemistry of the UKE and results were send to us for evaluation. The relevant parts of questionnaires were evaluated by me during data analysis.

While CDT is highly specific for excessive alcohol consumption, it has some disadvantages (see 3.2.1.3). Some individuals show genetic variations leading to differences in CDT expression and making CDT analysis useless [27]. Furthermore, as mentioned in the introduction CDT takes several weeks of excessive alcohol consumption to distinctly increase and poorly reacts to short-term increase or decrease of alcohol ingestion.

It has been already supposed by several authors [110,111] that PEth might be favorable to indicate excessive drinking to CDT. Our study, in which very high PEth and CDT concentrations were observed supports this thesis and thorough discussion is found in the paper. Other than in previous studies [110] that investigated CDT and PEth, different drinking categories, based on participants statements were looked at and several homologues besides PEth 16:0/18:1 were included into evaluation. Finally, the results proved that PEth was superior to both CDT and MCV as marker for excessive alcohol consumption.

Additionally, the topic of influence of hct on PEth analysis is addressed in the paper. It has been discussed if and to which extend hct can impact PEth quantification, because PEth is found mainly in the erythrocytes. Hct is the calculated volume percentage of red blood cells in blood, which further consists of plasma, containing different proteins, white blood cells and thrombocytes. The extend of hct influence is rather difficult to evaluate, as more potential physiological factors might influence individuals PEth-synthesis. In the presented paper, we calculated a linear regression analysis to find out whether within a cohort with positive PEth concentrations a dependency on hct

could be seen. No correspondence was found. That again is only one aspect of hct influence on PEth-analysis and does not give direct information on individuals with varying hct. In chapter 7 further ideas for research and discussions on influence of hct and other physiological parameters will be discussed.

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Investigating the use of PEth, CDT and MCV to evaluate alcohol consumption in a cohort of homeless individuals– A comparison of different alcohol biomarkers

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ABSTRACT

In a cohort including individuals with suspected high alcohol consumption, the concentrations of the indirect alcohol biomarkers carbohydrate-deficient transferrin (CDT) and mean corpuscular volume (MCV) and the direct alcohol biomarker phosphatidylethanol (PEth) were investigated. Blood alcohol concentration (BAC) was analysed as a marker for acute alcohol ingestion.

In addition to questions about subjective alcohol consumption behaviour, 147 homeless persons underwent a physical examination with blood sampling. BAC, PEth, CDT and MCV were determined in the blood samples. Special focus was on the comparison of PEth and CDT for indicating excessive alcohol consumption.

BAC was measured above 0.1‰ in 39 blood samples (0.1–2.5‰, median 0.75‰). PEth was detected in all of them. Overall, PEth was positive (≥10 ng/ml) in 104 samples (71%) (11–5687 ng/ml, median 650 ng/ml) with 68 (46%) being above the cut-off for excessive alcohol consumption (210 ng/ml). In 26 subjects PEth was the only positive alcohol biomarker. CDT was ≥ 1.7% in 66 cases (47%) (1.8–22.2%, median 4.4%) and ≥ 2.5% in 52 (35%) cases. MCV was elevated (≥95 fl) in 58 subjects (39%). CDT and PEth concentrations showed a significant positive correlation (spearman's correlation coefficient $\rho = 0.77$, $p < 0.001$). PEth concentrations were significantly higher in samples that were also CDT positive than solely PEth positive ($p = 0.004$). PEth did not indicate excessive alcohol consumption (< 210 ng/ml) in eight and two cases in which CDT was ≥ 1.7% and ≥ 2.5%, respectively. On the other hand, CDT was < 1.7% and < 2.5% in ten and 18 cases, respectively, in which PEth was above cut-off for excessive alcohol consumption. Taking the self-reports of the participants into consideration, PEth's sensitivity for detecting excessive alcohol consumption was 100% (10 ng/ml) and 94% (210 ng/ml) and CDT's was 88% (1.7%) and 75% (2.5%).

In individuals of the investigated cohort unusually high concentrations of the alcohol consumption markers PEth and CDT were quantified, which proves the assumption of chronic excessive alcohol consumption in parts of the cohort. PEth was the marker that was positive most often and was more sensitive for excessive alcohol consumption than CDT.

1. Introduction

An elevated risk for health problems is associated with alcohol consumption. It is generally known that the health risks are related to the amount of alcohol consumed [1]. Wood et al. (2018) proposed 100 g ethanol (EtOH)/week as a lower threshold for harmful drinking [2]. Excessive alcohol consumption is defined as 50 g EtOH/day [3]. In the Global Status Report on Alcohol and Health (2018), the World Health Organisation (WHO) presented that more impoverished individuals suffer from more significant harm from a certain amount of alcohol consumption than wealthier individuals. This phenomenon is reflected in the "harm per litre" scale.

Consequently, the WHO recommended the initiation of screening and intervention programs for harmful drinking in order to lower the burden and consequences of excessive alcohol consumption [4]. In several countries, the number of homeless people increased in the past years [5]. In 2018 it was estimated that about 678,000 homeless individuals lived in Germany [6]. Hamburg, the second-largest city in

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Germany with a population of about 1.8 million, was the location of about 6600 of them [7]. Homeless individuals have a high prevalence of substance use disorders, which is one reason for frequent premature deaths among this group [8,9]. Furthermore, recent results on this cohort indicated that chronic excessive alcohol consumption, objectified by carbohydrate-deficient transferrin (CDT ≥ 2.5%), is associated with less frequent physician visits [10]. Therefore, individuals with health issues caused or enhanced by chronic alcohol consumption are less likely to get treatment and intervention is more complicated.

In order to evaluate the nature of alcohol consumption in individuals, alcohol biomarkers can be measured from body fluids or keratinous tissue in addition to self-reports. Beside indirect biomarkers, direct alcohol biomarkers exist. These markers are metabolic products of EtOH which makes them highly specific [11]. One of the direct alcohol biomarkers, that has recently gained attention is phosphatidylethanol (PEth), an abnormal phospholipid consisting of a phosphoethanol headgroup with a variety of fatty acid chains attached to a glycerol backbone. It is synthesised by phospholipase D from phosphatidylcholine and EtOH, as EtOH's affinity to the enzyme is higher than that of water [11]. As the fatty acid chains can vary, different homologues of PEth exist. At least 48 homologues have been described [12]. Simultaneous quantification of six of these homologues via LCMS-MS has been validated [13]. PEth was shown to have a half-life of 3–10 days [14,15]. Previous studies reported sensitivity and specificity of 73–100% and 90–96%, respectively, to determine alcohol consumption in the previous 1–4 weeks [16].

CDT is categorised as an indirect alcohol marker. It its changed by alcohol and potentially its metabolites as they inhibit glycosylation and sialylation in the Golgi-apparatus of hepatocytes, increasing lower sialylated isoforms of CDT, while total-CDT is unchanged [11]. While being specific, CDT has deficiencies concerning its sensitivity. Therefore, this long-term marker is most suitable for the detection of excessive alcohol consumption. CDT showed a 40–79% sensitivity and a specificity of 57–99% in previous studies in patients with liver disease [16]. Mean corpuscular volume (MCV), a traditional alcohol biomarker, has been reported to lack diagnostic accuracy in different settings [17,18].

As proposed by Neumann et al., comparison of PEth with other alcohol biomarkers needs further investigations [19]. Therefore, PEth is compared to CDT in detail in this study while also looking at MCV and blood alcohol concentration (BAC), as a marker for acute alcohol ingestion. Furthermore, it is reported on drinking behaviour in a cohort of homeless individuals in Hamburg, comparing alcohol biomarker analysis with self-reported drinking behaviour.

2. Materials and methods

In the 'Hamburg survey of homeless individuals' persons without permanent residency but with access to specialised medical practices, lodging houses or shelters, were asked to participate. In total, 151 persons took part in the study. In this study, 147 cases were evaluated, as we excluded those without blood samples. A medical examination and blood sampling were conducted confidentially in the institution/shelter/practice between May 25th and June 3rd, 2020. If the participants could read and comprehend the questions independently, they were asked to fill out a questionnaire. However, most participants needed support with the questionnaires, so they were filled out via face-to-face interviews. Of all participants, 62 (42%) received a questionnaire with questions that concerned the subjective self-assessment of general alcohol consumption. Participants chose between the options that they never drink alcohol, that they occasionally consume alcohol defined as ≤ three standard drinks about once a week, that their alcohol consumption is regular-moderate (four to seven standard drinks per week) or regular-high (≥ eight drinks per week). All participants provided written informed consent prior to the investigations. All data collected was anonymized. A positive vote from the ethics committee of Hamburg Medical Association (PV 7333) was received. Participants received an expense allowance of 5€.

Blood was sampled into serum-gel tubes (Sarstedt, Nümbrecht, Germany) for analysis of blood alcohol concentration, CDT and MCV. For PEth analysis, blood was sampled into EDTA-tubes (BD, Heidelberg, Germany), from which 20 µL dried blood spots (DBS) were generated within 2–6 h after sampling.

PEth was analyzed from the DBS as whole. Detailed information about the sample preparation and method settings have previously been described [13]. Additional validation for a calibration range up to 6000 ng/ml was performed and passed. PEth-homologues 16:0/18:1, 16:0/18:2, 16:0/20:4, 18:0/18:1, 18:0/18:2 and 18:1/18:1 were simultaneously quantified. Furthermore, the haematocrit (hct) of all blood samples was determined (haematology-analyzer ADVIA 2020i, Siemens, Munich, Germany). The applied method for PEth quantification is validated for hcts between 20% and 60% concerning matrix effects and recovery. Concentrations ≥ 10 ng/ml were regarded positive. This low cut-off was chosen as it yields higher sensitivity and the aim was to compare positive alcohol biomarkers. Abstinence testing, for which a cut-off of 35 ng/ml has been suggested [20] on the other hand, was not of focus. For excessive drinking the PEth cut-off was 210 ng/ml. CDT was analyzed by HPLC using a commercially available, fully validated, and IVD-CE-labelled kit (CDT in blood ClinRep© Komplettkit 'CDT im Serum– HPLC', Recipe, München, Germany). Two cut-offs for CDT were evaluated, 2.5% for excessive alcohol consumption and 1.7% as a value for suspiciously excessive alcohol consumption. MCV was measured via flow cytometry. MCV concentrations of 95 fl or higher were considered abnormal. BAC was measured enzymatically (DRI®-Ethanol-Assay, Thermo Scientific, Fremont, California, USA) on a clinical-chemical analyzer (AU 480, Beckmann Coulter, Brea, California, USA). A decision limit of 0.1‰ (0.1 g/l) was applied for positive BAC.

3. Results

3.1. Participant characteristics

In total, 115 (79%) male, 31 (21%) female participants and one participant of unknown sex, with a median age of 47 years (20–86 years) were included. Some became homeless recently, while others had been homeless for several years (median number of months being homeless: 24, range: 1–720, n = 117). When asked which language was spoken most by the participants, 73 responded German, six English, 56 listed an East European language and seven another language (n = 142). Median Body-Mass-Index (BMI) of the participants was 24.9 kg/m^2 (17.1–41.1 kg/m^2).

3.2. Statements about alcohol consumption

All 62 participants who received the question about their drinking behaviour responded (100% response rate). Of those, ten (16%) claimed to never to consume alcohol, twelve (19%) stated to consume alcohol occasionally. A regular-moderate consumption was reported by 24 participants (39%) and a regular-high consumption by 16 (26%).

3.3. Alcohol consumption markers

In 39 (27%) samples, BAC was positive (≥ 0.1‰). In all of them PEth 16:0/18:1 was above 10 ng/ml. Altogether PEth 16:0/18:1 was ≥ 10 ng/ml in 104 cases (71%). Of all samples, CDT was ≥ 1.7% (cut-off for a suspiciously excessive consumption) in 66 (47%) and ≥ 2.5% (cut-off for chronic excessive drinking) in 52 (37%). PEth 16:0/18:1 was ≥ 10 ng/ml in all but one case with the lower CDT cut-off and all

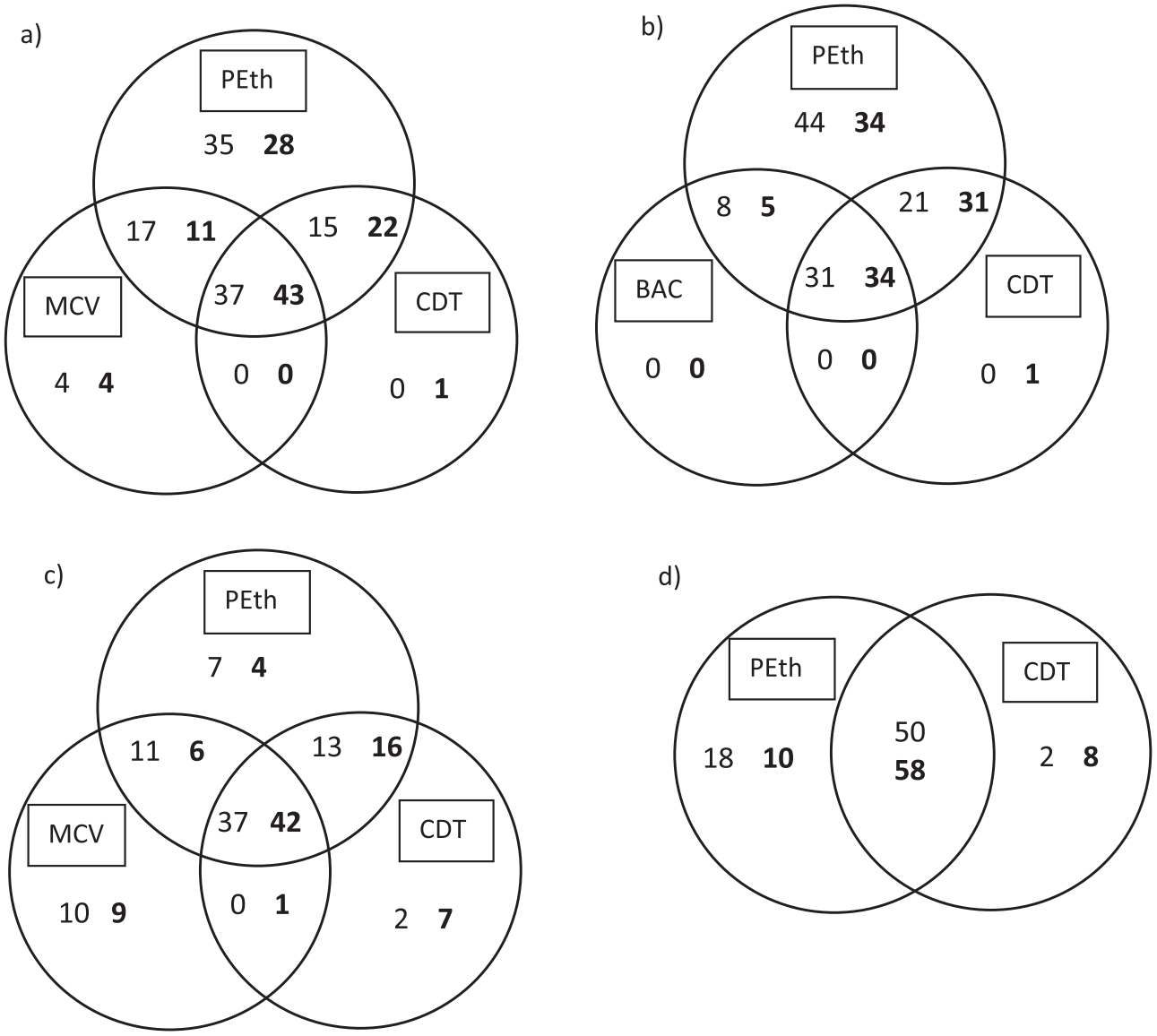

Fig. 1. Number of cases with positive biomarkers, each marker alone and combinations of markers, represented by overlapping areas. For bold numbers a CDT cut-off of 1.7% is applied; for plain numbers 2.5%. Comparison of a) PEth 16:0/18:1 (cut-off 10 ng/ml), CDT and MCV; b) PEth 16:0/18:1 (cut-off 10 ng/ml), CDT and BAC; c) PEth (cut-off 210 ng/ml), CDT and MCV; d) PEth (cut-off 210 ng/ml) and CDT.

with the higher one. But it was < 210 ng/ml (cut-off for excessive alcohol consumption) in eight samples with CDT ≥ 1.7% and two samples with CDT ≥ 2.5%. On the other hand of the 68 (46%) participants with PEth 16:0/18:1 ≥ 210 ng/ml, 18 did not exceed CDT of 2.5% and ten of 1.7% (Fig. 1d) The range of PEth-concentrations in those cases was 257–3000 ng/ml.

In 31 samples, PEth was the only positive (10 ng/ml) alcohol marker considering the higher CDT cut-off. PEth 16:0/18:1-concentration varied from 11 to 1368 ng/ml in those cases. When using the cut off 1.7% for CDT, PEth was the only positive (10 ng/ml) marker in 26 cases. PEth 16:0/18:1 was ≥ 210 ng/ml while the other markers were below cut-off in seven (CDT cut-off: 2.5%) and four (CDT cut-off: 1.7%) cases. Fig. 1 illustrates the number of positive cases by combination of different biomarkers. MCV was ≥ 95 fl in 58 cases, of which 54 were PEth positive as well. Of note, MCV, PEth and CDT were positive in 37 (CDT ≥2.5%) and 43 (CDT ≥ 1.7%) samples, respectively. In 26 of those samples, BAC was additionally positive.

Table 1 shows the measured ranges of PEth-homologue-, BAC-, CDT-, and MCV- concentrations, of cases exceeding the applied cut-offs.

Concentrations of PEth 16:0/18:1 (U=257, z = −8.1, p < 0.001), CDT (U=467, z = −6.8, p < 0.001), and MCV (U=849, z = −5.5, p < 0.001) were significantly higher in participants who had a positive BAC at the time of blood sampling.

PEth 16:0/18:1 -concentration was significantly higher in patients exceeding CDT of 2.5% (U=322, z = −8.7, p < 0.001) and 1.7% (U=394, z = −8.9, p < 0.001). A Boxplot is shown in Fig. 2, where a significant difference in medians is illustrated.

CDT and PEth 16:0/18:1 showed a significant correlation in the spearman rank analysis with a correlation coefficient of $\rho = 0.77$ ($p < 0.001$). All other homologues showed similar correlation coefficients to CDT with $\rho = 0.77$, $\rho = 0.75$, $\rho = 0.78$, $\rho = 0.78$ and $\rho = 0.79$ (all $p < 0.001$) for PEth 16:0/18:2, PEth 16:0/20:4, PEth 18:0/18:1, PEth 18:0/18:2 and PEth 18:1/18:1, respectively.

A scatterplot of the individuals' concentrations of CDT and PEth 16:0/18:1 is illustrated in Fig. 3. The dashed vertical line marks 210 ng/ml for PEth, the dashed horizontal lines mark 1.7% and 2.5% for CDT.

MCVs correlation was better to PEth ($\rho = 0.58$, $p < 0.001$) than to CDT ($\rho = 0.47$, $p < 0.001$).

Table 1
Overview of biomarker concentrations.

biomarker	range	mean concentration (95%-confidence interval)	median concentration
PEth 16:0/18:1 [ng/ml]	11–5687	1049 (821–1277)	650
16:0/18:2	10–4449	798 (622–974)	440
16:0/20:4	10–596	155 (125–186)	101
18:0/18:1	10–1482	279 (220–338)	188
18:0/18:2	11–1246	264 (210–318)	175
18:1/18:1 ≥ 10	10–543	124 (84–131)	110
16:0/18:1 ≥ 210	241–5687	1571 (1293–1848)	1335
BAC [‰]	0.1–2.5	1.1 (0.81–1.29)	0.75
CDT [%] ≥ 2.5	2.5–22.2	6.9 (5.8–8.1)	5.8
≥ 1.7	1.8–22.2	5.9 (4.8–5.9)	4.4
MCV [fl]	96–111	100 (98.6–100.5)	98

3.4. PEth-homologues

PEth 16:0/18:2 was the homologue with the highest concentration in 20% of the PEth positive samples; in all others, it was 16:0/18:1. The ranges of percental ratios of each homologue from total PEth (concentration of all measured homologues) is shown in Table 2.

Furthermore, we calculated ratios of all homologues to PEth 16:0/18:1. The average values of the ratios of 16:0/18:1 / 16:0/18:2 ($p < 0.001$), 16:0/18:1 / 16:0/20:4 ($p < 0.001$) and 16:0/18:1 / 18:0/18:2 ($p = 0.011$) were significantly higher in samples with CDT ≥ 2.5% than the ones < 2.5%. As an example: When calculating the ratio 16:0/18:1 / 16:0/18:2, the mean value for patients with CDT ≥ 2.5% is 1.4 and for the ones < 2.5% 1.1, meaning the homologues concentrations are on average closer in value when CDT is not above cut-off. Significant differences also occurred for ratios with 16:0/20:4 and 18:0/18:2. No significant difference was observed between samples with positive and negative BAC or between individuals who claimed regular or occasional consumption.

3.5. Hct

The participants' hct ranged from 27.2% to 52.0% (mean: 43.0%). Thus, all values were in the validated range and could be included in the analysis. Hct of the patients' blood did not significantly influence PEth concentration, as confirmed by linear regression analysis (corrected $r^2 = 0.03$, $p = 0.06$). As exact drinking amounts were not registered, a two-factor analysis including hct was not performed. When splitting the participants into three groups according to the hct: I) 27.6–39.9% (mean: 37%, n = 21), II) 40–46% (mean 43%, n = 59), and III) 46.1–52 (mean 48.3%, n = 28), and comparing the PEth concentrations between each group, no significant differences were observed. The scatterplot (Fig. 4) shows the random distribution of PEth 16:0/18:1-concentrations by hct. Random distribution also occurs when looking at cases of only one drinking behaviour category.

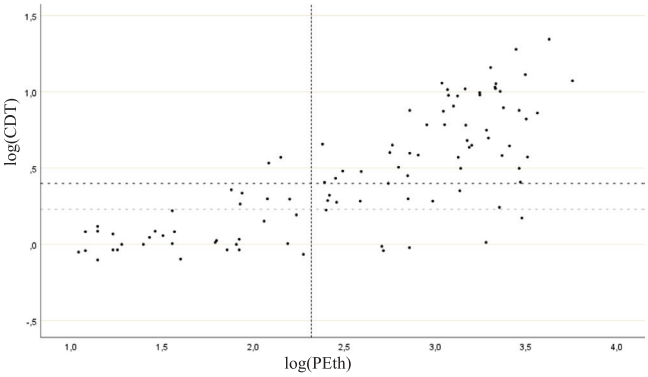

Fig. 3. Scatterplot of logarithmic CDT-concentrations against logarithmic PEth 16:0/18:1-concentrations (vertical line marks PEth 210 ng/ml; horizontal lines mark CDT 1.7% (grey) and 2.5% (black).

Table 2
Range and mean of percentile ratios of each measured PEth-homologue of the total PEth-concentration of all six homologue (* difference significant).

	percentile ratio of total PEth [%]		ratio with PEth 16:0/18:1	
PEth homologue	range	mean	CDT ≥ 2.5%	CDT < 2.5%
16:0/18:1	21–63	38		
16:0/18:2	14–56	31	1.4*	1.1*
16:0/20:4	0–24	6	9.0*	5.8*
18:0/18:1	0–24	10	4.2	4.1
18:0/18:2	0–22	10	4.1*	3.7*
18:1/18:1	0–9	4	11.4	10.1

3.6. Correspondence between alcohol markers and drinking behaviour

In Table 3, the total amounts of positive biomarkers are listed by self-reported drinking behaviour.

The self-reported drinking behaviour was dichotomised into two groups: I) never and occasional and II) regular moderate and regular

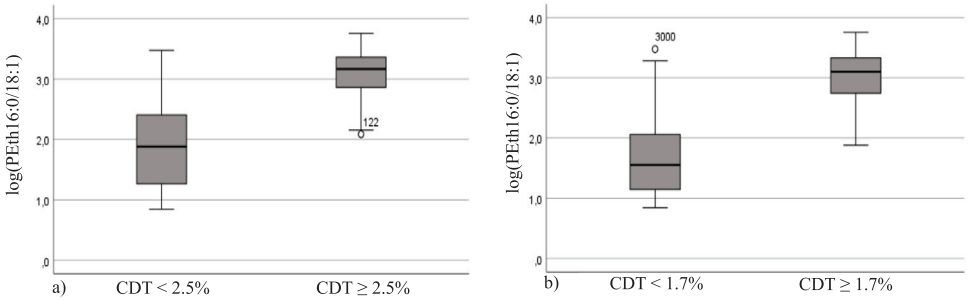

Fig. 2. Boxplots of logarithmic PEth 16:0/18:1-concentrations, comparing cases with positive CDT and negative CDT; a) cut-off CDT: 2.5% b) cut-off CDT: 1.7% (The numbers in the diagram are the de-logarithmized concentrations [ng/ml]).

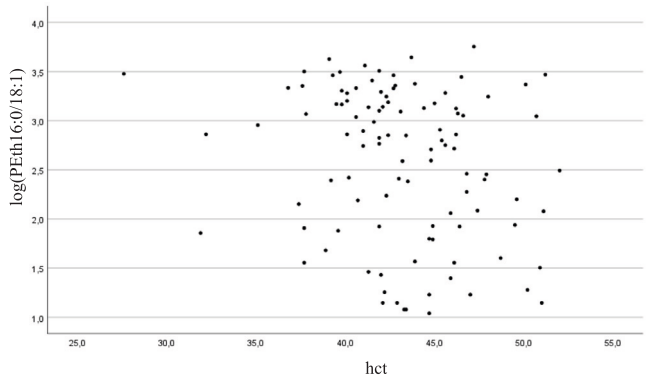

Fig. 4. Scatterplot of logarithmic PEth 16:0/18:1-concentrations by hct.

high. The medians for PEth 16:0/18:1, CDT, and MCV were as follows: I) 32, 1.2, and 90 and II) 1335, 3.8, 97, respectively. It was revealed by Mann-Whitney-*U*-Test, that the groups differ significantly concerning PEth 16:0/18:1- ($U=201$, $z=-3.5$, $p<0.001$), CDT- ($U=240$, $z=-2.8$, $p=0.004$), and MCV- ($U=174$, $z=-3.9$, $p<0.001$) concentrations.

Dividing between regular moderate and regular high drinking behaviour did not yield any significant differences in alcohol marker concentrations.

3.6.1. Sensitivity and specificity

As mentioned above, 62 participants gave a statement about their general alcohol consumption behaviour. When considering the 52 participants who chose at least an occasional alcohol consumption sensitivity of PEth 16:0/18:1, CDT (2.5%), CDT (1.7%), MCV and BAC was 83%, 60%, 71%, 60% and 43%, respectively. Specificity for PEth 16:0/18:1, CDT (2.5%), CDT (1.7%) was 50%, 90% and 80%, respectively. For all other markers specificity was 100%. N for calculation of specificity was only 9 though, so validity might not completely be reliable.

3.7. Comparison between sexes

A lot more males (n = 115) participated in the study than females (n = 31). Calculated by the chi^2-test, the samples of male participants were significantly more often positive for all alcohol biomarkers, except for CDT when applying the lower cut-off (PEth 16:0/18:1 (10 ng/ml): $p=0.004$; PEth 16:0/18:1 (210 ng/ml): $p=0.005$; CDT (2.5%): $p=0.005$; MCV: $p=0.015$; BAC: $p=0.006$). PEth 16:0/18:2 ($p=0.004$) and 18:0/18:1 ($p=0.04$) were also significantly more often above cut-off in men than woman. On the other hand, the amount of positive PEth 16:0/20:4, PEth 18:0/18:2 and PEth 18:1/18:1 samples did not significantly differ between the sexes. No difference occurred in the number of cases that were PEth ≥ 210 ng/ml but CDT < 2.5%. Absolute numbers and percentages are given in Table 4. Considering the positive cases, the concentrations of CDT, PEth and MCV did not differ significantly between the sexes (Mann-Whitney-U-Test), but medians were a lot higher in men (e.g. 709 vs 189 ng/ml for PEth 16:0/18:1, 4.5 vs 2.3% for CDT) With only four MCV concentrations above cut-off and four cases with CDT ≥ 2.5% in females, validity is limited. As only two women had positive BAC, statistical analysis was not carried out.

Self-reported consumption behaviour is also listed in Table 4. Although percentages for no or occasional alcohol consumption were higher in women and for regular consumption higher in men, the difference was not quite significant ($p=0.061$). In both sexes half of the participants who claimed to never drink alcohol were positive for PEth and one of the males additionally had a CDT-concentration of 2.5%.

ROC-AUCs for male participants were 0.69 for 16:0/18:1, 16:0/18:2, 18:0/18:1 and 0.68 for 16:0/20:4 18:0/18:2, 18:1/18:1 (positive n = 40). For females they were 0.87 for 18:0/18:1 and 18:0/18:2, 0.86 for 16:0/18:1 and 18:1/18:1 and 0.84 for 16:0/18:2 and 16:0/20:4 (positive n = 8).

4. Discussion

The cohort chosen for this study is interesting and informative, as the rate of positive alcohol biomarkers was high, and concentrations were rather elevated. Same as in other studies which evaluated different biomarkers in patients with liver diseases [21], heavy drinkers [22], patients in alcohol rehabilitation clinics for reduced drinking [23] and patients of occupational health centres [19], PEth was the marker that was positive most often compared to the other alcohol markers. It also exceeded the cut-off for excessive alcohol consumption in more cases than CDT.

CDT functions exclusively as a biomarker for chronic alcohol use. While PEth can indicate excessive alcohol consumption (cut-off: 210 ng/ml) as well, it can additionally detect lower amounts of alcohol consumption which is a general advantage of this direct alcohol biomarker, especially in the context of abstinence control programs. Overall, CDT and PEth correlated well in this study. However, concerning the analysis of excessive alcohol consumption some discrepancies could be observed. Other studies have already described the variable synthesis rates of PEth and CDT in individuals [19,24]. This might be the reason for the cases in which PEth 16:0/18:1 was only 122 ng/ml and 142 ng/ml, although CDT was 3.4% and 3.7%, respectively. The first participant was not asked about drinking behaviour; the second claimed to be an occasional drinker, what would apply to the PEth- concentration, but not to CDT's. Possibly the participant recently reduced drinking amounts, as CDT takes longer to decrease after EtOH reduction [25,26].

In a study by Årving et al. [24], almost 50% of the patients with PEth > 210 ng/ml had a CDT < 1.7%. In our study this only occurred in approximately 10%, but PEth 16:0/18:1-concentrations were very high with 520–3000 ng/ml in cases with CDT below cut-off. One reason could be the faster formation rate of PEth and the smaller amounts of EtOH causing PEth formation [27]. It needs to be noted that post-sampling formation of PEth in samples that contain EtOH, which was the case in seven of the 18 samples with CDT < 2.5% and PEth ≥ 210 ng/ml (with EtOH 0.1–1.6‰), has been described [28]. We used Whatmann#903 protein saver cards, which dried openly at room temperature. This procedure has been demonstrated to inhibit post-sampling of PEth effectively [28]. Details about post-sampling synthesis are not known very well, especially of authentic EtOH-positive samples. DBS were generated within two to six hours after

Table 3
Absolute numbers and percentage (in brackets) of positive alcohol biomarkers in each alcohol consumption category.

	PEth≥ 10 ng/ml	PEth≥ 210 ng/ml	CDT≥ 1.7%	CDT≥ 2.5%	MCV≥ 95fl	BAC≥ 0.1‰
never (n = 10)	4 (40)	1 (10)	2 (20)	1 (10)	0	0
occasionally (n = 12)	8 (67)	6 (50)	7 (58)	6 (50)	3 (25)	4 (33)
regular moderate (n = 24)	19 (79)	16 (67)	16 (67)	13 (54)	17 (71)	12 (50)
regular high (n = 16)	16 (100)	15 (94)	14 (88)	12 (75)	11 (69)	8 (50)

Table 4
Absolute numbers and percentage (in brackets) of positive alcohol biomarkers and self-reported consumption behaviour category by sex.

	PEth		CDT		MCV	BAC	alcohol consumption behaviour			
	≥ 10 ng/ml	≥ 210 ng/ml	≥ 1.7%	≥ 2.5%	≥ 95 fl	≥ 0.1‰	never	occasional	regular moderate	regular high
male	88 (77)	60 (52)	56(49)	47(41)	51(44)	36(31)	6(13)	8(17)	19(40)	14(30)
female	15(50)	7 (23)	9(30)	4(13)	6(20)	2(7)	4(29)	4(29)	4(29)	2(14)

blood sampling, so post sampling-effects in the EDTA-tubes in this small timeframe cannot be excluded. This might have influenced PEth values and is a limitation of the study.

Range and mean/median of PEth 16:0/18:1 -concentration of participants who claimed regular high alcohol consumption (range: 29–5687 ng/ml (mean:1840 ng/ml, median: 1362 ng/ml)) are in accordance with the values reported for heavy drinkers (231–3855 ng/ml (mean: 1864 ng/ml, median: 1542 ng/ml)) [22]. Values of occasional drinkers in this study (10–3133 ng/ml, mean: 682 ng/ml, median: 195 ng/ml) were not close to moderate drinkers of a previous study (5–119 ng/ml (median: 15 ng/ml)) [29]. The median was more in accordance with values found in patients of an outpatient clinic for reduced drinking (median: 161 ng/ml) [23].

Although the medians of PEth, CDT and MCV differed significantly between the participants who stated to never or occasionally drink alcohol and those that stated to drink regularly, a closer look reveals some discrepancies. First, some individuals who claimed to never drink alcohol presented positive PEth and/or CDT concentrations. This explains the low specificity in this study compared to others [16]. With both markers known to be specific, correctness of the statements must be doubted. Reasons for false statements could be fear of stigmatisation or misunderstanding of the question. Furthermore, some might have started abstinence more recently, as PEth and CDT might still be detectable for several weeks after onset of abstinence [30,31]. More studies correlating PEth values to detailed alcohol consumption statements, especially with subsequent blood samplings, would be helpful to gain more insight into dose-responses.

Of those claiming occasional alcohol consumption 50% had PEth 16:0/18:1- and CDT-concentrations above the cut-offs for excessive alcohol consumption. Here, participants might have either misjudged their alcohol intake or did not want to admit their excessive drinking behaviour. While PEth-concentrations could be influenced by higher amounts of alcohol during the days before the study date, as it increases after single high doses of ethanol [15], CDT does not respond to short-term increase of alcohol intake [11]. PEth synthesis and elimination are prone to inter individual differences, while groups generally reveal good dose-responses. This was also shown in other studies and could have influenced results in this study as well [23,32]. When taking statements of the participants into consideration, in cases with self-reported regular high consumption, sensitivity of PEth 16:0/18:1 (10 ng/ml) was 100% and all but one participant exceeded 210 ng/ml (94%). CDT was not always above the applied cut-offs; thus, CDT was less sensitive for high drinking amounts (88% for CDT of 1.7% and 75% for CDT of 2.5%).

4.1. Hct

Hct is discussed as a critical issue in PEth analysis, as some studies showed significant influence [33], while others did not [34]. Significant influence on the analytical method has been excluded for the used method [13] as it was shown that recovery and matrix effects do not differ depending on hct (20%, 40% and 60%) during the validation. But general physiological influences are rather difficult to evaluate, as many individual factors might impact PEth synthesis and dose-response. No correlation could be observed between PEth-concentrations and hct in linear regression analysis in this study.

4.2. Homologues

Analysis of multiple PEth-homologues did not yield any additional findings. Previous studies showed a faster initial synthesis rate and a shorter half-life of PEth 16:0/18:2 than PEth 16:0/18:1, thus the authors concluded that this could be used to specify information about ingestion times [27,35]. In this study no correspondence was found between cases with positive BAC, which indicates a recent alcohol intake and certain homologue ratios. As it was only asked about the general consumption behaviour in the current study, no information was given on exact consumption times. Thus, data on correlation to ingestion times could not be obtained.

4.3. Sexes

The percentage of women in this study (20%) is representative for the actual ratio of female homeless persons in Hamburg [36]. Men were significantly more often positive for alcohol biomarkers and median concentrations were higher compared to women. Therefore, it can be concluded that men consumed markedly more alcohol than women. The significant difference did not occur in the statements about drinking behaviour. The ROC-AUCs for all homologues were higher in women than in men, which speaks for the assumption, that PEth's diagnostic value might be more significant in females than in men.

5. Conclusion

The high alcohol biomarker concentrations in many cases suggest an excessive alcohol consumption behaviour of individuals in this cohort. PEth was the alcohol marker, that was positive most often and had the highest sensitivity in all drinking behaviour categories self-reported by the participants. Overall, PEth and CDT correlated well, but it was demonstrated that the applied cut-offs for excessive alcohol consumption of the biomarkers are not interchangeable in all cases. Results in this specific collective underline the advantage of including PEth into standard alcohol biomarker analysis. Still, further investigations concerning influences on the synthesis and elimination rates and the combination of different PEth-homologues are needed.

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CRediT authorship contribution statement

N. Aboutara: Methodology, Formal analysis, Investigation, Writing – original draught, Visualisation. **H. Jungen:** Investigation **A. Szewczyk:** Investigation. **A. Müller:** Supervision. **V. van Rüth:** Conceptualization, Methodology. **F. Bertram:** Writing – review & editing. **K. Püschel:** Conceptualization, Funding acquisition. **F. Heinrich:** Conceptualization, Writing – review & editing, Funding acquisition. **S. Iwersen-Bergmann:** Conceptualization, Writing – review & editing, Resources.

Declarations of interest

none.

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6. Further studies (unpublished)

6.1 Stability of six PEth- homologues in whole blood

With the increasing application of PEth as an alcohol biomarker in different settings, a comprehensive validation of the pre-analytical handling of samples for PEth analysis is essential. Different specimen are being used for PEth quantification today (whole blood, washed erythrocytes, DBS) which is one reason for potential variability. Extraction methods are another. Sample stability is also of outmost importance, as falsification of results and interpretations occur, if the analyte is only stable for a short time, but the samples are not handled appropriately. This applies to measurement from whole blood and DBS generated from whole blood. Principally this issue can be avoided by directly generating DBS from capillary blood on-site via volumetric sample devices. Concerning post-sampling synthesis of PEth from ethanol contained in the blood sample risk remains, even in some micro sampling devices as shown by Beck et al. [112]. Drying DBS by natural ventilation after application of blood to Whatman #903 filter paper proved to inhibit post-sampling synthesis and resulted in sample stability for at least up to three to nine months. For DBS generating from EDTA tubes stability is still an issue due to the transportation and storage time until DBS generating.

Faller et. al reported in 2016 that pre-analytical sample handling is critical due to poor stability of PEth in whole blood samples [102]. All previously carried out studies in this work (see 5) adjusted to that by reducing transportation and storage times in the best way possible or generating DBS on-site from capillary blood after a finger prick (see 6.2). More recent studies concluded that specimen processing is not as time critical as assumed and storage can take several days before analysis [113,114].

In the following study, which has been submitted (11.03.2022) and is currently under review with the *Journal of Toxicology* (JAT-22-3737), stability of six PEth homologues, of which some have not or rarely been investigated in context of stability testing, in authentic blood samples were thoroughly analyzed over 30 days, stored at room temperature. Results showed very inhomogeneous stability among the 62 samples and varying reduction rate of the different homologues. The conclusion to minimize transportation or storage, is based on the fact that analyte stability in some samples was not given even during the first days.

Investigations on stability of PEth 16:0/18:1, 16:0/18:2, 16:0/20:4, 18:0/18:1, 18:0/18:2 and 18:1/18:1 in authentic blood samples (at room temperature)

Nadine Aboutara, Hilke Jungen, Anne Szewzcyk, Alexander Müller, Stefanie Iwersen-Bergmann

Abstract

Phosphatidylethanol is a direct alcohol biomarker, used for monitoring individuals' drinking behavior that has gained recognition in clinical and forensic settings. The increasing application of the marker makes investigation of the preanalytical handling necessary and analyte stability deserves major attention. This study was carried out to investigate change of six PEth homologues' concentration, stored in authentic samples of EDTA blood over a course of 30 days at room temperature (n=62). Stability criteria of concentration being within 15% of the original concentration was fulfilled at mean for 10, 3, 2, 5, 2 and 7 days for PEth 16:0/18:1, 16:0/18:2, 16:0/20:4, 18:0/18:1, 18:0/18:2 and 18:1/18:1, respectively. For all homologues there were samples in which concentration had already declined by >15% on day 1. Overall, calculated concentration declines were very inhomogeneous, with inter-sample differences of 43-73% after 30 days. PEth 16:0/18:1, 16:0/20:4 and 18:0/18:2 declined at a greater extent than PEth 16:0/18:1. Blood alcohol concentration was measured >0.1‰ in 25 samples. Of the six samples that exceeded 115% of initial concentrations, three were positive for blood alcohol. The study results add to the previously reported varying information on PEth stability and firstly looks at the six homologues in comparison.

Due to the high scatter of stability among the samples and the observed poor stabilities in some of them it can be concluded that transportation and storage times, especially if cooling cannot be provided, must be kept short. If analyzing from dried blood, generating should preferably be conducted at the site of sampling.

Introduction

Over the past decades several alcohol biomarkers have been discovered and investigated [1]. They are widely used in the fields of forensic and clinical toxicology to evaluate and monitor individuals' drinking behavior. Phosphatidylethanol (PEth), a fraction of ethanol-modified phospholipids, is a highly specific (formed exclusively in the presence of ethanol) marker that allows detection of ethanol uptake for up to several weeks. Concentrations and detection windows are dependent on the drinking behavior and consumption amounts [2,3]. PEth is derived from phosphatidylcholine (PC) and produced enzymatically by the action of Phospholipase D in the presence of ethanol due to the high affinity of short-chained alcohols to the enzyme [1]. PEth is a group of diverse homologous compounds, based on the diversity of the precursor PC, consisting of a

phosphoethanol headgroup to which a variety of fatty acid chains are attached via a glycerol backbone (in sn-1 and sn-2 positions) [4]. 48 different homologues have been identified [5], while it has been reported by several authors that the most abundant ones are those with palmitic and oleic acid chains (16:0/18:1) and palmitic and linoleic acid chains (16:0/18:2) [6,7,8]. Helander et al. investigated half-lives of PEth homologues and reported 3.7–10.4 days, 2.7–8.5 days and 2.3–8.4 days for PEth 16:0/18:1, PEth 16:0/18:2 and PEth 16:0/20:4, respectively [9]. As PEth is more and more routinely used as an alcohol biomarker due to its good sensitivity and specificity [10], analyte stability and storage conditions are important issues. Especially due to the circumstance that samples often need to be transported to the analytical laboratory. Additionally, the samples might be stored at the site of sampling before the transportation is executed. Stability is recommended to be judged as maintained if the deviation from the original concentration is within ±15%, as described by Nowatzke et al. in their work about the evaluation of analyte stability [11]. Studies concerning PEth stability included different storage temperatures and PEth 16:0/18:1 [12,13] and 18:1/18:1 [14] were investigated. In a study by Faller et al. in 2013 it was observed, that PEth was unstable and had declined by about 30-70% on the second day of storage at 4°C, 20°C and 40°C, while being stable for eight days at -20°C and 30 days at -80°C. Another study concluded that PEth is stable for 28 days in authentic samples, irrespective of storage temperatures [12] and Dumitrascu et al. reported stability of PEth for up to 60 days in authentic samples [13]. The latter found a difference in the stability when comparing authentic and spiked samples after 30 days. Stability of PEth in dried blood spots has been evaluated and found to be remained for at least 30 days [14], 60 days [15] and nine months [16].

Due to the differences in stability of PEth that has been reported further data should be collected and discussed. To add to the existing data this study was carried out to thoroughly calculate stability of PEth in authentic samples at room temperature, with a wide range of initial concentrations. Furthermore, stability profiles of multiple homologues have been investigated. As many of the samples were tested positive for blood alcohol, the potential influence of post-sampling synthesis on the collected data is addressed.

Method and materials

Within a study that was carried out in our toxicological laboratory concerning a collective with a suspectedly high alcohol consumption, that had been given approval by the local ethics committee (PV7333) [17], the samples were stored at room temperature and DBS were generated on several date points subsequently (day 1,2,3,4,5,7,10,13,15,21 and 30 after sampling). On the blood sampling day (T0) DBS were generated within two to six hours, by pipetting 20 µl on to filter paper (Whatman #903, GE Healthcare, Buckinghamshire, UK) from blood collected in EDTA tubes (BD,

Heidelberg, Germany). Overall, 62 PEth-positive samples (>10 ng/ml) were stored, of which material was sufficient for DBS generating on eleven days. The EDTA blood was stored shielded from direct sun light and room temperature varied between 20-23 °C. Before each DBS generating the tubes were gently shaken. DBS were left to dry for at least three hours and then stored in Ziploc bags with a desiccant bag (Sigma-Aldrich, St. Louis, MO, USA) for a maximum of 45 days. PEth was analyzed from the DBS as whole (20µl). PEth-homologues 16:0/18:1, 16:0/18:2, 16:0/20:4, 18:0/18:1, 18:0/18:2 and 18:1/18:1 were simultaneously quantified on a LC-MS/MS (XEVO TQ-S) coupled to a UPLC (Acquity System) and a sample manager (Acquity I-class) (all from Waters, Milford, MA, USA). A detailed description about the sample preparation and settings of the method have previously been explained [18]. Additional validation for a calibration range up to 6000 ng/ml was performed and passed with in scope of the study these samples were part of [17]. All samples of one participant were analyzed within one run.

Blood alcohol concentration (bac) was measured from blood sampled into serum-gel tubes (Sarstedt, Nümbrecht, Germany) simultaneously to EDTA blood. It was analyzed enzymatically (DRI®-Ethanol-Assay, Thermo Scientific, Fremont, California, USA) on a clinical-chemical analyzer (AU 480, Beckmann Coulter, Brea, California, USA) and a decision limit of 0.1‰ (0.1 g/l) was applied for positive BAC.

For statistical analysis Microsoft Excel 2016 (Microsoft Corporation, WA, USA) and IBM SPSS Version 27 (IBM, Armonk, NY, USA) were used. It was looked at overall stability along the time frame of 30 days. Stability was defined if a maximum decrease in analyte concentration of 15% [11] of the initial concentration occurred.

Results

All used samples for stability testing were positive (> 10 ng/ml) for the homologues PEth 16:0/18:1 and 16:0/18:2 on T0 (n=62). PEth 18:0/18:1 and 18:0/18:2 were positive in 59 of the samples and PEth 16:0/20:4 and 18:1/18:1 in 56. Initial median concentrations and ranges were 1002 ng/ml (23-5273 ng/ml), 697 ng/ml (15-4079 ng/ml), 128 ng/ml (13-596 ng/ml), 264 ng/ml (15-1482 ng/ml), 223 ng/ml (14-1246 ng/ml) and 111 ng/ml (13- 520) ng/ml for PEth 16:0/18:1, PEth 16:0/18:2, PEth 16:0/20:4, PEth 18:0/18:1, PEth 18:0/18:2 and PEth 18:1/18:1, respectively. Table 1 summarizes the percentage of residual concentrations of all homologues found on each storage day. Furthermore, it contains how many of the samples were ≥85% of the initial concentration. The latter is also represented in figure 1.

Tab.1: Percentage of samples with ≥85% of the starting concentration and mean percentages (standard deviation (SD), ranges) of residual concentrations for all PEth-homologues on each day of storage

T	PEth 16:0/18:1		PEth 16:0/18:2		PEth 16:0/20:4		PEth 18:0/18:1		PEth 18:0/18:2		PEth 18:1/18:1	
	≥85% [%]	**Mean % of T0 (SD) range**	**≥85% [%]**	**Mean % of T0 (SD) range**	**≥85% [%]**	**Mean % of T0 (SD) range**	**≥85% [%]**	**Mean % of T0 (SD)**	**≥85% [%]**	**Mean % of T0 (SD) range**	**≥85% [%]**	**Mean % of T0 (SD) range**
1	97	97 (11) 80-123	86	95 (11) 70-113	81	89 (17) 58-116	95	97 (11) 81-118	82	93 (10) 68-113	96	98 (9) 71-117
2	95	96 (11) 80-123	81	90 (11) 61-112	64	85 (18) 44-115	92	94 (9) 75-119	74	91 (10) 57-112	94	95 (10) 74-115
3	88	89 (9) 72-112	64	85 (11) 61-109	57	82 (16) 47-105	88	89 (10) 72-113	62	83 (11) 57-112	87	91 (12) 63-119
4	82	91 (12) 65-123	59	84 (15) 52-114	46	81 (18) 44-105	83	89 (12) 66-116	54	82 (12) 56-107	78	90 (11) 64-112
5	78	89 (11) 67-120	51	81 (13) 53-111	32	76 (16) 42-108	75	87 (12) 64-116	48	80 (13) 55-105	74	87 (10) 63-110
7	70	87 (11) 68-118	36	78 (13) 51-111	18	70 (17) 23-105	71	83 (11) 55-115	29	76 (13) 48-106	67	86 (10) 59-109
10	63	87 (15) 43-118	16	71 (11) 46-96	9	61 (13) 21-83	67	83 (12) 58-117	8	70 (13) 41-98	49	81 (12) 41-96
13	36	77 (15) 43-113	13	65 (13) 35-93	6	58 (13) 33-94	24	75 (16) 41-107	4	64 (14) 36-101	27	74(14) 43-100
15	38	79 (12) 52-106	9	65 (13) 35-92	0	56 (12) 15-83	36	79 (13) 52-105	4	66 (13) 36-90	30	75 (17) 20-107
21	26	74 (11) 48-87	5	55 (15) 23-88	0	47 (12) 26-70	12	67 (18) 30-90	0	55 (15) 25-81	26	62 (21) 27-93
30	21	71 (15) 45-94	0	55 (13) 34-81	0	48 (13) 29-72	15	69 (17) 42-99	0	53 (16) 25-82	19	62 (21) 27-100

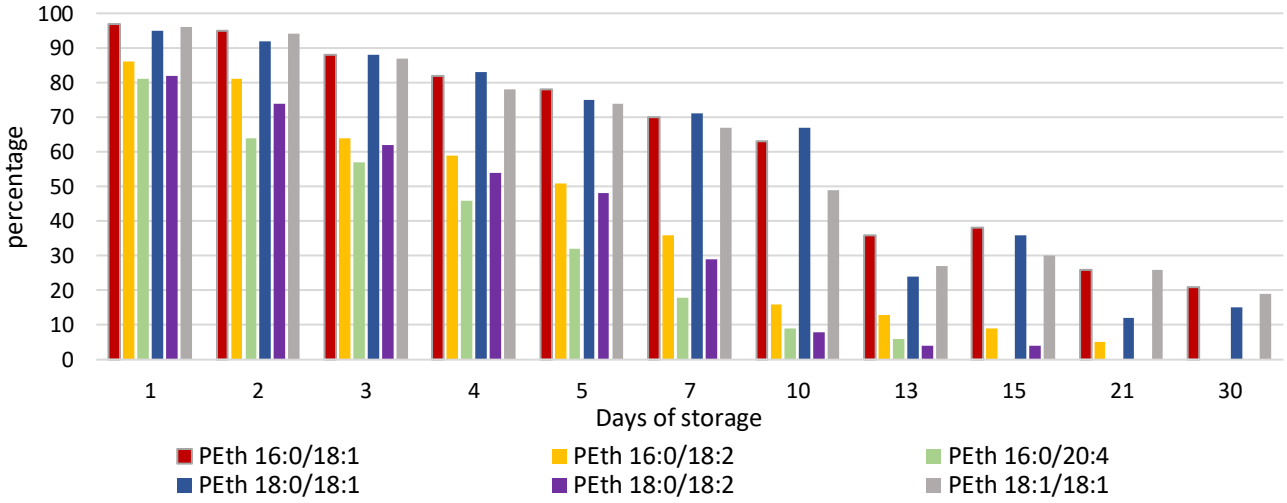

Fig. 1: Percentage of samples with ≥85% of the starting concentration [T0] of all homologues after certain days of storage

Figure 2 shows graphs representing the course of the measured mean residual concentrations in percent over the 30 days the samples were stored. Standard deviations are marked in grey, which in addition to the ranges reported in table 1 represent the rather high scatter of the individual analysis results.

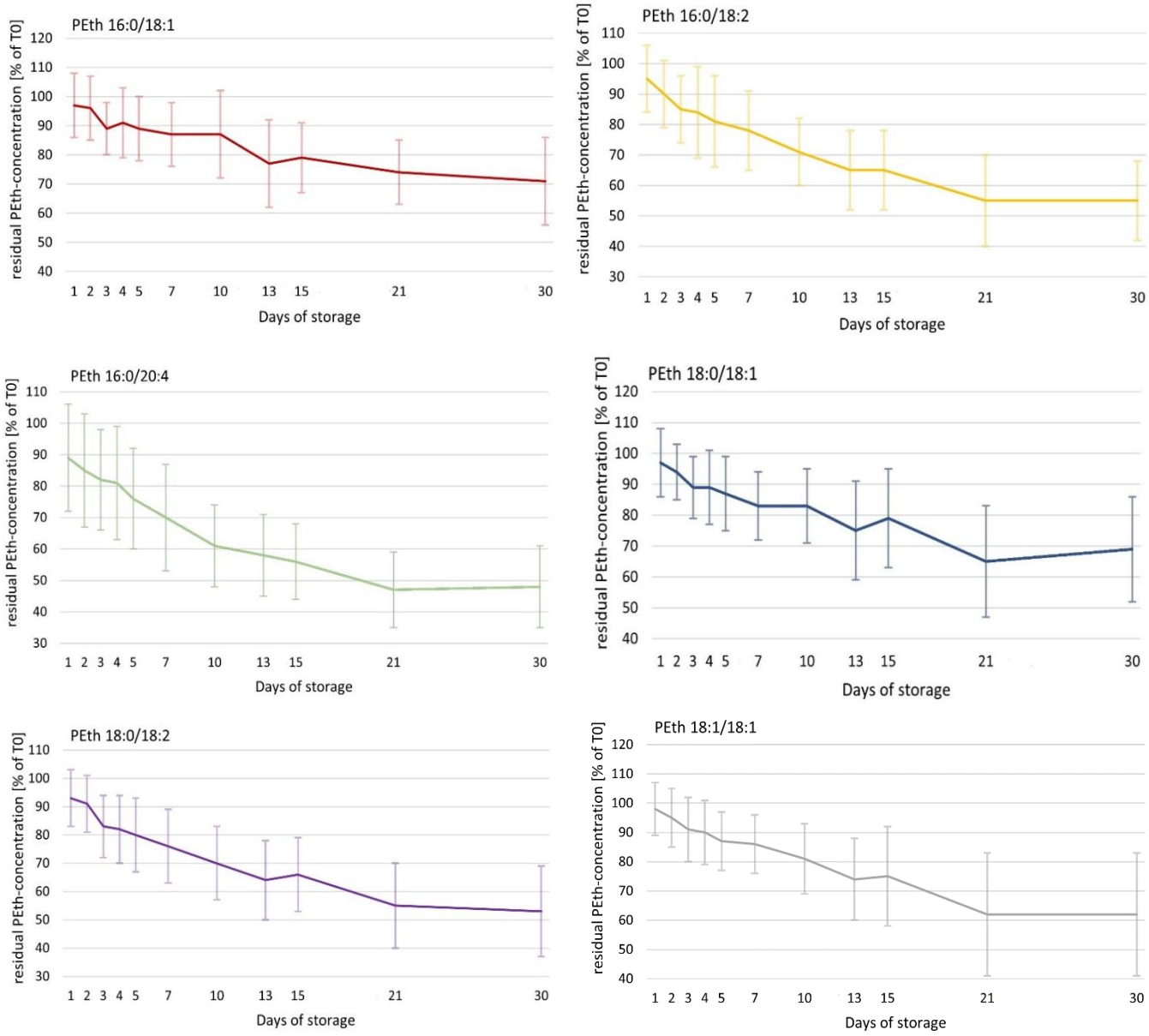

Fig.2: Mean residual concentrations in percent of starting concentration (T0) with marked SD

The central tendencies of the concentrations measured on day T0 and T1 differed significantly for all homologues, except PEth 18:1/18:1, calculated with the Wilcoxon test ($p=0.011$ for PEth 16:0/18:1, $p=0.002$ for PEth 16:0/18:2, $p=0.005$ for PEth 16:0/20:4, $p=0.018$ for PEth 18:0/18:1, $p=0.007$ for PEth 18:0/18:2). For PEth 18:1/18:1 significantly lower concentration was measured on T2 compared to T0 ($p<0.001$).

When looking at the decrease in concentration during the first, second, third and the last week following results were calculated:

a) all homologues' concentrations were significantly lower when comparing T1 and T7 (Wilcoxon test, p<0.001 for all homologues). The percentages of residual concentration were compared with the paired t-test, which yielded a significant mean difference of 11% (p=0.028) for PEth 16:0/18:1, 17% (p<0.001) for PEth 16:0/18:2, 20% (p=0.002) for PEth 16:0/20:4, 14% (p=0.008) for PEth 18:0/18:1, 18% (p=0.002) for PEth 18:0/18:2 and 12% (p=0.028) for PEth 18:1/18:1.

b) between T7 and T15 central tendency of all homologues' concentrations, except PEth 18:0/18:1 differed significantly (p=0.01 for PEth 16:0/18:1, p=0.001 for PEth 16:0/18:2 and p<0.001 for PEth 18:0/18:2 and 18:1/18:1). The percentages of residual concentration between the days declined by a mean of 8% (p=0.005) for PEth 16:0/18:1, 12% (p<0.001) for PEth 16:0/18:2, 14% (p<0.001) for PEth 16:0/20:4, 11% (p<0.001) for PEth 18:0/18:2 and 11% (p<0.001) for PEth 18:1/18:1. Residual concentrations of PEth 18:0/18:1 were lower by mean of 4% (p=0.74).

c) During the third week (T15 to T21) again all homologues' concentrations decreased significantly at central tendencies (p=0.004 for PEth 16:0/18:1, p<0.001 for PEth 16:0/18:2, 16:0/20:4, 18:0/18:1, 18:1/18:1 and p=0.003 for PEth 18:0/18:2). At mean the residual concentrations were 6% lower for PEth 16:0/18:1,10% for PEth 16:0/18:2, 10% for PEth 16:0/20:4, 15% for PEth 18:0/18:1, 11% for PEth 18:0/18:2 and 13% for PEth 18:1/18:1 (all p<0.001) on T21 compared to T15.

d) None of the homologues central concentrations decreased significantly during the last week of storage (T21 to T30). Small changes in mean residual concentration were found between -2% to +3%.

Figure 3 displays a graph including the progression of the residual concentration of all analyzed PEth-homologues.

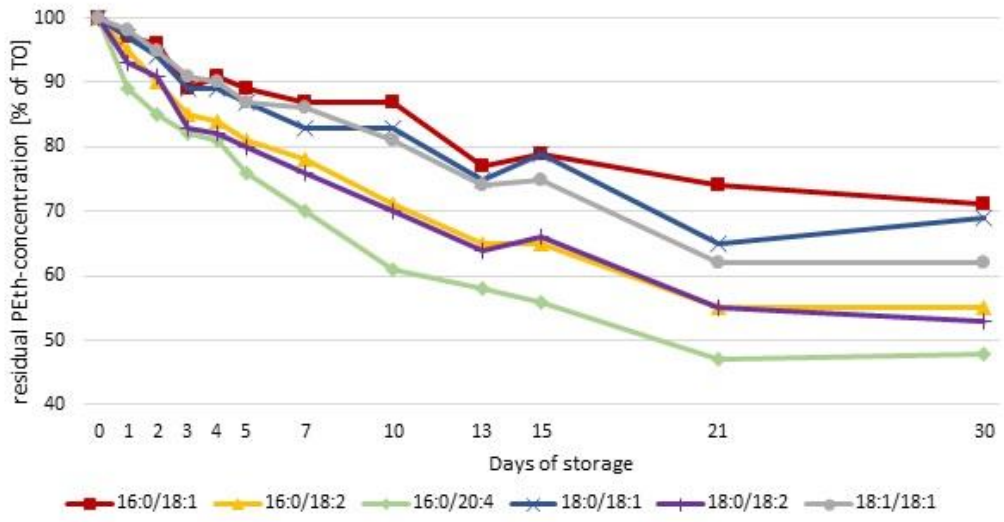

Fig. 3: Progression of the residual concentrations in percent of all analyzed PEth-homologues

When comparing the mean percentages of concentrations that were still found on the applied storage testing days of the homologues to the ones of the most researched homologue PEth 16:0/18:1 it was calculated that PEth 16:0/18:2 (T1: p=0.017, T2: p=0.001, T3: p=0.005, T4: p=0.002, T5: p=0.005, T7: p=0.009, T10-T30: p<0.001) and PEth 16:0/20:4 (T1-T2: p=0.002, T4: p=0.044, T4: p=0.004, T5: p=0.003, T7: p=0.009, T10-T30: p<0.001) had declined significantly more on every measured storage day. Reduction of concentration of PEth 18:0/18:2 differed significantly from the one of PEth 16:0/18:1 on all storage days, except for T1 (T2: p=0.001, T3: p=0.044, T4: p=0.044, T5: p=0.005, T7: p=0.008, T10-T30: p<0.001). The percentage of residual concentration of PEth 18:0/18:1 and 18:1/18:1 only differed significantly from PEth 16:0/18:1 on two days, the first on T5 (p=0.007) and T21 (p=0.001) and the second on T3 (p=0.011) and T10 (p=0.026).

Bac positive vs bac negative

Previous calculations were all obtained without differentiating between samples in which alcohol was detected and in which it was not. bac was positive in 25 cases of the 62 (40%) and ranged from 0.1 to 2.5 ‰. As samples were spotted on filter paper between two and six hours after blood sampling it is unknown if post-sampling synthesis might have occurred during that time, as little is known about the time frame and extent of this phenomenon. It was looked at the cases with positive and negative BAC in comparison, to evaluate if post-sampling synthesis could have influenced the findings on stability testing. Of all 62 cases, six exceeded the T0-concentration of PEth 16:0/18:1, PEth 16:0/18:2, PEth 18:0/18:1 or PEth 18:1/18:1 by > 15% at some time point during storage. Three of the samples were bac positive. Details on the days and percentages can be found in table 2.

Tab. 2: Cases in which the percentage of PEth concentration exceeded T0 by more than 15%, day on which this occurred, calculated percentages and analyzed bac are given

Case	bac	PEth 16:0/18:1 >115%	PEth 16:0/18:2 >115%	PEth 18:0/18:1 >115%	PEth 18:1/18:1 >115%
A	-	T1 (119%), T2 (117%)			
B	-	T1 (123%), T2 (121%), T3 (116%)			
C	1 ‰	T1 (120%), T3 (123%), T5 (118%), T7 (118%), T10 (118%)		T1 (119%), T3 (116%), T5 (115%), T10 (117%)	
D	1.8 ‰	T1 (120%), T2 (117%)	T1 (116%), T2 (117%)		
E	-			T1 (118%)	
F	0.6 ‰				T1 (117%), T3 (117%)

To calculate whether the number of samples that meet the requirement of containing at least 85% of the base concentration statistically differs depending on whether BAC was initially present or not, the chi^2-test was carried out. Significantly more samples that were originally BAC positive were ≥85% of the base 16:0/18:1 concentration than bac negative samples on T10 (79% vs 51%, p=0.016). On T5 significantly more samples met the criteria for stability for PEth 16:0/20:4 if BAC had been positive (43% vs 21%, p=0.035). For all other homologues no significant difference was calculated for any day.

If focus is not solely on meeting stability criteria of 15%, the mean residual concentration in percent can be compared between bac positive and bac negative samples. Interestingly, all homologues' mean residual concentrations in percent were significantly higher on T21 in samples that had been tested negative for BAC (p=0.034 for PEth 16:0/18:1, p<0.001 for 16:0/18:2, 16:0/20:4, 18:0/18:1, p=0.003 for PEth 18:0/18:2, p=0.02 for PEth 18:1/18:1) and T30 (p<0.001 for PEth 16:0/18:1, 16:0/18:2, 16:0/20:4, 18:0/18:1, p=0.003 for PEth 18:0/18:2, p=0.02 for PEth 18:1/18:1). On the other hand, mean residual concentrations in percent were significantly higher in bac positive samples on T4 for PEth 16:0/18:1 (p=0.009) and 18:1/18:1 (p<0.001), on T5 for PEth 16:0/18:1 (p=0.011) and 18:1/18:1 (p=0.027), on T7 for PEth 16:0/18:1 (p=0.026) and 16:0/20:4 (p=0.019) and on T10 for PEth 18:1/18:1 (p=0.037).

Discussion

This study was carried out to evaluate stability of different PEth homologues in samples at room temperature. When applying the criterion of the residual concentration remaining within ±15% of the base concentration for stability it is concluded that PEth 16:0/18:1 was stable for at least 10 days, which is a lot shorter than the 28 days that were reported by Skråstad et al. [12] at similar room temperatures (about 22°C). As the only difference is that the concentrations of PEth in samples of this study were higher, we looked separately at only the samples with maximum concentration of 500 ng/ml, because highest concentration in the other study was about 420 ng/ml. It was excluded that the shorter stability time at same storage conditions could have arisen from the difference in concentration, because the samples only with the lower concentrations were also below a mean of 85% at T10. The difference could be due to much larger sample size (n=62) compared to the other study (n=10). Faller et al. also conducted a study investigating analyte stability of PEth 16:0/18:1 and 18:1/18:1 [14]. The tested authentic samples (n=5) had declined by more than 15% at mean on day one or two for both homologues, with storage at 20°C. Data of this study therefore lies in between the high discrepancy of the two other mentioned studies, with PEth 18:1/18:1 being <85% of the initial concentration on T7 in this study. The other four homologues fulfilled the stability criteria for a shorter period. PEth 16:0/20:4 and 18:0/18:2 remained above the

stability limit only until T2, PEth 16:0/18:2 until T3 and PEth 18:0/18:1 until T5. While Skarstad et al. concluded that PEth samples can be sent to laboratories for analysis without a rush and can be stored for up to 28 days even at room temperature, our data does not support that thesis. A closer look at the data even prevents us from suggesting storage up to seven days, even if only quantifying PEth 16:0/18:1, as it was shown that although mean residual concentration remained >85% until T7, only 63% of the analyzed samples fulfilled the criteria if looking at samples individually. When comparing the mean absolute concentrations, all homologues were calculated to have significantly lower concentrations in the t-test on T1 compared to T0. Furthermore, even after one day of storage some samples declined below the set limit, as represented in table 1. There the wide range of residual percentages are outlined as well. These wide ranges and the fairly high SDs that were calculated additionally show how inhomogeneous the pattern of decline of PEth is among the samples.

The analyzed PEth homologues seem to have different stability patterns in authentic samples stored at room temperature as well. While PEth 18:0/18:1 and 18:1/18:1 were quite similar to PEth 16:0/18:1 in this study, judged by the comparison of mean residual concentrations, PEth 16:0/18:2, 16:0/20:4 and 18:0/18:2 declined faster (significantly less residual concentration on (almost) every storage day). Instability of analytes, such as PEth is suspectedly mainly caused by residual enzymatic activities in sampled blood, chemical hydrolysis and oxidation [19]. It can be suspected that molecules with certain fatty acid residues are more prone to hydrolysis than others, which results in the discrepancy in decrease between some homologues. These results are especially important if more than one homologue is quantified and needs to be kept in mind when looking at homologue patterns, that might be useful to indicate recency of alcohol consumption [8].

Overall, more samples were positive for bac than we had primarily expected. Studies that have investigated post-sampling synthesis of PEth all reported about the occurrence [6,20,21]. While an early study by Helander et al. in 2009 reported that PEth synthesis did not happen within in the first 24 hours of storage after spiking ethanol to PEth-free blood, Schröck et al. described a linear formation over the first seven hours after sampling. The first study measured PEth in half of the samples with up to about 520 ng/ml [6]. The second additionally calculated synthesis rates of about 1.4, 11 and 21 ng/ml per hour for samples that were spiked to 0.1, 1 and 2 ‰, respectively [20]. A more recent study of the firstly mentioned working group found post-sampling synthesis in stored whole blood and whole blood applied to some commercial sampling devices during the time of drying [21]. The DBS technique (drying openly on Whatman filter paper) applied in this experimental set-up was judged as capable of inhibiting post-sampling and degeneration of PEth. In this study six samples exceeded 115% of the initial concentrations. As only half of them were bac positive, it cannot be concluded that post sampling synthesis leads to more failings of the

requirement for stability. Throughout the storage time only two homologues revealed a higher number of samples that were >85% of the base concentration at one time point each if BAC had initially been positive in the sample (PEth 16:0/18:1 on T10 and PEth 16:0/20:4 on T5). This might be a coincidental finding as the time points seem rather random.

Overall, the PEth concentrations in BAC negative samples were significantly lower than in bac positive ones. Generally, a higher absolute concentration results in a lower percental change of concentrations than in samples with lower T0. If a certain amount of PEth is synthesized per hour (linear increase [20]) consequently the influence on percental calculation is smaller on samples with a high PEth concentration to start with and thus more likely to not exceed 115% of base concentration.

There is no plausible explanation we can give for the fact that all homologues residual concentrations were higher in BAC negative than BAC positive samples on the last two time points. The lower reduction of PEth 16:0/18:1 and 18:1/18:1 on T4 to T10 could be plausible if applying the theory of delayed post-sampling synthesis. But with the general high scatter of concentration decrease among the samples and the different homologues, this interpretation seems rather audacious. However, to ensure that this was not caused due to the significant concentration difference between BAC positive and negative samples, we compared the BAC negative samples by concentrations above and below 500 ng/ml concerning the remaining residual concentrations in percent. No significant difference was found for any homologue on any storage day.

While this study supplies further comprehensive data on the stability on PEth and includes multiple homologues, limitations need to be pointed out. The time frame until DBS generating for T0 was up to 6 hours, thus very early decrease in PEth or post-sampling synthesis is not included into the evaluation. In further studies these starting conditions should be kept as short as possible, preferably generating DBS on filter paper at the site of sampling. Furthermore, the only storage condition that was investigated was at room temperature. The studies on post-sampling synthesis that are discussed here were all carried out by spiking blood with ethanol, while the ethanol in the samples of our study was the authentic consequence of recent alcohol consumption. It would be helpful to further investigate the difference between authentic and spiked ethanol in samples.

Conclusion

Results of stability analysis of PEth vary a lot between different studies. The stability pattern of the samples used in this study was highly scattered, looking at single samples, some homologues remained stable for the whole storage time in some samples, while in others stability was not

remained after one day of storage. Therefore, we tend to recommend short times between blood sampling and sample analysis or generating of DBS for storage.

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6.2 Consumption of low amounts of alcohol- investigations on cut-off finding

To further contribute to the ongoing discussion about suitable cut-offs for PEth, a study with participants who consumed 20 g of alcohol on three consecutive evenings was carried out. The study was proposed to the 'Bund gegen Alkohol in Straßenverkehr' (B.A.D.S.) who showed interest and decided to provide financial support. This prospective study aimed at investigating the synthesis of PEth 16:0/18:1 and 16:0/18:2 on each day following small alcohol intake and the development of PEth concentrations during three subsequent days without further intake. A total of 86 participants took part in the study of which 21 belonged to the control group, that consumed one liter of alcohol-free beer/sparkling wine. Before starting the drinking experiment, participants had to stay abstinent from alcohol until individual PEth concentration was below 7 ng/ml. All participants were 'social drinkers' who consume alcohol rarely or moderately according to self-report. This was supported by their monitored PEth concentration during the abstinence phase since the highest concentration found was 60 ng/ml PEth 16:0/18:1.

Cut-offs were already discussed in the paper 'Phosphatidylethanol in patients with liver diseases of different etiologies: Analysis of six homologues and comparison with other alcohol markers' (5.2) in retrospective in patients with liver diseases [105]. There specificity of the low cut-off 10 ng/ml was 100% if not only looking at the past four weeks but a longer timeframe. This could be seen in this study as-well as most participants reached concentrations <LOQ within three to four weeks of abstinence. In some participants PEth concentrations took longer than four weeks to become <LOQ in this collective as well though. In the group that consumed 'alcohol-free' beverages, PEth concentration did not increase in any blood sample. In 5.2 it was concluded that cut-off should be lowered (from 35 ng/ml) in favor of increased sensitivity. This drinking study supports the suggestion. Especially if detection of small alcohol amounts is demanded, cut-off of 10 ng/ml proved a lot better than 20 ng/ml. 35 ng/ml PEth 16:0/18:1 and 16:0/18:2 was exceeded by very few participants after consumption of 20 g of alcohol on three days. The two studies (5.2 and 6.2) together suggest a confident benefit of applying a cut-off of 10 ng/ml of PEth. Sensitivities for the cut-offs are compared, central tendencies of the two PEth homologues are analyzed and influencing participant characteristics are investigated in the manuscript 'PEth 16:0/18:1 and 16:0/18:2 after consumption of low doses of alcohol- a contribution to cut-off discussion' that is currently under review at the Journal *Drug Testing and Analysis (*DTA-22-0102, submitted on 18.03.2022*)*.

PEth 16:0/18:1 and 16:0/18:2 after consumption of low doses of alcohol- a contribution to cut-off discussion

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Abstract

Phosphatidylethanol has gained recognition as a direct alcohol biomarker. While different cut-offs have been suggested and applied there is no consensus on suitable cut-off concentrations of PEth for differentiating between abstinence and alcohol consumption today. For investigating this objective, 75 participants consumed 20 g of ethanol on three consecutive days. Blood was sampled on each following day. Starting concentrations were <7 ng/ml for both homologues. PEth 16:0/18:1 yielded a sensitivity of 25%, 45% and 49% and PEth 16:0/18:2 of 40%, 61% and 71% for the consumption days, respectively, when applying a cut-off of 10 ng/ml. PEth 16:0/18:1 reached >20 ng/ml in five samples overall. Sensitivity of PEth 16:0/18:2 >20ng/ml was a lot better with 7%, 24% and 35% after the drinking days, respectively. Overall, PEth 16:0/18:1 was > 35 ng/ml in one sample and PEth 16:0/18:2 in three samples. Significantly more women had PEth 16:0/18:1 concentrations > 10 ng/ml after three days of consuming 20 g of alcohol (p=0.02) and PEth 16:0/18:2 > 10 ng/ml after the second (p=0.023) and the third (p=0.002) drinking event.

Consumption of one liter of 'alcohol free' beverage on three days did not impact PEth concentrations (n=21).

While the response rates of PEth to alcohol uptake are subject to strong interindividual differences, the study results suggest, that PEth cut-off should be lowered for better detection of consumption of low to medium amounts of alcohol. Furthermore, it is advantageous to include analysis of both PEth 16:0/18:2 and 16:0/18:1 over only analyzing the latter.

1. Introduction

Phosphatidylethanol (PEth) is a direct alcohol biomarker that has gained importance in the past years. It has been shown to be beneficial alone or in combination with other alcohol biomarkers in different settings and patient collectives[1,2,3].

PEth, an abnormal phospholipid consisting of a phosphoethanol headgroup with a variety of fatty acid chains attached to a glycerol backbone, is only synthesized by phospholipase D from phosphatidylcholine if ethanol (EtOH) is present in the body[4]. PEth has been found to accumulate after continuing alcohol uptake[4,5]. The composition of fatty acid chains varies, like in the precursor molecule, so different homologues of PEth exist[6]. The most abundant homologues are the ones with palmitic and oleic (16:0/18:1) and palmitic and linoleic acid chains (16:0/18:2)[6,7].

Several studies have investigated the half-life of PEth[8,9]. Helander et al.[10] recently specified between homologues and reported half-lives of 3.7–10.4 days and 2.7–8.5 days for PEth 16:0/18:1 and 16:0/18:2, respectively. Therefore, depending on the alcohol consumption behavior and resulting PEth concentrations, it might have a detection window of several weeks[11].

In a review on PEth analysis in patients with liver diseases sensitivity of 73–100% and specificity of 90–96% were reported to determine alcohol consumption in the previous one to four weeks. Reduced sensitivity with 58-92% but higher specificity with 98-100% for different consumption amounts in the past four to twelve weeks were recently presented by our working group[12]. Overall PEth has been shown to have a good dose-response to ethanol[13,14]. Still, substantial interindividual differences in PEth synthesis and elimination exist[15,16].

Several clinical or forensic settings demand monitoring of alcohol consumption behavior of individuals, such as monitoring health professionals with substance use disorders, patients before or after liver transplantation, during pregnancy or during detoxification programs[9,17-20]. With the improved sensitivity by application of mass spectrometry techniques for PEth analysis, the biomarker has received attention in context of abstinence control[21,22]. What is ongoing is the search for a reliable cut-off for abstinence testing, that delivers the best possible combined results for sensitivity and specificity. In the United States there is general consensus to apply a cut-off of 20 ng/ml to distinguish between conscious alcohol consumption and abstinence or incidental exposures[23]. Suggestion from a Swedish working group for a cut-off of 35 ng/ml have also been widely established[24], while other studies raise the question if these cut-offs are rather conservative and could be lowered[8,21,25].

Aim of this study was to systematically investigate the concentrations of PEth 16:0/18:1 and 16:0/18:2 after consumption of small amounts of alcohol (typically contained in half a liter of beer). It was tested whether a) minimal alcohol concentration that might be contained in alcohol-free-declared beverages (max. 0.5%) can increase PEth concentrations (samplings AF1, AF2, AF3) and b)

if consumption of low amounts of alcohol generates PEth-concentration above the different applied cut-offs 10, 20 and 35 ng/ml and to analyze cumulation effects after three days of ingestion of 20 g of alcohol (samplings A1, A2, A3).

2. Materials and methods

2.1 Beverage consumption

The drinking experiment started after an abstinence phase of at least three weeks resulting in PEth concentrations <7 ng/ml. This was individually verified by sampling and analyzing blood during this time. The participants were then contacted if their concentrations were low enough to start. Then the participants consumed a) one liter of alcohol-free beer/sparkling wine within one hour or b) 20 g of alcohol (e.g., 500 ml of 5 vol.-% beer, 200 ml of 12.5 vol.-% wine) within 30 minutes on three consecutive evenings. Each following day the participants came to the site of blood sampling (Department of Legal Medicine, Hamburg) between 7:30 am and 4 pm. Some participants that were willing to be abstinent for three more days after part (b) came for additional blood samplings (E1, E2, E3) on the three days following the last sampling day of (b).

2.2 Sampling and analysis of blood

Blood was sampled by the laboratory staff. Participants were asked to wash their hands with warm water to increase blood flow. Capillary blood was then gained from the fingertip: a safety lancet was used to prick the finger, then a 20 µl capillary (Minivette, Sarstedt, Nümbrecht, Germany) was filled and then emptied on the filter paper (Whatman #903, GE healthcare, Chicago, IL, USA) to volumetrically generate dried blood spots (DBS). DBS were left to dry for at least three hours and then stored in ziploc bags with a desiccant bag until analysis. PEth 16:0/18:1 and 16:0/18:2 were analyzed from the DBS as previously described in detail[26]. In brief: one spot was used as a whole (20 µl), extracted with 1 ml methanol (containing the deuterated internal standards) in a disposable glass tube on a sample shaker. 800 µl was transferred into a vial and evaporated. After reconstitution in MeOH and acetate buffer (90:10 [vol%]) and vortexing the sample analyses was carried out on a tandem-quadrupole mass spectrometer (XEVO TQ-S; Waters, Milford, MA, USA). Limit of detection (LOD) for both analyzed PEth homologues is 3.9 ng/ml. Limit of quantification (LOQ) is 8.6 ng/ml for PEth 16:0/18:1 and 6.2 ng/ml for PEth 16:0/18:2.

2.3 Study participants

General inclusion criteria were that the participants were at least 18 years old and did not have any health or mental conditions that require abstinence from alcohol. Female participants had to confirm that they were neither pregnant nor breast feeding. The study was approved by the local ethic committee (2021-100742-BO-ff) and financial support was provided by the B.A.D.S. The participants received an allowance after successfully terminating the study protocols.

Statistical analyses were carried out with Microsoft Excel 2016 (Microsoft Corporation, WA, USA) and IBM SPSS Version 27 (IBM, Armonk, NY, USA). For statistical comparison of ratios of sample amounts chi^2- or McNemar-tests were applied. For calculations comparing mean values or tendencies t-tests, Mann-Whitney-U- and Wilcoxon-tests were used.

3. Results

3.1 Study participants

Formerly, n=81 participants were recruited for the drinking experiment with ingestion of low amounts of alcohol on three consecutive days. Of those, six had to be excluded because they did not stay abstinent before the drinking events long enough (n=2), did not drink the alcohol as described in the description (n=2) or did not come to the appointment for blood sampling (n=2). Of those included (n=75) 72% (n=54) were female. Additional blood was sampled of 39 participants on the three days after the last sampling day after the third consumption day. The group that consumed alcohol-free beer consisted of n=22 persons. Of those seven were male (32%). Participants aged between 19 and 69 years (37.1 ±11.4) and had a mean body mass index (BMI) of 24.1 kg/m^2(SD±4.1, range 18.0-38.9 kg/m^2).

3.2 Starting points

55% of the subjects in the AF-group had a PEth 16:0/18:1 concentration <LOD (defined as 0 for calculations) at the starting point (AF0) and in the alcohol-group it was 36% (A0). Regarding PEth 16:0/18:2 concentration, 86% and 69% were <LOD at AF0 and A0, respectively. Numbers and percentages of samples that were >LOD can be found in table 1.

Because it took longer than anticipated for some participants to reach concentration <7 ng/ml, this concentration was chosen for being eligible for starting the experiments instead being <LOD.

3.3 Alcohol-free beverages

None of the 21 cases showed a notable increase in PEth 16:0/18:1 or 16:0/18:2 concentrations after consumption of the first, the second or the third liter of alcohol-free beer/sparkling wine. The number of samples with PEth concentrations <LOD remained similar (tab. 1)

Tab. 1: Absolute numbers and percentages of samples that were >LOD for PEth 16:0/18:1 and 16:0/18:2 during the three consumption days of 'alcohol-free' beverages (n=21), the three consumptions of 20 g of alcohol (n=75) and the three subsequent days (n=39)

Sampling day	PEth 16:0/18:1		PEth 16:0/18:2	
	n > LOD	*%*	*n > LOD*	*%*
AF0	10	45	3	14
AF1	10	45	3	14
AF2	8	38	4	18
AF3	8	38	4	18
A0	48	64	24	31
A1	68	91	62	83
A2	70	93	67	89
A3	70	93	68	91
E1	35	90	35	90
E2	35	90	35	90
E3	32	82	30	78

3.4 Consumption of 20 g of alcohol

Numbers and percentages of samples that were >LOD on each study day are listed in table 1. On each of the days after consumption of 20 g of alcohol, significantly more samples were measured > LOD than on A0 for both PEth homologues ($p<0.001$ for A0 compared to A1, A2, A3). Between A1, A2 and A3 no significant increase in samples >LOD was calculated.

Table 2 shows the mean PEth concentrations that were measured on each sampling day during the alcohol consumption phase and the subsequent three days of abstinence in the samples that were > LOQ (8.6 ng/ml).

Tab. 2: Overview over PEth concentrations of samples that were >LOQ for PEth 16:0/18:1 and 16:0/18:2 during the three consumption days of 20g of alcohol and three subsequent blood samplings; total n: 75 for A1-A3, 39 for E1-E3

sampling day	PEth 16:0/18:1		PEth 16:0/18:2	
	n > 8.6 ng/ml	*mean (sd) concentration; range*	*n > 8.6 ng/ml*	*mean (sd) concentration; range*
A1	30	11.6 (2.9); 8.9-21.5	36	15.2 (5.8); 8.7-31.7
A2	40	13.1 (2.9); 8.7-19.3	50	18.3 (7.1); 9.0-39.3
A3	50	14.3 (6.2); 8.8-42.3	61	18.4 (8.2); 9.4-43.0
E1	20	13.1 (2.4); 9.2-17.1	30	18.3 (7.9); 9.0-42.9
E2	20	12.7 (2.8); 8.8-19.1	27	16.7 (7.4); 9.1-41.0
E3	16	11.6 (2.3); 8.9-17.0	18	14.1 (4.5); 9.0-25.0

The difference in mean concentrations on A1 to A2 and A2 to A3 was not significant for PEth 16:0/18:1, but the mean increase of A3 compared to A1 was ($p=0.026$). Mean concentration of PEth 16:0/18:2 was significantly higher on A2 than on A1 ($p<0.001$), but there was almost no change in

mean concentration between A2 and A3. Between A3 and E1 both homologue's mean concentration did not significantly change. For PEth 16:0/18:1 there was also no significant decrease from E1 to E2 or from E2 to E3, but if comparing A3 to E3 (p=0.021) and E1 to E3 (p=0.004). Mean concentration of PEth 16:0/18:2 decreased significantly between the three abstinence days, from E1 to E2 (p=0.002) and from E2 to E3 (p=0.01).

Figure 1 shows the development of the two PEth homologue's concentration over the course of the study time. Standard deviations are marked and demonstrate the rather high scatter of the values around the mean concentrations. This represents the interindividual differences in dose response of PEth to ethanol. The graphs in fig. 1 allow the interpretation, that PEth 16:0/18:2 concentration increases at a higher rate during consumption days and decreases at higher rate on the following abstinence days than PEth 16:0/18:1. To be able to integrate A0 into the graph, for this data point, mean concentrations were quantified via extrapolation in the samples with PEth 16:0/18:1 or 16:0/18:2 > LOD.

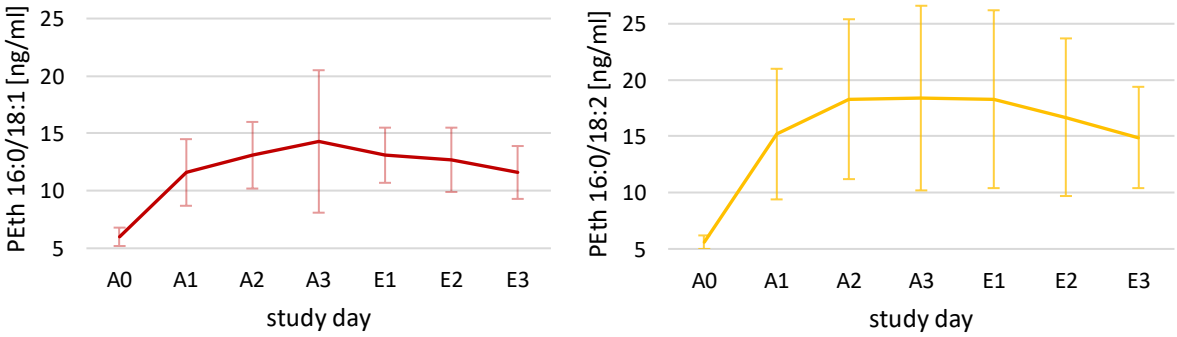

Fig. 1: Mean PEth concentrations (with standard deviations) during three days of consumption of 20 g of alcohol and three subsequent days of abstinence, quantification for A0 (if concentrations >LOD) via extrapolation

In the samples that were >LOQ for both homologues, on A1 (n=26) concentration of PEth 16:0/18:2 was higher than PEth 16:0/18:1 in 22 cases (85%). On A2 (n=39) it were 38 cases (97%) and on A3 (n=47) 42 (90%). When quantifying both homologues on A0 via extrapolation if concentrations are >LOD, only two samples had a higher PEth 16:0/18:2 than 16:0/18:1 concentration (n=24) (8%).

Central tendencies of PEth 16:0/18:2 concentrations were significantly higher than PEth 16:0/18:1's throughout all consumption days (p<0.001; z=-3.5 for A1, z=-4.7 for A2, z=-3.8 for A3).

3.5 Cut-offs

Table 3 shows absolute numbers and percentages of samples that were quantified above 10 ng/ml, 20 ng/ml and 35 ng/ml, three suggested cut-offs for PEth 16:0/18:1, during consumption of alcohol free and alcoholic beverages. Specificity of both homologues for every applied cut-off was 100%

(for consumption of 1 L of alcohol-free beer/sparkling wine consumed within one hour). The percentages in the table represent the sensitivities of the cut-offs, as all participants consumed 20 g of alcohol on three evenings. Calculations reveal that PEth 16:0/18:2 is more sensitive for short term low alcohol uptake than PEth 16:0/18:1. Both homologues show an increase of sensitivity over the course of the three consumption days. Although direct comparison between A3 and E1 is imprecise, because not all participants were included in the abstinence phase after the consumption phase, it can be suspected, that sensitivity decreases over the course of abstinence (E1-E3).

Tab.3: Overview over samples with PEth 16:0/18:1 or 16:0/18:2 concentration above the three applied cut-offs on any sampling day; absolute number and percentage (in brackets); total n: 21 for AF0-AF3, 75 for A0-A3, 39 for E1-E3

sampling day	**PEth 16:0/18:1**						**PEth 16:0/18:2**					
	>10 ng/ml		*>20 ng/ml*		*>35 ng/ml*		*>10 ng/ml*		*>20 ng/ml*		*>35 ng/ml*	
	n	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*
AF0	0	0	0	0	0	0	0	0	0	0	0	0
AF1	0	0	0	0	0	0	0	0	0	0	0	0
AF2	0	0	0	0	0	0	0	0	0	0	0	0
AF3	0	0	0	0	0	0	0	0	0	0	0	0
A0	0	0	0	0	0	0	0	0	0	0	0	0
A1	19	25	1	1	0	0	30	40	5	7	0	0
A2	34	45	0	0	0	0	46	61	18	24	1	1
A3	37	49	5	7	1	1	51	71	26	35	3	4
E1	17	44	0	0	0	0	26	67	11	28	1	1
E2	16	41	1	3	0	0	25	64	6	15	1	1
E3	13	33	0	0	0	0	17	44	3	8	0	0

3.6 Sexes

More women participated in the study than men, as the voluntary application rate of men was a lot lower than the one of women. Concerning the compliance during the study, women and men had to be excluded equally (three each).

While the ratio of samples that were > LOD on A0 was equal for males and females (62%), significantly more samples of women than of men had detectable PEth concentrations on A2 (PEth 16:0/18:1: 98% vs 86%, p=0.033; PEth 16:0/18:2: 96% vs 81%, p=0.032) and A3 (PEth 16:0/18:1: 98% vs 86%, p=0.033; PEth 16:0/18:2: 98% vs 81%, p=0.009).

Taking the concentrations into consideration, that could be quantified (>LOQ), the concentration of PEth 16:0/18:1 in samples from females was higher than in samples from males (table 3), but the difference was only short under significant on A2 (p=0.048, z=-2). Difference of PEth 16:0/18:2's concentration in samples from woman and men was significant on A2 (p=0.047, z=-2) and A3 (p=0.039, z=-2.1) (table 3). Figure 2 displays the concentrations of males and females in comparison

in box plots. Here it can also be seen that all maximum concentrations were analyzed in samples of women after consumption of alcohol.

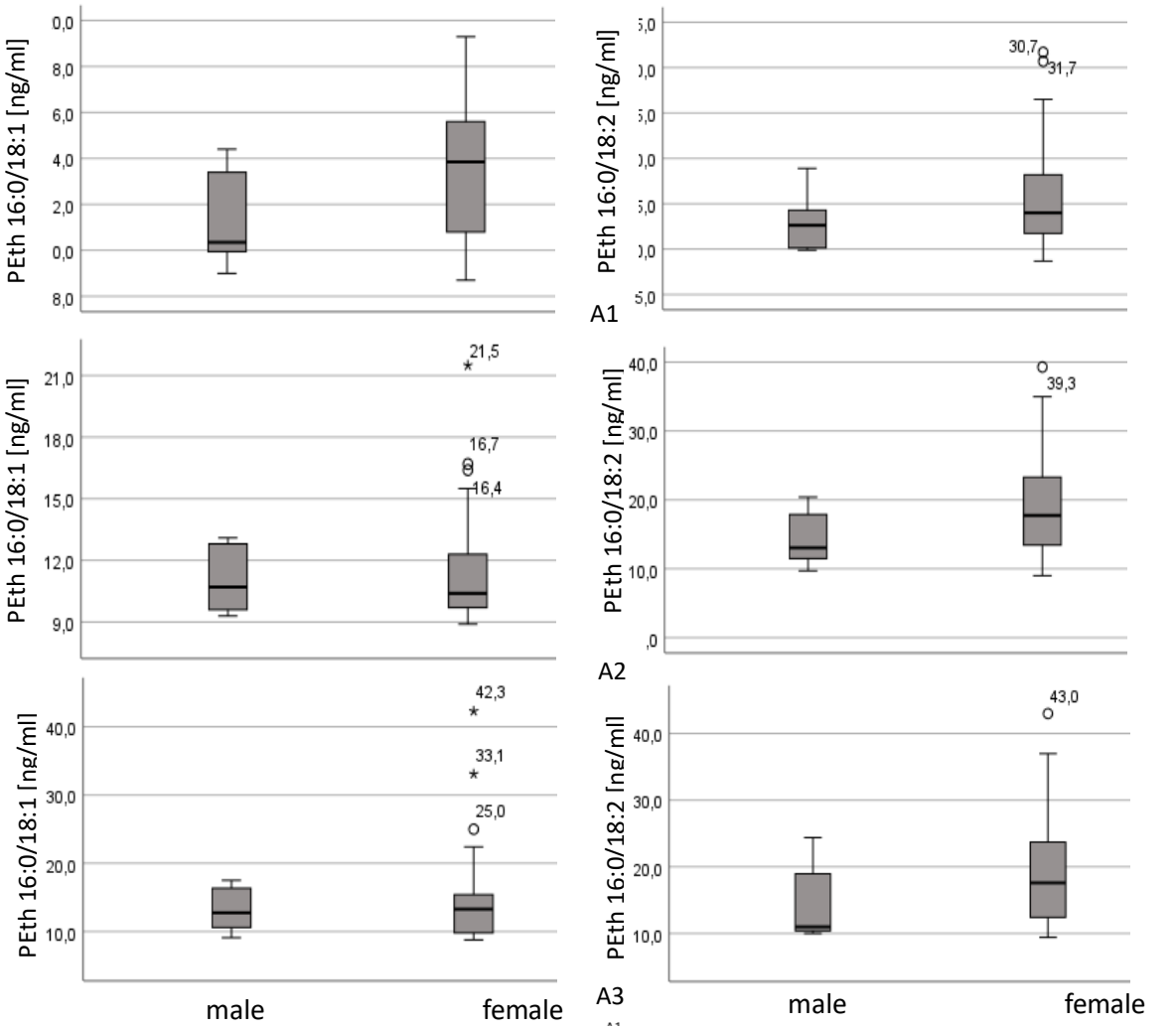

Fig. 2: Box-plots representing PEth 16:0/18:1 and 16:0/18:2 concentrations in samples from male and female participants on days A1, A2, A3 (outliers labelled with PEth concentration)

Looking at the different applied cut-offs the percentage of samples from female participants exceeded the one of males on every sampling day (table 4). The difference of the ratio of samples >10 ng/ml PEth 16:0/18:1 was significant on A3 (p=0.02) and >10 ng/ml 16:0/18:2 on A2 (p=0.023) and A3 (p=0.002). Furthermore, all samples in which PEth 16:0/18:1 was quantified >20 ng/ml or 35 ng/ml on any sampling day came from female participants. So did all samples that were >35 ng/ml PEth 16:0/18:2 and the five samples that were >20 ng/ml on A1. Significantly more samples that were >20 ng/ml PEth 16:0/18:2 on A2 (p=0.012) and A3 (p=0.016) came from female participants, with overall 17 of 18 (94%) and 23 of 26 (88%) samples being from women on A2 and A3, respectively.

Tab. 4: Samples in which PEth 16:0/18:1 or 16:0/18:2 concentrations exceed the three applied cut-offs (10, 20 and 35 ng/ml) divided by sex, total n males: 21, total n females: 54

		PEth 16:0/18:1				**PEth 16:0/18:2**			
		male		*female*		*n male (%)*		*n female (%)*	
		n	*%*	*n*	*%*	*n*	*%*	*n*	*%*
A1	> 10 ng/ml	4	19	15	28	6	29	25	46
	> 20 ng/ml	0	0	1	2	0	0	5	9
	> 35 ng/ml	0	0	0	0	0	0	0	0
A2	> 10 ng/ml	6	29	29	53	9	43	38	17
	> 20 ng/ml	0	0	0	0	1	4	17	31
	> 35 ng/ml	0	0	0	0	0	0	1	2
A3	> 10 ng/ml	6	29	31	58	10	48	45	83
	> 20 ng/ml	0	0	9	17	3	14	23	43
	> 35 ng/ml	0	0	2	4	0	0	3	6

Significant differences were found between the sexes in the same tendencies if looking at overweight and normalweight participants separately (data not shown), to exclude influence of BMI differences between the sexes (see below).

3.7 Body weight and BMI

According to the classification by the World Health Organization (WHO)[27] two participants were underweighted (<18.5 kg/m^2), 49 were normal weight (18.5-24.9 kg/m^2), 19 in the range of pre-obesity (25-29.9 kg/m^2) and five obese (>29.9 kg/m^2). No correlation occurred between the PEth homologue's concentrations and the BMI of the participants on A1 and A2 and only a weak correlation on A3 (PEth 16:0/18:1: ρ=-0.25, p=0.029; PEth 16:0/18:2: ρ=-0.24, p=0.024). Correlation between bodyweight and PEth 16:0/18:1 was moderate on A2 (ρ=-0.35, p=0.003) and weak on A3 (ρ=-0.27, p=0.022). Between bodyweight and PEth 16:0/18:2 it was moderate on A2 (ρ=-0.39, p<0.001) and A3 (ρ=-0.33, p=0.004).

In the following participants that were normalweight (NW) (BMI < 25 kg/m^2) and overweight (OW) (BMI ≥ 25 kg/m^2) are compared. Mean PEth 16:0/18:1 concentration did not differ significantly between NW and OW patients on any study day. Mean PEth 16:0/18:2 concentration differed significantly after three days of alcohol consumption (A3) (p=0.022, z=-2.3).

For PEth 16:0/18:1 no difference in sample number with concentrations >LOD was found on any of the days after alcohol consumption (A1-A3). Significantly more of the NW participants' PEth 16:0/18:2 concentration was >LOD on A3 (p=0.02) (98 vs 83 %) than of OW ones. Also, on A3 significantly more NW participants were above the cut-offs of 10 ng/ml PEth 16:0/18:1 (p=0.003) and 10 ng/ml (p=0.013) and 20 ng/ml (p=0.018) PEth 16:0/18:2.

It needs to be pointed out that significantly more of the male participants were OW (57%) than of the women (22%) (p=0.003). Thus, the male ratio in the OW group is 50% and in the NW group 17%.

That is why analysis was also carried out regarding BMI differences in women and men separately, to eliminate the additional criteria of sex. In samples of females there was no significant difference of mean PEth 16:0/18:1 or 16:0/18:2 concentration on any study day between NW and OW participants. Neither was there a difference in samples above LOD or any of the applied cut offs between the two groups. In samples from male participants the difference of concentrations of PEth 16:0/18:2 between NW and OW participants could also not be found. But in more of the NW samples a PEth 16:0/18:1 concentration was quantitated > 10 ng/ml on A3, with five of nine of the NW men compared to one of eleven OW men (p=0.018). On all other days no difference in ratios of samples > cut-offs occurred when comparing NW and OW men.

After dividing the male and female participants into two weight groups, divided by being above or below median body weight (68 kg for woman, 86 kg for men), central tendency of PEth 16:0/18:2 concentration was lower in lighter than heavier men on A3 (p=0.005, z=-2.7). The lower central tendency of PEth 16:0/18:1 of lighter women was just significant than in heavier women on A2 (p=0.047, z=-2).

Concerning the correlation of BMI to PEth concentrations, none of them was significant on any sampling day if looking at men and women separately. Correlation was rather strong between weight and PEth 16:0/18:2 in male participants (ρ=0.45, p=0.04).

4. Discussion

Overall PEth responded to the uptake of 20 g of ethanol on consecutive days, as with each consumption day significantly, more samples had detectable (>LOD) PEth concentrations. A quantifiable increase of mean concentrations occurred after three days of consuming 20 g of alcohol for PEth 16:0/18:1 and after one day of consumption for PEth 16:0/18:2. The latter did not change at mean after ongoing consumption. Possibly, elimination of PEth 16:0/18:2 balanced out further increase in concentration that could occur due to new synthesis after drinking. The high ranges of PEth concentrations among the collective highlights the interindividual dose response of PEth to ethanol, as some participants did not show any change in PEth concentrations while others reached >20 ng/ml after initially being <LOD.

4.1 Cut-offs

Prospective studies with controlled alcohol consumption amount and study settings are very helpful when evaluating biomarkers since retrospective comparisons of self-reported consumption behavior and results of biomarker testings are generally biased by underreporting or false estimations by subjects[29]. Especially for the investigation about sensitivities and cut-offs it yields helpful answers if participants consume defined amounts of alcohol. As described in the introduction, different cut-offs for PEth analysis have been proposed over the past years. While a

Swedish working group suggested a cut- off of 35 ng/ml for abstinence testing, which was adopted in several studies[18,29], American and Belgian laboratories apply a lower cut-off of 20 ng/ml[16]. The latter cut-off has also been used regularly in studies[12,19,30]. In other studies decision limits of 10 ng/ml[8,11] or 8 ng/ml[31,32] have been used. With lowering a cut-off there is always the risk of increasing the number of false positives. That is why analysis of specificity is important besides the sensitivity. Here different reports have been given, as summarized in the introduction. For patients with liver diseases specificities of 90-96% have been calculated[1]. As the calculations based on self-report and some patients rely on a potential liver transplantation, which requires previous abstinence, underreporting in this setting is a potential source of bias[19]. As PEth is only built if ethanol is present in the body a high specificity is generally expected, but there is only little literature on the influence of incidentally consumed alcohol, such as consumption of alcohol-free beer which in Germany is allowed to contain up to 0.5 vol.-% of ethanol. Specificity in this setting was 100% for participants who consumed one liter of 'alcohol-free' beer or sparkling wine on three consecutive days. Concerning PEth, Reisfield et al. conducted a study with 15 participants using an ethanol containing mouthwash (21.6% ethanol) daily for twelve or thirteen days (gargling for 30 seconds four times a day)[23]. In a second study the authors investigated 15 participants with intense (24-100 times per day with 1.75 ml) use of hand sanitizer (60% ethanol) for twelve or thirteen days[36]. None of the participants of the two studies reached PEth > 20 ng/ml. Two participants of the hand sanitizer study exceeded 10 ng/ml though (11 ng/ml and 13 ng/ml) measured on the last study day. After intense use of mouthwash one participant had a PEth 16:0/18:1 concentration of 12 ng/ml on day 13, after the concentration being 4 ng/ml on the sampling day seven days prior. Those findings contradict application of 10 ng/ml as a cut-off, although in the presented study 51% of the participants who consumed a total of 60 g of alcohol over three days, did not exceed 10 ng/ml. In addition, the usage of ethanol-containing hygiene products in the studies of Reisfield was very intense. For a cut-off of 20 ng/ml there have been no reports of clinical false positive cases and it has been dismissed that incidental alcohol exposure can raise PEth concentration > 20 ng/ml[36,37]. Stöth et al. recently conducted a study in which six women consumed between 13.8 and 30.1 g and six men between 20.0 and 45.3 of alcohol in a single drinking event[38]. In multiple subsequent blood samplings and analyses PEth 16:0/18:1 and 16:0/18:2 never exceeded 20 ng/ml.

Sensitivity of this cut-off was only 7% in the presented study for consumption of 20 g on three days. The cut-off 35 ng/ml seems to be not applicable for detecting low to medium consumption amounts, as it was exceeded by only one participant after consuming 20 g of alcohol on three days. It would be interesting to investigate a longer time span than three days to see if continuous uptake of low amounts of alcohol elevates PEth >35 ng/ml in more individuals or if elimination and synthesis rate balance each other out. Results of the presented study revealed that a low cut-off of

10 ng/ml yields by far best sensitivity, with almost 50% for PEth 16:0/18:1, which is the most tested homologue today.

Evaluation of the participants that came for blood samplings for three additional days while not consuming any alcohol after the drinking phase, revealed that sensitivity (cut-off 10 ng/ml) was 11% and 23% lower on the third day (E3) compared to the first day (E1) after abstinence onset for PEth 16:0/18:1 and 16:0/18:2, respectively. This shows the potentially short detection time of PEth for consumption of low amounts of alcohol after consumption is stopped. A limitation of the study is that PEth was tested during an elimination phase of only three days, so no statement about the time span in which PEth could be detected can be given. But the information from this study is in accordance with results after single consumption of higher amounts of alcohol (amounts to yield 1 ‰), in which PEth 16:0/18:1 was detectable (>10 ng/ml) for three to twelve days and PEth 16:0/18:2 for one to eight days.

Again, as already reported in manifold[14,16,39,40] this study highlights the variabilities in interindividual synthesis rates of PEth. That increases difficulty to establish a set cut-off and finalize the growing consensus of judging values below a certain cut-off as complying with abstinence or uptake of minimal alcohol amounts[16,17].

It needs to be kept in mind that when applying a certain cut-off, PEth can still be positive depending on the consumption behavior before the sampling. In this study it took several weeks (more than seven) for some participants to reach concentrations < 10 ng/ml or <LOD, although they were in the moderate range with their self-reported alcohol consumption and had concentrations between 23-60 ng/ml at the beginning of the abstinence phase. Such long elimination times in some individuals has previously been reported[8,9,11]. This shows that individuals claiming abstinence of several weeks should not be doubted due to PEth > cut-off. Unfortunately, two of the participants whose PEth concentration did not reach < 10 ng/ml after seven weeks of abstinence withdrew from the study so the elimination could not be observed further. Van Uytfanghe et al. recently presented a model to verify abstinence, even when PEth 16:0/18:1 concentration is > cut-off (applied 20 ng/ml)[16].

4.2 Homologues

In this study the two most abundant homologues were quantified[6]. It has been published by Javors et al. [41] and Hill-Kapturczak et al.[42] that PEth 16:0/18:2 synthesis rate is higher and half-life shorter than of PEth 16:0/18:1. In their drinking studies mean PEth 16:0/18:2 concentration exceeded PEth 16:0/18:1 concentration directly after drinking alcohol until about two days after the drinking event, when it fell below PEth 16:0/18:1 concentration[41] and mean AUC was higher for PEth 16:0/18:2 than 16:0/18:1[41,42]. The findings of the presented study are in accordance with that, as mean concentration between A1 and A2 differed by more for PEth 16:0/18:2 (3.1 ng/ml) than

16:0/18:1 (1.5 ng/ml). Decrease of mean concentration from E1 to E3 of PEth 16:0/18:2 (3.4 ng/ml) was also higher than of PEth 16:0/18:1 (1.5 ng/ml). Furthermore, while only two samples showed concentrations of PEth 16:0/18:2 > 16:0/18:1 before the drinking phase, it were > 80% on the first and >90% on the second consumption day, which also highlights the difference in synthesis rates of the two homologues. Schröck et al. on the other hand found higher PEth 16:0/18:1 than PEth 16:0/18:2 concentrations in most participants of a study with controlled alcohol consumption (amounts to reach a blood alcohol concentration (bac) of 1‰) at a single drinking event[11]. In a study with patients with liver disease that was carried out by our working group, indications were found that a smaller ratio of PEth 16:0/18:2 to 16:0/18:1 are found in patients whose last consumption was during the week before the sampling instead of more than a week before the sampling[8]. The findings in this study support this theory further. Looking at the ratios of PEth 16:0/18:1 to 16:0/18:2 could also be helpful for judging if rather low (but > cut-off) concentrations are caused by recent consumption of alcohol or by a drinking event that lies further back with high consumption amounts, with multiple half times having passed. Furthermore, sensitivity of PEth 16:0/18:2 was higher for alcohol uptake of 20 g on every day after consumption and every cut-off for up to 30% compared to PEth 16:0/18:1. This leads us to the conclusion, that it will be useful to quantify the two homologues in abstinence testing and drinking monitoring to increase sensitivity and to potentially provide information about the onset of abstinence. This is in accordance with the previously mentioned findings of Hill-Kapturczak et al[42].

It needs to be noted that currently there are no commercially available external quality controls or proficiency testing that include quantification of other homologues than PEth 16:0/18:1, today.

4.3 Sexes

As expected, women exceeded the applied cut-offs more often than men after consumption of 20 g of ethanol on day two and three. Due to the averagely higher body fat women have a lower distribution volume for ethanol which leads to higher bac after consumption of equal amounts of alcohol compared to men resulting in higher PEth concentrations[4]. Additionally, body weight of woman is averagely lower. The number of samples from men that were quantifiable was only six (on A1) and eight (on A2 and A3), which should be kept in mind when evaluating the statistical results.

Several studies in collectives of patients with liver disease[1], in detoxification clinics[44] and patients with unhealthy alcohol use[40], sex was reported not to influence the diagnostic performance of PEth 16:0/18:1. In Hahn et al.'s study PEth sensitivity was calculated against score of the Audit-C, which implemented different cut-offs for men and women, thus excluding the sex difference[40]. In the study previously carried out by our working group in a collective of patients with liver disease, PEth 16:0/18:1's sensitivity did not differ between sexes, but PEth 16:0/18:2's did[8]. Possibly the overall

higher consumption amount compared to the low amounts in this current study influenced the incongruent results concerning PEth 16:0/18:1. The retrospectively self-reported consumption amount could also have biased results, as described in the original work[8]. In this controlled drinking experiment it was decided consciously to have all participants consume the same amount of alcohol (contained in 500 ml beer) to reference results to the absolute amount of consumed alcohol instead of an alcohol dose per kilogram bodyweight. During abstinent testing in practice, subjects with positive PEth values often state the justification: I only drank one beer. Furthermore, sensitivity differences between the sexes could be evaluated. Sensitivity in authentic patients undergoing PEth monitoring might not necessarily differ between the sexes in real-life scenarios, due to the averagely higher alcohol consumption amount of men[45].

4.4 BMI and body weight

In this study the influence of BMI on synthesis and elimination of PEth 16:0/18:1 and 16:0/18:2 was found to be of minor importance. For the latter homologue a significant difference in concentration was calculated after three days of consumption of 20 g of ethanol between NW and OW participants. This seems to potentially be caused by the uneven distribution of women and men in the compared groups with BMI above and below 25 kg/m^2, as no significant difference was calculated when looking at the sexes separately. The correlation between BMI and PEth concentrations on A3 was not significantly found if evaluating the sexes separately. Body weight on the other hand also shows a correlation in only the male participants to PEth 16:0/18:2, so it could be a stronger impacting factor than BMI. This should further be investigated in studies with controlled drinking amounts.

In their individual participant data meta-analysis, Hahn et al. reported that BMI influences PEth sensitivity[40]. To our knowledge no studies that included controlled consumption of small amounts of alcohol were part of that analysis, so more data concerning this objective, how greatly PEth concentrations after certain alcohol consumption, are influenced by BMI and body weight are needed to extend findings of the presented study.

5. Conclusion

Consumption of one liter of an 'alcohol-free' beverage on three consecutive evenings did not elevate PEth 16:0/18:1 and 16:0/18:2 concentration. For consumption of small amounts of EtOH the low cut-off of 10 ng/ml of PEth was by far more sensitive than 20 or 35 ng/ml. Still, it only detected 50% of the participants as positive on the day after three consumption events of 20 g alcohol. Therefore, leaving half of the participants who consumed alcohol undetected even directly on the day after consumption. Consequently, in settings in which consumption of small amounts of alcohol needs to be detected it could be recommended to apply 10 ng/ml as cut-off. Further studies

investigating influence of incidental alcohol uptake are needed. The quantification of the two homologues 16:0/18:1 and 16:0/18:2 together increases sensitivity and might have the potential to make conclusions about recency of alcohol uptake.

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7. Discussion

While in chapter 5 and 6 of this work, thorough and detailed discussion are already presented, this chapter is intended to provide a summarized overarching discussion. In addition, future perspectives and outlooks are addressed.

The analytical method that was validated in the scope of this work enables analysis of six different homologues of the direct alcohol biomarker PEth. The method was successfully established into routine analysis in the Toxicology of the Department of Legal Medicine in Hamburg, where it is applied as an alcohol biomarker in abstinence testing and alcohol consumption monitoring of liver transplant candidates and patients with liver disease, in authority ordered alcohol monitoring in context of custody or probation affairs and consumption monitoring of patients of forensic psychiatric clinics after temporary releases. Chromatographic separation of the analytes was achieved. The developed method was henceforth used in all studies presented in this work. While a calibration range of 10-1000 ng/ml was sufficient for the study regarding abstinence testing in a cohort of patients with liver disease, it needed to be extended immensely (to 6000 ng/ml) for the study with a cohort of homeless individuals. The run time of seven minutes makes the method applicable for routine analysis as it allows satisfactory sample throughput.

While validation of the method was carried out according to current international guidelines, the use of DBS demanded for special investigation of hct. These were directed according to the proposals for DBS assays by the Official International Association for Therapeutic Drug Monitoring and Clinical Toxicology Guideline [115]. Blood with three different hct was therefore used for evaluating recovery, matrix effects, precision and accuracy. Validating the method regarding different hct levels (about 20 %, 40 % and 60 %) ruled out that hct influences the analytical aspect of PEth measurement, especially extraction and influences from the matrix. Cutting out the whole spot that has been volumetrically generated, instead of punching out parts of it, allows to neglect further inspection of influence that hct has on the DBS creation (e.g., uneven distribution on the filter paper due to varying viscosity, volcano effect). These analytical aspects are independent from the physiological effects hct can have on PEth measurement as a marker for alcohol consumption. Since PEth synthesis and storage occurs in red blood cells, an impact of hct can be assumed but investigation of hct effects on PEth concentration on a physiological level are difficult as there are numerous different physiological factors which can be poorly standardized. One would need one patient to consume the exact same amount of alcohol under a similar setting (consumption time and duration, same kind of beverage, same food intake etc.), while having differing hct levels. As hct is physiologically set to narrow variations [37] this setting would be almost impossible to establish. Additionally, when comparing PEth concentrations of groups with differing hct level, after

consumption of alcohol under the exact same setting, influence of all other possible physiological effects would not be possible to exclude during analysis which would make assumption of valid statements of hct effect on PEth levels rather vague. Particularly problematic are the general interindividual differences in PEth synthesis and elimination, for example caused by individually varying occurrence of the involved enzymes or the absorption rate that have been described in different studies [81,116].

A common problem in quantitative analysis is the difference in authentic and fortified samples. For quantitative analysis a native standard of the analyte is externally added to the analyte- free matrix to create a calibration curve. For PEth analysis with the established method this means the standards are mixed with whole blood and DBS are created from this after some incubation time. The externally added PEth is most likely not incorporated into the erythrocytes like the PEth that is synthesized in-vivo. Therefore, recovery might be unequal between authentic and spiked samples. This has also been interpreted as a reason for the divergent stability of authentic and spiked PEth samples [102,113].

In PEth molecules the relative position of the fatty acyl chain on the glycerol backbone can vary between sn-2- and sn-3-position. Depending on the constitution altering fragmentation patterns may occur, due to the sn-2-position being a preferred fragmentation site. It has been described that majority of the analyzed diacyl glycerophospholipids of biological origin show a significantly higher regio-isomeric purity than the synthetic standards [117]. Luginbühl et al. investigated the regio-isomeric purity of three different reference materials (the ones from Echelon, used in this work were not included) and a difference in mean concentrations was found if calibration was obtained with a regio-isomerically pure versus an impure standard [118]. While in the study it is stated that it remains unclear if the overestimation of concentrations is caused by the constitution of acyl chains on sn-positions or if it can be judged as expected variation when comparing reference material from different manufacturers, this is an aspect that research should be further devoted to.

The study in 6.1 addressed stability testing of the six PEth homologues included in the analytical method and it was concluded that sample transportation and storage time should not exceed one day, as in some samples PEth concentration had decreased significantly after storage until the day after blood sampling.

Sampling of capillary blood is a suitable collection technique in different settings, as it is less invasive and does not call for a staff member specially trained for venous blood sampling. An additional benefit of the procedure in context of PEth analysis is the improvement of stability and elimination of post-sampling synthesis, as drying on filter paper defies these effects. Kummer et al. thoroughly compared PEth concentration measured from DBS created from venous and capillary blood and

found no marked deviations [119]. Results of our laboratory are in accordance with that, as in DBS generated from capillary blood taken from the fingertip with a micro pipette (20 µL) and DBS generated from venous blood sampled at the same time point (n=49) no significant deviations were found and highest percental difference between PEth concentrations obtained from the different DBS types was 19% (results not shown). The sampling from the fingertip makes the application of PEth easier and extends the areas in which it can be applied.

As multiple different extraction methods have been presented for PEth analysis from DBS and some laboratories measure PEth from whole blood, external quality controls and participation in proficiency testing are of outmost importance to secure the comparability of different methods for PEth quantification. However, currently available certified quality controls are containing PEth 16:0/18:1 only, as it is the most analyzed and best researched homologue. While it has been found that it is one of the most abundant homologues some time ago [78,80], this work shows the advantage of measuring multiple PEth homologues. Especially the simultaneous quantification of PEth 16:0/18:1 and the second most abundant homologue PEth 16:0/18:2 has proven to be advantageous in some contexts, beyond functioning as a plausibility check for PEth 16:0/18:1. An inclusion of this homologue into inter-laboratory assessments is desirable.

The drinking study with participants who started off with PEth concentrations <LOQ, principally revealed the additional benefit of analyzing PEth 16:0/18:2 as well as PEth 16:0/18:1, as sensitivity of the latter was between 15-20% lower than that of PEth 16:0/18:2 on every day after the consumption of 20 g alcohol (cut-off 10 ng/ml). In the experiment PEth 16:0/18:2 concentration was higher than of PEth 16:0/18:1 in most samples after the first, second and third intake of 20 g of alcohol with 85 %, 97 % and 90 %, respectively. In all other cohorts investigated in this work, PEth 16:0/18:1 exceeded PEth 16:0/18:2 in the majority of samples with 80 % of the homeless individuals and 84 % of the patients with liver disease. This shows that varying distribution of homologues can occur depending on the drinking behavior and time point of blood sampling, due to differing synthesis and elimination rates.

The occurrence of the homologues of the precursor molecule PC surely also influences the PEth homologues' distribution between individuals. Influence of nutrition has also been suspected [82]. Therefore, further studies that analyze more than one or two homologues in different cohorts need to be carried out, ideally while documenting drinking behavior, to investigate if using homologue-ratios can be helpful for specifying certain drinking patterns, time of the drinking event or abstinence onset, as suggested by the results of 5.2 and 6.2. Especially the question, if positive PEth concentrations are a consequence of an excessive consumption that lies further back (up to several weeks) or a moderate or low more recent consumption could be approached.

In 5.2 and 5.3 PEth performed well in comparison to other alcohol biomarkers. This is in accordance with previous studies that compared the biomarkers [63]. At the beginning of this work (mid 2017) PEth was very rarely included in routine biomarker analysis in Germany. Since then, it has gained growing recognition, especially due to its promising diagnostic performance. Even though PEth was the biomarker that was positive most often in several studies, beside 5.2 and 6.2, combination of different alcohol biomarkers is state of the art today [63,106]. PEth analysis complements analysis of Etg in urine and in hair, as uEtg detects small alcohol intake during the days before the appointment and hEtg can be used to examine consumption during the previous three month.

Urine for Etg analysis is essentially sampled under visual control, to obstruct tampering of the sample by handing in foreign or fake urine or adding substances that destroy Etg in the sample by the tested person. This might be very uncomfortable for the subject, potentially leading to miction dysfunction and the inability to urinate. Furthermore, urine can quite easily be influenced by dilution via consumption of large amounts of water before the appointment. Main problem with hair analysis is sample availability, as hair needs to be of sufficient length and amount (especially difficult in bald men) and imperatively without any chemical or cosmetic treatment (bleaching, coloring, perming, thermal straightening). Additionally, subjects often refuse hair sampling due to the fear of negative impact on their appearance or due to religious conviction (Sikhs). Consequently, sample availability is not always a given, unlike for PEth analysis. Furthermore, hEtg concentration can be influenced for example by reduced hair growth or kidney function impairment [77].

It needs to be noted that, other than in the studies in this work (5.1-5.3, 6.2), routine monitoring of PEth in authentic patients is applied over a longer period of time and sampling can be carried out every one to four weeks in practice. This allows the monitoring of adapted drinking behavior as the delta between different sampling dates, which is unaffected by potential physiological pharmacokinetic factors, can be evaluated.

Another objective of this work was to contribute to the PEth cut-off discussion. So far it only concerns PEth 16:0/18:1, as that is the most commonly tested homologue. Abstinence evaluation results suggest that 35 ng/ml as cut-off is a lot less suitable than 20 ng/ml or 10 ng/ml. As discussed in 5.2 and 6.2 only little research has been presented on PEth caused by incidental alcohol intake. Still, as the aim of supporting and finding adequate therapy for patients requires the detection of any ongoing alcohol consumption or relapse, it seems justified to use 10 ng/ml as cut-off, since sensitivity was markedly higher than that of the other applied cut-offs. In the cohort of patients with liver diseases this cut-off of PEth 16:0/18:1 detected 58 % of those who consumed more than two standard drinks (24g) per week during the last month, which is 19 % more than by 20 ng/ml and 29 % more than by 35 ng/ml. After consumption of the amount of alcohol equal to five standard drinks (distributed over three days) PEth 16:0/18:1 was >10 ng/ml in 49 % of the participants and

>20 ng/ml and >35 ng/ml in 7% and 1%, respectively. Meanwhile specificity was not lowered compared to higher cut-offs. The cut-off of 210 ng/ml PEth 16:0/18:1 detected excessive alcohol consumption better in the patients with liver disease (5.2) and in the cohort of homeless individuals than CDT. This is in accordance with results obtained by Arving et al [110] and Neumann et al. [111], that show a distinctly higher sensitivity of PEth than of the other tested alcohol biomarkers sensitivity of PEth. In the patients with liver diseases PEth 16:0/18:1 detected 53% of those who consumed more than two standard drinks per week during the last three months, which is about 16% more than detected by hEtg, and 75% of those who consumed that amount in the week before blood sampling, of which uEtg detected 28%.

Therefore, PEth has proven to be a beneficial biomarker for alcohol consumption with high specificity and acceptable sensitivity for low amounts of alcohol. Further investigations on continuing ingestion of small amounts of alcohol would be desirable to get more information on consumption amounts that produce positive (above cut-off) PEth concentrations in individuals.
Additionally, studies on the development of PEth concentrations over the course of several weeks referred to the alcohol consumed during that time, preferably recorded in a drinking diary, would provide useful information for further interpretation of the delta of PEth concentration between sampling dates.
Furthermore, the method presented in this work (5.1) to quantify six homologues of PEth simultaneously can be used to get more insight into homologue distribution and potential factors contributing to altering homologue patterns between individuals, as little has been published on the kinetics of other homologues but PEth 16:0/18:1 and 16:0/18:2.

All in all, the compilation of investigations on PEth in this work, demonstrates why this alcohol biomarker should have a place in routine analysis for abstinence testing and alcohol consumption monitoring, all while being aware of the shortcomings and acknowledging the need for further research and reports about the physiological impact factors, preanalytical handling and application in different clinical and forensic settings.

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9. Appendix

9.1 List of the used hazardous substances according to GHS

The following reagents and solvents were used and are labeled according to GHS (EC CLP Regulation No. 1272/2008):

Substance	**pictogram**	**H-code**	**P-code**
Acetic acid	GHS02, GHS05	226, 314	280, 301+331+331, 307+310 305+351+338
Acetone	GHS02, GHS07	225, 319, 336	210, 233, 240, 241, 242, 305+351+338
Acetonitrile	GHS02, GHS07	225, 302+312+332, 319	210, 280, 301+312, 303+361+353, 304+340+312, 305+351+338
Chloroform	GHS06, GHS08	302, 315, 319, 331, 336, 351, 361d, 372, 412	201, 273, 301+312+330, 302+352, 403+340+311, 308+311
Dichloro methane	GHS07, GHS08	315, 319,336, 351,	201, 202, 261, 302+352, 305+351+338, 308+313
Ethanol	GHS02, GHS07	225	233, 240, 241, 242, 243,280, 303+361+353, 370+378, 403+235, 502
Formic acid	GHS01, GHS05, GHS06	226, 302, 314, 331	210, 280, 303+361+353, 304+340+310, 305+351+338
n-Hexan	GHS02, GHS08, GHS07, GHS09	225, 304, 315, 336, 361f, 373, 411	202,210, 273,303+361+353,331
Isopropanol	GHS02, GHS07	225, 319, 336	210, 233, 280, 305+351+ 338,
Methanol	GHS02, GHS06, GHS08	225, 301+311+331, 370	210, 260, 280, 301+310, 308+311, 403+233

9.2 AUDIT

9.2.1 AUDIT questionnaire

The Alcohol Use Disorders Identification Test: Self-Report Version

PATIENT: Because alcohol use can affect your health and can interfere with certain medications and treatments, it is important that we ask some questions about your use of alcohol. Your answers will remain confidential so please be honest. Place an X in one box that best describes your answer to each question.

Questions	0	1	2	3	4	
1. How often do you have a drink containing alcohol?	Never	Monthly or less	2-4 times a month	2-3 times a week	4 or more times a week	
2. How many drinks containing alcohol do you have on a typical day when you are drinking?	1 or 2	3 or 4	5 or 6	7 to 9	10 or more	
3. How often do you have six or more drinks on one occasion?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily	
4. How often during the last year have you found that you were not able to stop drinking once you had started?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily	
5. How often during the last year have you failed to do what was normally expected of you because of drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily	
6. How often during the last year have you needed a first drink in the morning to get yourself going after a heavy drinking session?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily	
7. How often during the last year have you had a feeling of guilt or remorse after drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily	
8. How often during the last year have you been unable to remember what happened the night before because of your drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily	
9. Have you or someone else been injured because of your drinking?	No		Yes, but not in the last year		Yes, during the last year	
10. Has a relative, friend, doctor, or other health care worker been concerned about your drinking or suggested you cut down?	No		Yes, but not in the last year		Yes, during the last year	
					Total	

9.2.2 AUDIT-C questionnaire

1. **How often do you have a drink containing alcohol?**
   - ☐ Never
   - ☐ Monthly or less
   - ☐ 2-4 times a month
   - ☐ 2-3 times a week
   - ☐ 4 or more times a week
2. **How many standard drinks containing alcohol do you have on a typical day?**
   - ☐ 1 or 2
   - ☐ 3 to 4
   - ☐ 5 to 6
   - ☐ 7 to 9
   - ☐ 10 or more
3. **How often do you have six or more drinks on one occasion?**
   - ☐ Daily or almost daily
   - ☐ Weekly
   - ☐ Monthly
   - ☐ Less than monthly
   - ☐ Never

9.3. Supporting information

9.3.1 Supporting information for 'Analysis of six different homologues of phosphatidylethanol from dried blood spots using liquid chromatography–tandem mass spectrometry (5.1)'

(originally an excel document)

	16:0/18:2 [ng/ml]	**16:0/18:1 [ng/ml]**	**16:0/20:4 [ng/ml]**	**18:0/18:1 [ng/ml]**	**18:0/18:2 [ng/ml]**	**18:1/18:1 [ng/ml]**	**total PEth (n=6) [ng/mL]**
P1	497,6	1597,2	162,5	300,1	166,8	234,9	2959,1
P2	687,9	1140,5	78,7	289,4	504,6	108,7	2809,8
P3	489,4	1184,4	51,7	291,5	218,2	444	2679,2
P4	299,9	764,4	79,9	277,2	177,6	139,1	1738,1
P5	431,6	648,6	89,6	178,4	243,2	114,6	1706
P6	161,2	772,2	20	256,2	121	157,4	1488
P7	400,8	543,1	74,9	152,6	193,3	69,1	1433,8
P8	311,1	696,1	73,9	153,7	117,3	32,9	1385
P9	340,5	477,6	60,8	133,7	239	49,9	1301,5
P10	417,5	473,1	29,6	82,6	110,1	44,3	1157,2
P11	234,7	426	33,3	72,8	55,8	68,2	890,8
P12	177,1	350,4	37,6	123	76,6	42,5	807,2
P13	179,8	329,6	81,1	73,2	57,8	44,8	766,3
P14	205,5	274,6	36,1	64,9	67,5	27,9	676,5
P15	172,9	193,5	37,1	62	112,3	20,9	598,7
P16	169,6	229,9	41,3	57,2	72,1	24	594,1
P17	105	252,5	26,7	56,5	95,4	24,8	560,9

P18	118,7	238,6	46,6	55,3	43,3	32,9	535,4
P19	60,9	178,9	9,2	79,4	78,2	15,5	422,1
P20	76,2	183,4	42,3	53,4	43,9	17,2	416,4
P21	93,4	146,3	21,8	60,4	72,4	17,5	411,8
P22	60,5	183,1	9	53,3	40,4	20,3	373,6
P23	102,3	115,5	22,5	37	53,6	7	337,9
P24	116,4	99	14,9	19,1	56,4	17,5	323,3
P25	54,4	115,4	11,9	37	35,1	18,1	271,9
P26	48,8	120,7	16,7	17,1	17,2	14,3	234,8
P27	62,9	61,2	15,9	21	20,3	26	207,3
P28	54,8	69,5	12,2	21,6	28,1	6,8	193
P29	50,9	53,6	17,2	17,2	21,2	7,1	167,2
P30	37,9	39,1	29,5	17,7	16,9	15,5	156,6
P31	33,4	40,1	11	19,2	24,8	10,7	139,2
P32	42,2	33,1	16,7	15,7	21,4	9,7	138,8
P33	39,3	38,4	16	15,1	22,6	<LOQ	131,4
P34	28,9	53	13,6	<LOQ	12,2	10,3	124,8
P35	25,9	28,7	15,9	14	12,5	8,5	105,5
P36	25,8	32,1	11,1	12,9	21,2	<LOQ	103,1
P37	25,5	36,3	8,7	11,7	18,1	<LOQ	100,3
P38	15,7	28,1	7,1	9,4	9,4	14	83,7
P39	27,8	25,4	15,7	8,5	<LOQ	<LOQ	78,2
P40	21,5	32,2	7,7	10,1	<LOQ	<LOQ	71,5
P41	22,2	19	<LOQ	7,9	13,8	<LOQ	68,4
P42	15,5	32,7	<LOQ	8,6	<LOQ	<LOQ	56,8
P43	16,3	23,5	14,8	<LOQ	<LOQ	<LOQ	60,7
P44	20	16,5	9,7	7,4	<LOQ	<LOQ	58,2
P45	16,6	13,7	12,9	8,7	<LOQ	<LOQ	54,7
P46	18,3	20,6	8,9	<LOQ	<LOQ	<LOQ	47,8
P47	11,8	18,8	9	7,8	<LOQ	<LOQ	47,4
P48	13	14,4	10,2	8,5	<LOQ	<LOQ	46,1
P49	20,7	21	<LOQ	<LOQ	<LOQ	<LOQ	41,7
P50	16	13,7	9,1	<LOQ	<LOQ	<LOQ	38,8
P51	15,2	12	<LOQ	8	<LOQ	<LOQ	35,2
P52	7,4	19,1	<LOQ	<LOQ	<LOQ	<LOQ	26,5
P53	10,7	14,5	<LOQ	<LOQ	<LOQ	<LOQ	25,2
P54	8,9	13,5	<LOQ	<LOQ	<LOQ	<LOQ	22,4
P55	9,6	12,5	<LOQ	<LOQ	<LOQ	<LOQ	22,1
P56	8,4	11,9	<LOQ	<LOQ	<LOQ	<LOQ	20,3
P57	6,2	12,6	<LOQ	<LOQ	<LOQ	<LOQ	18,6

9.3.2 Supporting information for 'Phosphatidylethanol in patients with liver diseases of different etiologies: Analysis of six homologues and comparison with other alcohol markers' (5.2)

Tab. S1: hEtG and PEth concentrations and self-reported alcohol consumption of cases in which PEth and hEtg were both positive (/ means no statement was made)

hEtg [pg/mg]	PEth 16:0/18:1 [ng/ml]	Consumption last three months [g/week]	Consumption last four weeks [g/week]	Consumption last week [g/week]
50	44	118	336	0
29	24	60	0	0
80	764	216	216	84
53	13	/	/	/
155	772	780	492	0
139	183	216	216	0
114	115	60	24	24
111	473	216	216	132
18	62	/	/	/
84	426	/	492	12
292	176	1788	1788	0
17	649	/	/	/
26	140	/	/	/

Tab. S2: CDT and PEth concentrations and self-reported alcohol consumption in cases with CDT ≥1.7% (/ means no statement was made)

CDT [%]	PEth 16:0/18:1 [ng/ml]	Consumption last three months [g/week]	Consumption last four weeks [g/week]	Consumption last week [g/week]
2.4	1141	780	/	/
1.9	330	1236	1232	288
2.7	473	216	216	132
3.2	183	216	216	0
5.2	221	/	/	/

9.4 Validation protocols

Validierungsprotokoll

Seite:	2 von 7	**Institution:**	
Gültig ab:		**Methode:**	

1. Arbeitsbereich und Kalibrationsmodell

1.1 TARGET — **Messsignal:** 281 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	14.793	60.202	122	266	622	1064	1255			
MESSUNG 2	14.488	64.053	128	269	637	1053	1256			
MESSUNG 3	15.217	63.856	126	267	634	1076	1256			
MESSUNG 4	15.92	67.598	126	265	633	1065	1261			
MESSUNG 5	15.222	62.218	127	266	621	1080	1274			
MESSUNG 6	13.761	60.039	116	263	600	1078	1245			
Mittelwert	14.90016667	62.99433333	124	266	624	1069	1258			
SD	0.738	2.8352	4.4081	2.2036	13.59555086	10.18281764	9.3146			
Varianz	0.5446	8.0382	19.4314316	4.8559	185	104	86.7624547			
Werte	6.0	6.0	6.0	6.0	6.0	6.0	6.0			
Ausreisser-Test nach Grubbs										
Extremwert	13.761	67.598	116	263	600	1053	1274			
Prüfwert	1.544	1.624	1.789	1.533	1.795	1.563	1.701			
Signifikanz 95%										
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Signifikanz 99%										
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.4529	**Prüfwert**	0.27	**a**	1,28235	**A**	-0.00006
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	1,21112	**B**	1.34129
Bestanden?	ja	**Bestanden?**	ja	**R**	0,9991	**C**	-4.399149
				Rest-SD	24.0116	**R**	0.9991
						Rest-SD	25.979731

1.2 QUALIFIER — **Messsignal:** 255 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	6.315	19.726	38.369	78.216	189.238	292.477	371.657			
MESSUNG 2	6.292	20.164	37.536	80.988	188.775	291.111	370.188			
MESSUNG 3	6.216	18.643	36.429	79.244	186.753	292.908	369.243			
MESSUNG 4	6.164	19.319	37.756	77.562	185.590	294.080	369.850			
MESSUNG 5	6.250	20.469	37.370	78.801	188.283	294.298	371.013			
MESSUNG 6	6.242	18.936	36.946	75.051	184.264	295.590	372.623			
Mittelwert	6.247	19.54283333	37.401	78.31033333	187	293	371			
SD	0.054	0.708	0.668	1.974	1.96	1.575	1.249			
Varianz	0.003	0.501	0.447	3.895	3.841	2.48	1.559			
Werte	6	6	6	6	6	6	6			
Extremwert	6.164	20.469	36.429	75.051	184.264	291.111	372.623			
Prüfwert	1.530	1.308	1.454	1.652	1.473	1.460	1.490			
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.3061	**Prüfwert**	0.04	**a**	0.367283	**A**	-0.000002
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	2,26432	**B**	0.369325
Bestanden?	ja	**Linear?**	ja	**R**	0,9999	**C**	2.070003
				Rest-SD	2.0357	**R**	0.9999
						Rest-SD	2.2639

Validierungsprotokoll

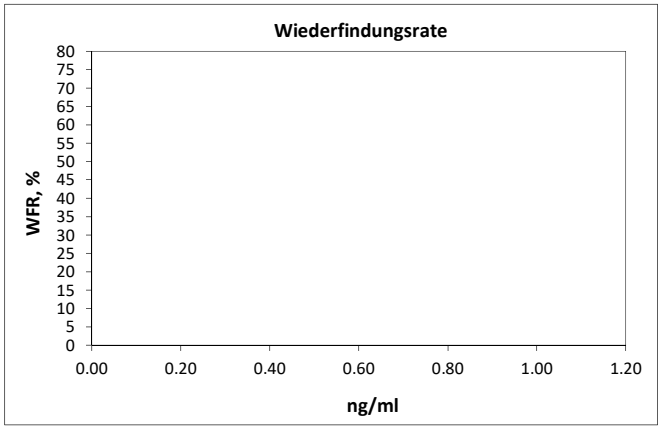
Seite:	3 von 7	**Institution:**	
Gültig ab:		**Methode:**	

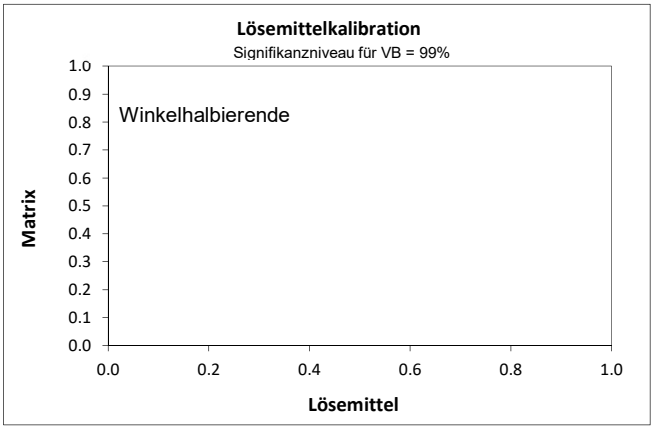
1.3 LÖSEMITTELKALIBRATION

Signifikanz	%
Konzentration	
Lösemittel (xo)	
Matrix (xm)	

WFR gemessen	
WFR berechnet	

Wiederfindungsfunktion **xm = ao * xo + bo**	**Ausreißer-F-Test**	**Linearitäts-Test**	**Varianzenhomogenitäts-F-Test**
ao	**Kalibrator Nr.**	**Prüfwert**	**RSD Grundkalibration**
bo	**Prüfwert**	**Kritischer-Wert**	**RSD Wiederfindung**
R	**Kritischer-Wert**	**Bestanden?**	**Prüfwert**
Rest-SD	**Ausreisser?**		**Kritischer-Wert**
			Bestanden?





2. Genauigkeit

2.1. Level 1

QC-Sollwert: 40 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	38.973	40.104	39.958	39.597	38.332	38.142	35.438	37.512	36.58	
2	41.694	39.269	37.404	40.523	37.701	41.317	36.987	40.678	38.029	
3	40.514	41.672	40.634		37.464	40.333	37.215	37.289	38.961	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	40.39	40.35	39.33	40.06	37.83	39.93	36.55	38.49	37.86	
BIAS, %	1.0	0.9	-1.7	0.2	-5.4	-0.2	-8.6	-3.8	-5.4	
SD	1.36	1.22	1.70	0.65	0.45	1.63	0.97	1.90	1.20	
RSD, %	3.4	3.0	4.3	1.6	1.2	4.1	2.6	4.9	3.2	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	38.94	**SD**	0.16	**SD**	1.41
SD	1.72	**RSD, %**	0.4	**RSD, %**	3.6
RSD, %	4.4				

Richtigkeit		**95%-Intervall**	OK
Abw.	-1.06	**Faktor**	2.43
Bias, %	-2.7	**ß-Toleranz**	34,46944 bis 41,32877
		Prüfbereich (30%)	28,0 bis 52,0
		Prüfbereich (40%)	24,0 bis 56,0

Validierungsprotokoll

Seite:	4 von 7	**Institution:**	
Gültig ab:		**Methode:**	

2.2. Level 2

QC-Sollwert: 400 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	399.7	435.3	421.3	421.5	407.3	411.9	417.8	407.8	425.5	
2	417.6	426.1	397.9	438.4	417.2	394.8	425.8	413.5	428.5	
3	385.4	432.2	407.9		412.2	381.8	427.4	429.8	431.4	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	400.93	431.22	409.01	429.93	412.26	396.17	423.68	417.01	428.47	
BIAS, %	0.2	7.8	2.3	7.5	3.1	-1.0	5.9	4.3	7.1	
SD	16.14	4.71	11.75	11.98	4.96	15.06	5.15	11.44	2.98	
RSD, %	4.0	1.1	2.9	2.8	1.2	3.8	1.2	2.7	0.7	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	416.00	**SD**	2.91	**SD**	13.03781633
SD	14.96536884	**RSD, %**	0.7	**RSD, %**	3.1
RSD, %	3.6				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	16.00	**Faktor**	2.429
Bias, %	4.0	**ß-Toleranz**	400,97291 bis 464,31461
		Prüfbereich (30%)	280,0 bis 520,0

2.3. Level 3

QC-Sollwert: 700.0 **Einheit:** ng/ml

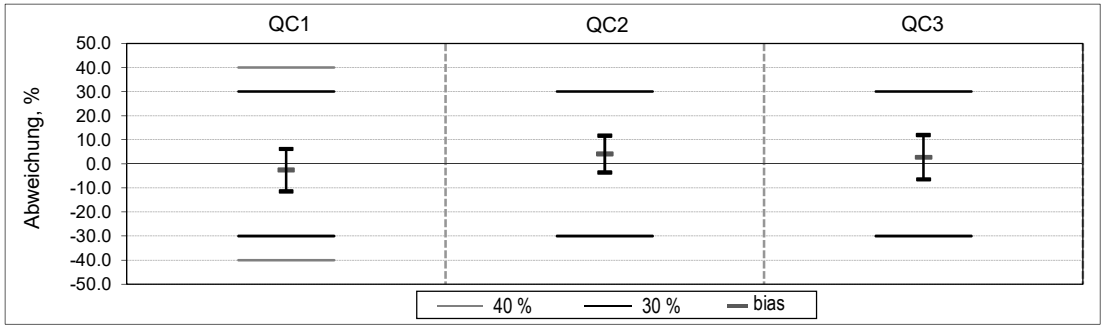
MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	653.8	701.7	658.8	708.1	743.7	690.2	727.6	745.8	776.6	
2	685.3	709.3	767.6	715.3	781.5	658.1	748.0	757.1	761.5	
3	683.5	736.7	670.8		699.1	710.0	721.1	733.8	754.7	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	674.2	715.9	699.1	711.7	741.4	686.1	732.3	745.6	764.3	
BIAS, %	-3.7	2.3	-0.1	1.7	5.9	-2.0	4.6	6.5	9.2	
SD	17.71	18.40	59.64	5.10	41.27	26.17	14.06	11.64	11.16	
RSD, %	2.6	2.6	8.5	0.7	5.6	3.8	1.9	1.6	1.5	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	719.23	**SD**	1.24	**SD**	27.17
SD	37.43	**RSD, %**	0.2	**RSD, %**	3.8
RSD, %	5.2				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	19.22723077	**Faktor**	2.431
Bias, %	2.7	**ß-Toleranz**	672,95125 bis 805,01392
		Prüfbereich (30%)	490,0 bis 910,0

Validierungsprotokoll

Zusammenfassung 95%-Intervall



Zusammenfassung	QC1	QC2	QC3
ß-Toleranz	34,46944 bis 41,32877	400,97291 bis 464,31461	672,95125 bis 805,01392
%	- 11,47 bis 6,15	-3,61 bis 11,61	-6,4 bis 11,9
Prüfbereich (30%)	28,0 bis 52,0	280,0 bis 520,0	490,0 bis 910,0
Prüfbereich (40%)	24,0 bis 56,0		

3. Grenzwerte

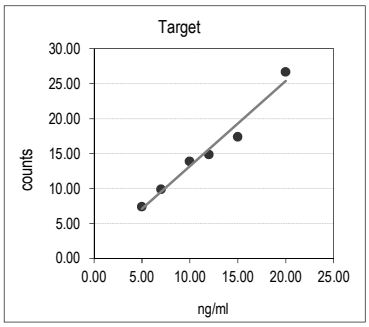
3.1 Bestimung nach DIN 32645

	Kalibrator	Target	Qualifier
Einheit			
1	5.0	7.400	2.600
2	7.0	9.900	3.300
3	10.0	13.900	4.700
4	12	14.900	5.200
5	15	17.400	6.100
6	20	26.700	9.300
7			
8			
9			
10			

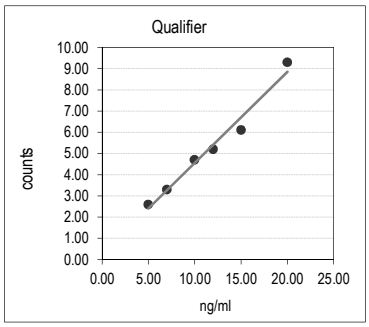
	6	6
Signifikanz	99	99
k-Wert	3	3
Ausreißer-F-Test		
Kalibrator Nr.	5	5
Prüfwert	7.9	7.78
Krit.-Wert 99%	34.11	34.11
Ausreißer	nein	nein
Linearitäts-Mandel-Test		
Prüfwert	1.98	3.35
Krit.-Wert 99%	34.11	34.11
Bestanden?	ja	ja

a	1.216054	0.429431
b	1.048718	0.261538
R	0.9855	0.9881
Rest-SD	1.28	0.4094

	Grenzwerte	
Nachweisgrenze	4.35	3.94
Erfassungsgrenze	8.71	7.88
Bestimmungsgrenze	8.60	7.61



Lineare Kalibration



Lineare Kalibration

Validierungsprotokoll

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Gültig ab:		**Methode:**	

3.2 Bestimmung mittels Alternativmethode

3.2.1 Nachweisgrenze aus Signal/Rauschverhältnis

	Dotiert ng/ml	Target 0	Untergrund 0	Target S/N	Q1 0	Untergrund 0	Q1 S/N	Q2 0	Untergrund 0	Q2 S/N
1										
2										
3										
4										
5										
6										
			NG-1	0.0		NG-2	0.0		NG-3	0.0

Nachweisgrenze :	**0.0**

3.2.2 Bestimmungsgrenze

Vorgabe	ng/ml

MESSUNG	Konz.
1	
2	
3	
4	
5	
6	

Wiederholpräzision		Bias	
Mittelwert		Bias	
SD		Bias, %	
RSD, %			
Krit. Wert, %	**20**	**Krit. Wert, %**	**20**

Beurteilung	**---**	Kriterium: RSD und bias<20%

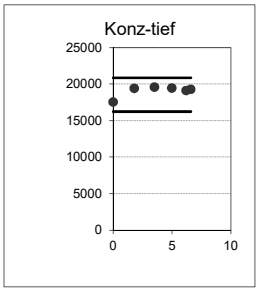
4. WIEDERFINDUNG

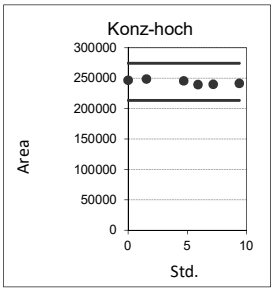
4.1 WIEDERFINDUNG

	QC-untere		QC-obere	
Konzentration	**ng/ml**		**ng/ml**	
	Lösemittel	**Matrix**	**Lösemittel**	**Matrix**
MESSUNG				
1				
2				
3				
4				
5				
6				
Mittelwert				
SD				
RSD, %				
N (Werte)				
Wiederfindung,%				
RSD, %				

4.2 STABILITAT

	Konz-tief Zeit	QC-Pool	Konz-hoch Zeit	QC-Pool
MESSUNG	0.00	17552	0.00	246484
	1.80	19429	1.55	248521
	3.50	19571	4.70	245565
	5.00	19449	5.90	239273
	6.20	19138	7.20	239940
	6.60	19285	9.40	241426
Werte	6		6	
min. Wert	17552		239273	
max. Wert	19571		248521	
Spanne	2019		9248	
Spanne, %	10.3	%	3.7	%
Prüfwert	25.0	%	25.0	%
Steigung	0.0021		-0.0007	
Abschnitt	-36.6929		183.0175	
R	0.62268		-0.79692	
Beurteilung	**OK**		**OK**	





Kriterium: Spanne < Prüfwert

Validierungsprotokoll

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Gültig ab:		**Methode:**	

4.3 MATRIXEFFEKTE - LC/MS/(MS)

	QC1	40		QC2	700	
	Reinsubstanz	**Extrakt**	**Matrix**	**Reinsubstanz**	**Extrakt**	**Matrix**
MESSUNG	201152.0	183879.0	142103.0	2903392.0	3083278.0	2187063.0
	199923.0	197657.0	153033.0	2796618.0	2979882.0	1711529.0
	177247.0	189052.0	160016.0	2785820.0	3144947.0	3288296.0
	180911.0	195075.0	144894.0	2828688.0	2973562.0	2550666.0
	190916.0	196400.0	152396.0	2830708.0	2973562.0	2755022.0
	188624.0	180415.0	167075.0	2852826.0	2563254.0	2351467.0
Mittelwert	189795.5	190413.0	153252.8	2833008.7	2953080.8	2474007.2
SD	9701.2	7135.3	9292.6	42241.1	203610.5	534160.7
Varianz	94112694.7	50911886.0	86352816.6	1784310484.3	41457229403.4	285327643849.4
Anzahl	6.0	6.0	6.0	6.0	6.0	6.0

Wiederfindung,%	80.5	83.8
RSD, %	6.8	17.0
Matrixeffekt,%	100.3	104.2
RSD, %	6.5	7.6
Beurteilung	Ok	Ok

Kriterium: Recovery >=50%
SD <=25%
Matrixeffekte: 75-125%

Anmerkungen

Validierungsprotokoll

Seite:	2 von 7	**Institution:**	
Gültig ab:		**Methode:**	

1. Arbeitsbereich und Kalibrationsmodell

1.1 TARGET — **Messsignal:** 279 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	6.817	22.306	44.4	91.925	219	348	429			
MESSUNG 2	7.145	22.741	42.989	91.468	219	351	431			
MESSUNG 3	7.168	21.424	43.548	90.78	218	354	434			
MESSUNG 4	6.842	22.506	43.998	92.293	217	349	429			
MESSUNG 5	6.847	22.971	44.858	89.198	219	351	435			
MESSUNG 6	6.99	23.457	43.724	94.226	220	353	436			
Mittelwert	6.968	22.5675	43.9195	91.64833333	219	351	433			
SD	0.1582	0.6876	0.6572	1.669	1.014	2.5226	3.0502			
Varianz	0.025	0.4728	0.432	2.7854	1.0283	6.3635	9.3038			
Werte	6.0	6.0	6.0	6.0	6.0	6.0	6.0			
Ausreisser-Test nach Grubbs										
Extremwert	7.168	21.424	44.858	94.226	217	348	436			
Prüfwert	1.263	1.663	1.428	1.544	1.716	1.388	1.225			
Signifikanz 95%										
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Signifikanz 99%										
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.4558	**Prüfwert**	1.93	**a**	0,43253	**A**	-0.00001
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	2,41043	**B**	0.445073
Bestanden?	ja	**Bestanden?**	ja	**R**	0,9999	**C**	1.216033
				Rest-SD	2.2549	**R**	1.0
						Rest-SD	2.0693

1.2 QUALIFIER — **Messsignal:** 255 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	3.003	9.165	18.126	37.658	88.543	143.024	166.278			
MESSUNG 2	3.374	9.768	16.977	36.939	86.374	135.716	171.416			
MESSUNG 3	3.462	9.231	17.237	35.237	85.217	140.994	167.889			
MESSUNG 4	3.404	9.766	18.129	35.628	86.152	139.566	164.888			
MESSUNG 5	3.002	8.955	17.199	37.362	85.474	134.661	172.335			
MESSUNG 6	3.659	10.397	18.241	36.274	88.022	136.516	169.217			
Mittelwert	3.317	9.547	17.6515	36.51633333	86.63033333	138	169			
SD	0.263	0.532	0.571	0.968	1.358	3.292	2.895			
Varianz	0.069	0.283	0.326	0.936	1.845	10.84044257	8.381			
Werte	6	6	6	6	6	6	6			
Extremwert	3.659	10.397	16.977	35.237	88.543	143.024	164.888			
Prüfwert	1.298	1.598	1.181	1.322	1.408	1.401	1.307			
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.4779	**Prüfwert**	4.26	**a**	0.168718	**A**	-0.00001
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	1,70798	**B**	0.177979
Bestanden?	ja	**Linear?**	ja	**R**	0,9998	**C**	0.826437
				Rest-SD	1.323	**R**	0.9999
						Rest-SD	1.0287

Validierungsprotokoll

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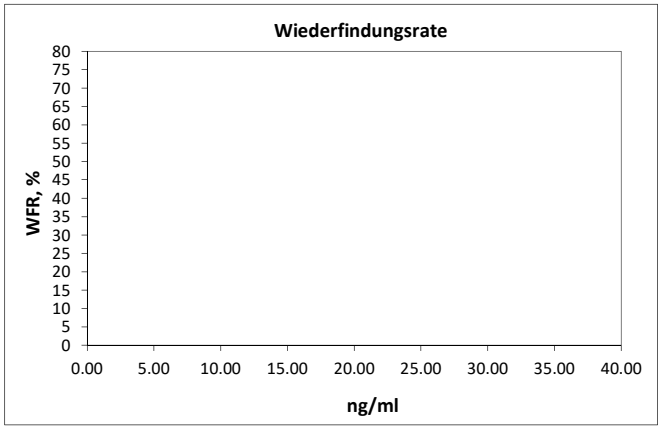
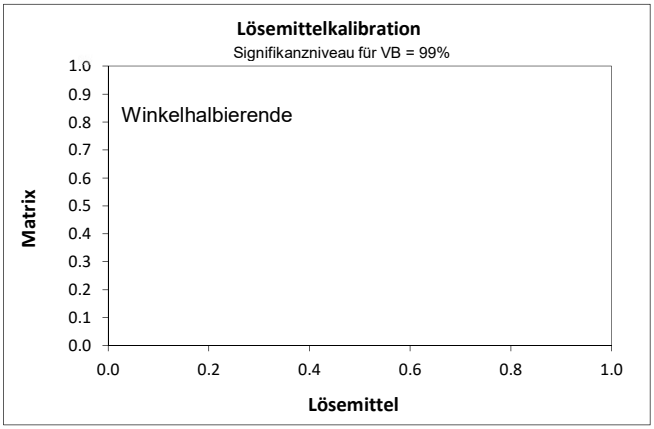
1.3 LÖSEMITTELKALIBRATION

Signifikanz	%
Konzentration	36
Lösemittel (xo)	
Matrix (xm)	

WFR gemessen	
WFR berechnet	

Wiederfindungsfunktion xm = ao * xo + bo	**Ausreißer-F-Test**	**Linearitäts-Test**	**Varianzenhomogenitäts-F-Test**
ao	**Kalibrator Nr.**	**Prüfwert**	**RSD Grundkalibration**
bo	**Prüfwert**	**Kritischer-Wert**	**RSD Wiederfindung**
R	**Kritischer-Wert**	**Bestanden?**	**Prüfwert**
Rest-SD	**Ausreisser?**		**Kritischer-Wert**
			Bestanden?





2. Genauigkeit

2.1. Level 1

QC-Sollwert: 40 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	37.944	42.529	38.824	35.016	38.268	35.294	36.367	34.355	33.853	
2	39.835	42.079	35.657	35.608	32.253	39.987	38.003	39.965	37.492	
3	38.584	40.562	40.603		38.113	35.719	39.312	33.993	35.894	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	38.79	41.72	38.36	35.31	36.21	37.00	37.89	36.10	35.75	
BIAS, %	-3.0	4.3	-4.1	-11.7	-9.5	-7.5	-5.3	-9.7	-10.6	
SD	0.96	1.03	2.51	0.42	3.43	2.60	1.48	3.35	1.82	
RSD, %	2.5	2.5	6.5	1.2	9.5	7.0	3.9	9.3	5.1	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	37.54	**SD**	0.10	**SD**	2.02
SD	2.67	**RSD, %**	0.3	**RSD, %**	5.4
RSD, %	7.1				

Richtigkeit		**95%-Intervall**	OK
Abw.	-2.46	**Faktor**	2.431
Bias, %	-6.1	**ß-Toleranz**	0030 bis 0040
		Prüfbereich (30%)	0028 bis 0052
		Prüfbereich (40%)	0024 bis 0056

Validierungsprotokoll

Seite:	4 von 7	**Institution:**	
Gültig ab:		**Methode:**	

2.2. Level 2

QC-Sollwert: 400 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	405.5	443.0	422.1	407.2	392.8	435.3	385.2	361.4	388.6	
2	416.0	433.8	409.4	437.0	406.4	428.5	409.4	382.0	390.4	
3	393.2	424.6	405.1		422.2	420.8	380.0	380.7	407.1	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	404.89	433.82	412.20	422.07	407.12	428.18	391.50	374.72	395.39	
BIAS, %	1.2	8.5	3.1	5.5	1.8	7.0	-2.1	-6.3	-1.2	
SD	11.39	9.18	8.80	21.08	14.71	7.29	15.69	11.55	10.18	
RSD, %	2.8	2.1	2.1	5.0	3.6	1.7	4.0	3.1	2.6	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	407.22	**SD**	5.11	**SD**	20.20431052
SD	20.81944796	**RSD, %**	1.3	**RSD, %**	5.0
RSD, %	5.1				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	7.22	**Faktor**	2.429
Bias, %	1.8	**ß-Toleranz**	00365 bis 00464
		Prüfbereich (30%)	00280 bis 00520

2.3. Level 3

QC-Sollwert: 700.0 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	655.5	761.8	649.6	746.9	697.1	758.0	772.3	753.6	754.8	
2	694.1	768.4	699.3	746.9	735.2	748.6	785.4	749.5	734.6	
3	654.8	753.2	679.8		678.0	785.1	792.9	734.4	684.6	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	668.1	761.1	676.3	746.9	703.4	763.9	783.5	745.8	724.7	
BIAS, %	-4.6	8.7	-3.4	6.7	0.5	9.1	11.9	6.5	3.5	
SD	22.50	7.61	25.04	0.01	29.08	18.93	10.43	10.11	36.15	
RSD, %	3.4	1.0	3.7	0.0	4.1	2.5	1.3	1.4	5.0	

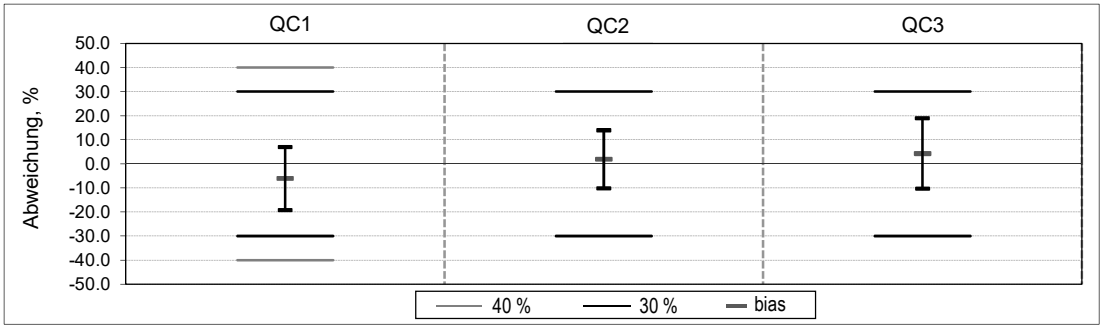
Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	729.79	**SD**	0.00	**SD**	43.81
SD	43.11	**RSD, %**	0.0	**RSD, %**	6.0
RSD, %	5.9				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	29.7875	**Faktor**	2.431
Bias, %	4.3	**ß-Toleranz**	00654 bis 00867
		Prüfbereich (30%)	00490 bis 00910

Validierungsprotokoll

Seite:	5 von 7	**Institution:**	
Gültig ab:		**Methode:**	

Zusammenfassung 95%-Intervall

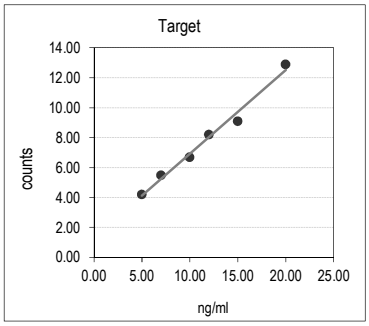


Zusammenfassung	QC1	QC2	QC3
ß-Toleranz	0030 bis 0040	00365 bis 00464	00654 bis 00867
%	- 019 bis 007	-010 bis 014	-10 bis 19
Prüfbereich (30%)	0028 bis 0052	00280 bis 00520	00490 bis 00910
Prüfbereich (40%)	0024 bis 0056		

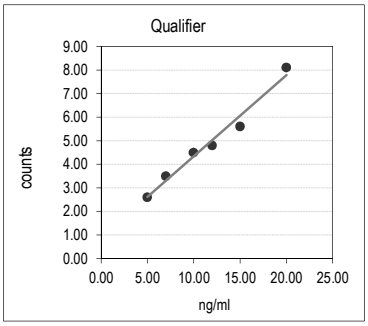
3. Grenzwerte

3.1 Bestimung nach DIN 32645

	Kalibrator	Target	Qualifier
Einheit			
1	5.0	4.200	2.600
2	7.0	5.500	3.500
3	10.0	6.700	4.500
4	12	8.200	4.800
5	15	9.100	5.600
6	20	12.900	8.100
7			
8			
9			
10			



Lineare Kalibration



Lineare Kalibration

	6	6
Signifikanz	99	99
k-Wert	3	3
Ausreißer-F-Test		
Kalibrator Nr.	5	5
Prüfwert	9.51	5.36
Krit.-Wert 99%	34.11	34.11
Ausreißer	nein	nein
Linearitäts-Mandel-Test		
Prüfwert	1.54	1.5
Krit.-Wert 99%	34.11	34.11
Bestanden?	ja	ja

a	0.558528	0.344147
b	1.34359	0.892308
R	0.9928	0.9881
Rest-SD	0.411	0.3273

	Grenzwerte	
Nachweisgrenze	3.04	3.93
Erfassungsgrenze	6.09	7.87
Bestimmungsgrenze	6.19	7.59

Validierungsprotokoll

Seite:	6 von 7	**Institution:**	
Gültig ab:		**Methode:**	

3.2 Bestimmung mittels Alternativmethode

3.2.1 Nachweisgrenze aus Signal/Rauschverhältnis

	Dotiert ng/ml	Target 0	Untergrund 0	Target S/N	Q1 0	Untergrund 0	Q1 S/N	Q2 0	Untergrund 0	Q2 S/N
1										
2										
3										
4										
5										
6										
			NG-1	0.0		NG-2	0.0		NG-3	0.0

Nachweisgrenze :	**0.0**

3.2.2 Bestimmungsgrenze

Vorgabe	ng/ml

MESSUNG	Konz.
1	
2	
3	
4	
5	
6	

Wiederholpräzision		Bias	
Mittelwert		Bias	
SD		Bias, %	
RSD, %			
Krit. Wert, %	**20**	**Krit. Wert, %**	**20**

Beurteilung	**---**	Kriterium: RSD und bias<20%

4. WIEDERFINDUNG

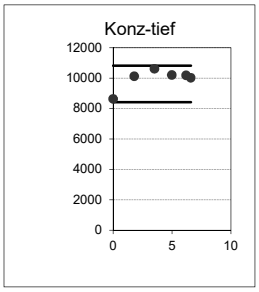
4.1 WIEDERFINDUNG

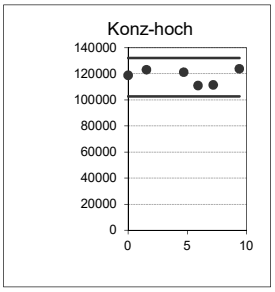
	QC-untere		QC-obere	
Konzentration	**ng/ml**		**ng/ml**	
	Lösemittel	**Matrix**	**Lösemittel**	**Matrix**
MESSUNG				
1				
2				
3				
4				
5				
6				
Mittelwert				
SD				
RSD, %				
N (Werte)				

Wiederfindung,%		
RSD, %		

4.2 STABILITAT

	Konz-tief Zeit	QC-Pool	Konz-hoch Zeit	QC-Pool
MESSUNG	0.00	8620	0.00	118837
	1.80	10127	1.55	123098
	3.50	10611	4.70	121187
	5.00	10198	5.90	111023
	6.20	10181	7.20	111428
	6.60	10007	9.40	123785
Werte	6		6	
min. Wert	8620		111023	
max. Wert	10611		123785	
Spanne	1991		12762	
Spanne, %	18.8	%	10.3	%
Prüfwert	25.0	%	25.0	%
Steigung	0.0023		-0.0001	
Abschnitt	-19.4524		17.7408	
R	0.61997		-0.17735	
Beurteilung	**OK**		**OK**	





Kriterium: Spanne < Prüfwert

Validierungsprotokoll

4.3 MATRIXEFFEKTE - LC/MS/(MS)

	QC1	40		QC2	700	
	Reinsubstanz	**Extrakt**	**Matrix**	**Reinsubstanz**	**Extrakt**	**Matrix**
MESSUNG	118554.0	108411.0	67320.0	1724246.0	1833977.0	1506578.0
	118510.0	120455.0	75403.0	1735429.0	1764343.0	1264217.0
	106560.0	112514.0	79819.0	1706844.0	1796239.0	1626954.0
	108826.0	116452.0	72978.0	1701427.0	1718274.0	1140230.0
	114213.0	118723.0	76198.0	1698997.0	1637523.0	1334450.0
	114221.0	110788.0	85387.0	1689809.0	1384529.0	1344156.0
Mittelwert	113480.7	114557.2	76184.2	1709458.7	1689147.5	1369430.8
SD	4933.1	4730.4	6121.6	17088.1	163953.9	173547.1
Varianz	24335771.9	22377102.2	37473504.6	292004692.3	26880881841.5	30118595064.2
Anzahl	6.0	6.0	6.0	6.0	6.0	6.0

Wiederfindung,%	66.5	81.1
RSD, %	6.1	11.4
Matrixeffekt,%	100.9	98.8
RSD, %	6.0	9.0
Beurteilung	Ok	Ok

Kriterium: Recovery >=50%
SD <=25%
Matrixeffekte: 75-125%

Anmerkungen

Validierungsprotokoll

Seite:	2 von 7	**Institution:**	
Gültig ab:		**Methode:**	

1. Arbeitsbereich und Kalibrationsmodell

1.1 TARGET — **Messsignal:** 303 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	2.9	15	29	60.8	146	245	280			
MESSUNG 2	2.8	15.5	30.4	60.7	146	231	300			
MESSUNG 3	3.1	14.6	29.1	60	144	247	287			
MESSUNG 4	2.9	15.7	29.558	61.2	147	251	289			
MESSUNG 5	2.7	15.2	31.2	58.3	146	246	298			
MESSUNG 6	2.8	15.8	30.3	61.6	148	239	290			
Mittelwert	2.867	15.3	29.92633333	60.43333333	146	243	291			
SD	0.1366	0.4561	0.8559	1.1742	1.4949	7.1355	7.2136			
Varianz	0.0187	0.208	0.7326	1.3787	2.2347	50.915	52.0359316			
Werte	6.0	6.0	6.0	6.0	6.0	6.0	6.0			
Ausreisser-Test nach Grubbs										
Extremwert	3.1	14.6	31.2	58.3	144	231	280			
Prüfwert	1.708	1.535	1.488	1.817	1.628	1.731	1.432			
Signifikanz 95%										
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Signifikanz 99%										
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.4839	**Prüfwert**	-3.99	**a**	000,000	**A**	-0.00001
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	000,001	**B**	0.308519
Bestanden?	ja	**Bestanden?**	ja	**R**	00,001	**C**	-0.631992
				Rest-SD	0.0000	**R**	0.9996
						Rest-SD	3.9008

1.2 QUALIFIER — **Messsignal:** 255 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	2.038	8.303	15.727	32.047	78.814	132.602	153.155			
MESSUNG 2	2.032	7.903	16.436	31.717	77.901	128.413	157.608			
MESSUNG 3	1.958	7.847	15.896	31.684	76.477	128.319	153.068			
MESSUNG 4	2.025	7.987	16.262	32.592	77.423	132.235	156.301			
MESSUNG 5	2.088	7.578	16.110	31.590	78.525	131.903	154.615			
MESSUNG 6	2.031	7.742	15.887	32.526	77.581	129.660	157.005			
Mittelwert	2.029	7.893	16.053	32.026	77.78683333	131	155			
SD	0.042	0.245	0.265	0.441	0.837	1.96	1.964			
Varianz	0.002	0.06	0.07	0.195	0.701	3.842	3.858			
Werte	6	6	6	6	6	6	6			
Extremwert	1.958	8.303	16.436	32.592	76.477	128.319	157.608			
Prüfwert	1.701	1.669	1.443	1.283	1.565	1.124	1.179			
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.442	**Prüfwert**	0.35	**a**	0.157566	**A**	-0.000007
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	000,000	**B**	0.16392
Bestanden?	ja	**Linear?**	ja	**R**	00,001	**C**	-0.251
				Rest-SD	2.2723	**R**	0.9995
						Rest-SD	2.4336

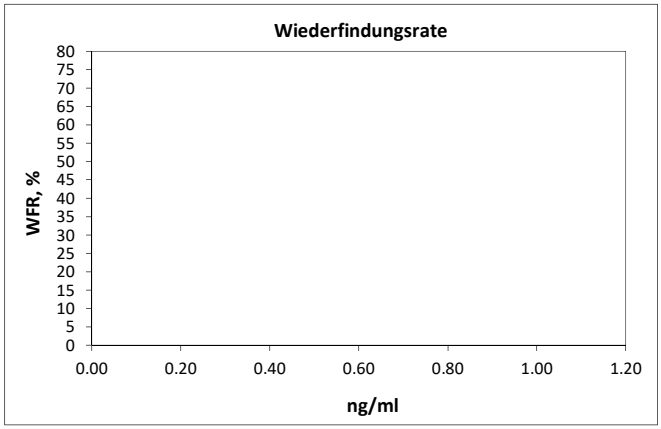
Validierungsprotokoll

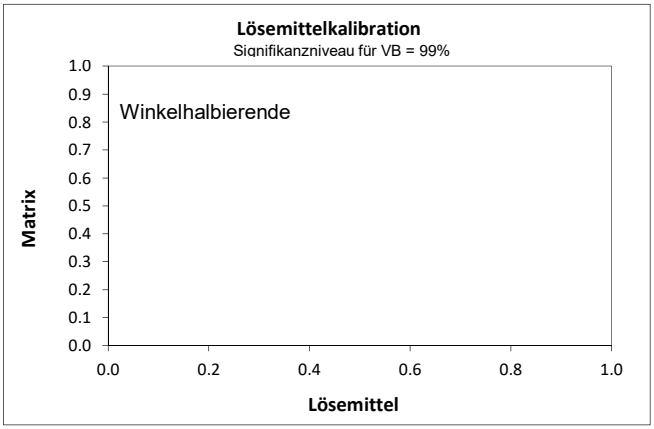
Seite:	3 von 7	**Institution:**	
Gültig ab:		**Methode:**	

1.3 LÖSEMITTELKALIBRATION

Signifikanz	%
Konzentration	
Lösemittel (xo)	
Matrix (xm)	
WFR gemessen	
WFR berechnet	

Wiederfindungsfunktion xm = ao * xo + bo	**Ausreißer-F-Test**	**Linearitäts-Test**	**Varianzenhomogenitäts-F-Test**
ao	**Kalibrator Nr.**	**Prüfwert**	**RSD Grundkalibration**
bo	**Prüfwert**	**Kritischer-Wert**	**RSD Wiederfindung**
R	**Kritischer-Wert**	**Bestanden?**	**Prüfwert**
Rest-SD	**Ausreisser?**		**Kritischer-Wert**
			Bestanden?





2. Genauigkeit

2.1. Level 1

QC-Sollwert: 40 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	40.637	41.381	40.108	40.053	40.239	35.141	37.346	36.222	35.762	
2	43.428	42.971	35.694	39.995	35.468	40.416	41.378	40.075	37.304	
3	40.78	42.569	39.376		38.367	38.908	41.443	37.93	38.36	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	41.62	42.31	38.39	40.02	38.02	38.16	40.06	38.08	37.14	
BIAS, %	4.0	5.8	-4.0	0.1	-4.9	-4.6	0.1	-4.8	-7.1	
SD	1.57	0.83	2.37	0.04	2.40	2.72	2.35	1.93	1.31	
RSD, %	3.8	2.0	6.2	0.1	6.3	7.1	5.9	5.1	3.5	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	39.28	**SD**	0.01	**SD**	1.76
SD	2.38	**RSD, %**	0.0	**RSD, %**	4.5
RSD, %	6.1				

Richtigkeit		**95%-Intervall**	OK
Abw.	-0.72	**Faktor**	2.431
Bias, %	-1.8	**ß-Toleranz**	0034 bis 0043
		Prüfbereich (30%)	0028 bis 0052
		Prüfbereich (40%)	0024 bis 0056

Validierungsprotokoll

Seite:	4 von 7	**Institution:**	
Gültig ab:		**Methode:**	

2.2. Level 2

QC-Sollwert: 400 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	411.9	458.5	436.2	352.4	320.2	342.3	407.3	432.1	433.6	
2	420.2	461.6	425.2	374.6	321.2	400.6	408.3	434.8	434.9	
3	406.7	450.5	435.7		353.4	386.3	424.3	421.3	423.9	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	412.93	456.85	432.36	363.53	331.61	376.42	413.30	429.42	430.84	
BIAS, %	3.2	14.2	8.1	-9.1	-17.1	-5.9	3.3	7.4	7.7	
SD	6.77	5.75	6.22	15.71	18.88	30.39	9.52	7.13	6.00	
RSD, %	1.6	1.3	1.4	4.3	5.7	8.1	2.3	1.7	1.4	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	406.86	**SD**	3.81	**SD**	41.68730088
SD	39.92747054	**RSD, %**	0.9	**RSD, %**	10.2
RSD, %	9.8				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	6.86	**Faktor**	2.43
Bias, %	1.7	**ß-Toleranz**	00313 bis 00515
		Prüfbereich (30%)	00280 bis 00520

2.3. Level 3

QC-Sollwert: 700.0 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	662.4	793.5	662.4	716.3	750.9	715.7	713.0	721.4	759.7	
2	719.2	788.0	719.8	710.8	727.4	746.3	762.4	752.1	759.4	
3	681.3	818.2	666.4	718.6	743.7	751.2	743.7	742.3	750.0	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	687.6	799.9	682.9	715.2	740.7	737.7	739.7	738.6	756.4	
BIAS, %	-1.8	14.3	-2.4	2.2	5.8	5.4	5.7	5.5	8.1	
SD	28.93	16.06	32.02	3.98	12.06	19.21	24.95	15.66	5.53	
RSD, %	4.2	2.0	4.7	0.6	1.6	2.6	3.4	2.1	0.7	

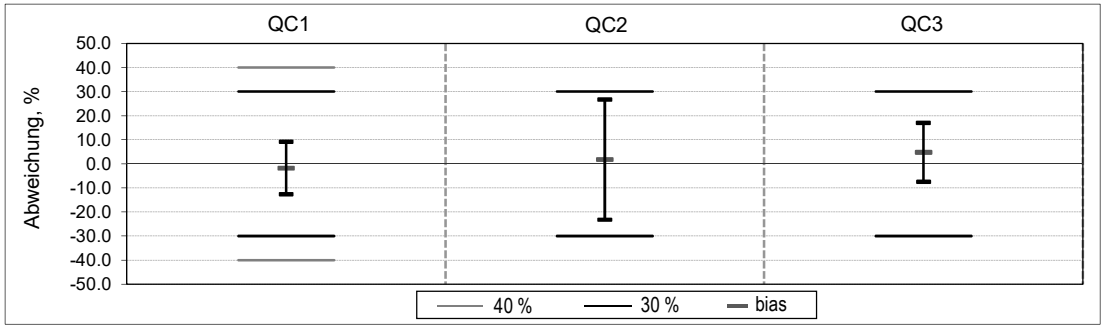
Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	733.19	**SD**	0.00	**SD**	36.90
SD	37.88	**RSD, %**	0.0	**RSD, %**	5.0
RSD, %	5.2				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	33.19118519	**Faktor**	2.431
Bias, %	4.7	**ß-Toleranz**	00678 bis 00858
		Prüfbereich (30%)	00490 bis 00910

Validierungsprotokoll

Seite:	5 von 7	**Institution:**	
Gültig ab:		**Methode:**	

Zusammenfassung 95%-Intervall

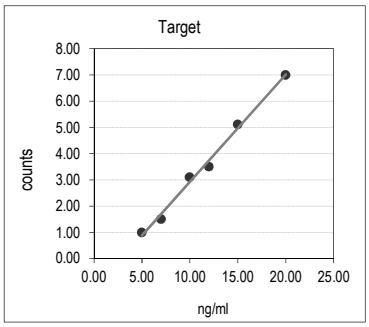


Zusammenfassung	QC1	QC2	QC3
ß-Toleranz	0034 bis 0043	00313 bis 00515	00678 bis 00858
%	- 013 bis 009	-023 bis 027	-07 bis 17
Prüfbereich (30%)	0028 bis 0052	00280 bis 00520	00490 bis 00910
Prüfbereich (40%)	0024 bis 0056		

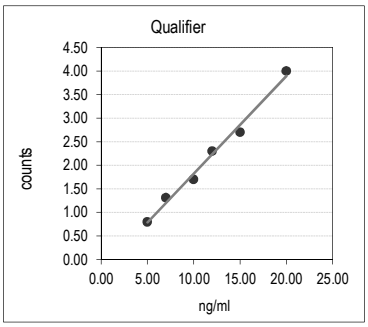
3. Grenzwerte

3.1 Bestimung nach DIN 32645

	Kalibrator	Target	Qualifier
Einheit			
1	5.0	1.000	0.800
2	7.0	1.500	1.310
3	10.0	3.100	1.700
4	12	3.500	2.300
5	15	5.120	2.700
6	20	7.000	4.000
7			
8			
9			
10			



Lineare Kalibration



Lineare Kalibration

	1	1
Signifikanz	99	99
k-Wert	3	3
Ausreißer-F-Test		
Kalibrator Nr.	4	5
Prüfwert	2.19	3.05
Krit.-Wert 99%	34.11	34.11
Ausreißer	nein	nein
Linearitäts-Mandel-Test		
Prüfwert	0.02	1.21
Krit.-Wert 99%	34.11	34.11
Bestanden?	ja	ja

a	0.409833	0.207057
b	-1.17641	-0.246154
R	0.9967	0.9948
Rest-SD	0.204	0.1297

	Grenzwerte	
Nachweisgrenze	2.67	3.36
Erfassungsgrenze	5.33	6.72
Bestimmungsgrenze	7.67	9.40

Validierungsprotokoll

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Gültig ab:		**Methode:**	

3.2 Bestimmung mittels Alternativmethode

3.2.1 Nachweisgrenze aus Signal/Rauschverhältnis

	Dotiert ng/ml	Target 0	Untergrund 0	Target S/N	Q1 0	Untergrund 0	Q1 S/N	Q2 0	Untergrund 0	Q2 S/N
1										
2										
3										
4										
5										
6										
			NG-1	0.0		NG-2	0.0		NG-3	0.0

Nachweisgrenze :	**0.0**

3.2.2 Bestimmungsgrenze

Vorgabe	ng/ml

MESSUNG	Konz.
1	
2	
3	
4	
5	
6	

Wiederholpräzision		Bias	
Mittelwert		Bias	
SD		Bias, %	
RSD, %			
Krit. Wert, %	**20**	**Krit. Wert, %**	**20**

Beurteilung	**---**	Kriterium: RSD und bias<20%

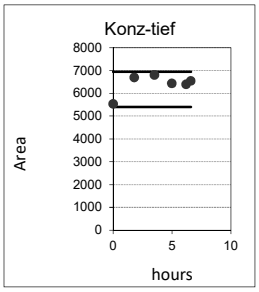
4. WIEDERFINDUNG

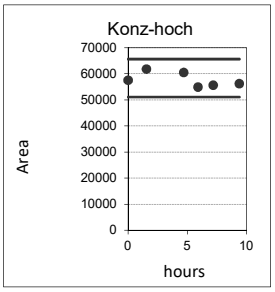
4.1 WIEDERFINDUNG

	QC-untere		QC-obere	
Konzentration	**ng/ml**		**ng/ml**	
	Lösemittel	**Matrix**	**Lösemittel**	**Matrix**
MESSUNG				
1				
2				
3				
4				
5				
6				
Mittelwert				
SD				
RSD, %				
N (Werte)				
Wiederfindung,%				
RSD, %				

4.2 STABILITAT

	Konz-tief Zeit	QC-Pool	Konz-hoch Zeit	QC-Pool
MESSUNG	0.00	5540	0.00	57473
	1.80	6694	1.55	61725
	3.50	6798	4.70	60423
	5.00	6437	5.90	54920
	6.20	6390	7.20	55571
	6.60	6541	9.40	56153
Werte	6		6	
min. Wert	5540	Area	54920	Area
max. Wert	6798	Area	61725	Area
Spanne	1258	Area	6805	Area
Spanne, %	18.5	%	11.0	%
Prüfwert	25.0	%	25.0	%
Steigung	0.0030		-0.0007	
Abschnitt	-15.0855		46.2780	
R	0.51224		-0.56633	
Beurteilung	**OK**		**OK**	





Kriterium: Spanne < Prüfwert

Validierungsprotokoll

4.3 MATRIXEFFEKTE - LC/MS/(MS)

	QC1			QC2		
	Reinsubstanz	**Extrakt**	**Matrix**	**Reinsubstanz**	**Extrakt**	**Matrix**
MESSUNG	64397.0	71790.0	38850.0	1351054.0	1401158.0	885689.0
	62351.0	72162.0	38990.0	1291135.0	1282503.0	796944.0
	64709.0	73232.0	40367.0	1310575.0	1278950.0	958434.0
	66762.0	71215.0	39888.0	1276467.0	1251573.0	794142.0
	63448.0	73735.0	37981.0	1263231.0	1205993.0	806066.0
	65763.0	71030.0	43386.0	1256056.0	1163216.0	794748.0
Mittelwert	64571.7	72194.0	39910.3	1291419.7	1263898.8	839337.2
SD	1578.6	1089.0	1896.5	35191.0	81307.8	68216.6
Varianz	2492038.3	1185940.4	3596589.9	1238406726.3	6610955219.8	4653499229.8
Anzahl	6.0	6.0	6.0	6.0	6.0	6.0

Wiederfindung,%	55.3	66.4
RSD, %	3.2	4.8
Matrixeffekt,%	111.8	97.9
RSD, %	3.2	3.7
Beurteilung	Ok	Ok

Kriterium: Recovery >=50%
SD <=25%
Matrixeffekte: 75-125%

Anmerkungen

Validierungsprotokoll

Seite:	2 von 7	**Institution:**	
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1. Arbeitsbereich und Kalibrationsmodell

1.1 TARGET — **Messsignal:** 283 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	7.112	24.127	48.553	106	247	419	503			
MESSUNG 2	7.171	24.35	46.998	104	249	413	514			
MESSUNG 3	7.236	23.231	45.769	102	253	422	506			
MESSUNG 4	7.231	25.283	47.602	105	253	422	513			
MESSUNG 5	6.782	25.653	48.904	103	247	418	509			
MESSUNG 6	6.976	25.496	49.565	105	251	418	512			
Mittelwert	7.085	24.69	47.8985	104	250	419	509			
SD	0.1766	0.9476	1.3893	1.433	2.6447	3.071	4.4146			
Varianz	0.0312	0.898	1.93	2.0534	6.9944	9.4308	19.4890423			
Werte	6.0	6.0	6.0	6.0	6.0	6.0	6.0			
Ausreisser-Test nach Grubbs										
Extremwert	6.782	23.231	45.769	102	247	413	503			
Prüfwert	1.714	1.54	1.533	1.345	1.206	1.737	1.453			
Signifikanz 95%										
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Signifikanz 99%										
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.4774	**Prüfwert**	-3.99	**a**	000,001	**A**	0.0
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	-000,001	**B**	0.510481
Bestanden?	ja	**Bestanden?**	ja	**R**	00,001	**C**	-0.376289
				Rest-SD	0.0000	**R**	0.9997
						Rest-SD	5.9972

1.2 QUALIFIER — **Messsignal:** 281 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	2.693	9.574	20.391	42.623	103.399	167.766	207.864			
MESSUNG 2	2.721	9.783	19.616	43.233	104.818	168.963	211.176			
MESSUNG 3	2.735	9.375	19.589	42.684	106.348	168.397	208.730			
MESSUNG 4	2.718	9.849	19.180	42.748	106.707	168.835	208.133			
MESSUNG 5	2.688	10.053	20.460	42.938	105.783	169.559	209.228			
MESSUNG 6	2.736	10.594	20.535	42.170	103.362	167.541	210.677			
Mittelwert	2.715	9.871	19.96183333	42.73266667	105	169	209			
SD	0.02	0.424	0.571	0.353	1.456	0.764	1.354			
Varianz	0.0	0.18	0.326	0.125	2.119	0.583	1.834			
Werte	6	6	6	6	6	6	6			
Extremwert	2.688	10.594	19.180	42.170	103.362	169.559	211.176			
Prüfwert	1.326	1.705	1.369	1.593	1.173	1.373	1.384			
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.4101	**Prüfwert**	0.63	**a**	0.209923	**A**	-0.000003
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	000,000	**B**	0.212762
Bestanden?	ja	**Linear?**	ja	**R**	00,001	**C**	-0.303522
				Rest-SD	0.7885	**R**	1.0
						Rest-SD	0.8191

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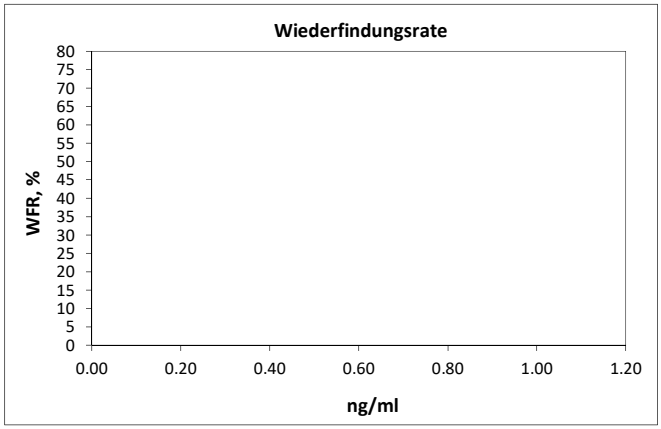
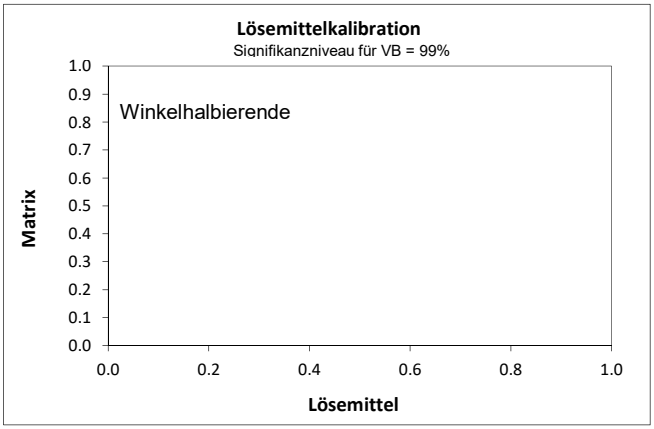
1.3 LÖSEMITTELKALIBRATION

Signifikanz	%
Konzentration	
Lösemittel (xo)	
Matrix (xm)	
WFR gemessen	
WFR berechnet	

Wiederfindungsfunktion **xm = ao * xo + bo**	**Ausreißer-F-Test**	**Linearitäts-Test**	**Varianzenhomogenitäts-F-Test**
ao	**Kalibrator Nr.**	**Prüfwert**	**RSD Grundkalibration**
bo	**Prüfwert**	**Kritischer-Wert**	**RSD Wiederfindung**
R	**Kritischer-Wert**	**Bestanden?**	**Prüfwert**
Rest-SD	**Ausreisser?**		**Kritischer-Wert**
			Bestanden?





2. Genauigkeit

2.1. Level 1

QC-Sollwert: 40 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	40.637	41.381	40.108	40.053	40.239	35.141	37.346	36.222	35.762	
2	43.428	42.971	35.694	39.995	35.468	40.416	41.378	40.075	37.304	
3	40.78	42.569	39.376		38.367	38.908	41.443	37.93	38.36	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	41.62	42.31	38.39	40.02	38.02	38.16	40.06	38.08	37.14	
BIAS, %	4.0	5.8	-4.0	0.1	-4.9	-4.6	0.1	-4.8	-7.1	
SD	1.57	0.83	2.37	0.04	2.40	2.72	2.35	1.93	1.31	
RSD, %	3.8	2.0	6.2	0.1	6.3	7.1	5.9	5.1	3.5	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	39.28	**SD**	0.01	**SD**	1.76
SD	2.38	**RSD, %**	0.0	**RSD, %**	4.5
RSD, %	6.1				

Richtigkeit		**95%-Intervall**	OK
Abw.	-0.72	**Faktor**	2.431
Bias, %	-1.8	**ß-Toleranz**	0034 bis 0043
		Prüfbereich (30%)	0028 bis 0052
		Prüfbereich (40%)	0024 bis 0056

Validierungsprotokoll

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2.2. Level 2

QC-Sollwert: 400 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	411.9	458.5	436.2	352.4	320.2	342.3	407.3	432.1	433.6	
2	420.2	461.6	425.2	374.6	321.2	400.6	408.3	434.8	434.9	
3	406.7	450.5	435.7		353.4	386.3	424.3	421.3	423.9	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	412.93	456.85	432.36	363.53	331.61	376.42	413.30	429.42	430.84	
BIAS, %	3.2	14.2	8.1	-9.1	-17.1	-5.9	3.3	7.4	7.7	
SD	6.77	5.75	6.22	15.71	18.88	30.39	9.52	7.13	6.00	
RSD, %	1.6	1.3	1.4	4.3	5.7	8.1	2.3	1.7	1.4	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	406.86	**SD**	3.81	**SD**	41.68730088
SD	39.92747054	**RSD, %**	0.9	**RSD, %**	10.2
RSD, %	9.8				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	6.86	**Faktor**	2.43
Bias, %	1.7	**ß-Toleranz**	00313 bis 00515
		Prüfbereich (30%)	00280 bis 00520

2.3. Level 3

QC-Sollwert: 700.0 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	662.4	793.5	662.4	716.3	750.9	715.7	713.0	721.4	759.7	
2	719.2	788.0	719.8	710.8	727.4	746.3	762.4	752.1	759.4	
3	681.3	818.2	666.4		743.7	751.2	743.7	742.3	750.0	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	687.6	799.9	682.9	713.6	740.7	737.7	739.7	738.6	756.4	
BIAS, %	-1.8	14.3	-2.4	1.9	5.8	5.4	5.7	5.5	8.1	
SD	28.93	16.06	32.02	3.90	12.06	19.21	24.95	15.66	5.53	
RSD, %	4.2	2.0	4.7	0.5	1.6	2.6	3.4	2.1	0.7	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	733.75	**SD**	0.95	**SD**	37.65
SD	38.51	**RSD, %**	0.1	**RSD, %**	5.1
RSD, %	5.2				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	33.75430769	**Faktor**	2.431
Bias, %	4.8	**ß-Toleranz**	00678 bis 00861
		Prüfbereich (30%)	00490 bis 00910

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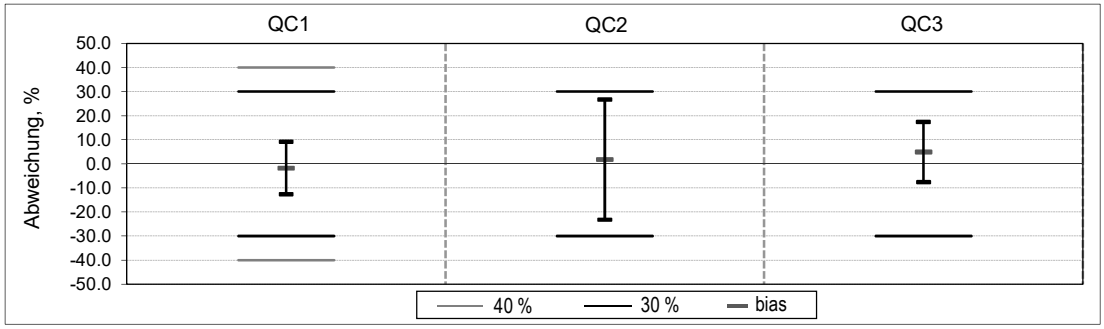
Zusammenfassung 95%-Intervall

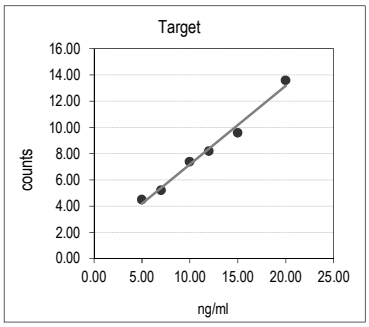


Zusammenfassung	QC1	QC2	QC3
ß-Toleranz	0034 bis 0043	00313 bis 00515	00678 bis 00861
%	- 013 bis 009	-023 bis 027	-08 bis 17
Prüfbereich (30%)	0028 bis 0052	00280 bis 00520	00490 bis 00910
Prüfbereich (40%)	0024 bis 0056		

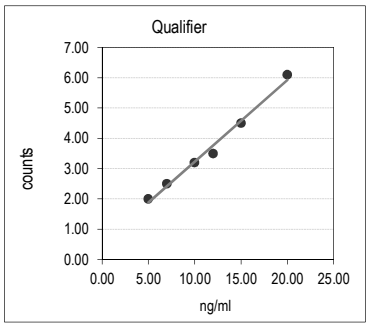
3. Grenzwerte

3.1 Bestimung nach DIN 32645

	Kalibrator	Target	Qualifier
Einheit			
1	5.0	4.500	2.000
2	7.0	5.200	2.500
3	10.0	7.400	3.200
4	12	8.200	3.500
5	15	9.600	4.500
6	20	13.600	6.100
7			
8			
9			
10			



Lineare Kalibration



Lineare Kalibration

	6	6
Signifikanz	99	99
k-Wert	3	3
Ausreißer-F-Test		
Kalibrator Nr.	5	4
Prüfwert	4.78	5.97
Krit.-Wert 99%	34.11	34.11
Ausreißer	nein	nein
Linearitäts-Mandel-Test		
Prüfwert	2.6	10.5
Krit.-Wert 99%	34.11	34.11
Bestanden?	ja	ja
a	0.598997	0.269565
b	1.194872	0.533333
R	0.9933	0.9941
Rest-SD	0.427	0.1802

	Grenzwerte	
Nachweisgrenze	2.95	2.76
Erfassungsgrenze	5.89	5.53
Bestimmungsgrenze	6.07	5.85

Validierungsprotokoll

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3.2 Bestimmung mittels Alternativmethode

3.2.1 Nachweisgrenze aus Signal/Rauschverhältnis

	Dotiert ng/ml	Target 0	Untergrund 0	Target S/N	Q1 0	Untergrund 0	Q1 S/N	Q2 0	Untergrund 0	Q2 S/N
1										
2										
3										
4										
5										
6										
			NG-1	0.0		NG-2	0.0		NG-3	0.0

Nachweisgrenze :	**0.0**

3.2.2 Bestimmungsgrenze

Vorgabe	ng/ml

MESSUNG	Konz.
1	
2	
3	
4	
5	
6	

Wiederholpräzision		Bias	
Mittelwert		Bias	
SD		Bias, %	
RSD, %			
Krit. Wert, %	**20**	**Krit. Wert, %**	**20**

Beurteilung	**---**	Kriterium: RSD und bias<20%

4. WIEDERFINDUNG

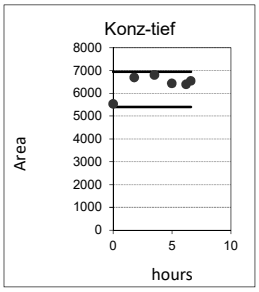
4.1 WIEDERFINDUNG

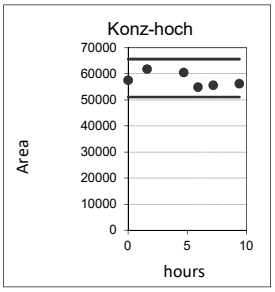
	QC-untere		QC-obere	
Konzentration	**ng/ml**		**ng/ml**	
	Lösemittel	**Matrix**	**Lösemittel**	**Matrix**
MESSUNG				
1				
2				
3				
4				
5				
6				
Mittelwert				
SD				
RSD, %				
N (Werte)				
Wiederfindung,%				
RSD, %				

4.2 STABILITAT

	Konz-tief Zeit	QC-Pool	Konz-hoch Zeit	QC-Pool
MESSUNG	0.00	5540	0.00	57473
	1.80	6694	1.60	61725
	3.50	6798	4.70	60423
	5.00	6437	5.90	54920
	6.20	6390	7.20	55571
	6.60	6541	9.40	56153

Werte	6		6	
min. Wert	5540	Area	54920	Area
max. Wert	6798	Area	61725	Area
Spanne	1258	Area	6805	Area
Spanne, %	18.5	%	11.0	%
Prüfwert	25.0	%	25.0	%
Steigung	0.0030		-0.0007	
Abschnitt	-15.0855		45.9840	
R	0.51224		-0.56368	
Beurteilung	**OK**		**OK**	





Kriterium: Spanne < Prüfwert

Validierungsprotokoll

4.3 MATRIXEFFEKTE - LC/MS/(MS)

	QC1			QC2		
	Reinsubstanz	**Extrakt**	**Matrix**	**Reinsubstanz**	**Extrakt**	**Matrix**
MESSUNG	192964.0	272947.0	216880.0	2800477.0	3688800.0	3499808.0
	199217.0	274714.0	224901.0	2848552.0	3641121.0	3392108.0
	186764.0	263304.0	224563.0	2698041.0	3572936.0	3650109.0
	187602.0	268327.0	226020.0	2718092.0	3600043.0	3139424.0
	198985.0	282060.0	223893.0	2829974.0	3618024.0	3024856.0
	185627.0	276396.0	243018.0	2785800.0	3623749.0	3126754.0
Mittelwert	191859.8	272958.0	226545.8	2780156.0	3624112.2	3305509.8
SD	6149.1	6517.6	8703.0	60331.5	39266.6	245887.9
Varianz	37811991.8	42479156.4	75742347.8	3639891407.6	1541867735.0	60460866371.4
Anzahl	6.0	6.0	6.0	6.0	6.0	6.0

Wiederfindung,%	83.0	91.2
RSD, %	3.4	6.9
Matrixeffekt,%	142.3	130.4
RSD, %	1.8	2.2
Beurteilung	Prüfen	Prüfen

Kriterium: Recovery >=50%
SD <=25%
Matrixeffekte: 75-125%

Anmerkungen

Validierungsprotokoll

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1. Arbeitsbereich und Kalibrationsmodell

1.1 TARGET — **Messsignal:** 283 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	14.793	60.202	122	266	622	1064	1255			
MESSUNG 2	14.488	64.053	128	269	637	1053	1256			
MESSUNG 3	15.217	63.856	126	267	634	1076	1256			
MESSUNG 4	15.92	67.598	126	265	633	1065	1261			
MESSUNG 5	15.222	62.218	127	266	621	1080	1274			
MESSUNG 6	13.761	60.039	116	263	600	1078	1245			
Mittelwert	14.90016667	62.99433333	124	266	624	1069	1258			
SD	0.738	2.8352	4.4081	2.2036	13.59555086	10.18281764	9.3146			
Varianz	0.5446	8.0382	19.4314316	4.8559	185	104	86.7624547			
Werte	6.0	6.0	6.0	6.0	6.0	6.0	6.0			
Ausreisser-Test nach Grubbs										
Extremwert	13.761	67.598	116	263	600	1053	1274			
Prüfwert	1.544	1.624	1.789	1.533	1.795	1.563	1.701			
Signifikanz 95%										
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Signifikanz 99%										
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.4529	**Prüfwert**	0.27	**a**	1,28235	**A**	-0.00006
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	1,21112	**B**	1.34129
Bestanden?	ja	**Bestanden?**	ja	**R**	0,9991	**C**	-4.399149
				Rest-SD	24.0116	**R**	0.9991
						Rest-SD	25.979731

1.2 QUALIFIER — **Messsignal:** 279 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	6.315	19.726	38.369	78.216	189.238	292.477	371.657			
MESSUNG 2	6.292	20.164	37.536	80.988	188.775	291.111	370.188			
MESSUNG 3	6.216	18.643	36.429	79.244	186.753	292.908	369.243			
MESSUNG 4	6.164	19.319	37.756	77.562	185.590	294.080	369.850			
MESSUNG 5	6.250	20.469	37.370	78.801	188.283	294.298	371.013			
MESSUNG 6	6.242	18.936	36.946	75.051	184.264	295.590	372.623			
Mittelwert	6.247	19.54283333	37.401	78.31033333	187	293	371			
SD	0.054	0.708	0.668	1.974	1.96	1.575	1.249			
Varianz	0.003	0.501	0.447	3.895	3.841	2.48	1.559			
Werte	6	6	6	6	6	6	6			
Extremwert	6.164	20.469	36.429	75.051	184.264	291.111	372.623			
Prüfwert	1.530	1.308	1.454	1.652	1.473	1.460	1.490			
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.3061	**Prüfwert**	0.04	**a**	0.367283	**A**	-0.000002
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	2,26432	**B**	0.369325
Bestanden?	ja	**Linear?**	ja	**R**	0,9999	**C**	2.070003
				Rest-SD	2.0357	**R**	0.9999
						Rest-SD	2.2639

Validierungsprotokoll

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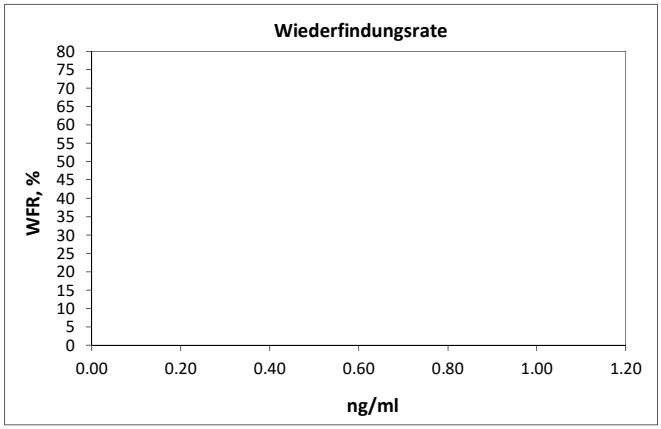
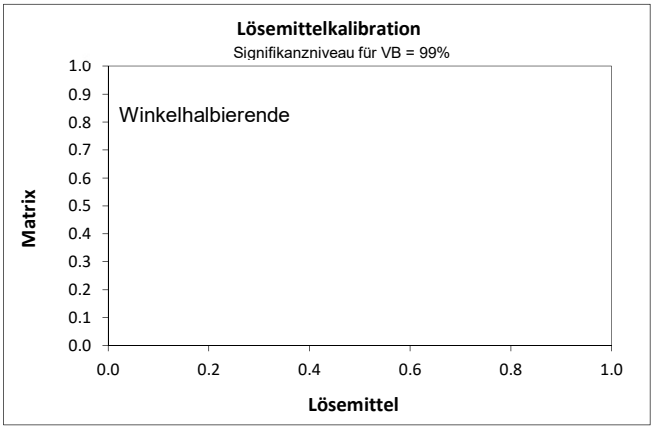
1.3 LÖSEMITTELKALIBRATION

Signifikanz	%
Konzentration	
Lösemittel (xo)	
Matrix (xm)	

WFR gemessen	
WFR berechnet	

Wiederfindungsfunktion xm = ao * xo + bo	**Ausreißer-F-Test**	**Linearitäts-Test**	**Varianzenhomogenitäts-F-Test**
ao	**Kalibrator Nr.**	**Prüfwert**	**RSD Grundkalibration**
bo	**Prüfwert**	**Kritischer-Wert**	**RSD Wiederfindung**
R	**Kritischer-Wert**	**Bestanden?**	**Prüfwert**
Rest-SD	**Ausreisser?**		**Kritischer-Wert**
			Bestanden?





2. Genauigkeit

2.1. Level 1

QC-Sollwert: 40 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	38.973	40.104	39.958	39.597	38.332	38.142	35.438	37.512	36.58	
2	41.694	39.269	37.404	40.523	37.701	41.317	36.987	40.678	38.029	
3	40.514	41.672	40.634		37.464	40.333	37.215	37.289	38.961	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	40.39	40.35	39.33	40.06	37.83	39.93	36.55	38.49	37.86	
BIAS, %	1.0	0.9	-1.7	0.2	-5.4	-0.2	-8.6	-3.8	-5.4	
SD	1.36	1.22	1.70	0.65	0.45	1.63	0.97	1.90	1.20	
RSD, %	3.4	3.0	4.3	1.6	1.2	4.1	2.6	4.9	3.2	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	38.94	**SD**	0.16	**SD**	1.41
SD	1.72	**RSD, %**	0.4	**RSD, %**	3.6
RSD, %	4.4				

Richtigkeit		**95%-Intervall**	OK
Abw.	-1.06	**Faktor**	2.43
Bias, %	-2.7	**ß-Toleranz**	34,46944 bis 41,32877
		Prüfbereich (30%)	28,0 bis 52,0
		Prüfbereich (40%)	24,0 bis 56,0

Validierungsprotokoll

Seite:	4 von 7	**Institution:**	
Gültig ab:		**Methode:**	

2.2. Level 2

QC-Sollwert: 400 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	399.7	435.3	421.3	421.5	407.3	411.9	417.8	407.8	425.5	
2	417.6	426.1	397.9	438.4	417.2	394.8	425.8	413.5	428.5	
3	385.4	432.2	407.9		412.2	381.8	427.4	429.8	431.4	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	400.93	431.22	409.01	429.93	412.26	396.17	423.68	417.01	428.47	
BIAS, %	0.2	7.8	2.3	7.5	3.1	-1.0	5.9	4.3	7.1	
SD	16.14	4.71	11.75	11.98	4.96	15.06	5.15	11.44	2.98	
RSD, %	4.0	1.1	2.9	2.8	1.2	3.8	1.2	2.7	0.7	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	416.00	**SD**	2.91	**SD**	13.03781633
SD	14.96536884	**RSD, %**	0.7	**RSD, %**	3.1
RSD, %	3.6				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	16.00	**Faktor**	2.429
Bias, %	4.0	**ß-Toleranz**	400,97291 bis 464,31461
		Prüfbereich (30%)	280,0 bis 520,0

2.3. Level 3

QC-Sollwert: 700.0 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	653.8	701.7	658.8	708.1	743.7	690.2	727.6	745.8	776.6	
2	685.3	709.3	767.6	715.3	781.5	658.1	748.0	757.1	761.5	
3	683.5	736.7	670.8		699.1	710.0	721.1	733.8	754.7	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	674.2	715.9	699.1	711.7	741.4	686.1	732.3	745.6	764.3	
BIAS, %	-3.7	2.3	-0.1	1.7	5.9	-2.0	4.6	6.5	9.2	
SD	17.71	18.40	59.64	5.10	41.27	26.17	14.06	11.64	11.16	
RSD, %	2.6	2.6	8.5	0.7	5.6	3.8	1.9	1.6	1.5	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	719.23	**SD**	1.24	**SD**	27.17
SD	37.43	**RSD, %**	0.2	**RSD, %**	3.8
RSD, %	5.2				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	19.22723077	**Faktor**	2.431
Bias, %	2.7	**ß-Toleranz**	672,95125 bis 805,01392
		Prüfbereich (30%)	490,0 bis 910,0

Validierungsprotokoll

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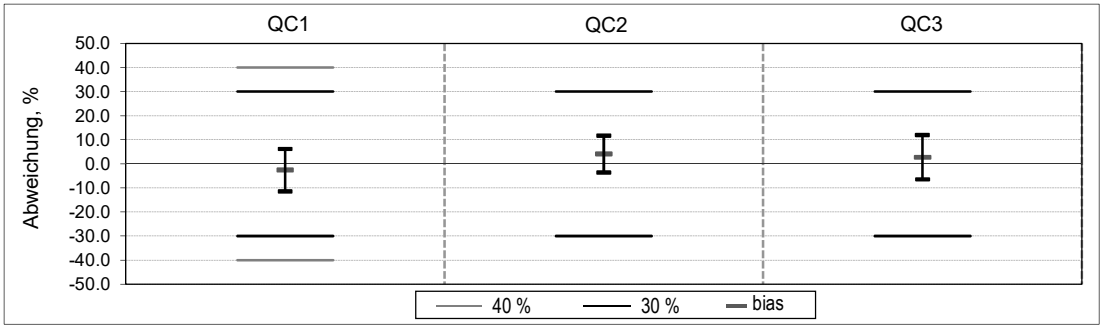
Zusammenfassung 95%-Intervall

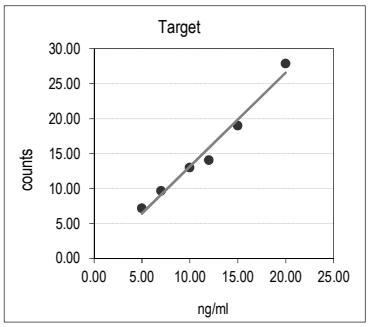


Zusammenfassung	QC1	QC2	QC3
ß-Toleranz	34,46944 bis 41,32877	400,97291 bis 464,31461	672,95125 bis 805,01392
%	- 11,47 bis 6,15	-3,61 bis 11,61	-6,4 bis 11,9
Prüfbereich (30%)	28,0 bis 52,0	280,0 bis 520,0	490,0 bis 910,0
Prüfbereich (40%)	24,0 bis 56,0		

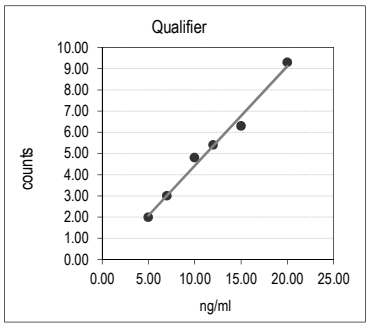
3. Grenzwerte

3.1 Bestimung nach DIN 32645

	Kalibrator	Target	Qualifier
Einheit			
1	5.0	7.200	2.000
2	7.0	9.700	3.000
3	10.0	13.000	4.800
4	12	14.100	5.400
5	15	19.000	6.300
6	20	27.900	9.300
7			
8			
9			
10			



Lineare Kalibration



Lineare Kalibration

	6	6
Signifikanz	99	99
k-Wert	3	3
Ausreißer-F-Test		
Kalibrator Nr.	4	5
Prüfwert	3.7	8.63
Krit.-Wert 99%	34.11	34.11
Ausreißer	nein	nein
Linearitäts-Mandel-Test		
Prüfwert	12	0.03
Krit.-Wert 99%	34.11	34.11
Bestanden?	ja	ja
a	1.342809	0.468896
b	-0.292308	-0.258974
R	0.9883	0.9939
Rest-SD	1.269	0.3177

	Grenzwerte	
Nachweisgrenze	3.91	2.80
Erfassungsgrenze	7.82	5.60
Bestimmungsgrenze	7.54	5.90

Validierungsprotokoll

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3.2 Bestimmung mittels Alternativmethode

3.2.1 Nachweisgrenze aus Signal/Rauschverhältnis

	Dotiert ng/ml	Target 0	Untergrund 0	Target S/N	Q1 0	Untergrund 0	Q1 S/N	Q2 0	Untergrund 0	Q2 S/N
1										
2										
3										
4										
5										
6										
			NG-1	0.0		NG-2	0.0		NG-3	0.0

Nachweisgrenze :	**0.0**

3.2.2 Bestimmungsgrenze

Vorgabe	ng/ml

MESSUNG	Konz.
1	
2	
3	
4	
5	
6	

Wiederholpräzision		Bias	
Mittelwert		Bias	
SD		Bias, %	
RSD, %			
Krit. Wert, %	**20**	**Krit. Wert, %**	**20**

Beurteilung	**---**	Kriterium: RSD und bias<20%

4. WIEDERFINDUNG

4.1 WIEDERFINDUNG

	QC-untere		QC-obere	
Konzentration	**50**	**ng/ml**		**ng/ml**

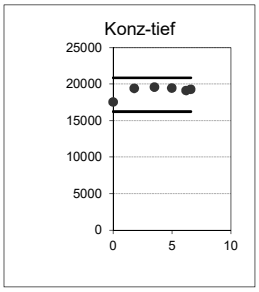
MESSUNG	Lösemittel	Matrix	Lösemittel	Matrix
1	177172.0	128664.0	2555757.0	2493011.0
2	151437.0	119246.0	3531457.0	2667969.0
3	170005.0	121469.0	2400409.0	2686753.0
4	150496.0	123723.0	2325750.0	2446316.0
5	157503.0	122517.0	2516684.0	2622116.0
6	154089.0	114491.0	2897854.0	2624091.0
Mittelwert	160117	121685	2704652	2590043
SD	10952	4718	450459	97658
RSD, %	6.8	3.9	16.7	3.8
N (Werte)	6	6	6	6

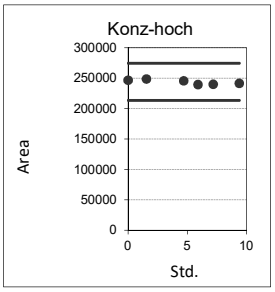
Wiederfindung,%	76.0	95.8
RSD, %	4.1	13.0

4.2 STABILITAT

	Konz-tief Zeit	QC-Pool	Konz-hoch Zeit	QC-Pool
MESSUNG	0.00	17552	0.00	246484
	1.80	19429	1.55	248521
	3.50	19571	4.70	245565
	5.00	19449	5.90	239273
	6.20	19138	7.20	239940
	6.60	19285	9.40	241426

Werte	6		6	
min. Wert	17552		239273	
max. Wert	19571		248521	
Spanne	2019		9248	
Spanne, %	10.3	%	3.7	%
Prüfwert	25.0	%	25.0	%
Steigung	0.0021		-0.0007	
Abschnitt	-36.6929		183.0175	
R	0.62268		-0.79692	
Beurteilung	**OK**		**OK**	





Kriterium: Spanne < Prüfwert

Validierungsprotokoll

4.3 MATRIXEFFEKTE - LC/MS/(MS)

	QC1	40		QC2	700	
	Reinsubstanz	**Extrakt**	**Matrix**	**Reinsubstanz**	**Extrakt**	**Matrix**
MESSUNG	219464.0	227291.0	161170.0	3596642.0	3614730.0	3106134.0
	247428.0	239833.0	159996.0	3604112.0	3737338.0	2942415.0
	220865.0	228673.0	162225.0	3540798.0	3570417.0	3241564.0
	221646.0	235135.0	166095.0	3517241.0	3627754.0	2823929.0
	221109.0	230787.0	162358.0	3559390.0	3563477.0	2774757.0
	216205.0	225920.0	177399.0	3545811.0	3544876.0	2801319.0
Mittelwert	224452.8	231273.2	164873.8	3560665.7	3609765.3	2948353.0
SD	11425.5	5285.8	6468.8	33718.2	70035.1	188753.1
Varianz	130541115.8	27939154.6	41845684.6	1136918948.3	4904910408.7	35627738894.8
Anzahl	6.0	6.0	6.0	6.0	6.0	6.0

Wiederfindung,%	71.3	81.7
RSD, %	3.9	5.4
Matrixeffekt,%	103.0	101.4
RSD, %	3.1	1.6
Beurteilung	Ok	Ok

Kriterium: Recovery >=50%
SD <=25%
Matrixeffekte: 75-125%

Anmerkungen

Validierungsprotokoll

Seite:	2 von 7	**Institution:**	
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1. Arbeitsbereich und Kalibrationsmodell

1.1 TARGET — **Messsignal:** 281 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	4.5	15.2	32.4	71.1	277	326	503			
MESSUNG 2	4.2	15.7	32.4	69.3	268	335	514			
MESSUNG 3	4.5	15.1	31.8	69.9	279	322	506			
MESSUNG 4	4.5	15.8	33.3	71.1	291	320	513			
MESSUNG 5	4.1	15.9	32.7	69.4	285	342	509			
MESSUNG 6	4.0	15.2	30.6	69.1	268	328	512			
Mittelwert	4.3	15.48333333	32.2	69.98333333	278	329	509			
SD	0.228	0.3545	0.923	0.9042	9.0685	8.5165	4.397			
Varianz	0.052	0.1257	0.852	0.8177	82.23766667	72.53066667	19.33366667			
Werte	6.0	6.0	6.0	6.0	6.0	6.0	6.0			
Ausreisser-Test nach Grubbs										
Extremwert	4.0	15.9	30.6	71.1	291	342	503			
Prüfwert	1.316	1.175	1.733	1.235	1.391	1.605	1.452			
Signifikanz 95%										
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Signifikanz 99%										
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.4674	**Prüfwert**	-3.99	**a**	000,000	**A**	0.00003
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	-000,010	**B**	0.45998
Bestanden?	ja	**Bestanden?**	ja	**R**	00,001	**C**	-7.188867
				Rest-SD	0.0000	**R**	0.9871
						Rest-SD	38.627647

1.2 QUALIFIER — **Messsignal:** 463 — **Messgröße:** — **Einheit:** ng/ml

Konzentration	10.0	50.0	100.0	200.0	500.0	800.0	1000.0			
MESSUNG 1	0.220	0.810	1.810	4.100	9.100	15.600	18.400			
MESSUNG 2	0.170	0.780	1.710	3.600	9.200	14.900	18.500			
MESSUNG 3	0.210	0.850	1.720	3.700	8.900	14.500	17.700			
MESSUNG 4	0.170	0.870	1.830	4.000	9.300	14.700	18.200			
MESSUNG 5	0.160	0.850	1.810	3.700	9.200	15.300	18.600			
MESSUNG 6	0.210	0.820	1.670	3.600	8.700	14.200	19.000			
Mittelwert	0.19	0.83	1.758	3.783	9.067	14.86666667	18.4			
SD	0.026	0.033	0.066	0.214	0.225	0.516	0.434			
Varianz	0.001	0.001	0.004	0.046	0.051	0.267	0.188			
Werte	6	6	6	6	6	6	6			
Extremwert	0.160	0.780	1.670	4.100	8.700	15.600	17.700			
Prüfwert	1.150	1.521	1.329	1.482	1.629	1.420	1.614			
Tabellenwert	1.822	1.822	1.822	1.822	1.822	1.822	1.822			
Straggler?	nein	nein	nein	nein	nein	nein	nein			
Tabellenwert	1.944	1.944	1.944	1.944	1.944	1.944	1.944			
Ausreißer?	nein	nein	nein	nein	nein	nein	nein			

Cochran-Test (Varianzenhomogenität) (Signifikanz 99%)		**Mandel-F-Test auf Linearität** (Signifikanz 99%)		**Lineare Kalibrationsfunktion** Y = a•x + b		**Quadratische Kalibrationsfunktion** Y = A•x² + B•x + C	
Prüfwert	0.4786	**Prüfwert**	0.0	**a**	0.018483	**A**	0.0
Tabellenwert	0.4866	**Tabellenwert**	21.19	**b**	000,000	**B**	0.018447
Bestanden?	ja	**Linear?**	ja	**R**	00,001	**C**	-0.035098
				Rest-SD	0.1077	**R**	0.9999
						Rest-SD	0.1204

Validierungsprotokoll

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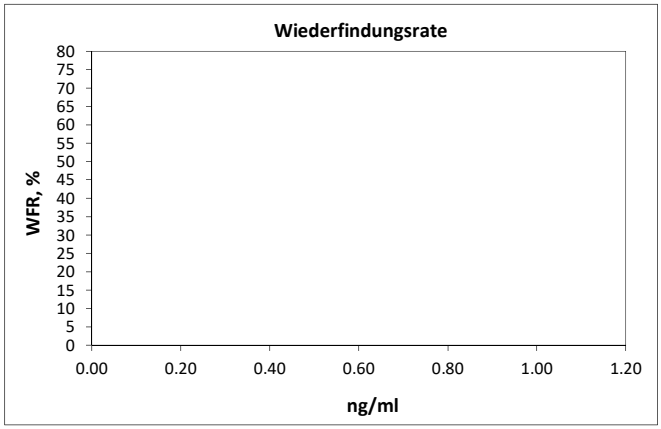
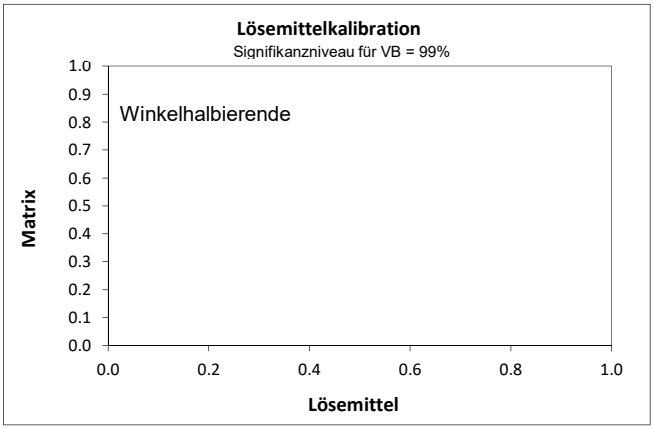
1.3 LÖSEMITTELKALIBRATION

Signifikanz	%
Konzentration	
Lösemittel (xo)	
Matrix (xm)	

WFR gemessen	
WFR berechnet	

Wiederfindungsfunktion **xm = ao * xo + bo**	**Ausreißer-F-Test**	**Linearitäts-Test**	**Varianzenhomogenitäts-F-Test**
ao	**Kalibrator Nr.**	**Prüfwert**	**RSD Grundkalibration**
bo	**Prüfwert**	**Kritischer-Wert**	**RSD Wiederfindung**
R	**Kritischer-Wert**	**Bestanden?**	**Prüfwert**
Rest-SD	**Ausreisser?**		**Kritischer-Wert**
			Bestanden?





2. Genauigkeit

2.1. Level 1

QC-Sollwert: 40 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	38.429	39.321	43.75	41.815	42.023	43.862	36.757	35.46	33.535	
2	37.165	41.852	39.426	43.801	40.134	41.713	37.065	37.532	34.891	
3	38.728	43.24	41.745		42.185	40.608	37.988	36.49	34.983	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	38.11	41.47	41.64	42.81	41.45	42.06	37.27	36.49	34.47	
BIAS, %	-4.7	3.7	4.1	7.0	3.6	5.2	-6.8	-8.8	-13.8	
SD	0.83	1.99	2.16	1.40	1.14	1.65	0.64	1.04	0.81	
RSD, %	2.2	4.8	5.2	3.3	2.8	3.9	1.7	2.8	2.4	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	39.40	**SD**	0.34	**SD**	2.55
SD	3.06	**RSD, %**	0.9	**RSD, %**	6.5
RSD, %	7.8				

Richtigkeit		**95%-Intervall**	OK
Abw.	-0.60	**Faktor**	2.43
Bias, %	-1.5	**ß-Toleranz**	0033 bis 0045
		Prüfbereich (30%)	0028 bis 0052
		Prüfbereich (40%)	0024 bis 0056

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2.2. Level 2

QC-Sollwert: 400 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	459.2	385.7	386.0	391.8	384.9	417.4	413.4	428.2	431.6	
2	407.9	387.2	362.9	431.1	386.6	425.6	420.4	417.8	433.1	
3	391.2	381.7	414.2		409.7	420.8	427.4	405.7	419.4	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	419.47	384.86	387.72	411.43	393.69	421.25	420.37	417.24	428.04	
BIAS, %	4.9	-3.8	-3.1	2.9	-1.6	5.3	5.1	4.3	7.0	
SD	35.42	2.85	25.69	27.80	13.85	4.12	7.00	11.27	7.49	
RSD, %	8.4	0.7	6.6	6.8	3.5	1.0	1.7	2.7	1.7	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	409.26	**SD**	6.74	**SD**	16.94836525
SD	21.70665039	**RSD, %**	1.6	**RSD, %**	4.1
RSD, %	5.3				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	9.26	**Faktor**	2.426
Bias, %	2.3	**ß-Toleranz**	377,62648 bis 459,84509
		Prüfbereich (30%)	280,0 bis 520,0

2.3. Level 3

QC-Sollwert: 700.0 **Einheit:** ng/ml

MESSUNG	Tag 1	Tag 2	Tag 3	Tag 4	Tag 5	Tag 6	Tag 7	Tag 8	Tag 9	Tag 10
1	679.2	637.5	661.6	716.9	735.6	756.5	706.9	647.0	716.1	
2	659.5	640.4	637.2	#BEZUG!	757.0	755.3	704.4	712.4	715.1	
3	665.7	636.4	642.5	726.6	693.8	776.5	710.8	640.7	663.1	
4										
5										
6										
7										
8										
9										
10										
Mittelwert	668.2	638.1	647.1	721.7	728.8	762.7	707.4	666.7	698.1	
BIAS, %	-4.5	-8.8	-7.6	3.1	4.1	9.0	1.1	-4.8	-0.3	
SD	10.05	2.04	12.84	6.86	32.12	11.90	3.22	39.72	30.29	
RSD, %	1.5	0.3	2.0	1.0	4.4	1.6	0.5	6.0	4.3	

Kenndaten	**OK**	**Wiederholpräzision**		**Laborpräzision**	
MW (ges.)	692.11	**SD**	1.66	**SD**	44.61
SD	43.81	**RSD, %**	0.2	**RSD, %**	6.4
RSD, %	6.3				

Richtigkeit		**95%-Intervall**	**OK**
Abw.	-7.89	**Faktor**	2.431
Bias, %	-1.1	**ß-Toleranz**	00576 bis 00793
		Prüfbereich (30%)	00490 bis 00910

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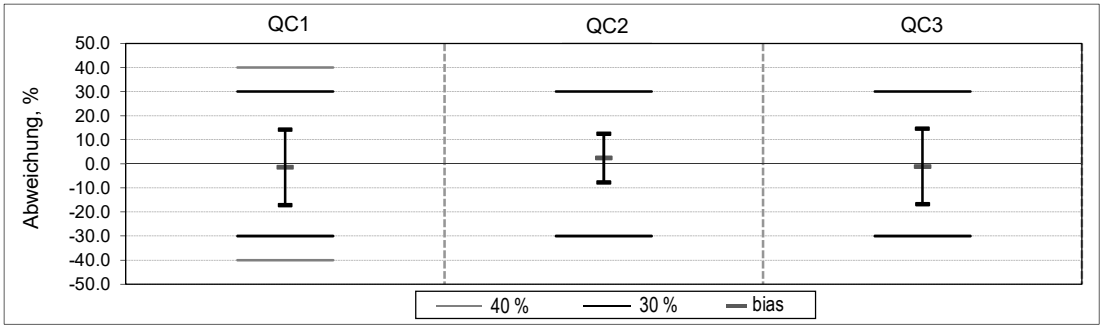
Zusammenfassung 95%-Intervall

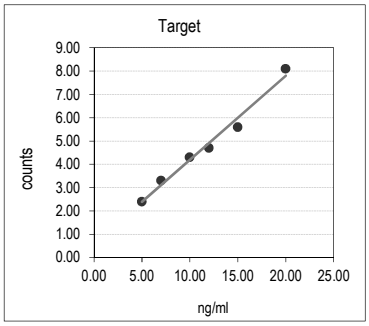


Zusammenfassung	QC1	QC2	QC3
ß-Toleranz	0033 bis 0045	377,62648 bis 459,84509	00576 bis 00793
%	- 017 bis 014	-7,73 bis 12,36	-17 bis 15
Prüfbereich (30%)	0028 bis 0052	280,0 bis 520,0	00490 bis 00910
Prüfbereich (40%)	0024 bis 0056		

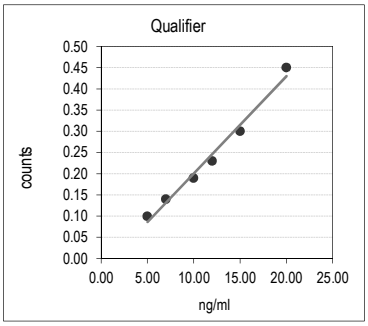
3. Grenzwerte

3.1 Bestimung nach DIN 32645

	Kalibrator	Target	Qualifier
Einheit			
1	5.0	2.400	0.100
2	7.0	3.300	0.140
3	10.0	4.300	0.190
4	12	4.700	0.230
5	15	5.600	0.300
6	20	8.100	0.450
7			
8			
9			
10			



Lineare Kalibration



Lineare Kalibration

	6	6
Signifikanz	99	99
k-Wert	3	3
Ausreißer-F-Test		
Kalibrator Nr.	5	6
Prüfwert	4.7	31.76
Krit.-Wert 99%	34.11	34.11
Ausreißer	nein	nein
Linearitäts-Mandel-Test		
Prüfwert	2.08	54.92
Krit.-Wert 99%	34.11	34.11
Bestanden?	ja	nein
a	0.360535	0.02291
b	0.587179	-0.028462
R	0.9914	0.9919
Rest-SD	0.292	0.0179

Grenzwerte		
Nachweisgrenze	3.35	3.23
Erfassungsgrenze	6.69	6.47
Bestimmungsgrenze	6.59	6.44

Validierungsprotokoll

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3.2 Bestimmung mittels Alternativmethode

3.2.1 Nachweisgrenze aus Signal/Rauschverhältnis

	Dotiert ng/ml	Target 0	Untergrund 0	Target S/N	Q1 0	Untergrund 0	Q1 S/N	Q2 0	Untergrund 0	Q2 S/N
1										
2										
3										
4										
5										
6										
			NG-1	0.0		NG-2	0.0		NG-3	0.0

Nachweisgrenze :	**0.0**

3.2.2 Bestimmungsgrenze

Vorgabe	ng/ml

MESSUNG	Konz.
1	
2	
3	
4	
5	
6	

Wiederholpräzision		Bias	
Mittelwert		Bias	
SD		Bias, %	
RSD, %			
Krit. Wert, %	**20**	**Krit. Wert, %**	**20**

Beurteilung	**---**	Kriterium: RSD und bias<20%

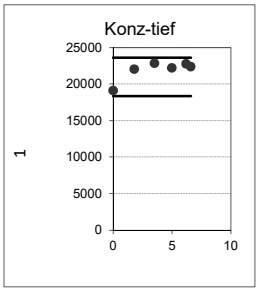
4. WIEDERFINDUNG

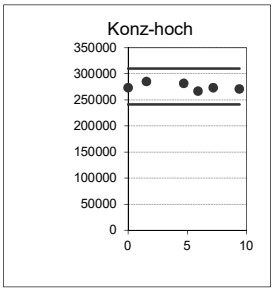
4.1 WIEDERFINDUNG

	QC-untere		QC-obere	
Konzentration	**ng/ml**		**ng/ml**	
	Lösemittel	**Matrix**	**Lösemittel**	**Matrix**
MESSUNG				
1				
2				
3				
4				
5				
6				
Mittelwert				
SD				
RSD, %				
N (Werte)				
Wiederfindung,%				
RSD, %				

4.2 STABILITAT

	Konz-tief Zeit	QC-Pool	Konz-hoch Zeit	QC-Pool
MESSUNG	0.00	19128	0.00	273088
	1.80	22074	1.55	284754
	3.50	22868	4.70	281513
	5.00	22248	5.90	266399
	6.20	22801	7.20	273311
	6.60	22412	9.40	271024
Werte	6		6	
min. Wert	19128	1	266399	1
max. Wert	22868	1	284754	1
Spanne	3740	1	18355	1
Spanne, %	16.4	%	6.4	%
Prüfwert	25.0	%	25.0	%
Steigung	0.0014		-0.0002	
Abschnitt	-27.1109		69.4465	
R	0.76495		-0.45754	
Beurteilung	**OK**		**OK**	





Kriterium: Spanne < Prüfwert

Validierungsprotokoll

4.3 MATRIXEFFEKTE - LC/MS/(MS)

	QC1			QC2		
	Reinsubstanz	**Extrakt**	**Matrix**	**Reinsubstanz**	**Extrakt**	**Matrix**
MESSUNG	274399.0	252443.0	165222.0	3596642.0	3634730.0	2500613.0
	281520.0	260943.0	158104.0	3604112.0	3737338.0	2642415.0
	251338.0	252506.0	166316.0	3540795.0	3570417.0	2541564.0
	264052.0	257380.0	176619.0	3517241.0	3627754.0	2423929.0
	258081.0	261913.0	172528.0	3559390.0	3563477.0	2374757.0
	260886.0	260335.0	186571.0	3545811.0	3544876.0	2501319.0
Mittelwert	265046.0	257586.7	170893.3	3560665.2	3613098.7	2497432.8
SD	11077.8	4239.1	9979.3	33718.6	70790.5	93214.6
Varianz	122717082.0	17969732.3	99585535.1	1136942791.0	5011294408.7	8688955574.6
Anzahl	6.0	6.0	6.0	6.0	6.0	6.0

Wiederfindung,%	66.3	69.1
RSD, %	3.7	2.0
Matrixeffekt,%	97.2	101.5
RSD, %	4.4	1.6
Beurteilung	Ok	Ok

Kriterium: Recovery >=50%
SD <=25%
Matrixeffekte: 75-125%

Anmerkungen

10. Acknowledgement and thanks

I want to thank Alex Müller sincerely for the supervision and support during the last years. I am grateful he always had an open door and ear for my ideas and questions and I enjoyed the interesting discussions we had. The knowledge he provided is remarkable and I really appreciate the trust and support he showed towards me. Without his great thoughts and ideas and constructive criticism, the thesis would have not been as it is. Beyond the professional exchange I will also remember the fun talks about sports, camping, biking and much more.

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11. Eidesstattliche Versicherung

Hiermit versichere ich Eides statt, die vorliegende Dissertation selbst verfasst und keine anderen als die angegebenen Hilfsmittel benutzt zu haben. Die eingereichte schriftliche Fassung entspricht der auf dem elektronischen Speichermedium. Ich versichere, dass diese Dissertation nicht in einem früheren Promotionsverfahren eingereicht wurde.

Nadine Aboutara Hamburg, 27.03.2022