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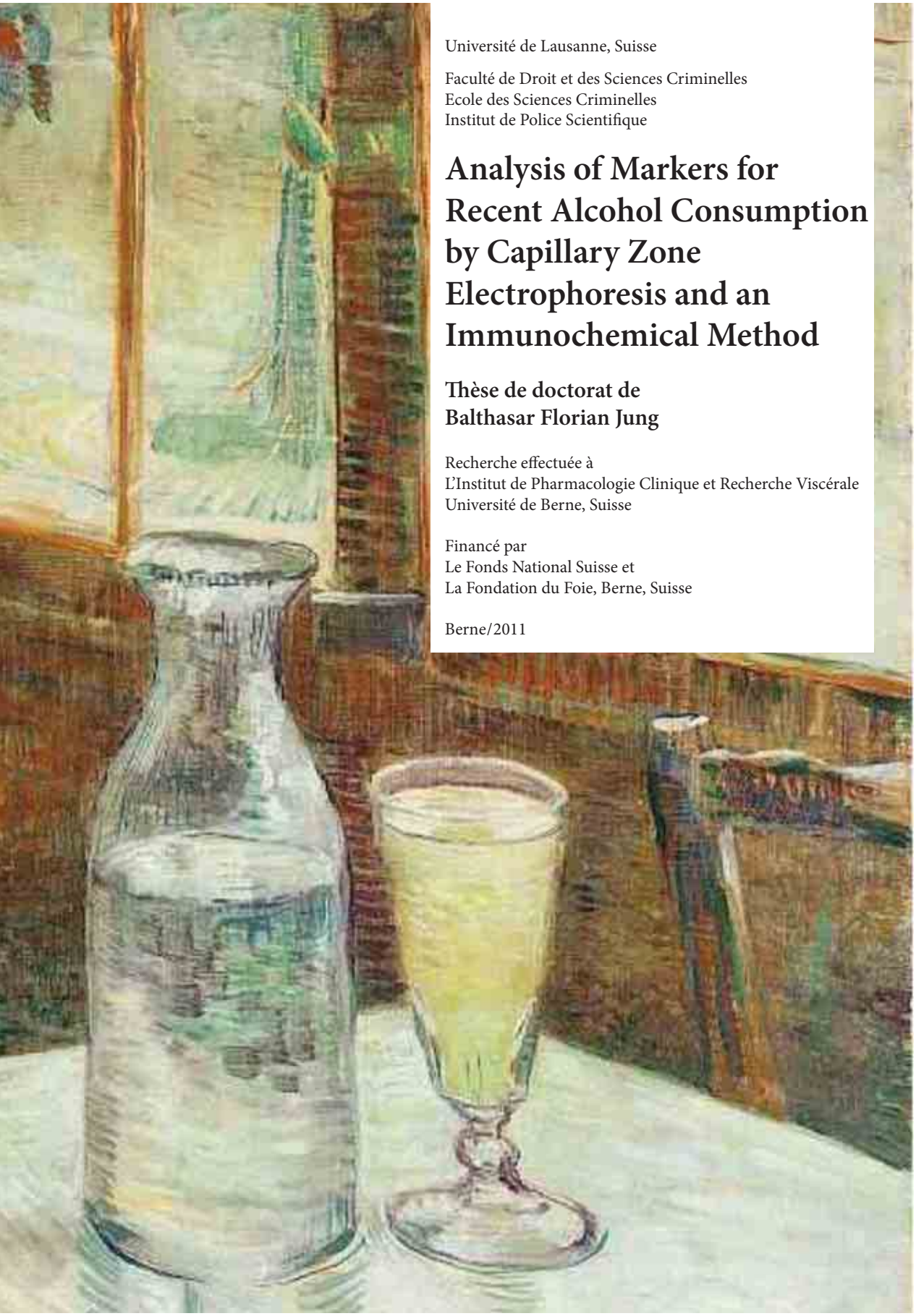
# Analysis of Markers for Recent Alcohol Consumption by Capillary Zone Electrophoresis and an Immunochemical Method

Jung Balthasar Florian



Université de Lausanne, Suisse

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# Analysis of Markers for Recent Alcohol Consumption by Capillary Zone Electrophoresis and an Immunochemical Method

**Thèse de doctorat de**
**Balthasar Florian Jung**

Recherche effectuée à
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*Cover picture "Still Life with Absinthe" by Vincent van Gogh (1887)*
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University of Lausanne, Switzerland

Faculty of Law and Criminal Sciences
School of Criminal Sciences
Forensic Science Institute

# Analysis of Markers for Recent Alcohol Consumption by Capillary Zone Electrophoresis and an Immunochemical Method

**PhD thesis of Balthasar Florian Jung**

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**IMPRIMATUR**

A l'issue de la soutenance de thèse, le Jury autorise l'impression de la thèse de M. Balthasar Jung, candidat au doctorat en sciences forensiques, intitulée

« Analysis of Markers for Recent Alcohol Consumption by Capillary Zone Electrophoresis and an Immunochemical Method »

Le Président du Jury

Professeur Franco Taroni

Lausanne, le 18 février 2011

**To alcohol!**
**The cause of, and solution to, all of life's problems.**

*Homer Jay Simpson from the animated television series The Simpsons (Episode 171, 1997)*
*Created and produced by Matt Groening*

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# RÉSUMÉ

Le but de ce travail doctoral était le développement de méthodes analytiques pour la détermination d'éthyl glucuronide et d'éthyl sulfate. Ces deux substances sont des métabolites directs de l'éthanol qui peuvent être détectées pendant des heures jusqu'à des jours dans des fluides corporels, après que léthanol ait été complètement éliminé du corps humain. Ce sont donc des marqueurs de consommation récente d'alcool.

La majorité des expériences ont été effectuées en utilisant l'électrophorèse capillaire. Il était envisagé de fournir des méthodes utilisables dans des laboratoires de routine. Des méthodes électrophorétiques ont été développées et optimisées pour la détermination d'éthyl sulfate dans le sérum et l'urine ainsi que pour l'éthyl glucuronide dans le sérum. L'éthyl glucuronide urinaire a pu être déterminé par un immunoassay commerciale qui a en plus été adapté avec succès pour des échantillons de sérum. Avec toutes ces méthodes d'analyse il était possible d'observer les deux marqueurs de consommation d'alcool récente, même une consommation aussi basse qu'un verre de boissons alcooliques.

Finalement, une étude englobant plus de 100 échantillons aété effectuée avec l'ambition de déterminer les valeurs de référence pour l'éthyl glucuronide dans le sérum et l'urine. De plus, la nécessité de normaliser les échantillons d'urine par rapport à la dilution a été investiguée. Grâce à cette étude des valeurs de cut-off et une base statistique pour l'interprétation probabiliste ont pu être proposées.

# 1
# INTRODUCTION

## 1.1 Alcohol Markers

Alcohol [1] consumption is a serious issue for mankind in many different facets. In 2002 for instance, alcohol was estimated to cause 6.4 % of all deaths in Europe [1]. Not only acute intoxication, but also the long-term exposure to alcohol in persons suffering from the alcohol dependence syndrome [2] have severe influence on the human body. The global scale of health issues associated with alcohol has prompted the World Health Organization (WHO) to start the discussion about alcohol dependence in the 1960s. Today, the WHO describes the "Mental and behavioural disorders due to use of alcohol", e.g. acute intoxication, harmful use, dependence syndrome, withdrawal state and more in Chapter V – F10 of the International Classification of Diseases (ICD-10, [2]).

In order to treat persons with problematic patterns of alcohol consumption, physicians must be able to identify individuals with alcohol related problems. The major problem diagnosing alcohol dependence is the patient's characteristic denial. The anamnesis and examinations including specific questionnaires might not be sufficient to estimate the pattern of a person's alcohol consumption. The required objective indicators for the estimation of someone's drinking behavior can be provided by "markers of alcohol consumption". These substances which can be found in many different matrices of the body may serve for clinical and forensic cases, e.g. relapse drinking, driver's license-suspension cases, crimes committed under the influence of alcohol, post-mortem cases. Hence, the evidence provided by alcohol markers may be incriminating as well as exculpatory for an individual.

The presence of different substances in the body can be related to alcohol consumption. The following list comprises the most commonly used alcohol markers. Some of these substances will later be discussed in detail.

[1] *In this text the term alcohol is used as a synonym for the chemically correct term ethanol.*

[2] *Alcohol dependence syndrome is the medical correct term for what is commonly known as "alcoholism".*

- ethanol
- 5-hydroxytryptophol (5-HTP)
- fatty acid ethyl ester (FAEE)
- ethyl glucuronide (EtG)
- ethyl sulfate (EtS)
- aspartate and alanine aminotransaminases (AST / ALT)
- β-hexosaminidase (HEX)
- carbohydrate-deficient transferrin (CDT)
- phosphatidyl ethanol (PEth)
- mean corpuscular volume (MCV)
- γ-glutamyltransferase (GGT)

It is important to differentiate between markers for recent and chronic alcohol intake [3-7]. Markers for chronic alcohol consumption detect long term exposure to alcohol, while markers for recent alcohol consumption can record one single intake of alcohol. However, there is no distinct separation between these two groups of substances. Depending on the evaluated situation or on the matrix available for analysis, some of the substances mentioned above might serve as marker for recent and/or chronic alcohol consumption.

The example of a fictitious car accident should be helpful for the understanding of the terminology: As soon as the police reaches the scene, the driver will be tested for alcohol intoxication, usually determined by breath analysis of ethanol. Positive results may be confirmed by a blood sample.

If the accident was a hit and run case, where some hours later the police arrests the person suspected having caused the accident, it is possible that no alcohol is found in his body. However, in this case, an investigation can focus on markers for recent alcohol consumption in order to prove that the suspect recently consumed ethanol. Such substances might be EtG, EtS, and FAEE.

After a car accident under influence of alcohol intoxication, the court may decide to suspend the driver's license. For regaining the license,

the court can request a proof that the individual does not suffer from the alcohol dependence syndrome. The driver could be regularly tested by a physician over a certain period of time for markers of chronic alcohol consumption. Markers used in this case might be CDT, MCV or GGT. Other substances in body fluids or different matrices (e.g. EtG in hair) can also be used for this purpose.

It is important to understand that the general drinking behavior of an individual can only be outlined having the information of a variety of different alcohol markers. Furthermore, it is crucial to define the hypothesis to evaluate before testing an individual with regard to the considered assumption. If a random test is performed without defining the purpose of the analysis, the outcome might be trivial and not helpful for the interpretation of the obtained data.

As seen in the example above, alcohol itself is the most evident marker for recent alcohol consumption which can be determined in many different sample matrices and by various analytical techniques. Nevertheless, it rapidly gets metabolized and thus only stays in the body for a rather short period of time. The main pathway of detoxification (> 90 %) is its oxidation to acetaldehyde by alcohol dehydrogenase (ADH), cytochrome P450 (particularly CYP2E1) and catalase. Finally, acetic acid is formed after further metabolism by aldehyde dehydrogenase (ALDH) [8-10]. Less than 10 % of the ingested alcohol leave the body unchanged through urine, sweat or breath [11, 12].

A minor part of the alcohol gets metabolized by non-oxidative pathways. Although the contribution of these pathways is not relevant for the detoxification, the resulting metabolites can be found in the human body. These non-oxidative metabolites are specific for the presence of alcohol, i.e. they are only formed in presence of alcohol. Figure 1 illustrates the normal alcohol metabolism in Caucasians.

The direct alcohol metabolites usually possess half-lifes (first-order kinetics) and remain longer in the body than alcohol itself (zero-order

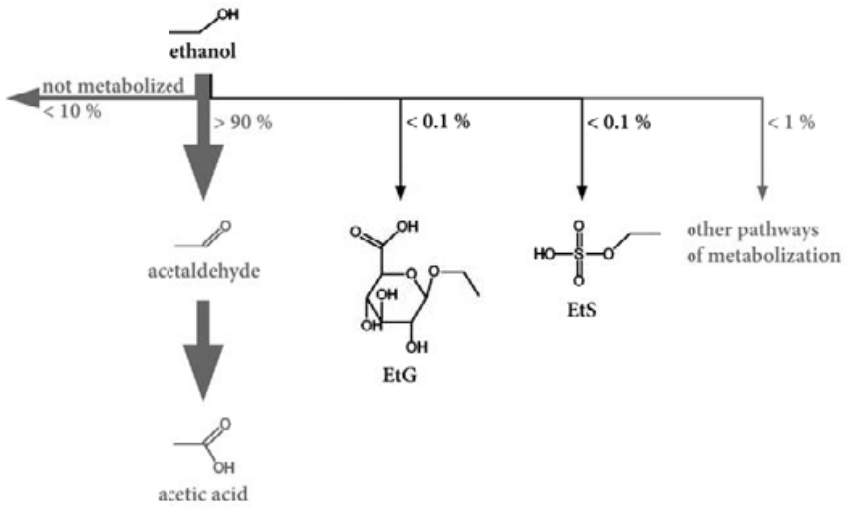



***Figure 1*** *Normal metabolic pathway of ethanol in Caucasians*

kinetics). So they can be used as markers for alcohol consumption. In today's practice, four such substances are used for forensic and clinical applications:
FAEE get formed by the reaction of alcohol with free fatty acids [13-16]. PEth is a phospholipid also only produced in presence of alcohol [8, 17, 18]. Less than 0.1 % of the ingested alcohol undergoes glucuronidation or sulfonation [3] to form EtG and EtS, respectively [19-24]. This work focuses on the determination of EtG and EtS in serum and urine. These two direct metabolites of alcohol are discussed in detail below in Chapter 1.2.

[3] *The term sulfonation is the appropriate descriptor for the conjugation reaction where a sulfonate and not a sulfate from the coenzyme of the cytosolic sulfotransferases, the 3'-Phosphoadenosine-5'-phosphosulfate, is transferred to the xenobiotics [54]. In this text this term is used rather than the often misused term sulfation.*

Markers for chronic alcohol consumption often depict metabolic changes caused by the toxic effect of long time exposure to ethanol. Generally, these substances are less specific for alcohol consumption than the recent alcohol markers. Such a marker for chronic alcohol consumption was studied intensively in the laboratories of the Institute of Clinical Pharmacology and Visceral Research (ICPVR) in Bern, and an electrophoretic method for the determination of carbohydrate-deficient transferrin (CDT) was introduced to the affiliated routine laboratory [25-29]. Only excessive intake of alcohol over a long period of time (> 50 to 80 g per day; > 2 weeks) results in increased levels of CDT [30, 31]. The half-life of CDT is roughly one week. Thus, CDT permits to demonstrate a person's chronic alcohol consumption even after weeks of alcohol abstinence, at a time when markers for recent alcohol consumption provide a negative result [32].

# 1.2 Ethyl Sulfate and Ethyl Glucuronide

As mentioned above, the two markers for recent alcohol consumption, EtS and EtG, are both direct metabolites of ethanol (for their structural formula refer to Figure 1 in Chapter 1.1). The two molecules are similar in terms of formation (conjugation reactions), size, half-life and elimination from the body. Nevertheless, they need to be discussed separately in order to provide an accurate introduction of the two alcohol markers.

In the early 1950s Kamil et al. discovered that alcohol does not exclusively get metabolized by oxidation and that a small fraction of the ingested alcohol gets conjugated with glucuronic acid [33, 34] to form EtG. However, it took until the 1990s, when Schmitt et al. recognized the potential of EtG as a marker for alcohol consumption and developed methods for its determination in serum and in hair [35-37]. Since then, the analysis of EtG in biological samples has attracted considerable attention.

Eighteen human Uridine Diphosphate-Glucuronosyltransferase-glucuronosyltransferases (UGT) have been described up to date [38]. Twelve of these are known to conjugate alcohol with glucuronic acid, derived from the cofactor UDP-glucuronic acid. The two most frequent isoforms are UGT1A1 and 2B7 [39, 40]. Although glucuronidation generally is an important way of detoxification of exogenous and endogenous substances, it metabolizes alcohol only to a minor extent (< 0.1 %) [19-21, 23, 24, 39]. EtG peaks in serum about 2 h after the maximum concentration of alcohol was reached (Figure 2 A) and then undergoes renal excretion due to good water solubility of the conjugated compound.

Not only serum, urine and hair samples were analyzed for EtG, but also other matrices like tissues, bone marrow and sweat. Besides the determination of EtG in body fluids, its analysis in hair samples is in the focus of research [24]. The Society of Hair Testing published a consensus on hair testing for chronic excessive alcohol consumption [41], showing the worldwide interest in this subject. The segmentation of hair and the determination of embedded alcohol metabolites is a promising perspective for the future.

Some UGT enzymes are known to express polymorphisms, but only the polymorphism of the UGT1A1 appears to translate into functional significance (e.g. Gilbert's disease) [42, 43]. To the knowledge of the author, no study evaluating the ability of alcohol glucuronidation by such phenotypes has been published. Anyhow, any functional difference in UGT1A1 is most likely masked by the other UGT isoforms [40]. In addition to the genetic impact and factors age and gender, large differences in the extent of glucuronidation may occur in the same individual. Intra-individual variability depends on many factors like hormonal status, disease state or exposure to xenobiotics [44, 45]. However, the extent of EtG production in the human body after alcohol consumption differs a lot so that up to date it is impossible to conclude on the amount and chronology of previous alcohol consumption. In presence of ethanol though, EtG appears to be formed in the human body in any case.

EtG was observed to be stable upon storage in urine and serum samples. When kept frozen at -20 °C, the EtG concentration did not undergo relevant alterations for months [46-50]. Stability is necessary in order to use the substance as alcohol marker. Furthermore, its non-volatile nature does not require special sample handling. However, it has been shown that EtG is sensitive to bacterial hydrolysis, particularly when specimens are infected by Escherichia coli. This microorganism is also able to form EtG from alcohol in urinary samples [51, 52]. Since Escherichia coli is the most common source of urinary tract infections in humans [51], one has to bear in mind that due to the presence of bacterial β-glucuronidase in samples, both, too low (breakdown of EtG) or too high (reverse reaction) urinary EtG results may occur.

A few years later than EtG, i.e. in the late 1950s, EtS was first reported as the product of the conjugation of activated sulfate and alcohol in rat liver [53]. Later on, Bonte and Bernstein described the sulfonation of alcohol in humans [55, 56, 57]. However, it is only in the last few years that EtS has started to attract the interest of the research in the field of marker for recent alcohol consumption in humans.

A total of eleven different cytosolic sulfotransferases (SULTs) have been identified in humans, with nine of them able to do in vitro sulfo-conjugation with alcohol. Nevertheless, only SULT1A3 and 1B1 are thought to be the key enzymes of in vivo sulfonation [22, 58]. All human SULT genes express genetic polymorphisms, however only few of them have functional genetic polymorphisms (none for SULF1A3 and 1B1 in Caucasians) [22, 58-62]. Today it is known that just a minor part of the ingested alcohol (< 0.1 %, exponentially rising with the elevation of the consumed dose of alcohol [22]) undergoes a sulfonate conjugation. It is still unknown though, which SULT isoforms are responsible for alcohol sulfonation in vivo.

Alike glucuronidation, sulfonation underlies a large intra-individual variability. Many different factors are responsible for these differences like hormonal state, diseases, nutrition, medicaments or obesity [63]. Thus, the inter-individual variations, e.g genetics and age, which may reach a similar level as the intra-individual variations, do not need special consideration. Furthermore, low or non-existing alcohol sulfonation of one isoform will get compensated in vivo by other SULTs [22]. Therefore, the extent of EtS production in the human body after alcohol consumption differs a lot so that it is very difficult, yet impossible, to conclude on the amount and chronology of previous alcohol consumption. Nevertheless, it is important to note that EtS seems to always be formed in the human body in presence of ethanol.

The peak concentration of EtS in serum is usually reached shortly before EtG achieves its maximum concentration, roughly 1.5 h to 2 h after the peak serum alcohol concentration (Figure 2 A). Nonetheless, the amount of alcohol metabolized to EtS, as well as its kinetics and renal excretion are very similar to EtG (half-life of about 3 to 6 h) [22, 64, 65]. Thus, EtS also stays detectable in body fluids for a considerable period of time after the complete elimination of alcohol from the body [19, 21, 63, 66, 67] (refer to Figure 2 B for urinary EtG and EtS concentrations).

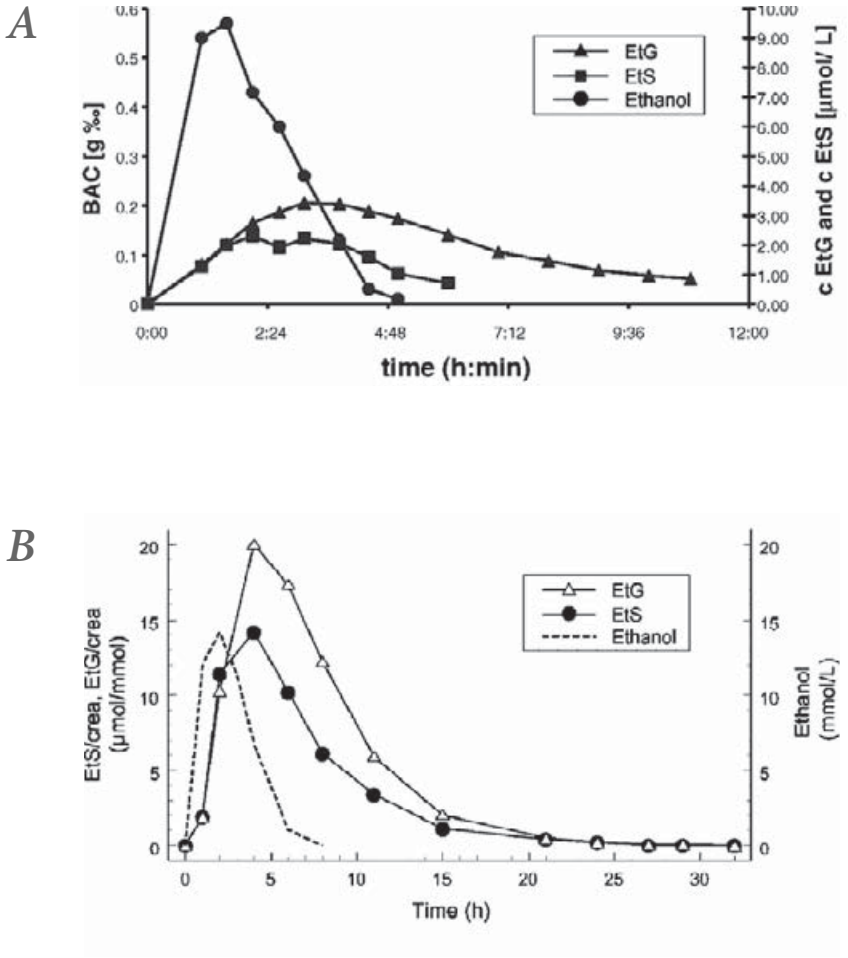



***Figure 2*** *A: Concentration-time profiles for ethanol in blood (BAC), serum EtS and EtG after alcohol ingestion. Blood samples were collected from a healthy male volunteer after ingestion of 0.52 g ethanol per kg body weight. Figure from [63].*
*B: Concentration-time profiles for urinary ethanol, EtS and EtG after alcohol ingestion. Urine samples were collected from a healthy male volunteer after ingestion of 0.5 g ethanol per kg body weight over 30 min in fasting state. EtS and EtG are expressed in relation to urinary creatinine (crea) to compensate for urine dilution. Figure from [21].*

Unlike EtG, EtS has never been reported to become hydrolyzed by bacterial action in urine. Also it seems that alcohol does not get sulfonized in urine by Escherichia coli or other common bacteria responsible for urinary tract infections [51, 52]. Urinary EtS is stable for more than 3 weeks when stored frozen [46, 47], and even when stored at room temperature, no relevant alterations of the EtS concentration were observed [64].

Summing up, EtG and EtS are well suited and established biomarkers for recent alcohol consumption. As direct metabolites of alcohol, they are specific for the presence of alcohol in the human body. Sensitivity of EtG to bacterial alteration is a drawback in certain cases, while resistance of EtS to such alterations seems to be advantageous.

The two metabolites are characterized by considerably longer elimination times than alcohol. So they can be detected in body fluids for hours to days after the complete elimination of alcohol itself. Unlike the metabolism of ethanol (zero-order), the elimination kinetics of EtG and EtS (first-order) can be described by half-lifes (roughly 3 to 6 h). Generally, EtS and EtG concentrations in urine and serum samples correlate well (Figures 2 A and B) [19, 21, 68]. In some body fluids though, only one of the direct metabolites was detectable after a certain period of time [21, 63, 64]. Due to their different metabolic pathways, EtS and EtG may be used simultaneously, thereby providing a better sensitivity to recent alcohol consumption. This can be very beneficial for forensic, as well as for clinical cases.

Long term exposure to alcohol often is harmful for the individual's liver. Thus, it is important to note that the two discussed metabolites of ethanol also form in persons suffering from severe liver diseases. Unlike the oxidative pathway, even at late stages of liver cirrhosis, the conjugation reactions (e.g. glucuronidation and sulfonation) still work efficiently [69-71]. Furthermore, these reactions are not limited to the hepatic system, but are also working in other organs. So it has been shown that liver cirrhosis is not an influencing factor of EtG levels in urine [72].

Since EtG, by the exception of bacterial interference, and EtS are only formed in the living body, their determinations in samples of post-mortem cases might provide important evidence for the elucidation of the source of ethanol present in the sample: was the alcohol ingested prior to death or was it produced by enzyme activity of bacteria, yeast and molds during the putrefaction of the body. Many publications focus on the use of EtG and EtS in post-mortem cases [68, 73-76]. Nevertheless, more specific studies are needed in order to be able to use these two direct metabolites of alcohol for such cases.

Although EtG and EtS have been detected in non-negligible quantities in alcoholic beverages [46], to date, no dietary ethanol-free source of the two alcohol markers is known. The issue of EtS and EtG formed from endogenous ethanol is discussed in Chapter 6.3.3 below.

Thus, EtG and EtS are potent markers for recent alcohol consumption, covering a clinically and forensically important time window from the complete elimination of ethanol in the body until the disappearance of the two marker substances. Nevertheless, it is important to note that so far it is impossible to make conclusions on the amount and chronology of an individual's alcohol ingestion.

# 1.3 Capillary Zone Electrophoresis

Most of the experiments of this work were done by capillary zone electrophoresis (CZE). CZE which gained a lot of credits in the last 25 years [77-83] is a technique for the separation of charged molecules in a conductive liquid medium under the influence of an electric field. The electrophoretic migration velocity of a particle ($v_p$) is given by

*Equation 1* $$v_p = \mu_p E$$

where $\mu_p$ is the electrophoretic mobility of the particle and $E$ is the electric field strength. Equation 2 clarifies that when two species in a sample have different mobilities (e.g. different charges or frictional forces [4]), they will separate from one another as they migrate in an electric field. The electrophoretic mobility of the particle is given by

*Equation 2* $$\mu_p = \frac{z}{6\pi\eta r}$$

where $z$ is the charge of the particle, $r$ is its Stokes radius and $\eta$ is the viscosity of the medium. In other words, in a liquid medium and under the influence of an electric field, small and highly charged molecules will migrate faster than cumbrous molecules with low charge.

The electric field is generated by the application of a high voltage (dimension of kV) between two electrodes in the conductive liquid. The charged particles migrate toward the electrode of opposite charge. The application of the high voltage provokes two issues, namely joule heating in the electrolyte and electrolysis at the electrodes. The latter issue is solved by introducing an aqueous buffer solution as conductive liquid. This running buffer (RB) prevents pH variations due to electrolysis and other possible factors and thus ensures stable conditions of the system. The problematic joule heating on the other hand puts some limits to electrophoretic separation. Due to heating, diffusion of the particles raises and the RB eventually would begin to boil, making a reproducible process impossible. This is why separation

[4] *The frictional force depends on the viscosity of the medium as well as the size and shape of the particle under consideration.*

is done in a thin capillary with an inner diameter (I.D.) between 25 and 100 µm and an outer diameter of about 200 to 400 µm. The heat generated inside the capillary is dissipated through the capillary walls.

Capillaries are usually fabricated from fused silica; other materials have been used in the past as well, though. For easier handling, the stability of the fused silica capillaries usually is enhanced by an external coating with a polymer (usually polyimide). Thermostating systems of the capillary, as depicted schematically in Figure 3, provide better heat dissipation and thus allow to push the limits of maximum voltage. Nevertheless, the generated power inside the system must be monitored and the applied voltage may need adaption in order to achieve reproducible separations.

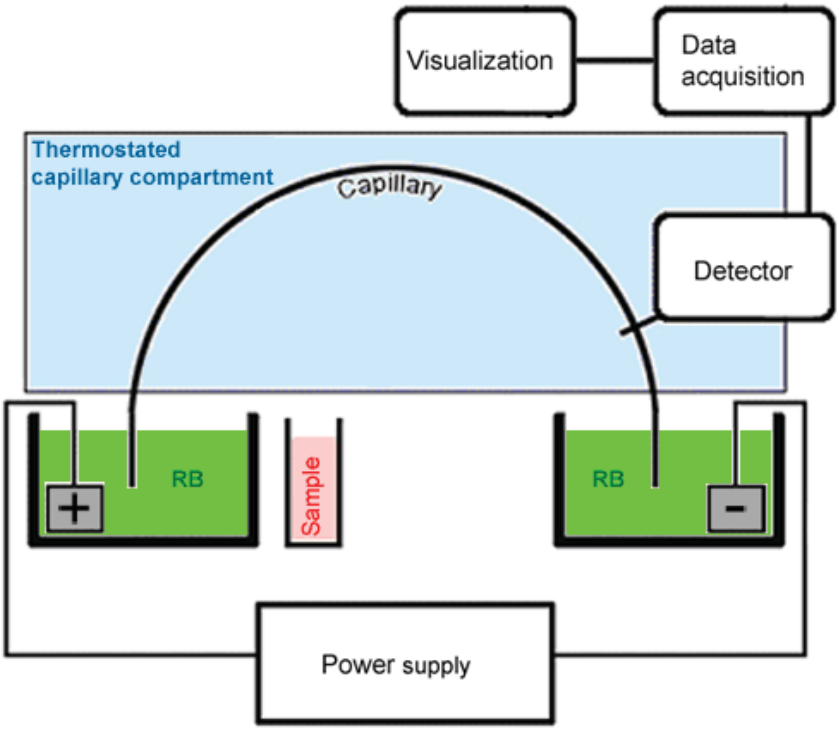



***Figure 3*** *Diagram of a CZE instrument (positive polarity).*

Figure 3 shows that in practice, CZE is a system composed of two buffer vials connected by a capillary. The whole system is filled with the RB and the electrodes are placed in the two vials. Prior to separation, the sample (in the order of nL) needs to be introduced into the capillary. This is done by placing one side of the capillary, the inlet, into the sample vial and applying pressure or vacuum, respectively for hydrodynamic injection, or electric power for electrokinetic injection. For the separation, the inlet end of the capillary is then placed back into the inlet buffer vial and the voltage is applied. With the anode on the inlet side of the capillary, the instrument is called to work with positive polarity; the opposite arrangement is called negative polarity (indicated with negative voltage). As described above, separated by differing electrophoretic mobilities, analytes migrate toward the electrode of opposite charge.

Theoretical determinations of the mobility may be inaccurate due to the lack of knowledge of the exact values of some parameters. However, the mobility can be determined experimentally by observing the time required for an analyte to reach the detector. The mobility is given by

*Equation 3* $$\mu = \frac{L_{eff} L_{tot}}{t_d U}$$

where $L_{eff}$ is the length of the capillary from the inlet to the detection site, $L_{tot}$ is the total length of the capillary, $t_d$ is the detection time of the analyte and $U$ is the applied voltage.

Uncharged molecules do not migrate electrophoretically. Nevertheless, in some cases they pass the detector. This is due to the movement of the entire electrolyte solution inside the capillary. This phenomenon is called electroosmotic flow (EOF). This flow can be determined experimentally injecting an uncharged substance and measuring the detection time of the compound. Uncharged particles are only moved forward by the stream of the EOF.

The mobility of the EOF can also be calculated according to

*Equation 4* $$\mu_{eof} = \frac{\varepsilon\zeta}{4\pi\eta}$$

where $\varepsilon$ is the relative permittivity of the buffer solution, $\zeta$ is the Zeta-potential [5] and $\eta$ is the viscosity of the electrolyte.

EOF is a consequence of the surface charge on the interior capillary wall. Figure 4 shows the cross-section of a capillary filled with RB. EOF results from the effect of the applied electric field on the solution doublelayer at the inner surface of the capillary [84]. The water of the hydration of the ions will get carried along, resulting in a continuous flow, called EOF, propelling all molecules inside the capillary.



***Figure 4*** *Cross-section of a fused silica capillary filled with an electrolyte solution. Figure from [84].*

It is possible to modify or even suppress the EOF in many different ways, e.g. raising the viscosity of the RB or applying a coating in order to change the surface charge. For certain applications no EOF or reversed EOF might be beneficial or even necessary for a good

[5] *The Zeta-potential is the potential difference close to the inner capillary wall that is built up by the counterions of the electrolyte solution by forming a diffuse double-layer in order maintain the charge balance.*

resolution of the analytes. EOF modifications are further discussed in the chapters about the experimental work executed.

Furthermore the flow profile of a system driven by EOF is flat, i.e. has a plug like form. This is distinctly different compared to pressure-driven flow which has a laminar flow profile. In addition, in CZE there is no mass transfer between phases. Thus, for this technique, very high separation efficiencies are observed with diffusion being the major limiting factor.

The observed velocity of an analyte in an electric field ($v$) is the sum of the particle's velocity ($v_p$) and the EOF's velocity ($v_{EOF}$):

*Equation 5* $v = v_p + v_{EOF} = (\mu_p + \mu_{EOF})E$

If none of the two velocities is equal to zero, the directions of the migration of the particle and the EOF may be the same or opposite. Hence, if the EOF towards the detector is strong enough, even analytes migrating in the opposite direction can eventually reach the detector.

Besides the high separation efficiencies discussed above, other advantages of CZE can be mentioned, e.g. the small injection volumes required, the economy and ecology of the technique, and the fast run times. Furthermore, sample preparation of body fluids for CZE is often very simple; even direct injections of the samples have been described in the literature [73]. Since in body fluids (i.e. aqueous solutions with pH > 6) conjugated metabolites occur as charged molecules, CZE represents an ideal technique for their separation.

## 1.4 CZE with Indirect and MS Detection

In the chapter above it has been shown that CZE is a very suitable technique for the separation of small ions in body fluids. However, in order to determine the concentration of an analyte in a sample, the substance needs to be registered by a detector. Many different detection techniques may be used in combination with CZE, e.g. (laser-induced) fluorescence, amperometry, radiometry or refractive index detectors.

For the most part of this work a UV detector (filter or diode array detector) was used. The measurement of the absorbance is done directely on the capillary, across its wall. For that purpose a "detection window" has to be fabricated by removing the outer coating of the capillary, leaving the transparent fused silica only. The signals from the detector are sent to a computer, where the data is processed and displayed as an electropherogram, which is a plot of detector response versus run time.

Measuring absorbance, UV detectors record absorbing analytes passing the detection window. Such direct detection is often used with CZE-methods. For EtS and EtG, however, this approach fails because the two substances do not absorb UV light and thus do not produce a measurable signal. If the analytes of interest are non-absorbing substances, indirect UV detection featuring an absorbing electrolyte may be used. For this case, the term background electrolyte (BGE) rather than RB will be used in this text. Applying UV detection, the displacement of the BGE by the analytes is observed, resulting in electropherograms with negative peaks. For this work the data has been mirrored so that "regular" electropherograms with positive peaks are depicted.

An alternative to indirect UV detection is conductivity detection. This technique is particularly suited for compounds that are elsewise difficult to detect, e.g. inorganic ions. Conductivity detection often provides better sensitivity than methods featuring indirect UV detection.

Another very potent detection technique in combination with CZE is the use of a mass spectrometer (MS). In this case, the detection is not accomplished on the capillary but at its end. Both, CZE and MS, being powerful analytical techniques, their ability of separation is quite complementary (e.g. substances of identical weight might be separated

by CZE and vice versa coeluting substaces might be distinguished by MS by their difference in weight or way of fragmentation).

The challenge of CZE-MS analysis is to couple the liquid phase separation technique with a vacuum detection technique. However, the low amounts of electrolyte solution eluting from a capillary during electrophoretic separation simplifies the task. A stable and commercialized ionization source for CZE-MS coupling is the electrospray ionization (ESI; Figure 5). The ESI is particularly suited for the ionization of polar and charged substances, which CZE generally deals with. However, the drawback is the challenging management of the electrical fields of the CZE system on one hand and of the ESI on the other hand. The electrode at the interface of the two instruments is actually shared and the method requires an intensive optimization procedure in order to find the best settings of all parameters.

Since CZE is a liquid-phase separation technique, an interface must be used to couple CZE with MS (Figure 5). The flow-rates from the separation capillary (about 0 to 100 nL $min^{-1}$) are much lower than the flow-rates observed in LC (in the low mL $min^{-1}$ range). The MS-interfaces are usually designed for the coupling of LC-MS and cannot operate at that low flow-rates. This problem is often solved by the addition of a make-up liquid, e.g. sheath liquid added coaxially to the separation capillary. A sheath gas is usually applied in this system in order to support the nebulization of the solution.

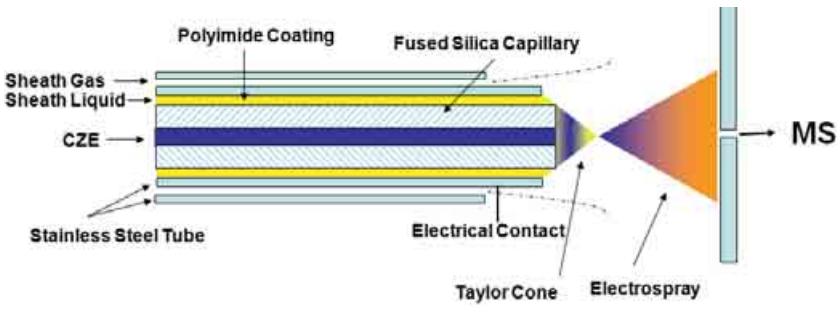



*Figure 5* *Diagram of a CZE-MS interface and ESI. Figure by Andrea Baldacci.*

## 1.5 Enzyme Immunoassay

The ultimate principle of an immunoassay is the analysis of a substance in a liquid phase based on the interaction of an antigen (analyte) with an antibody. Immunoassays make use of the high specificity and binding strength of such interactions.

If the assay uses an antibody or antigen labeled with an enzyme, it is called enzyme immunoassay (EIA). The reaction of a substrate catalyzed by this enzyme is used to confirm the presence of the analyte. The transformation is typically observed by a color change. Often the rate of the reaction is measured dynamically, i.e. at defined periods of time after the start of the reaction.

As mentioned above, antibodies or antigens can be labeled. In the case of a competitive immunoassay, a labeled competitor-antigen is used, i.e. a synthetic compound which has a similar structure as the analyte and which also binds to the antibody. The analyte and the labeled antigen compete for the binding sites of the antibodies. The enzymes (labels) are either inactive when bound by the antibody and active when free in solution, or vice versa. Therefore, the emitted signal may be directly or indirectly proportional to the concentration of the analyte.

# 2
# GOALS

Due to the worldwide spread of alcohol related problems, it is important to be able to detect recent alcohol consumption for forensic as well as clinical cases. The aims of the doctoral thesis were (i) to develop analytical methods for the determination of two markers of recent alcohol consumption, namely EtS and EtG, in serum and urine, (ii) to test the assays for routine suitability with large data sets of real samples from different individuals, and (iii) to introduce suitable methods at the associated routine laboratory of the ICPVR. The methods should be based on CZE featuring indirect detection and MS detection as well as on an EIA.

In order to achieve the last part of the goals, a study for the determination of the upper reference limit for EtG in urine and serum needed to be performed. The statistical evaluation with a considerable number of real samples should further allow the interpretation of patient samples using the frequentist approach.

# 3
# OVERVIEW OF THE EXPERIMENTAL WORK

## 3.1 Instrumentation and Samples

CZE measurements were performed on the P/ACE MDQ, P/ACE System 5010 and ProteomeLab PA 800 (Beckman Coulter, Fullerton, CA, USA) equipped with UV detectors (filter or diode array detector, DAD). MS was done on a Finnigan LCQ ion trap instrument (Finnigan MAT, San Jose, CA, USA) that was equipped with an ESI ionization source and sheath liquid interface (Finnigan). A PrinCE CZE system (Lauerlabs, Emmen, The Netherlands) was coupled to the ESI. For the automated immuno-chemical methods a Mira Plus analyzer from ABX Diagnostics (Diatools, Dietikon, Switzerland) was used.

Urine and serum samples used for this work stemmed from volunteers which gave their consent and provided information about recent alcohol consumption. Samples called "real samples" refer to test samples which were not spiked with the analytes of interest and served to demonstrate the suitability of the developed methods for case work. The author adapted the nomenclacture generally used in the literature and is aware of the fact that any sample discussed in this work actually is real.

## 3.2 Prediction of CZE Separations, Statistical Treatment and Data Presentation

The PeakMaster 5.2 software developed by the research group of Dr. Bohuslav Gaš (Charles University Prague, Czech Republic) was used to calculate effective mobilities and to investigate analyte separability [85, 86]. Simul 5, a software developed by the same group, was used to simulate CZE experiments and to optimize separation conditions [87]. Both programs are available for free download on internet (http://www.natur.cuni.cz/~gas/; last visited 27/01/2010).

Calculations, statistical analysis and data presentation was done using the PASW Statistics 17 and SigmaPlot Scientific Graphing Softwares (SPSS Inc., Chicago, Illinois, USA), R (R Foundation for Statistical Computing, Vienna, Austria; www.R-project.org, last visited: 15/08/09), and GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, California USA).

## 3.3 Discussed Data

Selected data obtained during the studies of EtG and EtS have been published in scientific journals:

- B. Jung, J. Caslavska, W. Thormann
  *Determination of ethyl sulfate in human serum and urine by capillary zone electrophoresis*
  Journal of Chromatography A 1206 (2008) 26

- B. Jung, J. Caslavska, W. Thormann
  *Determination of ethyl glucuronide in human serum by capillary zone electrophoresis and an immunoassay*
  Journal of Separation Science 32 (2009) 3497

- B. Jung
  *Preuve de consommation d'alcool récente grâce à la détermination du glucuronide et du sulfate d'éthyle dans des fluides corporels*
  Revue internationale de criminologie et de police technique et scientifique 62 (2009) 29

- C. Lanz, B. Jung, J. Caslavska, V. Deiss, W. Thormann
  *Alcohol markers reveal relapse drinking episode*
  Chimia 63 (2009) 522

- J. Caslavska, B. Jung, W. Thormann
  *Analysis of ethyl glucuronide and ethyl sulfate in serum and urine by CE-ESI-MS$^n$*
  Electrophoresis, accepted

These articles are attached to this document as Appendices I to V. In this work, the published data will be summarized in order to allow the reader to get an overview of the complete work which was done. Unpublished data will be discussed in more detail. For the sake of clarity, EtS and EtG are treated in separate chapters. The discussions of the two alcohol markers are structured in separate sections about

urine and serum, as well as the different techniques used for their determination. The basic goal of the work was to develop methods for the determination of both markers for recent alcohol consumption in serum and urine. Table 1 summarizes the achievements.

| Alcohol marker | Method | Urine | Serum |
|---|---|---|---|
| **EtS** | **CZE-UV** | Validated methods for quantitative analysis *Chapter 4.1* | Validated methods for quantitative analysis *Chapter 5.1* |
| | **CZE-MS** | Methods for qualitative analysis *Chapter 4.2* | Method for qualitative analysis *Chapter 5.2* |
| **EtG** | **CZE-UV** | Preliminary experiments *Chapter 6.1* | Validated methods for quantitative analysis *Chapter 7.1* |
| | **CZE-MS** | Methods for qualitative analysis *Chapter 6.2* | Method for qualitative analysis *Chapter 7.2* |
| | **EIA** | Evaluated method for quantitative analysis *Chapter 6.3* | Validated method for quantitative analysis *Chapter 7.3* |

***Table 1*** *Overview of the methods for EtS and EtG discussed in this work.*

# 4

# DETERMINATION OF URINARY ETHYL SULFATE

# 4.1 Ethyl Sulfate in Urine by CZE with Indirect Detection

The first published method for the analysis of EtS in body fluids was featuring liquid chromatography coupled with a mass spectrometer (LC-MS) [21]. Shortly thereafter, many publications followed describing methods for the detection of EtS, often determined simultaneously with EtG, in serum and urine by LC-MS or LC-$MS^2$ [22, 46, 47, 51, 52, 63, 65, 68, 73, 88]. In 2006, Esteve-Turrillas et al. published a method for the determination of urinary EtS by CZE [73]. The results were promising, however, they did not satisfy the requirements of the practical issues arising during routine use of a screening method.

## 4.1.1 Normalization of Urine Samples

The major problem of the work of Esteve-Turrillas et al. was that all urine samples were diluted 1:5 with water [73]. This does not take into account the very large variations in urine concentrations which may differ more than 100-fold in healthy individuals [89, 90]. Additionally, urine dilution can even be influenced by diseases or intentionally [23, 50, 91, 92]. With variations of urine concentration is meant that the components dissolved in the matrix occur at differing concentrations. Small variations may not be problematic for urinary analysis, stronger variations, however, may even make the application of certain analytical methods impossible. Furthermore, urinary samples should be normalized in regard to their concentration for qualitative comparison of the detected amount of analyte.
Generally, normalization of urine samples is done in regard to their creatinine concentration. Other possibilities to standardize the dilution of urine include conductivity, osmolality or density [89, 90, 93].
The conductivity of urine samples was found to correlate with the creatinine level [94]. The correlation of the conductivity and creatinine levels in urine samples obtained during the experiments for this work is discussed in Appendix VII.
When working with CZE, the conductivity of the samples can have a major impact on the separation. Hence, for the sake of reproducibility, it is advantageous to apply samples with similar conductivity. For the method described by Esteve-Turrillas et al. [73], particularly the fast

migrating anions covering a large part of the anions present in urine, e.g. chloride, sulfate and nitrate, should be present in similar amounts in the samples in order to provide comparable electropherograms.
In order to account for the dilution factor of the urine as well as to provide samples which are suitable for the analysis by CZE, a method for the normalization of urine samples in regard to their conductivity was developed. The procedure was then tested on a large set of samples from different individuals with the CZE method described above.
The adjustment to a specific conductivity was simple, fast and cost-effective, and was done prior to analysis.
Two strategies were investigated. Initially, urines were diluted with water to a conductivity of 5 mS $cm^{-1}$, mixed 1:1 with the internal standard (I.S.; vinylsulfonic acid, 8 mg $L^{-1}$) solution and analyzed. Some samples however, possessed conductivities < 5 mS $cm^{-1}$. For these samples this approach did not work. Furthermore, it was found that samples up to about 15 mS $cm^{-1}$ could be analyzed by CZE, with sharpest peaks resulting at a conductivity of 10 mS $cm^{-1}$ [95]. Thus, for the second approach, the conductivity of the urines was adjusted to 10 mS $cm^{-1}$ employing a urine aliquot according to

*Equation 6* $$V = \frac{\kappa_a}{\kappa} V_a$$

where $V$ is the urine aliquot [µL], $V_a$ is the sample volume after reconstitution [µL], $\kappa$ is the conductivity of the urine [mS $cm^{-1}$] and $\kappa_a$ a is the conductivity of the adjusted sample after reconstitution [mS $cm^{-1}$] [95]. With samples possessing similar characteristics it was possible to optimize the electrophoretic method. The same principle may also be adapted for the normalization in regard to urinary creatinine or another measurable parameter characterizing the concentration of urine.

### 4.1.2 Capillaries Coated with a Cationic Surfactant

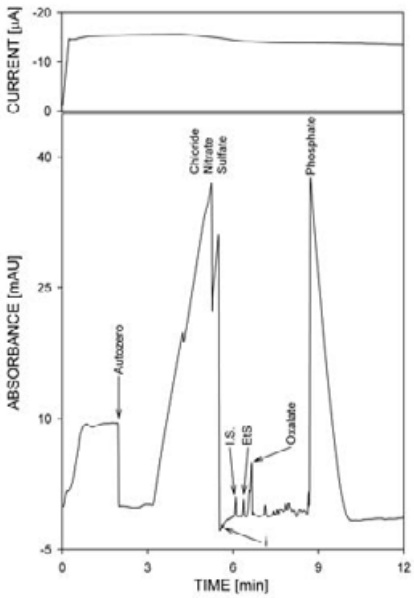



*Figure 6* *Typical electropherogram and temporal behavior of the current for the analysis of a blank urine (10 mS cm$^{-1}$) that was spiked with EtS (8 mg L$^{-1}$) and I.S. (vinylsulfonic acid, 8 mg L$^{-1}$) in a CTAB coated 50 µm I.D. capillary ($L_{tot}$ 60.2 cm; $L_{eff}$ 50.0 cm). The BGE consisted of 15 mM maleic acid, 1 mM phthalic acid and 25 µM CTAB (pH set to 2.20 by addition of NaOH). Experiments were done on a P/ACE MDQ with the detection wavelength set to 215 nm and a separation voltage of -19 kV. Key: i refers to an impurity of the I.S. Figure from [95].*

The BGE consisted of 15 mM maleic acid, 1 mM phthalic acid and 25 µM cetyltrimethyl-ammonium bromide (CTAB), a cationic surfactant. Both maleic acid and phthalic acid are absorbing compounds. The latter absorbs stronger in the region of the detection wavelength (215 nm). However, it is not well soluble and could not be added at higher amounts to electrolyte solution. The absorbing compounds of the BGE enabeled CZE featuring indirect detection.

The pH of the BGE was lowered (pH 2.2 instead of 2.5) in order to achieve better separation from matrix peaks. EtS is a strong acid (pKa < -3) [73, 96]. Therefore, at pH 2.2 the substance is completely deprotonated. This allowed the separation from the majority of the organic acids in the sample which were uncharged at such low pH. The uncharged compounds migrate much slower than EtS or migrate even with the EOF. Hence, EtS is detected in a zone between chloride and phosphate with almost no other substances around (Figure 6).

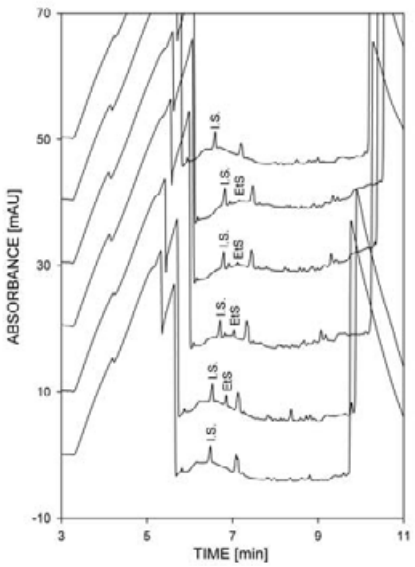



***Figure 7*** *Electropherograms obtained with the urines of a female individual who consumed 0.462 g of ethanol per kg body mass within 30 min. Urines (n=6) were sampled immediately before ethanol intake, and 3.5, 9.5, 11.5, 18 and 20.5 h after the beginning of ethanol intake (from bottom to top, and displayed with a y-axis offset for the sake of clarity). The urines were adjusted to a conductivity of 10 mS $cm^{-1}$, mixed with the I.S. (vinylsulfonic acid, 8 mg $L^{-1}$) and analyzed in a 50 µm I.D. fused silica capillary ($L_{tot}$ 60.2 cm; $L_{eff}$ 50.0 cm) using a BGE consisting of 15 mM maleic acid, 1 mM phthalic acid and 25 µM CTAB (pH set to 2.20 by addition of NaOH). Experiments were done on a P/ACE MDQ with the detection wavelength set to 215 nm and a separation voltage of -19 kV. Figure from [95].*

For the analysis of urine this fact is very important since an uncountable number of compounds can be present in this matrix at varying amounts, depending on nutrition, health state, age and many other factors concerning the sampled individual [89, 90].

Figure 7 illustrates the performance of the method, with the electropherograms of a series of six normalized urines. The urines were collected during more than 20 h from an individual who ingested about 32 g of ethanol in a short period of time. Prior to alcohol ingestion, and 20.5 h after the end of the consumption, no EtS was found in the samples. In the other urine samples the chronological rise and decrease of EtS can be observed. The ethanol content of these samples has not been determined. However, ingested at such amounts, alcohol itself is expected to only be detectable in urine for 3 to 5 h after the end of consumption.

### 4.1.3 Capillaries Coated with a Polycation

The handling of BGE containing CTAB can be quite tedious. The surfactant leads to strong foaming of the aqueous solution. Furthermore, in order to stabilize the system, long application of high voltage (-19 kV for at least 180 min) was required in the beginning of every working day. In addition, the 25 µM concentration CTAB in the BGE is known to be within a critical range in which a small change in the concentration of the surfactant strongly influences EOF [97]. For these reasons, a coating using poly(diallyldimethylammonium chloride) (PDADMAC, a polycation with a typical molecular mass from 200 to 350 kDa) in the BGE was evaluated as well. As CTAB, this substance is used to modify EOF [98-102]. The experience gained from tests with this coating substance for the development of CZE assays for EtG in serum was very helpful for the work described in this chapter. Experiments featuring dynamic coatings will therefore be discussed more in detail in Chapter 7.1.
CTAB and PDADMAC are used as dynamic wall coatings. They need to be reconditioned regularly, since they dynamically interact with the inner capillary wall and are not permanently bound to the capillary surface. Here, CTAB is comprised in the BGE, so that the coating is continuously reconditioned. PDADMAC, on the other hand, is applied to the capillary by a rinsing with a polycation-solution prior to filling the capillary with the BGE without PDADMAC for separation [95].

Aiming for better sensitivity for the detection of EtG, PDADMAC was used as a dynamic coating for capillaries with larger I.D. (100 µm) compared to the CTAB-coated capillaries (50 µm).

### 4.1.4 Assay Specification, Validation and Evaluation

Calibration curves in urine were calculated with five different concentrations (2, 5, 15, 35 and 100 mg $L^{-1}$ EtS). Most of the expected EtS concentrations in real samples are around the lower calibration points. Therefore, the suitability of different weighted linear regression models (based upon x, 1/x and $1/x^2$) were compared according to

Karnes and March [103]. The relative concentration residuals for each calibration point were calculated as

*Equation 7* $$\%RCR = 100\frac{RC - NC}{NC}$$

where *RC* represents the interpolated concentration (using the respective calibration curve) and *NC* is the nominal concentration. The sum of all *%RCR* (n=5) was found to be smallest for the $1/x^2$ weighted linear regression (for CTAB and PDADMAC coatings) and was therefore applied in this work.

The methods featuring CTAB (urine at 5 and at 10 mS $cm^{-1}$) as well as PDADMAC (only for urine at 5 mS $cm^{-1}$) were validated in terms of precision and accuracy, and showed to be robust assays allowing the monitoring of EtS in urine samples of persons who consumed as little as one standard drink of an alcoholic beverage (approximatively 14 g of ethanol) [95]. It was possible to monitor EtS after such moderate alcohol consumption thanks to the introduction of the normalization step during sample preparation and the optimization of the method leading to a more than 8-fold lower limit of quantitation (LOQ) than that reported by Esteve-Turrillas et al. [73].
It is further important to note that no EtS has been detected in any sample considered as blank (> 48 h of abstinence from alcohol).

The comparison of the two methods did not favor one of these.
Only the comparison of the limit of detection (LOD) showed better sensitivity for the method using the PDADMAC coated capillary (100 µm I.D.). For urine at 5 mS $cm^{-1}$, EtS could be detected at 0.5 mg $L^{-1}$ in the system featuring PDADMAC, while applying the method with CTAB in the BGE, the LOD was 1 mg $L^{-1}$. Nevertheless, urine samples adjusted to 10 mS $cm^{-1}$ could only be determined with CTAB as dynamic coating (LOD 0.4 mg $L^{-1}$ EtS).
In the PDADMAC coated capillary EOF was higher, so that the compounds were no longer baseline separated. Thus this method could not be used for the determination of urinary EtS [95].

### 4.1.5 BGE with Organic Modifiers for the Separation of Concentrated Samples

Since the dynamic coating featuring PDADMAC was more convenient to handle and sensitivity for urinary EtS in samples with a conductivity of 5 mS $cm^{-1}$ was slightly better than for CTAB-coated capillaries, further experiments were done with the goal of optimizing the method so that urine samples at 10 mS $cm^{-1}$ could be analzed.
It was aimed to increase the run time and by this way enhancing separation of the analytes. Two different approaches were tested, (i) the use of a longer capillary and (ii) the addition of an organic modifier to the BGE.
The first approach with the increase of the total length of the capillary up to 70 cm did not result in a baseline separated EtS peak for urine adjusted to 10 mS $cm^{-1}$.
The second approach provided promising results. Additions of organic modifiers to the BGE were evaluated, including isopropanol, methanol and acetonitrile (ACN). With the addition of ACN to the BGE, longer migration times of EtS were observed. The mobilities of the analytes, however, were influenced by this organic modifier in a way which made its use in this system impossible for the purpose of better resolution of the analytes. The results of the other experiments are listed in Table 2.
Isopropanol added to the BGE at 20 % had the strongest effect on the EOF of the tested configurations and the results were very reproducible. Thus, this BGE was studied more in detail. After rising the applied voltage from -9 kV to -15 kV, detection times of EtS could be reduced to about 8.6 min and the run time with isopropanol in the BGE was only slightly longer that the other methods for the determination of EtS in urine discussed above. The resulting current was lower than 15 µA and thus also the power was reasonably low (< 0.375 W $m^{-1}$).
Lacey et al. have experimentally determined the temperature changes in a 100 µm I.D. (235 µm outer diameter) capillary using an aqueous RB with 50 mM borate [104]. They found that the temperature change of the electrolyte inside of the capillary was 6.4 °C per 1 W $m^{-1}$ resulting during separation. In the system discussed above (0.375 W $m^{-1}$) the temperature within the capillary can roughly be estimated to

| Organic modifier Concentration | $t_{d\ (EtS)}$ | EOF [$cm^2\ V^{-1}\ s^{-1}$] | Resolution (EtS-Oxalate) |
|---|---|---|---|
| **None** | 9.26 min (RSD 0.06 %) | $-3.66 \cdot 10^{-4}$ | 1.14 (RSD 1.83 %) |
| **Isopropanol 10%** | 10.01 min (RSD 1.15 %) | $-3.20 \cdot 10^{-4}$ | 1.06 (RSD 9.87 %) |
| **Isopropanol 20%** | 19.04 min (RSD 1.44 %) | $-1.59 \cdot 10^{-4}$ | 1.82 (RSD 0.26 %) |
| **Methanol 10%** | 10.93 min (RSD 3.17 %) | $-2.75 \cdot 10^{-4}$ | 1.15 (RSD 4.21 %) |
| **Methanol 20%** | 13.33 min (RSD 0.33 %) | $-2.34 \cdot 10^{-4}$ | 1.47 (RSD 4.49 %) |

***Table 2*** *Influence of different organic modifiers in the BGE on the separation of urinary EtS (the mean of 3 runs for each experiment are indicated). Prior to the addition of the solvents, BGE was composed of 15 mM maleic acid and 1 mM phthalic acid (pH set to 2.20 by addition of NaOH). Experiments of urine at 10 mS $cm^{-1}$ spiked with EtS 20 mg $L^{-1}$ and I.S. 8 mg $L^{-1}$ were performed using a 100 µm I.D. PDADMAC-coated capillary ($L_{tot}$ 60.2 cm; $L_{eff}$ 50.0 cm) on a P/ACE MDQ with the detection wavelength set to 215 nm and a separation voltage of -9 kV. Resolution between EtS and Oxalate was calculated as the difference of their detection times divided by half the sum of the width of the two peaks at 5 % of their height. Key: $t_{d\ (EtS)}$ refers to detection time of EtS.*

rise about 2.5 °C. Therefore, the rise of the separation voltage is not supposed to be problematic for the isporopanol containing BGE. The method provided regular electropherograms (Figure 8), confirming the theoretical suggestions. Furthermore, LOD was evaluated to be 0.25 mg $L^{-1}$, which is somewhat lower than the LOD of the validated and published methods (LOD 0.4 mg $L^{-1}$ EtS for urine at 10 mS $cm^{-1}$ analyzed in the CTAB-coated capillary [95]). These achievements justify a future validation of the method and its application to a large set of real samples.

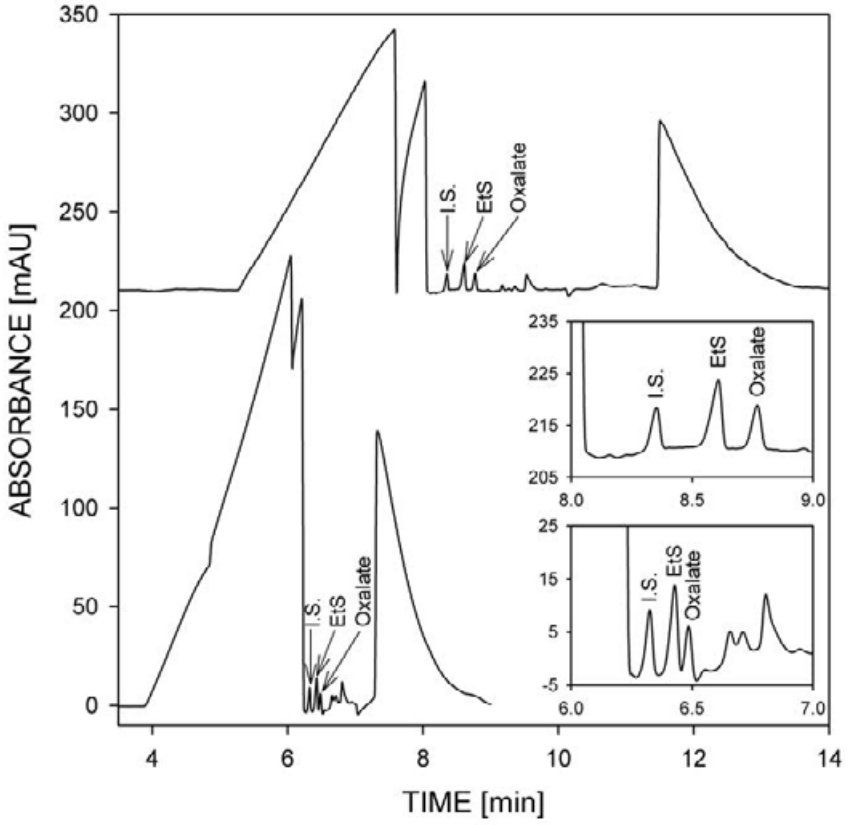



***Figure 8*** *Urine sample adjusted to 10 mS cm$^{-1}$ and spiked with 20 mg L$^{-1}$ EtS and 8 mg L$^{-1}$ I.S. (vinylsulfonic acid), analyzed in a PDADMAC coated capillary (100 µm I.D.; $L_{tot}$ 60.2 cm; $L_{eff}$ 50.0 cm) using a P/ACE MDQ with the detection wavelength set to 215 nm (DAD). The bottom graph with its corresponding lower inset was produced using the plain BGE (15 mM maleic acid and 1 mM phthalic acid adjusted to pH 2.20 by addition of NaOH) and with a separation voltage of -9 kV. The upper graph (displayed with a y-axis offset for the sake of clarity) with its corresponding upper inset was achieved using the same method as before but with 20 % isopropanol added to the BGE and applying a separation voltage of -15 kV.*

### 4.1.6 Conclusions

In conclusion, with the introduction of the normalization of the samples prior to the analysis, regular electropherograms for more than 35 urines collected from different individuals were obtained. Urinary EtS could be determined in every analyzed sample. Furthermore, a robust coating with PDADMAC was developed providing reproducible data. Long conditioning in the beginning of the working day as for the CTAB-coating was thereby avoided.

It has been shown that the dynamic coating featuring PDADMAC can be used in combination with organic modifiers. First experiments with 20 % isopropanol added to the BGE provided reproducible data suggesting an LOD more than 10-fold lower than the method featuring the CTAB-coated 50 µm capillary. These results are promising for the monitoring of EtS in urine by CZE.

## 4.2 Ethyl Sulfate in Urine by CZE-MS

More specific peak identification than discussed above for UV detection was obtained by coupling the CZE system to an ESI-MS. Ideally, positive results from a screening test should be confirmed by another method preferably featuring MS detection, e.g. CZE-MS or LC-MS. The goal of our work featuring the MS detector was to explore the possibility of identifying alcohol markers in body fluids by CZE-MS and CZE-$MS^2$ in order to confirm positive results from the CE-UV screening method.

### 4.2.1 Assay Specifications

A Finnigan LCQ ion trap mass spectrometer equipped with a ESI interface was used in the negative ion mode for the analysis of EtS. In order to avoid contamination of the MS equipment by non-volatile compounds, the BGE for CZE made of maleic acid and phthalic acid needed to be substituted by a volatile electrolyte solution. Heitmeier et al. [105] used a 50 mM ammonium acetate buffer adjusted to pH 9.8 with ammonium hydroxide for the analysis of paracetamol and its metabolites (e.g. paracetamol sulfate and glucuronide) by CZE prior to detection by MS. Some years ago, an assay for the analysis of furosemide in human urine was developed at our laboratory [106]. Caslavska et al. used 20 mM ammonium acetate adjusted to pH 9.5 with triethylamine as RB for CZE. The RB used for the work discussed below was a combination of these two methods, more precisely a solution of 20 mM ammonium acetate adjusted to pH 9.5 with ammonium hydroxide.

The dynamic coatings which have been used in assays featuring CZE with UV-detection (CTAB and PDADMAC, refer to Chapters 4.1, 5.1, and 7.1) were not employed for the methods involving the MS detector. Due to the dynamic equilibrium of the coating substances between the RB and the capillary wall, the large molecules (surfactant and polycation) risk to get swept out of the capillary into the MS, even if they are not present in the RB for separation. The ESI-MS could get contaminated with the non-volatile coating substances. Therefore an

uncoated fused-silica capillary (75 µm I.D.; $L_{tot}$ 80 cm) was used for the separation by CZE. The geometric arrangement of the PrinCE sampler did not allow the use of shorter capillaries.
Samples were introduced hydrodynamically by the application of a positive pressure (0.2 min at 70 mbar). Since the CZE instrument was operated with normal polarity (25 kV), the resulting EOF swept the anionic analytes towards the ESI-MS, while they electrophoretically migrated in the opposite direction.
Immediately following the beginning of the application of high voltage on the CZE instrument, the sheath gas ($N_2$, 40 arbitrary units) of the ESI was turned on. The suction induced on the outlet of the capillary possibly enhanced the flow of the electrolyte system towards the MS interface [107, 108]. Furthermore, in order to allow the analytes of interest to reach the detector within reasonable time, a constant pressure of 5 mbar was applied to the inlet side of the capillary during separation.

The sheath liquid was composed of isopropanol (60 % v/v) and water (40 % v/v). 0.5 % (v/v) ammonia was added to the water prior to mixing it with isopropanol. Benavente et al. compared different compositions of sheath liquid. They showed that best results were obtained with isopropanol as organic solvent and that ammonia supported the formation of negatively charged ions [109]. The sheath liquid was infused at a flow rate of 5 µL $min^{-1}$ using a 500 µL Hamilton syringe. The sheath gas ($N_2$) pressure was maintained at 40 arbitrary units for the whole run. No auxiliary gas flow was used.
The applied ionization spray voltage was -3.8 kV. In order to avoid interferences with sample injection, the voltage applied to the spray needle was turned on shortly after the injection of the sample. Additionally, the sheath gas was turned off during the injection as well. The temperature of the heated capillary was kept at 200 °C.

Full scan mass spectra were collected in the m/z range of 60 to 300 Th. Automatic gain control was employed summing three microscans for each full mass spectrum scan and allowing maximum injection times of 200 ms. Mass traces were reconstructed using an isolation width of

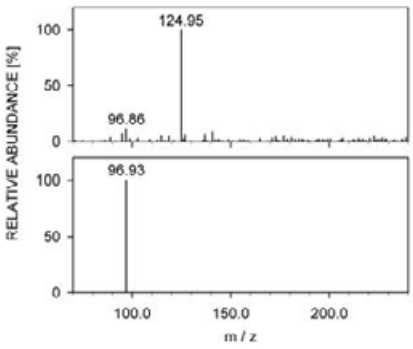



***Figure 9*** *Full scan mass spectrum at 9.60 min (top; NL: 1.19·10⁶) and MS² of m/z 125 Th (bottom; NL: 3.42·10⁴) of an EtS standard solution (1.54 mg L⁻¹ EtS in 10-fold diluted RB). Tandem mass spectrum spectrum was obtained applying a collision energy of 35. Other method parameters are described in the text.*

1 Th. For the tandem MS mode, experiments were performed targeting selectively the m/z ratios of interest with an isolation width of 2 Th. The instrument was tuned using a solution of 20 mg $L^{-1}$ EtS in RB (pH 9.5).

Prior to injection, the urine was diluted to a conductivity of about 3 mS $cm^{-1}$. No matrix interferences were observed when blank urine samples were analyzed. Figure 9 shows the mass spectrum of an EtS standard solution injected directely to the ESI-MS. Negative ionization of EtS resulted in a characteristic mass spectrum, showing one major signal for the molecular ion $[M-H]^-$ (m/z 125 Th).
Two minor signals representing $HSO_4^-$ (m/z 97 Th) and $[M-H]^-$ with the 34S isotope (m/z 127 Th; observed relative abundance 6.4 %; theoretical relative abundance 3.993 to 4.655 % [110]). The presence of the sulfat ion is probably due to fragmentation which occurred during ionization.
The specific transition of m/z 125 Th → m/z 97 Th during fragmentation was also observed in work featuring HPLC-$MS^2$ [22, 64].

### 4.2.2 Analysis of Real Samples

The analysis of urine from an individual who ingested approximately 1.43 g of ethanol per kg body mass in a 11 h time period before sampling (10-fold diluted) provided the mass traces for m/z 125 Th (deprotonated EtS) and m/z 221 Th (deprotonated EtG) depicted in Figure 10.

Even if this chapter focuses on the detection of urinary EtS, it is interesting to note that the mass trace at m/z 221 Th also revealed the presence of EtG in the same urine sample (for the spectra of EtG refer to Figure 17 in Chapter 6.2.2).
The mass spectrum for EtS is shown in Figure 11. The most abundant ion of the mass spectrum at 11.36 min was m/z 125 Th, corresponding

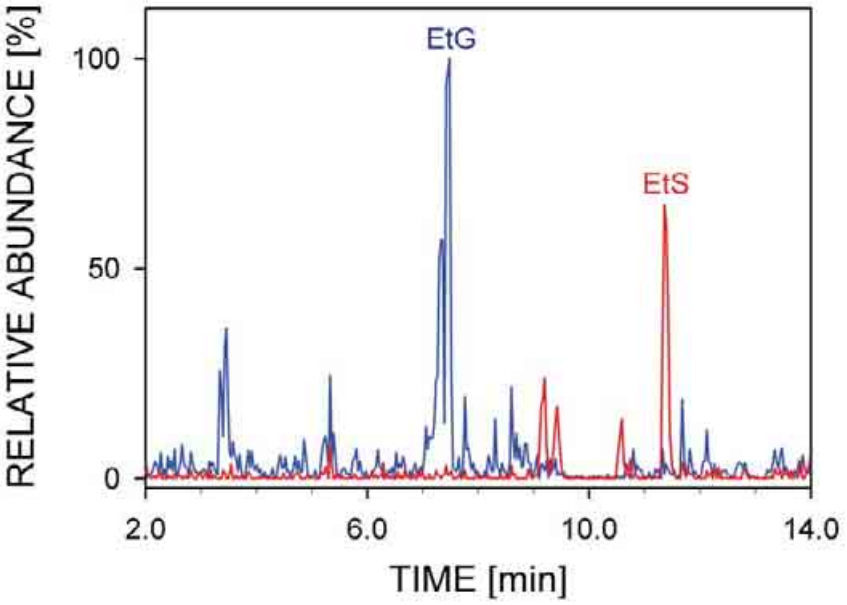



***Figure 10*** *Mass traces for m/z 125 Th (red) and 221 Th (blue) of urine from an individual who ingested approximately 1.43 g of ethanol per kg body mass in a 11 h time period before sampling (10-fold diluted). The maximal abundance of EtG at the time 7.41 min (NL: $6.51 \cdot 10^5$) corresponds to 100 %. The experimental conditions are described in the text.*

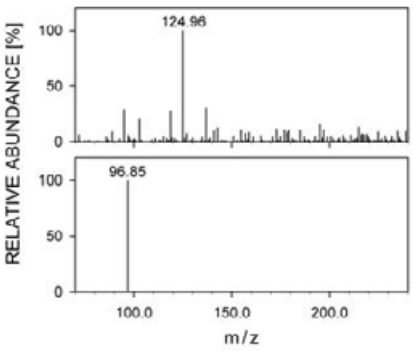



*Figure 11* *Full scan mass spectrum at 11.36 min (top; NL: $4.24 \cdot 10^5$) and $MS^2$ of m/z 125 Th (bottom; NL: $1.54 \cdot 10^4$) of a urine sample from an individual who ingested approximately 1.43 g of ethanol per kg body mass in a 11 h time period before sampling (10-fold diluted). Tandem mass spectrum spectrum was obtained applying a collision energy of 35. Other method parameters are described in the text.*

to $[M-H]^-$. After fragmentation of the deprotonated EtS ion, the fragment ion of m/z 97 Th ($HSO_4^-$) was observed. These ions correspond to those of EtS (Chapter 4.2.1). Hence, EtS was identified to be present in this urinary sample.

The same positive urine sample was analyzed using the CZE method described in Chapter 4.1.4. In urine of 5 mS $cm^{-1}$, EtS concentrations of 6.25 and 5.91 mg $L^{-1}$ were found with CTAB and PDADMAC as dynamic coating agent in the BGE, respectively.

### 4.2.3 Sample Preparation by Solid Phase Extraction

In order to allow the analysis of samples containing lower amounts of EtS, sample preparation by solid phase extraction (SPE) was studied. The extraction of the analyte allowed getting rid of ions of high concentratations present in urine, without diluting the sample. Therefore, the risk of ion suppression during the ionization process due to co-eluting compounds should be reduced.

The same EtS positive urine sample as mentioned in Chapter 4.2.2 was extracted by SPE (Strata X-AW, refer to Chapter 5.1.2 and [95]) following the manufacturer's instructions. The matrix could be simplified and cleaner electropherograms and mass spectra were obtained (Figure 12). The mass spectrum shows the detected ions of

m/z 125 Th (EtS) and 97 Th ($HSO_4^-$). Thus, EtS was also identified in the extracted sample. The abundance was about 10 times higher than for the analysis of plain urine. This corresponded to the difference in the dilution factor of the two samples. Similar results were obtained in some other real samples from individuals who ingested alcohol shortly before sampling as well as in spiked urines.

Urine samples extracted using the Strata weak anion exchange column were analyzed for EtG as well. However, no EtG was found. By contrast, EtS was identified in urine samples prepared using a SPE column designed for the extraction of EtG from urine. These results are discussed more in detail in Chapter 6.2.3 and illustrated with Figure 20.

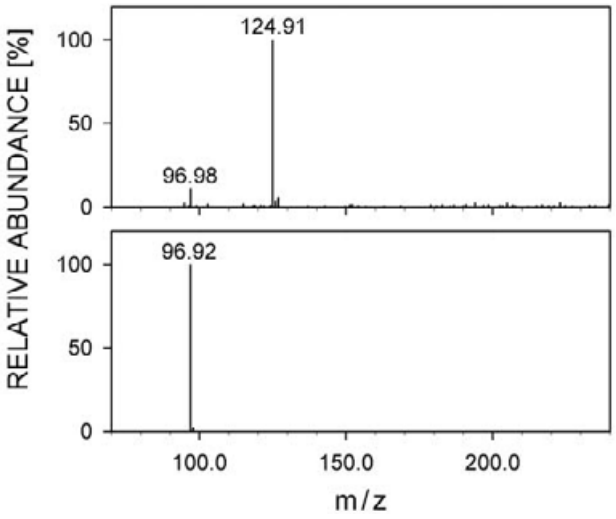



***Figure 12*** *Full scan mass spectrum at 10.72 min (top; NL: $3.79 \cdot 10^6$) and $MS^2$ of m/z 125 (bottom; NL: 8.58·104) of a urine sample from an individual who ingested approximately 1.43 g of ethanol per kg body mass in a 11 h time period before sampling. EtS was extracted from the sample by SPE (Strata X-AW, extraction in accordance to the instructions of the manufacturer) and reconstituted with water to its original volume. Tandem mass spectrum was obtained applying a collision energy of 35. Other method parameters are described in the text.*

### 4.2.4 Conclusions

EtS was identified by CZE-MS in plain urine as well as in extracts. The option to simplify and concentrate the samples using SPE is very helpful for the purpose of the development of a reproducible and sensitive method for the analysis of EtS by CZE-MS. Furthermore, EtS and EtG could be analyzed simultaneously in diluted plain urine.

The potential of the method for the confirmation of positive results from other assays has been shown. However, a thorough validation of the method is needed before its application to patient samples.

# 5
# DETERMINATION OF ETHYL SULFATE IN SERUM

# 5.1 Ethyl Sulfate in Serum by CZE with Indirect Detection

For clinical and forensic applications it is important to be able to determine EtS not only in urine but also in serum. A drawback of blood sampling is the invasive character of the procedure. Nevertheless, the surveillance during sampling is clearly better compared to urine sampling, because trained medical personnel is taking the blood sample. Thus, data obtained from blood samples is more reliable than those obtained from urinary samples. Furthermore, serum samples tell the observer the actual state of the body at a certain time, like a snapshot, which allows the establishment of kinetic studies. The amounts of the major compounds in blood are quite predictable [111], while urine samples vary much more and underlie more factors which might make the analysis and interpretation of the data more difficult (e.g. dilution of the urine, last voiding of the bladder, impaired renal excretion).
The biggest drawback of serum samples from an analytical point of view, however, is that the analytes of interest are often present in lower amounts compared to urine samples.

To the knowledge of the author, no method for the analysis of EtS in serum had been published before the work discussed in this chapter was started. In the meantime, different assays featuring LC-MS have been published [47, 63, 88]. It was aimed to develop a method for the determination of EtS in serum by CZE.
The base of the work was the assay for urinary EtS discussed above in Chapter 4.1. The same coatings (CTAB and PDADMAC) and BGE as used for analysis of EtS in urine were used and the validated assay has been published [95]. As mentioned in the paper, different ways of sample preparation were tested prior to the validation of the method featuring solid phase extraction (SPE).

## 5.1.1 Removal of Proteins from Serum

Analysis of plain serum and serum diluted 1:1 with water resulted in electrophoretic separation of EtS from most of the matrix compounds. Nevertheless, the reproducability of the method was not satisfying, resulting in irreproducible electropherograms after about 10 runs

done on the same capillary. It was assumed that the reason for this was the presence of the proteins in serum. Rinsing the capillary with 1 M NaOH did not reconstitute the capillary in a way that reproducible data could be produced. The equilibrium of CTAB and the inner wall of the capillary was disturbed by every rinsing with the strong base.

Thus, the goal of the following work was to find a way to get rid of parts of the matrix of the serum, notably the proteins, applying a suitable sample preparation. First attempts were done by ultrafiltration (UF) using the "Ultrafree 0.5 Centrifugal Filter and Tube" system (Millipore Corporation, Biomax 5k NMWL Membrane 0.5 mL Vol, Billerica, MA, USA). During UF, with the help of centrifugal force, the sample is forced to pass a membrane of a defined cut-off in regard of particule size. In this case, a cut-off level of 5 kD was used, so that most of the proteins are retained in the membrane. The analytes which are not protein-bound are recovered in the filtrate.
The recovery for EtS at 7 mg $L^{-1}$ was 78.8 % (n=6). When analyzing spiked ultrafiltrates of serum with the system featuring the CTAB-coated capillary discussed in Chapter 4.1, good repeatability for the detection time of EtS was observed (RSD 0.21 %, n=6). Nevertheless, the drawback was that this method was not sensitive enough for the detection of EtS in samples collected from persons who recently consumed alcoholic beverages.

Therefore, it was aimed to concentrate the samples applying protein precipitation (PP) with ACN, followed by evaporation to dryness, and reconstitution in water. It was observed that the concentration of phosphate and other non-identified compounds was reduced. The removal of phosphate during ACN precipitation has also been observed in experiments with EtG [112]. Although reproducible data for the detection time of EtS resulted (for n=6 recovery 68.3 % at 7 mg $L^{-1}$ EtS, RSD 0.39 %), the high chloride concentration in the samples did not allow to concentrate the samples due to the overlap of the chloride peak with the anions of interest.

### 5.1.2 Chloride Elimination and Solid Phase Extraction

A selective way for the elimination of chloride from aqueous solutions is its precipitation with silver ions. However, the use of silver nitrate could not solve the problem because nitrate as well passes the detector shortly before EtS in the studied electrophoretic system. The commercially available silver acetate was found to be the ideal compound for chloride elimination because acetate is uncharged at pH 2.2 and migrates therefore with the EOF, passing the detector much later than EtS. The combination of chloride precipitation by silver acetate after protein precipitation was promising, but did result in noisy and not reproducible electropherograms. Furthermore, the peaks were observed to get broader with rising amounts of acetate in the sample. When concentrating the samples up to 500 % of the original concentration, noisy electropherograms resulted. Additionally, some samples showed EtS peaks with shoulders.
For this reason, SPE using the Strata X-AW column (33 µm Polymeric Weak Anion, 60 mg / 3 mL; Phenomenex, Torrance, CA, USA) was applied. This weak anion exchange procedure applied after the chloride elimination with silver acetate, in combination with the optimized CZE method, led to reproducible data for a large number of real samples [95].

### 5.1.3 Assay Specification, Validation and Evaluation

EtS determinations in serum were done using the same capillaries and BGE as for the analysis of urinary EtS (Chapter 4.1), namely a PDADMAC-coated capillary (100 µm I.D.; $L_{tot}$ 60.2 cm; $L_{eff}$ 50.0 cm) with BGE consisting of 15 mM maleic acid and 1 mM phthalic acid (pH 2.20 by addition of NaOH), and a CTAB-coated capillary (50 µm I.D.; $L_{tot}$ 60.2 cm; $L_{eff}$ 50.0 cm) with BGE made of 15 mM maleic acid, 1 mM phthalic acid and 25 µM CTAB (pH 2.20 by addition of NaOH).

Calibration curves in serum were calculated with five different concentrations (0.2, 0.5, 1.5, 3.5 and 10 mg $L^{-1}$ EtG). As for the calibration of urinary EtS (Chapter 4.1.4), different weighted linear regression models (based upon x, 1/x and $1/x^2$) were compared

according to Karnes and March [103]. The sum of all %RCR (n=5) was found to be smallest for the $1/x^2$ weighted linear regression (for CTAB and PDADMAC coatings) and was therefore also applied for EtS determination in serum [95]. Calibration data (n=5) of the two assays resulted in mean slopes of 0.542 (5.2 % RSD) and 0.546 (5.7 % RSD) as well as mean y-intercepts of 0.002 (0.031 SD) and -0.025 (0.019 SD) mg $L^{-1}$ EtS for the CTAB and PDADMAC capillaries, respectively. The F values were 1819 and 3564, respectively.
The LOD of 0.1 mg $L^{-1}$ EtS (s/n about 3) and LOQ of 0.4 mg $L^{-1}$ (< 4 % RSD) were found to be the same for both capillaries (n=3). Further validation data of the two assays is depicted in Table 3. Based on this data, no method could be considered superior.

| **Capillary coating** | **EtS [mg $L^{-1}$]** | **Precision [% RSD]** | | **Accuracy [% of nominal value]** | |
|---|---|---|---|---|---|
| | | **Intraday** | **Intraday** | **Intraday** | **Intraday** |
| **CTAB** | 1 | 2.2 | 6.4 | 101.2 | 105.5 |
| | 4 | 1.4 | 4.5 | 104.5 | 106.4 |
| | 8 | 1.9 | 6.4 | 101.2 | 103.3 |
| **PDADMAC** | 1 | 4.2 | 1.7 | 94.8 | 99.3 |
| | 4 | 2.4 | 2.0 | 100.8 | 101.2 |
| | 8 | 1.1 | 1.7 | 105.3 | 103.7 |

***Table 3*** *Validation data of the two assays for EtS determination in serum.*

### 5.1.4 Analysis of Real Samples

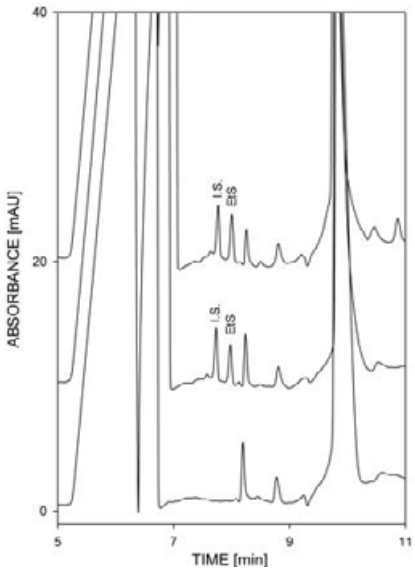



*Figure 13* *Electropherograms of the analysis of serum extracts prepared from blank serum, blank serum spiked with EtS (1.5 mg $L^{-1}$) and I.S. (vinylsulfonic acid, 1.5 mg $L^{-1}$), and a real sample with an EtS serum concentration of 1.70 mg $L^{-1}$ (from bottom to top, and displayed with a y-axis offset for the sake of clarity). Separation of the SPE extracts (Strata X-AW, refer to text and [95] for detailed extraction method) was performed on a P/ACE MDQ instrument (detection wavelength 214 nm, -9 kV) using a PDADMAC-coated capillary (100 µm I.D.; $L_{tot}$ 60.2 cm; $L_{eff}$ 50.0 cm) and with BGE consisting of 15 mM maleic acid and 1 mM phthalic acid (pH 2.20 by addition of NaOH). Figure from [95].*

A total of 14 sera were analyzed using the two methods discussed above. No EtS was found in 5 blank sera (> 48 h of abstinence from alcohol). 4 samples were taken from individuals who consumed only a small amount of alcohol prior to sampling (approximatively 14 g of ethanol; blood sampling within 2 h after alcohol intake). 3 of these samples resulted in positive EtS detection. In one case, EtS was not detected with any of the two electrophoretic methods.
The remaining 5 sera were taken after a dinner accompanied by many alcoholic beverages. In these samples EtS concentrations between 0.26 and 1.95 mg $L^{-1}$ were determined.

The values obtained for these 5 samples using the capillaries coated with CTAB and PDADMAC were found to correlate well ($y = 0.96x + 0.02$, $r = 0.996$) [95].

Typical electropherograms of serum samples prepared with chloride precipitation followed by SPE are shown in Figure 13.

### 5.1.5 Conclusions

The validated methods featuring the two different coating procedures resulted in very similar data. They allowed the monitoring of EtS in samples of persons who consumed as little as one standard drink of alcoholic beverage (approximatively 14 g of ethanol).
It is important to note that EtS was not detected in any sample considered as blank (> 48 h of abstinence from alcohol). Therefore, no false-positive identification resulted.

## 5.2 Ethyl Sulfate in Serum by CZE-MS

As for urinary EtS, it was aimed to detect EtS in serum samples from persons who consumed alcoholic beverages prior to sampling using CZE-MS and therefore confirming the positive results obtained with the CZE-UV screening method. The same CZE-MS method as for urine samples was applied. Refer to Chapter 4.2 for method specifications and CZE-MS and CZE-$MS^2$ data of a standard sample.

### 5.2.1 Assay Specifications and Sample Preparation by Solid Phase Extraction

As seen above, protein containing samples are problematic for the analysis by CZE. In the case of the method discussed here the problem is particularly pronounced. In contrast to the CZE assays featuring optical detection, the separation was done in uncoated fused-silica capillaries. Thus, due to the opposite charge, the proteins would tend to stick to the inner capillary wall. In order to avoid the proteins of the serum causing non reproducible data or even blocking the capillary, samples were extracted by SPE (Strata X-AW, refer to Chapter 5.1.2 and [95]). The advantage of SPE was that the samples were not only freed from the proteins and other potentially interfering compounds but, if needed, they could also be concentrated.

No EtS or matrix interferences were found in blank samples (> 48 h of alcohol abstinence). In blank sera spiked with EtS in the range of 1 mg $L^{-1}$, the ions of m/z 125 Th and 97 Th were detected.

### 5.2.2 Analysis of Real Samples

CZE-MS and CZE-MS2 data obtained with a serum sample from an individual who ingested more than 1.5 g of ethanol per kg body mass in a 12 h time period before sampling is presented in Figure 14. The abundance (NL: $3.91 \cdot 10^5$) was lower than for the mass spectra of EtS in extracted urine and about as high as for EtS in plain urine.

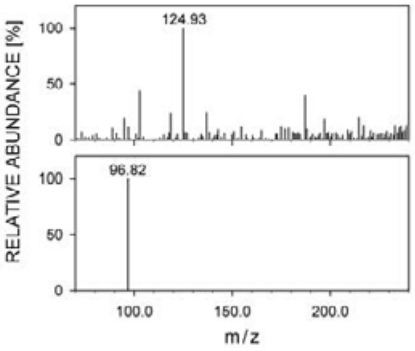



***Figure 14*** *Full scan mass spectrum at 9.10 min (top; NL: $3.91 \cdot 10^5$) and $MS^2$ of m/z 125 Th (bottom; NL: $1.39 \cdot 10^4$) of a serum sample from an individual who ingested more than 1.5 g of ethanol per kg body mass in a 12 h time period before sampling. The serum was extracted by SPE (for detailed method for EtS extraction with the Strata X-AW column refer to Chapter 5.1.2 or [95]) and reconstituted with water to 25 % of its original volume. Tandem mass spectrum spectrum was obtained applying collision energy of 35. Other method parameters are described in the text.*

Some non-identified ions reach relative abundances of almost 50 %. However, the most abundant ion of the mass spectrum at 9.10 min was m/z 125, corresponding to $[M-H]^-$ of EtS. After the fragmentation of this ion, one fragment with m/z 97 ($HSO_4^-$) was observed. This confirms the presence of EtS in this sample.
EtS was also identified by CZE-MS in other sera from individuals who ingested alcoholic beverages prior to sampling as well as in blank sera spiked with EtS.
No EtG was detected in the sample after SPE with the Strata X-AW column.

The serum sample of Figure 14 was analyzed using the CZE methods described in Chapter 5.1. EtS concentrations of 1.86 and 1.70 mg $L^{-1}$ were found with the methods featuring CTAB and PDADMAC as dynamic coating agents, respectively.

Sensitivity was roughly investigated with sera spiked with EtS. The analyte of interest was identified in a sample containing 0.2 mg $L^{-1}$ EtS ($NL_{MS}$: $1.30 \cdot 10^5$; $NL_{MS^2}$: $4.67 \cdot 10^3$).

### 5.2.3 Conclusions

With the discussed CZE-MS and CZE-$MS^2$ method, EtS was identified in sera from individuals who recently consumed alcoholic beverages.
The method has a potential for the confirmation of the presence of EtS in serum samples. However, the method needs to be validated before its application to patient samples.

# 6

# DETERMINATION OF URINARY ETHYL GLUCURONIDE

# 6.1 Ethyl Glucuronide in Urine by CZE with Indirect Detection

The first published method (1990s) for the analysis of urinary EtG was featuring GC-MS [36]. Method development for the determination of EtG in urine using this technique is going on until today [113]. In the meantime many methods applying LC-MS, immunochemical assays, and other techniques have been described in the literature, though [20, 23, 50, 52, 114-117]. To the knowledge of the author, no method for the analysis of EtG in urine by CZE has been published yet.

## 6.1.1 Assay Specifications

The experience gained during the studies of the CZE assay for EtG in serum (refer to Chapter 7.1) provided an advanced starting point for the aimed development of a method for urinary EtG featuring CZE with indirect detection.
A capillary with a linear polyacrylamide (LPA) coating was used (permanent coating with almost no EOF). Applying the BGE made of 10 mM nicotinic acid with a pH adjusted to 4.4 by the addition of about 6 mM ε-aminocaproic acid (EACA), a noisy pattern with many unidentified peaks covering the EtG peak. Hence, an organic modifier evaluated during the development of the assay for EtG determination in serum was added to the BGE, namely 5 % (v/v) isopropanol. This lead to an EOF reduction and therefore enhanced the separability of the anions (refer to Chapter 7.1.12 for a detailed discussion).

## 6.1.2 Ethyl Glucuronide Extraction

Injecting plain urine, many matrix peaks migrated close to EtG or even overlapped its peak. Thus, the matrix needed simplification by suitable sample preparation. For the first attempts of sample preparation SPE was tested using Strata X-AW columns (33 µm Polymeric Weak Anion, 60 mg / 3 mL; Phenomenex, Torrance, CA, USA) and Clean Screen ETG extraction columns (200 mg proprietary

carbon sorbent, 3 mL, United Chemical Technologies, Bristol, PA, USA; http://www.amchro.com/PDFs/SPE/EthylGlucuronideFlyer.pdf, last visited: 27/01/2010).
The first column is filled with a polymeric sorbent for anion exchange. The same product and method was used for sample preparation for EtS in serum (refer to Chapter 5.1.2 and [95]). The second column is based on a proprietary carbon packing material developed for the extraction of EtG for LC-MS and GC-MS samples. Following the manufacturers' instructions, the matrices could be simplified with both SPE procedures, however, the interfering peaks in the electropherograms were not eliminated. The perspective of getting rid of all disturbing matrix compounds by optimizing the SPE methods was not given.

Thus, further efforts were focused on liquid-liquid extraction (LLE). The two methods described below were tested:

- 1 mL of urine (5 mS $cm^{-1}$) was spiked with the EtG (10 mg $L^{-1}$) and I.S. (2,4-dimethylglutarate, 20 mg $L^{-1}$) and then acidified with 25 µL of hydrochloric acid 1 M (the resulting pH of the sample was about 2). 5 mL of ethyl acetate were added and the solution was extracted for 10 min. After centrifugation for 5 min at about 16000·g the organic phase (upper phase) was collected, evaporated under air and finally reconstituted with 1 mL of water.

- 1 mL of urine (5 mS $cm^{-1}$) was spiked with the EtG (10 mg $L^{-1}$) and I.S. (2,4-dimethylglutarate, 20 mg $L^{-1}$) and then acidified with 15 µL of hydrochloric acid 1 M (the resulting pH of the sample was about 5.5). 5 mL of dichloromethane were added and the solution was extracted for 10 min. After centrifugation for 5 min at about 16000·g the organic phase (upper phase) was collected, evaporated under air and finally reconstituted with 1 mL of water.

LLE with ethyl acetate provided non-reproducible electrophoretic data with wild patterns. The approach with dichloromethane on the other hand, resulted in reproducible data, although the electropherograms were noisy. Thus, the samples extracted by LLE with dichloromethane were used for further work.

### 6.1.3 Analysis of Real Samples

The pKa value of EtG (3.21) is close to the values of many organic acids occuring in urine, e.g. malonic acid, benzoic acid, tartaric acid, citric acid, and mandelic acid. Since some of these substances could

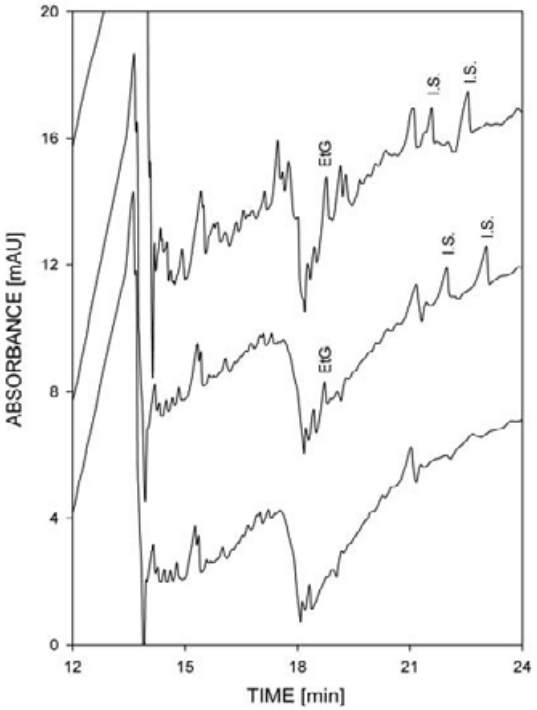



***Figure 15*** *Data obtained with a blank spot urine, a blank urine spiked with 10 mg $L^{-1}$ EtG and 20 mg $L^{-1}$ I.S. (2,4-dimethylglutarate), and a real spot urine sample (1.5 h after the consumption of 0.658 g of ethanol per kg body mass; EtG concentration not determined) are displayed with a y-axis offset for the sake of clarity (from bottom to top). Samples were adjusted to a conductivity of 5 mS $cm^{-1}$ as described in Chapter 4.1.1 and [95] prior to preparation by LLE with dichloromethane (reconstitution of sample to the same volume as prior to LLE). Analysis was done by CZE (ProteomeLab PA 800 with UV detection at 214 nm and at -15 kV) featuring a homemade 100 µm LPA-coated capillary ($L_{tot}$ 60.2 cm; $L_{eff}$ 50.0 cm) and BGE prepared from a 10 mM nicotinic acid solution that was adjusted to pH 4.40 via addition of EACA (about 6 mM), and completed by the addition of isopropanol (5 % v/v). The two diastereomers of the I.S. are separated by this method, resulting in two discrete peaks (for explanations refer to Chapter 7.1.1).*

not be removed from the samples, EtG did not migrate well resolved with this CZE method. The bad resolution of EtG can be seen in Figure 15, showing electropherograms of different urine samples. Despite the noisy pattern, the LOD was found to be about 0.5 mg $L^{-1}$ EtG.
It was intended to optimize the electrophoretic method in order to achieve better separation for EtG. Variations of the BGE's pH in the range of 3.4 to 5.4 did not provide better resolution of the analyte of interest than at pH 4.4. Therefore, efforts for the development of a CZE method for urinary EtG featuring optical detection were abandoned.

### 6.1.4 Conclusions

It was not possible to develop a CZE method for the detection of urinary EtG. Urine contains too many organic acids migrating close to EtG. These disturbing matrix compounds could be eliminated neither by SPE nor by LLE. More laborious sample preparation would be required in order to achieve satisfying resolution of EtG. However, with extensive sample preparation the advantages of CZE would be neglected.
Therefore, further work was focused on assays featuring other techniques, namely CZE-MS and above all, an immunochemical approach (refer to Chapters 6.2 and 6.3).

## 6.2 Ethyl Glucuronide in Urine by CZE-MS

### 6.2.1 Assay Specifications

The same method as for urinary EtS was applied for the analysis of urinary EtG, with the exception that the target ion for the fragmentation in the CZE-$MS^2$ mode (m/z 221 Th). A directly injected aqueous standard solution of EtG provided the spectra depicted in Figure 16. The characteristic mass spectrum shows one major signal representing the molecular ion $[M-H]^-$ (m/z 221 Th) and a minor signal with m/z 222 Th explained by the occurence of 13C isotopes (relative abundance 0.98 to 1.15 % [110]).
The observed ions in the tandem MS mode corresponded to the m/z found by Janda et al. in their studies of the analysis of EtG in hair applying HPLC-$MS^2$ [118]: 203 (-$H_2O$), 159 (-$H_2O$, -CO2), 129 (-$H_2O$, -$CO_2$, -$C_2H_6$), 113 (-$H_2O$, -$CO_2$, -$C_2H_5OH$), 95 (2 times -$H_2O$, -$CO_2$, -$C_2H_5OH$), 85 (-$H_2O$, -$CO_2$, -$C_2H_5OH$, -CO), and 75 Th were observed at relative abundances of 100, 14.6, 3.2, 22.6, 2.1, 26.2, and 18.6 %, respectively.

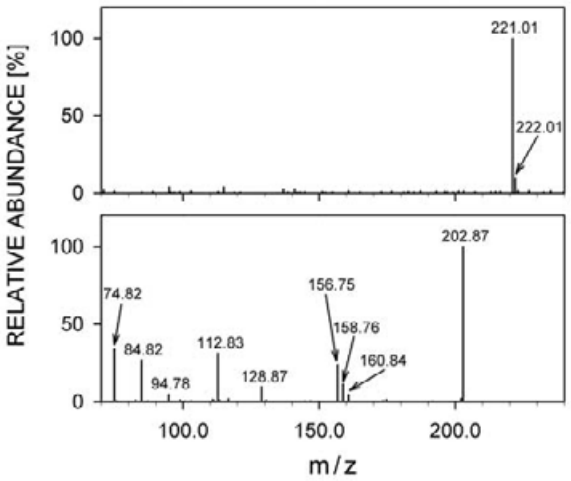



*Figure 16* *Full scan mass spectrum at 6.59 min (top; NL: $2.66 \cdot 10^6$) and $MS^2$ of m/z 125 Th (bottom; NL: $4.67 \cdot 10^5$) of an EtG standard solution (1.73 mg $L^{-1}$ EtG in 10-fold diluted RB). Tandem mass spectrum spectrum was obtained applying a collision energy of 35. Other method parameters are described in the text.*

### 6.2.2 Analysis of Diluted Urine

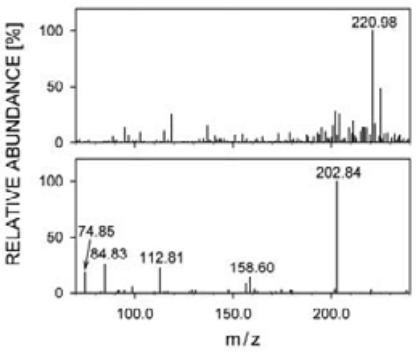



*Figure 17* *Full scan mass spectrum at 7.48 min (top; NL: $6.51 \cdot 10^5$) and $MS^2$ of m/z 221 (bottom; NL: $1.16 \cdot 10^5$) of a urine sample from an individual who ingested approximately 1.43 g of ethanol per kg body mass in a 11 h time period before sampling (10-fold diluted). Tandem mass spectrum was obtained applying a collision energy of 35. Other method parameters are described in the text.*

As shown in Chapter 4.2.2, EtG was found in plain urine diluted 10-fold to an approximative conductivity of 3 mS $cm^{-1}$ (Figure 10). The corresponding spectra for EtG are depicted in Figure 17.

The most abundant ion of the mass spectrum at 7.48 min was m/z 221, corresponding to $[M-H]^-$ of EtG. Some other ions with relative abundances up to almost 64 % (m/z 251, outside of the range of Figure 17) were registered. The fragmentation of the deprotonated EtG ion produced ions of m/z corresponding to those of the EtG standard solution. This confirmed the presence of EtG in the sample.

### 6.2.3 Analysis of Urine Extracts

According to the work about EtS, it was aimed to extract EtG from urine in order to enhance the sensitivity of the method and to achieve reproducible data. The instrument settings were the same as described above.

It was observed that EtG could not be extracted from urine using the Strata X-AW SPE columns. Thus, another SPE method was applied. The Clean Screen ETG columns designed for the extraction of EtG from urine (refer to Chapter 6.1.2) were used following the manufacturer's instructions. The same spot urine sample was investigated as in Chapters 4.2.2, 4.2.3, and 6.2.2. Applying the EIA

described in Chapter 6.3, an EtG concentration of 165.03 mg $L^{-1}$ was determined for this urine sample. The conductivity of the sample was found to be 25.7 mS $cm^{-1}$ (for instrumentation and method used refer to [95]).

The mass traces of this sample depicted in Figure 18 show that besides EtG a small amount of EtS was also recovered from urine using the Clean Screen ETG columns. The relative abundance of the EtS peak only amounted to approximatively 2.4 % of the EtG peak, though.

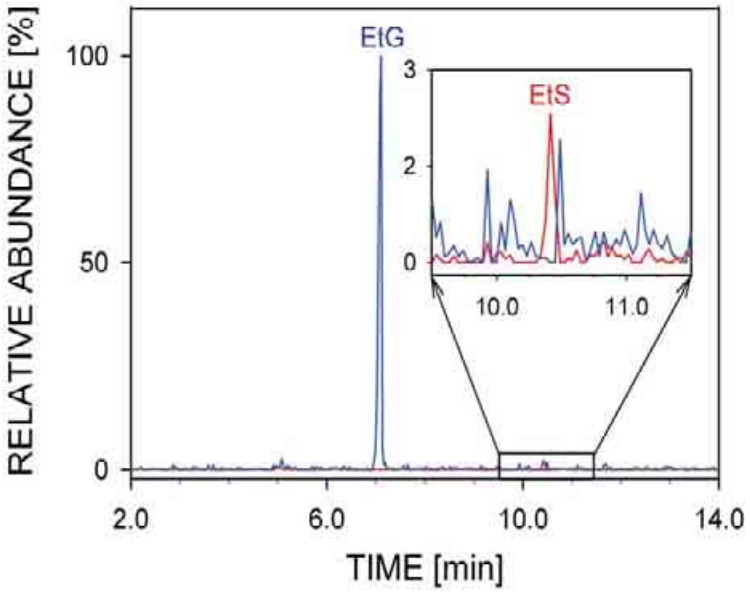



***Figure 18*** *Mass traces for m/z 221 Th (blue; NL: 5.46·10⁶) and 125 Th (red; NL: 1.26·10⁵) of urine from an individual who ingested approximately 1.43 g of ethanol per kg body mass in a 11 h time period before sampling. Analyte extraction from urine was done using the Clean Screen ETG SPE columns (extraction performed according to the manufacturer's instructions and reconstituted with water to 125 % of its original volume). The maximal abundance of EtG at the time 7.11 min corresponds to 100 %. Inset: Zoom on the time frame between 9.5 and 11.5 min in order to visualize the peak of the mass trace at m/z 125. The experimental conditions are described in the text.*

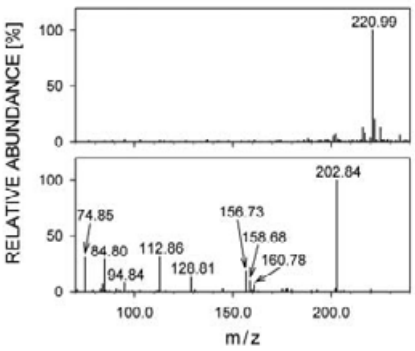



***Figure 19*** *Full scan mass spectrum at 7.11 min (top; NL: 5.46·10⁶) and MS² for m/z 221 (bottom; NL: 2.90·10⁵) of urine from an individual who ingested approximately 1.43 g of ethanol per kg body mass in a 11 h time period before sampling. The sample was extracted using the Clean Screen ETG SPE columns (extraction performed according to the manufacturer's instructions) and reconstituted with water to 125 % of its original volume. Tandem mass spectrum was obtained applying a collision energy of 35. Other method parameters are described in the text.*

The full scan mass spectrum in Figure 19 shows much less disturbing signals than observed for the analysis of plain urine (Figure 17). Although the sample is 8-fold more concentrated, the sample matix appears much simpler and clean CZE-MS data is provided.
The abundance of EtG achieved with the extracted sample was about 10 times higher than with the diluted urine. The factor roughly corresponds to the difference in sample dilution.

Tandem mass spectra for the ion with m/z 221 Th revealed ions with the typical m/z ratios for EtG (Figure 19). Hence, EtG has been identified in urine extracts.

The presence of EtS in the urine extracts was also investigated. The full scan mass spectrum at 10.41 min shown in Figure 20 includes an ion with m/z 232 Th. Its abundance is about 1.4 times higher than the one of m/z 125 Th. The $MS^2$ of the parent ion (m/z 125 Th) showed the typical ion (m/z 97 Th) observed during the fragmentation of EtS (refer to Chapters 4.2.1).

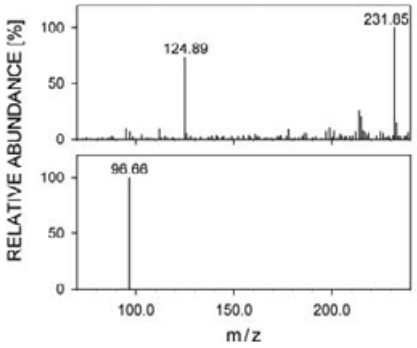



*Figure 20* *Full scan mass spectrum at 10.41 min (top; NL: $1.73 \cdot 10^5$ for non-identified ion with m/z 232) and $MS^2$ of m/z 125 (bottom; NL: $9.74 \cdot 10^3$) of urine from an individual who ingested approximately 1.43 g of ethanol per kg body mass in a 11 h time period before sampling. The sample was extracted using the Clean Screen ETG SPE columns (extraction performed according to the manufacturer's instructions) and reconstituted with water to 125 % of its original volume. Tandem mass spectrum was obtained applying a collision energy of 35. Other method parameters are described in the text.*

Therefore, also the presence of EtS has been demonstrated in serum. However, the abundance of the deprotonated EtS ion was generally about 3 times lower than in the data obtained from the direct analysis of diluted urine (Chapter 4.2.2).

In one run the presence of both alcohol markers (EtG and EtS) was confirmed in an extract from urine stemming from an individual who ingested a large amount of alcohol before sampling. Similar results were obtained for some other urine samples taken after recent alcohol consumption as well as for spiked urine samples. No EtG or EtS was found in any blank sample (> 48 h of alcohol abstinence).

### 6.2.4 Conclusions

EtG was identified in urine samples using CZE-MS. Furthermore, EtS was found using the same analytical methods (injecting plain urine and extracts). The discussed CZE-MS and CZE-$MS^2$ methods have the potential to confirm the positive results obtained with other assays. They need to be validated, though.

# 6.3 Ethyl Glucuronide in Urine by an Enzyme Immunoassay

## 6.3.1 Assay Specifications

Microgenics (Fremont, CA, USA) developed an enzyme immunoassay (EIA) for urinary EtG and introduced it to the market as a kit called "DRI Ethyl Glucuronide Assay" in 2007. The assay is based on the competition between the analyte (EtG) and EtG labeled with an enzyme for the binding sites on the anti EtG antibodies. Only the enzymes of the free labeled EtG are active and transfer the substrate to its product. The rate of the reduction of the coenzyme in this reaction is measured dynamically by photometry. Therefore, the emitted signal is directly proportional to the concentration of EtG in the urinary sample.

The kit is schematically explained in Figure 21 and comprises the following solutions:

- Reagent 1:
  Mouse monoclonal anti EtG antibody, glucose-6-phosphate (G6P, substrate), and nicotinamide adeninedinucleotide ($NAD^+$, coenzyme)

- Reagent 2:
  Glucose-6-phosphate dehydrogenase (G6PDH) labeled EtG

- 5 calibrators:
  Urine spiked with 0, 0.1, 0.5, 1.0, and 2.0 mg $L^{-1}$ EtG

- 4 control samples:
  Negative and positive control for cut-off at 0.5 mg $L^{-1}$ EtG (urine containing 0.375 and 0.625 mg $L^{-1}$ EtG, respectively), as well as negative and positive control for cut-off at 1.0 mg $L^{-1}$ EtG (urine containing 0.750 and 1.250 mg $L^{-1}$ EtG, respectively)

The automated assay has a test range from 0 to 2 mg $L^{-1}$ EtG (with an automatic 1:10 dilution step with water for samples with EtG concentrations above 2 mg $L^{-1}$). The performance of the kit with a Mira Plus analyzer and its suitability for the introduction to the routine laboratory of the ICPVR (University of Bern) was evaluated and is discussed below.

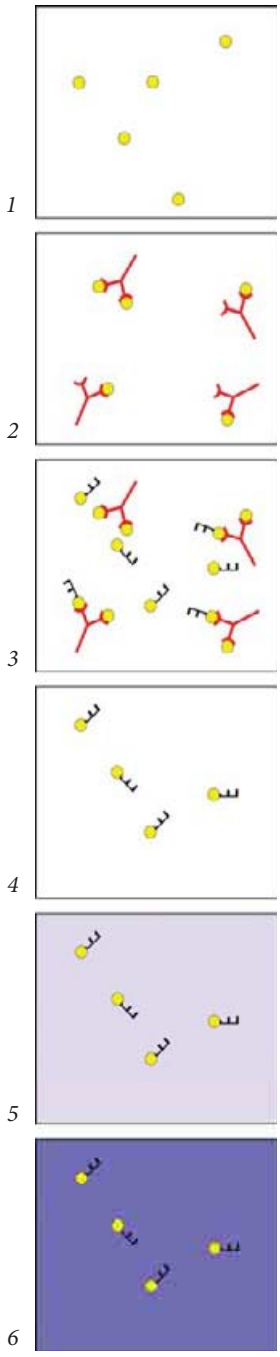



***Figure 21*** *Operating mode of the "DRI Ethyl Glucuronide Assay" from Microgenics.*

*1: Sample containing an unknown amount of EtG (yellow dots) or no EtG.*

*2: The first reagent containing the anti EtG antibodies is added to the sample. If EtG is present in the sample it binds to the antibody. The same reagent contains glucose-6-phosphate (G6P) and nicotinamide adenine dinucleotide ($NAD^+$).*

*3: The second reagent contains EtG labeled with glucose-6-phosphate dehydrogenase (G6PDH). The labeled EtG competes with the analyte (EtG) for the binding sites of the antibody.*

*4: Only the enzyme labeled EtG which is free, i.e. not bound to the antibody, is active and converts G6P into 6-phosphoglucono-δ-lactone.*

*5 and 6: The co-enzyme $NAD^+$ is reduced to NADH during the metabolism of G6P. NADH is measured photometrically at 340 nm.*

### 6.3.2 Assay Validation

Precision data were achieved using the four control samples from the EIA kit. Intraday precision (n=6, injections from the same vial) was found to be 6.99 %, 6.03 %, 1.79 %, and 1.39 % for 0.375, 0.625, 0.750, and 1.250 mg $L^{-1}$ EtG, respectively. As recommended by the kit manufacturer the system was calibrated by measuring the five calibrators in duplicates (measuring twice the same sample out of the same vial). With only one measurement per calibration level, the intraday precision increased to 4.2 % for the 0.750 mg $L^{-1}$ EtG control sample (n=6, injections from the same vial). Hence, it was decided to follow the manufacturer's instructions and to calibrate by averaging two values for each calibration level. Samples on the other hand were always only determined by a single injection.

In order to determine the LOQ of the assay samples containing 0.05 and 0.02 mg $L^{-1}$ EtG were prepared and injected. The precision achieved (n=6) was 11.5 % and 65.7 %, respectively. Thus, samples down to 0.05 mg $L^{-1}$ were accepted as quantitatively determined.

Interday precision (n=6) with daily calibrations was determined to be 3.58 % and 1.73 % at 0.75 mg $L^{-1}$ EtG and 1.25 mg $L^{-1}$ EtG, respectively. For economic reasons (with the perspective of a future routine application of the test), it was evaluated if the calibration of the system could be done on a weekly basis instead of calibrating the system at the beginning of every working day. Thus, precision was determined with a calibration done only on the first day of the validation period of 6 consecutive days. Under these circumstances, interday precision (n=6) was found to be 8.13 %, 5.75 %, 3.4 %, 2.71 % for 0.375, 0.625, 0.75, and 1.25 mg $L^{-1}$ EtG, respectively. In accordance with the CAC/GL codex guidelines of the WHO [119] the precision ( < 15 % RSD) fulfilled the general criteria for attributes in drug controlling. Therefore, the data suggests that weekly calibration is sufficient for EtG determinations using the DRI kit. However, the following data were produced with daily calibrations.

The data advocated the introduction of the kit to the routine laboratory. Nevertheless, doubt arose from the manufacturer's vague indications concerning the proposed cut-off levels. It is suggested that either 0.5 mg $L^{-1}$ or 1 mg $L^{-1}$ should be used as separator of positive

and negative results [120]. Unfortunately it is not declared on what data Microgenics bases their recommendations. Moreover, up to date, no cut-off level for urinary EtG has been generally accepted.

For routine testing of patients, a reliable reference limit is crucial. Thus, it was aimed to determine the reference limit using a collective of blank samples from volunteers. The data from the blank urines should further allow frequentist interpretation (see below) of future unknown samples, and possibly detect factors responsible for false positives. Another important aspect of the study was to evaluate the necessity of normalization of the samples taking in account the dilution factor of the urine. For explications about the normalization of urine samples refer to Chapter 4.1.1 or Appendix VII.

### 6.3.3 Upper Reference Limit

The establishment of the reference limit is required because ethanol can be formed endogenousely in the human body from acetaldehyde by a reaction mediated by alcohol dehydrogenase [121-123] or derived from the gut flora [124, 125]. Endogenous ethanol concentrations up to 1 mg $L^{-1}$ have been observed [124, 126]. In addition, alcohol can be ingested accidentely, e.g. through fruits, "alcoholfree" beverages, medicaments or hygene products (e.g. mouthwash or hand sanitizers) [127-130]. These ethanol traces in the human body will also produce very low amounts of its metabolites which may be detected by sensitive analytical methods.
The necessity to differentiate whether the detected EtG is completely or partially of endogenous or exogenous origin is known from other compounds of forensic interest, e.g. GHB or nandrolone [131, 132]. Generally, this issue is very challenging. Since the goal of determining alcohol markers is to detect deliberate alcohol consumption, it was attempted to achieve a statistical elucidation of the level of EtG in individuals who recently did not consume any alcoholic beverage.

For the elucidation of the upper reference limit of EtG in urine, samples were collected following a well defined protocol. For the documents related to the study refer to the Appendix VI. The collective of volunteers for the study was not selected with specific requirements. The goal was to reach a balanced profile of the general Swiss population. The only exclusion criterion was alcohol consumption in the 72 h prior to sampling. The study was blinded and approved by the local ethics committee.

A total of 92 volunteers participated in the study (35 males with mean age 38.2 y, median 35 y, range: 19 – 62 y; 57 females with mean age 33.7 y, median 29.5 y, range 16 – 64 y). Urines were collected not earlier than 72 h after the last intake of an alcoholic beverage. The volunteers filled out a detailed questionnaire and signed an informed consent.
A second urine donation per volunteer was accepted, but not earlier than two weeks after the first donation. 9 males and 6 females provided two urine samples under these conditions, resulting in a total of 107 urine samples.
106 of these were stored frozen at -20 °C for some days until their analysis, while one sample was directely analyzed without being frozen and within 1 h after sampling. Prior to analysis the samples were thawed, vortexed and centrifuged for 10 min at about 16000·g. 250 µL of the urine (supernatant) were placed in a sample cup and put in the Mira Plus analyzer. EtG and creatinine determinations were done from the same vial.

Creatinine was determined using the Crea Jaffe kit from Diatools (Villmergen, CH). For the calibration and quality control of the assay, the Diacal Crea and Diacon Urin were used, respectively. In alkaline environment creatinine and picric acid form the so called Meisenheimer complex. The red color emerging during this Jaffe reaction is measured photometrically at 510 nm.

Additionally, the conductivity of all urine samples was measured using a conductivity meter model 101 from Orion Research (Cambridge, USA) equipped with a model PW 9510/65 cell from Philips (Eindhoven, Netherlands).

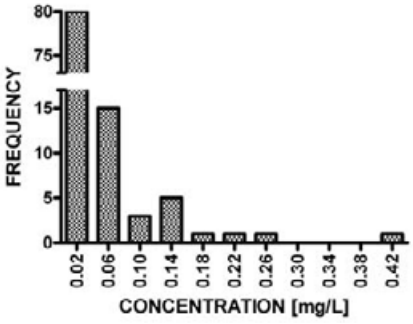



*Figure 22* *Histogram of EtG concentrations in all blank urine samples from the reference limit study (n=107).*

Most of the values were lower than the LOQ. For routine applications EtG levels below 0.05 mg $L^{-1}$ must be indicated as < LOQ. However, in order to provide more interesting data for the statistical discussion, the exact values obtained were used even if they were < LOQ. The results for the determination of the upper reference limit were not biased by this procedure.

The range of EtG concentrations measured in urine was 0 to 0.411 mg $L^{-1}$ (mean 0.034 mg $L^{-1}$; median 0.014 mg $L^{-1}$). Figure 22 shows the histogram of all blank urine samples analyzed, while Figure 23 depicts the same values in gender specific boxplots.
In this work, all boxplots indicate the interquartile range, the lowest and highest value measured as well as the median.
The 97.5$^{th}$ percentile (90 % confidence interval) was determined to be 0.237 mg $L^{-1}$ (0.220 – 0.253 mg $L^{-1}$).

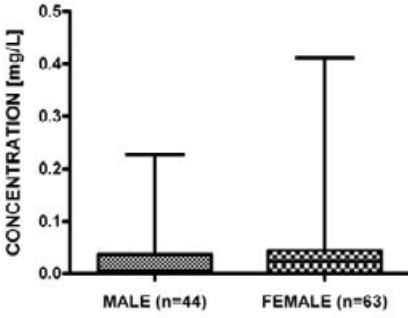



*Figure 23* *Gender specific boxplots of EtG concentration in all blank urine samples from the reference limit study (n=107).*

One outlier (0.411 mg $L^{-1}$ EtG) could be identified with the Dixon-Reed-method (suspected value or group of values is considered as outlier if the distance to the next closest value is > 1/3 of the range of all values [133]). The sample was

reanalyzed so that an analytical error could be excluded. Furthermore, the assessment of the volunteer's questionary did not provide any reason to exclude the outlier from the statistics. Therefore this sample was not excluded from the statistical evaluation.

The t-test did not reveal a relevant difference between EtG concentrations in women and men (P=0.5935).
It is interesting to note that all values from the study samples were below the lower cut-off level proposed by the manufacturer, namely 0.5 mg $L^{-1}$ EtG. The levels defined by the manufacturer, 0.5 and 1.0 mg $L^{-1}$ EtG, seem to be chosen rather conservatively.

Investigation of the intra-individual variability of urinary EtG was done by sampling an individual 5 times within 64 days (7 to 34 days between two consecutive samples). The male volunteer followed the procedure described above and the first two samples were included for the determination of the reference limit. EtG-levels ranged from 0.024 to 0.13 mg $L^{-1}$ (RSD 76.13 %). The variation of the EtG-levels found in the same individual is quite high and the samples seem to fit into the collective of the other blank samples collected.
Therefore, the donation of two samples from some volunteers was retrospectively accepted and the samples were not excluded from the statistical evaluation.

### 6.3.4 Upper Reference Limit with Normalization using Creatinine Concentration

It was further investigated whether the dilution factor of urine samples needs to be considered for EtG-testing and if it is necessary to normalize the data. Appendix VII is devoted to the discussion of the correlation of the two ways of urine normalization studied in this work, namely urinary creatinine concentration and conductivity.

Normalization using creatinine was done by dividing the EtG concentration (mg $L^{-1}$) of a sample by its creatinine concentration (mol $L^{-1}$). The range of normalized EtG concentrations measured in

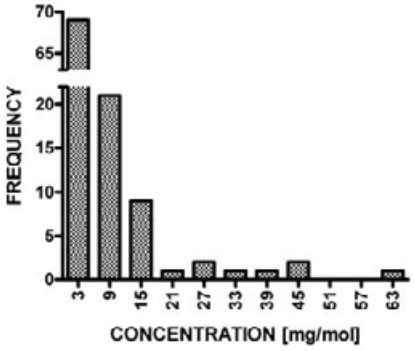



*Figure 24* *Histogram of EtG concentration in all blank urine samples from the reference limit study normalized using creatinine (n=107).*

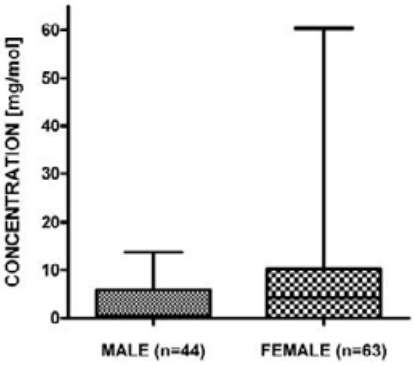



*Figure 25* *Gender specific boxplots of EtG concentration in all blank urine samples from the reference limit study normalized using creatinine (n=107).*

urine was 0 to 60.4 mg $mol^{-1}$ (mean 6.3 mg $mol^{-1}$; median 2.4 mg $mol^{-1}$; n=107). It is interesting to note that the highest value of a sample (60.4 mg $mol^{-1}$, female volunteer; refer to Figures 24 and 25) actually was low in EtG (0.013 mg $L^{-1}$). Its very low creatinine concentration (212 µM), however, was responsible for the high normalized value.

The 97.5$^{th}$ percentile (90 % confidence interval) was determined to be 42.9 mg $mol^{-1}$ (14.5 – 71.3 mg $mol^{-1}$). The confidence interval is very large, almost covering the whole range of the normalized EtG values from the blank samples study. In other words, the real value of the 97.5$^{th}$ percentile of the distribution is actually not known. The uncertainty is too high. Thus, the use of the determined reference limit for EtG normalized with creatinine makes no sense from a statistical point of view. No outlier was identified with the Dixon-Reed-method, which is also due to the strongly scattered data.

The reason for the statistical spread can be explained by the ample differences in creatinine concentrations within the

collective of blank samples. Moreover, the difference of the values for EtG normalized with creatinine between the groups of female and male volunteers is quite obvious (Figure 25). The visual observations are confirmed by the t-test which revealed a significant difference between the data stemming from women and men (P=0.0063).

Urinary creatinine concentration is a measure for the glomerular filtration (without reabsorption) of the kidney, providing a useful indication of the concentration of the urine. However, creatinine is filtered from the blood, where it is transported after its formation during the break-down of creatine in the muscle. Thus, creatinine occurs at different concentrations in blood, depending on factors like gender, muscular mass or race of the sampled individual. This results in the large variety of creatinine concentrations in urine samples, especially between the groups of women and men.
The reference limit including both genders is not recommended to be used for the interpretation of EtG testing. If normalization with creatinine is done for routine use, gender specific reference limits need to be applied.
Thus, separate statistics for the two genders were evaluated. The $97.5^{th}$ percentile (90 % confidence interval) was determined to be 50.8 mg $mol^{-1}$ (46.3 – 55.3 mg $mol^{-1}$) and 13.8 mg $mol^{-1}$ (12.0 – 15.6 mg $mol^{-1}$) for women (n=63) and men (n=44), respectively.
The confidence intervals for both reference limits are reasonably narrow, so that the use of the gender specific reference limits can be advocated. Also for these separately treated groups, no outlier was identified with the Dixon-Reed-method.

Intra-individual variability of urinary EtG concentrations normalized by creatinine was investigated as discussed above for EtG without normalization. The same 5 samples from a male volunteer were used as described above (Chapter 6.3.3). Normalized EtG ranged from 2.2 to 12.2 mg $mol^{-1}$ (RSD 59.05 %).
The variation of these values is somewhat smaller than for the EtG concentration without respecting the dilution factor of the urine. However, the variation of the EtG-levels found in the same individual is quite high and seem to fit into the distribution of the samples collected for the reference limit study. Thus, as for the interpretation

without normalization, no reason was found for the exclusion of the duplicate donations of some volunteers. Hence, they were included in the study.

### 6.3.5 Upper Reference Limit with Normalization using Conductivity

Normalization using conductivity was calculated by dividing the EtG concentration (mg $L^{-1}$) by the conductivity (mS $cm^{-1}$) of the urine samples. For the sake of ease of readability of the numbers, the obtained values were then multiplied by a factor of 1000 and expressed in mg cm $L^{-1}$ $S^{-1}$. The range of normalized EtG concentrations measured in urine was 0 to 17.8 mg cm $L^{-1}$ $S^{-1}$ (mean 2.5 mg cm $L^{-1}$ $S^{-1}$; median 1.2 mg cm $L^{-1}$ $S^{-1}$) and is represented by Figure 26. Using the Dixon-Reed-method, no outlier was identified.

The 97.5$^{th}$ percentile (90 % confidence interval) was determined to be 13.7 mg cm $L^{-1}$ $S^{-1}$ (12.7 – 14.7 mg cm $L^{-1}$ $S^{-1}$). The confidence interval of the reference limit is clearly narrower than it was observed above for EtG normalized by creatinine.

Conductivity is a measure of the ability of a material to conduct an electric current. In this case the material is an aqueous solution, namely urine. The number of ions in the solution is responsible for the conductivity of urine.
In the body, the ions in the blood are filtered in the kidney. However, they get reabsorbed by the kidney in order to regulate the blood pressure by adjusting the content of the electrolytes and the acid-base homeostasis. Nutrition, e.g. the ingestion of varying amounts of sodium ions, may strongly influence the excretion of ions and so the conductivity of urine. Thus, urinary conductivity and creatinine concentration do not provide the same information of the urine's dilution factor. The differences in the results from the different ways of normalization were expected and confirmed.

The comparison of the boxplots of the female and male group indicates only a small difference between the two collectives (Figure 27). The t-test confirmed that the difference between women and men was

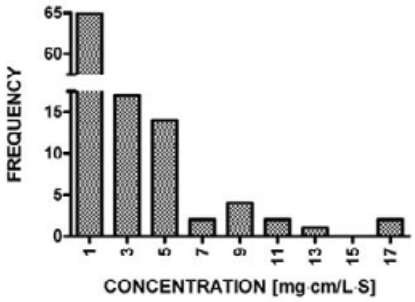



*Figure 26* *Histogram of EtG concentration in all blank urine samples from the reference limit study normalized using conductivity (n=107).*

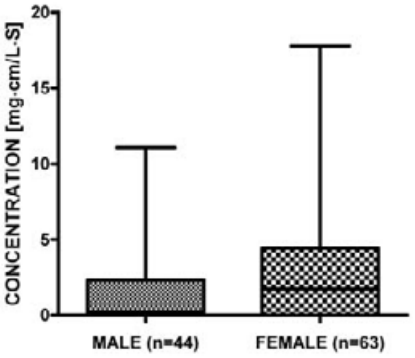



*Figure 27* *Gender specific boxplots of EtG concentration in all blank urine samples from the reference limit study normalized using conductivity (n=107).*

not significant (P=0.0583). Separate statistics for men and women were evaluated. The 97.5$^{th}$ percentile (90 % confidence interval) was determined to be 17.6 mg cm $L^{-1}$ $S^{-1}$ (16.2 – 19.0 mg cm $L^{-1}$ $S^{-1}$) and 10.9 mg cm $L^{-1}$ $S^{-1}$ (9.7 – 12.1 mg cm $L^{-1}$ $S^{-1}$) for women (n=63) and men (n=44), respectively.
No outlier was identified with the Dixon-Reed-method for the data obtained from women, while the data from men revealed 3 outliers: 11.1, 9.5, and 8.7 mg cm $L^{-1}$ $S^{-1}$. These 3 samples were reanalyzed so that an analytical error could be excluded. Furthermore, the evaluation of the volunteer's questionnaires did not provide any reason to exclude these samples from the statistics. So, these 3 samples were kept included in the calculations for the reference limit.

Intra-individual variability of urinary EtG concentrations normalized by conductivity was investigated as well using the same 5 samples from a male volunteer as discussed above. EtG normalized by conductivity ranged from 1.3 to 6.4 mg cm $L^{-1}$ $S^{-1}$ (RSD 43.34 %). It is interesting to note that the variation of the normalized values by conductivity is smallest of the

three ways of EtG determination in urine discussed in this work. The levels almost vary 5-fold and seem to fit into the distribution of the samples collected for the reference limit study.
Thus, as for the other two ways of EtG determination in urine, the duplicate donations of some volunteers of the blank urine study were accepted for this work.

### 6.3.6 Classification of Real Samples According to Upper Reference Limit

The parameters of a screening test, which are of most interest, are its sensitivity and specificity. Sensitivity measures the proportion of actual positives which are correctly identified as such. In the case of EtG testing as defined by the blank urine study this is the percentage of individuals who consumed alcoholic beverages within the last 72 h and who are identified as positive applying the EIA. In other words, sensitivity is the proportion of true positive test results. Specificity measures the proportion of negatives which are correctly identified. Here, this means the percentage of individuals who did not drink within the last 72 h and who are tested negative using the EIA. In other words, specificity is the proportion of true negative test results.

Ideally, a test's sensitivity and specificity is equal to 1. It is almost impossible to reach maximal sensitivity and specificity in reality.
In order to give an idea of the sensitivity and specificity of the test discussed in this chapter, applying the different reference limits evaluated above, the reference limits with and without normalization, were applied to real samples with known or estimated recent alcohol consumption of the sampled individuals (refer to Tables 4 and 5). The general and gender specific reference limits are used for EtG testing with and without normalization, respectively.

Surprisingly, the classifications of the samples with regard to the evaluated reference limits all correspond to each other. If the reference limit including the data collected of both genders was applied instead, sample RS-22 would get classified negative for EtG normalized by creatinine and conductivity. This sample was taken after an abstinence

| Nr | Gender | Amount of last ethanol ingestion [g / kg body weight] | Duration of last ethanol consumption [h] | Time between last ethanol ingestion and sampling [h] | EtG [mg $L^{-1}$] | Creatinine [mM] | Conductivity [mS $cm^{-1}$] |
|---|---|---|---|---|---|---|---|
| RS-1 | male | blank | — | > 48 | 0.139 | 22.30 | 27.20 |
| RS-2 | male | blank | — | > 48 | 0 | 34.97 | 29.60 |
| RS-3 | female | blank | — | > 48 | 0.055 | 15.10 | 25.40 |
| RS-4 | male | 0.737 | 5 | 17 | 10.622 | 9.32 | 18.90 |
| RS-5 | female | 0.160 | 1 | 2 | 18.697 | 4.16 | 25.90 |
| RS-6 | female | 0.218 | 1 | 2 | 5.722 | 4.13 | 7.30 |
| RS-7 | female | 0.185 | 1 | 2 | 34.477 | 13.23 | 26.30 |
| RS-8 | male | 1.430 | 11 | 0 | 165.030 | 19.47 | 25.70 |
| RS-9 | female | blank | — | 42.5 | 0.167 | 4.67 | 12.40 |
| RS-10 | female | 0.462 | 0.5 | 3.5 | 13.074 | 4.58 | 9.41 |
| RS-11 | female | 0.462 | 0.5 | 9.5 | 33.550 | 15.40 | 24.40 |
| RS-12 | female | 0.462 | 0.5 | 11.5 | 6.388 | 0.44 | 22.10 |
| RS-13 | female | 0.462 | 0.5 | 18 | 2.540 | 10.17 | 23.80 |
| RS-14 | female | 0.462 | 0.5 | 20.4 | 0.297 | 3.13 | 9.71 |

***Table 4 (part 1/2)*** *Measured values for EtG, creatinine and conductivity in 28 real samples with the time and amount of the last alcohol consumption prior to sampling (individual estimations by volunteers). Methods and instrumentation of analyses are described in Chapter 6.3. Samples 9 to 16, 17 to 21, and 22 to 28 are 3 series of consecutive samples taken from a female and two male volunteers.*

| Nr | Gender | Amount of last ethanol ingestion [g / kg body weight] | Duration of last ethanol consumption [h] | Time between last ethanol ingestion and sampling [h] | EtG [mg $L^{-1}$] | Creatinine [mM] | Conductivity [mS $cm^{-1}$] |
|---|---|---|---|---|---|---|---|
| RS-15 | female | 0.462 | 0.5 | 23.2 | 0.218 | 6.50 | 18.50 |
| RS-16 | female | 0.462 | 0.5 | 42.5 | 0.162 | 3.32 | 15.40 |
| RS-17 | male | 0.658 | 0.5 | 5.7 | 61.545 | 9.22 | 15.70 |
| RS-18 | male | 0.658 | 0.5 | 9.8 | 42.967 | 12.47 | 22.90 |
| RS-19 | male | 0.658 | 0.5 | 12.1 | 4.483 | 3.06 | 6.12 |
| RS-20 | male | 0.658 | 0.5 | 17.2 | 5.722 | 4.06 | 6.96 |
| RS-21 | male | 0.658 | 0.5 | 27.5 | 0.324 | 2.44 | 16.10 |
| RS-22 | male | blank | — | > 48 | 0.357 | 11.72 | 31.20 |
| RS-23 | male | 0.875 | 0.5 | 1.7 | 1.118 | 1.46 | 3.09 |
| RS-24 | male | 0.875 | 0.5 | 7 | 195.789 | 16.24 | 17.20 |
| RS-25 | male | 0.875 | 0.5 | 9.7 | 74.656 | 15.02 | 24.80 |
| RS-26 | male | 0.875 | 0.5 | 18 | 21.180 | 12.98 | 21.50 |
| RS-27 | male | 0.875 | 0.5 | 24 | 3.482 | 8.87 | 26.50 |
| RS-28 | male | 0.875 | 0.5 | 28 | 0.743 | 8.20 | 18.10 |

***Table 4 (part 2/2)*** *Measured values for EtG, creatinine and conductivity in 28 real samples with the time and amount of the last alcohol consumption prior to sampling (individual estimations by volunteers). Methods and instrumentation of analyses are described in Chapter 6.3. Samples 9 to 16, 17 to 21, and 22 to 28 are 3 series of consecutive samples taken from a female and two male volunteers.*

| Nr | EtG | | | EtG normalized with creatinine | EtG normalized with conductivity |
|---|---|---|---|---|---|
| | DRI 1 mg $L^{-1}$ | DRI 0.5 mg $L^{-1}$ | Evaluated (including all samples) f & m: 0.237 mg $L^{-1}$ | Evaluated (gender specific) f: 50.8 mg $mol^{-1}$ m: 13.8 mg $mol^{-1}$ | Evaluated (gender specific) f: 17.6 mg cm $L^{-1}$ $S^{-1}$ m: 10.9 mg cm $L^{-1}$ $S^{-1}$ |
| RS-1 | neg | neg | neg | neg | neg |
| RS-2 | neg | neg | neg | neg | neg |
| RS-3 | neg | neg | neg | neg | neg |
| RS-4 | pos | pos | pos | pos | pos |
| RS-5 | pos | pos | pos | pos | pos |
| RS-6 | pos | pos | pos | pos | pos |
| RS-7 | pos | pos | pos | pos | pos |
| RS-8 | pos | pos | pos | pos | pos |
| RS-9 | neg | neg | neg | neg | neg |
| RS-10 | pos | pos | pos | pos | pos |
| RS-11 | pos | pos | pos | pos | pos |
| RS-12 | pos | pos | pos | pos | pos |
| RS-13 | pos | pos | pos | pos | pos |
| RS-14 | neg | neg | pos | pos | pos |

***Table 5 (part 1/2)*** *Classification for 28 real urine samples by the application of the gender specific reference limits obtained from the blank urine study (refer to text for details). Samples 9 to 16, 17 to 21, and 22 to 28 are 3 series of consecutive samples taken from a female and two male volunteers. Key: f and m refer to female and male, respectively.*

| Nr | EtG | | | EtG normalized with creatinine | EtG normalized with conductivity |
|---|---|---|---|---|---|
| | DRI 1 mg $L^{-1}$ | DRI 0.5 mg $L^{-1}$ | Evaluated (including all samples) f & m: 0.237 mg $L^{-1}$ | Evaluated (gender specific) f: 50.8 mg $mol^{-1}$ m: 13.8 mg $mol^{-1}$ | Evaluated (gender specific) f: 17.6 mg cm $L^{-1}$ $S^{-1}$ m: 10.9 mg cm $L^{-1}$ $S^{-1}$ |
| RS-15 | neg | neg | neg | neg | neg |
| RS-16 | neg | neg | neg | neg | neg |
| RS-17 | pos | pos | pos | pos | pos |
| RS-18 | pos | pos | pos | pos | pos |
| RS-19 | pos | pos | pos | pos | pos |
| RS-20 | pos | pos | pos | pos | pos |
| RS-21 | neg | neg | pos | pos | pos |
| RS-22 | neg | neg | pos | pos | pos |
| RS-23 | pos | pos | pos | pos | pos |
| RS-24 | pos | pos | pos | pos | pos |
| RS-25 | pos | pos | pos | pos | pos |
| RS-26 | pos | pos | pos | pos | pos |
| RS-27 | pos | pos | pos | pos | pos |
| RS-28 | neg | pos | pos | pos | pos |

***Table 5 (part 2/2)*** *Classification for 28 real urine samples by the application of the gender specific reference limits obtained from the blank urine study (refer to text for details). Samples 9 to 16, 17 to 21, and 22 to 28 are 3 series of consecutive samples taken from a female and two male volunteers.*
*Key: f and m refer to female and male, respectively.*

of more than 24 h. The moment of the last alcohol intake is not known exactly, though. Since the pre-test classification is missing for this case and because the sample was interpretated using reference levels evaluated with samples from persons who did not drink for at least 72 h, it can not be stated with certitude which classification actually is correct, the positive or the negative one.
Applying the cut-off levels proposed by Microgenics (1 or 0.5 mg $L^{-1}$), samples RS-14, RS-21 and RS-22 produce negative outcomes with both limits. RS-28 is only classified negatively with the 1 mg $L^{-1}$ cut-off. Thus, the cut-off levels indicated by the manufacturer result in a considerable loss of sensitivity and accordingly should not be used for urinary EtG determination. Our statistically evaluated reference limits are more precise than the – apparently arbitrarily defined – cut-off levels proposed by the manufacturer.

### 6.3.7 Reference Limit Based on ROC Analysis

The use of the sensitivity and specificity data for the establishment of a reference limit is the more modern method than the calculation of the 97.5$^{th}$ percentile. The advantage of the latter method is that only negative samples are required for the determination of the limit. Nevertheless, it is accepted that 2.5 % of the samples produce false-positive results. It is therefore logic to note that 2 out of the 107 samples from the blank urine study were measured with values above the reference limit of 0.237 mg $L^{-1}$ EtG. The same holds for the normalized values.

For the determination of the sensitivity, a large number of positive samples is required. In the case of alcohol markers, it is not ethical to produce a large number of positive individuals, because this would require volunteers to consume large amounts of ethanol.
Nevertheless, a receiver operating characteristic (ROC) analysis for the determination of the reference limit with the evaluation of sensitivity and specificity data was done. A ROC is a graphical plot of the sensitivity versus (1 – specificity) for a binary classifier system. In other words, the true-positives are plotted versus the false-positives.

The advantage compared to the traditional way (e.g. 97.5$^{th}$ percentile) is, that prior to determining the reference limit, the desired sensitivity or specificity can be defined. Or the minimal demand for one of the parameters may be fixed, e.g. 99 % sensitivity. Furthermore the ROC provides a graph which is easy to interpret for the ideal definition of a test's reference limit: The best possible method for EtG testing would yield a point in the upper left corner of the ROC space, representing 100 % sensitivity and 100 % specificity. As mentioned above, in reality this is almost impossible to achieve.

It is important to note, that the establishment of a ROC requires a large number of negative and positive samples. In our case, there is a lack of positive samples. Furthermore the unambiguous pre-test classification of the samples is mandatory. In the collective of samples available for this work, unfortunately this is not given in every case. Some individuals provided more than two urine samples. Nevertheless a ROC was produced for the determination of urinary EtG and shown in Figure 28.

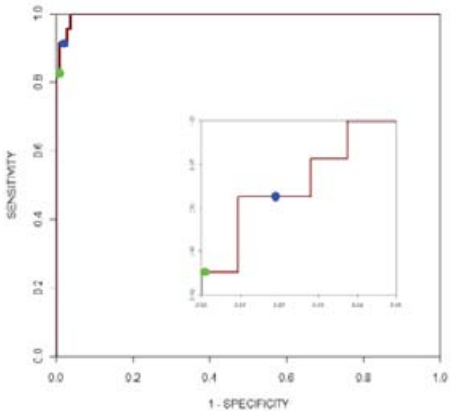



***Figure 28*** *ROC curve of urinary EtG determinations applying the DRI assay for 107 blank samples and 28 samples taken after alcohol consumption. The x-axis (1 - specificity) corresponds to the rate of false-positives; the y-axis (sensitivity) represents the rate of true-positives.*
*Blue dot: Reference limit at 0.237 mg L$^{-1}$ EtG, respectively (sensitivity 0.913; specificity 0.981). Green dot: Reference limit at 0.5 mg L$^{-1}$ EtG (sensitivity 0.826; specificity 1). Inset: Close view of the upper left corner of the ROC; axis biased.*

With the low number of samples taken after alcohol consumption (n=23), a ROC is obtained which passes close to the upper left corner. Thus, the positive and negative samples are quite well separated from each other.
The blue dot in Figure 28 represents the threshold set at the upper limit of the confidence interval of the determined 97.5$^{th}$ percentile of the distribution of the blank samples (0.237 mg $L^{-1}$). It is clearly visible that sensitivity is better than for the reference limit of 0.5 mg $L^{-1}$ (green dot). The loss of specificity going along with it (1 to 0.981) needs to be discussed. The reference limit proposed by Microgenics (0.5 mg $L^{-1}$) may be applied, when 2 false-positive results per 100 sampeled individuals are considered to be too high. However, using this reference limit, the classification of some samples will certainly be in favor of the tested individual. The choice taken by a person with forensic training is probably not the same choice taken by a person with medical training. What ever might be the needs for the definition of the reference limit, the ROC assists in understanding the effect of the reference limit on the sensitivity and specificity of an assay.

### 6.3.8 Interpretation of Patient Samples with Reference Limit

The DRI-kit for EtG determination has been introduced to the routine laboratory of the ICPVR according to the instructions of the manufacturer. It was decided to head for maximum specificity and to apply the reference limit of 0.5 mg $L^{-1}$ EtG. The urine samples are not normalized in regard to their concentration, since the data available up to date did not show a worse performance of the interpretation of the test results without normalization. Furthermore, the same reference limit can be applied for female and male individuals.

For economical reasons it has been shown that it is possible to calibrate the instrument only once a week. However, up to date patient samples have been stored frozen (-20 °C) until their analysis which have been done sporadically (roughly on a weekly basis). Thus, the instrument was calibrated before each session.

The routine laboratory released the test results of patient samples binarily classified (positive or negative) for urinary EtG. Although the results were not normalized in respect to the dilution factor of the urine, urinary creatinine and conductivity were determined. This allowed the further evaluation of the impact of urine concentration on the outcomes and to possibly adapt the way of data interpretation and communication of the results in the future.

Urine samples from patients of the outpatient clinic of the ICPVR (n=37) were analyzed and evaluated with the different reference limits. Samples were only taken when the treating physician had doubts about the patient's declaration of alcohol abstinence. Since the gender of the patients is not known, only the general reference limits (including all samples) were used for the interpretation of the samples (Table 6).

In 7 out of the 37 patient samples, the test result did not correspond for all reference limits. In 3 of these cases, the samples were classified negatively by the 0.5 and 1 mg $L^{-1}$ cut-off level proposed by Microgenics and positively by all evaluated reference limits. Obviousely these discrepancies are due to the less sensitive reference limits.
The 4 other samples, namely PS-14, PS-22, PS-24 and PS-33, were all classified negative when normalized using creatinine and positive when normalized using conductivity. Without normalization, 2 of these samples were tested negative and 2 were tested positive. The value for urinary EtG of PS-22 exactly corresponded to the reference limit (0.237 mg $L^{-1}$) and thus was classified as negative. Since the pattern of alcohol consumption of the tested patients is not known, it is not sure, which result is the correct one for these samples. Furthermore, in the case of normalization with urinary creatinine, PS-14, PS-22, PS-24 and PS-33 would classify positive if the reference limits specific for men would be applied, and negative using the reference limits specific for women. The same is true for PS-22 and PS-33 in the case of normalization by conductivity.
Thus, ignoring the gender of the patients provoked discrepancies in some cases. It is this important to receive the information about the patients' gender.

| Nr | EtG [mg $L^{-1}$] | Creatinine [mM] | Conductivity [mS $cm^{-1}$] | EtG | | | EtG normalized with creatinine | EtG normalized with conductivity |
|---|---|---|---|---|---|---|---|---|
| | | | | DRI 1 mg $L^{-1}$ | DRI 0.5 mg $L^{-1}$ | Evaluated 0.237 mg $L^{-1}$ | Evaluated 42.9 mg $mol^{-1}$ | Evaluated 13.7 mg cm $L^{-1}$ $S^{-1}$ |
| PS-1 | 0.109 | 5.33 | 13.35 | neg | neg | neg | neg | neg |
| PS-2 | 0.025 | 4.67 | 10.90 | neg | neg | neg | neg | neg |
| PS-3 | 0.027 | 9.41 | 24.40 | neg | neg | neg | neg | neg |
| PS-4 | 0.044 | 5.63 | 11.50 | neg | neg | neg | neg | neg |
| PS-5 | 0.306 | 6.14 | 8.45 | neg | neg | pos | pos | pos |
| PS-6 | 0 | 2.63 | 15.20 | neg | neg | neg | neg | neg |
| PS-7 | 0.062 | 5.66 | 13.30 | neg | neg | neg | neg | neg |
| PS-8 | 7.115 | 5.49 | 12.20 | pos | pos | pos | pos | pos |
| PS-9 | 0.132 | 14.39 | 12.45 | neg | neg | neg | neg | neg |
| PS-10 | 588.450 | 10.84 | 19.90 | pos | pos | pos | pos | pos |
| PS-11 | 7.949 | 7.16 | 17.15 | pos | pos | pos | pos | pos |
| PS-12 | 0.127 | 8.29 | 22.05 | neg | neg | neg | neg | neg |
| PS-13 | 0.022 | 4.15 | 12.50 | neg | neg | neg | neg | neg |
| PS-14 | 0.458 | 26.55 | 17.05 | neg | neg | pos | neg | pos |
| PS-15 | 0.015 | 24.43 | 28.90 | neg | neg | neg | neg | neg |
| PS-16 | 0.125 | 4.85 | 11.10 | neg | neg | neg | neg | neg |
| PS-17 | 192.058 | 4.41 | 12.20 | pos | pos | pos | pos | pos |
| PS-18 | 0.112 | 14.96 | 18.60 | neg | neg | neg | neg | neg |
| PS-19 | 1.784 | 22.61 | 17.40 | pos | pos | pos | pos | pos |

***Table 6 (part 1/2)*** *Test results from 37 patient urine samples of the outpatient clinic of the ICPVR applying different reference limits with and without normalization. The gender of the patients and their real drinking patterns are unknown. "Evaluated" stands for the calculated reference limits including all samples of both genders.*

| Nr | EtG [mg $L^{-1}$] | Creatinine [mM] | Conductivity [mS $cm^{-1}$] | EtG | | | EtG normalized with creatinine | EtG normalized with conductivity |
|---|---|---|---|---|---|---|---|---|
| | | | | DRI 1 mg $L^{-1}$ | DRI 0.5 mg $L^{-1}$ | Evaluated 0.237 mg $L^{-1}$ | Evaluated 42.9 mg $mol^{-1}$ | Evaluated 13.7 mg cm $L^{-1}$ $S^{-1}$ |
| PS-20 | 0 | 4.50 | 12.15 | neg | neg | neg | neg | neg |
| PS-21 | 8.406 | 3.83 | 18.10 | pos | pos | pos | pos | pos |
| PS-22 | 0.238 | 11.93 | 14.05 | neg | neg | neg | neg | pos |
| PS-23 | 0.095 | 8.99 | 15.70 | neg | neg | neg | neg | neg |
| PS-24 | 0.384 | 14.99 | 18.45 | neg | neg | pos | neg | pos |
| PS-25 | 4.642 | 3.03 | 12.20 | pos | pos | pos | pos | pos |
| PS-26 | 0.105 | 16.90 | 21.65 | neg | neg | neg | neg | neg |
| PS-27 | 0.049 | 13.53 | 9.45 | neg | neg | neg | neg | neg |
| PS-28 | 0.548 | 12.69 | 15.40 | pos | pos | pos | pos | pos |
| PS-29 | 0 | 13.17 | 17.50 | neg | neg | neg | neg | neg |
| PS-30 | 0.088 | 6.82 | 20.55 | neg | neg | neg | neg | neg |
| PS-31 | > 20 | 19.94 | 17.30 | pos | pos | pos | pos | pos |
| PS-32 | > 20 | 17.05 | 8.31 | pos | pos | pos | pos | pos |
| PS-33 | 0.145 | 8.06 | 9.86 | neg | neg | neg | neg | pos |
| PS-34 | 0.737 | 11.78 | 11.10 | neg | pos | pos | pos | pos |
| PS-35 | 0.664 | 2.60 | 12.20 | neg | pos | pos | pos | pos |
| PS-36 | 0 | 7.89 | 19.10 | neg | neg | neg | neg | neg |
| PS-37 | 35.675 | 22.94 | 19.80 | pos | pos | pos | pos | pos |

***Table 6 (part 2/2)*** *Test results from 37 patient urine samples of the outpatient clinic of the ICPVR applying different reference limits with and without normalization. The gender of the patients and their real drinking patterns are unknown. "Evaluated" stands for the calculated reference limits including all samples of both genders. Exact EtG concentration has not been determined for PS-31 and PS-32.*

### 6.3.9 Frequentist approach

Some of the test results discussed in the previous chapter are just above or below the reference limit. Such cases may occur when applying a distinct reference limit. It is imaginable that the samples from two different individuals provide EtG values which are very close to each other. If one of these values is just above and the other one is just below the reference limit, then the classification of the samples is completely contrary. Different test interpretations may result for similar evidence in a binary classifier system. In order to avoid such disagreements, the interpretation of the results applying the Bayes' theorem (Equation 8) is strongly recommended [134, 135].
The frequentist approach is based on data, which can be used to describe the power of evidence. The theorem deals with conditional probabilities, e.g. it can be used to calculate the probability that an individual actually did consume alcohol prior to testing, given observed evidence:

*Equation 8*
$$P(RAC \mid X) = \frac{P(X \mid RAC)\, P(RAC)}{P(X)}$$

where *P(X)* is the probability to measure the value X for EtG, *P(RAC)* is the probability of recent alcohol consumption of an individual, *P(X| RAC)* is the probability to measure the value X for EtG given the fact of recent alcohol consumption of an individual, and *P(RAC| X)* is the probability of recent alcohol consumption of an individual given the measured value *X* for EtG.

The theorem can be easily transformed for the expression in odds (Equation 9) and likelihood ratio (LR) by dividing the term by *P(RAC'| X)*. Expressed in words *P(RAC'| X)* is the probability that an individual recently did not consume alcohol given the measured value *X* for EtG.

*Equation 9*
$$\frac{P(RAC \mid X)}{P(RAC' \mid X)} = \frac{P(X \mid RAC)\, P(RAC)\, P(X)}{P(X \mid RAC')\, P(RAC')\, P(X)} \Leftrightarrow O(RAC \mid X) = LR(RAC \mid X)\, O(RAC)$$

*O(RAC)* are the (prior) odds of recent alcohol consumption by itself. They need to be defined by the expert, e.g. judge or physician, prior

to knowing the outcome of the test. Typically, in clinical practice EtG-testing is only ordered by the physician when he doubts about the patient's assertion of alcohol abstinence. In this case, the prior odds will be chosen greater than 1 because the probability of the patient having recently consumed alcohol is estimated higher than the probability of the patient having not ingested any alcoholic beverage the last three days. In a legal case where no history or other facts about the tested person are known, the prior odds may also be set equal to 1 or even in favour of the individual, between 0 and 1. Multiplication of the prior odds with the LR provides the posterior odds. These correspond to the odds given the evidence, which is the measured value *X* for urinary EtG concentration.

The LR corresponds to the probability of finding the value *X* for EtG given recent alcohol consumption divided by the probability of finding the value *X* for EtG given abstinence from alcohol for the last three days. The corresponding probabilities for measured values *X* for EtG can be determined with the help of the theoretical distributions for EtG concentrations in blank samples and in samples stemming from persons who recently consumed alcohol. For continuous distributions *f(x)* can be defined. The function represent the probability density with the following properties (Equations 10 and 11):

*Equation 10* $f(x) \geq 0 \quad , \forall\, x \in R$

*Equation 11* $\int f(x)\, dx = 1$

Considering an interval $I=\{x;\ x+dx\}$, the probability P can be found with Equation 12 below.

*Equation 12* $f(X)\, dx = P(X \in I)$

If the distributions for EtG values measured in samples taken after recent alcohol consumption $f_{RAC}(x)$ and after alcohol abstinence $f_{Blank}(x)$ can be found, the LR can be determined applying Equation 13:

*Equation 13* $$LR(RAC \mid X) = \frac{P(X \mid RAC\ )}{P(X \mid RAC')} = \frac{f_{RAC}(x)\, dx}{f_{Blank}(x)\, dx} = \frac{f_{RAC}(x)}{f_{Blank}(x)}$$

### 6.3.10 Determination of Likelihood Ratio for Urinary Ethyl Glucuronide

The values obtained from the study with blank samples described above allowed the estimation of $f_{Blank}(x)$. The normality test (Kolmogorov-Smirnov) indicated that the EtG concentration (without normalization as well as normalized by creatinine or conductivity) of the set of blank samples from the study was not normally distributed. The pattern of the distribution rather suggested a Poisson distribution (Equation 14):

*Equation 14* $$f(x,\lambda) = \frac{\lambda^x e^{-\lambda}}{x!} \quad , x > 0$$

Calculations have been done using the R software. For the programmation the gamma distribution was adopted (Equation 15).

*Equation 15* $$f(x,k,\theta) = x^{k-1}\frac{e^{-\frac{x}{\theta}}}{\theta\Gamma(k)} \quad , x > 0$$

If the shape $k$ of the gamma distribution is equal to 1, then the term corresponds to the Poisson distribution. For the following work the gamma function (with $k$=1) will be used.
The likelihood function for $N$ independent and identically distributed observations $(x_1, \ldots, x_n)$ is

*Equation 16* $$L(p_1, \ldots, p_k) = \Pi_{i=1}^{N} f(x_i, p_1, \ldots, p_k)$$

The most likely estimated parameters of the distribution $f$ maximize the likelihood function $L$. Zeros of the partial derivates provide the maximum likelihood estimator of the parameters $p_i$. It is common to use the log likelihood function $l$ that transforms the product of $L$ into a sum:

*Equation 17* $$l(p_1, \ldots, p_k) = \Sigma_{i=1}^{n} \log\left( f(x_i, p_1, \ldots, p_k)\right)$$

If $k$=1, the maximum of the gamma distribution with respect to $\theta$ is then found to be

*Equation 18* $$\theta_{max} = \frac{1}{N}\Sigma_{i=1}^{n} x_i = \bar{x}$$

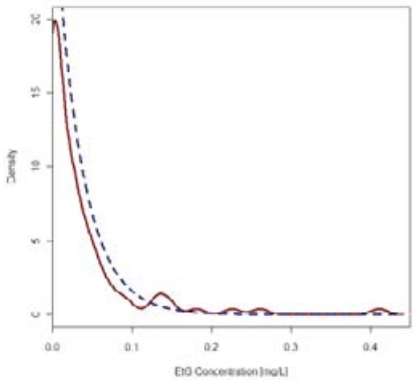



***Figure 29*** *Experimental and theoretical distribution of the urinary EtG values measured in the study with blank samples (n=107). Red graph: Experimentally evaluated and smoothened (bandwidth 0.01 mg $L^{-1}$) sample density. Blue graph: Theoretical density (gamma distribution with k=1 and θ=0.034).*

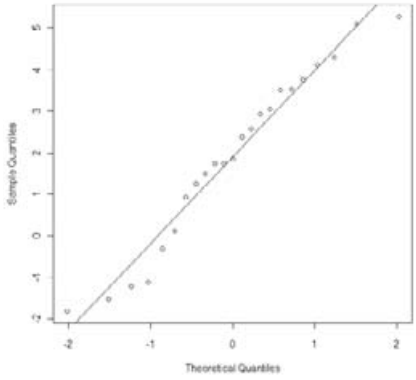



***Figure 30*** *Q-Q plot of randomized normally distributed data versus the logarithm of urinary EtG concentrations in 23 real samples taken after alcohol consumption. Correlation: slope 2.09; y-axsis intercept 1.9; r 0.984; F 631; $p < 2.2 \cdot 10^{-16}$.*

Thus, the best estimation for $\theta$ corresponds to the mean of the measured EtG values.

Figure 29 shows the distribution of the EtG levels in the blank samples from the study discussed above as well as the theoretical density. For the sake of vividness the experimental data has been smoothened applying a Gaussian distribution around the measured values for EtG (bandwith 0.1 mg $L^{-1}$).

The estimation of the distribution of EtG values sampling individuals after alcohol consumption is more challenging. The distribution strongly depends on the population from which the samples are taken. So it is obvious that in a group of persons suffering from the alcohol dependence syndrome, higher EtG concentrations are expected to be found than in a group of recreational consumers of alcoholic beverages. This data needs to be assessed for the groups from which individuals are tested. Since here this specific data is missing, the best approximation is given by the data obtained from volunteers sampeled after declared ethanol ingestion. At our laboratory 23 such urine samples have been

analyzed and described above, namely RS-4 to RS-8, RS-10 to RS-21, and RS-23 to RS-28. The Q-Q plot in Figure 30 shows the quantiles of the logarithm of the EtG concentrations these real samples plotted against the quantiles of normally distributed randomized data.

The Q-Q analysis reveals a strong correlation between the two distributions and thus suggests a logarithmic-normal distribution of the EtG values in the analyzed real samples:

*Equation 19* $$f(x, \mu, \sigma) = \frac{1}{x\sigma\sqrt{2\pi}} e^{-\frac{(\ln x - \mu)^2}{2\sigma^2}} \quad , x > 0$$

The maximum likelihood estimation of the log-normal distribution's parameters can be done analogous to the procedure for the gamma distribution discussed above (Equations 15 to 17):

*Equation 20* $$\mu_{max} = \frac{1}{N} \sum_{i=1}^{N} \ln(x_i) = \overline{\ln(x)}$$

*Equation 21* $$\sigma_{max} = \frac{1}{N} \sum_{i=1}^{N} \left(\ln(x_i) - \mu_{max}\right)^2 = \overline{\left(\ln(x) - \ln(x)\right)^2}$$

Thus the best estimations for $\mu$ and $\sigma$ are the mean and the standard deviation of the ln of the values, respectively. Logically, these parameters correspond to the y-axis intercept and slope of the regression line in the Q-Q plot, respectively. Figure 31 shows the distribution of the EtG levels in the real samples taken after alcohol consumption as well as the theoretical density.

As for the data of the blank samples, a good fit has been found for the distribution of the urines from individuals sampeled after alcohol consumption. The LR for measured amounts of urinary EtG can be calculated as described in Equation 22:

*Equation 22* $$LR(EtG_{Urine}) = \frac{f_{Log\text{-}normal}(x = c(EtG), \mu = 1.9, \sigma = 2.114)}{f_{Gamma}(x = c(EtG), k = 1, \theta = 0.034)}$$

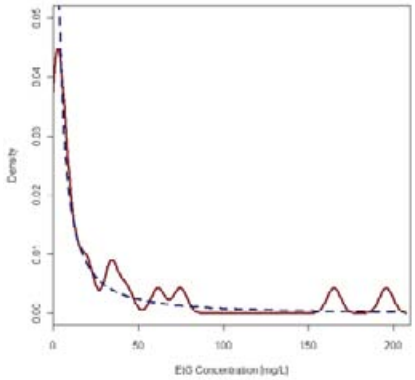



*Figure 31* *Experimental and theoretical distribution of the urinary EtG values measured in 23 real urine samples taken after alcohol consumption.*
*Red graph: Experimentally evaluated and smoothened (bandwidth 4 mg $L^{-1}$) sample density.*
*Blue graph: Theoretical density (log-normal distribution with $\mu = 1.9$ and $\sigma = 2.114$).*

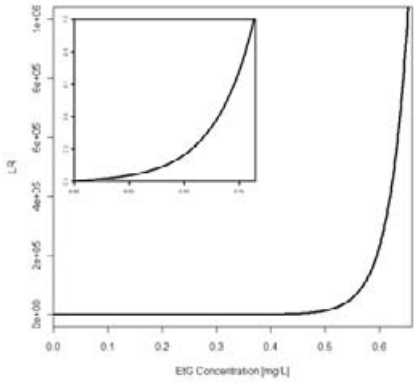



*Figure 32* *Urinary EtG concentration plotted against the resulting LR. Inset: Zoom on the plot for EtG concentrations resulting in LR ≤ 1.*

It is important to note that for $x \to \infty$ the LR approximately corresponds to the exponential function. Thus, the LR is strongly rising for large values of EtG. This makes sense in case very high EtG levels are found. In such cases it is much more probable that the sample stems from an individual who recently consumed alcohol than from an individual who did not ingest alcohol during the last 3 days. The urinary EtG concentration plotted against the resulting LR is depicted in Figure 32.

For measured EtG concentrations of 0.5 mg $L^{-1}$, which corresponds to the proposed reference limit, a LR of 13422.7 is resulting. This means that it is more than 10000 times more probable finding 0.5 mg $L^{-1}$ EtG in a sample that stems from an individual who recently consumed alcohol than determining the same EtG concentration in a sample from an person who did not drink alcohol for at least the last 3 days. The hypothesis that the tested subject recently did drink alcohol is supported.

### 6.3.11 Determination of Likelihood Ratio for Normalized Urine Samples

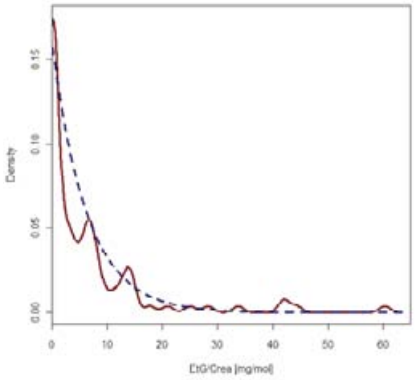



*Figure 33* *Experimental and theoretical distribution of the urinary EtG values of the study with blank samples normalized with creatinine (n=107).*
*Red graph: Experimentally evaluated and smoothened (bandwidth 1 mg mol$^{-1}$) sample density.*
*Blue graph: Theoretical density (gamma distribution with k=1 and θ = 6.293).*

The parameters could also be evaluated for urinary EtG values normalized by creatinine and conductivity. The results are shown in Figures 33 to 38 below. In order to take into account the differences between the two genders, specific evaluation for the female and male group should be done. However, since the genders of the patients (sample PS-1 to PS-37) are not known and because only a low number of real samples was available, this evaluation was not done here. The frequentist approach is explained above in Chapter 6.3.10 and analogously has been applied for the present chapter.

The Q-Q plots for urinary EtG concentrations obtained from samples taken after alcohol consumption (n=23) normalized by creatinine and conductivity resulted in slopes of 1.744 and 1.95, y-axis intercepts of 7.004 and 6.062, r of 0.973 and 0.974, F of 377 and 391, and p of $6.78 \cdot 10^{-15}$ and $4.689 \cdot 10^{-15}$, respectively. Thus, as for the samples without correction for the dilution factor, the log-normal distribution was used to describe the theoretical distribution of the expected EtG concentrations.

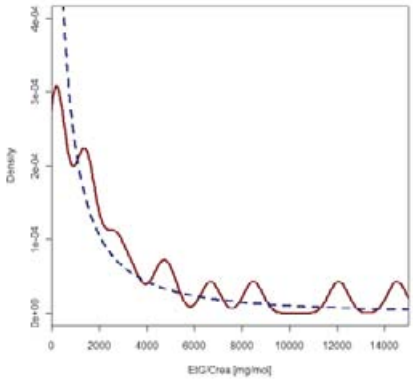



***Figure 34*** *Experimental and theoretical distribution of the urinary EtG values measured in 23 real samples taken after alcohol consumption, normalized with creatinine. Red graph: Experimentally evaluated and smoothened (bandwidth 400 mg mol$^{-1}$) sample density. Blue graph: Theoretical density (log-normal distribution with $\mu = 7.004$ and $\sigma = 1.782$).*

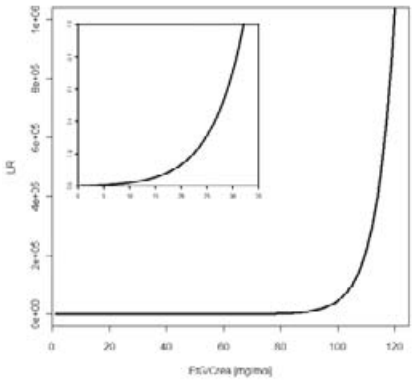



***Figure 35*** *Urinary concentration of EtG normalized with creatinine plotted against the resulting LR. Inset: Zoom on the plot for EtG concentrations resulting in LR ≤ 1.*

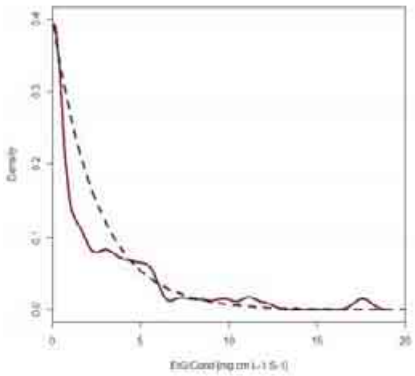
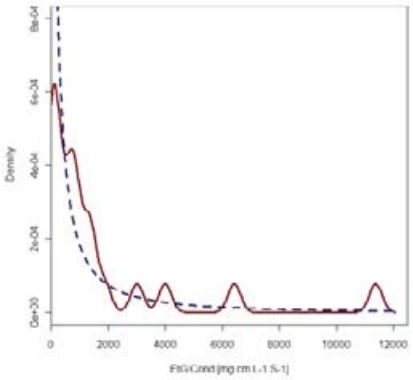


*Figure 36* *Experimental and theoretical distribution of the urinary EtG values of the study with blank samples normalized with conductivity (n=107).*
*Red graph: Experimentally evaluated and smoothened (bandwidth 0.45 mg cm $L^{-1}$ $S^{-1}$) sample density.*
*Blue graph: Theoretical density (gamma distribution with k=1 and $\theta$ = 2.5).*

*Figure 37* *Experimental and theoretical distribution of the urinary EtG values measured in 23 real samples taken after alcohol consumption, normalized with conductivity.*
*Red graph: Experimentally evaluated and smoothened (bandwidth 225 mg cm $L^{-1}$ $S^{-1}$) sample density.*
*Blue graph: Theoretical density (log-normal distribution with $\mu$ = 6.062 and $\sigma$ = 1.991).*



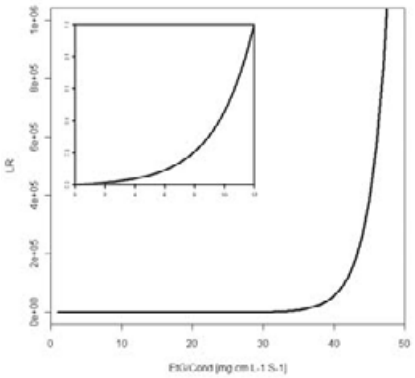



*Figure 38* *Urinary concentration of EtG normalized with conductivity plotted against the resulting LR. Inset: Zoom on the plot for EtG concentrations resulting in LR ≤ 1.*

### 6.3.12 Binary Classifier versus Frequentist Approach

When applying frequentist approach, similar evidence, e.g. samples with EtG concentrations which only differ slightly from each other, will be interpreted alike. Applying a binary classifier (e.g. based on the reference level), however, may produce contrary classifications for similar evidence close to the limit. Such cases have been discussed in the previous chapters. Furthermore, it has been shown that the weight of the evidence of two samples may be completely different, although they both have been classified "just" positive in a binary classifier system. A closer look on sample PS-33 shows that normalized with regard to its conductivity it is tested positive (14.706 mg cm $L^{-1}$ $S^{-1}$; cut-off:13.7 mg cm $L^{-1}$ $S^{-1}$). The LR for urinary EtG normalized with conductivity is 2.908 (Table 7). This is clearly less than the LR of of PS-35 for example with the same normalization (54.426 mg cm $L^{-1}$ $S^{-1}$; LR $1.535 \cdot 10^7$). Thus, even if the prior odds of patient 35 are defined to be 1000 times smaller than for patient 33, the posterior odds of patient 35 are about 5000 times higher than for patient 33.

| Nr | EtG [mg $L^{-1}$] | Creatinine [mM] | Conductivity [mS $cm^{-1}$] | LR | | |
|---|---|---|---|---|---|---|
| | | | | EtG | EtG normalized with creatinine | EtG normalized with conductivity |
| PS-1 | 0.109 | 5.33 | 13.35 | 0.215 | 0.146 | 0.222 |
| PS-2 | 0.025 | 4.67 | 10.90 | $1.646 \cdot 10^{-2}$ | $7.213 \cdot 10^{-3}$ | $1.755 \cdot 10^{-2}$ |
| PS-3 | 0.027 | 9.41 | 24.40 | $1.731 \cdot 10^{-2}$ | $2.881 \cdot 10^{-3}$ | $7.851 \cdot 10^{-3}$ |
| PS-4 | 0.044 | 5.63 | 11.50 | $3.112 \cdot 10^{-2}$ | $1.302 \cdot 10^{-2}$ | $3.582 \cdot 10^{-2}$ |
| PS-5 | 0.306 | 6.14 | 8.45 | 55.52 | 17.19 | $1.252 \cdot 10^{4}$ |
| PS-6 | 0 | 2.63 | 15.20 | $< 6.485 \cdot 10^{-5}$ | $< 10^{-6}$ | $< 10^{-6}$ |
| PS-7 | 0.062 | 5.66 | 13.30 | $5.542 \cdot 10^{-2}$ | $2.607 \cdot 10^{-2}$ | $5.303 \cdot 10^{-2}$ |
| PS-8 | 7.115 | 5.49 | 12.20 | $> 10^{6}$ | $> 10^{6}$ | $> 10^{6}$ |
| PS-9 | 0.132 | 14.39 | 12.45 | 0.407 | $1.772 \cdot 10^{-2}$ | 0.574 |
| PS-10 | 588.450 | 10.84 | 19.90 | $> 10^{6}$ | $> 10^{6}$ | $> 10^{6}$ |
| PS-11 | 7.949 | 7.16 | 17.15 | $> 10^{6}$ | $> 10^{6}$ | $> 10^{6}$ |
| PS-12 | 0.127 | 8.29 | 22.05 | 0.358 | $5.913 \cdot 10^{-2}$ | $8.348 \cdot 10^{-2}$ |
| PS-13 | 0.022 | 4.15 | 12.50 | $1.429 \cdot 10^{-2}$ | $6.868 \cdot 10^{-3}$ | $1.259 \cdot 10^{-2}$ |
| PS-14 | 0.458 | 26.55 | 17.05 | $4.057 \cdot 10^{3}$ | $8.329 \cdot 10^{-2}$ | $3.272 \cdot 10^{2}$ |
| PS-15 | 0.015 | 24.43 | 28.90 | $1.010 \cdot 10^{-2}$ | $3.493 \cdot 10^{-4}$ | $3.877 \cdot 10^{-3}$ |
| PS-16 | 0.125 | 4.85 | 11.10 | 0.341 | 0.360 | 0.761 |
| PS-17 | 192.058 | 4.41 | 12.20 | $> 10^{6}$ | $> 10^{6}$ | $> 10^{6}$ |
| PS-18 | 0.112 | 14.96 | 18.60 | 0.233 | $1.221 \cdot 10^{-2}$ | $9.284 \cdot 10^{-2}$ |
| PS-19 | 1.784 | 22.61 | 17.40 | $> 10^{6}$ | $1.665 \cdot 10^{3}$ | $> 10^{6}$ |

***Table 7 (part 1/2)*** *Determined values and calculated LR for urinary EtG in 37 patient samples without normalization, and with normalization using creatinine and conductivity.*

| Nr | EtG [mg $L^{-1}$] | Creatinine [mM] | Conductivity [mS $cm^{-1}$] | LR | | |
|---|---|---|---|---|---|---|
| | | | | EtG | EtG normalized with creatinine | EtG normalized with conductivity |
| PS-20 | 0 | 4.50 | 12.15 | < $6.485 \cdot 10^{-5}$ | < $10^{-6}$ | < $10^{-6}$ |
| PS-21 | 8.406 | 3.83 | 18.10 | $3.359 \cdot 10^{3}$ | > $10^{6}$ | > $10^{6}$ |
| PS-22 | 0.238 | 11.93 | 14.05 | 7.948 | 0.1318 | 6.751 |
| PS-23 | 0.095 | 8.99 | 15.70 | 0.145 | $2.3957 \cdot 10^{-2}$ | $9.468 \cdot 10^{-2}$ |
| PS-24 | 0.384 | 14.99 | 18.45 | 502.2 | 0.3477 | 31.3 |
| PS-25 | 4.642 | 3.03 | 12.20 | > $10^{6}$ | > $10^{6}$ | > $10^{6}$ |
| PS-26 | 0.105 | 16.90 | 21.65 | 0.1917 | $8.931 \cdot 10^{-3}$ | $5.693 \cdot 10^{-2}$ |
| PS-27 | 0.049 | 13.53 | 9.45 | $3.646 \cdot 10^{-2}$ | $3.976 \cdot 10^{-3}$ | $6.447 \cdot 10^{-2}$ |
| PS-28 | 0.548 | 12.69 | 15.40 | $5.244 \cdot 10^{4}$ | 5.966 | $9.797 \cdot 10^{3}$ |
| PS-29 | 0 | 13.17 | 17.50 | < $6.485 \cdot 10^{-5}$ | < $10^{-6}$ | < $10^{-6}$ |
| PS-30 | 0.088 | 6.82 | 20.55 | 0.116 | $3.721 \cdot 10^{-2}$ | $4.407 \cdot 10^{-2}$ |
| PS-31 | > 20 | 19.94 | 17.30 | > $10^{6}$ | > $10^{6}$ | > $10^{6}$ |
| PS-32 | > 20 | 17.05 | 8.31 | > $10^{6}$ | > $10^{6}$ | > $10^{6}$ |
| PS-33 | 0.145 | 8.06 | 9.86 | 0.596 | $9.507 \cdot 10^{-2}$ | 2.908 |
| PS-34 | 0.737 | 11.78 | 11.10 | > $10^{6}$ | $1.283 \cdot 10^{2}$ | > $10^{6}$ |
| PS-35 | 0.664 | 2.60 | 12.20 | $1.422 \cdot 10^{6}$ | > $10^{6}$ | > $10^{6}$ |
| PS-36 | 0 | 7.89 | 19.10 | < $6.485 \cdot 10^{-5}$ | < $10^{-6}$ | < $10^{-6}$ |
| PS-37 | 35.675 | 22.94 | 19.80 | > $10^{6}$ | > $10^{6}$ | > $10^{6}$ |

***Table 7 (part 2/2)*** *Determined values and calculated LR for urinary EtG in 37 patient samples without normalization, and with normalization using creatinine and conductivity.*

In other samples, e.g. PS-10, PS-17 and PS-32, the computer has been pushed to its limits and the exact value for the LR could not be calculated. It can be stated that the LR is greater than $10^{300}$. The exact value for the LR is not relevant in these cases, because even with very small prior odds, e.g. in the order of one to a trillion, which means that the probability of the individual having recently consumed alcohol is about $10^{12}$ times smaller than the probability of the individual not having consumed alcohol during the last three days, knowing the test result, the posterior odds are anyway beyond all doubt. In such a case any physician or judge would be convinced that the patient actually recently ingested alcohol.
PS-32 and 33 have not been further diluted to determine the exact concentration of EtG. Assuming that the determined level is 20 mg $L^{-1}$ EtG, the result is in favour of the tested person. The LR of greater than $10^{66}$ for all three methods (with or without normalization) are certainly providing a good basis for the interpretation and the decision to be taken by the expert in charge.

The application of the described model to samples where no EtG was found is problematic. The computer system provides a LR of zero for samples with an EtG concentration of 0 mg $L^{-1}$. Since the LR cannot be zero,
in these cases the LR is indicated to be smaller than the LR for 0.0001 mg $L^{-1}$ EtG, which corresponds to the smallest output unit of the EIA. These values are set to 0.001 mg $mol^{-1}$ and $10^{-6}$ mg cm $L^{-1}$ $S^{-1}$ for values normalized by creatinine and conductivity, respectively.

It is probable that the distribution of the samples stemming from persons who consumed alcohol is not log-normal distributed for EtG concentrations close to 0 mg $L^{-1}$. The distribution will at one point rather merge into a Poisson distribution as it has been shown for the collective of blank samples. This would solve the problem of the LR producing values of zero for samples with EtG concentration of 0 mg $L^{-1}$. Nevertheless, the evaluation of this region can not be done with the small number of data available. Until further studies are done and better fitted distributions are established, samples with no EtG detected are treated as described above. Doing so, the resulting LR is

strongest for conductivity normalized samples, followed by samples normalized with creatinine. The smallest LR for urine samples without normalization ($< 6.485 \cdot 10^{-5}$) is clearly less strong than the two others. Nevertheless, with prior odds close to 1 or lower the posterior odds are low. However, it is clear that EtG concentrations close to 0 mg $L^{-1}$ may occur with or without recent alcohol consumption. In other words, the discriminatory power of low EtG concentrations is rather weak. The influence of the evidence is expressed much stronger with samples determined to contain high EtG concentrations.

Interestingly enough, Table 7 suggests that the same samples as above for the discussion concerning the reference limit are worth a closer look, especially PS-14, 22, 24 and 33. These samples provide LR > 1 or LR < 1, dependent on their way of normalization.
Nevertheless, often the values for the LR are rather close to each other (maximal about 50000-fold difference for PS-14). The impact of the evidence on the posterior odds is rather modest. Thus it depends on the prior odds, if a clear statement can be given. However, with prior odds close to 1, the posterior odds will slightly favour or derogate the alternative propositions for a tested individual. Nonetheless, in this case, additional evidence is needed in order to conclude on the individual's pattern of recent alcohol consumption.

Striking differences between the LR are further observed for PS-5, 28 and 34. However, for these samples LR greater than 1 is resulting in any case, favorizing the hypothesis that the individuals actually did consume alcohol recently. Depending on the dilution factor of the samples, the differences of the LR's power are immense in some cases, e.g. more than $10^{16}$-fold for PS-19. Such discrepancies are observed in other samples of this collective, especially in the case of elevated urinary creatinine levels (PS-19: 22.613 mM).

The prior probabilities should always be considered when testing an individual. Notably physicians are aware of the occurrence of false-positive results. Therefore, they do not test patients for all possible diseases. A test is only significant if the prior probability, e.g. the prevalence is considered. So, patient 14 was tested negative for EtG

with the cut-off level suggested by the manufacturer of the DRI kit (0.5 mg $L^{-1}$). The calculated LR is $4.057 \cdot 10^3$, e.g. the probability to find the concentration of 0.458 mg $L^{-1}$ EtG in an individual that recently consumed alcohol is more that 4000 times larger than the probability to find the same amount of EtG in an individual given no ethanol ingestion for the last three days. This information is certainly more meaningful than a simple negative result. Depending on the prior odds, the interpretation of the test outcome may be very different to the one achieved using a reference limit.

The LR approach further allows the combination of multiple evidence, e.g. the results of different tests for recent alcohol consumption (as long as they are independent). Based on the prior probability of a hypothesis its posterior probability can be determined by multiplication of all LR determined by the analysis of the different markers.

### 6.3.13 Conclusions

The validated immunochemical assay is a convenient screening method for urinary EtG. The discussion of different ways for the normalization of urine concentration as well as of possible approaches for the interpretation of results provided suggestions suitable for handling the analytical issues of urine analysis.

Independent of the method for data interpretation, it is of great importance for routine controls to inform the tested individual in advance about the possibility of accidental alcohol ingestion. Wrong accusations may be avoided if the tested subject is aware and attentive to potentially alcohol containing products.

# 7

# DETERMINATION OF ETHYL GLUCURONIDE IN SERUM

# 7.1 Ethyl Glucuronide in Serum by CZE with Indirect Detection

The research group of Professor Thormann in Bern was involved in projects about EtG prior to the start of the work presented here. A method for the determination of EtG in serum by CZE was published in collaboration with a research group in Brno (Czech Republic) [136]. The method featured the analysis of serum 1:1 diluted with water. The simple sample preparation and analysis are the striking advantages of the method. Analysis was performed in a linear polyacrylamide (LPA) coated capillary. The polymer is covalently bound to the inner surface of the fused-silica capillary. This stable coating is therefore a permanent coating.

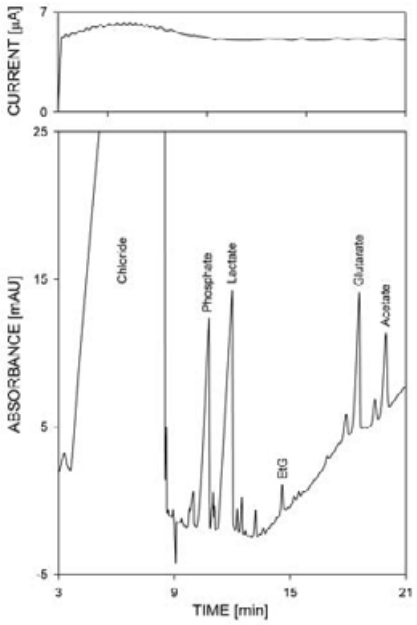



***Figure 39*** *Typical electropherogram obtained with the Brno homemade LPA-coated capillary (100 µm I.D.) on a P/ACE System 5010. A BGE prepared from a 10 mM nicotinic acid solution that was adjusted to pH 4.40 via the addition of ε-aminocaproic acid (EACA, about 6 mM) was used. The serum sample was spiked with EtG (5 mg $L^{-1}$), diluted 1:1 with water and injected for 5 sec at 0.5 psi. For the separation, the voltage was set to -15 kV (resulting current < 7 µA), and the optical detection was performed at 214 nm (filter).*

LPA-coated capillaries with an internal diameter of 100 µm were prepared by group members of Dr. Ludmila Křivánková in Brno (Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic) following the adopted Hjertén method [137] and sent to our laboratory.
The method for the analysis of EtG by CZE mentioned above was tested using these capillaries. Up to 400 runs could be performed on each of the homemade capillary. Figure 39 shows a typical electropherogram obtained.

The experiments using this CZE method with samples from different individuals revealed the following problems: (i) matrix peaks interfering with the EtG peak in some serum samples, (ii) time consuming coating procedure, and (iii) method did not feature an I.S. Thus, it was aimed to develop a method for the determination of EtG in serum by CZE with the focus on a future introduction to routine use.

### 7.1.1 Search for an Internal Standard

The first issue tackled was the search for an appropriate I.S. The PeakMaster 5.2 software was used to support the investigations. The freeware program predicts parameters of BGE and analyte peaks. The simulations can be displayed as electropherograms. PeakMaster contains a large database of compounds with their pKa values as well as their mobilities. However, the list needed to be completed by some substances and their parameters, e.g. EtG (pKa 3.21, $\mu$ $-23.5 \cdot 10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$).
The data was compiled from the literature [138-142] and the internet (http://research.chem.psu.edu/brpgroup/pKa_compilation.pdf, last visited: 17/11/2009), or was estimated by the author.

Many candidates could be eliminated with the help of the informatic tool, while 2,4-dimethylbenzoate, 3-bromobutyrate, 4-bromobutyrate and mandelate were experimentally evaluated by CZE. All these substances interfered with different matrix peaks. Glutarate was predicted to be a promising candidate, but it is present endogenously in the human body and was thus not suitable [143]. Most of its commercially available derivates were observed in experiments to occur endogenously or to interfere with matrix peaks of the serum samples, notably 2-methylglutarate and 2,2-dimethylglutarate.

Another derivate tested was 2,4-dimethylglutarate (2,4-DMGA), which usually is not present in the human body, neither endogenously nor from external sources. It passed the detector shortly after EtG, between

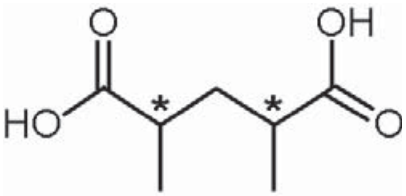



***Figure 40*** *I.S. used for the electrophoretic method for the determination of EtG in serum: 2,4-dimethylglutarate (2,4-DMGA).*

glutarate and glutamate, and was baseline-separated from any matrix-peak. This substance has two chiral centers (Figure 40). The two resulting pairs of isomers have opposite configurations at one of the chiral centres. The pairs are not mirror images of each other. The so called diastereomers may have different physical and chemical properties as it is the case for 2,4-DMGA. The two diastereomers are separated by the applied CZE method, yielding two discrete peaks (Figures 15, 42, 43, 45, and 47 to 50).

The resolution of the two diastereomers of 2,4-DMGA was a critical aspect for this method, since the slower migrating peak of the I.S. was comigrating with L-ascorbic acid (vitamin C), which was present in large amounts in some samples, depending on the nutrition of the sampeled individual. Therefore, only the faster migrating diastereomer was used for the standardization of the results.

### 7.1.2 Effects of pH Variations on Analyte Resolution

After the introduction of an I.S. to the assay for EtG determination in serum, the issue concerning the matrix peaks interfering with the analyte of interest was tackled. In order to solve this problem, it was necessary to know what compounds actually migrated close to or with EtG. The study of the literature provided many candidates for endogenous compounds, especially carboxylic acids and amino acids, e.g. glutarate, benzoate, succinate, aspartate, hydroxybutyrates, propionic acid, isovaleric acid, valeric acid, caproic acid, oxalic acid, pyruvic acid, malic acid, acetoacetic acid or citric acid [143]. Employing the PeakMaster 5.2 software, it was possible to evaluate which substances might interfere with EtG during the electrophoretic separation (Figure 41).

The findings obtained from the simulations were experimentally confirmed and succinate could be identified to comigrate with EtG having a BGE at about pH 4.40. Benzoate, in reality absorbing stronger than the BGE and therefore resulting in a peak in the opposite direction, was also confirmed not to be baseline separated from the EtG-peak at higher concentrations. Thus, the BGE needed

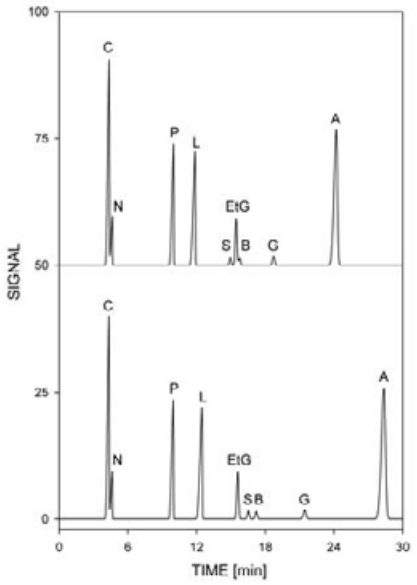



***Figure 41*** *Simulation of electropherograms created with the PeakMaster 5.2 software showing the interference of EtG and succinate between pH 4.3 (lower graph) and pH 4.4 (upper graph). The simulation implied the application of -15 kV and featured a BGE made of 10 mM nicotinic acid, about 6 mM EACA and sodium as counter ion. Key: C, N, P, L, S, B, G and A refer to chloride, nitrate, phosphate, lactate, succinate, benzoate, glutarate and acetate, respectively.*

modifications in order to provide the separation of EtG from other matrix peaks in real samples. As for the simulation data, experiments with a BGE of pH 4.30 provided better resolution of EtG. This is why the pH was adjusted 0.1 unit lower than proposed by Mrázková et al. [136]. The simulations and the experiments by CZE clearly revealed the strong impact of small pH variations on the mobility of certain analytes.

### 7.1.3 Ethyl Glucuronide Extraction from Serum

The change of the pH of the BGE to 4.30 allowed avoiding comigration of EtG and succinate. However, at large amounts of succinate, the two peaks were not baseline separated. Furthermore, the method required very precise pH adjustment and was therefore not robust.
In order to avoid the interference between EtG and succinate or benzoate, two approaches were taken into consideration: (i) sample preparation involving the elimination of the disturbing compounds from the sample matrix and (ii) further optimization of the BGE.

First, the extraction of EtG from serum was investigated. SPE has been shown to be a potent method for the extraction of anions occurring in low concentrations in serum (Chapter 5.1.2 and [95]). Since the water soluble EtG has a pKa value of 3.21, the SPE columns listed in Table 8 were tested.

The disposable Clean Screen ETG columns from United Chemical Technologies (UCT, Bristol, PA, USA) were developed and introduced for the extraction of EtG from urine for the analysis by LC-MS or GC-MS (http://www.amchro.com/PDFs/SPE/EthylGlucuronideFlyer.pdf, last visited: 27/01/2010). The methods for LC-MS and GC-MS just differ for details like the amount of conditioning solution or the way of drying the sample. However, the extraction method for the preparation of urine samples for LC-MS was adapted for serum samples analysed by CZE [112]. The conditioning with 2 mL of formic acid 1 % (in water, v/v) was the same. Prior to the sample load, the column was not

| Name | Manufacturer | Type | Result |
|---|---|---|---|
| Oasis MAX | Waters | Mixed-mode anion-exchange and reversed-phase sorbent | Poor EtG recovery (< 10 %) |
| Isoelute NH2 | Biotage | Weak anion-exchange | Poor EtG recovery (< 10 %) |
| Oasis HLB | Waters | Reversed-phase (hydrophilic-lipophilic balance sorbent) | EtG not recovered |
| Bond Elut PBA | Varian | Covalent retention, highly specificity for cis-diol | Matrix not suitable for CZE. Recovery could not be determined due to not reproducible electropherograms. |
| Strata X-AW | Phenomenex | Polymeric weak anion-exchange | Poor EtG recovery (< 10 %) |
| Strata SAX | Phenomenex | Strong anion-exchange | EtG not recovered |
| Strata X | Phenomenex | Reversed-phase | EtG not recovered |
| Clean Screen ETG | UTC | Active carbon blend, designed for extraction of EtG from urine | Good EtG recovery (72.9 – 99.4 %) |

***Table 8*** *Tested SPE columns for the extraction of EtG from human serum. The method for the extraction using the Clean Screen ETG column is described in the text. The other SPE columns have been tested according to the standard procedures from the manufacturers.*

dried. Instead of 1 mL urine mixed with 50 µL formic acid, 500 µL serum mixed with 100 µL I.S. solution (100 mg $L^{-1}$ 2,4-DMGA in water), 250 µL water (or EtG dissolved in water for spiking of the sample), and 100 µL formic acid were loaded on the column. Without the dilution with water, the sample was too viscous to pass the column. In order to reach pH of about 2, the amount of formic acid to add needed adaptation because of the natural pH-buffer system of the blood.
After the sample passed the column, a washing step with 2 mL water followed, before the column was dried for 10 min at 2 kPa. Elution was done with a filter (Millex-GP 0.22 lm Filter Unit, Millipore, Bedford, MA, USA) placed under the column. This was done in order to avoid leaking of the carbon filling which could not be sorted out by the manufacturer. The column was rinsed with 2 mL formic acid 1 % (in MeOH, v/v). Additionally, the filter alone was rinsed with 1 mL of the same solution. The eluates were combined and evaporated to dryness under stream of air. It was observed that even an extensive stream of air did not have an influence on the EtG concentration in the sample. Thus, air was used instead of nitrogen gas as recommended by the manufacturer. Samples were then reconstituted with 500 µL water.

These columns clearly provided the best recovery for EtG (n=2 at each level: 72.9, 91.1 and 99.4 % for 12, 6 and 1.5 mg $L^{-1}$ EtG, respectively [112]) as well as nice and reproducible electropherograms. Figure 42 depicts the electropherograms obtained from a blank serum, a blank serum spiked EtG and I.S. (2,4-DMGA) and a real sample from a volunteer who ingested ethanol prior to sampling. Serum extracts obtained using the Clean Screen ETG columns were also used for other applications throughout this work. Serum extracts obtained using the Clean Screen ETG columns were also used for other applications throughout this work (Chapters 7.1.7, 7.1.9, 7.1.10, 7.2, and 7.3.1 as well as Figures 44, 46, 47, and 51).

For economic reasons and in order to investigate whether the carbon filling was somehow modified by UCT for the extraction of EtG or

not, it was aimed to replace the commercial columns with home-made columns. However, EtG could not be extracted using ordinary active carbon as column filling.

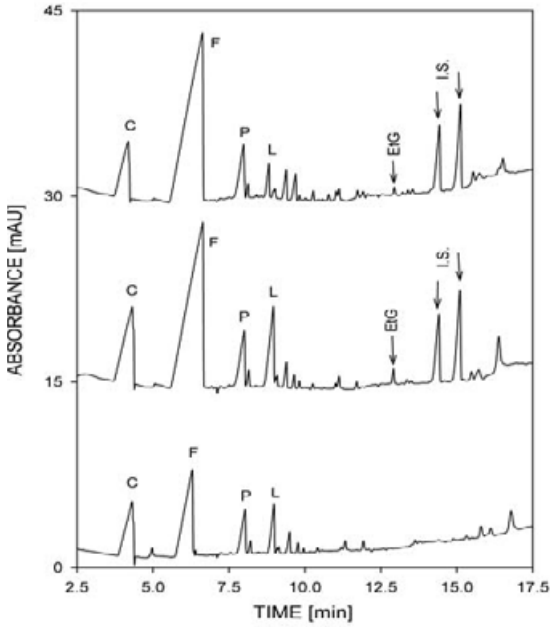



***Figure 42*** *Electropherograms of sera extracted with the Clean Screen ETG SPE column (refer to text or [112] for the detailed description of the extraction method), reconstituted with water to their original volume and analyzed on a P/ACE MDQ with UV detection at 215 nm featuring a LPA-coated capillary (75 µm I.D.; $L_{tot}$ 60.0 cm; $L_{eff}$ 50.0 cm). Separation was done applying -24 kV and using a BGE composed of 10 mM nicotinic acid, about 11.5 mM EACA (pH 4.65) and 10 % ACN.*
*Data were obtained with a blank serum, a blank serum spiked with 1.5 mg $L^{-1}$ EtG and 20 mg $L^{-1}$ I.S. (2,4-DMGA), and a sample taken from a volunteer who had consumed alcohol recently before sampling (EtG concentration 1.01 mg $L^{-1}$ determined using the CZE method published by Jung et al. [112]) are displayed with a y-axis offset for the sake of clarity (from bottom to top). Key: C, F, P and L refer to chloride, formate, phosphate and lactate, respectively. Figure from [112].*

As all the other tested columns, the Clean Screen ETG column has a major drawback: It is not specific to EtG and thus other anions elute from the column together with EtG, above all succinate. However, 2,4-DMGA has a high recovery as well and can be used as I.S. for the extraction.

The Clean Screen ETG column was not advantageous compared to plain serum for the use in the CZE method using the LPA-coated capillary. Concentration of the samples during extraction was useless, because the interfering matrix compounds were concentrated as well. However, the matrix could be simplified so that less proteins and fast migrating anions (e.g. chloride and sulfate) were present in the eluate. Even if the SPE extraction was not advantageous here, it was very useful for other CZE methods discussed below, especially for the work featuring CZE-MS (Chapter 7.2).

### 7.1.4 CZE in Capillaries with Permament Coating and Search for Sensitivity Enhancement

The preparation of the LPA-coated capillaries is a time consuming process. Furthermore, batch to batch differences are quite large in some cases. This is why before heading toward the development of a BGE providing better separation of EtG from matrix compounds, different capillary coatings were evaluated. The goal was to evaluate whether the homemade LPA-coated capillary could be replaced by another capillary.
First, the commercially available Guarant capillary I.D. 50 µm (Alcor Bioseparations, Palo Alto, CA, USA) with a very low EOF ($< 0.3 \cdot 10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$) was tested [112]. As the LPA-coating, the hydrophylic and hydrolytically stable wall coating made of a neutral polysaccharide of the Guarant capillary is a permanent coating. The data produced showed much more noise compared to the LPA-coated capillary and consequently the LOD was > 1 mg $L^{-1}$ (LPA-coated capillary 0.15 mg $L^{-1}$). No further work was done to elucidate the reasons for this difference.

In order to reach better sensitivity of the electrophoretic method, the enhancement of the optical detection was investigated. As discussed above, EtG is a nonabsorbing substance. Therefore, it was necessary to revert to indirect detection for the work featuring a UV-detector. For the CZE method discussed in this chapter, the aromatic heterocyclic nicotinate is the UV absorbing substance enabling indirect detection [144].

With the goal to enhance sensitivity, strongly absorbing compounds were added to the BGE, namely squaric acid (SQA) and 2,5-dihydroxy-1,4-benzoquinine (DHBQ). These two vinylogous carboxylic acids have been described in a US Patent to strongly absorb UV light [145] and to reach their maximum absorption at 270 nm and 320 nm, respectively. The substances may serve the purpose of enhancing absorption, even if present at low amounts in BGE. At low concentrations, no precipitation of the poorly soluble compounds and only negligible disturbances of the electrophoretic system were expected.
Both buffer additives were tested in separate electrolyte solutions each. They were added at a concentration of 3 mM to the BGE made of nicotinic acid 10 mM and about 6 mM EACA (pH 4.40) and tested in the two capillaries with no EOF discussed in this chapter, i.e. LPA-coated (Polymicro Technologies) and Guarant capillary (Alcor Bioseparations).

With the Diode Array Detector, the spectra were recorded from 190 to 350 nm (data rate 4 Hz). The maximum absorbance of SQA was confirmed to be at 270 nm. However, the observed maximum absorbance for DHBQ was at 295 nm. This is lower than the 320 nm indicated by Yengoyan et al. [145].
Some electropherograms showed similar patterns as without the addition of SQA or DHBQ to the BGE. Nevertheless, both methods featuring the buffer additives lacked in reproducibility. Above all, the baseline was very unstable so that after some runs (n>5) the pattern produced during previous runs was no longer observed. Generally, the electropherograms were very noisy. The LOD obtained with the two vinylogous carboxylic acid compounds in the BGE was more than

10-fold higher compared to the LOD achieved with the BGE without the two absorbing substances. Hence, research about sensitivity enhancement based on the addition of absorbing substances other than nicotinate was abandoned.

### 7.1.5 CZE in Uncoated Capillaries

Since the capillaries with permanent coatings did not yield satisfying results, it was aimed to use a bare fused silica capillary (100 µm I.D.) without modifying its inner wall. With the discussed BGE, a strong EOF towards the inlet resulted. Thus, a short capillary ($L_{eff}$ 20 cm; $L_{tot}$ 27 cm) was used in order to keep the detection time low. The exact EOF was not determined because the uncharged EOF marker (acetone) did not reach the detector in this system. A current of 10.5 µA was observed at a separation voltage of -15 kV. The electropherograms showed flat and large peaks so that EtG could not be detected at concentrations in the low mg $L^{-1}$ range (detection time fot EtG about 3 min). The system was unstable and sensitivity was insufficient, thus this approach was abandoned.

### 7.1.6 CZE in Dynamically Coated Capillaries using CTAB

Further tests then involved dynamic capillary coatings. As mentioned above, in this case the coating substances interact dynamically with the inner wall of the capillary by physical adsorption. Therefore the coating needs to be renewed regularily in order to achieve reproducible data.
As it has been discussed for EtS in urine and serum (Chapters 4.1 and 5.1), dynamic coatings systems can also include the coating substance in the BGE during separation. This was the case for this method featuring CTAB as well.
The positively charged part of CTAB attaches to the negatively charged capillary wall, while the uncharged part points towards the center of

the capillary. Since it has been shown that the EOF can be modified by the addition of varying concentrations of CTAB to the BGE, the application of this cationic surfactant for the suppression of the EOF was tested.
The conditioning before applying the coating was the same for all dynamic coatings: the capillary was rinsed with NaOH 1 M, NaOH 0.1 M, and water for 30 min at 20 psi, each.
The washing of the 100 µm I.D. capillary (10 min at 20 psi) and the separation were performed using the BGE containing CTAB. Adding CTAB at a concentration of 25 µM to the BGE made of nicotinic acid 10 mM and about 6 mM EACA (pH 4.40) provided a system with nearly no EOF. The exact EOF was not determined (EOF marker did not pass the detector within 40 min) and the current was lower than 5.2 µA. No matrix peak was found to comigrate with EtG. Nevertheless, the system was not robust at this concentration of CTAB. This has also been shown in other work [97]. The EOF varied strongly with minor changes of the CTAB concentration. Moreover, before every working day the system needed a large number of runs or the application of about 3 h of high voltage to the capillary filled with BGE before the EOF was stable. Nevertheless, the system was convenient due to its simplicity. Thus, the approach discussed by Chiari et al. [146] with the addition of both, CTAB (50 µM) and polyvinyl alcohol (PVA, 0.05 % w/v), to the BGE was tested. The capillary used was also a fused-silica capillary of 57 cm total length and an I.D. of 100 µm.
PVA increases the viscosity of the BGE and thereby decreases the EOF (Chapter 1.3, Equation 4). The BGE was adjusted to pH 4.50 and electropherograms with patterns comparable to those obtained with the LPA-coated capillaries resulted (EOF marker did not pass the detector within 40 min). The method settings as well as typical electropherograms are depicted in Figure 43. However, as the capillary regularly blocked after a few runs when analyzing serum samples [112].

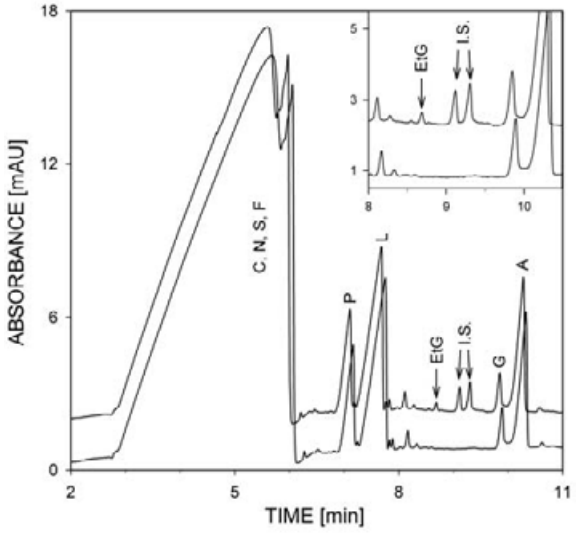



***Figure 43*** *Electropherograms obtained on a P/ACE System 5010 with a dynamically coated capillary ($L_{eff}$ 50 cm, $L_{tot}$ 57 cm, 100 µm I.D.). A BGE prepared from a 10 mM nicotinic acid solution that was adjusted to pH 4.50 via the addition of ε-aminocaproic acid (EACA, about 7.5 mM) was used. CTAB (50 µM) and PVA, (0.05 % w/v) were added to the BGE as EOF modifying substances. A blank serum sample diluted 1:1 with water (bottom) and the same sample spiked with 5 mg $L^{-1}$ EtG and I.S. (20 mg $L^{-1}$, 2,4-DMGA) prior to the 1:1 dilution with water (top) were injected for 5 sec at 0.5 psi. For the separation, the voltage was set to -20 kV, and the optical detection was performed at 214 nm (filter). Key: C, N, S, F, P, L, G and A refer to chloride, nitrate, sulfate, formate, phosphate, lactate, glutarate and acetate, respectively.*

### 7.1.7 CZE in Dynamically Coated Capillaries using PDADMAC

Further tests involved a PDADMAC coating (see also Chapters 4.1.3 and 5.1) applied to a 100 µm I.D. fused-silica capillary. This polycation is an often used substance for the establishment of dynamic coatings [95, 98-102]. An amalgam of some of these published methods adapted to the available instruments and settings, personal communications with Dr. J.B. Schlenoff (Florida State University Tallahassee), as well as own experience led to the method described below. The main issue

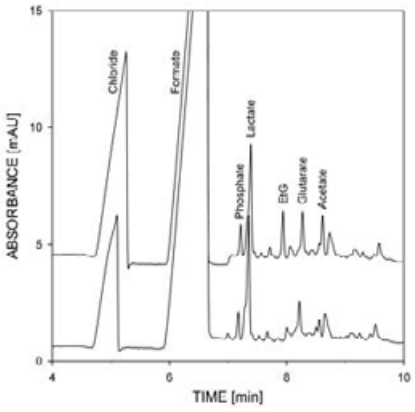



***Figure 44*** *Electropherograms obtained with the capillary coated with a single PDADMAC layer ($L_{eff}$ 63 cm, $L_{tot}$ 70 cm, 100 µm I.D.) on a P/ACE System 5010. A BGE prepared from a 10 mM nicotinic acid solution that was adjusted to pH 4.40 via the addition of ε-aminocaproic acid (EACA, about 6 mM) was used. A blank serum sample (bottom) and the same sample spiked with 2 mg $L^{-1}$ EtG (top) were extracted with the Clean Screen ETG SPE column (refer to the text for the precise description of the method for extraction), reconstituted with water to their original volume and injected for 5 sec at 0.5 psi. For the separation, the voltage was set to -15 kV (current about 4 µA), and the optical detection was performed at 214 nm (filter).*

was that the reproducible rinsing of the capillary with the viscous polymere. Furthermore, the concentration of PDADMAC in the coating solution influenced the density of the charge of the inner capillary wall leading to variations in the EOF.

Many factors have been described to have an impact on the formation of the PDADMAC-layer, e.g. ionic strength of the coating solution, polyelectrolyte molecular weight, concentration and deposition time [147-153]. The influence of the ionic strength of the coating solution on the layer could not be confirmed experimentally and therefore, no salt was added to the coating solution of the final method. Since it was observed, that in this system, the BGE at pH 4.40 provided better resolution for EtG than at pH 4.30, the higher pH was applied for the following experiments.

The coating of the 100 µm I.D. capillary was done by rinsing it with PDADMAC 0.5 % (w/w) in BGE (pH 4.40) for 10 min at 20 psi, followed by the application of -12.5 kV with PDADMAC 0.5 % (w/w) in the BGE. It has been shown that the coating

can be stabilized by the application of high voltage [151, 153]. This was observed to be true in experiments done at our laboratories (for detailled study with the PEI-PSS-PDADMAC multiple layer coating refer to Chapter 7.1.9). The coating was renewed before every run by a 30 sec rinsing (20 psi) with PDADMAC 0.5 % in BGE. Before sample injection, the capillary was rinsed with BGE (2 min, 20 psi). The EOF was determined to be $-29.7 \cdot 10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$ (current 5.5 μA). The coating was stable and provided quite reproducible data (detection time for EtG 5.3 min (1.43 % RSD, n=5)). Nevertheless, in some serum samples, interferences between EtG and matrix peaks occurred.

Thus, a longer capillary ($L_{tot}$ = 70 cm) was then used with the goal of better separation of the analytes of interest. Detection time of EtG was then about 8 min, however, it comigrated with succinate at pH from 4.30 to 4.50 (Figure 44). In samples containing no detectable amounts of succinate, the LOD for EtG was determined to be about 0.05 mg $L^{-1}$.

Unfortunately, the EOF slightly rose with every run, so that the method was not validated. Nevertheless, the PDADMAC-coating served for the studies about the optimization of the BGE discussed below (Figure 48 and 49 in Chapter 7.1.12). Due to its simple fabrication, this coating was very useful for quick evaluations of new BGE compositions.

### 7.1.8 CZE in Multi Layer Coated Capillaries using PDADMAC-PSS-PDADMAC

It was investigated whether the EOF could be stabilized with the addition of multiple layers to the PDADMAC coating. Multiple layer coatings are formed with alternating layers of a polycation and a polyanion. Due to the opposite charges of the layers, they physically attract each other, and thus attach to the inner wall of the capillary. Multiple layer coatings have been shown to be more enduring than single layer coatings [149, 154, 155].

First experiments were done featuring PDADMAC and poly(4-styrenesulfonic acid) (PSS, mean molecular mass ~75 kDa), which have been used as coating substances by other authors [148, 149, 153, 156, 157]. The first layer of PDADMAC was attached to the inner surface of the capillary the same way as it was done for the single PDADMAC coating (Chapter 7.1.7). The following layer, featuring the polyanion, was introduced by rinsing the capillary with PSS 0.02 % (w/w) in BGE (pH 4.40) for 5 min at 10 psi followed by the application of -15 kV for 30 min. Then the capillary was rinsed with water (5 min, 10 psi). This procedure was repeated alternatively with the polycation (PDADMAC 0.02 %) and the polyanion until the 7 layers of PDADMAC were completed.

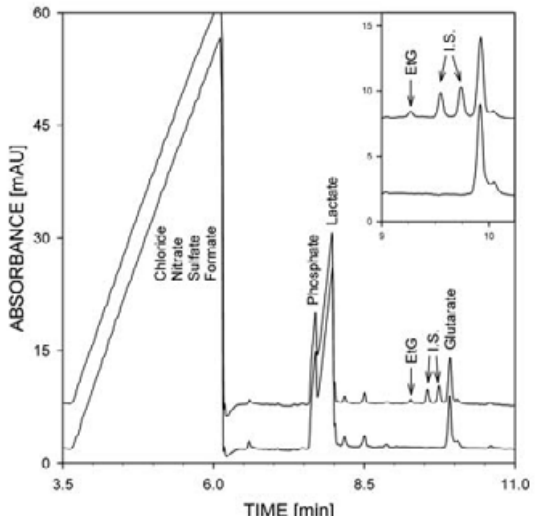



***Figure 45*** *Electropherograms obtained with the PDADMAC-PSS-PDADMAC multiple layer (7 layer of PDADMAC, 6 layers of PSS; refer to the text for the precise description of the coating procedure) coated capillary ($L_{eff}$ 60 cm, $L_{tot}$ 50 cm, 100 µm I.D.) on a ProteomeLab PA 800. A BGE prepared from a 10 mM nicotinic acid solution that was adjusted to pH 4.40 via the addition of ε-aminocaproic acid (EACA, about 6 mM) was used. A blank serum sample (bottom) and the same sample spiked with 5 mg $L^{-1}$ EtG and 20 mg $L^{-1}$ 2,4-DMGA as I.S. (top) mixed 1:1 with water and injected for 6 sec at 0.5 psi. For separation, voltage was set to -20 kV, and optical detection was performed at 214 nm.*

Prior to each run the capillary was rinsed with BGE (30 sec, 40 psi) followed by a rinsing with 0.02 % PDADMAC in BGE (24 sec, 1 psi) and another rinsing with BGE only (90 sec, 40 psi). Separation of the analytes was done without the addition of coating substance to the BGE.

The PDADMAC-PSS-PDADMAC coating provided similar patterns of the electropherograms as the separations performed using the LPA-coated capillary (Figure 45). However, the runs were somewhat faster due to the presence of the negative EOF (migration time of EtG about 9.3 min). The intraday-reproducibility of detection times was good (RSD < 0.4 %, n=5), while the differences between working days were clearly larger (RSD 3.9 %, n=4). The method was not stable enough with the perspective of future routine use.

### 7.1.9 CZE in Coated Capillaries featuring PEI

Due to the lack of stability of the PDADMAC-PSS-PDADMAC coating, another multiple layer coating was tested: the first polycation-layer was replaced by polyethyleneimine (PEI) for both, the single layer coating and the multiple layer coating. PEI is said to adhere stronger to the capillary surface than other polycations (personal communications with Dr. J.B. Schlenoff).

The coating procedure was the same as for PDADMAC described above but with 5 % (w/w) PEI in the BGE (pH 4.40). The single PEI-coating resulted in an EOF of $-35 \cdot 10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$ and a current of less than 5 µA. The pattern of the electropherograms was very similar to the data obtained with the PDADMAC-coated capillary. EtG was not resolved from some compounds of the matrix like succinic acid and benzoic acid (Figure 46). Therefore the LOD could not be determined.

The method provided reproducible data for about 20 runs (RSD < 1 %). Thereafter, the EOF was observed to change in an unpredictable manner.

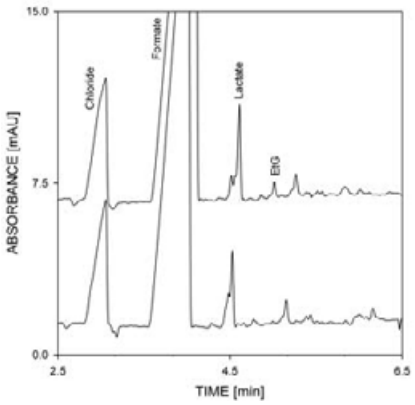



***Figure 46*** *Electropherograms obtained with the PEI-PSS-PDADMAC multiple layer (1 layer of PEI, 6 alterning layers of PSS and PDADMAC) coated capillary ($L_{eff}$ 50 cm, $L_{tot}$ 57 cm, 100 µm I.D.) on a P/ACE System 5010. A BGE prepared from a 10 mM nicotinic acid solution that was adjusted to pH 4.40 via the addition of ε-aminocaproic acid (EACA, about 6 mM) was used. A blank serum sample (bottom) and the same sample spiked with 1 mg $L^{-1}$ EtG (top) were extracted with the Clean Screen ETG SPE column (refer to the text for the precise description of the method for extraction), reconstituted with water to their original volume and injected for 5 sec at 0.5 psi. For the separation, the voltage was set to -15 kV, and the optical detection was performed at 214 nm (filter).*

The multiple layer coating (PEI-PSS-PDADMAC) was very stable. When high voltage was applied during the coating step of each layer, the RSD of the detection time of EtG was less than 0.4 % (for n=5 and n=15). When no voltage was applied, the detection times varied more (RSD 1.86 % for n=5). EOF and current were $-45 \cdot 10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$ and 5 µA, respectively. Although the stability of this multiple layer coating was satisfying, the baseline of the electropherograms of the single and multiple layer coating showed more noise than for the PDADMAC-PSS-PDADMAC coating. This led to a worse sensitivity for EtG and therefore it was decided not to continue the work with this method.

Other multiple layer coatings including PEI were evaluated, e.g. featuring PEI as last layer in two different systems. First a PEI-PSS-PEI multiple layer coating introduced analogous to the coating discussed in the section above, but replacing PDADMAC with PEI. For the second experiment PSS was replaced by dextransulfate (DS), in order

to produce a PEI-DS-PEI multiple layer coating. In both systems featuring top layers composed of PEI, wild patterns were observed which could not be reproduced.

Multiple coatings with different numbers of layers of polybrene and DS, as described by Katayama et al. [155], were tested as well. The coating procedure was analogous to the PDADMAC-PSS-PDADMAC multilayer coating described above. The electropherograms were very noisy and the general pattern was not reproducible. Hence, the studies about multiple layer coatings including polybrene or DS were abandoned.

### 7.1.10 Short End Injection

Some of the tested capillary coatings could possibly replace the LPA-coated capillaries for the determination of EtG in serum, e.g. coatings featuring PDADMAC or CTAB-PVA. However, none of the methods provided better performance than the LPA coating. In order to investigate other coatings, it was considered to use short end injection. This means that the inlet and outlet side of the capillary are inversed and the separation takes place on the short side of capillary, where the distance to the detector is minimal. This allowed applying systems featuring normal polarity, which would result in long detection times with the standard setup.

Under normal polarity, the negatively charged analytes migrate away from the detector. However, with a negatively charged coating of the inner wall of the capillary, an EOF in the direction of the detector is produced. If the absolute velocity of the EOF is larger than the absolute velocity of the analytes, then these are sweeped towards the detector. The order the sample ions passing the detector is inversed compared to the systems featuring reversed polarity discussed above. Experiments were done on the ProteomeLab PA 800 which has a $L_{eff}$ of 10 cm on the short end of the capillary.

The first experiments were done featuring capillaries with multiple PDADMAC-PSS layers. The coating procedure for the layers was exactly the same as the method described above applying these two polyions (Chapter 7.1.8). The pH of the BGE was 4.40. The only difference to the prior multiple coating was that as last layer (n=6 for the first analysis) was made of PSS. Like this, a pH independent negative surface of the inner capillary wall was fabricated.

The coating was renewed by introducing two new layers before every run. This was done by consecutive rinsings at 20 psi with NaOH 0.1 M (1 min), water (1 min), PDADMAC 0.5 % (w/w) in BGE (1 min), air (30 sec), water (30 sec), PSS 0.5 % (w/w) in BGE (1 min), air (30 sec), water (30 sec), and finally PSS 0.05 % (w/w) in BGE (2 min). The last solution corresponded to the BGE used for the separation.
The EOF of this system was determined injecting diluted BGE at the capillary end opposite to the detector ($49 \cdot 10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$). The maximum current was observed to be 5.4 μA.

With the voltage set to 15 kV, EtG passed the detector between 6 and 10 min. The general pattern of the electropherograms was similar for all runs, however, EOF was varying a lot. Furthermore, spikes of the signal were often observed and the LOD was > 5 mg $L^{-1}$ when injecting a sample plug of 0.25 psi sec. Thus, this method was considered unqualified for the purpose of EtG determinations in serum.

Then, two different double coating kits from Analis (Namur, Belgium) were tested with 100 μm I.D. capillaries, namely the CEofix-CDT kit and the CEofix-MS kit. The proprietary reagents of the solutions are not known to the author. However, it is known that different polycations and polyanions are featured in these kits. The manufacturer describes the introduction of the negative layer as last layer in the capillary.
The first kit was developed for the analysis of CDT by CZE [26]. At our laboratory this double coating was studied intensively in the past. It was observed that the mobility of the analytes of interest was larger than the mobility of the EOF. Thus, the separation was done under

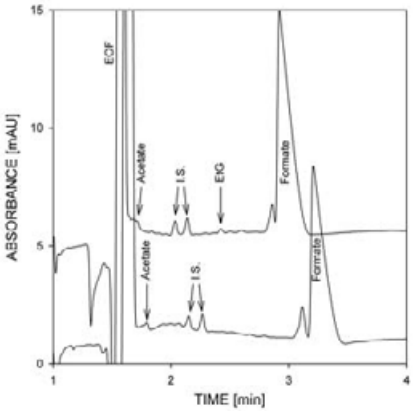



***Figure 47*** *Electropherograms from blank serum spiked with I.S. (2,4-DMGA) 10 mg $L^{-1}$ (bottom graph), and blank serum spiked with EtG 1 mg $L^{-1}$ and I.S. 10 mg $L^{-1}$ (top graph) are displayed with a y-axis offset for the sake of clarity.*
*Prior to injection the samples were extracted with Clean Screen ETG SPE columns and reconstituted with water to 20 % of their their original volume (refer to the text for the precise description of the method for extraction).*
*Analysis featuring short-end injection were executed on a ProteomeLab PA 800 with UV detection at 214 nm and using a 50 µm I.D. capillary coated with the reagents of the CEofix-MS kit from Analis ($L_{tot}$ 110.0 cm; $L_{eff}$ 10.0 cm). BGE consisted of the polyanion solution of the kit mixed 1:24 with 10 mM nicotinic acid and about 6 mM EACA (pH 4.40). Injection of the SPE-extracts was achieved by the application of 10 psi sec. A voltage of 25 kV was applied for the separation.*

reversed polarity. With the BGE used (pH 4.40, described above), it was not possible to stabilize the EOF. In addition, this coating was tested with the injection performed on the long end of the capillary (normal injection) and with the voltage set to reversed polarity. As in short end injection, here the EOF could not be stabilized neither. The extent of variations was so elevated that the detection times of EtG were from 17 min up to more than 25 min (end of run at 25 min).

The CEofix-MS kit was only tested with the short end injection setup (capillary: $L_{eff}$ 10 cm, $L_{tot}$ 110 cm, I.D. 50 µm). The coating was applied by rinsing (40 psi) the capillary consecutively with NaOH 0.1 M (2 min), water (1 min), "initiator" (polycation, 2 min), "separation solution" (polyanion in BGE 1:24, 4 min), NaOH 0.1 M (2 min), water (1 min), and "initiator" (2 min).

Every 20 runs, as well as at the beginning of each working day, this coating procedure was repeated. Before injecting a sample, the capillary was rinsed with the "separation solution" for 1.5 min at 40 psi.

Samples were injected for 20 sec at 0.5 psi (about 5.5 % of $L_{eff}$). As above, the runs were performed applying reversed polarity. The EOF was observed to be $29 \cdot 10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$ (-15 kV; current about 1 µA). The coating was very stable for many runs (n=5, RSD 1.04 %).

In order to simplify the matrix and to reach better sensitivity, besides serum diluted 1:1 with water also SPE-extracts prepared with the Clean Screen ETG columns were injected (Figure 47). The LOD of EtG determined for this method was comparable to that obtained with the LPA-coated capillary (0.15 mg $L^{-1}$), but unfortunately, EtG again comigrated with some matrix peaks. Tests with other BGE compositions and varying pH, e.g. mandelic acid 10 mM adjusted to pH 3.5 with about 6 mM EACA, did not provide better separation of the analytes.

### 7.1.11 Choice of Capillary Coatings for Further Work

In conclusion, some coating methods were found to be possible alternatives for the homemade LPA-coating, above all the systems featuring CTAB-PVA and the CEofix-MS kit from Analis. Nevertheless, due to the periodical blocking of the capillaries applying BGE containing CTAB and PVA, this system was not further investigated. The costly CEeofix-MS kit was not advantageous compared to the LPA coating. Therefore, the kit was not considered for further experiments as well.

Polymicro Technologies (Phoenix, AZ, USA) introduced a LPA-coated capillary (75 µm I.D.) to the market. The commercially produced capillary represented an attractive alternative to the own fabrication of capillaries. Therefore, these capillaries were tested in further experiments described below.

The capillary coating featuring PDADMAC did not provide constant EOF and attempts for the stabilization of the EOF with multiple

layer coatings revealed other issues. However, due to its convenient fabrication and for economical reasons, this coating was used for the development of an appropriate BGE for the separation of EtG in serum along with the comercially available LPA-coated capillary.

### 7.1.12 Background Electrolytes with Organic Modifier

Recapitulatory, different alternatives for the LPA-coated capillary were found and an I.S. has been introduced. However, after the tested methods for EtG extraction from serum, different anions comigrating with EtG were still present in the samples. Therefore, the goal was to develop an electrophoretic method able to separate EtG and the I.S. from the other ions in the sample.

In previous work, different BGE compositions have been evaluated, i.e. combinations of different coions and counterions as well as pH variations in the range of 3.2 to 4.5 [136, 158]. It has been shown that particularly changes in pH have a strong impact on the isotachophoretic stacking [6] of EtG in presence of components occurring in the sample plug at high concentrations (from 0.5 up to about 100 mM), e.g. chloride, lactate, phosphate or acetate. In the system featuring BGE with nicotinate as coion and EACA as counter-ion (pH in the range of 4.30 to 4.40), the mentioned ions of the sample matrix act as leading ions while nicotinate of the BGE acts as terminator [111, 136].

As mentioned above, pH variations of the BGE may affect the sensitivity of the analytical method. However, sensitivity was

[6] *Sample stacking is the process of concentrating diluted samples inside the capillary during electrophoretic separation. In isotachophoetic stacking the sample zone is sandwiched between a leader and a terminator, i.e. ions with higher and lower mobility, respectively. When voltage is applied, the analyte will be stacked and then separated by electrophoresis.*

immolated for the sake of better separation of EtG from matrix peaks since resolution is crucial for the development of a method with a potential routine application in future.

It was not possible to adjust the pH so that EtG was baseline-separated from matrix-peaks. Thus, different organic modifiers, namely iso-propanol, methanol and ACN, were added to the BGE at concentrations from 0 to 20 %. Capillaries coated with the single PDADMAC coating described above (Chapter 7.1.7) were used. The basic BGE was composed of 10 mM nicotinic acid and about 6 mM EACA (pH 4.40). The solvents decrease the EOF because

| Organic modifier | Concentration | $t_{d\ (EtG)}$ |
|---|---|---|
| None | — | 6.8 min |
| Isopropanol | 5 % | 9.6 min |
| | 10 % | 9.9 min |
| | 15 % | 13.3 min |
| | 20 % | 16.3 min |
| Methanol | 5 % | 8.6 min |
| | 10 % | 9.5 min |
| | 15 % | 10.4 min |
| | 20 % | 11.2 min |
| Acetonitrile | 5 % | 8.1 min |
| | 10 % | 8.2 min |
| | 15 % | 8.7 min |
| | 20 % | 8.3 min |

***Table 9*** *Influence of different organic modifiers in the BGE on the separation of EtG in serum. One run was assessed for each level. Prior to the addition of the solvents, BGE was composed of 10 mM nicotinic acid and about 6 mM EACA (pH 4.40). The same serum sample spiked with 20 mg $L^{-1}$ EtG was analyzed on a ProteomeLab PA 800 CZE instrument with UV detection at 214 nm and featuring a capillary coated with a single PDADMAC layer ($L_{eff}$ 63 cm, $L_{tot}$ 70 cm, 100 µm I.D.). Separation was done at -18 kV. Key: $t_{d\ (EtG)}$ refers to detection time of EtG.*

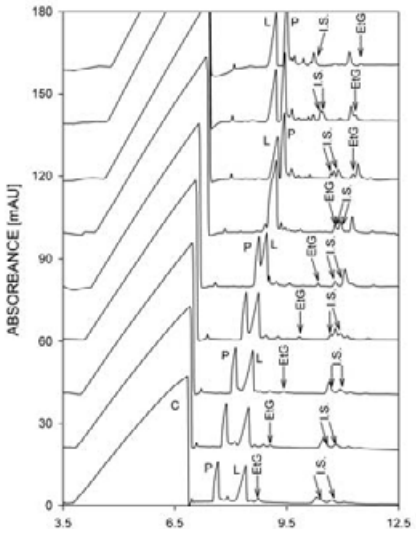



***Figure 48*** *Effect of pH on the electrophoretic separation of EtG (8 mg $L^{-1}$) and the I.S. (12 mg $L^{-1}$, 2,4-DMGA) in plain serum. The study was performed on a P/ACE MDQ with UV detection at 215 nm, featuring a PDADMAC coated 100 µm I.D. capillary ($L_{tot}$ 70.0 cm; $L_{eff}$ 60.0 cm) and the voltage for separation was set to -18 kV. The BGE was composed of 10 mM nicotinic acid, 12.5 % ACN and EACA at different concentrations (from about 4 to 40 mM) in order to adjust the BGE to different pH.*
*The electropherograms obtained from BGE pH values from 4.30 to 5.10 (from bottom to top) in steps of 0.10 pH units are displayed with a y-axis offset for the sake of clarity.*
*Key: C, P and L refer to chloride, phosphate and lactate, respectively. Figure from [112].*

hydroorganic mixtures have higher viscolity compared to water alone (refer to Equation 4 in Chapter 1.3). Table 9 shows the detection times for EtG with the three different solvents added to the BGE. The expected prolongation of the run times was observed.

The methods with less EOF did not improve the resolution of the compounds. Mixing BGE with ACN, though, was observed to additionally influence the electrophoretic mobilities of some analytes due to solvation. The combination of the two parameters of the BGE was investigated, i.e. its concentration of ACN and its pH. The strong influence of pH variations and the concentration of ACN on the mobility of the analytes are shown in Figure 48 and 49.

It was found that the required separations between EtG, the I.S. and matrix compounds was achieved using a BGE made of 10 mM nicotinic acid, about 11.5 mM EACA (pH 4.65), and 8 to 10 % ACN. Since ACN is prone to evaporation, it was added at 10 % to the BGE for further work in order to ensure good resolution even after a slight loss of ACN.

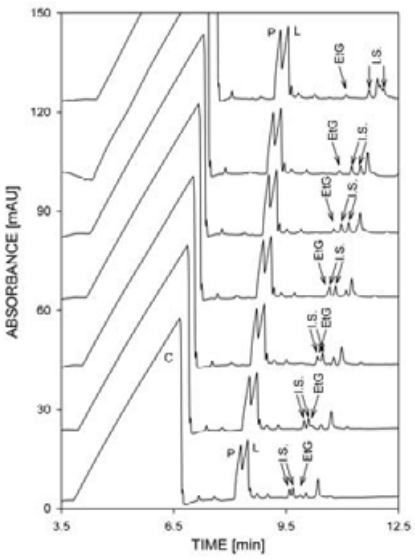



***Figure 49*** *Effect of ACN concentration on the mobility of EtG (8 mg $L^{-1}$) and I.S. (20 mg $L^{-1}$, 2,4-DMGA) in plain serum. The study was performed on a P/ACE MDQ with UV detection at 215 nm and featuring a PDADMAC coated 100 µm I.D. capillary ($L_{tot}$ 70.0 cm; $L_{eff}$ 60.0 cm). The applied voltage for separation was -18 kV and the BGE was composed of 10 mM nicotinic acid, about 13 mM EACA (pH 4.70) and various amounts of ACN. The electropherograms obtained with an ACN concentration of 0 % to 15 % (v/v) (from bottom to top) in steps of 2.5 % are displayed with a y-axis offset for the sake of clarity.*
*Key: C, P and L refer to chloride, phosphate and lactate, respectively. Figure from [112].*

While the cheap and easy to fabricate PDADMAC single layer coating was used for the basic method development, the commercially available LPA-coated capillary (75 µm I.D.) was used for the evaluation of the sample pretreatment and method validation because it provided stable EOF (Chapter 7.1.14).

The ACN containing BGE did not only enhance resolution of the analytes but also extended the separation time. Since at -15 kV the observed currents were low (< 2.5 µA), the applied voltage was increased to -30 kV for the validated method. This resulted in maximum currents of about 5.5 µA (power < 0.28 W $m^{-1}$) and reduced the migration times of EtG from 20.5 to 10.2 min.

### 7.1.13 Removal of Proteins from Serum

The drawback of the developed method featuring the commercially available LPA-coated capillary was that the ACN containing BGE seemed to impede the reproducibility after about 10 to 15 runs done on the same capillary. A wavy baseline was often observed. It was assumed that protein deposits in the capillary are responsible for these disturbances. Therefore, it was intended to get rid of the proteins by rinsing the capillary with an aqueous solution of urea (5 M). Urea disrupts the noncovalent bonds in the proteins and so increases the solubility of some proteins. However, rinsing the capillary with urea (40 psi for 3 min) between the runs did not improve reproducibility.

Thus, an incompatibility between a proteinaceous sample and the ACN containing BGE was assumed. Consequently, different sample preparation procedures were tested in order to eliminate the proteins from the sample prior to analysis, namely UF, SPE and PP.

After UF some peaks appeared with one migrating close to EtG (refer to [112] for details of UF method). This issue was not further investigated. EtG extraction from serum using the Clean Screen ETG SPE columns has been discussed above in Chapter 7.1.3. The matrix could be simplified and most of the proteins were eliminated from the sample. PP with ACN provided reproducible data. Finally, PP was used for the following work due to its simplicity, the short working time required and the lower cost.

### 7.1.14 Assay Specification, Validation and Evaluation

The developed method using the LPA-coated capillary, featuring an I.S. and providing good resolution for EtG from matrix compounds was validated.

Separation was done using the LPA-coated capillary ($L_{eff}$ 60 cm, $L_{tot}$ 50 cm, 75 µm I.D., Polymicro Technologies). Prior to injection, the samples underwent a PP by mixing 100 µL serum with 200 µL of the I.S. solution (10 mg $L^{-1}$ 2,4-DMGA in ACN). 240 µL of the

supernatant from the vortexed and centrifuged (10 min at about 16000·g) sample were transferred to another vial, evaporated to dryness under a constant stream of air (35 to 40°C) and reconstituted with 160 µL of water.

The BGE was prepared from a 10 mM nicotinic acid solution that was adjusted to pH 4.65 via addition of EACA (about 11.5 mM) and completed by the addition of ACN (10 % v/v).
Before each run, the capillary was rinsed with BGE for 4 min (30 psi). Injection was done by the application of vacuum for 16 sec at 0.5 psi. To improve reproducibility, a plug of BGE was added behind the injected sample (0.1 min at 0.1 psi). A voltage of -30 kV (reversed polarity) was applied for separation. Under these conditions, EtG and the two diastereomers of the I.S. passed the detector at about 10.3, 11.3 and 11.8 min, respectively.
At the end of each working day the capillary was washed with water for 20 min (30 psi) followed by drying with air for 10 min (30 psi).

Calibration was done with serum spiked at 5 levels (1, 2, 4, 8 and 16 mg $L^{-1}$ EtG) and prepared by PP analogous to the other samples. Differently weighted linear regression models were tested according to Karnes and March [103] as discussed above for EtS in urine and serum (Chapters 4.1.4 and 5.1.3). The $1/x^2$ weighted linear regression provided the lowest RCR (69.8 %) over the 6 days of validation. 1/x and x provided 74.4 and 125.8 % RCR, respectively. Thus the $1/x^2$ weighted linear regression was applied for validation and analysis of real samples [112].
Calibration revealed linear relationships. Over the 6 days of validation, the mean of the slopes of the calibration curves was 0.0656 (SD 0.0038). The mean y-intercept was calculated to be 0.0083 (SD 0.0086). The F coefficient was always larger than 690.

Intraday precision (n=6) was 8.7, 2.5 and 0.5 % for 1.5, 6 and 12 mg $L^{-1}$ EtG, respectively. For the same EtG levels, interday precision (n=6) was 10.0, 2.0 and 3.7 %, respectively.

The LOQ was found to be 0.5 mg $L^{-1}$ and the LOD was determined to be 0.25 mg $L^{-1}$ (s/n about 3) [112]. The sensitivity of this method approximately corresponds to the sensitivity reached at our laboratories applying the method described by Mrázková et al. [136].

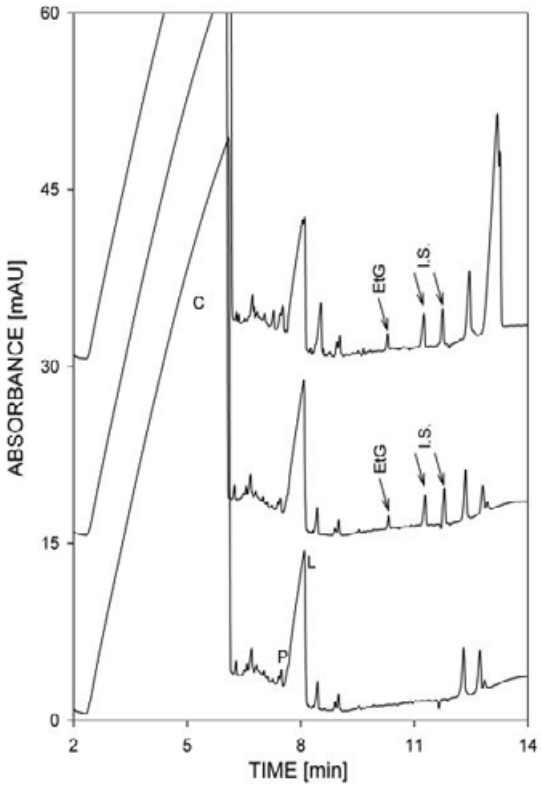



***Figure 50*** *Typical electropherograms of serum samples after protein precipitation analyzed in a LPA-coated capillary. Experiments were performed on a P/ACE MDQ with UV detection at 215 nm, featuring a LPA-coated 75 µm I.D. capillary ($L_{tot}$ 60.0 cm; $L_{eff}$ 50.0 cm) and the voltage for separation was set to -30 kV. The BGE was composed of 10 mM nicotinic acid, about 11.5 mM EACA (pH 4.65) and 10 % ACN (v/v). The data from a blank serum, a blank serum spiked with 4 mg $L^{-1}$ EtG and 20 mg $L^{-1}$ I.S. (2,4-DMGA), and a real serum sample (determined EtG concentration 4.72 mg $L^{-1}$) are displayed with a y-axis offset for the sake of clarity (from bottom to top).*
*Key: C, P and L refer to chloride, phosphate and lactate, respectively. Figure from [112].*

### 7.1.15 Analysis of Real Samples

Over 30 sera from more than 10 different persons were analyzed [112]. It is important to note that every sample could be analyzed successfully (EtG was always well resolved from matrix peaks). Furthermore, the results of the analysis corresponded to the qualitative expectations of EtG concentration; most notably no EtG was found in any sample from volunteers who did not consume alcohol for at least 48 h.
The data shown in Figure 50 are those obtained for a blank serum, a blank serum spiked with 4 mg $L^{-1}$ EtG and 20 mg $L^{-1}$ 2,4-DMGA, and a real serum sample taken from an individual who consumed alcohol prior to sampling.

It has been shown that the method is suitable for the monitoring of alcohol consumption as low as one standard drink containing 12 g of ethanol (LOQ 0.5 mg $L^{-1}$), although sensitivity was neglected for the sake of resolution of the analytes.

### 7.1.16 Conclusions

The goal to develop and validate a CZE method for the determination of EtG in serum was achieved. It has been shown that good resolution of EtG from matrix peaks was given in a large number of real samples. CZE featuring optical detection was succesfully applied for the determination of EtG in serum.

A lower LOD would be beneficial in order to extend the period of time during which prior alcohol consumption can be detected. The concentration of the sample during sample preparation may raise the sensitivity of the method. An enhancement of the sensitivity of about 3-fold is expected.
Even if the limits of CZE with optical detection have been revealed, the reliable method featuring an I.S. is a candidate for future routine application.

The extensive studies about the determination of EtG in serum provided important know-how for other work about the detection of markers for recent alcohol consumption in body fluids. The experience gained here provided ideas and starting points for the research on other topics, e.g. detection of EtS in body fluids by CZE or for the work featuring CZE-MS.

# 7.2 Ethyl Glucuronide in Serum by CZE-MS

## 7.2.1 Assay Specifications and Extraction of Ethyl Glucuronide

Samples classified as positive applying a screening assay should ideally be confirmed by a technique featuring an MS detector. EtG had successfully been detected in urinary samples using CZE-MS (Chapter 6.2). The same CZE-MS method was used for the analysis of EtG in serum. The proteins contained in serum (about 8 % w/v), however, risked interfering with the electrophoretic separation process, with the interface or with the MS. Therefore, serum samples needed adequate pretreatment. In addition, EtG concentrations in serum have been observed to be lower than 1 mg $L^{-1}$ in most of the analyzed real samples. The concentration of the sample during sample preparation was aimed for the sake of sensitivity of the analytical method.

Good results have been obtained for the analysis of EtS extracted from serum samples using SPE (Strata X-AW column, Chapter 5.2.1). It has been shown though, that this SPE column is not suitable for the extraction of EtG from body fluids (Chapter 4.2.3). Another SPE method using the Clean Screen ETG columns has been developed for the extraction of EtG from serum (Chapter 7.1.3, Figure 42). Good reproducibility of the extracts was achieved. Thus, the Clean Screen ETG extraction columns were used for the preparation of serum samples for the analysis by CZE-MS.

## 7.2.2 Analysis of Real Samples

No matrix interferences were observed when blank urine samples were analyzed. 4 sera taken after alcohol consumption have been analyzed and EtG was found in each of them. For the following illustration, the same sample as discussed above for the detection of EtS in serum has been used, namely the serum from an individual who ingested more than 1.5 g of ethanol per kg body mass in a 12 h time period before sampling. The same sample has before been analyzed using the CZE method described in Chapter 7.1.14 and [112]. An EtG concentration of 4.37 mg $L^{-1}$ was determined. The analysis by EIA (detailed method description in Chapter 7.3.3 and [112]) provided an EtG level of 3.22 mg $L^{-1}$.

The mass trace for m/z 221 Th showed a large peak at 7.05 min. The ion with m/z 221 Th in the full scan mass spectrum corresponded to $[M-H]^-$ of EtG (Figure 51). The fragmentation of the parent ion with m/z 221 Th produced the same m/z signals as the EtG standard solution analyzed before (Chapter 6.2). Thus, the presence of EtG in this real sample has been proven.

Similar results were obtained in other real samples from individuals who recently ingested ethanol as well as in blank sera spiked with EtG. This marker for recent alcohol consumption was also identified in a real sample which only contained 0.156 mg $L^{-1}$ EtG (determined applying the EIA described in Chapter 7.3 and [112]).

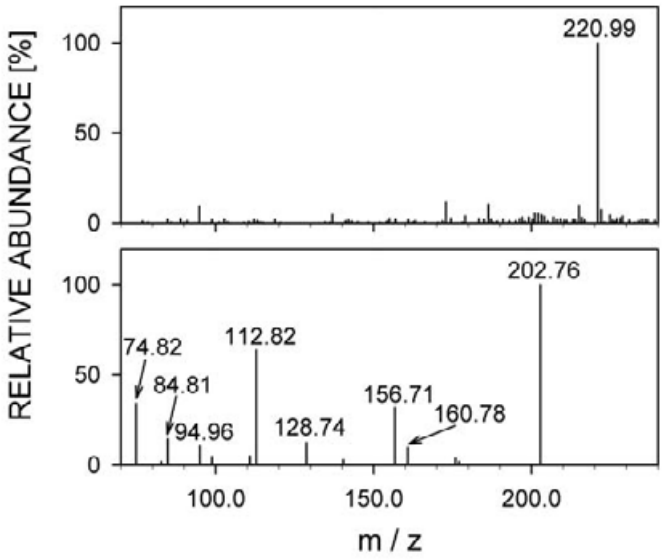



***Figure 51*** *Full scan mass spectrum at 7.05 min (top; NL: $5.1 \cdot 10^5$) and $MS^2$ of m/z 221 (bottom; NL: $4.23 \cdot 10^4$) of a serum sample from an individual who ingested more than 1.5 g of ethanol per kg body mass in a 12 h time period before sampling. The sample was extracted with the Clean Screen ETG SPE column (refer to Chapter 7.1.3 or [112] for the detailed description of the extraction method) and reconstituted with water to 25 % of the original volume. Tandem mass spectrum obtained commencing from the m/z 221 Th ion and applying a collision energy of 35. Other method parameters are described in the text.*

The data discussed above (Figure 51) was further studied and the mass trace for m/z 125 (EtS) was evaluated. The mass trace as well as the full mass spetrum were very noisy and did not provide valuable information about the analyte of interest. However, the $MS^2$ data at 12.66 min of the ions with m/z 125 Th revealed fragments of m/z 97, corresponding to the m/z of $HSO_4^-$. This is the typical fragment of EtS (Chapter 4.2.1). However, its abundance was rather low (NL: $2.18 \cdot 10^3$).

### 7.2.3 Conclusions

In conclusion, CZE-MS allowed identifying EtG in sera from individuals who consumed alcoholic beverages shortly before sampling. The samples were extracted applying a simple SPE method which was developed during this work (Chapter 7.1.3 and [112]).

CZE-MS could potentially be used to confirm positive results from screening assays. The results obtained in this section are a promising initiation for future identification of EtG in unknown serum samples.

# 7.3 Ethyl Glucuronide in Serum by an Enzyme Immunoassay

In Chapter 6.3, the results obtained with the DRI Ethyl Glucuronide Assay for the determination of urinary EtG have been discussed. The EIA was specifically designed for the analysis of urine samples. The present chapter deals with the adaptation, validation and further evaluation of the assay for serum samples.

## 7.3.1 Assay Specifications and Adaption to Serum Samples

When plain or diluted serum samples were injected to the Mira Plus analyzer instead of urinary samples, no reproducible results were obtained using the DRI Ethyl Glucuronide Assay. It was assumed that the reason was the G6PDH of the sample. Thus, the main issue for the adaption of the test for serum samples was to eliminate G6PDH from the matrix. This enzyme is found in erythrocytes of whole blood and may be liberated into the serum when the red blood cells are destroyed (e.g. during centrifugation).
Samples prepared by SPE (Clean Screen ETG, refer to Chapter 7.1.3 or [112] for the detailed description of the extraction method) did not provide better data. Therefore, the serum samples were ultrafiltered prior to their analysis. The 5 kDa membrane used for UF retained the interfering enzymes and it was demonstrated that with this sample preparation reproducible results for the serum analysis of EtG by EIA were produced (refer to Chapter 7.3.3 for validation data).

## 7.3.2 Fabrication of Calibrators and Control Samples

When applying spiked blank serum for calibration, too high or too low values for EtG were found in control samples. Different reproducible biases were observed dependent on the serum used for the fabrication of the calibrators and control samples. This was particularly obvious when analyzing blank samples, where often negative EtG concentrations resulted. Thus, it was assumed that small amounts of EtG possibly being present in the blank sera were

responsible for the biases of the response. Hence, a matrix was needed, which was similar to serum in terms of ionic strength and protein concentration, but which certainly did not contain any EtG. Therefore, the calibrators and controls were fabricated by spiking home-made Krebs-Ringer solution containing 5 % bovine serum albumin.

### 7.3.3 Assay Validation

For the sake of ease of comparison of the EtG concentrations determined in urine and serum samples, results are given here in the same unit as for urinary EtG, namely in mg $L^{-1}$.

The recovery of the UF was assessed with samples containing 1.5, 0.39, and 0.23 mg $L^{-1}$ and was determined to be above 97 % for each level.In accordance to the EIA for urinary EtG, the calibration levels used were 0, 0.1, 0.5, 1.0, and 2.0 mg $L^{-1}$ EtG [112]. Control samples were Krebs-Ringer solution containing 5 % bovine serum albumin spiked with EtG at 0.23 and 0.39 mg $L^{-1}$. These concentrations were chosen due to the proposed upper reference limit of 0.31 mg $L^{-1}$ EtG in serum by Zimmer et al. [114]. This limit was calculated with the results from an immunochemical screening procedure.

Repetitive analysis of the same ultrafiltered sample revealed reproducible data. RSD values (n=6) for 0.23 and 0.39 mg $L^{-1}$ EtG levels were determined to be 6.94 and 3.58 %, respectively. Intraday precision (6 different samples analyzed on the same day) was 4.40 % RSD and 4.07 % RSD for 0.23 and 0.39 mg $L^{-1}$, respectively. Interday precision (6 different samples analyzed on 6 consecutive days) was 4.93 % RSD and 3.93 % RSD for 0.23 and 0.39 mg $L^{-1}$, respectively. More detailed information about the validation of the method has been published [112].

The values for EtG found in blank sera (Chapters 7.3.4 and 7.3.5) were lower than expected and thus also clearly below the levels of the control samples. In order to characterize the precision of the assay

at low EtG levels, the data of the calibrators and a sample containing 0.05 mg $L^{-1}$ EtG were evaluated. For each calibrator only the values of the second runs of the duplicate determinations were used. The mean values over 6 days of validation for the calibrators at 0, 0.1, 0.5, 1.0 and 2.0 mg $L^{-1}$ were 0.0039 (0.006 mg $L^{-1}$ SD), 0.1011 (9.96 % RSD), 0.5117 (1.99 % RSD), 0.9919 (1.90 % RSD) and 1.9889 mg $L^{-1}$ (0.87 % RSD), respectively. All accuracies were between 91.3 and 102.4 %.

The sample containing 0.05 mg $L^{-1}$ EtG was made of Krebs-Ringer solution containing 5 % bovine serum albumin. The mean value and precision of 6 consecutive analyses of ultrafiltered aliquots were 0.0457 mg $L^{-1}$ and 10.96 % RSD, respectively. Therefore, the LOQ for routine applications was 0.05 mg $L^{-1}$ EtG. As for the discussion of urinary EtG the statistical discussions were made using the exact values provided by the test, even if these were lower than the LOQ.

### 7.3.4 Analysis of Real Samples and Comparison with CZE Results

A set of 29 real serum samples was collected. They were taken from individuals who estimated their own alcohol consumption of the last days prior to sampling. The values obtained from these samples will be discussed in detail below. The range of the measured EtG concentrations using the EIA ranged from < LOQ to 4.672 mg $L^{-1}$.
Two series of consecutive serum samples taken from a female and a male volunteer (four samples each) who ingested defined amounts of alcohol in a short period of time prior to sampling were included in this collective. Figure 52 shows the time course of the EtG serum concentrations of these two individuals. The peak values of EtG were measured at 2 and 4 h after the beginning of alcohol ingestion for the samples stemming from the female and male volunteer, respectively. Prior to ethanol ingestion the two individuals had < LOQ (female) and 0.027 mg $L^{-1}$ EtG (male) in their blood.

The two developed assays for the determination of EtG in serum, EIA and CZE (Chapter 7.1.14), were compared using the 29 real samples discussed above. The value of 7 samples could be determined by CZE

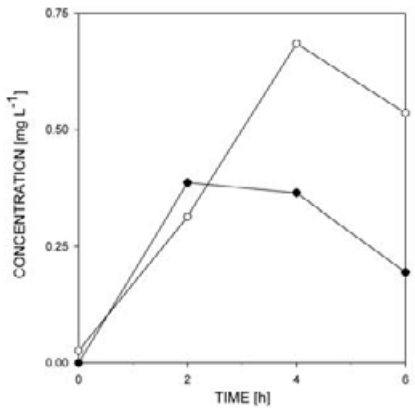



*Figure 52* *Two series of 4 consecutive serum samples each were taken from a female (●) and a male (○) volunteer ingesting 0.462 and 0.658 g of ethanol per kg body mass within 30 min, respectively. Samples were taken prior to alcohol consumption 2 h, 4 h, and 6 h after the beginning of alcohol ingestion. EtG was determined using the validated EIA described in Chapter 7.3.3 and [112].*

(LOQ 0.5 mg $L^{-1}$). The correlation of these samples with the values obtained by EIA resulted in a linear relationship with $r > 0.999$ ($y=0.996\ x - 0.026$; where x and y refer to the values obtained by CZE and EIA, respectively). For the values above 0.5 mg $L^{-1}$, the data of both methods correlate well which suggests that both assays are suitable for the determination of EtG in serum samples.

The optimal tool for the analysis of the agreement between two different assays for the determination of the same analyte would be the Bland-Altman plot, also called bias plot or difference plot in the literature [159]. It is constructed assigning the mean of the two measurements as the x-axis value, and the difference between the two values as the y-axis value.
However, the plot with only 7 data points (Table 10), for which EtG could be determined by both methods was not meaningful. As soon as more data of positive samples is available, the Bland-Altman plot should be established in order to visualize the possible bias between the two methods for the determination of EtG in serum.

In 7 other samples, EtG was detected using CZE, but could not be quantified (LOD 0.25 mg $L^{-1}$ < x < LOQ 0.5 mg $L^{-1}$). Analyzed with the EIA, these samples provided values for EtG between 0.156 and 0.386 mg $L^{-1}$ (mean 0.281 mg $L^{-1}$).
In 13 blank samples (more than 2 days of ethanol abstinence prior to sampling) and 2 samples taken after the consumption of a low amount of alcohol, EtG was not detected by CZE, whereas EIA provided data between 0 and 0.204 mg $L^{-1}$ (mean 0.021 mg $L^{-1}$ EtG). Hence, the values below the LOD of the CZE method were determined to be below 0.25 mg $L^{-1}$ by the EIA as well.

The results of the EtG determinations by the two methods are listed in Table 10.

| | EtG [mg $L^{-1}$] | |
|---|---|---|
| | **EIA** | **CZE** |
| Samples taken after alcohol abstinence of > 48 h | 0 | n.d. |
| | 0 | n.d. |
| | 0 | n.d. |
| | 0 | n.d. |
| | 0 | n.d. |
| | 0 | n.d. |
| | 0 | n.d. |
| | 0.002 | n.d. |
| | 0.005 | n.d. |
| | 0.010 | n.d. |
| | 0.024 | n.d. |
| | 0.026 | n.d. |
| | 0.028 | n.d. |
| Samples taken after recent alcohol consumption | 0.030 | n.d. |
| | 0.156 | < LOQ |
| | 0.190 | < LOQ |
| | 0.195 | < LOQ |
| | 0.204 | n.d. |
| | 0.314 | < LOQ |
| | 0.364 | < LOQ |
| | 0.369 | < LOQ |
| | 0.386 | < LOQ |
| | 0.482 | 0.517 |
| | 0.685 | 0.690 |
| | 0.942 | 1.011 |
| | 0.942 | 1.020 |
| | 0.965 | 1.019 |
| | 1.315 | 1.244 |
| | 4.672 | 4.722 |

***Table 10*** *Comparison of the EtG values in serum obtained by EIA and CZE (LOQ: 0.5 mg $L^{-1}$). For the analytical methods refer to Chapters 7.3.3 and 7.1.14. Key: n.d. = not determined.*

The EIA provides values for any EtG concentration, whereas very low concentrations of EtG can not be differentiated by the CZE assay. This is why it is important to define an upper reference limit for EtG determinations by EIA. Only samples containing an EtG concentration above a certain level should be classified as positives.

### 7.3.5 Upper Reference Limit

During the study described above for the determination of the reference limit of urinary EtG (Chapter 6.3.3 and Appendix VI), serum samples were collected as well. The same inclusion and exclusion criteria were applied for the collection, but women were excluded during pregnancy or lactation. Most of the participants donated urine and serum samples on the same day, some volunteers provided duplicate donations of serum samples as well. This resulted in a collective of 114 serum samples from 32 males (mean age 38.6 years, median 36 years, range 21–62 years) and 70 females (mean age 33.7 years, median 28 years, range 16–64 years).
Analogous to urinary EtG, the intra-individual variability of EtG in serum was investigated. The 5 serum samples from one individual were taken on the same days as the urine samples were collected (refer to Chapter 6.3.3).

Levels for EtG in serum ranged from 0 to 0.0389 mg $L^{-1}$ (RSD 105.6 %). Interestingly, the variation of EtG in serum was somewhat larger than in urine. As in urine, EtG concentration strongly varied in serum stemming from one individual (0.0154 mg $L^{-1}$, 105.6 % RSD; range: 0 to 0.0389 mg $L^{-1}$). Therefore, the donation of two samples per volunteer was accepted with a two weeks gap between the two donations. 7 male and 5 female participants provided two serum samples under these conditions.

With the validated method for EtG determination featuring the EIA, each of the 114 serum samples of the reference limit study could successfully be analyzed. Since no outlier was identified by

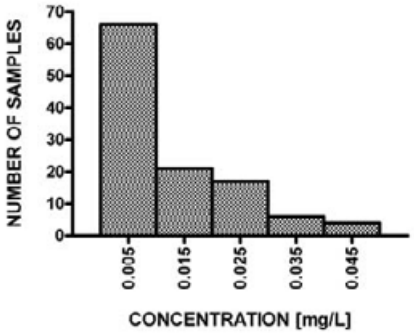



*Figure 53* *Histogram representing the distribution of the EtG concentration in 114 blank serum samples used for the determination of the reference level of EtG in serum by EIA (detailed method description in Chapter 7.3.3). Figure from [112].*

the Dixon-Reed-method, the complete dataset was included for the determination of the reference limit. The Kolmogorov-Smirnov test indicated a non-normal distribution of the data represented in Figure 53.

The range of the obtained data was 0 to 0.0445 mg $L^{-1}$ (mean 0.0111 mg $L^{-1}$, 110.6 % RSD; median 0.0064 mg $L^{-1}$). The 97.5th percentile (90 % confidence interval) was determined to be 0.0434 mg $L^{-1}$ (0.0402 – 0.0466 mg $L^{-1}$).

The unpaired t-test revealed that the difference between the groups of the males (n=39) and females (n=75) was not statistically significant (P=0.1539). The boxplots in Figure 54 shows the distributions of the two genders.

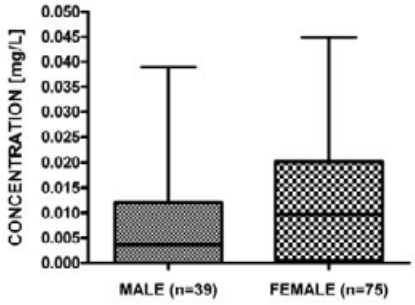



*Figure 54* *Gender specific boxplots of the EtG concentration determined in 114 blank serum samples used for the determination of the reference level of EtG in serum by EIA (detailed method description in Chapter 7.3.3).*

As shown above for the whole collective, also in the specific groups for women and men, no outlier was detected using the Dixon-Reed-method.

In accordance to the CAC/GL codex guidelines of the WHO [119] the precision (< 20 % RSD for EtG 0.05 mg $L^{-1}$, and < 15 % RSD for EtG ≥ 0.1 mg $L^{-1}$) as well as accuracy data (70 to 110 %

for EtG 0.05 mg $L^{-1}$ and 80 to 110 % for EtG ≥ 0.1 mg $L^{-1}$) of the calibrators and the sample containing 0.05 mg $L^{-1}$ EtG discussed above (Chapter 7.3.3) fulfilled the general criteria.
Consequently, for routine use the reference limit corresponding to the LOQ of 0.05 mg $L^{-1}$ is suggested for EtG testing in serum using the EIA from Microgenics. In the following text, however, the calculated value of 0.0434 mg $L^{-1}$ EtG will be discussed.

### 7.3.6 Classification of Real Samples according to Upper Reference Limit

Applying the evaluated reference limit (Chapter 7.3.5) to the 114 samples from the blank serum study, only one sample resulted in a positive outcome, since the value of the two next highest samples corresponded exactly to 0.0434 mg $L^{-1}$.
The reference limit was also applied to the 29 real serum samples mentioned above. It was observed that no serum sample from persons who did not drink alcohol for at least 48 h prior to sampling provided a positive result. 15 out of 16 individuals who admitted recent alcohol consumption (within the last 2 days) were tested positive. One female volunteer consuming half a glass of wine 1 h prior to sampling provided a serum sample containing 0.03 mg $L^{-1}$ EtG and so was tested negative (Table 10, Chapter 7.3.4).

### 7.3.7 Reference Limit Based on ROC Analysis

The graphical establishment of a ROC for this case is not very helpful and results in a curve comprising only one step in the upper left corner. This step is due to the only sample providing a false-negative observation at a reference limit above 0.03 mg $L^{-1}$ EtG. If the limit is defined above 0.045 mg $L^{-1}$ EtG, no false-positive classification results (range of the negative samples from < LOQ to 0.0449 mg $L^{-1}$ EtG).
The ROC suggests defining the reference limit at 0.045 mg $L^{-1}$ in order to achieve a specificity of 1 and a sensitivity of 0.9375, rather than a

specificity of 0.991 without improving the sensitivity if the evaluated reference limit of 0.0434 mg $L^{-1}$ was applied (Chapter 7.3.5). The next higher value measured for a positive sample was 0.156 mg $L^{-1}$ EtG. It is interesting to note how clearly the groups of positive and negative samples are separated from each other.

To conclude, the reference limit of 0.045 mg $L^{-1}$ for EtG determinations by EIA represents an effective differentiation of samples stemming from individuals who recently consumed alcoholic beverages and those who did not. The limited dataset from persons who consumed alcohol prior to sampling, however, is not sufficient to properly discuss the sensitivity of the assay. Many more samples are needed in order to establish a statistically correct distribution of the positive samples.

### 7.3.8 Frequentist Approach

As for urinary EtG determinations by EIA (refer to Chapter 6.3.9), the LR based interpretation of EtG test results in serum has been evaluated. The procedure was done analogous and the same models for the theoretical distributions fitted the experimental distributions in serum (Figures 55 to 58).

No patient sample was collected. Thus, the frequentist approach was not applied to samples with unknown pattern of alcohol consumption. However, the procedure is the same as for urinary samples (Chapter 6.3.12). The LR-function for serum samples is discussed below and has been calculated (Equation 23) so that it can readily be applied to samples stemming from patients or suspects.

*Equation 23*
$$LR(EtG_{Serum}) = \frac{f_{Log\text{-}normal}\left(x = c(EtG),\ \mu = -0.863,\ \sigma = 1.131\right)}{f_{Gamma}\left(x = c(EtG),\ k = 1,\ \theta = 0.011\right)}$$

The plot of the LR-function (Figure 58) shows that EtG concentrations around the determined reference level (0.045 mg $L^{-1}$ EtG) also provide LR close to 1. For serum EtG concentrations above 0.045 mg $L^{-1}$, the

LR rises exponentially, e.g. to about $10^6$ for 0.2 mg $L^{-1}$ EtG. Any future opportunity, when further samples of individuals who recently consumed alcohol can be collected, should absolutely be utilized.

A larger size of such a collective of positive samples would allow the establishment of a more precise statistical evaluation. Nevertheless, until more data is available, the collectives discussed here allow the best possible interpretation of unknown samples. The power of the frequentist approach for the interpretation of evidence has

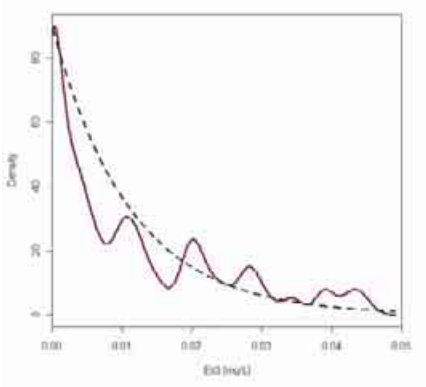


***Figure 55*** *Experimental and theoretical distribution of the EtG values of the study with blank serum samples (n=114).*
*Red graph: Experimentally evaluated and smoothened (bandwidth 0.0014 mg $L^{-1}$) sample density.*
*Blue graph: Theoretical density (gamma distribution with k=1 and θ=0.011).*

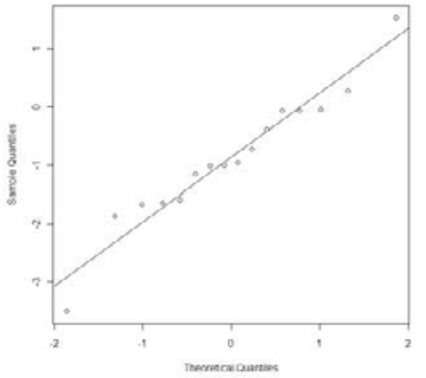


***Figure 56*** *Q-Q plot of randomized normally distributed data versus the logarithm of EtG concentrations in 16 real serum samples taken after alcohol consumption. Correlation: Slope 1.106; y-axsis intercept -0.863; r 0.971; F 232; p $4.212 \cdot 10^{-10}$.*

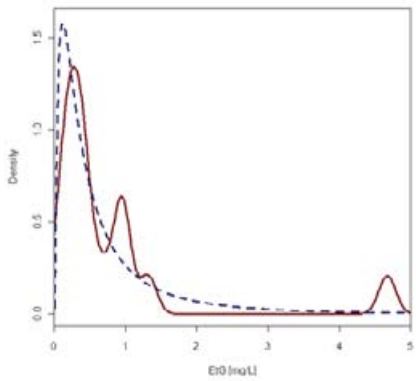



***Figure 57*** *Experimental and theoretical distribution of EtG values measured in 16 real serum samples taken after alcohol consumption. Red graph: Experimentally evaluated and smoothened (bandwidth 0.12 mg $L^{-1}$) sample density. Blue graph: Theoretical density (log-normal distribution with $\mu = -0.863$ and $\sigma = 1.131$).*

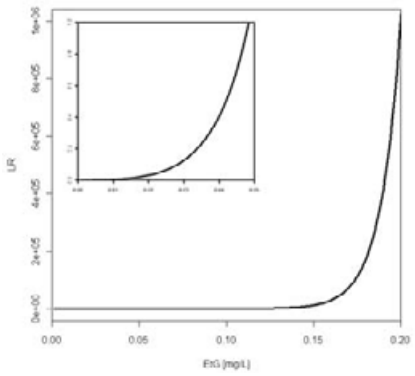



***Figure 58*** *EtG concentration in serum plotted against the resulting LR. Inset: Zoom on the plot for EtG concentrations resulting in LR ≤ 1.*

### 7.3.9 Conclusions

The validated EIA for EtG in serum is a convenient assay for the fast screening of a large number of samples. However, it must be emphasized that it is a screening method and thus false results may occur with any of the discussed methods for interpretation of evidence. False-positives have not been identified in the large set of samples analyzed for this work. Nonetheless, elsewhere it has been shown that with a metabolite of chloral hydrate, a sedative and hypnotic drug, false-positive results can occur during the determination of urinary EtG using the discussed EIA [160]. Therefore, confirmation of EtG-positive results should be done with another method.

# 8
# GENERAL CONCLUSIONS

Analytical methods for the determination of EtS and EtG in serum and urine have been developed, validated and tested for routine suitability with real samples. CZE with indirect UV detection has been shown to be a powerful tool for the screening for alcohol markers in body fluids. An immunochemical screening assay for the determination of urinary EtG has been evaluated and a method for the application of the EIA for serum samples has been developed. With these tests, EtG and EtS may be detected in body fluids after as little as a single intake of an alcoholic standard drink and after the complete disappearance of ethanol from the body. CZE-MS was successfully used to confirm positive results from the screening tests.

EtG and EtS determinations in body fluids are usually done using LC-MS or GC-MS. LC-MS is reported to perform with LOQs for EtG between 0.05 mg $L^{-1}$ and 0.1 mg $L^{-1}$ in serum as well as urine [20, 23, 161]. Methods featuring GC-MS were about half as sensitive [49, 162]. The EIA with the determined LOQ of 0.05 mg $L^{-1}$ can compete with the sensitivity of these analytical methods. The CZE assays with indirect UV detection were almost 10-fold less sensitive than EIA and the chromatographic methods mentioned above. Earlier attempts using conductivity detection instead of indirect UV detection made in our laboratories did not provide better sensitivity and were therefore not further evaluated.

In order to provide the base for EIA data interpretation a study for the determination of the upper reference limit for EtG in urine and serum has been established. Based on this data set, patient samples with unknown patterns of alcohol consumption prior to sampling were tested for recent alcohol consumption applying the calculated reference limits as well as the frequentist approach. It is fundamental to note that the term “recent” is rather vague and necessitates an unambiguous definition in order to allow the statistical interpretation of test results. In the protocol of the mentioned study “recent alcohol consumption” has been defined as a deliberate ingestion of ethanol within the last 72 h prior to sampling.

It is also important to state that the goal of the work was not to find information about on the level of ethanol intoxication of a tested individual for a specific time point, e.g. the time of a crime. The large intra- and inter-individual variations in human ethanol metabolism do not allow precise conclusions on ethanol blood concentrations based on measured EtG or EtS levels in body fluids.

However, the hypothesis that an individual recently, e.g. within the last 72 h prior to sampling, and deliberately consumed ethanol can be tested against the alternative proposition. Even if the exact amount of ingested alcohol or the resulting blood ethanol concentration of an individual cannot be determined, the information provided by EtS and EtG testing may be very useful in forensic as well as in clinical cases. For example, the denial of recent alcohol consumption of a person suspected in a hit and run accident may be disproved.

In conclusion, EtS and EtG have been shown to be very potent markers for recent alcohol consumption. The research on the analytical as well as statistical level allowed the successful introduction of the developed EIA for the determination of EtG to the routine laboratory of the ICPVR. Hence, the studies done for this PhD thesis contribute to fight the worldwide burden of alcohol related problems.

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# 10
# APPENDICES

# Appendix I
# B. Jung et al., J Chromatogr A

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# Determination of ethyl sulfate in human serum and urine by capillary zone electrophoresis

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ABSTRACT

The use of capillary zone electrophoresis (CZE) with indirect absorbance detection for the analysis of ethyl sulfate (EtS) in serum and urine was investigated. EtS is a direct metabolite of ethanol employed as marker for recent alcohol consumption. Fused-silica capillaries of 60 cm total length were either coated with cetyltrimethylammonium bromide (CTAB, 50 μm I.D. capillary) or poly(diallyldimethylammonium chloride) (PDADMAC, 100 μm I.D. capillary) to allow CZE analyses to be performed with reversed polarity. At pH 2.2 with a maleic acid/phthalic acid background electrolyte, both approaches provided reliable EtS serum levels down to 0.2 mg $L^{-1}$ (1.6 μM) for the analysis of solid-phase extracts that were prepared after chloride precipitation. Analysis of urines diluted to a conductivity of 5 S $m^{-1}$ and analyzed in the two capillary formats resulted in limits of quantification (LOQs) of 2 and 1 mg $L^{-1}$, respectively. With urines adjusted to 10 S $m^{-1}$ via dilution or condensation, an LOQ of 0.6 mg $L^{-1}$ (4.8 μM) was obtained in the CTAB coated capillary whereas in the PDADMAC-coated capillary of equal length not all matrix components were resolved from EtS. The developed assays are robust and suitable to monitor EtS in samples of individuals who consumed as little as one standard drink of an alcoholic beverage containing about 14 g of ethanol.



## 1. Introduction

Ethyl sulfate (EtS, sulfovinic acid, for structure refer to insert in Fig. 1) is a non-volatile, water soluble, stable upon storage, direct metabolite of ethanol. Less than 0.1% of the ingested ethanol is excreted as EtS in urine [1–3]. For an ethanol intake during a short time period, it was reported that EtS reaches its maximum concentration in serum about 3 h after the beginning of alcohol consumption [1,3–5]. Thus, EtS can be detected for an extended time period in serum (in the range of hours) and in urine (in the range of hours to days) after complete elimination of ethanol such that it can be employed as marker substance for recent alcohol consumption. Together with ethyl glucuronide (EtG, [3,4,6,7]), it covers a clinically and forensically important time window between short-term markers, such as ethanol itself, and long-term markers, such as carbohydrate-deficient transferrin (CDT, [8–11]).

The analysis of EtS in body fluids has attracted considerable attention in the past few years. Methods based upon LC–MS and LC–MS–MS with negative electrospray ionization have been developed for the determination of EtS in urine [1–5,12–15] and serum [4]. These efforts revealed that a calibration range for EtS in serum should be between 0.1 and 10 mg $L^{-1}$ (0.8–79.3 μM). For urinary concentrations of EtS the range between 0.5 and 100 mg $L^{-1}$ (4.0–792.8 μM) is of interest. To the knowledge of the authors no cut-off level has been proposed yet for EtS. Esteve-Turrillas et al. reported a capillary zone electrophoresis (CZE) method with indirect UV absorption detection for urinary EtS with a lower limit of quantitation (LOQ) of 5 mg $L^{-1}$ [16]. No CZE-based assay for EtS in serum has been reported thus far. CZE with optical detection is an attractive technology that is less expensive than instrumentation featuring a MS and has successfully been employed for the monitoring of other alcohol markers, namely CDT [8,10] and EtG [6,7] in serum. The CZE conditions of Esteve-Turrillas et al. [16] for analysis of EtS in urine were adopted and optimized for better resolution and sensitivity using different capillary coatings, capillary inner diameters and urine concentrations. Furthermore, the determination of EtS in human serum after chloride precipitation and solid-phase extraction (SPE) was studied in the same CZE buffer. Data obtained with urinary and serum EtS assays using fused-silica capillaries coated with cetyltrimethylammonium bromide (CTAB) and

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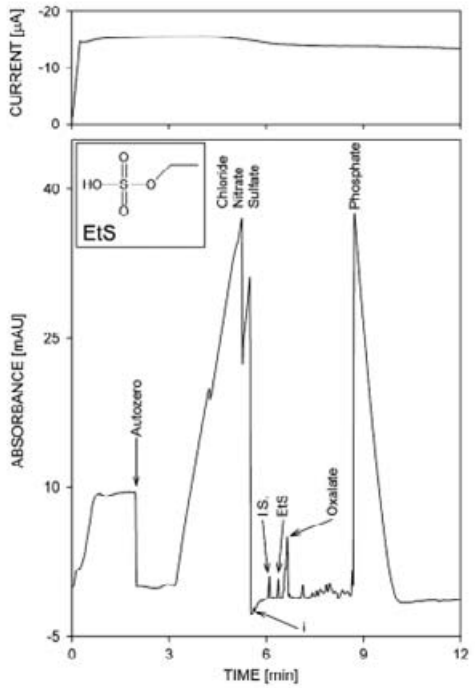


**Fig. 1.** Typical electropherogram and temporal behavior of the current for the analysis of a blank urine (10 S m$^{-1}$) that was spiked with EtS and I.S. (8 mg L$^{-1}$ each) in the CTAB-coated capillary. Key: i refers to an impurity of the I.S. Insert: Chemical structure of EtS.

poly(diallyldimethylammonium chloride) (PDADMAC) are compared and shown to be suitable to monitor EtS on the mg L$^{-1}$ level in both body fluids.

## 2. Experimental

### 2.1. Chemicals and samples

All chemicals used were of highest analytical purity. Sodium EtS was purchased from TCI Europe (Zwijndrecht, Belgium). Vinylsulfonic acid sodium salt [25%, w/w aqueous solution; internal standard (I.S.)] and PDADMAC (typical molecular mass 200–350 kDa, 20%, w/w aqueous solution) were from Aldrich (Milwaukee, WI, USA). Maleic acid, phthalic acid, sodium chloride and sodium oxalate came from Fluka (Buchs, Switzerland). sodium sulfate, ammonium nitrate, disodium hydrogenphosphate, isopropanol, sodium hydroxide (NaOH), formic acid and ammonia solution (32%, w/w) were from Merck (Darmstadt, Germany) and CTAB was from Sigma (St. Louis, MO, USA). Silver acetate (SAC) was obtained from Acros Organics (Geel, Belgium). Acetonitrile (ACN), methanol (MeOH) and dichloromethane were from VWR (Leuven, Belgium). Bidistilled water was used throughout. Sera and urines used in this work stemmed from members of our department which gave their consent. Three individuals ingested known amounts of ethanol within about 30 min and urines were collected immediately before and up to 28 h after alcohol consumption. All others were asked to roughly estimate the quantity and time of their last intake of alcoholic beverages.

### 2.2. CZE Instrumentation and methods

CZE measurements were performed using the instruments P/ACE MDQ and ProteomeLab PA 800 (Beckman Coulter, Fullerton, CA, USA) with the UV detectors set to indirect detection at 214 nm (filter) and 215 nm (diode array detector), respectively. The cartridge temperature was set to 15 °C, the sample storage to 5 °C. Fused-silica capillaries were from Polymicro Technologies (Phoenix, AZ, USA) and had a total length of 60.2 cm (50 cm to the detector). The I.D.s were 50 μm (for experiments using CTAB) and 100 μm (for experiments using PDADMAC) whereas the outer diameters were 375 μm in both cases. Bare fused-silica capillaries were treated prior to their first use with 1 M NaOH, 0.1 M NaOH and water (50 μm I.D. capillaries: 30 min at 20 psi each (1 psi = 6894.76 Pa); 100 μm I.D. capillaries: 10 min at 30 psi each). Solute quantitation was based upon multi-level internal calibration using corrected peak areas (peak areas divided by detection time) for data evaluation.

#### 2.2.1. CZE in 50 μm I.D. capillaries coated with CTAB

The background electrolyte (BGE) consisted of 15 mM maleic acid, 1 mM phthalic acid and 25 μM CTAB and is similar to that used previously by Esteve-Turrillas et al. [16]. The pH was set to 2.20 by addition of NaOH. At the beginning of every working day, the capillary was rinsed with the BGE for 10 min at 20 psi followed by stabilization with application of −19 kV for 90 min. Before each run, the capillary was flushed with the BGE for 5 min at 30 psi. Sample injection was effected with vacuum for 29 s at 0.5 psi. A plug of BGE was added behind the injected sample (0.02 psi min). A voltage of −19 kV (reversed polarity) was applied for the separation. At the end of each working day the capillary was washed with water for 20 min at 30 psi and the ends of the capillary were left in vials filled with water. Electroosmotic flow (EOF) was measured by injecting diluted BGE (1:1 with water) as sample.

#### 2.2.2. CZE in 100 μm I.D. capillaries coated with PDADMAC

The PDADMAC coating [17–25] was applied after conditioning with NaOH by flushing the capillary for 5 min at 30 psi with a solution containing 0.1% (w/w) PDADMAC in BGE. The BGE was composed of 15 mM maleic acid and 1 mM phthalic acid that was adjusted to pH 2.20 by addition of NaOH. Following the application of the coating, it was stabilized under a voltage of 12.5 kV for 180 min, a procedure that was performed once and not repeated at the beginning of a new working day. Before each run, the capillary was flushed with 0.1% (w/w) PDADMAC in BGE for 30 s at 20 psi followed by a flush of BGE without the cationic polymer (2 min at 20 psi). Sample was injected by pressure for 10 s at 0.5 psi. A plug of BGE was added behind the injected sample (0.01 psi min) before application of −9 kV (reversed polarity). At the end of each run the capillary was washed with water for 30 s at 40 psi. At the end of a working day the capillary was washed with water for 20 min at 30 psi and dried with air for 10 min at 30 psi. EOF was measured by injecting diluted BGE (1:1 with water) as sample.

### 2.3. Sample preparation

#### 2.3.1. Urine samples

Two strategies for normalization of the urine concentration were investigated. Initially, urines were diluted to a conductivity of 5 S m$^{-1}$, mixed 1:1 with the I.S. solution (8 mg L$^{-1}$) and analyzed by CZE. In a second approach, the conductivity of the urine

was adapted to a specific conductivity employing a urine aliquot according to

$$V = \frac{\kappa_a}{\kappa} V_a \quad (1)$$

where $V$ is the urine aliquot in μL, $V_a$ is the sample volume after reconstitution in μL, $\kappa$ is the conductivity of the urine in S m$^{-1}$ and $\kappa_a$ is the conductivity of the adjusted sample after reconstitution in S m$^{-1}$. If not stated otherwise, $\kappa_a$ was 10 S m$^{-1}$. The urine aliquot, 20 μL of I.S. solution (100 mg L$^{-1}$) and 250 μL ACN were mixed and evaporated to dryness with air at a maximum temperature of 45 °C. If needed, additional ACN was added in order to ensure fast evaporation of the fluid (10–60 min). If not stated otherwise, the residue was reconstituted in 250 μL water and analyzed by CZE.

*2.3.2. Serum samples*

Serum (500 μL) and I.S. (50 μL at 15 mg L$^{-1}$) were mixed. Then 1 mL of SAC (50 mM) was added, vortexed and the sample was put on ice for 10 min prior to centrifugation (10 min at about 1000 × *g*). The silver chloride precipitate was disposed. 10 μL of concentrated formic acid was added to the supernatant prior to its application on the SPE column (Strata X-AW 33 μm Polymeric Weak Anion, 60 mg/3 mL; Brechbühler, Schlieren, Switzerland) which was previously conditioned with 1 mL MeOH and equilibrated with 1.5 mL 50 mM formic acid at pH 4.0 (adjusted with NaOH). The column was washed with 1 mL water and 1 mL MeOH and dried at 100 mbar vacuum for 1 min. The sample was eluted using 1 mL 5% (v/v) ammonia in MeOH. The eluent was evaporated to dryness with air at maximum 35 °C (about 20 min) and reconstituted in 125 μL water.

*2.4. Determination of pH and conductivity*

The pH was measured using a pH meter model 1.744.0010 from Metrohm (Herisau, Switzerland) and the conductivity was determined using a conductivity meter model 101 from Orion Research (Cambridge, MA, USA) equipped with a model PW 9510/65 cell from Philips (Eindhoven, The Netherlands).

## 3. Results and discussion

*3.1. CZE of EtS in capillaries coated with polycations*

With the pH 2.5 BGE used by Esteve-Turrillas et al. [16], which was composed of 15 mM maleic acid, 1 mM phthalic acid, NaOH and 50 μM CTAB, matrix peaks were detected close to (urine) or within (serum extract) EtS. The same was found to be true for an unidentified impurity of the I.S. Thus, the pH of the BGE was reduced to 2.2 which provided a good separation between EtS, I.S., oxalate and the unidentified peaks. Furthermore, the EOF was adapted via a reduction of the CTAB concentration to 25 μM. This provided data with a low EOF towards the anode (electroosmotic mobility: $-8.8 \times 10^{-9}$ m$^2$ V$^{-1}$ s$^{-1}$). Peaks were identified by spiking with standards and reanalysis of the spiked sample. A typical electropherogram and the temporal behavior of the current are presented in Fig. 1. Furthermore, in order to reach a better sensitivity, the use of a 100 μm I.D. capillary was tested with the optimized BGE. These efforts, however, were abandoned as no reproducible results were obtained with the dynamic CTAB coating.

The 25 μM concentration of CTAB in the BGE is within a critical range in which a small change in the concentration of the polycation strongly influences the resulting EOF [26,27]. Thus, the search for a capillary coating which is less prone to EOF deviations and which does not require a long stabilizing time interval at the beginning of a working day was undertaken. Capillaries of 100 μm I.D. coated with the cationic polymer PDADMAC [17,18] were tested and compared with the performance of the dynamic coating of 50 μm I.D. capillaries using CTAB. The capillary was rinsed with the BGE containing 0.1% (w/w) PDADMAC whereas the electrophoretic run was executed without PDADMAC in the BGE. The applied voltage was set to −9 kV (current: about −28 μA) in order to obtain comparable detection times for EtS in both configurations as well as to keep the resulting current and hence joule heating reasonably low. With PDADMAC, the resulting positive charge of the capillary wall led to a strong EOF in the direction of the anode (electroosmotic mobility: $-36.6 \times 10^{-9}$ m$^2$ V$^{-1}$ s$^{-1}$). The noncovalently bound layer of PDADMAC was reconditioned before each run (cf. Section 2.2.2) and provided reproducible results (see below). It is also imaginable to coat the capillary with successive ionic-polymer layer coatings in order to save time by getting rid of the regeneration step of the coating [18,21,22,25,28].

Reproducibility of detection times was assessed for the I.S. independent of sample matrix (adjusted urine or serum extract) interday RSD ($n = 5$) values for the CTAB and PDADMAC approaches were <4.3 and <7.3%, respectively. Relative detection times of EtS (ratio of detection times of EtS and I.S.) varied less than 1% in all cases which demonstrates the robustness of the approaches used in this work. The calibration curves were calculated with five different concentrations for serum (0.2, 0.5, 1.5, 3.5 and 10 mg L$^{-1}$) and urine (2, 5, 15, 35 and 100 mg L$^{-1}$). Since most of the expected EtS concentrations in real samples are around the lowest calibration points, the suitability of different weighted linear regression models (based upon $x$, $1/x$ and $1/x^2$) were compared according to Karnes and March [29]. The relative concentration residuals for each calibration point were calculated as

$$\%\mathrm{RCR} = 100 \times \frac{\mathrm{RC} - \mathrm{NC}}{\mathrm{NC}} \quad (2)$$

where RC represents the interpolated concentration (using the particular calibration curve) and NC is the nominal concentration. For both coated capillaries, as well as for analysis of serum and urine, the sum of all %RCR was found to be smallest for the $1/x^2$ weighted linear regression and largest for the $x$ weighted linear regression. Thus, the $1/x^2$ weighted linear regression was used in this work.

*3.2. Urine assays*

Esteve-Turrillas et al. [16] proposed to dilute every urine sample 1:5 with water. This sample preparation is very simple and convenient. It does, however, not account for the large range of urine concentrations which can differ up to 100-fold [30,31]. A wide concentration range of the 29 urine samples studied during the present work was also observed. Conductivity was determined to range from 3.09 to 31.2 S m$^{-1}$. Thus, urine should be normalized to a urine property, such as the creatinine concentration [30] or conductivity [32]. The studied CZE methods require samples which contain a similar amount of ions. Particularly the fast migrating anions, such as chloride, sulfate and nitrate, which cover a large part of the anions present in urine, should be present in similar amounts in the samples in order to guarantee comparable electropherograms. Thus, as the conductivity of urine samples was found to correlate with the creatinine level [32], the urines were adjusted to a specific conductivity which led to comparable electropherograms.

In a first step, all samples and calibrators were diluted to 5 S m$^{-1}$ and mixed 1:1 with the I.S. solution (8 mg L$^{-1}$). Typical electropherograms obtained in CTAB- and PDADMAC-coated capillaries are presented in Fig. 2 (lower set of graphs) and Fig. 3, respectively. Electropherograms of samples which were taken after a dinner accompanied by many alcoholic beverages are shown as top graphs of the triplets. Calibration graphs were found to be linear and characterized with small $y$-intercepts (Table 1) for both coating

**Table 1**
Calibration data ($n = 5$)[a]

| Capillary coating | Sample | Slope | | y-Intercept | | r | | F | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SD | Mean (mg L$^{-1}$) | SD (mg L$^{-1}$) | Mean | SD | Mean | SD |
| CTAB | Urine (5 S m$^{-1}$) | 0.095 | 0.001 | −0.021 | 0.020 | 0.999 | 0.001 | 3005 | 3235 |
| | Urine (10 S m$^{-1}$) | 0.099 | 0.004 | 0.006 | 0.020 | 0.999 | <0.001 | 28792 | 42199 |
| | Serum | 0.542 | 0.028 | 0.002 | 0.031 | 0.998 | 0.002 | 1819 | 1312 |
| PDADMAC | Urine (5 S m$^{-1}$) | 0.096 | 0.007 | −0.007 | 0.038 | 0.998 | 0.003 | 2546 | 2014 |
| | Serum | 0.546 | 0.031 | −0.025 | 0.019 | 0.999 | <0.001 | 3564 | 2070 |

[a] Except for the serum analyses in the PDADMAC-coated capillary, data were monitored on the P/ACE MDQ. Ratio of corrected peak area and concentration were taken as $y$ and $x$ values, respectively, and employed for linear regression analysis.

**Table 2**
Validation data ($n = 5$) of the assays[a]

| Capillary coating | Sample | EtS (mg L$^{-1}$) | Precision (RSD (%)) | | Accuracy (% of nominal value) | |
|---|---|---|---|---|---|---|
| | | | Intraday | Interday | Intraday | Interday |
| CTAB | Urine (5 S m$^{-1}$) | 10 | 3.3 | 3.8 | 99.5 | 99.8 |
| | | 40 | 2.5 | 2.7 | 97.8 | 98.7 |
| | | 80 | 0.9 | 2.3 | 99.1 | 99.1 |
| | Urine (10 S m$^{-1}$) | 10 | 1.0 | 4.2 | 91.4 | 98.7 |
| | | 40 | 1.5 | 2.9 | 94.4 | 99.0 |
| | | 80 | 1.4 | 2.7 | 96.7 | 99.6 |
| | Serum | 1 | 2.2 | 6.4 | 101.2 | 105.5 |
| | | 4 | 1.4 | 4.5 | 104.5 | 106.4 |
| | | 8 | 1.9 | 6.4 | 101.2 | 103.3 |
| PDADMAC | Urine (5 S m$^{-1}$) | 10 | 0.5 | 0.8 | 98.9 | 99.1 |
| | | 40 | 1.3 | 2.5 | 102.1 | 99.6 |
| | | 80 | 2.6 | 1.2 | 102.1 | 100.5 |
| | Serum | 1 | 4.2 | 1.7 | 94.8 | 99.3 |
| | | 4 | 2.4 | 2.0 | 100.8 | 101.2 |
| | | 8 | 1.1 | 1.7 | 105.3 | 103.7 |

[a] Except for the serum analyses in the PDADMAC-coated capillary, data were monitored on the P/ACE MDQ.

methods. Using three concentration levels, the assay performance in CTAB- and PDADMAC-coated capillaries was found to be good and comparable (Table 2). The LOQ's were determined to be 2 and 1 mg L$^{-1}$, respectively (Table 3), values that are somewhat too high for patient screening. The same is true for the 5 mg L$^{-1}$ LOQ reported by Esteve-Turrillas et al. [16].

With the CTAB coating, 7 out of 29 urines revealed an EtS peak that could be quantitated. Using the PDADMAC coating, EtS could be quantitated in 5 additional urines (EtS values between 1.0 and 2.0 mg L$^{-1}$). Four of these urines revealed a tiny detectable EtS peak in the CTAB-coated capillary. Using the PDADMAC coating, one real sample with a conductivity higher than 5 S m$^{-1}$ did not reveal an expected EtS peak. Furthermore two urines had a conductivity lower than 5 S m$^{-1}$. The concentration of these urines was not altered and the I.S. was added directly. The result had to be calculated considering the actual conductivity of the sample, so that the amount of EtS could be estimated in respect to a conductivity of 5 S m$^{-1}$. The samples with a lower salt concentration than 5 S m$^{-1}$ resulted in slight shifts to shorter detection times.

The electrophoretic method was found to work well for urines up to 10 S m$^{-1}$ (Fig. 1). From the 29 samples analyzed, however, 7 had a conductivity lower than 10 S m$^{-1}$. Thus, a second approach comprising evaporation of a urine aliquot (cf. Eq. (1)) to dryness and reconstitution in 250 μL water (final conductivity of 10 S m$^{-1}$) was investigated (Fig. 2, upper set of graphs). In order to accelerate evaporation, 250 μL of an organic solvent was added. Tests were performed with isopropanol, ACN, dichloromethane and MeOH. ACN was found to be best suited to accelerate the evaporation. Furthermore, it is interesting to note that the analysis of 10 S m$^{-1}$ samples revealed sharpest peaks for EtS when compared with data generated in the 2.5–15 S m$^{-1}$ range (data not shown). Assay calibration (Table 1) and validation (Table 2) were executed for 10 S m$^{-1}$ samples using the CTAB coating. Calibration graphs were found to be linear and characterized with small y-intercepts (Table 1). Precision and accuracy data were determined to be comparable to

**Table 3**
LOQ and LOD ($n = 3$) data of the assays[a]

| Capillary coating | Sample | LOQ | | | LOD (mg L$^{-1}$) s/n ≈ 3 |
|---|---|---|---|---|---|
| | | (mg L$^{-1}$) | RSD (%) | (% of nominal value) | |
| CTAB | Urine (5 S m$^{-1}$) | 2.0 | 1.3 | 101.7 | 1.0 |
| | Urine (10 S m$^{-1}$) | 0.6 | 8.7 | 104.4 | 0.4 |
| | Serum | 0.2 | 3.2 | 99.9 | 0.1 |
| PDADMAC | Urine (5 S m$^{-1}$) | 1.0 | 5.2 | 90.5 | 0.5 |
| | Serum | 0.2 | 4.0 | 98.2 | 0.1 |

[a] Except for the serum analyses in the PDADMAC-coated capillary, data were monitored on the P/ACE MDQ.

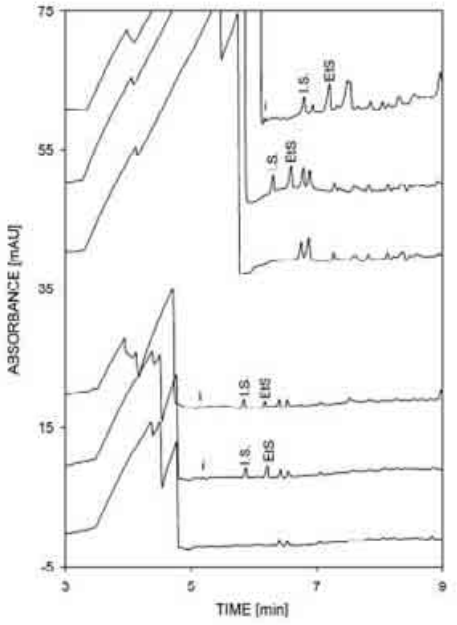


**Fig. 2.** Analysis in a CTAB coated capillary of urines diluted to a conductivity of 5 S m$^{-1}$ (lower triplet of graphs) and urines adjusted to a conductivity of 10 S m$^{-1}$ (upper triplet of graphs). From bottom to top: blank urine, blank urine spiked with EtS (15 mg L$^{-1}$) and I.S. (8 mg L$^{-1}$), and a real sample (EtS: 6.71 mg L$^{-1}$ in the 5 S m$^{-1}$ sample). Key as for Fig. 1.

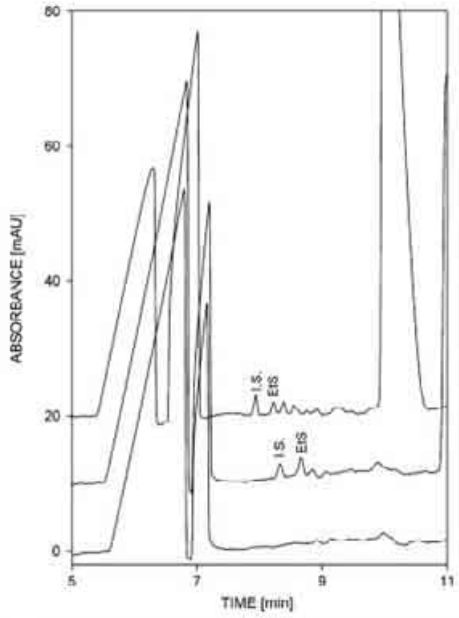


**Fig. 3.** Analysis in a PDADMAC-coated capillary of urines diluted to a conductivity of 5 S m$^{-1}$. From bottom to top: blank urine, blank urine spiked with EtS (15 mg L$^{-1}$) and I.S. (8 mg L$^{-1}$), and a real sample (EtS concentration: 5.91 mg L$^{-1}$).

those obtained with the first approach (Table 2). LOQs and limits of detection (LODs), however, were somewhat better (Table 3). This improvement is essential as many positive urines have EtS levels around 1–2 mg L$^{-1}$. It is important to realize that the urine adjustment to a uniform conductivity level is done prior to the analysis. With assays using post-analysis normalization, it is possible that intentional urine dilution obtained via drinking of water provides a urinary analyte concentration below the LOD, this resulting in a false-negative outcome [33]. Using the PDADMAC approach, the EtS peak was not baseline separated from a peak of the urine matrix. Thus, this coating was not evaluated with urine adapted to 10 S m$^{-1}$. Longer capillaries or capillaries with a smaller I.D. would have to be employed for complete resolution.

With the adjustment of the urine to 10 S m$^{-1}$, 15 out of the 29 analyzed real urine samples revealed an EtS peak that could be quantitated. The consumption of a small amount of ethanol, such as a glass of wine (approximatively 14 g of ethanol), and urine collection 2–3 h after the beginning of the ethanol ingestion, resulted in EtS levels between 2.5 and 4.5 mg L$^{-1}$ (urines of three test persons). In the urines of a subject consuming 0.875 g ethanol per kg body mass, the highest EtS amount found was 38.69 mg L$^{-1}$ (urine collected after 7 h) and EtS could be detected in urines that were collected up to 24 h after the start of ethanol consumption (Fig. 4). No EtS was detected in the sample collected thereafter. Lower EtS levels were monitored after ingestion of 0.462 g ethanol per kg body mass (Fig. 4). Note that an insufficient number of samples was collected for proper description of the excretion peak of EtS. For that example, EtS could be quantitated up to the urine collected 11.5 h after the beginning of ethanol intake, whereas a tiny EtS peak was detected

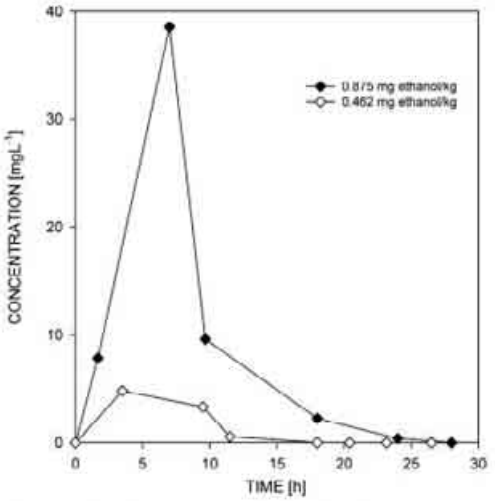


**Fig. 4.** EtS concentration in urines collected from a male person who ingested 0.875 g ethanol per kg body mass and a female individual who consumed 0.462 g ethanol per kg body mass. The urines were adjusted to a conductivity of 10 S m$^{-1}$ and analyzed in the CTAB-coated capillary.

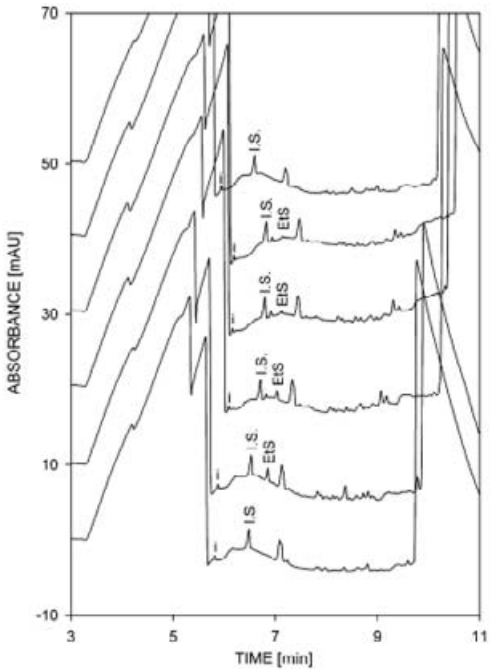


**Fig. 5.** Electropherograms obtained with the urines of a female individual who consumed 0.462 g ethanol per kg body mass. Urines were sampled immediately before the ethanol intake, and 3.5, 9.5, 11.5, 18 and 20.5 h after the beginning of ethanol intake (from bottom to top, respectively, and displayed with a y-axis offset). The urines were adjusted to a conductivity of 10 S $m^{-1}$ and analyzed in the CTAB-coated capillary.

at 18 h as is shown with the electropherograms presented in Fig. 5. These data suggest that the urine adaptation to a conductivity of 10 S $m^{-1}$ is best suited for clinical purposes. Sample preparation is simple, fast and cost-effective. Moreover, the creatinine level of the urine or any other property allowing a normalization of the concentration of the samples (e.g. osmolality, density and specific gravity [30,32]) could be used. For that purpose, however, Eq. (1) must be adapted accordingly. In principle, both capillary coatings can be employed. Practical criteria, such as the time interval required for capillary equilibration at the beginning of the day and the buffer volume needed for capillary rinsing, should be considered for the selection of the coating method. The use of the 100 μm I.D. capillary provides a somewhat better detection limit which is certainly valuable for patient screening. With the PDADMAC coating, however, a longer capillary would have to be employed for proper analysis of urines adjusted to 10 S $m^{-1}$.

### 3.3. Serum assays

In order to get rid of parts of the serum's matrix and to concentrate the samples to reach lower LODs, it was necessary to simplify the sample matrix. Attempts using ultrafiltration or protein precipitation with ACN followed by evaporation to dryness and reconstitution did not provide satisfying CZE data. SPE using a weak anion exchange column provided a good recovery of the analytes. However, the high chloride content of the extract did not allow a concentration of the sample. Thus chloride was precipitated with SAC and removed prior to SPE. As the chloride concentration in serum is very stable around 100 mM, an equal amount of SAC was added to the sample. Using the conditions given in Section 2.3.2 provided EtS recoveries ($n = 5$) of 88.4, 86.5 and 83.7% for serum spiked with 1.0, 4.0 and 8.0 mg $L^{-1}$ of EtS, respectively (about a 3.5-fold concentration). Analyses were executed in capillaries coated with CTAB and PDADMAC and selected electropherograms are presented in Figs. 6 and 7, respectively.

Calibration graphs were found to be linear and characterized with small y-intercepts (Table 1). Intraday and interday precision data ($n = 5$) for the experiments with CTAB and three EtS concentrations (1.0, 4.0 and 8.0 mg $L^{-1}$ in serum) were lower than 2.2 and 6.4%, respectively (Table 2). Corresponding values for the PDADMAC-coated capillary were 4.2 and 2.0%, respectively (Table 2). All accuracy values were between 94.8 and 106.4% (Table 2). The LOQ (0.2 mg $L^{-1}$) and the LOD (0.1 mg $L^{-1}$) were the same for both methods (Table 3). It is important to note that the analyses in the PDADMAC-coated capillary were performed on the PA 800 instrument which featured a diode array detector set to 215 nm whereas those in the CTAB-coated capillary were generated on the MDQ which was equipped with a 214 nm bandpass filter. A potential difference in the sensitivity of the two detector assemblies was not evaluated. Looking at the data obtained with the 5 S $m^{-1}$ urines (Table 3), however, it can be assumed that the sensitivity obtained with the MDQ is somewhat better. For serum samples both methods were characterized by very similar

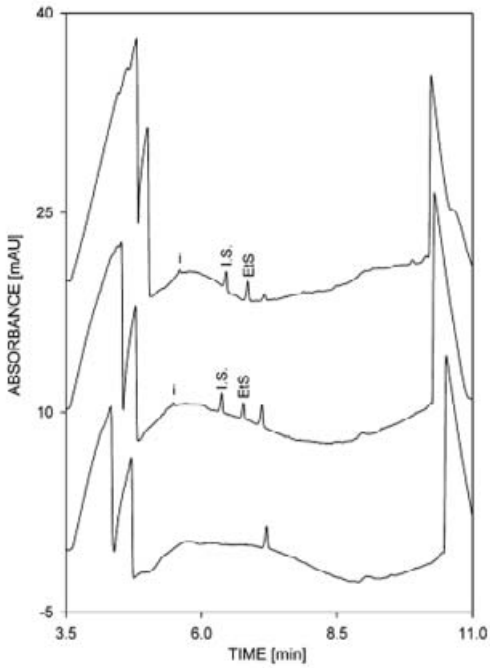


**Fig. 6.** Analysis in a CTAB-coated capillary of serum extracts prepared from blank serum, blank serum spiked with EtS (1.5 mg $L^{-1}$) and I.S. (1.5 mg $L^{-1}$), and a real sample with an EtS serum concentration of 1.86 mg $L^{-1}$ (bottom to top graphs, respectively).

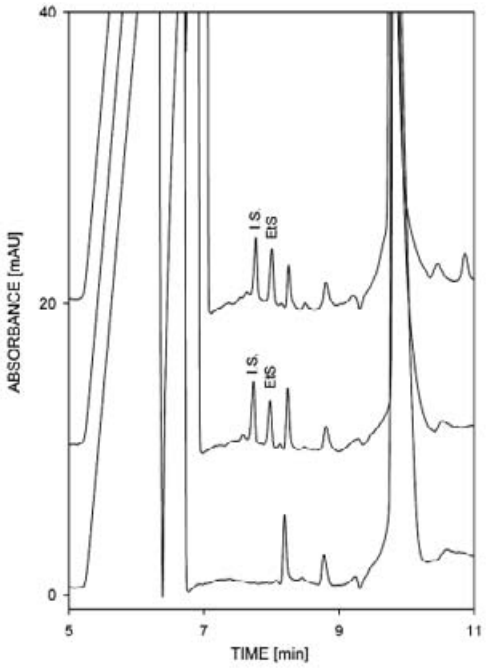


**Fig. 7.** Analysis in a PDADMAC-coated capillary of serum extracts prepared from blank serum, blank serum spiked with EtS (1.5 mg $L^{-1}$) and I.S. (1.5 mg $L^{-1}$), and a real sample with an EtS serum concentration of 1.70 mg $L^{-1}$ (bottom to top graphs, respectively).

performances. Based on the method validation data, no method can be considered as being superior.

A total of 14 sera were analyzed. Five were blank sera which did not reveal any EtS using the two methods. A small amount of ethanol intake, such as a glass of wine (approximatively 14 g of ethanol) and blood withdrawal about 2 h after the beginning of the ethanol ingestion, resulted in positive EtS detection in three out of four sera. The three EtS peaks were lower than the LOQ but were above the LOD. In one case EtS could not be detected with any of the two methods. In five samples which were taken after a dinner accompanied by many alcoholic beverages, higher amounts of EtS were found (for examples see top graphs in Figs. 6 and 7). EtS concentrations between 0.26 and 1.95 mg $L^{-1}$ were monitored and the values obtained by the two methods were found to correlate well ($y = 0.96x + 0.02$, $r = 0.996$).

## 4. Conclusions

The data presented in this paper demonstrate robust approaches for the CZE determination of EtS in urine diluted to 5 S $m^{-1}$, urine adjusted to a conductivity of 10 S $m^{-1}$ and extracted serum. The sample matrices were chosen to account for the variability of urine concentration, to enhance sensitivity and to be able to analyze all samples with the same CZE configuration which features reversed polarity and indirect UV detection. Two capillary coatings were evaluated, a 50 μm I.D. fused-silica capillary dynamically coated with CTAB (25 μM CTAB added to the BGE) and a 100 μm I.D. capillary coated with PDADMAC. Reproducible data down to mg $L^{-1}$ EtS levels were obtained with both capillary treatments. The LOQ of the serum assays is 0.2 mg $L^{-1}$. With adjustment of the urine to 10 S $m^{-1}$ the LOQ with the CTAB-coated capillary could be reduced to 0.6 mg $L^{-1}$. These sensitivities were found to be sufficient to detect EtS in samples of humans who consumed as little as one alcoholic drink.

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# Appendix II
# B. Jung et al., J Sep Sci

Appendix II depicts the following publication as it was printed by the journal (10 pages).

B. Jung, J. Caslavska, W. Thormann
*Determination of ethyl glucuronide in human serum by capillary zone electrophoresis and an immunoassay*
Journal of Separation Science 32 (2009) 3497

J. Sep. Sci. 2009, 32, 3497–3506



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Original Paper

# Determination of ethyl glucuronide in human serum by capillary zone electrophoresis and an immunoassay

Ethyl glucuronide (EtG) is a marker of recent alcohol consumption. For the optimization of the analysis of EtG by CZE with indirect absorbance detection, the use of capillaries with permanent and dynamic wall coatings, the composition of the BGE, and various sample preparation procedures, including dilution with water, ultrafiltration, protein precipitation, and SPE, were investigated. Two validated screening assays for the determination of EtG in human serum, a CZE-based approach and an enzyme immunoassay (EIA), are described. The CZE assay uses a coated capillary, 2,4-dimethylglutaric acid as an internal standard, and a pH 4.65 BGE comprising 9 mM nicotinic acid, ε-aminocaproic acid and 10% v/v ACN. Proteins are removed *via* precipitation with ACN prior to analysis and the LOQ is 0.50 mg/L. The EIA is based upon commercial reagents which are promoted for the determination of urinary EtG. Krebs–Ringer solution containing 5% BSA is used as a calibration matrix. All samples are ultrafiltered prior to analysis of the ultrafiltrate on a Mira Plus analyzer. Assay calibration ranged between 0 and 2 mg/L and the upper reference limit was determined to be 0.05 mg/L. Both assays proved to be suitable for the analysis of samples from different individuals. For EtG levels above 0.50 mg/L, good agreement was observed for the comparison of the results of the two methods.

**Keywords:** Alcohol marker / Capillary zone electrophoresis / Ethyl glucuronide / Immunoassay / Serum

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## 1 Introduction

Less than 0.1% of ingested ethanol is metabolized non-oxidatively to ethyl glucuronide (EtG) which is excreted in urine. EtG reaches its maximum concentration in serum about 2 h after the peak concentration of ethanol [1, 2] and can be detected for an extended time period in serum (in the range of hours) and in urine (in the range of hours to days) after complete elimination of ethanol [3–5]. EtG was observed to be stable upon storage [6, 7]. Hence, it is a potent marker substance for recent alcohol consumption. EtG and another ethanol metabolite, ethyl sulfate (EtS [8–13]), cover a clinically and forensically important time window between the complete elimination of ethanol in the body and the disappearance of these ethanol metabolites.

The determination of EtG in biological samples has attracted considerable attention during the past decade. For the analysis of EtG in urine and serum, methods based on GC-MS [14–16], LC-MS [1, 2, 5, 12, 15–19] and ELISA [20, 21] have been developed. Furthermore, CZE [22, 23] and ITP-CZE [24] were applied to the determination of EtG in serum, and a commercial enzyme immunoassay (EIA) for urinary EtG became recently available and was found to be suitable for rapid urine screening [3, 25, 26]. The literature suggests that the calibration range for EtG in serum has to be in the low mg/L range. Zimmer *et al.* [21] suggest an upper reference limit for EtG in serum of 0.31 mg/L whereas Høiseth *et al.* [1] report an administrative cut-off of 0.09 mg/L. Although cut-off levels for urinary EtG of 0.5 mg/L or 0.5 mg EtG/g creatinine

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**Abbreviations:** **2,4-DMGA,** 2,4-dimethylglutaric acid; **EACA,** ε-aminocaproic acid; **EIA,** enzyme immunoassay; **EtG,** ethyl glucuronide; **EtS,** ethyl sulfate; **G6PDH,** glucose-6-phosphate dehydrogenase; **I.S.,** internal standard; **LPA,** linear polyacrylamide; **MeOH,** methanol; **PDADMAC,** poly(diallyldimethylammonium chloride); **PP,** protein precipitation; **PVA,** polyvinyl alcohol; **UF,** ultrafiltration

have been proposed [1, 3, 25, 27–30], no cut-off level has been generally accepted yet.

For the analysis of non-absorbing anions in aqueous samples, such as serum and urine, CZE with indirect optical detection is an attractive technology. CZE has successfully been applied to the monitoring of EtS in urine [9], EtS in serum and urine [8] and EtG in serum [22–24]. Using the previously described assay for serum EtG at pH 4.3 [22] with patient samples revealed (i) a potential interference between succinate and EtG and (ii) that benzoate migrated shortly behind EtG and at higher quantities it was not baseline separated from the analyte of interest. Furthermore, analyses were performed without an internal standard (I.S.) and in home-made linear polyacrylamide (LPA) coated capillaries. Thus, efforts were undertaken in our laboratory (i) to test the suitability of other coated capillaries, (ii) to improve the composition of the BGE, (iii) to use internal calibration with a deliberately added I.S., and (iv) to test different sample pretreatment procedures. Other activities were focused on the use of the commercial EIA reagents (DRI EtG Assay, Microgenics, Fremont, CA, USA) for the determination of EtG in serum which included the search for a suitable sample matrix, assay validation, and the determination of the upper reference limit *via* analysis of sera of volunteers that did not consume any alcohol for at least 3 days prior to blood collection.

In this paper, CZE data obtained with different dynamic and permanent capillary coatings, a BGE providing higher resolution through elevation of the pH to 4.65 and addition of 10% ACN, and serum dilution, ultrafiltration (UF), protein precipitation (PP), and SPE for sample preparation are reported. The CZE assay performed in a commercial LPA-coated capillary, with PP as sample preparation and 2,4-dimethylglutaric acid (2,4-DMGA) as I.S., was validated. Data obtained with real samples were compared to those obtained using the EIA with ultrafiltered serum and having ultrafiltered Krebs–Ringer solution containing 5% BSA as a calibration matrix.

## 2 Materials and methods

### 2.1 Chemicals

All chemicals used were of highest analytical purity. EtG was purchased from Medichem (Steinenbronn, Germany) and 2,4-DMGA (mixture of D, L, and meso) from Acros Organics (Geel, Belgium). ACN and methanol (MeOH) were from VWR (Leuven, Belgium). Nicotinic acid, polyvinyl alcohol (PVA; mean molecular mass ~15 kDa), urea, sodium chloride, glutaric acid, sodium benzoate, L-lactic acid, and oxaloacetic acid were from Fluka (Buchs, Switzerland). Isopropanol, formic acid, sodium sulfate, sodium acetate, sodium dihydrogenphosphate, potassium dihydrogenphosphate, potassium chloride, magnesium sulfate, sodium hydrogencarbonate, calcium chloride, and L-glutamic acid were from Merck (Darmstadt, Germany). ε-Aminocaproic acid (EACA), CTAB, L-ascorbic acid, and succinic acid (disodium salt) were from Sigma (St.Louis, MO, USA). Poly(diallyldimethylammonium chloride) (PDADMAC, typical molecular mass 200–350 kDa, 20% w/w aqueous solution) and poly(4-styrenesulfonic acid) (PSS, mean molecular mass ~75 kDa, 18% w/w aqueous solution) came from Aldrich (Milwaukee, USA). BSA was from Calbiochem (San Diego, CA, USA). Bidistilled water was used throughout this work.

### 2.2 Serum samples

Two sets of samples were used in this work. Twenty nine sera were from members of our department which gave their consent and provided information about recent alcohol consumption. The sera used for the elucidation of the upper reference limit of the EIA were from 102 volunteers (32 males with mean age 38.6 years, median 36 years, range 21–62 years; 70 females with mean age 33.7 years, median 28 years, range 16–64 years) whose blood was drawn not earlier than 3 days after the last intake of an alcoholic beverage. The volunteers filled out a detailed questionnaire and signed an informed consent. Exclusion criteria were ethanol consumption in the 72 h prior to sampling, pregnancy, or lactation. A second serum donation *per* volunteer was accepted, but not earlier than 2 weeks after the first donation. Seven males and five females provided two serum samples under these conditions. The study was blinded and approved by the local ethics committee.

### 2.3 CZE instrumentation and methods

CZE measurements were performed on the P/ACE MDQ (Beckman Coulter, Fullerton, CA, USA) with the UV detector set to indirect detection at 214 nm (DAD or filter). The cartridge temperature was kept at 20°C, the sample storage at 5°C. LPA-coated capillaries with 75 μm id and bare fused-silica capillaries of 100 μm id (both from Polymicro Technologies, Phoenix, AZ, USA) were employed. Prior to first use, the LPA-coated capillaries were flushed with BGE for 15 min at 15 psi (1 psi = 6894.76 Pa), while bare fused-silica capillaries were pretreated with 1 M NaOH, 0.1 M NaOH, and water (30 min at 15 psi each).

#### 2.3.1 CZE in LPA-coated capillaries

A 75 μm id capillary with a total length of 60 cm (50 cm effective length) was used. The BGE was prepared from a 10 mM nicotinic acid solution that was adjusted to pH 4.65 via addition of EACA (~11 mM) and completed by the addition of ACN (9:1 v/v). Before each run, the

 

capillary was flushed with the BGE for 4 min at 30 psi. The injection was effected by application of vacuum for 16 s at 0.5 psi. To improve reproducibility, a plug of BGE was added behind the injected sample (0.1 min at 0.1 psi). If not stated otherwise, a voltage of −30 kV (reversed polarity) was applied. At the end of each working day the capillary was washed with water for 20 min at 30 psi followed by drying with air for 10 min at 30 psi. The EOF was measured by injecting diluted BGE (1:10 with water) as a sample. Quantitation was based upon multi level internal calibration using corrected peak areas (peak areas divided by detection time) for data evaluation.

#### 2.3.2 CZE in CTAB/PVA coated capillaries

The BGE composition was 10 mM nicotinic acid adjusted to pH 4.5 with EACA, 0.05% w/v PVA, and 50 μM CTAB. The 100 μm id capillary with a total length of 60 cm was used. At the beginning of a working day, the capillary was rinsed with the BGE for 10 min at 20 psi followed by stabilization with application of ~20 kV for 30 min. Before each run, the capillary was flushed with the BGE for 5 min at 40 psi. The injection was effected with a vacuum for 5 s at 0.5 psi. A plug of BGE was added behind the injected sample (0.1 min at 0.1 psi). A voltage of ~20 kV was applied for the separation. At the end of each working day the capillary was washed with water for 30 min at 30 psi and the ends of the capillary were left in vials filled with water.

#### 2.3.3 CZE in PDADMAC coated capillaries

The PDADMAC coating was applied after conditioning with NaOH by flushing the capillary (100 μm id and 70 cm total length) for 10 min at 20 psi with a solution containing 0.5% w/w PDADMAC in BGE (10 mM nicotinic acid and adjusted to pH 4.5 with EACA; without ACN). Following the application of the coating, it was stabilized under a voltage of −15 kV for 180 min. Before each run, the capillary was flushed consecutively with the BGE (1 min at 20 psi), 0.1% w/w PDADMAC in BGE (0.7 min at 20 psi) followed by another flush of BGE (2 min at 20 psi). The sample was injected by pressure for 6 s at 0.5 psi. A plug of BGE was added behind the injected sample (0.1 min at 0.1 psi) before application of −18 kV. At the end of each run the capillary was washed with water for 0.5 min at 40 psi. At the end of a working day the capillary was washed with water for 15 min at 40 psi and dried with air for 5 min at 40 psi.

### 2.4 Sample preparation for CZE

#### 2.4.1 Serum calibrators and controls

If not stated differently, standard solutions of EtG dissolved in ACN (16 mg/L for calibration and 20 mg/L for the control samples) were prepared. Aliquots of these solutions were pipetted into Eppendorf vials, evaporated to dryness and reconstituted with blank human serum.

#### 2.4.2 Protein precipitation

The 2,4-DMGA (I.S.) was dissolved in ACN at a concentration of 10 mg/L. Two hundred microliters of this solution were mixed with 100 μL of serum, vortexed, and centrifuged for 10 min at about 16 000 × g. Two hundred and forty microliters of the supernatant were transferred to another vial, evaporated to dryness under a constant stream of air at a temperature of 35–40°C (<10 min), and reconstituted with 160 μL water.

#### 2.4.3 SPE

Clean Screen ETG extraction columns (200 mg proprietary carbon sorbent, 3 mL volume, United Chemical Technologies, Bristol, PA, USA) were conditioned with 2 mL of 1% v/v formic acid. Five hundred microliters of serum were spiked with aqueous solutions of EtG (calibrators and controls only) and I.S. and diluted with water to a total volume of 950 μL. Fifty microliters of formic acid were added to the sample prior to loading onto the extraction column. The column was washed with 2 mL water and dried at 2 kPa for 2 min. Elution was effected with 2 mL of 1% v/v formic acid in MeOH. For this step, a filter (Millex-GP 0.22 μm Filter Unit, Millipore, Bedford, MA, USA) was connected to the column end to retain leaking carbon filling. The filter was separately rinsed with 1 mL of the elution solution. The column eluate and the filter rinse were combined, evaporated to dryness at a temperature of 35–40°C under a steady stream of air (<30 min) and reconstituted in 500 μL water.

#### 2.4.5 Ultrafiltration

Four hundred microliters of serum were put in an Ultrafree 0.5 Centrifugal Filter and Tube (Millipore Corporation, Biomax 5 k NMWL Membrane 0.5 mL Vol, Billerica, MA, USA) and centrifuged for 12 min at about 16 000 × g.

### 2.5 Enzyme immunoassay for EtG in serum

Immunochemical EtG determinations were performed on a Mira Plus analyzer (ABX Diagnostics, Diatools, Dietikon, Switzerland). The EIA reagents were purchased as a kit (DRI EtG Assay, Microgenics, Fremont, CA, USA). Two independent standard solutions of 10 mg/L EtG dissolved in MeOH were used for the preparation of calibration and control samples. Aliquots of these solutions were pipetted into Eppendorf vials, evaporated to dryness (air, 35–40°C) and reconstituted with home-made Krebs–Ringer solution containing 5% BSA. If not stated otherwise, the samples were ultrafiltered (see Section 2.4.5) and the ultrafiltrate was analyzed according to the instructions of the kit manufacturer. The logit/log4 cali-

 

bration mode was applied. Calibrators were analyzed in duplicates whereas controls and samples were analyzed once only. Samples containing more EtG than the highest calibrator (2 mg/L EtG) were automatically ten-fold diluted and reanalyzed.

### 2.6 Prediction of CZE separations and statistical treatments

Effective mobilities were calculated and analyte separability was investigated using Peakmaster 5.1 (www.natur.cuni.cz/gas). The PASW Statistics 17 (SPSS, Chicago, IL, USA) program was used for the statistical analysis of the upper reference level of EtG in serum and its confidence interval. GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA, USA) was employed to check for outliers by the Grubb's method and to test for normality according to the Kolmogorov–Smirnov procedure.

## 3 Results and discussion

### 3.1 CZE of EtG in serum

#### 3.1.1 CZE in capillaries with a dynamic wall coating

The first approach tested comprised CTAB and PVA in the running buffer and was based on a coating method of Chiari *et al.* [31]. Both agents have been described by other authors to influence the EOF when used separately [8, 9, 32] or in combination [33]. While CTAB decreases the zeta potential or even reverses its prefix, PVA increases the viscosity of the BGE and thereby decreases EOF. Having a BGE composed of 10 mM nicotinic acid adjusted to pH 4.5 with EACA, 0.05% PVA and 50 μM CTAB resulted in a low EOF toward the anode [32] (the EO peak associated with buffer change at the original sample location did not reach the detector within 40 min). The applied samples comprised human serum diluted 1:1 with water. Using newly prepared capillaries, patterns comparable to those observed in LPA-coated capillaries [22, 23] were monitored (data not shown). Data could, however, not be reproduced after a few runs as the capillary became blocked. For the removal of possible protein deposits, the capillary was flushed with 5 M urea and BGE (2.5 min at 40 psi each) before each run. Blocking of the capillary could thereby be avoided. Migration times of EtG, however, were not reproducible enough between different days (change of >5% from day to day) and the urea crystallized on top of the vials such that the instrument needed intensive cleaning.

The dynamic coating based on the polycation PDADMAC was previously used for the determination of EtS in serum and urine [8]. The EOF toward the anode was strong ($-29.7 \times 10^{-9}$ m$^2$/Vs). Baseline separation of the analytes of interest was achieved during the first runs on a new capillary, but the EOF slightly increased with every run and could not be completely stabilized. As a result, peaks became not well separated. Nevertheless the method allowed studying the effects of different organic solvents as buffer additives and variations of the BGE pH (Figs. 1 and 2). While isopropanol and MeOH concentrations between 5 and 20% slightly modified the EOF but not the relative migration of the compounds of interest (data not shown), the addition of ACN influenced the electrophoretic mobilities of selected analytes and provided the required separations between EtG, the I.S. and endogenous compounds (Fig. 1). Separability of EtG from endogenous compounds and the I.S. is strongly dependent on the pH of the BGE (Fig. 2). Resolution of the two diastereomers of 2,4-DMGA was also a critical aspect for the selection of buffer properties. Many other compounds were tested as candidates for I.S., including 2,2-DMGA, glutarate, methacrylate, mandelate, 3-bromobutyrate, and 4-bromobutyrate. They could be excluded using Peakmaster or were found to comigrate with

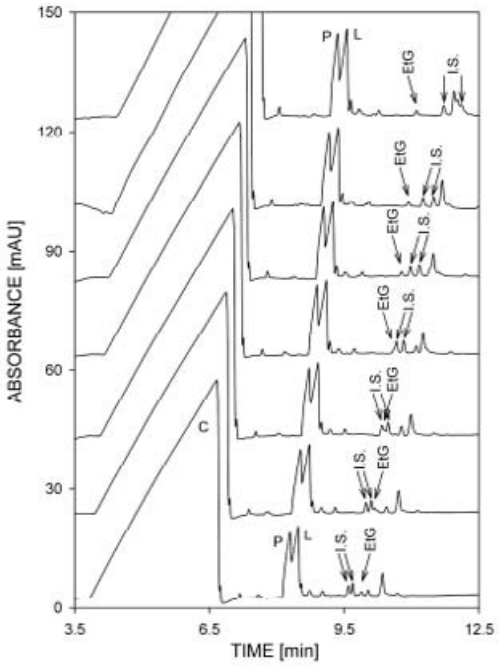


**Figure 1.** Effect of ACN content on the mobility of the analytes (EtG 8 mg/L and I.S. 20 mg/L). The study was performed in a PDADMAC coated capillary using BGEs composed of 10 mM nicotinic acid, EACA (pH 4.70) and various amounts of ACN. Electropherograms resulting from an ACN amount of 0% (bottom graph) to 15% v/v (top graph) in steps of 2.5% are displayed with a *y*-axis offset. C, P, and L refer to chloride, phosphate, and lactate, respectively.

 

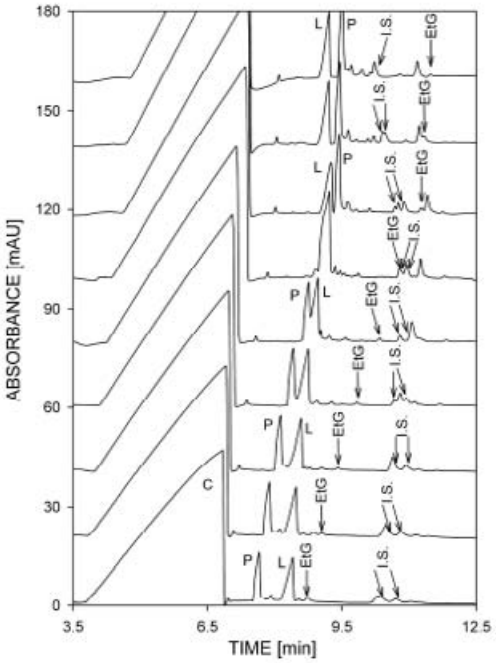


**Figure 2.** Effect of pH on the electrophoretic separation of EtG (8 mg/L) and the I.S. (12 mg/L). Study performed in a PDADMAC coated capillary using BGEs composed of 10 mM nicotinic acid, 12.5% ACN and EACA at different concentrations. Electropherograms resulting from BGE pH values between 4.30 (bottom graph) and 5.10 (top graph) in steps of 0.1 pH units are displayed with a *y*-axis offset. C, P, and L refer to chloride, phosphate, and lactate, respectively.

endogenous compounds. Using 2,4-DMGA, best conditions were found to be at a pH of 4.65 and at an ACN concentration of 8–10%. Due to the risk of evaporation of ACN prior to analysis, the amount of 10% was chosen for further work.

In order to stabilize the EOF, the use of a multilayer coating formed with alternating layers of PDADMAC (polycation, total of seven layers) and PSS (polyanion, six layers) [34–36] was studied. The separation of the analytes was similar to the method using the PDADMAC coating. The intraday-reproducibility of detection times was good (RSD <0.4%, $n = 5$), while the differences between working days were too large (RSD 3.9%, $n = 4$) for the method being used in a routine setting. No improvements were observed for the analysis of SPE extracts. Thus, configurations with dynamic capillary coatings were not further investigated.

### 3.1.2 CZE in capillaries with permanent coating

Home-made LPA-coated capillaries have previously shown to perform well for the determination of EtG in serum [22, 23]. Thus, the optimized BGE reported in Section 3.1.1 (10 mM nicotinic acid adjusted to pH 4.65 with EACA and mixed 9:1 with ACN) was used together with commercially available 75 µm id LPA-coated capillaries. An applied voltage of −30 kV (current <6.2 µA, power <0.31 W/m) provided detection times for EtG of about 10.4 min and an electroosmotic mobility of $(8.3 \times 10^{-9}$ $m^2/Vs$. Using sera diluted 1:1 with water, interday RSD values of detection times were better (RSD <1%, $n = 5$) than those obtained with the dynamically coated capillaries. EtG was detected free from interferences and the two diastereoisomers of 2,4-DMGA were baseline-separated.

Originally, all sera were diluted 1:1 with water [22, 23]. No problems in reproducibility were observed in the BGE

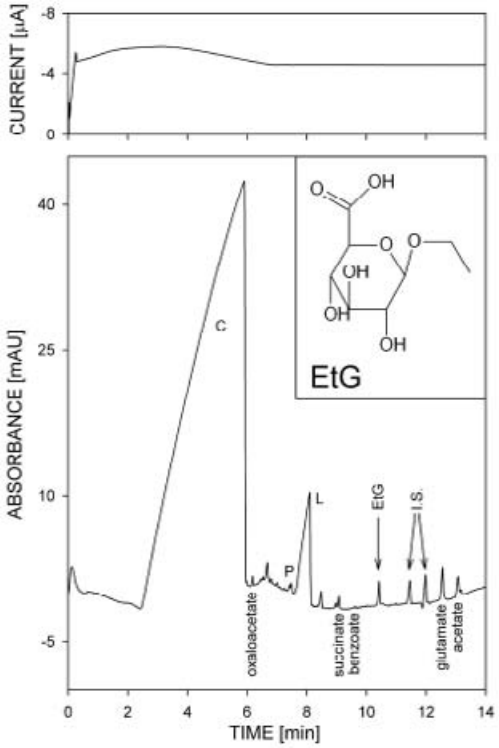


**Figure 3.** Typical electropherogram (lower graph) and temporal behavior of the current (upper graph) obtained for the analysis of a blank serum spiked with EtG (12 mg/L) and I.S. (20 mg/L) after PP and analysis in a LPA-coated capillary at −30 kV using a BGE composed of 10 mM nicotinic acid, EACA (pH 4.65), and 10% ACN. Conditions as in Sections 2.3.1 and 2.4.2. The inset in the lower panel depicts the chemical structure of EtG. C, P, and L refer to chloride, phosphate, and lactate, respectively. The positions of other identified anions are marked under the trace. Sulfate and nitrate are comigrating with chloride.

 

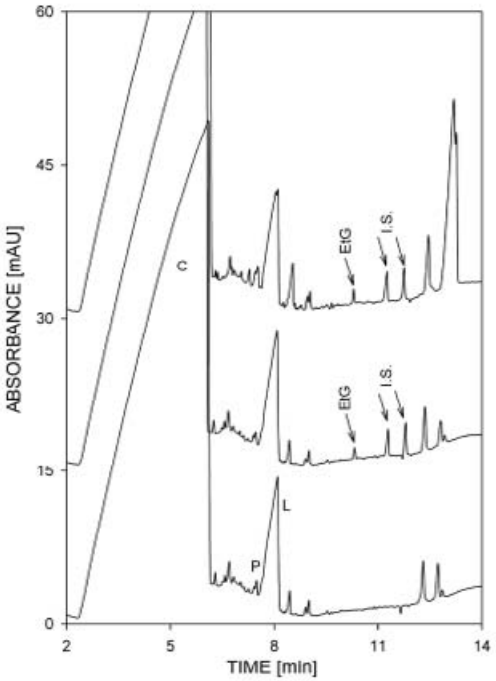


**Figure 4.** Electropherograms of sera after PP analyzed in a LPA-coated capillary. Data of blank serum, blank serum spiked with 4 mg/L EtG and 20 mg/L I.S., and a real sample (determined EtG concentration 4.72 mg/L, I.S. 20 mg/L) are displayed with a *y*-axis offset (from bottom to top, respectively). Other conditions as for Fig. 3.

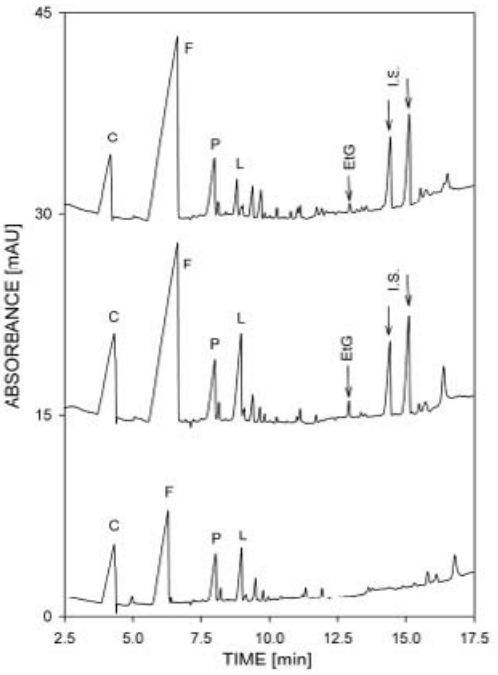


**Figure 5.** Electropherograms of sera extracted with SPE and analyzed in a LPA-coated capillary at −24 kV using a BGE composed of 10 mM nicotinic acid, EACA (pH 4.65) and 10% ACN. Data obtained with a blank serum, a blank serum spiked with 1.5 mg/L EtG and 20 mg/L I.S., and a real sample (EtG concentration 1.01 mg/L) are displayed with a *y*-axis offset (from bottom to top, respectively). C, F, P, and L refer to chloride, formate, phosphate, and lactate, respectively.

used by Mrázková *et al.* [22] (pH 4.3, no addition of ACN). With the ACN containing BGE, however, it was problematic to ensure the reproducibility over a large number of injections. A wavy baseline was often observed. The capillary was therefore flushed with 5 M urea between runs (4 min at 30 psi), an approach which did not provide an improvement but suggested a possible incompatibility between a proteinaceous sample and the ACN containing BGE. Thus, three different sample preparation procedures were tested, namely UF, PP, and SPE. After UF some peaks appeared with one migrating close to EtG (data not shown) and this issue was not further investigated. With PP using ACN and reconstitution of the dried supernatant in water, nice and reproducible electropherograms were obtained (Fig. 3). Succinate, benzoate, glutamate, and acetate do not interfere with the detection of EtG and the I.S. The data shown in Fig. 4 are those obtained for a serum blank, a blank spiked with 4 mg/L EtG and 20 mg/L 2,4-DMGA, and a real sample. This procedure led to a reduced amount of phosphate in the sample and otherwise no change in the detector response compared to that obtained with 1:1 diluted serum. It is worth mentioning that the same dilution of the sample was applied. The recovery of EtG was determined at three concentrations ($n = 3$ for each level), namely 1.5 mg/L (recovery 85.66 ± 14.44%), 6 mg/L (85.58 ± 0.49%), and 12 mg/L (87.85 ± 6.26%). The reproducibility of detection times was assessed for the I.S. and the relative detection time (ratio of detection times of EtG and I.S.). Intraday RSD ($n = 18$) were found to be 2.19 and 2.37%, respectively. Interday RSD ($n = 18$) values were 2.30 and 2.43%, respectively.

Using SPE with disposable Clean Screen ETG columns provided nice electropherograms as well (Fig. 5). These columns have been developed and recommended for the extraction of EtG in urine by its manufacturer. To the knowledge of the authors, no publication showed the suitability of these SPE columns for serum. The data presented in Fig. 5 are those obtained for a blank serum, a blank spiked with 1.5 mg/L EtG and 20 mg/L 2,4-DMGA,

 

and a sample of a healthy volunteer who ingested ~1.37 g ethanol per kilogram body mass in a 10 h time period before blood sampling. The matrix could be simplified, notably the amount of the fast migrating anions in the sample could be reduced. The recovery was determined to be 99.6 ± 13.1, 91.1 ± 8.6, and 72.6 ± 9.1% for 1.5, 6, and 12 mg/L EtG, respectively ($n$ = 2 at each level). The I.S. also could be extracted from the SPE-column and showed a recovery of 72.8% at 20 mg/L ($n$ = 6). The reproducibility of the extraction method was not investigated further as PP was considered to be the more favorable approach due to its simplicity and the lower cost.

Other coated capillaries with a low or no EOF were also tested. The Guarant capillary (Alcor Bioseparations, Palo Alto, CA, USA) which has a hydrophilic and hydrolytically stable permanent wall coating with a very low EOF ($<0.3 \times 10^{-9}$ m$^2$/V/s) did not provide good data with the investigated configuration featuring indirect detection. The buffer tested was at pH 4.3 and did not include any ACN. Compared to LPA-coated capillaries, the noise was higher and LOD >1 mg/L EtG. No further work was executed to elucidate the reasons for this difference.

### 3.1.3 Validation of EtG assay with PP in the LPA-coated capillary

Based on the studies reported in Section 3.1.2, the EtG assay in an LPA coated capillary and using PP was validated. The BGE was made up fresh every day from standard solutions of nicotinic acid and EACA (100 mM each). Also PP of the blank sample, the five calibrators (1, 2, 4, 8, and 16 mg/L) and the three controls (1.5, 6, and 12 mg/L) were done daily. In some real samples, the slower migrating diastereomer of the I.S. was found to comigrate with unidentified matrix peaks. Thus, only the peak of the faster migrating diastereomer was used to standardize the results. Furthermore, the $1/x^2$ weighted linear regression provided best results and was used for data evaluation as was described previously for EtS [8]. Calibration revealed linear relationships. Over the 6 days of validation, the mean of the slopes of the calibration curves was 0.0656 (SD 0.0038). The mean $y$-intercept was calculated to be 0.0083 (SD 0.0086). Coefficients $r$ and $F$ were always larger than 0.999 and 690, respectively. Accuracy and precision data are summarized in Table 1. The LOQ was found to be 0.5 mg/L and the LOD was determined to be 0.25 mg/L (s/n ≈ 3).

The robustness of the CZE assay was tested by varying the different parameters of the BGE. Differences in the concentration of nicotinic acid did not change the general pattern of the electropherogram and only minor influences in the migration time intervals were observed. Changes of the amount of ACN in the BGE (8–12%) led to minor changes in the mobility of some analytes, mostly pronounced for the two peaks of the I.S. Nevertheless the separation was still efficient under these conditions. For a pH between 4.6 and 4.7 the analytes of interest migrated separately. However, at pH variations of more than 0.1 pH units. EtG and the I.S. are no longer separated from each other or from matrix peaks. Proper pH adjustment is crucial for this method.

**Table 1.** Validation data of the CZE assay for EtG in serum ($n$ = 6)

| Concentration (mg/L) | | 1.5 | 6 | 12 |
|---|---|---|---|---|
| Intraday | Accuracy (% of nominal value) | 102.4 | 130.2 | 100.8 |
| | Precision (% RSD) | 8.73 | 2.49 | 0.47 |
| Interday | Accuracy (% of nominal value) | 99.5 | 126.4 | 101.7 |
| | Precision (% RSD) | 9.96 | 2.00 | 3.66 |

### 3.2 EIA for EtG in serum

The recently introduced EIA kit reagents for the determination of urinary EtG were used. They include mouse monoclonal anti EtG antibodies, glucose-6-phosphate, nicotinamide adenine dinucleotide (NAD) and EtG labeled with glucose-6-phosphate dehydrogenase (G6PDH). Upon incubation with a sample, the unbound EtG tracer produces NADH which can be followed photometrically. For clinical and forensic purposes it is often more suitable to analyze EtG in serum than in urine (surveillance of sampling, no intentional alteration such as dilution of the sample, representation of the actual state, and hence the possibility to describe the kinetics of the metabolism). Attempts using plain serum instead of urine as sample failed as the obtained results were not reproducible. G6PDH, a cytosolic enzyme mainly found in erythrocytes, may be present in small amounts in serum and thereby influence the rate determining NAD to NADH reaction. Thus, the use of different sample pretreatment procedures was investigated. Employing SPE with Clean Screen EtG extraction columns and reconstitution of the dried extract in water (half of volume compared to the amount of serum applied to the extraction column) did not reveal better data. With UF and analysis of the ultrafiltrate, however, reproducible data were obtained (data not shown). G6PDH with a molecular mass of more than 50 kDa is believed to become completely retained with the 5 kDa membrane used for UF.

The use of ultrafiltered human blank serum as calibration matrix revealed acceptable calibration graphs. Analysis of blank sera, however, did not provide any values (print out result: < calc range), which indicated that the blank human serum used for calibration contained a small amount of EtG. Although interday and intraday precision data were acceptable (RSD <10% in both cases for an EtG spiking level of 0.23 mg/L; $n$ = 6), calibration with a matrix containing EtG was obviously not ideal for

 

**Table 2.** Validation data of the EIA for EtG in serum ($n = 6$)

| Concentration (mg/L) | | 0.23 | 0.39 |
|---|---|---|---|
| Intraday | Accuracy (% of nominal value) | 94.4 | 96.2 |
| | Precision (% RSD) | 4.40 | 4.07 |
| Interday | Accuracy (% of nominal value) | 93.5 | 97.4 |
| | Precision (% RSD) | 4.93 | 3.93 |

the determination of low EtG serum levels (*e.g.*, EtG concentrations <0.50 mg/L). Thus, the use of Krebs–Ringer solution containing 5% BSA as a substitute for human serum was investigated and found to be suitable for calibration. This solution does not contain any EtG and is similar to human serum in terms of its ionic composition and protein content. Calibration levels used were blank and samples fortified with 0.1, 0.5, 1.0, and 2.0 mg/L of EtG. Samples were ultrafiltered prior to analysis. Recovery was assessed with samples containing 1.5, 0.39, and 0.23 mg/L and were determined to be 97.1 ± 2.4, 100.0 ± 8.1, and 104.1 ± 7.1%, respectively ($n = 2$ for each level). Repetitive analysis of the same ultrafiltered sample revealed reproducible data. RSD values ($n = 6$) for 0.23 and 0.39 mg/L EtG levels were determined to be 6.94 and 3.58%, respectively. Intra- and interday accuracy and precision data for the two controls were determined to be good (Table 2). Furthermore, a good repeatability was also observed for the calibration data. For the calibrators containing 0.1, 0.5, 1.0, and 2.0 mg/L EtG, RSD values for the mean of duplicate determinations ($n = 6$) were 3.50, 1.02, 0.46, and 0.07%, respectively. Comparative values for the first samples were 9.66, 1.04, 1.37, and 1.79%, respectively. These data suggest that EtG levels as low as 0.1 mg/L can be accurately determined.

The upper reference level for EtG in serum was elucidated according to the guidelines provided in the protocol of the National Committee on Clinical Laboratory Standards (NCCLS) [37], the approved recommendations of the International Federation of Clinical Chemistry (IFCC) and the International Committee for Standardization in Haematology (ICSH) [38, 39, 40, 41] and using the validated assay conditions with ultrafiltered Krebs–Ringer solution containing 5% BSA as calibrators. A total of 114 blank sera were collected and stored at −20°C until analysis by EIA. The obtained data (mean, 0.0111 mg/L; median, 0.0064 mg/L; range, 0–0.0445 mg/L) are presented as a frequency diagram and a boxplot (Fig. 6). The Kolmogorov–Smirnov test indicated a nonparametric distribution of the data and no outlier was identified by the Grubb's method. Thus, all 114 data points were included for the establishment of the reference level. The 97.5th percentile (90% confidence interval) was determined to be 0.0434 mg/L (0.0402–0.0466 mg/L). Only one blank from the reference sample set, whose EtG content was found to be 0.0449 mg/L, resulted in an EtG level higher than 0.0434 mg/mL. These

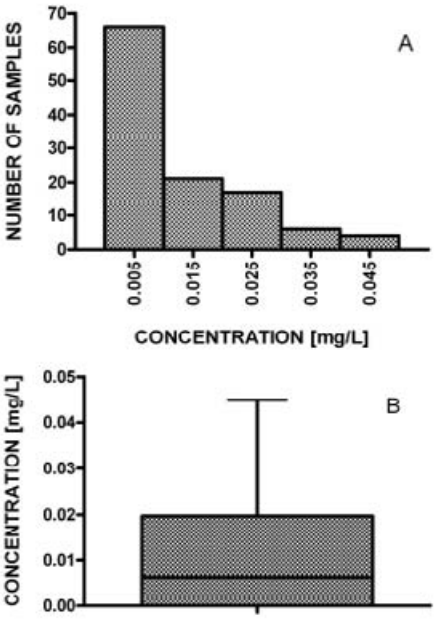


**Figure 6.** EIA data of 114 reference sera presented as (A) frequency diagram with a bin width of 0.01 mg/L and (B) boxplot with median, 25 and 75% percentiles, and highest value.

data suggest that an upper reference limit of 0.05 mg/L could be used. Using this limit, no positive result was obtained for the analysis of 13 sera that were from persons who did not drink alcoholic beverages at least 2 days prior to blood collection. On the other hand, all but one sample taken after ethanol consumption tested positive.

### 3.3 Determination of EtG in real samples by CZE and EIA

A total of 29 real samples were analyzed by CZE and EIA. In 13 blank samples (more than 2 days of ethanol abstinence prior to sampling) no EtG could be detected by CZE, whereas EIA provided data between 0 and 0.028 mg/L (mean, 0.007 mg/L EtG), values that are below the upper reference limit of 0.05 mg/L. In two samples, which were taken after ethanol consumption, no EtG was detected by CZE. EIA provided EtG levels of 0.030 and 0.204 mg/L. In seven other samples, which were taken after ethanol consumption, EtG could be detected by CZE, but not quantified. These samples resulted in a mean value of 0.281 mg/L EtG (range, 0.156–0.386 mg/L) by EIA. In three samples of this group, the sera stemmed from persons who ingested about 2 dL of white wine (~20 g ethanol). In these samples EtG concentrations between 0.156

 

and 0.204 mg/L were found by EIA. CZE could determine EtG in seven samples taken after ethanol ingestion and revealed EtG values between 0.517 and 4.722 mg/L. Corresponding EIA values ranged between 0.482 and 4.672 mg/L. The correlation with the values obtained by EIA resulted in a linear relationship with $r > 0.999$ ($y = 0.996x \pm 0.026$; where $x$ and $y$ refer to the values obtained by CZE and EIA, respectively). The electropherogram of the sample with the highest EtG level (4.72 mg/L) is shown as top graph in Fig. 4.

For values above 0.5 mg/L, the data of both methods correlate well which suggests that both assays are suitable for the determination of EtG in serum samples. The validated EIA method is a convenient assay for the fast screening of a large amount of samples. However, in order to avoid false-positive results such as those obtained with a metabolite of chloral hydrate [25], the confirmation of EtG-positive results should be confirmed with another method. Furthermore, for the analysis of EtG in urine, true false-positive results of EtG were demonstrated in urine after use of mouthwash [18] and contact with hand sanitizer [28, 42] containing ethanol. With the 13 blank serum samples mentioned above, EtG was not detected by CZE, and EIA provided very low EtG levels. Hence, there was no false-positive result. The data suggest that the cut-off level of 0.31 mg/L suggested by Zimmer *et al.* [21] should not be applied to the EIA method described. An upper reference value between 0.05 and 0.10 mg/L should be used instead which is comparable to that previously suggested by Høiseth *et al.* [1].

## 4 Concluding remarks

In addition to optimization efforts for the determination of EtG by CZE in coated capillaries, this paper describes two validated screening assays for the determination of EtG in human serum. CZE performed in a commercial 75 μm id LPA-coated fused-silica capillary with a BGE containing 10% ACN and PP prior to sample injection provides reproducible data down to mg/L EtG levels. The LOQ of the CZE assay is 0.5 mg/L, a sensitivity which was found to be suitable for the monitoring of ethanol consumption in a wide variety of samples. The EIA is based upon use of the commercially available DRI reagents. It is ten-fold more sensitive than the CZE assay and requires UF of the sample prior to analysis. An upper reference limit of 0.05 mg/L EtG is proposed for the analysis of EtG in serum by EIA. This limit was found to be suitable to recognize EtG in serum after consumption of a standard drink containing 12 g ethanol. Questionable screening data should be confirmed using another method, preferably LC-MS, which is reported to be able to recognize EtG levels down to the upper reference limit of the EIA assay [1, 2, 7, 19].

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*The authors declared no conflict of interest.*

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# Appendix III
# B. Jung, RICPTS

Appendix III depicts the following publication as it was printed by the journal (3 pages).

B. Jung
*Preuve de consommation d'alcool récente grâce à la détermination du glucuronide et du sulfate d'éthyle dans des fluides corporels*
Revue internationale de criminologie et de police technique et scientifique 62 (2009) 29

**Preuve de consommation d'alcool récente grâce à la détermination du glucuronide et du sulfate d'éthyle dans des fluides corporels**
par *Balthasar Jung*

La démonstration de consommation d'alcool chez un individu peut poursuivre plusieurs buts. En pratique clinique, afin de procéder à un diagnostic du syndrome de dépendance à l'alcool, le médecin a besoin d'indicateurs objectifs. Ces derniers peuvent être apportés par de nombreux marqueurs biologiques. Pour vérifier si une personne suspectée de consommation d'alcool dans une situation particulière a réellement bu ou non, c'est d'autres indices qu'il faut rechercher. Avant de choisir une méthode d'analyse, il est donc important de bien définir quel type d'information on souhaite obtenir. Des marqueurs spécifiques sont utilisés selon la question posée: les marqueurs de consommation d'alcool chronique informent seulement sur une exposition à l'alcool à long terme (CDT, VGM, GGT (7) etc.) alors que les marqueurs de consommation d'alcool récente sont à même de dévoiler une seule et unique consommation d'alcool (5-HTOL, EEAG (8) etc.). Le glucuronide et le sulfate d'éthyle (ethyl glu-

curonide, EtG; ethyl sulfate, EtS) sont des métabolites directs (non oxydatifs) de l'alcool et font partie des marqueurs de consommation d'alcool récents. Bien que moins de 0.1 % de l'alcool consommé soit transformé en EtG et EtS, ils peuvent être détectés dans des fluides corporels encore pendant des heures, voire des jours après que l'alcool ait été complètement éliminé du corps.

Le but de ce travail est de développer des méthodes analytiques pour déterminer la présence et la quantité de l'EtG et EtS dans le sérum et l'urine. Les deux analytes se présentant comme anions dans des échantillons biologiques aqueux, l'électrophorèse capillaire (CE) est un outil particulièrement bien adapté. La figure (figure 3) comportant un extrait de six électropherogrammes indique que, grâce à l'EtS, même une consommation modérée d'alcool peut encore être démontrée dans l'urine pendant plus d'une demi-journée après l'élimination complète de l'alcool (dans ce cas pendant au moins 18 h après la consommation). Ingéré en de telles quantités, l'alcool lui-même est estimé être détectable dans l'urine seulement pendant 3 à 5 h après la consommation.

En outre de problèmes analytiques, l'interprétation des résultats pose également des difficultés. EtG et EtS sont spécifiques à alcool. Chaque individu est porteur d'un peu d'alcool endogène. Des concentrations minimes des deux

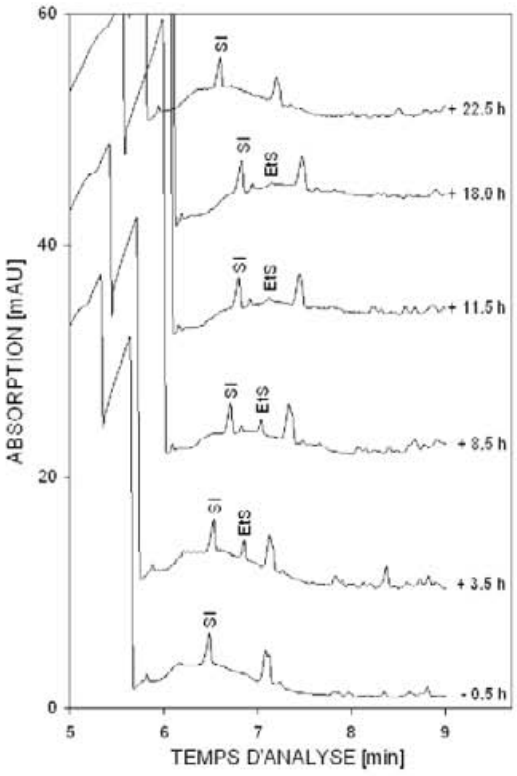


**Figure 3**
Extrait d'électropherogrammes obtenu de six urines consécutives qui proviennent d'un individu féminin qui a consommé 0.462 g éthanol par kg de poids du corps en 30 min. Les échantillons ont été collectés immédiatement avant jusqu'à 20.5 h après le début de la consommation d'alcool (du bas en haut, imprimé avec décalage axial des ordonnées pour permettre l'observation du développement temporel de la concentration d'EtS). Aucun EtS n'a été détecté dans les urines collectées avant l'ingestion d'alcool et 20.5 h plus tard. Les autres échantillons contiennent tous de l'EtS prouvant la consommation d'alcool préalable. SI: standard interne (Jung *et al.* 2008).

métabolites peuvent donc également apparaître dans le corps sans que l'individu n'ait consommé d'alcool. Par ailleurs, l'alcool peut être ingéré accidentellement en mangeant des desserts ou des fruits fermentés, en avalant des médicaments ou encore au travers de produits d'hygiène. Il est crucial de choisir soigneusement le seuil de discernement (cut-off) pour éviter ces „vrais faux positifs". Pour cela, une approche probabiliste qui se base sur la statistique Bayesienne semble à cet égard la plus appropriée aux besoins forensiques.

Un test immunochimique pour la détermination d'EtG dans l'urine a récemment été introduit sur le marché (DRI Ethyl Glucuronide Assay, Microgenics Corporation, Fremont, USA). Le défi sera d'adapter ce test à la matrice du sérum qui permet une meilleure surveillance lors de l'échantillonnage et qui fournit un instantané de l'état du corps. Une vision d'avenir serait qu'un grand nombre d'échantillons de personnes suspectées d'avoir consommé de l'alcool puissent être passées à un test de dépistage (screening). Contrairement à la pratique clinique, les résultats de ce test ne suffisent souvent pas lorsqu'ils sont effectués dans un contexte forensique. Une confirmation des résultats positifs est absolument nécessaire pour réduire le risque d'erreur. Cette vérification pourrait se faire en déterminant l'EtG et/ou EtS par CE. La possibilité de mesurer ces deux métabolites de l'alcool dans des fluides corporels et l'interprétation des résultats permettra de dévoiler une consommation d'alcool préalable, même lorsque l'alcool lui-même a été éliminé complètement du corps.

# Appendix IV
# C. Lanz et al., Chimia

Appendix IV depicts the following publication as it was printed by the journal (1 page).

C. Lanz, B. Jung, J. Caslavska, V. Deiss, W. Thormann
*Alcohol markers reveal relapse drinking episode*
Chimia 63 (2009) 522

doi:10.2533/chimia.2009.522

# Highlights of Analytical Chemistry in Switzerland

## Division of Analytical Chemistry

A Division of the Swiss Chemical Society

### Alcohol Markers Reveal Relapse Drinking Episode

Christian Lanz[a], Balthasar Jung[a], Jitka Caslavska[a], Veronika Deiss[b], and Wolfgang Thormann*[a]

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**Keywords:** Alcohol marker · Capillary electrophoresis · CDT · Ethyl sulfate

Alcohol abuse and alcohol dependence are major public health problems. Because of the known ability of alcohol abusers to hide their addiction and the importance of an early diagnosis of alcohol abuse, assays for the verification of alcohol intake are required. Direct ethanol metabolites (ethyl glucuronide and ethyl sulfate) and changes in the isoform pattern of transferrin (Tf) present in serum are biomarkers for recent and chronic excessive alcohol intake, respectively. The two ethanol metabolites are non-volatile, water soluble and stable. They can be detected in serum and urine hours after complete elimination of the ingested alcohol. The microheterogeneity of Tf isoforms changes upon chronic consumption of large amounts of ethanol: the relative amount of desialylated isoforms becomes higher compared to the pattern of healthy teetotalers and social drinkers. Carbohydrate-deficient transferrin (CDT) encompasses Tf isoforms with zero (asialo-Tf) and two (disialo-Tf) sialic acid residues in the carbohydrate side chains of the molecule.

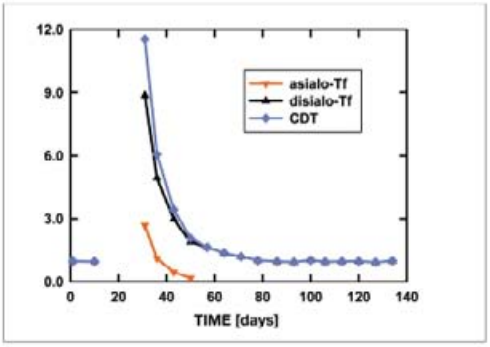
Longitudinal monitoring of individuals with a history of alcohol abuse is important for the early detection of relapse drinking after abstinence and in legal cases. Alcohol, alcohol metabolites, and CDT levels should therefore be determined on a regular basis over an extended period of time. In sera of a patient collected over a period of more than four months, alcohol could not be detected. The same was true for ethyl sulfate in the first sample collected after a three-week break. Analysis of CDT, however, revealed about a twelve-fold increase of CDT after the three-week interval without sample collection. CDT levels before the break and again in the samples collected later than five weeks after the break were normal. Sera were analyzed by a capillary electrophoresis assay for which the upper reference value was determined to be 1.70%. Asialo-Tf could be unambiguously detected in the first four samples after the break. The presence of asialo-Tf and the high amounts of disialo-Tf and thus CDT monitored and the characteristic decay of these levels thereafter unambiguously revealed an episode of relapse drinking of this patient. **This example demonstrates that the capillary electrophoresis assay applied to samples from patients with suspected chronic excessive alcohol consumption provides a solid basis to identify alcohol abusers.**

Received: May 15, 2009

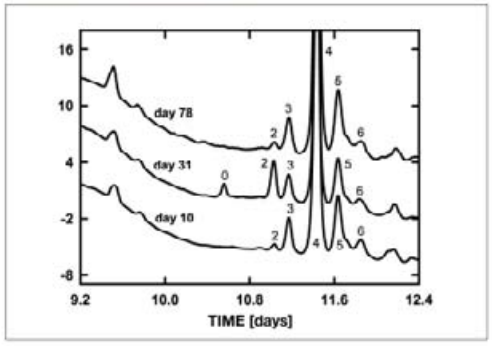
**References**

C. Lanz, M. Kuhn, V. Deiss, W. Thormann, *Electrophoresis* **2004**, *25*, 2309.
B. Jung, J. Caslavska, W. Thormann, *J. Chromatogr. A* **2008**, *1206*, 26.

How much is too much?




CDT, disialo-Tf and asialo-Tf serum levels determined by capillary electrophoresis in samples of a patient collected over a 132-day period.




Electropherograms showing the transferrin patterns of three sera. 0, asialo-Tf; 2, disialo-Tf; 3, trisialo-Tf; 4, tetrasialo-Tf; 5, pentasialo-Tf; 6, hexasialo-Tf. Data at days 10 and 78 show normal transferrin patterns whereas that at day 31 is typical for an alcohol abuser.

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# Appendix V
# J. Caslavska et al., Electrophoresis

Appendix V depicts the following manuscript as its acceptance for publication was confirmed by the journal (13 pages).

J. Caslavska, B. Jung, W. Thormann
*Analysis of ethyl glucuronide and ethyl sulfate in serum and urine by CE-ESI-MSn*
Electrophoresis, accepted

**Short communication**

# Confirmation analysis of ethyl glucuronide and ethyl sulfate in human serum and urine by CZE-ESI-MS$^n$ after intake of alcoholic beverages

Jitka Caslavska, Balthasar Jung, Wolfgang Thormann*

Department of Clinical Pharmacology and Visceral Research, University of Bern, Bern, Switzerland.

**Short title:** Analysis of ethyl glucuronide and ethyl sulfate by CZE-MS$^n$

**Keywords:** capillary electrophoresis, mass spectrometry, alcohol marker

**Abbreviations:** CDT, carbohydrate deficient transferrin; CZE, capillary zone electrophoresis; EOF, electroosmotic flow; ESI, electrospray ionization; EtG, ethyl glucuronide; EtS, ethyl sulfate; MS, mass spectrometry; SPE, solid phase extraction; TOF, time-of-flight

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**Abstract**

Capillary zone electrophoresis (CZE) coupled to sheath liquid based electrospray ionization (ESI) and multiple-stage ion trap mass spectrometry ($MS^n$) was used for confirmation analysis of ethyl glucuronide (EtG) and ethyl sulfate (EtS) in human serum and urine collected after intake of alcoholic beverages. Electrophoretic separations were performed in uncoated fused-silica capillaries using a pH 9.5 ammonium acetate background electrolyte and normal polarity. MS detection of EtG and EtS occurred after negative ionization using a spray liquid containing 0.5 % (v/v) ammonia in isopropanol/water (60 : 40 %, v/v). CZE-MS and CZE-$MS^2$ results obtained after injection of solid phase extracts for EtG and EtS and of diluted urine confirmed the presence of EtG and EtS in samples whose levels were previously determined by CZE with indirect UV detection. Detection limits of each compound were estimated to be around 2.0 (injection of diluted urine) and 0.2 µg/mL (extracts).

The exploration of markers linked to alcohol consumption and abuse has attracted a great deal of attention during the past three decades. Alcohol intake leads to the formation of direct metabolites, such as ethyl glucuronide (EtG, for chemical structure see Figure 1) and ethyl sulfate (EtS, Figure 1), and ethanol induced changes of the levels of endogenous compounds, including liver enzymes, carbohydrate deficient transferrin (CDT), the urinary ratio of 5-hydroxytryptophol and 5-hyroxyindolacetic acid and cytokines [1-5]. EtG is formed by conjugation of ethanol with activated glucuronic acid in the presence of membrane-bound mitochondrial uridine diphosphate glucuronyltransferase, whereas EtS is formed via sulfonation of ethanol in presence of sulfotransferases. Less than 0.1 % of the ingested ethanol is excreted in urine as EtG or EtS. For an ethanol intake during a short time period, it was reported that the two direct metabolites reach their maximum concentrations in serum about 3 h after the beginning of alcohol consumption. They can be detected for an extended time period in serum (in the range of hours) and in urine (in the range of hours to days) after complete elimination of ethanol and thus represent marker substances for recent alcohol consumption. They cover a clinically and forensically important time window between short term markers, such as ethanol itself, and long term markers, such as CDT [1,2, 6-12].

EtG and EtS are non-volatile, water soluble and stable. For their determination in biological samples, different analytical methods have been developed, including LC–MS/MS with negative electrospray ionization (ESI) [8-13] and capillary zone electrophoresis (CZE) with indirect UV detection [14-19]. EtG in serum and urine has also been determined by GC-MS [6]. Furthermore, EtG in urine [20] and serum [19] can be monitored with an immunoassay. The CZE methods are based on the use of fused-silica capillaries whose inner walls were permanently coated with linear polyacrylamide [16-18,19] or dynamically coated with a polycation [14,15,19]. They were shown to be suitable to detect the two ethanol metabolites in urines of volunteers who ingested a known amount of alcohol and in urines of patients with an unknown history of alcohol consumption. For confirmation analysis, MS detection rather than indirect UV absorption is required. CZE hyphenated with negative ESI to ion trap $MS^n$ has previously been used for the monitoring of selected drugs and/or their metabolites in diluted or extracted urines of patients or volunteers, including paracetamol glucuronide and paracetamol sulfate [21], furosemide [22], γ-hydoxybutyric acid [23] and the 3O-glucuronide of lorazepam [24], and of the alcohol marker phosphatidylethanol in extracts of whole blood [25]. Furthermore, CZE hyphenated with negative ESI to time-of-flight (TOF) MS has been applied to the selective determination of urinary sulfates, sulfonates and phosphates using an

acidic pH buffer [26]. In the latter work, CZE-ESI-TOF was employed to measure EtS in spiked urine and SPE extracted fortified urine. No CZE-$MS^n$ investigations dealing with the analysis of EtS and EtG in serum and urine collected after ethanol ingestion or in patient samples have been reported thus far.

In this work we have focused on analysis of EtG and EtS in serum and urine of samples collected after ethanol ingestion. A Prince C650 capillary electrophoresis sampler (Prince Technologies, Emmen, NL) connected to a commercial coaxial sheath liquid ESI (Finnigan, San Jose, CA, USA) and a LCQ ion trap MS (Finnigan) with a 75 µm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 80 cm length was used. The sheath gas ($N_2$) pressure was 30 arbitrary units, the sheath liquid comprised 60 % (v/v) isopropanol with 0.5 % (v/v) of $NH_3$ and was infused with a flow rate of 3 µL/min, the temperature of the MS capillary was 200 $^0$C and the spray potential was -3.7 kV. Full scan mass spectra were collected in the m/z range of 60 to 300 Th using automated gain control. Double stage ($MS^2$) experiments were performed targeting selectively the m/z ratios of the compounds of interest with an isolation width of 2 Th and a collision energy of 35 %. XCalibur 1.0 software (Finnigan) was used for instrumental control, data acquisition and data evaluation. Urinary EtG levels were also determined with an enzyme immunoassay using the DRI Ethyl Glucuronide Assay reagents (Microgenics, Fremont, CA, USA) on a Mira Plus analyzer (ABX Diagnostics, Diatools, Dietikon, Switzerland).

With the CZE-MS setup, separation of EtS (pKa < -3.0 [14], mobility 4.86 $10^{-8}$ $m^2$/Vs [14], molecular mass 126.13 g/mol) and EtG (pKa 3.21 [16], mobility 2.35 $10^{-8}$ $m^2$/Vs [16], molecular mass 222.3 g/mol) was effected at 25 kV with a buffer composed of 20 mM ammonium acetate adjusted to pH 9.5 with ammonia. Sample injection occurred via application of 70 mbar constant pressure during 12 s and the sheath gas flow was interrupted during that time interval. The configuration used is characterized with a strong electroosmotic flow towards the cathode which allows the two anions of interest to be monitored at the cathodic capillary end. In order to allow the analytes to reach the detector within a reasonable time, an additional cathodic buffer flow generated via application of constant pressure of 5 mbar at the anodic side was used. EtG and EtS of a standard sample were thereby detected after about 6.6 min and 9.6 min, respectively (Figure 1), and were characterized by their MS spectra which provided the $[M-H]^-$ ions with m/z 221 (EtG) and 125 (EtS) together with the isotopic patterns resulting from $^{13}$C (relative abundance 1.08 %, both compounds), $^{18}$O (0.2 %, both compounds), $^{34}$S (4.4 %, EtS only) and $^{33}$S (0.8 %, EtS only). Fragmentation of the

$[M-H]^-$ ions resulted in well known $MS^2$ spectra. Major fragments observed for EtG with m/z 203 ($-H_2O$), 159, 129, 113, 85 and 75 are the same as those previously reported with $LC/MS^2$ [6,24]. The same is true for the m/z 125 to 97 ($HSO_4^-$) transition observed in the case of EtS [7,8]. A small amount of the $HSO_4^-$ fragment is formed during ionization and thus found in the MS data. Reproducibility was assessed via analysis of the same sample at 5 different days. Typical RSD values of EtG and EtS detection times were 3.38 and 4.94 %, respectively. Corresponding values for peak heights were 21.05 and 64.41 %, respectively. The latter data indicate that an internal standard comigrating with the compound of interest (deuterated analog) should be used for quantitation.

The data presented in Figure 2 were obtained with a serum of an individual who ingested more than 1.5 g of ethanol per kg body mass during a 12 h period before sample collection. EtG and EtS levels in this serum were determined to be 4.37 µg/mL and 1.86 µg/mL, respectively, using the CE assays with indirect UV detection reported in Refs. 19 and 15, respectively. Due to the large differences in physicochemical parameters of the two compounds, two different solid phase extraction (SPE) methods were used. EtG was extracted with Clean Screen ETG SPE columns (3 mL column with proprietary carbon packing material from UCT, Bristol, PA, USA) and the conditions recommended by its manufacturer. A Strata X-AW SPE column (weak anion exchange resin, Phenomenex, Torrance, CA, USA) was employed to recover EtS as reported elsewhere [15]. The compounds were extracted from 0.5 mL serum and reconstituted in 0.125 mL water prior to analysis under the conditions described above for the standards. CZE-MS analysis of the extracts confirmed the presence of both direct ethanol markers (Figure 2). MS and $MS^2$ spectra of the EtG and EtS peaks were found to correspond well with those of the standards (compare spectra of Figures 1 and 2). Unambiguous confirmation of EtG and EtS in sera containing about 0.5 µg/mL of these compounds was also possible and analysis of sera from individuals who did not ingest any alcohol during at least 48 h prior to blood collection did not reveal any peaks for the two compounds (data not shown). Thus, the data reveal that the presence of the two compounds of interest can easily be confirmed for µg/mL serum levels.

The data presented in Figures 3 and 4 were obtained for a urine sample of an individual who ingested about 1.43 g of ethanol per kg body mass during an 11 h period before urine collection. EtG and EtS levels in this urine were determined to be 146.7 µg/mL (immunoassay) and 32.2 µg/mL (CE assay of [15]), respectively. CZE-MS analysis of 10-fold

diluted urine (sample with a conductivity of about 3 mS/cm, see [15]) confirmed the presence of both direct ethanol markers (Figure 3). MS and $MS^2$ spectra of the EtG and EtS peaks were found to correspond well with those of the standards (compare spectra of Figures 1 and 3). Interday reproducibility was assessed via analysis of the same sample at different days. Data comparable to those mentioned above for the standards were obtained. For such a confirmation analysis, urinary analyte concentrations larger than about 2 µg/mL are required. Thus, in order to have a lower detection limit, the use of SPE was evaluated. EtG and EtS were separately extracted with the same two columns as described for serum. 1 mL aliquots of urine were applied and the dried residues were reconstituted in 1 mL water. The two SPE methods allowed unambiguous recovery of the two compounds (Figure 4). Compared to analysis of diluted urine, cleaner electropherograms and MS data were obtained (compare data of Figures 3 and 4). It is worth mentioning that a small response of EtS was also detected in the Clean Screen extract, whereas no EtG was found in the Strata X-AW extract. Using these SPE procedures, detection limits could be enhanced by about an order of magnitude. No further efforts were undertaken to quantitate urinary EtG and EtS via use of internal standards. Analysis of blank urines did not reveal any peaks that could be allocated to the two compounds of interest (data not shown).

To our knowledge this is the first attempt to use CZE-MS for confirmation analysis of EtG and EtS in serum and urine collected after alcohol intake. Under the investigated conditions the sensitivity achieved was found to be sufficient for confirmation of the presence of these compounds in serum and urine down to about 200 ng/mL. This is somewhat less sensitive compared to established LC-MS/MS approaches [9-13] and to the recent CZE-ESI-TOF investigation for ETS in spiked urine [26]. Lower detection limits for EtG and EtS would certainly be beneficial for use in clinical and forensic settings. Nano-electrospray ionization is reported to provide better spray stability, smaller signal suppression effects and thus lower detection limits compared to the commercial sheath liquid based interface employed in our laboratory [28-31]. More work is required to assess the use of such instrumental setups, the impact of all involved parameters such that optimized CE-MS conditions are obtained for analysis of EtG and EtS in body fluids, and quantitation of the compounds of interest. The announcement of novel and robust commercial sheathless ESI interfaces from the key manufacturers of CE equipment will lead to improved setups and open the possibility for a widespread adoption of CZE-MS in clinical and forensic laboratories.

**Acknowledgements**

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**Figure 1:** Mass traces for EtG and EtS together with the chemical structures (on the left) and extracted MS spectra (upper graphs on the right) and $MS^2$ spectra (lower graphs on the right) obtained with a standard containing 17.3 µg/mL EtG and 15.4 µg/mL EtS dissolved in 10-fold diluted running buffer. Full scan mass spectra were collected in the m/z range of 60 to 300 Th using automated gain control. $MS^2$ data were collected with an isolation width of 2 Th and a collision energy of 35 %.

**Figure 2:** Mass traces for EtG and EtS together with extracted MS and $MS^2$ spectra of the two compounds obtained after SPE of a serum sample using Clean Screen ETG (for extraction of EtG, top graphs) and Strata X-AW (for extraction of EtS, bottom graphs) SPE columns. Other experimental conditions as for Figure 1.

**Figure 3:** Mass traces for EtG and EtS together with extracted MS and $MS^2$ spectra of the two compounds obtained for analysis of a 10-fold diluted urine of an individual who ingested about 1.43 g of ethanol per kg body mass during an 11 h period prior to urine collection. Experimental conditions as for Figure 1.

**Figure 4:** Mass traces for EtG and EtS together with extracted MS and $MS^2$ spectra of the two compounds obtained after SPE of the same urine as for Figure 3 using Clean Screen ETG (for extraction of EtG, top graphs) and Strata X-AW (for extraction of EtS, bottom graphs) SPE columns. Other experimental conditions as for Figure 1.

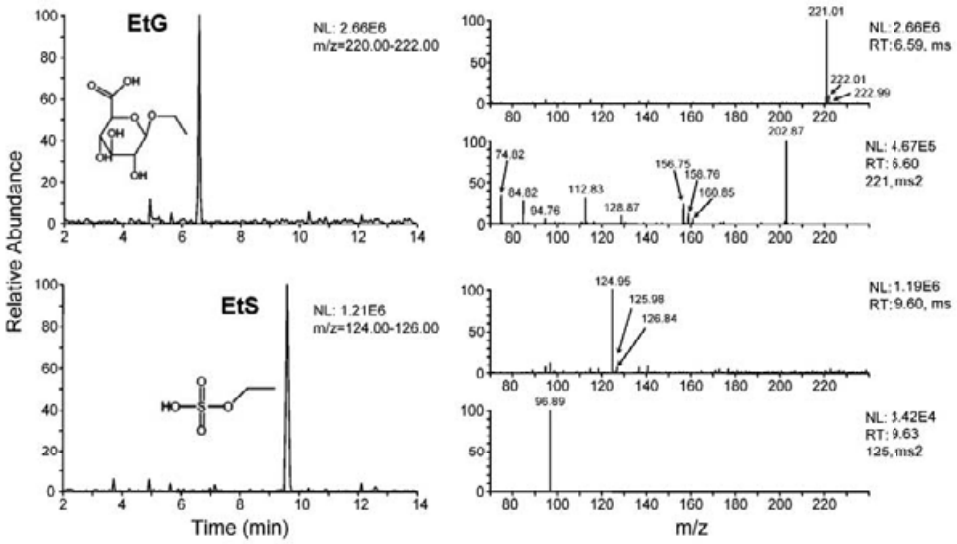
EtG
NL: 2.66E6
m/z=220.00-222.00
EtS
NL: 1.21E6
m/z=124.00-126.00
Relative Abundance
Time (min)
NL: 2.66E6
RT:6.59, ms
NL: 4.67E5
RT: 6.60
221, ms2
NL:1.19E6
RT:9.60, ms
NL: 3.42E4
RT: 9.63
125, ms2
m/z

Figure 1

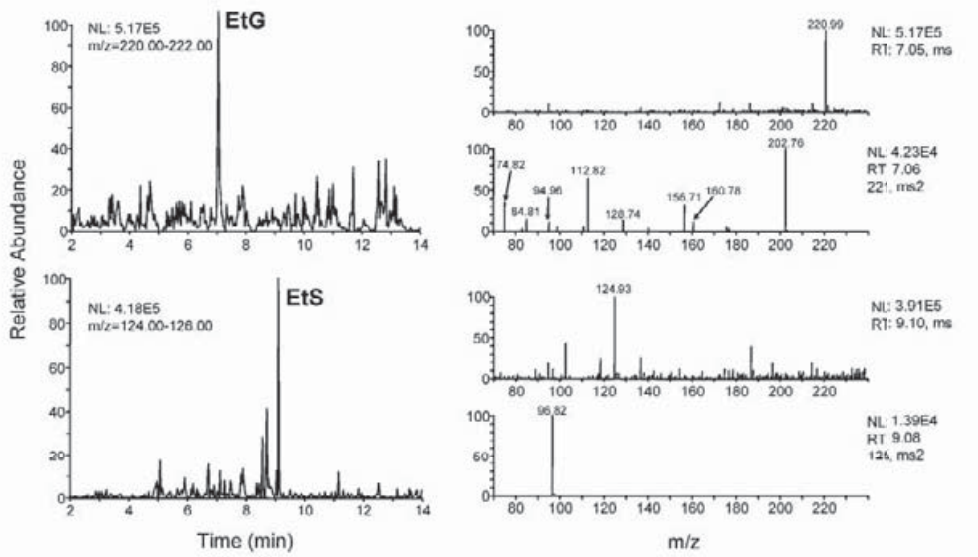
EtG
NL: 5.17E5
m/z=220.00-222.00
EtS
NL: 4.18E5
m/z=124.00-126.00
Relative Abundance
Time (min)
220.99
NL: 5.17E5
RT: 7.05, ms
202.76
74.82
112.82
94.96
84.81
128.74
156.71
160.78
NL: 4.23E4
RT 7.06
221, ms2
124.93
NL: 3.91E5
RT: 9.10, ms
96.82
NL: 1.39E4
RT 9.08
124, ms2
m/z

Figure 2

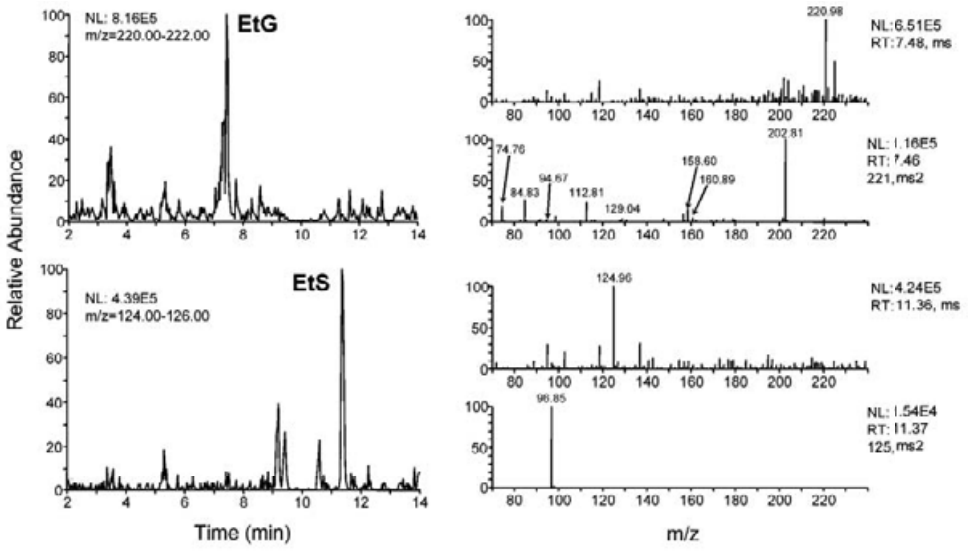
NL: 8.16E5
m/z=220.00-222.00
EtG
NL: 4.39E5
m/z=124.00-126.00
EtS
Relative Abundance
Time (min)
220.98
NL:6.51E5
RT:7.48, ms
202.81
74.76
84.83
94.67
112.81
129.04
158.60
160.89
NL: 1.16E5
RT: 7.46
221,ms2
124.96
NL:4.24E5
RT:11.36, ms
96.85
NL: 1.54E4
RT: 11.37
125,ms2
m/z

Figure 3

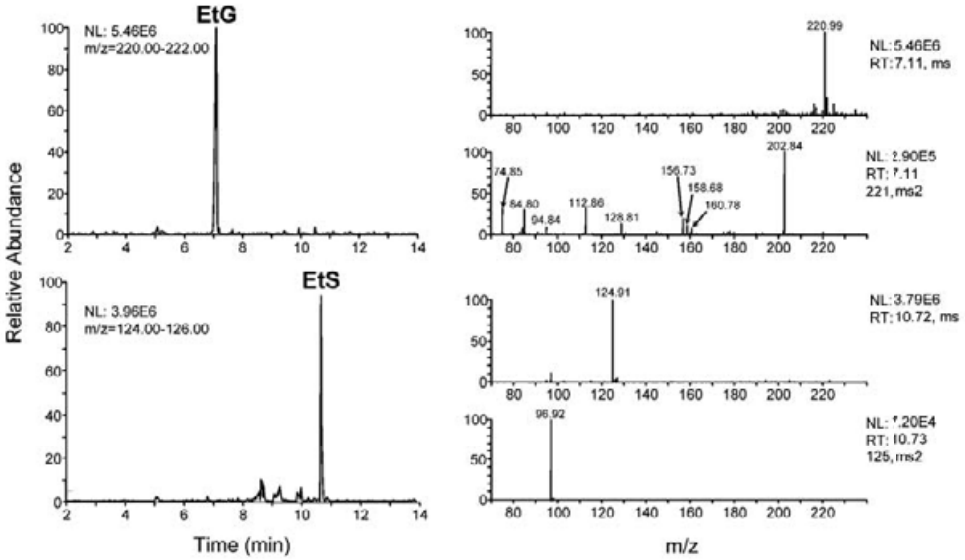
EtG
NL: 5.46E6
m/z=220.00-222.00
EtS
NL: 3.96E6
m/z=124.00-126.00
Relative Abundance
Time (min)
220.99
NL:5.46E6
RT:7.11, ms
202.84
74.85
64.80
94.84
112.86
128.81
156.73
158.68
160.78
124.91
NL:3.79E6
RT:10.72, ms
96.92
m/z

Figure 4

# Appendix VI
# Reference Level Study Documents

For the elucidation of the upper reference limit of EtG in urine and serum, samples were collected strictly following the protocol.

Appendix VI depicts the study protocol (3 pages) followed by the questionnaire to fill by the volunteers participating in the study (8 pages).

# Studienprotokoll

## Ermittlung des Referenzbereichs für Ethylglukuronid (EtG) an gesunden Personen mit einem Immunoassay

### Hintergrund

EtG ist ein direktes Stoffwechselprodukt von Alkohol, das durch die Konjugation mit Glukuronsäure entsteht. Weniger als 0.1 % des konsumierten Alkohols wird auf diesem Weg metabolisiert und später im Urin ausgeschieden [1, 2]. EtG kann während Stunden bis Tagen nach der vollständigen Elimination von Alkohol in Körperflüssigkeiten nachgewiesen werden [3, 4]. Dies macht EtG zu einem potenten Marker für kürzlichen Alkoholkonsum, der auch einen einmaligen Alkoholkonsum nachweisen kann.

Die Bestimmung von EtG wird in Ergänzung zur Anamnese und eventuell anderen Laboruntersuchungen (z.B. CDT) als Hilfsmittel der klinischen Chemie zur Identifizierung bzw. zum Ausschluss eines vorherigen Alkoholkonsums verwendet. Weiter dient die Bestimmung von EtG der frühzeitigen Erkennung eines Rückfalls von Patienten, die unter einem Alkoholproblem leiden.

### Ziel der Bestimmung des Referenzbereiches von EtG an gesunden, freiwilligen Personen

EtG kann in Serum und Urin mittels Kapillarelektrophorese und einem kommerziellen Immunoassay bestimmt werden. Die Bestimmungsmethoden sollen nun in die Routineanalytik der Untersuchungsabteilung des Institutes für Klinische Pharmakologie und Viszerale Forschung der Universität Bern eingeführt werden. Um die Interpretation der Ergebnisse von Patientenproben zu ermöglichen, muss der Referenzbereich bekannt sein, also die Werte des interessierenden Analyten (EtG) bei gesunden Menschen, die in den letzten Tagen keinen Alkohol konsumiert haben. Die Resultate von Patienten werden dann mit dem Referenzbereich verglichen.

Jeder Mensch produziert endogen Alkohol. Weiter kann Alkohol in kleinen Dosen unbewusst z.B. durch Fruchtsäfte, Hygieneprodukte oder Medikamente aufgenommen werden. Aber nur das durch den wissentlichen Konsum von alkoholischen Getränken gebildete EtG soll als positives Testresultat interpretiert werden.

Weiter soll abgeklärt werden, ob es notwendig ist, die Analysen-Resultate für EtG hinsichtlich der Konzentration des Urins anzupassen. Dazu wird die Kreatinin-Konzentration im Urin gemessen. Die Kreatininausscheidung im Urin ist beim gesunden Menschen relativ konstant, so dass dem Verdünnungsfaktor des Urins ein objektiver Wert zugeordnet werden kann.

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**Studiendesign**

Es wird keine bestimmte Auswahl der Studienteilnehmer angestrebt. Die Teilnehmer werden nicht behandelt und eine Kontrollgruppe wird nicht benötigt, da das Ziel der Studie ausschliesslich die Bestimmung des Referentwertes bei Alkoholabstinenz ist. Die Studie wird anonymisiert, indem jedem Teilnehmer eine Nummer zugeordnet wird und nur der Studienarzt im Besitz der Namen- und Nummernliste ist. Sollten mit den durchgeführten Analysenmethoden Auffälligkeiten festgestellt werden, kann der Studienarzt jederzeit die Teilnehmerliste entblinden und den Teilnehmer allenfalls kontaktieren und informieren. Sämtliche für diese Untersuchung erhobenen Daten werden streng vertraulich behandelt und nur zu Studienzwecken verwendet.

**Zu erhebende Messwerte**

Die entnommenen Körperflüssigkeiten werden ausschliesslich zur Bestimmung von Alkoholmarkern und der Konzentration des Urins verwendet. Das EtG wird in den biologischen Proben mit einem kommerziellen Immunoassay (DRI Ethyl Glucuronide Assay, Microgenics Corporation) und / oder einer kapillarelektrophoretischen Methode gemessen. Die Konzentration des Urins wird anhand seiner Kreatinin-Konzentration immunochemisch oder anhand einer der Messung seiner Leitfähigkeit bestimmt.

**Statistische Auswertemethoden**

Es wird angenommen, dass die Werte für EtG in alkoholabstinenten Personen nicht normalverteilt sind. Falls sich aufgrund der vorliegenden Studie zeigen sollte, dass es sich entgegen der Annahme um parametrische Daten handelt, würde die Statistik noch präziser ausfallen als geplant und dies wäre vorteilhaft für die Bestimmung des Referenzbereichs. Um eine statistisch relevante Aussage betreffend den Referenzbereich von EtG bei Personen, die keinen Alkohol getrunken haben, machen zu können, werden mindestens 120 Proben benötigt [5]. Es wird somit angestrebt, jeweils 120 Urin- und Serumproben zu erhalten. 60 Proben werden benötigt, um eventuelle Unterschiede in Untergruppen aufzudecken (Mann / Frau; Vitamine / keine Vitamine; unwissentliche Alkoholaufnahme / totale Alkoholabstinenz). Im Rahmen der vorliegenden Studie wird aber nur der Referenzbereich der totalen alkoholabstinenten Population definiert. Weitere Studien wären allenfalls nötig, um die Referenzbereiche von Untergruppen zu bestimmen.

Ausreisser werden nach der „D/R > 1/3"-Regel identifiziert und von der Bestimmung des Referenzbereichs ausgeschlossen [6]. Es wird untersucht, ob allfällige falsch-positive Resultate analytische Gründe haben, oder ob der Teilnehmer tatsächlich im Körper EtG gebildet hat.

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**Ein- und Ausschlusskriterien von Patienten**

Die erwachsenen Teilnehmer müssen vertrauenswürdig sein, so dass ein wissentlicher Alkoholkonsum in den letzten 72 Stunden vor der Probenentnahme ausgeschlossen werden kann. Ausschlusskriterien sind vom Studienarzt vermuteter, kürzlicher Alkoholkonsum des Teilnehmers, oder wenn der Teilnehmer selbst angibt, in den letzten 72 Stunden vor der Probenentnahme Alkohol getrunken zu haben.

Blut wird keinen Frauen während der Schwangerschaft oder der Stillzeit entnommen. Von der Abgabe von Urin ist diese Gruppe jedoch nicht ausgeschlossen. Falls sich eine Blutentnahme als problematisch erweist, wird die Entnahme sofort abgebrochen und auf die Serumprobe verzichtet

Pro Person sind zwei Teilnahmen erlaubt. Die zweite Abgabe darf aber frühestens eine Woche nach der ersten erfolgen.

**Risiken**

Die Blutentnahme erfordert die oberflächliche Punktion einer Vene am Arm oder an der Hand. Dies kann zu einem Bluterguss oder in seltenen Fällen zu einer oberflächlichen Venenentzündung führen. Bei der uriniert der Teilnehmer in einen speziellen Becher, welchen er vom Studienverantwortlichen erhält. Die selbständige Entnahme der Urinprobe ist gesundheitlich unbedenklich.

**Visitenplanung**

Die Teilnehmer müssen nur einmal erscheinen, damit das Ausfüllen der Einverständniserklärung und des Fragebogens sowie die Probenentnahme erfolgen können.

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# Information für Versuchsteilnehmer

## Ermittlung des Referenzbereichs für Ethylglukuronid (EtG) an gesunden Personen mit einem Immunoassay

Teilnehmernummer: _____ *(Wird vom Studienverantwortlichen vor Ort zugeteilt)*

Name: _____ Vorname: _____

Telefonnummer: _____

Gesucht werden freiwillige Personen zur Gewinnung von Serum und Urin zur Ermittlung des Referenzbereiches von EtG.

Benötigt wird ein Serumröhrchen (9 ml) venösen Bluts und Urin (10 mL).

**Hintergrund**

EtG ist ein direktes Stoffwechselprodukt von Alkohol, das durch die Konjugation von Alkohol und Glukuronsäure entsteht. Weniger als 0.1 % des konsumierten Alkohols wird auf diesem Weg metabolisiert und später im Urin ausgeschieden. EtG kann während Stunden bis Tagen nach der vollständigen Elimination von Alkohol in Körperflüssigkeiten nachgewiesen werden. Dies macht EtG zu einem wichtigen Marker für kürzlichen Alkoholkonsum, der auch einen einmaligen Alkoholkonsum nachweisen kann.

Die Bestimmung von EtG wird in Ergänzung zur Anamnese und eventuell anderen Laboruntersuchungen (z.B. CDT) als Hilfsmittel der klinischen Chemie zur Identifizierung bzw. zum Ausschluss eines vorherigen Alkoholkonsums verwendet. Weiter dient die Bestimmung von EtG der frühzeitigen Erkennung eines Rückfalls von Patienten, die unter einem Alkoholproblem leiden.

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**Ziel der Bestimmung des Referenzbereiches von EtG an gesunden, freiwilligen Personen**

EtG kann in Serum und Urin mittels Kapillarelektrophorese und einem kommerziellen Immunoassay bestimmt werden.
Die Bestimmungsmethoden sollen nun in die Routineanalytik der Untersuchungsabteilung (Labor 5) des Institutes für Klinische Pharmakologie und Viszerale Forschung der Universität Bern eingeführt werden. Um die Interpretation der Ergebnisse von Patientenproben zu ermöglichen, muss der Referenzbereich bekannt sein, also die Werte des interessierenden Analyten (EtG) bei gesunden Menschen, die in den letzten Tagen keinen Alkohol konsumiert haben. Jeder Mensch produziert endogen (in seinem eigenen Körper) Alkohol. Weiter kann Alkohol in kleinen Dosen unbewusst z.B. durch Fruchtsäfte, Hygieneprodukte oder Medikamente aufgenommen werden. Aber nur das durch den wissentlichen Konsum von alkoholischen Getränken gebildete EtG soll als positives Testresultat interpretiert werden.

Weiter soll abgeklärt werden, ob es notwendig ist, die Analysen-Resultate für EtG hinsichtlich der Konzentration des Urins anzupassen. Dazu wird die Kreatinin-Konzentration im Urin gemessen. Die Kreatininausscheidung im Urin ist beim gesunden Menschen relativ konstant, so dass dem Verdünnungsfaktor des Urins ein objektiver Wert zugeordnet werden kann.

Die Resultate von Patienten werden später mit dem Referenzbereich verglichen. Mit Ihrer Hilfe wird es möglich sein, diese Analysenmethode in die Routineanalytik einzuführen.

**Vertraulichkeit**

Sämtliche für diese Untersuchung erhobenen Daten werden streng vertraulich behandelt und nur zu Studienzwecken verwendet. Die Daten werden anonymisiert und sind nur Fachleuten zur wissenschaftlichen Auswertung zugänglich.

**Teilnahme an der Studie zur Bestimmung des Referenzbereiches von EtG**

Die Teilnahme an dieser Studie ist freiwillig. An der Bestimmung des Referenzbereichs von EtG können gesunde Männer und Frauen teilnehmen. Pro Person sind zwei Teilnahmen erlaubt. Die zweite Probenabgabe darf aber frühestens eine Woche nach der ersten erfolgen.

**Untersuchungsablauf**

1. Besprechung mit dem Studienarzt und Ausfüllen der Einverständniserklärung und des Fragekatalogs
2. Blutentnahme
3. Abgabe der Urinprobe an Studienarzt

Die Blutentnahme (1 Serumröhrchen venöses Blut, 9 ml) erfolgt durch ärztliches, medizinisches oder pflegerisches Personal. Der Urin wird vom Teilnehmer selbst in einem Urinröhrchen gesammelt, verschlossen und zur Beschriftung dem Studienverantwortlichen übergeben.

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Universität Bern

5. Februar 2009

Die Testperson soll mindestens seit 72 Stunden vor der Probenentnahme keinen Alkohol konsumiert haben. Der Zeitbedarf für die Blutentnahme und die Urinprobe beträgt rund 10 Minuten. Die gespendeten Körperflüssigkeiten werden ausschliesslich zur Bestimmung von Alkoholmarkern und der Konzentration des Urins verwendet. Es ist für die Teilnehmer auch möglich, nur Blut oder nur Urin zu spenden.

Personen, die bereit sind, an der Studie teilzunehmen, wenden sich zur Blut- und/oder Urinabgabe bitte an folgende Personen vom Institut für Klinische Pharmakologie und Viszerale Forschung der Universität Bern, Murtenstrasse 35, 3010 Bern, Stock F

- Dr. med. G. Stirnimann, Labor 3 (F818a), 031 632 87 27, guido.stirnimann@ikp.unibe.ch

  oder

- Jeannine Joneli, Labor 5 (F821), 031 632 35 98, jeannine.joneli@ikp.unibe.ch

  oder

- Balthasar Jung, Labor 4 (F820), 031 632 35 94, balthasar.jung@ikp.unibe.ch

Ohne Voranmeldung können Probenentnahmen am Mittwoch- und Freitagnachmittag zwischen $13^{15}$ und $16^{30}$ Uhr erfolgen. Es können auch zu anderen Zeiten Proben entnommen werden. Dazu wird um eine Absprache mit einer der drei obenstehenden Personen gebeten.

**Risiken**

Die Blutentnahme erfordert die oberflächliche Punktion einer Vene am Arm oder an der Hand. Dies kann zu einem Bluterguss oder in seltenen Fällen zu einer oberflächlichen Venenentzündung führen. Bei der Entnahme der Urinprobe uriniert der Teilnehmer in einen speziellen Becher, welchen er vom Studienverantwortlichen erhält. Die Urinabgabe ist gesundheitlich unbedenklich.

**Kontaktperson**

Bei Unklarheiten oder Fragen betreffend die hier beschriebene Studie können Sie sich jederzeit an die untenstehende Kontaktperson wenden:

Balthasar Jung
Institut für Klinische Pharmakologie und Viszerale Forschung
Universität Bern
Murtenstrasse 35 (Maurice E. Mueller Haus)
3010 Bern

Tel. 031 632 35 94 (Insel intern 235 94)
079 672 48 51

e-Mail: balthasar.jung@ikp.unibe.ch

Bern, 5. Februar 2009 Balthasar Jung

# Einverständniserklärung

## Ermittlung des Referenzbereichs für Ethylglukuronid (EtG) an gesunden Personen mit einem Immunoassay

Der Teilnehmer erklärt mit seiner Unterschrift, dass er freiwillig an der Untersuchung teilnimmt, und dass er zuvor die Information für Versuchsteilnehmer (Seiten 1 – 3, 5. Februar 2009) sorgfältig durchgelesen und verstanden hat. Das Probematerial darf nur für die auf dem Informationsblatt beschriebenen Analysen verwendet werden. Dem Teilnehmer ist bewusst, dass er zu jedem Zeitpunkt der Studie Fragen zu allfälligen Unklarheiten stellen kann und sich jederzeit ohne Begründung von der Studie zurückziehen darf.

Der Teilnehmer ist einverstanden, den Fragebogen gewissenhaft auszufüllen und

- O Blut und Urin abzugeben.
- O nur Blut abzugeben.
- O nur Urin abzugeben.

| Teilnehmernummer | Name |
|---|---|
| _____ | _____ |
| Datum und Unterschrift | Datum und Unterschrift |
| _____ | _____ |
| Versuchsteilnehmer | Probenabnehmende(r) Arzt, Pflegefachfrau, Medizinische Praxisassistentin oder Biomedizinische Analytikerin |

**Wichtige Information**

Bei der Probenvorbereitung und den Messungen besteht die Möglichkeit, dass bestimmte Auffälligkeiten auftauchen könnten. Solche Abweichung vom normalen Muster können als Ursache sowohl banale Gesundheitsstörungen als auch gravierende, abklärungsbedürftige Erkrankungen haben. Wir müssen daher von Ihnen wissen, wie wir uns verhalten sollen, falls bei Ihren Proben Auffälligkeiten festgestellt werden:

Ich will benachrichtigt werden, falls meine Probe(n) eine mit den durchgeführten Analysenmethoden feststellbare Auffälligkeit zeigt / zeigen.

Ja O Nein O

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Universität Bern

# Fragenkatalog

**Bitte Teilnehmernummer oben auf jedem Blatt des Fragenkatalogs vermerken!**

**Angaben zu den Probenentnahmen**

- Welche Probe(n) möchten Sie abgeben?

  Blut O Urin O

- Haben Sie für diese Studie zu einem früheren Zeitpunkt schon einmal Körperflüssigkeit(en) abgegeben?

  Nein O

  Blut O Wenn ja, wann? _____

  Urin O Wenn ja, wann? _____

**Allgemeine Angaben**

- Nationalität: _____

- Ethnie: Kaukasisch O Asiatisch O Schwarz O

  Hispanisch O Andere: _____

- Geschlecht: Mann O Frau O

- Alter: _____ Jahre

- Gewicht: _____ kg Körpergrösse: _____ cm

- Sind Sie Raucher? Ja O Nein O

- Wann haben Sie zuletzt vor der Blutentnahme etwas gegessen oder etwas anderes getrunken als Wasser?

  Datum: _____ Uhrzeit: _____

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Universität Bern

5. Februar 2009

**Angaben zur Gesundheit und zur Einnahme von Medikamenten**

- Fühlen Sie sich gesund? Ja O Nein O

- Waren Sie kürzlich krank? Ja O Nein O

  Wenn ja, wann? _____ und was? _____

- Hatten Sie jemals eine Harnwegsinfektion?

  Ja O Nein O

  Wenn ja, wann zum letzten Mal? _____

- Leiden Sie an einer systemischen (chronischen) Erkrankung (z.B. Erkrankung von Leber, Nieren, Herz oder Lunge, Diabetes oder andere metabolische Störungen)

  Ja O Nein O

- Wurde bei Ihnen eine Erbkrankheit festgestellt (insbesondere Gilbert-Syndrom, Crigler-Najjar-Syndrom)?

  Ja O Nein O

  Wenn ja, welche? _____

- Nehmen Sie regelmässig Medikamente ein (vom Arzt verschriebene oder selbst gekaufte), bei Frauen inklusive Hormonpräparate zur Empfängnisverhütung oder andere Hormonpräparate?

  Ja O Nein O

  Wenn ja, welche? _____

- Haben Sie in den letzten 72 Stunden vor der Blutentnahme ein Medikament eingenommen (auch nur einmalige Einnahme von Schmerzmitteln, Antiallergika, Medikamente gegen Erkältungen, homöopatische und spagyrische Essenzen usw.)?

  Ja O Nein O

  Wenn ja, welche? _____

- Nehmen Sie zur Zeit ein Vitaminpräparat ein?

  Ja O Nein O

- Führen Sie eine spezielle Diät durch?

  Ja O Nein O

  Wenn ja, welche? _____

*Nur Frauen:*
- Sind Sie schwanger oder in der Stillzeit?

  Ja O Nein O

**Angaben zum Alkoholkonsum vor der Probenentnahme**

- Haben Sie in den letzten 72 Stunden vor der Blut- / Urinentnahme Alkohol getrunken?

  Ja O Nein O

- Haben Sie in den letzten 72 Stunden vor der Blut- / Urinentnahme Produkte (z.B. Medikamente, Pralinen, alkoholfreies Bier) zu sich genommen, von welchen Sie wissen, dass sie kleine Mengen Alkohol enthalten (können)?

  Ja O Nein O

- Haben Sie in den letzten 2 Wochen Alkohol getrunken?

  Ja O Nein O

  Wenn ja, wann hat der letzte Alkoholkonsum stattgefunden

  _____ und

  wie viel ungefähr? _____ Bier

  _____ Wein

  _____ Schnaps

  _____ _____ (andere)

# Fallstudienbericht

Der Fallstudienbericht ist von der Person auszufüllen, welche die Serumprobe entnommen und/oder die Urinprobe entgegen genommen hat. Bitte kein Feld leer lassen, sondern allenfalls abstreichen.

Teilnehmernummer: _____

Blutentnahme:

Datum: _____ Zeit: _____

Negative Vorfälle / Beobachtungen: _____

_____

Falls das Blut zentrifugiert und in Röhrchen abgegossen wurde:

Datum: _____ Zeit: _____

Urinprobe vom Teilnehmer erhalten:

Datum: _____ Zeit: _____

Negative Vorfälle / Beobachtungen: _____

Name

_____

| Datum und Unterschrift | Datum und Unterschrift |
|---|---|
| _____ | _____ |
| Versuchsteilnehmer | Probenabnehmende(r) Arzt,<br>Pflegefachfrau,<br>Medizinische Praxisassistentin oder<br>Biomedizinische Analytikerin |

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# Appendix VII
# Determination of Urinary Concentration

During the development of assays for urinary EtG and EtS, the need for normalization of urine samples was obvious. However, it was not clear whether urinary creatinine concentration and conductivity are comparably appropriate for this task. Therefore, a study about urine concentration has been executed and is discussed in Appendix VII.

## Need for Normalization of Urinary Samples

Substances of clinical or forensic interest as well as their metabolites often are excreted in urine. Urine samples are therefore frequently analyzed and the compounds of interest are quantified.
As mentioned in the chapters discussing the analysis of urine samples, the concentration of urine can vary a lot, even more than 100-fold [89, 90]. In order to express the results of analytical determinations objectively, the dilution factor should be considered and the results should undergo normalization. Different approaches of such a standardization of urine are used in clinical and forensic practice, e.g. creatinine concentration, conductivity, osmolality or density [23, 50, 89-91, 93, 94].
Some authors have preferences for one of the parameters for normalization or the scientists choose one approach dependent on practical reasons, e.g. ease of handling or economical reasons.

## Data for Comparison of Different Ways of Urine Normalization

Comparisons of the different approaches are very rare and it is not clear to what extent the methods correlate. A rough study about the normalization of urine samples using different parameters had been done at our laboratories in Bern a couple of years ago in the context of a study about the determination of GHB in human urine by CZE, published by Baldacci et al. [94].
The correlation of two different parameters describing the concentration of urine, creatinine and conductivity, were found to correlate (r=0.885). However, the number of samples was rather low (n=8) and stemmed from one and the same person. Given the large number of urine samples (n=107) collected under controlled conditions during the reference limit study for the EIA (Chapter 6.3.3 and Appendix VI), another study was done using the same set of samples with the goal to elucidate the correlation of the creatinine concentration and the conductivity of the urine samples. The instrumentation and methods used for creatinine and conductivity determination are explained in Chapter 6.3.3 and [95].

28 real samples from healthy volunteers were included in the statistical work. Together with the samples from the study with blank urines they add up to a set of 135 samples called “urine from healthy volunteers”. 37 samples from patients of the hepatological outpatient clinic of the ICPVR are also used for some evaluations. The different groups have been described in detail in Chapter 6.3.

### Stability of Creatinine in Urine

Since for routine applications, stability of the measured compounds is a prerequisite, it was further investigated whether creatinine is stable in a urine sample at room temperature. Urine from a male volunteer was collected on a morning. The evening before the volunteer consumed about 3 alcoholic standard drinks. EtG was measured on the first (0.818 mg $L^{-1}$) and last day (0.807 mg $L^{-1}$) only.
Creatinine concentration was immediately determined and then reanalyzed for 7 consecutive days at the same time of the day. During these 7 days the sample was stored at a constant temperature between 25 and 27 °C. The mean creatinine concentration of the 7 measurements was 7946.7 µM (1.63 % RSD). No drift in the creatinine concentration was observed. Thus, creatinine was shown to be stable in urinary samples, even if stored at room temperature for several days.

### Differences of Creatinine Excretion in Women and Men

The renal creatinine excretion is known to be different for women and men [143]. For the samples of the reference limit study, the t-test confirmed a significant difference (P=0.0001) of urinary creatinine concentrations in men (n=44) and women (n=63).
Including the set of 28 other samples mentioned above from which some were collected after recent alcohol consumption, the difference between men (n=59) and women (n=76) is clearly significant as well (P<0.0001).

Figures 59 and 60 depict the histogram of all samples and boxplots of the gender-specific distributions of the urinary creatinine concentration. Including all samples, the mean urinary creatinine concentrations of women, men and all subjects were 7016 µM (72.3 % RSD), 11855 µM (62.5 % RSD) and 9131 µM (72.6 % RSD), respectively.

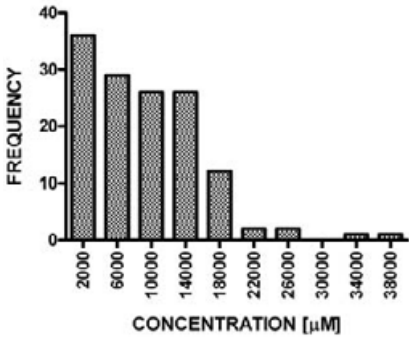


***Figure 59*** *Histogram of the creatinine concentration in urine from healthy volunteers (n=135; bin width 4000 µM).*

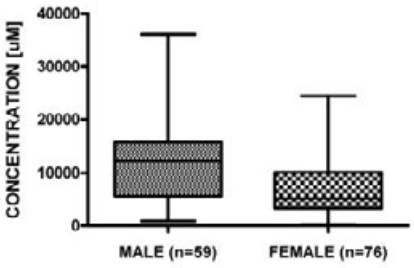


***Figure 60*** *Gender-specific boxplots of creatinine concentrations in urine from healthy volunteers.*

## Differences of Urinary Conductivity in Women and Men

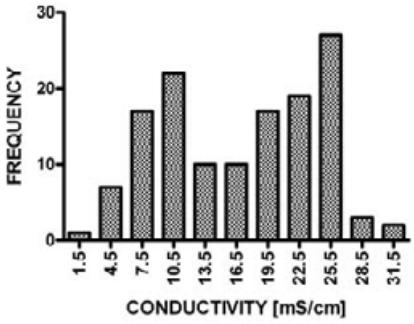


*Figure 61* *Histogram of the conductivity in urine from healthy volunteers (n=135; bin width 3 mS cm$^{-1}$).*

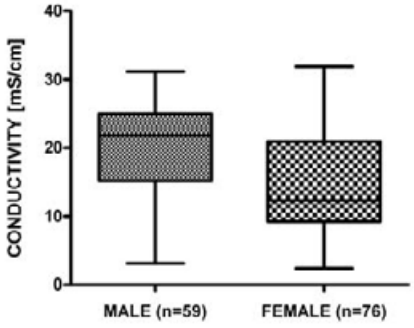


*Figure 62* *Gender-specific boxplots of the conductivity in urine from healthy volunteers.*

Analogous to creatinine, the evaluation was also executed for the conductivity of urine. Instrumentation and methodology of the measurements were described in Chapter 6.3.3 and [95].
Figures 61 and 62 show the histogram of all samples and boxplots of the gender-specific distributions. For the samples of the reference limit study with and without the 28 real samples, the t-test indicated a significant difference of the conductivity of urine in women and men: P<0.0001 (men n=44, women n=63) and P=0.0001 (men n=59, women n=76), respectively.
The mean conductivities of urine of women (n=76), men (n=59) and all subjects (n=135) were 14.68 mS cm$^{-1}$ (48.3 % RSD), 19.59 mS cm$^{-1}$ (36.5 % RSD) and 16.83 mS cm$^{-1}$ (44.5 % RSD), respectively.

## Correlation of Urinary Creatinine and Conductivity

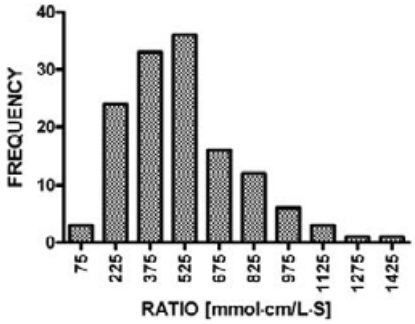


*Figure 63* *Histogram of the ratio of the creatinine concentration of urine and its conductivity (bin width 150 mmol cm $L^{-1}$ $S^{-1}$). The samples stemmed from healthy volunteers (n=135).*

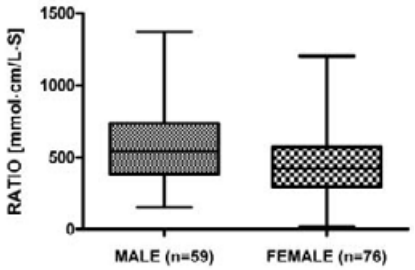


*Figure 64* *Gender-specific boxplots of the ratio of the creatinine concentration and its conductivity in urine from healthy volunteers.*

Furthermore, the ratio of the two parameters for the determination of the dilution factor of urine was investigated in regard to differences between the male and female groups.
Figures 63 and 64 show the histogram of all samples and boxplots of the gender-specific distributions. The mean values of creatinine concentration divided by the conductivity of urine of women (n=76), men (n=59) and all samples (n=135) were 460.5 (50.2 % RSD), 577.3 (43.6 % RSD) and 511.5 mmol cm $L^{-1}$ $S^{-1}$ (48.2 % RSD), respectively.
A significant difference (P=0.0486) between the two genders was determined for the 107 samples of the reference level study. Including the 28 other samples, the t-test indicated a significant difference of the ratio of urinary creatinine and conductivity in women and men (P=0.0058, men n=59, women n=76). Nevertheless, the differences between the groups of female and male individuals were less pronounced than if considering one of the parameters alone.

The correlation of the samples' creatinine concentration and conductivity is illustrated in

Figure 65. The fit of the linear regression curves are described by r of 0.747, 0.764 and 0.693 for all 135 data points, female and male data, respectively.
The data evaluated for the present work show a lower linear correlation compared to those reported by Baldacci et al. (r=0.885) [94]. The 8 data points of the latter work are included in Figure 62. The fact that these 8 urines stem from the same indivisual might explain the linear relationship found by Baldacci et al.

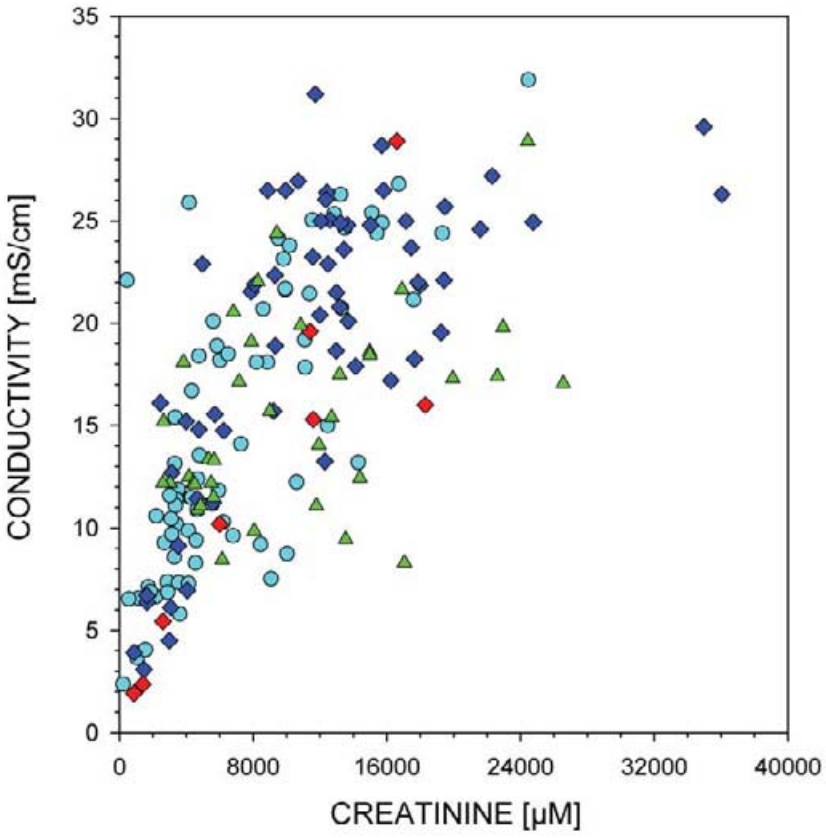


***Figure 65*** *Scatter plot of the urinary creatinine concentration versus conductivity of urine from healthy volunteers (total n=135; females ○ n=76; and males ◆ n=59), outpatient clinic samples (gender unknown ▲ n=37), and a male volunteer who ingested a dose of 25 mg sodium GHB per kg body mass (◆ n=8; data taken from Baldacci et al. [94]).*

For the present work, samples from more than 90 different persons have been analyzed. The 37 patient samples, collected from a group of individuals following medical treatment, reveal data which appear not to fit the distribution of the healthy volunteers (Figure 65). The t-test confirmed this observation (P=0.0011; 135 healthy volunteers and 37 persons following medical treatment) for the ratio of the creatinine concentration and the conductivity of the urine samples. Nevertheless, the difference of the two groups was not significant for creatinine (P=0.2700) and conductivity (P=0.2885), evaluated as single parameters.

Other regressions than the linear one fitted the data better (e.g. power function: Conductivity = $-4.091+0.8828 \cdot c(\text{Creatinine})^{0.3544}$, r=0.8930). However, the data indicates that these two ways for urine normalization do not correlate well. Too many factors like nutrition, gender, muscular mass, race or health state influence urinary conductivity and creatinine concentration [89, 90].

## Conclusions

The linear correlation between urinary creatinine and conductivity could not be confirmed with the large data set available for this study. However, both of the two parameters have been shown to serve for the purpose of normalizing urine samples. None of them is “right” or “wrong”. The production of urine in the body is very complex, so that urinary concentration cannot be described by a single factor only. Each parameter gives an indication for the dilution factor of urine samples, though, and helps to approximatively define the concentration of urine.
Since there is no optimal method for any case, the choice of the method for normalization of urine samples rather depends on the analytical technique used for the determination of the compound of interest and practical considerations. Above all, it is important not to forget to consider urinary concentration for the interpretation of urine samples.