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# Extensive bile acid profiling by the use of Orbitrap LC - MS

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# Directory

# 1 Introduction

## 1.1 Part I: Biological background

### 1.1.1 Structure and function of bile acids

Bile acids consist of a steroid backbone with three 6- rings and one five ring, on which the 5 carbon- atoms- long side chain is positioned. This gives the molecule its lipophilic characteristic. However, the carboxygroup on the side chain and the various hydroxygroups (positioned as described and depicted in "1.1.4 Synthesis of bile acids" below) give its hydrophilic trait, resulting in an amphiphilic molecule as a whole. On the carboxygroup, conjugation with the amino acids taurine or glycine via anhydride formation can occur - again increasing its polarity and water solubility.
This amphiphilic character allows the main function of bile acids: the role as an emulsifier. This enables forming of micelles of lipids and therefore not only digestion of these, yet also the transportation from the intestinal tract throughout the organism.
As will be described in circulation of bile acids, they can also be sulfated, glucuronidated and glucosaminidated which further increases water solubility. Sulfation happens preferably on the C'3 atom, however also cases of conjugation and sulfation on the C'6 and C'7 atoms have been reported. Glucuronidation also takes place on the C'3 or C'6 atom, depending on the position of the bile acid's hydroxygroup [1]. As also on the C'7 atom hydroxygroups are possible, also here sugar moieties have been found. [2] Up to 1992, N-acetylglucosaminidation was thougt to be more selective: in vivo, it has only been found on the C'7 atom in a β configuration [3]. In 1997, also C'3 and C'6 N-acetylglucosaminidations of bile acids were reported [2]. However, these modification do not serve for mediation between water and lipids, but rather facilitated accumulation in urine and therefore excretion.

### 1.1.2 Circulation of bile acids

The in the human liver daily produced 0.2–0.6 g of bile acids are secreted into bile and stored in the gallbladder. Here, bile acids (ca.67% of bile) are solved in mainly water (ca. 95%), pospholipids (ca. 22%), cholesterol (ca. 4%) together with

electrolytes, proteins, bilirubin and its catabolic structures and secretoric IgA. [4] Already at this stage, a small percentage can pass the tissue barrier and enter the sinusoid bloodstream.

Another small percentage is reabsorbed already in the cholangiocytes and recycled back to

hepatocytes due to the so called cholangiohepatic shunt.

The stored bile acids are excreted into the intestinal tract, where they are, as explained more detailled in "1.1.4 Synthesis of bile acids" below, bacterially modified into secondary bile acids or deconjugated.

The excretion is enabled by the contraction of the gallbladder - induced postprandial - and, as described in "1.1.4.2 Excursion: the effects of ursodeoxycholic acid" below, enhanced by ursodeoxycholic acid.

On a more detailed perspective however, not all bile acids have to undergo the indirect way of being stored in the gallbladder - two terms explain the distinctive ways: the gallbladder-dependent and the gallbladder-independent enterohepatic circulation of bile acids.

The direct passage of bile acids into the duodenum makes up only 20-25% of the total bile acid flow in healthy individuals. Of course, with an increasing ejection volume of the gallbladder also the gallbladder- dependent enterohepatic circulation of bile acid increases and determines the concentration of bile acids in the gallbladder [5].

The bile acids that pass into the duodenum and therefore into the intestinal tract in order to fullfill their task (descibed in "1.1.1 Structure and functions of bile acids" above) are absorbed in the upper intestine by passive diffusion to a small degree. Most bile acids (about 95%) are absorbed in the terminal ileum actively by the "apical sodium dependent bile acid transporter" - or short ASBT. As its affinity differs between bile acids, this is at least the cause for primary bile acids. Only a small percentage of the secondary bile acids are reabsorbed here. Deoxycholic acid, e.g., is reabsorbed by passive transport. Of course, a small amount (ca. 0.5g /day in humans) is defecated with stool. This amount is replenished by de novo synthesis in the liver to maintain a constant bile acid pool of ca. 3g and gets recycled 4 to up to 12 times per day.

The bile acids that are absorbed, however, diffuse across the enterocyte to the basolateral membrane and are excreted into portal blood circulation. They finally

reach the sinusoid of hepatocytes again where they can be reprocessed. One reprocessing event is of course the reconjugation with glycine or taurine. These bile acids are lead back to the gallbladder, closing the circuit of enterohepatic circulation. [6]

A certain amount of bile acids are sulfated on the 3-C atom, hydroxylated, glucuronidated or even N-acetylglucosaminated as a process of the hepatic first pass effect in order to increase its polarity and therefore water solubility for secretion with urine. These modified bile acids are transported in the bloodstream and are filtered through the kidneys' glomeruli. As described, all bile acids are at one point transported in the bloodstream and, as absorption from hepatocytes is not excerted completely, they are, too, affected by renal filtration. However, the presence of the ASBT also in the proximal tubulus of the nephron's Henle's loop avoids excessive excretion of reusable bile acids: Its affinity is again proven to differ between different bile acids, as described in "5.6.1 Deglucuronidation and desulfation of unconjugated and conjugated ursodeoxycholic acids" below. Not surprisingly, ASBT -knock out genotype mice show an interrupted enterohepatic bile acid circulation and a malabsorption of these [7].

The sulfated, glucuronidated and N-acetylglucosaminidated forms are successively excreted with small amounts of bile acids which were unaffected by the livers' first pass effect in urine.

### 1.1.3 Regulation of the bile acids' formation and circulation - or the additional functions of bile acids

This circulation is of course an important physiological route for the recycling of bile acids and the absorption of nutrients and lipids. In addition, it plays an important role in the regulation of the entire organisms' lipid metabolism:

Both unconjugated and conjugated bile acids have been shown to bind the ligand-binding domain of the nuclear receptor "farnesoid X receptor"- or short FXR (as mentioned in "excursion: the effects of lithocholic acid"). This results in an alteration of its conformation and allows the sequestration of the retinoid X receptor. This heterodimer is now able to bind to inverted repeats (AGGTCA-like sequence with one

nucleotide spacing) in the promotor areas of its target genes, thus inducing their transcription. The transcription products have different targets:

For one, they can inhibit CYP7A1 [8]. In addition, they have also been found to impede CYP27A1 [9] and CYP8B1 [10]- the latter one being a human species specific effect only [11].

This suggests to be a very potent control mechanism of bile acid synthesis in a direct manner by the synthesis products itself: The conservative pathway, by which approximately 80% of bile acid formation takes place, can be obstructed by the inhibition of CYP7A1 as well as the alternative pathway, which accounts to the formation of the remaining 20%. This pathway can even be obstructed twice by the inhibition of CYP27A1 as well as CYP8B1 thus also impeding the synthesis of oxysterols.

In addition to this feedback mechanism, the activation of the FXR results in the transcription of BAT and BACS (described in "1.1.4 Synthesis of bile acids" below) and therefore also promoting conjugation of bile acids with glycine or taurine [12]. This implies that bile acids themselves also regulate their conjugation and therefore hindering a possible accumulation of lipophilic ones and therefore preventing toxic conditions. Of course, conjugated bile acids are also important for properly emulsifying different lipids in an aqueous environment.

Moreover, the increased conjugation of bile acids is accompanied by an increased secretion of these as well as unconjugated bile acids into the bile stored in the gallbladder. This is accomplished by the increased transcription of the gene encoding the "canalicular bile salt export pump"- or short BSEP in the canalicular membrane.

Interestingly, the circulation of bile acids seems to be regulated differently in different species: whilest activation of the FXR resulted in an inhibition of the ASBT (described in "1.1.2 Circulation of bile acids" above) in rabbits, it showed an activation in mice. In humans, no significant effect could be described [13].

The activation of FXR also induces an increased bile acid secretion into the intestine, as it activates the major bile acid efflux transporter: OST, consiting of the units OSTα and OSTβ, located in the basolateral (sinusoid) membrane [14] of the intestine as well as in the sinusoid membrane of hepatocytes [15]. This effect has been proven in patients with primary biliary cirrhosis and in bile duct-ligated rats and mice [16]. This shows yet another mechanism of the activated FXR to protect hepatocytes from the toxic effects induced by increased concentrations of lipophilic bile acids.

Another putative mechanism, this time concerning cells located in the illeum, is:
It has been shown that activated FX receptors increase the concentrations of the "ileal bile acid binding protein" - or IBABP [17]. The mechanism is putative as the physiological function of IBAP is not yet proven but thought to be the binding of bile acids and lower their concentration in the affected area.

Yet, bile acids can regulate their metabolism not only by the activation of FXR, but also by several other receptors:
The secondary bile acid lithocholic acid (as shall be described in "1.1.4.1 Excursion: the effects of lithocholic acid" below) e.g. can bind and activate the receptors PXR - or "pregnane X receptor"- and VDR - or "vitamin D receptor".
Both nuclear receptors bind, when activated, to the promotor region of the CYP7A1 gene, repressing its transcription. As we can recall, also the activated FXR represses the conservative pathway of bile acid synthesis. A mistaken accounting of this function to PXR is expelled by the fact, that "Guggulsterone", an FXR antagonist and PXR agonist, inhibits CYP7A1 transcription by activating PXR [18]. Interestingly, PXR also impedes the activation of CYP27A1 (the alternative pathway of bile acid formation) just like FXR in liver cells. However, it activates it in intestinal cells. This suggests that the primary bile acid formation in the liver is suppressed, whereas the efflux of cholesterol from the intestine and HDL formation leading to its transport back to the liver is promoted [19].
This means, that besides the obvious indirect cholesterol controlling mechanism bile acids can provide, they regulate its metabolism also through other processes.
In addition to the activation of FXR and PXR, also the activation of VDR allows an inhibition of the CYP7A1 transcription and therefore an obstruction of the conservative pathway of bile acid synthesis. [6]

Bile acids can also bind and activate TGR-5 [20]. This receptor differs from the above mentionened as it is a cytoplasm bound G-protein coupled receptor, transfering its activation via the second messenger cAMP and inducing various processes this way:
Activation modulates energy expenditure by controlling the activity of type 2 deiodinase and the subsequent activation of thyroid hormone in brown adipose tissue and muscle. In fact, pharmacological intervention with a diet containing 0.5% of the primary bile acid cholic acid has been shown to efficiently attenuate diet induced

obesity in mice [21]. Therefore, the semi-synthetic cholic acid (CA) derivative, 6α-ethyl-23(S)-methyl-cholic acid (EMCA, INT-777) was even tested as a promising possible treatment for diabetes and obesity [22].
In addition to the above-described effect, the TGR-5 receptor is also expressed in enteroendocrine L-cells [23] as well as enteroendocrine cell lines like the STC-1 cells [24]. These can secrete incretin and "glucagon-like peptide-1"- or GLP-1 postprandially. The importance of GLP-1 regarding these metabolic syndromes is shown by the fact that by the increase of its half life or activation of its receptor, type II diabetes is treatable [25]. Possibly, the activation of TGR-5 could also induce secretion of GLP-1 and therefore be an additional target for treatment of metabolic diseases [22].
In addition to its important roles in energy homeostasis and glucose metabolism, the activated TGR-5 receptor is an effective tumor suppressor and in fact was also suggested to serve as an attractive therapeutic tool for the treatments of human liver cancer. In this context, it was also shown that it inhibits inflammatory responses mediated by the factor NF-κB [26] as well as synthesis of proinflammatory cytokines. Therefore, it majorly contributes to the attenuation of the development of atherosclerosis [27].

We now understand that the bile acids' act as emulsifiers provided by their amphiphatic character, which enables formation of micelles and therefore promoting breakdown and transportation of lipids as well as other nutrients and vitamins throughout the organism is only one important role. They furthermore act as versatile signalling molecules, inducing various different hormonal responses.

### 1.1.4 Synthesis of bile acids

Catabolic routes of cholesterol include conversion into steroid hormones by endocrine tissue [28], conversion into vitamin D3 in the skin, liver, and kidney [29] and production of oxysterols by the lung [30] and the brain [31].
These can be shunt into a bile acid producing enzyme cascade via two different oxysterol 7-α hydroxylases, one beeing part of the CYP 39A1 complex and one of the CYP7B1. The latter one is part of the so called "alternative pathway" of bile acid synthesis, being initialized by oxysterolformation of cholesterol by the enzyme

CYP27A1. The name indicates, that a vast majority (up to 90%) of bile acid synthesis is initialized by a different pathway: Here, cholesterol is directly hydroxylated at the 7 C Atom in an α-configuration without the oxysterol intermediates. However, the different possible initiation steps join back together in the same pathway at the enzyme 3-β Hydroxy-$\Delta^5$-C27-Steroid Oxidoreductase. The second downstream enzyme from this stage on is 3-oxo-Δ4-steroid 5-β-reductase. This enzyme is shown to catalyze the formation of coprostanone out of 4-cholesten-3-one [32]. The 3-α Hydroxysteroid dehydrogenase then allows this 3' ketone to form coprostanoles. After being hydroxylated again, coprostanoles are an important bile acid intermediate. In fact, dihydroxy- and trihydroxycoprostanic acid will also be part of analysis in this work as described in "1.1.6 Bile acid precursors: coprostanic acids" below. After a side chain shortening via β - oxidation, which takes place in peroxisomes, the simplest bile acid is formed: chenodeoxycholic acid. An additional hydroxylation at the 12 C atom results in formation of the second primary bile acid: cholic adic [33].

However, this is not one simple reaction cascade as simplified in Fig.1 below: cholic acid is predominantly directly produced in the "conservative pathway" leading to the alternative pathway including CYP 27A1 and CYP8B1 from 7α- Hydroxycholesterol. When the shunt to CYP8B1 is avoided, the conservative pathway, starting from CYP7A1, leads to the production of Chenodeoxycholic acid. Chenodeoxycholic acid is, however, also the downstream production step of the alternative pathway induced by CYP27A1 leading to CYP7B1 [6].

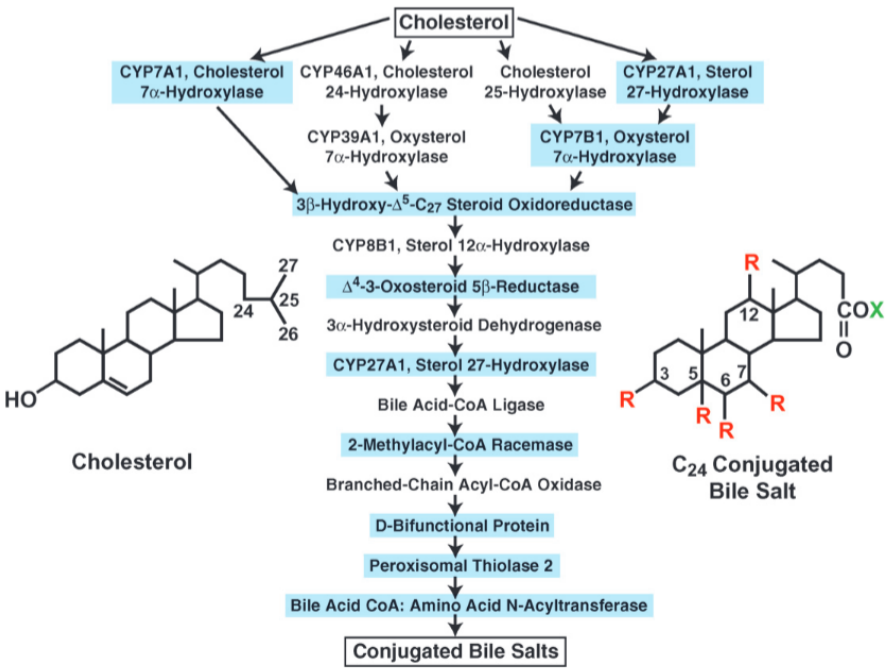
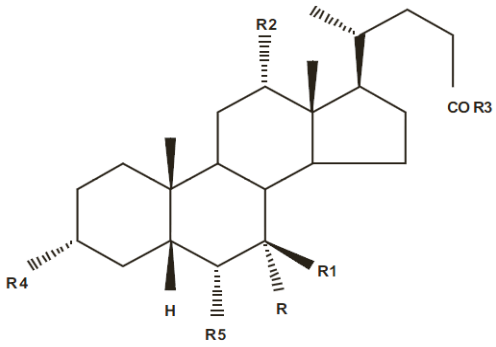



*Fig.1: Scheme of enzymes involved in bile acid formation* [34]*; Fig 1*

The primary bile acids, still in the hepatocytes, are partially excreted into the bile. A large amount, however, is conjugated with taurine or glycine. The reason behind this is to drastically increase polarity and therefore water solubility- e.g. the pKa is reduced from ca. 6 to 2 for cholic- to taurocholic acid and 4 for glycocholic acid. This, of course, hinders diffusion through lipid layers, causing them also to be less likely to permit cell membranes. Yet primarily the conjugation step serves to increase secretion into bile and therefore lessen the bile acids' toxicity induced by overaccumulation in hepatocytes.

The enzymes involved in this conjugation step is of course an aminoacyltransferase, the bile acid: amino acid transferase - or BAT- after an activation step of the bile acid by bile acid:CoA synthase -or BACS [6], [33].

After secretion into the intestinal tract, primary bile acids are bacterially modified. As this following formation of bile acids does not take place in the liver but in the gut by microbial enzymes, the resulting bile acids are called secondary bile acids.




| | R | R1 | R2 | R3 | R4 | R5 | |
|---|---|---|---|---|---|---|---|
| CA | -OH | -H | -OH | -OH | -OH | -H | primary bile acids |
| CDCA | -OH | -H | -H | -OH | -OH | -H | |
| DCA | -H | -H | -OH | -OH | -OH | -H | secondary bile acids |
| LCA | -H | -H | -H | -OH | -OH | -H | |
| UDCA | -H | -OH | -H | -OH | -OH | -H | |
| HCA | -OH | -H | -H | -OH | -OH | -OH | |
| HDCA | -H | -H | -H | -OH | -OH | -OH | |
| Glycine Conjugated | | | | $-NHCH_2CO_2^-$ | | | |
| Taurine Conjugated | | | | $-NH(CH_2)_2SO_3^-$ | | | |
| Sulfated | | | | | $-SO_3O^-$ | | |

*Fig.2: Structure of bile acids; structures shown are the two primary bile acids (CA, CDCA) as well as four secondary bile acids (LCA, UDCA, HCA, HDCA); also the position of conjugation with taurine and glycine as well as of sulfatation is presented* [35]*;*

Litocholic acid is synthesized by 7-dehydroxylation of chenodeoxycholic acid. This resulting bile acid with just one hydroxygroup is, of course, relatively apolar resulting in different complications:

### 1.1.4.1 Excursion: the effects of lithocholic acid

In fact, lithocholic acid and its precursor chenodeoxycholic acid have been shown to be highly toxic when fed to rabbits, rhesus monkeys and baboons [36]. Administration of lithocholic acid to rodents is proven to cause intrahepatic cholestasis [37]–[39].
However, in man and chimpanzees it can be detoxified via hydroxylation and sulfation and are therefore less toxic. Lithocholic acid itself was shown to induce its own detoxification by activating nuclear receptors to promote transcription of genes coding for sulfotransferase [36].

In addition, lithocholic acid and its 3 - keto metabolite binds to the "pregnane X receptor" - or PXR. This leads to its activation and furthermore to the expression of genes involved in biosynthesis, transport and metabolism of bile acids including 7 - α - cholesterol hydroxylase (CYP7A1, see Fig.1 above) and the natrium- independent organic anion transporter 2 (Oatp2). All these processes induced by the activation of the PXR receptor contribute to the protection against severe liver damage. Also FXR - the "farnesoid x receptor" - is, allthough usually activated by a distinctive set of bile acids, also activated by binding of lithocholic acid. This would enhance the possibility that both contribute to the removal of lithocholic acid from the body when its concentrations reach pathophysiological levels [39]. The role of bile acids regarding as ligands to these receptors was already explained in "1.1.3 Regulation of the bile acids' formation and circulation - or the additional functions of bile acids" above.
Elevated levels of lithocholic acid could be attributed to patients suffering from chronic cholestatic liver disease [40].
As will be shown in the course of this work, lithocholic acid is excreted mainly by defecation with stool in an unconjugated state - even in healthy human subjects. Neither the unconjugated form nor the glycine or taurine conjugate could be observed in high concentrations in urine. This also includes glucuronidated and sulfated forms. The only exception to this is a relatively high concentration of 8 nmol/L of 3-sulfated lithocholic acid in one sample. This may be due to a detoxification step of excessive amounts of lithocholic acid - as will be stated, the healthy state of the patients from whom urine samples were taken can not be guaranteed.
Due to this toxicity, it is believed that the evolution of secondary trihydroxy bile acids in higher evolved vertebrates may have occurred to decrease the formation of lithocholic acid [36].

7-dehydroxylation of cholic acid leads to the dihydroxycholanic acid deoxycholic acid. Epimerisation of the 7- hydroxygroup of chenodeoxycholic acid from cis- to trans configuration leads to ursodeoxycholic acid. This single simple step results in a more polar bile acid with astonishing biological consequences:

### 1.1.4.2 Excursion: the effects of ursodeoxycholic acid

Ursodeoxycholic acid is able to cause a gallbladder contraction, or with other words, increases gallbladder contractility: Initially, ursodoexycholic acid was thought to directly impair gallbladder contractility, together with more hydrophobic bile salts. E.g. a study of 1985 suggests, that ursodoexycholic acid added to a cholesterol enriched diet in ground squirrels strongly impaired in vitro gallbladder contractility in response to cholecystokinin levels. However, this study was probably biased by the cholesterol enriched diet or other bile acids [41].
As Gomez G described, the bile salt taurocholate in fact strongly impairs gallbladder contractility, increases pancreatic growth, decreases the concentration of cholecystokinin receptors in the gallbladder muscle as well as decreases meal-stimulated plasma levels of cholecystokinin in vivo in guinea pigs, whereas the bile acid sequestrant cholestyramine had the complete opposite effect.
However, ursodoexycholic acid itself actually significantly increases acetylcholine and cholecystokinine levels significantly and therefore enhances the contractile potential of the smooth muscle cells in the gallbladder wall. Treatment with ursodoexycholic acid also led to a reduced biliary cholesterol saturation [42].

As was found out later, ursodeoxycholic acid can act on various different levels to prevent or meliorate different cholestatic disorders: It was shown that ursodoexycholic acid can protect injured cholangiocytes from the toxic effects bile acids can excert. This, of course, is of importance in early stage primary biliary cirrhosis and primary sclerosing cholangitis.
In addition to the above- described effect on smooth muscle cells in the gallbladder's wall, impaired hepatocellular secretion can also be affected by post transcriptional mechanisms including stimulation of synthesis, targeting and insertion of key transporters in apical membranes. This is relevant in more advanced cholestasis.
Of course, in case of intrahepatic cholestasis of pregnancy, these described effects could also be crucial for rapid relief of priuritus, as it is also the case for several drug-induced cholestasis cases.
Ursodoexycholic acid is also proven to stimulate calcium-dependent secretion of chloride and bicarbonate ions. This could affect the process of cystic fibrosis. As it

also counteracts bile acid induced hepatocyte apoptosis, it is important for all states of cholestasis that are characcterized by hepatocellular bile acid retention [43].

Of course, ursodoexycholic acid is also effectively used to treat pain resulting of gallstones. In addition, it has been shown to be an effective treatment for biliary pancreatitis [44]. The underlying mechanisms include an impaired gallbladder motility and therefore painreduction [45] (as a result of the increased acetylcholine and cholecystokinine levels) yet still allows a better excretion of bile acids and therefore a positive effect on the cholesterol levels [46] - and therefore also reduces the formation of cholesterol crystals. Also, ursodoexycholic acid has been shown to decrease the mucin contents in bile [47], allowing a better fluidity.

However, in case of acute cholecystitis ursodoexycholic acid does not reduce biliary symptoms in highly symptomatic patients. In case of patients with symptomatic gallstones [48], cholecystectomy is critical.

Interestingly, ursodoexycholic acid was also shown to be beneficial in heart failure in humans [49]. The increase in hepatocellular bile acid secretion and therefore the lowering of endogenous serum bile acid levels caused by ursodoexycholic acid counteracts the effect of primary bile acids not only on fetal hearts but also on the adult myocardium. Both are susceptible to bile acid induced arrhythmias. In addition, , ursodoexycholic acid itself lacks this effect.

Incubation with taurocholic acid has shown an enhanced NCX-inward current density and resting membrane potential depolarisation. In addition, it gives rise to afterdepolarisation in adult cardiomycytes.

A very probable explanation for the induction of myocard arrythmias in vitro as well as in vivo in men is the inhibition of the cardiac sodium–calcium exchanger NCX.

This exchanger is proven to be affected by negatively charged amphiphilic molecules [50]. As we know, these traits can fit to bile acids: The steroid backbone allows embedding in the lipid bilayer [51] and if the environment's pH value lies below the pKa value of the certain bile acid, it is present in an anionic state.

Now of course a lower pKa value - as it is the case for ursodeoxycholic acid - leads to a higher degree of protonisation and therefore a lower charge under physiological conditions which in turn lessens the interaction with the NCX exchanger. This implies, that bile acids with a low pKa value like ursodeoxycholic acid affects the exchanger less than e.g. lithocholic acid. However, the pKa value lies very close for all

unconjugated bile acids (5 - 6.5 [52]) and this effect would therefore only contribute to yet not cause the affinity to the NCX exchanger.
More importantly, polar bile acids like ursodeoxycholic acid are less likely to embed in the lipid bilayer because of this trait.
In fact, ursodeoxycholic acid conjugates were even shown to protect cholesterol- rich plasma membranes from toxic effects of more hydrophobic bile acids [53].

In addition to these dehydroxylation and epimerisation processes of the steroid structure, primary bile acids are also deconjugated from taurine and glycine (for position on the bile acid side chain see Fig.2 above).

### 1.1.5 Metabolic disorders and diseases associated with bile acids

Considering that their metabolism is involved lipid digestion, lipid and cholesterol transport, their turnover, their excretion and balance in general as well as different effects on glucose metabolism and in inflammatory responses it is not surprising, that they could be associated with many different metabolic disorders and diseases:
7α-hydroxy-4-cholesten-3-one - or short C4- is a biosynthetic bile acid precursor formed from 7α-hydroxycholesterol in the conservative pathway. Its downstream product is majorly the primary bile acid cholic acid, however, through the formation of the intermediate 5β-cholestane-3α,7α-diol it can also yield synthesis of the other primary bile acid chenodeoxycholic acid. Therefore, its concentration resembles the activity of the bile acid synthesis pathway [54]. [55]
It has been shown to be present in serum in a significantly increased concentration in the case of metabolic syndrome, which consists of an accumulation of metabolic risk factors for cardiovascular disease including abdominal obesity, elevated blood pressure, impaired fasting glucose or overt diabetes mellitus type 2, hypertriglyceridemia and low high density lipoprotein cholesterol levels as well as disturbances in the regulation of metabolism, inflammation and coagulation.
C4 has also been shown to be elevated in diabetes mellitus type 2. In both cases, disturbances in the bile acid metabolism have also been reported [56]. [55]

Of course, an increase of C4 can also resemble bile acid malabsorption or bile acid diarrhea [57], as in both cases bile acid synthesis has to be increased to compensate the elevated loss.

Interestingly, in the case of diabetes mellitus type II, the baseline of the total unconjugated as well as glycine and taurine conjugated bile acid concentration in serum was not significantly elevated compared to healthy and non - obese individuals. However, they showed an elevated post prandial response, consisting of a significantly increased peak concentration of unconjugated and glycine conjugated bile acids. Taurine conjugates, again, were not significantly elevated [58].

Surprisingly, elevated serum concentrations of certain bile acids were also observed in cases related to cardiac diseases, especially arrhythmias [59]. As it turned out, they are not only possible biomarkers for different heart related diseases - but very much involved in their emergence:

In opposite to the described effects of ursodeoxycholic acid in regard to myocard arrhythmias (see "1.1.4.2 Excursion: the effects of ursodeoxycholic acid" above), high levels of taurocholic acid induces arrhythmias in adult human atria. Atrial fibrillation, the most common observed heart arrhythmia, is associated with elevated concentrations of conjugated bile acids except ursodeoxycholic acid conjugates.

In combination with the in " Excursion: the effects of ursodeoxycholic acid" explained observations of ursodeoxycholic acid's effect this suggests, that high levels of arrhythmogenic bile acids and low levels of the protective bile acids create a milieu with a decreased arrhythmic threshold and therefore facilitated arrhythmic events [51]. The putative mechanism behind the arrhythmogenicity of certain bile acids is also described in "1.1.4.2 Excursion: the effects of ursodeoxycholic acid " above.

The case of gallstone disease is especially interesting: the complete bile acid pool has been shown to be consistently decreased in comparison to healthy individuals. However, the rate of bile acid synthesis has been proven to be elevated. This leads to the conclusion, that fecal excretion of bile acids is higher and reabsorption is diminished. The higher rate of synthesis is of course performed in order to compensate the higher loss, also demanding more cholesterol in the liver and hepatic VLDL [60].

It also implies higher serum concentrations of the bile acid precursor C4 as well as the cholesterol precursor lathosterol as reported in [61].

### 1.1.6 Bile acid precursors: coprostanic acids

In this work, the two bile acid precursors di- and trihydroxycoprostanic acid will be included in the bile acid analysis due to their potential as diagnostic tools for peroxisomal disorders.
Both coprostanic acids have a similar structure to their downstream products cholic- and chenodeoxycholic acid. However, the side chain is still two carbon atoms longer. It is shortened through peroxisomal β- oxidation as mentioned in "1.1.4 Synthesis of bile acids" above.
In healthy individuals, these analytes are therefore present in very low concentrations due to the fact that upon formation, they are transformed into the known primary bile acids. However, patients with either a peroxisome biogenesis defect or a specific bile acid β- oxidation defect accumulate these DHCA and THCA in blood and bile. Measurements of DHCA and THCA in body fluids contribute to the diagnosis of such defects, both postnatal and prenatal [62].

## 1.2 Part II: Methods

### 1.2.1 Thin layer chromatography (TLC)

The thin layer chromatography is, like the paper - and the electrochromatography, a planar method of chromatography. They all result in an "inner chromatogram", where analytes are separated as they cover different distances in the same time - in contrast to the "outer chromatogram" of e.g. the HPLC. Here, the analytes are separated as they appear at different times on the end of the chromatography column.
The analyte is applied at the base of the planar stationary phase, which, mostly consisting of silica gel, cellulose or aluminium oxide, can be the carrier itself or mounted on glass, plastic or metal (mostly aluminium). The carrier with the stationary phase is then placed in a closed chamber (mostly consisting of glass), which is saturated with the vapour of the mobile phase. The mobile phase, of course, covers the floor of the chamber. It is important to consider that it should not drench the spot of appliance on the stationary phase.

Capillary forces allow the mobile phase to travel up the stationary phase. Depending on the analytes' different solubilities in the mobile phase as well as their interaction with the stationary phase, they are carried for different distances - separation is therefore achieved.
When the solvent front reaches about to thirds of the carrier plate, the plate is removed and analytes are detected using

- luminescence traits of the analyte (in case of organic molecules this shall be fluorescence, in case of anorganic compounds phosphorescence)
- fluoresceing indicators like pyrene derivates, fluorescein, morine or rhodamin B. These allow the detection of analytes under UV light.
- oxidizing reagents like $HNO_3$, $KMnO_4$ or $H_2SO_4$. The oxidation of organic compounds appear as dark spots on the carrier plate when heated.
- group - specific - reagents like ninhydrin which allows the visualisation of $NH_2$ groups, Iron (III) -chloride for phenoles, anilinpthalate for reducing sugars or complexing agents in case of metal ions.

As it is a very simple form of liquid chromatography, easily quickly and cheaply performed, it is often used as a preliminary step for column chromatography (as done in this work). Furthermore, the thin layer chromatography is an often used screening method in chemical, clinical, industrial, pharmaceutical, biochemical and biological laboratories. [63]

### 1.2.2 High performance liquid chromatography (HPLC)

The classical liquid chromatography consists of a liquid mobile phase and solid stationary phase, packed in glass columns of about 1 -5 cm inner radius and 50 - 100cm length. The particle size of the stationary phase lies between 150 -200 µm, allowing a flow rate of about 1ml / min. Separation of analytes is time consuming and only of limited efficacy. Therefore it is nowadays used for preparative purposes only.
Analytical separation of analytes in liquid chromatography was extended by the "high performance liquid chromatography" at the end of the 1960ies by introducing a much smaller particle size of the stationary phase (3 - 10 µm). At the same flow rates, this resulted in a drastic increase of the generated pressure (up to 15MPa) and had therefore higher demands on the column material as well as the pump and

connective tubes. Almost all modules had to be changed. A scheme of them arranged in a HPLC system is depicted below:

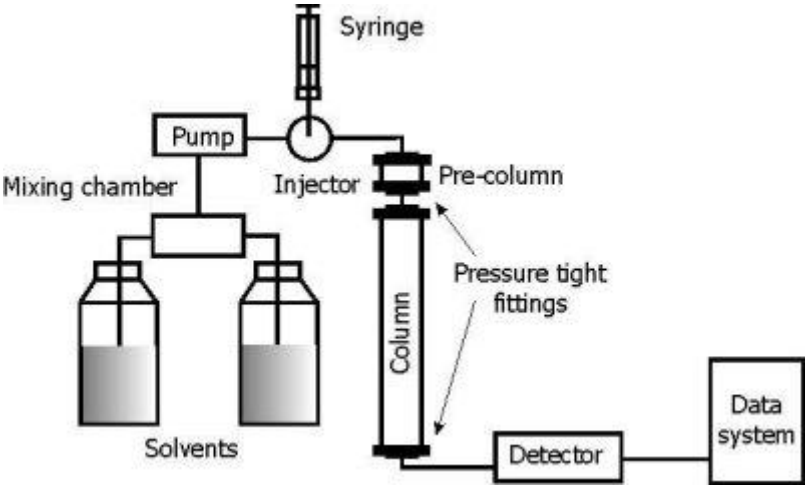



*Fig.3: Scheme of HPLC system* [64]*;*

1.2.2.1 Solvents:

The solvents (mobile phase) are stored in a reservoir of glass or stainless steel. Solved gases (e.g. nitrogen or oxygen) have to be expelled in order to prevent unwanted pressure fluctuations and a negative effect on chromatographic performance. This is done by incubation of the solvents in an ultrasonic bath or introducing noble gases like helium. In addition, a degasser integrated in the pump assists this purpose.

Suspended matter is avoided by using fresh solvents and a millipore filter.

The different eluents can be constantly used in the same amounts throughout the run (isocratic elution), or in different compositions over the course of the run (gradient elution). An optimized gradient allows a shorter elution of the analytes, as well as narrower peaks and the same width throughout the run.

The gradient can be mixed either at the pressure side of the pump - also called "high pressure gradient" or at the suction side of the pump - "low pressure gradient". A gradient generated by low pressure is usually less exact, as volume contraction of the solvents plays a more important role here.

1.2.2.2 Pump:

The pump is the driving force. It must be able to generate a pressure of up to 15MPa. In addition, it must meet the following requirements:

- a low resting pulsation
- chemical resistance (e.g. to avoid corrosion)
- constant performance between 0.1 and 10 ml /min
- reproducibility and control over the flow

Pumps can be devided into piston pumps with a long stroke and piston pumps with a short stroke. Piston pumps with a long stroke work like a syringe: a relatively large volume (about 200 ml) of the mobile phase is sucked in and pressed into the HPLC system without pulsation. During this time, of course, no delivery can take place.
For this reason, a short stroke is preferred. They allow a smaller internal volume and a high pressure at the pump's exit. More importantly, the flow remains constant regardless of the backpressure exerted by the chromatography column or the viscosity of the solvents. In order to reduce pulsation, they are mostly used in combination - working with a phase displacement of 180°.

1.2.2.3 Injection system:

The injection system allows the appliance of the sample into the HPLC system.
At the time of injection, the pump carries the preferred flow of eluents onto the chromatography column on a separate canal. After injection, a switch is engaged which allows the transportation of the injected sample volume onto the chromatography column. When the sample is loaded, the switch is engaged again and the eluent directly flows to the column again.

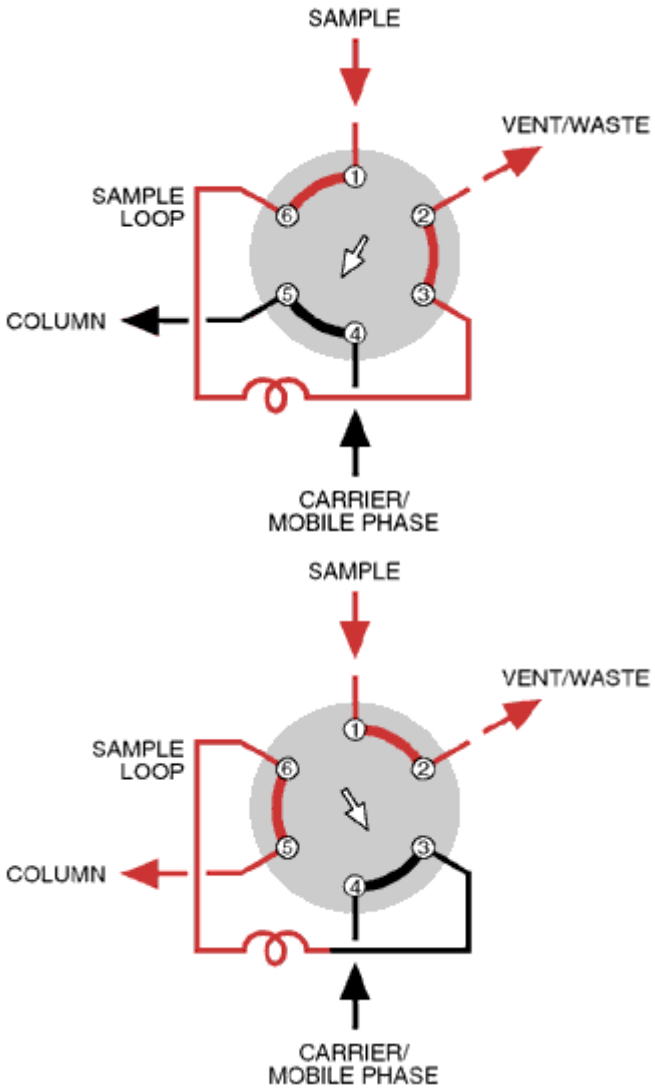



*Fig.4: Mechanism of sample appliance in a HPLC system; Left: time of appliance; Right: carrying of the sample material to the chromatography column* [65]*;*

This allows the mantainance of a constant pressure and the injection of defined sample quantities without introducing of air.

1.2.2.4 Columns:

The described small particle size of the stationary phase packed into the chromatography columns results in a high theoretical plate number (in case of 3 µm particle size up to 100.000 theoretical plates per meter). Standard columns reach a length of ca. 250 mm and an inner diameter of about 4.6 mm. However, in order to

reduce the consumption of the expensive HPLC grade eluents, inner diameters can be reduced to 1mm and length to as short as 30 mm.

1.2.2.5 The principles:

Columns are available with many different stationary phases, the principle of separation being the ones known from chromatography so far:

- adsorption
- partition
- ion - exchange
- size exclusion
- or even affinity

Mostly, a combination of different principles is the case (at least to a certain degree). The adsorption chromatography columns are divided into *normal phase* or *reverse phase* columns. The latter principle is currently the most used analytical method.

1.2.2.6 Detectors:

Detectors used in HPLC are:

- photometric detectors (such as UV detector - the most used detector in HPLC)
- fluorescence detectors (lower limit of detection than a UV detector)
- refractometer (less sensitive than a UV detector, also very sensitive towards temperature)
- electrochemical detectors (problematic with surfice active substances)
- and of course spectrometric detectors (one being the mass spectrometer, allowing an accurate and reliable detection as well as characterisation of the analyte) [66]

### 1.2.3 Electrospray ionization (ESI)

In order to charge the analytes for mass spectrometric detection, they need to be ionized. To achieve this, there are various different techniques. As analytes in this presented work are relatively large, polar and separated by HPLC, they were ionized by electrospray ionization.

Ionization occurrs, when the liquid (consisting of mobile phase, analytes and ionization modifiers like acetic acid) passes through a capillary tube with a low flow rate of between 1 - 500 µl/min. A strong electric field applied between the capillary and a counter electrode causes a charge accumulation at the liquid surface located at the end of the capillary. The initially almost spherical droplet now elongates and when the pressure exceeds the surface tension (described by the Rayleigh equation), highly charged droplets burst out. At this stage, they measure a relatively large diameter of about 1.5 µm and carry up to 50,000 elementary charges. A "Taylor cone" is formed, droplets divide and explode as a cause of charge repulsion, leading to smaller droplets and producing a spray. The droplets now have a diameter of only 0.1 µm and carry about 300 elementary charges.
In order to direct the dispersing droplets, a gas is injected coaxially. This allows the passage through a heated capillary, causing removal of the last solvent molecules.
Two different theories describe the process of charge accumulation and solvent removal: The "charged residue model" describes a the consisted removal of solvent, until a small droplet remains with a diameter of about 1nm and a single charged analyte molecule. The "ion evaporation model" describes bigger and multiply charged droplets. However, single charged analyte molecules can desorb into the gas phase.
From the heated capillary, the charged analytes are again concentrated through a lens and reach the mass spectrometer in a certain angle through the skimmer as depicted in Fig.5 below.

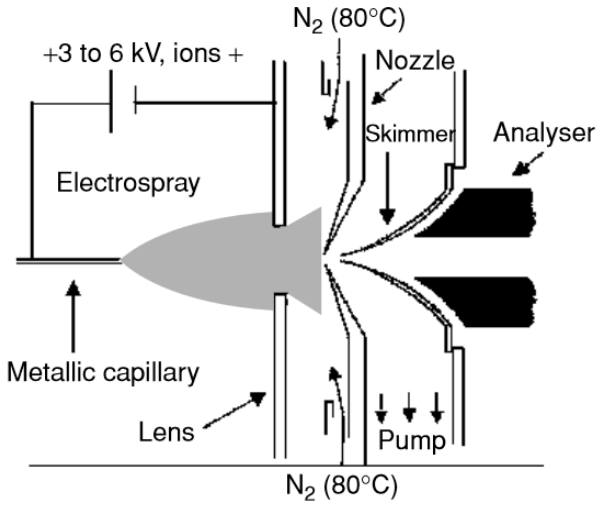



*Fig.5: Scheme of electrospray ionization; in this case using inert nitrogen gas for desolvation instead of a heated capillary* [67]*;*

Of course, large molecules with various ionizable sites like proteins will be multiply charged. On the other hand, also small molecules without any ionizable site can be charged for mass spectrometric analysis, as formation of sodium, chloride, ammonium, acetate or other adducts takes place (depending on ionization modifier added and the analyte itself). [68]

### 1.2.4 Orbitrap mass spectrometer

There are various different principles of mass spectrometric detection of ions. A very sensitive one is the orbitrap.

The orbitrap consists of an inner central spindle - shaped electrode (diameter of about 8mm) and an outer barrel - shaped electrode (diameter of about 20mm), which is severed by a thin gap. This gap allows the tangential introduction of the ionic analytes into the orbitrap. Subsequently, they begin to oscillate in intrical spirals around the inner electrode, their path of course being determined by their mass : charge ratio. Ions of a selected mass range can be "trapped" and analysed here.

The detection of the ions itself is achieved by Fourier transormation: the charged analytes induce a current in the detectorplates, depending on their mass : charge ratio. The broadband current is then converted into the individual frequencies and intensities, allowing the calculation of a mass spectrum.

1.2.4.1 The compartments:

The instruments can consist of different compartments and differ in the way they are arranged. The first commercially available orbitrap mass spectrometer e.g. has a linear ion trap - or LIT. It allows ion storage and ejection of them or only selected ones, including ones produced in MS/MS operations in the LIT itself. When ions are lead to the orbitrap where a high resolution spectrum can be aquired, the LIT can still operate and e.g. aquire low resolution spectra at the same time.
It also features various lenses in order to regulate the number of ions injected into the LIT as well as the orbitrap.
The resolution attained as well as the dynamic range is dramatically higher than the ones of a Q-TOF instrument. The resolution is also higher for high masses than of the similar FTICR instruments. Here, the charged analytes are trapped by a magnetic field instead of charged electrodes. This is due to the fact, that the resolution of the orbitrap mass spectrometer is inversely proportional to $(m/z)^{1/2}$, whereas the resolution of the FTICR mass spectrometer is inversely proportional to m/z. With a rising mass : charge ratio, the resolution of the orbitrap mass spectrometer declines slower. However, the resolution for lower masses is lower. [69]

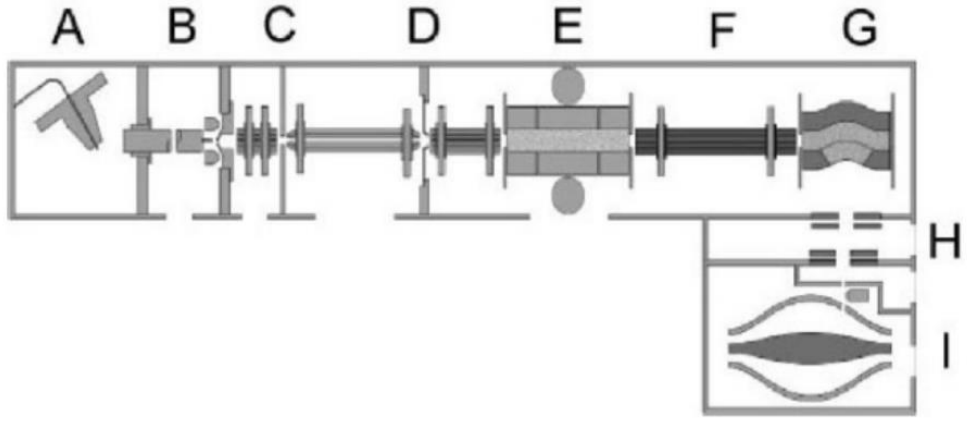



*Fig.6: Scheme of the first commercially available orbitrap MS (presented by Thermo Electron corporation); it features A) an atmospheric pressure ESI source, B)multipole focusing devices, C) gating lens for limiting the ions passing to the LIT, D) focusing octapole, E) linear ion trap (LIT) including two detectors, F) focusing multipole, G) a bent quadrupole - or "C trap" again for ion storage (bundles of ions), H) trajectory*

*with focusing electrodes and repellers from which ions are inserted laterally into the I) orbitrap* [70]*;*

The orbitrap used in the presented work is, as described in "3.2.3 Equipment for mass spectrometric analysis" below, a Thermo scientific Q- Exactive Orbitrap. It shows a different configuration and usage of compartments:

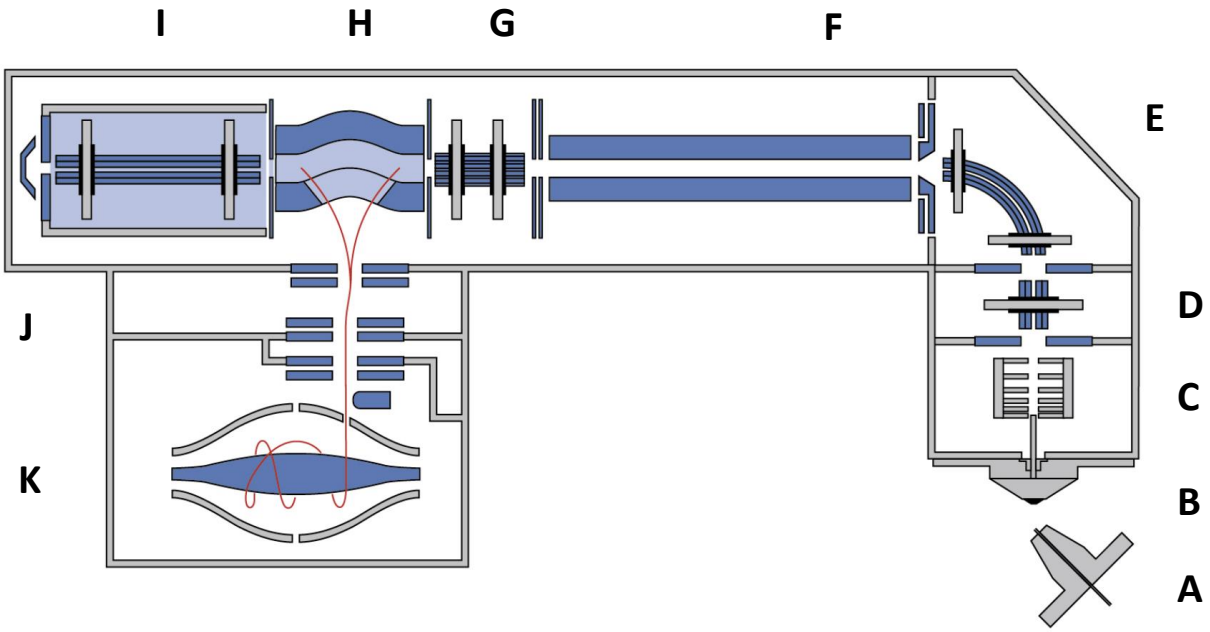



*Fig.7: Scheme of the Thermo scientific Q- Exactive Orbitrap; it features A) ionization source, B), ion transfer tube, C) S lens, D) injection flatapole, E) bent flatapole, F) quadrupole mass filter, G) octopole, H) C-trap (bent quadrupole), I) HCD collision cell, J), Z - lens,trajectory with focusing electrodes and repellers from which ions are inserted laterally into the K) orbitrap* [71]*;*

The ionization source is variable: an ESI source as well as an AP-SMALDI10, NSI, DESI and LTP can be used. The ions pass the ion transfer tube and are focussed by the S lens. Via injection multipole, they reach the bent flatapole, where collisional cooling takes place. After another lens, they enter the hyperbolic quadrupole, which allows an accurate selection of different mass / charge ions. They pass the octapole and reach the C - trap, where ions can intermediately be stored and the desired mass /charge ions can be released into the Z -lens, from where they enter the orbitrap in order to be analyzed. Alternatively, the can also be sent into the HCD collision cell.

Filled with nitrogen, this compartment allows fragmentation with different collision energies of the ions.

With this arrangement, many different operations are possible. One example is "multiplexing" between MS and MS/MS operations: Certain ions can be selected in the quadrupole and shown in the same spectrum (the quadrupole has to switch rapidly between the different narrow mass ranges). Subsequently, they can be fragmented in rapid succession and the resulting ions can again be filtered and characterized in joint analysis in the orbitrap. [71], [72]

# 2. Aim of the project

The routine analysis of bile acids at the laboratory for metabolic disorders at the LKH Graz is done by preparation of bile acids from patient serum, their chromatographic separation as well as mass spectrometric determination by ESI - MS. The mass spectrometer used for this purpose was an API 2000 triple Quad MS.
However, as there is a need of a more sensitive as well as a more accurate and reliable detection of bile acids, the mass spectrometer was replaced by the Thermo Q Exactive Orbitrap MS. This allows a 100 fold increased sensitivity towards bile acids as well as an accuracy of 6ppm.
In addition to these changes, the routine analysis of bile acids at the LKH Graz should meet the following improvements:

- As there is a need of better chromatographic separation between certain analytes (especially unconjugated chenodeoxycholic - and deoxycholic acid), chromatography should be optimized. In addition, a shorter runtime would of course be very beneficial for routine analysis.

- The spectrum of internal standards as well as natural targets for the mass spectrometric quantification of bile acids should be enlarged. This would allow the analysis and quantification of additional bile acids as well as a more accurate quantification.

  As all purchasable standards were arleady part of the method, this implies synthesis as well as purification and quantification of 32 bile acid standards.

- Additionally, bile acids should not only be able to be purified from patient serum, but alternatively also from stool, urine as well as bile.

# 3. Materials

## 3.1 Chemicals

### 3.1.1 general chemicals

| **Chemical** | **Brand** | **Type** |
|---|---|---|
| Distilled water | Fresenius Kabi | Aqua bidest. |
| Methanol | Merck | For analysis |
| Ethanol | | |
| Isopropanol | | |
| Hexane | | |
| Chloroform | | |
| Ethyl acetate | | |
| isooctane | | |

### 3.1.2 Chemicals used in HPLC/MS

| **Chemical** | **Brand** | **Type** |
|---|---|---|
| Distilled water | Sigma Aldrich | HPLC grade |
| Methanol | Sigma Aldrich | Gradient grade |
| Formic acid | Merck | For analysis |
| Acetic acid | Merck | For analysis |
| Ammonium acetate | Merck | For analysis |
| Calibration solution for positive mode (caffeine) | Pierce | LTQ Velos ESI positive Ion calibration solution |

| | | |
|---|---|---|
| Calibration solution for negative mode | Pierce | ESI negative Ion calibration solution |

### 3.1.3 Chemicals used for synthesis of bile acid standards

| **Chemical** | **Brand** | **Type** |
|---|---|---|
| All purchasable bile acid standards used except α-muricholic acid | Sigma Aldrich | Bile acid standard |
| α-muricholic acid | Steraloids | Bile acid standard |
| Glycine | Merck | Amino acid |
| Double - $^{13}C_2$ - labeled glycine | Sigma Aldrich | Amino acid |
| Taurine | CDN Isotopes | Amino acid |
| Quadruple - H2 - labeled taurine | CDN Isotopes | Amino acid |
| Trimethylchlorosilane | Pierce | Derivatizing reagent |
| N - (3 Dimethylaminopropyl) - N'-ethylcarbodiimide hydrochloride | Sigma Aldrich | Coupling reagent |
| K2CO3 | Merck | K2CO3 |

## 3.2 Equipment

### 3.2.1 General laboratory equipment

| **Equipment** | **Brand** | **Type** |
|---|---|---|
| Stripping apparatus | Pierce | Reactivyp III combined with Reactitherm III |
| Rotary evaporator | Büchi | Rotovapor EL130 |
| Vortex | IKA | MS3 basic |

| | | |
|---|---|---|
| Centrifuge | Beckman | GS - 15 R Centrifuge |
| precision scale | Rauch | Radwag Radon |
| pH indicator | Radiometer Copenhagen | PHM 82 Standard pH Meter |
| pH indicator stripes | Lactan | pH-Test 0-14 PT |

### 3.2.2 Equipment for chromatography

| **Equipment** | **Brand** | **Type** |
|---|---|---|
| **Thin layer chromatography** | | |
| Silica plates | Merck | 20 x 20 mm, Aluminum sheet |
| Glass chamber | / | |
| **Normal phase chromatography** | | |
| Silica gel | Macherey Nagel | 0.063 - 0.2mm / 70 -230 mesh ASTM |
| Glass pasteur pipettes | Assistent | No. 567/1, 5 x 150 mm, |
| **Solid phase extraction (SPE)** | | |
| Negative pressure chamber for SPE | Varian | / |
| SPE column, C18 reverse phase | Varian | Bond elute |
| **High performance liquid chromatography (HPLC)** | | |
| HPLC column (used in the optimized method) | Macherey Nagel | Nucleoshell 2.7µm, 50mm, C18 reverse phase |
| HPLC column | Phenomenex | kinetex, 2.6µm, 100x 3 mm, C18 reverse phase |
| HPLC column | Thermo | Hypersil Gold, 1.9 µm, 100 x 2.1mm, |

| | | |
|---|---|---|
| | scientific | C18 reverse phase |
| HPLC column | Agilent | Zorbax, 2.1 µm, 50mm, C18 reverse phase |
| HPLC column | Phenomenex | EZ: faast, 4 µm, 250 x 2mm, C18 reverse phase |
| HPLC column | Phenomenex | kinetex, 2.6 µm, 100 x 60mm, Pentafluorobenzyl phase |
| HPLC column | Waters | Symmetry, 3.5 µm, 1 x 150mm, C8 phase |
| Autosampler for HPLC | Thermo scientific | / |
| Syringe of Autosampler | Thermo scientific | Hamilton syringe |
| HPLC Pump | Accela | 1250 |
| HPLC Column compartment | Maylab | Mistraswitch |

### 3.2.3 Equipment for mass spectrometric analysis

| **Equipment** | **Brand** | **Type** |
|---|---|---|
| ESI Source | Thermo scientific | HESI II Probe |
| Mass spectrometer | Thermo scientific | Q Exactive Orbitrap |

# 4. Methods

## 4.1 Optimization of the chromatographic separation of bile acids

Bile acids, be it purified from serum be it from urine or stool, were solved in 200 µl of the second eluent used (see below). From these, 10 µl were injected per run to the chromatography column. The chromatography column used at this stage was the "phenomenex kinetex", 2.6µm 100x 3 mm with a stationary C18 reverse phase.
The first eluent consisted of MS grade distilled water with 1.2 % (v/v) formic acid as an ionization enhancer and 0.38 % (w/v) of ammonium acetate. The second eluent used consisted of MS grade methanol with the same modifiers in the same amounts. Both eluents were degassed in an ultrasonic bath for 5 minutes before usage.

The gradient of the eluents before optimization is depicted in Tab.1 below.

| Time [min] | First eluent [%] | Second eluent [%] | Flow rate [µl/min] |
|---|---|---|---|
| 0 | 60 | 40 | 400 |
| 1 | 60 | 40 | 400 |
| 9 | 30 | 70 | 400 |
| 13 | 30 | 70 | 400 |
| 15 | 5 | 95 | 400 |
| 18 | 5 | 95 | 400 |
| 21 (start of reequilibration) | 60 | 40 | 400 |
| 25 (end of reequilibration) | 60 | 40 | 400 |

*Tab.1: gradient as well as flow rate of both eluents used for chromatographic separation of bile acids before optimization of the method; First eluent: MS grade distilled water with 1.2 % (v/v) formic acid and 0.38 % (w/v) of ammonium acetate*

In order to optimize chromatography, the column was switched (a "Macherey Nagel nucleoshell", 2.7µm 50 mm, with a stationary C18 reverse phase was now used). The eluents remained the same, however, the flow rate was increased to 500 µl/min and the gradient was changed as observable in Tab.2 below.

| Time [min] | First eluent [%] | Second eluent [%] | Flow rate [µl/min] |
|---|---|---|---|
| 0 | 60 | 40 | 500 |
| 1 | 60 | 40 | 500 |

| | | | |
|---|---|---|---|
| 9 | 30 | 70 | 500 |
| 11 | 35 | 65 | 500 |
| 12 | 0 | 100 | 500 |
| 15 | 0 | 100 | 500 |
| 19 (start of reequilibration) | 60 | 40 | 500 |
| 23 (end of reequilibration) | 60 | 40 | 500 |

*Tab.2: Gradient as well as flow rate of both eluents used for chromatographic separation of bile acids after optimization of the method; First eluent: MS grade distilled water with 1.2 % (v/v) formic acid and 0.38 % (w/v) of ammonium acetate.*

The container of the chromatography column was set to 25°C.

## 4.2 Settings of the electrospray ionization process (ESI)

Settings of the electrospray ionization were not adapted, as any change resulted in a loss of the analytes' intensity.
Also, a different ionization modifier was tested (as described in "4.1 Optimization of the chromatographic separation of bile acids" above, the formic acid in the eluents). And indeed, the usage of acetic acid resulted in a higher yield of free bile acid ions. However, bile acid adduct ions are lower when run with acetic acid and it is impossible to expell formic acid from the chromatographic system as it is part of other methods. The usage of another ionization modifier would therefore result in formation of acetic acid as well as formic acid adducts and therefore cause confusion and the necessity of the observation of additional masses. Formic acid therefore remained the ionization modifier of choice. The resulting bile acid formic acid adduct ions can be considered in the analysis by simply adding 46.0058 to the different bile acid masses. This allows an even more sensitive analysis, since both the free bile acid ions as well as the adduct bile acid ions are detected. The increase is up to 60 % in case of an electrospray voltage setting of 3 and depending on the different bile acids.

| | |
|---|---|
| Sheath gas flow rate | 40 |
| Aux gas flow rate | 10 |
| Sweep gas flow rate | 0 |

| | |
|---|---|
| Spray voltage [kV] | 3.00 |
| Capillary temperature [°C] | 350 |
| S-lens RF level | 50 |
| Aux gas heater temp [°C] | 300 |

*Tab.3: ESI settings of the bile acid method;*

## 4.3 Settings of the mass spectrometer

Bile acids were analysed in negative mode. As there are many different masses to consider (the five major bile acids cholic -, chenodeoxycholic -, ursodeoxycholic -, deoxycholic-, lithocholic acid as well as the soon - to - be - approved therapeutic norursodeoxycholic acid as well as the mouse specific α-,β-,γ-,and ω-muricholic acid and hyodeoxycholic acid as well as all of their taurine and glycine conjugates and in case of low signal also all of their formic acid adducts) the bile acids were analysed in full scan mode.
The window was set to 370 m/z to 530 m/z, resulting in a resolution of 70,000.

## 4.4 Synthesis of bile acid standards

Cholic-, deoxycholic-, chenodeoxycholic-, lithocholic-, ursodeoxycholic-, norursodeoxycholic-, hyodeoxycholic, α -, β-, γ-muricholic-, the precursors dihydroxycoprostanic- and trihdroxycoprostanic acid as well as all of their taurine and glycine conjugates were used as standards.
From these, tauronorursodeoxycholic-, tauro γ-muricholic-, taurodihydroxycoprostanic-, taurotrihydroxycoprostanic as well as glyconorursodeoxycholic-, glyco α-, β-, γ-muricholic-, glycodicoprostanic and glycotricoprostanic acid had to be synthesised as they were not purchasable like the others.
D4-deoxycholic-, D4- lithocholic-, D3-dicoprostanic-, D3- tricoprostanic-, D4 – taurine conjugates of cholic-, deoxycholic-, chenodeoxycholic-, lithocholic-, ursodeoxycholic-, norursodeoxycholic-, hyodeoxycholic, α -, β-, γ-muricholic- as well as taurine and glycine conjugates of D3-dicoprostanic- and D3- tricoprostanic acid were used as internal standards.

From these, only D4-deoxycholic-, D4- lithocholic-, D3-dicoprostanic- and D3-tricoprostanic acid were purchasable. D4 - taurine conjugates of cholic-, deoxycholic-, chenodeoxycholic-, lithocholic-, ursodeoxycholic-, norursodeoxycholic-, hyodeoxycholic, α -, β-, γ-muricholic- as well as taurine and glycine conjugates of D3-dicoprostanic- and D3- tricoprostanic acid had therefore also to be synthesised. Synthesis of $^{13}C_2$ labeled glycine conjugates of cholic-, deoxycholic-, chenodeoxycholic-, lithocholic-, ursodeoxycholic-, norursodeoxycholic-, hyodeoxycholic, α -, β-, and γ-muricholic acid was not successfull.

For a better overview, see Tab.4 below.

| | natural target | | | internal standard | | |
|---|---|---|---|---|---|---|
| | **unconjugated** | **tauro** | **glyco** | **unconjugated** | **tauro** | **glyco** |
| **Cholic acid** | C | TCDC | GC | - | **TCDC-d4** | GC-d4 |
| **Deoxycholic acid** | DC | TDC | GDC | DC-d4 | **TDC-d4** | GDC-13C |
| **Chenodeoxycholic acid** | CDC | TCDC | GCDC | - | **TCDC-d4** | GCDC-d4 |
| **Lithocholic acid** | LC | TLC | GLC | LC-d4 | **TLC-d4** | GLC-13C |
| **Ursodexcholic acid** | UDC | TUDC | GUDC | - | **TUDC-d4** | GUDC-13C |
| **nor-Ursodexcholic acid** | nor-UDC | **Tnor-UDC** | **Gnor-UDC** | - | **Tnor-UDC-d4** | Gnor-UDC-13C |
| | | | | | | |
| **Hyodeoxycholic acid** | HYO | THYO | GHYO | - | **THYO-d4** | GHYO-13C |
| **alpha-Muricholic acid** | aMU | TaMU | **GaMU** | - | **TaMU-d4** | GaMU-13C |
| **beta-Muricholic acid** | bMU | TbMU | **GbMU** | - | **TbMU-d4** | GbMU-13C |
| **gamma-Muricholic acid** | gMU | **TgMU** | **GgMU** | - | **TgMU-d4** | GgMU-13C |
| | | | | | | |
| **Dihdroxycoprostanic acid** | DICOPR | **TDICOPR** | **GDICOPR** | DICOPR-d3 | **TDICOPR-d3** | GDICOPR-13C |
| **Trihydroxycoprostanic acid** | TRICOPR | **TTRICOPR** | **GTRICOPR** | TRICOPR-d3 | **TTRICOPR-d3** | GTRICOPR-13C |

*Tab.4: overview of the complete bile acid standard spectrum used in this work; bile acid standards marked in black: purchasable; bile acid standards marked in red: not purchasable and therefore newly synthesised, purified and their concentration determined; bile acid standards marked in grey: synthesis not successfull;*

Synthesis was done by conjugation of the different bile acid standard with glycine, taurine or D4 taurine (see Fig.8 below).

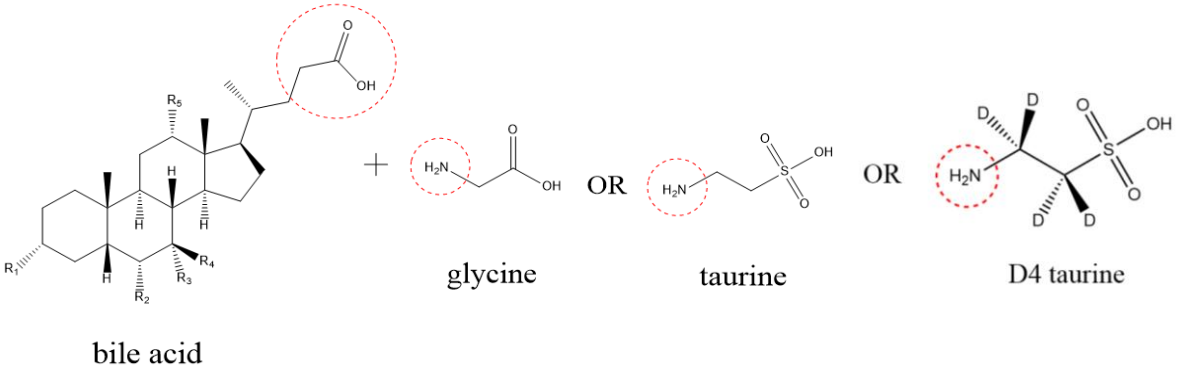


*Fig.8: Scheme of bile acid conjugation with glycine or taurine; Drawn with Chemdraw Ultra 13.0*

### 4.4.1 Synthesis of taurine and D4 taurine conjugated bile acid standards

Synthesis of taurine and D4 taurine conjugated bile acid standards could be achieved as described by Mills et al. [73].
2.5 µmol of the bile acid standard already solved in methanol was dried under a stream of nitrogen , 50µmol of taurine (0.00625g) or D4 taurine (only 0.005g per standard left) was added and 25µmol EDC (0.005g) and solved in 100µl of buffer (0.1M pyridine hydrochlorid in water, pH 5) and 50 µl of distilled water. Conjugation was allowed to occur at room temperature for 12 h.
Conjugation effecitivity varied among different bile acids, showing eg. 84.02 % for tauronorursodeoxycholic acid and 98.3% for tauro-γ-muricholic acid. Purity was determined by ESI-Orbitrap MS. However, as evaluation would be very inaccurate, only intensities were compared. Dilution for the LC-MS Orbitrap was 1:2500

### 4.4.2 Synthesis of glycine conjugated bile acid standards

As already described by Mills et al.[73], glycine conjugation can not occurr under the same conditions as taurine conjugation, as peptide linkage between the glycines amino group and another glycine‘s carboxygroup is faster to occur than peptide linkage between the bile acid’s carboxygroup and the glycine’s aminogroup – the glycines carboxygroup must therefore be protected- e.g. by esterification of the glycine’s carboxygroup with methanol.

#### 4.4.2.1 Esterification of glycine with methanol

Esterification was achieved using the method described by Jiabo Li and Yaowu Shai [74]:
0.2 mol (21.728g or 18.599 ml) of trimethylchlorosilane is added to 0.1 mol (7.75 g) of glycine whilest stirring. 100ml of methanol is then added and the resulting solution is stirred at room temperature for 24h. No further work is specified.
As these quantities exceed our requirements, only a thousandth was synthesized.

As described in "9.1 Esterification of glycine with methanol", wrong usages of TMCS or methanol result in a drastically reduced yield of esterification. This does not only show the significance of proper silylation of the glycine in order to „activate“ it for methylation, but also shows the critical conditions under which this takes place.

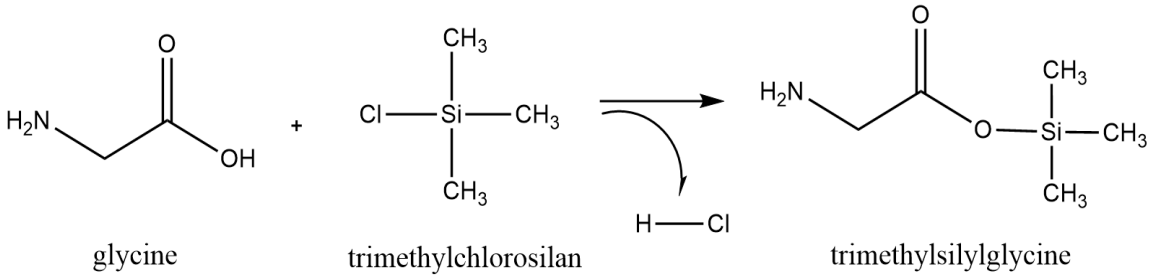



*Fig.9 a) silylationreaction of glycine with trimethylchlorosilan (TMCS) -"activation" of glycine*

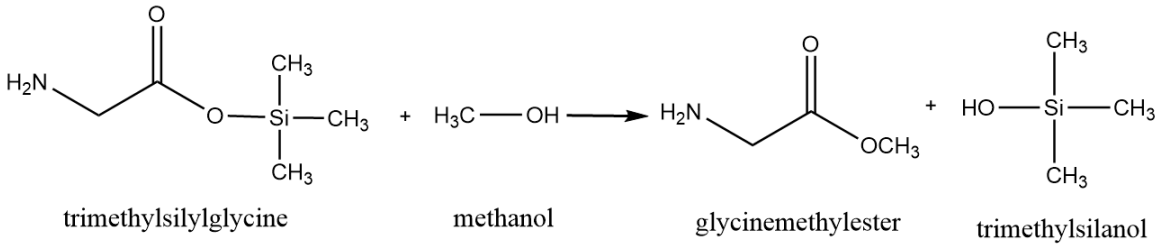



*Fig.9 b) subsequent esterification of glycine with methanol*

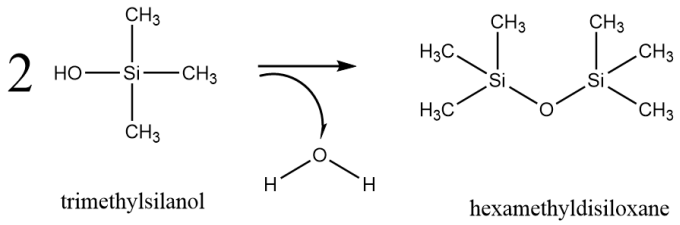



*Fig.9 c) hydrolysis of the remaining trimethylsilanol to hexametyldisiloxane*

*All figures were drawn with chembiodraw ultra 13.0*

As 2.5µmol of standards were used for synthesis of taurine conjugated bile acids, 2.5 µmol of standards should also be used for glycinemethylester conjugation. In relation,

taurine was used in a high excess (50µmol)- therefore glycinemethylester should be too. As the methylesterification did obviously not occurr to 100%, 58.8 µmol glycine was initially used-this was obviously calculated for an esterification yield of 85% instead of > 90%. However, as neither glycine nor TMCS or methanol is expensive and amounts can be „lost“ considering the small volumes that were pipetted (sticking on surfaces e.g.), a slightly bigger batch was used.

Per conjugation of one standard, this led to:
0.00462 g glycine (75 µmol) is silylated with 190,81µl (150 µmol) of TMCS for 30 minutes at room temperature while stirring. 75 µl of methanol is then slowly added and incubated for 24 h to let the conjugation process occurr.

#### 4.4.2.2 Conjugation

Conjugation of different bile acid standards with glycinemethylester could again be achieved as described in Mills et al.[73]: 50-75 µmol of glycinemethylester (depending on yield, pipetting errors etc. see above) solved in methanol was dried under a stream of nitrogen and incubated with 2.5 µmol of bile acid standard (also solved in methanol and dried under a stream of nitrogen ), 0.1 µl buffer solution (0.1M pyridine hydrochloride, pH 5) and 50µl of distilled water over night instead of just two hours. As both the glycinemethylester and the bile acid was not placed dry into the vial but solved in methanol and subsequently dried under a stream of nitrogen , it was advisable to make sure it was solved in the buffer solution and distilled water by scratching material of the vial’s wall and vortexing the sample.
While incubating, the mixture was magnetically stirred. It is crucial that this step happens in a glas vial instead of plastics as various glycine conjugated bile acids and their methylesters bind to plastic surfaces, resulting in a more or less (depending on the different polarities of conjugates) high loss of synthesis products. Especially glycoursodeoxycholic acid and glycodeoxycholic acid has shown to be affected.
This could be observed in a routine analysis of glycine conjugated bile acids of different patients at the LKH Graz. Of course natural fluctuations occurr, yet these diminished concentrations lead to the hypothesis of a non metabolic loss. To confirm the hypothesis, the same samples were analysed using glas vials as well as „lo bind“

plastic vials from eppendorf. In addition, controls were used in which only a known dilution of glycine conjugated standards were used.

#### 4.4.2.3 Saponification of glycine methylester conjugates

After conjugation, the protective methylgroup was removed again from glycine conjugates. This was achieved by adding a 10%$K_2CO_3$ solution to a boiling ethanol solution of the glycinemethylester conjugates as described by Mills et al.[73]. However, certain adjustements and steps had to be elaborated as they were not described.
1ml of HPLC grade ethanol was added to the conjugates, vortexed and heated at 80° C for 15 minutes. Up next, 1 ml of the 10% $K_2CO_3$ solution was slowly dripped on the boiling solution, resulting in an alkaline hydrolysis of the methylester.
After an incubation time of 15 minutes, the remaining ethanol was removed under a stream of nitrogen and 2ml of distilled water and 1ml of 0.1M pyridine hydrochloride buffer (pH 5) were added. At this point, the pH value of the mixture was adjusted to about 5 ("about" 5, as it could only be measured by pH indicator stripes) with ca 125µl of 38% HPLC grade HCl.

## 4.5 Purification of bile acid standards

### 4.5.1 Purification of taurine and D4 taurine conjugated bile acid standards

Taurine conjugated standards were solved in 5 ml of distilled water and purified using a Solid phase extraction: C 18 columns (bought from Varian Bond elute) were applied on a negative pressure chamber and preconditioned by applying 5ml of distilled water followed by 5ml of methanol and again 5ml of distilled water. Taurine conjugates were loaded onto the column and rinsed with 5 ml of distilled water, allowing to discard the EDC product as well as the unconjugated taurine. Taurine was not recollected, however for financial reasons D4 taurine could be reused by collecting the wash steps and evaporating the water using a rotary evaporator.

Taurine conjugates were then eluted with 5ml of methanol : distilled water 50:50 or, depending on the different polarities with methanol : distilled water : 60:40. Unreacted bile acids were eluted with methanol.

### 4.5.2 Purification of glycine conjugated bile acid standards

The in " Saponification of glycine methylester conjugates" described pH value reajustment was done in order to make sure unconjugated standards were present in a protonated state. Yet, glycine conjugated bile acids should still be present in a majorly deprotonated and therefore anionic state. This is due to the fact that the pH value of 5 lies below the pKa value of unconjugated bile acids (pKa value of unconjugated bile acids lies at 7.25 ± 0.85 [75]), yet still above the pKa value of glycine conjugated bile acids, as their pKa value lies at 5.85 ± 1.5 [75]. This was supposed to result in different properties of interaction with the stationary phase of the solid phase extraction columns between unconjugated and conjugated bile acids- allowing a successful purification:

The anionic state of the glycine conjugates resulted in less interaction with the reverse C18 stationary phase because of the higher charge. In addition, of course, this charge caused a better solubility in water and allowed them therefore to be eluted with the concentration of methanol : distilled water 50:50.

The unconjugated bile acids, on the other hand, were present in a protonated state and are therefore less charged - inducing more interaction with the stationary phase. Of course, being less charged, they were a little less soluble in water and eluted therefore better with the ratio of methanol : distilled water 60: 40.

This step was crucial for the successfull solid phase extraction using C18 reversed phase columns, as described in "Supplemental, purification of glycine conjugated bile acid standards".

However, as already described by J.L. Turumin observable in Tab.5 below, polarities of bile acids vary greatly. Glycine conjugates are of course more polar than unconjugated bile acids, yet they still lie very close in comparison to the far more polar taurine conjugates. This makes purification of glycine conjugates much more difficult.

For these reasons, separation after Mills et al. [73] (see "4.5.1 Purification of taurine and D4 taurine conjugated bile acid standards" above) was possible after the adjustment of the pH value from about 13 to about 5, yet not optimal as a part of

glycine conjugated bile acids still elute with a relatively high concentration of methanol, with which unconjugated ones already partially elute. For a more detailled description see "9.3 Purification of glycine conjugated bile acid standards" below.
As the method allowed a purification of bile acid standards (yet with a high loss of product) and no alternative worked better (as described in "9.3 Purification of glycine conjugated bile acid standards " below), it still was the method of choice.

## 4.6 Determination of the synthesised standards' concentration

In order to be able to calculate the concentration of a naturally ocurring bile acid in a sample, the intensity of detection or better said the area of the peak it provokes on detection is compared to a series of dilution of natural standards with known concentration as well as with added internal standards with known concentration (these have to be distinguished from the naturally ocurring analytes and are therefore isotopically labeled).
It is therefore crucial for every following sample that is measured with the newly synthesised standards, that its concentration is known.
However, determination of newly synthesised and purified standards has shown to be callenging, as one does not have -so to speak- the standards for the new standards. In addition, their concentrations vary greatly as for some standards a smaller batch was needed (base material was limited) or the synthesis was simply less effective.
First, all newly synthesised and purified standards were analysed separatly in the same dilution (1:100 after purification) in order to gather their elution times for identification purpose.
Second, they all were pipetted together in a dilution that seemed appropriate to the single ones just by observing their intensity of detection with the same dilution from the first step. This is done as a raw step of equilibration between the concentration of the different standards, as in order to be accurately determined they need to lie in the same range of intensity.
Up next, two internal standards (D4 glycocholic acid and D4 deoxycholic acid) that have been used in routine analysis for bile acid determination were added to the

synthesised standard mixture- of course again in a concentration that should more or less match the concentrations of the different synthesised standards.
The peaks were integrated and compared with xCalibur 2.3, dilution steps had to be considered and the concentration could be determined roughly and only internally (as of course no dilution series with already known concentrations could be matched - only dilution series of the same analyte can be matched).

Up next, the best possible stock concentration and volume for every single standard was calculated. It was not possible to have the same stock concentration for every standard, as concentrations varied greatly and even volumes differed sometimes.
This was due to a large difference in the pKa value between some unconjugated and conjugated bile acids (Glycodicoprostanic, Glycotricoprostanic, Taurotricoprostanic, D4 Taurocholic, D4 Taurodeoxycholic, D4 Taurolithocholic, D3 Taurodicoprostanic and D3 Taurotricoprostanic acid and Taurotricoprostanic acid), allowing them to be eluted with a higher methanol concentration and still not being contamined with the unconjugated counterpart.
This can be understood from the hydrophobic indices in literature (unfurtunately, hydrophobic indices of a few bile acids can not be found).

**Hydrophilic-hydrophobic index (HHI) of bile acids in mammals (2).**

| Bile acids | HHI of bile acids | Mammals |
|---|---|---|
| β-Hyocholic acid (β-HCA) | –0.60 | rats |
| α-Muricholic acid (α-MCA) | –0.51 | rats |
| β- Muricholic acid (β-MCA) | –0.40 | rats |
| Murideoxycholic acid (MDCA) | –0.33 | rats |
| Ursodeoxycholic acid (UDCA) | –0.17 | bears |
| α-Hyocholic acid (α-HCA) | –0.03 | pigs |
| Hyodeoxycholic acid (HDCA) | +0.09 | pigs |
| Cholic acid (CA) | +0.23 | human |
| Chenodeoxycholic acid (CDCA) | +0.83 | human |
| Deoxycholic acid (DCA) | +0.98 | human, primates, rabbits |
| Lithocholic acid (LCA) | +1.23 | human |

*Tab.5: different polarities of unconjugated bile acids* [76]*, Table 1*

Of course, slight differences in efficiency of purification (due to material differences etc.) can distort this theory.
As taurine is more polar than glycine, separation of taurine conjugated bile acids from their unconjugated bile acid was easier and always effective- on the contrast to the

separation of glycine conjugated bile acids from their unconjugated bile acid (as comprehensible from the sections: "4.5.1 Purification of taurine conjugated bile acid standards" and especially "9.3 Purification of glycine conjugated bile acid standards").

In order to delute the standards for the calculated concentrations, they were dried under a stream of nitrogen and resuspended in methanol. As also water was present (we recall the elution with methanol to distilled water 50:50 and/or 60:40), methanol was gradually added to the drying process in order to gradually remove the remaining water. Of course, this method is time consuming and not very gentle. Therefore, larger volumes of water were expelled using a rotary evaporator. In this case, a vacuum concentrator or "speedvac" would be ideal in order to gently remove water over night, yet the one available was not suited for these volumes.

The newly concentrated standards were now reevaluated in order to determine the concentration with higher precision. Basically, the process was the same: calculation of the concentration by comparing the peakareas of the synthesised standards with the peakareas of internal standards with known concentrations and of course recalling the dilution step. Yet this process was needed not to just reassure concentrations, but rather correcting them- as we can see in Tab.9 in "5.2 Determination of the synthesised standards' concentration" below.

## 4.7 Bile acid purification from human stool samples

Bile acid purification was partially already described in the previous work of the master project lab course "mass spectrometric analysis of bile acids and their precursors". However, as the work was continued afterwards and the preparation and analysis of stool samples is an integral part of the newly developed and adapted methods of bile acid analysis it will be presented completely in this work.

### 4.7.1 Sample collection and first preparation of stool samples

The preparation of bile acids from stool was already perfomed by L. Humbert et al. [35]-. It could therefore mostly be adopted:

In the presented case, 100 mg of freeze- dried stool, collected from 54 different subjects, were incubated with 2ml of NaOH for 60 minutes, followed by an addition of 4ml of distilled water. Up next, stool was homogenized. This coincedes with the "S1 protocol" as described by Humbert et al. However, the deproteinization step of the "S2 protocol" was also added: 4ml of acetonitrile (80%v/v) were added and incubated for 20 minutes at room temperature. Now, 100 µl of the four internal standards (D4 lithocholic acid, D4 glycocholic acid, D4 deoxycholic acid and D4 glycochenodeoxycholic acid) were added in a concentration of 2mmol/L to the mixture, followed by vortexing for 1 minute. Up next, the mixture was centrifuged for 20 minutes at 20,000g at 20°C.

In contrast to the protocols described by Humbert et al. [35], the supernatant was now dried under a stream of nitrogen at 50° C and the residue was resolved in 4 ml of ammonium acetate. Before being directly purified with solid phase extraction, the mixture was frozen and stored at -20°C.

### 4.7.2 Solid phase extraction of prepared stool samples

Again, C18 Varian Bond Elut SS – SAX. 500mg 3ml cartridges were used for solid phase extraction. After being connected to a negative pressure system, they were preconditioned with 5ml of distilled water, 5ml of methanol and again 5 ml of distilled water.

The unfrozen samples were now loaded onto the cartridges.

Hydrophilic material as salts etc. were discarded by washing with 20ml of distilled water. In order to remove lipophilic material like neutral lipids, 10 ml of hexane were applied. An additional washing step with 20ml of distilled water was added. Bile acids were now eluted with 2ml of methanol.

The protocol of L.Humbert et al recommends 5ml of methanol, however after the second milliliter, all bile acids have been eluted as we can see in Fig.10 below. As preparation of bile acids from stool should be optimized (concerning performance as

well as time), a lesser usage of methanol which has to be dried under a stream of nitrogen afterwards is superior.

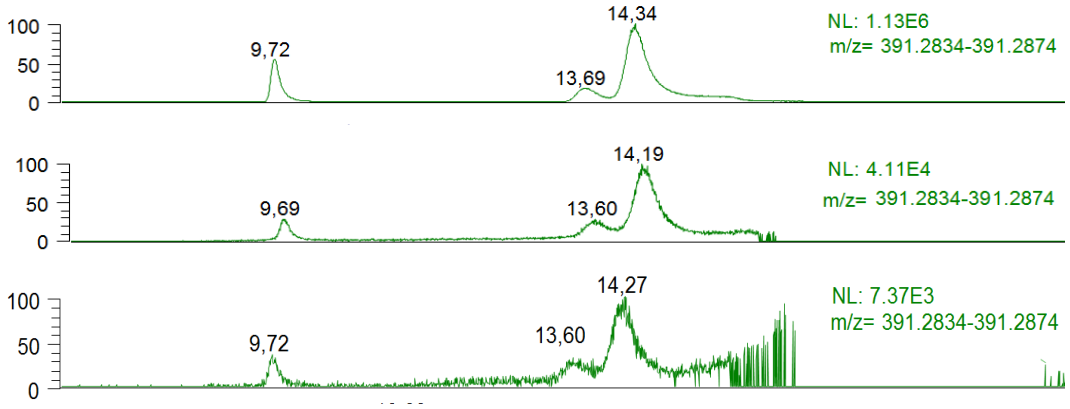



*Fig.10: Elution of bile acids with methanol at the purification with solid phase extraction; unconjugated ursodeoxycholic-, chenodeoxycholic- and deoxycholic acid are demonstrated as example; first eluting milliter: top chromatogram, second eluting milliliter: middle chromatogramm; third eluting milliliter: bottom chromatogram;*

As announced, the eluted bile acids were dried under a stream of nitrogen at 50°C and resolved in 100 µl. As still small particles were observable, an additional centrifugation step was added. However, the HPLC column was still clogged after applying the first samples. In "Supplemental, Troubleshooting with a clogged HPLC column" the attempt of unclogging the column is described.

In order to avoid this situation for the future, samples were diluted 1:100 - which is still concentrated enough for analytes to be detected as well as evaluated. Of course, even material of diluted samples will add up in the course of time resulting in an impairment of the column. For this reason, a washing step is run after ten stool samples. This washing step consists of purging the column with chloroform: methanol 2:1 for 30 minutes. This suffices to discard all lipophilic material accumulated in the chromatographic system. Chloroform is not used purely as this is not recommended for reverse phase chromatography.

Before injecting the next sample, the column is of course reequilibrated with water : methanol 60:40. For the detailed method see Tab.6 below:

| Time [min] | First eluent [%] | Second eluent [%] | Third eluent [%] | Flow rate [µl/min] |
|---|---|---|---|---|
| 0 | 0 | 0 | 100 | 300 |
| 20 | 0 | 0 | 100 | 300 |
| 23 | 0 | 100 | 0 | 300 |
| 33 | 60 | 40 | 0 | 300 |
| 35 | 60 | 40 | 0 | 300 |

*Tab.6: Eluent gradient and flow rate of the purging method; minute 0-20: actual purging in order to remove lipophilic substances; minute 20-23: transition to the next step; minute 23 -33: additional purging with pure methanol and transition to the reequilibration process; minute 33-35:reequilibration of the chromatography column; For consistency of the first two eluents see "4.1 Optimization of the chromatographic separation of bile acids" above; third eluent: HPLC grade chloroform and MS grade methanol in the ratio 2:1;*

## 4.8 Bile acid purification from human urine samples

In order to purify bile acids from human urine, different aspects have to be taken into consideration: Besides lipophilic substances, of course a lot of hydrophilic substances , salts and proteins have to extracted first.

As already described by Humbert L et al.[35], bile acids themselves are present only scarcely in unconjugated forms (only cholic- and deoxycholic acid). Also glycine and taurine conjugated bile acids, even though more polar, are also scarce in concentration (and again, only the conjugated forms of cholic- and deoxycholic acid are detectable).

As described in the introduction "1.1.2 Circulation of bile acids", they are rather present glucoronidated, sulfated or even glucosaminidated form in order to be better soluble in water. As described, these conjugation processes are mostly performed on the position 3'C. Sulfation or glucoronidation on this position does not compete with conjugation of glycine or taurine on the carboxygroup, and therefore sulfated and glucoronidated forms of glycine- or taurine conjugated bile acids are also present in urine. The large variety of bile acids therefore impose a challenge for analyzation.

Glucoronidated and sulfated bile acids could be detected with ESI- MS as described by L. Humbert et al. [35]. In fact, the direct measurment will also be tested in this work.

However, as for direct measurment every possible combination of glucuronide and/or sulfate on the different possible position (as also described in the introduction "1.1.2 Circulation of bile acids" C'3, C'6, C'7 and C'12) from all unconjugated, glycine - as well as taurine conjugated bile acid would have to be considered, complete analysis of glucuronidated and sulfated bile acids is not possible this way. In addition, as polarities differ greatly to unconjugated or even glycine- and taurine conjugated bile acids, a different method of HPLC separation is needed and - more importantly - a more time consuming one. This, of course, is not for the purpose of routine analysis. Also, the information in which form the bile acid is present (sulfated, glucoronidated or free) is of marginal importance for routine analysis. Therefore, sulfates and glucoronides should be split off enzymatically.

### 4.8.1 Sample collection and first preparation of bile acids from urine

Samples used were collected urine samples of 24 hours from different patients whose background (including health issues and medical attention) was unknown to the author for ethical reasons. Concentrations and occurrence of certain bile acids can therefore deviate from data published by Humbert et al. [35], where concentration of bile acids in urine samples collected in 24 hours from healthy patients are presented.

| | Serum (nmol/l) | Urine (nmol/l) | Stool (nmol/g) |
|---|---|---|---|
| TUDCA-3S | nd | 53.90 ± 36.19 | 0.34 ± 1.21 |
| GUDCA-3S | 37.87 ± 73.21 | 201.25 ± 246.55 | 0.75 ± 1.58 |
| UDCA-3S | nd | 25.05 ± 38.87 | 1.43 ± 3.38 |
| TUDCA | nd | nd | 0.30 ± 0.37 |
| TLCA-3S | 53.27 ± 23.98 | 108.6 ± 95.39 | 0.77 ± 0.63 |
| GUDCA | 99.62 ± 204.16 | nd | 2.39 ± 2.1 |
| CA-3S | nd | nd | 0.20 ± 0.26 |
| GLCA-3S | 76.34 ± 53.96 | 185.73 ± 151.63 | 1.26 ± 1.24 |
| THDCA | nd | nd | nd |
| TCA | 41.15 ± 29.21 | 16.29 ± 11.82 | 5.78 ± 4.32 |
| GCA | 112.81 ± 115.51 | 47.69 ± 51.04 | 10.15 ± 7.51 |
| CDCA-3S | nd | 19.33 ± 40.55 | 1.06 ± 1.20 |
| DCA-3S | nd | 49.53 ± 85.42 | 9.36 ± 15.51 |
| UDCA | 57.91 ± 110.71 | nd | 27.05 ± 61.13 |
| HCA | nd | nd | 6.71 ± 4.46 |
| TCDCA | 83.43 ± 90.08 | nd | 6.03 ± 5.00 |
| GCDCA | 705.03 ± 680.16 | nd | 22.28 ± 15.65 |
| HDCA | nd | nd | nd |
| TDCA | 47.89 ± 65.63 | nd | 4.32 ± 5.81 |
| LCA-3S | nd | nd | 7.76 ± 9.24 |
| GDCA | 255.25 ± 293.31 | nd | 19.19 ± 13.69 |
| CA | 169.12 ± 336.08 | 124.84 ± 193.22 | 44.71 ± 47.79 |
| TLCA | nd | nd | 0.51 ± 0.40 |
| GLCA | nd | nd | 6.68 ± 18.49 |
| CDCA | 177.84 ± 295.01 | nd | 54.8 ± 72.07 |
| DCA | 242.90 ± 300.96 | 19.01 ± 15.32 | 1920.10 ± 1390.50 |
| LCA | nd | nd | 1016.60 ± 647.31 |
| Total | 2160 ± 1797 | 853 ± 532 | 3171 ± 2095 |

nd, not detectable. Results are means values ± SD.

*Fig.11: Concentrations of different bile acids in urine, serum and stool samples Humbert et al.* [35]

Simple purification from urine was already done as described by Humbert et al. [35], where also different protocolls were compared for an optimizated purification. The protocoll using acetonitril for deproteinization („L3“) turned out to be the most suitable. As the amount of bile acids contained in 1 ml of 24h collective urine should very well suffice for analysis with the sensitive Orbitrap MS, only half of the described quantity was used- therefore only 5ml of acetonitrile was added for deproteinization. After incubation for 20 minutes at room temperature, the precipitated protein was removed by centrifuging for 15 minutes at 4000g and taking only the supernatant. This was dried under a stream of nitrogen  while heated with 50°C.

### 4.8.2 Solid phase extraction of prepared urine samples

After resuspension with 4ml of an ammonium acetate buffer (15mmol/l, pH 5.3), analytes were loaded onto C18 reverse phase columns, which were mounted on a

negative pressure chamber and preconditioned with 5ml distilled water, 5ml of methanol and again 5ml of distilled water.
Salts, sugars and other hidrophilic material was removed by washing the column with 20ml of distilled water, lipophilic material was removed by a subsequent wash with 10ml of hexane. After an additional washing step with 20 ml of distilled water, bile acids were eluted with 5ml of methanol.

### 4.8.3 Enzymatic deglucuronidation and desulfation of bile acids in urine

#### 4.8.3.1 Choosing the suited enzymes

No specific sulfatases were used in this work. The reason being is, that sulfatases are too structure specific - considering the already described high diversity of bile acids in biological samples these enzymes are not suitable for the use of desulfation [77], [78]. As described by griffith, solvolysis of sulfates is a possibility -yet for routine analysis unpractical. In addition, most protocols resulted in changes of bile acid structure: Usage of dimethoxypropane or acetone showed formation of acetonides from vicinal cis glycol structures, whereas ethyl acetate even caused transesterification. [78]
As already described by Volkmar Graef et al., the β glucuronidase of molluscs also contains a sulfatase function - in contrast to the bacterial β glucuronidases like the one from E. coli [79].
Therefore, incubation of samples with the β- glucuronidase from Helix pomatia will result in higher concentrations of measured bile acids than of the ones incubated with deglucuronidase from E. coli - besides possible differences in efficiencies of the enzymes.
In order to be able to deduce the amount of sulfated and the amount of glucuronidated bile acids without actually measuring them directly, data of samples incubated with the two different enzymes need to be compared.

As it was the case for the evaluation of stool samples, it shall be reminded that the generation of data regarding sulfate, glucuronide or every other concentration of bile acids in urine is not performed for medicinal and biological investigation purpose, but

for validation of the established method. In order to achieve this, values have, of course, to be compared with values from literature.

Of course, the experiment should still be conducted under ideal conditions for both enzymes. Still, the mentioned possible differences in efficiency of deglucuronidation of the two enzymes could obviously affect the calculation of sulfates and glucuronides.
In the report of Volkmar Graef et al., we can in fact observe differences in the two enzymes' efficiencies:
Samples from human urine were reported to be incubated with the β glucuronidase of E. Coli, resulting in an effective deglucuronidation of the substrates analysed (p-Nitrophenylglucuronide, Phenolphthaleinglucuronide, Estriolglucuronide, Pregnanediolglucuronide and 17- Hydroxycorticosteroidglucuronide). In fact, the yields for p- Nitrophenylglucuronide, Estriolglucuronide and 17-Hydroxycorticosteroidglucuronide were higher for samples incubated with the β glucuronidase from E.Coli than with the β glucuronidase from Helix pomatia (both incubated at their optimal pH value).
In addition, also the substrates Androsterone, Etiocholanolone, Dehydroepiandrosterone, Pregnanediol, Pregnanetriol, Estriol and 17-Hydroxycorticosteroids, freed of deglucuronidaseinhibitors like saccharo 1,4- lactone, gluconic acid and saccharic acid showed a better deglucuronidation when incubated with the β glucuronidase of E. coli then with the β glucuronidase from Helix pomatia.
Estriol glucuronide was deglucuronidated fastest with the glucuronidase from E. coli, reaching the maximal hydrolysis after two hours. Of course, the efficiency and rate of hydrolysis can depend on the substrate, as described and also proven in the conducted experiment. [79]

Even if the β glucuronidase of E. Coli is not able to desulfate bile acids, both enzymes will be tested in order to be able to calculate the amount of sulfated and glucuronidatd bile acids.

#### 4.8.3.2 Buffer for incubation and the optimal pH value

After the methanol was dried off under a stream of nitrogen , purified bile acids were resuspended in 500µl of sodium acetate buffer (0.1mol/l, pH 5.4) in order keep the pH value in an optimal range for the enzyme to remain active. 0.1 M Sodium acetate buffer was already used by Toshiaki Momose et al.[2] as well as noted by Michael Court [80] and therefore selected as the buffer of choice.

The optimal pH value for the first enzyme used (β-Glucuronidase from Helix pomatia Type H-1, sigma aldrich) lies at 4.5 for glucoronidase activity yet at 6.2 for sulfatase activity [81]. The report by Toshiaki Momose et al.[2] reassured the ideal pH value for an incubation with β-Glucuronidase from Helix pomatia.
For the second enzyme used (β-Glucuronidase from Escherichia coli Type X-A) only glucuronidase activity is reported and no ideal pH value, however activity was measured by Sigma aldrich at pH 6.8 [81]. Also, Volkmar Graef et al. reported an incubation of different steroid glucuronides with the β-Glucuronidase from Escherichia coli at a pH value of 6.5 [79].
In order to hit the proper pH value for both the glucuronidase and sulfatase activity of the β-Glucuronidase from Helix pomatia as well as the glucoronidase activity of Escherichia coli, a pH value of 5.4 was chosen (take into consideration that to 500µl of buffer, 200µl of enzyme solved in distilled water are added, which will increase the pH value to 5.45).

#### 4.8.3.3 Quantity of enzyme needed

In order to determine how much enzyme suffices to completely desulfate and/or deconjugate bile acids, different sources have been checked:

Toshiaki Momose et al.[2] e.g. used 0.1 units of β glucuronidase from Helix pomatia for 100µg of bile acid glucoside standards. This indication is not ideal for our purpose, as the concentration remains unknown and we do not use bile acid glucoside standards but a natural occurring mixture of unconjugated bile acids, conjugated ones, sulfated and glucuronidated ones. As they have very different specific weights, this makes the ratio of enzyme to substrate an estimation. However, calculating from the information given:

As one used substrate was the standard HDCA-3-Glc with a specific weight of 571.73 g/mol, of which 100µg were incubated with 0.1 unit of β glucuronidase – this equals 57.173 nmol of substrate per 0.1 unit.
In the work form Eva Pump [82], a stock solution was used solving 2.23 g of enzyme in 10ml of distilled water, of which 10µl were incubated with 300µl of buffer with 200µl of a bile acid dilution of 0.1mmol/L.
This equals 0.02 nmol bile acid standards and- given the fact that 2.23 g of enzyme equal 669,000 units of β glucuronidase but only 22,300 units of sulfatase, incubated with 66,900 units of β glucuronidase and 2,230 units of sulfatase. This equals 300,000 units of β glucuronidase and 10,000 units sulfatase per gram (!).
We can see, vastly different approaches have been made. As we not only want our substrate to be surely digested- but also not to use the expensive enzymes in far exceeding concentrations especially for routine purpose, a third approach had to be made. In addition, these calculations can be inaccurate as different batches have varying specific activites, which have not been mentioned by the authors. Also, as already described, the substrates used are different. Therefore, besides possible different digestion effectivities of the enzyme, also concentrations are hard to be calculated.

On the manufacturers homepage of the used enzyme, the unit is defined by: “One Sigma or modified Fishman unit will liberate 1.0 µg of phenolphthalein from phenolphthalein glucuronide per hr at 37°C at pH 5.0 (30 min assay) [81].”

Of course, the substrates complete mass has to be estimated:
In Humbert et al. [35] a concentration varying between 643.39mmol/L and 1337.99 mmol/L of sulfated bile acids is stated for healthy patients, glucuronides have not been measured.
As 1ml of urine will be used for our measurement and estimating about 450 g/mol as a median specific mass (as we can see in they range from 455.2467 to 544.2586) we can expect max. 600µg of sulfated bile acids, glucuronides are present in a lower concentration.
600 units should thereby be used. Given the fact that by definition, these should be digested in an hour and incubation will take place between two and >12 hours (over night), even high bile acid levels of e.g. patients with biliary obstruction should be

measured correctly. In addition, pH levels are not ideal for sulfatase function as decribed above.
As the used batch of β -glucuronidase from Helix pomatia type H1 has a specific activity of 449,300 units /g, 0.0013509 g should be used per sample.
Of course, the same amount should be used of the β -glucuronidase from E. Coli. The used batch has a specific activity of 1,134,600 units/g. 0.0010698 g should be used per sample.

#### 4.8.3.4 Optimal temperature

As noted by Michael Court and Volkmar Graef et al. as well as tested by Toshiaky Momose et al., 37°C is the ideal incubation temperature for both enzymes. However, as no comparing data is shown, differences in enzyme activity were questioned (as it states the necessity of the usage of a sample limiting water bath in routine analysis) – incubation was therefore tested at both 37°C and room temperature (24°C).

#### 4.8.3.5 Optimal incubation time

Even though over night incubation was reported by Michael Court [80] as well as by Eva Pump [82]“, and incubation times between 0-25h were tested by Toshiaki Momose et al [2]. as well as 24h were used in the case of deglucuronidation of different steroids with both enzymes by Volkmar Graef et al.[79], optimal incubation time was not clear as well- as there is a lack of data showing comparisons between different incubation times with β-Glucuronidase from Helix pomatia as well as Escherichia coli at 37° C of *biological samples* regarding bile acids.
The substrate used by Toshiaki Momose et al. for enzymatic digestion with β-Glucuronidase from Helix pomatia were unconjugated Hyodeoxycholic acid 3- Beta-d-glucuronide as well as unconjugated hyodeoxycholic acid 6-Beta-d-glucuronide, unconjugated murideoxycholic acid 3-Beta- d-glucuronide and unconjugated chenodeoxycholic acid 3- Beta- d glucuronide. Of course, data with different glucoronidated bile acid standards used is a more accurate depiction of biological samples. Yet already the time needed to deglucuronidate these standards varied

greatly, indicating a differing affinity of the β-Glucuronidase from Helix pomatia towards different bile acids as well as a differing efficacy for glucoronides on different positions:
HDCA 3-GlcA was deglucuronidated faster than MDCA 3-GlcA or CDCA 3-GlcA at the same temperature with the same concentrations used. Also, HDCA 3-GlcA was deglucuronidated faster than HDCA 6-GlcA [2].
This large difference could also be affirmed by our conducted experiment. This indicates that not only different unconjugated bile acids and in addition with differing glucuronid positions are deglucuronidated with a different rate and efficacy by the β-glucuronidase from Helix pomatia, yet probably also glycine and taurine conjugates. Extrapolation of the reported methods (especially regarding the incubation time) established with four standards to biological samples is therefore not possible.
In addition, the sulfatase activity of the β-glucuronidase from Helix pomatia was not covered by the above mentionened works or elsewhere neither towards sulfated bile acid standards nor the complex variety of sulfated bile acids in biological samples.

Therefore, the effect of sample incubation after two hours as well as ca. 12 (over night) was observed.

### 4.8.4 Direct measurment of sulfated bile acids

As already described in "4.8 Bile acid purification from human urine samples" above, direct measurement of sulfated bile acid is not the purpose of the established method. In fact, it would demand another method of chromatography to optimally elute the far more polar sulfated bile acids as well as conjugated and unconjugated non sulfated bile acids. In addition, elution time, peakform and the higher mass range is a problem.
However, in order to evaluate the integrity of the method of extraction, purification and analysis, comparison with published data is crucial. Therefore, direct measurement of different sulfated bile acids was performed with success:
UDCA-3S, GUDCA-3S, CDCA-3S, GCDCA-3S, DCA-3S, GDCA-3S, GCA-3S, LCA-3S and GLCA-3S were detected and measured.

However, the mass of sulfated taurine conjugated bile acids were too high for the used method- yet as observable in the Fig. 11 above, as well as deductable from Fig. 25, 26, 27, and 29, they are present in very small amounts.

As we can see in Fig.12 below, peaks are broad because of the analytes' high polarity. They can still be integrated and therefore the amount can be calculated, yet for routine usage an adaptation of the gradient would definitely be recommended. Elution times were of course shorter than of non-sulfated bile acids, yet detectable. The mass range for detecting all sulfated bile acids would also need to be increased by at least 70m/z- which of course lowers the accuracy of detection. The m/z window for the full scan mode is currently set from 390 (391.28 are the first masses to be analysed) to 570 (556.33 being the highest mass analysed).

As sulfated bile acids have a 79.9568 higher mass than their non-sulfated counterparts, sulfated glycine conjugated as well as sulfated unconjugated bile acids could be detected.

We can see, that identification of sulfated bile acids is not easy, as there are no reference retention times. However, they will very likely have the same order of elution as their unsulfated counterparts.

#### 4.8.4.1 Mass range of sulfated bile acids and their chromatogram:

##### 4.8.4.1.1 Unconjugated sulfated bile acids:

| **Bile acid:** | **Mass** | **Retention time** |
|---|---|---|
| S-hyodeoxycholic acid | 471.2422 | ? |
| S-ursodeoxycholic acid | 471.2422 | 6,50 |
| S-chenodeoxycholic acid | 471.2422 | 7,33 |
| S-deoxycholic acid | 471.2422 | 8,28 |
| S-α-muricholic acid | 487.2371 | ? |
| S-β-muricholic acid | 487.2371 | ? |
| S-γ-muricholic acid | 487.2371 | ? |
| S-cholic acid | 487.2371 | ~5,27 |
| S-lithocholic acid | 455.2473 | ? |
| S-norursodeoxycholic acid | 457.2260 | ? |

*Tab.7: Masses of unconjugated sulfated bile acids as well as their elution times with the method used*

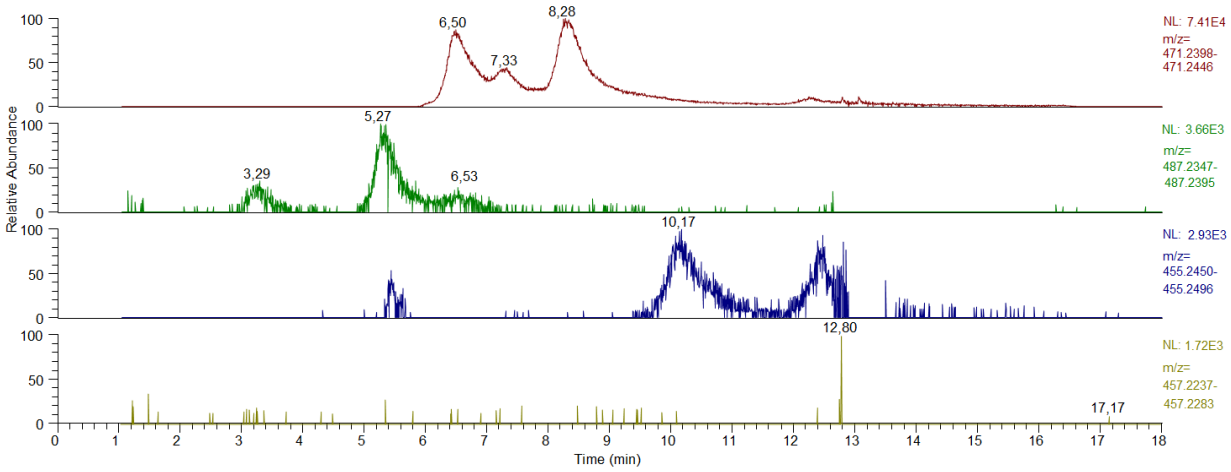



*Fig.12: Chromatogram of sulfated unconjugated bile acids of Sample 3 (incubated without any enzyme); for masses and identification of peaks see Tab.7 above;*

Looking at the "red" chromatogram and taking into consideration, that hyodeoxycholic acid is present only in scarce amounts in humans, the first peak resembles the sulfated ursodeoxycholic acid, the second peak matches the sulfated chenodeoxycholic acid while the third matches the sulfated deoxycholic acid.

Identification of the "green" chromatogram is more difficult. Three peaks are observable, whereas the third is very shallow. Extrapolating from the unsulfated standards, the last peak would be the sulfated cholic acid. This would suggest the presence of another isomere at 3,29, which seems strange as muricholic acids, as already described, are not usually present in humans. Yet, intensity is very low and even the highest peak does only show little distinction from the background noise. It can therefore not be stated that more than one sulfated bile acid with the mass 487.2371 is present in this sample.

We must not forget, that the gradient obviously is not adapted for such polar bile acids, causing the analyte therefore not to elute in a single sharp peak.

The peak in the "blue" chromatogram resembles the sulfated lithocholic acid. However, its low intensity does not distinguish it from background signal - no sulfated lithocholic acid is present.

Also in the "yellow" chromatogram only background signal can be detected. This makes sense, as no sulfated norursodeoxycholic acid was administered.

#### 4.8.4.1.2 Glycine conjugated sulfated bile acids:

| **Bile acid:** | **Mass** | **Retention time** |
|---|---|---|
| S-glycohyodeoxycholic acid | 528.2636 | ? |
| S-glycoursodeoxycholic acid | 528.2636 | 6,46 |
| S-glycodeoxycholic acid | 528.2636 | 12,36 |
| S-glycochenodeoxycholic acid | 528.2636 | 9,35 |
| S-glyco-α-muricholic acid | 544.2586 | ? |
| S-glyco-β-muricholic acid | 544.2586 | ? |
| S-glyco-γ-muricholic acid | 544.2586 | ? |
| S-glycocholic acid | 544.2586 | 6,86 |
| S-glycolithocholic acid | 512.2687 | 12,37 |
| S-glyconorursodeoxycholic acid | 434.3106 | ? |

*Tab.8: Masses of glycine conjugated sulfated bile acids as well as their elution times with the method used*

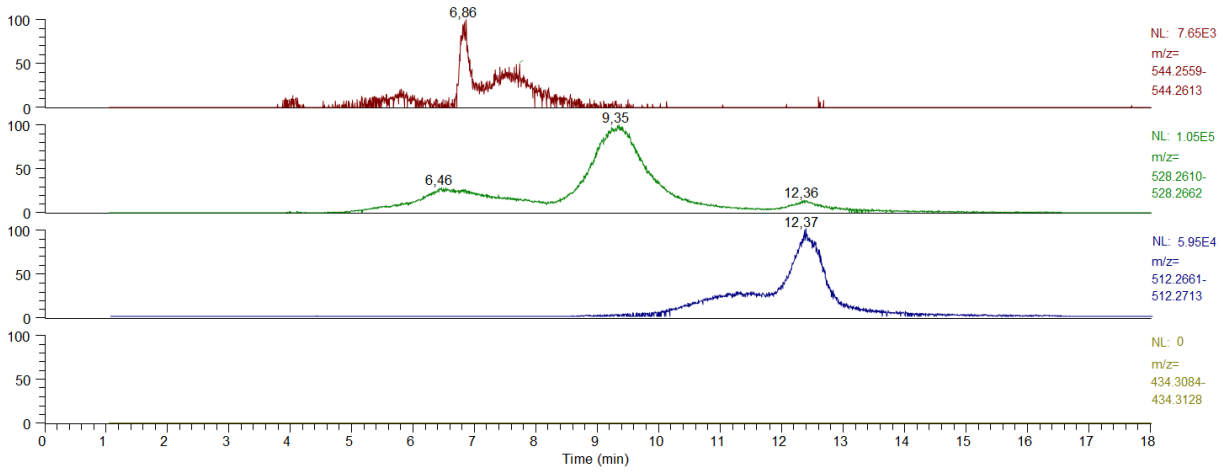



*Fig.13: Chromatogram of sulfated glycine conjugated bile acids of Sample 3 (incubated without any enzyme); for masses and identification of peaks see Tab.8 above;*

Concerning the sulfated glycine conjugated bile acids, identification is a little easier as we can see in Fig.13: The only peak in the "red" chromatogram - even if distorted and very low - resembles the sulfated glycine conjugated cholic acid.

The three peaks in the "green" chromatogram belong to sulfated glycoursodeoxycholic acid, sulfated glycochenodeoxycholic acid and sulfated glycodeoxycholic acid.
Interestingly, a certain amount of sulfated glycine conjugated lithocholic acid is present- this is the only analyte representing the peak in the "blue" chromatogram. It is a good example of how peaks are eluting in the course of minutes rather than sharp and fast.
Of course, as we can see in the "yellow" chromatogram, no sulfated glycine conjugated norursodeoxycholic acid can be detected.

Evaluation of directly analysed sulfated bile acids was conducted manually and "more simple" than for the usual unsulfated bile acids: As, of course, no sulfated internal standards for internal quantification and sulfated natural standards for quantification with the dilution series were available, other standards were chosen:

For the calculation of the area ratio, peaks of the internal standard D4 Glycocholic acid were integrated and compared with the peaks of the sulfated bile acids. The concentration is now usually calculated by entering the calculated area ratio as the y value into the linear equation of the corresponding natural standard. This linear equation is calculated by analysis of a dilution series. The x value corresponds to the concentration of the analysed substance. In this case, the linear equation of the glycocholic acid standard dilution series was used.

$$y = 0.369647 * x + 0.0109701$$
$$R^2 = 0.99$$

## 4.9 Preparation and analysis of bile acids from mouse bile

As described by Humbert et al.[35], preparation of bile acids from human bile can be achieved using the same extraction protocol as for human urine. As explained, the most efficient one is the protocol also used in this work, using acetonitrile for denaturation of proteins. The only adjustment is that, since less bile is needed as bile acids are more concentrated here, also less acetonitrile is needed. As described, the

highly sensitive Orbitrap Ms allows the usage of less urine (1 instead of 2ml) and therefore also only 5 instead of 10ml of acetonitrile for the denaturation step. This of course abbreviates routine analysis, especially the drying step with a stream of nitrogen.
The same should apply to bile samples: 500 µl instead of 1ml suffice, and therefore only 2ml of acetonitrile is needed for denaturation. The rest of the protocoll should not need modification and can be found in [35].

The protocol used for extraction of bile acids from human body fluids should also apply to mouse body fluids. Yet the composition differs: As already presented by de aguiar vallim et al.[83] observable in Fig.14 below, there seems to be a larger variety of bile acids in mice.

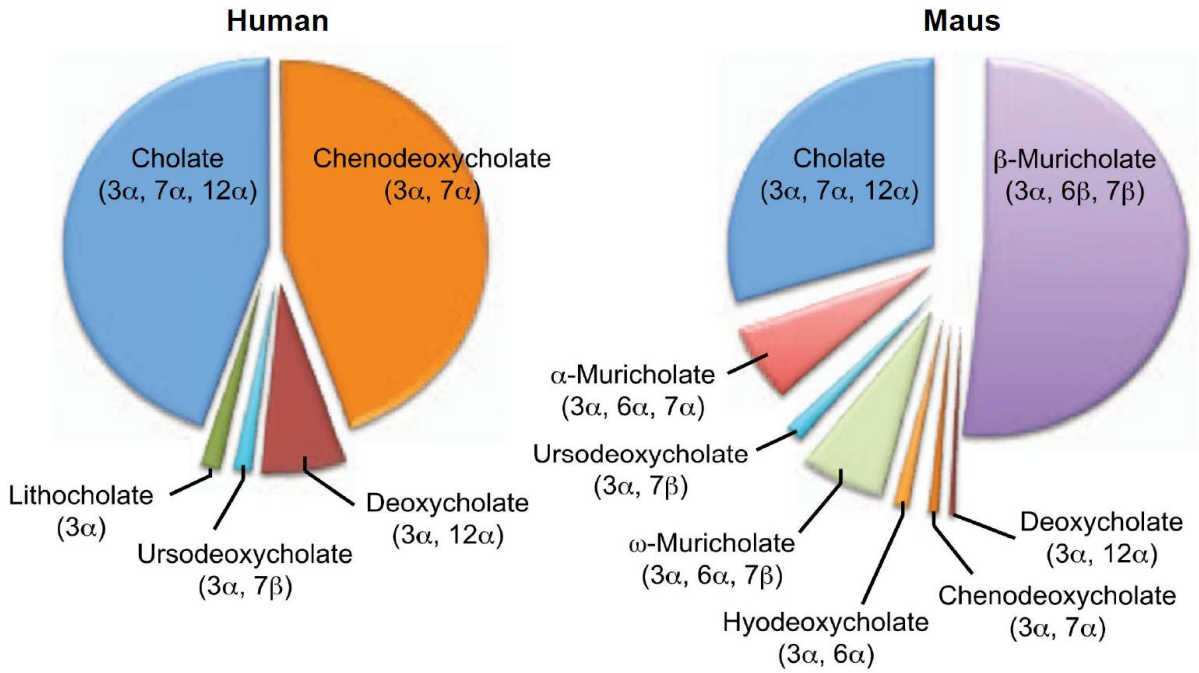



*Fig.14: Comparison of bile acids between human and mice* [83]

Especially muricholic acids are present in mice in high concentrations, though are, as described in "5.5 Example of a chromatography of bile acids purified from urine" below, only rarely and when, only scarcely detectable in humans. Yet the unconjugated γ - muricholic acid as well as all glycine - and taurine conjugated bile acids have not been investigated for this scheme: It should therefore be looked further into the distribution of bile acids in mice.

α-, β -, γ- and ω- muricholic acid could be purchased and, as described above, conjugated with taurine, D4 taurine and glycine. As, unfortunately, ω- muricholic acid was not available at the time conjugation was conducted (and the masterthesis is limited), conjugated standards of it were not available. As we can see in Tab.4 above, glycine and taurine conjugated standards of human bile acids (including hyodeoxycholic acid) were available.
The difficulty of the next step lies in the establishment of a chromatographic method which allows the simultaneous analysis of all listed bile acids, while keeping the analytes' peaks separated and evaluable.

As we can see in Fig.15 below, α- and β- muricholic acid have very similar polarities and elute - at least when using the recently established method - almost at the same time. However, in unconjugated form they are still separated and individually integrable. This is not the case for taurine conjugates (and D4 taurine conjugates, for that matter): Here, the peaks of tauro-α- and tauro-β- muricholic acid merge together - they can not be evaluated.
The identification of the four isomeres is possible as the elution times with this method have been noted when standards were analyzed separatly (as described in "4.6 Determination of the bile acid standards' concentration" above).
With this method, glycine conjugated α- and β- muricholic acid can not be separated well enough for a correct evaluation as well. It is important to note that other bile acids can be detected and evaluated well.

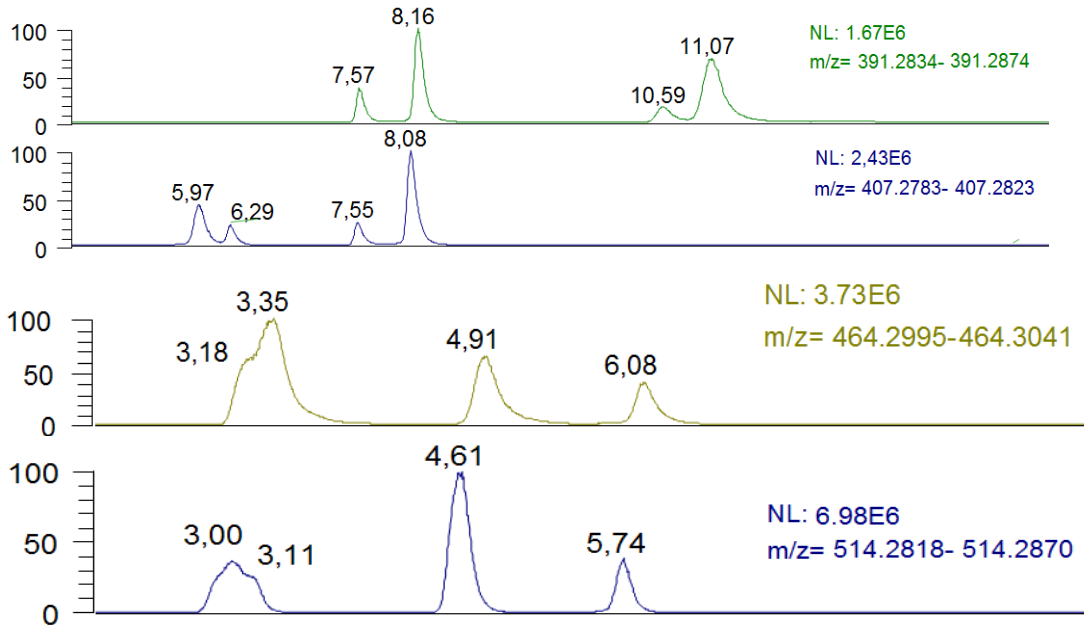



*Fig.15: Chromatography of α- and β- muricholic acid as well as their taurine and glycine conjugates using the Macherey nagel c18 Nucleoshell, 50mm; 2.7µm column and the recently established gradient (see for more detailed information)*
*unconjugated hyodeoxy-, ursodeoxy-, chenodeoxy- and deoxycholic acid: top first; unconjugated α-, β - and γ- muricholic as well as cholic acid: top second; glycine conjugated muricholic acids: third; taurine conjugated α-, β - and γ- muricholic acids: bottom; indications are given in order of elution;*

The method had therefore to be modified again:

### 4.9.1 Establishing a chromatography protocol for mouse bile acids

The goal was separation of glycine- and taurine conjugated α- and β- muricholic acid in order to be individually integrable while maintaining the chromatographic performance for every other bile acid.
Before optimization could occur, a sample with every needed bile acid standard, including all muricholic acids, had to be mixed - of course in the same concentrations. At this stage, the unconjugated ω- muricholic acid was still not available. A possible interference with the α-, β - and γ- muricholic as well as cholic acid (all have the same mass) could therefore not be analysed let alone optimized.

At first it was checked if an adaptation of the gradient without changing the column could have a positive impact on the separation:
As it was clear from the beginning, that the gradient was already optimized to a high degree and slight switching would very likely provoke a downgrade of the analytes' elution, only the equilibration gradient was switched from 60:40 distilled water: methanol to, 50:50, 40:60 and 30:70
Of course, a less polar gradient resulted in a faster elution of every bile acid. The early eluting bile acids were therefore directly solved in the mobile phase - hindering interaction with the column.
For this reason a more polar equilibration gradient was tried: 70:30
However, this also resulted in an inproper elution.

Up next, as muricholic acids are relatively polar bile acids and elute early with a higher concentration of water, a short implemented increase in methanol was thought alter the elution - and probably different for the different muricholic acids.

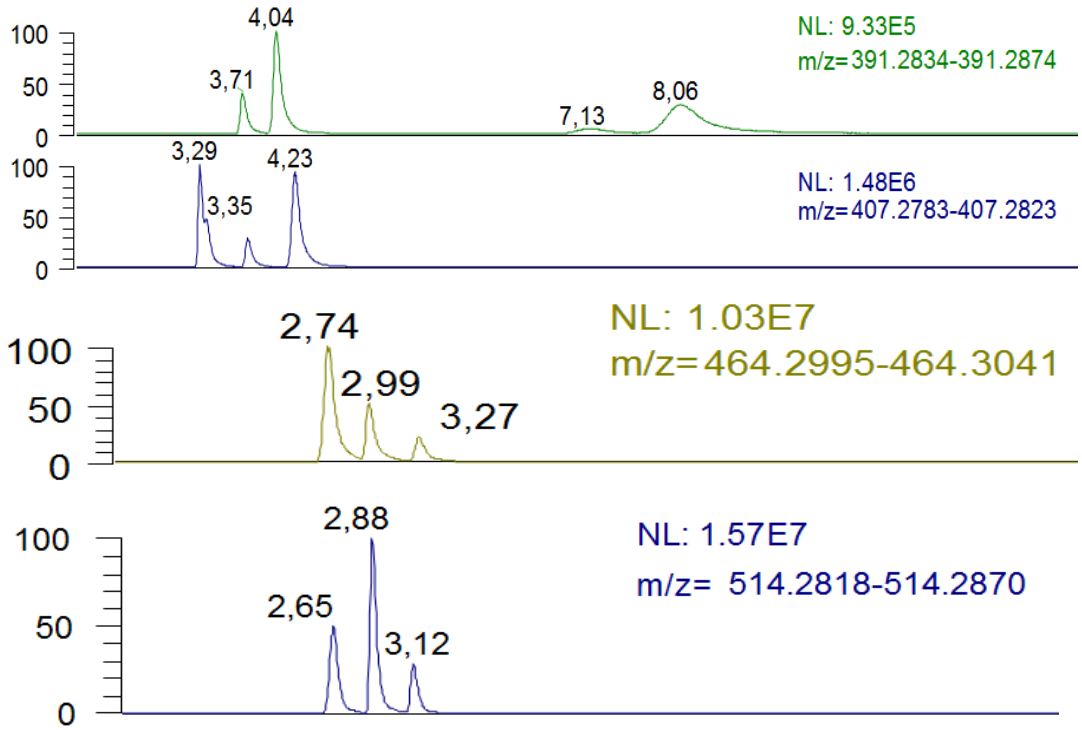



*Fig.16: Chromatography of α- and β- muricholic acid as well as their taurine and glycine conjugates Altered gradient, using a Macherey nagel c18 Nucleoshell, 50mm; 2.7µm column*

*unconjugated ursodeoxy-, hyodeoxy- chenodeoxy- and deoxycholic acid: top first; unconjugated α-, β - and γ- muricholic as well as cholic acid: top second; glycine conjugated muricholic and cholic acids: third; taurine conjugated α-, β - and γ- muricholic and cholic acids: bottom; indications are given in order of elution;*

As we can see, this was not the case. This also caused the peaks of the unconjugated muricholic acids to merge, which previously were separated. In addition, the peak forms of chenodeoxycholic- and deoxycholic acid suffered severely. Glycine and taurine conjugated muricholic and cholic acids eluted early and were not separated better.

However, a higher concentration of distilled water for a short period of time is already implemented in the established method. As repeatedly described, the gradient was already optimized, there is no room for further elaboration.

For this reason, columns were exchanged. Of course, during the optimization process various columns had been already tried out. These rather short C18 columns were therefore left out.

Instead, a second C18 Macherey nagel Nucleoshell, 50mm; 2.7µm column was added behind the first one with a short connection tube. This was thought to further separate the analytes and therefore achieve a better chromatographic performance. As we can see in Fig.17 below, this was not the case.

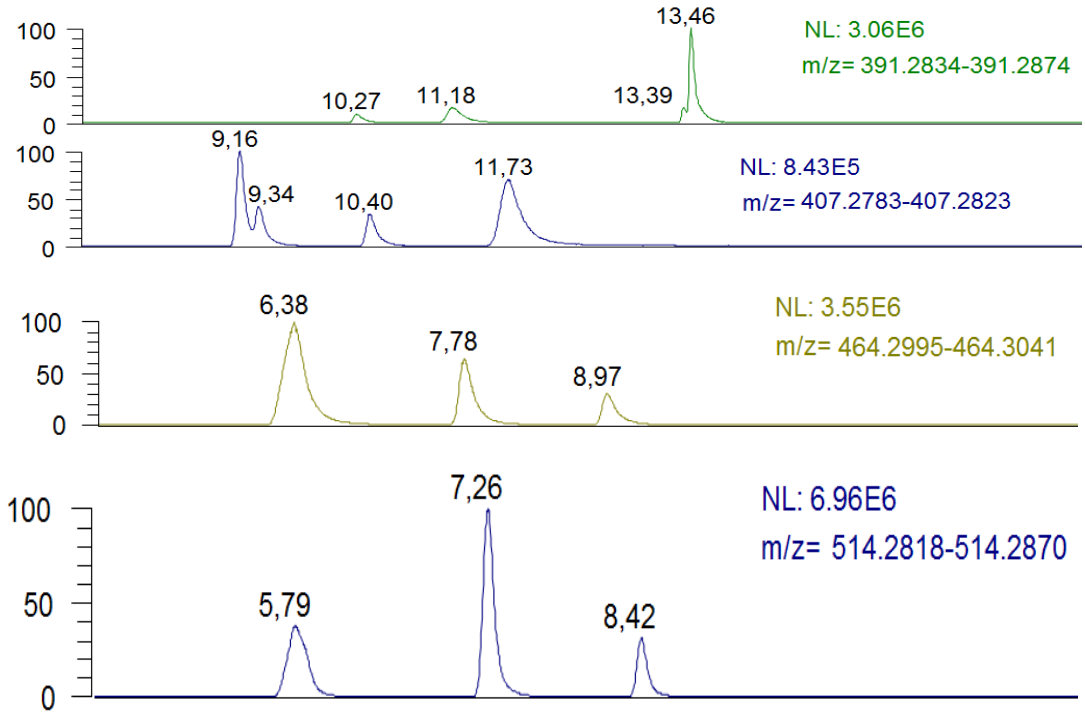



*Fig.17: Chromatography of muricholic acid standards using two C18 Macherey nagel Nucleoshell, 50mm; 2.7µm column switched in series*
*unconjugated ursodeoxy-, hyodeoxy- chenodeoxy- and deoxycholic acid: top first; unconjugated α-, β - and γ- muricholic and cholic acids: top second; glycine conjugated muricholic and cholic acids: third; taurine conjugated α-, β - and γ- muricholic and cholic acids: bottom; indications are given in order of elution;*

Chromatographic performance did actually suffer. The reason for this is firstly the non adapted gradient for the later elution times- which interestingly did not deviate as much from the single column method as thought.
Mostly, however, because of the increasing dead volume which impairs the analytes to stay separated on the transmission to the second column. As we can see, the peaks of unconjugated chenodeoxycholic- and deoxycholic acid almost merge together. Unconjugated α- and β -muricholic acids, which were separated well enough for analysis with a single C18 Macherey nagel Nucleoshell, 50mm; 2.7µm column, now elute almost simultaneously. Concerning glycine and taurine conjugated α- and β -muricholic acid we can see, that besides from a slightly crooked peak form we can't even see that two different isomeres elute (see peaks at time 6,38 and 5,79, respectively in Fig.17).

Up next, a long C8 column from Waters was installed (1 x 150mm; 3.5 µm). The stationary more polar C8 phase suggested a stronger interaction with more polar bile acids, like α- and β- muricholic acids. This would lead to a higher separation performance than with a less polar stationary phase - at least for certain bile acids. However, as the column was very old and had not been used for years, the column material was most certainly dried out. The pressure increased dramatically as soon as a solvent reached it- it was clogged and could not be used.

That's why another column was tried: A C18 Phenomenex (250 x 200mm; 4 µm). In part of the chromatographic optimization process for bile acids, a C18 phenomenex column was already tested- showing a poor performance. However, this C18 phenomenex is 12 cm longer than the previous one and therefore allows a significantly increased interaction process between the analyte and the stationary phase - possibly enough for a separation of α- and β- muricholic acids.

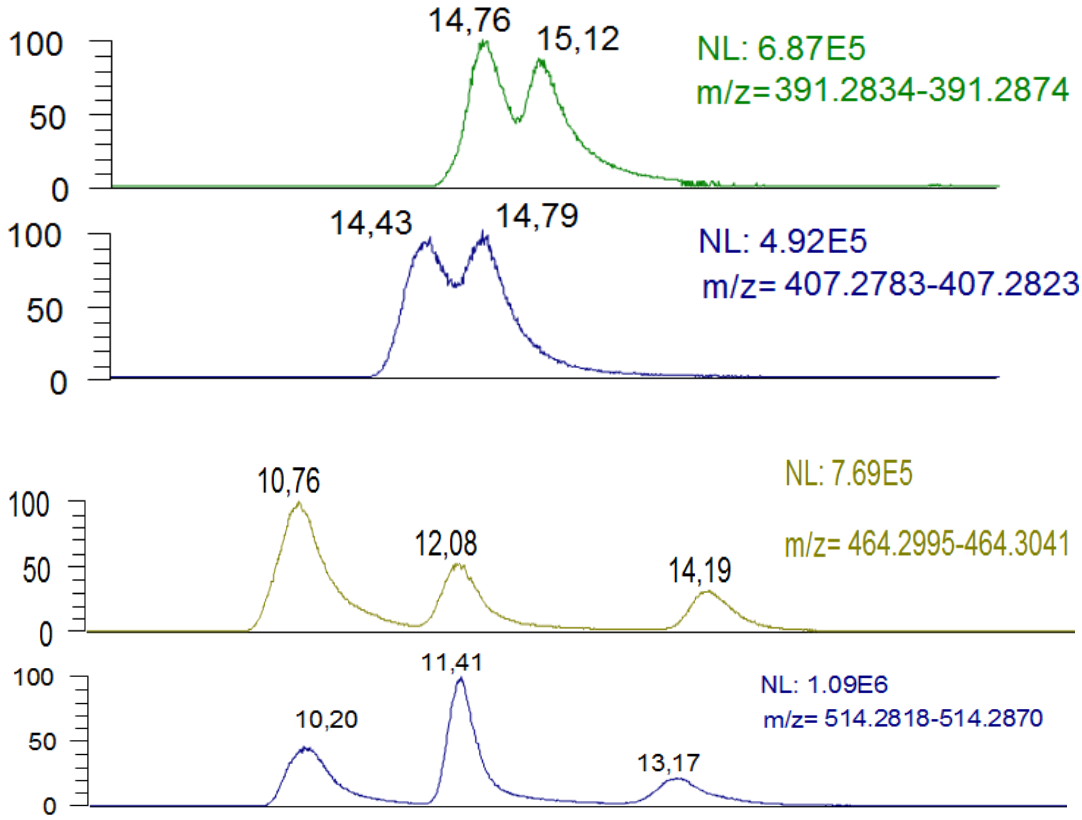



*Fig.18: Chromatography of muricholic acid standards using a C18 Phenomenex (250 x 200mm; 4 µm) column; unconjugated chenodeoxy- and deoxycholic acid: top first; unconjugated muricholic acids: top second; glycine conjugated α-, β - and γ-*

*muricholic and cholic acid: third; taurine conjugated α-, β - and γ- muricholic and cholic acids: bottom; indications are given in order of elution;*

As we can see in Fig.18 above, this was not the case. In addition, besides the broadened peaks, we can see that not even the unconjugated muricholic acids are separated. In fact, we can't even see four peaks. Interestingly, also the unconjugated chenodeoxycholic acid could be separated from the deoxycholic acid. This column is therefore not recommended for bile acid separation. Needless to say, also glycine and taurine conjugated muricholic and cholic acids are not separated. Again, the first peak does not show any sign that two different isomeres elute.

A Phenomenex Kinetex PFP (pentafluorophenyl) column was tested next. The effect of a completely different stationary phase was hard to estimate: would bile acids interact via pi stacking bonds or just with the polar fluor residues? Could it even cause bile acids to elute in a different order?

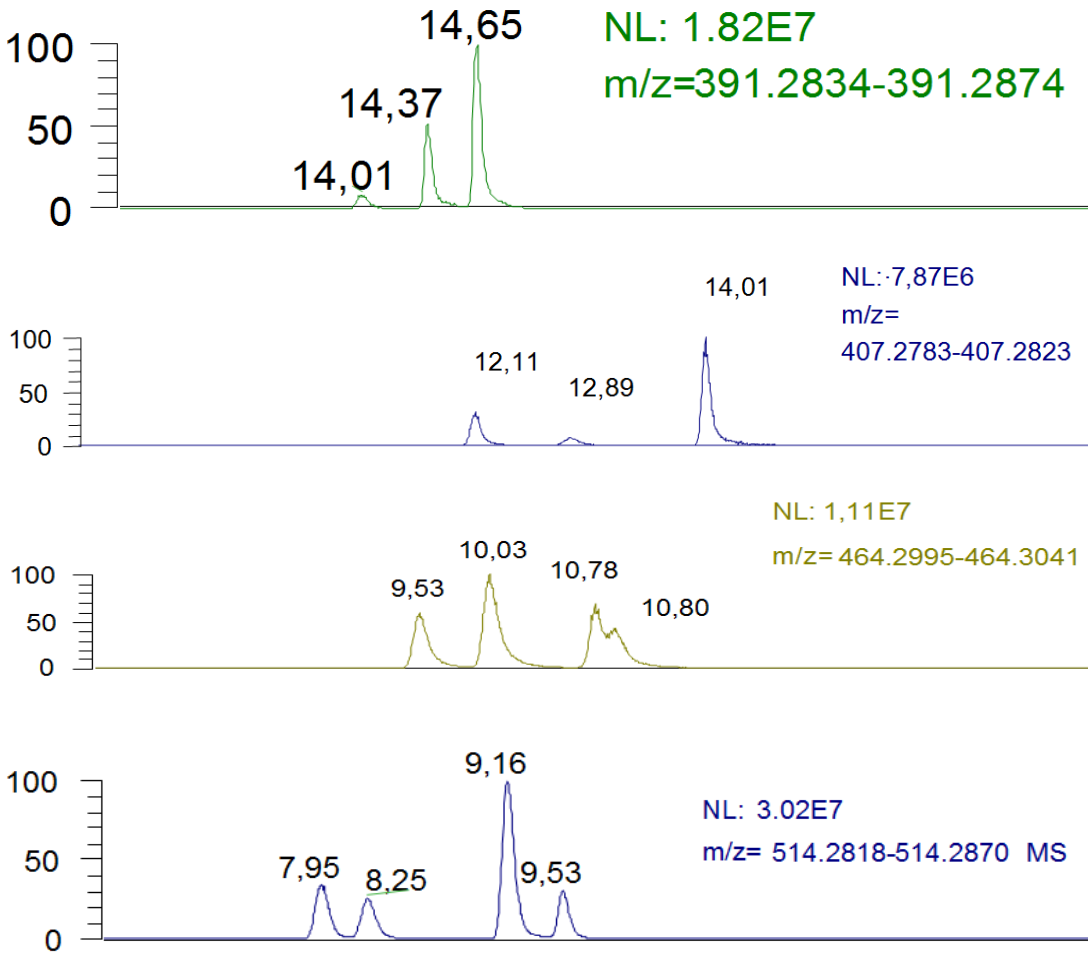


*Fig.19: Chromatography of muricholic acid standards using a Phenomenex Kinetex PFP column*
*unconjugated chenodeoxycholic and deoxycholic acids: top first; unconjugated α-, β - and γ- muricholic- and cholic acids: top second; glycine conjugated α-, β - and γ- muricholic- and cholic acids: third; taurine conjugated α-, β - and γ-muricholic- and cholic acids: bottom; indications are given in order of elution;*

The order of elution probably remained the same. In order to prove it, standards would need to be analyzed separatly with this column.
It is clear however, that taurine (and D4 taurine) conjugates of muricholic acids were indeed separated well. In addition, other taurine conjugates are eluting properly and are evaluable.
However, glycine conjugates could not be separated as well as taurine conjugates: Two peaks are overlapping. Interestingly, the pattern is different from the results with the C18 columns:
As we can recall from Fig. 16, 17 and 18 above, the first two peaks overlap almost completely. These are, as already described, proven to be glyco α- and β- muricholic acid. In Fig. 19 we can see, that when using Phenomenex Kinetex PFP column, the first two peaks of the muricholic-or cholic acids are separated well. However, the following two peaks overlap. As the order of elution is not proven, there are two possibilites:

- either the order of elution has changed. This would mean that α- and β- muricholic acids are still not able to be separated well, however better than with the C18 Macherey nagel column.
- or the order of elution remained the same. This would suggest that α- and β- muricholic acids could be separated effectively, yet γ- muricholic - and cholic acid are now overlapping.

Interestingly, the last two eluting unconjugated muricholic acids or cholic acid now overlap as well. As we can remember, this was not the case with C18 columns. As we can see in Fig. 19 above, which peaks overlap is not clear at first sight. However, comparing the intensities between Fig 16 or 17 and Fig.19 while recalling that the

same sample is used, we understand that the last peak in Fig. 19 has to contain more than one analyte.
Again, the order is not proven. Given the fact that α- and β- muricholic acid behave very similar and have similar polarities, a change in order of elution seems now very likely.
The analytes have not been identified by checking their retention times separatly as it is of not much use: As both glycine conjugated as well as unconjugated muricholic or cholic acids can still not be evaluated properly, this column can not be used for analysis.

As no other columns were available at this time, the investigation was stopped. Further columns have to be tested to establish a working chromatography for muricholic acids. A very promising aspect is a more polar stationary phase like C8 or even C3, for the reasons already explained above. At this time it would be advisable to include the ω- muricholic acid standard to observe other possible elution interferences. In addition it would be very promising to include glycine as well as taurine conjugated ω- muricholic acid standards, synthesised purified and prepared in a stock solution as described in this work- in order to complete the bile acid standard package.

# 5 Results and Discussion

## 5.1 Esterification of glycine with methanol

In order to evaluate the esterification of glycine with methanol, the glycinemethylester solved in methanol was analyzed with ESI- Orbitrap MS, in positive mode and compared with values from literature. The mass detected for the free glycine (M+H) was 76.039. The mass detected for glycine methylester (M+H) 90.0553).

Jiabo Li et al. [74] (from whom the method was adapted) achieved an esterification yield of 96 %. The hydrochloride salt was measured, which resulted from the

evaporation with a rotary epavorator. The amount was calculated with 1H-NMR (300 MHz) and 13C-NMR (75 MHz) and LC-MS (Bruker ESQUIRE-LC).

Gros, L et al. achieved an esterification yield of 89%, allthough here, glycine was esterified with methanol using thionyl chloride instead of trimethylchlorosilane. Therefore, the resulting methyl 2 - aminoethanoate was measured by GCMS in positive mode, [M-HCl]+. EI was used for ionization. [84]

The esterification in this conducted work was achieved to a degree between 99.99 and 99.999 % (measured was the glycinemethylester with a mass of 90.0553 and the free glycine with a mass of 76.0398 detected as [M+H]+ in positive mode, by using LC-MS with an ESI ionization source):

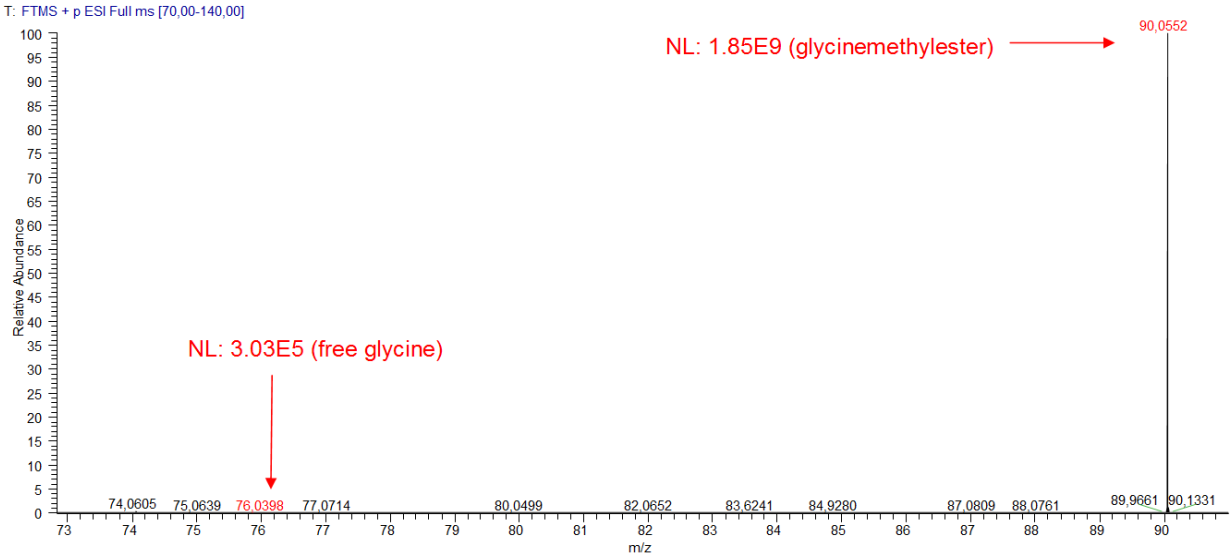



*Fig.20: Esterification of free glycine with methanol, high yield. Mass detected for free glycine: 76.0398; Mass detected for esterified glycine: 90.0552; M+H, positive mode;*

## 5.2 Determination of the synthesised standards' concentration

As we can recall from "4.6 Determination of the synthesised standards' concentration", the concentration of the newly synthesised and purified standards was determined twice internally. The results are observable in Tab.9 below:

| | C calculated 1st step [nmol/ml] | C final determination [nmol/ml] | Volume [ml] |
|---|---|---|---|
| Glyconorursodeoxycholic acid | 0.5 | 0.46 | 1.011 |
| Glycoalphamuricholic acid | 50 | 47.36 | 2.873 |
| Glycobetamuricholic acid | 50 | 40.54 | 6.333 |
| Glycogammamuricholic acid | 50 | 68.37 | 1.332 |
| Glycohyodeoxycholic acid | 5 | 4.98 | 1.250 |
| Glycodicoprostanic acid (10inst. of 5ml) | 50 | 22.77 | 7.030 |
| Glycotricoprostanic acid (10inst. of 5ml) | 50 | 22.05 | 5.354 |
| | | | |
| D4 Tauronorursodeoxycholic acid | 50 | 43.46 | 4.500 |
| D4Tauroalphamuricholic acid | 500 | 240.71 | 3.403 |
| D4Taurogammamuricholic acid | 500 | 298.94 | 1.927 |
| D4Taurobetamuricholic acid | 50 | 44.43 | 5.949 |
| D4Taurohyodeoxycholic acid | 50 | 38.53 | 8.580 |
| D4Taurocholic acid(10inst. of 5ml) | 500 | 271.54 | 3.146 |
| D4Tauroursodeoxycholic acid | 500 | 173.59 | 1.154 |
| D4Taurodeoxycholic acid(10inst. of 5ml) | 2000 | 412.72 | 5.925 |
| D4Taurochenodeoxycholic acid(10inst. of 5ml) | 500 | 206.38 | 3.048 |
| D4Taurolithocholic acid(10inst. of 5ml) | 5 | 1.77 | 3.216 |
| D3Taurodicoprostanic acid(10inst. of 5ml) | 5 | 4.91 | 6.873 |
| D3Taurotricoprostanic acid(10inst. of 5ml) | 500 | 343.33 | 1.283 |
| | | | |
| Tauronorursodeoxycholic acid | 50 | 53.52 | 3.653 |
| Taurogammamuricholic acid | 500 | 595.11 | 1.790 |
| Taurodicoprostanic acid | 50 | 76.94 | 1.011 |
| Taurotricoprostanic acid (10inst. of 5ml) | 500 | 131.19 | 5.109 |

*Tab.9: First calculation (second column) compared to the second calculation and final determination of the synthesised bile acid standards' concentration (third column); Volumes are depicted in the fourth column; Green: only slight deviations from the second to the first calculation; Yellow: deviating values; Red: strong deviations;*

Comparing the first calculation of the concentrations (second column in Tab.9 )with the final corrected concentration (third column in Tab.9) we can observe a fairly high deviation- ranging from less than 0.1 nmol (e.g. D3 Taurotricoprostanic acid, Glycohyodeoxycholic acid or Glyconorursodeoxycholic acid) up to 369 nmol, which is over 50% of the initial calculation (Taurotricoprostanic acid).

The major reason for the sometimes large deviation is definetely the large gap of the concentration / intensity between the internal known standards and some of the newly synthesised standards in the first calculation. As described in "4.6 Determination of the synthesised standards' concentration" above, it was not easy to estimate and calculate the concentration of the newly synthesised standards and bring them into the range of the known internal standards. However, the real problem was, that the indications of the concentration of the known internal standards were unclear (dilution step was missing). This resulted in the usage of 10nmol of internal

standard per vial of 200 µl instead of 0.2 nmol per 200 µl. The range of concentration of all different standards is therefore very high: Between 0.5nmol/L (Glyconorursodeoxycholic acid) and 50 x $10^3$ nmol/L for the internal standards, with intensities ranging from $10^3$ to $10^8$, respectively. Of course, this lead to an imprecision in calculation by comparison. Recalling that it is not possible to match an analyzed dilution series of natural standards- as they were just newly synthesised- this is a major hindrance.

The next reason is the fact, that standards had to be dried under a stream of nitrogen. As they contained water which had to be gradually removed - prolonging the harsh process as described in "4.6 Determination of the synthesised standards' concentration" above - a certain amount of analytes were lost. Using the rotary evaporator for certain standards (which contained the most water) was definetely a more preserving process. However, standards had to be refilled in different vials for this process and even again after resolving them in methanol. Even when washing the vials with methanol, a certain loss of analytes is inevitable.

Another factor might be pipetting errors when resolving them in methanol to reach the aimed concentrations after the first calculation.

However, as all these factors can be excluded for the second calculation of the concentration as they were not dried or refilled, the range of the concentration between all standards matched in the second calculation and they were not resolved. Therefore, a third evaluation of the concentration will not be necessary.

The stock solutions of the synthesised purified and defined standards were labelled and stored at -20°C.

## 5.3 Example of a chromatography of bile acids purified from stool

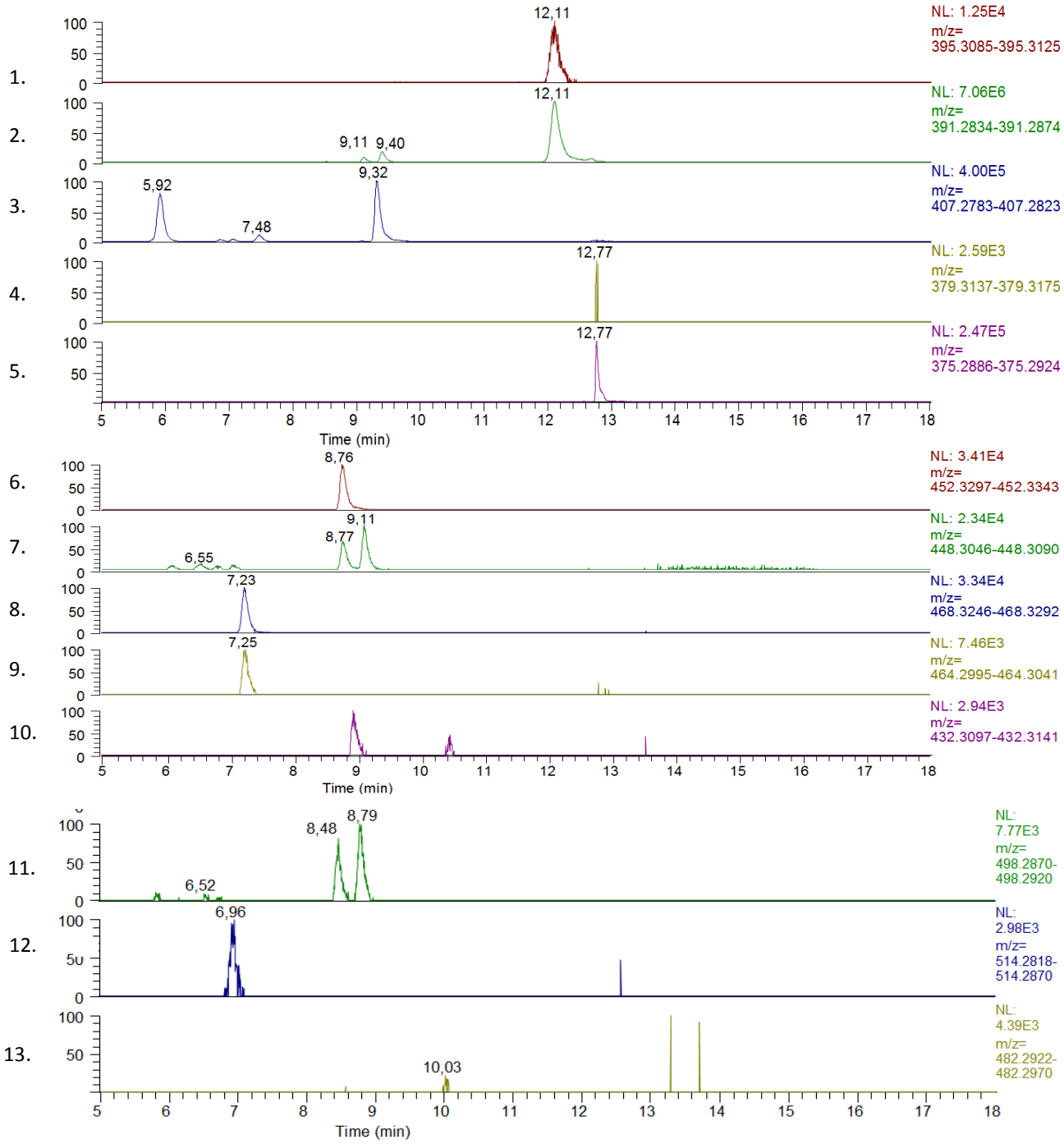



*Fig.21: Example of bile acids purified from stool; chromatogram of unconjugated bile acids: top (line 1.-5.); chromatogram of glycine conjugated bile acids: middle (line 6.-10.); chromatogram of taurine conjugated bile acids: bottom (line 11.-14.);*

### 5.3.1 Unconjugated bile acids purified from stool

In Fig.21 in the "1. line" we can see, that the internal standard D4 deoxycholic acid was partially lost in the course of purification. This loss has therefore to be

considered when calculating the concentration of the bile acids found in the stool samples. The retention time of this standard assures that the peak in the "2. line" at 12,11 resembles deoxycholic acid. Chenodeoxycholic acid would elute immediately before. It can be assumed that in this sample, no chenodeoxycholic acid is present. The two small peaks at 9,11 and 9,40 can be accounted to ursodeoxycholic and hyodeoxycholic acid, respectively. Allowing a few seconds of tolerance, the elution times coincede with the standard ones and the ones observed in the chromatography of bile acids extracted from urine (see fig).

However, the standard of hyodeoxycholic acid, as well as its glycine and taurine conjugated forms, were not part of the standard mixture as it was thought to not play a role in human bile acid profiling. This conception seems widely spread: In the comparison of bile acid profiles between mice and men by de Aguiar Valim et al. [83], as we can see in Fig.14, hyodeoxycholic acid was only analysed in mouse samples.

As we can observe in the chromatograms of bile acids purified from stool and especially urine (see Fig.23 below) however, hyodeoxycholic acid and in particular its glycine and taurine conjugate is very well present in high concentrations in humans. As the standards are now synthesised and ready to use, they will be incorporated in routine analysis from now on.

Hyodeoxycholic acid and its glycine and taurine conjugate was not retrospectively evaluated, as the acurracy of the determination of their concentration is drastically impaired by the absence of the standards in the dilution series. The same of course applies to the evaluation of hyodeoxycholic acid and its glycine and taurine conjugate in urine.

The peak at 9,32 in the "3. line" identifies as cholic acid. The peaks in front of it could be accounted to isomers such as α-, β -, γ- or ω- muricholic acid. However, occurrence of these has not been reported in human stool. In general, they are present in humans only rare and in scarce amounts. In addition, the first eluting muricholic acid, α- muricholic acid, elutes about one minute later than the here appearing prominent peak at 5,92. It can therefore be attributed to different still unknown isomeres - as it is the case for bile acids extracted from urine (see fig). For a description of possible isomers concerning the mass 391.28 as well as 407.28, see "bile acids purified from urine".

The "4. line" shows the internal standard D4 lithocholic acid. Interestingly, it was added in the same concentration as D4 deoxycholic acid - however the loss seems

advanced. The loss could be accounted to the washing step. However, the difference can not be explained, especially as the natural occurring lithocholic acid was detected in high amounts (see "5. line").

### 5.3.2 Glycine conjugated bile acids purified from stool

The "6. line" shows the internal standard D4 glycodeoxycholic acid. In the "7. line" we can see glycodeoxycolic acid eluting at 9,11 and glycochenodeoxycholic acid eluting right before that. The four low isomeric peaks in front of them are hard to be attributed. However, as their intensities are not clearly above the background signal, they can't be evaluated as present.
The "8. line" shows the internal standard D4 glycocholic acid - affirming the identity of glycocholic acid in the "9. line".
In the "10. line" we can see, that glycolithocholic acid is not present in this sample.

### 5.3.3 Taurine conjugated bile acids purified from stool

In this chromatogram, no significant amount of taurine conjugated bile acids can be detected.

## 5.4 Evaluation of stool samples:

### 5.4.1 Taurine conjugated bile acids:

As we can observe in Tab.10 below, this coincedes with the concentrations of taurine conjugated bile acids of the remaining stool samples: The mean value shows that overall, taurine conjugates are present -if ever - only scarcely in stool of healthy human subjects. In general, this can be affirmed by the findings of L. Humbert et al. However, taurine conjugates of chenodeoxycholic and deoxycholic acids have been shown to be present in amounts of ca 5 nmol/g, whereas in this conducted experiment they were detected in only negligible amounts.

### 5.4.2 Glycine conjugated bile acids:

Glycine conjugated bile acids are, in general, present in a minimally higher concentration than taurine conjugated ones - reaching a maximum of 2.9 nmol/g as a median value for glycocholic acid. The values presented of L. Humbert et al. also show a significantly higher concentration of glycine conjugated bile acids.
Also, Glycochenodeoxycholic acid was, with a median concentration of 22.28 nmol/g, the highest concentrated glycine conjugated bile acid in stool. This does not coincede with the values of this presented work. Here, glycocholic acid is present in the highest concentration.

### 5.4.3 Unconjugated bile acids:

Unconjugated bile acids in general were found to be the highest concentrated ones in the literature as well as in this presented work. Lithocholic acid, of course, has shown the highest concentration of 709.03 nmol/g as a median value. After Humbert et al. [35] , lithocholic acid is also highly concentrated (1016.60 nmol/g as a median value). Interestingly however, unconjugated deoxycholic acid almost doubles this value- whereas in this presented work the concentration of unconjugated deoxycholic acid is less than 60% of the unconjugated lithocholic acid's concentration.
In addition, the concentration of bile acids extracted from stool found by Humbert et al.[35] are in general higher as previously mentionened.
As will be explained in the evaluation of urine samples, the interindividual dispersion of bile acid concentration is very large. This can be understood from the large standard deviations in this conducted work as well as by L. Humbert et al [35] (shown in Tab.10).
In addition, this was also described by Carine Steiner et al.: the study already mentioned in "1.1.5 Metabolic disorders and diseases associated with bile acids" above, observed an interindividual 24 hour variation of 42% to 70 % in case of C4, 23% to 91% in case of conjugated bile acids and 49% to 90% in case of unconjugated bile acids in the serum of four healthy probands. Also, conjugated bile acids were shown to increase drastically postprandially, wheareas C4 changed

interindividually the most between 20:00 and 1:00 and unconjugated bile acids between 3:00 and 8:00 [55]. Neither this postprandial effect, nor the diurnal rhythm were considered in the study of Humbert et al. [35] or in the sample collection of this work.

All these restraints, of course, limit the validity of the comparison between this and Humbert et al's work- even if the sample size suffices in both cases (the sample size of this work is 54, whereas Humbert et al's work almost doubles it with 100).

In both cases, healthy individuals were compared. Also, in both cases analytes were not derivatized as high performance liquid chromatography was used to separate analytes. Sample preparation was adapted from the paper and besides the few adaptations described in "4.7.1 Sample collection and first preparation of stool samples" above, does not differ.

What then could cause the observed deviations?

Besides differences caused by the different equipment also possible differences in the evaluation process could be accounted. However, a more important factor is, that allthough in both cases 100mg dried feces were used for sample preparation, this quantity can not always be granted in this conducted work as for some samples, not enough material was present. This can, of course, lead to lower results for some samples and lower the concentration of the calcuated median value.

However, the strong differences in single bile acids like the unconjugated deoxycholic acid or glycochenodeoxycholic acid hint more towards an unhealthy state of probands used in either one of the works.

It is important to note that analysis was not done in order to claim medicinal significance and fathom the differences in biological processes- in this case concerning the bile acid metabolism. For this purpose these values do not withstand for the above stated reasons, nor was it the purpose of this project.

The purpose was to establish a method which allows successfull preparation of bile acids from stool as well as their mass spectrometric analysis. In order to prove the method, values produced by it were compared with values from literature. As we could see, the concentrations measured with the presented method fit very well into the range presented in literature and therefore the method can be accounted as working.

### 5.4.4 Bile acid distribution in stool samples

Concerning the bile acid distribution between unconjugated, glycine conjugated and taurine conjugated bile acids differences can be observed between unconjugated and conjugated bile acids:

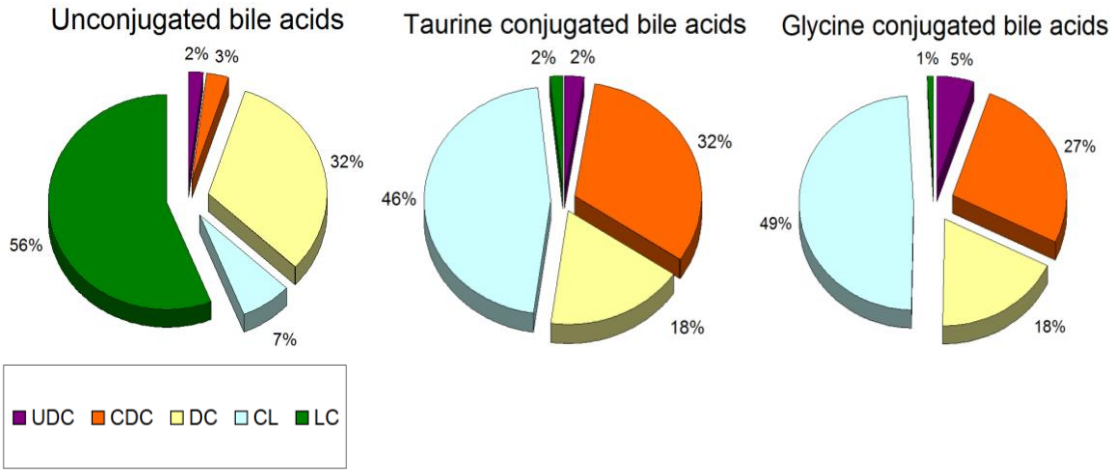



*Fig.22: Bile acid distribution between unconjugated, glycine conjugated and taurine conjugated bile acids in human stool;*

Lithocholic acid is, as stated above, the highest concentrated bile acid in stool. In the case of unconjugated bile acids it makes up as much as 56 % of the total unconjugated bile acids. The relative quantity of lithocholic acid is drastically reduced in the case of taurine or glycine conjugated bile acids.

Also the relative quantity of deoxycholic acid shrinks from 32% to 18%.

The share of cholic acid and chenodeoxycholic acid, interestingly the two primary bile acids, drastically increases in the case of conjugated bile acids. This can be explained due to the fact that, of course, primary bile acids are conjugated to a higher degree than secondary bile acids. As described in the introduction, secondary bile acids are not produced until they reach the ileum. For conjugation, they must be transported back to the liver- whereas primary bile acids are already synthesised there, prone to the conjugation process.

Ursodeoxycholic acid seems to be the only constant bile acid. As described, the distribution between glycine and taurine conjugated bile acids is the same. The marginal observed differences (increase of ursodeoxycholic acid in glycine

conjugated bile acids) can be explained due to the small total amounts detected. An outlier has therefore a high impact on the median value and the total distribution.

| **Bile acids**<br>**Calculated amount [nmol/g]** | 1. UDC | 2. TUDC | 3. GUDC | 4. CDC | 5. TCDC | 6. GCDC | 7. DC |
|---|---|---|---|---|---|---|---|
| S1 | 0.19 | 0.00 | 0.20 | 0.00 | 16.22 | 3.81 | 96.56 |
| S2 | 5.34 | 0.00 | 0.68 | 32.83 | 1.13 | 6.25 | 235.97 |
| S3 | 0.74 | 0.02 | 0.08 | 0.00 | 0.16 | 0.74 | 671.53 |
| S4 | 38.31 | 0.00 | 0.47 | 35.37 | 0.13 | 0.93 | 260.96 |
| S5 | 1.38 | 0.00 | 0.21 | 10.20 | 0.59 | 4.47 | 31.97 |
| S6 | 251.93 | 0.56 | 0.48 | 69.01 | 0.46 | 1.37 | 1323.65 |
| S7 | 25.99 | 0.11 | 1.00 | 47.84 | 0.68 | 4.06 | 119.67 |
| S8 | 23.07 | 0.05 | 0.19 | 76.22 | 0.31 | 2.49 | 1100.44 |
| S9 | 88.43 | 0.13 | 0.21 | 58.78 | 0.14 | 0.41 | 281.00 |
| S10 | 0.00 | 0.03 | 0.11 | 0.00 | 0.14 | 1.27 | 266.09 |
| S11 | 0.00 | 0.01 | 0.11 | 1.34 | 0.16 | 1.34 | 68.58 |
| S12 | 3.58 | 0.03 | 0.11 | 0.00 | 0.17 | 0.40 | 123.12 |
| S13 | 32.44 | 0.05 | 0.11 | 80.74 | 0.44 | 0.93 | 1162.96 |
| S14 | 7.14 | 0.01 | 0.02 | 28.67 | 0.06 | 0.33 | 154.62 |
| S15 | 48.90 | 0.02 | 0.14 | 84.95 | 0.22 | 0.93 | 1439.94 |
| S16 | 9.97 | 0.06 | 0.45 | 5.62 | 0.24 | 1.47 | 75.65 |
| S17 | 2.45 | 0.00 | 0.10 | 4.07 | 0.08 | 0.37 | 77.66 |
| S18 | 0.00 | 0.00 | 0.11 | 0.36 | 0.15 | 0.64 | 148.17 |
| S19 | 0.39 | 0.00 | 0.00 | 0.19 | 0.10 | 0.13 | 21.91 |
| S20 | 109.66 | 0.70 | 4.77 | 464.48 | 2.45 | 14.84 | 626.38 |
| S21 | 0.00 | 0.03 | 0.19 | 0.00 | 0.25 | 0.99 | 1165.95 |
| S22 | 21.31 | 0.05 | 1.12 | 9.62 | 0.13 | 1.21 | 39.01 |
| S23 | 0.91 | 0.00 | 0.05 | 0.00 | 0.20 | 1.03 | 270.84 |
| S24 | 0.00 | 0.01 | 0.06 | 6.65 | 0.80 | 1.91 | 41.70 |
| S25 | 1.19 | 0.00 | 0.01 | 3.21 | 0.06 | 0.21 | 73.69 |
| S26 | 0.00 | 0.01 | 0.31 | 0.00 | 0.29 | 2.40 | 602.96 |
| S27 | 0.00 | 0.00 | 0.15 | 1.21 | 0.34 | 2.32 | 424.20 |
| S28 | 0.00 | 0.00 | 0.00 | 0.00 | 0.07 | 0.19 | 7.92 |
| S29 | 0.00 | 0.00 | 0.00 | 0.49 | 0.08 | 0.27 | 218.17 |
| S30 | 0.00 | 0.00 | 0.00 | 0.00 | 0.03 | 0.05 | 78.76 |
| S31 | 0.00 | 0.00 | 0.07 | 0.71 | 0.09 | 1.11 | 453.99 |
| S32 | 4.98 | 0.00 | 0.02 | 176.88 | 0.23 | 0.94 | 23.04 |
| S33 | 105.03 | 0.12 | 0.25 | 403.55 | 0.46 | 1.41 | 2005.02 |
| S34 | 0.00 | 0.00 | 0.03 | 15.23 | 0.09 | 0.56 | 106.46 |
| S35 | 31.54 | 0.01 | 0.39 | 17.96 | 0.19 | 1.76 | 748.80 |
| S36 | 1.88 | 0.00 | 0.04 | 0.00 | 0.14 | 1.65 | 2016.50 |
| S37 | 13.69 | 0.04 | 0.42 | 6.56 | 0.25 | 1.52 | 505.83 |
| S38 | 1.56 | 0.00 | 0.02 | 0.75 | 0.03 | 0.15 | 18.57 |
| S39 | 11.35 | 0.02 | 0.66 | 37.77 | 0.40 | 5.24 | 470.81 |
| S40 | 284.51 | 0.03 | 0.51 | 286.55 | 0.15 | 1.28 | 1655.90 |
| S42 | 4.75 | 0.02 | 0.48 | 5.54 | 0.21 | 1.67 | 365.77 |
| S43 | 0.00 | 0.04 | 0.19 | 2.89 | 0.47 | 1.08 | 376.77 |
| S44 | 0.00 | 0.02 | 0.19 | 1.10 | 0.13 | 0.90 | 28.55 |
| S45 | 0.65 | 0.00 | 0.01 | 7.07 | 0.11 | 0.31 | 83.75 |
| S50 | 26.19 | 0.09 | 0.45 | 40.18 | 0.73 | 3.20 | 103.95 |
| S51 | 1.71 | 0.00 | 0.08 | 7.46 | 0.13 | 0.37 | 38.31 |
| S52 | 149.69 | 0.14 | 1.11 | 103.24 | 0.21 | 1.03 | 194.75 |
| S53 | 0.74 | 0.00 | 0.01 | 0.60 | 0.08 | 0.34 | 96.07 |
| S54 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| S55 | 2.61 | 0.00 | 0.01 | 3.60 | 0.14 | 0.41 | 202.99 |
| S56 | 0.00 | 0.00 | 0.00 | 0.00 | 0.07 | 0.32 | 17.96 |
| S57 | 0.55 | 0.00 | 0.04 | 0.80 | 0.28 | 1.97 | 324.55 |
| S58 | 0.00 | 0.00 | 0.01 | 6.17 | 0.38 | 0.64 | 404.52 |
| S59 | 0.00 | 0.00 | 0.01 | 0.00 | 0.01 | 0.31 | 197.84 |
| **MV ± SD** | **24.35 ± 57.38** | **0.04 ± 0.12** | **0.30 ± 0.68** | **39.75 ± 92.62** | **0.58 ± 2.20** | **1.59 ± 2.27** | **400.94 ± 509.24** |
| **MV ± SD after L. Humbert et al.** | **27.05 ± 61.13** | **0.3 ± 0.37** | **2.39 ± 2.1** | **54.8 ± 72.07** | **6.03 ± 5.00** | **22.28 ± 15.65** | **1920.10 ± 1390.50** |

| Bile acids<br>Calculated amount [nmol/g] | 8. TDC | 9. GDC | 10. CL | 11. TC | 12. GC | 13. LC | 14. TLC | 15. GLC | TOTAL |
|---|---|---|---|---|---|---|---|---|---|
| S1 | 0.00 | 0.07 | 702.54 | 21.46 | 30.61 | 0.26 | 0.05 | 0.04 | **755.04** |
| S2 | 0.44 | 3.25 | 45.10 | 2.54 | 18.73 | 74.97 | 0.04 | 0.05 | **145.11** |
| S3 | 0.08 | 1.44 | 6.32 | 0.17 | 1.13 | 389.25 | 0.01 | 0.22 | **398.61** |
| S4 | 0.16 | 0.38 | 143.11 | 0.28 | 0.83 | 49.41 | 0.01 | 0.03 | **194.19** |
| S5 | 0.03 | 0.37 | 5.38 | 0.24 | 1.99 | 150.95 | 0.02 | 0.02 | **159.01** |
| S6 | 1.45 | 1.06 | 35.72 | 0.71 | 1.00 | 5399.66 | 0.13 | 0.04 | **5439.77** |
| S7 | 0.08 | 0.47 | 29.98 | 0.42 | 3.59 | 183.83 | 0.00 | 0.00 | **218.37** |
| S8 | 0.61 | 2.14 | 52.47 | 0.38 | 3.59 | 638.07 | 0.11 | 0.12 | **697.49** |
| S9 | 0.32 | 0.18 | 43.08 | 0.24 | 0.21 | 349.55 | 0.05 | 0.01 | **393.65** |
| S10 | 0.07 | 0.73 | 4.92 | 0.20 | 1.88 | 1340.22 | 0.02 | 0.06 | **1348.10** |
| S11 | 0.08 | 0.73 | 2.84 | 0.13 | 0.95 | 1717.36 | 0.01 | 0.07 | **1722.14** |
| S12 | 0.11 | 0.40 | 0.71 | 0.17 | 0.17 | 590.79 | 0.01 | 0.09 | **592.46** |
| S13 | 0.15 | 1.05 | 105.43 | 0.23 | 0.75 | 0.00 | 0.01 | 0.02 | **107.64** |
| S14 | 0.05 | 0.20 | 9.39 | 0.08 | 0.22 | 398.89 | 0.00 | 0.00 | **408.83** |
| S15 | 0.24 | 1.07 | 58.95 | 0.17 | 0.53 | 543.76 | 0.01 | 0.04 | **604.78** |
| S16 | 0.06 | 0.36 | 1.94 | 0.10 | 0.56 | 84.23 | 0.00 | 0.00 | **87.26** |
| S17 | 0.02 | 0.10 | 4.61 | 0.12 | 0.33 | 87.27 | 0.00 | 0.00 | **92.44** |
| S18 | 0.03 | 0.53 | 1.99 | 0.16 | 0.70 | 5684.50 | 0.00 | 0.02 | **5687.93** |
| S19 | 0.04 | 0.05 | 0.27 | 0.12 | 0.08 | 50.09 | 0.00 | 0.00 | **50.64** |
| S20 | 1.13 | 6.77 | 994.89 | 5.94 | 35.13 | 72.29 | 0.04 | 0.05 | **1116.23** |
| S21 | 2.49 | 2.24 | 5.74 | 0.44 | 1.09 | 1205.02 | 0.28 | 0.13 | **1217.43** |
| S22 | 0.00 | 0.97 | 173.52 | 0.04 | 1.65 | 0.00 | 0.00 | 0.00 | **176.18** |
| S23 | 0.24 | 2.37 | 3.04 | 0.40 | 1.17 | 332.40 | 0.00 | 0.11 | **339.73** |
| S24 | 0.01 | 0.06 | 4.54 | 0.65 | 1.54 | 2683.35 | 0.00 | 0.05 | **2690.21** |
| S25 | 0.02 | 0.13 | 1.16 | 0.03 | 0.10 | 429.36 | 0.00 | 0.00 | **430.80** |
| S26 | 0.11 | 1.25 | 6.69 | 0.22 | 1.79 | 931.46 | 0.00 | 0.07 | **941.59** |
| S27 | 0.16 | 1.32 | 3.74 | 0.31 | 1.44 | 739.04 | 0.00 | 0.07 | **746.08** |
| S28 | 0.03 | 0.05 | 0.20 | 0.06 | 0.11 | 13.39 | 0.00 | 0.00 | **13.84** |
| S29 | 0.07 | 0.79 | 1.04 | 0.11 | 0.23 | 342.56 | 0.00 | 0.05 | **344.84** |
| S30 | 0.01 | 0.15 | 0.40 | 0.02 | 0.04 | 126.18 | 0.00 | 0.00 | **126.80** |
| S31 | 0.04 | 0.77 | 4.62 | 0.08 | 0.63 | 529.99 | 0.00 | 0.00 | **536.13** |
| S32 | 0.20 | 0.76 | 918.92 | 0.66 | 1.86 | 13.41 | 0.00 | 0.00 | **935.81** |
| S33 | 1.09 | 1.53 | 365.13 | 0.48 | 1.04 | 330.64 | 0.14 | 0.11 | **700.15** |
| S34 | 0.05 | 0.35 | 5.27 | 0.19 | 1.02 | 546.92 | 0.00 | 0.00 | **553.81** |
| S35 | 0.09 | 2.83 | 6.44 | 0.08 | 0.73 | 705.46 | 0.00 | 0.24 | **715.88** |
| S36 | 4.11 | 2.74 | 3.14 | 0.17 | 0.58 | 3737.00 | 0.61 | 0.13 | **3748.48** |
| S37 | 0.90 | 2.55 | 7.52 | 0.36 | 1.63 | 1707.52 | 0.06 | 0.07 | **1720.62** |
| S38 | 0.00 | 0.01 | 0.35 | 0.03 | 0.06 | 14.78 | 0.00 | 0.00 | **15.24** |
| S39 | 0.27 | 3.72 | 38.02 | 1.09 | 15.18 | 603.76 | 0.01 | 0.04 | **662.09** |
| S40 | 0.09 | 1.33 | 379.36 | 0.19 | 1.81 | 451.48 | 0.00 | 0.02 | **834.28** |
| S42 | 0.07 | 0.92 | 6.20 | 0.30 | 2.20 | 0.00 | 0.00 | 0.09 | **9.78** |
| S43 | 0.27 | 1.37 | 3.87 | 0.71 | 1.89 | 453.47 | 0.01 | 0.13 | **461.73** |
| S44 | 0.08 | 0.67 | 1.51 | 0.18 | 1.01 | 115.63 | 0.00 | 0.00 | **119.08** |
| S45 | 0.19 | 0.53 | 31.27 | 0.16 | 0.33 | 27.05 | 0.00 | 0.00 | **59.53** |
| S50 | 0.19 | 0.61 | 47.25 | 1.63 | 5.48 | 224.40 | 0.00 | 0.00 | **279.57** |
| S51 | 0.11 | 0.54 | 13.52 | 0.15 | 1.79 | 63.68 | 0.00 | 0.00 | **79.80** |
| S52 | 0.16 | 0.33 | 422.49 | 0.25 | 1.66 | 0.00 | 0.00 | 0.00 | **424.90** |
| S53 | 0.09 | 0.49 | 3.15 | 0.20 | 0.47 | 217.07 | 0.00 | 0.01 | **221.47** |
| S54 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 258.03 | 0.00 | 0.00 | **258.03** |
| S55 | 0.12 | 0.35 | 2.16 | 0.18 | 0.42 | 575.72 | 0.01 | 0.01 | **578.98** |
| S56 | 0.03 | 0.20 | 0.53 | 0.08 | 0.28 | 333.81 | 0.00 | 0.04 | **334.97** |
| S57 | 0.20 | 2.19 | 3.90 | 0.48 | 1.49 | 0.00 | 0.00 | 0.06 | **8.31** |
| S58 | 0.54 | 1.00 | 3.60 | 1.06 | 1.54 | 1539.42 | 0.00 | 0.00 | **1547.16** |
| S59 | 0.00 | 0.49 | 0.91 | 0.03 | 0.17 | 1230.37 | 0.00 | 0.00 | **1231.96** |
| **MV ± SD** | **0.32 ± 0.68** | **1.05 ± 1.20** | **87.39 ± 215.03** | **0.83 ± 2.99** | **2.88 ± 6.76** | **709.03 ± 1185.49** | **0.03 ± 0.09** | **0.04 ± 0.05** | **1269 ± 2076** |
| **MV ± SD after L. Humbert et al.** | **4.32 ± 5.81** | **19.19 ± 13.69** | **44.71 ± 47.79** | **5.78 ± 4.32** | **10.15 ± 7.51** | **1016.60 ± 647.31** | **0.51 ± 0.4** | **6.68 ± 18.49** | **3140 ± 2292** |

*Tab.10: Concentration of unconjugated, taurine and glycine conjugated bile acids purified from stool inclusive comparison with the values from L. Humbert et al.* [35]. *Stool was collected from 54 different healthy subjects*

# 5.5 Example of a chromatography of bile acids purified from urine

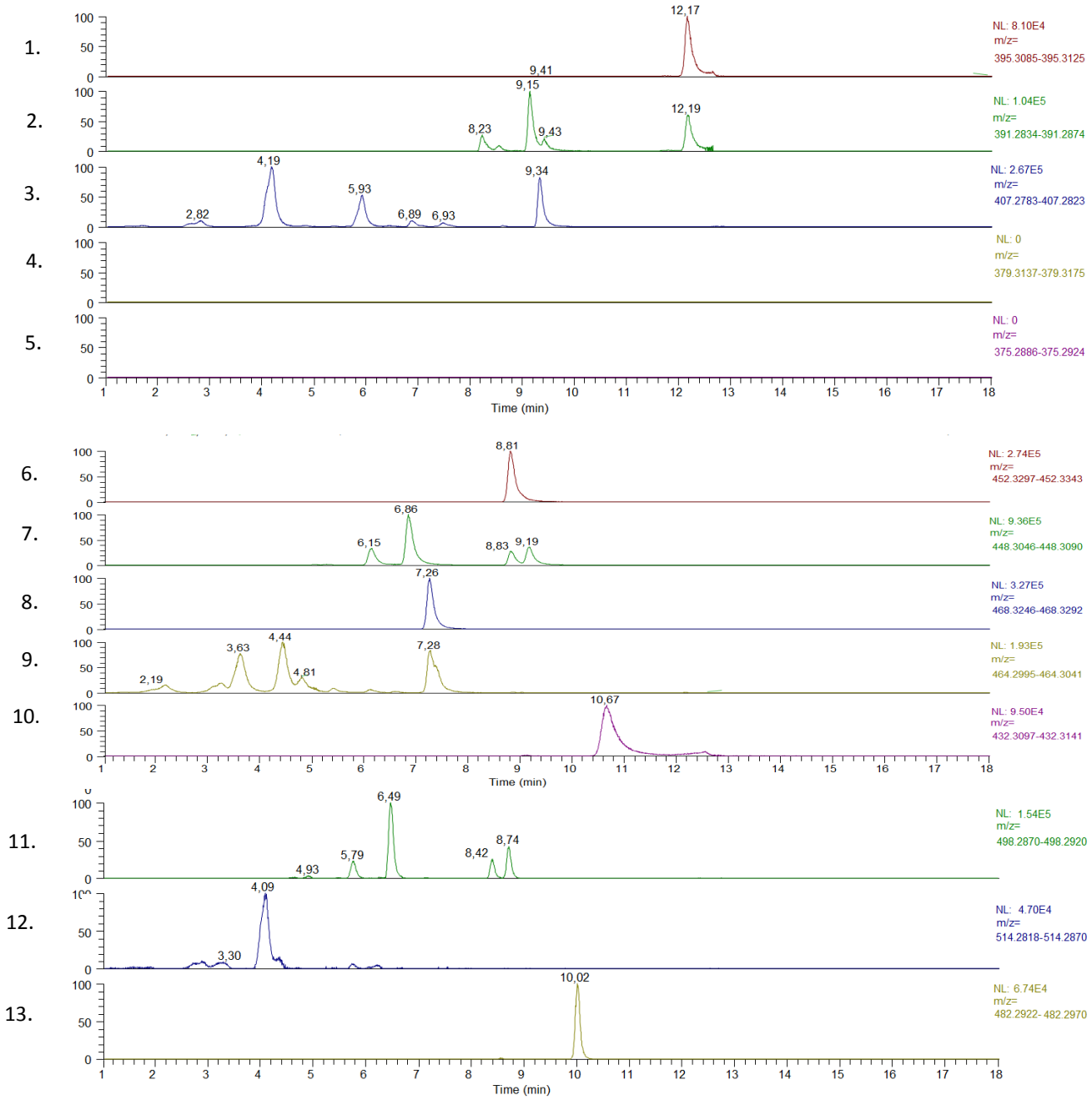



*Fig.23: Example of bile acids purified from urine; chromatogram of unconjugated bile acids: top (line 1.-5.); chromatogram of glycine conjugated bile acids: middle (line 6.-10.); chromatogram of taurine conjugated bile acids: bottom (line 11.-13.);*

Fig.23 shows an example of the successful preparation of bile acids from human urine and subsequent chromatography and mass spectrometric analysis of these.

### 5.5.1 Unconjugated bile acids purified from urine

Looking at the top chromatogram, we can see that the peak of 12,19 in the "2. line" resembles the unconjugated deoxycholic acid - as the internal standard D4 deoxycholic acid elutes at the same time (see "1. line"). As we already know, chenodeoxycholic acid would elute immediately before - at about 11,80. Looking at this chromatogram we can see, that it is present only in scarce amounts. As there still are four isomeric peaks observable, this would indicate that ursodeoxycholic- as well as the in human very scarcely appearing (see Fig.11 by Humbert et al. [35] below) hyodeoxycholic acid would be present. The usual retention time of ursodeoxycholic acid does indeed match with the high peak we can observe at 9,15 in this chromatogram. Hyodeoxycholic acid usually has a retention time of 9,50 - indicating that in this chromatogram it resembles the small peak at 9,43. As described in "5.3 Example of a chromatography of bile acids purified from stool " above, even though the standards hydoexycholic acid and its glycine and taurine conjugates were available, they were not used for analysis for the above stated reasons. Retrospective evaluation without the standard dilution series would be inaccurate and was therefore not performed.
The small peaks preceding ursodeoxycholic acid can not be identified with the used standards. Other isomeres of this mass are not well known, however there are many combinations of hydroxygroup - positioning left of the same molecular formula. Slight differences in hydroxygroup - positioning are very likely, however the specific structure can not be identified at this time.
A hindering factor to proper elution which would result in an appearance of numerous peaks of the same analyte is not likely. Therefore, the identified peaks are safely assigned to its analytes and can be evaluated separatly. The same of course applies to the example chromatogram of bile acids exctracted from stool, see Fig.21 above.

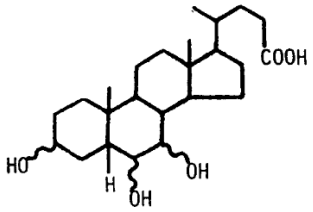
It is safe to say that the peak at 9,34 in the "3. line" resembles cholic acid, as the retention time coincides with the expected time. Again, numerous peaks - in this case

five- are observable in front. Assuming that every peak would belong to a different isomer, we would be of one analyte short as there are only 4 isomeres of cholic acid known- α-, β -, γ- and ω- muricholic acid. All are of course very rare in humans - yet detectable in urine as stated by Junichi Goto et al [85].

α- muricholic acid, which could very well be present in human urine, is not matching to any peak with a usual retention time of 7,80. All other muricholic acids usually elute even later, indicating that the prominent peaks we can observe at 4,19 or 5,39 do not represent muricholic acids.

This does further show the large variety of bile acids and that many structures are not yet identified, leave alone determined their chemical properties or biological functions.

After Takashi Iida et al. [86], there are eight possible configuration isomeres of muricholic acids. Here, the hydroxygroups are positioned at 3, 6 and 7. Four of these, of course, being the documented α-, β -, γ- and ω- muricholic acid. This leaves four other possible analytes.




| | C-3 | C-6 | C-7 |
|---|---|---|---|
| **1** | α | α | α |
| **2** | α | α | β |
| **3** | α | β | α |
| **4** | α | β | β |
| **5** | β | α | α |
| **6** | β | α | β |
| **7** | β | β | α |
| **8** | β | β | β |

*Fig.24: Configuration isomers of muricholic /cholic acids* [86]*;*

Concerning constitution isomers, this implies another seven possible configuration isomers just of cholic acid. Here, hydroxygroups are positioned at 3, 7 and 12. Other rearrangements of hydroxygroups are also possible:

| C-3 | C-6 | C-7 | C-12 |
|---|---|---|---|
| √ | √ | √ | x |
| √ | √ | x | √ |
| √ | x | √ | √ |
| x | √ | √ | √ |

*Tab.11: Possible constitution isomers of muricholic/ cholic acids;*

Again, each having eight configuration isomers. In total, this means that there are 32 isomers of which only five are described.

The same of course applies to the isomers of ursodeoxycholic-, hyodeoxycholic-, chenodeoxycholic- and deoxycholic acid.

Considering that in this case the two hydroxygroups can be lined up in α-or β-, this leaves four possibilities of every configuration isomer. Also taking into account that they can be positioned at 3, 6, 7 and 12 of the steroid backbone this leaves 6 different constitution isomers. Therefore, 24 different isomers of dihydroxycholan-24-oic acids are possible - four of which represent ursodeoxycholic-, hyodeoxycholic-, chenodeoxycholic- and deoxycholic acid. This leaves 20 other possible analytes in the same mass range.

In the "4. line" no peak is observable. This is not suprising, as no internal D4 lithocholic acid standard was added. In the "5. line" we can see, that no lithocholic acid is present in the sample.

### 5.5.2 Glycine conjugated bile acids purified from urine

The peak of the internal standard "D4 glycochenodeoxycholic acid" in the "6. line" affirms the usual retention time. However, as we can see in the "7. line", it is still difficult to identify glycochenodeoxycholic- and glycodeoxycholic acid. The latter small peak of course belongs to glycodeoxycholic acid.

Also, the earlier isomeres are not easy to identify:

The high peak at 6,86 could very well be the usually rare glycohyodeoxycholic acid with a usual retention time of 6,83. In addition, glycoursodeoxycholic acid elutes earlier at about 6,06 - the identity of the small first peak in the "7. line" is therefore resolved. As they both are clearly separated, the the large peak at 6,86 is not an

offshoot of the glycoursodeoxycholic acid. In addition, this also coincides with the evaluation of unconjugated bile acids - even if hyodeoxycholic acid's glycine conjugate is present in a much higher concentration.
The internal standard D4 glycocholic acid in the "8. line" affirms, that the peak at 7,28 in the "9. line" is glycocholic acid. The preceding peaks are -as it is the case with the unconjugated cholic /muricholic acids - hard to identify. Considering the evaluation of the unconjugated ones and checking the usual retention times for glycine conjugated muricholic acids (starting at 4,80 for glyco α- muricholic acid) it is clear, that no glycine conjugated muricholic acids are present in this sample. The structures remain, as it was the case with the unconjugated ones, unknown.
In the "10. line" we can see, that a low amount of glycolithocholic acid is present.

### 5.5.3 Taurine conjugated bile acids purified from urine

As we can see, up to the synthesis, purification and preparation of new bile acid standards, no internal taurine conjugated bile acid standards were part of the method.
After synthesis of these, we know that a D4 taurine conjugated deoxycholic acid usually elutes at about minute 9 with the same method. However, again two small peaks are detectable in this range (see "11. line"). The attribution of the peak at 8,42 to taurochenodeoxycholic acid is therefore very likely.
Again, the peak at 6,49 can be attributed to taurohyodeoxycholic acid, as the synthesised standard also usually elutes at 6,56. The peak at 5,79 of course resembles tauroursodeoxycholic acid.
In the "12. line" we can only see one prominent peak at 4,09. At first sight we assume that it should be attributed to the first eluting tauro muricholic acid- tauro α- muricholic acid as it appears very early. However, as it was also the case for the unconjugated and glycine conjugated ones, even for tauro α- muricholic acid this is too early. Taurocholic acid, the one that should be present individually and in the highest concentration of this mass, usually elutes at 6,80 hence the confusion. As no other prominent peak is observable, the appearance of tauromuricholic acids is unlikely as it appears even too early for muricholic acids, retention times have clearly been distorted. Yet, the peak can be attributed to taurocholic acid.

In the "13. line" we can see, that taurolithocholic acid is present in a low concentration.

## 5.6 Evaluation of urine samples

| Bile acids calculated amount in nmol/L | 25°C | | | | | | 37°C | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S1 No enzyme 2h | S1 E.coli 2h | S1 Helix p. 2h | S2 No enzyme 2h | S2 E.coli 2h | S2 Helix p. 2h | S3 No enzyme overnight | S3 E. coli 2h | S3 Helix p. 2h | S3 E.coli overnight | S3 Helix p. overnight | S4 No enzyme overnight | S4 E. coli 2h | S4 E.coli overnight | S4 Helix p. 2h | S4 Helix p. overnight |
| 1. Ursodeoxycholic | 117.383 | 34.295 | 92.495 | 36.007 | 181.759 | 174.192 | 3.578 | 53.870 | 39.501 | 53.075 | 45.642 | 4.165 | 24.646 | 21.263 | 33.486 | 69.300 |
| 2. Tauroursodeoxycholic | 0.528 | 1.207 | 0.199 | NF | 1.047 | 0.541 | 0.074 | 3.103 | 6.717 | 3.901 | 15.550 | NF | 8.324 | 9.077 | 16.433 | 46.564 |
| 3. Glycoursodeoxycholic | 44.737 | 7.269 | 18.131 | 74.574 | 130.730 | 183.791 | 26.407 | 104.146 | 141.985 | 134.141 | 302.178 | 9.035 | 78.710 | 94.896 | 152.277 | 379.392 |
| 4. Chenodeoxycholic | 561.016 | 179.137 | 373.501 | 95.507 | 160.547 | 140.548 | 12.232 | 12.389 | 220.627 | 11.834 | 46.212 | 3.851 | 10.270 | 9.106 | 18.472 | 24.401 |
| 5. Taurochenodeoxycholic | 9.076 | 2.502 | 2.285 | 2.587 | 4.853 | 7.212 | 0.707 | 1.384 | 7.731 | 1.545 | 13.286 | 0.291 | 1.195 | 1.189 | 6.570 | 12.561 |
| 6. Glycochenodeoxycholic | 272.474 | 74.413 | 118.967 | 50.827 | 116.235 | 139.769 | 6.703 | 28.703 | 117.436 | 25.311 | 206.704 | 5.828 | 16.386 | 15.781 | 51.236 | 96.680 |
| 7. Deoxycholic | 331.983 | 101.576 | 216.326 | 96.863 | 340.963 | 309.598 | 17.227 | 82.965 | 115.742 | 100.463 | 111.086 | 6.278 | 6.106 | 4.154 | 8.187 | 7.065 |
| 8. Taurodeoxycholic | 10.193 | 2.433 | 2.889 | 2.447 | 4.877 | 7.692 | 0.632 | 1.645 | 7.259 | 1.588 | 17.612 | 0.209 | 0.428 | 0.966 | 1.600 | 4.025 |
| 9. Glycodeoxycholic | 310.436 | 55.488 | 131.908 | 38.143 | 135.715 | 186.587 | 6.544 | 39.399 | 110.577 | 43.832 | 197.353 | 4.558 | 8.751 | 7.225 | 14.343 | 24.094 |
| 10. Cholic | 283.872 | 453.680 | 590.138 | 1605.560 | 2505.151 | 2564.855 | 149.835 | 230.543 | 353.994 | 256.193 | 376.192 | 54.347 | 80.960 | 76.266 | 148.137 | 194.140 |
| 11. Taurocholic | 4.992 | 3.257 | 2.800 | 0.129 | 0.128 | 0.275 | 0.110 | NF | 0.179 | NF | NF | 1.018 | 3.369 | 2.961 | 4.004 | 5.706 |
| 12. Glycocholic | 201.222 | 97.987 | 132.599 | 114.245 | 123.247 | 142.151 | 86.270 | 88.285 | 130.549 | 88.946 | 167.579 | 8.775 | 18.378 | 16.757 | 20.249 | 29.788 |
| 13. Lithocholic | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF |
| 14. Taurolithocholic | 16.053 | 3.384 | 4.539 | 2.599 | 1.752 | 10.881 | 0.570 | 0.351 | 6.781 | 0.397 | 20.157 | 0.164 | 0.140 | NF | 0.211 | 0.538 |
| 15. Glycolithocholic | 509.590 | 101.437 | 235.390 | 51.946 | 42.551 | 120.170 | 7.519 | 6.442 | 39.436 | 9.713 | 137.270 | 5.620 | 2.139 | 1.627 | 2.186 | 2.565 |

*Tab.12: Results of preparation and subsequent deglucuronidation and desulfatation of bile acids in human urine samples. Sample 1 and 2 are incubated for 2 hours with β-Glucuronidase from Helix pomatia Type H-1 and β-Glucuronidase from Escherichia coli Type X-A at 25°C and a pH of 5.44. Samples 3 and 4 are incubated for 2 hours and overnight with the same enzymes at 37°C also at a pH of 5.44.*

*Concentrations were calculated with Xcalibur 2.3, using the integrated detected peakareas of the different bile acids and peakareas of the corresponding internal standards. Concentrations are presented in nmol/l.*

| Directly measured sulfated bile acid calculated amount in nmol/L | 25°C | | | | | | 37°C | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S1 No enzyme 2h | S1 E.coli 2h | S1 Helix p. 2h | S2 No enzyme 2h | S2 E.coli 2h | S2 Helix p. 2h | S3 No enzyme overnight | S3 E. coli 2h | S3 Helix p. 2h | S3 E.coli overnight | S3 Helix p. overnight | S4 No enzyme overnight | S4 E. coli 2h | S4 E.coli overnight | S4 Helix p. 2h | S4 Helix p. overnight |
| **1. Ursodeoxycholic** | 41.32 | 201.83 | 111.91 | 263.56 | 293.30 | 230.03 | 240.0601825 | 199.982162 | 192.9708887 | 199.916644 | 180.5761995 | 52.16219391 | 64.95113728 | 52.64531744 | 62.41505826 | 41.14869157 |
| **3. Glycoursodeoxycholic** | 12.20 | 23.30 | 20.69 | 48.28 | 56.31 | 48.77 | 25.21081667 | 20.3445837 | 18.10728532 | 20.59298187 | 6.960190807 | 14.00431662 | 13.70876609 | 12.67943682 | 8.624432075 | 1.313553507 |
| **4. Chenodeoxycholic** | 10.86 | 41.98 | 41.88 | 543.80 | 656.47 | 383.87 | 202.4956129 | 168.097341 | 97.00262962 | 128.710674 | 4.506239493 | 45.61031579 | 59.17037727 | 45.46955819 | 58.83675279 | 37.50561577 |
| **6. Glycochenodeoxycholic** | 4.66 | 8.19 | 7.16 | 119.06 | 136.54 | 137.50 | 41.80707956 | 33.8655659 | 20.6637161 | 32.87750324 | 6.317787796 | 10.28403518 | 9.413322482 | 8.005671174 | 5.961694499 | 1.495034359 |
| **7. Deoxycholic** | 2.89 | 9.12 | 8.82 | 56.96 | 73.82 | 54.47 | 81.93159949 | 66.4619733 | 62.11451598 | 62.0173174 | 49.90445578 | 7.008691487 | 4.42685393 | 2.862581255 | 5.26029458 | 2.264205276 |
| **9. Glycodeoxycholic** | 1.05 | 0.57 | 0.57 | 11.69 | 13.70 | 16.18 | 5.136512314 | 4.08062752 | 3.318309069 | 4.143921087 | 1.005524123 | 1.320231446 | 1.115754379 | 0.906453554 | 0.931615997 | 0.16770236 |
| **12. Glycocholic** | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF |
| **13. Lithocholic** | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF | NF |
| **15. Glycolithocholic** | NF | 0.01 | NF | 0.07 | 0.30 | 0.13 | 0.055876555 | 0.04750973 | 0.033104177 | 0.037086706 | 0.055962631 | NF | 0.04467488 | 0.060017775 | 0.055319218 | 0.056064573 |

*Tab.13: Results of directly measured sulfated unconjugated and glycine conjugated bile acids.Taurine conjugated bile acids could not be measured with the method used. Sample 1 and 2 are incubated for 2 hours with β-Glucuronidase from Helix pomatia Type H-1 and β-Glucuronidase from Escherichia coli Type X-A at 25°C and a pH of 5.44. Samples 3 and 4 are incubated for 2 hours and overnight with the same enzymes at 37°C also at a pH of 5.44.*

*Concentrations were calculated with Xcalibur 2.3, using the integrated detected peakareas of the different bile acids and peakareas of the corresponding internal standards. Concentrations are presented in nmol/l.*

For better and faster understanding of the possible conclusions, the following graphs will show the part of the data more comprehensible:

### 5.6.1 Deglucuronidation and desulfation of unconjugated and conjugated ursodeoxycholic acids

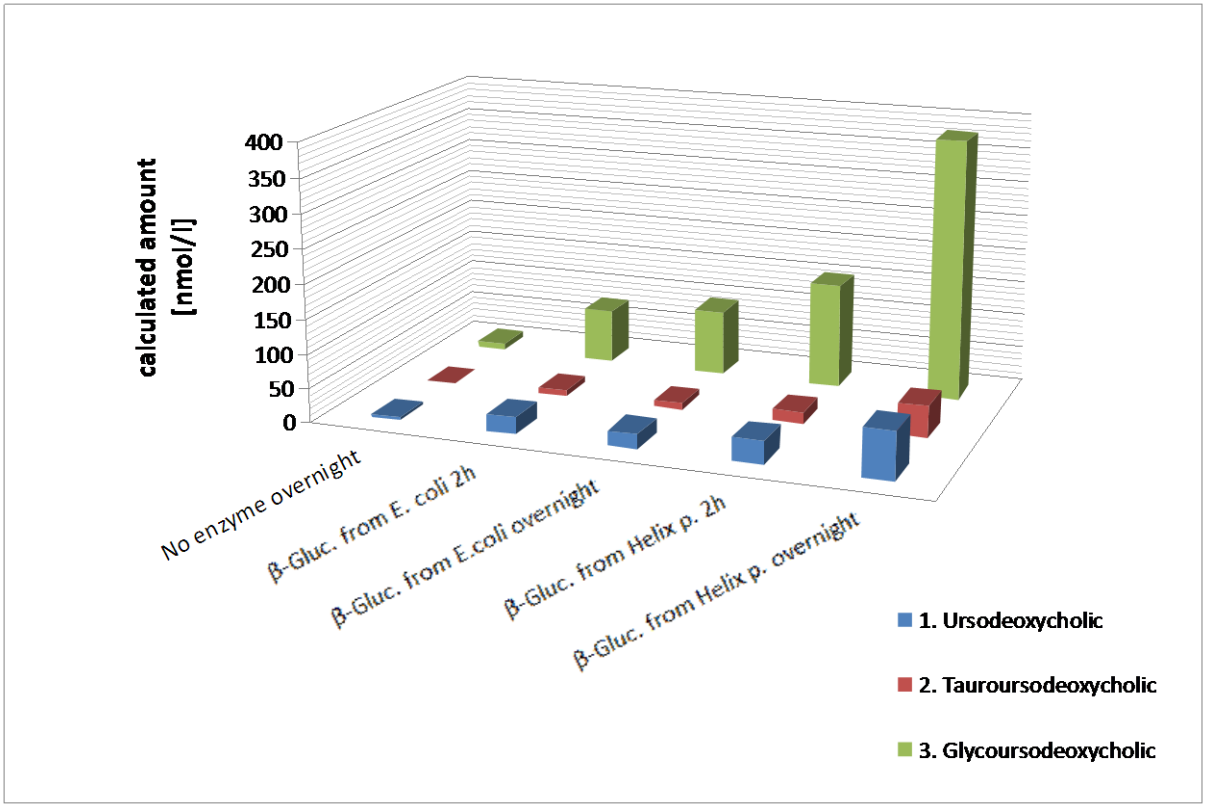



*Fig.25: Deglucuronidation and desulfation of unconjugated and conjugated ursodeoxycholic acids, purified from sample 4 and incubated at 37°C for 2 hours or over night with β glucuronidase of E. Coli or of Helix pomatia. Concentrations are presented in nmol/ml.*

*S4 No enzyme overnight:* No enzyme was added (control). Incubation over night , *S4 E. coli 2h:* β-Glucuronidase from Escherichia coli was added. Incubation for 2 hours , *S4 E. coli overnight:* β-Glucuronidase from Escherichia coli was added. Incubation over night , *S4 Helix p. 2h:* β-Glucuronidase from Helix pomatia was added.

Incubation for 2 hours *, S4 Helix p. overnight:* β-Glucuronidase from Helix pomatia was added. Incubation over night *.*

The first noticeable thing is, that glycine conjugated ursodeoxycholic acids are excreted in much higher amounts than taurine conjugated ones or unconjugated ones.
Looking closer, glycine conjugated ursodeoxycholic acid that was detected without any enzyme added in the incubation over night, is not present in a much higher concentration than taurine conjugated ones or unconjugated ones. Yet, the concentrations are rising after deglucuronidation with the E. coli derived enzyme was achieved and are again dramatically higher after desulfation and deglucuronidation was achieved with the Helix pomatia derived enzyme.
They are now present in a much higher concentration of up to 42 times of the non deglucuronidated and desulfatated glycine conjugated ursodeoxycholic acid. This shows that most of glycine conjugated ursodeoxycholic acids in urine are present in sulfated forms, which coincides with the findings of Humbert et al. [35] see Fig.11 above.
In addition to these findings, we can also conclude that also a noticeable proportion of glycine conjugated ursodeoxycholic acids are excreted in a glucuronidated form.
Comparing the bars of incubation with the β glucuronidase from Helix pomatia for 2 hours with the ones of the incubation over night, we can also see that ursodeoxycholic acid - be it in unconjugated or taurine or glycine conjugated state- could not be deglucuronidated or desulfated effectively after two hours. Both enzymes, at least with the concentration used (600 units per millilitre), take a longer period of time- even if the pH value is set and the incubation temperature is optimal.

Ursodeoxycholic acids are excreted only scarcely in serum and stool (see Fig.11 above) and only show higher concentrations when administered for therapeutic use. Analyzing the samples had therefore risen the suspicion that it had been administered to the patients.
As we can see in Fig.11 above, especially sulfated and glycine conjugated ursodeoxycholic acids do naturally ocurr in urine. This again provokes the question why ursodeoxycholic acid is only scarcely found in serum yet in high amounts in urine, as bile acids excreted with urine should only be the serum filtered ones. An

explanation for this would be a reconfiguration of bile acids past the renal filtration. As described in "1.1.4 Synthesis of bile acids" above, ursodeoxycholic acid is usually synthesized by epimerization of the 7 cis-hydroxygroup of the primary bile acid chenodeoxycholic acid to a 7 trans configuration. This happens in the gut by microbial enzymes.

Transformation of bile acids after circulation in the bloodstream has already been observed. In one case, ursodeoxycholic acid that was administered orally has been shown to be isomerised into isoursodeoxycholic acid by epimerization of the 3-α-hydroxygroup [87]. This process has also been shown vice versa in vivo (epimerization of the 3-β-hydroxygroup [88]). In addition, the administered ursodeoxycholic acid has been shown to be hydroxylated at the C1, C5, C6, C12, and the C21 as well as C22 atom of the side chain [87]. This demonstrates a fraction of the possibilities of how versatile bile acids could be isomerised into - in this case- ursodeoxycholic acid.

However, this does still not explain why the composition of ursodeoxycholic acid and its various conjugates differs that much between serum and urine, as latter ones should just be filtered out of the serum. (Observable in Fig.11).

The answer does not lie in an isomerisation process - as this takes place partially in the liver and partially by mycrobial gut enzymes. In addition, isomerisation of certain bile acids into another one would still cause an equal composition throughout the different body fluids. The explanation is a bile acid specific transporter in the proximal tubulus of the kidney, already mentioned in "1.1.2 Circulation of bile acids" above:

In an observation, taurochenodeoxycholate was perfused within a protein free medium into an isolated rat kidney preparation. This caused a decreased reabsorption of taurocholate, chenodeoxycholate 3,7-disulfate and chenodeoxycholate 7-monosulfate in the proximal tubulus and thus increasing the renal excretion. This alter in renal reabsorption as a cause of an interaction between bile acids explains the different composition of bile acids in urine compared with that in serum or plasma [89]. This shows that bile acids prepared from urine are not in the same composition and concentration as bile acids prepared from serum. In fact, only 10-30% of the bile acids present in plasma are not protein bound and therefore target of renal filtration [90].

In addition, a nearly complete reabsorption of the filtered bile acids in the proximal tubulus causes a different composition of bile acids in urine than in blood. Certain bile

acids (or conjugation forms of bile acids) are therefore reabsorbed to a high degree, whereas others are concentrated in the urine.
This imposes an important reason for the analysis of bile acids extracted from a patient's urine: Comparing the composition and concentration of certain bile acids between serum and urine could possibly lead to a diagnosis of renal complications. Of course, the depth of a complication in the reabsorption of bile acids in the renal proximal tubulus needs to be further explained by medical investigations. A second possible diagnostic target could be complications of the sodium dependent bile salt transporter -or ASBT- as a malfunction (e.g. genetical) would of course lead to an impaired reabsorption both in the illeum and in the kidney and thus affecting the excretion levels of bile acids. This can be predicted as both ASB transporter (the ones localized in the brush border membrane of proximal tubular cells as well as the ones in the ileum) are encoded by the same gene (SLC10A2) and even the same transcript [91].

The identity of bile acids which are more likely to be reabsorbed and the ones that are avoided by the ASBT are deductable from Fig.11 presented by Humbert et al. and from Tab.15 of this work:

**Bile acid and their conjugate form concentrated in urine:**

As already described by C L Corbett et al. [92] and explained in "1.1.2 Circulation of bile acids" above, bile acids are excreted with urine predominantly in a sulfated form. In case of liver cirrhosis, renal clearance of sulfated bile acids has in fact been found to be elevated 20 - 200 fold [93].
It is not suprising therefore, that after Humbert et al. [35] (see Fig.11) GUDCA-3S, TLCA-3S and GLCA-3S have been found in urine in much higher concentrations than in serum. As a matter of fact, TUDCA-3S, UDCA-3S, CDCA-3S and DCA-3S have only been found in urine. These observations were made in samples of healthy patients.

**Bile acid and their conjugate form reabsorbed to a high degree:**

TCA, GCA, CA and DCA was found in urine as well as in serum of healthy patients. Yet the concentration was lower in urine than in serum.

In addition, GUDCA, UDCA, TCDCA, GCDCA, TDCA, GDCA and CDCA has been found in serum and not urine. These bile acids are therefore partially -or, as in the latter case - completely reabsorbed in the ASB transporters of the proximal tubulus in the kidneys.

However, data presented in this work shows, that CDC can be found in human urine. Even low concentrations of UDCA, GDCA, GCDCA and GDA were found in urine in the conducted experiment. However, TCDCA and TDCA could not be found as well.

How can these findings be explained? First of all, as already mentioned, the healthy status of the patients observed in this conducted experiment can not be guaranteed. E.g. a case of liver cirrhosis or biliary obstruction could not only lead to altered bile acid concentrations, but, as a source of this, to an impaired reabsorption of bile acids from the ASBT. This, of course, could cause certain bile acids to be present in the urine even if they would be reabsorbed to 100% in a healthy subject.

In addition, the threshold values of concentrations as well as the evaluation can differ. E.g. TCDCA and TDCA were found to a minimal degree in the conducted experiment. Yet it is also a question of the background height to "trust" this data. Last but not least, the mass spectrometric machinery used in the presented work is much more sensitive than the one used in previous literature. This allows a more sensitive analysis of low bile acid concentrations.

### 5.6.2 Deglucuronidation and desulfation of unconjugated and conjugated chenodeoxycholic acids

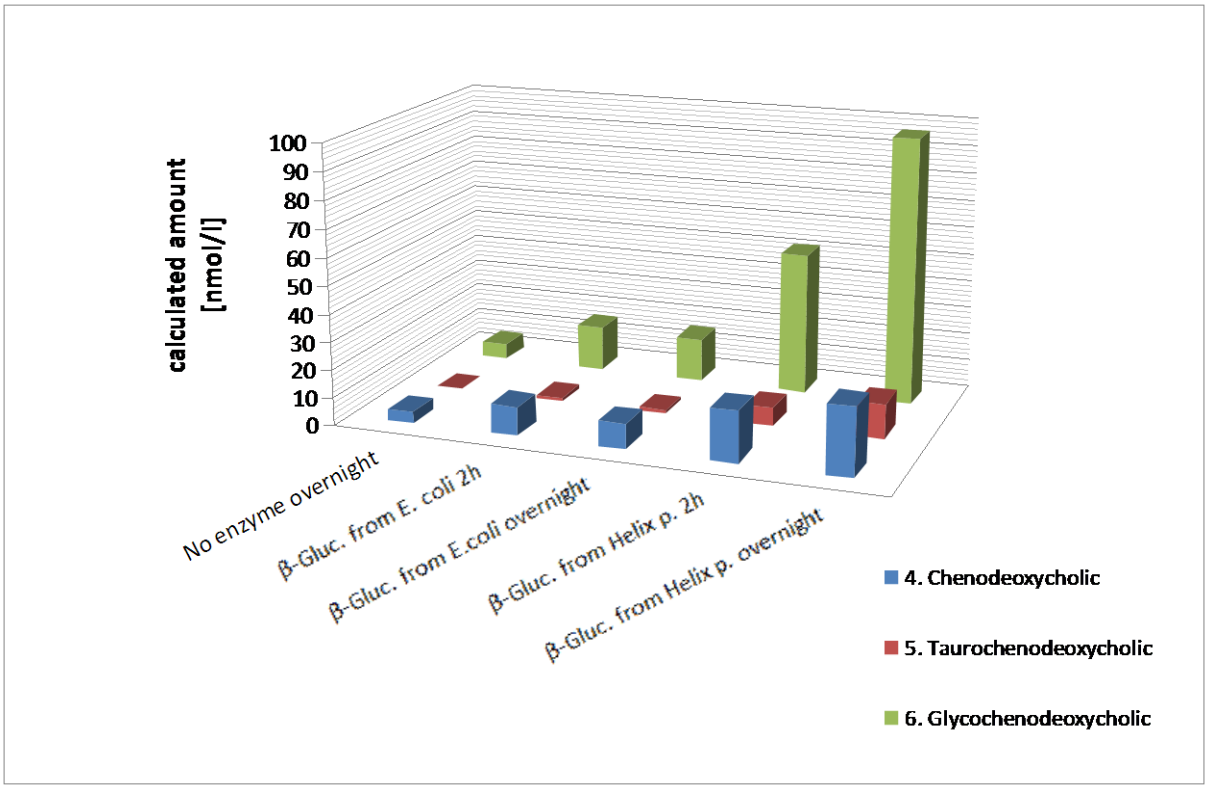



*Fig.26: Deglucuronidation and desulfation of unconjugated and conjugated chenodeoxycholic acids. Concentrations are presented in nmol/ml.*

*S4 No enzyme overnight:* No enzyme was added (control). Incubation over night , *S4 E. coli 2h:* β-Glucuronidase from Escherichia coli was added. Incubation for 2 hours , *S4 E. coli overnight:* β-Glucuronidase from Escherichia coli was added. Incubation over night , *S4 Helix p. 2h:* β-Glucuronidase from Helix pomatia was added. Incubation for 2 hours , *S4 Helix p. overnight:* β-Glucuronidase from Helix pomatia was added. Incubation over night

Again, glycine conjugates of the bile acid were excreted in much higher concentration than taurine conjugated ones. Unconjugated chenodeoxycholic acids were again excreted in higher amounts than taurine conjugated ones. Again we can see, that glycine conjugated chenodeoxycholic acids have to be present especially in sulfated forms, as concentrations are much higher after the incubation with the β glucuronidase of Helix pomatia. The incubation over night yields much higher

concentrations, indicating also for the glycine conjugated chenodeoxycholic acid, that one or more sulfated forms need more than two hours to be digested.
The share of sulfated forms of unconjugated and taurine conjugated chenodeoxycholic acids is, again, much lower.
The results of the incubation with the β glucuronidase of E. Coli reveal, that chenodeoxycholic acid- be it unconjugated or conjugated with glycine or taurine- are only scarcely excreted in glucuronidated forms.
Interestingly, they also show a decline in concentration rather then a rise as observed for glycoursodeoxycholic acid!
This does not only show that after two hours deglucuronidation of chenodeoxycholic acids was completely successful (in contrast to desulfation with β glucuronidase of Helix pomatia), but also that most probably, as already described by Volkmar Graef et al. [79], after reaching maximal hydrolysis, the steroid backbone is attacked enzymatically by different enzymes contained in the preparation of the β glucuronidase of E. Coli. As explained by Volkmar Graef et al. [79], different steroids are also hydrolysed while being stored in urine itself. In case of this experiment, this origin of steroid hydrolysing enzymes is not very likely, as a protein denaturing step with acetonitrile as well as removal with a solid phase extraction is part of the purification of bile acids from urine.
Not only have hydrolisation reactions of steroids because of contaminations in β glucuronidase preparations of bacteria and molluscs been reported [79], but also transormations of steroids:
Estron and estradiol to various other steroids [94], estron to estratiodiol [95]and vice versa [96].

For routine analysis, this means that an ideal time for desulfation (as seen, over night) is not an ideal time for deglucuronidation, as contaminations of the β glucuronidase of Helix pomatia can lead to hydrolysis and/or transformations of deglucuronidated bile acids- leading to lower concentrations than ocurring in the patient. Yet, as this preparation of enzyme is still the best commercially available option and the deviation is, as observable in Tab.12 and Fig.27, 28 and 29, not that high, it will still be used for routine analysis.

### 5.6.3 Deglucuronidation and desulfation of unconjugated and conjugated deoxycholic acids

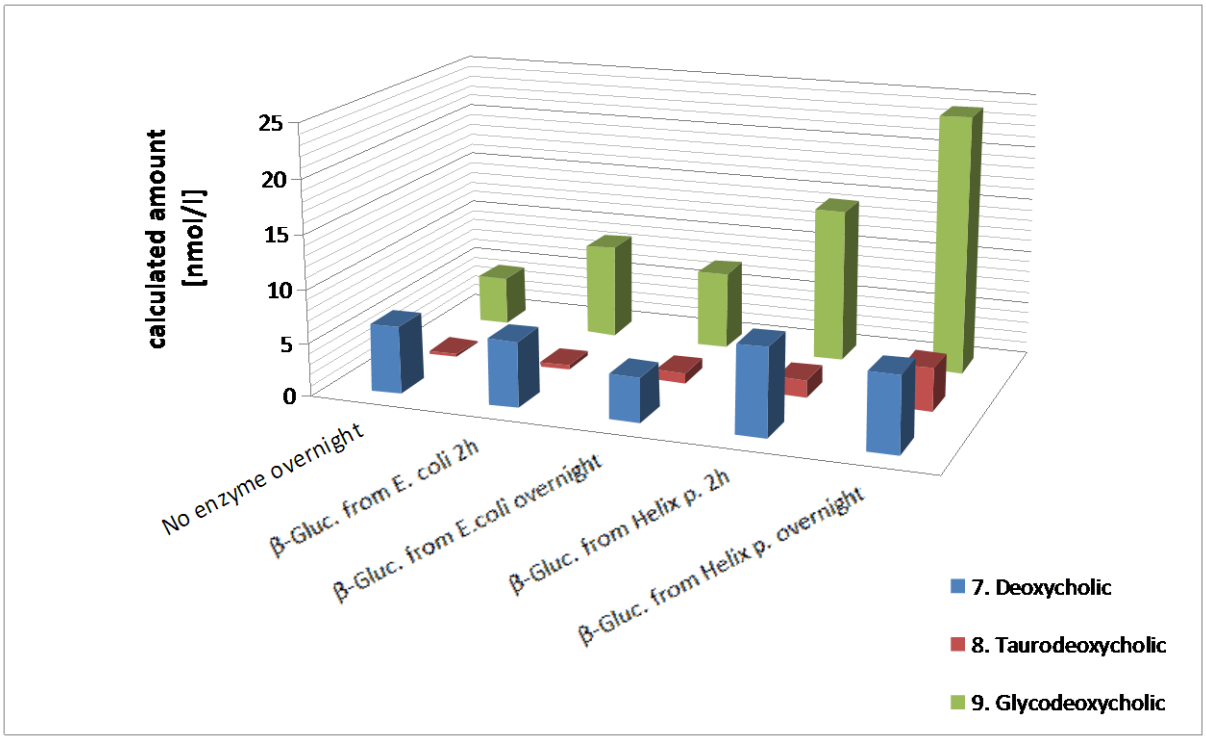



*Fig.27: Deglucuronidation and desulfation of unconjugated and conjugated deoxycholic acids. Concentrations are presented in nmol/ml.*

*S4 No enzyme overnight:* No enzyme was added (control). Incubation over night , *S4 E. coli 2h:* β-Glucuronidase from Escherichia coli was added. Incubation for 2 hours , *S4 E. coli overnight:* β-Glucuronidase from Escherichia coli was added. Incubation over night , *S4 Helix p. 2h:* β-Glucuronidase from Helix pomatia was added. Incubation for 2 hours , *S4 Helix p. overnight:* β-Glucuronidase from Helix pomatia was added. Incubation over night

Also the concentration of glycine conjugated deoxycholic acids exceed unconjugated and taurine conjugated ones. Again, taurine conjugated ones are almost not present at all.

The incubation with the β glucuronidase from E.Coli over night does not change this- indicating that they are not even present in glucuronidated forms. Sulfated taurine conjugated bile acids on the other hand are present in small amounts. Again, an

incubation of two hours is not sufficient for complete desulfation, be it for taurine conjugated or glycine conjugated ones.

Interestingly, unconjugated deoxycholic acids do not seem to be present much in sulfated or glucuronidated forms as the concentrations of enzymatically digested samples do not exceed the negative control. Or, better said, it is hard to estimate as, as decribed above, hydrolysis and/or transformation of deglucuronidated and desulfated bile acids by impurification of the enzyme preparation lead to a decline in concentration.

This leads to another observation: Not only do ideal incubation times vary between desulfatation and deglucuronidation, but also between the different bile acids. It is therefore impossible to adapt the incubation time in order to abolish the negative effect of hydrolysis and transformation of bile acids without sacrificing time needed for the ideal digestion of other bile acids.

The different rates of desulfation of the differing bile acid sulfates, depending on the position of the sulfate, were already described by Parmentier and Eyssen in 1975 [97]. This shows, that also protocols using solvolysis instead of a sulfatase meet these challenges.

Again, sulfated forms - at least sulfated forms of glycine conjugated deoxycholic acids- are present in much higher concentrations than glucuronidated forms.

### 5.6.4 Deglucuronidation and desulfation of unconjugated and conjugated cholic acids

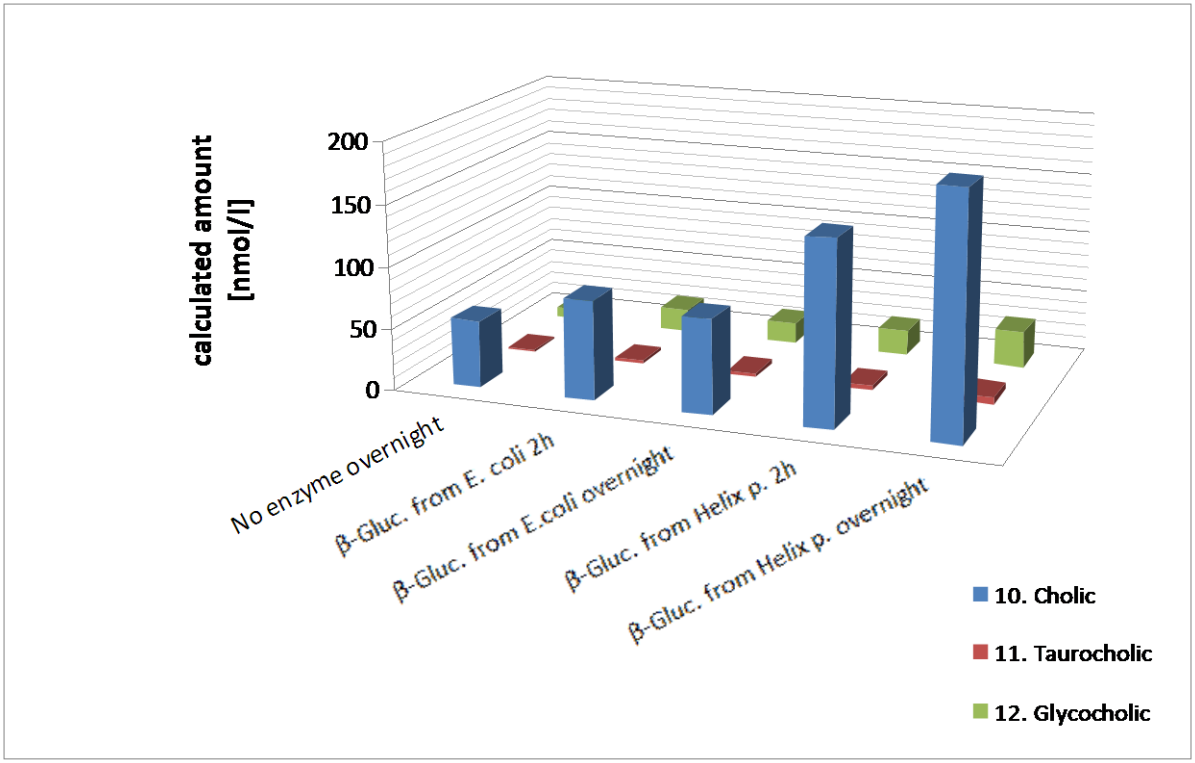



*Fig.28: Deglucuronidation and desulfation of unconjugated and conjugated cholic acids. Concentrations are presented in nmol/ml.*

*S4 No enzyme overnight:* No enzyme was added (control). Incubation over night , *S4 E. coli 2h:* β-Glucuronidase from Escherichia coli was added. Incubation for 2 hours , *S4 E. coli overnight:* β-Glucuronidase from Escherichia coli was added. Incubation over night , *S4 Helix p. 2h:* β-Glucuronidase from Helix pomatia was added. Incubation for 2 hours, *S4 Helix p. overnight:* β-Glucuronidase from Helix pomatia was added. Incubation over night

Cholic acids differ from other observed bile acids in the aspect that they are the only one occuring in an unconjugated form in the highest concentration. No taurine conjugated cholic acid could be detected, not even a glucuronidated or sulfated one. Glycine conjugated cholic acids were only found in scarce amounts, with glucuronidated or sulfated forms taking no part.

Again, a decline of concentration was observable for deglucuronidation of bile acids with the β glucuronidase derived from E. Coli, indicating that an overnight incubation indeed is too long. No decline is observable for the incubation with the β glucuronidase from Helix pomatia, which does not mean that no hydrolysis or transformation of bile acids is taking place. As the desulfatation process is not finished after two hours, a possible decline of bile acids is masked by the rising concentration of desulfated bile acids.

### 5.6.5 Deglucuronidation and desulfation of unconjugated and conjugated lithocholic acids

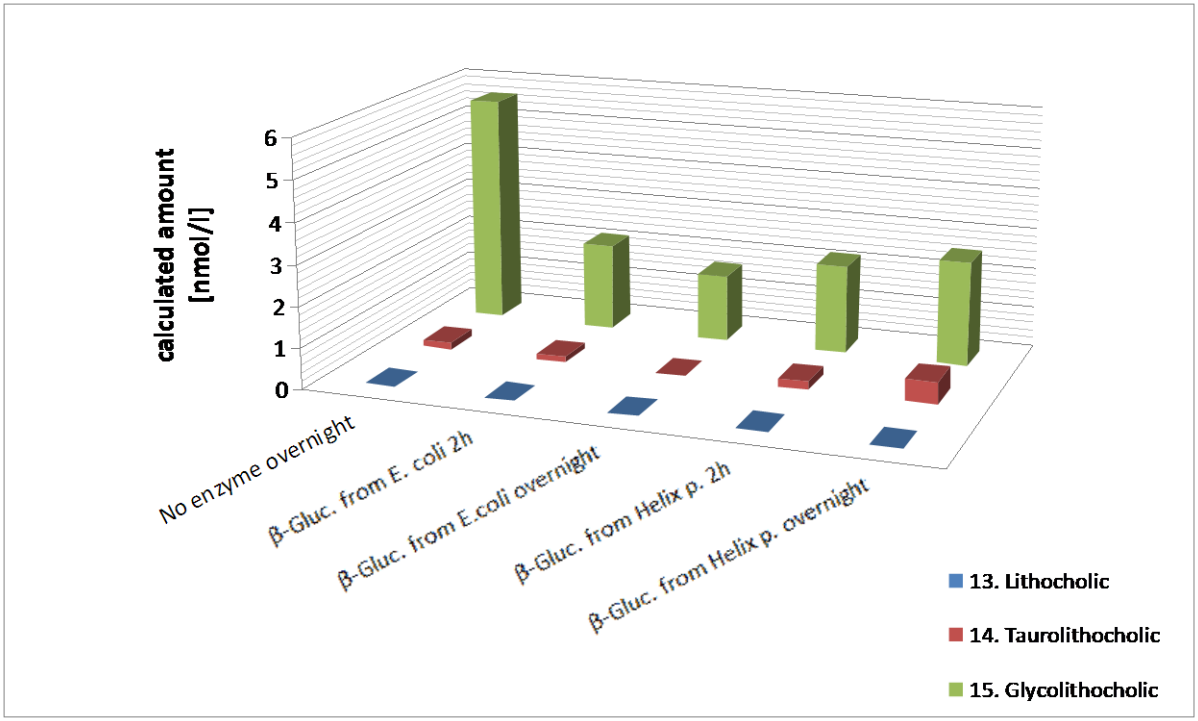



*Fig. 29: Deglucuronidation and desulfation of unconjugated and conjugated lithocholic acids. Concentrations are presented in nmol/ml.*

*S4 No enzyme overnight:* No enzyme was added (control). Incubation over night , *S4 E. coli 2h:* β-Glucuronidase from Escherichia coli was added. Incubation for 2 hours , *S4 E. coli overnight:* β-Glucuronidase from Escherichia coli was added. Incubation over night , *S4 Helix p. 2h:* β-Glucuronidase from Helix pomatia was added. Incubation for 2 hours , *S4 Helix p. overnight:* β-Glucuronidase from Helix pomatia was added. Incubation over night

Lithocholic acids are only present in human urine in a glycine conjugated form. Interestingly, any incubation with an enzyme lead to a lower concentration than of the negative control. This would indicate that the incubation itself very likely was not successfull. However, as lithocholic acids were measured in the same sample as every other bile acid, this is not a possible explanation. This would mean that glycine conjugated-, non - sulfated- and non - glucuronidated lithocholic acid seems to be the only form of lithocholic acid present in the human urine (apart from a trace amount of a sulfated taurine conjugated lithocholic acid- at least in the observed sample). Interestingly, Humber et al. found no (!) unsulfated glycolithocholic acid, but a sulfated form of glycolithocholic acid (even in relatively high amounts) and a sulfated form of a taurolithocholic acid.

However, looking at Tab.13 above we can see, that in the sample of patient 3 (see S3 Helix pomatia over night), high amounts of sulfated glycine and taurine conjugated lithocholic acids could be observed, in contrast to other lithocholic acids! For a clearer depiction of the amount of sulfated bile acids in the sample see Tab.15 below.

This again coincedes with the literature. Patient 4 has almost throughout every bile acid only half of the concentration of patient 3, yet in this case he shows a strong deviation from the literature. Any interpretation of medical issues though lies beyond the competence of the author.

This shows, again, the high variability of concentrations of different forms of bile acids in different patients.

### 5.6.6 Concentration of glucuronidated and sulfated bile acids in urine

Taurine conjugates, even if they are more polar and therefore better soluble in water, are present in urine in far inferior concentrations than glycine conjugated bile acids. In addition, even unconjugated bile acids are present in a higher concentration than taurine conjugated ones. This imposes that glucuronidation and sulfation plays a more important role for increasing water solubility in bile acids than conjugation with taurine or glycine, of course as a cause of the hepatic first pass effect described in "1.1.2 Circulation of bile acids" above.

In order to precise the proportions of sulfates and glucuronides in urine, we need to take a closer look at the concentrations of table and calculate the amount of sulfated and glucuronidated bile acids:
For an overview of the total concentration of bile acids found in the samples as well as the concentrations without glucuronidated bile acids or sulfated bile acids see Tab.14 below.

The complete calculated concentration of bile acids in sample 3 was 1,656 nmol/l whilest in sample 4 a concentration of 896 nmol/l was found. Considering the high variability between the two samples, it is hard to make assumptions about the concentrations. Analysis of more samples and observing the median, as it was done by L. Humbert et al. in Fig.11 above would be beneficial. Yet, as observable in this figure's high standard deviations, also a large sample quantity and calculation of a median value is only of limited validity and significance for medical assumptions. As also explained in "5.4 Evaluation of stool samples, unconjugated bile acids" above, the variablity of bile acid concentration between different patients, even in a healthy state is just too high.
Considering that a healthy state of the patients is not assured as explained, a comparison with literature therefore has its drawbacks. In addition, not all the same analytes were observed, as shall be explained in the different paragraphs:

#### 5.6.6.1 Indirectly calculated concentrations of glucuronidated bile acids in urine

Glucuronidated bile acids were not observed by Humbert et al. As explained, values of glucuronidated bile acids were calculated indirectly in the conducted experiment:
Data derived from the incubation without any enzyme over night was subtracted from the data derived from the incubation with the β glucuronidase of E. Coli- only the concentrations of glucuronidated bile acids should therefore remain (of course, neglecting the efficiency of the enzyme).

#### 5.6.6.2 Indirectly calculated concentrations of sulfated bile acids in urine:

The data published by Humbert et al. contains twelve different sulfated bile acids (UDCA-3S, GUDCA-3S, TUDCA-3S, CDCA-3S, GCDCA-3S, DCA-3S, GDCA-3S,

TDCA-3S, CA-3S, GCA-3S, TCA-3S and LCA-3S), which were directly measured by Esi-Ms.

The data presented in this work contains every sulfated bile acid that could be desulfated by the β glucuronidase of Helix pomatia- not only will the different bile acids exceed the number of the analytes analysed by Humbert et al., but also bile acids that are sulfated also on a position other than 3 (be it 6, 7 or even 12). The drawback in this case is, that the complete concentration of sulfated bile acids had to be calculated indirectly:

Data derived from incubation with the β glucuronidase of E. Coli over night was subtracted from the data derived from the incubation with the β glucuronidase of Helix pomatia over night. As both contain values of non glucuronidated and glucuronidated bile acids, the concentration of sulfated bile acids derived from the incubation with the β glucuronidase of Helix pomatia should remain (again, neglecting the different efficiencies of the enzymes).

| | [nmol/L] | |
|---|---|---|
| **Concentration of bile acids without glucuronidated forms after L. Humbert et al.** | **853 ± 532** | |
| **Concentration of bile acids found without glucuronidated forms in the conducted experiment** | 1656.82 | 896.82 |
| **Concentration of sulphated bile acids after L. Humbert et al.** | **643.39 ± 694.6** | |
| **Concentration of sulphated bile acids found in the conducted experiment (indirect calculation)** | 933.31 | 635.02 |
| **Concetration of glucuronidated bile acids after L. Humbert et al.** | **not measured** | |
| **Concentration of glucuronidated bile acids found in the conducted experiment (indirect calculation)** | 413.21 | 163.41 |

*Tab. 14: Comparison of bile acid concentrations withouth glucuronidated forms as well as concentrations of sulfated bile acids and glucuronidated forms found in human urine between data from this work and data published by Humbert et al. [35]*

In Tab.14 we can see, that bile acids are present in a far higher concentration (more than double) in a sulfated form than in a glucuronidated one. This reassures that sulfated bile acids are crucial for analysis of bile acids in urine. The usage of an enzyme with sulfatase function is therefore inevitable – the β glucuronidase of Helix pomatia is the enzyme of choice even without taking into consideration possible differences of efficiencies between the two enzymes.

As we can see in Tab.14 above, the concentration of total bile acids found in sample 4 without the glucuronidated forms lies well within the range of the concentration of healthy patients described by L. Humbert et al. The total concentration of bile acids found in sample 3 is ca. 250 nmol/L above the limit. As in general (see fig and ), bile acid concentrations were very high for this patient, an unhealthy status is very probable.
As observable in Tab.14, also sulfated bile acids of both samples seem to lie well within the range of healthy bile acid concentrations proposed by Humbert et al., even if, as already described above, the presented total concentration of sulfated bile acids calculated indirectly contains more analytes than the directly measured ones published by Humbert et al.

Limitations:

It shall be reminded, that these values only have limited accuracy regarding the different efficiencies of the two different enzymes affecting the indirect calculations, even though they were incubated over night and therefore being able to exert full potential. Additionally, as described in "5.6.2 Deglucuronidation and desulfation of unconjugated and conjugated chenodeoxycholic acids" above, these values include a small degree of bile acid degradation by impurities in the enzyme preparation. In general, this is not a direct measurement of glucuronidated and sulfated bile acids but an indirect calculation.

| Bile acids<br>Calculated amount [nmol/l] | S3 Helix p. overnight | S3 No enzyme overnight | S3 E.coli overnight | S3 Sulfated b.a. | S3 glucuronidated b.a. |
|---|---|---|---|---|---|
| 1. Ursodeoxycholic | 45.64 | 3.58 | 53.08 | **0.00** | **49.50** |
| 2. Tauroursodeoxycholic | 15.55 | 0.07 | 3.90 | **11.65** | **3.83** |
| 3. Glycoursodeoxycholic | 302.18 | 26.41 | 134.14 | **168.04** | **107.73** |
| 4. Chenodeoxycholic | 46.21 | 12.23 | 11.83 | **34.38** | **0.00** |
| 5. Taurochenodeoxycholic | 13.29 | 0.71 | 1.55 | **11.74** | **0.84** |
| 6. Glycochenodeoxycholic | 206.70 | 6.70 | 25.31 | **181.39** | **18.61** |
| 7. Deoxycholic | 111.09 | 17.23 | 100.46 | **10.62** | **83.24** |
| 8. Taurodeoxycholic | 17.61 | 0.63 | 1.59 | **16.02** | **0.96** |
| 9. Glycodeoxycholic | 197.35 | 6.54 | 43.83 | **153.52** | **37.29** |
| 10. Cholic | 376.19 | 149.83 | 256.19 | **120.00** | **106.36** |
| 11. Taurocholic | NF | 0.11 | NF | **0.00** | **0.00** |
| 12. Glycocholic | 167.58 | 86.27 | 88.95 | **78.63** | **2.68** |
| 13. Lithocholic | NF | NF | NF | **0.00** | **0.00** |
| 14. Taurolithocholic | 20.16 | 0.57 | 0.40 | **19.76** | **0.00** |
| 15. Glycolithocholic | 137.27 | 7.52 | 9.71 | **127.56** | **2.19** |

| Bile acids<br>Calculated amount [nmol/l] | S4 No enzyme overnight | S4 E.coli overnight | S4 Helix p. overnight | S4 Sulfated b.a. | S4 glucuronidated b.a. |
|---|---|---|---|---|---|
| 1. Ursodeoxycholic | 4.16 | 21.26 | 69.30 | **48.04** | **17.10** |
| 2. Tauroursodeoxycholic | NF | 9.08 | 46.56 | **37.49** | **9.08** |
| 3. Glycoursodeoxycholic | 9.03 | 94.90 | 379.39 | **284.50** | **85.86** |
| 4. Chenodeoxycholic | 3.85 | 9.11 | 24.40 | **15.29** | **5.25** |
| 5. Taurochenodeoxycholic | 0.29 | 1.19 | 12.56 | **11.37** | **0.90** |
| 6. Glycochenodeoxycholic | 5.83 | 15.78 | 96.68 | **80.90** | **9.95** |
| 7. Deoxycholic | 6.28 | 4.15 | 7.07 | **2.91** | **0.00** |
| 8. Taurodeoxycholic | 0.21 | 0.97 | 4.02 | **3.06** | **0.76** |
| 9. Glycodeoxycholic | 4.56 | 7.23 | 24.09 | **16.87** | **2.67** |
| 10. Cholic | 54.35 | 76.27 | 194.14 | **117.87** | **21.92** |
| 11. Taurocholic | 1.02 | 2.96 | 5.71 | **2.75** | **1.94** |
| 12. Glycocholic | 8.77 | 16.76 | 29.79 | **13.03** | **7.98** |
| 13. Lithocholic | NF | NF | NF | **0.00** | **0.00** |
| 14. Taurolithocholic | 0.16 | NF | 0.54 | **0.00** | **0.00** |
| 15. Glycolithocholic | 5.62 | 1.63 | 2.57 | **0.94** | **0.00** |

*Tab.15: Concentration of bile acids detected in Sample 3 and 4 as well as of sulfated bile acids (calculated by subtracting naturally occurring bile acids and deglucuronidated bile acids from the ones incubated with β glucuronidase from Helix pomatia) and of glucuronidated bile acids (calculated by subtracting naturally occurring bile acids from the ones incubated with β glucuronidase from E.coli*

### 5.6.6.3 Directly measured sulfated bile acid concentrations

The sum of directly measured sulfated bile acids was not comparable to the data published by L. Humbert et al., as with the method used it was not possible to detect sulfated forms of taurine conjugated bile acids. However, concentrations of individual sulfated bile acids detected in this work as well as the ones published by Humbert et al. [35] could be compared and are listed below:

| Direct measurement of sulphated bile acid | Humbert et al. [nmol/L] | Conducted experiment | |
|---|---|---|---|
| | | Sample 3 [nmol/L] | Sample 4 [nmol/L] |
| UDCA-3S | 25.05 ± 38.87 | 103.84 | 17.38 |
| GUDCA-3S | 201.25 ± 246.55 | 123.97 | 105.31 |
| TUDCA-3S | 53.9 ± 36.19 | *not measured* | *not measured* |
| CDCA-3S | 19.33 ± 40.55 | 44.44 | 10.04 |
| GCDCA-3S | not detected | 464.34 | 105.65 |
| TCDCA-3S | *not measured* | *not measured* | *not measured* |
| DCA-3S | 49.53 ± 85.42 | 108.64 | 2.93 |
| GDCA-3S | *not measured* | 28.01 | 6.26 |
| TDCA-3S | *not measured* | *not measured* | *not measured* |
| CA-3S | not detected | 1.57 | 2.76 |
| GCA-3S | *not measured* | 2.48 | not detected |
| TCA-3S | *not measured* | *not measured* | *not measured* |
| LCA-3S | not detected | 7.51 | not detected |
| GLCA-3S | *not measured* | 80.11 | 0.00 |
| TLCA-3S | *not measured* | *not measured* | *not measured* |
| HDCA-3S | *not measured* | not detected | not detected |

*Tab.16: Comparison of directly measured sulfated bile acids between data from the conducted experiment and literature [35]*

As we can see, sulfated forms of hyodeoxycholic - as well as glycolithocholic- , glycocholic- and glycodeoxycholic acid were additionally measured in this conducted experiment.

Concerning the comparison of the directly measured sulfated bile acids in Tab.16 above, it is very hard to make assumptions. Not only is the standard deviation of the data published by Humbert et al. very high, but also the variance between the two analysed samples in the presented work. Again, for a proper comparison a way higher sample quantity would be needed. In general, the concentrations observed do fit in the range presented by Humbert et al., which is what this comparison was about in the first place. The preparation of bile acids from urine until the enzyme digestion step is therefore working, and additionally the analysis of sulfated bile acids by direct measurment with ESI-MS therefore possible.

One exception of this was ursodeoxycholic acid in sample 3: it is elevated ca. 3 times of the in the literature presented median value and ten times the concentration of the other

analysed sample. As mentioned, the health status and the medical assistance of the subjects were unknown to the author.

However, observing the the generally very high concentrations of the other bile acids in this subject which suggests a form of obstruction and recalling the therapeuthic effects of ursodeoxycholic acid described in "1.1.4.2 Excursion: effects of ursodeoxycholic acid", the source of its in human unnatural high concentration lies most certainly in the medical administration.

In general we can observe that the concentrations from sample 4 are below average comparing them to the values presented by Humbert et al. - yet still in the range. Concentrations of sample 3 however, do mostly exceed the average values. In fact, even trace amounts of sulfated lithocholic acid were detected. This coincedes with the indirect calculation of sulfated bile acids in Tab.15 above.

By comparing the concentrations of the direct measurements of sulfated bile acids and the indirect calculated concentrations we can see, that the range seems to fit. Of course, indirect calculated concentrations are higher. This can be attributed to the fact, that bile acids are also present sulfated on multiple sites as well as sulfated and glucuronidated forms etc.

These forms can not be measured directly - or, better said, not all combinations can be analysed at the same time. This is an advantage of the enzymatic hydrolysis of sulfated and glucuronidated bile acids before analysis.

### 5.6.7 Bile acid distribution in urine samples

As it was the case for stool samples, an illustration of the bile acid distribution of unconjugated, glycine conjugated and taurine conjugated bile acids would be an interesting comparison. However, as not enough samples could be analysed in order to calculate useful median values, this would not show a veritable bile acid distribution.

### 5.6.8 Conclusion for the protocol of enzyme digestion of bile acids purified from urine samples for routine analysis

In general, the usage of an enzyme for desulfation is recommended for routine analysis of bile acids instead of solvolysis protocols. Due to their impracticability. In addition, they also

differ in the rate of desulfation for different bile acid sulfates and cause even more degradation and transformation of bile acids.

As mentioned, the β-glucuronidase derived from Helix pomatia needs to be used for enzyme digestion of bile acids purified from urine. Even though, as described in "4.8.3.1 Choosing the suited enzymes" above, the enzyme derived from E.Coli is able to deglucuronidate faster and more efficiently. This process has shown to be completed in just two hours, under the given circumstances of 37°C and a pH value of 5.4. However, it is not able to excert desulfation of bile acids, whereas the enzyme of Helix pomatia can.

The incubation should take place over a time of about 12 hours. As we could observe in the data of Tab.12 or, more comprehensible, in Fig.25, 26, 27 and 28, the process of desulfation takes this time.

This is recommended even though a decline in the case of incubation with the β-glucuronidase from E.coli shows the impact of impurities in the enzyme preparation resulting in degradation of bile acids. This degradation process will also take place in the incubation with the β-glucuronidase from Helix pomatia, as it is nowhere near as pure as the preparation from E. coli. As desulfation still takes place while bile acids are already degraded to a certain degree, it is hard to observe. The incubation time of about 12 hours is therefore a trade off between full desulfation and progressed degradation of bile acids to a small degree, yet still the best option.

As explained in "4.8.3.2 Buffer for incubation and the optimal pH value" above, pH value of 5.4 is the best option.

As explained in "4.8.3.3 Quantity of enzyme needed" above, the quantitiy of β-glucuronidase from Helix pomatia used should be 600 units per millilitre of urine used.

The direct analysis of sulfated bile acids was possible and allowed a comparison with literature - showing that the values fit into the range and indicating that the purification of bile acids from urine up to the enzymatic digestion step does work,

even though not enough urine samples were analysed to generate a median value for better comparison.

The concentrations of sulfated and glucuronidated bile acids generated with the method of enzyme digestion and subsequent indirect calculation is of course not comparable with the values from literature. As observable in the graphs however, deglucuronidation and desulfation under the right circumstances was possible. Also, the indirect calculated values seem to fit into the range of directly measured sulfated bile acids. The protocoll is suitable for usage in routine analysis.

# 6 Outlook for the future

It remains to say, that a direct comparison of bile acid concentration between stool - and urine-, as well as bile- and serumsamples of the same patients would be very interesting in order to evaluate the different methods of bile acid preparation.
Yes, comparison of data generated by the extraction of bile acids out of the different body fluids has been made as we can see in Fig.11 by Humbert et al. Yet, only median values of patients and not the bile acid concentrations of the same patient at the same time of stool, urine, bile and serum. This would also reassure or question theories of bile acid circulation, filtration and modulation (e.g. the properties of the ASB transporter in the proximal tubulus of the nephrons Henle's loop as well as the ones located in the terminal illeum, allowing the individual reabsorption of the different bile acids).
A practical example would be the diagnosis of gallstone disease:
As explained in "1.1.5 Metabolic disorders and diseases associated with bile acids" above, patients show higher bile acid concentrations in stool, as the reabsorption is impaired. To compensate this, the rate of bile acid synthesis is elevated - therefore serum concentrations are increased. Contemporary analysis of bile acids in serum as well as in stool could diagnose this condition well. A promising target here would also be the serum analysis of C4, as (as also described in "1.1.5 Metabolic disorders and diseases associated with bile acids" above) it resembles the rate of bile acid synthesis.
In addition, bile acid distribution should be compared between stool, serum, urine and bile. Also, comparison between humans and mice would be interesting. As described, the distribution in serum was already described by de Aguilar Valim et al -also between mice and humans. However, it is in need of enhancement concerning glycine and taurine conjugates. The distribution of bile acids in stool could be illustrated in this work, yet for a proper evaluation the sample size of urine samples was too low, and -as described above- bile samples of humans were not available and the complete analysis of bile acids in mice still meets the described challenges.

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# 8 Aknowledgements

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Of course a special thank goes to the whole team of laboratory assistants from the special routine laboratory of metabolism disorders of the LKH Graz and especially Maria Schäffer for the help in sample preparation as well as on other numerous occasions.
Furthermore i would like to express my gratitude to Dr. Gerald Rechberger, who enabled the work by introducing the author to the supervisor and the project - and in general for encouraging collaboration with extrauniverisitary sectors.
Of course i also want to express my appreciation to all my friends and colleagues for their support and even helping me getting access to hard attainable literature.

# 9 Supplemental

## 9.1 Esterification of glycine with methanol

This was first tried with EDC as a coupling reagent, using the same concentrations as in the taurine conjugation steps (as in both cases water needs to be expelled)-25µmol (0.005g) EDC, 50 µmol (0.00375 g) glycine and 1ml methanol was used.
The ester could not be detected with TLC using a standard mobile phase for separating amino acids (Butanol: glacial acetic acid: distilled water 4:1:1) and a silicagel plate as a stationary phase. The glycine and glycinemethylester were tried to mark with Ninhydrin (this should color the aminogroups) as well as the oxidizing reagent phosphomolybdic acid. (For a more detailed explanation of the TLC see ).
As no glycinemethylester could be detected, the result was reassured using LC-MS in positive mode (expecting a mass of 90.0553 for the glycinemethylester and 76.0398 for the free glycine, M+H). No chromatography column was used as no separation was required for this analysis. As even with this highly sophisticated method no ester was found, esterification was sure to not be successfull.

For this reason, esterification was tried with 3N methanolic HCL as a catalyst: 13µl 3N methanolic HCL was added to 50µmol glycine and 1 ml methanol and incubated for at least 12 h at room temperature as well as 80°C. Again, no esterification could be detected.

As described in "4.4.2.1 Esterification of glycine with methanol and results, esterification of glycine with methanol " above, esterification was finally achieved using the method

described by Jiabo Li [74]. However, at the initial stage a miscalculation lead to a lower usage of TMCS (25µl instead of 185µl) which resulted in a much lower yield (ca. 10%) of glycinemethylester.

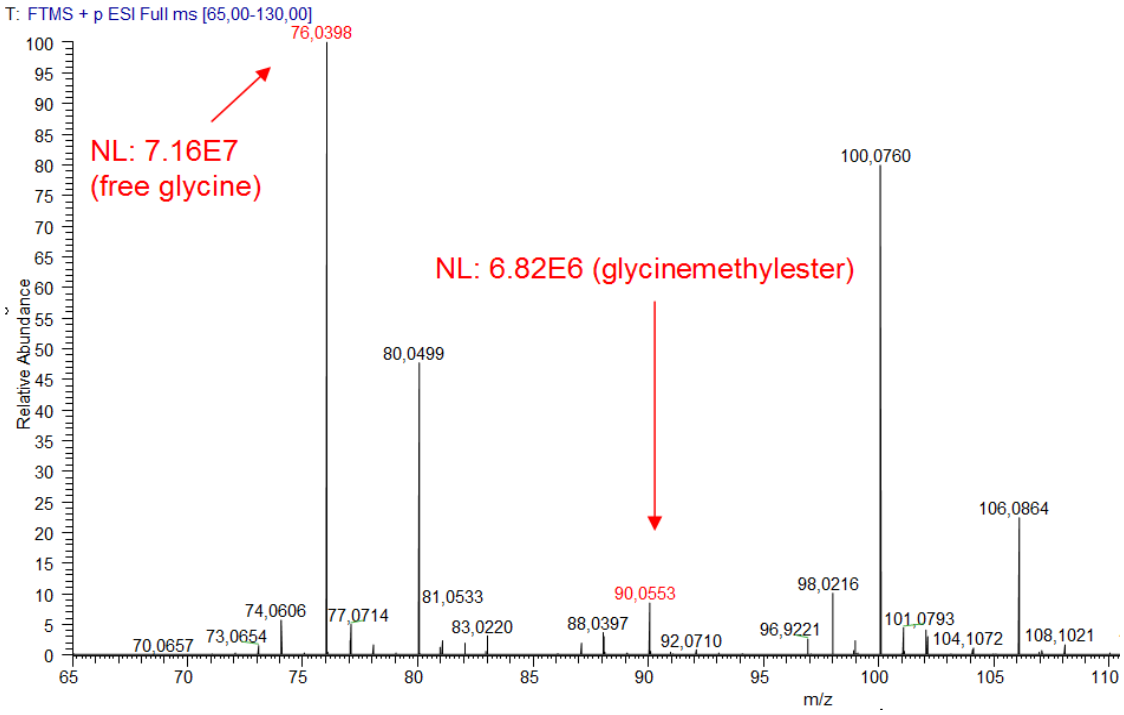



*Fig.30.: Esterification of free glycine with methanol, low yield; Mass detected for free glycine: 76.0398; Mass detected for esterified glycine: 90.0553; M+H, positive mode;*

Also, heating the batch did not result in a higher solubility of glycine and a better esterification process.

However, as described, with the proper amount of TMCS and a longer incubation period for it to silylize the glycine (30 minutes), a much higher yield was achieved as described.

Before achieving a yield > 90%, purification of the glycinemethylester was tried using ethylacetate and water in order to remove unsoluble glycine but also the byproduct of silylation - hexamethyldisilyloxane (see Fig.9 c) which could impair subsequent conjugation: The conjugation product was first dried under a stream of nitrogen in order to remove methanol, which would act as a compatibilizer between the phases of water and ethylacetat. Next, 2 ml of distilled water and 3 ml of ethylacetate were added. Glycine solubilizes in water, while the more apolar glycine methylester should solubilize in ethylacetete. This phase was therefore taken off and again extracted with 2ml of distilled water. Obviously, the glycinemethylester was not completely soluble in ethylacetate. As ethylacetate is the most polar solvent able to provoke a phase separation with water and,

as already described, methanol would act as a compatibilizer, this method of purification does not work.

When using this method on a well optimized esterification example -e.g. Fig.20 above- this resulted in a loss of intensity of factor 10 for the free glycine. Yet it also caused a loss of intensity of factor 1000 for the glycinemethylester!

Of course, the mass difference between the methylester and the free form of glycine is too low for a size exclusion chromatography. In addition, establishing a chromatography method (like ion exchange chromatography e.g.) is not worth the effort as esterification can be optimized (as described in "4.4.2.1 Esterification of glycine with methanol" above) to a degree which renders purification redundant.

## 9.2 Synthesis of $^{13}C_2$ marked glycine conjugated bile acid standards

Of course, bile acid standard conjugation with $^{13}C_2$ labeled glycine should work the same way conjugation with glycine, taurine and D4 taurine worked. However, synthesis was not achieved:

Esterification of the double $^{13}C_2$ labeled glycine with methanol could be achieved. On the first try, a simple calculation mistake led to a low yield of $^{13}C_2$ glycine methyl ester, as we can see below.

| | |
|---|---|
| expected mass for free unlabeled glycine: | 76.0398 |
| expected mass for unlabeled glycinemethylester: | 90.0553 |
| expected mass for free labeled $^{13}C_2$ glycine: | 78.0462 |
| expected mass for labeled $^{13}C_2$ glycinemethylester: | 92.0619 |

All indications are in positive mode and M+H.

Masses found:

| **Analytes** | **Mass range** | **Intensity** |
|---|---|---|
| free unlabeled glycine | 76.0394-76.0402 | 4.97 E4 |
| unlabeled glycinemethylester | 90.0548-90.0558 | 7.98 E5 |
| free labeled $^{13}C_2$ glycine | 78.0458-78.0466 | 4.22 E4 |
| labeled $^{13}C_2$ glycinemethylester | 92.0614-92.0624 | 6.16 E6 |

*Tab.17: Esterification of $^{13}C_2$ labeled glycine with methanol; not enough TMCS and methanol used;*

0.046 g (which equals about 0.6 mmol) of $^{13}C_2$ labeled glycine was used. This was correct as it suffices for the eight planned glycine conjugations, since only halve of the standards could be used for synthesis of internal standards (standards were limited). This means, that for these standards, only one fourth of the by Mills et al. [73] proposed quantities can be used for synthesis!

Only 147.3 µl of TMCS and only 580 µl of methanol were used because of a wrong deducation.
Interestingly, the $^{13}C_2$ labeled glycine was obviously not very pure as we can detect a large amount of non $^{13}C_2$ labeled glycine, in a free as well as esterified state (see table).
By checking the indications of Jiabo Li et al. [74] again, double the amount of TMCS should be used. As TMCS has a density of 0.854 g/ml at room temperature, this implies a volume of 1.5265 ml or 1,526.5 µl (1.2 mmol) instead of 147.3.
Concerning methanol, 600 µl should have been used instead of 580.
As $^{13}C_2$ labeled glycine is rather expensive and both the other two substances used are volatile, methanol and the remaining TMCS -or now rather hexamethyldisiloxane (see Fig.9c) above)- was dried off under a stream of nitrogen . As TMCS and methanol are not a very limited supply, the correct quantities were simply added slowly and again incubated over night while stirring. The usage of the correct quantity of TMCS for proper silylation clearly had a critical impact, as already described in "4.4.2.1 Esterification of glycine with methanol" above and observable in Tab.18 below:

For expected masses see indications above.

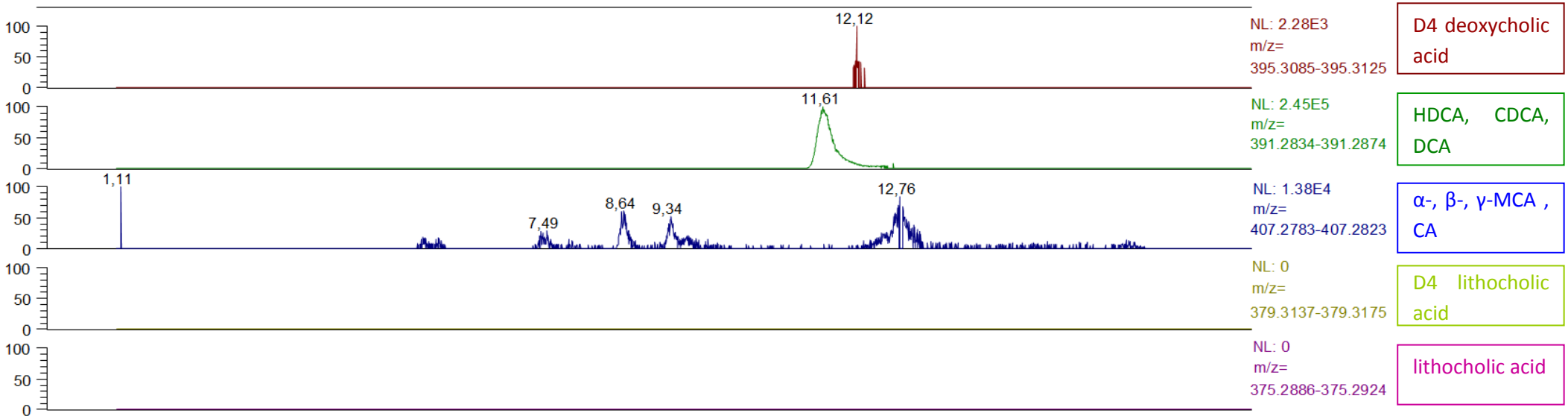
| **Analytes** | **Mass range** | **Intensity** |
|---|---|---|
| free unlabeled glycine | 76.0394-76.0402 | 3.46 E4 |
| unlabeled glycinemethylester | 90.0548-90.0558 | 1.79 E5 |
| free labeled $^{13}C_2$ glycine | 78.0458-78.0466 | 2.85 E4 |
| labeled $^{13}C_2$ glycinemethylester | 92.0614-92.0624 | 3.66 E8 |

*Tab.18: Esterification of $^{13}C_2$ labeled glycine with methanol; proper quantities of TMCS for silylation and methanol for esterification used;*

As we can see in Tab.18, $^{13}C_2$ labeled glycine is now present in a two powers of ten higher concentration than before. This shows again how critical the silylation step is for proper esterification. The quantity of methanol was of course also adjusted to the value proposed by Jabio Li et al. [74], however only a fraction of the quantity is needed.
The silylation step was therefore the critical point of adaption in this esterification process. Interestingly, the amount of free $^{13}C_2$ labeled glycine has only halved with the improved esterification.
In addition, the concentration of the unlabeled glycinemethylester decreased by more than three quarters, while the concentration of the free unlabeled glycine stayed the same.
The result of an increased esterification process would let us expect an increase of the concentration of the $^{13}C_2$ labeled glycinemethylester as well as of the non labeled glycinemethylester. The free $^{13}C_2$ labeled glycine as well as the unlabeled glycine should decrease -as it gets methylated- roughly by the same rate.
The high accuracy of the Orbitrap MS and the reassured calculations of the masses do not leave room to question the identity of the observed masses. A more probable doubt lies in the fact that the intensities range not far from the limit of detection (less than one power of ten order of magnitude). This would render the intensities of the free glycines dubious and thus indicate that the impurity - the concentration of unlabeled glycines - lies three powers of ten lower than the $^{13}C_2$ labeled glycine rather than only one. This coincides with the manufacturers claims of the $^{13}C_2$ labeled glycine.

Nonetheless, $^{13}C_2$ labeled glycine was successfully esterified with methanol and could now be conjugated with the different bile acids the same way it was done before (as described in "4.4.1 Synthesis of taurine or D4 taurine conjugated bile acid standards").

For a matter of length, only some conjugation trials will be shown in this work, as none worked.


100
50
0
12,12
NL: 2.28E3
m/z=
395.3085-395.3125
D4 deoxycholic acid
11,61
NL: 2.45E5
m/z=
391.2834-391.2874
HDCA, CDCA, DCA
1,11
7,49
8,64
9,34
12,76
NL: 1.38E4
m/z=
407.2783-407.2823
α-, β-, γ-MCA , CA
NL: 0
m/z=
379.3137-379.3175
D4 lithocholic acid
NL: 0
m/z=
375.2886-375.2924
lithocholic acid

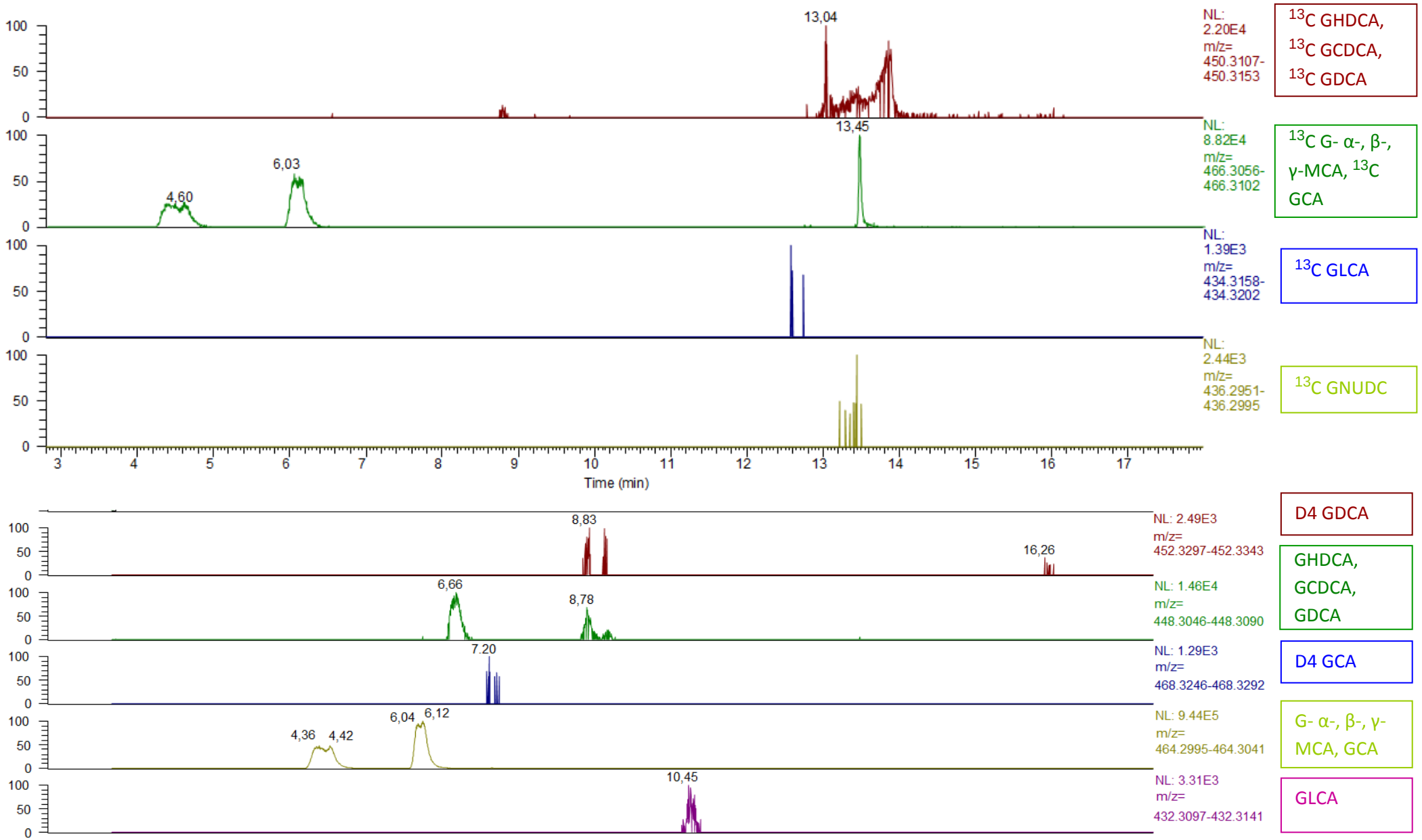
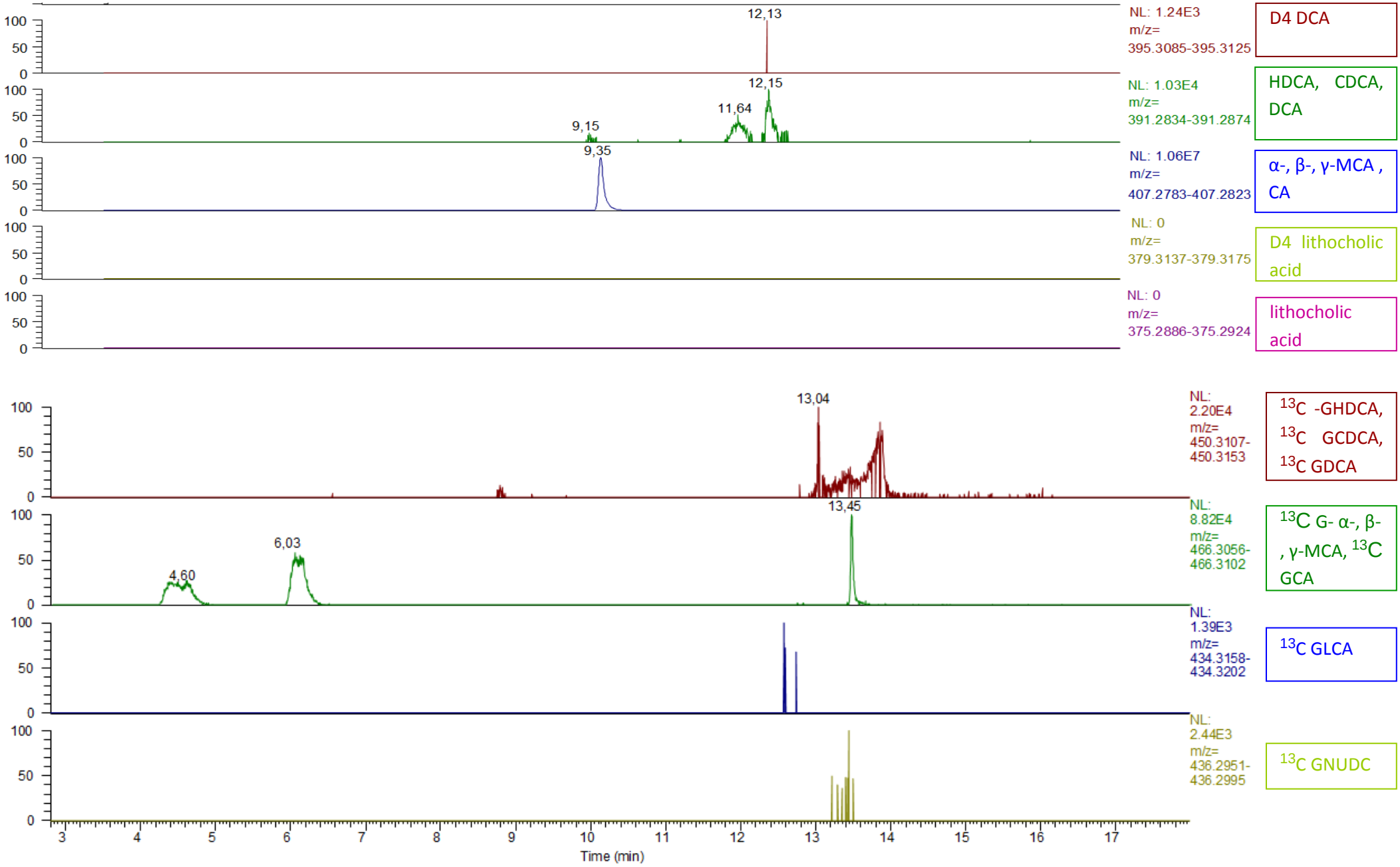

13,04
NL: 2.20E4 m/z= 450.3107-450.3153
13C GHDCA, 13C GCDCA, 13C GDCA
13,45
6,03
4,60
NL: 8.82E4 m/z= 466.3056-466.3102
13C G- α-, β-, γ-MCA, 13C GCA
NL: 1.39E3 m/z= 434.3158-434.3202
13C GLCA
NL: 2.44E3 m/z= 436.2951-436.2995
13C GNUDC
Time (min)
8,83
16,26
NL: 2.49E3 m/z= 452.3297-452.3343
D4 GDCA
6,66
8,78
NL: 1.46E4 m/z= 448.3046-448.3090
GHDCA, GCDCA, GDCA
7.20
NL: 1.29E3 m/z= 468.3246-468.3292
D4 GCA
6,04
6,12
4,36
4,42
NL: 9.44E5 m/z= 464.2995-464.3041
G- α-, β-, γ-MCA, GCA
10,45
NL: 3.31E3 m/z= 432.3097-432.3141
GLCA

*Fig.31: Analysis of $^{13}C_2$ glycine conjugated cholic acid; result of unconjugated bile acids: top; result of $^{13}C_2$ labeled glycine conjugated bile acids: middle; result of glycine conjugated bile acid: bottom;*
*red chromatogramm: D4 deoxycholic acid*
*green chromatogramm: (in order)*
*blue chromatogramm: α- muricholic-/ β- muricholic / γ-muricholic or cholic acid (in order)*
*D4 Lithocholic acid*
*Lithocholic acid*
*Dilution was halve of the usual dilution, 1:50 (yet lower dilutions have also been checked)*

As we can see in the example of "$^{13}C_2$ Glycocholic", only a small peak of what appears to be $^{13}C_2$ labeled glycine conjugate could be observed. The peak at 13,45 can however not be the $^{13}C_2$ conjugate, as it elutes far too late and in addition, this same peak was observed also in other conjugation trials (see the two other examples below).
Interestingly, also no unconjugated cholic acid, but an unconjugated chenodeoxycholic acid could be detected. In addition, two non labeled glycocholic /muricholic acids could be detected. They both show the known slight doublepeak described in "purification of glycine conjugated bile acid standards". By comparison to fig, and recalling that the $^{13}C_2$ marked glycine conjugates were saponificated and their pH value still set to 13, this is explained.


12,13
NL: 1.24E3
m/z=
395.3085-395.3125
D4 DCA
12,15
11,64
9,15
NL: 1.03E4
m/z=
391.2834-391.2874
HDCA, CDCA, DCA
9,35
NL: 1.06E7
m/z=
407.2783-407.2823
α-, β-, γ-MCA , CA
NL: 0
m/z=
379.3137-379.3175
D4 lithocholic acid
NL: 0
m/z=
375.2886-375.2924
lithocholic acid
13,04
NL:
2.20E4
m/z=
450.3107-
450.3153
13C -GHDCA, 13C GCDCA, 13C GDCA
13,45
6,03
4,60
NL:
8.82E4
m/z=
466.3056-
466.3102
13C G- α-, β-, γ-MCA, 13C GCA
NL:
1.39E3
m/z=
434.3158-
434.3202
13C GLCA
NL:
2.44E3
m/z=
436.2951-
436.2995
13C GNUDC
Time (min)

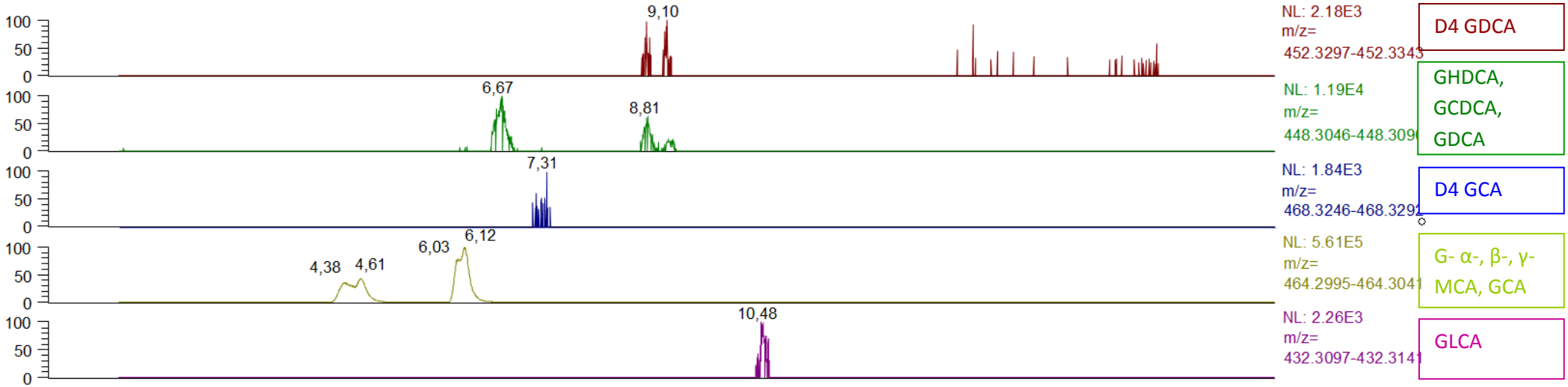
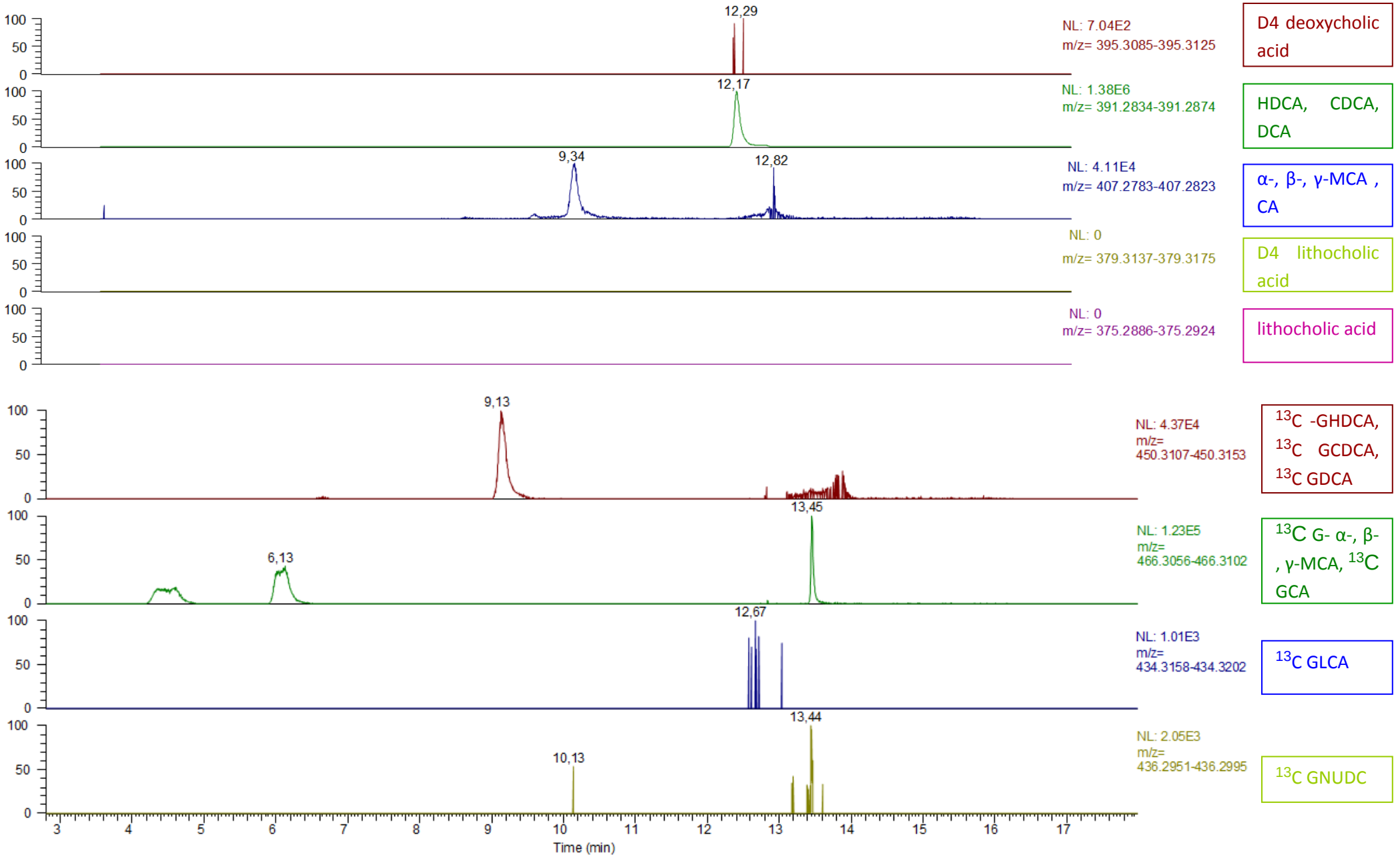



*Fig.32: Analysis of $^{13}C_2$ glycine conjugated deoxycholic acid; result of unconjugated bile acids: top; result of $^{13}C_2$ labeled glycine conjugated bile acids: middle; result of glycine conjugated bile acid: bottom;*

As we can see in the example of "$^{13}C_2$ Glycodeoxycholic", again no $^{13}C_2$ labeled glycine conjugate could be detected. Also, the same distinctive peaks in the chromatogram of $^{13}C_2$ Glycine conjugated α-, β-, γ-muricholic as well as cholic acids showed up, affirming their identity as artefacts. Again, the bile acid used for synthesis, in this case deoxycholic acid, could not be detected- yet unconjugated cholic acid could!

Also, the same two glycine conjugated cholic/muricholic acids were detected. As already described above, the doublepeak originates from the pH value being in the range of the glycine conjugated standards' pKa value- causing them two be present in two different charged states.


12,29
NL: 7.04E2
m/z= 395.3085-395.3125
D4 deoxycholic acid
12,17
NL: 1.38E6
m/z= 391.2834-391.2874
HDCA, CDCA, DCA
9,34
12,82
NL: 4.11E4
m/z= 407.2783-407.2823
α-, β-, γ-MCA , CA
NL: 0
m/z= 379.3137-379.3175
D4 lithocholic acid
NL: 0
m/z= 375.2886-375.2924
lithocholic acid
9,13
NL: 4.37E4
m/z= 450.3107-450.3153
13C -GHDCA, 13C GCDCA, 13C GDCA
13,45
6,13
NL: 1.23E5
m/z= 466.3056-466.3102
13C G- α-, β-, γ-MCA, 13C GCA
12,67
NL: 1.01E3
m/z= 434.3158-434.3202
13C GLCA
13,44
10,13
NL: 2.05E3
m/z= 436.2951-436.2995
13C GNUDC
Time (min)

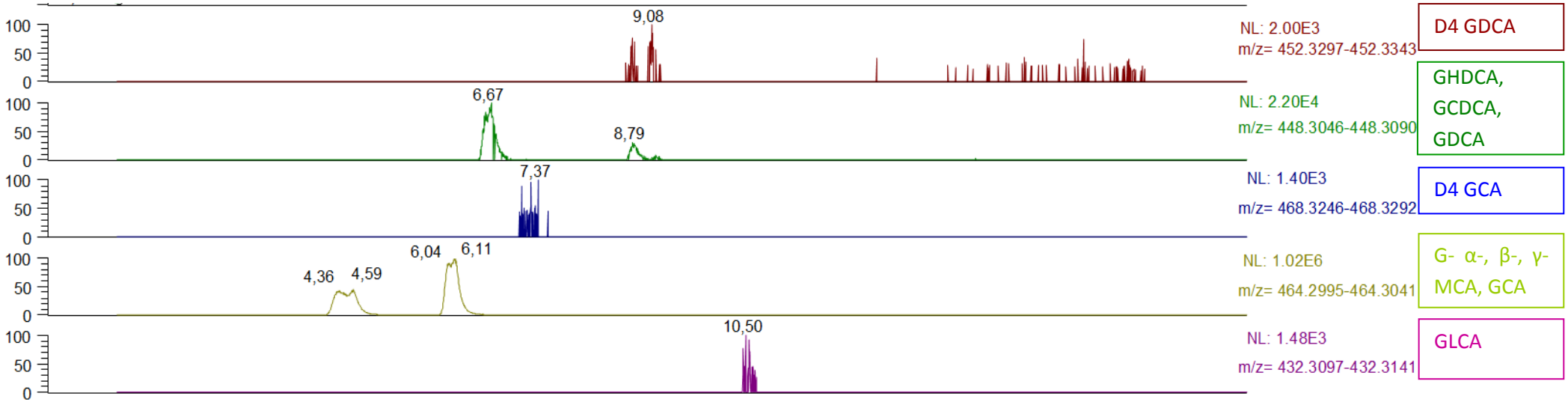



*Fig.33: Analysis of $^{13}C_2$ glycine conjugated chenodeoxycholic acid; result of unconjugated bile acids: top; result of $^{13}C_2$ labeled glycine conjugated bile acids: middle; result of glycine conjugated bile acid: bottom;*

As we can see in the example of "$^{13}C_2$ Chenodeoxycholic acid", finally a $^{13}C_2$ conjugated bile acid standard could be detected - $^{13}C_2$ glycochenodeoxycholic acid. The mass with 450.3133 would fit into range- however, the peak is not very intense and distorted.
Also, an unconjugated chenodeoxycholic acid is present in high amount, which would suggest an at least partial conjugation.
Again, the two glycine conjugated cholic/muricholic acids were detected.
There peak in the mass range of the unlabeled glycochendeoxycholic acid - which in case of a successfull conjugation would not be surprising as unlabeled glycine was present and even esterified to a certain degree, as explained above- can not resemble glycochenodeoxycholic acid. Its elution is far too early, and besides that, below the limit of quantification.

Regarding these problems the first suggestion would be a wrong calculation of the $^{13}C_2$ marked- glycine conjugates. They were therefore checked various times and reassured.
The second suggestion would be a contamined column. However, it was purged well before and after each sample run. Negative controls clearly show no sign of carrying over material.
In addition, to rule out other errors of the analysis itself, samples were also analysed with a thermo scientific API 2000 triple quad: no difference could be observed.
As even the unconjugated bile acid standards did not match the incubation, it stands to reason that the standards were simply added into the wrong vials. However, this would not explain why conjugation did not occurr, and even other bile acids conjugated with unlabeled glycine appear. Latter could be explained by a simple contamination. However, these were not found in all samples. Another explanation would be that no coupling reagent (EDC) or even the $^{13}C_2$ glycinemethylester were not added or not working correctly. Yet this would not explain why in the case of "$^{13}C$ Chenodeoxycholic acid" a trace of $^{13}C_2$ labeled glycine conjugated bile acid could be detected.
A next explanation could be the usage of contamined glas vials.
In addition, one mistake could happen - errare humanum est- yet in order to explain the observed pattern many different mistakes and also improbabilities would have had to sum up- and it still does not clear every detail.
A definetely hindering fact is, that, as already mentioned, only one fourth of the quantities proposed by mills et al. could be used. As synthesis had to be performed in glas vials (as desribed above a large quantity binds to the surface of plastics) which are rather large,

cohesion of the small quantities and therefore complete interaction between the substances may not have been granted.
There still is no known plausible explanation for the outcome of the $^{13}C_2$ labeled glycine conjugations.
Purification with SPE of course was not performed.

## 9.3 Purification of glycine conjugated bile acid standards

### 9.3.1 Purification of glycine conjugated bile acid standards with solid phase extraction (SPE)

As described in "Methods, purification of glycine conjugated bile acid standards", the adjustment of the pH value to five was critical in order for the glycine conjugated bile acid standards to be present in an anionic state, while the unconjugated ones are already protonated. However, this fact could not be identified immediately. Instead, a byproduct of conjugation like an EDC product was thought to hinder separation- as even various different concentrations of methanol : distilled water did not show any separation of the polar glycine conjugated bile acids from the more apolar unconjugated ones.
This is why also purification of the conjugation product was tried, again (see "supplemental, esterification of glycine with methanol"), with distilled water and acetonitrile. The water, in which the EDC product is soluble, was again extracted with acetonitrile to reduce losses of the conjugation product- as it does not completely solve in acetonitrile.
However, LC-MS analysis showed that the loss is inevitably high. Again, this method could not be a method of choice.

Before identifying the important step of pH value readjustment before solid phase extraction of bile acid standards described in "methods, purification of glycine conjugated bile acid standards" an anionic state of the glycine conjugates as well as unconjugated standards at a non-adjusted pH level of 13 led to an impaired separation of non-conjugated from conjugated ones: The negative charges of the unconjugated bile acids' carboxylate- and hydroxygroups led to a higher polarity and therefore a lower affinity towards the reverse phase stationary phase and an earlier elution with less water.
As we can see in Fig 34, both the unconjugated and the glycine conjugated α-muricholic acid elute almost completely with a concentration of methanol : distilled water 50:50 when the pH value is not adjusted- no analyte is left to be eluted with a higher concentration of

methanol. Separation is not possible and purification of the glycine conjugated standard therefore not achieved.

E.g.: glyco α-muricholic acid

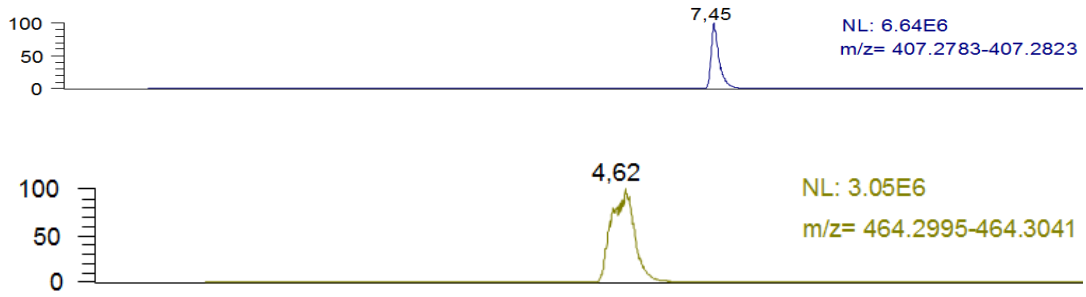



*Fig.34: Elution of glyco α-muricholic acid with methanol : distilled water 50:50; Contamination with unconjugated α-muricholic acid: above; glyco α-muricholic acid: below; pH value not adjusted*

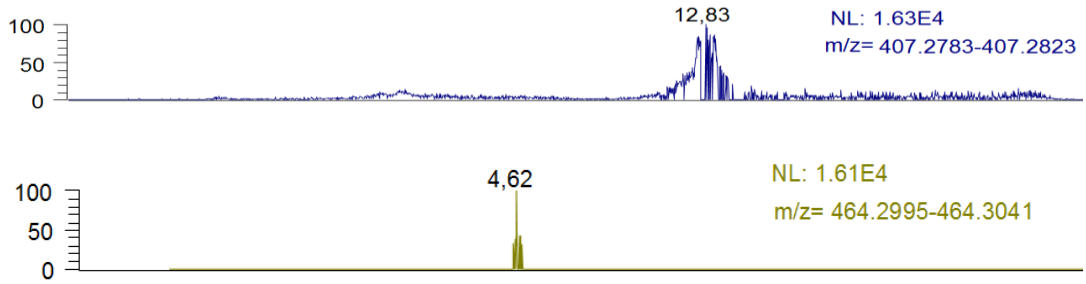



*Fig.35: Elution of glyco α-muricholic acid with methanol : distilled water 60:40; Contamination with unconjugated α-muricholic acid: above; glyco α-muricholic acid: below; pH value not adjusted*

However, also a readjustment of the pH value to five did result in certain complications:

As described in "Methods, purification of glycine conjugated bile acid standards " and observable in fig, separation of glycine conjugated α-muricholic acid from the unconjugated one was possible adjusting the pH value to ca. five and using this concentration of methanol : distilled water. Impurity lies at approximately 1%.
The theory described in "4.5.2 Purification of glycine conjugated bile acid standards" above did therefore mostly apply to the conducted tests: unconjugated bile acids did indeed elute with higher concentrations of methanol, allowing a successful purification of glycine conjugated bile acid standards. It did not apply as the pH value of 5 lies within the range of the glycine conjugated bile acid standard's pKa value, resulting, as described and

observable in Fig.34, in elution of more than 50% of the glycine conjugated α-muricholic acid with the concentration methanol : distilled water 60:40. This is when also a major part of the unconjugated α-muricholic acid elutes, rendering this second solution useless.
However, a further shift of the pH value towards the acidic milieu would result in glycine conjugated bile acid standards to be present in a complete protonated state - causing them to elute even less with methanol : water 50 : 50. As described, a shift towards the basic milieu would result in unconjugated bile acid standards to be present in an unprotonated and therefore charged state. This would allow them to elute with a concentration of methanol : water 50 : 50 and therefore contaminate this purification step. Certain compromises have therefore to be made with this method.

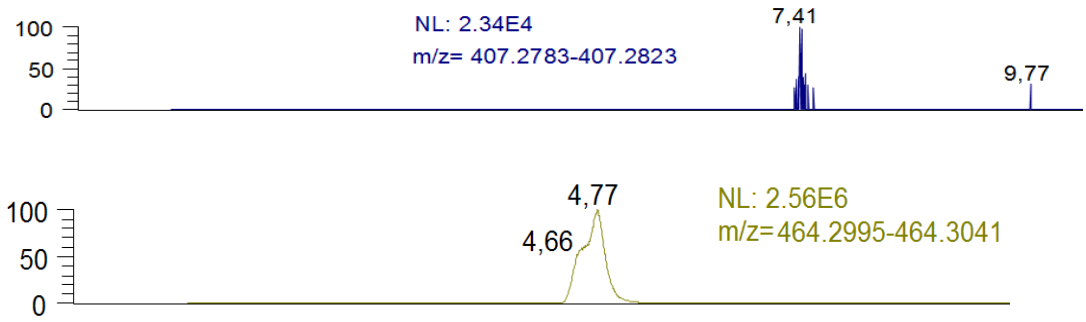



*Fig.36: Elution of glyco α-muricholic acid with methanol : distilled water 50:50; Contamination of unconjugated α-muricholic acid: above; glyco α-muricholic acid: below; pH value adjusted to 5*

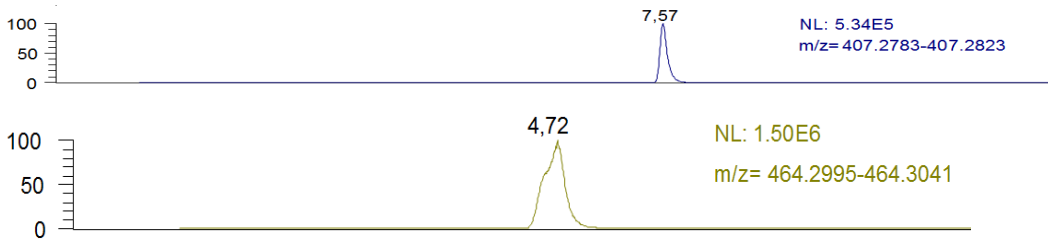



*Fig.37: Elution glyco α-muricholic acid with methanol : distilled water 60:40; Contamination of unconjugated α-muricholic acid: above; glyco α-muricholic acid: below; pH value adjusted to 5*

Another fact we notice when observing the glycine conjugated standard in Fig.36 and 37 is the double peak. The peaks are separated 0.11 seconds with the used gradient. Even the

clear peakform in fig does not implicate neither a simple distortion nor two different isomeres of the analyte.
Assuming It's a matter of isomers, it can be ruled out that two different bile acid standards have been used in the conjugation as this would result in a larger separation of the two peaks. In addition, the α-muricholic acid standard has already been analyzed chromatographically, showing only the distinctive peak. This proves its purity and would suggest two different isomers of the molecule it gets conjugated with. As this was obtained commercially, only a racemate would be an explanation. However, as we are speaking of glycine in this case, there are no different enantiomers as it does not contain a chiral carbon atom.
The double peak can therefore be attributed to a condition resulting in an altered elution. As we can observe critical differences between the chromatographic behaviour of α-muricholic acid purified at a strong basic pH value- ca 13- (see Fig.34 and 35) and an acidic pH value of about 5 (see Fig.36 and 37), this suggests a dependency from the pH value. Moreover, the doublepeak at the low pH value indicates that the analyte is present in two differently charged states: one part is completely protonated and therefore charged more- leading to a weaker interaction with the apolar stationary phase of the HPLC column and therefore an earlier elution time, whereas the carboxy- and hydroxygroups of the other part are not yet completely protonated- resulting in more interaction with the stationary reverse phase and less solubility with the earlier, more polar gradient. This causes the slightly later elution time.
This is due to the adjusted pH value lying within the range of the pKa value of the analyte. This is confirmed by the fact, that not the complete fraction of the glycine conjugated α-muricholic acid elutes with the concentration of methanol : water 50 : 50 in the purification step.

Because of these complications and in order to further optimize the separation, also normal phase chromatography was tried:

### 9.3.2 Normal phase chromatography of glycine conjugated bile acid standards

#### 9.3.2.1 Thin layer chromatography test

For initial tests, Thin layer chromatography with silicaplates were used and different mobile phases. A small glas chamber was filled with 12ml of mobile phase and incubated for a

few minutes in order to be vapour saturated. A Merck silicagel with the dimensions 4.5 cm x 12 cm was initally used.
On the 6th trial (5. Mobile phase composition) a bigger chamber, 30ml of mobile phase and a larger plate (6 cm x 11.5 cm) were used.
Analytes were applied two times in identical volumes (6µl). After separation, the plate was cut in half longitudinally.
On the first half, analytes were detected by spraying them with either Ninhydrin (in order to detect glycine and glycine- conjugated standards) or phosphomolybdic acid (oxidizing reagent, used e.g. in Fig.38 left below) and heating them on a heating plate for 3 minutes at 120° C.
In order to identify them with certainty and to determine the concentration, putative retention spaces were moisturized with distilled water on the second half of the plate and the silicagel was scratched off and captured in a vial. 500µl of HPLC grade methanol were added in order to solve glycine conjugated and unconjugated bile acid standards. The solution was diluted 1:10 and analyzed with ESI-MS.
Note that for TLC, the pH value of the analytes was not yet adapted to 5 as this problem was not yet identified. In the case of normal phase chromatography this caused a better interaction between the analytes and the mobile phase. Also, the glycinemethylester was still purified using acetonitrile and distilled water (see "9.1 Esterification of glycine with methanol " above). The analyte used was glyco-β-muricholic acid, which was synthesised with a non optimized glycinemethylester before conjugation and therefore containing a high amount of unconjugated β-muricholic acid:

Intensity of the unconjugated β-muricholic acid: 1.63 E7

Intensity of the actual glyco-β-muricholic acid: 3.48 E6

1. mobile phase:

Butanol: glacial acetic acid: distilled water 4:1:1

This is a known composition to separate amino acids and was already tried for analysis of the glycine esterification process (see "9.1 Esterification of glycine with methanol" above).
As glycine is the most polar one, it should stay bound to the stationary phase very close to the spot of appliance. More apolar amino acids solve better in the mobile phase and are therefore „carried“ further. This should in this case also apply to the glycine conjugated standards. But as we can see in Fig.38, both unconjugated and conjugated bile acid

standards do not interact with the stationary phase -or not well enough. They are carried up until shortly before the solvent front while not being separated. The very bright spot close to the location of sample appliance (proximity is of course depending on the different mobile phases) we can see in the figures is the EDC Product. The middle of the thin grey line beneath it is the spot of appliance. The black line on the upper end of the TLC shows the solvent front.

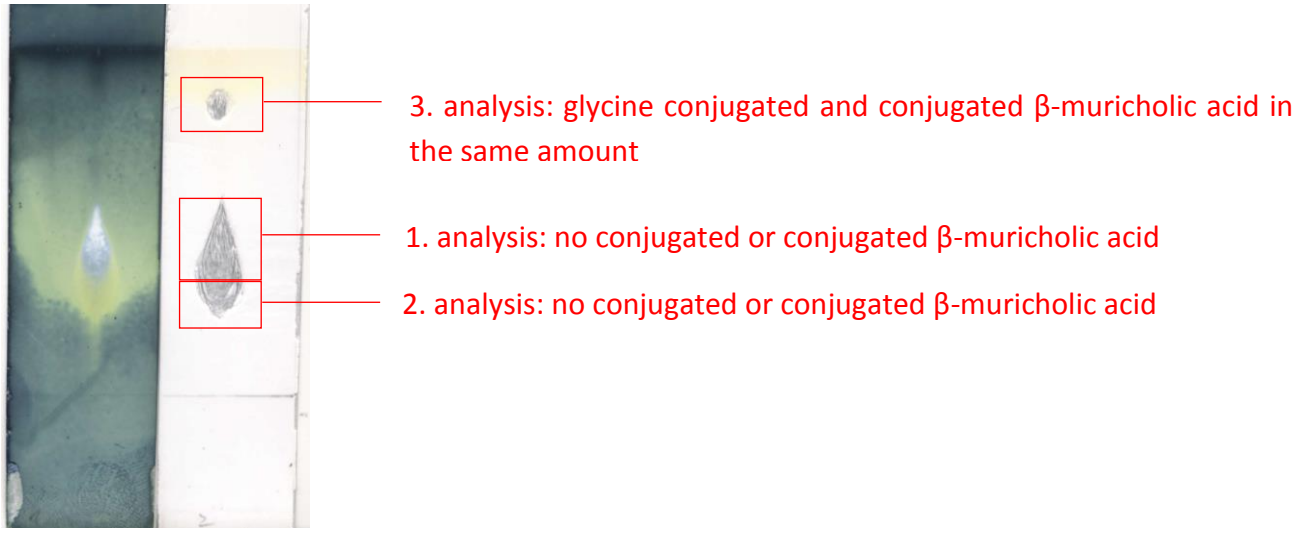



*Fig.38: TLC of glycine conjugated β-muricholic acid containing unconjugated β-muricholic acid; Butanol: glacial acetic acid: distilled water 4:1:1 used as mobile phase; analysis done by subsequent ESI-MS;*

2. mobile phase:

Butanol: glacial acetic acid: distilled water 10:1:1

The composition is similar to the 1. one, yet a bit more apolar. It was already used by Frosch and Wagener [98].
As we can see in Fig.39, unconjugated as well as glycine conjugated standards could be bound to the stationary phase shortly below the solvent front. Even if the theory did apply and the unconjugated β-muricholic acid was carried further, they could not be separated effectively.

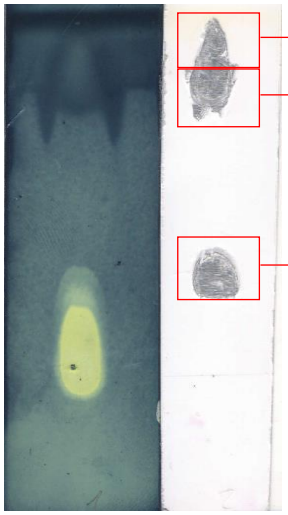



*Fig.39: TLC of glycine conjugated β-muricholic acid containing unconjugated β-muricholic acid; Butanol: glacial acetic acid: distilled water 10:1:1 used as mobile phase; analysis done by subsequent ESI-MS;*

3. mobile phase:

Methanol: chloroform: distilled water 15:7:1

A different composition was tried. As we can see in Fig.40, the EDC Product was bound stronger by the stationary phase. Bile acid standards, on the other hand, are highly soluble in methanol. This causes them to not interact enough with the stationary phase and thus being carried up to the solvent front– resulting in a very poor separation between glycine conjugated and unconjugated ones.

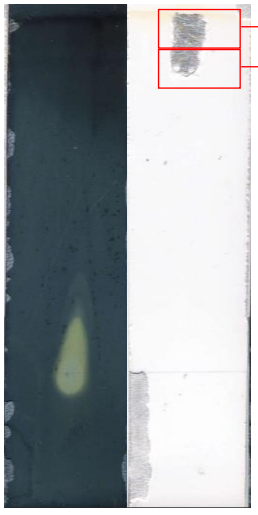



*Fig.40: TLC of glycine conjugated β-muricholic acid containing unconjugated β-muricholic acid; Methanol: chloroform: distilled water 15:7:1 used as mobile phase; analysis done by subsequent ESI-MS;*

4. mobile phase:

Methanol: chloroform: distilled water 4:1:1

As the 3rd composition was too apolar, a more polar approach was tried- with less chloroform and methanol. This caused the EDC product to bind slightly better, yet the glycine conjugated and unconjugated bile acid standard could still not be separated as they both were again carried up until the solvent front.

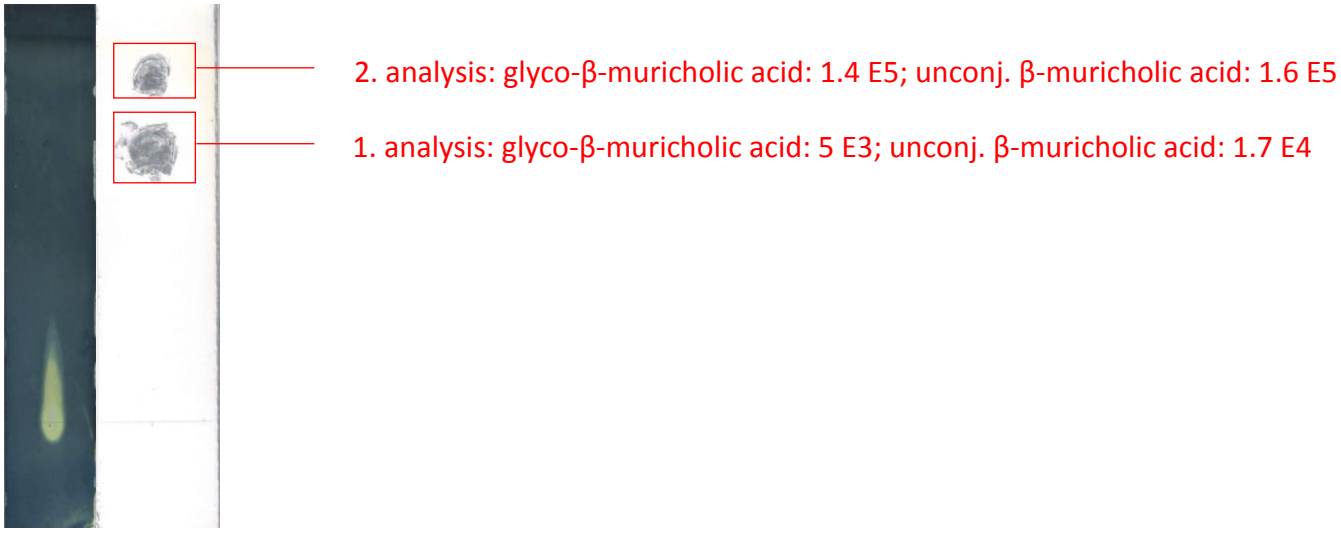



*Fig.41: TLC of glycine conjugated β-muricholic acid containing unconjugated β-muricholic acid; Methanol: chloroform: distilled water 4:1:1 used as mobile phase; analysis done by subsequent ESI-MS;*

5. mobile phase:

Ethanol: isopropanol: Isooctan: ethylacetate 25:10:10:10

As described by Satindra K. et al. [99], this composition allows a separation of unconjugated bile acids from glycine as well as taurine conjugated ones. On the first try, all bile acid standards were still on the solvent front- indicating that the length was simply not sufficing for separation. As mentionend above, a larger chamber and plate was used- this time resulting in an acceptable separation, as seen in Fig.42

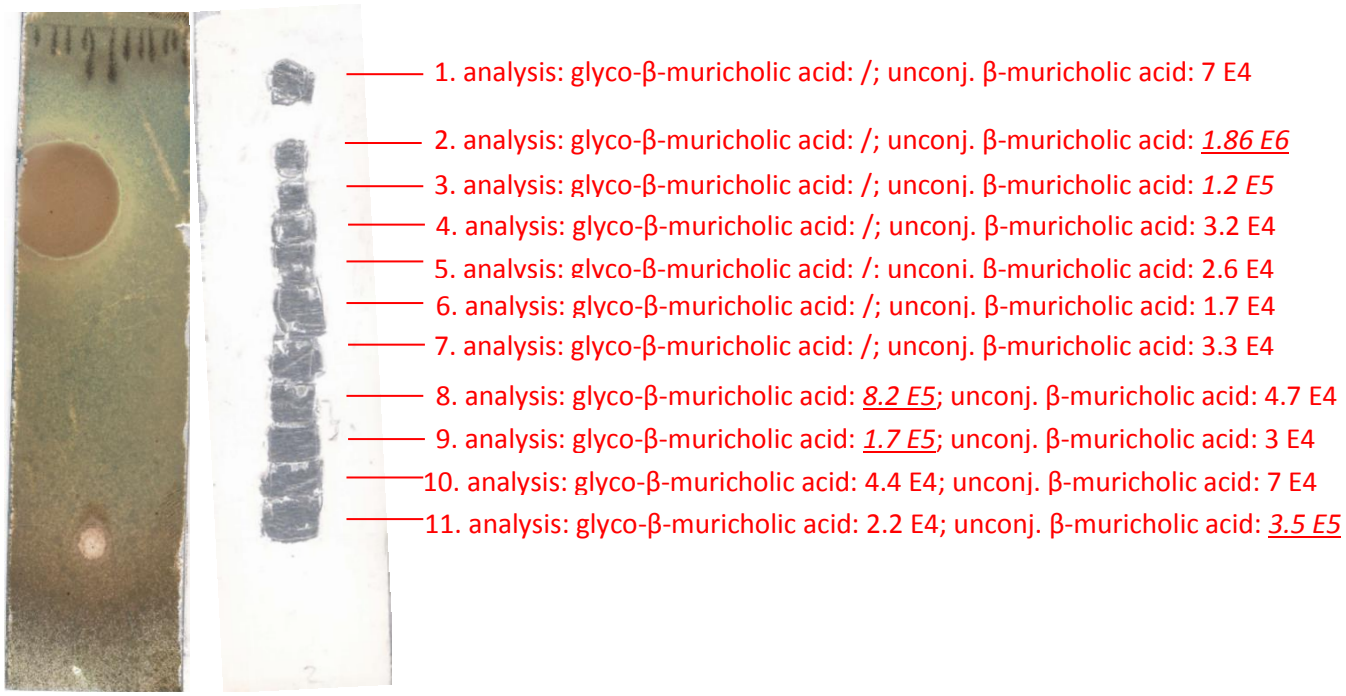



*Fig.42: TLC of glycine conjugated β-muricholic acid containing unconjugated β-muricholic acid; Ethanol: isopropanol: Isooctan: ethylacetate 25:10:10:10 used as mobile phase; analysis done by subsequent ESI-MS;*

Interestingly, unconjugated β-muricholic acid was detected in first traces not far from the spot of appliance as we can see in "11. analysis" in Fig.42. However, a strong majority was carried a lot further to the points of "3. analysis" and "2. analysis", demonstrating that the first amount was simply not solved in the mobile phase yet boun to the EDC product.
The glycine conjugated β-muricholic acid could be bound earlier (see "9. analysis" and "8. analysis" in Fig.42) by the stationary phase, finally resulting in a separation between glycine conjugated and unconjugated bile acid standard.

As analytical separation seemed to work, a preparative TLC was tried. Yet, even when using a 12 cm broad plate, as 252 µl of synthesis product had to be loaded onto the plate instead of 6µl, a continuous band had to be applied thrice- of course dried between applying. This resulted in an overload – analytes could not be separated. Another hypothesis – but less likely - is, that there still is a product inhibiting separation as seen in the TLC above- affecting larger quantities of course more than analytical quantities.

### 9.3.2.2 Normal phase chromatography with columns

As mentioned above, even when the problem with the pH value was identified and separation with C18 columns improved, it was still not optimal.

As the analytical TLC with the 5. Composition still seemed promising, normal phase columns were made using long glass pasteur pipettes (10cm length, 0.5 cm diameter) and filling them with a volume of 500µl of silica gel (Macherey Nagel 60, 0.063-0.2mm / 70-230 mesh). In order for the gel not to pass through the opening of the pasteur pipettes, a small piece (ca 1cm²) of filter paper was set in- of course the mobile phase was still able to pass through.

The silicagel was slurried with the 5. mobile phase (composition see above) and the analytes were applied. They were still solved in 1ml ethanol (from the heating process), 1ml of 10% $K_2CO_3$ (saponification) and HCl from acidification.

20ml of the 5. Mobile phase were applied, every 2 ml were fractioned and analyzed with ESI-MS. As we can see, no bile acid standards could be detected in a significant amount except at the beginning – where the analytes were applied. This is unlikely to be due to an excessive dilution, but rather because of a too strict binding of the analytes to the stationary phase. Note that the salt form of the analytes interacts stronger with a polar stationary phase- the opposite problem to the separation with the c18 reverse phase columns. Also, the mobile phase used is much more apolar, which of course results in less solubility of the glycine conjugate in it.

| **Fraction** | **Uncon.Deoxycholic (391.28)** | **Glycodeoxycholic (448.32)** |
|---|---|---|
| Fr.1 (2ml) | 2 E5 | 2.7 E4 |
| Fr.2 (2ml) | 2.4 E4 | 4.9 E3 |
| Fr.3 (2ml) | 2.6 E4 | / (<4 E3) |
| Fr.4 (2ml) | / (<3 E3) | / (<4 E3) |
| Fr.5 (2ml) | / (<3 E3) | / (<4 E3) |
| Fr.6 (2ml) | / (<3 E3) | / (<4 E3) |
| Fr.7 (2ml) | / (<3 E3) | / (<4 E3) |
| Fr.8 (2ml) | / (<3 E3) | / (<4 E3) |
| Fr.9 (2ml) | / (<3 E3) | / (<4 E3) |
| Fr.10 (2ml) | / (<3 E3) | / (<4 E3) |
| Fr.11 (2ml) | / (<3 E3) | / (<4 E3) |

*Tab.19: Results of normal phase chromatography using Ethanol: isopropanol: Isooctan: ethylacetate 25:10:10:10, glycine conjugates majorly present in an anionic state, unconjugated standard present protonated;*

For this reason, the test was repeated without the reajustment of the pH value.
This time, water from the saponificated standard was extracted using a rotary evaporator- $K_2CO_3$ remained at the bottom as a salt. The analytes were then solved in 3ml of the 5. Mobile phase and applied on the column. Again, every 2ml was one fraction, 10 fractions were collected. In order to make sure glycine conjugated standards elute, an 11th fraction was collected using 100% methanol.

| **Fraction** | **Uncon.Hyodeoxycholic(391.28)** | **Glycohyodeoxycholic(448.32)** |
|---|---|---|
| Fr.1 (3ml) | 7.2 x E4 | / (1.9 x E3) |
| Fr.2 (2ml) | 1.3 x E5 | / (2.1 x E3) |
| Fr.3 (2ml) | 1.33 x E4 | / (1.9 x E3) |
| Fr.4 (2ml) | 2.1 x E4 | / (1.6 x E3) |
| Fr.5 (2ml) | 5.58 x E4 | / (1.55 x E3) |
| Fr.6 (2ml) | 6.8 x E4 | / (1.2 x E3) |
| Fr.7 (2ml) | 1.42 x E5 | / (1.67 x E3) |
| Fr.8 (2ml) | 1.3 x E5 | / (2.78 x E3) |
| Fr.9 (2ml) | 6.92 x E4 | / (2.8 x E3) |
| Fr.10 (2ml) | 1.07 x E5 | / (5 x E3) |
| Fr.11 MeOH (2ml) | 5.44 x E5 | 6.31 x E6 |

*Tab.20 Normal phase chromatography to separate glycine conjugated hyodeoxycholic acid (448.32) from unconjugated hyodeoxycholic acid (391.28). Mobile phase used: Ethanol: isopropanol: Isooctan: ethylacetate 25:10:10:10; pH value set to about 13; all analytes therefore present protonated and less charged.*

As we can see, no significant amount of glycine conjugated bile acid (in this case glycohyodeoxycholic acid) could be eluted with the mobile phase, even when using 21 ml. This shows, that even the protonated and less charged form interacts too strong with the stationary polar normal phase. In contrast to the test with TLC, in which the same stationary and mobile phase were used as well as the same state of the analyte,

separation of unconjugated from glycine conjugated bile acids could not be achieved with the used mobile phase. This is due to the higher amount of surface of the stationary phase the analyte is exposed to in the column as opposed to the thin layer chromatography.
The hypothesis that simply conjugation did not occur is not plausible as glycine conjugated hyodeoxycholic acid could be eluted in high concentration when using 100% methanol.

However, unconjugated bile acids were partially eluted with Ethanol: isopropanol: Isooctan: ethylacetate 25:10:10:10 – Using 2 ml of methanol eluted the majority.
This would suggest the usage of a higher quantity of the 5. Mobile phase – allowing to elute the unconjugated bile acid completely and subsequently the elution of a purified glycine conjugated bile acid with only methanol. Yet the 21ml used already resulted in a time consuming method- considering the density of the column and no negative pressure applied. For complete elution of unconjugated bile acids, a multiple quantum would be needed. As no automatization is possible this method can not compete with the purification using a reverse phase C18 column.

Another normal phase chromatography was tested, this time using another composition of mobile phase:
The tests of purification with acetonitril and water indicated that bile acids are not completely soluble in acetonitrile, but unconjugated ones being better soluble than the glycine conjugated ones.
To approve this and make use of this fact, the mobile phase constituted in a declining ratio of acetonitrile : methanol starting with 10:1 and ending with 1:10 (6 fractions consisting of 2ml each). This should elute unconjugated ones earlier then glycine conjugated ones.
As bile acid sandards are highly soluble in methanol and therefore easy to elute, they were again applied in an anionic form in order to increase affinity to the stationary phase and therefore also the interaction time. This should increase separation performance.
In order to remove the water used in the saponification step (which would affect the silicagel), again a rotary evaporator was used.

| **Fraction** | **Uncon.Hyodeoxycholic(391.28)** | **Glycohyodeoxycholic(448.32)** |
|---|---|---|
| Fr.1 | 3.8 x E5 | / |
| Fr.2 | 9.7 x E5 | / |
| Fr.3 | 1.5 x E6 | 1 x E5 |

| | | |
|---|---|---|
| Fr.4 | 5.2 x E5 | 8 x E4 |
| Fr.5 | 9 x E4 | 8.2 x E4 |
| Fr.6 | 8.7 x E3 | 1.6 x E4 |
| Fr.7 | / (< 3 x E3) | / (3 x E3) |
| Fr.8 | / (< 3 x E3) | / (6 x E3) |
| Fr.9 | / (< 3 x E3) | / (< 3 x E3) |

*Tab.22: Normal phase chromatography to separate glycine conjugated hyodeoxycholic acid (448.32) from unconjugated hyodeoxycholic acid (391.28). Mobile phase used: declining ratio of acetonitrile : methanol; pH value set to 5; glycine conjugates therefore majorly present in an anionic state, unconjugated standard present protonated;*

As we can see, unconjugated standards could in fact be eluted using acetonitrile/ only low concentration of methanol. The glycine conjugated standard in general eluted with a higher concentration. Separation was therefore partially achieved, yet not effectively enough as the glycine conjugated bile acid already eluted in the loading step. This can be explained due to the small amount of methanol used (as explained they are not completely soluble in 100% acetonitrile). It is therefore not the method of choice for chromatographic separation of glycine conjugated bile acids from unconjugated bile acids.

As described above, solid phase extraction with the c18 cartridges as described remained the best possible way to achieve purification of glycine conjugated bile acid standards.

## 9.4 Troubleshooting with a clogged HPLC column

As described in "Methods, purification of bile acids from stool" the used protocol of bile acid extraction from patient stool lead to a clogged HPLC column after injection into the system for chromatographic and mass spectrometric analysis. In order to remove the material which engorged the column, several methods have been tried:
At first, the column was purged with the usual gradient of distilled water : methanol 50:50 at a flow rate of 500 µl/min for over two hours. In addition, the column was purged with pure methanol for 30 minutes (in order to elute apolar substances). After running a sample with bile acid standards it was clear, that this did not solve the problem.

The column was then purged with acetonitrile against the running direction ata flow rate of 100µl/min for 12 hours, then for 6 hours with hexane, for 6 hours withacetonitrile, for 3 hours with methanol and for 3 hours with distilled water. For a more detailed description of the troubleshooting process see "Mass spectrometric analysis of bile acids and their precursors, Project lab course WS 2013 Biochemistry and molecular Biomedicine", "4.4. Analyzing bile acids from stool samples and Troubleshooting with C18 reverse phase column Macherey Nagel nucleoshell 2.7 µm, 50 mm."

As all the effort of "cleaning" the column (again, the column of the optimized method was used- C18 Macherey nagel Nucleoshell, 50mm; 2.7µm) did not show any improvement in chromatographic performance, the column was exchanged.