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METABOLOMIC ANALYSIS OF BILE ACIDS IN VARIOUS BIOLOGICAL SAMPLES

Diploma thesis

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Consultant: prof. Seppo Auriola

Hradec Králové 2018 Pavlína Hadravská

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METABOLOMICKÁ ANALÝZA ŽLUČOVÝCH KYSELIN V RŮZNÝCH BIOLOGICKÝCH VZORCÍCH

Diplomová práce

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V Hradci Králové Pavlína Hadravská

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ABSTRACT

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Title of Diploma thesis: **Metabolomic analysis of bile acids in various biological samples**

This thesis aimed to establish a suitable qualitative and quantitative analysis of selected bile acids in mouse liver, human plasma and especially in human hair. Nine selected bile acids were analyzed (cholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, lithocholic acid, taurocholic acid, taurodeoxycholic acid, glycocholic acid and glycochenodeoxycholic acid). An identification and a quantification of the bile acids have been performed by UHPLC with two types of hybrid mass spectrometers (Quadrupole connected to Time of Flight and Quadrupole connected to Orbitrap). We tried to develop a method that allows a detection of both unconjugated bile acids and their glycine- and taurine-conjugates in biological samples. To develop and optimize this method, mouse liver and human plasma were used. Afterwards, the method was used to detect bile acids in human hair, because the bile acids could serve as potencial biomarkers for the cholestasis. Hair samples obtained from different pacients were used for analysis: hair samples from mothers suffering from obstetric cholestasis and from their neonates, and from men without any information about their diagnosis. All studied bile acids, except chenodeoxycholic and lithocholic acid, were detected in hair samples obtained from the neonates. Some of them were also found in hair obtained from the mothers suffering from obstetric cholestasis. The hair samples from the men were bile acids negative. The developed method will be used for further analyses of bile acids in various biological samples.

Key words: bile acids, UHPLC, MS, mouse liver, human plasma, human hair, cholestasis

ABSTRAKT

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Název diplomové práce: **Metabolomická analýza žlučových kyselin v různých biologických vzorcích**

Cílem práce bylo vyvinout vhodnou metodu pro kvalitativní a kvantitativní analýzu vybraných žlučových kyselin v myších játrech, lidské plazmě a především v lidských vlasech. Analyzováno bylo devět vybraných žlučových kyselin (kyselina cholová, deoxycholová, chenodeoxycholová, ursodeoxycholová, lithocholová, taurocholová, taurodeoxycholová, glykocholová a glykochenodeoxycholová). Identifikace a kvantifikace žlučových kyselin byly provedeny pomocí UHPLC přístroje se dvěma typy hybridních hmotnostních spektrometrů (kvadrupólovým analyzátorem spojeným s analyzátorem doby letu a s kvadrupólovým analyzátorem spojeným s orbitrapem). Snažili jsme se vyvinout metodu, která umožňuje detekci jak nekonjugovaných žlučových kyselin, tak i jejich glycinových a taurinových konjugátů v biologických vzorcích. K vývoji a optimalizaci této metody jsme využili myší játra a lidskou plazmu. Poté byla tato metoda použita k detekci žlučových kyselin v lidských vlasech, neboť žlučové kyseliny by mohly být potenciálními biomarkery pro cholestázu. Pro analýzu byly použity vzorky vlasů od různých pacientů, a to vzorky vlasů matek trpících intrahepatální cholestázou těhotných a vzorky jejich novorozenců, a vzorky od mužů bez informace o jejich diagnóze. Všechny žlučové kyseliny, kromě kyseliny chenodeoxycholové a lithocholové byly nalezeny ve vlasech získaných od novorozenců. Některé kyseliny byly objeveny i ve vlasech matek trpících intrahepatální cholestázou těhotných. Ve vlasech mužů nebyly nalezeny žádné žlučové kyseliny. Vyvinutá metoda bude využívána pro další analýzy žlučových kyselin v různých biologických vzorcích.

Klíčová slova: žlučové kyseliny, UHPLC, MS, myší játra, lidská plazma, lidské vlasy, cholestáza

CONTENTS

1 INTRODUCTION

Bile acids are the major components of the bile. There are two major primary bile acids, cholic acid and chenodeoxycholic acid, which are synthesized from cholesterol in the liver hepatocytes. Primary bile acids are covalently linked to glycine or taurine, to form the glyco- or tauro-conjugates. The secondary bile acids, deoxycholic acid and lithocholic acid, are produced from the primary bile acids by the intestinal bacterial flora in the colon. There are also some minor bile acids, such as ursodeoxycholic acid, a 7β-epimer of chenodeoxycholic acid.

Bile acids play many important physiological roles in humans and animals. The conjugated forms of bile acids in the bile assist as lipid solubilizers to facilitate the digestion and absorption of the fats and fat soluble-vitamins in the intestine. They are amphipathic molecules containing both regions, a polar and a non-polar region, which results in their ability to act as detergents.

A cholestasis is a disorder of production of the bile. Intrahepatic cholestasis of pregnancy, also known as obstetric cholestasis, is a reversible form of cholestasis predominantly of the third trimester of pregnancy characterized by pruritus and changes in the liver enzymes. The serum concentration of total bile acids in blood circulation of mother is increased. Genetic predispositions, hormonal changes in pregnancy and environmental factors are important factors in the pathogenesis of the disease. The obstetric cholestasis disappears after delivery, usually in few days.

The accumulated bile acids in mother's circulation cross the placenta to the fetus. The concentration of bile acids in amniotic fluid and in cord blood is increased.

Nowadays, the (Ultra)High Performance Liquid Chromatography coupled to the Mass Spectrometry represents the method of choice for the analysis of bile acids and also their conjugates in biological samples.

Up to now, the analysis of bile acids has been studied in biological fluids, in blood and urine, to determine bile acids as potential biomarkers for liver diseases, such as for the cholestasis. We studied the hair, other biological material, using the UHPLC-MS. The hair obtained from the mothers with intrahepatic cholestasis of pregnancy and their neonates, and the hair from men without any information about their diagnosis were analyzed. The hair is a stable structure and it is easy to collect the hair samples for the analysis. The hair can reflect exposure of endogenous compounds and also environmental exposures for many weeks or months prior to the analysis.

2 AIMS OF STUDY

- To set up a suitable qualitative and quantitative UHPLC-MS analytical method for detection of selected bile acids in various biological samples, including human hair.
- To quantify the concentration of individual bile acids in hair of mothers with intrahepatic cholestasis of pregnancy, in hair of their newborns and in hair of men without cholestasis.
- To evaluate the suitability of the hair samples as a biological material.

3 THEORETICAL PART

3.1 Bile Acids

Bile acids (BAs), the major components of the bile, are the terminal products of cholesterol metabolism in the liver. There are three major classes of BAs depending on the terminal polar group (alcohol or acid) and the length of the side chain (C-24 or C-27): C-24 bile acids, C-27 bile acids and C-27 bile alcohols (Ridgway and McLeod 2015). The BAs studied in this thesis belong to the C-24 bile acids group. Therefore, the theoretical part is focused on the C-24 BAs and their conjugates, namely cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA).

Table 1. *Selected characteristics of the nine bile acids studied in this thesis.*

	Bile acid	**Abbreviation**	**Molecular formula**	**Molar mass* g mol^{-1}**
1	Cholic acid	CA	$C_{24}H_{40}O_5$	408.2876
2	Deoxycholic acid	DCA	$C_{24}H_{40}O_4$	392.2927
3	Chenodeoxycholic acid	CDCA	$C_{24}H_{40}O_4$	392.2927
4	Ursodeoxycholic acid	UDCA	$C_{24}H_{40}O_4$	392.2927
5	Lithocholic acid	LCA	$C_{24}H_{40}O_3$	376.2977
6	Taurocholic acid	TCA	$C_{26}H_{45}NO_7S$	515.2917
7	Taurodeoxycholic acid	TDCA	$C_{26}H_{45}NO_6S$	499.2968
8	Glycocholic acid	GCA	$C_{26}H_{43}NO_6$	465.3090
9	Glycochenodeoxycholic acid	GCDCA	$C_{26}H_{43}NO_5$	449.3141

*calculated by Agilent MassHunter Qualitative analysis B.07

3.1.1 Structural Properties

The chemical structure of BAs is derived from cholesterol. BAs are the water-soluble compounds characterized by a 5β-steroid ring (the four-ring perhydrocyclopentanophenanthrene nucleus) carrying a pentanoic acid substituent at C-17. They bear one to three hydroxyl groups at positions 3, 7 and 12 of the steroid cycle. The number, position and orientation of the hydroxyl groups differ the BAs. There are two major primary BAs in humans, CA and CDCA. In the structure of CA, there are three

hydroxyls at positions 3α, 7α and 12α of the steroid nucleus, whereas CDCA (3α, 7α-OH), DCA (3α, 12α-OH) and UDCA (3α, 7β-OH) bear two hydroxyl groups in different positions (as shown in brackets). LCA carries only one hydroxyl group at position 3α of the steroid nucleus. DCA and LCA are called as secondary BAs (Ridgway and McLeod 2015).

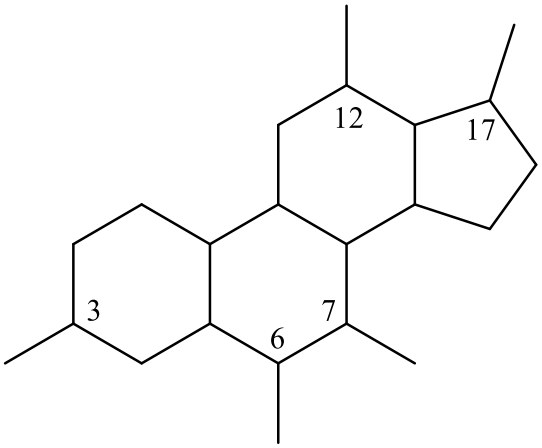


Figure 1. *The structure of the four-ring perhydrocyclopentanophenanthrene nucleus with marked positions of the substituents.*

The hydroxyls can be in the positions 3, 7, 12 (or at C-6 in the structure of muricholic acid in mice and rats), pentanoic acid substituent at C-17.

Table 2*. Position and orientation of the hydroxyl groups of the free BAs studied in this thesis (Ridgway and McLeod 2015).*

Bile acid	**C-3**	**C-7**	**C-12**
CA	α-OH	α-OH	α-OH
DCA	α-OH	-	α-OH
CDCA	α-OH	α-OH	-
UDCA	α-OH	β-OH	-
LCA	α-OH	-	-

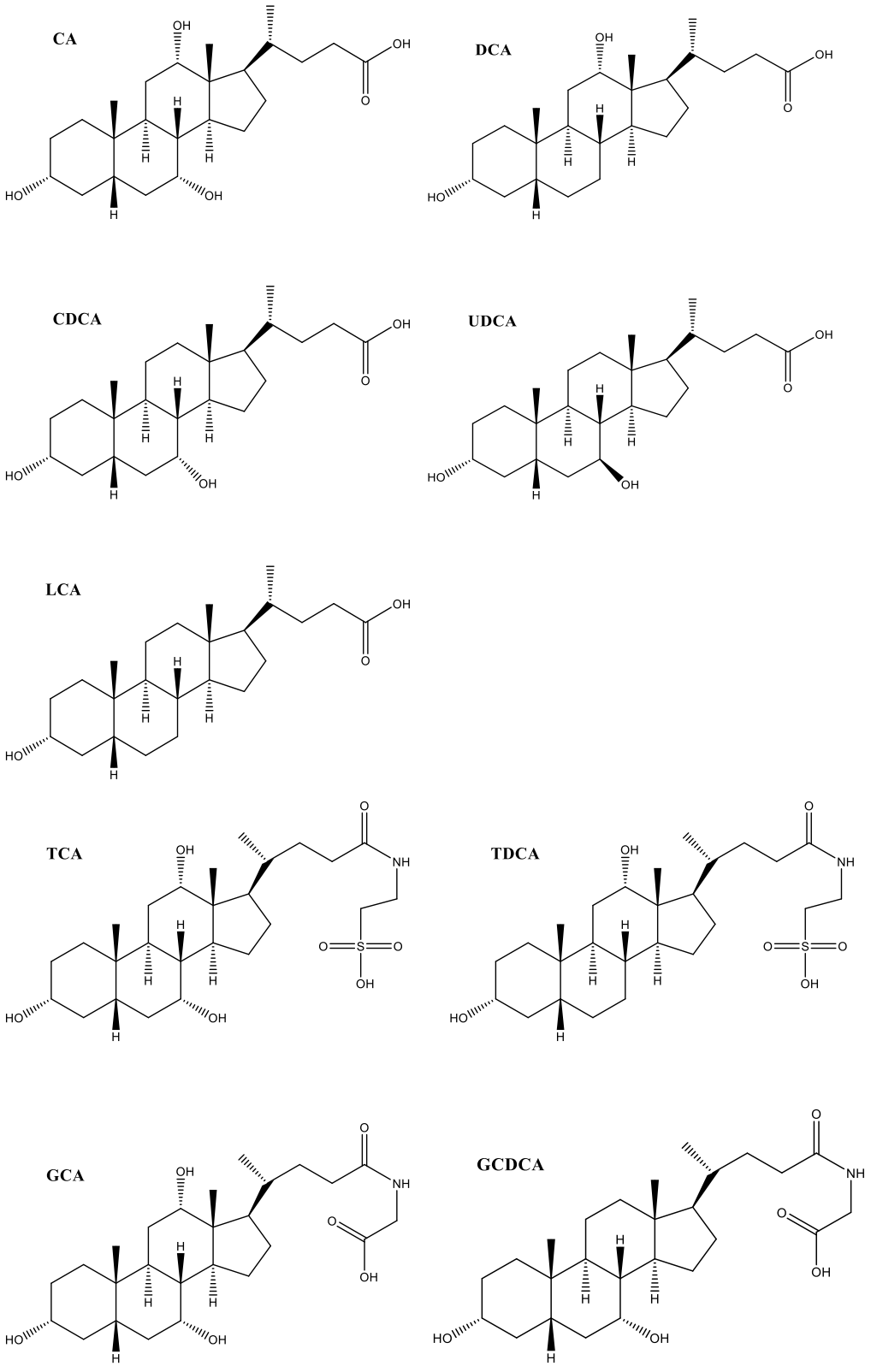


***Figure 2**. The structural formulas of BAs and their conjugates studied in this thesis: cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA).*

These amphipathic molecules contain both regions, a polar and a non-polar region. The hydroxyl groups and carboxyl group at the C-24 often reside on the same side of the molecule to form a hydrophilic surface, whereas the carbon steroid nucleus forms the hydrophobic face. This molecular structure results in their ability to behave as detergents (Ridgway and McLeod 2015).

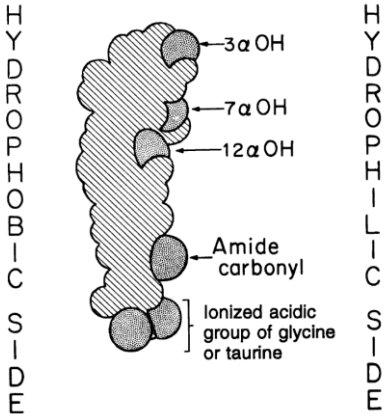


Figure 3. *The space filling model of the conjugated CA showing its amphipathic structure with both regions, the hydrophobic side and the hydrophilic side (Hofmann and Hagey 2008).*

3.1.2 Physical Properties

The physical properties of the BAs are influenced by the molecular structure. The aqueous solubility depends on the number of hydroxyl groups. The hydrophilicity is rising in the progression from monohydroxy to trihydroxy BAs. A position and an orientation of these hydroxyls are also important for the resulting properties. The molecules with hydroxyl groups in α-orientation are less hydrophilic than molecules with β-OH. A conjugation to taurine or glycine also affects the solubility. Thus, the taurine-conjugates are more soluble than glycine-conjugated forms, which are in turn more soluble than unconjugated BAs (Ridgway and McLeod 2015).

3.1.3 Biosynthesis

The BAs are syntetized from cholesterol, a C-27 sterol with one hydroxyl group at C-3 and a double bond at C-5/6 position. A conversion of cholesterol into the BA includes following steps: a hydroxylation of the steroid nucleus at C-7 position, a shortening of the side chain (from the C8 isooctane side chain to the C5 isopentanoic

side chain), an epimerization of the β-hydroxyl group to the 3α conformation, and a saturation of the steroid nucleus (induces a *cis*-configuration at the junction of the A/B rings resulting in a kink in the planar steroid nucleus) (Ridgway and McLeod 2015).

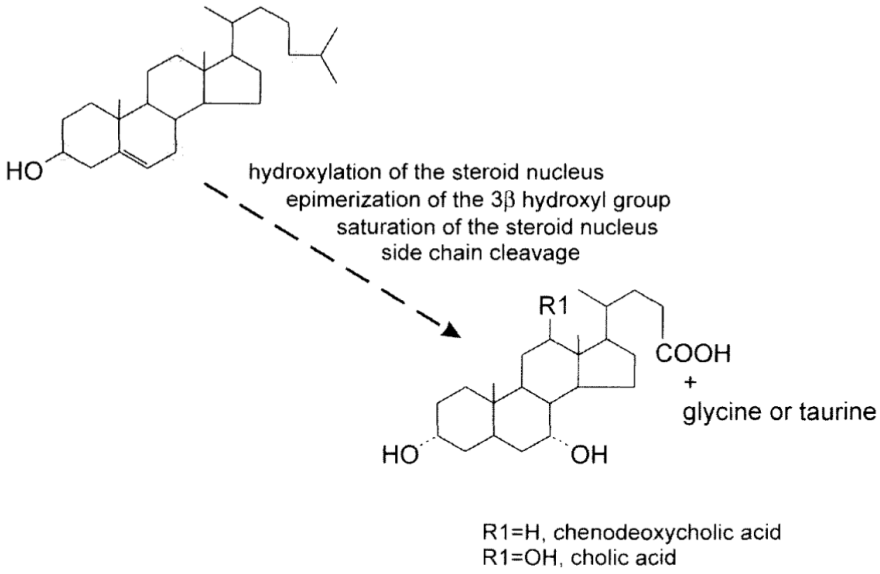


Figure 4. *The brief conversion of cholesterol into the BAs and the conjugation with glycine or taurine (Vance and Vance 2002).*

The BA biosynthesis is the major pathway in the cholesterol catabolism. The liver is the only organ that has all enzymes required for the *de novo* synthesis of the primary BAs in humans (Chiang 2009). The two major primary BAs, CA and CDCA, are synthesized from cholesterol in the liver hepatocytes. One or two hydroxyl groups are added to the steroid nucleus of cholesterol. In contrast to humans, muricholic acid (MCA; 3,6,7-OH) is also primary BA in rats and mice (Russell 2003).

Two pathways of the biosynthesis of the primary BAs are known, namely a classical pathway (that favours the biosynthesis of CA) and an indirect pathway called alternative (that favours the biosynthesis of CDCA in humans and 6-hydroxylated BA muricholic acid in mice and rats) (Russell 2003).

The initial step of the classical pathway includes an α-hydroxylation of C-7 of the steroid nucleus catalyzed by a cholesterol 7α-hydroxylase (CYP7A1), that belongs to the microsomal cytochrome P450 enzymes, expressed only in the hepatocytes. This reaction is the rate-limiting step for BA synthesis. The subsequent step of BA biosynthesis is catalyzed by a 3β-hydroxy-Δ5-C27-steroid oxidoreductase (HSD3B7) to convert the 7α-hydroxycholesterol to a 7α-hydroxy-4-cholestene-3-one (often

abbreviated as C4). For the synthesis of CA, which bears three hydroxyl groups, a sterol 12α-hydroxylase (CYP8B1) is then required. Within the synthesis of dihydroxy-BAs, the C4 directly undergoes the steroid ring isomerisation and saturation reactions. Further, the sterol side chain oxidation is catalyzed by a mitochondrial sterol 27-hydroxylase (CYP27A1) and after that, 3 carbons are trimmed from the side chain to yield the C-24 BAs (Ridgway and McLeod 2015).

In the alternative pathway, the first step involves a hydroxylation of the cholesterol side chain by different enzymes that are presented in the liver or other tissues, such as in the brain or lungs. This hydroxylation results in the formation of oxysterols. The formation of a 27-hydroxycholesterol is catalyzed by an enzyme sterol 27-hydroxylase (CYP27A1) which is in addition to the liver also expressed in other tissues. Other minor pathways can be initiated by a sterol 25-hydroxylase in the liver or sterol 24-hydroxylase (CYP46A1) in the brain. The next step of this indirect pathway includes the 7α-hydroxylation of oxysterols. The hydroxylation of the 25- and 27-hydroxycholesterols is catalyzed by a 7α-hydroxylase (CYP7B1), whereas the 24-hydroxycholesterol formed in the brain is transported to the liver and the 7α-hydroxylation is caused by the CYP39A1. The classical and alternative pathways converge at the enzymatic step for isomerisation of the steroid ring. In the alternative pathway, the oxysterols are metabolized mainly to CDCA (Ridgway and McLeod 2015).

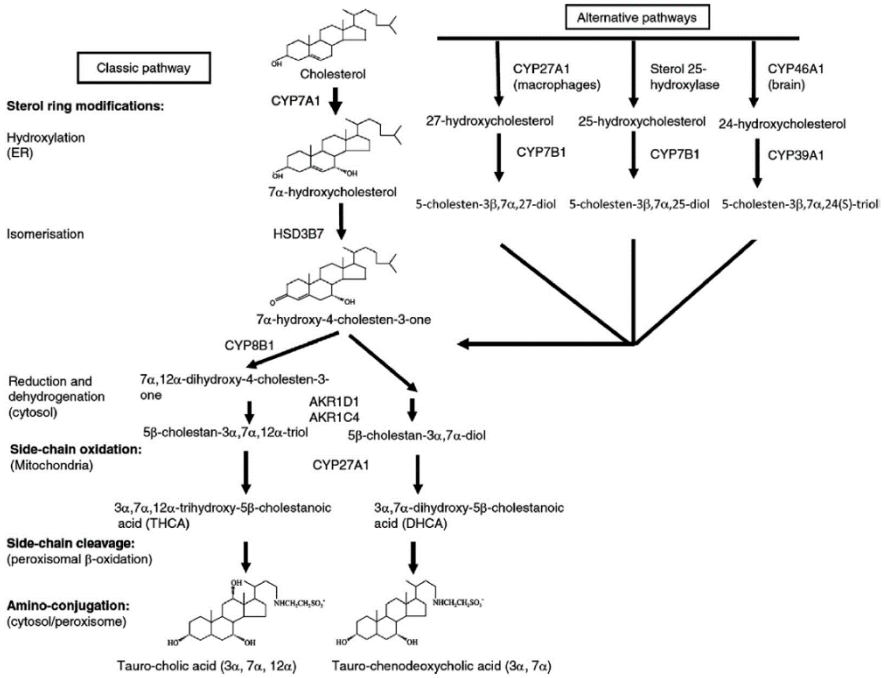


Figure 5. *The schematic representation of BA biosynthesis. Both pathways are shown, namely the classical pathway and the alternative pathway (Chiang 2013).*

3.1.4 Conjugation

A carboxyl group of the primary BAs formed from cholesterol is then covalently linked to the endogenous acids, mainly to glycine or taurine, to form glyco- or tauro-conjugates. The preference for glycine or taurine conjugation varies between different species. In humans, a ratio of the glycine- to taurine-conjugates is approximately 3:1. In mice, however, free BAs tend to conjugate to the taurine rather than to the glycine. The amidation with these hydrophilic moieties at C-24 results in a decrease of the toxicity and in an increase of the water-solubility for the secretion into the bile. These conjugates are then excreted via bile into the intestine to facilitate the digestion and absorption of the fat nutrients through the forming of micelles with phospholipids (Ridgway and McLeod 2015).

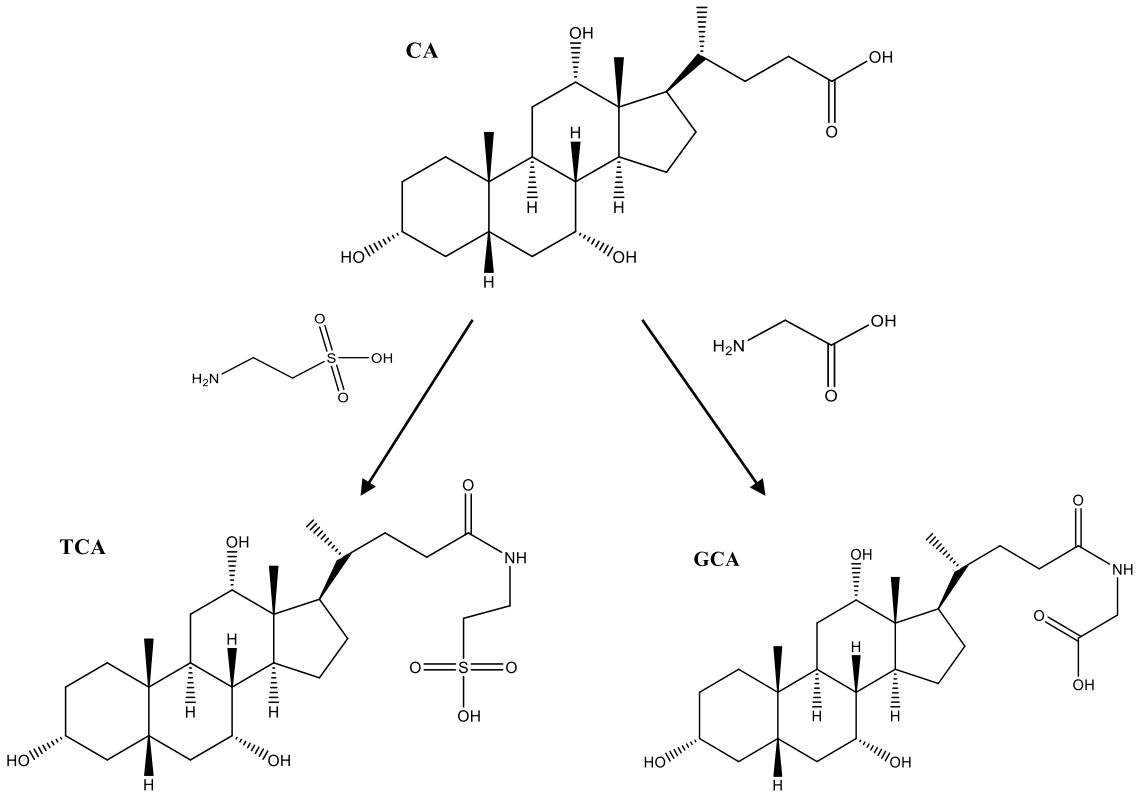


***Figure 6**. The scheme of the amidation of cholic acid (CA) with taurine and glycine to form taurocholic acid (TCA) and glycocholic acid (GCA).*

Besides the amidation with glycine or taurine, BAs can be also conjugated as 3-sulfated metabolites, with the glucuronic acid or they can be found as glucosides, galactosides or N-acetylglucosamides. The formation of conjugates is depending on the structure of BAs and on the conjugation moiety as well (Griffiths and Sjövall 2010).

A sulfation mediated by sulfotransferase 2A1 is the major metabolic pathway to eliminate BAs in humans. The sulfation increases the solubility of BAs, decreases the absorption in the intestine and enhances the excretion to the feaces and urine (Alnouti et al. 2008). For example, LCA (the toxic 7-deoxy derivate of CDCA) undergoes C-24 amidation together with C-3 sulfation. The sulfation decreases the toxicity of LCA because it is becoming a non-resorb form and is then excreted in the faeces. Thus, BAs can be also simultaneously conjugated at different positions (e.g. the amidation is often combined with the sulfation) (Jenkins and Hardie 2008).

The secondary BAs, DCA and LCA, are produced in the colon by the intestinal bacterial flora from the primary BAs through a removal of the glycine or taurine (deconjugation) and a dehydroxylation at C7 by a 7α-dehydroxylase. CA is converted into DCA and CDCA into LCA (Jenkins and Hardie 2008).

There are also some minor BAs that have important functions. One of them is UDCA, a 7β-epimer of CDCA, which was firstly found and isolated from a bear bile of Ursidae family (as its name suggest). However, it can be produced by the colonic bacteria from CDCA in humans as well (Jenkins and Hardie 2008). UDCA plays a role in the cholesterol regulation through reducing the intestinal absorption and biliary secretion of cholesterol. This BA is characterized by hepatoprotective activities and helps to modify the composition of the bile with decreasing levels of endogenous hydrophobic BAs, while increasing the proportion of nontoxic hydrophilic BAs. Nowadays, UDCA is used as a solvent of the cholesterol gallstones and also as a treatment for cholestatic liver diseases (Angulo 2002).

3.1.5 Brief Summary of their Function in Organism

BAs perform a variety of indispensable physiological functions in humans and animals. The conversion of cholesterol into BAs plays an important role in the cholesterol homeostasis, specifically in the regulation of an absorption and an elimination of cholesterol. The conjugated forms of BAs in the bile assist as lipid solubilizers to facilitate the digestion and absorption of the fats and fat soluble-vitamins in the intestine. BAs are also signal molecules with diverse paracrine and endocrine functions. They are ligands for G-protein coupled receptors (such as TGR5) and several nuclear receptors (such as the farnesoid-X-receptor and pregnane-X-receptor). Through an activation of the various signaling pathways, they control not only their own metabolism but also regulate metabolism of lipids, glucose, and energy homeostasis (Ridgway and McLeod 2015).

3.1.6 Enterohepatic Circulation

BAs are stored in the gallbladder until the fats in the duodenum signal its contraction (via cholecystokinin) and empty its contents into the small intestine. After the BAs facilitate intestinal fat digestion and absorption, they are reabsorbed in the ileum into the blood and returned back via portal circulation to the liver to inhibit their own synthesis and are then resecreted into the bile (Chiang 2013, Jenkins and Hardie 2008).

This recycling, that most of BAs (95%) undergo several times each day, is known as the enterohepatic circulation. About 5% of BAs (500-600 mg/day) go to the colon to be eliminated in the faeces. This is the major way cholesterol is lost from the body. The BAs are then replaced by the new synthesis in the liver (Chiang 2013, Jenkins and Hardie 2008).

3.2 Intrahepatic Cholestasis of Pregnancy

Intrahepatic cholestasis of pregnancy (ICP), also known as obstetric cholestasis, is a reversible type of cholestasis. It is the most common pregnancy-related liver disorder. It frequently develops in the third trimester of the pregnancy and disappears shortly after the delivery, mostly within few days (Lammert et al. 2000).

The most common symptom for this liver disease is a pruritus (itching), which commonly starts in palms and sole, with changes in the liver enzymes (alanine transaminase). The serum concentration of total BAs is increased. The pruritus is more severe at night and leads to a discomfort for the mothers. A jaundice (icterus) occurs only in 10-15% of cases (Pusl and Beuers 2007).

The etiology is not completely clear. It is a multifactorial disorder. The genetic factors, hormonal changes in pregnancy and also environmental factors play important roles in the pathogenesis of the disease. The BAs or their toxic metabolites are shown to induce contraction of the chorionic veins of the placenta, and myometrial sensitivity of healthy women to oxytocin was increased after an incubation with cholic acid (Pusl and Beuers 2007).

A defect in the production of the bile leads to a rise of serum BA levels. These accumulated BAs are deposited within the skin, causing the intensive pruritus. The increase of serum total BAs in combination with typical pruritus is highly suggestive of the diagnosis of ICP. In ICP, CA is raised more than CDCA. The CA/CDCA ratio is increased (4:1). In normal pregnancies of the same gestational age and also in non-pregnant women, the CA/CDCA ratio is 1.5. This situation reflects the fact that CDCA (as less polar BA) is preferably sulfated and excreted in the urine. The glycine- or taurine-conjugates, mainly TCA, are also increased (Lammert et al. 2000).

There is an increased flux of BAs through the placenta from the mother to the fetus. The concentration of BAs in amniotic fluid and in cord blood is increased. Fetal BA balance depends on the placental transfer capacity for BAs (Lammert et al. 2000).

ICP increases the risk of spontaneous preterm birth, fetal bradycardia, fetal distress and fetal loss (Pusl and Beuers 2007).

The aim of the pharmacologic treatment in ICP is to alleviate the symptoms and improve the fetal outcome. Currently, UDCA is the most effective treatment for ICP. The pruritus was significantly reduced, and serum alanine transaminase and endogenous serum total BA levels were lowered after UDCA therapy. The delivery shifted closer to the term. UDCA seems to be well tolerated by pregnant women and no adverse effects in mothers or newborns have been observed (Pusl and Beuers 2007).

3.3 Hair Metabolomics

Recently, hair has become an attractive biological material used as a source of the biomarkers. Hair grows slowly and can reflect exposure of both endogenous compounds and environmental exposures for many weeks or months prior to the sample collection. An analysis of hair brings advantages and also disadvantages. On the one hand, hair is a stable structure and easy to collect. The collection of hair is non-invasive and the samples can be stored at the room temperature. Hair analysis can assess not only current exposure, but also reflect a long-term retrospective history of individual exposure, even if the exposure has already been ended. In contrast to hair, an analysis of blood requires an invasive sampling, an immediate treatment of samples and the metabolome is changing in time. It can also be influenced by the recent dietary intake. On the other hand in terms of disadvantages of the hair samples, the exogenous contamination could interfere with the analysis (Sulek el al. 2014, He et al. 2016).

Up to now, hair analysis has been applied in many fields, such as in forensic toxicology, in drug abuse studies or in clinical toxicology (Nakahara 1999). Information about hair metabolomics can be used as a possible approach to get biomarkers for identifying of various diseases. It has already been studied by GC-MS in gestational diabetes mellitus and fetal growth restriction (Sulek el al. 2014, He et al. 2016).

Previous studies reported metabolomic profiles of BAs in biological fluids, such as in blood and urine. These compounds were shown to be the potential biomarkers for diverse liver diseases (e.g. primary biliary cirrhosis, cholestasis). In this thesis, the hair samples were used to study the profile of BAs and to evaluate BAs as potential biomarkers for obstetric cholestasis.

3.4 (Ultra)High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is the major analytical technique used to separate various compounds that are present in a sample, and further to an identification and a quantification (Kazakevich and Lobrutto 2007). The components contained in the sample distribute differently between two immiscible phases according to their affinity to these phases. One of them is known as a stationary (immobile) phase which is located in a chromatographic column, and the other one is called a mobile phase constituted by a moving solvent which carries the analyte through the column. The process of partition depends on the formation of interaction between the analytes dissolved in the mobile phase and the surface of the stationary phase (Arsenault and McDonald 2007). Each component reacts differently with the particles of the stationary phase which means that each component is differently long retained in the column and has different retention time. The compounds with low affinity to the stationary phase are more likely to be carried by the mobile phase and migrate faster through the column. On the contrary, the analytes retained in the column more strongly elute later from the column and have longer retention time (LC/GC´s CHROMacademy [online], cit. 2017-10-05).

3.4.1 Components and Basic Principles

The sample must be soluble in the mobile phase. This is the basic condition for the HPLC analysis (LC/GC´s CHROMacademy [online], cit. 2017-10-05). The mobile phase, free from the air removed by a vacuum degasser, is continously pumped through the system under high pressure by the pumps which are used to generate a flow rate. The sample is injected into the flowing mobile phase by an injector or autosampler and carried onto the stationary phase which has a large surface area for an effective separation. The compounds contained in the sample are separated in the column according to their preference for the stationary phase or for the mobile phase (Kazakevich and Lobrutto 2007).

In the HPLC system, a detector is then needed to detect the separated compounds eluted from the HPLC column. The detector is a component of the HPLC system responsible for a conversion of a physical or chemical attribute into a measurable signal corresponding to the concentration of the identity. Several types of detectors have been developed to detect a presence of many different compounds. For example, an UV-visible detectors can be used for the molecules that can absorb photons in the UV or visible region. If the compound is fluorescent, a fluorescence detector is taken into the consideration for detection. The molecules can also fluoresce using a derivatization. The

molecules with groups capable of oxidation or reduction can be detected based on the electrochemical detection, where the column is connected to a cell containing electrodes, where a reaction takes place. Nowadays, the combination of HPLC and mass spectrometer as the detector presents a modern technique for large number of applications. This combination is applied for qualitative and as well for quantitative analysis of complex mixtures of compounds, where the structural information is needed (Swartz 2010, Arsenault and McDonald 2007). The detector is connected with a computer to generate a chromatogram which is a visual output of the chromatography (a detector response plotted against the time of retention), and subsequently analyze the data (Kazakevich and Lobrutto 2007).

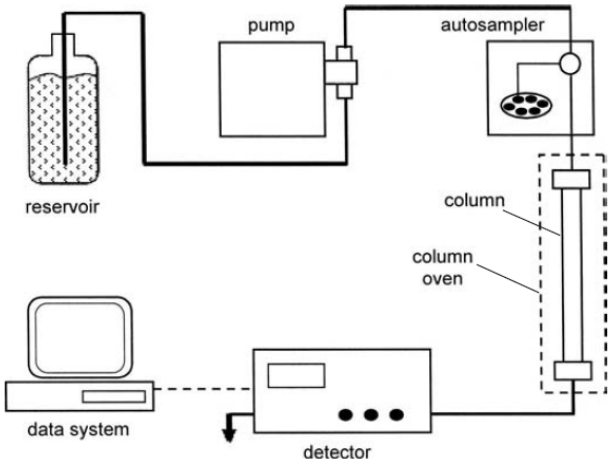


Figure 7. *The components of the HPLC system (Snyder et al. 2010).*

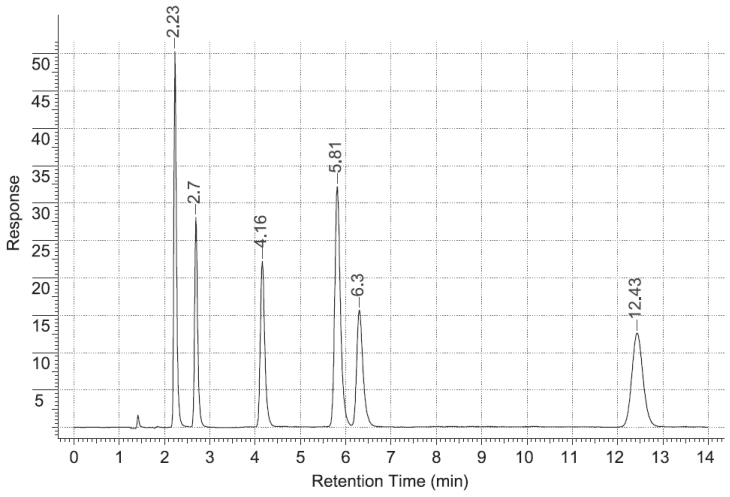


Figure 8*. The chromatogram of a mixture of compounds, the detector response plotted against the time of retention (Kazakevich and Lobrutto 2007).*

The polarity, ionic character and molecular size are the primary characteristic of compounds which can be used for HPLC separation. A mode using a polar stationary phase (the most frequent is unbonded silica particles) with much less polar mobile phase (mixture of less-polar organic solvents such as hexane, chloroform) is known as a Normal-Phase (NP) chromatography. A Reversed-Phase (RP) chromatography consists of the non-polar stationary phase (chemically modified silica mostly with C18, C8 alkyl chains) and the mobile phase which is usually composed of a polar mixture of water or buffer (to modify a pH of the mobile phase) with an organic solvent (such as acetonitrile, methanol). The concentration of organic modifier is important factor for the retention of the analytes. Today, RP chromatography is the most frequently used mode (Arsenault and McDonald 2007, Snyder et al. 2010).

HPLC analysis can be performed in two modes of mobile phase elution. If the composition of the mobile phase is constant during the analysis, the separation is termed an isocratic elution. Mostly, the sample contains analytes with a wide range of polarities and the isocratic elution may not provide adequate separation. In this case, a gradient elution is needed. In this elution mode, the composition of the mobile phase must be changed throughout the separation process. For example, at the beginning of the separation the gradient starts at 20% of ACN as an organic modifier and is gradually increased at 80% of ACN. The mobile phase composition is mixed by the pumping system (LC/GC´s CHROMacademy [online], cit. 2017-10-05).

Ultra-High Performance Liquid Chromatography (UHPLC) is a variation of HPLC which provides an increase in resolution and speed of analysis by reducing the particle diameter. Smaller particles (less than 2 μm) are packed into the shorter columns. The pressure in the column is significantly increased as a response to the size reduction of particles. The separation efficacy of analysis is improved (Kazakevich and Lobrutto 2007).

3.5 Mass Spectrometry

Mass Spectrometry (MS) is one of the most significant analytical methods and is used for both qualitative and quantitative analysis thanks to its sensitivity (femto-gram amounts may be detected by certain mass analyzers) and also its selectivity. This technique is based on the formation of ions from analyte molecules. The formed ions are then separated in the mass analyzer according to their m/z value, where m is the mass of the ion (Da) and z is the charge (e), known as the mass to charge ratio (LC/GC´s CHROMacademy [online], cit. 2017-10-05).

Mass spectrometer can be online connected to the HLPC (abbreviated as LC-MS). The HPLC is used as a separative technique and the MS as a detector for the separated compounds.

The mass spectrometer consists of the following components: an ionization source, one or more mass analyzers, a detector, a data processing system. It is useful to consider which instrumentation should be used for each analysis in order to get reproducible results (LC/GC´s CHROMacademy [online], cit. 2017-10-05, Hoffmann and Stroobant 2007).

3.5.1 Components and Basic Principles

A HPLC eluent, contained the analytes dissolved in the mobile phase, is sprayed into an ion source in the atmospheric region, where the neutral molecules are converted to the ions. Formed ions are then accelerated into the mass analyzer and separated according to their mass to charge ratio (*m/z* value) using electric and/or magnetic fields. The most popular analyzers include: a Quadrupole, a Time of Flight, an Ion Trap and a Magnetic Sector. Eventually, the system can contain more analyzers of the same type or their combination to get fragment ions from the selected precursor ions for further identification of analytes (tandem mass spectrometry, MS/MS). After the separation process, a detection takes place. The ions reach the detector producing an electrical current and the signal of each ion may also be amplified in order to make it detectable. The most used detectors are: an electron multiplier, a dynode, a photodiode and a multi-channel plate. The mass analyzer and detector are operated under high vacuum. The last component to end this analysis is the data processing system to produce a mass spectrum, an output of the analysis (LC/GC´s CHROMacademy [online], cit. 2017-10-05, Hoffmann and Stroobant 2007).

The mass spectrum is a plot of the ion abundance against the *m/z* value. The most intensive peak in the mass spectrum is called the base peak and is set to the relative abundance of 100%. All other ions have abundances measured to the intensity of this peak (Hoffmann and Stroobant 2007).

3.5.2 Ion Source

An ionization in the ion source is a process whereby the analyzed neutral molecules are ionized to produce ions prior to the analysis. Charged species are produced as a gas phase by various ionization methods. During this process, electrons are removed or added to the atoms or molecules to produce the ions (anions can be

achieved in the negative ion mode, while cations in the positive ion mode). In addition, the ions can be also formed via association with other molecules that carry a charge (e.g. a proton). Multiply charged ions can be also obtained (LC/GC´s CHROMacademy [online], cit. 2017-10-05).

Some of these ionization techniques are soft and produce the ions of the molecular species, whereas other techniques are very energic to cause a fragmentation of the molecules (Hoffmann and Stroobant 2007). The most commonly used ionization techniques for HPLC analysis coupled to MS are: an Electrospray Ionization (ESI), an Atmospheric Pressure Chemical Ionization (APCI) and an Atmospheric Pressure Photo Ionization (APPI), together termed as the Atmosperic Pressure Ionization (API) techniques working under atmospheric pressure conditions. All of them belong to the soft ionization techniques (Holčapek: Ionizační Techniky [online], cit. 2017-10-22).

None of the ionization techniques is universal. Therefore, it is necessary to choose an effective ion source for each analysis to get high sensitivity and reproducible spectra (Holčapek: Ionizační Techniky [online], cit. 2017-10-22).

3.5.2.1 *Ionization Technique: Electrospray Ionization*

The Electrospray Ionization (ESI) is a soft ionization technique usually used for the analysis of polar molecules. The process consists of three main parts: formation of the charged droplets, release of the ions from the droplets, and transferring the ions from the ion source to the mass analyzer (Holčapek: Ionizační Techniky [online], cit. 2017-10-22).

At first, the analytes contained in the HPLC eluent are transferred into a gas phase ions. The eluent passes into the ion source through a nebuliser, surrounded by nitrogen gas and held at high voltage (4-5 kV, relative to a counter electrode), resulting in a creation of the droplets that are electrically charged. The droplets possess either a positive or a negative charge depending on the polarity of the nebuliser. Positive or negative ion mode can be selected according to the nature of the studied species. A solvent is then evaporated from the fine spray of droplets as a result of an application of a heated drying gas (mostly nitrogen that passes in the opposite direction to the passage of droplets) (Downard 2004).

During the evaporation process, the electrical charge density at the surface of the droplets is increasing. The increase in charge to surface area ratio results in a Coulombic explosion, which means that the repulsive forces between the ions overcome the critical point known as Rayleigh Instability Limit of the surface tension of the droplet. This Coulombic fission results in the production of smaller droplets (Downard 2004).

After evaporation of all solvent, the ions are attracted into the mass analyzer through the capillary. The ions of macromolecules produced by this ionization technique are usually multiply-charged ions, whereas the molecules with lower molecular weight tend to form singly-charged ions (protonated molecules in the positive ion mode or deprotonated molecules in the negative ion mode) (Lee et al. 2014).

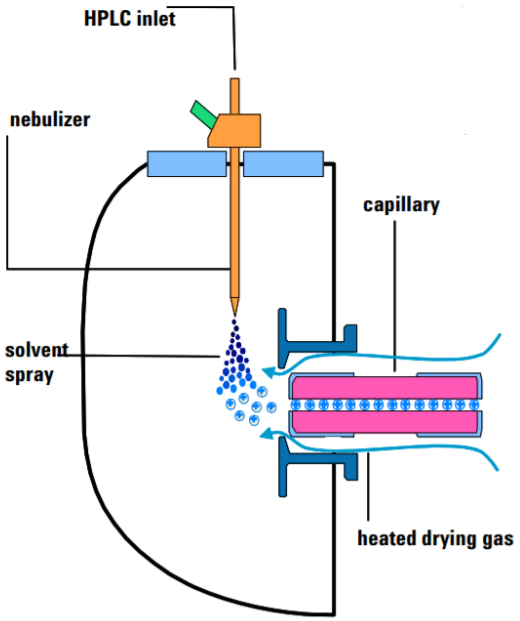


Figure 9. *The scheme of the Electrospray Ionization technique (Agilent Technologies 2014).*

3.5.3 Mass Analyzers

After the ions have been formed in the ion source, the mass analyzer is used to separate the ions or fragments of the ions based on their mass to charge ratio through the application of electric or magnetic fields. There are many popular types of mass analyzers, such as: Quadrupole, Ion Trap, Time of Flight, Magnetic Sector. In addition, mass analyzers can be coupled together for use in tandem mass spectrometry where the fragment ions are achieved by the collision-induced dissociation (CID) induced by an inert gas. An instrument consisted of different types of the mass analyzers is known as a hybrid mass spectrometer (Downard 2004).

3.5.3.1 Quadrupole

The Quadrupole (Q) consists of four parallel equidistant rods. The ions are separated based on the stability of their trajectories in the oscillating electric fields that are applied to the rods. Alternating radiofrequency current (RF) is applied to all rods. Additionally, positive direct current (DC) voltage is put to two opposite rods. To the other opposite rods, negative DC voltage is applied (Holčapek: Hmotnostní Analyzátory [online], cit 2017-11-06).

The ions are accelerated out of the ion source into the center of the rods where they oscillate. For a specific ratio of DC and RF, the oscillations are stable only for ions with a specific *m/z* value, which means that these ions are able to pass through the quadrupole without touching the rods and reach the detector. This is called as non-collisional or stable trajectory. Collisional or unstable trajectory, on the other hand, is a situation where the ions do not reach the detector and are caught by the rods. All ions go gradually through the mass analyzer and reach the detector due to the changes of DC voltage and RF amplitude, while their ratio stays constant (LC/GC´s CHROMacademy [online], cit. 2017-10-05, Holčapek: Hmotnostní Analyzátory [online], cit 2017-11-06).

3.5.3.2 Time of Flight and Hybrid Quadrupole-Time of Flight

As the name suggests, Time of Flight (TOF) mass analyzer separates the ions based on the measurement of the time that ions take to „fly“ from the ion source to the detector. The separation of ions takes place in a field-free region, so-called a flight tube of a set length. Therefore, either electric or magnetic fields are not needed in this part of instrument. The ions are accelerated to the flight tube by application of a high accelerating potecial and they reach the detector in different flight times. The speed at which the ions fly through the tube depends on their *m/z*. All ions achieve the same kinetic energy, however, the ions with a low *m/z* value „fly“ faster down the flight tube than the ions with a higher *m/z* ratio. Scanning the broad mass range of all ions is very fast (Downard 2004, Holčapek: Hmotnostní Analyzátory [online], cit 2017-11-06).

The TOF mass analyzer is frequently connected to the Quadrupole to form a hybrid mass spectrometer (Q-TOF) that performs the MS/MS analysis. The Quadrupole selects the precursor ions that are fragmented in a collision cell into the product ions and the TOF is used to produce the spectra (Hoffmann and Stroobant 2007).

3.5.3.3 Orbitrap

The Orbitrap is the latest type of mass analyzers invented by Makarov and launched on the market by Thermo Fisher Scientific in 2005. It is based on a completely new concept. The Orbitrap is an electrostatic ion trap consisting of two specially shaped electrodes, an inner (central) electrode and an outer electrode. The outer electrode has the shape of a barrel split into two parts separated by a small gap and the central electrode is a spindle-shaped (Hoffmann and Stroobant 2007).

There is neither RF nor magnetic field to hold the ions inside. Only DC voltages are applied. Electrostatic voltage of several kilovolts is applied to the central electrode, while the outer electrode is at ground potential (Makarov 2000).

The instrument is equipped with a storage device, known as C-Trap, for pulse injection of the formed ions. The ion packets are injected through the gap in external electrode into the space between the central and outer electrode (Thermo Fisher Scientific 2017). The injected ions rotate around the central electrode on elliptical trajectories. In addition, at the same time the ions oscillate back and forth along the *z* axis with a frequency (axial direction). Different ions oscillate at different frequencies, resulting in their separation. These oscillations in the axial direction are then detected as an image current induced on the outer electrode and the so-called Fourier transform is used to obtain the mass spectra (Makarov 2000).

The Orbitrap is commonly connected to other mass analyzers to form hybrid analyzers. The Orbitrap used in our study is connected to the Quadrupole mass filter used for precursor ion selection (abbreviated as Q-Orbitrap).

Orbitrap's benefits include sub-1-ppm mass accuracy and extremely high mass resolution (Makarov 2000).

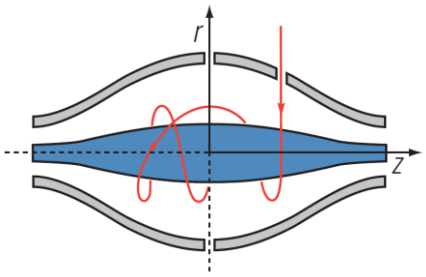


Figure 10. *An example of stable ion trajectory (Thermo Fisher Scientific 2017).*

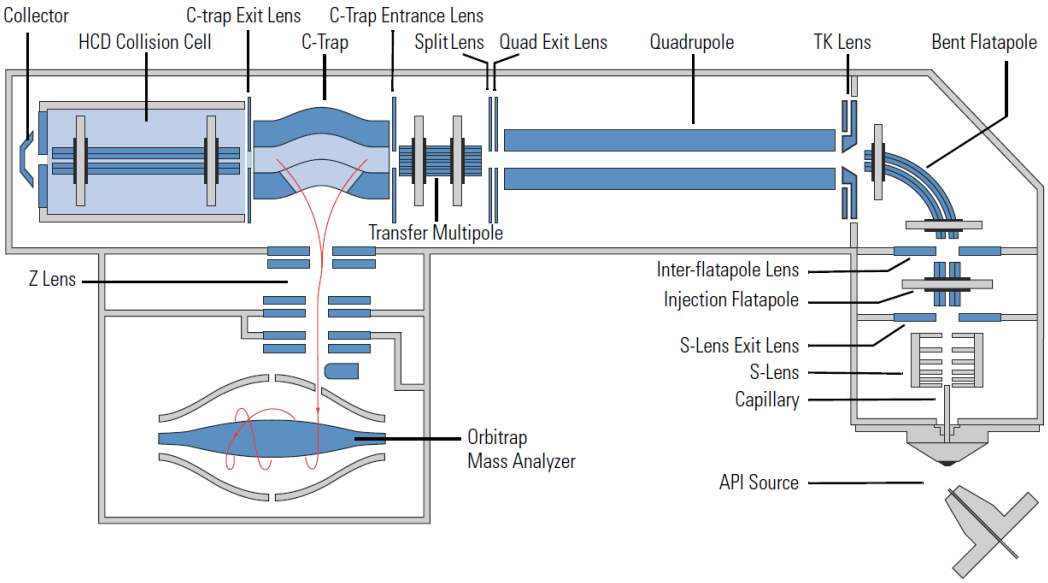


Figure 11. *The scheme of the Orbitrap connected to the Quadrupole (Thermo Fisher Scientific 2017).*

3.5.4 Detectors

After the ions pass through the mass analyzer, they continue to the detector to be detected and transformed into a measurable signal. Several types of detectors are in use such as a Faraday cup, an Electron Multipliers and a Microchannel Plate Electron Multipliers (LC/GC´s CHROMacademy [online], cit. 2017-10-05).

Some detectors, such as Faraday cup, are able to generate an electric current proportional to the abundance when an ion hits the surface. The others, such as Electron Multiplier detectors, are based on the generating the secondary electrons from the inicial ions, which are further amplified to give an electronic current (Hoffmann and Stroobant 2007, LC/GC´s CHROMacademy [online], cit. 2017-10-05).

3.6 HPLC-MS in Bile Acid Analysis

Nowadays, the HPLC separation coupled to MS represents the method of choice for detailed analysis of BAs and their conjugates in biological samples. Before the HPLC has been developed, gas chromatography (GC) was an available separation technique. GC is compared to HPLC a time-consuming technique for BA separation, but more specific for BA isomers. The samples must undergo multiple steps preparation, including a derivatization of BAs to their volatile and thermostable form, and the analysis of conjugated forms requires a hydrolysis of conjugates into the unconjugated form prior to

their analysis. These steps could be a potential source of errors (Griffiths and Sjövall 2010, Roda et al. 1998).

HPLC coupled to MS allows simultaneously analysis of both conjugated and non-conjugated BAs within a single run. The conjugated BAs presented in biological samples can be analyzed directly without preliminary hydrolysis procedures. Thus, the separation of BAs by HPLC on a C18 RP column coupled to MS analysis using negative ESI mode belongs to the most effective technique to detect and identify BAs (Griffiths and Sjövall 2010, Roda et al. 1998).

The analysis of free BAs and their conjugates has always presented difficulties due to their structural similarity, low concentration in biological samples and the existence of isomeric forms. There are only small structural differences between the isomeric forms therefore it is necessary to set optimum HPLC-MS conditions to detect BAs in biological samples and to achieve effective characterization of BA isomers (Tagliacozzi et al. 2003).

4 EXPERIMENTAL PART

4.1 Materials and Methods

4.1.1 Chemicals and Reagents

- BA standards were obtained from Merck KGaA (Darmstadt, Germany), products of Sigma-Aldrich (St. Louis, MO, USA)
    - Sodium cholate hydrate (≥ 99%)
    - Sodium deoxycholate (≥ 97%)
    - Chenodeoxycholic acid (≥ 97%)
    - Ursodeoxycholic acid (≥ 99%)
    - Lithocholic acid (≥ 95%)
    - Taurocholic acid sodium salt hydrate (≥ 95%)
    - Sodium taurodeoxycholate hydrate (≥ 95%)
    - Glycocholic acid hydrate (≥ 97%)
    - Sodium glycochenodeoxycholate (≥ 97%)
- Internal standards (ISs) were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada)
    - Cholic acid-d5
    - Deoxycholic acid-d5
- Acetonitrile and Fetal Bovine Serum (FBS) were obtained from VWR International (Finland)
    - Acetonitrile, HiPerSolv CHROMANORM, for LC-MS (min. 99,90%)
    - FBS South America, Charcoal Stripped, Biowest
- Other chemicals were purchased from Thermo Fisher Scientific (Finland)
    - Methanol, CHROMASOLV LC-MS Ultra, Honeywell Riedel-de Haën (min. 99,90%)
    - Formic acid, Eluent additive for LC-MS, Honeywell Fluka (98%)
    - 2-Propanol, CHROMASOLV LC-MS, Honeywell Riedel-de Haën (min. 99,90%)
- De-ionized water was prepared by a Milli-Q system – PURELAB Ultra Water Purification Systems, Model ULXXXANM2, ELGA LabWater

4.1.2 Samples

- Mouse liver – donated from Dr. Mikko Gynther, Ph.D, University of Eastern Finland
- Human plasma – donated from Kuopio University Hospital
- Human hair
    - Hair samples of two men (marked as young and old hair) donated from Dr. Merja Häkkinen, Ph.D, University of Eastern Finland
    - Hair samples of two mothers with intrahepatic cholestasis of pregnancy and their neonates (marked as Mother 1 and Baby 1; Mother 2 and Baby 2) donated from Kuopio University Hospital

4.1.3 Instrumentation

- Agilent 1290 Infinity LC System, UHPLC (Agilent Technologies, Santa Clara, CA, USA)
- Agilent 6540 Q-TOF LC-MS System (Agilent Technologies, Santa Clara, CA, USA)
- Eppendorf Centrifuge 5804 R (Eppendorf AG, Hamburg, Germany)
- Homogenizer, Type X 1020 (Ystral GmbH, Dottingen, Germany)
- Multi Reax Test Tube Shaker (Heidolph, Schwabach, Germany)
- Nitrogen Evaporator, 24 Position N-EVAP 112 (Organomation Associates, Inc., Berlin, MA, USA)
- Q Exactive Focus Hybrid Quadrupole–Orbitrap High Resolution MS/MS System (Thermo Scientific, Bremen, Germany)
- Vanquish Flex binary UHPLC System (Thermo Scientific, Bremen, Germany)
- Vortex-Genie 2 (Scientific Industries, Inc., Bohemia, NY, USA)
- Weights MT5 (Mettler Toledo, Columbus, OH, USA)
- Weights Sartorius BP221S (Sartorius AG, Göttingen, Germany)

4.1.4 Preparation of Stock Solutions of Standards

The stock solutions of individual BA standards were prepared in acetonitrile (ACN) at a concentration of 1 mg/ml. Dissolving of bile salts was facilitated using water/ACN (1:9, v/v). The concentrations of 100 µg/ml were prepared from these stock solutions by dilution with ACN.

4.1.5 Preparation of Internal Standards

Deuterated cholic acid (CA-d5) and deoxycholic acid (DCA-d5) served as Internal Standards (ISs) for the analysis performed by LC-Q-Orbitrap. 0.1 mg of each IS was dissolved in 10 ml of 50% ACN at a concentration of 10 µg/ml. These stock solutions of both ISs were mixed at a concentration of 5 µg/ml per each IS. The mixture was further diluted in 50% ACN at a concentration of 1000 ng/ml. This solution was added to the samples for LC-MS analysis performed by LC-Q-Orbitrap.

4.1.6 Preparation of Working Solutions and Calibration Curves

The stock solutions of 100 µg/ml of each BA standard were further diluted with 50% ACN to give solutions at a concentration of 45 µg/ml. These nine solutions of BA standards were mixed to give a concentration of 5 µg/ml per each analyte. The working solutions were prepared by dilution with 50% ACN in a range of 1-5000 ng/ml (1, 5, 10, 20, 50, 100, 250, 500, 1000, 5000 ng/ml).

For the construction of calibration curve in a range of 0.1-500 ng/ml (0.1, 0.5, 1, 2, 5, 10, 25, 50, 100, 500 ng/ml) for the analysis by LC-Q-Orbitrap, 10 µl of working solution were mixed with 100 µl of 50% ACN and 10 µl of IS mixture (1000 ng/ml).

The calibration curves were also prepared in charcoal serum. 10 µl of working solution were mixed with 100 µl of charcoal serum and 10 µl of IS mixture (1000 ng/ml). A 300 µl of ice-cold ACN were added by vortexing for protein precipitation. The samples were then centrifuged at 14 000 rpm, at 4°C, for 5 min. The supernatants were filtered to a vial tube and stored at 4°C until measurement.

4.1.7 Sample Preparation

4.1.7.1 Preparation of Mouse Liver Samples

Mouse liver was frozen in liquid nitrogen and stored at -80°C until the sample preparation. Two different methods were used for the preparation of liver samples.

In the first method, mouse liver was mechanically homogenized with water (1:3, w/v) using homogenizer. An extraction was performed by protein precipitation using methanol (MeOH). Therefore, 100 µl of liver homogenate were diluted in 300 µl of 80% MeOH and vortexed for approximately 10 min. After 10 min of centrifugation at 13 000 rpm, at 4°C, the supernatant was filtered to a vial tube and stored at 4°C until the analysis. The rest of homogenate was stored in an Eppendorf tube at -80°C.

In the second way of preparation, liver was frozen in liquid nitrogen and crushed. The liver powder was reconstituted in 80% MeOH in a ratio of 1:3 (w/v), vortexed for approximately 10 min, centrifuged at 13 000 rpm, at 4°C, for 10 min and the supernatant was filtered to a vial tube and stored at 4°C until time of analysis. For the analysis by LC-Q-Orbitrap, the liver homogenate was ten times diluted. Then, 100 µl of diluted liver homogenate were mixed with 10 µl of 50% ACN and 10 µl of IS mixture (1000 ng/ml).

4.1.7.2 Preparation of Plasma Samples

Plasma was obtained from healthy volunteers using standard venipuncture techniques, randomized and stored at -80°C until the sample preparation, which was performed as follows: A simple protein precipitation using ice-cold ACN was used. A 300 µl of ice-cold ACN were added by vortexing to 100 µl of plasma in an Eppendorf tube. The samples were then centrifuged at 14 000 rpm, at 4°C, for 5 min. The supernatants were filtered to a vial tube and stored at 4°C until measurement.

To the samples prepared for the analysis performed by LC-Q-Orbitrap, 10 µl of IS mixture (1000 ng/ml) were added before addition of 300 µl of ACN.

4.1.7.3 Preparation of Hair Samples

Hair samples obtained from mothers with ICP and their neonates were provided by Kuopio University Hospital. The hair has been collected since May 2017 within the project Kuopio Birth Cohort (KuBiCo) launched in 2012.

Other hair samples were obtained from 23 years old man (young hair) and 60 years old man (old hair) without any information about their diagnosis.

Hair collection

Hair was collected from the mothers and their neonate(s) on the first or second day after the parturition by midwives trained for the study in Kuopio University Hospital. Hair was cut with fine scissors as close to the scalp as possible from the posterior vertex of mother and from the occipital area of the head of neonate. Scissors and all other

equipment were washed with 80% denaturated ethanol before and after hair sample was cut and the sample was transmitted into aluminium foil and envelope where one hair sample was storaged and identified only with mother's or neonate's own research code.

The hair samples from the men were cut in the barber shop (more detailed information is not known).

Hair washing and extraction procedure

The first step of preparation of hair samples consisted of washing the hair. The washing step was used to remove possible external contaminants, such as cosmetic product residues, sweat, sebum or other surface contaminants that could interfere with the analysis and could cause a background noise. Decontamination of hair was performed using isopropanol as a washing solution.

In the case of hair obtained from male patients, 15-20 mg of whole, non-pulverized hair in an Eppendorf tube were washed in 1.5 ml of isopropanol and vortexed for 2 min at room temperature. Isopropanol was removed using Pasteur pipette and the rest of isopropanol was evaporated under a nitrogen evaporater at 40°C for 1 hour. For extraction, 1.5 ml of MeOH were added to hair samples and incubated by vortexing for 18 hours at room temperature. After 18 hours, the MeOH extract was transferred to the new Eppendorf tube. 50 µl of hair extract in 3 replicates were used for LC-MS analysis performed by LC-Q-TOF and the rest of extract was frozen at -80°C. During the procedure, a blank sample was prepared in the same way.

For analysis performed by LC-Q-Orbitrap, IS mixture was added to the prepared MeOH extract: 100 µl of hair extract were mixed with 10 µl of 50% ACN and 10 µl of IS mixture (1000 ng/ml).

In the case of mother's and baby's hair, 6-9 mg of hair strands were weighed out. The preparation process was identical as described above, only with a difference in addition of IS mixture. IS mixture was added before the MeOH extraction started. These hair samples were measured using only LC-Q-Orbitrap.

4.1.8 LC-MS Conditions

The analysis of standards and biological samples (mouse liver, human plasma and human hair) was performed using two instruments, UHPLC connected to Q-TOF mass spectrometer and UHPLC connected to Q-Orbitrap mass spectrometer.

4.1.8.1 LC-Q-TOF

BA analysis of mouse liver, human plasma and human hair was performed by UHPLC system (Agilent 1290 Infinity LC System, Agilent Technologies, Santa Clara, CA, USA) which was connected to the Q-TOF mass spectrometer (Agilent 6540 Q-TOF LC-MS System, Agilent Technologies, Santa Clara, CA, USA) using two chromatographic techniques: reversed phase (RP) and hydrophilic interaction chromatography (HILIC), both combined with positive and negative mode of electrospray ionization (ESI).

The RP column used in this study was a Zorbax Eclipse XDB-C18 Rapid Resolution HD (100 mm x 2.1 mm I.D., 1.8 µm particles; Agilent Technologies, Santa Clara, CA, USA). The column temperature was set at 50°C. A gradient elution was used with water (eluent A) and methanol (eluent B), both containing 0.1% (v/v) of formic acid. A flow rate used for these eluents was 0.4 ml/min. The injection volume of sample solutions was 2 µl. The run time of the whole process was 16.50 minutes.

Table 3. *The examined gradient used for RP chromatography. The eluent A consisted of 0.1% formic acid in water and eluent B of 0.1% formic acid in methanol.*

Time (min)	Eluent A (%)	Eluent B (%)
0.00	98	2
10.00	0	100
14.50	0	100
14.51	98	2
16.50	98	2

The HILIC separation was performed on an Acquity UPLC BEH Amide column (100 mm x 2.1 mm I.D., 1.7 µm particles; Waters Corporation, Milford, MA, USA). The column temperature was 45°C. The eluents A and B were 50% (v/v) and 90% (v/v) ACN, respectively, both containing 20 mM of pH 3 ammonium formate, at a flow rate of 0.6 ml/min. The gradient separation is shown in Table 4. The injection volume of all sample solutions was 2 µl. The run time of this method was 12.50 minutes.

Table 4. *The examined gradient used for HILIC chromatography. Eluent A 50% (v/v) ACN and eluent B 90% (v/v) ACN, both containing 20 mM of pH 3 ammonium formate.*

Time (min)	Eluent A (%)	Eluent B (%)
0.00	0	100
2.50	0	100
10.00	100	0
10.01	0	100
12.50	0	100

The mass spectrometer ion source conditions are shown in Table 5. From each precursor scan, four most abundant ions were selected for fragmentation with a collision energy of 10, 20 and 40 V in subsequent injections. Continuous mass axis calibration was performed by monitoring two reference ions from an infusion solution throughout the runs. The data were acquired and processed by Agilent MassHunter Qualitative Analysis B.07 (Agilent Technologies). To compare the data, a METLIN Metabolomics Database and an UEF PCDL library (PCDL Manager B.07.) were used.

Table 5. *Applied MS parameters for Q-TOF.*

Parameters	Values – Negative mode	Values – Positive mode
Gas temperature (nitrogen)	325°C	325°C
Gas flow rate	10 l/min	10 l/min
Nebulizer pressure	45 psi	45 psi
Sheath gas temperature	350°C	350°C
Sheath gas flow rate	11 l/min	11 l/min
Capillary voltage	3500 V	3500 V
Nozzle voltage	1000 V	1000 V
Fragmentor voltage	100 V	100 V
Collision energy	10, 20, 40 eV	10, 20, 40 eV
Mass range	*m/z* 50-1600	*m/z* 65-1600
Reference ions	*m/z* 112.98558700, 966.00072500	*m/z* 121.05087300, 922.00979800
Exclude ions	*m/z* 44.9999, 68.9977, 112.985587, 966.000725, 1033.988109	*m/z* 121.050873, 922.009798
Isolation Width MS/MS	Narrow (~1.3 amu)	Narrow (~1.3 amu)

4.1.8.2 LC-Q-Orbitrap

The analysis was also performed by UHLPC (Vanquish Flex binary UHPLC System, Thermo Scientific, Bremen, Germany) connected to the Q-Orbitrap mass spectrometer (Q Exactive Focus Hybrid Quadrupole–Orbitrap High Resolution MS/MS System, Thermo Scientific, Bremen, Germany) equipped with a HESI mode.

For the analysis, 10 µl of the sample solution were injected on the RP column Zorbax Eclipse XDB-C18 Rapid Resolution HD (100 mm x 2.1 mm I.D., 1.8 µm particles; Agilent Technologies, Santa Clara, CA, USA). The column temperature was set at 40°C. The mobile phases flow rate was 0.4 ml/min and consisted of water (eluent A) and methanol (eluent B), both containing 0.1% (v/v) of formic acid, delivered with the following gradient conditions shown in Table 6. The run time of the analysis was 16.50 minutes.

Table 6. *The examined gradient used for RP chromatography. Water as eluent A and methanol as eluent B, both containing 0.1% (v/v) of formic acid.*

Time (min)	Eluent A (%)	Eluent B (%)
0.00	98	2
10.00	0	100
14.50	0	100
14.51	98	2
16.50	98	2

The mass spectrometer conditions used for this method are shown in Table 7. MS/MS analysis was not performed. Data assessment was determined by Xcalibur Software (Thermo Scientifics). A mass tolerance window of ± 5 ppm around the theoretical *m/z* of each analyte was used to construct the chromatograms.

Table 7. *Applied MS conditions for Q-Orbitrap.*

Parameters	**Values**
Ion mode	Negative
Spray voltage	3500 V
Capillary temperature	350°C
Sheat gas flow rate	35 (arbitrary units)
Auxiliary gas flow rate	15 (arbitrary units)
Spare gas flow rate	2 (arbitrary units)
Probe heater temperature	300°C
S-lens RF Level	60 (arbitrary units)
Mass range	*m/z* 100-600
Ion source	HESI*

*HESI – heated-electrospray ionization (abbreviation used by Thermo Fisher Scientific Inc.)

5 RESULTS

5.1 Summary of Used Methods and Samples

The analysis of BA standards and biological samples (mouse liver, human plasma and human hair) was performed using two instruments, UHPLC connected to Q-TOF and afterwards, UHPLC connected to Q-Orbitrap.

The data obtained from LC-Q-TOF were used for identification of BAs. For the analysis performed by LC-Q-Orbitrap, the samples were spiked with ISs to obtain the data not only for identification, but also for quantification.

First, BA analysis of biological samples was performed by LC-Q-TOF. Prior to the hair analysis, the method was tested on mouse liver and human plasma samples. The profiles of BAs were obtained. Information about retention times and *m/z* values obtained by measuring the standard solutions were used for identification of BAs in biological samples.

After the optimalization of the used method, the analysis of human hair obtained from two men was carried out. The data assessment was focused not only on the BA detection, but also on the entire hair metabolome for future analysis of the hair for the KuBiCo project (data not shown).

The Kuopio University Hospital provided us hair samples obtained from mothers with ICP and their neonates. To achieve a higher accuracy and precision, an analysis by LC-Q-Orbitrap was applied for all samples.

Not all samples were measured with all instruments and methods.

Table 8. *Summary of biological samples analyzed in this study and methods used for the analysis of BAs and other compounds.*

Sample / **Instrument**	**Mouse liver**	**Human plasma**	**Human hair (men)**	**Human hair (mothers + neonates)**
LC-Q-TOF				
RP	neg	neg	neg/pos	-
HILIC	-	-	neg/pos	-
LC-Q-Orbitrap RP	neg	neg	neg	neg

*neg = negative ionization mode, pos = positive ionization mode

Nine BA standards were available for the analysis. Identification of nine corresponding BAs in biological samples was provided based on the information about

retention times and *m/z* obtained by measuring the standard solutions. Searching for these nine BAs, other isomers with the same *m/z* were observed. Their identification was provided based on the information from previous studies. For the certain identification of other BAs found in biological samples, all BA standards are needed.

For the identification of other compounds found in human hair, data in PCDL Library (UEF) or METLIN Metabolomics Database were used.

5.2 Measurement of Standards of Bile Acids

BA standards were measured by LC-Q-TOF and LC-Q-Orbitrap. Achieved retention times and mass spectra information were used for identification of BAs found in biological material (mouse liver, human plasma and human hair).

5.2.1 Retention Behavior of Standards by LC-Q-TOF

The retention of free BAs and their conjugates studied in this thesis on the RP C18 column was determined as a function of their hydrophobicity/hydrophilicity balance. The retaining depended on the interaction of BAs with the hydrophobic particle surface of the stationary phase. Therefore, CA as a tri-hydroxy BA was eluted earlier than di-hydroxy BAs because it tended to be carried by the polar mobile phase more than retained on the hydrophobic stationary phase.

However, for resulting retention time, the position and stereochemistry of hydroxyl groups were also important. Hydroxyl groups with the α-orientation are located below the steroid nucleus, whereas β-OH groups are oriented above the steroid nucleus (Ridgway and McLeod 2015). Thus, UDCA (di-hydroxy BA) was eluted earlier than CA (tri-hydroxy BA) due to its 7β-OH group which is located above the hydrophobic surface. This conformation led to weakening of the bond to the hydrophobic stationary phase. The elution of di-hydroxy BAs was in following order: UDCA, CDCA and DCA.

LCA was retained on the C18 column longer than di-hydroxy and tri-hydroxy BAs because of the presence of only one hydroxyl group, therefore increased preference for stationary phase.

The separation of free BAs and their corresponding conjugates was observed as follows: the glycine-conjugated BAs were the first to be eluted, followed by taurine-conjugates and unconjugated forms (for CA-derived molecules: GCA < TCA < CA).

Table 9. *The retention times of BA standards using C18 column, performed by LC-Q-TOF.*

	Bile acid	Formula	RT (min)
1	CA	$C_{24}H_{40}O_5$	8.981
2	DCA	$C_{24}H_{40}O_4$	9.601
3	CDCA	$C_{24}H_{40}O_4$	9.536
4	UDCA	$C_{24}H_{40}O_4$	8.682
5	LCA	$C_{24}H_{40}O_3$	10.029
6	TCA	$C_{26}H_{45}NO_7S$	8.576
7	TDCA	$C_{26}H_{45}NO_6S$	9.594
8	GCA	$C_{26}H_{43}NO_6$	8.455
9	GCDCA	$C_{26}H_{43}NO_5$	8.953

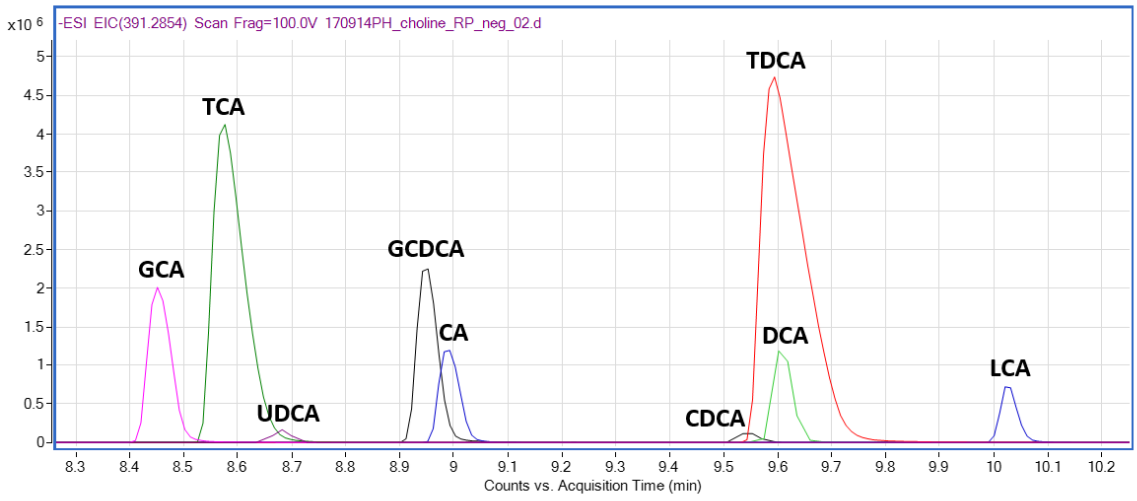


Figure 12*. Extracted Ion Chromatogram (EIC) of nine BA standards determined by Agilent MassHunter Qualitative analysis B.07.*

5.2.2 MS Data and Fragmentation Pathways of Standards by Q-TOF

The MS data of BAs were obtained using ESI in negative mode. In mass spectra of all BA standards, the predominant $[M-H]^-$ ions were achieved. In spectra of CA, CDCA, UDCA and LCA, the abundant $[M-H+HCOOH]^-$ ions were observed, formed by adduction with formic acid. However, DCA did not provide this adduct. This knowledge could be used for differentiation of these three isomers studied in this thesis (DCA, CDCA and UDCA). For these isomers and CA, an adduct with two molecules of water was typical. All free BAs and as well their corresponding glycine- and taurine-conjugates exhibited abundant $[2M-H]^-$ ions.

Further information was obtained via CID where MS/MS analysis was employed to achieve more specific fragment ions for each analyte. The free BAs mainly underwent an elimination of H_2O, CO_2, HCOOH.

The CA preffered neutral loss of formic acid and water molecule giving an ion at *m/z* 343.2624. Whereas, a *m/z* value of 345.2779 was achieved by loss of carbon dioxide and water molecule. The ion at *m/z* 289.2161 was formed by elimination of side chain of steroid nucleus (101 Da) and hydroxyl group (17 Da).

Using collision energy of 40V, three isomeric BAs showed different fragment ions resulting in their easier differentiation. The $[M-H]^-$ ion of CDCA inclined to produce $[M-H-H_2O]^-$ ion at *m/z* 373.2666. The $[M-H]^-$ of DCA preffered neutral loss of HCOOH (*m/z* 345.2793), whereas the UDCA exhibited no further fragmentation. In a similar way as UDCA, LCA was also resistant to fragmentation.

$[M-H]^-$ ions of taurine-conjugates, in our study TCA and TDCA, produced ions corresponding to taurine fragment moiety at *m/z* 79.9574, 106.9808, 124.0074 (*m/z* calculated by MassHunter).

A *m/z* value of 74.0248 (loss of glycine, *m/z* calculated by MassHunter) was universal for glycine conjugates, specifically for studied GCA and GCDCA. GCA underwent an elimination of formic acid and H_2O molecule giving an ion at *m/z* 400.2850 and an elimination of CO_2 and H_2O molecules to form ion at *m/z* 402.3014. The same elimination of CO_2 and H_2O molecules was observed for GCDCA at *m/z* 386.3049. In addition, in MS/MS spectrum of GCDCA, the ion at *m/z* 330.2435 was attributed to loss of side chain of steroid nucleus and hydroxyl group.

Table 10. *Fragmentation pathways of nine BA standards performed by Q-TOF.*

Bile acid	*Calc *m/z* $[M-H]^-$	MS *meas *m/z*	Formed ions	MS/MS of $[M-H]^-$ CID 40 V *meas *m/z*	Formed ions
CA	407.2803	407.2793	$[M-H]^-$	289.2161	$[M-H-101-OH]^-$
		443.2560	$[M-H+2H_2O]^-$	343.262	$[M-H-HCOOH-H_2O]^-$
		453.2844	$[M-H+HCOOH]^-$	345.2779	$[M-H-CO_2-H_2O]^-$
		815.5677	$[2M-H]^-$	407.2796	$[M-H]^-$
DCA	391.2854	391.2840	$[M-H]^-$	345.2793	$[M-H-HCOOH]^-$
		427.2606	$[M-H+2H_2O]^-$	391.2838	$[M-H]^-$
		783.5759	$[2M-H]^-$		
CDCA	391.2854	391.2848	$[M-H]^-$	373.2666	$[M-H-H_2O]^-$
		427.2610	$[M-H+2H_2O]^-$	391.2842	$[M-H]^-$
		437.2900	$[M-H+HCOOH]^-$		
		783.5777	$[2M-H]^-$		
UDCA	391.2854	391.2849	$[M-H]^-$	391.2845	$[M-H]^-$
		427.2609	$[M-H+2H_2O]^-$		
		437.2898	$[M-H+HCOOH]^-$		
		783.5771	$[2M-H]^-$		
LCA	375.2905	375.2898	$[M-H]^-$	375.2890	$[M-H]^-$
		421.2953	$[M-H+HCOOH]^-$		
		751.5881	$[2M-H]^-$		
TCA	514.2844	514.2835	$[M-H]^-$	79.9570	$[SO_3]^-$
		1029.5764	$[2M-H]^-$	106.9806	$[CH_2CHSO_3]^-$
				124.0069	$[H_2NC_2H_4SO_3]^-$
				514.2834	$[M-H]^-$
TDCA	498.2895	498.2893	$[M-H]^-$	79.9571	$[SO_3]^-$
		997.5882	$[2M-H]^-$	106.9801	$[CH_2CHSO_3]^-$
				124.0069	$[H_2NC_2H_4SO_3]^-$
				498.2885	$[M-H]^-$
GCA	464.3018	464.3011	$[M-H]^-$	74.0246	$[H_2NCH_2COO]^-$
		929.6115	$[2M-H]^-$	400.2850	$[M-H-HCOOH-H_2O]^-$
				402.3014	$[M-H-CO_2-H_2O]^-$
				464.3011	$[M-H]^-$
GCDCA	448.3068	448.3069	$[M-H]^-$	74.0245	$[H_2NCH_2COO]^-$
		897.6228	$[2M-H]^-$	330.2435	$[M-H-101-OH]^-$
				386.3049	$[M-H-CO_2-H_2O]^-$
				448.3058	$[M-H]^-$

*calc – calculated *m/z* $[M-H]^-$ in MassHunter, meas – measured *m/z*

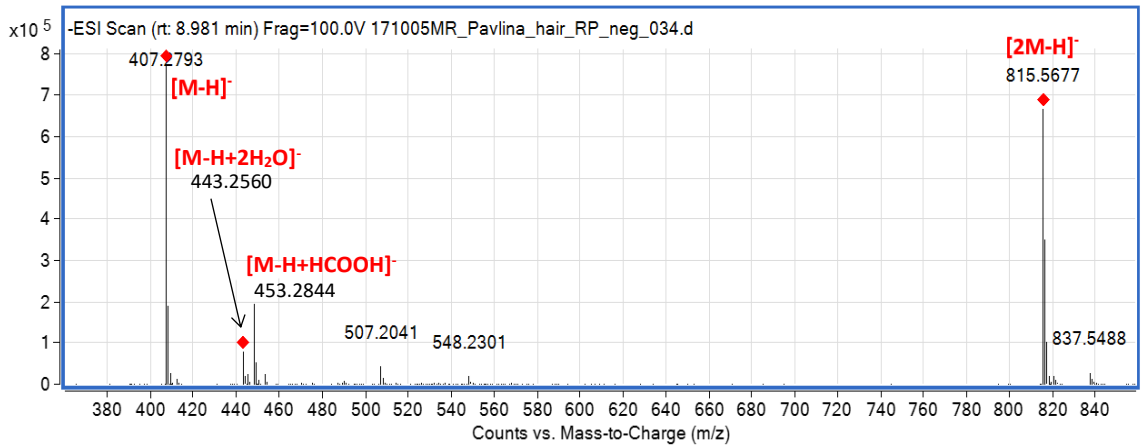


***Figure 13**. Mass spectrum of CA.*

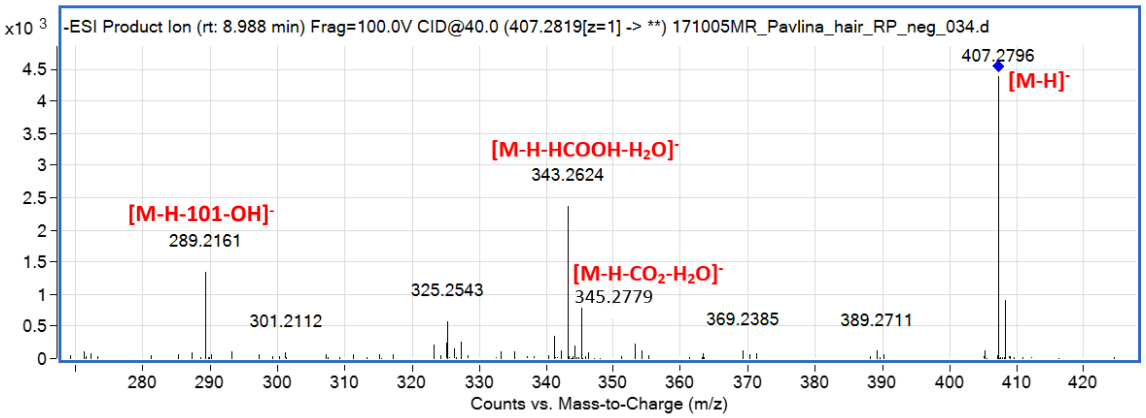


***Figure 14.** Fragmentation pathways of [M-H]- of CA.*

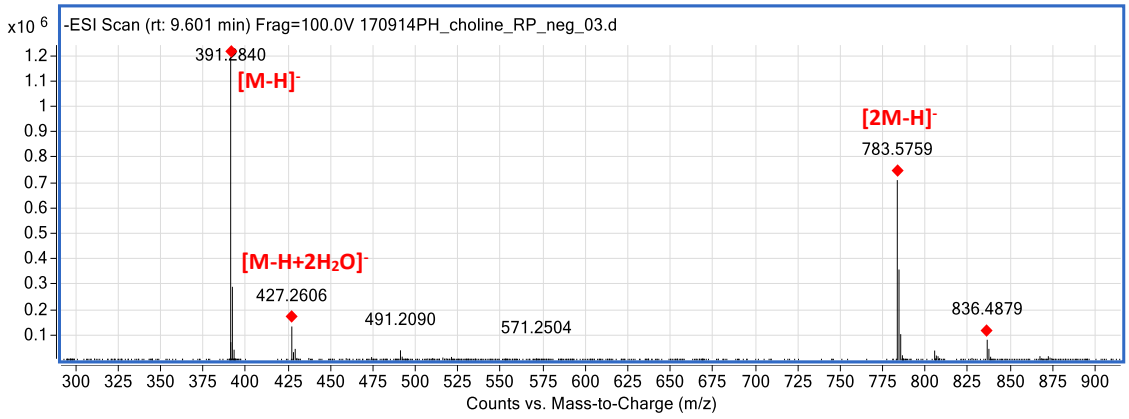


***Figure 15.** Mass spectrum of DCA.*

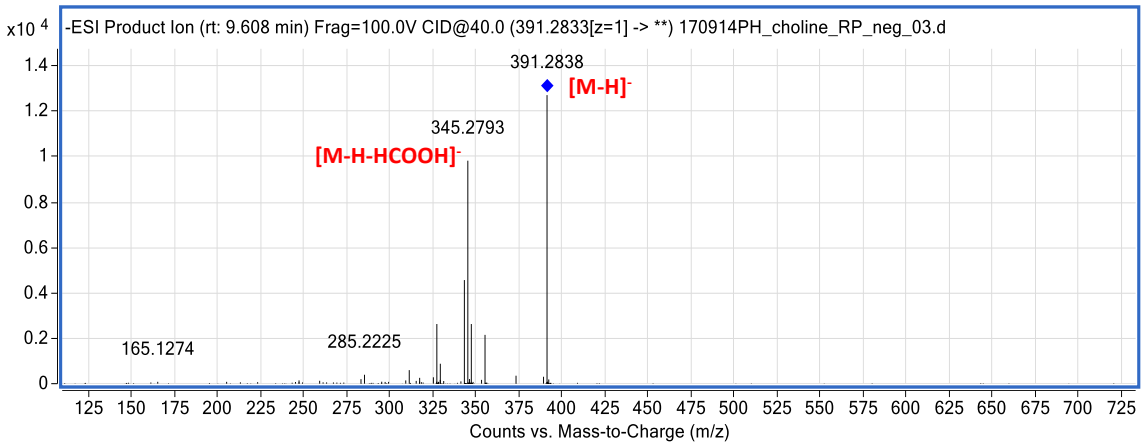


Figure 16. *Fragmentation pathways of $[M-H]^-$ of DCA.*

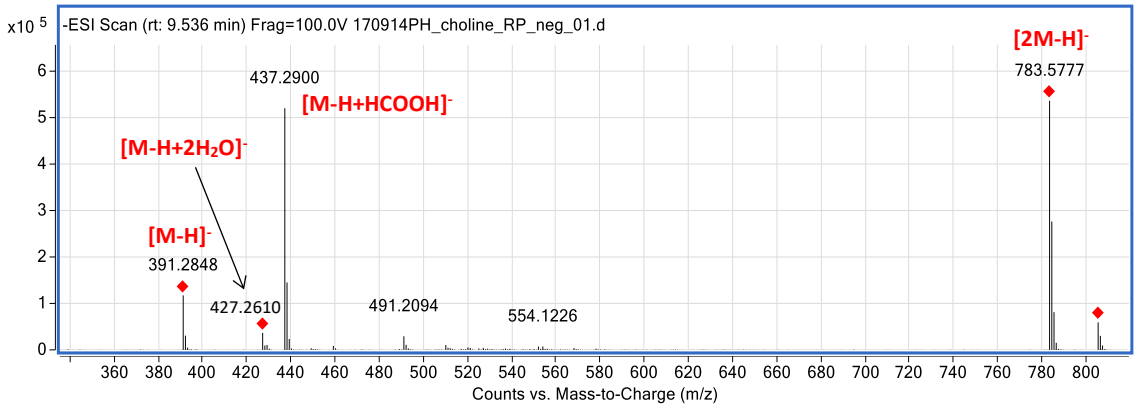


Figure 17. *Mass spectrum of CDCA.*

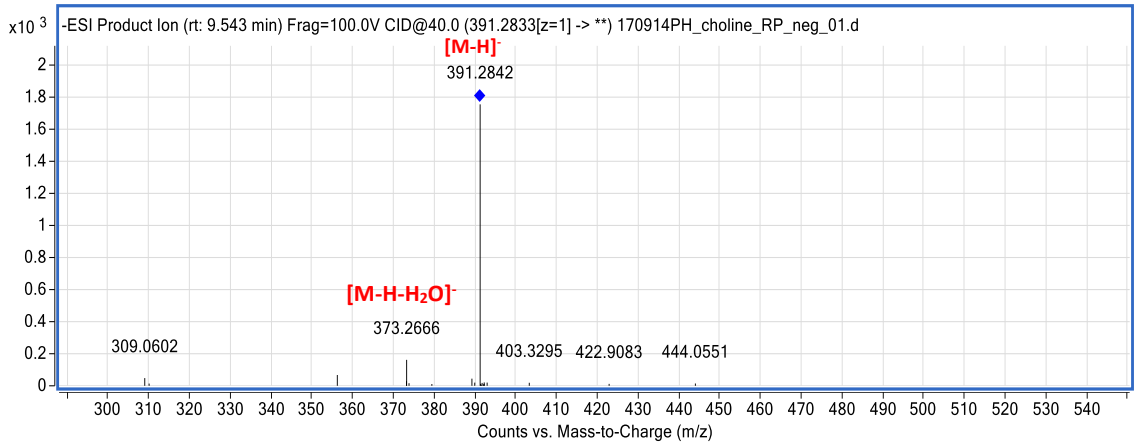


Figure 18*. Fragmentation pathways of $[M-H]^-$ of CDCA.*

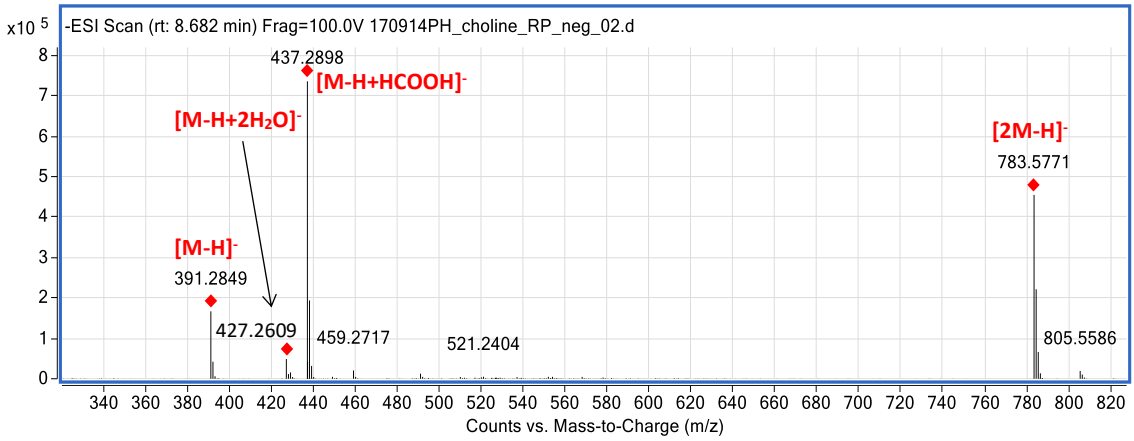


Figure 19. *Mass spectrum of UDCA.*

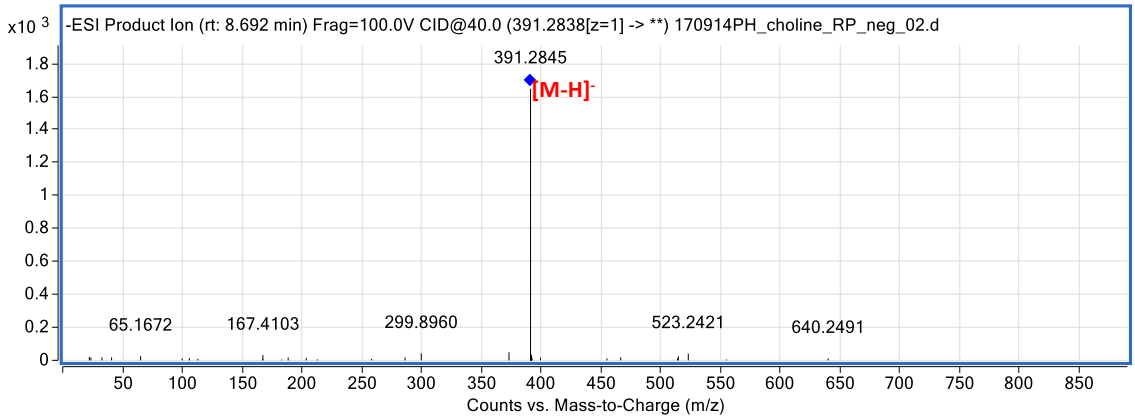


Figure 20. *Fragmentation pathways of [M-H]- of UDCA.*

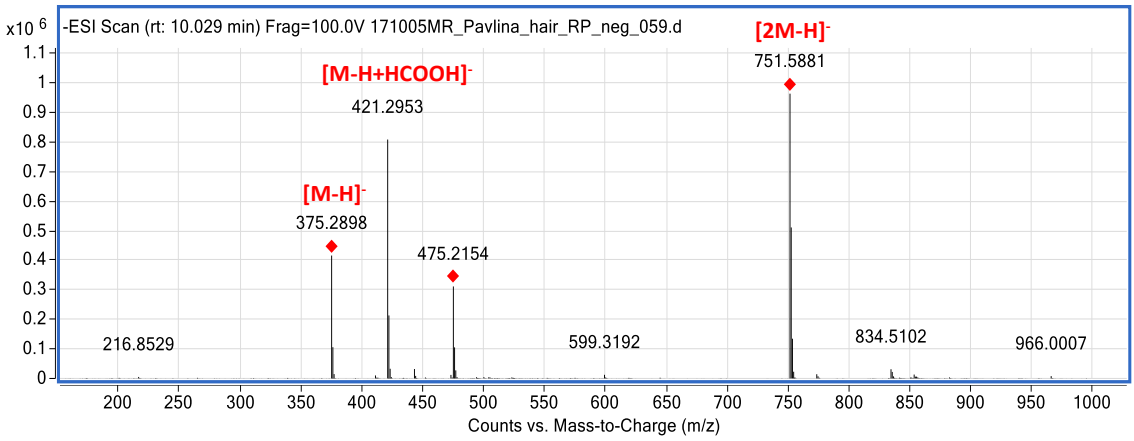


Figure 21. *Mass spectrum of LCA.*

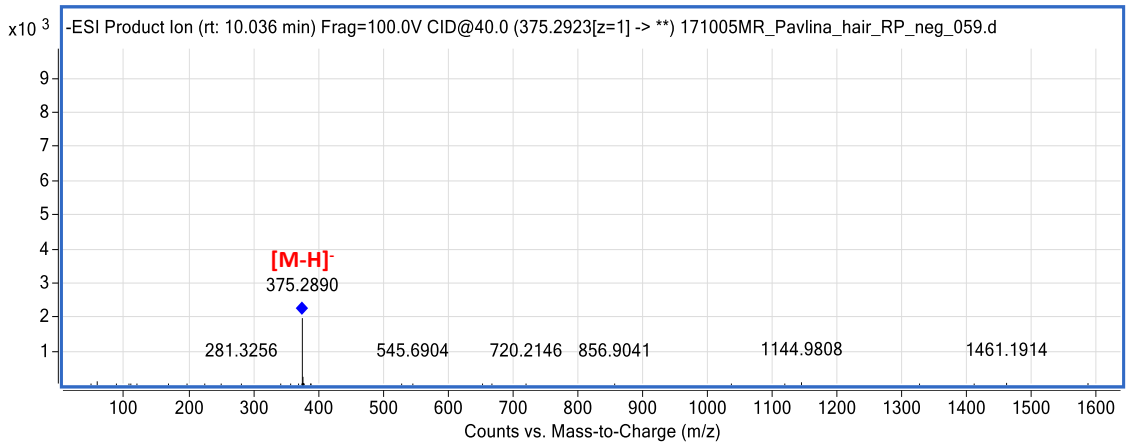


Figure 22. *Fragmentation pathways of [M-H]- of LCA.*

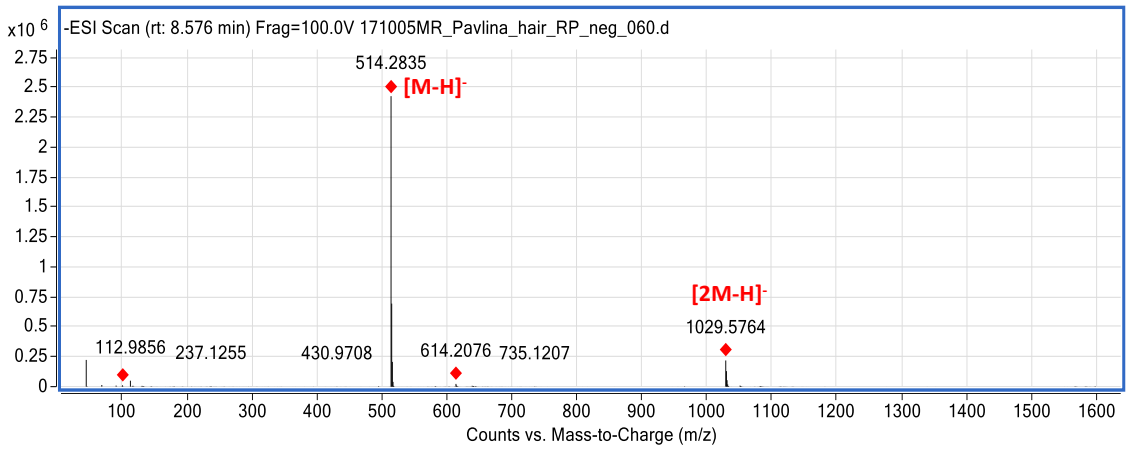


Figure 23. *Mass spectrum of TCA.*

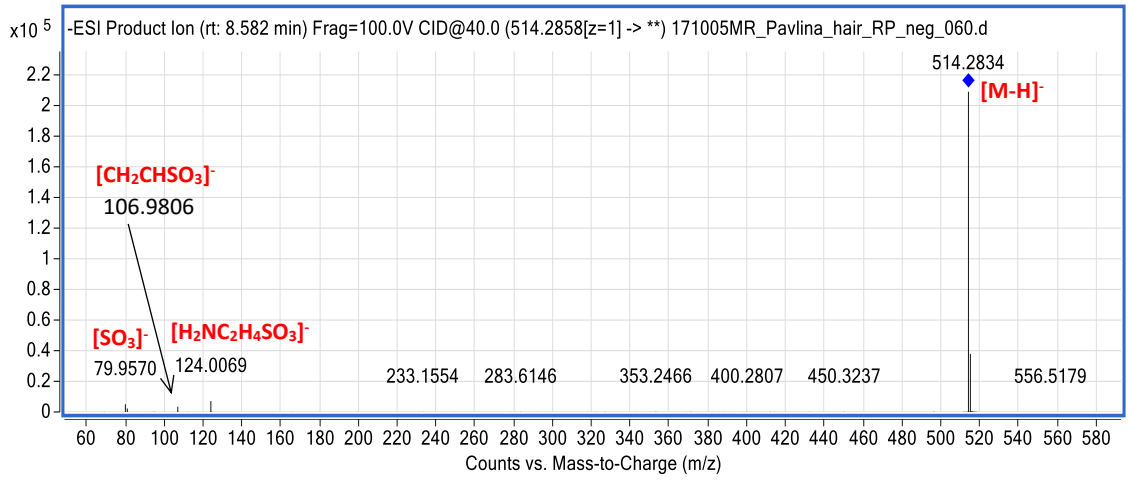


Figure 24. *Fragmentation pathways of [M-H]- of TCA.*

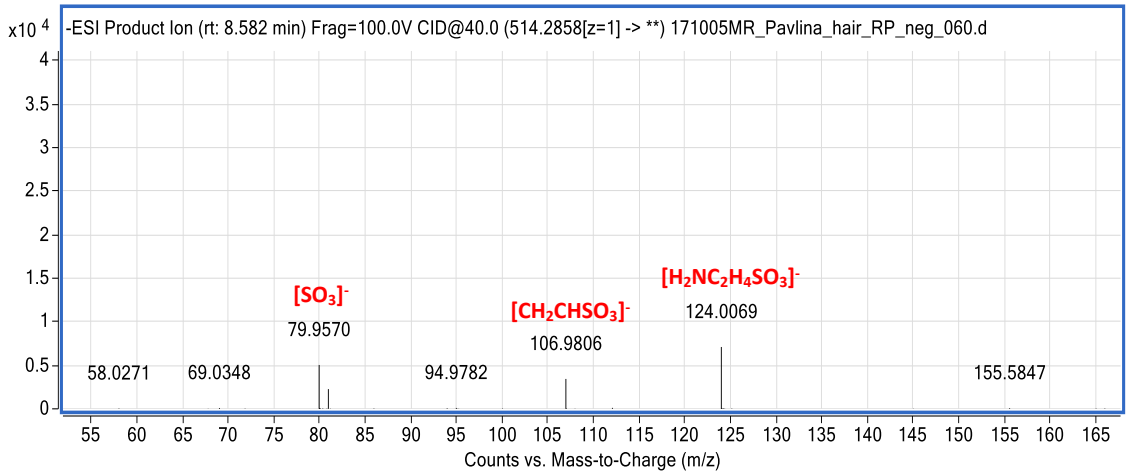


Figure 25. *Fragmentation of taurine moiety.*

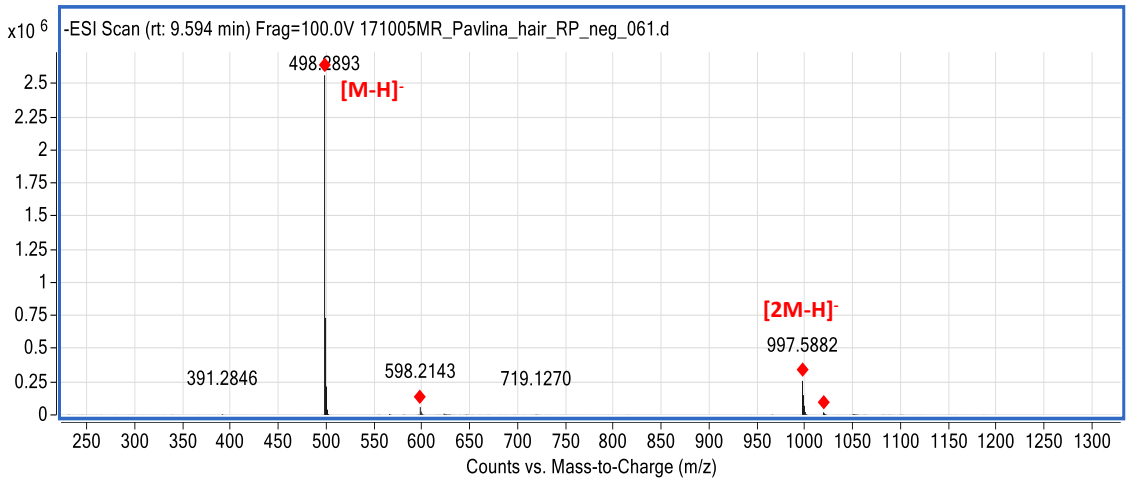


Figure 26. *Mass spectrum of TDCA.*

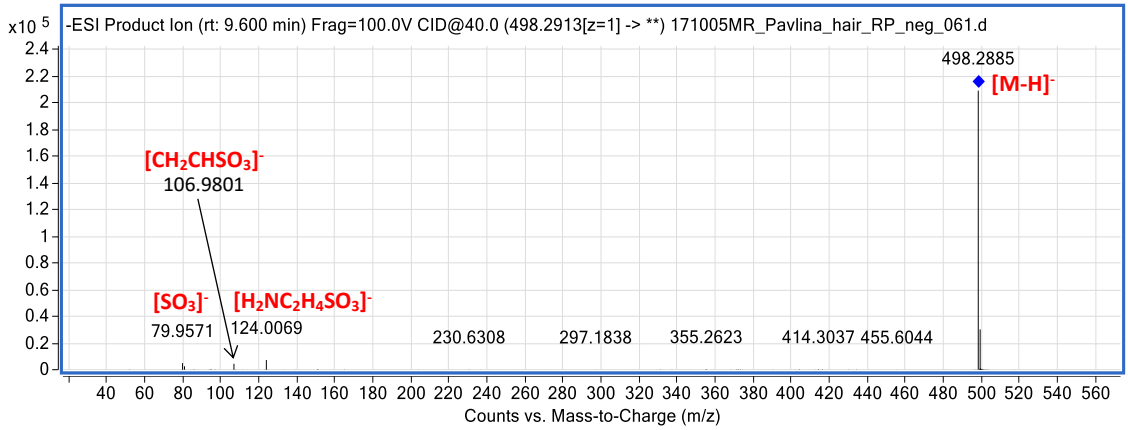


Figure 27. *Fragmentation pathways of [M-H]- of TDCA.*

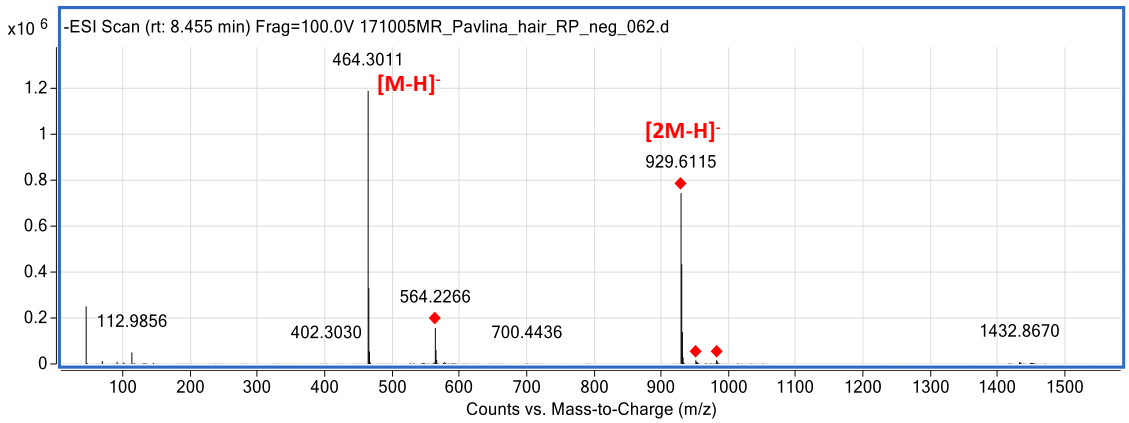


Figure 28. *Mass spectrum of GCA.*

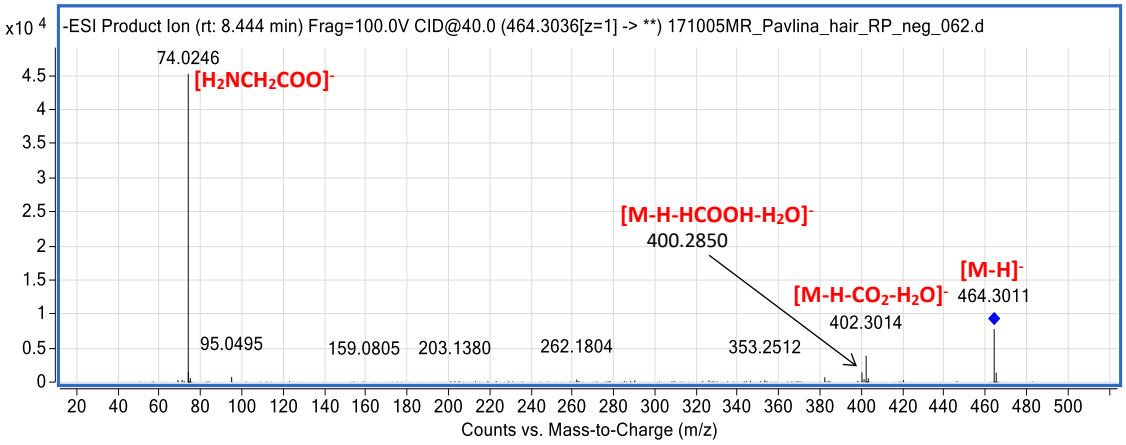


Figure 29. *Fragmentation pathways of [M-H]- of GCA.*

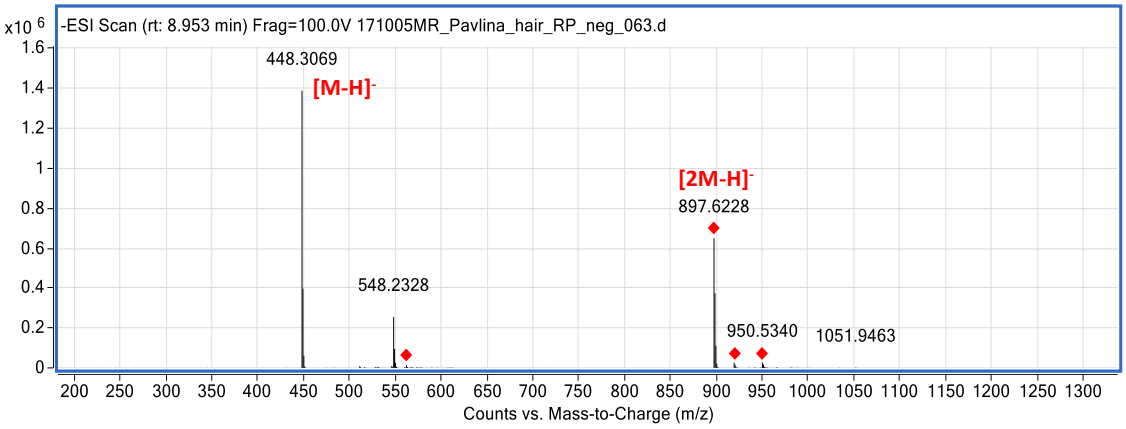


Figure 30. *Mass spectrum of GCDCA.*

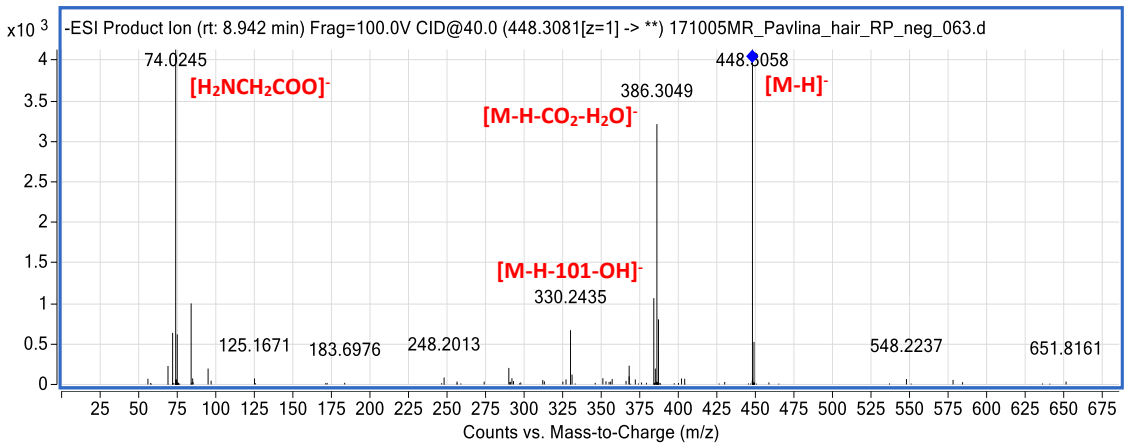


Figure 31. *Fragmentation pathways of [M-H]- of GCDCA.*

5.2.3 Analysis performed by LC-Q-Orbitrap

BA standards were measured by UHPLC connected to Q-Orbitrap as well. Retention times and *m/z* values were used for identification of BAs in biological samples. The elution order of BA standards using LC-Q-TOF and LC-Q-Orbitrap was similar. The only difference was observed for CDCA and TDCA showing that TDCA was eluted earlier than CDCA.

The adducts (specifically the dimers) were not observed in mass spectra of BA standards because the mass range was set at 100-600 *m/z*. Compared to data achieved by Q-TOF, a difference was observed in spectrum of DCA. The adduct with formic acid was also formed.

Table 11. *Retention times of BA standards and ISs using C18 column.*

	Bile acid	Formula	RT (min)
1	CA	$C_{24}H_{40}O_5$	9.26
2	DCA	$C_{24}H_{40}O_4$	9.87
3	CDCA	$C_{24}H_{40}O_4$	9.80
4	UDCA	$C_{24}H_{40}O_4$	8.91
5	LCA	$C_{24}H_{40}O_3$	10.35
6	TCA	$C_{26}H_{45}NO_7S$	8.74
7	TDCA	$C_{26}H_{45}NO_6S$	9.68
8	GCA	$C_{26}H_{43}NO_6$	8.72
9	GCDCA	$C_{26}H_{43}NO_5$	9.20
10	CA-d5	$C_{24}H_{35}D_5O_5$	9.25
11	DCA-d5	$C_{24}H_{35}D_5O_4$	9.87

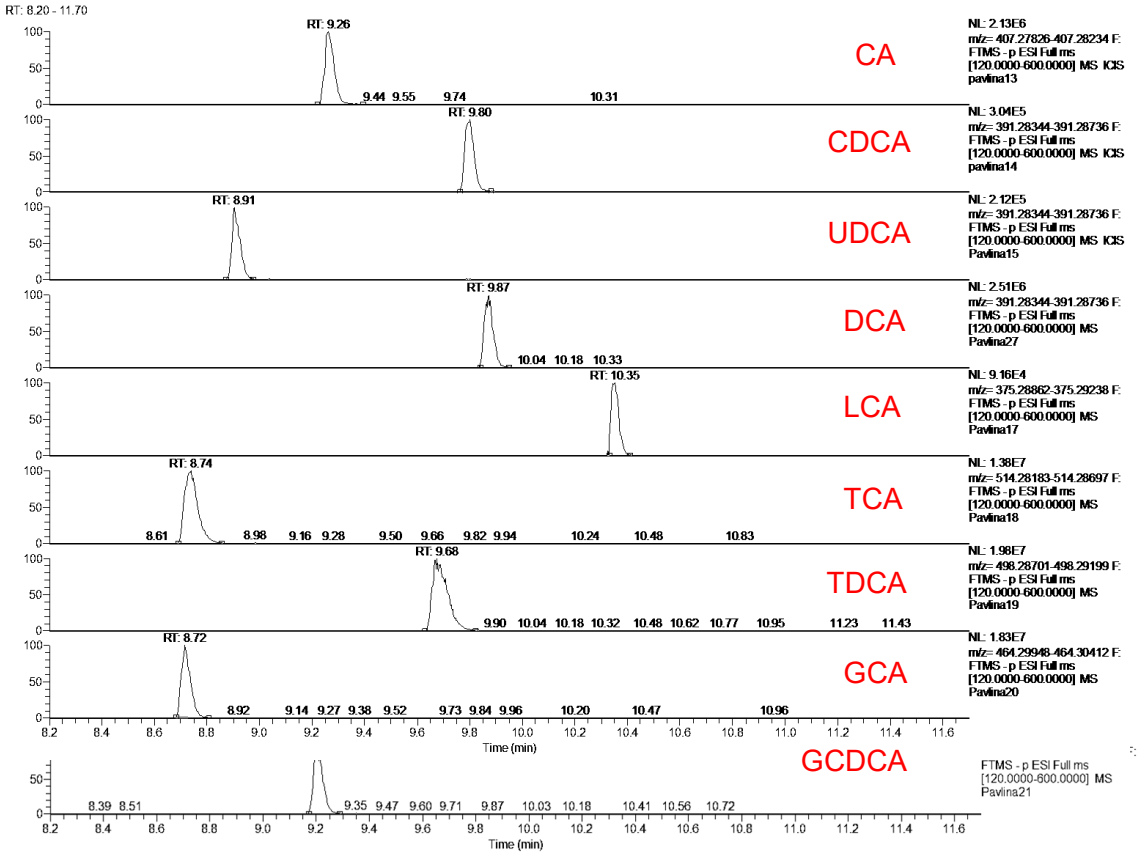


***Figure 32**. Chromatograms of BA standards determined by Xcalibur Software (Thermo Scientifics).*

Table 12. *MS information about BA standards, data obtained by Q-Orbitrap.*

	Bile acid	*Calc *m/z* [M-H]$^-$	MS *meas *m/z*	Formed ions	Delta ppm
1	CA	407.2803	407.2794	[M-H]$^-$	0.538
			443.2568	[M-H+2H$_2$O]$^-$	-
			453.2853	[M-H+HCOOH]$^-$	-
2	DCA	391.2854	391.2847	[M-H]$^-$	0.981
			427.2617	[M-H+2H$_2$O]$^-$	-
			437.2902	[M-H+HCOOH]$^-$	-
3	CDCA	391.2854	391.2841	[M-H]$^-$	-0.578
			427.2618	[M-H+2H$_2$O]$^-$	-
			437.2905	[M-H+HCOOH]$^-$	-
4	UDCA	391.2854	391.2840	[M-H]$^-$	-0.808
			427.2621	[M-H+2H$_2$O]$^-$	-
			437.2905	[M-H+HCOOH]$^-$	-
5	LCA	375.2905	375.2892	[M-H]$^-$	-0.404
			421.2956	[M-H+HCOOH]$^-$	-
6	TCA	514.2844	514.2836	[M-H]$^-$	0.642
7	TDCA	498.2895	498.2888	[M-H]$^-$	0.873
8	GCA	464.3018	464.3008	[M-H]$^-$	0.249
9	GCDCA	448.3068	448.3065	[M-H]$^-$	1.651
10	CA-d5	412.3117	412.3108	[M-H]$^-$	0.498
			448.2881	[M-H+2H$_2$O]$^-$	-
			458.3167	[M-H+HCOOH]$^-$	-
11	DCA-d5	396.3168	396.3158	[M-H]$^-$	0.404
			432.2930	[M-H+2H$_2$O]$^-$	-
			442.3216	[M-H+HCOOH]$^-$	-

*calc – calculated *m/z* [M-H]$^-$ in MassHunter, meas – measured *m/z*

5.3 Analysis of Mouse Liver

The analysis of mouse liver was performed using both instruments, UHPLC connected to Q-TOF and UHPLC connected to Q-Orbitrap. The samples were measured in negative ionization mode using RP chromatography. The aim was to test and optimize the method for BA analysis.

Two ways of homogenization were applied during the preparation of liver samples to find the better process. These two homogenates were measured by LC-Q-TOF.

Searching for nine BA standards, other isomers with the same *m/z* were noticed. Their identification was provided based on the information from previous studies (Alnouti et al. 2008). These found isomers are also mentioned. For the accurate identification, all BA standards would be needed.

5.3.1 Difference in Homogenization Process

In chromatograms of mouse liver samples, the relative abundance of BAs was influenced by the method used for the liver homogenization (Figure 33). Therefore, BA analysis was processed analyzing the data of samples prepared by homogenzation in liquid nitrogen.

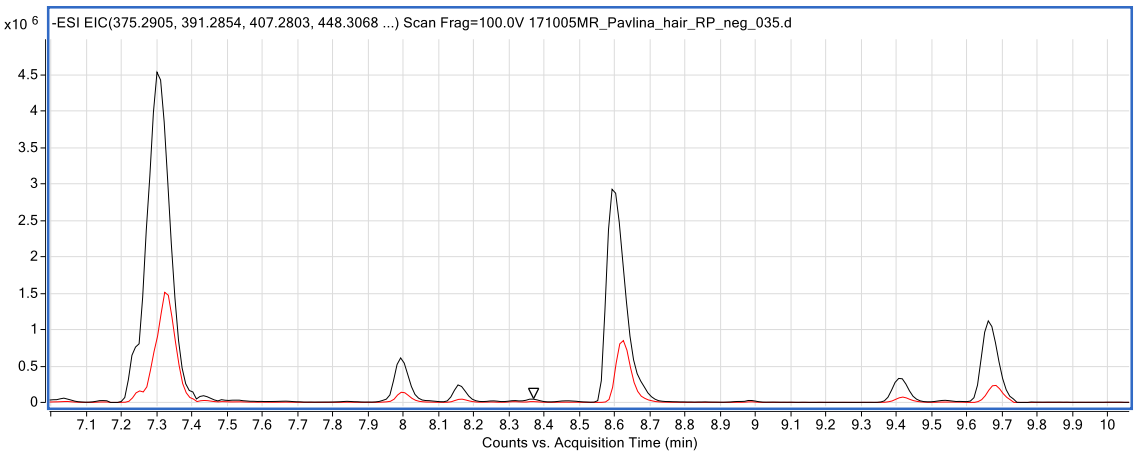


Figure 33. *Combined chromatogram of mouse liver samples.*
A difference in height of signals between homogenization in liquid nitrogen (black) and mechanical homogenization in deionized water (red).

5.3.2 Analysis of Mouse Liver performed by LC-Q-TOF

We could verify nine BAs based on the knowledge of retention times and fragmentation pathways of BA standards that we used.

As Alnouti et al. (2008) have reported, free BAs, especially CDCA, UDCA, DCA, are presented in low concentration in mouse liver. In mass spectrum of DCA, we were able to observe an $[M-H]^-$ ion and an adduct with two molecules of water, whereas in spectra of CDCA and UDCA, only the adducts with formic acid were observed. We could not achieve the fragmentation of ions because of the low signal of peaks. Even without fragmentation, we could identify these isomers based on the comparison to the retention times and *m/z* values of BA standards.

In mass spectrum of LCA, the same ions as in the spectrum of LCA standard were obtained, $[M-H]^-$ ion and an adduct with formic acid. However, $[M-H]^-$ ion exhibited no further fragmentation.

The predominant ion $[M-H]^-$ of CA could yield the same fragments as CA standard, the $[M-H-HCOOH-H_2O]^-$, $[M-H-101-OH]^-$ and $[M-H-CO_2-H_2O]^-$ ions. The retention time was almost identical compared to the retention time of standard.

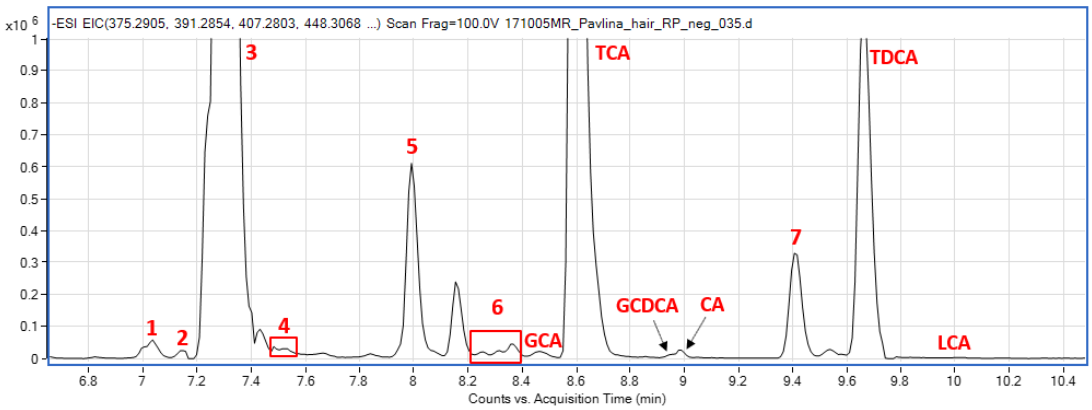
Taurine-conjugates were the most common BAs presented in the mouse liver. In mass spectra, taurine-conjugates exhibited abundant $[M-H]^-$ ions together with minor $[2M-H]^-$ ions. In the MS/MS spectra acquired by CID of 40V, the $[M-H]^-$ ions inclined to produce the taurine fragments.

The major $[M-H]^-$ ions were also observed in spectra of glycine-conjugates. Glycine moiety was noticed after the fragmentation of $[M-H]^-$ ions. In MS/MS spectra of studied glycine-conjugates, also other fragment ions were achieved. It was helpful for their confirmation based on the knowledge of the MS/MS and the retention times of standards.

Table 13. *Nine BAs found and verified in mouse liver sample based on their retention times and m/z values compared to the information achieved by measuring BA standards.*

Bile acid	**RT (min)**	**MS *meas *m/z***	**Formed ions**	**MS/MS CID 40V *meas *m/z***	**Formed ions**
CA	8.989	407.2797	$[M-H]^-$	289.2192	$[M-H-101-OH]^-$
				343.2651	$[M-H-HCOOH-H_2O]^-$
				345.2762	$[M-H-CO_2-H_2O]^-$
				407.2768	$[M-H]^-$
DCA	9.579	391.2862	$[M-H]^-$	-	-
		427.2342	$[M-H+2H_2O]^-$	-	-
CDCA	9.518	437.2893	$[M-H+HCOOH]^-$	-	-
UDCA	8.653	437.2916	$[M-H+HCOOH]^-$	-	-
LCA	10.016	421.2947	$[M-H+HCOOH]^-$	-	-
		low signal of 375	$[M-H]^-$	-	-
TCA	8.592	514.2830	$[M-H]^-$	79.9569	$[SO_3]^-$
				106.9807	$[CH_2CHSO_3]^-$
				124.0071	$[H_2NC_2H_4SO_3]^-$
				514.2828	$[M-H]^-$
		1029.5740	$[2M-H]^-$	514.2827	$[M-H]^-$
				1029.5766	$[2M-H]^-$
TDCA	9.660	498.2890	$[M-H]^-$	79.9569	$[SO_3]^-$
				106.9801	$[CH_2CHSO_3]^-$
				124.0065	$[H_2NC_2H_4SO_3]^-$
				498.2889	$[M-H]^-$
		997.5857	$[2M-H]^-$	498.2876	$[M-H]^-$
GCA	8.450	464.3012	$[M-H]^-$	74.0238	$[H_2NCH_2COO]^-$
				400.2849	$[M-H-HCOOH-H_2O]^-$
				402.2954	$[M-H-CO_2-H_2O]^-$
				464.3013	$[M-H]^-$
GCDCA	8.948	448.3040	$[M-H]^-$	74.0239	$[H_2NCH_2COO]^-$
				386.3041	$[M-H-CO_2-H_2O]^-$
				448.3046	$[M-H]^-$

*meas – measured *m/z*



1, 2, 3 – TMCA isomers; 4 – GMCA isomers; 5 - TUDCA, 6 – MCA isomers; 7 – TCDCA

Figure 34. *EIC of mouse liver homogenate prepared in liquid nitrogen.*
BAs studied in this thesis are marked with acronyms. Not all BAs are visible in this figure, see the detailed chromatogram below (Figure 35). Other significant peaks marked with numbers belong to the other BA derivates presented in mouse liver.

Due to the lack of other BA standards, we could not confirm all BA derivates found in mouse liver sample. Standards of all these isomers are needed to differentiate them based on their unique retention time. Three isomers with the same *m/z* values, taurine-conjugates of DCA, CDCA and UDCA, produced the identical fragment ions of the $[M-H]^-$ precursor ion, i.e. fragments of taurine moiety. Therefore, it was not possible to differentiate them just based on their mass spectra. TUDCA and TCDCA were identified based on the knowledge of their retention behavior from previous study (Alnouti el al. 2008).

In contrary to humans, muricholic acid (MCA) is also primary BA in rodents, presented as α, β and ω isomers (Russell 2003). Four peaks with the *m/z* 407 were obtained in the spectra of mouse liver (Figure 35). One of these peaks belonged to CA based on the similar retention time as standard. The other three peaks could belong to the isomers of MCA (α, β, ω). Because of the absence of MCA standards, we could not certainly verify these derivates. The same difficulty occured for its corresponding taurine- and glycine-conjugates.

Table 14. *Predicted BA derivates found in mouse liver.*

Bile acid	RT (min)	MS *meas *m/z*	Formed ions	MS/MS CID 40V *meas *m/z*	Formed ions
MCA isomers	8.256	407.2795	$[M-H]^-$	407.2783	$[M-H]^-$
		453.2846	$[M-H+HCOOH]^-$	407.2789	$[M-H]^-$
	8.317	407.2785	$[M-H]^-$	-	-
		453.2842	$[M-H+HCOOH]^-$	-	-
	8.368	407.2795	$[M-H]^-$	-	-
		453.2845	$[M-H+HCOOH]^-$	-	-
TMCA isomers	7.035	514.2833	$[M-H]^-$	-	-
	7.147	514.2830	$[M-H]^-$	-	-
	7.300	514.2845	$[M-H]^-$	-	-
		1029.5784	$[2M-H]^-$	-	-
GMCA isomers	7.432	464.2997	$[M-H]^-$	-	-
	7.483	464.3000	$[M-H]^-$	74.0230	$[H_2NCH_2COO]^-$
TUDCA	7.992	498.2885	$[M-H]^-$	79.9570	$[SO_3]^-$
				106.9807	$[CH_2CHSO_3]^-$
				124.0056	$[H_2NC_2H_4SO_3]^-$
				498.2873	$[M-H]^-$
TCDCA	9.406	498.2893	$[M-H]^-$	79.9581	$[SO_3]^-$
				106.9791	$[CH_2CHSO_3]^-$
				124.0055	$[H_2NC_2H_4SO_3]^-$
				498.2883	$[M-H]^-$

*meas – measured *m/z*

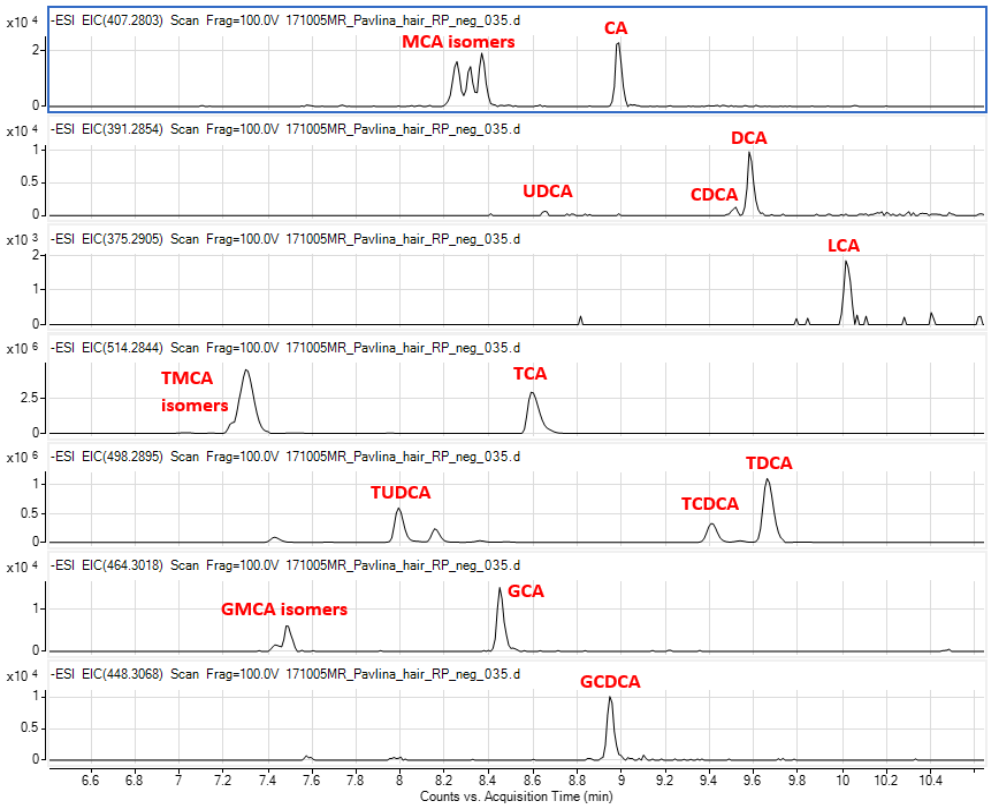


Figure 35. *The chromatograms of BAs found in mouse liver.*

5.3.3 Analysis of Mouse Liver performed by LC-Q-Orbitrap

Mouse liver sample was analyzed also by LC-Q-Orbitrap on C18 column, in negative ionization mode. CID was not used, no fragmentation data was obtained. BA standards were measured with the same method and the obtained retention times and *m/z* values were used for identification of BAs presented in mouse liver.

Only $[M-H]^-$ ions of BAs were noticed in mass spectra, no adducts. CDCA, LCA and GCDCA were not found in mouse liver sample.

Searching for TDCA, five peaks with the *m/z* 498 were observed. The retention time of TDCA standard was not identical to any peak. However, based on the retention behaviour of TDCA measured by LC-Q-TOF, the peak at 9.82 min presumably belonged to TDCA. The retention time was shifted by 0.14 min compared to the retention time of standard.

Table 15. *BAs and ISs found and verified in mouse liver based on their retention times and m/z compared to BA standards.*

Bile acid	RT (min)	*Meas *m/z* [M-H]$^-$	Area	Delta ppm
CA	9.25	407.2804	288 854	3.018
DCA	9.88	391.2858	21 318	3.869
CDCA	*ND	-	-	-
UDCA	8.90	391.2848	3 679	1.211
LCA	*ND	-	-	-
TCA	8.77	514.2835	246 249 935	0.409
TDCA	9.82	498.2890	13 707 072	1.174
GCA	8.71	464.3008	227 674	0.313
GCDCA	*ND	-	-	-
CA-d5	9.25	412.3111	10 026 594	1.177
DCA-d5	9.87	396.3164	8 495 146	1.716

*meas – measured *m/z*, ND – not detected

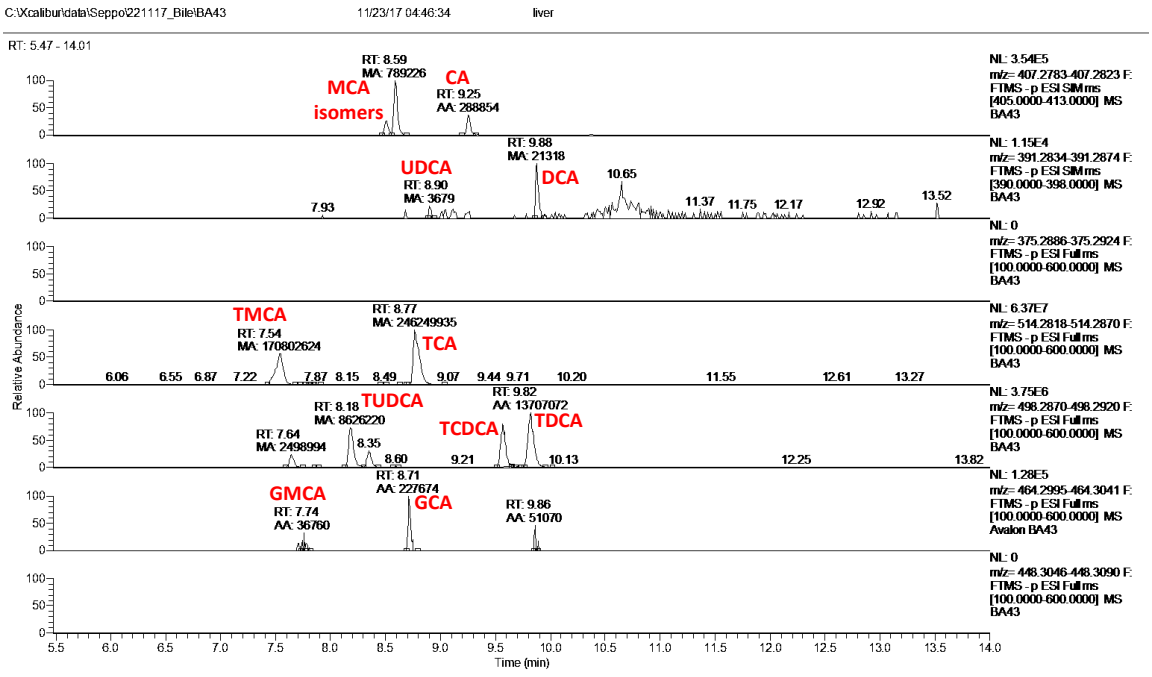


Figure 36. *EIC of BAs found in mouse liver.*

As already mentioned above, due to the lack of standard solutions for all BA derivates, some peaks were identified based on the previous studies (Alnouti et al. 2008) and the information obtained by LC-Q-TOF.

Table 16. *Predicted BA derivates found in mouse liver.*

Bile acid	**RT (min)**	***Meas *m/z* [M-H]⁻**	**Area**	**Delta ppm**
MCA	8.51	407.2800	223 617	1.889
isomers	8.59	407.2800	789 226	1.962
TMCA	7.54	514.2833	170 802 624	0.059
GMCA	7.74	464.3010	36 760	0.637
TUDCA	8.18	498.2888	8 626 220	0.873
TCDCA	9.56	498.2887	9 578 416	0.692

*meas – measured *m/z*

5.4 Analysis of Human Plasma

The analysis of human plasma was performed using both instruments, UHPLC connected to Q-TOF and UHPLC connected to Q-Orbitrap. The samples were measured in negative ionization mode using RP chromatography to verify the method in human plasma and identify BA profiles.

5.4.1 Analysis of Human Plasma performed by LC-Q-TOF

The retention behavior and mass data were compared to the information obtained from standard solutions and used for identification. The ions were produced by deprotonation as $[M-H]^-$ ions. The same situation occured as in mouse liver: the adducts with formic acid were observed in mass spectra of CDCA and UDCA, whereas in spectrum of DCA an adduct with two water molecules was determined. UDCA did not give $[M-H]^-$ ion at *m/z* 391 and no further fragmentation of the adduct ion, but we could still identify this BA based on its retention time and the formed adduct compared to the standard.

The MS/MS spectra were obtained using collision energy of 40V. The fragment ions formed from the $[M-H]^-$ ion of CA were not obtained because of the low signal. The identification of CA was supported by similar retention time of CA standard. DCA was verified based on the identical retention time and fragment ions compared to the standard. $[M-H]^-$ ion underwent an elimination of HCOOH to form an ion at *m/z* 345.2779. CDCA in plasma sample eluted at the same time as CDCA standard and fragmentation of the adduct ion constituted an $[M-H]^-$ ion at *m/z* 391.2838. This information was helpful for identification. $[M-H]^-$ ion did not yield fragmentation due to the low signal.

As Tagliacozzi et al. (2003) have reported, LCA is present in low concentration in human plasma. The concentration in our sample was below the limit of detection (LOD).

In plasma samples, taurine fragments were not formed from the $[M-H]^-$ ion of TCA and TDCA because of the low signal. The retention time of TDCA was shifted by -0.2 min, in contrast to TDCA standard. Based on the knowledge of the retention behavior of this conjugates and also unconjugated form, we could presumably determine this peak at the time 9.391 min as TDCA.

Glycine-conjugates, especially GCDCA, were identified as the most common BA form in human plasma. Glycine-conjugates studied in this thesis, GCA and GCDCA, were identified according to their retention times and fragmentation. These conjugates inclined to eliminate the glycine moiety and gave characteristic fragment ions at *m/z* 402.2999 (GCA) and *m/z* 386.3011 (GCDCA) formed by losses of water and carbon dioxide molecules.

Table 17. *BAs verified in human plasma based on their retention times and fragment ions compared to standards.*

Bile acid	**RT (min)**	**MS *meas *m/z***	**Formed ions**	**MS/MS CID 40 V *meas *m/z***	**Formed ions**
CA	9.026	407.2797	$[M-H]^-$	-	-
DCA	9.602	427.2541	$[M-H+2H_2O]^-$	-	-
		391.2840	$[M-H]^-$	345.2779	$[M-H-HCOOH]^-$
				391.2834	$[M-H]^-$
CDCA	9.542	437.2895	$[M-H+HCOOH]^-$	391.2838	$[M-H]^-$
		391.2835	$[M-H]^-$	-	-
UDCA	8.662	437.2890	$[M-H+HCOOH]^-$	-	-
LCA	*ND				
TCA	8.476	514.2837	$[M-H]^-$	514.2845	$[M-H]^-$
TDCA	9.391	498.2878	$[M-H]^-$	-	-
GCA	8.476	464.3004	$[M-H]^-$	74.0247	$[H_2NCH_2COO]^-$
				402.2999	$[M-H-CO_2-H_2O]^-$
				464.2979	$[M-H]^-$
GCDCA	8.970	448.3062	$[M-H]^-$	74.0244	$[H_2NCH_2COO]^-$
				386.3011	$[M-H-CO_2-H_2O]^-$
				448.3074	$[M-H]^-$

*meas – measured *m/z*, ND – not detected

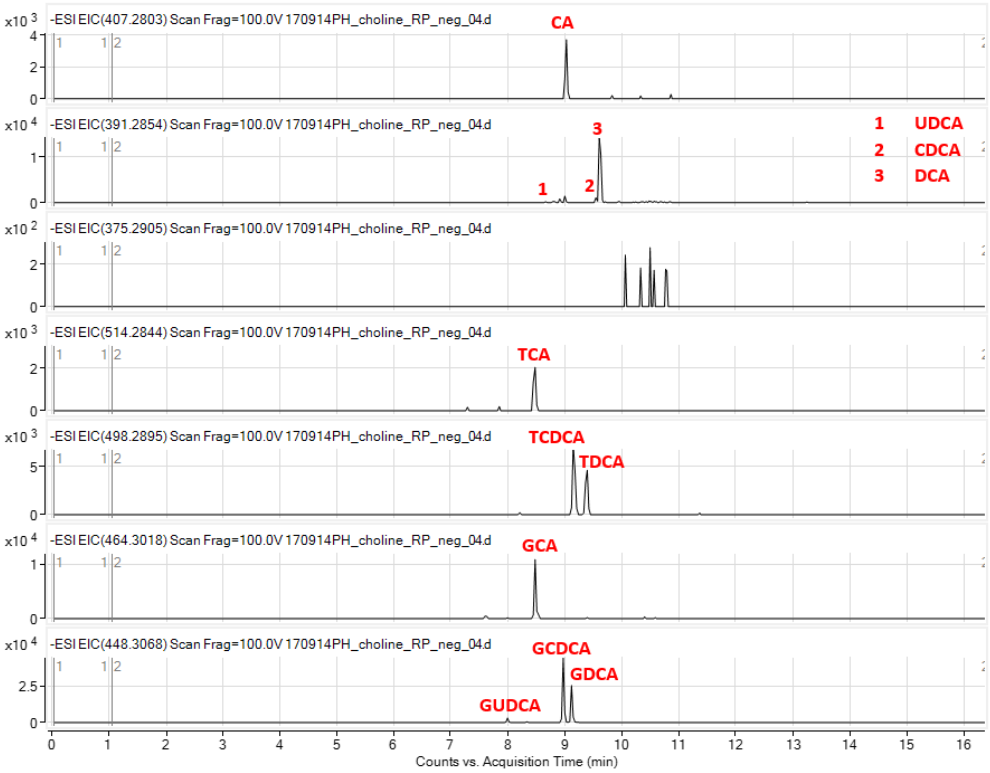


Figure 37. *The chromatograms of BAs found in human plasma.*

Three other conjugates were detected in the chromatogram searching for BAs with appropriate standards, i.e. GDCA, GUDCA and TCDCA. Due to the absence of standards we could not absolutely verify them. The identification was performed based on the accurate mass and typical fragments found for glycine and taurine molecules. Retention time of TCDCA was compared to the retention time in PCDL library (UEF), which was 9.2 min.

Table 18. *Predicted BAs found in human plasma.*

Bile acid	**RT (min)**	**MS *meas *m/z* [M-H]⁻**	**MS/MS CID 40V *meas *m/z***	**Formed ions**
GDCA	9.116	448.3055	-	-
GUDCA	7.993	448.3056	74.0238	$[H_2NCH_2COO]^-$
			448.2947	$[M-H]^-$
TCDCA	9.145	498.2874	79.9567	$[SO_3]^-$
			106.9825	$[CH_2CHSO_3]^-$
			124.0057	$[H_2NC_2H_4SO_3]^-$
			498.2876	$[M-H]^-$

*meas – measured *m/z*

5.4.2 Analysis of Human Plasma performed by LC-Q-Orbitrap

All samples were measured in fullscan, no fragmentation data were obtained. Retention times and *m/z* acquired from standard solutions were used for identification.

LCA was also not detected by LC-Q-Orbitrap.

Table 19. *BAs found and verified in human plasma based on their retention times and mass spectra compared to standards.*

Bile acid	RT (min)	*Meas *m/z* $[M-H]^-$	Area	Delta ppm
CA	9.25	407.2799	1 779 374	1.741
DCA	9.87	391.2852	5 476 001	2.387
CDCA	9.80	391.2851	179 405	2.080
UDCA	8.90	391.2854	9 890	2.847
LCA	*ND	-	-	-
TCA	8.78	514.2835	734 800	0.409
TDCA	9.78	498.2888	1 591 190	0.933
GCA	8.71	464.3006	4 456 292	-0.139
GCDCA	9.20	448.3060	15 143 769	0.491
CA-d5	9.25	412.3111	2 045 463	1.177
DCA-d5	9.87	396.3160	2 800 625	0.858

*meas – measured *m/z*, ND – not detected

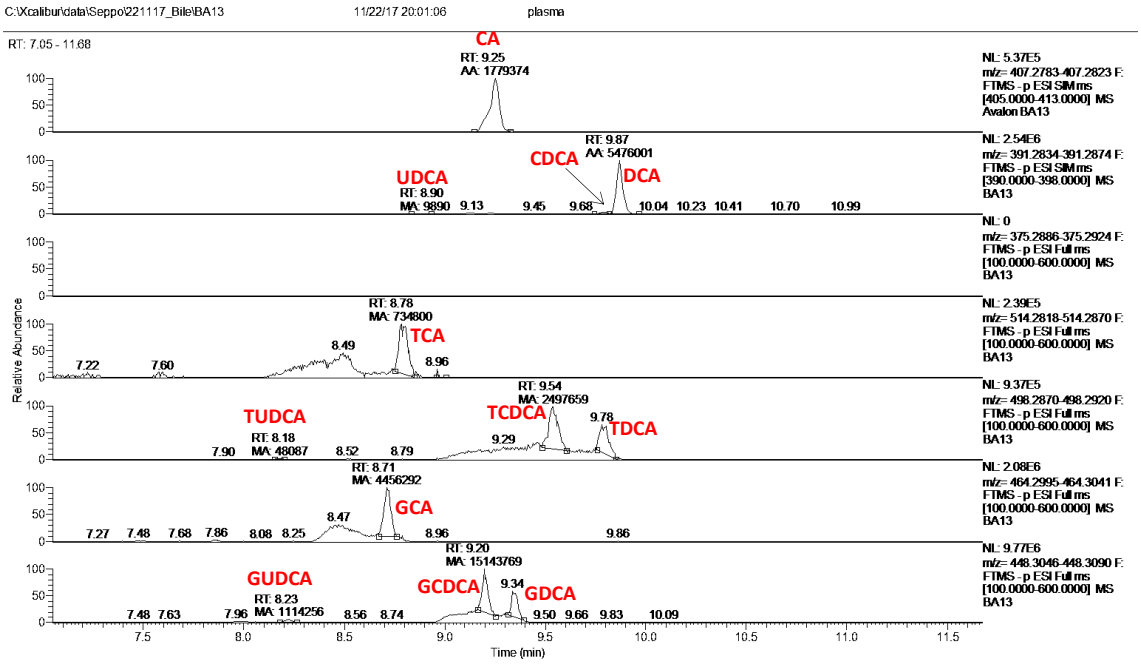


Figure 38. *EIC of BAs found in human plasma.*

Searching for BAs with appropriate standards, other glycine- and taurine-conjugates were also observed by LC-Q-Orbitrap.

Table 20. *Predicted BA derivates found in human plasma.*

Bile acid	RT (min)	*Meas *m/z* $[M-H]^-$	Area	Delta ppm
GUDCA	8.23	448.3059	1 114 256	0.424
GDCA	9.34	448.3060	10 251 713	0.625
TUDCA	8.18	498. 2897	48 087	2.699
TCDCA	9.54	498.2887	2 497 659	0.632

*meas – measured *m/z*

5.5 Analysis of Human Hair

5.5.1 Hair Samples Obtained from Men

The analysis of human hair obtained from two men without any information about their diagnosis was performed by UHPLC connected to Q-TOF and afterwards using UHPLC connected to Q-Orbitrap. For hair metabolite profiling by LC-Q-TOF, data processing was focused also on other compounds for the KuBiCo project, not only on the BA analysis. Therefore, HILIC and RP chromatography, positive and negative ionization mode were set (data not shown).

LC-Q-Orbitrap analysis was used to achieve a higher accuracy and precision. The data processing was focused only on the BAs. For that reason, the analysis was performed on C18 column, in negative ionization mode.

5.5.1.1 Analysis performed by LC-Q-TOF

BAs were not found in these hair samples.

Analyzing the hair metabolome and searching for other compounds, retention times and fragment ions of the found compounds were compared to the data in the UEF PCDL library or to mass spectra in METLIN Metabolomics Database (data not shown).

Carboxylic acids and fatty acids were found in data obtained from LC-Q-TOF with RP column and measured in negative ionization mode. Moreover, intensive peak of lauryl sulfate, a surfactant contained in most of hair products, was observed in negative mode.

Assessment of data measured using RP chromatography and positive ionization mode showed exogenous exposure of the hair. For example phtalates, diethyltoluamide (DEET), cocamidopropyl betaine and other compounds were found as residues of the personal care products. Another compound found in both hair samples was verified as caffeine. The hair from the older man contained cortisol, which also provides better ionization profile in positive mode than in negative mode. Cortisol was found only in the hair sample obtained from older man, whereas the young hair was cortisol negative.

HILIC chromatography in positive mode allowed an identification of amino acids, such as L-histidine, L-isoleucine, L-leucine and others.

5.5.1.2 Analysis performed by LC-Q-Orbitrap

The hair samples were also measured by LC-Q-Orbitrap. The RP chromatography and negative ionization mode were set. The data assessment was focused on the analysis of BAs.

CA, DCA and their ratio are the most important in the ICP. For that reason, a narrow range of *m/z* was set for scanning these two BAs. For other BAs studied in this thesis, a wilde range of 100-600 *m/z* was set, because the setting of the narrow range is time-consuming.

BAs were not found using LC-Q-Orbitrap either. Some peaks were noticed but only with low amount of areas. We could not determine these peaks as BAs, but only as a noise.

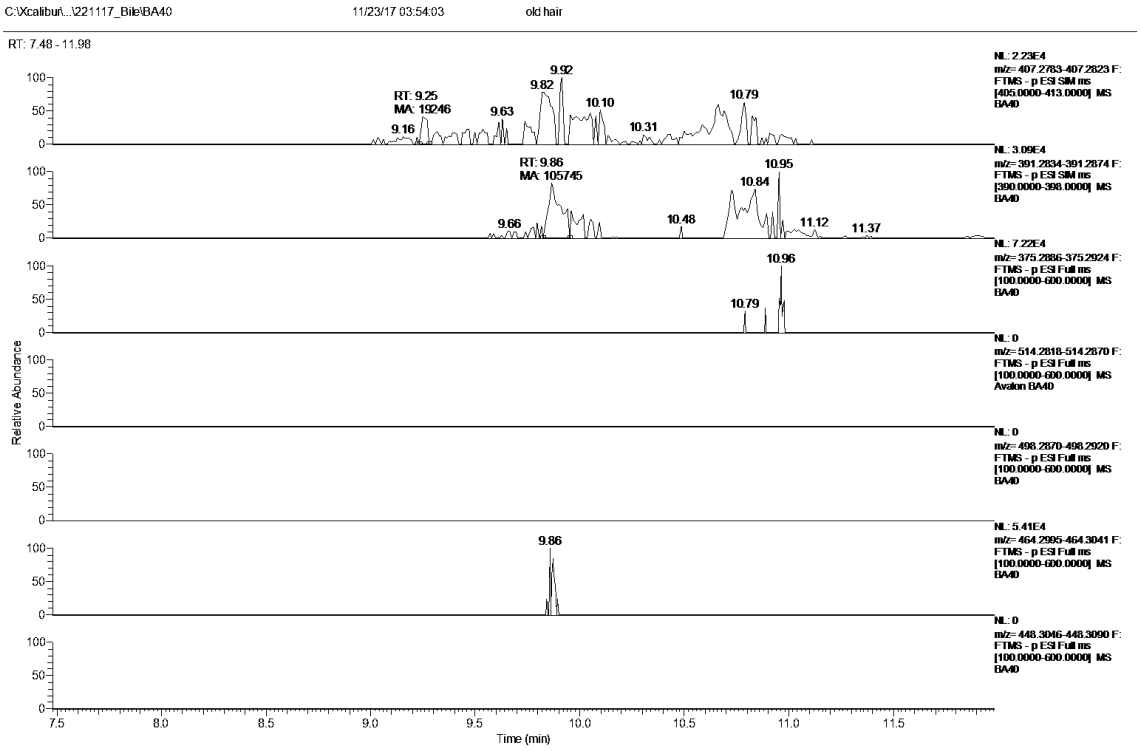


Figure 39. *EIC of old hair sample.*

5.5.2 Hair Samples obtained from Mothers and Neonates

Hair samples obtained from mothers with ICP and their neonates were measured by LC-Q-Orbitrap. The analysis was performed setting RP chromatography and negative ionization mode. Found BAs were verified based on the retention times and *m/z* of BA standards.

GCA and TCA were verified in hair sample obtained from Mother 1, whereas CA, DCA, GCA and GCDCA were found in hair sample from Mother 2.

All BAs, except CDCA and LCA, were observed in hair samples obtained from the neonates. Predicted isomers of tauro- and glyco-conjugates were noticed as well.

Table 21. *BAs and ISs found and verified in the hair of Mother 1.*

Bile acid	RT (min)	*Meas *m/z* [M-H]$^-$	Area	Delta ppm
TCA	8.79	514.2841	10 708	1.595
GCA	8.72	464.3024	2 766	3.738
CA-d5	9.25	412.3112	592 688	1.151
DCA-d5	9.86	396.3161	648 034	1.161

*meas – measured *m/z*

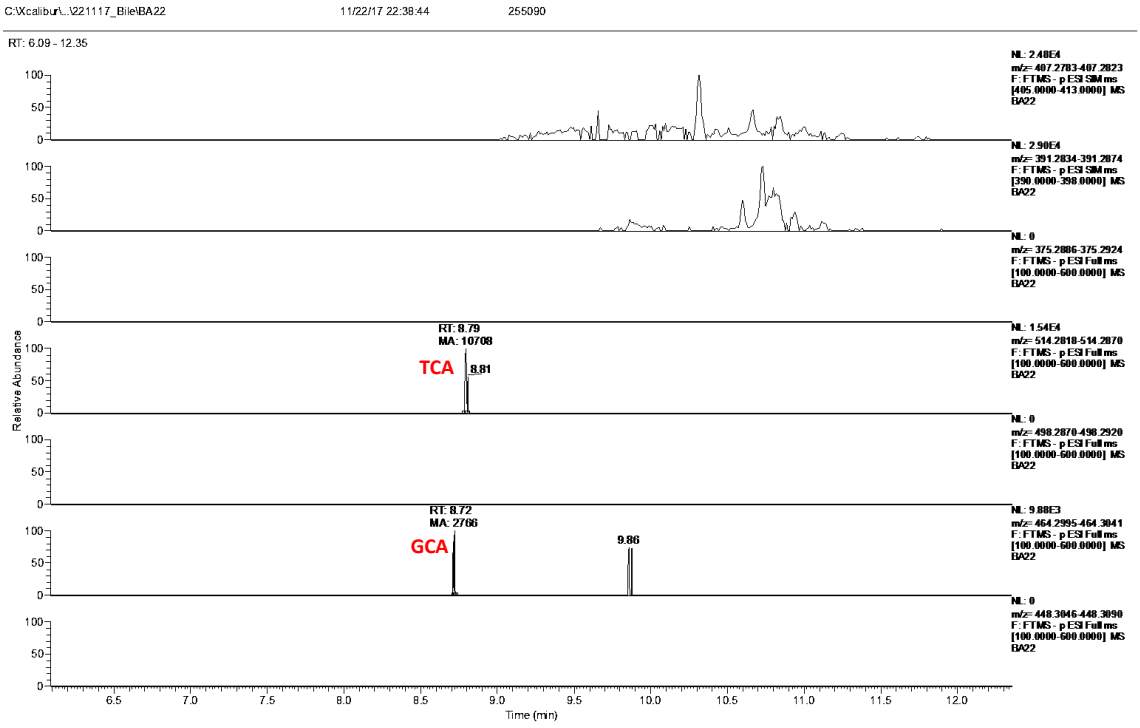


Figure 40. *EIC of hair sample obtained from Mother 1.*

Table 22. *BAs and ISs found and verified in the hair of Baby 1.*

Bile acid	RT (min)	*Meas *m/z* [M-H]$^-$	Area	Delta ppm
CA	9.25	407.2799	189 911	1.815
DCA	9.87	391.2858	12 598	3.792
CDCA	*ND	-	-	-
UDCA	8.91	391.2849	3 103	1.441
LCA	*ND	-	-	-
TCA	8.79	514.2831	351 825	-0.427
TDCA	9.79	498.2887	58 630	0.632
GCA	8.71	464.3008	11 676 762	0.378
GCDCA	9.20	448.3058	139 259	0.023
CA-d5	9.25	412.3113	705 002	1.614
DCA-d5	9.87	396.3163	770 627	1.539

*meas – measured *m/z*, ND - not detected

Table 23. *Predicted BAs found in the hair of Baby 1.*

Bile acid	RT (min)	*Meas *m/z* [M-H]$^-$	Area	Delta ppm
TUDCA	8.17	498.2883	160 193	0.111
TCDCA	9.54	498.2890	265 076	1.174
GUDCA	8.22	448.3062	447 157	0.959
GDCA	9.36	448.3062	59 733	0.893

*meas – measured *m/z*

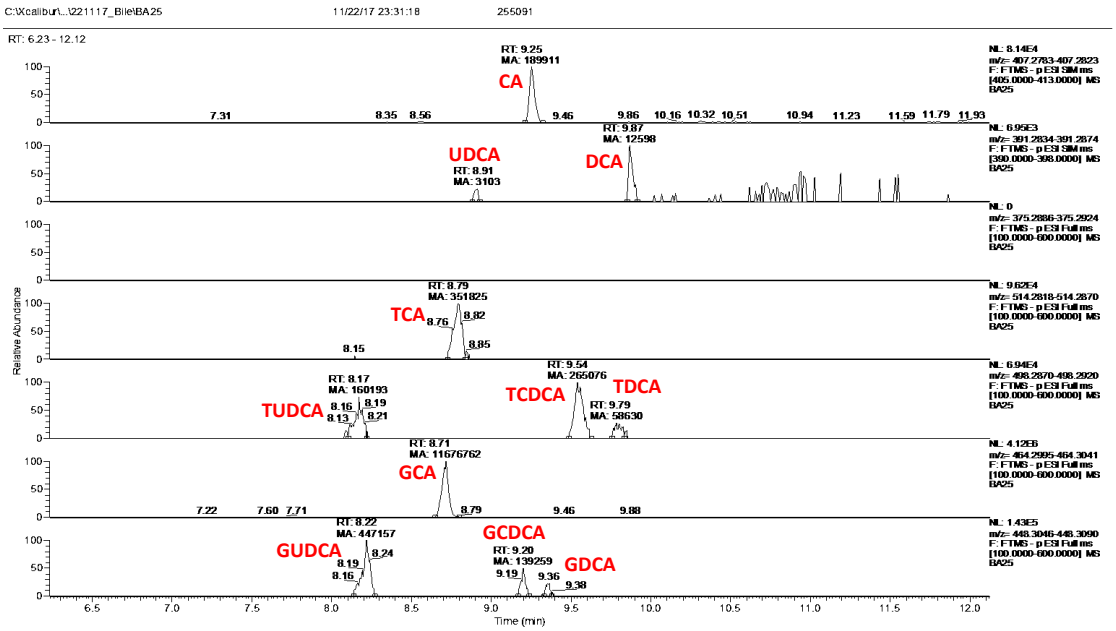


Figure 41. *EIC of hair sample obtained from Baby 1.*

Table 24. *BAs and ISs found and verified in the hair of Mother 2.*

Bile acid	RT (min)	*Meas *m/z* [M-H]$^-$	Area	Delta ppm
CA	9.25	407.2793	3 871	0.244
DCA	9.87	391.2862	12 712	4.789
GCA	8.72	464.3018	14 093	2.360
GCDCA	9.19	448.3053	3 121	-1.070
CA-d5	9.25	412.3112	813 617	1.541
DCA-d5	9.87	396.3162	778 484	1.388

*meas – measured *m/z*

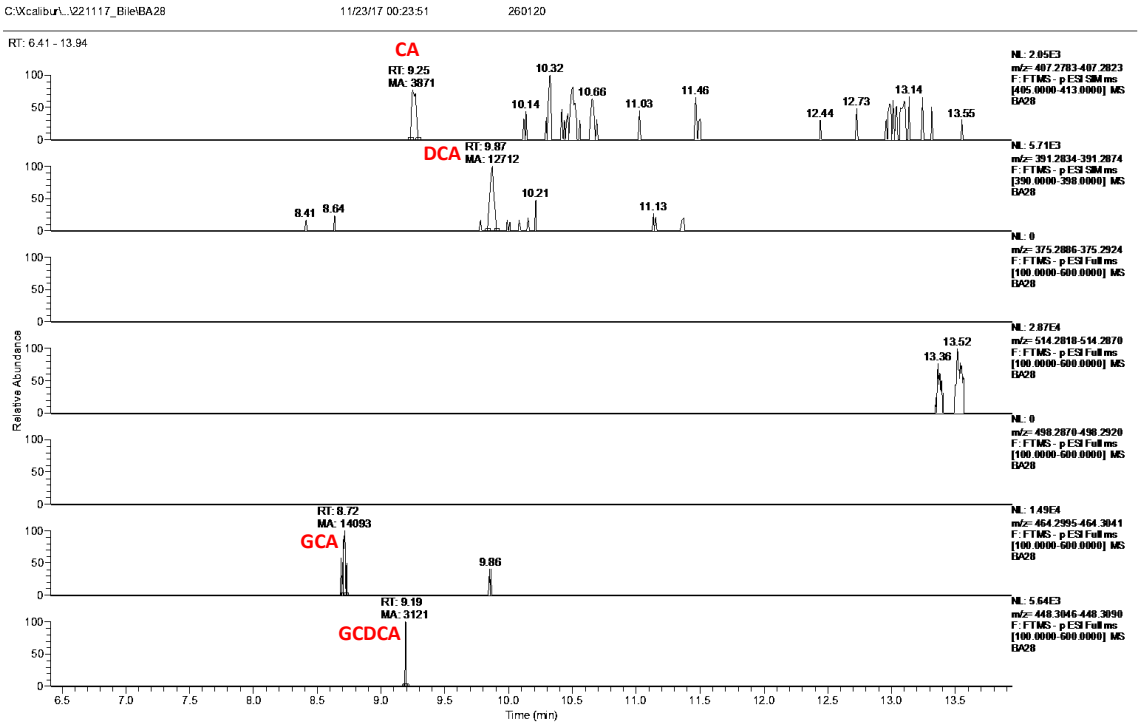


Figure 42. *EIC of hair sample obtained from Mother 2.*

Table 25. *BAs and ISs found and verified in the hair of Baby 2.*

Bile acid	RT (min)	*Meas *m/z* [M-H]$^-$	Area	Delta ppm
CA	9.25	407.2801	43 700	2.110
DCA	9.87	391.2856	9 408	3.307
CDCA	*ND	-	-	-
UDCA	8.91	391.2856	2 005	3.230
LCA	*ND	-	-	-
TCA	8.81	514.2841	332 710	1.595
TDCA	9.83	498.2889	46 626	1.053
GCA	8.71	464.3005	418 248	-0.333
GCDCA	9.20	448.3062	234 914	1.093
CA-d5	9.25	412.3114	607 847	1.978
DCA-d5	9.87	396.3163	592 604	1.464

*meas – measured *m/z*, ND – not detected

Table 26. *Predicted BAs found in the hair of Baby 2.*

Bile acid	RT (min)	*Meas *m/z* [M-H]$^-$	Area	Delta ppm
TUDCA	8.17	498.2884	146 995	0.070
TCDCA	9.55	498.2890	341 293	1.174
GUDCA	8.23	448.3062	428 812	1.026
GDCA	9.35	448.3060	80 897	0.625

*meas – measured *m/z*

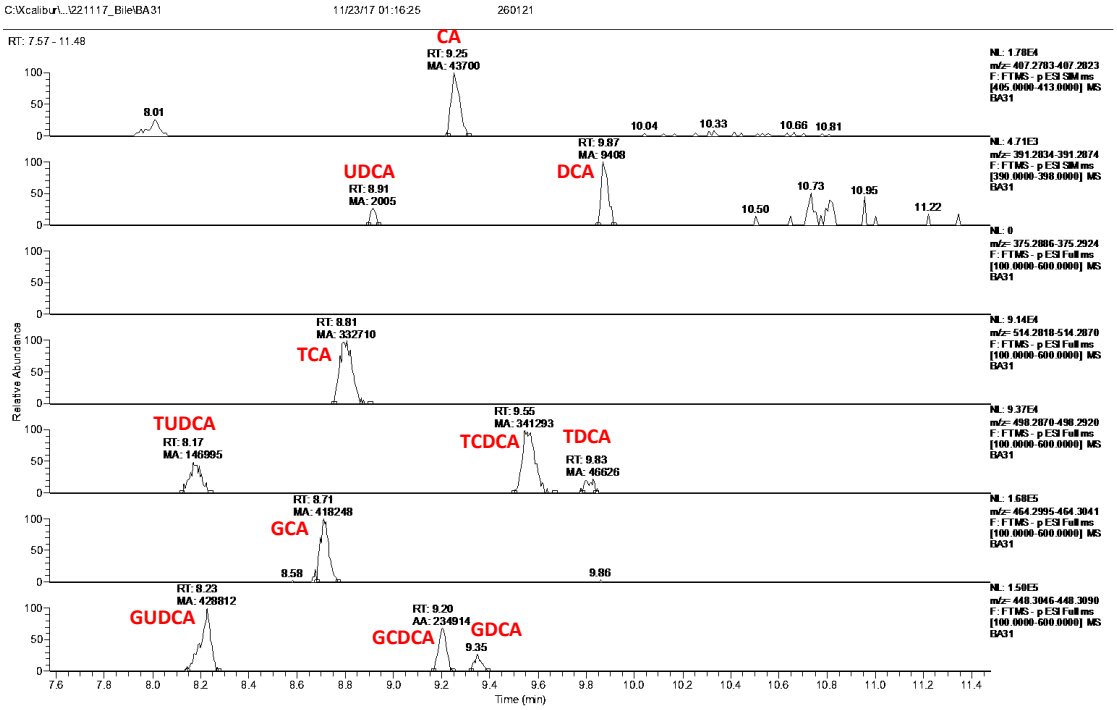


Figure 43. *EIC of hair sample obtained from Baby 2.*

5.6 Levels of Bile Acids in Biological Samples

The quantitative analysis of BAs in biological samples was performed by LC-Q-Orbitrap. The samples were mixed with ISs. The calibration curves were prepared in 50% ACN and in charcoal serum. After analyzing the data, the calibration curves in ACN were used for the quantification.

Table 27. *BA concentrations in mouse liver and human plasma.*

	Concentration µg/g of liver	**Concentration ng/ml**
	Mouse liver	**Human plasma**
CA	0.163	90.824
DCA	0.038	215.986
CDCA	-	92.227
UDCA	0.243	12.263
LCA	-	-
TCA	51.957	22.963
TDCA	1.832	17.813
GCA	0.099	123.591
GCDCA	-	285.269

Table 28. *BA concentrations in human hair obtained from mothers with ICP and their neonates.*

	Concentration µg/g of hair			
	Mother 1	**Baby 1**	**Mother 2**	**Baby 2**
CA	-	6.370	0.301	1.748
DCA	-	0.548	0.471	0.499
CDCA	-	-	-	-
UDCA	-	2.923	-	2.459
LCA	-	-	-	-
TCA	0.890	6.691	-	6.756
TDCA	-	1.025	-	0.969
GCA	0.261	205.519	0.412	8.189
GCDCA	-	2.532	0.422	4.680

6 DISCUSSION

This thesis aimed to establish a suitable qualitative and quantitative method for the analysis of selected BAs in mouse liver, human plasma and especially in human hair. To develop and optimize the method, mouse liver and human plasma were used. Afterwards, this method served for the analysis of BAs in human hair. The hair obtained from the mothers with ICP and their neonates, and the hair from men without any information about their diagnosis were tested within the analysis.

In the last years, BAs are frequently studied for understanding their functions in humans and animals. They are important prognostic and diagnostic indicators for hepatobiliary and intestinal dysfunction.

Nowadays, the HPLC coupled to MS is the most significant technique for BA profiling in biological samples. In literature, different conditions are set to analyze BAs and their glycine- and taurine-conjugates.

We optimized the method for BA profiling by LC-Q-TOF, which is commonly used at Department of Pharmaceutical Chemistry (UEF). This method, using a C18 column and water/methanol gradient, well separated all BAs, including the isomeric BAs, within 16.50 minutes. All BAs were studied in negative ESI mode. Although, in some studies (Alnouti et al. 2008) they performed the analysis of glycine-conjugated BAs in positive ionization mode, however, these conjugates provided good ionization profiles in negative ionization mode.

Regarding the formic acid was used as an ionization modifier in HPLC solvents, the adducts with formic acid were observed. Other adducts of BAs were formed by adduction with water molecules. To achieve the fragment ions for further identification, the CID was used. Collision energies of 10V, 20V and 40V were tested by LC-Q-TOF. The collision energy of 40V proved to be the most effective energy to achieve the fragment ions for better identification. Thus, the identification of isomeric BAs was facilitated by different fragment ions after CID. The free BAs underwent an elimination of water molecules, formic acid and carbon dioxide. The conjugated BAs inclined to eliminate the taurine or glycine fragments.

The achieved retention times and MS data were compared to the previous studies. The elution order was similar, the differences are caused by different conditions set for the analysis.

For the high mass resolution and high mass accuracy, the BA analysis was performed by LC-Q-Orbitrap. During the analyses performed by LC-Q-TOF and LC-Q-Orbitrap, the same C18 column and the same conditions were set, except the column temperature. The retention times were shifted by approximately ± 0.3 min. It could be

attributed to the different temperature which was set during the analyses (50°C for LC-Q-TOF, 40°C for LC-Q-Orbitrap). The elution order of BA standards using LC-Q-TOF and LC-Q-Orbitrap was similar. The only difference was observed for CDCA and TDCA - TDCA was eluted earlier than CDCA. The only difference in mass spectra was observed in spectrum of DCA, where the adduct with formic acid was also formed.

Searching for studied BAs in biological samples, also other BA derivates with the same mass were detected (TCDCA, TUDCA, GDCA, GUDCA, MCA, TMCA, GMCA). Their structure was predicted based on the retention behaviour of free BAs and the exact mass and glycine or taurine fragments. For the accurate identification, all BA standards would be needed.

The quality of the data was better when using LC-Q-Orbitrap. We obtained clean chromatograms with lower background and the peaks were resolved well with the exception of human plasma, where the peaks were difficult to resolve. The plasma contains many compounds, that can harm the separation as biological disturbance. A dilution of plasma samples could help better separation.

For the quantification of BAs, two calibration curves were prepared, in 50% ACN and in charcoal serum. For data assessment, the calibration curve prepared in 50% ACN was used. For the calibration curve prepared in charcoal serum, the same situation occurred as for the real human plasma - biological disturbance causing that the peaks were not resolved. The charcoal serum was propably not completely stripped, also biological BAs were observed.

Two methods were used for the preparation of the mouse liver homogenate. After the analysis of both samples, the relative abundance was higher in the chromatogram of liver homogenate prepared in liquid nitrogen. Therefore, the liver homogenate for the analysis performed by LC-Q-Orbitrap was prepared in liquid nitrogen. The concentrations of taurine-conjugates were higher than the concentrations of free BAs and glycine-conjugates. Alnouti et al. (2008) have reported the same results. The taurine-conjugates were the most common BAs in mouse liver, followed by the unconjugated and glycine-conjugated BAs. In contrast to humans, the enzyme responsible for the conjugation of BAs in mice is specific to taurine rather than glycine (Alnouti et al. 2008). The exact concentrations found in mouse liver are influenced by the analytical method, by the method used for the collection and preparation of the liver and by the individual animal.

Glycine-conjugates, especially GCDCA, were the most common BAs found in human plasma. Tagliacozzi et al. (2003) acquired the same results.

In the patients with ICP, the serum levels of taurine- and glycine-conjugates are increased, especially TCA and GCA. Also serum level of CA is increased. In hair sample

of Mother 1, the only two found BAs were TCA and GCA. In the hair of Mother 2, GCA and GCDCA were observed, together with CA and DCA. In the hair of neonates, glycine-conjugates were also the most common BAs, together with CA and TCA in higher concentration.

The baby's hair seems to be a better material for the analysis. All BAs, except CDCA and LCA, were identified in the hair of both neonates. The analysis of adult hair is influenced using personal care products, which may cause background in the analysis. For example, a high peak of lauryl sulfate was observed in hair samples of adults. Lauryl sulfate is a detergent and surfactant contained in many personal care products, such as soaps or shampoos. The hair could be influenced by the sunlight or by the sauna. The presence of this contamination may harm the ionization of other compounds and suppress the signals. The baby's hair was collected on the first or second day after the parturition. Therefore, the hair should be free from these personal care products and could provide a good material for the analysis.

The concentrations of BAs found in babies's hair were relatively high. In the ICP, there is an increased flux of BAs through the placenta from the mother to the fetus. The concentration of BAs in amniotic fluid and in cord blood is increased. Fetal BA balance depends on the placental transfer capacity for BAs. The BAs found in hair samples of the neonates could come from their own blood circulation and also from the amniotic fluid and from the cord blood. The next aim could be to analyze the cord blood and determine where the BAs come from.

7 CONCLUSION

- The developed UHPLC-MS method can be used for the BA analysis in biological samples, including human hair.
- Nine BAs and two ISs were separated and analyzed by LC-Q-Orbitrap in a single run. The data was used for the quantification of BAs in biological samples. BAs were not found in the hair obtained from the men withnout the cholestasis. However, BAs were successfully detected in the hair from the mothers with intrahepatic cholestasis of pregnancy and in the hair from their neonates.
- The hair obtained from the neonates seems to be a better material for the analysis, because of the absence of personal care products, which can harm the analysis of the hair of adults.

8 ABBREVIATIONS

ACN	Acetonitrile
BA	Bile acid
CA	Cholic acid
CA-d5	Deuterated Cholic acid
CDCA	Chenodeoxycholic acid
CID	Collision-induced dissociation
DC	Direct Current
DCA	Deoxycholic acid
DCA-d5	Deuterated Deoxycholic acid
EIC	Extracted Ion Chromatogram
ESI	Electrospray Ionization
FBS	Fetal Bovine Serum
GC	Gas Chromatography
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GMCA	Glycomuricholic acid
HESI	Heated-Electrospray Ionization
HILIC	Hydrophilic Interaction Chromatography
HPLC	High Performance Liquid Chromatography
ICP	Intrahepatic Cholestasis of Pregnancy
IS	Internal Standard
LCA	Lithocholic acid
LC-MS	Liquid Chromatography-Mass Spectrometry
m/z	Mass to charge ratio
MCA	Muricholic acid
MeOH	Methanol
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
Q	Quadrupole
Q-Orbitrap	Quadrupole-Orbitrap
Q-TOF	Quadrupole-Time of Flight
RF	Radiofrequency Current
RP	Reversed Phase chromatography
TCA	Taurocholic acid

TDCA	Taurodeoxycholic acid
TMCA	Tauromuricholic acid
TOF	Time of Flight
UDCA	Ursodeoxycholic acid
UHPLC	Ultra-High Performance Liquid Chromatography

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