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Development and validation of a GC-MS based method to analyse faecal bile acids

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"Gratitude can transform common days into thanksgivings, turn routine jobs into joy, and change ordinary opportunities into blessings" — William Arthur Ward

ABSTRACT

Background: About 5% of the bile acids (BAs) escape enterohepatic recirculation in the terminal ileum and enter the large intestine where they are further metabolized by the colonic microbiota and finally excreted in faeces. The amount and composition of faecal bile acids has been associated to several disease states.

Aim: to develop a Gas Chromatography – Mass Spectrometry (GC-MS) based method that allows the quantification of bile acids (BA) in faecal samples and to validate this method.

Methods: BAs were esterified and silylated to increase their volatility. Standard solutions of cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), litocholic acid (LCA) and usodeoxycholic acid (UDCA) were prepared and used to optimize chromatographic parameters using hyodeoxycholic acid (HDCA) as internal standard. After optimizing the esterification (type of catalyst, temperature and time) and silylation (type and amount of reagent, temperature and time) the optimised method was validated. Intraday and interday precision and accuracy of calibration points was assessed as well as the limit of detection (LOD) and limit of quantification (LOQ). Similarly, precision and accuracy were determined for freeze dried stool samples and dried faecal water.

Results: Baseline separation of the 5 BAs and internal standard was achieved on an apolar Rxi-1MS column with split injection (split flow 0.25 ml/min) using a He-flow of 1.5 ml/min. Esterification was optimal using HCl 12N as catalyst and heating for 2 h at 60°C whereas the best conditions for silylation were 100 µl of HMDS+TMCS+Pyridine (3:1:9) and heating for 30 min at 55°C. The intraday and interday precision and accuracy was evaluated for each BA and concentration. LOD was 0.05 µg and LOQ was 0.1 µg for most BAs. However, for faecal samples, precision and accuracy were low, despite an additional sonication step and reflux in ethanol to improve the solubilisation of the BAs.

Conclusions: Although the performance of the developed method was satisfying for BA standard solutions, its application to faecal samples needs further optimization of the clean-up of the samples prior to derivatisation.

RESUMO

Background: Cerca de 5% dos ácidos biliares escapa à recirculação enterohepática no íleo terminal e entra no intestino grosso onde eles são posteriormente metabolizados pelo microbioma colónico e finalmente excretado nas fezes. A quantidade e composição dos ácidos biliares fecais tem sido associada a vários estados de doenças.

Objetivo: desenvolver um método baseado em cromatografia gasosa – espectrometria de massa que permita a quantificação de ácidos biliares em amostras fecais e validar este mesmo método.

Métodos: Os ácidos biliares foram esterificados e sililados para aumentar a sua volatilidade. Soluções padrão de ácido cólico, ácido desoxicólico, ácido quenodesoxicólico, ácido litocólico, ácido ursodesoxicólico foram preparadas e usadas para otimizar parâmetros cromatográficos, usando o ácido hiodesoxicólico como padrão interno. Após a otimização da esterificação (tipo de catalisador, temperatura e tempo) e sililação (tipo e quantidade de reagente, temperatura e tempo), o método otimizado foi validado. A precisão intra-dia e inter-dia e a exatidão dos pontos de calibração foram avaliadas bem como o limite de deteção e o limite de quantificação. Semelhantemente, a precisão e exatidão foram determinadas para amostras de fezes liofilizadas e para água fecal liofilizada.

Resultados: A separação da linha de base dos 5 ácidos biliares e do padrão interno foi atingida com a coluna apolar Rxi-1MS com injeção em modo split (fluxo do split 0.25 ml/min) usado com fluxo de He a 1.5 ml/min. A esterificação foi ótima usando HCl 12N como catalisador a uma temperatura de 60°C, durante 2 h, enquanto que as melhores condições para a sililação foram 100 µl de HMDS+TMCS+Pyridine (3:1:9) a uma temperatura de 55°C, durante 30 min. A precisão, intra- e inter-dia, e a exatidão foram avaliadas para cada ácido biliar e cada concentração. O limite de deteção foi 0.05 µg e o limite de quantificação foi 0.1 µg para a maioria dos ácidos biliares. Contudo, para as amostras fecais, a precisão e exatidão foram baixas, apesar do passo adicional da sonicação e refluxo em etanol para melhorar a solubilização dos ácidos biliares.

Conclusões: Apesar do desempenho do método desenvolvido ter sido satisfeito para as soluções padrão dos ácidos biliares, a aplicação para as amostras fecais necessita posteriormente de uma otimização na limpeza das amostras antes da derivatização.

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List of Abbreviations

B

BA	Bile Acid

C

CA	Cholic Acid
CDCA	Chenodeoxycholic Acid
CV	Coefficient of Variation

F

FW	Faecal Water

D

DCA	Deoxycholic Acid

E

EI	Electron Impact

G

G	Gravity
GC	Gas Chromatography
GI	Gastrointestinal

H

HDCA	Hyodeoxycholic Acid

L

LCA	Lithocholic Acid
LOD	Limit of Detection
LOQ	Limit of Quantification

I

IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome

M

MS	Mass Spectrometry
m/z	Mass-to-charge Ratio

S

S	Stool
STDEV	Standard Deviation

U

UDCA	Ursodeoxycholic Acid

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CHAPTER 1

LITERATURE REVIEW

1.1. GUT MICROBIOME

The gut microbiome consists of an aggregate genome of trillions of microorganisms residing in the human gastrointestinal (GI) ecosystem (Ghaisas, Maher, & Kanthasamy, 2016).

Forbes and co-workers (2016), defined the term "microbiota" as "the population of microbes at a particular anatomical niche" and "microbiome" as "the collective genes encoded by all microbes of that particular niche".

In the healthy human gut most bacteria belong to four predominant bacterial phyla: Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria (Tap et al., 2009).The phylum Bacteroidetes represents one of the most abundant genera in the gut (Huttenhower et al., 2012).

Some studies revealed that the gut composition is influenced by several host factors, including quantity and quality of diet, lifestyle, use of antibiotics, and genetic background (Sanduzzi Zamparelli et al., 2016). Nevertheless, even with this diversity in microbial composition, recent studies revealed that the functional part of the healthy microbiome is relatively stable (Forbes, Van Domselaar, & Bernstein, 2016).

For quite a long time, it has been thought that the development of the GI microbiota only started at birth, with exposure of the infant to its mother's microbiota in the birth canal or to the mother's skin in case of caesarean section. However, very recent studies reported that, already in utero, microbes pass from the mother to the foetus through the placenta (Sanduzzi Zamparelli et al., 2016). After weaning, the composition of the microbiota becomes more diverse and more stable and resembles that in adulthood (Claesson et al., 2012). With ageing, numbers of bifidobacteria decline.

Within the GI tract, both the numbers and the diversity of bacteria increase from the stomach to the colon, with the terminal ileum being the site where prevalent species change from aerobes to anaerobes (Mondot, de Wouters, Doré, & Lepage, 2013). The colon harbours the densest microbial population with up to 1012 cfu/g. In addition, microbial populations on mucosal surfaces significantly differ from that in the lumen (Li et al., 2015). Microbes at the mucosa surface are closer to the intestinal epithelium, and may have a

greater influence on the immune system whereas luminal microbes might be more crucial for energy and metabolic interactions. As a consequence, studies of the gut microbiota that use faecal material may not reflect the totality of viable microbes, and do not provide a complete overview of the portfolio of microbes (Forbes et al., 2016).

The association between the gut microbiome and host immunity implicates a bidirectional correlation between microbes and the host innate and adaptive immune system. The balance between pro- and anti-inflammatory mechanisms is critical for gut immune homeostasis and is directly affected by the commensal microbial communities of the gut (Forbes et al., 2016).

Disturbance of the gut microbiome not only affects intestinal diseases, such as colorectal cancer (CLC), ulcerative colitis (UC) and inflammatory bowel disease (IBD), but also more systemic diseases such as diabetes, metabolic syndrome, atopy and cystic fibrosis (Aries, Crowther, Drasar, Hill, & Williams, 1969; Degirolamo, Modica, Palasciano, & Moschetta, 2011; Ghaisas et al., 2016; M. J. Hill et al., 1975; Walkera & Lawley, 2013). Furthermore, the gut microbiome has been implicated in various type-2 diabetes (T2D)-related complications, including diabetic retinopathy, kidney toxicity, atherosclerosis, hypertension and diabetic foot ulcers (Zhang & Zhang, 2013). Also patients with celiac disease, have been shown to display GI microbiome abnormalities compared with healthy individuals (Fujimura & Slusher, 2010; Nadal, Donant, Ribes-Koninckx, Calabuig, & Sanz, 2007). Besides, disturbance of the gut microbiome can also be related to central nervous system (CNS) disorders, such as Parkinson's and Alzheimer's diseases and autism (Ghaisas et al., 2016). Also autoimmune disorders such as lupus, multiple sclerosis, psoriasis, rheumatoid arthritis, the allergic disorders and asthma have been associated with aberrations in the human gut microbiome (Fujimura & Slusher, 2010).

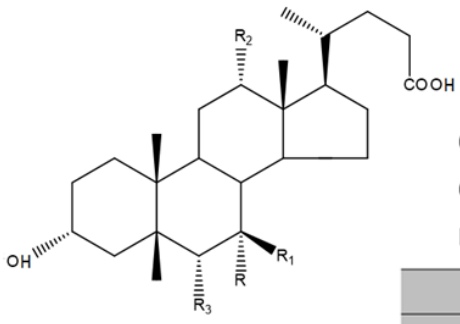
As a consequence, the gut microbiota has become an important target for promoting health, longevity, and potentially revolutionize the treatment of some diseases. Modulation of the microbiota can be achieved with interventions with prebiotics, probiotics or antibiotics. Management of the gut microbiome holds considerable potential in the domain of preventive medicine, having as the biggest challenge to control external factors as environmental and dietary influences, and to better understand genomic interactions between the host and the gut microbiome (Ghaisas et al., 2016).

Dysbiosis occurs when pathological imbalances in gut bacterial colonies precipitate disease and has been linked to the dysmetabolism of bile acids (BAs) in the gut (Sagar, Cree, Covington, & Arasaradnam, 2015).

1.2. Bile Acids

Bile acids (BAs) are synthesized from cholesterol in the liver and their production is the primary pathway for cholesterol catabolism. This conversion occurs exclusively in hepatocytes by a cascade of 12 reactions catalysed by different enzymes (Chiang, 2002).

All BAs contain the same apolar sterol core structure that is substituted with hydroxyl groups at different positions and have a side chain that ends in a polar carboxyl group, Figure 1.1 (Humbert et al., 2012). These amphipathic nature of BAs is crucial to the function that they execute which is to facilitate solubilisation of lipids and their further absorption in the gastrointestinal tract. As amphipathic molecules, BAs also have powerful detergent properties (Houten, Watanabe, & Auwerx, 2006).



CA – Cholic Acid

CDCA – Chenodeoxycholic Acid

DCA – Deoxycholic Acid

LCA – Lithocholic Acid

UDCA – Ursodeoxycholic Acid

HCA – Hyocholic Acid

HDCA – Hyodeoxycholic Acid

	CA	CDCA	DCA	LCA	UDCA	HCA	HDCA
R	-OH	-OH	-H	-H	-H	-OH	-H
R1	-H	-H	-H	-H	-OH	-H	-H
R2	-OH	-H	-OH	-H	-H	-H	-H
R3	-H	-H	-H	-H	-H	-OH	-OH

Figure 1.1. General chemical structure of BAs (adapted from Humbert et al., 2012)

Bile acids are synthesised in the liver and stored in the gallbladder. The BAs are conjugated with glycine or taurine to decrease their toxicity and increase solubility for secretion into bile (Degirolamo et al., 2011). After a meal, they flow into the duodenum and

intestine. Per day, between 0.2g to 0.6g of BAs is synthesised and secreted in healthy humans and on average 0.5g is excreted in faeces. It is estimated that 95% of BAs are reabsorbed from the gastrointestinal tract into the circulation, mainly via active transport in the terminal ileum through the apical sodium-dependent bile acid transporter (ASBT). A small amount is reabsorbed by passive diffusion in the upper intestine to the portal blood and is recycled to the liver. The same ASBT is present in the kidneys and prevents urinary excretion of bile acids that have undergone glomerular filtration (Cai & Chen, 2014; Chiang, 2013; Houten et al., 2006).

BAs that have been reabsorbed are transported to the liver via the portal blood and are taken up at the basolateral (sinusoidal) membrane and exported again at the apical (canalicular) membrane of the hepatocytes into the bile canaliculus (transhepatic BA flux) (Houten et al., 2006). This cycle is known as the enterohepatic recirculation of BAs (Figure 1.2). On average, BAs are recycled 4 to 12 times a day (Houten et al., 2006). The enterohepatic circulation of BAs is an important circuit not only for regulation of BAs synthesis, cholesterol homeostasis and absorption of nutrients, but also for the regulation of whole-body lipid metabolism (Chiang, 2009).

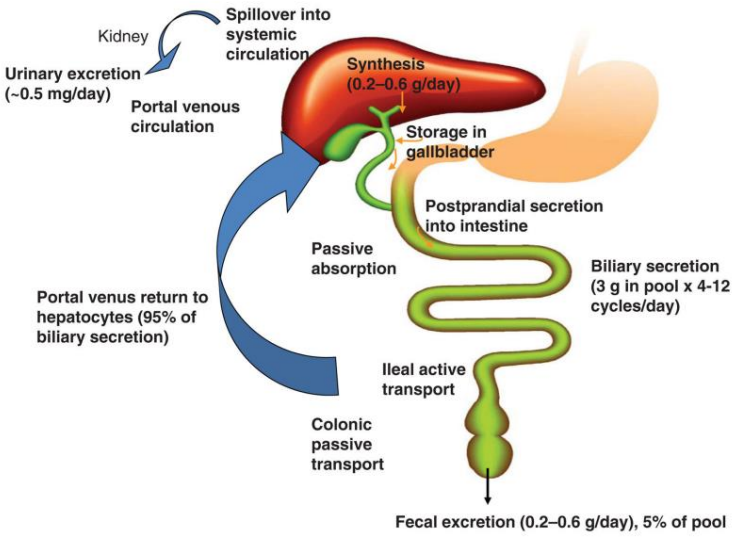


Figure 1.2. Entheropatic Circulation of BAs (adapted from Chiang, 2013)

Approximately 5% of BAs escapes reabsorption, and enters the colon where the BAs

undergo modifications by the intestinal microbiota (Batta et al., 1999). The glycine or taurine conjugates are hydrolysed by bacterial bile salt hydrolyses. The primary bile acids are converted into secondary bile acids by removal of the hydroxyl group on the 7α-position by bacterial enzymes (Cai & Chen, 2014). In this way CA is converted to DCA and CDCA is converted to LCA. CDCA can also be converted to other secondary BAs, including HCA, UDCA, MDCA, and others (Chiang, 2009; Li & Chiang, 2015). The UDCA, in humans, is not epimerised during the hepatocyte transport, being found in few percentage in the biliary bile acids in majority of people. (Hofmann & Hagey, 2008; Humbert et al., 2012)

Besides their role in the absorption, transport, and distribution of lipid soluble vitamins and dietary fats, BAs also regulate bile acid and cholesterol metabolism, through signalling molecules that activate nuclear receptors such as the farnesol X receptor (FXR). In addition, BAs induce the cytochrome P450 3A (CYP3A) family of cytochrome P450 enzymes that allow the detoxification of bile acids, drugs and xenobiotics in the liver and intestine, and also promote hepatocyte apoptosis (Chiang, 2002, 2009; Monte, Marin, Antelo, & Vazquez-Tato, 2009). Furthermore, BAs are also known to facilitate intestinal calcium absorption and to modulate pancreatic enzyme secretion (Koop et al., 1996; Monte et al., 2009).

The BAs toxicity is determined by hydrophobicity which depends on the number, position and orientation (stereochemistry) of the hydroxyl groups. UDCA is the most hydrophilic and LCA is the most hydrophobic BA (magnitude of hydrophobic BAs: UDCA<CA<CDCA< DCA<LCA). The BAs hydrophobicity are linked to their intrinsic toxicity, with the more hydrophobic BAs being more toxic (Degirolamo et al., 2011).

In healthy humans, secreted BAs, are composed of about 30% to 40% of CA, 30% to 40% of CDCA, 20% to 30% of DCA, and a trace amount of LCA (Ajouz, Mukherji, & Shamseddine, 2014; Chiang, 2013).

Diet composition, in particular high fat intake, has repeatedly been shown to influence the levels and composition of BAs in the colon, which explains the relevance of the analysis of faecal BAs in metabolic diseases, such as obesity, type 2 diabetes or hyperlipidaemia (M. J. Hill et al., 1975; Houten et al., 2006; Thomas, Pellicciari, Pruzanski, Auwerx, & Schoonjans, 2008).

In patients with cirrhosis faecal DCA and LCA (secondary BAs) concentrations were

significantly lower than in control subjects. This same study (G Kakiyama et al., 2013) showed that the primary BAs were higher in advanced cirrhotics and secondary BAs were lower, suggesting that there is an association with cirrhosis and the decrease of conversion of primary to secondary BAs.

Recent studies revealed an increase in primary BAs and a decrease in secondary BAs in IBD and IBS patients compared with healthy controls (Bajor, Törnblom, Rudling, Ung, & Simrén, 2015; Duboc et al., 2012; Slattery, Niaz, Aziz, Ford, & Farmer, 2015).

The secondary BAs levels are considered as cytotoxic and genotoxic and have been related with several disorders and diseases. The role of BAs in colorectal cancer risk and the mechanism of their effect have been the subject of many studies in this field (Degirolamo et al., 2011; Pearson, Gill, & Rowland, 2009). Actually, it is known that secondary BAs increase proliferation of colonic cells (Ochsenkühn et al., 1999), and induce apoptosis, being necessary to prevent mutated cells replicating for the future generations (Bernstein et al., 1999).

Bile acids can also induce DNA damage, as DNA breaks were reported to directly correlate with bile acid concentrations (Venturi, Hambly, Glinghammar, Rafter, & Rowland, 1997). Secondary, but not primary, BAs have recently been shown to exert adverse effects on epithelial barrier function, an endpoint thought to be related to tumour promotion (Hughes, Kurth, McGilligan, McGlynn, & Rowland, 2008). Significant evidence suggests that increased concentrations of DCA may be associated with colon polyp formation and large bowel cancer, acting as co-carcinogen in colon cancer (Batta et al., 1999).

1.3. Gas Chromatography-Mass Spectrometry

1.3.1. Gas Chromatography

Chromatography is a technique that separates compounds in a mixture, while they are transported by a mobile phase over a stationary phase. For gas chromatography (GC), the mobile phase is an inert gas, which transports the analyte of interest but does not interact with it. The stationary phase can either be a microscopic layer of a liquid on a solid support, called gas-liquid chromatography, or a solid, called gas-solid chromatography

(Neves, 1980; Skoog, Holler, & Nieman, 1997). In gas-liquid chromatography, which is the most commonly used type of GC, separation of the compounds is based on differences in boiling point, whereas gas-solid chromatography is based on differences of adsorption capacity (Neves, 1980).

In general, a standard chromatography system comprises a source of carrier gas and valves for regulating the flow rate, an injector, a column that is put in an oven, a detector, and a recorder (Figure 1.3) (Neves, 1980; Skoog et al., 1997). The carrier gas should be pure and chemically inert and the most commonly gasses are helium, nitrogen and hydrogen. The choice of carrier gas is most often determined by the type of detector that is used in combination with the GC (Neves, 1980; Skoog et al., 1997). Helium is the most common gas as it is non-flammable and works with a large number of detectors. However, worldwide availability of helium has become critical in recent years, resulting in increasing prices. Therefore, chromatographers increasingly switch to the use of hydrogen gas.

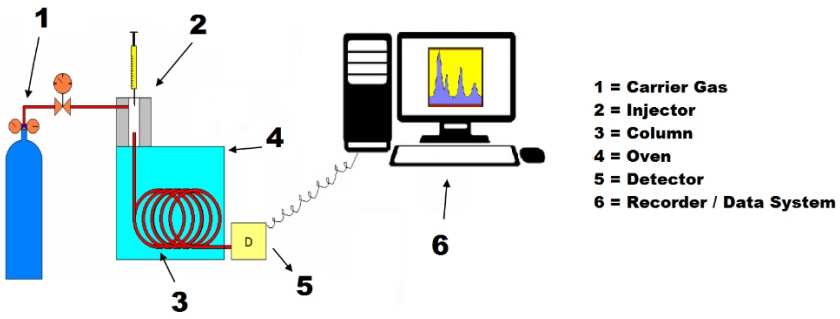


Figure 1.3. A general schematic of GC (adapted from Skoog et al., 1997)

Although manual injection of a sample is possible, the use of an autosampler is recommended as it provides better reproducibility and is more time-efficient. The injection of the sample is an important aspect of the analytical process that can influence the efficiency of the chromatography. A slow injection of a large amount of sample induces extension of the bands, which results in a bad resolution (Skoog et al., 1997).

Most injection systems make use of a micro syringe. The sample can be injected as gas or as liquid, but in the latter case, the sample is vaporized before it comes onto the column. The micro syringe is contained between heated metal blocks that have a sufficiently

high temperature to quickly vaporize the sample without decomposing the sample (Neves, 1980; Skoog et al., 1997). Most injectors allow split and splitless injection mode (Figure 1.4). Split injection is normally applied for the analysis of compounds in high concentrations. The presence of a split line and a split valve, allows that just a part of the injected sample enters the column, whereas the remainder is discharged through the split line. In this way, it is possible to obtain a chromatogram with very sharp peaks due to the rapid sample transfer onto the column (Thermo Scientific, 2014).

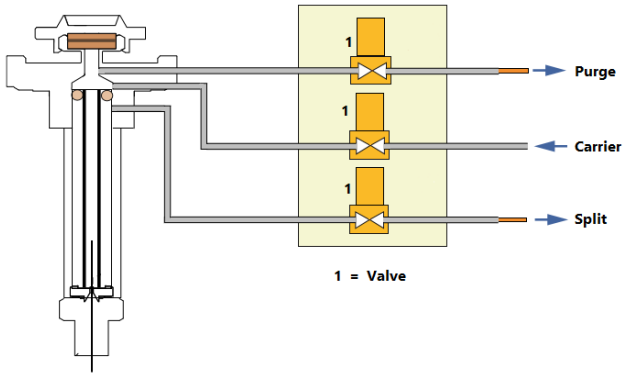


Figure 1.4. A schematic of the injection system of GC, showing particularly the split line (adapted from Thermo Scientific, 2014)

In splitless mode, the split valve is closed and the entire sample enters the column, which is appropriate for compounds with low concentrations (Thermo Scientific, 2014). The column, localised inside a specialised oven, is the place where the separation occurs. The carrier gas transports the sample from the injector into the column head. Within the column, the different components of the mixture are partitioned between the stationary phase and the mobile phase allowing the separations of the compounds (Burchfield & Storrs, 1962; Neves, 1980; Skoog et al., 1997).

Nowadays, mainly capillary columns are used which have a small internal diameter (0.25 until 0.5 mm) and a length between 12 to 300 m. To fit in the oven, the columns are wound in 10 to 30 cm of diameter (Raulin et al., 1999; Skoog et al., 1997). Parameters that need to be taken into account when selecting a GC column include the type of stationary phase, the internal diameter, the film thickness and the column length. Non-polar stationary phases separate components predominantly based on boiling point whereas stationary phases that contain phenyl and/or cyanopropyl groups rather separate based on differences in molecular dipole moment. Columns with smaller internal diameter allow more

efficient separation resulting in a better resolution compared to broader columns. Columns with a thick film (requiring also a larger internal diameter) are appropriate for samples with large variation in solute concentrations as they prevent overloading of the column. The thicker the film, the greater the loading capacity. Increasing the length of the column might increase the resolution. However, the shortest column that provides the required resolution should be selected.

The oven temperature needs to be precisely controlled, as the rate at which the components pass through the column is directly proportional to the temperature of the column. Higher temperatures result in higher rates but also in less interaction with the stationary phase of the column and thus less separation. Most methods use a temperature program which means that the temperature is gradually increased during the analysis to allow adequate separation of highly volatile compounds and acceptable retention times for slowly eluting compounds.

Compounds that elute from the column pass to the detector. The time that a compound takes to pass from the injector to the detector is entitled the retention time (Burchfield & Storrs, 1962; Raulin et al., 1999).

Most commonly used GC detectors are Thermal Conductivity Detector (TCD), Flame Ionization Detector (FID), Electron Capture Detector (ECD) and mass spectrometers (Raulin et al., 1999). From those detectors, only the MS provides structural information about the analytes.

The electronic signal produced in the detector is sent to a recorder/ data system and is used to construct a plot with the relative abundance (Y-axis) as a function of the retention time (X-axis), which is called the chromatogram. The retention time can be useful for the identification of the compounds, when compared to a reference library.

1.3.2. Mass Spectrometry

Mass Spectrometry (MS) separates compounds according to their mass-to-charge (m/z) ratios after ionization (Hill, 1969). A mass spectrometer generally consists of an ion source, a mass analyzer, a detector and a recorder/ data system (Davis & Frearson, 1987; De Hoffmann, Charette, & Stroobant, 1996). The ion source converts the electrically neutral

molecules into ions, by capturing or removing electrons or protons, resulting in a charged molecule (Davis & Frearson, 1987; Mikkelsen & Cortón, 2004). There are different ion sources that ionize the analytes in different ways. The most common ionization techniques are electron impact (EI), chemical ionization (CI), field ionization (FI), field desorption (FD) and fast atom bombardment (FAB) (Davis & Frearson, 1987; De Hoffmann et al., 1996). Only EI will be discussed here as this is the type of ionization that was used in this project. An EI source is composed of an ionization chamber, a heated filament, an anode, and lenses (Figure 1.5). Compounds that elute from the GC column, enter the ionization chamber of the ion source which is contained under vacuum. The filament is heated by an electric current to emit electrons (De Hoffmann et al., 1996). In this way, a current of electrons is created in the ionization chamber. When those electrons collide or pass very close to the gaseous compounds, an energy transfer can occur, resulting in the expelling of an electron from the compounds and the formation of a positive ion. Theoretically, it is also possible to produce a negatively charged ion, the probability of electron capture is about 100 times less than that of electron removal (Davis & Frearson, 1987; De Hoffmann et al., 1996).

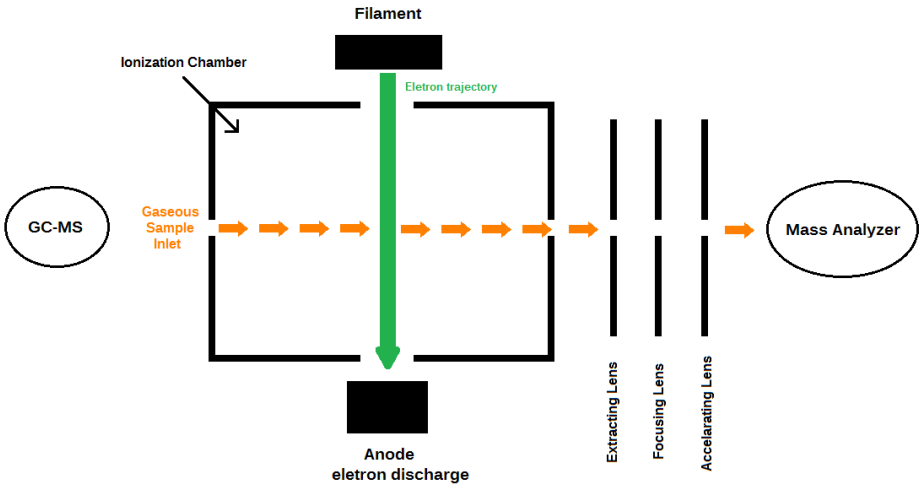


Figure 1.5. A schematic of an ion source of MS (adapted from De Hoffmann et al., 1996)

This type of ionization induces a high degree of fragmentation of the ions that is characteristic for the respective compounds and is called hard ionization. Careful analysis of the fragmentation patterns and comparison to mass spectral libraries allows structural elucidation and identification of unknown compounds (De Hoffmann et al., 1996). A series

of lenses positioned outside the ionization chamber extract the ions from the ionization chamber and accelerates them into the mass analyzer (De Hoffmann et al., 1996).

The mass analyzer separates the ions according their m/z ratios (Davis & Frearson, 1987; Mikkelsen & Cortón, 2004) and is characterized by the upper mass limit, the transmission and the resolution. The upper mass limit is the highest m/z ratio value that can be determined. The number of molecules that reached the detector divided by the number of ions produced in the source gives the transmission number. The resolution, also known as resolving power, indicates the capacity to distinguish signals with a small mass difference (De Hoffmann et al., 1996). Different types of mass analyzers are available. In this project, a single quadrupole was used and therefore limit the description to this type of analyzer. The quadrupole analyzer is composed of four parallel rods (Figure 1.6). The diagonal rods are electrically connected, divided into two pairs, creating an electrical field (De Hoffmann et al., 1996; Mikkelsen & Cortón, 2004).

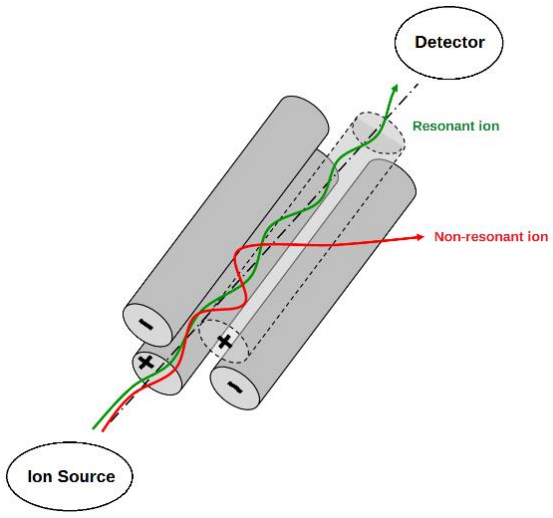


Figure 1.6. A schematic of a quadrupole analyzer of MS (adapted from De Hoffmann et al., 1996)

A radio frequency (RF) alternating field is created between the 4 rods that selectively stabilises or destabilises the paths of the ions passing through the quadrupole. Only the ions with a specific m/z ratio (resonant ions) are able to pass through the quadrupole for a specific voltage applied on the rods, whereas the other ions have unstable trajectories and collide with the rods (non-resonant ions). A quadrupole mass analyzer can be used in Singe Ion Monitoring (SIM) modus in which one m/z ion is continuously monitored by

keeping the applied voltages constant, or in Full Scan mode (FSM), in which a range of m/z values is measured by continuously varying the applied voltages (De Hoffmann et al., 1996; Mikkelsen & Cortón, 2004).

After the mass analyser, the separated ions reach the detector and the number of ions with a specific mass is counted. The resulting mass spectrum is a graph that contains the number of the ions with different masses that run through the detector (Davis & Frearson, 1987; Mikkelsen & Cortón, 2004).

1.4. Sample Preparation and validation: theoretical points

1.4.1. Sample Preparation

The BAs that will be analysed in this study are CA, CDCA, DCA, LCA and UDCA (structures shown in Figure 1.1). However, those bile acids are not volatile (Table 1.1) and therefore, require derivatisation prior to injection into a GC-MS system.

Table 1.1. Boiling Point of BAs (CA, CDCA, DCA, LCA, UDCA)

BA	CA	CDCA	DCA	LCA	UDCA
Boiling Point (°C)	200-201	165-167	174-176	183-188	203

Normally, the derivatisation process turns the sample sufficiently volatile to be eluted at reasonable temperatures without thermal decomposition or molecular rearrangement. The derivate, in general, is less polar, more volatile and more thermally stable (Orata, 2012; Sigma Aldrich, 2011). The derivatisation can also reduce analyte adsorption in the GC, this means decrease the adhesion of the analyte to an active surface of column wall and the solid support, which can improve the symmetry and the shape of the peak (Orata, 2012; Sigma Aldrich, 2011). Common derivatisation reactions for GC applications are classified into three types: alkylation, acylation and silylation (Sigma Aldrich, 2011).

Alkylation is mostly used as the first step for further derivatisations or as a method of protection of certain active hydrogens in a sample molecule (Orata, 2012). The alkylation

consists in the replacement of active hydrogen by an alkyl group (Sigma Aldrich, 2011). The most popular alkylation reaction is esterification. In this process a carboxylic acid is treated with an alcohol and an acid catalyst, to form an ester, releasing a water molecule (Figure 1.7). The ester is more volatile than the carboxylic acid (Sigma Aldrich, 2011).

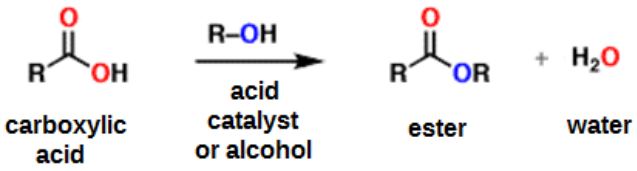


Figure 1.7. Representation of a reaction in esterification process, also known as Fischer esterification (adapted from Sigma Aldrich, 2011)

The most popular alkylation reaction is esterification. In this process a carboxylic acid is treated with an alcohol and an acid catalyst, to form an ester, releasing a water molecule (Figure 1.7). The ester is more volatile than the carboxylic acid. Figure 1.8 shows the esterification of CA to its butyl ester.

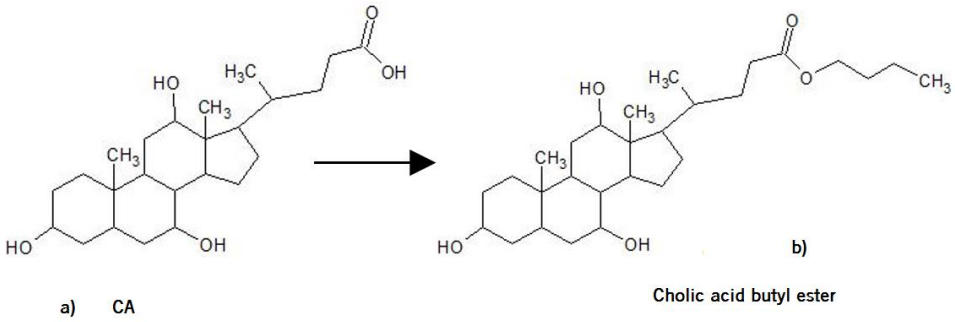


Figure 1.8. Representation of reaction in esterification process for Cholic Acid

Derivatisation by acylation converts functional groups with active hydrogen such as –OH, -SH, and –NH into esters, thioesters and amides, respectively (Sigma Aldrich, 2011).

Silylation reactions introduce a silyl group into the analyte, usually in substitution for an active hydrogen in the compound. Nearly all functional groups like hydroxyl groups, carboxylic acids, thiols, phosphates or amines can be silylated, typically by replacing a proton with a trimethylsilyl group (Orata, 2012). The Figure 1.9 represented the CA and the CA butyl ester TMS ether.

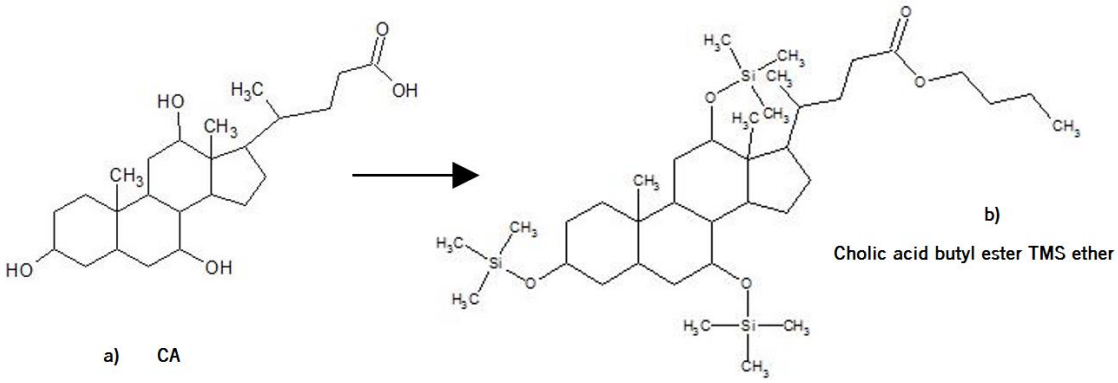


Figure 1.9. Representation of a – Cholic Acid; b – Cholic Acid after suffer esterification and silylation processes

1.4.2. VALIDATION PROCEDURE

In 1987 the Food and Drug Administration, from United States, defined validation as a "process of establishing through documented evidence a high degree of assurance that a specific process will consistently produce a product that meets its predetermined specifications and quality attributes" (FDA, 2010).

The validation procedure of an analytical method involves a number of experiments to demonstrate that the method is precise and accurate. In addition, limits of quantitation (LOQ) and limits of detection (LOD) were determined (FDA, 2015; Houben, 2010).

- **LINEARITY**

To quantify BA concentrations in unknown samples, calibration curves are constructed by plotting the peak area ratios of each BA to the internal standard for different amounts of BA. The results are fit to a straight line using linear regression analysis. Such calibration curves are characterised by a slope, an intercept and a regression coefficient (r^2). The slope represents the sensitivity of the method whereas the intercept gives an indication of the background signal. The value of r^2 represents the adjustment of a generalized linear statistical model. The r^2 should be as close as possible to 1.

Reproducibility of calibration curves is assessed by repeating the experiments on 3 different days allowing the calculation of the average, standard deviation (STDEV) and coefficient of variation (CV) of the three variables.

- **PRECISION**

The precision of a method is related to its repeatability and indicates the degree to which repeated measurements under unchanged conditions show the same results. The precision of a method is often indicated by its CV. The CV is the STDEV of the results divided by the average of the same results, and multiplied by 100. For a good precision, the CV should be low (≤10%). To assess the precision, both the repeatability within one day and the repeatability over several days is evaluated.

- **INTRADAY VARIABILITY (VARIABILITY IN 1 DAY)**

The variability on the measurements within one day is calculated for standard solutions of BA in different concentrations and for real samples. Three replicates of each standard/sample are prepared and analysed on the same day to allow calculating the CV. This experiment is repeated on a separate day.

- **INTERDAY VARIABILITY (VARIABILITY ON DIFFERENT DAYS)**

The interday variability evaluates the consistency of the results on different days. Therefore, standard BA solutions and samples were analysed on 3 different days to allow calculating the CV on these averages.

- **ACCURACY**

Accuracy indicate the closeness between the measured value and the true value (Figure 1.10).

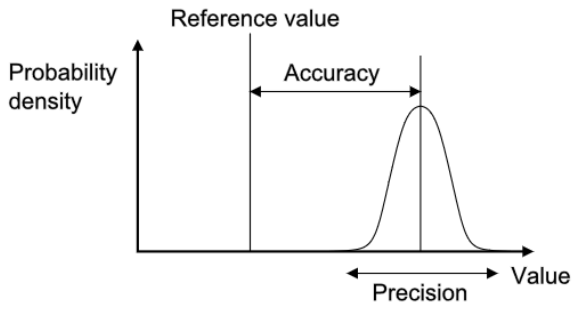


Figure 1.10. Schematic representation of accuracy and precision (Pekaje, 2007)

For standard solutions, it is the deviation of the measured value from the true value on the calibration curve, expressed as relative error.

For unknown samples, accuracy of the measurements is estimated from recovery experiments. This implicates that the concentration of each BA is measured in the sample as such (before spike) after which the sample is spiked with a known concentrations of the component and measured again (after spike). The difference in concentration between the sample after and before spike divided by the added concentration in the spike and multiplied by 100 is the recovery of the sample and should be between 80 and 120%.

- **LIMIT OF QUANTIFICATION**

The limit of quantification (LOQ) reveals the lowest concentration of an analyte that can be reliably quantified. The LOQ can be defined as the concentration at which the CV≤15% (Armbruster & Pry, 2008).

Alternatively, to the LOQ is defined as the concentration that corresponds to 10-20 times the noise level (Figure 1.11) (Huber, 2007).

- **LIMIT OF DETECTION**

The limit of detection (LOD) indicates the lowest concentration of an analyte that can be detected (Armbruster & Pry, 2008; Shrivastava, 2011). It is defined as the concentration that corresponds to a signal that is 2-3 times the height of the noise level, Figure 1.11 (Huber, 2007; Shrivastava, 2011; Skoog et al., 1997).

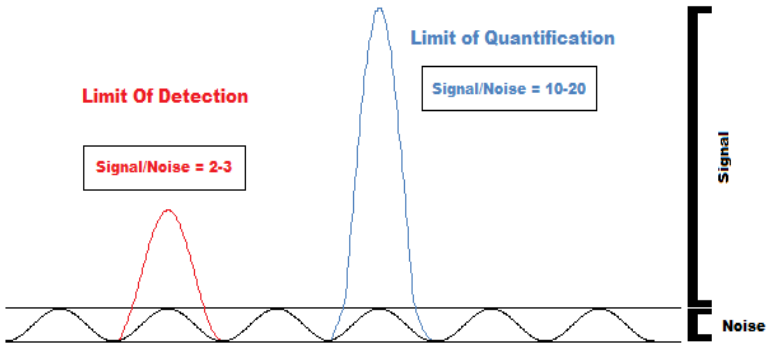


Figure 1.11. A schematic representation of LOD and LOQ basing on the noise level

CHAPTER 2

MOTIVATION AND OBJECTIVES

2.1. MOTIVATION

About 5% of bile acids (BAs) escape to enterohepatic recirculation. This escape means that the BAs are not reabsorbed in the terminal ileum going directly to the large bowel, where they are metabolized and end up excreted in faeces.

The BAs, as mentioned previously, have been implicated in a number of diseases such as obesity, type 2 diabetes, hyperlipidemia, cirrhosis, inflammatory bowel disease (IBD) and inflammatory bowel syndrome (IBS). Another diseases like large bowel cancer, colon and colorectal cancer are also related with BAs. For these extensive association with disorders and diseases, it is important analyse faecal BAs in order to use them as a biochemical markers. Theses markers might be in further a promising way to diagnosis the development of these disorders and hopefully prevent them.

There are already some studies that used HPLC, GC-MS and LC-MS to analyse faecal BAs, (Batta et al., 1999; Courillon, Gerhardt, Myara, Rocchiccioli, & Trivin, 1997; Genta Kakiyama et al., 2014), however none of these methods are validate. The validation process allows the reproducibility of the method, this means that if the same conditions of one method are made, the results obtained have to be the same, for equivalent samples.

2.2. OBJECTIVES

The main purpose of this dissertation is to develop and validate an analytical procedure for the analysis of faecal bile acids using GC-MS.

The first priority of this work was to obtain valid chromatograms, with correct shape peaks, which appear well separate and with good internal standard. After this goal achieved, the further steps were the optimization of all the procedures that occur before the GC injection.

As bile acids are not volatile as such, a derivatisation step was included in the sample preparation protocol. The derivatisation processes, esterification and silylation, were optimised by varying conditions like the amount of reagent, reaction time and temperature.

After obtaining a developed method, the precision and accuracy of calibration points have to be inside of the defined parameters, and posteriorly the precision and accuracy of faecal samples also have to correspond to the parameters established to consider this method valid.

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemical Reagents and Solutions

CA 97%, CDCA ≥97%, DCA 98.5%, UDCA 99% and LCA 98% were purchased from Sigma Aldrich (Germany). HDCA 98% (TCI Chemicals, Tokyo) was used as internal standard.

Hydrochloric acid 37% fuming (HCl) and ethanol absolute were obtained from Merck KGaA (Germain) and butanol-(1) 99.5% was purchased from Chem-Lab NV (Belgium). Hexane Chromasolv for HPLC ≥97% (GC) was obtained from Sigma-Aldrich (Germany).

Sodium Hydroxide (NaOH) was purchased from VWR Chemicals (Belgium) and the Supplier was Aldon Corporation SE.

The mixtures used for derivatisation HMDS+TMCS+Pyridine (Hexamethyldisilazane, Trimethylchlorosilane, Pyridine (3:1:9)) and N,O-bis(trimethylsilyl)acetamide, Trimethylchlorosilane, N-trimethylsilyimidazole (BSA+TMCS+TMSI; 3:2:3) were purchased from Sigma Aldrich (Germany) whereas N,O-bis(trimethylsilyl)trifluoroacetamide with 1% Trimethylchlorosilane (BSTFA+TMCS) was obtained from Grace Davison Discovery Science.

3.2. Sample Preparation

3.2.1. Preparation of stock solutions and faecal samples

Standard stock solutions were prepared by dissolving each BA in butanol with a concentration of 10 µg/µl for each compound. These standard stock solutions were further diluted 1:10 with butanol to obtain a concentration of 1 µg/µl.

A mixed standard solution was made containing the 5 bile acids, CA, CDCA, DCA, DCA, UDCA and LCA. The concentration of each compound in this solution was 0.1 µg/µl. For the reflux experiment, solutions of the BA with the same concentrations were prepared in ethanol. These mixed solutions were further diluted with butanol or ethanol, respectively, to obtain different concentrations of standard solutions to prepare calibration curves.

To validate the method, faecal samples collected at the University Hospital UZ Leuven were either freeze-dried during 70 h (Alpha 1-4 LSC, Christ) (freeze dried stool), or centrifuged at 50000 × G at 4°C for 2 h (Optima LE-80K Ultracentrifuge, Beckman) to

prepare faecal water (FW) that was further dried under a N_2 atmosphere.

3.2.2. Sample preparation

To each sample, or standard solution (0.1 µg/µl), 100 µl of internal standard [0.1 µg/µl] was added and each sample was then diluted with butanol up to a total volume of 200 µl.

The samples were sonicated for 5 min to 90 min (Ultrasonic Cleaner, VWR, Leuven, Belgium).

3.2.3. Esterification

The endstanding carboxyl function of the BAs was converted to an ester using butanol as an alcohol in the presence of an acid catalyst (50 µl HCl 12N or 20 µl H_2SO_4 36N). The amount of butanol ranged between 50 µl and 200 µl. After adding the acid catalyst, all samples were vortexed for 5 s, to homogenise the mixture. The solutions were subsequently incubated at 60°C or 70°C for 30 min to 4 h. After cooling, the samples were centrifuged at 1500 × G at room temperature for 5 min. These samples were dried with a N_2 stream until complete dryness.

3.2.4. Silylation

Silylation of the hydroxyl groups of the BAs was performed by addition of 25-100 µl of a silylation mixture (HMDS+TMCS+Pyridine (3:1:9), BSTFA+TMCS or BSA+TMCS+TMSI) to the dry residue. The samples were heated at 55°C, 60°C or 70°C for 30 min to 24 h. After centrifugation of the samples for 5 min, at 1500 × G at room temperature, samples were dried with a N_2 stream until complete dryness.

3.2.5. Extraction

The derivatised BAs were extracted using 200 µl of hexane. After centrifugation of the organic layer, the samples were analysed on a GC-MS.

3.2.6. REFLUX

To increase the solubilisation of the BAs in faecal samples, faecal samples were suspended in 5mL of absolute ethanol to which 400µl of NaOH 0.15M was added. After sonication, the solutions were refluxed in a block heater for 1 h at 80 °C. After centrifugation 10 min at 1500 × g, the supernatant was transferred to new vials and dried at 80°C for 40 min. Subsequently, samples were esterified, silylated and extracted as described above.

The recovery tests were performed by analysing the faecal samples as such and after spiking with different amounts of mixed standard solution (0.2 µg and 10 µg of BA).

3.2.7. GC-MS ANALYSIS

The gas chromatograph used was a Trace 1300 from Thermo Scientific and the mass spectrometer used was a DSQ II from Thermo Scientific.

During this project, two GC-columns were used. The first column was a HP-5MS, (5%-Phenyl)-methylpolysiloxane with 30 m of length, 0.25 mmID, 0.25 µm df, from Agilent J&W. The second column was an Rxi-1ms, Crossbond® 100% dimethyl polysiloxane with 30 m of length, 0.25 mmID, 0.25 µm df, from Restek.

The autosampler was a robotic GC pal system from Interscience.

Helium (>99.9996%) was used as a carrier gas with a constant flow of 1.0 ml/min or 1.5 ml/min. Mass spectrometric detection was performed either in full scan mode from m/z 59 to m/z 590 at 2 scans/s or in single-ion-mass mode for masses m/z 215.00, m/z 253.00 and m/z 255.00.

3.3. LINEARITY AND DETECTION LIMITS

To evaluate the linearity, calibration curves were made with different concentrations of mixed standard solution (solution with 5 bile acids, CA, CDCA, DCA, UDCA and LCA). Amounts of BAs varied from 0.05 µg to 50 µg.

Recovery tests were performed by analysing the faecal samples as such and after spiking with different amounts of mixed standard solution (1 µg and 10 µg of BA).

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Instrument Method Development

Standard solutions of bile acids (20 µg of each BA) were esterified with butanol by addition of 50 µl HCl 12N and incubation for 4 h at 60°C and subsequently silylated with 100 µl of HMDS+TMCS+Pyridine (3:1:9) and incubation for 30 min at 55°C.

The GC-MS method described by (Keller & Jahreis, 2004) was used as the starting point. The GC-column was a HP-5MS column (Courillon, Gerhardt, Myara, Rocchiccioli, & Trivin, 1997) and the temperature program ranged from 150°C to 298°C as depicted in Figure 4.1. For this first method, the helium flow was set at 1.0 ml/min and samples were injected in a splitless mode. The MS was operated in full scan.

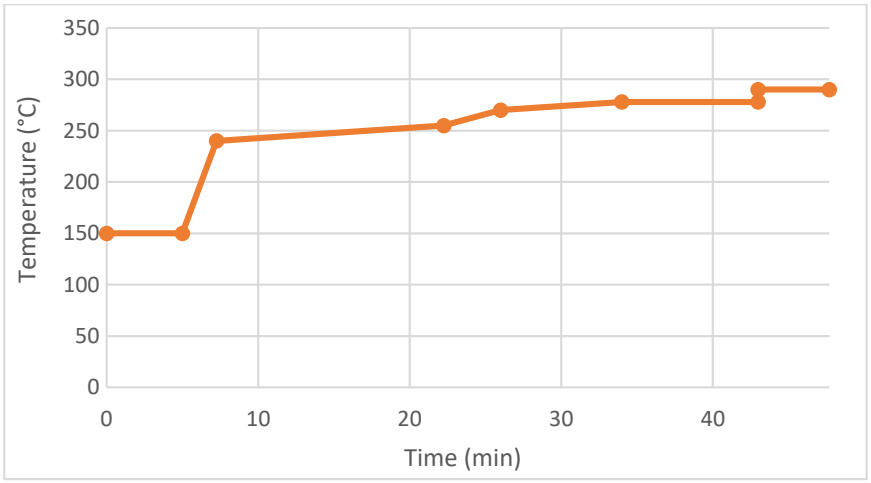


Figure 4.1. Temperature program data of first method

The chromatograms, obtained under the conditions described for method 1, of CA, HDCA (internal standard) and of a mixture solution are presented in Figure 4.2.

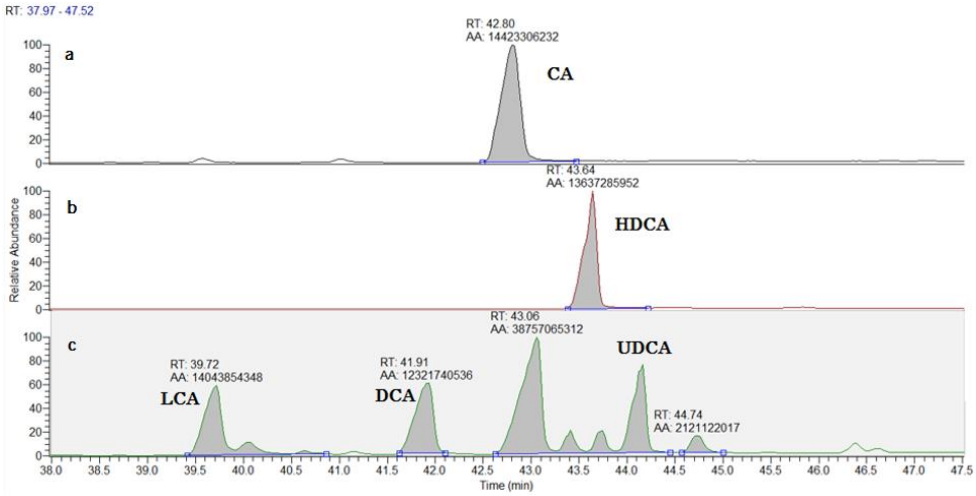


Figure 4.2. GC chromatograms resultants of initial experiments analysis in a range of retention time since 37.97 until 47.52 for a – CA; b – HDCA; c – mixture (LCA, DCA, CDCA, CA and UDCA)

It is clear from those chromatograms that the shape of the peaks was not symmetric with fronting in all BA peaks. In addition, the peaks had a rather long retention time with the first peak of interest only eluting from the column after more than 39 min. Furthermore, CA and CDCA were not separated and coeluted from the column when analysing the mixture of BAs.

Therefore, the temperature program was modified for this second method by accelerating the increase in temperature, to reduce the retention time, as shown in Figure 4.3, In addition, the helium flow was increased from 1.0 ml/min to 1.5 ml/min.

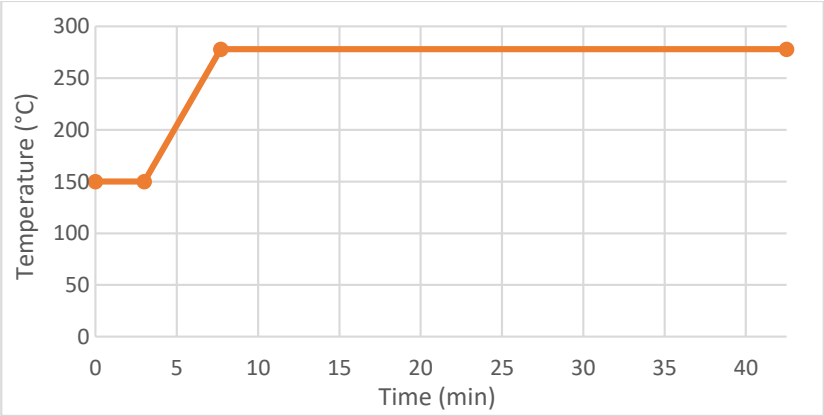


Figure 4.3. Temperature program data of second method

Figure 4.4 shows the combination of the individual chromatogram in just one chromatogram, obtained with the second method settings.

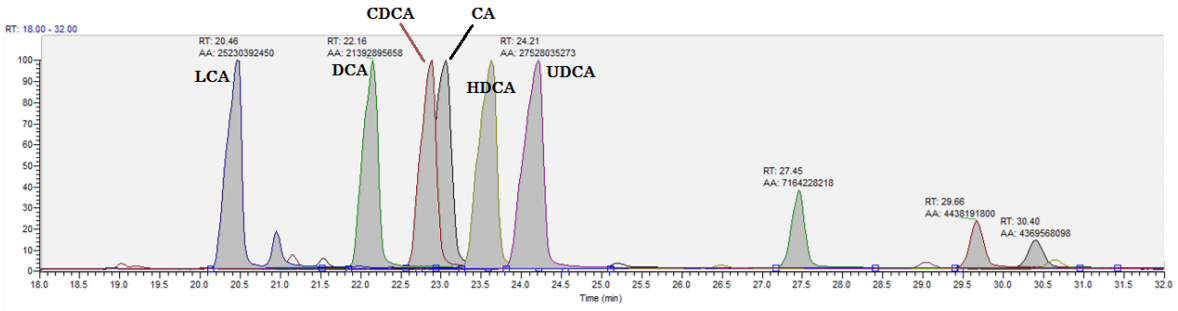


Figure 4.4. GC chromatogram obtained due combination of individual chromatograms (CA, CDCA, DCA, LCA and UDCA) and HDCA chromatogram analysed in a range of retention time from 18.00 to 32.00

These modifications on the temperature program resulted in a clearly more symmetric peak shape and shorter retention times. Under these conditions, LCA elutes already after

20 min from the column.

Unfortunately, although separation between CDCA and CA was improved, the separation between both compounds was still far from a baseline separation.

A further possibility to get smaller peaks and improve separation is to change the injection modus from splitless to split.

However, applying a split flow of 50 ml/min or 25 ml/min did not further improve nor deteriorate the separation between CA and CDCA. To prevent overloading of the column and contamination of the ion source, the use of split injection with a split flow of 25 ml/min was kept in all further experiments.

4.1.1. Column HP-5MS versus Column Rxi-1MS

Since the CA and CDCA peaks were overlapping, the analytical column was switched from a HP-5MS column (Courillon et al., 1997), which contained 95% of polysiloxane, to a more apolar Rxi-1MS column containing 100% polysiloxane (Batta et al., 1999). Figure 4.5 shows the chromatograms obtained with the HP-5MS and the Rxi-1MS columns.

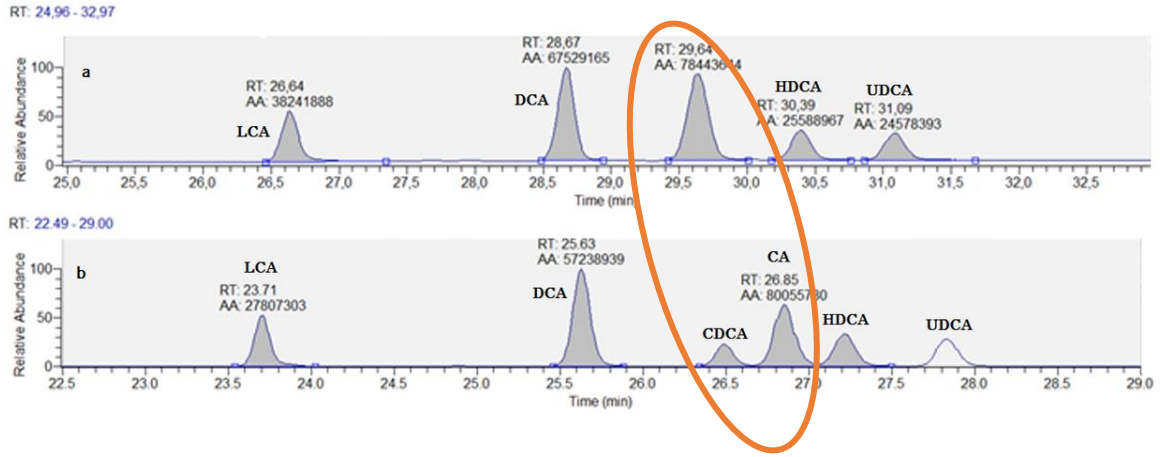


Figure 4.5. Analysis of a BA mixture on a HP 5MA column – a; Rxi 1MS column – b; using second method

It is clear that the Rxi-1MS analytical column solved the problem of overlapping CA and CDCA peaks.

Using this column, all peaks were separated and could be identified and quantified. As there was no further need to measure in full scan mode, all further experiments were

performed in single ion monitoring mode. Reducing the number of ions to be measured results in improved sensitivity.

4.2. Optimization of the derivatisation procedure

4.2.1. Standard Solutions

4.2.1.1. Conditions of Esterification

Three aspects were taken into consideration during the esterification: the temperature the time and the type of acid catalyst.

- **Temperature and Time**

The impact of the temperature and time during the esterification reaction is shown in Figure 4.6. It was possible to observe that extending the time up to 24 h did not improve the esterification and even resulted in lower peak areas for CDCA and CA.

Since the results are difficult to analyse just with Figure 4.6, a statistical analysis was made. The two parameters, temperature and time, were evaluated separately with F-test in the one-way anova.

For temperature, after analyzing Table 4.1 and Figure 4.7 it is possible to conclude that independently of BAs, there are no statistically significant differences between any pair of means at the 95.0% confidence level. Therefore, and taking into consideration the savings energy, the lowest temperature, i.e. 60°C, was selected to be used in all further experiments.

According with Table 4.2 and Figure 4.8, for time, it is possible to see that there are statistically significant differences with three groups: - 2 h and 4 h; 0.5, 1, 1.5, 6 and 16 h; and 24 h. Since the intensity of the area is bigger for 2 and 4 h hours category, the optimized time chosen was the lowest value 2 h.

To conclude, for esterification the optimized temperature was 60°C and the optimized time was 2 h.

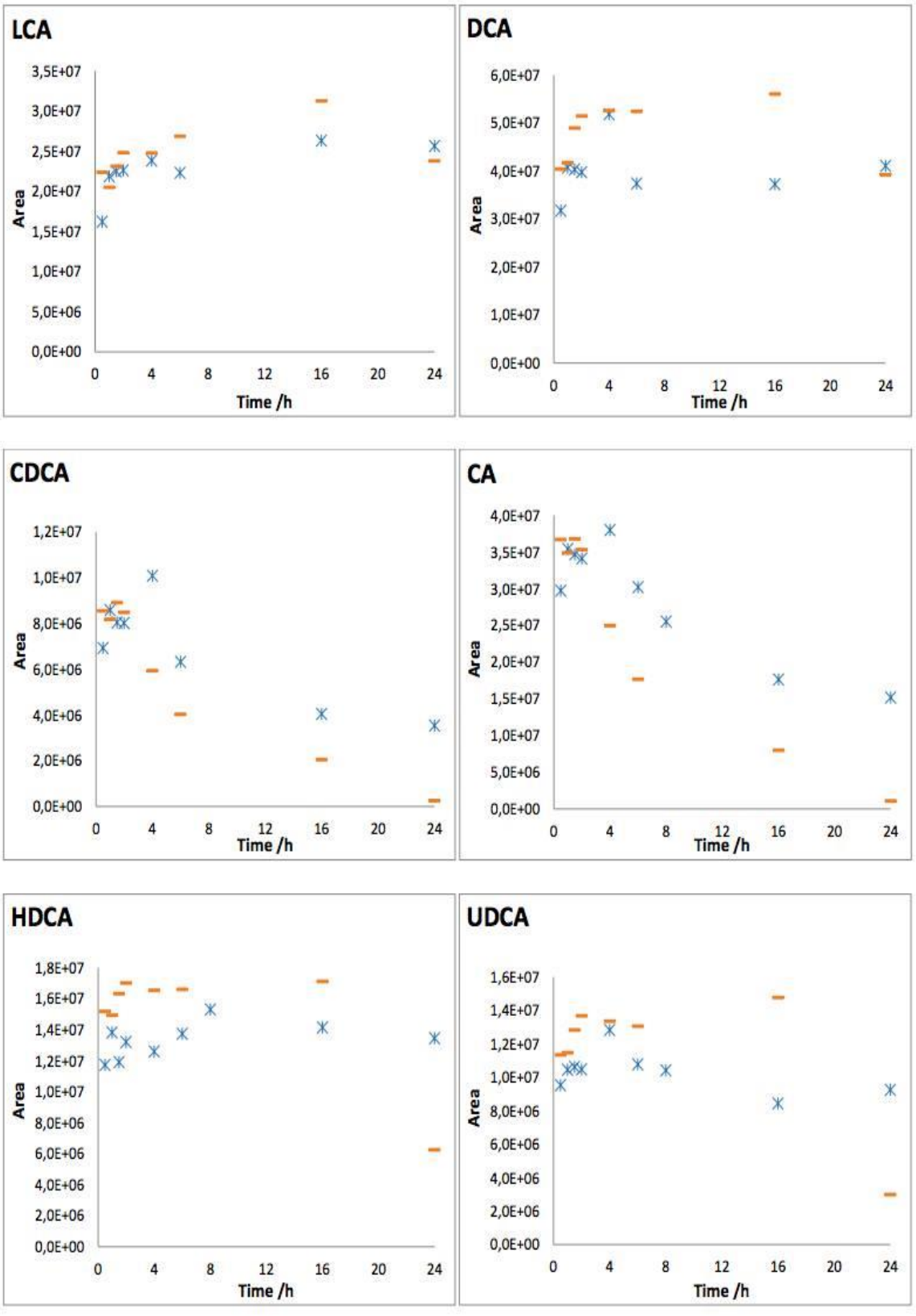


Figure 4.6. Peak areas, obtained by GC-MS, of the individual BAs as a function of time (30 min-24h) and temperature (60°C or 70°C) during esterification. The blue points represent the experiment at 60°C and the orange ones represent at 70°C

Temperature	Count	Mean	Homogeneous Groups
60	96	2.03974E7	X
70	96	2.15819E7	X

Table 4.1. F-test table that indicated the statistically significant difference to different temperatures in esterification, for a confidence level of 95.0%

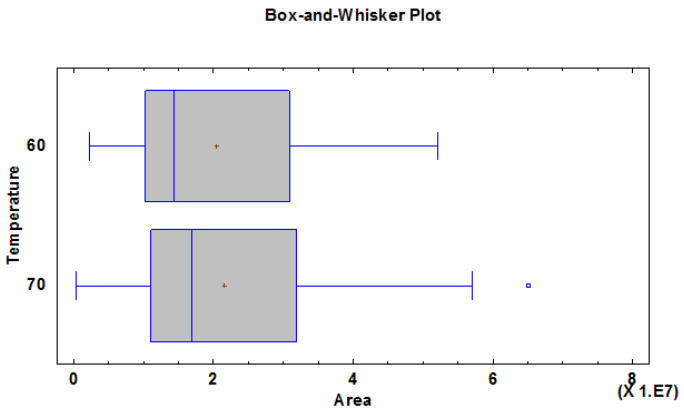


Figure 4.7. Box and Whisker Plot of different temperatures in esterification, for a confidence level of 95.0%

Time	Count	Mean	Homogeneous Groups
24	24	1.51575E7	X
16	24	1.97602E7	XX
0.5	24	2.00436E7	XX
6	24	2.09535E7	XX
1	24	2.18845E7	XX
1.5	24	2.29203E7	XX
2	24	2.32524E7	X
4	24	2.39452E7	X

Table 4.2. F-test table that indicated the statistically significant difference to different times in esterification, for a confidence level of 95.0%

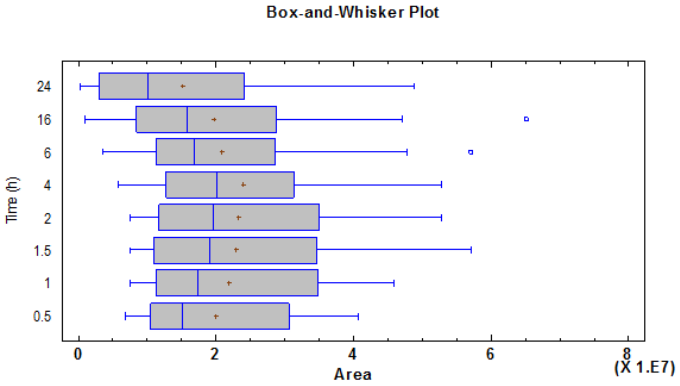


Figure 4.8. Box and Whisker Plot of different times in esterification, for a confidence level of 95.0%

- **ACID CATALYST**

The esterification of acids with alcohols requires a strong (non-carboxylic) acid as catalyst. As in many cases (Batta et al., 1999; Birk, Dippold, Wiesenberg, & Glaser, 2012; Courillon et al., 1997; Keller & Jahreis, 2004) the HCl 12N was tested, in parallel with H_2SO_4 36N.

The results (Figure 4.9) demonstrate that H_2SO_4 as a acid catalyst clearly decreased the efficiency of the esterification reaction. Only a small peak of LCA could be observed in the chromatogram whereas the remaining BAs were not esterified. No further experiments were performed using H_2SO_4, and HCl was used in all further experiments.

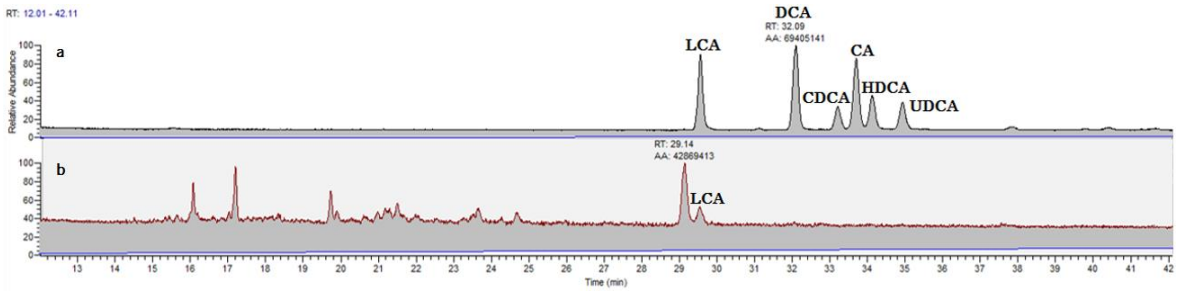


Figure 4.9. GC chromatograms of a mixture standards solution of BAs using a – HCl; b – H_2SO_4 for esterification

4.2.1.2. Conditions of Silylation

A few parameters are important to optimise the conditions of silylation. Four of them have been tested, which are the type and amount of silylation reagent, the temperature, and the time of silylation.

- **Silylation Reagent**

i. HMDS+TMCS+Pyridine (3:1:9) versus BSTFA+1%TMCS

The HMDS+TMCS+Pyridine (3:1:9) solution had been described in previous studies to silylate BA (Batta et al., 1999; Keller & Jahreis, 2004). The efficiency of HMDS+TMCS+Pyridine (3:1:9) and BSTFA+1%TMCS was compared to silylate a mixed standard solution of BAs. The resulting chromatograms are presented in Figure 4.10.

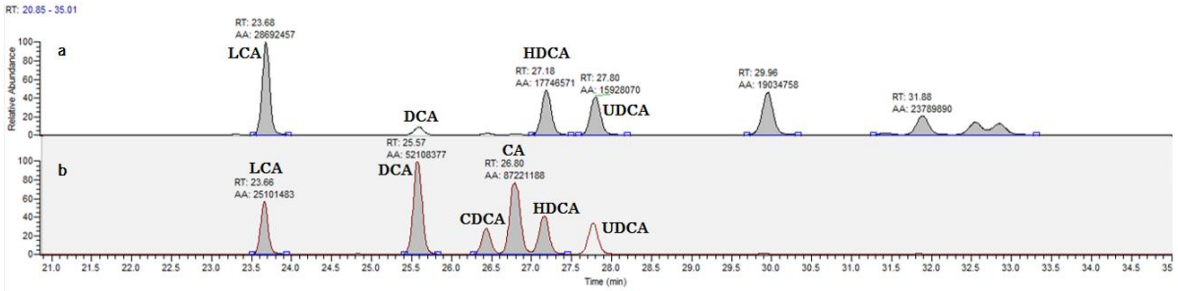


Figure 4.10. GC chromatograms of a mixed standard solution of BAs obtained with a – BSTFA+1%TMCS; b – HMDS+TMCS+Pyridine (3:1:9) as silylating reagent

When BSTFA+1%TMCS was used as silylating reagent, the DCA peak was clearly smaller than when using HMDS+TMCS+Pyridine (3:1:9). No peak at the retention time of CDCA nor CA was observed whereas additional peaks eluted in the last part of the chromatogram.

Figure 4.11 shows the chromatograms of the individual BAs after derivatisation with BSTFA+1%TMCS.

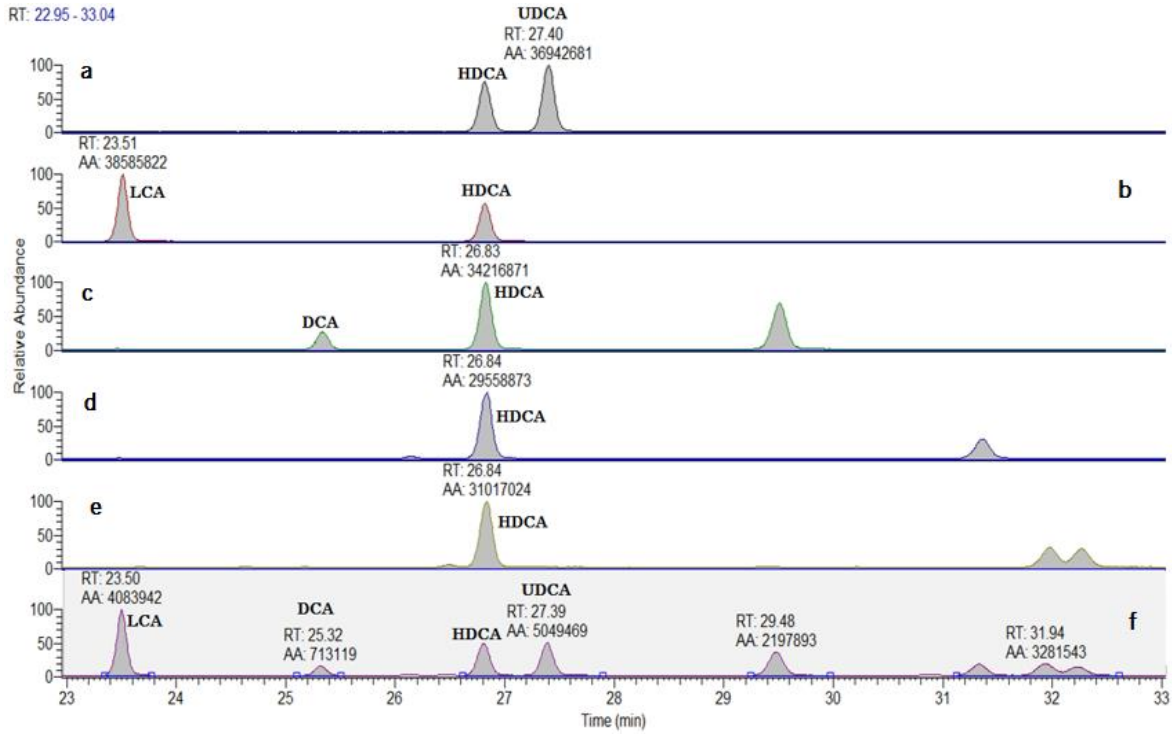


Figure 4.11. GC chromatograms of standard solutions of BAs (a – UDCA; b – LCA; c – DCA; d – CDCA; e – CA; f – mixed standard solution) obtained with BSTFA+1%TMCS as silylating reagent

It is likely that BSTFA+1%TMCS is a less strong silylating reagent compared to HMDS+TMCS+Pyridine (3:1:9), resulting in silylation of only one or two hydroxyl groups. Those mono- or di-silylated derivatives of the BAs are more polar and less volatile than the tri-silylated analogue and therefore have a longer retention time on the GC-column (Drozd, 1981; Kataoka, 1996). Increasing the temperature to 70°C and extending the duration of incubation to 24 h did not improve the efficiency of the silylation reaction as is shown in Figure 4.12.

As already mentioned, with these results, even raising the temperature and the time, it is possible to conclude that BSTFA+1%TMCS solution is not capable, not strong enough, to silylate all the hydroxyl groups. For this reason, the HMDS+TMCS+Pyridine (3:1:9) was used in further experiments.

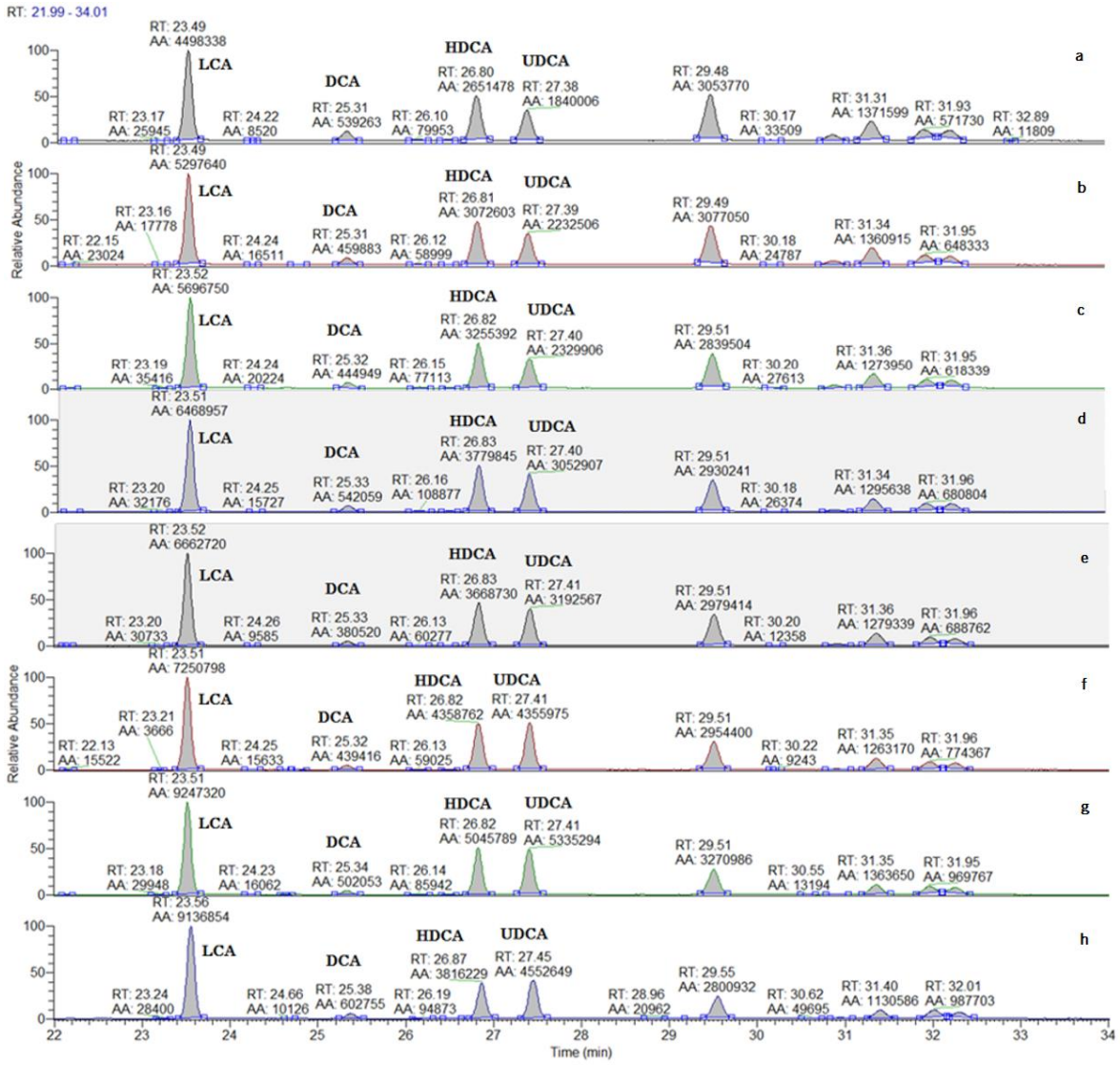


Figure 4.12. GC chromatograms of a mixed standard solutions of BAs using BSTFA+1%TMCS as silylating reagent at 70°C for a – 0.5 h; b – 1 h; c – 1.5 h; d – 2 h; e – 4 h; f – 6 h; g – 8 h; h – 24 h

ii. HMDS+TMCS+Pyridine (3:1:9) versus BSA+TMCS+TMSI (3:2:3)

According to Suzuki et al., (1997), the addition of TMSI, to the mixture of BSA+TMCS might increase the silylating capacity for even strongly hindered hydroxyl groups. Figure 4.13 shows the chromatograms of the mixed standard solution of BAs and the individual solutions using BSA+TMCS+TMSI as silylating reagent.

After observing the results (Figure 4.13), it was possible to confirm that the addition of TMSI to the silylating reagent improved the derivation of CA and CDCA, but resulted in multiple peaks for UDCA. Therefore, the original reagent HMDS+TMCS+Pyridine (3:1:9) was kept in all further experiments.

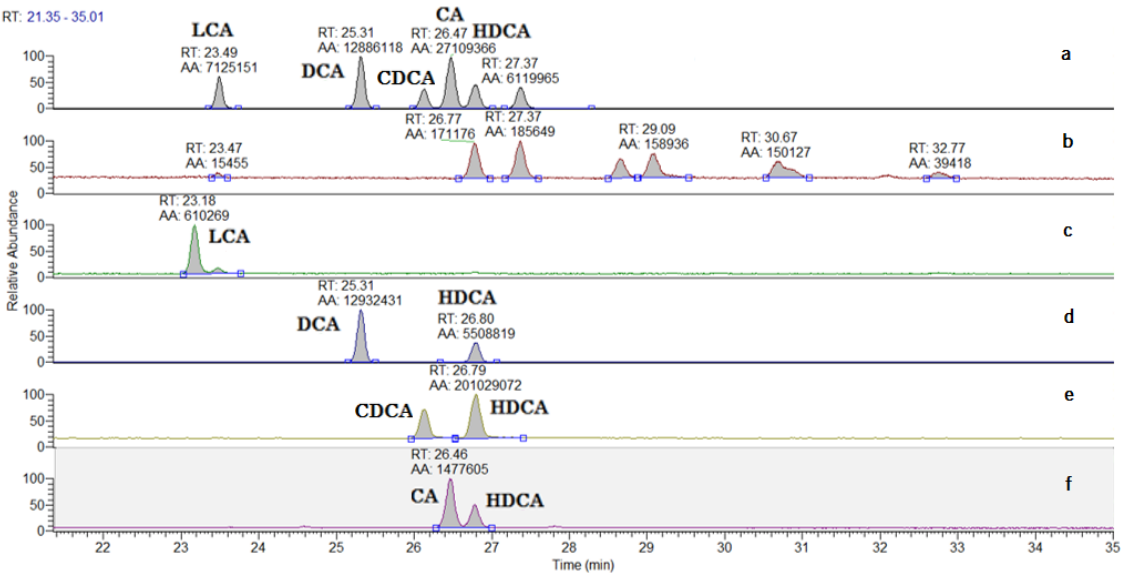


Figure 4.13. GC chromatograms with BSA+TMCS+TMSI for a – mixture; b – UDCA; c – LCA; d – DCA; e – CDCA; f – CA

- **AMOUNT OF SILYLATION REAGENT**

The amounts of silylation reagent were varied from 25 µl to 100 µl. The areas of each BA increased with increasing amount of silylating reagent (Figure 4.14).

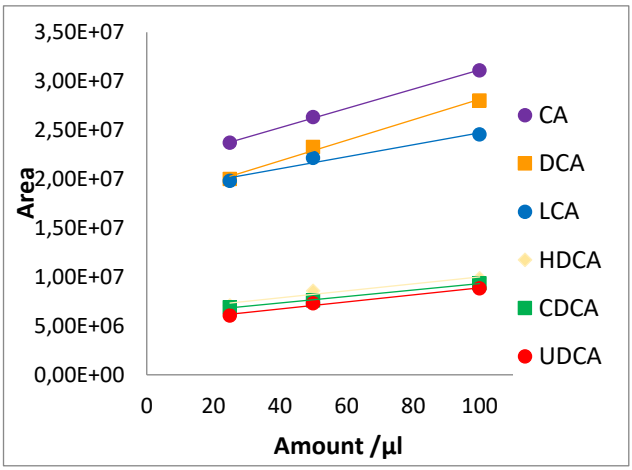


Figure 4.14. Impact of different amounts of HMDS:TMCS:Pyridine on the peak areas of the resulting BA derivatives

After analysing all the results, it is possible to conclude that 100 µl is the amount more favourable when compared with 25 µl and 50 µl. Therefore, all further experiments were performed with 100 µl of silylating reagent.

- **TEMPERATURE AND TIME**

The temperature of the silylation reaction was varied between 55°C and 70°C. For each incubation temperature, time of incubation ranged from 30 min, to 8 h (Figure 4.15).

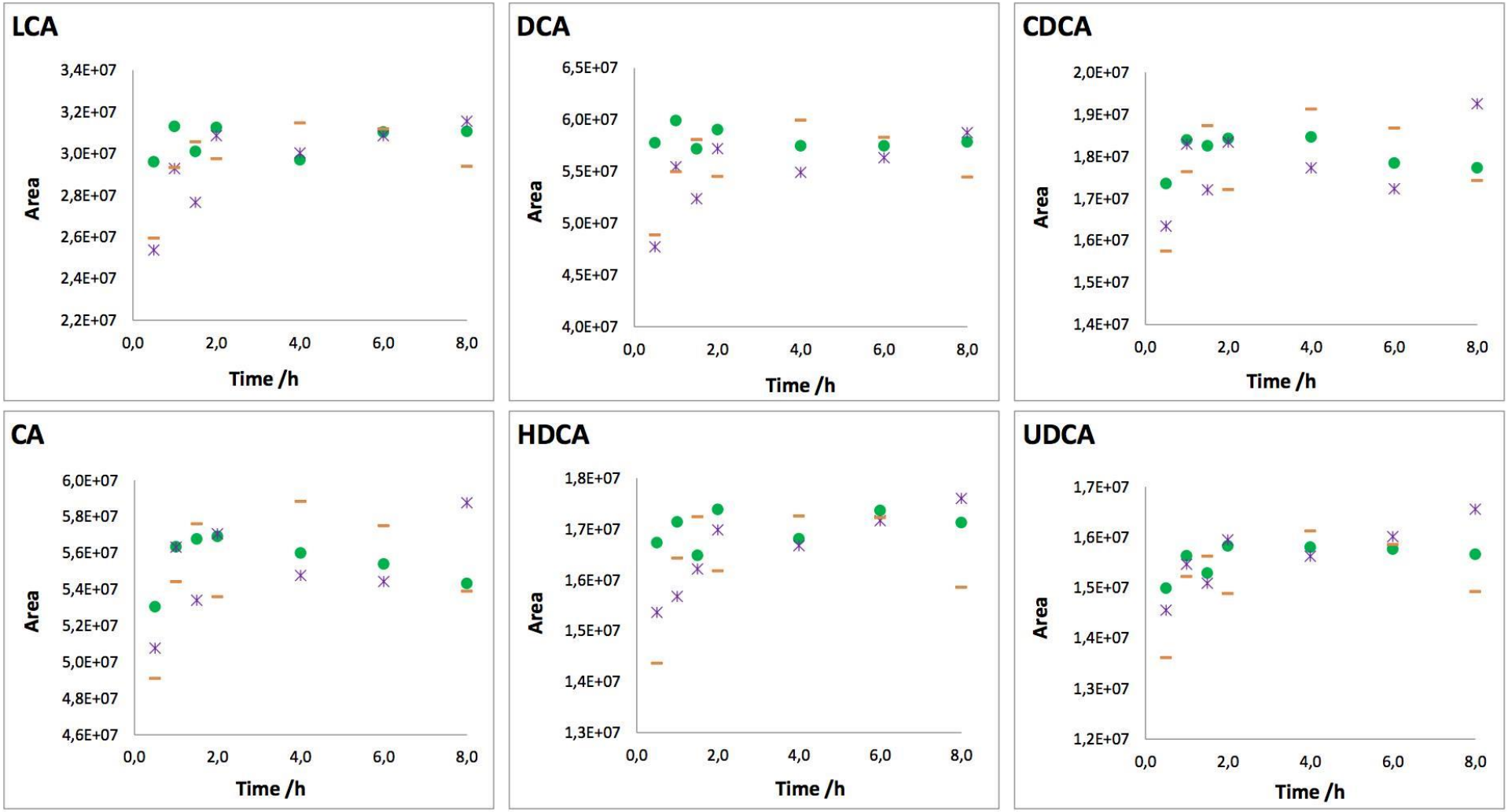


Figure 4.15. Peak areas, obtained by GC-MS, of the individual BAs as a function of time (30 min-8h) and temperature (50°C, 60°C or 70°C) during silylation. The green symbols represent the 55°C, the purple symbols show the results for 60°C and the orange symbols symbolize the 70°C

As the results are not consistently for all BAs, it was made a statistical analysis. The Table 4.3 and Figure 4.16 represent the statistical analysis results for temperature and Table 4.4 and Figure 4.17 represent the same results for time. It is possible to see that, independently of BAs, for both parameters there are no statistically significant differences between any pair of means at the 95.0% confidence level. For this reason, it was decided to choose the lowest temperature for economic reasons (saving energy) and the lowest time.

Temperature	Count	Mean	Homogeneous Groups
60	84	3.15096E7	X
70	78	3.16466E7	X
55	84	3.24816E7	X

Table 4.3. F-test table that indicated the statistically significant difference to different temperatures in silylation, for a confidence level of 95.0%

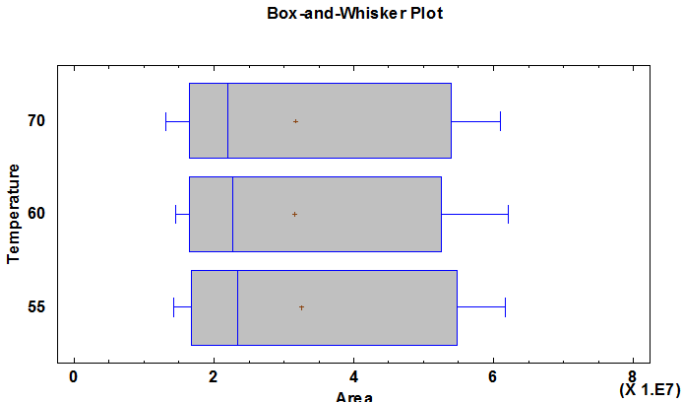


Figure 4.16. Box and Whisker Plot of different temperatures in silylation, for a confidence level of 95.0%

Time	Count	Mean	Homogeneous Groups
0.5	36	2.9295E7	X
1	36	3.15684E7	X
2	36	3.23026E7	X
1.5	36	3.23912E7	X
6	36	3.25399E7	X
4	36	3.26011E7	X
8	30	3.26189E7	X

Table 4.4. F-test table that indicated the statistically significant difference to different times in silylation, for a confidence level of 95.0%

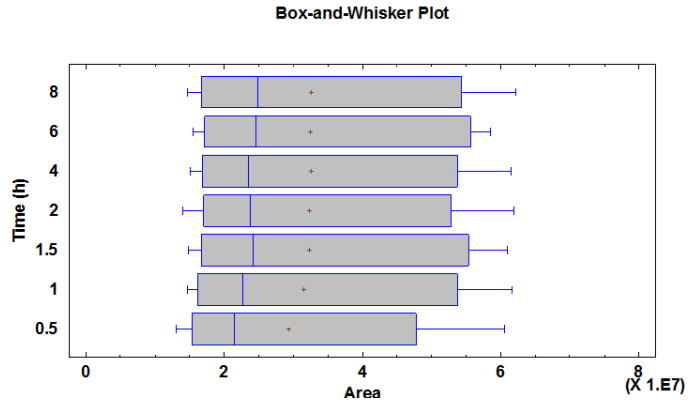


Figure 4.17. Box and Whisker Plot of different times in silylation, for a confidence level of 95.0%

To conclude, for silylation the optimized temperature was 55°C and the optimized time was 30 min.

4.2.2. FAECAL SAMPLES: ADDITION OF A SONICATION STEP

Lyophilised faecal samples were suspended in butanol and subjected to sonication prior to esterification in an attempt to increase the efficiency of the derivatisation procedure.

Figure 4.18 shows the impact of duration of this sonication step.

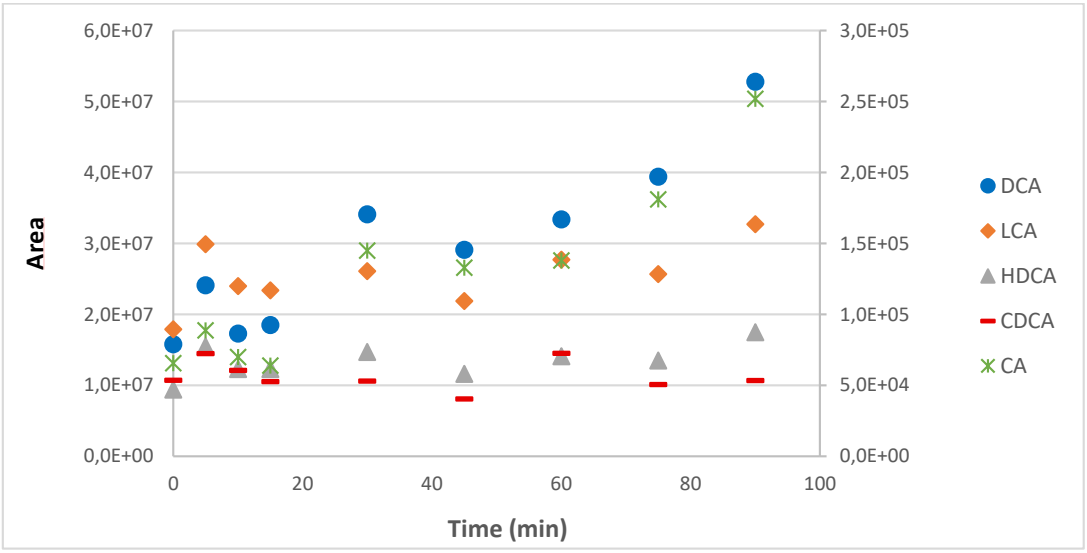


Figure 4.18. Results of areas obtained in different times of sonication in CA, CDCA, DCA, LCA and HDCA. The CA and CDCA have the values of right scale

Increasing the time of sonication increased the area of the resulting BA derivatives. A maximal increase was observed after 90 min of sonication. Therefore, this sonication for 90 min was included in the preparation of all samples and standard solutions for the validation process.

4.3. Validation Procedure

4.3.1. Standard Solutions

- **Precision (intraday and interday variability)**

The precision of the measurement of the solutions was evaluated by analysing each dilution three times on the same day (intraday variability). For each BA and each amount of BA, the CV was calculated (Table 4.5). This analysis was repeated on three consecutive days. The mean value for each BA per day was used to calculate the CV over three days reflecting the interday variability (Table 4 6).

For the solutions with amounts of BAs up to 0.5 µg, the intraday variability exceeded the accepted limit of 10% on at least 1 day. Nevertheless, the interday variability remained below 10% for all BAs and all amounts except for 0.05 µg.

Table 4.5. Intraday variability (n=3) on measurement of individual bile acids, expressed as CV. The measurements that fulfil the criterion (CV≤10%) are coloured in green

Amount of BA	CV of three measurements performed on the same day														
	CA			CDCA			DCA			LCA			UDCA		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
0.05	70	14	1	56	26	6	81	21	4	90	12	6	67	14	22
0.1	15	10	13	11	7	10	8	8	17	6	3	19	22	12	6
0.25	19	5	1	12	5	1	15	6	8	9	6	5	14	6	3
0.5	12	1	5	14	3	4	11	1	3	7	6	3	9	3	3
0.75	9	2	5	10	1	5	10	2	5	3	1	5	13	3	4
1	6	6	3	6	5	4	5	5	7	4	3	5	5	3	2
2.5	3	7	11	3	7	11	2	9	10	4	11	8	2	8	9
5	7	4	1	8	5	1	4	5	0	8	6	1	4	4	0
7.5	5	2	2	7	3	6	9	2	1	8	5	6	5	3	2
10	8	4	4	5	5	5	3	5	2	2	8	1	2	4	1
25	11	5	8	13	9	9	6	5	11	3	8	12	8	4	9
50	7	5	3	8	6	3	5	7	4	7	9	6	4	6	5

Table 4 6. Interday variability (n=3) on measurement of individual bile acids, expressed as CV. The measurements that fulfil the criterion (CV≤10%) are coloured in green and the measurement that do not fulfil the criterion are coloured in red

BA	Parameters	Amount of BA (µg)											
		0.05	0.1	0.25	0.5	0.75	1	2.5	5	7.5	10	25	50
CA	Average	0.078	0.109	0.253	0.519	0.819	1.157	2.452	4.975	7.794	11.954	30.762	54.201
	STDEV	0.013	0.003	0.014	0.011	0.021	0.019	0.027	0.076	0.087	0.048	1.516	4.369
	CV	16	3	6	2	3	2	1	2	1	0	5	8
CDCA	Average	0.073	0.104	0.251	0.517	0.796	1.100	2.341	5.063	7.874	11.134	27.524	53.616
	STDEV	0.011	0.001	0.019	0.011	0.023	0.020	0.029	0.056	0.164	0.119	0.854	0.345
	CV	15	1	7	2	3	2	1	1	2	1	3	1
DCA	Average	0.077	0.108	0.271	0.544	0.845	1.331	2.512	5.209	8.271	11.860	28.340	56.998
	STDEV	0.020	0.007	0.018	0.015	0.020	0.024	0.015	0.021	0.171	0.117	1.034	0.424
	CV	27	7	7	3	2	2	1	0	2	1	4	1
LCA	Average	0.082	0.107	0.265	0.543	0.854	1.164	2.518	5.195	8.286	11.947	28.666	57.162
	STDEV	0.023	0.008	0.019	0.015	0.024	0.017	0.013	0.051	0.142	0.098	0.462	0.194
	CV	28	8	7	3	3	1	1	1	2	1	2	0
UDCA	Average	0.071	0.107	0.256	0.511	0.797	1.129	2.424	5.011	7.947	11.366	29.206	53.794
	STDEV	0.008	0.005	0.005	0.019	0.010	0.014	0.021	0.071	0.195	0.145	0.918	0.374
	CV	12	5	2	4	1	1	1	1	2	1	3	1

- **LIMITATION OF QUANTIFICATION AND DETECTION**

The limit of quantification (LOQ) is the lowest amount of compound with a CV below 15%. The interday CV fulfilled this criterion for all BAs at an amount of 0.1 µg and was even 0.05 µg for UDCA (Table 4 6).

The limit of detection (LOD) was determined based on the signal-to-noise ratio (SN). The LOD is defined as the concentration that corresponds 2-3 times to the height of the noise level. Figure 4.19 shows a chromatogram of a solution containing 0.05 µg of each BA.

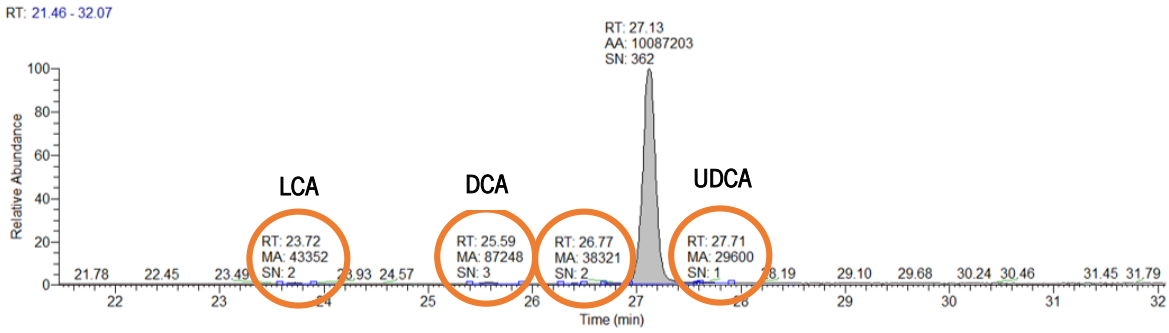


Figure 4.19. GC chromatogram of standard solution containing 0.05 µg of each BA

- **LINEARITY**

The measurements presented in Table 4.7 were used to construct three different calibration curves in different concentration ranges, each characterized by a slope, intercept and regression coefficient (r^2). These curves were prepared on 3 different days. The results can be found in Table 4.7.

The minimal value of the regression coefficient, r^2, was 0.9788 and the majority of the values were higher than 0.99 indicating a good linearity of the calibration curves.

Table 4.7. Characteristics of calibration curves for each BA constructed on 3 different days

BAs	Stock Solutions (μg)	Day 1			Day 2			Day 3		
		slope	intercept	r^2	slope	intercept	r^2	slope	intercept	r^2
LCA	0.05 – 1	0.0935	0.0017	**0.9955**	0.0963	-0.0005	**0.9963**	0.0940	-0.0001	**0.9980**
	0.5 – 10	0.1652	-0.1093	**0.9889**	0.1737	-0.1123	**0.9883**	0.1634	-0.1056	**0.9919**
	5 – 50	0.2835	-0.9602	**0.9995**	0.2341	-0.4151	**0.9998**	0.2658	-0.7827	**0.9994**
DCA	0.05 – 1	0.1434	0.0034	**0.9972**	0.1451	-0.0001	**0.9959**	0.1442	0.0007	**0.9988**
	0.5 – 10	0.2578	-0.1593	**0.9914**	0.2669	-0.1739	**0.9881**	0.2552	-0.1568	**0.9928**
	5 – 50	0.4382	-1.4523	**0.9995**	0.4155	-1.2083	**0.9989**	0.3943	-0.9643	**0.9986**
CDCA	0.05 – 1	0.0357	-0.0001	**0.9970**	0.0354	-0.0004	**0.9952**	0.0348	-0.0002	**0.9991**
	0.5 – 10	0.0584	-0.0221	**0.9940**	0.0603	-0.0262	**0.9913**	0.0581	-0.0230	**0.9950**
	5 – 50	0.0829	-0.1660	**0.9999**	0.0819	-0.1203	**0.9998**	0.0764	-0.0797	**0.9974**
CA	0.05 – 1	0.0984	-0.0009	**0.9945**	0.0975	-0.0023	**0.9913**	0.0941	-0.0016	**0.9971**
	0.5 – 10	0.2183	-0.1716	**0.9827**	0.2198	-0.1783	**0.9788**	0.2226	-0.1914	**0.9801**
	5 – 50	0.2429	-0.3819	**0.9864**	0.2468	-0.4091	**0.9825**	0.2512	-0.4364	**0.9870**
UDCA	0.05 – 1	0.0404	-0.0005	**0.9978**	0.0400	-0.0012	**0.9923**	0.0388	-0.0009	**0.9967**
	0.5 – 10	0.0736	-0.0417	**0.9941**	0.0763	-0.0497	**0.9871**	0.0726	-0.0449	**0.9922**
	5 – 50	0.0946	-0.0851	**0.9981**	0.0895	-0.0287	**0.9983**	0.0855	-0.0058	**0.9919**

- **INTRADAY**

The CV on the slope, intercept and regression coefficient values on the 3 days was calculated as a measure of the interday variability (Table 4.8).

The CV of slope and r^2 values was consistently lower than 10%. However, the variability of the intercept was higher for all BAs in the low-range calibration curve and for all BAs but CA in the high range calibration curve.

Table 4.8. CV of the slope, intercept and r^2 value of calibration curves constructed on 3 days. The measurements that fulfil the criterion (CV≤10%) are coloured in green and the measurement that do not fulfil the criterion are coloured in red

Stock Solution (µg)			CA	CDCA	DCA	LCA	UDCA
0.05 – 1	slope	Average	0.0967	0.0353	0.1442	0.0946	0.0397
		STDEV	0.0023	0.0004	0.0009	0.0015	0.0008
		CV	**2.33**	**1.27**	**0.59**	**1.58**	**2.04**
	intercept	Average	-0.0016	-0.0002	0.0013	0.0004	-0.0008
		STDEV	0.0007	0.0002	0.0018	0.0012	0.0004
		CV	**-41.91**	**-86.39**	**139.42**	**328.84**	**-42.58**
	r^2	Average	0.9943	0.9971	0.9973	0.9966	0.9956
		STDEV	0.0029	0.0020	0.0014	0.0013	0.0029
		CV	**0.29**	**0.20**	**0.14**	**0.13**	**0.29**
0.5 – 10	slope	Average	0.2202	0.0589	0.2600	0.1674	0.0742
		STDEV	0.0022	0.0012	0.0061	0.0055	0.0019
		CV	**0.99**	**2.05**	**2.36**	**3.28**	**2.53**
	intercept	Average	-0.1805	-0.0238	-0.1633	-0.1091	-0.0454
		STDEV	0.0101	0.0021	0.0093	0.0034	0.0040
		CV	**-5.57**	**-9.02**	**-5.68**	**-3.09**	**-8.83**
	r^2	Average	0.9805	0.9935	0.9908	0.9897	0.9911
		STDEV	0.0020	0.0019	0.0024	0.0020	0.0036
		CV	**0.20**	**0.19**	**0.24**	**0.20**	**0.36**
5 – 50	slope	Average	0.2470	0.0804	0.4160	0.2611	0.0898
		STDEV	0.0042	0.0035	0.0219	0.0250	0.0046
		CV	**1.69**	**4.37**	**5.27**	**9.59**	**5.10**
	intercept	Average	-0.4091	-0.1220	-1.2083	-0.7193	-0.0399
		STDEV	0.0273	0.0432	0.2440	0.2780	0.0408
		CV	**-6.66**	**-35.39**	**-20.19**	**-38.65**	**-102.30**
	r^2	Average	0.9853	0.9990	0.9990	0.9996	0.9961
		STDEV	0.0024	0.0014	0.0005	0.0002	0.0036
		CV	**0.25**	**0.14**	**0.05**	**0.02**	**0.36**

- **ACCURACY**

Table 4.9 shows the accuracy of the calibration points which is expressed as the relative error between the measured value and the true value. The results are the mean values for the measurements on 3 days and triplicate samples. Only the lowest amount of BAs (0.05 µg) display a relative error above 10% whereas the accuracy is within the limits of 10% deviation for all other amounts of BA.

Table 4.9. Mean relative error of each calibration point and each BA. The measurements that fulfil the criterion (CV≤10%) are coloured in green and the measurement that do not fulfil the criterion are coloured in red

Concentration points	BAs				
	LCA	DCA	CDCA	CA	UDCA
0.05	-44	-34	-36	-40	-29
0.1	6	5	4	2	1
0.25	7	5	7	9	6
0.5	5	4	4	7	6
0.75	0	1	1	2	3
1	-2	-2	-2	-4	-3
2.5	8	8	10	10	10
5	8	6	3	10	8
7.5	3	3	2	7	3
10	5	-4	-3	-7	-4
25	0	0	-2	-10	-7
50	0	0	0	3	1

4.3.2 Faecal Samples

- **Precision**

Three different freeze-dried stool samples (S1, S2, S3) and three faecal water samples (FW1, FW2, FW3) that were dried under N_2 were used for the validation of the method. The samples were analysed as such and again after spiking with 1 µg of each BA (spike 1) and with 10 µg of each BA (spike 10). The average and SD (Annex A - Average and Standard Deviation values for faecal samples), and the CV (Table 4.10) reflect the intraday precision.

It is clear that the precision of both stool samples and faecal water is inferior to that of the standard solutions as only 38 out of the 80 BAs measurements displayed a CV below 10%.

Table 4.10. Intraday variability (n=3) on measurement of individual bile acids, expressed as CV

Samples	CA	CDCA	DCA	LCA	UDCA
S1	9	38	11	3	16
S1 spike 1	24	11	15	3	3
S1 spike 10	112	130	76	5	24
S2	39	39	23	5	10
S2 spike 1	9	8	4	5	5
S2 spike 10	11	12	15	3	2
S3	1	9	20	17	11
S3 spike 1	16	23	9	6	5
S3 spike 10	85	85	74	12	5
FW1	28	14	13	13	1
FW1 spike 1	62	46	36	25	3
FW1 spike 10	58	40	46	17	7
FW2	10	16	23	19	31
FW2 spike 1	8	13	4	4	4
FW2 spike 10	1	3	1	1	1
FW3	139	135	93	15	5
FW3 spike 1	55	53	38	5	13
FW3 spike 10	42	38	29	3	5

- **ACCURACY**

The accuracy of the BAs measurement in faecal samples was calculated from the recovery of the spiked samples and is shown in Table 4.11.

Table 4.11. Recovery of spiked (0.2 µg and 10 µg) stool samples and faecal water samples

Samples	CA	CDCA	DCA	LCA	UDCA
S1 spike 1	109.67	92.00	66.27	1.014.28	163.12
S1 spike 10	47.19	81.04	0.85	125.65	103.17
S2 spike 1	2.453.42	1.241.18	665.82	267.66	225.92
S2 spike 10	170.86	137.71	144.59	150.71	122.65
S3 spike 1	1.767.36	1.187.45	197.15	259.89	435.64
S3 spike 10	717.27	510.72	91.48	137.89	119.01
FW1 spike 1	9.66	1.02	28.43	430.65	192.72
FW1 spike 10	4.48	8.14	27.70	185.02	121.89
FW2 spike 1	766.18	299.48	200.55	231.68	162.99
FW2 spike 10	170.20	143.28	148.35	142.08	115.85
FW3 spike 1	174.72	176.92	212.36	276.65	169.97
FW3 spike 10	30.66	5.95	77.89	156.26	124.70

In the majority of the cases, recovery values were out of the acceptable range (between 80 and 120%) which is most likely due to the imprecision of the measurements.

- **REFLUX**

The high imprecision of the faecal samples compared to the standard solutions might be attributable to matrix effects and unreproducible derivatisation and extraction of the BAs. Therefore, it was investigated whether solubilisation of the BAs in ethanol by refluxing the dried faecal (water) samples prior to sonication and derivatisation, could improve the precision and accuracy of the BA quantification. A freeze dried stool sample was analysed as such and after spiking with 0.2 µg and 10 µg of each BA. Table 4.12 shows the intraday precision of the samples whereas the accuracy is shown in Table 4.13.

Table 4.12. Intraday precision of a freeze dried stool samples before and after spiking, expressed as CV (n=3)

	CV (n=3)				
	CA	CDCA	DCA	LCA	UDCA
no spike	113	-	12	5	122
spike 0.2	22	21	11	4	5
spike 10	18	47	16	6	4

Table 4.13. Recovery of a spiked (0.2 µg and 10 µg) stool samples that was refluxed in ethanol prior to derivatisation

	% recovery				
Samples	CA	CDCA	DCA	LCA	UDCA
spike 0.2	11,95	662,82	332,73	728,71	182,53
spike 10	58,85	63,12	163,48	139,12	108,16

Although the results indicate some improvement compared to the values obtained without reflux, the precision and accuracy are still not satisfactory.

CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

5.1. General Conclusions and Future Perspectives

The aim of the present study was to develop and validate a reliable protocol to analyse bile acids in faecal samples. The five most prominent bile acids were selected to develop the chromatographic method. Baseline separation was achieved using an apolar 100% polysiloxane column. Bile acids need to be derivatised at the carboxyl function and hydroxyl functions to render them sufficiently volatile for analysis using gas chromatography. Esterification of the carboxyl function with butanol proceeded optimally in the presence of HCl 12N and heating for 2 h at 60°C. The most appropriate silylating reagent was HMDS+TMCS+Pyridine (3:1:9) and heating for 30 min at 55°C was sufficient to obtain adequate derivatisation.

When moving from standard solutions to faecal samples, an additional sonication step for 90 min proved to be efficient in increasing the peak area of the derivatised bile acids.

The validation of the method using standard bile acid solutions resulted in good intraday and interday precision and accuracy. The limit of detection (LOD) and limit of quantification (LOQ) values were sufficiently low to allow quantification of the bile acids in faecal samples. Nevertheless, precision and accuracy in faecal samples (either freeze dried stool samples or dried faecal water) were unacceptably low which might be attributed to matrix effects hindering appropriate derivatisation and extraction of the bile acids.

Solubilisation of the bile acids by refluxing the faecal samples in ethanol prior to derivatisation improved the precision and accuracy of the measurement but not to a level that can be considered as appropriate.

To further develop this method into a suitable and valid method for faecal bile acid measurement, additional efforts need to be done to improve the sample preparation and clean up the faecal samples prior to derivatisation. An alternative solution might be to switch the analytical platform used and move to LC-MS/MS. In this case, no prior derivatisation of the samples is required although some clean-up and upconcentration of the faecal samples might be necessary.

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ANNEXES

A. Average and Standard Deviation values for faecal samples that reflect the intraday precision

The following Tables (Table A.1 and Table A.2) show the average and standard deviation (SD) of the faecal samples.

Table A.1. Average values of faecal samples using the optimized GC-MS method

Samples	CA	CDCA	DCA	LCA	UDCA
S1	0.018	0.106	7.218	10.681	0.009
S1 spike 1	0.120	0.142	7.315	9.784	0.069
S1 spike 10	0.933	0.587	7.239	12.836	0.713
S2	3.310	0.593	1.546	0.684	0.154
S2 spike 1	5.591	1.074	2.527	0.921	0.237
S2 spike 10	6.621	1.410	5.234	3.269	0.992
S3	37.052	7.782	0.016	0.047	2.430
S3 spike 1	35.409	7.322	0.306	0.277	2.591
S3 spike 10	23.152	4.754	2.349	2.412	3.243
FW1	0.005	0.010	0.311	0.468	0.079
FW1 spike 1	0.014	0.013	0.352	0.849	0.150
FW1 spike 10	0.092	0.058	1.017	3.642	0.911
FW2	26.953	1.445	0.005	0.011	0.002
FW2 spike 1	27.665	1.561	0.301	0.216	0.062
FW2 spike 10	30.251	2.294	3.789	2.448	0.793
FW3	4.962	0.553	0.462	0.158	0.074
FW3 spike 1	4.800	0.485	0.775	0.402	0.137
FW3 spike 10	4.368	0.589	2.449	2.838	0.926

Table A.2. Standard Deviation of faecal samples using the optimized GC-MS method

Samples	CA	CDCA	DCA	LCA	UDCA
S1	0.002	0.041	0.814	0.349	0.001
S1 spike 1	0.029	0.016	1.131	0.283	0.002
S1 spike 10	1.047	0.765	5.500	0.593	0.173
S2	1.300	0.230	0.357	0.034	0.015
S2 spike 1	0.489	0.089	0.094	0.043	0.011
S2 spike 10	0.738	0.170	0.787	0.107	0.022
S3	0.337	0.669	0.003	0.008	0.273
S3 spike 1	5.718	1.698	0.026	0.017	0.118
S3 spike 10	19.650	4.018	1.747	0.289	0.146
FW1	0.001	0.001	0.041	0.059	0.001
FW1 spike 1	0.009	0.006	0.126	0.214	0.005
FW1 spike 10	0.053	0.023	0.469	0.621	0.062
FW2	2.685	0.228	0.001	0.002	0.001
FW2 spike 1	2.141	0.205	0.012	0.009	0.002
FW2 spike 10	0.266	0.062	0.053	0.014	0.007
FW3	6.887	0.745	0.431	0.023	0.004
FW3 spike 1	2.661	0.257	0.293	0.021	0.018
FW3 spike 10	1.838	0.226	0.699	0.098	0.042