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Chromatography of metabolites in plasma and urine following oral administration of anthocyanin rich capsules

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Abstract

Anthocyanins (ACs) are powerful antioxidants widely distributed in fruits and vegetables. Several international studies suggest that anthocyanins have positive effects on the health, including various chronic diseases. To be able to study the possible anthocyanin mechanisms in the human body, it is of importance to know their metabolism. MEDOX® is an AC rich product made from bilberries and blackcurrant and consists of 17 different anthocyanins with delphinidin-3-O-β-glucopyranoside and cyanidin-3-O-β-glucopyranoside as the main constituents. There are only 5 different aglycone structures of these 17 anthocyanins; delphinidin, cyanidin, peonidin, petunidin and malvidin. An extensive metabolism of ACs is indicated following oral administration of this supplement. Free and conjugated benzoic acids (BAs) with functional groups corresponding to the anthocyanin B-ring structure have been recognized as metabolites in urine and plasma. Correspondingly, gallic acid, protochatechuic acids, vanillic acid, syringic acid and 3, 4-dihydroxy-5-methoxybenzoic acid have been suggested as metabolites of delphinidin, cyanidin, peonidin, malvidin and petunidin respectively. Hydroxy benzoic acids are relatively polar compounds, and derivatization is therefore usually necessary prior to gas chromatography mass spectrometry (GCMS) analysis.

The objective of this thesis was to develop a robust analytical method for determination of these BAs in addition to 4-hydroxybenzoic acid which is a suggested metabolite of the AC pelargonidine. Liquid-liquid extraction (LLE) was chosen as the best sample extraction method as compared to solid phase extraction (SPE) and solid phase analytical derivatization (SPAD) with respect to recovery, reproducibility and sample purity. LLE was also less time consuming than SPE. The recoveries of BAs in urine ranged from 62 – 121 %, and recoveries in plasma from 37 – 158 %. All BAs were identified and quantified in urine and plasma after oral administration of MEDOX® in increased levels compared to before intake. All metabolites were detected as both free BAs and in conjugated form linked with glucuronic acid, in both urine and plasma. Results obtained from the work of this thesis suggest that the ACs in this product metabolize to their corresponding BAs.

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Christina Helén Nilsen, Stavanger 2010

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Abbreviations

3OMGA	-	3-O-methylgallic acid
4OMGA	-	4-O-methylgallic acid
4CL	-	4-coumarate-CoA ligase
AA	-	Amino acid
AC	-	Anthocyanin
ACCase	-	Acetyl CoA carboxylase
AcN	-	Acetonitrile
ACT	-	Acyltransferase
ADP	-	Adenosine diphosphate
ANS	-	Anthocyanidin synthase
ATP	-	Adenosine triphosphate
BA	-	Benzoic acid
BSA	-	N,O-bis(trimethylsilyl) acetamide
BSTFA	-	N,O- bis(trimethylsilyl) trifluoroacetamide
C3G	-	Cyanidin-3-O-β-glucopyranoside
C4H	-	Cinnamate 4-hydroxylase
CE	-	Capillary electrophoresis
CHI	-	Chalcone isomerase
CHI	-	Chalcone synthase
CI	-	Chemical ionization
CoA	-	Coenzyme A
Conc.	-	Concentration
D3G	-	Delphinidin-3-O-β-glucopyranoside
DFR	-	Dihydroflavonol 4-reductase
d-GA	-	Deuterated gallic acid (GA)
d-HBA	-	Deuterated 4-hydroxybenzoic acid (HBA)
DHMBA	-	3,4-dihydroxy-5-methoxybenzoic acid
DR	-	Derivatization reagent
EDTA	-	Ethylenediaminetetraacetic acid
EI	-	Electron ionization
EtAc	-	Ethyl acetate
Eq.	-	Equation
F3H	-	Flavonone 3-hydroxylase
F3´H	-	Flavonoid 3´-hydroxylase
F3´5´H	-	Flavonoid 3´, 5´-hydroxylase
FI	-	Field ionization
Fig.	-	Figure
GA	-	Gallic acid
GC	-	Gas chromatography
GCMS	-	Gas chromatography mass spectrometry
GLC	-	Gas-liquid chromatography
GSC	-	Gas-solid chromatography
GT	-	Glycosyltransferase
HBA	-	4-hydroxybenzoic acid
HCl	-	Hydrochloric acid

HMBA	-	4-(hydroxymethyl) benzoic acid
HMDS	-	hexamethyldisilazane
HPLC	-	High performance liquid chromatography
Ill.	-	Illustration
IS	-	Internal standard
LLE	-	Liquid liquid extraction
LOD	-	Limit of detection
LOQ	-	Limit of quantification
m/z	-	Mass to charge ratio
MAT	-	Malonyltransferase
MeOH	-	Methanol
MPI	-	Multi photon ionization
MS	-	Mass spectrometry
MTBE	-	Methyl tert-butyl ether
OH	-	Hydroxy-group
P	-	Phosphate
PAL	-	Phenylalanine ammonia-lyase
PCA	-	Protocatechuic acid
PEP	-	Phosphoenol pyruvate
PI	-	Photo ionization
PP	-	Polypropylene
QP	-	Quadrupole
RIC	-	Reconstructed ion chromatogram
RP	-	Reversed phase
S/N	-	Signal-to-noise ratio
SA	-	Syringic acid
SPAD	-	Solid phase analytical derivatization
SPE	-	Solid phase extraction
Std.	-	Standard
Tab.	-	Table
TIC	-	Total ion current
TMCS	-	Trimethylchlorosilane
TMS	-	Trimethylsilyl
TMSI	-	N-trimethylsilylimidazole
UV	-	Ultra violet
UV/Vis	-	Ultra violet/visible
VA	-	Vanillic acid

1. Introduction

Flavonoids are a common term of numerous polyphenolic compounds found ubiquitously in nature. Flavonoids are plant pigments giving color to certain fruits and flowers. The perhaps most intriguing aspect of flavonoids are their antioxidant function which holds a potential health benefit for humans. The main dietary sources of these antioxidants for humans are fruits, vegetables and various beverages. Based on their chemical structure flavonoids are categorized into [1-3]:

- Flavones
- Flavanones
- Flavonols
- Isoflavones
- Catechins
- Anthocyanidins
- Chalcones

Anthocyanins (Fig. 1) are a part of the vast flavonoid family, consisting of an aglycone (anthocyanidin) part and a glycone (sugar) part. They are a group of naturally occurring compounds which are powerful antioxidants. The anthocyanins (ACs) are responsible for the color of many fruits, vegetables, and are also found in grain products. As a result, the ACs participate in a typical everyday life as a part of the human diet. The intake of anthocyanins in humans has been estimated to be 180-215 mg/day, based on numbers from the United States of America. This estimate is considerably higher than the intake of other flavonoids, including quercetin, kaempferol, myricetin, apigenin and luteolin, which is approximately 23 mg/day [4, 5].

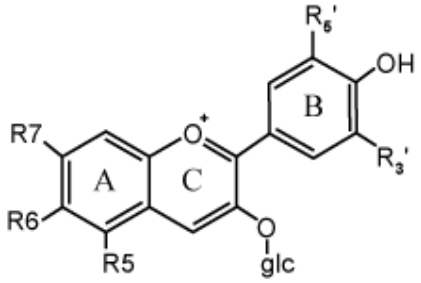


Figure 1. Anthocyanin structure

ACs are perhaps best known as color giving plant pigments, however they have other more important features. In plants anthocyanins contribute to the immune system protecting them from harmful ultraviolet (UV) rays, and play an important part in the pollination process [6]. The main objective for the interest in ACs considering humans is the alleged health benefits due to their antioxidant effect. Because of the possibility of ACs being source of several health aspects, they have been the target of a number of research projects and studies [7, 8].

In this thesis, potential metabolites of the anthocyanins containing the six most prominent aglycone structures have been studied (Table 1). Five of these aglycone structures with different sugars attached, are found in the blueberry and blackcurrant based product MEDOX® which has been used as supplement for oral administration to study a possible

metabolism of the anthocyanins. The sixth anthocyanin, pelargonidin, should not be found in this product. The R5, R6 and R7 groups are the same for all these compounds: OH, H and OH respectively [3].

Table 1. Most common aglycone structures

Aglycone	R5´	R3´
Delphinidin	OH	OH
Cyanidin	OH	H
Peonidin	OCH_3	H
Petunidin	OH	OCH_3
Malvidin	OCH_3	OCH_3
Pelargonidin	H	H

Benzoic acids (BAs) consist of a benzene ring directly bonded to a carboxyl group (Fig. 2). BAs can also be called phenolic acids in some cases and vice versa, depending on the structure. Benzoic acids always have the acidic group bound directly to the benzene ring; this is not always true for phenolic acids. Phenolic acids have a hydroxy (OH) group bound directly to the benzene ring, which is not always the case for benzoic acids. The compounds sharing these qualities and can be referred to as both, are also often called hydroxy-benzoic acids [9].

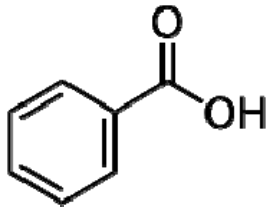


Figure 2. Benzoic acid structure

Based on research on anthocyanins and metabolic research of ACs, benzoic acids are suggested to be metabolites of anthocyanins. The BA metabolite's functional groups correspond to the anthocyanin B-ring structure. These benzoic acids can be found either as free or conjugated acids, where in the latter case deconjugation might be necessary for a quantitative analysis. The conjugated acids are usually linked to glucuronic acid (glucuronidated) and/or sulphuric acid. Methylation is also a common metabolic route, allowing some BAs to transform into others [10-15].

The benzoic acids analyzed in this thesis is corresponding to the six most common aglycone structures of anthocyanins (Table 2). All the BAs share the qualities qualifying their structures as both benzoic and phenolic acids.

Table 2. Analyzed benzoic acids and their parent compounds

BA (chemical name)	BA (abbreviation)	BA (common name)	Parent compound
3,4,5-trihydroxybenzoic acid	GA	Gallic acid	Delphinidin
3,4-dihydroxybenzoic acid	PCA	Protocatechuic acid	Cyanidin
4-hydroxy-3-methoxybenzoic acid	VA	Vanillic acid	Peonidin
3,4-dihydroxy-5-methoxybenzoic acid	DHMBA	-	Petunidin
4-hydroxy-3,5-methoxybenzoic acid	SA	Syringic acid	Malvidin
4-hydroxybenzoic acid	HBA	-	Pelargonidin

The structures of the suggested hydroxy-benzoic acid metabolites of the selected anthocyanins are corresponding to the different AC's B-ring structure (Fig. 3).

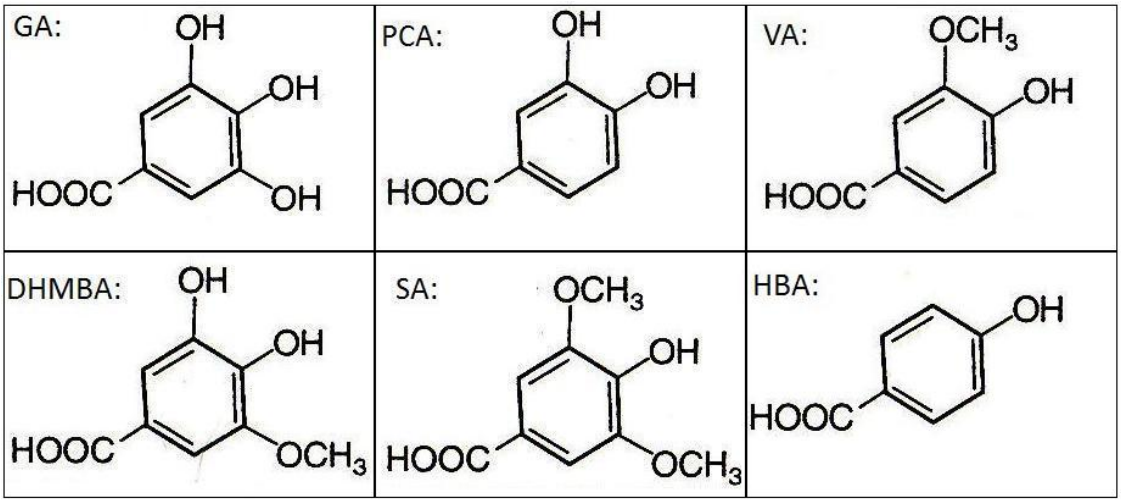


Figure 3. Structure of the benzoic acid metabolites analyzed in this thesis

MEDOX® (Fig. 4) is a unique and natural anthocyanin product containing 17 different anthocyanins (Biolink Group AS, Norway).

Figure 4. MEDOX® product

MEDOX® is a product combining bilberries (*Vaccinum myrtillus*) and blackcurrant (*Ribes nigrum*). The molecules believed to have the highest biological potential among ACs are the ones possessing a so-called "ortho-dihydroxy phenol" structure. Two of these are cyanidin-3-

O-β-glucopyranoside (C3G) and delphinidin-3-O-β-glucopyranoside (D3G), which are vastly overrepresented in MEDOX®. In fact C3G and D3G accounts for 91 % of the 80 g AC content in MEDOX® capsules [16, 17].

Of the 17 different anthocyanins found in MEDOX®, there are only five different aglycone structures (Table 3).

Table 3. Anthocyanins found in MEDOX®

Aglycone structure	Variations			
	3-O-β-glucopyranoside	3-O-β-galactopyranoside	3-O-α-arabinopyranoside	3-O-β-rutinoside
Delphinidin	x	x	x	x
Cyanidin	x	x	x	x
Peonidin	x	x	x	
Petunidin	x	x	x	
Malvidin	x	x	x	

Preparation of samples prior to analysis is necessary to make the sample material compatible with the instrument utilized. Complex matrixes such as urine and plasma are unfit for direct analysis because they contain an excess of biological materials such as protein, salt and cell products, making a purification step essential. By applying an extraction method for purification, the sample is also allowed to be concentrated. Sometimes extraction alone is insufficient and an additional modification is needed, such as derivatization. Derivatization is performed on a sample material to make it suitable for the following analysis [18].

Common sample preparation methods for ACs in biological samples are liquid-liquid extraction (LLE) and solid phase extraction (SPE) following pretreatment of deconjugation in most cases [7, 8, 10, 19-23].

Chromatography is defined as a process for separating compounds in a mixture. The term chromatography is collective for several separation techniques, for which the principle is to separate analytes by distribution over two phases, a mobile phase and a stationary phase. The mobile phase is either a liquid or gas; hence the names liquid chromatography (LC) and gas chromatography (GC) [18].

For the separation, detection and analysis including identification and quantification of the compounds in this thesis, capillary gas chromatography coupled with mass spectrometry (GCMS) was chosen. GCMS was considered a good choice due to the techniques separation and identification qualities [24, 25]. Various separation techniques have been used for separation and identification of phenolic compounds, mainly HPLC and GC [7, 8, 10, 19-23]. Capillary electrophoresis (CE) has also been utilized for separation of phenolic compounds, however it is not common [26]. A common advantage when applying techniques such as HPLC and CE, compared to GC, is that these methods do not usually require a derivatization step before quantitative analysis which can be quite time consuming. This argument is contributing to the choice of HPLC as the most commonly used method for qualitative and quantitative analysis of plant phenolic compounds. The detector principles used with HPLC is typically UV, electrochemical and fluorescence [10, 15, 18]. Because the UV/Vis spectrum does not supply sufficient identifying power, and HPLC does often not provide sufficient separating performance, GCMS is a good choice of method for analysis of phenolic compounds [24, 25, 27].

The overall aim of the thesis is the development of a robust chromatographic method for determination of selected metabolites in urine and plasma following oral administration of MEDOX®. The selected metabolites are free and glucuronic acid conjugated benzoic acids:

- 3,4,5-trihydroxybenzoic acid (**GA**)
- 3,4-hydroxybenzoic acid (**PCA**)
- 4-hydroxy-3-methoxybenzoic acid (**VA**)
- 3,4-dihydroxy-5-methoxybenzoic acid (**DHMBA**)
- 4-hydroxy-3,5-dimethoxybenzoic acid (**SA**), and:
- 4-hydroxybenzoic acid (**HBA**)

The first five of these benzoic acids, respectively, are expected metabolites from the MEDOX® supplement; whereas the last acid (HBA) is an expected metabolite from pelargonidine which is an anthocyanin found in strawberries amongst others.

Based on the results acquired by the use of the developed optimized method, there should be possible to discuss whether these benzoic acids really are metabolites of the anthocyanins found in MEDOX®.

2. Theory

2.1 Anthocyanins

Anthocyanins are of great interest because of their alleged antioxidant activity. They are part of the flavonoid family which are synthesized from phenylalanine and are characterized by a benzo- γ-pyrone structure [2]. The skeletal carbon structure (Fig. 5) possessing 15 carbon atoms; two benzene rings joined by a linear three carbon chain, can be described as C_6-C_3-C_6, containing three phenolic rings called A, B and C. The C-ring can also rarely consist of two carbon atoms. Usually the B-ring is in 2 position of the C-ring; however it can also be located at position 3 and 4 [2, 28].

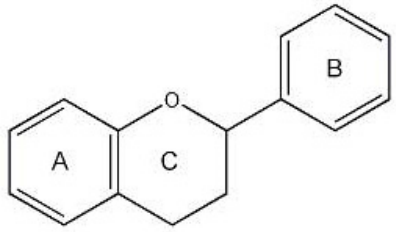


Figure 5. General flavonoid structure

Phenols and polyphenols, including flavonoids and ACs, are effective antioxidants since the radical products of these molecules are resonance stabilized and thus relatively stable [29]. It is believed that ACs have positive effects on several health aspects also in a preventive way. The metabolism and bioactivity of these antioxidants are two features of great importance to study to understand their effect.

2.1.1 Biosynthesis and metabolism

The anthocyanin biosynthesis is also often called the flavonoid pathway since other flavonoids are also synthesized by this pathway. The ACs are assembled by two separate biosynthesis paths which result in the amino acid (AA) phenylalanine (Fig. 6) and malonyl-coenzyme A (coA) (Fig. 7) which are both essential in the joined synthesis of anthocyanins [3, 6, 29-31].

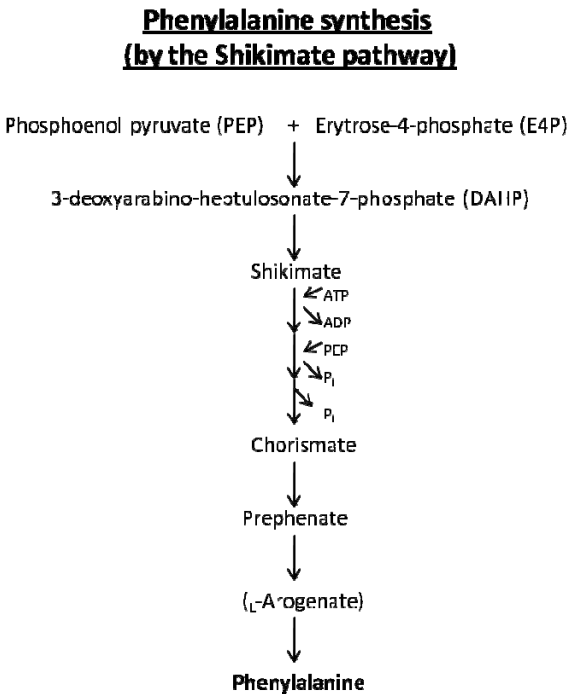


Figure 6. Synthesis of the amino acid phenylalanine, the starting point of AC biosynthesis

The abbreviations ATP, ADP and P in the figure above (Fig. 6) stand for adenosine triphosphate, adenosine diphosphate and phosphate respectively. ATP functions as a coenzyme and is often used for energy transfer in the cells for metabolism. ATP is transformed to ADP by phosphorylation [29, 30]. The Shikimate pathway leads from PEP and E4P to chorismate. Chorismate is thereafter used as substrate to form the aromatic AAs phenylalanine, tyrosine and tryptophan. Only phenylalanine is involved in the synthesis of flavonoids and ACs. Phenylalanine synthesis from L-Arogenate is the characteristic route in higher plants. Some bacteria form the aromatic AAs directly from prephenate [3, 32].

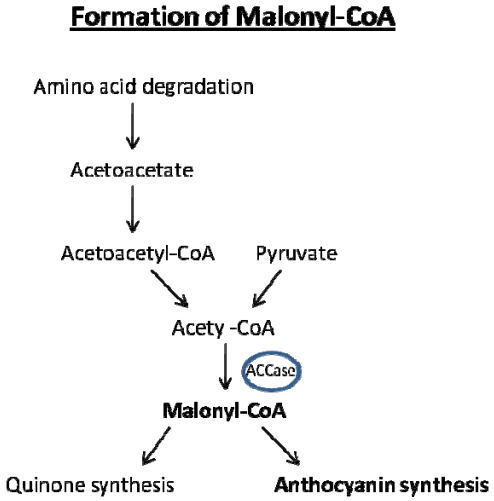


Figure 7. Synthesis of Malonyl-CoA, which is needed for AC synthesis

Acetyl-CoA carboxylase (ACCase) is the enzyme which transforms Acetyl-CoA into Malonyl-CoA which is used further for AC synthesis [29, 30]. The two separate streams resulting in phenylalanine and malonyl-CoA meet and are enzymatically joined together by chalcone synthase (CHS), generating a transitional chalcone which is isomerized to the flavonone naringenin. Anthocyanin production happens in plants through a series of enzymatically assisted steps which constitutes the anthocyanin biosynthesis (Fig. 8) [3, 6, 31].

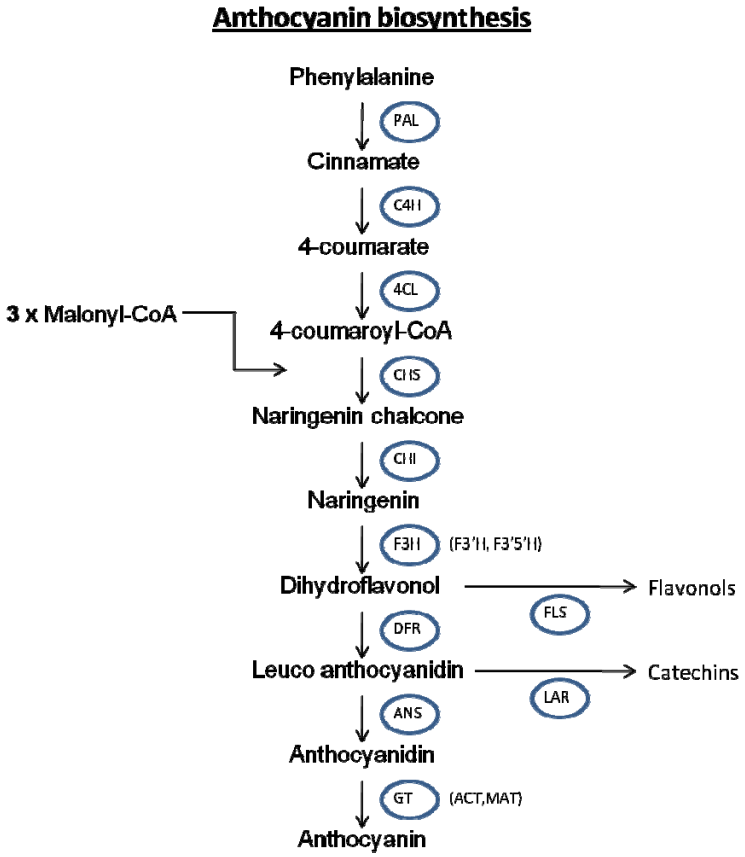


Figure 8. Anthocyanin (flavonoid) biosynthesis, abbreviations explained in the text

Phenylalanine ammonia-lyase (PAL) is the first enzyme involved in the biosynthetic pathway of ACs. PAL leads to the cleavage of the amino-group of phenylalanine which is released as ammonium which is utilized in glutamine synthetase. Glutamate and glutamine serve as nitrogen donor to chorismate in the pathway leading to phenylalanine. Transcription of the PAL gene is enhanced by light, plant growth regulation as wounding (excision) and different types of stress. For instance have stressful light conditions such as high UV-radiation proved to increase flavonoid and AC production in plants which serve as the plants protection from UV damage. It has also been suggested that nutritional depletion in plants also enhance the transcription of the PAL gene enabling AC synthesis. The other enzymes shown between the steps in the figure (Fig. 8) is cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavonone 3-hydroxylase (F3H), flavonoid 3´-hydroxylase and flavonoid 3´,5´-hydroxylase (F3´H, F3´5´H), flavonol synthase (FLS), leucoanthocyanidin reductase (LAR), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), glycosyltransferase (GT), acyltransferase and malonyltransferase (ACT,MAT) [3, 6, 31].

The anthocyanidin forms are unstable and by glycosylation they become anthocyanins and are stabilized. There are a great number of sugar conjugations possible but the most common are: glucose, galactose, rhamnose, arabinose and xylase. Glycosylation of the anthocyanidin is what forms the AC since it is where the sugar group is added. If acylation or malonylation occur, it happens after the glycosylation. Anthocyanins are water soluble pigments caused by the presence of sugar. In case the sugar moiety is hydrolyzed or lost, the solubility will decrease and the aglycone will be destabilized or degraded. Acylation and malonylation increase the water solubility of the molecule [3, 6, 33].

Keppler et al. employed a new in vitro model system, to investigate the question of deglycosylation, ring scission, and other bacterial degradation pathways of anthocyanins. They used a naturally composed gut flora directly isolated from the caecum of freshly slaughtered pigs, excluding any aerobic atmosphere. Their results clearly show that anthocyanidin glycosides were hydrolyzed extensively by the intestinal micro flora depending on the sugar moiety. After cleavage of the protective 3-glycosidic linkage, the released aglycones are very unstable under physiological conditions in the intestine at neutral pH and degrade spontaneously into phenolic acids and aldehydes. Anthocyanidins were released by hydrolysis of ACs. The decay of the aglycone (anthocyanidin) cyanidin led to protocatechuic acid (PCA), peonidin degraded into vanillic acid (VA), and syringic acid (SA) was detected as the degradation product of malvidin. There was also O-demethylation, with the formation of two other metabolites; SA was O-demethylated into gallic acid (GA), and VA converted to PCA. The cleavage of methyl groups and liberation of free hydroxyl groups modulate the antioxidant properties of these phenolic compounds. It is believed that, because of their higher chemical and microbial stability, phenolic acids (hydroxy-benzoic acids) identified as AC metabolites, might be mainly responsible for the observed antioxidant activities and other physiological effects in vivo and not just the anthocyanins themselves [11].

2.1.2 Biological effects

Studies show that anthocyanins have substantial bioactivity, including antioxidant activity, and therefore may have beneficial effects on human health [11, 21]. Dietary ACs' presumed ability to influence biological systems could be partly due to their characteristic ability to form complexes with macromolecules, combined with their polyphenolic nature. ACs is showed to have a role as scavenger of harmful free radical which cause oxidative damage to nucleic acids, proteins and lipids, in addition to have potential interaction with biological systems. ACs have been shown to have reactivity towards both reactive oxygen and nitrogen in vitro [7, 14, 25]. Reports of health beneficial effects have been shown in humans and also experimental animals. The effects of ACs are many and diverse, ranging from antioxidant behavior to cancer prevention and inhibition. Vision improvement is one function of ACs where they have a regenerating effect of rhodopsin which is a pigment of the retina for formation of photoreceptor cells. Apoptotic effects (programmed cell death) of anthocyanidins and ACs in cancer cells have been reported both in vitro and in vivo, in addition to tumor decrease, and inhibition of tumorigenesis in rats. Anticarcinogenic effects altering metabolic activation of carcinogens are also reported [8, 11, 14, 22, 34, 35]. There are also studies suggesting that ACs have protective and preventive effects on cardiovascular diseases and atherosclerosis. Atherosclerosis is a condition causing chronic inflammatory response due to thickening of the arterial wall as the result of for instance cholesterol and other fatty materials. Other functions of the health beneficial effects of ACs are suggested to be anti-

inflammatory effects, reduced risk of stroke and diabetes, anti-obesity effects and anti-mutagenic action against various mutagens. In addition to this they are also believed to protect against DNA damage and prevent low-density lipoprotein oxidation and inhibition of platelet aggregation [10, 35-37].

As ACs play a natural part in the daily diet of humans, these discoveries are of high interest and importance to pursue. Knowledge of ACs for prevention of chronic diseases is of great importance for humans as ACs are easily available as food and beverages. To be able to study these effects further also other aspects of these phenolic compounds are needed to research. The bioavailability and metabolism of the anthocyanins are necessary to study in order to understand more of the biological effects of ACs due to dietary intake.

2.1.3 Bioavailability

To begin to understand the health benefits caused by anthocyanins and the extent of these, there is a need to understand more concerning the bioavailability of ACs in humans. In order to exert an effect in vivo, a dietary compound has to reach tissues, in the native or metabolized form, in a dose sufficient to yield biological effects [12]. To achieve an effect in a target organ or tissue (except the GI tract), bioactive components must be bioavailable. Evaluating the health benefits of ACs in humans, the bioavailability, including the absorption, distribution, metabolism and excretion, must be known [21, 36]. ACs have two absorbance maxima, 279-280 nm and 510-540 nm [5], a fact which is utilized when trying to detect these compounds. The most common naturally occurring ACs are the following: 3-O-glucosides or 3, 5-di-O-glucosides of cyanidin, delphinidin, peonidin, petunidin and malvidin. Several studies have shown that ACs are absorbed as glycosides in humans, and in experimental animals such as rats [1, 5, 21, 22, 37, 38]. The intact glycosidic forms have indeed been recovered in plasma and urine after oral administration, with one drawback [4]. The bioavailability was thought to be very low and considering the metabolism was not, and is still not, fully understood, the phenolic acid metabolites were not considered at that point. The recovery, of the formerly only known, metabolites (glucuronidated and methylated compounds) in urine and plasma has been found to be below 1 %, whereas up to 99 % if the ingested anthocyanins is eliminated in the gut [12].

The supposed low bioavailability of anthocyanins contradicted the explanation of the antioxidant properties of AC rich food as a major health benefit contributor. The results of low absorption values compared to the ingested dosage in humans and animals led to the indication of an extensive biotransformation of the ACs after oral ingestion and absorption. In a study, protocatechuic acid (PCA) was retrieved as the main metabolite of cyanidin glycosides (CyG). These results showed consistency with former studies, recovering less than 1 % methylated or glucuronidated AC in blood and urine. PCA, however, was found as the main metabolite in serum and it accounted for 44 % of ingested CyG in the following 6 hour period after ingestion. No PCA was recovered in urine, and the native AC and glucuronidated and methylated compounds seemed to be most common. In other studies, PCA has been found in trace amounts in both plasma and urine, unsuspectingly of the source. One study found PCA in concentrations 8-fold higher than that of the native AC itself. In another study, however, PCA was suggested not to be the metabolite of CyG. Despite these discrepancies, PCA was reported as an AC metabolite, and gave way for the possibility of other phenolic acids as metabolites [11-14, 21].

2.2 Chromatography

Chromatography is a common name for several separation techniques in chemical analysis. Even though there are different forms of chromatography, they are all based on the principle that the compounds separated are dispersed between two phases; one of them is mobile while the other is stationary. Depending on the method, the mobile phase can either be a liquid or a gas, inspiring the names liquid- (LC) and gas chromatography (GC).

Chromatography can separate gases and volatile substances by GC, non-volatile chemicals and materials of high molecular weight by LC, and also by the inexpensive planar thin layer chromatography (TLC). GC and LC are both column chromatography methods.

In one single process, chromatography can separate a mixture into its individual components, and with an appropriate detector connected identify and quantify each compound. There are of course some restrictions and a need for sample preparation work before an analysis. A column chromatography system consists of the following main components: Mobile phase, pump, column, detector and data treatment (Fig. 9).

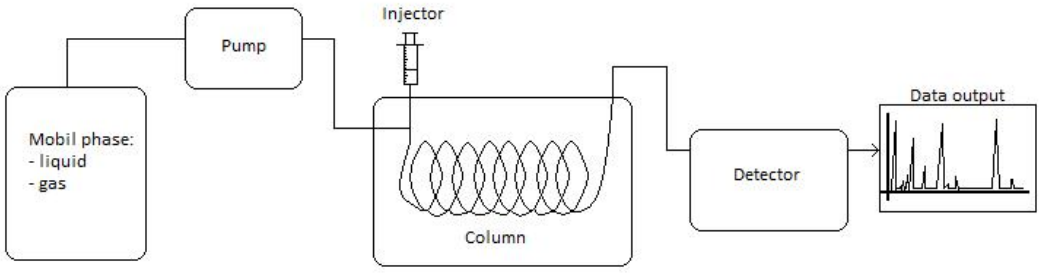


Figure 9. Column chromatography system

The selection of the right combination of several separation factors is crucial to the separation of a mixture of compounds:

- Stationary and mobile phase
- Length and diameter of column
- Mobile phase velocity
- Sample size

The properties of a compound determine the velocity of which the compound moves through the column. The differences between the diverse compounds in a sample make them migrate at different velocities. It is the compounds equilibrium distribution between the two phases that determine the migration velocity. Without this difference there would be no separation.

A sufficient separation is the goal when using chromatography. Resolution (R) is a quantitative measurement for separation. When R has the value of 1, there is 2 % overlap between two peaks. A higher value than 1 indicates a better separation, and a lower value means poorer separation.

In order to detect the compounds separated with chromatography, a suitable detector is needed. There are several detector possibilities, depending on chromatographic technique and compounds of interest [18].

2.2.1 Gas chromatography

Gas chromatography use gas as its mobile phase which is usually called the carrier gas and is stored in a high pressure cylinder. To be able to analyze compounds with GC, the compounds need to be volatile and stable at the temperatures employed by the system. There are two kinds of gas chromatography:

- Gas-solid chromatography (**GSC**)
- Gas-liquid chromatography (**GLC**)

GLC is the chromatography type used for most GC analyses. There are several nonvolatile compounds that can be separated with both GC and LC but these need to be derivatized before GC separation. The capillary columns in GC have as a rule far higher separation efficiencies than LC columns. Consequently, GC can handle multi-component mixtures more easily.

The purpose of the carrier gas in the chromatographic system is to transport volatile substances through the column. The most common gases used are helium, nitrogen and hydrogen, which are all inert and will not react with the sample or the stationary phase.

Most samples analyzed with GC are fluids which are injected with a syringe often in volumes of 1 µl or less. There are three types of columns for GC; preparative, analytical and capillary [18].

2.2.2 Detectors

For GC there are several detectors which can be employed, and these can all be classified within two main types:

1) Concentration sensitive detectors
2) Mass sensitive detectors

More than 15 years ago, some of the most commonly used detectors were the thermal conductivity detector (TCD) and the flame ionization detector (FID), type 1 and 2 respectively. During the last two decades mass spectrometry (MS) has increased its impact, and is today the most commonly used detector in combination with gas chromatography (GCMS) [18].

2.2.3 Mass spectrometry

A mass spectrometer (MS) is a mass selective detector and fits in under type 2 of GC detectors. A mass spectrometer consists of an ion source, a mass analyzer and a detector. One of the most frequently used ion sources are electron ionization (EI). When the separated

compounds enter the EI ion source, a bombardment of electrons hit the molecules resulting in formation of charged molecules and charged fragments (Fig. 10). The charged molecules and/or fragments enter the mass analyzer by the force of electrically charged lenses. Both negatively and positively charged ions are formed in the ion source. However, it is not common to detect negative ions due to the increased sensitivity for positive ionization mode (EI+). Fragmentation is the result of the excess energy in the molecule [18, 39, 40].

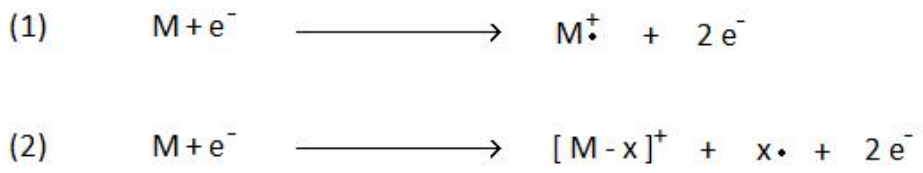


Figure 10. Illustration of EI reaction creating charged molecules and ion fragments

Several different types of ionization and various mass analyzers can be chosen. The selection of these components is dependent on the use of the MS. The purpose of MS is to separate the ions based on their mass-to-charge ratios (m/z) and to detect these qualitatively and quantitatively [18, 40].

Apart from EI, there are other common ionization methods in the gas phase, such as [40]:

- chemical ionization (CI)
- field ionization (FI)
- photo ionization (PI)
- multi photon ionization (MPI)

Mass analyzers differ with regard to how m/z values are separated, and can be used alone or in combination (MSMS and MS^n). The most commonly used mass analyzers are [18, 39, 40]:

- Magnet instrument
Ions with different m/z values are separated in a magnetic field by varying the field.

- Quadrupole instrument
The analyzer consists of four parallel circular rods. Ions are separated in the quadrupole based on the stability of their trajectories in the oscillating electric fields that are applied to the rods. In GCMS combination, mainly quadrupole instruments are used because their mass area covers the molecular weights for the compounds which can be applied to GC.

- Ion trap instrument
The separation happens by a corresponding principle as in the quadrupole instrument. In contrast to a regular quadrupole instrument, the ion source and the analyzer is combined in one common unit.

- Time-of-flight (TOF) instrument
Often used in combination with HPLC but not for GC. Ions accelerate in pulses before they are led through a drift tube where they receive the same kinetic energy and move through the tube with a velocity equivalent to the square root of their mass.

- Ion cyclotron resonance
The ions are accelerated in a cyclotron and separation occur based on cyclotron frequency of the ions in a fixed magnetic field.

Using a mass spectrometer as detector enables structural information, and the possibility of identification, of the analytes in addition to quantification. There are two modes one can obtain mass spectra with; scan mode or single ion monitoring (SIM) mode. Scan is usually used for identification and SIM for quantification. Continuously scanning the magnetic field exponentially downward from high to low mass keeping the accelerate voltage constant is called "scan" mode. Scan is typically chosen to obtain a mass spectrum of the whole m/z range, and produces mass spectral peaks of constant width. Peak width is inversely proportional to the resolving power, which is the ability of the MS to separate ions of adjacent mass number. The mass spectrum is a histogram plotted as relative intensity of ions as a function of m/z values.

When components are known or suspected to be found, the full spectrum is not necessary to detect. The MS instrument can also be programmed to detect only one or a few masses, this is called SIM mode. SIM mode requires that the spectrum of the target compounds are known so that the most characteristic masses can be chosen; selecting the right masses for SIM improves the sensitivity. When masses are chosen for SIM detection, a qualifier (identifier) and quantifier ion is chosen. The identifier ion should ideally be unique for a compound, and the quantifier ion should be the most prominent peak in the mass spectra with the highest intensity compared to the other compound peaks. These criteria ought to be followed to achieve a good foundation for a SIM method [18, 40].

2.2.3.1 Typical fragmentation of benzoic acid trimethylsilyl derivatives by electron ionization

The combination of GC and MS (or MSMS) is a powerful analytical technique for identification and quantification of compounds in complex mixtures. By coupling GC directly to the MS, the carrier gas containing the analytes enters directly into the ion source. Ionization can occur since the analytes are volatile and in gas phase.

Sample preparation prior to GCMS analysis of compounds such as BAs involves derivatization to make the compounds compatible with the instrument. A common derivatization method is silylation creating trimethylsilyl derivatives (TMS) of the compounds in question. By the use of EI as ionization mode, there are several typical ion fragments found in the mass spectra (Table 4).

Table 4. Common ions/ion fragments found in mass spectra of BA TMS derivatives

Ions/ion fragments	Explanation
$[M]^+$	Molecular ion (positive charge)
$[M-15]^+$	Loss of a methyl group via α-cleavage
$[M-30]^+$	Loss of a formaldehyde molecule
$[M-59]^+$	Subsequent loss of CO_2 after rearrangement
$[M-89]^+$	Loss of trimethylsiloxyl (TMSO)
$[M-177]^+$	Loss of TMSO-$Si(CH_3)_4$

The molecular ion is usually a predominant peak in all BA mass spectra. For TMS esters generation of the fragments $[M-15]^+$ and $[M-59]^+$ are common. The fragment $[M-30]^+$ is produced by derivatives possessing a methoxy group on the phenyl ring. $[M-89]^+$ is an established fragmentation pathway for carboxylic acids, because the acylium cation formed is a stable species. The fragment $[M-177]^+$ is most common for derivatives with two or more TMS groups attached in adjacent positions [24, 41].

Other than the m/z values for the molecular ions and ion fragments, the fragment m/z 73 is commonly observed as the base peak in mass spectra for TMS derivatives, representing the TMS group. The fragment m/z 147 can also be found in such mass spectra, representing the structure $[(CH_3)_2Si=O-Si(CH_3)_3]^+$ which means there is two or more TMS groups present in the molecule [24].

2.2.4 Internal standard

An internal standard (IS) is a substance added to a sample in a known quantity to make it possible to correct certain factors. The IS is an essential component in developing a robust analytical method, and its role is to function as a correction for:

- loss during sample preparation
- differences in injected volume
- differences during a chromatographic analysis
- change in response factor

During sample preparation there can be loss of sample volume during several steps, for instance by use of pipettes and evaporation of solvent. The internal standard has to be added to the sample solution before the preparation starts.

More than one internal standard may be added to the sample, in case there are multiple substances with different chemical properties which are supposed to be detected in a sample. In order to quantify compounds in a sample, a standard calibration curve is needed. A standard curve is prepared by an analysis of known amounts of the substances and the internal standards. The area ratio of substance/IS or peak height ratio of substance/IS is plotted against the concentration ratio[18].

Equation for a linear calibration curve is:

x = (y-b) / a **(1)**

Concentration of target compounds are found by this equation:

Conc. compound = (Conc. IS x Compound area) / IS area **(2)**

There are several criteria that needs to be filled when selecting an internal standard [18]:

- it has to be separated from the rest of the substances in a given sample, either by time in the chromatogram or by mass in the mass spectra

- the retention time must be similar to the chosen substance that is going to be quantified
- similar chemical properties as the chosen substance, which is especially important throughout the sample preparation step such as extraction and derivatization
- it must not be present in the sample
- it has to be stable and not react with anything in the sample (or the stationary/mobile phase)
- it needs to be of high purity

A good choice in internal standard is therefore a substance as similar to the substance you want to quantify as possible. One possibility is to utilize an isotopically labeled version of the target substance. Because of the abundance in hydrogen atoms in organic molecules, deuterium is generally the preferred isotope to use to make isotopically labeled internal standards. A deuterated IS is nearly identical to the substance itself except for its molecular weight which will increase with +1 for each deuterium attached to the substance. Ideally the deuterated IS has the same recovery, very close retention time and the same ionization response, as the unlabeled substance [42].

2.3 Sample preparation

Sample preparation is performed before analysis to make a sample suitable for the selected analytical technique. However, before the sample preparation methods can be done, the sample matrix often needs to be somewhat adjusted. Before extraction, a sample might need to be adjusted to the right pH value for the compounds to be able to move to the right phase. Often, and especially in biological matrixes, compounds are conjugated with highly polar molecules, such as glucuronic acid or sulfuric acid. When the total amount of a compound is required, deconjugation by hydrolysis might be a necessary pretreatment step [10, 15, 18].

Sample preparation is used to purify and concentrate the compounds of interest prior to analysis. The purification usually includes separation of target compounds from the matrix and transference to a solvent which is compatible with the analytical method.

Sample preparation is usually necessary because of the following reasons [18]:

- the sample matrix is incompatible with the separation method, and would hamper good analytical quality
- the compounds of interest cannot be analyzed with a satisfactory result because of interference of other compounds in the sample
- the concentration of the compounds are below the instrumental limits of detection

The most frequent used sample preparation methods are two extraction techniques [18]:

- liquid-liquid extraction (**LLE**)
- solid phase extraction (**SPE**)

In some cases a simple extraction method for sample preparation is not sufficient prior to analysis of the compounds. Derivatization is chemical modification of a compound, and is often used to increase volatility prior to GC analysis or improve LC detection limits by addition of an appropriate chromophore. The derivatization process chemically modifies one

compound to produce a new compound which has the suitable properties for the analysis [43]. In high pressure liquid chromatography (HPLC) derivatization is not a necessity but it can be utilized to enhance sensitivity and sometimes also affect the selectivity and solubility. For GC, which is the chromatography method used in this thesis, derivatization is used to make compounds more volatile and less polar.

2.3.1 Liquid-liquid extraction (LLE)

Liquid-liquid extraction is an extraction method based on how compounds distributes in two immiscible liquids. Usually, one phase is an aqueous hydrophilic solution, and the other a hydrophobic organic solvent. The sample is the hydrophilic solution and the solvent added needs to have the right properties so that the compounds of interest will transfer over to this phase. To efficiently extract the desired compounds from one phase to another, a sequence of two or three extractions with the chosen solvent is performed [18, 44].

The dispersion of a compound in two immiscible liquids can be well described by the equilibrium constant (K) for the given compound at a fixed temperature, ion strength and pH [18].

$$\mathbf{K_{LLE} = [I]_{L1} / [I]_{L2}} \qquad \mathbf{(3)}$$

where $[I]_{L1}$ and $[I]_{L2}$ is the two immiscible liquids. If the characteristics of one solvent change the equilibrium constant K changes likewise. To extract a compound from one phase to another the K value is optimized by the choice of solvent, pH and ion strength.

The density of a solvent should not have a higher value than water for the practical benefit of an extraction. It should also be noted that several extractions with smaller volumes yields higher efficiency than extracting once with an equivalent large volume. To concentrate a sample after extraction, the sample is often evaporated, calling for a volatile solvent to be employed for extraction [18, 44].

2.3.2 Solid phase extraction (SPE)

In contrast to LLE, the compound is in the case of solid phase extraction not transferred to another liquid phase but to a solid surface, a sorbent. SPE is used with the same purpose as LLE, to isolate, purify and concentrate compounds in liquids [18, 45].

Equilibrium is formed when the compound is distributed between the liquid sample and the sorbent, either by adsorption to the surface or penetration of the outer layers of the molecules on that surface. An equilibrium constant can also be described for SPE as for LLE:

$$\mathbf{K_{SPE} = [I]_{S1}/[I]_{L1}} \qquad \mathbf{(4)}$$

However, if the dispersion process occurs in a SPE column packed with a sorbent, which is usually the case, a different approach to the equilibrium constant is needed and a different equation applies:

$$\mathbf{k = 1/(1-k')} \text{ or: } \mathbf{V_0/V_R} \qquad \mathbf{(5)}$$

where V_0 and V_R is the void volume in the column and the retention volume respectively [45].

If a compound is to be isolated on the surface of a sorbent, the surface needs to have chemical groups which will have a stronger interaction with the compound than the interaction between the compound and the liquid.

There are different packing materials and extraction principles with SPE, the most common are [18, 45, 46].

- Reversed phase extraction
Used to extract non-polar or weakly polar compounds. It separates analytes based on their polarity, retaining compounds by hydrophobic interactions. The extraction is based on interaction between the carbon-hydrogen bonds in the compound and the carbon-hydrogen bonds on the sorbent. It involves a polar or moderately polar sample matrix and a nonpolar stationary phase (solid phase).

- Normal phase extraction
Used to extract polar compounds from non-aqueous solutions. It involves a nonpolar liquid phase (sample matrix) and a polar solid phase. The principle is based on polar interactions between the compounds and sorbent such as hydrogen bonds, dipole-dipole interaction and other interactions caused by positive and negative charge differences. Normal phase extraction retains compounds based on hydrophilic interaction.

- Ion exchange
Used to isolate ionized compounds from aqueous solutions. The extraction is based on the ionic interactions between sorbent. Anion exchange is based on positively charged functional groups interacting and retaining negatively charged anions (acids), while cation exchange has negatively charged functional groups interacting and retaining positively charged cations (bases).

Silica based sorbents are perhaps the most commonly used sorbent for reversed phase, normal phase and ion exchange columns. Its characteristics inhibit swelling and shrinking in various solvents resulting in faster equilibration in new solvents. The surface chemistry of silica based packing material is the presence of hydroxide groups (silanols), and it is an inorganic polymer with the general formula $(SiO_2)_x$. Pore size is usually around 60 Angstrom (Å), prohibiting large molecules from entering the pores, however the pore size ranges from 50-500 Å [18, 45].

Polymer based sorbents are organic polymer materials opposed to silica sorbents which are inorganic. An organic polymer sorbent eliminates problems of highly active sites found on silica and other oxides. The organic compound is usually a polymerization of styrene or methyl methacrylate. To make the polymer useful for SPE, it has to be crosslinked with another compound; typical crosslink agents are divinylbenzene and ethylene dimethacrylate. The pH range for polymer based packing materials is 1-14 because of short interaction time between sorbent and solvent [45].

The solid phase extraction is carried out by 4 steps: conditioning of the column, application of sample, washing of column and finally elution of the target compounds. The condition step is performed to activate the column sorbent and preparing it for the sample. If the sorbent is not conditioned before sample application, the functional groups will be compressed on the

surface and the active surface area will be very little. This is however more relevant for silica based columns than for the polymer based columns. Thus, the goal of conditioning is to increase the surface area. Thereafter, washing of the column is performed by a solution of similar composition as the sample. Washing of the column is typically done to get rid of the conditioning solvents. When the sample is applied to the column, the target compounds will interact with the packing material and become retained while other molecules and impurities pass through. By adding an additional wash step, components with weak interaction to the sorbent gets washed out. Target compounds can be eluted from the column by applying an elution solvent which should not be strong enough to elute pollutants. It is also of great importance when using silica based columns to not let the column dry between the conditioning, washing and sample application steps, to prevent packing material to collapse thus spoiling the extraction process [18, 45].

2.3.3 Derivatization

To make compounds compatible with chromatographic analysis, derivatization is often needed. Derivatization is especially performed prior to GC, to convert water soluble and temperature labile compounds suited for GC analysis. By derivatizing compounds certain properties will increase:

- volatility
- detectability
- stability

By optimizing the derivatization method to increase these properties, the peak shape, intensity and resolution will be better.

There are common types of derivatization prior to GC-analysis:

- silylation
- alkylation
- acylation

Silylation is the most commonly used derivatization method in GC, allowing a wide variety of compounds to be silylated and a large number of reagents are available. The derivatization method used in this thesis is silylation. The silyl derivatives are more volatile and more thermally stable than the original compounds. Trimethylsilyl (TMS) groups will replace active hydrogens on the compounds treated with the derivatization reagent. Trace alcohol will also react with the derivatization reagent leading to insufficient amounts of derivatization reagent to fully derivatize the target compounds. This fact makes it crucial for the end result to dry the sample and solvent adequately before derivatization. The reagents are sensitive to moisture and because of its derivatization mechanism only aprotic organic solvents can be used. The ranking of functional groups after silyl accepting properties are: alcohols > phenols > carboxylic acids > amines > amides [18, 43, 47].

There are several silylating reagents, including N-trimethylsilylimidazole (TMSI/TMSIM), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), N,O-bis(trimethylsilyl) acetamide (BSA), trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS). The reagents are ranked in that order respectively with regard to their silyl donor properties [18, 43]. BSTFA and

BSTFA+TMCS are the preferred reagents for trimethylsilylation of alcohols, alkaloids, amines, biogenic amines, carboxylic acids, phenols, and steroids. The use of BSTFA and BSTFA+TMCS as the most common derivatization reagents of phenolic acids prior to GCMS analysis seem to correspond to literature [19, 24, 25, 41, 48, 49]. TMCS is typically mixed with other silylating reagents to increase their reactivity and work as a catalyst. TMCS is rarely used alone in analytical applications [50, 51].

The mechanism of BSTFA (and TMCS) as silylating reagent, where an active hydrogen is replaced by an alkylsilyl group - TMS, is shown in Fig. 11 [47, 51].

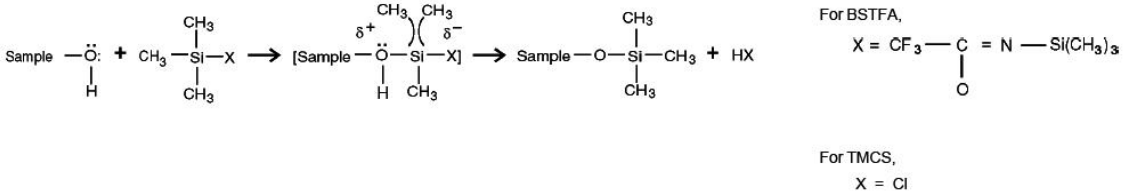


Figure 11. Silylation mechanism of reagents BSTFA and TMCS

Certain chemicals, other than TMCS, can be used to enhance the derivatization result when added to the derivatization reaction. Examples are chemicals such as the solvents pyridine and acetonitrile which both have positive effects as catalysts. A catalyst is used to increase the reactivity of the reagent [24]. Pyridine is also used to catch HCl formed from the derivatization processes where chloride is part of the reagent. The free nitrogen on the pyridine molecule reacts with the chloride ion.

2.3.4 Solid phase analytical derivatization (SPAD)

SPAD is an example of the combination of two sample preparation methods in one. The target compounds are adsorbed to the packing material, followed by addition of derivatization reagent, and finally elution of the derivatized compound. By merging SPE and derivatization, time will be saved as both steps are time-consuming.

3. Materials and methods

3.1 Chemicals

Acetic acid, acetone, acetonitrile (AcN), ethyl acetate (EtAc), hydrochloric acid (HCl) fuming 37 %, methanol (MeOH), sodium sulphate (anhydrous) and tert-butyl methyl ether (MTBE) were purchased from MERCK (Darmstadt, Germany). β-glucuronidase with 5 % sulfatase activity (Type HP-2 from Helix pomatia), pyridine (anhydrous) and sodium acetate (anhydrous) were bought from Sigma-Aldrich (Steinheim, Germany). The derivatization reagents N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and BSTFA + trimethylchlorosilane (TMCS) 99:1 (Sylon BFT), were obtained from Supelco (Sigma-Aldrich). All chemicals are of HPLC purity or better.

3.1.1 Solutions

Abbreviations and purities for benzoic acids (BAs) are spelt out in Table 5. Stock solutions were prepared for all six benzoic acids in addition to HMBA which was used as an internal standard for the GC system. Stock solutions of single compounds GA, PCA, VA, DHMBA, SA, HBA and HMBA were made by dissolving BAs in 1 mL 100 % MeOH with concentrations ranging from 5.79 to 13.33 mg/mL. Concentrations were calculated by weighing both BA and solvent on an analytical balance (accuracy of ± 0.01 mg), as well as considering BA purity. Deuterated internal standards of GA and HBA were prepared in the same way, with concentrations 8.86 and 11.45 mg/mL respectively. All user and standard solutions were prepared from these stock solutions. Accurate concentrations for all stock solutions are found in Appendix 1.

Table 5. Purity and distributor of the benzoic acids and the internal standards

Chemical name	Abbreviation	Common name	Purity (%)	Distributor
3,4,5-trihydroxybenzoic acid	GA	Gallic acid	100	Sigma
3,4-dihydroxybenzoic acid	PCA	Protocatechuic acid	100	Fluka
4-hydroxy-3-methoxybenzoic acid	VA	Vanillic acid	99.40	Fluka
3,4-dihydroxy-5-methoxybenzoic acid	DHMBA	-	99	Fluorochem
4-hydroxy-3,5-methoxybenzoic acid	SA	Syringic acid	99.25	Fluka
4-hydroxybenzoic acid	HBA	-	99	Aldrich
4-(hydroxymethyl)benzoic acid	HMBA	-	100	Aldrich
3,4,5-trihydroxybenzoic-2,6-d2 acid	d-GA	Deuterated gallic acid	99.30	CDN Isotopes
4-hydroxybenzoic-2,3,5,6-d4 acid	d-HBA	-	98.80	CDN Isotopes

PBS buffer was prepared (0.1 M, pH 7.2), using BupH™ Phosphate Buffered Saline Pack from Pierce, containing 0.1 M sodium phosphate and 0.15 M sodium chloride.

The enzyme solution was prepared by mixing 100 µL β-glucuronidase with 5 % sulphatase activity (H-2, Helix pomatia, 131 700 units/mL) with 2900 µL sodium acetate buffer (0.4 M,

pH 4.95).

3.2 Standard calibration curve

Standard solutions, diluted with MTBE, were prepared from the stock solutions made of all BA and IS, to make a standard calibration curve (Appendix 2). Ten calibration standards, containing a mix of six BAs, two deuterated quantitation internal standards and one GCMS internal standard were prepared in nine concentrations ranging from 5.29 to 1654.52 µg/L, in addition to a calibration blank (Table of accurate concentrations found in Appendix 3). The internal standard were added to all standards with constant concentration; 197.06, 183.53 and 172.32 µg/L for HMBA, d-HBA and d-GA in that order.

The standard calibration curves used throughout the analyses made in this thesis were linear (eq. 1 in 2.3.5). BA concentrations were found by the principle of eq. 2 in section 2.3.5:

Conc. BA = (Conc. BA IS x BA area) / BA IS area **(6)**

Several calibration curves were prepared throughout the analysis work because fresh standard solutions were made every second month to obtain reliable and reproducible results. Three internal standards were used in the analyses, two were the deuterated substances corresponding to the unlabeled substances GA and HBA; d-GA and d-HBA respectively, and the third was HMBA. The two isotopically labeled internal standards were used as BA IS and labeled IS for the standard calibration curve which the BAs are quantified by. The third IS, HMBA, called the GCMS IS was used to keep track of the instrument stability. If the GCMS IS concentration changes noticeably between analyses, the instrument sensibility is changing and gives an indication that a new calibration curve should be made.

3.3 Sampling

All samples analyzed for the utilization part of this thesis has been stored at - 20ºC or - 72ºC at different periods of time before analysis. Samples used for method development has either been stored at -20ºC or collected shortly before sample preparation.

3.3.1 Urine samples

Urine samples were given by volunteers (n=13) at different periods of time. An important aspect of analyzing urine samples is that they need to be comparable. To be able to compare BA concentrations in different urine samples, either the total urinary volume within each sampling period or the creatinine level was measured.

The urine sampling can be divided into three categories:

- **Method development**
  Spot urine from healthy volunteers (n=5) was used as matrix for development and evaluation of the best sample preparation method. Urine used from method development was mainly collected shortly before preparation. For method

development considering deconjugation samples stored at – 20 ºC was utilized because of known levels of creatinine.

- **Oral administration of MEDOX®**
  For samples that were to be analyzed after oral administration of Medox® capsules, two kinds of sampling were employed:

  1) Spot urine sampling
  Ten healthy volunteers, ranging from 24 to 60 years of age, were orally administered 6 capsules of Medox®. Before and two hours after ingestion of the capsules, spot urine samples were collected. The volunteers did not follow any diet restrictions. The urine samples from this experiment are from 2007 and have been stored at -20 ºC.

  2) 6 hour sampling
  Two healthy female volunteers were orally administered 8 Medox® capsules. Urine were sampled before and 1, 2, 3 and 6 hours after administration. Total urinary volume was measured for each time interval, as well as creatinine levels. No diet restrictions were followed by the volunteers. All collected urine samples were stored at -20 ºC.

- **Oral administration of delphinidin-3-O-β-glucopyranoside**
  One healthy male volunteer was orally administered 500 mg delphinidin-3-O-β-glucopyranoisde powder, swallowed with water. One urine sample was collected before ingestion of the delphinidin powder. Urine samples were also collected at each of the following 8 hours after administration. The volunteer followed a low anthocyanin diet for 48 hours prior to the administration and during the sampling period. All samples were acidified (1 mL 6 M HCl to 25 mL urine), aliquoted and stored at -72 ºC. The samples from this delphinidin experiment have been stored since 2007.

3.3.2 Plasma samples

Plasma (EDTA) samples were collected from healthy volunteers (n=4) in a similar manner as the urine samples. All blood samples were collected in two 10 ml EDTA vials, and 15 minutes after collection they were centrifuged for 10 minutes at 2000 g and 4 ºC to separate the plasma from the remaining blood cells. Plasma from both vials was mixed to a homogenous sample.

The sampling can be divided into the same three categories as the urine samples were:

- **Method development**
  Plasma samples used for method development had been stored at -20 ºC.

- **Oral administration of MEDOX®**
  For plasma samples, one kind of sampling considering oral administration of Medox® was applied. Blood was collected identically to the 6 hour sampling type for urine, with the same two female volunteers administered 8 Medox® capsules. Samples were drawn before ingestion and then 1, 2, 3 and 6 hours following administration. All plasma samples were stored at - 20ºC.

- **Oral administration of delphinidin-3-O-β-glucopyranoside**
  The same healthy male volunteer as for urine sampling was orally administered 500 mg delphinidin-3-O-β-glucopyranoside powder swallowed with water. A blood sample was drawn before the ingestion of delphinidin. Subsequent blood samples were collected after time intervals: 15, 30, 45, 60 and 90 minutes, and 2, 3, 4, 5, 6, 7 and 8 hours. Plasma was acidified (40 µL 6 M HCl to 1000 µL plasma) and stored as 1 mL aliquots at – 72 ºC. These samples have been stored since 2007.

3.4 Sample pretreatment

Pretreatment of samples before preparation step included pH adjustments and enzymatic reactions. Both urine and plasma samples were adjusted to pH 2 by addition of 1 M HCl before extraction by LLE, SPE and SPAD.

3.4.1 Deconjugation

Some samples were deconjugated before sample preparation; this step was performed before acidification of samples because of optimal enzyme reaction at pH levels 4.5 – 5.

Deconjugation of a sample involved adding 300 µL enzyme solution to 500 µL urine or plasma. The composition of the enzymatic mixture was 10 µL enzyme + 290 µL sodium acetate buffer (see 3.1.1). The mix was incubated for 2 hours at 37 ºC.

3.5 Sample preparation

All frozen samples were thawed in the refrigerator and subsequently went through a pretreatment step before sample preparation. The sample preparation consists of an extraction method and derivatization. Between the extraction and derivatization step, the sample was concentrated by evaporation either by nitrogen flow or vacuum centrifugation (except for in SPAD where the two preparation steps are combined).

All three extraction methods (LLE, SPE and SPAD) were compared with the consideration of recovery values, peak resolution and execution.

3.5.1 Derivatization

BA sample extracts and standards were derivatized by the final optimized method as follows:

Sample extracts or standards were derivatized in a mix of 25 % AcN, 10 % BSTFA and 65 % MTBE, by heating at 60 ºC for one hour.

The optimized method described above was achieved by variation of the following parameters:

- **Solvent**
  MTBE, ethyl acetate and cyclohexane were tested as solvents in the derivatization method.

- **Derivatization vial**
  Polypropylene (PP) vials and glass vials were compared to see if there was a difference in the result.

- **Derivatization reagent**
  Two different derivatization reagents were tested: BSTFA and BSTFA+TMCS (99:1).

- **Derivatization method**
  Two different setups for the derivatization performance were applied.

- **Derivatization catalyst**
  The solvents pyridine and acetonitrile were added to the process in various concentrations to compare the improving effects on the derivatization (Table 6).

Table 6. Different concentrations of AcN and pyridine tested for improvement of derivatization efficiency

Organic solvents	Concentrations (%)						
	1	5	10	20	25	30	50
Acetonitrile		x	x	x	x	x	x
Pyridine	x	x	x	x			

3.5.2 LLE

Acidified urine and plasma, as well as enzyme treated and acidified urine and plasma, were extracted by the optimized LLE method as follows:

Liquid-liquid extraction (Fig. 12) was carried out in the same matter throughout the project, except for change of solvent.

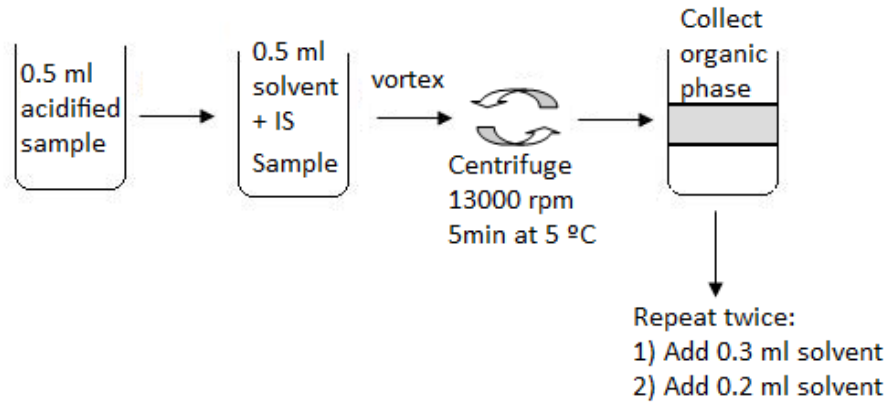


Figure 12. Illustration of the liquid-liquid extraction method

The following extraction solvents were compared:

- EtAc
- MTBE
- Diethyl ether
- MTBE with 25 % AcN
- MTBE with 20 % MeOH

3.5.3 SPE

Acidified urine and plasma, as well as enzyme treated and acidified urine and plasma, were extracted by the optimized SPE method as follows:

The SPE method (Fig. 13) was performed as illustrated, with change in elution solvent.

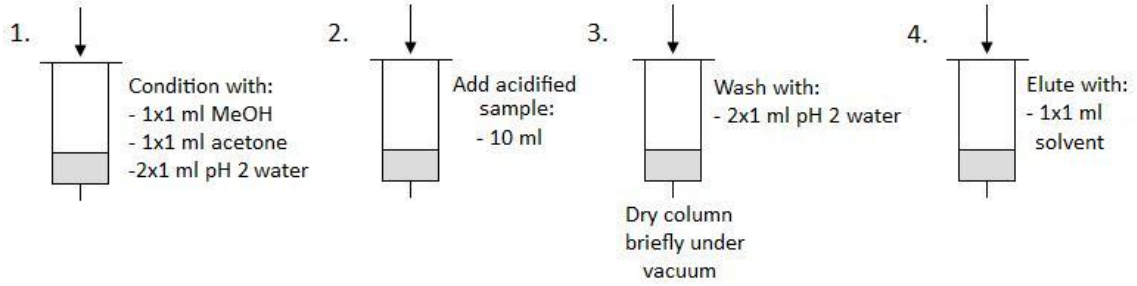


Figure 13. Illustration of the solid-phase extraction method

The variables in the SPE method include:

- **Column packing material (RP column):**

  1) ***Silica based RP column*** (Discovery DSC-18, Supelco. 1 mL/100mg. Polymerically bonded, octadecyl (18 %C), endcapped), and:

  2) ***Polymeric RP column*** (Strata-X 8B-S100-TAK, Phenomenex. 1mL/30mg Surface modified styrene divinylbenzene polymeric surface).

- **Elution solvents:**
  Three solvents were used for elution: EtAc, MeOH and 90 % acetone.

3.5.4 SPAD

SPAD was examined as a method combining the two sample preparation steps; extraction and derivatization. Acidified urine and plasma samples were extracted using this method. The SPAD method (Fig. 14) is almost identical to the SPE method from the last section (3.5.3) except for the derivatization step which has been added after the washing step.

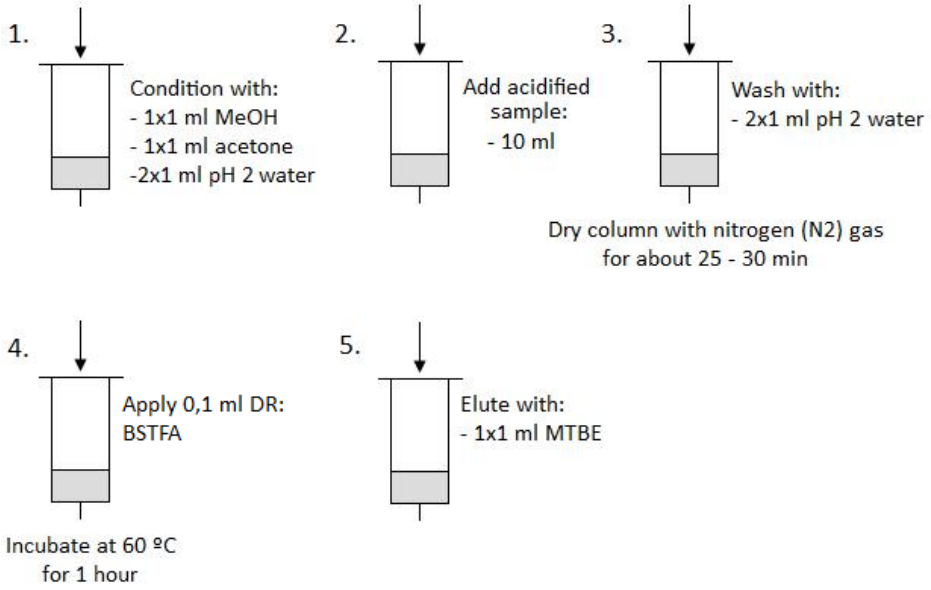


Figure 14. Illustration of the solid-phase analytical derivatization method

Two different column types where applied when performing SPAD:

- Anionic exchange column (Oasis Max, Waters)
- Reversed phase (RP) column (Discovery DSC-18, Supelco)

3.6 Gas chromatography mass spectrometry (GCMS) analysis

The instrument combination used for sample analysis in this thesis was gas chromatography coupled with a mass spectrometry using electron ionization: GC-EI-MS. The analytical equipment consisted of a Shimadzu GCMS-QP2010 (QP – quadrupole) and an AOC-5000 autosampler (Bergman, Norway), controlled by Shimadzu GCMS Labsolutions (Version 2.50 SU1). Samples and standards were injected in splitless mode with a sampling time of 1 min. Helium was used as carrier gas and the initial column used during most of the project was Factor Four capillary column, 50 m x 0.25 mm with 0.25 µm film thickness (Varian, Netherlands). The Factor Four column was replaced towards the end of the utilization analyses to CP-SIL 8 CB-MS, 50 m x 0.25 mm with 0.25 µm film thickness (Varian, Netherlands). A more complete list of instrumental settings can be found in Appendix 4 (Instrumental settings GCMS).

A GC method is composed by time and temperature adjustments. The ramp, meaning how much increase in degrees Celsius will occur per minute, is adjusted in the GC method. By elongating or shortening the temperature interval, a compounds retention time will change. One GC method was chosen, and only changed slightly during the analysis work due to column cutting. The column was replaced towards the end of the project; a new GC method compatible with BA separation and the new column was then made.

To detect the compounds separated by GC, a mass spectrometer is coupled with it. The initial analyses of single compounds of GA, PCA, VA, DHMBA, SA, HBA, HMBA, d-GA and d-

HBA was made in SCAN mode. The range was set to 70 – 500 m/z. After determination of identifier (qualifier) and quantifier ions, SIM mode was proceeded with for all analyses.

3.7 Limit of detection (LOD) / limit of quantification (LOQ)

The limit of detection (LOD) is the quantity or concentration of the smallest amount considered detectable for an analytical method. The limit of quantification (LOQ) is the smallest quantity or concentration considered reliable enough to measure the quantity.

The LOD (eq. 7 & 8) and LOQ (eq. 9 & 10) values are calculated by means of the signal-to-noise (S/N) ratio and the knowledge of the injected concentration. In GCMS, the LOD value is defined to be 3 times higher than the background noise ($S/N \geq 3$), and LOQ 10 times higher ($S/N \geq 10$). Since there is more uncertainty and difficulty in the quantification of a compound than the detection of it, the S/N value needs to be higher for LOQ determination.

Determination of LOD:

f = S/N / 3 **(7)**

LOD = injected concentration / f **(8)**

Determination of LOQ:

f = S/N / 10 **(9)**

LOQ = injected concentration / f **(10)**

The post analysis program for the GC can give an estimation of the S/N values for all compounds, or the S/N value can be determined by observation and comparison of the height of the peak and the surrounding noise.

To determine LOD and LOQ, the standard solution of lowest concentration (std. 2) for the standard calibration curve was diluted to find S/N values corresponding to the LOD and LOQ limits.

The standard deviation (SD) for each compound's LOD and LOQ values was calculated (eq. 11). Where N is the number of values of which the mean and SD is calculated from, and $\bar{x}$ represents the mean value.

$$SD\ (\sigma) = \sqrt{\frac{1}{N} \times \sum_{i=1}^{N} (X_i - \bar{X})^2} \qquad \textbf{(11)}$$

3.8 Recovery

Recovery is used to indicate the yield of an analyte in a preconcentration or extraction stage in an analytical method [52]. Recovery (eq. 12) is determined by dividing the obtained concentration by analysis with the concentration which the sample was spiked with before undergoing the process leading up to the analysis. In this case the sample goes through an extraction and derivatization step. To get the recovery percentage, the result is multiplied with 100. SD (eq. 11) has also been calculated for all recovery values.

$$\text{Recovery \%} = \frac{\text{Conc. compound quantified by analysis}}{\text{Conc. compound added to sample}} \text{ x } 100 \qquad \textbf{(12)}$$

3.9 Utilization

BA concentrations in urine samples have to be corrected with regard to either creatinine levels or total urinary volume to be comparable (eq. 13 & eq. 14).

Correction of concentration taking creatinine levels into account:

$$\frac{\text{Conc. compound (µg/L)}}{\text{Creatinine level (mmol/L)}} = \text{µg compound / mmol creatinine} \qquad \textbf{(13)}$$

Correction due to total urinary volume:

$$\textbf{Conc. compound (µg/L) x total volume (L) = µg compound} \qquad \textbf{(14)}$$

4. Results and discussion

4.1 Gas chromatography mass spectrometry (GCMS)

4.1.1 Mass spectrometry

Initially, TMS derivatives of single BAs were injected and their mass spectra recorded in full scan mode (m/z 70 – 500). The full scan spectra were used for selection of ions for quantitative SIM analyses of a mix of all target compounds.

Identifier and quantifier ions were chosen based on the evaluation of the mass spectras for each individual compound (Fig. 15). Some mass spectra contain a limited number of prominent ions (Fig. 15 upper), which ease the selection of quantifier ion and identifier ion, whereas other spectra have several alternatives (Fig. 15 lower). The mass spectrum for gallic acid is easier to determine ions for than for instance syringic acid, both shown in the example. When a compounds mass spectrum contains multiple ion alternatives, the most prominent and unique ions are generally chosen.

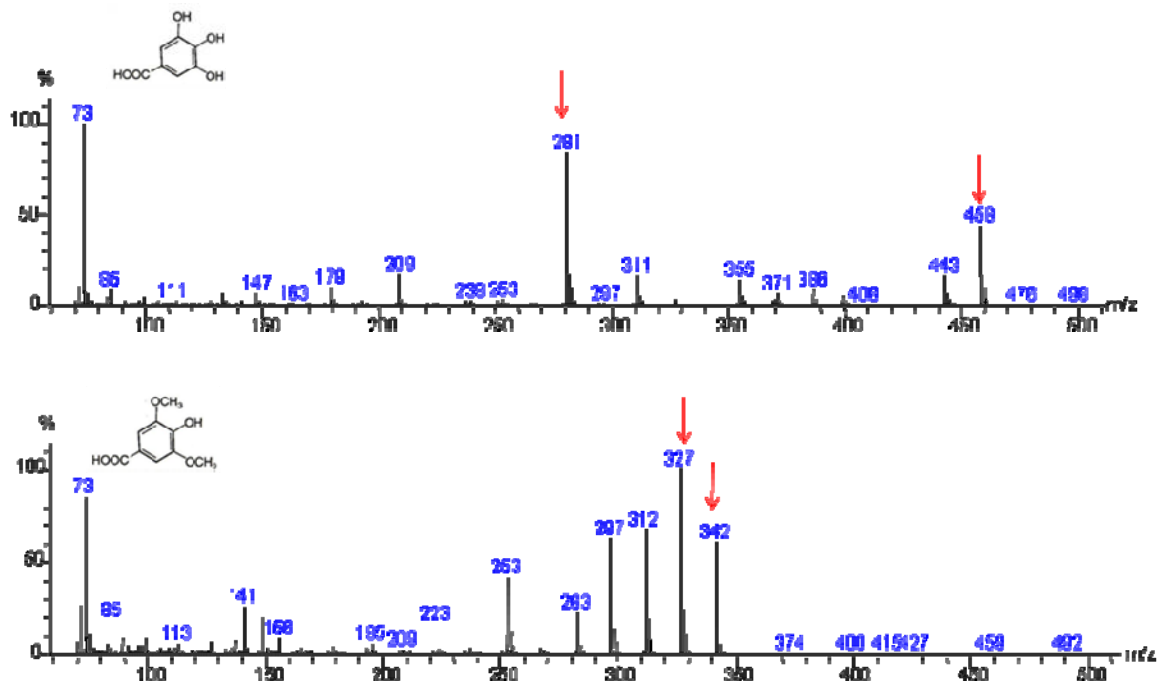


Figure 15. Mass spectra of GA (upper) and SA (lower) with arrows showing selected identifier and quantifier ions.

Mass spectra for the remaining compounds not represented in Fig. 15 are found in Appendix 5.

Selected quantifier and identifier ions are presented in Table 7.

Table 7. Quantifier and identifier ions for each individual compound analyzed in the thesis

	Ions	
Compound	Quantifier	Identifier
GA	281	458
PCA	193	370
VA	297	312
DHMBA	223	400
SA	327	342
HBA	267	282
HMBA	281	296
d-GA	283	460
d-HBA	271	286

The ions selected as quantifier and identifier ions for all BAs correspond to the general fragmentation patterns found in literature [19, 24, 25, 41]. The three BAs with two or more TMS groups present in adjacent positions on the benzene ring, GA, PCA and DHMBA, all have a prominent peak at $[M-177]^+$ which is a common fragment for those components. This is also the case for the IS d-GA. The remaining BAs, containing only TMS groups in non adjacent positions, all have quantifier ions based on the $[M-15]^+$ fragment. As can be observed in the SA mass spectrum (Fig. 15), other common ion fragments such as $[M-30]^+$, $[M-59]^+$ and $[M-89]^+$ are also found. The $[M-15]^+$ fragment, however, has highest intensity and one of the most unique masses found compared to the other BAs chromatograms, and is therefore chosen, as is the case for the other BAs too.

All the 17 selected ions were used for quantitative analysis in SIM mode. Since the detector sensitivity decreases with an increasing number of simultaneously acquired SIM ions, the number of ions in one acquisition window is limited to 10. Therefore, the selected ions were acquired in two chromatographic time windows (Fig. 16). By specifying which masses the detector will recognize in SIM mode, unwanted peaks with similar retention times will be excluded and thereby improves the background noise.

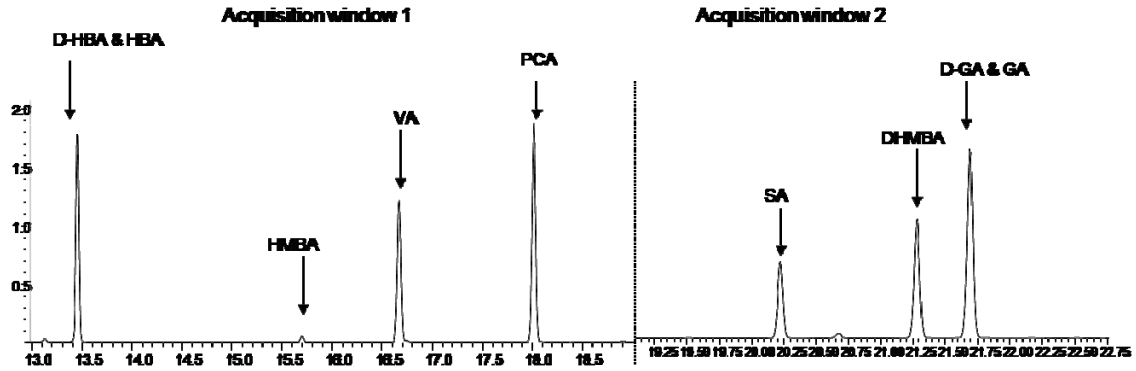


Figure 16. Chromatogram showing two acquisition (time) windows

The acquisition windows end and start where there is sufficient time between two peaks. The ions for the first 5 compounds are programmed to be acquired in the first window, and the

ions of the remaining 4 compounds are registered in the next window. The order in which the compounds appear in the chromatogram is presented in section 4.1.2.

4.1.2 Gas chromatography

The retention time of each compound varies depending on which GC method is used, and changes if the column is shortened (by end-capping) or if a new column of different length is installed. However, even if the retention time changes due to various causes, the sequence of the compounds in the chromatogram will not change.

The GC method used, with small modifications due to column-cutting or change of column, is shown in Table 8:

Table 8. BA SIM method 1

Ramp ºC/min	Temperature ºC	Holdtime min
-	50	1
25	160	0
3	215	0
15	300	5

Even though the analytes can be separated in a GCMS analysis, the goal is to achieve optimized results. To achieve this, several factors can be adjusted to improve the separation of the compounds. A good separation means having a high efficiency and resolution value. The separation can be altered either by making changes in the GCMS methods or making changes in the sample preparation before analysis.

Initially, the temperature programs were changed to find out if the separation improved (Fig. 17). The method which resulted in shortest retention times was chosen, presented by the upper chromatogram in the figure.

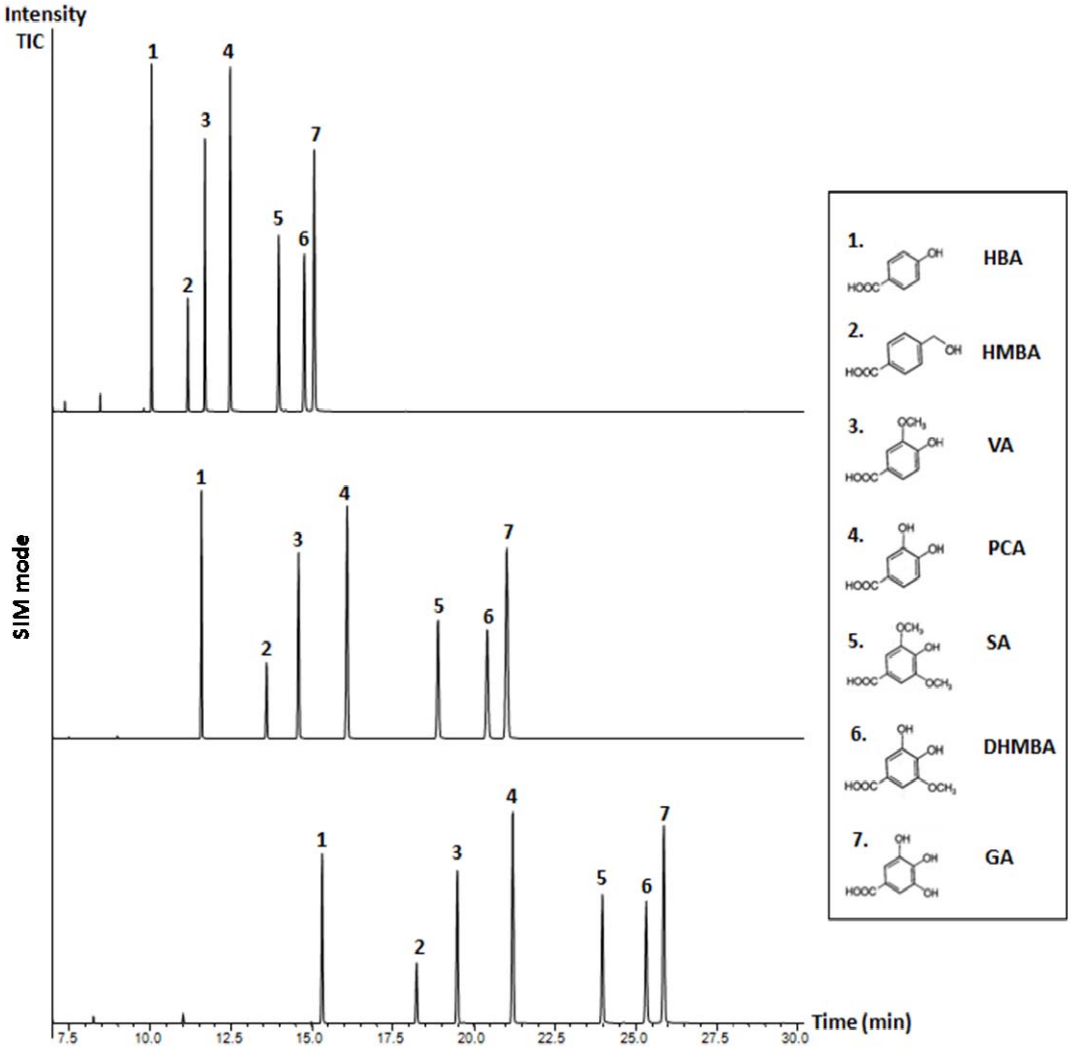


Figure 17. Difference in chromatograms caused by change in GC temperature program

The peak separation of the three methods compared with regards to changes in the temperature program is similar, and the method chosen for further analysis results in higher sample throughput. The intensity of the peaks in the upper chromatogram in Fig. 17 is also higher as compared to the two other methods due to narrower peaks. The time period separating the peaks in the middle and lower chromatogram are longer, however, since the resolution for the first is very good that method was chosen.

When the GC column was replaced towards the end of the experimental work, the retention times shifted and made it necessary to alter the GC method slightly. The retention times was pushed forward by approximately 2 minutes, making the new method slightly longer than the previous (Table 9).

Table 9. BA SIM method 2

Ramp ºC/min	Temperature ºC	Holdtime min
-	50	1
25	160	0
2.5	215	0
15	300	4

The sequence of the compounds analyzed was determined by first analyzing each separate compound and thereafter a mixture of all BA and IS (Table 10).

Table 10. Sequence of the compounds

Sequence	Compound
1	d-HBA
2	HBA
3	HMBA
4	VA
5	PCA
6	SA
7	DHMBA
8	d-GA
9	GA

The two deuterated internal standards, d-HBA and d-GA, have very similar retention time to the corresponding BAs HBA and GA respectively, due to their small difference in mass. Therefore there will only seem as though there is one peak for d-HBA + HBA and d-GA + GA in the total ion current (TIC) chromatogram.

4.2 Sample preparation

Two sample preparation methods, LLE and SPE, have been compared with respect to extraction efficiency of BAs. Derivatization has also been optimized to give the best possible result considering derivatization efficiency and chromatographic performance. A third extraction method combining SPE and derivatization was also tested because it would mean a less time consuming sample preparation step. A poster presenting some of the results for method development of sample preparation techniques was enrolled to the 2010 Symposium of Chromatography in Sandefjord (Appendix 6).

LLE was chosen as the best extraction method compared to both SPE and SPAD based on recovery and chromatographic peak separation. SPAD was excluded for further analyses early in the method development process due to poor results. LLE and SPE was explored more thoroughly, however LLE yielded better recovery of all BAs as well as a better chromatographic separation of peaks (Fig. 18). LLE is also a less labor demanding method taking less time to perform. Results for each sample preparation method are presented in the following sections.

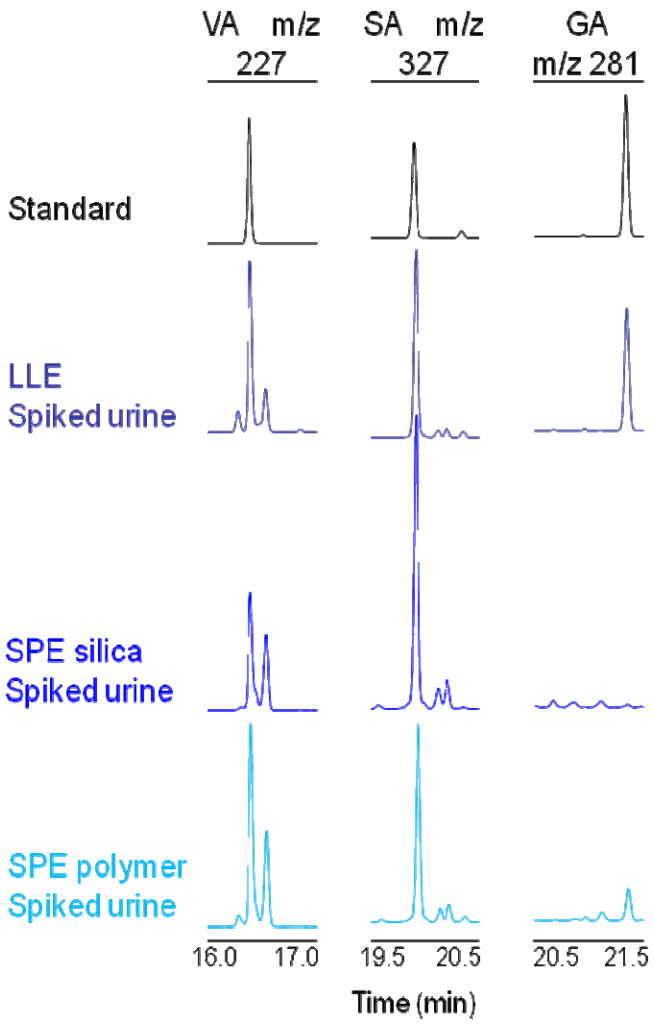


Figure 18. Comparison of peak shape of three BA using LLE and two different SPE columns

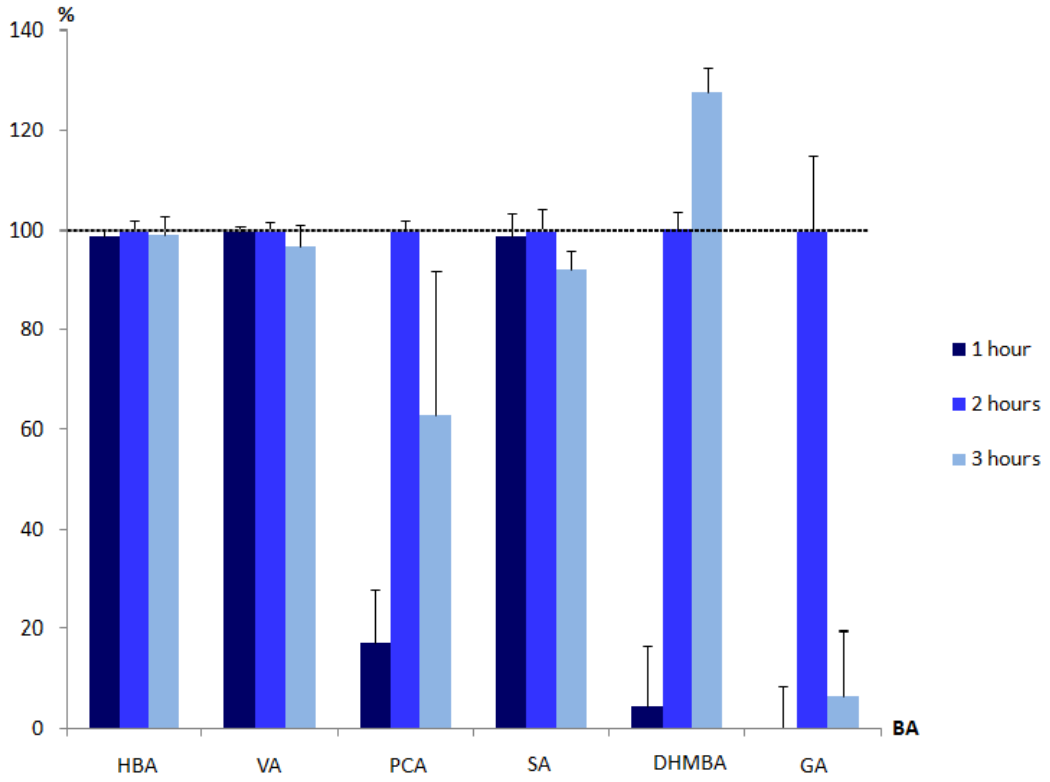
Recovery percentage for each compound has been determined for LLE, SPE and SPAD. Recovery for both LLE and SPE using different extraction solvents have been acquired and for LLE both with and without deconjugation of samples. For SPE and SPAD recovery results have been determined in urine samples, whilst for LLE recovery results have been determined in both urine and plasma matrices being the method of choice. Recovery values for each extraction method are presented under their respective section and accordingly discussed and compared in the following recovery section (4.2.5).

For the utilization part of the results, the BA concentrations in different samples needed to be compared. Urine samples are often of different densities and concentrations which need to be taken into account before comparison of the BA concentrations. To be able to compare the BA concentrations in urine samples before and after ingestion of MEDOX® capsules, creatinine levels were used as a measure. For the samples given before and after intake of delphinidin-3-O-β-glucopyranoside powder, total urinary volume was used for comparison.

Creatinine levels give an indication of how concentrated a urine sample is. Creatinine is a break down product of creatine phosphate in the muscles. The kidneys filter creatinine out of the blood and the creatinine leaves the body in the urine [53]. Depending on how much fluids a person has consumed, the creatinine levels will vary. Morning urine has an especially high value, and if a person drinks a lot which they will in the case of these types of experiments in this thesis, the creatinine levels will drop.

With regard to the sample pretreatment, the composition of the enzyme solution is based on similar studies. The solution used in this thesis, is an approximation of the method used by Nurmi et al. 2006 [15] with 2500 U β-glucuronidase/mL urine. The sample material used in the experiments are 0.5 ml (= 1250 units/ 0.5 mL). The β-glucuronidase with 131700 U/mL equals 132 U/µL, and it is therefore used 10 µL of enzyme to every 0.5 mL sample deconjugated. Nurmi et al. used a sodium acetate buffer with a pH of 4.1, which is a little lower than the optimal pH for deconjugation of 4.5 – 5, and the buffer made for this experiment is therefore of a higher pH value.

Also different deconjugation times have been tested to optimize the deconjugation treatment of the samples. There was some difference in incubating the samples at 37 ºC for 1, 2 and 3 hours for most BAs (Fig. 19). A slight increase in recovery was obtained for allowing the samples to incubate for 2 hours compared to 1 hour for HBA, VA and SA. For the remaining 3 BAs, PCA, DHMBA and GA, there was a vast difference. No significant improvement was however found by increasing the incubation with another hour from 2 to 3 hours of incubation, only decrease for most compounds if anything. 2 hours incubation time was based on these results considered the best choice for deconjugation.



Figur 19. Effect of different incubation times for deconjugation

The effect is measured by the difference in concentration found after the three time spans. In Fig. 19, the effect given by 2 hours of incubation is set as 100 % being the overall best incubation time.

4.2.1 Derivatization

The optimized derivatization method as presented in the materials & methods section 3.7.1 is as follows: 100 µL BSTFA, 250 µL AcN and 650 µL MTBE added to the evaporated sample to a total of 1 mL.

Derivatization of the analyzed benzoic acids was necessary to be able to analyze them by GCMS. The derivatization increases the volatility of the BAs and their thermal stability, by converting protonic functional groups into thermally stable nonpolar groups. The derivatization was optimized by altering a number of factors and comparing the chromatographic result of each of the alternatives. The following aspects were examined: solvents, derivatization reagent, derivatization catalyst, derivatization method and vials for derivatization.

4.2.1.1 Derivatization solvents

MTBE was chosen as the solvent giving best results for derivatization.

A mixture of the six benzoic acids and the GC IS, HMBA, was analyzed by GCMS and the solvent presenting the best chromatographic result was chosen. By comparing the three different solvents MTBE, ethyl acetate and cyclohexane, MTBE gives the best result. It is important to have a method where the resolution is the best possible, with good peak separation, peak shape and as little background noise achievable.

By comparing the resulting chromatograms (Fig. 20) for each derivatization solvent, it is clearly MTBE that is the best choice to pursue for further derivatizations. There is less background noise than for the other two solvents, and the peak shape is sharper for all BAs with perhaps an exception for GA which has some tailing. The peaks in the MTBE chromatogram show in general little or no tailing, with an exception for GA while cyclohexane gives tailing both in front and the back of the BA peaks. Ethyl acetate also give tailing in addition to as an excess of background noise and other peaks with the same intensities as the BA peaks.

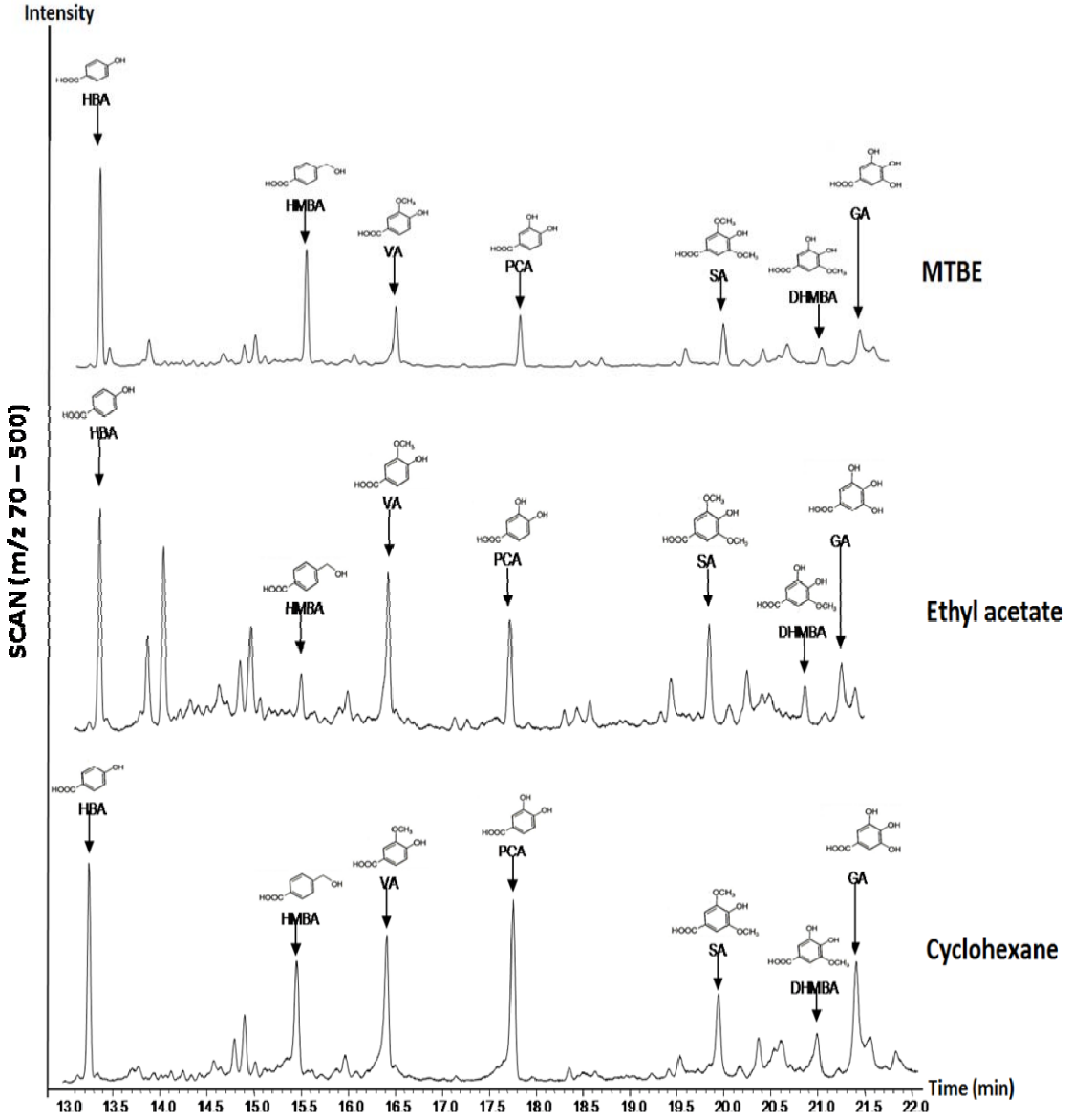


Figure 20. Chromatographic result of different derivatization solvents

4.2.1.2 Derivatization vials

Polypropylene (PP) vials were used for derivatization initially but the chromatogram showed a lot of noise giving reason to believe the vials could be of importance to the result. The PP vials are supposed to be inert but somewhere in the derivatization process these vials interfere with the end result. Glass vials were tested to compare the difference in chromatographic appearance. Glass vials gave a visibly better result than PP vials (Fig. 21).

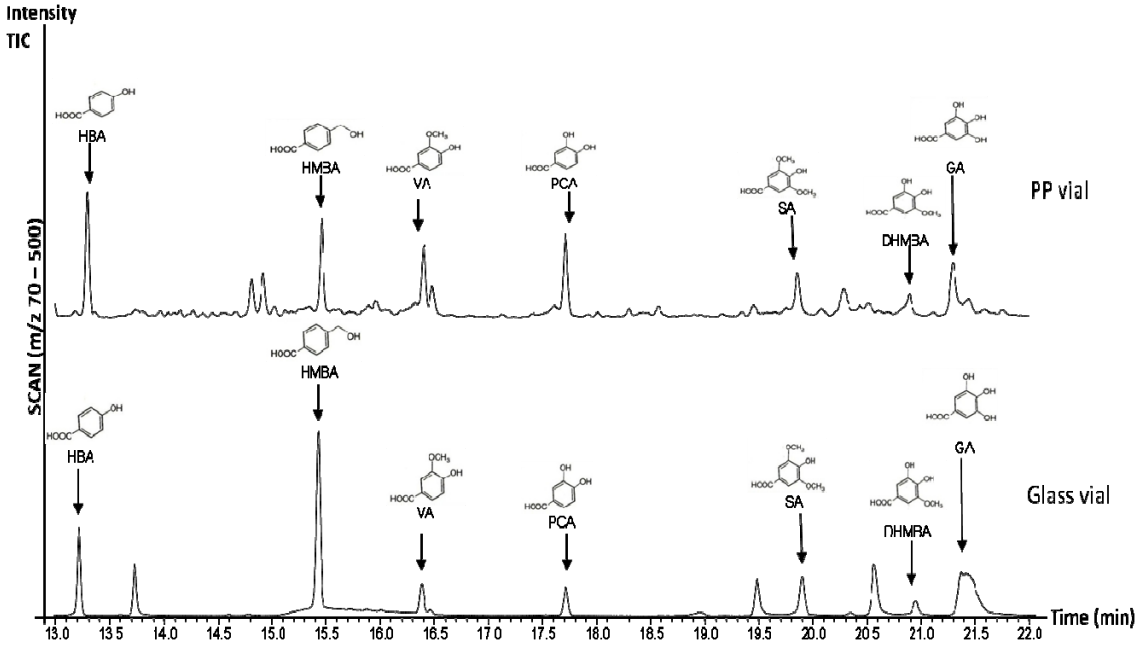


Figure 21. The use of PP vials vs. glass vials

After testing the significance of vial application, it became clear that PP vials could be used for the initial steps of sample preparation, including the extraction, without interfering with the results. Switching to glass vials before evaporation and derivatization of the sample material was however a necessary effort to improve the result.

4.2.1.3 Derivatization reagents

Two derivatization reagents (DR) were evaluated: BSTFA and BSTFA+TMCS. The selected DR for further analyses was BSTFA.

BSTFA+TMCS was tested because TMCS is often found to be a catalyst to increase TMS donor potential. In this case, however, BSTFA alone gave a better result for the intensity of the BA peaks (Fig. 22).

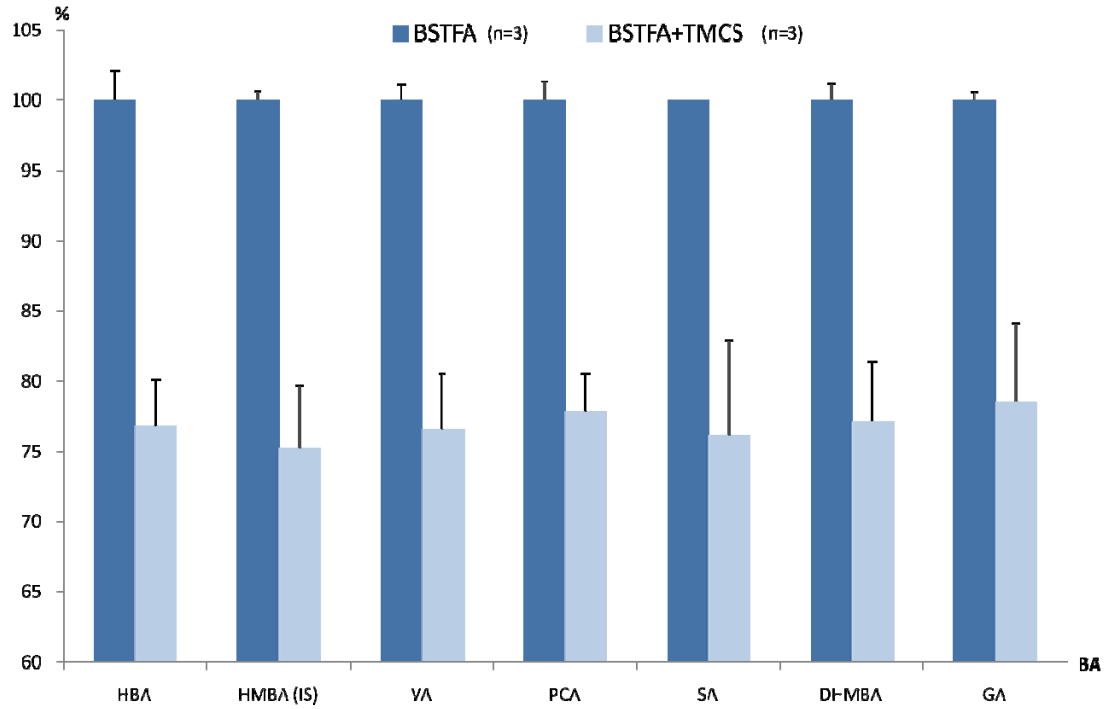


Figure 22. Difference in peak area by using BSTFA vs. BSTFA+TMCS as DR

In the figure above (Fig. 22), BSTFA is set as 100 % as it gave the best results, for illustration of the difference between the two reagents.

4.2.1.4 Derivatization method

Two setups for the derivatization methods have been applied and compared to choose the best way to perform the derivatization step of sample preparation (Fig. 23).

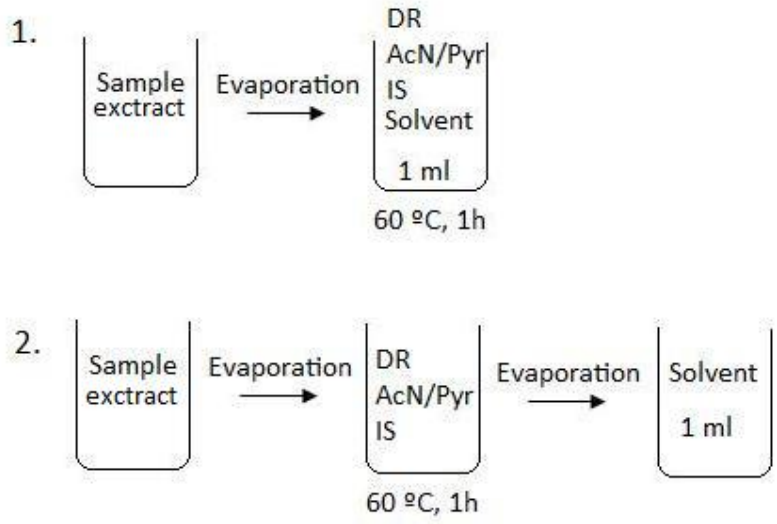


Figure 23. Two derivatization methods

Method 1 (Fig. 23 upper) show a derivatization method where evaporation of sample material is done before derivatization, whereas method 2 (Fig. 23 lower) apply evaporation both before and after the heat treatment.

Method 1 has been utilized throughout the project, this is because the methods do not differ much in result (Fig. 24) and it is less time consuming. The intensity of the peaks given by using derivatization method 1 is slightly higher than those of method 2.

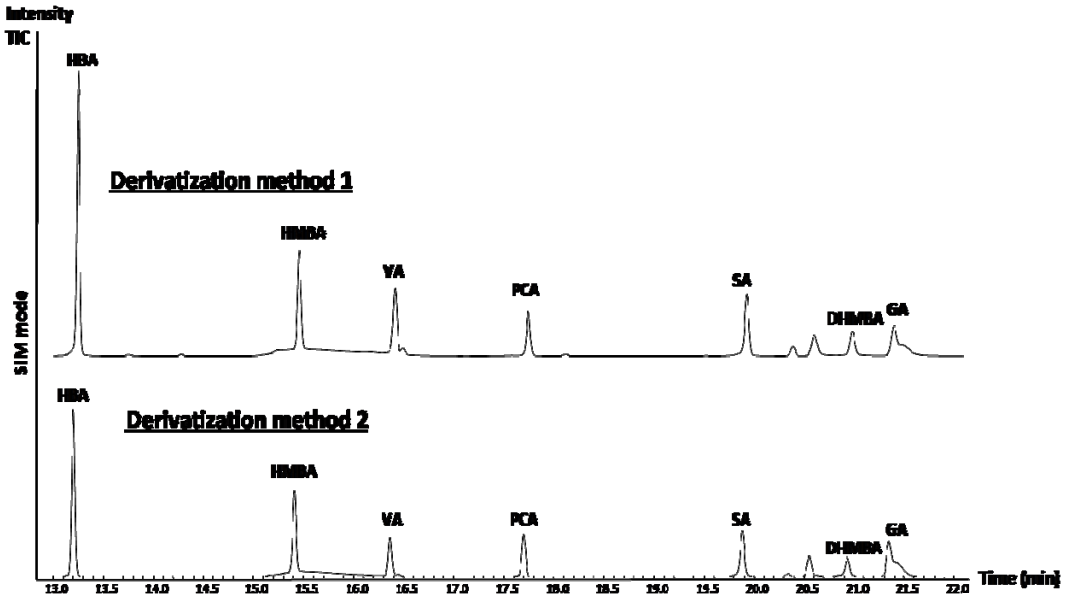


Figure 24. Difference in derivatization methods presented by their chromatograms

The chromatograms shown in Fig. 24 are the result of derivatization before any derivatization catalysts was added to the method, which could explain why there is some tailing.

In the final procedure, an additional drying step was added prior to derivatization to avoid excess moisture. Anhydrous sodium sulphate was added to the sample extract after evaporation. The rest of the derivatization process was carried out in the same matter.

4.2.1.5 Derivatization catalysts

Acetonitrile (AcN) or pyridine was added separately in several concentrations to improve the outcome of the derivatization (Fig. 25). AcN was chosen as the best catalyst to the derivatization reaction of the two, and 25 % was proven to be the most effective concentration. The different concentrations used for derivatization with pyridine were: 1, 5, 10 and 20 %. Concentrations compared for acetonitrile was initially 5, 10, 20, 25, 30 and 50 %, of which 20 – 30 % AcN gave best results, leading to further analysis of 20, 25 and 30 % AcN to find the best concentration for derivatization.

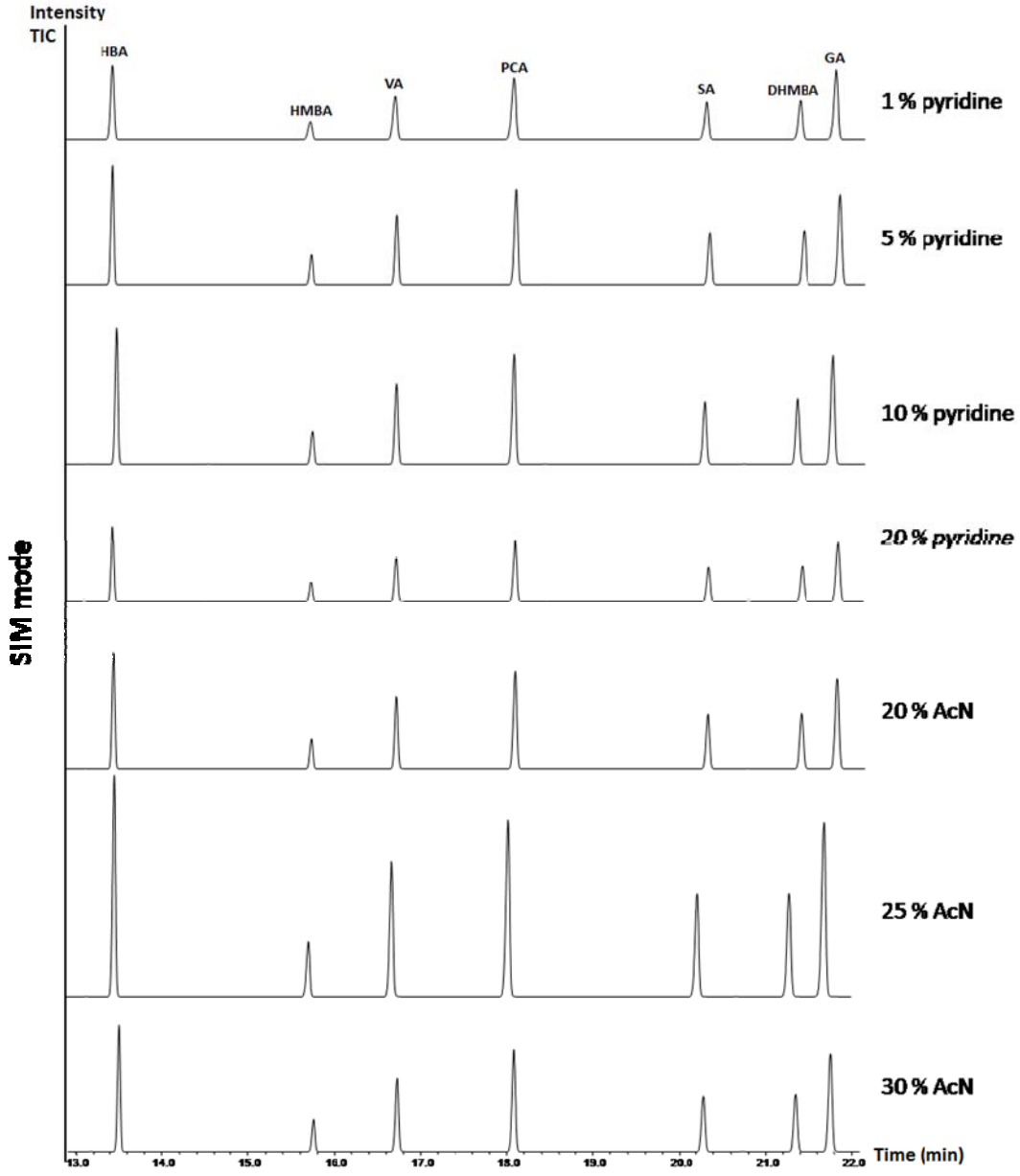


Figure 25. Effect on peak intensity using different concentrations of pyridine and AcN for derivatization

10 % pyridine for derivatization gave the best result for pyridine added to the derivatization process (Fig. 26) and 25 % AcN gave the best result of the acetonitrile concentrations tested (Fig. 27). The peak intensity and peak area are both higher for 25 % AcN and it was therefore chosen as the best derivatization catalyst.

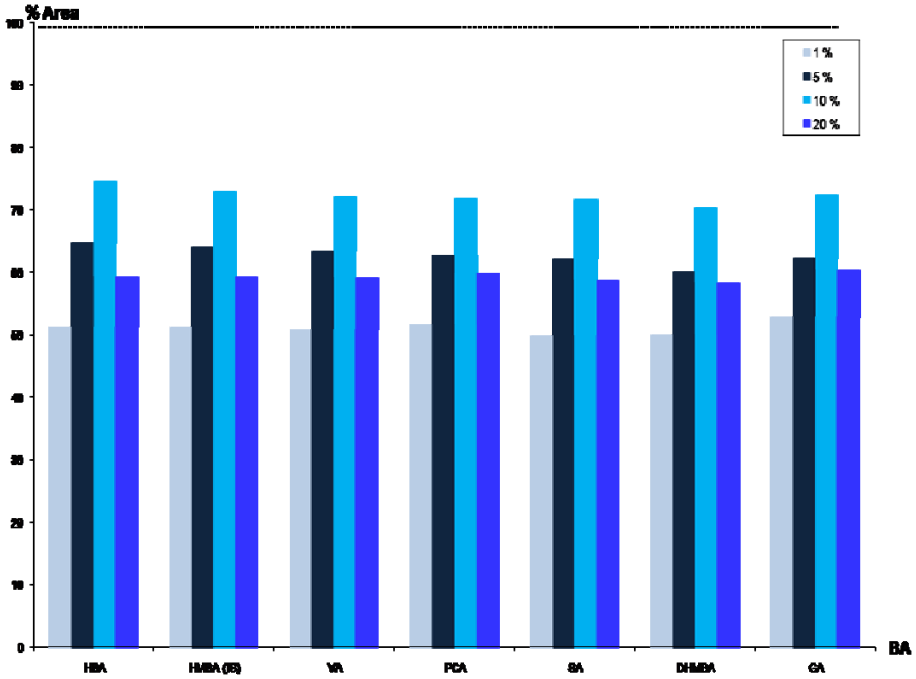


Figure 26. Difference in derivatization result by the addition of various amounts of pyridine

The y-axis of the figure above (Fig. 26) represents the peak area (%) compared to the best acetonitrile concentration which is set to 100 %. From the figure it is clear to see that 10 % pyridine gives the best result, however compared to AcN the effect is visibly lower.

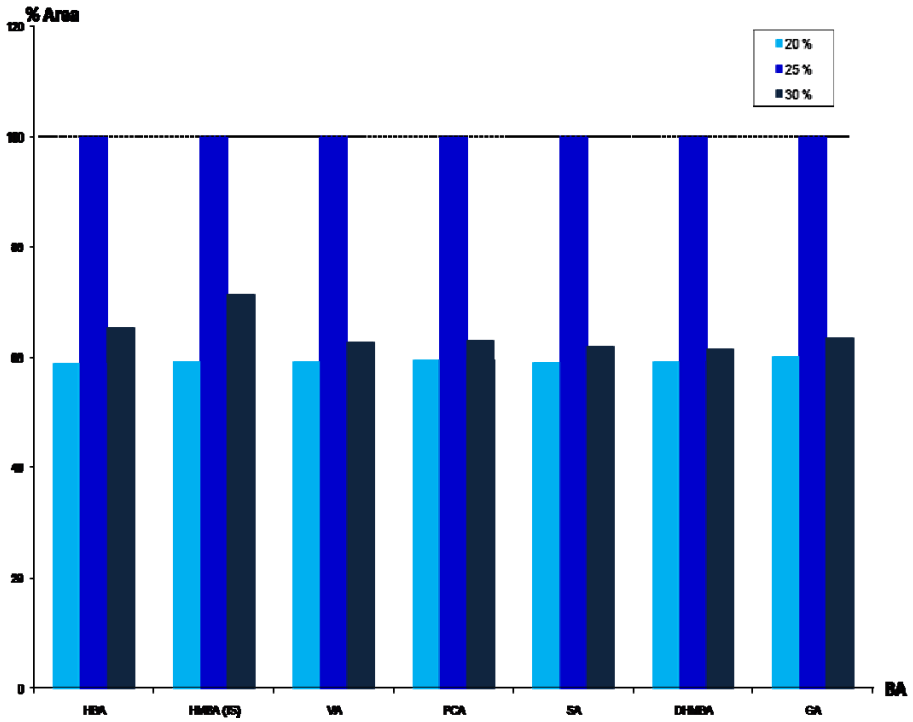


Figure 27. Peak area effect caused by different amounts of AcN for derivatization

The effect seen in Fig. 27 above show that 25 % acetonitrile is evidently the best derivatization catalyst.

4.2.2 Liquid-liquid extraction (LLE)

LLE was chosen as the best sample extraction method compared to SPE (4.2.3) and SPAD (4.2.4). The best extraction solvent for LLE was found to be ethyl acetate (EtAc) based on extraction efficiency and reproducibility.

The other solvents evaluated were: MTBE and diethyl ether. Chromatograms for the samples extracted with the different solvents are presented in Fig. 28.

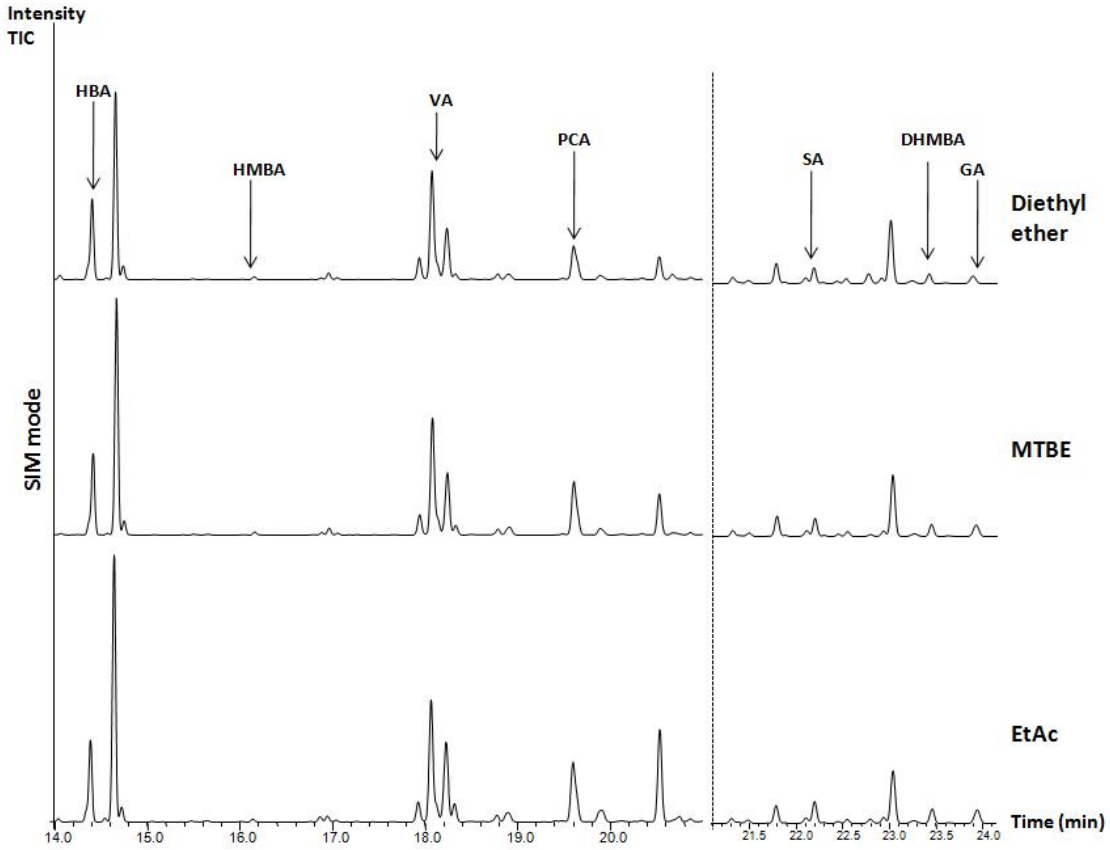


Figure 28. Effect of LLE extraction solvents: Ethyl acetate, MTBE and diethyl ether

There is very little difference in the chromatographic appearance comparing samples extracted with EtAc, MTBE and diethyl ether. The BA peak intensities are highest for EtAc, then slightly lower for MTBE in comparison and accordingly slightly lower for diethyl ether compared to MTBE.

Addition of a polar solvent to the extraction solvent was made in an attempt to modify the phases for a possible improvement of extraction yield. MTBE w/ 25 % AcN and MTBE w/ 20

% MeOH were compared with the other solvents. However, both the added solvents, AcN and MeOH, modified the water phase instead and there was a significant decrease in recovery and lack of reproducibility for the obtained results.

All recovery experiments considering extracting solvents were performed on urine samples which were not deconjugated. The recovery results for the target compounds are presented in Table 11.

Table 11. Recovery results for the six target BAs in urine applying different extraction solvents for LLE

	Recovery (%)±SD			
BA	Ethyl acetate (n=5)	MTBE (n=5)	Diethyl ether (n=4)	MTBE w/ 20 % MeOH (n=3)
HBA	89 ± 5	88 ± 3	85 ± 4	67 ± 13
VA	100 ± 6	97 ± 6	92 ± 4	25 ± 35
PCA	95 ± 4	91 ± 2	82 ± 2	20 ± 48
SA	105 ± 6	100 ± 3	91 ± 3	20 ± 33
DHMBA	94 ± 6	88 ± 2	72 ± 2	19 ± 34
GA	121 ± 4	114 ± 1	113 ± 3	65 ± 26

Based on the recovery results, EtAc was chosen as the best extraction solvent and used to determine the recovery for BAs in both urine and plasma, using the optimized LLE extraction method. Recovery yield for both matrixes have also been obtained for both deconjugated samples and samples not treated with enzyme. Any presence of the target BAs in urine and plasma samples before spiking is subtracted before calculation of recovery.

- **Recovery results for BA in urine:**

  5 parallels (n=5) of spiked urine samples extracted with LLE by EtAc was analyzed with regard to each BA. The recovery results presented are the calculated average (mean), and the standard deviation is also given for each value. Recovery values for samples both undergoing deconjugation and for samples not treated with enzyme is presented in Table 12.

  Table 12. Recovery (%) for BA in urine, n= 5

	Recovery (%) ± SD	
BA	Urine	Urine (deconjugated)
HBA	89 ± 5	96 ± 12
VA	100 ± 6	107 ± 30
PCA	95 ± 4	79 ± 6
SA	105 ± 6	73 ± 19
DHMBA	94 ± 6	62 ± 3
GA	121 ± 4	121 ± 4

  HBA and VA have both elevated recovery values after deconjugation compared to in the samples which are not enzyme treated. These BAs also have higher standard deviation values as compared to the not enzymatically treated urine which is the case for most BAs after deconjugation here. The elevated recovery values in addition to

recovery values over 100 % could most likely be explained by interfering peaks which cannot be separated from the BA peaks. Some BAs, PCA, SA and DHMBA have lower recovery % after deconjugation. The addition of a deconjugation step means an extra sample preparation step which increases the possibility of BA loss before analysis.

- **Recovery results for BA in plasma:**

  3 parallels (n=3) of spiked plasma samples was analyzed for all target BAs, and recovery results for each compound is presented as the calculated mean. The SD is also specified for each recovery value. Recovery for the six BAs is presented in Table 13 with recovery results for both deconjugated samples and samples which have not been given any enzyme treatment.

Table 13. Recovery (%) for BA in plasma, n=3

	Recovery (%) ± SD	
BA	Plasma	Plasma (deconjugated)
HBA	57 ± 3	60 ± 1
VA	105 ± 5	116 ± 3
PCA	63 ± 3	55 ± 3
SA	139 ± 7	158 ± 3
DHMBA	64 ± 6	37 ± 2
GA	80 ± 3	81 ± 2

In plasma there is a little higher recovery % for the deconjugated samples on the whole compared to not enzymatically treated samples, except for PCA and DHMBA which are lower. This could indicate some loss of these compounds during that sample pretreatment step, however there could also be loss of these in a later preparation step. High recovery values, over 100 %, could be the result of interfering peaks. In the case of the plasma samples, SA and VA are the two BAs with highest recovery results which are both over 100 %.

4.2.3 Solid phase extraction (SPE)

To achieve the best possible recovery results for an extraction method, an alternative method to LLE was examined: Solid phase extraction (SPE). Comparison of the two methods considering recovery results made LLE the best method, and SPE was therefore not used for further sample preparation.

The components of SPE tested for improvement of the outcome were: column packing material and elution solvents. The results were compared based on recovery results and chromatographic peak appearance.

Two SPE packing materials were tested; silica based column material vs. polymeric based (Table 14).

Table 14. Difference in peak area using silica- and polymer based column sorbents

	Peak area					
SPE sorbent	**HBA**	**VA**	**PCA**	**SA**	**DHMBA**	**GA**
Silica	167544	1167828	14608	2174580	251384	4764
Polymer	2830608	1967784	1683328	1610080	2986324	198132

Based on the areas from Table 14, polymer based packing material for the RP column give best results. SA is the only compound with higher peak area using the silica based packing material than the polymer based packing material. However, polymeric is a better choice on the whole. Another advantage using polymer based RP columns is that it has higher pH range than the silica based, and the packing material does not collapse if the column dries up between SPE steps.

When the optimal column sorbent (packing material) was determined, different elution solvents was tested to find the solvent giving the best results. 90 % acetone was chosen as the best elution solvent based on recovery results. MeOH and EtAc were also tested as extraction solvents. EtAc was eliminated early in the process due to poor results (Fig. 29).

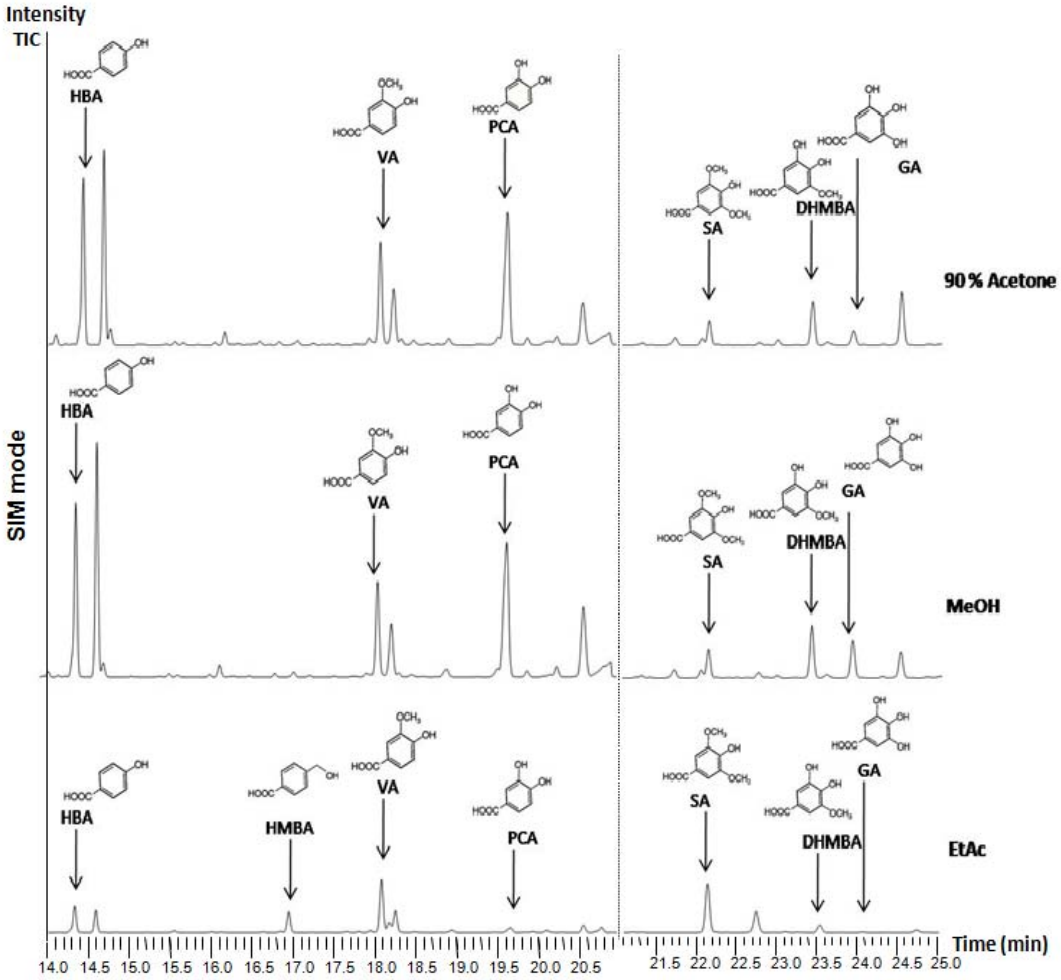


Figure 29. Peak shape and separation using different elution solvents for SPE

Ethyl acetate as elution solvents gave poor chromatographic results regarding peak resolution. 90 % acetone and MeOH, for which the chromatographic result and peak intensities was very similar, was proceeded with further to obtain recovery results (Table 15).

Table 15. Recovery (%) results for SPE using different extraction solvents

	Recovery % ± SD	
BA	90 % Acetone (n=5)	MeOH (n=5)
HBA	36 ± 4	34 ± 6
VA	74 ± 5	73 ± 13
PCA	79 ± 7	73 ± 14
SA	125 ± 17	120 ± 7
DHMBA	104 ± 8	101 ± 5
GA	43 ± 2	53 ± 14

4.2.4 Solid phase analytical derivatization (SPAD)

Because sample preparation before GCMS analysis is quite time consuming, solid phase analytical derivatization was examined as an alternative method. SPAD involves the combination of SPE and derivatization in one method. Because SPAD gave very unstable and poor results for extraction, it was excluded for further analysis early in the method development procedure.

Two column types were tested initially to find the column giving best results for extracting the compounds (Fig. 30).

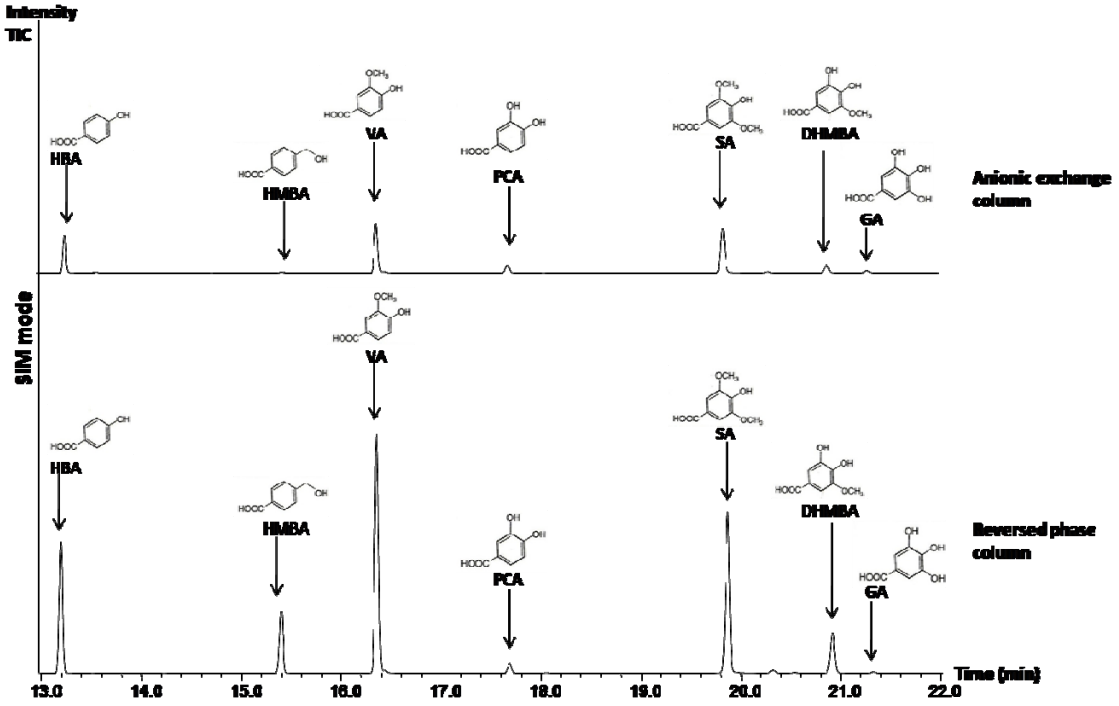


Figure 30. Anionic exchange column vs. reversed phase column for SPAD

Because the reversed phase column gave best results of the two column types, it was used for both SPAD and SPE during the rest of the method development process. The SPAD testing different columns was performed by applying BAs in 0.05 M HCl to the RP column and BAs in PBS buffer to the anionic exchange column. Recovery results for SPAD were acquired using RP column (Table 16).

Table 16. Recovery (%) results for SPAD

BA	Recovery (%) ± SD
HBA	45 ± 33
VA	n.a.
PCA	1 ± 1
SA	n.a.
DHMBA	n.a.
GA	n.a.

n.a.: *not available due to major interferences or loss of internal standards during sample preparation.*

As the recovery values from the table above (Table 16) indicates, SPAD is a very unstable sample preparation method for BAs with very poor reproducibility. When internal standards are poorly derivatized or extracted they will spoil the credibility of the analysis results. If the sample material does not contain the internal standards or very little of these due to a bad sample preparation method but contain some of the target compounds, it will seem as though these exist in very high amounts in the sample. The peaks for the internal standards are very low and barely above the detection limit. Because the internal standards are very poorly recovered all the other recovery values will not be credible since they cannot be properly calculated.

Considering the unstable results and poor reproducibility of SPAD as sample preparation, it was excluded for the rest of the method development as LLE and SPE proved to be better and more stable sample preparation methods.

In SPAD more than in the SPE process, drying of the column is very important because the derivatization reagent is applied directly to the column. The derivatization reagent used in silylation replaces the protonic groups with a trimethylsilyl (TMS) group. Any presence of water or solvents like alcohols might cause an incomplete derivatization. Also in SPAD the column cannot be allowed to dry between the first three steps of the extraction process for silica based columns. Because the columns were allowed to dry a long time and sufficient amounts of the derivatization reagent was added to the column before heat treatment, it leads to the assumption that BAs are not suitable for SPAD.

4.2.5 Recovery

By comparing the recovery results and the reproducibility (the standard deviation is a measure of this) for the three sample extraction methods, LLE, SPE and SPAD (Table 17), it is safe to

say that LLE is the best sample extraction method for the six target compounds: HBA, VA, PCA, SA, DHMBA and GA.

Table 17. Recovery (%) results for LLE, SPE and SPAD in urine for the best method

BA	Recovery (%) ± SD		
	LLE (n=5)	SPE (n=5)	SPAD (n=3)
HBA	89 ± 5	36 ± 4	45 ± 33
VA	100 ± 5	74 ± 5	n.a.
PCA	95 ± 4	79 ± 7	1 ± 1
SA	105 ± 6	125 ± 17	n.a.
DHMBA	94 ± 6	104 ± 8	n.a.
GA	121 ± 4	43 ± 2	n.a.

n.a.: *see Table 16.*

SPAD was excluded for further method development quite early in the process. LLE and SPE was continued with to determine which sample extraction method to pursue further. SPE with polymer based column proved to give better recovery results than SPE silica based column. LLE was in the end chosen to be the best overall sample extraction method for all the BAs considering recovery and chromatographic results in peak separation and peak intensities. In the figure below (Fig. 31) recovery differences in LLE, SPE-RP (silica) and SPE-RP (polymer) is displayed together with the standard deviation.

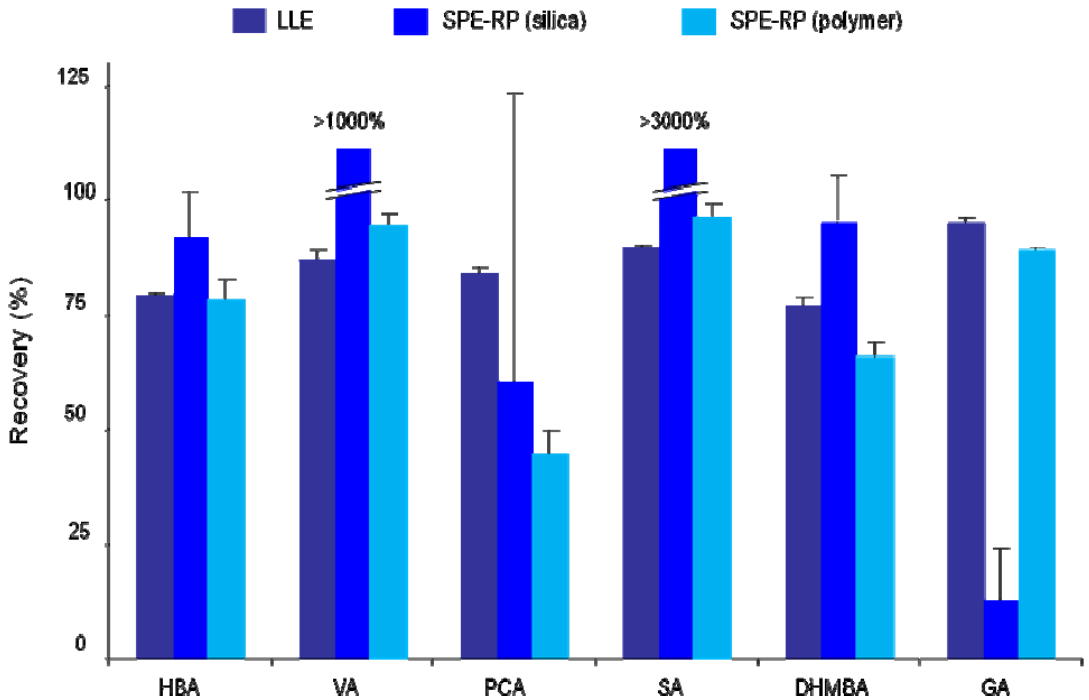


Figure 31. Difference in BA recovery (%) using LLE, SPE-RP (silica) and SPE-RP (polymer), n=3

4.3 Limits of detection (LOD) and limits of quantification (LOQ)

Standard 2 for the standard calibration curve was diluted 1:2, 1:5 and 1:10 in MTBE to determine LOD and LOQ by evaluating the S/N values. The signal stands out from the noise surrounding it (Fig. 32).

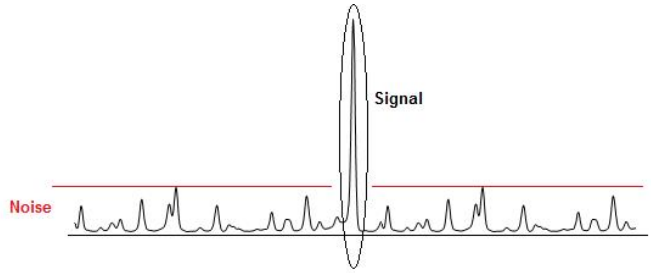


Figure 32. Illustration of signal and noise

The S/N values used for LOD and LOQ determination was not calculated by the GCMS post analysis program but determined by observation of the signal and surrounding noise in the chromatogram. LOD and LOQ values for all BAs are presented in Table 18.

Table 18. Limits of detection and quantification for all BA TMS derivatives

BA	LOD (µL), n=4, n=5 for SA	LOQ (µL), n=5, n=10 for PCA
HBA	0.514 ± 0.127	1.706 ± 0.223
VA	0.479 ± 0.106	1.548 ± 0.240
PCA	0.531 ± 0.062	1.984 ± 0.798
SA	1.155 ± 0.169	3.280 ± 0.296
DHMBA	0.267 ± 0.028	1.048 ± 0.161
GA	0.486 ± 0.027	2.676 ± 0.106

LOD for HBA, VA, PCA, DHMBA and GA was determined from the 1:10 dilution of std. 2, where SA was determined from the 1:5 dilution. LOQ for HBA, VA, PCA and DHMBA was determined from the 1:5 dilution, while SA and GA were determined from the 1:2 dilution. In addition to determining LOQ for PCA with 1:5, the 1:2 dilution was also used. The limits of detection and quantification are quite low for all BAs, allowing for very small amounts of all compounds to be detected in the urine and plasma samples that were to be analyzed for the utilization part of the thesis work.

4.4 Utilization

To be able to discuss whether the selected BAs; GA, PCA, VA, DHMBA and SA, really could be metabolites of the different anthocyanins found in MEDOX, a number of volunteers were orally administered MEDOX® capsules. Urine and plasma samples were collected from the volunteers both before ingestion and for a time interval after to be able to see if there was an increase in the target compounds. Delphinidin-3-O-β-glucopyranoside powder was also administered to one volunteer to see if there would be an increase in GA which is the

suggested metabolite for this anthocyanin.

A MEDOX® capsule, containing 80 mg anthocyanins, was dissolved in 100 ml 0,01 M HCl (800 mg AC/L) and extracted with LLE and derivatized before analysis by GCMS to determine the BA content in the capsule itself. The result showed that there were < 0.1 ‰ BAs in the MEDOX® capsule compared to the AC concentration. It is likely to assume that the BA content in MEDOX® is not significant for the analysis results. However, the possibility of these BA contributing to the excreted BA levels after oral administration of MEDOX® cannot be excluded completely due to little knowledge of BA absorption efficiency.

Benzoic acids are believed metabolites of anthocyanins, as well as they are used as food preservatives on their own. Since the volunteers administered MEDOX® were not under any diet restrictions, BAs could also originate from other food sources. The volunteer who ingested the delphinidin powder was following a low anthocyanin diet; still, anthocyanins are part of a vast number of dietary sources making total avoidance difficult.

4.4.1 Urine samples

Urine samples from all volunteers before ingestion of MEDOX® was analyzed, and the total concentration of the six BAs as well as the concentration of the BAs in their free form was found. The distribution of the compounds is presented in Fig. 33.

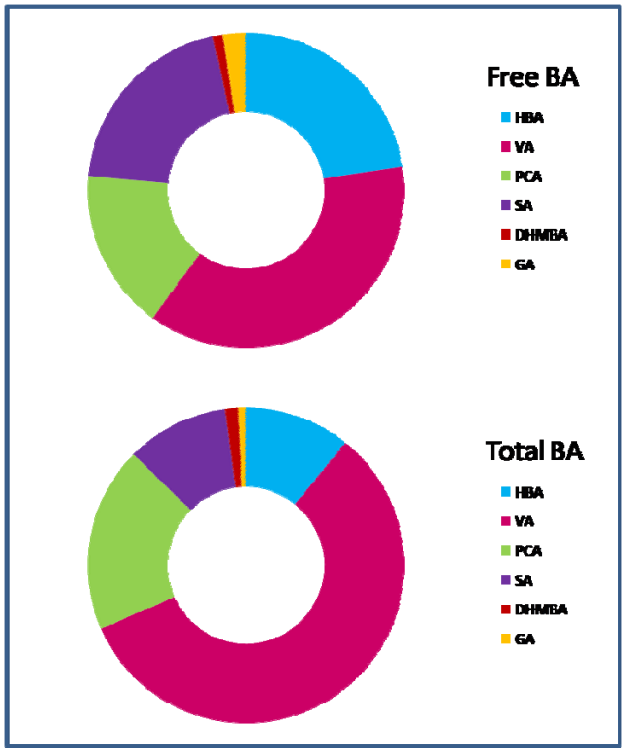


Figure 33. Free and total BA in urine before administration of MEDOX®

There is both free and conjugated BA in urine before ingestion of MEDOX®. The figure (Fig. 33) is based on numbers of analyzed urine from 12 volunteers as regards to the creatinine

concentration in the urine samples. As the figure shows, VA and HBA are main constituents excreted in urine compared to the other analyzed BA, both in free and conjugated form. GA and DHMBA are only found in very small amounts.

4.4.1.1 Oral administration of MEDOX®

- <u>Spot urine sampling:</u>

10 volunteers were administered 6 MEDOX® capsules (480 mg anthocyanins) to see whether there would be an increase in the suggested BA metabolites of the anthocyanins in the product. The change in BA concentration is presented as the difference between the concentration 2 hours after ingestion and the concentration before; all values have been corrected to creatinine levels. Positive values indicate increase in BA concentration after ingestion and negative values show decrease. The values in the diagrams represent µg BA/mmol creatinine. Change in concentration of free BA is presented in Fig. 34.

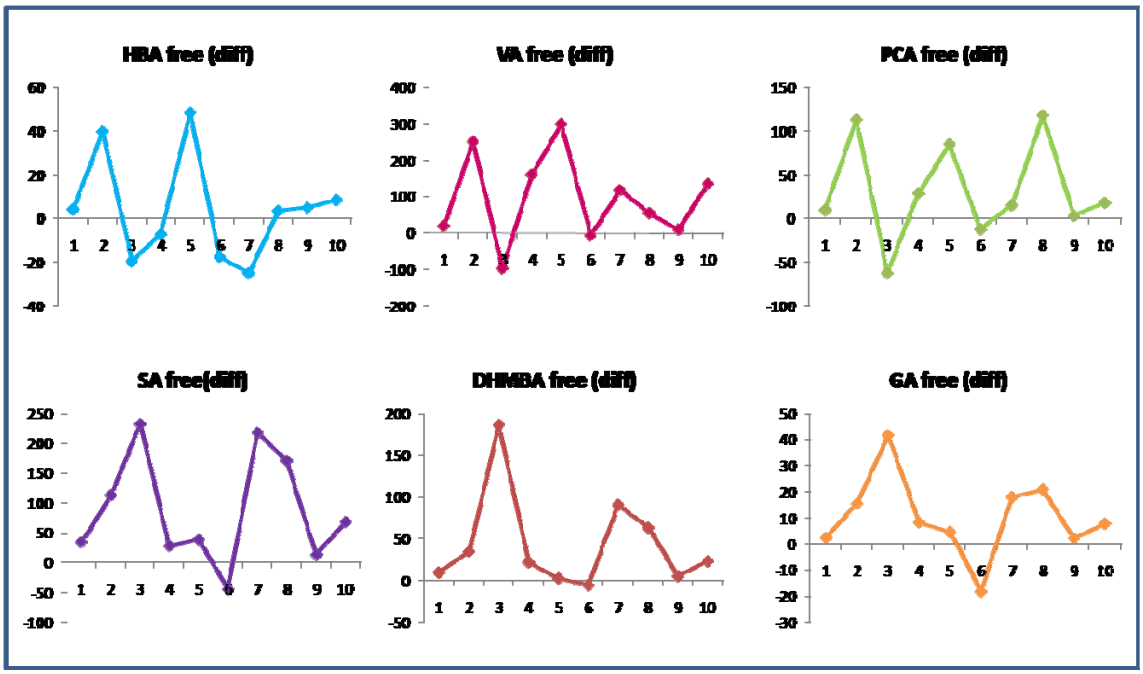


Figure 34. Change in free BA in urine 2 hours after MEDOX® ingestion (positive value show increase), n=10

HBA should not be found in the urine due to MEDOX® ingestion since pelargonidine is not one of the five anthocyanins found in the product. There is a decrease in BA after 2 hours for the sixth volunteer and also for VA and PCA for the third volunteer. In general there is an increase in the 5 BAs for all volunteers. The change in total amount of BA shows the same tendency (Fig. 35).

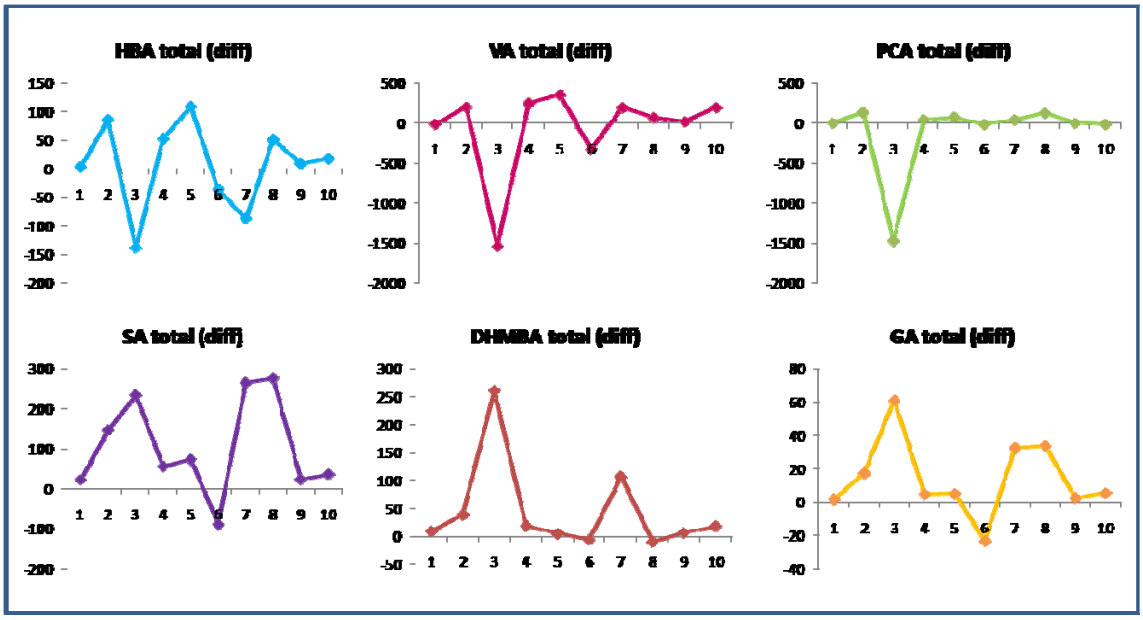


Figure 35. Change in total BA concentration in urine 2 hours after MEDOX® ingestion (positive value show increase), n=10

HBA should not be found in the urine due to ingestion to MEDOX® so the presence of this BA is most likely due to the volunteers' diet or metabolism of other compounds. There is generally an increase of all BAs 2 hours following oral administration of MEDOX®. Volunteer number 3 have a decrease of VA and PCA compared to the concentration at time zero meaning before ingestion, however the following three BAs SA, DHMBA and GA increase for this volunteer. For the sixth volunteer though, all BA decrease in concentration after 2 hours. That BAs decrease in concentration 2 hours after ingestion could mean that it will take longer for the compounds to be excreted in the urine, since there is an increase for the majority of volunteers this is probably not the case. Another reason could be that the volunteers have a high concentration of these compounds already in their urine due to their diet. Fruits and also coffee which are normal constituents to an average diet, contain ACs and BAs. General increase for all BA indicated that these could be metabolites from their corresponding anthocyanins in MEDOX®. All volunteers were responsible for labeling their own samples, which lead to another possibility for the decrease of BAs for volunteer 6 after ingestion of MEDOX®. The creatinine levels for this volunteer were also higher after the 2 hours following ingestion than before, which is generally not the case. If the samples were mixed and the before and 2 hours after test is in reality the opposite, there was also an increase in all BAs for this volunteer.

- <u>6 hour sampling:</u>

2 healthy female volunteers were administered 8 MEDOX® capsules (640 mg anthocyanins) and the BA concentration was analyzed the following 6 hours to observe a possible increase in BAs in addition to getting an idea of how fast the compounds are excreted into urine. Total amount of BA excreted in the urine during the 6 hour period following administration was 2778 µg for volunteer 1, where HBA, VA and PCA together makes up for 92 % of the total BA concentration. Total excreted BA in urine for volunteer 2 was 1939 µg where HBA, VA and PCA constitute 81 % of the total BA concentration.

Analyzed BA concentrations were corrected according to creatinine levels and so y-axis values in the diagrams are presented as µg BA/mmol creatinine. Both volunteers showed increase in all BAs after MEDOX® consumption (Fig. 36 and Fig. 37).

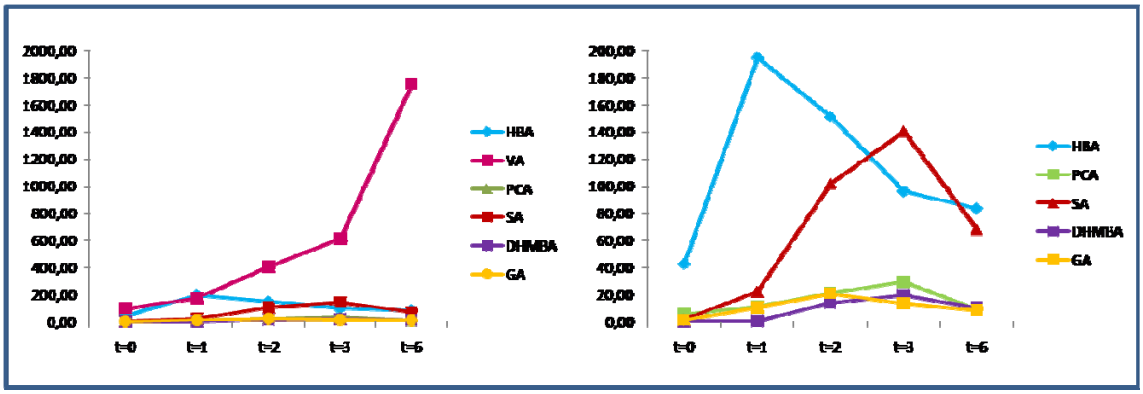


Figure 36. Increase in total BA content in urine after oral administration of MEDOX®, volunteer 1

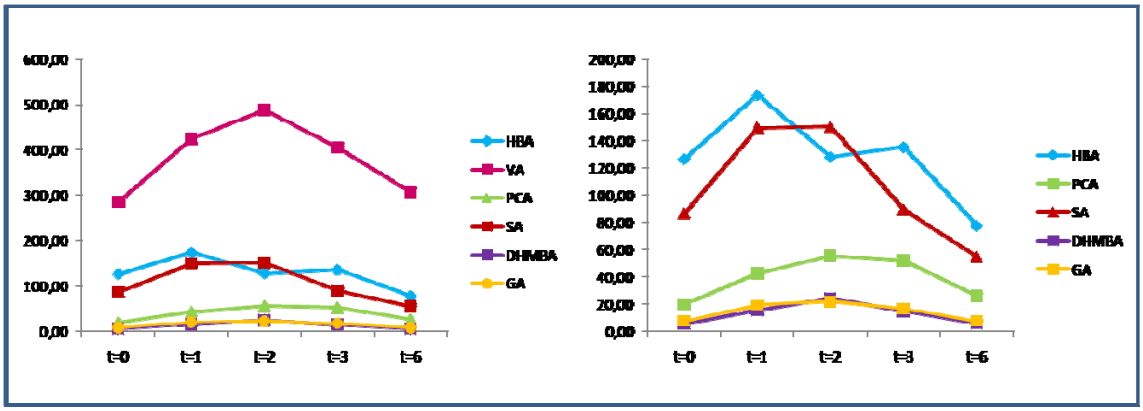


Figure 37. Increase in total BA content in urine after oral administration of MEDOX®, volunteer 2

Volunteer 1 (Fig. 36) showed a major increase in VA which kept increasing even until the sixth hour after ingestion. Volunteer 2 (Fig. 37) also had higher amounts of VA excreted in the urine compared to the other 5 analyzed BAs, though not as much increase as volunteer 1 and VA conc. started to decrease after 2 hours. This might have something to do with the food consumed by the first volunteer during the experiment most likely since the increase is so dramatic. This volunteer consumed food containing chocolate during the experiment and chocolate contain the flavoring agent vanillin which is also used in other foods, beverages and pharmaceuticals. Dietary vanillin is converted in the liver to the urinary metabolite VA in both conjugated and free form [54]. This is probably the reason for the drastic increase over time of this metabolite.

All BAs increased for both volunteers compared to the start concentration measured for the urine before intake of MEDOX® (Table 19). HBA also increased and had its peak concentration 1 hour after consumption for both volunteers. This BA should not increase due to MEDOX® administration; however it could be a result from the normal diet of the

volunteers, or perhaps a metabolite of one of the other BAs.

Table 19. Times for peak concentration of BAs in urine for both volunteers

BA	Volunteer 1 (t=)	Volunteer 2 (t=)
HBA	1	1
VA	6	2
PCA	3	2
SA	3	2
DHMBA	3	2
GA	2	2

There is an increase in all BAs for both volunteers indicating they are indeed metabolites of the anthocyanins in MEDOX®. There are some differences in which times the BA concentrations are highest when comparing the two. This could be due to difference in metabolism, or due to interference from other dietary components which are excreted as these BAs. The times are similar, 2 and 3 hours as time for peak concentration for most BA, whereas VA stands out from the rest. Stating an exact time for peak concentration is difficult because there was an hour between collections of each sample and it could occur right before or after one of these time periods.

4.4.1.2 Oral administration of delphinidin-3-O-β-glucopyranoside

Urine samples acquired from the male volunteer who was administered delphinidin-3-O-β-glucopyranoside powder (500 mg of the compound) were made comparable by adjusting values to the total urinary volume at each time period. The urine samples were collected for an 8 hour period at each hour following consumption of the powder. The delphinidin anthocyanin is suggested to metabolize to GA. All BA was analyzed in the urine samples following administration of the powder, and there was observed increase in the concentrations of GA, SA and DHMBA (Fig. 38). Total excreted concentration of these three compounds in the 8 hour period was 2476.35 µg.

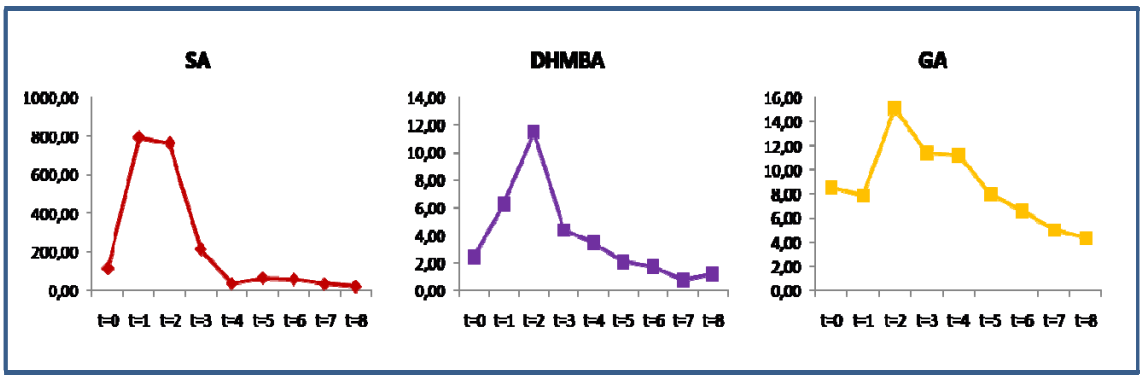


Figure 38. Changes in total SA, DHMBA and GA concentration in urine after ingestion of delphinidin powder

The volunteer did not ingest the anthocyanins malvidin and petunidin, which is suggested to metabolize into SA and DHMBA respectively. GA has three hydroxy groups in positions 3, 4 and 5, DHMBA has a similar construction except there is a methoxy group in position 5

instead and SA has methoxy groups in position 3 and 5. By methylation GA could transform into the BA DHMBA by one methylation and SA by two methylation reactions. Since there is an increase in all these three BAs after consumption of delphinidin, this metabolic route might be a possibility. There has been evidence of demethylation of SA into GA [11]; therefore the opposite should also be a possibility.

In research work done on gallic acid metabolism 3-O-methylgallic acid, 4-O-methylgallic acid and 3,4-O-dimethylgallic acid has been found [20, 55]. The first of those to metabolites are isomeric structures of DHMBA which would receive the name 5-O-methylgallic acid if it was named as a gallic acid metabolite. 3, 4-O-dimethylgallic acid has the same molecular formula as SA except SA has its methyl groups in third and fifth positions. A. Booth et.al identified SA as a metabolite of 3-O-methylgallic acid in 1959, and also 4-O-methylgallic acid as a metabolite of GA [56]. Since 3OMGA is a metabolite of GA it could be possible it metabolizes further to SA in that way since. DHMBA has perhaps not been considered a metabolite of GA since only 3OMGA and 4OMGA has been found. Nevertheless, what is not searched for is usually not found either.

Since GA as well as SA and DHMBA increased in concentration in urine after administration of delphinidin-3-O-β-glucopyranoside, there is indeed a possibility that SA and DHMBA could be metabolites of GA.

4.4.2 Plasma samples

Distribution of free and total (free + conjugated) BAs in plasma before ingestion of MEDOX® /delphinidin powder is illustrated in Fig. 39.

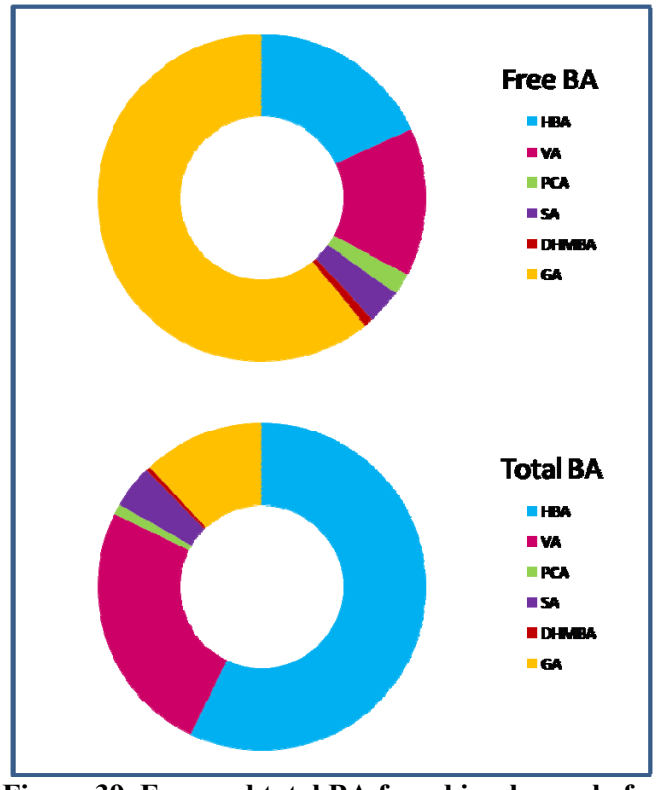


Figure 39. Free and total BA found in plasma before ingestion of MEDOX®

The numbers of which Fig. 39 has been based on are obtained by plasma analysis from 3 volunteers. In plasma, similarly as in urine, HBA and VA are two of the BAs that are found in the largest portions compared to the rest of the BAs. In plasma however, GA which were only found in very small amounts in urine both in free and conjugated form, contributes to 61 % of the total concentration of the six BAs found in free form plasma. For total BA concentrations, GA accounts for 12 % of the total BA concentrations found of the six target compounds.

Concentrations of free and conjugated BA in plasma are spelt out in Table 20:

Table 20. Concentration of free BA and free + conjugated BA in plasma

BA	Free BA (µg/L)	Total BA (µg/L)
HBA	5.59 ± 2.39	107.56 ± 15.82
VA	4.52 ± 2.36	47.60 ± 3.00
PCA	0.65 ± 0.25	1.94 ± 0.46
SA	1.06 ± 0.57	7.92 ± 1.24
DHMBA	0.28 ± 0.19	0.84 ± 0.58
GA	18.74 ± 7.88	22.61 ± 7.22

Evaluating the concentrations in Table 20 gives reason to think that there is mostly BA in conjugated form in plasma. At least the three BAs HBA, VA and SA, seems to be mostly in conjugated form since their concentration of free compounds is very low. The BAs GA, DHMBA and PCA, however, do not have the same intense increase in concentration comparing free and total BA concentration. The compound in free form makes up for a big part of the total concentration, at least for GA where the free compound represents nearly the whole total concentration.

4.4.2.1 Oral administration of MEDOX®

BA concentrations in plasma samples from the two female volunteers were analyzed in a 6 hour period to see if there would be an increase in the target compounds (Fig. 40 and Fig. 41).

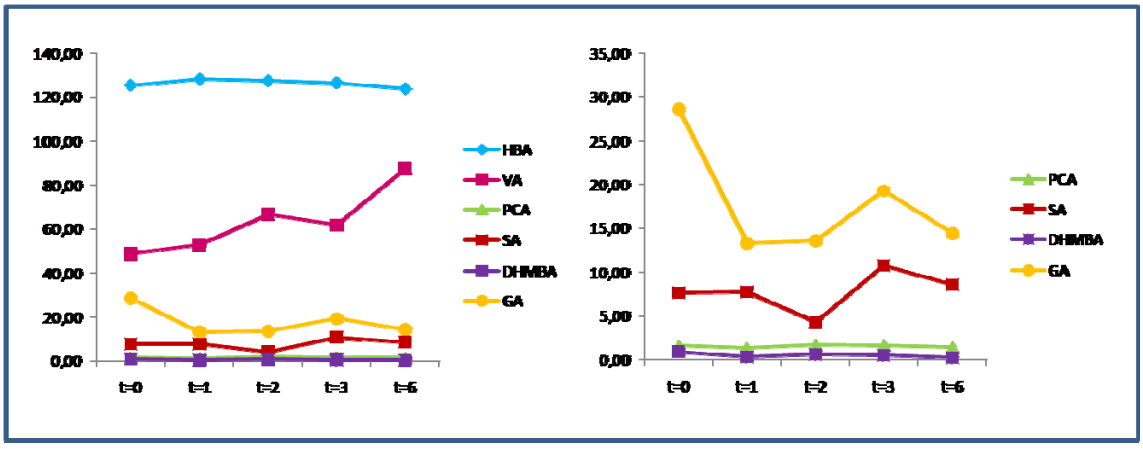


Figure 40. Total BA content in plasma 1, 2, 3 and 6 hours following oral administration of MEDOX®, volunteer 1

Change in BA concentration in volunteer 1 is presented in the figure above (Fig. 40) where the second diagram shows the four BAs of lowest concentrations to illustrate the change over time better. HBA exist in the highest concentration in plasma both before and after ingestion of MEDOX® capsules, since this BA is not believed to be a metabolite of any anthocyanin constituents in the product, it is likely caused by the regular diet or the metabolism of other substances. VA is also continuously increasing in plasma as it was in urine for this volunteer and can be explained by the same reason, that the diet is probably the cause. For DHMBA and GA there is a reduction in concentration from time zero and after consumption of MEDOX®. There is however an increase in SA which could indicate the possibility of this BA as a metabolite of GA and possibly also of DHMBA.

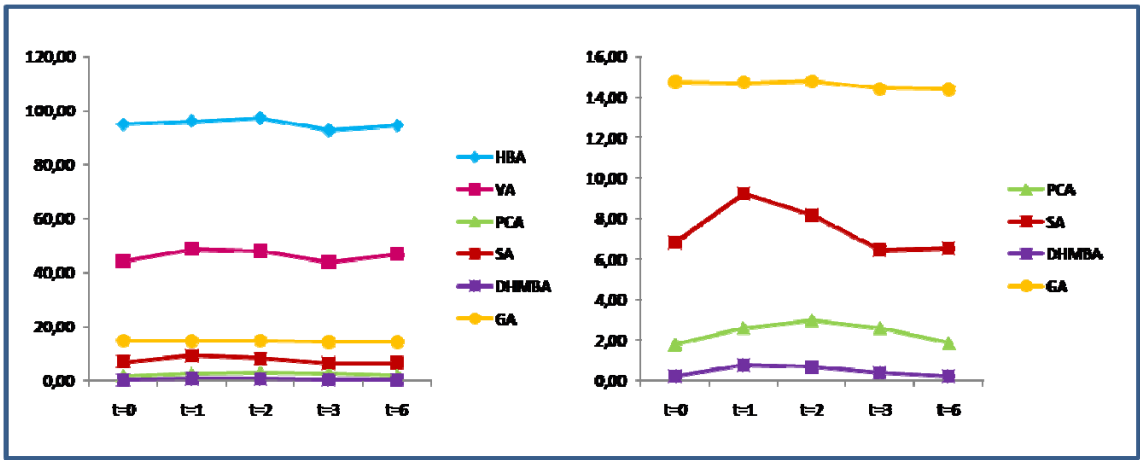


Figure 41. Total BA content in plasma 1, 2, 3 and 6 hours following oral administration of MEDOX®, volunteer 2

In the figure (Fig. 41) presenting total BA content in the plasma samples from volunteer 2, there is an increase in all BA. GA has a quite equal concentration throughout all the time period without much increase or decrease. The second diagram show only PCA, SA, DHMBA and GA because these BA is found in quite lower concentrations than VA and HBA and is thus presented in a better way in a diagram showing only these four.

Peak concentration was reached at different times for the two volunteers for most BA except PCA and SA; all times are laid out in Table 21.

Table 21. Time for peak total concentration of the BAs for both volunteers

BA	Volunteer 1 (t=)	Volunteer 2 (t=)
HBA	1	2
VA	6	1
PCA	2	2
SA	1	1
DHMBA	0	1
GA	0	2

SA has its first peak concentration time for volunteer at 1 hours after consumption, however it has a new peak with an even higher concentration at 3 hours even though there was a decrease

from t=1 to t=2, this new peak concentration could be the result of something the volunteer has consumed in the course of the experiment. If time zero is ignored for DHMBA and GA, their peak concentration is reached after 2 and 3 hours respectively.

4.4.2.2 Oral administration of delphinidin-3-O-β-glucopyranoside

Plasma samples given from the male volunteer who was administered delphinidin-3-O-β-glucopyranoside powder were analyzed to observe change in BA concentration over a certain time period. The urine samples were collected for an 8 hour period following consumption of the powder. All BA was analyzed in the urine samples even though GA is the suggested metabolite of delphinidin. SA and DHMBA was found in increased levels in urine after delphinidin administration and are also interesting to evaluate in plasma. Changes in concentrations of GA, SA and DHMBA are presented in Fig. 42 below.

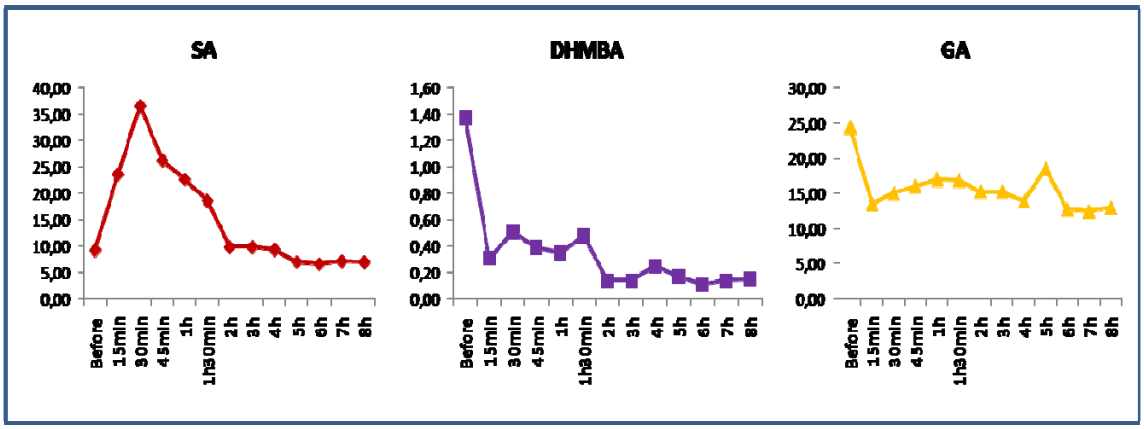


Figure 42. Changes in total SA, DHMBA and GA concentration in urine after ingestion of delphinidin powder

SA was the only BA which was found in a higher concentration after intake of delphinidin even though GA is the main metabolite in this case. This adds to the idea that SA could be metabolized from GA. That neither DHMBA nor GA is found in increasing amounts is curious and does not agree with the results analyzed for urine. Similar tendencies of analyzed GA and DHMBA concentrations could also be observed in the two other volunteers who were administered MEDOX®.

4.4.3 Summary and comparison of urine and plasma results

GA and PCA should be the most abundant BA metabolites after supplementation of MEDOX® theoretically since they are the corresponding metabolites of delphinidin and cyanidin, respectively, which are the main constituents of the product. However, based on the obtained results, VA and HBA are the two BAs found in highest concentrations in urine as both free and conjugated acids both before and after intake of MEDOX®, in plasma GA is actually one of the BAs found in highest concentrations both before and after intake. VA and HBA as the main BAs found excreted in urine is corresponding to what Nurmi et al. found in their research after ingestion of an oregano extract which contains many of the same compounds [15]. They however, did not detect gallic acid in the urine after supplementation even though it was expected. GA was again failed to be found in a new research project

published by the same group in 2009 where delphinidin glycosides were the main AC in their bilberry-lingonberry purée [10]. In the results from this utilization part of the thesis, GA was found in urine both in free and conjugated forms in urine after administration and in increasing amounts from before intake though not in vast amounts.

Nurmi et.al[10] suggested that GA was metabolized further to SA by methylation, which could indeed be the case. Studying the trend of increase in urine following oral administration of MEDOX® SA is increasing in much higher concentrations than GA, which somehow seems odd since malvidin is not found in the same amounts in MEDOX® as delphinidin. The SA increase observed could very well be the result of GA methylation and as a metabolite of the malvidin anthocyanin. In plasma, however, GA is found in larger quantities than SA although GA is decreasing after intake. Since SA increase after intake in opposite of GA, this could also be an indication of transformation of GA to SA. Also seeing that DHMBA is found in small concentrations and sometimes also decrease after intake could suggest that also this BA undergo methylation to SA to some extent. DHMBA however, is not the metabolite of one of the main constituents in MEDOX® and should therefore not be retrieved in the same degree as GA after ingestion. The experiments analyzing BAs in both urine and plasma after intake of delphinidin powder support the idea of GA metabolizing to SA and also to DHMBA.

PCA is not found in very high amounts in whether urine or plasma after intake, just like GA. In urine it is found in higher concentrations than GA but in plasma the numbers are turned. VA and HBA are the main constituents excreted in both urine and plasma and are found in the highest amounts. VA could be a metabolite of PCA by methylation, and that could explain the reason for the drastic increase in VA after intake of MEDOX®. It is also suggested that HBA is metabolized from PCA; this would happen if PCA lost a hydroxy group. Similar tendencies of BA findings have been described by Nurmi et.al in 2006 after oregano extract ingestion. They have also stated that small amounts of HBA have been found in rat urine after been given PCA [15]. This could also explain why increase in HBA concentration has been observed after ingestion of the supplement, even though pelargonidine which is its corresponding AC is not found in MEDOX®. VA is the main constituent found in urine and HBA in plasma, these are also the cases before consumption of the AC rich product.

The overall distribution of BAs in plasma and urine correspond to each other, except that GA is found in higher concentrations in plasma than in urine compared to PCA, SA and DHMBA which also correspond to the figures showing distribution of BAs in urine and plasma before ingestion of supplements. It seems as though they increase corresponding to that model, which is also true for the main BA constituents found in both urine and plasma before and after intake. BAs are also found in lower concentration in plasma than in urine. This could be explained by the fact that the urine is "stored" in the body for a while before it is excreted and thereby the compounds are allowed to up concentrate and the concentrations are thus higher here.

Total excreted concentration of BAs in urine and plasma seem to be quite low in comparison to the ingested amount of ACs which is 80 mg per MEDOX® capsule. There is however an increase in BA after intake of these capsules suggesting that these compounds really are metabolites of their corresponding ACs. Excretion of BAs in the 6 hour period following intake of MEDOX® was ~ 2 – 3 mg in urine. The excretion of these metabolites probably continues a while longer adding to the concentrations but the peak concentration is mainly after 1-2 hours in plasma and 1-3 hours in urine for the BAs.

Because ACs and also BAs alone are found in other dietary sources than the supplements given the volunteer subjects, all BA excreted is probably not due to MEDOX®. However, the increase propose metabolism of the ACs in MEDOX® to their corresponding BAs which was the aim to observe by applying the developed method in this thesis. The results even suggest further metabolism between the BAs themselves into each other by either methylation or loss of a hydroxy group. Total concentration of excreted BAs GA, DHMBA and SA increased from < 20 % to 54.3 % in urine and 82.9 % in plasma following administration of delphinidin which only strengthen the ideas about BA metabolism. Increase in BA concentration in plasma and urine after intake of MEDOX® due to the existing (very low) BA concentrations in the capsule is unlikely but cannot be excluded.

HBA, VA and SA increase most in free to total concentration in plasma, suggesting these are found mostly in conjugated form. In urine all BA increase quite much in concentration considering free and conjugated compounds suggesting the majority of BAs in urine are conjugated. GA and DHMBA are the two BAs that show the least difference in increase from free to total concentration of BA.

5. Conclusion

A robust analytical method consisting of sample preparation and a chromatographic method was developed with good recovery (%) results for all six BA analyzed. LLE was chosen as the best sample extraction technique with ethyl acetate as extraction solvent. Derivatization of all samples, with BSTFA as derivatization reagent and the addition of 25 % acetonitrile as derivatization catalyst, was performed on all samples prior to analysis by GCMS. GCMS proved to be an accurate and fitting analytical separation and detection combination with achieved low limits of detection and quantification for all target compounds. A linear standard calibration curve was prepared with two deuterated internal standards with the same BA structure as GA and HBA, to be able to quantify the BAs. The finished analytical method for identification and quantification of the six BA yielded good results for all compounds (Table 22). Linearity range is given as the highest concentration for each BA that was implemented in the calibration curve for linearity, and recovery results are for non-enzyme treated samples.

Table 22. Analytical characteristics of BAs given the method for the GCMS analysis

BA	Recovery % (urine)	Recovery % (plasma)	Linearity (R^2)	Linearity range (µg/L)	LOD (µg/L)	LOQ (µg/L)
HBA	89 ± 5	57 ± 3	0.996444	1405.22	0.514 ± 0.127	1.706 ± 0.223
VA	100 ± 5	105 ± 5	0.997416	1175.27	0.479 ± 0.106	1.548 ± 0.240
PCA	95 ± 4	63 ± 3	0.991396	1385.97	0.531 ± 0.062	1.984 ± 0.798
SA	105 ± 6	139 ± 7	0.998321	1085.22	1.155 ± 0.169	3.280 ± 0.296
DHMBA	94 ± 6	64 ± 6	0.998191	1058.22	0.267 ± 0.028	1.048 ± 0.161
GA	121 ± 4	80 ± 3	0.990978	827.31	0.486 ± 0.027	2.676 ± 0.106

Identified BAs increased in quantity compared to baseline excretion, which suggest the absorbed AC compounds in MEDOX® were extensively metabolized to their corresponding BAs. Further metabolism resulting in certain BAs metabolizing to other BAs were also discussed due to interesting observations. GA is suggested to metabolize to SA and DHMBA by methylation, and PCA is suggested to metabolize to VA and also HBA. HBA is observed in increased amounts after intake of MEDOX® even though the parent compound of pelargonidine is not found in the product, which could be explained by the possible metabolic pathway from PCA.

HBA and VA are seen as the major BA constituents in both urine and plasma, GA is also one of the most abundant BAs found in plasma. In both matrixes before ingestion of MEDOX® or delphinidin powder, HBA and VA are still the most abundant BAs; however small amounts of the remaining BA are also found in both free and conjugated form. From the results, it seems that BAs are mostly in conjugated form in urine, and HBA, VA and SA are mostly found as conjugated compounds in plasma, but the concentrations of GA, PCA and DHMBA do not increase much by enzyme treatment.

The conclusion of this thesis work suggests that ACs in MEDOX® metabolize into their corresponding BAs. This is based on increased levels of all BA after supplementation, however one cannot say with full certainty that the increase originates exclusively from the intake of MEDOX®/delphinidin powder based on variety in results in addition to no diet restrictions for most volunteers. An idea for further research is to apply strict diet restrictions.

The obstacle of applying such restrictions is the abundance of ACs in food and their widespread nature. Study of absorption and metabolism of these compounds is challenging because a number of parent compounds usually convert into an assortment of metabolites. Since the BA metabolites are excreted relatively fast after ingestion, their antioxidant effect is most probably of a brief nature as well. They are also most likely absorbed in the small intestine due to their rapid excretion.

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Appendices

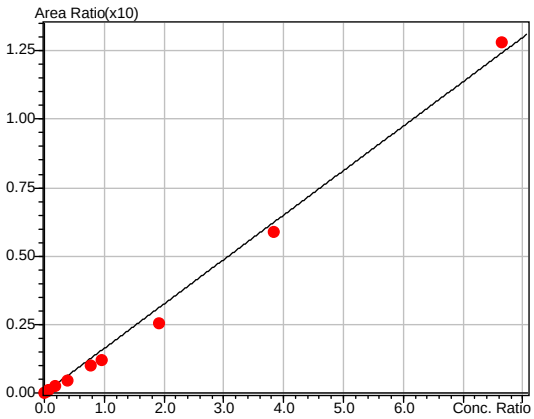
Appendix 1. Concentrations of stock solutions

Chemical name	Abbreviation	MW	Purity	Innveid	20 mM HCl in MeOH	20 mM HCl in MeOH	20 mM HCl in MeOH			Purity corr
			%	mg 5-10 mg	Dissolved in mg	Dissolved in ml	Dissolved in ml	Conc mg/ml	Conc mg/l	Conc mg/l
3,4,5-trihydroxybenzoic acid	GA	170,12	99	12,26	1159,00	1,4671	1,47	8,35669	8356,687	**8273,120**
3,4-dihydroxybenzoic acid	PCA	154,12	97	9,73	1075,94	1,3619	1,36	7,14417	7144,172	**6929,846**
4-hydroxy-3-methoxybenzoic acid	VA	168,15	97	6,55	854,15	1,0812	1,08	6,05807	6058,069	**5876,327**
3,4-dihydroxy-5-methoxybenzoic acid	DHMBA	184,15	99	5,79	856,00	1,0835	1,08	5,34357	5343,575	**5290,139**
4-hydroxy-3,5-dimethoxybenzoic acid	SA	198,18	97	6,02	850,17	1,0762	1,08	5,59394	5593,940	**5426,122**
4-hydroxybenzoic acid	HBA	138,12	99	13,26	1475,98	1,8683	1,87	7,09725	7097,251	**7026,278**
4-(hydroxymethyl)benzoic acid	HMBA	168,15	99	13,33	1322,60	1,6742	1,67	7,96212	7962,120	**7882,499**
4-hydroxybenzoic-2,3,4,6-d4 acid	d4-HBA	142,12	99	11,45	1219,83	1,5441	1,54	7,41538	7415,378	**7341,224**
3,4,5-trihydroxybenzoic-2,6-d2 acid	d2-GA	172,12	99	8,86	1005,29	1,2725	1,27	6,96257	6962,568	**6892,942**

Appendix 2. Standard calibration curves

Standard calibration curves and curve information for each BA:

HBA:

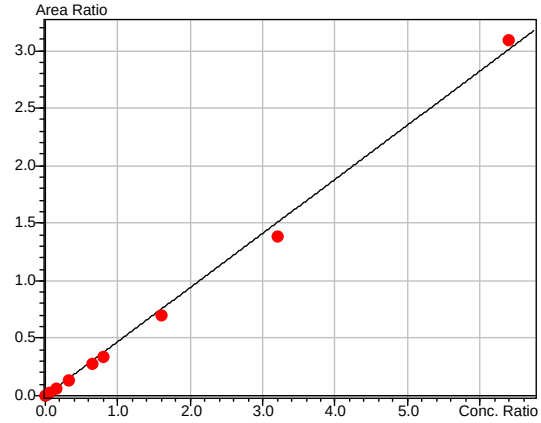


Y = 1.624695X - 0.0
R^2 = 0.9964444
R = 0.9982206

Internal Standard
Curve: Linear
Origin: Force Through
Weighting Method: None

Mean RF : 1.222034
RF SD : 0.4537548
RF %RSD : 37.13110

VA:

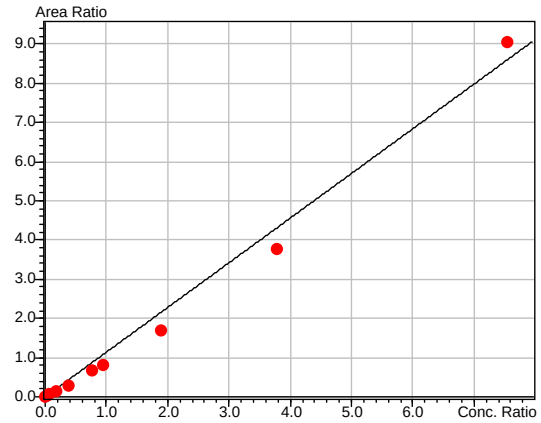


Y = 0.4707241X - 0.0
R^2 = 0.9974157
R = 0.998707

Internal Standard
Curve: Linear
Origin: Force Through
Weighting Method: None

Mean RF : 0.4042758
RF SD : 0.1482374
RF %RSD : 36.66739

PCA:

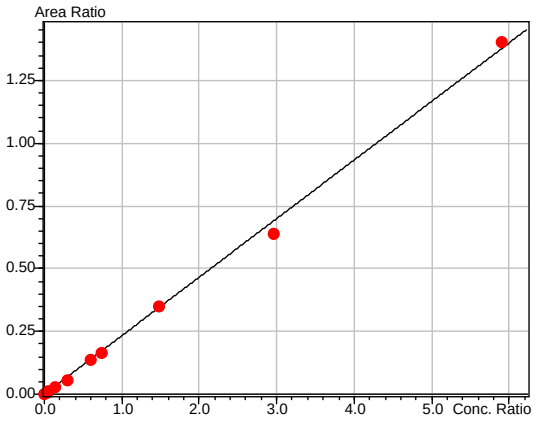


Y = 1.139965X - 0.0
R^2 = 0.9913962
R = 0.9956888

Internal Standard
Curve: Linear
Origin: Force Through
Weighting Method: None

Mean RF : 0.7958557
RF SD : 0.3094732
RF %RSD : 38.88559

SA:

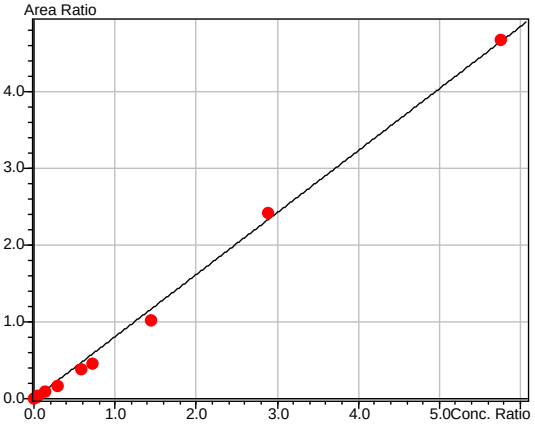


Y = 0.2331914X - 0.0
R^2 = 0.998321
R = 0.9991602

Internal Standard
Curve: Linear
Origin: Force Through
Weighting Method: None

Mean RF : 0.187665
RF SD : 0.0098439
RF %RSD : 37.21732

DHMBA:

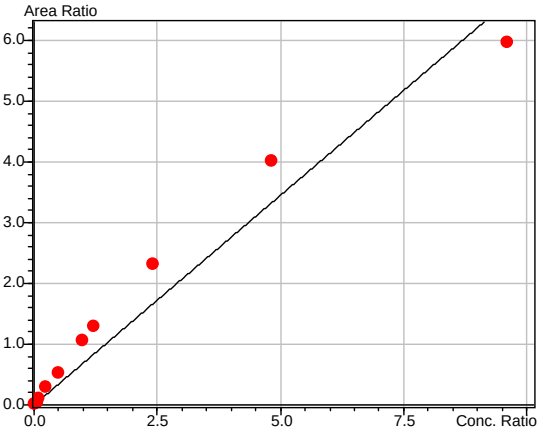


Y = 0.8069609X - 0.0
R^2 = 0.9981911
R = 0.9990951

Internal Standard
Curve: Linear
Origin: Force Through
Weighting Method: None

Mean RF : 0.5935286
RF SD : 0.2302034
RF %RSD : 38.78555

GA:



Y = 0.690161X - 0.0
R^2 = 0.9662904
R = 0.9830007

Internal Standard
Curve: Linear
Origin: Force Through
Weighting Method: None

Mean RF : 0.9295451
RF SD : 0.3737176
RF %RSD : 40.20435

* The tenth calibration standard was eliminated to make GA's standard curve more linear. R2 = 0.990978.

Scans of the standard calibration curve printouts from the GCMS post analysis program:

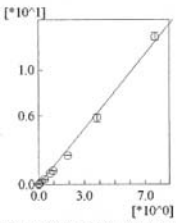
C:\GCMSsolution\Data\090902 GJON\SIM Februar\BA sim 100210.qgm

Calibration

ID#:1 Mass:271.00 Name:4-hydroxybenzoic-2,3,5,6-d4 acid
f(x)=?
rr2=0.000000

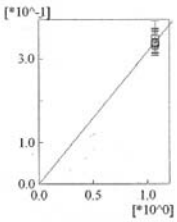
ISTD

ID#:2 Mass:267.00 Name:4-hydroxy benzoic acid
f(x)=1.624695*x+0.000000
rr2=0.996444



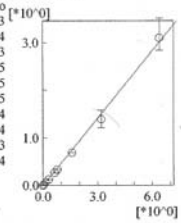
#	Conc. Ratio (ug/L)	Mean Area Ratio
1	0.000	0.01
2	0.038	0.05
3	0.077	0.10
4	0.191	0.23
5	0.383	0.46
6	0.766	0.98
7	0.957	1.22
8	1.914	2.55
9	3.828	5.87
10	7.657	12.83

ID#:3 Mass:281.00 Name:4-(hydroxymethyl) benzoic acid
f(x)=0.315793*x+0.000000
rr2=0.000000



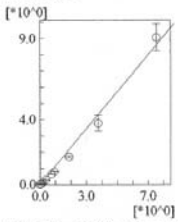
#	Conc. Ratio (ug/L)	Mean Area Ratio
1	1.074	0.33
2	1.074	0.34
3	1.074	0.33
4	1.074	0.35
5	1.074	0.35
6	1.074	0.35
7	1.074	0.34
8	1.074	0.34
9	1.074	0.33
10	1.074	0.34

ID#:4 Mass:297.00 Name:4-hydroxy-3-methoxy benzoic acid
f(x)=0.470724*x+0.000000
rr2=0.997416



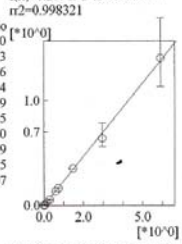
#	Conc. Ratio (ug/L)	Mean Area Ratio
1	0.000	0.00
2	0.032	0.02
3	0.064	0.03
4	0.160	0.07
5	0.320	0.13
6	0.640	0.27
7	0.800	0.34
8	1.601	0.70
9	3.202	1.39
10	6.404	3.10

ID#:5 Mass:193.00 Name:3,4-dihydroxy benzoic acid
f(x)=1.139965*x+0.000000
rr2=0.991396



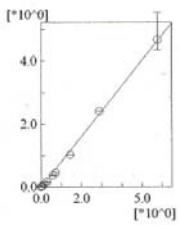
#	Conc. Ratio (ug/L)	Mean Area Ratio
1	0.000	0.00
2	0.038	0.03
3	0.076	0.06
4	0.189	0.14
5	0.378	0.29
6	0.755	0.65
7	0.944	0.80
8	1.888	1.69
9	3.776	3.75
10	7.552	9.07

ID#:6 Mass:327.00 Name:4-hydroxy-3,5-dimethoxy benzoic acid
f(x)=0.233191*x+0.000000
rr2=0.998321



#	Conc. Ratio (ug/L)	Mean Area Ratio
1	0.000	0.00
2	0.030	0.01
3	0.059	0.01
4	0.148	0.03
5	0.296	0.05
6	0.591	0.13
7	0.739	0.16
8	1.478	0.35
9	2.957	0.64
10	5.913	1.40

ID#:7 Mass:223.00 Name:3,4-dihydroxy-5-methoxy benzoic acid
f(x)=0.806961*x+0.000000
rr2=0.998191



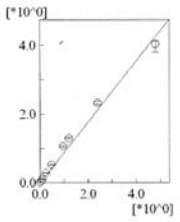
#	Conc. Ratio (ug/L)	Mean Area Ratio
1	0.000	0.00
2	0.029	0.02
3	0.058	0.03
4	0.144	0.08
5	0.288	0.17
6	0.593	0.38
7	0.721	0.46
8	1.441	1.02
9	2.882	2.41
10	5.765	4.68

ID#:8 Mass:283.00 Name:3,4,5-trihydroxy benzoic -2,6-d2 acid
f(x)=?
rr2=0.000000

ISTD

1 / 2

ID#:9 Mass:281.00 Name:3,4,5-trihydroxy benzoic acid
f(x)=0.885616*x+0.000000
r2=0.990978



#	Conc. Ratio (ug/L)	Mean Area Ratio
1	0.000	0.02
2	0.048	0.05
3	0.096	0.12
4	0.240	0.29
5	0.480	0.53
6	0.960	1.07
7	1.200	1.30
8	2.401	2.33
9	4.801	4.03

Appendix 3. Preparation of standard solutions and their concentrations

Preparation of standards:

	Std 1	User 3 Std 2	User 3 Std 3	User 3 Std 4	User 3 Std 5	User 3 Std 6	User 2 Std 7	User 2 Std 8	User 2 Std 9	User 2 Std 10
	µl	µl	µl	µl	µl	µl	µl	µl	µl	µl
BA mix	0	10	20	50	100	200	5	10	20	40
BAIS	50	50	50	50	50	50	50	50	50	50
GCIS	50	50	50	50	50	50	50	50	50	50
ADD BA mix, BAIS and GCIS to all 10 vials. Evaporate to dryness and derivatize. NB! One calibration curve was prepared without evaporation										
BSTFA	200	200	200	200	200	200	200	200	200	200
Acetonitril	500	500	500	500	500	500	500	500	500	500
MTBE	1300	1300	1300	1300	1300	1300	1300	1300	1300	1300
Total	**2000**	**2000**	**2000**	**2000**	**2000**	**2000**	**2000**	**2000**	**2000**	**2000**
Dilution BA mix	-	200	100	40	20	10	400	200	100	50
Dilution BAIS mix	40	40	40	40	40	40	40	40	40	40
Dilution GCIS mix	40	40	40	40	40	40	40	40	40	40

Concentration of standards:

	60% MeOH	6% MeOH	0,12% MeOH												
	user 1	user 2	user 3		User 3	User 3	User 3	User 3	User 3	User 2	User 2	User 2	User 2	User 2	User 2
	Conc	Conc	Conc	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9	Std 10		
	mg/l	mg/l	mg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l		
GA	827,31	82,731	1,6546	0	8,27	16,55	41,37	82,73	165,46	206,83	413,66	827,31	1654,62		
PCA	692,98	69,298	1,3860	0	6,93	13,86	34,65	69,30	138,60	173,25	346,49	692,98	1385,97		
VA	587,63	58,763	1,1753	0	5,88	11,75	29,38	58,76	117,53	146,91	293,82	587,63	1175,27		
DHMBA	529,01	52,901	1,0580	0	5,29	10,58	26,45	52,90	105,80	132,25	264,51	529,01	1058,03		
SA	542,61	54,261	1,0852	0	5,43	10,85	27,13	54,26	108,52	135,65	271,31	542,61	1085,22		
HBA	702,61	70,261	1,4052	0	7,03	14,05	35,13	70,26	140,52	175,65	351,31	702,61	1405,22		
	user 1	user 2													
	fort 1:10	fort 1:100													
HMBA	788,250	7,882		197,06	197,06	197,06	197,06	197,06	197,06	197,06	197,06	197,06	197,06		
	user 1	user 2													
	fort 1:10	fort 1:100													
d4-HBA	734,122	7,341		183,53	183,53	183,53	183,53	183,53	183,53	183,53	183,53	183,53	183,53		
d2-GA	689,294	6,893		172,32	172,32	172,32	172,32	172,32	172,32	172,32	172,32	172,32	172,32		

Appendix 4. Instrumental settings, GCMS

GC:
Column oven temperature: 50 ºC
Injection temperature: 280 ºC
Injection mode: Splitless
Sampling time: 1.00 min
Carrier gas: Helium (He)
Primary pressure: 500 - 900
Flow control mode: Linear velocity
Pressure: 122.7 kPa
Total flow: 32.3 mL/min
Column flow: 1.40 mL/min
Linear velocity: 35 cm/sec
Purge flow: 3.0 mL/min
Split ratio: 20.0

Column:
Name: Factor Four/ CP-SIL 8 CB-MS
Film thickness: 0.25 µm
Length: 50 m
Inner diameter: 0.25 mm

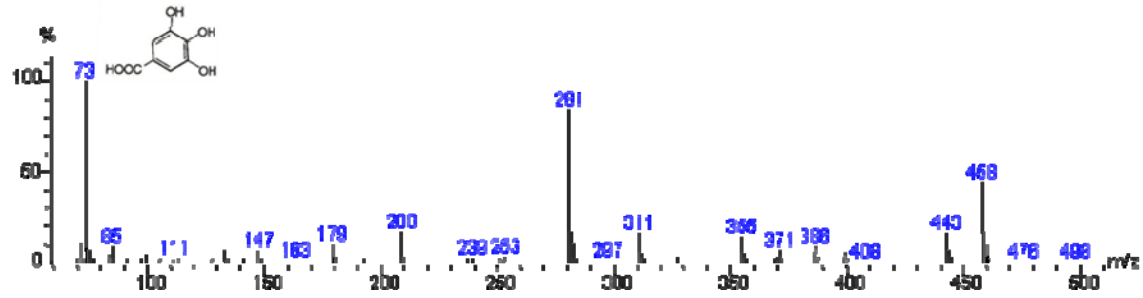
MS:
Ion source temperature: 240 ºC
Interface temperature: 300 ºC
Solvent cut time: 7 min
Start time: 7 min
End time: 42 min
Acquisition mode: SCAN/SIM
Event time: 0.15 sec
Scan speed: 3333
Start m/z: 70
End m/z: 500

Ionization method: EI

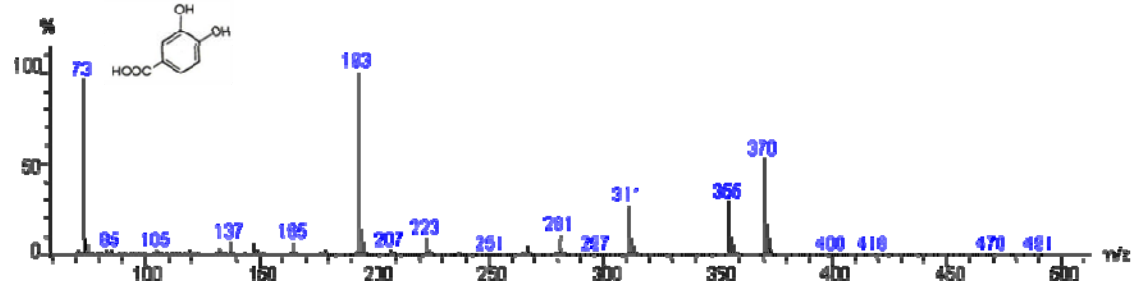
Appendix 5. Mass spectra of all benzoic acids and internal standards

Benzoic acids:

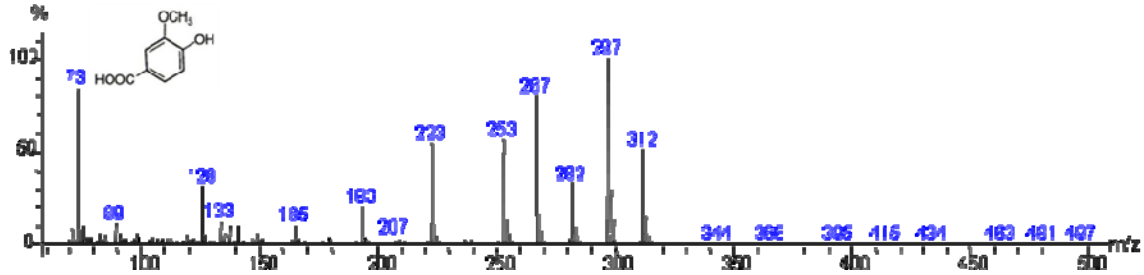
Gallic acid (GA):



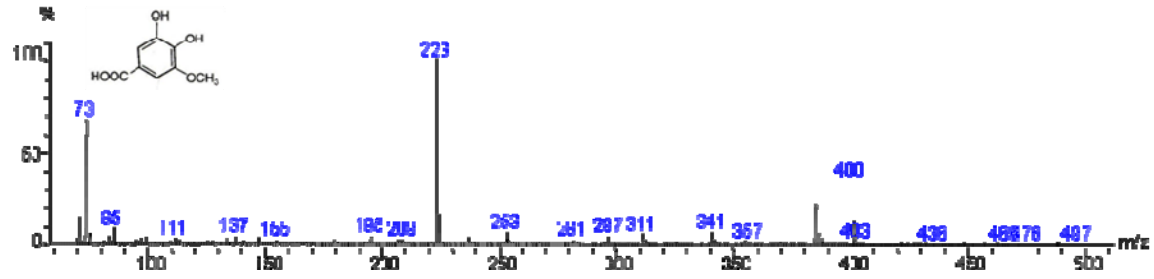
Protocatechuic acid (PCA):



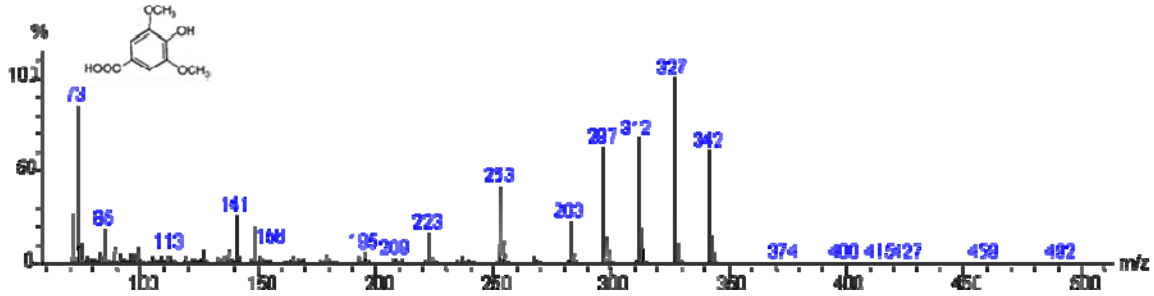
Vanillic acid (VA):



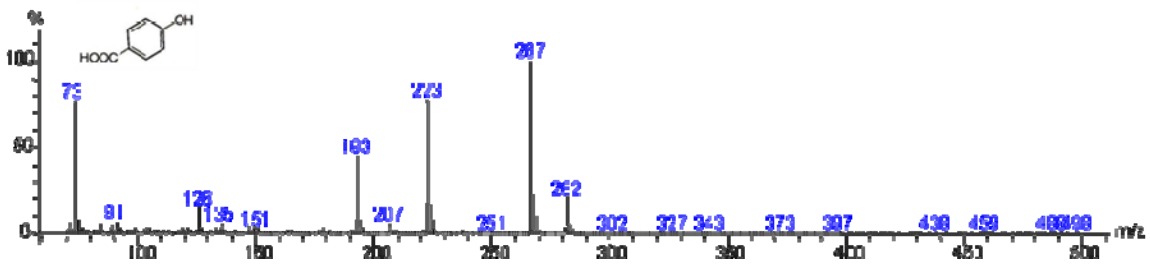
DHMBA:



Syringic acid (SA):

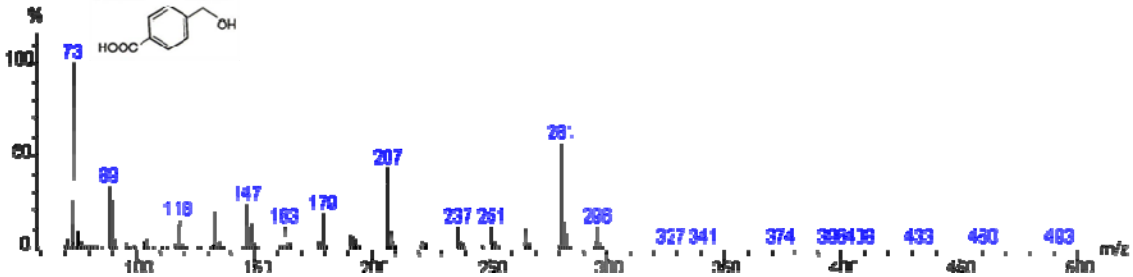


HBA:

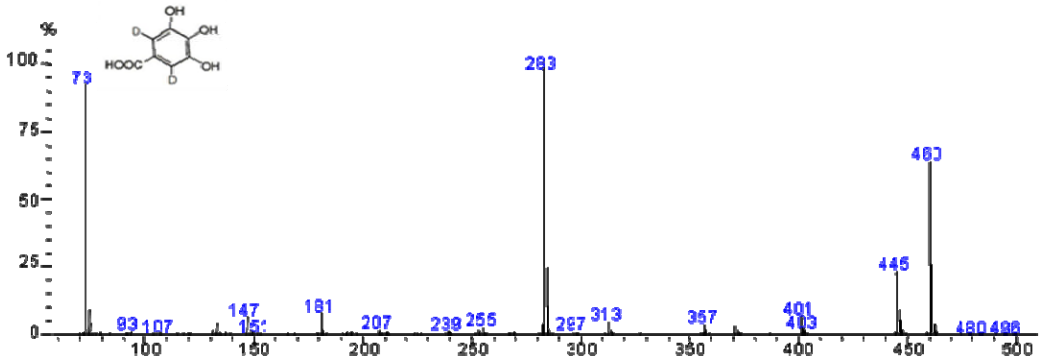


<u>Internal standards:</u>

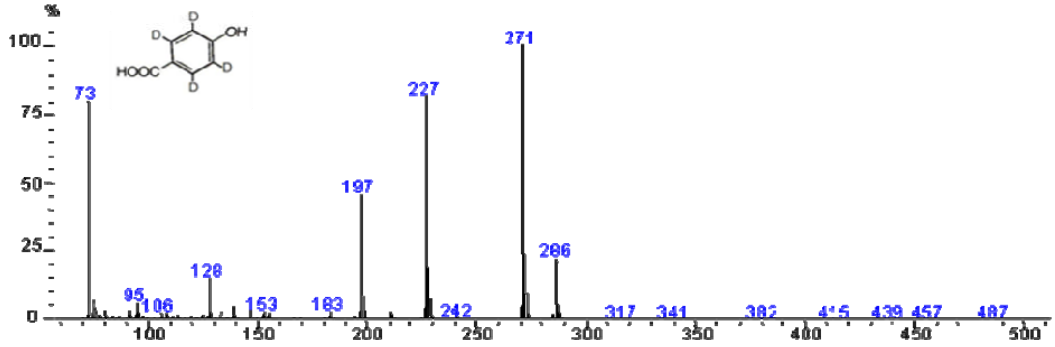
HMBA:



d-Gallic acid (d-GA):



d-HBA:


%
100
75
50
25
0
D
D
OH
HOOC
D
D
73
95
106
128
153
183
197
227
242
271
286
317
341
382
415
439
457
487
100
150
200
250
300
350
400
450
500

Appendix 6. Poster presenting sample preparation, admitted to "Kromatografisymposiet" in Sandefjord 2010

Sample preparation for GCMS analysis of hydroxybenzoic acids in urine

Nilsen CH[a,b], Nævdal A[c] and Jonsson G[a].

[a]Department of Medical Biochemistry, Stavanger University Hospital, Stavanger, Norway; [b]University of Stavanger, Department of Mathematics and Natural Sciences, Stavanger, Norway; [c]International Research Institute of Stavanger, IRIS-Biomiljø, Randaberg, Norway

Stavanger University Hospital
Stavanger Hospital Trust

University of Stavanger

IRIS

Introduction

Anthocyanins are powerful antioxidants widely distributed in fruits and vegetables[1]. They belong to the flavonoid family and consist of an aglycone (anthocyanidin, Fig. 1) and a glycone (sugar)[1] moiety. Their antioxidant capacity is strong, and may explain their beneficial health effects. MEDOX® is an anthocyanin rich product made from bilberries and blackcurrant, and contains five different aglycone structures[2] (Table 1).
Several studies report very low absorption and urinary excretion of intact anthocyanins[3]. Additionally, the recovery of anthocyanins in faeces is very low. This indicate an extensive biotransformation after oral ingestion. One suggested metabolic route result in the formation of free and conjugated benzoic acids (Fig.1)[4]. The benzoic acid substitution pattern correspond to the aglycone B-ring structure. Accordingly, MEDOX® will metabolize to five different benzoic acids.
Sample purification and derivatization of acidic protons facilitate gas chromatography mass spectrometry (GCMS) analysis of hydroxybenzoic acids in urine. Two methods have been compared for extraction of hydroxybenzoic acids in urine samples; Liquid liquid extraction (LLE) and solid phase extraction (SPE), both followed by trimethylsilyl (TMS) derivatization. The samples were analyzed by GCMS, and the methods evaluated with respect to recovery and clean-up properties.

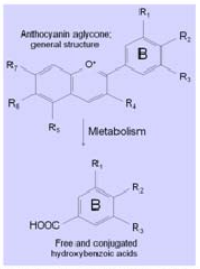


Figure 1. Anthocyanins metabolise to benzoic acids

Table 1. Anthocyanin aglycones and their respective benzoic acid metabolites

R_1, R_2, R_3	Aglycone	Benzoic acid (potential metabolite)	Common name	Abbreviation
H, OH, H	Pelargonidin*	4-hydroxybenzoic acid	-	HBA
OCH_3, OH, H	Peonidin	4-hydroxy-3-methoxybenzoic acid	Vanillic acid	VA
OH, OH, H	Cyanidin	3,4-dihydroxybenzoic acid	Protocatechuic acid	PCA
OCH_3, OH, OCH_3	Malvidin	4-hydroxy-3,5-dimethoxybenzoic acid	Syringic acid	SA
OH, OH, OCH_3	Petunidin	3,4-dihydroxy-5-methoxybenzoic acid	-	DHMBA
OH, OH, OH	Delphinidin	3,4,5-trihydroxybenzoic acid	Gallic acid	GA

*Present in strawberries (not in MEDOX®)

Material and methods

Sample material: Acidified urine (pH < 2) from a healthy volunteer. For recovery studies, aliquots of the urine were spiked with a mix of six hydroxybenzoic acids (0.62 – 0.72 µg/ml urine).
Solid phase extraction (SPE): Two *SPE* reversed phase (RP) columns were compared; Silica based sorbent (Discovery DSC-18, Supelco) and polymer based sorbent (Strata-X 8B-S100-TAK, Phenomenex). Acetone (90%) was used as eluent).
Liquid liquid extraction (LLE): Three *LLE* solvents were compared; ethyl acetate, tert-butylmethylether (MTBE), and MTBE mixed with 20% methanol.
TMS-derivatization: The extract was evaporated to dryness and derivatized before GCMS analysis (electron impact ionisation); 0.1 ml N,O-bis(trimethylsilyl)trifluoroacetamide (B-STFA, Supelco), 0.25 ml acetonitril and 0.65 ml MTBE was added to the dry extract and the samples heated for 1 hr at 60° C.

Results and discussion

LLE, using ethyl acetate as solvent, extracts all six hydroxybenzoic acids efficiently (Fig. 2). The recoveries range between 77 and 95%. Silica based SPE resulted in poor recovery for the most polar benzoic acid, GA (13%), and very high recoveries for VA (>1000%) and SA (>3000%) due to unknown interfering peaks (Fig. 2). Polymeric based reversed phase SPE resulted in recoveries comparable to LLE (66-96%), except for PCA (44%). The extraction reproducibility, expressed by standard deviation, is poor for silica based SPE as compared to LLE and polymer based SPE (Fig. 2). Ethyl acetate was used as an alternative SPE eluent to 90% acetone without improvement (not shown). A more polar eluent, such as methanol and acetonitril, may increase the recovery, but has not been tested yet.

LLE has improved or similar clean-up properties as compared to the two tested SPE procedures (Fig. 3). In urine, there were no naturally occurring peaks interfering with HBA, PCA and DHMBA (not shown). Ion chromatograms of VA, SA and GA show minor chromatographic interference of other peaks (Fig. 3). LLE was considered to be the most promising sample preparation method by comparison of recoveries and clean-up properties. Additionally, LLE was less labour demanding than SPE.

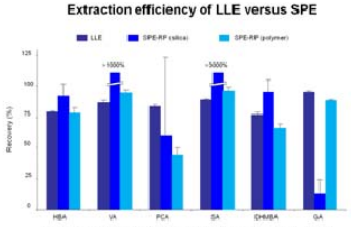


Figure 2. Recovery (%) of six hydroxybenzoic acids using different extraction methods ($n = 3$)

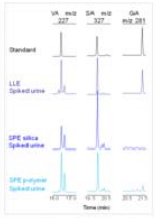


Figure 3. Ion chromatograms of VA, SA and GA extracted from urine by three different methods.

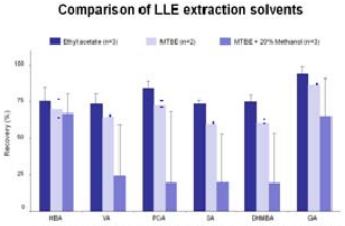


Figure 4. LLE recovery (%) of six hydroxybenzoic acids using different extraction solvents.

LLE extraction efficiency and reproducibility with ethyl acetate was compared with MTBE and MTBE:methanol (4:1) as solvents (Fig. 4). The recoveries were slightly reduced for all six hydroxybenzoic acid by use of the hydrophobic MTBE. A significant decrease in recovery and lack of reproducibility was achieved when methanol was added to modify the phases. This is due to increased solubility of hydroxybenzoic acids in the methanol containing water phase.

Conclusion

LLE, using ethyl acetate as solvent, resulted in improved recovery and reproducibility as compared with SPE. The clean-up properties of LLE and SPE was comparable.
Two alternative LLE solvents to ethyl acatate, MTBE and MTBE:methanol (4:1) did not increase the recovery of hydroxybenzoic acids from urine samples.

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