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Establishment of a Metabolite Extraction Method with MS-based Metabolite Profiling of Invasive Ductal Carcinoma Xenografts

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Preface

This master thesis was carried out as a collaboration between the Department of Biotechnology and the Department of Circulation and Medical Imaging at the Norwegian University of Science and Technology spring 2012.

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This master thesis was written by me alone, but the laboratory work was carried out in collaboration with master student Ine Pedersen. Therefore, I would especially like to thank Ine Pedersen for all support, great collaboration, good friendship and laughter. We are quite a team!

Finally, I am thankful for the love and support from friends and family. My mother, father and sister for their belief in me and what I can achieve. Most importantly, I am deeply grateful to my dear Øystein, for the love, patience and overwhelming optimism.

Abstract

Breast cancer is a complex disease comprising subtypes with varying clinical behavior, biological features and treatment response. Breast cancer heterogeneity characterized by different subtypes, is responsible for the high mortality among breast cancer patients, since patients with identical diagnosis can have very different prognosis. Metabolite profiles and biomarkers can hopefully be used for the improved diagnosis and optimal therapeutic treatment.

The scope of this study was split in two parts. The first aim was to develop an optimal method for the complete extraction of polar and non-polar metabolites, from invasive ductal carcinoma xenografts. The optimization experiments were performed with tissue samples of a basal-like xenograft model (MAS98.12), and a Precellys 24 homogenizer equipped with a cooling unit. Polar metabolites were detected by absolute quantification analysis by gas-chromatography triple quadrupole mass spectrometry (GC-QqQ-MS) after methyl chloroformate (MCF) derivatization. Non-polar metabolites were detected as fatty acid methyl esters (FAMEs) by quantitative analysis by GC-Q-MS (single quadrupole GC-MS). TMS derivatization was also evaluated, but MCF derivatization was concluded to be a more sensitive method for the polar extracts than TMS derivatization. The complete extraction was achieved after three homogenization rounds with methanol and chloroform, respectively.

The second aim was to use the optimal method in metabolite profiling experiments of luminal-like (MAS98.06) and basal-like (MAS98.12) xenografts, in addition to metabolite profiling of basal-like xenografts treated with a cancer drug called MK-2206. Polar metabolite profiles were obtained by absolute quantitative MCF GQ-QqQ-MS and compared by principle component analysis (PCA) and Student‘s t-tests. The statistical analyses showed that the MAS98.12 xenograft has significant higher concentrations of lactate and glycine compared to the MAS98.06 xenograft, while the MAS98.06 xenograft has significant higher concentrations of O-acetyl-L-serine and aspartate. The untreated MAS98.12 xenograft has significant higher concentration of lactate than the MK-2206 treated MAS98.12 xenograft. Classification of breast cancer subtypes can therefore be made based on the polar metabolite profiles. Non-polar metabolite profiles were not found after flow injection (FIA) MS of a non-polar extract, much due to a limited research time.

Both the extraction method and the metabolite profiles need further validation in the search for biomarkers, that can serve as prognostic tools from the development of breast cancer and determination of prognosis to the establishment of personalized treatment.

Sammendrag

Brystkreft er en kompleks sykdom med ulike subtyper med varierende klinisk oppførsel, biologiske særpreg og respons på behandling. Den høye dødeligheten blant brystkreftpasienter skyldes i all hovedsak uensartetheten i brystkreft, karakterisert av de ulike subtypene. Metabolittprofiler og biomarkører kan forhåpentligvis brukes til bedre diagnotisering og optimal behandling.

Hensikten med denne studien var delt i to. Det første målet var å utvikle en optimal metode for fullstendig ekstraksjon av polare og upolar metabolitter fra invasive duktale carcinoma xenografter. Optimaliseringseksperimentene ble utført med vevsprøver fra en basal-like xenograft model (MAS98.12), og en Precellys 24 homogenisator utstyrt med en kjøleenhet. Polare metabolitter ble detektert ved absolutt kvantifisering med gasskromatografi trippelkvadrupol massespektrometri (GC-QqQ-MS) etter derivatisering med metylkloroformat (MCF). Upolare metabolitter ble detektert som fettsyremetylestere (FAMEs) ved kvantifisering med GC-Q-MS (singelkvadrupol GC-MS). Derivatisering med TMS ble også evaluert, men derivatisering med MCF ble konkludert til å være en mer sensitiv metode for de polare ekstraktene enn derivatisering med TMS. Fullstendig ekstraksjon av polare og upolare metabolitter ble oppnådd etter tre homogeniseringsrunder med henholdsvis metanol og kloroform.

Det andre målet var å bruke den optimale metoden for å finne metabolittprofilene til luminal-like (MAS98.06) og basal-like (MAS98.12) xenografter, i tillegg til å finne metabolittprofilen til basal-like xenografter behandlet med en kreftmedisin kalt MK-2206. Polare metabolittprofiler ble funnet etter absolutt kvantitativ analyse med MCF GC-QqQ-MS og sammenlignet med prinsipal komponent analyse (PCA) og Student‘s t-tester. De statistiske analysene viste at MAS98.12 xenograftet har signifikant høyere konsentrasjoner av laktat og glycine sammelignet med MAS98.06 xenograftet. MAS98.06 xenograftet har signifikant høyere konsentrasjon av O-acetyl-L-serine og aspartate. Det ubehandlede MAS98.12 xenograftet har signifikant høyere konsentrasjon av laktat sammelignet med MAS98.12 xengraftet behandlet med MK-2206. Klassifisering av subtyper av brystkreft kan derfor gjøres basert på de polare metabolittprofilene. Upolare metabolittprofiler ble ikke funnet etter flow injection (FIA) MS av et upolar ekstrakt, mye på grunn av begrenset forskningstid.

Det er et behov for ytterligere validering av både den optimale ekstraksjonsmetoden og metabolittprofilene for å finne biomarkører som kan fungere som prognostiske verktøy, fra utviklingen av brystkreft og bestemmelsen av prognose til etableringen av personlig behandling.

List of abbreviations

AMDIS	Automated mass spectra deconvolution and identification system
CI	Chemical ionization
DMSO	Dimethyl sulfoxide
DRS	Deconvolution Reporting Software
EI	Electron impact , electron ionization
ER	Oestrogen receptor
ESI	Electrospray ionization
FAME	Fatty acid methyl ester
FIA	Flow injection analysis
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HR MAS	High resolution magic angle spinning spectroscopy
HVL	Heavy labeled
ISTD	Internal standard
LC	Liquid chromatography
MCF	Methyl chloroformate
MRM	Multiple reaction monitoring
MRS	Magnetic resonance spectroscopy
MS	Mass spectrometry
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
MTBE	Methyl-*tert*-butyl ether
MTBSTFA	N-(*tert*-butyldimethylsilyl)-N-methyltrifluoroacetamide
NMR	Nuclear magnetic resonance
PC	Principal component
PCA	Principal component analysis
PI3K	Phosphatidylinositol-3-kinase
pmol/pM	Pico mole (10^{-12} mole)/pico molar (10^{-12} molar)
PP	Polypropylen
Q-TOF	Quadrupole - time-of-flight
RSD	Relative standard deviation
RT	Retention time
SD	Standard deviation
STD	Standard
TgI	Target ion
TMCS	Trimethylchlorosilane
TMS	Trimethylsilane

Contents

1 Introduction

1.1 Breast cancer

Breast cancer is a heterogeneous and complex disease [1]. The majority of cancer deaths in women worldwide is caused by breast cancer, even though the mortality has been reduced [2, 3]. The breast cancer heterogeneity is represented with subtypes varying in clinical behavior, biological features and treatment response [1].

The female breast is mainly made up of the milk-producing glands (the lobules) and the tubes that carry the milk from the lobules to the nipple (the ducts) (Figure 1.1). The stroma, consisting of fatty and connective tissue, surrounds the ducts, the lobules, and the blood and lymphatic vessels. Two types of epithelial cells are found in the mammary gland, the basal cells and the luminal cells [4]. The luminal cells line the surface of the ducts and are responsible for the milk secretion, while the basal cells surround the luminal cells and have both epithelial and contractile muscle properties [5].

The most common type of breast cancer is invasive ductal carcinoma, a cancer that begins in the cells in the ducts and invades the surrounding blood and lymphatic vessels [6]. Breast cancer can begin in the luminal cells or the basal cells, called luminal-like and basal-like breast cancer, respectively. Classification of breast tumors based on gene expression patterns has shown that breast cancer can be divided in two groups, based on the expression of the genes coding for the oestrogen receptor (ER) and the genes involved in the ER pathway [4]. ER-positive breast cancer is characterized by the expression of the ER genes and high expression of many of the genes expressed by luminal breast cells, while ER-negative breast cancer fails to express the ER genes [4]. The luminal-like and the basal-like cancer are considered ER-positive and ER-negative, respectively, and the basal-like cancer has a more aggressive clinical behavior and a more poor prognosis than the luminal-like cancer [1, 7].

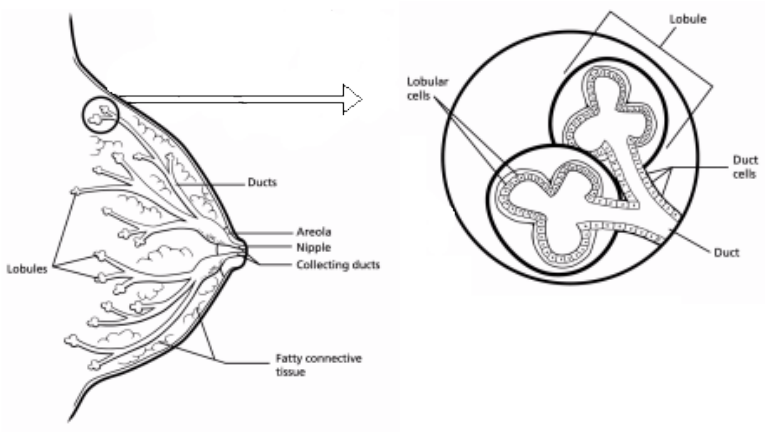


Figure 1.1: *The normal structure of the female breast showing the organization of the lobules and the ducts with their epithelial cells. The stroma, fatty and connective tissues, surrounds the lobules and the ducts.*

1.2 Metabolomics and breast cancer

Identification together with qualitative and quantitative measurement of low-molecular weight metabolites, in a biochemical network in a cell or tissue, is known as metabolomics [8]. Metabolites, molecules such as amino- and non-amino organic acids, sugars and lipids, represent the functional fingerprints in a cell, as they represent events downstream of transcription and translation of the genome [9]. Because of this, metabolomics can reveal and identify specific metabolite changes by comparing unique metabolite profiles from different phenotypes. These metabolite changes can thereafter reflect and identify specific diseases [10, 11], for example breast cancer. Upregulation of both the glycolysis, thus increased formation of lactate, and the choline phospholipid metabolism are known dominant metabolite changes seen in breast cancer [12]. Other metabolite changes seen in breast cancer are related to the citric acid cycle and the fatty acid metabolism [12]. These metabolite changes identified by metabolite profiles of breast cancer can characterize the breast cancer subtype, the development of cancer and possible prognostic factors [13]. Comparison of metabolite profiles and prognostic factors, such as hormonal status of oestrogens and lymph node status, are important for the identification of breast cancer. Establishment of personalized treatment is also important, since patients with the same diagnosis can have different prognosis due to the heterogeneity of breast cancer [14, 15]. Endocrine therapy can suppress tumor growth if receptors for hormones are present [15]. Breast cancer patients with no metastasis in the lymph nodes and small tumors are

predicted to be cured, while patients with the opposite scenario are predicted to have a high risk for recurring cancer disease [15].

Increased knowledge about abnormalities in lipid metabolism seen in diseases like diabetes and Alzheimer‘s disease, has attracted more attention towards the field of lipidomics [16]. Lipidomics involves the identification and the quantification of lipids in a biochemical network [16]. Since metabolite changes in fatty acid metabolism have been shown in breast cancer, the identification of lipid profiles together with metabolite profiles are highly appreciated [12, 16].

Metabolomics and lipidomics utilizing mass spectrometry (MS)-based tools involve several steps before metabolite and lipid profiles can be obtained. As shown in Figure 1.2, a cell or tissue must first be quenched immediately after sample harvesting to stop the biochemical processes taking place in the sample. After quenching, the metabolites are extracted into an appropriate solvent by disrupting the cell walls, to make them accessible to the analytical tool. Thirdly, samples are often concentrated by removal of the extraction solvent, because many metabolites are present in concentrations below the detection level of the analytical tool. Then, the resulting metabolite and lipid profiles obtained after metabolite analysis can be compared, and specific diseases can perhaps be identified. [10, 17]

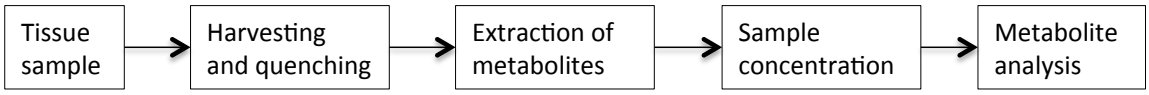


Figure 1.2: *Schematic outline of the experimental steps in metabolomics and lipidomics.*

1.3 Future clinical approaches of metabolomics and breast cancer research

1.3.1 Breast cancer xenografts

Mice models have proven to be good approaches for modeling human cancer for research [18, 19]. Bergamaschi *et al.* have described a procedure, where primary mammary tumor specimens collected from patients were implanted into immunodeficient mice, to obtain two breast cancer xenograft models (MAS98.06 and MAS98.12) [19]. Both the xenografts maintained the morphological characteristics of the original tumor, and they were classified as invasive grade III ductal carcinoma [19] (Figure 1.3). The MAS98.06 xenograft and the MAS98.12 xenograft were categorized as luminal-like breast cancer and basal-like cancer, respectively (section 1.1) [20].

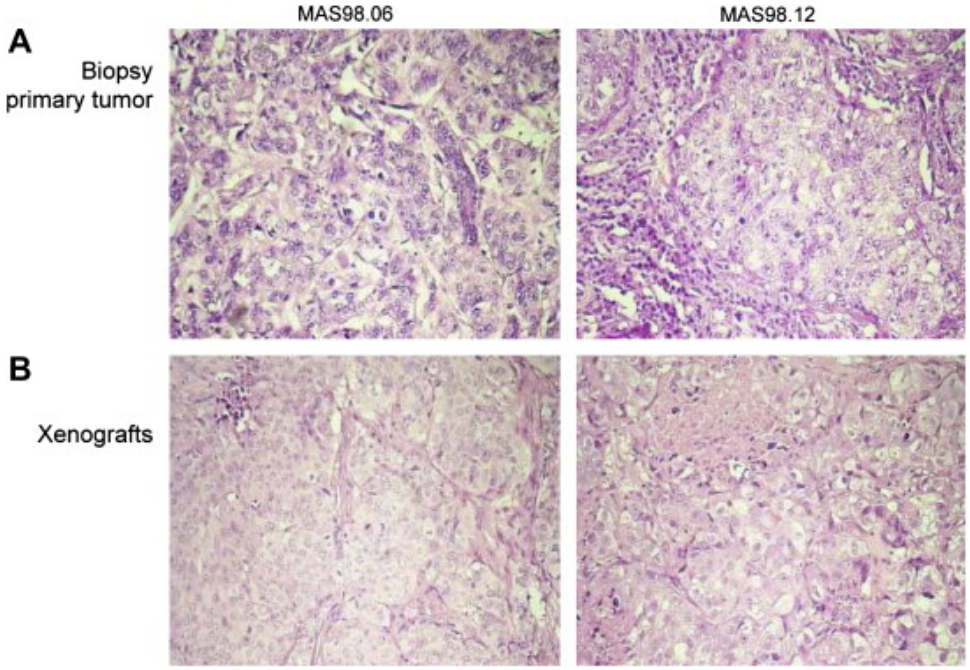


Figure 1.3: *Histopathological analysis of the MAS98.06 and the MAS98.12 (a) primary tumors and (b) xenografts performed by Bergamaschi [19]. Both the xenografts maintained the morphological characteristics of the primary tumor.*

1.3.2 Breast cancer treatment with MK-2206

The MAS98.12 xenograft model has been tested with a cancer drug called MK-2206, an allosteric Akt (Protein Kinase B, PKB) inhibitor [21]. Akt is activated by growth factors or survival factors through phosphatidylinositol-3-kinase (PI3K), and the abnormal activation of PI3K is an essential step in the initiation and maintenance of human tumors to inhibit apoptosis [21]. The PI3K/Akt pathway regulates cellular functions such as metabolism, cell proliferation and cell survival (Figure 1.4) [22]. Indeed, Akt is an antiapoptotic factor, and activation and overexpression of Akt is often associated with resistance to cancer treatment, such as chemotherapy or radiotherapy [21]. The MK-2206 drug is tested in the MAS98.12 xenograft since activation of the PI3K/Akt pathway has been associated with the basal-like breast cancer, which identifies the pathway as a potential therapeutic target [22]. Research by Moestue *et al.* have shown that the MK-2206 drug restrains both signaling and cell division in the cancer cells (Figure 1.5) [23].

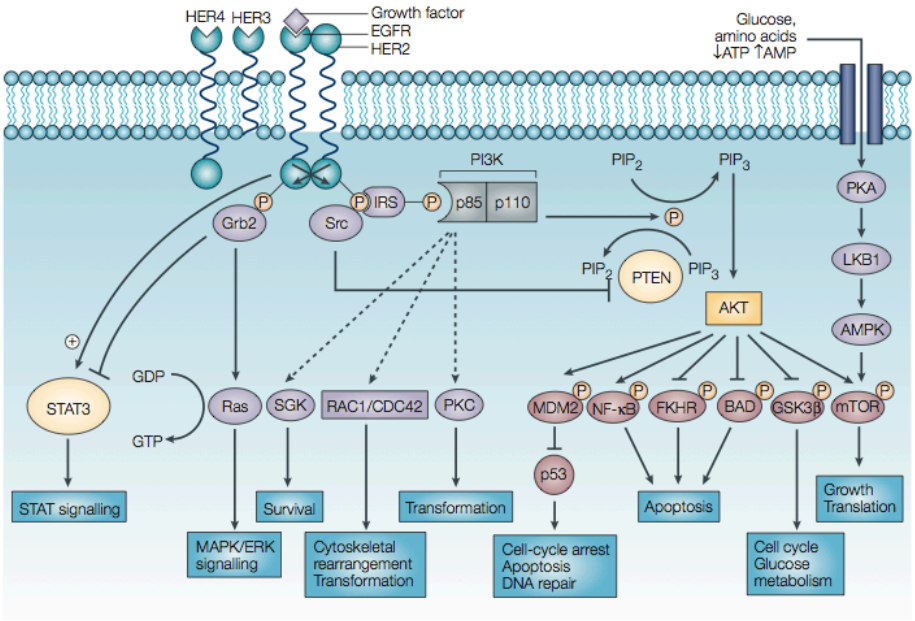
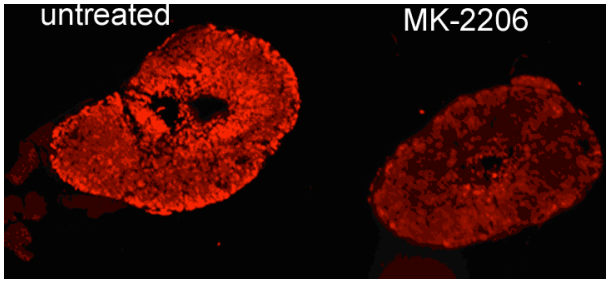
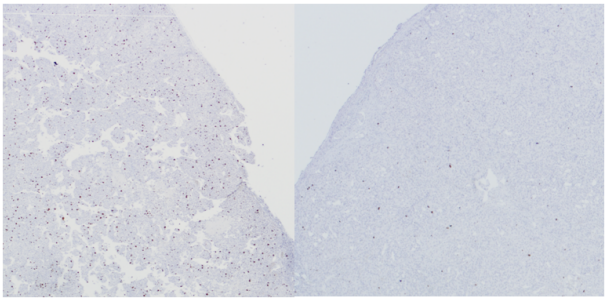


Figure 1.4: *Illustration of the signaling in the PI3K/Akt pathway [24]. The pathway inhibits the cell cycle, glucose metabolism and apoptosis, but activates cell growth and translation, through activation of Akt.*



(a)



(b)

Figure 1.5: *Effects of the MK-2206 drug in the MAS98.12 xenograft. The MK-2206 drug restrains both (a) the signaling and (b) the cell division in the cancer cells by inhibiting the PI3K/Akt pathway given in Figure 1.4 [23]. (a) The signaling, stained with red, has a reduced intensity in the xenograft treated with the MK-2206 drug (right) compared to the untreated xenograft (left). (b) The cancer cells in untreated xenograft (left) divide because of an active cell cycle, but the cell division is restrained in the xenograft treated with the MK-2206 drug (right).*

1.3.3 Breast cancer biomarkers

Specific biomarkers identified from metabolite profiles of breast biopsies taken from breast cancer patients, can hopefully be used to detect the presence and determine the prognosis of cancer. Cancer cells possess an unique metabolite profile that can be used to develop and identify these specific biomarkers. The Her2/neu protein and the oestrogen receptor (ER) are examples of known protein biomarkers in breast cancer. Prognosis, prediction and follow-up for breast cancer patients can be revealed by the use of biomarkers, since biomarkers give the opportunity to follow the cell metabolism from before to after therapeutic treatment [12]. However, breast cancer patients with identical diagnosis may have very different prognosis, even with the same prognostic factors (section 1.2) [14]. Prognostic factors such as lymph node status and hormonal status are in fact considered insufficient and inaccurate in the development of complete and adequate personalized treatment. The need for supplementary predictors of tumor aggressiveness and treatment response, based on tumor phenotypes and metabolite profiles, are therefore

considered necessary in the course of finding the right treatment for individual patients [25]. Such responses can be associated with single metabolites as biomarkers, but they are more likely associated with ratios of biomarkers, or metabolite profiles [25]. By identifying such metabolite profiles and biomarkers, patients with serious prognosis can be separated from patients with a good prognosis, and patient response of a specific treatment can be revealed, making the personalized treatment more beneficial. In addition, metabolite profiles and biomarkers can possibly lead to more prognostic tools and more efficient diagnostic, together with early detection of cancer and the prediction of survival [14].

1.4 Extraction of metabolites

1.4.1 General background

After harvesting and quenching of tissue samples (Figure 1.2), intracellular metabolites should be extracted. Extraction of metabolites can be achieved with mechanical and non-mechanical methods for the disruption of the cell walls and leakage of metabolites into an extraction solvent [17]. Mechanical extraction methods include sonication, pressure extraction, grinding with mortar and pestle, and homogenization with a beads-based or a rotor-based homogenizer. Non-mechanical methods include the use of high temperatures or extreme pH, in addition to the use of enzymatic, chemical or physical reagents.

Extracting all classes of metabolites from tissues is not straightforward, due to the large variety of metabolite differences in both physical and chemical properties [9, 12]. In addition, the level of each metabolite varies depending on the physiological, developmental and pathological state of the tissue [17], and the extraction of metabolites from tissues is normally a labor-intensive step [11]. Any extraction method must fulfill three requirements for it to be considered an ideal method [26]. First, an ideal extraction method must give completeness to ensure that all metabolites are completely accessible for further analysis by an analytical tool. Second, conversion of the metabolites during the extraction must be prevented. The metabolite concentrations have a fast turnover, meaning that the concentrations change rapidly [17]. To prevent degradation and maintain the physiological state of the tissue after harvesting, enzymes involved in the biochemical processes within the tissue must be inactivated. This is mainly done by quenching the tissue by freezing or cooling, often in liquid nitrogen (N_2), immediately after harvesting. At last, the extraction method must not be able to extensively degrade the metabolites.

Different extraction methods have been tested over the recent years, but a reliable ex-

traction method for the complete extraction of metabolites from breast cancer tissues has, however, not yet been developed. Extraction methods combining mechanical and non-mechanical approaches have previously been tested in preliminary experiments in a project work by Madsen, in hope of finding an optimal method [27]. Madsen demonstrated that the preferred polar metabolite extraction is accomplished by utilizing a homogenizer and methanol as the extraction solvent. This conclusion was made based on both the extraction efficiency, hence the number and amount of metabolites present after extraction, and the reproducibility and feasibility of the method. A homogenizer acts by disrupting the cell walls in tissues, thereby causing extraction of metabolites into an appropriate extraction solvent [28]. Both a Precellys 24 (beads-based) homogenizer and an Ultra-Turrax (rotor-based) homogenizer gave promising results, while boiling ethanol, freezing-thawing, sonication and grinding with mortar and pestle were considered non-ideal methods [27]. Extraction of metabolites with a beads-based homogenizer was considered ideal if equipped with a cooling unit, in order to prevent degradation reactions during extraction. One important advantage of the beads-based homogenizer compared to the rotor-based homogenizer is the ability to extract up to 24 samples simultaneously, making the extraction both rapid and simple [10]. Another advantage is the use of the same plastic tube in the homogenizer and the centrifuge, which will minimize the loss of samples throughout the experimental procedure. This will also be extremely important if more than one homogenization round is necessary for the complete metabolite extraction.

1.4.2 Extractable metabolites

Both polar and non-polar metabolites can be extracted from tissues, if the extraction solvents have the necessary chemical and physical properties. Amino acids, organic acids, sugars and sugar derivatives are polar metabolites that can be identified in breast cancer tissues, while lipids, fatty acids, oestrogens and sterols are examples of non-polar metabolites.

The metabolism of choline is known to be upregulated in breast cancer [12, 20]. Choline can be metabolized in breast cancer cells by two major pathways, the biosynthesis of phosphocholine and phosphatidylcholine or the synthesis of glycine via betaine in the choline oxidative pathway (Figure 1.6a) [20, 12]. Glycine is mainly biosynthesized through glycolysis from the intermediate 3-phosphoglycerate [29], but it is known to be synthesized from choline in breast cancer cells [20, 12]. Lactate (Figure 1.6b), a non-amino organic acid formed through the glycolysis and pyruvate, is upregulated in breast cancer [12]. Since

the glycolysis is upregulated, the concentration of glucose is reduced. Research by Sitter *et al.* demonstrated that poor prognosis among breast cancer patients is characterized with higher concentrations of both glycine and lactate [15].

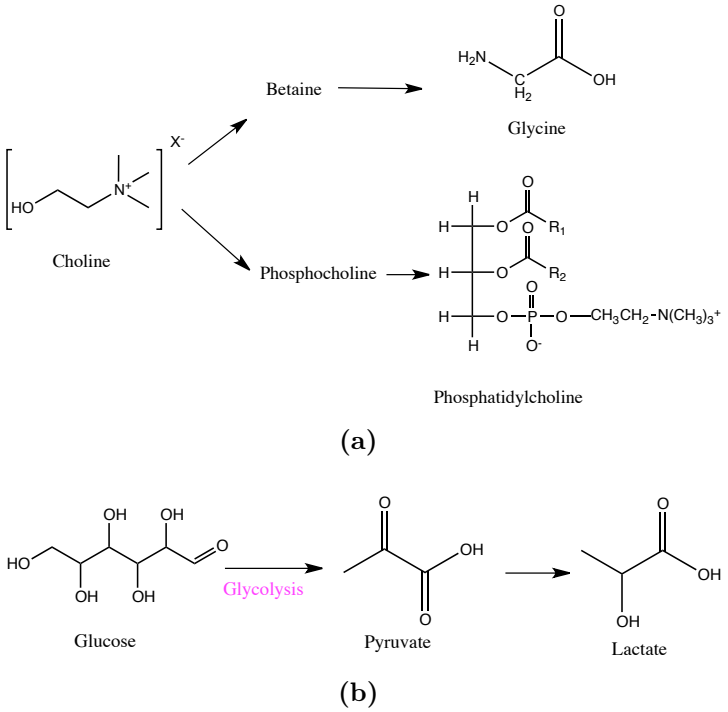


Figure 1.6: *(a) Metabolism of choline, either to glycine via betaine in the choline oxidative pathway or to phosphatidylcholine via phosphocholine. R_1 and R_2 represent fatty acids; (b) Formation of lactate from glucose and pyruvate in the glycolysis.*

Lipids are a group of molecules that are both functionally and chemically diverse. They are mainly hydrophobic (non-polar), making them soluble in non-polar solvents such as chloroform and ethers [30]. Lipids play multiple functional roles in biochemical networks, as they are involved in signaling and energy storage and as structural components of cell membranes [31]. The chemical structure of lipids range from the simple components fatty acids to the storage lipids triacylglycerols and the membrane lipids phospholipids (Figure 1.7). Since lipids can be structural components of cell membranes, membrane lipids are amphipathic, having both hydrophilic (polar) and hydrophobic regions. In fact, most lipids reside in cell membranes, where they form the lipid bilayer [32, 33]. Membrane lipids in mammalian cells consist mainly of sterols and phospholipids, for example phosphatidylcholine [31]. Lipids act as energy storage, where fatty acids are the most reduced form of chemical energy. The majority of fatty acids in cells are part of lipids, since free fatty acids are toxic to cells [31]. Lipids are involved in the regulation of several cellular functions, for example cell proliferation and apoptosis, as signaling molecules [31]. In

cancer cells, processes involving lipids are accelerated leading to uncontrolled cell growth and cancerous growth [31]. Disturbances in fatty acid metabolism are seen in breast cancer [12].

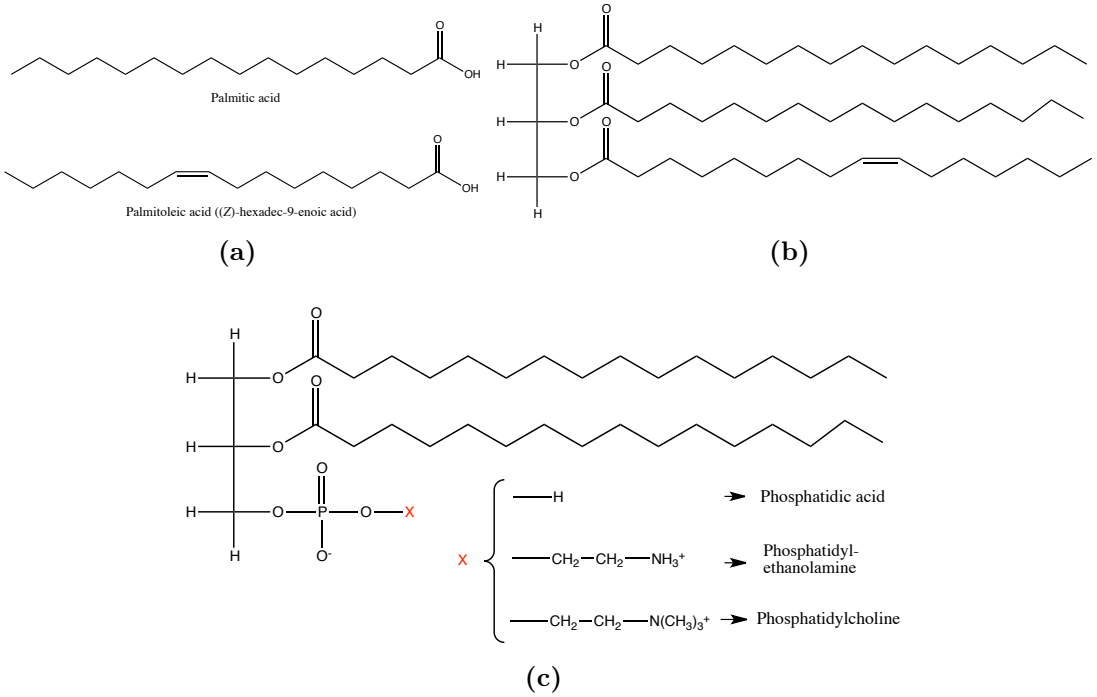


Figure 1.7: *Illustration of lipids: (a) saturated and unsaturated fatty acids illustrated by palmitic acid and palmitoleic acid, respectively; (b) an example of a triacylglycerol with its glycerol backbone linked to three fatty acids; (c) an example of a phospholipid with its glycerol backbone linked to two fatty acids and a head-group substituent (X). Possible head-group substituents giving different phospholipids are shown.*

Oestrogens and their oxidative metabolites are assumed to be involved in the development of breast cancer. They are carcinogens, thus, they affect both tumor initiation and cell proliferation [12].

1.5 Metabolite analysis and interpretation

After extraction of metabolites and evaporation of the extraction solvent to concentrate the metabolites to a detectable level (Figure 1.2), the metabolites are to be identified and measured by analytical tools. After metabolite analysis, the resulting data can be processed and interpreted.

1.5.1 Analytical tools

Nuclear magnetic resonance (NMR) and mass spectrometer (MS) are two analytical tools used to measure metabolites, and their techniques are well established. In NMR analysis, key nuclei of compounds are influenced by a magnetic field, and the magnetic influence is measured and recorded as a spectrum [12]. The spectrum is then used in the accurate determination of a molecule based on the height and position of different peaks. NMR is a rapid, cost effective and reproducible analysis technique. Metabolite analysis by NMR can detect and identify a wide range of compounds, both polar and non-polar metabolites, from biofluids, extracts or intact tissues. Intact tissues can be analyzed in a non-destructive manner by high resolution magic angle spinning magnetic resonance spectroscopy (HR MAS MRS) [34]. NMR is also very useful in the characterization of unknown compounds [35]. However, NMR has a lower sensitivity and requires more expensive instrumentation than MS [12].

MS has been shown to be a major tool for analysis and measurement of metabolites [8]. MS can be coupled to chromatographic setups, for example liquid-chromatography (LC) or gas-chromatography (GC), to provide extreme powerful systems for the measurement of metabolites (Figure 1.8a) [8]. Analysis with both LC-MS and GC-MS involves a chromatographic step, where the metabolites are separated either in the liquid or the gas phase, respectively [34]. After separation, the metabolites are ionized by the ion source in the MS, and the resulting charged particles are separated according to their mass-to-charge (m/z) ratio (Figure 1.8b) [12, 34]. The m/z ratio is thereafter used to identify the metabolites with an appropriate software tool. MS-based tools are considered more sensitive than NMR tools, since MS-based tools can detect metabolites at picogram levels compared to microgram level for NMR [14].

Sample preparation, involving metabolite extraction, can be time-consuming and critical for the metabolite analysis by GC-MS [34]. GC-MS systems can analyze a wide range of volatile compounds, but semi-volatile and non-volatile compounds must be derivatized prior to analysis (section 1.5.3) [35]. GC systems can be coupled to single-quadrupole

and triple-quadrupole MS, here termed GC-Q-MS and GC-QqQ-MS, respectively. GC-Q-MS systems provide nominal-mass information, while GC-QqQ-MS systems result in very detailed fragmentation information and both a higher selectivity and a higher level of molecular specificity than GC-Q-MS systems [35, 36]. Thus, GC-QqQ-MS systems are considered more sensitive than GC-Q-MS systems.

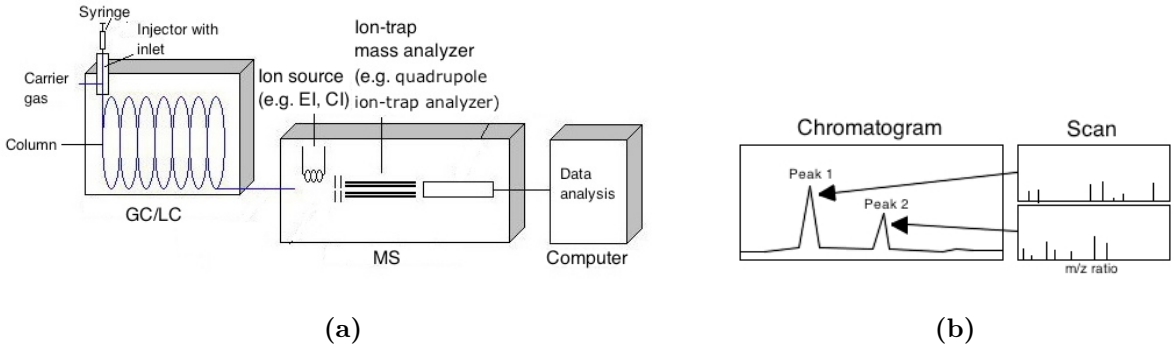


Figure 1.8: *Illustration of: (a) a schematic setup of LC-MS or GC-MS; (b) a chromatogram and scan for two compounds represented by two peaks.*

LC-MS systems provide identification and quantification of both polar and non-polar metabolites, in both targeted and non-targeted analysis [35]. The sensitivity of LC-MS systems is compatible with the sensitivity of GC-MS systems. Electrospray ionization (ESI) is the most widely used ionization technique, both in positive and negative mode, and it is suitable for a broad range of metabolites [35]. Important advantages of metabolite analysis by LC-MS systems are the ability to detect and identify metabolites in complex biological samples, and to analyze samples without the need for derivatization. Quadrupole - time-of-flight (Q-TOF) and high-performance liquid chromatography (HPLC) systems can be used as powerful metabolite analysis tools.

1.5.2 Metabolite analysis by analytical tools

Polar metabolites, such as amino- and non-amino organic acids, sugars and sugar derivatives, are often analyzed and identified with GC-MS systems, but also with NRM-systems. Lipids can either be analyzed by LC-MS systems after direct infusion of non-polar extracts, as fatty acid methyl esters (FAMEs) with GC-MS systems [37], or with NMR. The direct infusion of non-polar extracts into for example a Q-TOF system, also called flow injection MS analysis (FIA MS), provides a shotgun lipidomics approach. Such an approach comprises a high-throughput screening tool, where non-polar extracts are injected directly into the Q-TOF without a chromatographic separation [38].

1.5.3 Derivatization procedures for GC-MS systems

One important limitation for the use of GC-MS systems in metabolite analysis is the need for volatile samples, in order to separate the metabolites on a GC column. Prior to metabolite analysis, sample extracts must therefore be derivatized at the functional groups to increase the volatility and thermal stability. An ideal derivatization method should hold some requirements; it should be efficient, simple and rapid. [35]

Derivatization with silylation reagents The most frequent and classical derivatization method used in metabolomics is based on silylation reagents [35, 36]. With silylation (TMS derivatization), a silyl group ($[Si(CH_3)_3]$) is replaced by the active hydrogen of a functional group (Figure 1.9). The reagents used to introduce silyl groups are derivatives of trimethylsilane ($(CH_3)_3SiH$, TMS), such as N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and N-(*tert*-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) [35]. The derivatization method does not hold the three requirements needed for it to be an ideal derivatization method; it is not efficient, simple or rapid [35]. Other important disadvantages of silylation are the injection of unreacted derivatization reagents into the column of the GC-MS, reducing the lifetime of the column, and formation of unstable TMS derivatives [35, 36, 39]. However, stability of TMS derivatives can be improved by the protocol developed by Lien *et al.* [40].

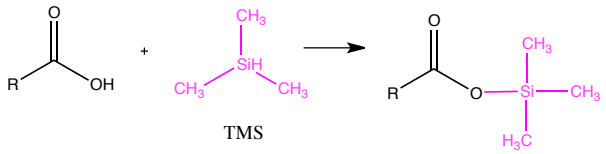


Figure 1.9: *Illustration of a derivatization reaction of an organic acid with trimethylsilane (TMS).*

Derivatization with chloroformate derivatives Another derivatization method with increased popularity is the use of alkyl chloroformate derivatives, such as methyl chloroformate (MCF, $C_2H_3ClO_2$), in esterification reactions (Figure 1.10) [35]. Amino acids and organic acids can be derivatized with chloroformate derivatives, to give reproducible and stable metabolites [35]. The use of chloroformate derivatives has several advantages over silylation, as the reactions are rapid and easy to automate. In addition, the reagents costs are negligible compared to silylation [35]. The reagents in MCF derivatization create high sensitivity and stability towards the derivatized metabolites. There are therefore very little unreacted derivatization reagents injected into the column of the GC-MS, as it is for silylation [35, 36].

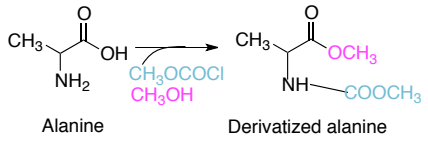


Figure 1.10: *Illustration of a derivatization reaction of alanine with methyl chloroformate (MCF) and methanol.*

Derivatization of fatty acids and lipids to fatty acid methyl esters Fatty acids are usually analyzed by GC-MS systems after conversion to the more volatile fatty acid methyl esters (FAMEs) [37]. Preparation of FAMEs involves extraction of lipids from tissues, breaking of ester bonds in lipid extracts resulting in fatty acids, and formation of fatty acid methyl esters. Breaking of ester bonds and formation of methyl esters can be combined in one step by alcoholysing the lipids in the extract directly by acid or base in methanolic solutions, a reaction termed methylation (Figure 1.11) [41]. The most used reagent in methylation of lipids to FAMEs is hydrogen chloride in methanol (methanolic HCl), which is shown to esterify all fatty acids at approximately the same rate [41].

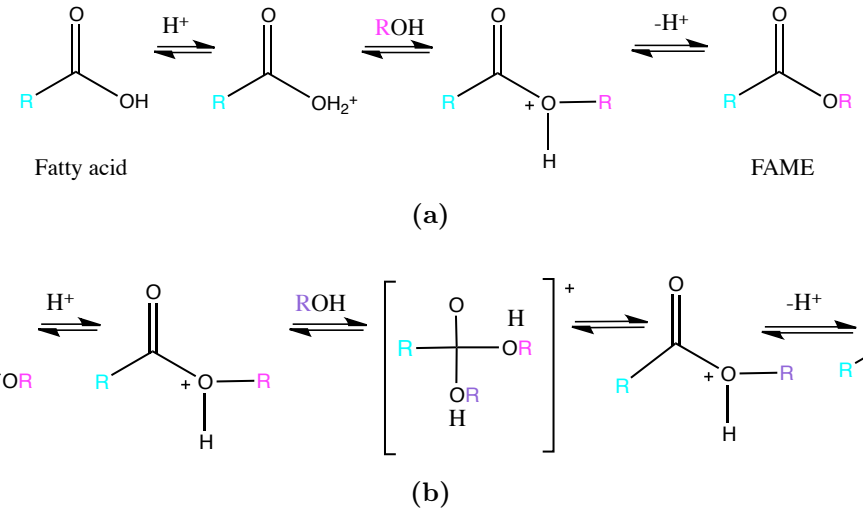


Figure 1.11: *Illustration of the methylation reactions from (a) fatty acids and (b) lipids to FAMEs with acid in methanolic solution.*

1.5.4 Data handling and interpretation of results

After analysis of sample extracts with an analytical tool, the resulting data sets will contain tremendous information of the metabolites found in those samples. Data processing and analyzing software are used to identify metabolites by peak assignment and integration [8]. Data obtained after data processing are normally normalized, to adjust for variability in the analytical methodology, sample preparation and biological material. Internal standards are therefore frequently added during the sample preparation, often prior to metabolite extraction, and subsequent derivatization. Such internal normalization, or standardization, is preferred to minimize both the variation between samples and the loss of samples in the sample preparation [35, 42]. Variability in biological material is often the main source of variations when performing metabolite analyses, and biological variations can be as high as 40% [42]. Biological replicates are often preferred over technical replicates, and biological replicates can therefore be considered more important.

Complete interpretation of data sets from several samples can be time-consuming and even impossible without statistical analysis. Classical comparison of two different samples is mainly done by a Student‘s t-test, which compare the means of each sample group [43]. Analysis of variance (ANOVA) is a generalization of the Student‘s t-test and can be used to compare the means of several sample groups [43]. ANOVA is a typical statistical approach for comparing variance within each group and variance between each group. If only two groups are to be compared, ANOVA and Student‘s test will give the same result. Principal component analysis (PCA), a basic non-discriminating multivariate data analysis, can identify similarities and differences between samples based on a data set. The purpose of multivariate analysis is to detect and model a possible phenomena, based on the information of the detected metabolites in the tissue samples [14, 44]. PCA can therefore be used to identify and detect trends and groups in a data set, containing for example breast cancer tissue samples and metabolites identified in those samples. By performing a PCA, score and loading plots are generated, where the score plot and the loading plot show the objects (tissue samples) and the variables (metabolites) of the data sets, respectively (Figure 1.12). The corresponding score and loading plots are complementary, which together provides the most valuable information about the samples [44]. When interpreting the results from score and loading plots, single metabolites or ratio of metabolites that are conspicuous can be identified. Therefore, possible biomarkers and metabolite profiles of importance can be detected, leading to a further understanding of specific and critical pathways [14].

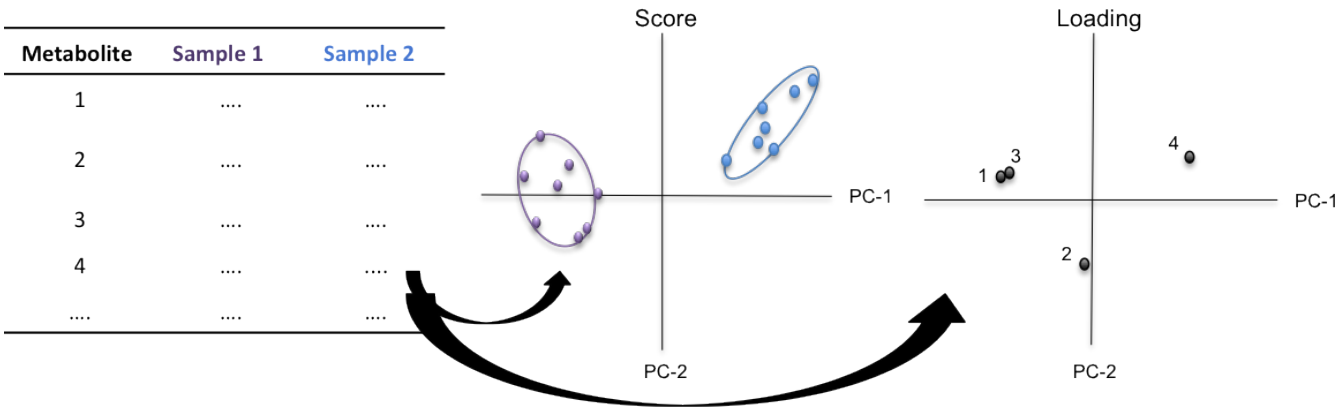


Figure 1.12: *Illustration of principal component analysis (PCA) on a data set of two samples and four metabolites. Two principal components, PC-1 and PC-2, describe the model. Sample 1 correlates with metabolite 1 and 3, and sample 2 correlates with metabolite 4. Metabolite 2 is significant only to PC-2, and metabolite 2 is not correlated to neither sample 1 nor sample 2.*

1.6 Scope of study

The scope of this master thesis was split in two parts. In the first part, the aim was to develop an optimal method for the complete extraction of both polar and non-polar metabolites from basal-like (MAS98.12) breast cancer xenografts. Polar metabolite analysis was performed after MCF derivatization by the GC-QqQ-MS system, while the non-polar metabolite analysis was performed after conversion of lipids to fatty acid methyl esters (FAMEs) by the GC-Q-MS system. In agreement with Römisch-Margl *et al.* [11], preliminary research by Madsen has demonstrated that a beads-based homogenizer could be an optimal method if equipped with a cooling unit (section 1.4.1) [27]. It is preferred to maintain the same physiological state in the samples throughout the experimental procedure, in order to prevent conversion and degradation reactions of the metabolites. This can be achieved if the samples are placed in a cool environment during both the homogenization and the rest of the extraction. The extraction method was therefore developed with a Precellys 24 (beads-based) homogenizer equipped with a Cryolys cooling unit. Figure 1.13 provides an overview of the experimental set up in the development of an optimal extraction method, where the polar and non-polar metabolite extractions were evaluated separately. The polar metabolite extraction was evaluated with 60% methanol as the extraction solvent, and the non-polar metabolite extraction was evaluated with chloroform. Both evaluations were performed in two steps (Figure 1.13).

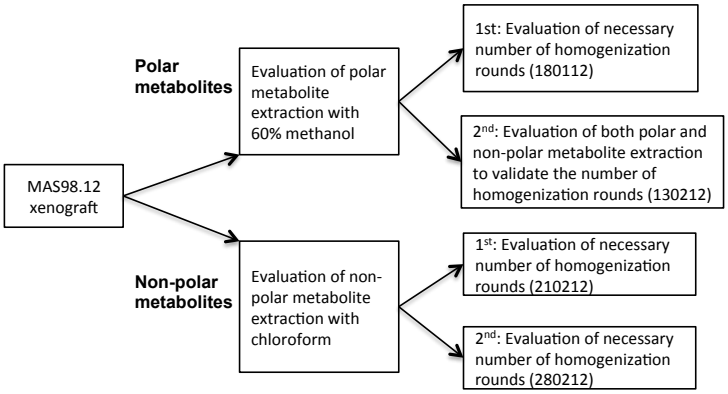


Figure 1.13: *Schematic outline of the optimization experiments. The date for each extraction is given in parenthesis.*

The second aim was to use the optimal extraction method to obtain metabolite and lipid profiles, and possibly identify valid biomarkers, of the MAS98.06 and MAS98.12 xenografts. Samples of the MK-2206 treated MAS98.12 xenograft were also extracted, to investigate metabolite differences between untreated and treated breast cancer xenografts. Figure 1.14 provides an overview of the experimental set up in the metabolite profiling experiments. The first parameter to evaluate was the standard deviations for the polar metabolites from three technical replicates of three tissue samples from the MAS98.06 and MAS98.12 xenografts. Thereafter, extraction of all three xenografts were performed to evaluate the metabolite profiles. The polar metabolite profiles were evaluated after MCF derivatization and absolute quantitative analysis by the GC-QqQ-MS system. PCA and Student‘s t-test were used as statistical analysis tools to compare the polar metabolite profiles of the MAS98.06 and the MAS98.12 xenografts, as well as the untreated and treated MAS98.12 xenografts. The analysis of non-polar metabolites as lipids were performed by direct infusion (FIA MS) of one non-polar extract by the Q-TOF LC-MS system.

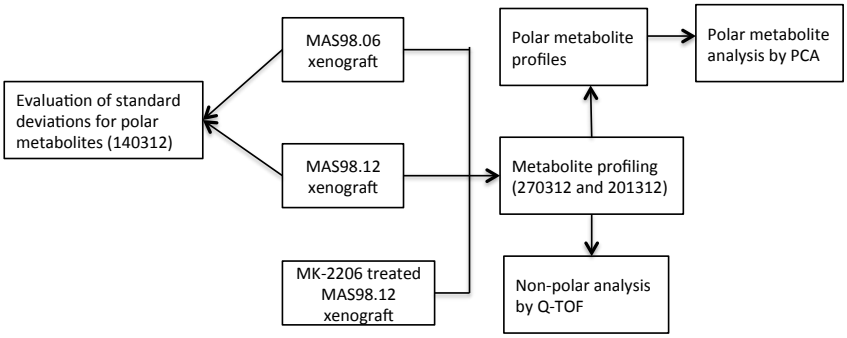


Figure 1.14: *Schematic outline of the metabolite profiling experiments. The date for each extraction is given in parenthesis.*

2 Materials and methods

2.1 Tissue samples from breast cancer xenografts

Tissue samples of luminal-like, basal-like and MK-2206 treated basal-like xenograft models (MAS98.06, MAS98.12 and MK-2206 treated MAS98.12 xenografts, respectively) were collected from an existing biobank administered by the MR cancer group at the Department of Circulation and Medical Imaging at St. Olavs hospital in Trondheim. The xenografts were approved by The National Animal Research Authority. The breast cancer xenografts were frozen at $-196°C$ in liquid nitrogen until metabolite extraction.

2.2 Optimization of the beads-based extraction method

The extraction of polar and non-polar metabolites using a beads-based homogenizer is chosen based on the work done by both Römisch-Margl *et al.* and Madsen (see Figure 1.13 in section 1.6 for an overview of the experimental set up) [11, 27]. The optimization experiments were performed with tissue samples of the MAS98.12 xenograft in the weight range 30 ± 10 mg. The tissue samples were placed in 2 mL Cryo tubes filled with 0.5 ± 0.05 g ceramic (zirconium oxide) beads with a diameter of 1.4 mm (Bertin Technologies) (Figure 2.1a). The homogenizations were performed with a Precellys 24 homogenizer equipped with a Cryolys cooling unit (Bertin Technologies) (Figure 2.1b). The Cryolys was filled with liquid nitrogen, that cools the air that diffuses into the homogenizer. If a break was made in the course of the experimental procedure, the Cryo tubes with the tissue samples were placed in an ethanol bath holding $-20°C$.

(a) **(b)**

Figure 2.1: *Homogenization equipments: (a) Cryo tube filled with ceramic beads; (b) Precellys 24 homogenizer equipped with a Cryolys cooling unit.*

2.2.1 The 1^{st} evaluation of the polar metabolite extraction

The number of homogenization rounds necessary for the complete extraction of the polar metabolites contained in tissue samples, was evaluated with methanol as the polar extraction solvent. Precooled 60% methanol (400 μL) was added to three tissue samples (Table 2.1). The samples were homogenized for three intervals of 20 sec at 5,500 rpm, with 30 sec pause between each homogenization interval. After homogenization, the samples were centrifuged for 5 min at 5,000 rpm and -9°C in an Eppendorf 5804R centrifuge. Three technical replicates of each sample was made by transferring 100 μL of the polar extracts to polypropylen (PP) tubes (5 mL, length 75 mm length, outer diameter 12 mm, article number 212-1821, VWR).

The remaining pellets were homogenized in two more rounds, as described above. In total, the tissue samples were exposed to three rounds of homogenization, and technical replicates were made from each homogenization round. All technical replicates were dried in a vacuum concentrator (Savant SPD2010 SpeedVac concentrator, Thermo Electron Corporation) until completely dry (approximately 1-2 hours), and the dried samples were stored at -80°C for further sample preparation and absolute quantitative analysis by the GC-QqQ-MS system.

Table 2.1: *Samples used in the 1^{st} evaluation of the polar metabolite extraction. The tumor identification, given by the MR cancer group at the Department of Circulation and Medical Imaging at St. Olavs hospital in Trondheim, and the sample weights are given.*

Sample name	Tumor identification	Weight [mg]
180112-1	20.11.08 MAS98.12 1-1V	38.9
180112-2		28.7
180112-3		32.8

2.2.2 The 2^{nd} evaluation of the polar metabolite extraction

A flow chart of the experiment is shown in Figure 2.2, where the aim was to validate the number of homogenization rounds found in the first evaluation further. The extraction of polar metabolites from three tissue samples (Table 2.2) were performed as described in section 2.2.1, but 10 μL 1 mM d_4-succinate was added as an internal internal before the first homogenization round. The polar extracts of each sample were pooled in the same centrifuge tube (15 mL) after the homogenization rounds. Three technical replicates to be derivatized with MCF (1) were made for each sample by transferring 100 μL from the

centrifuge tube to three PP tubes (5 mL).

The remaining pellets after the three rounds of homogenization were exposed to three subsequent homogenization rounds with the same Precellys homogenizer, to extract the lipids present in the tissue samples. For extraction of lipids, 400 μL chloroform was added as an extraction solvent before each homogenization round. The pellets were homogenized for three intervals of 20 sec at 5,500 rpm, with 30 sec pause between each homogenization interval, in each round. After each homogenization round, the samples were centrifuged for 5 min at 5,000 rpm and -9°C in an Eppendorf 5804R centrifuge, and the resulting non-polar extracts were discarded.

To check for possible remaining polar metabolites in the tissue samples after extraction with both methanol and chloroform, the remaining pellets were exposed to one homogenization round with the Precellys homogenizer (three intervals of 20 sec at 5,500 rpm with 30 sec pause between each homogenization interval). Before the homogenization round, 400 μL precooled 60% methanol and 10 μL 1 mM d_4-succinate were added to each sample as an extraction solvent and an internal extraction standard, respectively. After homogenization and subsequent centrifugation (5 min at 5,000 rpm and -9°C) with an 5804R Eppendorf centrifuge, three technical replicates to be derivatized with MCF (2) were made for each sample by transferring 100 μL of the polar extracts to three PP tubes (5 mL).

All technical replicates were dried in a vacuum concentrator (Savant SPD2010 SpeedVac concentrator, Thermo Electron Corporation) until completely dry (approximately 1-2 hours), and the dried samples were stored at -80°C for further sample preparation and analysis.

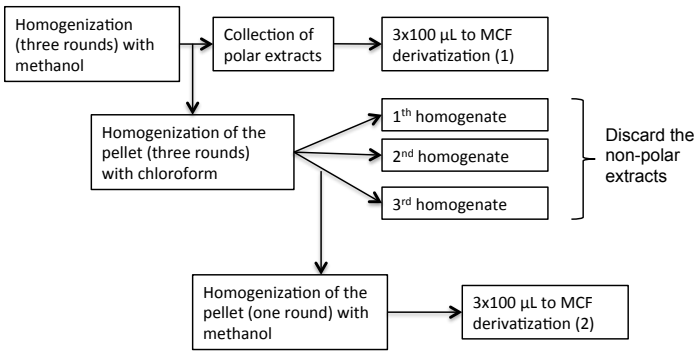


Figure 2.2: *Flow chart over the 2nd evaluation of the polar metabolite extraction.*

Table 2.2: *Samples used in the 2^{nd} evaluation of the polar metabolite extraction. The tumor identification, given by the MR cancer group at the Department of Circulation and Medical Imaging at St. Olavs hospital in Trondheim, and the sample weights are given.*

Sample name	Tumor identification	Weight [mg]
130212-1	21.11.08 MAS98.12 2-4V	28.7
130212-2		26.8
130212-3		33.9

2.2.3 The 1^{st} evaluation of the non-polar metabolite extraction

A flow chart of the experiment is shown in Figure 2.3, where the aim was to find the necessary number of homogenization rounds for the complete extraction of the non-polar metabolites. The extraction of polar metabolites from three tissue samples (Table 2.3) were performed as described in section 2.2.2, where three homogenization rounds were applied and the polar extracts for each sample were pooled in the same centrifuge tube (15 mL). One internal standard was added prior each homogenization round, with 10 μL 1 mM d_4-succinate, 10 μL 10 mM d_8-valine and 10 μL 10 mM d_3-alanine in the first, second and third homogenization round, respectively.

Three technical replicates of 100 μL to be derivatized by silylation (TMS derivatization) was made for each sample, by transferring 100 μL of each polar extract to three GC-MS vials together with 25 μL d_{27}-myristic acid (3 mg/mL in a 2:5:2 (v/v/v) water:methanol:isopropanol solution).

The remaining pellets after the three homogenization rounds were exposed to five subsequent homogenization rounds with the same Precellys homogenizer, to extract the lipids present in the tissue samples. For extraction of lipids, 400 μL chloroform was added as an extraction solvent together with 10 μL 10 mM d_{27}-myristic acid as internal standard, before each homogenization round. The pellets were homogenized for six intervals of 20 sec at 6,500 rpm, with 30 sec pause between each homogenization interval, in each round. After each homogenization round, the samples were centrifuged for 5 min at 5,000 rpm and -9°C in an Eppendorf 5804R centrifuge, and the resulting non-polar extracts were taken off into five separate PP tubes (5 mL). Three technical replicates to be analyzed for FAMEs (fatty acid analysis) were made for each sample, by transferring 100 μL of the non-polar extracts to three GC-MS vials. However, only one replicate of sample 210212-1 from all five homogenization rounds was analyzed.

All technical replicates were dried in a vacuum concentrator (Savant SPD2010 SpeedVac

concentrator, Thermo Electron Corporation) until completely dry (approximately 1-2 hours), and the dried samples were stored at −80°C for further sample preparation and analysis.

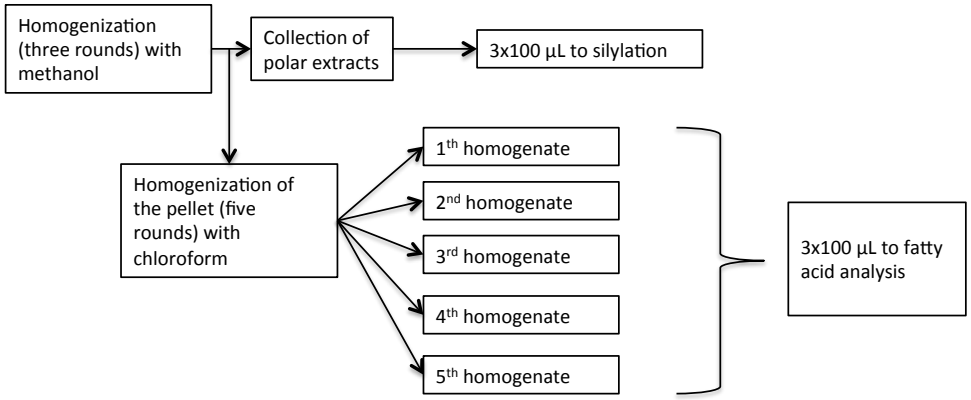


Figure 2.3: *Flow chart over the 1st evaluation of the non-polar metabolite extraction.*

Table 2.3: *Samples used in the 1st evaluation of the non-polar metabolite extraction. The tumor identification, given by the MR cancer group at the Department of Circulation and Medical Imaging at St. Olavs hospital in Trondheim, and the sample weights are given.*

Sample name	Tumor identification	Weight [mg]
210212-1	21.11.08 MAS98.12 2-4V	28.6
210212-2		27.4
210212-3		25.9

2.2.4 The 2nd evaluation of the non-polar metabolite extraction

A flow chart of the experiment is shown in Figure 2.4. The aim of this experiment was both to confirm the need for a separate non-polar metabolite extraction in the homogenizer, and to evaluate the number of homogenization rounds necessary for the complete extraction of the non-polar metabolites. The extraction of polar metabolites from two tissue samples (Table 2.4) were performed as described in the first part of section 2.2.3. After pooling the polar extracts obtained after homogenization and centrifugation in centrifuge tubes (15 mL), 2 mL chloroform was added to each of the two tubes, and the solutions were kept for partition (approximately 5 min) before centrifugation (5 min at 5,000 rpm and −9°C). The resulting two-phase systems consisted of one polar (upper) phase and one non-polar (lower) phase. Three technical replicates, to be derivatized with MCF, were made for each polar phase, by transferring 100 μL from the centrifuge tube to

three PP tubes (5 mL). Two technical replicates to be analyzed for FAMEs were made for each non-polar phase, by transferring 100 μL from the centrifuge tube to two PP tubes (5 mL).

The remaining pellets were exposed to five subsequent homogenization rounds with the same Precellys homogenizer, to extract the lipids present in the tissue samples. For extraction of lipids, 800 μL chloroform was added as an extraction solvent together with 10 μL 10 mM d_{27}-myristic acid as an internal standard, before each homogenization round. The pellets were homogenized for three intervals of 20 sec at 6,500 rpm, with 30 sec pause between each homogenization interval, in each homogenization round. After each homogenization round, the samples were centrifuged for 5 min at 5,000 rpm and -9°C in an Eppendorf 5804R centrifuge, and the resulting non-polar extracts were taken off into five separate PP tubes (5 mL). Two technical replicates to be analyzed for FAMEs (fatty acid analysis) were made for each sample, by transferring 100 μL of the supernatants to two GC-MS vials.

All technical replicates were dried in a vacuum concentrator (Savant SPD2010 SpeedVac concentrator, Thermo Electron Corporation) until completely dry (approximately 1-2 hours), and the dried samples were stored at -80°C for further sample preparation and analysis.

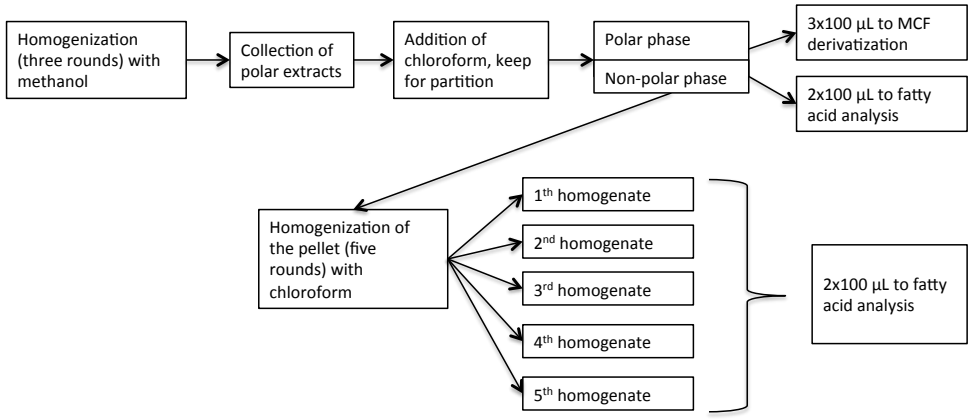


Figure 2.4: *Flow chart over the 2nd evaluation of the non-polar metabolite extraction.*

Table 2.4: *Samples used in the 2nd evaluation of the non-polar metabolite extraction. The tumor identification, given by the MR cancer group at the Department of Circulation and Medical Imaging at St. Olavs hospital in Trondheim, and the sample weights are given.*

Sample name	Tumor identification	Weight [mg]
280212-1	21.11.08 MAS98.12 2-1V	28.1
280212-2		39.8

2.3 Method development for extracting polar and non-polar metabolites from invasive ductal carcinoma xenografts suitable for mass spectrometry metabolite analysis

The optimal extraction method was developed by in collaboration with Ine Pedersen in March 2012, based on the optimization experiments (section 2.2). The protocol (see Figure 2.5 for a simplified experimental outline) is given as a point-by-point description for a perspicuous outline:

1. Cut and weigh tumor samples to obtain tissue samples of 30 ± 10 mg. Keep on ice.
2. Place tissue samples in precooled (−20°C) 2 mL Cryo tubes containing 0.50 g of ceramic (zirconium oxide) beads with a diameter of 1.4 mm.

   Part 1 - Extraction of polar metabolites
3. Add 400 μL precooled (−20°C) 60% methanol and 10 μL 10 mM internal standard (for example d_3-alanine).
4. Homogenize tissue samples for three intervals of 20 sec at 5,500 rpm using a Precellys 24 tissue homogenizer equipped with a Cryolys cooling unit (Bertin Technologies). Set the homogenizer to pause for 30 sec in between intervals.
5. Centrifuge the samples for 5 min at 5,000 rpm and −9°C using an Eppendorf 5804R centrifuge.
6. Remove and store polar extracts in tubes until later.
7. Repeat steps 3-6 twice, but use different internal standards (for example d_4-succinate and d_8-valine in the second and third homogenization round, respectively).
8. Pool the polar extracts from all three homogenization rounds and mix on a whirlmixer (≈ 10 sec) to ensure homogeneous solutions.
9. Make technical replicates of 100 μL in PP tubes (5 mL) for MCF derivatization.

   Part 2 - Extraction of non-polar metabolites
10. Add 800 μL precooled (−20°C) chloroform and 10 μL 10 mM internal standard (for example d_{31}-palmitic acid) to the Cryo tubes containing beads and cell pellets.

11. Homogenize tissue samples for three intervals of 20 sec at 6,500 rpm using a Precellys 24 tissue homogenizer equipped with a Cryolys cooling unit (Bertin Technologies). Set the homogenizer to pause for 30 sec in between intervals.
12. Centrifuge the samples for 5 min at 5,000 rpm and $-9°C$ using an Eppendorf 5804R centrifuge.
13. Remove and store non-polar extracts in tubes until later.
14. Repeat steps 10-13 twice, but use different internal standards (for example d_{35}-stearic acid in the second homogenization round).
15. Pool the non-polar extracts from all three homogenization rounds and mix on a whirlmixer ($\approx$ 10 sec) to ensure homogeneous solutions.
16. Make technical replicates of 100 μL in GC-MS vials for fatty acid derivatization to FAME.

Part 3 - Derivatization and GC-MS analysis

17. Dry the technical replicates in a vacuum concentrator (SpeedVac) until completely dry (approximately 1-2 hours).
18. Store dried samples at $-80°C$ for further sample preparation and analysis by analytical tools. Polar metabolites can be analyzed by MCF GC-MS, and non-polar metabolites can be analyzed as fatty acids methyl esters (FAMEs) by GC-MS.

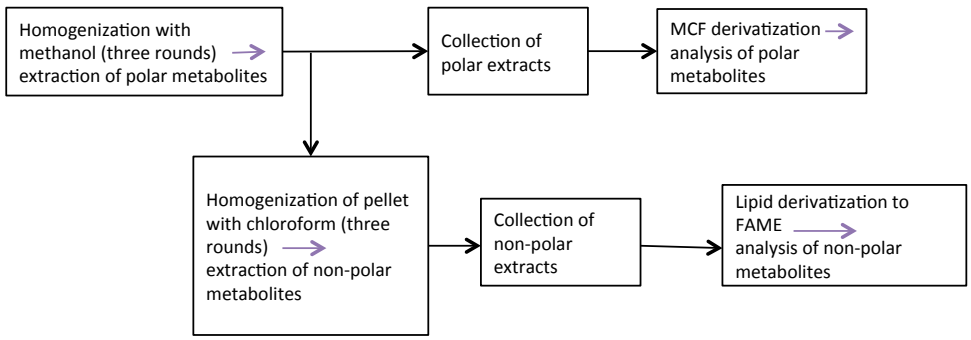


Figure 2.5: *Simplified schematic outline of the optimal method for extracting both polar and non-polar metabolites from breast cancer xenografts.*

2.4 Metabolite profiling of untreated and treated breast cancer xenografts

Metabolite profiling experiments were performed with the optimal extraction method (section 2.3), to obtain metabolite profiles of luminal-like, basal-like and MK-2206 treated basal-like xenografts (MAS98.06, MAS98.12 and MK-2206 treated MAS98.12, respectively) (see Figure 1.14 in section 1.6 for an overview of the experimental set up). Heavy labelled d_3-alanine, d_4-succinate and d_8-valine were used as internal standards (10 μL 10 mM) in the extraction of polar metabolites.

2.4.1 Evaluation of standard deviations for the polar metabolites

Three tissue samples of both the luminal-like (MAS98.06) and basal-like (MAS98.12) xenografts were extracted (Table 2.5), to evaluate the standard deviations for polar metabolites in tissue samples of the same xenografts. Non-polar metabolite extraction was not performed. Three technical replicates were made of each of the polar extracts, which were analyzed absolute quantitative by MCF GC-QqQ-MS.

Table 2.5: *Samples used in the evaluation of standard deviations for the polar metabolites contained in the MAS98.06 (Lum) and MAS98.12 (Bas) xenografts. The tumor identification, given by the MR cancer group at the Department of Circulation and Medical Imaging at St. Olavs hospital in Trondheim, and the sample weights are given.*

Sample name	Tumor identification	Weight [mg]
140312-Lum1	07.11.08 MAS98.06 6-2V	22.8
140312-Lum2		28.1
140312-Lum3		25.7
140312-Bas1	21.11.08 MAS98.12 2-1V	28.1
140312-Bas2		24.8
140312-Bas3		33.4

2.4.2 Extraction of basal-like and luminal-like breast cancer xenografts

Six tissue samples of both luminal-like (MAS98.06) and basal-like (MAS98.12) xenografts were extracted (Table 2.6), in order to evaluate and compare metabolite profiles of the two xenografts based on six biological replicates. One technical replicate was made of each of the polar extracts, and the replicates were analyzed absolute quantitative by MCF

GC-QqQ-MS. The non-polar extracts were stored at −80°C, and only the extract of the sample 270312-Lum6 was analyzed by FIA MS with a Q-TOF LC-MS system.

Table 2.6: *Samples of the MAS98.12 (Bas) and MAS98.06 (Lum) xenografts extracted to obtain metabolite profiles. The tumor identification, given by the MR cancer group at the Department of Circulation and Medical Imaging at St. Olavs hospital in Trondheim, and the sample weights are given.*

Sample name	Tumor identification	Weight [mg]
270312-Bas1	18.11.08 MAS98.12 2-3H	23.1
270312-Bas2	18.11.08 MAS98.12 2-2H	35.3
270312-Bas3	18.11.08 MAS98.12 2-2V	32.7
270312-Bas4	21.11.08 MAS98.12 2-4H	38.4
270312-Bas5	21.11.08 MAS98.12 2-5V	33.8
270312-Bas6	17.11.08 MAS98.12 1-3V	28.1
270312-Lum1	04.11.08 MAS98.06 4-1V	32.2
270312-Lum2	07.11.08 MAS98.06 6-2H	32.4
270312-Lum3	07.11.08 MAS98.06 5-4H	32.4
270312-Lum4	07.11.08 MAS98.06 6-1V	24.2
270312-Lum5	07.11.08 MAS98.06 6-2V	30.5
270312-Lum6	03.02.10 MAS98.06 LA11u H1 EMH	29.0

2.4.3 Extraction of MK-2206 treated basal-like breast cancer xenografts

Twelve tissue samples of MK-2206 treated basal-like (MAS98.12) xenografts were extracted (Table 2.7), in order to evaluate and compare metabolite profiles of untreated and treated MAS98.12 xenografts. One technical replicate was made of each of the polar extracts, and the replicates were analyzed absolute quantitative by MCF GC-QqQ-MS. The non-polar extracts were stored at −80°C, but not analyzed.

Table 2.7: *Samples of MK-2206 treated MAS98.12 (BasT) xenografts extracted to obtain metabolite profiles. The tumor identification, given by the MR cancer group at the Department of Circulation and Medical Imaging at St. Olavs hospital in Trondheim, and the sample weights are given.*

Sample name	Tumor identification	Weight [mg]
200312-BasT1	Basal 4-5V MK2206	25.8
200312-BasT2		32.5
200312-BasT3	Basal 5-1H MK2206	29.8
200312-BasT4		25.7
200312-BasT5	Basal 5-2H MK2206	27.0
200312-BasT6		36.6
200312-BasT7	Basal 4-2H MK2206	24.7
200312-BasT8		32.5
200312-BasT9	Basal 4-3V MK2206	27.0
200312-BasT10		23.6
200312-BasT11	Basal 4-1V MK2206 29.11.10	34.0
200312-BasT12		36.1

2.5 Polar metabolite analysis by MCF derivatization for a GC-QqQ-MS system

2.5.1 Quantitative metabolite analysis

Dried polar extracts, obtained in the second evaluation of the polar metabolite extraction and the second evaluation of the non-polar metabolite extraction (section 2.2.2 and section 2.2.4, respectively), were derivatized with MCF prior to quantitative analysis with a GC-QqQ-MS system.

Preparation of STD-curve samples A dilution series of a MCF standard-mix (STD-mix, 1.408 mM) (see table A.1 in Appendix A for compound list) with four dilutions (1:1, 1:3, 1:10 and 1:100) was made to make the four points in a standard curve, needed for quantification. All dilutions were made by diluting the wanted volume of the MCF STD-mix with 1 M sodium hydroxide (NaOH), in order to make the following concentrations; 0.4693 mM, 0.1408 mM and 0.01408 mM for the 1:3, 1:10 and 1:100 dilutions, respectively.

After preparation of the STD-curve samples, 100 μL of the 1:1, 1:3, 1:10 and 1:100 dilutions was added to four different PP tubes (5 mL) together with 333 μL methanol and 67 μL pyridine. The remaining derivatization protocol involved a MCF derivatization method developed by Villas-Bôas and co-workers [45]. After addition of methanol and pyridine, the solutions were mixed on a whirlmixer for 5 sec. The reactions were started by the addition of 80 μL MCF, and the solutions were mixed for 60 sec. Immediately after 60 sec of mixing, 400 μL chloroform was added to separate the MCF derivatives from the reactive solution. After addition of chloroform, the solutions were mixed for 10 sec followed by the addition of 400 of a μL 50 mM sodium bicarbonate ($NaHCO_3$) solution and subsequent mixing for 10 sec. The lower (chloroform) phases were transferred into new clean PP tubes, and the chloroform phases were dried by adding 3-4 spatula spoons of anhydrous sodium sulphate (Na_2SO_4). The chloroform phases were thereafter mixed for 5 sec. The water-free chloroform phases were transferred to four GC-MS vials with inserts, and the derivatized STD-curve samples were injected into the GC-QqQ-MS system.

MCF derivatization of dried samples Dried polar extracts were dissolved in 10 μL 10 mM heavy labelled d_5-glutamate, an internal derivatization standard, and 390 μL 1 M NaOH by vortexing. The remaining derivatization was conducted as for the derivatization of the STD-curve samples in the previous section.

2.5.2 Absolute quantitative metabolite analysis

To obtain a absolute quantitative metabolite analysis of the dried polar extracts not analyzed quantitative, a deuterized internal standard (ISTD) was prepared to spike the STD-curve and derivatized samples as developed by Kvitvang *et al.* [36]. Derivatization of STD-curve and dried samples were conducted as described in section 2.5.1. Prior to spiking, the water-free chloroform phases of the STD-curve samples and derivatized samples were transferred and stored in GC-MS vials without inserts.

Preparation of ISTD for spiking of STD-curve samples and dried samples The ISTD was prepared by transferring 100 μL of the 1:3 diluted MCF-STD mix, together with 300 μL 1 M NaOH, 333 μL deuterized methanol (d_4-MeOH), 67 μL pyridine and 80 μL deuterized MCF (d_3-MCF), to four different PP tubes (5 mL). The remaining preparation was conducted as the MCF derivatization described in section 2.5.1, but all four chloroform phases were pooled in the same PP tube before drying with Na_2SO_4.

Spiking of STD-curve samples and derivatized samples with ISTD The STD-curve samples and the derivatized samples were spiked with ISTD, to obtain absolute quantitative analysis. The spiking of the STD-curve samples was performed by transferring 30 μL of the ISTD to four GC-MS vials, before 170 μL of each STD-curve sample was added (creating a total volume of 200 μL in each GC-MS vial). The spiked STD-sample solutions were mixed carefully with a pipette, prior injection into the GC-QqQ-MS system. The spiking of the derivatized samples was performed as the spiking of the STD-curve samples.

2.5.3 GC-QqQ-MS instrumentation and data handling

Both absolute quantitative and quantitative analysis of polar extracts derivatized with MCF were performed with an Agilent 7890A GC system coupled to an Agilent 7000 GC/MS Triple Quad (Agilent Technologies). d_5-glutamate was used for retention time locking, which enables retention time correction as columns are cut during maintenance operations. A J&W Scientific capillary column (30 m x 250 μm x 0.25 μm with 10 m dura guard, Agilent 122-5532G) was used. 1 μL derivatized sample was injected in pulsed splittless mode with a GC PAL CTC Analytics injection system, where the injector temperature was set to 290°C. The metabolites were separated with a 20°C $(\text{min})^{-1}$ temperature gradient in the GC oven from 40°C (3 min hold time) to 320°C (3 min hold time), creating a run time of 23 min. Methane reagent gas was used for positive chemical ionization. The MS was operated in MRM mode, with 19 cycles $(\text{sec})^{-1}$ for the absolute quantification and 78 cycles $(\text{sec})^{-1}$ for the quantification.

Agilent Mass Hunter Quantitative Analysis for QqQ (version B.04.00/Build 4.0.225.0, Agilent Technologies) was utilized to identify metabolites from a library of 75 metabolites.

2.6 Polar metabolite analysis by TMS derivatization for a GC-Q-MS system

2.6.1 TMS derivatization protocol

A modified version of the TMS derivatization (silylation) procedure developed by Fiehn *et al.* was used [46]. Dried polar extracts obtained in the first evaluation of the non-polar metabolite extraction (section 2.2.3), were dissolved in 20 μL of a 4% methoxyamine HCl solution in pyridine, and the samples were then shaken carefully for 90 min at 30°C.

After shaking, 20 μL of each solution was transferred to new GC-MS vials, and 180 μL of a solution with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and 1 % trimethylchlorosilane (TMCS) was added. The samples were shaken carefully for 30 min at 37°C. The derivatized samples were transferred to inserts, and the inserts were placed back into the vials after cooling to room temperature ($\approx$ 10 min).

Three standards, d_{27}-myristic acid, FAME and a standard mix (10 mM of fructose, glucose, lactose, maltose and raffinose in equal volumes to a final concentration of 2 mM), were prepared and derivatized in the same way as the polar extracts, but with varying volumes of the reagents (Table 2.8).

Table 2.8: *An overview over the different samples and solutions derivatized with silylation. The volumes are given in μL.*

	Dried polar extracts	**Blank sample**	**d_{27}-myristic acid**	**FAME**	**Standard mix**
d_{27}-myristic acid	25	-	25	25	-
Standard mix	-	-	-	-	75
4% methoxyamine HCl in pyridine	20	50	50	50	100
Volume transferred to new vials after 90 min shaking at 30°C	20	20	20	20	50
MSTFA + 1% TMCS	180	180	180	150	180

2.6.2 GC-Q-MS instrumentation and data handling

The analysis of polar extracts derivatized with TMS was performed with an Agilent 7890A GC system coupled to an Agilent 5975 inert Mass Selective Ion/quadrupole mass spectrometer (Agilent Technologies). d_{27}-myristic acid was used for retention time locking, which enables retention time correction as columns are cut during maintenance operations. A J&W Scientific capillary column (30 m x 250 μm x 0.25 μm Agilent 122-5532G DB-5MS+DG) was used. 1 μL sample was injected in splitt mode (1:5) with a GC PAL CTC Analytics injection system, where the injector temperature was set to 250°C. The metabolites were separated with a 10°C $(\text{min})^{-1}$ temperature gradient in the GC oven from 60°C (1 min hold time) to 325°C (10 min hold time), creating a run time of 37.5 min. EI was used as the ion source operating at 70 eV. The data acquisition method was run in constant pressure mode with an operating pressure of 0.4461 bar. The MS

was operated in scan mode (start after 5.9 min, mass range 50-600 a.m.u. at 2.66 scans $(\text{sec})^{-1}$).

An automated mass spectra deconvolution and identification system (AMDIS, version 2.65) was utilized to identify metabolites accurately, by deconvoluting overlapping chromatographic peaks obtained by the GC-Q-MS [47]. Together with AMDIS, ChemStation (MSD ChemStation E.02.00.493, Agilent Technologies) was used to identify metabolites from an assembled library of about 1,000 metabolites.

2.7 Non-polar metabolite analysis as fatty acid methyl esters for a GC-Q-MS system

Non-polar extracts containing lipids were analyzed as fatty acid methyl esters (FAMEs) by a GC-Q-MS system. To convert the lipids to FAMEs, a derivatization protocol was adapted from Christie, but the protocol was modified [41].

2.7.1 Derivatization of lipids to FAMEs

Dried non-polar extracts were dissolved in 100 μL methanol in 1.25 M hydrogen chloride (MeOH/1.25 M HCl), and 10 μL 10 mM d_{27}-myristic acid was added as an internal standard to each GC-MS vial. The solutions were mixed on a whirlmixer and set to react on a heating block at 50°C for 16-24 hours. After 16-24 hours, the solutions were transferred to glas tubes, and 100 μL of MQ-grade water was added to each solution followed by the addition of 300 μL hexane. The solutions were vortexed on a whirlmixer for 10 sec to ensure proper mixing. The upper (hexane) phases were transferred to new glas tubes, and 2-3 spatula spoons of Na_2SO_4 were added to dry the hexane phases. The water-free hexane phases were transferred to GC-MS vials with inserts, prior to injection and analysis by the GC-Q-MS system.

A blank sample consisting of MeOH/1.25 M HCl, a sample with d_{27}-myristic acid and a FAME standard (see Table A.2 in Appendix A for compound list) were also prepared and injected into the GC-Q-MS system without further preparation.

2.7.2 GC-Q-MS instrumentation and data handling

The analysis of FAMEs was performed with an Agilent 7890A GC system coupled to an Agilent 5975 inert Mass Selective Ion/quadrupole mass spectrometer (Agilent Technologies). d_{27}-myristic acid was used for retention time locking, which enables retention time correction as columns are cut during maintenance operations. A J&W Scientific capillary column (30 m x 250 μm x 0.25 μm, Agilent 122-5532G DB-5MS+DG) was used. 2 μL sample was injected in splitt mode (1:5) with a GC PAL CTC Analytics injection system, where the injector temperature was set to 290°C. The metabolites were separated with a 10°C $(\text{min})^{-1}$ temperature gradient in the GC oven from 45°C (2 min hold time) to 325°C (5 min hold time), creating a run time of 35 min. EI was used as the ion source operating at 70 eV. The data acquisition method was run in constant pressure mode with an operating pressure of 0.82563 bar. The MS was operated in scan mode (start after 6 min, mass range 50-550 a.m.u. at 1.5 scans $(\text{sec})^{-1}$).

An automated mass spectra deconvolution and identification system (AMDIS, version 2.65) was utilized to identify the FAMEs accurately, by deconvoluting overlapping chromatographic peaks obtained by the GC-Q-MS [47]. Together with AMDIS, ChemStation (MSD ChemStation E.02.00.493, Agilent Technologies) was used to identify the FAMEs. A quantitative Deconvolution Reporting Software (DRS)-method was created to make an assembled library of four FAMEs (methyl hexadecanoate, methyl octadecanoate, d_{27}-myristic acid and unknown), detected in the non-polar phases in the second evaluation of the lipid extraction (see Figure D.1 in Appendix D for the chromatogram).

2.8 Non-polar metabolite analysis as lipids for a Q-TOF LC-MS system

Qualitative analysis of non-polar metabolites, as lipids, was performed with one non-polar extract (270312-Lum6) obtained in the metabolite profiling experiments (section 2.4.2).

2.8.1 Sample preparation

An aliquot of the non-polar extract (500 μL) was transferred to a PP-tube (5 mL) and dried in a vacuum concentrator (Savant SPD2010 SpeedVac concentrator, Thermo Electron Corporation) until completely dry (approximately 1 hour). The dried extract was dissolved in 100 μL dichloromethane. The resulting lipid solution was transferred to

a LC-MS vial with insert, prior direct infusion (FIA MS) and analysis by the Q-TOF LC-MS system. A vial with dichloromethane was also injected into the Q-TOF LC-MS system to serve as a blank sample.

2.8.2 Q-TOF LC-MS instrumentation and data handling

The lipid analysis was performed with an Agilent 1200 Series coupled to an Agilent 6510 Q-TOF LC-MS (Agilent Technologies). The Q-TOF LC-MS system was equipped with a dual ESI ion source operating at 325°C in positive mode. The lipid solution (10 μL) was injected by direct infusion at a flow rate of 0.2 mL $(\text{min})^{-1}$, and the run time was set to 2 min. 100% methanol was used as the mobile phase and the fragmentor was operating at 100 V. Ion fragments were detected in the mass range 100-1 500 a.m.u. at 1.03 spectra $(\text{sec})^{-1}$. Two reference masses were used (121.050873 m/z and 922.009798 m/z).

Agilent Mass Hunter Qualitative Analysis for QqQ (version B.04.00, Agilent Technologies) was utilized to analyze and identify non-polar metabolites (lipids).

2.9 Statistical analysis

2.9.1 Standard deviation

Reproducibility is evaluated in terms of standard deviation. Standard deviations (SD) (Equation (2.1)) and relative standard deviations (RSD) (Equation (2.2)) were calculated for the detected metabolites in each experiment, based on the sample standard deviation formula.

$$SD = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2} \tag{2.1}$$

$$RSD = \frac{SD}{\overline{x}} \cdot 100\% \tag{2.2}$$

2.9.2 Student‘s t-test

Student‘s t-test was applied to compare polar metabolite profiles of the luminal-like (MAS98.06) xenografts with the basal-like (MAS98.12) xenograft, as well as for the comparison of the polar metabolite profiles of the untreated MAS98.12 and the MK-treated

MAS98.12 xenografts. The Student‘s t-test calculations were made by using the built-in formula for student‘s t-testing in Excel (T.TEST). An two-tailed test was applied to each comparison, since information about the interactions between the xenografts was unknown. A Type 3 test was applied, since the variances were assumed to be unequal. Results from the student‘s t-testing indicate the probability of having observed t-values under the null hypothesis (the means of the metabolites in the xenografts are equal) by using the t-distribution. The significant levels were set to 5%. Thus, p-values < 0.05 mean significant differences between the xenografts based on their metabolites.

2.9.3 Principal component analysis

The interpretation of the results from the metabolite profiling experiments was performed with principal component analysis (PCA), a multivariate analysis, using the Unscrambler software (version X10.1, CAMO software AS, Oslo, Norway). The data imported to Unscrambler were denominated as pM (mg tissue sample)$^{-1}$. The data were weighted by scaling with the inverse of the standard deviation, to prevent over-dominance and unduly influence of the model. Score, loading, influence and explained variance plots were generated.

3 Results and discussion

3.1 Optimization of the beads-based extraction method

Preliminary results by Madsen (not shown) have shown that extraction with a Precellys 24 (beads-based) homogenizer can be an optimal method, if equipped with a cooling unit [27]. It was therefore chosen to establish a metabolite extraction method with the Precellys 24 homogenizer integrated with a Cryolys cooling system, to homogenize tissue samples of a basal-like breast cancer model (MAS98.12 xenograft) (section 1.6).

3.1.1 Evaluation of the polar metabolite extraction

Evaluation of the polar metabolite extraction was performed in two steps. First, the number of homogenization rounds necessary for the complete extraction with 60% methanol as the extraction solvent, was evaluated. Second, to validate the number of necessary homogenization rounds further, tissue pellets were investigated for remaining polar metabolites after both polar and non-polar metabolite extraction in the homogenizer.

The 1^{st} evaluation of the polar metabolite extraction

The evaluation of the number of homogenization rounds necessary for complete extraction of polar metabolites was performed with 60% methanol, as the extraction solvent (see section 2.2.1 for the experimental procedure). Three tissue samples were exposed to three homogenization rounds, and the three resulting polar extracts of each sample were analyzed absolute quantitative by MCF GC-QqQ-MS.

When comparing the total concentration of the polar metabolites after three homogenization rounds, the majority of metabolites are extracted by more than 95% after three homogenization rounds (Figure 3.1). The metabolites not shown in Figure 3.1 are extracted by less than 95% of the total concentration. These metabolites showed low and varying concentrations, and such low-abundant metabolites can be near the lowest detectable level of the GC-QqQ-MS system. These metabolites are assumed not to be valid biomarkers, and they are therefore not included in the evaluation. Since the majority of the metabolites are extracted by more than 95% after three homogenization rounds, three homogenization rounds with 60% methanol are considered necessary for the complete extraction of the polar metabolites.

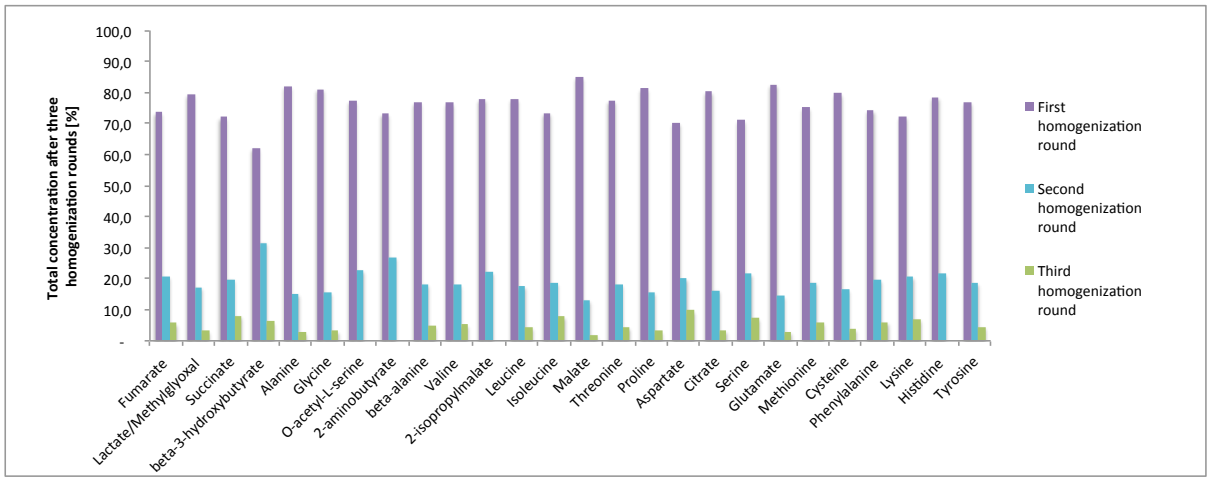


Figure 3.1: *Evaluation of the polar metabolite extraction to find the necessary number of homogenization rounds. Polar metabolites extracted by more than 95% of the total concentration after three homogenization rounds are given, where metabolites extracted by less than 95% are excluded from the figure.*

In addition to the excellent extraction results, the extraction of polar metabolites using the beads-based homogenizer was both rapid and simple. An important aspect with the procedure was the use of the same Cryo tube in all three homogenization rounds and in the centrifugations, therefore preventing loss of samples. In addition, the low temperatures in both the homogenizer and the centrifuge are assumed to prevent degradation reactions.

The 2^{nd} evaluation of the polar metabolite extraction

In the second attempt to optimize the polar metabolite extraction, the aim was to investigate the pellets after both the polar and the non-polar metabolite extraction for remaining polar metabolites. There are significantly less polar metabolites present after both extractions than before the non-polar metabolite extraction (Table C.4 in Appendix C). The majority of the polar metabolites found before the non-polar metabolite extraction are in fact not found after the non-polar extraction. It was therefore concluded once again that three homogenization rounds give complete extraction of polar metabolites.

3.1.2 Evaluation of the non-polar metabolite extraction

Due to the excellent results from the extraction of polar metabolites with the beads-based homogenizer (section 3.1.1), it was decided to evaluate the extraction of non-polar

metabolites in a separate step in the homogenizer, after the polar metabolite extraction. Chloroform was chosen as the extraction solvent, since it has the necessary chemical and physical properties to extract the non-polar metabolites. Fatty acid methyl esters (FAMEs) were assumed to be representative of lipids. Evaluation of the non-polar metabolite (lipid) extraction was performed in two steps. In the first evaluation, chloroform was added to the tissue pellets after the extraction of polar metabolites, to evaluate the number of necessary homogenization rounds for the complete extraction. In the second evaluation, two extraction methods was investigated. First, an extraction method based on the classical extraction by Folch *et al.* was evaluated [48]. This classical method was tested, since it is the most frequently used method for the simultaneous extraction of both polar and non-polar metabolites. Second, further evaluation of the extraction in the Precellys 24 homogenizer, providing separate extraction of polar and non-polar metabolites, was performed.

The 1^{st} evaluation of the non-polar metabolite extraction

In the first attempt to optimize the lipid extraction, the aim was to find the necessary number of homogenization rounds for the complete extraction of lipids. Chloroform was added to the tissue pellets present after the polar metabolite extraction, as a lipid extraction solvent in equal volume as methanol. The tissue pellets were exposed to five homogenization rounds at 6,500 rpm for the extraction of lipids. The bulk of cellular lipids are localized in cell membranes [33]. The homogenization intensity (rpm) was therefore increased compared to the polar metabolite homogenization (6,500 rpm compared to 5,500 rpm), to ensure that the tissues and cells were completely broken down. The approximately responses for the detected FAMEs in the non-polar extracts vary in all five homogenization rounds (Table B.7 in Appendix B). The responses were not gradually reduced, and the extraction of lipids was therefore not considered complete. This variation in response can be caused by a saturated chloroform-phase. To accomplish a complete lipid extraction, the use of a larger volume of chloroform was therefore decided to be tested.

The 2^{nd} evaluation of the non-polar metabolite extraction

In the second evaluation of the non-polar metabolite extraction, both a simultaneous and a separate extraction of polar and non-polar metabolites were tested.

1. Simultaneous extraction of polar and non-polar metabolites

   An extraction method based on the classical extraction by Folch *et al.* was evaluated, where polar and non-polar metabolites are extracted simultaneously [48]. After the extraction of polar metabolites with 60% methanol, chloroform was added to the extracts and the resulting solutions were kept for partition and then centrifuged. After centrifugation, a two-phase (biphasic) system was observed, with an upper polar phase and a lower non-polar phase. The polar phases were analyzed quantitative by MCF GC-QqQ-MS (see Table C.5 in Appendix C for the results), while the non-polar phases were analyzed quantitative by FAME GC-Q-MS (data not shown). There were no FAMEs detected in the non-polar phases, as opposite to the satisfying results for the polar metabolites. The apparently explanation for the lack of FAMEs in the non-polar phases is the use of 60% methanol as the extraction solvent in the three homogenization rounds, before addition of chloroform. The non-polar metabolites have a poor solubility in 60% methanol, and they will therefore remain in the tissue pellets (consisting of tissue parts and cell debris) after the homogenization rounds. The need for a separate extraction of non-polar metabolites in the homogenizer, after extraction of polar metabolites, is therefore confirmed.

2. Separate extraction of polar and non-polar metabolites

   The first paramount objective in this master thesis was to find an optimal method for the complete extraction of both polar and non-polar metabolites, consequently the extraction of all metabolites in tissue samples of the MAS98.12 xenograft (section 1.6). Due to the above mentioned results, it was decided to evaluate the extraction of the non-polar metabolites in a separate step in the homogenizer, after the polar metabolite extraction, further. The aim was therefore to find the necessary number of homogenization rounds when altering the volume of chloroform, due to the results from the first evaluation of the non-polar metabolite extraction. The volume of chloroform was doubled compared to the volume of methanol, giving 2:1 chloroform/methanol ratio [48], and the non-polar extracts were analyzed quantitative by FAME GC-Q-MS. When comparing the total amounts (mg tissue

sample)$^{-1}$) of FAMEs after five homogenization rounds, the three FAMEs are considered completely extracted from the tissue samples after three rounds since more than 95% of the FAMEs are extracted (Figure 3.2).

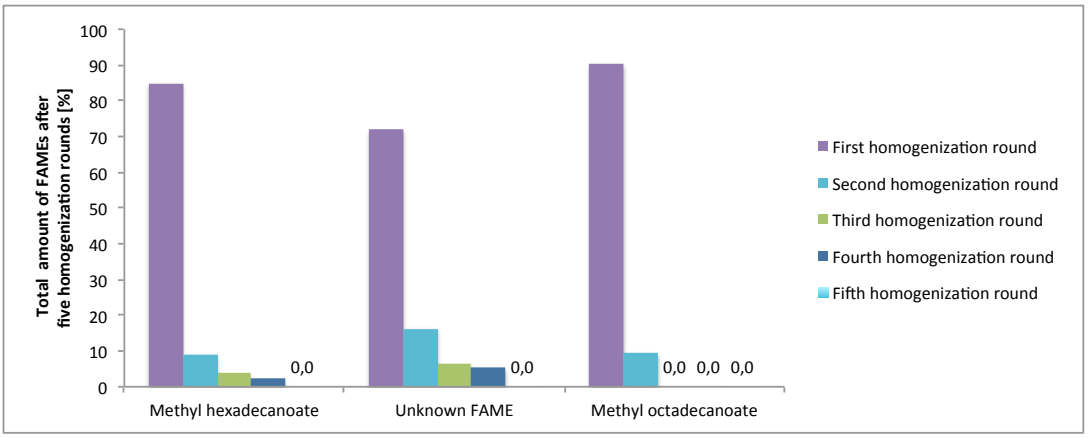


Figure 3.2: *The 3rd evaluation of the non-polar metabolite extraction, where a separate extraction of polar and non-polar metabolites was evaluated. More than 95% of the three FAMEs are extracted after three homogenization rounds. There are no FAMEs observed in the non-polar extracts obtained after the fifth homogenization round.*

Comments to the 2nd evaluation of the non-polar metabolite extraction

As compared with the results from the first evaluation of the lipid extraction, less FAMEs were detected in the second evaluation. The lost FAMEs are assumed to be FAMEs in very low concentrations, perhaps FAMEs with concentrations near the lowest detectable level of the GC-Q-MS system. It could therefore be beneficial to concentrate the non-polar extracts further, either in the drying process in the vacuum concentrator and/or in the preparation of the FAMEs (section 2.7.1), to see if the number of FAMEs increases. However, the aim was to find the number of homogenization rounds necessary for the complete extraction of FAMEs, and three homogenization rounds are concluded necessary based on the complete extraction of the three FAMEs. The low-abundant FAMEs were therefore not included in the quantitative DRS-method (section 2.7.2). The chromatogram showing the four FAMEs included in the DRS-method is given in Figure D.1 in Appendix D. An unknown peak assumed to be a FAME was detected in the analysis, but its identity was not found (see Figure D.2 in Appendix D for the scan of the unknown FAME).

An important aspect to consider is the possible presence of polar and amphipathic lipids in the tissue samples. Glycolipids and phospholipids are localized in cell membranes (section 1.4.2) and have amphipathic nature. Such lipids will have a slight affinity towards 60%

methanol, and they may therefore be found in the polar extracts. The polar extracts should therefore be analyzed for polar and amphipathic lipids. A loss of polar and amphipathic lipids into the polar extracts are therefore probable, but hopefully negligible compared to the total lipid concentration.

3.1.3 Comparison of MCF derivatization and TMS derivatization

TMS derivatization was adapted from Fiehn *et al.* [46], but had to be modified for the polar extracts obtained from the MAS98.12 xenografts. Originally, 50 μL 4% methoxyamine HCl in pyridine is supposed to be added to dried sample extracts. However, after performing a TMS derivatization with 50 μL 4% methoxyamine HCl in pyridine on five technical replicates obtained in the first evaluation of the lipid extraction, the resulting chromatogram showed no responses (data not shown). This was also seen in preliminary research by Madsen (results not shown), where it was assumed that the polar metabolite concentrations were below the detection level of the GC-Q-MS system [27]. Therefore, the derivatization procedure was modified to increase the concentrations, by adding 20 μL 4% methoxyamine HCl in pyridine to the four remaining technical replicates. The resulting TMS derivatization was successful (see Table B.6 in Appendix B for the raw data and Table C.6 in Appendix C for the results).

Amino acids and organic acids are supposed to be derivatized with both TMS and MCF [35]. There are many more metabolites detected with MCF derivatization than with TMS derivatization, maximum 53 and 19 metabolites, respectively (Table 3.1). Some of the metabolites detected after MCF derivatization are, however, in low abundance. An explanation for the detection of low abundance metabolites derivatized with MCF can be the GC-QqQ-MS system, since such a triple quadrupole GC-MS is known to be more sensitive than a single quadrupole GC-MS (section 1.5.1) [35]. Such low abundance metabolites will probably not be relevant as biomarkers, but they are still included in the comparison of the TMS and the MCF derivatization (section 3.1.1).

Table 3.1: *Comparison of MCF derivatization and TMS derivatization for the polar metabolites in polar extracts obtained in the optimization experiments. The metabolites detected in each method is given by the symbol* $\surd$.

/Metabolite	MCF derivatization	TMS derivatization	/Metabolite	MCF derivatization	TMS derivatization
4-methylvalerate	√		Aspartate	√	
Malonate	√		Citrate	√	√
Pyruvate	√		5-aminovalerate	√	
3-methyl-oxovalerate	√		Anthralinate	√	
Fumarate	√	√	Serine	√	√
Lactate/Methylglyoxal	√	√	Allantoin	√	
Succinate	√		Glutamate	√	√
Citraconate/Itaconate	√		N-Acetyl-L-glutamate	√	
Benzoate	√		Methionine	√	
Glyoxylate	√		Hydroxyproline	√	
beta-3-hydroxybutyrate	√		Cysteine	√	
Alanine	√		Phenylalanine	√	
Glycine	√	√	Putrescine	√	
O-acetyl-L-serine	√		Hippurate	√	
Phenylacetate	√		4-imidazoleacrylate	√	
2-aminobutyrate	√		Histamine	√	
m-Toluate	√		Ornithine	√	
beta-alanine	√		Lysine	√	
Adipate	√		Histidine	√	
Salicylate	√		Tyrosine	√	
Alpha-ketoglutarate	√	√	Thryptophane	√	
Valine	√		Urea		√
2-isopropylmalate	√		Phosphoric acid		√
beta-hydroxypyruvate	√		Creatinine		√
alpha-ketoadipate	√		Glycerol 1-phosphate		√
Leucine	√		O-phosphocolamine		√
Isoleucine	√		Methyl-beta-D-galactopyranoside		√
Malate	√	√	D-glucose		√
Oxaloacetate	√		D-allose		√
Threonine	√		Maltose		√
Proline	√	√	Cholesterol		√
L-homoserine	√				

Nine metabolites are common to both derivatization methods, but the results of these metabolites are not completely comparable. The metabolites identified with TMS derivatization are not identified absolute quantitative as for the MCF derivatization. But, the number of metabolites are comparable, and it seems that the MCF derivatization, as the GC-QqQ-MS system, is more sensitive than the TMS derivatization. However, ten metabolites are unique for the TMS derivatization, and these can be exploited in further research as potentially important metabolites.

3.2 Summary of critical and important aspects with the optimal extraction method

From the results of the optimization experiments, an optimal method for extracting both polar and non-polar metabolites was developed (see section 2.3 for the protocol). This method combines mechanical and non-mechanical extraction methods in an elegant way. The method is rapid and simple, and an important aspect is the use of the same Cryo tube throughout the whole experimental procedure, which prevents the loss of sample extracts. The beads-based (Precellys 24) homogenizer is equipped with a cooling unit that uses liquid nitrogen to cool air, which then diffuses into the homogenizer. The temperature observed in the homogenizer was always below 0°C, which is a very important result. In addition, centrifugations were performed at −9°C. Due to the low temperatures throughout the experimental procedure, it is assumed that degradation reactions as a consequence of heat generation are prevented.

Complete extraction of polar and non-polar metabolites was achieved after three homogenization rounds with 60% methanol and chloroform, respectively. The non-polar metabolites are assumed to be completely extracted with the optimal method, since FAMEs (expected to represent the lipids) are completely extracted. A loss of polar and amphipathic lipids into the polar extracts are expected, but the loss is hopefully negligible (section 3.1.2). There will most certainly be losses of metabolites during the extractions, due to the high chemical diversity and high concentration range of all the metabolites [42]. Loss of metabolites is therefore inevitable, but expected to be constant and negligible compared to the total concentration. The extraction of polar metabolites is nevertheless considered complete and optimal, and polar metabolite profiles can therefore be elucidated without the risk for underestimation of the polar metabolite concentrations.

The optimal extraction method was developed with tissue samples of the MAS98.12 xenograft, but the method is assumed to be valid for xenografts of other breast cancer models, such as the MAS98.06 and MK-2206 treated MAS98.12 xenografts. The tissue samples used in the optimization experiments were in the range 30 ± 10 mg. This weight range was suitable for the available xenografts obtained from St. Olavs hospital, which ranged from 30 mg to 130 mg. To minimize the number of xenografts, the xenografts were cut in desirable sizes to satisfy the weight range. In this way, more xenografts than necessary were not used. This was also preferred since the number of available xenografts were limited. But, most importantly, the use of the same weight range for all experiments was suitable to obtain correct and similar normalization patterns against the tumor weights in the data handling. The normalization patterns are most certainly

not linear, making the normalization difficult for different tumor weights.

The reproducibility of an extraction method on breast cancer xenografts is crucial, especially since small changes in the metabolite concentrations reflect considerable changes in the cell metabolism [35]. The standard deviations of technical replicates for both the polar and non-polar metabolites are quite varying, ranging from 20-30% up to 50-70% (see Tables in Appendix C). Variation in sample preparation, involving quenching, extraction, concentration and derivatization, contributes to the observed standard deviations, together with variations in instrumental performance and biological materials. The tissue samples used to calculate the standard deviations for the polar metabolites, as for the non-polar metabolites, are from the same tumor. The tissue samples are therefore not biological replicates. Ideally, the metabolite concentrations would therefore be very similar if the tumors were homogeneous. In order to extract and analyze a representative piece of a tumor, the tumor should be cut as pizza-slices to obtain parts that represent all the biological properties present in the tumor. But, biological materials will most certainly always contribute to quite high standard deviations (see section 1.5.4). The developed method is therefore considered reproducible and qualified to be recognized as an optimal extraction method.

3.3 Polar metabolite profiling of breast cancer xenografts

Xenografts of two breast cancer models, luminal-like (MAS98.06) and basal-like (MAS98.12) breast cancer, were extracted with the optimal method (section 2.3) and analyzed absolute quantitative by MCF GC-QqQ-MS, to investigate similarities and differences in their polar metabolite content. A breast cancer model of the basal-like subtype treated with a cancer drug called MK-2206 was also analyzed, to evaluate the effects of the treatment compared to the untreated subtype.

3.3.1 Evaluation of standard deviations for the polar metabolites

Technical replicates should ideally be very similar, resulting in low standard deviations. The standard deviations for the polar metabolites found in three technical replicates of three tissue samples, from both the MAS98.06 and MAS98.12 xenografts, range from below 10% to over 90%, even with cutting tumors as pizza-slices (section 3.2) (see Table E.4, Table E.5 and Table E.6 in Appendix E). The evident varying standard deviations indicate that the polar extracts were heterogeneous, when pipetting out the aliquots to the technical replicates. After each homogenization round with methanol and subsequent

centrifugation, the polar extracts were pooled (section 2.3). The pooled extracts were vortexed before the aliquots were transferred to new PP tubes, to make the technical replicates. The extracts should perhaps be vortexed before transferring each aliquot, to obtain more homogeneous solutions.

The MAS98.12 xenograft seems to have a lower variability than the MAS98.06, meaning that the MAS98.12 xenograft can be considered more homogeneous. When comparing the standard deviations of glycine in both xenografts found in this work with the research by Moestue *et al.*, where intact tissues were analyzed by HR MAS MRS, the standard deviations are comparable [20]. This means that, even though the standard deviations are high, they can be considered representative of the biological variations in these tumor models. High standard deviations are frequently observed in metabolomics, due to the physiological challenges in the extraction of metabolites [35]. The metabolites contained in tumors show a high diversity, both in physical and chemical properties [9, 12]. According to Villas-Bôas *et al.*, the number of biological replicates are often preferable compared to technical replicates [42]. One could therefore extract and analyze tissue samples from several mice and tumors (biological replicates), instead of tissue samples from the same tumor (technical replicates). It was therefore concluded to analyze only one technical replicate in the subsequent experiments. This was preferred since the beads-based homogenizer used in this work, had a limitation in the number of samples that could be homogenized simultaneously. The homogenizer could homogenize a total of 12 samples, when the 2 mL Cryo tubes are used.

3.3.2 Polar metabolite profiles

The polar metabolite profiles of the MAS98.06 (luminal-like), the MAS98.12 (basal-like) and the MK-2206 treated MAS98.12 (treated basal-like) xenografts are given in Table 3.2. There are most metabolites extracted from the treated MAS98.12 xenograft (Table 3.2). 43 polar metabolites are detected in the treated MAS98.12 xenograft, while 35 and 34 polar metabolites are detected in the untreated MAS98.12 and MAS98.06 xenografts, respectively. The difference in the number of polar metabolites has no clear explanation. One possible explanation can be the differences in the biological properties of the xenografts, the treated MAS98.12 xenograft can be easier to homogenize and extract compared to the other two xenografts. Another explanation can be variations in the GC-QqQ-MS system. The extraction of the treated MAS98.12 xenograft was performed on a different day than the extraction of both the MAS98.06 and MAS98.12 xenografts. It is impossible to completely remove such instrumental variations, but one can limit the

variations by analyzing samples in a random order at the same day. Another possible explanation can be the d_3-MCF used in the derivatization of the ISTD (section 2.5.2). It was recognized after the metabolite profile experiments, that the d_3-MCF was out of date. When analyzing the results with Mass Hunter Quantitative Analysis for QqQ, it was seen that the chromatograms of the ISTD were unusual. Many of the peaks were not considered completely correct, but the results were still used due to the lack of time to get a new d_3-MCF. Finally, the MK-2206 drug used to treat the mice with the MAS98.12 xenograft model was dissolved in dimethyl sulfoxide (DMSO) and cyclodextrin [23]. The metabolites only detected in the treated MAS98.12 xenograft can perhaps be degradation products resulting from the use of DMSO and cyclodextrin. Nevertheless, the metabolites unique for the MK-2206 treated MAS98.12 xenograft (Table 3.2) are assumed to be low abundance metabolites. Their concentrations can be near the lowest detectable level in the GC-QqQ-MS system. The sensitivity of the analytical instrument is therefore the most probable reason for the difference in the number of polar metabolites. The results, hence the metabolite profiles, were assumed representative of the xenografts, but they should be considered temporary.

Table 3.2: *Metabolite profiles of the polar metabolites found in the MAS98.06 (luminal-like), the MAS98.12 (basal-like) and the MK-2206 treated MAS98.12 (treated basal-like) xenografts. The order of the metabolites are given in approximately decreasing concentrations. The data and standard deviation for each metabolite are given in pM (mg tissue sample)$^{-1}$. Relative standard deviations (RSD, %) are given in parentheses.*

Xenograft/Metabolite	Luminal-like	Basal-like	Treated basal-like
Lactate/Methylglyoxal	59,886 ± 23,501 (39.2%)	101,489 ± 29,027 (28.6%)	59,643 ± 19,573 (32.8%)
Glutamate	36,735 ± 11,160 (30.4%)	106,700 ± 27,821 (26.1%)	38,592 ± 13,231 (34.3%)
O-acetyl-L-serine	33,629 ± 6,752 (20.1%)	16,943 ± 6,573 (38.8%)	37,027 ± 19,236 (52.0%)
alpha-ketoglutarate	-	-	20,194 ±16,415 (81.3%)
Allantoin	32,652 ± 10,904 (33.4%)	72,088 ± 31,164 (43.2%)	20,305 ± 15,565 (76.7%)
Alanine	23,503 ± 5,227 (22.2%)	19,397 ± 6,351 (32.7%)	22,134 ± 10,650 (48.1%)
Glycine	17,326 ± 4,416 (25.5%)	60,867 ± 16,809 (27.6%)	65,596 ± 17,166 (26.2%)
Salicylate	12,742 ± 5,295 (41.6%)	11,574 ± 3,522 (30.4%)	1,410 ± 837 (59.3%)
Proline	15,265 ± 4,651 (30.5%)	17,547 ± 3,326 (19.0%)	6,813 ± 2,195 (32.2%)
Threonine	10,473 ± 2,561 (24.5%)	9,844 ± 3,023 (30.7%)	6,848 ± 1,802 (26.3%)
N-glycyl-L-proline	-	-	6,089 ± 2,301 (37.8%)
Glyoxylate	3,388 ± 1,438 (42.5%)	8,031 ± 5,105 (63.6%)	4,611 ± 1,698 (36.8%)
Aspartate	8,233 ± 3,270 (39.7%)	1,569 ± 571 (36.4%)	712 ± 531 (74.5%)
Citrate	6,628 ± 2,633 (39.7%)	15,829 ± 3,779 (23.9%)	3,566 ± 1,145 (32.1%)
Serine	4,347 ± 2,726 (62.7%)	3,633 ± 1,252 (34.5%)	1,940 ± 445 (22.9%)
Lysine	4,277 ± 1,581 (37.0%)	10,331 ± 2,906 (28.1%)	10,236 ± 3,315 (32.4%)
Valine	3,608 ± 1,341 (37.2%)	3,414 ± 870 (25.5%)	4,965 ± 1,238 (24.9%)
Oxaloacetate	-	-	4,502 ± 1,803 (40.0%)
N-Acetyl-L-glutamate	-	-	3,241 ± 1,410 (43.5%)
Tyrosine	2,293 ± 875 (38.2%)	2,673 ± 646 (24.2%)	2,684 ± 795 (29.6%)
Malate	1,819 ± 1,204 (66.2%)	3,298 ± 1,117 (33.9%)	3,120 ± 1,179 (37.8%)
Succinate	3,111 ± 774 (24.9%)	3,001 ± 492 (16.4%)	1,685 ± 533 (31.6%)
Pyruvate	1,457 ± 857 (58.8%)	2,503 ± 1,096 (43.8%)	2,206 ± 769 (34.8%)
Fumarate	1,197 ± 404 (33.7%)	2,154 ± 587 (27.3%)	1,488 ± 429 (28.8%)
Phenylalanine	1,630 ± 513 (31.5%)	1,584 ± 513 (32.4%)	2,147 ± 654 (30.5%)
Isoleucine	-	-	1,493 ± 402 (26.9%)
Citramalate	859 ± 272 (30.3%)	1,447 ± 386 (26.6%)	418 ± 127 (30.3%)
Methionine	1,453 ± 433 (29.8%)	1,296 ± 281(21.7%)	1,150 ± 379 (33.0%)
Hippurate	1,256 ± 352 (28.0%)	1,302 ± 285 (21.9%)	1,194 ± 368 (30.8%)
Ornithine	1,125 ± 686 (61.0%)	647 ± 306 (47.4%)	1,371 ± 601(43.9%)
Phenylacetate	977 ± 407 (41.6%)	1,042 ± 241 (23.1%)	1,058 ± 413 (39.0%)
Leucine	975 ± 344 (35.3%)	617 ± 292 (47.3%)	2,988 ± 936 (31.3%)
m-Toluate	569 ± 207 (36.4%)	550 ± 107 (19.5%)	133 ± 56 (42.0%)
beta-hydroxypyruvate	563 ± 172 (30.6%)	1,059 ± 370 (34.9%)	918 ± 242 (26.4%)
Benzoate	484 ± 143 (24.9%)	753 ± 185 (24.6%)	428 ± 147 (34.5%)
2-isopropylmalate	451 ± 118 (26.1%)	694 ± 126 (18.1%)	342 ± 125 (36.6%)
3-methyl-oxovalerate	260 ± 94 (36.3%)	643 ± 123 (19.1%)	952 ± 333 (34.9%)
Citraconate/Itaconate	-	540 ± 156 (29.0%)	681 ± 195 (28.7%)
5-aminovalerate	-	-	614 ± 246 (40.0%)
beta-3-hydroxybutyrate	-	-	269 ± 182 (67.9%)
4-methylvalerate	179 ± 53 (29.7%)	275 ± 69 (25.0%)	154 ± 56 (36.2%)
2-aminobutyrate	245 ± 80 (32.9%)	269 ± 67 (25.0%)	148 ± 40 (27.1%)
Adipate	-	-	121 ± 44 (36.2%)

3.3.3 Comparison of the polar metabolite concentrations in the polar metabolite profiles

The metabolites with their concentrations and standard deviations detected in polar extracts of the MAS98.06, MAS98.12 and MK-2206 treated MAS98.12 xenografts (luminal-like, basal-like and treated basal-like, respectively), are given in Table 3.2 in section 3.3.2. Student‘s t-tests are applied to compare the MAS98.06 and MAS98.12 xenografts, and the untreated and treated MAS98.12 xenografts (Table E.10 in Appendix E). ANOVA was not performed since this study aims to compare only groups in pair (section 1.5.4).

The significant levels of the Student's t-tests were set to 5%, and metabolites with p-values < 0.05 are therefore significant different. Metabolites with p-values between 5% and 10% can be considered baseline significant.

To compare the polar metabolite profiles, the ratio of the metabolites found in the xenografts to be compared are given in a $\log_2$-scale. Figure 3.3 and Figure 3.4 show the comparison of the MAS98.06 and MAS98.12 xenografts, and the comparison of the untreated and treated MAS98.12 xenografts, respectively. As for both figures, $\log_2 < 0$ means a higher metabolite concentration in the untreated MAS98.12 xenograft compared to the two other xenografts.

Figure 3.3 shows that the MAS98.06 xenograft has lower concentrations of most of the polar metabolites compared to the MAS98.12 xenograft. There are, however, some metabolites present in higher concentrations in the MAS98.06 xenograft, for example O-acetyl-L-serine, leucine, aspartate and ornithine. The difference in the concentrations of O-acetyl-L-serine and aspartate in the two xenografts is indeed significant (Table E.10 in Appendix E). Lactate, glycine and glutamate are examples of metabolites that are present in significant higher concentrations in the MAS98.12 xenograft, compared to the MAS98.06 xenograft.

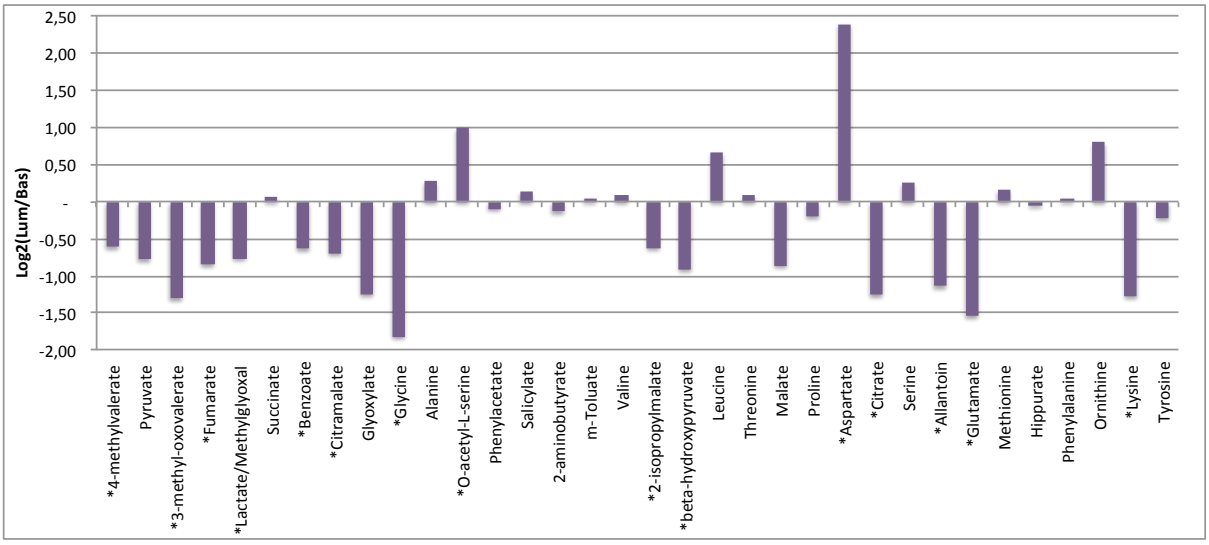


Figure 3.3: *The comparison of the MAS98.06 and the MAS98.12 xenografts, hence the luminal-like and the basal-like breast cancer, respectively. The data are given as the $\log_2$-ratio of the polar metabolites found in the MAS98.06 xenograft (Lum) compared to the polar metabolites found in the MAS98.12 xenograft (Bas). $\log_2 < 0$ means a higher concentration in the MAS98.12 xenograft compared to the MAS98.06 xenograft. Metabolites marked with * have significant different concentrations in the two xenografts. Citraconate/Itaconate is excluded from the figure, since it was only found in the MAS98.12 xenograft.*

Figure 3.4 shows that the treated MAS98.12 xenograft has lower concentrations of most of the polar metabolites compared to the untreated MAS98.12 xenograft. But, some metabolites are present in higher concentrations in the treated xenograft, for example 3-methyl-oxovalerate, O-acetyl-L-serine, valine, leucine and ornithine. The difference in concentrations of these five metabolites is significant (Table E.10 in Appendix E). The untreated MAS98.12 xenograft has a significant lower concentration of lactate than the MK-2206 treated xenograft.

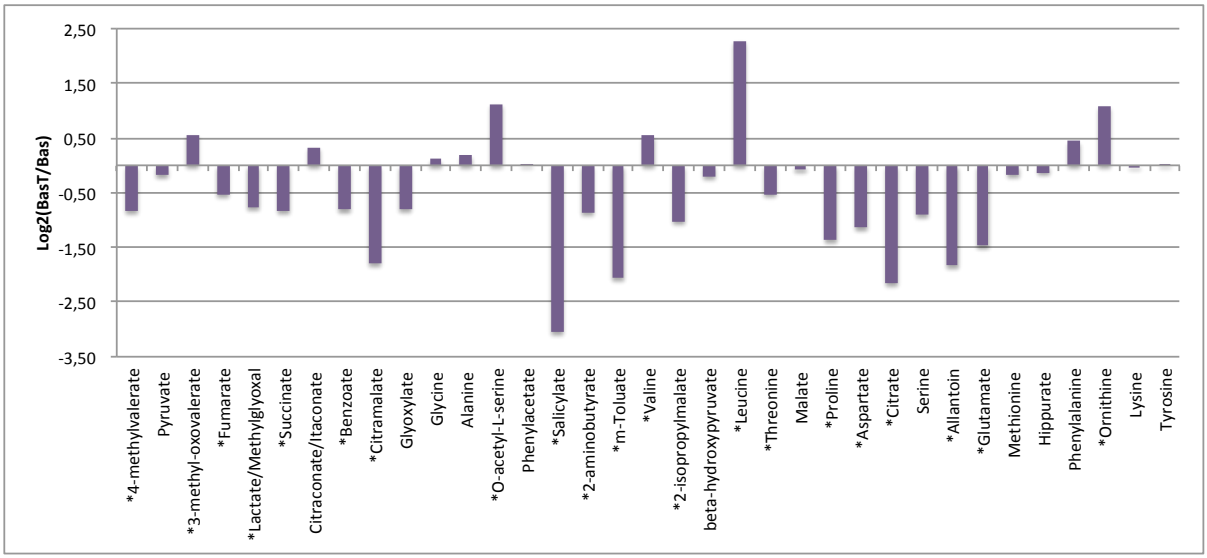


Figure 3.4: *The comparison of the untreated and the MK-2206 treated MAS98.12 xenografts, hence the basal-like and the treated basal-like breast cancer, respectively. The data are given as the log_2-ratio of the polar metabolites found in the treated MAS98.12 xenograft (BasT) compared to the polar metabolites found in the untreated MAS98.12 xenograft (Bas). $log_2 < 0$ means a higher concentration in the untreated MAS98.12 xenograft compared to the treated MAS98.12 xenograft. Metabolites marked with * have significant different concentrations in the two xenografts. The untreated MAS98.12 xenograft has a significant lower concentration of lactate than the MK-2206 treated xenograft. Metabolites only found in one xenograft are excluded from the figure.*

3.3.4 PCA of polar metabolite profiles of breast cancer xenografts

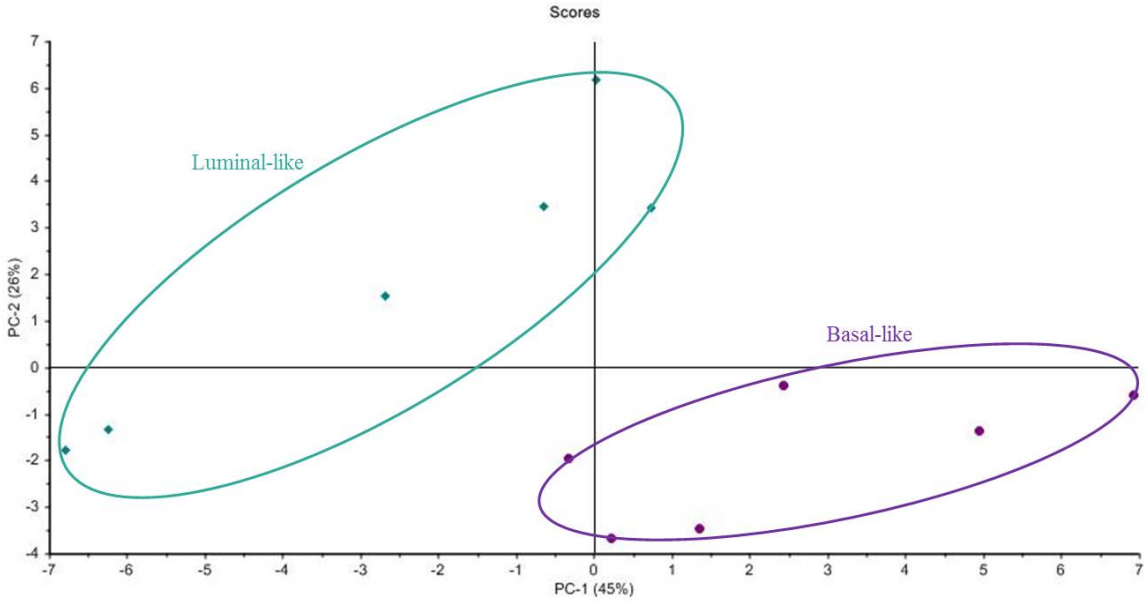
The polar metabolite profiles of the MAS98.06, MAS98.12 and MK-2206 treated MAS98.12 xenografts were compared by PCA, by using the Unscrambler software. The data imported into Unscrambler were weighted (see Figure E.1 and Figure E.3 in Appendix E).

Comparison of luminal-like and basal-like breast cancer xenografts

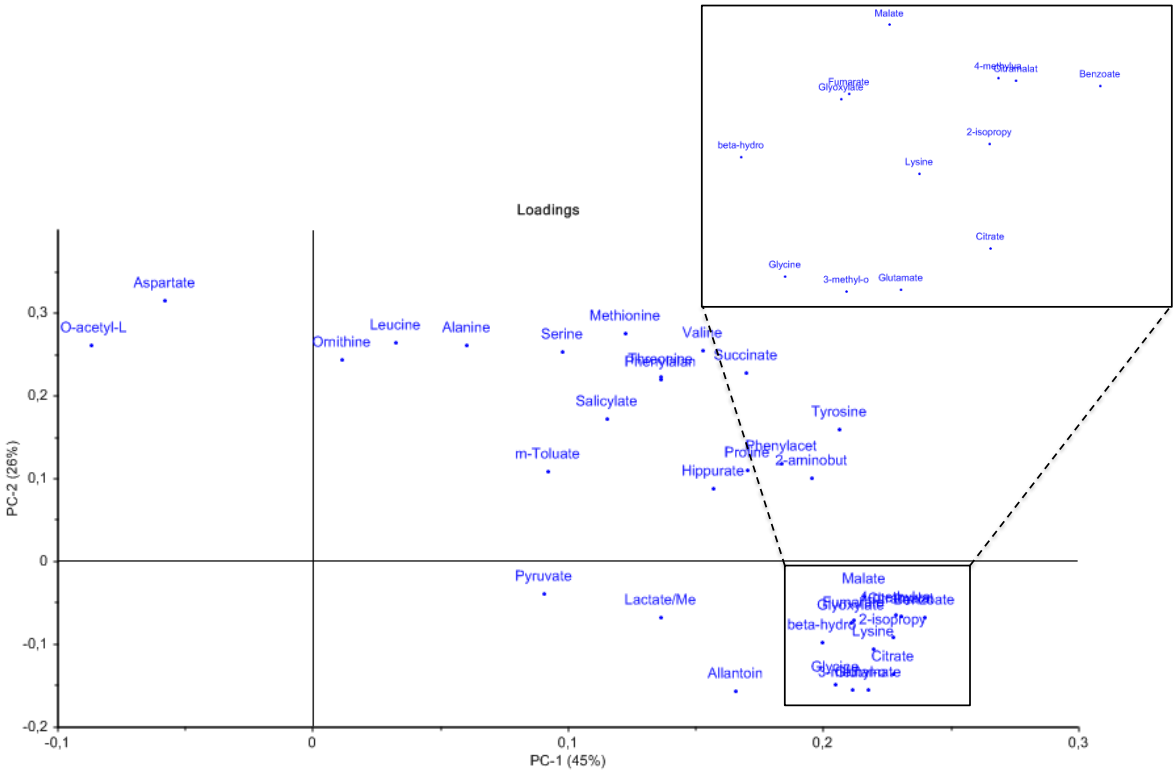
Polar metabolite profiles of six tumors from six mice (six biological replicates) of the MAS98.06 (luminal-like) xenograft and the MAS98.12 (basal-like) xenograft were compared with PCA. The score and loading plots obtained by PCA are shown in Figure 3.5, where the two principal components, PC-1 and PC-2, together describe 71% of the total variance.

The MAS98.06 and MAS98.12 xenografts are clustered in two separate groups, as indicated in Figure 3.5a. It is therefore a clear differentiation between the two xenografts based on the detected polar metabolites and their concentrations. The separation of the two xenografts and the avoidance of outliers (Figure E.2b in Appendix E) indicate that a model of each xenograft will be appropriate. PCA can therefore be used to classify breast cancer tissues based on the two xenografts [44]. However, the scattering in the two xenograft clusters is quite large. This scattering is assumed mainly to be biological variations, but variations in both sample preparation and analytical instruments will also contribute to the scattering. As the score plot shows, the luminal-like cancer (MAS98.06 xenograft) seems to have more biological variations than the basal-like cancer (MAS98.12 xenograft). The basal-like cancer can perhaps be considered more homogeneous than the luminal-like cancer, as also concluded section 3.3.1.

The loading plot in Figure 3.5b shows that pyruvate and malate are significantly only to PC-1, while ornithine are significantly only to PC-2. The MAS98.12 xenograft is characterized by higher concentrations of for example lactate, pyruvate, malate and glycine, compared to the MAS98.06 xenograft. The MAS98.06 xenograft is characterized by higher concentrations of metabolites such as leucine, ornithine, O-acetyl-L-serine, aspartate and alanine, than the MAS98.12 xenograft.



(a)



(b)

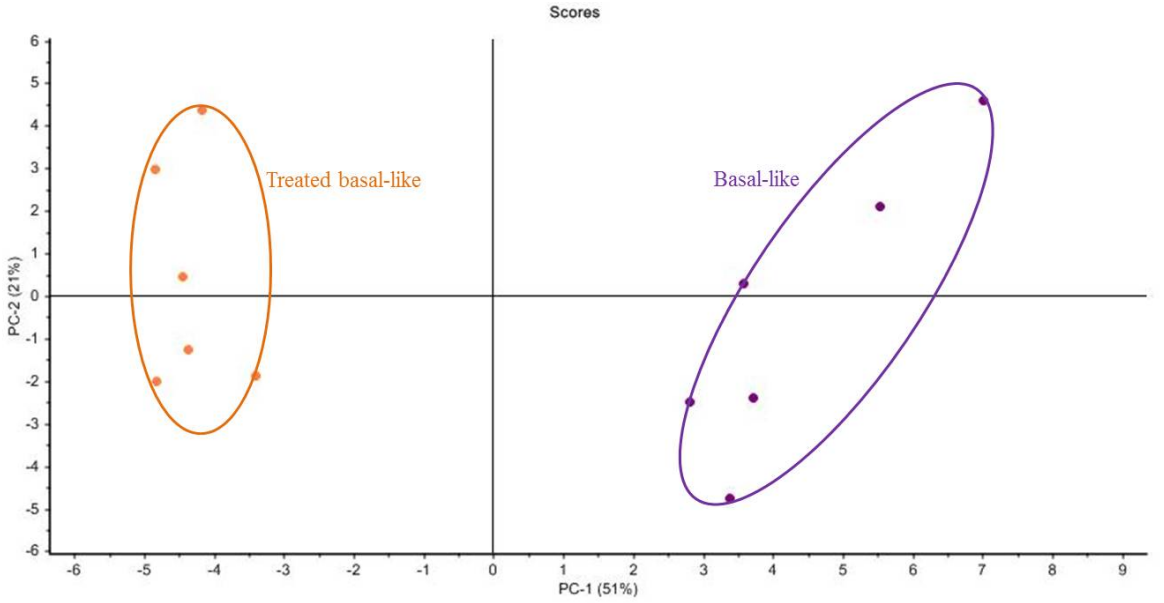
Figure 3.5: *PCA of the MAS98.06 (luminal-like) and MAS98.12 (basal-like) xenografts: (a) the score plot; (b) the loading plot.*

Comparison of untreated and treated basal-like breast cancer xenografts

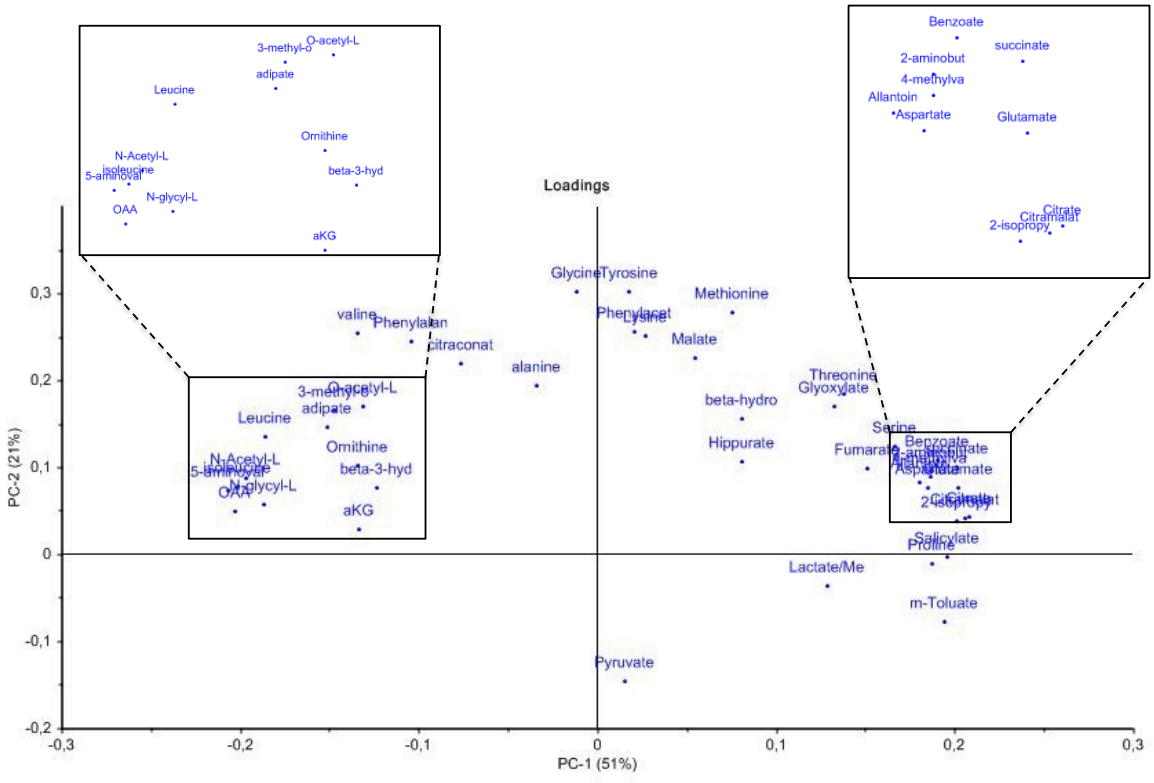
Polar metabolite profiles of six tumors from six mice (six biological replicates) of the untreated and the MK-2206 treated basal-like (MAS98.12) xenograft were compared with PCA. The score and loading plots obtained by PCA are shown in Figure 3.6, where the two principal components, PC-1 and PC-2, together describe 72% of the total variance.

The untreated and MK-2206 treated MAS98.12 xenografts are clustered in two separate areas, as indicated in Figure 3.6a. It is therefore a distinct differentiation between the untreated and treated xenografts. The separation of the two xenografts and the avoidance of outliers (Figure E.4b in Appendix E) indicate that a model proposed by PCA will be appropriate [44]. As for the comparison of the MAS98.06 and MAS98.12 xenografts, the scattering caused mainly by biological variations in the two clusters is quite large. However, the untreated and treated MAS98.12 xenografts cluster in areas further apart, meaning that they are more dissimilar. In addition, the scattering in each cluster indicates that the biological variations between the samples can be reduced compared to the comparison between the MAS98.06 and the MAS98.12 xenografts (Figure 3.5a).

The loading plot in Figure 3.6b shows that PC-1 distinguishes the untreated and treated xenografts. The untreated xenograft has higher concentrations of metabolites such as lactate, fumarate, serine, glutamate and threonine, while the treated xenograft has higher concentrations of leucine, ornithine, O-acetyl-L-serine and valine. The eight metabolites only detected in the treated xenograft (Table 3.2 in section 3.3.2) are of course only positively correlated to the treated xenograft. The significant difference between lactate in the two xenografts is consistent with the results from Moestue *et al.* [23].



(a)



(b)

Figure 3.6: *PCA of the untreated and MK-2206 treated MAS98.12 (basal-like and treated basal-like, respectively) xenografts: (a) the score plot; (b) the loading plot. Each point of the treated MAS98.12 xenografts in the score plot is the average of two samples (two technical replicates) of the same xenograft.*

3.3.5 Conclusions of the polar metabolite profiles

The MAS98.12 xenograft has a more aggressive behavior and is related to a more poor prognosis than the MAS98.06 xenograft [1, 7]. The difference between the concentration of both glycine and lactate in the MAS98.06 and MAS98.12 xenografts is significant, where the two metabolites are found in higher concentrations in the MAS98.12 xenograft. The PCA results demonstrates this strong association. This is indeed in agreement with research by Sitter *et al.*, where it was shown that patients with a poor prognosis have higher concentrations of both glycine and lactate compared to patients with a good prognosis (section 1.4.2) [15]. Moestue *et al.* have also shown that the MAS98.12 xenograft has a higher concentration of glycine than the MAS98.06 xenograft, as opposite to the concentration of phosphocholine [20]. The significant difference in glycine concentration in the two xenografts is suggested to be caused by a difference in the formation of glycine and the choline routing [20]. Choline can be metabolized to either glycine or phosphocholine and phosphatidylcholine (Figure 1.6a in section 1.4.2). The formation of glycine from choline via betaine is assumed to be upregulated in breast cancer cells, making the metabolic flux towards glycine formation higher than the flux towards the production of phosphatidylcholine [20]. Such a abnormality in the metabolism is well known in cancer. The MAS98.06 xenograft has significant higher concentrations of metabolites such as O-acetyl-L-serine and aspartate compared to the MAS98.12 xenograft.

Research by Moestue *et al.* has indicated that the MK-2206 treated MAS98.12 xenograft has a lower concentration of lactate compared to the untreated MAS98.12 xenograft [23]. This is further demonstrated in this work. Lactate is found in significant higher concentration in the untreated xenograft than in the treated xenograft, and the PCA results demonstrates this strong association. The formation of lactate from glucose and pyruvate in the glycolysis is known to be upregulated in breast cancer cells (Figure 1.6b in section 1.4.2) [12]. Metabolism of glucose to lactate is preferable in cancer cells, since the glucose metabolism (glycolysis) and cell cycle is inhibited through the PI3K/Akt pathway (Figure 1.4 in section 1.3.2). The MK-2206 drug inhibits the PI3K/Akt pathway, and therefore activates glucose metabolism through the glycolysis, and not the formation of lactate. The treated xenograft has indeed lower concentrations of most of the polar metabolites compared to the untreated xenograft. However, the treated xenograft has significant higher concentrations of 3-methyl-oxovalerate, O-acetyl-L-serine, valine, leucine and ornithine compared to the untreated xenograft. The untreated xenograft has a higher concentration of glycine, but the difference between the two xenografts is not significant (Figure 3.4 in section 3.3.3 and Table E.10 in Appendix E). In the model proposed by PCA, glycine is only significant to PC-2, and none of the xenografts correlate

with glycine (Figure 3.6 in section 3.3.4). Therefore, the PCA model fails for glycine, but the rest of the results from the metabolite profiles coincide with the metabolite analysis by PCA.

By performing PCA on the polar metabolite profiles of the MAS98.06, the untreated MAS98.12 and the treated MAS98.12 xenografts, a possible phenomena can be detected [44]. An appropriate model of each xenograft can therefore be made, showing the characteristics of each xenograft based on the detected metabolites. Since the model proposed by PCA mostly coincide with the metabolite profiles, they are both considered acceptable and representative of the xenografts. The results from PCA can therefore be used to classify breast cancer tissues based on the luminal-like, basal-like and MK-2206 treated xenografts, by interpreting metabolite profiles into Unscrambler. Effects on the MK-2206 treatment can also be revealed by the metabolite profiles and PCA.

3.4 Non-polar metabolite profiling of breast cancer xenografts

The experiments to obtain non-polar metabolite profiles, meaning lipid profiles, of the luminal-like (MAS98.06), basal-like (MAS98.12) and the MK-2206 treated basal-like xenografts were performed using the optimal method (section 2.3). After extraction of the tissue sample 270312-Lum6, the non-polar extract was dried and dissolved in dichloromethane prior high resolution flow injection MS analysis (FIA MS) by a Q-TOF LC-MS system. The analysis was performed without a chromatographic separation and with positive electrospray ionization (ESI) mode.

The mass spectra of a blank sample of dichloromethane and the non-polar extract are given in Figure 3.7, and these spectra are assumed to be representative of the composition of the samples. By overlapping the spectra, the spectrum of the sample extract shows a higher intensity than the spectrum of the blank sample. The sample extract is therefore assumed to consist of lipids. This assumption is further validated by Figure F.1 in Appendix F, where scans of the sample extract and the blank sample at two different time points are given. The scans indicate the presence of lipids in the sample extract, since the m/z ratios of the sample extract are greater than the m/z ratios of the blank sample. But, it seems that the concentration level of the lipids is close to the detection level of the Q-TOF system, since the height of the peaks is fairly low. In addition, the assumed peak from 0.4 min to 1.2 min in the spectrum of the sample extract is clearly not a well defined peak. Due to the insufficient results compromising both low lipid concentrations and poor peaks, only one non-polar extract was analyzed. The identity of the masses found in greater abundance in the sample extract compared to the blank

sample, were unfortunately not found due to lack of research time.

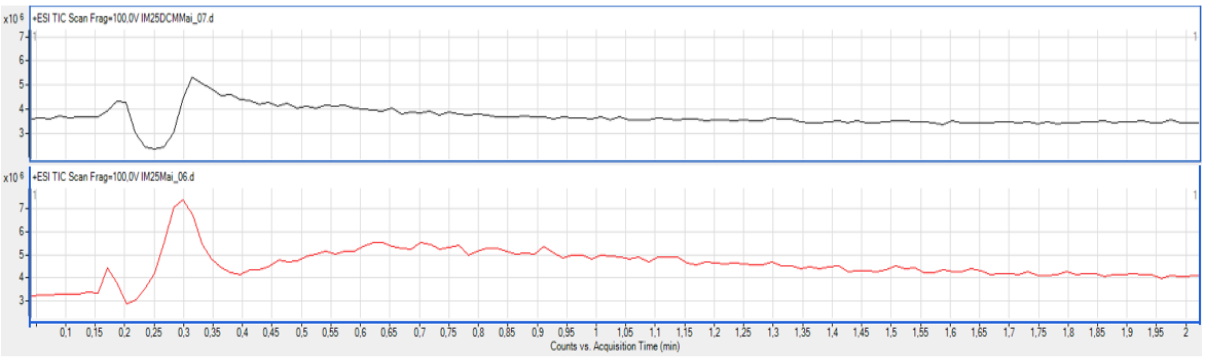


Figure 3.7: *Mass spectra of the blank sample (dichloromethane) (top) and the non-polar extract of sample 270312-Lum6 (bottom), after flow injection mass spectrometry analysis (FIA MS) by positive ESI mode by a Q-TOF LC-MS system.*

The apparently low concentrations of lipids seen with the analysis by the Q-TOF system are in agreement with the results from the FAME analysis by the GC-Q-MS system. Only three peaks representing three FAMEs were detected by GC-Q-MS, together with the peak representing the internal standard (d_{27}-myristic acid) (section 2.7.2 and section 3.1.2). Since both analyses yielded poor results, it is assumed that the concentrations of the lipids present in the non-polar extracts are low and near the lowest detection level of both instruments. Analysis of lipids in the non-polar extracts must therefore be optimized to be considered acceptable. It is preferred to analyze the non-polar extracts as lipids and not FAMEs, since lipid profiles would be highly appreciated in further understanding of breast cancer. The first aspect that can be evaluated is the concentration levels, the concentration of the non-polar extracts can perhaps be increased even further (section 3.1.2). Second, the analysis by the Q-TOF system was performed in positive ESI mode, which ionizes cationic regions of lipids, e.g. phosphatidylcholines [38]. Lipid analysis can also be performed in negative ESI mode, which ionizes anionic regions of lipids, e.g. phosphatidylinositols [38], but negative ESI mode is approximately 100 times less sensitive than a positive ESI mode. If negative ESI mode is used, the concentration of the non-polar extracts must therefore be greatly increased. In addition, there are lipids that are classified as neutral or poorly ionizable, e.g. triacylglycerol, and these lipids will not be ionized with ESI alone. To ionize such lipids, salts can be added to the extracts to form adduct ions [38]. Finally, the instrumental conditions of the Q-TOF system or another analytical system, as appropriate collision energies and ionization parameters, can be modified and hopefully optimized [38].

3.5 Potential biomarkers

Polar metabolite profiles of the two breast cancer xenografts representing the luminal-like and basal-like breast cancer (MAS98.06 and MAS98.12, respectively), were found after extraction and analysis by MCF GC-QqQ-MS (Table 3.2 in section 3.3.2). In addition, a polar metabolite profile of the MK-2206 treated MAS98.12 xenograft was discovered. These metabolite profiles were assumed representative of the xenografts, but temporary (section 3.3.2). Metabolites can serve as biomarkers for the detection of breast cancer, but they can also be indicators of tumor aggressiveness and treatment response. Before targeting metabolites as biomarkers for breast cancer, metabolite profiles of both cancerous and non-cancerous (healthy) tissues should be revealed [14]. Differentiation between cancerous and non-cancerous tissues are also considered necessary for the increased understanding of the development and the progression of cancer [14]. By comparing metabolite profiles of cancerous and non-cancerous tissues, metabolites or ratio of metabolites that are conspicuous can perhaps be targeted as biomarkers. Therefore, metabolites can not be targeted as biomarkers for detection of breast cancer in this work since only cancerous tissues have been extracted and analyzed. However, possible biomarkers for tumor aggressiveness and treatment response based on the difference between the MAS98.06 and MAS98.12 xenografts and the difference between the untreated and treated MAS98.12 xenografts, respectively, can be observed. In addition, there are some metabolites that can be promising in further research for breast cancer biomarkers.

Citraconate/itaconate is only detected in the MAS98.12 xenograft, but the concentration is fairly low (Table 3.2 in section 3.3.2). As stated in section 3.1.1, metabolites in low concentrations will possibly have varying concentrations in different xenograft samples, but citraconate/itaconate may be an important metabolite if the same trend is seen in more experiments. Glutamate is present in significant higher concentration in the MAS98.12 xenograft compared to the MAS08.06 xenograft, while O-acetyl-L-serine and aspartate are present in significant higher concentration in the MAS98.06 xenograft. Glutamate, O-acetyl-L-serine and aspartate can therefore potentially be important indicators of differences in tumor aggressiveness, if the same trend is seen in more experiments. Glycine and lactate are known to be present in high concentrations in breast cancer tissues, and poor prognosis is related to higher concentrations of both glycine and lactate compared to good prognosis (section 1.4.2). Both glycine and lactate are found to be present in significant higher concentrations in the MAS98.12 xenograft compared to the MAS98.06 xenograft (section 3.3.5), and the MAS98.12 xenograft is related to poor prognosis. Thus, the ratio glycine/choline and/or the ratio lactate/glucose can perhaps serve as biomarkers, both for the detection of breast cancer and as indicators of tumor aggressiveness.

The MAS98.12 xenograft treated with the cancer drug MK-2206 is shown to have generally lower concentrations of most of the polar metabolites, compared to the untreated xenograft. Lactate is found in significant higher concentration in the untreated xenograft, and lactate can therefore potentially be a biomarker for a positive treatment response. The other metabolites found in lower concentrations in the treated xenograft can also be biomarkers for such treatment, if the same trend is seen in more experiments.

Non-polar metabolite profiles were not found, due to the insufficient results from the non-polar metabolite analysis (section 3.4). Lipids that possibly can serve as biomarkers are therefore not found. However, choline metabolism is shown to be directed in two different routes in breast cancer, and differences in phosphocholine and phosphatidylcholine concentrations compared to the concentration of glycine in the two breast cancer xenografts can therefore potentially be important (section 3.3.5) [20].

3.6 Comparison of MS- and NMR-based systems for metabolite analysis

Both MS and NMR systems are used as analytical tools in metabolomics (section 1.5.1). MS are often coupled to LC or GC, while NMR systems involve approaches such as magnetic resonance spectroscopy (MRS) and high resolution magic angle spinning spectroscopy (HR MAS) MRS. One important difference between MS and NMR systems is the instrumental sensitivity, comprising both the concentration and the number of metabolites [34]. MS-based approaches have a much lower concentration threshold than NMR (section 1.5.1) [14], in addition to an ability to identify around 1,000 metabolites compared to 20-200 metabolites for NMR-based approaches [34]. This gains MS-based approaches, such that metabolites present in picogram levels in tissues can be detected, identified and quantified. Sensitivity of an analytical tool is extremely important, since many metabolites are present in very low concentrations in different tissues. In fact, metabolites can be present in concentrations below or near the lowest detection levels of the analytical tools. For GC-MS analysis, extracts are often concentrated by evaporating the extraction solvent, to increase the concentrations to detectable levels (section 1.2). GC-MS systems generally have the ability to identify more metabolites than NMR-system [34]. However, work done by Sitter *et al.* shows that more than 30 metabolites are usually detected in intact human breast cancer tissue by HR MAS MRS [49]. This is comparable with the number of polar metabolites detected in tissue extracts by GC-QqQ-MS systems (see Table 3.2 in section 3.3.2). Many of the polar metabolites (mostly amino acids) detected with HR MAS MRS are also detected with GC-QqQ-MS. Non-polar metabo-

lites are also detected with HR MAS MRS, but the HR MAS MRS analysis can not be compared with the FIA MS, since the results were insufficient and the identity of lipids remains unsolved (section 3.4).

Metabolite analysis with HR MAS MRS has the advantage of being non-destructive, meaning that intact breast cancer tissues can be analyzed directly [34]. Important disadvantages of metabolite analysis of breast cancer tissues by GC-MS systems are the need for both metabolite extraction and derivatization, prior to metabolite analysis [35, 17]. Extraction and derivatization are two time-consuming procedures, and loss of metabolites throughout the experimental procedures is anticipated and almost always inevitable. To correct for such loss, internal standards are added before both the extraction and derivatization (section 1.5.4) [35, 42]. But, the loss of metabolites are only minimized, not completely eliminated. Therefore, one should perform metabolite extractions several times to prevent underestimation of metabolite concentrations. Metabolite analysis by LC-MS systems generally eliminates the need for derivatization, such that tissue extracts can be analyzed directly. Ion suppression resulting in reduced detection capability, underestimation of metabolite concentrations and prevented detection of metabolites, is a crucial disadvantage of LC-MS systems [34]. However, the sensitivity of LC-MS systems is comparable with the sensitivity of GC-MS systems.

Another important advantage of NMR-based approaches is the rapid analysis time, approximately 10 min compared to about 30 min with GC-MS systems [34]. This contributes to an overall more rapid NMR-procedure, since tissues must be extracted and derivatized prior analysis by GC-MS systems.

NMR-based approaches are most frequently used in resent research in tumor metabolomics [34]. But, since MS-based approaches present higher sensitivity, both GC-MS and LC-MS systems have been more popular as metabolite analysis tools. A combined approach of NMR- and MS-based systems can therefore be of great value when performing metabolite analyses, since such a combination can offer the best coverage of all the metabolites present in tissues.

3.7 Recommendations of future work

3.7.1 Optimal extraction method

A method for extracting both polar and non-polar metabolites was developed and recognized as an optimal method, even with the expected loss of polar and amphipathic

lipids and the high and varying standard deviations (see section 3.2). It can therefore be beneficial to consider a few more aspects to validate the optimal method further.

Non-polar metabolite extraction The non-polar metabolite extraction was developed and performed in the Precellys 24 homogenizer with chloroform, in a separate step after the polar metabolite extraction. A simultaneous extraction of both polar and non-polar metabolites in the homogenizer by addition of methanol and chloroform to tissue samples, can be evaluated. However, it is assumed that such an approach will result in a three-phase system, with an emulsion (middle-phase) layer containing non-extractable proteins and perhaps metabolites with amphipathic properties [27, 50]. If such an approach is to be evaluated, the emulsion layer should be analyzed for metabolites lost from the polar and non-polar phases.

The DRS-method developed to identify FAMEs (section 2.7.2) should be extended to include more than one internal standard. Ideally, internal standards should be added in the extractions, as well as in the derivatization (section 1.5.4) [35, 42]. d_{31}-palmitic acid and d_{35}-stearic acid can be included as internal standards in the DRS-method.

The identity of the unknown FAME (Figure D.2 in Appendix D) can also be of importance, since abnormalities in fatty acid metabolism are seen in breast cancer [12].

The polar extracts should be analyzed for polar and amphipathic lipids, since it is assumed that such lipids can be concentrated in the polar extracts with 60% methanol (section 3.1.2). The conversion of lipids to FAMEs happened over a time range from 16 to 24 hours (section 2.7.1). A time series for the conversion of FAMEs could be performed, to detect the time for best results.

Non-polar metabolite extraction solvent Chloroform was used as the lipid extraction solvent. Matyash *et al.* claim that lipid extraction with methyl-*tert*-butyl ether (MTBE) allows both faster and cleaner lipid recovery compared to chloroform. In addition, MTBE is non-carcinogenic and non-toxic, which provides a great reduction in health risks. MTBE, or another ether, could therefore be evaluated as a lipid extraction solvent.

TMS derivatization The derivatization with TMS was modified to be suitable for tissue samples of the MAS98.12 xenograft. Even though the modification was a success, TMS-labeled metabolites should be analyzed absolute quantitative to obtain concentrations instead of peak area (mg tissue)$^{-1}$. In the evaluation of TMS derivatization, ten metabolites were found to be unique for that derivatization (see section 3.1.3). These ten

metabolites can possibly be important metabolites for breast cancer metabolite profiles, and more research can therefore be beneficial.

Evaluation of the tissue weight range The tissue samples used both in the optimization experiments and the metabolite profiling experiments were in the weight range 30 ± 10 mg. As stated in section 3.2, this weight range was suitable for the available xenografts collected from St. Olavs hospital. With a metabolite profile for breast cancer available, biomarkers found in breast cancer tissues from biopsies can hopefully be used to determine the presence of cancer and predict the prognosis. Tissue samples from breast biopsies are commonly smaller than 30 ± 10 mg. It is therefore necessary to evaluate the tissue weight range suitable for the optimal extraction method.

Analysis of lipids The complete extraction of non-polar metabolites was evaluated in terms of FAMEs, assumed representative of lipids. The non-polar extracts should nevertheless be analyzed for lipids, perhaps by a LC-MS system.

Evaluation of other tissues The optimal extraction method was developed with tissue samples of the MAS98.12 xenograft, thus xenografts of the basal-like breast cancer. The method was assumed to be valid for the MAS98.06 and MK-2206 treated MAS98.12 xenografts. Tissue samples of the MAS98.06 and MK-2206 treated MAS98.12 xenografts should therefore be evaluated to confirm the optimal method on those xenografts. The extraction method should also be applied to other types of tissues, for example breast biopsies from cancer patients and other cancer tissues, to validate the method even further as an optimal extraction method. It could be beneficial if the optimal extraction method could be applied to other types of cancer tissues, such that other metabolite profiles can be revealed and the understanding of other cancer types can be increased.

3.7.2 Metabolite profiles and biomarkers

By using the optimal extraction method, polar extracts of the MAS98.06, MAS98.12 and MK-2206 treated MAS98.12 xenografts were analyzed absolute quantitative by the GC-QqQ-MS system and compared by PCA. Unfortunately, non-polar metabolite profiles of the xenografts were not obtained, much due to lack of research time. There are however aspects of both the polar and non-polar metabolite analyses that can be evaluated, in hope of finding trustworthy metabolite and lipid profiles and potential biomarkers.

Evaluation of standard deviations for the polar metabolites The standard deviations of technical replicates for the polar metabolites extracted from both the MAS98.06 and MAS98.12 xenografts were evaluated (section 3.3.1). The results showed both varying and high standard deviations, nevertheless, they were considered acceptable. Sample preparation, derivatization and metabolite analysis by an analytical tool contribute to the standard deviations. In order to minimize the instrumental variations, samples can be analyzed in a random order at the same day.

Biological replicates are considered more preferable than technical replicates [42], and it was therefore decided to analyze only one technical replicate in the metabolite profiling experiments (section 3.3.1). However, if low standard deviations are of importance, the sample extracts could be vortexed before transferring each aliquot to new PP tubes, to make the technical replicates. This can hopefully result in technical replicates that are completely representative of the sample extracts, in addition to more homogeneous sample extracts.

Evaluation of the standard deviations of non-polar metabolites when an optimal analysis method is found or developed, could also be of interest.

Analysis of lipids Analysis of the non-polar extracts for lipids by FIA MS gave unfortunately deceptive results, since the identity of lipids remains unsolved (section 3.4). In order to identify the lipids in breast cancer xenografts, the analysis should be optimized based on the concentration level and the instrumental analysis method. When an optimal analysis is revealed, one can hopefully identify the different lipids by the different m/z ratios obtained in the analysis. The MassHunter Qualitative Analysis software has a built-in function that can find compounds by molecular feature, meaning compounds with chromatographic profiles. This analysis approach to identify lipids can be time-consuming, but PCA can be utilized if differences between xenografts are the major goal.

Repetition of both the extractions and the metabolite analyses To completely validate the metabolite profiles of the different xenografts, the experiments should be repeated. By repeating metabolite extraction and analysis, preferable by analyzing tissue extracts in a random order by analytical tools at the same day, day-to-day variations can be limited. The true metabolite profiles can thereafter hopefully be revealed.

Evaluation of other tissues and biomarkers Single metabolites or ratio of metabolites can not be targeted as biomarkers for the detection of breast cancer without the

presence of metabolite profiles of both cancerous and non-cancerous tissues (section 3.5) [14]. Therefore, metabolite extraction and analysis of healthy breast tissues, that is healthy tissues from mice, must be performed. When metabolite and lipid profiles of breast cancer xenografts and healthy tissues are known, metabolite extraction and analysis of breast biopsies, both cancerous and non-cancerous, can be performed to evaluate differences in breast cancer between humans and animals due to biological factors.

To validate the potential biomarkers for tumor aggressiveness and treatment response (section 3.5), the experiments should be repeated to see if the same trends are observed.

Analysis of tissue extracts with NMR-based approaches For a complete comparison between metabolite analysis by NMR- and MS-based approaches, tissue extracts obtained by the use of the optimal method should also be analyzed by NMR. Analysis by NMR can reveal other important metabolites not detected by MS-systems, which possibly can serve as biomarkers. As stated in section 3.6, a combined NMR- and MS-based analysis has the opportunity to cover all the metabolites found in the breast cancer xenografts. Even more reliable and complete metabolite profiles of the MAS98.06, MAS98.12 and MK-2206 treated MAS98.12 xenografts can thereby be provided.

3.8 Other remarks

In should be noted that the MCF STD-mix used in the first MCF derivatizations of polar extracts was discarded, because of poor responses in the metabolite analysis by the GC-QqQ-MS system. A new MCF STD-mix containing slightly fewer polar metabolites was prepared, since the removed metabolites had poor peaks in the chromatogram, which made their detection difficult and uncertain. The number of metabolites derivatized with MCF from the metabolite profiling experiments is therefore slightly reduced compared to the optimization experiments. However, most of the metabolites removed from the MCF STD-mix were low abundance metabolites, and they are assumed not to be the most important metabolites in potential polar metabolite profiles.

4 Conclusion

An optimal method for the extraction of both polar and non-polar metabolites from invasive ductal carcinoma xenografts with a beads-based homogenizer was developed. The optimal method combines mechanical and non-mechanical extraction methods in an elegant way, where the homogenization and the extraction occur simultaneously. Complete extraction of polar and non-polar metabolites is achieved in two separate steps. The polar metabolite extraction is completed after three homogenization rounds with 60% methanol as the extraction solvent, and the non-polar metabolite extraction is completed in three subsequent homogenization rounds with chloroform.

Polar metabolite profiles of the MAS98.06, MAS98.12 and MK-2206 treated MAS98.12 xenografts were obtained after absolute quantitative analysis by MCF GC-QqQ-MS, and thereafter compared by PCA and Student‘s t-tests. The statistical analyses showed that the MAS98.12 xenograft has significant higher concentrations of lactate and glycine compared to the MAS98.06 xenograft, while the MAS98.06 xenograft has significant higher concentrations of O-acetyl-L-serine and aspartate. The untreated MAS98.12 xenograft has significant higher concentration of lactate than the MK-2206 treated MAS98.12 xenograft.

Non-polar metabolite profiles of the MAS98.06, MAS98.12 and MK-2206 treated MAS98.12 xenografts were not obtained after FIA MS by the Q-TOF LC-MS system, much due to lack of research time.

More work is needed before reliable metabolite and lipid profiles are obtained. The polar metabolite profiles are considered acceptable, but they should be validated further with repetition of the experiments. The non-polar metabolite profiling should gain more work, especially the metabolite analysis method. When an complete optimal extraction method and complete metabolite profiles are obtained, comparison of cancerous and non-cancerous (healthy) tissues, both from xenografts and biopsies, can hopefully reveal conspicuous metabolites or ratio of metabolites that can serve as biomarkers.

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Appendices

A Compound lists of standards

Table A.1 provides the information about the polar metabolites in the MCF standard-mix, while Table A.2 gives the information about the FAMEs in the FAME standard.

Table A.1: *An overview over the metabolites in the MCF standard-mix used for analysis by GC-QqQ-MS. The retention time (RT) for each metabolite is given together with the multiple reaction monitoring (MRM) target ions (TgIs) and MRM TgIs of their corresponding internal standards (ISTD MRM TgI).*

Metabolite	RT [min]	MRM TgI	ISTD MRM TgI	Metabolite	RT [min]	MRM TgI	ISTD MRM TgI
4-Methylvalerate	6.438	43.0	43.0	Proline	10.836	83.8	87.0
Malonate	6.769	68.8	69.0	Oxaloacetate (OAA)	10.847	68.8	68.9
Pyruvate	6.967	56.8	60.0	Aspatate	11.345	74.8	81.0
(+-)-3-Methyl-2-oxovalerate	7.220	67.0	67.0	Citrate	11.381	100.8	61.9
Fumarate	7.740	84.8	88.0	5-aminovalerate	11.412	55.0	55.0
d_4-succinateHVL	7.782	57.8	57.9	Threo-beta-hydroxyaspartate	11.583	81.9	85.0
Lactate/Methylglyoxal	7.803	30.8	61.9	Serine	11.819	99.7	102.8
Succinate	7.813	54.8	62.0	Anthranilate	11.827	89.9	89.9
Citraconate/Itaconate	8.369	41.0	42.0 / 62.0	Allantoin	11.922	41.9	45.1
Benzoate	8.491	77.0	77.0	N-Acetyl-L-Glutamate	12.169	84.0	84.0
Citramalate	8.584	43.0	43.0	d_5-GlutamateHVL	12.177	102.9	106.0
Glyoxylate	8.687	75.0	53.0	Glutamate	12.204	97.8	100.9
beta-3-hydroxybutyrate	8.696	41.0	41.0	Hydroxyproline	12.323	81.8	85.0
d_3-AlanineHVL	8.711	60.8	108.0	Methionine	12.342	60.8	61.0
Glycine	8.720	43.8	47.1	Beta-Methylamino-L-alanine	12.679	98.0	115.9
Alanine	8.727	101.9	105.1	2-oxobutyrate	12.809	67.0	95.0
O-Acetyl-L-Serine	8.735	42.0	42.0	Cysteine1	12.917	88.7	62.0
Nicotinate	8.875	77.9	77.9	Isocitrate	13.003	75.0	80.9
Phenylacetate	9.185	91.0	91.0	Putrescine	13.006	55.0	55.0
Salicylate	9.378	121.0	121.0	Hippurate	13.069	104.9	105.0
2-Aminobutyrate	9.422	57.1	60.0	Phenylalanine	13.126	127.9	128.0
m-Toluate	9.460	91.0	91.0	Phenylpyruvate	13.139	115.0	62.0
Beta-Alanine	9.495	44.0	47.0	2,4-Diaminobutyrate	13.465	70.0	73.0
OH-Glutarate	9.659	98.7	101.9	4-imidazoleacrylate	13.503	107.0	110.1
Adipate	9.679	73.0	76.0	Cadaverine	13.642	69.0	69.0
d_8-ValineHVL	9.777	61.9	62.0	4-Aminobenzoate	13.742	90.8	94.0
Valine	9.825	70.9	74.0	Histamine	13.963	109.1	67.9
alpha-ketoglutarate (aKG)	9.848	55.0	55.0	p-Coumarate	14.092	132.9	136.0
2-Isopropylmalate	9.943	43.0	43.0	Ornithine	14.256	128.0	131.1
beta-hydroxypyruvate	10.049	74.9	81.1	N-Glycyl-L-Proline	14.708	70.0	70.0
alpha-ketoadipate	10.363	68.9	61.9	Lysine	14.833	142.0	145.0
Leucine	10.466	43.1	46.8	Ferulate	15.104	176.0	175.9
Isoleucine1	10.497	68.9	36.0	Histidine	15.154	150.0	153.1
gamma-aminobutyrate	10.544	69.0	62.0	Tyrosine	15.736	158.0	161.0
Isoleucine2	10.548	68.9	36.0	2,6-diaminopimelate	15.844	139.9	143.1
Threonine	10.621	55.8	55.8	Tryptophane	16.713	185.0	185.2
Malate	10.703	0.8	81.0	Cysteine2	17.543	73.9	-
L-Homoserine	10.718	30.0	61.1	Serotonin	18.184	157.1	157.0

Table A.2: *An overview over the fatty acid methyl esters (FAMEs) in the FAME standard used for analysis by GC-Q-MS. The retention time for each FAME observed in the analysis by GC-Q-MS is given, together with the identification of their corresponding fatty acids.*

FAME	**Retention time [min]**	**Corresponding fatty acid**
Methyl octanoate	10.231	Caprylic acid (C8:0 fatty acid)
Methyl nonanoate	11.864	Pelargonic acid (C9:0 fatty acid)
Methyl decanoate	13.364	Capric acid (C10:0 fatty acid)
Methyl dodecanoate	16.230	Lauric acid (C12:0 fatty acid)
Methyl tetradecanoate	18.730	Myristic acid (C14:0 fatty acid)
Methyl hexadecanoate	21.007	Palmitic acid (C16:0 fatty acid)
Methyl octadecanoate	23.096	Stearic acid (C18:0 fatty acid)
Methyl eicosanoate	25.040	Arachidic acid (C20:0 fatty acid)
Methyl docosanoate	26.795	Behenic acid (C22:0 fatty acid)
Methyl tetracosanoate	28.429	Lignoceric acid (C24:0 fatty acid)
Methyl hexacosanoate	29.928	Hexacosanoic acid (C26:0 fatty acid)
Methyl octacosanoate	31.517	Octacosanoic acid (C28:0 fatty acid)
Methyl triacontanoate	33.383	Melissic acid (C30:0 fatty acid)

B Raw data from the optimization experiments

The optimization experiments were performed with tissue samples of the MAS98.12 xenograft and the Precellys 24 homogenizer. The raw data from the MCF derivatization, the TMS derivatization and the derivatization of lipids to FAMEs are given in section B.1, section B.2 and section B.3, respectively.

B.1 MCF derivatization of polar metabolites

The raw data from the MCF derivatization are given in pmol (μL injected sample)$^{-1}$ after the absolute quantitative analysis using the Mass Hunter Quantitative Analysis for QqQ software. But, the raw data given in Table B.4 are given in response, due to the lack of a standard curve. Table B.1, B.2 and B.3 show the raw data of three tissue samples from the first evaluation of the polar metabolite extraction. Table B.4 shows the raw data from the second evaluation of the extraction of the polar metabolites, while Table B.5 shows the raw data from the second evaluation of the non-polar metabolite extraction.

Table B.1: *Raw data from the MCF derivatization on the polar extracts of sample 180112-1, where the number of necessary homogenization rounds was evaluated (the first polar metabolite extraction evaluation). Raw data are given in pmol (µL injected sample)$^{-1}$.*

	180112-1 1^{st} round			**180112-1 2^{nd} round**			**180112-1 3^{rd} round**		
Replicate/Metabolite	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
4-methylvalerate	0.10	0.10	0.08	0.08	0.08	0.09	0.07	0.06	0.07
Malonate	0.14	0.14	0.13	0.13	0.14	0.17	0.17	0.15	0.18
Pyruvate	1.56	1.78	1.76	0.82	0.83	0.93	0.53	0.44	0.48
3-methyl-oxovalerate	0.32	0.35	0.33	0.14	0.13	0.14	0.13	0.15	0.16
Fumarate	1.55	1.68	211.90	0.44	0.41	0.48	0.12	0.12	0.14
Lactate/Methylglyoxal	351.49	364.95	351.87	81.45	77.37	87.88	18.19	15.66	15.22
Succinate	3.17	3.53	3.07	0.89	0.81	0.71	0.41	0.39	0.48
Benzoate	0.20	0.20	0.15	0.15	0.20	0.15	0.13	0.13	0.15
beta-3-hydroxybutyrate	4.98	4.99	5.10	1.44	1.20	1.29	0.44	0.44	0.51
Alanine	39.46	40.88	39.28	7.82	7.35	8.33	1.59	1.46	1.48
Glycine	46.52	53.23	46.61	9.37	8.97	10.53	2.11	2.00	2.01
O-acetyl-L-serine	17.28	16.55	14.59	4.35	4.49	4.57	-	-	2.00
Phenylacetate	0.08	0.08	0.08	0.07	0.07	0.08	0.06	-	0.06
2-aminobutyrate	0.16	0.15	0.16	0.07	0.06	0.06	0.03	0.03	0.03
m-Toluate	0.20	0.22	0.13	0.16	0.14	0.17	0.11	0.11	0.13
beta-alanine	3.54	3.54	3.73	0.91	0.88	1.02	0.25	0.24	0.24
Adipate	0.27	0.20	0.17	0.24	0.23	0.23	0.20	0.24	0.20
Valine	4.25	4.27	4.40	1.11	1.12	1.10	0.36	0.33	0.31
2-isopropylmalate	0.28	0.28	0.28	-	-	-	-	-	-
beta-hydroxypyruvate	0.75	0.84	0.63	0.28	0.24	0.42	0.14	0.14	0.13
alpha-ketoadipate	0.81	0.80	0.74	0.65	0.79	0.60	0.81	0.74	0.57
Leucine	3.83	3.46	3.84	0.87	0.87	1.02	0.27	0.23	0.24
Isoleucine	2.15	2.00	2.00	0.56	0.56	0.53	0.25	0.21	0.25
Malate	3.89	3.04	3.18	0.63	0.55	0.40	0.09	0.08	0.05
Oxaloacetate	2.58	1.99	1.58	0.34	0.34	0.23	0.52	-	0.92
Threonine	6.74	7.98	8.00	1.36	1.49	1.97	0.45	0.44	0.44
Proline	10.79	10.82	11.25	2.32	2.22	2.32	0.50	0.47	0.46
L-homoserine	0.36	0.30	0.22	0.16	0.15	0.17	0.16	0.15	0.13
Aspartate	0.70	0.67	0.60	0.18	0.17	0.19	0.14	0.10	0.11
Citrate	6.00	7.10	4.76	1.18	1.06	0.92	0.24	0.21	0.30
5-aminovalerate	0.21	0.20	0.18	0.10	0.10	0.09	0.13	0.14	0.13
Anthralinate	0.08	0.08	0.09	0.09	0.10	0.11	0.13	0.14	0.12
Serine	3.83	3.55	3.91	1.23	1.10	1.07	0.60	0.37	0.33
Allantoin	34.03	2.23	14.75	7.89	7.11	6.21	4.40	4.63	5.39
Glutamate	52.79	56.32	45.30	8.80	7.75	6.11	1.84	1.58	1.93
N-Acetyl-L-glutamate	1.54	1.52	1.36	0.64	0.54	0.48	0.36	0.39	0.39
Methionine	0.89	0.93	0.95	0.24	0.24	0.25	0.09	0.08	0.08
Cysteine	28.37	26.00	24.45	5.36	4.96	5.59	1.19	1.17	1.14
Phenylalanine	2.09	2.06	2.16	0.59	0.57	0.64	0.21	0.19	0.19
Putrescine	0.75	0.65	0.69	0.37	0.37	0.37	0.46	0.35	0.40
Hippurate	0.56	0.47	0.56	0.42	0.57	0.54	0.53	0.54	0.61
4-imidazoleacrylate	0.09	0.09	0.09	0.06	0.06	0.06	0.09	0.05	0.04
Histamine	1.76	2.03	1.55	1.12	0.97	1.16	1.96	2.27	3.10
Ornithine	0.53	0.50	0.53	0.21	0.23	0.20	0.18	0.12	0.12
Lysine	8.75	8.75	9.01	2.72	2.62	2.66	0.98	0.85	0.95
Histidine	0.40	0.37	0.48	0.13	0.13	0.13	-	-	-
Tyrosine	1.42	1.49	1.59	0.38	0.38	0.41	0.11	0.11	0.10
Thryptophane	0.54	0.66	0.76	0.27	0.25	0.27	0.14	0.13	0.12

Table B.2: *Raw data from the MCF derivatization on the polar extracts of sample 180112-2, where the number of necessary homogenization rounds was evaluated (the first polar metabolite extraction evaluation). Raw data are given in pmol (μL injected sample)$^{-1}$.*

	180112-2 1^{st} round			**180112-2 2^{nd} round**			**180112-2 3^{rd} round**		
Replicate/Metabolite	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
4-methylvalerate	0.05	0.06	0.06	0.06	0.06	0.05	0.05	0.05	0.05
Malonate	0.23	0.21	0.22	0.21	0.25	0.20	0.16	0.15	0.16
Pyruvate	1.72	1.47	1.71	0.55	0.81	0.52	0.41	0.31	0.35
3-methyl-oxovalerate	0.19	0.22	0.24	0.11	0.19	0.11	0.15	0.07	0.09
Fumarate	1.17	1.01	1.24	0.37	0.50	0.38	0.12	0.09	0.11
Lactate/Methylglyoxal	628.97	575.31	619.09	145.88	194.68	140.92	32.22	25.45	27.24
Succinate	4.95	4.63	6.86	1.61	2.16	1.69	0.64	0.56	0.59
Benzoate	0.16	0.14	0.16	0.17	0.18	0.15	0.13	0.11	0.13
beta-3-hydroxybutyrate	4.07	4.40	4.39	1.30	1.72	1.30	0.52	0.36	0.37
Alanine	45.49	42.55	43.79	8.61	11.85	8.86	1.90	1.51	1.81
Glycine	42.00	40.21	41.06	8.73	12.19	9.09	2.12	1.74	2.10
O-acetyl-L-serine	15.39	14.26	15.91	4.95	5.84	4.66	-	1.97	-
Phenylacetate	-	-	-	-	-	-	-	-	-
2-aminobutyrate	0.13	0.12	0.12	0.05	0.06	0.05	-	0.03	-
m-Toluate	0.09	0.10	0.10	0.12		0.10	0.09	0.10	0.08
beta-alanine	3.08	2.95	2.97	0.76	1.01	0.77	0.23	0.20	0.22
Adipate	0.38	0.30	0.27	0.32	0.32	0.30	0.24	0.16	0.19
Valine	5.14	4.51	4.65	1.16	1.54	1.19	0.37	0.32	0.37
2-isopropylmalate	0.35	0.33	0.35	-	-	0.25	-	-	-
beta-hydroxypyruvate	0.62	0.71	0.68	0.26	0.31	0.25	-	0.16	0.17
alpha-ketoadipate	9.36	9.78	9.54	2.94	3.75	2.79	1.02	0.84	1.00
Leucine	4.91	5.02	4.86	1.12	1.42	1.14	0.30	0.24	0.32
Isoleucine	2.56	2.14	2.29	0.60	0.83	0.64	0.27	0.24	0.29
Malate	2.14	1.62	1.72	0.30	0.46	0.33	0.07	0.05	0.05
Oxaloacetate	1.38	1.26	1.68	0.37	0.48	0.51	-	-	0.78
Threonine	7.19	1.70	7.43	1.63	2.43	1.88	0.41	0.40	0.44
Proline	11.78	10.40	11.28	2.35	3.30	2.43	0.55	0.43	0.54
L-homoserine	0.24	0.25	0.27	0.19	0.21	0.15	0.15	0.11	0.15
Aspartate	0.81	0.61	0.91	0.23	0.30	0.26	0.10	0.09	0.07
Citrate	3.19	2.29	4.70	0.88	1.15	1.10	0.19	0.15	0.22
5-aminovalerate	0.17	0.16	0.17	0.15	0.15	0.14	0.15	0.13	0.13
Anthralinate	0.08	0.05	0.06	0.08	0.11	0.09	0.11	0.11	0.10
Serine	4.92	3.81	4.66	1.50	1.91	1.51	0.56	0.49	0.48
Allantoin	16.88	69.95	19.05	8.53	8.15	7.45	5.72	5.16	5.50
Glutamate	44.25	35.27	57.36	10.00	13.13	11.13	2.18	1.71	2.03
N-Acetyl-L-glutamate	1.23	0.99	1.34	0.63	0.83	0.65	0.46	0.35	0.34
Methionine	1.06	1.02	1.06	0.29	0.37	0.29	0.09	0.08	0.09
Cysteine	30.18	27.71	30.32	6.93	8.82	7.50	1.95	1.52	1.75
Phenylalanine	2.46	2.39	2.43	0.70	0.83	0.68	0.22	0.19	0.22
Putrescine	0.73	0.68	0.72	0.48	0.68	0.42	0.51	0.50	0.45
Hippurate	0.33	0.38	0.49	0.52	0.52	0.55	0.43	0.48	0.53
4-imidazoleacrylate	0.06	0.05	0.06	0.04	0.08	0.04	0.04	0.02	0.03
Histamine	1.25	0.59	1.32	0.88	1.59	1.35	-	-	-
Ornithine	0.78	0.71	0.73	0.27	0.40	0.27	0.17	0.13	0.14
Lysine	10.45	9.32	9.53	2.75	3.69	2.85	1.03	0.77	0.89
Histidine	0.52	0.43	0.44	0.13	0.18	0.16	-	-	-
Tyrosine	1.76	1.74	1.86	0.46	0.61	0.44	0.12	0.10	0.10
Thryptophane	0.57	0.67	0.94	0.23	0.27	0.19	-	-	-

Table B.3: *Raw data from the MCF derivatization on the polar extracts of sample 180112-3, where the number of necessary homogenization rounds was evaluated (the first polar metabolite extraction evaluation). Raw data are given in pmol (µL injected sample)$^{-1}$.*

	180112-3 1^{st} round			**180112-3 2^{nd} round**			**180112-3 3^{rd} round**		
Replicate/Metabolite	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
4-methylvalerate	0.05	0.05	0.06	0.05	0.05	0.05	0.05	0.05	0.05
Malonate	0.16	0.16	0.18	0.15	0.15	0.16	0.13	0.15	0.19
Pyruvate	0.88	0.96	1.13	0.35	0.45	0.55	0.24	0.25	0.36
3-methyl-oxovalerate	0.35	0.33	0.31	0.07	0.09	0.08	0.08	0.08	0.09
Fumarate	1.06	1.18	1.10	0.20	0.23	0.22	0.05	0.07	0.09
Lactate/Methylglyoxal	353.74	409.84	380.38	40.61	51.52	46.78	6.65	10.28	13.84
Succinate	2.96	3.59	2.87	0.55	0.62	0.57	0.24	0.34	0.43
Benzoate	0.11	0.11	0.11	0.11	0.12	0.11	0.09	0.11	0.13
beta-3-hydroxybutyrate	3.55	4.59	4.06	0.66	0.77	10.47	0.24	0.41	0.73
Alanine	33.73	40.19	34.94	3.96	4.67	4.35	0.58	0.87	1.19
Glycine	41.62	48.30	43.83	5.42	6.51	6.02	0.93	1.36	1.83
O-acetyl-L-serine	11.35	12.13	12.16	2.72	3.04	2.97	1.38	1.66	-
Phenylacetate	0.05	-	-	-	-	-	0.04	-	-
2-aminobutyrate	0.12	0.14	0.13	0.03	0.04	0.03	0.02	-	-
m-Toluate	0.09	0.08	0.08	0.09	0.09	0.08	0.08	0.09	-
beta-alanine	3.01	3.52	3.23	0.49	0.59	0.55	0.12	0.16	0.21
Adipate	0.13	0.15	0.13	0.21	0.22	0.22	0.18	0.27	0.38
Valine	3.92	4.69	4.19	0.65	0.78	0.72	0.17	0.24	0.30
2-isopropylmalate	0.25	0.30	0.25	0.23	-	-	-	-	-
beta-hydroxypyruvate	0.85	0.92	0.90	0.16	0.19	0.18	0.13	0.15	-
alpha-ketoadipate	0.38	0.34	0.40	0.49	0.50	0.50	0.33	0.36	0.48
Leucine	3.21	3.82	3.43	0.50	0.69	0.57	0.12	0.15	0.21
Isoleucine	1.84	2.19	2.10	0.34	0.42	0.38	0.16	0.19	0.22
Malate	1.56	1.90	1.65	0.14	0.20	0.15	0.02	0.03	0.04
Oxaloacetate	1.95	2.96	2.09	0.48	0.38	0.42	-	-	-
Threonine	7.01	8.07	7.25	0.93	1.22	1.09	0.24	0.31	0.39
Proline	10.72	12.75	11.41	1.28	1.70	1.45	0.20	0.30	0.42
L-homoserine	0.17	0.23	0.17	0.19	0.15	0.13	0.12	0.19	0.14
Aspartate	0.49	0.50	0.45	0.09	0.09	0.10	0.05	0.08	0.08
Citrate	3.67	4.31	2.93	0.42	0.55	0.44	0.09	0.11	0.11
5-aminovalerate	0.14	0.15	0.16	0.12	0.12	0.11	0.10	0.11	0.14
Anthralinate	0.06	0.05	0.06	0.05	0.06	0.07	0.11	0.10	0.08
Serine	3.48	3.77	3.67	0.64	0.88	0.83	0.17	0.29	0.42
Allantoin	72.83	15.26	11.55	6.14	5.60	5.97	23.07	5.21	5.61
Glutamate	37.94	48.66	36.40	4.11	5.24	4.18	0.82	1.05	1.33
N-Acetyl-L-glutamate	0.95	1.12	0.86	0.36	0.46	0.51	0.25	0.41	0.46
Methionine	0.93	1.09	0.97	0.18	0.19	0.18	0.05	0.07	0.08
Cysteine	23.28	27.89	24.83	3.17	3.71	3.53	0.57	0.84	1.25
Phenylalanine	1.99	2.37	2.14	0.40	0.46	0.42	0.12	0.14	0.18
Putrescine	0.46	0.54	0.56	0.40	0.38	0.37	0.29	0.33	0.54
Hippurate	0.56	0.40	0.42	0.35	0.38	0.38	0.40	0.42	0.41
4-imidazoleacrylate	0.04	0.04	0.03	0.02	0.02	0.03	0.02	0.02	0.03
Histamine	1.36	1.10	1.18	0.78	-	-	1.12	-	-
Ornithine	0.53	0.58	0.56	0.16	0.20	0.20	0.09	0.11	0.16
Lysine	8.12	9.52	8.81	1.77	2.28	2.01	0.55	0.89	1.18
Histidine	0.45	0.49	0.43	0.08	0.07	0.09	-	-	-
Tyrosine	1.41	1.70	1.53	0.24	0.29	0.26	0.05	0.08	0.10
Thryptophane	0.55	0.73	0.62	0.14	0.19	0.17	0.09	-	-

Table B.4: *Raw data from the MCF derivatization on the polar extracts obtained in the second evaluation of the polar metabolite extraction. The polar extracts of the samples obtained after the non-polar metabolite extraction are termed with "check". Raw data are given in response (peak area).*

	130212-1		130212-2			130212-3			130212-check-1			130212-check-2			130212-check-3		
Replicate/Metabolite	**1**	**2**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
4-methylvalerate	1,206	818	278	-	383	123	186	-	-	-	-	-	-	-	-	-	-
Pyruvate	11,102	11,068	9,753	12,762	15,043	19,096	20,020	20,824	463	373	467	338	485	393	457	573	322
3-methyl-oxovalerate	4,302	3,757	2,409	2,380	1,467	3,197	3,116	2,728	-	-	1,404	-	-	-	697	627	-
Fumarate	71,677	62,026	30,383	39,774	38,138	54,059	57,637	48,505	-	-	-	-	-	-	-	-	-
d_4-succinateHVL	75,616	67,737	56,974	60,162	67,176	56,058	48,431	50,689	123,843	116,998	124,898	133,770	137,598	121,166	111,483	124,372	110,579
Lactate/Methylglyoxal	423,137	381,375	187,550	239,859	256,813	316,071	337,297	310,381	10,666	8,937	10,482	10,077	7,798	6,895	10,347	7,923	7,858
Succinate	72,974	66,961	36,949	47,463	49,577	55,555	56,218	45,494	17,034	5,119	20,815	16,215	20,552	13,917	-	-	-
Benzoate	22,939	16,585	8,094	8,144	6,581	6,659	5,745	5,380	-	-	-	-	-	-	-	-	-
Alanine	181,057	133,412	81,386	87,799	115,504	101,127	89,768	109,185	-	-	-	-	-	-	-	-	-
Glyoxylate	7,135	3,494	2,781	2,398	3,153	2,648	3,098	2,749	-	-	-	-	-	-	-	-	-
Glycine	830,784	671,270	461,867	520,195	624,093	646,022	642,489	664,309	39,468	39,938	35,535	35,882	37,163	33,421	33,536	34,261	33,271
O-acetyl-L-serine	24,820	21,018	16,208	17,405	19,737	20,284	19,684	19,080	2,737	2,559	2,360	2,519	2,917	2,469	2,570	2,444	2,501
2-aminobutyrate	6,209	4,787	3,443	3,005	3,551	3,043	2,932	2,954	-	-	-	-	-	-	-	-	-
Salicylate	16,139	7,828	5,056	4,420	4,505	4,026	2,839	2,742	-	-	-	-	-	-	-	-	-
beta-alanine	35,274	24,602	17,326	17,627	25,343	24,613	24,821	27,842	-	-	-	-	-	-	-	-	-
Adipate	25,623	19,453	17,652	18,707	24,096	19,718	17,915	20,061	-	-	-	-	-	-	-	-	-
Valine	63,369	48,571	36,137	37,735	44,036	47,689	46,319	48,896	2,289	2,656	1,988	2,084	2,366	2,094	2,119	2,122	2,136
beta-hydroxypyruvate	4,100	3,363	3,464	3,940	5,088	5,484	5,596	5,708	392	450	366	317	377	336	352	320	436
Leucine	17,133	13,547	10,254	10,987	13,574	14,538	14,407	15,572	-	-	-	-	-	-	-	-	-
Isoleucine	3,090	2,279	1,094	1,487	2,267	1,923	2,032	2,269	-	-	-	-	-	-	-	-	-
Malate	50,138	42,493	37,650	41,190	46,691	32,643	32,971	31,912	1,421	1,813	1,105	1,171	1,536	1,329	925	1,105	976
Oxaloacetate	563	531	302	768	622	601	554	549	-	-	-	-	-	-	-	-	-
Threonine	5,583	5,389	2,559	3,050	2,898	4,251	2,816	3,243	-	-	-	-	-	-	-	-	-
Proline	50,611	38,391	21,246	22,854	27,069	34,485	32,266	34,913	-	-	-	-	-	-	-	-	-
Aspartate	494	455	279	334	475	428	314	474	-	-	-	-	-	-	-	414	411
Citrate	15,619	15,281	9,309	9,629	10,390	12,398	9,020	11,812	-	-	-	-	-	-	-	-	-
Serine	9,258	8,416	6,911	7,838	9,669	8,090	8,146	7,732	339	285	305	384	328	367	389	313	278
Glutamate	270,122	307,009	189,283	225,542	245,820	241,841	167,785	219,409	3,316	3,555	2,817	2,005	2,430	1,964	2,456	2,976	2,495
N-Acetyl-L-glutamate	4,550	4,524	4,036	4,122	4,612	3,911	2,743	3,561	5,182	4,943	4,110	4,010	4,666	3,715	3,881	4,062	4,018
Methionine	52,821	43,855	30,510	33,918	42,041	45,551	41,061	46,284	554	543	375	271	382	285	527	394	389
Cysteine	29,809	29,516	17,285	21,931	30,652	43,921	38,393	43,109	-	-	-	-	-	-	-	-	-
Phenylalanine	32,302	27,644	18,998	22,213	24,510	29,061	25,157	28,638	544	445	436	245	292	202	583	435	384
Histamine	333	297	153	176	165	186	152	262	-	-	-	-	-	-	-	-	-
p-coumarate	1,859	1,374	696	744	637	668	418	494	-	-	-	-	-	-	-	-	-
Lysine	11,877	14,536	7,465	10,855	10,052	10,313	9,103	10,835	-	-	-	-	-	-	-	-	-
Citraconate/itaconate	6,345	5,394	4,386	4,292	4,480	3,825		4,189									
Allantoin	30,251	31,039	24,510	28,188	29,898	26,929	16,955	25,201	30,410	31,023	24,323	22,944	27,878	21,986	22,253	23,718	23,744

Table B.5: *Raw data from the MCF derivatization on the polar extracts obtained in the second evaluation of the non-polar metabolite extraction. Raw data are given in pmol (µL injected sample)$^{-1}$.*

	280212-1			280212-2		
Replicate/Metabolite	**1**	**2**	**3**	**1**	**2**	**3**
Malonate	3.94	3.05	3.68	3.40	3.22	3.27
Pyruvate	11.73	11.13	11.34	17.01	15.46	12.75
3-methyl-oxovalerate	8.53	8.44	8.11	9.11	9.49	8.13
Fumarate	14.08	14.66	13.71	15.80	16.17	14.93
d_4-succinateHVL	54.65	58.00	56.55	56.49	62.73	56.20
Lactate/Methylglyoxal	130.05	142.16	138.25	194.66	192.09	175.06
Succinate	9.42	9.89	9.56	11.54	12.86	11.55
Citraconate/Itaconate	7.34	7.79	7.19	8.79	8.34	9.07
Benzoate	4.01	3.91	3.57	3.50	3.57	3.26
Glyoxylate	33.37	36.14	32.92	34.32	35.80	34.95
beta-3-hydroxybutyrate	0.00	5.03	3.52	0.00	0.00	0.00
d_3-alanineHVL	77.22	85.22	81.47	84.79	88.00	83.72
Glycine	98.71	113.99	107.35	127.89	134.35	127.89
O-acetyl-L-serine	61.64	70.20	65.36	80.59	85.98	78.91
Alanine	5.88	7.46	6.72	10.17	11.48	10.00
Phenylacetate	0.63	0.52	0.39	0.39	0.33	0.30
Salicylate	30.15	23.46	23.95	23.94	19.33	19.62
2-aminobutyrate	3.33	3.32	3.23	3.74	3.80	3.54
m-Toluate	0.17	0.12	0.08	0.07	0.07	0.05
beta-alanine	26.75	30.17	28.47	37.25	38.31	36.43
Adipate	7.31	8.80	9.46	8.79	9.35	8.58
d_8-valineHVL	59.43	65.77	65.04	63.95	65.07	61.56
Valine	6.56	7.75	7.57	10.33	10.88	9.85
Alpha-ketoglutarate	43.74	46.66	43.82	68.60	64.07	56.80
beta-hydroxypyruvate	25.23	26.92	24.83	26.98	27.42	24.69
Leucine	18.12	19.53	19.31	23.54	24.39	23.29
Isoleucine	19.29	21.24	20.76	25.79	25.98	24.99
Threonine	51.18	59.75	53.50	66.63	69.09	67.22
Malate	50.91	64.64	58.64	71.11	71.42	63.32
Proline	62.26	68.23	67.02	80.90	82.34	77.81
Citrate	33.60	36.69	37.25	43.69	46.65	44.71
Serine	37.64	40.58	39.51	43.43	43.49	40.51
Allantoin	76.10	82.83	80.12	85.00	98.96	92.56
d_5-GlutamateHVL	711.35	801.99	779.16	839.02	1052.22	958.72
Glutamate	99.65	110.06	108.32	126.32	144.84	134.74
Methionine	11.81	20.66	13.92	16.11	26.25	17.93
Phenylalanine	26.37	27.53	27.49	31.51	32.59	31.15
Cysteine	-	99.58	-	99.58	99.58	-

B.2 TMS derivatization of polar metabolites

TMS derivatization was applied to four of the technical replicates obtained in the first evaluation of the non-polar metabolite extraction. The raw data are given in Table B.6 as peak area for each detected metabolite.

Table B.6: *Raw data from the TMS derivatization on four technical replicates obtained in the first evaluation of the non-polar metabolite extraction. Raw data for the sample extracts and blank samples are given in peak area.*

Sample	**Blank**			**210212-2**	**210212-3**		
Replicate/Metabolite	**1**	**2**	**3**	**3**	**1**	**2**	**3**
L-(+) lactic acid	-	-	-	2,213,730	2,477,960	2,627,620	2,504,960
Urea	-	-	-	613,172	635,031	710,652	687,498
L-serine	-	-	-	-	41,643	48,492	46,947
Glycerol	834,986	759,963	674,495	1,409,160	1,360,390	1,441,060	1,419,290
Phosphoric acid	-	-	-	6,668,050	7,764,480	8,694,750	8,535,380
L-proline	-	-	-	-	154,663	187,594	156,845
Glycine	-	-	-	1,980,930	2,260,610	2,718,750	2,366,180
Porphine	420,799	376,832	342,033	352,304	363,060	316,833	336,729
Fumaric acid	-	-	-	30,174	33,287	33,690	30,593
D-malic acid	-	-	-	99,821	117,536	126,737	108,039
L-glutamic acid (dehydrated)	-	-	-	340,927	400,570	431,866	437,915
L-glutamic acid 1	-	-	-	602,858	853,561	989,755	966,027
Creatinine	-	-	-	24,293	37,132	38,082	41,748
Alpha-ketoglutaric acid	-	-	-	49,984	61,144	60,239	62,167
L-glutamic acid 2	-	-	-	109,748	136,913	141,230	134,235
Glycerol 1-phosphate	-	-	-	145,192	176,206	206,267	183,342
O-phosphocolamine	-	-	-	103,291	142,467	175,087	150,956
Citric acid	-	-	-	21,870	27,773	35,979	36,507
d_{27}-myristic acid	50,435	-	-	43,437,800	42,082,300	48,998,600	48,631,300
Myristic acid	55,973	-	-	-	-	-	-
Methyl-beta-D-galactopyranoside	-	-	-	173,827	152,321	172,399	221,242
D-glucose	-	-	-	390,654	253,770	295,122	306,242
D-allose	-	-	-	68,127	43,481	51,489	51,023
Palmitic acid	203,514	51,472	155,705	282,263	387,472	458,024	468,329
Oleic acid	-	-	44,963	30,460	83,452	100,629	102,135
Stearic acid	91,861	28,746	104,709	139,116	208,260	273,442	283,309
Maltose	-	-	-	235,024	166,163	197,849	278,249
Cholesterol	-	-	-	637,707	730,234	842,769	749,360

B.3 FAME analysis of non-polar metabolites

The formation of FAMEs from lipids was conducted with two non-polar extracts. Table B.7 shows the raw data of the non-polar extracts of sample 210212-1 in the first evaluation of the non-polar metabolite extraction, and the raw data are given in approximately response. Table B.8 and Table B.9 show the raw data given in peak area for the second evaluation of the lipid extraction for sample 280212-1 and sample 280212-2, respectively.

Table B.7: *Raw data from the FAME analysis of the non-polar extracts of sample 210212-1, obtained in the first evaluation of the non-polar metabolite extraction. Raw data are given in approximately response for each detected FAME in the five homogenization rounds.*

Sample	**210212-1 1^{st} round**	**210212-1 2^{nd} round**	**210212-1 3^{rd} round**	**210212-1 4^{th} round**	**210212-1 5^{th} round**
Replicate/FAME	**1**	**1**	**1**	**1**	**1**
Methyl nonanoate	1,500	-	-	-	-
d_{27}-myristic acid	1,000,000	-	-	2,000,000	1,000,000
Methyl tetradecanoate	10,000	-	-	-	-
Unknown 1	6,000	-	-	-	-
Methyl hexadecanoate	400,000	150,000	160,000	120,000	400,000
Unknown 2	150,000	220,000	240,000	250,000	100,000
Methyl octadecanoate	160,000	90,000	90,000	75,000	17,000
Unknown 3	20,000	-	-	-	-
Unknown 4	10,000	-	-	-	-

Table B.8: *Raw data from the FAME analysis on the non-polar extracts of sample 280212-1, obtained in the second evaluation of the non-polar metabolite extraction. Raw data are given in peak area for each detected FAME in the five homogenization rounds.*

	280212-1 1^{st} round		**280212-1 2^{nd} round**		**280212-1 3^{rd} round**		**280212-1 4^{th} round**		**280212-1 5^{th} round**	
Replicate/FAME	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**2**	**3**
Methyl hexadecanoate	113,341	105,393	14,498	31,254	-	-	-	9,351	-	-
Unknown (RT 21.107)	158,782	143,505	35,700	57,368	-	18,478	9,544	15,431	-	-
Methyl octadecanoate	68,064	68,340	5,945	16,270	-	-	-	-	-	-
d_{27}-myristic acid	6,988,020	7,737,240	11,803,400	6,408,190	4,157,550	6,788,310	8,725,850	10,075,900	10,778,100	10,780,900

Table B.9: *Raw data from the FAME analysis on the non-polar extracts of sample 280212-2, obtained in the second evaluation of the non-polar metabolite extraction. Raw data are given in peak area for each detected FAME in the five homogenization rounds.*

	280212-2 1^{st} round		**280212-2 2^{nd} round**	**280212-2 3^{rd} round**	**280212-2 4^{th} round**	**280212-2 5^{th} round**	
Replicate/FAME	**1**	**2**	**1**	**1**	**1**	**1**	**2**
Methyl hexadecanoate	338,670	337,975	13,572	12,632	-	-	-
Unknown (RT 21.107)	265,909	261,279	34,888	26,363	-	-	-
Methyl octadecanoate	219,969	223,070	-	-	-	-	-
d_{27}-myristic acid	11,596,700	11,281,900	7,652,700	11,341,100	6,513,320	11,246,200	5,861,710

C Results from the optimization experiments

The results from the optimization experiments with the Preceellys 24 homogenizer are based on the raw data given in Appendix B. Section C.1, section C.2 and section C.3 show the results from the MCF derivatization, the TMS derivatization and the derivatization of lipids to FAMEs, respectively.

C.1 MCF derivatization of polar metabolites

The results from the MCF derivatization are given in pM (mg tissue sample)$^{-1}$, except the raw data given in Table C.4, where the results are given in response (mg tissue sample)$^{-1}$. Normalization against internal standards was made if internal standards were added before the extraction, before derivatization, or before both. After internal standard normalization, the resulting data were normalized against the volume of the polar extracts and the weight of the tissue samples. Table C.1, Table C.2 and Table C.3 show the results from the first evaluation of the polar metabolite extraction for one, two and three homogenization rounds, respectively. Table C.4 show the results from the second evaluation of the polar metabolite extraction. Table C.5 show the results from the second evaluation of the non-polar metabolite extraction.

Table C.1: *Results from the MCF derivatization on the polar extracts exposed to one homogenization round in the evaluation of the number of necessary homogenization rounds (the first evaluation of the polar metabolite extraction). The results are given in pM (mg tissue sample)$^{-1}$. Averages and standard deviations are given.*

	1^{st} homogenization round					
Sample /Metabolite	**180112-1**	**180112-2**	**180112-3**	**Average**	**Standard deviation**	**Standard deviation [%]**
4-methylvalerate	112	91	76	93	18	19.5
Malonate	167	358	234	253	97	38.2
Pyruvate	2,055	2,679	1,424	2,053	628	30.6
3-methyl-oxovalerate	403	354	469	409	58	14.1
Fumarate	1,950	1,869	1,600	1,807	183	10.1
Lactate/Methylglyoxal	430,795	996,586	547,093	658,158	298,800	45.4
Succinate	3,943	8,982	4,506	5,811	2,761	47.5
Benzoate	223	247	155	209	48	22.9
beta-3-hydroxybutyrate	6,076	7,025	5,831	6,311	630	10.0
Alanine	48,236	72,053	52,057	57,449	12,791	22.3
Glycine	59,023	67,372	63,964	63,453	4,198	6.6
O-acetyl-L-serine	19,521	24,906	17,046	20,491	4,018	19.6
Phenylacetate	96	-	23	59	52	86.7
2-aminobutyrate	190	197	182	190	7	4.0
m-Toluate	223	159	121	168	51	30.7
beta-alanine	4,363	4,918	4,666	4,649	278	6.0
Adipate	260	523	198	327	173	52.9
Valine	5,211	7,815	6,119	6,382	1,322	20.7
2-isopropylmalate	337	565	382	428	121	28.3
beta-hydroxypyruvate	897	1,100	1,276	1,091	190	17.4
alpha-ketoadipate	951	15,675	541	5,722	8,622	150.7
Leucine	4,487	8,089	5,002	5,859	1,948	33.2
Isoleucine	2,481	3,819	2,932	3,077	681	22.1
Malate	4,076	3,000	2,447	3,174	828	26.1
Oxaloacetate	2,481	2,358	3,346	2,728	538	19.7
Threonine	9,160	8,919	10,679	9,586	954	10.0
Proline	13,255	18,288	16,683	16,075	2,571	16.0
L-homoserine	352	415	274	347	71	20.4
Aspartate	791	1,270	687	916	311	34.0
Citrate	7,198	5,568	5,218	5,994	1,057	17.6
5-aminovalerate	239	277	214	243	32	13.0
Anthralinate	106	103	79	96	14	15.0
Serine	4,555	7,320	5,218	5,698	1,444	25.3
Allantoin	20,569	57,871	47,652	42,031	19,276	45.9
Glutamate	62,266	74,816	58,825	65,302	8,417	12.9
N-Acetyl-L-glutamate	1,783	1,949	1,402	1,711	281	16.4
Methionine	1,119	1,713	1,429	1,420	297	20.9
Hydroxyproline	189,998	188,479	259,130	212,535	40,359	19.0
Cysteine	31,785	48,212	36,349	38,782	8,479	21.9
Phenylalanine	2,545	3,985	3,108	3,213	726	22.6
Putrescine	845	1,166	748	920	219	23.8
Hippurate	641	655	660	652	10	1.6
4-imidazoleacrylate	112	94	56	87	28	32.3
Histamine	2,154	1,728	1,741	1,874	242	12.9
Ornithine	629	1,217	798	881	303	34.4
Lysine	10,690	16,014	12,653	13,119	2,693	20.5
Histidine	503	759	653	638	128	20.1
Tyrosine	1,814	2,925	2,223	2,321	562	24.2
Thryptophane	791	1,192	904	962	207	21.5

Table C.2: *Results from the MCF derivatization on the polar extracts exposed to two homogenization rounds in the evaluation of the number of necessary homogenization rounds (the first evaluation of the polar metabolite extraction). The results are given in pM (mg tissue sample)$^{-1}$. Averages and standard deviations are given.*

	2^{nd}homogenization round					
Sample /Metabolite	**180112-1**	**180112-2**	**180112-3**	**Average**	**Standard deviation**	**Standard deviation [%]**
4-methylvalerate	98	91	75	88	12	13.7
Malonate	181	357	221	253	92	36.4
Pyruvate	1,041	1,022	643	902	224	24.9
3-methyl-oxovalerate	169	224	117	170	54	31.6
Fumarate	532	683	311	508	187	36.9
Lactate/Methylglyoxal	99,480	263,163	66,431	143,025	105,347	73.7
Succinate	974	2,986	835	1,599	1,204	75.3
Benzoate	202	271	164	212	54	25.6
beta-3-hydroxybutyrate	1,587	2,358	5,695	3,213	2,184	68.0
Alanine	9,479	16,023	6,210	10,571	4,997	47.3
Glycine	11,641	16,404	8,579	12,208	3,943	32.3
O-acetyl-L-serine	5,408	8,445	4,178	6,010	2,196	36.5
Phenylacetate	86	-	-	86	-	-
2-aminobutyrate	76	82	51	69	16	23.7
m-Toluate	191	175	126	164	34	20.6
beta-alanine	1,134	1,388	783	1,102	304	27.6
Adipate	282	513	316	371	125	33.6
Valine	1,341	2,124	1,032	1,499	563	37.5
2-isopropylmalate	-	134	109	121	18	14.6
beta-hydroxypyruvate	376	452	252	360	101	28.0
alpha-ketoadipate	821	5,180	713	2,238	2,548	113.9
Leucine	1,113	2,009	840	1,321	611	46.3
Isoleucine	665	1,136	544	781	313	40.0
Malate	635	600	231	488	224	45.9
Oxaloacetate	370	740	610	574	188	32.7
Threonine	1,946	3,247	1,550	2,248	888	39.5
Proline	2,766	4,417	2,118	3,100	1,185	38.2
L-homoserine	193	301	219	238	56	23.7
Aspartate	215	434	134	261	155	59.4
Citrate	1,273	1,709	677	1,220	518	42.5
5-aminovalerate	116	243	163	174	64	37.0
Anthralinate	122	148	89	120	30	24.9
Serine	1,372	2,690	1,119	1,727	843	48.8
Allantoin	8,551	13,193	8,468	10,070	2,704	26.9
Glutamate	9,138	18,724	6,469	11,444	6,445	56.3
N-Acetyl-L-glutamate	671	1,155	638	821	289	35.2
Methionine	295	515	260	357	138	38.7
Hydroxyproline	74,878	51,663	96,682	74,408	22,513	30.3
Cysteine	6,417	12,709	4,976	8,034	4,113	51.2
Phenylalanine	728	1,207	613	849	315	37.1
Putrescine	444	863	549	619	218	35.2
Hippurate	617	865	534	672	173	25.7
4-imidazoleacrylate	74	88	38	66	26	39.0
Histamine	1,309	2,088	371	1,256	860	68.5
Ornithine	259	516	271	348	145	41.7
Lysine	3,225	5,080	2,901	3,735	1,176	31.5
Histidine	153	255	116	175	72	41.4
Tyrosine	473	830	380	561	237	42.3
Thryptophane	316	378	240	312	69	22.1

Table C.3: *Results from the MCF derivatization on the polar extracts exposed to three homogenization rounds in the evaluation of the number of necessary homogenization rounds (the first evaluation of the polar metabolite extraction). The results are given in pM (mg tissue sample)$^{-1}$. Averages and standard deviations are given.*

	3rd homogenization round					
Sample	**180112-1**	**180112-2**	**180112-3**			
/Metabolite				**Average**	**Standard deviation**	**Standard deviation [%]**
4-methylvalerate	83	82	71	79	7	8.4
Malonate	200	258	219	225	30	13.2
Pyruvate	582	587	406	525	103	19.7
3-methyl-oxovalerate	177	171	122	157	30	19.0
Fumarate	150	172	96	139	39	28.1
Lactate/Methylglyoxal	19,791	46,407	14,715	26,971	17,023	63.1
Succinate	512	981	484	659	279	42.3
Benzoate	166	205	160	177	24	13.8
beta-3-hydroxybutyrate	564	685	656	635	64	10.0
Alanine	1,826	2,856	1,263	1,982	807	40.7
Glycine	2,466	3,256	1,971	2,564	648	25.3
O-acetyl-L-serine	805	1,077	1,455	1,112	326	29.3
Phenylacetate	49	-	20	35	20	58.5
2-aminobutyrate	41	15	11	22	16	72.4
m-Toluate	143	153	126	141	13	9.5
beta-alanine	299	357	229	295	64	21.8
Adipate	259	319	396	325	69	21.1
Valine	403	577	343	441	122	27.6
2-isopropylmalate	-	-	-	-	-	-
beta-hydroxypyruvate	164	182	205	184	20	11.1
alpha-ketoadipate	856	1,562	559	992	515	51.9
Leucine	299	475	231	335	126	37.7
Isoleucine	288	440	272	333	93	28.0
Malate	88	87	41	72	27	36.8
Oxaloacetate	581	427	-	504	109	21.6
Threonine	534	681	450	555	117	21.1
Proline	575	828	444	615	195	31.7
L-homoserine	177	223	213	205	24	11.7
Aspartate	142	140	99	127	24	19.0
Citrate	301	305	150	252	88	35.1
5-aminovalerate	159	224	171	185	34	18.7
Anthralinate	159	175	139	158	18	11.6
Serine	520	834	422	592	215	36.3
Allantoin	5,817	8,954	16,208	10,326	5,330	51.6
Glutamate	2,157	3,237	1,530	2,308	863	37.4
N-Acetyl-L-glutamate	461	628	537	542	84	15.5
Methionine	102	143	93	113	27	23.5
Hydroxyproline	39,117	52,150	58,506	49,924	9,884	19.8
Cysteine	1,411	2,851	1,272	1,845	874	47.4
Phenylalanine	237	341	209	262	69	26.5
Putrescine	489	800	554	615	164	26.7
Hippurate	676	789	587	684	101	14.8
4-imidazoleacrylate	73	47	37	52	19	35.7
Histamine	2,953	-	538	1,746	1,708	97.9
Ornithine	172	239	171	194	39	20.0
Lysine	1,121	1,474	1,253	1,282	178	13.9
Histidine	-	-	-	-	-	-
Tyrosine	129	172	107	136	33	24.4
Thryptophane	154	-	44	99	78	78.9

Table C.4: *Results from the MCF derivatization on the polar extracts obtained in the second evaluation of the polar metabolite extraction. The polar extracts obtained after the non-polar metabolite extraction are termed with "check". The results are given in response (mg tissue sample)$^{-1}$. Averages and standard deviations are given.*

							130212			130212-check		
/Metabolite	130212-1	130212-2	130212-3	130212-check-1	130212-check-2	130212-check-3	Average	Standard deviation	Standard deviation [%]	Average	Standard deviation	Standard deviation [%]
4-methylvalerate	1,259	577	319	-	-	-	718	486	67.6	-	-	-
Pyruvate	13,790	21,842	41,400	219	238	243	25,677	14,199	55.3	233	13	5.6
3-methyl-oxovalerate	5,013	3,638	6,245	-	-	-	4,965	1,304	26.3	-	-	-
Fumarate	83,164	62,980	110,651	-	-	-	85,598	23,928	28.0	-	-	-
Lactate/Methylglyoxal	500,413	397,915	665,661	5,048	4,841	4,703	521,330	135,093	25.9	4,864	174	3.6
Succinate	87,041	77,922	108,624	7,210	9,906	-	91,196	15,767	17.3	8,558	1,907	22.3
Benzoate	24,584	13,270	12,283	-	-	-	16,712	6,835	40.9	-	-	-
Alanine	195,603	165,563	207,265	-	-	-	189,477	21,516	11.4	-	-	-
Glyoxylate	6,611	4,846	5,868	-	-	-	5,775	887	15.4	-	-	-
Glycine	934,291	934,073	1,348,812	19,286	20,809	18,192	1,072,392	239,387	22.3	19,429	1,315	6.8
O-acetyl-L-serine	28,512	31,026	40,784	1,285	1,545	1,353	33,441	6,483	19.4	1,394	135	9.7
2-aminobutyrate	6,839	5,815	6,167	-	-	-	6,274	521	8.3	-	-	-
Salicylate	14,907	8,131	6,635	-	-	-	9,891	4,408	44.6	-	-	-
beta-alanine	37,243	35,065	53,374	-	-	-	41,894	10,001	23.9	-	-	-
Adipate	28,037	35,158	39,849	-	-	-	34,348	5,947	17.3	-	-	-
Valine	69,628	68,571	98,703	1,163	1,279	1,148	78,967	17,100	21.7	1,197	72	6.0
beta-hydroxypyruvate	4,642	7,265	11,595	203	201	199	7,834	3,512	44.8	201	2	0.9
Leucine	19,083	20,247	30,748	-	-	-	23,359	6,425	27.5	-	-	-
Isoleucine	3,339	2,820	4,300	-	-	-	3,486	751	21.5	-	-	-
Malate	57,617	73,003	67,361	728	789	541	65,994	7,784	11.8	686	129	18.8
Oxaloacetate	681	984	1,178	-	-	-	947	250	26.4	-	-	-
Threonine	6,825	4,948	7,122	-	-	-	6,298	1,179	18.7	-	-	-
Proline	55,360	41,388	70,220	-	-	-	55,656	14,418	25.9	-	-	-
Aspartate	590	633	840	-	-	223	688	133	19.4	-	-	-
Citrate	19,220	17,055	22,952	-	-	-	19,742	2,983	15.1	-	-	-
Serine	10,993	14,201	16,555	156	211	176	13,916	2,791	20.1	181	28	15.3
Glutamate	358,980	384,203	434,474	1,625	1,251	1,427	392,552	38,433	9.8	1,434	187	13.1
N-Acetyl-L-glutamate	5,644	7,427	7,055	2,388	2,422	2,153	6,709	940	14.0	2,321	147	6.3
Methionine	60,133	61,918	91,791	247	183	236	71,281	17,785	25.0	222	34	15.4
Cysteine	36,901	40,632	86,629	-	-	-	54,721	27,696	50.6	-	-	-
Phenylalanine	37,287	38,221	57,229	239	144	253	44,245	11,254	25.4	212	59	27.8
Histamine	392	287	415	-	-	-	364	68	18.6	-	-	-
p-coumarate	2,011	1,207	1,092	-	-	-	1,437	501	34.8	-	-	-
Lysine	16,429	16,500	20,894	-	-	-	17,941	2,558	14.3	-	-	-
Citraconate/itaconate	7,302	7,652	8,303	-	-	-	7,752	508	6.6	-	-	-
Allantoin	38,123	48,035	47,717	14,389	14,231	12,548	44,625	5,633	12.6	13,723	1,020	7.4

Table C.5: *Results from the MCF derivatization on the polar extracts obtained in the second evaluation of the non-polar metabolite extraction. The results are given in pM (mg tissue sample)$^{-1}$. Averages and standard deviations of each detected metabolite in the two samples are given.*

			280212		
/Metabolite	**280212-1**	**280212-2**	**Average**	**Standard deviation**	**Standard deviation [%]**
Malonate	5,120	3,342	4,231	1,258	29.7
Pyruvate	16,426	15,274	15,850	814	5.1
3-methyl-oxovalerate	12,045	9,027	10,536	2,134	20.3
Fumarate	20,387	15,840	18,114	3,215	17.8
Lactate/Methylglyoxal	197,167	189,753	193,460	5,243	2.7
Succinate	13,867	12,139	13,003	1,222	9.4
Citraconate/Itaconate	10,723	8,847	9,785	1,327	13.6
Benzoate	5,519	3,487	4,503	1,437	31.9
Glyoxylate	49,204	35,487	42,346	9,699	22.9
beta-3-hydroxybutyrate	6,162	-	6,162	-	-
Glycine	153,735	131,770	142,753	15,532	10.9
O-acetyl-L-serine	94,725	82,913	88,819	8,353	9.4
Alanine	9,632	10,692	10,162	749	7.4
Phenylacetate	740	343	542	281	51.9
Salicylate	37,258	21,242	29,250	11,325	38.7
2-aminobutyrate	4,743	3,744	4,244	706	16.6
m-Toluate	179	62	121	82	68.1
beta-alanine	41,015	37,826	39,420	2,254	5.7
Adipate	12,285	9,022	10,654	2,307	21.7
Valine	10,507	10,491	10,499	11	0.1
Alpha-ketoglutarate	64,475	63,995	64,235	340	0.5
beta-hydroxypyruvate	36,978	26,710	31,844	7,261	22.8
Leucine	27,361	24,052	25,706	2,340	9.1
Isoleucine	29,442	25,925	27,683	2,487	9.0
Threonine	78,989	68,544	73,766	7,386	10.0
Malate	83,673	69,528	76,600	10,003	13.1
Proline	94,875	81,418	88,146	9,515	10.8
Citrate	51,659	45,612	48,635	4,276	8.8
Serine	56,555	43,039	49,797	9,557	19.2
Allantoin	114,827	93,395	104,111	15,155	14.6
Glutamate	152,767	137,097	144,932	11,081	7.6
Methionine	22,284	20,360	21,322	1,360	6.4
Phenylalanine	39,100	32,171	35,636	4,899	13.7
Cysteine	143,500	100,900	122,200	30,123	24.7

C.2 TMS derivatization of polar metabolites

The results from the TMS derivatization (Table C.6) on four of the technical replicates obtained in the first evaluation of the non-polar metabolite extraction, are given in peak area (mg tissue sample)$^{-1}$. Metabolites detected in both the blank samples and the sample extracts were not included in the results in Table C.6. Normalization was made against the internal standard d_{27}-myristic acid. After internal standard normalization, the resulting data were normalized against the weight of the tissue samples to give peak area (mg tissue sample)$^{-1}$.

Table C.6: *Results from the TMS derivatization on the polar extracts obtained in the first evaluation of the non-polar metabolite extraction. The results are given as peak area (mg tissue sample)$^{-1}$. Average and standard deviation of each detected metabolite in the four technical replicates are given.*

			210212		
/Metabolite	210212-2	210212-3	Average	Standard deviation	Standard deviation [%]
L-(+) lactic acid	85,319	96,505	90,912	7,910	8.7
Urea	23,632	25,730	24,681	1,483	6.0
L-serine	-	1,733	1,733	-	-
Phosphoric acid	256,993	316,234	286,613	41,890	14.6
L-proline	-	6,313	3,156	4,464	141.4
Glycine	76,347	92,882	84,614	11,692	13.8
Fumaric acid	1,163	1,240	1,202	55	4.5
D-malic acid	3,847	4,472	4,160	442	10.6
L-glutamic acid (dehydrated)	13,140	16,085	14,612	2,082	14.3
L-glutamic acid 1	23,235	35,507	29,371	8,678	29.5
Creatinine	936	1,482	1,209	386	31.9
Alpha-ketoglutaric acid	1,926	2,330	2,128	286	13.4
L-glutamic acid 2	4,230	5,234	4,732	710	15.0
Glycerol 1-phosphate	5,596	7,159	6,377	1,105	17.3
O-phosphocolamine	3,981	5,921	4,951	1,372	27.7
Citric acid	843	1,262	1,052	296	28.2
Methyl-beta-D-galactopyranoside	6,699	6,877	6,788	125	1.8
D-glucose	15,056	10,797	12,927	3,012	23.3
D-allose	2,626	1,844	2,235	553	24.7
Maltose	9,058	8,065	8,562	702	8.2
Cholesterol	24,578	29,396	26,987	3,407	12.6

C.3 FAME analysis of non-polar metabolites

The results from the FAME analysis on the non-polar extracts obtained in the second evaluation of the non-polar metabolite extraction, are given in Table C.7 and Table C.8. Table C.7 shows the results from the first and the second homogenization round, while Table C.8 gives the results from the third and the fourth homogenization round. Results from the fifth homogenization round are excluded, since it is clear from Table B.8 and Table B.9 in Appendix B that no FAMEs are detected in that round. The results are given as peak area (mg tissue sample)$^{-1}$ for each detected FAME. Normalization was made against the internal standard d_{27}-myristic acid. After internal standard normalization, the resulting data were normalized against the weight of the tissue samples to give peak area (mg tissue sample)$^{-1}$.

Table C.7: *Results from the FAME analysis on the non-polar extracts of the samples 280212-1 and 280212-2, obtained in the second evaluation of the non-polar metabolite extraction. The results are given in peak area (mg tissue sample)$^{-1}$ for each detected FAME in the first and the second homogenization round. Average and standard deviation of each detected metabolite in the two samples are given.*

	1st homogenization round					**2nd homogenization round**				
Sample	**280212-1**	**280212-2**				**280212-1**	**280212-2**			
/FAME			**Average**	**Standard deviation**	**Standard deviation [%]**			**Average**	**Standard deviation**	**Standard deviation [%]**
Methyl hexadecanoate	4,511	6,327	5,419	1,284	23.7	763	379	571	271	47.5
Unknown (RT 21.107)	6,234	4,930	5,582	923	16.5	1,552	975	1,264	408	32.3
Methyl octadecanoate	2,813	4,143	3,478	940	27.0	370	-	370	-	-

Table C.8: *Results from the FAME analysis on the non-polar extracts of the samples 280212-1 and 280212-2, obtained in the second evaluation of the non-polar metabolite extraction. The results are given in peak area (mg tissue sample)$^{-1}$ for each detected FAME in the third and the fourth homogenization round. Average and standard deviation of each detected metabolite in the two samples are given.*

	3rd homogenization round					**4th homogenization round**				
Sample	**280212-1**	**280212-2**				**280212-1**	**280212-2**			
/FAME			**Average**	**Standard deviation**	**Standard deviation [%]**			**Average**	**Standard deviation**	**Standard deviation [%]**
Methyl hexadecanoate	-	238	238	-	-	151	-	151	-	-
Unknown (RT 21.107)	513	497	505	11	2.2	403	-	403	-	-
Methyl octadecanoate	-	-	-	-	-	-	-	-	-	-

D FAME analysis by GC-Q-MS

The experimental protocol of the FAME analysis of non-polar extracts obtained in the optimization experiments is given in section 2.7.

D.1 Chromatogram from the FAME analysis

Figure D.1 shows the chromatogram of the FAME analysis by the GC-Q-MS system, where four FAMEs are detected (the internal standard d_{27}-myristic acid, methyl hexadecanoate, methyl octadecanoate and an unknown FAME). The scan of the unknown FAME is given in Figure D.2. Table D.1 gives an overview of the identification and retention time of the four FAMEs. The two peaks in the chromatogram identified as FAMEs (methyl hexadecanoate and methyl octadecanoate) were identified due to the presence in the FAME standard (Table A.2 in Appendix A), while the peak of the internal standard was identified based on the chromatogram resulting from the injection of only d_{27}-myristic acid (chromatograms of the FAME standard and the d_{27}-myristic acid are not shown). The peak of the unknown FAME was not present in neither the FAME standard or the blank sample (chromatogram of the blank sample is not shown), but based on the scan in Figure D.2 it is assumed to be a FAME. The peaks in the chromatogram not identified as FAMEs were shown to be present in the blank sample, and therefore not targeted as FAMEs.

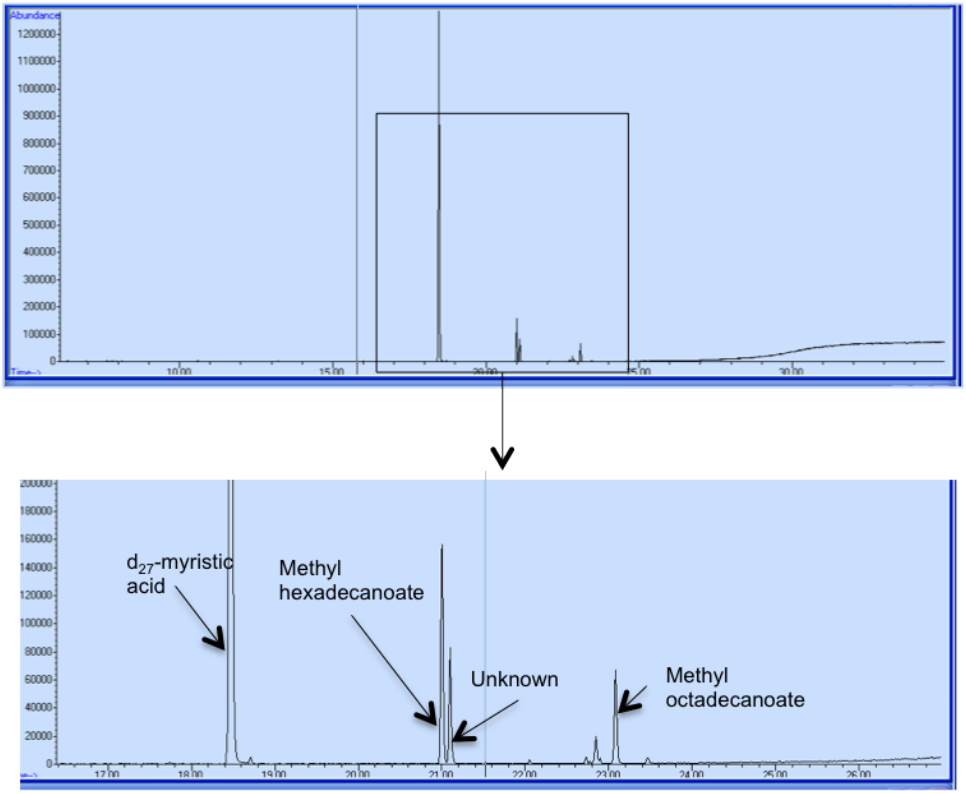

Figure D.1: *Chromatogram from the FAME analysis by the GC-Q-MS system. The peaks representing the internal standard d_{27}-myristic acid, methyl hexadecanoate, methyl octadecanoate and the unknown FAME are indicated by narrows.*

Table D.1: *Identification and retention time of the four FAMEs detected in the FAME analysis by the GC-QqQ-MS system.*

FAME	**Retention time [min]**	**Comment**
d_{27}-myristic acid	18.468	Internal standard
Methyl hexadecanoate	21.007	Methyl ester of palmitate (C16:0 fatty acid)
Unknown	21.107	Assumed to be a FAME
Methyl octadecanoate	23.093	Methyl ester of stearate (C18:0 fatty acid)

D.2 Scan of the unknown FAME

The scan of the unknown FAME detected in the non-polar extracts obtained in the second evaluation of the non-polar metabolite extraction, is given in Figure D.2. The FAME is detected at 21.107 min.

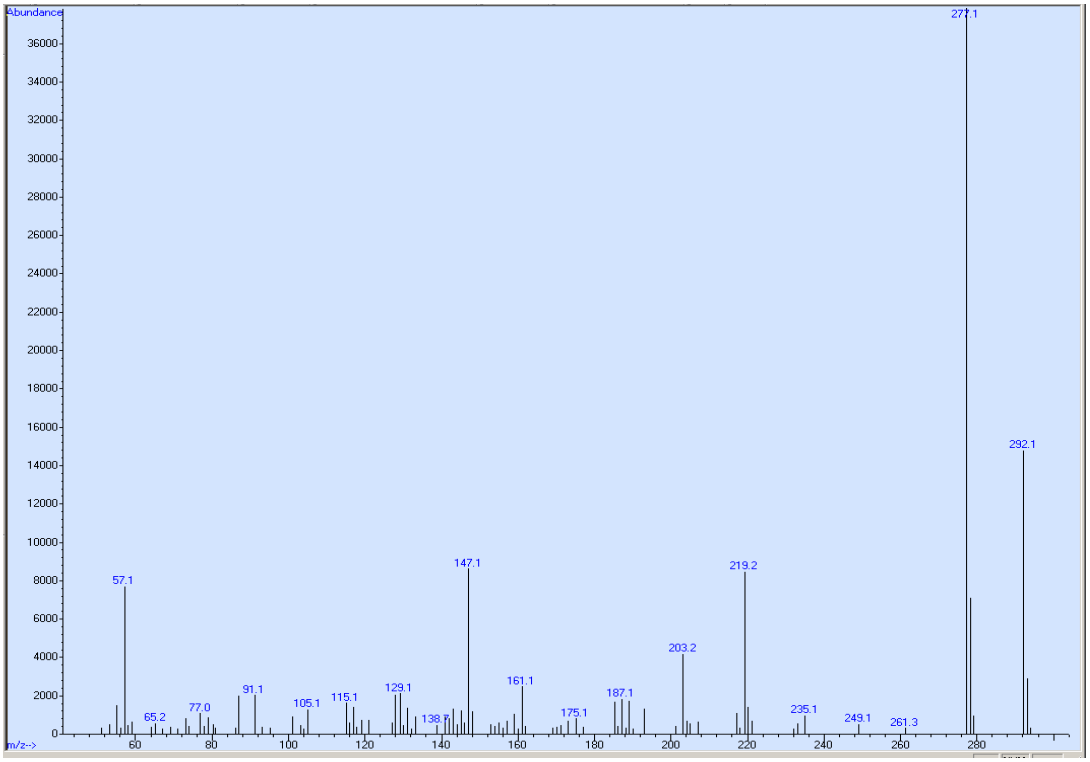

Figure D.2: *The scan of the unknown FAME detected at 21.107 min.*

E Polar metabolite profiling of breast cancer xenografts by GC-QqQ-MS

The metabolite extraction of luminal-like, basal-like and MK-2206 treated basal-like xenografts (MAS98.06, MAS98.12 and MK-2206 treated MAS98.12 xenografts, respectively) were performed as described in section 2.4. The polar extracts were analyzed absolute quantitative by MCF GC-QqQ-MS.

E.1 Raw data from the polar metabolite profiling

The raw data from the MCF derivatization on the polar extracts obtained in the metabolite profiling experiments are given in pmol (μL injected sample)$^{-1}$, after the absolute quantitative analysis using the Mass Hunter Quantitative Analysis for QqQ software. Table E.1 shows the raw data from the evaluation of the standard deviations of the MAS98.06 and MAS98.12 xenografts. Table E.2 show the raw data of the MAS98.06 and MAS98.12 xenografts, while Table E.3 shows the raw data of the MK-2206 treated MAS98.12 xenograft.

Table E.1: *Raw data from the MCF derivatization on the polar extracts of the MAS98.06 (Lum) and MAS98.12 (Bas) xenografts, where the standard deviations of each xenograft were evaluated. Raw data are given in pmol (µL injected sample)$^{-1}$.*

Sample	**140312-Bas1**			**140312-Bas2**			**140312-Bas3**			**140312-Lum1**			**140312-Lum2**			**140312-Lum3**		
Replicate/Metabolite	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
4-methylvalerate	0.14	0.12	0.13	0.13	0.12	0.12	0.13	0.13	0.13	0.12	0.13	0.14	0.14	0.13	0.14	0.13	0.14	0.15
Pyruvate	1.03	1.36	3.44	0.70	1.23	1.04	1.77	1.96	1.65	0.08	1.03	1.80	1.30	1.54	1.40	2.07	1.74	3.30
3-methyl-oxovalerate	0.28	0.22	0.32	0.18	0.21	0.20	0.22	0.24	0.21	0.15	0.23	0.23	0.40	0.24	0.31	0.26	0.29	0.47
Fumarate	1.35	1.20	1.70	0.62	0.91	0.79	1.40	1.59	1.37	1.20	0.59	0.77	0.84	0.76	0.72	0.75	0.80	0.97
Lactate/Methylglyoxal	74.14	54.43	98.56	42.36	52.76	46.74	70.39	73.85	54.24	44.24	39.62	52.14	53.83	43.77	57.10	52.70	55.23	65.43
Succinate	1.46	1.31	1.52	0.67	1.24	1.12	0.98	1.08	0.92	0.73	1.20	1.86	1.81	1.38	1.03	1.47	1.49	1.91
Citraconate/Itaconate	0.47	0.32	0.48	0.27	0.26	0.24	0.29	0.28	0.23	0.17	0.23	0.26	0.27	0.21	0.25	0.24	0.24	0.25
Benzoate	0.86	0.73	0.64	0.54	0.69	0.64	0.56	0.65	0.54	0.64	0.57	0.61	0.63	0.55	0.52	0.55	0.56	0.74
Citramalate	0.19	0.18	0.15	0.12	0.14	0.14	0.14	0.14	0.12	0.13	0.13	0.14	0.13	0.12	0.11	0.12	0.11	0.12
Glyoxylate	1.94	1.76	1.49	2.19	1.80	1.65	1.47	1.21	0.83	-	0.61	1.48	1.08	1.46	1.49	1.25	1.08	1.98
beta-3-hydroxybutyrate	0.42	0.44	0.59	0.40	0.48	0.41	0.53	0.53	0.54	0.86	0.80	0.92	1.06	1.01	1.02	1.30	1.18	1.32
Glycine	61.93	55.53	95.73	30.58	37.55	30.04	46.69	49.74	33.30	11.46	10.28	18.03	13.74	12.65	10.97	13.21	10.21	14.40
Alanine	13.86	12.50	20.25	6.91	9.03	7.73	10.81	11.64	9.71	19.47	19.23	28.20	23.74	22.32	22.94	23.90	23.36	22.27
O-acetyl-L-serine	6.45	5.86	11.54	2.21	4.07	3.06	5.41	6.21	5.33	11.52	10.04	18.73	11.24	13.83	14.04	13.52	13.37	21.20
Nicotinate	0.55	0.51	0.63	0.45	0.45	0.48	0.59	0.75	0.87	-	-	-	-	-	-	-	-	-
Phenylacetate	0.16	0.25	0.13	0.17	0.15	0.14	0.15	0.19	0.15	0.17	0.17	0.18	0.26	0.20	0.19	0.20	0.21	0.24
Salicylate	2.29	1.02	1.92	0.85	2.56	2.05	2.69	1.99	2.07	2.03	2.48	1.97	3.84	1.38	2.88	3.23	2.79	4.97
2-aminobutyrate	0.27	0.25	0.30	0.18	0.20	0.20	0.22	0.24	0.21	0.32	0.30	0.35	0.35	0.33	0.37	0.33	0.32	0.39
m-Toluate	0.19	0.17	0.15	0.15	0.18	0.16	0.17	0.17	0.15	0.16	0.17	0.17	0.21	0.20	0.21	0.24	0.21	0.23
Beta-alanine	3.68	3.05	4.59	2.24	2.56	2.25	3.00	3.46	2.96	0.48	0.45	0.50	0.53	0.51	0.54	0.44	0.48	0.37
OH-Glutarate	0.42	0.39	0.42	0.24	0.25	0.31	0.31	0.37	0.18	-	-	-	-	-	-	-	-	-
Adipate	0.26	0.24	0.21	0.18	0.23	0.20	0.23	0.21	0.20	0.20	0.19	0.22	0.21	0.22	0.25	0.18	0.20	0.21
Valine	4.58	2.09	3.09	1.24	1.61	1.41	1.90	2.07	1.58	3.01	2.72	3.98	3.48	3.47	3.79	3.30	3.26	4.24
alpha-ketoglutarate	3.94	3.92	6.89	2.72	2.76	2.69	2.99	2.89	2.31	-	-	-	-	-	-	-	-	-
beta-hydroxypyruvate	0.98	0.84	2.00	0.39	0.46	0.44	0.66	0.68	0.51	0.09	0.46	0.59	0.55	0.44	0.65	0.48	0.64	0.52
alpha-ketoadipate	0.40	0.17	0.17	0.18	0.21	0.20	0.20	0.16	0.15	0.19	0.15	0.16	0.15	0.12	0.16	0.12	-	0.20
Leucine	0.75	0.63	0.97	0.32	0.49	0.39	0.60	0.70	0.51	0.70	0.66	1.12	1.02	0.96	1.05	0.96	0.96	1.29
gamma-aminobutyrate	0.99	0.98	0.90	0.93	0.90	0.87	1.02	1.03	1.04	1.16	0.97	1.04	1.01	1.27	1.18	1.21	1.36	1.18
Threonine	3.30	3.13	4.69	1.59	2.11	1.99	2.81	3.03	2.56	2.77	2.90	4.74	4.28	4.04	4.25	3.96	4.21	5.23
Malate	4.00	3.24	5.98	1.42	1.76	1.48	2.76	2.91	2.17	2.29	1.82	2.64	2.17	1.80	2.33	1.90	1.81	2.47
Proline	12.50	11.00	17.36	6.54	8.72	7.51	10.41	11.53	9.22	9.98	10.45	15.76	12.20	11.49	12.04	13.28	13.01	17.25
Aspartate	0.72	0.55	0.65	0.24	0.34	0.36	0.44	0.40	0.33	2.96	2.23	3.82	3.79	2.96	2.30	2.53	2.60	3.32
Citrate	4.00	4.43	5.13	1.80	2.84	2.94	3.29	3.89	3.33	0.79	1.42	3.14	2.68	1.88	1.55	2.04	2.08	2.52
Serine	1.87	1.74	2.89	0.80	1.00	1.01	1.69	2.01	1.51	10.30	2.12	2.79	3.04	2.23	2.46	2.35	2.51	2.77
Anthralinate	0.15	0.10	0.10	0.07	0.10	0.09	0.09	0.08	0.09	0.09	0.11	0.09	0.09	0.05	0.07	0.07	0.06	0.09
Allantoin	68.33	64.91	43.38	37.10	43.65	48.54	32.67	41.03	29.22	73.12	35.77	45.70	40.03	58.50	25.21	28.39	32.43	37.70
N-Acetyl-L-glutamate	5.49	4.14	3.57	3.06	4.69	4.54	3.25	3.77	2.97	7.85	3.97	4.74	5.03	2.80	2.46	3.85	2.91	4.78
Glutamate	69.47	63.97	86.69	26.97	40.98	39.86	52.37	55.07	45.00	25.39	19.10	29.45	28.94	22.18	17.96	21.46	24.37	24.56
Methionine	0.64	0.54	0.77	0.33	0.45	0.39	0.52	0.59	0.48	0.58	0.66	0.88	0.80	0.77	0.81	0.76	0.79	0.99
Hippurate	0.15	0.15	0.14	0.10	0.16	0.17	0.23	0.25	0.30	0.23	0.20	0.22	0.23	0.28	0.25	0.34	0.31	0.43
Phenylalanine	1.05	0.95	1.37	0.56	0.78	0.66	0.88	1.02	0.82	1.06	0.99	1.42	1.27	1.22	1.29	1.14	1.11	1.52
p-coumarate	0.07	0.06	0.04	0.02	0.03	0.02	0.02	0.02	0.02	-	-	-	-	-	-	-	-	-
Ornithine	1.71	1.39	1.79	1.06	1.10	1.11	1.38	1.53	1.18	1.88	2.24	1.88	1.99	1.85	2.16	2.00	2.17	2.43
Lysine	6.13	5.59	7.53	3.53	3.93	3.69	5.03	5.52	4.51	3.94	4.20	4.45	4.55	4.12	4.68	4.50	4.53	5.77

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Table E.1 – *Continued from previous page*

Sample	140312-Bas1			140312-Bas2			140312-Bas3			140312-Lum1			140312-Lum2			140312-Lum3		
Replicate/Metabolite	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Tyrosine	1.78	1.14	1.81	0.87	1.06	1.12	1.31	1.26	1.12	1.23	1.48	1.53	1.47	1.43	1.44	1.36	1.04	1.52
Cysteine	11.22	8.34	8.21	10.53	7.44	8.38	10.46	9.19	6.14	9.08	6.80	4.10	7.53	6.41	5.98	4.99	4.22	5.14
d_3-alanineHVL	19.11	16.77	27.14	14.09	18.22	15.97	14.00	14.98	12.17	13.80	12.73	19.51	15.63	14.71	15.83	15.27	15.28	20.12
d_4-succinateHVL	32.24	24.21	16.38	15.75	28.71	25.93	18.69	23.12	18.32	14.51	20.73	21.88	19.46	15.77	12.04	20.42	20.77	23.42
d_8-valineHVL	28.52	19.13	11.46	19.52	25.20	21.65	17.60	19.47	15.82	26.95	20.87	14.99	17.87	16.87	18.45	18.62	19.13	25.63
d_5-GlutamateHVL	576.04	388.86	326.13	297.77	362.54	397.35	266.43	298.07	225.82	470.27	289.70	343.42	338.42	217.05	172.84	235.38	240.40	285.29

Table E.2: *Raw data from the MCF derivatization on the polar extracts obtained in the extraction of the MAS98.06 (Lum) and MAS98.12 (Bas) xenografts. Raw data are given in pmol (μL injected sample)$^{-1}$.*

Sample/Metabolite	270312-Bas1	270312-Bas2	270312-Bas3	270312-Bas4	270312-Bas5	270312-Bas6	270312-Lum1	270312-Lum2	270312-Lum3	270312-Lum4	270312-Lum5	270312-Lum6
4-methylvalerate	0.15	0.16	0.15	0.14	0.13	0.16	0.16	0.15	0.14	0.12	0.12	0.15
Pyruvate	0.36	0.99	1.56	2.32	1.39	2.14	1.49	1.51	1.74	0.71	0.72	0.39
3-methyl-oxovalerate	0.27	0.40	0.47	0.31	0.36	0.33	0.18	0.23	0.20	0.23	0.19	0.19
Fumarate	1.07	1.05	0.91	1.97	1.26	0.91	0.90	0.91	1.18	0.89	0.95	0.78
d_4-succinateHVL	19.51	18.92	20.22	18.58	18.69	19.83	19.75	20.40	21.01	21.85	19.08	19.08
Lactate/Methylglyoxal	28.39	85.47	84.99	62.00	40.33	49.52	36.02	53.55	72.59	36.09	50.98	36.00
Succinate	1.46	1.66	1.78	1.99	1.65	1.37	2.59	2.46	2.84	1.88	2.49	2.73
Citraconate/Itaconate	0.25	0.36	0.26	0.24	0.37	0.27	-	-	-	-	-	-
Benzoate	0.41	0.45	0.39	0.40	0.40	0.39	0.40	0.37	0.39	0.37	0.38	0.38
Citramalate	0.83	0.77	0.76	0.77	0.73	0.80	0.62	0.57	0.87	0.69	0.68	0.85
Glyoxylate	6.78	4.54	3.35	3.01	4.56	2.32	2.73	1.90	2.54	3.27	2.45	2.86
d_3-alanineHVL	12.48	12.97	14.72	11.23	12.26	11.25	11.81	12.57	10.49		12.89	14.81
Glycine	32.30	29.67	40.79	47.40	32.75	19.17	13.75	14.45	16.21	10.60	12.54	15.83
Alanine	7.30	7.04	15.12	17.01	12.73	6.60	18.59	18.80	20.15	15.10	22.18	18.64
O-acetyl-L-serine	5.81	5.06	13.30	18.48	10.13	5.91	22.93	24.30	24.18	24.80	39.84	29.44
Phenylacetate	0.54	0.73	0.65	0.61	0.50	0.40	0.72	0.78	0.75	0.92	0.56	0.77
Salicylate	6.01	9.82	7.23	7.47	4.04	4.19	11.64	8.18	10.45	10.86	6.77	10.88
2-aminobutyrate	0.14	0.16	0.16	0.12	0.14	0.15	0.15	0.24	0.22	0.18	0.21	0.14
m-Toluate	0.19	0.38	0.31	0.31	0.26	0.39	0.44	0.44	0.43	0.50	0.31	0.55
d_8-valineHVL	13.58	16.77	13.86	13.70	13.56	12.13	13.46	13.15	13.05	14.33	13.42	14.36
Valine	1.67	1.59	2.51	2.45	1.94	1.19	2.68	2.96	4.13	2.23	2.73	2.27
2-isopropylmalate	0.33	0.35	0.39	0.41	0.39	0.40	0.34	0.45	0.34	0.31	0.39	0.33
beta-hydroxypyruvate	0.40	0.66	0.55	0.64	0.86	0.39	0.45	0.53	0.42	0.42	0.40	0.43
Leucine	0.42	0.20	0.32	0.30	0.41	0.29	0.64	0.91	1.15	0.42	0.94	0.73
Threonine	4.55	4.28	7.87	7.60	5.79	2.88	7.32	8.45	9.08	7.66	9.45	8.28
Malate	1.89	1.70	1.38	2.64	2.04	1.15	1.12	1.07	1.43	2.30	1.17	0.95
Proline	5.97	11.31	14.46	10.42	8.73	8.57	11.59	10.56	14.77	11.23	12.21	11.98
Aspartate	1.03	0.80	0.80	0.84	0.83	0.70	6.70	4.92	9.74	5.16	5.26	7.23
Citrate	7.57	8.63	8.75	9.57	10.41	6.86	5.74	5.83	4.45	5.71	4.62	4.21
Serine	1.72	1.30	2.41	3.07	2.46	1.13	2.75	2.95	7.03	2.36	2.39	2.83

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Table E.2 – *Continued from previous page*

Sample/Metabolite	270312-Bas1	270312-Bas2	270312-Bas3	270312-Bas4	270312-Bas5	270312-Bas6	270312-Lum1	270312-Lum2	270312-Lum3	270312-Lum4	270312-Lum5	270312-Lum6
Allantoin	32.66	44.71	38.08	30.54	61.14	24.77	23.29	16.21	19.02	20.12	57.39	33.55
d_5-glutamateHVL	305.42	332.34	320.99	263.46	319.30	257.89	269.58	251.42	244.26	275.70	205.34	211.98
Glutamate	54.89	54.06	56.96	71.86	68.35	42.90	31.81	27.64	27.78	27.98	25.19	33.57
Methionine	0.60	0.58	0.93	0.93	0.74	0.53	1.21	1.10	1.49	0.84	1.05	1.29
Hippurate	0.65	0.74	0.60	0.78	0.64	0.85	0.86	1.07	0.99	1.01	1.07	0.97
Phenylalanine	0.77	0.83	1.48	0.99	0.68	0.55	1.39	1.27	1.65	0.96	1.13	1.36
Ornithine	0.21	0.43	0.30	0.28	0.60	0.29	0.57	1.09	1.78	0.44	0.79	0.76
Lysine	5.48	6.73	7.87	5.38	4.78	3.76	3.84	3.27	4.55	2.51	2.86	3.11
Tyrosine	1.35	1.37	2.06	1.79	1.30	1.00	1.81	1.89	2.59	1.43	1.48	1.55

Table E.3: *Raw data from the MCF derivatization on the polar extracts obtained in the extraction of the MK-2206 treated MAS98.12 (BasT) xenograft. Raw data are given in pmol (μL injected sample)$^{-1}$.*

Sample/Metabolite	200312-BasT1	200312-BasT2	200312-BasT3	200312-BasT4	200312-BasT5	200312-BasT6	200312-BasT7	200312-BasT8	200312-BasT9	200312-BasT10	200312-BasT11	200312-BasT12
4-methylvalerate	0.10	0.10	0.11	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Pyruvate	1.18	1.49	2.38	0.82	2.09	2.65	1.39	1.98	0.97	1.31	1.79	0.81
3-methyl-oxovalerate	0.64	0.69	0.73	0.46	0.51	0.52	0.68	0.77	0.55	0.68	0.80	0.71
Fumarate	0.90	1.39	0.98	0.66	0.79	1.93	0.74	1.55	0.60	0.85	1.09	1.30
d_4-succinateHVL	10.91	10.53	10.94	10.87	10.88	10.37	11.55	12.53	9.69	12.52	10.04	10.19
Lactate/Methylglyoxal	33.75	46.87	58.18	43.33	20.06	50.05	30.05	51.79	24.28	47.00	53.74	38.97
Succinate	1.00	1.31	1.14	1.24	0.92	1.66	0.93	1.19	0.80	1.09	1.18	1.32
Citraconate/Itaconate	0.49	0.42	0.64	0.33	0.47	0.56	0.38	0.53	0.46	0.47	0.50	0.38
Benzoate	0.29	0.28	0.29	0.29	0.29	0.29	0.27	0.29	0.25	0.31	0.28	0.30
Citramalate	0.27	0.28	0.28	0.27	0.28	0.37	0.24	0.30	0.26	0.28	0.26	0.30
Glyoxylate	2.43	2.80	2.77	2.61	2.29	3.63	2.52	3.31	6.02	4.63	3.07	2.26
beta-3-hydroxybutyrate		-	0.24	0.30	-	-	0.12	0.25	0.07	-	0.13	0.14
d_3-alanineHVL	15.01	15.53	17.62	14.31	16.38	16.77	14.80	18.03	14.05	17.33	15.37	13.93
Glycine	44.85	46.79	55.36	33.50	34.77	61.11	33.95	61.36	33.58	41.02	50.84	52.51
Alanine	13.13	16.22	31.93	20.44	13.75	18.51	7.09	13.50	10.91	13.38	11.15	11.49
O-acetyl-L-serine	25.12	34.00	51.35	35.52	16.85	28.60	11.19	21.82	18.05	23.76	17.53	17.54
Phenylacetate	0.82	0.80	0.82	0.51	0.71	0.77	0.72	0.78	0.63	0.85	0.62	0.46
Salicylate	0.85	1.05	1.07	0.41	0.91	1.22	1.50	1.29	0.68	1.21	1.02	0.23
2-aminobutyrate	0.09	0.10	0.14	0.09	0.10	0.11	0.09	0.14	0.11	0.10	0.10	0.08
m-Toluate	0.09	0.08	0.07	0.10	0.10	0.08	0.10	0.09	0.09	0.07	0.09	0.09
Adipate	0.08	0.09	0.09	0.07			0.08	0.08	0.08		0.08	0.08
d_8-valineHVL	11.82	14.84	13.74	16.97	14.75	15.72	13.22	16.73	13.36	17.47	15.30	12.47
Valine	3.32	3.50	4.49	2.85	2.93	4.16	2.55	4.35	3.02	3.33	3.64	3.16
alpha-ketoglutarate	10.60	8.07	6.46				25.30	14.88	7.43	11.32	20.36	12.51
2-isopropylmalate	0.20	0.20	0.24	0.22	0.23	0.20	0.26	0.23	0.25	0.23	0.25	0.23
beta-hydroxypyruvate	0.59	0.71	0.80	0.41	0.69	1.03	0.46	0.85	0.41	0.60	0.57	0.61
Leucine	1.77	2.17	2.90	2.34	1.61	2.86	1.24	2.30	1.60	1.95	1.91	2.11

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Table E.3 – *Continued from previous page*

Sample/Metabolite	**200312-BasT1**	**200312-BasT2**	**200312-BasT3**	**200312-BasT4**	**200312-BasT5**	**200312-BasT6**	**200312-BasT7**	**200312-BasT8**	**200312-BasT9**	**200312-BasT10**	**200312-BasT11**	**200312-BasT12**
Isoleucine	0.87	1.00	1.48	0.97	1.09	1.39	0.56	1.17	0.87	1.08	0.93	1.07
Threonine	4.69	5.71	5.23	3.81	4.60	6.17	3.68	6.14	3.48	4.08	4.69	4.57
Malate	2.82	2.83	2.39	1.12	2.21	3.90	1.15	2.76	1.71	1.70	2.00	1.91
Proline	4.92	4.96	5.09	3.42	3.51	4.95	4.59	6.67	3.34	4.11	6.06	4.65
Oxaloacetate	2.16	2.61	3.61	2.85	2.78	3.15	1.97	4.53	2.73	2.26	3.01	6.05
Aspartate	0.32	0.21	0.31	0.43	1.30	0.60	0.35	-	0.34		0.31	-
Citrate	2.30	2.28	2.55	2.47	2.14	2.58	2.12	2.90	1.92	2.33	2.51	2.80
5-aminovalerate	0.40	0.34	0.35	0.47	0.40	0.43	0.35	0.36	0.38	0.41	0.37	0.59
Serine	1.27	1.69	1.53	1.00	1.35	2.06	0.72	1.43	1.38	1.23	1.32	1.43
Allantoin	9.90	11.32	9.59	28.01	8.98	9.17	9.83	10.78	8.04	11.54	9.35	27.37
N-Acetyl-L-glutamate	2.23	-	2.05	2.41	1.89	-	2.00	2.03	1.45	2.37	1.56	2.03
d_5-GlutamateHVL	119.16	111.48	113.83	135.10	117.13	115.23	131.55	116.43	86.29	137.71	96.10	112.79
Glutamate	26.23	27.67	24.15	18.12	21.96	31.12	26.32	37.50	17.95	23.95	27.54	34.13
Methionine	0.92	0.92	1.05	0.66	0.54	0.92	0.57	0.95	0.60	0.73	0.82	0.78
Hippurate	0.73	0.81	0.71	0.76	0.81	0.89	0.79	0.86	0.79	0.80	0.92	0.83
Phenylalanine	1.67	1.63	1.93	1.17	1.09	1.79	1.05	1.79	1.17	1.41	1.59	1.49
Ornithine	1.17	0.95	0.67	0.56	1.52	1.45	0.79	0.86	1.00	0.71	1.00	0.57
N-glycyl-L-proline	4.47	3.01	4.51	-	-	3.80	3.43	4.39	3.54	5.13	5.76	4.76
Lysine	7.94	7.26	8.12	4.65	4.18	7.19	5.35	8.98	5.98	7.98	9.48	7.96
Tyrosine	1.86	2.07	2.45	1.72	1.50	2.01	1.36	2.04	1.59	1.88	1.82	1.70

E.2 Results from the polar metabolite profiling

The results from the polar metabolite profiling of breast cancer xenografts are based on the raw data given in section E.1.

The results from the MCF derivatization are given in pM (mg tissue sample)$^{-1}$. Normalization was made both against the three internal standards added before extraction, and the internal standard added before derivatization. After internal standard normalization, the resulting data were normalized against the volume of the polar extracts and the weight of the tissue samples to give pM (mg tissue sample)$^{-1}$. The results from the evaluation of the standard deviations of the MAS98.06 and MAS98.12 xenografts are given in Table E.4, Table E.5 and Table E.6. Table E.7 and Table E.8 show the results of the MAS98.12 and MAS98.06 xenografts, respectively. Table E.9 gives the results of the MK-2206 treated MAS98.12 xenograft.

Table E.4: *Results from the MCF derivatization on the polar extracts of the samples 140312-Bas1 and 140312-Bas2 (samples of the basal-like (MAS98.12) xenograft), where the standard deviations for the polar metabolites were evaluated. The results are given in pM (mg tissue sample)$^{-1}$. Averages and standard deviations are calculated from the technical replicates of each sample.*

Sample	**140312-Bas1**						**140312-Bas2**					
Replicate	**1**	**2**	**3**				**1**	**2**	**3**			
/Metabolite				**Average**	**Standard deviation**	**Standard deviation [%]**				**Average**	**Standard deviation**	**Standard deviation [%]**
4-methylvalerate	409	369	447	408	39	9.5	347	154	274	258	97	37.7
Pyruvate	3,129	4,245	11,502	6,292	4,547	72.3	1,858	1,590	2,378	1,942	400	20.6
3-methyl-oxovalerate	837	672	1,068	859	199	23.2	472	276	451	400	107	26.9
Fumarate	4,084	3,757	5,686	4,509	1,033	22.9	1,640	1,182	1,817	1,546	328	21.2
Lactate/Methylglyoxal	224,529	169,832	329,893	241,418	81,356	33.7	112,703	68,354	106,929	95,995	24,112	25.1
Succinate	4,411	4,075	5,093	4,526	518	11.5	1,789	1,613	2,567	1,990	508	25.5
Citraconate/Itaconate	1,409	988	1,603	1,333	314	23.6	711	339	560	537	187	34.9
Benzoate	2,610	2,281	2,150	2,347	237	10.1	1,440	896	1,464	1,266	321	25.4
Citramalate	570	559	492	540	42	7.8	321	187	322	276	78	28.1
Glyoxylate	5,871	5,484	4,975	5,443	449	8.2	5,817	2,332	3,778	3,975	1,751	44.0
beta-3-hydroxybutyrate	1,267	1,364	1,959	1,530	375	24.5	1,064	620	938	874	229	26.2
Glycine	187,563	173,249	320,437	227,083	81,163	35.7	81,366	48,654	68,713	66,244	16,495	24.9
Alanine	41,966	38,991	67,768	49,575	15,826	31.9	18,392	11,699	17,674	15,922	3,675	23.1
O-acetyl-L-serine	19,527	18,280	38,631	25,479	11,407	44.8	5,887	5,275	7,010	6,057	880	14.5
Nicotinate	1,676	1,605	2,101	1,794	268	14.9	1,206	581	1,090	959	333	34.7
Phenylacetate	477	766	439	561	179	31.9	449	198	313	320	126	39.3
Salicylate	6,943	3,176	6,438	5,519	2,045	37.1	2,268	3,313	4,700	3,427	1,220	35.6
2-aminobutyrate	819	795	991	868	107	12.3	472	257	461	397	121	30.5
m-Toluate	580	530	510	540	36	6.7	389	228	361	326	86	26.4
beta-alanine	11,152	9,518	15,362	12,011	3,016	25.1	5,969	3,321	5,148	4,813	1,355	28.2
OH-Glutarate	1,264	1,205	1,406	1,291	103	8.0	639	324	713	559	207	37.0
Adipate	791	734	692	739	50	6.8	492	303	467	421	102	24.3
Valine	13,870	6,514	10,357	10,247	3,679	35.9	3,301	2,089	3,225	2,872	679	23.6
alpha-ketoglutarate	11,940	12,234	23,060	15,745	6,337	40.2	7,236	3,576	6,149	5,654	1,880	33.2
beta-hydroxypyruvate	2,981	2,625	6,694	4,100	2,254	55.0	1,045	601	1,001	882	245	27.8
alpha-ketoadipate	1,218	528	570	772	387	50.1	486	269	459	405	118	29.1
Leucine	2,259	1,962	3,260	2,494	680	27.3	852	630	903	795	145	18.3
gamma-aminobutyrate	3,002	3,071	3,012	3,028	37	1.2	2,465	1,161	1,992	1,873	660	35.3
Threonine	9,991	9,754	15,706	11,817	3,370	28.5	4,236	2,734	4,549	3,840	970	25.3
Malate	12,119	10,101	20,013	14,077	5,238	37.2	3,774	2,283	3,379	3,146	773	24.6
Proline	37,866	34,315	58,104	43,429	12,833	29.5	17,409	11,298	17,184	15,297	3,465	22.7
Aspartate	2,187	1,712	2,172	2,024	270	13.3	637	446	814	633	184	29.1
Citrate	12,128	13,811	17,172	14,370	2,568	17.9	4,788	3,675	6,718	5,060	1,540	30.4
Serine	5,671	5,422	9,684	6,926	2,392	34.5	2,128	1,294	2,302	1,908	539	28.2
Anthralinate	465	301	326	364	88	24.2	182	128	196	169	36	21.5
Allantoin	206,958	202,512	145,182	184,884	34,454	18.6	98,706	56,550	111,034	88,763	28,570	32.2
N-Acetyl-L-glutamate	16,633	12,908	11,952	13,831	2,473	17.9	8,146	6,072	10,392	8,203	2,160	26.3
Glutamate	210,411	199,599	290,175	233,395	49,469	21.2	71,749	53,090	91,190	72,010	19,051	26.5
Methionine	1,923	1,677	2,578	2,059	466	22.6	877	584	894	785	174	22.2

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Table E.4 – *Continued from previous page*

Sample	140312-Bas1						140312-Bas2					
Replicate	**1**	**2**	**3**				**1**	**2**	**3**			
/Metabolite				**Average**	**Standard deviation**	**Standard deviation [%]**				**Average**	**Standard deviation**	**Standard deviation [%]**
Hippurate	451	453	469	458	10	2.1	262	206	396	288	97	33.8
Phenylalanine	3,192	2,954	4,592	3,579	885	24.7	1,489	1,007	1,508	1,335	284	21.3
p-coumarate	219	202	126	182	49	27.0	64	37	46	49	13	27.4
Ornithine	5,182	4,322	5,978	5,161	828	16.0	2,817	1,427	2,541	2,262	736	32.6
Lysine	18,575	17,434	25,191	20,400	4,188	20.5	9,402	5,098	8,452	7,651	2,261	29.6
Tyrosine	5,383	3,546	6,058	4,996	1,300	26.0	2,320	1,368	2,570	2,086	634	30.4
Cysteine	33,971	26,032	27,474	29,159	4,229	14.5	28,024	9,644	19,167	18,945	9,192	48.5

Table E.5: *Results from the MCF derivatization on the polar extracts of the samples 140312-Bas3 and 140312-Lum1 (samples of the basal-like (MAS98.12) and luminal-like (MAS98.06) xenografts, respectively), where the standard deviations for the polar metabolites were evaluated. The results are given in pM (mg tissue sample)$^{-1}$. Averages and standard deviations are calculated from the technical replicates of each sample.*

Sample	140312-Bas3						140312-Lum1					
Replicate	**1**	**2**	**3**				**1**	**2**	**3**			
/Metabolite				**Average**	**Standard deviation**	**Standard deviation [%]**				**Average**	**Standard deviation**	**Standard deviation [%]**
4-methylvalerate	289	243	275	269	24	8.8	623	267	330	407	190	46.7
Pyruvate	3,874	3,673	3,393	3,647	242	6.6	408	2,039	4,310	2,252	1,959	87
3-methyl-oxovalerate	482	442	429	451	28	6.2	808	464	557	610	178	29.2
Fumarate	3,072	2,974	2,816	2,954	129	4.4	6,418	1,161	1,855	3,145	2,856	90.8
Lactate/Methylglyoxal	154,345	138,446	111,517	134,769	21,649	16.1	235,997	78,577	124,981	146,519	80,890	55.2
Succinate	2,139	2,017	1,894	2,017	122	6.1	3,881	2,386	4,454	3,574	1,068	29.9
Citraconate/Itaconate	644	521	471	545	89	16.4	931	454	630	672	241	35.9
Benzoate	1,238	1,220	1,121	1,193	63	5.3	3,396	1,135	1,468	2,000	1,220	61
Citramalate	300	270	238	269	31	11.5	695	267	335	432	230	53.2
Glyoxylate	3,222	2,261	1,713	2,399	764	31.8	-	1,205	3,549	2,377	1,657	69.7
beta-3-hydroxybutyrate	1,165	990	1,102	1,086	89	8.2	4,583	1,591	2,213	2,796	1,579	56.5
Glycine	102,382	93,253	68,477	88,037	17,544	19.9	61,151	20,383	43,210	41,581	20,433	49.1
Alanine	23,697	21,814	19,968	21,826	1,865	8.5	103,839	38,129	67,595	69,854	32,913	47.1
O-acetyl-L-serine	11,860	11,643	10,966	11,490	466	4.1	61,471	19,905	44,892	42,089	20,924	49.7
Nicotinate	1,296	1,408	1,794	1,500	262	17.4	-	-	-	-	-	-
Phenylacetate	319	348	304	323	22	6.8	885	334	435	551	293	53.2
Salicylate	5,909	3,722	4,262	4,631	1,139	24.6	10,826	4,922	4,711	6,819	3,471	50.9
2-aminobutyrate	477	456	438	457	20	4.3	1,686	587	832	1,035	577	55.7
m-Toluate	367	321	302	330	33	10.1	874	332	401	536	295	55.0
beta-alanine	6,570	6,491	6,094	6,385	255	4.0	2,562	884	1,204	1,550	891	57.5
OH-Glutarate	677	687	379	581	175	30.1	-	-	-	-	-	-

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Table E.5 – *Continued from previous page*

Sample	140312-Bas3						140312-Lum1					
Replicate	**1**	**2**	**3**				**1**	**2**	**3**			
/Metabolite				**Average**	**Standard deviation**	**Standard deviation [%]**				**Average**	**Standard deviation**	**Standard deviation [%]**
Adipate	496	399	412	435	53	12.1	1,067	370	531	656	365	55.6
Valine	4,164	3,887	3,255	3,769	466	12.4	16,068	5,391	9,534	10,331	5,383	52.1
alpha-ketoglutarate	6,560	5,410	4,742	5,571	920	16.5	-	-	-	-	-	-
beta-hydroxypyruvate	1,448	1,279	1,048	1,258	201	16.0	484	921	1,403	936	459	49.1
alpha-ketoadipate	444	303	309	352	80	22.7	1,018	307	394	573	388	67.7
Leucine	1,324	1,315	1,044	1,228	159	13.0	3,749	1,312	2,685	2,582	1,221	47.3
gamma-aminobutyrate	2,226	1,938	2,148	2,104	149	7.1	6,172	1,927	2,492	3,530	2,305	65.3
Threonine	6,161	5,683	5,265	5,703	448	7.9	14,779	5,761	11,366	10,635	4,553	42.8
Malate	6,042	5,464	4,453	5,320	804	15.1	12,210	3,616	6,322	7,383	4,394	59.5
Proline	22,818	21,615	18,967	21,133	1,970	9.3	53,239	20,724	37,773	37,245	16,264	43.7
Aspartate	968	742	683	798	150	18.9	15,795	4,419	9,158	9,791	5,714	58.4
Citrate	7,207	7,284	6,851	7,114	231	3.2	4,203	2,815	7,526	4,848	2,421	49.9
Serine	3,704	3,776	3,106	3,529	368	10.4	54,916	4,199	6,683	21,933	28,592	130.4
Anthralinate	203	142	185	177	32	18.0	460	217	204	294	144	49.0
Allantoin	71,640	76,926	60,075	69,547	8,618	12.4	390,010	70,939	109,555	190,168	174,142	91.6
N-Acetyl-L-glutamate	7,131	7,072	6,109	6,771	574	8.5	41,875	7,875	11,351	20,367	18,707	91.9
Glutamate	114,826	103,243	92,525	103,531	11,154	10.8	135,447	37,869	70,602	81,306	49,662	61.1
Methionine	1,146	1,102	988	1,079	81	7.5	3,071	1,312	2,112	2,165	881	40.7
Hippurate	513	463	610	529	75	14.2	1,209	403	525	713	435	61.1
Phenylalanine	1,930	1,919	1,689	1,846	136	7.4	5,663	1,960	3,406	3,676	1,866	50.8
p-coumarate	42	35	32	36	5	14.4	-	-	-	-	-	-
Ornithine	3,037	2,872	2,423	2,777	318	11.4	10,035	4,442	4,499	6,325	3,213	50.8
Lysine	11,034	10,353	9,274	10,220	888	8.7	20,991	8,329	10,668	13,329	6,737	50.5
Tyrosine	2,877	2,353	2,297	2,509	320	12.8	6,568	2,932	3,670	4,390	1,922	43.8
Cysteine	22,934	17,225	12,633	17,597	5,161	29.3	48,429	13,495	9,818	23,914	21,310	89.1

Table E.6: *Results from the MCF derivatization on the polar extracts of the samples 140312-Lum2 and 140312-Lum3 (samples of the luminal-like (MAS98.06) xenograft), where the standard deviations for the polar metabolites were evaluated. The results are given in pM (mg tissue sample)$^{-1}$. Averages and standard deviations are calculated from the technical replicates of each sample.*

Sample	140312-Lum2						140312-Lum3					
Replicate	**1**	**2**	**3**				**1**	**2**	**3**			
/Metabolite				**Average**	**Standard deviation**	**Standard deviation [%]**				**Average**	**Standard deviation**	**Standard deviation [%]**
4-methylvalerate	440	238	174	284	139	48.8	131	141	109	127	16	13.0
Pyruvate	4,054	2,733	1,751	2,846	1,156	40.6	2,164	1,810	2,427	2,134	310	14.5
3-methyl-oxovalerate	1,239	419	387	682	483	70.9	272	303	349	308	39	12.7

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Table E.6 – *Continued from previous page*

Sample	**140312-Lum2**						**140312-Lum3**					
Replicate	**1**	**2**	**3**				**1**	**2**	**3**			
/Metabolite				**Average**	**Standard deviation**	**Standard deviation [%]**				**Average**	**Standard deviation**	**Standard deviation [%]**
Fumarate	2,627	1,352	898	1,626	897	55.1	782	831	714	775	59	7.6
Lactate/Methylglyoxal	167,454	77,802	71,592	105,616	53,643	50.8	54,974	57,486	48,190	53,550	4,809	9.0
Succinate	5,630	2,455	1,286	3,124	2,248	72.0	1,530	1,549	1,404	1,494	79	5.3
Citraconate/Itaconate	854	372	317	514	295	57.4	255	252	187	231	38	16.6
Benzoate	1,947	975	648	1,190	676	56.8	573	581	548	567	17	3.1
Citramalate	399	217	140	252	133	52.8	120	120	90	110	17	15.6
Glyoxylate	3,352	2,597	1,863	2,604	744	28.6	1,301	1,127	1,455	1,294	164	12.7
beta-3-hydroxybutyrate	3,312	1,799	1,275	2,129	1,057	49.7	1,354	1,231	970	1,185	196	16.5
Glycine	42,758	22,479	13,755	26,331	14,880	56.5	13,783	10,625	10,602	11,670	1,830	15.7
Alanine	73,848	39,667	28,767	47,427	23,521	49.6	24,933	24,313	16,405	21,884	4,755	21.7
O-acetyl-L-serine	34,954	24,584	17,600	25,712	8,732	34.0	14,107	13,913	15,616	14,545	933	6.4
Nicotinate	-	-	-	-	-	-	-	-	-	-	-	-
Phenylacetate	796	351	242	463	293	63.3	209	213	177	200	20	9.9
Salicylate	11,939	2,448	3,609	5,999	5,177	86.3	3,367	2,904	3,664	3,312	383	11.6
2-aminobutyrate	1,083	580	461	708	330	46.7	345	332	287	321	31	9.5
m-Toluate	655	357	262	424	205	48.4	245	220	170	212	38	18.1
beta-alanine	1,634	902	681	1,072	499	46.5	460	495	276	410	118	28.8
OH-Glutarate	-	-	-	-	-	-	-	-	-	-	-	-
Adipate	655	389	314	453	179	39.6	192	205	152	183	27	14.9
Valine	10,834	6,165	4,747	7,249	3,185	43.9	3,447	3,397	3,119	3,321	176	5.3
alpha-ketoglutarate	-	-	-	-	-	-	-	-	-	-	-	-
beta-hydroxypyruvate	1,718	785	815	1,106	530	48.0	496	663	385	515	140	27.2
alpha-ketoadipate	460	207	206	291	146	50.4	130	-	144	137	10	7.4
Leucine	3,168	1,714	1,311	2,065	977	47.3	1,005	997	953	985	28	2.8
gamma-aminobutyrate	3,157	2,251	1,484	2,297	838	36.5	1,262	1,414	868	1,181	282	23.9
Threonine	13,330	7,180	5,324	8,611	4,190	48.7	4,134	4,384	3,851	4,123	267	6.5
Malate	6,762	3,198	2,923	4,295	2,142	49.9	1,984	1,888	1,818	1,897	84	4.4
Proline	37,949	20,432	15,103	24,494	11,953	48.8	13,848	13,538	12,703	13,363	592	4.4
Aspartate	11,799	5,256	2,881	6,646	4,619	69.5	2,641	2,703	2,442	2,595	136	5.2
Citrate	8,325	3,337	1,939	4,533	3,357	74.1	2,125	2,164	1,854	2,048	169	8.3
Serine	9,465	3,960	3,090	5,505	3,457	62.8	2,447	2,616	2,039	2,367	297	12.5
Anthralinate	274	95	82	150	107	71.5	77	63	66	68	7	10.8
Allantoin	124,528	103,994	31,612	86,712	48,809	56.3	29,610	33,757	27,769	30,378	3,067	10.1
N-Acetyl-L-glutamate	15,649	4,981	3,085	7,905	6,773	85.7	4,016	3,030	3,519	3,522	493	14.0
Glutamate	90,033	39,432	22,517	50,661	35,131	69.3	22,383	25,364	18,086	21,944	3,659	16.7
Methionine	2,486	1,375	1,012	1,625	768	47.3	788	821	726	779	48	6.2
Hippurate	711	506	318	512	197	38.5	351	325	315	330	19	5.8
Phenylalanine	3,952	2,165	1,621	2,580	1,220	47.3	1,188	1,152	1,119	1,153	35	3.0
p-coumarate	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine	6,199	3,290	2,712	4,067	1,869	46.0	2,088	2,255	1,788	2,044	237	11.6
Lysine	14,148	7,321	5,864	9,111	4,423	48.5	4,692	4,711	4,248	4,550	262	5.8
Tyrosine	4,576	2,547	1,801	2,975	1,436	48.3	1,415	1,087	1,121	1,208	180	14.9
Cysteine	23,440	11,401	7,504	14,115	8,307	58.9	5,201	4,392	3,787	4,460	709	15.9

Table E.7: *Results from the MCF derivatization on the polar extracts obtained in the extraction of the MAS98.12 xenograft (Bas). The results are given in pM (mg tissue sample)$^{-1}$. Average and standard deviation of each detected metabolite in the six samples are given.*

	270312-Bas1	270312-Bas2	270312-Bas3	270312-Bas4	270312-Bas5	270312-Bas6	270312-Bas		
/Metabolite							Average	Standard deviation	Standard deviation [%]
4-methylvalerate	391	258	246	182	276	295	275	69	25.0
Pyruvate	921	1,596	2,526	3,124	2,879	3,972	2,503	1,096	43.8
3-methyl-oxovalerate	688	643	761	419	734	611	643	123	19.1
Fumarate	2,787	1,706	1,475	2,654	2,604	1,697	2,154	587	27.3
Lactate/Methylglyoxal	73,673	138,459	137,952	83,452	83,388	92,013	101,489	29,027	28.6
Succinate	3,784	2,687	2,891	2,674	3,420	2,551	3,001	492	16.4
Citraconate/Itaconate	651	581	426	323	757	503	540	156	29.0
Benzoate	1,071	729	630	536	832	719	753	185	24.6
Citramalate	2,147	1,249	1,236	1,043	1,514	1,495	1,447	386	26.6
Glyoxylate	17,599	7,354	5,430	4,056	9,431	4,318	8,031	5,105	63.6
Glycine	83,824	48,059	66,206	63,792	67,707	35,613	60,867	16,809	27.6
Alanine	18,947	11,403	24,551	22,900	26,317	12,265	19,397	6,351	32.7
O-acetyl-L-serine	15,079	8,199	21,593	24,874	20,938	10,976	16,943	6,573	38.8
Phenylacetate	1,411	1,184	1,058	823	1,027	752	1,042	241	23.1
Salicylate	15,591	15,914	11,740	10,058	8,363	7,778	11,574	3,522	30.4
2-aminobutyrate	370	255	259	161	292	280	269	67	25.0
m-Toluate	506	609	511	412	533	726	550	107	19.5
Valine	4,327	2,579	4,068	3,294	4,012	2,207	3,414	870	25.5
2-isopropylmalate	845	566	633	558	815	747	694	126	18.1
beta-hydroxypyruvate	1,041	1,070	886	861	1,768	726	1,059	370	34.9
Leucine	1,088	321	512	405	847	531	617	292	47.3
Threonine	11,797	6,938	12,770	10,234	11,974	5,352	9,844	3,023	30.7
Malate	4,895	2,755	2,232	3,547	4,219	2,138	3,298	1,117	33.9
Proline	15,479	18,328	23,470	14,020	18,055	15,927	17,547	3,326	19.0
Aspartate	2,668	1,303	1,299	1,129	1,706	1,306	1,569	571	36.4
Citrate	19,654	13,973	14,196	12,878	21,525	12,747	15,829	3,779	23.9
Serine	4,461	2,099	3,915	4,131	5,090	2,100	3,633	1,252	34.5
Allantoin	84,740	72,436	61,809	41,103	126,411	46,028	72,088	31,164	43.2
Glutamate	142,415	87,578	92,463	96,724	141,314	79,707	106,700	27,821	26.1
Methionine	1,552	942	1,510	1,247	1,539	986	1,296	281	21.7
Hippurate	1,676	1,205	968	1,051	1,326	1,587	1,302	285	21.9
Phenylalanine	2,004	1,346	2,398	1,332	1,409	1,013	1,584	513	32.4
Ornithine	555	691	487	376	1,236	536	647	306	47.4
Lysine	14,210	10,900	12,772	7,239	9,875	6,993	10,331	2,906	28.1
Tyrosine	3,504	2,214	3,351	2,414	2,692	1,862	2,673	646	24.2

Table E.8: *Results from the MCF derivatization on the polar extracts obtained in the extraction of the MAS98.06 xenograft (Lum). The results are given in pM (mg tissue sample)$^{-1}$. Average and standard deviation of each detected metabolite in the six samples are given.*

	270312-Lum1	270312-Lum2	270312-Lum3	270312-Lum4	270312-Lum5	270312-Lum6	270312-Lum		
/Metabolite							Average	Standard deviation	Standard deviation [%]
4-methylvalerate	245	189	197	214	111	120	179	53	29.7
Pyruvate	2,287	1,852	2,368	1,278	643	316	1,457	857	58.8
3-methyl-oxovalerate	272	284	277	412	166	152	260	94	36.3
Fumarate	1,379	1,120	1,613	1,594	842	633	1,197	404	33.7
Lactate/Methylglyoxal	55,158	65,877	99,076	64,528	45,372	29,304	59,886	23,501	39.2
Succinate	3,967	3,027	3,881	3,358	2,216	2,218	3,111	774	24.9
Citraconate/Itaconate	-	-	-	-	-	-	-	-	-
Benzoate	616	457	526	658	338	310	484	143	29.4
Citramalate	943	702	1,188	1,239	606	691	895	272	30.3
Glyoxylate	4,179	2,333	3,466	5,841	2,181	2,328	3,388	1,438	42.5
Glycine	21,055	17,782	22,117	18,958	11,160	12,881	17,326	4,416	25.5
Alanine	28,466	23,132	27,505	27,002	19,738	15,173	23,503	5,227	22.2
O-acetyl-L-serine	35,122	29,898	33,003	44,335	35,456	23,962	33,629	6,752	20.1
Phenylacetate	1,098	965	1,028	1,650	496	625	977	407	41.6
Salicylate	17,828	10,070	14,261	19,418	6,021	8,853	12,742	5,295	41.6
2-aminobutyrate	237	297	303	325	191	115	245	80	32.9
m-Toluate	676	537	592	886	274	449	569	207	36.4
Valine	4,103	3,637	5,640	3,990	2,430	1,851	3,608	1,341	37.2
2-isopropylmalate	516	556	470	547	345	270	451	118	26.1
beta-hydroxypyruvate	688	654	579	751	359	346	563	172	30.6
Leucine	983	1,125	1,569	750	835	591	975	344	35.3
Threonine	11,210	10,395	12,399	13,688	8,406	6,740	10,473	2,561	24.5
Malate	1,713	1,320	1,952	4,114	1,044	771	1,819	1,204	66.2
Proline	17,751	12,988	20,153	20,078	10,869	9,749	15,265	4,651	30.5
Aspartate	10,262	6,059	13,287	9,217	4,686	5,884	8,233	3,270	39.7
Citrate	8,786	7,171	6,067	10,208	4,108	3,430	6,628	2,633	39.7
Serine	4,215	3,626	9,590	4,222	2,131	2,299	4,347	2,726	62.7
Allantoin	35,671	19,943	25,954	35,963	51,076	27,303	32,652	10,904	33.4
Glutamate	48,720	34,008	37,916	50,026	22,417	27,323	36,735	11,160	30.4
Methionine	1,847	1,359	2,033	1,500	931	1,048	1,453	433	29.8
Hippurate	1,320	1,318	1,354	1,797	957	788	1,256	352	28.0
Phenylalanine	2,127	1,565	2,252	1,725	1,002	1,108	1,630	513	31.5
Ornithine	877	1,338	2,429	782	705	620	1,125	686	61.0
Lysine	5,884	4,017	6,212	4,479	2,544	2,528	4,277	1,581	37.0
Tyrosine	2,772	2,325	3,531	2,548	1,321	1,264	2,293	875	38.2

Table E.9: *Results from the MCF derivatization on the polar extracts obtained in the extraction of the MK-2206 treated MAS98.12 xenograft (BasT). The results are given in pM (mg tissue sample)$^{-1}$. Average and standard deviation of each detected metabolite in the twelve samples are given.*

	200312-BasT1	200312-BasT2	200312-BasT3	200312-BasT4	200312-BasT5	200312-BasT6	200312-BasT7	200312-BasT8	200312-BasT9	200312-BasT10	200312-BasT11	200312-BasT12	200312-BasT		
/Metabolite													Average	Standard deviation	Standard deviation [%]
4-methylvalerate	229	127	136	222	156	105	254	83	122	167	91	156	154	56	36.2
Pyruvate	2,632	1,835	3,061	1,724	3,167	2,761	3,385	1,708	1,153	2,168	1,619	1,262	2,206	769	34.8
3-methyl-oxovalerate	1,422	844	943	969	768	540	1,655	661	651	1,135	726	1,115	952	333	34.9
Fumarate	2,014	1,716	1,263	1,391	1,197	2,010	1,796	1,337	709	1,406	987	2,030	1,488	429	28.8
Lactate/Methylglyoxal	75,234	57,718	74,891	91,421	30,427	52,081	73,080	44,727	28,852	78,018	48,499	60,762	59,643	19,573	32.8
Succinate	2,240	1,609	1,467	2,613	1,395	1,729	2,251	1,031	955	1,808	1,067	2,058	1,685	533	31.6
Citraconate/Itaconate	1,087	515	821	698	718	579	922	457	549	780	452	596	681	195	28.7
Benzoate	639	350	371	616	443	305	646	251	297	508	252	460	428	147	34.5
Citramalate	608	342	359	574	424	386	592	259	312	460	232	471	418	127	30.3
Glyoxylate	5,419	3,452	3,565	5,500	3,480	3,776	6,133	2,860	7,156	7,683	2,773	3,530	4,611	1,698	36.8
beta-3-hydroxybutyrate	-	-	311	640	-	-	285	217	87	-	120	222	269	182	67.9
Glycine	99,976	57,617	71,264	70,690	52,721	63,587	82,574	52,996	39,895	68,082	45,875	81,879	65,596	17,166	26.2
Alanine	29,270	19,970	41,097	43,130	20,848	19,258	17,239	11,655	12,959	22,214	10,058	17,914	22,134	10,650	48.1
O-acetyl-L-serine	55,998	41,866	66,100	74,950	25,557	29,757	27,208	18,848	21,447	39,430	15,816	27,347	37,027	19,236	52.0
Phenylacetate	1,835	991	1,055	1,071	1,081	802	1,747	674	752	1,410	559	721	1,058	413	39.0
Salicylate	1,889	1,292	1,376	859	1,383	1,268	3,643	1,114	802	2,006	922	365	1,410	837	59.3
2-aminobutyrate	204	127	178	180	145	113	218	122	126	160	86	118	148	40	27.1
m-Toluate	205	99	86	210	147	81	234	79	107	124	79	140	133	56	42.0
Adipate	177	109	121	150	-	-	189	67	89	-	70	118	121	44	36.2
Valine	7,411	4,314	5,782	6,013	4,440	4,332	6,196	3,759	3,594	5,530	3,280	4,925	4,965	1,238	24.9
alpha-ketoglutarate	23,625	9,933	8,309	-	-	-	61,526	12,847	8,831	18,781	18,377	19,515	20,194	16,415	81.3
2-isopropylmalate	436	248	304	467	353	208	626	199	295	380	221	362	342	125	36.6
beta-hydroxypyruvate	1,324	876	1,024	874	1,048	1,073	1,112	734	491	999	515	952	918	242	26.4
Leucine	3,940	2,668	3,734	4,944	2,442	2,973	3,016	1,982	1,907	3,233	1,721	3,298	2,988	936	31.3
Isoleucine	1,929	1,227	1,900	2,047	1,652	1,451	1,359	1,008	1,033	1,801	841	1,670	1,493	402	26.9
Threonine	10,447	7,037	6,727	8,034	6,979	6,418	8,961	5,307	4,138	6,776	4,231	7,122	6,848	1,802	26.3
Malate	6,285	3,486	3,082	2,367	3,345	4,054	2,800	2,384	2,037	2,819	1,805	2,983	3,120	1,179	37.8
Proline	10,957	6,111	6,557	7,224	5,316	5,155	11,165	5,761	3,972	6,814	5,465	7,254	6,813	2,195	32.2
Oxaloacetate	4,823	3,209	4,649	6,018	4,210	3,281	4,786	3,916	3,243	3,748	2,712	9,432	4,502	1,803	40.0
Aspartate	715	257	401	912	1,979	627	839	-	400	-	282	-	712	531	74.5
Citrate	5,130	2,805	3,282	5,212	3,247	2,684	5,157	2,506	2,278	3,862	2,265	4,359	3,566	1,145	32.1
5-aminovalerate	901	423	453	988	609	452	842	308	453	680	334	926	614	246	40.0
Serine	2,834	2,085	1,973	2,120	2,043	2,142	1,758	1,238	1,637	2,038	1,191	2,223	1,940	445	22.9
Allantoin	22,078	13,937	12,347	59,101	13,613	9,544	23,905	9,308	9,557	19,152	8,442	42,683	20,305	15,565	76.7
N-Acetyl-L-glutamate	4,976	-	2,636	5,088	2,863	-	4,854	1,757	1,727	3,937	1,406	3,165	3,241	1,410	43.5
Glutamate	58,462	34,072	31,089	38,235	33,307	32,384	64,027	32,390	21,327	39,752	24,850	53,212	38,592	13,231	34.3
Methionine	2,052	1,128	1,350	1,396	826	961	1,381	821	718	1,210	740	1,219	1,150	379	33.0
Hippurate	1,632	998	917	1,600	1,222	922	1,913	746	940	1,323	828	1,288	1,194	368	30.8
Phenylalanine	3,721	2,010	2,478	2,474	1,651	1,863	2,546	1,543	1,387	2,337	1,438	2,318	2,147	654	30.5

Continued on next page

Table E.9 – *Continued from previous page*

	200312-BasT1	**200312-BasT2**	**200312-BasT3**	**200312-BasT4**	**200312-BasT5**	**200312-BasT6**	**200312-BasT7**	**200312-BasT8**	**200312-BasT9**	**200312-BasT10**	**200312-BasT11**	**200312-BasT12**	**200312-BasT**		
/Metabolite													**Average**	**Standard deviation**	**Standard deviation [%]**
Ornithine	2,608	1,168	861	1,175	2,301	1,504	1,927	745	1,183	1,184	906	883	1,371	601	43.9
N-glycyl-L-proline	9,961	3,704	5,804	-	-	3,957	8,348	3,789	4,204	8,509	5,198	7,417	6,089	2,301	37.8
Lysine	17,707	8,943	10,447	9,819	6,337	7,479	13,021	7,760	7,102	13,253	8,554	12,413	10,236	3,315	32.4
Tyrosine	4,146	2,545	3,159	3,637	2,275	2,089	3,300	1,762	1,894	3,116	1,639	2,649	2,684	795	29.6

E.3 Results from the Student‘s t-tests

Student‘s test was applied to compare the MAS98.06 and MAS98.12 xenografts, as well as for the comparison of the untreated and MK-2206 treated MAS98.12 xenografts (section 2.9.2). Table E.10 gives the results from the student‘s t-tests. The significance level was set to 5%, hence p-values < 0.05 represent significance differences.

Table E.10: *Results from the Student‘s t-tests applied for the comparison of the polar metabolites detected in the luminal-like (MAS98.06) and basal-like (MAS98.12) xenografts, as well as the comparison of the untreated and MK-2206 treated basal-like (MAS98.12) xenografts. p-values < 0.05 indicates significant difference between xenografts based on their polar metabolite concentrations.*

	Comparison of MAS98.12 and MAS98.06		**Comparison of untreated and treated MAS98.12**	
/Metabolite	**p-value**	**Comment**	**p-value**	**Comment**
4-methylvalerate	0.0240	Significant	0.0052	Significant
Pyruvate	0.0973	Not significant	0.5699	Not significant
3-methyl-oxovalerate	0.0002	Significant	0.0118	Significant
Fumarate	0.0096	Significant	0.0397	Significant
Lactate/Methylglyoxal	0.0220	Significant	0.0143	Significant
Succinate	0.7751	Not significant	0.0003	Significant
Citraconate/Itaconate	-	-	0.1231	Not significant
Benzoate	0.0193	Significant	0.0053	Significant
Citramalate	0.0186	Significant	0.0010	Significant
Glyoxylate	0.0774	Not significant	0.1651	Not significant
Glycine	0.0010	Significant	0.5883	Not significant
Alanine	0.2505	Not significant	0.5063	Not significant
O-acetyl-L-serine	0.0015	Significant	0.0053	Significant
Phenylacetate	0.7423	Not significant	0.9203	Not significant
Salicylate	0.6638	Not significant	0.0007	Significant
2-aminobutyrate	0.5775	Not significant	0.0051	Significant
m-Toluate	0.8449	Not significant	0.0001	Significant
Valine	0.7734	Not significant	0.0083	Significant
2-isopropylmalate	0.0061	Significant	0.0002	Significant
beta-hydroxypyruvate	0.0204	Significant	0.4270	Not significant
Leucine	0.0813	Not significant	≈0.0000	Significant
Threonine	0.7059	Not significant	0.0612	Significant
Malate	0.0521	Not significant	0.7612	Not significant
Proline	0.3537	Not significant	0.0002	Significant
Aspartate	0.0037	Significant	0.0063	Significant
Citrate	0.0009	Significant	0.0004	Significant
Serine	0.5778	Not significant	0.0200	Not significant
Allantoin	0.0254	Significant	0.0079	Significant
Glutamate	0.0009	Significant	0.0012	Significant
Methionine	0.4759	Not significant	0.3747	Not significant
Hippurate	0.8057	Not significant	0.5037	Not significant
Phenylalanine	0.8786	Not significant	0.0677	Not significant
Ornithine	0.1635	Not significant	0.0038	Significant
Lysine	0.0022	Significant	0.9513	Not significant
Tyrosine	0.4146	Not significant	0.9747	Not significant

E.4 PCA of polar metabolite profiles of breast cancer xenografts

The polar metabolite profiles given in Table 3.2 in section 3.3.2 were analyzed by PCA with the software Unscrambler. Score and loading plots are given in Figure 3.5 and Figure 3.6 in section 3.3.4 for the comparison of the MAS98.06 and MAS98.12 xenografts, and the comparison of the untreated and MK-2206 treated MAS98.12 xenografts, respectively.

Comparison of luminal-like and basal-like breast cancer xenografts

Luminal-like (MAS98.06) and basal-like (MAS98.12) xenografts were compared by their polar metabolite profiles. Figure E.1 shows the loading plot when the data imported into Unscrambler were unweighted. PC-1 was used to describe only glycine and O-acetyl-L-serine unweighted, and O-acetyl-L-serine, lactate, allantion and glutamate dominated the model proposed by PCA. It was therefore determined to weight the data, to prevent such over-dominance and unduly influence of the model (section 2.9.3). Figure E.2 shows the explained variance and influence plots from the PCA. From Figure E.2a, it is concluded that the maximum number of principal components (PCs) are two, since the explained variance (red line) is reduced for more than two PCs. Figure E.2b indicates that there are no outliers, meaning samples that deviates extremely from the rest of the data set, in the model proposed by PCA. There are therefore no biological replicates excluded from the model.

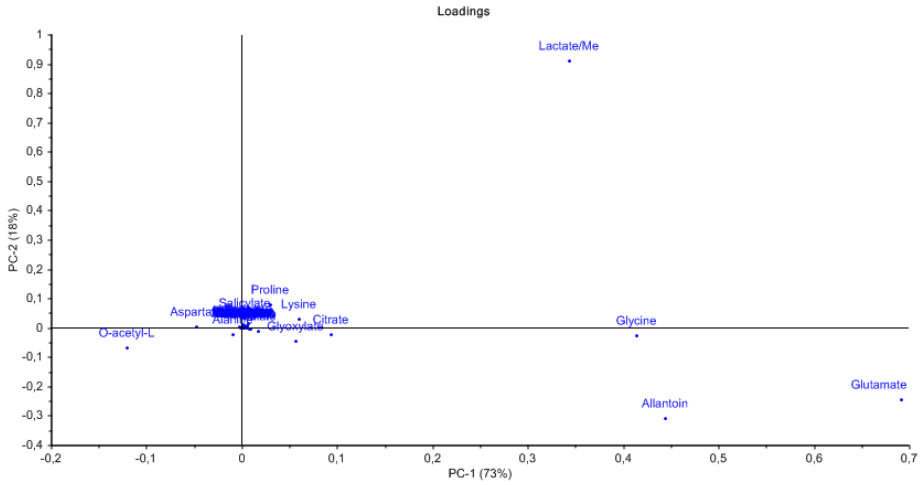
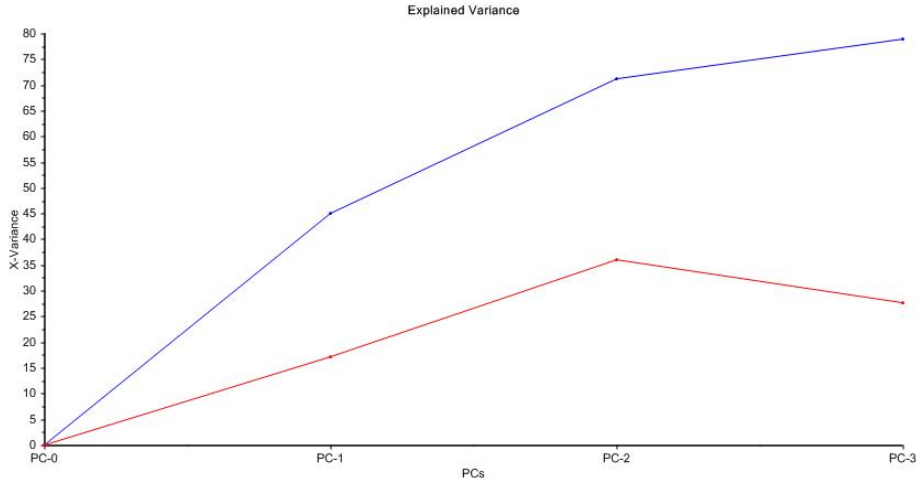
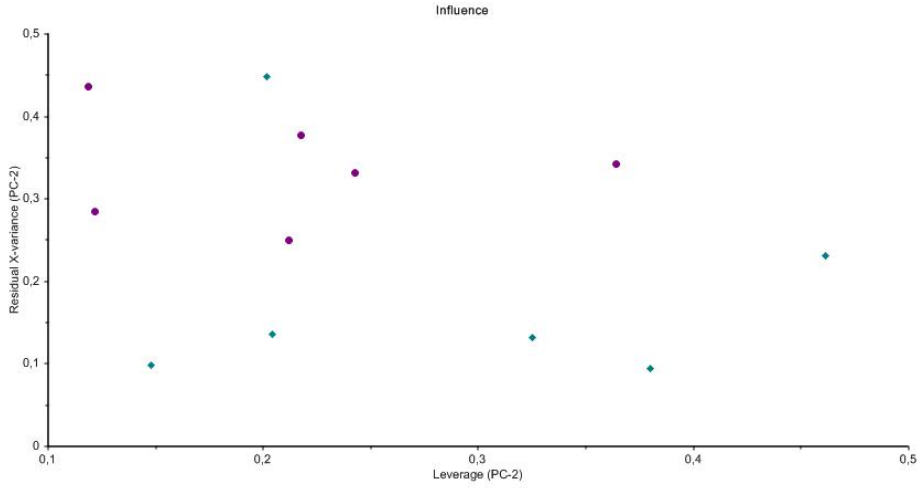


Figure E.1: *Loading plot of the comparison of the MAS98.06 and MAS98.12 xenografts, when the data imported to Unscrambler were unweighted. O-acetyl-L-serine, glycine, lactate, allantoin and glutamate dominated the model.*



(a)



(b)

Figure E.2: *PCA of the MAS98.06 (luminal-like) and MAS98.12 (basal-like) xenografts: (a) the explained variance plot; (b) the influence plot. The purple dots represent the MAS98.12 xenograft, while the turquoise diamonds represent the MAS98.06 xenograft.*

Comparison of untreated and treated basal-like breast cancer xenografts

The untreated and MK-2206 treated basal-like (MAS98.12) xenografts were compared by their polar metabolite profiles. Figure E.3 shows the loading plot when the data imported into Unscrambler were unweighted. PC-1 was used only to describe cysteine unweighted, and cysteine dominated the model proposed by PCA. It was therefore determined to weight the data, to prevent such over-dominance and unduly influence of the model (section 2.9.3). Figure E.4 shows the explained variance and influence plots from the PCA.

From Figure E.4a, it is concluded that the maximum number of principal components (PCs) are two, since the explained variance (red line) is reduced for more than two PCs. Figure E.4b indicates that there are no outliers, meaning samples that deviates extremely from the rest of the data set, in the model proposed by PCA. There are therefore no biological replicates excluded from the model.

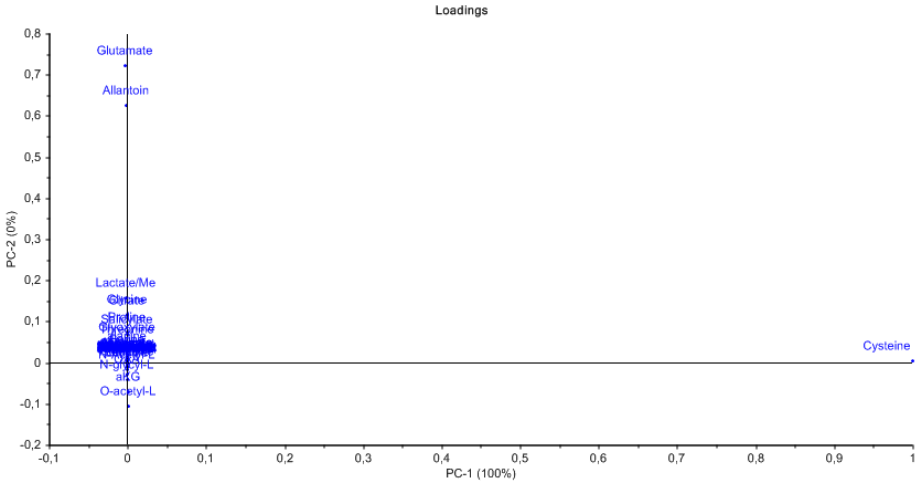
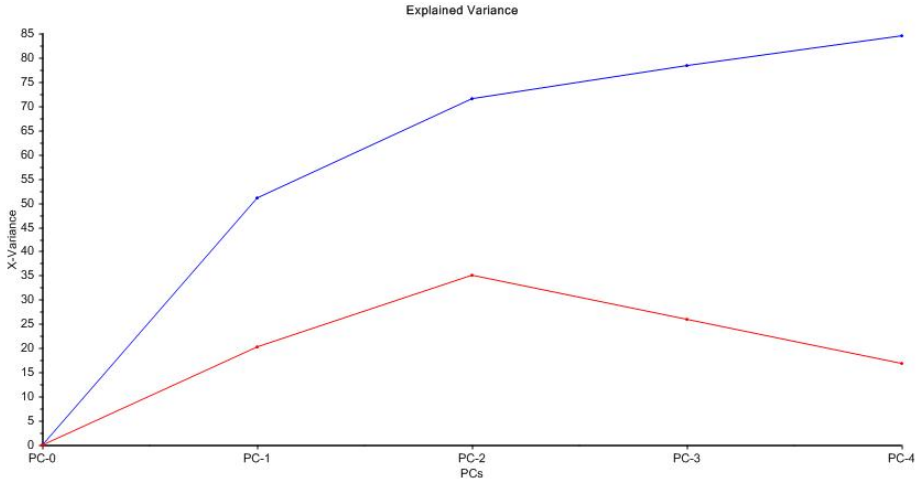
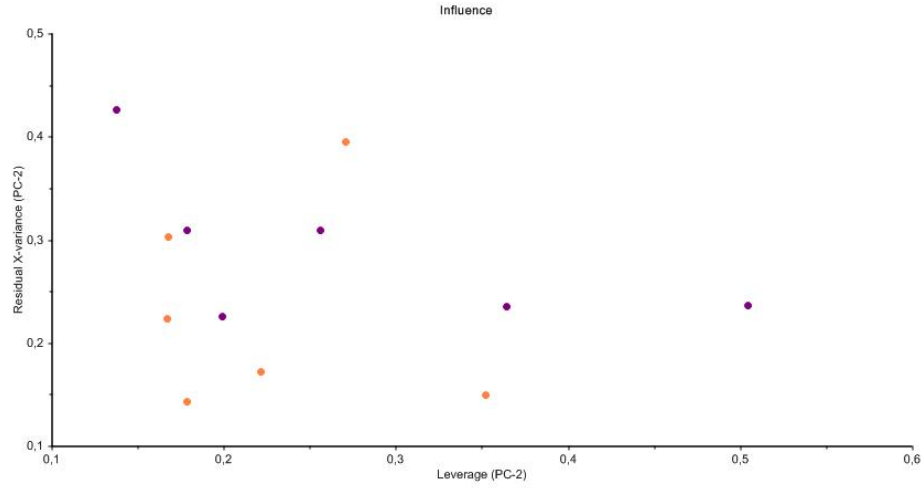


Figure E.3: *Loading plot of the comparison of the untreated and MK-2206 treated MAS98.12 xenografts, when the data imported to Unscrambler were unweighted. Cysteine dominated the model, since PC-1 was used to describe only cysteine.*



(a)

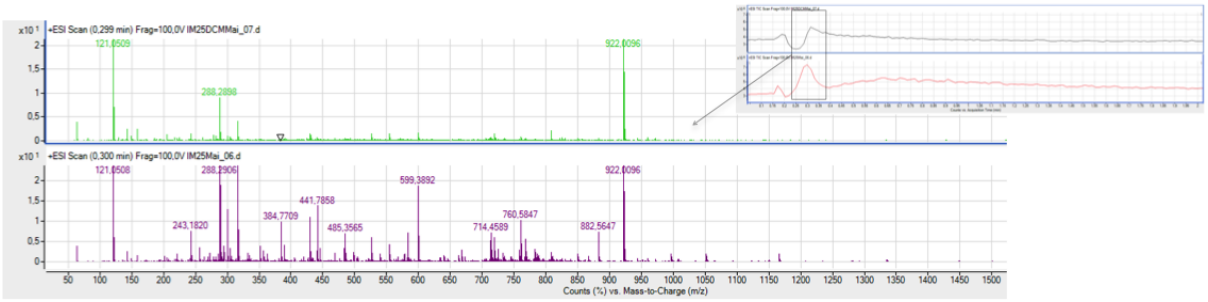


(b)

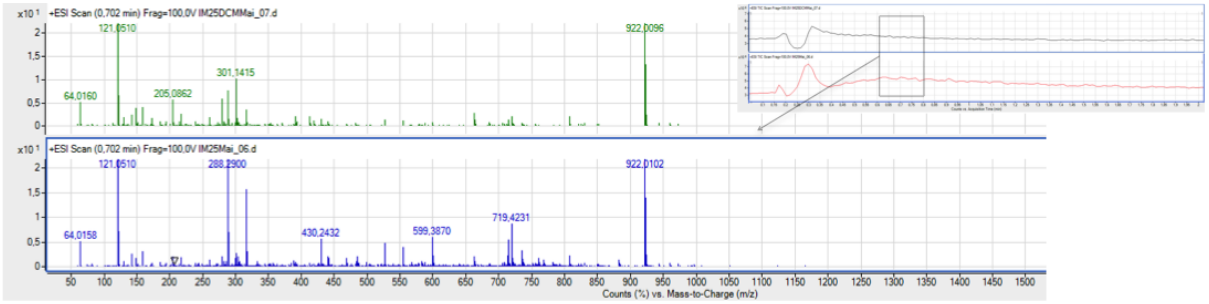
Figure E.4: *PCA of the untreated and MK-2206 treated MAS98.12 (basal-like) xenografts: (a) the explained variance plot; (b) the influence plot. The purple dots represent the untreated MAS98.12 xenograft, while the orange dots represent the MK-2206 treated MAS98.12 xenograft. Each point of the treated MAS98.12 xenografts in the score plot is the average of two samples (two technical replicates) of the same xenograft.*

F Non-polar metabolite profiling of breast cancer xenografts by Q-TOF

The non-polar extract of sample 270312-Lum6 and a blank sample consisting of dichlorometane were analyzed by FIA MS by a Q-TOF LC-MS system, as described in section 2.8. The mass spectra from the analysis are given in Figure 3.7 in section 3.4. Figure F.1 shows the scans of the sample extract and the blank sample at two different time points.



(a)



(b)

Figure F.1: *Scans from the FIA MS of the non-polar extract of sample 270312-Lum6 and a blank sample (dichloromethane) by a Q-TOF LC-MS system: (a) scans of the blank sample (top) and the sample extract (bottom) from the peak at 0.3 min shown in the spectra; (b) scans of the blank sample (top) and the sample extract (bottom) from the assumed to be a second peak (approximately 0.4-1.2 min) in the spectrum of the sample extract. The m/z ratios 121.05 and 922.01 are the reference masses.*