NTNU – Trondheim
Norwegian University of
Science and Technology

A Study of Metabolites in Breast Cancer Xenografts

Optimisation of Extraction Method for MS-based Analysis and Investigation of Subtypes using Metabolite Profiling and Isotope Labelling

Ine Pedersen

Chemical Engineering and Biotechnology
Submission date: June 2012
Supervisor: Per Bruheim, IBT
Co-supervisor: Siver A. Moestue, ISB

Norwegian University of Science and Technology
Department of Biotechnology

Preface

This Master's thesis was based on collaboration between the Department of Biotechnology and the Department of Circulation and Medical Imaging at the Norwegian University of Science and Technology (NTNU). The thesis was carried out for the Master of Technology degree in Chemical Engineering and Biotechnology in the spring of 2012. I would like to express my sincere gratitude to my supervisor, Associate Professor Per Bruheim at the Department of Biotechnology for his expert guidance and full support. Grateful acknowledgements are also due to PhD Candidate Siver Moestue and Research Scientist Tone Bathen at the Department of Circulation and Medical Imaging for their valuable advice and for providing xenograft tissue. I wish to express my sincere appreciation to PhD Candidate Hans Fredrik N. Kvitvang for his assistance and patience. Special thanks go to Senior Engineer Kåre Kristiansen and PhD Candidate Stina K. Lien for their help in the laboratory.

This report was written by me alone but the laboratory work was carried out together with Master student T. Marita Madsen. I would like to express gratitude to her for encouragement, support and for making the laboratory work an enjoyable experience.

Contents

Abstract

The most common cancer among women in Europe and the United States is breast cancer. However, treatment remains a major challenge. If prediction of prognosis and treatment response become more reliable, treatment can be improved. An approach to overcome this challenge is to establish a technique for identification of subtypes. Metabolite profiling of breast cancer has shown potential to identify subtype. The aim was divided into three parts, including metabolite extraction and profiling and isotope labelling of metabolites.

The first aim was to optimise a method for extraction of polar and non-polar metabolites for mass spectrometry-based analysis. To achieve the aim, beads-based homogenisation of xenograft tissue in 60% methanol solution and in chloroform was performed in a series of experiments. Requirements were fulfilled, including complete extraction, simple performance and high reproducibility. Method was therefore stated successfully optimised.

The second aim was to obtain and compare metabolite profiles of luminal-like and basal-like subtypes of breast cancers. To achieve the aim, polar metabolites were extracted from xenograft tissue using the optimised method prior to gas chromatography mass spectrometry (GC-MS) analysis. Profiles comprising more than 30 metabolites and their concentration were obtained. For comparison of subtypes, data analysis including $\log_2$ ratios, principal component analysis (PCA) and Student's t-test were used. All data analyses indicated differences in metabolite concentrations between subtypes. 15 metabolites were found by Student's t-test to significantly differ in concentration between subtypes, including lactate, glycine, citrate, lysine and aspartate. Therefore, metabolite profiling is a potential tool for identification of subtype. Furthermore, metabolites shown to significantly differ may provide insight into metabolic changes in breast cancers that remain poorly understood.

The third aim was to investigate metabolic pathways in luminal-like subtype, possessing reduced tumour growth rates due to treatment. To achieve the aim, ^{13}C-labelled glucose was injected into xenograft models prior to tumour excision, extraction, GC-MS analysis and calculation of summed fractional labelling (SFL). 8.3% lactate, 2.2% citrate and 1.6% fumarate were found labelled using SFL. ^{13}C labelling was therefore shown retained throughout glycolysis, to enter the tricarboxylic acid cycle (TCA) cycle and to give rise to TCA intermediates.

Sammendrag

Brystkreft er den vanligste kreftformen blant kvinner i Europa og i USA. Likevel er optimal behandling en stor utfordring. Bedre behandling kan oppnås med mer nøyaktige metoder for å stille prognose og forutsi respons på behandling. Utfordringen kan angripes ved å etablere en metode for identifisering av brystkreftsubtype. Metabolittprofiler har vist potensiale til å identifisere subtype. Formålet med oppgaven ble delt inn i tre deler; ekstraksjon av metabolitter, metabolittprofiler og isotopmerking av metabolitter.

Det første formålet var å optimalisere en ekstraksjonsmetode for polare og upolare metabolitter for massespektroskopi-basert analyse. En rekke eksperimenter ble utført med perle-basert homogenisering av xenograft vev i en 60% metanol-løsning og i kloroform. Metodekravene; fullstendig ekstraksjon, enkel gjennomførelse og høy reproduserbarhet ble innfridd og metoden erklært optimal.

Det andre formålet var å lage og sammenligne metabolittprofiler av luminal-like og basal-like subtyper av brystkreft. Polare metabolitter ble ekstrahert fra xenograft vev ved hjelp av den optimaliserte metoden i forkant av GC-MS analyse. Profilene inneholdt mer enn 30 metabolitter. Dataanalyser som $\log_2$ forhold, prinsipal komponent analyse (PCA) og Students t-test ble brukt til å sammenligne subtyper. Dataanalysene indikerte forskjeller i metabolittkonsentrasjoner mellom subtyper. 15 metabolitter ble funnet signifikant forskjellige i subtyper ved hjelp av Students t-test blant annet laktat, glysin, citrat, lysin og aspartat. Dette viser at metabolittprofiler har potensiale til å kunne identifisere subtype. Dessuten kan de signifikant forskjellige metabolittene bidra til økt kunnskap om metabolske forandringer i brystkreft som man per dags dato vet lite om.

Det tredje formålet var å undersøke metabolske spor i luminal-like subtype med redusert tumor vekst rate som følge av behandling. ^{13}C-merket glukose ble injisert inn i xenograft mus før fjerning av tumour, ekstraksjon av metabolitter, GC-MS analyse og beregning av summed fractional labelling (SFL). 8.3% laktat, 2.2% citrat og 1.6% fumarat ble funnet merket. Det ble dermed vist at ^{13}C-merkinga ble beholdt gjennom hele glykolysen, gikk inn i sitronsyresyklusen (TCA) og resulterte i TCA-mellomprodukter.

1 Introduction

1.1 Breast cancers and prospects for improvement of treatment

The most common cancer among women in Europe and the United States is breast cancer and as many as one in every ten Norwegian women are diagnosed with the disease at some time during their lives [1, 2]. However, optimal treatment remains a major challenge. An approach to overcome this challenge is to establish better techniques for the prediction of prognosis and treatment response. If these techniques become more reliable, treatment can be tailored specifically to individual patients. However, the establishment of better techniques are dependent on a deeper understanding of the biology of breast cancers [1, 3].

Cancers arise from abnormalities in cell function. Consequently, cells proliferate in an uncontrolled manner causing a mass of growing tissue, a tumour. Tumours are classified as either benign (non-cancerous) or malignant (cancerous). A benign tumour is rarely dangerous because it grows in a confined local area. In contrast, a malignant tumour is capable of spreading and may therefore spread to and invade neighbouring tissues [4].

Nearly all breast cancers are carcinomas. Breast carcinoma arises from epithelial cells constituting the lining layer of the breast. The two main groups are ductal and lobular carcinoma. Ductal carcinoma starts in the ducts, tubes that transport milk to the nipple. Lobular carcinoma has its origin in the lobules, glands where milk production occurs [4]. The anatomy of the female breast, including lobules (arrow 3) and ducts (arrow 6), is illustrated in Figure 1.1.

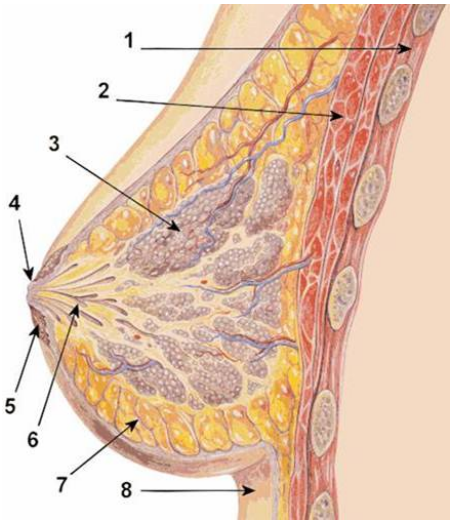

Figure 1.1: *Cross-section scheme of the female breast illustrating chest wall (arrow 1), chest muscles (2), lobules (3), nipple (4), areola (5), duct (6), fatty tissue (7) and skin (8)* [5].

Five subtypes have been defined, including luminal-like and basal-like subtypes, named after the epithelial cells in which the cancer arises. If techniques for prediction of prognosis and treatment response include identification of subtype, they become more reliable. However, the usage of subtypes remains limited [6, 3]. More insight into subtypes may be provided by investigating metabolites contained in subtypes [3].

1.2 Metabolite profiling

Metabolites are low-molecular-weight compounds used by or formed by cells in association with their metabolism. A cell's metabolism involves bioprocesses required for proliferation and survival [7]. Due to the abnormal cell function of cancers, metabolic changes occur [8]. Breast cancers have shown increased concentrations of lactate, glycine and choline metabolites [9, 3]. Metabolite profiles are able to show metabolic changes. Xenograft mouse models were exploited because they are considered unique tools for investigation of human breast cancers [1]. To obtain such a profile, metabolites have to be extracted from tissue and quantitated using an analytical technique [8, 10, 3].

1.3 Extraction of metabolites

To extract metabolites from tissue, cells and tissue are broken up to enable metabolites to liberate to an extraction solvent. Most commonly, this is achieved by grinding tissue with mortar and pestle [11, 10]. Beads-based and rotating blade homogenisers can also be employed to break up tissue. Several extraction methods including mortar and pestle, beads-based and rotating blade homogenisation, freezing-thawing, boiling ethanol and ultrasonication were previously evaluated for their suitability for xenograft tissue. Beads-based homogenisation equipped with cooling was shown superior to the other methods [12].

Figure 1.2: *The beads-based homogeniser used in this project, a Precellys 24 tissue homogeniser. Cryo tubes containing beads are seen loaded into homogeniser.*[13].

At the time tissue is broken up, metabolites can liberate to extraction solvents in which they are soluble. Methanol mixed with water has shown to be a suitable solvent for polar metabolites and chloroform for non-polar metabolites [11, 14]. Often, these solvents are in contact with tissue simultaneously and a biphasic system is obtained [15][14, 10].

1.4 Analytical techniques

Metabolites are commonly analysed using nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS). MS is coupled with gas chromatography (GC) and liquid chromatography (LC) in order to separate metabolites in extracts. GC requires chemical derivatisation of metabolites. In contrast, extracts can be injected directly into LC. MS-based approaches, however, are more sensitive than NMR and provides therefore access to metabolites with lower concentrations [16, 10, 7, 11]. In addition to obtain metabolite profiles, the aforementioned analytical techniques can be used to investigate for instance the passage of an isotope through pathways [9].

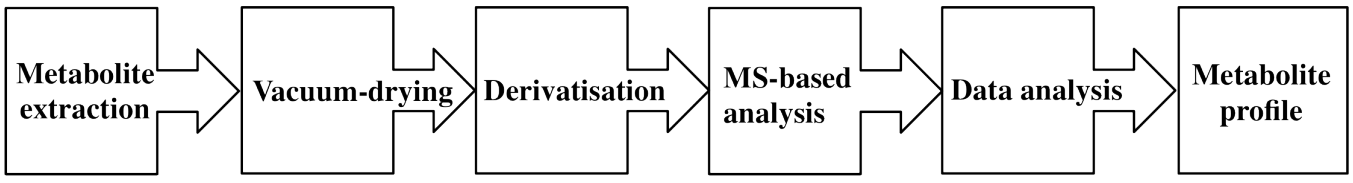

Figure 1.3: *Flowchart of experimental procedures performed in order to obtain metabolite profile of tissue.*

1.5 Aim

This project includes three aims, all investigating the metabolites in breast cancer xenograft tissue.

The first aim is to optimise a method for extraction of polar and non-polar metabolites for MS-based analysis. An optimised method requires complete extraction, simple performance and high reproducibility. For the reason that beads-based homogenisation has shown potential to fulfill these requirements, beads-based homogenisation of tissue directly in extraction solvent is attempted optimised [12]. Extraction solvents of polar and non-polar metabolites include a 60% methanol solution and chloroform, respectively.

The second aim is to obtain and compare metabolite profiles of luminal-like and basal-like subtypes of breast cancer. To achieve the aim, the optimised method can be used to extract metabolites from xenograft tissue prior to MS-based analysis. Data analysis of profiles may show differences between subtypes.

The third aim is to investigate metabolic pathways in treated luminal-like subtype using isotope labelling. Carbon 13-labelled glucose had been injected into xenograft mice and the passage of ^{13}C is tracked obtaining summed fractional labelling (SFL) from GC-MS data.

2 Experimental methods

2.1 Xenograft mouse models

The MR Cancer Group at the Department of Circulation and Medical Imaging at NTNU provided the tumours after approval by the National Animal Research Authority in Norway. Xenograft mouse models were established by serial transplantation of human breast cancer isolates in severe combined immunodeficient (SCID) female mice. Prior to transplantation, isolates were classified as invasive grade III ductal carcinoma. Tumours were excised from xenograft models at the time tumour diameter reached 15 mm. Immediate storage on liquid nitrogen was performed to halt biological activity [17].

Xenograft models used in this study, MAS98.06 and MAS98.12, represent luminal-like and basal-like subtypes of ductal carcinoma, respectively. Luminal-like subtype has been charcterised as estrogen receptor positive (ER+) and basal-like subtype as estrogen receptor negative (ER-). Basal-like subtype has demonstrated a worse prognosis than luminal-like subtype [17, 3, 1].

Drinking water was supplied with estrogen and given to mice bearing luminal-like tumours to promote growth of tumours [3]. However, some of the mice with luminal-like tumours were not given estrogen. In consequence, their tumours possessed reduced growth rates. The low abundance of estrogen in breast tissue was meant to resemble treatment with tamoxifen. Moreover, 29 mg [1-^{13}C]glucose was injected into mice ten minutes prior to tumour excision. In this project, tissue from these mice are therefore referred to as treated [18, 19]. Full tumour names are shown in Table B.1.

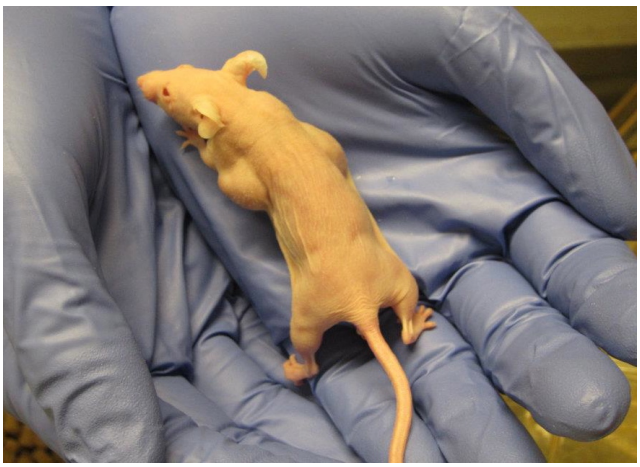

Figure 2.1: *Xenograft mouse model bearing two tumours of ductal carcinoma.*

2.2 Extraction of polar and non-polar metabolites

To optimise extraction method for xenograft tissue (2.1) for MS-based analysis, a beads-based homogeniser, more specifically a Precellys 24 tissue homogeniser (Bertin Technologies) was employed. Homogeniser was coupled with a Cryolys cooling system (Bertin Technologies) to ensure low temperatures. Method was developed for cancerous tissue within the range of 30±10 mg. 0.50 g of ceramic (zirconium oxide) beads with a diameter of 1.4 mm were used [14]. Optimisation experiments extracted metabolites exclusively from luminal-like subtype. At last, optimised method was performed for different purposes, including metabolite profiling and investigation of isotope labelling.

2.2.1 Extraction of polar metabolites

Tumours were cut and weighed to obtain tissue samples of 30±10 mg. Tissue samples were placed in precooled (-20°C) 2 ml cryo tubes containing beads. 400 µl 60% methanol and 10 µl internal standard (10 mM d3-alanine) were added to tubes. Tissue samples were homogenised for three intervals of 20 sec at 5500 rpm using the beads-based homogeniser. Homogeniser was set to pause for 30 sec in between intervals. Samples were centrifuged for 5 min at 5000 rpm and -9 °C. Extracts were removed and three technical replicates of 100 µl were obtained in 5 ml polypropylene (PP) tubes.

Experimental procedure described in previous paragraph was repeated twice to obtain a second and a third extraction round. d5-glutamate was used as internal standard for second round. Third round lacked internal standard. Polar extracts were derivatised using MCF prior to GC-QqQ-MS analysis.

2.2.2 Combining extraction of polar and non-polar metabolites

Attempts to combine extraction of polar and non-polar metabolites were made. To achieve complete extraction of non-polar metabolites, number of extraction rounds and volume of chloroform were investigated.

Three extraction rounds in 400 µl chloroform

To extract polar metabolites, experimental procedure previously described was performed (2.2.1). Some changes were made; internal standards for first and second extraction round were 10 mM d3-alanine and 1 mM d4-succinate, respectively. Extracts were pooled and technical replicates obtained from pool of extracts.

To extract non-polar metabolites, 400 µl precooled (-20 °C) chloroform and 10 µl 10 mM internal standard (d5-glutamate) were added to cryo tubes containing beads and homogenised tissue. Tissue samples were homogenised for three intervals of 20 sec at 5500 rpm using tissue homogeniser. Homogeniser was set to pause for 30 sec in between intervals. Samples were centrifuged for 5 min at 5000 rpm and -9 °C. Extracts were removed and three technical replicates of 100 µl were obtained in vials.

Experimental procedure described in previous paragraph was repeated twice to obtain a second and a third extraction round. No internal standard were added.

To investigate whether polar metabolites remained in tissue, a new extraction round in methanol solution was performed. 400 µl precooled (-20 °C) 60% methanol and 10 µl internal standard (1 mM d4-succinate) were added to tubes containing beads and homogenised tissue. Tissue samples were homogenised for three intervals of 20 sec at 5500 rpm using tissue homogeniser. Homogeniser was set to pause for 30 sec in between intervals. Samples were centrifuged for 5 min at 5000 rpm and -9 °C. Extracts were removed and three technical replicates of 100 µl were obtained in 5 ml PP tubes. Polar extracts were derivatised using MCF prior to GC-Q-MS analysis.

Five extraction rounds in 400 µl chloroform

Experimental procedure as described in 2.2.2 was performed with some changes. 1 mM d4-succinate, 10 mM d3-alanine and 10 mM d8-valine were added prior to first, second and third extraction round, respectively. Polar extracts were derivatised using TMS prior to GC-Q-MS analysis.

To extract non-polar metabolites, five extraction rounds in chloroform was performed with settings as previously. Three technical replicates of 100 µl were obtained in vials each round. Non-polar extracts were derivatised using HCl in methanol prior to GC-Q-MS analysis. No last extraction round in methanol solution as previously described was performed.

Biphasic system prior to five extraction rounds in 800 µl chloroform

Experimental procedure described in 2.2.2 was performed as previously up until extraction of non-polar metabolites. Then, 2 ml chloroform was added to pool of polar extracts (of approximately 1 ml) to obtain a biphasic system of approximately 2:1 volume-to-volume (v/v) chloroform/60% methanol solution. After 5 min of separation, three technical replicates of 100 µl of both phases were obtained. Methanol phase was derivatised using MCF prior to GC-QqQ-MS analysis and chloroform phase was derivatised using HCl in methanol prior to GC-Q-MS analysis.

Cell pellet was homogenised in 800 µl chloroform for three intervals of 20 sec at 6500 rpm. Homogeniser was set to pause for 30 sec in between intervals. Samples were centrifuged for 5 min at 5000 rpm and -9 °C. Extracts were removed and three technical replicates of 100 µl obtained in vials.

Experimental procedure described in previous paragraph was repeated twice. Non-polar extracts were derivatised using HCl in methanol prior to GC-Q-MS analysis.

2.2.3 Optimised extraction method

A few adjustments were made to experimental procedure described in 2.2.2 to obtain the optimised method that is described in detail below.

Tumours were cut and weighed to obtain tissue samples of 30±10 mg. Tissue samples were placed in precooled (-20°C) 2 ml cryo tubes containing 0.50 g of ceramic (zirconium oxide) beads with a diameter of 1.4 mm. To maintain low temperatures, tubes were kept on ice.

To extract polar metabolites, homogenisation of tissue in a 60% methanol solution was performed. 400 µl 60% methanol and 10 µl 10 mM internal standard (d3-alanine) were added to tubes. Tissue samples were homogenised for three intervals of 20 sec at 5500 rpm using the beads-based homogeniser. Homogeniser was set to pause for 30 sec in between intervals. Samples were centrifuged for 5 min at 5000 rpm and -9 °C. Extracts were removed and stored in tubes until later.

Experimental procedure described in previous paragraph was repeated twice with the only change being addition of other internal standards (d4-succinate and d8-valine for second and third round, respectively). Extracts were pooled and vortexed. Three technical replicates of 100 µl obtained in 5 ml polypropylene (PP) tubes. Pool of extracts were vortexed in between obtaining replicates.

To extract non-polar metabolites, homogenisation of tissue in chloroform was performed. 800 µl chloroform and 10 µl 10 mM internal standard (d31-palmitic acid) were added to tubes containing beads and cell pellet. Tissue samples were homogenised for three intervals of 20 sec at 6500 rpm. Homogeniser was set to pause for 30 sec in between intervals. Samples were centrifuged for 5 min at 5000 rpm and -9 °C. Extracts were removed and stored until later.

Experimental procedure described in previous paragraph was repeated twice with the only change being addition of another internal standards (d35-stearic acid). Extracts were pooled. Three technical replicates of 100 µl were obtained in GC-MS vials.

Polar extracts obtained from luminal-like and basal-like subtypes were derivatised using MCF prior to GC-QqQ-MS analysis. Polar extracts obtained from treated luminal-like subtypes were also derivatised using MCF but analysed using GC-Q-MS.

2.3 Derivatisation

Prior to derivatisation, extracts were vacuum-dried by a Savant SPD2010 SpeedVac concentrator (Thermo Electron Corporation).

2.3.1 MCF derivatisation of polar extracts

Samples were derivatised using an established protocol for methyl chloroformate (MCF) derivatisation, frequently described in literature [20]. However for an adaption to tissue, some changes were made. 390 µl 1M sodium hydroxide (NaOH), 10 µl 10 mM internal standard (d5-glutamate), 333 µl methanol and 67 µl pyridine were added to vaccum-dried samples in PP tubes. The mixture was voretexed for 5 sec. To start the derivatisation

reaction, 80 µl MCF was added followed by 1 min of vortexing. 400 µl chloroform was added and mixture vortexed for 10 sec. Then, 400 µl 50 mM sodium bicarbonate ($NaHCO_3$) solution was added followed by 10 sec of vortexing. Lower chloroform phase was transferred to another PP tube and dried by adding a small portion of anhydrous sodium sulfate (Na_2SO_4). After vortexing for 5 sec, water-free chloroform phase was transferred to a GC vial with insert and analysed using triple quadrupole GC-MS. Standard curve samples were obtained by making a dilution serie of a 1.408 mM MCF standard mix (std mix) in 1 M NaOH. 100 µl std mix dilutions of 1:1, 1:3, 1:10 and 1:100 were made to obtain a four point standard curve. Derivatisation was performed in PP tubes using the same procedure as samples.

Absolute quantitation

Absolute quantitation was enabled by spiking samples with the 1.408 mM MCF std mix, separately derivatised with deuterated derivatisation reagents. To obtain std mix for spiking, often referred to as deuterised internal std mix (d3-istd), 100 µl 1:3 diluted MCF std mix was derivatised following the same procedure as samples but using 300 µl NaOH and deuterated derivatisation reagents, respectively MCF and MeOH. The derivatisation of 100 µl 1:3 diluted MCF std mix was repeated three times. All the derivatised std mixes were mixed before 170 µl derivatised sample solution was spiked with 30 µl std mix in an insert. Sample solution and std mix were carefully mixed with a pipette. Likewise, 170 µl derivatised standard curve samples were spiked with 30 µl of internal std mix. MCF derivatised samples were analysed using GC-QqQ-MS.

2.3.2 TMS derivatisation of polar extracts

25 µl internal standard (3mg/ml d27-myristic acid in a 2:5:2 (v/v/v) water:methanol:isopropanol) was added to samples in vials and empty vials, labelled d27 and FAME. 75 µl 2 mM std mix was added to empty vials, labelled std1 and std2. Vials were vacuum-dried. 10 µl 1:100 FAME R1 solution (10 mM and equal volumes of fructose, glucose, lactose, maltose and raffinose) was added to FAME vial. 20 µl 4% methoxyamine HCl in pyridine was added to samples, 50 µl to d27, FAME and to an empty vial, labeled blank, and 100 µl to std1 and std2. Vials were shaken at 30 ^{o}C for 90 min. 20 µl of samples,

d27, FAME and blank were transferred to new vials, 50 µl of std1 and std2. 180 µl MSTFA+1%TMCS was added to samples, d27, std1, std2 and blank. 150 µl to FAME. Vials were shaken at 37 ^{o}C for 30 min. Then cooled down to room temperature. Solutions were transferred to inserts and placed back into vials. Sequence order: 2xblank, d27, FAME, std1, samples, blank, std2. TMS derivatised samples were analysed using GC-Q-MS.

2.3.3 HCl in methanol derivatisation of non-polar extracts

Preparation of FAMEs from lipids and free fatty acids was performed with some changes to an experimental procedure described in literature[21]. 100 µl 1.25 M hydrogen chloride (HCl) in methanol and 10 µl 10 mM internal standard (d27-myristic acid) were added to vacuum-dried samples in vial. The mixture was mixed and set to react overnight on a heating block of 50 ^{o}C. In addition, derivatisation reagent was also added to a vial containing 10 µl super standard mix.

The next day, samples were transferred to glass tubes. 100 µl MQ-grade water and 300 µl hexane were added to tubes followed by 10 sec vortexing. Upper layer hexane phase was transferred to a PP tube and a small portion of anhydrous (Na_2SO_4) added. After vortexing for 5 sec, water-free hexane phase was transferred to vial with insert and analysed using a GC-Q-MS.

2.4 GC-MS analysis

2.4.1 Triple Quadrupole GC-MS

A Triple Quadrupole GC-MS (GC-QqQ-MS) system was employed. The GC-MS system included an Agilent 7890A GC coupled with an Agilent 7000B QqQ MS. A Triple-Axis HED-EM Detector was used. The GC was operated under a constant pressure of 1 bar and with helium as the carrier gas. d5-glutamate was used as a standard for retention time (RT) locking of method. Injection of 1 µl sample was performed in the pulsed splitless mode into an inlet of 290 ^{o}C. The GC compartment was equipped with a 30 m x 250 µm x 0.25 µm Agilent J&W 122-5532G DB-5MS+DG capillary column. Its temperature was set at 325 ^{o}C. Run time was 20 min including 3 min hold time at 40 ^{o}C, temperature gradient of 20 ^{o}C/min to 320 ^{o}C and 3 min hold time. Post run was set to 5 min. Methane reagent gas was used for positive chemical ionisation (PCI). Multiple reaction monitoring (MRM) was applied. 19 cycles/sec and 78 cycles/sec were obtained by performing absolute quantitation and semi-quantitation, respectively. Std mix dilutions and d3-istd were analysed twice, prior and subsequent to samples. The most concentrated dilution was the first to be analysed.

2.4.2 Single Quadrupole GC-MS

A Single Quadrupole GC-MS (GC-Q-MS) was employed. The GC-MS system was an Agilent 7890A GC coupled with an Agilent 5975 inert Mass selective detector (MSD)/MS system. The GC compartment was equipped with a 30 m x 250 mm x 0.25 mm Agilent J&W 122-5532G DB-5MS+DG capillary column. Electron impact (EI) source operated at 70 eV. Method settings were chosen according to derivatisation method.

Subsequent to TMS derivatisation

d27-myristic acid was used as a standard for RT locking of method. Injection of 1 µl sample was performed in the split mode using 1:5 split. Inlet temperature was set to 250 ^{o}C. Run time was 37.5 min including 1 min hold time at 60 ^{o}C, temperature gradient of 10 ^{o}C/min to 325 ^{o}C and 10 min hold time. Post run was set to 1 min. The scan mode started after 5.9 min with a mass range of 50-600 a.m.u. at 2.66 scans/sec.

Subsequent to FAME analyis of lipids

d27-myristic acid was used as a standard for RT locking of method. Injection of 2 µl sample was performed in the split mode using 1:5 split. Inlet temperature was set to 290 ^{o}C. Run time was 35 min including 2 min hold time at 45 ^{o}C, temperature gradient of 10 ^{o}C/min to 325 ^{o}C and 5 min hold time. Post run was set to 1 min . The scan mode started after 6 min with a mass range of 50-550 a.m.u. at 1.5 scans/sec.

Subsequent to MCF derivatisation of isotopically labelled metabolites

d5-glutamate was used as a standard for retention time (RT) locking of method. Injection of 2 µl sample was performed in the splitless mode. Inlet temperature was set to 290 ^{o}C. Run time was 35 min including 7.5 min hold time at 45 ^{o}C, temperature gradient of 10 ^{o}C/min to 300 ^{o}C and 2 min hold time. Post run was set to 1 min. The scan mode started after 6 min with a mass range of 50-550 a.m.u. at 2.5 scans/sec.

2.5 Q-TOF LC-MS analysis

A Quadrupole Time-of-Flight (Q-TOF) LC-MS was employed. The LC-MS system was an Agilent 6510 Q-TOF LC-MS including an Agilent 1200 Series LC. 500 µl non-polar extracts were vaccum-dried and dissolved in 100 µl dichloromethane (DCM). 10 µL of the DCM solution was directly infused into the MS compartment of the Q-TOF LC-MS. Flow was 0.2 mL/min. Electrospray ionisation (ESI) was carried out in positive ion mode with a gas temperature of 325 ^{o}C. Fragmentor was set at 100 V. Mass-to-charge range was chosen as 100-1500 m/z. Reference masses were 121.050873 m/z and 922.009798 m/z. 1.03 spectra was obtained per sec. Run time was 2 min.

2.6 Software for identification and quantitation

2.6.1 Chemstation for GC-Q-MS

Identification and semi-quantitation subsequent to GC-Q-MS analysis were enabled using Chemstation software, version E. 02.00.493 (Agilent Technologies) in combination with Automated Mass Spectral Deconvolution and Identification Software (AMDIS) (National Institute of Standards and Technology (NIST)).

Construction of a semi-quantitative DRS method

For the semi-quantitation of FAMEs obtained from fatty acids and lipids, a Deconvolution Reporting Software (DRS) method was constructed. AMDIS and Chemstation libraries were made separately and then linked together. Compounds and their corresponding RT obtained from a previous run were added to an empty AMDIS library. Among added compounds were the corresponding methyl esters of palmitate, stearate, d27-myristic acid and "unknown RT 21.107". A tab file was made in order to make the Chemstation part. Target and qualifier ions were chosen for each compound based on the mass spectrum from the previous run. Target and qualifier ions, and also the calculated ratio of these ions were added to tab file. The constructed DRS method was performed on chloroform extracts subsequent to GC-Q-MS analysis. Peak area was obtained.

2.6.2 Masshunter for GC-QQQ-MS and Q-TOF LC-MS

Identification and absolute quantitation subsequent to GC-QqQ-MS analysis were enabled using Masshunter Workstation software - Qualitative Analysis, version B.04.00 (Agilent Technologies). Data obtained from Q-TOF LC-MS analysis were also investigated using Masshunter.

3 Results and discussion

A method for extraction of polar and non-polar metabolites from breast cancer xenograft tissue for GC-MS analysis was optimised (3.1). Metabolite profiles of luminal-like and basal-like subtypes of ductal carcinoma were obtained and compared (3.2). The passage of carbon 13 of glucose through pathways in luminal-like subtype was investigated (3.3). Non-polar extracts were analysed using LC-MS for investigation of lipids (3.4). At last, recommendations for future work were proposed (3.5).

3.1 Optimisation of a method for metabolite extraction

Beads-based homogenisation of tissue in polar and non-polar solvents was chosen as the basis for extraction. In combination with cooling, beads-beating directly in solvents has previously shown the potential to completely extract metabolites [12]. A methanol solution and chloroform were used as extraction solvents for polar and non-polar metabolites, respectively. Method was developed for xenograft tissue within the range of 30±10 mg. Method requirements included complete extraction of metabolites, simple performance and reproducible method. In order to fulfill the requirements several aspects of method were investigated; extraction of polar metabolites (3.1.1), extraction of non-polar metabolites (3.1.2), combining extraction of polar and non-polar metabolites (3.1.3), sample preparation of polar extracts (3.1.5) and reproducibility of method (3.1.4). Conclusions throughout method development were summarised (3.1.6) and at last, protocol for the optimised extraction method was obtained (3.1.7).

3.1.1 Extraction of polar metabolites

To extract polar metabolites, beads-based homogenisation of tissue in 60% methanol solution was performed. Metabolite concentrations were obtained by performing MCF derivatisation and GC-QqQ-MS analysis. Extraction round was defined as the experimental procedure including three 20 sec intervals of homogenisation, centrifugation and collection of extract. The number of extraction rounds needed in order to completely extract polar metabolites was investigated.

Complete extraction with three extraction rounds in 60% methanol solution

Three extraction rounds in 400 µl 60% methanol solution were performed. Metabolite concentrations were obtained each round. To investigate whether metabolites were completely extracted, percentage concentrations were calculated. The calculation of percentage concentration was based on the assumption that metabolites were completely extracted with three extraction rounds. Even if assumption is incorrect, percentage concentrations still serve as a good indication of completeness. Polar metabolites that achieved complete extraction with three extraction rounds are shown in Figure 3.1. Raw data, including incompletely extracted metabolites, are shown in Table C.8.

In this project, extraction was stated complete for the round obtaining 5% concentration. 16 polar metabolites obtained a percentage concentration of 5% or less in third extraction round and were therefore stated completely extracted. These metabolites were all high-abundance metabolites, contained in high concentrations in xenograft tissue. Several metabolites were not completely extracted. However, incomplete extraction was seen for low-abundance metabolites. Optimisation experiments were chosen to focus on some metabolites and for this reason, complete extraction was stated for polar metabolites. Therefore, three extraction rounds were shown to achieve complete extraction of polar metabolites.

Reassurance of complete extraction

Another experiment was conducted to reassure that three extraction rounds achieve complete extraction of polar metabolites. It was investigated whether chloroform has the ability to break up more cells and thus enable more polar metabolites to leak out. Tissue was therefore homogenised in chloroform subsequent to the apparent complete extraction. To check for remaining polar metabolites, an extraction round in 60% methanol solution was performed. Percentage concentration was calculated for the last extraction round, the check round, and shown in Table 3.1 for metabolites previously stated as completely extracted. Corresponding data are shown in Table C.9.

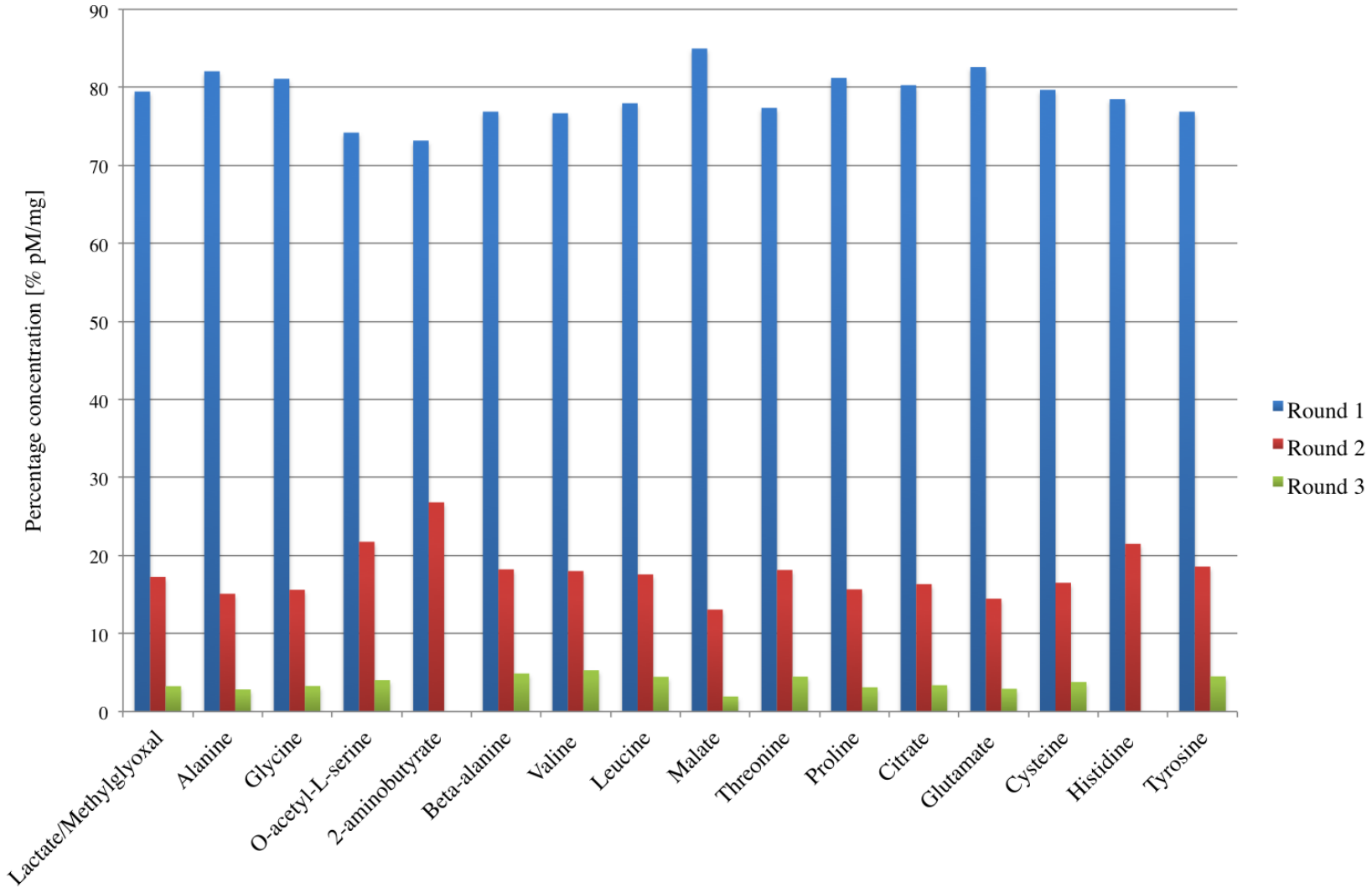

Figure 3.1: *Percentage concentration of polar metabolites obtained from each of the three extraction rounds of beads-based homogenisation in 60% methanol solution. Only metabolites completely extracted with three extraction rounds were included in the graph.*

Table 3.1: *Percentage concentration [% pM/mg] to check for remaining polar metabolites obtained in extraction round in 60 % methanol solution subsequent to the apparent complete extraction and homogenisation in chloroform.*

Metabolite	**Percentage concentration**
Lactate/Methylglyoxal	0.9
Alanine	-
Glycine	1.8
O-acetyl-L-serine	4.0
2-aminobutyrate	-
Beta-alanine	-
Valine	1.5
Leucine	-
Malate	1.0
Threonine	-
Proline	-
Citrate	-
Glutamate	-
Cysteine	-
Histidine	-
Tyrosine	-

All metabolites previously stated as completely extracted were obtained in so small concentrations or not obtained at all, that complete extraction with three extraction rounds was reassured. Cells were therefore adequately broken up during three extraction rounds in 60% methanol solution. Further breakage of cells using chloroform was not necessary as complete extraction of polar metabolites was already achieved. The 60% methanol solution was shown suitable as a polar extraction solvent.

Conclusion

Complete extraction of polar metabolites is achieved with three extraction rounds in 400 µl 60% methanol solution. Therefore three extraction rounds, each round including three intervals of 20 sec, are implemented in experimental procedure. Extracts were pooled in order to simplify method.

3.1.2 Extraction of non-polar metabolites

Beads-based homogenisation of tissue in chloroform was performed in order to extract non-polar metabolites. The number of extraction rounds needed to completely extract non-polar metabolites was investigated. Metabolites were quantitated by performing hydrogen chloride in methanol derivatisation and GC-Q-MS analysis. Extraction round was defined as previously (3.1.1).

Incomplete extraction with five extraction rounds in 400 µl chloroform

Five extraction rounds, set at 6500 rpm, of tissue in 400 µl chloroform was performed subsequent to polar metabolite extraction. Approximate values of metabolite abundance from chromatogram are shown in Figure 3.2. Corresponding data are shown in Table E.1.

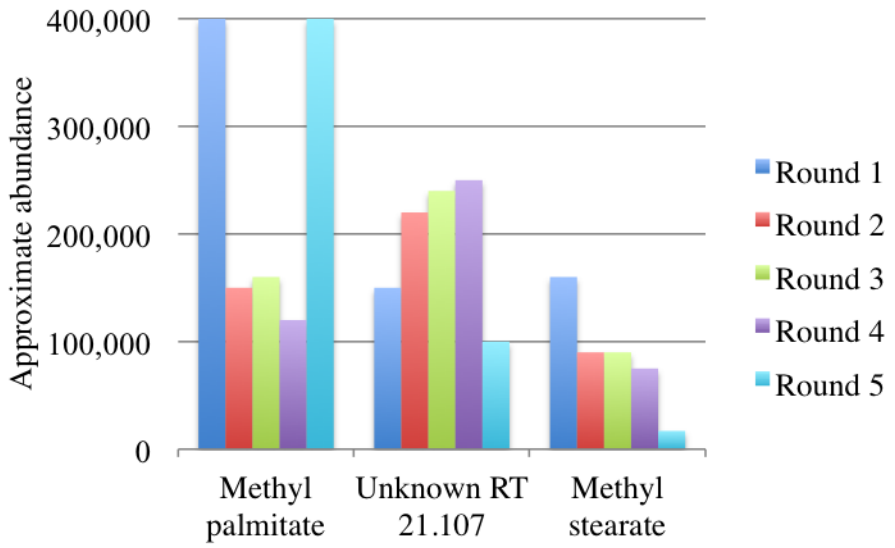

Figure 3.2: *Approximate abundance of non-polar metabolites obtained in each of the five extraction rounds in 400 µl chloroform.*

The extraction of non-polar metabolites was clearly incomplete as about the same abundances were obtained each round. This is likely due to saturation of chloroform. In order to extract more non-polar metabolites into chloroform, an increase in volume is necessary. Alternatively, number of extraction rounds can be increased.

Only three compounds were obtained with GC-Q-MS analysis. More compounds are likely to be obtained with a more sensitive analysis. However, optimisation experiments made use of GC-Q-MS analysis of non-polar extracts for investigation of complete extraction.

Complete extraction with five extraction rounds in 800 µl chloroform

To address the issue of saturation, the volume of chloroform was increased to 800 µl. The alternative of increasing number of extraction rounds leads to a more labour-intensive method and was therefore not chosen. Five extraction rounds in 800 µl chloroform was performed subsequent to polar metabolite extraction. Response of metabolites, given in peak area, was obtained and normalised against tissue weight as shown in Figure 3.3. Percentage response was obtained using the same assumption as for polar metabolites (3.1.1). Data are shown in Table E.3 and mass spectra of unidentified compound in Figure E.1.

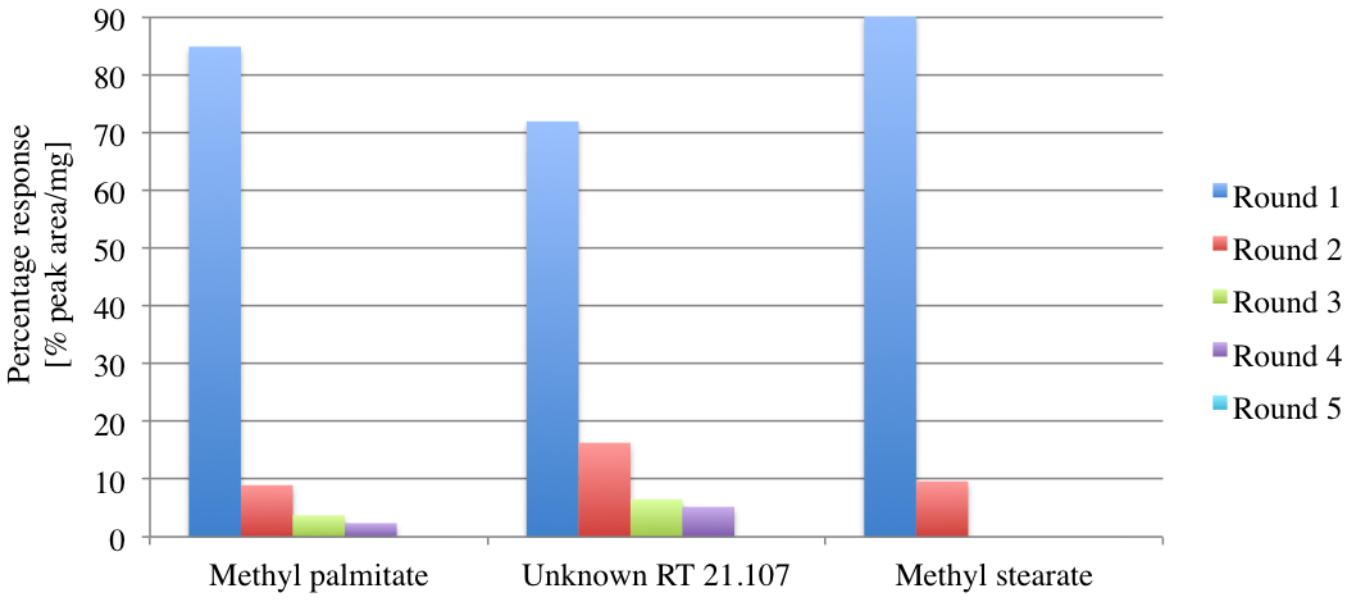

Figure 3.3: *Percentage response, given in percentage peak area per tissue, obtained from each of the five extraction rounds in chloroform subsequent to polar metabolite extraction.*

All non-polar metabolites obtained percentage response of about 5% in the third extraction round. Round four obtained responses even lower and round five no responses at all. Therefore, extraction was stated complete with three extraction rounds. Chloroform was shown suitable as a non-polar extraction solvent. Although complete extraction was shown for fatty acids, complete extraction was assumed for other non-polar metabolites.

Conclusion

Complete extraction of non-polar metabolites is achieved with three extraction rounds in 800 µl chloroform. Therefore three extraction rounds, each round including three intervals of 20 sec, are implemented in experimental procedure. Extracts were pooled in order to simplify method.

3.1.3 Combining extraction of polar and non-polar metabolites

Complete extraction of polar and non-polar metabolites was previously achieved (3.1.1 and 3.1.2). For this reason, approaches to combine and implement extractions of polar and non-polar metabolites into a single method were investigated. An approach to separately extract polar and non-polar metabolites was investigated. Separate extraction was achieved by performing polar metabolite extraction prior to and separately from non-polar metabolite extraction. Furthermore, an approach to simultaneously extract metabolites was investigated. Simultaneous extraction involved a biphasic system of methanol solution and chloroform.

Separate extraction

Extraction of polar metabolites was performed prior to and separately from extraction of non-polar metabolites for a separate extraction. For the reason that polar metabolites were the first to be extracted, performance of extraction was identical to previous (3.1.1). Polar metabolites are therefore completely extracted using the separate approach. The subsequent extraction of non-polar metabolites was also stated complete because previous investigation (3.1.2) was also performed subsequent to polar metabolite extraction. Some

non-polar metabolites may be extracted into polar extracts thus complete extraction into chloroform is not achieved. However, this applies to a few out of many non-polar metabolites. Therefore the approach to separately extract metabolites is stated complete for polar and non-polar metabolites.

Simultaneous extraction

A biphasic system of methanol solution and chloroform was obtained to enable simultaneous extraction of polar and non-polar metabolites. Chloroform was added to polar extract of methanol/water. The biphasic system was obtained subsequent to vortexing and centrifugation. Both phases were collected and analysed using GC-QqQ-MS and GC-Q-MS for methanol/water phase and chloroform phase, respectively. Polar metabolites obtained by analysis of methanol/water phase are shown in Table C.7. Low standard deviations of technical replicates indicate an homogenous methanol/water phase thus reproducible quantitation of polar metabolites.

Non-polar metabolites, however, were not obtained by analysing chloroform phase. An insensitive analysis could have been the explanation. However, a more likely explanation is that non-polar metabolites remain in tissue. Tissue was not present in the biphasic system because chloroform was exclusively added to extracts. The biphasic system described in literature, however, includes tissue [15, 14, 10].

An advantage of the simultaneous approach is that non-polar metabolites, with fairly polar charcteristics, cannot be lost in polar extracts.

Conclusion

The approach to separately extract polar and non-polar metabolites was shown to achieve complete extraction. Separate extraction was therefore implemented in experimental procedure. In contrast, simultaneous extraction obtaining a biphasic system did not achieve complete extraction.

3.1.4 Method reproducibility and variation

Variation in metabolite concentrations were investigated for the reason that method is required reproducible. As a measure of variation, standard deviation based on concentrations of all technical replicates was calculated. Standard deviations obtained for basal-like and luminal-like subtypes based on three technical replicates are listed in Table C.10 and Table C.11, respectively.

Standard deviations obtained from basal-like subtype were generally around 20%. In contrast, tissue samples of luminal-like subtype resulted in great differences in standard deviation. Two tissue samples of luminal-like subtype were associated with high standard deviations of about 40%, another with much lower. These high standard deviations represent great heterogeneity of extracts likely to be due to inadequate mixing. A reduction of standard deviations are required in order to state a reproducible and optimised method. To address this problem, extracts were vortexed in between the making of replicates. With this change in experimental procedure, adequate mixing was assumed. Therefore, method was stated reproducible.

In addition to variation created by extraction method, some variation is expected created by analysis. Day-to-day variation of analytical instruments are likely to affect the sensitivity of analysis which in turn affects concentration. Moreover, variation due to biological diversity cannot be avoided. However, it is assumed smaller than the variation created by experimental procedures. If not, experimental procedures would be highly inadequate. Similarily, there are always going to be some heterogeneity associated with tissue samples (3.2.2).

3.1.5 Sample preparation for polar extracts

Derivatisation methods for GC-QqQ-MS analysis of polar metabolites were evaluated in terms of their suitability to derivatise metabolites in breast cancer xenografts.

Class and number of metabolites obtained with MCF and TMS derivatisation of polar extracts were compared as shown in Table 3.2. The basis of the comparison is optimisation experiments, given in Table C.7 to Table C.11. Metabolites only occasionally obtained are excluded from comparison.

Table 3.2: *Metabolites obtained performing MCF and TMS derivatisation on polar extracts. Class of metabolites are included.*

Metabolites	**MCF**	**TMS**
Amino acids		
Lactate/Methylglyoxal	x	x
Glutamate	x	x
O-acetyl-L-serine	x	
Alanine	x	
Glycine	x	x
Proline	x	x
Threonine	x	
Aspartate	x	
Serine	x	x
Lysine	x	
Valine	x	
Malate	x	x
Fumarate	x	x
Phenylalanine	x	
Methionine	x	
Leucine	x	
Cysteine	x	
Non-amino organic acids		
Salicylate	x	
Citrate	x	x
Glyoxylate	x	
Succinate	x	
Pyruvate	x	
Phenylacetate	x	
Beta-hydroxypyruvate	x	
Benzoate	x	
3-methyl-oxovalerate	x	
Citraconate/Itaconate	x	
4-methylvalerate	x	
Malonate	x	
2-aminobutyrate	x	
Urea		x
Phosphoric acid		x
alpha-ketoglutarate		x
Sugars		
D-glucose		x
D-allose 2		x
Maltose 1		x
Sugar alcohols		
Glycerol 1-phosphate		x
Sterols		
Cholesterol		x
Other		
Allantoin	x	
Creatinine		x
O-phosphocolamine		x
Methyl-beta-D-galactopyranoside		x

MCF and TMS derivatisation obtained 31 and 19 metabolites, respectively. TMS obtained sugar alcohol and sugars not obtained with MCF. The number of metabolites obtained with MCF was considerably greater than the number obtained with TMS. Therefore, MCF is associated with a more sensitive analysis of breast cancer xenografts. MCF was considered suitable for this purpose and was therefore implemented in experimental procedure of extraction method.

3.1.6 Optimised extraction method

Method requirements were shown fulfilled. Consequently, method was stated optimised. Complete extraction was achieved for polar and non-polar metabolites using an approach to separately extract metabolites. The first aim of this project was achieved.

3.1.7 Protocol

Extraction of polar and non-polar metabolites from breast cancer xenograft tissue for MS-based analysis

---

1. Cut and weigh tumours to obtain tissue samples of 30±10 mg.
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.
3. Keep tubes on ice to maintain low temperatures.

Polar metabolite extraction

4. Add 400 µl 60% methanol and 10 µl internal standard to tubes.
5. Homogenise tissue samples for three intervals of 20 sec at 5500 rpm using Precellys 24 tissue homogeniser coupled with Cryolys cooling system (Bertin Technologies).
6. Centrifuge tissue samples at 5000 rpm and -9 °C for 5 min.
7. Remove extracts and store in tubes until later.
8. Repeat 4-7 twice to obtain a second and a third extraction round but add other internal standards.
9. Pool extracts obtained from same tissue sample. Vortex pooled extracts.
10. Make three technical replicates of 100 µl in 5 ml polypropylene (PP) tubes. Vortex pooled extracts in between the making of replicates.

Non-polar metabolite extraction

11. Add 800 µl chloroform and 10 µl internal standard to tubes containing beads and cell pellet.
12. Homogenise tissue samples for three intervals of 20 sec at 6500 rpm.
13. Centrifuge tissue samples at 5000 rpm and -9 °C for 5 min.
14. Remove extracts and store in tubes until later.
15. Repeat 11-14 twice to obtain a second and a third extraction round but add other internal standards.
16. Pool extracts obtained from same tissue sample. Vortex pooled extracts.
17. Make three technical replicates of 100 µl in vials. Vortex pooled extracts in between the making of replicates.

Sample preparation and analysis

18. Vacuum-dry polar and non-polar extracts.
19. Derivatise vacuum-dried polar extracts using MCF.
20. Analyse MCF-derivatised samples using GC-QQQ-MS.
21. Derivatise vacuum-dried non-polar extracts using HCl in methanol.
22. Analyse non-polar extracts using GC-Q-MS.

3.2 Metabolite profiling of polar extracts

Metabolite profiles of luminal-like and basal-like subtypes comprising metabolite concentrations were obtained (3.2.1). To obtain profiles, xenograft tissue from SCID mice was extracted following the optimised method and polar extracts analysed using GC-QqQ-MS. Furthermore, profiles were compared using $\log_2$ ratios, principal component analysis (PCA) and Student's t-test (3.2.2).

3.2.1 Metabolite profiles of luminal-like and basal-like subtypes

Six biological replicates, each producing one technical replicate, were the basis of metabolite profiles shown in Table 3.3. To represent uncertainty in concentration, standard deviation between biological replicates, in picomolar/mg tissue (pM/mg) and in percentage, was included. Raw data are shown in Table C.12.

Metabolite profiles of luminal-like and basal-like subtypes comprise of 34 and 35 polar metabolites, respectively. The polar metabolites include amino and non-amino organic acids. Citraconate/itaconate was only found in basal-like subtype. Lactate and glycine, metabolites that have been noted to increase in breast cancers, were obtained in profiles [3].

Standard deviations range widely and are generally higher than 30%. This indicates high heterogeneity of tissue samples. Moreover, heterogeneous tissue samples indicate heterogeneous tissue. Heterogeneity of samples was attempted reduced by cutting tumours in a certain way. The average of standard deviations obtained for luminal-like and basal-like subtypes was 36% and 30%, respectively. The higher value obtained for luminal-like subtype indicates that tissue samples of luminal-like subtype are more heterogeneous than tissue samples of basal-like subtype. Therefore, it was suggested that luminal-like subtype possesses a more heterogeneous tissue than basal-like subtype. The suggestion is in accordance with earlier studies [17].

Table 3.3: *Metabolite profiles of luminal-like and basal-like subtypes of breast cancer comprising polar metabolites, their concentration and corresponding standard deviation, given in pM/mg tissue. Percentage standard deviation is given in paranthesis.*

Metabolite	**Concentration [pM/mg]**					
	Luminal-like			**Basal-like**		
Lactate/Methylglyoxal	59,886	± 23,501	(39%)	101,489	± 29,027	(29%)
Glutamate	36,735	± 11,160	(30%)	106,700	± 27,821	(26%)
O-acetyl-L-serine	33,629	± 6,752	(20%)	16,943	± 6,573	(39%)
Allantoin	32,652	± 10,904	(33%)	72,088	± 31,164	(43%)
Alanine	23,503	± 5,227	(22%)	19,397	± 6,351	(33%)
Glycine	17,326	± 4,416	(25%)	60,867	± 16,809	(28%)
Salicylate	12,742	± 5,295	(42%)	11,574	± 3,522	(30%)
Proline	15,265	± 4,651	(30%)	17,547	± 3,326	(19%)
Threonine	10,473	± 2,561	(24%)	9,844	± 3,023	(31%)
Glyoxylate	3,388	± 1,438	(42%)	8,031	± 5,105	(64%)
Aspartate	8,233	± 3,270	(40%)	1,569	± 571	(36%)
Citrate	6,628	± 2,633	(40%)	15,829	± 3,779	(24%)
Serine	4,347	± 2,726	(63%)	3,633	± 1,252	(34%)
Lysine	4,277	± 1,581	(37%)	10,331	± 2,906	(28%)
Valine	3,608	± 1,341	(37%)	3,414	± 870	(25%)
Tyrosine	2,293	± 875	(38%)	2,673	± 646	(24%)
Malate	1,819	± 1,204	(66%)	3,298	± 1,117	(34%)
Succinate	3,111	± 774	(25%)	3,001	± 492	(16%)
Pyruvate	1,457	± 857	(59%)	2,503	± 1,096	(44%)
Fumarate	1,197	± 404	(34%)	2,154	± 587	(27%)
Phenylalanine	1,630	± 513	(31%)	1,584	± 513	(32%)
Citramalate	895	± 272	(30%)	1,447	± 386	(27%)
Methionine	1,453	± 433	(30%)	1,296	± 281	(22%)
Hippurate	1,256	± 352	(28%)	1,302	± 285	(22%)
Ornithine	1,125	± 686	(61%)	647	± 306	(47%)
Phenylacetate	977	± 407	(42%)	1,042	± 241	(23%)
Leucine	975	± 344	(35%)	617	± 292	(47%)
m-Toluate	569	± 207	(36%)	550	± 107	(19%)
Beta-hydroxypyruvate	563	± 172	(31%)	1,059	± 370	(35%)
Benzoate	484	± 143	(30%)	753	± 185	(25%)
2-isopropylmalate	451	± 118	(26%)	694	± 126	(18%)
3-methyl-oxovalerate	260	± 94	(36%)	643	± 123	(19%)
Citraconate/Itaconate		-		540	± 156	(29%)
4-methylvalerate	179	± 53	(30%)	275	± 69	(25%)
2-aminobutyrate	245	± 80	(33%)	269	± 67	(25%)

3.2.2 Comparison of metabolite profiles

Metabolite profiles were compared in order to find differences and similarities in metabolite content between subtypes. Results from $\log_2$ ratios, Student's t-test and PCA are shown in Table 3.4.

Log_2 ratios

Log_2 ratios of concentrations in metabolite profiles were obtained and presented in Figure 3.4. Ratios were calculated with concentration in luminal-like subtype as the nominator and with concentration in basal-like subtype as the denominator. As a consequence, positive ratios indicated a higher concentration in luminal-like than in basal-like subtypes. Negative ratios indicated the opposite. It was assumed that ratios with absolute values of 0.5 indicates a difference between subtypes. Therefore, the coloured area of the plot illustrates $\log_2$ ratios not associated with a difference in concentration.

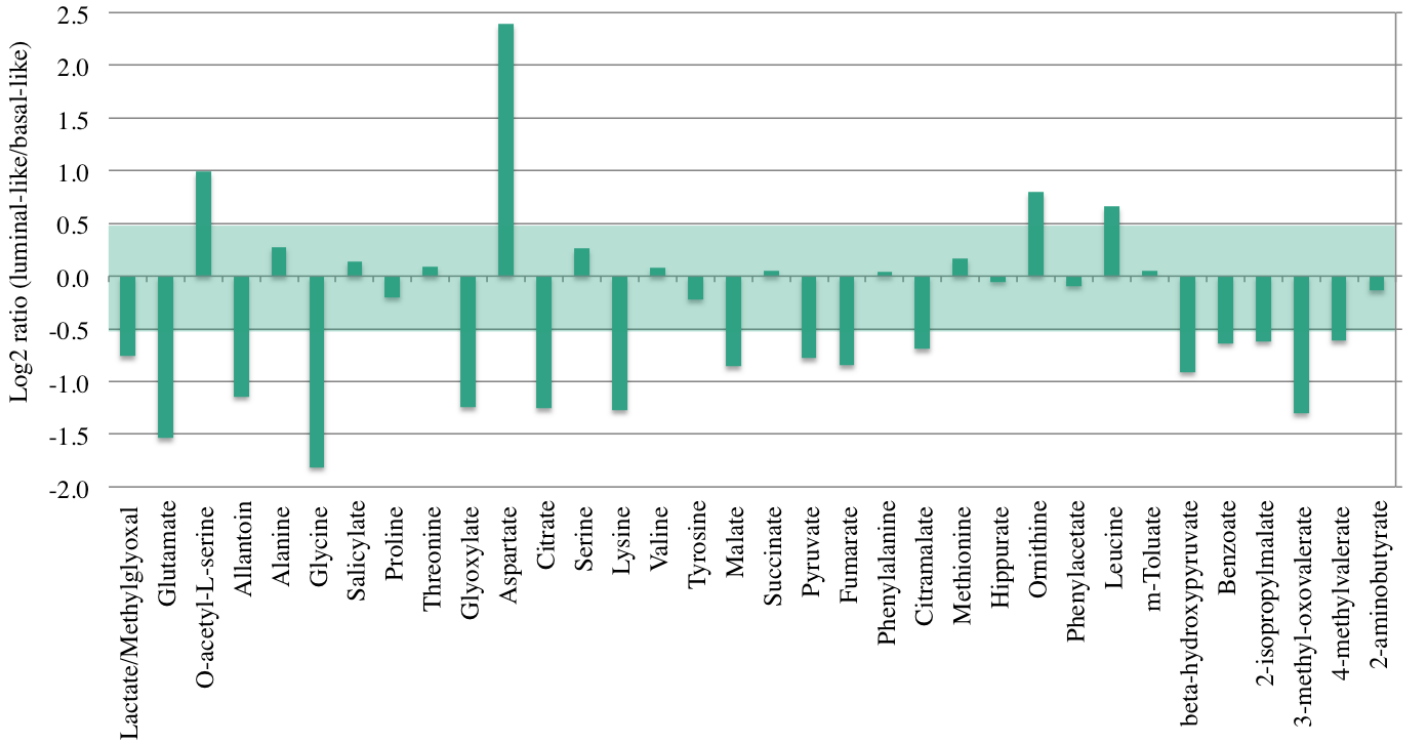

Figure 3.4: *Log_2 ratio of concentration in luminal-like subtype to basal-like subtype. Difference in concentration was assumed for ratios greater than absolute value of 0.5.*

20 metabolites were found to differ in concentration between subtypes. Most of these metabolites were contained in higher concentrations in basal-like than in luminal-like subtypes, for instance glycine. This finding has previously been described in literature [3]. Metabolites that were suggested by $\log_2$ plot to be contained in different concentrations between subtypes are shown in Table 3.4.

Principal component analysis (PCA)

PCA analysis was performed on concentrations used to obtain metabolite profiles, shown in Table C.12. A score plot of principal component 1 (PC-1) versus principal component 2 (PC-2) is shown in Figure 3.5. Each data point represents a biological replicate. Lines were drawn to link biological replicates in order to investigate for grouping of subtypes.

Score plot showed a grouping of subtypes into two distinct groups. The two distinct groups that appears in score plot indicate considerable differences between subtypes. Otherwise, these groups would have overlapped. Biological replicates of luminal-like subtype were seen spread in a greater extent than biological replicates of basal-like subtype. Luminal-like subtype is therefore associated with greater standard deviations and higher heterogeneity of tissue samples and tissue compared to basal-like subtype. This is in accordance with previous discussion of standard deviation and heterogeneity (3.2.1).

A loading plot of PC-1 versus PC-2 is shown in Figure 3.6. Each data point represents the concentration of a specific metabolite. Loading plot was compared with score plot in order to suggest correlations between metabolites and subtypes. Correlation with subtype was shown in Table 3.4 as it highlights difference between subtypes. Position of metabolite in loading plot coinciding with position of subtype in score plot suggests positive correlation between metabolite and subtype. Several metabolites were shown to positively correlate with basal-like subtype and a few with luminal-like subtype. Metabolites found contained in higher concentrations in luminal-like subtype than in basal-like subtype were compared with results of $\log_2$ ratios in Figure 3.4. Metabolites found contained in higher concentrations in basal-like subtype than in luminal-like subtype were also compared. All correlations found by PCA coincided with conclusions drawn from $\log_2$ ratios.

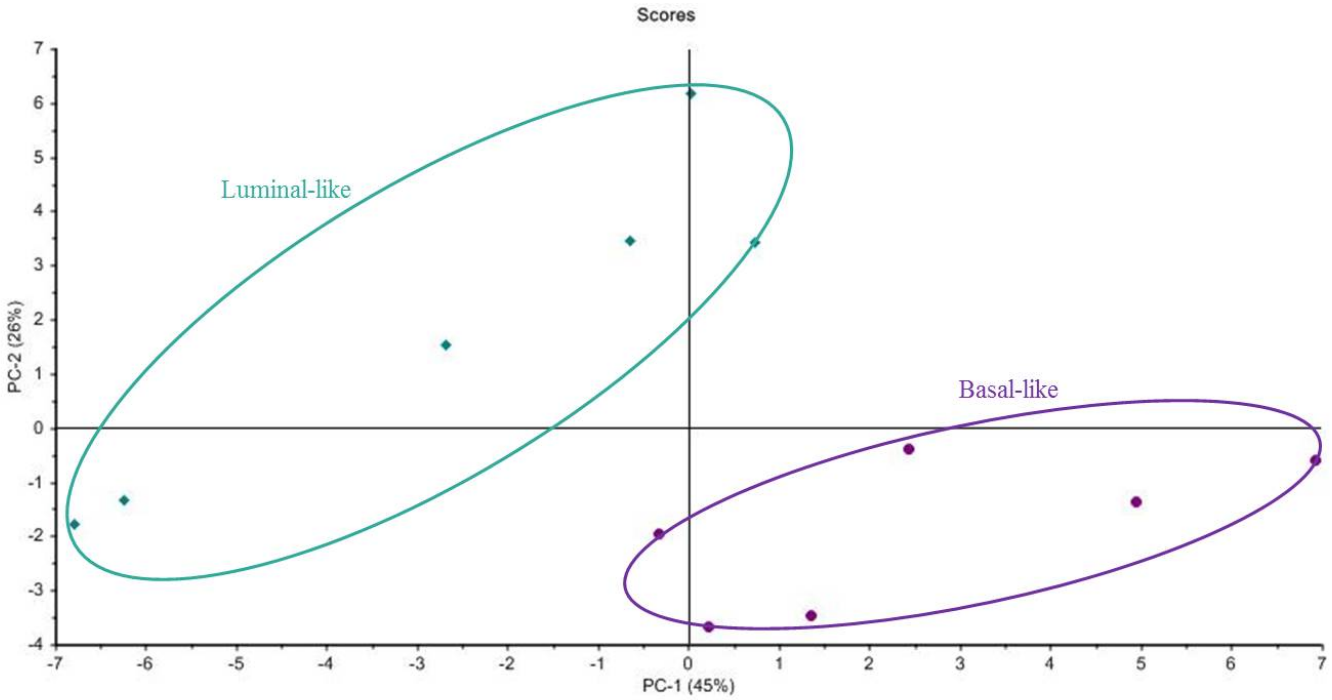

Figure 3.5: *Score plot based on metabolite concentrations of six biological replicates of basal-like and luminal-like subtypes obtained by performing PCA analysis.*

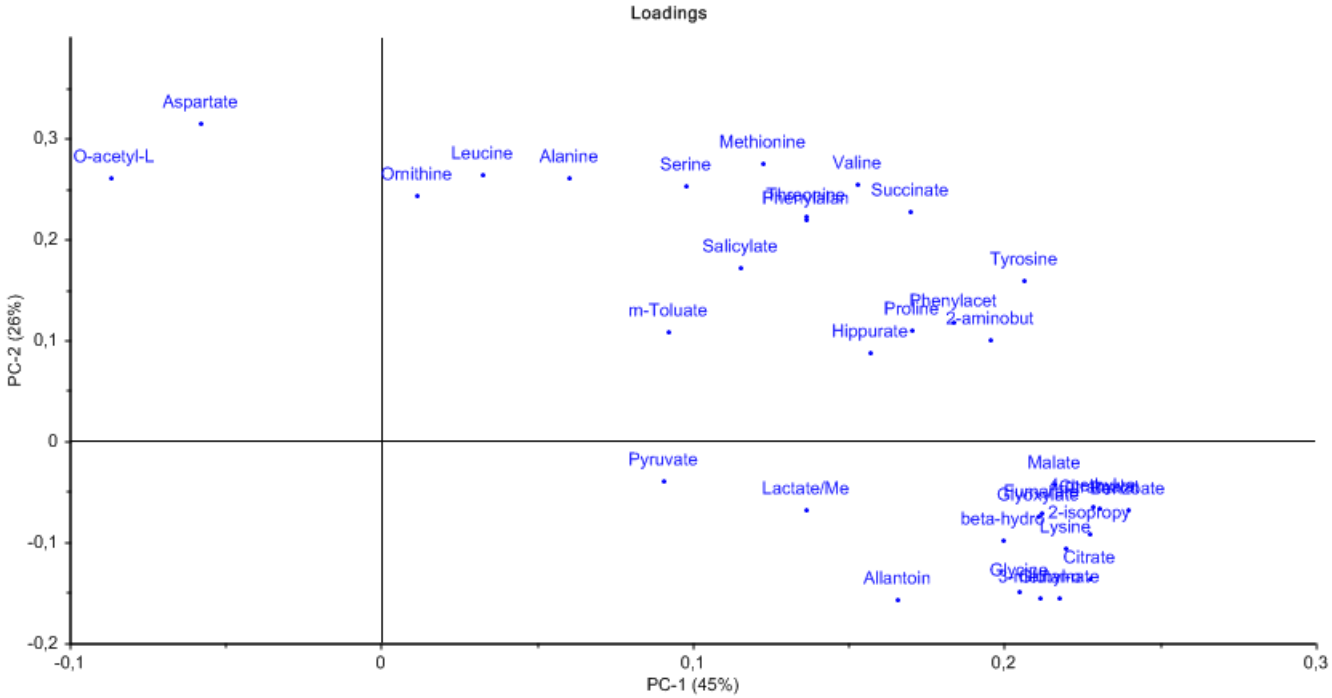

Figure 3.6: *Loading plot based on metabolite concentrations of six biological replicates of basal-like and luminal-like subtypes obtained by performing PCA analysis.*

Student's t-test

Metabolites that were shown to significantly differ in concentration between subtypes using Student's t-test are shown in Table 3.4. Student's t-test was performed on concentrations used to obtain metabolite profiles, shown in Table C.12. Probability describing likeliness of concentrations to obtain the same average was obtained. Student's t-test is therefore a great indication of whether a metabolite is contained in different concentrations in subtypes. Because two subtypes giving rise to two data sets were investigated, Stundent's t-test rather than analysis of variance (ANOVA) was chosen. Significance level was set to 5% meaning that a significant difference in concentration is stated for metabolites obtaining this probability. All metabolites associated with a significant difference between subtypes were also suggested contained in different concentrations by $\log_2$ ratios and PCA analysis.

Differences and similarities in metabolite content between subtypes

Results of $\log_2$ ratios, PCA analysis and Student's t-test are shown in Table 3.4. Log_2 ratios and PCA analysis suggested that 20 metabolites differed in concentration between subtypes. However, significant difference were shown for 14 metabolites using Student's t-test. These metabolites can possibly be exploited in order to identify subtypes. Metabolite profiles were therefore shown a potential tool for identification of subtype.

Whether a difference was obtained due to a higher or a lower concentration in luminal-like subtype compared to basal-like subtype was readily seen in Figure 3.4. Log_2 ratios were shown in accordance with results from PCA analysis. Lactate, glycine, citrate and lysine were some of the metabolites associated with a significant difference and a higher concentration in basal-like subtype. Generally, basal-like subtype contained higher concentrations than luminal-like subtype. O-acetyl-L-serine and aspartate were some of the metabolites associated with a significant difference and a higher concentration in luminal-like than basal-like subtypes. In addition to possibly identify subtype, these metabolites may provide insight into metabolic changes in breast cancers. Furthermore, some of these metabolites can potentially serve as metabolic markers for prognosis and treatment response.

Table 3.4: *Results of log_2 ratios, PCA and Student's t-test highlighting differences and similarities in metabolite concentration between subtypes. x represents difference in concentration, blank similar concentration and n/a not applicable. p-values associated with the t-test are shown in parantheses.*

Metabolite	**Data analysis**		
	Log_2 ratio	**PCA**	**Student's t-test**
Lactate/Methylglyoxal	x	x	x (2.2%)
Glutamate	x	x	
O-acetyl-L-serine	x	x	x (0.1%)
Allantoin	x	x	x (2.5%)
Alanine			
Glycine	x	x	x (0.1%)
Salicylate			
Proline			
Threonine			
Glyoxylate	x	x	
Aspartate	x	x	x (0.4%)
Citrate	x	x	x (0.1%)
Serine			
Lysine	x	x	x (0.2%)
Valine			
Tyrosine			
Malate	x	x	
Succinate			
Pyruvate	x	x	
Fumarate	x	x	x (1.0%)
Phenylalanine			
Citramalate	x	x	x (1.9%)
Methionine			
Hippurate			
Ornithine	x	x	
Phenylacetate			
Leucine	x	x	
m-Toluate			
Beta-hydroxypyruvate	x	x	x (2.0%)
Benzoate	x	x	x (1.9%)
2-isopropylmalate	x	x	x (0.6%)
3-methyl-oxovalerate	x	x	x (0.0%)
Citraconate/Itaconate	n/a	n/a	x (0.0%)
4-methylvalerate	x	x	x (2.4%)
2-aminobutyrate			

3.3 Investigation of metabolic pathways using isotope labelling

Metabolic pathways of treated luminal-like subtype, shown to possess reduced tumour growth rates, was investigated using carbon-13 (^{13}C)-labelled glucose. Carbon 1 of glucose was replaced by ^{13}C, giving rise to [1-^{13}C]glucose. Injection of [1-^{13}C]glucose into xenograft models was performed 10 min prior to tumour excision and halting of biological activity. Polar metabolites were extracted and analysis using GC-Q-MS.

To track the passage of ^{13}C through metabolic pathways, summed fractional labelling (SFL) was calculated directly from mass spectrum. See Appendix G, Equation G.1. SFL given in percentage and corrected for naturally occuring ^{13}C is shown in Table 3.5 for lacate, citrate, proline and fumarate.

Table 3.5: *Summed fractional labelling (SFL) given in percentage for metabolites obtained in treated luminal-like subtype. Possible positions of ^{13}C in metabolite are included.*

Metabolite	**Possible position of ^{13}C**	**SFL [%]**
Lactate	C-3	8.3
Citrate	C-4	2.2
Proline	C-4	0
Fumarate	C-4	1.6

Metabolic pathways of glycolysis and tricarboxylic acid cycle (TCA) are illustrated in Figure 3.7. Formation of lactate and proline were also illustrated. Red coloured carbons represent ^{13}C. The passage of ^{13}C shown in Figure 3.7 is only a suggestion based on literature [8].

SFL indicated ^{13}C labelling of 8.3% lactate, 2.2% citrate and 1.6% fumarate. Given this, it can be concluded that ^{13}C is retained throughout glycolysis. Moreover, ^{13}C enters the TCA cycle and gives rise to ^{13}C-labelled TCA intermediates such as citrate and fumarate. This is in accordance with previous studies [9]. Figure 3.7 suggests that carbon 3 of lactate and carbon 4 of citrate and fumarate were labelled as a result of [1-^{13}C]glucose.

No labelling was obtained for proline. It took 10 minutes from injection to excision, and this period may be inadequate to give rise to labelled proline. Furthermore, a very small fraction of alpha-ketoglutarate is likely to be labelled as only 2.2% citrate was obtained. Alpha-ketoglutarate can form other TCA inermediates in addition to proline. This reduces the possibility of labelled proline. SFL of glutamate, alanine and succinate were not calculated because their corresponding deuterised internal standards were previously added to extracts.

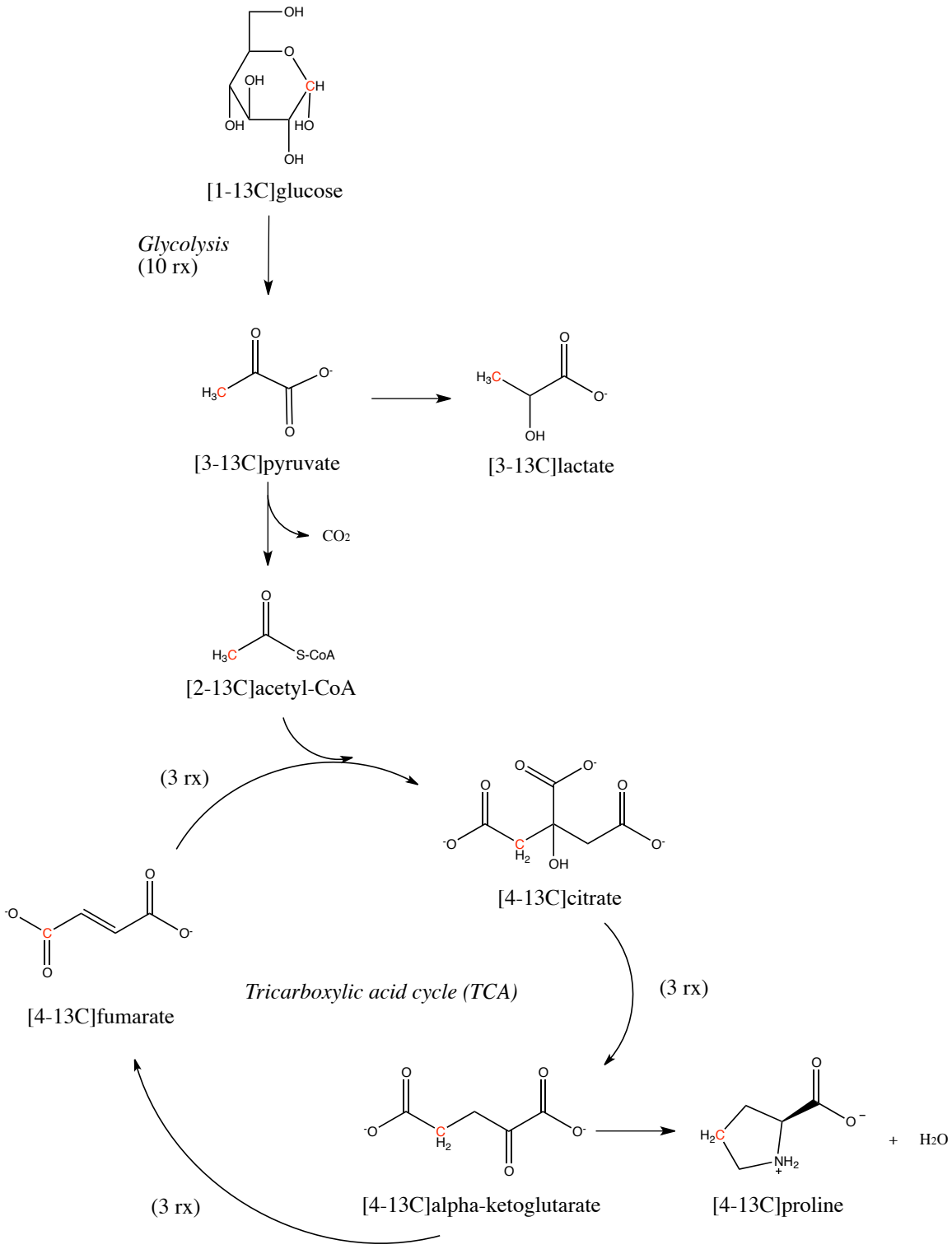

Figure 3.7: *Metabolic pathways in glycolysis, TCA cycle and in formation of lacate and proline drawn with ChemBioDraw Ultra software. Number of successive reactions (rx) is shown beside arrows, for arrows that represent more than one reaction. Possible passage of* ^{13}C *from glucose labelled in position 1 is shown using red coloured carbons to represent* ^{13}C*.*

SFL of metabolites in untreated luminal-like subtype may differ from the ones obtained above for the treated luminal-like subtype. Moreover, differences in metabolic pathways between subtypes may be noted using isotope labelling.

3.4 Q-TOF LC-MS analysis of non-polar extracts

Non-polar extracts were vaccum-dried, redissolved in dichloromethane (DCM) and infused directly into MS compartment of Q-TOF LC-MS. Total ion current (TIC) plot, showing mass scan data collected over time, were obtained using electrospray ionisation (ESI) in positive ion mode. Figure 3.8 shows TIC plots of blank (DCM) and sample obtained from non-polar extract.

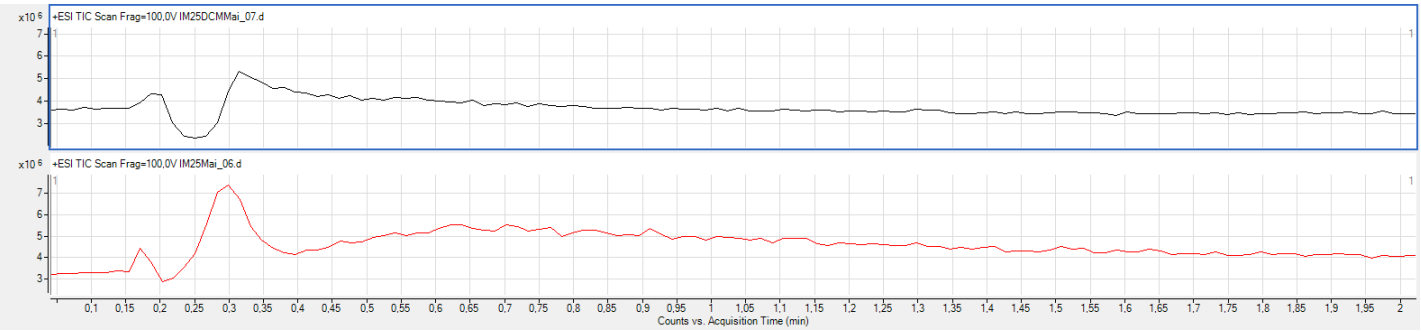

Figure 3.8: *TIC plots of blank at the top and sample at the bottom showing mass scan data collected over time.*

The peaks obtained after 0.3 min in blank and sample are probably due to DCM. However, peak in sample is greater than the one in blank and may therefore contain non-polar metabolites as lipids. Sample obtained a slight increase of abundance from 0.4 min and on. This increase is not shown in blank and is therefore likely to represent non-polar metabolites. Mass fragments of peak and of the slight increase are shown in F.1 and F.2, respectively. Injection of more concentrated samples and adjustment of LC-MS method settings are assumed to lead to more peaks in TIC plot of non-polar extracts.

3.5 Recommendations for future work

3.5.1 Loss of lipids in polar extracts

The approach to separately extract polar and non-polar metabolites was included in the optimised method (3.4). However, it is recommended to investigate a possible loss of lipids in polar extracts. Alternatively, if complete extraction is achieved by simultaneous extraction using a biphasic system, optimised method can include simultaneous extraction instead. Loss of lipids is not of any great concern for extractions using biphasic system.

3.5.2 Analysis of non-polar extracts

Optimisation was based on fatty acid analysis of non-polar extracts using GC-Q-MS. However, low sensitivity leading to detection of only three non-polar metabolites indicates inadequacy of method. An approach to overcome this problem, is to make samples more concentrated and to adjust method settings of GC-Q-MS.

Investigation of lipids in non-polar extracts can be performed using LC-MS analysis.

3.5.3 Metabolite profiles

Metabolite profiles were obtained using six biological replicates. To obtain more accurate profiles, extraction of more biological replicates are recommended. Prior to future extraction experiments, expiring dates of MCF and deuterated-MCF should be checked. It is suggested that an expired deuterated-MCF was the reason GC-QqQ-MS analysis obtained unexpected shapes of peaks. These peaks made data handling more difficult. For this reason, cysteine was excluded. Expiring date was assumed longer for MCF than deuterated-MCF, thus it was concluded that deuterated-MCF was expired [22].

To increase the number of metabolites presented in metabolite profiles, non-polar metabolites is suggested quantitated and included. Metabolite profiles representing more metabolite classes are desired.

In order to start using metabolite profiles in clinical settings, comprehensive investigation remains.

4 Conclusion

Optimal treatment of breast cancer remains a major challenge. To improve treatment, prediction of prognosis and treatment response have to become more reliable. Metabolite profiling of breast cancer tissue can potentially contribute to better techniques for prediction. The aim of this project was divided into three parts: Metabolite extraction and profiling, and isotope labelling of metabolites.

The first aim was to optimise a method for extraction of polar and non-polar metabolites for MS-based analysis. Beads-based homogenisation of tissue in 60% methanol solution and in chloroform was shown to fulfill method requirements, including complete extraction, simple performance and high reproducibility. Consequently, extraction method was successfully optimised.

The second aim was to obtain and compare metabolite profiles of breast cancer subtypes. Profiles comprised of polar metabolites and their concentration, given in pM/mg tissue, were obtained for luminal-like and basal-like subtypes using the optimised extraction method in combination with GC-MS analysis. Log_2 ratios, PCA analysis and Student's t-test were used to compare metabolite profiles, hence subtypes. Log_2 ratios and PCA pointed out that basal-like subtype is generally associated with higher concentrations than luminal-like subtype. PCA obtained a grouping of subtypes, indicating considerable differences in metabolite content between subtypes. 15 metabolites were found by Student's t-test to significantly differ in concentration between subtypes, including lactate, glycine, citrate, lysine, o-acetyl-L-serine and aspartate. Metabolite profiling is therefore a potential tool for identification of subtype. Established metabolite profiles may contribute to a more reliable prediction of prognosis and treatment response. Lactate and glycine have previously been noted to increase in breast cancers. The 13 other metabolites found to differ significantly, may increase the knowledge of metabolic changes in breast cancers and possibly serve as potential metabolic markers of breast cancer.

The third aim was to investigate metabolic pathways of treated luminal-like subtype using isotope labelling. Injection of [1-^{13}C]glucose into xenograft models prior to tumour excision, extraction and GC-MS analysis was performed. Summed fractional labelling (SFL) indicated that ^{13}C was retained throughout glycolysis and that it entered the TCA cycle and gave rise to ^{13}C-labelled TCA intermediates.

References

[1] T. Vargo-Gogola and J.M. Rosen. Modelling breast cancer: one size does not fit all. *Nature Reviews Cancer*, 7(9):659–672, 2007.

[2] Cancer Registry of Norway, http://www.kreftregisteret.no/no/Registrene/Kreftstatistikk/. Viewed 04.03.2012.

[3] S. Moestue, E. Borgan, E. Huuse, E. Lindholm, B. Sitter, A.L. Børresen-Dale, O. Engebraaten, G. Mælandsmo, and I. Gribbestad. Distinct choline metabolic profiles are associated with differences in gene expression for basal-like and luminal-like breast cancer xenograft models. *BMC cancer*, 10(1):433, 2010.

[4] W. M. Becker, L. J. Kleinsmith, J. Hardin, and G. P. Bertoni. *The World of the Cell.* Pearson International Edition, 7th edition, 2009, p. 24, 43, 67, 159, 757, 758, 761, 774.

[5] 9ma, http://www.9ma.com/body/2012/01/79100-The-anatomic-breast.html. Viewed 15.02.2012.

[6] Parker J Hastie T Marron JS Nobel A et al: Sorlie T, Tibshirani R. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA*, 100:8418–8423, 2003.

[7] M.R. Mashego, K. Rumbold, M. De Mey, E. Vandamme, W. Soetaert, and J.J. Heijnen. Microbial metabolomics: past, present and future methodologies. *Biotechnology letters*, 29(1):1–16, 2007.

[8] D. L. Nelson and M. M Cox. *Lehninger Principles of Biochemistry.* Number p. 529, 621, 861-862, 865-866. W. H. Freeman and Company, 5th edition, 2008.

[9] K. Glunde, L. Jiang, S.A. Moestue, and I.S. Gribbestad. Mrs and mrsi guidance in molecular medicine: targeting and monitoring of choline and glucose metabolism in cancer. *NMR in Biomedicine*, 24(6):673–690, 2011.

[10] H. Wu, A.D. Southam, A. Hines, and M.R. Viant. High-throughput tissue extraction protocol for nmr-and ms-based metabolomics. *Analytical biochemistry*, 372(2):204–212, 2008.

[11] M. Staahlman, C. S. Ejsing, K. Tarasov, J. Perman, J. Boren, and K. Ekroos. High-throughput shotgun lipidomics by quadrupole time-of-flight mass spectroscopy. *Journal of Chromatography B*, 877:2664–2672, 2009.

[12] I. Pedersen. *Evaluation and Optimisation of Metabolite Extraction Methods for Breast Cancer Xenografts for GC-MS analysis.* NTNU Student Project TMT4105, 2011.

[13] R. Verollet. A major step towards efficient sample preparation with bead-beating. *Biotechniques*, 44(6):832–833, 2008.

[14] W. Römisch-Margl, C. Prehn, R. Bogumil, C. Röhring, K. Suhre, and J. Adamski. Procedure for tissue sample preparation and metabolite extraction for high-throughput targeted metabolomics. *Metabolomics*, 8:133–142, 2012.

[15] E.G. Bligh and W.J. Dyer. A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology*, 37(8):911–917, 1959.

[16] J.L. Griffin and R.A. Kauppinen. Tumour metabolomics in animal models of human cancer. *Journal of proteome research*, 6(2):498–505, 2007.

[17] A. Bergamaschi, G.O. Hjortland, T. Triulzi, T. Sørlie, H. Johnsen, A.H. Ree, H.G. Russnes, S. Tronnes, G.M. Mælandsmo, O. Fodstad, et al. Molecular profiling and characterization of luminal-like and basal-like in vivo breast cancer xenograft models. *Molecular Oncology*, 3(5):469–482, 2009.

[18] K. Lindberg, L.A. Helguero, Y. Omoto, J.Å. Gustafsson, and L.A. Haldosén. Estrogen receptor b represses akt signaling in breast cancer cells via downregulation of her2/her3 and upregulation of pten: implications for tamoxifen sensitivity. *Breast Cancer Research*, 13:R43, 2011.

[19] J.F. Forbes, J. Cuzick, A. Buzdar, A. Howell, J.S. Tobias, M. Baum, et al. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the atac trial. *The lancet oncology*, 9(1):45–53, 2008.

[20] S.G. Villas-Boas, D.G. Delicado, M. Akesson, J. Nielsen, et al. Simultaneous analysis of amino and nonamino organic acids as methyl chloroformate derivatives using gas chromatography-mass spectrometry. *Analytical biochemistry*, 322(1):134–138, 2003.

[21] K. Ichihara and Y. Fukubayashi. Preparation of fatty acid methyl esters for gas-liquid chromatography. *Journal of lipid research*, 51(3):635–640, 2010.

[22] http://worldaccount.basf.com/wa/NAFTA/Catalog/ChemicalsNAFTA/doc4/BASF/PRD/30037055/pdf?title=.&asset_type&=msds/pdf&language=EN&validArea=US&urn=urn:documentum:ProductBase_EU:09007af880093849.pdf. Viewed 29.03.2012.

[23] C. Cannizzaro, B. Christensen, J. Nielsen, and U. Stockar. Metabolic network analysis on phaffia rhodozyma yeast using 13 c-labeled glucose and gas chromatography-mass spectrometry. *Metabolic Engineering*, 6(4):340–351, 2004.

A List of abbreviations

Abbreviations used in report are listed in table A.1 and A.2.

Table A.1: *Abbreviations used in report*

Word	**Abbreviation**
Automated Mass Spectral Deconvolution and Identification Software	AMDIS
Average	avg
Basal-like	bas
Carbon 13	^{13}C
Carbon 13 at position 1	[1-^{13}C]
Deconvolution Reporting Software	DRS
Dichloromethane	DCM
Deuterised internal standard	d3-istd
Deuterised methyl chloroformate	d3-MCF
Deviation	dev
Electron impact	EI
Electrospray ionisation	ESI
Estrogen receptor negative	ER-
Estrogen receptor positive	ER+
Fatty acid methyl esters	FAME
Gas chromatography	GC
Gas chromatography mass spectrometry	GC-MS
Glucose	Glc
High Energy Dynode Detector - Electron Multiplier	HED-EM
Hydrogen chloride	HCl
Id est	i.e.
Liquid chromatography	LC
Liquid chromatography mass spectrometry	LC-MS
Luminal-like	lum
Mass selective detector	MSD
Mass spectrometry	MS
Mass-to-charge	m/z
Methyl chloroformate	MCF
Methyl tert-betyl ether	MTBE
Millimetre	mm
Micro	µ
Minute	min
Molar	M
Milli-Q-grade	MQ
National Institute of Standards and Technology	NIST
Norwegian University of Science and Technology	NTNU
Not applicable	n/a
Nuclear magnetic resonance	NMR
Principal component analysis	PCA
Principal component	PC
Pico	p

Table A.2: *Abbreviations used in report continued*

Word	**Abbreviation**
Polypropylene	PP
Positive chemical ionisation	PCI
Quadrupole Time-of-Flight	Q-TOF
Reaction	rx
Retention time	RT
Second	sec
Severe combined immunodeficient	SCID
Single Quadrupole	Q
Sodium sulfate	Na_2SO_4
Standard	std
Summed fractional labelling	SFL
Total ion current	TIC
Tricarboxylic acid cycle	TCA
Trimethylsilyl/-ation	TMS
Triple Quadrupole	QqQ
Volume	V
Volume-to-volume	v/v

B Tumour and sample names

Table B.1 relates sample names to full tumour names, specific to each individual tumour. Section in report where experimental procedure is described is also given.

Sample name includes date of extraction and a number representing each individual sample. Samples extracted following the optimised method include subtype. Bas and lum denote basal-like and luminal-like subtypes, respectively. T represents treatment. Tumour name includes date of excision, subtype and location of mouse in cage. Treated xenograft models are not associated with any date but type of treatment. No supply of estrogen is denoted as -estrogen and injection of ^{13}C-labelled glucose into xenograft models as 13C Glc.

Table B.1: *Sample name, full tumour name and experimental procedure of extraction.*

Sample name	**Full tumour name**	**Experimental**
180112 1-3	20.11.08 MAS98.12 1-1V	2.2.1
130212 1-3, 1 check-3 check	21.11.08 MAS98.12 2-4V	2.2.2
210212 1-3	21.11.08 MAS98.12 2-4V	2.2.2
280212 1-2	21.11.08 MAS98.12 2-1V	2.2.2
140312 bas 1-bas 3	21.11.08 MAS98.12 2-1V	2.2.3
140312 lum 1-lum 3	07.11.08 MAS98.06 6-2V	2.2.3
210312 lumT 1	MAS98.06 10-6 -estrogen 13C Glc	2.2.3
210312 lumT 2-3	MAS98.06 10-3 -estrogen 13C Glc	2.2.3
210312 lumT 4-5	MAS98.06 10-1 -estrogen 13C Glc	2.2.3
210312 lumT 6-7	MAS98.06 10-2 -estrogen 13C Glc	2.2.3
210312 lumT 8-9	MAS98.06 10-4 -estrogen 13C Glc	2.2.3
210312 lumT 10-12	MAS98.06 10-5 -estrogen 13C Glc	2.2.3
270312 bas 1	18.11.08 MAS98.12 2-3H	2.2.3
270312 bas 2	18.11.08 MAS98.12 2-2H	2.2.3
270312 bas 3	18.11.08 MAS98.12 2-2V	2.2.3
270312 bas 4	18.11.08 MAS98.12 2-4H	2.2.3
270312 bas 5	21.11.08 MAS98.12 2-5V	2.2.3
270312 bas 6	17.11.08 MAS98.12 1-3V	2.2.3
270312 lum 1	04.11.08 MAS98.06 4-1V	2.2.3
270312 lum 2	07.11.08 MAS98.06 6-2H	2.2.3
270312 lum 3	07.11.08 MAS98.06 5-4H	2.2.3
270312 lum 4	07.11.08 MAS98.06 6-1V	2.2.3
270312 lum 5	07.11.08 MAS98.06 6-2V	2.2.3
270312 lum 6	LAI1u H1 MRI MAS98.06 03.02.10 EMH	2.2.3

C MCF derivatisation of polar extracts

C.1 Raw data

Raw data were obtained in picomol per microliter injected sample. However, Table C.3 is an exception with raw data in peak area per microliter injected sample.

Table C.1: *Metabolite concentration of injected sample [pmol/µl] of methanol/water phase in the biphasic system obtained subsequent to three extraction rounds in 60 % methanol. Each tissue sample obtained three technical replicates.*

Metabolite	**Sanple name** 280212					
	1 1	1 2	1 3	2 1	2 2	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
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	-	5.03	3.52	-	-	-
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
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
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	-
d5-Glutamate	711.35	801.99	779.16	839.02	1,052.22	958.72
d8-Valine	59.43	65.77	65.04	63.95	65.07	61.56
d4-Succinate	54.65	58.00	56.55	56.49	62.73	56.20
d3-Alanine	77.22	85.22	81.47	84.79	88.00	83.72

Table C.2: *Metabolite concentration of injected sample [pmol/µl] of polar extracts obtained in each of the three extraction rounds in 60% methanol. Concentrations are an average of three technical replicates.*

Sample name 180112	1 r1	1 r2	1 r3	2 r1	2 r2	2 r3	3 r1	3 r2	3 r3
4-methylvalerate	0.09	0.08	0.07	0.06	0.06	0.05	0.05	0.05	0.05
Malonate	0.14	0.15	0.16	0.22	0.22	0.16	0.16	0.15	0.15
Pyruvate	1.70	0.86	0.48	1.63	0.62	0.36	0.99	0.45	0.28
3-methyl-oxovalerate	0.03	0.01	0.01	0.01	0.01	0.00	0.02	0.00	0.00
Fumarate	1.61	0.44	0.12	1.14	0.42	0.10	1.12	0.22	0.07
Lactate/Methylglyoxal	356.11	82.23	16.36	607.79	160.50	28.30	381.32	46.30	10.26
Succinate	3.26	0.81	0.42	5.48	1.82	0.60	3.14	0.58	0.34
Benzoate	0.18	0.17	0.14	0.15	0.17	0.13	0.11	0.11	0.11
Beta-3-hydroxybutyrate	5.02	1.31	0.47	4.28	1.44	0.42	4.06	3.97	0.46
Alanine	39.87	7.84	1.51	43.94	9.77	1.74	36.28	4.33	0.88
Glycine	48.79	9.62	2.04	41.09	10.00	1.99	44.58	5.98	1.37
O-acetyl-L-serine	16.14	4.47	0.67	15.19	5.15	0.66	11.88	2.91	1.01
Phenylacetate	0.08	0.07	0.04	-	-	0.00	0.02	-	0.01
2-aminobutyrate	0.16	0.06	0.03	0.12	0.05	0.01	0.13	0.04	0.01
m-Toluate	0.18	0.16	0.12	0.10	0.11	0.09	0.08	0.09	0.09
Beta-alanine	3.61	0.94	0.25	3.00	0.85	0.22	3.25	0.55	0.16
Adipate	0.21	0.23	0.21	0.32	0.31	0.19	0.14	0.22	0.28
Valine	4.31	1.11	0.33	4.77	1.30	0.35	4.27	0.72	0.24
2-isopropylmalate	0.11	0.11	0.08	0.07	0.07	0.07	0.06	0.06	0.06
Beta-hydroxypyruvate	0.74	0.31	0.14	0.67	0.28	0.11	0.89	0.18	0.14
Alpha-ketoadipate	0.79	0.68	0.71	9.56	3.16	0.95	0.38	0.50	0.39
Leucine	3.71	0.92	0.25	4.93	1.23	0.29	3.49	0.59	0.16
Isoleucine	2.05	0.55	0.24	2.33	0.69	0.27	2.04	0.38	0.19
Malate	3.37	0.52	0.07	1.83	0.37	0.05	1.71	0.16	0.03
Oxaloacetate	2.05	0.31	0.48	1.44	0.45	0.26	2.33	0.43	-
Threonine	7.57	1.61	0.44	5.44	1.98	0.42	7.44	1.08	0.31
Proline	10.96	2.29	0.48	11.15	2.69	0.50	11.63	1.48	0.31
L-homoserine	0.29	0.16	0.15	0.25	0.18	0.14	0.19	0.15	0.15
Aspartate	0.65	0.18	0.12	0.77	0.26	0.09	0.48	0.09	0.07
Citrate	5.95	1.05	0.25	3.40	1.04	0.19	3.64	0.47	0.10
5-aminovalerate	0.20	0.10	0.13	0.17	0.15	0.14	0.15	0.11	0.12
Anthralinate	0.09	0.10	0.13	0.06	0.09	0.11	0.06	0.06	0.10
Serine	3.76	1.13	0.43	4.46	1.64	0.51	3.64	0.78	0.29
Allantoin	17.00	7.07	4.81	35.29	8.05	5.46	33.21	5.90	11.30
Glutamate	51.47	7.55	1.78	45.63	11.42	1.97	41.00	4.51	1.07
N-Acetyl-L-glutamate	1.47	0.55	0.38	1.19	0.70	0.38	0.98	0.44	0.37
Methionine	0.93	0.24	0.08	1.04	0.31	0.09	1.00	0.18	0.06
Hydroxyproline	157.06	61.90	32.33	114.95	31.51	31.81	180.61	67.39	40.78
Cysteine	26.27	5.30	1.17	29.40	7.75	1.74	25.34	3.47	0.89
Phenylalanine	2.10	0.60	0.20	2.43	0.74	0.21	2.17	0.43	0.15
Hippurate	1.90	1.85	1.99	1.52	1.90	1.76	1.70	1.44	1.55
Putrescine	0.70	0.37	0.40	0.71	0.53	0.49	0.52	0.38	0.39
4-imidazoleacrylate	0.09	0.06	0.06	0.06	0.05	0.03	0.04	0.03	0.03
Histamine	1.78	1.08	2.44	1.05	1.27	-	1.21	0.26	0.37
Ornithine	0.52	0.21	0.14	0.74	0.31	0.15	0.56	0.19	0.12
Lysine	8.84	2.67	0.93	9.77	3.10	0.90	8.82	2.02	0.87
Histidine	0.42	0.13	-	0.46	0.16	0.00	0.46	0.08	-
Tyrosine	1.50	0.39	0.11	1.78	0.51	0.11	1.55	0.27	0.07
Thryptophane	0.65	0.26	0.13	0.73	0.23	-	0.63	0.17	0.03

Table C.3: *Metabolite response per injected sample [peak area/µl] of polar extracts obtained with three extraction rounds and obtained in a check round subsequent to extraction of non-polar metabolites. Response is an average of three technical replicates.*

Sample name	130212 1	130212 2	130212 3	130212 1 check	130212 2 check	130212 3 check
4-methylvalerate	1,012	331	154	-	-	-
Pyruvate	11,085	12,519	19,980	434	405	451
3-methyl-oxovalerate	4,030	2,085	3,014	-	-	-
Fumarate	66,851	36,098	53,400	-	-	-
Lactate/Methylglyoxal	402,256	228,074	321,250	10,028	8,257	8,709
succinate	69,967	44,663	52,422	14,323	16,895	-
Benzoate	19,762	7,606	5,928	-	-	-
alanine	157,235	94,896	100,027	-	-	-
Glyoxylate	5,315	2,777	2,832	-	-	-
Glycine	751,027	535,385	650,940	38,314	35,489	33,689
O-acetyl-L-serine	22,919	17,783	19,683	2,552	2,635	2,505
2-aminobutyrate	5,498	3,333	2,976	-	-	-
Salicylate	11,983	4,660	3,202	-	-	-
beta-alanine	29,938	20,099	25,758	-	-	-
adipate	22,538	20,152	19,231	-	-	-
valine	55,970	39,303	47,634	2,311	2,181	2,125
beta-hydroxypyruvate	3,731	4,164	5,596	403	344	369
Leucine	15,340	11,605	14,839	-	-	-
Isoleucine	2,684	1,616	2,075	-	-	-
Malate	46,315	41,844	32,509	1,446	1,345	1,002
OAA	547	564	568	-	-	-
Threonine	5,486	2,836	3,437	-	-	-
Proline	44,501	23,723	33,888	-	-	-
Aspartate	474	363	405	-	-	413
Citrate	15,450	9,776	11,077	-	-	-
Serine	8,837	8,139	7,989	310	360	327
Glutamate	288,565	220,215	209,678	3,229	2,133	2,642
N-Acetyl-L-glutamate	4,537	4,257	3,405	4,745	4,130	3,987
Methionine	48,338	35,489	44,299	491	312	437
Cysteine	29,663	23,289	41,808	-	-	-
Phenylalanine	29,973	21,907	27,619	475	246	468
Histamine	315	165	200	-	-	-
p-coumarate	1,616	692	527	-	-	-
Lysine	13,207	9,457	10,084	-	-	-
Citraconate/itaconate	5,869	4,386	4,007	-	-	-
Allantoin	30,645	27,532	23,028	28,585	24,269	23,238
d5-Glutamate	3,170,025	2,822,124	2,228,402	3,305,454	2,829,390	2,748,690
d4-Succinate	71,677	61,437	51,726	121,913	130,845	115,478

Table C.4: *Metabolite concentration of injected sample [pmol/µl] of polar extracts obtained by performing optimised extraction method on tissue of basal-like-subtype.*

Sample name 140312	bas 1 1	bas 1 2	bas 1 3	bas 2 1	bas 2 2	bas 2 3	bas 3 1	bas 3 2	bas 3 3
4-methylvalerate	0.14	0.12	0.13	0.13	0.12	0.12	0.13	0.13	0.13
Pyruvate	1.03	1.36	3.44	0.70	1.23	1.04	1.77	1.96	1.65
3-methyl-oxovalerate	0.28	0.22	0.32	0.18	0.21	0.20	0.22	0.24	0.21
Fumarate	1.35	1.20	1.70	0.62	0.91	0.79	1.40	1.59	1.37
Lactate/Methylglyoxal	74.14	54.43	98.56	42.36	52.76	46.74	70.39	73.85	54.24
Succinate	1.46	1.31	1.52	0.67	1.24	1.12	0.98	1.08	0.92
Citraconate/Itaconate	0.47	0.32	0.48	0.27	0.26	0.24	0.29	0.28	0.23
Benzoate	0.86	0.73	0.64	0.54	0.69	0.64	0.56	0.65	0.54
Citramalate	0.19	0.18	0.15	0.12	0.14	0.14	0.14	0.14	0.12
Glyoxylate	1.94	1.76	1.49	2.19	1.80	1.65	1.47	1.21	0.83
Beta-3-hydroxybutyrate	0.42	0.44	0.59	0.40	0.48	0.41	0.53	0.53	0.54
Glycine	61.93	55.53	95.73	30.58	37.55	30.04	46.69	49.74	33.30
Alanine	13.86	12.50	20.25	6.91	9.03	7.73	10.81	11.64	9.71
O-acetyl-L-serine	6.45	5.86	11.54	2.21	4.07	3.06	5.41	6.21	5.33
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
Salicylate	2.29	1.02	1.92	0.85	2.56	2.05	2.69	1.99	2.07
2-aminobutyrate	0.27	0.25	0.30	0.18	0.20	0.20	0.22	0.24	0.21
m-Toluate	0.19	0.17	0.15	0.15	0.18	0.16	0.17	0.17	0.15
Beta-alanine	3.68	3.05	4.59	2.24	2.56	2.25	3.00	3.46	2.96
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
Valine	4.58	2.09	3.09	1.24	1.61	1.41	1.90	2.07	1.58
aKG	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
Alpha-ketoadipate	0.40	0.17	0.17	0.18	0.21	0.20	0.20	0.16	0.15
Leucine	0.75	0.63	0.97	0.32	0.49	0.39	0.60	0.70	0.51
Gamma-aminobutyrate	0.99	0.98	0.90	0.93	0.90	0.87	1.02	1.03	1.04
Threonine	3.30	3.13	4.69	1.59	2.11	1.99	2.81	3.03	2.56
Malate	4.00	3.24	5.98	1.42	1.76	1.48	2.76	2.91	2.17
Proline	12.50	11.00	17.36	6.54	8.72	7.51	10.41	11.53	9.22
Aspartate	0.72	0.55	0.65	0.24	0.34	0.36	0.44	0.40	0.33
Citrate	4.00	4.43	5.13	1.80	2.84	2.94	3.29	3.89	3.33
Serine	1.87	1.74	2.89	0.80	1.00	1.01	1.69	2.01	1.51
Anthralinate	0.15	0.10	0.10	0.07	0.10	0.09	0.09	0.08	0.09
Allantoin	68.33	64.91	43.38	37.10	43.65	48.54	32.67	41.03	29.22
N-Acetyl-L-glutamate	5.49	4.14	3.57	3.06	4.69	4.54	3.25	3.77	2.97
Glutamate	69.47	63.97	86.69	26.97	40.98	39.86	52.37	55.07	45.00
Methionine	0.64	0.54	0.77	0.33	0.45	0.39	0.52	0.59	0.48
Hippurate	0.15	0.15	0.14	0.10	0.16	0.17	0.23	0.25	0.30
Phenylalanine	1.05	0.95	1.37	0.56	0.78	0.66	0.88	1.02	0.82
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
Lysine	6.13	5.59	7.53	3.53	3.93	3.69	5.03	5.52	4.51
Tyrosine	1.78	1.14	1.81	0.87	1.06	1.12	1.31	1.26	1.12
d3-alanine	19.11	16.77	27.14	14.09	18.22	15.97	14.00	14.98	12.17
d4-succinate	32.24	24.21	16.38	15.75	28.71	25.93	18.69	23.12	18.32
d8-valine	28.52	19.13	11.46	19.52	25.20	21.65	17.60	19.47	15.82
d5-glutamate	576.04	388.86	326.13	297.77	362.54	397.35	266.43	298.07	225.82

Table C.5: *Metabolite concentration of injected sample [pmol/µl] of polar extracts obtained by performing optimised extraction method on tissue of luminal-like subtype.*

Sample name 140312	lum 1 1	lum 1 2	lum 1 3	lum 2 1	lum 2 2	lum 2 3	lum 3 1	lum 3 2	lum 3 3
4-methylvalerate	0.12	0.13	0.14	0.14	0.13	0.14	0.13	0.14	0.15
Pyruvate	0.08	1.03	1.80	1.30	1.54	1.40	2.07	1.74	3.30
3-methyl-oxovalerate	0.15	0.23	0.23	0.40	0.24	0.31	0.26	0.29	0.47
Fumarate	1.20	0.59	0.77	0.84	0.76	0.72	0.75	0.80	0.97
Lactate/Methylglyoxal	44.24	39.62	52.14	53.83	43.77	57.10	52.70	55.23	65.43
Succinate	0.73	1.20	1.86	1.81	1.38	1.03	1.47	1.49	1.91
Citraconate/Itaconate	0.17	0.23	0.26	0.27	0.21	0.25	0.24	0.24	0.25
Benzoate	0.64	0.57	0.61	0.63	0.55	0.52	0.55	0.56	0.74
Citramalate	0.13	0.13	0.14	0.13	0.12	0.11	0.12	0.11	0.12
Glyoxylate	0.00	0.61	1.48	1.08	1.46	1.49	1.25	1.08	1.98
Beta-3-hydroxybutyrate	0.86	0.80	0.92	1.06	1.01	1.02	1.30	1.18	1.32
Glycine	11.46	10.28	18.03	13.74	12.65	10.97	13.21	10.21	14.40
Alanine	19.47	19.23	28.20	23.74	22.32	22.94	23.90	23.36	22.27
O-acetyl-L-serine	11.52	10.04	18.73	11.24	13.83	14.04	13.52	13.37	21.20
Nicotinate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phenylacetate	0.17	0.17	0.18	0.26	0.20	0.19	0.20	0.21	0.24
Salicylate	2.03	2.48	1.97	3.84	1.38	2.88	3.23	2.79	4.97
2-aminobutyrate	0.32	0.30	0.35	0.35	0.33	0.37	0.33	0.32	0.39
m-Toluate	0.16	0.17	0.17	0.21	0.20	0.21	0.24	0.21	0.23
Beta-alanine	0.48	0.45	0.50	0.53	0.51	0.54	0.44	0.48	0.37
OH-Glutarate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Adipate	0.20	0.19	0.22	0.21	0.22	0.25	0.18	0.20	0.21
Valine	3.01	2.72	3.98	3.48	3.47	3.79	3.30	3.26	4.24
aKG	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Beta-hydroxypyruvate	0.09	0.46	0.59	0.55	0.44	0.65	0.48	0.64	0.52
Alpha-ketoadipate	0.19	0.15	0.16	0.15	0.12	0.16	0.12	0.00	0.20
Leucine	0.70	0.66	1.12	1.02	0.96	1.05	0.96	0.96	1.29
Gamma-aminobutyrate	1.16	0.97	1.04	1.01	1.27	1.18	1.21	1.36	1.18
Threonine	2.77	2.90	4.74	4.28	4.04	4.25	3.96	4.21	5.23
Malate	2.29	1.82	2.64	2.17	1.80	2.33	1.90	1.81	2.47
Proline	9.98	10.45	15.76	12.20	11.49	12.04	13.28	13.01	17.25
Aspartate	2.96	2.23	3.82	3.79	2.96	2.30	2.53	2.60	3.32
Citrate	0.79	1.42	3.14	2.68	1.88	1.55	2.04	2.08	2.52
Serine	10.30	2.12	2.79	3.04	2.23	2.46	2.35	2.51	2.77
Anthralinate	0.09	0.11	0.09	0.09	0.05	0.07	0.07	0.06	0.09
Allantoin	73.12	35.77	45.70	40.03	58.50	25.21	28.39	32.43	37.70
N-Acetyl-L-glutamate	7.85	3.97	4.74	5.03	2.80	2.46	3.85	2.91	4.78
Glutamate	25.39	19.10	29.45	28.94	22.18	17.96	21.46	24.37	24.56
Methionine	0.58	0.66	0.88	0.80	0.77	0.81	0.76	0.79	0.99
Hippurate	0.23	0.20	0.22	0.23	0.28	0.25	0.34	0.31	0.43
Phenylalanine	1.06	0.99	1.42	1.27	1.22	1.29	1.14	1.11	1.52
Ornithine	1.88	2.24	1.88	1.99	1.85	2.16	2.00	2.17	2.43
Lysine	3.94	4.20	4.45	4.55	4.12	4.68	4.50	4.53	5.77
Tyrosine	1.23	1.48	1.53	1.47	1.43	1.44	1.36	1.04	1.52
d3-alanine	13.80	12.73	19.51	15.63	14.71	15.83	15.27	15.28	20.12
d4-succinate	14.51	20.73	21.88	19.46	15.77	12.04	20.42	20.77	23.42
d8-valine	26.95	20.87	14.99	17.87	16.87	18.45	18.62	19.13	25.63
d5-glutamate	470.27	289.70	343.42	338.42	217.05	172.84	235.38	240.40	285.29

Table C.6: *Metabolite concentration of injected sample [pmol/µl] of polar extracts obtained by performing optimised extraction method on tissue of basal-like and luminal-like subtypes.*

Sample name 270312	bas 1	bas 2	bas 3	bas 4	bas 5	bas 6	lum 1	lum 2	lum 3	lum 4	lum 5	lum 6
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	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	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	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
Allantonin	32.66	44.71	38.08	30.54	61.14	24.77	23.29	16.21	19.02	20.12	57.39	33.55
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
d4-succinate	1.07	1.05	0.91	1.97	1.26	0.91	0.90	0.91	1.18	0.89	0.95	0.78
d3-alanine	6.78	4.54	3.35	3.01	4.56	2.32	2.73	1.90	2.54	3.27	2.45	2.86
d8-valine	0.19	0.38	0.31	0.31	0.26	0.39	0.44	0.44	0.43	0.50	0.31	0.55
d5-glutamate	305.42	332.34	320.99	263.46	319.30	257.89	269.58	251.42	244.26	275.70	205.34	211.98

C.2 Normalised data

Normalisation against internal standards, dilutions, volume analysed and tissue weight was performed on raw data. As a result, metabolite concentrations are given in picomole per mg (pM/mg) tissue.

Table C.7: *Metabolite concentration per tissue mass [pM/mg] of methanol/water phase in the biphasic system obtained subsequent to three extraction rounds in 60% methanol. Each of the three technical replicates are shown.*

Sanple name 280212	1 1	1 2	1 3	2 1	2 2	2 3	Std dev 1	Std dev 2
Tissue weight [mg]		28.05			39.82			
Malonate	8,204	4,341	5,851	3,463	2,230	3,086	26	18
Pyruvate	24,430	15,860	18,050	17,327	10,707	12,020	19	21
3-methyl-oxovalerate	17,767	12,029	12,904	9,278	6,573	7,661	18	14
Fumarate	29,328	20,892	21,816	16,094	11,196	14,075	16	15
Lactate/Methylglyoxal	270,907	202,620	220,063	198,266	133,034	165,022	13	16
Succinate	19,616	14,096	15,221	11,752	8,904	10,884	15	11
Citraconate/Itaconate	15,293	11,107	11,444	8,955	5,773	8,547	15	18
Benzoate	8,355	5,573	5,679	3,560	2,472	3,072	20	15
Glyoxylate	69,517	51,505	52,408	34,958	24,791	32,947	14	14
Beta-3-hydroxybutyrate	-	7,174	5,599	-	-	-	12	-
Glycine	205,628	162,464	170,871	130,264	93,044	120,561	10	14
O-acetyl-L-serine	128,402	100,050	104,043	82,084	59,545	74,391	11	13
Alanine	12,239	10,627	10,697	10,362	7,950	9,429	7	11
Phenylacetate	1,310	739	627	393	226	286	34	23
Salicylate	62,803	33,442	38,127	24,387	13,384	18,498	29	24
2-aminobutyrate	6,935	4,726	5,139	3,809	2,634	3,341	17	15
m-Toluate	348	176	129	69	46	48	43	19
Beta-alanine	55,715	42,999	45,316	37,935	26,535	34,345	12	14
Adipate	15,232	12,545	15,060	8,949	6,474	8,086	9	13
Valine	13,661	11,046	12,043	10,524	7,532	9,288	9	13
Alpha-ketoglutarate	91,111	66,506	69,758	69,867	44,373	53,548	14	19
Beta-hydroxypyruvate	52,557	38,374	39,520	27,477	18,988	23,272	15	15
Leucine	37,743	27,839	30,736	23,974	16,888	21,952	13	14
Isoleucine	40,189	30,276	33,041	26,265	17,992	23,557	12	15
Threonine	106,623	85,165	85,162	67,861	47,849	63,369	11	14
Malate	106,055	92,127	93,344	72,429	49,459	59,695	6	16
Proline	129,706	97,244	106,675	82,401	57,028	73,350	12	15
Citrate	69,995	52,294	59,297	44,495	32,306	42,147	12	13
Serine	78,415	57,839	62,894	44,231	30,121	38,187	13	15
Allantoin	158,537	118,049	127,527	86,578	68,533	87,252	13	11
Glutamate	207,588	156,858	172,426	128,664	100,310	127,019	12	11
Methionine	24,609	29,442	22,157	16,404	18,177	16,901	12	4
Phenylalanine	54,932	39,244	43,765	32,095	22,569	29,366	14	14
Cysteine	-	141,927	-	101,424	68,965	-	-	19

Table C.8: *Metabolite concentration per tissue mass [pM/mg] of polar extracts obtained in each of the three extraction rounds in 60% methanol.*

Sample name 180112	1 r1	1 r2	1 r3	2 r1	2 r2	2 r3	3 r1	3 r2	3 r3
Tissue weight [mg]	38.9			28.7			32.8		
4-methylvalerate	112	98	83	91	91	82	76	75	71
Malonate	167	181	200	358	357	258	234	221	219
Pyruvate	2,055	1,041	582	2,679	1,022	587	1,424	643	406
3-methyl-oxovalerate	31	9	9	22	12	8	36	5	5
Fumarate	1,950	532	150	1,869	683	172	1,600	311	96
Lactate/Methylglyoxal	430,795	99,480	19,791	996,586	263,163	46,407	547,093	66,431	14,715
Succinate	3,943	974	512	8,982	2,986	981	4,506	835	484
Benzoate	223	202	166	247	271	205	155	164	160
Beta-3-hydroxybutyrate	6,076	1,587	564	7,025	2,358	685	5,831	5,695	656
Alanine	48,236	9,479	1,826	72,053	16,023	2,856	52,057	6,210	1,263
Glycine	59,023	11,641	2,466	67,372	16,404	3,256	63,964	8,579	1,971
O-acetyl-L-serine	19,521	5,408	805	24,906	8,445	1,077	17,046	4,178	1,455
Phenylacetate	96	86	49	0	-	-	23	-	20
2-aminobutyrate	190	76	41	197	82	15	182	51	11
m-Toluate	223	191	143	159	175	153	121	126	126
Beta-alanine	4,363	1,134	299	4,918	1,388	357	4,666	783	229
Adipate	260	282	259	523	513	319	198	316	396
Valine	5,211	1,341	403	7,815	2,124	577	6,119	1,032	343
2-isopropylmalate	139	136	98	110	116	108	93	87	88
Beta-hydroxypyruvate	897	376	164	1,100	452	182	1,276	252	205
Alpha-ketoadipate	951	821	856	15,675	5,180	1,562	541	713	559
Leucine	4,487	1,113	299	8,089	2,009	475	5,002	840	231
Isoleucine	2,481	665	288	3,819	1,136	440	2,932	544	272
Malate	4,076	635	88	3,000	600	87	2,447	231	41
Oxaloacetate	2,481	370	581	2,358	740	427	3,346	610	-
Threonine	9,160	1,946	534	8,919	3,247	681	10,679	1,550	450
Proline	13,255	2,766	575	18,288	4,417	828	16,683	2,118	444
L-homoserine	352	193	177	415	301	223	274	219	213
Aspartate	791	215	142	1,270	434	140	687	134	99
Citrate	7,198	1,273	301	5,568	1,709	305	5,218	677	150
5-aminovalerate	239	116	159	277	243	224	214	163	171
Anthralinate	106	122	159	103	148	175	79	89	139
Serine	4,555	1,372	520	7,320	2,690	834	5,218	1,119	422
Allantoin	20,569	8,551	5,817	57,871	13,193	8,954	47,652	8,468	16,208
Glutamate	62,266	9,138	2,157	74,816	18,724	3,237	58,825	6,469	1,530
N-Acetyl-L-glutamate	1,783	671	461	1,949	1,155	628	1,402	638	537
Methionine	1,119	295	102	1,713	515	143	1,429	260	93
Hydroxyproline	189,998	74,878	39,117	188,479	51,663	52,150	259,130	96,682	58,506
Cysteine	31,785	6,417	1,411	48,212	12,709	2,851	36,349	4,976	1,272
Phenylalanine	2,545	728	237	3,985	1,207	341	3,108	613	209
Hippurate	2,304	2,234	2,404	2,492	3,116	2,893	2,441	2,064	2,226
Putrescine	845	444	489	1,166	863	800	748	549	554
4-imidazoleacrylate	112	74	73	94	88	47	56	38	37
Histamine	2,154	1,309	2,953	1,728	2,088	-	1,741	371	538
Ornithine	629	259	172	1,217	516	239	798	271	171
Lysine	10,690	3,225	1,121	16,014	5,080	1,474	12,653	2,901	1,253
Histidine	503	153	-	759	255	-	653	116	-
Tyrosine	1,814	473	129	2,925	830	172	2,223	380	107
Thryptophane	791	316	154	1,192	378	-	904	240	44

Table C.9: *Metabolite response per tissue mass [peak area/mg] of polar extracts obtained with three extraction rounds and obtained in a check round subsequent to non-polar metabolite extraction.*

Sample name	130212 1	130212 2	130212 3	130212 1 check	130212 2 check	130212 3 check	% in check
Tissue weight [mg]	28.67	26.79	33.93	28.67	26.79	33.93	
4-methylvalerate	1,259	577	319	-	-	-	-
Pyruvate	13,790	21,842	41,400	219	238	243	0.90
3-methyl-oxovalerate	5,013	3,638	6,245	-	-	-	-
Fumarate	83,164	62,980	110,651	-	-	-	-
Lactate/Methylglyoxal	500,413	397,915	665,661	5,048	4,841	4,703	0.92
Succinate	87,041	77,922	108,624	7,210	9,906	-	-
Citraconate/itaconate	7,302	7,652	8,303	-	-	-	-
Benzoate	24,584	13,270	12,283	-	-	-	-
Alanine	195,603	165,563	207,265	-	-	-	-
Glyoxylate	6,611	4,846	5,868	-	-	-	-
Glycine	934,291	934,073	1,348,812	19,286	20,809	18,192	1.78
O-acetyl-L-serine	28,512	31,026	40,784	1,285	1,545	1,353	4.00
2-aminobutyrate	6,839	5,815	6,167	-	-	-	-
Salicylate	14,907	8,131	6,635	-	-	-	-
beta-alanine	37,243	35,065	53,374	-	-	-	-
Adipate	28,037	35,158	39,849	-	-	-	-
Valine	69,628	68,571	98,703	1,163	1,279	1,148	1.49
beta-hydroxypyruvate	4,642	7,265	11,595	203	201	199	2.50
Leucine	19,083	20,247	30,748	-	-	-	-
Isoleucine	3,339	2,820	4,300	-	-	-	-
Malate	57,617	73,003	67,361	728	789	541	1.03
OAA	681	984	1,178	-	-	-	-
Threonine	6,825	4,948	7,122	-	-	-	-
Proline	55,360	41,388	70,220	-	-	-	-
Aspartate	590	633	840	-	-	223	-
Citrate	19,220	17,055	22,952	-	-	-	-
Allantoin	38,123	48,035	47,717	14,389	14,231	12,548	23.52
Serine	10,993	14,201	16,555	156	211	176	1.28
Glutamate	358,980	384,203	434,474	1,625	1,251	1,427	-
N-Acetyl-L-glutamate	5,644	7,427	7,055	2,388	2,422	2,153	25.70
Methionine	60,133	61,918	91,791	247	183	236	-
Cysteine	36,901	40,632	86,629	-	-	-	-
Phenylalanine	37,287	38,221	57,229	239	144	253	-
Histamine	392	287	415	-	-	-	-
P-coumarate	2,011	1,207	1,092	-	-	-	-
Lysine	16,429	16,500	20,894	-	-	-	-

Table C.10: *Metabolite concentration per tissue mass [pM/mg] of polar extracts obtained by performing optimised extraction method on tissue of basal-like subtype.*

Sample name 140312	bas 1 1	bas 1 2	bas 1 3	bas 2 1	bas 2 2	bas 2 3	bas 3 1	bas 3 2	bas 3 3	Std dev [%]	Std dev [%]	Std dev [%]
Tissue weight [mg]		22.8			28.13			25.7		bas 1	bas 2	bas 3
4-methylvalerate	409	369	447	347	154	274	289	243	275	8	31	7
Pyruvate	3,129	4,245	11,502	1,858	1,590	2,378	3,874	3,673	3,393	59	17	5
3-methyl-oxovalerate	837	672	1,068	472	276	451	482	442	429	19	22	5
Fumarate	4,084	3,757	5,686	1,640	1,182	1,817	3,072	2,974	2,816	19	17	4
Lactate/Methylglyoxal	224,529	169,832	329,893	112,703	68,354	106,929	154,345	138,446	111,517	28	21	13
Succinate	4,411	4,075	5,093	1,789	1,613	2,567	2,139	2,017	1,894	9	21	5
Citraconate/Itaconate	1,409	988	1,603	711	339	560	644	521	471	19	29	13
Benzoate	2,610	2,281	2,150	1,440	896	1,464	1,238	1,220	1,121	8	21	4
Citramalate	570	559	492	321	187	322	300	270	238	6	23	9
Glyoxylate	5,871	5,484	4,975	5,817	2,332	3,778	3,222	2,261	1,713	7	36	26
Beta-3-hydroxybutyrate	1,267	1,364	1,959	1,064	620	938	1,165	990	1,102	20	21	7
Glycine	187,563	173,249	320,437	81,366	48,654	68,713	102,382	93,253	68,477	29	20	16
Alanine	41,966	38,991	67,768	18,392	11,699	17,674	23,697	21,814	19,968	26	19	7
O-acetyl-L-serine	19,527	18,280	38,631	5,887	5,275	7,010	11,860	11,643	10,966	37	12	3
Nicotinate	1,676	1,605	2,101	1,206	581	1,090	1,296	1,408	1,794	12	28	14
Phenylacetate	477	766	439	449	198	313	319	348	304	26	32	6
Salicylate	6,943	3,176	6,438	2,268	3,313	4,700	5,909	3,722	4,262	30	29	20
2-aminobutyrate	819	795	991	472	257	461	477	456	438	10	25	3
m-Toluate	580	530	510	389	228	361	367	321	302	5	22	8
Beta-alanine	11,152	9,518	15,362	5,969	3,321	5,148	6,570	6,491	6,094	21	23	3
OH-Glutarate	1,264	1,205	1,406	639	324	713	677	687	379	7	30	25
Adipate	791	734	692	492	303	467	496	399	412	6	20	10
Valine	13,870	6,514	10,357	3,301	2,089	3,225	4,164	3,887	3,255	29	19	10
Alpha-ketoglutarate	11,940	12,234	23,060	7,236	3,576	6,149	6,560	5,410	4,742	33	27	13
Beta-hydroxypyruvate	2,981	2,625	6,694	1,045	601	1,001	1,448	1,279	1,048	45	23	13
Alpha-ketoadipate	1,218	528	570	486	269	459	444	303	309	41	24	19
Leucine	2,259	1,962	3,260	852	630	903	1,324	1,315	1,044	22	15	11
Gamma-aminobutyrate	3,002	3,071	3,012	2,465	1,161	1,992	2,226	1,938	2,148	1	29	6
Threonine	9,991	9,754	15,706	4,236	2,734	4,549	6,161	5,683	5,265	23	21	6
Malate	12,119	10,101	20,013	3,774	2,283	3,379	6,042	5,464	4,453	30	20	12
Proline	37,866	34,315	58,104	17,409	11,298	17,184	22,818	21,615	18,967	24	18	8
Aspartate	2,187	1,712	2,172	637	446	814	968	742	683	11	24	15
Citrate	12,128	13,811	17,172	4,788	3,675	6,718	7,207	7,284	6,851	15	25	3
Serine	5,671	5,422	9,684	2,128	1,294	2,302	3,704	3,776	3,106	28	23	9
Anthralinate	465	301	326	182	128	196	203	142	185	20	18	15
Allantoin	206,958	202,512	145,182	98,706	56,550	111,034	71,640	76,926	60,075	15	26	10
N-Acetyl-L-glutamate	16,633	12,908	11,952	8,146	6,072	10,392	7,131	7,072	6,109	15	22	7
Glutamate	210,411	199,599	290,175	71,749	53,090	91,190	114,826	103,243	92,525	17	22	9
Methionine	1,923	1,677	2,578	877	584	894	1,146	1,102	988	18	18	6
Hippurate	451	453	469	262	206	396	513	463	610	2	28	12
Phenylalanine	3,192	2,954	4,592	1,489	1,007	1,508	1,930	1,919	1,689	20	17	6
P-coumarate	219	202	126	64	37	46	42	35	32	22	22	12
Ornithine	5,182	4,322	5,978	2,817	1,427	2,541	3,037	2,872	2,423	13	27	9
Lysine	18,575	17,434	25,191	9,402	5,098	8,452	11,034	10,353	9,274	17	24	7
Tyrosine	5,383	3,546	6,058	2,320	1,368	2,570	2,877	2,353	2,297	21	25	10

Table C.11: *Metabolite concentration per tissue mass [pM/mg] of polar extracts obtained by performing optimised extraction method on tissue of luminal-like subtype.*

Sample name 140312	lum 1 1	lum 1 2	lum 1 3	lum 2 1	lum 2 2	lum 2 3	lum 3 1	lum 3 2	lum 3 3	Std dev [%]	Std dev [%]	Std dev [%]
Tissue weight [mg]	28.09			24.76			33.44			lum 1	lum 2	lum 3
4-methylvalerate	623	267	330	440	238	174	131	141	109	38	40	11
Pyruvate	408	2,039	4,310	4,054	2,733	1,751	2,164	1,810	2,427	71	33	12
3-methyl-oxovalerate	808	464	557	1,239	419	387	272	303	349	24	58	10
Fumarate	6,418	1,161	1,855	2,627	1,352	898	782	831	714	74	45	6
Lactate/Methylglyoxal	235,997	78,577	124,981	167,454	77,802	71,592	54,974	57,486	48,190	45	41	7
Succinate	3,881	2,386	4,454	5,630	2,455	1,286	1,530	1,549	1,404	24	59	4
Citraconate/Itaconate	931	454	630	854	372	317	255	252	187	29	47	14
Benzoate	3,396	1,135	1,468	1,947	975	648	573	581	548	50	46	3
Citramalate	695	267	335	399	217	140	120	120	90	43	43	13
Glyoxylate	-	1,205	3,549	3,352	2,597	1,863	1,301	1,127	1,455	93	23	10
Beta-3-hydroxybutyrate	4,583	1,591	2,213	3,312	1,799	1,275	1,354	1,231	970	46	41	14
Glycine	61,151	20,383	43,210	42,758	22,479	13,755	13,783	10,625	10,602	40	46	13
Alanine	103,839	38,129	67,595	73,848	39,667	28,767	24,933	24,313	16,405	38	40	18
O-acetyl-L-serine	61,471	19,905	44,892	34,954	24,584	17,600	14,107	13,913	15,616	41	28	5
Phenylacetate	885	334	435	796	351	242	209	213	177	43	52	8
Salicylate	10,826	4,922	4,711	11,939	2,448	3,609	3,367	2,904	3,664	42	70	9
2-aminobutyrate	1,686	587	832	1,083	580	461	345	332	287	46	38	8
m-Toluate	874	332	401	655	357	262	245	220	170	45	40	15
Beta-alanine	2,562	884	1,204	1,634	902	681	460	495	276	47	38	23
Adipate	1,067	370	531	655	389	314	192	205	152	45	32	12
Valine	16,068	5,391	9,534	10,834	6,165	4,747	3,447	3,397	3,119	43	36	4
Beta-hydroxypyruvate	484	921	1,403	1,718	785	815	496	663	385	40	39	22
Alpha-ketoadipate	1,018	307	394	460	207	206	130	-	144	55	41	71
Leucine	3,749	1,312	2,685	3,168	1,714	1,311	1,005	997	953	39	39	2
Gamma-aminobutyrate	6,172	1,927	2,492	3,157	2,251	1,484	1,262	1,414	868	53	30	19
Threonine	14,779	5,761	11,366	13,330	7,180	5,324	4,134	4,384	3,851	35	40	5
Malate	12,210	3,616	6,322	6,762	3,198	2,923	1,984	1,888	1,818	49	41	4
Proline	53,239	20,724	37,773	37,949	20,432	15,103	13,848	13,538	12,703	36	40	4
Aspartate	15,795	4,419	9,158	11,799	5,256	2,881	2,641	2,703	2,442	48	57	4
Citrate	4,203	2,815	7,526	8,325	3,337	1,939	2,125	2,164	1,854	41	60	7
Serine	54,916	4,199	6,683	9,465	3,960	3,090	2,447	2,616	2,039	106	51	10
Anthralinate	460	217	204	274	95	82	77	63	66	40	58	9
Allantoin	390,010	70,939	109,555	124,528	103,994	31,612	29,610	33,757	27,769	75	46	8
N-Acetyl-L-glutamate	41,875	7,875	11,351	15,649	4,981	3,085	4,016	3,030	3,519	75	70	11
Glutamate	135,447	37,869	70,602	90,033	39,432	22,517	22,383	25,364	18,086	50	57	14
Methionine	3,071	1,312	2,112	2,486	1,375	1,012	788	821	726	33	39	5
Hippurate	1,209	403	525	711	506	318	351	325	315	50	31	5
Phenylalanine	5,663	1,960	3,406	3,952	2,165	1,621	1,188	1,152	1,119	41	39	2
Ornithine	10,035	4,442	4,499	6,199	3,290	2,712	2,088	2,255	1,788	41	38	9
Lysine	20,991	8,329	10,668	14,148	7,321	5,864	4,692	4,711	4,248	41	40	5
Tyrosine	6,568	2,932	3,670	4,576	2,547	1,801	1,415	1,087	1,121	36	39	12

Table C.12: *Metabolite concentration per tissue mass [pM/mg] of polar extracts obtained by performing optimised extraction method on tissue of basal-like and luminal-like subtypes.*

Sample name 270312	bas 1	bas 2	bas 3	bas 4	bas 5	bas 6	lum 1	lum 2	lum 3	lum 4	lum 5	lum 6	% std dev	% std dev
Tissue weight [mg]	23.12	35.25	32.73	38.38	33.77	28.12	32.15	32.41	32.35	24.19	30.52	28.95	bas	lum
4-methylvalerate	391	258	246	182	276	295	245	189	197	214	111	120	25	30
Pyruvate	921	1,596	2,526	3,124	2,879	3,972	2,287	1,852	2,368	1,278	643	316	44	59
3-methyl-oxovalerate	688	643	761	419	734	611	272	284	277	412	166	152	19	36
Fumarate	2,787	1,706	1,475	2,654	2,604	1,697	1,379	1,120	1,613	1,594	842	633	27	34
Lactate/Methylglyoxal	73,673	138,459	137,952	83,452	83,388	92,013	55,158	65,877	99,076	64,528	45,372	29,304	29	39
Succinate	3,784	2,687	2,891	2,674	3,420	2,551	3,967	3,027	3,881	3,358	2,216	2,218	16	25
Citraconate/Itaconate	651	581	426	323	757	503	-	-	-	-	-	-	29	-
Benzoate	1,071	729	630	536	832	719	616	457	526	658	338	310	25	29
Citramalate	2,147	1,249	1,236	1,043	1,514	1,495	943	702	1,188	1,239	606	691	27	30
Glyoxylate	17,599	7,354	5,430	4,056	9,431	4,318	4,179	2,333	3,466	5,841	2,181	2,328	64	42
Glycine	83,824	48,059	66,206	63,792	67,707	35,613	21,055	17,782	22,117	18,958	11,160	12,881	28	25
Alanine	18,947	11,403	24,551	22,900	26,317	12,265	28,466	23,132	27,505	27,002	19,738	15,173	33	22
O-acetyl-L-serine	15,079	8,199	21,593	24,874	20,938	10,976	35,122	29,898	33,003	44,335	35,456	23,962	39	20
Phenylacetate	1,411	1,184	1,058	823	1,027	752	1,098	965	1,028	1,650	496	625	23	42
Salicylate	15,591	15,914	11,740	10,058	8,363	7,778	17,828	10,070	14,261	19,418	6,021	8,853	30	42
2-aminobutyrate	370	255	259	161	292	280	237	297	303	325	191	115	25	33
m-Toluate	506	609	511	412	533	726	676	537	592	886	274	449	20	36
Valine	4,327	2,579	4,068	3,294	4,012	2,207	4,103	3,637	5,640	3,990	2,430	1,851	25	37
2-isopropylmalate	845	566	633	558	815	747	516	556	470	547	345	270	18	26
Beta-hydroxypyruvate	1,041	1,070	886	861	1,768	726	688	654	579	751	359	346	35	31
Leucine	1,088	321	512	405	847	531	983	1,125	1,569	750	835	591	47	35
Threonine	11,797	6,938	12,770	10,234	11,974	5,352	11,210	10,395	12,399	13,688	8,406	6,740	31	24
Malate	4,895	2,755	2,232	3,547	4,219	2,138	1,713	1,320	1,952	4,114	1,044	771	34	66
Proline	15,479	18,328	23,470	14,020	18,055	15,927	17,751	12,988	20,153	20,078	10,869	9,749	19	30
Aspartate	2,668	1,303	1,299	1,129	1,706	1,306	10,262	6,059	13,287	9,217	4,686	5,884	36	40
Citrate	19,654	13,973	14,196	12,878	21,525	12,747	8,786	7,171	6,067	10,208	4,108	3,430	24	40
Serine	4,461	2,099	3,915	4,131	5,090	2,100	4,215	3,626	9,590	4,222	2,131	2,299	34	63
Allantoin	84,740	72,436	61,809	41,103	126,411	46,028	35,671	19,943	25,954	35,963	51,076	27,303	43	33
Glutamate	142,415	87,578	92,463	96,724	141,314	79,707	48,720	34,008	37,916	50,026	22,417	27,323	26	30
Methionine	1,552	942	1,510	1,247	1,539	986	1,847	1,359	2,033	1,500	931	1,048	22	30
Hippurate	1,676	1,205	968	1,051	1,326	1,587	1,320	1,318	1,354	1,797	957	788	22	28
Phenylalanine	2,004	1,346	2,398	1,332	1,409	1,013	2,127	1,565	2,252	1,725	1,002	1,108	32	31
Ornithine	555	691	487	376	1,236	536	877	1,338	2,429	782	705	620	47	61
Lysine	14,210	10,900	12,772	7,239	9,875	6,993	5,884	4,017	6,212	4,479	2,544	2,528	28	37
Tyrosine	3,504	2,214	3,351	2,414	2,692	1,862	2,772	2,325	3,531	2,548	1,321	1,264	24	38

D TMS derivatisation of polar extracts

D.1 Raw data

Raw data were obtained in peak area per microliter injected sample.

Table D.1: *Metabolite response [peak area] of polar extracts obtained by performing optimised extraction method prior to TMS derivatisation and GC-Q-MS analysis.*

Sample name	Blank 1	Blank 2	Blank 3	210212 2 3	210212 3 1	210212 3 2	210212 3 3
Lactate/Methylglyoxal	-	-	-	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
Fumarate	-	-	-	30,174	33,287	33,690	30,593
D-Malate	-	-	-	99,821	117,536	126,737	108,039
L-Glutamate 3	-	-	-	340,927	400,570	431,866	437,915
L-Glutamate 1	-	-	-	602,858	853,561	989,755	966,027
Creatinine	-	-	-	24,293	37,132	38,082	41,748
alpha-ketoglutarate	-	-	-	49,984	61,144	60,239	62,167
L-Glutamate 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
Citrate	-	-	-	21,870	27,773	35,979	36,507
Methyl-beta-D-galactopyranoside	-	-	-	173,827	152,321	172,399	221,242
D-glucose	-	-	-	390,654	253,770	295,122	306,242
D-allose 2	-	-	-	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 1	-	-	-	235,024	166,163	197,849	278,249
Cholesterol	-	-	-	637,707	730,234	842,769	749,360
Myristic acid	55,973	-	-	-	-	-	-
d27-Myristic acid	50,435	-	-	43,437,800	42,082,300	48,998,600	48,631,300

D.2 Normalised data

Normalisation against internal standards and tissue weight was performed and response per mg tissue obtained.

Table D.2: *Metabolite response per tissue mass [peak area/mg] of polar extracts obtained by performing optimised extraction method prior to TMS derivatisation and GC-Q-MS analysis.*

Sample name	210212 2 3	210212 3 1	210212 3 2	210212 3 3
Tissue weight [mg]	27.35	25.94	25.94	25.94
Lactate/Methylglyoxal	85,319	103,937	94,658	90,921
Urea	23,632	26,636	25,601	24,954
L-Serine	-	1,747	1,747	1,704
Phosphoric acid	256,993	325,679	313,221	309,802
L-Proline	-	6,487	6,758	5,693
Glycine	76,347	94,821	97,941	85,883
Fumarate	1,163	1,396	1,214	1,110
D-Malate	3,847	4,930	4,566	3,921
L-Glutamate 1	23,235	35,802	35,655	35,063
L-Glutamate 2	4,230	5,743	5,088	4,872
L-Glutamate 3	13,140	16,802	15,558	15,895
Creatinine	936	1,557	1,372	1,515
alpha-ketoglutarate	1,926	2,565	2,170	2,256
Glycerol 1-phosphate	5,596	7,391	7,431	6,655
O-phosphocolamine	3,981	5,976	6,307	5,479
Citrate	843	1,165	1,296	1,325
Methyl-beta-D-galactopyranoside	6,699	6,389	6,211	8,030
D-glucose	15,056	10,644	10,632	11,115
D-allose 2	2,626	1,824	1,855	1,852
Maltose 1	9,058	6,970	7,127	10,099
Cholesterol	24,578	30,629	30,360	27,199

E HCl in methanol derivatisation of non-polar extracts

E.1 Raw data

Raw data were obtained in peak area per microliter injected sample.

Table E.1: *Approximate metabolite responses [abundance] of non-polar extracts obtained in each of the five extraction rounds in chloroform.*

Sample name	210212 1 r1	210212 1 r2	210212 1 r3	210212 1 r4	210212 1 r5
Methyl nonanoate	1,500	-	-	-	-
d27-Myristic acid	1,000,000	-	-	2,000,000	1,000,000
Methyl myristate	10,000	-	-	-	-
Unknown RT 20.774	6,000	-	-	-	-
Methyl palmitate	400,000	150,000	160,000	120,000	400,000
Unknown RT 21.107	150,000	220,000	240,000	250,000	100,000
Methyl stearate	160,000	90,000	90,000	75,000	17,000
Unknown RT 24.407	20,000	-	-	-	-
Unknown RT 24.562	10,000	-	-	-	-

Table E.2: *Metabolite response [peak area] of non-polar extracts obtained in each of the five extraction rounds in chloroform. Response is an average of two technical replicates.*

Sample name	**Tissue weight [mg]**	Methyl palmitate	Unknown RT 21.107	Methyl stearate	d27-myristic acid
280212 1 r1		109,367	151,144	68,202	7,362,630
280212 1 r2		22,876	46,534	11,108	9,105,795
280212 1 r3	28.05	-	9,239	-	5,472,930
280212 1 r4		4,676	12,488	-	9,400,875
280212 1 r5		-	-	-	10,779,500
280212 2 r1		338,323	263,594	221,520	11,439,300
280212 2 r2		13,572	34,888	-	7,652,700
280212 2 r3	39.82	12,632	26,363	-	11,341,100
280212 2 r4		-	-	-	6,513,320
280212 2 r5		-	-	-	8,553,955

E.2 Normalised data

Raw data were normalised against internal standards and tissue weight to obtain peak area per mg tissue.

Table E.3: *Metabolite response per tissue mass [peak area/mg] of non-polar extracts obtained in each of the five extraction rounds in chloroform.*

Sample name	280212 r1	280212 r2	280212 r3	280212 r4	280212 r5
Methyl palmitate	5,419	571	238	151	-
Unknown RT 21.107	5,582	1,264	505	403	-
Methyl stearate	3,478	370	-	-	-

E.3 Unidentified compound

Scan of unknown compound RT 21.107 is shown in figure E.1.

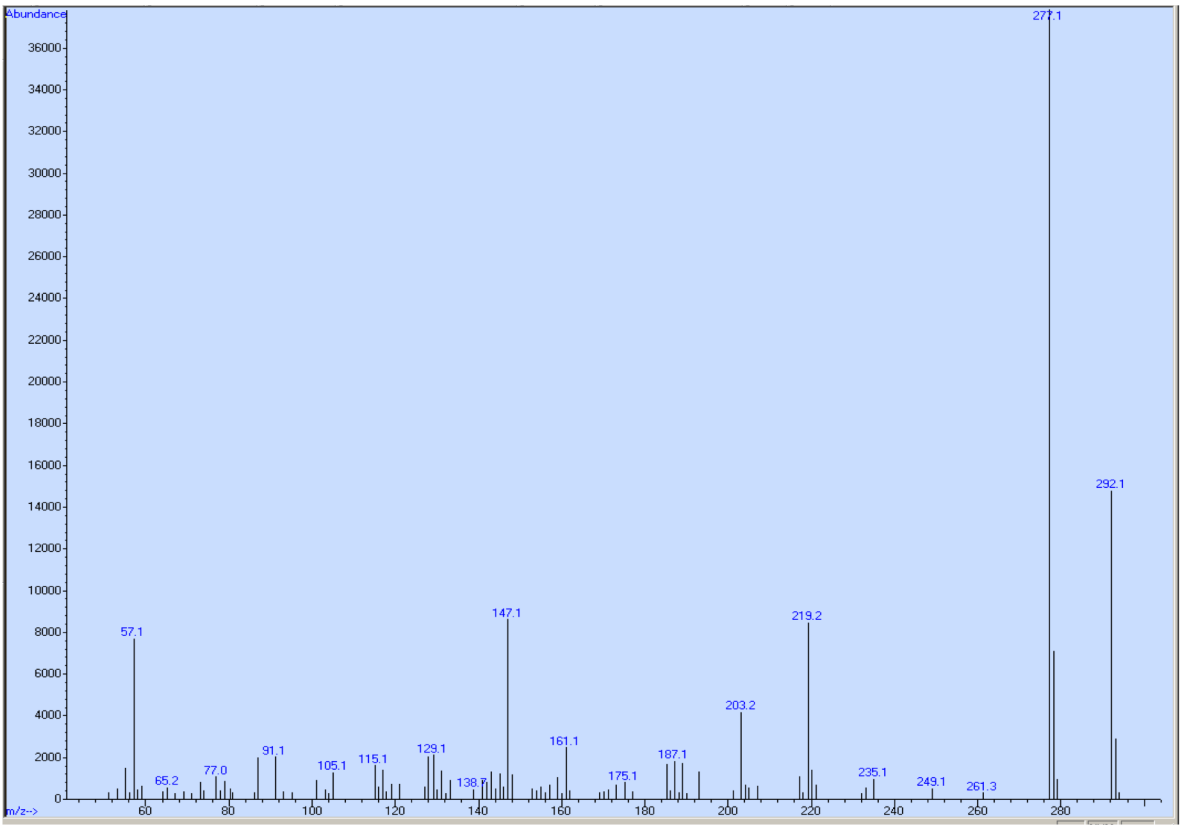

Figure E.1: *MS scan for unknown compound with retention time 21.107 obtained with GC-Q-MS analysis of non-polar extracts.*

F Lipid analysis of non-polar extracts

Q-TOF LC-MS analysis with direct infusion was performed. Scan at 0.299 min and 0.300 min for DCM blank and sample, respectively, obtained with ESI in positive ion mode is shown in Figure F.1.

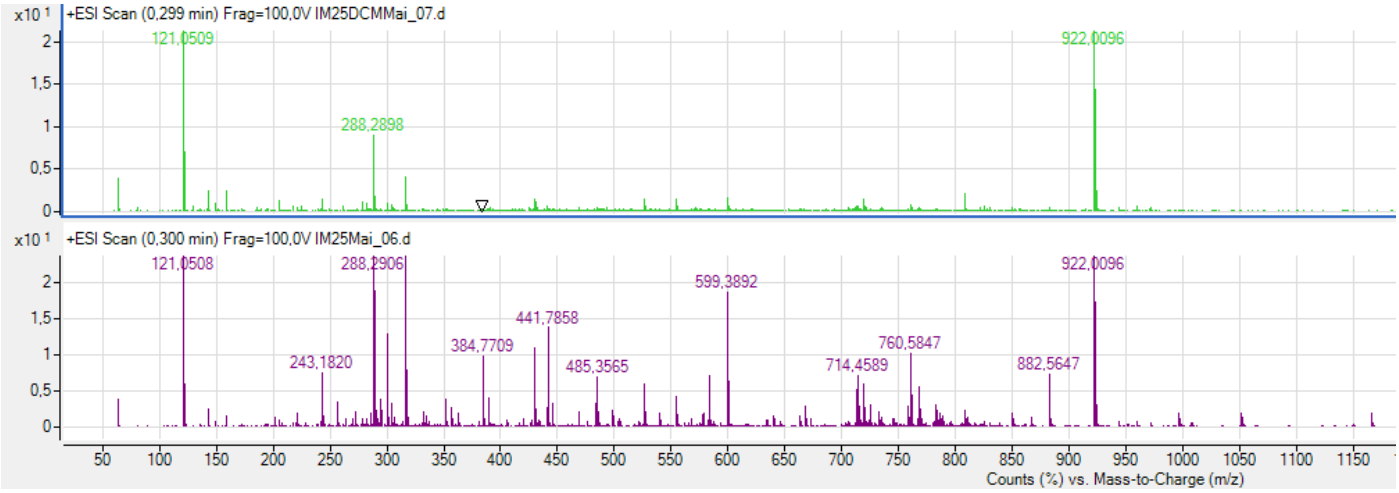

Figure F.1: *Scan of mass fragments, showing abundance as a function of m/z, for DCM blank at the top and sample at the bottom obtained at 0.3 min with ESI in positive ion mode.*

Scan at 0.702 min for DCM blank and sample obtained with ESI in positive ion mode is shown in Figure F.2.

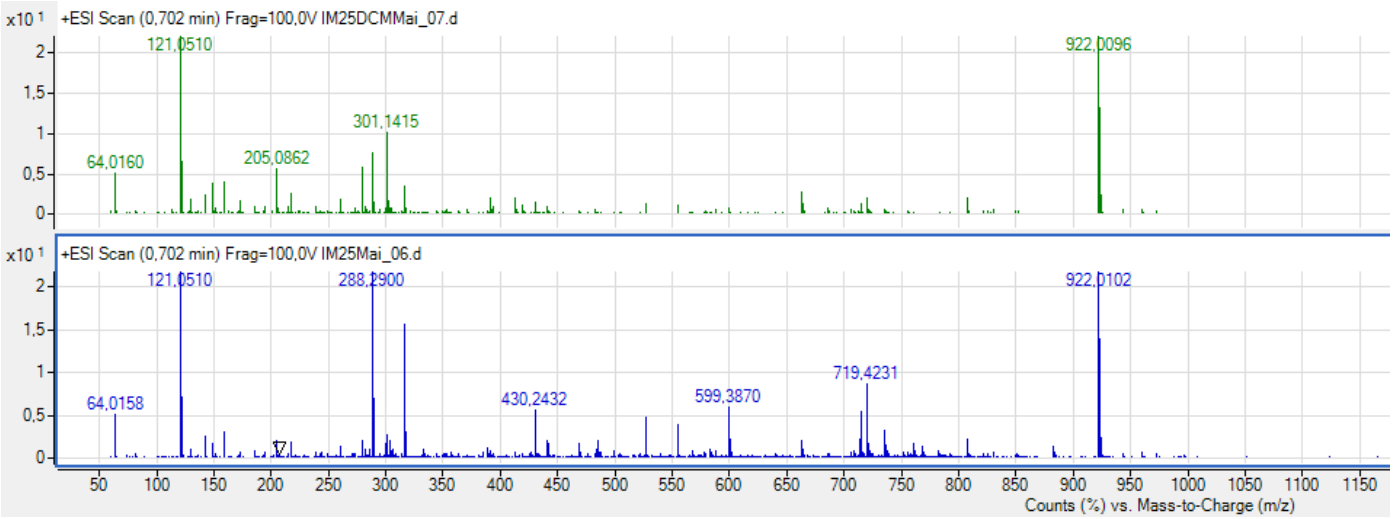

Figure F.2: *Scan of mass fragments, showing abundance as a function of m/z, for DCM blank at the top and sample at the bottom obtained at 0.702 min with ESI in positive ion mode.*

Mass fragments at 121.05 m/z and 922.01m/z represent the reference masses. The comparison of blank and sample in Figure F.1 and Figure F.2 showed that several mass fragments were obtained in sample but not in blank. These may represent lipids contained in non-polar extracts.

G Isotope labelling experiment

Isotope labelling with ^{13}C was investigated by calculating summed fractional labelling (SFL). SFL of metabolites can be obtained using Equation G.1 [23].

$$SFL = \frac{\sum i \times I_i}{\sum I_i} \tag{G.1}$$

for i=0 and up until n C-atoms in the fragment. I is the intensity of isotopomer. An approach to correct for naturally occurring ^{13}C, is to calculate SFL for unlabelled metabolites and subtract it from SFL for possibly labelled metabolites. Raw data and calculated SFL are shown in Table G.1.

Table G.1: *Summed fractional labelling (SFL) calculated with intensities of C 12, C 13 and C 14 obtained from GC-MS analysis.*

		Intensity	Sample name 210312					
Metabolite	Fragments [m/z]	Non-labelled	LumT 1	LumT 2	LumT 4	LumT 6	LumT 8	LumT 10
Lactate	103	365440	349184	173120	146432	247488	200896	466240
	104	15953	50096	26824	23256	34560	26968	62832
	105	3896	4756	2310	1791	2786	1998	4784
Proline	128	186944	228352	202496	584000	312896	307264	428224
	129	12978	16083	12639	40544	20624	21920	30448
	130	1076	1311	1471	3541	1709	1924	2208
Citrate	101	228160	7199	10480	7206	10506	10570	31688
	102	11877	500	575	730	971	928	1802
Fumarate	113	80744	6511	6299	7300	7275	7028	16384
	114	15562	1206	1219	1845	1601	1467	3854
	C-atoms							
% SFL lactate	1-3		8.6	9.4	9.5	7.9	7.3	7.4
% SFL proline	2-5		0.1	-0.3	0.1	-0.4	0.3	0.0
% SFL citrate	2-4		1.5	0.3	4.3	3.5	3.1	0.4
% SFL fumarate	1-4		-0.5	0.1	4.0	1.9	1.1	2.9

H Data analysis

H.1 Standard deviation

In this project, Excel's function STDEV.S, estimating standard deviation based on sample, was used. STDEV.S uses Equation H.1 where x is a quantity, $\overline{x}$ is the average value of quantity and n is number of values obtained from quantity.

$$STDEV.S = \sum \frac{(x - \overline{x})^2}{(n-1)} \tag{H.1}$$

H.2 Student's t-test

Student's t-test was performed on metabolite concentrations of six biological replicates of each subtype, the same data used to obtain metabolite profiles. The probabilities associated with Student's t-test are shown in Table H.1. The probability of metabolite concentration in luminal-like and basal-like subtypes to obtain the same average concentration was obtained. Excel's function TTEST was used. Tail was set to 2 for a two-tailed distribution. Type was set to 3 for the reason that variances were assumed to differ between subtypes. Significance level was set to 5% meaning that a significant difference in concentration between subtypes was stated for metabolites obtaining a probability of 5% or less.

Table H.1: *Probability associated with Student's t-test obtained for polar metabolites in luminal-like and basal-like subtypes.*

Metabolite	**Probability [%]**
4-methylvalerate	2.4
Pyruvate	9.7
3-methyl-oxovalerate	0.0
Fumarate	1.0
Lactate/Methylglyoxal	2.2
Succinate	77.5
Citraconate/Itaconate	0.0
Benzoate	1.9
Citramalate	1.9
Glyoxylate	7.7
Glycine	0.1
Alanine	25.1
O-acetyl-L-serine	0.1
Phenylacetate	74.2
Salicylate	66.4
2-aminobutyrate	57.7
m-Toluate	84.5
Valine	77.3
2-isopropylmalate	0.6
Beta-hydroxypyruvate	2.0
Leucine	8.1
Threonine	70.6
Malate	5.2
Proline	35.4
Aspartate	0.4
Citrate	0.1
Serine	57.8
Allantoin	2.5
Glutamate	0.1
Methionine	47.6
Hippurate	80.6
Phenylalanine	87.9
Ornithine	16.4
Lysine	0.2
Tyrosine	41.5

H.3 Principal component analysis (PCA)

In order to compare luminal-like and basal-like subtypes, PCA analysis was performed using Unscrambler X multivariate analysis software. PCA analysis was performed on concentrations used to obtain metabolite profiles, shown in Table C.12. Loading plot without weighting of concentrations is shown in Figure H.1.

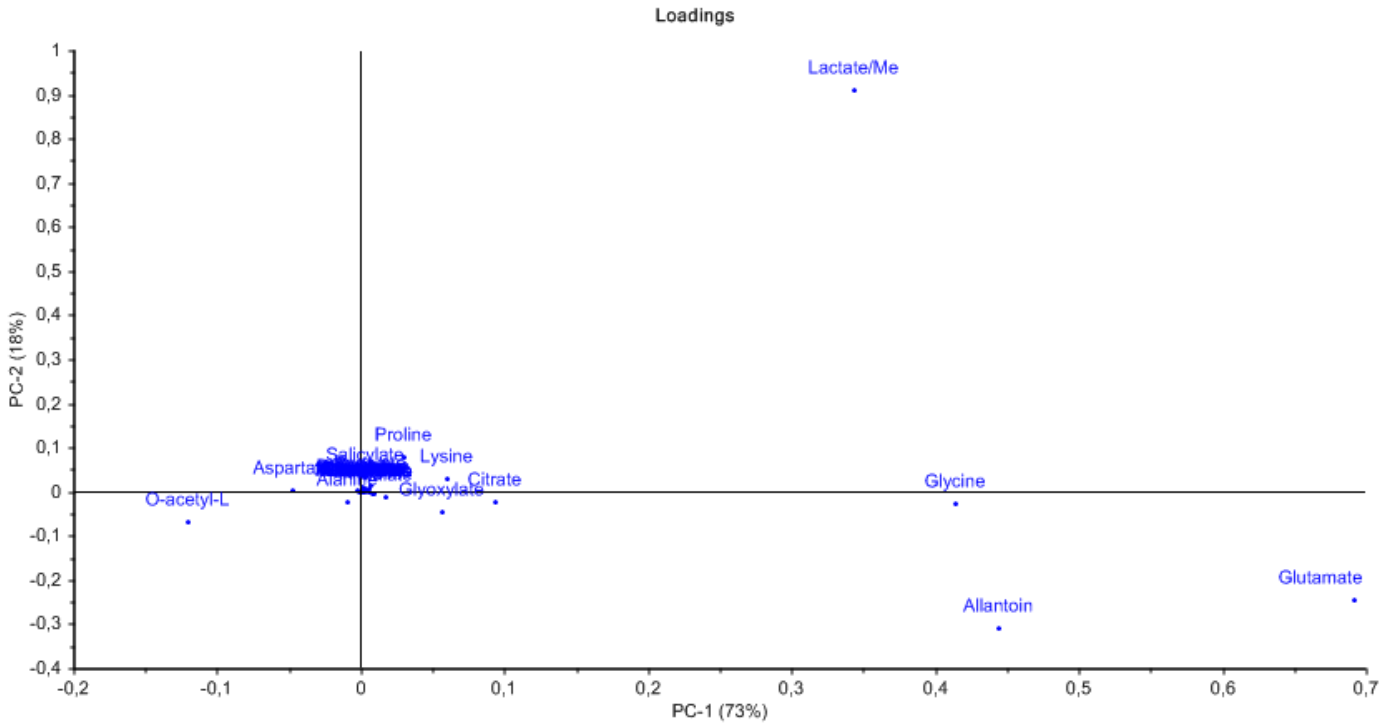

Figure H.1: Loading plot of PC-1 versus PC-2 without weighting.

Five metabolites (o-acetyl-L-serine, lactate, glycine, allantonin, glutamate) were seen to explain all the variance between subtypes. O-acetyl-L-serine and glycine are only explained by principal component 1 (PC-1). For this reason, concentrations were weighted.

Subsequent to weighting of concentrations, PCA analysis was performed. Influence plot is shown in Figure H.2. Data points seen in influence plot represent biological replicates.

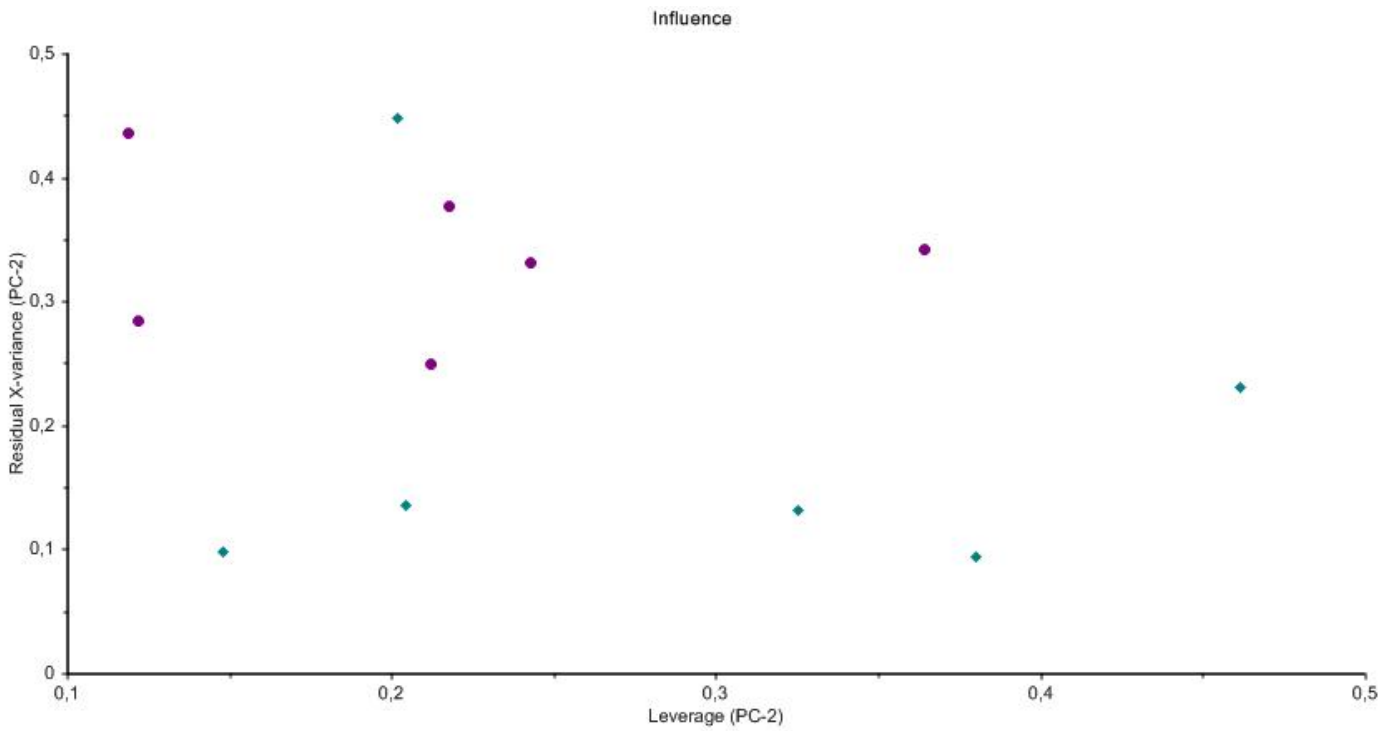

Figure H.2: *Influence plot showing to what extent data points, i.e. biological replicates, spread.*

Biological replicates were spread evenly out in influence plot. No outliers were seen thus no data needed to be excluded for PCA analysis.

Explained variance plot is shown in Figure H.3.

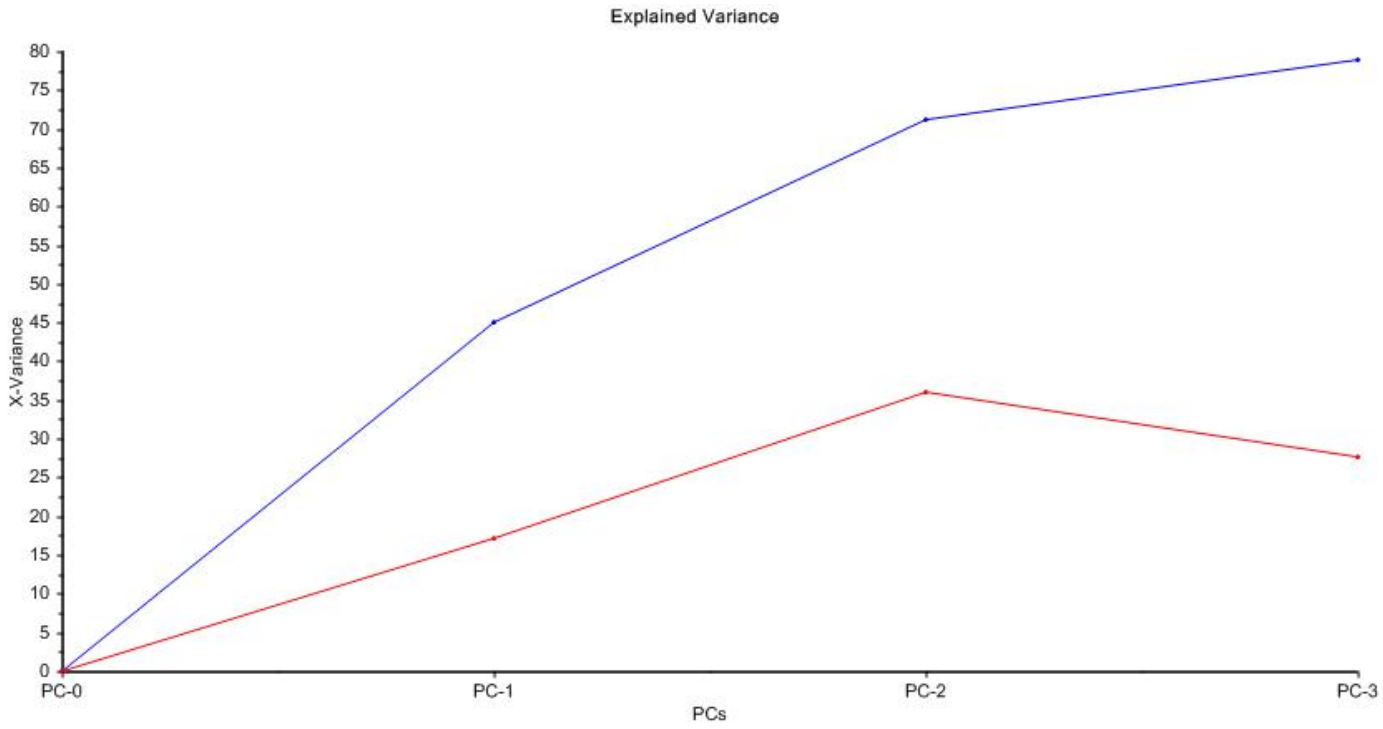

Figure H.3: *Explained variance plot showing PCs up to PC-3.*

Optimum number of principal components (PCs) was two as seen in explained variance plot. PC-1 and PC-2 explain around 70% of the variance in concentrations between subtypes. Morover, other PCs obtained a decrease in explained variance as shown by a decreasing red line. PC-1 and PC-2 were therefore used.

Loading plot in Figure 3.6 was enlarged in order to see the metabolites shown to positively correlate with luminal-like subtype. The enlargement is shown in Figure H.4.

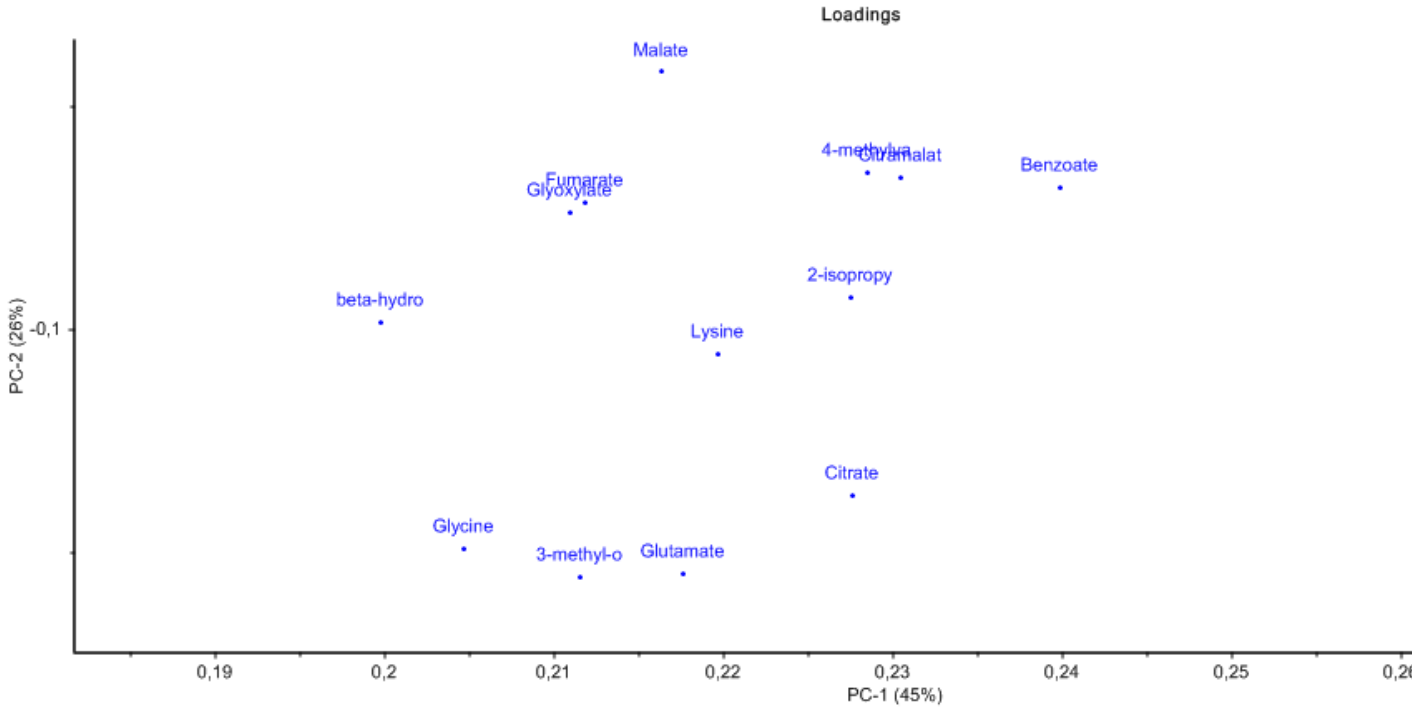

Figure H.4: *Enlargement of loading plot obtained using PCA analysis.*