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MAMMALIAN CELL CULTURES AS MODELS FOR METABOLOMIC STUDIES

By

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LIST OF ABBREVIATIONS

A

ATP: adenosine-5-triphosphate

AMBIC: ammonium bicarbonate

ANOVA: analysis of variance

AcCoA: acetyl-coenzyme A

AKG: 2-oxoglutarate

ACT: cis-aconitate

B

BHK cells: baby hamster kidney cells

BSA: bovine serum albumin

BCA: bicinchoninic acid

C

CE-MS: capillary electrophoresis mass spectrometry

CHO: chinese hamster ovary cells

CV: coefficient of variance

CIT: citrate

D

DMSO: dimethyl sulfoxide

DHAP: dihydroxy-acetone-phosphate

E

EC: energy charge

ESI: electro-spray ionisation

E4P: erythrose-4-phosphate

F

FBS: fetal bovine serum

F6P: fructose-6-phosphate

FUM: fumarate

G

GC-MS: gas chromatography mass spectrometry

GA: phosphate-activated glutaminase

G6P: glucose-6-phosphate

GAP: glyceraldehyde-3-phosphate

H

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HSP: heat shock proteins

hs: heat shock

hsh: heat shock, repair

I

IS: internal standards

ISOCIT : isocitrate

L

LC-MS: liquid chromatography mass spectrometry

LDH: lactate dehydrogenase

M

MDCK cells: madin–Darby canine kidney

MRM: multiple reaction monitoring

MTT: 4,5-dimethylthiazol-2-y1]-5-diphenyltetrazolium bromide

MSTUS: mass spectrometry total useful signal

MAL: malate

N

NMR: nuclear magnetic resonance

NaCl: sodium chloride

NEAA: non-essential amino acids

NAD: nicotinamide adenine dinucleotide

NADH: nicotinamide adenine dinucleotide reduced

NADP: nicotinamide adenine dinucleotide phosphate

NADPH: nicotinamide adenine dinucleotide phosphate reduced

O

OXA: oxaloacetate

P

PPP: pentose phosphate pathway

PENSTREP: penicillin /streptomycin

PBS: phosphate buffer saline

PCA: principle component analysis

PDH: pyruvate dehydrogenase

3PG: 3-phosphoglycerate

2PG: 2-phosphoglycerate

PEP: phosphoenolpyruvate

PYR: pyruvate

6PG: 6-phosphogluconate

R

R5P: ribose-5-phosphate

Ribu5P: ribulose-5-phosphate

S

SUC: succinate

T

TCA: tricarboxylic acid cycle

X

Xylu5P: xylulose-5-phosphate

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ABSTRACT

The use of cultured cells in metabolomic studies is receiving more and more attention. There are many advantages when using cultured cells in metabolomic studies, for example cultured cells can easily be manipulated for the purpose of the experiment. This creates many opportunities for metabolomics studies, for example cell cultures can offer an alternative manner of drug testing. Even though the use of cultured cells in metabolomic studies is very promising and they create many opportunities for metabolomic research, there are still challenges that create obstacles in this research. One of the challenges is that present analytical technologies do not always fully meet the requirements for metabolomics. There is, however, much effort going into optimising the methods concerning cultured cells and metabolomics, but there is a lack of attention when it comes to the sample preparation which is initiated by quenching. The aim of this study was to investigate cultured cells as models for metabolomics investigations and to standardise a proper quenching method for a metabolomics analysis of mammalian cultured cells.

A quenching method adapted from the literature was evaluated for the cell line used in this study, namely HeLa. Metabolites of the central carbon metabolism were targeted, using a published list. This method was tested for its effectiveness by introducing the samples to waiting periods (0, 3, 6 and 24 hours) before extraction after immediate quenching. Results indicated that the entire metabolism under study was not effectively quenched. The optimum composition and temperature for this quenching method were also investigated by comparing three different quenching methods derived from the literature. The results were contradicting. Cell cultures were exposed to two perturbations (environmental and genetic) to investigate if these perturbations can be captured and measured by using metabolomics as an instrument. There was a significant difference between control groups and the groups exposed to the different perturbations. The results gained from this study indicate that it is definitely possible to use cultured cells in metabolomics studies.

Keywords: cultured cells, mammalian, metabolomics, quenching, perturbations

OPSOMMING

Die gebruik van sel kulture in metabolomika kry meer en meer aandag. Daar is baie voordele vir die gebruik van sel kulture in metabolomiese studies, byvoorbeeld selkulture in vitro kan maklik gemanipuleer word vir die doel van die eksperiment, wat dan baie geleenthede skep vir metabolomiese studies. 'n Voorbeeld van so 'n geleentheid is dat dit 'n alternatiewe manier skep vir die toets van medikasie. Al is die gebruik van sel kulture in metabolomiese studies baie belowend en dit skep soveel geleenthede vir metabolomiese studies is daar nog uitdagings wat hierdie tipe navorsing kniehalter. Een van hierdie uitdagings is dat die huidige analitiese tegnologie nie voldoen aan al die noodsaaklikhede vir hierdie tipe metabolomiese studies nie. Daar word wel baie navorsing gedoen wat gerig is om die metodes vir sel kulture en metabolomika te optimaliseer maar daar is 'n te kort aan aandag wanneer dit kom by die stap wat monster voorbereiding behels, hierdie stap word geïnisieer deur die metabolisme te staak ("quenching").

Die doel van hierdie studie is om selkulture te ondersoek vir die geskiktheid daarvan vir metabolomiese studies en ook om 'n behoorlike metode te standardiseer vir die onmiddellike staking van die metabolisme vir die metabolomiese analisering van soogdier selkulture.

'n Aangepaste metode uit die literatuur om die metabolisme te staak, was getoets vir die sellyn wat gebruik is in hierdie studie, naamlik HeLa-selle. Metaboliete van die sentrale koolstofmetabolisme was geteiken, 'n lys opgestel deur Luo et al., was gebruik. Die metode is getoets vir sy effektiwiteit deur die monsters bloot te stel aan wagperiodes (0, 3, 6 en 24 uur) na onmiddellike staking van die metabolisme voordat ekstraksie uit gevoer is. Die resultate wys dat die hele geteikende metabolisme nie volledig gestaak was nie. Die optimale samestelling en temperatuur vir hierdie metode was verder ondersoek deur drie verskillende metodes vir die staak van die metabolisme, gevind in die literatuur te vergelyk. Die eindresultaat was teenstrydig. Die sel kulture was verder booltgestel aan twee stressors (omgewings en geneties) om vas te stel of hierdie stressors vasgevang en gemeet kan word deur van metabolomika as instrument gebruik te maak. Daar was 'n duidelike verskil tussen die kontrrole groep en groepe blootgestel aan die verskillende stressors. Resultate

verwerf uit hierdie studie wys hoe dit definitief moontlik is om sel kulture te gebruik in metabolomika studies.

Sleutel woorde: sel kulture, soogdier, metabolomika , staak, stressors

Chapter 1: Introduction

There is a very important place for the use of cell cultures in metabolomics studies. However, the use of cell cultures in metabolomics studies is still in its infancy and up until recently body fluids was mostly used in metabolomic studies concerning human biology and health (Cuperlovic-Culf, et al, 2010). There are, however, many advantages in using cell cultures and they add to the holistic understanding of the function and properties of a cell (Cuperlovic-Culf, et al. 2010). One example of the advantages in using cell cultures is that they can easily be manipulated for the purpose of an experiment, which is not possible for cells in vivo (Jacoby & Paston, 1979). The challenges of using cell cultures in metabolomics studies are that the present analytical technologies do not always meet the requirements for metabolomics and the complexity of metabolites complicates the complete and absolute metabolite analysis (Álvarez-Sánchez & Priego-Capote, 2010).

There is much effort going into optimising the methods concerning metabolomics and cell cultures but there is a lack of attention when it comes to sample preparation which includes quenching and extraction. Thus, the sample preparation step is a major challenge in the development and optimisation of using cell cultures in metabolomics studies (Álvarez-Sánchez & Priego-Capote, 2010). The reason why this drawback is such a big challenge is because sample preparation is vital for this type of studies, since the efficiency of all the analytical methods that are used in metabolomics relies on this step. As sample preparation is initiated with quenching, this study was designed firstly to investigate cell cultures as models for metabolomics studies but also to optimise a quenching method for the cell cultures used in this study, namely, HeLa cells.

Chapter 2 consists of a literature review with a short discussion of the metabolism and metabolomics and how metabolomics can be used as a tool to study the metabolism. Cell cultures are defined and also the advantages, disadvantages and challenges of using cell cultures in metabolomics studies. The application of cell cultures in metabolomics studies is further discussed with examples of different fields using metabolomics to analyse cell cultures like pharmacokinetics. Quenching is also discussed as the initiating step of sample preparation, the importance of this

step for a true snapshot of the metabolism and also the requirements for quenching. The methods and materials used in every experiment as well as the principle of every method are discussed in chapter 3. The results gained from the experiments are presented and discussed in chapter 4. Chapter 4 ends with a summary of the results and also obstacles identified in this study with suggestions for future research. Chapter 5 is a brief discussion of this study where the literature and results are integrated to form a final conclusion with reference to the use of cell cultures as models for metabolomics studies.

Chapter 2: Literature review

Introduction

This chapter begins with a short description of the metabolism. A description of metabolomics follows, which is the analysis of all the small molecular weight metabolites in a biological sample (biological fluid, cell or organism) under a given set of physiological conditions (Álvarez-Sánchez & Priego-Capote, 2010). The different strategies or approaches that exist for performing metabolomics as well as the techniques available are also presented. Four strategies are described, namely: targeted analysis, global metabolomics, footprinting and finally fingerprinting. The analytical techniques that are frequently used are distinguished from those that are less frequently used. The frequently used techniques are: NMR (nuclear magnetic resonance), LC-MS (liquid chromatography mass spectrometry) and GC-MS (gas chromatography mass spectrometry). The techniques less frequently used are: CE-MS (capillary electrophoresis mass spectrometry), LC-NMR, LC-NMR-MS and LC-ESI-MS/MS. These techniques can be used to analyse different sample types including those derived from cell cultures.

Cell cultures are defined as a technique that consist of the isolation of cells from tissues or whole organs derived from humans, animals, plants or microbes and the maintenance of these cell cultures *in vitro* (Wilson & walker, 2005:71). The different cell types used in cell cultures can be divided in one of two categories which is primary cell cultures or a cell line. Cells can also be described according to their morphology of functional characteristics. The three basic morphologies are epithelial-like cells, lymphoblast-like cells and fibroblast-like cells. The two basic culture systems that is used for growing cells are adherent cells (require attachment for growth) and suspension cells (does not require attachment for growth). The advantages and disadvantages of using cell cultures are discussed as well as the application and challenges of using cultured cells in metabolomic studies. The discussion consists of the possibilities in using cell cultures in metabolomics studies and also the challenges that these studies face like the analytical techniques that do not meet the requirements for these studies.

Two studies involving cultured cells of microorganisms are discussed to show some of the sampling methods used in metabolomics in cultured cells. The following five studies discussed are studies involving mammalian cell culture and metabolomics. The last three studies illustrate the applicability of these studies.

Because quenching is the initiating step of sample preparation which is of great importance, this step will be discussed in detail. The definition of quenching is the sudden and complete termination of the metabolism by inhibiting endogenous enzyme activity. This step is important because quenching provides a valid snapshot of the metabolic state at a given time enabling one to obtain a reliable metabolic profile (Faijes, *et al.* 2007). A quenching method should meet certain requirements, which will also be mentioned. There is also a discussion of strategies for quenching and previous studies of quenching and cultured cells. The chapter ends with the aims and objectives set for this study.

2.1 Metabolomics

2.1.1 Metabolism

The metabolism is the total network of all the chemical reactions that is present in a cell with the purpose of maintaining life. It is a process where one metabolite is changed into another through a sequence of enzymatic reactions (Soga, *et al.* 2009). The enzymatic reactions form a metabolic network and within this network lie the central carbon metabolism, consisting of the following metabolic pathways: glycolysis, the pentose phosphate pathway, the tricarboxylic acid (TCA) cycle and corresponding cofactors. The central carbon metabolism has key functions in the processes of substrate degradation, energy and cofactor regeneration, and biosynthesis precursor supply (Soga, *et al.* 2009).

It is essential to study metabolism, since it is well-known that metabolism plays an essential role in human physiology (Mo & Palson, 2008). The metabolic function is vital for understanding aging, nutrition and disease states and its progression. More specifically it is of great interest to study the central carbon metabolism, not only because of the key role it plays as mentioned above, but also because this part of

the metabolism has a broad biological relevance due to its presence in almost all organisms (Kiefer, *et al.* 2008). The central carbon metabolism is also very susceptible to changes in the environmental conditions of the cell and will change substantially in reaction to these changes (Büscher, *et al.* 2009). This part of the metabolism is also sensitive to genetic modification or perturbations, and data obtained where their concentration dynamics was affected by these changes is also of great interest (Luo, *et al.* 2007). Moreover, the application of studying the central carbon metabolism in mammalian cultured cells has only recently gained interest (Sellick, *et al.* 2009). One example of such an application is that cell culture models were developed as an alternative way of drug testing (Khoo & Al-Rubeai, 2007).

Metabolomics is one of the new 'omics' technologies that can be used to study the metabolism. There are a number of strategies that can be followed and techniques that can be used which will be discussed in the next section. These strategies explain how metabolomics can be used to study the metabolome as a whole (untargeted) or just specific pathways in the metabolome (targeted).

2.1.2 What is metabolomics?

Metabolomics is a more recent 'omics' technique compared to others like proteomics and transcriptomics. The first definition for metabolomics was given by Oliver *et al.* in 1998 (Khoo & Al-Rubeai, 2007). Metabolomics is the 'omics' technique to study the metabolome (Roux, *et al.* 2010) and it aims to narrow the gap between genotype and phenotype by giving more insight into biological processes as it is the end result of the interactions between genotype and environment. Metabolomics can thus be defined as the analysis of all the small molecular weight metabolites in a biological sample (biological fluid, cell or organism) under a given set of physiological conditions (Álvarez-Sánchez & Priego-Capote, 2010). Two terms in this regard need to be described, namely metabonomics and metabolomics. Metabolomics is the measurement of the global pool of metabolites in a cell; metabonomics on the other hand is the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification (Roux, *et al.* 2010). These terms have an interchangeable nature because both refer to the multi-component study of metabolites in a biological system. The term

metabolomics will be used in this study (Cuperlovic-Culf, *et al.* 2010). The purpose of using the metabolomics approach is that it is a data driven approach with the aim to add to the better understanding of how biological processes work and interact (Roux, *et al.* 2010).

2.1.3 The different strategies for metabolomic studies

There are different strategies or approaches that can be followed when the experimental design is planned for a metabolomics study, the first involves a targeted analysis, where the aim is to study one or more metabolites of a small group which is chemically similar or belong to a specific metabolic pathway. The second is global metabolomics, in which a wide range of metabolites is studied by using either one analytical platform, or by combining complementary analytical platforms, such as GC-MS, LC-MS, capillary electrophoresis (CE)-MS or NMR. The purpose of global metabolomics is to obtain a complete profile of the metabolome. Metabolic fingerprinting, whichever strategy is followed, is a rapid high throughput method which is used to analyse biological samples that offer metabolite fingerprints that is used to classify and screen the sample. The final strategy is metabolite footprinting with the purpose of studying metabolites in extracellular fluids (exometabolome) (Álvarez-Sánchez & Priego-Capote, 2010).

2.1.4 Analytical techniques available for metabolomics studies

The step in the experimental design following sample preparation is to choose the appropriate analytical technique. The appropriate technique is mainly dependent on the characteristics of the metabolites involved and the aim of the study. There are several techniques available for doing metabolomics (Table 2.1)

Table 2.1: The different analytical techniques used in metabolomics studies:

Techniques	Advantages	Disadvantages	Most common use	Referenced article
NMR ¹	Non-invasive, non-destructive, very discriminatory and a high throughput method	Low sensitivity compared to mass spectrometry techniques	Applicable to relatively crude samples, thus requires larger amount of sample	(Roux, <i>et al.</i> 2010); (Khoo & Al-Rubeai, 2007)
GS-MS ^{2&3}	High sensitivity and selectivity	Require longer analysis times (because of the gas chromatographic separation times) and are restricted to samples that are volatile or samples that can be derivatised	To analyse samples that are volatile or can be derivitised	(Khoo & Al-Rubeai, 2007); (Roux, <i>et al.</i> 2010).
LC-MS ²	Derivatisation is not necessary thus this method can analyse higher mass ranges and this method can also analyse samples that can't easily be derivatised	Low chromatographic resolution compared to GC-MS	Analyse samples with high mass ranges and samples that cannot be derivatised	(Khoo & Al-Rubeai, 2007)

1. One of the first techniques used in metabolomic studies is nuclear magnetic resonance (NMR) (Roux, *et al.* 2010).

2. A very valuable platform to use is combined instruments because they provide an extra dimension to the analyses of metabolites since analysis of compounds are based on different characteristics, e. g. retention time or different physical properties, for example mass. In addition, they add extra structural information for metabolite identification. The two most frequent combined technologies used in metabolomic studies are LC-MS and GC-MS.

3. When it comes to metabolite detection and quantification, the gold standard is considered to be the GC-MS (Khoo & Al-Rubeai, 2007) and was the first separative method that was able to be combined to a mass spectrometer (Roux, *et al.* 2010).

Other platforms available which are less frequently used include CE-MS (capillary electrophoresis-mass spectrometry), which is used generally to separate a wide range of cationic and anionic compounds, nucleotides and coenzyme metabolites. Metabolite quantification and identification is ideally done by the MS. LC-NMR as well as LC-NMR-MS methods have also been developed (Khoo & Al-Rubeai, 2007). To follow the metabolism of yeast cells an approach was used ^{13}C isotopes together with LC-ESI-MS/MS instruments to eliminate drawbacks like linear responses or matrix effects (Khoo & Al-Rubeai, 2007).

The techniques described in the above section can be used to analyse different sample types, for example, body fluids such as blood, urine and sputum and also samples derived from tissues or cell cultures. Since the use of cell cultures in metabolomics studies is still in its developing stages, a description of cell cultures seems appropriate.

2.2 Cell Cultures

2.2.1 The nature of cell cultures

There are many different uses for cell cultures because they are very good models for studying intracellular processes like protein synthesis and drug metabolism, e. g. the mechanisms of cell-cell interactions, genetics and drug metabolism and actions (Wilson & Walker, 2005:71)

2.2.2 The different cell types used in cell cultures

Different cell types are used in cell cultures and they can be grouped into one of two categories, namely primary cell cultures or cell lines. Cells that are directly derived from tissues subsequent to enzymatic dissociation, or from tissue fragments, are referred to as primary cell cultures (Wilson & Walker, 2005:81). The advantage of using primary cell culture is that it retains their characteristics and thus reveals the exact activity of the cell type *in vivo*. Their disadvantages are that the isolation of these cells can be a difficult process and a heterogeneous population of cells may be obtained. Another disadvantage is that they have a limited lifespan (Wilson &

Walker, 2005:82). Primary cell cultures are derived from various tissues and the cell type is usually defined by the source of tissue used, for example hepatocytes are isolated from liver tissue (Wilson & Walker, 2005:82). Cells can also be described according to their morphology or functional characteristics. Three basic morphologies exist, namely epithelial-like cells, which are cells that attach to a substrate and their appearance are flattened and they have a polygonal shape; lymphoblast-like cells that normally don't attach to substrate and thus remain in suspension and is spherical in shape. Lastly fibroblast-like cells which do attach to substrate and their appearance are elongated and bipolar; in heavy cultures they can form swirls (Ryan, 2008). Continuous cell lines on the other hand consist of a single cell type and their life span is infinite. They usually gain this ability via transformation of these cells by numerous ways, for example treatment with carcinogens or exposure to viruses (Wilson & Walker, 2005:82). A disadvantage of cell lines is that when cells are transformed, several of their *in vivo* characteristics usually are lost. There are a number of advantages when working with cell lines. Less serum is required for growth than primary cell cultures, their doubling time is also shorter and they do not necessarily require attachment for growth.

2.2.3 Two basic cell culture systems

Two basic culture systems are used for growing cells and are differentiated according to the growth characteristics of cells, namely those cells that require attachment for growth and those who don't. Cells that do require attachment for growth are called adherent cells and those who don't are called suspension cells (Ryan, 2008)

2.2.4 Advantages and disadvantages of cell cultures

In the general cell cultures have many advantages. They provide a continuous supply of homogenous cellular material for biochemical experiments. Cells *in vitro* can be easily manipulated for the purpose of the experiment whereas this is not possible with cells *in vivo*. Cells can also be stored in a deep frozen state and doing so there is no alteration to their growth rate or genetic composition and they can be revived whenever needed. It is far more economical to use cell cultures instead of

rearing animals and doing experiments with animals (Jacoby & Paston, 1979: 439). In addition there is no requirement for ethical approval (Cuperlovic-Culf, *et al.* 2010). There are also a number of disadvantages when using cell cultures, and includes the need for specialized equipment, their sensitivity for varying environmental conditions, e.g. power failure. Another disadvantage is that cell cultures are very prone to infections which can make the experiments involving cell cultures very time consuming (Ryan, 2008).

2.3 Metabolomics and cultured cells

2.3.1 The application and challenges of using cultured cells in metabolomic studies

The application of metabolomics in cultured cells is of great importance in a variety of fields. Up until now, body fluids was mostly used in metabolomic studies concerning human biology and health, as these studies had mostly a holistic focus point in terms of biological systems (Cuperlovic-Culf, *et al.* 2010). However, for a more holistic understanding of the function and properties of a cell, there is a requirement for appropriate information of specific types of cells under a variety of conditions, which is vital for this holistic understanding. This can be accomplished by shifting the focus so that, instead of focusing on the whole organism, the focus can be on smaller parts like cultured cells. This can complement the results of the whole system (Cuperlovic-Culf, *et al.* 2010). In the field of pharmacokinetics and drug testing, *in vitro* cultured cell models were developed as an alternative way of drug testing (Khoo & Al-Rubeai, 2007). In plant cell cultures, metabolomics can be used to determine secondary metabolites that are of great importance. Examples of such metabolites are isoflavone and taxol which is proven effective pharmaceutical ingredients (Khoo & Al-Rubeai, 2007). Another very interesting application is the analysis of the cell phenotype for optimisation of cell culture and bioreactor conditions (Cuperlovic-Culf, *et al.* 2010). Because of the tightly connected complex metabolic networks in living cells, perturbations that are induced at the level of the transcriptome and proteome can filter down to the metabolite level. Thus a further application of using cultured cells in metabolomic studies is the investigation of the effects of mutagenesis and genetic aberrations (Khoo & Al-Rubeai, 2007).

Despite of all the possibilities, metabolomics still faces some challenges in the area of mammalian cultured cells. These challenges include the current analytical technologies, which do not always fully meet the requirements for metabolomics. For example it is at present still impossible to identify and quantify all the metabolites present in a biological sample (Khoo & Al-Rubeai, 2007). Although it is believed that the number of metabolites in a cell is approximately 1000 in *E. coli*, 3000 in humans and 200 000 in plants, the exact number is not known (Büscher, *et al.* 2009). The complexity of metabolites, because of their diverse chemical properties, also complicates obtaining a complete or absolute metabolite analysis (Khoo & Al-Rubeai, 2007). Thus the functioning conditions of applied metabolomics methods need to be optimised properly to achieve the goal of comprehensive approaches. One way of addressing these problems is to integrate various analytical techniques, for example LC-MS and GC-MS (Álvarez-Sánchez & Priego-Capote, 2010).

Although much effort is going into optimising the methods used in metabolomics, there is a lack of attention when it comes to sample preparation. This includes quenching (the rapid stopping of the metabolism) and extraction of metabolites from the sample. The sample preparation step is very important as the efficiency of all the analytical methods that are used in metabolomics relies on this step which is usually initiated with quenching. This is specifically true when one is working with cultured cells because they are susceptible to enzyme action and quenching offers an opportunity to obtain a valid snapshot of the metabolic state of a cell culture at a given time (Álvarez-Sánchez & Priego-Capote, 2010). The sample preparation step is the drawback in the total development of the analytical methods concerning metabolomics (Álvarez-Sánchez & Priego-Capote, 2010). The shortcoming of sample preparation is the requirement for conventional approaches because of the heterogeneity of the samples.

The protocols for quenching cultured cells need to be optimised for each individual cell line (Álvarez-Sánchez & Priego-Capote, 2010). The extraction procedure can involve the addition of foreign reagents that may have an influence on the metabolism of the cells. Some extraction procedures can be time consuming which can cause sample degradation (Cuperlovic-Culf, *et al.* 2010). In addition, when the hands-on time of the experiment tends to get to long, the high-throughput screening

and experimentation become challenging. It is also a difficult task to obtain the exact number of cultured cells needed for the experiment (Cuperlovic-Culf, *et al.* 2010).

2.3.2 Advantages of using cultured cells in metabolomic studies

Despite these challenges, there are still major advantages in using metabolomics as an instrument to study cultured cells. Metabolomics has been used in a wide range of studies, which includes functional genomics, pharmacogenomics, biomarker discovery, integrative systems biology and recently cell cultures (Goodacre, *et al.* 2004). One advantage is when there is a change in the quantity of an individual enzyme which does not necessarily have a great impact on the metabolic fluxes, but does have a great effect on the concentration of several other metabolites (Goodacre, *et al.* 2004). Thus when there is a change introduced in the cell culture, for example a change in the environment or energy substrate, this change can be measured using metabolomics. Another advantage in using metabolomics as a tool for the different study types mentioned before is that it reflects the cellular processes at a functional level closer than genomics. This is because the metabolome is further down the 'omics' cascade and close to the functional phenotype (Goodacre, *et al.* 2004). A great advantage in using cell cultures for metabolomic studies is that some very difficult issues in other metabolomic applications, like the variation between individual subjects, sample times, problems concerning population control and many other factors like gender, age, health status, the environmental exposure and the aid of different tissues are not applicable when working with cultured cells. When the focus of the study is on a specific cell type it can decrease variability which will give a background that is more constant. This constant background makes it easier to detect subtle metabolic changes (Cuperlovic-Culf, *et al.* 2010).

2.3.3 Previous metabolomics studies involving cultured cells

Two studies will be discussed where the metabolomics approach was followed to study cultured cells of microorganisms. This is to illustrate some of the sampling methods used in metabolomics when cultured cells are studied. In the first case the sampling methods currently used for metabolomics were investigated (Bolten, *et al.* 2007). This includes methods with cell separation (cold methanol quenching and

fast filtration) and without cell separation (liquid nitrogen quenching and fast heating of the whole broth). The methods without cell separation were investigated by analysing metabolite levels in the medium that have the potential to interfere. In this study researchers used different bacteria that are either model organisms or production strains in biotechnology, *Bacillus subtilis*, *C. glutamicum*, *Escherichia coli*, *Gluconobacter oxydans*, *Pseudomonas putida*, and *Zymomonas mobilis*. To account for a possible influence of the structure of the cell wall, Gram-positive and Gram-negative bacteria were included (Bolten, *et al.* 2007). Intracellular metabolites from various pathways were analysed, namely glycolysis, the pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle and the biosynthesis of amino acids (Bolten, *et al.* 2007). They concluded that the key to accurate and valid metabolomics is appropriate sampling. At that time there was no conclusion on a final design for an ideal sampling method. Certain key points were identified to address in future developments. These key points are factors that influence metabolite leakage, the effect of sampling time or a validity check of the data. In a second study the value of labelled internal standards (IS) in a metabolomics study was demonstrated, when using liquid chromatography electron spray ionisation coupled to mass spectrometry (LC-ESI-MS/MS). The data was compared with conventional ^{12}C -based methods to illustrate the advantages of the LC-ESI-MS/MS approach, when the glycolytic and TCA intermediates of *Saccharomyces cerevisiae* are analysed, in both steady-state and transient conditions (Wu, *et al.* 2005).

Although mammalian cells will be used in this study, these two studies illustrate different sampling methods when metabolomics is used as an instrument to study cell cultures in general. They also address key points for future development. Furthermore these studies analysed the metabolites of the central carbon metabolism which will also be the focus of this study.

Because the use of mammalian cultured cells in metabolomic studies are still in its infancy, only a small number of studies have been reported thus far. The first study that will be discussed illustrates a straightforward and robust chromatographic method to determine and quantify more than 25 intracellular metabolites of the energy metabolism of mammalian cells in culture (Ritter, *et al.* 2006). They used an on-line electrolytic eluent generator to make this method trustworthy and convenient

and it only requires water for eluent generation. For the sample preparation Ritter and co-workers used a one step to quench and extract ion method of the cells. They used a $-70\text{ }^{\circ}\text{C}$ methanol/formic acid mixture for the Madin–Darby canine kidney cells (MDCK) and for the BHK (Baby Hamster Kidney) cells they used methanol/tricine mixtures. With this method they managed to detect a broad range of intracellular metabolites that are present as intermediates in the TCA cycle and glycolysis and also nucleotides (Ritter, *et al.* 2006). The online electrolytic eluent generation system made it possible to use complex gradient programs and this evidently increased the separation of these compounds (Ritter, *et al.* 2006).

Another study was done on single Islets of Langerhans where a microscale method for metabolomic analysis was used (Ni, *et al.* 2008). They illustrated sample preparation (initiated with snap freezing in liquid nitrogen as the quenching method) separation and detection. For detection Ni and co-workers also used LC-ESI-MS/MS like in the previous study but they used it in the negative ion mode (Ni, *et al.* 2008). In this study the intermediates of the glycolysis pathway and TCA cycle was targeted. The limits of detection for these targeted metabolites were in the concentration range of low nano-molar to low micro-molar, which indicated that the sensitivity for detection is suitable for a variety of intracellular metabolites in a complex biological system. Ni and co-workers also tested the reproducibility of the method by changing the fuel, in this case glucose, and they concluded that this method was sufficient to identify big relative variations in metabolite concentrations concerning fuel changes, without internal standards (Ni, *et al.* 2008).

In the previous two studies mammalian culture cells were analysed using the metabolomics approach. These studies illustrate different sampling and analysing methods for studying mammalian cultured cells, when the energy metabolism is under study.

The following three examples serve to demonstrate the potential of using mammalian cultured cells in metabolomics-based investigations. A study was done by Pastural *et al* where they looked into the hypothesis which states that the manifestation of the pathology and symptoms of autism is the outcome of metabolic toxicity and deregulation, regardless of what factors initially caused this disease.

They used two different cell cultures, namely astrocyte and hepatocytes, to do a comparison of the effects of glutamate toxicity *in vitro* to changes in biomarkers seen in the serum samples of autistic subjects. They concluded that in autism there exist a common metabolic phenotype and this phenotype can be measured, without difficulty, in a blood sample (Pastural, *et al.* 2009). In another study a cell culture model system was developed for routine testing of substances inducing oxidative stress (Ritter, *et al.* 1999). They concluded that substances like 2-tert-butylperoxy-2-methylpropane (tBuOOH) and H₂O₂ can induce biological effects which are discriminating and with their experimental set-up these effects can be detected. This system is considered to be a valuable indicator for continued research on the mechanisms of oxidative stress (Ritter, *et al.* 1999). In the third study it was investigated whether a set of paired samples of normal colon tissue and colorectal cancer tissue, of individual patients, could be profiled with a GC-MS (Denkert, *et al.* 2008). The reason being to see if molecular changes in tumour tissue can be detected and interpreted and also if metabolic patterns linked with different biological entities can be detected. Denkert *et al.* concluded in their study that fresh-frozen tumour tissue of colon cancer can be used to detect metabolic signatures and individual metabolites. Furthermore these changes can also be associated with applicable biochemical pathways. They strongly recommend the metabolomics approach, which is complementary to transcriptomics and proteomics, when analyses of alterations in the malignant phenotype are required (Denkert, *et al.* 2008).

These studies not only illustrate the potential of using cell cultures in metabolomics studies but also emphasise the advantages of cell cultures previously mentioned. It is clear from these studies that research involving establishing biomarkers, profiling of diseases and oxidative stress can be done on mammalian cell cultures using the metabolomics approach. This study will further evaluate if mammalian cell cultures, especially cell cultures derived from human cells, can be used as models for metabolomics studies. For example can a perturbation induced (genetically or environmental) be measured using this approach.

2.4 Quenching

2.4.1 Quenching and its importance

The sample preparation step is initiated by quenching and comprises the instantaneous and complete terminated of the metabolism in a cell culture which is achieved by terminating all endogenous enzyme activity. This is a very important step in metabolomic studies as sample representativeness can only be accomplished when the metabolism is rapidly and completely stopped (Álvarez-Sánchez & Priego-Capote, 2010; Fajjes, *et al.* 2007; Danielsson *et al.* 2010). Processes in the metabolism are rapid as its duration varies between milliseconds to minutes (Khoo & Al-Rubeai, 2007). Take for example those reactions occurring in glycolysis and even more so, those reactions involving ATP, which include fluxes of millimoles per litre per second (Winder, *et al.* 2008). This gave rise to the common rule of thumb to perform quenching of cells in culture very fast, if it is possible, in less than one second (Ewald, *et al.* 2009). Quenching provides a valid snapshot of the metabolism of a cell culture at a given time enabling one to obtain a reliable metabolite profile (Fajjes, *et al.* 2007). This immediate stopping of the metabolism is of cardinal importance when it comes to analysing cell cultures, when the aim is to reveal the metabolic profile (Álvarez-Sánchez & Priego-Capote, 2010).

Quenching is cell line and sample specific. In addition, cell composition and size can also influence the effectiveness of the quenching method as well as the amount and rate of metabolite leakage (Sellick, *et al.* 2009). Therefore, different quenching methods have been developed in different laboratories (Sellick, *et al.* 2009; Spura, *et al.* 2009). This study focuses on establishing a standard quenching method for our laboratory. The following requirements need to be accomplished by a quenching strategy (Sellick, *et al.* 2009; Spura, *et al.* 2009; Álvarez-Sánchez & Priego-Capote, 2010):

1. The metabolism must be terminated faster than the metabolic reactions taking place in the sample.
2. Of cardinal importance is the efficiency of the quenching method, because many primary metabolites has got turnover rates in the area of mM/s.
3. It is further important to maintain sample reliability during the quenching process,

especially in case of cells where it is important to limit the leakage of intracellular metabolites.

4. The chemical and physical properties as well as the concentration of the metabolites should not be significantly altered by quenching.

5. After quenching, the sample should be compatible with the analytical steps that follow.

6. Another vital requirement is that the quenching procedure is reproducible

2.4.2 Strategies for quenching

The most frequently applied quenching strategies are based on rapid alteration of the sample conditions (cell environment) which is generally the pH or temperature (Spura, *et al.* 2009; Álvarez-Sánchez & Priego-Capote, 2010). Quenching can be accomplished by adding either an alkali or an acid and thereby changing the pH to extreme values. The limitation of this method is that the number of metabolites detected is decreased because of metabolite degradation resulting from a low pH (Khoo & Al-Rubeai, 2007). Another problem with adding acids to decrease the pH is that it causes problems for the subsequent analytical processes, since the acidic solvents have to be removed prior to these processes (Khoo & Al-Rubeai, 2007). In terms of changing the temperature quenching is mainly accomplished by lowering the temperature. Temperatures below -20 °C are usually preferred, assuming that the cold shock does not limit sample reliability (Álvarez-Sánchez & Priego-Capote, 2010). This cold shock can be achieved by liquid nitrogen which is -196 °C, freeze clamping (Khoo & Al-Rubeai, 2007) or adding a pre-cooled organic solvent (Álvarez-Sánchez & Priego-Capote, 2010). Although the liquid nitrogen is thought of as the easiest way to quickly terminate the metabolism of the cells (Khoo & Al-Rubeai, 2007) it has the limitation of damaging the cell envelopes with the formation of ice crystals during freeze and thaw cycles.

The method mostly used is cold methanol quenching where the metabolism of a cell culture is terminated in less than one second. The composition of this quenching solution is usually a water-methanol mixture which is pre-cooled to very low

temperatures, generally -40°C (Álvarez-Sánchez & Priego-Capote, 2010). The reasons why methanol is such a good organic solvent to prepare quenching solutions with are because of its low freezing point, the fact that it is miscible with water and aqueous-methanol solutions have a low viscosity (Álvarez-Sánchez & Priego-Capote, 2010). The drawback of using methanol as the organic solvent is that it can cause metabolite leakage (Álvarez-Sánchez & Priego-Capote, 2010). This leakage is most likely dependent on two factors, namely cell wall and membrane structure (Winder, *et al.* 2008). There are a number of cold methanol quenching strategies that includes an additive, like tricine, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) or ammonium carbonate, to control the ionic strength in order to limit damage to the cell envelopes (Álvarez-Sánchez & Priego-Capote, 2010). An alternative way to reduce leakage of metabolites is to keep the contact time between the cells and organic solvent as short as possible (Sellick, *et al.* 2009; Winder, *et al.* 2008; Ewald, *et al.* 2009). However, a more recent study done by Danielsson *et al.* proved that for adherent cells the sample preparation process can actually be facilitated by metabolite leakage. This is possible by linking the quenching and extraction steps in the sample preparation process; because cell isolation and rinsing is performed without difficulty before the quenching step, this is not true for suspension cells (Danielsson, *et al.* 2010). A quenching method that is not often used is the fast increasing of temperature to create a heat shock. This can be achieved by adding ethanol which is 90°C . The reason why this method is not a very popular option is because it has the potential to degrade thermolabile metabolites as well as to enhance the cell permeability.

2.4.4 Previous quenching studies involving cultured cells

Five studies of quenching of cultured cells are compared in table 2.2.

Table 2.2: A comparison of five studies involving quenching and cultured cells

Study	Cell line	Quenching method	Findings	Referenced article
¹ A novel quenching method for microbial cell cultures, which is capable of coping with variations in the operating environment	<i>Pseudomonas fluorescents</i> (Gram(-) bacterium), <i>Streptomyces coelicolor</i> (Gram(+) bacterium) and <i>Saccharomyces</i> (yeast cell representative)	Four quenching solutions were compared: glycerol water, glycerol-saline, glycerol-mannitol and methanol-water solution	Glycerol-saline solution is the best quenching solution for quenching microorganisms	Villas-bo & Bruheim, 2007
² Development of a quenching and extraction method for <i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>	Four different quenching solutions were compared, and each contained 60% methanol	It appears that the most appropriate quenching buffer is ammonium carbonate solution	Fajjes, <i>et al.</i> 2007
³ Development and optimisation of a standard method for quenching and extraction of metabolites in <i>E. coli</i> cells to do a global metabolite analysis	<i>E. coli</i> cells	Three quenching solutions were compared	It was found that the 60% methanol (-48°C) gave the best recovery of metabolites	(Winder, <i>et al.</i> 2008).
⁴ Optimised a new Quenching method for three different organisms	<i>Saccharomyces cerevisiae</i> (Eukaryote), <i>Corvnebacterium glutamicum</i> (Gram(+)), <i>Escherichia coli</i> (Gram(-) prokaryote)	40% Ethanol with added 0.8% (w/v) NaCl (-20°C) compared with a cold methanol solvent	Best results were produced by the cold ethanol solution	Spura, <i>et al.</i> 2009
⁵ Comparison of four different quenching methods to test the efficiency of each method	Chinese hamster ovary cells (CHO) which are mammalian cells	Four different quenching methods were compared	Quenching in 60% methanol (-40°C) with 0.85% ammonium carbonate (AMBIC) gave a metabolite profile that is representative of a physiological status	Sellick, <i>et al.</i> 2009

1 This method is based on a quenching method that uses a cold glycerol-saline solution as the quenching agent; this reduces the leakage of intracellular metabolites throughout the quenching process (Villas-bo & Bruheim, 2007). To prove Sillias and co-workers findings, they compared three representative microbes' intracellular metabolite profiles. They quenched these samples with a cold glycerol-saline solution and also with a cold methanol-water solution which were the negative control. Sillias and coworkers compared four different quenching methods, these methods includes a glycerol water solution which was prepared with pure glycerol and bidistilled water, glycerol-saline solution which was prepared with pure glycerol and sodium chloride solution (13.5 g/L), a glycerol-mannitol solution which was compared with pure glycerol and aqueous mannitol solution (44 g/L) and the final solution was a methanol-water solution which was prepared with analytical-grade methanol and bidistilled water, all of these quenching solutions were prepared to a final ratio of 3:2 (v/v) (Villas-bo & Bruheim, 2007). They prepared the washing solutions in a very similar manner as the quenching solutions except for the final ratio which was 1:1 (v/v) for the washing solutions (Villas-bo & Bruheim, 2007). Sillias and co-workers concluded that the glycerol-saline solution is the best quenching solution for quenching microorganisms. The reasons for this conclusion is that this method is the only quenching method for microorganisms that offers such good recovery of intracellular metabolites and can further at the same time, eliminate the interference of extracellular compounds (Villas-bo & Bruheim, 2007).

2 In this study Fajjes and co-workers used the EC (energy charge) value to indicate proper halting of the metabolism (Fajjes, *et al.* 2007). There was only two quenching solutions that produced less than 10% cell leakage, they were, one that contained 70mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and the other one containing 0.85% (w/v) ammonium carbonate (pH5.5) (Fajjes, *et al.* 2007). The EC for the cells quenched by these 2 quenching solutions gave a high value indicating good quenching (Fajjes, *et al.* 2007). For metabolomic studies on *L. plantarum* it appears that the most appropriate quenching buffer is ammonium carbonate solution. This is because this ammonium carbonate solution gave minimum leakage of metabolites, a high EC value and all components of this quenching buffer is removed without any trouble during freeze-drying (Fajjes, *et al.* 2007).

3 This study is the first comparing different quenching methods for *E. coli* cells (Winder, *et al.* 2008). Winder and co-workers compared three quenching solutions which have never been tested for *E. coli* cells (Winder, *et al.* 2008). The supernatant was also analysed to monitor metabolite leakage during the quenching process (Winder, *et al.* 2008). Winder and co-workers found that there does occur leakage of metabolites in *E. coli* cells, this is more prominent during the hot ethanol quenching (Winder, *et al.* 2008). Including a buffer in the quenching solution does not have a positive effect throughout the methanol quenching of *E. coli* cells, the buffer system used in this study is based on tricine (Winder, *et al.* 2008). To conclude the findings of Winder and co-workers, it was found by them that the 60% methanol (-48 °C) gave the best recovery of metabolites (Winder, *et al.* 2008). They also advise that the footprint and supernatants must be monitored subsequent to quenching for a proper calculation of intracellular metabolites (Winder, *et al.* 2008).

4 In a study done by Spura *et al.* they optimised a new Quenching method for three different organisms (Spura, *et al.* 2009). The optimisation of the quenching process includes the concentration and type of alcoholic compound, the quantity of salt and the temperature during quenching (Spura, *et al.* 2009). Spura and co-workers states that these are the most vital parameters for the effectiveness of a quenching method (Spura, *et al.* 2009). They reduced the concentration of alcohol in order to prevent severe damage to the cell membrane before the extraction takes place (Spura, *et al.* 2009). The results were compared with two different methods (Spura, *et al.* 2009). One method served as a nonquenched basis, which is a standard method and the other method is one that is a very widely applied quenching method which uses cold methanol as the quenching solution (Spura, *et al.* 2009). The best results were produced by the cold ethanol solution and the results also emphasised that the method is applicable to different types of organisms and can further be applied for routine uses (Spura, *et al.* 2009).

5 They applied these quenching methods to Chinese hamster ovary cells (CHO) which are mammalian cells (Sellick, *et al.* 2009). They also tested whether the quenching process is important in sample preparation, which in this study is the sample preparation of the intracellular metabolites from suspension cultured mammalian cells (Sellick, *et al.* 2009). Sillias and coworkers measured a collection of labile metabolites and the results showed that quenching in 60% methanol (-40 °C) with 0.85% ammonium carbonate (AMBIC) gave a metabolite profile that is representative of a physiological status (Sellick, *et al.* 2009)

2.5 The aim of this study

The main aim of this study is to evaluate the use of cultured cells in metabolomics investigations.

To achieve these goals, the following objectives were formulated for this study:

- Determine the optimum composition of the quenching solution
- Determine the optimum time frame for quenching
- Determine the optimum quenching temperature
- Determine the repeatability of the quenching method
- Determine if a perturbation induced can be detected using metabolomics

Chapter 3: Materials and Methods

Introduction

A standard method was chosen for quenching of the metabolism, disrupting the cell membranes and extracting the metabolites. This method was chosen for a number of reasons. Firstly, it was applicable for the cell line used in this study, i.e. HeLa cells, which are adherent cells. The same cell line was used in the method which we have adapted from. The second reason is that this cell line is suitable to study the targeted metabolites selected for this study, namely the central carbon metabolism (Danielsson, *et al.* 2010). The targeted metabolites are a list of metabolites found in the central carbon metabolism as compiled by Luo et al (Luo, *et al.* 2007). This method will then be optimised for the quenching of HeLa cells.

3.1. Culturing of cells

3.1.1 Principle of method

The culturing conditions depend on the nature of a specific cell line, e.g. an adherent cell line or a suspension cell line. An adherent cell line was chosen for this study because it is an easier cell line to work with. The growth media is easily removed as the cells are attached to the surface which also cancels out the issue regarding metabolite leakage.

3.1.2 Materials used

- DMEM media (HyClone medium; Thermo Scientific)
- Penicillin /streptomycin (penstrep) (Lonza)
- Non-essential amino acids (NEAA) (Lonza)
- L-Glutamine (Lonza)
- Fetal bovine serum (Lonza)
- Trypsine (Lonza)
- PBS (Sigma)
- Trypan blue (Sigma)

3.1.3 Method

Cells were cultured in 75cm² flasks (Nunc) in DMEM medium containing 5.5ml NEAA, 2mM L-Glutamine, 10% FBS and 5.5ml penstrep at 37°C in a humidified atmosphere containing 5% CO₂. To prevent over growth, the cells were passaged at a 80 – 90% confluency, every second day. The medium was just changed when the confluency was not reached 80-90% to provide nutrients to enhance the growth of the cells. New medium was then added, usually 15ml per 75cm² flask, except when the cells required more.

The experiments were done in six well plates (Nunc) even though the cells were cultured in the flasks, as it was established that the metabolite profile is more repeatability when using the six well plates. Thus before an experiment was done, cells had to be passaged and then seeded into six well plates. We had to seed 300 000 cells in each well and let them grow for 48 hours to reach the optimum confluency (80%). In order to get 300 000 cells from the flasks, cells in the flask(s) had to be counted and the correct micro litres of media and cells had to be transferred in each well as was calculated. The counting of the cells was done according to the Trypan blue exclusion method: The principle of this method is that live cells have intact cell membranes and will exclude the dye and it will not enter the cells cytoplasm, but cells which do not have intact cell membranes (dead cells) will on the other hand not exclude the dye and will have a blue colour when seen under the microscope. The cells were counted with a hemocytometer. Cells are trypsinated, after which the contents of the flask (medium, cells and trypsin) was transferred to a 10ml tube. A mixture is prepared, for counting of cells, comprising of 15µl of PBS, 25µl trypan blue and 10µl cells. This mixture was then used for counting of the cells on the hemocytometer. Ten micro litres of this mixture was placed on each side of the hemocytometer. The hemocytometer was then placed under a light microscope. The cells were counted in 5 of the 9 blocks (the 4 corner blocks and the middle one) and this was done two times for each side of the hemocytometer to get a mean value. The number of cells per micro litre was calculated and the appropriate number of micro litres was transferred to each well.

3.2. Quenching of Cells

3.2.1 Principle of method

In this study 100% methanol (-80 °C) was used to quench the metabolism of the cells. The different reasons why methanol is such a good organic solvent to prepare quenching solutions with, is that it is soluble in water, has a low freezing point and aqueous-methanol solutions have a low viscosity (Álvarez-Sánchez & Priego-Capote, 2010). A more recent study done by Danielsson proved that for adherent cells metabolite leakage can actually facilitate the process of sample preparation, by linking quenching and extraction, because the isolation of cells as well as the rinsing is without difficulty performed prior to quenching (Danielsson, *et al.* 2010).

3.2.2 Materials used

- 100% methanol (-80 °C) (Merck)
- PBS (phosphate-buffered saline) (Sigma)

3.2.3 Method

The six well plates containing the cultured cells were placed on ice immediately after incubation and the growth medium removed from each well. The cells were then washed twice with 2ml of ice cold PBS. Then 1000µl of the 100% methanol (-80 °C) was added to each well.

3.3. Harvesting of cells

3.3.1 Principle of method

HeLa cells were used to standardise the method. For this study, cells were scraped from the bottom of the growth chamber as trypsination changes the metabolic profile. This enzyme (trypsin) markedly changes the physiological state of cells due to its interaction with membrane proteins (Teng, *et al.* 2008).

3.3.2 Method

The lid of the six well plate was removed partially so that only two wells were exposed at a time. The cells were scraped in the quenching solution (100% methanol) and transferred to a 2ml Eppendorf tube.

3.4 Disrupting the cell membranes

3.4.1 Principle of method

In order to isolate the intracellular metabolites from the cells, the cell membrane needs to be disrupted. In the paper of Danielsson three disrupting methods were compared (vibration mill, snap-freezing and direct extraction by vortexing for 5 minutes) at the following two quenching solvent concentrations respectively: 100% methanol and 82% aqueous methanol. They found that the vibration mill with the 82% methanol gave the best results (Danielsson, *et al.* 2010). This method was tested and it was found that for our cell line (HeLa cells) the best results were gained if the cells were disrupted with the vibration mill in the quenching solution (100% methanol).

3.4.2 Materials used

- Glass beads (Retsch)

3.4.3 Method

The glass beads were placed in the Eppendorf tubes containing the sample and were then vibrated in the vibration mill (Retsch® MM400) for 5 minutes at 30Hz.

3.5 Extracting the intracellular metabolites

3.5.1 Principle of method

The extraction method depends on the characteristics of the targeted metabolites. Danielsson and co-workers found in their study that with 82% aqueous methanol extracting solution a broader range of metabolic pathways are covered, which include glycolysis, amino acids, and TCA cycle intermediates (Danielsson, *et al.* 2010). This is because most of the polar metabolites have a negative charge thus with an increase in methanol concentration their yield will decrease. On the other hand, levels of sugar phosphates decreased with increasing water, the reason for

this can be that with increasing water content, as a result of the modulation of the pH with the fraction of the organic solvent, the hydrolysis of sugar phosphates will increase during evaporation

3.5.2 Materials used

- 100% methanol (Merck)
- Milli Q water (Millipore)

3.5.3 Method

The sample consists of the cells and the 1000 μ l quenching solution. The extraction was done by adding 640 μ l 100% methanol plus 360 μ l milli Q water to get a final volume of 2000 μ l, thus a 82% aqueous methanol solution. The extracts were then placed on ice for 20 minutes. The samples were then centrifuged for 20 minutes at 17400 x g where after the supernatants were transferred to an Eppendorf tube and placed in a Savant ISS110 Speedvac Concentrator (Thermo Electron Corporation) to evaporate the methanol under vacuum. The remaining water in the Eppendorf tubes were dried under vacuum and kept in the -80 $^{\circ}$ C freezer until used. The pellets were used for protein concentration determination as described in section 3.7.

3.6 LC-MS metabolite analysis of the supernatants

3.6.1 Principle of method

For the metabolomics part of the study, it was decided to use LC-MS/MS with a semi-targeted metabolomics approach. The metabolites that were targeted were representative of the central carbon metabolism, which includes glycolysis, the pentose-phosphate-pathway, tricarboxylic acid cycle (TCA) and cofactors. A list of metabolites compiled by Luo, et al (2007) (See Table 3.2.) was selected for the targeted analysis.

3.6.2 Materials used

- 1% Acetic acid (Sigma Aldrich)
- 100% Acetonitrile (ACN)

3.6.3 The LC-MS/MS method

After the internal metabolites were extracted with the water and methanol two- phase extraction (see section 3.2.2), the metabolites were analyzed by using the LC-MS/MS method. Samples that were dried under vacuum were redissolved in 50µl 1% acetic acid in water. High-performance liquid chromatography was performed on an Agilent 1200 series LC equipped with a C18-Aqua column (150mm x 2.10mm, particle size 5µm) from Agilent (Santa Clara, CA, USA). Mobile phase A consisted of 1% acetic acid in water (H₂O) and mobile phase B of 100% acetonitrile (ACN). The column temperature was maintained at 25 °C and the flow rate at 0.2ml/min. The samples (10µl) were injected and the mobile phase composition was changed from 0% of B to 100% of B over 15min, after which the percentage of B was maintained at 100% for 3min. The percentage of B was changed back to 0% over 4min and the column re-equilibrated for 6min. The MS/MS analysis was performed on an Agilent 6410 Triple Quad (Santa Clara, CA, USA) in negative electro-spray ionisation (-ESI). MS conditions were optimised with the MassHunter optimiser software from Agilent in multiple reaction monitoring (MRM) mode, with the transitions being monitored that are listed in table 3.1. Data analysis of the extracted MRM abundances was performed with the Agilent MassHunter Qualitative analysis software.

Table 3.1: Selected metabolites and their optimised fragmentation parameters for the Agilent 6410 QQQ operated in negative ionisation electrospray.

Compound	[M-H]-	Main prod ion	Fragmentor (V)	Collision Energy (V)	
Sugar phosphates					
Dihydroxy-acetone-phosphate	169.0	97	-[H ₂ PO ₄]-	80	30
Glyceraldehyde-3-phosphate	169.0	97	-[H ₂ PO ₄]-	80	30
Glycerate 2-phosphate	185.0	79	-[PO ₃]-	80	40
3-Phosphoglycerate	185.0	79	-[PO ₃]-	80	40
Erythrose-4-phosphate	199.0	97	-[H ₂ PO ₄]-	80	4
Ribose-5-phosphate	229.0	97	-[H ₂ PO ₄]-	85	4
Ribulose-5-phosphate	229.0	97	-[H ₂ PO ₄]-	85	4
Xylulose-5-phosphate	229.0	97	-[H ₂ PO ₄]-	86	4
Fructose-6-phosphate	259.0	97	-[H ₂ PO ₄]-	96	8
Glucose-6-phosphate	259.0	97	-[H ₂ PO ₄]-	180	8
6-Phosphogluconate	275.0	79	-[PO ₃]-	107	50
Fructose-1,6-diphosphate	339.0	97	-[H ₂ PO ₄]-	127	16
Organic acids					
Pyruvate	87.0	43	-CO ₂	65	4
Fumarate	115.0	71	-CO ₂	55	4
Succinate	117.0	73	-CO ₂	65	8
Oxaloacetate	131.0	87	-CO ₂	175	8
Malate	133.0	115	-H ₂ O	70	4
2-Oxoglutarate	145.0	101	-CO ₂	65	4
Phosphoenol pyruvate	167.0	79	-[PO ₃]-	75	8
<i>Cis-aconitate</i>	173.0	85	-2CO ₂	65	4
Citrate	191.0	87	-	85	12
Isocitrate	191.0	73	-	85	12
Coenzyme					
Acetyl-coenzyme A	808.3	79	-[PO ₃]-	154	36
Redox cofactors					
Nicotinamide adenine dinucleotide	662.3	540	Nicotinamide	120	25
Reduced nicotinamide adenine dinucleotide	664.3	79	[PO ₃]-	120	25
Nicotinamide adenine dinucleotide phosphate	742.2	620	-Nicotinamide	120	30
Reduced nicotinamide adenine dinucleotide phosphate	744.3	79	[PO ₃]-	120	30

Luo, B., Groenke, K., Takors, R., Wandrey, C. and Oldiges, M. 2007. Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by liquid chromatography–mass spectrometry. *Journal of Chromatography A*, 1147: 153-164.

3.7 Protein content of the cell pellet

3.7.1 Principle of method

The pellet was used for the determination of protein concentration, because a sufficient protein precipitation was obtained with the selected concentration of methanol in the extracting solution. The pellet was resuspended in 67 μ l PBS and vortexed for 20 seconds. The protein concentration was determined with BCATM protein assay kit from Thermo Scientific. The product of this assay is a purple-coloured reaction and this product is formed by the chelation of two molecules of BCA with one cuprous ion. A strong absorbance at 562nm is exhibited by the water-soluble complex and this is almost linear with rising protein concentrations over a broad range, 20-2000 μ g/ml. This method does not have a true end-point thus the colour continues to develop. Nevertheless after incubating the sample, the rate of colour development is adequately slow. There are a number of things responsible for the colour formation with BCA: the macromolecular structure of protein, the amount of peptide bonds present and the presence of four specific amino acids, namely cysteine, cystine, tryptophan and tyrosine. Protein concentrations are usually determined with reference to standards of a common protein, for example bovine serum albumin (BSA).

3.7.2 Materials used

- Bicinchoninic acid (BCA) (Thermo Scientific).
- Cuprous sulphate (Thermo Scientific).
- Albumin Standard Ampules, 2mg/ml containing bovine serum albumin (BSA) at 2.0mg/ml (Thermo Scientific)
- PBS (Sigma)

3.7.3 Method

The pellet was resuspended in 67 μ l of PBS, this was used for the protein concentration determination so that the metabolite abundance can be quantified with reference to the amount of protein in each well. The protein assay was done in a 96 well plate (Corning Black) and a reaction mixture was prepared according to the

number of wells used. This mixture contains the cuprous sulphate (CuSO_4) and the BCA in the following ratio: $4\mu\text{l}$ CuSO_4 and $196\mu\text{l}$ BCA for each well. Thus the number of wells used multiplied with the amount of CuSO_4 and BCA needed for each well gives the final volume of the mixture. Of this mixture, $200\mu\text{l}$ was added to standard and sample wells. For the standard series BSA was used in this study. The standard BSA dilution series was prepared in the 96-well plate with the final concentration of $2\mu\text{g}/\mu\text{l}$ (2 mg/ml). The series are as follows, well A1-A6, 0, 4, 8, 12, 16, and $20\mu\text{g}/\mu\text{l}$. Ten μl of sample was added to each well. Each well was then filled up with PBS to a final volume of $20\mu\text{l}$. The mixture was then added to each well. All samples were analyzed in duplicate. The reaction takes 30 – 45 minutes but is faster when it is incubated at 37°C . Following incubation, the protein concentration was determined with a BioTEC plate reader (Synergy HT)

3.8 Variations on the quenching method

3.8.1 The principle of method

The variations on the quenching method were done in order to standardise a quenching method for the cell line used in this study (HeLa cells). This is important because quenching is cell line specific (Sellick, *et al.* 2009). The quenching solution composition and volume were changed as well as the quenching temperature.

3.8.2 Materials used

- AMBIC (Ammonium bi-carbonate) (AnalaR)
- Methanol (100%) (Merck)
- Milli Q H_2O (Millipore)

3.8.3 Method

The standard method was followed for the culturing, harvesting and the disruption of the cells. The only variation was for the quenching step. Three different quenching methods were compared. The first method was 100% methanol at -80°C . The second method was 100% methanol at room temperature and the third method was 100% methanol with added AMBIC (0.85%). These quenching solutions were added

to the six well plates as previously described (Section 3.2). The quenching volume remained the same.

3.9 Optimum time frame for extraction (waiting periods)

3.9.1 The principle of the method

To establish whether the enzymes catalysing the reactions of the metabolism under study was effectively quenched, the respective experimental groups (named 0h, 3h, 6h en 24h) were exposed to different waiting periods (0, 3, 6 en 24 hours). Quenching was done immediately but the different experimental groups were exposed to the respective waiting periods before the extraction of metabolites was performed. This was done to detect if the metabolite profile changes dramatically with time even though immediate quenching was performed on the cells.

3.9.2 Method

The cells were cultured under standard conditions discussed previously (Section 3.1). The sample preparation was also done as discussed previously (Section 3.2 - 3.5) except for the extraction that was only done after the respective waiting periods. There were four different experimental groups namely 0h (control), 3h, 6h and 24h. Thus after immediate quenching the respective groups was exposed to the different waiting periods. For the control (0h) extraction was done immediately after quenching was performed, the 3h group was exposed to a 3hour waiting period before extraction. The 6h group was exposed to a 6 hour waiting period and the 24h group was exposed to a 24 hour waiting period before extraction was done. During the waiting period the samples were stored at room temperature in the quenching solution. After extraction metabolite analysis was done as discussed in paragraph 3.6.

3.10 Effect of different perturbations

3.10.1 The principle of the method

The HeLa cells were exposed to two perturbations, one was a genetic change and the other one was an environmental change. This was done to detect changes in the metabolism of the HeLa cells when they are exposed to different perturbations.

The first perturbation was to compare the energy metabolism of a transfected HeLa cell line namely HeLa-Tts (TTS) with a normal HeLa cell line (N). This was done to establish what the effect of a genetic change is on the energy metabolism of these cells. When a cell line is transformed, the cellular characteristics is often altered, which is visible in the production of unpredicted proteins. This can be ascribed to silencing of certain genes and activating of others (Esteller, 2008). In these experiments, the transfected cells contain the pTTS-Neo vector. Transfection of the cells was performed by Etesia van Dyk from our laboratory for another study (Van Dyk, 2005). The second perturbation was a heat shock to detect changes in the energy metabolism of HeLa cells when there is a change in the environment. Previous studies states that the effect of elevated temperatures (42°C) causes a certain group of genes to be expressed, namely heat shock protein genes (Abravanya, *et. al.* 1991). The expression of these genes causes several proteins to be synthesised, these proteins are termed heat shock proteins (hsp) in eukaryotic cells and prokaryotic cells. The function of these proteins is largely unknown (Kumar, *et. al.* 1995). A previous study revealed that disruption of the energy metabolism is one of the early and possibly critical subcellular events in the response of the cells to hyperthermia (Lunec & Cresswell, 1983). The study done by Lunec and coworkers also presented evidence that respiration is depressed by hyperthermia as the oxygen consumption decreases and this is a general phenomenon but is more marked in tumour cells.

3.10.2 Materials

- HeLa-tTS cells (Clontech, cat. # 630928)

3.10.3 Method

For the first perturbation the standard method was used for both samples one containing normal HeLa cells(N) and the other containing transfected HeLa cells namely HeLa-tTS cells (TTS). The results were compared to detect changes in the energy metabolism of the two different cell lines and to establish what the effect of a genetic change is on the energy metabolism. For the second perturbation (heat shock) cells were exposed to an elevated temperature (42 °C) by placing the six well

plates in a water bath at a controlled temperature of 42°C for an hour. One of the samples was immediately quenched and metabolites extracted as described in the standard method. The other sample was placed in an incubator (37°C) to recover for an hour where after the standard method was followed for quenching and extraction of metabolites. The control sample was not exposed to the heat shock.

3.11 MTT assay

3.11.1 Principle of method

The MTT assay is a colorimetric assay that is used to measure viability, proliferation and activation of cells. The principle of the MTT assay is that in live cells the metabolically active mitochondrial dehydrogenase enzymes will convert the yellow water soluble substrate (MTT) to form deep purple coloured formazan salts that is insoluble in water. When the insoluble crystals have been dissolved, the concentration of the formazan product can be colorimetrically quantified.

3.11.2 Reagents and chemicals

- MTT (4,5-dimethylthiazol-2-y1]-5-diphenyltetrazolium bromide) (Sigma)
- PBS (Sigma)
- DMSO (dimethyl sulfoxide) (Merck)
- 100% Acetic Acid (Sigma Aldrich)

3.11.3 Method

HeLa cells were seeded in a 96 well plate (Corning Black). Twenty five thousand cells were seeded in each well. Cells were seeded in two separate plates, one plate served as the positive and negative control and the other one was used for the heat shock as described in section 3.9. For the positive control, cells were exposed to 100% acetic acid. A gradient exposure was done so that the first positive control received 3µl, the second 6µl and the third 12µl of acetic acid for 30 minutes. Following the heat shock and the exposure to acetic acid, cells were washed twice with PBS to remove the serum and medium. The MTT-solution was then added to

each well (20µl of a 5mg/ml solution) and the plates were incubated at 37°C for 5 hours to allow the formation of the formazan crystals. Following the incubation period, 200µl of DMSO was added to dissolve the crystals. Optical density was measured with a BioTEC plate reader (Synergy HT) at a wavelength of 560nm. The background absorption was measured at 630nm and subtracted automatically by the plate reader.

3.12 Statistical Analysis

3.12.1 Method

The targeted LC-MS/MS data was extracted with Agilent's MassHunter. The metabolite levels between samples were normalized using the MSTUS (mass spectrometry total useful signal) normalisation method from Warrack *et al.* (2009). Missing value imputation was done after data normalization. Missing values were replaced with the group mean (nearest neighbour imputation) and the data log transformed before statistical analysis to make it more normally distributed. PCA was done to assess and visualise the overall variance in the metabolic profiles of the experimental groups. ANOVA was done to find metabolites that differed significantly between the experimental groups. Pearson's correlation analysis was done to assess change of metabolite levels over time.

Chapter 4: Results and Discussion

Introduction

This chapter contains a description and discussion of the results obtained from all the experiments done. Briefly, the first experiments were done to determine the best quenching method for the HeLa cell line and to determine the best time for extraction of the metabolites after immediate quenching of the culture. There were four different groups, namely 0, 3, 6, and 24 hour groups each representing the respective waiting period that the cultured cells were exposed to. Each group was quenched immediately, but extraction was only done after the respective waiting period (zero hours, which was the control group, three, six and 24 hours). Results obtained from this experiment are presented in a PCA score plot which assessed the overall variance in the metabolic profiles of the experimental groups, a correlation analysis between metabolite levels and the waiting period to indicate if there were metabolites that degrade (decrease) or form (increase) over time. The quenching solution was then investigated, i.e. the composition of the quenching solution for a specific number of cells. Different quenching solutions were compared for a specific number of cells of the same batch of HeLa cells. To represent the results obtained from this experiment a PCA analysis was used to visualize the variation in the replicates (Figure 4.2.1). The coefficient of variance (CV) for each metabolite was also determined and the sum of the distribution of the CVs plotted for each quenching method. This was done to assist the PCA results (Figure 4.2.2) because PCA is just a visualisation tool and for that reason does not allocate values to the variance. Finally, experiments were done to determine the effect of two different perturbations on the metabolite profile of the cultured cells. The first perturbation entailed the transfection of the cells with an expression vector. This perturbation was done to determine whether a change in the genetics of a cell culture would have an effect on the metabolic profile. The comparison of the metabolic profiles of normal HeLa cells (HeLa N) and transfected HeLa cells (HeLa- Tts) revealed a definite difference in the metabolic profiles. The second perturbation was a heat shock experiment. Cells were heat shocked at 42 °C for an hour. One group was placed back into the 37°C incubator to recover (HSH h group) for an hour and another group was quenched immediately after the heat shock (HS group) (Chapter

3, Section 3.8). The PCA score plot show a definite difference between the control (0h) and heat shock groups while no difference was observed between the two different heat shock groups.

4.1 Determination of the optimum time (waiting periods) for quenching

Metabolite extraction was performed 0, 3, 6 and 24 hours after quenching to determine if the metabolic profile of the cells changes over time after quenching of the metabolism.

The metabolite levels between samples were normalised using the MSTUS (mass spectrometry total useful signal) normalisation method (Warrack, *et al.* 2009). Missing value imputation was done after data normalisation. Missing values were replaced with the group mean (nearest neighbour imputation) and the data log transformed before statistical analysis. PCA was done to assess the overall variance in the metabolic profiles of the experimental groups and also the changes. ANOVA was done to find metabolites that differed significantly between the experimental groups. Correlation analysis was also performed to assist the ANOVA. Correlation between metabolite levels and the waiting period was done to find metabolites that degrade (decrease) or form (increase) over time.

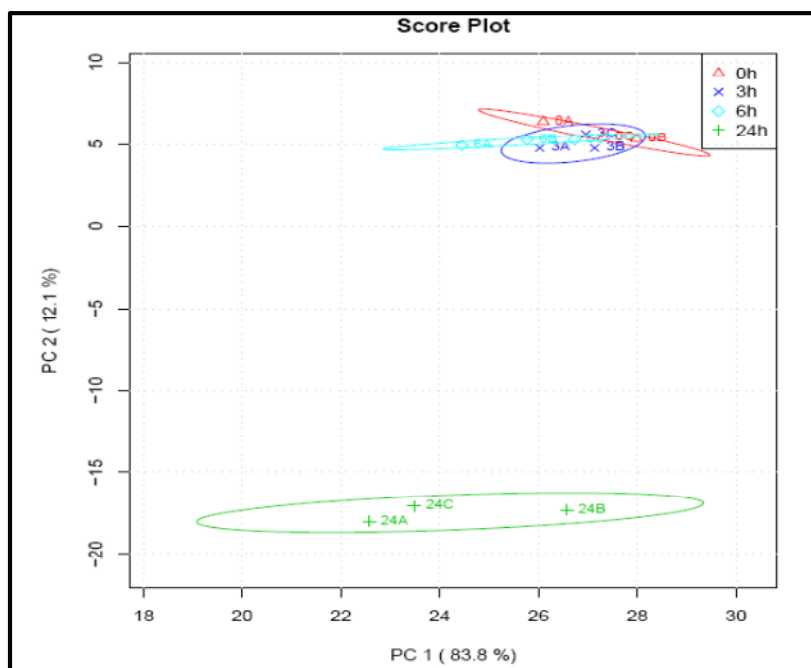


Figure 4.1.1: PCA score plot of the log transformed metabolic data from the 0, 3, 6 and 24 hour waiting groups. The colours represent the groups as follow: Red, 0h hour group which is the control group, thus extraction was done immediately after quenching; blue, the 3 hour group; turquoise, 6 hour; and green, 24 hour group.

It is evident from Figure 4.1.1 (PCA score plot) that the 0, 3 and 6 hour waiting period groups, which were extracted after the respective waiting period after quenching, grouped together while the scores of the 24 hour group clustered separately. Regarding the intra-group variance, it is well visualised by the confidence ellipse, which is very similar between the 0, 3 and 6 hour waiting period groups. The variance in the 24 hour waiting period group is without doubt larger. This separate grouping of the 24 hour period group and also the higher intra-group variance, imply a definite change waiting in the metabolic profile of this group.

In order to indicate which metabolites differed between the groups and also those that were responsible for the separate grouping of the 0, 3 and 6 and the 24 hour waiting period groups, the ANOVA test was done. The metabolites listed in Table 4.1.1 were identified by ANOVA to differ significantly between the waiting period groups. Fisher's LSD post-hoc indicates between which groups the respective metabolite differ significantly. The results gained by the ANOVA and post-hoc analysis correlated with the PCA results as the respective metabolites differed considerably between the 0, 3 & 6 hour waiting groups and 24 hour waiting group.

Only two metabolites differed significantly between the other groups. X5P was markedly different between the 3 and 6 hour waiting groups. PEP was markedly different between the 0 and 6 hour waiting groups.

Table 4.1.1: Important metabolites identified by ANOVA and post-hoc analysis.

	p-value¹	Fisher's LSD
6PG	0.000000000688	0h – 24h; 3h – 24h; 6h – 24h
NAD	0.000000000794	0h – 24h; 3h – 24h; 6h – 24h
X5P	0.00000000806	24h – 0h; 24h – 3h; 24h – 6h; 6h – 3h
PEP	0.000000435	0h - 24h; 0h - 6h; 3h - 24h; 6h - 24h
MAL	0.00000299	0h - 24h; 3h - 24h; 6h - 24h
CIT	0.00000431	0h - 24h; 3h - 24h; 6h - 24h
3PG/2PG	0.0000236	0h - 24h; 3h - 24h; 6h - 24h
FBP	0.000199	0h - 24h; 3h - 24h; 6h - 24h
R5P	0.000645	0h - 24h; 3h - 24h; 6h - 24h
NADP	0.002009	24h - 0h; 24h - 3h; 24h - 6h
ISOCIT	0.009909	24h - 0h; 6h - 0h; 24h - 3h
DHAP/GAP	0.01298	24h - 0h; 24h - 3h; 24h - 6h
F6P/G6P	0.040696	24h - 0h; 24h - 3h

¹ the p-value indicates statistical differences between metabolites of the respective groups. A $p > 0.05$ indicates metabolites of no statistical relevance and a $p < 0.05$ indicates that these metabolites do differ statistically between the two groups.

To establish which of the respective metabolites changed considerably over time, a correlation analysis was done. Figure 4.1.2 is a representation of the results obtained by the correlation analysis. The pink bars indicate positive correlation and the blue bars indicate negative correlation. The changes in respective metabolite levels did not show good correlation to the elapsed time. Hence, the differences seen in the metabolite levels cannot be (predominantly) linked to degradation over time. In fact, of the 23 metabolites tested, 11 gave a positive correlation between metabolite concentration and time which indicate an increase of certain metabolites during the waiting period. An example of this pattern is shown in Figure 4.1.3 A for NADP. These results were against expectations since a decrease in metabolite levels were expected during the waiting period. If the enzymes were not completely quenched, there would be an increase in specific metabolite levels and a decrease in

others. This depends on where the specific metabolite is situated in the metabolic pathway. If the substrate levels decrease and the product levels increase then the enzyme catalysing those reactions were not effectively quenched. An increase in specific metabolites during the 24 hour waiting period would thus signify that enzymatic activity was not fully quenched during this time.

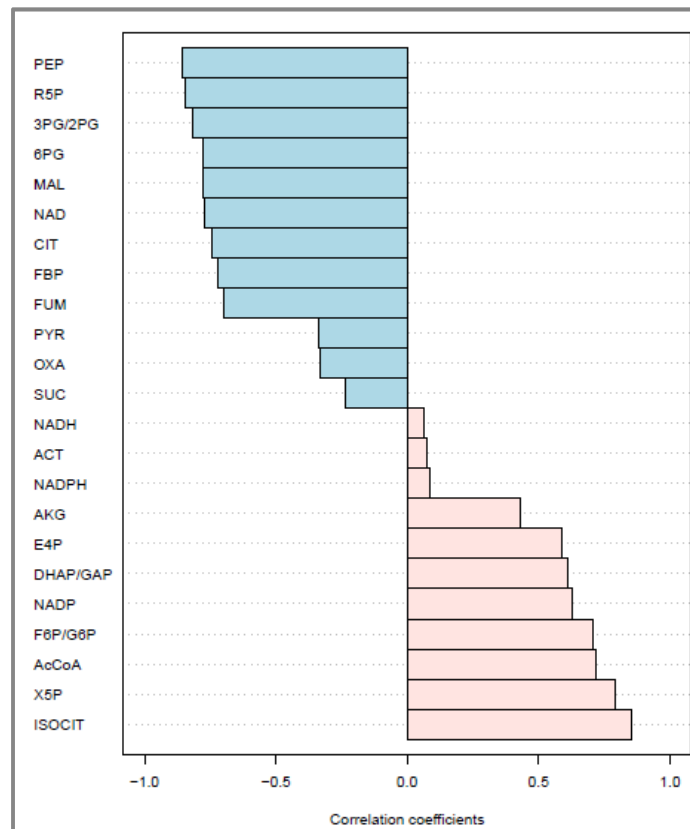


Figure 4.1.2: Correlation with the waiting periods of selected metabolites. Pink indicates metabolites with positive correlation and light blue metabolites with negative correlation. Metabolite abundance correlated with increasing time (zero, three, six, 24 hours).

The following figures give an indication of which enzymes were not efficiently quenched; these line graphs compare the relative concentration of the metabolites over the respective time period. The line graphs thus show how the products of specific reactions increase with time while the substrate decreases with time; this is an indication that the enzymes catalysing these reactions were not effectively quenched.

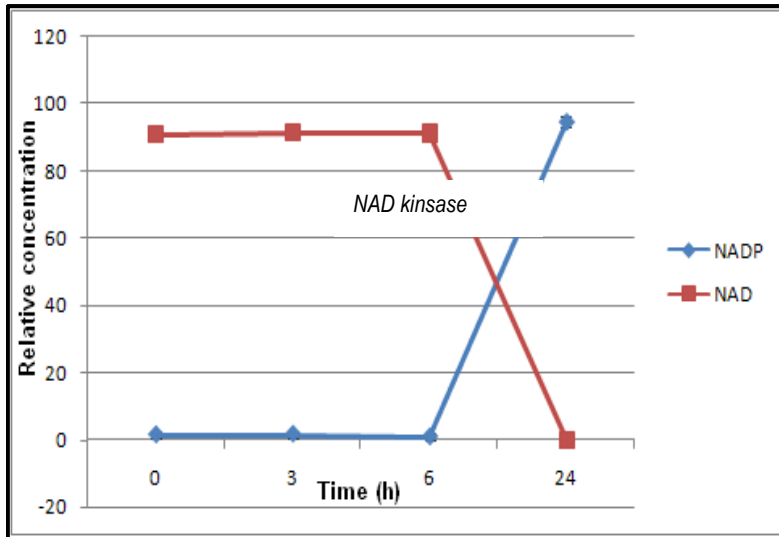


Figure 4.1.3 A

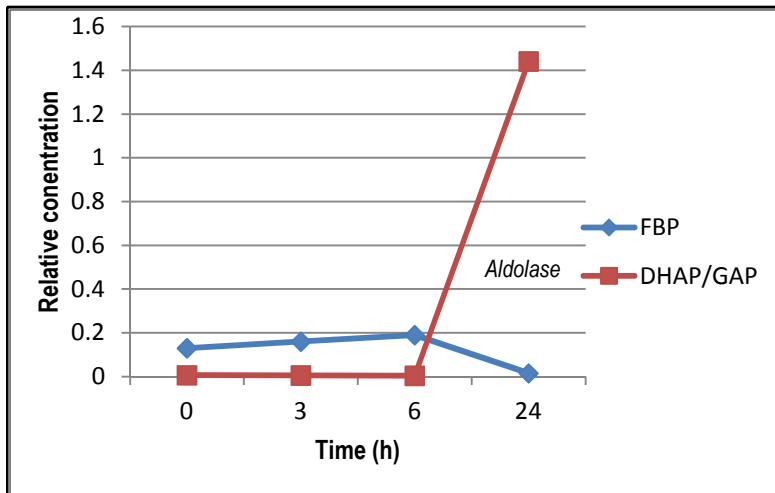


Figure 4.1.3 B

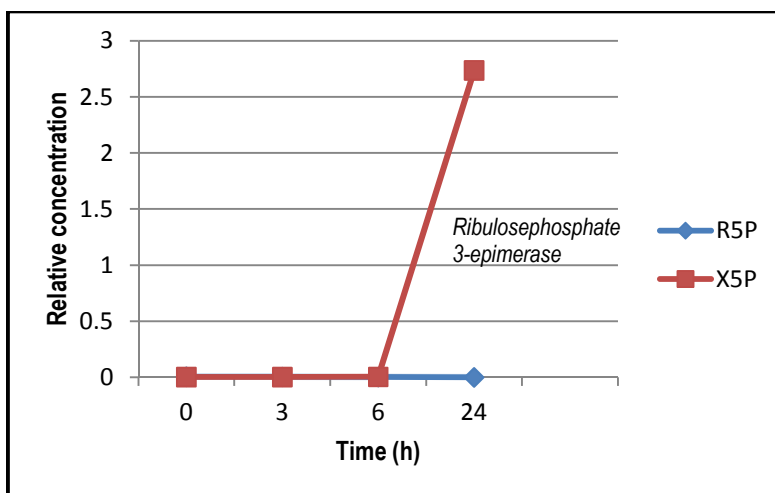


Figure 4.1.3 C

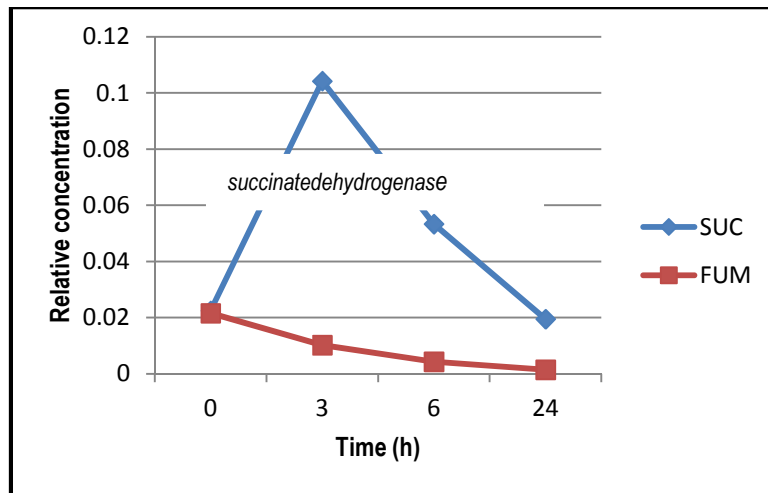


Figure 4.1.3 D

Figure 4.1.3

A: Line chart showing the changes in NADP and NAD concentration over time catalysed by the enzyme NAD kinase.

B: Changes in the concentration of FBP and DHAP/GAP over time. It represents the reaction Fructose1,6-biphosphate (FBP) ↔ Dihydroxyacetonephosphate (DHAP) and Fructose1,6-biphosphate (FBP) ↔ glyceraldehydes-3-phosphate (GAP) catalysed by the enzyme Aldolase.

C: The concentration of R5P (Ribulose5-phosphate) and X5P (xylulose5-phosphate) change over time. It represents the reaction R5P ↔ X5P catalysed by the enzyme Ribulosephosphate-3-epimerase.

D: Line chart showing the change in concentration of SUC (succinate) and FUM (fumarate) over time. It represents the reaction SUC ↔ FUM catalysed by the enzyme succinate dehydrogenase. Each point in figures (4.1.3 A – D) represents group mean ± standard deviation. Error bars are not visible in figures due to scale.

Figures 4.1.3 A – 4.1.3 D represent four different reactions of the energy metabolism where substrate, product and enzyme are present in each figure. These figures show how the substrate of each reaction is used to form the product catalysed by the respective enzyme. The Y-axis of each figure represents relative concentration and the X-axis represent a time period of zero to 24 hours. From these figures it would thus be clear if the product and substrate concentrations of each reaction increased or decreased with time.

It is evident from figures 4.1.3 B and 4.1.3 C that these enzymes were not effectively quenched since the substrate of each reaction decreased over time and the product increased over time. Thus the reaction did not stop completely after quenching. Figure 4.1.3 D show the possibility that the enzyme that is responsible for making

succinate, namely succinyl CoA synthetase, was still active for some time before degeneration started for both the enzymes involved (succinate dehydrogenase and succinyl CoA synthetase).

Because of the fast turnover rate of most of the metabolites in the energy metabolism, especially glycolysis, it seems to be a difficult task to quench the metabolism completely. Of the 23 reactions measured 12 were effectively quenched and 11 were not effectively quenched. When the metabolites that was identified by ANOVA and post-Hoc analysis to be important (Table 4.1.1) is taken in consideration, then most of them in this table is metabolites of the glycolysis pathway. Ideally, the whole metabolism must be quenched so that the metabolites extracted from the cell culture resemble the true metabolite profile of the specific cells under study, in that particular moment of quenching, so that metabolomics tools can reveal the true changes if a change was induced. Of paramount importance is the nature and composition of the quenching solution used for a specific number of cells. The next section will discuss this important factor.

4.2 Determine the composition of quenching solution for a specific number of cells (variation of quenching)

With the purpose of establishing which quenching method give the most consistent results, three different quenching methods described in the literature (refer to Chapter 2 Section 2.4.4), were compared. They are: 100% methanol at -80°C , 100% methanol with a 0.85% AMBIC at -80°C and 100% methanol at room temperature (Chapter 3 Section 3.8 variation on the quenching method). The experiments were done with HeLa cells. Statistical analysis was done to examine the variation between the replicate samples, so that the method that produced less variation (and thus higher repeatability) could be identified.

PCA was used to visualize the variation in the replicates (Figure 4.2.1). The CV for each metabolite was determined and the sum of the distribution of the CVs plotted for each quenching method to assist the PCA results (Figure 4.2.2). The same normalisation, missing value imputation and data pre-treatment methods were used

as described above (Paragraph 4.1). The metabolite CVs were calculated from the normalised data.

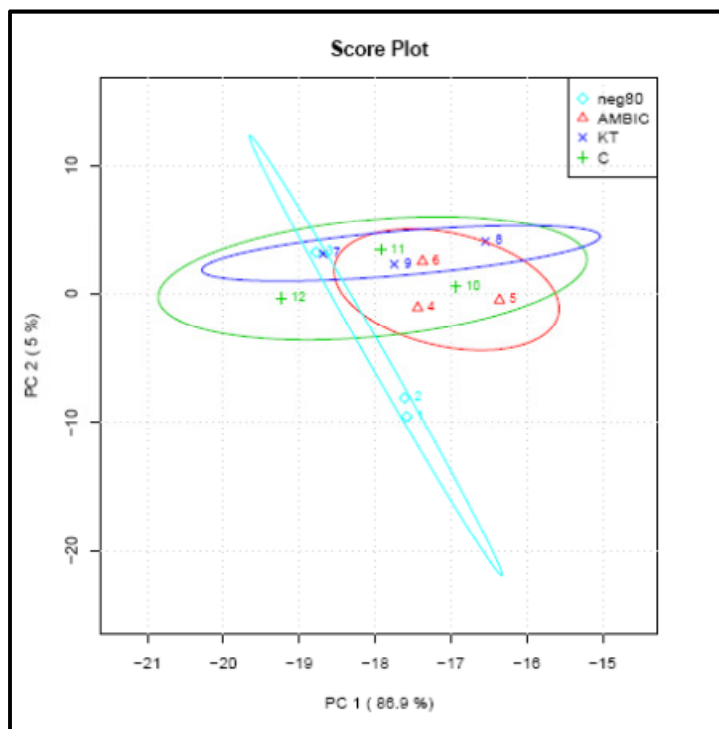


Figure 4.2.1: PCA score plot of the log transformed metabolic data from the different quenching methods. Turquoise, neg80 (100% methanol at -80°C); red, AMBIC (100% methanol with a 0.85% AMBIC at -80°C); blue, KT (100% methanol at room temperature); and green, C (control group, which was the standard method, also 100% methanol at -80°C).

The PCA score plot (Figure 4.2.1) of the different quenching methods indicates that the metabolic profiles obtained from the respective methods is very similar as there is no clear separate grouping for the different methods. The 95% confidence ellipse shows the intra group variance between the groups. According to the multivariate analysis the least variation was seen in the AMBIC group and the second least in the KT group. The repeatability of these two groups was also better when compared to the -80°C and C groups, respectively. PCA was used to investigate the overall variance in the replicates when the different quenching methods were compared. However, this does not allocate values to the variance as it is merely a visualization tool. For this reason it was seen fit to determine the distribution of the metabolite CVs. Figure 4.2.2 shows the sum of the CV distribution of the different quenching methods.

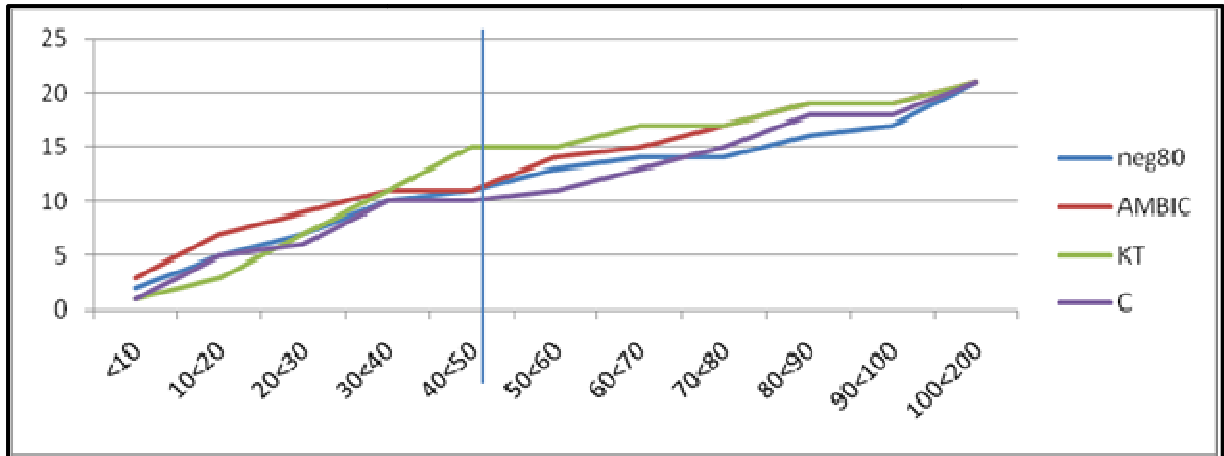


Figure 4.2.2: Sum of the distribution of the metabolite CVs. The 50% cut-off is indicated by the vertical line.

The X-axis represents the CV range and the Y-axis the number of metabolites with 21 being the total. The chart lines therefore indicate the total sum of detected metabolites with CV smaller than X. A rapid increase in a chart line would thus indicate a more precise method. The vertical line in the chart indicate the CV = 50% cut-off line which is often used to indicate precision. Metabolites with CV < 50% were precisely measured while those with CV > 50 are considered unstable and unreliable.

The line representing the AMBIC quenching method (refer back to Chapter 3 Section 3.8) increased quickly which indicate this method had more metabolites (of the 21 detected) with CV in the low range. More than half (52%) of the detected metabolites had a CV < 50% which would indicate this method to be more precise than the other methods. Nevertheless, 71% of the metabolites in the KT method (refer to Chapter 3 Section 3.8) had a CV below this 50% cut-off and therefore seems to be the more precise method. The control method performed worst, as less than half (48%) of the detected metabolites had a CV < 50%. The lines representing the different methods are all very close to each other, the explanation for this is that these methods have more or less the same repeatability on this small scale of 21 metabolites detected in the central carbon metabolism.

It can be concluded from the previous two experiments that quenching should in fact be very rapid as it should be faster than the most rapid reaction in the metabolism under study. Quenching in this case was still not 100% even though the rule of thumb for quenching was followed, that is, that it should be less than a second if possible (refer to Chapter 2 Section 2.4.1). Most of the reactions in the glycolysis pathway have turnover times of millimoles per litre per second (Winder, *et al.* 2008). Thus quenching should be even more rapid when this pathway is studied. The quenching method was, however, still able to completely quench the major part of the metabolism. The quenching method with the best repeatability, according to the CVs of the metabolites of the different methods, was the KT method (100% methanol at room temperature). The PCA, however, indicates that the method with the less intra-group variance is the AMBIC method (100% methanol with a 0.85% AMBIC at –80°C). Therefore it is debatable if the KT method is the best quenching method. The reason for the different results can be because of the temperature differences of these two methods. None of the quenching methods did, however, change the metabolism of the HeLa cells since the metabolic profiles for each method was very similar. This is a vital requirement for quenching (refer to Chapter 2 Section 2.4.1).

Another important influencing factor to mention is the effect of methanol on the enzymatic activity. Organic solutions are known to lower (or even inactivate) the activity of numerous enzymes, hence its use as quenching agents (Doukyu and Ogino, 2010). The reasons for this low activity (or inactivation) of the enzymes can be divided into three groups: first the water molecules present in the enzymes are removed or replaced with solvent molecules and cause the deformation and denaturation of the enzyme structure. The organic molecules can bind to specific sites on the enzymes (such as the catalytic site) which will cause the enzyme to lose its activity. In two-phase systems the interfacial or surface tension of solvents can destroy the tertiary structure of enzymes. This is not the only effect that organic solutions have on enzyme activity. In organic solutions enzymes can catalyse a wide range of reactions that is not possible in aqueous media, they change the thermodynamic equilibria in such a way that it favours synthesis over hydrolysis, they cause the suppression of water-dependent side reactions and they alter the substrate-, region- and stereo-specificity of the enzymes (Ogino & Ishikawa, 2001).

There is a necessity for an enzyme to be active in organic solvents and this is that a minute amount of water is vital for catalytic activity of the enzymes. Reason being, as mentioned earlier when the water is completely removed the conformation of the enzyme is severely distorted and the enzyme is inactivated (Zhu, *et al.* 2011). This could also have influenced the results as there is a wash step before quenching and a small amount of water could still be present when the methanol was introduced during the quenching step. This can help to explain why the reactions were not completely quenched and why certain metabolite levels increased after the respective waiting periods. The organic solvent, in this case methanol, could have incorporated the changes (mentioned earlier in this paragraph) into the enzymes so that it was still active but with varied catalytic activity as when in an aqueous media.

4.3 Effect of different perturbations on the metabolite profile of cultured cells

4.3.1 Effect of transfection on metabolite profiles

This perturbation was chosen to investigate the effect that genetic changes will have on the energy metabolism of cultured HeLa cells. Another important factor under investigation is the question: if there is a change in the energy metabolism, can it be seen using metabolomics as a tool? The standard method was used for both samples, one containing normal HeLa cells (N) and the other containing transfected HeLa cells namely HeLa-tTS cells (TTS). Transfected cells contains a vector namely, vector ptTS-Neo vector. HeLa-tTS cells were purchased from Clontech (cat. # 630928). Transfection of the cells was performed by Etesia van Dyk from our laboratory for another study (Van Dyk, 205).

Differences in metabolite profiles were examined with PCA. The same procedures were used as described in the previous section. Student's t-test was used to identify metabolites that differed significantly between the experimental groups (N and TTS). The log transformed data was used for this test to ensure a more normal distribution.

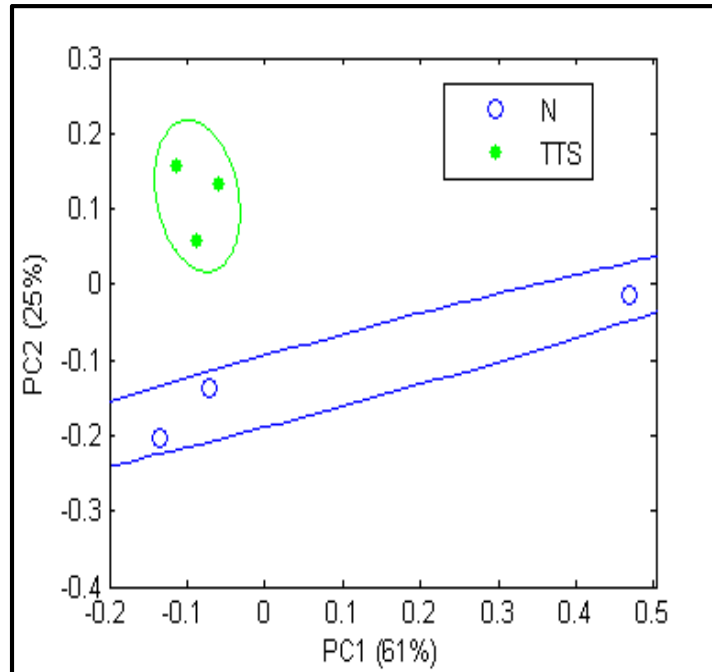


Figure 4.3.1: PCA score plot comparing the metabolite profiles of the normal HeLa cells (N) and HeLa-TTS cells (TTS)

The PCA score plot (Figure 4.3.1) indicates that the metabolic profile of the two groups (normal and transfected HeLa cells) differed significantly. This is evident from the separate grouping of the two groups. Regarding the intra-group variance, the normal HeLa cells have an obvious larger intra-group variance because of the smaller ellipse in this figure. In order to understand the separate grouping and the difference in the metabolic profiles of these two groups, the Student's t-test was used to identify the metabolites that differed significantly between these two groups. The results are given in Table 4.3.1.

Table 4.3.1: Important metabolites identified with the Student's t-test.

	P -value
MAL	0.00327
ACT	0.00566
FUM	0.01331
ISOCIT	0.01351
FBP	0.01485
NAD	0.02777
NADPH	0.03935
X5P	0.04753

That there are differences in the metabolism of the normal and transfected HeLa cells is very clear, but there can be speculations on what caused these differences. In another study that was done in our laboratory by Jean du Toit (Du Toit, 2010), differences in the global DNA methylation levels of normal and HeLa cells were observed. It is clear from this study that the global DNA methylation for transfected HeLa cells is 17.91% and only 14.84% for normal HeLa cells. When a cell line is transformed, the cellular characteristics is often altered, this is visible in the production of unpredicted proteins. This can be ascribed to silencing of certain genes and activating of others (Esteller, 2008). This alteration in the global DNA methylation levels and the cellular characteristics can possibly have a big impact on the energy metabolism and might be the cause of the difference in energy metabolism of the transfected HeLa cells compared with the normal HeLa cells.

Concerning the energy metabolism, it was reported that very little glucose carbon enters the citrate acid cycle in HeLa cells, only about five% of the 85% glucose carbon that becomes pyruvate, 80% lactate plus five% acetyl CoA, enters the TCA cycle (Reitzer, *et al.* 1979). They also found that the concentration of three TCA cycle intermediates, namely glutamate, α -ketoglutarate and malate, stay relative constant as a function of sugar. This suggest that these three metabolites of the TCA cycle are not maintained from the metabolism of sugar because when the energy source is changed to either fructose and galactose, very little sugar carbon is

actually metabolised through the glycolytic pathway (Reitzer, *et al.* 1979). Even in the presence of glucose these authors found that glutamine is still providing 70% of the ATP, this fact concludes that glutamine is most likely to be the major energy source of HeLa cells. Reitzer and co-workers also found that the most important function for sugar in mammalian cultured cells is most likely to provide carbon for the pentose cycle metabolism and that HeLa cells use one part of the TCA cycle as a linear sequence of reactions with a significantly different flow rate from the reactions in another part of this cycle.

Glutamine →glutamate→ α -ketoglutarate→succinate→malate→pyruvate→lactate

It was shown in another study that the HeLa cells contain all the enzymes of the TCA cycle (Barban & Shultze, 1956). These authors also suggested from results obtained with isotope experiments, that the TCA cycle is an important metabolic pathway for supplying these cells with the necessary energy for their requirements. From this research it is also clear that the HeLa cells can metabolise α -ketoglutarate to succinate like in the previous study (Barban & Shultze, 1956). In a more recent study Rissignol and co-workers compared the metabolism of HeLa cells that was grown in a galactose medium versus HeLa cells grown on glucose medium (Rossignol, *et al.* 2004). They found that there was a two-fold increase in the endogenous respiratory rate of the HeLa cells that was grown on the galactose medium. They had a number of possible reasons for this finding: firstly it could be due to a general stimulation of mitochondrial biogenesis, secondly it could be due to increased synthesis of respiration related enzymes and lastly this could also be due to an increased flux of the oxidation of glutamine through an existing pathway namely TCA cycle and respiratory chain. A second change in the metabolism was observed by Rossignol and coworkers and this was an increased in citrate synthase protein expression. This observation could be an indication of an induced expression of TCA cycle enzymes. They also proposed an explanation for this occurrence; this explanation is based on the fact that in galactose medium these cells derive their energy from glutamine. This glutamine is catabolised via the mitochondrial glutamase enzyme into the product glutamate. Glutamate is then converted to α -ketoglutarate via the enzyme glutamate dehydrogenase which is then processed in the TCA cycle (Rossignol, *et al.* 2004). Glutamine and glucose are known energy sources for tumour and proliferating cells (Matès, *et al.* 2009).

As was mentioned in the previous studies, this study also suggest that the main degradation pathway for glutamine is inside the mitochondria (initiated by the enzyme phosphate-activated glutaminase) and that HeLa cells can actually adjust their mitochondrial network structurally and functionally to only gain their energy through the breakdown of glutamine (Matès, *et al.* 2009). In this paper the authors also observed a very interesting difference between a transformed and non-transformed cell line, which is the change noted in the pyruvate dehydrogenase (PDH) complex levels (Matès, *et al.* 2009). Another interesting suggestion is made in this paper: this is that the selection energy substrate by the tumour cells is dependent on certain mutations that affect oncogenic signalling pathways. They give an example that explains the novel role of the overexpressed oncogene Myc on the glutamine metabolism of a tumour. This oncogene apparently represses miR-23a/b microRNAs which results in greater expression of mitochondrial GA (phosphate-activated glutaminase) which leads to an up-regulation of the glutamine catabolism. Glutamine degradation in tumour cells is activated by Myc in three different ways, namely to induce glutamine transporters like ASCT2 and SN2, to induce GA (which delaminates glutamine into glutamate, which in turn results in its capture intracellular) and lastly to induce lactate dehydrogenase (LDH) which has the function to convert pyruvate that is derived from glutamine into lactate. According to them the breakdown of glutamine can refill the TCA cycle via α -ketoglutarate and this in turn generates a vigorous production of NADPH which is needed for cell growth (Matès, *et al.* 2009). This suggests that Myc overexpression reprograms the mitochondria to be dependent on glutaminolysis for the energy requirements and thus viability of these cells (Matès, *et al.* 2009).

From these studies it is clear that there are definite differences in the metabolism between transformed and non-transformed cells. The current study not only supports these observations but also gives credence to the use of cell cultures in metabolomic studies. It is thus possible that, when HeLa cells are transformed, a change in the energy metabolism occurs that help these transformed cells to only use glutamine as energy source. One possibility would be the overexpression of the Myc gene in HeLa- tTs cells. This hypothesis can further be investigated as the intra-variation of HeLa-tTS cells are much less than the HeLa normal cells which can

possibly be that the HeLa-tTs cells only uses glutaminolysis pathway for energy production and not both glycolysis and glutaminolysis.

The exposure of the HeLa cells to an environmental perturbation, i.e. a heat shock, will be described in the next section.

4.3.2 Heat shock

In order to further evaluate the use of cell cultures in metabolomics studies, a heat shock intervention experiment was done to study the effect of a metabolic stressor on the HeLa cells. With this experiment it was aimed to assess whether induced biological variance (the heat shock) would exceed the technical variance from quenching and extraction of the metabolites. If the technical variance, which was introduced by the quenching and extracting methods (refer to Chapter 3 Section 3.2 and 3.5 respectively) was too big, it will be virtually impossible task to detect the induced biological variance. This can make the use of cell cultures in this type of research questionable. PCA was used to analyse the data and to visualise the metabolic data of the experimental groups in a simplified manner. PCA was performed as described previously.

The metabolic differences among the experimental groups are shown in the PCA score plot (Figure 4.3.2). The separate grouping of the 0h group from the hs (heat shock) and hs h (heat shock, repair) groups is clear, indicating noticeable differences in their metabolic profiles. However, this result also indicates that there is not much difference between the hs and hs h groups. The differences between the control (0h) group and the other two groups (hs and hs h) may indicate that enough metabolic variation was induced in the HeLa cells when the shock intervention was performed. There is little difference between the hs and hsh groups. This was confirmed by ANOVA which identified only two metabolites that differ between them, which are FBP and DAHP/GAP.

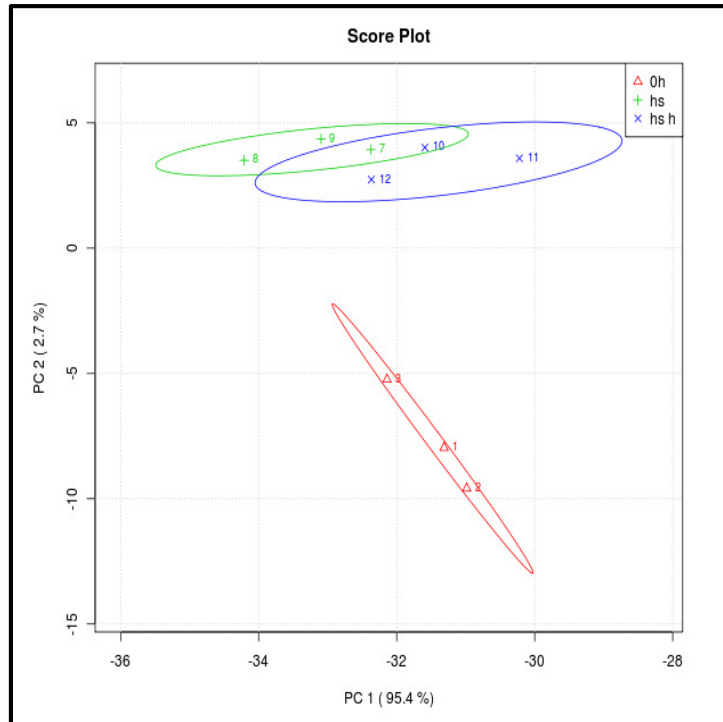


Figure 4.3.2: PCA score plot comparing the metabolite profiles of the HeLa cells after the heat shock intervention. Red, 0h (control group); green, hs (heat shock); and blue, hs h (heat shock repair).

In order to determine which metabolites caused the separate grouping of the respective groups, ANOVA and post-hoc analyses were done. The results are listed in Table 4.3.2. This result correlates with the PCA as there is not much difference between the hs and hs h groups, as there is only two metabolites that caused the separate grouping of these two groups, which are FBP and DAHP/GAP. On the other hand the metabolites that caused the separate grouping of the 0h group (which was the control) from the hs h and hs groups were ISOCIT, 3PG/2PG, FBP, NAD and ACT. There were only two metabolites extra for the hs and 0h groups separately, DHAP/GAP and OXA. There was also only two extra metabolites for the hs h and 0h groups separately, X5P and R5P.

Table 4.3.2: important metabolites identified by ANOVA and post-hoc analysis results

Metabolite	p.value ¹	Fisher's LSD
ISOCIT	0.000000624	0h - hs; 0h - hs h
3PG/2PG	0.000000785	hs - 0h; hs h - 0h
FBP	0.000518	hs - 0h; hs h - 0h; hs h - hs
NAD	0.014489	hs - 0h; hs h - 0h
ACT	0.015292	0h - hs; 0h - hs h
X5P	0.016748	hs h - 0h
DHAP/GAP	0.028065	hs - 0h; hs - hs h
R5P	0.031443	hs h - 0h
OXA	0.032788	0h - hs

¹ the p-value indicates statistical differences between metabolites of the respective groups. A $p > 0.05$ indicates metabolites of no statistical relevance and a $p < 0.05$ indicates that these metabolites does differ statistically between the two groups

Previous studies that looked into the effect of heat shock on the metabolism of cells showed that heat shock has a very definite effect on cellular respiration and glycolysis. The disruption of the energy metabolism is one of the initial and probably critical subcellular events when a cell launches a response to increased temperatures (Lunec & Cresswell, 1983). John and Susan R Creswell also suggests that the heat shock proteins that is coded by the heat shock protein genes which are activated during or after a heat shock, example Hsp70 gene in HeLa cells, may play a role in the energy metabolism. There is also a shift in Hsp70 levels when HeLa cells are starved of glutamine so that these cells cannot express normal amount of Hsp70 (Roth, 2008). This also contributes to the hypothesis that heat shock proteins may play a role in the energy metabolism (Lunec & Cresswell, 1983).

In order to represent the holistic effect of the heat shock on the energy metabolism, two charts of the energy metabolism were designed (Figure 4.3.3 A and 4.3.3 B). Only the metabolites that were identified by the ANOVA and post-hoc analysis to be important are represented in these charts.

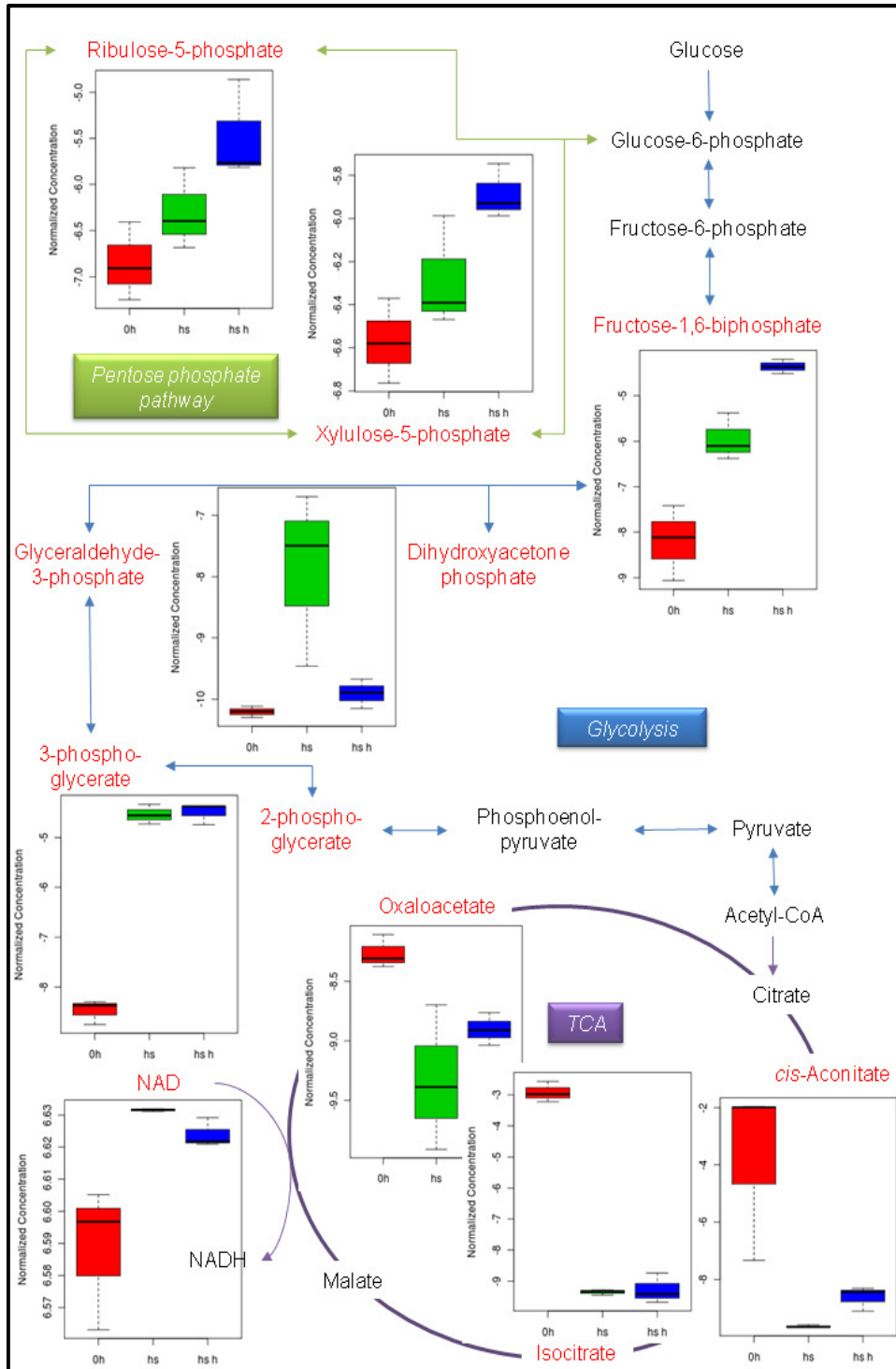


Figure 4.3.3 A

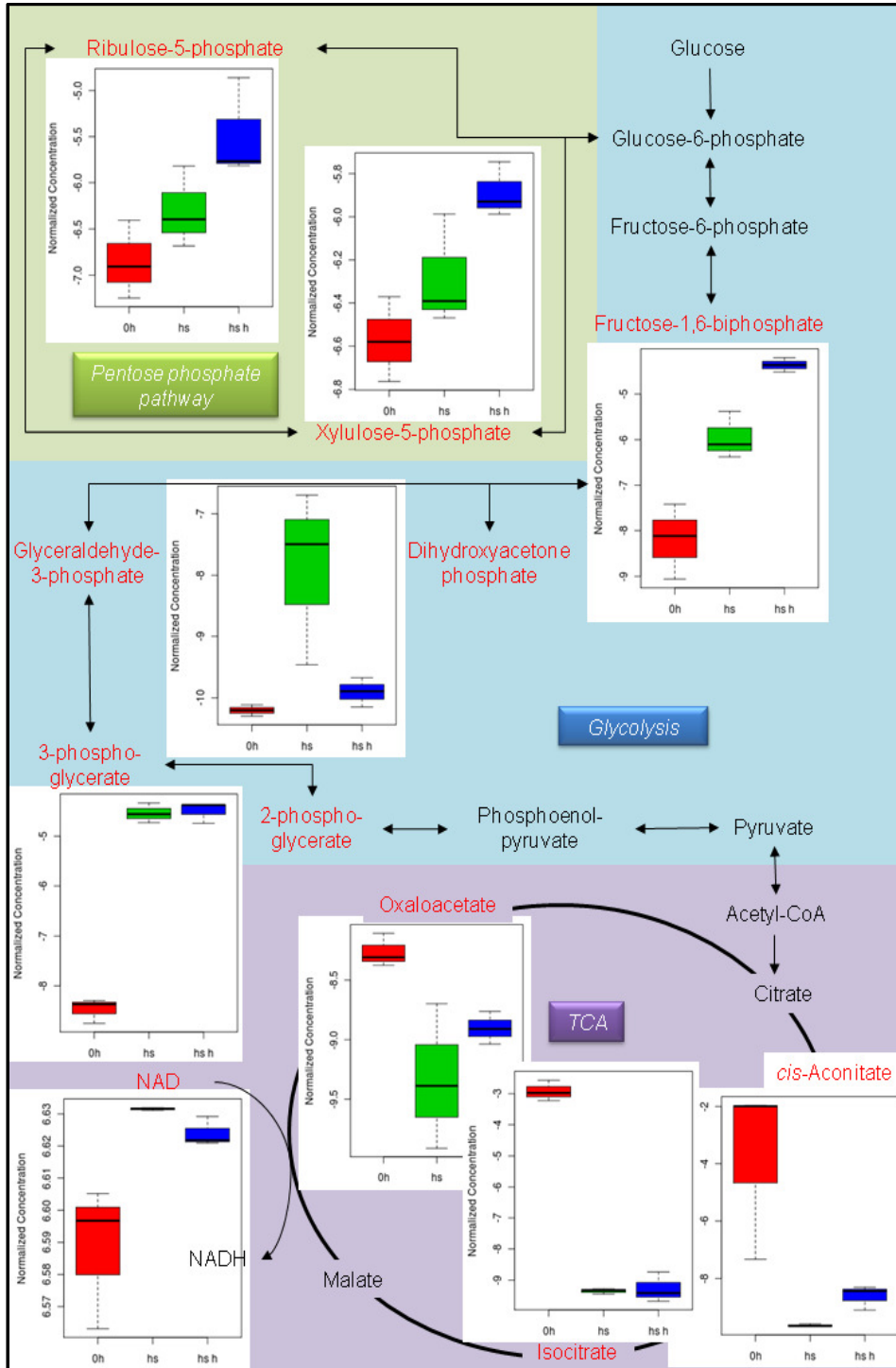


Figure 4.3.3 B

Figure 4.3.3: A metabolic chart to indicate the metabolites that differed significantly (that was identified by ANOVA and post-hoc analysis) in the respective metabolic pathways (glycolysis, PPP and TCA cycle) in comparison with the control (0h) and heat shock repair (hs h) groups, when the heat shock (hs) was introduced. Box and whisker plots are shown for each of the experimental groups (0h; hs; hs h) for each metabolite to indicate the differences between the respective groups. Red, 0h (control); green, hs (heat shock); and blue, hs h (heat shock repair). B: The same

metabolic chart as in A, but the metabolic pathways are colour coded in this figure. Green, PPP; blue, glycolysis; and purple, TCA cycle.

It is clear from these figures (4.3.3 A and B), that there is a definite change induced in the energy metabolism of the cells by the heat shock treatment. This change is seen in all the pathways of the energy metabolism that is represented in these charts (Figure 4.3.3 A and B) namely the PPP, glycolysis and the TCA cycle. The box and whisker plots were very useful to compare the distribution of the data obtained between the respective groups. Because of the little space they take up, it was possible to represent these differences on a metabolic chart to show the effect on the entire metabolism under study. When the heat shock was introduced the profile changed significantly in relation to the control (0h) as represented by the red and green box and whisker plots. The group that was allowed a repair period (hs h), represented by the blue box and whisker plots, is very similar to the heat shock group (hs).

In a paper concerning the metabolism of tumour cells, the authors state that in tumour cells the FBP metabolite is normally abundant in the tumour cell cytoplasm and this is a product of anaerobic glycolysis (Diaz-Ruiz, *et al.* 2009). Accumulation of this intermediate of glycolysis inhibits the TCA cycle. It is clear from Figures 4.3.3 A and 4.3.3 B that this metabolite is abundant in the heat shock group but is decreased in the control group. This can possibly be that the heat shock halted the metabolism and that the enzyme activity was much lower during the heat shock. It is also clear from Figures 4.3.3 A and 4.3.3 B that NAD is abundant in the heat shocked cells but the level is significantly decreased in the control cells. This observation not only supports the hypotheses that during the heat shock the metabolism was halted, but is also the best example of this phenomenon that occurs during the heat shock as it clearly demonstrates the difference in the metabolite concentration between heat shocked and normal cells. The high concentration of metabolites in the heat shocked cells compared with the normal cells can to a large degree be explained by this hypothesis. The only exception was for the TCA cycle. In this part of the metabolism the metabolite concentration was actually decreased compared to the normal HeLa cells. This can possibly be that the build-up of some of the other metabolites inhibited the TCA cycle, for example the FBP metabolite as

stated previously in this paragraph. A second possibility is that the heat shock could have increased the activity of the TCA cycle enzymes while inhibiting the enzymes of the other two pathways under study. The heat shock intervention did in fact induce a change in the levels of the metabolites of the energy metabolism investigated in this study and this was measured using metabolomics technology.

A MTT assay was done to measure the effect of the heat shock on the viability of the cells (Section 3.10). It is clear from these results (Figure 4.3.4) that the HeLa cells were viable after the heat shock was administered. This follows from the comparison of the heat shock cells (represented by the light blue column in Figure 4.3.4) with the negative control cells (represented by the dark blue column in Figure 4.3.4). For the positive control 100% acetic acid was used in different volumes namely three, six and 12 μ l respectively. The viability of the HeLa cells did in fact decrease as the volume of the acetic acid increased which is clear by the descending red, green and purple columns in Figure 4.3.4. The negative control (HeLa cells cultured under normal conditions and not exposed to a stressor) show a good viability of the HeLa cells, which is represented by the dark blue column in Figure 4.3.4. If the principle of the MTT assay is taken in consideration, that in live cells the metabolically active mitochondrial dehydrogenase enzymes will convert the yellow water soluble substrate (MTT) to form deep purple coloured formazan salts, then it seems that this part of the metabolism was actually more active. This is probably part of the heat shock response launched by the cell in order to maintain life. This result also correlates with the previous heat shock results (Figure 4.3.3 A and B plus discussion) as the TCA cycle metabolites was in fact lowered after the heat shock, which can possibly indicate an increase in enzyme activity in the TCA cycle. This can possibly also be in agreement with the literature that was previously mentioned in this chapter in Section 4.3 by Mates and co-workers that HeLa cells can actually adjust their mitochondrial network structurally and functionally to only gain their energy by the breakdown of glutamine. HeLa cells can use glutamine under stressful conditions as their sole energy substrate.

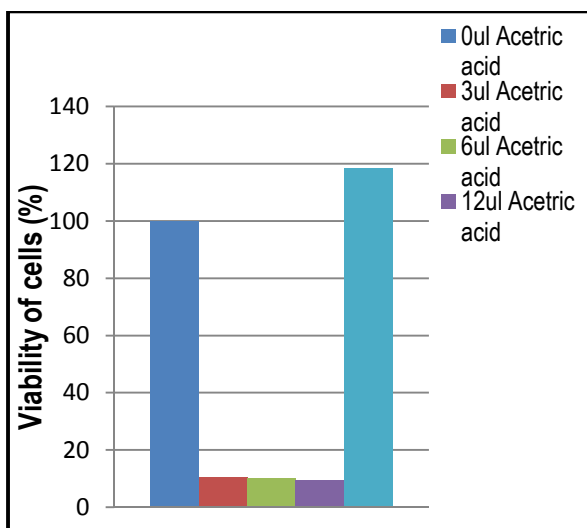


Figure 4.3.4: Effect of heat shock on cell viability. MTT assay to show the amount of viable HeLa cells after the cells were exposed to a heat shock (42°C for 60 minutes). Dark blue, negative control; red, green and purple, positive control; and light blue, heat shocked cells. 100% acetic acid was used for the positive control

4.4 In summary

With the two perturbations that the HeLa cells were exposed to, namely the transfection with an expression vector (Section 4.3.1) and the heat shock (Section 4.3.2), the HeLa cells were exposed to a genetic and an environmental change, respectively. It is clear from this work that both these perturbations had a definite effect on the energy metabolism and it was possible to capture and measure these changes using cultured cells as a model for metabolomics studies. There are still obstacles that should be overcome in using metabolomics as a tool in cell culture studies. These obstacles lie within the sample preparation methods. The standard quenching method (described in Chapter 3 Section 3.2 for adherent mammalian cell cultures) was tested to establish how effective it will be when used to quench HeLa cells. This method did not quench the entire metabolism under study because some of the metabolites studied showed an increase in metabolite levels which indicate that the enzymes catalysing those reactions were not effectively quenched. These results were obtained from the waiting period experiment (Section 4.1). For the first six hours the metabolism did not change but after 24 hours there was a definite change in the metabolism compared to the control. A quenching method that does not require the steps to remove the media and to wash the cells might help to overcome this drawback, especially when the energy metabolism is under study.

These steps take additional time before quenching can actually be performed and this can influence the effectiveness of the quenching method. Different compositions of the quenching method were tested for less variation and better repeatability. There was contradiction about which method gave the best results (Section 4.2). The quenching solution as an organic solvent can also influence the activity of the enzymes if it does not completely quench the reactions, as mentioned in the conclusion of Section 4.2. This can also contribute to the understanding of the results obtained in this study. A quenching method that will give even less variation and better repeatability than the ones tested in this study will give even more precise results. Concerning the volume of the quenching solution for a specific amount of cells it was established, using the quenching solution described in the standard quenching method (Chapter 3, Section 3.2.), that the least variation is found when cells are cultured in six well plates and that one ml of the quenching solution gave the best results (results are discussed in the honours project of Angelique Lewies, (Lewies, 2010). Despite these obstacles it was observed in this study, using the standard quenching and extracting methods (Chapter 3, Section 3.2. and 3.5) that it is possible to see a change in the metabolism of cultured HeLa cells using metabolomics as a tool. The cultured HeLa cells were exposed to two perturbations, one was a genetic change and the other one was a change in the environment (Section 4.3). Both of these perturbations did have an effect on the energy metabolism and this change was captured and measured using metabolomics.

Chapter 5: Final Discussion

Metabolomics aims to narrow the gap between genotype and phenotype and can be defined as the analysis of all the small molecular weight metabolites in a biological system (biological fluid, cell or organism) under a given set of physiological conditions (B. Álvarez-Sánchez & F. Priego-Capote 2010). The ultimate goal of metabolomics is to identify and quantify all of the metabolites in a given system in an unbiased, reproducible way. This presents an analytical challenge because of the wide diversity of metabolites and their varied physico-chemical properties and abundances. Various approaches can be followed to investigate subsets of the metabolome, i. e. metabolite profiling, targeted analysis, metabolite fingerprinting and metabolite footprinting. Targeted analysis, which is restricted to metabolites of a particular enzyme system, aims to qualitatively and quantitatively study one or more chemically or functionally related metabolic pathways (Goodacre et al, 2004; Álvarez-Sánchez et al, 2010).

The reason for pursuing the metabolomics approach to study the metabolism is, is that it is primarily a data driven approach that aims to add to a better understanding of how biological processes work and interact (Roux, et al. 2010). On the other hand, it is of great interest to study the metabolism because of the essential role it plays in human physiology. The metabolism is also very important when it comes to the understanding of disease states and the progression of diseases, nutrition and also aging. The central carbon metabolism, glycolysis, the PPP and the TCA cycle, is of great importance to the cell, because of the following reasons:

1. It has key functions in the processes of substrate degradation, energy and cofactor regeneration, and biosynthesis precursor supply (Soga, et al. 2009)
2. It has a broad biological relevance (Kiefer, et al. 2008)
3. It is sensitive to changes in the environmental conditions as well as genetic changes or perturbations.

It is, therefore, a prime part of the metabolism of a cell to study that reflects the total metabolism of the cell. When using the metabolomics approach to study the central carbon metabolism, there are different sample types and analysis strategies to use.

This is thoroughly discussed in the literature review. For this study the targeted approach was followed and cell cultures were chosen for the sample type (Álvarez-Sánchez & Priego-Capote, 2010).

There is a number of advantages in using cell cultures, i. e. a continuous supply of homogenous cellular material is readily available they can easily be manipulated to fit the purpose of the experiment, they can be stored in a deep frozen state and it is more economical to use than *in vivo* systems (Jacoby & Paston, 1979: 439).

The application of cell cultures in metabolomics studies is of great importance to contribute to a more holistic understanding of the function and properties of living systems (Cuperlovic-Culf, et al. 2010). A variety of fields are using cell cultures in metabolomics studies for example in pharmacokinetics and drug testing, *in vitro* cultured cell models were developed as an alternative way of drug testing (Khoo & Al-Rubeai, 2007). The challenges that metabolomics face in the area of cultured cells are the present analytical technologies do not fully meet the requirements for metabolomics. The complexity of metabolites also complicates obtaining the complete metabolite analysis (Khoo & Al-Rubeai, 2007). The sample preparation step is also a major challenge in the development of analytical methods concerning metabolomics. Sample preparation includes quenching and extraction of the metabolites and there is a lack of attention when it comes to optimising sample preparation (Álvarez-Sánchez & Priego-Capote, 2010). Besides these challenges there are still major advantages in using cell cultures in metabolomics studies, for example when there is a change introduced in the cell culture, for example when there is a variation introduced in the environment or energy substrate, this change can be measured using metabolomics.

The sample preparation step is initiated by quenching to terminate the metabolism of the cells immediately and this termination is very important to obtain a valid snapshot of the metabolism (Faijes, et al. 2007).

The aim of this study was to evaluate the use of cultured cells in metabolomics investigations and to standardise a proper quenching method for a metabolomics analysis of mammalian cultured cells. HeLa cells were chosen for this study and these cells were cultured for all the experiments. A standard method for quenching of the metabolism, disrupting the cell membranes and extracting the metabolites was

adapted from the literature (Danielsson, et al. 2010). The quenching method was tested for its effectiveness in quenching the metabolism of HeLa cells used in this study. Different compositions of the quenching method were also tested for better repeatability. As mentioned previously the targeted approach was chosen for this study and the metabolites of the central carbon metabolism were the target. The list of metabolites was compiled by Leo et al. (Luo, et al. 2007).

Firstly, the optimum time frame for extraction was determined to also determine the effectiveness of the quenching method (Chapter 4, paragraph 4.1). Cells were cultured in six well plates (as explained in chapter 3) until the optimum confluency (80%) was reached. Cells were then immediately quenched and harvested. Extraction of metabolites was performed after the different waiting periods, namely zero, three, six and 24 hours after quenching. For the control group (0h) extraction was immediately performed. For the other three groups 3h, 6h and 24h, extraction was performed three hours, six hours and 24 hours after quenching. The results gained from this experiment were normalised using MUSTUS. PCA was done to assess the overall variance and changes in the metabolic profiles of experimental groups (Figure 4.1.1). It was found that the 0h, 3h, and 6h groups clustered together while the 24h group clustered separately from the other groups. The intra-group variance was also higher in the 24h group. This implied that there was a definite change in the metabolic profile of this group. The ANOVA test was done to identify which metabolites caused the separate clustering of the 0h, 3h 6h waiting period groups and the 24h waiting period group (Table 4.1.1). A correlation analysis was done to establish which of the metabolites changed considerably over time (Figure 4.1.2). It was established that some of the metabolites that are products in the respective metabolic pathways did in fact increase over time while their substrates decreased. This suggests that the quenching method did not completely quench these metabolic reactions.

In spite of this drawback of the quenching method, it was still possible to differentiate between different experimental metabolic effectors, e. g. heat shock and transfection of the cells by an expression vector (See 4.3). The following experiment was done to determine the composition and temperature of the quenching solution for a specific amount of cells (variation in the quenching method) (Chapter 4, paragraph 4.2). Three quenching methods described in the literature were compared, they are:

100% methanol at -80°C (neg80), 100% methanol with a 0.85% AMBIC at -80°C (AMBIC) and 100% methanol at room temperature (KT). The results obtained were statistically analysed to determine which method gave less variation and thus better repeatability. Normalisation and PCA was done as described for the previous experiment. PCA indicated that the different groups clustered together, thus the metabolic profiles for the different experimental groups was very similar (Figure 4.2.1). According to the multivariate analysis the least variation was seen in the AMBIC group and the second least in the KT group. These two groups also had better repeatability compared to the other groups. The distribution of the metabolite CVs was determined to allocate values to the variance as the PCA is just a visualisation tool. A rapid increase in a chart line indicates a more precise method. The vertical line in the chart (Figure 4.2.2) indicate the CV = 50% cut-off line which is often used to indicate precision. This revealed that 71% of the metabolites in the KT method had a CV below the 50% cut-off and therefore seems to be the more precise method.

Thus quenching should be rapid, less than a second if possible. It should be more rapid than the fastest reaction under study and in this study the central carbon metabolism was targeted, which includes glycolysis. Glycolysis has reactions which have turnover times of millimoles per litre per second (Winder, et al. 2008). When this part of the metabolism is under study quenching should perhaps be even more rapid and complete. The quenching method with the best repeatability according to the CVs of the metabolites of the different methods was the KT method. The PCA, however, indicates that the method with the less intra-group variance is the AMBIC method. Therefore it is debatable if the KT method is the best quenching method. Another factor that could have influence the effectiveness of the quenching method is the effect of the quenching agent in this study methanol. Organic solvents are used as quenching agents because they are known to inactivate the enzymes (Doukyu & Ogino, 2010). They can, however, have different effects on enzyme activity: in organic solutions enzymes can catalyse a wide range of reactions that is not possible in aqueous media (Ogino & Ishikawa, 2001). It is, however, vital that there is a small amount of water present in the organic solvent for the enzymes to be active (Zhu, et al. 2011). If this was true for the experiments done in this study then

the methanol, could have incorporated the changes into the enzymes so that it was still active but with varied catalytic activity as when in an aqueous media.

Two perturbations were introduced in the HeLa cell culture to investigate whether these perturbations can be captured and measured using metabolomics as a tool (Chapter 4, paragraph 4.3). The standard quenching method (as adapted from the literature, chapter 3, 3.2–3.5) was used in this experiment. The first perturbation was to evaluate the effect of transfection to establish the effect of a genetic change on the energy metabolism of the HeLa cells (Chapter 4, paragraph 4.3.1). Transfected HeLa cells contained a vector namely, vector ptTS-Neo vector. The metabolisms of normal HeLa cells (N) and transfected HeLa cells (TTS) were compared. PCA score plot indicated a definite difference in the metabolic profiles of the two experimental groups as they clustered separately on this plot (Figure 4.3.1). It is also clear from the PCA analysis that the intra-group variance is higher in the normal HeLa cells than in the transfected HeLa cells. To contribute to the understanding of the separate clustering of the two experimental groups, a student t-test was performed. From this test it is clear which of the metabolites differed significantly between the two groups (Table 4.3.1). It is speculated in this study what the causes are for the differences in the metabolisms of transfected HeLa cells and normal HeLa cells. One of the possibilities is that DNA methylation can cause these differences since it is shown that the DNA methylation levels do differ for these two groups of cells (Du Toit, 2010). Another possibility is that, when the cells are transfected, they change the primary energy pathway so that they only derive their energy from the breakdown of glutamine. This speculation is based on the observation that HeLa cells can adjust their mitochondrial network structurally and functionally to obtain their energy through the breakdown of glutamine (Matès, et al. 2009). Further it is also known that tumour cells select their energy substrate depending on certain mutations that effect oncogenic signalling pathways (Matès, et al. 2009). Thus, it is possible that during transfection, the HeLa cells could change their mitochondrial network and /or their choice of energy substrate, perhaps to only glutamine, in reaction to the genetic change.

The second perturbation was to assess what a change in the environment would have on the energy metabolism of the HeLa cells (Chapter 4, paragraph 4.3.2). The HeLa cells were exposed to a heat shock, which is also a metabolic stressor (Lunec

& Cresswell, 1983). With this perturbation it was aimed to establish if the induced biological variance would exceed the technical variance from quenching and extraction of the metabolites. If the technical variance, which was introduced by the quenching and extracting methods, was too big, it will be virtually impossible task to detect the induced biological variance. There were three experimental groups, a control group (0h), a heat shock group (hs) and a heat shock repair group (hsh). The 0h group was not exposed to the heat shock, the hs group was exposed to a 42°C heat shock for an hour and the hsh group was exposed to the same heat shock but was exposed to a recovery period for an hour in the incubator at 37°C. PCA indicated a clear difference between the different experimental groups (Figure 4.3.2). The 0h group clustered separately from the hs and the hsh groups which clustered almost together. This imply that the metabolic profiles of the 0h group differed remarkably from the hs and hsh groups and on the other hand that the metabolic profiles of the hs and hsh groups were very similar. This could possibly indicate that the induced biological variance did exceed the technical variance. ANOVA was done to indicate which of the metabolites was responsible for the separate clustering of the different experimental groups (Table 4.3.2).

It is clear from these results that heat shock has a definite effect on the energy metabolism. This was also observed in study where the disruption of the energy metabolism was one of the initial, and probably critical, subcellular events when a cell launches a response to increased temperatures (Lunec & Cresswell, 1983). They state further that heat shock proteins may play a role in this change seen in the energy metabolism.

Two charts of the energy metabolism was designed to give a more holistic presentation of the effect of heat shock on the metabolism, only the metabolites identified by ANOVA was included in these charts (Figure 4.3.3 A & B). To compare the distribution of the data obtained from the different groups, box and whisker plots were included on these charts. It is clear from these charts that the metabolic profiles of the control cells compared to the hs and hsh cells differed significantly. The metabolite concentration of the hs cells is mostly increased compared to the control cells with the exception of the TCA cycle where the metabolite concentration is decreased in the hs cells compared to the control cells. The conclusion was drawn that the heat shock could have increased the activity of the TCA cycle

enzymes while inhibiting the enzymes of the other two pathways under study. The heat shock intervention did in fact induce a change in the levels of the metabolites of the energy metabolism investigated in this study and this was measured using metabolomics technology.

To measure the effect that heat shock has on the viability of the HeLa cells a MTT assay was done (Figure 4.3.4). It was clear from the results gained from this experiment that the cells were viable after the heat shock was administered. The results also show a very interesting phenomenon this is that if the principle of this assay is taken in consideration then it indicates that the TCA cycle of the HeLa cells was actually more active than in the negative control cells. This correlates with the previous results obtained from the heat shock experiment and can possibly indicate that this is part of the heat shock response launched by the cell in order to maintain life.

It is clear from this work that both these perturbations had a definite effect on the energy metabolism and it was possible to capture and measure these changes using cultured cells as a model for metabolomics studies. The obstacles identified by this study in using metabolomics as a tool in cell culture studies lies within the sample preparation methods. The standard quenching method was tested to evaluate its effectiveness in quenching HeLa cells. As seen in the results this quenching method did not quench the entire metabolism under study. A possible outcome for this drawback is to design a quenching method that does not require the steps to remove the media and to wash the cells. Concerning the different compositions of the quenching solution, there was contradiction of which method gave the best results. However a quenching method that will give even less variation and better repeatability than the ones tested in this study will give even more precise results.

The optimum quenching volume for a specific amount of cells was determined in our laboratory by Angelique Lewies in her honours study (Lewies, 2010). The optimum composition and temperature for the quenching solution was despite the effort not determined in this study as it gave contradicting results when three different compositions and 2 different temperatures were compared. The repeatability of the quenching method as adapted from the literature did not give optimum values and this was identified as an obstacle as discussed in the previous paragraph. The

optimum time frame for quenching was however determined and it should in fact be less than a second especially when certain pathways of the energy metabolism is under study, like glycolysis. The perturbations induced into the environment and genes of the cell cultures could be captured and measured using metabolomics as an instrument which make it definitely capable to use cell cultures as models for metabolomics studies. Their effectiveness as models for metabolomics studies can however greatly improve by bridging the obstacles identified in this study.

To summarise, the goals formulated for this study were achieved to a large extent. Concerning the quenching method, the optimum composition and temperature were not determined because of contradicting results, the repeatability was not satisfactory and the optimum time frame should in fact be less than a second. This study showed that cell cultures can definitely be used as models for metabolomics investigations by determining if a perturbation induced to the cell cultures can be detected using metabolomics as an instrument. Two perturbations was induced, one genetically and one environmental. Both could be captured and measured using metabolomics. The obstacles identified lies within sample preparation. A quenching method that does not require a wash step and step to remove media could give more repeatable results and this could improve the use of cultured cells in metabolomics. The repeatability of experiments involving cultured cells and metabolomics can also be improved by optimising the infrastructure in which these experiments are performed. By, for example, designing a specific laboratory for such experiments where you culture, harvest, prepare and analyse the cultured cells in the same laboratory.

Chapter 6: Article

The use of mammalian cell cultures in metabolomics studies was investigated. Unwanted variance was noted after transfection and quenching. The results obtained from this study are presented here in the form of a first draft manuscript that was prepared for submission to the Metabolomics journal.

Web address of the Metabolomics journal

<http://www.springer.com/life+sciences/biochemistry+%26+biophysics/journal/11306>

Mammalian cell cultures as models for metabolomic studies: taking note of unwanted variance after transfection and quenching

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Abbreviated title:

Unwanted variance in mammalian cell culture models used in metabolomics studies

Abstract

There are many advantages when using cell cultures in metabolomic studies as cell cultures can easily be manipulated for the purpose of the experiment. This creates many opportunities for metabolomic studies. However, the use of cell cultures also holds certain drawbacks and risks which, if not noted, could lead to inaccurately interpreted results and biological information. To append previously reported drawbacks and risks of cell cultures in metabolomic investigations, the authors studied the metabolic changes that occur after quenching and transfection. Changes in metabolite levels of the central carbon metabolism 0, 3, 6 and 24 hours after quenching was investigated. Changes in these targeted metabolites after transfection of HeLa cells with plasmid DNA was also studied. It was found that a sample preparation window of up to three hours after quenching is suitable with the selected quenching method as no marked metabolic variance in the central carbon metabolism was detected. Transfection of cells with plasmid DNA did, however, introduce unwanted variance which is hypothetically linked to epigenetic alterations such as DNA methylation. These unwanted changes in the central carbon metabolism should be accounted for when using transfected cells and when working outside the optimal sample preparation window.

Key words: metabolomics, cell cultures, unwanted variance, transfection, DNA methylation

1. Introduction

Metabolomics aims to narrow the gap between genotype and phenotype and can be defined as the analysis of all the small molecular weight metabolites in a biological system (biological fluid, cell or organism) under a given set of physiological conditions (Álvarez-Sánchez *et al.*, 2010). This presents an analytical challenge because of the wide diversity of metabolites and their varied physico-chemical properties and abundances (Khoo & Al-Rubeai, 2007). Furthermore, the metabolome of biological systems are in constant flux with metabolite reaction half lives generally less than a second. This means that metabolic changes can be observed which is not related to the research question (Dunn *et al.*, 2005). Hence, the experimental design and setup must be intensely controlled on each level in order to limit the introduction of unwanted technical or biological variance. The use of cell cultures in metabolomics research is therefore advantageous as their environment can be meticulously controlled, thereby limiting unwanted biological variation (Chaudry, 2006; Cuperlovi-Culf *et al.*, 2010). However, cell cultures too present some challenges which, if not addressed, could introduce unwanted technical variance that is unspecific and irrelevant to the study. Awareness around cell harvesting, quenching and metabolite extraction have for this reason increased over the years (Dietmair *et al.*, 2010; Teng *et al.*, 2009) leading to improved methods and techniques for studying cell cultures. Conversely, several aspects remain unnoticed and/or unstated which could jeopardize the validity of cell culture's use in certain metabolomics studies. Firstly, the effect of the time-lapse after quenching on the metabolic profile remains un-investigated. What is the workable time window after quenching before metabolic fluxes resumes? Secondly, the metabolite variance produced with the transfection of plasmid DNA to cell cultures. The effect of the above mentioned factors on specific metabolites of the central carbon metabolism of cultured HeLa cells were investigated.

2. Materials and methods

2.1. Culturing conditions and transfection

Cells were cultured in 75cm² flasks (Nunc) in DMEM medium containing 5.5ml NEAA, 2mM L-Glutamine, 10% FBS and 5.5ml penstrep at 37°C in a humidified atmosphere containing 5% CO₂. For each experiment 300 000 cells were seeded in each well of six well plates and grown for 48 hours to reach 80% confluency. The cells

were scraped in the quenching solution and transferred to a 2ml Eppendorf tube. To monitor the effect of transfection, HeLa cells were transfected with ptTS-Neo vector (Clontech) as described elsewhere (van Dyk, 2011) which is commonly used to create cell knock-down models.

2.2. Quenching

The six well plates containing the cultured cells were placed on ice and the growth medium removed from each well. The cells were then washed twice with 2ml of ice cold PBS. Then 1000 μ l of the quenching solution and 100% cold methanol (-80°C) was added to each well (Danielsson, *et al.* 2010). For the time-lapse investigation, metabolite extraction was performed 0, 3, 6 and 24 hours after quenching to determine if the metabolic profile of the cells changes over time after quenching of the metabolism. Metabolite extraction of transfected cells followed immediately after quenching.

2.3. Metabolite extraction

Metabolite extraction was done by adding 640 μ l 100% methanol plus 360 μ l milli Q water to get a final volume of 2000 μ l, thus an 82% aqueous methanol solution. The extracts were then placed on ice for 20 minutes. The samples were centrifuged for 20 minutes at 17400 x g after which the supernatants were transferred to an Eppendorf tube. The samples were dried under vacuum in a Savant ISS110 Speedvac Concentrator (Thermo Electron Corporation) and kept at -80°C until use (Danielsson, *et al.* 2010).

2.4. LC-MS/MS instrumentation and analysis

The extracted intracellular metabolites were analyzed with LC-MS/MS. The multiple reaction monitor method described by Luo *et al.* (2007) was used to monitor metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle. Dried samples were re-dissolved in 50 μ l 1% acetic acid in water. High-performance liquid chromatography was performed on an Agilent 1200 series LC equipped with a C18-Aqua column (150mm x 2.10mm, particle size 5 μ m) from Agilent (Santa Clara, CA, USA). Mobile phase A consisted of 1% acetic acid in water and mobile phase B of 100% acetonitrile. The column temperature was maintained at 25 °C and the flow rate at 0.2ml/min. The samples (10 μ l) were injected and the mobile phase composition was changed from 0% of B to 100% of B over 15 minutes, after which the percentage of B was maintained at 100% for 3 minutes. The percentage of B was changed back to 0% over 4 minutes and the column re-equilibrated for 6

minutes. The MS/MS analysis was performed on an Agilent 6410 Triple Quad (Santa Clara, CA, USA) in negative electro-spray ionisation (-ESI).

2.5. Data pre-processing, pre-treatment and statistical analysis

The targeted LC-MS/MS data was extracted with Agilent's MassHunter Qualitative analysis software. The metabolite levels between samples were normalized using the MSTUS (mass spectrometry total useful signal) normalisation method from Warrack *et al.* (2009). Missing value imputation was done after data normalization. Missing values were replaced with the group mean (nearest neighbour imputation) and the data log transformed before statistical analysis to make it more normally distributed. PCA was done to assess and visualise the overall variance in the metabolic profiles of the experimental groups. ANOVA and Student's t-test were used to find metabolites that differed significantly between the experimental groups. Pattern analysis using Pearson's correlation analysis was done to assess change of metabolite levels over time. All statistical tests (except PCA) were done using the MetaboAnalyst web service (Xia *et al.*, 2009; www.metaboanalyst.ca). PCA was done using the statistical toolbox in Matlab (Mathworks, Natick, MA, USA).

3. Results and Discussion

3.1. Metabolic changes after quenching

It is important in metabolomics studies to limit unwanted or irrelevant variance in order to find meaningful differences between different samples. It is, therefore, necessary to eliminate any factor that can interfere with obtaining meaningful results. One such factor is the time elapsed after quenching and extraction of the samples, in this case cultured cells. To ascertain whether the time elapsed after quenching had any effect on the metabolic profile in the respective experimental groups, metabolite extraction was performed at 0, 3, 6 and 24 hours after quenching of the cultured cells. PCA of the results was done to assess the overall variance in the metabolic profiles of the experimental groups. Fig. 1 shows the PCA score plot of the log transformed metabolic data from the time-lapse groups. The 0, 3 and 6 hour groups clustered close together while the 24 hour group clustered completely separate. The intra-group variance visualised with the confidence ellipses is similar in the 0, 3 and 6 hour sample groups while this variance is evidently greater in the 24 hour time-lapse group. The separate grouping of the cells that were processed 24 hours after quenching indicate a definite change in their metabolic profile in comparison to the other samples. One-way ANOVA was performed to highlight the metabolites that differed markedly between the groups followed by Pearson's correlation analysis of all the

metabolites with the time-lapse pattern. The metabolites that differed markedly between the groups are listed in Table 1 along with their respective correlation coefficients.

The results obtained by the ANOVA correspond with the PCA results as the majority of the listed metabolites differed between the 24 hour time-lapse group and the other sample groups. Only two metabolites differed significantly between the other groups, i.e. xylulose-5-phosphate (X5P) was markedly different between the 3 and 6 hour groups and phosphoenolpyruvate was markedly different between the 0 and 6 hour groups. The majority of the compounds significantly different between the 0 - 6 and 24 hour sample groups were lower in the latter group, implying metabolite degradation. This is supported by their relatively strong inverse correlation with the elapsed time. However, X5P, NADP, isocitrate (ISOCIT), dihydroxyacetone phosphate/glyceraldehydes-3-phosphate and fructose-6-phosphate/glucose-6-phosphate levels were higher in the 24 hour samples which indicate the opposite. Correlation of these compounds with the elapsed time was relatively strong positive indicating formation of these compounds over time. Hence, the differences seen in the metabolite levels cannot be (predominantly) linked to a time dependent degradation. Fig. 2 illustrates the relationship between several of these compounds which further show that enzyme activity could have resumed after some time. For example, the average acetyl-CoA abundance drops over time while the average citrate abundance increase, implying that acetyl-CoA is converted to citrate by citrate synthase. However, since the metabolite levels in each culture were not monitored over time, this theory is built on the assumption that the different cell colonies have small biological variance.

3.2. Effect of cell transfection on metabolite profiles

Transfection of cell cultures to over-express the cloned genes or to create knock-down models is widely used in the scientific world. When such cell cultures are used in metabolomics research it could give valuable information about gene function or dysfunction. However, there are certain aspects of such cell culture models that should be minded and which, if not accounted for, could give irrelevant variance during metabolite measurements. One of these aspects is the effect of transfection on the metabolic profiles of such cells. We have transfected HeLa cells with the pTTS-Neo vector to eventually create knock-down cultures of fumaroyl acetoacetate hydrogenase (FAH), the enzyme defective in tyrosinemia type I (van Dyk, 2011). The central carbon metabolism of the cells transfected with an empty expression vector were compared to the normal, untransfected cells.

The PCA score plot with 90% confidence ellipses are shown in Fig. 3. The control cells (N) grouped separately from the transfected cells (TTS) and showed less intra-group variance. From a multivariate point of view it is thus clear that the investigated metabolome differed between the control and transfected HeLa cells. Student's t-test was used to identify metabolites that differed significantly between the experimental groups. The results are shown in Table 2. Most of the compounds that differed markedly between the experimental groups are found in the tricarboxylic acid cycle while glycolysis and the pentose phosphate pathways were less affected. All the metabolites that differed markedly between the experimental groups were lower in the transfected cells except for fructose-1,6-biphosphate, fumarate and malate. Also, intermediates of the tricarboxylic acid cycle were found to differ most between the transfected and control cells. The reason for this particular profile is, however, unclear but may reside in deviations in the regulation of gene expression. In another study in our laboratory it was found that the global DNA methylation in transfected HeLa cells is 17.91% and only 14.84% for normal HeLa cells (du Toit, 2011). This alteration in the global DNA methylation levels and the concomitant deviation in cellular metabolic characteristics can possibly have a significant impact on the energy metabolism and might be the cause of the difference in energy metabolism between the transfected and the normal HeLa cells (Cyr & Domann, 2011).

4. Conclusion

It was observed for bacterial cells that metabolite extraction should quickly follow quenching to retain an accurate snapshot of the metabolome of the investigated system (Faijes, *et al.* 2007). The results seen in this investigation verified this principle for a eukaryotic cell line (HeLa), at least for the metabolites of the central carbon metabolic pathways. The one drawback in using adherent cell cultures over, for example, tissue samples, for metabolomics research is that the cells must be harvested before metabolite extraction. Depending on the method used, this task could take up to several minutes. While the results indicate that the targeted metabolite levels remained unchanged for at least three hours after quenching, this result is linked to the quenching method used in this investigation. This time window could be very different for other quenching methods not to mention other parts of the metabolome. It is thus important to control, or at least acknowledges, the possible variance that such waiting periods could introduce.

Cultured cells are frequently used to produce recombinant proteins of value and these protein expression systems are going through a rigorous preparation and control process to ensure high producing stable production of the proteins (Barnes *et al.*, 2003). It is, however, seldom that a study is done to verify the effect of cloning as such in these processes since it is known that epigenetic events, as was observed in this study, are introduced by cloning and that it has a definite effect on the stability of protein expression (Osterlehner *et al.*, 2011). These effects can be ascribed to silencing of certain genes and activating of others, resulting in deviations in protein expression (Esteller, 2008) and the consequent change in metabolic profiles. Metabolic changes introduced by the transfection process should therefore not be mistaken as changes relevant to a specified genetic intervention.

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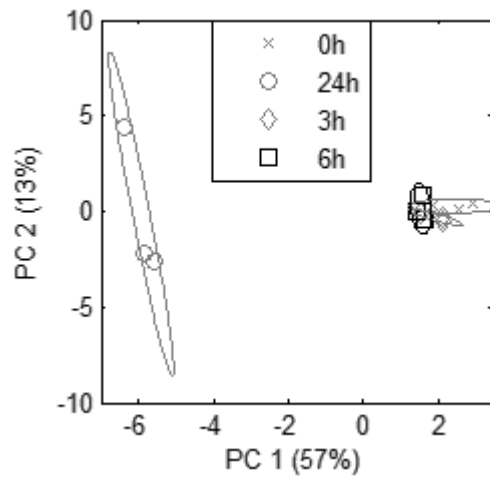


Fig 1: PCA score plot of the metabolic data from the 0, 3, 6 and 24 hour time-lapse groups. Grouping is shown with 90% confidence ellipses.

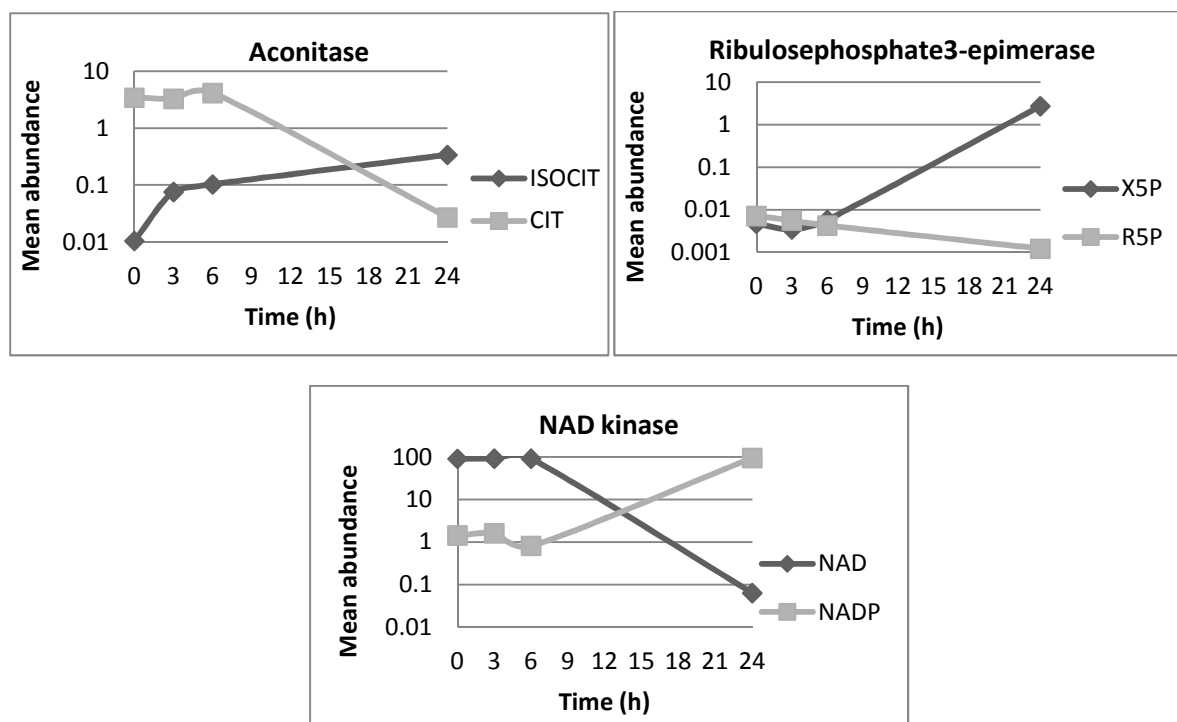


Fig 2: The possible involvement of enzymes in illustrated metabolite relationships over time. Aconitase converts citrate to isocitrate. Ribulose phosphate 3-epimerase converts ribulose-5-phosphate to xylulose-5-phosphate. NAD kinase converts NAD to NADP.

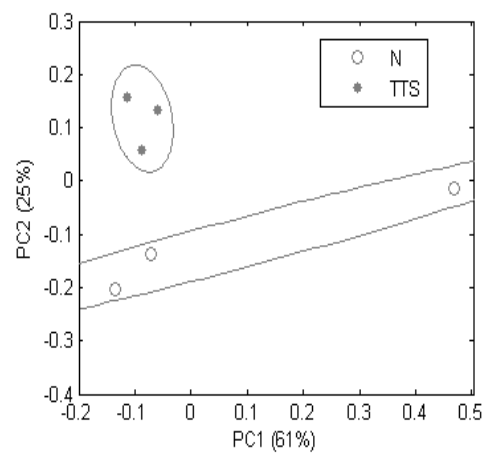


Fig 3: PCA score plot comparing the metabolite profiles of the control HeLa cells (N) and HeLa-TTS cells (TTS)

Table 1: Metabolites significantly different between the time-lapse groups and their correlation with the waiting period.

Metabolites	$p^{\#}$	Fisher's LSD ^Δ	r^*
6-Phosphogluconate	< 0.001	0h – 24h; 3h – 24h; 6h – 24h	-0.77639
NAD	< 0.001	0h – 24h; 3h – 24h; 6h – 24h	-0.77286
Xylulose-5-phosphate	< 0.001	24h – 0h; 24h – 3h; 24h – 6h; 6h – 3h	0.79073
Phosphoenolpyruvate	< 0.001	0h - 24h; 0h - 6h; 3h - 24h; 6h - 24h	-0.85859
Malate	< 0.001	0h - 24h; 3h - 24h; 6h - 24h	-0.77461
Citrate	< 0.001	0h - 24h; 3h - 24h; 6h - 24h	-0.74107
3-phosphoglycerate / 2-phosphoglycerate	< 0.001	0h - 24h; 3h - 24h; 6h - 24h	-0.81846
Fructose-1,6-biphosphate	< 0.001	0h - 24h; 3h - 24h; 6h - 24h	-0.71815
Ribose-5-phosphate	< 0.001	0h - 24h; 3h - 24h; 6h - 24h	-0.84566
NADP	0.002009	24h - 0h; 24h - 3h; 24h - 6h	0.62787
Isocitrate	0.009909	24h - 0h; 6h - 0h; 24h - 3h	0.85535
dihydroxyacetone phosphate/ glyceraldehydes-3-phosphate	0.01298	24h - 0h; 24h - 3h; 24h - 6h	0.61105
fructose-6-phosphate/ glucose-6-phosphate	0.040696	24h - 0h; 24h - 3h	0.706

[#] the p-value indicates statistical differences between metabolites of the respective groups. A $p < 0.05$ was considered statistically significant.

^Δ Indicate in which groups the respective compound was significantly different. The group given first in a comparison have a higher mean abundance of the respective compound.

* Pearson's correlation coefficient. An absolute $r > 0.7$ are considered significant.

Table 2: Metabolites differing significantly between the control and transfected HeLa cells as identified with the Student's t-test.

Metabolites	<i>p</i>
Malate	0.00327
Acetyl-CoA	0.00566
Fumarate	0.01331
Isocitrate	0.01351
Fructose-1,6-biphosphate	0.01485
NAD	0.02777
NADPH	0.03935
Xylulose-5-phosphate	0.04753