

An analytical method to investigate the metabolites and co-factors of the methionine-homocysteine cycle in women using combined oral contraceptives and non-users

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ABSTRACT

Biotransformation protects the body from endogenous and exogenous substances like combined oral contraceptives (COCs). During biotransformation, estrogen undergoes methylation with the assistance of catechol-O-methyltransferase (COMT) to limit the production of carcinogenic products. Effective COMT activity is crucial to limit the formation of these carcinogenic products. The importance of estrogen methylation is demonstrated by the fact that women with polymorphisms in the COMT-pathway, which negatively affect the methylation rate, are more prone to develop breast cancer. The conversion of catechol estrogens to methoxy-estrogens seems to be reduced in COC users. However, whether this is the result of inhibition of the COMT enzyme or due to the limited availability of co-factors is not known. Analytes that form part of the methionine-homocysteine cycle, such as betaine, S-adenosyl-methionine (SAM), and 5-methyl-tetrahydro folic acid (5-MTHFA,) act as methyl group donors during the methylation of catechol estrogens. The methionine-homocysteine cycle is a multi-step cycle including a significant number of metabolites and co-factors. To determine how the methionine-homocysteine cycle is influenced by COCs (and other xenobiotics), an analytical method is required to successfully quantify all the metabolites and co-factors of the methionine-homocysteine cycle.

During the first part of this study, the focus was to implement and further optimise a liquid chromatography-electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) method to quantify 18 metabolites and co-factors of the methionine-homocysteine cycle. This method included the use of a C18 column together with perfluoroheptanoic acid (PFHpA) as an ion-pairing agent. Cystine and homocystine were added to this method, and sample preparation was optimised. The LC-ESI-MS/MS method was successfully developed and partially validated by assessing parameters such as linearity, repeatability, precision, accuracy, and stability. The analytes of interest gave good linearity and repeatability, although some analytes showed less than ideal precision and accuracy, particularly those that were investigated at low concentrations.

The final part of this study focused on implementing the LC-ESI-MS/MS method to determine how COC use affects the methylation metabolism in pre-menopausal women. Serum samples obtained for the estrogen biotransformation and oxidative stress status (eBOSS) study were used and included COC users and non-users. The method was successfully applied to these samples and indicated a significant difference in the levels of betaine, choline, dimethyl glycine (DMG), total cysteine, and ratios such as betaine:choline and DMG:betaine when the two groups were compared. These results suggest that COC use may affect the methionine-homocysteine cycle. Further investigation is needed to better understand the results obtained and the potential impact it may have.

Keywords: methionine-homocysteine cycle, liquid chromatography, mass spectrometry, ion-pairing

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LIST OF ABBREVIATIONS

×g	Relative centrifugal force
µm	Micrometre
µmol	Micromole
3-PG	3-Phosphoglycerate
5-methyl-THF	5-Methyl- tetrahydrofolate
5-MTHF	5-Methyltetrahydrofolate
ABC	ATP-binding cassette
Acetyl-CoA	Acetyl coenzyme A
ACN	Acetonitrile
AJSESI	Agilent Jet Stream ESI
API	Atmospheric pressure ionisation
ATP	Adenosine triphosphate
BHMT	Betaine homocysteine methyltransferase
BMI	Body mass index
BRCA	Breast cancer gene
C	Carbon
C18	Octadecylsilane
Ca ²⁺	Calcium ion
CBS	Cystathionine β-synthase
CE	Catechol estrogens
CI	Chemical ionisation
CO ₂	Carbon dioxide
CoA	Coenzyme A
COCs	Combined oral contraceptives
COMT	Catechol-O-methyltransferase
COOH	Carboxylic acid
CTH	Cystathionine γ-lyase
CV	Coefficient of variance
CYP	Cytochrome P450
DHF	Dihydrofolate
DMG	Dimethylglycine
DMGD	DMG-dehydrogenase
dMRM	Dynamic MRM
DNA	Deoxyribonucleic acid
DRSP	Drospirenone

DTT	Dithiothreitol
E1	Estrone
E2	Estradiol
E3	Estriol
EE	Ethinyl estradiol
EI	Electron impact ionisation
ESI	Electrospray ionisation
eV	Electron volts
FA	Formic acid
FAB	Fast atom bombardment
FAD	Flavin adenine dinucleotide
FC	Fold change
FMN	Flavin mononucleotide
GC	Gas chromatography
GCS	Glycine cleavage enzyme system
GNMT	Glycine N-methyltransferase
GPx	Glutathione peroxidase
GS	GSH synthetase
GSH	Glutathione in its reduced form
GSSG	Glutathione in its oxidised form
GT	Glutathione transferase
H ₂ O	Water
HCA	Homocysteic acid
HCl	Hydrogen chloride
HER-2	Human growth factor receptor 2
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
L	Litre
LC	Liquid chromatography
LLE	Liquid liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
M	Molar
m/z	Mass to charge ratio
MAT	Methionine adenosyl transferase
MeOH	Methanol

mg/ml	Milligram per millilitre
min	Minutes
mM	Milli molar
mm	Millimetre
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MT	Methyltransferases
MTR	Methionine synthetase
N ₂	Nitrogen
NAD	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NAPQI	N-Acetyl-p-benzoquinoneimine
ng	Nanograms
NH ₂	Amino radical
NH ₃	Ammonium
NH ₄ OAc	Ammonium acetate
NNMT	Nicotinamide-N-methyltransferase
O	Oxygen
OH	Hydroxide
PA	4-Pyridoxic acid
PAPS	Phosphoadenosine phosphosulfate
PCA	Principle component analysis
PFHpA	Perfluoroheptanoic acid
PLP	Pyridoxal-5'-phosphate
PLS-DA	Partial least squares discriminant analysis
PMP	Pyridoxamine 5'-phosphate
PNP	Pyridoxine 5'-phosphate
POMT	Phenol O-methyltransferase
ppm	Parts per million
psi	Pounds per square inch (6.89kPa)
PTFE	Polytetrafluoroethylene
Q1	First quadrupole
Q3	Third quadrupole
QQQ	Triple quadrupole
r ²	Correlation coefficient
RBC	Red blood cells

RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S	Sulfur
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-methionine
SARDH	Sarcosine dehydrogenase
SHMT	Serine hydroxymethyltransferase
SLC	Solute carrier
SNPs	Genetic polymorphisms
SO ³⁻	Sulfur trioxide
SPE	Solid phase extraction
SULT	Sulfotransferase
TCEP	Tris(2-carboxyethyl) phosphine
THF	Tetrahydrofolate
THF	Tetrahydrofolate
UDP-GA	Uridine-5'-diphospho- α -glucuronic acid
UGT	Uridine 5'-diphospho-glucuronosyltransferase
V	Volts

CHAPTER 1 INTRODUCTION

1.1 Background and problem statement

The process of biotransformation protects the body from endobiotic and xenobiotic substances. Natural endogenous and synthetic exogenous hormones are examples of such substances. Estrogen is the primary sex hormone in women and is produced from cholesterol in the ovaries (Garrett & Grisham, 2017). Synthetic forms of this hormone are often included in hormonal products, such as combined oral contraceptives (COCs). During biotransformation, these compounds are transformed into water-soluble compounds to facilitate excretion. Endogenous and exogenous estrogens can undergo sulfation, hydroxylation and glucuronidation (Raftogianis *et al.*, 2000). Estrogen can be hydroxylated to form catechol estrogens with the help of different CYP450 isoforms (Thomas & Potter, 2013). These catechol estrogens are defined as potentially carcinogenic because they can lead to the formation of quinone-DNA adducts that may induce DNA damage and initiate carcinogenesis (Raftogianis *et al.*, 2000; Yager, 2012). Therefore, inactivation of the catechol estrogens to prevent DNA-adduct formation, is of great importance. Methylation is the most active catechol estrogen detoxification pathway. The process of methylation leads to the formation of methoxyestrogens that can be easily excreted (Raftogianis *et al.*, 2000; Yager, 2012). The enzyme catechol-O-methyltransferase (COMT) is responsible for the methylation of catechol estrogens and transfers the methyl group from S-Adenosyl-Methionine (SAM) to the hydroxyl group of catechol estrogens (Raftogianis *et al.*, 2000; Thomas & Potter, 2013; Yager, 2012). Various factors can disturb this methylation process, and thus, estrogen biotransformation homeostasis. Polymorphisms in the gene that encodes for the enzyme COMT may lead to variations in enzyme activity (Cavalieri *et al.*, 2006). Some polymorphisms might cause COMT activity to decrease, leading to a possible increase in estrogen quinones and depurinating DNA adduct formation, subsequently increasing the risk of tumour formation. Because SAM donates a methyl group to COMT during methylation (biotransformation) (Yager, 2012), insufficient COMT activity and ineffective methylation might influence SAM levels and other metabolites of the methionine-homocysteine cycle. Therefore, the regulation of SAM and COMT is crucial to ensure that this biotransformation process proceeds as needed. The SAM necessary for the methylation of catechol estrogens is generated in the methionine-homocysteine cycle. During this cycle, the amino acid methionine is converted to SAM (Jančová & Šiller, 2012). After SAM has donated its methyl group, the metabolite S-adenosyl-homocysteine (SAH) is formed. During an enzymatic reaction, SAH is converted to homocysteine (Gropper & Smith, 2012). This amino acid can be subjected to trans-sulfuration or re-methylation (Blom & Smulders, 2011; Gropper & Smith, 2012). During trans-sulfuration,

homocysteine reacts with serine, enzymes and co-factors such as vitamin B₆ to produce cysteine (Blom & Smulders, 2011; Gropper & Smith, 2012). Re-methylation is the process where homocysteine is converted back to methionine (Espina *et al.*, 2015; Mato *et al.*, 2015; Nekrassova *et al.*, 2003; Newton *et al.*, 2010; Rehman *et al.*, 2020). This is a vitamin B₁₂ dependent reaction (Gropper & Smith, 2012; Mato *et al.*, 2015; Rasmussen & Møller, 2000). During this process, a methyl group is donated by the folate cycle, which is closely linked to the methionine-homocysteine cycle (Blom & Smulders, 2011; Gropper & Smith, 2012). There are a large number of metabolites and co-factors involved in the methionine-homocysteine cycle. The regulation of all these metabolites and co-factors is critical to ensure the effectiveness of the cycle.

During the eBOSS (estrogen, biotransformation and oxidative stress status) study, the biotransformation profile and redox state of pre-menopausal women using COCs containing drospirenone and ethinyl estradiol were analysed and compared to non-users. The preliminary results obtained from the eBOSS study indicated that the use of COCs alters the biotransformation homeostasis and the level of metabolites associated with the methionine-homocysteine cycle (e.g., glutathione). At the biotransformation and oxidative stress status (BOSS) laboratory of the North-West University (NWU), the urinary hormone metabolite profile of clinically referred patients is routinely analysed. Problems with the methylation metabolism are reflected by low levels of the methoxy-estrogens and increased levels of the glutathione conjugates and DNA adducts. After these patients have been treated with a co-factor cocktail that promotes the detoxification of the catechol estrogens and the quinones, their urinary hormone profiles change, often showing better methylation and less adduct formation.

1.2 Problem statement

The conversion of catechol estrogens to methoxy-estrogens seems to be reduced in COC users. However, it is not known whether this is the result of inhibition of the COMT enzyme, or due to limited availability of co-factors. In order to determine how the methionine-homocysteine cycle is influenced by the use of COCs (and other xenobiotics), an analytical method is required to successfully quantify metabolites and co-factors of the methionine-homocysteine cycle.

1.3 Research aims and objectives

1.3.1 Aim

The aim of this study is to develop a serum-based liquid chromatography-electrospray tandem mass spectrometry method to quantify the metabolites and co-factors of the methionine-

homocysteine cycle in order to investigate the effect of combined oral contraceptives on this pathway.

1.3.2 Objectives

The following objectives will be used to achieve the aim stated:

1. Successfully implement the method developed by Guiraud *et al.* (2017).
2. Optimise the method of Guiraud *et al.* (2017).
3. Partially validate the method to ensure the reliability and effectiveness of the method by studying parameters like linearity, precision, accuracy, and stability.
4. Application of the developed and validated method on samples from the eBOSS study including a group of combined oral contraceptive users and a control group, to determine whether COC use has any effect on the metabolites and co-factors in the methionine-homocysteine cycle.

1.4 Experimental design

During this study, the experimental design in Figure 1.1 was used to achieve the objectives listed in Section 1.3.2.

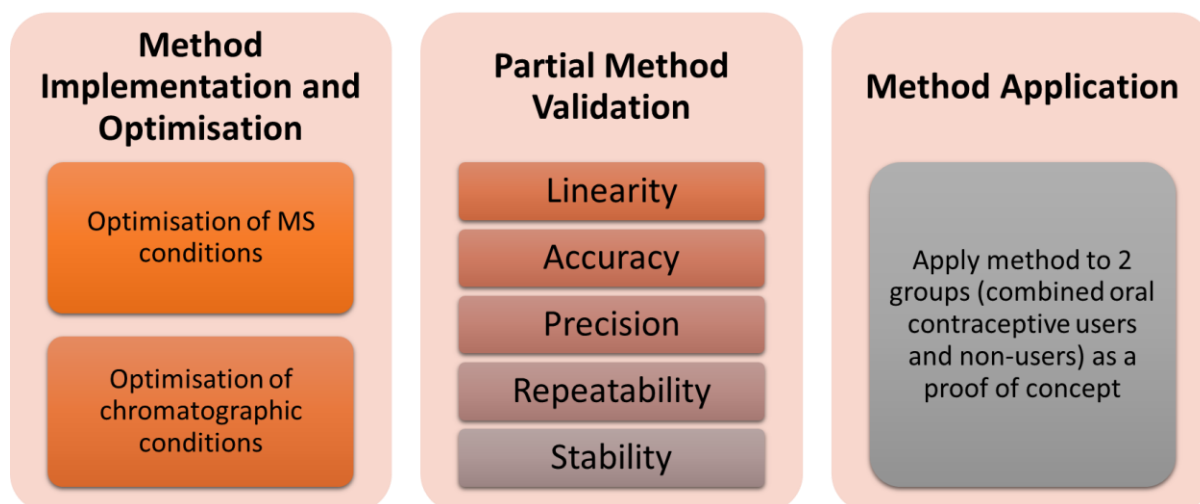


Figure 1-1 Experimental design to achieve the aim and objectives

1.5 Outline of dissertation

The five chapters of this dissertation can be described as follows. Chapter 1 (Introduction) gives an overview of the study, problem statement, describes the relevance, and the aim and

objectives of the study. Chapter 2 (Literature Review) includes an overview of the relevant literature and serves to provide the necessary background information and place the current study in context of the wider field. It includes literature on biotransformation, estrogen metabolism, the methionine-homocysteine cycle, combined oral contraceptives, cancer, and methods previously used for quantification. Chapter 3 (Methodology) describes all the materials and methods that were used during this study. This was done in a way that ensures the reader understands the processes that were followed to complete this study. In Chapter 4 (Results and Discussion), the results obtained during the study were displayed by means of figures and tables. Chapter 5 (Method application) includes the results obtained after the method was applied to a group of combined oral contraceptive users and non-users. After the statistical analysis of the data, these results were processed and discussed from a biochemical perspective. Chapter 6 (Conclusion and Future Prospects) includes conclusive remarks on the study conducted. After the thorough assessment of these conclusions, future recommendations were proposed. The reference list was compiled according to the NWU Harvard referencing style.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

In this chapter, the focus will be on providing literature that will help in understanding the connection between biotransformation (especially of estrogens), the methionine-homocysteine cycle, the effect of exogenous estrogen use, and the possible connection thereof with breast cancer. Analytical methods of investigating the methionine-homocysteine cycle will also be discussed.

2.2 Biotransformation

Biotransformation is the enzymatic transformation of chemical compounds (from endogenous and exogenous sources) to water-soluble chemicals in the liver of a living organism to facilitate excretion. The physiochemical properties of the foreign chemical are transformed from favouring absorption across biological membranes to favouring elimination (Merriam-Webster, 2020; Parkinson & Ogilvie, 2008). These chemicals can become inactive, active or toxic during reaction with the enzymes that catalyse biotransformation reactions (Phang-Lyn & Llerena, 2020). Xenobiotics can be defined as chemical compounds that are foreign to a living organism (Chen, 2020; Merriam-Webster, 2020). The foreign chemicals to which humans are exposed can include pharmaceutical drugs, natural chemicals, steroids, pesticides and pollutants (Chen, 2020; Parkinson & Ogilvie, 2008). Exposure to these chemicals can lead to their accumulation to toxic levels (Parkinson & Ogilvie, 2008). Biotransformation of xenobiotics serves as a detoxification mechanism (Parkinson & Ogilvie, 2008). Therefore, the biotransformation of these foreign compounds is of great importance. When the body is overexposed to a xenobiotic, the expression of the specific biotransformation enzymes involved can be upregulated, resulting in more efficient elimination of the xenobiotic that caused upregulation (Parkinson & Ogilvie, 2008). Reactive oxygen species (ROS) can be produced during the biotransformation of certain xenobiotics (Parkinson & Ogilvie, 2008). Oxidative stress can cause cell toxicity. The toxic effects of ROS can be limited by glutathione, glutathione transferase and glutathione peroxidase (Chen, 2020; Jefferies *et al.*, 2003; Parkinson & Ogilvie, 2008). Factors such as age, sex, nutritional status, disease, medication and genetic factors can lead to variation in drug metabolism (Chen, 2020; Parkinson & Ogilvie, 2008).

Hydrolysis, reduction, oxidation and conjugation are the four categories of biotransformation reactions. The enzymes involved in biotransformation are dependent on the type of reaction (Parkinson & Ogilvie, 2008). Some xenobiotics can be transformed non-enzymatically, for

example, during the conjugation with glutathione. Biotransformation can be divided into different phases. Hydrolysis, reduction and oxidation reactions are classified as Phase I reactions. These reactions expose or introduce a functional group that can be converted to a water-soluble conjugate (Chen, 2020; Parkinson & Ogilvie, 2008). Conjugation reactions are classified as Phase II reactions (Chen, 2020; Parkinson & Ogilvie, 2008).

2.2.1 Phase 0

Phase 0 biotransformation can be described as the absorption of foreign compounds across the basolateral membrane of the liver cells (Chen, 2020; Nortje *et al.*, 2016; Petzinger & Geyer, 2006). Solute carrier (SLC) transporters are embedded in the basolateral membrane and assist with the absorption of these foreign compounds. These carriers can facilitate the absorption of compounds with different chemical structures (Petzinger & Geyer, 2006).

2.2.2 Phase I

After Phase 0 biotransformation, Phase I reactions transform the parent compound into more polar metabolites by exposing or introducing a functional group (Parkinson & Ogilvie, 2008; Phang-Lyn & Llerena, 2020). This reaction occurs primarily during hepatic circulation. Oxidation, reduction and hydrolysis are the three types of Phase I reactions, with oxidation being the most common (Parkinson & Ogilvie, 2008; Phang-Lyn & Llerena, 2020). This reaction can produce active metabolites and these may become substrates for phase II biotransformation (Phang-Lyn & Llerena, 2020). Cytochrome P450 (CYP) enzymes are of great importance during these reactions. CYPs form a superfamily of enzymes containing heme as a co-factor (Jančová *et al.*, 2010; Parkinson & Ogilvie, 2008; Phang-Lyn & Llerena, 2020). These enzymes catalyse the oxidation of xenobiotics, the initial step of xenobiotic biotransformation. CYPs can be located in the inner membrane of the mitochondria or in the endoplasmic reticulum of the cells (Phang-Lyn & Llerena, 2020; Werck-Reichhart & Feyereisen, 2000).

2.2.3 Phase II

During phase II biotransformation, a polar metabolite is formed by adding endogenous hydrophilic groups to form water-soluble, inactive metabolites ready for excretion (Chen, 2020; Parkinson & Ogilvie, 2008; Phang-Lyn & Llerena, 2020). Compounds that undergo phase II biotransformation include the original compound, and toxic or nontoxic metabolites formed during phase I biotransformation (Phang-Lyn & Llerena, 2020). These compounds and metabolites have either -OH, -NH₂ or -COOH groups (Jančová & Šiller, 2012; Phang-Lyn & Llerena, 2020) to which certain molecules can be conjugated. Conjugation reactions serve as

a detoxifying step for foreign compounds during which a high-energy intermediate is produced. During these conjugation reactions, the activated conjugating agent combines with a substrate or amino acid to yield a conjugation product (Jančová & Šiller, 2012). These reactions include glucuronidation, sulfonation, methylation and amino acid conjugation (Chen, 2020; Jančová *et al.*, 2010; Jančová & Šiller, 2012). The conjugation reactions require cofactors such as adenosine triphosphate (ATP), phosphoadenosine phosphosulfate (PAPS), and acetyl coenzyme A (acetyl-CoA) and co-enzymes to help assist the detoxification of foreign compounds (Bachmann, 2009). Some examples of co-factors needed during conjugation reactions are uridine-5'-diphospho- α -glucuronic acid (UDP-GA) during glucuronidation, S-adenosylmethionine (SAM) during methylation, and glycine and taurine during amino acid conjugation (Parkinson & Ogilvie, 2008). Specific enzymes are of importance during the catalysis of phase II conjugation reactions, including sulfotransferase during sulfonation, and methyltransferase during methylation (Parkinson & Ogilvie, 2008). If the activity of these enzymes are decreased, due to the presence of a polymorphism, it can lead to an increase in the toxic effects of clinical drugs because of a decrease in the detoxification of the exogenous compounds (Jančová *et al.*, 2010; Jančová & Šiller, 2012). For the purpose of this study, more emphasis will be placed on O-methylation and especially the enzyme COMT in this literature review.

2.2.3.1 Methylation and COMT

As mentioned previously, methylation is an example of a Phase II conjugation reaction. This reaction is involved in the metabolism of endogenous compounds such as neurotransmitters, nucleic acids and drugs. S-adenosyl-methionine (SAM), formed in the methionine-homocysteine cycle from methionine and ATP, is the co-factor that is essential for the formation of methyl conjugates (Jančová & Šiller, 2012). During methylation, water-soluble metabolites are formed when a methyl group is transferred from SAM, known as the methyl donor, to endogenous compounds. Compounds can undergo N-, O-, S- and arsenic-methylation (Jančová & Šiller, 2012). Different enzymes are responsible for these methylation reactions (Parkinson & Ogilvie, 2008). Nicotinamide-N-methyltransferase (NNMT) is an example of a N-methyltransferase that is responsible for the N-methylation of nicotinamide. The enzyme S-methyltransferase catalyses S-methylation of anticancer and immunosuppressive thiopurines (6-mercaptopurine) (Jančová *et al.*, 2010; Jančová & Šiller, 2012). During O-methylation, the enzymes phenol O-methyltransferase (POMT) and COMT are responsible for the methylation of phenols and catechols, respectively. Catecholamine neurotransmitters (epinephrine, norepinephrine and dopamine) and catechol estrogens are inactivated by COMT (Jančová & Šiller, 2012) (Figure 2.1).

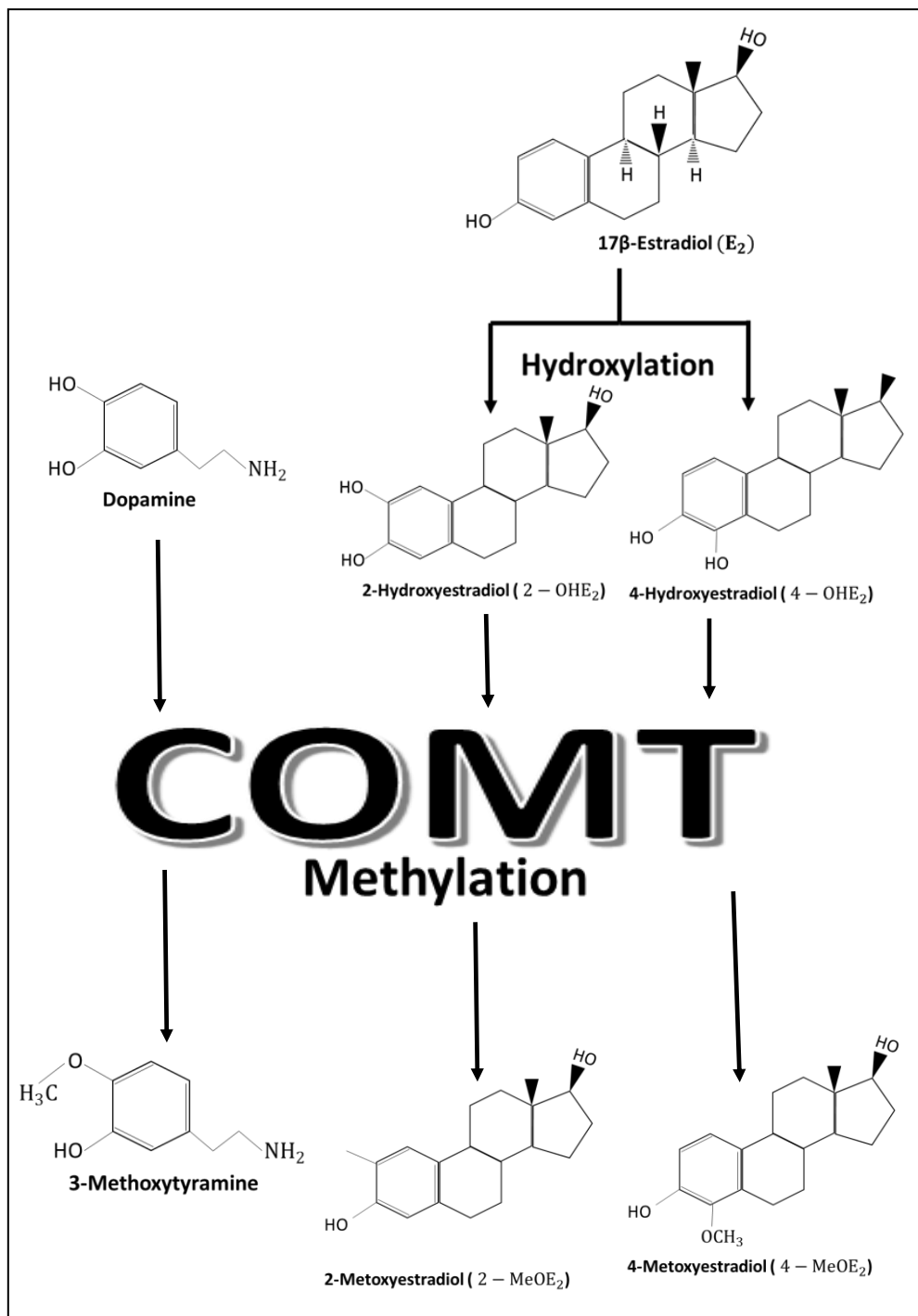


Figure 2-1 Methylation, a Phase II biotransformation pathway, with the help of the COMT enzyme. Catechol metabolites like catecholamines and -estrogens are methylated during Phase II biotransformation. During the methylation of catechol estrogens, methoxyestrogens are formed. Adapted from Yager (2012).

2.2.4 Phase III

During Phase III biotransformation, the metabolites can undergo further metabolism and excretion with the help of transporters (Chen, 2020). Examples of transporters involved in this phase include ATP-binding cassette (ABC) and SLC transporters (Chen, 2020; Döring & Petzinger, 2014; Phang-Lyn & Llerena, 2020). Energy (ATP) is required during transportation of the chemical from one side of the cell membrane to the other by ABC (Chen, 2020; Phang-Lyn & Llerena, 2020). A SLC transporter can transport the metabolite across the cellular membrane (Chen, 2020; Phang-Lyn & Llerena, 2020).

2.3 Methionine-homocysteine cycle

In this section, the methionine-homocysteine cycle will be explained in detail. The importance of the cycle and the connection to other parts of the metabolism is emphasised. A detailed explanation of important metabolites and co-factors of the methionine-homocysteine cycle, responsible for the optimal performance of the methionine-homocysteine cycle, is given.

2.3.1 Overview

In order to maintain the balance between folate metabolism, methionine metabolism and homocysteine trans-sulfuration, sufficient levels of the individual metabolites and co-factors involved are required (Figure 2.2). Folate (vitamin B₉) is a water-soluble B-vitamin that is present in numerous interconvertible forms (Warzyszynska & Kim, 2014), and forms part of the 13 essential vitamins (Greenberg *et al.*, 2011). The chemical structure of folate can be described as follows: a reduced pteridine moiety linked by a methylene group to a p-aminobenzoic acid attached to a glutamic acid (Gropper & Smith, 2012; Shane, 2010). Metabolically active folate that is present in the body has multiple glutamic acid residues (Gropper & Smith, 2012). Foliates can't be produced in the body and, therefore, need to be obtained from the diet (Gropper & Smith, 2012; Shane, 2010). Folate (a term for natural food folates, and folate found in dietary supplements and fortified foods (Pietrzik *et al.*, 2010)) is the reduced form of the vitamin that is naturally found in food (green vegetables, peanuts, strawberries and oranges, and liver) and biological tissue (Greenberg *et al.*, 2011; Gropper & Smith, 2012). Folic acid is known as the synthetic form of folate and is the oxidised form of the vitamin (Gropper & Smith, 2012; Shane, 2010; Warzyszynska & Kim, 2014). Folic acid is fully monoglutamylated, causing increased stability and bioavailability compared to folates (Gropper & Smith, 2012; Shane, 2010; Warzyszynska & Kim, 2014). Folic acid can be found in fortified foods (cereals, bread and grains) and supplements (Gropper & Smith, 2012), and is metabolised to reduced forms of folate, mostly 5-methyl- tetrahydrofolate (5-methyl-THF),

where it behaves as naturally reduced folates (Shane, 2010). Ingested folate and folic acid are reduced to form dihydrofolate (DHF) that is in turn reduced to tetrahydrofolate (THF). In the presence of serine and the co-factor vitamin B₂, THF produces 5,10-methylene THF (Figure 2.2). With the help of the enzyme methylene-THF-reductase, 5,10 – methylene THF forms 5-methyl-THF (5-MTHF) (Gropper & Smith, 2012)

The folate cycle is closely coupled to the methionine-homocysteine cycle via 5-MTHF (Zheng & Cantley, 2019). During the re-methylation of homocysteine back to methionine, in the presence of methionine synthetase (MTR) and co-factor vitamin B₁₂ (cobalamin), 5-MTHF is the methyl donor (Blom & Smulders, 2011; Shane, 2010). With the help of MTR and vitamin B₁₂, THF is produced after donating the methyl group (Gropper & Smith, 2012). This is a continuous process. Folates serve other important roles as well, for example: the derivatives of THF serve as donors of one-carbon units during the synthesis of purines, pyrimidines and amino acids (Gropper & Smith, 2012), and folates are also of great importance in DNA synthesis, the maintenance of DNA integrity, and the repair of DNA (Shane, 2010). The formation of 5-MTHF is important for DNA methylation and, because of the connection explained earlier, it is important for the regulation of the methionine-homocysteine metabolism (Greenberg *et al.*, 2011; Gropper & Smith, 2012; Shane, 2010). If there is a deficiency of MTR or insufficient amounts of vitamin B₁₂, folates will be trapped in the 5-methyl-THF form. This will cause lower concentrations of 5,10-methyleneTHF, which is known to be important for DNA synthesis (Zheng & Cantley, 2019). Elevated concentrations of homocysteine might also be seen. An imbalance in the folate cycle can, therefore, have a negative effect on important processes in the human body, such as the methionine-homocysteine cycle, which may also affect hormone methylation metabolism (Greenberg *et al.*, 2011; Shane, 2010).

The methionine-homocysteine cycle consists of a number of reactions involving sulfur-containing amino acids, water soluble B vitamins and other co-factors (Da Silva *et al.*, 2016). The co-factors obtained from the diet are essential for effective functioning of the methionine-homocysteine cycle (Guiraud *et al.*, 2017). In the methionine-homocysteine cycle, methionine is catabolised and converted to SAM by an adenosyl transferase enzyme (Figure 2.2, right side of figure). ATP is required during this reaction. SAM donates a methyl group and forms SAH with the help of a methyl transferase enzyme. The enzyme SAH hydrolase converts SAH to homocysteine. Homocysteine can be converted back to methionine, using a vitamin B₁₂- and folate- dependent reaction or a betaine-dependent reaction (Gropper & Smith, 2012). The vitamin B₁₂- and folate-dependent reaction is known as re-methylation and is the first step in the metabolism of homocysteine and dependent on the methionine synthase (MTR) enzyme (Figure 2.2) (Blom & Smulders, 2011; Gropper & Smith, 2012). The methyl group needed for

re-methylation is provided by methylcobalamin, which received the methyl group from 5-methyl-THF from the folate cycle discussed earlier (Blom & Smulders, 2011; Gropper & Smith, 2012). The metabolism of choline, folate and methionine comes together at the point of re-methylation of homocysteine. Re-methylation occurs when methionine concentrations are low (Rehman *et al.*, 2020). At high concentrations of methionine or an increased demand for cysteine, homocysteine undergoes trans-sulfuration (Rehman *et al.*, 2020). During trans-sulfuration homocysteine reacts with serine to produce cystathionine, which in turn, will be converted to cysteine (Gropper & Smith, 2012). Trans-sulfuration is a vitamin B₆-dependent reaction (Blom & Smulders, 2011; Gropper & Smith, 2012).

The methionine-homocysteine cycle contributes to the regulation of human health (Guiraud *et al.*, 2017). The products of the methionine-homocysteine cycle are important for energy production and detoxification (Bak *et al.*, 2019). Cysteine can be converted to glutathione or taurine and pyruvate (Figure 2.2). Glutathione is a powerful antioxidant and of great importance during Phase II detoxification of electrophilic compounds (Jančová & Šiller, 2012; Parkinson & Ogilvie, 2008). Pyruvate is important during the production of energy. Alterations in the metabolites and co-factors of the methionine-homocysteine cycle have been linked to cardiovascular disease, changes in cognitive function cancer, aging, regulation of gene expression and development (Da Silva *et al.*, 2016; Guiraud *et al.*, 2017). Late pregnancy complications, neurodegenerative disease, psychiatric disease and osteoporosis are diseases that can occur because of impaired folate and methionine-homocysteine metabolism (Blom & Smulders, 2011).

it can be classified as a non-proteinogenic amino acid. Homocysteine has two pathways of metabolism (Mato *et al.*, 2015). The first pathway is known as re-methylation, during which homocysteine is metabolised to methionine (Espina *et al.*, 2015; Mato *et al.*, 2015; Nekrassova *et al.*, 2003; Newton *et al.*, 2010; Rehman *et al.*, 2020). Re-methylation is favoured during low methionine levels. The second pathway is the trans-sulfuration pathway, where homocysteine is metabolised to cysteine, in the presence of excess methionine or increased cellular demand of cysteine (Espina *et al.*, 2015; Mato *et al.*, 2015; Nekrassova *et al.*, 2003; Newton *et al.*, 2010; Rehman *et al.*, 2020). Both re-methylation and trans-sulfuration are key steps in the methionine-homocysteine cycle and, therefore, homocysteine is of great importance to ensure that this cycle works efficiently.

Normal levels of homocysteine range between 3.5 – 15 µmol/L (Espina *et al.*, 2015; Newton *et al.*, 2010; Rasmussen & Møller, 2000). These levels can be influenced by different factors that include genetic factors, age, sex, menopausal status, physiological conditions and lifestyle conditions (including dietary factors, exercise routine and alcohol consumption) (Espina *et al.*, 2015; Newton *et al.*, 2010; Rasmussen & Møller, 2000). Therefore, it is expected that homocysteine levels will vary in individuals because of these factors that also differ from individual to individual. Homocysteine concentration between age groups and sex groups has been investigated. It has been proven that the total homocysteine increases with age, and it was also shown that men have higher homocysteine concentrations than women (Jacques *et al.*, 1999; Nygård *et al.*, 1995; Selhub *et al.*, 1993). Folic acid, vitamin B₆ and vitamin B₁₂ are important co-factors involved in the methionine-homocysteine cycle and, therefore, the availability thereof may influence the levels of homocysteine (Newton *et al.*, 2010). The intake of insufficient amounts of these co-factors results in an increase in homocysteine levels (Rehman *et al.*, 2020). As mentioned earlier, homocysteine is formed during the metabolism of methionine and, therefore, the methionine content of dietary protein may affect the levels of homocysteine in individuals.

The intracellular concentrations of homocysteine are tightly controlled. Folate-dependent re-methylation helps maintain optimal homocysteine concentrations in the cells. If the cellular capacity for this amino acid is exceeded, homocysteine is exported into the extracellular space (blood) until the intracellular levels are normal (Espina *et al.*, 2015).

Homocysteine is present in three forms in the blood (Figure 2.3). Together, these three forms are known as total plasma homocysteine, and include the reduced form (1-2%); the disulfide form (10-30%), in which a homocysteine molecule is bound to another homocysteine molecule; and the mixed disulfide form (70-80%), where the homocysteine molecule is bound to other thiols or proteins (Guiraud *et al.*, 2017; Nekrassova *et al.*, 2003; Newton *et al.*, 2010;

Rasmussen & Møller, 2000). The disulfides and mixed disulfides are formed during the oxidation of the -SH group in the presence of an electron acceptor. Homocysteine disulfides are known as homocystine (Newton *et al.*, 2010).

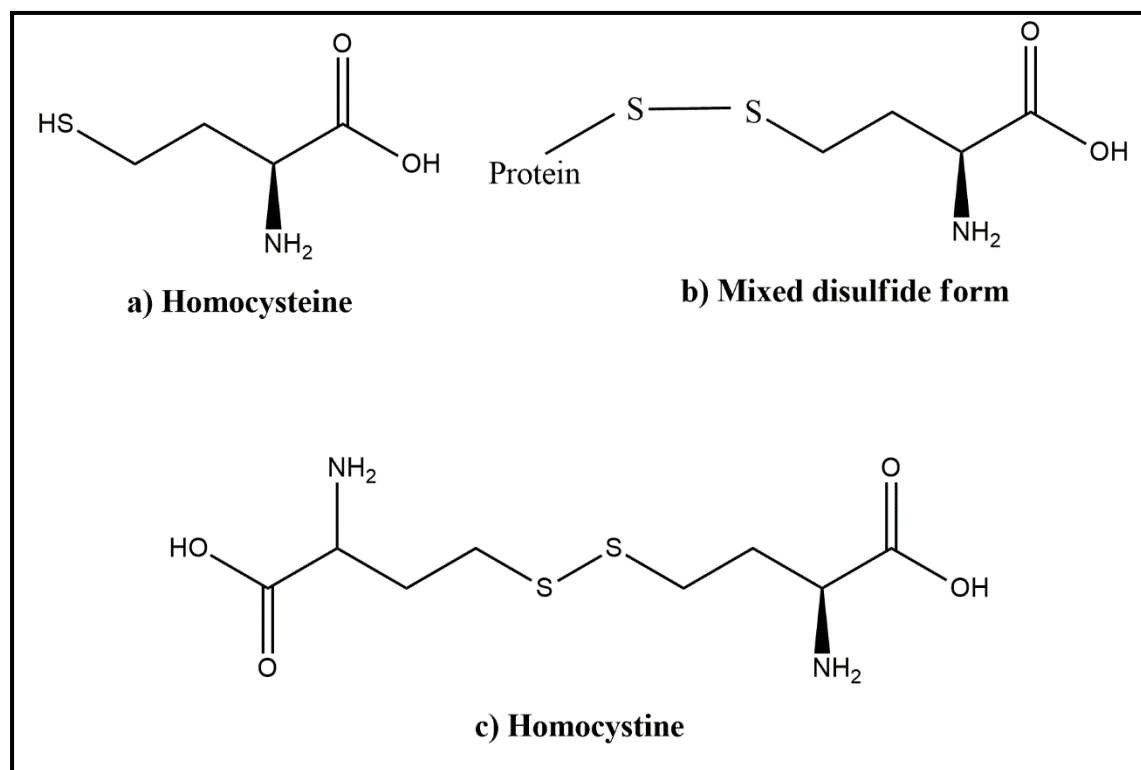


Figure 2-3 Different forms of Homocysteine. a) Is the structure of the reduced form called homocysteine, b) is the mixed disulfide form, and c) is the disulfide form, called homocystine. Adapted from Newton *et al.* (2010); Rasmussen and Møller (2000).

The presence of disulfides and mixed-disulfides makes it challenging to develop a reliable analytical method to quantify homocysteine (Guiraud *et al.*, 2017). Reduction of the disulfide bonds and protein precipitation are pre-treatment strategies that can be used to efficiently quantify the total homocysteine present in a sample.

2.3.2.2 Methionine

Methionine is one of the 20 common amino acids and can be classified as a sulfur containing, non-polar, neutral amino acid. Methionine is classified as an essential amino acid and needs to be obtained from dietary sources, for example, eggs, fish and meats (Gropper & Smith, 2012; Rasmussen & Møller, 2000; Schmidt *et al.*, 2016). This essential amino acid is absorbed rapidly (Gropper & Smith, 2012). As mentioned during the discussion of homocysteine (Section 2.2.3.1), the re-methylation of homocysteine produces methionine in a betaine-

dependent reaction or in a vitamin B₁₂ (cobalamin)- and folate-dependent reaction (Gropper & Smith, 2012; Mato *et al.*, 2015; Rasmussen & Møller, 2000). The metabolism of methionine occurs mainly in the liver (Gropper & Smith, 2012). Methionine is a precursor of succinyl-CoA, homocysteine, cysteine, creatine and carnitine (Figure 2.2) (Martínez *et al.*, 2017). By means of the enzyme methionine adenosyl transferase (MAT) and in the presence of ATP, methionine is converted to SAM (Figure 2.2) (Gropper & Smith, 2012). SAM inhibits the recycling of methionine because it is responsible for the inhibition of methylene-THF reductase activity, which facilitates the formation of 5-methyl-THF. This compound is essential during the formation of methionine from homocysteine (Figure 2.2) (Gropper & Smith, 2012). Normal levels of methionine can range between 23-38 µmol/L.

Methionine levels can be increased due to elevated methionine intake in the diet. A lack of vitamin cofactors, genetic polymorphisms (SNPs) in the genes of important enzymes and the fluctuation of ATP in the methionine-homocysteine cycle can cause dysfunction in this cycle and result in the excess production of homocysteine (Rehman *et al.*, 2020). This excess homocysteine production can increase the rate of re-methylation, causing a possible increase of methionine concentrations. A SNP in the MAT enzyme, for example, may cause elevated methionine levels because the conversion to SAM is reduced (Mudd, 2011). Transmethylation and transsulfuration are upregulated when methionine levels are elevated (Bertolo & McBreairty, 2013). On the other hand, insufficient methionine levels cause the downregulation of transsulfuration and upregulation of re-methylation to ensure methionine production (Bertolo & McBreairty, 2013). Dietary methionine deficiency can be treated by increasing dietary sources of methionine or supplementation (Bertolo & McBreairty, 2013).

2.3.2.3 Cysteine

Like methionine, cysteine is also one of the 20 common amino acids. This polar, sulfur-containing amino acid is classified as semi-essential, as it is obtained from the diet (poultry, eggs, beef and whole grains) but it can be made endogenously from cystathionine. The production of cysteine from cystathionine is part of the methionine-homocysteine cycle, (Figure 2.2, bottom) (Wang *et al.*, 2005). Vitamin B₆ and the enzyme cystathionine gamma-lyase (CTH) are needed during the production of cysteine (Figure 2.2). Because cysteine can be obtained from the diet, the production of cysteine during transsulfuration is less important (Stipanuk & Ueki, 2011).

Cysteine is an important component of glutathione (Lyons *et al.*, 2000; Salway, 2016), an important antioxidant, but can also be converted to taurine, sulfate and pyruvate (Gropper & Smith, 2012). The sulfur from the methionine is transferred to cysteine, whereafter the sulfur

atom is released by oxidation or excretion. Sulfate and taurine are excreted in the urine (Stipanuk & Ueki, 2011). Therefore, the production of cysteine can be seen as valuable in detoxification and energy production (Figure 2.2).

Normal levels of cysteine range between 271-392 $\mu\text{mol/L}$. Low levels of cysteine can cause slower growth, liver damage, muscle and fat loss, to name a few (Wang *et al.*, 2005). Increased methionine levels are required to replenish cysteine when there is insufficient intake through the diet (Bertolo & McBreaity, 2013).

2.3.2.4 S-adenosyl-methionine (SAM)

S-adenosyl-methionine (SAM) is defined as the active sulfonium form of methionine, which acts as a methyl donor during methylation reactions (Da Silva *et al.*, 2016; Gropper & Smith, 2012). For the formation of SAM, methionine reacts with ATP – a step, which forms a key part of the formation of homocysteine, as discussed earlier (Fontecave *et al.*, 2004; Merriam-Webster, 2021). The enzyme methionine adenosyl-transferase (MAT) assists during this reaction (Fontecave *et al.*, 2004; Rasmussen & Møller, 2000). The body cannot solely rely on dietary intake for sufficient SAM levels, and *de novo* synthesis is required. SAM is converted to s-adenosyl-homocysteine (SAH) during the removal or donation of the methyl group (Fontecave *et al.*, 2004; Rasmussen & Møller, 2000). DNA, RNA, proteins, phosphatidylethanolamine, glycine and guanidinoacetate can take up the methyl group during the formation of SAH (Mato *et al.*, 2015). These reactions are defined as methylation reactions and the enzyme group methyltransferases (MT) are involved in these reactions (Mato *et al.*, 2015). SAM is involved in the methylation of DNA (which is important for gene expression), as well as the methylation of hormones and neurotransmitters, thereby controlling the activity of the hormones and neurotransmitters (Fontecave *et al.*, 2004). Methylation is one of the three pathways for which SAM is an important precursor molecule: the other two pathways being transsulfuration and aminopropylation (Bottiglieri, 2002).

SAM plays a critical role during the regulation of methionine metabolism. High concentrations of SAM stimulate the transsulfuration pathway, with cysteine being the end product, and inhibits the re-methylation of homocysteine to methionine. SAM, therefore, facilitates the degradation of methionine (Fontecave *et al.*, 2004) and is present in every cell of the body (Bottiglieri, 2002). SAM acts as a co-factor and substrate for the methylation of DNA, protein, RNA and biosynthetic reactions of key brain metabolites (Fontecave *et al.*, 2004; Gropper & Smith, 2012). Therefore, it can be concluded that sufficient amounts of SAM are of great importance for efficient metabolism. Normal levels of SAM range between 65-150 nmol/L .

2.3.2.5 S-adenosyl-homocysteine (SAH/ AdoHcy)

As mentioned earlier, SAH is produced after the donation or removal of the methyl group from SAM (Gropper & Smith, 2012). SAH is produced as a by-product of methionine transmethylation reactions (Garibotto *et al.*, 2009; Mato *et al.*, 2015) and is known as the metabolic precursor of homocysteine (Garibotto *et al.*, 2009; Rasmussen & Møller, 2000; Wagner & Koury, 2007). SAH is hydrolysed by the enzyme SAH hydrolase to form homocysteine and adenosine (Fontecave *et al.*, 2004; Gropper & Smith, 2012; Mato *et al.*, 2015; Wagner & Koury, 2007). This reaction is reversible, and during equilibrium, the synthesis of SAH is favoured (Wagner & Koury, 2007). Increased levels of homocysteine would result in increased levels of SAH, causing the inhibition of SAM-dependent methyltransferases. SAH can, therefore, be classified as a competitive inhibitor for SAM-dependent methyltransferases (Garibotto *et al.*, 2009; Mato *et al.*, 2015; Struys *et al.*, 2000). Normal levels of SAH are ≈ 20 nmol/L (Wagner & Koury, 2007).

2.3.2.6 Choline

Choline can be made in the body via the phosphatidylethanolamine N-methyltransferase pathway (with the use of SAM) and can be obtained from the diet (Sanders & Zeisel, 2007). In its free form, choline can be found in small amounts in food, but it mostly forms part of the phospholipid lecithin, also known as phosphatidyl choline. Foods containing this phospholipid, include eggs, liver, muscle meat, wheat germ and legumes, to name a few (Sanders & Zeisel, 2007). Choline has several important functions in the body. It acts as a methyl donor and plays an important role in the formation of platelet aggregating factor, the secretion of very-low-density lipoproteins from the liver, and the synthesis of sphingomyelin, phosphatidyl choline and acetylcholine (neurotransmitter) (Sanders & Zeisel, 2007; Zeisel, 2006). Choline is important for the normal function of all cells (Sanders & Zeisel, 2007; Zeisel, 2006) and it ensures the structural integrity and signalling functions of the cell (Sanders & Zeisel, 2007). These metabolites are critical for the development of the brain of the foetus and helps maintain the levels of homocysteine during pregnancy (Sanders & Zeisel, 2007). Studies noted an increased likelihood for babies to develop neural tube defects when their mothers consumed less choline during pregnancy (Shaw *et al.*, 2004). Choline is connected to the methionine cycle as it serves as a precursor for betaine that can be used as a methyl donor (Sanders & Zeisel, 2007; Ueland *et al.*, 2007).

Choline deficiency can increase the development of carcinoma (Sanders & Zeisel, 2007). The requirement of choline differs for premenopausal and postmenopausal women. This is ascribed to estrogens that can be the mediator of increased activity of the enzyme PEMT and,

therefore, increased synthesis of choline, as studied in mice models (Sanders & Zeisel, 2007; Young, 1971; Zeisel, 2006).

2.3.2.7 Betaine

Betaine (trimethylglycine) is defined as a methylamine, as it consists of three chemically reactive methyl groups that are linked to a nitrogen atom (Obeid, 2013). Betaine is obtained from the diet through foods such as, wheat bran, beets and spinach, or it can be produced during the oxidation of choline in the liver and kidneys (Lever & Slow, 2010; Obeid, 2013). Betaine is an important osmolyte and it regulates cell volume and stabilises proteins (Lever & Slow, 2010; Obeid, 2013); it also acts as a methyl donor during the re-methylation of homocysteine to methionine (Lever & Slow, 2010). This pathway is mediated by the enzyme betaine homocysteine methyltransferase (BHMT) (Lever & Slow, 2010; Obeid, 2013). About 50% of the homocysteine methylation capacity of the liver is produced by this pathway (Lever & Slow, 2010). During the methylation of homocysteine, dimethylglycine (DMG) is produced (Obeid, 2013; Sanders & Zeisel, 2007). DMG and SAM inhibit the re-methylation pathway mentioned above and DMG controls the transfer of methylation groups (Obeid, 2013). These two main roles of betaine are not independent because the enzyme BHMT is osmoregulated (Lever & Slow, 2010). High tonicity can reduce the expression of BHMT, and betaine metabolism decreases when osmolyte concentrations need to be maintained (Lever & Slow, 2010). Betaine is also important during fat metabolism (Obeid, 2013) and is further connected to the metabolism of folate, choline, vitamin B₁₂ and methionine (Sanders & Zeisel, 2007). Finally, betaine is important during the production and maintenance of methionine and SAM (Lever & Slow, 2010).

2.3.2.8 Dimethylglycine (DMG)

Dimethylglycine (DMG) is produced from betaine by donating a methyl group to homocysteine by the enzyme BHMT (Kendall & Lawson, 2000; Obeid, 2013). DMG can be found in food such as cereal grains, seeds, beans and liver in low levels (Kendall & Lawson, 2000). During oxidative demethylation, DMG is converted to sarcosine and glycine with the help of the enzyme DMG-dehydrogenase with only a small amount being eliminated through the urine (Obeid, 2013). The two methyl groups are made available for SAM during transmethylation reactions in which folic acid, nicotinamide adenine dinucleotide (NAD⁺), flavin adenine dinucleotide (FAD) and vitamin B₁₂ act as essential factors (Kendall & Lawson, 2000). The enzyme DMG-dehydrogenase is a mitochondrial flavoprotein and folate-binding protein (Obeid, 2013). DMG-dehydrogenase produces 5,10-methylenetetrahydrofolate by transferring a one-carbon unit to tetrahydrofolate (Obeid, 2013). The concentration of DMG in the plasma

can be indicative of the use of betaine as a methyl donor (Obeid, 2013). Studies revealed that SAM concentrations show a positive correlation with plasma levels of choline and DMG but not with betaine (Obeid, 2013). DMG modulates T and B cells and their products and improves the antibody response. It impacts the immune modulation and oxidative processes and leads to better mental alertness and enhanced energy (Kendall & Lawson, 2000). DMG is important in the neurological system of the body because it acts as a precursor for an important neurotransmitter, glycine (Kendall & Lawson, 2000).

2.3.2.9 Cystathionine

This is a non-proteinogenic thioether containing amino acid and it is not present in significant amounts in the diet (Maclean *et al.*, 2012). It is formed during the reaction of homocysteine with serine as an intermediate during the transsulfuration pathway (Gropper & Smith, 2012; Maclean *et al.*, 2012). This reaction is catalysed by the vitamin B₆ (in its co-enzyme form, pyridoxal-5'-phosphate (PLP)) dependent enzyme, cystathionine β -synthase (CBS) (Gropper & Smith, 2012; Maclean *et al.*, 2012). Cystathionine is converted to cysteine, using the enzyme cystathionine γ -lyase (CTH), ammonia, and α -ketobutyrate (Gropper & Smith, 2012; Maclean *et al.*, 2012). CBS-deficient homocystinuria can be a result of a mutation in the enzyme CBS (Gropper & Smith, 2012; Maclean *et al.*, 2012). Individuals with this condition have high levels of blood homocysteine and methionine, and low levels of blood cysteine concentration (Gropper & Smith, 2012).

2.3.2.10 Glutathione

Glutathione (GSH) is synthesised from glutamate, cysteine and glycine in the liver (Jefferies *et al.*, 2003; Lu, 2013). It can exist in reduced (GSH) and oxidised (GSSG) form, with the reduced form being the predominant form (Lu, 2013). The synthesis of glutathione is divided into two steps. During the first step, γ -glutamylcysteine is produced from glutamate and cysteine (produced during transsulfuration) by the enzyme glutamate-cysteine ligase (GCL) (Lu, 2013). Thereafter, GSH is produced from γ -glutamylcysteine and glycine (Lu, 2013). This step is catalysed by the enzyme GSH synthetase (GS) (Lu, 2013). GSH has many important functions, including that it is the main intracellular antioxidant (Bottiglieri, 2002; Cooper & Hanigan, 2018; Jefferies *et al.*, 2003; Lu, 2013; Wang *et al.*, 2013); it modulates cell proliferation and immune response (Jefferies *et al.*, 2003; Lu, 2013); it helps regulate signal transduction within the cell (Jefferies *et al.*, 2003); and it defends the body against reactive electrophiles (Cooper & Hanigan, 2018; Jančová & Šiller, 2012). GSH degrades harmful molecules, such as reactive oxygen and nitrogen species, dietary oxidants and xenobiotics (Jefferies *et al.*, 2003). Reactive oxygen species (ROS) can include hydroxyl radicals,

superoxide anion, hydrogen peroxide and nitric oxide; these molecules can cause oxidative damage to lipids, DNA and proteins, and can cause peroxidation of lipoproteins (Jefferies *et al.*, 2003). Two types of enzymes are important during the inactivation of ROS or reactive nitrogen species (RNS) (Jefferies *et al.*, 2003; Lu, 2013): glutathione peroxidase (GPx) and GSH reductase. GPx catalyses the inactivation of ROS and RNS through the formation of GSSG. This is achieved by the formation of a disulfide bond between two GSH molecules (Jefferies *et al.*, 2003; Lu, 2013). Thereafter, GSH reductase catalyses the formation of two GSH molecules from GSSG (Jefferies *et al.*, 2003; Lu, 2013).

Some electrophilic xenobiotics (compounds that contain a C-, N- or S- atom) can potentially be harmful to the body (Cooper & Hanigan, 2018; Jančová & Šiller, 2012). During GSH conjugation, GSH launches a nucleophilic attack on these compounds to help defend the body against the potential harmful effect, by forming a less toxic molecule that will be excreted (Cooper & Hanigan, 2018; Jančová & Šiller, 2012; Jefferies *et al.*, 2003). Glutathione conjugation is a Phase II conjugation reaction. The enzyme glutathione transferase (GT) is responsible for these conjugation reactions (Jančová & Šiller, 2012; Parkinson & Ogilvie, 2008). This includes the detoxification of environmental carcinogens and reactive products produced after being metabolised by other enzymes (for example, after Phase I biotransformation reactions) (Parkinson & Ogilvie, 2008). During GSH S-conjugation, the conjugates are excreted into the bile or metabolised to a more water-soluble compound, mercapturic acid (Cooper & Hanigan, 2018; Jančová & Šiller, 2012). A good example of a GSH-conjugation reaction is the GSH-conjugation (biotransformation) of acetaminophen (paracetamol). During the first step, CYP450 helps convert paracetamol into an electrophilic, toxic compound N-Acetyl-p-benzoquinoneimine (NAPQI) (Athersuch *et al.*, 2018). Thereafter, NAPQI is metabolised to acetaminophen GSH-conjugate (GSH-conjugate) by the enzyme GT. The acetaminophen GSH-conjugate can be metabolised into cysteinyl acetaminophen (cysteine S-conjugate) with the help of enzyme transferase and peptidase enzymes (Athersuch *et al.*, 2018; Cooper & Hanigan, 2018; Jančová & Šiller, 2012). The mercapturate of acetaminophen is formed after the N-acetylation of the cysteinyl acetaminophen (Athersuch *et al.*, 2018; Cooper & Hanigan, 2018; Jančová & Šiller, 2012).

2.3.2.11 Sarcosine

Sarcosine (N-methylglycine) is a non-proteinogenic amino acid (Cernei *et al.*, 2013; Gropper & Smith, 2012). Sarcosine can be found in the diet in eggs, vegetables, poultry and other meats (Strzelecki *et al.*, 2015). The formation of sarcosine occurs in the mitochondria via two pathways (Cernei *et al.*, 2013). In the first pathway, phosphatidylethylamine is methylated by SAM to phosphatidylcholine that produces betaine (Cernei *et al.*, 2013; Strzelecki *et al.*, 2015).

Betaine is converted to DMG, which is then converted to sarcosine by the enzyme DMG-dehydrogenase (DMGD) (Cernei *et al.*, 2013; Gropper & Smith, 2012; Obeid, 2013; Sreekumar *et al.*, 2009). In the second pathway, sarcosine is produced from glycine during the transformation of the methyl group of SAM via the enzyme glycine N-methyltransferase (GNMT) (Cernei *et al.*, 2013; Strzelecki *et al.*, 2015). Sarcosine produces glycine when the methyl group is removed from sarcosine by the enzyme sarcosine dehydrogenase (SARDH) (Cernei *et al.*, 2013; Gropper & Smith, 2012; Sreekumar *et al.*, 2009; Strzelecki *et al.*, 2015).

GNMT is important in the regulation of SAM levels, and it affects DNA methylation by regulating gene expression (Cernei *et al.*, 2013). When SARDH is inactive, increased levels of sarcosine will be present (sarcosinemia) (Cernei *et al.*, 2013). An increased production of GNMT can cause cleavage of glycine to sarcosine, which leads to elevated levels of sarcosine in the urine (Cernei *et al.*, 2013). This can lead to an increased production of homocysteine and sarcosine, increasing the utilisation of SAM (Cernei *et al.*, 2013).

2.3.2.12 Glycine

Glycine is a nonessential amino acid and the simplest amino acid containing a single hydrogen side chain (Garrett & Grisham, 2013; Razak *et al.*, 2017; Wang *et al.*, 2013). The amount of glycine produced in the body is not enough for metabolic demands (Wang *et al.*, 2013); therefore, glycine intake is of importance. Glycine can be obtained from meat, fish, legumes and gelatine in the diet (Razak *et al.*, 2017), and it is produced from choline and serine (Wang *et al.*, 2013). Choline is converted to betaine (as discussed in 2.3.4.6 and 2.3.4.7), betaine is converted to DMG (as discussed in 2.3.4.7 and 2.3.4.8), and DMG is converted to sarcosine (as discussed in 2.3.4.8 and 2.3.4.10). In turn, sarcosine is converted to glycine by the enzyme sarcosine dehydrogenase (Gropper & Smith, 2012; Wang *et al.*, 2013). Methyl groups are generated during this conversion (Wang *et al.*, 2013). Sarcosine and glycine are interconvertible amino acids (Wang *et al.*, 2013). Sarcosine dehydrogenase controls the ratio of SAH to SAM (Razak *et al.*, 2017). In the folate cycle, serine gives a methyl group to 5,10-methylene-THF to produce glycine in the kidneys via the enzyme serine hydroxymethyltransferase (SHMT) (Gropper & Smith, 2012; Obeid, 2013; Razak *et al.*, 2017). This step is reversible. Glycine can also be generated by the catabolism of glutathione in the kidneys (Gropper & Smith, 2012). Glycine can be degraded via three pathways: conversion into glyoxylate via D-amino acid oxidase, conversion into serine by SHMT and deamination and decarboxylation by the glycine cleavage enzyme system (GCS) (Wang *et al.*, 2013). The GCS degrades glycine into NH_3 and CO_2 (Wang *et al.*, 2013).

Glycine has many beneficial effects. Glycine is responsible for the production of glutathione, proteins such as collagen and elastin, creatine, porphyrins, and glycocholate (bile salt) (Gropper & Smith, 2012; Obeid, 2013; Wang *et al.*, 2013). Most of the glycine present in the human body is used for protein synthesis, such as collagen and elastin, as previously mentioned (Razak *et al.*, 2017). Creatine is important in muscle and nerve cell energy metabolism (Kendall & Lawson, 2000; Wang *et al.*, 2013). Glycine has an important function in the central nervous system, where it acts as an inhibitory neurotransmitter, regulating behaviour, food intake and body homeostasis (Katzung, 2012; Kendall & Lawson, 2000; Wang *et al.*, 2013). Glycine also helps regulate immune function and the production of superoxide by modulating intracellular Ca^{2+} levels (Wang *et al.*, 2013). The pathways that generate RNA and DNA utilise glycine (Razak *et al.*, 2017). Glycine plays a key role in the digestion and absorption of lipids and lipid-soluble vitamins because it is the major amino acid for the conjugation of bile acids (Wang *et al.*, 2013). A glycine shortage can lead to immune response failure, low growth and abnormal nutrient metabolism (Wang *et al.*, 2013).

2.3.2.13 Serine

Serine is a non-essential amino acid with a polar hydroxyl group in the side chain (El-Hattab, 2016). Serine is derived from the anabolism of the glycolytic intermediate, 3-phosphoglycerate (3-PG), the diet, protein and phospholipid turnover, and via synthesis from glycine (El-Hattab, 2016; Wang *et al.*, 2013). The synthesis of serine from 3-PG is the major source of serine (El-Hattab, 2016). As mentioned in Section 2.4.3.12, glycine and serine are interconvertible via a reaction in the kidneys that involves donating single-carbon units (Gropper & Smith, 2012). For this reaction, THF and 5,10-methylene THF are essential coenzymes, and serine hydroxymethyltransferase is the enzyme required to catalyse the reaction (Gropper & Smith, 2012). In the methionine-homocysteine cycle, serine reacts with homocysteine to produce cystathionine, using the enzyme cystathionine synthase (Gropper & Smith, 2012; Sinha *et al.*, 2020). Serine is degraded either by conversion to glycine or by the enzyme serine dehydrogenase to produce pyruvate (El-Hattab, 2016).

Serine is important during the production of compounds such as cysteine, glycine, methionine, sphingolipids and phosphatidylserine (El-Hattab, 2016; Gropper & Smith, 2012). The formation of sphingolipids aids in the formation of sphingomyelin, which enables efficient synaptic transmission (Sinha *et al.*, 2020). Serine contributes to the redox homeostasis during the synthesis of glycine (El-Hattab, 2016). Serine is an important amino acid in protein synthesis and is involved in cellular homeostasis, proliferation and differentiation (El-Hattab, 2016). If serine levels are too high or too low, there can be hyper or hypo methylation of DNA

(Sinha *et al.*, 2020). Irregular levels of serine can affect the stress response, proliferation metabolism and the response to external stimuli (Sinha *et al.*, 2020).

2.3.2.14 Taurine

Taurine is a sulfur containing amino acid (Gropper & Smith, 2012). Humans are unable to synthesise sufficient levels of taurine and, therefore, are reliant on dietary sources (Huxtable, 1992; Schaffer & Kim, 2018). Seafood and meat are main sources of taurine in the diet (Schaffer & Kim, 2018). Taurine is produced in the liver at a slow rate (Gropper & Smith, 2012; Schaffer & Kim, 2018). In the methionine-homocysteine cycle, cysteine is converted to cysteine sulfate that can be used to produce taurine with the help of a decarboxylase enzyme (Gropper & Smith, 2012; Huxtable, 1992). The production of taurine from cysteine is a vitamin B₆ dependent reaction (Gropper & Smith, 2012). Taurine is excreted without any other further metabolism in the form of taurocholate and other bile salts (Huxtable, 1992; Schaffer & Kim, 2018).

Taurine exhibits antioxidant properties; in the retina, for example, where it helps maintain the structure and function of photoreceptor cells (Gropper & Smith, 2012; Schaffer & Kim, 2018). Taurine acts as an anti-inflammatory agent, neutralising the neutrophil oxidant, hypochlorous acid (Schaffer & Kim, 2018). Taurine decreases the generation of superoxide in the mitochondria (Gropper & Smith, 2012; Schaffer & Kim, 2018). Taurine can limit oxidative stress through the prevention of damage to antioxidant enzymes (Schaffer & Kim, 2018). These three activities sum up the antioxidant role of taurine.

It was reported that taurine is deficient in the hearts of patients suffering from heart failure (Jeejeebhoy *et al.*, 2002). Genes that are sensitive to taurine, contribute to cellular function (Schaffer & Kim, 2018). Taurine functions as a bile salt in the liver and intestine, and as an inhibitory neurotransmitter in the central nervous system (Gropper & Smith, 2012; Schaffer & Kim, 2018). Using taurine therapy may help restore mitochondrial protein biosynthesis, improve mitochondrial function, and reduce the production of superoxides (Schaffer & Kim, 2018). In body fluids such as plasma, taurine concentrations range from 10-100 µM (Huxtable, 1992).

2.3.3 Co-factors in the methionine-homocysteine cycle

The enzymes of the methionine-homocysteine cycle require co-factors to catalyse the formation of the metabolites described in Section 2.2.2.

The co-factors that are important in the methionine-homocysteine cycle are vitamin B₂, vitamin B₆ and 5-MTHF (Figure 2.2). These are water-soluble vitamins (Gropper & Smith, 2012; Powers, 2003). Vitamin B₆ serves as a co-factor for the formation of cystathionine from homocysteine, and cysteine from cystathionine, whereas vitamin B₂ helps with the formation of 5-MTHF (Midttun *et al.*, 2005). 5-MTHFA assists in the formation of methionine (Warzyszynska & Kim, 2014).

Vitamin B₆ can exist in seven forms, which are: pyridoxine, pyridoxine 5'-phosphate (PNP), pyridoxal, PLP, pyridoxamine, pyridoxamine 5'-phosphate (PMP), and 4-pyridoxic acid (PA) (Bisp *et al.*, 2002; Midttun *et al.*, 2005; Ueland *et al.*, 2015; Vrolijk *et al.*, 2017). These seven forms of vitamin B₆ exhibit equivalent vitamin activity because they are interconvertible in metabolism (Bisp *et al.*, 2002). Vitamin B₆ can be found in meat, nuts, fish, bananas and whole grains (Gropper & Smith, 2012; Vrolijk *et al.*, 2017). Pyridoxal and PLP are found in animal food products and pyridoxine, pyridoxamine, PNP and PMP are found in plants (Midttun *et al.*, 2007). In the diet, vitamin B₆ is present as pyridoxine, pyridoxal and pyridoxamine (Midttun *et al.*, 2007; Vrolijk *et al.*, 2017) that are phosphorylated by the enzyme pyridoxal kinase to produce pyridoxine phosphate, pyridoxal phosphate and pyridoxamine phosphate, respectively (Midttun *et al.*, 2007). Vitamin B₆ is important as a co-enzyme in reactions of the amino acid, carbohydrate, lipid, organic acid and fatty acid metabolism, and in the synthesis of neurotransmitters (Bisp *et al.*, 2002; Ueland *et al.*, 2015; Vrolijk *et al.*, 2017). The conversion of homocysteine to cysteine (transsulfuration) is a vitamin B₆ (pyridoxal 5'-phosphate) dependent reaction (Ueland *et al.*, 2015). Therefore, adequate vitamin B₆ is necessary to prevent elevated homocysteine concentrations. The majority of vitamin B₆ is converted to pyridoxal 5' phosphate in the liver (Vrolijk *et al.*, 2017). Pyridoxine phosphate oxidase assists in the oxidation of pyridoxamine phosphate and pyridoxine phosphate to pyridoxal phosphate (Midttun *et al.*, 2007). Excess pyridoxal is oxidised in the liver to form 4-pyridoxal acid (4-PA), which is excreted in the urine (Midttun *et al.*, 2007; Vrolijk *et al.*, 2017). A deficiency in vitamin B₆ can be due to a low intake, malabsorption, or the intake of drugs that inhibit the enzymes of the vitamin B₆ metabolism and usually goes hand in hand with the deficiency of other B-vitamins (Ueland *et al.*, 2015; Vrolijk *et al.*, 2017). Mutations causing defects in the vitamin B₆ pathway can cause vitamin B₆ deficiency (Ueland *et al.*, 2015). Controversial results on the effect of the use of oral contraceptives on vitamin B₆ levels has been reported (Wilson *et al.*, 2011).

Riboflavin (Vit B₂) is found in beef liver, steak, mushrooms, non-fat milk, dairy products, cereals, dark green vegetables and fatty fish (Gropper & Smith, 2012; Powers, 2003). Diets lacking dairy products and meat can lead to riboflavin deficiency (Powers, 2003). It consists

of a flavin (isoalloxazine ring) and a ribitol (sugar alcohol) side chain. Riboflavin can be found in free or protein-bound forms in food (Gropper & Smith, 2012). Only a small amount of the riboflavin found in food is free riboflavin (Powers, 2003). Riboflavin is primarily excreted in the urine (Gropper & Smith, 2012). The two coenzyme derivatives of riboflavin are flavin mononucleotide (FMN) and FAD (Gropper & Smith, 2012). Most of the riboflavin present in food is found in the FAD and FMN derivative forms (Powers, 2003). Before dietary riboflavin can be absorbed, FAD and FMN must be hydrolysed to riboflavin (Powers, 2003). FAD is converted to FMN by FAD pyrophosphatase and FMN is converted to riboflavin by the enzyme FMN phosphatase (Gropper & Smith, 2012). Riboflavin participates in redox reactions (an important reaction in the human metabolism) through FAD and FMN acting as electron carriers (Powers, 2003). Although some studies indicate that riboflavin deficiency can increase the risk of cancer at certain sites, controversy exists around this (Qiao, 1989; Rivlin, 1973). Riboflavin is important during the metabolism of homocysteine through re-methylation. FAD acts as a co-factor for the enzyme methylenetetrahydrofolate reductase that metabolises folate to the form that is used in homocysteine methylation (Powers, 2003). A mutation of the enzyme methylenetetrahydrofolate reductase (the 677C → T thermolabile variant) is associated with the increase in plasma homocysteine concentrations (Kang *et al.*, 1991). The metabolism of Vitamin B₆ is flavin-dependent and there is impaired synthesis of pyridoxal phosphate in riboflavin deficiency (Lakshmi & Bamji, 1974; Lakshmi & Bamji, 1976).

Folate (vitamin B₉) can be present in different forms and is the term for natural food folates, and folate found in dietary supplements and fortified foods (Pietrzik *et al.*, 2010). These folates function as enzyme cofactors and carry one-carbon molecules. Other B vitamins, such as vitamin B₂ and Vitamin B₆ are important during the homeostasis of one-carbon metabolism (see Figure 2.2) (Midttun *et al.*, 2005). Folic acid, dihydrofolate (DHF), tetrahydrofolate (THF), 5,10-methylene-THF, 5,10-methyl-THF and 5-methyl-THF are some of the intermediates that are important in the folate cycle (Pietrzik *et al.*, 2010; Warzyszyńska & Kim, 2014). Folates found in food are in the THF form, whereas folate in fortified foods and supplements contain folate in the form of folic acid or 5-MTHF. Green leafy vegetables, citrus fruit, legumes, dry cereals, and liver are sources of folate (Warzyszyńska & Kim, 2014). Folates that are consumed through food are converted to the 5-MTHF form. The conversion of homocysteine to methionine is a folate dependent reaction (Figure 2.2) and folate is of critical importance in this cycle (Cavalieri *et al.*, 1997; Pietrzik *et al.*, 2010). Folate is important during the synthesis of DNA and RNA, where it functions as a co-enzyme during single carbon transfer and cell division (Pietrzik *et al.*, 2010). During folate deficiency, homocysteine concentrations increase due to a decrease in the availability of 5-MTHF (Warzyszyńska & Kim, 2014).

From the foregoing discussion, it is clear that all these metabolites and co-factors have important functions in the body and that their levels need to be finely regulated. Deficiencies in the production of these metabolites and co-factors can lead to insufficient amounts of other compounds because of the fact that these metabolites and co-factors are closely linked. In order to detect disturbances or dysfunctions of the biochemical pathways in which they are involved, reliable methods for the accurate investigation and quantification of these metabolites are needed.

2.4 Estrogen metabolism and methylation

This section will explain estrogen metabolism, the importance of the COMT enzyme and the methionine-homocysteine cycle. It also focuses on the effect that exogenous estrogen use (COCs and hormone replacement therapy (HRT)) has and its connection to breast cancer.

2.4.1 Overview

Estrogen is the primary sex hormone for women and is produced from cholesterol in the ovaries (Garrett & Grisham, 2017). Proliferation and growth of cells responsible for secondary sexual characteristics of the female is promoted by the estrogens (Guyton & Hall, 2006). Estrogens play a critical role during reproduction, the menstrual cycle, fat metabolism and autoimmunity (Thomas & Potter, 2013). Estrogen is the collective name for estrone (E1), estradiol (E2) and estriol (E3). The enzyme aromatase is responsible for the production of estrone and estradiol. Aromatase consists of 503 amino acids and an iron-containing haem group (Thomas & Potter, 2013). Estrone is produced during aromatisation of androstenedione that is produced from cholesterol. Estradiol is produced during the aromatisation of testosterone that is produced by androstenedione (Cavalieri *et al.*, 2006; Thomas & Potter, 2013). Estriol is an oxidative product derived from estradiol and estrone (Guyton & Hall, 2006; Thomas & Potter, 2013). 17 β -Estradiol is the 17 β isomer of estradiol. Estrone and 17 β -estradiol are interconverted by the enzyme 17 β -estradiol dehydrogenase as can be seen in Figure 2.4 (Cavalieri *et al.*, 2006; Thomas & Potter, 2013). Estradiol is converted to estrone by a 16 α -hydroxyestrone intermediate (Thomas & Potter, 2013). Estrogens can be stored in the form of estrogen sulfate, synthesised by estrone sulfotransferases (Raftogianis *et al.*, 2000; Thomas & Potter, 2013). The enzyme steroid sulfatase can convert estrone sulfate to estrogen (Thomas & Potter, 2013).

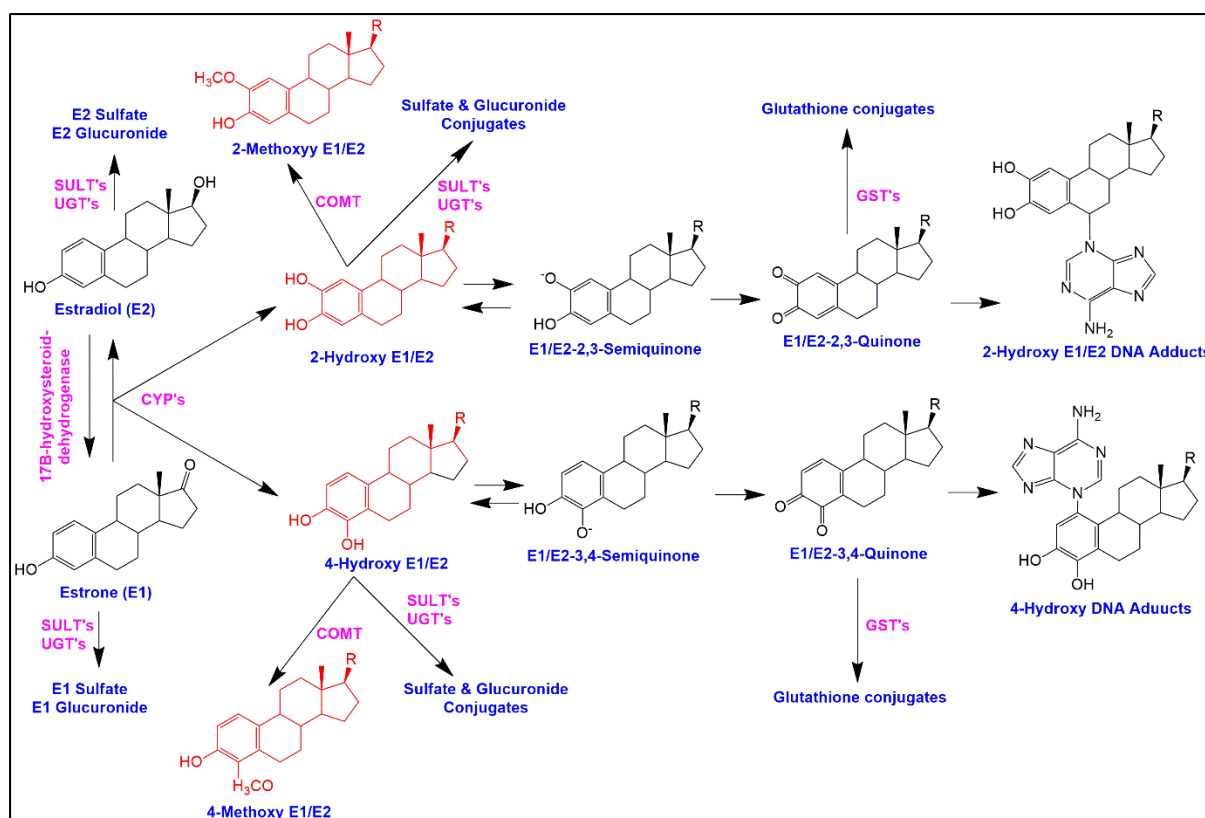


Figure 2-4 An illustration of estrogen metabolism and biotransformation.

2.4.2 Estrogen metabolism and biotransformation

Endogenous and exogenous E1 and E2 can undergo three reactions: sulfation, glucuronidation and hydroxylation (Raftogianis *et al.*, 2000). During sulfation, the enzyme sulfotransferase (SULT) transfers a SO_3^- group to E1 or E2 (Raftogianis *et al.*, 2000). The E1/E2 sulfate conjugates have a longer half-life than the parent estrogens (Raftogianis *et al.*, 2000). During glucuronidation, the enzyme uridine 5'-diphospho-glucuronosyltransferase (UGT) metabolises the conjugation of UDP glucuronic acid to the 3 or 17 β hydroxyl group of E1/E2 (Raftogianis *et al.*, 2000). The sulfate and glucuronidase conjugated products are the main form in which estrogens are excreted (Thomas & Potter, 2013). The hydroxylation of E1 and E2 leads to the formation of catechol estrogens (CE) (Parl *et al.*, 2009; Raftogianis *et al.*, 2000). Hydroxylation of E1 and E2 occurs at the 2-, 4- and 16-positions of the steroidal A ring leading to the production of 16 α -hydroxy-E1/E2, 2-hydroxy-E1/E2 and 4-hydroxy-E1/E2 (Raftogianis *et al.*, 2000; Thomas & Potter, 2013). Hydroxylation of estrone and estradiol is mediated by different isoforms of the CYP450 system (Thomas & Potter, 2013). These 2-hydroxy-E1/E2 and 4-hydroxy-E1/E2 are oxidised to form semi quinones and CE quinones (Raftogianis *et al.*, 2000). 2-Hydroxy-E1/E2 quinones and 4-hydroxy-E1/E2 quinones can lead to the formation of depurinating DNA adducts that have the potential to cause mutations (Figure 2.4) (Raftogianis *et al.*, 2000; Yager, 2012). These depurinating adducts can lead to

apurinic DNA sites and the formation of permanent mutations, causing tumourigenesis (Cavalieri *et al.*, 1997). During estrogen metabolism, catechol estrogens enter the redox cycle and become a source of ROS (Raftogianis *et al.*, 2000).

The inactivation of CE is of great importance. During biotransformation, these harmful metabolites are detoxified (Raftogianis *et al.*, 2000; Thomas & Potter, 2013). CE can be detoxified by methylation, glucuronidation and sulfation, and CE quinones can be detoxified by GSH conjugation (Raftogianis *et al.*, 2000; Thomas & Potter, 2013). Methylation is the most active CE detoxification pathway. During methylation, the enzyme COMT is used to transfer a methyl group from SAM, which is acting as the methyl donor, to the hydroxyl group of the CE (Raftogianis *et al.*, 2000; Thomas & Potter, 2013; Yager, 2012). This causes the formation of methoxyestrogens (Raftogianis *et al.*, 2000; Yager, 2012). Detoxification of the CEs via sulfation and glucuronidation occur to a lesser extent than methylation. During sulfation, SULTs catalyse the conjugation of CEs, and during glucuronidation, UGTs catalyse the conjugation of CE. CE quinones can undergo detoxification via conjugation with GSH, lowering the formation of CE quinones and the potential for DNA damage (Parl *et al.*, 2009; Raftogianis *et al.*, 2000). GSH conjugation can lower the possibility of oxidative stress and increase the formation of mercapturic acid that can easily be excreted (Raftogianis *et al.*, 2000). The risk of DNA damage caused by catechol estrogens can depend on the ability to conjugate the catechol estrogens and catechol estrogen quinones relative to the formation of catechol estrogens and catechol estrogen quinones (Raftogianis *et al.*, 2000).

2.4.3 Exogenous hormone use and breast cancer

2.4.3.1 Exogenous hormone use

COCs and HRT are the main types of exogenous hormones used by women. COCs (combinations of synthetic estrogen and progesterone) are hormonal drugs that are designed to be used by women for the purpose of birth control (Cooper & Mahdy, 2019; Rosendaal *et al.*, 2002). Different synthetic progestin forms that can be seen as the hormone that prevents ovulation, and therefore pregnancy, can be found in different types of the COC pills (Berg, 2015; Cooper & Mahdy, 2019; Rosendaal *et al.*, 2002). These include norethidrone acetate, norethidone, desogestrel, norgestrel and drospirenone (Petitti, 2003). Ethinyl estradiol is the synthetic estrogen present in most COCs (Berg, 2015; Rosendaal *et al.*, 2002). Estrogen is responsible for controlling menstrual bleeding (Cooper & Mahdy, 2019; Rosendaal *et al.*, 2002). These drugs (COCs) are also prescribed to be used for the treatment of acne, menstrual pains, irregular menstruation, fibroids and endometriosis-related pains (Ammembaal & Udupa, 2021; Cooper & Mahdy, 2019; De Leo *et al.*, 2016). HRTs are hormone replacement

drugs (consisting of estrogen with or without progesterone) (Hickey *et al.*, 2012; Nelson *et al.*, 2002) used by post-menopausal women to help relieve menopausal symptoms such as hot flashes and night sweats (Hickey *et al.*, 2012; Rosendaal *et al.*, 2002). HRT can prevent osteoporosis and the development of cardiovascular diseases (Rosendaal *et al.*, 2002).

A great number of pre – and post – menopausal women are exposed to exogenous hormones. The use of COCs and HRTs is not without risk and not all risks of the use thereof are fully known. There are some known adverse effects of COCs. Progestins can bind to androgen and glucocorticoid receptors and cause side-effects commonly associated with COC use, such as breakthrough bleeding, abdominal cramping, nausea, weight gain, headaches, breast tenderness and mood swings (Cooper & Mahdy, 2019; De Leo *et al.*, 2016; Erkkola & Landgren, 2005). Increased occurrence of acne and lower high-density lipoprotein cholesterol is caused by androgen progestins, while glucocorticoid progestins can cause bloating by causing the retention of salt and water (Berg, 2015). The use of COCs has an increased risk of venous thrombosis (the formation of blood clots) (Berg, 2015; Petitti, 2003; Rosendaal *et al.*, 2002). Estrogen receptors that are present in the liver are involved in blood clot production (Berg, 2015). The increased risk is because ethinyl estradiol is more potent than natural estradiol and, therefore, it remains in the body for a longer period. The ethinyl estradiol hyperactivates the ER-mediated clotting pathway in the liver, increasing the risk for the formation of blood clots (Berg, 2015; Petitti, 2003). Controversial evidence exists regarding the risk of breast cancer formation and the use of COCs (Berg, 2015; Petitti, 2003), but recent studies show that there might be a higher frequency of breast cancer formation in COC users (Ammemba & Udupa, 2021). The exogenous estrogens can stimulate estrogen receptors causing an increased risk for carcinogen formation (Ammemba & Udupa, 2021). It is, therefore, important to continue studying the possible link between COCs and breast cancer formation. Pulmonary embolism, stroke, cardiovascular disease, breast cancer and endometrial cancer are potential risks of HRT use (Hickey *et al.*, 2012). There is an increased risk of breast cancer development in women using HRT (estrogen and progestin) (Chen *et al.*, 2002; Hickey *et al.*, 2012; Million Women Study Collaborators, 2003). Increased exposure to endogenous or exogenous estrogen may disturb estrogen biotransformation homeostasis and increase carcinogen production, thereby increasing the risk of developing cancer (Parl *et al.*, 2009). Therefore, sufficient COMT activity is required to limit the formation of these genotoxic estrogen metabolites.

2.4.3.2 Cancer

Cancer is caused by the uncontrollable growth and division of cells (Cooper, 2000; Weinberg, 1996) that invade normal tissues and organs (Cooper, 2000). Proto-oncogenes and tumour

suppressor genes are responsible for the uncontrolled cell proliferation in human cancers (Rakoff-Nahoum, 2006; Weinberg, 1996; Yokota, 2000). Proto-oncogenes encourage growth, and when mutated, can become carcinogenic oncogenes, while tumour suppressor genes inhibit growth during the cell's lifetime and contribute to cancer when they are inactivated by mutations (Anderson *et al.*, 1992; Rakoff-Nahoum, 2006; Weinberg, 1996). When there is a mutation in a proto-oncogene, the growth stimulating pathway is overactive (Weinberg, 1996). Oncogenes can cause the overproduction of growth factors that can drive proliferation on the cells that produced them (Weinberg, 1996). There are many different types of cancers with different behaviours and treatment responses (Cooper, 2000). There are two types of genes that are involved in human carcinogens; the first type is genetically altered in diverse tumour types and the other type is altered in specific tumour types (Yokota, 2000). Tumours can be classified as benign (noncancerous) when the tumour does not invade surrounding tissue and does not spread through the body, or malignant (cancerous) when these tumours are able to invade surrounding tissue and spread throughout the body via metastasis (Cooper, 2000; Sinha, 2018; Weinberg, 1996). Metastasis is the spread of cancer cells via the lymphatic or circulatory system (Cooper, 2000; Sinha, 2018). Benign tumours can be removed through surgical procedures, but the spreading of malignant tumours makes this method of treatment hard (Carlson *et al.*, 2009; Cooper, 2000; Sinha, 2018). Treatment of malignant tumours include radiation therapy, chemotherapy and immunotherapy medication (Carlson *et al.*, 2009; Sinha, 2018).

Malignant tumours can become more and more aggressive over time (Sinha, 2018; Weinberg, 1996). Most cancers can be categorised into three main groups. The first is Carcinomas, which are malignancies of epithelial cells; cells from the surfaces of the body (Carbone, 2020; Cooper, 2000). Second is Sarcomas, which are solid tumours of connective tissue (Carbone, 2020; Cooper, 2000). Third is Leukaemias and Lymphomas; Leukaemias are produced from blood-forming cells and Lymphomas are produced from cells of the immune system (Cooper, 2000).

These tumours undergo different developmental stages (Weinberg, 1996). During the first stage, a normal cell has a genetic variation and its ability to proliferate is increased (Rakoff-Nahoum, 2006; Weinberg, 1996; Yokota, 2000). During the second stage, the cells that are produced look normal but overproduce, and another mutation can occur in one of these cells (Weinberg, 1996). The proliferated cells appear abnormal in shape and another mutation is caused in the cancerous cells that changes the behaviour of the cell during stage three (Weinberg, 1996). During stage four, abnormal growth and appearance further increases and is called in situ cancer if it has not spread to other tissue or organs. In stage five, tumours can

begin to invade nearby tissue and organs because of further genetic modifications and can be classified as malignant (Weinberg, 1996; Yokota, 2000). New tumours can be produced throughout the body (Weinberg, 1996). Tumours in their later stages have greater genetic alterations than tumours in their earlier stages (Yokota, 2000).

There are many endogenous and exogenous factors that can increase the risk for cancer development, including exposure to cancer causing substances (carcinogens) (Cooper, 2000; Wu *et al.*, 2018). Radiation (e.g. solar ultraviolet radiation) and chemical carcinogens (e.g. tobacco and aflatoxin) can cause cancer by damaging DNA and inducing mutations (Cooper, 2000; Wu *et al.*, 2018). Hormones such as estrogens can be seen as a risk factor for cancer development (Cooper, 2000; Wu *et al.*, 2018) because they can stimulate cell proliferation, thereby acting as tumour promoters (Cooper, 2000).

2.4.3.2.1 Breast cancer

Breast cancer is the most common cancer among women (Ammembal & Udupa, 2021). There are certain factors that can increase the possibility of developing breast cancer. These can include a family history of breast cancer (Carlson *et al.*, 2009), age, obesity, oral contraceptive use, alcohol use, age of first childbirth, age of menopause and radiation (Harris *et al.*, 1992; Endogenous Hormones Breast Cancer Collaborative Group, 2002). The incidence rates of women developing breast cancer increases during their fourth decade and after menopause (Harris *et al.*, 1992; Watkins, 2019). Genetic mutations can cause breast cancer (Carlson *et al.*, 2009). Mutations in the breast cancer (BRCA) 1 and 2 genes, which code for DNA repair proteins, are examples thereof (Carlson *et al.*, 2009; Watkins, 2019). There are four breast cancer subtypes: luminal A, luminal B, HER-2 positive and triple negative (basal) (Watkins, 2019). Human growth factor receptor 2 (HER-2) expression is also used to classify breast cancer (Watkins, 2019). The protein HER-2 promotes the growth of cancer cells. The luminal A subtype is estrogen and progesterone receptor positive and are mostly diagnosed at early stages and is HER-2 negative (Watkins, 2019). Luminal B is estrogen and progesterone receptor positive and can be HER-2 positive or negative (Carlson *et al.*, 2009; Watkins, 2019). The HER-2 subtype is estrogen and progesterone receptor negative (Watkins, 2019). Triple negative (basal) breast cancer subtype is estrogen and progesterone receptor and HER-2 negative. This subtype is diagnosed at a younger age than other sub-types and tends to be more aggressive (Watkins, 2019).

In a study done by the Endogenous Hormones Breast Cancer Collaborative Group (2002), the researchers found that postmenopausal women with high serum concentrations of sex hormones had about a two-fold higher risk of having breast cancer than postmenopausal

women with low serum concentrations of these sex hormones. Long-term therapy with a combination of estrogen and progesterone can lead to an increase in the risk of breast cancer (Cooper, 2000).

About 30% of women with breast cancer have a physical breast mass (Watkins, 2019). Other visible signs such as dimpling, blistering, sanguineous nipple discharge, and nipple retraction is associated with breast cancer (Watkins, 2019). A mammogram, ultrasound and tissue biopsy can be used as part of the diagnostic process of breast cancer (Watkins, 2019). After breast cancer diagnosis, it can be treated with surgery, chemotherapy, aromatase inhibitors and radiation therapy (Watkins, 2019).

2.5 The methionine-homocysteine cycle and cancer

It has been confirmed that estrogens are the primary risk factor for the development of breast cancer (Parl *et al.*, 2009; Endogenous Hormones Breast Cancer Collaborative Group, 2002). This is due to DNA damage caused by the oxidation products, catechol estrogens, and the stimulation of cell proliferation and gene expression via the estrogen receptor (Parl *et al.*, 2009). As described in Section 2.2, catechol estrogens are oxidised to form mutagenic quinones, potentially causing depurinating DNA adducts. Therefore, catechol estrogens are defined as carcinogenic (Cavalieri *et al.*, 2006). E2 also stimulates cell proliferation and gene expression through the ER, thereby contributing to breast cancer development (Parl *et al.*, 2009). Increased levels of 4-OHE2 have been observed in breast cancer patients, indicating that CYP1B1 activity is increased in breast cancer patients. This pathway leads to the development of depurinating DNA adducts (Rogan *et al.*, 2003). As described in Section 2.2, methylation of catechol estrogens to methoxy catechol estrogens acts as a protection mechanism, decreasing the formation of quinones. A decreased concentration of 2- and 4-methoxy-catechol estrogens has been reported in breast cancer patients in comparison to controls (Rogan *et al.*, 2003).

Women with polymorphisms in the COMT-pathway, which negatively affect the rate of methylation, are more prone to develop breast cancer (Parl *et al.*, 2009; Yager, 2012). Currently, 34 single nucleotide polymorphisms in COMT are known (Yager, 2012). These polymorphisms can cause a decreased or insufficient activity of COMT (Cavalieri *et al.*, 2006).

2.6 Quantification of the methionine-homocysteine cycle metabolites and co-factors

Throughout literature, the use of different types of matrices, analytical techniques and sample preparation methods used to quantify metabolites and co-factors of the methionine-

homocysteine cycle have been documented. The different methods are discussed in this section.

2.6.1 Suitable matrices

Quantification of metabolites of the methionine-homocysteine cycle has been done in different biological matrices. The quantification of these metabolites can help to understand and assess the methionine-homocysteine metabolism. Examples of these biological matrices are urine (Behringer *et al.*, 2019), blood plasma (Behringer *et al.*, 2019; Guiraud *et al.*, 2017; Midttun *et al.*, 2005; Struys *et al.*, 2000; Xie *et al.*, 2014), blood serum (Wada *et al.*, 2018), red blood cells (Da Silva *et al.*, 2016), cerebrospinal fluid (Guiraud *et al.*, 2017; Struys *et al.*, 2000) and saliva (Stachniuk *et al.*, 2016). The sensitivity, selectivity and reproducibility of analysis can be affected by sample collection, handling and storage (Issaq *et al.*, 2007). Furthermore, metabolite concentrations vary in different matrices. Therefore, choosing a suitable matrix is important for efficient analysis (Yu *et al.*, 2011).

2.6.1.1 Urine

Although the non-invasiveness and simple collection procedure of urine makes it a popular matrix for amino acid quantification (Gjerde *et al.*, 2011), urine is not the ideal matrix for quantifying homocysteine. This is because homocysteine is almost completely re-absorbed by the kidney tubules and less than 0.05% of the total cellular homocysteine is excreted in the urine (Rasmussen & Møller, 2000). Furthermore, the analysis of the relevant amino acids can be complicated by the presence of hydrophilic compounds in urine, which cause the formation of overlapping peaks (Deyl *et al.*, 1986; Harada *et al.*, 2020).

2.6.1.2 Blood

Blood consists of different components, like plasma, white blood cells, platelets and red blood cells (RBC). Plasma forms 60% of the blood. The cells present in blood make up the remaining 40%. As can be seen in Figure 2.5 (a), plasma mainly consists of water, ions, proteins, nutrients, waste and gases (Dean, 2005). RBC are the functional component of blood and these cells are responsible for the transportation of gasses and nutrients (Barbalato & Pillarisetty, 2019). Plasma is the liquid that forms when clotting is prevented (Issaq *et al.*, 2007). During the preparation of plasma, whole blood is collected into anticoagulant treated (EDTA) tubes (Gjerde *et al.*, 2011; Issaq *et al.*, 2007; Yu *et al.*, 2011). The anticoagulant will prevent blood clotting. Centrifugation will separate the cells and platelets from the plasma (Deyl *et al.*, 1986; Issaq *et al.*, 2007). Another popular matrix often used for metabolite quantification is serum. Serum is the liquid that remains after blood has clotted (Issaq *et al.*,

2007; Yu *et al.*, 2011) (Figure 2.5 (b)). Serum preparation is done in the following manner: whole blood is collected in a test tube (Issaq *et al.*, 2007). After collection, the tube is left at room temperature to induce coagulation (15-30 min). The clot that formed can be removed through centrifugation (Issaq *et al.*, 2007).

In previous studies, erythrocytes (RBC) have been used to quantify metabolites and co-factors of the methionine-homocysteine cycle. These cells are considered a good matrix because some metabolites are more abundant in RBC compared to plasma (Da Silva *et al.*, 2016; Monteiro *et al.*, 2014).

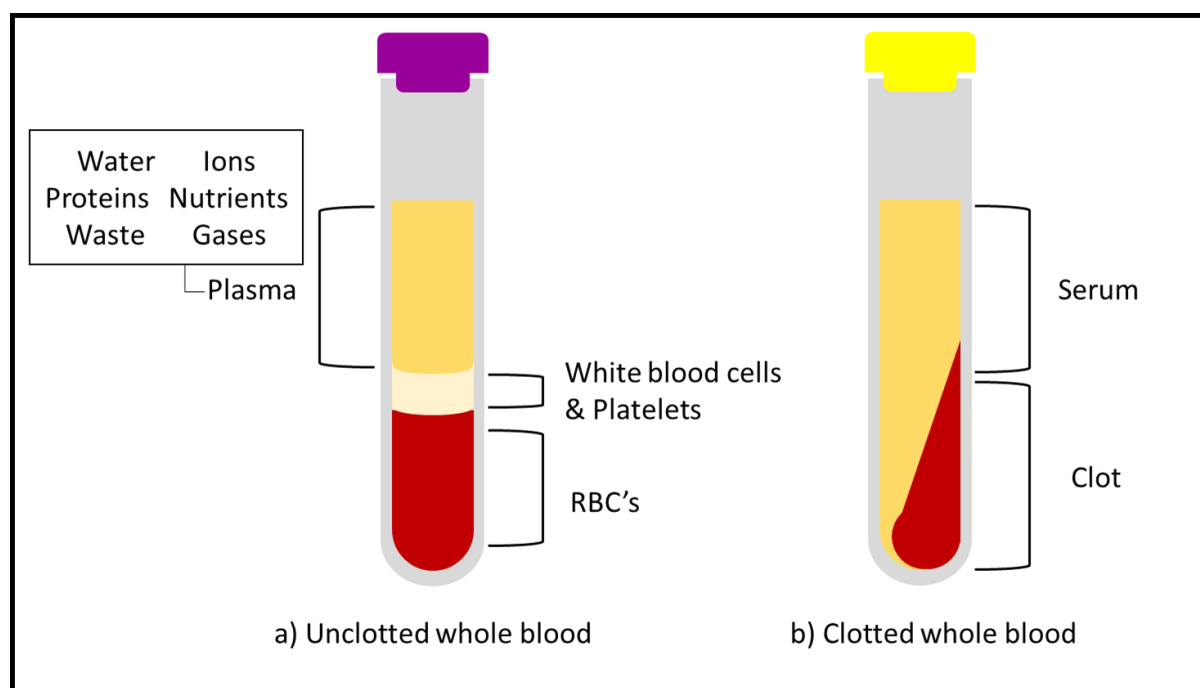


Figure 2-5 A visual comparison of the preparation of blood plasma and blood serum as a result of centrifuging. The spinning (centrifuging) of the blood samples causes the denser elements in the tube to sink, which causes the different components to separate. This eases the isolation process of the different components. a) To ensure the isolation of plasma, the presence of an anticoagulant is needed to prevent the formation of blood clotting. b) The isolation of serum requires blood tubes without an anticoagulant to ensure blood clotting. (Dean, 2005).

A study comparing plasma and serum metabolite profiles pointed out that plasma had a better reproducibility of metabolite concentration and that this may reflect on the less complicated collection procedure (no waiting for coagulation) (Yu *et al.*, 2011). Plasma seems to be more stable than serum (Luque-Garcia & Neubert, 2007). However, higher metabolite concentrations in serum were also observed and can be attributed to the volume displacement

effect. Serum has lower protein content than plasma, which may improve overall sensitivity (Yu *et al.*, 2011). Because of the possibility of anticoagulants interfering, serum is often preferred over plasma (Luque-Garcia & Neubert, 2007). When compared to RBC, the sample preparation of plasma is much simpler and quicker. The preparation of plasma and serum samples for biomarker analysis using mass spectrometry include solid phase extraction, protein precipitation and chemical reduction (Luque-Garcia & Neubert, 2007); while the preparation of red blood cells entails the vortexing of frozen samples, and the addition of sodium acetate and TCA. Thereafter, the samples are left on ice for protein precipitation, whereafter the sample is filtered (Da Silva *et al.*, 2016). After comparing LOD and LOQ in plasma and RBC, it was shown that plasma had higher metabolite concentrations than RBC (Da Silva *et al.*, 2016; Guiraud *et al.*, 2017). Yu *et al.* (2013) reported that plasma is preferred to quantify amino acids. Some disadvantages of blood matrices are the invasiveness of sample collection (Gjerde *et al.*, 2011) and the fact that when using plasma as a matrix it might cause compounds to co-elute because of the matrix effect (Guiraud *et al.*, 2017).

For the quantification of metabolites of the methionine-homocysteine cycle, a blood matrix is preferred, because all metabolites of the methionine-homocysteine cycle have increased detection in blood matrices. The assessment of plasma and serum are better for the detection of metabolites of the methionine-homocysteine cycle as they have less complicated preparation steps.

2.6.2 Current analytical techniques for the quantification of metabolites and co-factors of the methionine-homocysteine cycle

Methods used for quantifying the metabolites and co-factors of the methionine-homocysteine cycle include liquid chromatography (LC) or gas chromatography (GC) coupled with tandem mass spectrometry (MS), enzyme assays, immunoassays, and capillary electrophoresis (Ducros *et al.*, 2002). The most popular analytic technique used to quantify a comprehensive amount of the metabolites and co-factors of the methionine-homocysteine cycle is LC-MS/MS (Behringer *et al.*, 2019; Da Silva *et al.*, 2016; Golja *et al.*, 2020; Guiraud *et al.*, 2017; Su *et al.*, 2021). Most of the current studies analysed only a few of the metabolites and co-factors relevant to the methionine-homocysteine cycle (Cauci *et al.*, 2008; Golja *et al.*, 2020; Leklem *et al.*, 1977; Norouzi *et al.*, 2011; Su *et al.*, 2021; Weaving *et al.*, 2006). However, some groups of researchers analysed the methionine-homocysteine cycle more extensively. Behringer *et al.* (2019); Da Silva *et al.* (2016); Guiraud *et al.* (2017) analysed plasma and cerebrospinal fluid using a single analytical LC-MS/MS method to quantify up to 17 metabolites and co-factors of the methionine-homocysteine cycle in a study comparing Alzheimer patients and healthy subjects. Da Silva *et al.* (2016) analysed 13 metabolites of the methionine-

homocysteine cycle using red blood cells to assess the nutritional status of individuals. Another study managed to quantify only 10 thiol and thiolester metabolites for metabolic profiling using plasma, cells and urine as biological samples (Behringer *et al.*, 2019).

2.6.2.1 Chromatographic separation of analytes

Analytical methods such as LC/GC-MS are more reliable and sensitive (Coskun, 2016). In analytical methods, separation and detection methods are combined. GC and LC are separation methods used during the quantification of metabolites in biological fluids. The separated compounds can subsequently be identified with mass analysers such as quadrupole mass analysers or tandem quadrupole mass analysers (Jóźwik & Kałużna-Czaplińska, 2016). The use of coupled methods ensures immediate/direct detection of the separated, unique compounds (Jóźwik & Kałużna-Czaplińska, 2016). These analytical methods rely on the physical and chemical characteristics, ionisation behaviour, and the solubility of the different compounds (Garrett & Grisham, 2013).

Chromatographic techniques require the separation of analytes based on the manner in which an analyte distributes between two immiscible phases (Wilson & Walker, 2010). LC and GC are widely used in metabolic research (Jóźwik & Kałużna-Czaplińska, 2016; Newton *et al.*, 2010). Chromatographic techniques can separate multiple analytes present in a biological sample (Newton *et al.*, 2010). LC allows the identification and accurate quantification of a number of compounds in a matrix (Jóźwik & Kałużna-Czaplińska, 2016) and poses the following advantages: low detection limits, minimal sample preparation, successful quantification of analytes (Coskun, 2016) and high sensitivity and selectivity (Coskun, 2016; Jóźwik & Kałużna-Czaplińska, 2016; Wal *et al.*, 2010). LC systems are able to analyse a bigger variety of samples, including volatile and non-volatile samples, samples with a low or high molecular weight, that are stable at room temperature and soluble in a liquid. On the other hand, GC systems analyse volatile samples with a low molecular weight that are stable at high temperature. Accurate separation and identification of amino acids are achieved using LC (Coskun, 2016).

One of the challenges that are faced when attempting to analyse multiple metabolites with similar chemical properties, such as polar amino acids using liquid chromatography, is the fact that they react in the same way in the mobile and stationary phases. When a sample contains polar metabolites, the use of ion-pairing agents increases the retention of these polar analytes, such as amino acids when using reversed-phase chromatography (Dolan, 2008; Wilson & Walker, 2010). The addition of an ion-pairing agent increases the hydrophobic character of the analyte, thereby increasing the retention of the polar analytes to the non-polar stationary

phase of the reversed-phase column because of the dynamic interactions between the stationary phase, ion-pairing agent, and analyte (Wilson & Walker, 2010). Ion-pairing agents have a non-polar tail that binds to the non-polar stationary phase. This increases the hydrophilic character of the column causing better retention of polar compounds (Dolan, 2008). The use of ion-pairing agents comes with some precautions, including that the ion-pairing reagent can never be fully washed from the column (Dolan, 2008; Yerneni, 2017). Holčapek *et al.* (2004) reported that the signal intensity drops to 15-30% with the use of hept fluorobutanoic or perfluoroheptanoic acids (PFHpA).

Da Silva *et al.* (2016) compared the use of PFHpA and heptafluorobutyric acid as ion-pairing agents paired with an octadecylsilane (C18) column during the development of a method for the quantification of metabolites and co-factors of the methionine-homocysteine cycle. This method was later used by Guiraud *et al.* (2017) for application on plasma and cerebrospinal fluid. Gu *et al.* (2007) also used perfluoroheptanoic acid as an ion-pairing agent during the separation of protein amino acids on a C18 column. Petritis *et al.* (2002) reported that the use of perfluorinated carboxylic acids were efficient ion-pairing agents for the separation of amino acids and are volatile enough for tandem quadrupole mass spectrometry (MS/MS) detection.

2.6.2.2 Mass spectrometric detection of analytes

After the successful separation of the compounds in a sample, MS can be used to identify the compounds. During MS, the molecules of interest are subjected to an ionisation process in a source during which they are converted to gas ions. Thereafter, the ions are separated according to their mass-to-charge (m/z) ratio (Ho *et al.*, 2003; Wilson & Walker, 2010). The types of ionisation sources include, electron impact ionisation (EI), chemical ionisation (CI), fast atom bombardment (FAB), atmospheric-pressure ionisation (API) and electrospray ionisation (ESI) (Wilson & Walker, 2010). ESI is a popular ionisation process (Abou-Diwan, 2011; Behringer *et al.*, 2019; Da Silva *et al.*, 2016; Guiraud *et al.*, 2017) since it is known to be a soft ionisation technique during which the analyte is sprayed into an electric field. After this step, the mobile phase is evaporated and the ions are ejected from the charged droplet (Ho *et al.*, 2003; Wilson & Walker, 2010). A mass analyser analyses the ions that are formed according to their masses. The quadrupole mass analyser (MS) and tandem MS/MS are two of the analysers used. When using MS/MS different scan types can be used making the method of detection more selective (Ho *et al.*, 2003). This is achieved by changing the first quadrupole (Q1) and the third quadrupole (Q3) between scanning and static mode (Ho *et al.*, 2003; Wilson & Walker, 2010). The combination of LC, ESI and MS/MS is considered flexible and it requires minimal sample preparation (Taylor, 2005).

2.6.3 Sample preparation for LC-MS/MS

To ensure optimal separation and identification of the compounds of interest, efficient sample preparation is required. Sample preparation is performed to dissolve the analytes in an appropriate solvent, to remove any interfering matrix compounds (phospholipids, salts, proteins, nucleic acids and sugars) and to concentrate the analytes (Sargent, 2013; Wal *et al.*, 2010). The recovery of the analyte can be maximised by efficient sample preparation (Sargent, 2013). Sample preparation helps achieve selectivity (Verstraete *et al.*, 2019), sensitivity, and robustness of the analytical method being developed (Sargent, 2013). Liquid-liquid extraction (LLE), solid-phase extraction (SPE) and protein precipitation are some sample preparation methods that can be used before the sample is subjected to LC-MS/MS analysis (Sargent, 2013; Wal *et al.*, 2010). When comparing LLE and SPE, SPE is often considered a more efficient method of separation than LLE (Wal *et al.*, 2010). Protein precipitation is a simple preparation method when compared to LLE and SPE (Wal *et al.*, 2010). Different matrices require different sample preparation methods. Serum and plasma are known as clean matrices; therefore, protein precipitation with an organic solvent may be the preferred sample preparation method (Verstraete *et al.*, 2019). Urine, known as a dirty matrix, will require more extensive sample preparation like SPE.

During the process of protein precipitation, an organic solvent, which favours the solubility of the analyte and precipitation of proteins, is added to the sample (Sargent, 2013; Tomaiuolo *et al.*, 2009; Wal *et al.*, 2010). After the sample has been vortexed or mixed, it is centrifuged to allow for the precipitation of the unwanted proteins, and the supernatant can be removed for analysis (Sargent, 2013). Three to five volumes of the organic solvent added to plasma or serum help with the precipitation of large proteins (Zhao & Juck, 2018). Acetonitrile (ACN) and methanol (MeOH) are good choices for the organic solvent, as the analyte is soluble in these solvents (Guiraud *et al.*, 2017; Polson *et al.*, 2002; Verstraete *et al.*, 2019; Wal *et al.*, 2010; Weaving *et al.*, 2006; Zhao & Juck, 2018). The addition of an acid (for example, formic acid, FA) can help facilitate protein precipitation because it lowers the pH leading to the protein to be positively charged leading to the disruption of the water around the protein and causing easier protein precipitation (Da Silva *et al.*, 2016; Guiraud *et al.*, 2017). After the addition of the organic solvent, the supernatant, formed after centrifugation, can be subjected to LC-MS/MS analysis (Sargent, 2013; Wal *et al.*, 2010). In recent studies, sample preparation for the analyses of metabolites and co-factors of the methionine-homocysteine cycle included reduction of disulfide bonds and protein precipitation (Behringer *et al.*, 2019; Guiraud *et al.*, 2017). Because homocysteine is found in three forms in plasma, the disulfide bonds need to be chemically reduced to ensure the measurement of total homocysteine. Chemicals used for

the reduction of disulfide bonds include dithiothreitol (DTT), tris(2-carboxyethyl) phosphine (TCEP) and 2-mercaptoethanol (Behringer *et al.*, 2019; Guiraud *et al.*, 2017; Tomaiuolo *et al.*, 2009; Weaving *et al.*, 2006).

2.6.4 Internal standards

To aid in the qualification and quantification of the detection of the compounds of interest, internal standards like stable isotopically labelled internal standards are popular (Behringer *et al.*, 2019; Da Silva *et al.*, 2016; Guiraud *et al.*, 2017). Internal standards are compounds that can be added to a sample in a known concentration that helps with the qualitative and quantitative analysis of the compound/analyte of interest (Wal *et al.*, 2010). The addition of internal standards can help control variation in recovery, matrix effect and ionisation (Sargent, 2013). Internal standards can only be distinguished from the analyte in the mass spectra (Sargent, 2013; Zhou *et al.*, 2017). Internal standards should be added as early as possible to correct for the loss that occurs during sample preparation (Sargent, 2013). It has been proven that internal standards, specifically stable isotopically labelled internal standards, help compensate for matrix effects (Da Silva *et al.*, 2016; Guiraud *et al.*, 2017; Sargent, 2013). Other advantages of the use of stable isotopically labelled internal standards include the increase of accuracy, reproducibility and precision of the method (Behringer *et al.*, 2019; Da Silva *et al.*, 2016). Stable isotopically labelled internal standards have similar extraction recovery, ionisation response in ESI mass spectrometry, as well as similar chromatographic retention times (Wal *et al.*, 2010). The accuracy of detection of specific amino acids like SAM and SAH is also increased by using stable isotopes as internal standards (Struys *et al.*, 2000). The recovery during transfer and clean-up stages, variability of the injection volume and the variability in extraction efficiency can be accounted for by using stable isotopically labelled internal standards (Sargent, 2013) (Sargent, 2013).

2.6.5 Method validation

Method validation is an important step to ensure that the analytical method developed is suitable, reliable, consistent, and reproducible for its intended purpose (Chauhan *et al.*, 2015; Ravisankar *et al.*, 2015; Wal *et al.*, 2010). A sensitive and selective analytical method is of great importance during the quantification of metabolites (Wal *et al.*, 2010). There are three different types of method validations: full validation, partial validation and cross validation. During full validation, all the validation parameters are investigated. Partial validation entails the modification of an already validated analytical method. During cross validation, a comparison is made of validation parameters, using more than two analytical methods (Wal

et al., 2010). During the validation of a method, samples or standards similar to the unknown samples should be used (Papadoyannis & Samanidou, 2004).

CHAPTER 3 MATERIALS AND METHODS

3.1 Introduction

In this chapter, information regarding the analytical standards and instrumentation used is described. The preparation of standards and the methods used during the analysis of metabolites and co-factors of the methionine-homocysteine cycle are also described here.

3.2 Analytical standards, chemicals, and solutions

3.2.1 Analytical standards and chemicals

The following amino acid and amino acid derivative standards were purchased from Sigma Aldrich now Merck KGaA, Darmstadt, Germany (including product numbers): L-serine (S4500), taurine synthetic (T0625), L-cysteine (168149), L-cystine (30200), glycine (G7126), DL-homocysteine (44925), L-homocystine (H6010), L-methionine (M9625), -adenosyl-L-homocysteine (A9384), s-(5'-adenosyl)-L-methionine p-toluenesulfonate salt (A2408), cystathionine (C3633), and n,n-dimethylglycine (D1156). Analytical standards (including product numbers): DL-homocysteic acid (219746), betaine (61962), and choline (C7017) were purchased from Sigma Aldrich now Merck KGaA, Darmstadt, Germany. Co-factor standards (ID included), pyridoxine hydrochloride (vitamin B₆) (ZA01006362) and riboflavin (vitamin B₂) (ZA10015437) were supplied from Brenntag, Essen, Germany. 5-Methyl tetrahydrofolic acid glucosamine salt (0-F05) was provided by Gnosis by Lesaffre. The following isotopes were purchased from Cambridge Isotopes, Tewksbury, United States: d₄-L-cystine (DLM-9812-PK), d₈-DL-homocystine (DLM-3619-PK), methyl-d₃-methionine (DLM-431-PK), d₂-glycine (DLM-1674-PK), and d₅-phenylalanine (DLM-1258-PK). Ion-pairing agent perfluoroheptanoic acid (342041) and chemicals such as 32 % hydrochloric acid (HCl) (100319), sodium hydroxide (NaOH) (221465), L-ascorbic acid (A0278), and formic acid (94318) were purchased from Sigma Aldrich now Merck KGaA, Darmstadt, Germany. Methanol (MeOH) (67-56-1), high purity water (7732-18-5), and acetonitrile (75-65-8) were purchased from Honeywell, North-Carolina, United States. Ammonium acetate (NH₄OAc) (32301) was purchased from BDH limited, Poole, England.

3.2.2 Preparation of solutions for stock solutions

During the preparation of the analytical standard stock solutions, different solution types were used for the different standards. This was due to the difference in solubility of each standard. Six different types of solutions, as can be seen in Table 3.1, were used during the preparation of the analytical standards: 0.1 M HCl, 1 M HCl, MeOH/H₂O (10:90), MeOH/H₂O (50:50), 0.1

M NaOH, H₂O with 1% acetic acid, and NH₄OAc 10 mM, ascorbic acid 10%, and DTT 2% in H₂O (Table 3.1). The solutions were stored at 4°C.

Table 3-1 List of the metabolites and the solution type.

Stock solution type	Analyte
0.1 M HCl	L-Homocysteic acid L-Serine Glycine L-Methionine Pyridoxine hydrochloride (vitamin B6) L-Homocystine Cystathionine
1 M HCl	L-Cystine
MeOH/H ₂ O (10:90)	Taurine N,N-Dimethylglycine (DMG) Betaine
MeOH/H ₂ O (50:50)	Choline
0.1 M NaOH	Riboflavin (vitamin B6)
NH ₄ OAc 10 mM, 10% ascorbic acid, and 2% DTT	DL-Homocysteine L-Cysteine 5-Methyl tetrahydrofolic acid glucosamine salt (5-MTHFA)
H ₂ O + 1% Acetic Acid	S-Adenosyl-L-Homocysteine (SAH) S-(5'-Adenosyl)-L-Methionine p-toluenesulfonate salt (SAM)

3.2.3 Preparation of standard stock solutions and master mixes

Standard stock solutions and master mixes are needed for method implementation, development, and validation. Stock solution concentrations of 0.1 mg/ml (100 ppm) of all standards were prepared in the different solutions (see 3.2.2 and Table 3.1). The standard stock solution concentrations and solution types are from the protocol of Guiraud *et al.* (2017) and Da Silva *et al.* (2016). Furthermore, standard stock solutions of 0.01 mg/ml (10 ppm) and 0.001 mg/ml (1 ppm) were prepared by diluting the 0.1 mg/ml (100 ppm) solutions using the solution types specified in Table 3.1. All standard solutions were stored at -20°C until needed for method implementation, development, and validation. For method implementation and development, 10 ppm and 5 ppm master mixes were prepared. This was done by drying 100 µl of each sample (10 ppm or 5 ppm) under a constant nitrogen gas stream at 30°C and resuspending it in 1 ml of an acetonitrile and water mixture (5:95).

3.2.4 Preparation of internal standards stock solution and working solutions

To compensate for the loss that may occur during sample preparation and to help with the quantitative and qualitative analysis, five stable isotopically labelled internal standards were used (Oliveira *et al.*, 2010; Sargent, 2013; Wal *et al.*, 2010). Stock solutions with a concentration of 0.1 mg/ml (100 ppm) for all five isotopes were prepared. Methyl-d₃-methionine, d₂-glycine, and d₅-phenylalanine were prepared in methanol while d₄-L-cystine, and d₈-DL-homocystine were prepared in H₂O. Working solutions were prepared by diluting the stock solutions to prepare 0.01 mg/ml (10 ppm) and 0.001 mg/ml (1 ppm) solutions. The isotopes prepared in MeOH were stored at -20°C and those prepared in H₂O were stored at 4°C until needed for further analysis. A final working solution of the stable isotope internal standards was prepared in a 50 ml volumetric flask, resulting in the final concentrations as can be seen in Table 3.2 for each of the five isotopes. The isotope working solution was divided into smaller volumes and stored at -20°C until further analyses.

Table 3-2 Isotope concentration in working solution.

Isotope	Concentration (ng/ml)
Methyl-d ₃ -Methionine	52.288
d ₂ -Glycine	52.288
d ₂ -L-Cystine	78.431
d ₈ -DL-Homocystine	78.431
d ₅ -Phenylalanine	1.046

3.2.5 Preparation of working stock solutions, calibration standards, and quality control standards

To define the standard curve, nonzero concentrations covering the dynamic range of the assay was used (Meng & Bennett, 2012). A working stock solution including all of the 18 metabolites was prepared in ACN:H₂O (5:95), in a 50 ml volumetric flask using the standard stock solutions in Section 3.2.3. The working stock solution was divided into smaller volumes and stored at -20°C until needed for analysis. Nine calibration standards were prepared using 1:1 serial dilutions with ACN:H₂O (5:95) of the working stock solution. The standard series was prepared according to Guiraud *et al.* (2017) and the selected ranges were based on the physiological concentrations of the metabolites in serum (Table 3.3). The concentration range prepared was representative of the in-vial concentration. During method validation, four concentrations were chosen, lower limit of quantification (LLOQ), low concentration (LC), medium concentration

(MC), and high concentration (HC), to assess the accuracy, precision, repeatability, and stability. These four concentrations were prepared by diluting the working stock solution to the concentrations that can be seen in Table 3.8.

Table 3-3 The investigated calibration range of the metabolite and co-factors for serum analyses. These ranges were modified from ranges used by Guiraud *et al.* (2017).

Analyte	Investigated concentration range (ng/ml)
L-Homocysteic acid	0.613 – 156.863
Taurine	2.042 – 522.876
L-Serine	1.634 – 418.301
N, N-DMG	0.408 – 104.575
Betaine	1.634 – 418.301
Glycine	1.634 – 418.301
L-Cysteine	1.021 – 261.438
DL-Homocysteine	1.021 – 261.438
Riboflavin (Vitamin B2)	0.041 – 10.458
Choline	1.021 – 261.438
L-Methionine	2.042 – 522.876
Pyridoxine (Vitamin B6)	0.010 – 2614
5-Methyltetrahydro folic acid	0.061 – 15.686
L-Cystine	4.085 – 1045.752
Cystathionine	0.041 – 10.458
SAH	0.082 – 20.915
L-Homocystine	1.634 – 418.301
SAM	0.061 – 15.686

3.3 Instrumentation and materials

3.3.1 Instrumentation

For LC-MS/MS analysis, an Agilent 1290 infinity series high pressure liquid chromatography (HPLC) system was used, with a vacuum degasser (G1330B), a binary pump (G4220A), temperature-controlled column compartment (G1216C) and a temperature controlled autosampler (G4226A). This LC system was coupled to an Agilent 6460 triple quadrupole mass spectrometer (G6460A), with an Agilent Jet Stream ESI (AJSESI) source. These Agilent

instruments were manufactured by Agilent Technologies, Santa Clara CA, USA. For chromatographic separation of the analytes, a XSelect HSS T3 XP 2,5 micron 2,1 × 100 mm (186006151) column, purchased from Waters, were used. Other instrumentation used included a Heraeus multifuge X3R centrifuge (41160225); a BFC nitrogen evaporator, purchased from Stargate scientific; a WX vortex, purchased from Velp; a Modop Digital sonicator and a Mettler Toledo classic plus balance. Figure 3.1 schematically illustrates the instrumentation used during this study.

Agilent MassHunter workstation software was used for data acquisition and quantification of analytes. This software included LC/MS Data Acquisition software for 6400 series triple quadrupole (B.07.01), Quantitative Analysis software (B.08.00) and Qualitative Analysis for QQQ software (B.08.00). Agilent technologies MassHunter workstation Optimizer software for the 6400 series triple quadrupole was used for the optimisation of ionisation conditions (B.07.01) and Source and iFunnel Optimizer software for 6400 triple quadrupole source optimisation (B.07.01). For statistical analysis of the data, MetaboAnalyst 5.0 was used during this study.

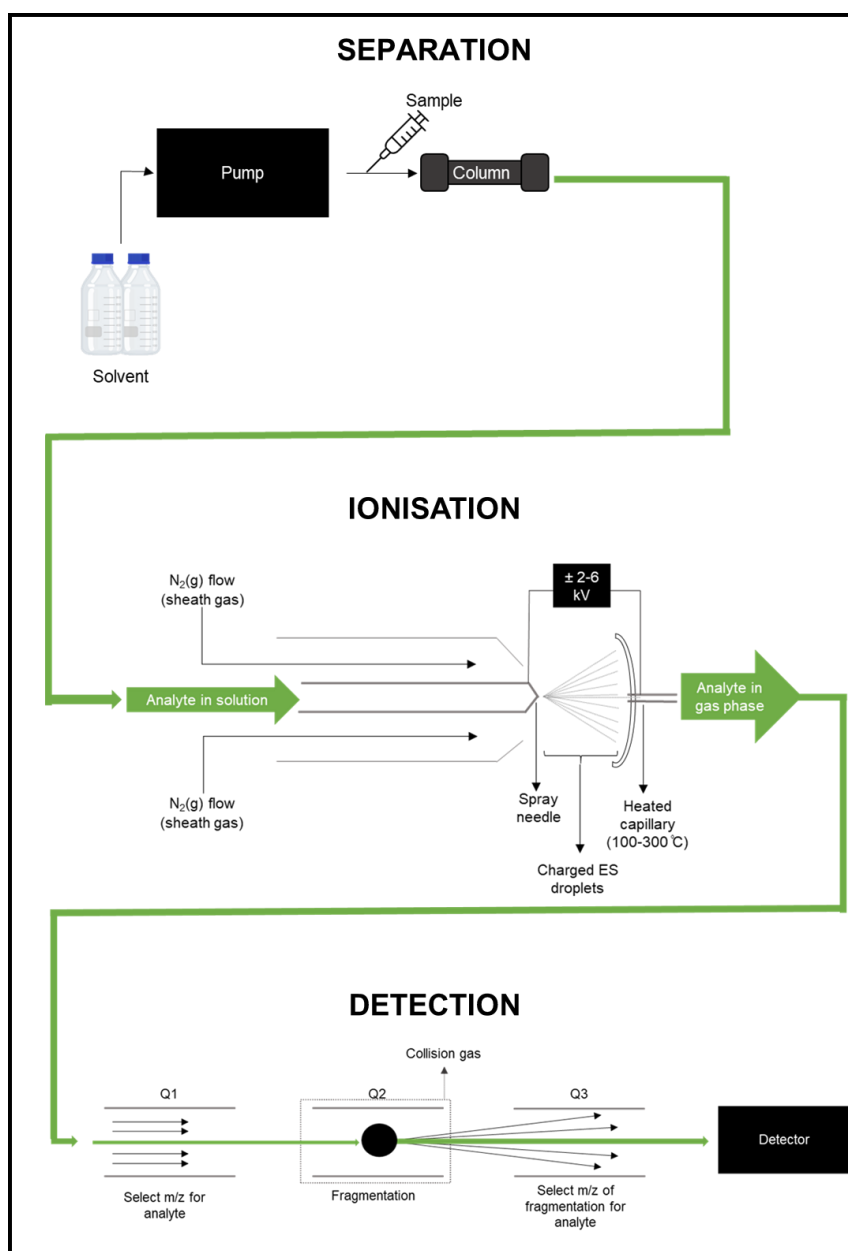


Figure 3-1 Schematic illustration of HPLC-ESI-MS/MS instrumentation used during this study, compiled from Ho *et al.* (2003); Wilson and Walker (2010). During liquid chromatography, analytes are separated based on their interaction with the stationary phase (column) and the mobile phase (solvent) (Wilson & Walker, 2010). After the separation of the analytes of interest, they are subjected to ESI. During this ionisation process, the analytes are sprayed into an electric field to form charged droplets (Ho *et al.*, 2003). These are selectively detected using a MS/MS system. Q1 and Q3 are set to scan for specific precursor ions and product ions (Wilson & Walker, 2010).

3.3.2 Materials

Merck (Johannesburg, South-Africa) was the supplier for 2 ml spin-X centrifuge filter tubes with a 0.22 µm nylon filter and 1.5 ml microcentrifuge tubes (HS4323). Two millilitres Agilent screw cap glass vials (5190-9062); 400 µl flat bottom inserts (5181-3377) and 9 mm blue screw caps with polytetrafluoroethylene (PTFE) septa (5185-5820) were purchased from Chemetrix (Johannesburg, South-Africa).

3.4 Method implementation and optimisation

3.4.1 Metabolites and co-factors analysed

Table 3.4 contains a list of the chemical properties of the metabolites and co-factors that were analysed during this study.

Table 3-4 **Chemical properties of the analytes of interest**

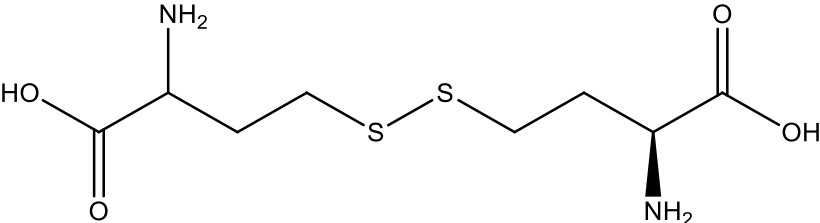
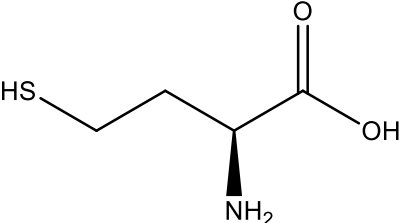
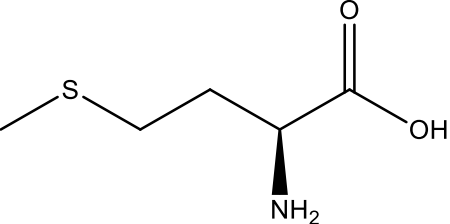
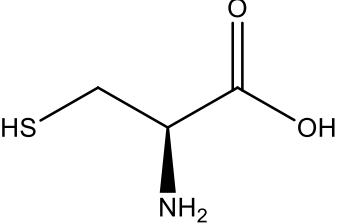
Metabolite	Formula	Molecular Mass (g/mol)	Structure
L-Homocystine	$C_8H_{16}N_2O_4S_2$	268.350	
DL-Homocysteine	$C_4H_9NO_2S$	135.180	
L-Methionine	$C_5H_{11}NO_2S$	149.210	
L-Cysteine	$C_3H_7NO_2S$	121.160	

Table 3-4 continued

Chemical properties of the analytes of interest

Metabolite	Formula	Molecular Mass (g/mol)	Structure
L-Cystine	$C_6H_{12}N_2O_4S_2$	240.300	
SAM	$C_{15}H_{22}N_6O_5S$	398.440	
SAH	$C_{14}H_{20}N_6O_5S$	384.412	

Table 3-4 continued

Chemical properties of the analytes of interest

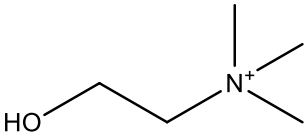
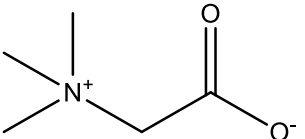
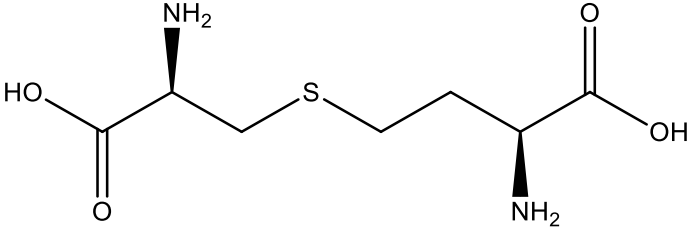
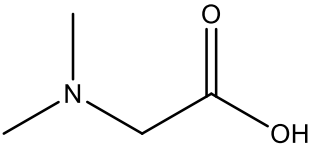
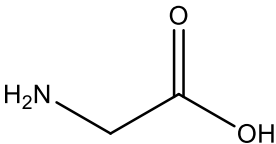
Metabolite	Formula	Molecular Mass (g/mol)	Structure
Choline	$C_5H_{14}NO$	104.171	
Betaine	$C_5H_{11}NO_2$	117.148	
Cystathionine	$C_7H_{14}N_2O_4S$	222.263	
N, N-DMG	$C_4H_9NO_2$	103.120	
Glycine	$C_2H_5NO_2$	75.070	

Table 3-4 continued

Chemical properties of the analytes of interest

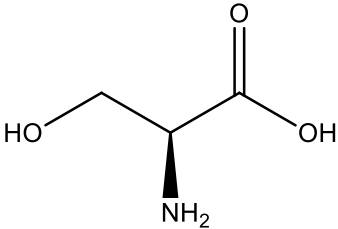
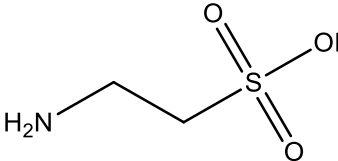
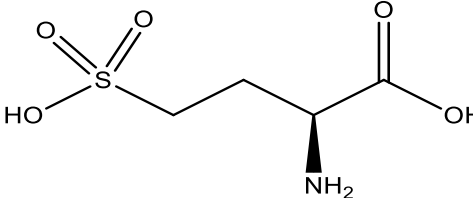
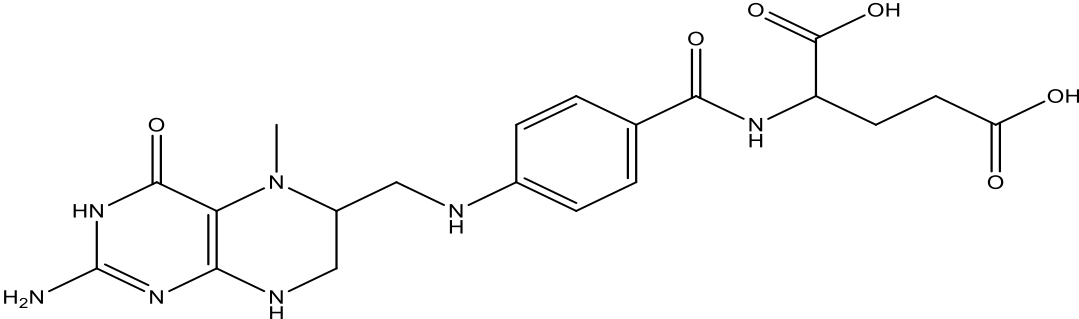
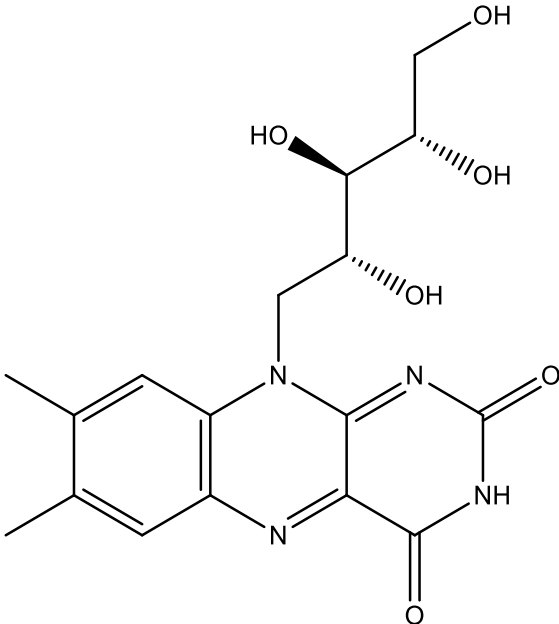
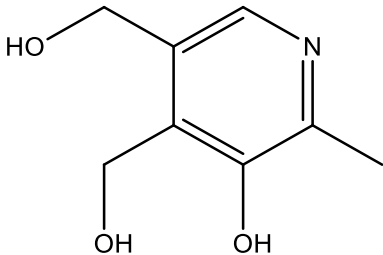
Metabolite	Formula	Molecular Mass (g/mol)	Structure
L-Serine	$C_3H_7NO_3$	105.090	
Taurine	$C_2H_7NO_3S$	125.150	
L-Homocysteic acid	$C_4H_9NO_5S$	183.180	
5-Methyltetrahydro folic acid	$C_{37}H_{59}N_7O_6$	459.460	

Table 3-4 continued

Chemical properties of the analytes of interest

Metabolite	Formula	Molecular Mass (g/mol)	Structure
Riboflavin (Vitamin B ₂)	C ₁₇ H ₂₀ N ₄ O ₆	376.360	
Pyridoxine (Vitamin B ₆)	C ₈ H ₁₁ NO ₃	169.180	

3.4.2 Mass spectrometry optimisation

3.4.2.1 Optimisation of ionisation conditions

To ensure that the optimal ionisation and multiple reaction monitoring (MRM) conditions for the instrumentation is used, direct infusion into the source was initially done. This analysis was done using the Agilent MassHunter Optimiser software (as mentioned in Section 3.3.1). During this analysis the chemical formula (as reported in Table 3.4) for each analyte was provided to the Agilent MassHunter Optimiser software. The software determined the best precursor ions, fragmentor voltage for each precursor ion, possible product ions, and the collision energy that is most optimal for each MRM transition by doing direct infusions. During this optimisation, 5 μ l of a 1 ppm standard stock solution of each analyte (see Section 3.2.3) was injected. An isocratic 50:50 composition of the mobile phases was used at a flow rate of 0.15 ml/min. Mobile phase A consisted of water with 0.1% formic acid (FA) and mobile phase B consisted of acetonitrile. During the optimisation of the ionisation conditions, Agilent Jet Stream electrospray ionisation (AJESI) was chosen as the ionisation source. Positive ionisation was used for all the analytes. Source parameters were set at default conditions according to the instrument manual. Nitrogen gas temperature was set at 300°C and the flow of the gas was 5 l/min. The nebuliser was set at 45 psi. Sheath gas heater temperature was set at 300°C and the sheath gas flow was at 11 l/min. The capillary voltage was set at 3500 V and the nozzle voltage was set at 0 V. During the optimisation of the ionisation conditions, the MS scan type was set to MS2 scan mode. The software was set to scan from 60 to 180 V for the fragmentor in steps of 5 V, and from 0 to 50 eV for the collision energy. Cell activator voltage was set to 4 V. The software only scanned for positive ions with a charge of 1 and the low mass cut off was set to 50 m/z for all analytes, except for glycine as it is a small molecule, and the product ion of underivatized glycine is smaller than 50 m/z.

3.4.2.2 Optimisation of source conditions

The optimisation of source parameters, such as drying gas flow (L/min), drying gas temperature (°C), nebuliser pressure (psi), capillary voltage (V), nozzle voltage (V), sheath gas flow (L/min) and sheath gas temperature (°C) for each analyte, ensures an increase in the sensitivity of the analytical method. AJS technology achieves a 5 to 10-fold improvement in the sensitivity of ESI-MS/MS. This is achieved by improving desolvation and spatial focusing of ions. Therefore, nitrogen sheath gas confines the nebulizer spray causing more effective drying of the ions and the concentration thereof (Mordehai & Fjeldsted, 2009). The optimisation of these parameters was done using the Agilent Source and iFunnel Optimizer software for 6400 triple quadrupole (as mentioned in Section 3.3.1). After the successful

optimisation of ionisation and MRM conditions were determined as described in Section 3.4.2.1, the optimal precursor ion, fragmentor, product ion, and collision energy were used to create MRM methods that were used by the iFunnel Optimizer software. During this optimisation, 5 µl of the 1 ppm standard stock solution (see Section 3.2.3) was injected using direct infusion. An isocratic 50:50 composition of the mobile phases was used, and the flow rate was kept at 0.15 ml/min. Mobile phase A consisted of water and 0.1% FA and mobile phase B consisted of acetonitrile.

For each of the parameters, a start value, end value, and step size were specified (Table 3.5). The software created an analysis worklist, analysing each parameter from start value to end value in step sizes (see Table 3.5). Each parameter value was injected in duplicate.

Table 3-5 A table of the start value, end value, and step size of the source parameters specified to the Agilent Source and iFunnel Optimizer software for the 6400 triple quadrupole.

Parameter	Start Value	End Value	Step Size
Sheath Gas Temperature (°C)	200	400	50
Sheath Gas Flow (L/min)	6	12	2
Drying Gas Temperature (°C)	200	350	25
Drying Gas Flow (L/min)	4	10	2
Nebulizer Pressure (psi)	20	50	5
Capillary Voltage (V)	2000	5000	500
Nozzle Voltage (V)	0	2000	500

3.4.3 Chromatographic optimisation

After the successful optimisation of MS conditions, the optimisation of chromatographic separation was done to ensure efficient separation of the analytes of interest. This requires careful selection of the appropriate column and mobile phases because the analytes are separated based on their interaction with both the stationary phase of the column and the mobile phases.

3.4.3.1 Column selection

According to Da Silva *et al.* (2016) the XSelect HSS T3 XP 2,5 micron 2,1 × 100 mm (186006151) column from Waters showed the best chromatographic separation for the analytes of interest. This reversed phase column is known for the improved retention of polar compounds when compared to normal C18 columns.

3.4.3.2 Mobile phase optimisation

For the analysis of the metabolites and co-factors of the methionine-homocysteine cycle, mobile phase A consisted of water with different concentrations of PFHpA. The purpose of this ion-pairing agent was to improve the separation of the polar analytes on the reversed phase C18 column. The ion-pairing agent increases the hydrophilic character of the column leading to increased retention of the polar compounds on the column (Dolan, 2008). The addition of mobile phase modifiers was evaluated. The gradient (the initial gradient can be seen in Table 3.6) of the mobile phases was adjusted to optimise the separation of the analytes and a flow rate of 0.4 ml/min was used.

Table 3-6 The composition of the initial mobile phase gradient that was used during separation

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
0.5	95	5
8	5	95
10	5	95
11	95	5
13	95	5

3.4.3.3 Injection volume optimisation

The optimisation of the volume that is injected onto the LC-MS/MS system is important to help ensure that the injection volume is sufficient for detection of the analytes of interest (Dolan, 2014). The injection volume was optimised by injecting 1 µl, 5µl, and 10 µl of a low (LC), medium (MC), and high (HC) concentration of the analytes onto the LC-MS/MS system in triplicate.

3.4.4 Addition of cystine and homocystine

Both homocysteine and cysteine can easily undergo oxidation to form homocystine and cystine. Samples used during this study were not treated with a disulfide reducing agent. Therefore, the method was adapted to include the detection of homocystine and cystine, the oxidised dimers for the amino acids, homocysteine and cysteine, respectively. Expanding the methionine-homocysteine profile to include these oxidised dimers can help achieve a better overview of the methionine-homocysteine profile. The ionisation and source conditions for both these analytes were done as described in Section 3.4.2.

3.4.5 Internal standard addition

Internal standards were added to help with the quantification and qualification of the compounds of interest (Oliveira *et al.*, 2010; Sargent, 2013 Wal *et al.*, 2010) as done by Behringer *et al.* (2019); Guiraud *et al.* (2017); Tomaiuolo *et al.* (2009); Weaving *et al.* (2006) in previous studies investigating the analytes and co-factors of the methionine-homocysteine cycle. Five deuterium-marked isotopes were used, including d₄-L-cystine, d₃-DL-homocystine, methyl-d₃-methionine, d₂-glycine, and d₅-phenylalanine. Table 3.7 shows a summary of the chemical properties of the internal standards. As was described in Section 3.4.2, the ionisation, MRM conditions, and source parameters for the internal standards were also optimised. This was done using the same software and method described in Section 3.4.2.

Table 3-7 Chemical properties of the internal standards

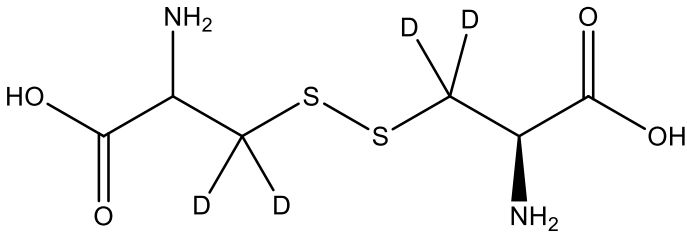
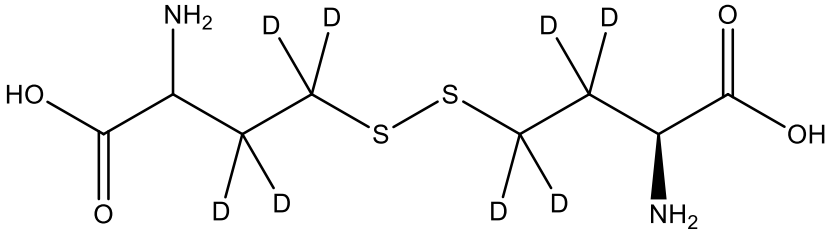
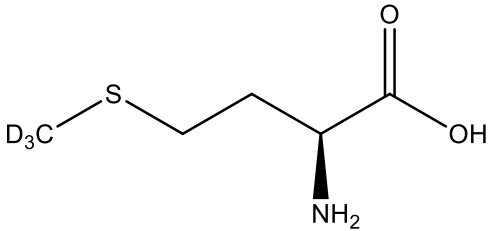
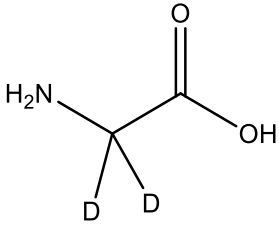
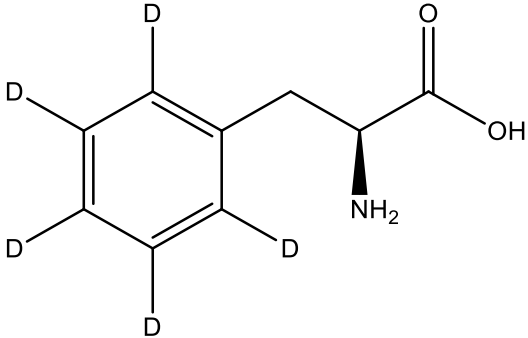
Internal Standard Name	Formula	Molecular Mass (g/mol)	Structure
d₄-L-Cystine	C ₆ H ₁₂ N ₂ O ₄ S ₂	244.32	
d₈-DL-Homocystine	C ₈ H ₁₆ N ₂ O ₄ S ₂	276.40	
Methyl-d₃-Methionine	C ₅ H ₁₁ NO ₂ S	153.22	
d₂-Glycine	C ₂ H ₅ NO ₂	77.08	

Table 3-7 continued

Chemical properties of the internal standards

Internal Standard Name	Formula	Molecular Mass (g/mol)	Structure
d ₅ -Phenylalanine	C ₉ H ₁₁ NO ₂	170.22	 The chemical structure of d₅-Phenylalanine is shown. It consists of a benzene ring with five deuterium (D) atoms at the 2, 3, 4, 5, and 6 positions. The ring is attached to a side chain consisting of a methylene group, a chiral center (marked with a wedge bond to the amino group), and a carboxylic acid group (COOH). The amino group is labeled NH₂.

3.4.6 Dynamic MRM

A dynamic MRM (dMRM) is an improvement of the conventional MRM method. During a dMRM, the mass spectrometer scans for the specific ion transitions at a specific retention time window that was determined during conventional MRM (Stone *et al.*, 2008). The dMRM mode improves selectivity because it doesn't waste cycle time by scanning for all the ion transitions, but only scans for specific ion transitions at specific times (Stone *et al.*, 2008). The sensitivity of the method also improves because the dwell times are increased because the method scans for less transitions during the specific windows. The MassHunter software determined the most optimal dwell time for each transition to ensure optimal cycle time. To assess if the dMRM would improve the results, a MC was analysed in triplicate using the conventional MRM and dMRM method. Chromatographic and qualitative data was assessed to conclude if the conventional or dMRM would be used.

3.5 Optimisation of sample preparation

It is known from literature that serum is known to be a relatively clean matrix and, therefore, the use of protein precipitation as a sample clean-up step is popular. Protein precipitation was achieved by altering the solubility of the protein (Burgess, 2009). During the addition of an organic solvent, the hydration layer of the proteins is destroyed, the repulsive forces between the protein molecules are reduced, and the solubility of the proteins are lowered. This causes proteins to precipitate out (Zhao & Juck, 2018). As discussed in Chapter 2, the addition of an organic solvent to the serum favours the solubility of the analytes and facilitates the precipitation of unwanted proteins present in the sample. Protein precipitation of serum samples are important to clean the sample, decreasing the number of unwanted compounds injected on the reversed phase column. According to literature, MeOH, ACN, and ethanol are popular organic solvents used for protein precipitation (Guiraud *et al.*, 2017; Zhao & Juck, 2018). Polson *et al.* (2003) reported that ACN was the most optimal for protein precipitation when compared to MeOH and ethanol. Literature states that 3-5 times the organic solvent is most optimal for protein precipitation (Kong, 2005). The use of an acid during protein precipitation lowers the pH, causing the protein to carry a positive charge (Da Silva *et al.*, 2016; Guiraud *et al.*, 2017). This causes a disruption of the hydration layer, causing the precipitation of the protein.

The protein precipitation protocol was based on the protocols used by Guiraud *et al.* (2017), Behringer *et al.* (2019), Liu *et al.* (2017), Ghassabian *et al.* (2014), Alshammari *et al.* (2015); Su *et al.* (2021). Protein precipitation of a serum sample with the use of cold MeOH, ACN, and ethanol with added 1% FA were assessed by adding 1 ml of the organic solvent to 200 µl of a

serum sample. Thereafter, the samples were vortexed to ensure that the solvents mix well with the sample. After thorough mixing of the organic solvent and the biological sample, the mixture was centrifuged at 9112 ×g and 4°C to ensure that the protein pellets precipitate. The supernatant was transferred to a filter microcentrifuge tube to eliminate any unwanted particles that might be present in the supernatant. The filtered supernatant was evaporated to dryness under a steady stream of N₂ at 35°C, after which it was resuspended in ACN:H₂O (5:95). The samples were transferred to glass vials for LC-MS/MS analyses. In the study by Guiraud *et al.* (2017), a disulfide reducing agent was added to the samples to aid in the accurate quantification of homocysteine and cysteine. The previously collected routine samples used for optimisation, and the eBOSS samples that were used for the proof of concept were not treated with a disulfide reducing agent.

3.6 Partial method validation

3.6.1 Introduction

Validation of an analytical method ensures that the analytical method used is reliable and reproducible (Wal *et al.*, 2010). During partial method validation, some validation parameters are determined to ensure the reliability and reproducibility of a modified analytical method that is already validated (Wal *et al.*, 2010). During this study, partial method validation was done by assessing parameters such as linearity, repeatability, accuracy, precision, and stability.

3.6.2 Linearity and lower limit of quantification

A calibration curve is a linear relationship between the concentration of an analyte and its response (Moosavi & Ghassabian, 2018). This curve is used to predict the unknown concentration of analytes that are present in a complicated matrix. To determine the linearity, and limit of quantification, a nine-point calibration range (Table 3.3 in Section 3.2.5) was prepared using 1:1 serial dilution of the working standard stock described in Section 3.2.5. These concentration levels were analysed in triplicate. When plotting the analyte response to the analyte concentration, a correlation coefficient (r^2) of between 0.98 and 1 is indicative of true linearity (Figure 3.2) when using the least squares regression method (Papadoyannis & Samanidou, 2004).

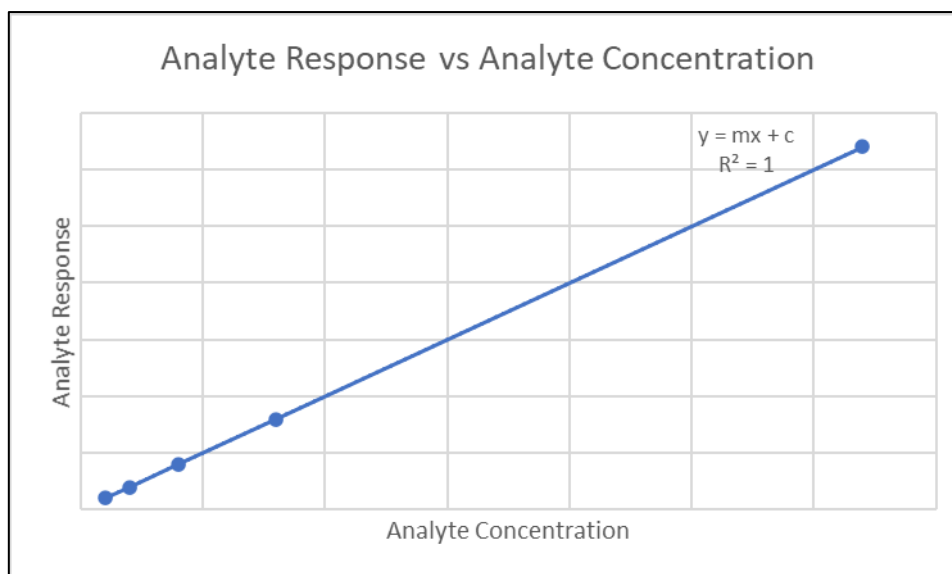


Figure 3-2 Representation of the ideal linearity when plotting the analyte response vs the analyte concentration.

For the purpose of this study the investigation of the lower limit of quantification (LLOQ) was of great importance while the investigation of the limit of detection (LOD) was not as important. LLOQ of an analytical standard refers to the lowest concentration of an analyte that can be accurately quantified when using the method (Papadoyannis & Samanidou, 2004) and was determined using a precision-based approach (Shrivastava & Gupta, 2011). During this approach the analyte concentration resulting in a coefficient of variance (CV) % of 20 % was used.

Equation 1 The formula used to determine the CV %

$$\text{CV\%} = (\text{Standard deviation} / \text{Mean}) \times 100$$

3.6.3 Precision and accuracy

Accuracy of a method can be described as how close the results obtained by the method are to the actual values (Papadoyannis & Samanidou, 2004; Ravisankar *et al.*, 2015). The precision of an analytic method refers to the degree of agreement of the measurements from different samples under the same analytical conditions (Chauhan *et al.*, 2015; Papadoyannis & Samanidou, 2004; Ravisankar *et al.*, 2015). To assess the precision and accuracy of the method, working standard stocks at 3 different concentrations (LC, MC, and HC), representative of the linear range and the LLOQ, was prepared as described in Section 3.2.5. The three concentration levels and LLOQ for each analyte can be seen in Table 3.8. Three replicates of these standards were prepared and analysed to determine intraday-precision and

accuracy. To determine the interday-precision and accuracy, these standards were prepared and analysed in triplicate over the course of three days. Precision was assessed based on the CV values and should be $\leq 15\%$, except for the LLOQ, where the CV values should be $\leq 20\%$ (see Equation 1); and the method accuracy was assessed using the mean values where the mean values should be within 15% of the nominal value, except for the LLOQ, where mean values should be within 20% (see Equation 2) (Lynch, 2016).

Table 3-8 The four concentration levels used for each analyte during the determination of accuracy and precision. The LLOQ was calculated as described in Section 3.6.2 and the remaining three concentrations were selected to be representative of the linear concentration range as reported in Section 4.7.2

Analyte	Concentration 1 (LLOQ) (ng/ml)	Concentration 2 (LC) (ng/ml)	Concentration 3 (MC) (ng/ml)	Concentration 4 (HC) (ng/ml)
L-Homocysteic acid	2.451	4.902	9.804	19.608
Taurine	32.680	65.359	130.719	261.438
L-Serine	3.268	13.072	26.144	52.288
N, N-DMG	0.817	3.268	6.536	26.144
Betaine	3.268	13.072	26.144	52.288
Glycine	3.268	13.072	52.288	209.150
L-Cysteine	4.085	16.340	32.680	65.359
DL-Homocysteine	2.042	4.085	16.340	65.359
Riboflavin (Vitamin B2)	0.163	1.307	2.614	5.229
Choline	2.042	8.170	16.340	65.359
L-Methionine	2.042	4.085	8.170	16.340
Pyridoxine (Vitamin B6)	0.041	0.082	0.163	0.327
5-Methyltetrahydro folic acid	1.961		3.922	7.843
L-Cystine	8.170	32.680	130.719	522.876
Cystathionine	0.163	0.654	2.614	5.229
SAH	2.614		5.229	10.458
L-Homocystine	3.268	13.072	26.144	52.288
SAM	0.980	1.961	3.922	7.843

Equation 2 The formula used to determine accuracy

$$\text{Accuracy} = \frac{\text{Relative response of the analyte calculates}}{\text{Relative response of the analyte in theory}} \times 100$$

3.6.4 Injection Repeatability

The injection repeatability was evaluated by injecting LLOQ, LC, MC, and HC samples in triplicate. Deviation in the relative response were assessed. This was done by determining the CV % of the responses. Values of ≤15% are normally viewed as acceptable.

3.6.5 Stability

The short-term and long-term stability of analytes needs to be determined because of possible decomposition during sample preparation, extraction and storage of prepared solvents (Meng & Bennett, 2012; Papadoyannis & Samanidou, 2004). For stability, a LLOQ, LC, MC, and HC (Table 3.8) were prepared in triplicate for four different conditions: immediate analysis, stored at room temperature (bench-top) for 24 hours, stored in the autosampler (4°C) for 24 hours, stored in the fridge (4°C) for 48 hours, and stored at -20°C for 7 days (one week). The response of the analytes obtained in each of the storage conditions were compared with the response of a freshly prepared standard. The stability was calculated as a percentage of the freshly prepared sample. A deviation below 15% is acceptable.

3.7 Statistical analysis

Stable labelled isotopes were used to quantify the metabolites and co-factors of the methionine-homocysteine cycle. Some of the analytes were relatively quantified. This was done using the relative response factor (the slope of the analyte of interest as seen after plotting the calibration curves). The calibration curve was compiled using the analyte response divided by the internal standard response, which was plotted against the concentration of the analyte divided by the internal standard concentration.

MetaboAnalyst was used to do a statistical analysis of the data obtained from the samples of the eBOSS samples (Section 5). Before importing the data obtained into MetaboAnalyst stable labelled isotopes were used for normalisation. Transformation and scaling were the data pretreatment processes used to convert the data to another scale. Using these pretreatment techniques decreased the possibility of the influence of factors such as differences in the concentration range of different analytes and technical variation (van den Berg *et al.*, 2006). During the data scaling, a scaling factor such as the standard deviation of the mean was used to place the variables on equal footing (van den Berg *et al.*, 2006). Pareto scaling was used

to scale the data set using the square root of the standard deviation as the scaling factor (van den Berg *et al.*, 2006). Transforming the data is done to correct skewed data. Log transformation removes heteroscedasticity of the data during statistical analysis (van den Berg *et al.*, 2006). Quality assessment of the data was done by investigating the presence of a batch effect, applying a QCCV filter, and assessing for outliers.

The samples of the eBOSS study were analysed in two batches, causing the possibility of a batch effect to arise due to analytical variance. A principal component analysis (PCA) was used to investigate if there was any prominent clustering of the data of the same batch. To remove variables that had no significant value in the data set a; QCCV filter was applied (Hackstadt & Hess, 2009). During this filtering step, features below a CV % of 35 % in the QC sample were removed. A PCA was used to investigate the presence of outliers. The preprocessing and filtering of the data provide a more simplified data set for univariate and multivariate statistical analysis.

During univariate analysis, only one variable is analysed at a time by using tests to compare different sets of samples. Both a t-test and a volcano plot were done. During the t-test, significant features were selected by using the p-value. The p-value threshold was set to 0.05, indicating that a feature with a p-value below 0.05 could indicate a rejection of the null hypothesis (Sellke *et al.*, 2001). The p-value was plotted against the fold change (FC) to create a volcano plot. FC describes the ratio of the means of the two groups (Ebrahimipoor & Goeman, 2021). During this analysis important features were selected using the criteria of an FC threshold of 1 and a p-value threshold of 0.05. The features are seen as more significant the further away they are from 0;0. Features towards the top are the most significant features (Ebrahimipoor & Goeman, 2021).

Multivariate statistical analysis is the investigation of multiple variables at the same time. Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) are two multivariate methods using projection to visualise and quantify the differences between two groups (Saccenti *et al.*, 2014). PCA and PLS-DA were used during the statistical analysis of the data obtained from the eBOSS samples. A PCA is an unsupervised method (meaning that it does not know about the different groups) during which the data is projected onto another dimension indicating the variation between the data while reducing the loss of data as much as possible (Jolliffe, 1990; Saccenti *et al.*, 2014). While PLS-DA is a supervised method (knows about the different groups), the data is projected onto another dimension to show variation in the data and the groups (Ruiz-Perez *et al.*, 2020; Saccenti *et al.*, 2014).

CHAPTER 4 METHOD IMPLEMENTATION, OPTIMISATION, AND VALIDATION

4.1 Introduction

In this chapter, the results obtained during the implementation and optimisation of the methods explained in chapter 3 are discussed. Before analytical methods can be applied to quantify certain metabolites in biological samples the development, optimisation, and validation of these methods are of great importance. The method developed and published by Guiraud *et al.* (2017) quantifies 17 metabolites and co-factors (of which two were Vitamin B₆ vitamers) of the methionine-homocysteine cycle by using a trifunctional C18 column with the support of an ion-pairing agent PFHpA for chromatographic separation and tandem mass spectrometry for the detection of the separated compounds. This method was implemented on the LC-MS/MS system mentioned in Section 3.2.

4.2 Mass spectrometry optimisation

The optimisation of ionisation, transitions, and source conditions are important to ensure optimal detection by the tandem mass spectrometer. Because the instrumentation used for this study differs from that used by Guiraud *et al.* (2017), the ionisation of the compounds can differ. For this reason, the MRM and source conditions of each metabolite and co-factors were optimised using the software mentioned in Section 3.3.1.

4.2.1 Optimisation of ionisation conditions

Optimal ionisation and MRM conditions were determined as described in Section 3.4.2.1. The conditions included precursor ions, fragmentor voltage for each precursor ion, the possible product ions, and collision energy that was most optimal for each transition. Five microliters of each of the 18 analytes were individually injected. The most abundant or the most unique precursor and product ion and the most optimal collision energy and fragmentor voltage, were chosen to use during MRM mode (Table 4.1). These optimal ionisation and MRM conditions were used to improve selectivity in the detection of the analytes of interest. The different transition results obtained by the Agilent MassHunter Optimizer software can be seen in Table A1 of Annexure A.

Table 4-1 Results of the optimisation of ionisation and MRM conditions. This table represents a summary of the results obtained for most abundant or the most unique precursor and product ion, and the most optimal collision energy and fragmentor voltage determined

Analyte	Formula	Polarity	Precursor (m/z)	Product (m/z)	Fragmentor voltage (V)	Collision energy (eV)
L-Homocystine	C ₈ H ₁₆ N ₂ O ₄ S ₂	Positive	269.07	136	89	4
DL-Homocysteine	C ₄ H ₉ NO ₂ S	Positive	136.05	90	60	16
L-Methionine	C ₅ H ₁₁ NO ₂ S	Positive	150.06	104.1	74	8
L-Cysteine	C ₃ H ₇ NO ₂ S	Positive	122.03	59	50	24
L-Cystine	C ₆ H ₁₂ N ₂ O ₄ S ₂	Positive	241.03	151.1	94	8
SAM	C ₁₅ H ₂₂ N ₆ O ₅ S	Positive	399.15	250.1	50	12
SAH	C ₁₄ H ₂₀ N ₆ O ₅ S	Positive	385.13	134	70	16
Choline	C ₅ H ₁₄ NO	Positive	104.12	60.1	70	16
Betaine	C ₅ H ₁₁ NO ₂	Positive	118.08	58.1	118	32
Cystathionine	C ₇ H ₁₄ N ₂ O ₄ S	Positive	223.08	134	79	8
N, N-DMG	C ₄ H ₉ NO ₂	Positive	104.07	58.1	79	12
Glycine	C ₂ H ₅ NO ₂	Positive	76.04	30.1	45	8
L-Serine	C ₃ H ₇ NO ₃	Positive	106.05	60.1	50	8
Taurine	C ₂ H ₇ NO ₃ S	Positive	126.02	108	89	8
L-Homocysteic acid	C ₄ H ₉ NO ₅ S	Positive	184.03	56.1	79	20
5-Methyltetrahydro folic acid	C ₂₀ H ₂₅ N ₇ O ₆	Positive	460.2	313.1	112	16
Riboflavin (Vitamin B2)	C ₁₇ H ₂₀ N ₄ O ₆	Positive	377.15	243.1	137	24
Pyridoxine (Vitamin B6)	C ₈ H ₁₁ NO ₃	Positive	170.08	152	89	12

4.2.2 Optimisation of source conditions

To ensure the optimal sensitivity of the method, the optimisation of each source parameter for each analyte is of importance. Section 3.4.2.2 describes the optimisation of the drying gas flow (L/min), drying gas temperature (°C), nebuliser pressure (psi), capillary voltage (V), nozzle voltage (V), sheath gas flow (L/min) and sheath gas temperature (°C), using the Agilent Source and iFunnel Optimizer software for the 6400 triple quadrupole. As described in Section 3.4.2.2,

a duplicate injection was done for each parameter. The raw data of each parameter for each analyte were processed to select the optimal settings. Table 4.2 is a representation of the average of the data of each parameter for each analyte (Table A2 contains all the results obtained during optimisation). The drying gas flow of 6 L/min, drying gas temperature of 300 °C, nebuliser pressure of 30 psi, capillary voltage of 2500 V, nozzle voltage of 500 V, sheath gas flow of 10 L/min, and a sheath gas temperature of 300 °C were the most optimal source conditions for each analyte (Table 4.2).

Table 4-2 Optimal source conditions for all analytes after optimisation of the source conditions

Source Parameter	Optimal condition
Sheath Gas Temperature (°C)	300
Sheath Gas Flow (L/min)	10
Drying Gas Temperature (°C)	300
Gas Flow (L/min)	6
Nebulizer Pressure (psi)	30
Capillary Voltage (V)	2500
Nozzle Voltage (V)	500

Subsequent analyses were all carried out using the MS parameters summarised in Table 4.2, unless stated otherwise.

4.3 Chromatographic separation

4.3.1 Column selection

The separation of the analytes of interest were done on the XSelect HSS T3 XP 2,5 micron 2,1 × 100 mm reversed phase column. This analysis was done based on the chromatographic method reported by Da Silva *et al.* (2016). During this analysis, 10 µl of a master mix (see Section 3.2.3), with a concentration of 1 ppm of each analyte, was injected in triplicate. Mobile phase A was water with 5 mM PFHpA and mobile phase B was acetonitrile. The mobile phase flow rate was set to 0.4 ml/min and the column temperature was set to 30°C. A gradient elution was used (see Table 4.3) and the total run time was 15 min. The MS/MS parameters were set as reported in Section 4.2.

Table 4-3 The initial mobile phase gradient used during separation

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
0,5	95	5
8	5	95
10	5	95
11	95	5
13	95	5

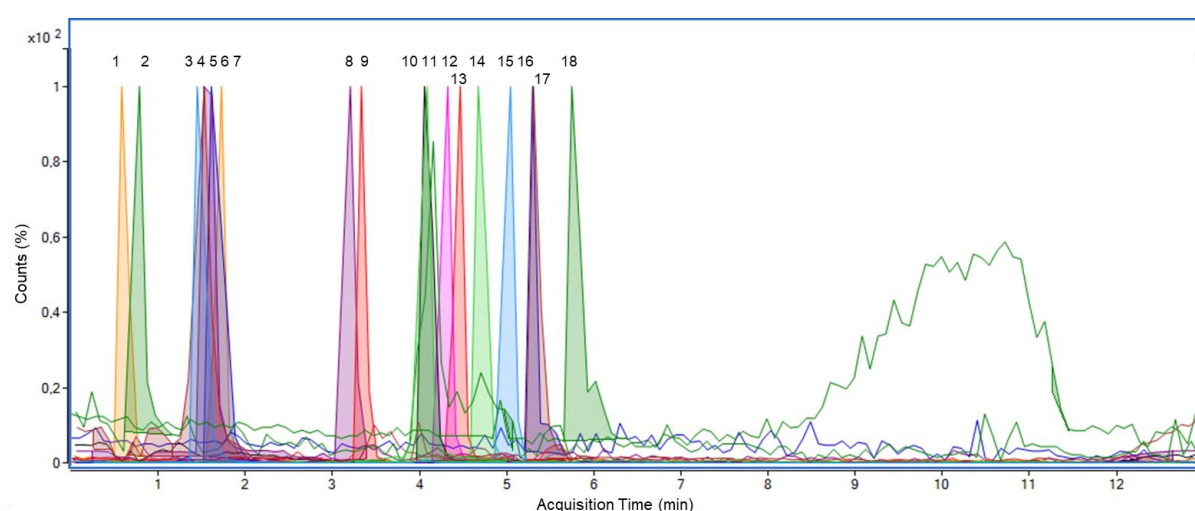


Figure 4-1 Chromatographic illustration of the separation of analytes on the XSelect HSS T3 XP 2,5 micron 2,1 × 100 mm column, including the retention time of each analyte (1) HCA, 2) Taurine, 3) DMG, 4) Serine, 5) Betaine, 6) Cysteine, 7) Glycine, 8) Riboflavin, 9) Homocysteine, 10) Choline, 11) Methionine, 12) Pyridoxine, 13) 5-MTHFA, 14) Cystine, 15) Cystathionine, 16) SAH, 17) Homocystine, and 18) SAM).

This analysis was done to determine the retention time (Table 4.4) of each analyte and to assess the separation of the analytes on the XSelect HSS T3 XP column. Figure 4.1 illustrates the chromatographic separation that was obtained. Because of the difference in response of the analytes of interest, all the chromatograms that followed in the dissertation were scaled to ensure a good illustration of chromatographic separation. From this chromatogram, it is clear that partial separation was obtained using the XSelect HSS T3 XP column with PFHpA as the ion-pairing agent and the selectivity of the MS lead to the identification of the compounds. The small particle size of the column's stationary material facilitates the use of high mobile phase flow rates, which results in much shorter analysis times. Mobile phase gradient adjustment

was considered to improve the separation of the analytes that elute close to each other. Cystine and homocystine forms part of the expansion of the methionine-homocysteine cycle (see Section 4.3.3). Furthermore, there might be some ionisation problems.

Table 4-4 Retention times (min) of each analyte when separated on the XSelect HSS T3 XP column using the liquid chromatography specifications described at the beginning of this section (including the mobile phase gradient in Table 4.3) and mass spectrometry specifications as discussed in Section 4.1.

Analytes	Retention time (min)
L-Homocysteic acid	0.582
Taurine	0.774
L-Serine	1.465
N, N-DMG	1.519
Betaine	1.568
Glycine	1.705
L-Cysteine	1.720
DL-Homocysteine	3.195
Riboflavin (Vitamin B2)	3.336
Choline	4.072
L-Methionine	4.077
Pyridoxine (Vitamin B6)	4.311
5-Methyltetrahydro folic acid	4.458
L-Cystine	4.692
Cystathionine	5.031
SAH	5.297
L-Homocystine	5.318
SAM	5.747

4.3.2 Mobile phase optimisation

The use of ion-pairing agents was reported to be critical for the separation of polar compounds (Gustavsson *et al.*, 2001) on the trifunctional C18 column (Da Silva *et al.*, 2016) but ion-pairing agents need to be used with caution, especially because of the effect of these agents on the separation characteristics of certain stationary materials (Dolan, 2008). The concentration of the ion-pairing agent used is of critical importance to achieve a balance between chromatographic retention and minimal signal suppression (Kostiainen & Kauppila, 2009). For this reason, the use of ion-pairing agents in the mobile phase was optimised to help achieve optimal separation of the compounds of interest. Formic acid was added to both mobile phase A and B to ensure optimal ionisation.

4.3.2.1 Ion-pairing agent optimisation

Although Da Silva *et al.* (2016) reported that the use of 5 mM PFHpA was optimal for the analysis of the analytes of interest it is known that ion-pairing agents can cause ionisation-suppression. It is also known that the separation characteristics of certain columns may be altered, using ion-pairing agents. Therefore, mobile phase A was prepared at three different concentrations: 3 mM, 4 mM and 5 mM in water. Triplicate injections (2 µl) of a 5 ppm master mix was done, using the three different concentrations of PFHpA in water. Mobile phase B consisted of acetonitrile. Retention time, peak shape, background noise, and peak intensity were considered during the assessment of optimal PFHpA concentration.

Figure 4.2 summarises chromatographic results obtained using 3 mM, 4 mM, and 5 mM PFHpA in water for mobile phase A. These results indicate that not one concentration of PFHpA was optimal for all 18 analytes, but most of the analytes showed good chromatographic profiles with the use of 4 mM or 5 mM PFHpA.

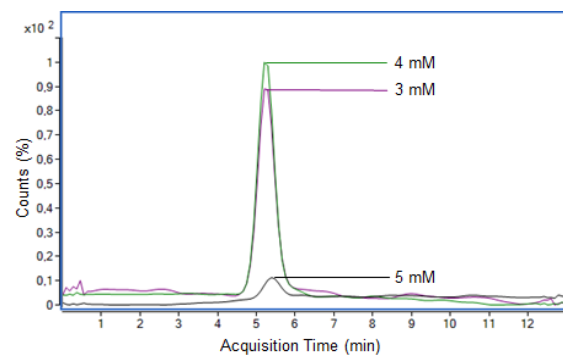
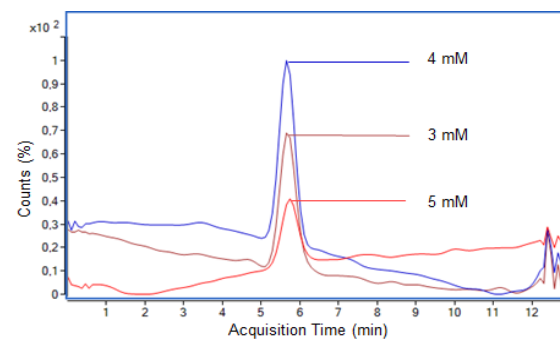
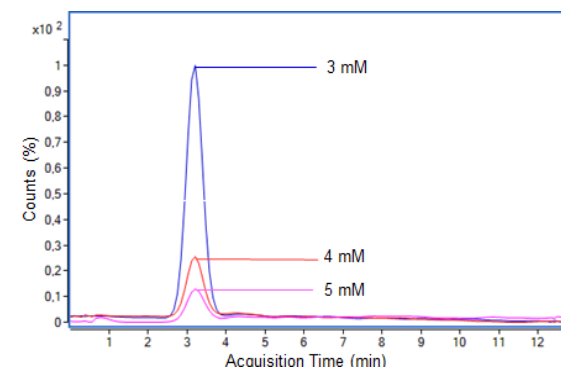
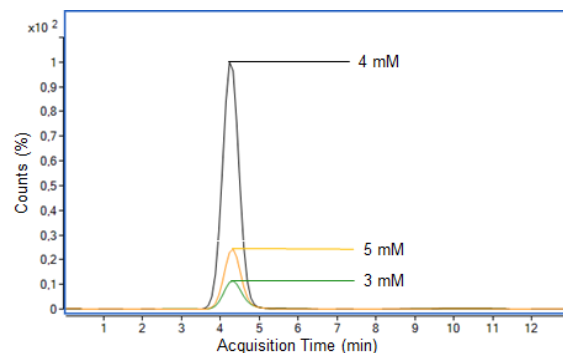
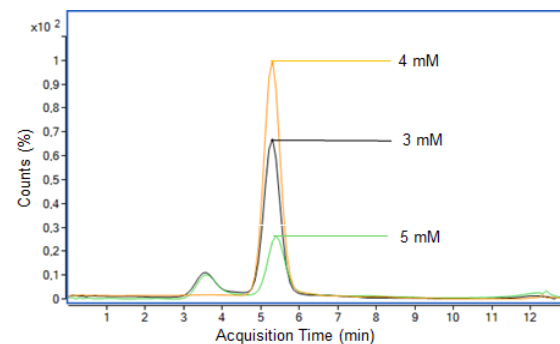
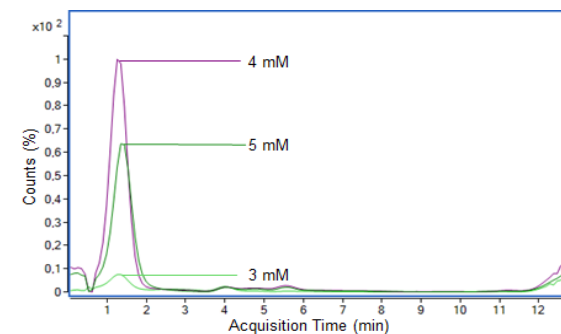
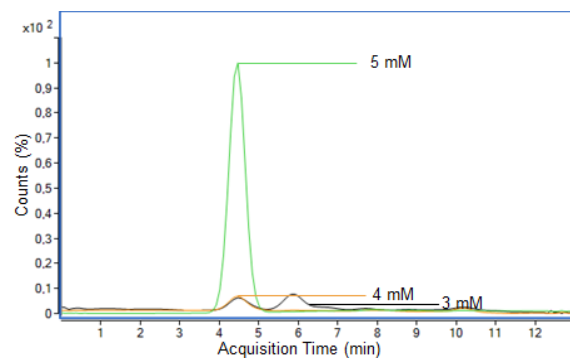
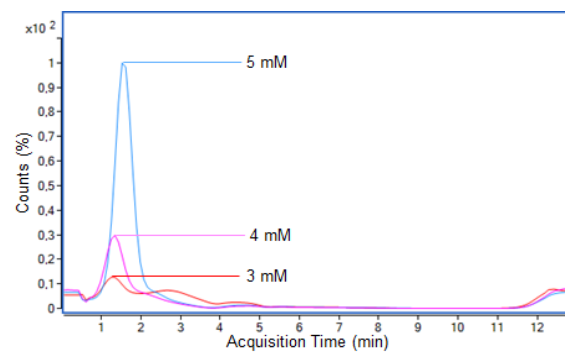
A) SAH**B) SAM****C) Riboflavin****D) Pyridoxine****E) Homocystine****F) Serine**

Figure 4-2 MRM chromatographic results of the 18 analytes of interest at 3, 4 and 5 mM PFHpA. A) SAH, B) SAM, C) Riboflavin, D) Pyridoxine, E) Homocystine, F) Serine, G) 5-MTHFA, H) Betaine, I) Choline, J) Cystathionine, K) Cysteine, L) Cystine, M) DMG, N) Glycine, O) Homocysteic acid, P) Homocysteine, Q) Methionine, R) Taurine.

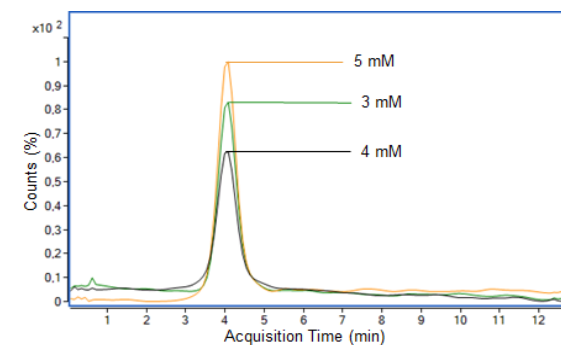
G) 5-MTHFA



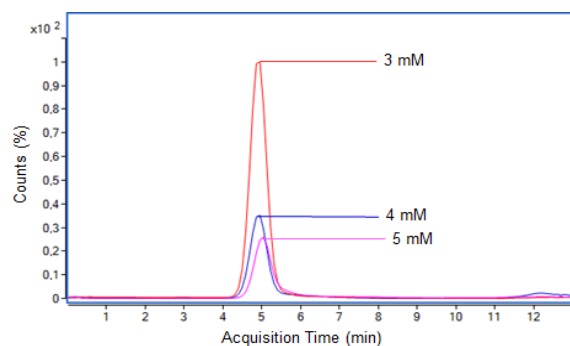
H) Betaine



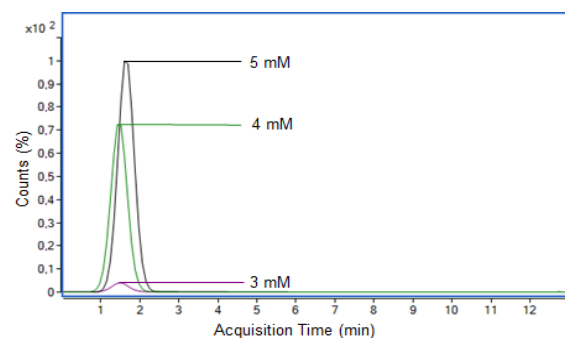
I) Choline



J) Cystathionine



K) Cysteine



L) Cystine

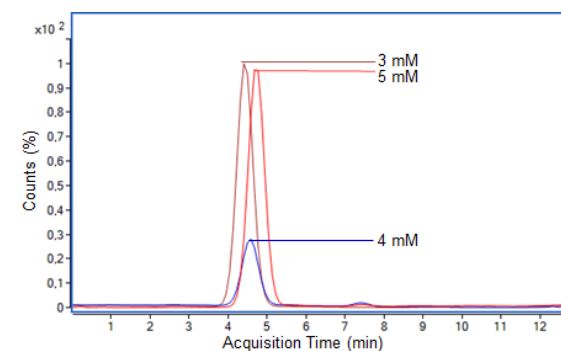
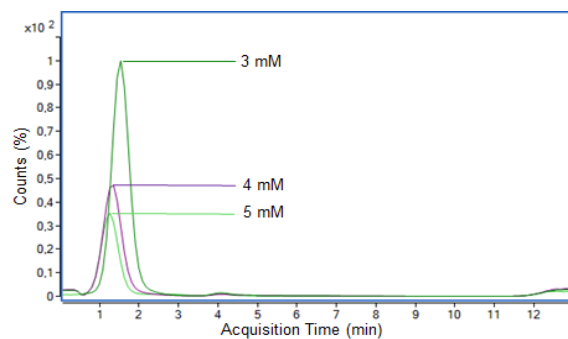


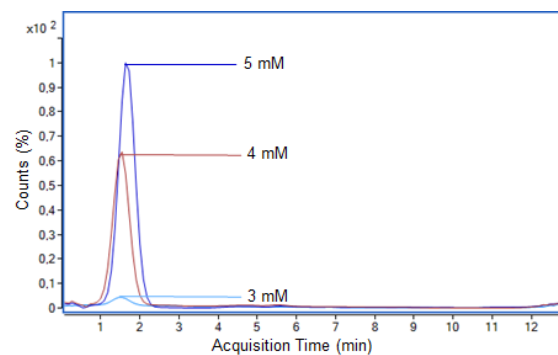
Figure 4 2 continued

MRM chromatographic results of the 18 analytes of interest at 3, 4 and 5 mM PFHpA. A) SAH, B) SAM, C) Riboflavin, D) Pyridoxine, E) Homocystine, F) Serine, G) 5-MTHFA, H) Betaine, I) Choline, J) Cystathionine, K) Cysteine, L) Cystine, M) DMG, N) Glycine, O) Homocysteic acid, P) Homocysteine, Q) Methionine, R) Taurine.

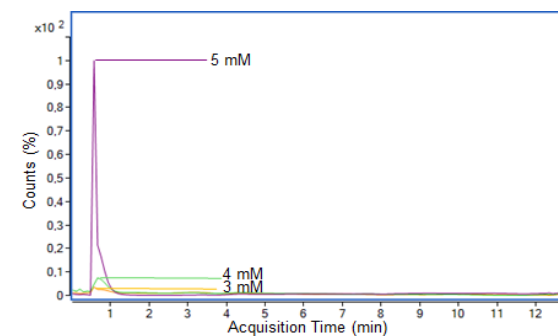
M) DMG



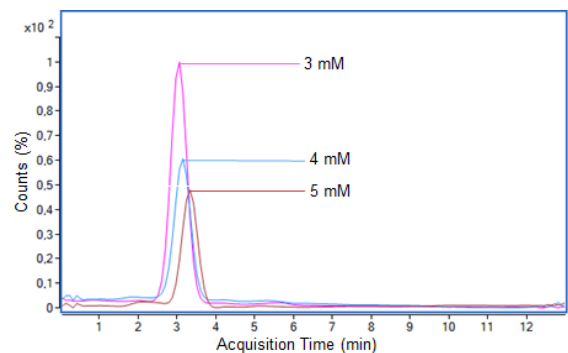
N) Glycine



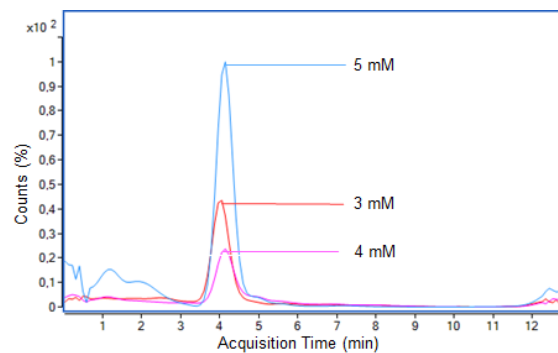
O) Homocysteic acid



P) Homocysteine



Q) Methionine



R) Taurine

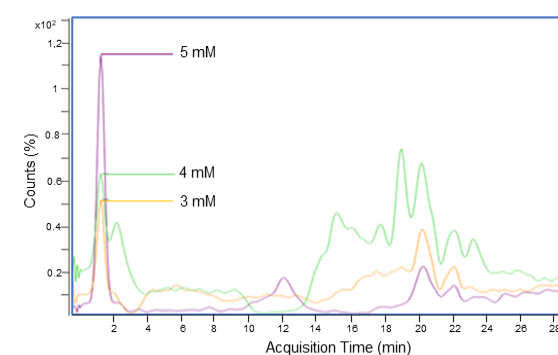


Figure 4 2 continued

MRM chromatographic results of the 18 analytes of interest at 3, 4 and 5 mM PFHpA. A) SAH, B) SAM, C) Riboflavin, D) Pyridoxine, E) Homocystine, F) Serine, G) 5-MTHFA, H) Betaine, I) Choline, J) Cystathionine, K) Cysteine, L) Cystine, M) DMG, N) Glycine, O) Homocysteic acid, P) Homocysteine, Q) Methionine, R) Taurine.

In Figure 4.2 (A), the chromatogram for SAH at the three different concentrations of PFHpA can be seen. Here it is clear that a concentration of 4 mM PFHpA is most optimal. This conclusion was made from visual comparison: the shape of the peak was better (less peak tailing). There was an increased peak intensity, and there was less baseline noise when compared with the 3 mM and 5 mM chromatogram. Analysis with 5 mM PFHpA showed decreased peak intensity, poor peak shape, and increased noise when compared with 3 or 4 mM. The chromatographic results for SAM are illustrated in Figure 4.2 (B). The green chromatogram illustrates a higher peak intensity, better peak shape, and the least background noise. The retention time stayed the same. These chromatograms indicated that 4 mM PFHpA was the most optimal for SAM. Chromatographic results for pyridoxine (Figure 4.2 D) showed that there was no difference in retention time, peak shape, or noise at the different concentrations PFHpA. However, there was a difference in peak intensity: using 4 mM PFHpA resulted in a more intense peak. In Figure 4.2 (E), the light and dark blue chromatograms showed a double peak for homocystine when 3 or 5 mM PFHpA was used. The chromatogram obtained after analysis with 5 mM had a slight increase in retention and noise. A concentration of 4 mM was considered most optimal for homocystine because it yielded a single peak and increased the peak intensity. In Figure 4.2 (F) the chromatograms indicate that there was a slight increase in the retention of serine with the use of 5 mM PFHpA. The peak shape at 3 mM PFHpA was not optimal when compared to the results obtained using 4 and 5 mM PFHpA and there was an increase in the noise. A concentration of 4 mM resulted in an increased peak intensity.

Figure 4.2 (G) is the chromatographic result of the effect of PFHpA concentrations on 5-MTHFA. Here it was concluded that 5 mM PFHpA was the most optimal as a concentration of 3 mM resulted in two peaks and 4 mM had increased baseline noise when compared to 5 mM PFHpA, which also resulted in an increased peak intensity. Betaine (Figure 4.2 H) had improved retention, peak shape, decreased noise and increased intensity at 5 mM PFHpA when compared to concentrations of 3 and 4 mM. In Figure 4.2 (I), Choline showed good results at all three concentrations, but an increased peak intensity with the use of 5 mM PFHpA. In Figure 4.2 (J), cystathionine had increased peak intensity with the use of 3 mM PFHpA but increased retention with the use of 5 mM PFHpA. For cysteine (Figure 4.2 K) the use of 5 mM was optimal because of increased retention, peak intensity, and better peak shape when compared to 3 and 4 mM PFHpA. Cystine (Figure 4.2 L) and DMG (Figure 4.2M) showed increased retention with an increase in PFHpA concentration. Both these analytes had increased peak intensity at a concentration of 5 mM. Results obtained from chromatograms for both glycine (Figure 4.2 N) and methionine (Figure 4.2 Q) indicated that there was increased retention and peak intensity with the use of 5 mM PFHpA, while lower

concentrations illustrated the presence of background noise. Although the chromatogram obtained for HCA show a poor chromatographic profile for all the concentrations PFHpA, 5 mM resulted in a better peak shape and increased peak intensity. The chromatographic profiles for taurine at the three different PFHpA concentrations can be seen in Figure 4.2 (N). In this chromatographic profile, it can be seen that 5 mM PFHpA was the most optimal for taurine. The use of 5 mM PFHpA resulted in better peak shape, better ionisation, and increased peak intensity.

When assessing the chromatographic profiles of riboflavin (Figure 4.2 C) and homocysteine (Figure 4.2 E), the most optimal concentration of PFHpA for these analytes is 3 mM. For homocysteine, retention on the column improved with an increase in the concentration of PFHpA, but there was a decrease in the area. The peak shapes were optimal at each concentration. The peak area decreased for riboflavin with an increase in concentration of PFHpA. The retention on the column was the same and the peak shape was good with the use of 3 mM and 4 mM PFHpA. Increasing the concentration to 5 mM, however, resulted in more background noise.

After testing the three concentrations of PFHpA, the results indicated that none of the concentrations tested were optimal for all the analytes of interest (see Table 4.5). In fact, some concentrations had a significant negative effect on the retention, peak shape and intensity, and background noise of the analytes.

Table 4-5 A summary of the results obtained after testing the most optimal concentration PFHpA for each analyte of interest.

Analyte	Most optimal PFHpA concentration	Second most optimal PFHpA concentration	PFHpA concentration giving a negative effect
L-Homocystine	4 mM		3 mM 5 mM
DL-Homocysteine	3 mM	4 mM 5 mM	
L-Methionine	5 mM	3 mM 4 mM	
L-Cysteine	5 mM	4 mM 3 mM	
L-Cystine	5 mM	4 mM 3 mM	
SAM	4 mM	3 mM 5 mM	
SAH	4 mM	3 mM 5 mM	
Choline	5 mM	3 mM 4 mM	
Betaine	5 mM	4 mM	3 mM
Cystathionine	3 mM	4 mM 5 mM	
N, N-DMG	3 mM	4 mM 5 mM	
Glycine	5 mM	4 mM	3 mM
L-Serine	4 mM	5 mM	3 mM
Taurine	5 mM		4 mM 3 mM
L-Homocysteic acid	5 mM	4 mM	3 mM
5-Methyltetrahydro folic acid	5 mM		4 mM 3 mM
Riboflavin (Vitamin B2)	3 mM	4 mM 5 mM	
Pyridoxine (Vitamin B6)	4 mM	5 mM 3 mM	

4.3.2.2 Method separation

The results discussed in Section 4.3.2.1 indicated that the analytes had better chromatographic profiles at different concentrations of PFHpA – SAM, SAH, pyridoxine, homocysteine, and serine had the best chromatographic profiles using 4 mM PFHpA. 5-MTHFA, betaine, choline, cysteine, cystine, DMG, glycine, HCA, methionine, and taurine had the best chromatographic profiles using 5 mM PFHpA (See Table 4.5). Riboflavin, cystathionine, and homocystine had the best chromatographic profiles with the use of 3 mM PFHpA. Because it is important to obtain optimal chromatographic profiles for all the analytes of interest, the use of a single concentration of PFHpA was reconsidered.

Twelve analytes had optimal chromatographic conditions using 5 mM PFHpA, four analytes using 4 mM PFHpA, and three metabolites were optimal with the use of a concentration of 3 mM PFHpA. These results suggest that the use of more than one concentration of PFHpA could improve the chromatographic separation of the analytes of interest. Throughout the optimisation of this method, it was important to keep the total analysis time in mind. The Agilent 1290 infinity series high pressure liquid chromatography consists of a binary pump and can, therefore, only switch between two different mobile phase A's. Considering all the results and limitations of the instrumentation, it was decided to split the method into two separate methods, Method A and Method B. During Method A, mobile phase A consisted of 4 mM PFHpA in water and during Method B, mobile phase A consisted of 5 mM PFHpA in water. The mass spectrometer was set to scan for SAM, SAH, homocystine, pyridoxine, serine, and riboflavin during Method A, while in Method B the mass spectrometer scanned for 5-MTHFA, betaine, choline, cystathionine, cysteine, cystine, DMG, glycine, HCA, methionine, taurine, and homocysteine. As reported in Table 4.5 and in Section 4.3.2.1, the chromatographic profile for riboflavin, cystathionine, and homocysteine was most optimal with the use of 3 mM PFHpA. However, the chromatographic profile for riboflavin at 4 mM, cystathionine at 5mM, and homocysteine at 5 mM also yielded adequate results. Therefore, these three metabolites were included in Method A and Method B, respectively.

During the first analysis done using the two different methods, 2 µl of a 5 ppm master mix was injected. The initial mobile phase gradient (Table 4.3) was adapted and used during both methods. Gradient 1 in Table 4.6 was used for Method A and Gradient 1 in Table 4.8 was used for Method B. Figures 4.3 and 4.4 illustrate the chromatographic profiles obtained using the two different methods.

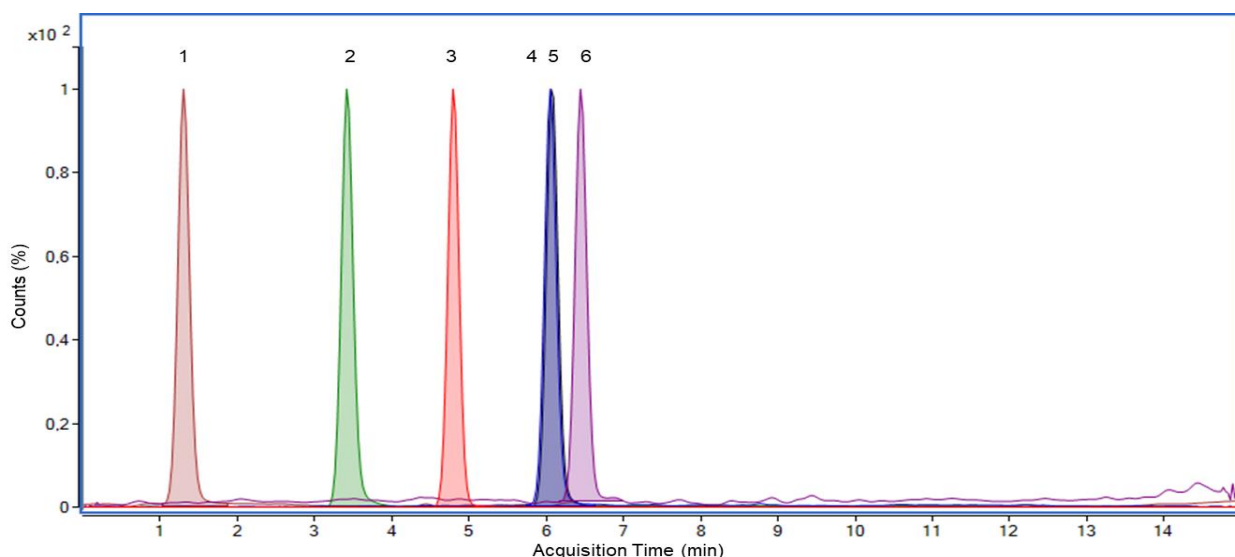


Figure 4-3 MRM chromatogram of the metabolites in Method A using Mobile Phase gradient 1 in Table 4.6 (1) Serine, 2) Riboflavin, 3) Pyridoxine, 4) SAH, 5) Homocystine, and 6) SAM) using gradient 1. The retention times for each analyte can be found in Table 4.7.

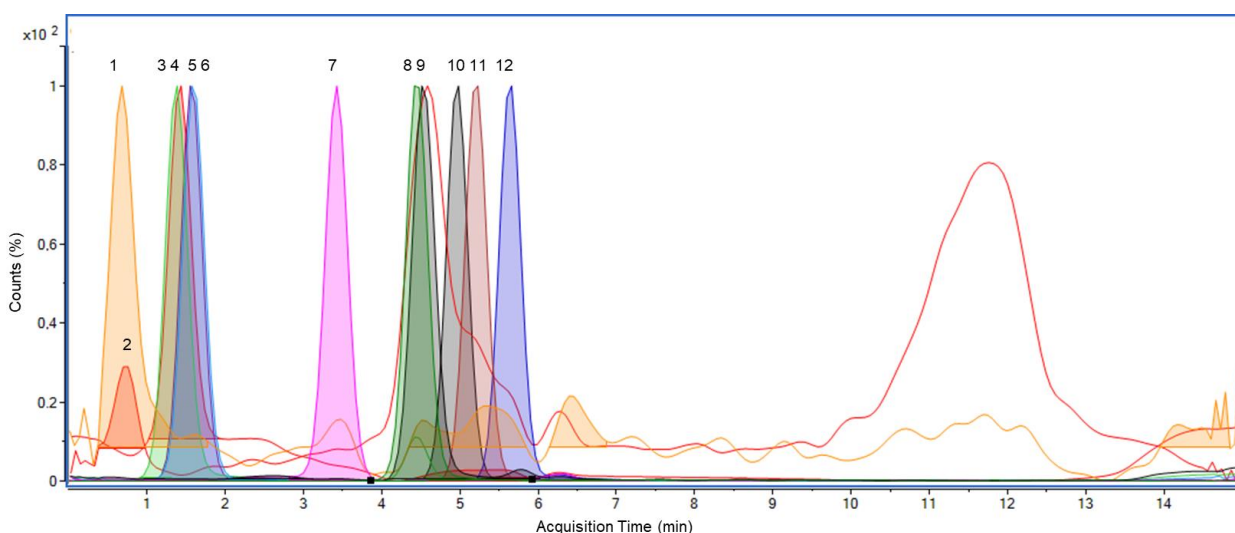


Figure 4-4 MRM chromatogram of the metabolites in Method B (1) HCA, 2) Taurine, 3) DMG, 4) Betaine, 5) Cysteine, 6) Glycine, 7) Homocysteine, 8) Choline, 9) Methionine, 10) 5-MTHFA, 11) Cystine, and 12) Cystathionine) using gradient 1. The retention times for each analyte can be found in Table 4.9.

In Figure 4.3, it can be seen that SAH and homocystine coelute, and in Figure 4.4 it can be seen that there are a few overlapping peaks. This includes DMG, betaine, glycine, and cysteine, and choline and methionine. The further optimisation of the mobile phase gradient needed to be done to improve peak separation.

4.3.2.3 Mobile phase gradient optimisation

After the separation of the methods the mobile phase gradients for Method A and B were optimised to ensure better chromatographic separation of the analytes. The different mobile phase gradients were determined progressively by using the initial gradient as a reference. Using the initial gradient composition of mobile phase B and the retention times obtained using the initial gradient, the specific mobile phase B % at which each analyte eluted was determined. This was done visually. This was done by adjusting the mobile phase compositions at different time periods.

4.3.2.3.1 Method A

During Method A, mobile phase A consisted of 4 mM PFHpA in water and the mass spectrometer was set to scan for SAM, SAH, homocystine, pyridoxine, serine, and riboflavin. As can be seen in Figure 4.3, good separation between the analytes of interest is obtained with the exception of homocystine and SAH which shows overlapping peaks. Therefore, 2 µl of a 5 ppm master mix was injected using three different mobile phase gradients (adjusted from gradient 1), as can be seen in Table 4.6.

Table 4-6 The six different mobile phase gradients used to optimise the separation of the analytes in Method A. The time in minutes and the percentage of mobile phase B is given.

Gradient 1		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
0.5	95	5
10	5	95
11	5	95
13	95	5
15	95	5
Gradient 2		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
1.5	95	5
5	43	57
7	40	60
8	5	95
10	5	95
11	95	5
Gradient 3		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
1.5	95	5
5	43	57
6	40.6	59.4
6.5	40.5	59.5
8	5	95
10	5	95
11	95	5

Table 4-6 continued

The six different mobile phase gradients used to optimise the separation of the analytes in Method A. The time in minutes and the percentage of mobile phase B is given.

Gradient 4		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
1.5	95	5
5	43	57
5.5	41	59
7	40	60
8	5	95
10	5	95
11	95	5

The retention times of the analytes of interest moved with a change in the composition of the mobile phase gradient. Table 4.7 indicates the shift in retention times that was observed using the gradients in Table 4.6, and Figures 4.5 – 4.7 illustrates the chromatographic profiles obtained during these analyses.

Table 4-7 Retention times of the analytes in Method A when using the different mobile phase gradients (Table 4.6).

Analyte	Retention Time (min)			
	Gradient 1	Gradient 2	Gradient 3	Gradient 4
Serine	1.301	1.060	0.998	0.998
Riboflavin	3.443	3.955	3.960	3.929
Pyridoxine	4.790	4.880	4.851	4.851
SAH	6.051	5.687	5.686	5.656
Homocystine	6.061	5.707	5.697	5.697
SAM	6.441	6.162	6.137	6.137

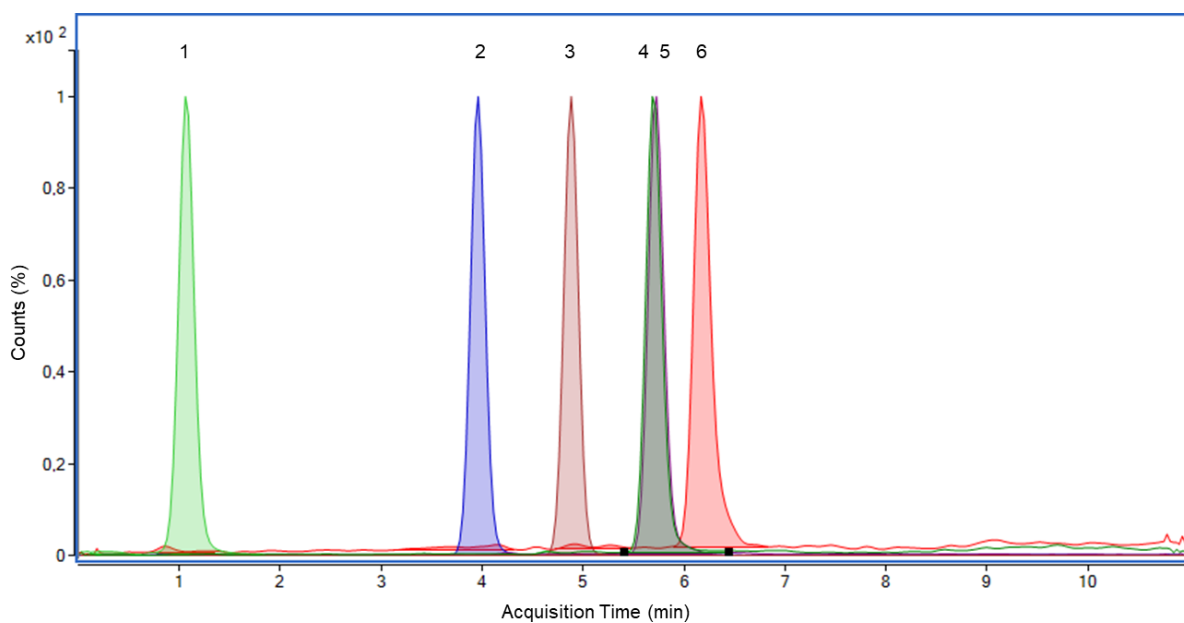


Figure 4-5 MRM Chromatographic profile of Method A, using gradient 2 (1) Serine, 2) Riboflavin, 3) Pyridoxine, 4) SAH, 5) Homocystine, and 6) SAM).

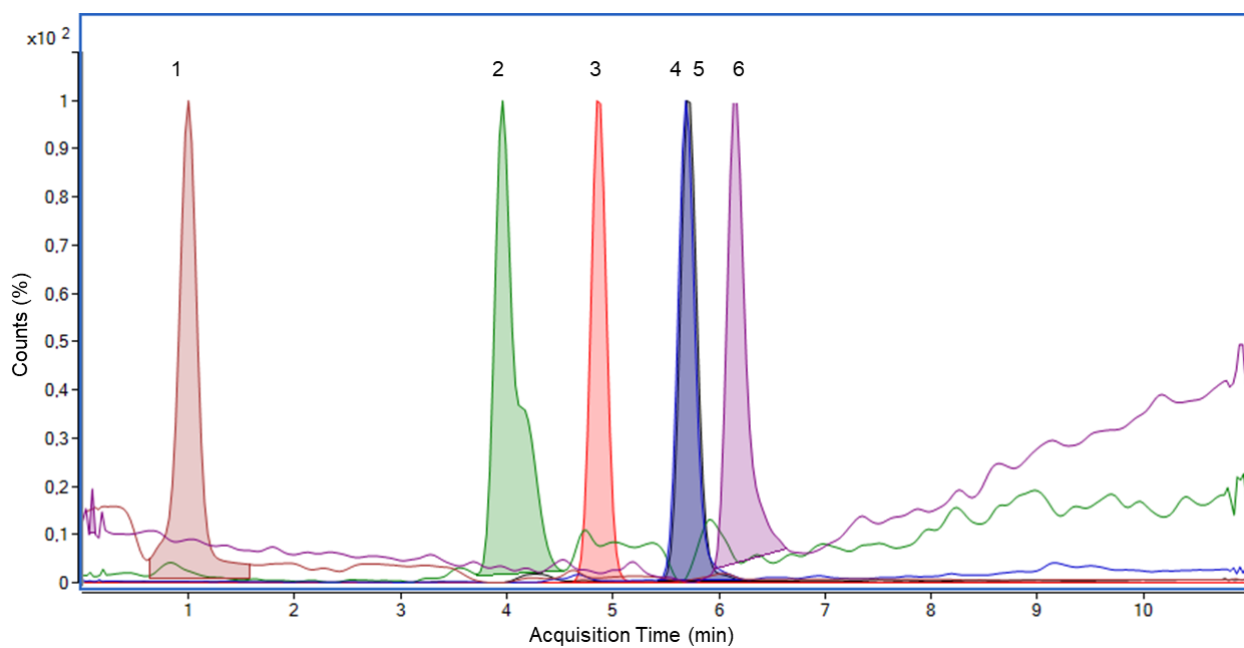


Figure 4-6 MRM Chromatographic profile of Method A, using gradient 3 (1) Serine, 2) Riboflavin, 3) Pyridoxine, 4) SAH, 5) Homocystine, and 6) SAM).

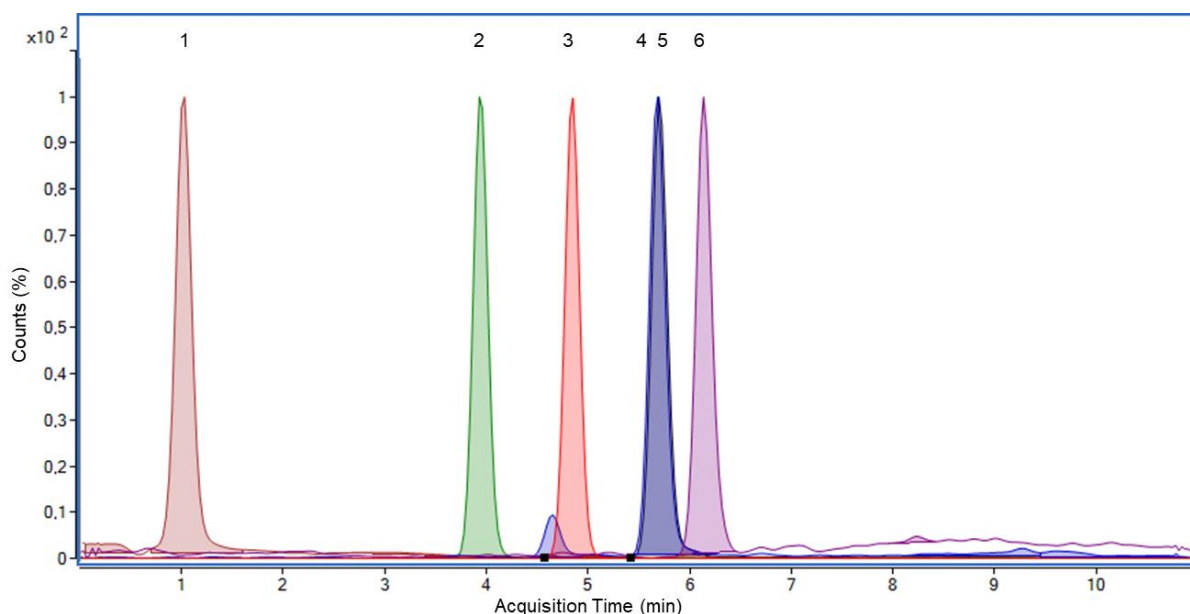


Figure 4-7 MRM Chromatographic profile of Method A, using gradient 4 (1) Serine, 2) Riboflavin, 3) Pyridoxine, 4) SAH, 5) Homocystine, and 6) SAM).

With the adjustment of the mobile phase gradient, a shift in retention times for all six analytes was observed but the peaks for SAH and homocystine still overlapped. This change in retention times for the six analytes can be seen in Table 4.7 and Figures 4.5 – 4.7. Since changing the gradient of Mobile phase B did not improve the separation between homocystine and SAH, gradient 1 was chosen for further analysis using Method A. This gradient also yielded the best results with regard to elution times as can be seen from the chromatographic profiles in Figures 4.3, 4.5 – 4.7, and in Table 4.7.

4.3.2.3.2 Method B

For Method B mobile phase A, consisted of 5 mM PFHpA in water and the mass spectrometer was set to scan for 5-MTHFA, betaine, choline cystathionine, cysteine, cystine, DMG, glycine, HCA, methionine, taurine, and homocystine. Figure 4.4 shows that there was an overlap between some analytes when using the gradient used by Guiraud *et al.* (2017) (Gradient 1 in Table 4.8). The overlapping peaks include DMG, betaine, and cysteine and homocystine, choline, and methionine. In an attempt to improve this, different mobile phase gradients were tested. Two microliters of a 5 ppm master mix was injected using four different mobile phase gradients (adjusted from gradient 1), as indicated in Table 4.8.

Table 4-8 The five different mobile phase gradients used to optimise the separation of the analytes in Method B. The time in minutes and the percentage mobile phase A and B is given.

Gradient 1		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
0.5	95	5
10	5	95
11	5	95
13	95	5
15	95	5
Gradient 2		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
1.5	95	5
2	85	15
4	62	38
5	53	47
8	5	95
10	5	95
11	95	5
Gradient 3		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
0.5	95	5
2	85	15
4	62	38
5	53	47
8	5	95
10	5	95
11	95	5

Table 4-8 continued

The five different mobile phase gradients used to optimise the separation of the analytes in Method B. The time in minutes and the percentage mobile phase A and B is given.

Gradient 4		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
0.5	95	5
1	85	15
1.5	82.5	17.5
2	82	18
2.5	81.5	18.5
3	81	19
4	62	38
5	53	47
8	5	95
10	5	95
11	95	5
Gradient 5		
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
1	95	5
1.5	81.5	18.5
42.5	80	20
4	62	38
5	53	47
8	5	95
10	5	95
11	95	5

As reported during the assessment of mobile phase gradients for Method A, the retention times of the analytes of interest shifted with a change in the composition of the mobile phase gradient. Table 4.9 indicates the shift in retention times that was observed using the mobile phase gradients in Table 4.8, and figures 4.4, 4.8 – 4.11 illustrate the chromatographic profiles obtained during this analysis.

Table 4-9 Retention times of the analytes analysed with Method B using the different mobile phase gradients (Table 4.8).

Analyte	Retention Time (min)				
	Gradient 1	Gradient 2	Gradient 3	Gradient 4	Gradient 5
HCA	0.684	0.684	0.623	0.684	0.684
Taurine	0.76	0.76	0.760	0.699	0.769
Betaine	1.438	1.134	1.134	1.074	1.195
DMG	1.387	1.144	1.084	1.084	1.144
Cysteine	1.555	1.251	1.251	1.129	1.251
Glycine	1.575	1.21	1.210	1.089	1.332
Homocysteine	3.428	2.577	2.517	2.152	2.638
Choline	4.421	4.724	4.664	3.995	4.603
Methionine	4.578	4.395	4.274	3.484	4.031
5-MTHFA	4.983	5.165	5.104	5.347	5.104
Cystine	5.17	5.291	5.231	5.474	5.352
Cystathionine	5.661	5.722	5.661	5.722	5.722

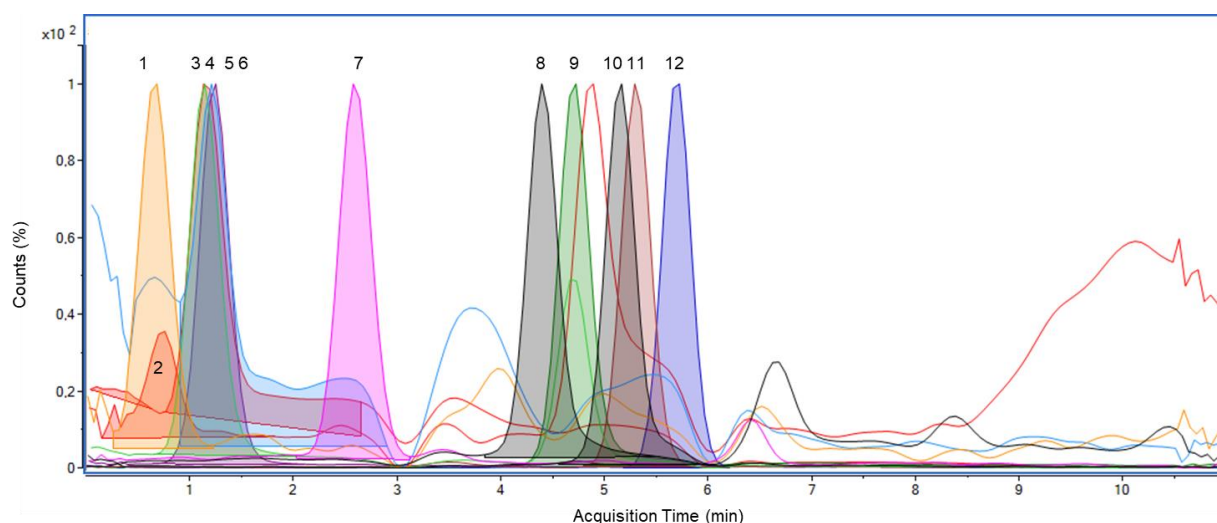


Figure 4-8 MRM chromatogram of the metabolites in Method B (1) HCA, 2) Taurine, 3) Betaine, 4) DMG, 5) Glycine, 6) Cysteine, 7) Homocysteine, 8) Methionine, 9) Choline, 10) 5-MTHFA, 11) Cystine, and 12) Cystathionine) using gradient 2.

Changing the mobile phase gradient for Method B resulted in a shift in the retention times for the 12 analytes analysed using this method. With the use of Gradient 2, separation, although not baseline separation, was obtained between choline and methionine. This can be seen

when comparing Figure 4.4 and 4.8. The retention time shift is indicated in Table 4.9. The retention time of choline shifted from 4.421 min to 4.724 min, and the retention time of methionine shifted from 4.578 min to 4.395 min. There was, however, no better chromatographic separation obtained between DMG, betaine, cysteine, and choline with the use of Gradient 2. During the assessment of Gradients 3 – 5 the challenge was to maintain the separation obtained between choline and methionine while improving the separation between DMG, betaine, and cysteine.

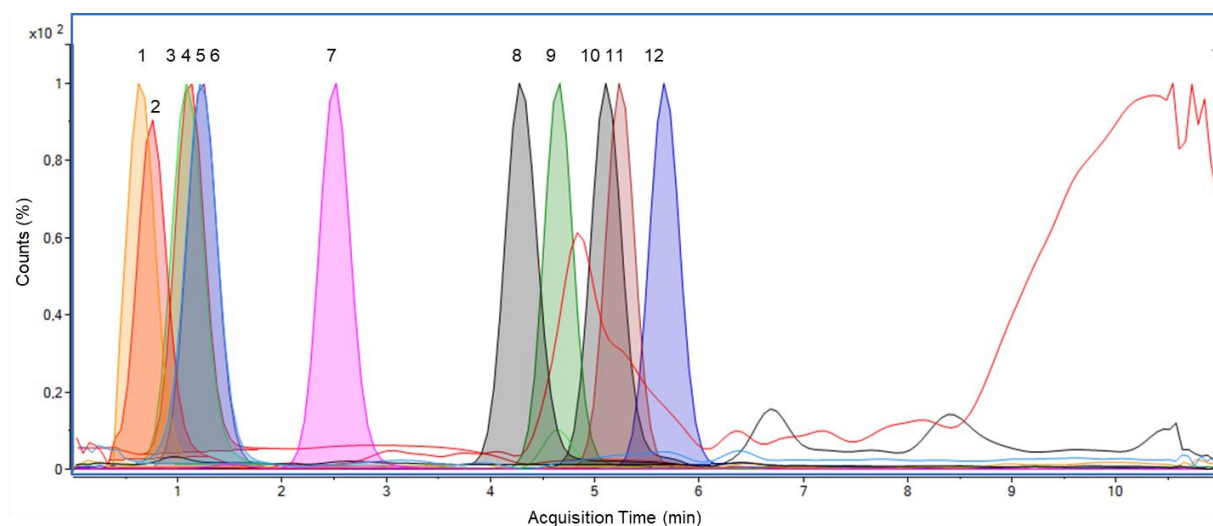


Figure 4-9 MRM chromatogram of the metabolites in Method B (1) HCA, 2) Taurine, 3) DMG, 4) Betaine, 5) Glycine, 6) Cysteine, 7) Homocysteine, 8) Methionine, 9) Choline, 10) 5-MTHFA, 11) Cystine, and 12) Cystathionine) using gradient 3.

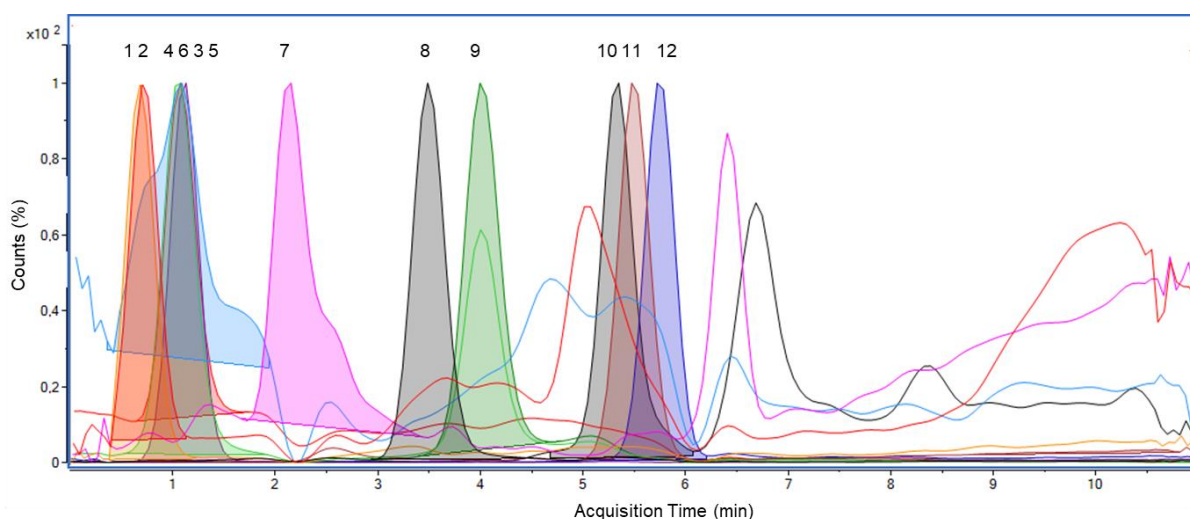


Figure 4-10 MRM chromatogram of the metabolites in Method B (1) HCA, 2) Taurine, 3) Betaine, 4) DMG, 5) Glycine, 6) Cysteine, 7) Homocysteine, 8) Methionine, 9) Choline, 10) 5-MTHFA, 11) Cystine, and 12) Cystathionine), using gradient 4

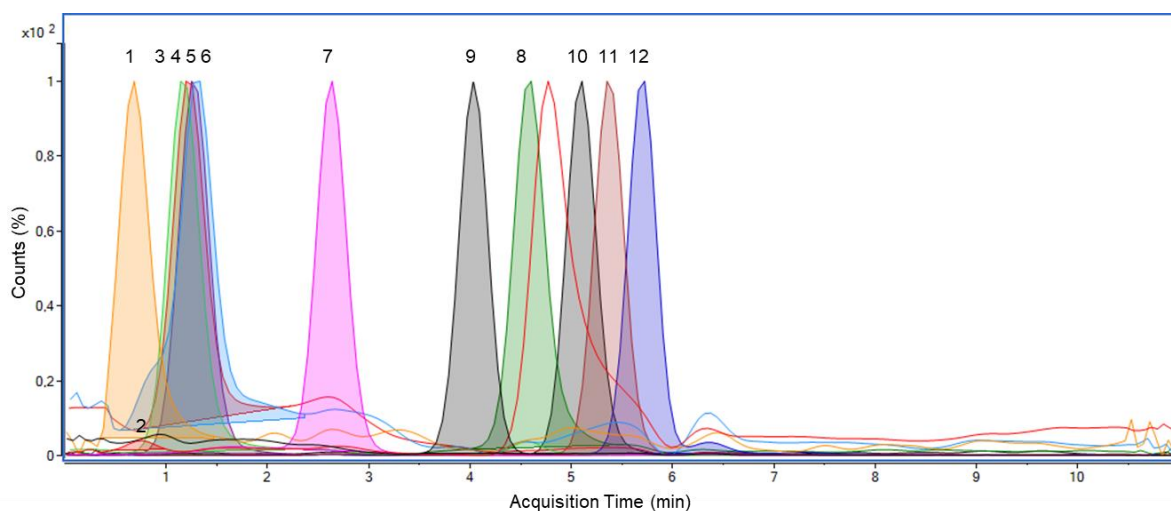


Figure 4-11 MRM chromatogram of the metabolites in Method B (1) HCA, 2) Taurine, 3) DMG, 4) Betaine, 5) Cysteine, 6) Glycine, 7) Homocysteine, 8) Methionine, 9) Choline, 10) 5-MTHFA, 11) Cystine, and 12) Cystathionine), using gradient 5.

Better chromatographic separation of DMG, betaine, cysteine, and glycine were not obtained with the use of Gradients 3 – 5. Therefore gradient 2 was chosen.

4.3.3 Addition of cystine and homocystine

Both homocysteine and cysteine can easily undergo oxidation to form homocystine and cystine, respectively. During other studies samples were treated with a disulfide bond reducing agent (Guiraud *et al.*, 2017) but samples used during this study were not treated in this way. Therefore, the method was adapted to include the detection of homocystine and cystine, the oxidised dimers for the amino acids homocysteine and cysteine, respectively. The addition of these analytes will help achieve a better overview of the methionine-homocysteine profile. The ionisation and source conditions for both these analytes were done as described in Section 4.2 and the optimal conditions can be seen in Table 4.1, 4.2, and A2.

To assess the chromatographic separation of cystine and homocystine on the Waters XSelect HSS T3 XP 2,5 micron 2,1 × 100 mm reversed phase column, 2 µl of a 5 ppm master mix was injected onto the column. Assessment of the effect of the ion-pairing agent on cystine and homocystine was investigated by using mobile phase A at 3 mM, 4 mM, and 5 mM PFHpA. In Figures 4.12 – 4.14, the chromatographic profiles of cystine and homocystine obtained using the three different concentrations of PFHpA, can be seen. Here it can be seen that the use of ion-pairing agents can have an influence on the signal intensity as concluded by Holčapek *et al.* (2004). The use of 4 mM PFHpA was most optimal for homocystine because the use of 3 mM and 5 mM PFHpA resulted in the presence of two peaks. The use of 4 mM also resulted in the highest peak intensity. For cystine, 5 mM PFHpA proved to be the most optimal ion-pairing concentration. This was due to the fact that it resulted in the highest peak intensity and best retention on the column.

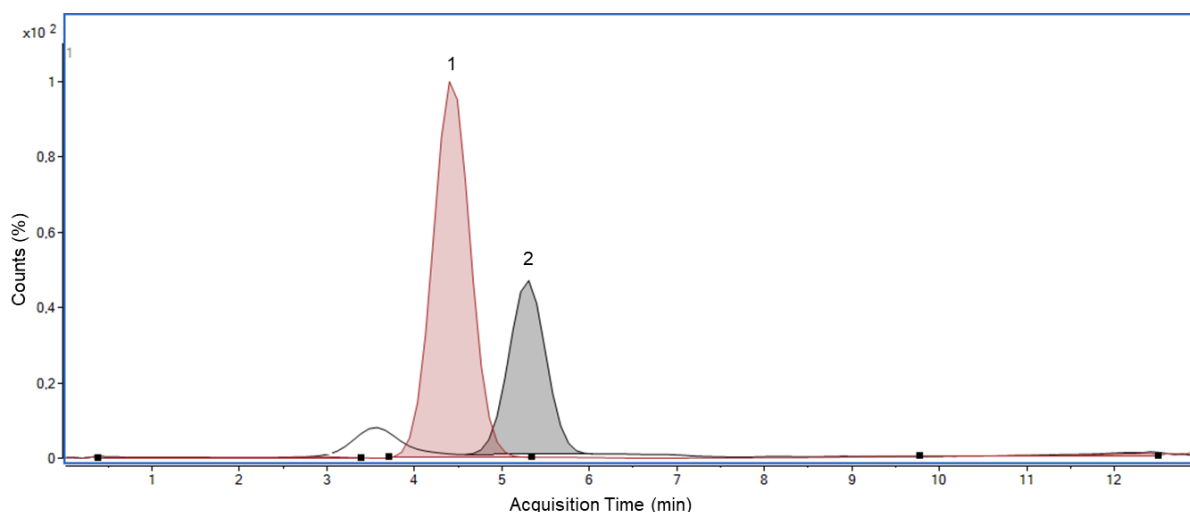


Figure 4-12 Chromatographic profile of the separation of 1) Cystine and 2) Homocystine on the Waters XSelect HSS T3 XP 2,5 micron 2,1 × 100 mm reversed phase column using 3 mM PFHpA in water as mobile phase A.

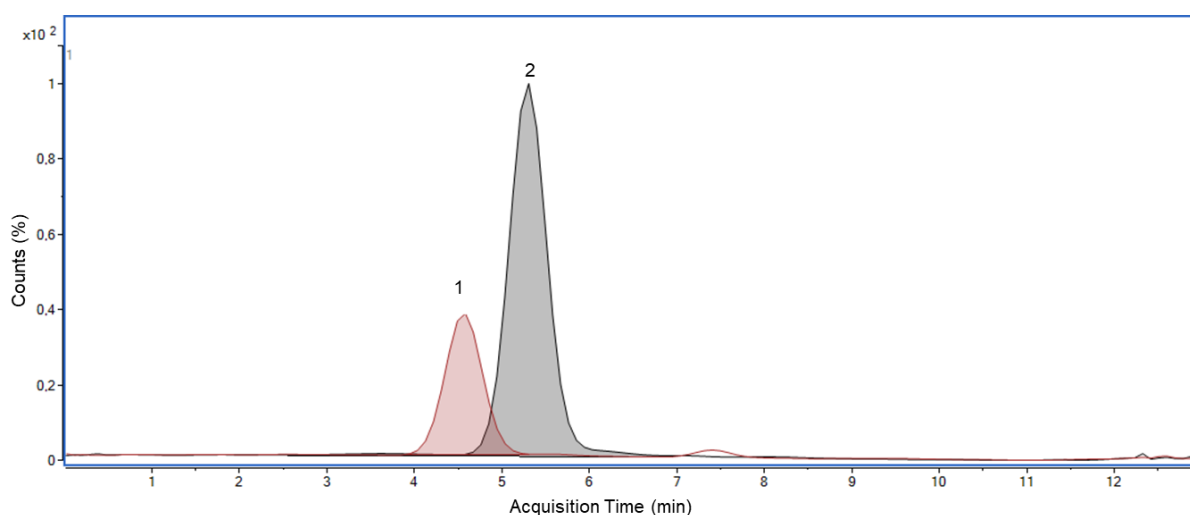


Figure 4-13 Chromatographic profile of the separation of 1) Cystine and 2) Homocystine on the Waters XSelect HSS T3 XP 2,5 micron 2,1 × 100 mm reversed phase column using 4 mM PFHpA in water as mobile phase A.

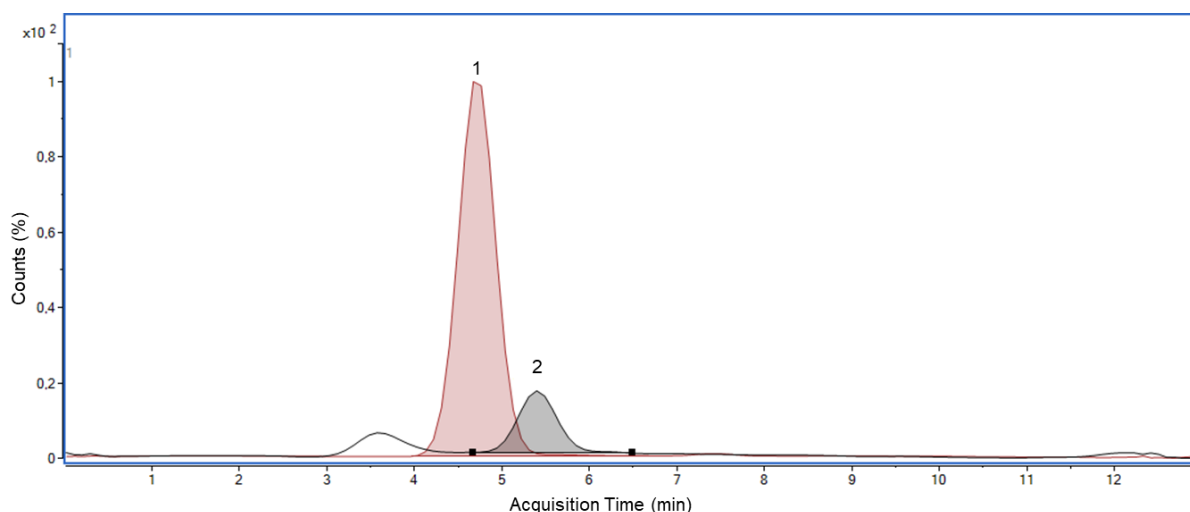


Figure 4-14 Chromatographic profile of the separation of 1) Cystine and 2) Homocystine on the Waters XSelect HSS T3 XP 2,5 micron 2,1 × 100 mm reversed phase column using 5 mM PFHpA in water as mobile phase A.

4.4 Re-optimisation of source conditions with an ion-pairing agent

Kostiainen and Kauppila (2009) indicated that the use of ion-pairing agents can suppress ionisation. Protonated molecules are masked by ion-pairing, which reduces the efficiency of the electrospray ionisation droplet to release protonated molecules to the gas phase (Kostiainen & Kauppila, 2009). Ion-pairing can lead to the reduction in charged separation at the tip of the electrospray ionisation sprayer leading to a decrease in the ionisation efficiency (Kostiainen & Kauppila, 2009). As mentioned in Chapter 2 and above, it is no secret that the use of ion-pairing agents comes with some possible disadvantages. One of these disadvantages includes the suppression of ionisation. This may result in a drop in signal intensity to 15-30%, as has been reported for Holčápek *et al.* (2004) with the use of PFHpA.

During initial mass spectrometry ionisation experiments, the ion-pairing agent was not added to mobile phase A. After considering the potential negative effect that ion-pairing may have on the ionisation of the analytes of interest, it was decided to optimise the source conditions again, this time with the addition of the ion-pairing agent of choice, PFHpA.

This experiment was conducted in the same manner as described in Sections 4.2.1 and 4.2.2, the only difference being the addition of 5 mM PFHpA to mobile phase A as done by Da Silva *et al.* (2016). As already described, the raw data of each parameter for each analyte was processed to select the most optimal parameters. The average was determined for each

parameter value. Table A3 gives a representation of the average of the data of each parameter for each analyte. Table 4.10 reports the most optimal source condition for each analyte.

Table 4-10 Optimal source conditions for all analytes after optimisation of the source conditions with PFHpA in mobile phase A.

Parameter	Optimal condition
Sheath Gas Temperature (°C)	300
Sheath Gas Flow (L/min)	10
Drying Gas Temperature (°C)	250
Gas Flow (L/min)	6
Nebulizer Pressure (psi)	50
Capillary Voltage (V)	3000
Nozzle Voltage (V)	0

When comparing the results obtained from the use of no PFHpA to 5 mM PFHpA in mobile phase A, the following was seen: there was an increase in capillary voltage from 2500 V to 3000 V and the nebulizer pressure increased from 30 psi to 50 psi, with the addition of the ion-pairing agent. The drying gas temperature also decreased from 300°C to 250°C. These changes indicate that the addition of ion-pairing agents can indeed affect ionisation.

4.5 Liquid Chromatography Optimisation

4.5.1 Injection volume

The LC-MS/MS method was further optimised by optimising the injection volume. This optimisation was done by injecting 1 µl, 5µl, and 10 µl of a low (LC), medium (MC), and high (HC) concentration analyte mix onto the LC-MS/MS system in triplicate. The samples were prepared by adding an internal standard mix to the standards and drying it under N₂ gas at 35°C, and resuspending it in 100 µl ACN:H₂O (5:95). When increasing the injection volume, the peak height and area increase to a point where the injection volume is too large (Dolan, 2014). When the injection volume is too large, the peak broadens and this may cause a decrease in resolution between adjacent peaks (Dolan, 2014).

Figures 4.15 and 4.16 show the results obtained after injecting 1 µl, 5µl, and 10 µl of the LC and MC, respectively. In these figures, it is clear that there is an increase in response with an increase in the injection volume, as would be expected. In Figure 4.17 it can be seen that there

is less of a difference in the response between the injection of 5 μl and 10 μl of the HC samples, when compared to the MC samples (Figure 4.16).

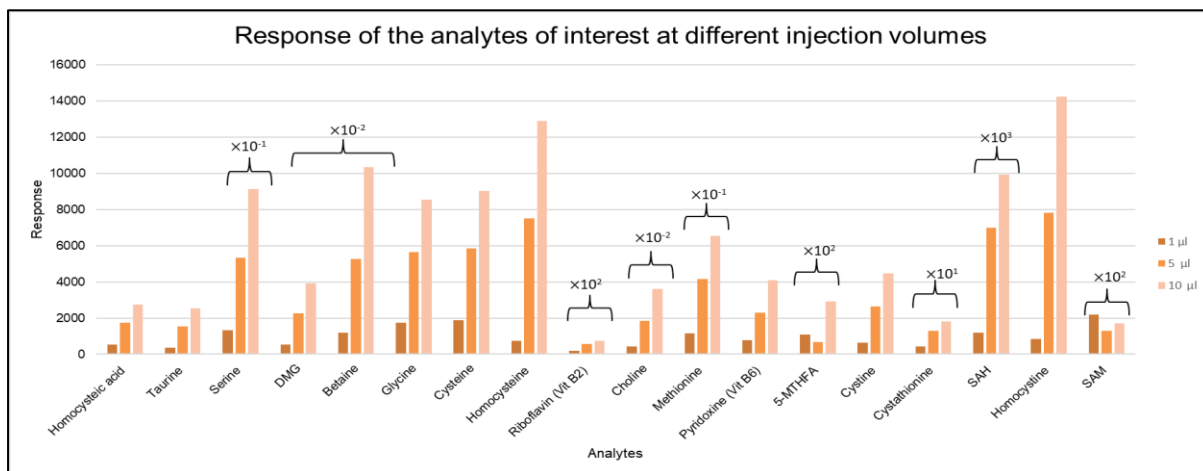


Figure 4-15 Visual comparison of the response (response obtained by the MS for each analyte of interest) of each analyte (LC) at different injection volumes. The responses of some analytes were adjusted to aid visual illustration of all analytes.

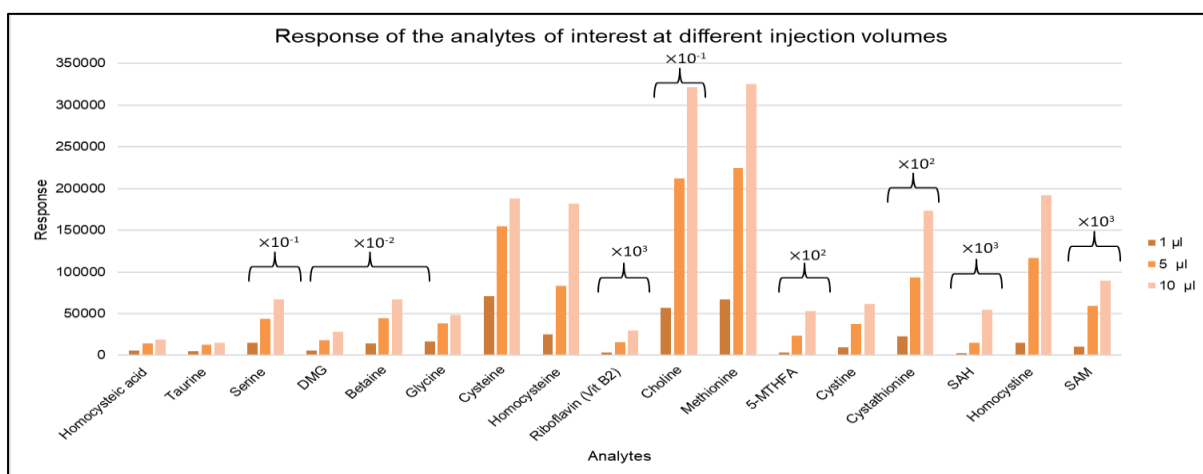


Figure 4-16 Visual comparison of the response of each analyte (MC) at different injection volumes. The responses of some analytes were adjusted to aid visual illustration of all analytes.

Figure 4.17 compares the response of a HC of the analytes when injecting 1 μl , 5 μl , and 10 μl . It can be seen that there was no increase in the response of all the analytes with an increase in injection volume. This is due to the fact that when analysing a higher concentration, the column is overloaded, and it results in suppression of the ionisation. Therefore, the injection of a higher volume into the LC-MS/MS system did not result in an increase in the response of all the analytes – especially those with a higher concentration. Figure 4.18 A and B shows the

response obtained for glycine and methionine at three different concentrations with different injection volumes. In these graphs it is clear that there was a decrease in response with an increase in injection volume when analysing a higher concentration of the analytes. This can also be seen for some of the other analytes such as serine, DMG, betaine, and cysteine (Figure A1 K, L, M, and N). However, for some analytes such as cystathionine, 5-MTHFA, riboflavin, HCA, taurine, and SAM (Figure A1 C, D, G, I, J, and Figure 4.18 D) there was an increase in response for all three different concentrations with an increase in injection volume. Finally, an injection volume of 5 μ l yielded the highest response for homocysteine, SAH, pyridoxine, choline, homocysteine, and cystine (Figure A1 A, B, E, F, H, and Figure 4.18 C) This is also illustrated in Figure 4.17.

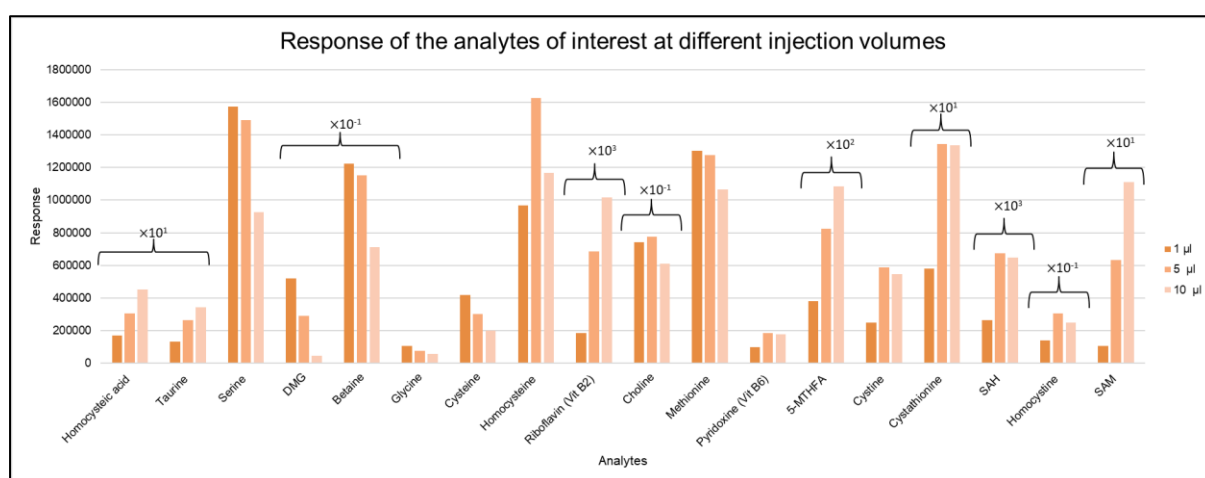


Figure 4-17 Visual comparison of the response of each analyte (HC) at different injection volumes. The responses of some analytes were adjusted to aid visual illustration of all analytes.

In Figure 4.19 (A & B), it can be seen how the area of the peaks decreased with an increase in injection volume. This is due to overloading of the column when injecting a bigger amount of the higher concentration of the standard. This overloading causes a suppression in ionisation and, therefore, the decrease in response illustrated in Figure 4.18 (A & B). The shape of the peaks is also of great importance. Ideal chromatographic peak shapes are symmetrical this is known as Gaussian-shaped peaks. In Figure 4.19 A it is evident that the peaks lost this Gaussian-shape with the injection of 5 μ l and 10 μ l. Overloading the column can cause this peak asymmetry (Stoll, 2021). From these results it is clear that at higher concentration, the injection of a smaller amount of sample onto the LC-MS/MS system resulted in a higher response for the analytes. Although an injection volume of 1 μ l proved to be most optimal, detection of the analytes was further improved by dissolving the dried samples in

500 μ l ACN:H₂O (5:95), instead of 100 μ l, and injecting 5 μ l onto the LC-MS/MS system (see Section 4.6).

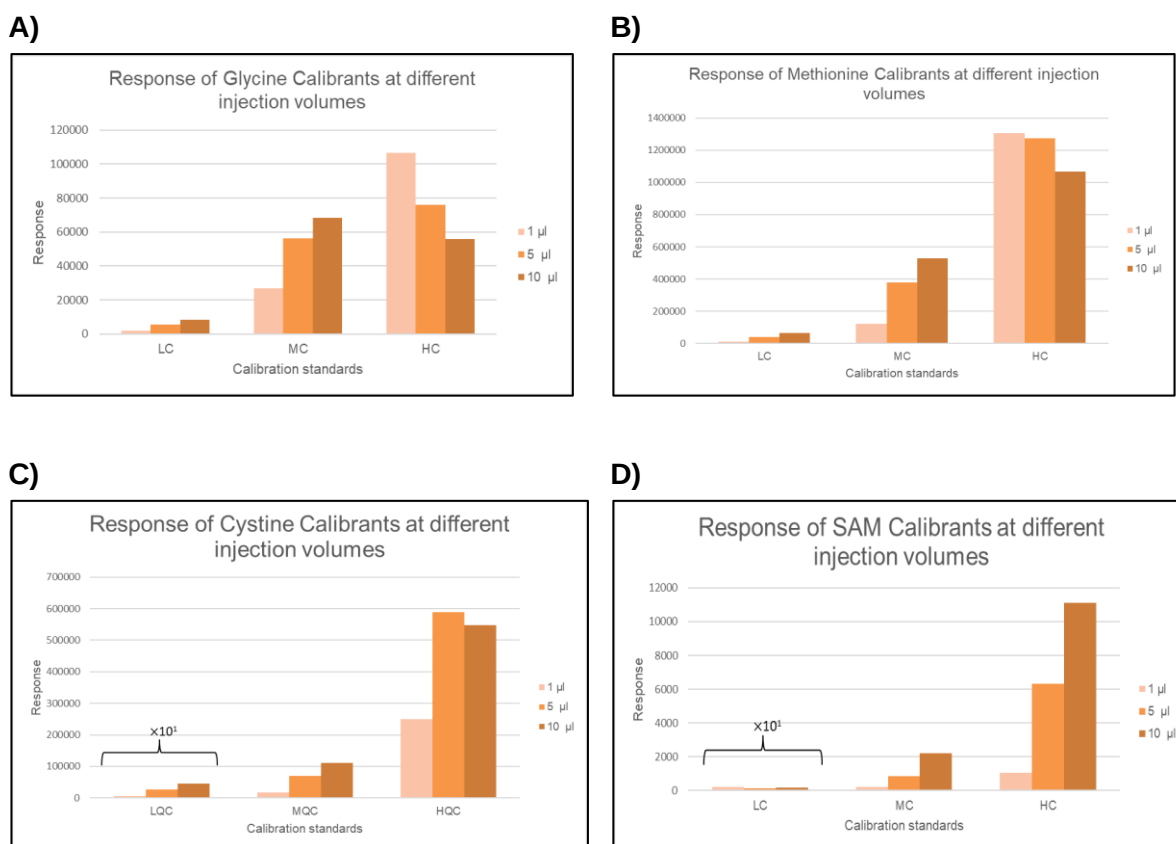


Figure 4-18 Response obtained after injecting three different volumes of three different concentration levels (LC, MC, and HC) of glycine (A), methionine (B), cystine (C), and SAM (D) onto the LC-MS/MS system.

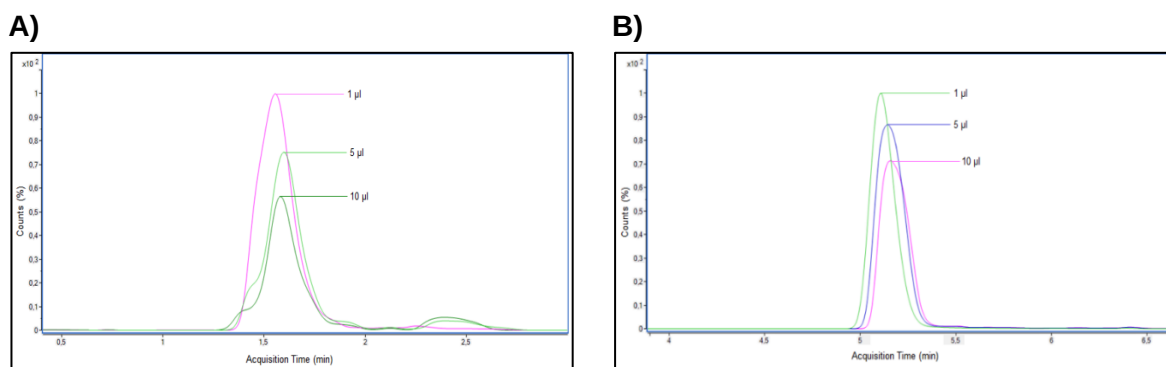


Figure 4-19 Chromatographic illustration of two examples where the influence of high injection volumes was clearly notable, namely in that of glycine (A) and methionine (B). During this analysis different volumes of a high concentration was injected onto the LC-MS/MS system.

4.5.2 Internal standards

The addition of internal standards to samples has advantages for quantitative and qualitative analysis (Wal *et al.*, 2010). It is used to control for variation in recovery, matrix effect, and ionisation (Sargent, 2013) and to compensate for the losses that occur during sample preparation (Oliveira *et al.*, 2010).

4.5.2.1 Optimisation of ionisation and source conditions

As described in Section 3.4.2.1 and 3.4.2.2, the determination of optimal ionisation, MRM, and source conditions for the five stable isotopes used as internal standards was done with the help of the Agilent MassHunter Optimizer and Agilent Source and iFunnel Optimizer software. The ionisation and MRM conditions included precursor ions, fragmentor voltage for each precursor ion, the possible product ions, and collision energy settings that were most optimal for each transition. The optimisation of source conditions included parameters such as drying gas flow (L/min), drying gas temperature (°C), nebuliser pressure (psi), capillary voltage (V), nozzle voltage (V), sheath gas flow (L/min) and sheath gas temperature (°C) for each analyte. Optimising these conditions ensures an increase in the sensitivity of the analytical method. The analysis was done in the same manner as for the 18 analytes of interest (see Section 4.2). The most abundant and/or the most unique precursor and product ion and the most optimal collision energy and fragmentor voltage were chosen for use during MRM mode and are presented in Table 4.11. These optimal ionisation and MRM conditions for the internal standards were used to help with the quantification of the analytes of interest. The source

parameters were most optimal at the same values determined for the 18 analytes of interest (Table 4.10).

Table 4-11 Results of the optimisation of ionisation and MRM conditions. This table represents a summary of the results obtained for all the transitions. Precursor ion, product ion, and collision energy and fragmentor voltage were determined using the Agilent MassHunter Optimizer software for the six isotopes used as internal standards.

Isotope	Formula	Polarity	Precursor (m/z)	Product (m/z)	Fragmentor energy (V)	Collision energy (eV)
d ₅ -Phenylalanine	C ₉ H ₆ D ₅ NO ₂	Positive	171.12	125.1	55	12
				106		32
				107.1		28
				79.1		44
d ₂ -Glycine	C ₂ H ₃ D ₂ NO ₂	Positive	77.04	32.1	35	8
d ₄ -L-Cystine	C ₆ H ₈ D ₄ N ₂ O ₄ S ₂	Positive	245.06	154	84	8
				122		16
				74.1		24
				76		36
Methyl-d ₃ -methionine	C ₅ H ₇ D ₃ NO ₂ S	Positive	153.08	136	75	4
				107		4
				56.1		16
				64.1		24
d ₈ -DL-Homocystine	C ₈ H ₈ D ₈ N ₂ O ₄ S ₂	Positive	277.12	140	79	4
				92.1		40
				94.1		24
				207.1		8

The most unique and abundant product ion with its corresponding collision energy for each isotope was used for detection of the isotopes by the tandem mass spectrometer. The chromatographic separation of the isotopes used can be seen in Figure 4.20.

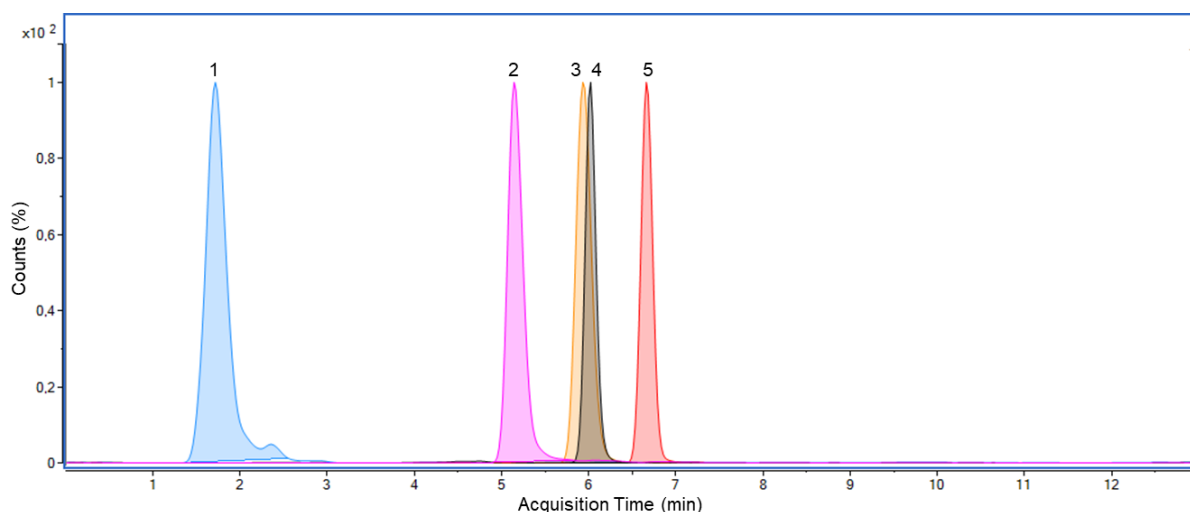


Figure 4-20 Chromatographic separation of the isotopes used during this method.
1) d₂-Glycine, 2) d₃-Methyl-Methionine, 3) d₄-L-Cystine, 4) d₅-Phenylalanine, and 5) d₈-DL-Homocystine.

4.5.3 Dynamic MRM

To improve the selectivity of a method, the instrument can be set to perform dynamic MRM (dMRM) instead of conventional MRM. During a dMRM, the mass spectrometer scans for the specific ion transitions at a specific retention time window that was determined during conventional MRM. The dMRM mode improves selectivity because it doesn't waste cycle time by scanning for all the ion transitions, but only scans for specific ion transitions at specific times. The MassHunter software determines the most optimal dwell time for each transition to ensure optimal cycle time.

During the setup of the dMRM, a data file of the conventional MRM method was used to import the retention times for each compound (as described in Section 3.4.6). The MassHunter software determines the average dwell time for each compound, using the cycle time and the concurrent MRMs (Figure 4.22) at certain retention times (Figure 4.21). Figure 4.22 illustrates that there was increased concurrent MRMs at the retention time ranges: 1.2-2.2 min and 5.2-7.22 min. The minimum average dwell time for each compound was 59, which was already an improvement from the dwell times of 50, using the conventional MRM method.

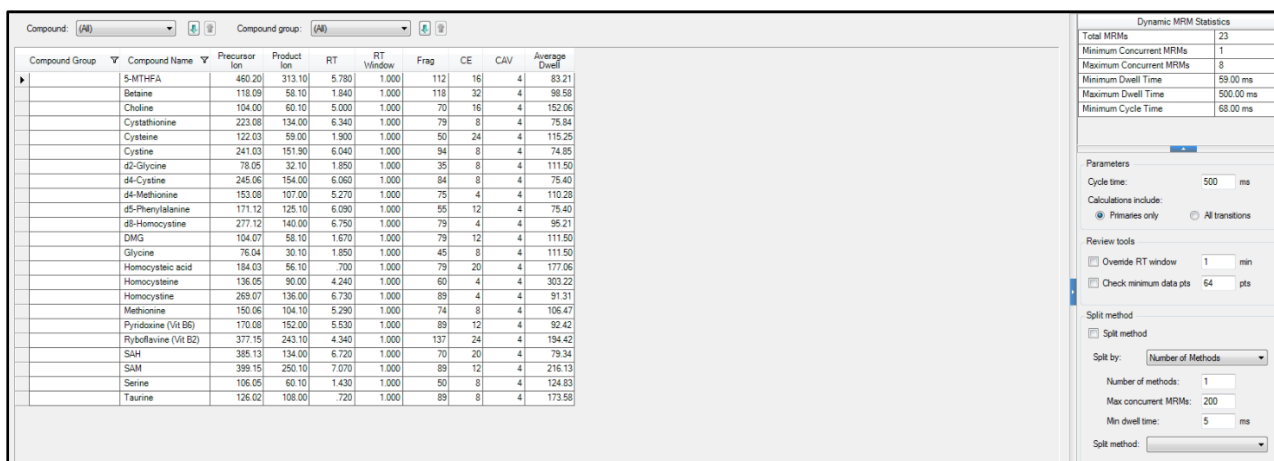


Figure 4-21 A screenshot of the dMRM viewer. The MassHunter software determines the average dwell time for each compound using the cycle time and the concurrent MRMs at certain retention times.

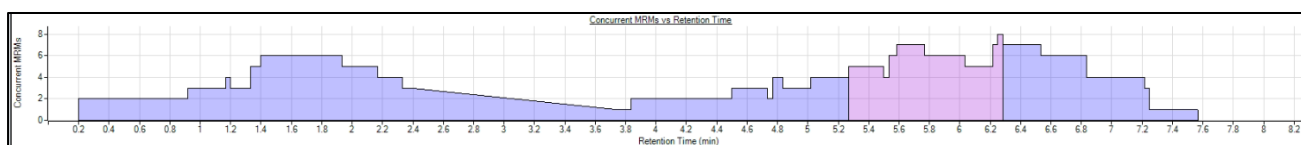


Figure 4-22 A screenshot of a plot of concurrent MRMs vs the retention time (min) of the compounds.

During this analysis the purpose was to improve the selectivity of the method. A MC was prepared in triplicate and analysed using both the conventional MRM and new dMRM method. Mass spectrometry and chromatographic conditions described in previous sections were used. The chromatographic and qualitative data obtained after using the two methods were assessed to determine which of the two yielded better chromatographic profiles and qualitative data.

A chromatographic profile of the MC sample using the conventional MRM and dMRM method can be seen in Figure 4.23 and Figure 4.24, respectively. Little to no difference was observed between the two methods. However, the use of a dMRM might prove to be more selective when analysing analytes in a matrix (such as serum) as it scans for specific transitions at specific time frames. dMRM was, therefore, applied in further analyses. Figure 4.25 illustrates the chromatographic difference for each analyte when the conventional MRM and dMRM was used. In this figure it can be seen that the dMRM method integrates the peaks at the specified retention time.

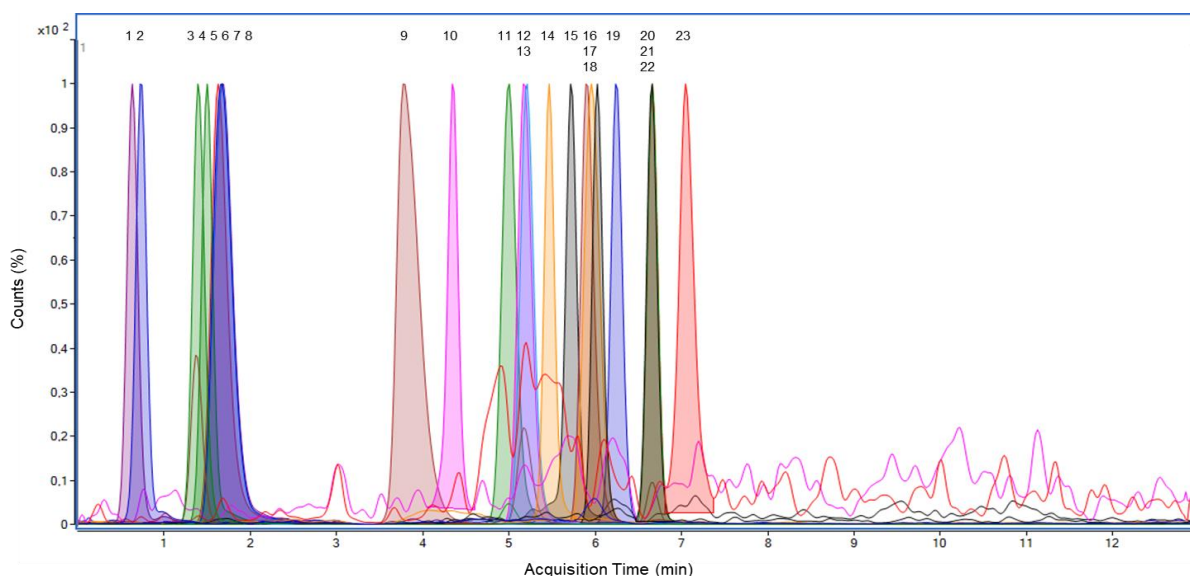


Figure 4-23 Chromatographic profile of the MRM method. 1) Homocysteic acid, 2) Taurine, 3) Serine, 4) DMG, 5) Betaine, 6) Cysteine, 7) d₂-Glycine, 8) Glycine, 9) Homocysteine, 10) Riboflavin, 11) Choline, 12) Methyl-d₃-Methionine, 13) Methionine, 14) Pyridoxine, 15) 5-MTHFA, 16) Cystine, 17) d₄-Cystine, 18) d₅-Phenylalanine, 19) Cystathionine, 20) SAH, 21) d₈-DL Homocysteine, 22) Homocysteine, and 23) SAM.

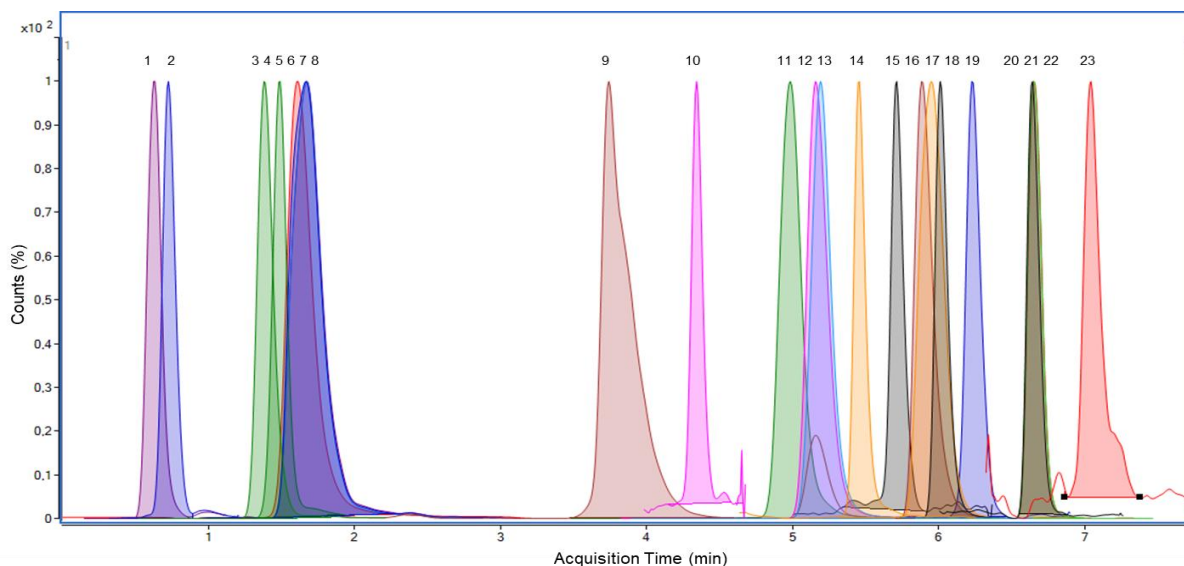
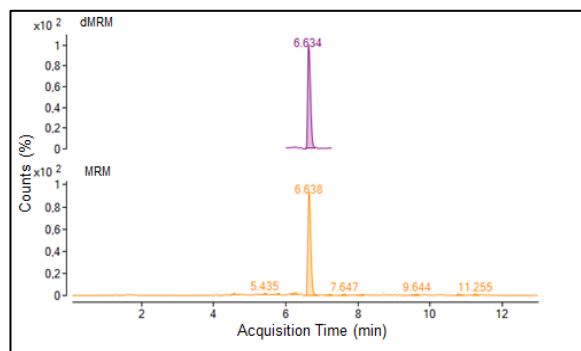
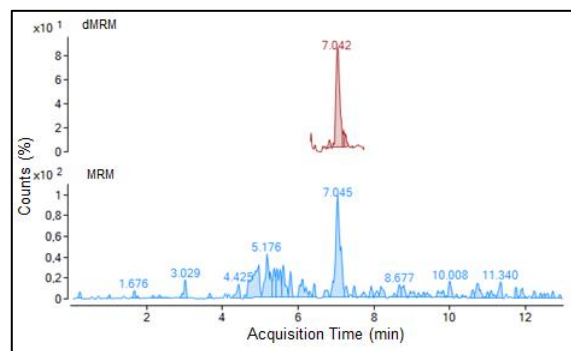


Figure 4-24 Chromatographic profile of the dMRM method. 1) Homocysteic acid, 2) Taurine, 3) Serine, 4) DMG, 5) Betaine, 6) Glycine, 7) Cysteine, 8) d₂-Glycine, 9) Homocysteine, 10) Riboflavin, 11) Choline, 12) Methyl-d₃-Methionine, 13) Methionine, 14) Pyridoxine, 15) 5-MTHFA, 16) Cystine, 17) d₄-Cystine, 18) d₅-Phenylalanine, 19) Cystathionine, 20) H SAH, 21) d₈-DL-Homocysteine, 22) Homocysteine, and 23) SAM.

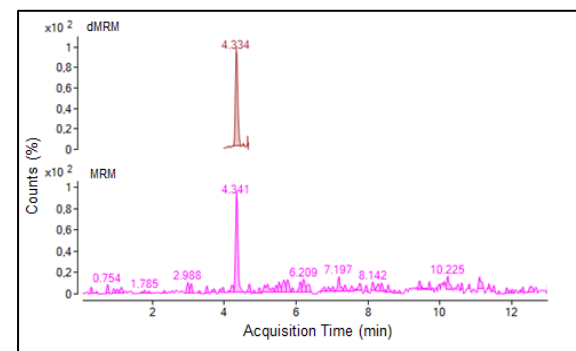
A) SAH



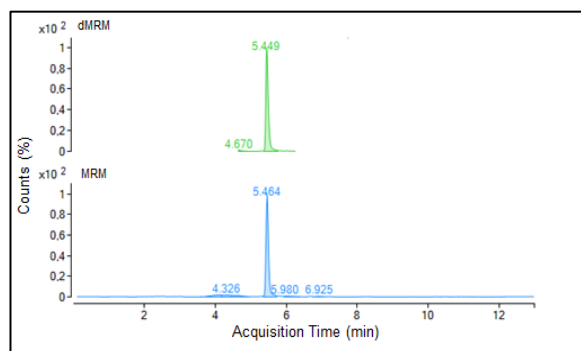
B) SAM



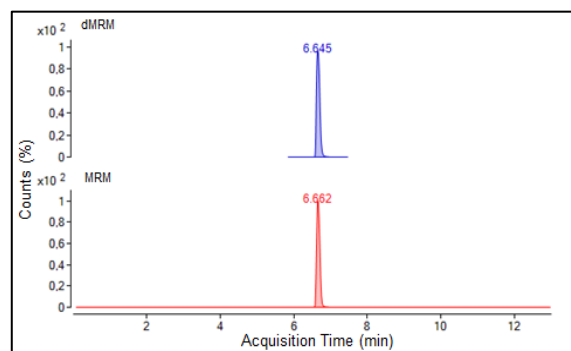
C) Riboflavin



D) Pyridoxine



E) Homocystine



F) Serine

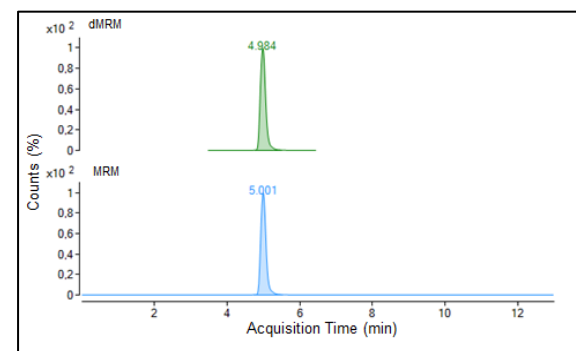
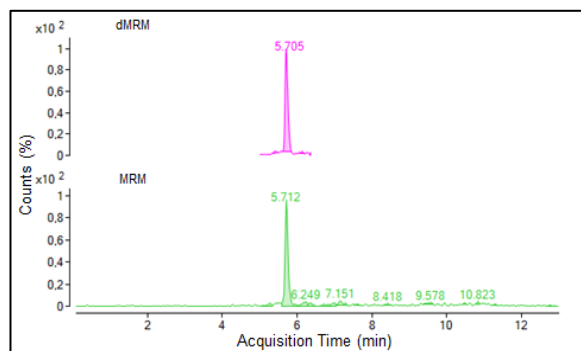
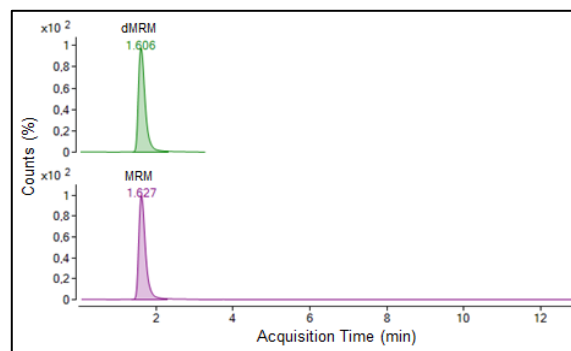


Figure 4-25 This figure compares the chromatographic profiles of the analytes of interest after applying conventional and dynamic MRM

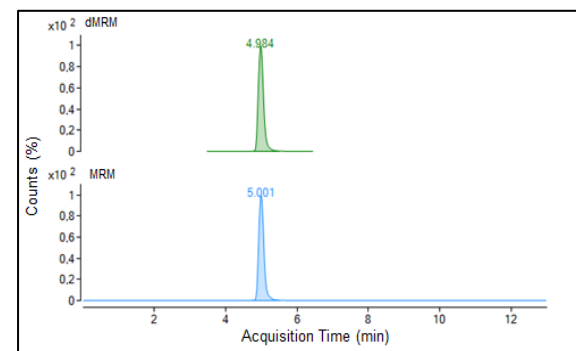
G) 5-MTHFA



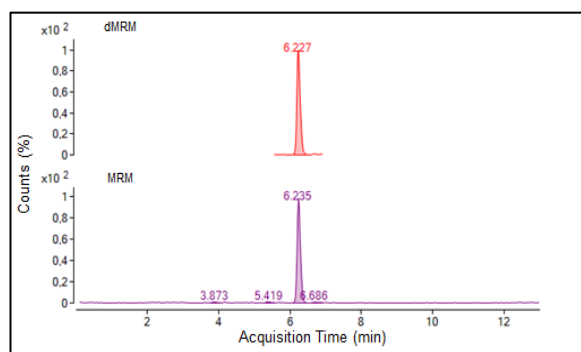
H) Betaine



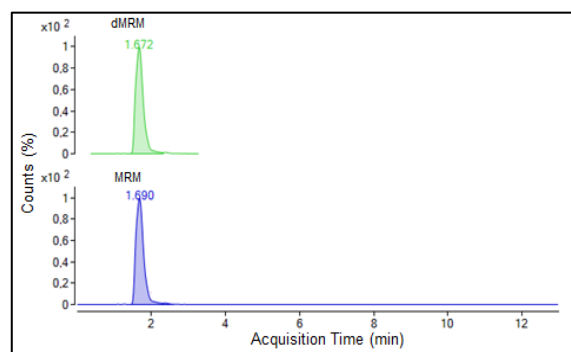
I) Choline



J) Cystathionine



K) Cysteine



L) Cystine

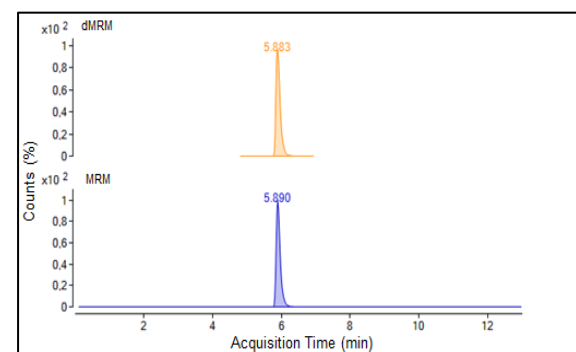
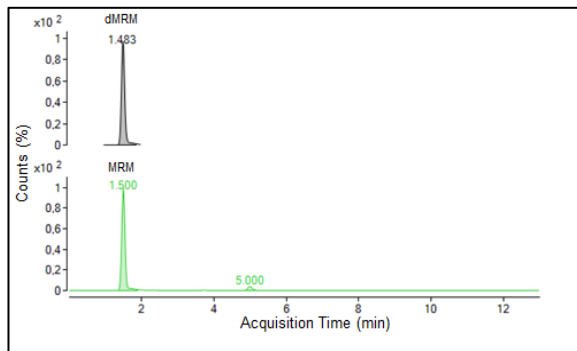


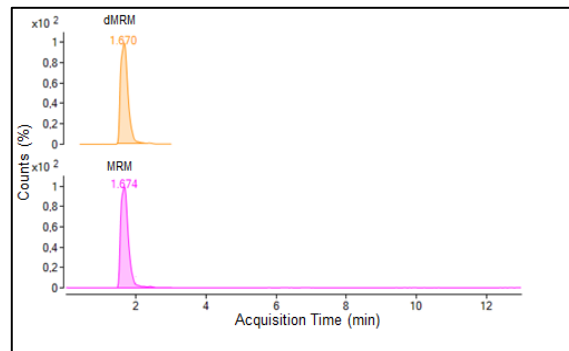
Figure 4 25 continued

This figure compares the chromatographic profiles of the analytes of interest after applying conventional and dynamic MRM

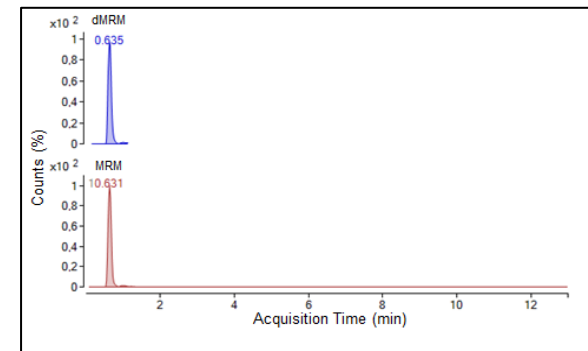
M) DMG



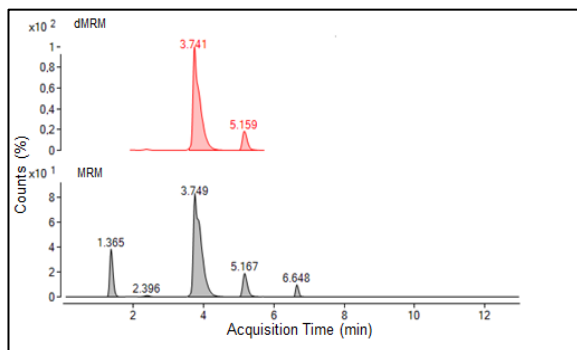
N) Glycine



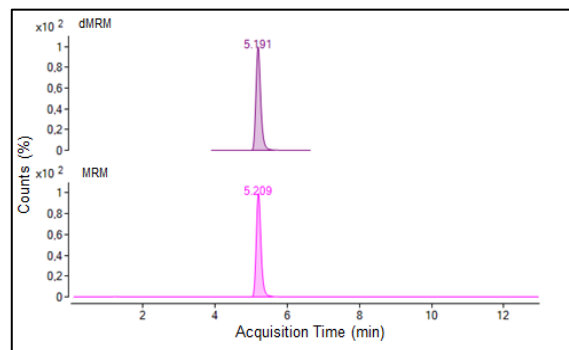
O) Homocysteic acid



P) Homocysteine



Q) Methionine



R) Taurine

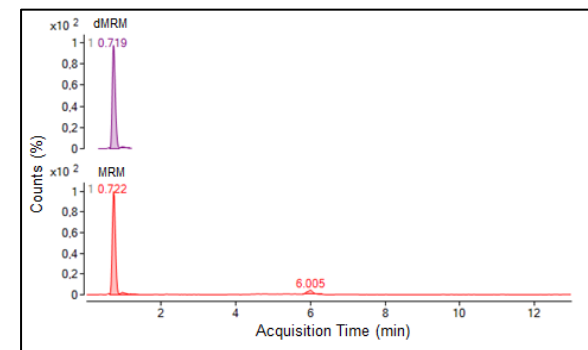


Figure 4 25 continued

This figure compares the chromatographic profiles of the analytes of interest after applying conventional and dynamic MRM

4.6 Optimisation of sample preparation

During the optimisation of this LC-MS/MS method, routine serum samples were used. These samples were collected in Vacutainer® tubes. Sample preparation was optimised from the protocol of Guiraud *et al.* (2017). During the procedure described by Guiraud *et al.* (2017) a disulfide bond reducing agent and protein precipitation solution were added to the plasma. Thereafter, the mixture was vortexed and centrifuged. The supernatant was transferred to vials for LC-MS/MS analysis. Since the serum samples used for method optimisation and application in this study were not treated with a disulfide bond reducing agent the use thereof was not optimised. Serum is seen as a relatively clean matrix therefore, protein precipitation is considered to be sufficient for sample clean-up (Verstraete *et al.*, 2019). To concentrate the sample, it is commonly evaporated to dryness and reconstituted in the mobile phase.

4.6.1 Protein precipitation

As mentioned in Section 3.5, cold ACN, MeOH, and ethanol with added 1% formic acid were assessed as protein precipitation solvents for serum samples. Five times the volume (1000 µl) of the organic solvent was added to 200 µl of a serum sample. Thereafter, the samples were vortexed for 1 min to ensure optimal mixing of the serum and organic solvent. The samples were then centrifuged at a speed of 9112 g for 15 min at 4°C. The supernatant was filtered to ensure that the sample did not contain any unwanted components. The supernatant was evaporated to dryness under N₂ at 35°C and resuspended in ACN: H₂O (9:95). The most optimal protein precipitation solvent was chosen based on visual inspection of the amount of protein precipitated. When precipitating with ACN, with 1 % FA, a precipitate immediately formed. After centrifugation of the samples the use of ACN with 1% FA resulted in a clearer supernatant and more compact precipitant compared to the other organic solvents. This was indicative of efficient protein precipitation. The use of MeOH as a precipitation solvent caused an increase in pressure on the column upon analysing the sample. For these reasons 1000 µl cold ACN with 1 % FA were used for protein precipitation of the serum samples.

4.6.2 Concentration of samples

In order to concentrate the samples, the prepared samples were evaporated to dryness under N₂ gas and resuspended in ACN:H₂O (5:95). After the injection volume was optimised in Section 4.5.1 it was clear that an injection volume of 1 µl was most optimal. However, to ensure optimal detection of the analytes of interest with the LC-MS/MS system samples were evaporated to dryness under N₂ gas and resuspended it in 500 µl ACN:H₂O (5:95) to inject 5 µl per sample.

4.7 Further optimisation of the LC-MS/MS method

4.7.1 Extension of run-time

To ensure that the sample injected onto the column is fully washed out of the column before the next injection, a serum sample was injected using a MS 2 scan method. During this scan method, the final gradient as mentioned earlier was used but the organic phase duration (mobile phase B) was extended to 10 min at 95% (previously it was 2 min) as can be seen in Table 4.12.

From the chromatographic profile presented in Figure 4.26 it is clear that compounds were still eluting from the column at an acquisition time of 10 min, when mobile phase B percentage would have been decreased under the original settings. After 16 min the baseline started to stabilise, and it appeared that no more compounds were eluting from the column anymore. Therefore, it was decided that mobile phase B be kept as 95% for a duration of 8 min.

Table 4-12 **Extended mobile phase gradient used to determine the optimal run-time. The yellow rows indicate the change in the gradient.**

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
1.5	95	5
2	85	15
4	62	38
5	53	47
8	5	95
18	5	95
19	95	5
21	95	5

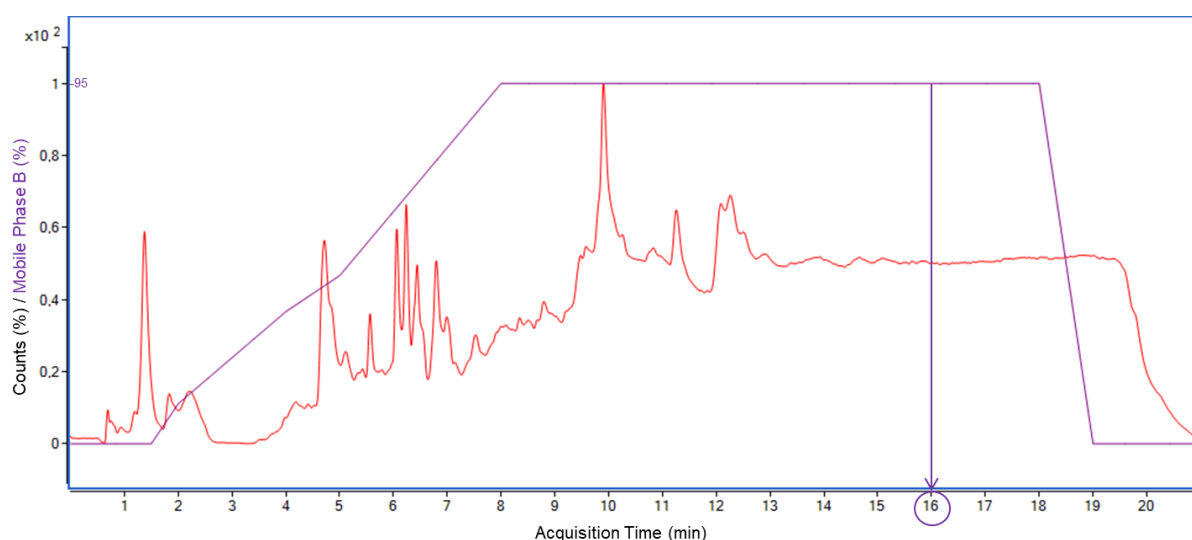


Figure 4-26 **Chromatographic profile of the MS 2 Scan method done on a serum sample using the mobile phase gradient in Table 4.12. In this figure, the purple line represents the mobile phase B % profile change during the MS 2 Scan.**

Before injection of a new sample, it is of great importance to equilibrate the column. Therefore, after assessing the most optimal time to elute all compounds from the column, an injection was done where mobile phase B was kept at 5% for 10 min (from an initial 2 min; Table 4.13) to assess the amount of time needed to equilibrate the column before the next injection.

The chromatographic profile obtained after this injection (Figure 4.27) indicated that the column was equilibrated for the next injection after 23 min. Therefore, the mobile phase

gradient was changed to stay at 5% mobile phase B for 4 min with a post time of 2 min. This added up to 6 min at 5% of mobile phase B.

Based on the results obtained, the final mobile phase gradient shown in Table 4.14 was used for subsequent analysis, with a post time of 2 min.

Table 4-13 Mobile phase gradient used to determine the time needed for equilibration before the next injection. The yellow rows indicate the change in the gradient.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
1.5	95	5
2	85	15
4	62	38
5	53	47
8	5	95
16	5	95
17	95	5
27	95	5

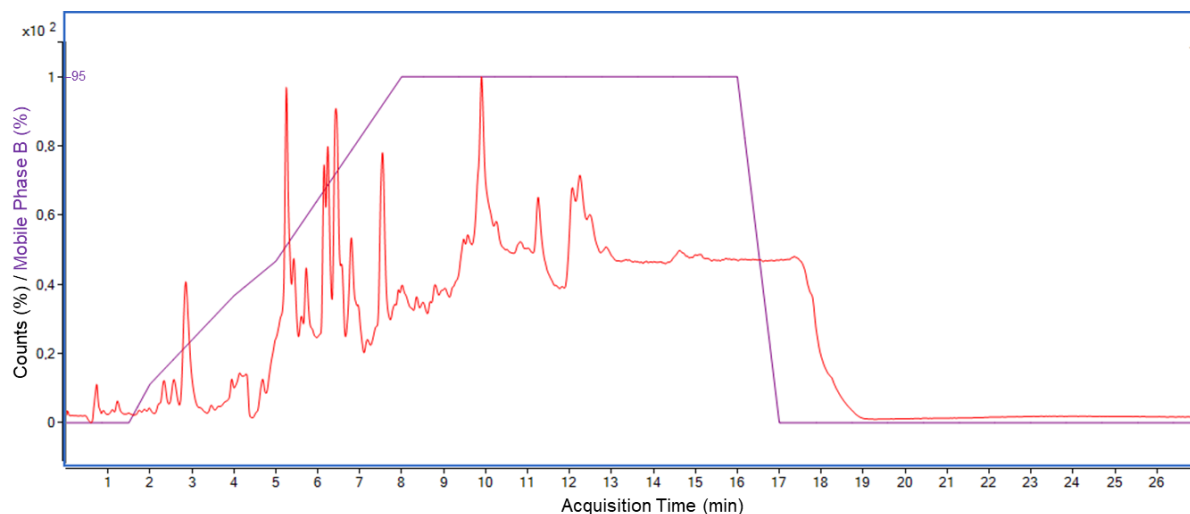


Figure 4-27 Chromatographic profile of the MS 2 Scan method done on a serum sample using the mobile phase gradient in Table 4.13. In this figure, the purple line represents the mobile phase B % profile change during the MS 2 Scan.

Table 4-14 Final mobile phase gradient used during the analysis of the analytes of interest.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
1.5	95	5
2	85	15
4	62	38
5	53	47
8	5	95
16	5	95
17	95	5
21	95	5

4.7.2 Adjustment of sample preparation method

4.7.2.1 Resuspension

Different ratios of ACN to H₂O were investigated for the resuspension of dried serum samples. The compositions were as follows: 100% ACN, 50:50 (ACN:H₂O), 40:60 (ACN:H₂O), 30:70 (ACN:H₂O), and 5:95 (ACN:H₂O). This was done to determine what composition would be most optimal to dissolve the metabolites of interest. To test these five compositions, five serum samples were prepared. This was done by adding 200 µl serum, 50 µl internal standard stock, and 1 ml ACN with 1% FA to a microcentrifuge tube, vortexing the sample, and filtering it using a filter microcentrifuge tube. The filtered sample was dried under N₂ gas at 35°C. After the samples were dried, they were resuspended in 500 µl of the five different ACN:H₂O compositions mentioned above.

For most analytes, the highest response was obtained when the composition of 30:70 (ACN:H₂O) was used (Table 4.15). When the results obtained from resuspending with 30:70 were compared with that of 5:95, as was done initially, a higher response was observed using 30:70 for all the analytes. A significant increase in response can be seen for cysteine, riboflavin, pyridoxine, and 5-MTHFA in particular. Therefore, it can be concluded that an increased amount of ACN is needed for the optimal solvation and ionisation of these analytes. It was speculated that during drying of the samples some of the analytes evaporated with the organic solvents.

Table 4-15 Responses of the metabolites and internal standards of interest when using five different compositions of ACN and H₂O to resuspend dried serum samples. The responses were colour coded for visual comparison. The colours used were red, orange, yellow, and green. Red being the lowest response and green being the highest response.

Analytes	100	50/50	40/60	30/70	5/95
	Response				
Betaine	142468	5894278	5639794	5823333	3660103
L-Homocysteic acid	6	21	1	2	1
Taurine	724	2196	2702	2567	2349
L-Cysteine	0	998	491	1936	194
Glycine	4123	123319	141253	150896	144093
L-Serine	7491	295455	341842	371599	272972
N,N-DMG	8	142575	136566	142756	103738
DL-Homocysteine	4	2	1	2	2
Choline	1610508	1219625	1160994	1285819	1183817
L-Methionine	121972	141070	136817	144025	84677
Riboflavin	5	54	82	58	1
Pyridoxine	8	971	1214	1049	4
L-Cystine	295	13580	15143	18491	1028
5-Methyltetrahydro folic acid	3	526	673	920	1
Cystathionine	1146	369	397	433	410
Homocystine	1050	793	777	842	551
SAH	6	8	7	9	0
SAM	170	97	139	131	59
d ₂ -Glycine	3	316	374	367	317
Methyl-d ₃ -Methionine	1483	2068	1839	2036	1310
d ₄ -L-Cystine	553	324	303	397	185
d ₅ -Phenylalanine	3690	3460	3328	3493	2421
d ₈ -DL-Homocystine	818	414	455	525	273

4.7.2.2 Investigation the possibility of excluding drying of samples

To investigate if the analytes were lost during evaporation, two other sample preparation methods were investigated. During sample preparation method 1 (Figure 4.28 A), 200 μ l of a HC standard stock was used. Fifty microliters of an internal standard stock and 1 ml ACN with 1% FA was added, and the samples were dried and resuspended in 500 μ l ACN:H₂O (30:70). Using 30:70, ACN:H₂O instead of 100% ACN, decreased the final percentage of ACN in each sample. During sample preparation method 2 (Figure 2.28 B), 50 μ l of an internal standard stock and 600 μ l ACN with 1% FA was added to 200 μ l of a HC. After this, the samples were not dried but diluted with water (1.15 ml) to ensure a final composition of 30:70, ACN:H₂O. This resulted in a more diluted sample of which 20 μ l was injected onto the LC-MS/MS.

Table 4.16 contains the responses obtained using the two different sample preparation methods explained in Figure 4.28 A – B. From Table 4.16, it is clear that using Sample Preparation Method 2 gave an improved response for most of the analytes and internal standards of interest.

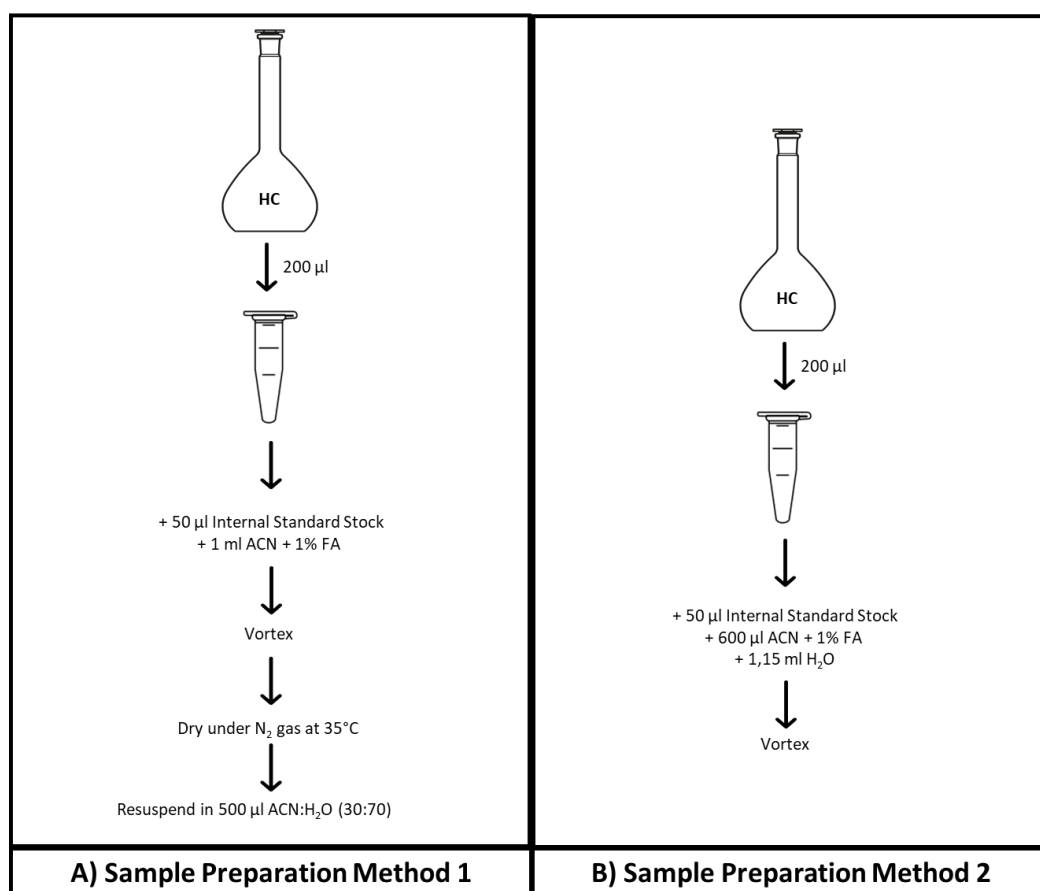


Figure 4-28 Sample preparation methods 1 and 2. The two different methods were used to investigate the effect that drying the samples under N₂ gas had.

Table 4-16 Responses of the metabolites and internal standards of interest when using two different sample preparation methods. The responses were colour coded for visual comparison. The colours used were red and green. Red being the lowest response and green being the highest response.

Analytes	Sample Preparation 1	Sample Preparation 2
	Response	
L-Homocysteic acid	1868	1788
Taurine	6975	3067
L-Serine	246495	269717
N, N-DMG	1799503	2130714
Betaine	6898036	11734623
Glycine	82352	100007
L-Cysteine	2237	5344
DL-Homocysteine	282	611
Riboflavin (Vitamin B2)	16731	171363
Choline	1941046	3212506
L-Methionine	554171	102152
Pyridoxine (Vitamin B6)	8898	33934
5-Methyltetrahydro folic acid	4206	7712
L-Cystine	377247	606974
Cystathionine	7364	10365
SAH	368	905
L-Homocystine	380	657
SAM	1802	1529
d ₂ -Glycine	52693	126775
Methyl-d ₃ -Methionine	2961	3899
d ₄ -L-Cystine	1917	3285
d ₅ -Phenylalanine	4018	5563
d ₈ -DL-Homocystine	41393	62610

4.7.2.3 Optimisation of injection volume

Because the sample preparation method used resulted in dilution of the sample, the injection volume had to be optimised. This was done by injecting 5 µl, 10 µl, 15 µl, and 20 µl of a stock

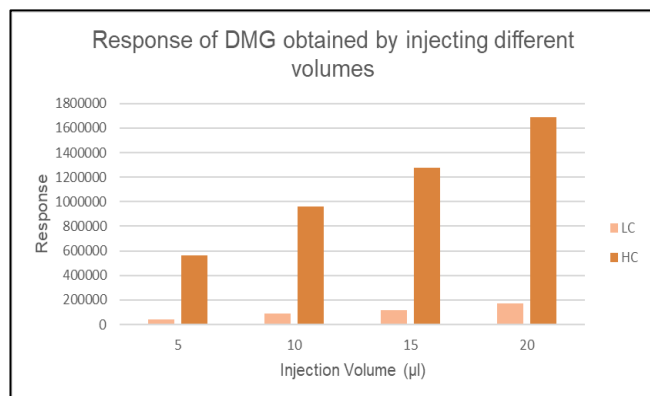
solution of a high and low concentration that had been prepared as described in Figure 4.28 B (sample preparation method 2).

Table 4.17, Figure 4.29, and Figure A2 present the data obtained during the optimisation of the injection volume. In Table 4.17, the response was colour coded, green being the highest response and red being the lowest response. For most analytes of interest, the injection of 15 μ l or 20 μ l yielded the highest response. It would be expected that a higher injection volume would yield a higher response, except when the column is overloaded. This could be especially relevant for metabolites that are present at higher concentrations, as was seen in Section 4.5.1. Figure 4.29 is a graphical representation of the responses obtained for the analytes of interest with an increase in injection volume. Here it can be seen that there was a clear increase in the response with an increase in injection volume. However, not all the analytes showed a proportional increase in response with an increase in injection volume. For example, DMG (Figure 4.29 A), methionine (Figure 4.29 B), and betaine (Figure 4.29 C). However, for analytes investigated at lower concentration, such as 5-MTHFA and SAH, a more proportional increase of response was seen with increased injection volume (Figure 4.29 D and E).

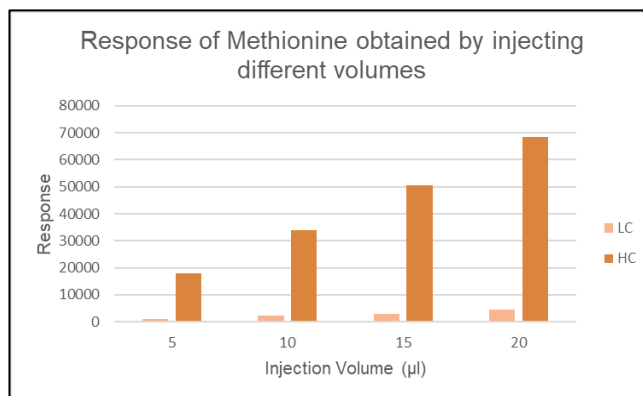
Table 4-17 Table summarising the response obtained for each analyte after injecting four different volumes of a HC and LC. The responses were colour coded for visual comparison. The colours used were red, orange, yellow, and green (red being the lowest response and green being the highest response).

	Homocysteic acid	Taurine	Serine	DMG	Betaine	Glycine	Cysteine	Riboflavin (Vit B2)	Homocysteine	Choline	Methionine	Pyridoxine (Vit B6)	5-MTHFA	Cystine	Cystathionine	SAH	Homocystine	SAM
HC 5 µl	830	2533	76377	566331	2858341	21818	28359	92	38361	1589240	17859	12552	402	68354	1576	197	12946	346
HC 10 µl	1388	2430	156209	958658	4795829	42618	51045	168	76265	2543433	33878	21011	1077	129796	2772	335	25525	530
HC 15 µl	1643	2265	223521	1276542	6804462	61143	70214	269	115665	3521752	50511	29855	2007	198543	4016	499	36900	715
HC 20 µl	1852	2408	279460	1687693	9407442	77722	88517	301	168157	4576237	68332	39498	2660	257601	5113	721	48857	992
LC 5 µl	168	489	5663	42411	264026	1631	2297	9	2576	168262	1078	3783	19	4581	399	49	1045	80
LC 10 µl	334	936	12799	92086	610850	3594	4752	9	5469	342559	2177	4874	83	8752	486	61	1892	93
LC 15 µl	401	1025	16952	118554	779191	5240	6490	11	7668	409052	3075	5298	104	12637	582	76	2525	114
LC 20 µl	503	1227	24532	168580	1102071	7012	9085	27	11097	613476	4505	6035	139	17899	675	78	3369	130

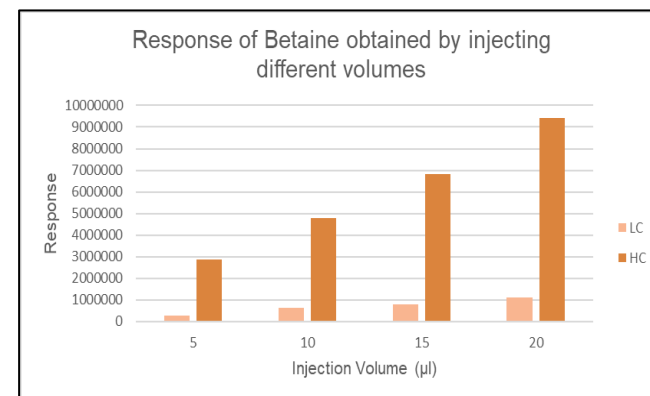
A) DMG



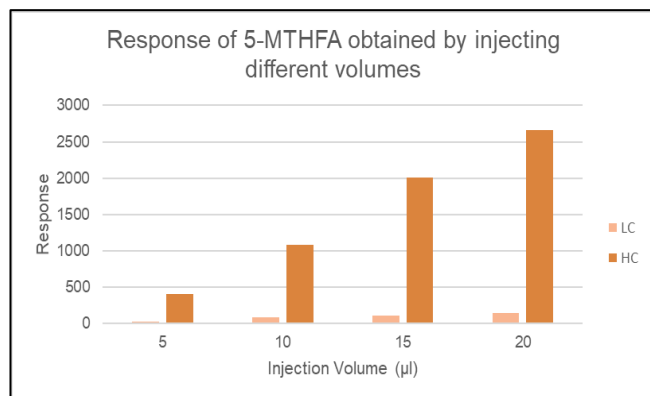
B) Methionine



C) Betaine



D) 5-MTHFA



E) SAH

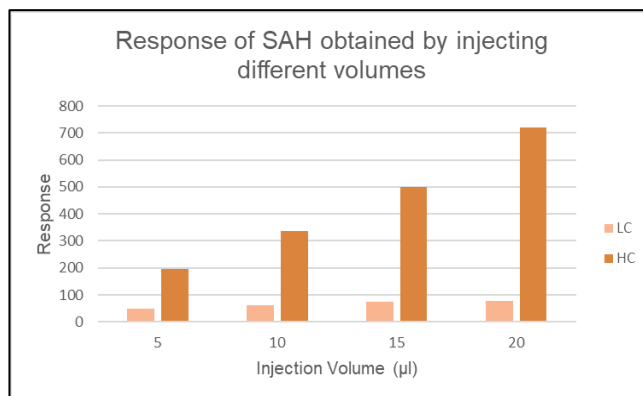


Figure 4-29 Graphical representation of the response obtained for all the analytes when different volumes were injected.

In order to choose the most optimal injection volume, another parameter was assessed, namely the chromatographic profile of each analyte. As mentioned before, peak shape would be affected negatively if the column is overloaded. This can include peak tailing, peak broadening, and a decrease in resolution (Dolan, 2005; 2014). In Figure 4.30 A and B, it can be seen that the peaks for DMG and betaine broadens with an increase in injection volume and when 20 μl is injected, the resolution of the peaks decreases, causing the presence of double peaks. Figure 4.30 B shows chromatographic profiles for methionine. The peak broadening can only be seen with an injection of 20 μl . Because of the peak tailing, broadening, and decrease in resolution, an injection volume of 10 μl was chosen to be used in the end.

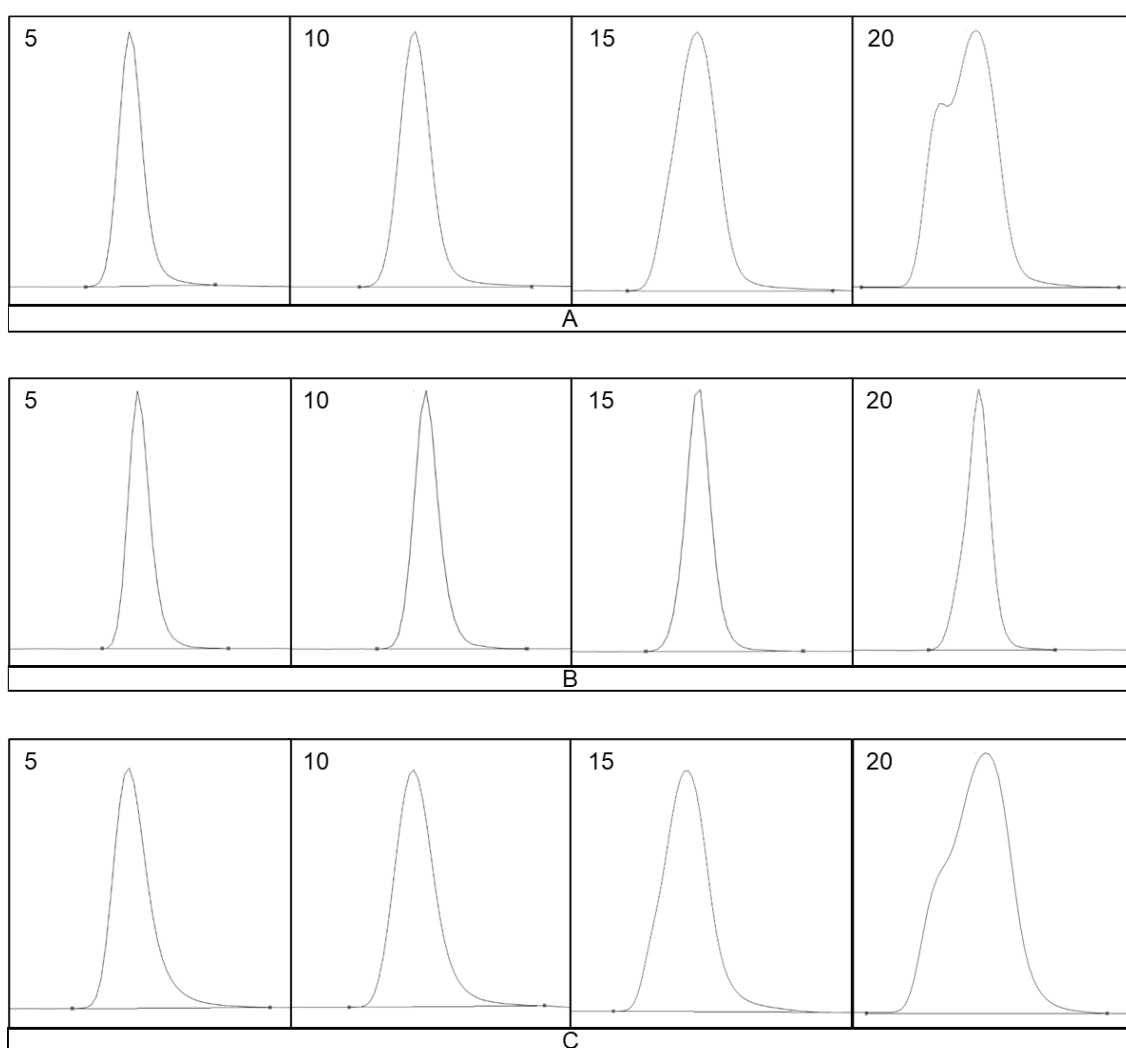


Figure 4-30 Graphic representation of the chromatographic peaks obtained for A) DMG, B) methionine, and C) betaine when different volumes of a HC were injected. Injection volumes (μl) are indicated in the top left corner of each square.

4.8 Summary of the final optimised method

4.8.1 Sample preparation

The sample preparation before LC-MS/MS analysis mainly consisted of protein precipitation and dilution. This was done to clean samples before they are subjected to further analysis. During this sample preparation, 600 µl ACN with 1 % FA were added to 200 µl of serum for the precipitation of proteins present in the sample. After 50 µl of an internal standard stock solution were added, the mixture was vortexed. After efficient mixing, the samples were centrifuged at 4°C for 15 min at a speed of 9112 ×g causing the precipitated proteins to accumulate at the bottom of the microcentrifuge tubes. The supernatant was transferred to 0.2 µm Nylon Spin-X filters. Eight-hundred microliters of the supernatant were added to 1 ml H₂O to ensure a composition of 30:70 ACN:H₂O and vortexed to ensure efficient mixing. The prepared sample was then transferred to vials for LC-MS/MS analysis.

4.8.2 LC-MS/MS analysis

4.8.2.1 Chromatographic conditions

During LC-MS/MS analysis, 10 µl of each of the samples was injected onto the Waters XSelect HSS T3 XP column that was kept at a temperature of 30°C. Mobile phase A consisted of H₂O with 5mM PFHpA and 0.1 % FA, and mobile phase B consisted of ACN with 0.1% FA. A flow rate of 0.4 ml/min was used with the mobile gradient given in Table 4.18 and a 2 min post time. Figure 4.31 illustrates the chromatographic separation of the final dMRM method.

Table 4-18 The final mobile phase gradient

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
1.5	95	5
2	85	15
4	62	38
5	53	47
8	5	95
16	5	95
17	95	5
21	95	5

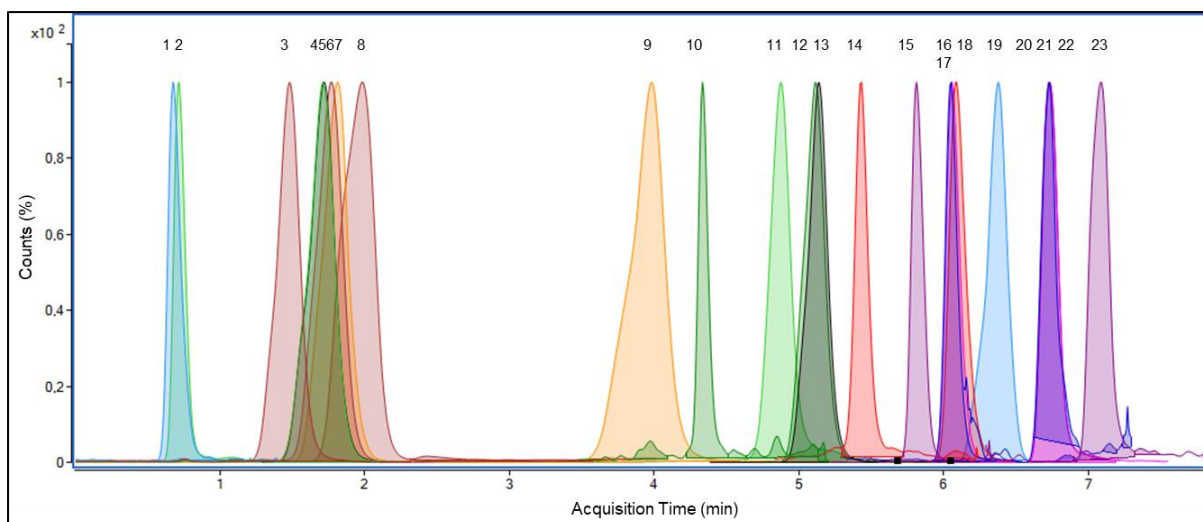


Figure 4-31 **Chromatographic profile of the final dMRM method. 1) Homocysteic acid, 2) Taurine, 3) Serine, 4) Glycine, 5) d₂-Glycine, 6) DMG, 7) Cysteine, 8) Betaine, 9) Homocysteine, 10) Riboflavin, 11) Choline, 12) Methyl-d₃-Methionine, 13) Methionine, 14) Pyridoxine, 15) 5-MTHFA, 16) d₅-Phenylalanine, 17) Cystine, 18) d₄-L-Cystine, 19) Cystathionine, 20) Homocystine, 21) SAH, 22) d₈-DL-Homocystine, and 23) SAM.**

4.8.2.2 Source conditions

The gas temperature was set to 250°C and the gas flow was set to 6 l/min. A nebulizer pressure of 50 psi, sheath gas temperature of 300°C, and sheath gas flow of 10 l/min were used. The capillary and nozzle voltages were set to 3000 and 0 V respectively. Dynamic MRM was done with the polarity, precursor ion, fragmentor energy, product ion, collision energy, and retention time for each analyte of interest as listed in Table 4.19.

Table 4-19 The final MRM data used during LC-MS/MS analysis

Analyte	Polarity	Precursor (m/z)	Product (m/z)	Fragmentor voltage (V)	Collision energy (eV)	Retention Time (min)
L-Homocystine	+	269.07	136	89	4	6.73
DL-Homocysteine	+	136.05	90	60	16	4.24
L-Methionine	+	150.06	104.1	74	8	5.29
L-Cysteine	+	122.03	59	50	24	1.9
L-Cystine	+	241.03	151.1	94	8	6.04
SAM	+	399.15	250.1	50	12	7.07
SAH	+	385.13	134	70	16	6.72
Choline	+	104.12	60.1	70	16	5
Betaine	+	118.08	58.1	118	32	1.84
Cystathionine	+	223.08	134	79	8	6.34
N, N-DMG	+	104.07	58.1	79	12	1.67
Glycine	+	76.04	30.1	45	8	1.85
L-Serine	+	106.05	60.1	50	8	1.43
Taurine	+	126.02	108	89	8	0.72
L-Homocysteic acid	+	184.03	56.1	79	20	0.7
5-Methyltetrahydro folic acid	+	460.2	313.1	112	16	5.78
Riboflavin (Vitamin B ₂)	+	377.15	243.1	137	24	4.34
Pyridoxine (Vitamin B ₆)	+	170.08	152	89	12	5.53
d ₅ -Phenylalanine	+	171.12	125.1	55	12	6.09
d ₂ -Glycine	+	78.05	32.1	35	8	1.85
d ₄ -L-Cystine	+	245.06	154	84	8	6.06
Methyl-d ₃ -Methionine	+	153.08	107	75	4	5.27
d ₈ -DL-Homocystine	+	277.12	140	79	4	6.75

4.8.3 Data analysis

The LC-MS/MS data obtained was imported into the MassHunter quantitative data analysis software. Each analyte was quantified relatively to the stable isotopes.

4.9 Partial method validation

4.9.1 Introduction

As discussed in Chapter 2, method validation is important to ensure the suitability, reliability, consistency, and reproducibility of the developed method (Chauhan *et al.*, 2015; Ravisankar *et al.*, 2015; Wal *et al.*, 2010). During this study, partial method validation was done by investigating parameters such as linearity, LLOQ, accuracy, precision, and stability.

4.9.2 Linearity and limit of quantification

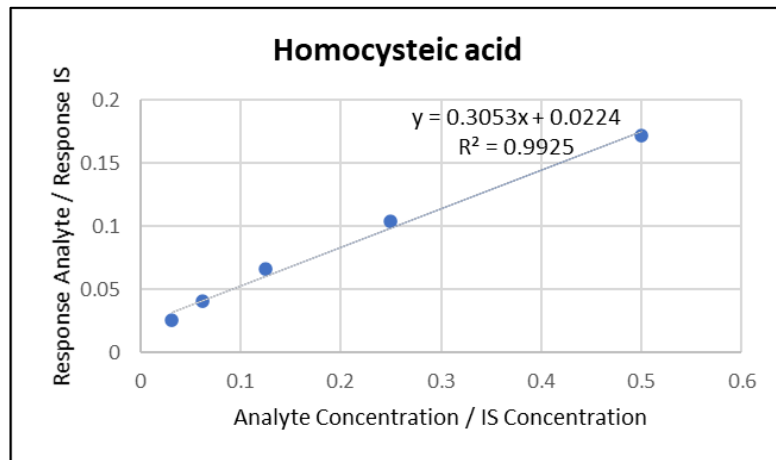
As described in Chapter 3, determining the linearity of each analyte is important to predict the concentration of analytes present in a complicated matrix (Moosavi & Ghassabian, 2018). The linearity of each analyte was investigated by analysing a nine-point calibration range in triplicate. Preparation of the calibration standards is described in Section 3.2.5. Lower concentration ranges were used for SAM, SAH, cystathionine, 5-MTHFA, riboflavin, and pyridoxine because they are present in lower concentrations in physiological samples. Fifty microliters of an internal standard stock (stable labelled isotopes) were added to the calibrants (see Table 3.2 for concentration of each isotope). Thereafter, 600 μ l ACN with 1 % FA were added and 800 μ l were mixed with 1 ml H₂O. The investigated concentration ranges, the linear range, and correlation coefficient (r^2) for each analyte are presented in Table 4.20. Calibration curves for all the analytes can be seen in Figure 4.32. In these graphs, the response of the analyte divided by the response of the internal standard was plotted against the analyte concentration divided by the internal standard concentration. Internal standards were used to compensate for any loss during sample preparation or suppression of ionisation. In Table 4.20, it can be seen that all the analytes of interest show r^2 values above 0.99, except taurine, having r^2 values of 0.986. In Figure 32 O it can be seen that cystathionine might have had a constant noise or signal present because the trendline doesn't go through the zero point.

Figure 4.33 shows calibration curves of methionine and homocysteic acid where response of the analyte was plotted against the analyte concentration, meaning that there was no compensation for loss of analyte with the use of the internal standards. When comparing Figures 4.32 (K and A) with 4.33 (A and B,) it can be seen that there was improved linearity with the use of internal standards because of the improvement of the r^2 values.

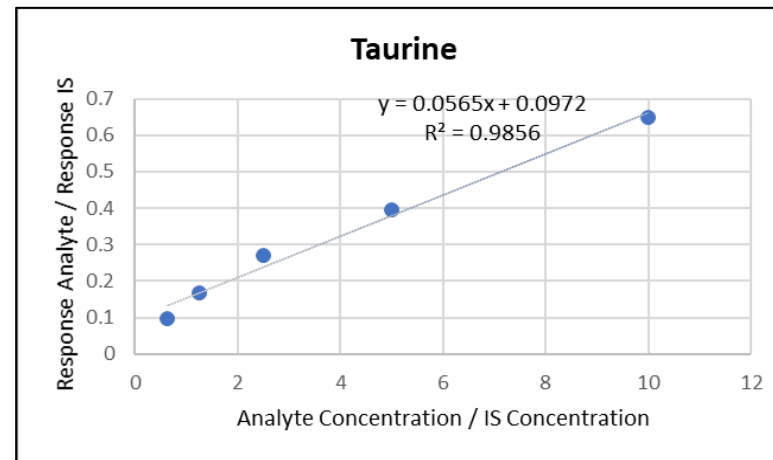
Table 4-20 A summary of the investigated concentration ranges, linear concentration ranges, and correlation coefficients for each analyte.

Analyte	Investigated concentration range (ng/ml)	Linear concentration range (ng/ml)	Correlation coefficient (R²)
L-Homocysteic acid	0.613 – 156.863	2.451 – 39.216	0.993
Taurine	2.042 – 522.876	32.680 – 522.876	0.986
L-Serine	1.634 – 418.301	1.634 – 104.575	0.999
N, N-DMG	0.408 – 104.575	0.408 – 52.288	0.999
Betaine	1.634 – 418.301	1.634 – 104.575	0.998
Glycine	1.634 – 418.301	1.634 – 418.301	0.999
L-Cysteine	1.021 – 261.438	2.042 – 130.719	0.998
DL-Homocysteine	1.021 – 261.438	1.021 – 130.719	0.997
Riboflavin (Vitamin B₂)	0.041 – 10.458	0.163 – 10.458	0.998
Choline	1.021 – 261.438	1.021 – 130.719	0.999
L-Methionine	2.042 – 522.876	2.042 – 32.680	0.999
Pyridoxine (Vitamin B₆)	0.010 – 2614	0.010 – 0654	0.999
5-Methyltetrahydro folic acid	0.061 – 15.686	0.245 – 15.686	0.997
L-Cystine	4.085 – 1045.752	4.085 – 1045.752	0.999
Cystathionine	0.041 – 10.458	0.082 – 10.458	0.998
SAH	0.082 – 20.915	0.163 – 20.915	0.998
L-Homocystine	1.634 – 418.301	1.634 – 104.575	1
SAM	0.061 – 15.686	0.061 – 15.686	0.992

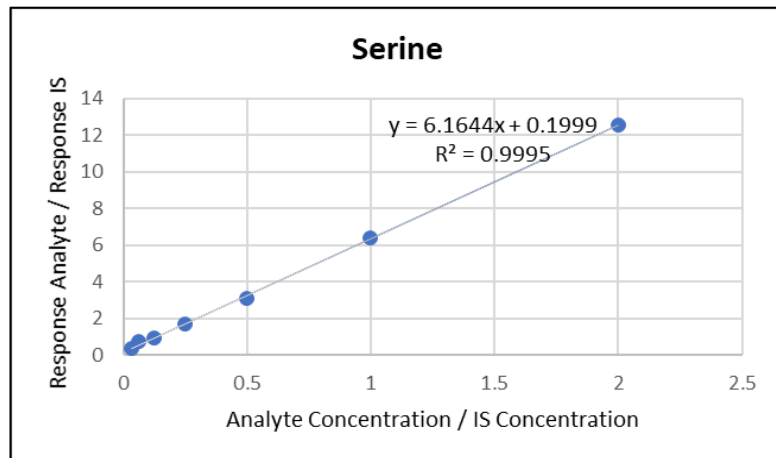
A)



B)



C)



D)

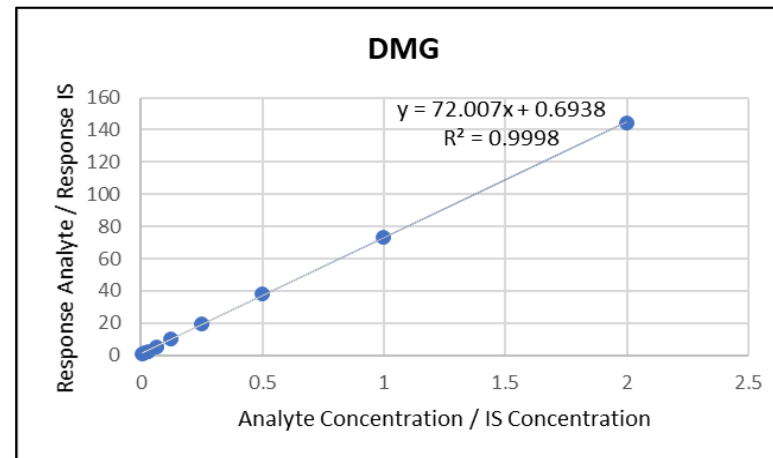
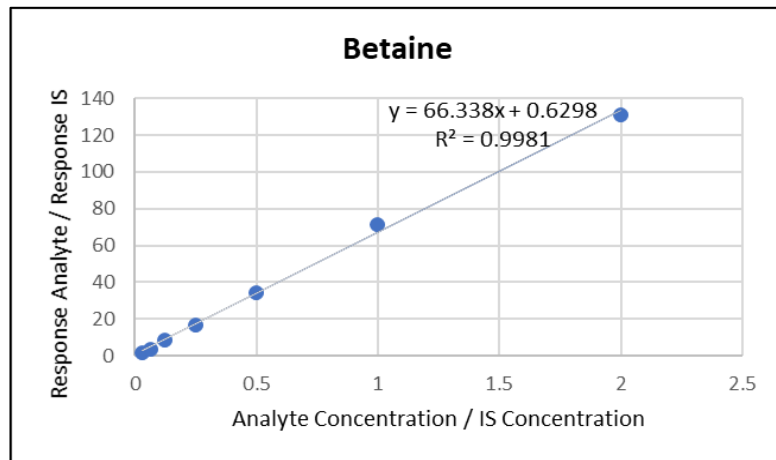
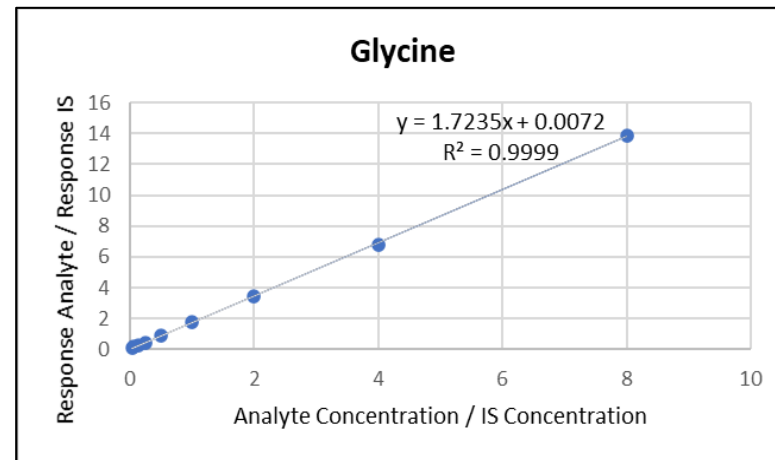


Figure 4-32 An illustration of the calibration curves of the 18 analytes of interest at the linear range.

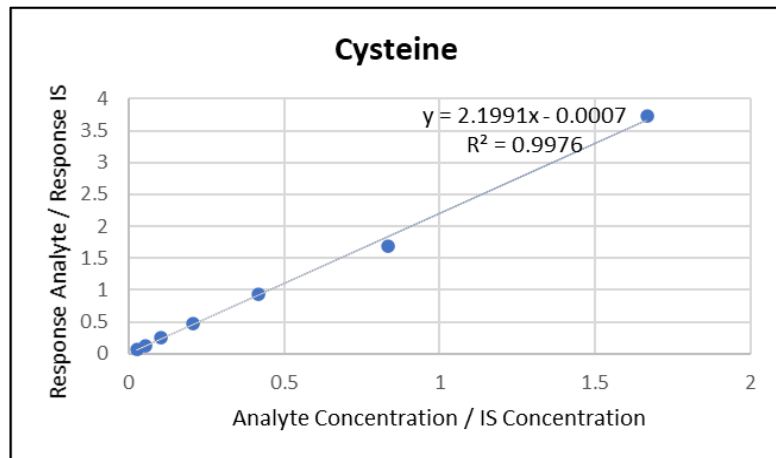
E)



F)



G)



H)

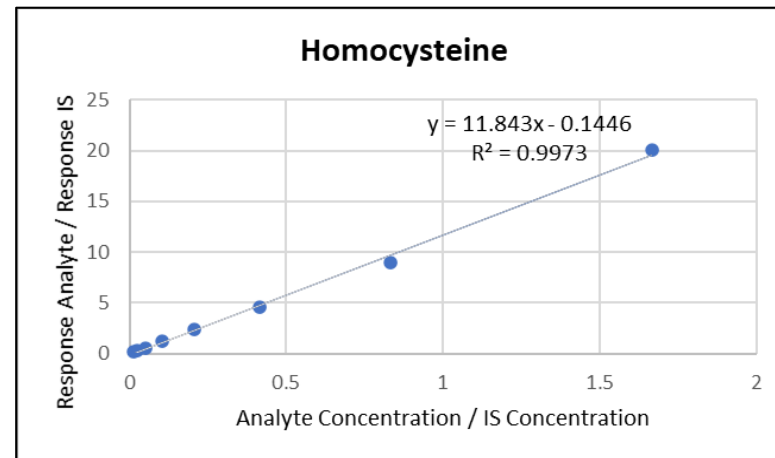
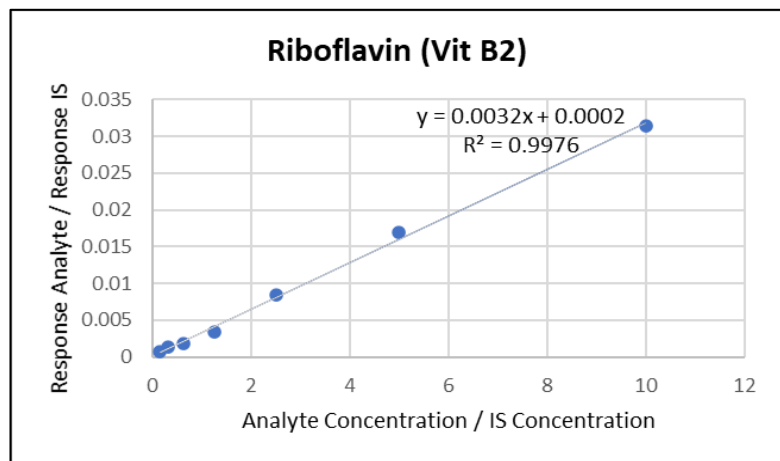


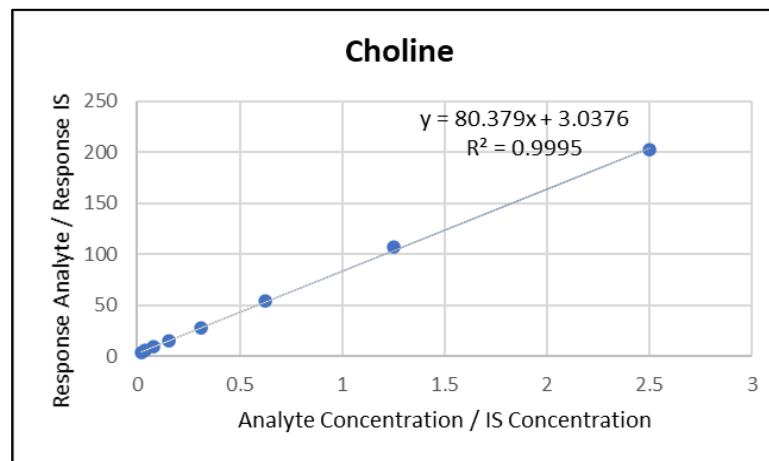
Figure 4 32 continued

An illustration of the calibration curves of the 18 analytes of interest at the linear range.

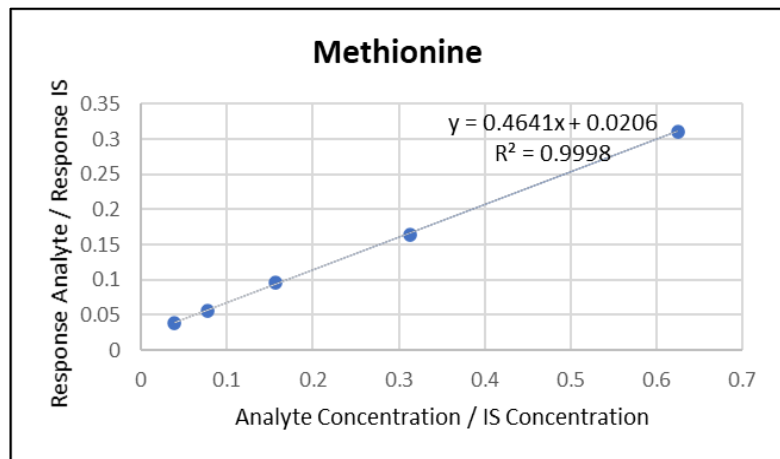
I)



J)



K)



L)

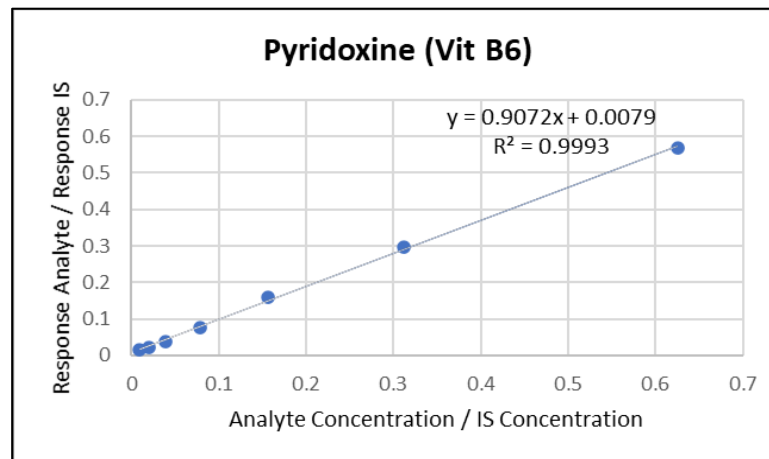
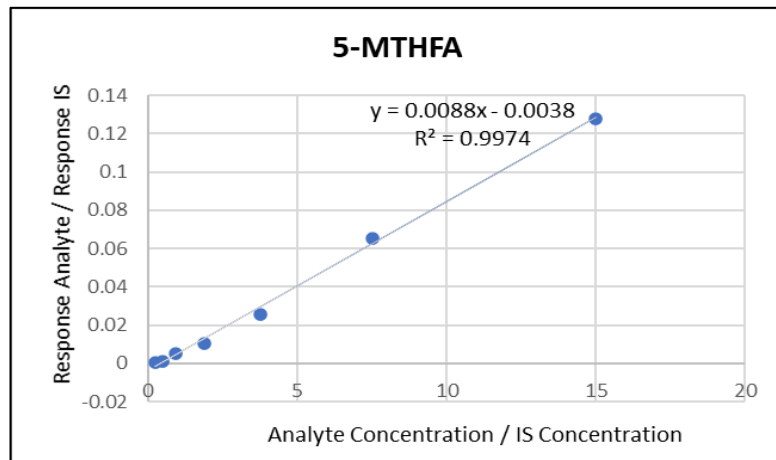


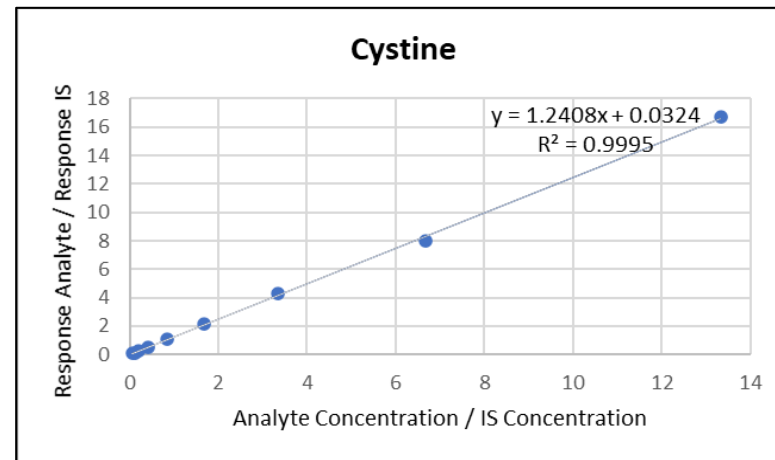
Figure 4 32 continued

An illustration of the calibration curves of the 18 analytes of interest at the linear range.

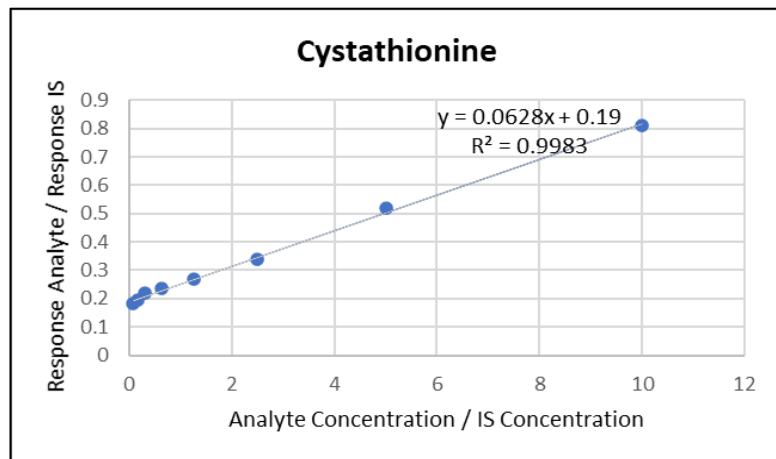
M)



N)



O)



P)

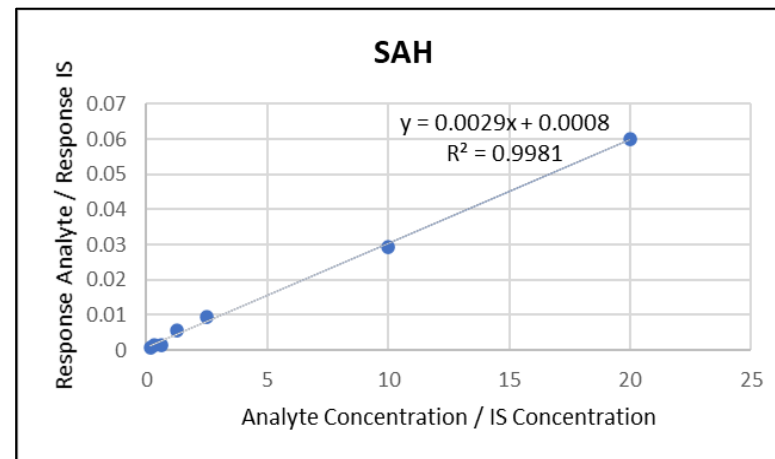
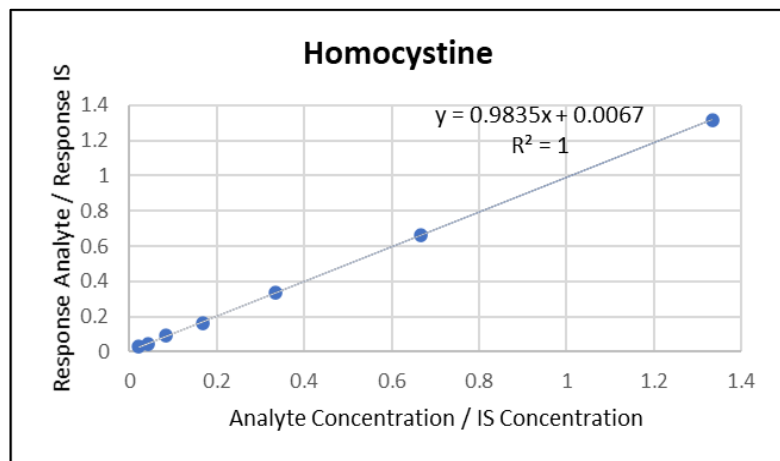


Figure 4 32 continued

An illustration of the calibration curves of the 18 analytes of interest at the linear range.

Q)



R)

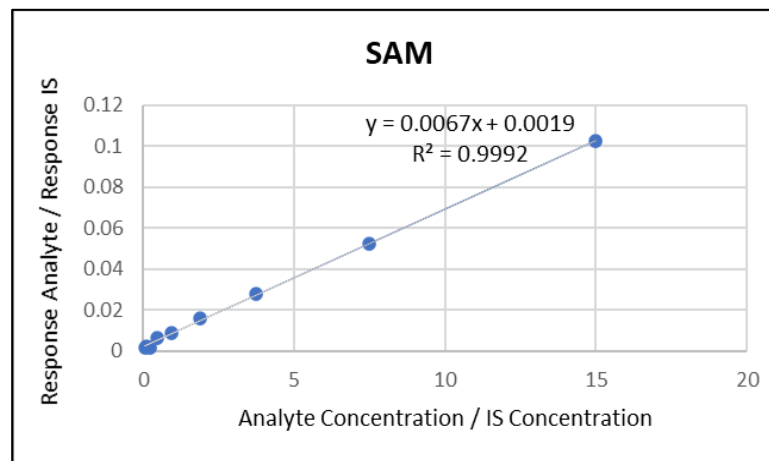
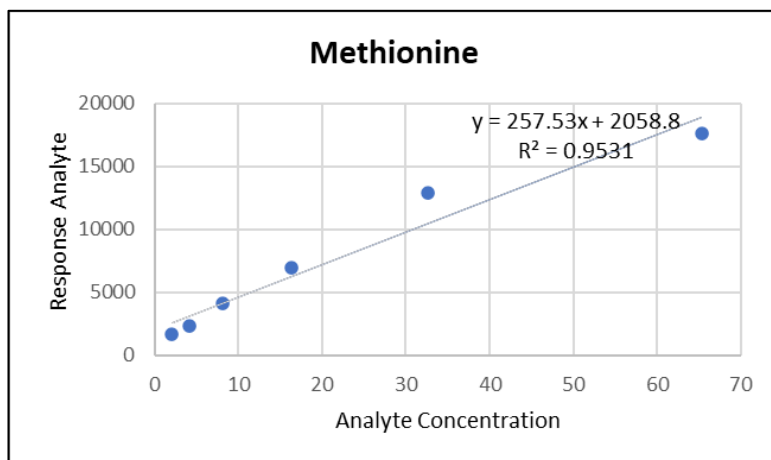


Figure 4 32 continued

An illustration of the calibration curves of the 18 analytes of interest at the linear range.

A)



B)

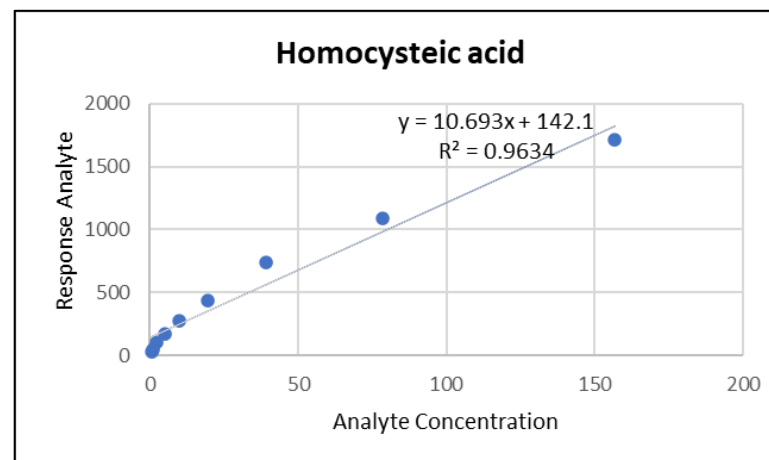


Figure 4-33 An illustration of the calibration curves of methionine and homocysteic acid without the use of internal standards for compensation of possible loss during sample preparation and ionisation suppression.

The LLOQ reported in Table 4.21 was determined using Equation 1 as described in Section 3.6.2, using the precision-based method. A CV of 20% was determined for SAM, SAH, 5-MTHFA, riboflavin, pyridoxine, and homocysteic acid. The LLOQ for the remaining analytes were chosen as the lowest or second lowest concentration in the linear range (reported in Table 4.20). This was done to ensure that the preparation of the selected LLOQ (made from the stock solution described in Section 3.2.5.) could be done with as little variation as possible.

Table 4-21 A summary of the LLOQ for each analyte.

Analyte	LLOQ (ng/ml)
L-Homocysteic acid	2.451
Taurine	32.680
L-Serine	3.268
N, N-DMG	0.817
Betaine	3.268
Glycine	3.268
L-Cysteine	4.085
DL-Homocysteine	2.042
Riboflavin (Vitamin B₂)	0.163
Choline	2.042
L-Methionine	2.042
Pyridoxine (Vitamin B₆)	0.041
5-Methyltetrahydro folic acid	1.961
L-Cystine	8.170
Cystathionine	0.163
SAH	2.614
L-Homocystine	3.268
SAM	0.980

4.9.3 Repeatability

In order to evaluate the repeatability of the autosampler injections, a LLOQ, LC, MC, and HC sample were injected in triplicate. The CV % for each analyte was calculated (see Equation 1) using the relative responses (see Equation 3 below). Table 4.22 summarises the CV % for each analyte at each of the investigated concentrations. In this table, it can be seen that all the CV % were below 15 %. This indicates that the autosampler had good injection repeatability.

Equation 3 The formula on how to determine the relative response

Relative Response = Response Analyte / Response IS

Table 4-22 Table with the CV % for the investigated concentrations for each analyte.

Analyte	LLOQ (CV %)	LC (CV %)	MC (CV %)	HC (CV %)
L-Homocysteic acid	22.43	6.26	2.54	2.74
Taurine	4.14	0.98	3.56	0.91
L-Serine	3.70	2.77	1.60	1.39
N, N-DMG	3.39	0.46	1.59	1.72
Betaine	2.43	0.31	0.41	1.60
Glycine	2.56	1.66	0.60	1.40
L-Cysteine	2.18	2.63	0.20	1.75
DL-Homocysteine	2.44	2.43	2.65	1.31
Riboflavin (Vitamin B ₂)	43.58	9.50	11.59	3.18
Choline	2.79	3.65	3.00	2.41
L-Methionine	0.61	1.15	1.64	2.42
Pyridoxine (Vitamin B ₆)	17.37	4.17	5.60	3.58
5-Methyltetrahydro folic acid	22.80		6.40	4.55
L-Cystine	4.41	1.95	0.89	1.64
Cystathionine	4.21	2.10	1.29	1.33
SAH	19.05		9.82	10.03
L-Homocystine	8.43	1.76	3.64	3.72
SAM	15.23	4.87	8.70	5.72

4.9.4 Precision and Accuracy

To assess the intraday and interday precision and accuracy, four different concentrations were investigated over the course of three days. Precision and accuracy were assessed both intraday and interday. During this analysis, the LLOQ, LC, MC, and HC were assessed in triplicate over the course of three days. To determine the precision, the CV % was calculated

(see Equation 1), the CV % should be $\leq 15\%$ except for the LLOQ where the CV values should be $\leq 20\%$. The accuracy of the method was determined using Equation 2 (see Section 3.6.3). Because of the high LLOQ (Table 4.21) and small linear range (Table 4.20 and Figure 4.31) that was determined for SAH and 5-MTHFA, only three concentrations were investigated for both these analytes (LLOQ, MC, and HC). Intraday precision and accuracy results is listed in Table 4.23. In this table it is shown that the accurate detection of some analytes deviates from the expected values, but the intraday precision was good with all CV % below 15% for LC, MC, and HC except for the LC of riboflavin. The LLOQ showed a CV % below 20% for all the analytes. This indicated that the analytical method is able to precisely detect the analytes of interest within the same day. The results obtained for intraday accuracy showed a mixture of good and less acceptable results. In Table 4.23 some metabolites presented with accuracy values that deviates from the acceptable norm. This also applied to the LLOQ and LC for some metabolites. The results of the interday precision and accuracy can be seen in Table 4.24. When looking at the results obtained for interday precision a decrease in the precise measurement was observed. These results indicated that the method is less precise when making measurements over the course of three days. The average interday accuracy results showed that the larger accuracy deviation was mainly observed in the lower concentration ranges (LLOQ, LC, and MC) and for specific metabolites. The interday accuracy measurements for serine, DMG, betaine, glycine, and cysteine were acceptable for the LC, MC, and HC of the analytes. All the concentrations investigated for DMG, cystine, cysteine, and homocystine showed acceptable results. The HC of the analytes, investigated in lower concentrations such as, riboflavin, pyridoxine, 5-MTHFA, and SAM showed acceptable accuracy. The interday accuracy between two batches (obtained over the course of two days) showed improved accuracy, as can be seen in Table A4. This was especially seen for betaine, homocysteine, choline, and SAH. Therefore, the samples analysed (Section 5) was divided into two batches to ensure precise and accurate analysis of the samples.

Table 4-23 **A summary of the intraday precision and accuracy of each analyte for all four concentrations.**

Analyte	LLOQ		LC		MC		HC	
	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
L-Homocysteic acid	14.77	277.781	10.92	206.264	2.86	170.898	3.63	136.168
Taurine	2.41	281.896	3.95	243.387	1.67	196.014	1.64	142.025
L-Serine	19.58	193.565	10.04	104.094	1.73	105.413	2.02	105.518
N, N-DMG	3.41	111.074	9.18	104.546	3.05	111.787	1.78	108.706
Betaine	5.78	94.527	8.60	99.154	1.97	106.485	2.59	108.406
Glycine	8.16	136.535	10.64	102.934	2.06	102.478	2.05	99.056
L-Cysteine	5.15	109.402	2.29	107.165	0.29	102.311	3.98	96.530
DL-Homocysteine	2.30	93.707	3.79	93.906	3.10	97.391	1.92	91.079
Riboflavin (Vitamin B ₂)	22.32	188.577	15.53	84.464	9.69	109.251	9.64	114.937
Choline	5.01	173.819	9.11	112.035	2.64	114.483	2.53	108.870
L-Methionine	2.65	210.859	1.19	152.294	1.51	132.139	8.65	109.510
Pyridoxine (Vitamin B ₆)	1.32	113.512	3.92	104.647	1.67	113.486	3.33	104.415
5-Methyltetrahydro folic acid	19.05	82.242			3.44	75.391	2.34	99.662
L-Cystine	1.69	105.462	9.38	98.862	0.69	105.080	0.38	97.803
Cystathionine	2.02	2019.579	4.15	586.871	2.38	220.482	1.01	164.405
SAH	17.65	133.741			8.26	79.621	2.29	95.685
L-Homocystine	1.93	116.537	12.15	92.646	2.75	102.962	1.00	98.609
SAM	17.70	148.291	11.91	128.460	8.31	116.279	2.27	107.149
Average	8.49	255.06	7.92	151.36	3.23	120.11	2.95	110.47

Table 4-24 A summary of the interday precision and accuracy of each analyte for all four concentrations.

Analyte	LLOQ		LC		MC		HC	
	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
L-Homocysteic acid	8.23	256.22	4.59	209.79	3.65	175.70	2.32	139.52
Taurine	10.10	310.99	11.12	258.90	10.26	207.20	13.52	155.57
L-Serine	20.43	160.63	4.64	108.18	6.11	107.18	4.40	103.03
N, N-DMG	5.25	107.10	1.27	105.39	0.74	110.93	2.45	106.10
Betaine	68.05	64.49	25.33	86.49	18.04	95.63	19.71	98.19
Glycine	8.47	131.24	2.01	104.78	0.88	102.98	2.55	101.25
L-Cysteine	6.55	110.47	7.29	108.57	5.18	103.77	8.57	98.37
DL-Homocysteine	12.76	82.55	15.46	81.23	15.15	84.99	17.31	78.99
Riboflavin (Vitamin B ₂)	14.60	174.59	46.35	92.17	41.25	111.56	28.75	113.65
Choline	15.46	191.11	15.78	131.34	13.97	127.98	13.06	117.66
L-Methionine	2.96	210.46	2.50	153.74	5.59	130.32	4.18	114.57
Pyridoxine (Vitamin B ₆)	6.46	122.22	6.79	113.54	3.53	118.23	4.98	109.70
5-Methyltetrahydro folic acid	14.27	79.26			14.84	78.06	5.25	102.09
L-Cystine	1.39	107.17	4.06	103.70	1.55	106.99	3.79	99.60
Cystathionine	26.82	1772.56	26.47	530.31	15.81	207.08	8.02	159.85
SAH	19.99	126.67			29.41	96.01	17.60	118.97
L-Homocystine	3.65	117.91	8.41	101.52	2.16	102.78	2.86	101.84
SAM	9.95	157.69	3.87	134.17	1.28	116.51	5.34	110.94
Average	14.19	237.96	11.62	151.49	10.52	121.33	9.15	112.77

4.9.5 Stability

To investigate the stability of the analytes, a LLOQ, LC, MC, and HC (Table 3.8) were prepared in triplicate for four different conditions: storage at room temperature (bench-top) for 24 hours, storage in the autosampler (4°C) for 24 hours, storage in the fridge (4°C) for 48 hours, and storage at -20°C for 7 days (one week). The response of the analytes obtained in each of the storage conditions were compared with the response of a freshly prepared standard. The stability was calculated as a percentage of the freshly prepared sample. A deviation below 15% is acceptable. Table 4.25 shows a summary of the average stability of the analytes at the four different concentrations investigated. Detailed tables of the stability of all analytes at the different storage conditions can be seen in Annexure A (Table A4 – A7). A good average stability can be seen at the different storage conditions. When looking at the results in Table A6 – A7 it can be concluded that storage at 4°C for 48 hours gave the best stability while storing the samples in the autosampler for 24 hours and at -20°C for 1 week led to decreased stability.

Table 4-25 A summary of the stability at four different storage conditions for the four concentrations investigated.

Storage conditions	LLOQ	LC	MC	HC
24 h Autosampler (% of freshly prepared sample)	80.976	78.254	83.553	80.639
24 h bench (% of freshly prepared sample)	91.145	84.037	92.189	90.444
48 h 4°C (% of freshly prepared sample)	93.202	90.090	101.241	97.817
1 week -20°C (% of freshly prepared sample)	115.513	104.005	103.101	110.118

CHAPTER 5 METHOD APPLICATION

5.1 Introduction

This chapter focusses on the application of the method in Section 4. In this chapter background about the samples used for method application and the results obtained during this analysis are discussed.

5.2 Ethical approval, participant recruitment, and sample collection

5.2.1 Ethical approval

Serum samples collected for the eBOSS study (ethics number NWU-00344-16-A1) were used to apply the developed and validated LC-MS/MS method summarised in Section 4.8. Ethical approval (see Annexure B) was obtained for this study in 2021 from the Health Research Ethics Committee of the NWU (NWU-00161-21-A1). The researchers involved in this study received appropriate ethics training.

5.2.2 Participant recruitments

Participants were recruited by placing advertisements or flyers at pharmacies, doctors' rooms, supermarkets, and shopping centres in the Potchefstroom/Klerksdorp area as well as placing the advertisements on social media platforms of the NWU. Recruitment was done from April 2017 until October 2019. Certain inclusion and exclusion criteria were applied by means of an eligibility questionnaire (Venter *et al.*, 2021). The inclusion criteria included:

- Participants that were healthy and pre-menopausal (18-35 years of age).
- Participants with healthy BMI (between 18.5-29.9).
- COC users had to be using formulations containing either 3 mg drospirenone (DRSP) and 0.03 mg ethinyl estradiol (EE), or 3 mg DRSP and 0.02 mg EE for at least three consecutive months.
- Non-COC users should not have used any hormonal contraceptives for at least four years prior to sample collection.

Participants were excluded if:

- They were pregnant or breastfeeding in the past six months.

- They had HIV and aids.
- They experienced irregular menstrual cycles.
- They smoked or used alcohol excessively.
- They had a history of peptic ulceration or tendency to hyperacidity.
- They had liver or kidney disease.
- They were using chronic medication

Eligible volunteers were invited to an information session and were given one-week after the session to decide if they wanted to take part in the study. Participants that decided to take part in this study met up with a research assistant to give informed consent.

5.2.3 Sample collection

Participants received a biotransformation and oxidative stress status (BOSS) test kit. The test was performed, and samples collected in the luteal phase of the participant's menstrual cycle. The venous blood samples relevant for this study were drawn using antecubital venepuncture and collected into serum tubes by a professional nurse. After allowing the blood to clot for 20 min. at room temperature, the samples were centrifuged at 1160 – 1800 ×g, for 10 min., at room temperature. Serum was aspirated and stored in 1 ml aliquots at -20°C.

5.2.4 eBOSS samples

The analysis of the eBOSS samples were done in two batches. Each batch contained 11 control samples and 12 COC users from a randomised samples list. These added up to 22 controls and 24 COC users.

5.3 Analytical materials and methods

A schematic layout of the method used to analyse the serum samples of the eBOSS-study is given in Figure 5.1. Before LC-MS/MS analysis of the samples, internal standard was added, and sample preparation was done by doing protein precipitation as described in Section 4.8.1. Liquid chromatography separation was done on the XSelect HSS T3 column. The column temperature was 30°C. Mobile phase A consisted of 5 mM PFHpA in water with 0.1% FA and mobile phase B consisted of 100% acetonitrile with 0.1% formic acid. The mobile phase flow rate was 0.4 ml/min and the injection volume was 10 µl. The mobile phase gradient shown in Table 5.1 was used and the total run time was 23 min. Retention times and final MRM data of

the analytes can be seen in Table 4.19. The mass spectrometry and liquid chromatography conditions discussed in Section 4.8.2 were used. The mass spectrometer was set to dynamic MRM (dMRM) mode and an EMV of 100 V, and CAV of 4 V were used.

Table 5-1 Mobile phase gradient

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	95	5
1.5	95	5
2	85	15
4	62	38
5	53	47
8	5	95
16	5	95
17	95	5
21	95	5

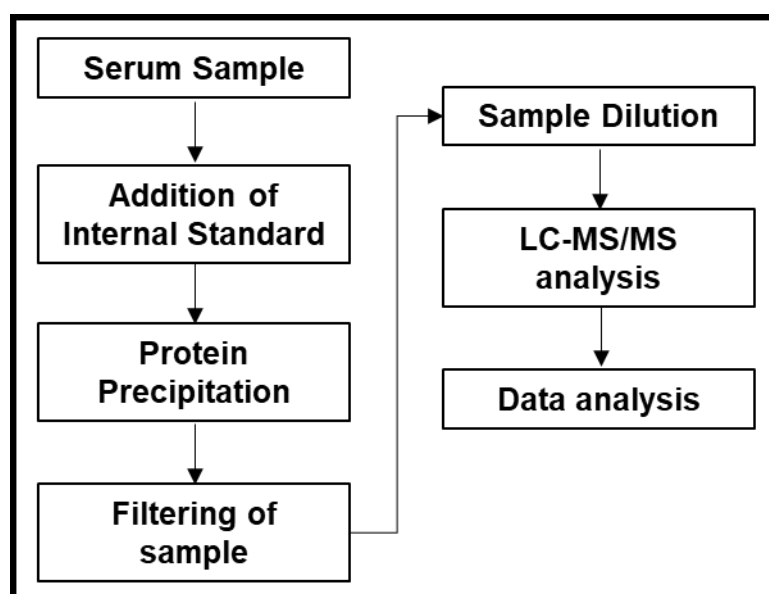


Figure 5-1 Schematic summary of the analytical method used to analyse the metabolites and co-factors of the methionine-homocysteine cycle in the eBOSS study serum samples.

5.4 Results and discussion

5.4.1 Pre-processing of statistical data

5.4.1.1 Normalisation, scaling, and transformation

The data obtained was normalised by using internal standards to determine the concentration of the analytes. Log transformation and Pareto scaling was applied to the data (described in Section 3.7). Figure 5.2 shows the results obtained before and after scaling and transformation was done. In this figure, it can be seen that after scaling and transformation, the distribution of the data was improved, and the skewness was reduced. According to a study done by Rios-Avila *et al.* (2015) the investigation of the ratios betaine:choline and DMG:betaine could help explain the difference in concentrations between the COC users and non-users. Therefore, both these ratios were determined and used during statistical analysis. Homocysteine, homocystine, cysteine, and cystine were analysed during this method and for statistical analysis total homocysteine and total cysteine were used which was the sum of the two different forms.

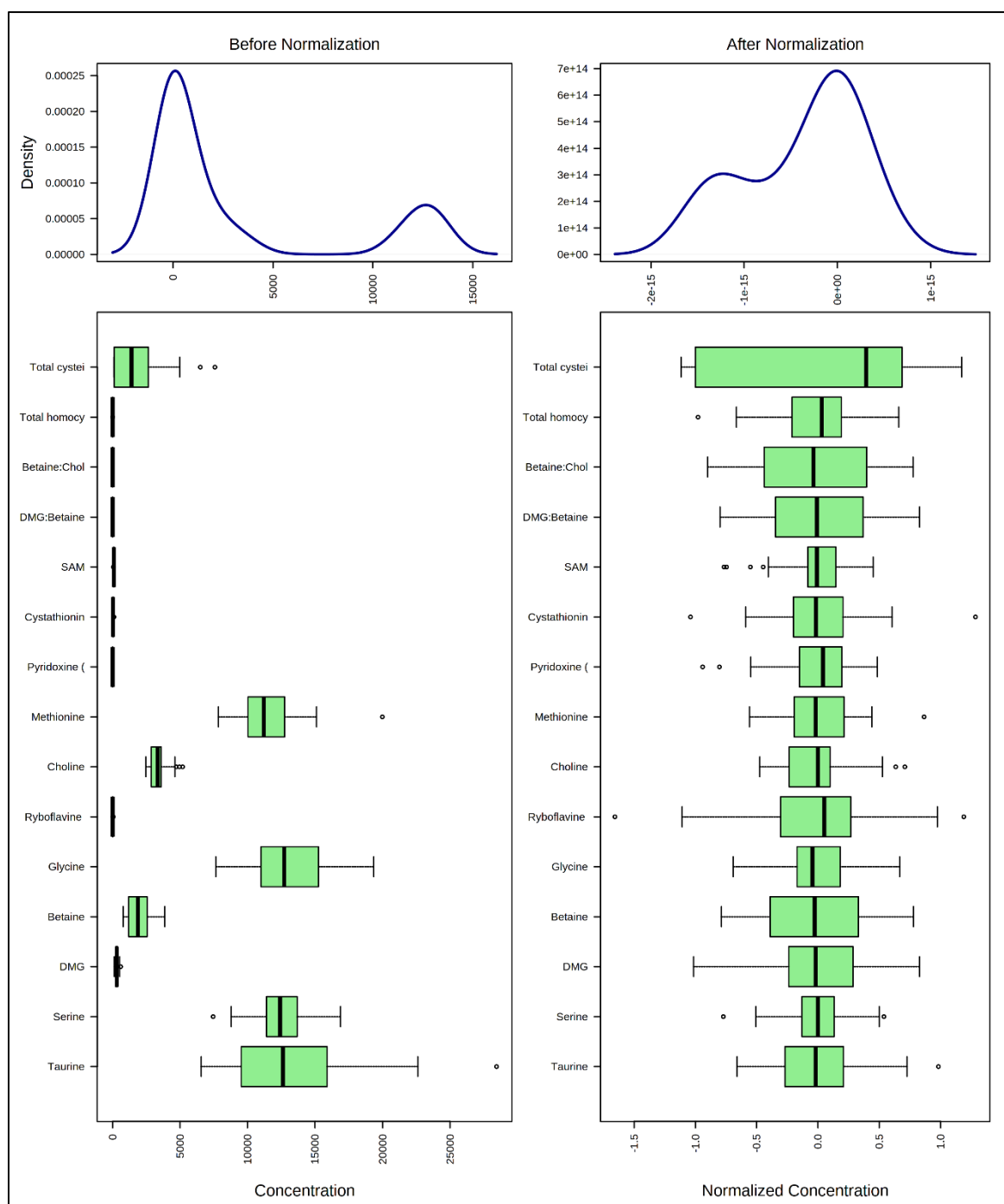


Figure 5-2 The distribution of the data before processing (on the left), and after Pareto scaling and Log transformation (on the right).

5.4.1.2 Quality assessment

5.4.1.2.1 Batch effect

As mentioned in Section 5.2.4 the samples were analysed in two different batches. This can cause a possible batch effect to arise. To assess this, MetaboAnalyst was used to perform a principal component analysis (PCA; Figure 5.3). The red circles represent the controls in batch

one (C1) and the green circles represent the users in batch one (U1), while the orange circles are representative of the controls in group two (C2), and the blue circles of the users in group two (U2). When looking at Figure 5.3, it can clearly be seen that there is no significant batch effect because the data does not show a clustering of the users and controls of the same batch. For all further statistical analysis, it was assumed that there was no batch effect present, and all batch indicators were removed.

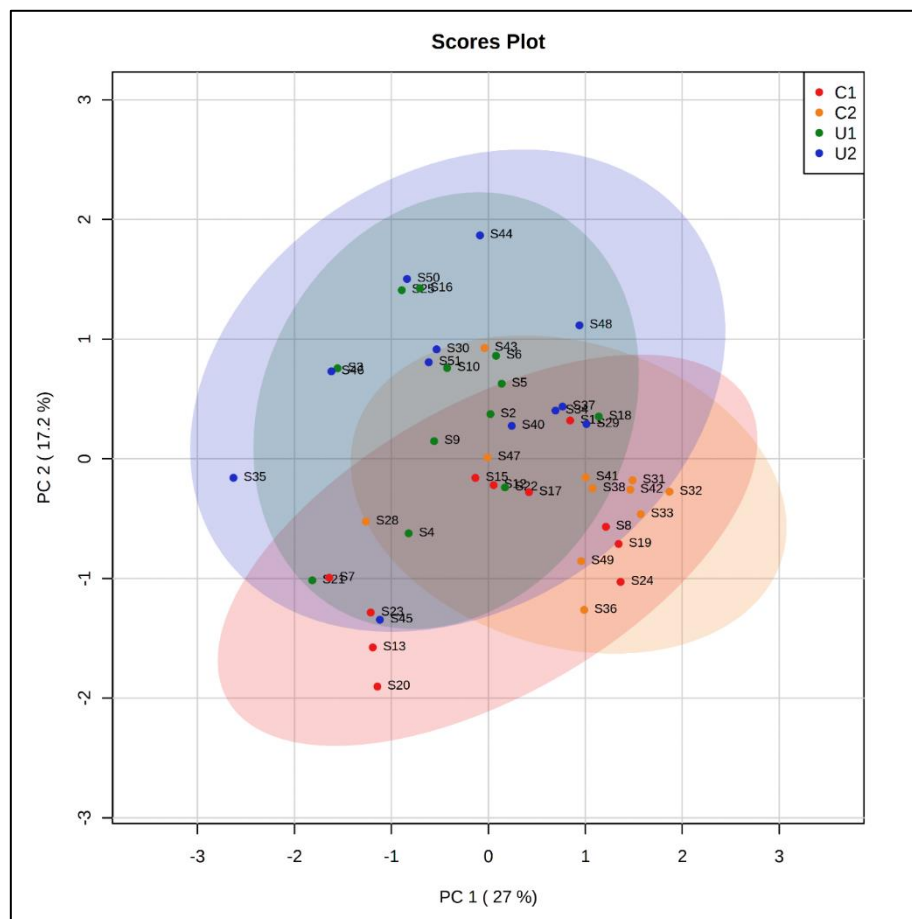


Figure 5-3 The scores plot between the selected principal components (PCs) (component 1 [x-axis] and component 2 [y-axis]) to assess the possibility of a batch effect. Batch one is represented by the red (control group) and green (user group) circles, while the orange (control group) and blue (user group) is representative of the samples analysed in batch two, as can be seen from the key in the top right corner of this figure.

5.4.1.2.2 Data filtering

During the processing of the data, data filtering was done to remove any features that might have insignificant value. One way to do data filtering is by filtering any features that have a

certain CV % in the QC samples. After assessing the possibility of a batch effect, data filtering was applied on the data. This was done by filtering features that had a CV % greater than 35% in the QC samples. This filtering step resulted in the removal of four features (analytes), namely SAH, 5-MTHFA, homocysteine, and homocysteic acid.

5.4.1.2.3 Outliers

During the process of statistical analysis of the two groups, the presence of outliers was assessed. After the data was filtered, a PCA (as can be seen in Figure 5.4) was done to determine if any outliers were present in the dataset. This indicated that samples S43 and S45 were outliers (as can be seen in Figure 5.4) - i.e., they fell outside the circle representing their respective groups. Therefore, these samples were excluded from the downstream analysis.

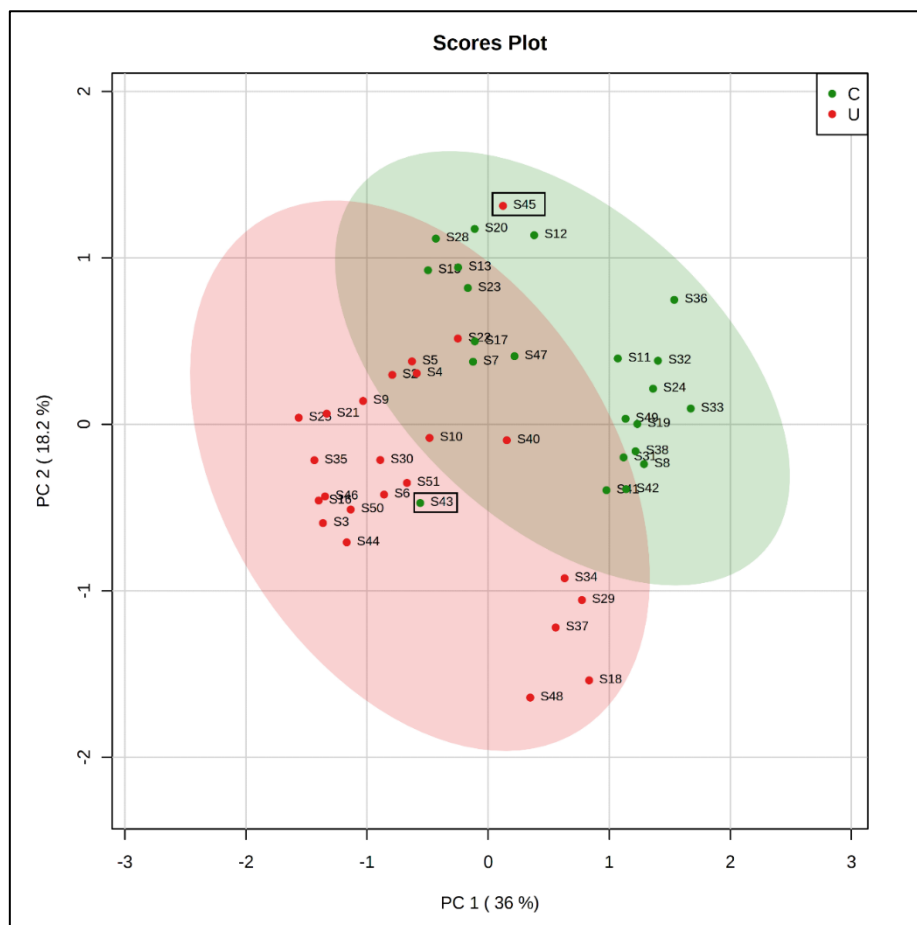


Figure 5-4 An illustration of the PCA scores plot showing the control group (green dots) and user group (red dots) when assessing for outliers. Samples S45 and S43 were identified as outliers.

5.4.2 Univariate statistics

In order to determine the possible differences between the control and combined oral contraceptive user group, univariate statistics were done. This statistical analysis was done using MetaboAnalyst. A t-test was performed, and a volcano plot constructed to analyse the data. According to the t-test, betaine, total cysteine, DMG, choline, betaine:choline, and DMG:betaine were important features because they had a FDR corrected p-value lower than 0.05, as can be seen in Table 5.2. The volcano plot in Figure 5.5 confirmed that, when taking the FC and p-value of the analytes into account, betaine, total cysteine, DMG, choline, betaine:choline, and DMG:betaine were indeed significantly different between control and user groups. This is because a p-value smaller than 0.05 and FC greater than 1.00 is seen as significant as mentioned in Section 3. Log transformation of the data makes it possible to plot features that were decreased and increased in a symmetrical way on the volcano plot. As indicated in Figure 5.5, betaine, betaine:choline, and DMG were higher in controls, while total cysteine, DMG:betaine, and choline were lower in controls. The difference between the concentrations of the control and user groups of betaine, total cysteine, DMG, choline, betaine:choline, and DMG:betaine is illustrated in Boxplots in Figure 5.6. Here it can be seen that betaine (Figure 5.6 A), betaine:choline (Figure 5.6 E), and DMG (Figure 5.6 C) concentrations were significantly lower in the user group, while the concentrations of total cysteine (Figure 5.6 B), choline (Figure 5.6 D), and DMG:betaine (Figure 5.6 F) were significantly higher in the user group. The DMG and total cysteine concentration fell within the range of the HC while betaine and choline fell above the HC investigated during partial method validation (Section 4.9).

Table 5-2 A table summarising the means and p-values of the significant features

Analyte	Mean Control (ng/ml)	Mean User (ng/ml)	p-value
DMG	341.917	267.062	0.006
Betaine	2619.246	1323.249	8.66E-15
Choline	3029.246	3649.471	0.0006
Total Cysteine	1245.402	2573.141	0.040
Betaine:Choline	0.865	0.375	5.78E-16
DMG:Betaine	0.138	0.209	6.25E-06

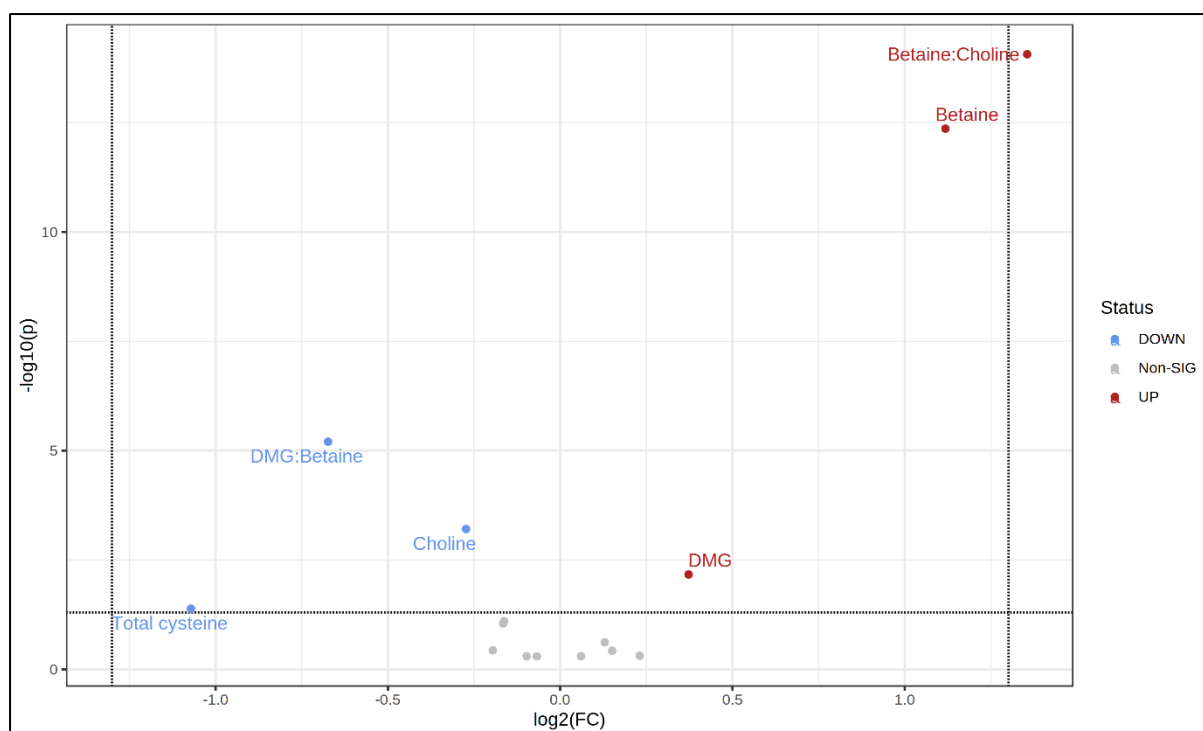


Figure 5-5 Volcano plot (log transformed p-value against the log transformed FC) of the data. The further the circles are from 0.0, the more significant they are. The red circles represent the features that are increased, the blue circles represent the features that are decreased, and the grey circles represent features that are seen as non-significant

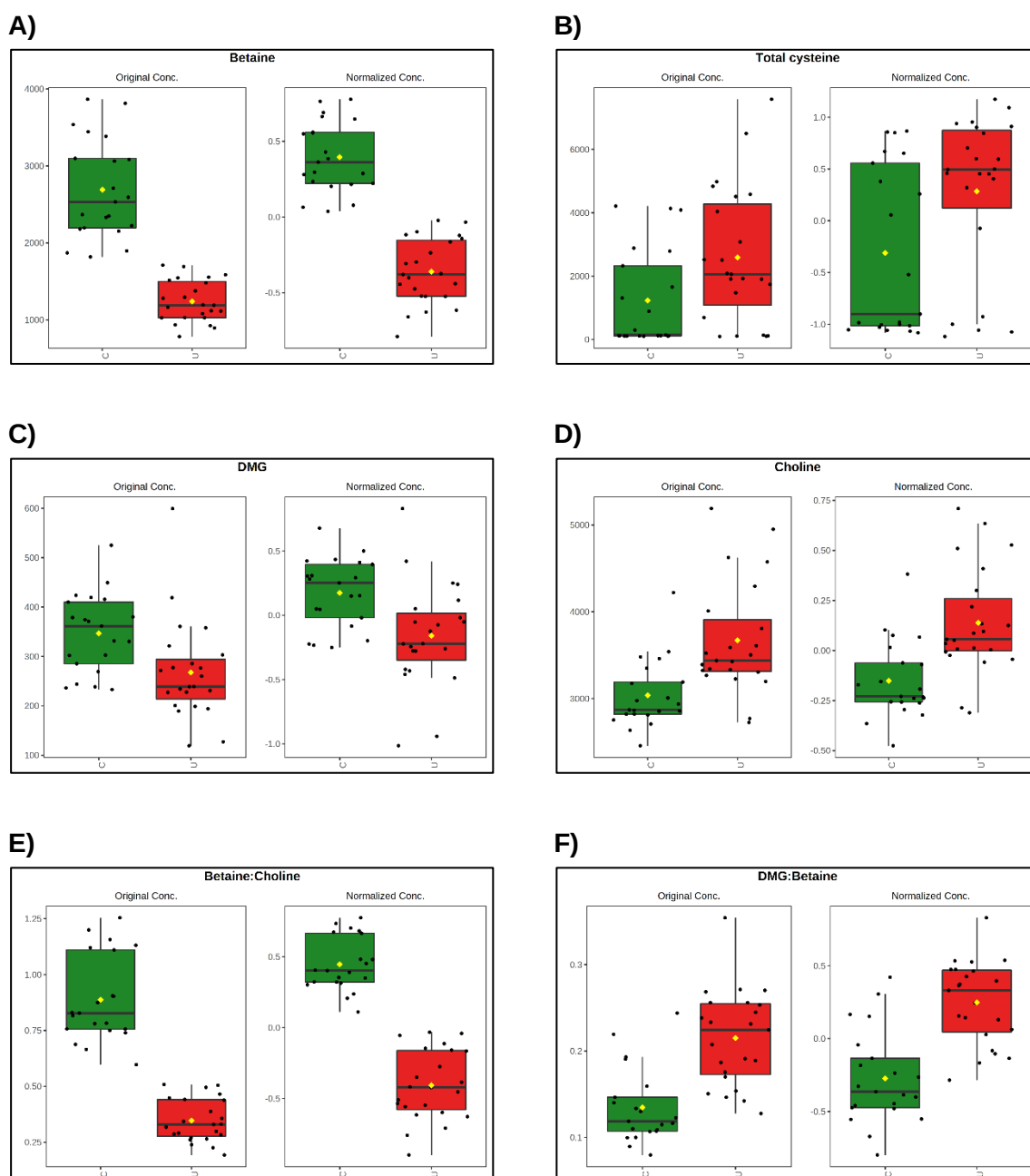


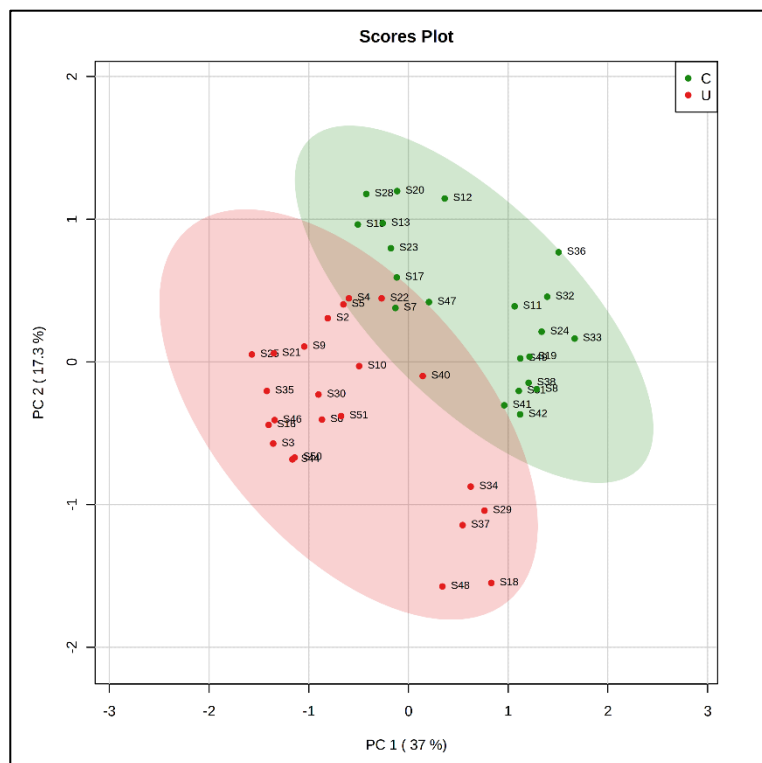
Figure 5-6 Boxplot graphs of A) Betaine, B) Total Cysteine, C) DMG, D) Choline, E) Betaine:Choline, and F) DMG:Betaine before and after the concentrations were normalised, scaled, and transformed. The green boxes represent the control group, and the red boxes are representative of the user group.

5.4.3 Multivariate statistics

Although the number of variates in this study was limited to 18, a multivariate approach was also followed to confirm the differences observed between the control and user group after applying univariate statistics. To do this, both a PCA and PLS-DA was done (see Section 3).

In Figure 5.7 A, it can be seen that, even though the analysis is unsupervised, there is a separation between the control and user groups. Figure 5.7 B confirms the separation between the groups using a supervised approach. The VIP scores plot in Figure 5.8 shows the important features that are responsible for the separation between the two groups. To correctly interpret the VIP scores plot, the VIP scores for components one and two were taken into account. Features were considered significant when the VIP score was ≥ 1 for component one and/or component two. After these criteria were applied, betaine, total cysteine, betaine:choline, and DMG:betaine were again identified as important features (see Table 5.3).

A)



B)

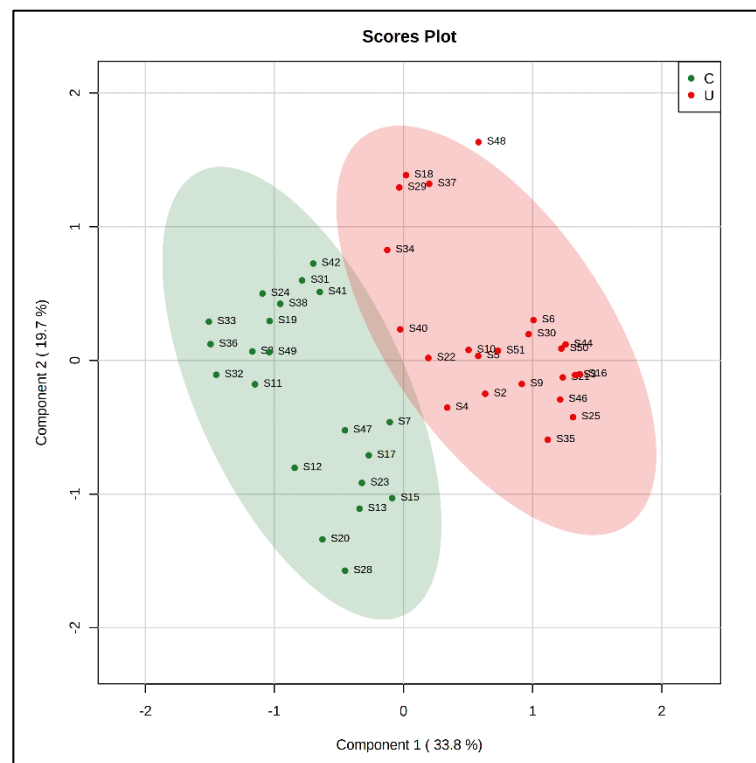


Figure 5-7 The scores plots of the results obtained after doing A) PCA and B) PLSDA. The red circles are representative of the user group while the green circles represent the control group.

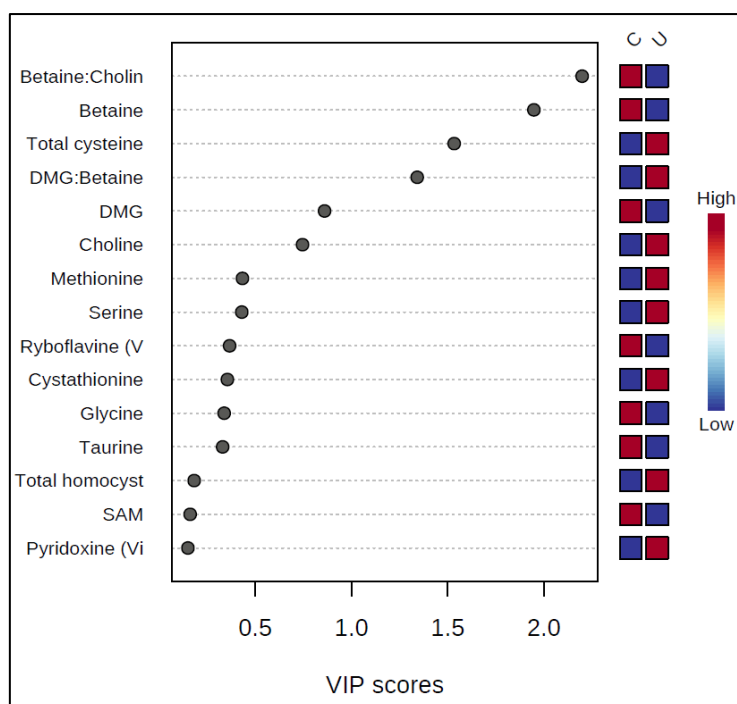


Figure 5-8 VIP scores plot that visually illustrates the VIP scores of the first component identified by PLS-DA. The red squares indicate that the feature is higher in that specific group and the blue squares indicate that the feature is lower in that specific group. The higher the VIP score (values indicated on the x-axis) the greater the significance of the feature (analyte) that is illustrated on the y-axis.

Table 5-3 A summary of the VIP scores for component 1 and 2 of the identified important features by using the criteria of ≥ 1 as mentioned earlier.

Analyte feature)	(significant	VIP Score Component 1	VIP Score Component 2
Betaine:Choline		2.197	2.062
Betaine		1.947	1.834
Total Cysteine		1.533	1.825
DMG:Betaine		1.341	1.245

5.4.4 Biochemical interpretation of the data

From the univariate and multivariate statistical analysis done in Sections 5.4.2 and 5.4.3, betaine, total cysteine, DMG, choline, DMG:betaine, and betaine:choline were identified as significant features. All of these analytes play vital parts in the methionine-homocysteine cycle.

According to the statistical analysis betaine, DMG, and betaine:choline was lower in the COC user group, while total cysteine, choline, and DMG:betaine were higher in the COC user group when compared to the control group. Betaine (also known as trimethylglycine) is known as an important methyl group donor during the re-methylation pathway of homocysteine (as seen in Figure 5.9). After betaine donates a methyl group to homocysteine, it forms DMG, which is further used as a methyl donor elsewhere. Both betaine and DMG showed lower levels in the COC user group. This can indicate a decrease in the availability of methyl group donors when COCs are used. The decreased levels of DMG could be ascribed to the decreased levels of betaine because DMG is produced from betaine, as seen in Figure 5.9. To get an indication of the production and utilisation of betaine, the ratio of betaine:choline and DMG:betaine was also investigated. The ratio of DMG:betaine was higher in the COC users than in the control group. The decreased levels of betaine, DMG:betaine, and increased levels of betaine:choline in the COC user group were consistent with the results obtained by Rios-Avila *et al.* (2015). Rios-Avila *et al.* (2015) also reported that they found no significant difference in choline and DMG between the COC users and non-users. This corresponds with the multivariate results obtained in this study. Therefore, using exogenous sex hormones seems to affect the production and use of betaine (Rios-Avila *et al.*, 2015). During the biotransformation of estrogen, methylation is an important biotransformation process that detoxifies catechol estrogens. Betaine donates methyl groups during this process. Lower levels of betaine in COC users could be ascribed to the extra dose of estrogens that undergo methylation in the COC users. There is, therefore, an increased load on the re-methylation pathway.

As explained in Section 4, the analysed samples were not treated with a reducing agent to prevent the formation of disulfide bonds and the method was optimised to detect cystine and homocystine. Therefore, total cysteine (the sum of cystine and cysteine) was used as a representative of cysteine as there was no way we could determine what amount of cysteine formed cystine. Total cysteine was significantly increased in the COC user group during this study. Swanepoel *et al.* (2020) reported that there was an increase in cysteine but a decrease in cystine concentrations of the COC users. The increase in the total cysteine concentration of the COC user group could be because of the decrease in betaine acting as a methyl donor during the re-methylation of homocysteine. Because homocysteine cannot successfully undergo re-methylation (due to the decrease in the methyl donor betaine) it is converted to cysteine by the transsulfuration pathway (see Figure 5.9). Although total homocysteine was not determined as a significant marker during univariate or multivariate statistics there is a tendency for total homocysteine to be increased in the COC user group (Figure 5.8). This supports the statement made previously that the transsulfuration pathway was favoured. The increased choline levels reported in COC users might be explained with the help of the results

obtained by Resseguie *et al.* (2007). During their study they investigated if phosphatidylethanolamine N-methyltransferase (PEMT), the enzyme responsible for the endogenous synthesis of choline, is regulated by estrogen. They found that estrogen treatment caused the increased activity of the enzyme PEMT, leading to an increased synthesis of choline (Resseguie *et al.*, 2007). The increased levels of choline in COC users can be ascribed to the fact that they take in increased levels of estrogen, which can lead to enhanced activity of PEMT causing an increase in choline levels as seen by Resseguie *et al.* (2007). Further investigation of the methionine-homocysteine cycle is needed to fully explain the results obtained.

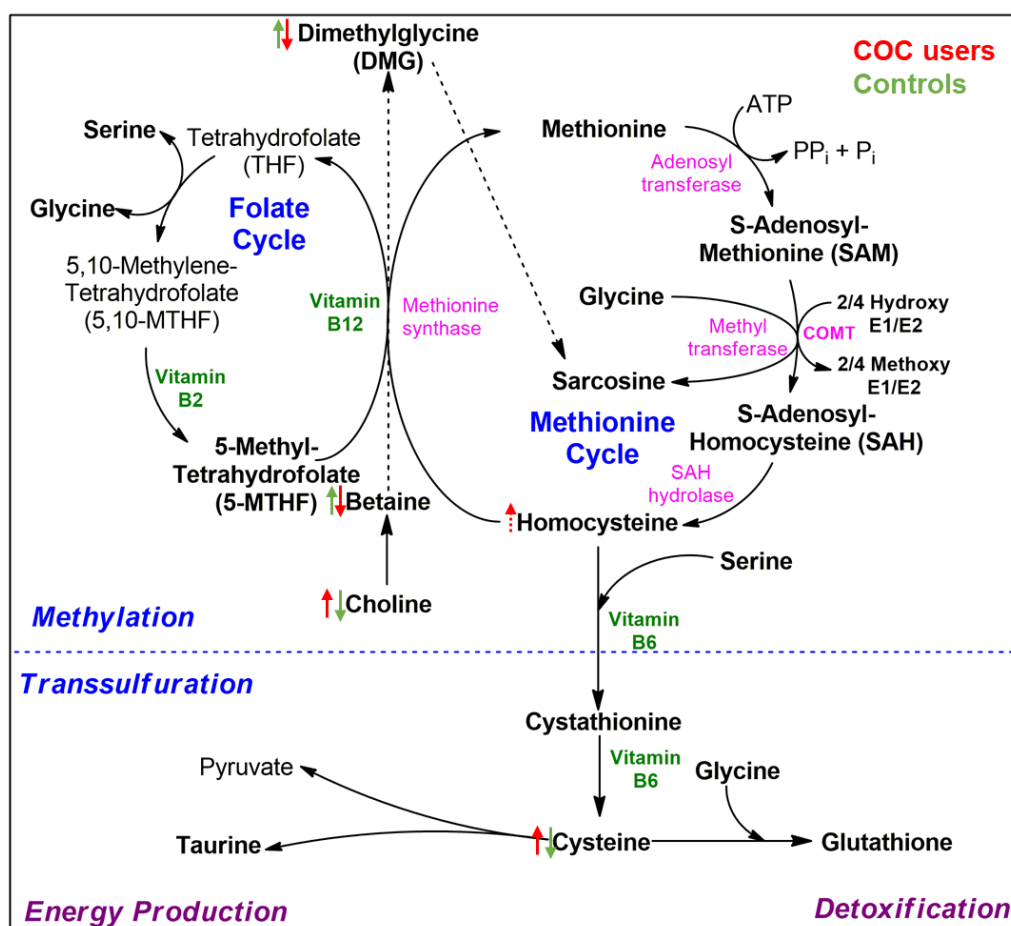


Figure 5-9 Metabolic pathway of the methionine-homocysteine cycle, illustrating the results obtained after the analysis of the eBOSS samples. The red arrows represent the COC user group while the green arrows are representative of the control group. Arrows showing upwards indicates an increase in the analyte while downward facing arrows indicate a decrease in the analyte. Modified from Gropper and Smith (2012); Guiraud *et al.* (2017).

CHAPTER 6 CONCLUSION AND FUTURE PROSPECTS

6.1 Conclusion

The **aim** of this study was to develop an LC-MS/MS method to quantify the metabolites and co-factors of the methionine-homocysteine cycle to investigate the effect of combined oral contraceptives on this pathway. The objectives discussed in Section 1 were used to help reach this aim. This section gives a critical discussion on the achievement of the objectives.

The **first objective** was to implement the method Guiraud *et al.* (2017) developed. Implementing the method entailed optimising the MS and the source conditions because of the difference in instrumentation. Chromatographic parameters were also implemented.

Optimising the implemented method by Guiraud *et al.*, (2017) was the focus of the **second objective**. Optimisation included sample preparation optimisation, mobile phase gradient optimisation, the inclusion of cystine and homocystine, and using a dMRM to improve sensitivity. While working towards this objective, some difficulties were encountered. These were mainly associated with the concentration difference between the analytes of interest and indicated that one might have to split the method in two, to accurately quantify both high and low concentrations of the analytes of interest.

Objective three focused on partially validating the implemented and optimised method by studying parameters like linearity, repeatability, precision, accuracy, and stability. Linearity for the analytes of interest was studied and achieved, except for taurine, which had a r^2 value of 0.986. Assessment of the repeatability of the autosampler injections showed good results with CV % below 15 % for all the analytes of interest. Intraday and interday precision and accuracy could be assessed successfully. The intraday precision showed promising results, indicating that the analytical method is able to detect the analytes of interest with precision, while the intraday accuracy showed mixed results, especially for the lower concentrations of the analytes. When studying the interday precision and accuracy it was seen that the precision and accuracy decreased when three days were assessed. The difference in concentration of the analytes investigated led to the decrease in precision and accuracy. Although the method's selectivity was not analytically verified the combination of high-resolution chromatography and triple quadrupole mass spectrometry ensures increased selectivity. Using stable labelled isotopes and a dMRM method also increases the method's selectivity.

The last objective was to apply the developed, optimised, and validated method to samples from the eBOSS study, which consists of a group of combined oral contraceptive users and a

control group. This was done to determine whether COC use has any effect on the metabolites and co-factors in the methionine-homocysteine cycle. The method was successfully applied to the samples and significant differences between the two groups were seen, although further investigation of the methionine-homocysteine cycle is needed to fully explain these observations.

6.2 Future prospects

Although we were able to implement and optimise a method to investigate the metabolites and co-factors of the methionine-homocysteine cycle in COC users and non-users, the following recommendations should be considered to improve the current method:

1. During the investigation of method validation, it became evident that the analytes grouped into two different concentration groups. When a combined investigation of the two groups was done, problems arose with the analytes of low concentrations. On these grounds the methods that would be developed in the future should compensate for these concentration differences. This will improve the detection of the analytes and the method's sensitivity. This can be done by separating the current method into two groups (analytes of higher concentration in one and analytes of lower concentration in another).
2. The selectivity of the method can be improved by adding a quantifying ion. This could improve the linearity of cystathionine.
3. The current sample preparation method can be further optimised to use a smaller serum volume. In the current method, 200 µl serum is used and a small fraction of the final sample volume is injected onto the column. Optimising the method to use a smaller sample volume combined with an improved sample extraction would be advantageous. This should be optimised by keeping the two suggested methods mentioned in the first prospect in mind.
4. The possibility of excluding the use of the ion-pairing reagent should be investigated. The use of an ion-pairing reagent can lead to ionisation problems. Using derivatisation might be an option as this will improve the sensitivity and retention on the C18 column. Gustavsson *et al.* (2001) reported that using PFHpA, as an ion-pairing agent, led to a decrease in repeatability and broadening of peaks.
5. Matrix effect should be assessed as a part of method validation.

6. The addition of more stable labelled isotopes should be considered to help accurately quantify all the analytes of interest. Using stable labelled isotopes could also have a carrier function which could be advantageous for the analytes in a lower concentration.
7. Expanding the sample preparation method to include the addition of a disulfide reduction reagent for a more accurate representation of homocysteine and cysteine should also be considered. This would have to be done during sample collection to ensure reduced oxidation.
8. The application of the method on a test group where the COC users can be their own control group can help investigate the COC effect on the users.
9. The addition of sarcosine and glutathione to the analytical method could aid in a better understanding of the influence COC use has on the methionine-homocysteine cycle.

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ANNEXURE A

Table A1 Results of the optimisation of ionisation and MRM conditions. This table holds all the results obtained during the optimisation of the MRM conditions. It includes the precursor ion, all the possible product ions, the most optimal collision energy, and fragmentor voltage determined using the Agilent MassHunter Optimizer software.

Metabolite	Formula	Polarity	Precursor (m/z)	Product (m/z)	Fragmentor energy (V)	Collision energy (eV)
L-Homocystine	<chem>C8H16N2O4S2</chem>	Positive	269,07	136	89	4
				88		36
				90		20
DL-Homocysteine	<chem>C4H9NO2S</chem>	Positive	136,05	90	60	16
				47,1		28
				118		0
				56,1		16
L-Methionine	<chem>C5H11NO2S</chem>	Positive	150,06	104,1	74	8
				56,1		8
L-Cysteine	<chem>C3H7NO2S</chem>	Positive	122,03	59	50	24
				81,2		0
				61,1		4
				64,1		0
L-Cystine	<chem>C6H12N2O4S2</chem>	Positive	241,03	151.9	94	8
				74		32
				120		20
SAM	<chem>C15H22N6O5S</chem>	Positive	399,15	250.1	50	12
				136		32
				97,1		36
SAH	<chem>C14H20N6O5S</chem>	Positive	385,13	134	70	16
				136		24

				88		52
Choline	<chem>C5H14NO</chem>	Positive	104,12	60,1	70	16
				58,1		32
				63,1		16
Betaine	<chem>C5H11NO2</chem>	Positive	118,08	58,1	118	32
				59,1		20
Cystathionine	<chem>C7H14N2O4S</chem>	Positive	223,08	134	79	8
				88		32
N, N-DMG	<chem>C4H9NO2</chem>	Positive	104,07	58,1	79	12
				63		16
Glycine	<chem>C2H5NO2</chem>	Positive	76,04	30,1	45	8
L-Serine	<chem>C3H7NO3</chem>	Positive	106,05	60,1	50	8
				65,1		0
				64,1		0
Taurine	<chem>C2H7NO3S</chem>	Positive	126,02	108	89	8
L-homocysteic acid	<chem>C4H9NO5S</chem>	Positive	184,03	56,1	79	20
5-Methyltetrahydro folic acid	<chem>C20H25N7O6</chem>	Positive	460,2	313,1	112	16
				180,1		44
				194,1		32
				152		60
Riboflavin (Vitamin B₂)	<chem>C17H20N4O6</chem>	Positive	377,15	243,1	137	24
				209,8		48
Pyridoxine (Vitamin B₆)	<chem>C8H11NO3</chem>	Positive	170,08	152	89	12
				114,5		8

Table A2 **Summary of the average calibration concentration for each parameter optimised during source optimisation. The data was colour coded for visual comparison. The colours used were red, yellow, and green (red being the lowest and green being the highest).**

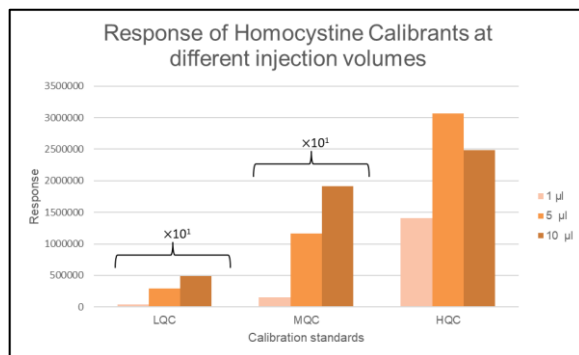
Metabolites	5-MTHFA	Betaine	Choline	Cystathionine	Cysteine	Cystine	DMG	Glycine	HCA	Homocysteine	Homocystine	Methionine	Pyridoxine	Riboflavin	SAH	SAM	Serine	Taurine
Average Calibration Concentration																		
Capillary Voltage																		
2000	100.119	99.590	100.534	100.475	97.831	99.893	100.138	99.591	98.386	97.911	100.651	98.917	99.101	100.168	96.652	101.113	99.104	97.438
2500	121.324	98.819	103.004	91.899	100.714	82.830	100.229	103.099	79.557	106.064	102.157	87.485	103.884	112.118	88.796	72.016	98.576	92.409
3000	135.225	92.965	102.728	59.187	102.267	62.548	89.733	108.369	45.162	104.919	104.552	77.399	95.027	118.748	70.089	130.981	99.453	85.165
3500	138.673	87.605	50.003	49.441	100.938	45.857	71.780	111.178	21.138	97.342	107.439	67.757	81.618	119.199	39.860	120.004	98.842	84.314
4000	135.004	86.572	0.544	40.527	96.637	38.934	57.315	116.078	19.796	87.073	105.802	56.218	70.821	119.507	23.863	98.725	96.577	80.662
4500	125.789	78.605	0.398	38.378	88.935	38.559	48.944	114.939	20.273	78.139	108.607	46.743	58.935	117.196	2.193	0.000	90.111	81.267
5000	111.658	71.047	84.825	34.778	82.734	42.467	44.446	108.357	22.562	70.060	0.000	39.371	54.258	125.448	2.006	0.000	81.382	40.842
Drying gas flow																		
4	99.040	97.644	100.304	101.427	98.174	103.978	102.021	98.567	100.773	96.571	100.233	99.624	103.270	98.031	99.614	100.531	98.831	98.525
6	132.834	96.758	106.038	85.769	104.291	105.534	99.914	109.293	98.050	105.038	104.904	99.270	102.840	113.016	77.170	103.285	107.994	97.034
8	190.055	94.833	108.302	82.362	116.526	104.318	95.235	125.689	96.259	128.107	105.147	96.939	101.733	127.565	75.528	90.649	117.576	91.767
10	206.705	87.306	108.363	75.835	131.509	100.664	87.070	145.690	93.656	130.705	105.803	96.926	75.604	137.935	83.103	72.849	122.604	85.417
Drying gas temperature																		
200	99.241	99.343	99.507	56.265	102.825	99.620	99.474	99.225	95.859	99.646	99.928	99.032	101.988	100.082	99.431	98.672	98.691	92.352
230	95.736	97.479	99.546	10.382	102.071	67.609	99.107	88.266	95.123	95.914	95.414	95.877	100.355	91.746	86.682	91.997	94.501	101.727
250	92.831	94.033	99.926	40.928	103.590	63.565	93.835	88.909	92.013	100.284	89.927	92.778	108.212	93.789	79.299	87.016	93.124	97.717
280	102.871	87.823	94.246	60.638	107.737	56.951	90.235	93.138	91.190	108.462	85.516	88.862	95.027	90.809	59.745	76.967	94.787	93.306
300	117.316	83.142	93.675	50.819	115.846	66.987	82.814	95.067	87.802	116.936	84.613	83.766	93.164	92.043	73.642	73.766	94.931	90.635
330	143.278	76.352	91.301	44.174	120.741	61.497	78.464	97.016	83.199	137.234	81.283	77.198	75.087	89.106	68.902	65.313	98.543	82.532
350	157.639	74.534	90.673	5.367	127.793	54.600	72.142	100.143	76.737	148.762	79.314	70.649	76.264	90.136	66.791	61.669	99.898	76.834
Nebulizer voltage																		
20	96.914	98.141	102.234	100.266	100.004	102.303	101.293	102.072	100.409	95.927	99.555	102.703	100.868	100.884	97.519	104.525	99.907	97.409
25	104.078	111.298	118.474	78.828	129.897	92.046	131.352	109.458	87.672	106.894	93.769	112.908	144.175	90.956	85.783	126.858	100.595	106.690
30	102.894	126.131	131.236	52.171	148.593	79.364	162.543	105.219	78.195	112.467	83.577	116.938	191.012	79.416	77.026	155.463	98.753	96.257
35	98.091	139.252	138.171	34.970	158.207	67.157	187.872	107.796	70.915	115.996	74.294	117.602	229.539	67.602	75.136	180.223	98.577	91.361
40	91.335	141.766	140.853	23.580	157.391	60.174	209.764	107.524	72.068	115.332	64.988	117.081	242.228	58.806	76.716	217.261	95.597	93.994
45	86.067	138.362	149.224	21.743	158.273	59.651	241.706	104.554	70.649	113.222	58.019	120.278	317.744	52.454	65.104	246.307	93.136	92.224
50	82.459	139.769	159.846	15.348	157.884	61.400	269.786	102.611	87.750	112.681	53.125	120.214	266.809	47.953	39.092	254.679	89.975	87.632
Nozzle voltage																		
0	98.323	99.223	127.411	137.186	97.791	96.264	99.770	101.547	103.895	99.666	77.424	98.902	101.328	100.227	92.750	105.269	117.780	92.035
500	203.117	99.148	91.131	147.071	211.249	159.656	102.990	68.649	247.998	204.581	107.188	67.023	69.673	168.290	107.431	49.299	284.858	113.811
1000	181.163	8.643	58.277	146.181	192.653	175.052	83.086	53.613	306.533	184.850	96.991	0.431	54.887	184.242	100.326	21.766	283.068	95.322
1500	159.826	7.821	0.000	134.307	171.513	190.293	35.713	48.795	354.850	160.304	98.555	0.736	44.904	190.485	58.088	17.878	253.780	80.477
2000	0.235	6.462	0.000	97.933	153.997	198.527	31.165	2.014	379.683	142.527	103.591	0.000	37.927	2.654	0.000	22.861	242.894	0.000
Sheath gas flow																		
6	94.727	99.189	100.882	96.492	98.464	97.230	101.858	93.130	98.945	97.211	93.496	89.845	99.102	93.313	95.132	98.223	99.146	89.304
8	87.652	102.137	105.541	98.424	119.827	98.455	107.666	107.666	105.755	100.537	95.605	106.299	113.335	104.985	99.382	106.514	106.829	87.356
10	103.017	108.622	111.045	110.769	159.768	110.240	104.037	129.625	121.852	123.172	107.843	141.556	124.953	131.519	117.175	98.810	114.118	108.515
12	151.648	116.665	111.902	118.885	202.678	130.240	100.207	137.155	138.250	167.812	116.571	173.290	127.183	160.139	134.966	92.447	122.687	116.161
Sheath gas temperature																		
200	95.942	99.081	99.703	70.954	105.833	97.469	95.869	96.612	100.507	101.143	120.414	88.073	120.283	100.531	121.295	139.814	102.309	100.815
250	87.493	101.315	96.296	59.688	139.238	111.129	76.709	129.829	111.566	123.664	134.045	125.216	136.975	118.719	174.644	151.512	121.178	125.578
300	148.066	103.490	98.574	126.226	197.772	120.463	67.386	163.597	132.135	182.951	125.376	183.232	131.859	174.960	212.356	142.497	128.160	160.534
350	277.049	112.161	104.517	148.135	191.514	100.134	54.721	183.567	145.449	227.008	97.941	226.114	132.347	278.137	156.757	110.204	138.759	188.036
400	438.537	124.968	100.573	100.042	147.156	127.606	48.727	199.838	159.938	194.418	110.008	245.684	136.371	450.922	209.086	70.009	148.064	205.132

Table A3

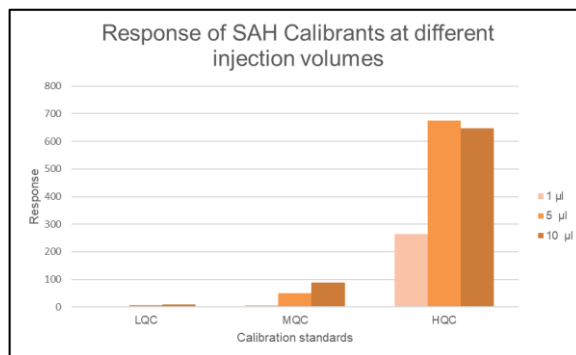
This table includes the average calibration concentration for each parameter analysed, for each analyte with the use of PFHpA in mobile phase A. The data was colour coded for visual comparison. The colours used were red, yellow, and green (red being the lowest and green being the highest).

Metabolites	5-MTHFA	Betaine	Choline	Cystathionine	Cysteine	Cystine	DMG	Glycine	HCA	Homocysteine	Homocystine	Methionine	Pyridoxine	Riboflavin	SAH	SAM	Serine	Taurine
Parameter values	Average Calibration Concentration																	
	Capillary Voltage																	
2000	100,119	99,590	100,534	100,475	97,831	99,893	100,138	99,591	98,386	97,911	100,651	98,917	99,101	100,168	96,652	101,113	99,104	97,438
2500	121,324	98,819	103,004	91,899	100,714	82,830	100,229	103,099	79,557	106,064	102,157	87,485	103,884	112,118	88,796	72,016	98,576	92,409
3000	135,225	92,965	102,728	59,187	102,267	62,548	89,733	108,369	45,162	104,919	104,552	77,399	95,027	118,748	70,089	130,981	99,453	85,165
3500	138,673	87,605	50,003	49,441	100,938	45,857	71,780	111,178	21,138	97,342	107,439	67,757	81,618	119,199	39,860	120,004	98,842	84,314
4000	135,004	86,572	0,544	40,527	96,637	38,934	57,315	116,078	19,796	87,073	105,802	56,218	70,821	119,507	23,863	98,725	96,577	80,662
4500	125,789	78,605	0,398	38,378	88,935	38,559	48,944	114,939	20,273	78,139	108,607	46,743	58,935	117,196	2,193	0,000	90,111	81,267
5000	111,658	71,047	84,825	34,778	82,734	42,467	44,446	108,357	22,562	70,060	0,000	39,371	54,258	125,448	2,006	0,000	81,382	40,842
Parameter values	Drying gas flow																	
4	99,040	97,644	100,304	101,427	98,174	103,978	102,021	98,567	100,773	96,571	100,233	99,624	103,270	98,031	99,614	100,531	98,831	98,525
6	132,834	96,758	106,038	85,769	104,291	105,534	99,914	109,293	98,050	105,038	104,904	99,270	102,840	113,016	77,170	103,285	107,994	97,034
8	190,055	94,833	108,302	82,362	116,526	104,318	95,235	125,689	96,259	128,107	105,147	96,939	101,733	127,565	75,528	90,649	117,576	91,767
10	206,705	87,306	108,363	75,835	131,509	100,664	87,070	145,690	93,656	130,705	105,803	96,926	75,604	137,935	83,103	72,849	122,604	85,417
Parameter values	Drying gas temperature																	
200	99,241	99,343	99,507	56,265	102,825	99,620	99,474	99,225	95,859	99,646	99,928	99,032	101,988	100,082	99,431	98,672	98,691	92,352
230	95,736	97,479	99,546	10,382	102,071	67,609	99,107	88,266	95,123	95,914	95,414	95,877	100,355	91,746	86,682	91,997	94,501	101,727
250	92,831	94,033	99,926	40,928	103,590	63,565	93,835	88,909	92,013	100,284	89,927	92,778	108,212	93,789	79,299	87,016	93,124	97,717
280	102,871	87,823	94,246	60,638	107,737	56,951	90,235	93,138	91,190	108,462	85,516	88,862	95,027	90,809	59,745	76,967	94,787	93,306
300	117,316	83,142	93,675	50,819	115,846	66,987	82,814	95,067	87,802	116,936	84,613	83,766	93,164	92,043	73,642	73,766	94,931	90,635
330	143,278	76,352	91,301	44,174	120,741	61,497	78,464	97,016	83,199	137,234	81,283	77,198	75,087	89,106	68,902	65,313	98,543	82,532
350	157,639	74,534	90,673	5,367	127,793	54,600	72,142	100,143	76,737	148,762	79,314	70,649	76,264	90,136	66,791	61,669	99,898	76,834
Parameter values	Nebulizer voltage																	
20	96,914	98,141	102,234	100,266	100,004	102,303	101,293	102,072	100,409	95,927	99,555	102,703	100,868	100,884	97,519	104,525	99,907	97,409
25	104,078	111,298	118,474	78,828	129,897	92,046	131,352	109,458	87,672	106,894	93,769	112,908	144,175	90,956	85,783	126,858	100,595	106,690
30	102,894	126,131	131,236	52,171	148,593	79,364	162,543	105,219	78,195	112,467	83,577	116,938	191,012	79,416	77,026	155,463	98,753	96,257
35	98,091	139,252	138,171	34,970	158,207	67,157	187,872	107,796	70,915	115,996	74,294	117,602	229,539	67,602	75,136	180,223	98,577	91,361
40	91,335	141,766	140,853	23,580	157,391	60,174	209,764	107,524	72,068	115,332	64,988	117,081	242,228	58,806	76,716	217,261	95,597	93,994
45	86,067	138,362	149,224	21,743	158,273	59,651	241,706	104,554	70,649	113,222	58,019	120,278	317,744	52,454	65,104	246,307	93,136	92,224
50	82,459	139,769	159,846	15,348	157,884	61,400	269,786	102,611	87,750	112,681	53,125	120,214	266,809	47,953	39,092	254,679	89,975	87,632
Parameter values	Nozzle voltage																	
0	98,323	99,223	127,411	137,186	97,791	96,264	99,770	101,547	103,895	99,666	77,424	98,902	101,328	100,227	92,750	105,269	117,780	92,035
500	203,117	99,148	91,131	147,071	211,249	159,656	102,990	68,649	247,998	204,581	107,188	67,023	69,673	168,290	107,431	49,299	284,858	113,811
1000	181,163	8,643	58,277	146,181	192,653	175,052	83,086	53,613	306,533	184,850	96,991	0,431	54,887	184,242	100,326	21,766	283,068	95,322
1500	159,826	7,821	0,000	134,307	171,513	190,293	35,713	48,795	354,850	160,304	98,555	0,736	44,904	190,485	58,088	17,878	253,780	80,477
2000	0,235	6,462	0,000	97,933	153,997	198,527	31,165	2,014	379,683	142,527	103,591	0,000	37,927	2,654	0,000	22,861	242,894	0,000
Parameter values	Sheath gas flow																	
6	94,727	99,189	100,882	96,492	98,464	97,230	101,858	93,130	98,945	97,211	93,496	89,845	99,102	93,313	95,132	98,223	99,146	89,304
8	87,652	102,137	105,541	98,424	119,827	98,455	104,357	107,666	105,755	100,537	95,605	106,299	113,335	104,985	99,382	106,514	106,829	87,356
10	103,017	108,622	111,045	110,769	159,768	110,240	104,037	129,625	121,852	123,172	107,843	141,556	124,953	131,519	117,175	98,810	114,118	108,515
12	151,648	116,665	111,902	118,885	202,678	130,240	100,207	137,155	138,250	167,812	116,571	173,290	127,183	160,139	134,966	92,447	122,687	116,161
Parameter values	Sheath gas temperature																	
200	95,942	99,081	99,703	70,954	105,833	97,469	95,869	96,612	100,507	101,143	120,414	88,073	120,283	100,531	121,295	139,814	102,309	100,815
250	87,493	101,315	96,296	59,688	139,238	111,129	76,709	129,829	111,566	123,664	134,045	125,216	136,975	118,719	174,644	151,512	121,178	125,578
300	148,066	103,490	98,574	126,226	197,772	120,463	67,386	163,597	132,135	182,951	125,376	183,232	131,859	174,960	212,356	142,497	128,160	160,534
350	277,049	112,161	104,517	148,135	191,514	100,134	54,721	183,567	145,449	227,008	97,941	226,114	132,347	278,137	156,757	110,204	138,759	188,036
400	438,537	124,968	100,573	100,042	147,156	127,606	48,727	199,838	159,938	194,418	110,008	245,684	136,371	450,922	209,086	70,009	148,064	205,132

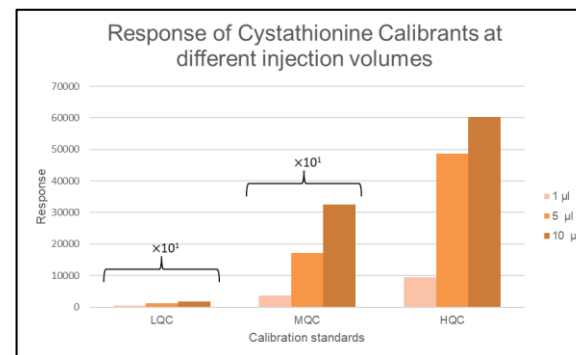
A) Homocystine



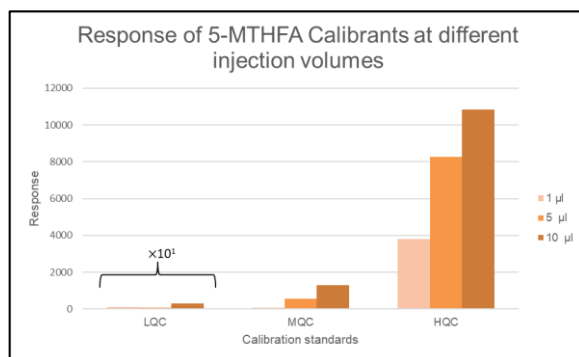
B) SAH



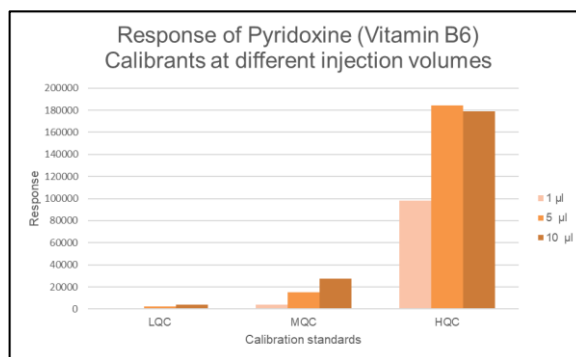
C) Cystathionine



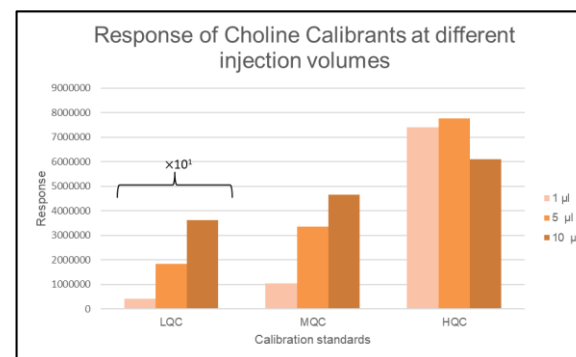
D) 5-MTHFA



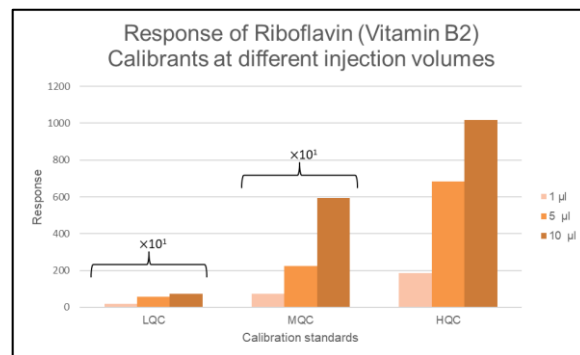
E) Pyridoxine (Vitamin B₆)



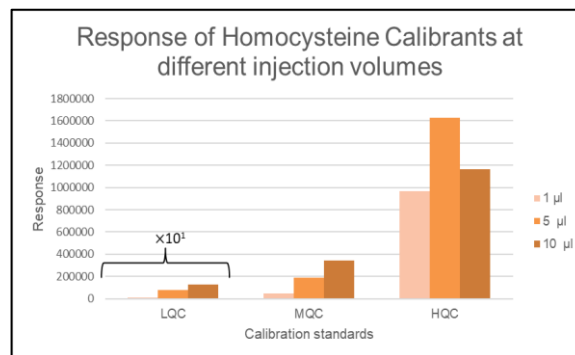
F) Choline



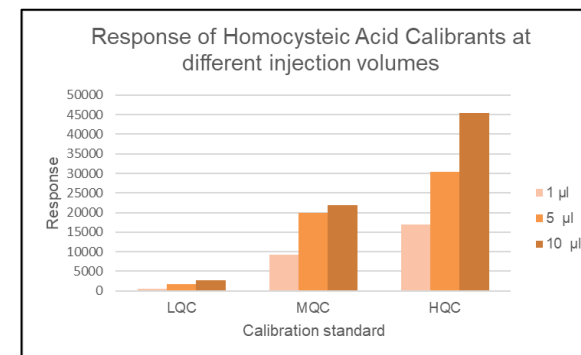
G) Riboflavin (Vitamin B₂)



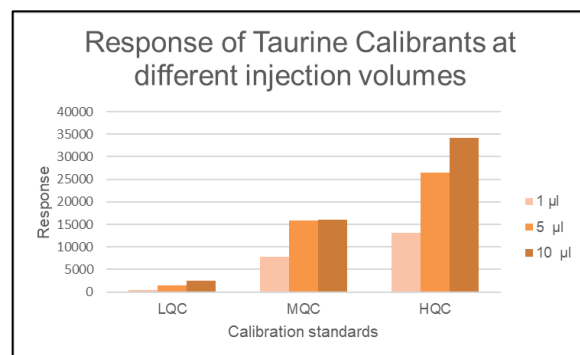
H) Homocysteine



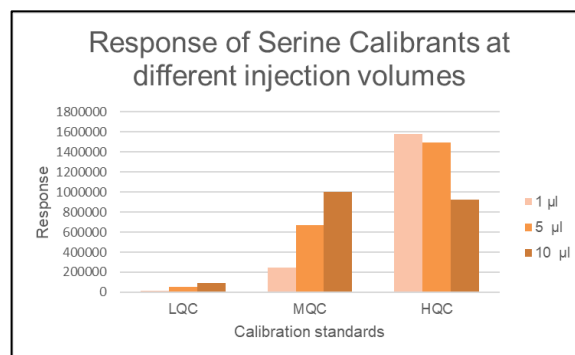
I) Homocysteic Acid



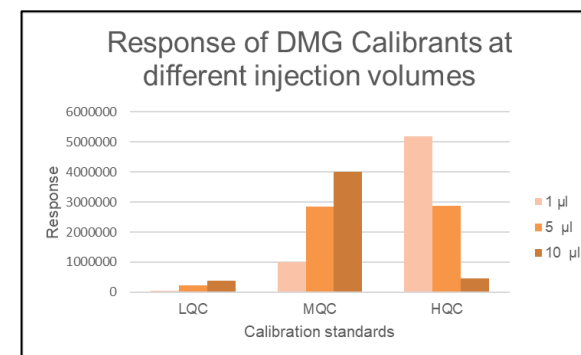
J) Taurine



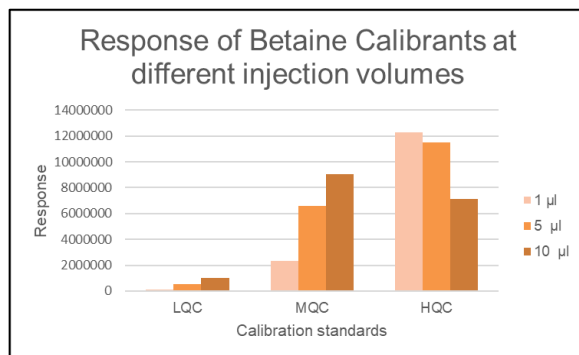
K) Serine



L) DMG



M) Betaine



N) Cysteine

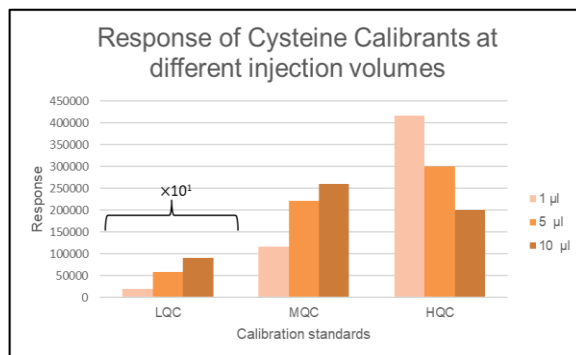
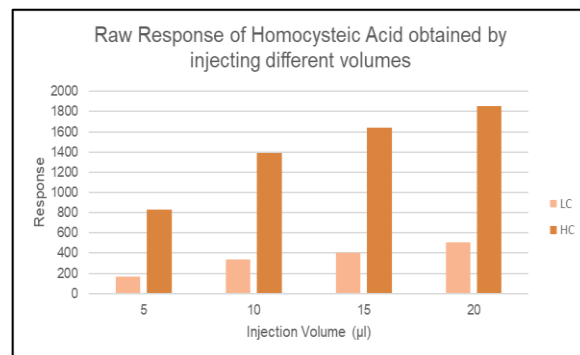
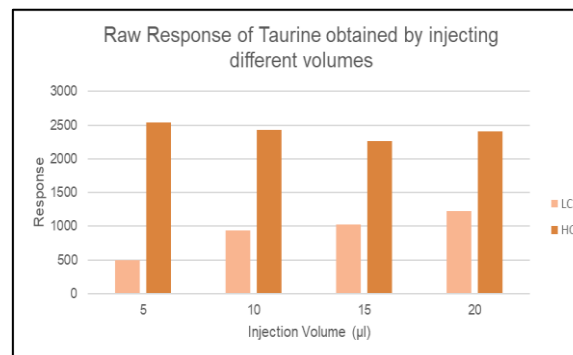


Figure A1 Visual representation of the response obtained after injecting three different volumes of three different concentration levels (LC, MC, and HC) onto the LC-MS/MS system. The responses of some analytes were adjusted to ensure visual illustration of all analytes.

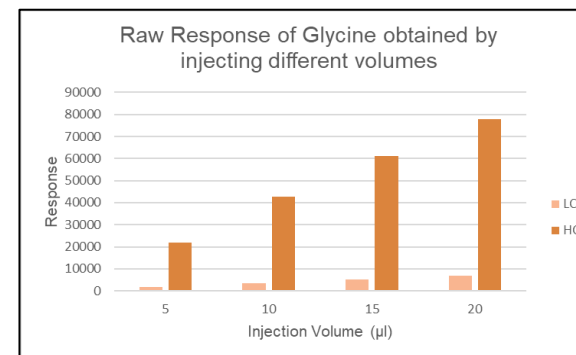
A) Homocysteic acid



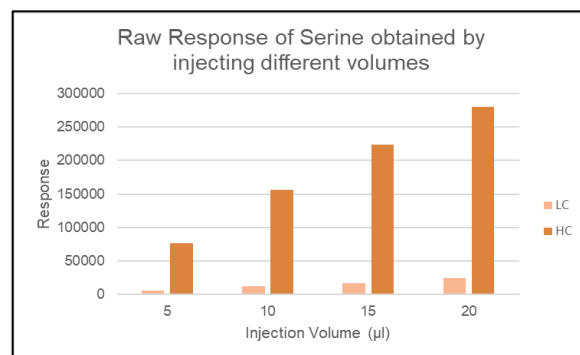
B) Taurine



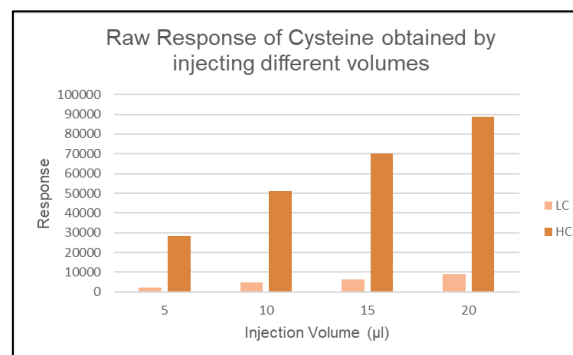
C) Glycine



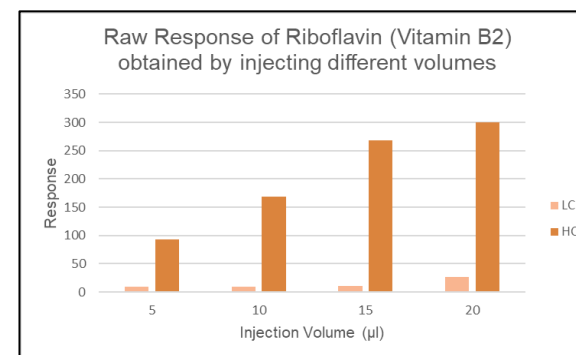
D) Serine



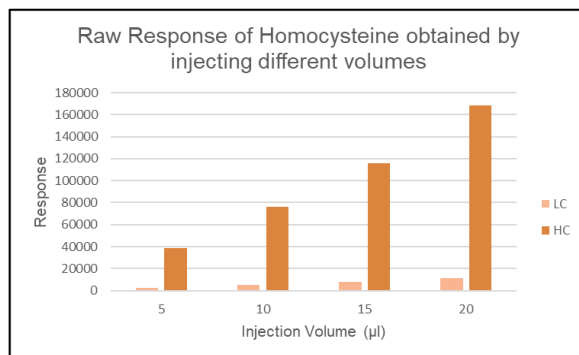
E) Cysteine



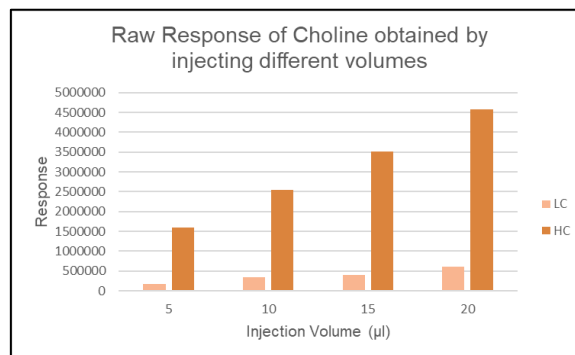
F) Riboflavin (Vitamin B₂)



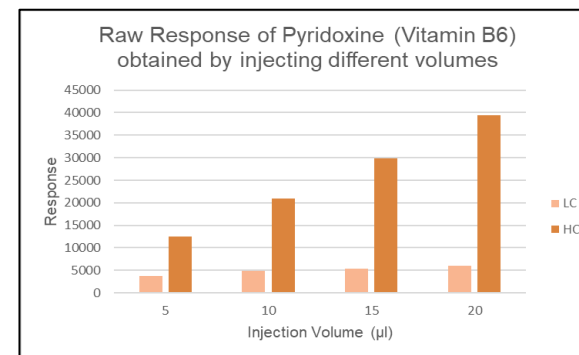
G) Homocysteine



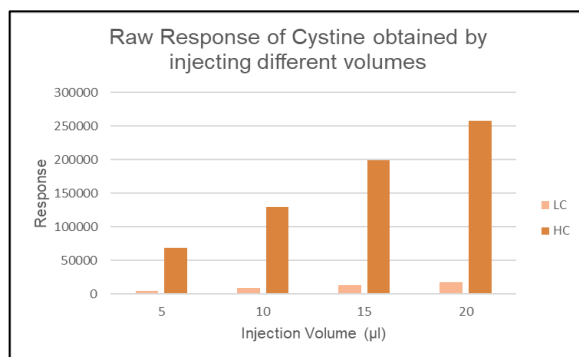
H) Choline



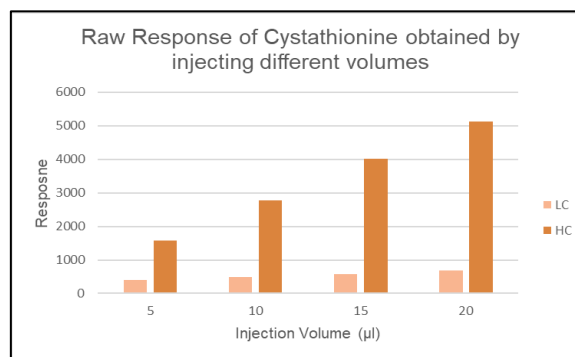
I) Pyridoxine (Vitamin B₆)



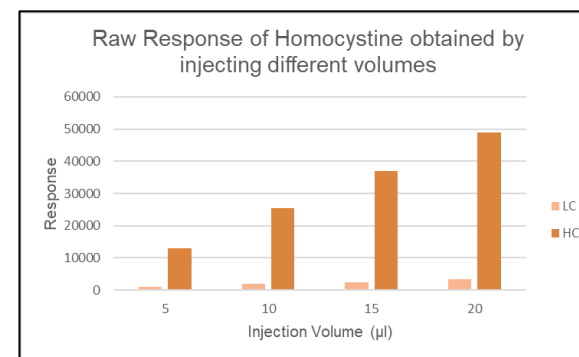
J) Cystine



K) Cystathionine



L) Homocystine



M) SAM

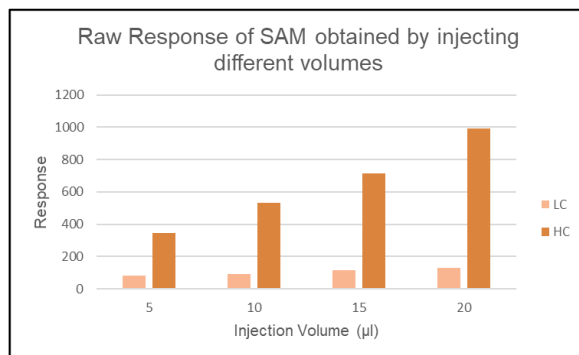


Figure A2 Graphical representation of the response obtained for all the analytes when different volumes were injected.

Table A4 **A summary of the interday precision and accuracy for two days**

Analyte	LLOQ		LC		MC		HC	
	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
L-Homocysteic acid	11.605	256.715	1.330	204.342	0.952	172.056	1.848	137.970
Taurine	5.979	294.340	0.641	242.288	0.775	194.946	1.415	143.461
L-Serine	13.251	176.982	6.286	118.496	5.797	109.918	0.175	105.649
N, N-DMG	0.977	110.312	1.597	105.740	0.591	111.322	3.462	106.108
Betaine	10.247	88.140	0.023	99.138	0.042	106.453	1.226	109.354
Glycine	1.122	137.627	0.953	103.633	0.028	102.458	1.102	99.834
L-Cysteine	3.704	106.610	3.873	104.308	2.129	100.794	4.168	93.767
DL-Homocysteine	10.139	87.438	10.438	87.452	8.880	91.638	7.634	86.414
Riboflavin (Vitamin B₂)	18.393	144.388	34.140	111.343	26.095	133.971	16.674	130.299
Choline	0.188	174.051	9.814	120.389	4.032	117.843	0.105	108.789
L-Methionine	1.862	213.671	2.647	155.199	2.312	134.335	5.895	114.274
Pyridoxine (Vitamin B₆)	8.993	121.221	8.677	111.487	4.711	117.396	3.279	106.893
5-Methyltetrahydro folic acid	5.409	85.513			13.070	83.068	5.834	103.950
L-Cystine	1.815	106.833	4.739	102.290	1.840	106.465	4.303	100.872
Cystathionine	1.868	2046.619	5.410	610.213	3.292	225.737	2.268	167.085
SAH	7.021	140.728			33.272	104.116	18.76596487	110.325
L-Homocystine	1.268	115.501	6.967	97.447	1.733	101.716	2.836	100.627
SAM	12.001	162.043	5.380	133.540	0.691	115.714	0.494	107.524

Table A5 **A summary of the stability results after storage in the autosampler for 24h for each analyte at four different concentrations**

Analyte	24 h Autosampler (% of freshly prepared sample)			
	LLOQ	LC	MC	HC
L-Homocysteic acid	64.834	66.471	59.854	55.805
Taurine	107.188	119.957	123.858	118.138
L-Serine	72.647	83.220	87.835	84.624
N, N-DMG	75.714	81.012	84.244	85.617
Betaine	11.384	57.775	77.629	62.274
Glycine	73.014	92.249	94.152	93.403
L-Cysteine	124.327	111.529	105.916	109.197
DL-Homocysteine	42.049	38.617	39.835	39.279
Riboflavin (Vitamin B₂)	69.079	27.490	57.029	49.945
Choline	141.563	145.417	133.918	130.437
L-Methionine	93.665	78.218	74.484	54.236
Pyridoxine (Vitamin B₆)	91.562	91.664	95.445	96.406
5-Methyltetrahydro folic acid	3.041	4.279	20.747	18.470
L-Cystine	102.789	94.182	92.933	93.027
Cystathionine	20.706	28.234	36.940	45.182
SAH	139.061	70.790	117.097	117.883
L-Homocystine	125.331	108.034	101.198	93.291
SAM	99.611	109.432	100.841	104.295
Average	80.976	78.254	83.553	80.639

Table A6 **A summary of the stability results after storage on the bench for 24h for each analyte at four different concentrations**

Analyte	24 h room temperature (% of freshly prepared sample)			
	LLOQ	LC	MC	HC
L-Homocysteic acid	64.881	67.516	64.991	60.281
Taurine	140.019	147.623	149.604	135.571
L-Serine	81.597	92.542	95.253	93.334
N, N-DMG	79.946	88.804	90.351	93.412
Betaine	12.342	59.040	78.405	62.481
Glycine	98.451	95.348	96.223	97.858
L-Cysteine	98.093	93.060	86.808	96.651
DL-Homocysteine	46.530	43.305	42.254	43.659
Riboflavin (Vitamin B₂)	114.030	48.488	76.870	95.429
Choline	164.184	163.708	142.807	139.948
L-Methionine	99.034	90.437	110.605	75.794
Pyridoxine (Vitamin B₆)	105.622	110.769	117.428	114.444
5-Methyltetrahydro folic acid	4.667	6.545	31.269	26.542
L-Cystine	104.507	94.022	94.682	96.853
Cystathionine	29.598	39.403	49.404	56.242
SAH	203.094	74.660	139.657	132.130
L-Homocystine	114.564	102.777	97.811	103.502
SAM	79.457	94.615	94.973	103.858
Average	91.145	84.037	92.189	90.444

Table A7 **A summary of the stability results after storage at 4°C for 48h for each analyte at four different concentrations**

Analyte	48 h 4°C (% of freshly prepared sample)			
	LLOQ	LC	MC	HC
L-Homocysteic acid	98.065	97.164	94.319	92.200
Taurine	140.277	137.457	148.211	143.537
L-Serine	97.230	106.037	107.014	106.399
N, N-DMG	95.265	112.395	112.554	114.447
Betaine	14.615	64.117	78.734	57.821
Glycine	92.992	95.978	96.833	96.461
L-Cysteine	103.137	95.866	95.646	96.436
DL-Homocysteine	84.380	78.291	74.502	74.637
Riboflavin (Vitamin B₂)	98.337	37.160	89.584	75.919
Choline	140.933	136.800	135.292	126.880
L-Methionine	102.857	101.491	99.585	99.968
Pyridoxine (Vitamin B₆)	102.248	95.342	105.362	105.721
5-Methyltetrahydro folic acid	14.662	21.134	99.044	85.154
L-Cystine	99.492	97.433	96.338	97.426
Cystathionine	62.746	68.569	78.739	81.504
SAH	134.730	66.406	104.582	98.431
L-Homocystine	113.849	102.226	97.431	97.119
SAM	81.829	107.750	108.560	110.641
Average	93.202	90.090	101.241	97.817

Table A8 **A summary of the stability results after storage at -20°C for 1 week for each analyte at four different concentrations**

Analyte	1 week h -20°C (% of freshly prepared sample)			
	LLOQ	LC	MC	HC
L-Homocysteic acid	68.191	76.734	72.849	63.790
Taurine	102.459	117.958	111.701	104.830
L-Serine	80.551	84.072	83.923	82.617
N, N-DMG	100.541	103.482	100.319	99.817
Betaine	126.364	161.400	165.029	161.556
Glycine	94.594	94.245	96.183	97.573
L-Cysteine	28.786	55.726	97.423	171.249
DL-Homocysteine	40.980	44.359	47.830	40.409
Riboflavin (Vitamin B₂)	49.499	41.886	97.682	78.476
Choline	186.842	188.766	168.262	163.093
L-Methionine	98.037	96.541	97.842	97.115
Pyridoxine (Vitamin B₆)	675.846	390.667	204.839	190.623
5-Methyltetrahydro folic acid	12.566	3.713	4.407	22.618
L-Cystine	115.217	111.258	104.420	101.209
Cystathionine	17.966	29.298	46.433	59.525
SAH	84.666	41.813	120.776	204.852
L-Homocystine	90.651	101.978	100.888	100.858
SAM	105.478	128.196	135.020	141.908
Average	115.513	104.005	103.101	110.118

ANNEXURE B



Private Bag X1290, Potchefstroom
South Africa 2520
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Web: <http://www.nwu.ac.za/>

North-West University Health Research Ethics Committee (NWU-HREC)

Tel: 018 299-1206
Email: Ethics-HRECApply@nwu.ac.za (for human studies)

21 May 2021

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Health Research Ethics Committee (NWU-HREC) on 21/05/2021, the NWU-HREC hereby approves your study as indicated below. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: An analytical method to investigate the metabolites and co-factors of the homocysteine methionine cycle in women using combined oral contraceptives and non-users (eBOSS-Methylation study)														
Principal Investigator/Study Supervisor/Researcher: Dr E Erasmus														
Student: T Jacobs - 27229084														
Ethics number:														
N	W	U	-	0	0	1	6	1	-	2	1	-	A	1
Institution			Study Number				Year			Status				
Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation														
Application Type: Single study														
Commencement date: 21/05/2021														
Expiry date: 31/05/2022														
Risk: Minimal														
Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of May annually until completion.														

General conditions: While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply: - The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-HREC: - Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and - without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study. - The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited. - Annually a number of studies may be randomly selected for active monitoring. - The date of approval indicates the first date that the study may be started. - In the interest of ethical responsibility, the NWU-HREC reserves the right to: - request access to any information or data at any time during the course or after completion of the study; - to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;

- *withdraw or postpone approval if:*
 - *any unethical principles or practices of the study are revealed or suspected;*
 - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
 - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
 - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information via Ethics-HRECApply@nwu.ac.za or 018 299 1206*

Special conditions of the research approval due to the COVID-19 pandemic:

Please note: Due to the nature of the study i.e. (laboratory work involving the metabolic analysis of previously collected biological samples), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

The NWU-HREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-HREC for any further enquiries or requests for assistance.

Yours sincerely,



Chairperson NWU-HREC

Current details: (23290522) G:\My Drive\0. Research and Postgraduate Education\0.1.5.4 Templates\0.1.5.4.2_NWU-HREC_EAL.docx
20 August 2019
File Reference: 0.1.5.4.2