Urinary 1,4-dihydroxynonene mercapturic acid (DHN-MA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) as markers of oxidative damage: The SABPA study

By

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Equipped with his five senses, man explores the universe around him and calls the adventure Science

~ Edwin Powell Hubble, The Nature of Science, 1954

This dissertation is dedicated to my son, Gunther,

who is expected in November 2010.

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ABSTRACT

The human body has evolved certain defence mechanisms to cope with the high occurrence of free radicals. These radicals are obtained endogenously from the mitochondria, peroxisomes, the cytochrome P450 (CYP 450) system and neutrophils, or exogenously from the environment. Lack of antioxidants and/or increased production of free radicals will result in oxidative stress, which has been implicated in certain human diseases such as hypertension, inflammation, ageing, autoimmunity, atherosclerosis, Parkinson's disease, cancer and diabetes.

Although the initial aim was to standardise a single assay to quantify both 8-OHdG and DHN-MA, this could not be achieved in this study due to the vast difference in the chemical properties of these two metabolites. Following the decision to use two separate assays for the quantification of the mentioned biomarkers, the 8-OHdG assay was standardised and validated. The intrabatch variation of the assay was 4.18% and the interbatch variation was 17.37%. Unfortunately, the DHN-MA assay could not be standardised within the time frame of this study due to experimental difficulties. Therefore, only urinary 8-OHdG and serum ROS levels were quantified.

Urinary 8-OHdG levels were measured in 409 participants (209 Caucasians, 101 males and 108 females and 200 Africans, 100 males and 100 females) from the SABPA study. After removal of outliers from the data matrix, the effect of gender and ethnicity was investigated on the measured urinary 8-OHdG levels. No significant difference in the urinary 8-OHdG levels between Caucasian males (n=87) and females (n=96) were observed ($p = 0.68$). A similar observation was made for the African males (n=86) and females (n=84), where no significant difference in 8-OHdG levels was detected ($p = 0.053$). Thus, from the results obtained in this study, it seems that urinary 8-OHdG levels are not influenced by gender. However, 8-OHdG levels were dramatically influenced by ethnicity. Caucasian males (n=87) excreted 70% higher amounts of 8-OHdG compared to African males (n=86) ($p < 0.001$). Caucasian females (n=96) also excreted larger urinary 8-OHdG amounts (42%) compared to African females (n=84) ($p < 0.001$). Therefore, it seems that urinary 8-OHdG levels are dramatically influenced by ethnicity. Finally, urinary 8-OHdG levels were compared to serum ROS levels, but no significant correlation between the measured metabolites was observed ($r = -0.045$). Hence, urinary 8-OHdG and serum ROS levels are not related in these subjects.

Even though the initial aim of this study was to standardise an analytical method to quantify both urinary 8-OHdG and DHN-MA, this could not be achieved due to time constraints.

However, an LC-MS/MS analytical assay was standardised and validated for the quantification of urinary 8-OHdG. The method proved reliable for the quantification of 8-OHdG from urine samples and can thus be used for further studies on oxidative DNA damage.

OPSOMMING

Die menslike liggaam beskik oor sekere verdedigings meganismes om die hoë voorkoms van vrye radikale te neutraliseer. Die vrye radikale kom endogeen voor vanuit die mitokondria, peroksisome, die sitochroom P450 sisteem (CYP450) en neutrofiele of eksogeen van die omgewing. ’n Tekort aan antioksidante en/of ’n verhoogde produksie van vrye radikale sal oksidatiewe stres tot gevolg hê, wat al geimpliseer is in veroudering en verskeie siekte-toestande soos, hipertensie, inflammasie, veroudering, outo-immuun-siektes, arterosklerose, Parkinson se siekte, kanker en diabetes.

Alhoewel die aanvanklike doel van hierdie studie was om een metode te standardiseer om beide 8-OHdG en DHN-MA te kwantifiseer, kon dit nie in die studie bereik word nie a.g.v. die groot verskil in die chemiese eienskappe van die twee metaboliete. Na besluitneming om die kwantifisering van die twee metaboliete te skei, is die 8-OHdG analise gestandardiseer en gevalideer. Die intra-groep variasie van die analise was 4.18% en die inter-groep variasie was 17.37%. Weens verskeie struikelblokke kon die DHN-MA analise nie gestandardiseer word binne die tydraamwerk van die studie nie. Daarom is net die urinêre 8-OHdG en serum ROS vlakke gekwantifiseer.

Urinêre 8-OHdG vlakke is in 409 deelnemers van die SABPA studie bepaal (209 Kaukasieërs, 101 manlik en 108 vroulik en 200 Afrikane, 100 manlik en 100 vroulik). Na die verwydering van uitskieters van die data matriks is die effek van geslag en etnisiteit op die gemete urinêre 8-OHdG vlakke ondersoek. Geen betekenisvolle verskil is waargeneem in die urinêre 8-OHdG vlakke in Kaukasieër-mans (n=87) en -vrouens (n=96) nie ($p = 0.68$). ’n Soortgelyke resultaat was waargeneem in Afrikaan-mans (n=86) en -vrouens (n=84), waar geen betekenisvolle verskil in 8-OHdG vlakke gevind was nie ($p = 0.053$). Die resultate van die studie toon dus dat urinêre 8-OHdG vlakke nie deur geslag beïnvloed word nie. Vervolgens is die effek van etnisiteit op urinêre 8-OHdG vlakke bestudeer. By Kaukasieër-mans (n=87) is 70% hoër 8-OHdG vlakke gemeet in vergelyking met Afrikaan-mans (n=86) ($p < 0.001$). Kaukasieër-vrouens (n=96) het ook meer 8-OHdG uitgeskei (42%) as Afrikaan-vrouens (n=84) ($p < 0.001$). Dit blyk dus dat urinêre 8-OHdG vlakke dramaties beïnvloed word deur etnisiteit. Laastens is bevind dat urinêre 8-OHdG vlakke geen korrelasie toon met serum ROS vlakke nie ($r = -0.045$). Gevolglik is geen verwantskap in hierdie studiegroep gevind tussen urinêre 8-OHdG en serum ROS vlakke nie.

Die oorspronklike doel van die studie was om een analitiese metode te standardiseer vir die kwantifisering van beide 8-OHdG en DHN-MA. Hierdie doelstelling kon egter nie bereik word nie

weens tydbeperkinge op die studie. ’n LC-MS/MS analise is gestandardiseer en gevalideer vir die kwantifisering van urinêre 8-OHdG vlakke. Die metode is betroubaar vir die kwantifisering van 8-OHdG vlakke in uriene en kan dus gebruik word vir verdere studies op oksidatiewe DNS-skade.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

°C	Degrees Celsius
%	Percentage
>	Greater than
<	Less than
®	Registered
™	Trademark
±	Plus minus
β	Beta
ω	Omega

Abbreviations

2’dG	2’-deoxyguanosine
2’dG^{15}N	2’-deoxyguanosine-N^{15}
4-HNE	trans-4-hydroxy-2-nonenal
4-HNE-MA	trans-4-hydroxy-2-nonenal mercapturic acid
4-HNE-MA-d3	trans-4-hydroxy-2-nonenal mercapturic acid-d3
8-OHAde	8-hydroxy-adenine
8-OHdA	8-hydroxy-2’-deoxyadenosine
8-OHGua	8-hydroxy-guanine
8-OHdG	8-hydroxy-2’-deoxyguanosine

A

ADP	Adenosine diphosphate
AKR	Aldo-keto reductase
Aq	Aqua
ATP	Adenosine Triphosphate

B

BER	Base excision repair

BDM	Brunner-Dette-Munk
BHT	Butylated Hydroxytoluene

C

CA	California
Cat. No.	Catalogue number
CE-ECD	Capillary electrophoresis-electrochemical detection
CE-MS	Capillary electrophoresis-mass spectrometery
CO_2	Carbon dioxide
CoA	Coenzyme A
cm	Centimeter
CuZn-SOD	Copper-Zinc superoxide dismutase
CYP 450	Cytochrome P450 system

D

DEPPD	N,N-diethyl-*para*-phenylenediamine
dGTP	Deoxyguanosine triphosphate
DHN	1,4-dihydroxynonene
DHN-MA	1,4-dihydroxynonene mercapturic acid
DHN-MA-d3	1,4-dihydroxynonene mercapturic acid-d3
DNA	Deoxyribonucleic acid

E

e.g.	For example
et al.	And others
etc.	et cetera
ETC	Electron transport chain
ELISA	Enzyme-linked immunosorbent assay
ECSOD	Extracellular superoxide dismutase

G

g	g force (9.80665 m/s^2)
g	gram

GCL	Glutamate cysteine ligase
GC-MS	Gas chromatography-mass spectrometry
GMP	Guanosine monophosphate
GSH	Glutathione
GSSG	Oxidised glutathione
GST	Glutathione-S-transferase

H

H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPLC-ECD	High-performance liquid chromatography-electrochemical detection

I

i.e.	that is

L

l	Liter
LC-MS/MS	Liquid chromatography- tandem mass spectrometry

M

µl	microliter
µM	micromolar
ml	milliliter
mg/ml	milligram/ milliliter
ml/min	milliliter/ minute
min	minutes
mM	Millimolar
mm Hg	millimeters of mercury
M	Molar
m/z	mass-to-charge-ratio
MDA	Malondialdehyde
MeCN	Acetonitrile

MeOH	Methanol
MnSOD	Mitochondrial-manganese superoxide dismutase
MRM	Multiple reaction monitoring
MS	Mass spectrometer
MS/MS	Tandem mass spectrometer

N

Nm	nanometer
nmol/g	nanomol/ gram
NER	Nucleotide excision repair
-NH_2	Amino group

O

O_2	Oxygen
$O_2^{\cdot -}$	Superoxide anion
$^1O_2$	Singlet oxygen
$OH^{\cdot}$	Hydroxyl radical
Ox-LDL	Oxidised low-density lipoprotein
OGG1	DNA glycosylase/lyase

P

PUFA	Polyunsaturated fatty acid

R

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSA	Republic of South Africa
RSD	Relative Standard Deviation

S

SABPA	Sympathetic Activity and Ambulatory Blood pressure in Africans
SB	Stable bond
SD	Standard deviation

SOD	Superoxide dismutase
SPE	Solid phase extraction
SST	Serum separator tube

T

TCA	Tricarboxylic acid cycle
Tg	Thymine glycol
TIC	Total ion chromatogram

U

USA	United States of America
UV	Ultraviolet

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CHAPTER 1

Introduction

Free radicals are very reactive and can react with and eventually damage other molecules in the human body. These radicals can originate from mitochondria, peroxisomes, the cytochrome P450 (CYP) system and neutrophils, or exogenously. The human body has specific mechanisms to counteract and defend itself against these free radicals. However, should the free radical levels become too elevated and exceed the defence capacity of the body, it leads to oxidative stress. Numerous data implicate increased formation of free radicals *in vivo* in disease development and progression such as hypertension, inflammation, ageing, autoimmunity, atherosclerosis, Parkinson's disease, cancer and diabetes. Therefore, the measurement of oxidative stress status could prove to be beneficial in studying the etiology of these diseases. One of the greatest needs in the field of free radical biology remains the development of reliable methods for measuring the oxidative stress status in humans. However, the quantification of free radicals proves troublesome as these molecules are very reactive. Therefore, the use of certain biomarkers of oxidative damage could give more reliable results as they are not as reactive as the free radicals themselves. Unfortunately no consensus exists regarding which biomarkers are the best to use.

In the Centre for Human Metabonomics at the North-West University, a need exists for the development of assays to successfully quantify biomarkers of oxidative damage to assess oxidative stress status in humans. Although the literature is not conclusive on the best biomarkers for DNA damage and lipid peroxidation, it was decided to use 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 1,4-dihydroxynonene-mercapturic acid (DHN-MA) as biomarkers of DNA damage and lipid peroxidation, respectively in this study. Therefore, the aim of this study was to develop a reliable assay to quantify biomarkers of oxidative damage (8-OHdG and DHN-MA) and to investigate the possible influence of gender and ethnicity on urinary 8-OHdG levels.

Chapter 2 contains a literature overview on ROS, the origin of ROS, damage caused by ROS, biomarkers of oxidative damage, as well as different ways of quantifying the relevant markers. The aim and objectives of this study are given at the end of Chapter 2, as well as the experimental approach. The participants used in this study (409 teachers from the Potchefstroom area in South Africa), sample collection and ethics approval are given in Chapter 3, as well as the ROS assay and the statistical analysis used. In Chapters 4 and 5, the

optimisation and validation of the 8-OHdG and DHN-MA assays respectively are given and discussed. The quantification of ROS and oxidative damage biomarkers are described and discussed in Chapter 6 before the concluding remarks and observations are given in Chapter 7. Raw data are presented in Appendix A.

CHAPTER 2

Literature review

2.1. Free radicals

Chemical species which possess one or more unpaired electrons are known as free radicals (Aruoma, 1998). These molecules are unstable and can react with, and consequently fragment, other molecules (Singh *et al*., 2009.) The term, reactive oxygen species (ROS) is used to describe free radicals such as superoxide anions ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH$), singlet oxygen ($^1O_2$) as well as some non-radicals like hydrogen peroxide (H_2O_2) (Wiseman & Halliwell, 1996; Aruoma, 1998). Since $O_2^{\cdot -}$ could react with nitric oxide ($NO^{\cdot}$) to produce peroxynitrite, a powerful oxidant, the oxidants derived from $NO^{\cdot}$ are termed reactive nitrogen species (RNS) (Turrens, 2003).

2.1.1. Sources of ROS

Reactive oxygen species in the body may originate from exogenous as well as endogenous sources. Endogenously, ROS are produced in the mitochondria, peroxisomes, the cytochrome P450 (CYP 450) system and neutrophils or could originate from exogenous sources such as UV radiation, drugs, etc. (Karithala & Soini, 2007). Irrespective of their origin, high ROS levels could have detrimental consequences for the cell (Cooke *et al*., 2003) if it is not sequestered by defence mechanisms.

2.1.1.1. Mitochondria

Mitochondria are better known as the power-generating units of the cell (Johannsen & Ravussin, 2009). They are plentiful where energy-requiring processes take place, such as cardiac muscle, and provide most of the energy required for cellular processes (Johannsen & Ravussin, 2009).

Respiration can be divided into three main pathways: glycolysis, the tricarboxylic acid cycle (TCA cycle) and the electron transport chain (ETC). During glycolysis, which occurs in the cytoplasm, glucose is catabolised to yield two molecules of pyruvic acid and two NADH molecules with a net gain of two ATP molecules. In the mitochondria, the enzyme system of the TCA cycle functions to break down acetyl Coenzyme A (CoA), derived from pyruvate (produced by glycolysis in the cytoplasm), fatty acids and amino acids. During this process, CO_2 is produced and NAD^+ and FAD^{2+} are reduced to form the electron donors NADH and FADH respectively (Duchen, 2004).

The enzyme pathway (electron transport chain) responsible for ATP generation through oxidative phosphorylation, consists of complexes I through V (Hatefi, 1985). The ETC consists of NADH-quinine oxidoreductase (Complex I), succinate dehydrogenase (Complex II), cytochrome bc_1 complex (Complex III), cytochrome c oxidase (Complex IV) and ATP synthase (Complex V) (Vedel *et al.,* 1999). The electron donors (NADH and $FADH_2$), produced during glycolysis and the TCA cycle, transfer their electrons to complex I and II of the ETC, respectively (Duchen, 2004). As the electrons pass through the ETC to molecular oxygen, the terminal electron acceptor (Kowaltowski *et al.,* 2009), protons are pumped from the mitochondrial matrix to the intermembrane space, establishing a proton gradient (Johannsen & Ravussin, 2009). This proton gradient generates a proton motive force (Bratic & Trifunovic, 2010). When protons diffuse back along this gradient they drive the synthesis of ATP through ATP synthase (Complex V) by the phosphorylation of ADP (Johannsen & Ravussin, 2009).

Approximately 90% of the total ROS produced in the cell, originates from the mitochondria (Bratic & Trifunovic, 2010) and is formed due to an electron "leak" that occurs mainly from complex I and III (St. Pierre *et al.,* 2002). This is because approximately 90% of the oxygen consumed by humans is used by the mitochondria (Nohl *et al.,* 2005). Should the ETC become saturated with electrons, the accumulated electrons from complex I and III could pass directly to O_2 to generate superoxide ($O_2^{\cdot -}$) (Turrens, 2003). Due to its reactivity, $O_2^{\cdot -}$ is transformed into H_2O_2 (Kowaltowski *et al.,* 2009) either through spontaneous dismutation or by superoxide dismutase, such as mitochondrial manganese SOD (Mn-SOD) as well as copper-zinc SOD (CuZn-SOD) (Weisiger & Fridovich, 1973; Nohl *et al.,* 2005). $O_2^{\cdot -}$ is also the primary ROS produced in the mitochondria (Lenaz, 1998; Bartosz, 2009). Should the resultant H_2O_2 not be metabolised by the mitochondrial antioxidant system, it could lead to the formation of hydroxyl radicals ($OH^{\cdot}$) which are highly reactive radicals (Cooke *et al*., 2003; Kowaltowski *et al.,* 2009). It is estimated that approximately 0.2 % of all the oxygen consumed by humans will eventually result in the formation of ROS (St-Pierre *et al.,* 2002).

2.1.1.2. Detoxification

Humans have become progressively more exposed to toxic compounds in the air, water and food, and people's ability to cope with these toxins, either obtained exogenously or endogenously, are of great importance to their health (Liska *et al.*, 2006). Detoxification enzymes in general, function sufficiently to minimise cellular damage (lipid peroxidation, DNA- and protein damage), however, dysfunction may occur should the system be overloaded or imbalanced (Liska *et al.*, 2006). Detoxification, also known as biotransformation, converts non-

polar xenobiotics into a more polar substance for excretion and it is mainly divided into two phases, Phase I and Phase II detoxification (Vander *et al.,* 1994).

Phase I detoxification is the first step in the elimination of non-polar xenobiotics, and depends on antioxidant support to be effective (Liska *et al.,* 2006). Here, the cytochrome P450 (CYP 450) family of enzymes is the first line of defence against xenobiotics (Liska, 1998) (Figure 2.1). In Phase I, a functional group (a hydroxyl-, carboxyl- or amino group) is exposed on the xenobiotic either through oxidation, hydrolation or reduction, which then needs to be further transformed by Phase II (Percival, 1997; Zamek-Gliszczynski *et al*., 2006) (Figure 2.1). This is necessary since the by-products of Phase I can sometimes be more toxic than the original substance (Percival, 1997; Liska, 1998). Many xenobiotics undergo Phase I oxidation before conjugation during Phase II (Crayford & Hutson, 1980). However, certain xenobiotics may undergo Phase II conjugation directly (Gram & Gilette, 1971).

Each CYP 450 reaction also leads to the formation of ROS, such as superoxide, peroxide or hydroxyl radicals (Liska *et al.,* 2006). Thus, an increase in toxin exposure increases CYP 450 activity which in turn increases ROS production and ultimately oxidative stress (Percival, 1997). Should the detoxification system be burdened or not functioning properly, intermediary metabolites may not be eliminated successfully. When these reactive oxygen intermediates accumulate, it may also contribute to oxidative stress (Percival, 1997).

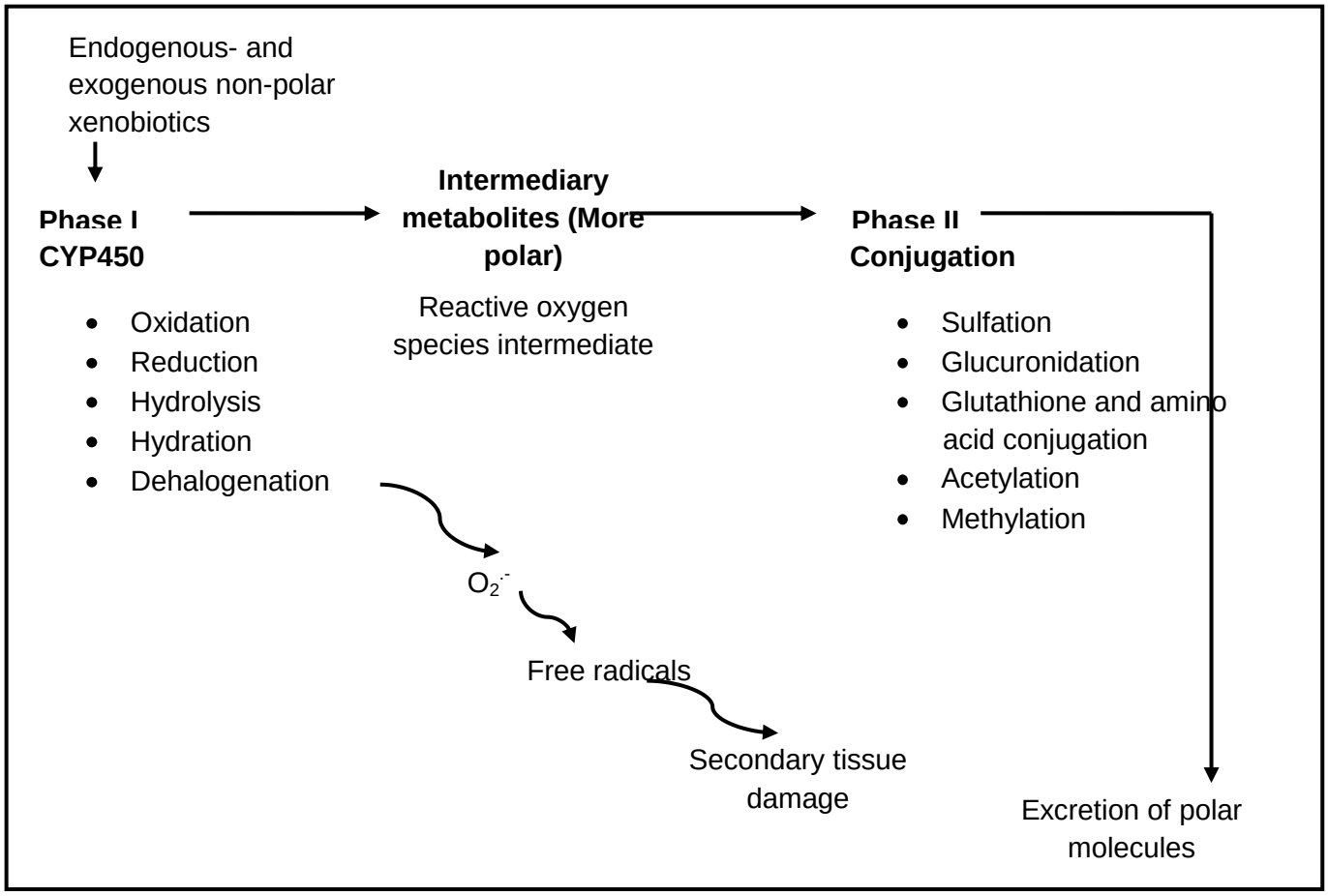

Figure 2.1: Phase I and II detoxification in the liver. Phase I is the first line of defence in the biotransformation process. The CYP 450 enzyme system exposes a functional group on the xenobiotic through one of CYP 450s' reactions. Phase II functions to conjugate a protecting agent onto the intermediary metabolites from Phase I to further increase the polar nature which is needed to eliminate the xenobiotic. Free radicals are produced during detoxification through Phases I and II (Adapted from Liska, 1998).

Phase II is better known as the conjugation pathway and depends on specific nutritional support to be fully functional (Liska *et al*., 2006). It functions by decreasing the activity and toxicity of a xenobiotic from Phase I (Liska *et al*., 2006). Here, xenobiotics from Phase I are further transformed by conjugating to a protecting agent, which makes the xenobiotic more polar and thus ready for excretion (Figure 2.1). The reactions for Phase II are sulfation-, glucuronidation-, acetylation-, methylation-, as well as glutathione- (GSH) and amino acid conjugation (Liska *et al.,* 2006) (Figure 2.1). The most prevalent of the conjugation reactions are sulfation-, glucuronide- and glutathione conjugation (Zamek-Gliszczynski *et al*., 2006). Although sulfation and glucuronide both conjugate with many of the same xenobiotics, glucuronide conjugation is most common at high concentrations when sulfation is inundated (due to co-substrate depletion or enzyme saturation) (Zamek-Gliszczynski *et al*., 2006). GSH conjugation is a vital Phase II conjugation reaction and its substrates include parent compound electrophiles, electrophilic

Phase I metabolites and some Phase II conjugates (Zamek-Gliszczynski *et al*., 2006). Both Phases I and II are imperative to detoxification, evidence suggests that induced Phase I and/or decreased Phase II reactions increase the risk of cancer and Parkinson's disease (LeCouteur *et al.,* 2002; Norrpa, 2004). Thus, it is important for these two phases of detoxification to work together, to successfully complete the detoxification process (Liska *et al.,* 2006).

2.1.1.3. Peroxisomes and neutrophils

Peroxisomes are present in all eukaryotic cells except erythrocytes (Fidaleo, 2009). Peroxisomes are involved in β-oxidation of very long chain fatty acids, prostaglandins and leukotrienes (Ferdinandusse *et al.,* 2002) as well as the biosynthesis of cholesterol, bile acids, dolichol, and ether lipids (Van den Bosch *et al.,* 1992). They also function to oxidize polyamines, uric acid and amino acids (Subramani *et al.,* 2000) and are involved in the detoxification of xenobiotics (Schrader & Fahimi, 2006). The respiratory pathway in peroxisomes reduce O_2 to H_2O_2, thus, peroxisomes are involved in the production of ROS and also in the scavenging of ROS through catalase (Fidaleo, 2009).

Neutrophils on the other hand are phagocytic and important for defence against pathogens. During their defence, neutrophils will produce substances such as lysozyme, peroxidases as well as ROS during an oxidative burst to destroy cells infected with viruses or bacteria (Rosen *et al*., 1995; Cooke *et al*., 2003), thereby contributing to the pool of ROS in the body.

2.1.2. Oxidative stress and free radical defence

Oxidative stress may be defined as an increased production of free radicals and a decreased antioxidant defence system (Blumberg, 2004). When there is a high ROS load present and defence mechanisms are overwhelmed, these accumulated radicals might damage macromolecules such as DNA, lipids and proteins (Halliwell, 1991; Bartosz, 2009).

Although ROS are normally produced during aerobic respiration, as stated earlier, defence mechanisms are usually in place to neutralise and keep ROS levels in balance (Renner *et al*., 2000). Enzymatic, non-enzymatic, endogenous and exogenous antioxidants are the defence mechanisms present to counteract increased ROS levels (Karithala & Soini, 2007).

The superoxide dismutase (SOD) class of enzymes are divided into three types, namely copper-zinc SOD (CuZnSOD) which is present in the cytoplasm, mitochondrial manganese SOD (MnSOD) found in the mitochondria and extracellular SOD (ECSOD) (Karithala & Soini, 2007; Bartosz, 2009). All of these SODs are capable of converting $O_2^{\cdot-}$ to H_2O_2 before catalase reduces hydrogen peroxide to water. Glutathione peroxidase reduces the formed hydrogen

peroxide, using glutathione (GSH) as substrate. Glutathione reductase reduces the formed GSSG again to form GSH (Karithala & Soini, 2007). In the presence of Fe^{2+}, the formed H_2O_2 produce $OH^{\cdot}$ (Singal *et al*., 1988) (Figure 2.2).

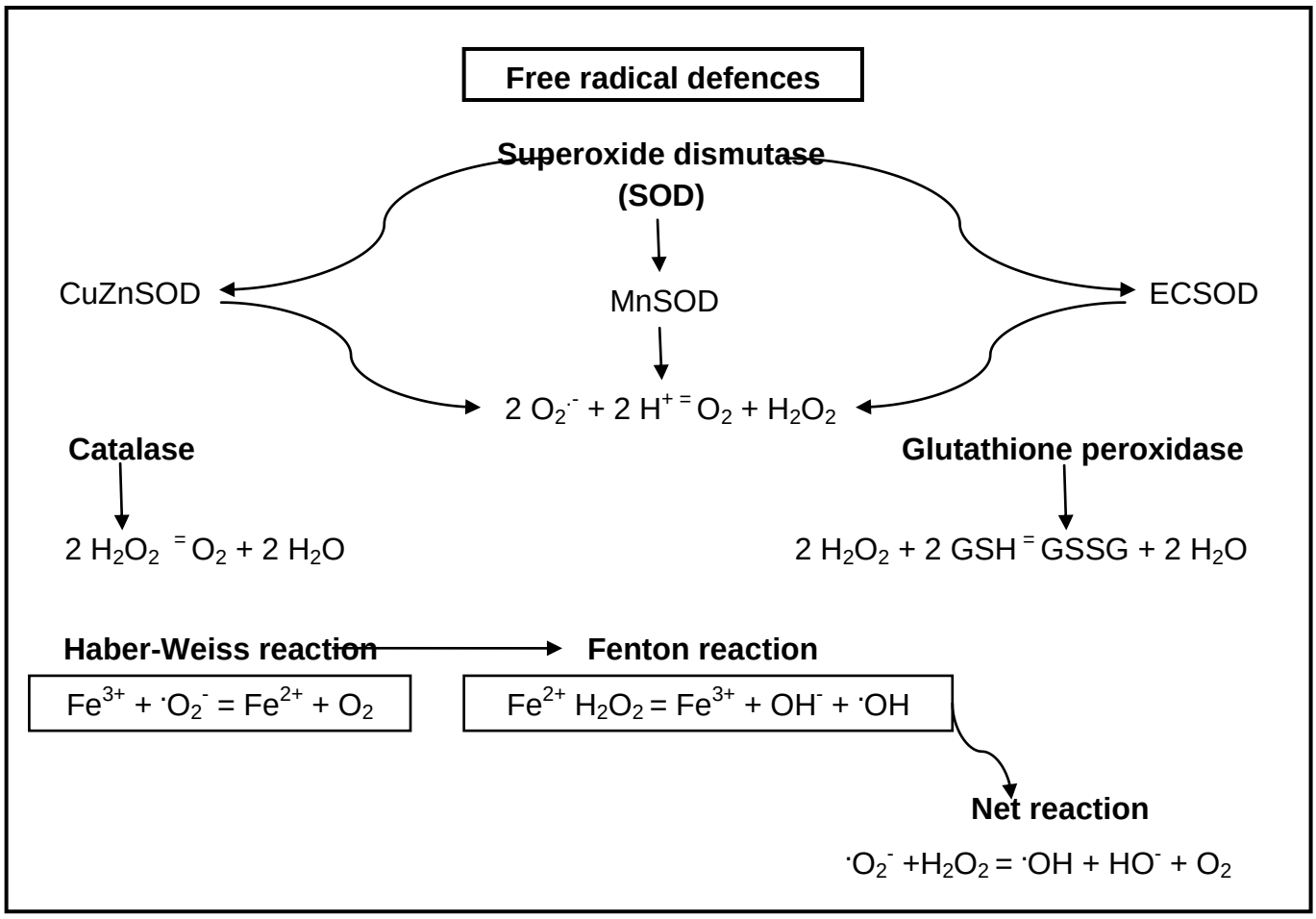

Figure 2.2: Free radical defence comprises of superoxide dismutase, catalase and glutathione peroxidase. The Haber-Weiss and Fenton reaction shows the formation of hydroxy radicals.

Glutathione (GSH) is a tripeptide, γ-glutamyl-cysteinyl-glycine and is synthesised from the amino acids cysteine, glycine, and glutamate (Kaplowitz, 1981; Cotgreave & Gerdes, 1998). GSH is involved in detoxifying xenobiotics and their metabolites (Kaplowitz, 1981; Lu, 2009). The synthesis of GSH depends on the availability of the rate-limiting amino acid and enzyme, cysteine and glutamate cysteine ligase (GCL) (Lu, 2009).

The substrates for glutathione conjugation are a broad spectrum of electrophiles (Zamek-Gliszczynski *et al*., 2006). Glutathione-S-transferase (GST) is a group of enzymes responsible for the conjugation of GSH with electrophiles. Its basic mechanism of action is that GSH conjugates to the electrophiles which are then metabolised further by cleavage of the glutamate and glycine residues. The resultant free amino acid group of the cysteinyl group is then acetylated to produce the final product, a mercapturic acid (Habig *et al*., 1974; Lu, 2009).

Intracellular GSH is difficult to deplete because of the high concentrations of GSH in the liver, but should this happen due to extremely high substrate concentrations, severe hepatoxicity may follow (Zamek-Gliszczynski *et al*., 2006). Thus, GSH is not only vital as an antioxidant but is also vital in detoxification and cell physiology (Kaplowitz, 1981).

2.1.3. Consequences of oxidative stress

Free radicals can modify DNA, activate cytoplasmic/nuclear signal transduction pathways, modify DNA polymerase activity and modulate gene expression and protein production, to name only a few (Cooke *et al.,* 2002). Therefore, these effects have led to oxidative stress being implicated in ageing and in human diseases such as hypertension, inflammation, autoimmunity, atherosclerosis, Parkinson's disease, cancer and diabetes (Aruoma, 1998).

Known characteristics of Diabetes mellitus include hyperglycaemia and insufficient insulin (Maritim *et al*., 2003). Oxidative stress has been implicated in diabetes development and progression (Baynes, 1991; Singh *et al.,* 2009), although the exact role of how oxidative stress accelerates diabetes is not completely understood. However, an increase in free radicals (Baynes, 1991), as well as a decrease in defence against free radicals (Halliwell & Gutteridge, 1990), have been shown to lead to insulin resistance, amongst others (Maritim *et al*., 2003).

Impaired mitochondrial function, iron content in the brain, lowered activity in the enzymatic defence mechanisms (in particular SOD) and reduced levels of GSH have been shown to be involved in the pathogenesis of Parkinson's disease patients (Jenner & Olanow, 1996). All of these are also linked to oxidative stress.

In cultured vascular smooth muscle cells, it was found that ROS induced the production of inositol triphosphate and reduced production of cyclic GMP, thus leading to vasoconstriction, i.e. hypertension (De Champlain *et al*., 2004). Oxidative damage to DNA is also considered an important factor in the development of cancer (Olinski *et al.,* 2003) since these lesions can alter the integrity of the genome (Jackson & Loeb, 2001). Atherosclerosis starts when the LDL in the body becomes oxidised by free radicals and then forms oxidised-LDL (ox-LDL). This ox-LDL damages the arterial wall, and the body's immune system then responds to the damage. The macrophages take up the ox-LDL, which then leads to cholesterol ester accumulation and foam cell formation (Witzum & Steinberg, 1991).

2.2. Biomarkers of oxidative damage

As ROS are short-lived, the measurement of certain biomarkers may give a better indication of oxidative status rather than only measuring the free ROS. These biomarkers may also be more stable than and not as reactive as ROS (Guéraud *et al.,* 2006). It should be kept in mind that no biomarker will always meet the requirements of an "ideal biomarker", however, some are better options than others (Dalle-Donne *et al*., 2006).

Biomarkers can be used to assess the degree of oxidative stress, to diagnose diseases earlier in their development, give an indication of disease progression and to determine whether an antioxidant therapy works efficiently (Dalle-donne *et al.,* 2006). The following should be kept in mind when choosing a biomarker: the biomarker chosen should be a major product of oxidative damage that could be implicated in the development of diseases, it should be stable (not an artificial product or lost during storage), it should represent a balance between oxidative damage generation and clearance and lastly it should not be influenced by the diet. The assay used for quantification of the biomarker should also be specific, reproducible and robust (Griffiths *et al.,* 2002).

2.2.1. DNA damage and 8-OHdG

Radicals produced within an individual, either naturally as a result of aerobic respiration, or from exogenous sources such as chemicals, drugs, air pollution cigarette smoke etc., puts DNA at risk of being damaged (Halliwell, 2000; Cooke *et al*., 2002). The effects of free radical damage on DNA include oxidation of guanine, cytosine, thymine and adenine (Cooke *et al.,* 2002), ring fragmentation, modifications of the sugar back-bone, strand breaks and covalent cross links with amino acids or other DNA bases (Breen & Murphy, 1995). Because guanine contains the lowest oxidation potential of the four bases found in DNA, it is more prone to oxidative damage (Chiou *et al.,* 2003; Peoples & Karnes, 2005). 8-Hydroxy-2'-deoxyguanosine (8-OHdG) is formed from a hydroxyl radical and a deoxyguanosine residue (Harri *et al.,* 2007). It is a well-known biomarker of oxidative stress (Cooke *et al*., 2003), most often studied (Chiou *et al.,* 2003) and also has mutagenic potential (Harri *et al*., 2007). During DNA replication, 8-OHdG may pair with adenine which then results in G to T substitutions, which could lead to the misreading of neighbouring bases (Harri *et al.,* 2007).

Defence mechanisms, such as antioxidants, reduce the interaction of radicals with macromolecules and in this case, DNA (Cooke *et al*., 2002). When DNA is oxidatively damaged by ROS, repair mechanisms are in place to safeguard the integrity of DNA for cellular survival (Chiou *et al*., 2003). However, the repair process responsible for urinary 8-OHdG levels is not

known yet (Evans *et al.,* 2010). The repair mechanisms for urinary 8-OHdG can include the following: sanitisation of the nucleotide pool (Nudix hydrolases), endonucleases (Cooke *et al.,* 2008), base excision repair (BER), nucleotide incision repair (NIR), nucleotide excision repair (NER) and/ or mismatch repair mechanisms (MMR) (Harri *et al*., 2007; Evans *et al.,* 2010). The products of excision repair are then transported from the cells and leave the body via the urine (Pilger *et al.,* 2002; Harri *et al*., 2007). Although Dalle-Donne *et al.,* (2006) reported that 8-OHdG may originate from the degradation of dGTP from the DNA precursor pool and thus not be representative of whole-body oxidative DNA damage, other authors differ. The more widely accepted view is that 8-OHdG in urine appears to be dependent on the rate of DNA damage *in vivo* and on the efficiency of the repair processes (Loft *et al.,* 1992) and is therefore representative of total body oxidative DNA damage (Halliwell & Whiteman, 2004). Patients treated with Adriamycin (a drug used in cancer treatment) showed an increase in uric acid, which is an indicator of cell turnover, although no increase in 8-OHdG levels was reported (Faure *et al*., 1998). 8-OHdG is not affected by diet because nucleosides are not absorbed from the intestinal tract (Wiseman & Halliwell, 1996). Thus, it was concluded that, according to the literature, 8-OHdG levels are not influenced by cell turnover or by the diet, however, more work needs to be done (Cooke *et al.,* 2008).

Additional markers used to assess oxidative DNA damage include 8-hydroxy-guanine (8-OHGua), 8-hydroxy-adenine (8-OHAde), 8-hydroxy-2'-deoxy-adenosine (8-OHdA) and thymine glycol. Adenine lesions were found to be less prevalent in DNA damage than guanine lesions (Burrows & Muller, 1998). After quantification of 8-OHdG and 8-OHdA, it was found that 8-OHdA levels were 15 times lower than 8-OHdG levels (Podmore *et al*., 2000). 8-OHGua is another marker that can be considered as a biomarker, however, it is influenced by the diet and thus its use as biomarker should be avoided (Wiseman & Halliwell, 1996; Kawai *et al*., 2007). Thymine glycol (Tg) was the first marker used to measure oxidative DNA damage, however, 8-OHdG is now more often used (Cooke *et al.,* 2002). Although Cathcart *et al.,* (1984) reported that the diet did not have an effect on thymine glycol levels, Simic (1994) reported that Tg was actually absorbed from the diet. Therefore the usage of Tg as biomarker of DNA damage was rejected and now 8-OHdG is most often used as a biomarker of oxidative damage on DNA. However, European standards committee on urinary DNA lesion analysis (ESCULA), still need to complete the validation of 8-OHdG as biomarker of oxidative stress (Cooke *et al.,* 2008).

Before 8-OHdG can be quantified in blood, DNA has to be isolated and degraded to generate free bases. Unfortunately, the chemical hydrolysis of DNA may lead to the artificial production of 8-OHdG, and as a consequence, to an overestimation of the 8-OHdG levels present (Collins *et al.,* 2004). When using urine as sample matrix, there is no artificial production of 8-OHdG and

no need for enzymatic digestion (Pilger *et al.,* 2002). However, as urine is a complex matrix, extensive cleanup procedures are usually required prior to quantification (Lin *et al.,* 2004). The use of urine, however, has many more advantages over the use of blood. Firstly, it is non-invasive. Secondly, it is easily collected and transported. Thirdly, there is no need for special storage conditions (Cooke, 2009). However, urine as sample matrix may be challenging due to the low levels of 8-OHdG found in urine (Harri *et al*., 2007). Because 8-OHdG was reported to remain stable in urine for over 10 years at -20 ºC (Loft *et al*., 2005) samples previously collected and stored can still be used to assess whole body DNA damage (Halliwell, 2000).

Numerous techniques have been employed to measure 8OHdG, including: gas chromatography- mass spectrometry (GC-MS); high performance liquid chromatography-electrochemical detection (HPLC-ECD); high performance liquid chromatography- tandem mass spectrometry (LC-MS/MS); enzyme-linked immunosorbent assay (ELISA); capillary electrophoresis– electrochemical detection (CE-ECD) and capillary electrophoresis- mass spectrometry (CE-MS). All of these methods have some limitations as well as advantages which have to be weighed against one another to determine which method/technique will give the most accurate results with regard to oxidative damage assessment using 8-OHdG as biomarker.

The use of GC-MS for the quantification of 8-OHdG carries a higher risk for artificial production of 8-OHdG due to the derivatisation step that is used for GC-MS analysis (Cadet *et al.,* 1997; Harri *et al.,* 2007; Chao *et al*., 2008). Pre-purification of the target metabolite via HPLC may be required before GC-MS analysis. On the other hand, LC-MS/MS methods to quantify 8-OHdG, have also encountered the same limitation as GC-MS with regard to the artificial oxidation of nucleosides present in the sample matrix (Chao *et al.,* 2008). Renner *et al*., (2000) reported that 8-OHdG was artificially produced from 2-deoxyguanosine (2'dG) during ionisation in the electrospray ion source of an LC-MS/MS. Therefore, it was deemed crucial to separate 2'dG and 8-OHdG via chromatography before entering the ionisation source. If only 8-OHdG is entering the mass spectrometer at a given time, without any 2'dG entering the mass spectrometer at the same time, no artificial 8-OHdG can be formed. Sufficient chromatographic separation of 2'dG and 8-OHdG prior to entering the ion source thus prevents the artificial oxidation (Renner *et al.,* 2000). Artificial oxidation of 8-OHdG from 2'dG is also much higher in DNA samples than urine samples, as the ratio of 2'dG is higher in DNA samples than urine (Weimann *et al.,* 2001).

HPLC-ECD is the most commonly used method for the detection of 8-OHdG. Conversely, a ten times increase in sensitivity for the quantification of 8-OHdG was reported using LC-MS/MS compared to HPLC-ECD (Peoples & Karnes, 2005). The ELISA assay used to quantify 8-OHdG is a very popular assay since it is easy to use, it requires no specialised equipment, numerous sample matrixes can be used, no sample pre-treatment is required and it is also a high-throughput technology (Cooke, 2009). However, the antibody, N45.1, used in the assay as it is highly specific for 8-OHdG, can lead to an overestimation of 8-OHdG levels as urea is also recognised by N45.1. This is because 8-OHdG and urea share a common –NH-CO-N- structure. This problem can be overcome by treating the sample with urease to remove the urea. Performing the ELISA assay at 4 $^{\circ}$C instead of 37 $^{\circ}$C reduced the recognition of the antibody with urea. However, it was found that although the analysis was done at 4 $^{\circ}$C, the 8-OHdG levels were still 1.5 fold higher compared to that found with HPLC-ECD. It was found that 8-hydroxy-guanine (8-OH-Gua) cross-reacts with N45.1 at 4 $^{\circ}$C which leads to higher 8-OHdG levels being reported (Song *et al*., 2009).

A method for the detection of 8-OHdG in urine via CE-ECD was also described (Mei *et al*., 2005). After comparing this method to a GC-MS method, it was concluded that both of these methods are suitable for detecting 8-OHdG in urine with sufficient accuracy. However, CE-ECD doesn't need a derivatisation step and thus is simpler. The method precision of CE-ECD is also better than the GC-MS method and the instrumentation of CE-ECD is cheaper. Nevertheless, because small volumes are used for detection in CE compared to HPLC, it resulted in lowered concentration sensitivity and reduced limits of detection (Peoples & Karnes, 2005). When CE is interfaced with MS/MS, it can also pose some problems. CE has a limited loading capacity of 1 µl and usually only 10-100 nl. These small volumes lead to small peaks being detected in the MS and prove problematic for MS/MS analysis (Dakna *et al*., 2009).

2.2.2. Lipid peroxidation and DHN-MA

Lipids, especially polyunsaturated fatty acids (PUFAs), are highly susceptible to reactions with free radicals (Rathahao *et al*., 2005). Lipid hydroperoxides are the major initial products produced when radicals react with, and consequently damage these lipids (Uchida, 2003). Lipid hydroperoxides produce certain breakdown products when decomposed, which, when compared to free radicals, are relatively stable, allows them to diffuse from the cell and damage targets far from their site of origin (Uchida, 2003). Therefore, these lipid peroxidation products are also known as second-toxic messengers of free radicals and can cause severe disturbances of cell functions, both at the genetic and biochemical levels (Srivastava *et al.,*

2000; Spies-Martin *et al*., 2002). Lipid peroxidation has been implicated in the development and progression of numerous diseases, such as cancer, atherosclerosis, and diabetes (Esterbauer *et al.,* 1991). Thus, it could be useful to measure these products of lipid peroxidation to determine the extent of damage so that proper intervention can be considered. However, the question remains: which product of lipid peroxidation is the better option to consider as a biomarker?

Malondialdehyde (MDA), F_2- isoprostanes, and trans-4-hydroxy-2-nonenal (4-HNE) are the lipid peroxidation products most often used as markers of oxidative damage on lipids (Peiro *et al*., 2005). However, the use of these metabolites has some limitations. Arachidonic acid is the only source of F_2- isoprostanes, therefore, it only represents the degradation of arachidonic acid (Peiro *et al.,* 2005). The main F_2- isoprostanes *in vivo* are 8-iso-prostaglandin $F_{2\alpha}$. This metabolite is only moderately stable at -20 $^{\circ}$C and requires the addition of butylated hydroxytoluene (BHT) to improve stability (Peiro *et al*., 2005). The MDA present in urine is partly due to the presence of oxidized PUFAs in the diet (Draper *et al*., 2000). Like F_2 – isoprostanes, this metabolite is also only moderately stable at -20 $^{\circ}$C and also requires addition of BHT (Peiro *et al*., 2005). 4-HNE is the primary aldehyde formed from lipid peroxidation (Esterbauer *et al.,* 1991). Unfortunately this metabolite is highly reactive (Rathahao *et al*., 2005) and can therefore not be considered a reliable marker of lipid peroxidation. However, 4-HNE is chemically reactive towards GSH (Völkel *et al.,* 2005) and when conjugation occurs, it finally gives rise to the end metabolite 1,4 dihydroxynonene mercapturic acid (DHN-MA). This metabolite is stable and can be considered as a biomarker of lipid peroxidation since it does not present the same shortcomings as its precursor, 4-HNE (Guéraud *et al*., 2006). DHN-MA remains stable during storage because its precursor, 4-HNE, is not present in urine (Alary *et al.,* 1995), and the synthesis of DHN-MA occurs enzymatically (Alary *et al.,* 2003). It is the main urinary product of exogenous 4-HNE in the rat and human (Alary *et al*., 1995). DHN-MA is considered to be a good and convenient biomarker of lipid peroxidation (Guéraud *et al*., 2006), compared to MDA or 8-iso-prostaglandin $F_{2\alpha}$ (Peiro *et al*., 2005). Due to the presence of 4-HNE in certain foods, the influence of these foods on 4-HNE levels measured in urine cannot be ruled out (Lang *et al.,* 1985; Draper *et al.,* 2000; Wilson *et al.,* 2002). However, when subjects are fasting, the use of hydroxy fatty acids (i.e. MDA, 4-HNE and DHN-MA) as markers of lipid peroxidation may be valid (Wilson *et al.,* 2002). In this study, 10 hour fasting baseline urine and serum samples were collected from the SABPA participants, thus excluding the diet as possible artificial influences on lipid peroxidation levels measured in urine.

The 4-hydroxy-2-alkenals are the most prominent lipid peroxidation aldehydes (Esterbauer *et al.,* 1991) and are formed from ω-6-PUFAs which are the PUFAs with the highest concentration

in mammalian tissues (Spies-Martin *et al*., 2002). They are electrophilic reagents which react with nucleophils such as sulfhydryl (-SH)-, and amino (-NH_2) groups as well as with the imidazole group of histidine (Spies-Martin *et al*., 2002). Arachidonic acid, as well as linoleic acid, is believed to be the potential precursors for 4-HNE as the nine carbons found in 4-HNE originate from the last nine carbons from the ω-6 essential fatty acids (Uchida, 2003).

The detoxification of 4-HNE involves several enzymatic reactions (Esterbauer *et al.,* 1991). These include glutathione-S-transferase (GST), aldehyde dehydrogenase and alcohol dehydrogenase (Uchida, 2003). GST catalyzes the conjugation of GSH to 4-HNE via Michael addition (Uchida, 2003; Kuiper *et al.,* 2008). NAD^+ dependent cytosolic and mitochondrial aldehyde dehydrogenase oxidizes 4-HNE to 4-hydroxy-2-nonenoic acid (HNA) which is the corresponding carboxylic acid (Alary *et al*., 1995; Alary *et al*., 2003). Aldo-keto reductase reduces 4-HNE to 1,4-dihydroxynonene (DHN) which is the corresponding alcohol (Kuiper *et al.,* 2008). When these conjugates leave the liver, glutamic acid and glycine are removed, leaving the cysteine conjugates which are acetylated giving rise to the mercapturic acid conjugates (Alary *et al*., 1995; Kuiper *et al.,* 2008) (Figure 2.3).

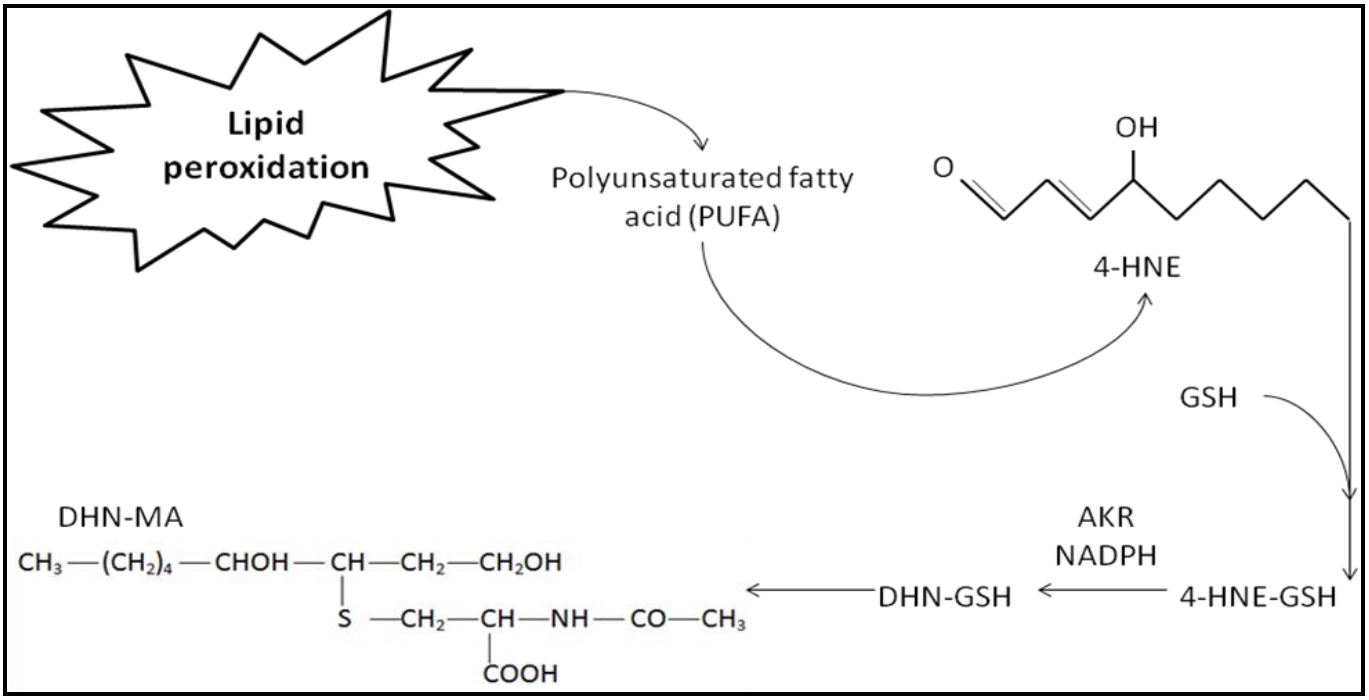

Figure 2.3: Formation of DHN-MA. Lipid peroxidation of polyunsaturated fatty acids leads to 4-HNE formation. After GSH conjugates to 4-HNE the formed 4-HNE-GSH is reduced to DHN-GSH through aldo-keto reductase (AKR) and eventually leads to DHN-MA formation. (Adapted from Peiro *et al.,* 2005).

DHN-MA is a physiological component of rat and human urine (Alary *et al*., 1998). Under non-pathological conditions, DHN-MA is present in the tissues of rat in the range of 0.1 to 3.0 nmol/g protein (Esterbauer *et al.,* 1991). These low DHN-MA levels reported is a consequence of low level lipid peroxidation occurring under physiological conditions (Alary *et al*., 1998). However,

when free radicals exceed the capacity of defence mechanisms in the rat, levels can reach 10 nmol/g protein (Esterbauer *et al.,* 1991). DHN-MA is very stable in urine and thus appears to be an appropriate biomarker of lipid peroxidation (Alary *et al*., 1998).

2.3. Aims and objectives

“One of the greatest needs in the field of free radical biology is the development of reliable methods for measuring oxidative stress status in humans” (Pryor & Godber, 1991). Thus, the aim of this study was to develop a single analytical method to successfully and reliably quantify urinary 8-OHdG and DHN-MA in human samples as a marker of oxidative stress status. As tandem mass spectrometry was available to this study, it was decided to use this platform for quantification of 8-OHdG and DHN-MA.

This study was divided into three main objectives:

i. Standardisation and validation of the LC-MS/MS assay for the simultaneous quantification of 8-OHdG and DHN-MA in human urine samples.
ii. Quantification of the urinary 8-OHdG and DHN-MA levels in a selected group of South African teachers.
iii. Investigating a possible correlation between urinary 8-OHdG-, DHN-MA- and serum ROS levels in a selected group of South African teachers.

2.4. Experimental approach

The basic experimental approach for this study was as follows:

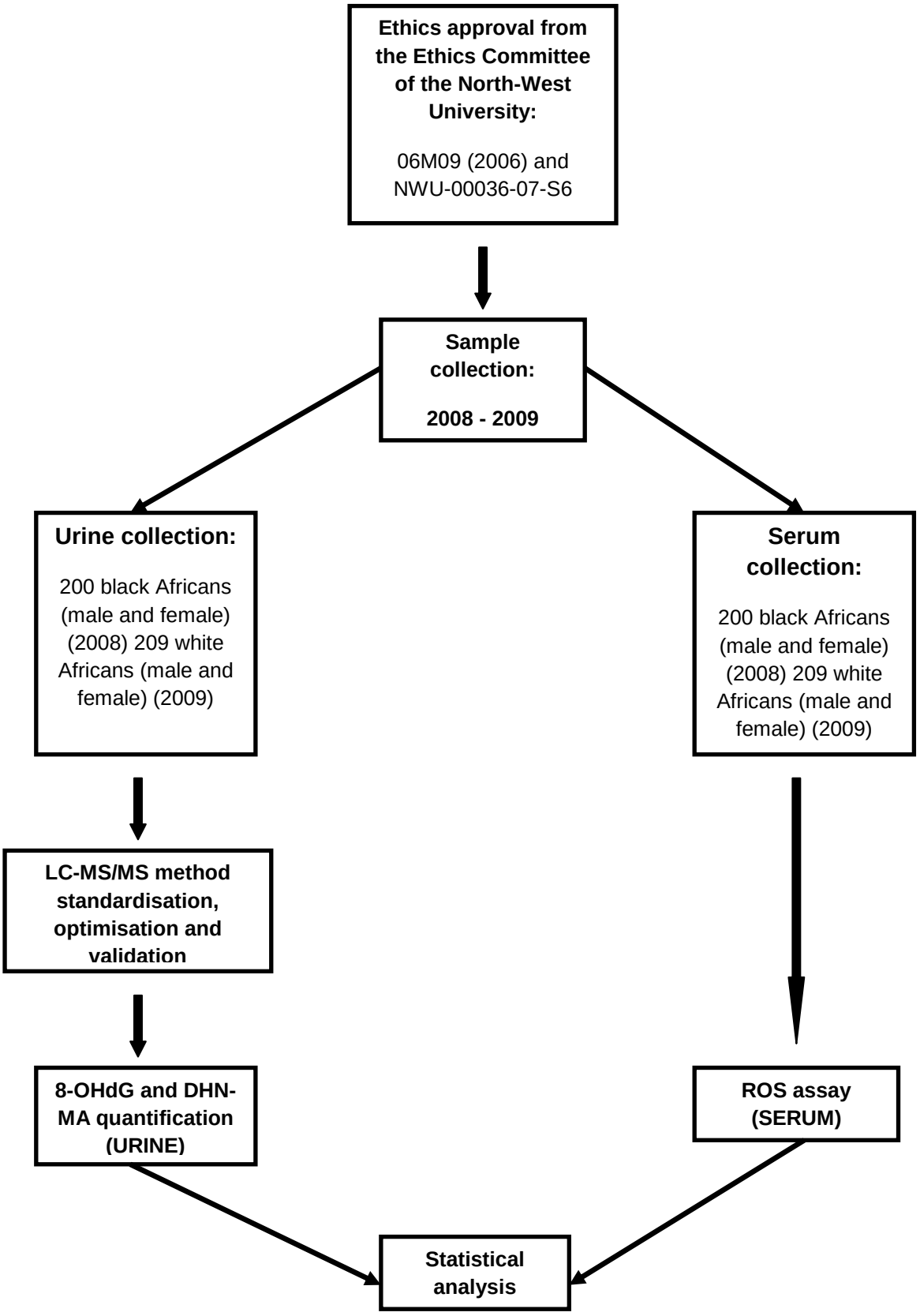

Figure 2.4: Visual representation of the strategy proposed for this study.

CHAPTER 3

Materials and methods

3.1. The SABPA study

3.1.1. Participants and methodological approach

This study forms part of the SABPA (**S**ympathetic activity and **A**mbulatory **B**lood **P**ressure in **A**fricans) study, which is mainly concerned with the effect of lifestyle and stress on hypertension in urbanised Africans. Potential participants completed a standard information questionnaire and were selected according to their responses in November 2007 for Phase I sample collection, and in November 2008 for Phase II sample collection. The inclusion criteria for the SABPA study included 209 Caucasian (101 males; 108 females) and 200 African (100 males; 100 females) teachers in the Potchefstroom area of the North-West province of South Africa. Age range between 25 and 60 years and a similar socio-economic status were also part of the inclusion criteria. Pregnancy, lactation, any acute/chronic medication (e.g. hypertension, tuberculosis, diabetes, coagulation factors, inflammation, epilepsy/mental disorders including psychotropic substance abuse or dependence) were used as exclusion criteria. Informed consent was obtained from the selected participants prior to the commencing of the study. For the SABPA study, sympathetic activity responses were measured. These were done at baseline level and after participants had been exposed to two laboratory stressors: colour-word conflict chart and the cold pressor test. However, 10 hour fasting baseline urine and serum samples were collected for the quantification of ROS, 8-OHdG and DHN-MA.

3.1.2. Ethics approval

This study was approved by the Ethics Committee of the North-West University under the title: ***"Development and standardisation of analytical techniques to determine oxidative stress and antioxidant capacity in humans"*** (06M09). As stated earlier this study also forms part of the SABPA study, which has ethics approval under the title: ***"SABPA, Sympathetic Activity and Ambulatory Blood Pressure in Africans"*** (NWU-00036-07-S6).

3.1.3. Sample collection and storage

The sample collection for the SABPA study was divided into two phases. During phase I, samples from 200 African males and females were collected from February to May of 2008. During phase II of this study, samples from 209 Caucasian males and females were collected. The collection of these samples began on February 2009 and ended in May of 2009. The samples collected included fasting baseline serum for the ROS assay and fasting baseline urine for 8-OHdG and DHN-MA quantification. These serum and urine samples were collected daily between 06:30 – 07:30.

An SST vacutainer was used for blood collection. The blood in the vacutainer was left to coagulate for 30 minutes. This was then centrifuged at 1000 x g for 10 minutes. Serum was collected and stored at -80 ºC until the day of analysis. Urine samples were collected in 30 ml polypropylene specimen containers and stored at -20 °C until the day of analysis. A urine sample was randomly selected for the optimisation and validation of the DHN-MA and 8-OHdG assay.

The ROS assay was performed on the serum samples within a six month period after samplecollection. However, the urine samples were stored at -20 °C until the analysis could be performed. Since the analytical methods for the quantification of 8-OHdG and DHN-MA had to be optimised and validated, it was essential that 8-OHdG and DHN-MA remains stable in the urine samples at -20 °C for an extended period. According to the literature, no artificial formation of DHN-MA is known to occur during the storage of urine, since its precursor, 4-HNE, is not present in urine and because the formation of DHN-MA cannot occur without enzyme action. DHN-MA can also not be easily degraded since it does not contain a chemical reactive group (Peiro *et al*., 2005). The metabolite 8-OHdG, was also found to have remained stable in urine for more than 10 years of storage at -20 °C (Loft *et al.,* 2006).

3.2. Creatinine values

Urinary levels of oxidative lesions are influenced by the efficacy of renal excretion of the metabolites. The use of urinary creatinine levels is necessary to correct for variations in the individual urine concentration (Cooke *et al.,* 2002). Therefore, the creatinine values are important in order to make it possible to compare urinary results with one another (Nakano *et al*., 2003).

The creatinine values of samples collected during February 2008 – May 2008, were determined in 2008. The creatinine values of samples collected in February 2009 – May 2009 were determined in 2009. The creatinine values were determined by Du Buisson & Associates (AMPATH, Potchefstroom). See Appendix A for the creatinine values of the SABPA participants.

3.3. Reactive oxygen species (ROS) assay

3.3.1. Basis of ROS assay

The ROS assay is a high throughput and automated analysis with high reproducibility and consistent accuracy (Hayashi *et al.,* 2007). The basis of this assay is that in an acidic medium, ROS will react with transition metals, such as iron, to form alkoxyl- and peroxyl radicals. The formed radicals will then oxidize N,N-diethyl-*para*-phenylenediamine (DEPPD) to its cation which is followed kinetically at 546 nm.

3.3.2. Reagents

Sodium acetate (anhydrous) (Cat. No. S2889), N,N-diethyl-*para*-phenylenediamine (DEPPD) (Cat. No. 168343) and hydrogen peroxide (H_2O_2) (Cat. No. H1009) were purchased from Sigma-Aldrich Co., USA. Ferrous sulphate (Cat. No. F106029) was purchased from Labchem, Edenvale, RSA.

3.3.3. Buffers and solutions

Standard curve: Hydrogen peroxide (0; 60; 120; 180; 240; 300 mg/L):

The concentration of the H_2O_2 stock solution was 8.8 M. A standard range of H_2O_2 solutions (0, 60, 120, 180, 240 and 300 mg/L respectively) were prepared by diluting the stock solution with milli Q water. The aliquots were stored at -8

Sodium acetate buffer [0.1 M]:

A 0.1 M sodium acetate buffer was prepared as follows: Anhydrous sodium acetate (4.1015 g) was added to 400 ml milli Q water. After adjusting the pH to 4.8 with glacial acetic acid, the buffer was stored at 2 – 8 °C. The buffer usually remains stable for approximately 6 months.

DEPPD solution (R1 [100 mM]):

A 100 mM DEPPD solution was prepared by adding 1.3117 g of DEPPD to 50 ml sodium acetate buffer (covered with foil), and it was stored at 2 – 8 ^{0}C. This reagent remains stable for a maximum of 4 weeks. As soon as a colour change from light pink to black occurs, the DEPPD solution needs to be discarded.

$FeSO_4$ solution (R2 [4.37 µM]):

Ferrous sulphate (0.0121 g) was dissolved in 1 ml sodium acetate buffer to give a concentration of 43.7 µM. This was then diluted to give a final concentration of 4.37 µM (covered with foil), and it was stored at 2 – 8 °C. This remains stable for approximately 6 months.

3.3.4. ROS assay

Serum samples were thawed on ice. Sodium acetate buffer (140 µl) was added to each well of the 96-well microtiter plate with a multichannel pipette. The standards/samples (2.5 µl) were then added to the 96-well microplate (standards in duplicate and samples in triplicate). The volume of R1:R2 (ratio, 1:25) required was calculated and mixed in a glass beaker (1000 µl R2 for each 40 µl R1). The R1:R2 solution (100 µl) was added to each well with a multichannel pipette. This was incubated at room temperature for 1 minute. The reaction was followed kinetically at 546 nm for 10 minutes (at 25 °C) with a BIO-TEK® 7 FL600 microplate fluorescence reader. The ROS levels in serum were then extrapolated from the H_2O_2 standard curve.

3.4. Statistical analysis and interpretation of data

All statistical analysis were carried out with Statistica Software (Version 9). Firstly the measured 8-OHdG data was tested for normal distribution using the Shapiro Wilk test. Normality was rejected ($p < 0.01$). Although sample sizes were large in all groups, non-parametric tests were employed for further data analysis based on the skewness in the data. Outliers were removed from the data matrix by the use of Tukey's method, which defines outliers as those data points falling outside the control limits. The control limits are defined as three interquartile ranges below the 25^{th} percentile or above the 75^{th} percentile (Tukey, 1977). Because initial investigation of the data revealed that the 8-OHdG levels might differ between some of the groups, each of the four groups were separately assessed to identify outliers in that group.

A BDM test was used to test the significance of the interaction between ethnicity and gender on 8-OHdG levels. The problem with the Kruskal-Wallis test is that, while it does not assume normality for groups, it assumes that the groups have the same distribution shape (homoscedasticity). Brunner *et al*., (1997) proposed a heteroscedastic version of the Kruscal-Wallis test which utilizes the *F*-distribution. This test, the Brunner-Dette-Munk test, is referred to as the BDM test throughout this study. Since no interaction between ethnicity and gender was found with the BDM test, the significance of the main effect can be investigated by pooling the data (i.e. ethnicity and gender). This means that the gender effect is investigated by ignoring ethnicity and vice versa. However, because the data is skewed and the ranges differ considerably between groups, pooling of data may not be advisable. Therefore, it was decided to analyse the main effect of gender on different levels of ethnicity and vice versa. Mann-Whitney U tests were used for this purpose while box plots were used for schematic representations of the data.

The existence of a correlation between urinary 8-OHdG and serum ROS levels was assessed with Spearman correlations. The following guidelines were used to interpret the correlation coefficient: a correlation coefficient is bounded between -1 and 1 where negative values indicate inverse relationships and positive values indicate direct relationships. Values close to zero indicate a small or no relationship between the given parameters while values close to 1 or -1 indicate a very strong relationship between the parameters.

CHAPTER 4

Optimisation and validation of the 8-OHdG assay

4.1. Introduction

Free radicals can cause DNA damage (Halliwell, 2000; Cooke *et al*., 2002). Although many oxidative DNA damage markers have been identified, adenine lesions were found to be less prevalent in DNA damage than guanine lesions (Burrows & Muller, 1998). Quantification of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-hydroxy-2'-deoxyadenosine (8-OHdA) revealed that 8-OHdA levels were 15 times lower than 8-OHdG levels (Podmore *et al*., 2000). This finding also correlates with the results obtained by Burrows & Muller (1998). Although 8-hydroxy-guanine (8-OHGua) is another metabolite that can be considered as a biomarker, its concentration in urine may be influenced by diet, compromising its value as a biomarker (Wiseman & Halliwell, 1996; Kawai *et al*., 2007). Thymine glycol (Tg) is another marker used to quantify oxidative DNA damage. Although Tg is formed from the repair of oxidised DNA, literature is contradictory on whether Tg is also absorbed from the diet (Cathcart *et al*., 1984; Simic, 1994), and therefore the use of Tg should also be limited. 8-OHdG is a well-known biomarker of oxidative stress (Cooke *et al*., 2003), it has mutagenic potential (Harri *et al*., 2007) and therefore it is most often studied (Chiou *et al.,* 2003). 8-OHdG is formed from the reaction of a hydroxyl radical and a deoxyguanosine residue and is excreted in urine as a result of DNA repair (Harri *et al.,* 2007). Many analytical methods have been used to successfully measure 8-OHdG in urine, such as GC-MS, HPLC-ECD, LC-MS/MS, ELISA, CE-ECD and CE-MS. Because LC-MS/MS appears to be a more sensitive and accurate analytical method to quantify 8-OHdG (Weimann *et al.,* 2001), and since LC-MS/MS technology was available to this study, it was decided to use LC-MS/MS to quantify 8-OHdG.

4.2. Chemicals, standard solutions and buffers

4.2.1. Chemicals

The 8-hydroxy-2'-deoxyguanosine (Cat No. 095K40974) and 2'-deoxyguanosine (Cat. No. 056K1400) were purchased from Sigma Aldrich Co., USA. The stable isotope 2'deoxyguanosine (2'dG^{15}N) (Cat. No.124303603) was purchased from Cambridge isotope, USA. Ammonium formate (Cat No. 70221) and formic acid (Cat. No. 33015) were purchased from Sigma, Aldrich Co., USA. Acetonitrile (MeCN) (Cat. No. 017-4) was purchased from Agilent, South Africa. Methanol (MeOH) (Cat. No. 1.06007.2500) was purchased from Merck Chemicals, South Africa.

4.2.2. Standard solutions and buffers

Standard solutions of 8-OHdG, 2'dG and 2'dG^{15}N were separately prepared in milli Q water with a final concentration of 1 mg/ml respectively. These standard solutions were used for optimising the mass spectrometer (MS) conditions (Section 4.3.2), the chromatographic conditions (Section 4.3.3) and calibrations (Section 4.6.1).

A 1 mM ammonium formate buffer (pH 3.75) was prepared by dissolving ammonium formate in milli Q water. The pH was adjusted to 3.75 with concentrated formic acid.

4.3. Optimisation of LC-MS/MS conditions for quantification of 8-OHdG in urine

4.3.1. Specifications of the LC-MS/MS

An Agilent 1200 series LC (Santa Clara, CA, USA) was used for sample handling as well as for mobile phase delivery. Samples (20 µl of each) were injected and a constant flow rate of 0.2 ml/min was maintained throughout the run. The MS/MS analysis was performed on an Agilent 6410 Triple Quadrupole mass spectrometer (Santa Clara, CA, USA) with positive ionisation.

4.3.2. Optimisation of the MS conditions

The first step in standardising the 8-OHdG assay was to optimise the MS conditions. For this optimisation, the MassHunter optimiser software from Agilent was used. MassHunter works on chromatographic and electrophoretic mass spectral data to extract information, reduce data complexity, eliminate potential interferences and generate a list of molecular features (Anon, 2005). MassHunter optimiser software provides a way to automatically optimise the data acquisition parameters for MRM mode (multiple-reaction monitoring) on an MS/MS instrument for each individual compound analysed. Specifically, it automates the selection of the precursor ions, the optimisation of the fragmentor voltage for each precursor ion, the selection of the optimal fragment ions, and the optimisation of collision energy values for each transition for a specified list of compounds (Anon, 2009).

For the optimisation of the MS conditions, 10 µl of each standard solution (8-OHdG, 2'dG and 2'dG^{15}N, 100 ng/ml respectively) was separately infused in the MS with 50% MeCN: 50% water and 0.1% formic acid as the mobile phase. The results obtained for the optimiser are given in Tables 4.1 to 4.3.

Table 4.1: The optimal electrospray tandem mass spectrometric conditions for the detection of 8-OHdG. MassHunter software was used to optimise the conditions for 8-OHdG detection with the LC-MS/MS.

Compound name	**Formula**	**Nominal mass**	**Polarity**	**Ion source**
8-OHdG	$C_{10}H_{13}N_5O_5$	283.2	Positive	ESI
Precursor ion	**Fragmentor**	**Product ion**	**Collision energy**	**Abundance**
284.2	90	168	12	302706
284.2	90	140	32	61640
284.2	90	112	40	26660
284.2	90	151	40	13941

Table 4.2: The optimal electrospray tandem mass spectrometric conditions for the detection of 2'dG. MassHunter software was used to optimise the conditions for 8-OHdG detection with the LC-MS/MS.

Compound name	**Formula**	**Nominal mass**	**Polarity**	**Ion source**
2'dG	$C_{10}H_{13}N_5O_4$	267.2	Positive	ESI
Precursor ion	**Fragmentor**	**Product ion**	**Collision energy**	**Abundance**
268.2	75	152.1	12	737
268.2	75	124	24	17
268.2	75	135.1	40	200
268.2	75	206.5	8	9

Table 4.3: The optimal electrospray tandem mass spectrometric conditions for the detection of 2'dG^{15}N. MassHunter software was used to optimise the conditions for 2'dG^{15}N detection with the LC-MS/MS.

Compound name	**Formula**	**Nominal mass**	**Polarity**	**Ion source**
2'dG^{15}N	$C_{10}H_{13}{}^{15}N_5O_4$	272.2	Positive	ESI
Precursor ion	**Fragmentor**	**Product ion**	**Collision energy**	**Abundance**
273.2	75	157	16	1324
273.2	75	139	36	414
273.2	75	115.3	20	17
273.2	75	210.4	0	11

From the optimiser reports it is clear that a product ion of 168 m/z and a precursor ion of 284.1 m/z are optimal for detection of 8-OHdG (Table 4.1) with the LC-MS/MS. A precursor of 268.1 m/z and a product ion of 152.1 m/z were selected for 2'dG detection (Table 4.2). For 2'dG^{15}N

detection, a precursor of 273.2 m/z and a product ion of 157 m/z were selected (Table 4.3). The precursor and product ions selected for 8-OHdG, 2'dG and 2'dG^{15}N quantification are consistent with those most often reported in the literature (Renner *et al.,* 2000; Weimann *et al.,* 2001; Harri *et al.,* 2007).

4.3.3. Chromatographic separation of 8-OHdG, 2'dG and 2'dG^{15}N

Since 8-OHdG can spontaneously form from 2'dG in the electrospray ion source of the mass spectrometer (Renner *et al*., 2000), it was concluded that chromatographic separation of 2'dG and 8-OHdG was essential. The chromatographic separation of 8-OHdG and 2'dG would prevent both metabolites from entering the mass spectrometer when 8-OHdG is quantified. This would prevent artificial formation of 8-OHdG from 2'dG, since no 2'dG molecules are eluting from the column when 8-OHdG elutes, as their retention times differ. Chromatographic separation was performed on a Zorbax SB-Aq (2.1 x 150 mm, 3.5 µm) column (Cat. No. USSQG 01048) purchased from Agilent. This type of column, with an alkyl reversed-phase bonded phase, is designed to retain hydrophillic compounds while using an aqueous mobile phase and is highly stable at low pH (pH < 4). The column was protected using a Zorbax SB-Aq (2.1 X 12.5 mm, 5 µm) (Cat. No. 82125-933) guard column, also purchased from Agilent. The flow rate was 0.2 ml/min and the injection volume was 20 µl. Electrospray ionisation was performed in the positive ion mode. Nitrogen gas was used as nebulising gas.

For chromatographic separation of 8-OHdG, 2'dG and 2'dG^{15}N a 1 mM ammonium formate buffer pH 3.75 was used as mobile phase A and 100% MeCN as mobile phase B. Initially the elution gradient was adjusted a couple of times to get optimal retention and sharp peaks on the chromatogram for 8-OHdG, 2'dG and 2'dG^{15}N (Figure 4.1 and 4.2). The optimal elution gradient is given in Table 4.4. Figure 4.1 illustrates the chromatographic separation of 8-OHdG and 2'dG standard solutions. Multiple reaction monitoring (MRM) was used to monitor and quantify 8-OHdG and 2'dG. The total ion chromatogram, as depicted in Figure 4.1, is the sum of both MRMs used for the detection of 8-OHdG and 2'dG.

Table 4.4: Mobile phase gradient timetable used for the chromatographic separation of 8-OHdG, 2'dG and 2'dG^{15}N. The mobile phases used for chromatographic separation were 1 mM ammonium formate buffer pH 3.75 (mobile phase A) and 100% MeCN as mobile phase B.

Time	B %	Flow
0	5	0.2
2	5	0.2
10	26	0.2
12	50	0.2
13	50	0.2
15	100	0.2
21	100	0.2
26	5	0.2
41	5	0.2

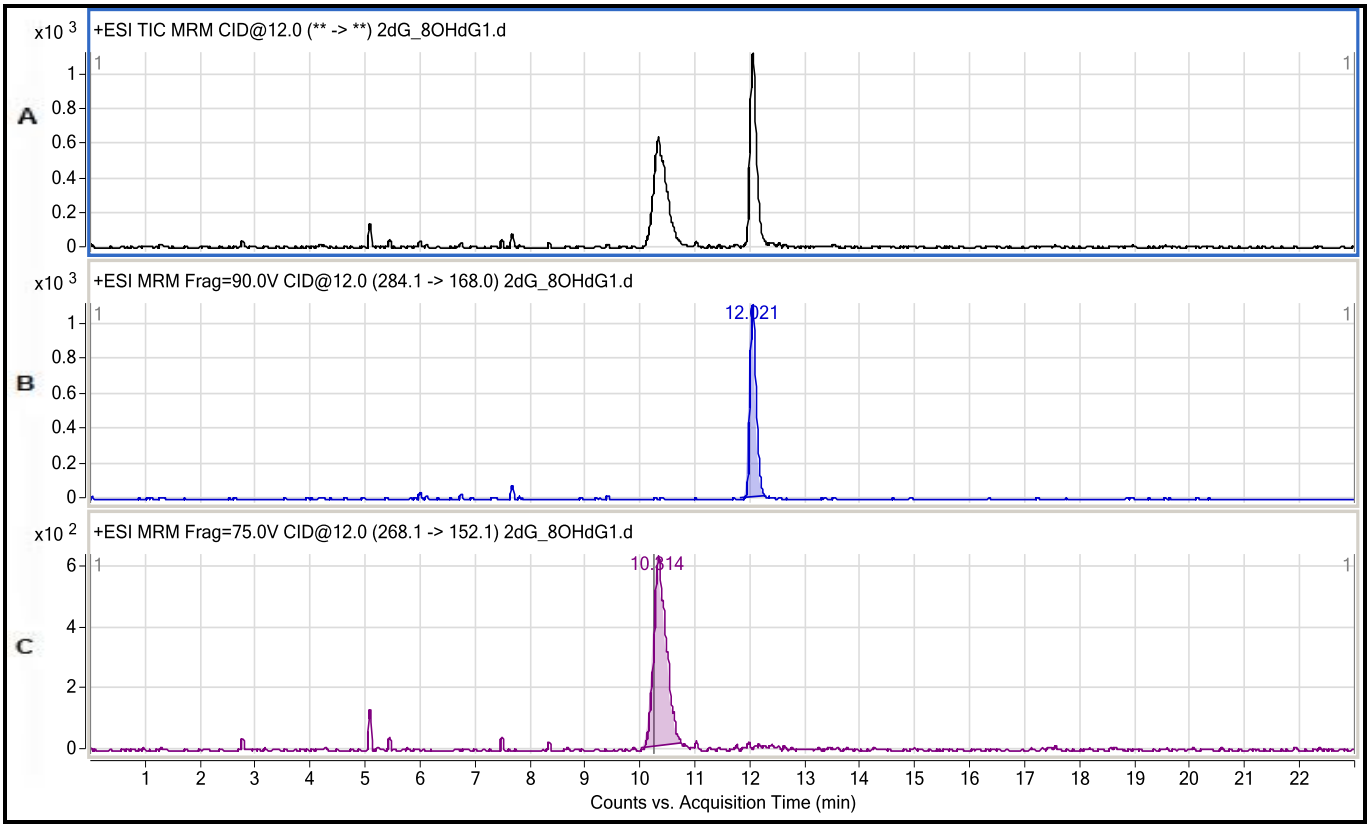

Figure 4.1: Chromatographic separation of 2'dG and 8-OHdG standard solutions: (A) the total ion chromatogram (TIC) illustrating retention times for both 2'dG and 8-OHdG (B) MRM of 8-OHdG and (C) MRM of 2'dG.

Chromatographic separation of 8-OHdG and 2'dG^{15}N is illustrated in Figure 4.2. Multiple reaction monitoring (MRM) was used to monitor and quantify 8-OHdG and 2'dG^{15}N. The total ion chromatogram, as depicted in Figure 4.2, is the sum of both MRMs used for the detection of 8-OHdG and 2'dG^{15}N.

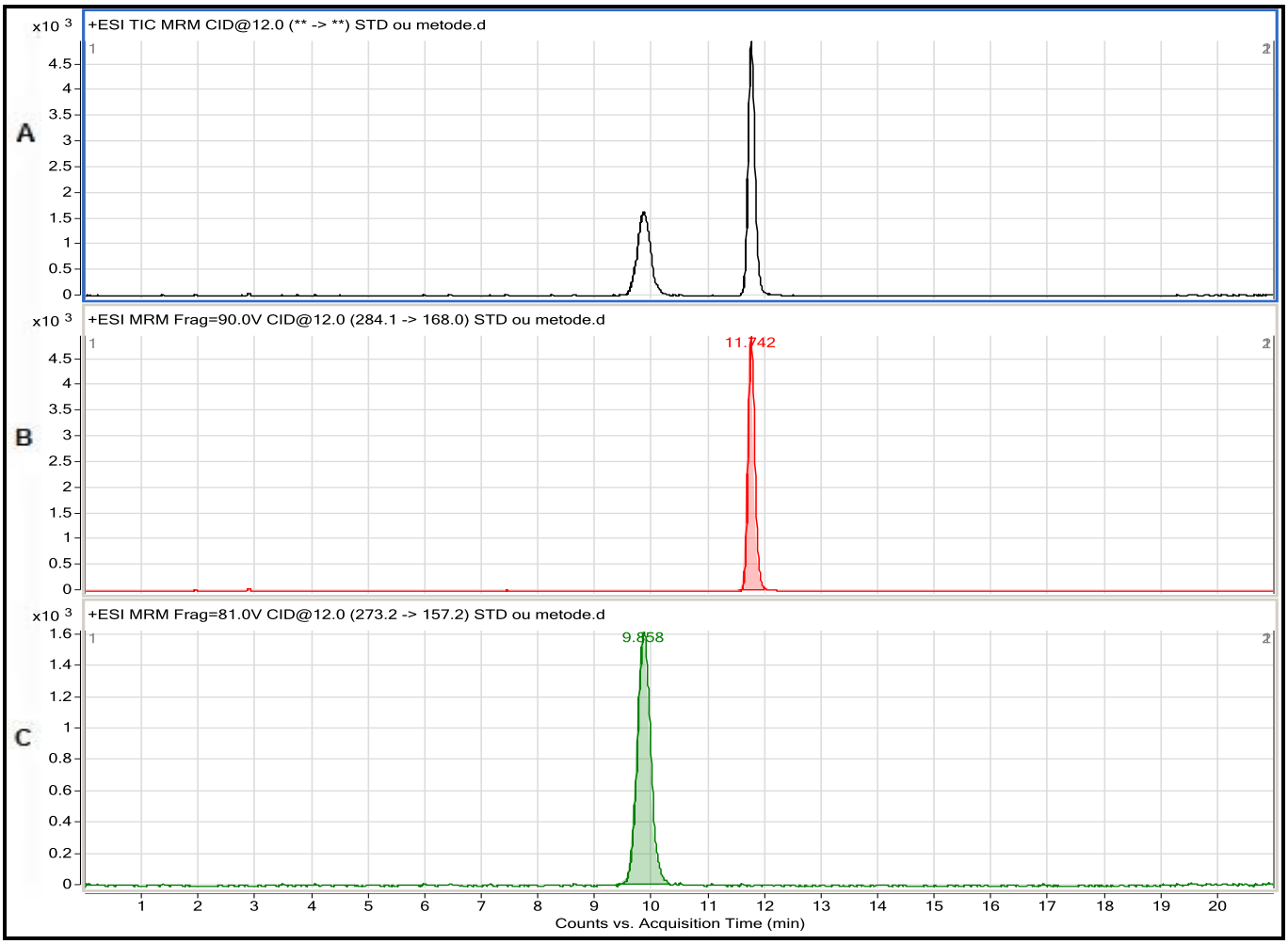

Figure 4.2: Chromatographic separation of 2'dG^{15}N and 8-OHdG standard solutions: (A) is the total ion chromatogram (TIC) illustrating retention times for both 2'dG^{15}N and 8-OHdG; (B) MRM of 8-OHdG and (C) MRM of 2'dG^{15}N.

When the gradient given in Table 4.4 is used for chromatographic separation, the biomarker 8-OHdG (284.1 – 168.0 m/z) has a retention time of ± 12 minutes (Figure 4.1 and 4.2), 2'dG (268.1 – 152.1 m/z) has a retention time of ± 10 minutes (Figure 4.1) and the isotope 2'dG^{15}N (273.2 – 157.2 m/z) has a retention time of ± 10 minutes (Figure 4.2). As mentioned previously, 8-OHdG can spontaneously form from 2'dG in the electrospray ion source of the mass spectrometer, therefore chromatographic separation of 8-OHdG and 2'dG is essential. The

chromatographic separation of 2'dG and 8-OHdG was sufficient to prevent any 2'dG from entering the MS at the same time as 8-OHdG (Figure 4.1).

4.4. Solid phase extraction (SPE) of 8-OHdG from urine

Although Weimann *et al.,* (2001) reported direct quantification of 8-OHdG in urine (without a clean-up step), this could not be achieved in this study (data not shown). The concentration of 8-OHdG in human urine was below the detection limit of the method and therefore a sample clean-up step was necessary. In an effort to increase the concentration of 8-OHdG, it was decided to use solid phase extraction (SPE). If a large volume of urine can be applied to the SPE column and the 8-OHdG retained on the column, a lot of contaminants can be washed from the column before the 8-OHdG is eluted in a small volume of mobile phase. This strategy would not only remove a lot of contaminants/metabolites from the sample, but would also increase the concentration of 8-OHdG to levels that can be detected and quantified.

A STRATA-X 200 mg/ 6 ml (Cat. No. 8B-S100-FCH) SPE column was chosen and purchased from Separations, Phenomenex, South Africa, for sample clean up. The STRATA-X 200 mg/ 6 ml SPE column, is a polymeric reversed phase SPE, designed to retain polar molecules. A vacuum manifold, (Visiprep^{TM}, from Supelco: Cat. No. 5-7030) at 125 mm Hg was used for solid phase extraction. The SPE column was preconditioned with 6 ml 100% methanol (MeOH) and then with 6 ml milli Q water. The sample to be analysed (3 ml urine, 200 µl 5 M ammonium formate buffer pH 3.75 and 50 µl 2'dG^{15}N, 100 ng/ml) was applied to the column, and the column was washed with 2 ml milli Q water. Elution of 8-OHdG was performed using 6 ml 40% MeOH:water as described by Hu *et al.,* (2004).

Retaining 8-OHdG and 2'dG^{15}N during the loading and washing steps of the SPE is essential for good reproducibility of the assay and was therefore investigated. A control urine sample was used to test whether all 8-OHdG and 2'dG^{15}N was retained by the SPE column during the loading and wash steps, and whether all the 8-OHdG and 2'dG^{15}N were removed from the SPE column during the elution step. After the SPE column had been conditioned as described previously, the control urine sample was loaded onto the SPE column (3 ml urine, 200 µl 5 M ammonium formate buffer pH 3.75 and 50 µl 2'dG^{15}N, 100ng/ml). The eluate from the loading step was collected in a 6 ml Medispo tube. The SPE column was washed with 2 ml milli Q water to remove some of the metabolites not of interest and the eluate was also collected in a new 6 ml Medispo tube. The 8-OHdG and 2'dG^{15}N were then eluted from the SPE column with 6 ml

40% MeOH: water. A second elution step with 6 ml 100% MeOH was used to determine whether 8-OHdG and 2'dG^{15}N remained on the SPE column after the initial elution step. The eluates from both these elution steps were separately collected in Medispo tubes.

All the collected fractions were evaporated under nitrogen on an AB 2000 Stargate Scientific Nitrogen gas dryer, for approximately 20 minutes before they were frozen and freeze dried overnight on a Virtis Manual Set freeze dryer. All the samples were reconstituted in 100 µl, 1 mM ammonium formate buffer pH 3.75 and analysed with the optimised 8-OHdG assay as described in Section 4.5. Firstly, the fractions collected during the loading and wash steps of the SPE column were evaluated. As can be seen from Figure 4.3, no 8-OHdG or 2'dG^{15}N was detected in the loading eluate (Figure 4.3, a) or the washing eluate (Figure 4.3, b).

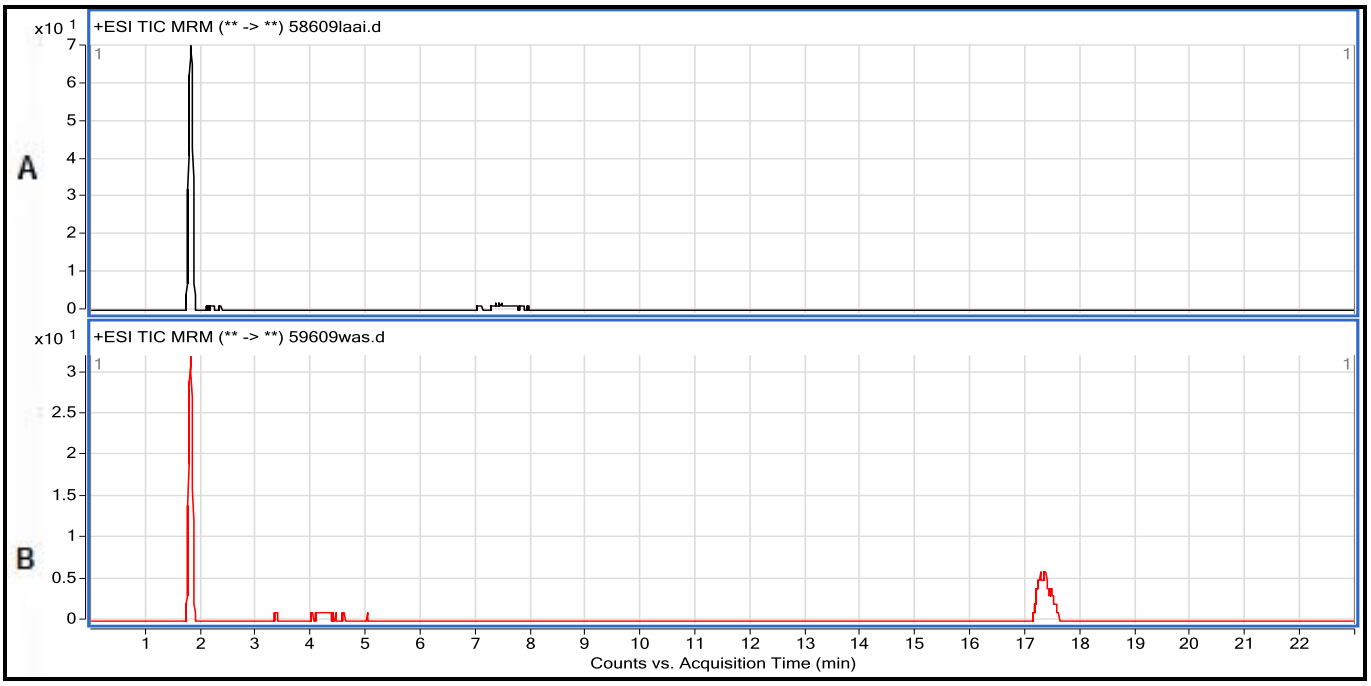

Figure 4.3: TIC of the loading and wash step of the SPE method. This chromatogram shows that no 8-OHdG (± 12 minutes) or 2'dG^{15}N (± 10 minutes) eluted from the column during the (A) loading (B) and washing of the STRATA-X SPE column.

The first and second elution steps were also evaluated to determine whether all the 8-OHdG and 2'dG^{15}N eluted from the SPE column with the first elution step. If any 8-OHdG or 2'dG^{15}N remained on the SPE column after the first elution step, a second elution step had to be introduced to the standard method. As can be seen from Figure 4.4, 8-OHdG and 2'dG^{15}N eluted from the SPE column within the first elution step. This was expected, as this step was designed to elute the wanted metabolites (8-OHdG and 2'dG^{15}N) from the SPE column.

However, if the first elution step was insufficient, the second elution step would also contain 8-OHdG and 2'dG^{15}N. This was not the case as can be seen in Figure 4.5, since no 8-OHdG or 2'dG^{15}N could be detected in the second elution step.

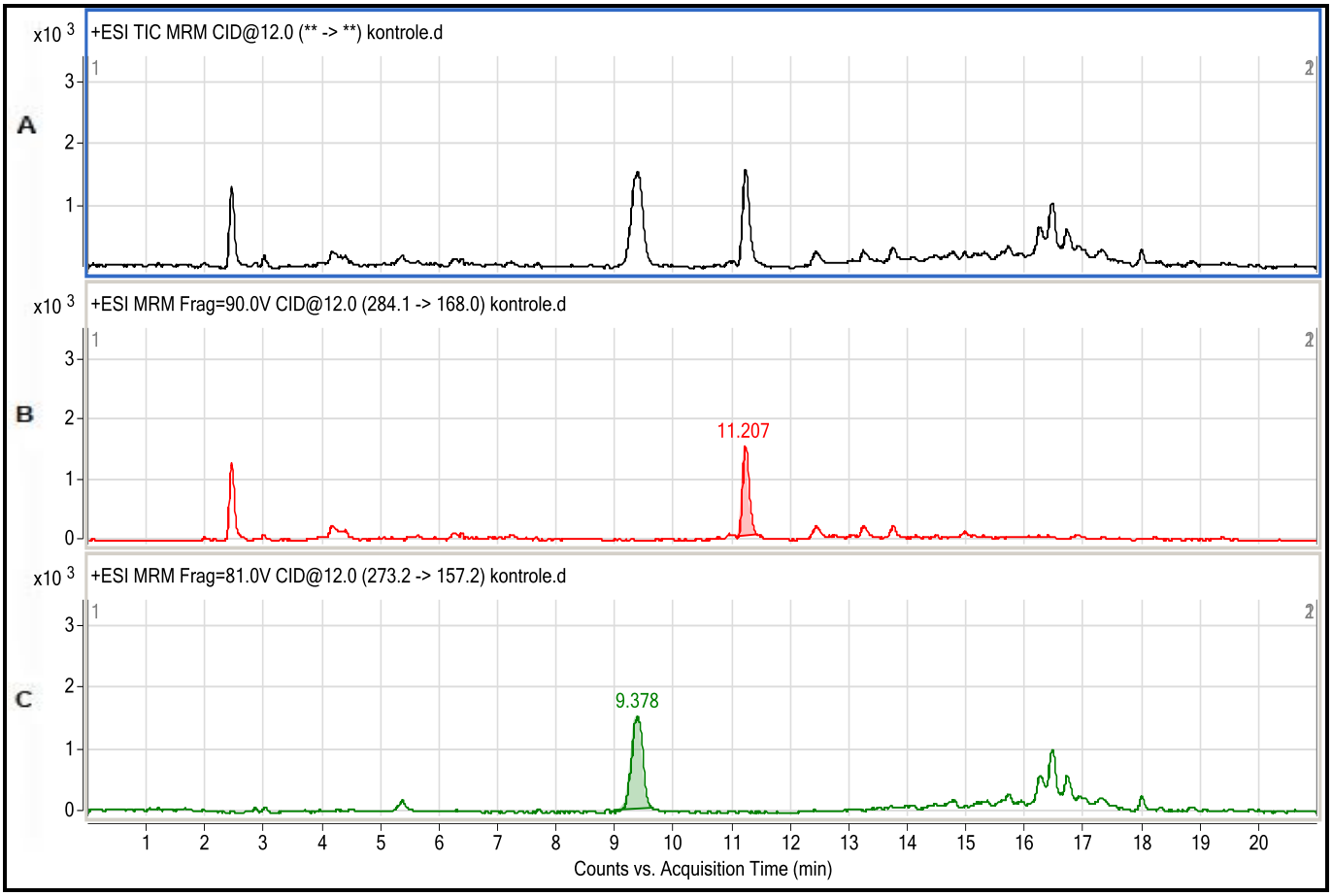

Figure 4.4: (A) TIC showing the elution of 8-OHdG and 2'dG^{15}N from the SPE column with the first elution step. The metabolites were eluted from the SPE column with 6 ml 40% MeOH during sample clean up (B) The MRM of 8-OHdG (C) The MRM of 2'dG^{15}N.

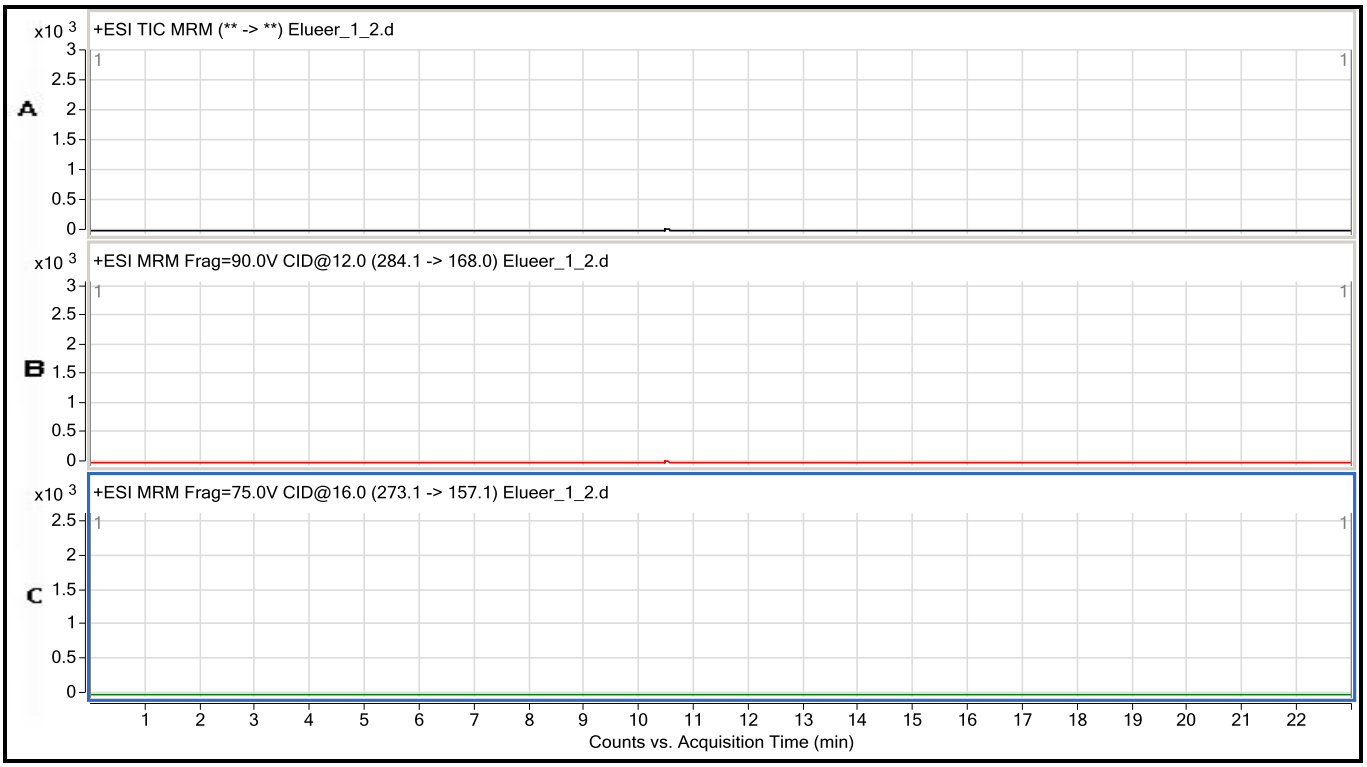

Figure 4.5: (A) TIC for 8-OHdG and 2'dG^{15}N from the SPE column with the second elution step. No 8-OHdG or 2'dG^{15}N was detected in the eluate from the second elution step (B) MRM for 8-OHdG (C) MRM for 2'dG^{15}N.

It is clear from the data obtained that the chosen SPE column, as well as the sample clean up method used, are sufficient in retaining the metabolites of interest during loading and washing. It also proves that the first elution, with 6 ml 40% MeOH is sufficient to elute all the 8-OHdG and 2'dG^{15}N from the SPE column, since no residual 8-OHdG and 2'dG^{15}N was eluted from the SPE column during the second elution with 6 ml 100% MeOH. It can therefore be concluded that the SPE process is sufficient to clean and concentrate the urine sample in order to detect and quantify 8-OHdG. No 8-OHdG or isotope is lost during the loading and washing steps, due to insufficient binding of the metabolites by the SPE column. However, all the 8-OHdG and 2'dG^{15}N are eluted from the SPE column by a single elution step, resulting in a concentrated sample where 8-OHdG can be successfully detected and quantified.

4.5. The optimised 8-OHdG assay

Urine samples which were collected in 2008 and 2009 (See Chapter 3) and stored at -20 ºC were thawed overnight in a fridge at 2 – 8 ºC. Each SPE column was preconditioned with 6 ml 100% MeOH and 6 ml milli Q water before the sample to be analysed (3 ml urine, 200 µl 5M

ammonium formate buffer pH 3.75 and 50 µl 2'dG^{15}N, 100 ng/ml) was loaded onto the column. After each loading step, each column was washed with 2 ml milli Q water. Elution of 8-OHdG was performed with 6 ml 40% MeOH: water. The eluted sample was dried under a gentle stream of nitrogen gas for ± 40 minutes at 37 °C on an AB 2000 Stargate Scientific Nitrogen gas dryer to remove the MeOH. After the nitrogen drying, the remainder of the sample was frozen at -80 °C for 2 hours. This was freeze dried overnight using a Virtis Manual Set freeze dryer. The dried sample was reconstituted in 1 mM ammonium formate buffer pH 3.75 (100 µl) prior to analysis. Chromatographic separation was performed on a Zorbax SB-Aq (2.1 x 150 mm, 3.5 µm) column and was protected with a Zorbax SB-Aq (2.1 X 12.5 mm, 5 µm) guard column. A 1 mM ammonium formate buffer pH 3.75 was used as mobile phase A and 100% MeCN as mobile phase B and the gradient selected can be seen in Table 4.4. The flow rate was 0.2 ml/min and the injection volume was 20 µl. Electrospray ionisation was performed in the positive ion mode. Nitrogen gas was used as nebulising gas. The tandem mass spectrometer was set to detect specific precursor and product ions for 8-OHdG (284.1 – 168.0 m/z) and the isotope 2'dG^{15}N (273.2 – 157.2 m/z). However, only the eluate fractions that contain the metabolites of interest (8-OHdG and 2'dG^{15}N) were directed to the mass spectrometer (time 5 minutes to 16 minutes). During the earlier (time 0 – 5 minutes) and later (time 16 – 41 minutes) part of the chromatographic separation, eluting compounds were directed to waste instead of going into the MS. During the analysis of the SABPA samples, a wash program (Table 4.5) was also introduced after every batch of 20 samples. Since the normal gradient used for 8-OHdG quantification (Table 4.4) only varied between 5 and 100% MeCN, some metabolites could theoretically remain on the chromatographic column. Therefore, the wash program was introduced to remove any residual metabolites from the column, and varied between 0 and 100% MeCN. For the wash step, 1 mM ammonium formate buffer pH 3.75 was used as mobile phase A and 100% MeCN was used as mobile phase B.

Table 4.5: Mobile phase gradient timetable for the wash program used after every batch of 20 samples analysed on the LC-MS/MS. The mobile phases used was 1 mM ammonium formate buffer pH 3.75 (mobile phase A) and 100% MeCN as mobile phase B.

Time	B %	Flow
0	5	0.2
2	0	0.2
14	0	0.2
24	100	0.2
30	100	0.2
40	5	0.2

4.6. Validation of the 8-OHdG quantification assay

Since method validation is an integral part in method development and it affects the quality of the data it ultimately produces (Peters *et al.,* 2007), the standardised method had to be validated before it could be used. The parameters assessed during the process of validation were specificity/selectivity, precision and linearity. The accuracy of the assay was not measured. However, the 8-OHdG data obtained from the SABPA participants (0.27 – 106.17 ng/mg creatinine), is more or less in the same range as the 8-OHdG levels reported in the literature, 0.49 – 14.29 ng/mg creatinine (Harri *et al.,* 2007).

4.6.1. Linearity of the 8-OHdG assay

In this study linearity of the 8-OHdG assay was investigated as follows: a calibration range, consisting of eight samples, was prepared, each containing 50 ng/ml 2'dG^{15}N. The samples also contained 0.5, 1, 2.5, 5, 10, 25, 50, 100 ng/ml 8-OHdG, respectively.

All the samples were prepared in 1 mM ammonium formate buffer pH 3.75 and analysed immediately. Twenty µl of each sample was injected onto the LC-MS/MS with the gradient given in Table 4.4. Mobile phase A consisted of 1 mM ammonium formate buffer pH 3.75 and mobile phase B contained 100% MeCN. A flow rate of 0.2 ml/min was maintained in positive ion mode during electrospray ionisation.

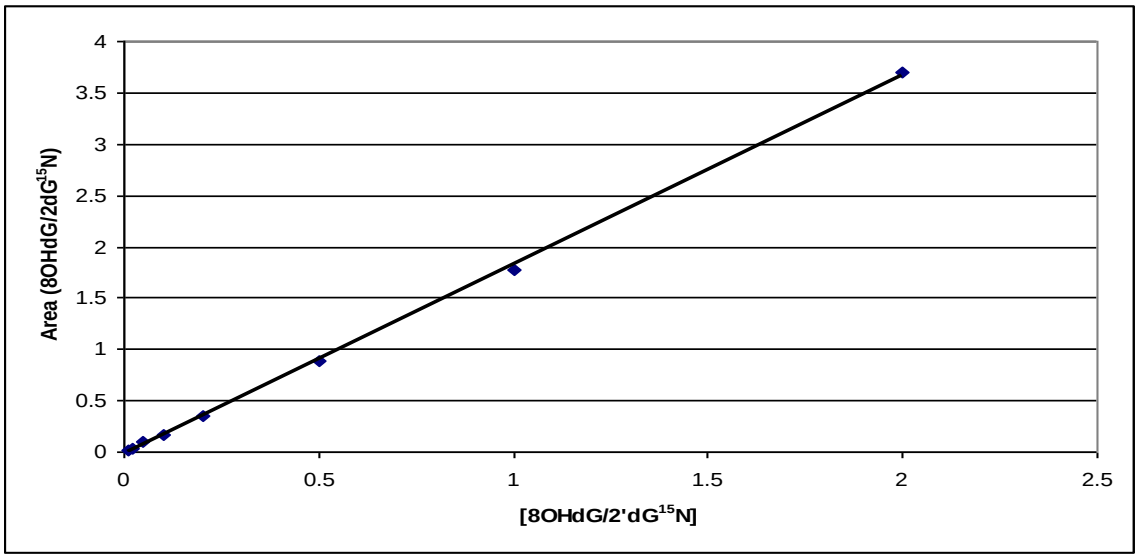

Figure 4.6: Calibration curve of 8-OHdG as obtained with the standardised 8-OHdG method. The [8-OHdG] ranged from 0.5 to 100 ng/ml while the [2'dG^{15}N] was 50 ng/ml in each sample (R^2 > 0.99).

As can be seen from Figure 4.6, the standardised 8-OHdG assay is linear over the concentration range measured (0.5 - 100 ng/ml) with a R^2 > 0.99. Even at the lower concentration range, the 8-OHdG assay is still linear (Figure 4.7).

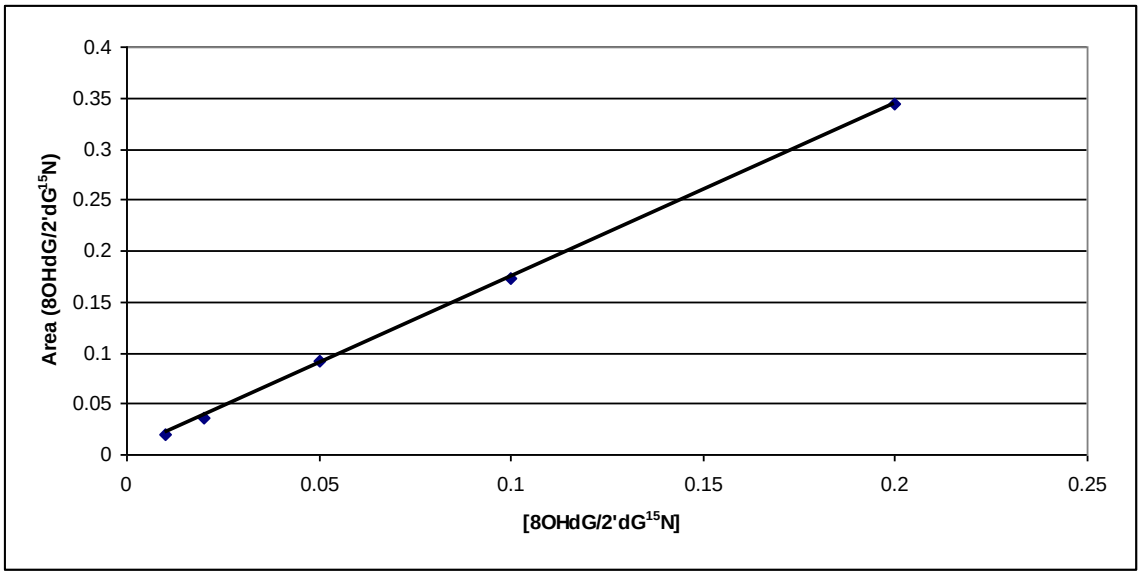

Figure 4.7: Calibration curve of 8-OHdG as obtained with the standardised 8-OHdG method in the lower concentration range.

4.6.2. Response factor of 8-OHdG to 2'dG^{15}N

The data used to demonstrate the linearity of the standardised 8-OHdG assay (section 4.6.1) were also used to calculate the response factor of 8-OHdG relative to the isotope used (2'dG^{15}N). The following equation was used:

Equation 4.1:

$$\text{Response factor} = \frac{\text{Response IS} \times [\text{IS}]}{\text{Response (analyte)} \times [\text{analyte}]}$$

Where:
Response IS = the response of the internal standard (isotope)
[analyte] = the concentration of the analyte
Response (analyte) = the response of the analyte
[IS] = the concentration of the internal standard (isotope).

The average response factor (RF) of 8-OHdG to 2'dG^{15}N is 0.55. Since the RF was obtained as the average over a broad concentration range (0.5 to 100 ng/ml 8-OHdG), the RF can be used to quantify [8-OHdG] over the specified concentration range.

4.6.3. Intrabatch and interbatch variation of the 8-OHdG quantification assay

Precision is the closeness of agreement between a series of measurements of the same homogenous sample (Peters *et al.,* 2007). Precision is usually measured in terms of imprecision and is expressed as relative standard deviation (RSD %). The precision of the newly standardised 8-OHdG assay was assessed by calculating the intra- and interbatch variations of the assay. The intrabatch variation gives the variation of the assay within a batch (variation on the same day), while the interbatch variation gives an indication of variation between different days. Since all the urine samples from this study would not be analysed in one day, but over a period of time, the interbatch variation should be considered more important.

A control urine sample was used to determine the intra- and interbatch variation of the 8-OHdG method. This urine sample was thawed overnight at 2 – 8 °C. Eight aliquots of 3 ml each were prepared and stored at -20 °C. For the intrabatch variation, four of these aliquots were thawed and prepared in a single batch with the optimised method (Section 4.5) before being analysed by LC-MS/MS. For the interbatch assay, four of the aliquots were separately assessed on four

consecutive days using the optimised method. Thus, these four samples were SPE extracted on separate days and were also analysed on the LC-MS/MS on separate days. The results obtained are given in Tables 4.6 and 4.7.

Table 4.6: Intrabatch variation of the 8-OHdG method. Mean = the mean of the four 8-OHdG concentrations measured with the standardised 8-OHdG method. SD = standard deviation and RSD = the relative standard deviation as a %

Sample #	**[8-OHdG]**
Intra1	40.79
Intra2	37.63
Intra3	41.09
Intra4	38.78
Mean	39.57
SD	1.65
RSD %	4.18

Table 4.7: Interbatch variation of the 8-OHdG method. Mean = the mean of the four 8-OHdG concentrations measured with the standardised 8-OHdG method. SD = standard deviation and RSD = the relative standard deviation as a %

Sample #	**[8-OHdG]**
Inter1	34.86
Inter2	40.97
Inter3	33.22
Inter4	26.64
Mean	33.92
SD	5.89
RSD %	17.37

For the intrabatch variation a RSD of 4.2% and for the interbatch variation a RSD of 17.4% was obtained. The higher than expected interbatch variation could be due to differences within the flow rate of the solvents on different days of the solid phase extraction process. A vacuum

manifold system was used to enhance the flow rate of organic solvent through the SPE. However, maintaining a constant flow rate over time (on different days) proved problematic. Although a constant flow rate was attempted each day, this was not always possible and could have contributed to the higher than expected interbatch variation.

4.7. Conclusions on the optimised 8-OHdG assay

After numerous trial and error efforts with regard to optimising the best conditions for the quantification of urinary 8-OHdG and a stable isotope, 2'dG^{15}N, using LC-MS/MS, an analytical assay was standardised. The 8-OHdG assay was validated with acceptable linearity (4.6.1) and precision (4.6.3) and performed satisfactory using human urine.

CHAPTER 5

Optimisation and validation of the DHN-MA assay

5.1. Introduction

Lipids, especially polyunsaturated fatty acids (PUFAs), are highly susceptible to reactions with free radicals (Rathahao *et al*., 2005). Since PUFAs are part of membrane phospholipids or lipoproteins, lipid peroxidation has been implicated in the development and progression of numerous diseases, such as cancer, atherosclerosis, diabetes and aging (Esterbauer *et al.,* 1991). Thus, it could be useful to quantify products of lipid peroxidation to determine the extent of lipid damage. One such lipid peroxidation product is 4-HNE. The latter is the primary aldehyde formed from lipid peroxidation (Esterbauer *et al.,* 1991). However, 4-HNE is chemically reactive towards GSH (Völkel *et al.,* 2005), and when conjugation occurs, it finally gives rise to the end metabolite 1,4-dihydroxynonene mercapturic acid (DHN-MA). This metabolite is relatively stable in urine and can be considered as a biomarker of lipid peroxidation (Alary *et al.,* 1998).

5.2. Chemicals, standard solutions and buffers

5.2.1. Chemicals

4-HNE-MA (Cat no. 32110) and its stable isotope, 4-HNE-MA-d3 (Cat no. 9000348) were purchased from Cayman Chemicals. Sodium borohydride (Cat no. S9125), sodium hydroxide (Cat. No. S8045), ammonium formate (Cat. No. 70221) and formic acid (Cat. No. 33015) were purchased from Sigma Aldrich Co., USA. HCl (Cat no. 1006660), ethyl acetate (Cat no. 1.09623.2500) and methanol (MeOH) (Cat. No. 1.06007.2500) were purchased from Merck Chemicals, South Africa. Acetonitrile (MeCN) (Cat. No. 017-4) was purchased from Agilent.

5.2.2. Standard solutions and buffers

A 1 mM ammonium formate buffer (pH 3.75) was prepared by dissolving the ammonium formate in milli Q water. The pH was adjusted to 3.75 with concentrated formic acid. This buffer was used as mobile phase A and MeCN was used as mobile phase B during chromatographic separation on the LC-MS/MS.

For the synthesis of DHN-MA and DHN-MA-d3, from 4-HNE-MA and 4-HNE-MA-d3 respectively, a 5 M sodium borohydride ($NaBH_4$) and a 1 N sodium hydroxide (NaOH) solution were prepared by dissolving sodium borohydride in milli Q water and sodium hydroxide in milli Q water respectively. A 5 N hydrochloric acid (HCl) solution was prepared by diluting concentrated HCl in milli Q water. For the quantification of DHN-MA and DHN-MA-d3, a 5 mg/ 10 ml H_2O acetaminophen mercapturic acid solution was prepared.

5.3. Synthesis of DHN-MA and DHN-MA-d3 from 4-HNE-MA and 4-HNE-MA-d3 respectively

Since DHN-MA and DHN-MA-d3 was not commercially available, it was necessary to synthesise and purify it. A modified version of the method described by Kuiper *et al*., (2008) was used for the synthesis of DHN-MA and DHN-MA-d3. DHN-MA was synthesised from the commercially available 4-HNE-MA and the stable isotope DHN-MA-d3 was synthesised from 4-HNE-MA-d3.

4HNE-MA in ethanol solution (100 µl, 1 mg/ml) was added to a clean Kimax tube and dried under a gentle stream of nitrogen with an AB 2000 Stargate Scientific nitrogen dryer. After the 4-HNE-MA had been dried, 1 ml of the 1 N NaOH and 10 µl of the 5 M $NaBH_4$ were added to the dried 4-HNE-MA in the Kimax tube. The mixture was sonicated at room temperature for 30 minutes. The pH was then lowered to pH 3 with 5 N HCl prior to extraction. Ethylacetate (1 ml) was added to the sample, and mixed on a rotor for 10 minutes at room temperature to extract all the formed DHN-MA from the water phase. The Kimax tube was centrifuged for 3 minutes at 2000 x g to ensure good phase separation. After centrifugation, the organic top phase was removed and added to a clean Kimax tube. This extraction of DHN-MA with ethylacetate was repeated twice. After each centrifugation step, the organic phase, containing DHN-MA, was added to the organic phase in the clean Kimax tube. The extracted sample was dried slowly under nitrogen at 20 °C. The dried sample was reconstituted in 1 mM ammonium formate buffer pH 3.75 (1 ml) and stored at -80 °C. The same conditions were used for the synthesis of DHN-MA-d3, except that 4-HNE-MA-d3 (100 µl, 1 mg/ml) was used as the substrate instead of 4-HNE-MA.

5.4. Optimisation of LC-MS/MS conditions for quantification of DHN-MA in urine

5.4.1. Specifications of the LC-MS/MS

An Agilent 1200 series LC (Santa Clara, CA, USA) was used for sample handling as well as for mobile phase delivery. Samples (20 µl of each) were injected and a constant flow rate of 0.2 ml/min was maintained throughout the run. The MS/MS analysis was performed on an Agilent 6410 Triple Quadrupole mass spectrometer (Santa Clara, CA, USA) in positive ionisation.

5.4.2. Optimisation of the MS conditions for 4-HNE-MA and 4-HNE-MA-d3 quantification

MassHunter optimiser software was used to optimise the MS conditions in order to quantify 4-HNE-MA and 4-HNE-MA-d3. MassHunter optimiser software provides a way to automatically optimise the data acquisition parameters of the mass spectrometer for MRM mode (multiple-reaction monitoring) of each individual compound to be analysed. It specifically automates the selection of the best precursor ions, the optimisation of the fragmentor voltage for each precursor ion, selection of the best fragment ions, and optimisation of collision energy values for each transition for a list of compounds specified. Ten µl of each standard solution (4-HNE-MA and 4-HNE-MA-d3) was separately infused in the MS (directly injected) with 50% MeCN: 50% water and 0.1% formic acid as the mobile phase. The results obtained for the MassHunter optimiser are given in Tables 5.1 and 5.2.

Table 5.1: The optimal electrospray tandem mass spectrometric conditions for the detection of 4-HNE-MA. MassHunter software was used to optimise the conditions for 4-HNE-MA detection with the LC-MS/MS.

Compound name	**Formula**	**Nominal mass**	**Polarity**	**Ion source**
4-HNE-MA	$C_{14}H_{25}NO_5S$	319.2	Positive	ESI
Precursor ion	**Fragmentor**	**Product ion**	**Collision energy**	**Abundance**
320.2	96	161.8	24	147
320.2	96	164.1	4	2761
320.2	96	273.8	0	145
320.2	96	80.2	32	533

Table 5.2: The optimal electrospray tandem mass spectrometric conditions for the detection of 4-HNE-MA-d3. MassHunter software was used to optimise the conditions for 4-HNE-MA-d3 detection with the LC-MS/MS.

Compound name	Formula	Nominal mass	Polarity	Ion source
4-HNE-MA-d3	$C_{14}H_{22}D3NO_5S$	322.2	Positive	ESI
Precursor ion	Fragmentor	Product ion	Collision energy	Abundance
323.2	65	164.1	4	803
323.2	65	122.1	20	319
323.2	65	83.1	36	119
323.2	65	282.2	4	43

From the optimiser reports (Tables 5.1 and 5.2) a precursor ion of 320.2 m/z and product ion of 164 m/z were selected for 4-HNE-MA and for 4-HNE-MA-d3, a precursor of 323.2 m/z and a product ion of 164.1 m/z were selected. These specific product ions were selected because their abundances were the highest of all product ions detected by the MassHunter software.

5.4.3. Optimisation of the MS conditions for DHN-MA and DHN-MA-d3 quantification

The newly synthesised DHN-MA and DHN-MA-d3 were used to optimise the mass spectrometric conditions for their detection and quantification. Although it was not certain at this point in time whether the syntheses were successful, mass spectrometry was the method of choice to examine the syntheses. However, before mass spectrometry could be employed to examine the syntheses, the MS conditions for the detection and quantification of DHN-MA and DHN-MA-d3 had to be optimised. Ten µl of each standard solution (DHN-MA and DHN-MA-d3) was separately infused in the MS with 50% MeCN: 50% water and 0.1% formic acid as mobile phase. The results obtained for the optimiser are given in Tables 5.3 and 5.4.

Table 5.3: The optimal electrospray tandem mass spectrometric conditions for the detection of DHN-MA. MassHunter software was used to optimise the conditions for DHN-MA detection with the LC-MS/MS.

Compound name	Formula	Nominal mass	Polarity	Ion source
DHN-MA	$C_{14}H_{27}NO_5S$	321.2	Positive	ESI
Precursor ion	Fragmentor	Product ion	Collision energy	Abundance
322.2	60	304.1	4	21037
322.2	60	162	16	9321
322.2	60	164	8	16978
322.2	60	130	16	5773

Table 5.4: The optimal electrospray tandem mass spectrometric conditions for the detection of DHN-MA-d3. MassHunter software was used to optimise the conditions for DHN-MA-d3 detection with the LC-MS/MS.

Compound name	Formula	Nominal mass	Polarity	Ion source
DHN-MA-d3	$C_{14}H_{24}D3NO_5S$	324.2	Positive	ESI
Precursor ion	Fragmentor	Product ion	Collision energy	Abundance
325.2	70	307.3	0	6308
325.2	70	164.1	8	8072
325.2	70	130	12	5711
325.2	70	162	16	4304

From the optimiser reports for DHN-MA and DHN-MA-d3, a precursor ion of 322.2 m/z and product ion of 304 m/z were selected for DHN-MA. However, the product ion of 304 m/z for DHN-MA proved problematic (Section 5.9.2) and therefore the 164 m/z was later used as the product ion of choice for DHN-MA detection. For DHN-MA-d3 a precursor of 325.2 m/z and a product ion of 164.1 m/z were selected. These specific product ions were selected because their abundances were the highest of all product ions detected by the MassHunter software**.**

5.4.4. Chromatographic separation

Since the initial aim was to standardise an analytical method for the simultaneous quantification of 8-OHdG and DHN-MA, the same chromatographic conditions were used for the DHN-MA assay as were used for the 8-OHdG assay (Chapter 4.3.3). Chromatographic separation was performed on a Zorbax SB-Aq (2.1 x 150 mm, 3.5 µm) column (Cat. No. USSQG 01048) purchased from Agilent. The column was protected with a Zorbax SB-Aq (2.1 X 12.5 mm, 5 µm) (Cat. No. 82125-933) guard column also purchased from Agilent. The flow rate was 0.2 ml/min and the injection volume was 20 µl. Electrospray ionisation was performed in the positive ion mode. Nitrogen gas was used as nebulising gas. The optimal elution gradient, as was found for

the 8-OHdG assay (Table 4.4), was used for chromatographic separation of DHN-MA and DHN-MA-d3 (Table 5.5).

Table 5.5: Mobile phase gradient timetable used for the chromatographic separation of 8-OHdG, 2'dG^{15}N, DHN-MA and DHN-MA-d3. The mobile phases used for chromatographic separation were 1 mM ammonium formate buffer pH 3.75 (mobile phase A) and 100% MeCN as mobile phase B.

Time	B %	Flow
0	**5**	**0.2**
2	**5**	**0.2**
10	**26**	**0.2**
12	**50**	**0.2**
13	**50**	**0.2**
15	**100**	**0.2**
21	**100**	**0.2**
26	**5**	**0.2**
41	**5**	**0.2**

Figure 5.1: Chromatogram of DHN-MA obtained when using the standard chromatographic conditions for DHN-MA separation (A) TIC of DHN-MA (B) MRM of DHN-MA. DHN-MA was observed at ± 18 minutes analysed on an LC-MS/MS.

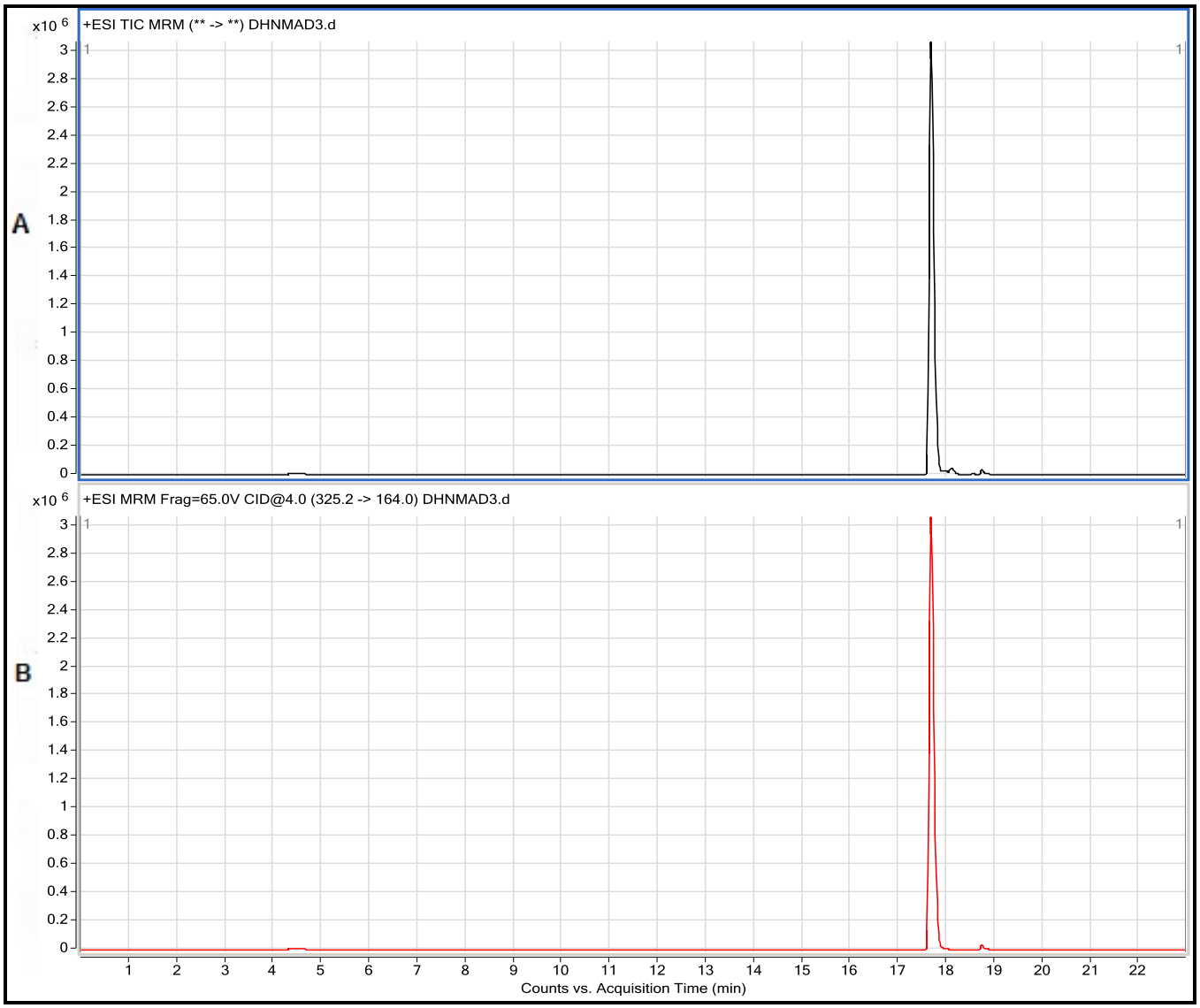

Figure 5.2: Chromatogram of DHN-MA-d3 obtained with chromatographic separation using the same standard chromatographic conditions as for DHN-MA separation. DHN-MA-d3 can be seen at ± 18 minutes analysed on an LC-MS/MS (A) TIC of DHN-MA-d3 (B) MRM of DHN-MA-d3.

DHN-MA (Figure 5.1) and DHN-MA-d3 (Figure 5.2) elute off the column at ± 18 minutes and the 2'dG^{15}N and 8-OHdG elute at ± 9 minutes and ± 11 minutes (Figure 4.2, Chapter 4) respectively. Thus, it will be possible to analyse both DHN-MA and 8-OHdG during the same run.

5.5. Purification of the synthesised DHN-MA and DHN-MA-d3

After the chemical synthesis of DHN-MA and DHN-MA-d3 (as described in Section 5.3), both were separately purified using a HPLC and a fraction collector. An Agilent 1200 series LC (Santa Clara, CA, USA) was used for sample handling as well as for mobile phase delivery. Forty fractions of 20 µl each of the synthesised DHN-MA were injected and a constant flow rate of 0.2 ml/min was maintained throughout the run. This was coupled to an Agilent 1200 series diode array detector (Santa Clara, CA, USA). Fraction collections were performed on an Agilent 1200 series analytical scale fraction collector (Santa Clara, CA, USA). The same mobile phases and gradient used for LC-MS/MS analysis (Section 5.2.2 and Table 5.5) were also used for the fractionation of DHN-MA and DHN-MA-d3.

The fraction collector (coupled to the HPLC) can be set to collect the fraction either based on time or based on the elution of a peak during the chromatographic run. Therefore, it works either in time based or in signal based mode. Due to the fact that DHN-MA has no chromatophore and can therefore not be detected using a UV detector, the DHN-MA was collected using a time-based strategy. However, the question remained at what retention time the DHN-MA would elute from the chromatographic column since that would ultimately determine the fraction to be collected. Caffeine was used to overcome the problem of DHN-MA not being detectable using a UV detection system. Caffeine (0.325 mg/L) was prepared and separated on the LC-MS/MS using the same mobile phases and chromatographic conditions used on the HPLC-UV. Therefore, one can expect the caffeine and DHN-MA to elute from the chromatographic column at approximately the same time as they would have eluted if they had been separated on the HPLC-UV system. Using the LC-MS/MS, the elution of both caffeine and DHN-MA could be monitored using mass spectrometry. After the elution times of both compounds had been determined using the LC-MS/MS (retention times of 16 minutes for caffeine and 18 minutes for DHN-MA), the same sample (containing both caffeine and DHN-MA) was analysed on the HPLC-UV. Although DHN-MA could not be detected using UV detection, caffeine could easily be detected. Since the retention time of caffeine was exactly the same on the LC-MS/MS and HPLC-UV (16 minutes), it was concluded that the retention time of DHN-MA should also be relatively constant. Therefore, it was decided to collect the fraction from 17.5 to 18.5 minutes. Although the exact retention time of DHN-MA remained unknown on the HPLC-UV, the fraction collected should contain the synthesised DHN-MA.

The 40 fractions collected were pooled and dried under nitrogen. The dried sample was reconstituted in 1 mM ammonium formate buffer pH 3.75 (1 ml). To determine whether 4-HNE-MA was indeed converted to DHN-MA the mass spectrometer was set to detect both DHN-MA and 4-HNE-MA (Sections 5.4.2 and 5.4.3) by direct infusion. Direct infusion was chosen instead of chromatographic separation as only two metabolites were monitored in the synthesised standard. Therefore, minimal interference with the detection of the two targeted compounds was expected. The results are depicted in Figure 5.3.

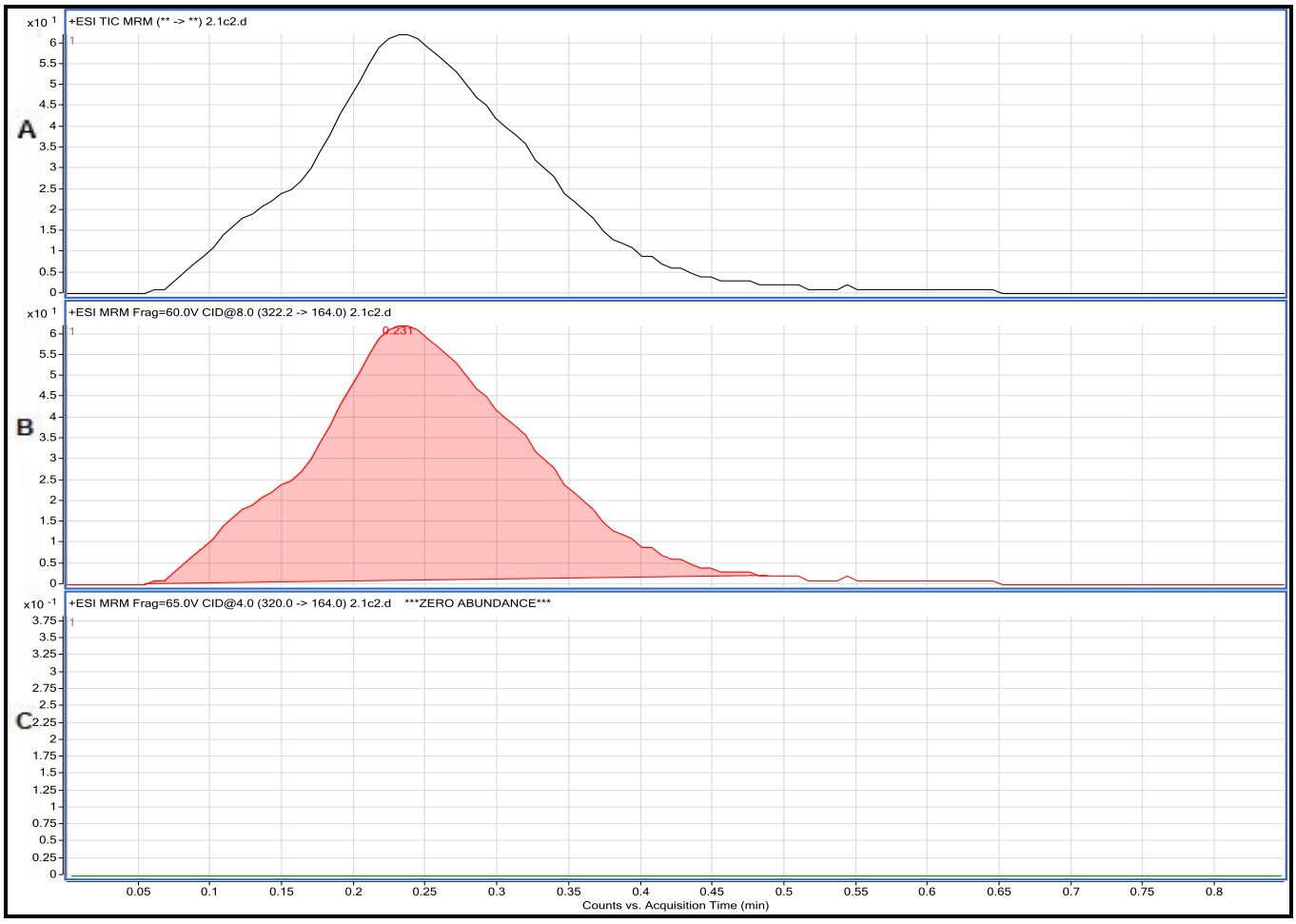

Figure 5.3: LC-MS/MS analysis of the synthesised DHN-MA by direct infusion on a LC-MS/MS (A) TIC of DHN-MA synthesis (B) MRM of DHN-MA (C) MRM of 4-HNE-MA.

From the results it is clear that the fractions collected contained DHN-MA. It is also clear that the synthesis contains DHN-MA only, since no 4-HNE-MA was detected. Since no 4-HNE-MA could be detected in the DHN-MA synthesis it can be concluded that an almost 100% conversion from 4-HNE-MA to DHN-MA was achieved. Therefore, the synthesised DHN-MA could be used as a standard to optimise and validate the DHN-MA quantification assay. The

synthesised DHN-MA-d3 was also purified as described for DHN-MA. An almost 100% conversion of 4-HNE-MA-d3 to DHN-MA-d3 was also obtained (data not shown).

5.6. Quantification of the synthesised DHN-MA and DHN-MA-d3

DHN-MA could not be quantified by weighing the synthesised DHN-MA as the sample quantity was too small to successfully weigh the sample even on a 6- decimal scale. Therefore, it had to be quantified by other means. It was decided to use acetaminophen mercapturic acid (AMA) to quantify DHN-MA and DHN-MA-d3, since it is also a mercapturic acid conjugate. The conditions for the quantification of AMA first had to be optimised on the LC-MS/MS. For the optimisation 10 µl of AMA (20 µg/ml) was directly infused in the MS with 50% MeCN, 50% water and 0.1% formic acid as the mobile phase. The results obtained for the optimiser are given in Table 5.6.

Table 5.6: The optimal electrospray tandem mass spectrometric conditions for the detection of acetaminophen mercapturic acid (AMA). MassHunter software was used to optimise the conditions for AMA detection on the LC-MS/MS.

Compound name	Formula	Nominal mass	Polarity	Ion source
AMA	$C_{13}H_{16}N_2O_5S$	312.1	Positive	ESI
Precursor ion	Fragmentor	Product ion	Collision energy	Abundance
313.1	102	208.1	16	175024
313.1	102	140.1	36	139353
313.1	102	166.1	28	118223
313.1	102	149.1	44	31314

As can be seen in Table 5.6, the mass spectrometric conditions for the detection of AMA were optimised and the product ion of 208.1 m/z was selected for the quantification of AMA since it had the highest abundance. AMA (20 µg/ml) was then analysed on the LC-MS/MS using the same chromatographic conditions (Section 5.4.4) to determine the retention time of AMA. Since

the retention time of AMA was ± 13 minutes (Figure 5.4), it was seen as satisfactory for the quantification of DHN-MA.

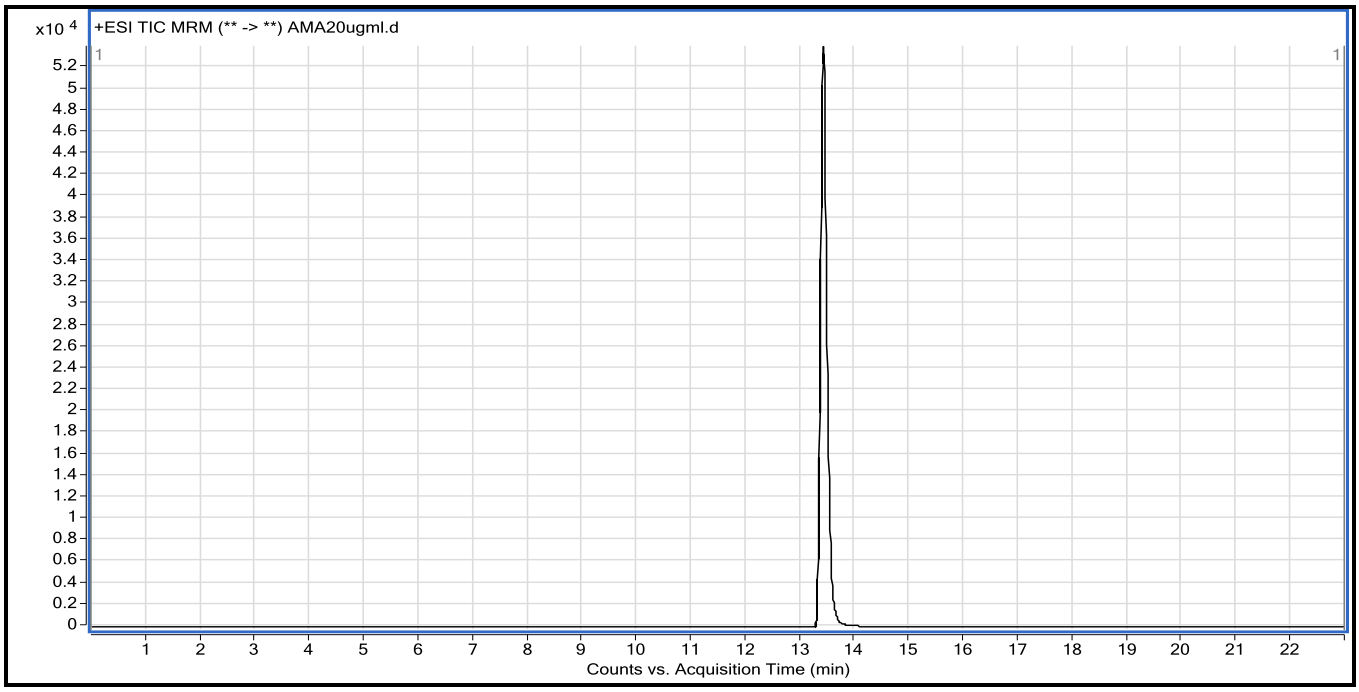

Figure 5.4: Chromatogrpahic separation of acetaminophen mercapturic acid (AMA). The TIC illustrates the retention time of AMA at ± 13 minutes using the standard chromatographic conditions used for the separation of 8-OHdG and DHN-MA.

The next step was to separate DHN-MA and AMA in the same sample using the LC-MS/MS. This was essential in order to quantify the synthesised DHN-MA solution. Twenty µl of a 10 x dilution of the synthesised DHN-MA was added to 20 µl of a 250 ng/ml AMA solution. These samples were analysed on the LC-MS/MS and the results obtained are depicted in Figure 5.5.

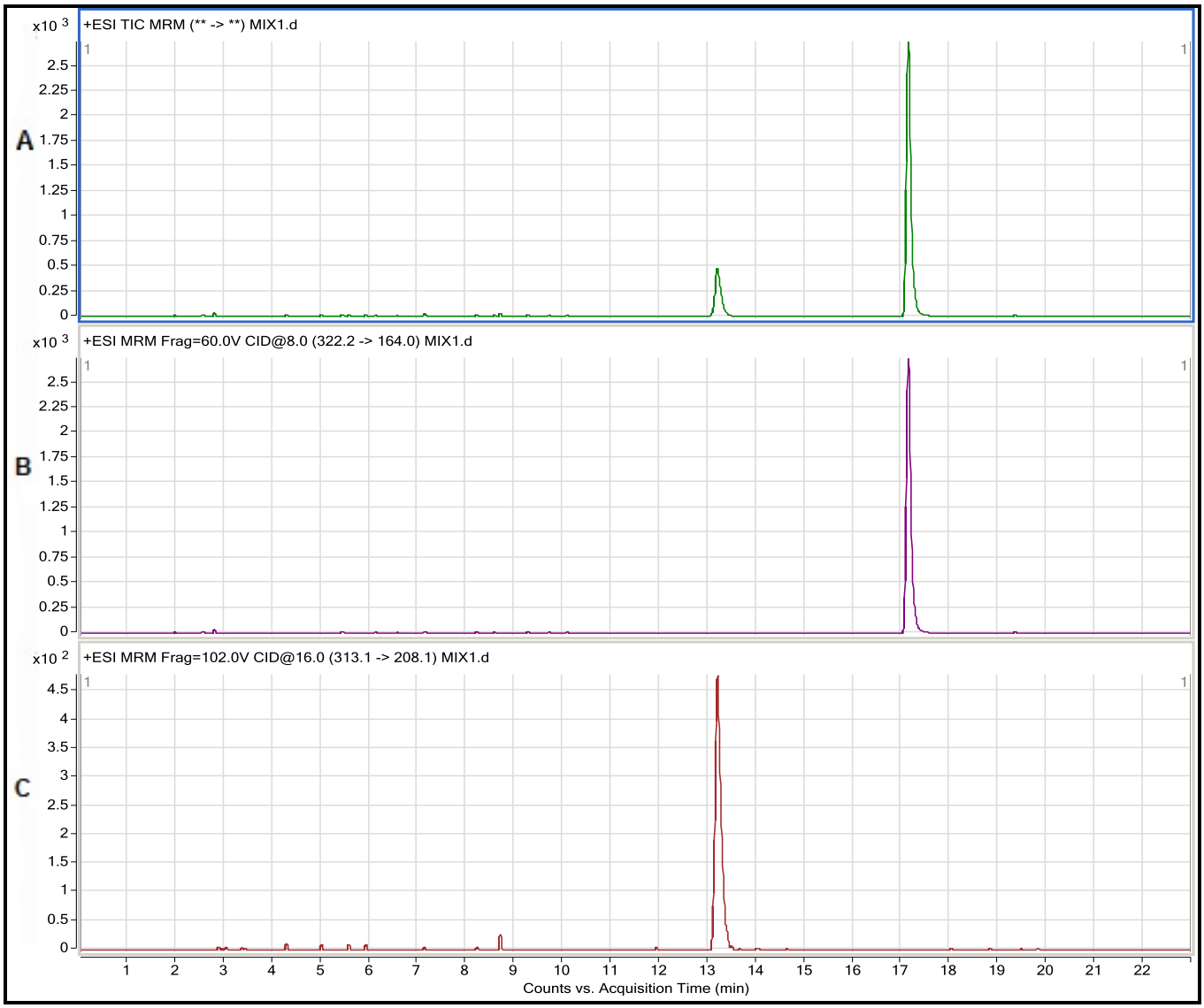

Figure 5.5: Chromatographic separation of AMA and DHN-MA on an LC-MS/MS (A) TIC of AMA (± 13 minutes) and DHN-MA (± 18 minutes) on an LC-MS/MS (B) MRM of DHN-MA on LC-MS/MS (C) MRM of AMA on LC-MS/MS.

From Figure 5.5 the relative concentration of the synthesised DHN-MA was calculated using Equation 5.1.

Equation 5.1: Response factor of DHN-MA to AMA.

$$\text{Response factor} = \frac{\text{Response IS} \times [\text{IS}]}{\text{Response (analyte)} \times [\text{analyte}]}$$

Where:
Response IS = the response of the internal standard (isotope)
[analyte] = the concentration of the analyte
Response (analyte) = the response of the analyte
[IS] = the concentration of the internal standard (isotope).

Since no response factor of DHN-MA to AMA could be calculated, the response factor was taken as one. Therefore, only the relative concentration of DHN-MA could be calculated and not the exact concentration. The relative concentration of the synthesised DHN-MA (dissolved in 1 ml 1 mM ammonium formate buffer pH 3.75) was calculated as 0.56 µg/ml. The same approach was used to determine the relative concentration of the synthesised DHN-MA-d3 (dissolved in 1 ml 1 mM ammonium formate buffer pH 3.75). This concentration was calculated as 9.08 µg/ml.

5.7. Solid phase extraction of DHN-MA from urine

As the initial aim was to develop a single assay to quantify both DHN-MA and 8-OHdG, the same method of sample cleanup used for 8-OHdG (Section 4.4) was used for DHN-MA. A vacuum manifold, (Visiprep™, from Supelco: Cat. No. 5-7030) was used at 125 mm Hg for solid phase extraction. Initially the SPE column was preconditioned with 6 ml 100% methanol (MeOH) and then with 6ml milli Q water. The sample to be analysed (3 ml urine, 200 µl 5M ammonium formate buffer pH 3.75, 50 µl 2'dG^{15}N, 100 ng/ml and 50 µl DHN-MA-d3, 0.908 ng/ml) was applied to the column and the column was washed with 2 ml milli Q water. Elution of DHN-MA was performed using 6 ml 40% MeOH: water.

5.8. The optimised DHN-MA assay

Each SPE column was preconditioned with 6 ml 100% MeOH and 6 ml milli Q water before the sample to be analysed (3 ml urine, 200 µl 5M ammonium formate buffer pH 3.75, 50 µl 2'dG^{15}N, 100 ng/ml and 50 µl DHN-MAd3, 0.908 ng/ml) was loaded onto the column. After the loading step, each column was washed with 2 ml milli Q water. Elution of DHN-MA was performed with 6 ml 40% MeOH: water. The eluted sample was dried under a gentle stream of

nitrogen gas for ± 40 minutes at 37 °C on an AB 2000 Stargate Scientific Nitrogen gas dryer to remove the MeOH. After the nitrogen drying, the remainder of the sample was frozen at -80 °C for 2 hours. This was freeze dried overnight using a Virtis Manual Set freeze dryer. The dried sample was reconstituted in 1 mM ammonium formate buffer pH 3.75 (100 µl) prior to analysis. Chromatographic separation was performed on a Zorbax SB-Aq (2.1 x 150 mm, 3.5 µm) column and was protected with a Zorbax SB-Aq (2.1 X 12.5 mm, 5 µm) guard column. A 1 mM ammonium formate buffer pH 3.75 was used as mobile phase A and 100% MeCN as mobile phase B. A gradient was used for chromatographic separation of DHN-MA (Table 5.5). The flow rate was 0.2 ml/min and the injection volume was 20 µl. Electrospray ionisation was performed in the positive ion mode. Nitrogen gas was used as nebulising gas. The MS/MS was set to detect specific precursor and product ions for DHN-MA (322.2 – 164 m/z) and DHN-MA-d3 (325.2 – 164.1 m/z).

5.9. Validation of the standardised DHN-MA assay

After the DHN-MA assay had been standardised on the LC-MS/MS, the next step was to validate the assay to confirm that it performed according to expectations. For the validation, the solid phase extraction process was evaluated and, the linearity of the assay as well as the performance of the assay when human urine samples were used instead of standards was evaluated.

5.9.1. Linearity of the DHN-MA assay

A dilution range of the newly synthesised DHN-MA and DHN-MA-d3 was prepared according to Table 5.7 and 5.8. A 100-, 125-, 167-, 250- and 500 x dilution range were prepared for each of the synthesised standards. These standards were used to test the linearity of the newly standardised DHN-MA assay (Figure 5.6 and 5.7).

Table 5.7: A dilution range of the synthesised DHN-MA used to evaluate the linearity of the DHN-MA assay. The table shows the final concentration of DHN-MA in each sample.

Sample Number	DHN-MA
1	0.0 ng/ml
2	1.1 ng/ml
3	2.2 ng/ml
4	3.3 ng/ml
5	4.5 ng/ml
6	5.6 ng/ml

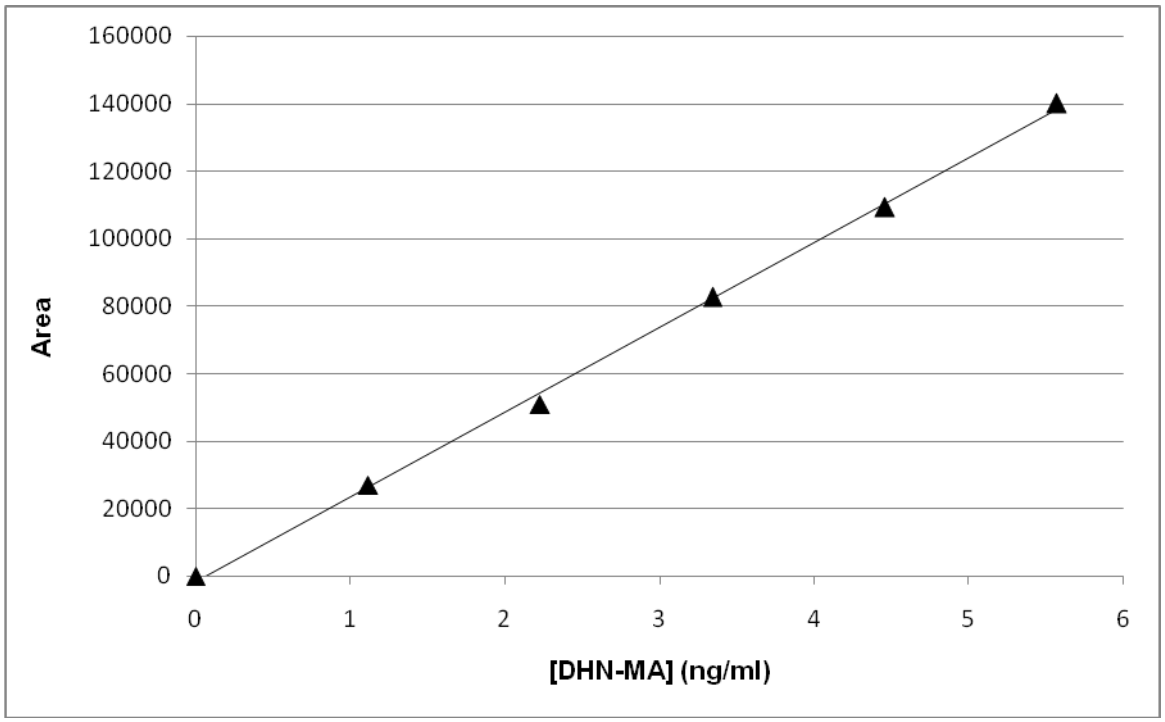

Figure 5.6: The linearity of the DHN-MA assay ($R^2 > 0.99$).

Table 5.8: A dilution range of the synthesised DHN-MA-d3 used to evaluate the linearity of the DHN-MA-d3 assay. The table shows the final DHN-MA-d3 concentration in each sample.

Sample Number	DHN-MA-d3
1	0.0 ng/ml
2	18.2 ng/ml
3	36.3 ng/ml
4	54.5 ng/ml
5	72.6 ng/ml
6	90.8 ng/ml

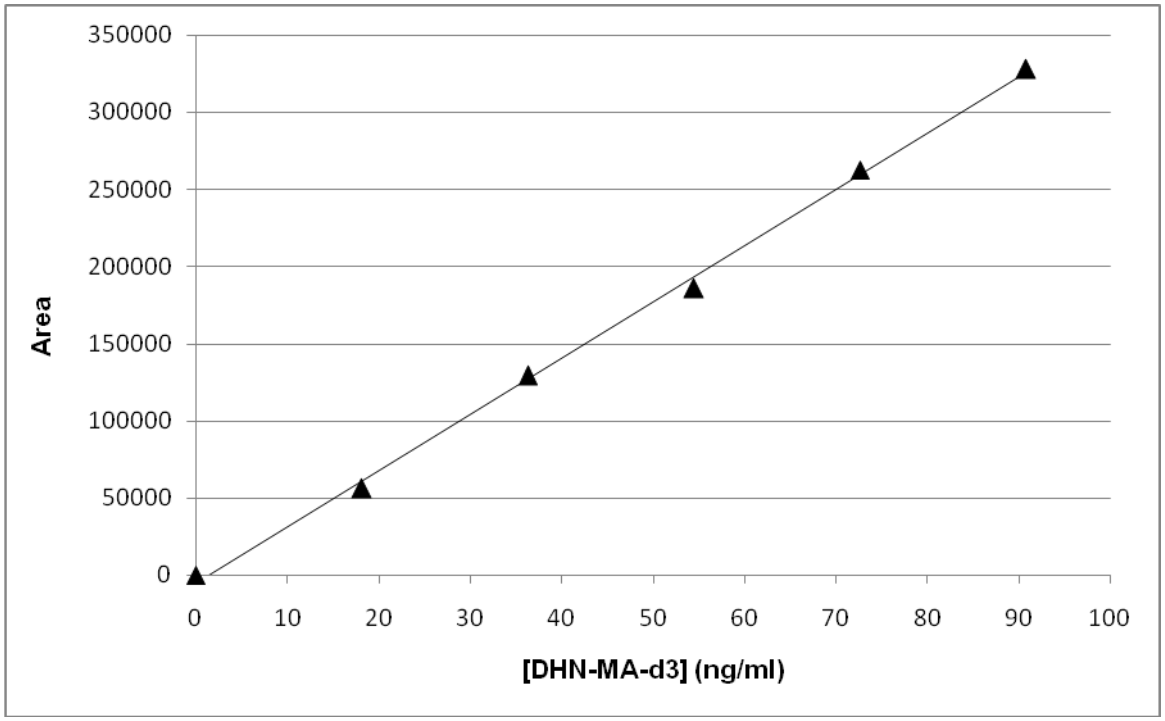

Figure 5.7: The linearity of the DHN-MA-d3 assay ($R^2 > 0.99$).

As can be seen from Figure 5.6 and 5.7, the newly standardised DHN-MA and DHN-MA-d3 are linear over a relatively wide concentration range. An $R^2 > 0.99$ was obtained for both DHN-MA and DHN-MA-d3.

5.9.2. Validation of the solid phase extraction (SPE) process for the DHN-MA assay

As the initial aim was to standardise a single assay for the quantification of DHN-MA and 8-OHdG, the same SPE clean-up process was used for DHN-MA as was used for 8-OHdG (Section 5.7). However, the binding of DHN-MA to the SPE column had to be evaluated. For this purpose, the synthesised DHN-MA (55.7 ng/ml) was used.

Initially, the SPE column was preconditioned with 6 ml 100% methanol (MeOH) and then with 6 ml milli Q water. The sample containing DHN-MA [(100 µl of the synthesised DHN-MA standard with a concentration of 55.7 ng/ml), ammonium formate buffer pH 3.75 (80 µl of a 5 M solution) and 820 µl milli Q water], was applied to the column and the loading eluate was collected for analysis. The SPE column was then washed with 2 ml milli Q water and the eluate was again collected. Elution of DHN-MA was performed using 6 ml 40% MeOH: water and the eluate collected. After metabolite elution, the column was again washed with 6 ml 100% MeCN to determine whether any DHN-MA remained on the SPE column after the initial elution step. All the collected eluates were analysed for the presence of DHN-MA using the optimised DHN-MA assay (Section 5.8) and the results obtained are depicted in Figures 5.8 to 5.11.

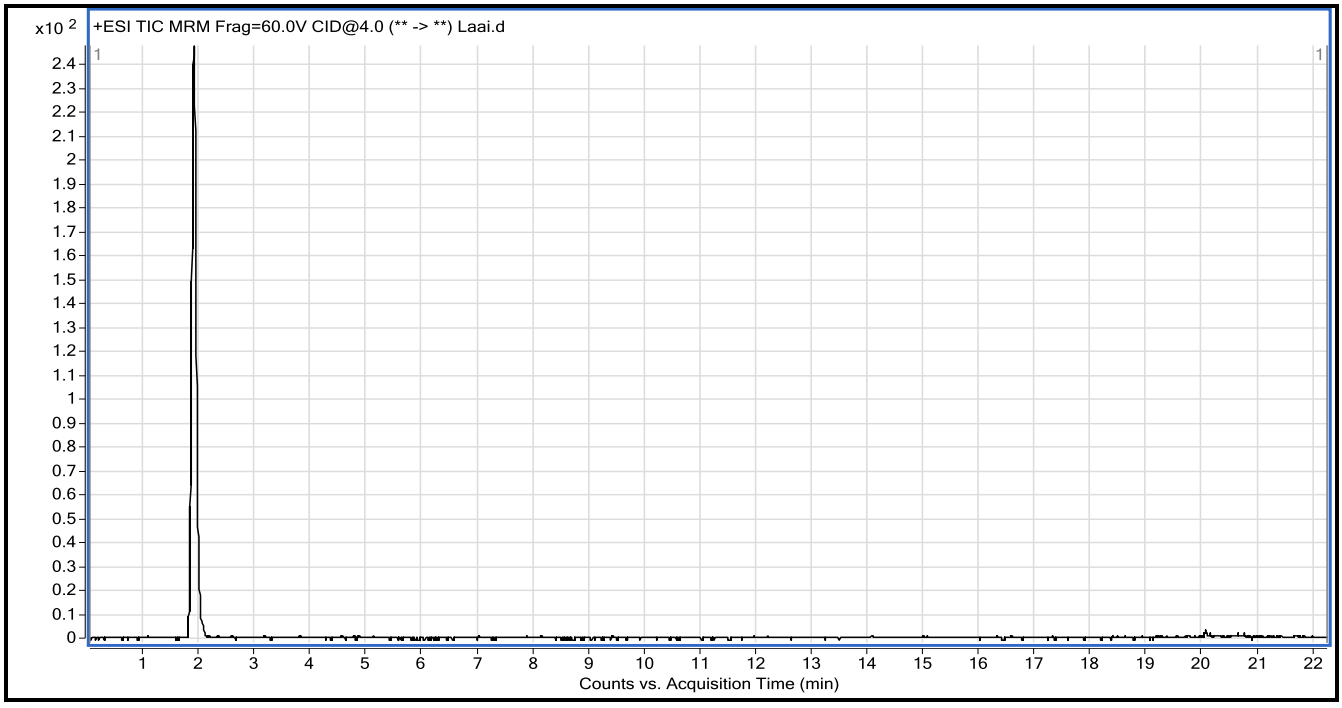

Figure 5.8: TIC of the eluate from the loading step of the SPE process. No DHN-MA (retention time ± 18 minutes) was detected.

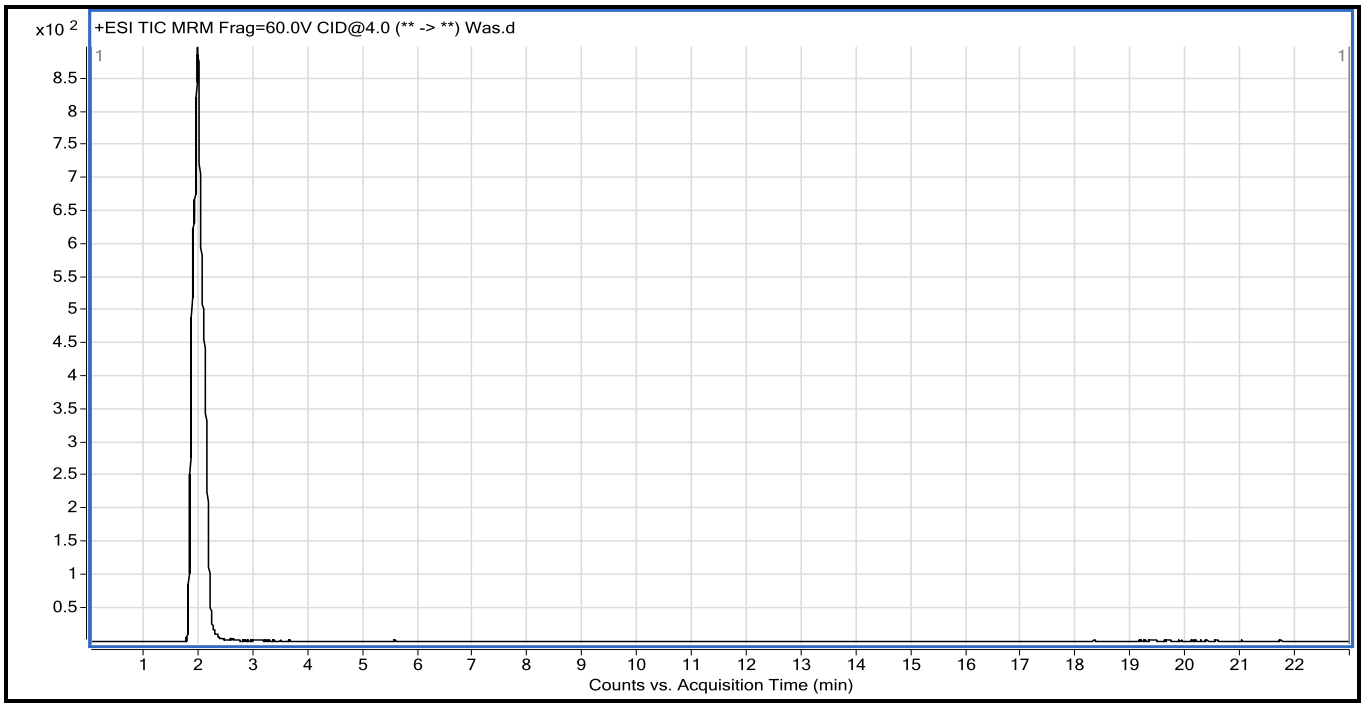

Figure 5.9: TIC of the first washing step of the SPE process. No DHN-MA was detected in the eluate of the wash step.

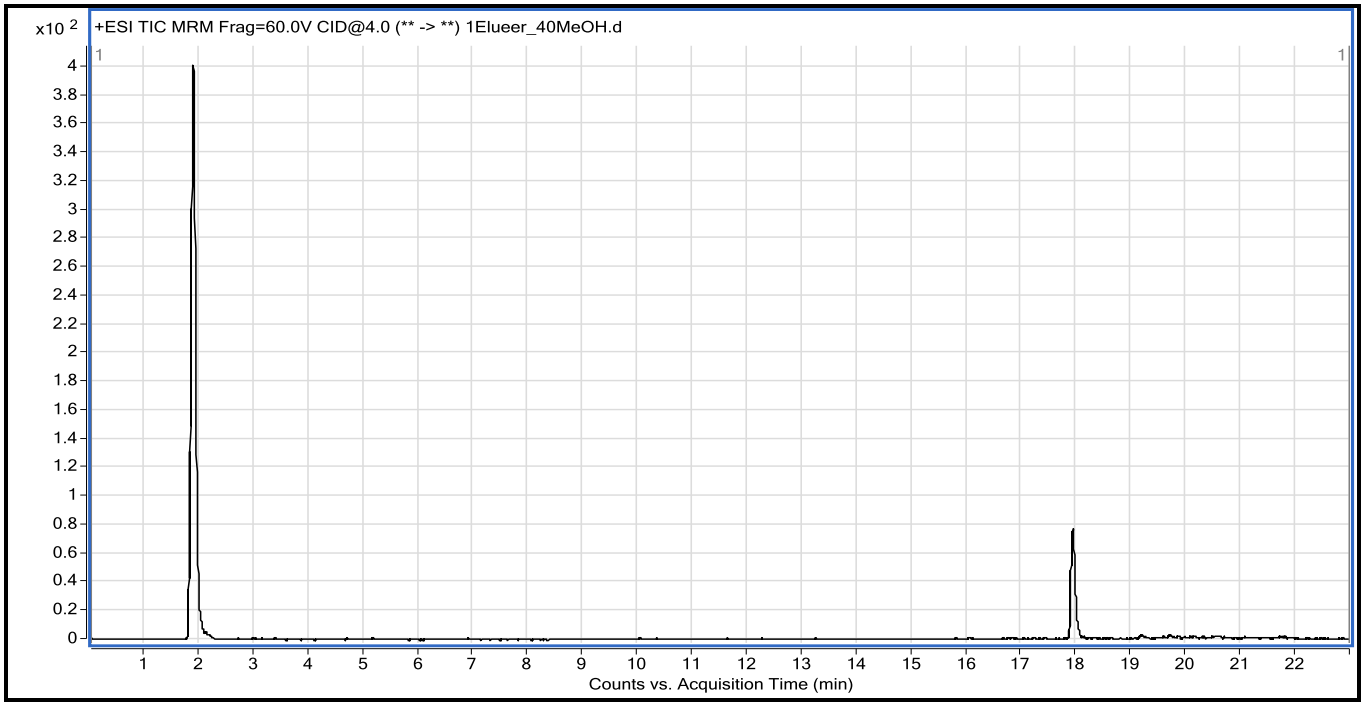

Figure 5.10: TIC of the first elution step with 6 ml 40% MeOH of the SPE process. DHN-MA was detected in the eluate with a retention time of approximately 18 minutes.

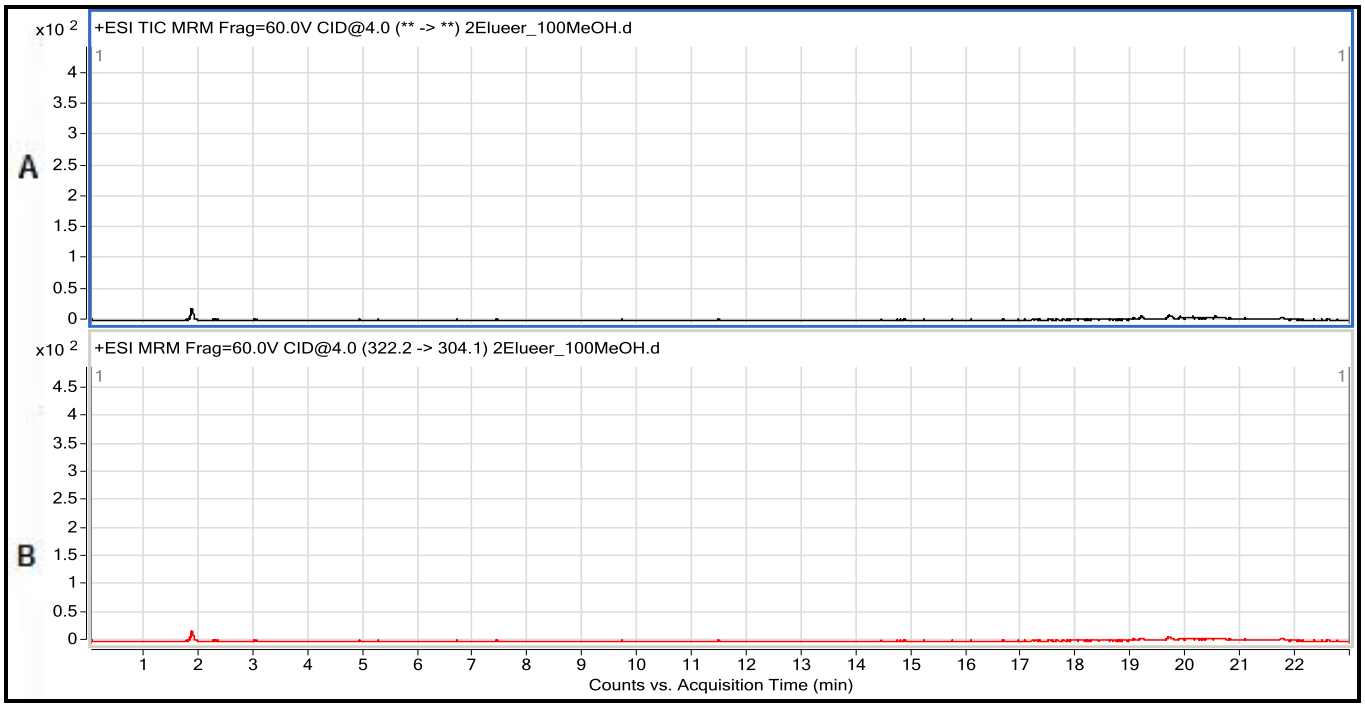

Figure 5.11: TIC of the second washing step with 6 ml 100% MeCN of the SPE process (A) TIC of second elution step of DHN-MA (B) MRM of DHN-MA.

As can be seen from Figures 5.8 and 5.9, no DHN-MA eluted from the SPE column. This means that the DHN-MA was retained on the SPE during the loading and washing steps. DHN-MA was detected in the elution fraction (Figure 5.10) as was expected. However, no DHN-MA was detected in the second elution eluate (Figure 5.11). From these data it is clear that a single elution step with 6 ml 40% MeOH: water was sufficient to remove all the DHN-MA from the SPE column. No DHN-MA remained on the SPE column after the first wash step and therefore no additional elution step was required.

Another concern was the metabolite that eluted at approximately 2 minutes on the chromatogram (Figures 5.8 to 5.11). Although the retention time differed dramatically from that of DHN-MA (retention time ± 18 minutes), the identity of this peak at ± 2 minutes was investigated.

The metabolite that elutes at ± 2 minutes was of concern since the LC-MS/MS was set to detect all metabolites with a precursor ion of 322 m/z and product ion of 304 m/z, at that stage. However, it was found that the peak at 2 minutes was not DHN-MA as its precursor ion and daughter ions differed from that of DHN-MA (Figure 5.12).

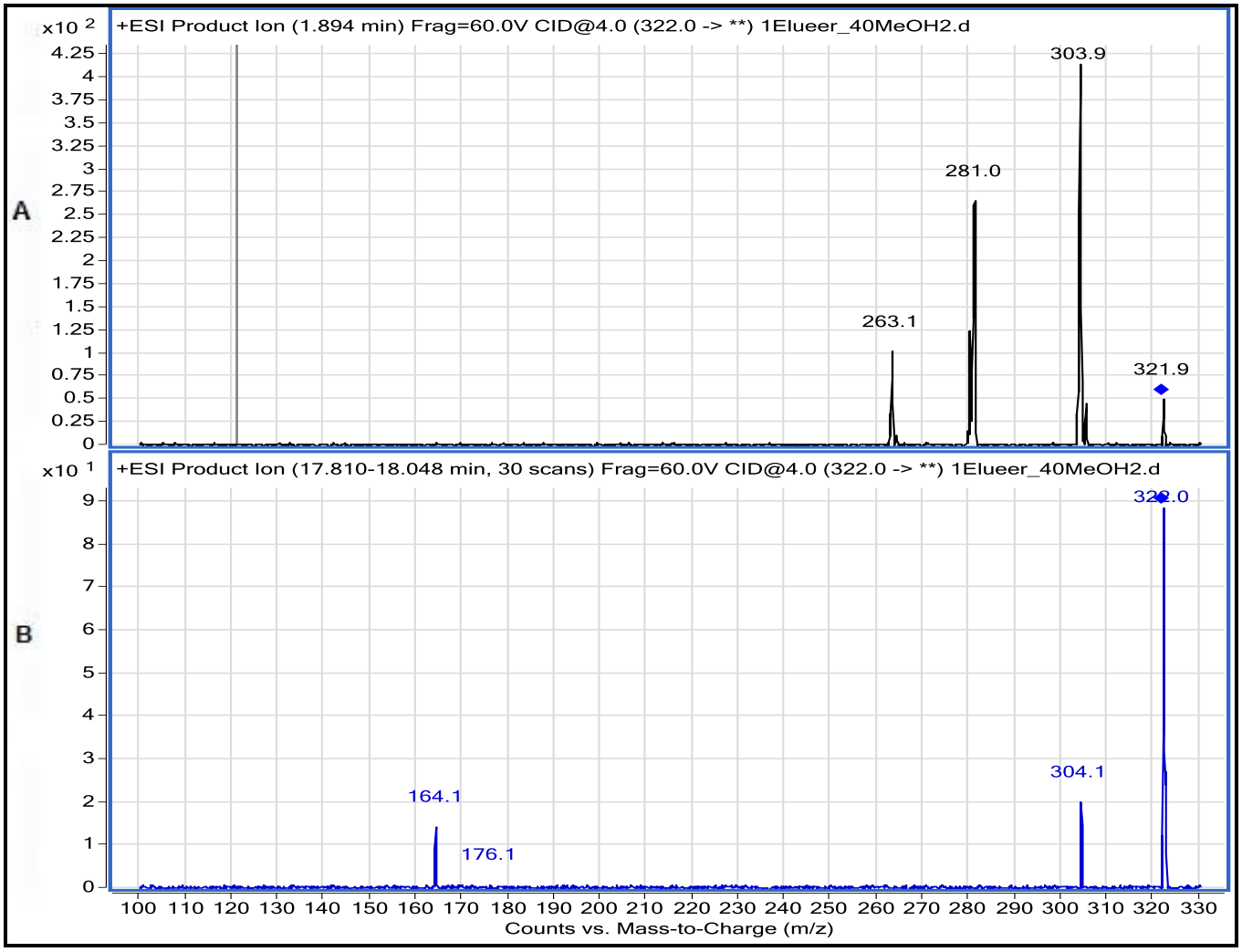

Figure 5.12: Chromatogram of the product ion spectra of the contaminant metabolite that eluted at approximately 2 minutes as well as the product ion spectra of DHN-MA. (A) Product ion scan of the contaminant. This spectrum shows a very similar precursor ion (321.9 m/z) to DHN-MA (322.0 m/z) as well as precursor ion (303.9 m/z) (B) Product ion scan of DHN-MA.

Although the contaminant had almost the same precursor ion (321.9 m/z) compared to that of DHN-MA (322.0 m/z) the product ions differed markedly. The product ions obtained for the contaminant were 303.9, 281.0 and 263.1 m/z. However, the product ions obtained for DHN-MA were 304.1, 164.0, 162.0 and 130.0 (Table 5.3). Therefore, the contaminant peak that eluted at approximately 2 minutes during the validation of the SPE process (Figures 5.8 to 5.11) cannot be DHN-MA and, consequently its presence could be ignored as it eluted far from the other metabolites of interest (DHN-MA and 8-OHdG).

An MRM was used to quantify DHN-MA, monitoring for a specific precursor and product ion. Initially, 322 m/z was used as the precursor ion and 304 m/z as the product ion since 304 m/z gave the highest abundance during optimisation (Table 5.3). However, Rathahao *et al*., (2005) suggested that a product ion of 304 m/z should not be used for the detection of DHN-MA but rather 164 m/z. Since the product ion of 304 m/z is formed by the elimination of water, and several urinary compounds containing a hydroxylated group could easily eradicate a water molecule, the loss of 18 m/z is not specific enough to use for an MRM and should be avoided. Therefore, it was decided to use a product ion of 164 m/z and precursor ion of 322 m/z for DHN-MA quantification, as also recommended by Alary *et al.,* (1998).

5.9.3. Measuring DHN-MA in human urine

It was concluded that the SPE method, optimised for 8-OHdG, worked well for DHN-MA in retaining the metabolite of interest during the SPE loading and washing steps. The elution step also performed well for eluting DHN-MA from the SPE column. The next step was to test the performance of the standardised DHN-MA assay on urine samples. For this purpose, a control urine sample (Section 3.1.3) was analysed and the results are depicted in Figure 5.13.

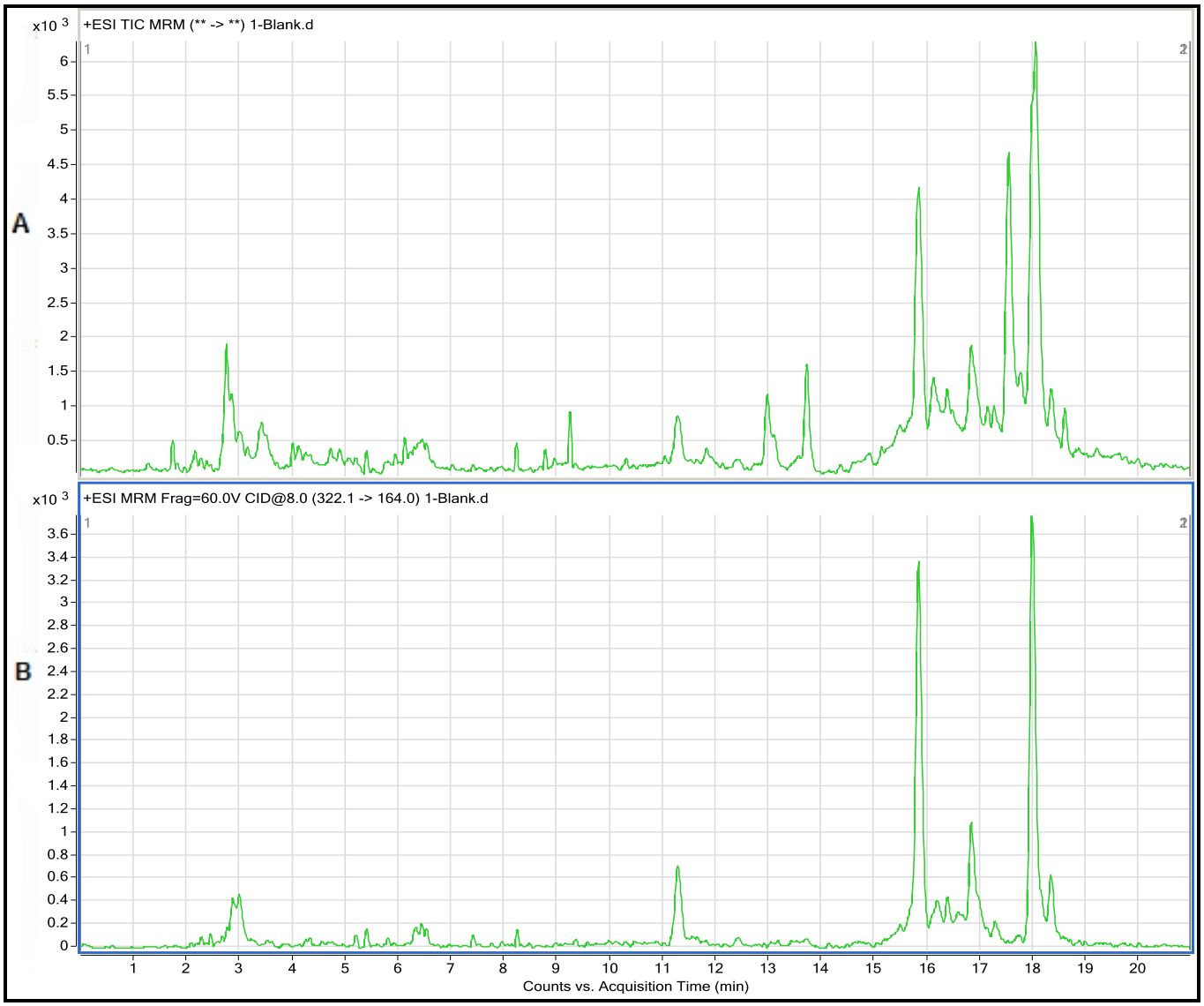

Figure 5.13: Chromatogram of a control urine sample (a) TIC showing co-eluting peaks at the retention time where DHN-MA elutes (± 18 minutes) (b) MRM of DHN-MA.

As can be seen in Figure 5.13, numerous other metabolites co-eluted with DHN-MA and also presented the same product and precursor ions. Since an MRM was used for DHN-MA detection, using a precursor ion of 322.2 m/z and a product ion of 164.1 m/z, any other metabolites displaying the same product and precursor ions will also be detected with the MRM. Thus, only a difference in retention time could be used to discriminate between DHN-MA and other metabolites displaying the same product and precursor ions. Since numerous metabolites with the same product and precursor ions eluted at approximately the same time as DHN-MA, it could not be said for certain which peak on the chromatogram presented DHN-MA. Another problem is that different samples might contain different contaminants eluting at approximately the same time as DHN-MA and having the same product and precursor ions as

DHN-MA. Therefore, numerous peaks might appear on the chromatogram at the time interval where DHN-MA usually elutes from the column, making quantification of DHN-MA in different urine samples basically impossible with the standardised method.

The reason for numerous metabolites eluting from the column at the same time as DHN-MA, is that DHN-MA elutes from the column only at 100% MeCN. This means that very little chromatographic separation still occurs in the sample and all the metabolites still on the column elute simultaneously when 100% MeCN is applied to the column. Therefore, new chromatographic conditions were needed where DHN-MA eluted from the column before the 100% organic mobile phase was reached.

5.10. Additional modifications to the DHN-MA assay

Since the newly standardised DHN-MA assay did not perform to satisfaction, additional modifications were considered. Firstly, different reverse phase chromatographic columns were tested in an effort to elute the DHN-MA from the column before 100 % organic mobile phase was applied. Secondly, a normal phase silica column was used for the chromatography of DHN-MA. Lastly, the effect of different pH ranges on the DHN-MA assay was evaluated.

5.10.1. The effect of different reverse phase chromatographic columns on the DHN-MA assay

The performance of different chromatographic columns was tested on the chromatography of DHN-MA. A Zorbax SB-C18 (2.1 mm x 15 cm) from Agilent (Cat. No. 883700.922) was selected as well as a Luna 5u C8 (2) 100A (150 x 2.00 mm, 5 µm) (Cat. No. 00F-4249-BO) from Phenomenex. The chromatographic separation of DHN-MA-d3 was tested on both these columns using the same gradient (Table 5.5) and mobile phases (1 mM ammonium formate buffer pH 3.75 and 100% MeCN) and compared to the separation obtained using the Zorbax SB-Aq column (used up to this point and also used for 8-OHdG chromatography). The results obtained are illustrated in Figures 5.14 and 5.15.

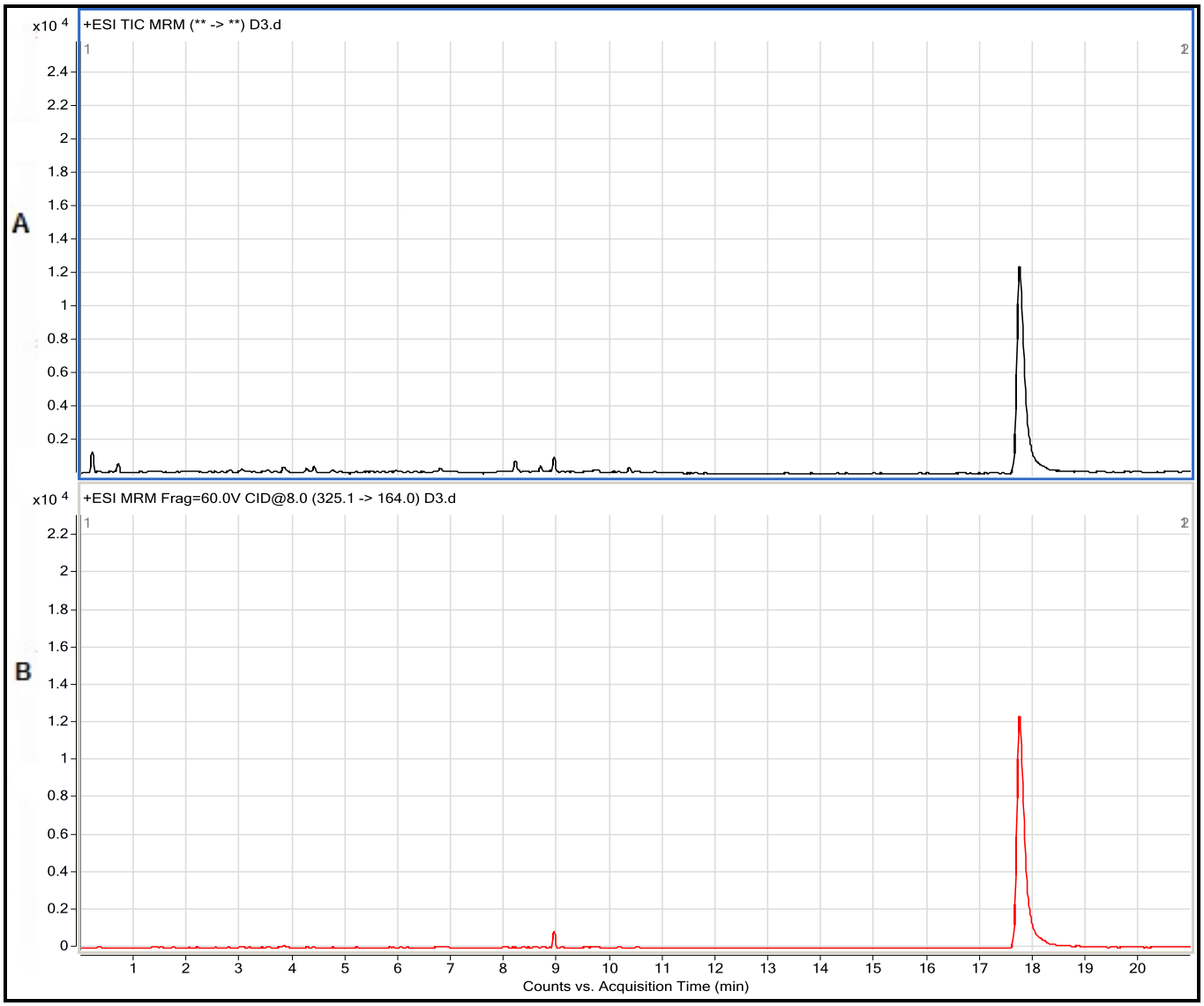

Figure 5.14: Chromatographic separation of DHN-MA-d3 on a C18 column (A) TIC obtained for DHN-MA-d3 (B) MRM of DHN-MA-d3. DHN-MA-d3 eluted from the column at ± 18 minutes.

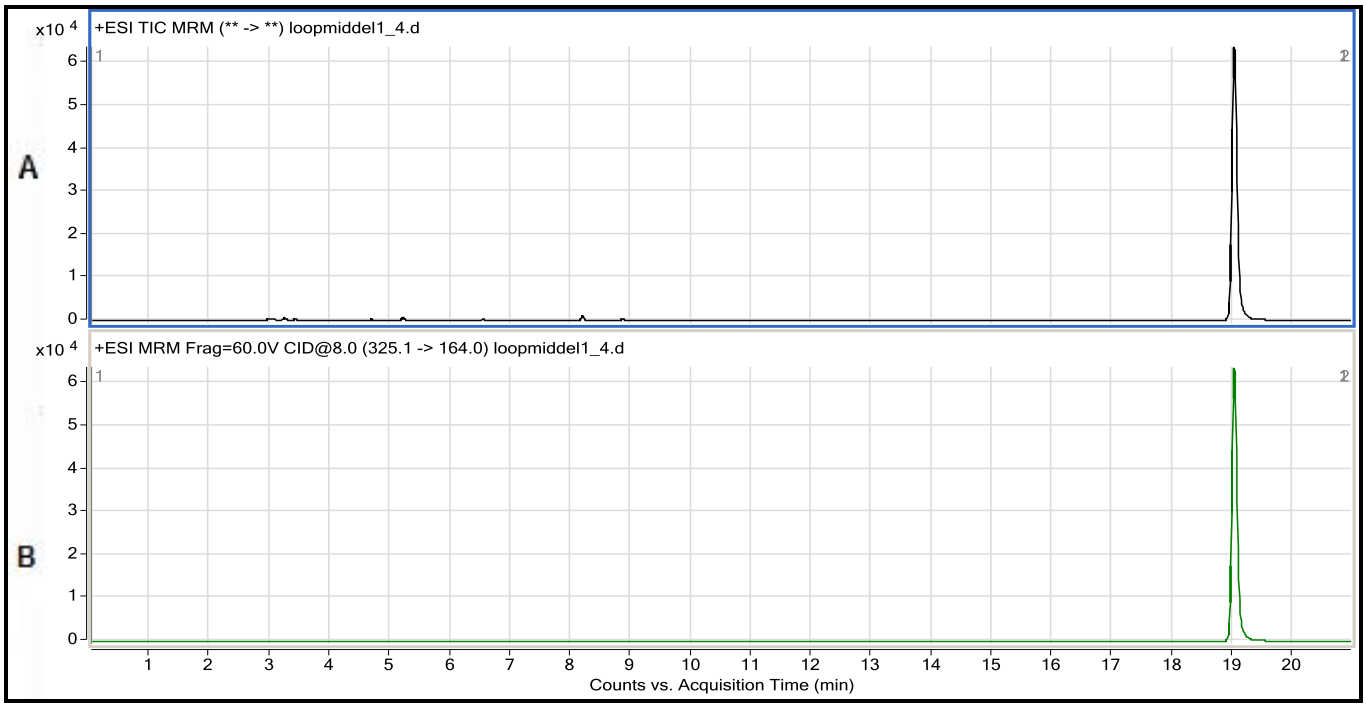

Figure 5.15: Chromatographic separation of DHN-MA-d3 using a C8 column (A) TIC of DHN-MA-d3 (B) MRM for DHN-MA-d3. DHN-MA-d3 eluted from the column at ± 19 minutes.

No signifficant difference was observed in the retention times of DHN-MA-d3 using the C18 column (Figure 5.14), the C8 column (Figure 5.15) or the Aqua column (Figure 5.2). Using all three columns, the DHN-MA-d3 only eluted from the column when 100% MeCN was applied. Since the aim was to get DHN-MA-d3 to elute from the column before 100% organic phase was applied, none of the tested columns proved satisfactory.

5.10.2. The effect of a normal phase chromatographic column on the DHN-MA assay

Next a normal phase Ascentis^{TM} Si (15 cm x 2.1 mm 5 µm) column from Supelco (Cat. No. 581509-U) was used for chromatographic separation of DHN-MA-d3. With this column, isocratic elution with mobile phases MeCN 100% (A) and 1 mM ammonium formate buffer pH 3.75 (B) were used. The following isocratic elutions were selected and tested: firstly 90% A and 10% B, secondly 80% A and 20% B, thirdly 95% A and 5% B and lastly 99% A and 1% B, and the results are given in Figure 5.16.

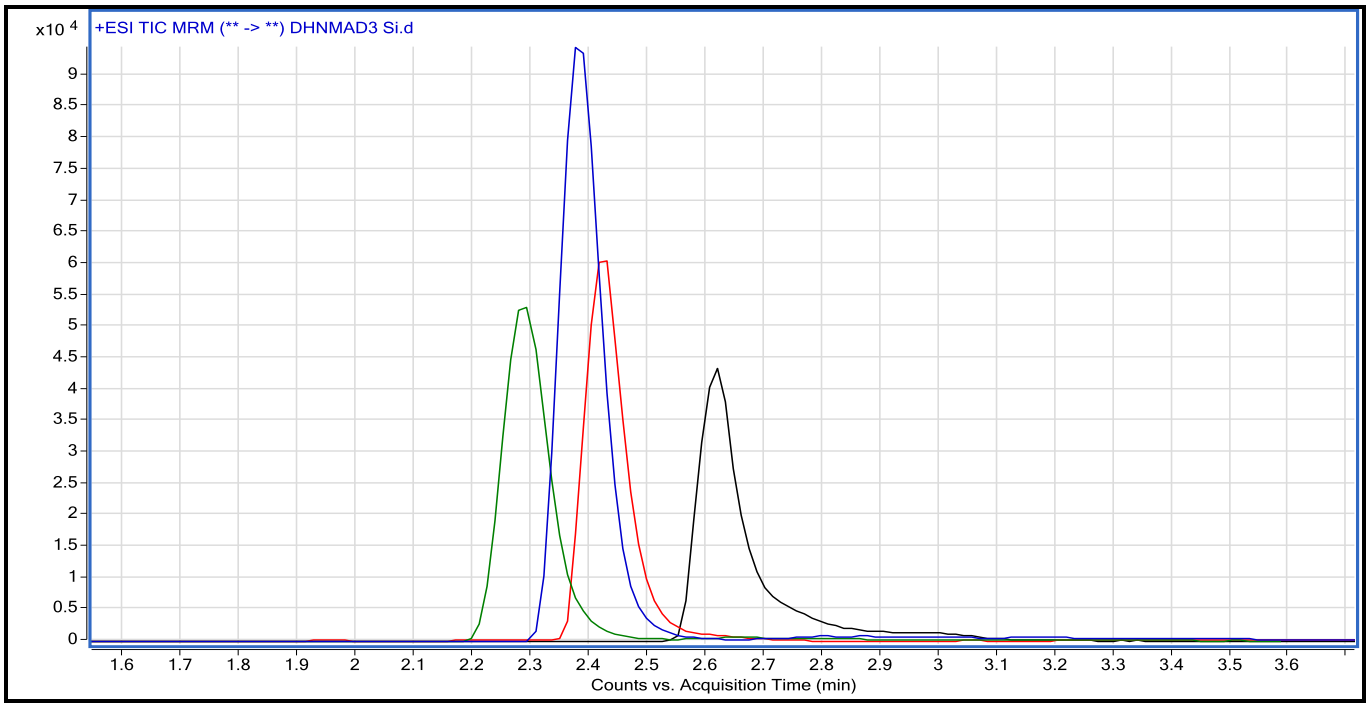

Figure 5.16: Overlay of chromatographic separation of DHN-MA-d3 on an Ascentis™ Si column using different concentrations mobile phases. The green peak was obtained with 80% mobile phase A and 20% mobile phase B. The blue peak is the result of 90% mobile phase A and 10% mobile phase B. With 95% mobile phase A and 5% mobile phase B, the red peak was obtained, while the black peak was obtained with 99% mobile phase A and 1% mobile phase B.

As can be clearly seen from Figure 5.16, the Ascentis™ Si column could not retain DHN-MA, not even when 99% MeCN (and 1 mM ammonium formate buffer pH 3.75) was used as mobile phase. Therefore, the idea of using a normal phase column instead of a reverse phase column was rejected.

5.10.3. The effect of pH on the chromatography of DHN-MA

A Zorbax SB-C18 (2.1 mm x 15 cm) from Agilent was again used and three different pH ammonium formate buffers (mobile phase B) were prepared (pH 5, 6 and 7.5 respectively). The standard gradient was used for chromatography (Table 5.5).

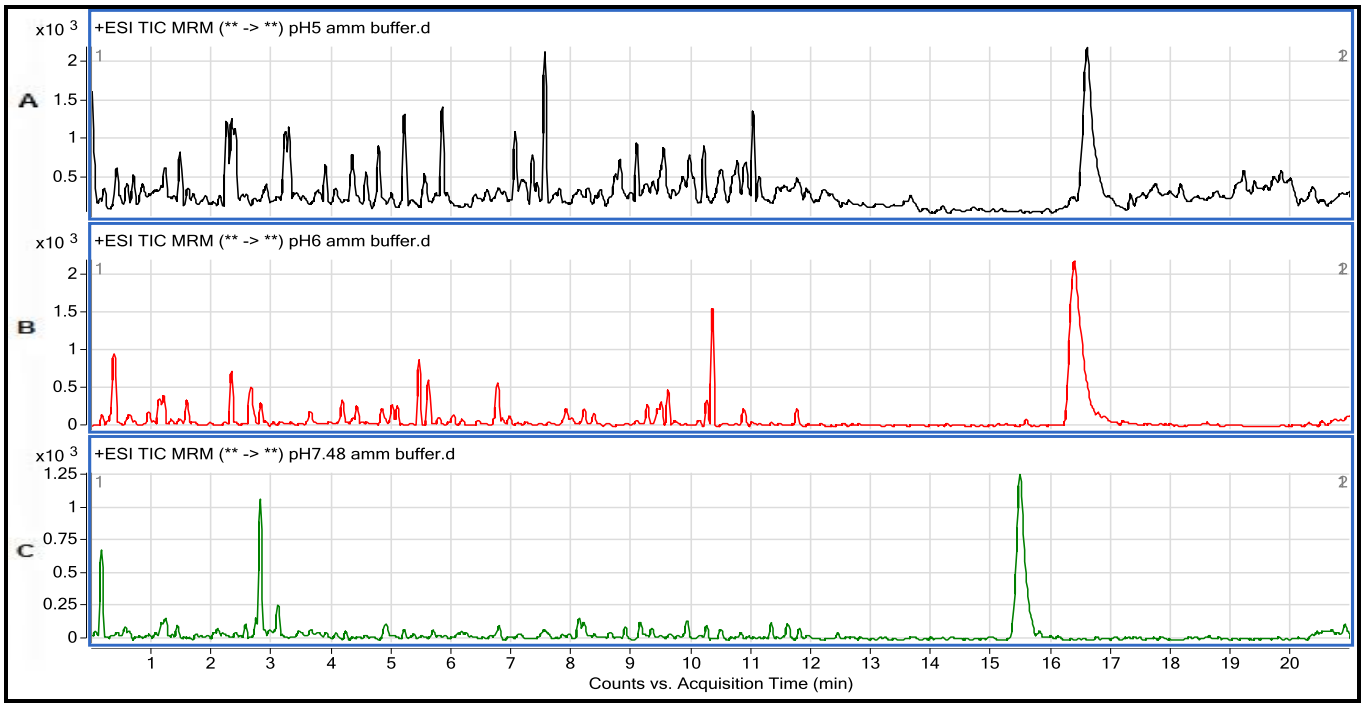

Figure 5.17: Chromatogram of DHN-MA-d3 obtained with the C18 column with different pH buffers of ammonium formate (a) TIC of DHN-MA-d3 obtained with an ammonium formate buffer of pH5 (b) TIC of DHN-MA-d3 obtained with a pH6 ammonium formate buffer and (c) TIC of DHN-MA-d3 obtained with a pH of 7.5 ammonium formate buffer.

Different pH ranges of the mobile phase, 1 mM ammonium formate buffer, show that a higher pH (Figure 5.17.c) does not retain DHN-MA-d3 as much as a mobile phase with a lower pH (Figure 5.17.a). However, DHN-MA-d3 eluted at ± 15 minutes with a pH of 7.5. At 15 minutes, the gradient (Table 5.5) is already on 100% MeCN, thus it does not solve the problem of co-eluting peaks.

5.11. Conclusions on the optimised DHN-MA assay

Although the initial aim was to optimise and standardise a single analytical assay to quantify both 8-OHdG and DHN-MA in urine samples, this was not possible within the scope and timeframe of this study. An analytical method was standardised and validated for the quantification of 8-OHdG using an LC-MS/MS and a stable isotope. As discussed in Section 4.6, the 8-OHdG assay was validated and performed satisfactory using human urine. However, the application of this method for the quantification of DHN-MA proved problematic.

Since DHN-MA and its stable isotope, DHN-MA-d3, was not commercially available, both had to be synthesised and purified. As the synthesis could only be performed on a very small scale,

the synthesised standards could not be dried and the weight determined effectively. Therefore, the two synthesised standards were dissolved in 1 ml, 1 mM ammonium formate buffer pH 3.75 respectively. Acetaminophen mercapturic acid (AMA) was commercially available and it was used for the relative quantification of DHN-MA and DHN-MA-d3. With the standards synthesised, purified and quantified, DHN-MA was used to test the performance of the standardised DHN-MA assay. The assay performed reasonably when standards were used, but when urine samples were used, the results were totally unreliable. Not only did numerous metabolites elute from the chromatographic column at the same time when DHN-MA did, but some of the eluting metabolites also displayed the same product and precursor ions used to detect DHN-MA. This was the result of DHN-MA only eluting from the column when 100% organic phase was applied. Because no gradient was applied any longer, metabolites did not elute form the column according to their polarities anymore and numerous metabolites eluted at once. This resulted in numerous peaks being detected around the DHN-MA peak and it could not be established for certain which peak represented DHN-MA in the sample.

Different approaches were followed in an effort to enhance the chromatographic separation of DHN-MA. Unfortunately, none were successful. Due to the time limitation on this study, it was decided to only use the standardised 8-OHdG assay to study the 409 participants described in Section 3.1.3. It was also realised that a single assay to quantify both 8-OHdG and DHN-MA may not be viable. A new approach is needed to successfully and reliably quantify DHN-MA.

CHAPTER 6

Results and discussion

6.1. Introduction

The aim of this study was to develop an LC-MS/MS assay to successfully and reliably quantify urinary 8-OHdG and DHN-MA as markers of oxidative stress and to calculate the correlation between ROS, 8-OHdG and DHN-MA. However, due to a number of difficulties with the quantification of DHN-MA (Chapter 5), it was decided to only quantify urinary 8-OHdG and serum ROS levels.

Participating study subjects in the SABPA study included 200 African (100 males and 100 females) and 209 Caucasian (101 males and 108 females) teachers in the Potchefstroom area of the North-West province of South Africa. Ten hour fasting baseline urine and serum samples were collected in 2008 and 2009 for the quantification of ROS, 8-OHdG and DHN-MA. The newly optimised and validated 8-OHdG assay (Section 4.5) was then used to quantify urinary 8-OHdG levels in all the SABPA participants, and the ROS assay (Section 3.3) was used to quantify serum ROS levels. The study was approved by the Ethics Committee of the North-West University (06M09 and NWU-00036-07-S6).

6.2. Urinary 8-OHdG levels

The newly optimised 8-OHdG assay (Section 4.5) was used to quantify urinary 8-OHdG levels for all the SABPA participants (Appendix A). Firstly, the Shapiro Wilk test for normality was applied to the data. From Figure 6.1 it is clear that normality was rejected ($p < 0.01$). Although sample sizes were large in all groups, non-parametric tests were employed for further data analysis based on the skewness in the data (Figure 6.1).

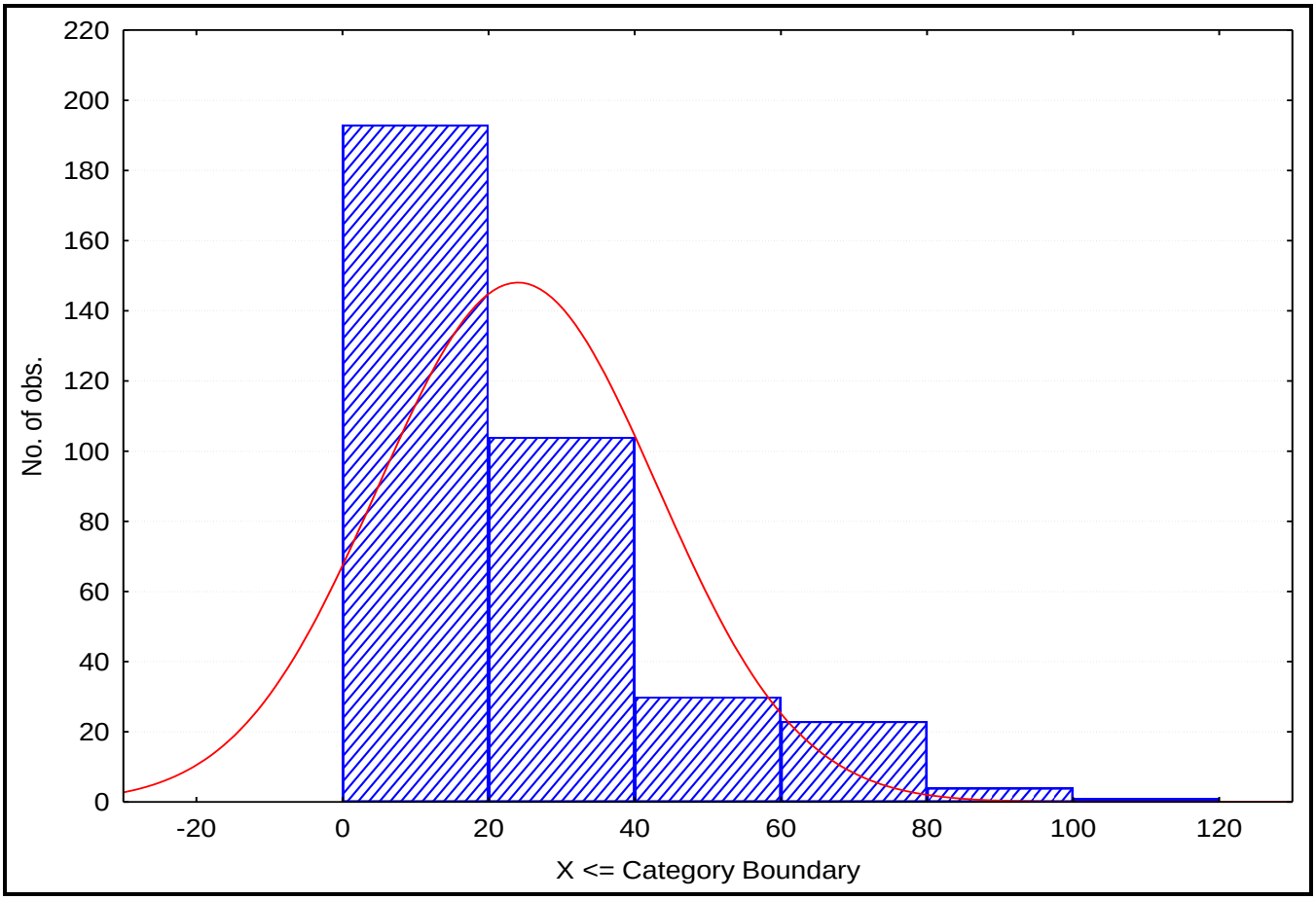

Figure 6.1: Distribution of the urinary 8-OHdG data. Since the data were skewed ($p < 0.01$), non-parametric tests were used to further analyse the data.

Before the data were analysed, outliers were removed by the use of Tukey's method, which defines outliers as those data points falling outside the control limits. The control limits are defined as three interquartile ranges below the 25th percentile or above the 75th percentile (Tukey, 1977). Because initial investigation of the data revealed that the 8-OHdG levels might differ between some of the groups, each of the four groups was separately assessed to identify outliers in that group. After removal of the outliers, 84 African females, 86 African males, 96 Caucasian females and 87 Caucasian males remained.

A BDM test was used to test the significance of the interaction between ethnicity and gender (Figure 6.2). Since no interaction was found ($p = 0.32$), the significance of the main effect could be investigated by pooling the data (i.e. ethnicity and gender). This means that the gender effect is investigated by ignoring ethnicity and vice versa. However, because the data are skewed (Figure 6.1) and the ranges differ considerably between groups, pooling of data may not be advisable. Therefore, it was decided to analyse the main effect of gender on different levels of ethnicity and vice versa. Mann-Whitney U tests were used for this purpose while box plots were used for schematic representations of the data.

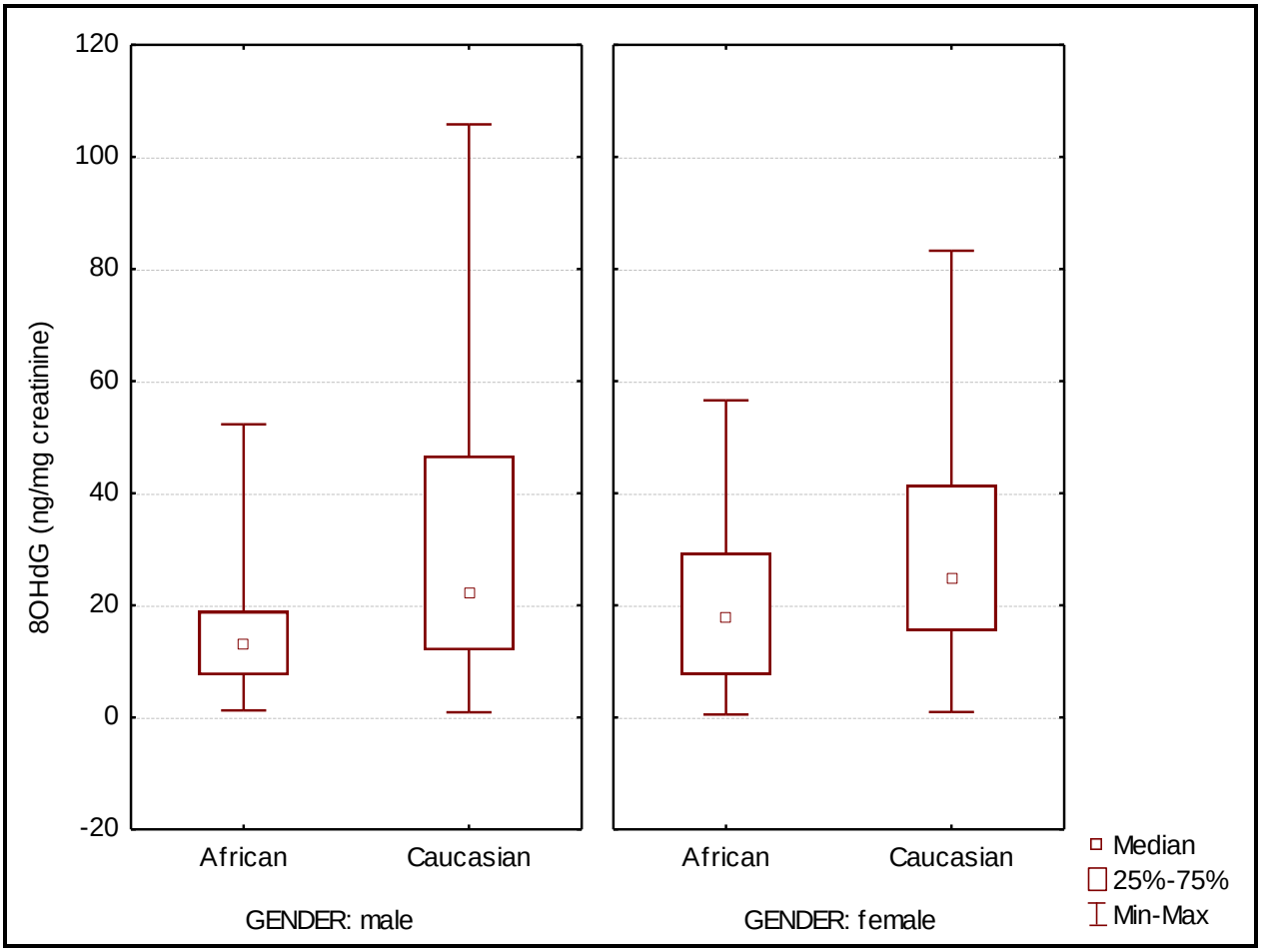

Figure 6.2: Schematic representation of the 8-OHdG data for the SABPA participants after outliers were removed from the data set by the use of Tukey's method. A BDM test was used to test the significance of the interaction between ethnicity and gender. No interaction was evident (p = 0.32).

6.2.1. The effect of gender on urinary 8-OHdG levels

The first step in comparing the different groups was to compare the measured 8-OHdG in males to that of the females for each ethnic group (Figures 6.3 and 6.4).

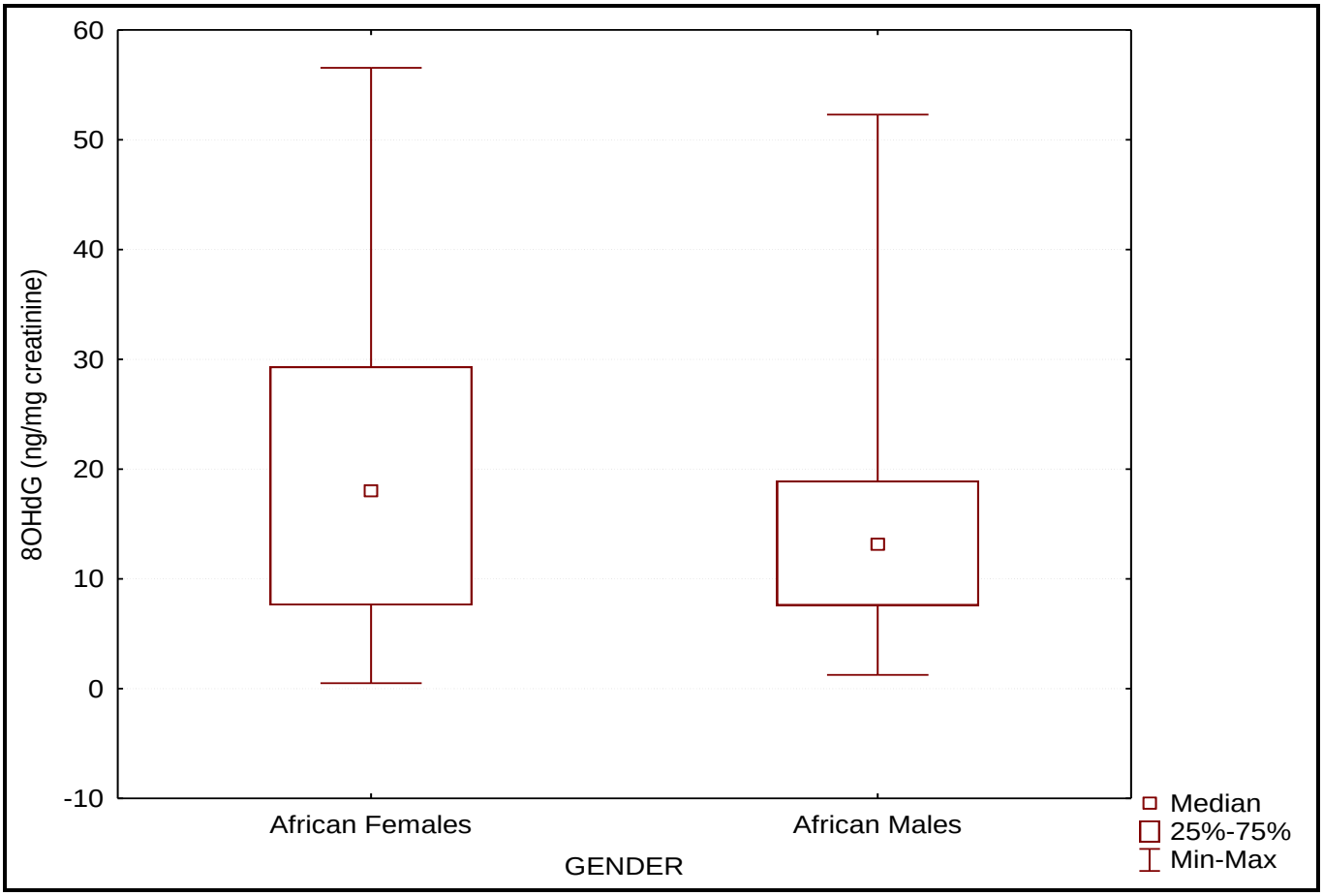

Figure 6.3: Urinary 8-OHdG levels of African females (n=84) and African males (n=86). p = 0.053.

For African men, the median urinary 8-OHdG level was 13.07 ng/mg creatinine and that of African females was 17.6 ng/mg creatinine. In the African male group, the 8-OHdG levels ranged from 0.99 to 52.38 ng/mg creatinine. In the African female group the 8-OHdG levels ranged between 0.27 and 56.63 ng/mg creatinine. A p-value of 0.053 was obtained, indicating that no statistical significant difference exist between African males and females regarding urinary 8-OHdG levels.

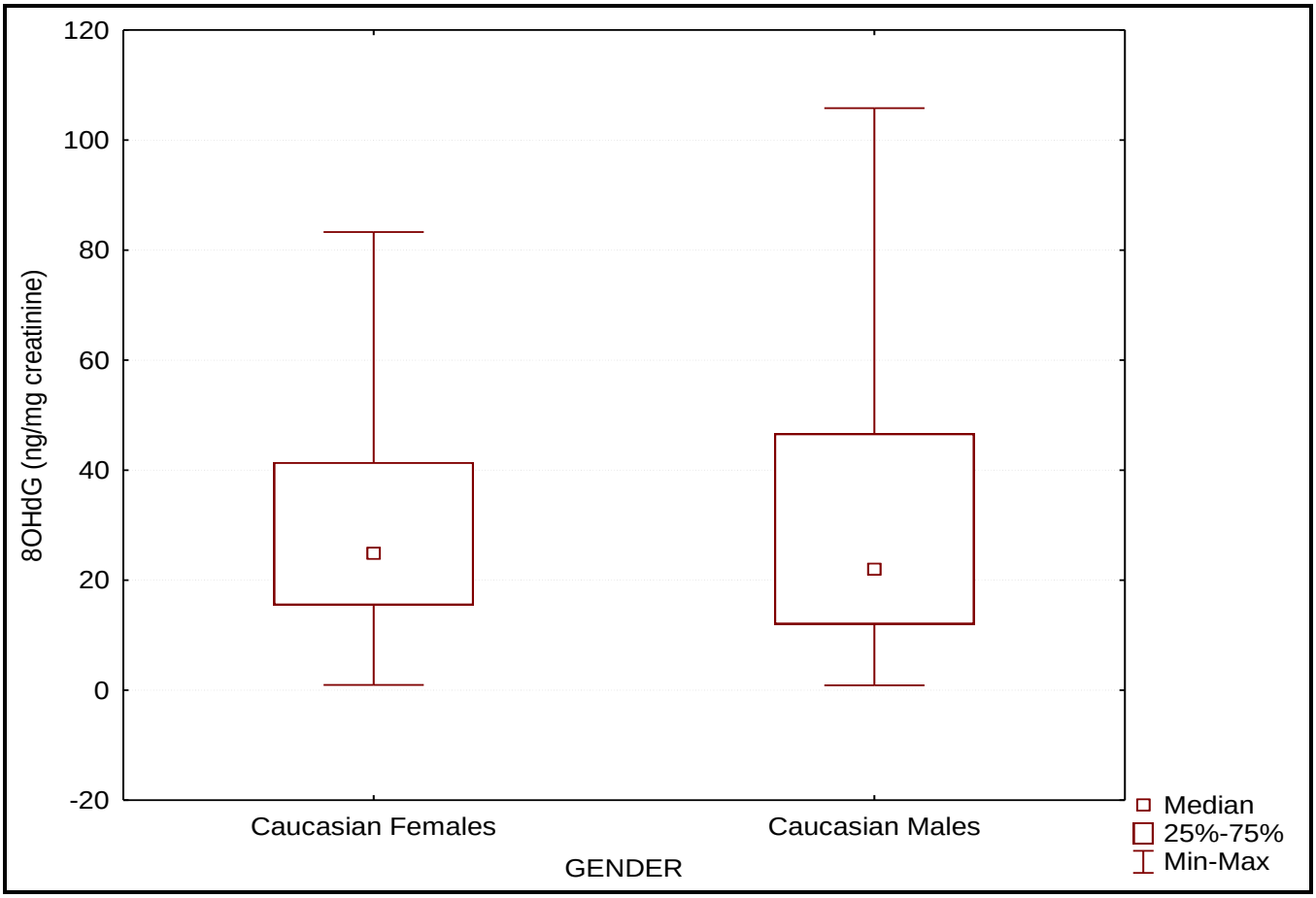

Figure 6.4: Urinary 8-OHdG levels of Caucasian females (n=96) and Caucasian males (n=87). p = 0.68.

For Caucasian men, the median urinary 8-OHdG level was 22.22 ng/mg creatinine and that of Caucasian females was 24.95 ng/mg creatinine. In the Caucasian male group, the 8-OHdG levels ranged from 0.54 to 106.17 ng/mg creatinine. In the Caucasian female group the 8-OHdG levels ranged between 0.54 and 83.50 ng/mg creatinine. A p-value of 0.68 was obtained with the Mann Whitney U test, indicating no statistically significant difference between Caucasian males and females regarding urinary 8-OHdG levels.

From the data obtained during this study no statistically significant difference between the urinary 8-OHdG levels between men and women from both ethnicities was evident.

Randomly selected Caucasian healthy subjects (52 females and 31 males) were included in the study done by Loft *et al.,* (1992), where slightly higher urinary 8-OHdG levels were found in males compared to females. The same result was obtained in a study done by Lin *et al.,* (2004). Here, 12 healthy non-smoking persons from Singapore were included in the study (seven males and five females). However, the differences observed in both studies were not statistically significant ($p > 0.05$) (Loft *et al.,* 1992; Lin *et al.,* 2004). Nakano *et al.,* (2003) found a slightly

higher level of urinary 8-OHdG levels in females (n=1254) compared to that of males (n=1253), but this difference was also not statistically significant. Thus, the results obtained in this study are consistent with the literature as no statistically significant difference was observed between urinary 8-OHdG levels of males and females for both ethnic groups.

6.2.2. The effect of ethnicity on urinary 8-OHdG levels

In order to determine the effect of ethnicity on 8-OHdG levels, Africans and Caucasians were compared for each gender group. The median with 25%-75% confidence intervals, as well as the spread (minimum and maximum values) for each parameter, is illustrated in Figures 6.5 and 6.6. Non-parametric comparisons between the groups were also made by using Mann-Whitney U tests.

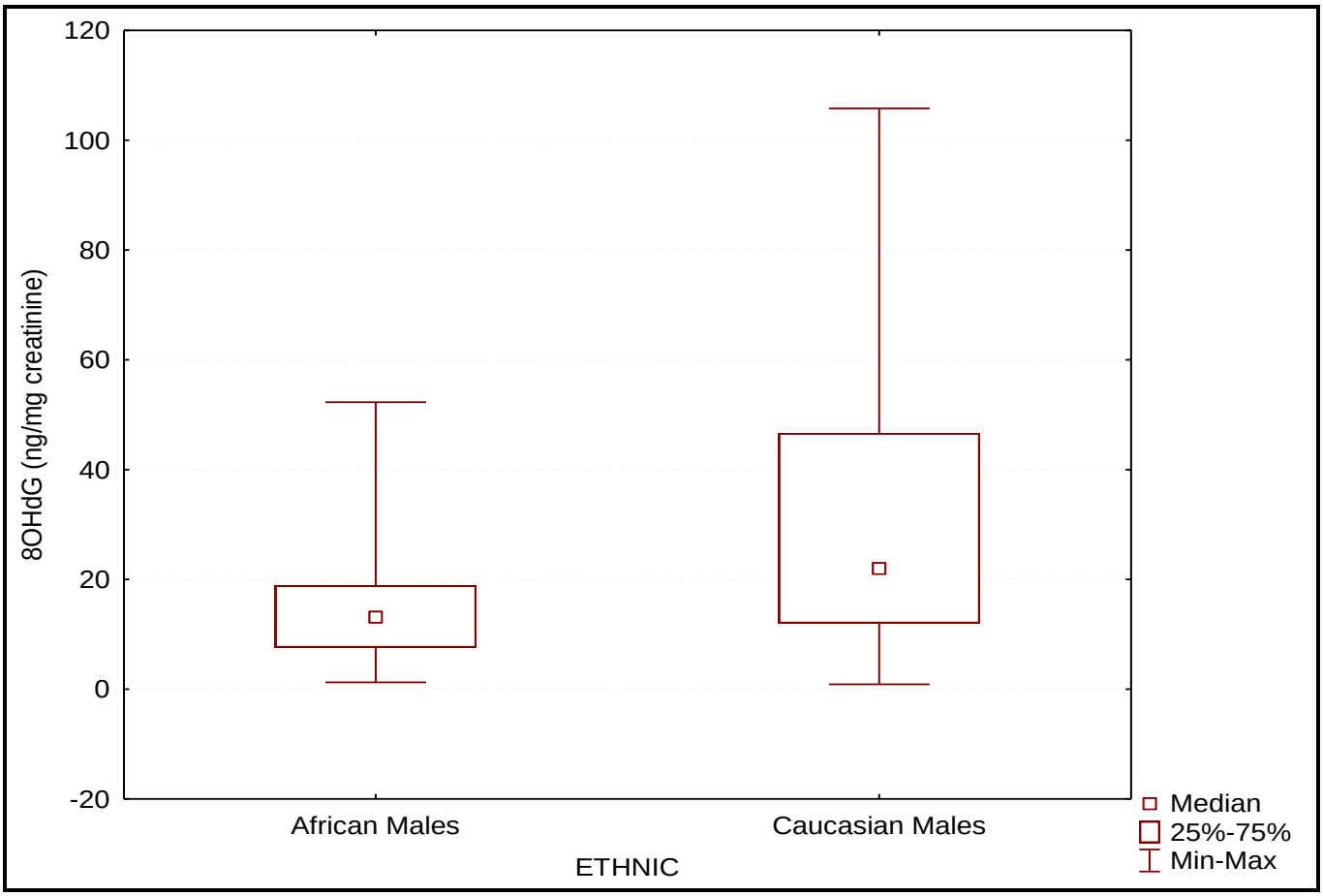

Figure 6.5: Urinary 8-OHdG levels of African males (n=86) and Caucasian males (n=87). $p < 0.001$.

The urinary 8-OHdG levels in the African male group (n=86) ranged between 0.99 and 52.38 ng/mg creatinine and the median was found to be 13.07 ng/mg creatinine. In the Caucasian male group (n=87), the median was 22.22 ng/mg creatinine and the 8-OHdG levels ranged between 0.53 – 106.17 ng/mg creatinine. The median of the Caucasian male group was ~70% higher than that of the African males. A p-value < 0.001 was obtained when these two groups

were compared using the Mann Whitney U test. This signifies that there is a statistical significant difference in urinary 8-OHdG levels in African and Caucasian males, with Caucasian males excreting higher amounts of 8-OHdG.

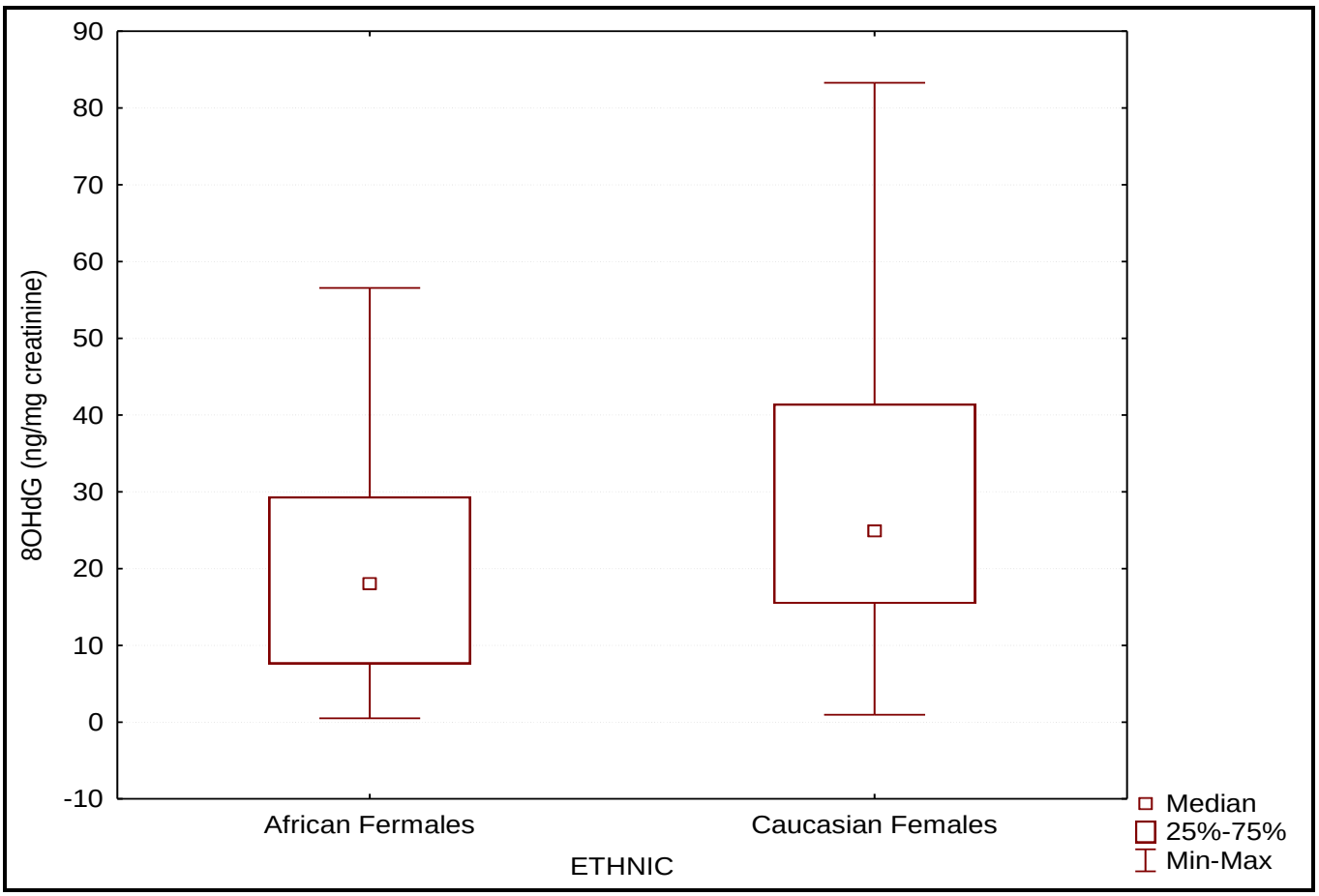

Figure 6.6: Urinary 8-OHdG levels of African females (n=84) and Caucasian females (n=96). $p < 0.001$.

In the African female group (n=84) the median was found to be 17.6 ng/mg creatinine. The median in the Caucasian female group was found to be 24.95 ng/mg creatinine. Thus, the median 8-OHdG value of the Caucasian females was ~42% higher than that of the African females. The urinary 8-OHdG levels in the African female group and Caucasian female group ranged between 0.27 – 56.63 ng/mg creatinine and 0.54 – 83.50 ng/mg creatinine, respectively. A p-value < 0.001 indicates a statistically significant difference in urinary 8-OHdG levels between African and Caucasian females.

In a study done by Huang *et al.,* (2000), a difference in the urinary 8-OHdG levels between African Americans and Caucasians was observed. A higher urinary 8-OHdG level was reported in Caucasians compared to that of African Americans ($p < 0.001$). The data obtained in this study also found higher 8-OHdG levels Caucasians than in Africans for both gender groups. The 8-OHdG levels in Caucasian males (0.53 – 106.17 ng/mg creatinine) were significantly

higher compared to African males (0.99 – 52.38 ng/mg creatinine). Caucasian females also had higher urinary 8-OHdG levels (0.54 - 83.50 ng/mg creatinine) compared to African females (0.27 – 56.63 ng/mg creatinine). Thus, the results obtained in this study are consistent with the results obtained by Huang *et al.,* (2000).

6.3. The correlation between urinary 8-OHdG and serum ROS levels

Serum ROS levels were measured using the method described in Section 3.3.4 and the results are given in Appendix A. Spearman correlations were used to assess the possible correlation between ROS and 8-OHdG levels measured in the SABPA participants. The data are depicted in Figure 6.7.

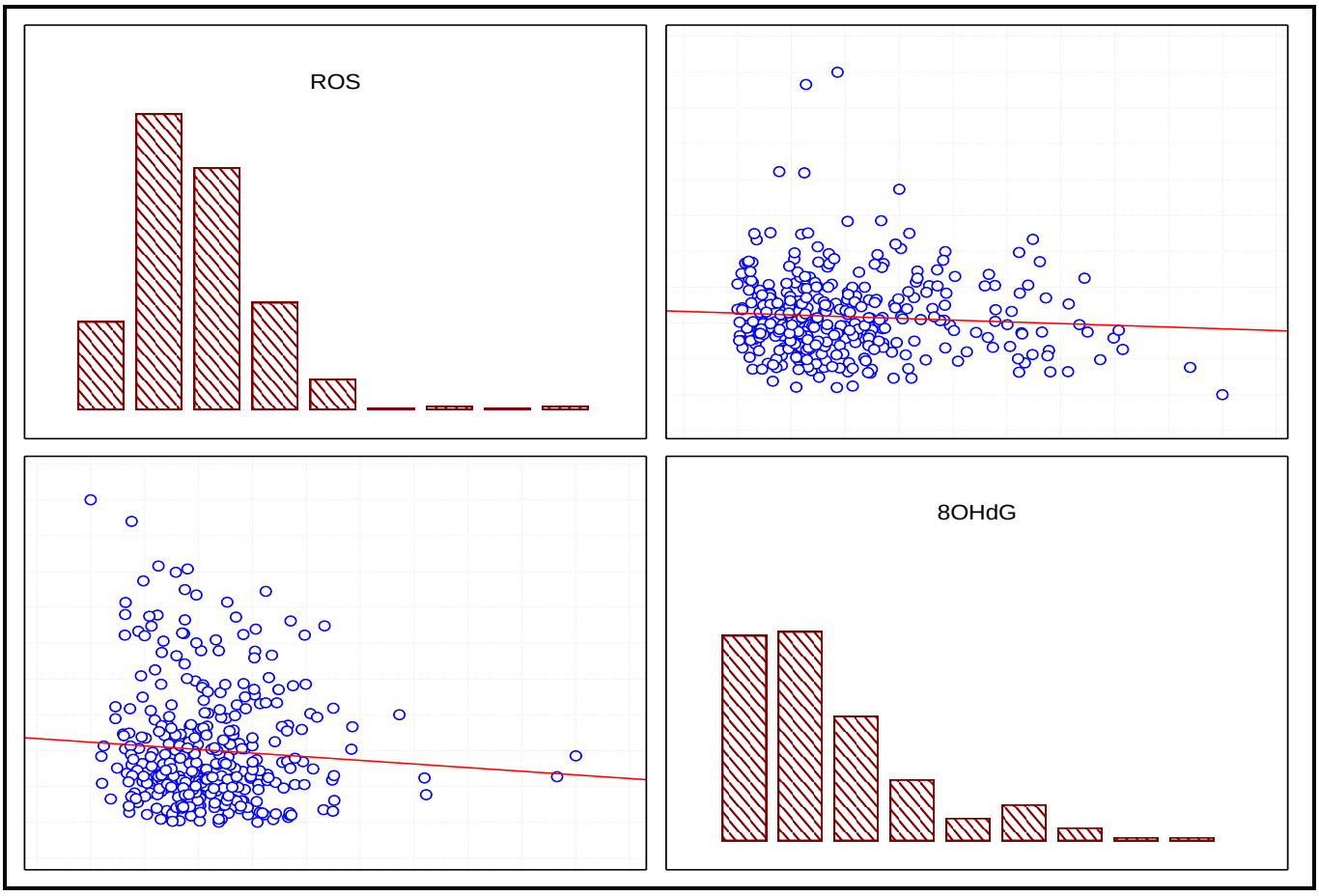

Figure 6.7: Scatter plot of 8-OHdG vs. ROS levels measured in the SABPA participants. A Spearman correlation coefficient indicated that no significant correlation exists between urinary 8-OHdG and serum ROS levels (r = -0.045).

As can be seen from Figure 6.7, no significant correlation was found between serum ROS levels and urinary 8-OHdG levels (r = -0.045). A correlation coefficient is bounded between -1

and 1 where negative values indicate inverse relationships and positive values indicate direct relationships. Values close to zero indicate a small or no relationship between the given parameters while values close to 1 or -1 indicate a very strong relationship between parameters. The small r-value indicates that these two markers are not related. However, in the literature it is widely accepted that free radicals in an individual can damage DNA (Halliwell, 2000; Cooke *et al*., 2002). This damage to DNA gives rise to the biomarker 8-OHdG which is excreted in the urine (Harri *et al.,* 2007). However, the data obtained in this study are in contrast to the literature. Since ROS causes damage not only to DNA, but also to proteins and lipids, it is unclear which of these macromolecules will be damaged the most by ROS. Repair mechanisms are also involved in the excision and repair of damaged DNA, resulting in 8-OHdG being excreted in the urine. Thus, it makes sense that no strong correlation between serum ROS and urinary 8-OHdG was observed in this study.

6.4. Discussion

In this study, urine and serum samples of the 409 participants from the SABPA project were used to determine serum ROS levels and urinary 8-OHdG levels. These 409 participants differed in ethnicity (Africans and Caucasians) and gender (males and females).

When urinary 8-OHdG levels from males and females were compared, no statistically significant difference was found for both the African and Caucasian ethnicities. The data obtained when males and females from both ethnicities were compared correlate with the literature, where no statistical significant difference was observed in the urinary 8-OHdG levels between males and females (Loft *et al.,* 1992; Nakano *et al.,* 2003; Lin *et al.,* 2004). However, when the urinary 8-OHdG levels in Africans and Caucasians were compared, a significant difference was observed. African males had significantly lower urinary 8-OHdG levels than Caucasian males ($p < 0.001$), and African females also had significantly lower urinary 8-OHdG levels compared to Caucasian females ($p < 0.001$). This is consistent with the results obtained in a study done by Huang *et al.,* (2000). This difference between Africans and Caucasians could be due to differences in genetic susceptibility, efficiency in detoxification and DNA repair or environmental factors (Huang *et al.,* 2000). As there are differences between human populations, a polymorphism in the DNA repair mechanism in one geographic or ethnic group could be less prevalent in another geographic or ethnic group. Differences in diet between Caucasians and Africans could also contribute to the differences seen between these 2 ethnicities.

No significant correlation between ROS and 8-OHdG levels was found in this study. However, in the literature it is clear that increased free radicals, such as in cancer patients, will lead to higher incidence of oxidative DNA damage, and consequently, to higher 8-OHdG levels (Olinski *et al.,* 2003; Mei *et al.,* 2005; Kuo *et al.,* 2007). However, certain factors could influence urinary 8-OHdG levels. Increased oxidative damage to DNA could lead to increased excretion of DNA base damage products, and decreased DNA repair activity could have lower excretion rates of DNA base damage products (Wiseman & Halliwell, 1996). Thus, urinary 8-OHdG levels are dependent on the rate of DNA damage *in vivo* and the efficacy of the DNA repair processes (Chiou *et al.,* 2003; Kuo *et al.,* 2007). In theory, high ROS levels and impaired defence mechanisms lead to oxidative damage, and the degree of the repair thereof leads to high or low urinary levels of the DNA damaged lesion. If the repair mechanism is compromised in any way it could influence the levels of excreted 8-OHdG. Therefore, a person with high urinary 8-OHdG levels and low ROS levels could be indicative of efficient repair mechanisms, and a person with low 8-OHdG levels and high ROS levels could indicate that repair mechanisms are impaired. This could lead to the result obtained in Figure 6.7, where it was concluded that serum ROS levels and urinary 8-OHdG does not correlate. Thus, it is recommended that in further studies, the efficiency of repair mechanisms (responsible for the excision of 8-OHdG) should also be investigated parallel to ROS and urinary 8-OHdG levels.

CHAPTER 7

Conclusion

7.1. Introduction

A great need exists in the development of methods for measuring oxidative stress status in humans. The reactivity of free radicals, however, is an immense drawback for use as an indicator of oxidative stress status. Therefore, more accurate results could be obtained when using biomarkers of oxidative damage instead. As there is uncertainty regarding which biomarkers are the better options, it was decided to use 8-OHdG and DHN-MA, as makers of oxidative damage. Thus, the aim of this study was to develop an analytical method to successfully and reliably quantify urinary 8-OHdG and DHN-MA as an indicator of oxidative stress status. As LC-MS/MS was available to this study, it was used as platform. The objectives of this study were to first standardise and validate an LC-MS/MS method for the quantification of urinary 8-OHdG and DHN-MA. Secondly, to quantify urinary 8-OHdG and DHN-MA levels in a selected group of South African teachers, and lastly to determine whether a correlation could be made between the urinary 8-OHdG and DHN-MA biomarkers of oxidative damage, and the serum ROS levels.

The strategy presented in Section 2.4 was followed. After ethics approval for this study had been obtained, 409 participants from the SABPA study (209 Caucasians, 101 males and 108 females and 200 Africans, 100 males and 100 females), were selected. Ten hour fasting, baseline urine and serum samples were collected from the 409 SABPA participants for the quantification of ROS, 8-OHdG and DHN-MA.

7.2. Optimisation of the 8-OHdG and DHN-MA assay

Before urinary 8-OHdG could be quantified, an assay needed to be optimised and validated on the LC-MS/MS. After numerous trial and error efforts with regard to optimising the best conditions for its quantification, an LC-MS/MS assay was developed for the quantification of urinary 8-OHdG. The optimised 8-OHdG assay was validated with acceptable linearity (R^2 > 0.99) and precision (intrabatch variation, RSD = 4.18% and interbatch variation, RSD = 17.37%). It was concluded that this optimised LC-MS/MS, 8-OHdG assay worked successfully and reliably in the quantification of urinary 8-OHdG.

Since a need exists in the Centre for Human Metabonomics at the North-West University, for assays to successfully quantify biomarkers of oxidative damage, this goal was achieved. However, this 8-OHdG assay is time-consuming as a sample cleanup step and long chromatographic run are employed. Therefore, its use in research will offer great results but its use for routine analyses should be limited, even though the assay works effectively.

For the optimisation of the DHN-MA assay, DHN-MA and its stable isotope needed to be synthesised as it was not commercially available. This then needed to be quantified, but due to the fact that synthesis could only be carried out on a small scale, it could not be quantified by successfully weighing the synthesised samples. Therefore, the synthesised DHN-MA and its isotope could only be relatively quantified. The synthesised, purified and relatively quantified DHN-MA and DHN-MA-d3 were then used to determine whether the optimised 8-OHdG assay performed effectively for the quantification of DHN-MA. The synthesised standards produced good results with the optimised 8-OHdG assay. However, when urine samples were used, problems were encountered. The DHN-MA could not be successfully and unequivocally quantified during the chromatographic run. This was due to the 100% MeCN needed to elute DHN-MA from the chromatographic column. During the time when DHN-MA elutes (± 18 minutes with 100% MeCN), there is no more chromatographic separation. Numerous non-polar metabolites present in the urine sample then elute simultaneously with DHN-MA. It was also observed that many metabolites in the urine sample have the same precursor and product ion as was used with DHN-MA which leads to a decrease in specificity. Due to this, the optimised 8-OHdG assay could not be duplicated for the quantification of DHN-MA as DHN-MA could not be distinguished in the chromatogram from other co-eluting peaks, which makes the data obtained very unreliable. Therefore, it was decided to separate the quantification of 8-OHdG and DHN-MA. Different chromatographic columns (C8, C18 and normal phase silica columns) were then considered, as well as differences in the pH ranges of the mobile phase (pH5, 6 and 7.48 of 1 mM Ammonium formate buffer). Still, DHN-MA only eluted with 100% MeCN which consequently did not solve the problem of co-eluting peaks. Time was an issue, thus no more strategies could be investigated.

7.3. The effect of ethnicity and gender on urinary 8-OHdG levels and the correlation of 8-OHdG with serum ROS levels

The 8-OHdG data obtained were then compared in groups as follows: Caucasian males vs. Caucasian females, African males vs. African females, Caucasian males vs. African males and

Caucasian females vs. African females. No significant difference in the urinary 8-OHdG levels in Caucasian males (n=87) and females (n=96) was observed ($p = 0.68$) neither was any significant difference in African males (n=86) and females (n=84) observed ($p = 0.053$). The data obtained are consistent with the literature, which found no statistically significant difference between males and females of both ethnicities. However, a significant difference was observed when urinary 8-OHdG levels in African males (n=86) and Caucasian males (n=87) were compared ($p < 0.001$), as well as Caucasian females (n=96) and African females (n=84) ($p < 0.001$). It was found that Caucasian males and Caucasian females excreted higher amounts of 8-OHdG in relation to African males and African females respectively. The difference seen in the urinary 8-OHdG levels between African and Caucasian males and African and Caucasian females was also obtained in another study (Huang *et al.,* 2000) and thus these results are also consistent with the literature.

Differences in the urinary 8-OHdG levels between Caucasian males and African males and Caucasian females and African females could possibly be explained by differences in their efficiency in detoxification and DNA repair or could be attributed by environmental factors. Since oxidative stress has been implicated in diseases such as hypertension, inflammation, ageing, autoimmunity, atherosclerosis, Parkinson's disease, cancer and diabetes, the results obtained in this study could contribute to an explanation why certain ethnicities have a predisposition to developing a certain disease. However, insufficient information regarding urinary 8-OHdG is available which was conducted in African and Caucasian males and females to draw sufficient conclusions. Thus, there can only be speculated as to why a difference exists.

It is widely accepted that increased levels of free radicals, and in this case ROS, will lead to higher incidence of oxidative DNA damage and consequently to higher urinary 8-OHdG levels. However, this was not the case in this study; as no significant correlation could be made between ROS and 8-OHdG levels it was concluded that ROS and 8-OHdG levels are not related. It was concluded that different factors could have an influence on the urinary 8-OHdG levels. Decreased DNA damage repair activity by base excision repair (BER) will lead to decreased excretion rates of DNA base damage products. Sufficient repair activity of damaged DNA will lead to increased levels of DNA base damage products. Therefore, it is believed that the urinary 8-OHdG levels are dependent on the rate of DNA damage *in vivo* and the efficacy of the repair processes. Thus, in theory, the BER mechanism can be considered the determining factor of urinary 8-OHdG levels. Accordingly, a person with high urinary 8-OHdG levels and low ROS levels could indicate efficient repair mechanisms, and a person with low 8-OHdG levels

and high ROS levels could indicate that repair mechanisms are impaired. This could explain why no correlation could be made between ROS and 8-OHdG levels. Nevertheless, care should be taken when commenting on urinary 8-OHdG levels as other contributing factors could influence results.

7.4. Recommendations for further studies

i. 8-OHdG assay

The optimised 8-OHdG assay was successful in quantifying urinary 8-OHdG, but its use for routine analysis is limited due to laborious and time consuming sample preparation. However, this optimised 8-OHdG assay could be further refined to determine whether the time needed for sample preparation and for chromatographic separation could be reduced to such an extent that would make this assay more acceptable for use in routine analysis.

ii. DHN-MA assay

It could be beneficial to determine the octanol: water partition coefficient of DHN-MA prior to optimising and validating an assay for its quantification. As was seen in this study, even though a metabolite is believed to act in a certain manner, it could react very differently from what was first believed. This could help in deciding which sample clean-up as well as chromatographic column and mobile phases would ultimately give the best result for its successful quantification. If the octanol: water partition coefficient had been determined for DHN-MA after its synthesis, it could have been established earlier that the quantification of 8-OHdG and DHN-MA should be separated and consequently more time could have been spent developing an LC-MS/MS assay for DHN-MA.

Seeing that DHN-MA and DHN-MA-d3 is not commercially available and they need to be synthesised, it is recommended to begin the synthesis with higher amounts of 4-HNE-MA and 4-HNE-MA-d3. Small quantities (1 mg) of 4-HNE-MA and 4-HNE-MA-d3 were used to synthesise DHN-MA and DHN-MA-d3 in this study, mainly because a concern existed that the synthesis might not work. However, it was established that the synthesis of DHN-MA and DHN-MA-d3 from 4-HNE-MA and 4-HNE-MA-d3 worked very well. Thus, the synthesis could be scaled up with larger quantities of 4-HNE-MA and 4-HNE-MA-d3 which could lead to more DHN-MA and DHN-MA-d3 being synthesised, which could then be successfully weighed and thus successfully quantified.

iii. Studies including Caucasians and Africans

As only a few studies were conducted where oxidative DNA damage was determined in Caucasian- and African-Americans, not sufficient information is available regarding oxidative stress status in Africans and Caucasians. Thus, a need exists for more studies to be conducted in Caucasians and Africans, where oxidative stress status is assessed.

iv. **ROS assay, repair mechanism, and gene knock-out experiments**

A possibility exists that the ROS assay used in this study is not as reliable as was first believed, which could explain why no correlation was found between the 8-OHdG and ROS levels. Thus, an improved assay could be used to quantify serum ROS levels.

As ROS and 8-OHdG levels does not correlate as was first suggested, it was concluded that other factors could influence the results obtained. One very important contributing factor was overlooked in the design of this study. This contributing factor is assumed to be the repair mechanism responsible for defending the integrity of the genome, by removing the oxidative damaged DNA ribonucleosides. However, the precise repair mechanism responsible for the excision of 8-OHdG remains elusive. Therefore, the use of gene knock-out experiments could be used to help clarify the repair mechanism/s responsible for excising 8-OHdG.

7.5. Conclusion

As DHN-MA could not be unequivocally quantified in urine, a different assay needed to be optimised and validated for its quantification. However, time was an issue and no more time could be spent on developing a different assay for the quantification of DHN-MA. Nonetheless, an LC-MS/MS assay was developed for the successful quantification of urinary 8-OHdG. The urinary 8-OHdG levels of the 409 SABPA participants were then quantified. This data obtained from the 8-OHdG assay were then correlated with the ROS levels. Thus, the aim and objectives of this study were partially reached.

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APPENDIX A

Table A1: Raw data of the 409 SABPA participants

SABPA participant	Ethnicity	Gender	Creatinine (mmol/L)	8-OHdG (ng/mg creatinine)	ROS (Units)
1	African	Female	14.18	32.2	75
2	African	Female	10.41	10.9	84
3	African	Male	2.51	11.8	99
4	African	Female	12.27	15.6	71
5	African	Male	5.41	11.2	79
6	African	Female	2.83	7.7	150
7	African	Male	6.29	10.2	90
8	African	Female	11.51	*196.5	142
9	African	Female	20.70	21.5	76
10	African	Female	5.86	38.8	106
11	African	Male	17.41	n.d.	93
12	African	Female	12.27	24.4	157
13	African	Female	4.17	21.0	115
14	African	Female	6.72	7.1	85
15	African	Female	14.12	7.3	115
16	African	Female	6.52	2.2	129
17	African	Male	15.04	15.6	69
18	African	Male	20.43	23.1	85
19	African	Female	3.72	12.8	132
20	African	Female	4.87	6.5	n.d.
21	African	Female	3.12	14.4	149
22	African	Male	6.23	10.7	n.d
23	African	Female	20.01	17.2	108
24	African	Male	28.53	24.3	104
25	African	Male	7.53	15.2	83
26	African	Female	6.88	3.0	118
27	African	Male	13.88	20.1	126
28	African	Male	10.08	3.1	85
29	African	Female	12.87	14.9	112
30	African	Male	9.85	*65.5	108
31	African	Female	21.13	32.2	129
32	African	Female	20.98	56.5	90
33	African	Male	5.37	13.8	69
34	African	Female	11.96	32.0	92
35	African	Female	13.04	7.5	109
36	African	Female	2.56	19.3	97
37	African	Female	3.13	8.0	104
38	African	Female	9.32	29.4	54
39	African	Female	2.34	37.6	110
40	African	Female	7.03	10.8	96
41	African	Male	6.33	3.1	65
42	African	Male	11.04	13.0	115
43	African	Female	14.92	34.5	101
44	African	Female	7.52	12.9	136
45	African	Male	11.54	8.5	89
46	African	Female	17.64	29.3	92
47	African	Female	12.48	42.1	114

48	African	Female	3.61	1.4	122
49	African	Male	6.39	12.6	91
50	African	Female	13.66	10.4	93
51	African	Female	6.58	15.6	112
52	African	Female	19.74	29.1	104
53	African	Female	4.31	3.8	116
54	African	Female	10.37	11.7	74
55	African	Female	4.59	30.9	135
56	African	Male	19.38	27.1	78
57	African	Male	10.00	6.2	99
58	African	Female	4.12	6.0	99
59	African	Female	8.00	21.8	102
60	African	Male	8.79	6.0	89
61	African	Female	14.22	5.7	80
62	African	Male	17.96	12.5	73
63	African	Male	6.04	43.9	113
64	African	Female	12.32	17.9	90
65	African	Female	2.26	14.2	119
66	African	Male	7.39	15.6	80
67	African	Male	8.42	16.6	56
68	African	Female	12.50	20.4	128
69	African	Male	6.18	8.1	49
70	African	Male	12.01	*57.5	41
71	African	Female	3.18	3.8	57
72	African	Female	12.07	1.0	80
73	African	Female	6.66	20.4	135
74	African	Female	9.64	21.5	132
75	African	Female	8.15	14.3	70
76	African	Female	20.37	22.8	98
77	African	Female	27.10	31.7	158
78	African	Female	7.59	4.9	107
79	African	Female	5.42	3.0	111
80	African	Female	9.81	5.3	103
81	African	Female	3.76	56.0	72
82	African	Male	13.06	13.9	63
83	African	Female	5.10	26.9	123
84	African	Female	1.38	19.9	84
85	African	Female	14.57	*75.9	67
86	African	Male	7.45	17.6	92
87	African	Female	3.91	43.9	124
88	African	Female	7.36	26.2	107
89	African	Male	6.46	2.1	76
90	African	Male	12.03	12.1	91
91	African	Male	5.06	17.9	92
92	African	Male	7.16	22.9	85
93	African	Male	12.03	3.5	102
94	African	Male	7.73	1.5	99
95	African	Male	7.73	28.1	65
96	African	Male	12.23	14.8	103
97	African	Male	5.07	3.0	87
98	African	Male	11.70	5.3	96
99	African	Male	17.09	17.9	113
100	African	Male	14.24	12.7	89
101	African	Male	8.89	*234.6	109
102	African	Male	2.45	19.7	96

103	African	Male	8.01	29.7	57
104	African	Male	9.15	38.9	76
105	African	Male	8.83	10.1	60
106	African	Male	10.54	*120.1	96
107	African	Male	8.20	14.6	71
108	African	Female	2.90	26.0	88
109	African	Female	4.33	24.5	81
110	African	Female	7.60	*82.4	112
111	African	Female	16.97	35.6	179
112	African	Male	7.92	25.5	46
113	African	Female	13.11	18.1	105
114	African	Female	7.56	23.7	87
115	African	Female	10.49	*82.4	109
116	African	Female	17.80	56.6	97
117	African	Female	4.35	*79.3	157
118	African	Female	6.03	39.3	116
119	African	Female	4.79	*97.4	66
120	African	Female	4.90	6.6	n.d.
121	African	Female	4.15	9.6	191
122	African	Female	11.19	36.0	139
123	African	Female	16.19	*80.9	114
124	African	Male	9.00	16.7	86
125	African	Male	8.45	7.6	108
126	African	Male	3.42	8.5	101
127	African	Male	10.31	14.0	89
128	African	Female	9.83	*68.1	147
129	African	Male	5.34	12.3	92
130	African	Male	6.00	24.5	109
131	African	Male	6.96	*62.9	81
132	African	Male	11.75	15.4	93
133	African	Male	9.45	32.0	84
134	African	Male	6.51	8.5	83
135	African	Male	31.42	45.8	109
136	African	Male	10.00	6.0	88
137	African	Female	10.98	31.9	126
138	African	Female	9.67	36.1	93
139	African	Male	19.90	7.1	84
140	African	Female	19.71	0.5	98
141	African	Female	9.46	n.d.	112
142	African	Male	5.85	36.4	91
143	African	Male	6.92	13.5	123
144	African	Male	9.42	19.3	58
145	African	Male	20.15	1.5	97
146	African	Female	9.82	*168.5	113
147	African	Female	7.56	19.2	103
148	African	Female	7.47	11.5	86
149	African	Male	9.82	13.6	81
150	African	Male	16.50	*52.9	97
151	African	Male	4.70	14.0	102
152	African	Female	8.37	15.0	190
153	African	Male	28.77	18.9	74
154	African	Male	17.51	*101.4	81
155	African	Male	11.24	34.0	69
156	African	Male	8.58	10.6	72
157	African	Male	9.74	11.7	127

158	African	Female	3.98	36.7	n.d.
159	African	Female	5.73	11.2	109
160	African	Female	3.28	19.7	63
161	African	Female	7.79	n.d.	111
162	African	Female	16.43	30.3	128
163	African	Male	5.18	n.d.	60
164	African	Female	6.54	5.4	111
165	African	Male	5.11	13.3	90
166	African	Male	7.99	4.4	74
167	African	Male	6.60	7.5	105
168	African	Male	9.32	*56.1	78
169	African	Male	5.98	*52.9	53
170	African	Female	10.68	4.7	145
171	African	Female	12.09	2.8	129
172	African	Male	12.03	7.0	61
173	African	Male	0.98	13.7	65
174	African	Male	7.55	2.6	85
175	African	Male	12.38	1.3	77
176	African	Male	8.04	22.1	45
177	African	Male	21.08	*286.7	69
178	African	Female	11.52	0.5	115
179	African	Male	3.66	52.3	82
180	African	Female	1.88	n.d.	77
181	African	Female	2.85	3.5	117
182	African	Female	2.55	9.0	58
183	African	Male	7.74	5.8	57
184	African	Male	6.45	5.8	107
185	African	Male	5.78	6.1	100
186	African	Female	13.80	29.8	84
187	African	Male	9.61	12.3	84
188	African	Female	4.99	24.7	62
189	African	Female	5.16	*81.3	94
190	African	Female	11.29	n.d.	101
191	African	Male	20.63	n.d.	94
192	African	Male	14.23	38.3	51
193	African	Male	9.27	10.2	80
194	African	Male	16.18	14.9	65
195	African	Male	12.73	15.4	74
196	African	Male	10.83	4.2	77
197	African	Male	5.78	11.2	115
198	African	Male	5.57	*61.3	69
199	African	Male	28.76	10.7	70
200	African	Male	29.32	28.4	63
201	Caucasian	Female	n.d.	n.d.	n.d.
202	Caucasian	Male	18.24	10.2	67
203	Caucasian	Male	9.20	13.2	65
204	Caucasian	Female	3.86	20.2	100
205	Caucasian	Female	8.09	12.0	106
206	Caucasian	Male	8.42	24.5	55
207	Caucasian	Male	16.89	13.2	45
208	Caucasian	Male	9.32	48.4	63
209	Caucasian	Male	8.71	16.3	71
210	Caucasian	Female	5.87	*129.3	100
211	Caucasian	Female	14.12	24.0	78
212	Caucasian	Male	17.60	25.5	58

213	Caucasian	Female	14.37	37.0	67
214	Caucasian	Female	4.20	13.7	103
215	Caucasian	Female	3.30	15.7	99
216	Caucasian	Male	7.48	14.2	72
217	Caucasian	Male	17.65	14.3	80
218	Caucasian	Male	6.72	105.8	40
219	Caucasian	Male	8.17	61.7	55
220	Caucasian	Female	13.33	34.9	142
221	Caucasian	Male	8.09	9.0	64
222	Caucasian	Female	6.51	29.7	73
223	Caucasian	Male	12.30	9.6	70
224	Caucasian	Male	23.50	3.3	76
225	Caucasian	Male	3.67	11.6	71
226	Caucasian	Female	5.32	16.2	119
227	Caucasian	Female	9.12	25.4	113
228	Caucasian	Female	8.42	45.6	137
229	Caucasian	Female	9.03	4.2	149
230	Caucasian	Female	7.23	3.7	129
231	Caucasian	Female	5.02	20.0	87
232	Caucasian	Female	2.16	*132.9	123
233	Caucasian	Male	9.56	14.6	91
234	Caucasian	Male	12.46	n.d.	63
235	Caucasian	Male	13.44	62.9	61
236	Caucasian	Female	1.61	60.1	96
237	Caucasian	Female	2.90	42.9	98
238	Caucasian	Female	17.12	n.d.	87
239	Caucasian	Male	6.92	*334.0	116
240	Caucasian	Male	12.76	66.6	82
241	Caucasian	Female	4.22	66.2	130
242	Caucasian	Male	6.41	18.8	67
243	Caucasian	Female	11.41	41.4	63
244	Caucasian	Female	2.74	41.5	109
245	Caucasian	Female	8.14	8.0	85
246	Caucasian	Female	7.11	15.4	250
247	Caucasian	Female	3.47	26.4	80
248	Caucasian	Female	12.30	*92.5	114
249	Caucasian	Female	21.41	n.d.	160
250	Caucasian	Female	7.59	7.6	88
251	Caucasian	Female	8.72	22.7	73
252	Caucasian	Female	8.22	18.1	130
253	Caucasian	Male	6.66	*309.2	83
254	Caucasian	Female	10.89	*186.3	218
255	Caucasian	Female	9.59	17.4	116
256	Caucasian	Female	7.38	14.2	81
257	Caucasian	Female	8.64	35.4	105
258	Caucasian	Male	10.44	46.7	87
259	Caucasian	Female	8.99	15.8	149
260	Caucasian	Female	6.91	12.2	85
261	Caucasian	Female	2.80	56.4	114
262	Caucasian	Female	4.19	28.2	113
263	Caucasian	Female	3.80	75.9	119
264	Caucasian	Female	3.46	30.6	104
265	Caucasian	Female	9.72	17.1	85
266	Caucasian	Female	1.56	15.2	120
267	Caucasian	Female	4.21	82.2	78

268	Caucasian	Female	6.42	34.8	99
269	Caucasian	Female	7.32	23.5	68
270	Caucasian	Male	1.48	68.4	55
271	Caucasian	Female	2.09	39.6	124
272	Caucasian	Female	3.26	17.6	113
273	Caucasian	Female	6.40	45.2	131
274	Caucasian	Male	11.68	*238.4	72
275	Caucasian	Female	5.08	63.6	114
276	Caucasian	Female	1.63	55.1	121
277	Caucasian	Female	3.52	59.1	87
278	Caucasian	Male	6.84	n.d.	78
279	Caucasian	Female	5.59	61.7	136
280	Caucasian	Male	8.44	8.2	60
281	Caucasian	Female	5.50	22.2	258
282	Caucasian	Female	5.07	45.5	100
283	Caucasian	Female	11.12	31.2	89
284	Caucasian	Female	3.82	45.4	90
285	Caucasian	Female	10.67	62.1	82
286	Caucasian	Female	3.41	3.3	123
287	Caucasian	Female	3.33	17.9	140
288	Caucasian	Female	3.21	*111.1	107
289	Caucasian	Female	10.31	7.6	101
290	Caucasian	Male	25.17	19.5	72
291	Caucasian	Male	15.77	8.7	79
292	Caucasian	Male	8.13	10.3	87
293	Caucasian	Female	18.84	37.8	149
294	Caucasian	Female	7.81	9.8	94
295	Caucasian	Female	9.75	14.5	101
296	Caucasian	Female	7.87	15.5	70
297	Caucasian	Female	7.59	19.6	101
298	Caucasian	Male	14.58	45.6	72
299	Caucasian	Male	14.55	5.1	70
300	Caucasian	Male	14.92	28.8	55
301	Caucasian	Female	3.70	64.7	145
302	Caucasian	Female	5.81	26.7	84
303	Caucasian	Female	3.00	61.8	109
304	Caucasian	Male	20.71	*566.2	68
305	Caucasian	Male	6.05	n.d.	108
306	Caucasian	Female	6.55	29.3	80
307	Caucasian	Female	10.61	13.0	74
308	Caucasian	Male	16.30	24.6	108
309	Caucasian	Male	7.98	1.6	71
310	Caucasian	Male	7.79	19.9	76
311	Caucasian	Male	1.52	29.0	78
312	Caucasian	Male	6.76	31.1	73
313	Caucasian	Male	17.44	17.5	61
314	Caucasian	Female	8.82	15.8	59
315	Caucasian	Female	10.35	14.5	92
316	Caucasian	Female	5.76	26.2	102
317	Caucasian	Female	10.89	6.8	96
318	Caucasian	Female	11.20	22.7	58
319	Caucasian	Female	7.33	28.9	73
320	Caucasian	Female	30.70	1.0	89
321	Caucasian	Female	29.82	30.4	102
322	Caucasian	Female	9.01	2.9	130

323	Caucasian	Male	19.16	16.0	72
324	Caucasian	Male	26.35	98.8	58
325	Caucasian	Female	3.53	32.5	85
326	Caucasian	Male	16.86	79.3	64
327	Caucasian	Male	17.84	15.7	77
328	Caucasian	Male	27.62	11.7	100
329	Caucasian	Female	8.53	62.3	81
330	Caucasian	Female	10.38	22.9	87
331	Caucasian	Female	21.55	21.1	59
332	Caucasian	Female	13.51	5.4	78
333	Caucasian	Male	22.39	72.3	55
334	Caucasian	Female	8.15	22.3	81
335	Caucasian	Female	33.34	26.0	103
336	Caucasian	Male	18.64	6.1	81
337	Caucasian	Female	24.65	18.0	76
338	Caucasian	Male	30.95	32.1	72
339	Caucasian	Male	5.87	40.3	91
340	Caucasian	Female	8.40	47.7	120
341	Caucasian	Male	8.16	13.8	57
342	Caucasian	Male	14.43	76.5	82
343	Caucasian	Male	12.61	68.2	70
344	Caucasian	Male	17.43	64.5	67
345	Caucasian	Female	7.59	27.4	99
346	Caucasian	Male	22.34	84.2	70
347	Caucasian	Male	12.54	72.4	101
348	Caucasian	Male	9.69	22.0	67
349	Caucasian	Female	4.81	54.9	79
350	Caucasian	Female	12.80	9.2	102
351	Caucasian	Female	6.45	12.1	76
352	Caucasian	Female	15.29	*187.1	89
353	Caucasian	Male	15.39	67.8	66
354	Caucasian	Female	14.27	83.3	84
355	Caucasian	Male	18.11	37.3	98
356	Caucasian	Female	9.38	n.d.	57
357	Caucasian	Male	11.64	5.6	85
358	Caucasian	Female	16.32	44.5	90
359	Caucasian	Female	6.50	26.1	75
360	Caucasian	Female	7.43	67.5	105
361	Caucasian	Female	9.89	3.8	89
362	Caucasian	Male	30.06	50.3	69
363	Caucasian	Male	19.57	15.4	105
364	Caucasian	Female	20.88	24.4	94
365	Caucasian	Female	14.08	n.d.	83
366	Caucasian	Male	18.62	31.4	91
367	Caucasian	Female	12.53	15.3	95
368	Caucasian	Female	16.71	n.d.	86
369	Caucasian	Female	6.32	39.6	119
370	Caucasian	Male	15.94	n.d	63
371	Caucasian	Female	5.98	28.1	87
372	Caucasian	Male	24.63	13.7	83
373	Caucasian	Female	17.01	24.0	97
374	Caucasian	Male	57.78	n.d.	106
375	Caucasian	Male	18.39	61.4	64
376	Caucasian	Male	19.41	35.1	75
377	Caucasian	Male	14.37	n.d.	89

378	Caucasian	Male	18.38	31.2	76
379	Caucasian	Male	19.78	54.2	113
380	Caucasian	Male	42.60	11.2	86
381	Caucasian	Male	21.66	11.8	81
382	Caucasian	Male	38.54	n.d.	70
383	Caucasian	Male	31.34	5.2	81
384	Caucasian	Male	22.13	9.5	82
385	Caucasian	Male	17.00	12.4	87
386	Caucasian	Male	17.64	18.2	52
387	Caucasian	Male	18.59	43.2	93
388	Caucasian	Male	14.57	n.d.	84
389	Caucasian	Male	27.07	20.2	113
390	Caucasian	Male	33.38	n.d.	92
391	Caucasian	Male	8.94	59.7	73
392	Caucasian	Male	14.15	47.5	83
393	Caucasian	Male	32.96	5.5	81
394	Caucasian	Male	29.44	74.8	87
395	Caucasian	Male	34.02	12.0	104
396	Caucasian	Male	17.17	9.6	84
397	Caucasian	Male	23.35	24.9	96
398	Caucasian	Male	23.98	0.9	77
399	Caucasian	Male	21.37	15.6	64
400	Caucasian	Male	20.89	34.4	51
401	Caucasian	Male	20.58	30.2	71
402	Caucasian	Male	17.43	29.0	92
403	Caucasian	Male	21.54	*367.2	86
404	Caucasian	Male	25.27	25.6	80
405	Caucasian	Male	27.44	24.8	83
406	Caucasian	Male	17.35	11.6	95
407	Caucasian	Male	32.35	21.5	80
408	Caucasian	Male	27.99	17.5	74
409	Caucasian	Male	22.56	37.6	57

n.d. = not detected; ***** = outliers that were later removed from the data matrix before statistical analysis were performed. The outliers were identified with Tukey's method as described in Section 6.2.