



NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

**The involvement of lipid and protein oxidation
in hypertension:
the SABPA study**

By

Karien Bothma, B.Sc. (Hons.)

*Dissertation submitted for the degree Magister Scientiae (M.Sc.) in
Biochemistry at the Potchefstroom Campus of the North-West University*

Supervisor: Dr. R Louw

School for Physical and Chemical Sciences, North-West University
(Potchefstroom Campus), South Africa.

Co-Supervisor: Mnr. L Erasmus

School for Physical and Chemical Sciences, North-West University
(Potchefstroom Campus), South Africa.

August 2012

Potchefstroom

“AD ASTARA PER ASPERA”
A ROUGH ROAD LEADS TO THE
STARS

Table of contents

Acknowledgements	VII
Abstract	VIII
List of Symbols and Abbreviations	X
List of Figures.....	XVIII
List of Tables.....	XXI
List of Equations.....	XXIII
Chapter 1: Introduction.....	1
Chapter 2: Literature overview	3
2.1. Introduction	3
2.2. Reactive oxygen species (ROS).....	3
2.3. Reactive nitrogen species (RNS)	5
2.4. Endogenous and exogenous sources of ROS and RNS	7
2.5. Oxidative stress.....	8
2.6. Lipid peroxidation	10
2.6.1. Introduction.....	10
2.6.2. Mechanism.....	10
2.6.3. Lipid peroxidation markers	13
2.6.3.1. Introduction.....	13
2.6.4.2. Malondialdehyde (MDA)	13

2.7. Protein oxidation.....	14
2.7.1. Introduction.....	14
2.7.2. Mechanism.....	15
2.7.3. Protein oxidation markers.....	18
2.7.3.1. Introduction.....	18
2.7.3.2. 3-Nitrotyrosine.....	18
2.8. Hypertension.....	20
2.9. Hypertension and oxidative stress.....	22
2.10. Study plan and approach.....	24
Chapter 3: Optimization of the TBARS assay.....	26
3.1. Introduction.....	26
3.2. Reagents, standards and solution.....	27
3.2.1. Reagents.....	27
3.2.2. Preparation of reagent mix.....	27
3.2.3. Preparation of standard.....	27
3.3. Optimization of TBARS assay.....	28
3.3.1. The effect of incubation time on the TBARS assay.....	28
3.3.2. The effect of incubation temperature on the TBARS.....	29
3.3.3. The effect of different volumes of urine on the TBARS assay.....	30
3.3.4. The effect of different volumes of reagent on the TBARS assay.....	31
3.4. The optimized TBARS assay.....	32

3.5. Validation of the optimized TBARS assay	33
3.5.1. Introduction.....	33
3.5.2. Intra-batch coefficient of variation.....	33
3.5.3. Inter-batch coefficient of variation.....	34
3.6. Conclusion on the optimized TBARS assay	35
Chapter 4: Optimization of 3-nitrotyrosine assay.....	36
4.1. Introduction	36
4.2. Reagents, standards and solutions	36
4.2.1. Reagents.....	36
4.2.2. Derivatization reagents.....	37
4.2.3. Internal standard (phenylalanine isotope)	37
4.2.4. Mobile phase for dissolving derivatized samples.....	37
4.2.5. Peroxynitrite	37
4.2.6. Phosphate buffer (1M, pH 7.4)	38
4.2.7. Tyrosine sock solutions	38
4.2.8. Mobile phase and ninhydrin colouring solution for thin layer chromatography	38
4.3. Quantification of 3-nitro-L-tyrosinewith LC-MS/MS.....	39
4.3.1. Butylation of 3-nitro-L-tyrosine standard.....	39
4.3.2. Optimization of MS condition for the quantification of 3-nitro-L-tyrosine standard.....	39
4.3.3. Chromatographic separation of 3-nitro-L-tyrosine standard	40
4.3.4. Derivatization of control urine sample	41

4.3.5. Quantification of 3NT in urine sample	42
4.4. Quantification of stereospecific derivatized 3-nitro-L-tyrosine standard with LC-MS/MS	45
4.4.1. Stereospecific butylation of 3-nitro-L-tyrosine standard.....	45
4.4.2. Chromatographic separation of stereospecific derivatized 3-nitro-L-tyrosine standard with LC-MS/MS	46
4.4.3. Stereospecific butylation and chromatographic separation of urine sample	46
4.4.4. Urine sample spiked with 3-nitro-L-tyrosine standard.....	47
4.5. Attempts to identify the contaminating peak/compound	49
4.5.1. Introduction.....	49
4.5.2. 3-Nitro-D-tyrosine	49
4.5.2.1. Synthesis of 3-nitro-D-tyrosine	49
4.5.2.2. Analysis of synthesized 3-nitro-D-tyrosine, 3-nitro-L-tyrosine and 3-nitro-DL- tyrosine	50
4.5.3. Other isomers of 3NT	52
4.5.3.1. Synthesis of nitro- <i>m</i> -tyrosine and nitro- <i>o</i> -tyrosine	52
4.5.2.2. Analysis of synthesized nitro- <i>m</i> -tyrosine and nitro- <i>o</i> -tyrosine.....	53
4.5.4. Determining the accurate mass of the unknown compound.....	54
4.5.4.1. Thin layer chromatography.....	54
4.5.4.2. Analyzes of unknown compound on the Q-TOF LC-MS.....	57
4.6. Conclusion	58
4.7. The optimized 3-nitrotyrosine assay	58

4.7.1. Derivatization of samples or standard	58
4.7.2. Chromatographic separation of the sample or standard.....	59
4.7.3. Quantification of 3NT.....	59
4.8. Linearity of the optimized 3-nitrotyrosine assay	62
4.9. Conclusion on the optimized 3-nitrotyrosine assay	64
Chapter 5: The involvement of 3-nitrotyrosine and TBARS in hypertension	65
5.1. Study group and ethical approval	65
5.2. Sample collection and other analyses	66
5.3. The effect of gender on urine 3NT and TBARS.....	68
5.4. Urinary TBARS and 3NT levels and hypertension.....	70
5.4.1. Introduction.....	70
5.4.2. The influence of categorical variables on urinary TBARS and 3NT levels.....	71
5.4.3. The influence of continuous variables on urinary TBARS and 3NT levels.....	72
5.4.3.1. Introduction.....	72
5.4.3.2. Correlation between TBARS and/or 3NT and continuous variables in males...	72
5.4.3.3. Correlation between TBARS and/or 3NT and continuous variables in females	73
5.5. Effect of analyses of covariance on urinary TBARS and 3NT levels	74
5.6. Discussion.....	77
Chapter 6: Conclusion	81
6.1. Introduction	81
6.2. Optimization of TBARS and 3-nitrotyrosine assay.....	81

6.3. Influence of lipid and protein oxidation markers on hypertension	83
6.4. Recommendations	84
6.5. Final conclusion.....	85
References	87

Acknowledgements

I am indebted to the following people for their love and support:

I want to thank the Lord, my Savior, for the talents He provided me with. For giving me the strength to complete this part of my studies. For all the wonderful people He put into my life and for carrying me when I stumble.

My study leaders, Dr Roan Louw and Mnr Lardus Erasmus, thank you for your guidance and advice during these past two years. Also thank you for your support, patience and lessons I learned from you.

Mnr Peet Jansen Van Rensburg, thank you for all your help with the LC/MS/MS work. Thank you for all the jokes and laughs we had.

To my Mom and Dad, thank you for giving me the opportunity to further my studies and for having faith in me. Thank you for your unconditional love. I will always be grateful for what you have provided me with and will never be able to say thank you enough. I love you very much!

To my brother Riaan, thank you for all your love, support and laughter when I needed it the most.

To my best friend Bianca, thank you for all your support, late night talks and all the coffee dates. See you at graduation!

To Stefan van Staden, thank you for the editing.

To the North-West University and the NRF, for their financial support.

Finally, to my boyfriend Dewald, thank you for all your patience and love. Thank you for understanding and being my “co-writer”. I am so glad you came into my life. Love you lots and lots.

Abstract

Oxidative stress, caused by increased levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and/or a decrease in antioxidant capacity, can result in the oxidation of various bio-molecules, such as proteins, lipids and deoxyribonucleic acid (DNA). These oxidized bio-molecules may contribute to pathologies such as cardiovascular diseases, neurodegenerative disorders and cancer. The Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study was initiated in 2008 to investigate the coping styles and catecholamine metabolic markers of Africans, contributing to their higher sympathetic output and poorer psychosocial wellbeing. This study forms part of the SABPA study, but with a specific aim to investigate lipid and protein oxidation markers in hypertensive Africans versus their normotensive counterparts.

Analytical methods for the quantification of specific lipid and protein oxidation markers were optimized and validated. Urine samples from 172 urbanized black South Africans were collected and 3-nitrotyrosine (3NT) and thiobarbituric acid reactive substances (TBARS) were quantified in these samples, using the optimized spectrophotometric and LC-MS/MS methods. Statistical analyses showed that in both males and females, TBARS and 3NT correlated with each other. In males, 3NT also correlated with physical activity level (PAL) and C-reactive protein (CRP), while TBARS also correlated with body mass index (BMI). In females 3NT correlated with BMI, while TBARS correlates with PAL. These correlations meant that they could influence the calculations of the true effect of 3NT and TBARS levels between normotensive and hypertensive subjects. After analyses of covariance (ANCOVA) analyses it was determined that the hypertensive male subjects had higher TBARS values than the normotensive male subjects did (p-value = 0.03) and the normotensive female subjects had higher 3NT levels compared to the hypertensive female subjects (p-value = 0.04).

These results partially supported the hypothesis that elevated concentrations of specific urinary lipid and protein oxidation markers will be observed in the

hypertensive test subjects compared to their normotensive counterparts. The results also indicated that there were indeed a difference in lipid and protein oxidation between hypertensive and normotensive subject.

Key words: reactive oxygen species, reactive nitrogen species, oxidative stress, lipid peroxidation, protein oxidation, malondialdehyde,3-nitrotyrosine, hypertension, SABPA.

List of Symbols and Abbreviations

β	:	beta
%	:	percentage
$^{\circ}\text{C}$:	degree Celsius
>	:	greater than
<	:	smaller than
\pm	:	plus/minus
4-HNE	:	4-hydroxynonenal
4-HHE	:	4-hydroxyhexenal
4-HDDE	:	4- hydroxydodecadienal
3NT	:	3-nitrotyrosine

A

AOX I	:	aldehyde oxidase
AU	:	absorbance units
ANCOVA	:	analyses of covariance

B

BP	:	blood pressure
BMI	:	body mass index
BH ₄	:	tetrahydrobiopterin

C

CH ₂	:	methylene group
-----------------	---	-----------------

CH [•]	:	methylene radical
C3	:	three-carbon
C18	:	eighteen-carbon
Cat	:	catalase
CO ₃ ²⁻	:	carbon trioxide
CV	:	coefficient of variance
cm	:	centimetre
Cu	:	copper
CRP	:	C-reactive protein
D		
DNA	:	deoxyribonucleic acid
DBP	:	diastolic blood pressure
DDAH	:	dimethylargininedimethylaminohydrolase
E		
eNOS	:	endothelial nitric oxide synthase
ELISA	:	enzyme-linked immunosorbent assay
eV	:	electron volt
ESI	:	electrospray ionization
F		
[Fe–S]	:	iron cluster
Fe	:	iron
Fe ²⁺	:	ferrous

Fe^{3+} : ferric

G

GPx : glutathione peroxidase

GS-SG : oxidised glutathione

GSH : reduced glutathione/glutathione

GC : gas chromatography

g : earth's gravitational acceleration

g : gram

GR : glutathione reductase

GGT : gamma glutamyl transferase

g/mol : gram per mole

GI : glycemic index

H

H_2O_2 : hydrogen peroxide

H_2O : water

H^+ : hydrogen atom

HOOH : lipid hydroperoxides

HPLC : high pressure liquid chromatography

HOCl^- : hypochlorous acid

HCl : hydrochloric acid

HIV : human immunodeficiency virus

I

iNOS : inducible nitric oxide synthase

IS	:	internal standard
IFG/IGT	:	impaired glucose regulation
J		
JHB	:	Johannesburg
K		
kg	:	kilogram
kcal/h	:	kilocalorie per hour
L		
LDL	:	low-density lipoprotein
LC-MS/MS	:	liquid chromatograph connected to a triple quadrupole mass analyzer
LC	:	liquid chromatograph
LOD	:	limit of detection
LOQ	:	limit of quantification
L	:	litre
Ltd	:	limited company
M		
μ	:	micro
MPO	:	myeloperoxidase
MDA	:	malondialdehyde
M ₁ dG	:	3-(2-deoxy-β-D-erythrpentofuranosyl)pyrimido[1,2α]purin- 10(3 <i>H</i>)-one
MeSOX	:	methionine sulfoxide

Me=O	:	oxo–metal complexes
m/v	:	mass to volume ratio
mM	:	millimolar
mmHg	:	millimetre of mercury
mL	:	millilitre
mg	:	milligram
MRM	:	multiple reaction monitoring
m	:	milli
<i>m/z</i>	:	mass-to-charge ratio
MS	:	mass spectrometry
mm	:	millimetre
mol	:	mole
M	:	molar
min	:	minute
N		
n	:	nano
NADPH oxidase	:	nicotinamide adenine dinucleotide phosphate-oxidase
NO [•]	:	nitric oxide
NO ₂ [•]	:	nitrogen dioxide
NO ₂	:	nitrite
NOS	:	nitric oxide synthase
nNOS	:	neural nitric oxide synthase
N ₂ O ₃	:	dinitrogen trioxide

<i>n</i> -3	:	omega 3
<i>n</i> -6	:	omega 6
NER	:	nucleotide excision repair pathway
nm	:	nanometre
N	:	normal
NaH ₂ PO ₄	:	sodium phosphate monobasic
NaHPO ₄	:	sodium phosphate dibasic
NaOH	:	sodium hydroxide
NaNO ₂	:	sodium nitrite
NWU	:	North-West University
Na ⁺	:	sodium
O		
O ₂	:	oxygen/dioxygen
O ₂ ⁻	:	superoxide anion
[•] OH	:	hydroxyl radical
ONOO ⁻	:	peroxynitrite
[•] OR	:	alkoxyl radical
P		
PUFAs	:	polyunsaturated fatty acids
psi	:	pressure per square inch
PP	:	polypropylene
pH	:	measurement of hydrogen ion concentration
PAL	:	physical activity level

pmol	:	picomole
pH	:	measures the hydrogen ion concentration
Q		
Q-TOF	:	quadrupole time-of-flight mass spectrometer
R		
ROS	:	reactive oxygen species
RNS	:	reactive nitrogen species
ROO [•]	:	peroxyl radical
R ²	:	R square value
S		
SABPA	:	Sympathetic Activity and Ambulatory Blood Pressure in Africans
SOD	:	superoxide dismutase
SBP	:	systolic blood pressure
STDEV	:	standard deviation
SMAC	:	sequential multiple analyzer computer
SPE	:	solid phase extraction
T		
TLC	:	thin layer chromatography
TBARS	:	thiobarbituric reactive substances
TCA	:	trichloroacetic acid
TIC	:	total ion chromatogram

TBA : thiobarbituric acid

U

UV : ultraviolet

U/L : units per litre

USA : United States of America

V

V : volts

W

w/w : weight to weight ratio

X

XO : xanthine oxidase

List of Figures

Figure 2.1. Negative effects and important biological role of ROS	5
Figure 2.2. Formation of NO^* , ONOO^- and NO_2^*	7
Figure 2.3. Endogenous and exogenous source of ROS	8
Figure 2.4. Causes and effects of oxidative stress	9
Figure 2.5. Mechanism of lipid peroxidation	12
Figure 2.6. Formation of M_1dG	14
Figure 2.7. Oxidation of the protein backbone.....	16
Figure 2.8. Protein oxidation by lipid peroxidation products as well as glycation- and glycoxidation products.....	17
Figure 2.9. Possible pathway of 3NT formation.....	19
Figure 2.10. Involvement of oxidative stress in hypertension	23
Figure 2.11. Experimental approach	25
Figure 3.1. TBA reacting with MDA	27
Figure 3.2. Effect of different incubation times on the TBARS assay	29
Figure 3.3. Effect of different incubation temperatures on the TBARS assay.....	30
Figure 3.4. Effect of different urine volume on the TBARS assay	31
Figure 3.5. Effect of different volumes of reagent on the TBARS assay.....	32
Figure 4.1. Chromatographic separation of butylated 3-nitro-L-tyrosine standard.....	41
Figure 4.2. Chromatographic separations of control urine sample A.....	42

Figure 4.3. Chromatographic separations of control urine sample B.....	43
Figure 4.4. Chromatographic separations of control urine sample C.....	43
Figure 4.5. Chromatographic separations of control urine sample D.....	44
Figure 4.6. Chromatographic separations of control urine sample E.....	44
Figure 4.7. Chromatographic separation of the 3-nitro-L-tyrosine standard, derivatized with R-(-2)-butanol:acetyl chloride	46
Figure 4.8. Chromatographic separation of a urine sample, derivatized with R-(-2)- butanol:acetyl chloride.....	47
Figure 4.9. Chromatographic separation of a stereospecific derivatized urine sample before it was spiked with stereospecific derivatized 3-nitro-L-tyrosine standard	48
Figure 4.10. Chromatographic separation of a stereospecific derivatized urine sample, spiked with stereospecific derivatized 3-nitro-L-tyrosine standard	49
Figure 4.11. Chromatographic separation of the synthesized 3-nitro-L-tyrosine derivatized with R-(-2)-butanol:acetyl chloride	51
Figure 4.12. Chromatographic separation of the synthesized 3-nitro-D-tyrosine derivatized with R-(-2)-butanol:acetyl chloride	51
Figure 4.13. Chromatographic separation of the synthesized 3-nitro-DL-tyrosine derivatized with R-(-2)-butanol:acetyl chloride	52
Figure 4.14. Chromatographic separation of the synthesized nitro- <i>o</i> -tyrosine standard derivatized with R-(-2)-butanol:acetyl chloride	53
Figure 4.15. Chromatographic separation of the synthesized nitro- <i>m</i> -tyrosine standard derivatized with R-(-2)-butanol:acetyl chloride	54
Figure 4.16. TLC plate used to determine the ideal amount of sample that could be loaded on to a TLC plate.....	55

Figure 4.17. Determining if 3NT could be extracted from urine by using TLC plates	57
Figure 4.18. Chromatographic separation of the internal standard, derivatized with R- (-2)-butanol:acetyl chloride.....	61
Figure 4.19. Chromatographic separations of IS in urine sample 1.....	62
Figure 4.20. Calibration curve of 3-nitro-L-tyrosine standard	63
Figure 4.21. Calibration curve of 3-nitro-L-tyrosine standard	63
Figure 5.1. Box plots of the 3NT levels in males and females.....	69
Figure 5.2. Box plots of the TBARS levels in males and females.....	70
Figure 5.3. ANCOVA for 3NT levels in male subjects	75
Figure 5.4. ANCOVA for 3NT levels in female subjects	75
Figure 5.5. ANCOVA for TBARS levels in female subjects	76
Figure 5.6. ANCOVA for TBARS levels in male subjects	77

List of Tables

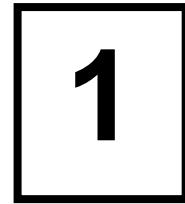
Table 2.1. Classification of hypertension according to The European Society of Hypertension	20
Table 3.1. Conditions for evaluating the effect of different incubation times on the TBARS assay	28
Table 3.2. Conditions for evaluating the effect of different incubation temperatures on the TBARS assay	29
Table 3.3. Conditions for evaluating the effect of different sample volume on the TBARS assay	30
Table 3.4. Conditions for evaluating the effect of different volumes of reagent on the TBARS assay	31
Table 3.5. Calculation of individual absorbance means of five samples assayed on the same day.....	34
Table 3.6. Calculation of single mean and STDEV using five individual mean values of the same sample	34
Table 3.7. Calculation of individual absorbance means of five samples assayed over a period of five days	35
Table 3.8. Calculation of single mean and STDEV using five individual mean values of the same sample over a period of five days	35
Table 4.1. The optimal MRM conditions for 3-nitro-L-tyrosine standard quantification	40
Table 4.2. Mobile phase gradient time table for the chromatographic separation of 3-nitro-L-tyrosine	40

Table 4.3. Areas of peak 1 and 2, before and after spiked with 0.1 mg/mL 3-nitro-L-tyrosinestandard.....	48
Table 4.4. Switching times of the LC-MS/MS between MS and waste	59
Table 4.5. The optimal MRM conditions for internal standard quantification	60
Table 5.1. Variables measured by co-investigators, which were used in the data set....	67
Table 5.2. Influence of categorical variables on urinary TBARS and 3NT levels in male subjects	71
Table 5.3. Influence of categorical variables on urinary TBARS and 3NT levels in female subjects	72
Table 5.4. Correlation of 3NT and TBARS with the continuous variables in male subjects	73
Table 5.5. Correlation of 3NT and TBARS with the continuous variables in the female subjects	73

List of Equations

Equation 2.1. Fenton Reaction	4
Equation 2.2. Haber–Weiss reaction	4
Equation 2.3. Enzymatic decomposition of H_2O_2 into H_2O and O_2 by catalase	5
Equation 2.4. Formation of ONOO^-	6
Equation 3.1. Equation for calculating coefficient of variation	33

Chapter 1



Introduction

Oxidative stress, a consequence of an increase in reactive oxygen species (ROS) and reactive nitrogen species (RNS) and/or a decrease in antioxidant capacity, result in the oxidation of bio-molecules such as lipids, protein and DNA. Oxidation of these bio-molecules is believed to contribute to aging and may contribute to the formation of pathologies such as cardiovascular diseases, neurodegenerative disorders and cancer. One such pathology, linked to oxidative stress, is hypertension.

In 2008, the Sympathetic activity and Ambulatory Blood Pressure in Africans study (SABPA) was initiated at the North-West University (Potchefstroom Campus) to investigate the association between cardiovascular function and psychological stress in urbanized black South Africans compared to Caucasians.

Problem statement and hypothesis

Initial results from the SABPA study showed that urbanized black South Africans have a higher prevalence for hypertension than their Caucasian counterparts. Little information on factors contributing to this epidemic is known, including the role of lipid and protein oxidation. Quantification of lipid peroxidation and protein oxidation markers can thus further our understanding of this epidemic. **It is therefore hypothesized that elevated concentrations of specific urinary lipid and protein oxidation markers will be observed in the hypertensive test subjects compared to their normotensive counterparts.** In order to test this hypothesis, the following aim and objectives were formulated:

Aim and objectives

The aim of this study is to investigate specific lipid and protein oxidation markers in urine samples of hypertensive Africans versus their normotensive counterparts.

The following objectives were formulated:

- 1) To optimize a spectrophotometric method to quantify lipid oxidation markers thiobarbituric reactive substances (TBARS) assay.
- 2) To optimize a LC/MS/MS method to quantify 3-nitrotyrosine (3NT).
- 3) To quantify 3NT and TBARS in a cohort of African test subjects.
- 4) To statistically investigate the potential involvement of the measured lipid and protein oxidation markers in hypertension in this cohort.

Chapter 2 contains a literature overview on ROS and RNS, sources of ROS, oxidative stress, lipid- and protein oxidation as well the biomarkers for quantifying lipid and protein oxidation. Information is also given on hypertension and a possible link between hypertension and oxidative stress. Chapter 2 concludes with a study plan and approach. Optimization of the TBARS assay is described and discussed in chapter 3. In chapter 4, the optimization of the 3-nitrotyrosine assay is described and discussed. Chapter 5 explores the involvement of lipid and protein oxidation markers in hypertension. In chapter 6 the conclusion as well as recommendations are given. The Harvard referencing style was used for referencing.

Chapter 2

2

Literature overview

2.1 INTRODUCTION

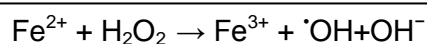
Free radicals are molecules or fragments of a molecule that have an unpaired electron on the atomic or molecular level. The breaking of covalent bonds between two atoms leaves each atom with an unpaired electron (Gutteridge, 1995). These unpaired electrons cause the molecules and fragments to be highly reactive, leading to the formation of other classes of free radicals as well as molecules that do not contain an unpaired electron. However, due to its high reactivity, the latter is included under the free radical group (Tremellen, 2008; Valko *et al.*, 2007; Gutteridge, 1995).

2.2 REACTIVE OXYGEN SPECIES (ROS)

One such class of free radicals is reactive oxygen species, which is better known as ROS. ROS is a term that is used to define free radical species that reacts with oxygen (O_2) (D'Autréaux and Toledano, 2007; Valko *et al.*, 2007). When O_2 receives an extra electron through means of oxidation, a superoxide anion (O_2^-) is formed. O_2^- is mainly formed as a by-product of respiration through the enzyme nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) (D'Autréaux and Toledano, 2007). This enzyme is a membrane-bound enzyme complex and is situated in plasma membranes as well as the membranes of phagosomes. Alternatively, O_2^- can be formed by means of iron-sulphur ([Fe-S]) clusters, which are situated in heme. During this process iron (Fe) is oxidized from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state. The O_2^- , which is bound to heme, is released during decomposition of the heme (Buonocore *et al.*, 2010; D'Autréaux and Toledano, 2007). O_2^- is known as the primary ROS and can react with other molecules directly or indirectly through enzymes, such as superoxide dismutase (SOD) xanthine oxidase (XO) and aldehyde oxidase (AOX I) or through metal catalyzed reactions (Buonocore *et al.*, 2010). These reactions give rise to secondary ROS such as the hydroxy radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) (Tremellen, 2008; Valko *et al.*, 2007).

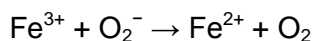
$\cdot\text{OH}$ forms when increased amounts of O_2^- and H_2O_2 are present during oxidative stress. $\cdot\text{OH}$ is one of the most reactive species of the ROS group. As a result of the instant reaction with other molecules at the formation site, $\cdot\text{OH}$ contains a short half-life time of about 10^{-9} seconds (Buonocore *et al.*, 2010; Valko *et al.*, 2007). $\cdot\text{OH}$ can be produced with two methods, namely through the Fenton reaction as well as the Haber–Weiss reaction. The primary produced ROS, O_2^- causes the [Fe–S] containing enzymes of the dehydratase-lyase family to release iron in the Fe^{2+} state. The Fe^{2+} is then used to produce $\cdot\text{OH}$ using the Fenton reaction (Equation 2.1). Under oxidative conditions, the high levels of O_2^- are used in the Haber–Weiss reaction, shown in Equation 2.2. This reaction is a combination of the Fenton reaction and the reduction of the Fe^{3+} through Fe^{2+} . The iron clusters are oxidized by the O_2^- , resulting in the formation of $\cdot\text{OH}$ from H_2O_2 (Buonocore *et al.*, 2010; Valko *et al.*, 2007).

Equation 2.1 Fenton Reaction



Equation 2.2 Haber–Weiss reaction

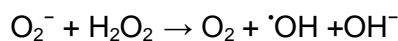
The first step is the reduction of ferric ion to ferrous:



The second step is the Fenton reaction:

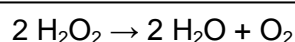


The netto reaction:



One of the non-radical ROS species is H_2O_2 . H_2O_2 is produced by the peroxisome, a location where there are high levels of O_2 consumption (Valko *et al.*, 2007). H_2O_2 gives way to the oxidation of a variety of bio-molecules and can contribute to high levels of $\cdot\text{OH}$ during the Haber–Weiss reaction (Equation 2.2). Prevention of toxic levels of H_2O_2 occurs through enzymes such as catalase (Cat) that decomposes H_2O_2 into water (H_2O) and O_2 (Equation 2.3).

Equation 2.3 Enzymatic decomposition of H_2O_2 into H_2O and O_2 by catalase



Although ROS has many negative effects such as the oxidation of bio-molecules, it also has important biological roles. These roles can be either regulatory, intracellular signalling or defence of nature. Both the negative effects as well as the important biological roles of ROS are summarized in Figure 2.1 (Buonocore *et al.*, 2010).

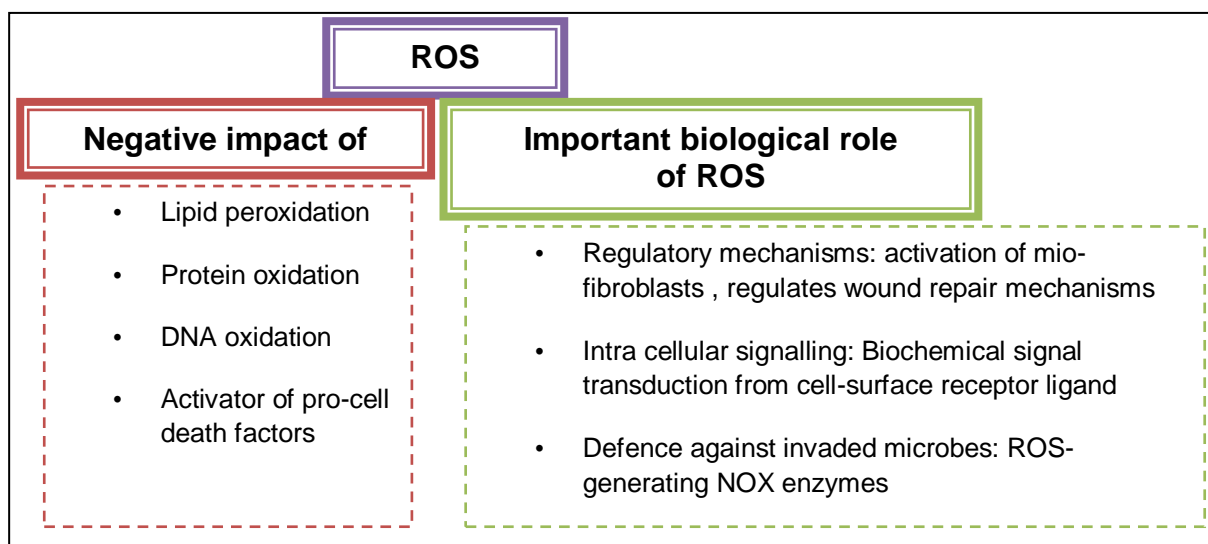


Figure 2.1 Negative effects and important biological role of ROS. ROS can have negative effects such as oxidation of lipids, proteins; DNA and it can acts as activator of pro-cell death factors. Important biological roles to ensure cellular function are regulatory roles, intracellular roles signalling and defence roles (adapted from Buonocore *et al.*, 2010).

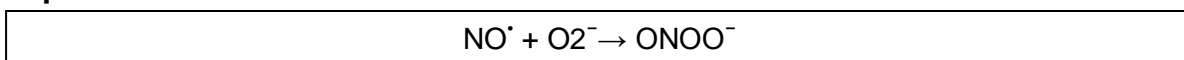
2.3 REACTIVE NITROGEN SPECIES (RNS)

Another class of free radical species that can be formed is known as reactive nitrogen species, referred to as RNS. RNS include molecules such as nitric oxide (NO^\bullet), peroxynitrite (ONOO^-) and nitrogen dioxide (NO_2^\bullet). These molecules are responsible for the oxidation of proteins, through the nitration of tyrosine molecules (Valko *et al.*, 2007). NO^\bullet is a molecule that is produced in biological tissues through nitric oxide synthase (NOS), where three different isoenzymes are distinguished namely nNOS (neural NOS), iNOS (inducible NOS) and eNOS (endothelial NOS). NOS converts arginine to citrulline which is achieved by means of a five electron oxidative reaction. This will ultimately result in the formation of NO^\bullet . Just as with ROS, NO^\bullet serves as a very important biological signalling molecule, by being involved in processes such as neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation (Valko *et al.*, 2007; Mohiuddin *et al.*, 2005). When NO^\bullet reacts with O_2 , nitrite (NO_2^-) is formed and is then converted into NO_2^\bullet through the enzyme myeloperoxidase (MPO)

(Mohiuddin *et al.*, 2005). As a result of its solubility in both aqueous and lipid media, NO[•] can diffuse across cytoplasm and plasma membranes. This means it can have effects on both neuronal transmission and synaptic plasticity in the central nervous system. It can also react with H₂O and O₂ in the extracellular environment, leading to the formation of nitrate as well as nitrite anions. As a result of antioxidant defence mechanism not functioning correctly, an excess of RNS in the system is referred to as nitrosative stress. Nitrosative stress can cause nitrosylation reactions, leading to the rearrangement of protein structures, which in turn can cause a decrease, or total loss of protein function (Valko *et al.*, 2007; Ridnour *et al.*, 2004; Klatt & Lamas, 2000).

During inflammatory processes, O₂⁻ and NO[•] can react with each other, which will ultimately result in the formation of ONOO⁻, a highly oxidative molecule (Equation 2.4). ONOO⁻ is a far more reactive oxidizing reagent than NO[•] and can react with bio-molecules such as lipids, proteins and DNA, resulting in lipid peroxidation, protein oxidation and DNA fragmentation, respectively (Carr *et al.*, 2000). ONOO⁻ reactions can also lead to the formation of NO₂[•], as well as the formation of other secondary RNS products such as dinitrogen trioxide (N₂O₃). Figure 2.2 summarizes the formation of NO[•], as well as NO₂[•] and ONOO⁻ (Augusto *et al.*, 2008; Mohiuddin *et al.*, 2005; Bian *et al.*, 1999).

Equation 2.4 Formation of ONOO⁻



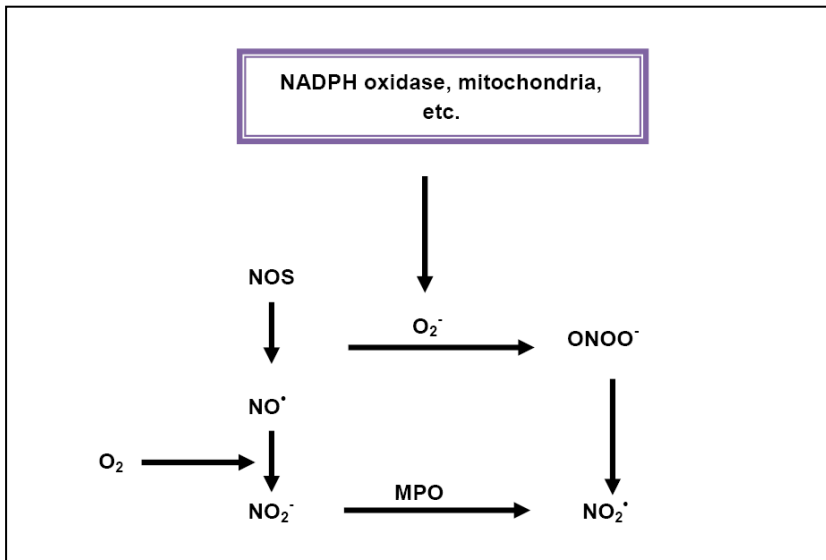


Figure 2.2 Formation of NO^{\bullet} , $ONOO^{\bullet -}$ and NO_2^{\bullet} . In this figure the two pathways of nitrogen dioxide (NO_2^{\bullet}) formation are illustrated. The first pathway is when nitric oxide (NO^{\bullet}) is produced from L-arginine by the enzyme nitric oxide synthase (NOS). NO^{\bullet} then reacts with $O_2^{\bullet -}$, resulting in the formation of peroxynitrite ($ONOO^{\bullet -}$). This is then converted to NO_2^{\bullet} . The second pathway is where NO^{\bullet} reacts with molecular oxygen resulting in the formation of nitrite (NO_2^-). NO_2^- is then converted to NO_2^{\bullet} by, means of myeloperoxidase (MPO). Both of these pathways result in tyrosine nitration (adapted from Mohiuddin *et al.*, 2005).

2.4 ENDOGENOUS AND EXOGENOUS SOURCE OF ROS AND RNS

ROS and RNS can be formed through various endogenous as well as exogenous sources. As previously mentioned, the formation of the primary ROS is through NADPH oxidase situated in plasma membranes and membranes of phagosomes. Other endogenous sources include mitochondrial leaking, respiratory burst, auto-oxidation, enzymatic oxidation, peroxisomal β -oxidation and phagocytic cells during inflammatory processes. Exogenous sources that contribute to the formation of ROS and RNS are ultraviolet (UV) irradiation, pollutants, cigarette smoke, ionizing radiation, xenobiotics and medication such as antibiotics (Young and Woodside, 2001). Figure 2.3 gives a summary of the endogenous and exogenous sources of ROS.

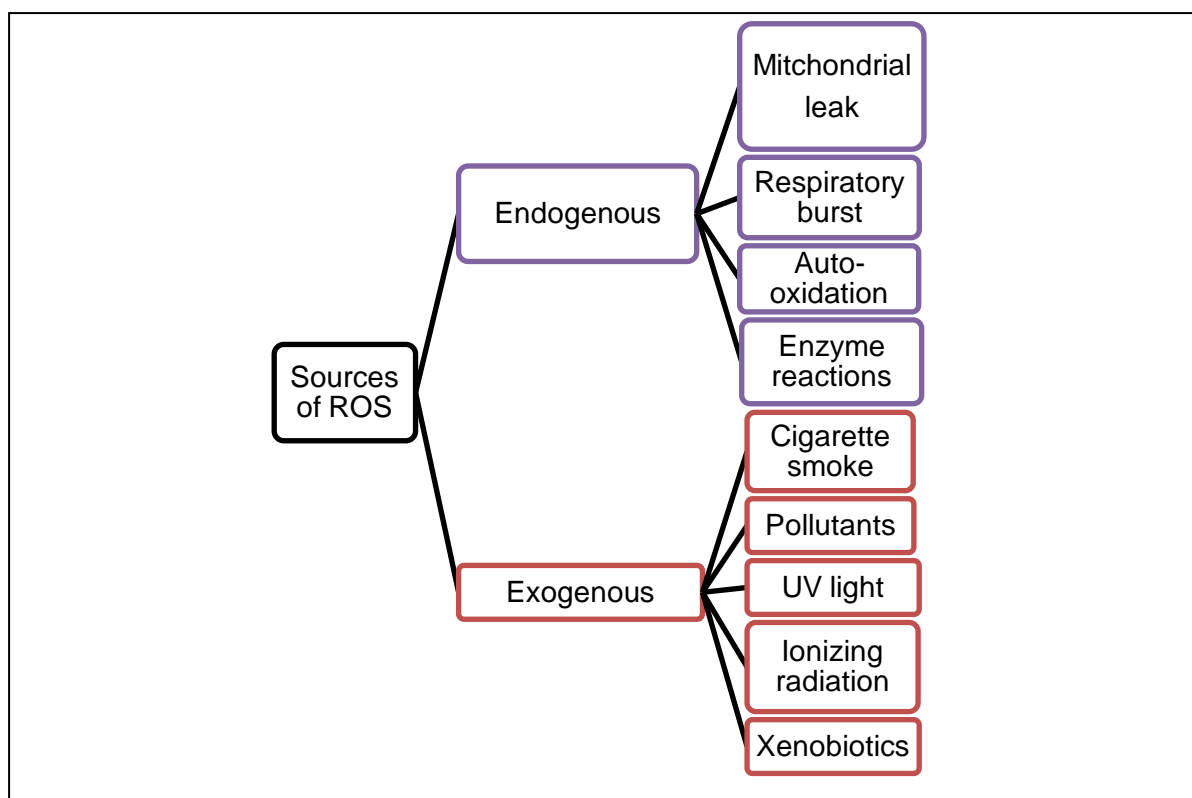


Figure 2.3 Endogenous and exogenous source of ROS. ROS can be formed endogenously through means of mitochondrial leaking, respiratory burst and through enzyme as well as auto-oxidation reactions. Exogenous formation of ROS can occur through cigarette smoke, pollutants, UV light, ionizing radiation and xenobiotics (adapted from Young and Woodside; 2001).

2.5 OXIDATIVE STRESS

Oxidative stress, or in the case of RNS, nitrosative stress, is a process that occurs when the oxidation and reduction state (redox state) of the body is disrupted. Causes of oxidative stress can be ascribed to an increase in ROS and other free radical production, which then overwhelms the body's antioxidant defence mechanism. It can also be as a result of the dysfunction of the antioxidant mechanisms leading to an antioxidant to ROS imbalance (Galley, 2011; Lavie and Lavie, 2009).

Because of oxidative stress, the body contains adaptive mechanisms which up-regulate defence systems. These mechanisms can offer either complete or partial protection. In the case of complete protection, no disease formation will take place where in turn partial protection would lead to damage of the cellular structures caused by oxidation of lipids, proteins as well as DNA (Galley, 2011; Valko *et al.*, 2007). This is believed to contribute to tissue injury, resulting in the necrosis or apoptosis of cells as well as disease formation (Dalle-Donne *et al.*, 2003).Figure 2.4

sums up the effects of complete and partial protection of the antioxidant defence mechanisms.

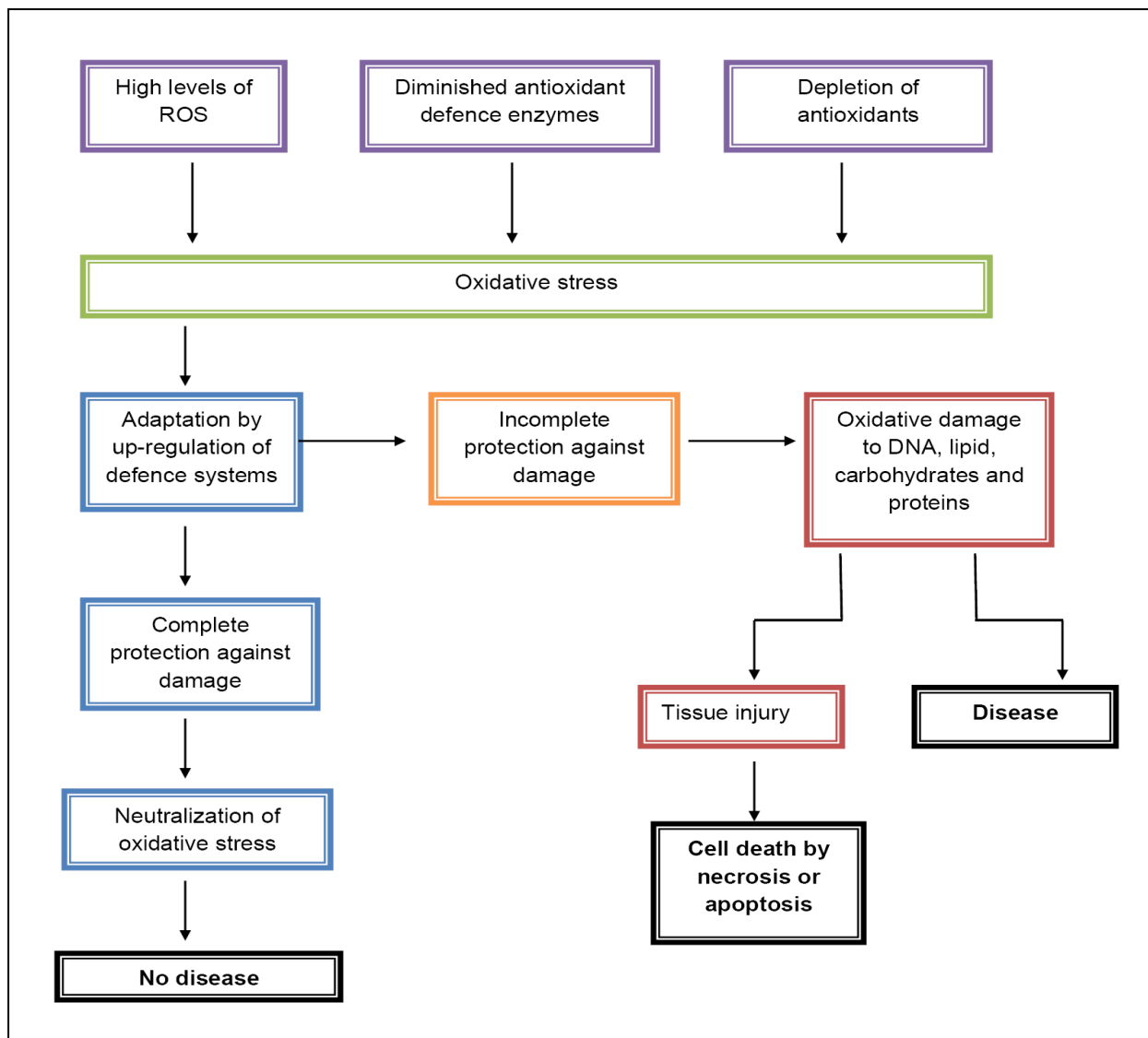


Figure 2.4 Causes and effects of oxidative stress. This figure summarizes the causes as well as the effects of oxidative stress. Oxidative stress can occur if there is an increase in ROS production, the antioxidant defence mechanisms functions incorrectly and if there is depletion in the amount of available antioxidants. Because of oxidative stress, an up regulation of adaptive defence mechanisms can occur. This results in complete or partial protection. Complete protection will lead to no formation of diseases. Whereas damage to the macromolecules such as carbohydrates, lipids and proteins can occur as a result of direct oxidative stress or partial protection by the up regulation of adaptive defence systems. This can lead to tissue injury, resulting in the necrosis or apoptosis of cells as well as disease formation (adapted from Dalle-Donne *et al.*, 2003).

The body's primary method of protecting itself against oxidative stress is by means of the antioxidant defence mechanisms. Antioxidant defence mechanism can be grouped into two defence systems; the enzymatic defence system and the non-

enzymatic defence system (Tremellen, 2008; Valko *et al.*, 2007). The enzymatic defence system includes enzymes such as catalase (Cat), glutathione peroxidase (GPx) and superoxide dismutase (SOD). SOD is responsible for converting O_2^- into H_2O_2 and O_2 , whereas Cat decomposes H_2O_2 into H_2O and O_2 . Along with Cat, GPx also decreases the H_2O_2 levels by catalyzing a reaction where two reduced glutathione (GSH) with H_2O_2 to form two H_2O molecules. This reaction is accompanied by the formation of oxidized glutathione disulfide (GS-SG). In the presence of NADPH, this cycle is then completed with GS-SG being reduced back to GSH through means of glutathione reductase (GR) (Tremellen, 2008; Prabhakar *et al.*, 2005; Saydam *et al.*, 1997). The non-enzymatic defence system includes ascorbic acid (vitamin C), tocopherol (vitamin E), carotenoids and flavonoids (Valko *et al.*, 2007). Both of these systems can be found in cellular membranes, in the plasma or in the cytosol (Pepe *et al.*, 2009; Wiernsperger, 2003).

2.6 LIPID PEROXIDATION

2.6.1 Introduction

As previously mentioned, one of the main targets of ROS during oxidative stress is lipids. The oxidation of lipids is referred to as lipid peroxidation, and was defined in the 1940's as an autoxidative free-radical chain reaction (Gutteridge, 1995). During this process, both ROS and RNS can react with polyunsaturated fatty acids (PUFAs) found in cellular membranes, liposomes and lipoproteins (Bochkov *et al.*, 2010; Pereira *et al.*, 2003). This can result in the alteration of the cells fluidity as well as membrane permeability, which may have biological consequence (Denicola and Radi, 2005). The end result of these consequences may cause the onset of formation of different pathologies including cancer, cardiovascular disease and neurodegenerative disorders such Alzheimer and Parkinson's disease (Dix and Aikens, 1992).

2.6.2 Mechanism

Lipid peroxidation consists of three steps; the initiation, the propagation and the termination (Higdon *et al.*, 2012; Schneider *et al.*, 2008; Dix and Aikens, 1992). The initiation step can be sub-divided into two steps. The first step is the triggering step, which involves the removal of a hydrogen (H^+) atom and secondly the formation of a

more stable conjugated diene (Pereira *et al.*, 2003). During the initiation step, ROS such as the $\cdot\text{OH}$, will react with the omega-3 ($n-3$) and -6 ($n-6$) PUFAs situated in cellular membranes. This results in the removal of the methylene group's (CH_2) H^+ atom, leaving the CH_2 with an unpaired electron. The unpaired CH_2 is referred to as a methylene radical (CH^\cdot). Due to the unpaired electron, the CH^\cdot is unstable and undergoes a conversion by molecular rearrangement to produce a conjugated diene that is a more stable form (Figure 2.5). The formed conjugated diene reacts with O_2 . This gives way to the formation of a highly reactive peroxy radical (ROO^\cdot) which enters the propagation phase (Gutteridge, 1995; Dix and Aikens, 1992). During the propagation phase, the ROO^\cdot can remove H^+ atoms from other PUFAs, forming different fatty acid radicals and lipid hydroperoxides (HOOH) as well as cyclic peroxide when it reacts with itself. The HOOH can undergo further reactions, resulting in the formation of reactive alkoxy radicals ($\cdot\text{OR}$) and $\cdot\text{OH}$, through Fe or Cu-catalyzed Fenton-like reactions. A free radical chain reaction mechanism follows, as these radicals as well as other fatty acid radicals follow the same pathway (Bochkov *et al.*, 2010; Gutteridge, 1995; Dix and Aikens, 1992). Termination of the free radical chain reaction mechanism occurs when two or more radical species react with each other to form non-radical species (Figure 2.5). This will be accomplished if the amount of radical species produced, are high enough to ensure a reaction between two radicals. Another way of termination can be through means of the antioxidant defence mechanisms, both enzymatic and non-enzymatic that acts as free radical scavengers (Dix and Aikens, 1992).

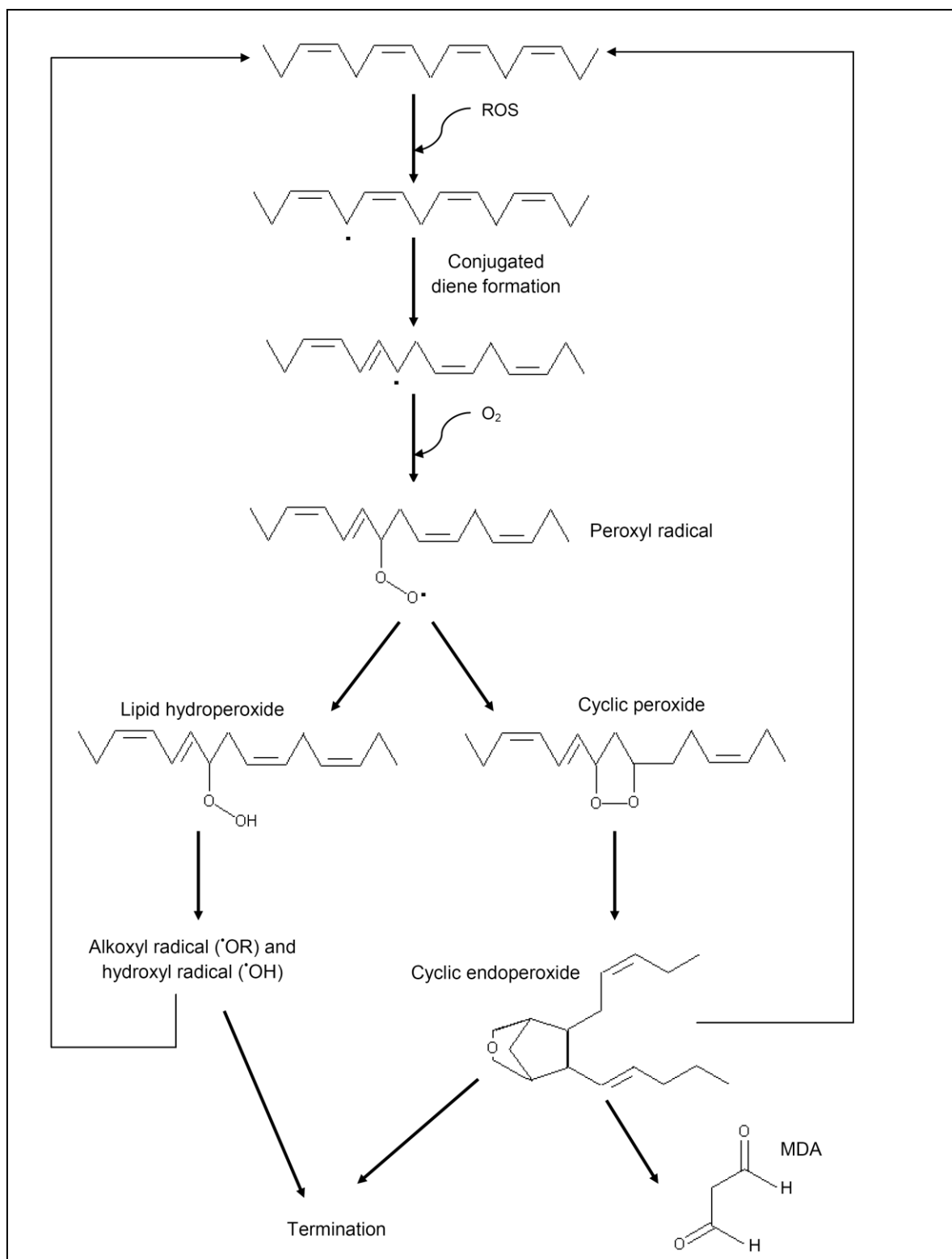


Figure 2.5 Mechanism of lipid peroxidation. Formation of an unstable methylene radical (CH[•]) takes place through the removal of the methylene group's hydrogen atom by ROS and/or RNS. The unstable CH[•] is then converted to a more stable form. The stable conjugated diene reacts with oxygen (O₂) to form a peroxy radical. The peroxy radical can form hydroperoxides and cyclic peroxides. The cyclic peroxides results in the formation of endoperoxides. The endoperoxide will finally give rise to the lipid oxidation marker, malondialdehyde (MDA). During the termination step of lipid peroxidation, the free radical chain reaction mechanism is stopped through two lipid radical reacting with each other or through antioxidant defence mechanisms (adapted from Pereira *et al.*, 2003).

2.6.3 Lipid peroxidation markers

2.6.3.1 Introduction

Because of lipid peroxidation of the *n*-3 and *n*-6 PUFAs, a variety of reactive products is formed. These products include malondialdehyde (MDA), F₂-isoprostane and the 4-hydroxyalkenals, 4-hydroxy-2*E*-nonenal (4-HNE), 4-hydroxy-2*E*-hexenal (4-HHE) and 4-hydroxydodecadienal (4-HDDE) (Kuiper *et al.*, 2010; Guichardant *et al.*, 2004; Guha and Moore, 2003). Quantification of these products can be used as an indication of the amount of lipid peroxidation that occurred. MDA is one of the most common markers of lipid peroxidation (Romero *et al.*, 1998; Esterbauer *et al.*, 1990) and will be discussed in more detail.

2.6.3.2 Malondialdehyde (MDA)

MDA is a three-carbon (C₃) molecule with a low molecular weight. It is a by-product, which is formed during the oxidation of *n*-3 PUFAs and is used as a global marker of lipid peroxidation (Guichardant *et al.*, 2004). During the oxidation of the *n*-3 PUFAs, the ROO[•] which is formed, is converted into a cyclic peroxide, and thereafter, into a cyclic endoperoxide. The cyclic endoperoxide is the structure that gives rise to the MDA (Figure 2.5). Apart from the oxidation of *n*-3 PUFAs, MDA can also be formed by means of other sources. These sources include formation as a by-product of the metabolism of arachidonic acid during the process of prostaglandin biosynthesis and the oxidative iron-dependent break down of amino acids, pentoses, hexoses and other carbohydrates (Grotto *et al.*, 2009; Singh *et al.*, 2001).

MDA can react with the functional groups of other molecules such as lipoproteins, DNA and proteins. One such an example is shown in Figure 2.6, where MDA can react with deoxyguanosine located in DNA. This reaction leads to the formation of the highly florescent M₁dG (3-(2-Deoxy-β-D-*erythro*-pentofuranosyl)pyrimido[1,2-α]purin-10(3*H*)-one). Reaction can also occur with the adenine and cytosine deoxynucleoside, but this is to a lesser extent. M₁dG is mutagenic to both bacterial and mammalian cells and causes base pair substitutions as well as frame shift mutations in repeated sequences and are repaired by the nucleotide excision repair pathway (NER) (Otteneder *et al.*, 2006; Singh *et al.*, 2001). Due to the fact that M₁dG is less prone to be formed artificially, it portrays a good marker for the indication of

oxidative damage to DNA, both *in vitro* and *in vivo* (Jeong *et al.*, 2008; Esterbauer *et al.*, 1990). MDA can also cause formation of amino-imino-propen crosslinks. This involves cross-linking between the amino groups of the guanosine base and the amino group of the cytosine base located on the DNA complementary strand. This leads to the modification of double-stranded DNA (Esterbauer *et al.*, 1990).

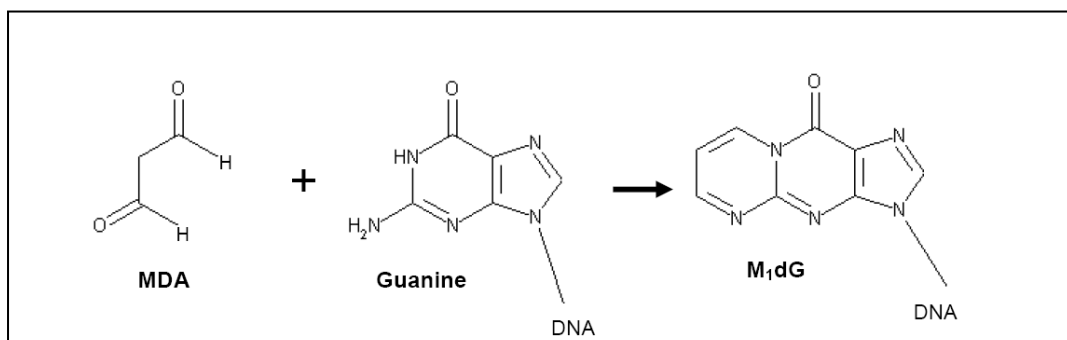


Figure 2.6 Formation of M₁dG. During M₁dG formation, MDA reacts with the guanine deoxynucleoside situated in the DNA (adapted from Singh *et al.*, 2001).

MDA can be measured either through colour reactions, fluorescent reactions or through spectrophotometric reactions. Chromatography can also be considered an option during which thin layer chromatography (TLC), high-pressure liquid chromatography (HPLC) or gas chromatography (GC) can be used. These methods can be used on either derivatized or underivatized molecules, depending on the method chosen. One of the most widely used methods for the detection of MDA is by means of the TBARS (thiobarbituric reactive substances) assay, which will be discussed in Chapter 3. The TBARS assay is a colorimetric analysis where an adduct is formed between the reagent and the MDA, resulting in the formation of a chromophore that can be measured spectrophotometrically at 532,nm (Esterbauer *et al.*, 1990). Over the past two decades, elevated levels of malondialdehyde have been associated with pathologies such as cancer, diabetes and Alzheimer's disease. Elevated concentrations of MDA have also been used to measure the toxicological effects of pollutants such as metal, solvents and xenobiotics (Grotto *et al.*, 2009).

2.7 PROTEIN OXIDATION

2.7.1 Introduction

Not only are lipids targets of ROS and RNS, but also the amino acids residues that are situated in the side chains of proteins. Oxidation of these amino acid residues is

referred to as protein oxidation. Protein oxidation can lead to the formation of protein-protein cross-linkage as well as peptide bond cleavage. This can lead to the fragmentation or rearrangement of the protein structures, dissociation of the protein subunits, unfolding of the protein structure (local and global) or exposure of the hydrophobic residues. All of these can cause the decrease or total loss of protein function, resulting in the formation of diseases (Berlett and Stadtman, 1997; Dean *et al.*, 1997).

2.7.2 Mechanism

Oxidation of proteins can occur through three reactions. The first reaction includes the direct oxidation by ROS such as $\cdot\text{OH}$ and is referred to as the oxidation of the protein backbone (Dean *et al.*, 1997). During oxidation of the protein backbone, a carbon-centred radical is formed. This radical can react with other carbon-centred radicals to form protein cross-linkage. It can also undergo further reactions with O_2 , resulting in the formation of alkylperoxyl radical intermediates. Again, these intermediates can undergo further reactions, producing alkylperoxide radicals, which in turn results in the production of alkoxy radicals. Alkoxy radicals are responsible for peptide bond cleavage and leads to the formation of hydroxyl protein derivatives. The intermediates that are formed during the oxidation process (alkyl, alkylperoxyl and alkoxy), are able to react with other amino acid residues located in the same protein structure or in other protein structure. This can then give rise to other new carbon-centred radicals that follow the same pathway, forming a chain reaction that mimics those of lipid peroxidation (Figure 2.7) (Berlett and Stadtman, 1997; Dean *et al.*, 1997).

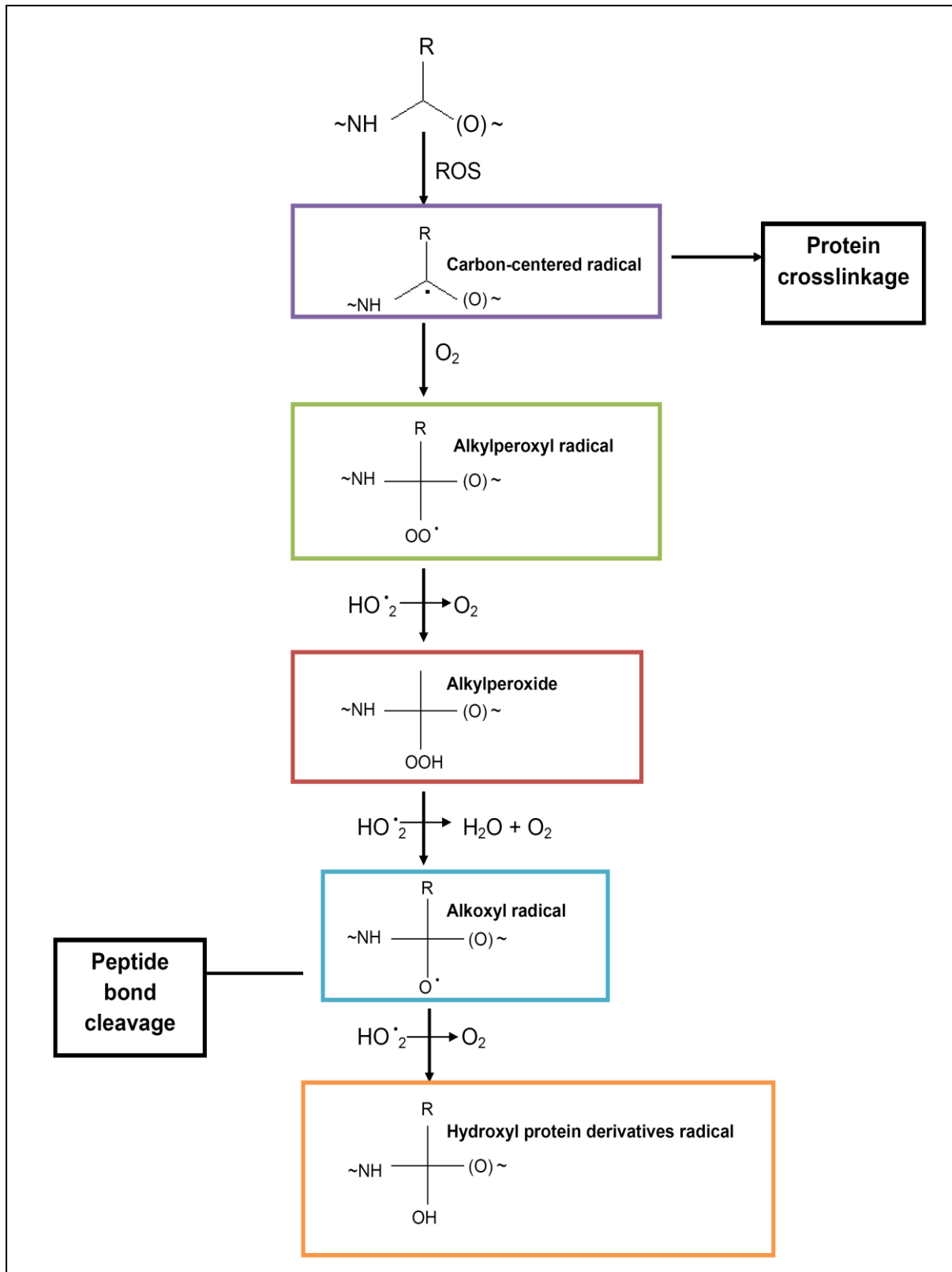


Figure 2.7 Oxidation of the protein backbone. The first step of protein backbone oxidation is the formation of the carbon-centred radical. Then an alkylperoxyl radical intermediate is formed, which leads to the formation of an alkylperoxide. The alkylperoxide gives rise to an alkoxy radical leading to the final product which is the hydroxyl protein derivative (adapted from Berlett and Stadtman, 1997; Dean *et al.*, 1997).

The second pathway by which protein oxidation can take place is through the reaction of proteins with other reactive carbonyl derivatives such as ketoamines, ketoaldehydes, and deoxyosones. These carbonyl derivatives are formed during processes such as glycation and glycoxidation (Figure 2.8 A). The third pathway entails the reaction of proteins with lipid peroxidation products such as MDA and 4-HNE (Figure 2.8 B and C) (Berlett and Stadtman, 1997). These reactions lead to the formation of a protein carbonyl, a marker that is used as an indication of protein oxidation (discussed in Section 2.1.3.3).

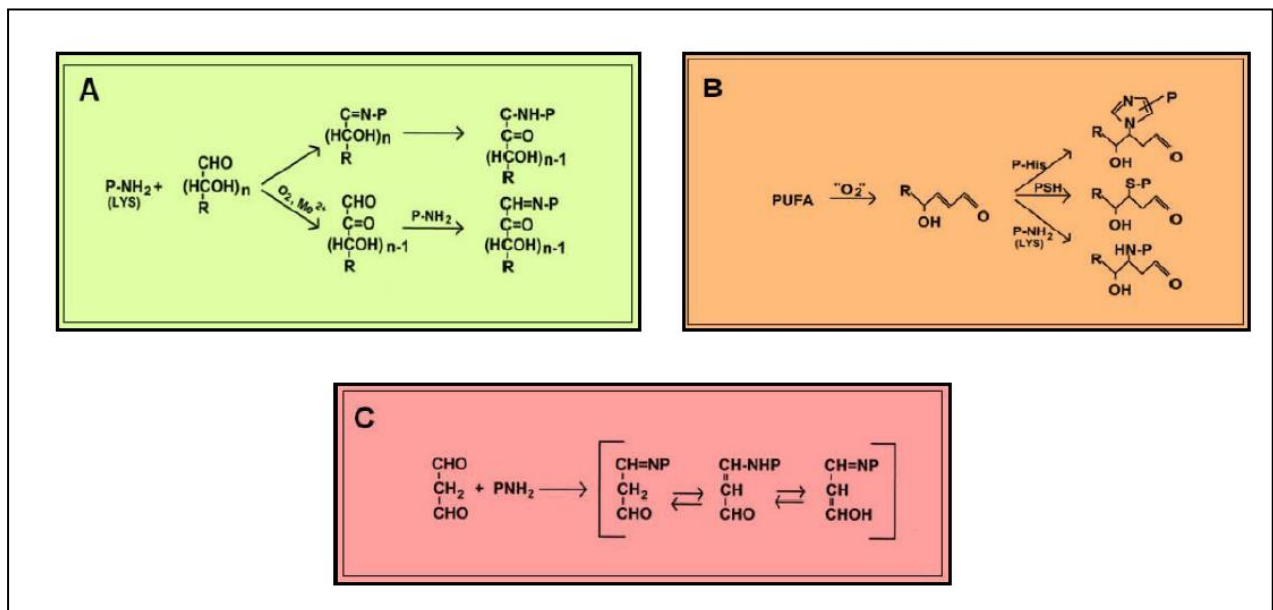


Figure 2.8 Protein oxidation by lipid peroxidation products as well as glycation- and glycoxidation products. This is an illustration of protein reacting with reducing sugars as a result of glycation and glycoxidation reactions. It also illustrates the reaction between protein and the lipid peroxidation markers 4-HNE and MDA (adapted from Berlett and Stadtman, 1997). A= reaction with sugars, B = reaction with 4-HNE and C= reaction with MDA.

All of the amino acids are susceptible to protein oxidation. However, some amino acids are more prone to protein oxidation than others are. The amino acids methionine and cysteine, which contains sulphur residues, is an example of last mentioned group. These amino acids can be targeted by any kind of ROS and are also the only amino acids that can be repaired through the repair enzymes disulfide reductases and methionine sulfoxides (MeSOX) reductases. Disulfide reductases and MeSOX reductase converts disulfides and MeSOX back to their reduced state (Dean *et al.*, 1997). Another group of amino acids that are more prone to oxidation is the hydrophobic aromatic amino acids (tryptophan), tyrosine and phenylalanine.

Oxidation of these amino acids results in the formation of 3,4-dihydroxyphenylalanine also known as dopa, 2-OH-phenylalanine (o-tyrosine) and 3-OH-phenylalanine (m-tyrosine) (Dean *et al.*, 1997).

2.7.3 Protein oxidation markers

2.7.3.1 Introduction

As with lipid peroxidation, protein oxidation also results in the formation of protein oxidation products. These products can be used to determine whether protein oxidation has occurred in a sample. Markers indicating the presence of protein oxidation include protein carbonyls as well as modified tyrosine molecules such as 3-nitrotyrosine (3NT) chlorotyrosine, dityrosine, trityrosine and 3-bromotyrosine. Of the markers mentioned, 3NT will be discussed further.

2.7.3.2 3-Nitrotyrosine

The modified tyrosine molecule, 3-nitrotyrosine (3NT), is a protein oxidation marker that is formed as a result of neutrophil activation upon inflammation and micro-organism invasion. During activation of these immune cells, antimicrobial systems located in the phagosomes discharges their contents. These antimicrobial systems can be enzymes such as myeloperoxidase (MPO) (Klebanof, 2005). Accompanied by the secretion of this enzyme, is an increase in the O_2 consumption. This process is known as oxidative burst. During this process, an increase in O_2^- and H_2O_2 production takes place. The enzymes utilize H_2O_2 as a co-substrate for the production of other radical products and the two main products, that are formed, are hypochlorous acid ($HOCl$) and NO_2^* (Podrez *et al.*, 1999).

During the formation 3NT, carbon trioxide (CO_3^{2-}), oxo-metal complexes ($Me=O$) or *OH reacts with tyrosine. This reaction leads to the formation of a tyrosyl radical. The tyrosyl radicals then undergoes dimerization, resulting in the formation of 3,3-dityrosine, which is another modified tyrosine molecule. The tyrosyl molecule can form 3NT if and when it reacts with NO_2^* , which is formed by MPO. This two possible pathways that the tyrosyl radicals follows, competes with each other. To lessen the competition, the reaction with NO^* is favoured as a result of stabilisation of the tyrosyl molecule. This is achieved through limiting the intra- and intermolecular dimerization.

Another pathway that also competes with 3NT formation, is the formation of 3-hydroxytyrosine which is formed from a tyrosine hydroxyl radical adduct (Radi, 2004; Mohiuddin *et al.*, 2005). 3NT can also be formed as a result from tyrosyl radical reacting with NO^\bullet . This leads to the formation of 3-nitrosotyrosine which is oxidized to 3NT through a two-electron oxidation reaction, using a tyrosine iminoxyl radical as an intermediate (Mohiuddin *et al.*, 2005; Radi, 2004) (Figure 2.9).

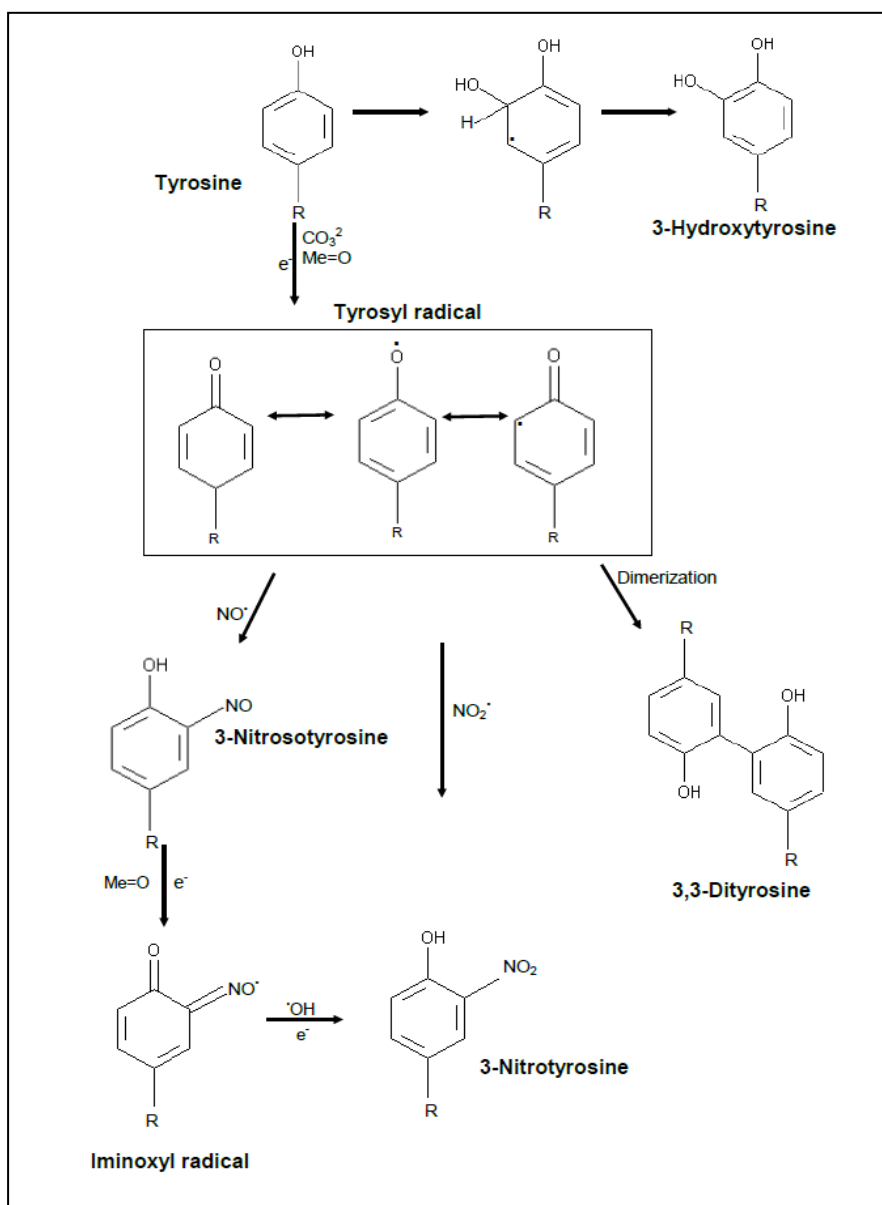


Figure 2.9 Possible pathway of 3NT formation. Tyrosine is converted to a tyrosyl radical through a reaction with molecules such as carbon trioxide (CO_3^{2-}), oxo-metal complexes (Me=O) or hydroxyl radicals (OH^\bullet). The tyrosyl radical can react with nitrogen dioxide (NO_2^\bullet) which will result in 3NT formation. The tyrosyl can also react with NO^\bullet forming 3-nitrosotyrosine which is converted to 3NT by forming tyrosine iminoxyl radical as an intermediate (adapted from Radi, 2004).

The 3NT that is formed in bound protein is believed to contribute to atherosclerosis. This is as a result of the protein that is bound to LDL (low-density lipoprotein). The tyrosine molecule then gets nitrated, resulting in oxidation of LDL. The oxidized LDL molecules undergo phagocytosis via macrophages. This results in the formation of cholesterol and foam cells, which are the major components of plaque that is responsible for the atherosclerosis formation. 3NT also plays a role in other inflammatory conditions such as lupus, arthritis, rheumatoid, pancreatitis, Crohn's disease and influenza (Mohiuddin *et al.*, 2005).

2.8 HYPERTENSION

Hypertension, also commonly known as high blood pressure (Giles *et al.*, 2009) is a medical condition that is believed to have a correlation with strokes, myocardial infarctions, heart as well as kidney failure and in some cases are believed to be involved in premature death (Siyad, 2011). Classifying hypertension is problematic as there are various classification systems with guidelines for blood pressure measurement, normal ranges, as well as cut-off points for hypertension. During this study, the classifying system of The European Society of Hypertension was used. As indicated by Table 2.1 this classifying system categorizes blood pressure (BP) to be normal when one has a systolic and diastolic blood pressure (SBP and DBP) of below 120 mmHg and 80mmHg, respectively. According to these guidelines a patient is diagnosed with hypertension when he or she has continues SBP and DBP readings of 140 mmHg and/or 90 mmHg or higher (O'Brien *et al.*, 2005).

Table 2.1 Classification of hypertension according to The European Society of Hypertension

Blood pressure classification	Systolic blood pressure (SBP) mmHg	Diastolic blood pressure (DBP) mmHg
Normal	< 120	< 80
Hypertension	>140	>90

Hypertension can be divided into three main groups: essential hypertension, hereditary hypertension and secondary hypertension. A patient is diagnosed with essential hypertension, also referred to by some as primary hypertension, when the

cause of the hypertension is unknown. This form of hypertension is responsible for nearly 90 % of all hypertension cases. Hereditary hypertension is as a result from genes associated with hypertension passed on through generations. Secondary hypertension is defined when a patient is diagnosed with hypertension and the cause is known. These causes are usually medical condition such as kidney or thyroid diseases, but can also be a result of certain medications such as cold medicine and oral contraceptive drugs (Siyad, 2011; Widmaier, 2006; Guyton and Hall, 2006; Chobanian *et al.*, 2003).

Hypertension is caused by a variety of factors of which the two main ones are sedentary lifestyle and obesity. Other risk factors contributing to the development of hypertension includes chronic levels of elevated psychological stress, high alcohol intake, smoking, high salt intake as well as age. People are also at risk to develop hypertension when they suffer from diabetes (Whitney and Rolfes, 2008). The main goal for treating hypertension is to lower blood pressure to a healthy level, which usually starts with changes in the patient's lifestyle. These lifestyle changes may include maintaining a healthy body weight through means of healthier eating and exercise, lowering stress levels and reducing smoking as well as alcohol and salt intake. In severe cases of hypertension, a combination of lifestyle changes as well as medication is needed in order to lower the blood pressure to an acceptable level. Medication that is used for the treatment of hypertension are diuretics, beta-adrenergic blockers, calcium channel blockers, as well as Angiotensin-converting enzyme inhibitors (Widmaier, 2006; Guyton and Hall, 2006; Chobanian *et al.*, 2003).

Hypertension is known as "the silent killer", as it is symptom free and in many cases is first detected when series complications have already developed (Siyad, 2011). Hypertension has become an epidemic and it is believed that almost half of cardiovascular diseases worldwide stem from hypertension (Mearns, 2012). Data generated in 2005, showed that in 2000 nearly 972 million people suffered from hypertension, of these 333 million people lived in economically developed countries and 639 million in economically developing countries. This number was estimated to increase to 1.56 billion people by 2025 (Kearney *et al.*, 2005). In Sub-Saharan Africa, hypertension is ranked second, after AIDS, as the disease that hosts the greatest health challenges for the continent (Opie and Seedat, 2005).

It was predicted in 1972 that developing populations would be more prone to hypertension, diabetes and stroke, due to an increase in both urbanization and socio-economic status of developing populations (Walker, 1972). This was seen in a longitudinal study where the blood pressures, heart rate, urinary electrolytes and sociological as well as anthropometric data were gathered over a period of 24 months after the population had migrated. This data was then compared to a control group that was both age and sex-matched, but rural based. The study showed that the people that had migrated had higher BP as opposed to the rural people whose BP was lower (Poulter *et al.*, 1985). In South Africa, during urbanization of Africans, a diet change takes place. The traditional diet that consisted of low GI carbohydrates is replaced with a diet that is high in fat and refined sugars. This adapted diet supports obesity, a risk factor for hypertension as well as other cardiovascular diseases. Accompanying the nutritional transition is increased levels of psychological stress, brought upon by modern society (Harmer *et al.*, 2010; Malan *et al.*, 2006).

2.9 HYPERTENSION AND OXIDATIVE STRESS

With hypertension being one of the most important risk factors that correlates with the development of various cardiovascular diseases, investigating mechanisms which is believed to contribute to the formation of hypertension have become of great importance. One such a mechanism is the role of oxidative stress contributing to hypertension (Rodrigo *et al.*, 2011).

Illustrated in Figure 2.10 is a proposed mechanism of how oxidative stress contributes to the development of hypertension. During oxidative stress, an increase in O_2^- takes place. Due to the high levels of O_2^- , a reaction between O_2^- and NO^\bullet occurs, resulting in the formation of $ONOO^-$. This reaction leads to the decrease in amount of NO^\bullet , which as previously mentioned, is one of the main vasodilators of blood vessels. In return, the $ONOO^-$ oxidize tetrahydrobiopterin (BH_4), one of the co-factors of eNOS. This lead to the uncoupling eNOS as well as inhibition of the enzyme dimethylargininedimethylaminohydrolase (DDAH), resulting in the production of more O_2^- which lead to a further decrease in NO^\bullet levels. Decreased levels of NO^\bullet can increase the sympathetic activity in the blood vessels leading to vasoconstriction which promotes hypertension. Decreased levels of NO^\bullet will also inactivate the renin-angiotensine system situated in the kidneys. This can result in

the retention of sodium (Na^+) as well as H_2O . Retention of these two compounds leads to increased arterial pressure, another factor that contributes to hypertension. As a result of the decreased levels of NO^* , the walls of the blood vessels thicken, also contributing to the formation of hypertension (Vaziri and Rodríguez-Iturbe, 2006).

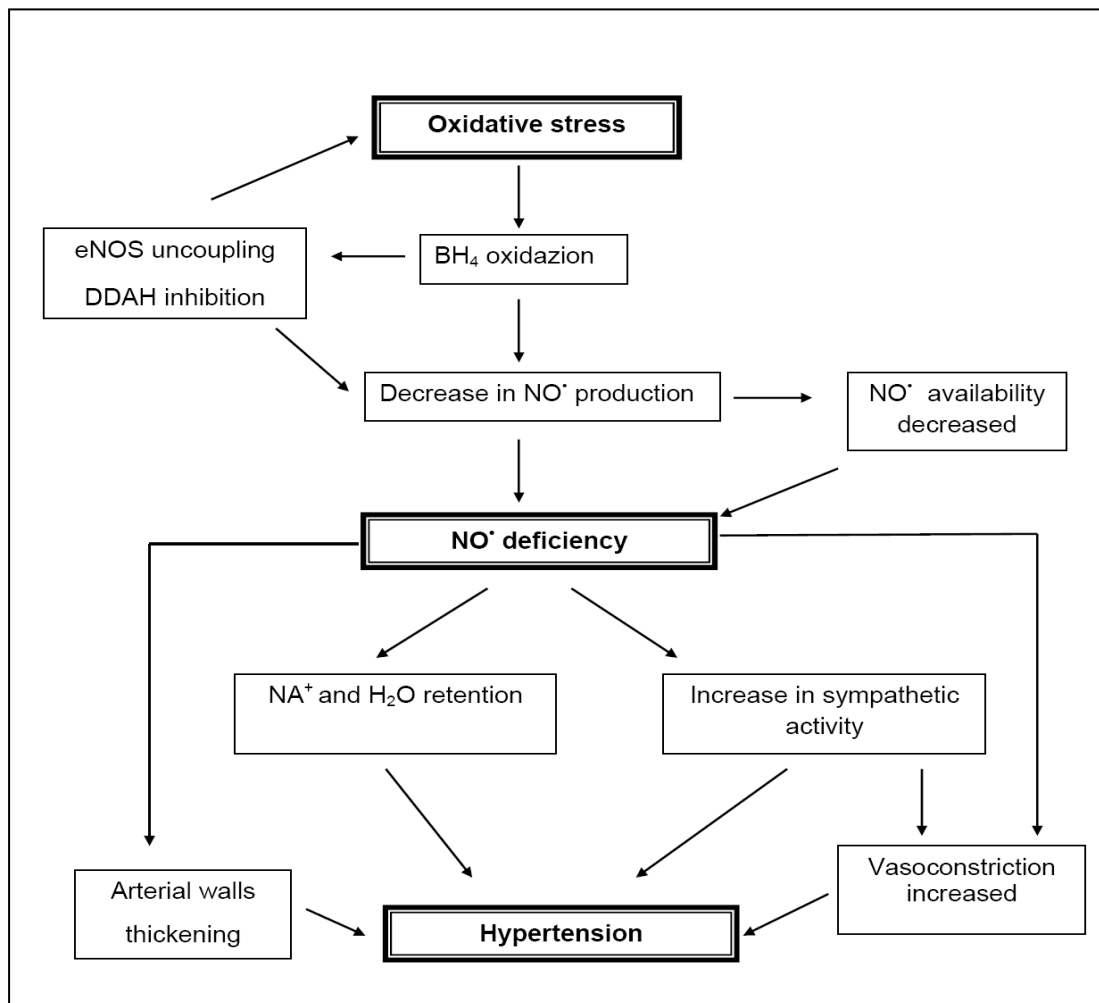


Figure 2.10 Involvement of oxidative stress in hypertension. With high levels of ROS, a reaction occurs between the nitric oxide (NO^*) and superoxide (O_2^-), resulting in the formation of hydrogen peroxide (H_2O_2). H_2O_2 oxidizes the co-factor of endothelial nitric oxide synthase (eNOS), tetrahydrobiopterin (BH_4), resulting in the uncoupling of eNOS as well as inhibition of dimethylargininedimethylaminohydrolase (DDAH). This will lead to an increase of O_2^- as well as a decrease in NO^* production. A decrease in NO^* , will lead to increased vasoconstriction, increased sympathetic activity as well as retention of sodium (Na^+) and water (H_2O) as well as thickening of the arterial walls. All of these contribute to the formation of hypertension (adapted from Vaziri and Rodríguez-Iturbe, 2006).

While some studies show no significant increase of oxidation markers in hypertensive patients, other studies indicates a strong correlation between oxidative stress and hypertension (Rodrigo *et al.*, 2007). Simic *et al.*, (2003), showed findings

of increased oxidation products in all classes of hypertension as well as decreased levels of the antioxidant enzyme catalase in the more severe hypertensive classes. A study done by Kedziora-Kornatowskan *et al.*, (2004) showed that there was an increase in both lipid and protein oxidation markers, decreased levels of nitric oxide as well as decreased levels of the antioxidants GSH and the antioxidant enzyme superoxide dismutase in elderly patients diagnosed with essential hypertension.

Since oxidative stress have previously been linked to hypertension (Kedziora-Kornatowskan *et al.*, 2004; Simic *et al.*, 2003) and urbanized black South Africans have a higher prevalence for hypertension (Hamer *et al.*, 2011), the aim of this study was to investigate specific lipid and protein oxidation markers in hypertensive Africans versus their normotensive counterparts. The following experimental approach was used for this investigation

2.10 STUDY PLAN AND EXPERIMENTAL APPROACH

To investigate the involvement of lipid and protein oxidation markers in hypertension, the study plan summarized in Figure 2.10 was followed. The first step entailed the optimization of the spectrophotometric as well as the LC/MS/MS method, using control urine samples. After optimization, the optimized spectrophotometric method (TBARS assay) as well as LC-MS/MS method (3-nitrotyrosine assay) was performed on the 172 black South African SABPA samples for quantification of the lipid peroxidation marker MDA and the protein oxidation marker 3NT. In the next step statistical analysis and data interpretation of the data was done. Finally a comparison was made between the data and a conclusion was drawn which either supported or discarded the hypothesis, described in Chapter 1.

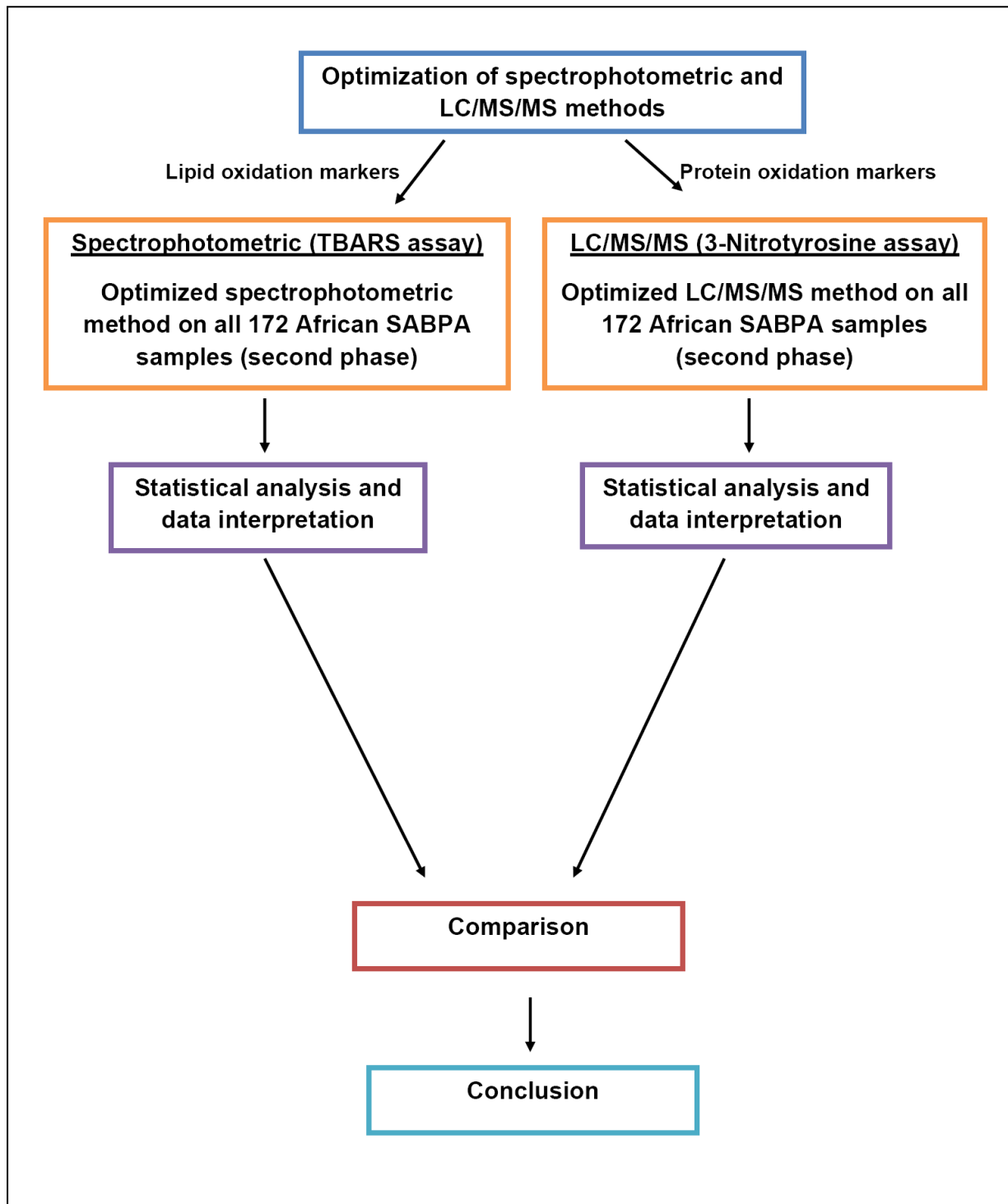


Figure 2.11 Experimental approach. In this figure an outline of the study plan is given. The first step is to do the optimization of the methods. There after the optimized methods is performed on the urine samples of the African teachers from the second phase of the SABPA study. This is followed by statistical analysis and data interpretation. Finally, a comparison is made between the two data sets and a conclusion is drawn.

Chapter 3

3

Optimization of TBARS assay

3.1 INTRODUCTION

During the oxidation of lipids, ROS and RNS can react with polyunsaturated fatty acids (PUFAs). As a result, a variety of reactive products is formed. One of these products, MDA, is the most common marker of lipid oxidation (Wood *et al.*, 2008; Romero *et al.*, 1998; Esterbauer *et al.*, 1990) and it was therefore decided to use MDA as an indication of lipid peroxidation. The TBARS assay (thiobarbituric acid reactive substances), is an assay, which is used to indicate whether lipid oxidation products are present in a sample. This assay was first used in 1958 to determine the degree of rancidity in food and has since then been developed to be used on biological samples such as urine and blood (Sinnhuber *et al.*, 1958). One of the main oxidation products that the TBARS assay measures is MDA. During the assay the TBA (thiobarbituric acid) reagent reacts with the MDA, resulting in the formation of a MDA-TBA TBARS adduct (Figure 3.1). The MDA-TBA adduct can be detected by fluorescence methods but the most common technique is through spectrophotometric methods, measuring at 532 nm. The TBARS assay does not exclusively react with MDA, but also with 4-hydroxynonenal (4HNE), another lipid peroxidation marker. However, the contribution of 4-HNE to the measured TBARS value is minimal in relation to that of MDA, that in this dissertation the TBARS assay will be an indication of MDA concentrations (Linden *et al.*, 2008). Thus in this dissertation, whenever there is referred to the TBARS assay and MDA, one has to keep in mind that the TBARS assay does not only quantify MDA concentration, but rather that of all thiobarbituric reactive substances, including 4-HNE. Although the TBARS assay is widely used and is regarded as the golden standard for analyzing the presence of lipid oxidation markers, it has its limitations and problems. It therefore should be used in combination with other screening methods to ensure accurate and clear results for diagnostic purposes (Wood *et al.*, 2008).

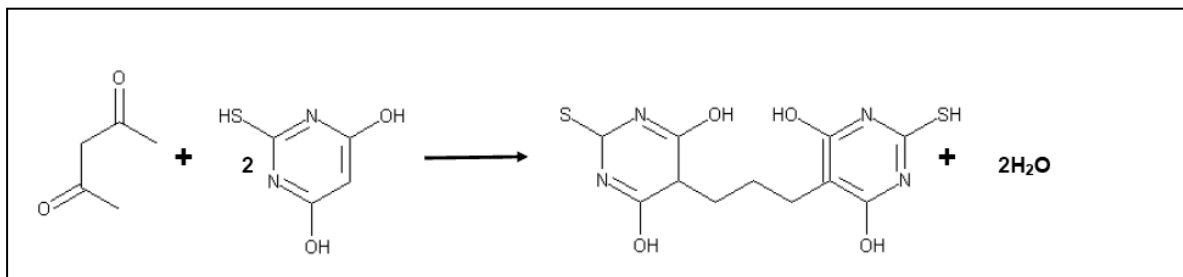


Figure 3.1 TBA reacting with MDA. The figure illustrates the formation of the TBA adduct. During the reaction one MDA molecule reacts with two TBA molecules, resulting in the formation of the MDA-TBA TBARS adduct. The formation of this adduct can be followed spectrophotometrically at 532 nm.

The basic structure of the TBARS assay is a TBARS method that was initially published by Buege and Aust, (1978). However, this method had to be optimized to ensure optimal performance in our laboratory. For the optimization of the TBARS method, the following conditions were evaluated: the effect of time, the effect of temperature, the effect of sample volume and the effect of reagent volume.

3.2 REAGENTS, STANDARDS AND SOLUTIONS

3.2.1 Reagents

Trichloroacetic acid (TCA) was purchased from Saarchem (Pty) Ltd (Merck, JHB, South Africa), 37 % hydrochloric acid (HCl) (Cat 43557-0) as well as tetramethoxypropane (108383) was purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A) and TBA was purchased from BDH AnalAR[®] (Merck; Darmstadt, Germany, product nr 30408).

3.2.2 Preparation of reagent mix

A fresh reagent mix was prepared each day. The reagent mix consisted of 15% TCA (m/v), 0.375% TBA (m/v) and 0.25 N HCl. For the preparation of 50 ml of reagent mix, 7.5 g of TCA and 0.1875 g of TBA was dissolved in 30 mL of Milli-Q water before 1.25 mL of 37% HCl solution was added. This was topped up to 50 mL with Milli-Q water.

3.2.3 Preparation of standards

For the preparation of the standard series, a 10 mM MDA stock solution was prepared by adding 16.64 μ L tetramethoxypropane to 10 mL Milli-Q water. This 10

mM stock solution was then used to prepare the following dilution range in water: 0, 0.98, 1.95, 3.91, 7.81, 15.63, 31.15, 62.5, 125 and 250 μ M MDA solution.

3.3 OPTIMIZATION OF THE TBARS ASSAY

3.3.1 The effect of incubation time on the TBARS assay

Most TBARS assays described in the literature use an incubation time between 30 minutes and 60 minutes before the reaction is analyzed spectrophotometrically. To determine the optimal incubation time of the TBARS assay, six identical MDA standard solutions (62.5 μ M in water) was used. The only difference between the six reaction mixtures was the different incubation times used for each reaction, ranging from 10 minutes to 60 minutes. The reagent volume, sample volume and temperature were kept constant as illustrated in Table 3.2.

Table 3.1 Conditions for evaluating the effect of different incubation times on the TBARS assay

Reaction	1	2	3	4	5	6
Sample volume	350 μ L	350 μ L	350 μ L	350 μ L	350 μ L	350 μ L
Reagent volume	700 μ L	700 μ L	700 μ L	700 μ L	700 μ L	700 μ L
Incubation time at 90°C	10 minutes	20 minutes	30 minutes	40 minutes	50 minutes	60 minutes

After the elapsed incubation time, the reactions were centrifuged at 15700 xg at room temperature for 4 minutes. An aliquot of the supernatant was removed (150 μ L) and placed in a 96 well U-bottom microplate, made from polystyrene. The absorbance was then measured spectrophotometrically at 532 nm with a Synergy multi-mode microplate Reader from BioTek. As illustrated in Figure 3.2, the lowest absorbance (at 532 nm) was measured in sample one (10 minute incubation). The absorbance at 532 nm increased with increased incubation time, but reached a maximum at 50 minutes and 60 minutes. The reactions incubated for 50 minutes and 60 minutes respectively, followed identical absorbance over the wavelengths measured as can be seen clearly from the graph. Since no clear difference in absorbance at 532 nm was observed between the 50 minutes and 60 minutes incubation time reactions, it was decided to use 60 minutes as the ideal incubation time for all future TBARS reactions.

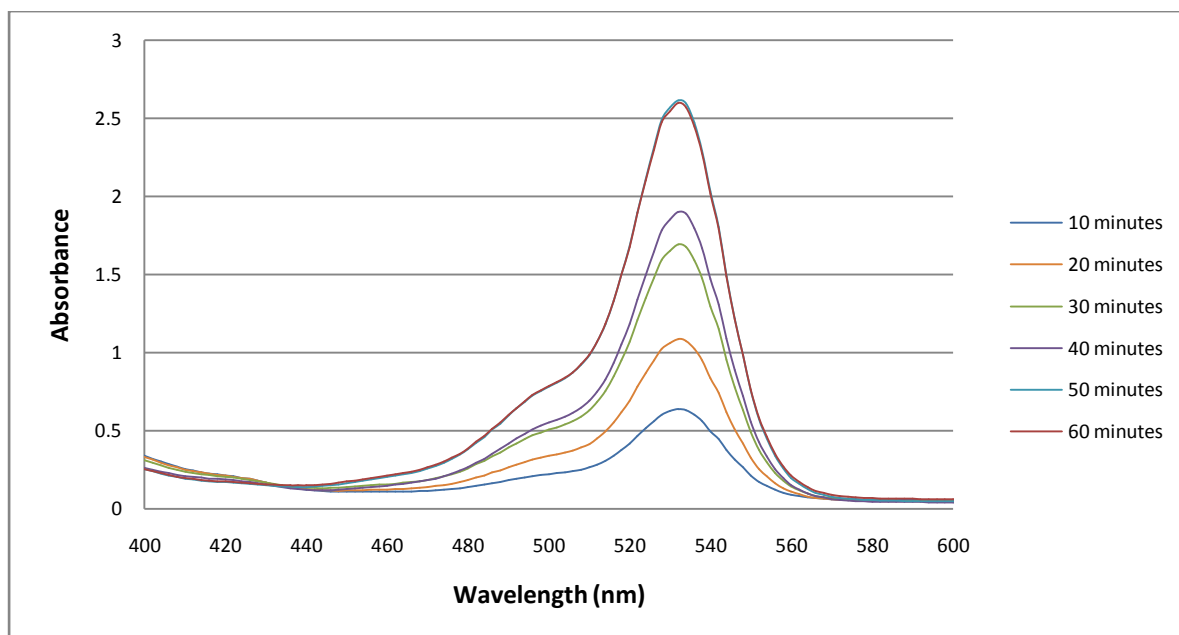


Figure 3.2 Effect of different incubation times on the TBARS assay. An MDA standard solution (62.5 μM in water) was used to prepare six identical reactions. The reactions were incubated for 10, 20, 30, 40, 50 and 60 minutes, respectively before the absorbance was measured spectrophotometrically.

3.3.2 The effect of incubation temperature on the TBARS assay

To determine the optimal incubation temperature for the TBARS assay, again six identical MDA standard solutions (62.5 μM in water) was used. The only difference between the six reaction mixtures was the different temperatures they were incubated at. Each MDA standard solution was incubated for 60 minutes, as determined by Section 3.3.1. The reagent volume and sample volume were kept constant as illustrated in Table 3.3.

Table 3.2 Conditions for evaluating the effect of different incubation temperatures on the TBARS assay

Reaction	1	2	3	4	5
Sample volume	350 μL	350 μL	350 μL	350 μL	350 μL
Reagent volume	700 μL	700 μL	700 μL	700 μL	700 μL
Incubation temperature	60°C	70°C	80°C	90°C	100°C

After incubation, the same procedure was followed as in Section 3.3.1. As illustrated in Figure 3.3, the lowest absorbance (at 532 nm) was measured in sample two (70°C incubation temperature). The absorbance at 532 nm varied between the various

temperatures but reached a maximum at 90°C. It was decided to use 90°C as the ideal incubation time for all future TBARS reactions.

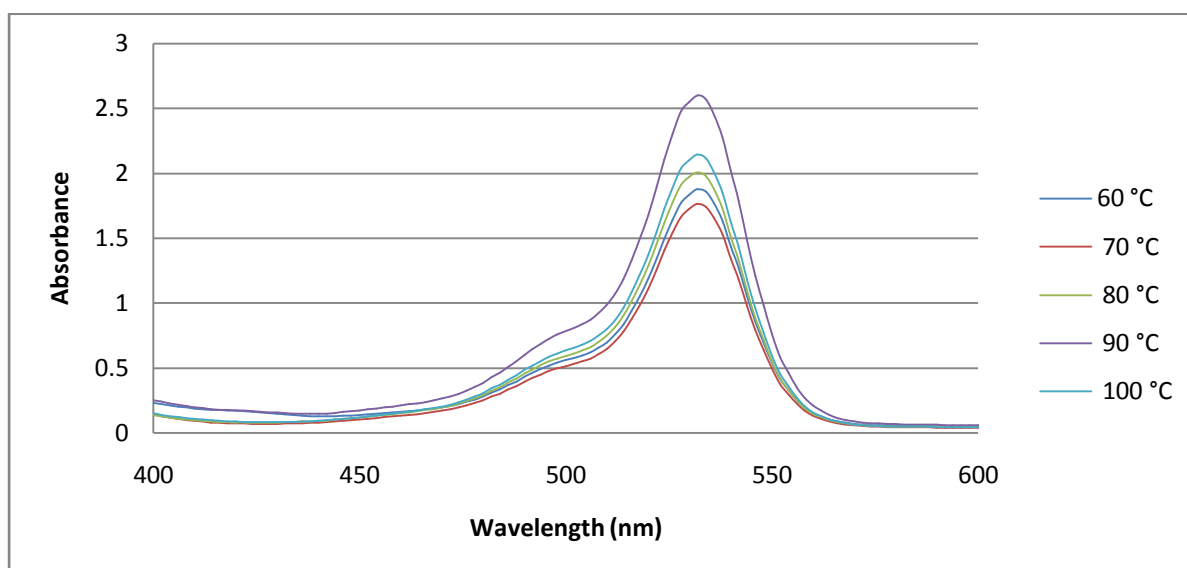


Figure 3.3 Effect of different incubation temperatures on the TBARS assay. An MDA standard solution (62.5 μM in water) was used to prepare six identical reactions. The reactions were incubated for 60 minutes, at 60, 70, 80, 90 and 100°C respectively before the absorbance was measured spectrophotometrically.

3.3.3 The effect of different volumes of urine on the TBARS assay

To determine the optimal amount of sample needed for the TBARS assay, different volumes of the same urine sample was assayed. Each reaction was incubated at 90°C for 60 minutes, as determined in Section 3.3.1 and 3.3.2. The different volumes were topped up with Milli-Q water so that the end volume was the same. The reagent volume was kept constant as illustrated in Table 3.4.

Table 3.3 Conditions for evaluating the effect of different urine volume on the TBARS assay

Reaction	1	2	3	4	5
Sample volume	350 μL	450 μL	550 μL	650 μL	750 μL
Reagent volume	700 μL	700 μL	700 μL	700 μL	700 μL

After incubation, the same procedure was followed as in sections 3.3.1 and 3.3.2. As illustrated in Figure 3.4, the lowest absorbance (at 532 nm) was measured in sample one (350 μL urine sample). The absorbance at 532 nm slightly increased with increased amount of sample volume, but reached a maximum at 650 μL and 750 μL .

The absorbance at 532 nm for the reactions that contained 650 μL and 750 μL of the sample, was almost identical for the two reactions, as can be seen clearly from the graph. Since no clear difference in absorbance at 532 nm was observed between using 650 μL and 750 μL of sample, it was decided to use 750 μL of sample as the ideal amount of sample for all future TBARS reactions.

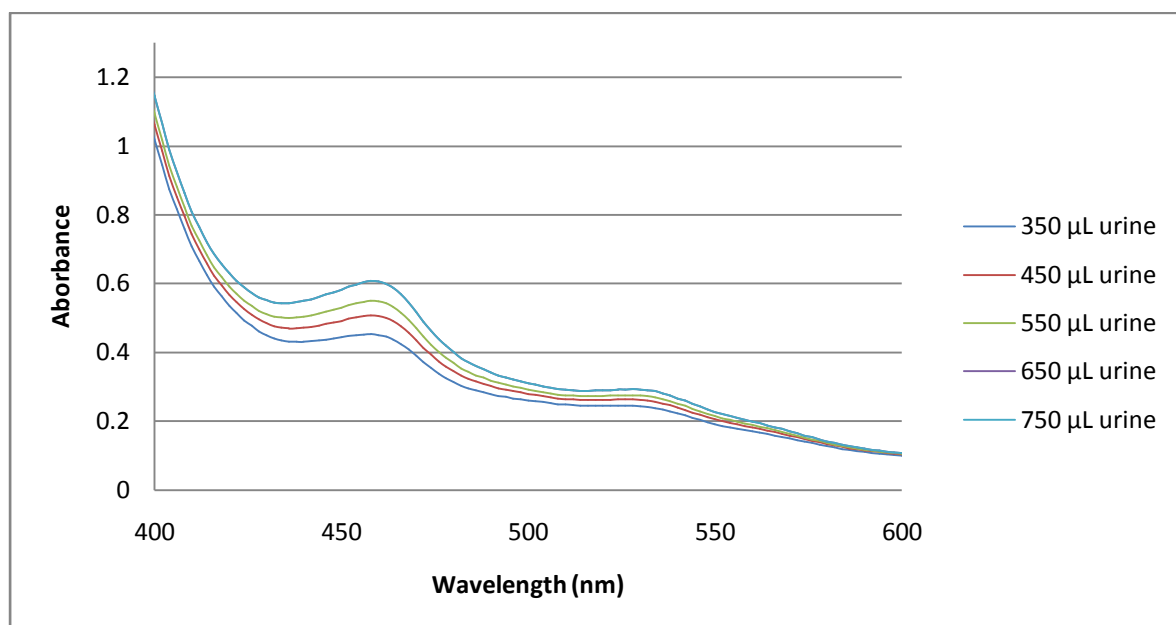


Figure 3.4 Effect of different urine volume on the TBARS assay. A urine sample was used to prepare six reactions with different sample volumes. The reactions were incubated for 60 minutes at 90°C before the absorbance was measured spectrophotometrically.

3.3.4 The effect of different volumes of reagent on the TBARS assay

To determine the optimal amount of reagent needed during the TBARS assay, five identical urine samples were assayed. The only difference between the five reactions was the different volumes of reagent used for each reaction, ranging from 800 μL to 1200 μL . Each reaction was incubated at 90°C for 60 minutes, as determined by section 3.3.1 and 3.3.2. The different volumes were topped up with Milli-Q water so that the end volume was the same. The sample volume was kept constant as illustrated in Table 3.5.

Table 3.4 Conditions for evaluating the effect of different volumes of reagent on the TBARS assay

Reaction	1	2	3	4	5
Sample volume	800 μL	800 μL	800 μL	800 μL	800 μL
Reagent volume	800 μL	900 μL	1000 μL	1100 μL	1200 μL

The same procedure that was used in section 3.3.1 and 3.3.2 was followed after the incubation was completed. As illustrated in Figure 3.5, the lowest absorbance (at 532 nm) was measured in sample one (800 μL reagent volume). The absorbance at 532 nm varied little, to almost nothing with the increase in reagent volume. Although the 800 μL volume theoretically had the lowest absorbance value, seen that there was almost no difference between the absorbance values, it was decided to use 800 μL as the ideal volume of reagent for all future TBARS reactions.

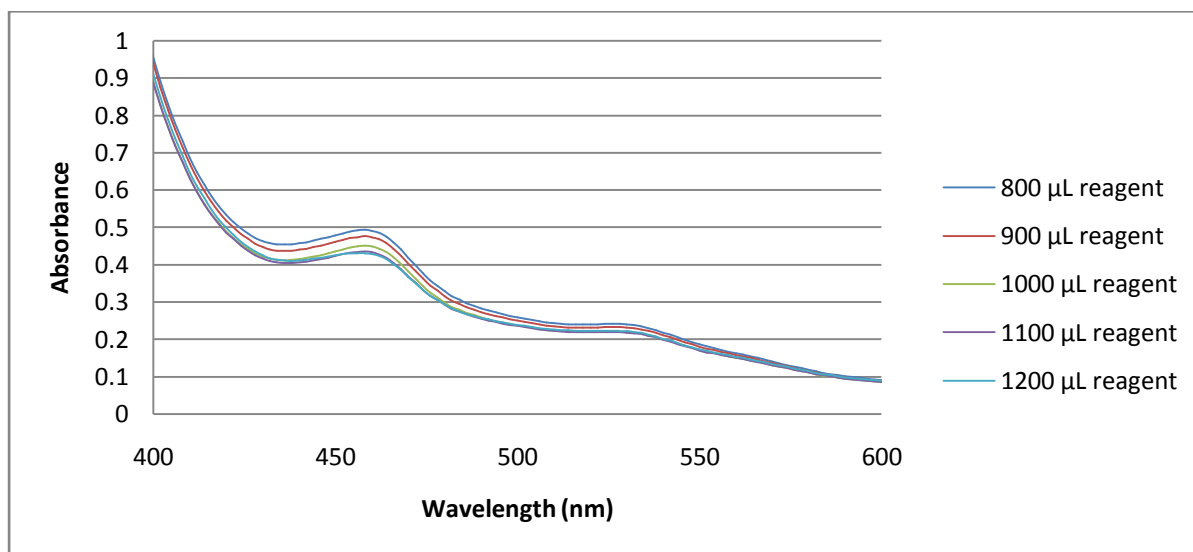


Figure 3.5 Effect of different volumes of reagent on the TBARS assay. A urine sample was used to prepare six reactions with different volumes reagent added. The reactions were incubated for 60 minutes at 90°C before the absorbance was measured spectrophotometrically.

3.4 THE OPTIMIZED TBARS ASSAY

The following dilution range of the MDA standards is used to construct a calibration range to quantify TBARS adducts in urine samples: 0, 0.98, 1.95, 3.91, 7.81, 15.63 and 31.15 μM MDA in water. Either a urine sample or MDA standard (800 μL) is added to 2 mL microcentrifuge tube (polypropylene (PP) with captive me, Z 628034 from Sigma Aldrich). To this, 800 μL reagent mix (15% TCA, 0.375% TBA and 0.25 N HCl) is added, followed by an incubation step for 60 minutes at 90°C. After incubation, the tubes are centrifuged at 15 700 $\times g$ for 4 minutes at room temperature. Supernatant (150 μL) is transferred to three consecutive wells (triplicates) in a 96 well plate (96 well U-bottom, microplates made from polystyrene). The absorbance is then measured spectrophotometrically at 532 nm with a Synergy multi-mode microplate Reader from BioTek. In literature, 600 nm is used as

background (Wu *et al.*, 2009; Vladimirof *et al.*, 1994). Therefore, the samples are also measured at 600 nm. The 532 nm-600 nm values will then be later used for calculations. The MDA standards on the plate is automatically used by the software to construct a calibration curve to calculate the concentration of the TBARS adducts in the urine samples, reported in μM (during this study, the R^2 -value of the linear calibration curve had to be above 0.99 or the entire plate of samples was repeated). The concentration of the MDA in the urine samples is reported in μM by the software. This needs to be converted to mg MDA/g creatinine. Thus, the creatinine values of the urine samples used in this study were determined by using the QuantiChrom™ Creatinine Assay Kit from BioAssay Systems. The method used was exactly as described by the manufacturers.

3.5 VALIDATION OF THE OPTIMIZED TBARS ASSAY

3.5.1 Introduction

Validation of the TBARS assay entailed measuring the intra- as well as the inter-batch coefficient of variance (intra-batch CV and inter-batch CV). The CV of a sample group is used as a statistical measurement to give an indication of the dispersion of your data points (George *et al.*, 2002; Kelley, 2007). The CV is calculated by the formula given in Equation 3.1.

Equitation 3.1 Equation for calculating coefficient variance

$$\%CV = \frac{[\text{Standard deviation (STDEV)}]}{\text{Mean of the data series}} \times 100$$

3.5.2 Intra-batch coefficient of variation

Calculating the intra-batch CV involved the analysis of five aliquots of a single urine sample on one day. The TBARS assay was performed as described in Section 3.4. During the analysis, all five samples were analyzed (in triplicate) in a single batch. Firstly, the mean of each of the five samples were calculated as they all were analyzed in triplicates (Table 3.6). After the mean was calculated, the standard deviation (STDEV) values were determined by using the five mean values (Table

3.7). Using the final mean and STDEV of the five samples, the intra-batch CV was calculated to be 10.52 % by using Equation 3.1.

Table 3.5 Calculation of individual absorbance means of five samples assayed on the same day

	Sample A	Sample A	Sample A	Sample A	Sample A
	0.186	0.215	0.168	0.172	0.175
	0.212	0.251	0.199	0.194	0.201
	0.216	0.253	0.203	0.201	0.201
Mean	0.205	0.240	0.190	0.189	0.192

Absorbance values is given in absorbance units (AU)

Table 3.6 Calculation of single mean and STDEV using five individual mean values of the same sample

Sample	Mean
Sample A	0.205
Sample A	0.240
Sample A	0.190
Sample A	0.189
Sample A	0.192
Mean	0.203
STDEV	0.021
CV	10.52

3.5.3 Inter-batch coefficient of variation

For the calculation of the inter-batch CV, a urine sample was analyzed each day over a period of five days, giving a total of five of the same urine samples. Just as with the intra-batch CV, the samples were analyzed according to the method described in Section 3.4. Each day one sample was incubated, centrifuged and analyzed. After all five samples had been analyzed, the 532 nm-600 nm values were exported to Microsoft Excel[®] and compiled into a spreadsheet. Using the same process as during the calculation of the intra-batch CV, each sample was analyzed in triplicate, from which the mean was calculated (Table 3.8). The mean value as well as the STDEV of the five mean values was then also calculated (Table 3.9). The inter-batch CV was calculated to be 11.64% by using Equation 3.1.

Table 3.7 Calculation of individual absorbance means of five samples assayed over a period of five days

Day	1	2	3	4	5
	0.186	0.152	0.149	0.187	0.173
	0.212	0.171	0.168	0.216	0.183
	0.216	0.172	0.168	0.219	0.186
Mean	0.205	0.165	0.162	0.207	0.181

Absorbance values is given in absorbance units (AU)

Table 3.8 Calculation of single mean and STDEV using five individual mean values of the same sample over a period of five days

Sample	Mean
Sample A Day 1	0.205
Sample A Day 2	0.165
Sample A Day 3	0.162
Sample A Day 4	0.207
Sample A Day 5	0.181
Mean	0.184
STDEV	0.021
CV	11.64%

3.6 CONCLUSION ON THE OPTIMIZED TBARS ASSAY

By using this quantification method, it was possible to quantify TBARS, in control urine samples, using MDA standards. This assay had an intra-batch CV of 10.52 % and an inter-batch CV of 11.64%. These CV's indicated that the optimized 96-well spectrophotometric method for the TBARS was successful and reliable for quantification of urinary TBARS. Therefore, the optimized TBARS assay was used for the quantification of TBARS in all of the 172 SABPA urine samples.

Chapter 4

4

Optimization of 3-nitrotyrosine assay

4.1 INTRODUCTION

Oxidation of amino acid residues in proteins leads to the formation of a variety of protein oxidation markers (Klebanof, 2005; Mohiuddin *et al.*, 2005; Radi, 2004). One such a marker is protein carbonyl, which can be quantified by using a chemical probe such as, 2,4-dinitrophenylhydrazine. However, due to the low repeatability of the protein carbonyl assay (Yan and Forster, 2011), it was decided not to use this assay as a measurement of protein oxidation in this study. Another protein oxidation marker is chlorotyrosine, which is formed when hypochlorous acid (produced by myeloperoxidase) reacts with amino acid residues in proteins. The quantification of chlorotyrosine as an indication of protein oxidation was also discarded due to the difficulty in measuring chlorotyrosine in human urine (Kato *et al.*, 2009). A third marker of protein oxidation, 3-nitrotyrosine (3NT), which is formed when peroxynitrite (ONOO^-) reacts with amino acid residues in proteins, have been linked to the formation of atherosclerosis (Upmacis, 2008). Increased levels of 3NT have also been associated with inflammatory conditions such as lupus, arthritis, rheumatoid, pancreatitis, Crohn's disease and influenza (Mohiuddin *et al.*, 2005). 3NT can be quantified by various analytical methods, including GC-MS, GC-MS/MS, stacking capillary electrophoresis, ELISA, LC-MS and LC-MS/MS (Tsikas, 2012; Ogino and Wang, 2007). With a variety of options available to quantify 3NT, it was decided to use an LC-MS/MS method for 3NT quantification as this technology was available in our laboratory and according to literature, LC-MS/MS is one of the more sensitive and accurate methods for quantification of 3NT (Tsikas *et al.*, 2012; Chen and Chiu, 2008).

4.2 REAGENTS, STANDARDS AND SOLUTIONS

4.2.1 Reagents

3-Nitro-L-tyrosine (85,191-4), D-tyrosine (855456), DL-*o*-tyrosine (93851), DL-*m*-tyrosine (T3629), L-tyrosine (T3754), formic acid (06440), R-(-)-2-butanol (236691),

HCl (43,557-0), methanol (494437), sodium phosphate monobasic (NaH_2PO_4) (S8282), sodium phosphate dibasic (Na_2HPO_4) (S7907), isatine (58240), lutidine (L3600), acetyl chloride (00990) and glacial acid (27221) was purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A). Sodium nitrite (NaNO_2) was purchased from Rochelle Chemicals. Sodium hydroxide (NaOH) (5823200), butan-1-ol (101988) and acetone (1022040) was purchased from Saarchem (Pty) Ltd (Merck, JHB, South Africa). Ninhydrin (6762) was purchased from Merck (Pty) Ltd South Africa. L-Phenyl-d5-alanine was purchased from C/D/N Isotopes, INC. Pointe-Claire, Quebec, Canada. Acetone was purchased from BDH AnalaR[®] (Merck; Darmstadt, Germany, product nr 100034Q). EZ:faast amino acid GC/MS analyses kit was purchased from Phenomenex (Promolab Pty Ltd T/A Separations, Randburg, Gauteng, South Africa).

4.2.2 Derivatization reagents

To prepare either of the derivatization reagents n-butanol:acetyl chloride or R-(-2)butanol:acetyl chloride, acetyl chloride was added to n or R-(-2)-butanol. The volume acetyl chloride added, equalled 25 % of the volume of the butanol used. All this was done while the butanol was kept on ice.

4.2.3 Internal standard (phenylalanine isotope)

For the preparation of the internal standard, a stock solution was prepared. This was done by adding 40.63 mg of the phenylalanine isotope (L-phenyl-d5-alanine) to 200 mL methanol. This stock solution had a concentration of 1.19 mM and was stored at -20°C . A dilution was made from the stock solution, by adding 400 μL of the stock solution to 100 mL methanol. The diluted solution had a final concentration of 4.77 μM .

4.2.4 Mobile phase for dissolving derivatized samples

The mobile phase that was used to dissolve the dried samples consisted of 10% acetonitrile, 90% water and 0.1% formic acid. To prepare this solution, 900 mL Milli-Q water was added to 100 mL acetonitrile. To this, 1 mL formic acid was added.

4.2.5 Peroxynitrite

Before the peroxynitrite was prepared, the following stock solutions were prepared: 0.6 N HCl was prepared by adding 0.6 mL of a 12 N (37%) HCl solution to

9.4 mL Milli-Q water. A 30% w/w H₂O₂ stock solution was diluted to a 0.7 M H₂O₂ solution by adding 0.715 mL of the stock solution to 9.285 mL Milli-Q water. A 0.6 M NaNO₂ solution was prepared by dissolving 0.414 g NaNO₂ in 10 mL Milli-Q water. 1.5 M NaOH was prepared by weighing off any number of NaOH pellets, dissolving the pellets in a small volume of Milli-Q water and then diluting the stock solution with Milli-Q water to a final concentration of 1.5 M NaOH solution. For the preparation of peroxyxynitrite, a method described by Hughes and Nicklin, (1970) was used. Firstly, all of the reagents were kept on ice at all times. In a glass beaker, 10 mL of ice cold 0.6 M NaNO₂ was stirred with a magnetic stirrer. Ice cold 0.6 N HCl (10 mL) and 0.7 M H₂O₂ (10 mL) were simultaneously added to the NaNO₂. Immediately after this, the 20 mL 1.5 M NaOH solution was added. The solution was transferred to two 50 mL tubes and stored at -20°C for approximately 24 hours for phase separation to occur, with peroxyxynitrite being mainly in the top phase after the freezing step.

4.2.6 Phosphate buffer (1M, pH7.4)

For preparation of the phosphate buffer, 14.12 g of NaHPO₄ was dissolved in 100 mL Milli-Q water. In a separate beaker, 6 g of NaH₂PO₄ was dissolved in 50 mL Milli-Q water. NaH₂PO₄ solution was then added to 80 mL of Na₂HPO₄ until the pH reached 7.4.

4.2.7 Tyrosine stock solutions

For the synthesis of the stereoisomers of 3NT, 1 mM stock solutions of L-tyrosine, D-tyrosine, DL-*o*-tyrosine and DL-*m*-tyrosine were prepared in Milli-Q water.

4.2.8 Mobile phase and ninhydrin colouring solution for thin layer chromatography

The mobile phase was prepared by adding 300 mL n-butanol to 300 mL acetone, 180 mL Milli Q water and 100 mL glacial acetic acid. The ninhydrin colouring solution was prepared by adding 1250 mg ninhydrin, 50 mg isatine and 5 mL lutidine to 500 mL acetone. This solution was prepared 24 hours prior to the plate development and it was stored at 4°C in an amber bottle.

4.3 QUANTIFICATION OF 3-NITRO-L-TYROSINE STANDARD WITH LC-MS/MS

4.3.1 Butylation of 3-nitro-L-tyrosine standard

The first step in optimizing the 3-nitrotyrosine assay was to determine the multiple reaction monitoring (MRM) conditions for 3NT. Before any optimization could be done, the 3-nitro-L-tyrosine standard first had to be derivatized. Derivatization of 3NT, improves sensitivity, selectivity, stability, gets rid of interfering compounds and it improves the retention time of 3NT as it reduces the polarity by binding to the carboxylic acid group. Derivatization was done by derivatizing the 3-nitro-L-tyrosine standard with n-butanol:acetyl chloride, and by doing so, converting them to their corresponding butyl ester forms. For derivatization of the 3-nitro-L-tyrosine standard, 100 μ L of a 1 mg/L 3-nitro-L-tyrosine standard stock solution was added to a 1.5 mL tube (1.5 mL micro tubes from PLASTIBRAND[®], Sigma-Aldrich, St. Louis, Mo., U.S.A). The tube was dried under a stream of nitrogen gas at 65°C for approximately 40 minutes. After the samples were dried, it was derivatized by adding 200 μ L n-butanol:acetyl chloride. The sample was incubated for 15 minutes at 65°C, after which it was dried again, using the same conditions as the previous drying step. The completely dried standard was then dissolved in 100 μ L mobile phase (10% acetonitrile, 90% water and 0.1% formic acid), and transferred to a vial with a tapered insert (2 mL clear vial with a screw cap and a 250 μ L pulled point glass insert, both from Agilent Technologies, Santa Clara, CA, USA).

4.3.2 Optimization of MS conditions for the quantification of 3-nitro-L-tyrosine standard

Most LC-MS/MS methods described in the literature uses a precursor and product ion combination of 283.13 m/z and 181 m/z , respectively, for the quantification of 3NT (Marvin *et al.*, 2003). To determine the optimal MRM condition on the LC-MS/MS (1290 infinity LC from the 1200 infinity series connected to a 6460 triple quadrupole mass analyzer, both from Agilent Technologies, Santa Clara, CA, USA), the derivatized 3-nitro-L-tyrosine standard was infused into the MS, using electrospray ionization (ESI) in positive mode. This was done without any chromatographic separation. MassHunter optimizer software from Agilent (Santa Clara, CA, USA) was used to determine the optimal MS conditions for quantifying 3NT (Table 4.1). The software varied the fragmentor and collision energy, which resulted in the formation

of various product ions, with various abundance levels. From Table 4.1 it is clear that the optimal precursor and product ion combination for the quantification of 3NT was 283.13 *m/z* and 181 *m/z*, respectively, as it was the combination with the highest product ion abundance.

Table 4.1 The optimal MRM conditions for 3-nitro-L-tyrosine quantification

Compound	Formula	Nominal mass	Polarity	Ion source
3-Nitro-L-tyrosine	C ₁₃ H ₁₈ N ₂ O ₅	282.13	Positive	ESI
Precursor ion	Product ion	Fragmentor	Collision energy	Abundance
283.13 <i>m/z</i>	181 <i>m/z</i>	101 V	12 V	3598
283.13 <i>m/z</i>	114.4 <i>m/z</i>	101 V	48 V	41
283.13 <i>m/z</i>	172.1 <i>m/z</i>	101 V	28 V	40
283.13 <i>m/z</i>	90.1 <i>m/z</i>	101 V	40 V	408

4.3.3 Chromatographic separation of 3-nitro-L-tyrosine standard

After optimization of the MRM conditions, the 3-nitro-L-tyrosine standard could be chromatographically analyzed. This was done by injecting 5 μ L derivatized 3-nitro-L-tyrosine standard (1 mg/L) onto a C18 column (Zorbax SB-18, 1.8 μ m, 2.1X50 mm, Agilent Technologies, Santa Clara, CA, USA) at a flow rate of 0.2 mL/min with mobile phases A (Milli-Q water and 0.1% formic acid)) and B (acetonitrile and 0.1% formic acid). A mobile phase gradient was used for chromatographic separation of 3NT (Table 4.2).

Table 4.2 Mobile phase gradient time table for the chromatographic separation of 3-nitro-L-tyrosine

Time (minutes)	% Mobile phase B
0	10
15	30
17	90
18	90
19	100
22	100
25	10

After the gradient, the column was equilibrated with 90% solvent A and 10% solvent B during a 4 minute post run time. The nebulizer was 20 psi, the sheath gas temperature and gas flow was 125°C and 3.0 L/min, respectively, and the column temperature was 25°C. Nitrogen was used as collision gas with collision energy

being 12 eV. As illustrated by Figure 4.1 the retention time for derivatized 3-nitro-L-tyrosine standard was 10.44 minutes.

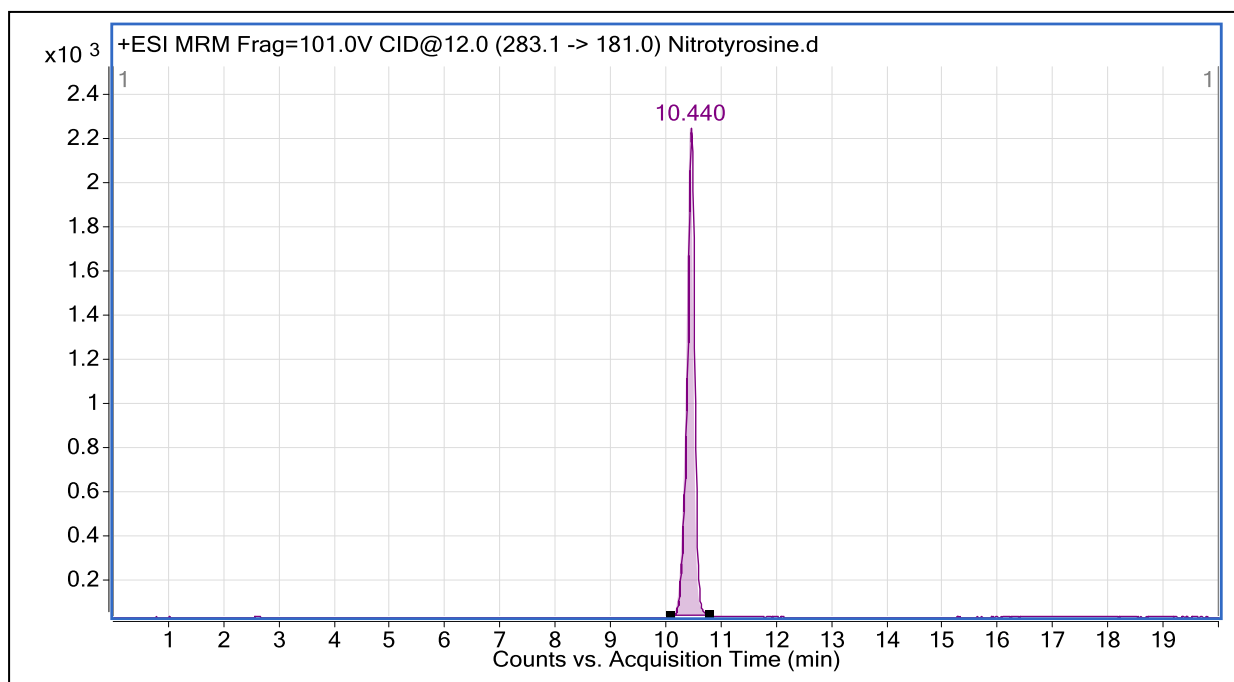


Figure 4.1 Chromatographic separation of butylated 3-nitro-L-tyrosine standard. The chromatogram illustrates the chromatographic separation of the butylated 3-nitro-L-tyrosine with a retention time of 10.44 minutes.

4.3.4 Derivatization of control urine samples

After the MRM and chromatographic separation conditions were optimized for the quantification of 3NT, the method was tested on control urine samples. The control urine samples were received from the Biotransformation and Oxidative Stress Status Laboratory of the Centre for human Metabonomics situated at the North-West University, Potchefstroom Campus. These samples were from patients that were referred to the laboratory by a physician. Although these samples had nothing to do with the SABPA study, it was readily available and therefore used to optimize the 3-nitrotyrosine assay. The samples were stored for approximately three months at -20°C. Five control urine samples (sample A-E) were aliquoted into five 1.5 mL tubes, after which the tubes were centrifuged at 15 700 x g at room temperature for 2 minutes. 100 µL of the supernatant from each tube was then transferred to a corresponding new 1.5 mL tube and 100 µL methanol was added to each tube, to enhance the drying under the nitrogen gas before derivatization. For the derivatization of these aliquots, the same method described in Section 4.3.1 was used. The samples were dried under nitrogen gas, butylated with 200 µL n-

butanol:acetyl chloride and again dried under nitrogen gas, all at 65°C. After the samples were dried again, it was dissolved in 100 µL mobile phase (10% acetonitrile, 90 % water and 0.1% formic acid). After the butylated samples were dissolved, they were centrifuged again at 15 700 x g at room temperature for 2 minutes to remove any crystals. After the centrifugation step, 90 µl of each supernatant was transferred to a vial with a tapered insert.

4.3.5 Quantification of 3NT in urine samples

3NT was quantified in control urine samples A-E as described in Section 4.3.2. As indicated by the blocks in Figures 4.2-4.6, it was possible to identify 3NT in all of the control urine samples. As can be seen from samples A-E, it is clear that the average retention time for 3NT was approximately 10.32 minutes, which is approximately 0.2 minutes earlier than the retention time of the 3-nitro-L-tyrosine standard. This is probably due to the matrix effect of the urine sample, compared to the standard being dissolved in only mobile phase. The retention times did not differ much between the samples, with the maximum drift being average retention time \pm 0.02 minutes. This retention time stability is of great importance for right peak identifications, which is critical for quantification of 3NT.

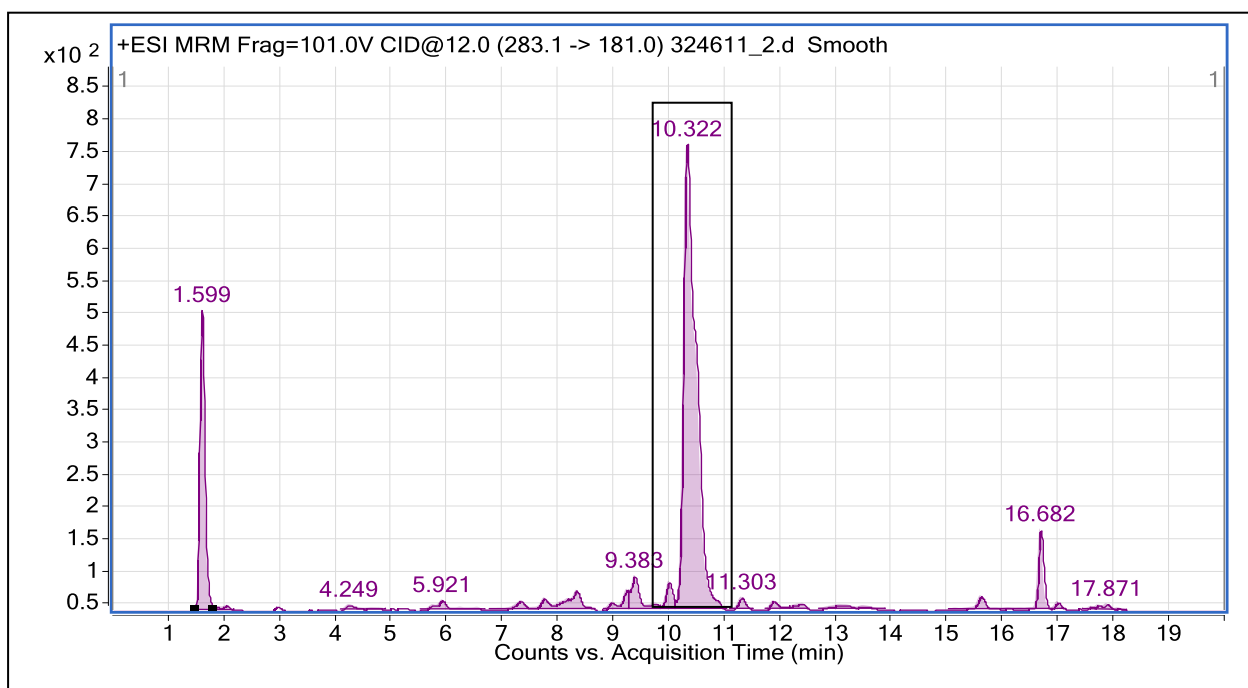


Figure 4.2 Chromatographic separations of control urine sample A. MRM of 3NT with retention time 10.32 minutes.

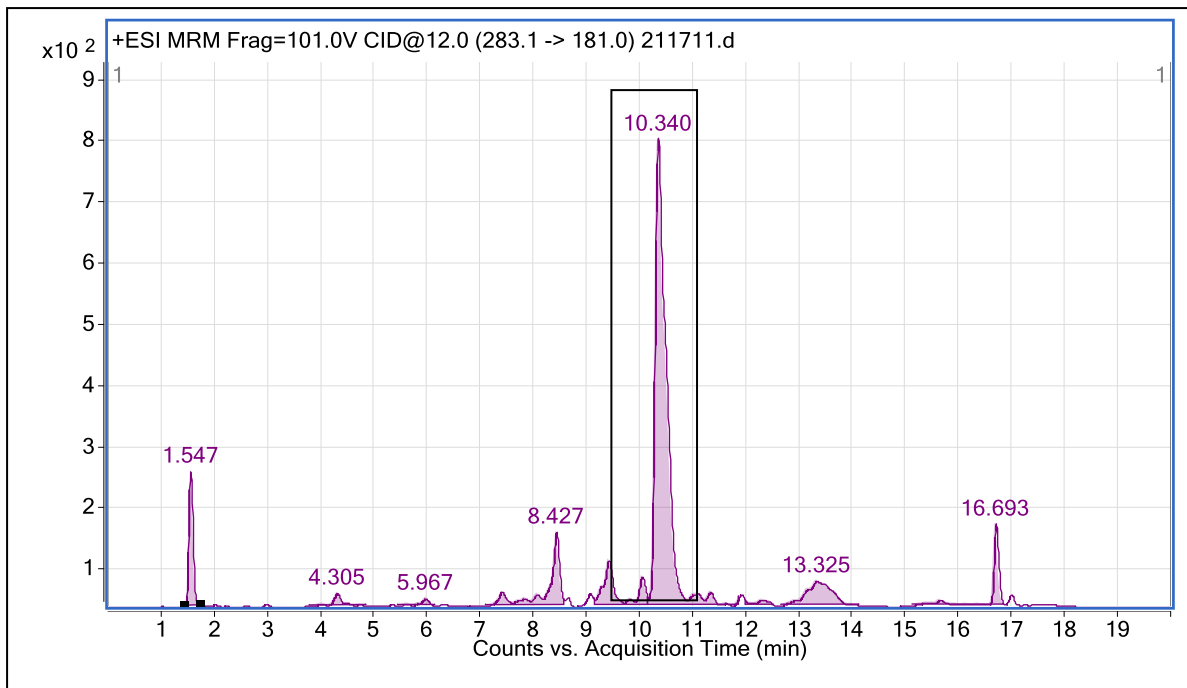


Figure 4.3 Chromatographic separations of control urine sample B. MRM of 3NT with retention time 10.34 minutes.

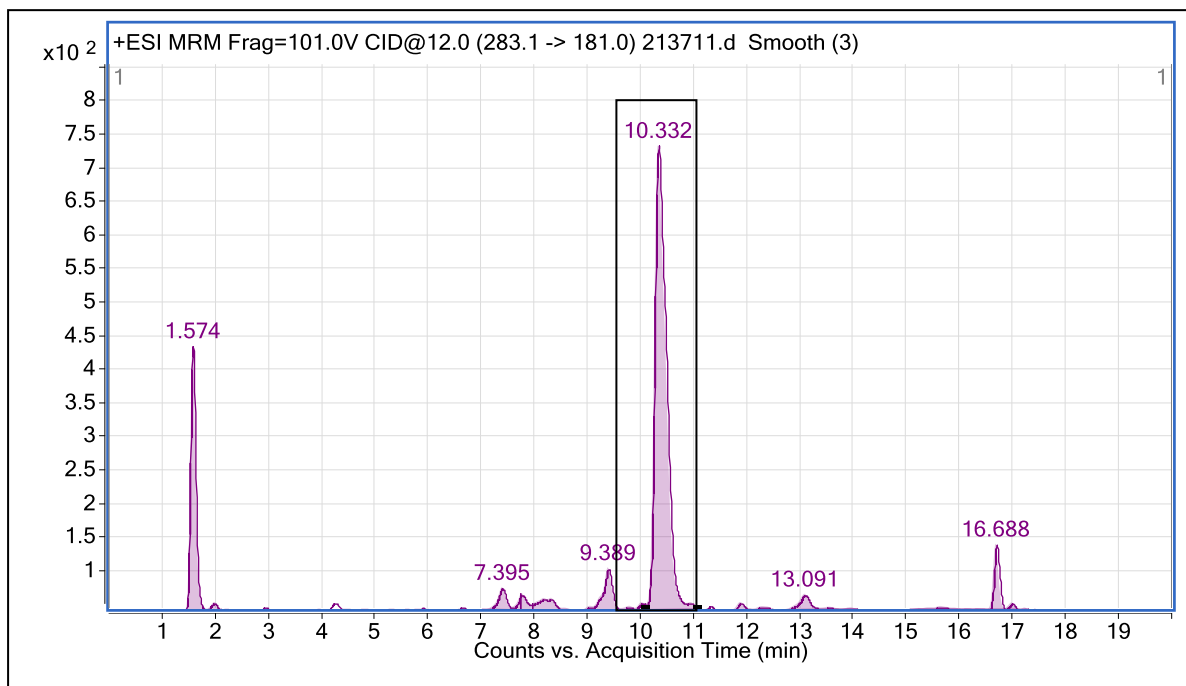


Figure 4.4 Chromatographic separations of control urine sample C. MRM of 3NT with retention time 10.33 minutes.

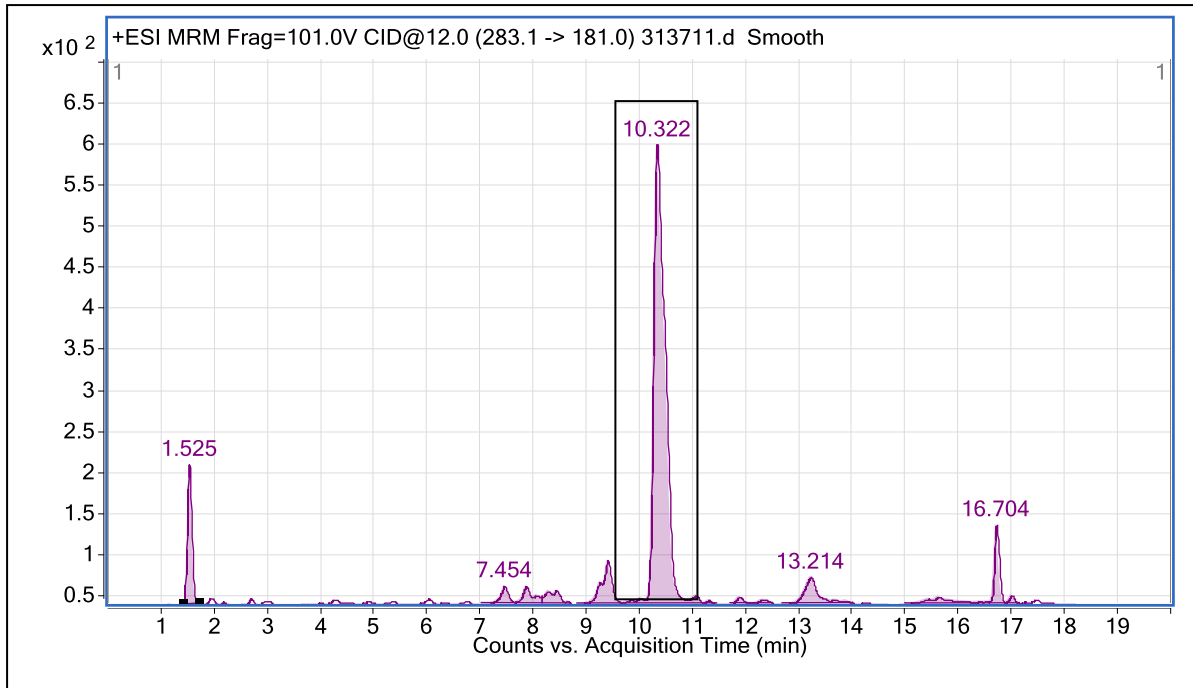


Figure 4.5 Chromatographic separations of control urine sample D. MRM of 3NT with retention time 10.32 minutes.

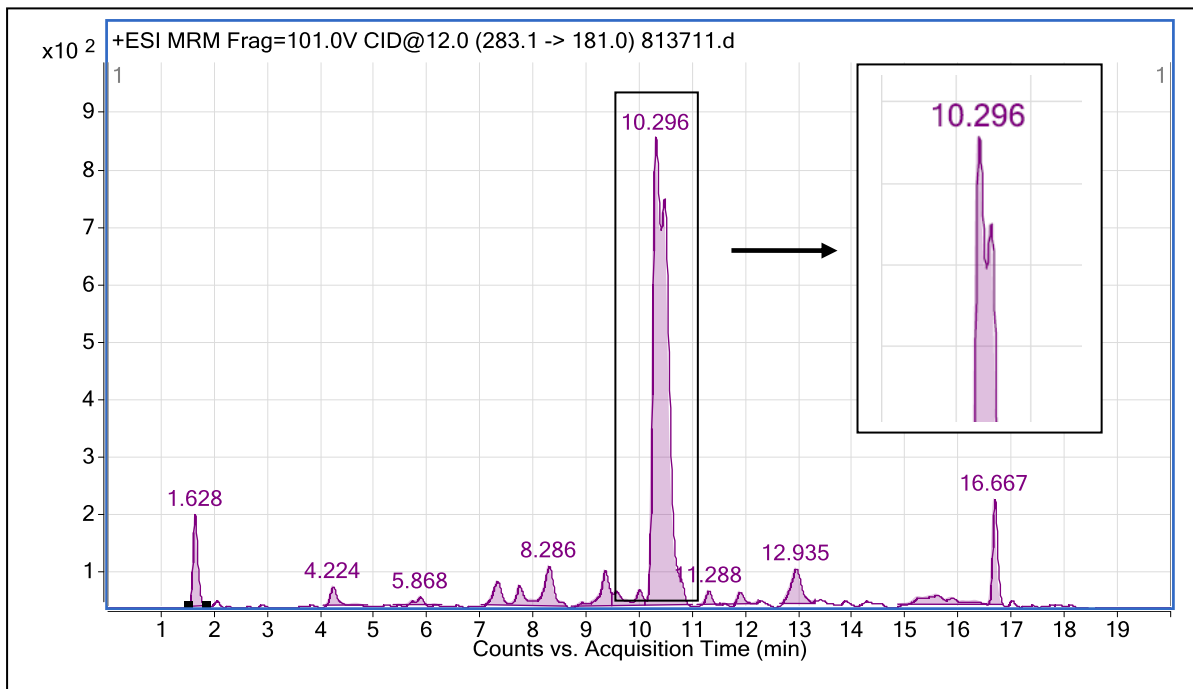


Figure 4.6 Chromatographic separations of control urine sample E. A MRM of 3NT with retention time 10.29 minutes.

After close inspection of the MRM spectra of 3NT in the control urine samples (especially sample E, Figure 4.6), it was noticed that there was an unknown compound that co-eluted with 3NT as a double peak was obtained. Under normal

conditions, this would not be a problem as the MS/MS adds selectivity, but in this case, the compound had the same precursor and product ion combination as 3NT. Apart from the double peak, co-elution was also supported by the fact that when 3-nitro-L-tyrosine standard was analyzed, no double peak was obtained. Co-elution with 3NT meant that ion suppression could be present as the method is in positive ionization mode. This could result in increased or decreased ionization of 3NT. Altered ionization of 3NT could lead to inaccurate determination of the limit of detection (LOD) and limit of quantification (LOQ) as well as linearity, and precision (Patel, 2011). For this reasons it was of great importance to try to separate the two peaks, determine which peak presents 3NT, and try to identify the unknown compound.

4.4 QUANTIFICATION OF STEREOSPECIFIC DERIVATIZED 3-NITRO-L-TYROSINE STANDARD WITH LC-MS/MS

4.4.1 Stereospecific butylation of 3-nitro-L-tyrosine standard

Since the unknown compound was detected with the same precursor and product ion combination as 3NT, and its retention time on the C18 column was very close to that of 3NT, it was hypothesized that the unknown compound could be a stereoisomer of 3NT, most probably 3-nitro-D-tyrosine, since D-tyrosine has been reported in human urine (Armstrong *et al.*, 1991). In order to separate the two possible enantiomers from each other, they first had to be converted to diastereomers through means of a chemical reaction. This was achieved by using R-(-2)-butanol:acetyl chloride instead of n-butanol:acetyl chloride. Just as 3NT, R-(-2)-butanol also contains a chiral centre. When adding a second rotating, chiral centre to a specific location on two enantiomers, they turn into diastereomers as the rotation of the second chiral centre disrupts the mirror image, a process known as chiral derivatization. By no longer being mirror images of each other, the two compounds can be separated from each other chromatographically (Lin *et al.*, 2011; Davankov, 1997). Before derivatizing the control urine sample E, 100 μ L containing 0.1 mg/L of the 3-nitro-L-tyrosine standard, was first derivatized with the R-(-2)-butanol:acetyl chloride. This was used to determine whether a change in derivatization reagent altered the chromatographic retention time. The same procedure was followed as described in Section 4.3.1, but instead of adding 200 μ L of n-butanol:acetyl chloride,

100 μ L of the R-(-2)-butanol:acetyl chloride was added. After the standard was dried it was again dissolved in 100 μ L of the mobile phase.

4.4.2 Chromatographic separation of the stereospecific derivatized 3-nitro-L-tyrosine standard

For chromatographic separation of the stereospecific derivatized 3-nitro-L-tyrosine standard, the same MRM condition, chromatographic separation method, column and mobile phase gradient was used as described in Section 4.3.3. As can be seen from the chromatogram in Figure 4.7, the derivatization reagent had an effect on the retention time. This is due to the shorter non-polar tail of the R-(-2)-butanol compared to the n-butanol. The retention time for the 3-nitro-L-tyrosine standard was 9.89 minutes.

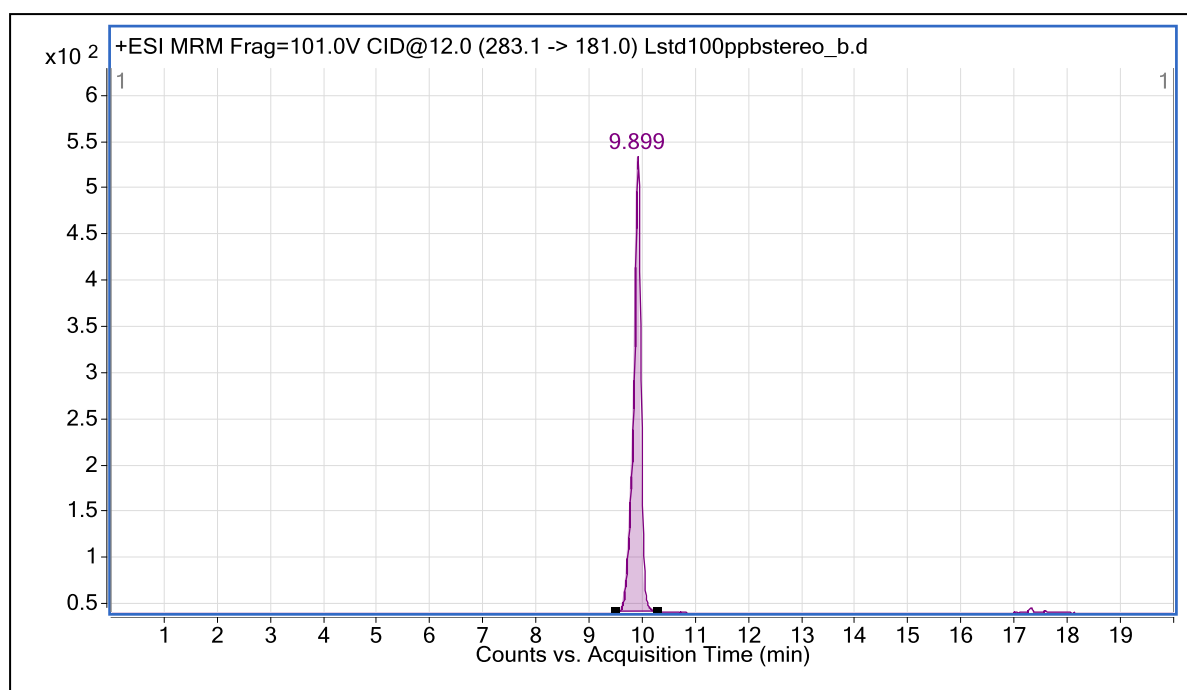


Figure 4.7 Chromatographic separation of the 3-nitro-L-tyrosine standard, derivatized with R-(-2)-butanol:acetyl chloride. MRM of chromatographic separation of the 3-nitro-L-tyrosine standard, derivatized with R-(-2)-butanol:acetyl chloride. The 3-nitro-L-tyrosine standard had a retention time of 9.89 minutes.

4.4.3 Stereospecific butylation and chromatographic separation of urine sample

After stereospecific derivatization of the 3-nitro-L-tyrosine standard, the control urine sample E was stereospecific derivatized. The same derivatization method was used

as described in Section 4.4.1. As illustrated in Figure 4.8, by using the R-(-2)-butanol:acetyl chloride instead n-butanol:acetyl chloride, the method was able to separate the co-eluting compound from 3NT. While normal derivatization using n-butanol:acetyl chloride resulted in very little separation of 3NT and the co-eluting compound (Figure 4.6), stereospecific derivatization using R-(-2)-butanol:acetyl chloride resulted in almost complete separation of 3NT from the co-eluting compound. From the chromatogram it was determined that the retention times of the two peaks differed slightly from each other. The first peak (peak 1) had a retention time of 9.40 minutes, and the second peak (peak 2) had a retention time of 9.75 minutes.

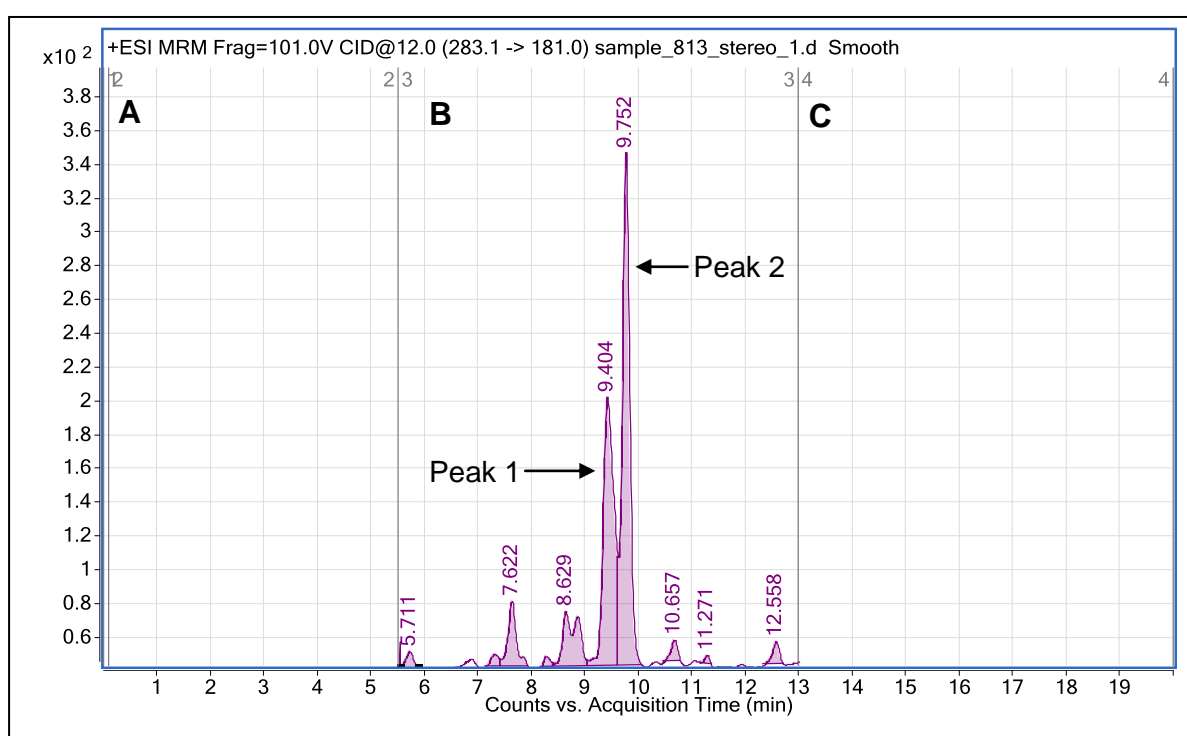


Figure 4.8 Chromatographic separation of a urine sample, derivatized with R-(-2)-butanol:acetyl chloride. MRM of chromatographic separation of sample E, derivatized with R-(-2)-butanol:acetyl chloride. Peak 1 has a retention time of 9.40 minutes and peak 2 has a retention time of 9.75 minutes. A+C = to waste, no data collection. B = to MS, data collection from 5.5 minutes to 13 minutes.

4.4.4 Urine sample spiked with 3-nitro-L-tyrosine standard

After separation of the co-eluting peak from 3NT, it was predicted that peak 2 corresponded to 3NT and peak 1 to the co-eluting compound, as the retention time of peak 2 was similar to that of the stereospecific derivatized 3-nitro-L-tyrosine standard. To confirm this, the stereospecific derivatized urine sample (control sample E) was spiked with 0.1 mg/L of the stereospecific derivatized 3-nitro-L-tyrosine

standard. As illustrated by Figures 4.9-10, the relative abundance of peak 2 increases relatively to the relative abundance of peak 1. As indicated by Table 4.3, peak 2 was 51.5% of the total peak (peak 1 and 2 combined) before the urine sample was spiked with 3-nitro-L-tyrosine standard. However, after the urine sample was spiked with the 3-nitro-L-tyrosine standard, peak 2 increased to 60.6% of the total peak (peak 1 and 2 combined). The relative abundance and height of peak 2 increased when the urine sample was spiked with the 3-nitro-L-tyrosine standard, while peak 1 did not seem to be affected by the additional 3-nitro-L-tyrosine standard. Thus it was concluded that peak 2 corresponded to 3NT and that peak 1 was indeed a contaminant that co-eluted with 3NT when the urine samples are derivatized with n-butanol:acetyl chloride.

Table 4.3 Areas of peak 1 and 2, before and after spiked with 0.1 mg/L 3-nitro-L-tyrosine standard

	Peak 1		Peak 2	
	Relative abundance	%	Relative abundance	%
Before spike	2231	48.5	2368	51.5
After spike	1889	39.4	2911	60.6

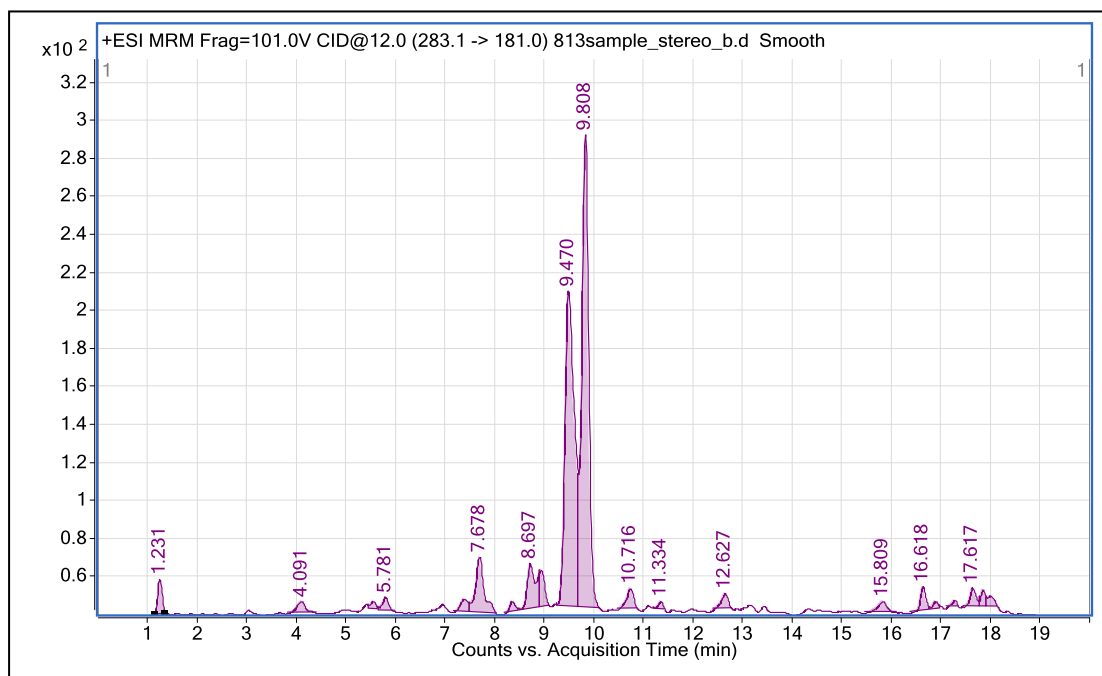


Figure 4.9 Chromatographic separation of a stereospecific derivatized urine sample before it was spiked with stereospecific derivatized 3-nitro-L-tyrosine standard. MRM of chromatographic separation of the stereospecific derivatized sample E, before spiked with 3-nitro-L-tyrosine standard. The relative abundance of peak 1 is 2231 (48.5%) and peak 2 is 2368(51.5%).

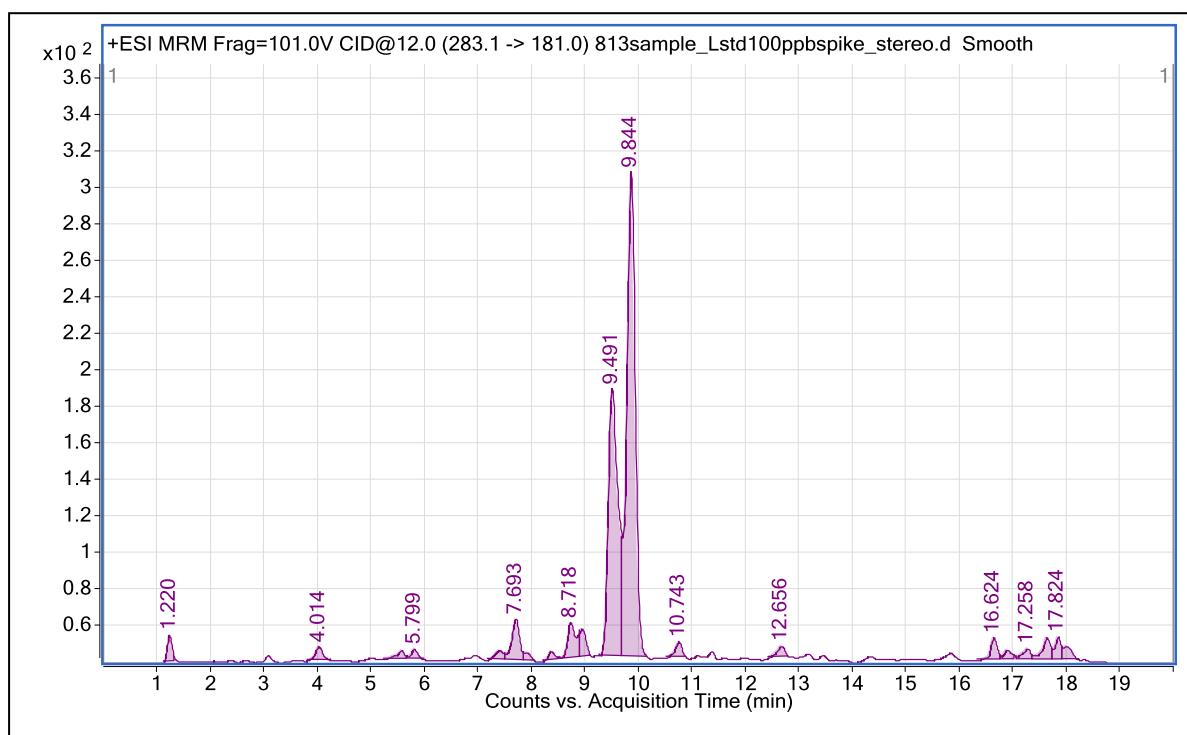


Figure 4.10 Chromatographic separation of a stereospecific derivatized urine sample, spiked with stereospecific derivatized 3-nitro-L-tyrosine standard. MRM of chromatographic separation of the stereospecific derivatized sample E, spiked with the stereospecific derivatized 3-nitro-L-tyrosine standard. The relative abundance of peak 1 is 1889(39.4%) and peak 2 is 2911 (60.6%).

4.5 ATTEMPTS TO IDENTIFY THE CONTAMINATING PEAK/COMPOUND

4.5.1 Introduction

From the results in Section 4.4.4, it was clear that peak 2 corresponded to 3NT and peak 1 not. Peak 1 might be an unknown compound or a possible isomer of 3NT. Due to the fact that the two compounds had the same precursor and product ion and with normal butylation had basically the same retention times it was hypothesized that the unknown compound might be 3-nitro-D-tyrosine.

4.5.2 3-Nitro-D-tyrosine

4.5.2.1 Synthesis of 3-nitro-D-tyrosine

Due to the fact that 3-nitro-D-tyrosine was not commercially available, it had to be synthesized by using D-tyrosine. Before the 3-nitro-D-tyrosine could be synthesized, peroxyxynitrite had to be prepared as described in Section 4.2.5. 3-Nitro-D-tyrosine, 3-nitro-L-tyrosine as well as 3-nitro-DL-tyrosine were synthesized by using D-tyrosine, L-tyrosine and DL-tyrosine, respectively. Synthesizing 3-nitro-L-tyrosine would indicate whether the synthesis worked as the retention time of the synthesized 3-

nitro-L-tyrosine had to be similar to that of previously analyzed purchased 3-nitro-L-tyrosine standard. If the first peak was 3-nitro-D-tyrosine, the retention of the synthesized 3-nitro-D-tyrosine had to be similar to that of peak 1. For the same reason, the synthesized 3-nitro-DL-tyrosine had to have the same double peaks that were present in the previously analyzed stereospecific derivatized control urine sample E (Figure 4.8).

For the synthesis of the stereoisomers of 3NT, 1 mM stock solutions of D-tyrosine, L-tyrosine and DL-tyrosine, respectively, were prepared in Milli-Q water. 100 μ L of the stock solution was added to 800 μ L Milli-Q water in a 1.5 mL tube. To this, 100 μ L of a 1M phosphate buffer (Section 4.2.6) was added as well as 50 μ L of the prepared peroxyxynitrite. The reactions were incubated at room temperature for 15 minutes, after which 100 μ L of each of the reactions were aliquoted into 1.5 mL tubes. The synthesized 3-nitro-D-tyrosine, 3-nitro-L-tyrosine as well as 3-nitro-DL-tyrosine were then derivatized in the same manner as described in Section 4.4.1.

4.5.2.2 Analysis of synthesized 3-nitro-D-tyrosine, 3-nitro-L-tyrosine and 3-nitro-DL-tyrosine

The synthesized 3-nitro-D-tyrosine, 3-nitro-L-tyrosine and 3-nitro-DL-tyrosine were analyzed as described in Section 4.3.3. As indicated by the block in Figure 4.11, the synthesis process was successful, as the retention time of the synthesized 3-nitro-L-tyrosine, 9.82 minutes, was similar to that of the retention time of chemically pure 3-nitro-L-tyrosine standard. The synthesized 3-nitro-D-tyrosine and 3-nitro-DL-tyrosine is indicated by the blocks in Figures 4.12-13 with retention times of 9.84 minutes and 9.89 minutes, respectively. Clearly, the retention times of 3-nitro-D-tyrosine and 3-nitro-DL-tyrosine are very similar to that of 3-nitro-L-tyrosine. Also, the synthesized 3-nitro-DL-tyrosine did not result in 2 peaks. Although it is possible, to separate D and L stereoisomers from each other, the chromatographic conditions used during this assay was not sufficient to separate 3-nitro-D-tyrosine and 3-nitro-L-tyrosine (results from Fig 4.13). Since the unknown compound separated from 3-nitro-L-tyrosine (Fig 4.8), it can be concluded that peak 1 (unknown compound) does not correspond to 3-nitro-D-tyrosine.

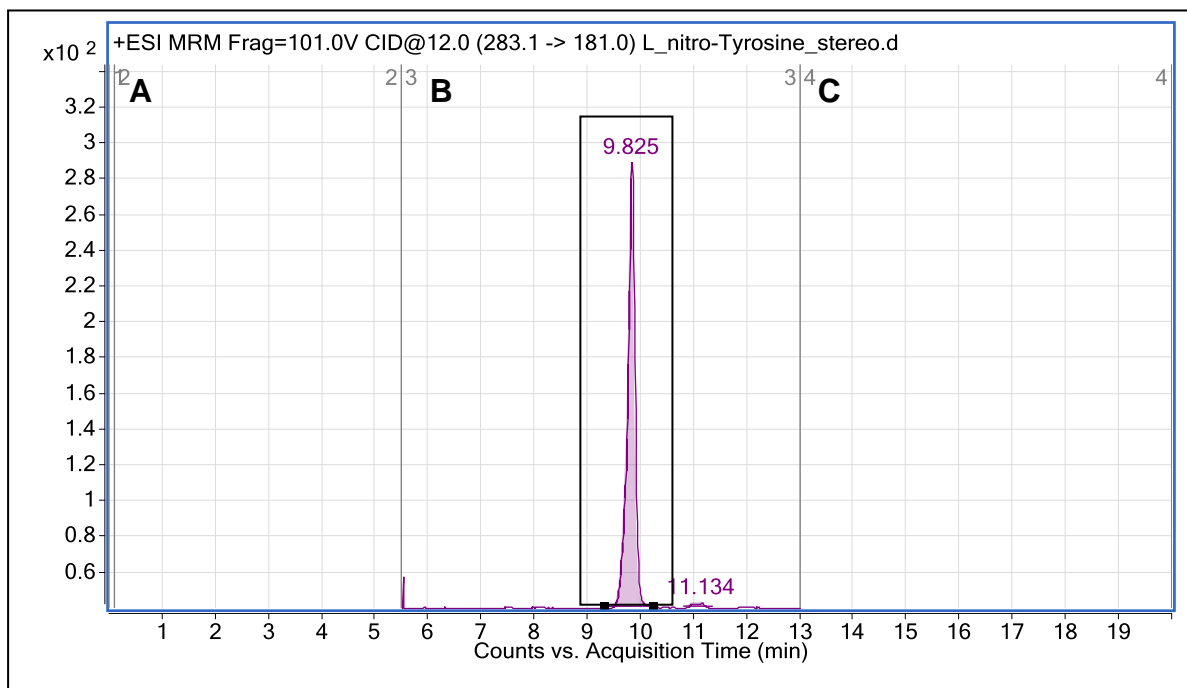


Figure 4.11 Chromatographic separation of the synthesized 3-nitro-L-tyrosine derivatized with R-(-2)-butanol:acetyl chloride. The chromatogram illustrates the MRM of the chromatographic separation of stereospecific derivatized, synthesized 3-nitro-L-tyrosine with a retention time of 9.82 minutes. A+C = to waste, no data collection. B = to MS, data collection from 5.5 minutes to 13 minutes.

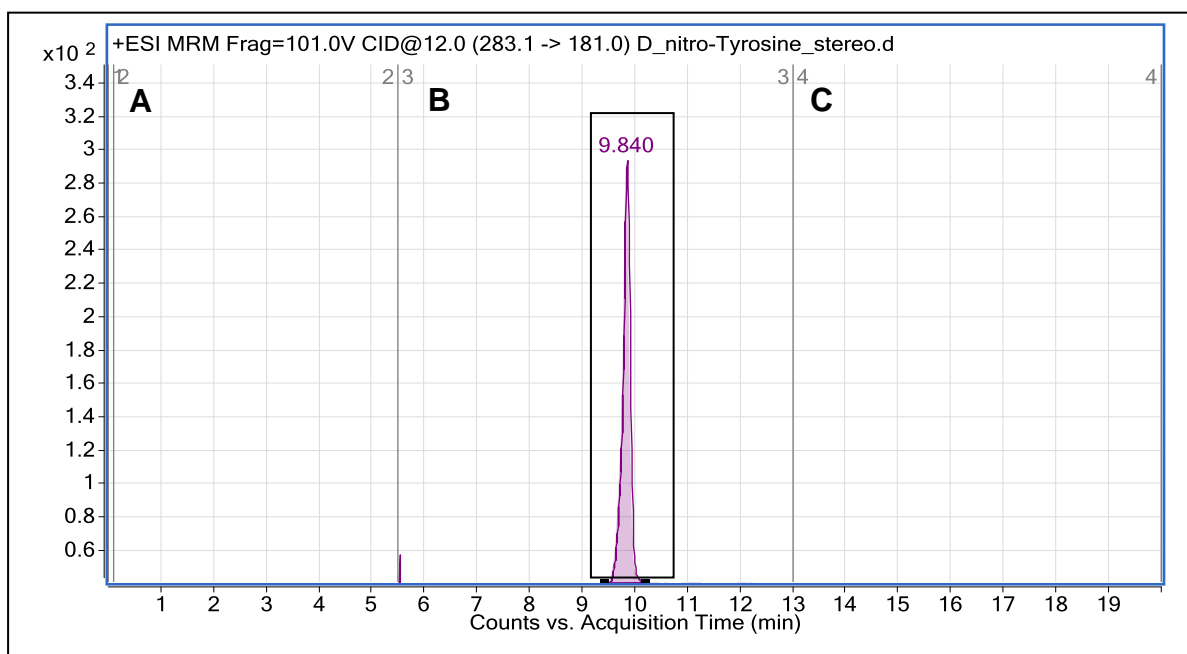


Figure 4.12 Chromatographic separation of the synthesized 3-nitro-D-tyrosine derivatized with R-(-2)-butanol:acetyl chloride. A chromatogram, showing the MRM for the chromatographic separation of the stereospecific derivatized synthesized 3-nitro-D-tyrosine, with a retention time of 9.84 minutes. A+C = to waste, no data collection. B = to MS, data collection from 5.5 minutes to 13 minutes.

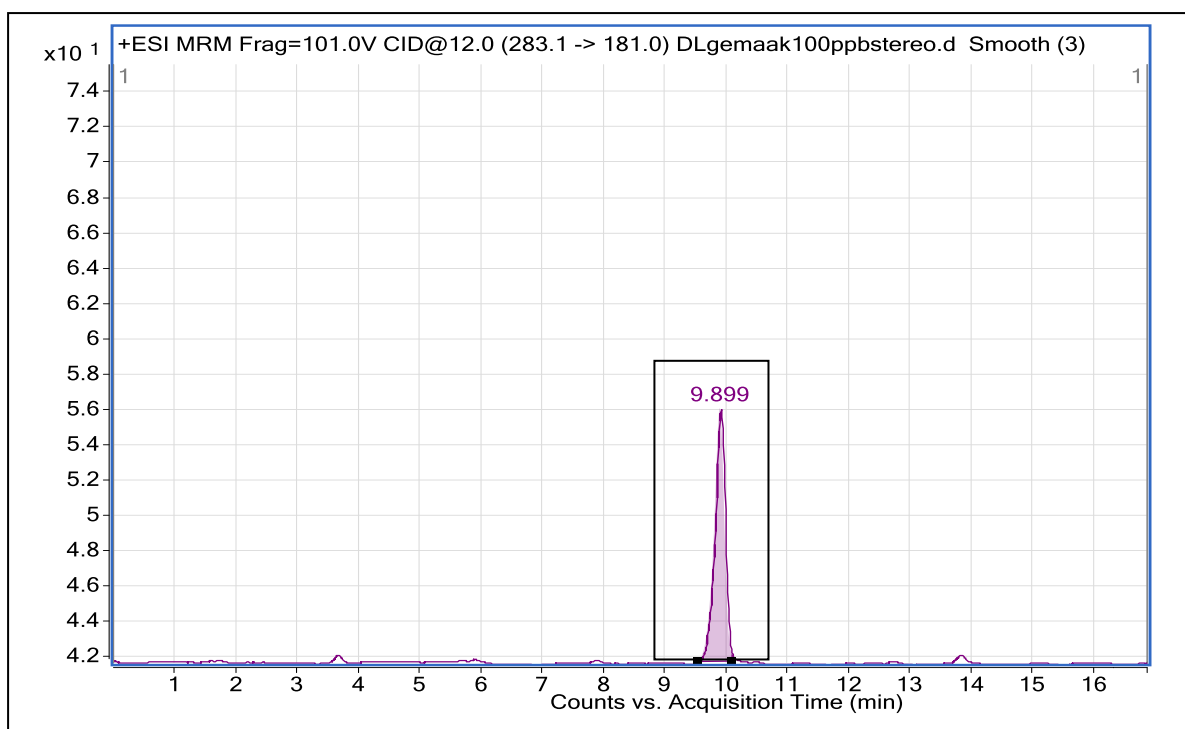


Figure 4.13 Chromatographic separation of the synthesized 3-nitro-DL-tyrosine derivatized with R-(-2)-butanol:acetyl chloride. The chromatogram illustrates the MRM for the chromatographic separation of stereospecific derivatized 3-nitro-DL-tyrosine with a retention time of 9.89 minutes.

4.5.3 Other isomers of 3NT

4.5.3.1 Synthesis of nitro-*m*-tyrosine and nitro-*o*-tyrosine

After it was established that peak 1 (the unknown compound) does not correspond to 3-nitro-D-tyrosine, the possibility of peak 1 being another isomer of 3NT still existed as peak 1 had the same precursor and product ion and nearly the same retention time as peak 2 under normal butylation conditions. In literature ortho-tyrosine as well as meta-tyrosine had been described to be present in the human body (Molnar *et al.*, 2005; Du *et al.*, 2004; Heinecke, 1999) and the possibility existed that these tyrosine's could be oxidized by ONOO⁻. It was therefore believed that these oxidized tyrosine isomers could theoretically result in nitro-*o*-tyrosine and nitro-*m*-tyrosine. Since these two compounds were not commercially available, it had to be synthesized from DL-*o*-tyrosine and DL-*m*-tyrosine.

Nitro-*m*-tyrosine and nitro-*o*-tyrosine were synthesized using the same method as described in Section 4.5.2.1. 1 mM stock solutions of DL-*o*-tyrosine and DL-*m*-tyrosine, respectively, were prepared in Milli-Q water. 100 μ L of the stock solution was added to 800 μ L Milli-Q water in a 1.5 mL tube. To this, 100 μ L of a 1M

phosphate buffer (Section 4.2.6) was added as well as 50 μL of the prepared peroxyxynitrite. The reactions were incubated at room temperature for 15 minutes, after which 100 μL of each of the reactions were aliquoted into 1.5 mL tubes. After the synthesis, the synthesized nitro-*o*-tyrosine and nitro-*m*-tyrosine were stereospecific derivatized as described in Section 4.4.1.

4.5.3.2 Analyses of synthesized nitro-*m*-tyrosine and nitro-*o*-tyrosine

After the synthesized nitro-*o*-tyrosine and nitro-*m*-tyrosine were stereospecific derivatized, they were analyzed in the same manner as described in Section 4.3.3. As indicated by Figures 4.14-4.15, the analysis of both nitro-*o*-tyrosine and nitro-*m*-tyrosine resulted in the formation of more than one peak. However, the retention times of either of these peaks did not correspond to that of the unknown compound (peak 1). The retention time of the nitro-*o*-tyrosine and nitro-*m*-tyrosine also did not correspond to the retention of the synthesized 3-nitro-D-tyrosine, 3-nitro-DL-tyrosine and 3-nitro-L-tyrosine or to the retention time of the chemically pure 3-nitro-L-tyrosine standard. Thus, it can be concluded that the unknown compound (peak 1) does not correspond to either nitro-*o*-tyrosine or nitro-*m*-tyrosine and was therefore not one of the 3NT isomers that could be synthesized.

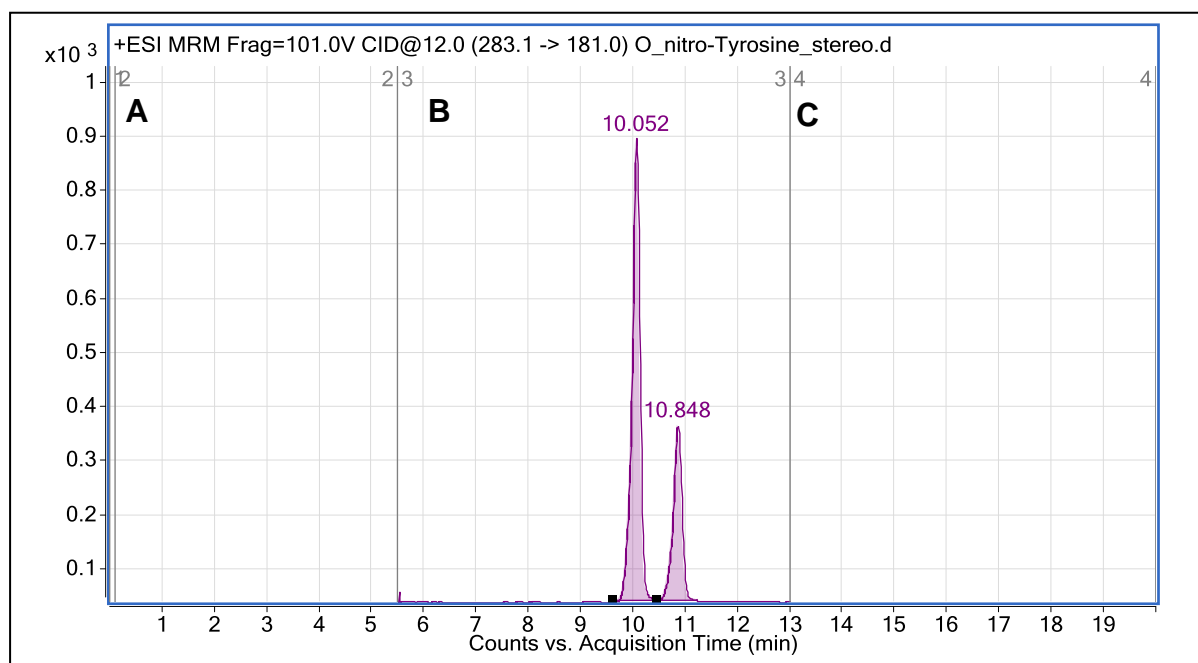


Figure 4.14 Chromatographic separation of the synthesized nitro-*o*-tyrosine standard derivatized with R-(-2)-butanol:acetyl chloride. MRM for the chromatographic separation of the stereospecific derivatized nitro-*o*-tyrosine with a retention time of 10.05 minutes. A+C = to waste, no data collection. B = to MS, data collection from 5.5 minutes to 13 minutes.

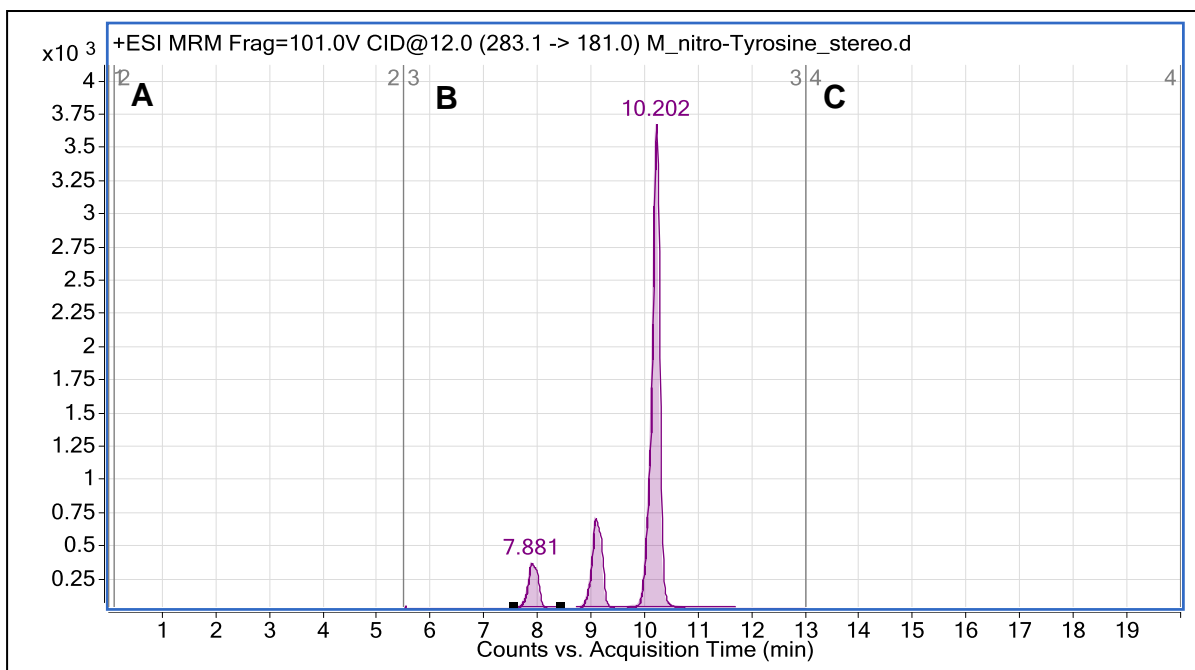


Figure 4.15 Chromatographic separation of the synthesized nitro-*m*-tyrosine standard derivatized with R-(-2)-butanol:acetyl chloride. MRM for the chromatographic separation of the stereospecific derivatized nitro-*m*-tyrosine with a retention time of 10.20 minutes. A+C = to waste, no data collection. B = to MS, data collection from 5.5 minutes to 13 minutes.

4.5.4 Determining the accurate mass of the unknown compound

4.5.4.1 Thin layer chromatography

By ruling out all the postulated isomers of 3NT in an attempt to identify the unknown compound (peak 1), the next step was to try and determine the accurate mass of peak 1 and determine whether it differed from the accurate mass of 3NT. To investigate the accurate mass, a 1200 series LC from Agilent Technologies (Santa Clara, CA, USA) connected to a 6510 quadrupole time-of-flight mass spectrometer (Q-TOF), also from Agilent Technologies, Santa Clara, CA, USA) was used. The Q-TOF is an instrument that is capable of measuring the accurate molecular mass of a compound, up to four decimals. However, direct analysis of derivatized control urine samples with the Q-TOF LC-MS was not possible since the level of 3NT in the urine is very low and below the detection limit of the Q-TOF LC-MS. Therefore, options were investigated to concentrate control urine sample E. It was decided to use thin layer chromatography (TLC), an old but very effective method in order to concentrate the sample. The first part was to determine how much urine could be loaded onto the TLC plate. A TLC plate (Merck (Pty) Ltd, JHB, South Africa, DC-Alufolien art 5552) 10 mm in width and 20 mm in length, was used with positions marked, 1 cm from the

bottom, 1 cm from the side and 1 cm between each other. On the first position, 5 μ L of the EZ:faast amino acid standard cocktail was loaded. This cocktail consisted of 23 amino acids (phenylalanine, threonine, leucine, glycine, aspartic acid, α -aminobutyric acid, α -aminoadipic acid, β -aminoisobutyric acid, histidine, lysine, proline, methionine, valine, alanine, glutamine, serine, ornithine, hydroxyproline, cystine, allo-isoleucine, sarcosine and tyrosine) and was used to have a slight indication of where 3NT might elute on the TLC plate, as the position of the tyrosine in the cocktail will be known. On the second position 5 μ L of the purchased 3-nitro-L-tyrosine standard (0.9 mg dissolved in 3 mL methanol) was loaded. The next positions received 1 μ L, 2 μ L, 3 μ L, 5 μ L, 10 μ L and 20 μ L of control urine sample E, respectively. After all the application positions were dried, the plate was inserted in a developing vessel in the mobile phase consisting of n-butanol, acetone, water and glacial acetic acid (Section 4.2.8) so that the level was just under the 1 cm bottom line. The plate was left in the developing vessel, until the mobile phase reached about 2 cm from the top. Once it reached the position, the plate was removed and left to dry. After the plate was completely dried, the entire plate was sprayed with the ninhydrin colouring reagent (Section 4.2.8). The plate was baked for 15 minutes at 45°C. As illustrated in Figure 14.16, purple spots (corresponding to amino acids) could be noticed on the plate. As indicated by Figure 4.16, in the 20 μ L urine spot, a small amount of 3NT could be observed. Therefore, it was decided to use 20 μ L per spot as the ideal amount of urine sample to load onto the TLC, in an attempt to concentrate the control urine sample E for analyses on the Q-TOF LC-MS.

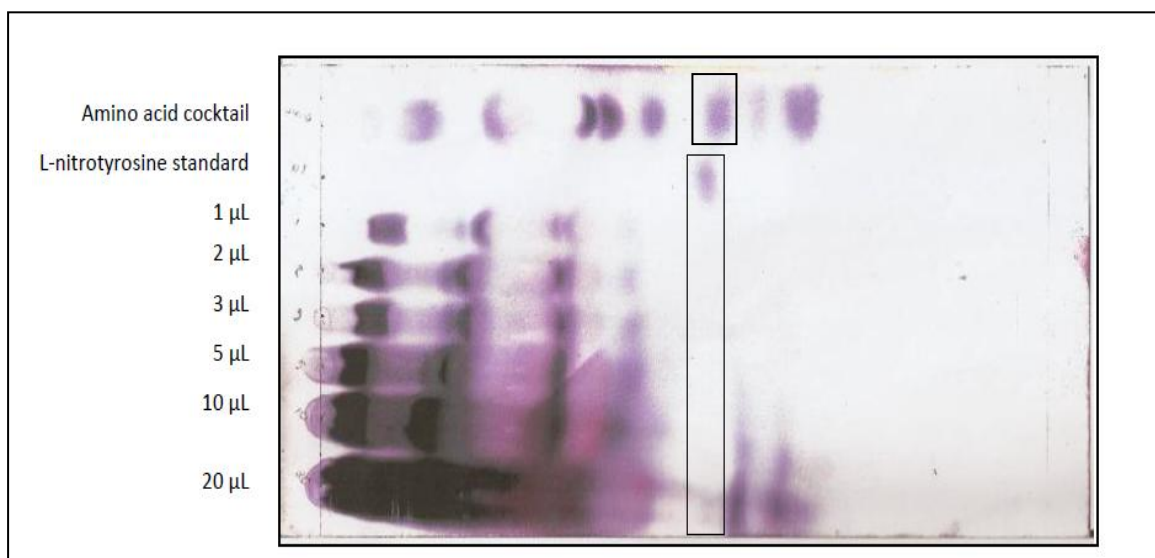


Figure 4.16 TLC plate used to determine the ideal amount of sample that could be loaded onto a TLC plate. A developed TLC plate, indicating the amino acid cocktail with tyrosine, in line 1, the purchased 3-nitro-L-tyrosine standard in line 2, and 1 μL , 2 μL , 3 μL , 5 μL , 10 μL and 20 μL of sample E in line 3, 4, 6, 7 and 8 respectively.

After the optimum amount of urine was determined per position, sample E was concentrated by loading six positions with 20 μL each. This was done for 12 TLC plates. Each plate also received 5 μL of the 3-nitro-L-tyrosine standard. The plates were developed as previously described. The only difference was that this time only the 3-nitro-L-tyrosine standard was covered with ninhydrin. This was then used as an indication where the 3NT in the samples was situated. The samples were not developed with ninhydrin, as ninhydrin irreversibly reacts with the amino acids. The area/bands that contained the 3NT were scraped off the TLC plates. The cellulose scrapings, containing the 3NT (and other metabolites that were not separated from the 3NT using the specific TLC conditions) were then washed, by adding a solution consisting of 20 % water, 80% methanol and 0.1% formic acid. The tubes were sonicated for about 1 hour after which it was centrifuged at 2500 $\times g$ for 10 minutes.

The supernatants were combined and dried under a stream of nitrogen gas, until approximately 1 mL remained. From this 100 μL supernatant, a 20 μL aliquot was stereospecific derivatized (Section 4.4.1), dissolved in 25 μL mobile phase and analyzed on the LC-MS/MS. This was done to determine whether the attempt to extract and concentrate the 3NT from control urine sample E was successful. As illustrated by Figure 4.17, 3NT was successfully extracted and concentrated by using the TLC plates. The TLC plates did not only extract and concentrate the 3NT, but

also the unknown contaminant corresponding to peak 1. The two peaks were detected with the MRM analysis and the retention times corresponded well to the retention times previously determined for the same urine sample (Fig 4.8).

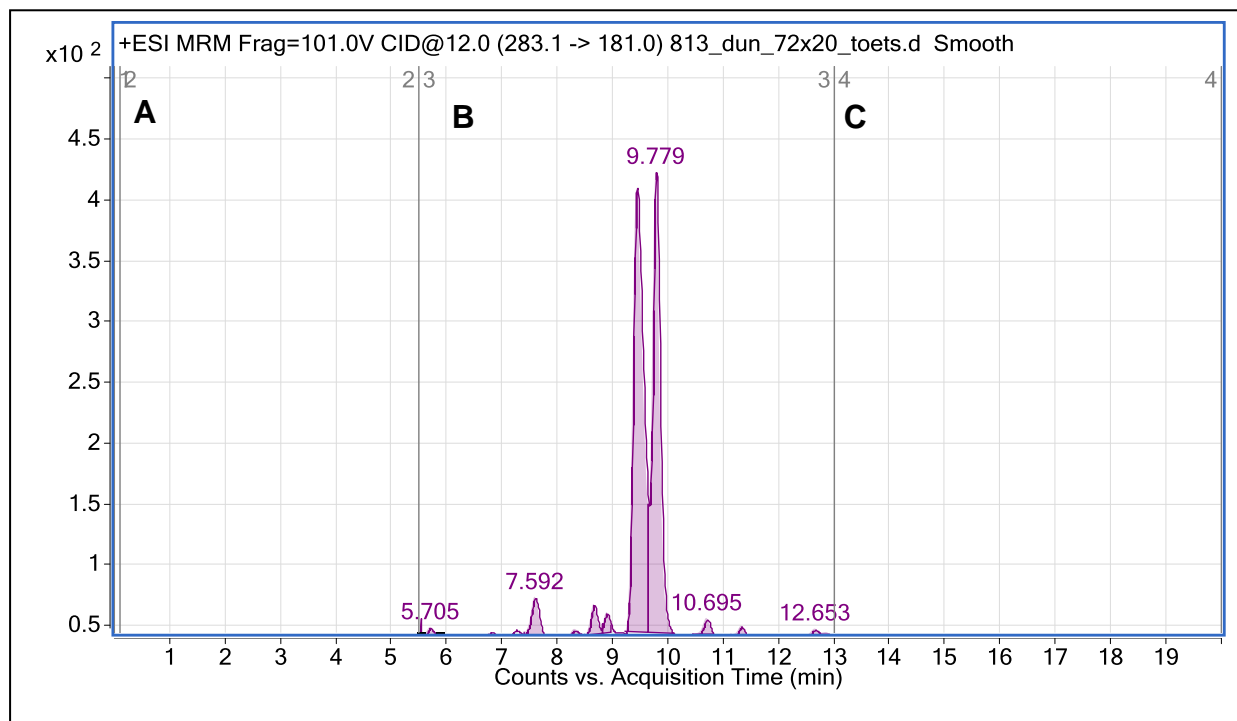


Figure 4.17 Determining if 3NT could be extracted from urine by using TLC plates. The chromatogram illustrates the MRM for the chromatographic separation of stereospecific derivatized, 3NT, concentrated by using the TLC plates with a retention time of 9.77 minutes. A+C = to waste, no data collection. B = to MS, data collection from 5.5 minutes to 13 minutes.

After it was determined that the 3NT was successfully extracted, the remaining 80 μ L was stereospecific derivatized, using the same method as described in Section 4.4.1, and dissolved in 30 μ L mobile phase. The dissolved, concentrated 3NT standard was then transferred to a vial with a tapered insert, ready to be analyzed on the Q-TOF LC-MS.

4.5.4.2 Analyzes of unknown compound on the Q-TOF LC-MS

In order to analyze control urine sample E on the Q-TOF LC-MS, the same separation method (chromatographic conditions) that was used in Section 4.3.3 was used on the Q-TOF LC-MS as the retention time of 3NT was known with the use of this method. This made the detection of 3NT easier. The same column as well mobile phases A and B, which was used in the LC-MS/MS analyses, also had to be used during the Q-TOF LC-MS analyses, which ensured that there was minimum

shift in retention time. Together with the concentrated sample, a 1mg/L 3-nitro-L-tyrosine standard was also analyzed. This was done to determine the accurate mass of 3NT. After analyzing the sample as well as the standard, using the method described in Section 4.3.3, it was determined that there was a difference in molecular mass between peak 1 and 3NT. The Q-TOF LC-MS was able to determine that the molecular mass of peak 1 (unknown compound) was 283.16 m/z and that of 3NT, 283.13 m/z

4.6 CONCLUSION

After numerous attempts to identify the unknown compound, through means of chromatographic separation and synthesis of 3NT isomers, the identity of the unknown compound could still not be ascertained. However, it was proved that peak 2 corresponded to 3-nitro-DL-tyrosine, as the retention time was similar to that of the purchased 3-nitro-L-tyrosine as well as the synthesized 3-nitro-L-tyrosine and 3-nitro-D-tyrosine. Although the Q-TOF LC-MS determined that there was a mass difference of 0.3 m/z between the two peaks, the concentration of 3NT in the concentrated sample was still close to the detection limit of the Q-TOF LC-MS to say with confidence that peak 1 had a different mass from peak 2. It was therefore decided to cease attempts at identifying the unknown compound due to time limitation on this study. By using the stereospecific derivatization, 3NT can be quantified in urine samples. As such this method could now be validated for the quantification of 3NT.

4.7 THE OPTIMIZED 3-NITROTYROSINE ASSAY

4.7.1 Derivatization of samples or standard

After thawing a urine sample, a 1 mL aliquot is centrifuged at 15 700 $\times g$ at room temperature for 2 minutes to remove any crystals. 100 μL of the supernatant is then transferred to a corresponding new 1.5 mL tube (1.5 mL micro tubes from PLASTIBRAND[®], Sigma-Aldrich, St. Louis, Mo., U.S.A). To the urine samples, 100 μL methanol is added. The mixture is dried under a stream of nitrogen gas at 65°C for 40 minutes and stereospecifically derivatized, by adding 100 μL R-(-)-butanol:acetyl chloride to the dried sample. The reaction is afterwards incubated at 65°C for 15 minutes. After incubation, the derivatized sample is dried again under a stream of nitrogen gas at 65°C for approximately 15-20 minutes. The completely

dried sample is then dissolved in 100 μ L mobile phase (10% acetonitrile, 90% water and 0.1% formic acid). The derivatized sample is again centrifuged at 15 700 xg at room temperature for 2 minutes to remove any residual crystals. The clear supernatant (90 μ L) is then transferred to vials with tapered inserts (2 mL clear vial with a screw cap and a 250 μ L pulled point glass insert, both from Agilent Technologies, Santa Clara, CA, USA).

4.7.2 Chromatographic separation of the sample or standard

For the analysis of a sample or a standard, a 1290 infinity LC from the 1200 infinity series connected to a 6460 triple quadrupole mass analyzer in positive electron spray ionization, both from Agilent Technologies (Santa Clara, CA, USA) is used. The sample injection volume is 5 μ L which is separated on a C18 column (Zorbax SB-18, 1.8 μ m, 2.1X50 mm, Agilent Technologies, Santa Clara, CA, USA), using a flow rate of 0.2 mL/min with mobile phases A (Milli-Q water and 0.1% formic acid) and B (acetonitrile and 0.1 % formic acid). A mobile phase gradient is used for chromatographic separation of 3NT (Table 4.1). For maximum sensitivity, the MS is used in MRM mode. Nitrogen is used as collision gas with collision energy being 12 eV. The precursor-product combination for derivatized3NT is 283.13 m/z to 181 m/z . To minimize unnecessary compounds entering the MS, the instrument is programmed to switch between MS and waste as illustrated in Table 4.4. The exclusion of the unnecessary compounds entering the ion source, not only ensures for a cleaner chromatogram, but also protects the electrospray interface against damage, caused by the unwanted salts and proteins in the biological matrix of the samples.

Table 4.4 Switching times of the LC-MS/MS between MS and waste

Time	Switch
0.0	To waste
5.5	To MS
13.0	To waste

4.7.3 Quantification of 3NT

For quantification of 3NT, a phenylalanine stable isotope (L-phenyl-d5-alanine) was used as internal standard (IS) since neither a tyrosine stable isotope nor a 3NT stable isotope was available. The chemical structure of L-phenyl-d5-alanine also closely resembles that of 3NT and therefore it was decided to use the L-phenyl-d5-alanine isotope as internal standard for 3NT quantification. By using an IS, the response of the 3NT could be normalized against the response of the IS. This would compensate for an increase or decrease in ion suppression and any variation during the preparation, injection and chromatographic separation steps (Jessome and Volmer, 2006). Therefore, the optimal MRM condition for the quantification of IS was determined by using the same optimization method as described in Section 4.3.2. As indicated by Table 4.5 the MassHunter optimizer software determined that the ideal precursor and product ion combination for the IS was 227.21 *m/z* and 125.1 *m/z*, respectively.

Table 4.5 The optimal MRM conditions for internal standard quantification

Compound	Formula	Nominal mass	Polarity	Ion source
Phenylalanine isotope	C ₁₃ H ₁₈ N ₂ O ₅	282.13	Positive	ESI
Precursor ion	Product ion	Fragmentor	Collision energy	Abundance
227.21 <i>m/z</i>	125.1 <i>m/z</i>	94 V	12 V	26037
227.21 <i>m/z</i>	106.1 <i>m/z</i>	94 V	40 V	879
227.21 <i>m/z</i>	79.1 <i>m/z</i>	94 V	50 V	1262
227.21 <i>m/z</i>	142.3 <i>m/z</i>	94 V	4 V	40

After the optimal MRM condition was determined for the IS, the IS was chromatographically separated by using the same method as described in Section 4.3.3. As indicated by Figure 4.18 the IS had a retention time of 10.05 minutes. When comparing the retention time of 3NT to the IS one can see that they elute very close to one another from the column.

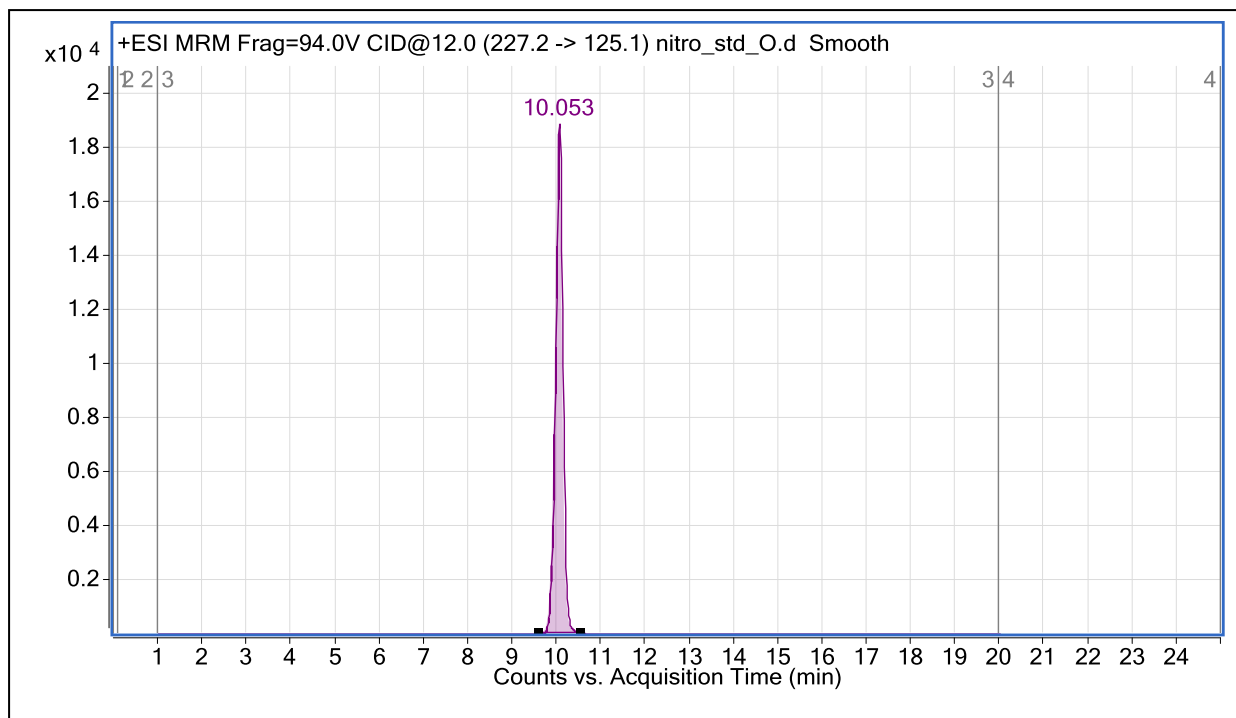


Figure 4.18 Chromatographic separation of the internal standard, derivatized with R-(-2)-butanol:acetyl chloride. MRM chromatogram showing the chromatographic separation of the internal standard (L-phenyl-d5-alanine) derivatized with R-(-2)-butanol:acetyl chloride. The internal standard had a retention time of 10.05 minutes.

After the MRM and chromatographic separation conditions were optimized for the IS, quantification of 3NT using the IS, was tested. A couple of urine samples were derivatized in the same manner as described in Section 4.7.1 but instead of adding 100 μ L pure methanol, 200 μ L methanol containing the IS was added. These samples were analyzed as described in Section 4.7.2. As indicated in Figure 4.19 co-elution of an unknown compound with the IS took place, when analyzed in a urine sample. This co-elution resulted in an asymmetric peak for the IS in some of the urine samples, opposed to a symmetric peak found when analyzing butylated L-phenyl-d5-alanine standard (Figure 4.18). This asymmetry meant that the peak could not be probably integrated. Changing the mobile phase gradient to achieve a better symmetric peak for integration was not an option as the current mobile phase gradient was optimum for the separation of 3NT from the unknown compound. As a result of the co-elution, 3NT could not be quantified using L-phenyl-d5-alanine.

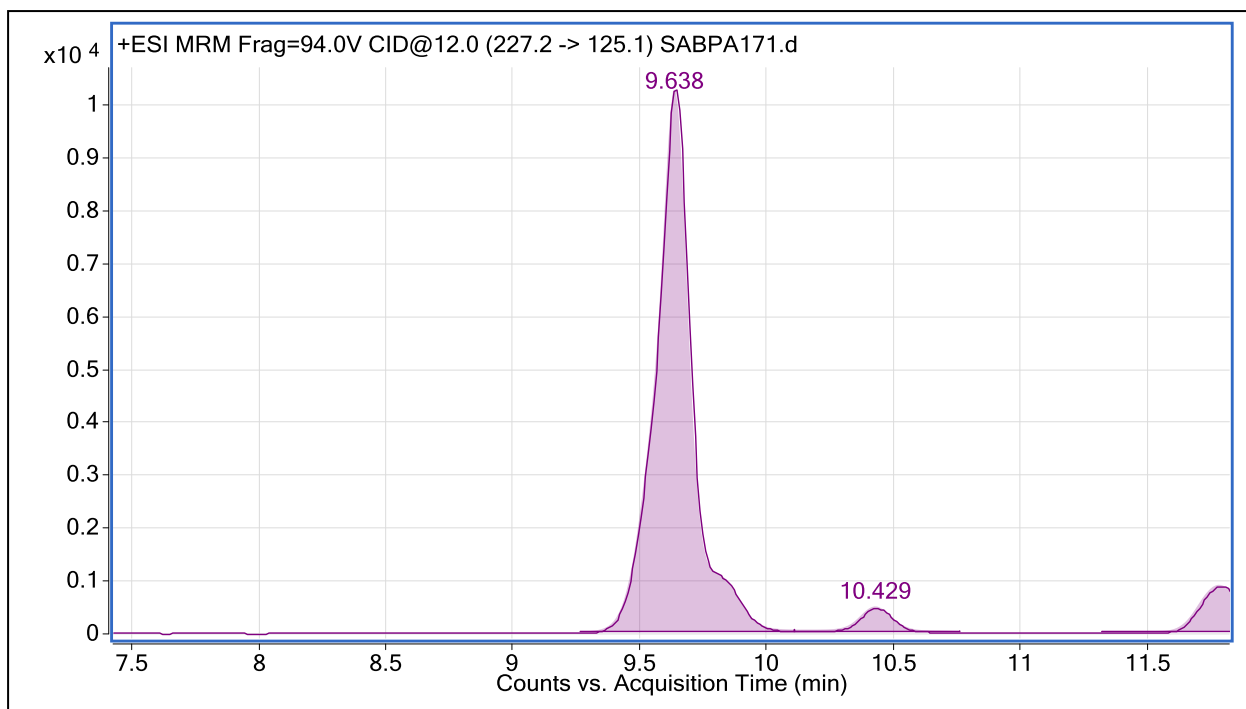


Figure 4.19 Chromatographic separations of IS in urine sample 1. MRM of IS with retention time 9.63 minutes. Co-elution with an unknown compound is visible, resulting in the formation of an asymmetric peak that is difficult to integrate.

Instead, quantification was done by using a calibration curve in order to quantify 3NT. The calibration curve ranged from 0.001-0.05mg/L L3-nitro-L-tyrosine standard. The standards were stereospecific derivatized as described in Section 4.4.1 and analyzed in the same manner as in Section 4.4.2. A response vs concentration curve was constructed through means of linear regression with response on the y-axis and concentrations of the 3-nitro-L-tyrosine standards on the x-axis, as illustrated in Section 4.8, Figures 4.20-4.21. These graphs had R^2 value of >0.99 (with $n=4$). The final 3NT values were expressed per mmol creatinine, thus the creatinine values of the urine were determined by using the QuantiChrom™ Creatinine Assay Kit from BioAssay Systems. The method used was exactly as described by the manufacturers.

4.8 LINEARITY OF THE OPTIMIZED 3-NITROTYROSINE ASSAY

To investigate the linearity of the 3-nitrotyrosine assay, a calibration curve was constructed. The calibration curve consisted of five samples 0.001, 0.01, 0.05, 0.1 and 0.5 mg/L 3-nitro-L-tyrosine standard. The samples were stereospecific derivatized and analyzed as described in Sections 4.4.1 and 4.4.2.

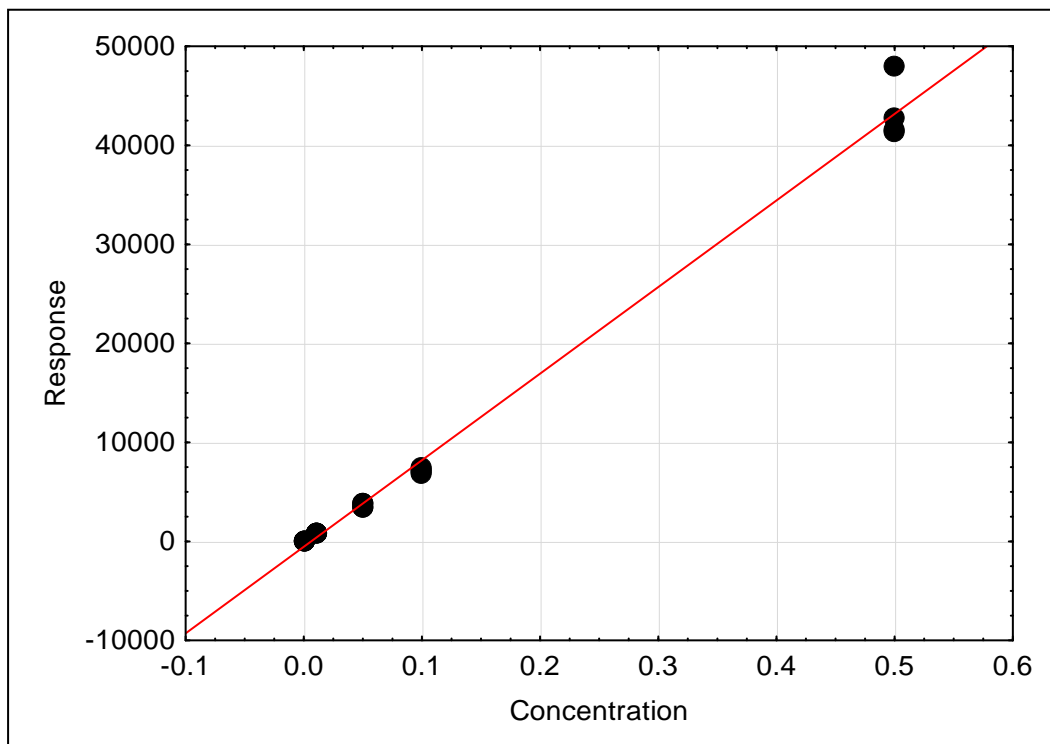


Figure 4.20 Calibration curve of 3-nitro-L-tyrosine standard. A calibration curve constructed from data obtained from the concentration range analyzed by the optimized 3-nitro-L-tyrosine standard method. The curve had a R^2 value of 0.99. $n = 4$.

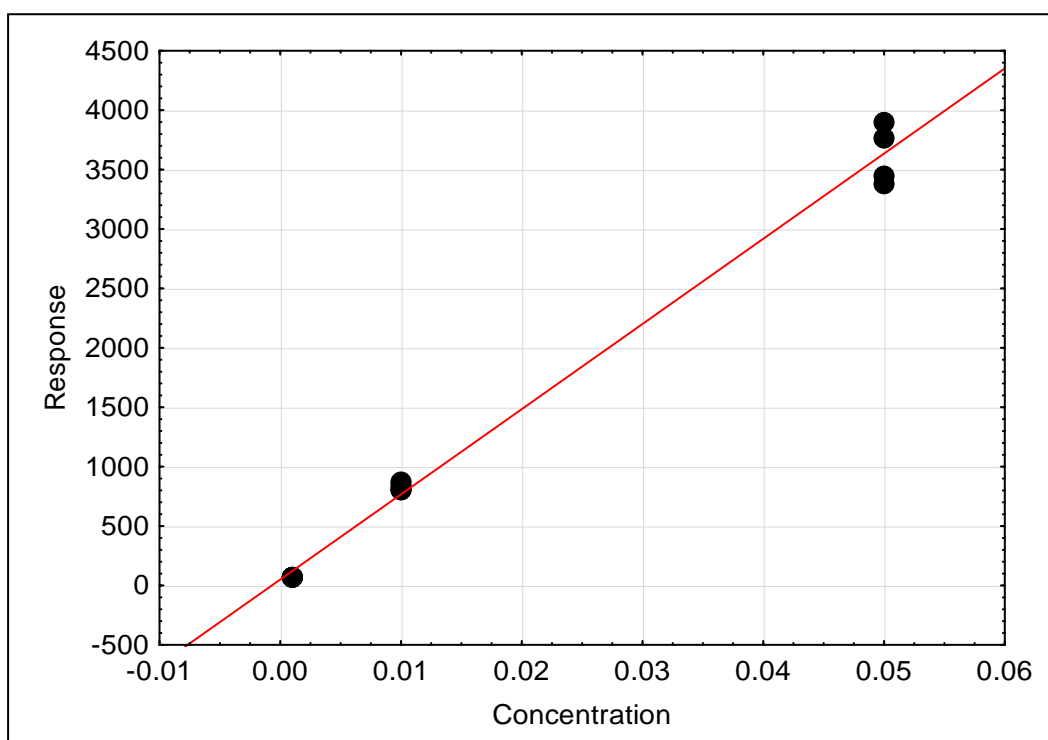


Figure 4.21 Calibration curve of 3-nitro-L-tyrosine standard. A calibration curve for the lower concentrations in the concentration range (0.001-0.05 mg/L) with a R^2 value of 0.99. $n = 4$.

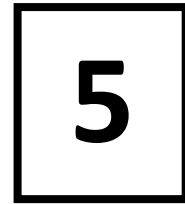
As can be seen from Figure 4.21, the calibration curve is linear over the concentration range 0.001-0.5 mg/L with an R^2 value of >0.99 ($n = 4$). This was also

the case for the lower concentration range, which also had a R^2 value of >0.99 ($n = 4$) (Figure 4.22).

4.9 CONCLUSION ON THE OPTIMIZED 3-NITROTYROSINE ASSAY

The co-elution of the unknown compound with 3NT in urine samples was not expected, thus many attempts were made to separate 3NT from the unknown compound. Although the identity of the unknown compound could not be identified, it was possible to identify which of the two peaks corresponded to 3NT and it was therefore decided to use this peak for quantification of 3NT. Usually an internal standard (IS) will be used for quantification, as it compensates for any loss of sample as well as ion suppression. In this case, the IS co-eluted with another compound, resulting in an asymmetric peak in some of the urine samples. This asymmetric peak made it difficult to integrate the IS peak accurately. Therefore, 3NT could not be quantified by using this IS. Instead, a calibration curve was constructed which ranged from 0.001-0.05mg/L 3-nitro-L-tyrosine standard. By using this quantification method, it was possible to quantify 3NT, using control urine samples. This assay had acceptable linearity (Section 4.8) and it was therefore decided to analyze and quantify all of the 172 SABPA samples by using this optimized method.

Chapter 5



The involvement of 3-nitrotyrosine and TBARS in hypertension

5.1 STUDY GROUP AND ETHICAL APPROVAL

As previously mentioned this study formed part of the SABPA study. The main aim of this larger study was to investigate the association between cardiovascular disease and psychological stress in urbanized black South Africans compared to their Caucasians counterparts. The SABPA study consists of two phases and the study group initially consisted of 209 Caucasian (101 males; 108 females) and 200 African (100 males; 100 females) teachers who worked in one of the four Kenneth Kaunda Education districts (Ventersdorp, Potchefstroom, Matlosana and Maquassi Hills) from the North West Province, South Africa. During the initial phase, samples from the 200 African teachers were collected (2008) and then from the 209 Caucasian teachers (2009). Phase two of the SABPS study was a follow-up study of phase one and samples were again collected from the same African and Caucasian teachers. Some of the teachers involved in the initial phase were, however, not available for the second phase. Thus, during the second phase, samples were collected from 172 African teachers in 2011 and 190 Caucasian teachers in 2012, all of which participated in the initial phase, resulting in a 86 % follow-up for the SABPA study. All participants were between the ages of 25 and 60 years, had a similar socio-economic status and more or less the same income. The exclusion criteria for the study was an oral temperature above 37°C, the use of chronic or acute medication, pregnancy, lactation, vaccination three months prior to the study and blood donors. The objectives, as well as procedures and risks of the study, were discussed with the participants prior to their recruitment and, if needed, were conducted in their home language. All the participants signed an informed consent form. Ethics approval for the SABPA study was obtained from the Ethics Committee of the North-West University under the title “SABPA, Sympathetic Activity and Ambulatory Blood Pressure in Africans” (NWU-00036-07-S6).

5.2 SAMPLE COLLECTION AND OTHER ANALYSES

The day prior to sampling, the participants were transported to the Metabolic Unit Research Facility of the North-West University (Potchefstroom Campus) where they were familiarized with the protocol. They received a standardized dinner and were woken up at approximately 05:45 the following morning after which testing started. Registered nurses collected fasting venous blood samples, anthropometric measurements as well as information about previously prescribed medication and medical conditions. 24-hour urine collection (with HCl as stabilizer), 24-hour ambulatory blood pressure monitoring as well as other cardiovascular measurements information were collected and assessed. After the blood and urine was collected, it was transported to the North-West University's Physiology Department, where all samples were aliquoted into smaller vials (1.5 mL eppendorf tubes) and stored at -80°C. As multiple research groups are working on the SABPA study, numerous other analyses were performed on the collected samples by co-investigators. Some of the variables that were tested for were used for this study's data set. Permission was obtained from co-investigators to use these measured variables in this dissertation. Table 5.1 lists the variable that was used in this study's data set as well as method of measurement.

Table 5.1 Variables measured by co-investigators, which were used in the data set

Variable	Method and unit
Age	Measured in years.
Physical activity level (PAL)	Physical activity level (PAL) was measured by using an ActicalR accelerometer (Montreal, Quebec).
GGT	Gamma glutamyltransferase (GGT) is a liver enzyme that is measured in the serum and is associated with high alcohol consumption. It was measured by using a Cobas Integra 400 plus (Roche, Basel, Switzerland) enzymatic colorimetric assay from and the unit and was measured in U/L.
CRP	Measuring high sensitivity serum C-reactive protein gives an indication of the degree of inflammation present. It was measured by using a Cobas Integra 400 plus (Roche, Basel, Switzerland) particle enhanced turbidimetric assay and was measured in mg/L.
Glucose	Fasting sodium fluoride serum was used to determine the glucose levels. It was measured by using an enzymatic reference method with hexokinase from Cobas Integra 400 plus (Roche, Basel, Switzerland) and was measured in mmol/L. Glucose levels were classified as normal (< 5.6 mmol/L), impaired glucose regulation (IFG/IGT) (5.6–6.9 mmol/L) and diabetes (7.0 mmol/L) (Genuth <i>et al.</i> , 2003).
Self report alcohol	Self-reported alcohol intake was determined by a self-reporting questionnaire on current consumption of alcoholic beverages.
Self report smoke	Self-reported smoking was determined by a self-reporting questionnaire on current smoking habits.
HIV	HIV status was determined with both the SD Bionline HIV-1/2 3.0 test (Standard Diagnostics Inc, Kyonggi-do, South Korea) and the Alere Determine HIV-1/2 test (Alere Medical Co. Ltd, Shiba, Japan).
Progesterone (pmol/L)	Progesterone was determined with an electrochemiluminescence immunoassay on the Elecsys 2010 (Roche, Basel, Switzerland).
Estradiol (pmol/L)	Estradiol was determined with an electrochemiluminescence immunoassay on the Elecsys 2010 (Roche, Basel, Switzerland).
BMI	To calculate BMI, height was measured, using a stadiometer while the participant's head was in the Frankfurt plane. Weight was determined to the nearest 0.1kg by making use of a Krups scale, while the participants had minimal clothes on. The calculated height and mass were then used to calculate BMI which is done by dividing the mass through square height.

Participants were classified as either hypertensive or normotensive according to the classification system of The European Society of Hypertension (Section 2.8). This classification system classifies a participant as hypertensive if he or she has continues systolic blood pressure (SBP) and diastolic blood pressure (DBP) of 140 mmHg and/or 90 mmHg or higher (O'Brien *et al.*, 2005).

For the statistical analysis the entire data set was used, including the outliers which were identified through Tukey's method. Tukey's method defines outliers as data points that fall outside the upper and lower control limits of the data set. These limits are three interquartile ranges below the 25th or above the 75th percentile (Tukey, 1977). Non-parametric analysis was used and therefore the influence of outliers is negligible. For the purposes of graphical presentation the outliers were excluded from the figures in this chapter.

5.3 THE EFFECT OF GENDER ON URINARY 3NT AND TBARS

During this study, the involvement of lipid and protein oxidation in hypertension was investigated on the 172 SABPA samples collected in 2011 (black South Africans). The optimized TBARS assay (Section 3.4) as well the optimized 3-nitrotyrosine assay (Section 4.7.) was performed on the 24-hour urine samples. The sample group consisted of 88 male (74 hypertensive and 14 normotensive) subjects and 84 female (49 hypertensive and 35 normotensive) subjects.

The first step in statistical analysis was to determine whether a difference in TBARS or 3NT levels existed between the two genders. However, before the data could be statistically analyzed, the distribution of the data (normal distribution or skewed) had to be determined. To determine if each group's data was distributed normally or not, descriptive statistics were performed, more specifically the Shapiro Wilk's W test. The Shapiro Wilk's W test indicated normality if a p-value higher than 0.05 was calculated. Both the female and male data sets were calculated to be non-normally distributed, as each group's data was skewed (p-value < 0.05). Theoretically, parametric statistics could be used in further analysis, as the sample group was large enough according to the central limit theorem (Le Cam, 1986; Rosenblatt, 1956). However, due to the skewness of the data set, non-parametric statistic was used to analyze the data.

The possible difference in TBARS and/or 3NT levels between males and females was determined by means of a Mann Whitney U test, using STATISTICA version 10.0. The Mann Whitney U test is the non-parametric version of the Student's T test and is used for analyzing categorical variables. This test is used on independently observed samples for an indication on whether a difference exists between the

groups that are being compared (Hart, 2001). As indicated by Figure 5.1, a significant difference in 3NT levels was detected between the males and females as indicated by the p-value of less than 0.05 ($p < 0.01$). The female group had a higher median 3NT of 12.44 nmol/mmol creatinine compared to the median of the males, which were 6.77 nmol/mmol creatinine. The 25% - 75% range for the females was 8.10-19.45 nmol/mmol creatinine which is higher than the range of the males which was 4.67-11.48 nmol/mmol creatinine. No statistical difference was observed in the TBARS levels between males and females (Figure 5.2, p-value = 0.707). The medians for the females and males were 0.21 $\mu\text{mol}/\text{mmol}$ creatinine and 0.18 $\mu\text{mol}/\text{mmol}$ creatinine, respectively. The 25% - 75% range for the females were 0.10-0.30 $\mu\text{mol}/\text{mmol}$ creatinine and for the men 0.11-0.29 $\mu\text{mol}/\text{mmol}$ creatinine.

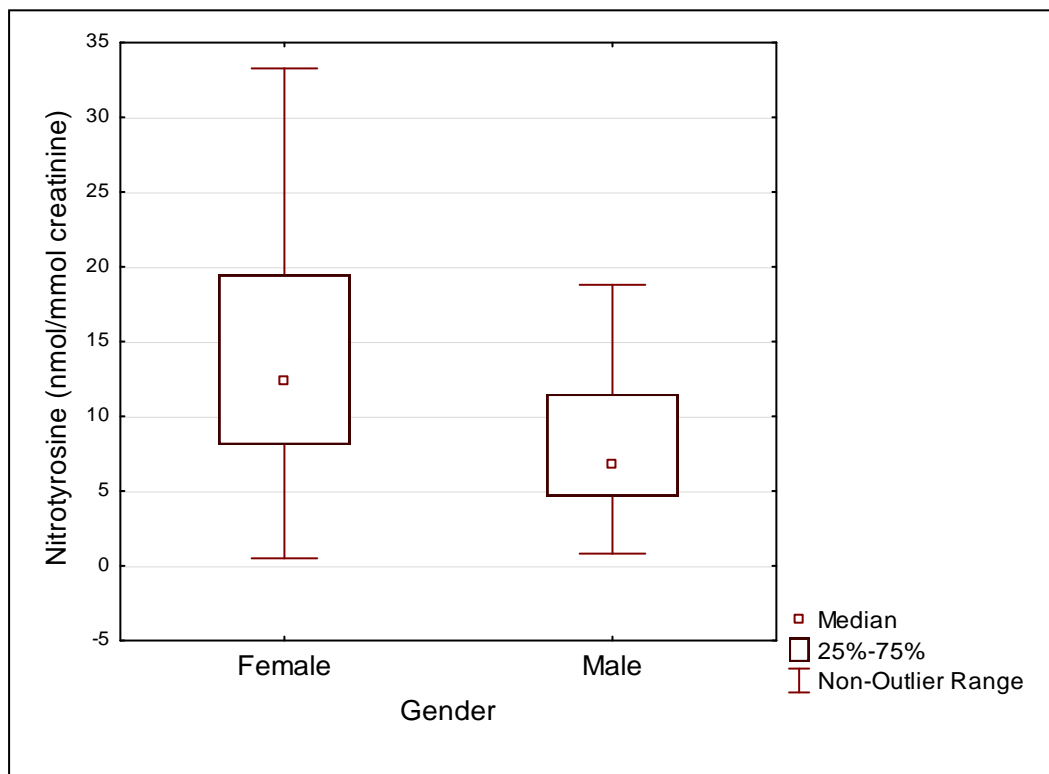


Figure 5.1 Box plots of the 3NT levels in males and females. The figure illustrates the difference in 3NT levels in males compared to female subjects. Statistical difference was indicated by a p-value < 0.05 .

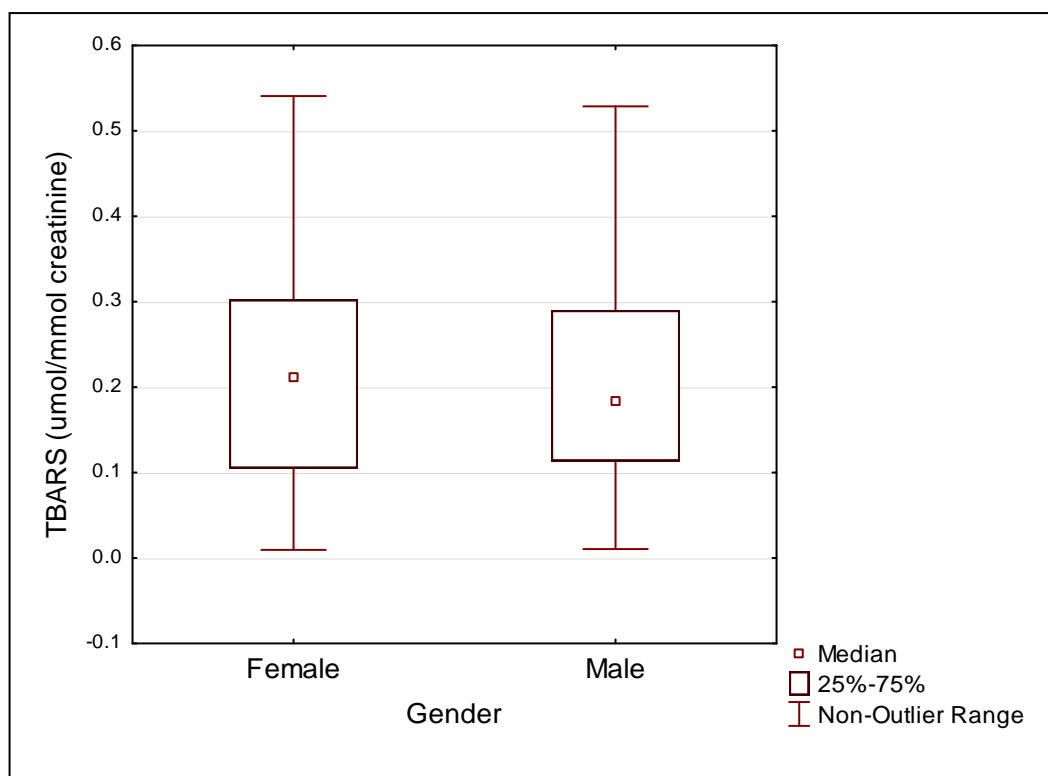


Figure 5.2 Box plots of the TBARS levels in males and females. This figure illustrates no difference in TBARS levels in males compared to female subjects. P-value was 0.70.

From this data, together with the fact that the majority of the SABPA data was divided in male and female groups, it was decided to analyze male and female data separately in this study. Another reason for this division is the effect of oxidative stress between genders. Although Sartori-Valinotti *et al.*, (2007), reported that neither human nor animal studies have definitely proven that there is a difference in oxidative stress levels between males and females, previous studies reported the opposite (Ide *et al.*, 2002; Collins *et al.*, 1998; Nielsen *et al.*, 1997). Therefore, dividing the data set into male and female will eliminate the possible influence that different oxidative stress levels in gender groups might have on the statistical analyses. The male and female groups were then further divided into normotensive and hypertensive subjects.

5.4 URINARY TBARS AND 3NT LEVELS IN HYPERTENSION

5.4.1 Introduction

Non-parametric statistics was used to determine if some of the other variables in the data set had a statistically significant influence on the TBARS and 3NT levels in the male and female subjects, respectively. In statistics, variables can be divided into

categorical and continuous variables. Categorical variables are variables that can be sub-divided into one or more sub group but has nointrinsic ordering among the categories. The categorical variables in this study include HIV, self report smoke and alcohol intake. Continuous variables are variables that, within the limit of a range, can have any number possible. The continuous variables in this study includes 3NT, TBARS, age, physical activity (PAL), gamma-glutamyl transferase (GGT), progesterone, estradiol, body mass index (BMI), C-reactive protein (CRP) and blood glucose levels. Each category had a different non-parametric test. For the categorical variables, the Mann Whitney U test was used and for the continuous variables, the Spearman’s correlation coefficient was used.

5.4.2 The influence of categorical variables on urinary TBARS and 3NT levels

As mentioned in Section 5.3 the Mann Whitney U test is the non-parametric version of the Student’s T test that was used for analyzing the categorical variables for an indication of whether any of the categorical variables had an effect on either the TBARS or 3NT levels. As indicated by Table 5.2 and 5.3, no statistical differences were observed in either of the categorical variables on TBARS or 3NT level in both male and female subjects. The only comparison that came close to a difference was self-report alcohol intake in females on 3NT level, which had a p-value of 0.06. Since 95% confidence level was used in this study, the alcohol intake in females was not regarded as statistically significant.

Table 5.2 Influence of categorical variables on urinary TBARS and 3NT levels in male subjects

Categorical variable	Males			
	Valid N Yes/Positive	Valid N No/Negative	TBARS p Value	3NT p Value
Self report alcohol	52	26	0.44	0.47
Self report smoke	20	60	0.32	0.49
HIV	14	75	0.17	0.20

This table shows the Mann Whitney U results after comparing the categorical variables with TBARS as well 3NT in the male subjects. No statistical differences were determined.

Table 5.3 Influence of categorical variables on urinary TBARS and 3NT levels in female subjects

	Females			
			TBARS	3NT
Categorical variable	Valid N Yes/positive	Valid N No/Negative	p Value	p Value
Self report alcohol	21	55	0.37	0.06
Self report smoke	5	72	0.50	0.21
HIV	7	75	0.84	0.42

This table shows the Mann Whitney U results after comparing the categorical variables with TBARS as well 3NT in the female subjects. No statistical differences were determined.

5.4.3 The influence of continuous variables on urinary TBARS and 3NT levels

5.4.3.1 Introduction

For continuous variables, the Spearman's correlation coefficient was calculated for both the TBARS and 3NT, in the male as well as in the female subjects. The Spearman's correlation coefficient is the non-parametric version of the Pearson correlation coefficient and is an indication if a good correlation exists between the variables that are being compared. The correlation is indicated by a value ranging from -1 to 1, with -1 being a negative correlation, 0 no correlation and 1 a positive correlation (Crawford, 2006).

5.4.3.2 Correlation between TBARS and/or 3NT and continuous variables in males

The Spearman's correlation coefficient results between TBARS/3NT and continuous variables are shown in Table 5.4. Highlighted in red, are the variables that correlate statistically significant with either TBARS or 3NT. In the male subjects, 3NT positively correlated with TBARS as well as PAL and negatively with CRP. TBARS also correlated negatively with BMI. These correlations had a p-value less than 0.05 which was <0.01, 0.01, 0.03 and <0.01, respectively as well as statistically significant Spearman's correlation coefficients of 0.33, 0.27, -0.23 and -0.31, respectively. No correlation existed between either 3NT or TBARS and the other continuous variables (age, GGT, progesterone, estradiol and glucose) as there Spearman and p-values were not statistically significant.

Table 5.4 Correlation of 3NT and TBARS with the continuous variables in male subjects

Variables	Valid N	TBARS		Valid N	3NT	
		Spearman R	p value		Spearman R	p value
TBARS				88	0.33	<0.01
Age	89	-0.02	0.83	88	-0.11	0.29
Physical activity level	86	0.11	0.30	85	0.27	0.01
GGT	85	0.11	0.30	84	0.02	0.83
Progesterone (pmol/L)	88	0.19	0.07	87	0.09	0.37
Estradiol (pmol/L)	88	-0.07	0.47	87	-0.01	0.92
BMI	89	-0.31	<0.01	88	-0.09	0.38
CRP	85	0.06	0.53	84	-0.23	0.03
Glucose	89	0.03	0.78	88	-0.17	0.09

This table shows the Spearman's correlation coefficient results after comparing the continuous variables with TBARS as well 3NT in the male subjects. TBARS and 3NT correlated. In addition 3NT also correlated with PAL and CRP, while TBARS correlated with BMI.

5.4.3.3 Correlation between TBARS/3NT and continuous variables in females

Just as seen in the males, a positive correlation exists between TBARS and 3NT in the female subjects, as indicated by Table 5.5. A correlation also exists between 3NT and BMI (negative correlation), as well as between TBARS and PAL (positive correlation). These correlations were indicated by p-values of 0.01, 0.04 and 0.04, respectively ($p < 0.05$) and Spearman's correlation coefficients of 0.26, -0.22 and 0.22, respectively.

Table 5.5 Correlation of 3NT and TBARS with the continuous variables in the female subjects

Variables	Valid N	TBARS		Valid N	3NT	
		Spearman R	p value		Spearman R	p value
TBARS	83				0.26	0.01
Age	83	0.16	0.14	83	0.05	0.65
Physical activity level	82	0.22	0.04	82	0.13	0.24
GGT	78	-0.12	0.27	78	-0.06	0.54
Progesterone (pmol/L)	80	-0.06	0.56	80	-0.08	0.45
Estradiol (pmol/L)	80	0.05	0.63	80	0.03	0.79
BMI	83	-0.01	0.91	83	-0.22	0.04
CRP	77	-0.14	0.20	77	-0.04	0.67
Glucose	81	-0.13	0.21	81	-0.19	0.08

This table shows the Spearman's correlation coefficient results after comparing the continuous variables with TBARS as well 3NT in the female subjects TBARS and 3NT correlated. In addition 3NT also correlated with BMI, while TBARS correlated with PAL.

From the statistics, it was determined that in females, 3NT levels correlates with TBARS levels as well as with BMI. On the other hand, TBARS correlates with PAL. In males the correlation between 3NT and TBARS was also observed along with 3NT's correlation with CRP and PAL and a correlation between TBARS and BMI. To determine the relationship of only the TBARS and 3NT on hypertension, in males and females, without the influence of other confounding variables, the next step was to do analyses of covariance, also known as ANCOVA.

5.5 EFFECT OF ANALYSES OF COVARIANCE ON URINARY TBARS AND 3NT LEVELS

Analysis of covariance (ANCOVA) is a statistical method of analyzing a data set when one or more factors influences the variables that are being compared. In this study, the main aim was to evaluate the levels of 3NT and TBARS in normotensive and hypertensive subjects, both male and female, and to determine whether there is a statistically significant difference between the two groups. As indicated by Figure 5.3, after ANCOVA adjusted for TBARS, CRP and PAL, no significant difference in the urinary levels of 3NT was observed between hypertensive and normotensive male subjects, which was indicated with a p-value of 0.93. A difference in 3NT levels where indeed observed in the female subjects after ANCOVA adjusted for TBARS and BMI. As illustrated by Figure 5.4 the normotensive female subjects had a higher mean 3NT of 15.85nmol/mmol creatinine compared to their hypertensive counterparts, which had a mean of 12.35 nmol/mmol creatinine.

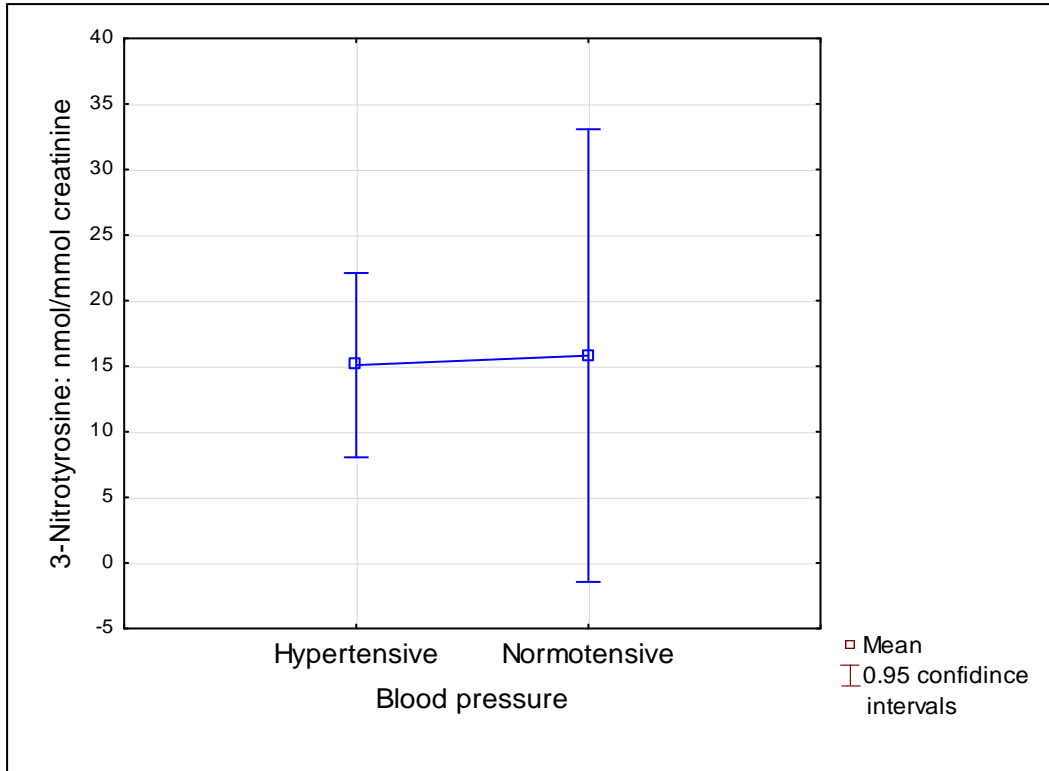


Figure 5.3 ANCOVA for 3NT levels in male subjects. As indicated by the figure, no difference in the 3NT levels existed between the normotensive and hypertensive male subjects.

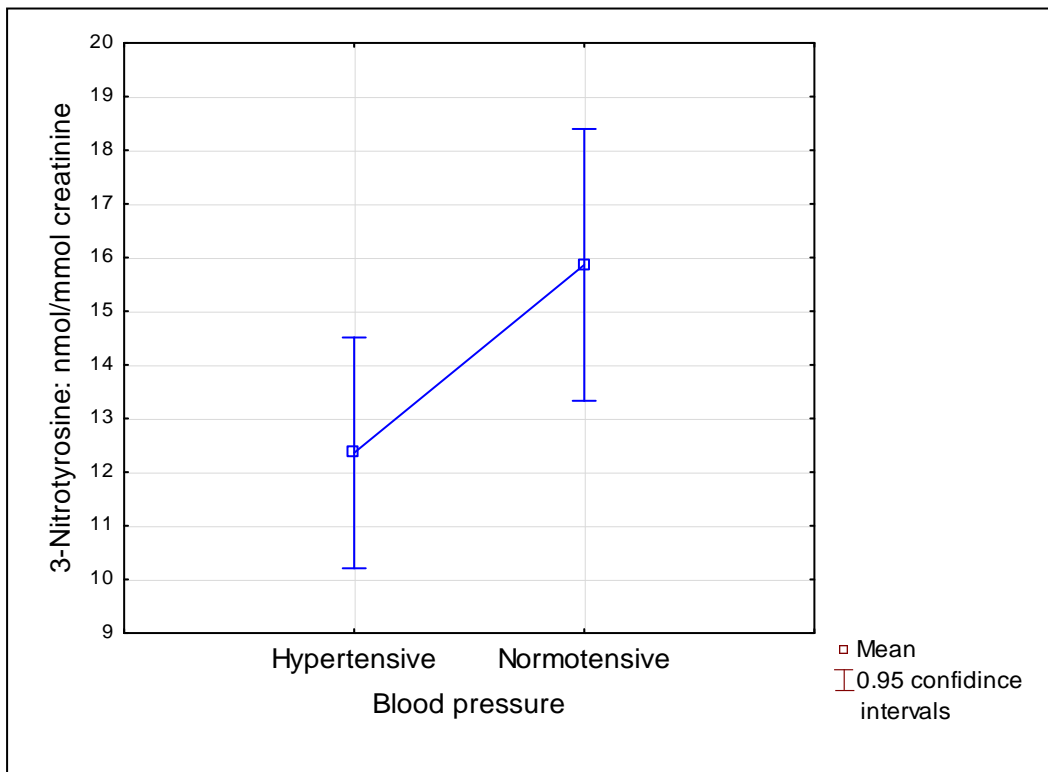


Figure 5.4 ANCOVA for 3NT levels in female subjects. As indicated by the figure a difference in the 3NT levels existed between the normotensive and hypertensive female subjects. Statistical difference was indicated with a p-value of 0.04.

From the ANCOVA analyses of the TBARS levels, there is no statistically significant difference between the normotensive and hypertensive groups in the female subjects, after ANCOVA adjusted for PAL, as indicated by the p-value of 0.31 in Figure 5.5. A difference was observed in the TBARS levels between normotensive and hypertensive male subjects, as illustrated in Figure 5.6. The hypertensive male subjects had a higher mean of 0.26 $\mu\text{mol}/\text{mmol}$ creatinine, after the ANCOVA analyses compensated for BMI, compared to the mean of the normotensive subjects of 0.14 $\mu\text{mol}/\text{mmol}$ creatinine. This statistical difference was indicated with a p-value of 0.03.

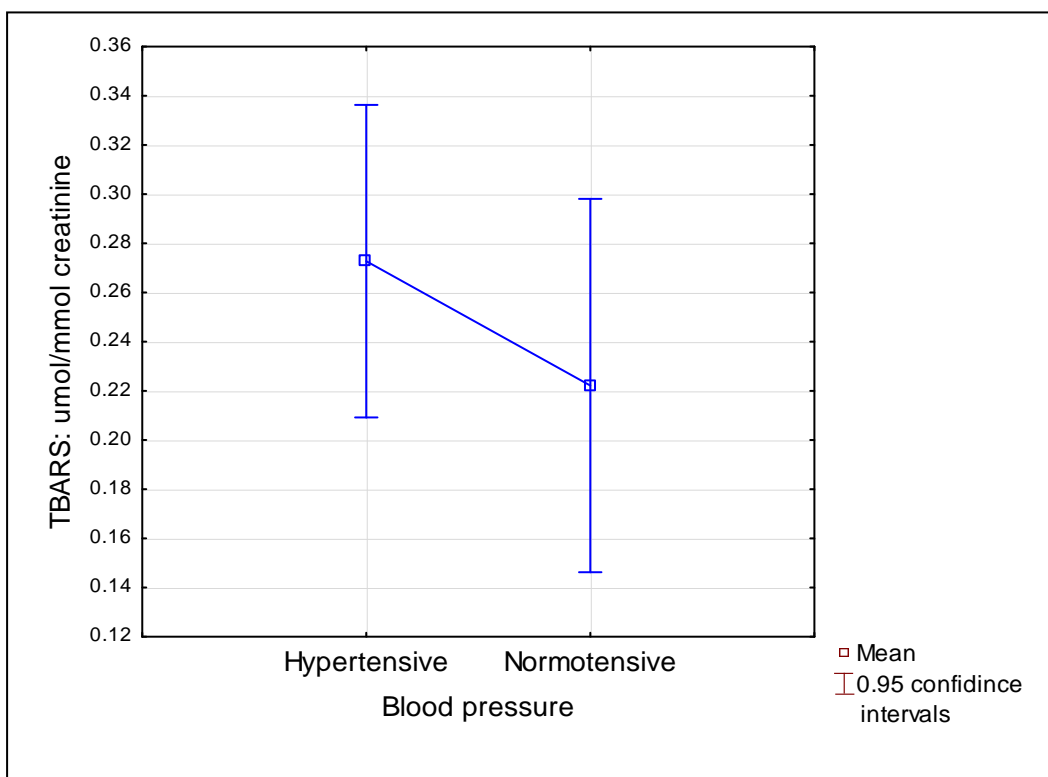


Figure 5.5 ANCOVA for TBARS levels in female subjects. As indicated by the figure no difference in the TBARS levels existed between the normotensive and hypertensive female subjects indicated by a p-value of 0.31.

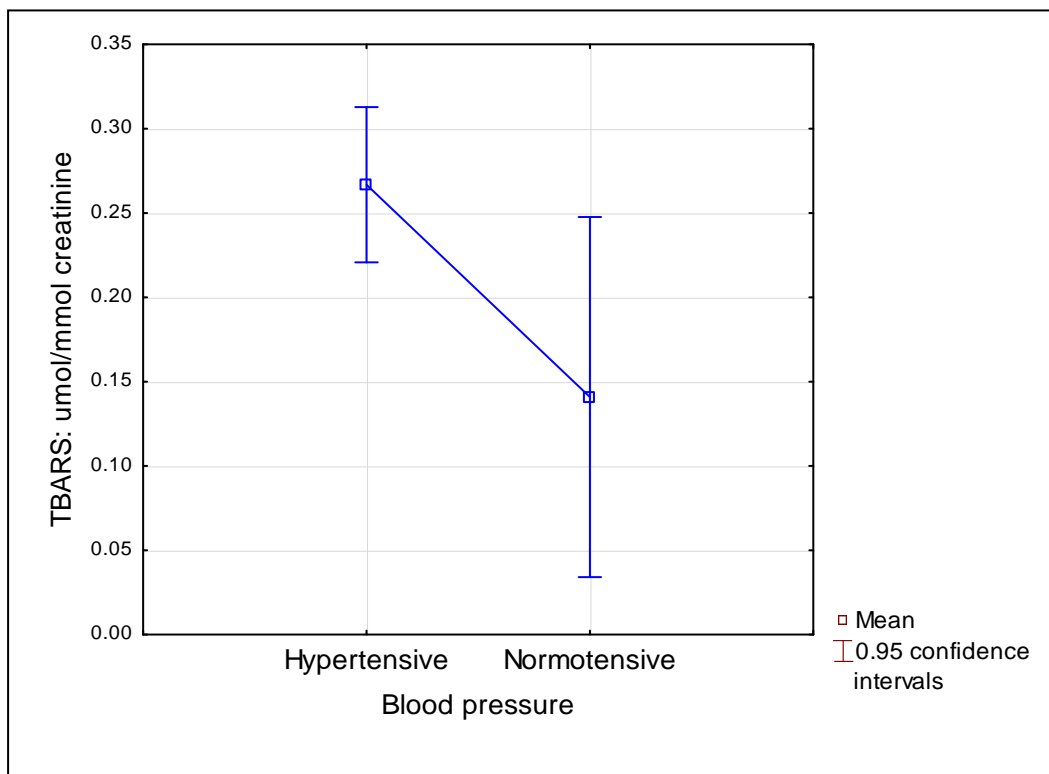


Figure 5.6 ANCOVA for TBARS levels in male subjects. As indicated by the figure a difference in the TBARS levels existed between the normotensive and hypertensive male subjects, and is indicated by a p-value of 0.03.

5.6 DISCUSSION

In conclusion, the Mann Whitney U test indicated that a difference existed between the 3NT levels of males and females and therefore it was decided to analyze the genders separately, as was done throughout SABPA study. These two groups were then sub-divided into normotensive and hypertensive subjects. The Shapiro Wilk's W test indicated that the data sets from both the males and females were not distributed normally. Due to the skewed data, non-parametric analyses were therefore used. The variables of the data set were divided into two groups categorical (HIV, self report smoke and alcohol intake) and continuous (age, PAL, GGT, CRP, BMI and glucose). For the categorical variables, the Mann Whitney U test was used and for continuous variables, the Spearman's correlation coefficient was used. These statistical methods calculated that in both males and females, TBARS and 3NT correlated. In males, 3NT also correlated with PAL and CRP, while TBARS also correlated with BMI. In females 3NT correlated with BMI, while TBARS correlates with PAL. These correlations meant that they could influence the

calculations of the true effect of 3NT and TBARS levels between normotensive and hypertensive subjects. To eliminate these influences, the ANCOVA statistical method was used. ANCOVA determined that the 3NT levels differed in females, with normotensive subjects having higher 3NT levels than the hypertensive female subjects. No difference was observed in the male subjects. ANCOVA also determined that the TBARS levels were higher in the hypertensive male subjects, but no difference was observed in the females.

When comparing the urinary TBARS levels between the hypertensive and normotensive male subjects, the hypertensive subjects had significantly higher TBARS levels than the normotensive subjects ($p < 0.05$). This was expected and could be ascribed to the fact that 74 of the male subjects were hypertensive and only 14 were normotensive. This is consistent with literature where elevated serum TBARS levels were found in hypertensive subjects compared to the controls (Sarkar *et al.*, 2011; Kamel *et al.*, 2005). A negative correlation existed between BMI and TBARS (Spearman's R-value = -0.317) for the male subjects. This is in contrast to literature where obesity is associated with elevated levels of lipid peroxidation (Vincent and Taylor, 2006). Another factor that was not measured but might contribute to the difference in TBARS levels is visceral fat accumulation (Palmieri *et al.*, 2006). A study done on 1,178 middle-aged Japanese men showed that increased visceral fat accumulation should rather be associated with circulating TBARS than the degree of obesity (Okauchi *et al.*, 2011). Although the glucose levels did not correlate with TBARS, the hypertensive males had a slightly higher glucose median of 5.40 mmol/L compared to the median of 5.20 mmol/L for the normotensive male subjects. Of the 74 hypertensive male subjects 36 had blood glucose levels of above 5.60 mmol/L. Although these values are border line for (IFG/IGT), literature indicated that men which had higher glucose levels had higher TBARS values (Menon *et al.*, 2004). It is therefore possible that this slightly higher glucose levels might have contributed to the increased TBARS levels in the hypertensive male subjects.

When comparing the urinary 3NT level in females the normotensive female subjects had significantly higher 3NT levels than the hypertensive female subject, which was not expected. This is inconsistent with literature that gives an indication of an

association of increased 3NT with hypertension (Zhou *et al.*, 2006; Turko and Murad, 2002; Moriel *et al.*, 2002; Roggensack *et al.*, 1999). As with the TBARS levels in males, there existed a negative correlation between the 3NT levels and BMI (Spearman 's R-value= -0.22). Again, this is in contrast with literature where obesity is associated with increased levels of protein oxidation (Dobrian *et al.*, 2001; Roberts *et al.*, 2000). Although the PAL did not have a statistical significant influence (p-value = 0.24) on the 3NT levels, the normotensive females had a slightly lower physical activity level median of 2 kcal/h compared to the median of 2.13 kcal/h for the hypertensive female subjects. This slightly lower physical activity level might contribute to the elevated 3NT levels in the normotensive subjects. This is consistent with a study where the effect of cardiorespiratory fitness on vascular regulation and oxidative stress in postmenopausal women were studied. They determined that the sedentary subjects had higher 3NT levels of 163 nmol/L compared to the exercising group whose 3NT levels were 157 nmol/L (Pialoux *et al.*, 2012). The higher levels of 3NT and TBARS in the normotensive female subjects might indicate that the normotensive group had higher oxidative stress than the hypertension group, which is also in contrast with literature (Grossman, 2008; Rodrigo *et al.*, 2007). The correlation between the TBARS and the 3NT levels, in both the male and female subjects (Spearman's R-values of 0.33 and 0.26, respectively) were not predicted as these markers are formed through different kinds of ROS. TBARS is formed through $\cdot\text{OH}$ and 3NT is formed through ONOO^- (Sections 2.6 and 2.7). This correlation is consistent with literature where increased levels of both TBARS and 3NT are associated with condition of oxidative stress (Caramori and Papi, 2012; Atukeren *et al.*, 2010; Alvarez *et al.*, 2008; White *et al.*, 2006).

In literature, average 3NT levels are reported to be 1.4 nmol/mmol creatinine, measured in healthy Japanese (Kato *et al.*, 2009) and 0.4 nmol/mmol creatinine in healthy Germans (Pham *et al.*, 2009). 3NT values in this study were determined to be 14.69 nmol/mmol creatinine. These values are between 10-40 times higher than reported in literature. A possible reason for this difference can be ascribed to different population that are being used, as values for this study are for black South Africans. A second possible reason is that although it was possible to separate the co-eluting compound from 3NT, the possibility exist that peak 2, which corresponded with 3NT, still contains other interfering compounds that were not separated from

peak 2, and therefore the values are higher than reported in literature (Tsikas *et al.*, 2005). Other reasons can be due to different methods used or artificial formation of 3NT that could have taken place during any of the preparation steps (Ogino and Wang *et al.*, 2007).

In literature average TBARS levels are reported to be 0.42 $\mu\text{mol}/\text{mmol}$ creatinine, measured in Nepalese (Jha *et al.*, 2007) and 7.71 $\mu\text{mol}/\text{mmol}$ creatinine in Taiwanese (Huang *et al.*, 2003). TBARS values in this study were determined to be 0.22 $\mu\text{mol}/\text{mmol}$ creatinine. These values are between 2-35 times lower than reported in literature. Again, this difference can be ascribed to the possible influence of different population on the TBARS levels. Other possible reasons for the difference between the TBARS values of the study and levels reported in literature might also be due to the different methodologies that were used for measurement of TBARS (Kamel *et al.*, 2005).

Although the values for both TBARS and 3NT, determined in this study, differed from values reported in literature, it will not influence data interpretation and the outcome of this study as the aim of this study was to investigate the involvement of TBARS and 3NT in hypertension. Thus, the test subjects were divided into hypertensive and normotensive. Since the TBARS and 3NT levels in the hypertensive group is only compared relative to the TBARS and 3NT levels of the normotensive group, relative quantification of these markers is sufficient to reach the aim of this study. Although the measured markers did not correspond to levels reported in literature, the aim set for this study could still be reached.

Chapter 6

6

Conclusions

6.1 INTRODUCTION

Results from the SABPA study showed that black South Africans had a higher prevalence for hypertension than their Caucasian counterparts did. This created the need to investigate the association between oxidative stress and hypertension in black South Africans. This Masters study formed part of the SABPA study and had a main aim of using optimized spectrophotometric as well as LC/MS/MS methods to evaluate the concentrations of specific lipid and protein oxidation markers in urine samples from black South African subjects of the SABPA study. To reach this aim, the following objectives had to be reached: firstly to optimize a spectrophotometric method to quantify lipid oxidation markers (TBARS assay). The second objective was to optimize an LC-MS/MS method to quantify 3NT. The third objective was to quantify 3NT and TBARS in a cohort of African test subjects. The final objective was to statistically investigate the potential involvement of the measured lipid and protein oxidation markers in hypertension in this cohort. A hypothesis was formulated that the hypertensive subjects would present with higher levels of lipid and protein oxidation markers compared to their normotensive counterparts. The hypothesis was investigated through the strategy outlined in Section 2.10.

6.2 OPTIMIZATION OF TBARS AND 3-NITROTYROSINE ASSAYS

Before quantification of urinary TBARS, a 96-well spectrophotometric method had to be optimized and validated. After several optimization steps, to determine the ideal method conditions for optimal performance in our laboratory, an optimized urinary TBARS 96-well spectrophotometric method was developed. The optimized TBARS method had an intra CV of 10.52% and a inter CV of 11.64%. This indicated that the optimized 96-well spectrophotometric method was successful and reliable for quantification of urinary TBARS and could be used in quantification of TBARS in the SABPA subjects.

For quantification of the urinary 3NT in the SABPA subjects, a LC-MS/MS method first had to be optimized. Unfortunately, during optimization certain challenges were encountered. When analyzing control urine samples, co-elution of 3NT and an unknown compound occurred. The co-elution with 3NT could cause altered ionization of 3NT and consequently lead to inaccurate determination of the LOD, LOQ, linearity and precision. For this reasons it was of great importance to attempt the separation of the two peaks present, determine which peak corresponded with 3NT and try to identify the unknown compound represented by the remaining peak. Separation of the co-eluting compound from 3NT were achieved through chiral derivatization, by substituting n-butanol:acetyl chloride with R-(-2)-butanol:acetyl chloride. Since the co-eluting compound had the same precursor and product ion combination and nearly the same retention time as 3NT, it was hypothesized that the co-eluting compound could be a stereoisomer of 3NT, most probably 3-nitro-D-tyrosine. Other possibilities included nitro-*m*-tyrosine and nitro-*o*-tyrosine. These stereoisomers of 3NT, were not commercially available and had to be synthesized. After analysis of these synthesized stereoisomers, it was concluded that the co-eluting peak was not 3-nitro-D-tyrosine, nitro-*m*-tyrosine or nitro-*o*-tyrosine as the retention time of the co-eluting peak did not correspond with the retention times of either of the stereoisomers. It was also determined that although it is possible to separate enantiomers it was not possible using the selected method as the synthesized 3-nitro-DL-tyrosine only presented with one peak instead of a double peak. With all the possible stereoisomer ruled out, the next step was to determine, whether a difference existed between the molecular masses of the co-eluting peak and 3NT. This was done by making use of a Q-TOF LC-MS. After the sample was concentrated, by using TLC plates, it was analyzed on the Q-TOF LC-MS and it was determined that the co-eluting peak had a molecular mass of 283.16 m/z compared to the molecular mass of 3NT which was 283.13 m/z. The concentration of 3NT in the concentrated sample was however very close to the detection limit of the Q-TOF LC-MS to say with confidence that peak 1 had a different mass from peak 2.

Due to time constraints within this study, it was decided to cease attempts at identifying the co-eluting peak, and rather to do quantification by using the peak that corresponded with 3NT. Initially it was decided to use phenylalanine isotope (L-phenyl-d5-alanine) as an internal standard for quantification of 3NT, but difficulty with

integration of the internal standard peaks, resulted in the decision to use a calibration curve instead for quantification of 3NT. To validate the optimized assay the linearity of the calibration curve was determined, which had a R^2 value of > 0.99 . It was therefore concluded that the optimized 3-nitrotyrosine assay could be used for quantification of 3NT in the urine of the SABPA subjects.

After quantifying TBARS and 3NT in the SABPA subjects it was determined that the TBARS values were 2-10 times lower and that the 3NT levels were 10-40 times higher than reported in literature. During this study the trend of TBARS as well as 3NT levels were investigated in hypertension. Therefore, relative quantification is sufficient and there is no need for absolute quantification. Possible reasons for the difference between the 3NT and TBARS values reported in literature and values determined in the SABPA subjects, might be the following: the first possible reason might be the difference in populations between the literature and this study's population (black South Africans). A second possible reason can be ascribed to artificial formation during any of the preparation steps. A third reason might be due to different methods that were used for measurement.

6.3 THE INVOLVEMENT OF LIPID AND PROTEIN OXIDATION MARKERS IN HYPERTENSION

Since literature suggests a possible gender effect on oxidative stress, it was decided to separate the data set into male and female groups. Each of these groups was again divided into normotensive and hypertensive sub-groups. Consequently, the data set consisted of hypertensive and normotensive male subjects as well hypertensive and normotensive female subjects. Statistical analyses of the data set showed that there is a significant difference in oxidation markers in both the male and female subjects. The male hypertensive subjects had significantly increased TBARS values compared to the normotensive male subjects (p -value = 0.03), while a significant increase in 3NT levels were observed in the normotensive female subjects compared to the hypertensive female subjects (p -value = 0.04). The increased TBARS values are consistent with literature where higher TBARS values are associated with hypertension. However, the increased 3NT levels in the female subjects are contrary to that reported in literature where increased 3NT levels are associated with hypertension, rather than normotensivity. A correlation also existed

in both the male and female subjects between TBARS and 3NT levels (Spearman's R-values of 0.33 and 0.26, respectively). Although this correlation was unexpected, it is consistent with literature where increased 3NT and TBARS are associated with oxidative stress conditions.

Oxidation of bio-molecules (lipids, proteins and DNA) might contribute to aging as well as pathologies such as cardiovascular diseases, neurodegenerative disorders and cancer. It was therefore hypothesized that there would be an increase in both the lipid and protein oxidation markers in the hypertensive male and female subjects. Although this was partially true in the hypertensive male subjects, the opposite was observed in the female subjects where the normotensive subject showed significantly higher 3NT values. The increased TBARS in the hypertensive male subjects could be ascribed to the fact that more than half of the hypertensive subjects had blood glucose levels of 5.60 mmol/L and above. This is consistent with literature where high blood glucose levels are associated with high TBARS levels. In the hypertensive males, TBARS had a negative correlation with BMI, which is contrary to literature reports. The increased 3NT levels were unexpected as it was hypothesized that the hypertensive female subjects would have higher oxidation markers. From the data, no possible reason could be given why the hypertensive female subjects had lower 3NT values than the normotensive female subjects as all the variables indicated that there should be an increase in the hypertensive subjects. Although there was no statistical difference in the TBARS values in the female subjects, the normotensive female subjects also had higher TBARS levels than the hypertensive female subjects. This indicates an overall higher level of oxidative stress in the normotensive female subjects compared to the males subjects where only lipid oxidation was increased. The contradictory findings in the female subjects indicates that the relationship between oxidative stress and hypertension is complex and still not fully understood and that factors that have been thought to be insignificant, might have a greater influence on lipid and protein oxidation than initially expected.

6.4 RECOMMENDATIONS

Currently the TBARS assay is successfully optimized for our laboratory with minimum preparation time, but should still be used in combination with other

oxidation marker method for an indication of oxidative stress. Due to the time limitation to this study, other possible derivatization reagents could not be explored. Other methods such as SPE columns could also be explored to increase the concentration of the sample for analysis on the Q-TOF LC-MS. SPE columns could also help in cleaning the sample, which might eliminate the co-eluting compound.

It would be extremely beneficial if the samples of both phases one and two of the SABPA study was analyzed in terms of lipid and protein oxidation markers. Firstly, this data could give an indication of what the reference values for lipid and protein oxidation markers are for South Africans, as current reference values are from other populations. Further analysis of the data from the two phases could give an indication of whether there exists a difference in lipid and protein oxidation between Africans and Caucasian. It could also give an indication whether there was an increase or decrease in oxidative stress in the subjects between the period of the initial sample collection and the follow up sample collection. Apart from the lipid and protein oxidation markers, it will also be beneficial to analyze the DNA oxidation markers as well as ROS and antioxidant levels. By combining this data with the data of the lipid and protein oxidation markers, a profile could be created which will give more information on the degree of oxidative stress in the subject than just the measurement of two oxidation markers.

6.5 FINAL CONCLUSION

Although the compound that co-eluted with 3NT could not be identified, it was possible to successfully quantify the TBARS as well as 3NT levels in all the black South African SABPA subjects from the second phase. The mean TBARS value was higher in the hypertensive male subject while the 3NT levels were significantly higher in the normotensive female subjects. The aim as well as objectives was reached in this study. The results partially supported the hypothesis in the male subjects where an association existed between hypertension and lipid oxidation but no association with protein oxidation. The hypothesis was totally rejected in the female subjects where protein oxidation was elevated in the normotensive subjects instead of the hypothesized hypertensive female subjects. These results indicate that there are indeed differences in lipid and protein oxidation between hypertensive and

normotensive subjects. However, a better indication of the trend can be obtained if the samples size is increased.

References

Armstrong, D., Duncan, J. & Lee, S. 1991, "Evaluation of D-amino acid levels in human urine and in commercial L-amino acid samples", *Amino acids*, vol. 1, no. 1, pp. 97-106.

Álvarez, M.C., Caldiz, C., Fantinelli, J.C., Garciarena, C.D., Console, G.M., De Cingolani, G.E.C. & Mosca, S.M. 2008, "Is Cardiac Hypertrophy in Spontaneously Hypertensive Rats the Cause or the Consequence of Oxidative Stress&quest", *Hypertension Research*, vol. 31, no. 7, pp. 1465-1476.

Atukeren, P., Yavuz, B., Soydinc, H.O., Purisa, S., Camlica, H., Gumustas, M.K. & Balcioglu, I. 2010, "Variations in systemic biomarkers of oxidative/nitrosative stress and DNA damage before and during the consequent two cycles of chemotherapy in breast cancer patients", *Clinical chemistry and laboratory medicine*, vol. 48, no. 10, pp. 1487-1495.

Augusto, O., Trindade, D.F., Linares, E. & Vaz, S.M. 2008, "Cyclic nitroxides inhibit the toxicity of nitric oxide-derived oxidants: mechanisms and implications", *Anais da Academia Brasileira de Ciências*, vol. 80, no. 1, pp. 179-189.

Berlett, B.S. & Stadtman, E.R. 1997, "Protein oxidation in aging, disease, and oxidative stress", *Journal of Biological Chemistry*, vol. 272, no. 33, pp. 20313-20316

Bian, K., Davis, K., Kuret, J., Binder, L. & Murad, F. 1999, "Nitrotyrosine formation with endotoxin-induced kidney injury detected by immunohistochemistry", *American Journal of Physiology-Renal Physiology*, vol. 277, no. 1, pp. F33-F40.

Bochkov, V.N., Oskolkova, O.V., Birukov, K.G., Levonen, A.L., Binder, C.J. & Stöckl, J. 2010, "Generation and biological activities of oxidized phospholipids", *Antioxidants & redox signaling*, vol. 12, no. 8, pp. 1009.

Buege, J.A. and Aust, A.D. 1978. Microsomal lipid peroxidation. *Methods in Enzymology*, vol. 52, pp. 302-310.

Buonocore, G., Perrone, S. & Tataranno, M.L. 2010, "Oxygen toxicity: chemistry and biology of reactive oxygen species", *Seminars in Fetal and Neonatal Medicine* Elsevier, pp. 186.

Caramori, G. & Papi, A. 2004, "Oxidants and asthma", *Thorax*, vol. 59, no. 2, pp. 170-173.

Carr, A.C., McCall, M.R. & Frei, B. 2000, "Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection", *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 7, pp. 1716-1723.

Chen, H.J.C. & Chiu, W.L. 2008, "Simultaneous detection and quantification of 3-nitrotyrosine and 3-bromotyrosine in human urine by stable isotope dilution liquid chromatography tandem mass spectrometry", *Toxicology letters*, vol. 181, no. 1, pp. 31-39.

Chobanian, A.V., Bakris, G.L., Black, H.R., Cushman, W.C., Green, L.A., Izzo, J.L., Jones, D.W., Materson, B.J., Oparil, S. & Wright, J.T. 2003, "Seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure", *Hypertension*, vol. 42, no. 6, pp. 1206-1252.

Collins, A.R., Gedik, C.M., Olmedilla, B., Southon, S. & Bellizzi, M. 1998, "Oxidative DNA damage measured in human lymphocytes: large differences between sexes and between countries, and correlations with heart disease mortality rates", *The FASEB journal*, vol. 12, no. 13, pp. 1397-1400.

Crawford, S.L. 2006, "Correlation and regression", *Circulation*, vol. 114, no. 19, pp. 2083-2088.

Dalle-Donne, I., Giustarini, D., Colombo, R., Rossi, R. & Milzani, A. 2003, "Protein carbonylation in human diseases", *Trends in molecular medicine*, vol. 9, no. 4, pp. 169-176.

D'Autréaux, B. & Toledano, M.B. 2007, "ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis", *Nature Reviews Molecular Cell Biology*, vol. 8, no. 10, pp. 813-824.

Davankov, V.A. 1997, "Analytical chiral separation methods", *Pure and Applied Chemistry*, vol. 69, pp. 1469-1474.

Dean, R., Fu, S., Stocker, R. & Davies, M.J. 1997, "Biochemistry and pathology of radical-mediated protein oxidation.", *Biochemical Journal*, vol. 324, no. Pt 1, pp. 1.

Denicola, A. & Radi, R. 2005, "Peroxynitrite and drug-dependent toxicity", *Toxicology*, vol. 208, no. 2, pp. 273-288.

Dix, T.A. & Aikens, J. 1993, "Mechanisms and biological relevance of lipid peroxidation initiation", *Chemical research in toxicology*, vol. 6, no. 1, pp. 2-18.

Dobrian, A.D., Davies, M.J., Schriver, S.D., Lauterio, T.J. & Prewitt, R.L. 2001, "Oxidative stress in a rat model of obesity-induced hypertension", *Hypertension*, vol. 37, no. 2, pp. 554-560.

Esterbauer, H. & Cheeseman, K.H. 1990, "[42] Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal", *Methods in enzymology*, vol. 186, pp. 407-421.

Galley, H. 2011, "Oxidative stress and mitochondrial dysfunction in sepsis", *British journal of anaesthesia*, vol. 107, no. 1, pp. 57.

Genuth, S., Alberti, K.G., Bennett, P., Buse, J., Defronzo, R., Kahn, R., Kitzmiller, J., Knowler, W.C., Lebovitz, H., Lernmark, A., Nathan, D., Palmer, J., Rizza, R., Saudek, C., Shaw, J., Steffes, M., Stern, M., Tuomilehto, J., Zimmet, P. & Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003, "Follow-up report on the diagnosis of diabetes mellitus", *Diabetes care*, vol. 26, no. 11, pp. 3160-3167.

Giles, T.D., Materson, B.J., Cohn, J.N. & Kostis, J.B. 2009, "Definition and classification of hypertension: an update", *The Journal of clinical hypertension*, vol. 11, no. 11, pp. 611-614.

Grossman, E. 2008, "Does increased oxidative stress cause hypertension?", *Diabetes care*, vol. 31, no. Supplement 2, pp. S185-S189.

Grotto, D., Maria, L.S., Valentini, J., Paniz, C., Schmitt, G., Garcia, S.C., Pomblum, V.J., Rocha, J.B.T. & Farina, M. 2009, "Importance of the lipid peroxidation biomarkers and methodological aspects for malondialdehyde quantification", *Química Nova*, vol. 32, no. 1, pp. 169-174.

Guha, I.N. & Moore, K. 2003, "F2-isoprostanes and the liver", *Prostaglandins & other lipid mediators*, vol. 72, no. 1-2, pp. 73-84.

Guichardant, M., Chantegrel, B., Deshayes, C., Doutheau, A., Moliere, P. & Lagarde, M. 2004, "Specific markers of lipid peroxidation issued from n-3 and n-6 fatty acids", *Biochemical Society transactions*, vol. 32, no. 1, pp. 139-140.

Gutteridge, J. 1995, "Lipid peroxidation and antioxidants as biomarkers of tissue damage", *Clinical chemistry*, vol. 41, no. 12, pp. 1819.

Guyton, A.C. and Hall, J.E., 2006. *Textbook of Medical Physiology*. (11th ed). Elsevier Saunders. pp. 220.

Hamer, M., Malan, L., Schutte, A., Huisman, H., van Rooyen, J., Schutte, R., Fourie, C., Malan, N. & Seedat, Y. 2011, "Conventional and behavioral risk factors explain differences in sub-clinical vascular disease between black and Caucasian South Africans: The SABPA study", *Atherosclerosis*, vol. 215, no. 1, pp. 237-242.

Hart, A. 2001, "Mann-Whitney test is not just a test of medians: differences in spread can be important", *Bmj*, vol. 323, no. 7309, pp. 391-393.

Huang, H.S., Ma, M.C., Chen, C.F. & Chen, J. 2003, "Lipid peroxidation and its correlations with urinary levels of oxalate, citric acid, and osteopontin in patients with renal calcium oxalate stones", *Urology*, vol. 62, no. 6, pp. 1123-1128.

Hughes, M. & Nicklin, H. 1970, "The chemistry of peroxonitrites. Part II. Copper (II)-catalysed reaction between hydroxylamine and peroxonitrite in alkali", *J.Chem.Soc.A*, pp. 925-928.

Ide, T., Tsutsui, H., Ohashi, N., Hayashidani, S., Suematsu, N., Tsuchihashi, M., Tamai, H. & Takeshita, A. 2002, "Greater oxidative stress in healthy young men compared with premenopausal women", *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 22, no. 3, pp. 438-442.

Jeong, Y.C., Walker, N.J., Burgin, D.E., Kissling, G., Gupta, M., Kupper, L., Birnbaum, L.S. & Swenberg, J.A. 2008, "Accumulation of M1dG DNA adducts after chronic exposure to PCBs, but not from acute exposure to polychlorinated aromatic hydrocarbons", *Free Radical Biology and Medicine*, vol. 45, no. 5, pp. 585-591.

Jessome, L.L. & Volmer, D.A. 2006, "Ion suppression: a major concern in mass spectrometry", *LCGC North America*, vol. 24, no. 5, pp. 498-510.

Jha, J., Maharjan, B., Adhikari, D., Vishwanath, P., Nagamma, T., Azhari, S. & Singh, P. 2007, "Cigarette smoke induced oxidative insult in local population of Pokhara.", *Kathmandu University Medical Journal*, vol. 6, no. 1, pp. 16-22.

Lilika, Z. 2008, "The significance of urinary markers in the evaluation of diabetic nephropathy", *Journal of Medical Biochemistry*, vol. 27, no. 3, pp. 376-382.

Kamel, A.A. 2005, "Evaluation of Serum Levels of Oxidized Low Density Lipoprotein Autoantibodies (Olab) and Soluble Urokinase Plasminogen Activator Receptor (Supar) as Possible Risk Factors in Essential Hypertension", *Journal of the Medical Research Institute*, vol. 26, no. 4, pp 307-315.

Kato, Y., Dozaki, N., Nakamura, T., Kitamoto, N., Yoshida, A., Naito, M., Kitamura, M. & Osawa, T. 2009, "Quantification of modified tyrosines in healthy and diabetic human urine using liquid chromatography/tandem mass spectrometry", *Journal of clinical biochemistry and nutrition*, vol. 44, no. 1, pp. 67.

Kearney, P.M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P.K. & He, J. 2005, "Global burden of hypertension: analysis of worldwide data", *The Lancet*, vol. 365, no. 9455, pp. 217-223.

Kedziora-Kornatowska, K., Czuczejko, J., Pawluk, H., Kornatowski, T., Motyl, J., Szadujkis-Szadurski, L., Szewczyk-Golec, K. & Kedziora, J. 2004, "The markers of oxidative stress and activity of the antioxidant system in the blood of elderly patients with essential arterial hypertension", *Cell Mol Biol Lett*, vol. 9, no. 4A, pp. 635-641.

Kelley, K. 2007, "Sample size planning for the coefficient of variation from the accuracy in parameter estimation approach", *Behavior Research Methods*, vol. 39, no. 4, pp. 755-766.

Klatt, P. & Lamas, S. 2000, "Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress", *European Journal of Biochemistry*, vol. 267, no. 16, pp. 4928-4944.

Klebanoff, S.J. 2005, "Myeloperoxidase: friend and foe", *Journal of leukocyte biology*, vol. 77, no. 5, pp. 598.

Kuiper, H.C., Langsdorf, B.L., Miranda, C.L., Joss, J., Jubert, C., Mata, J.E. & Stevens, J.F. 2010, "Quantitation of mercapturic acid conjugates of 4-hydroxy-2-nonenal and 4-oxo-2-nonenal metabolites in a smoking cessation study", *Free Radical Biology and Medicine*, vol. 48, no. 1, pp. 65-72.

Lavie, L. & Lavie, P. 2009, "Molecular mechanisms of cardiovascular disease in OSAHS: the oxidative stress link", *European Respiratory Journal*, vol. 33, no. 6, pp. 1467-1484.

Le Cam, L. 1986, "The Central Limit Theorem Around 1935", *Statistical Science*, , pp. 78-91.

Lin, C., Kuo, C.Y., Liao, K.S. & Yang, W.B. 2011, "Monosaccharide-NAIM derivatives for D-, L-configurational analysis", *Molecules*, vol. 16, no. 1, pp. 652-664.

Linden, A., Gülden, M., Martin, H.J., Maser, E. & Seibert, H. 2008, "Peroxide-induced cell death and lipid peroxidation in C6 glioma cells", *Toxicology in Vitro*, vol. 22, no. 5, pp. 1371-1376.

Malan, L., Schutte, A.E., Malan, N.T., Wissing, M.P., Vorster, H.H., Steyn, H.S., van Rooyen, J.M. & Huisman, H.W. 2006, "Specific coping strategies of Africans during urbanization: Comparing cardiovascular responses and perception of health data", *Biological psychology*, vol. 72, no. 3, pp. 305-310.

Mearns, B.M. 2012, "Hypertension: Tackling challenges in the diagnosis and management of hypertension in 2012", *Nature Reviews Cardiology*, vol. 9, no. 5, pp. 253-254.

Menon, V., Ram, M., Dorn, J., Armstrong, D., Muti, P., Freudenheim, J., Browne, R., Schunemann, H. & Trevisan, M. 2004, "Oxidative stress and glucose levels in a population-based sample", *Diabetic Medicine*, vol. 21, no. 12, pp. 1346-1352.

Mohiuddin, I., Chai, H., Lin, P.H., Lumsden, A.B., Yao, Q. & Chen, C. 2006, "Nitrotyrosine and chlorotyrosine: clinical significance and biological functions in the vascular system", *Journal of Surgical Research*, vol. 133, no. 2, pp. 143-149.

Moriel, P., Sevanian, A., Ajzen, S., Zanella, M., Plavnik, F., Rubbo, H. & Abdalla, D. 2002, "Nitric oxide, cholesterol oxides and endothelium-dependent vasodilation in plasma of patients with essential hypertension", *Brazilian journal of medical and biological research*, vol. 35, no. 11, pp. 1301-1309.

Nakao, A., Toyoda, Y., Sharma, P., Evans, M. & Guthrie, N. 2010, "Effectiveness of hydrogen rich water on antioxidant status of subjects with potential metabolic syndrome—an open label pilot study", *Journal of clinical biochemistry and nutrition*, vol. 46, no. 2, pp. 140.

Nielsen, F., Mikkelsen, B.B., Nielsen, J.B., Andersen, H.R. & Grandjean, P. 1997, "Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors", *Clinical chemistry*, vol. 43, no. 7, pp. 1209-1214.

O'Brien, E., Asmar, R., Beilin, L., Imai, Y., Mancia, G., Mengden, T., Myers, M., Padfield, P., Palatini, P. & Parati, G. 2005, "Practice guidelines of the European Society of Hypertension for clinic, ambulatory and self blood pressure measurement", *Journal of hypertension*, vol. 23, no. 4, pp. 697.

Ogino, K. & Wang, D.H. 2007, "Biomarkers of oxidative/nitrosative stress: an approach to disease prevention", *Acta Medica Okayama*, vol. 61, no. 4, pp. 181.

Okauchi, Y., Kishida, K., Funahashi, T., Noguchi, M., Ogawa, T., Okita, K., Iwahashi, H., Ohira, T., Imagawa, A. & Nakamura, T. 2011, "Cross-sectional and longitudinal study of association between circulating thiobarbituric acid-reacting substance levels and clinicobiochemical parameters in 1,178 middle-aged Japanese men—the Amagasaki Visceral Fat Study", *Nutrition & Metabolism*, vol. 8, no. 1, pp. 82.

Opie, L.H. & Seedat, Y.K. 2005, "Hypertension in sub-Saharan African populations", *Circulation*, vol. 112, no. 23, pp. 3562-3568.

Otteneder, M.B., Knutson, C.G., Daniels, J.S., Hashim, M., Crews, B.C., Rimmel, R.P., Wang, H., Rizzo, C. & Marnett, L.J. 2006, "In vivo oxidative metabolism of a major peroxidation-derived DNA adduct, M1dG", *Proceedings of the National Academy of Sciences*, vol. 103, no. 17, pp. 6665-6669.

Palmieri, V.O., Grattagliano, I., Portincasa, P. & Palasciano, G. 2006, "Systemic oxidative alterations are associated with visceral adiposity and liver steatosis in patients with metabolic syndrome", *The Journal of nutrition*, vol. 136, no. 12, pp. 3022-3026.

Patel, D. "Matrix effect in a view of LC/MS/MS: an overview ", *International Journal of Pharma and Bio Sciences*, vol. 2, no. 1. pp. 559-564.

Pepe, H., Balci, S.S., Revan, S., Akalin, P.P. & Kurtoglu, F. 2009, "Comparison of oxidative stress and antioxidant capacity before and after running exercises in both sexes", *Gender medicine*, vol. 6, no. 4, pp. 587-595.

Pereira, I.R.O., Bertolami, M.C., Faludi, A.A., Campos, M.F., Ferderbar, S., Lima, E.S., Aldrighi, J.M. & Abdalla, D.S.P. 2003, "Lipid peroxidation and nitric oxide inactivation in postmenopausal women", *Arquivos Brasileiros de Cardiologia*, vol. 80, no. 4, pp. 415-423.

Pham, V.V., Stichtenoth, D.O. & Tsikas, D. 2009, "Nitrite correlates with 3-nitrotyrosine but not with the F₂-isoprostane 15 (S)-8-iso-PGF_{2α} in urine of rheumatic patients", *Nitric Oxide*, vol. 21, no. 3, pp. 210-215.

Pialoux, V., Brown, A.D., Leigh, R., Friedenreich, C.M. & Poulin, M.J. 2009, "Effect of cardiorespiratory fitness on vascular regulation and oxidative stress in postmenopausal women", *Hypertension*, vol. 54, no. 5, pp. 1014-1020.

Podrez, E.A., Abu-Soud, H.M. & Hazen, S.L. 2000, "Myeloperoxidase-generated oxidants and atherosclerosis", *Free Radical Biology and Medicine*, vol. 28, no. 12, pp. 1717-1725.

Poulter, N.R., Khaw, K., Hopwood, B.E., Mugambi, M., Peart, W.S. & Sever, P.S. 1985, "Determinants of blood pressure changes due to urbanization: a longitudinal study", *Journal of hypertension. Supplement: official journal of the International Society of Hypertension*, vol. 3, no. 3, pp. S375-7.

Prabhakar, R., Vreven, T., Morokuma, K. & Musaev, D.G. 2005, "Elucidation of the mechanism of selenoprotein glutathione peroxidase (GPx)-catalyzed hydrogen peroxide reduction by two glutathione molecules: a density functional study", *Biochemistry*, vol. 44, no. 35, pp. 11864-11871.

Radi, R. 2004, "Nitric oxide, oxidants, and protein tyrosine nitration", *Proceedings of the National Academy of Sciences*, vol. 101, no. 12, pp. 4003.

Reed, G.F., Lynn, F. & Meade, B.D. 2002, "Use of coefficient of variation in assessing variability of quantitative assays", *Clinical and diagnostic laboratory immunology*, vol. 9, no. 6, pp. 1235-1239.

Ridnour, L.A., Thomas, D.D., Mancardi, D., Espey, M.G., Miranda, K.M., Paolocci, N., Feelisch, M., Fukuto, J. & Wink, D.A. 2004, "The chemistry of nitrosative stress induced by nitric oxide and reactive nitrogen oxide species. Putting perspective on stressful biological situations", *Biological chemistry*, vol. 385, no. 1, pp. 1-10.

Roberts, C.K., Vaziri, N.D., Wang, X.Q. & Barnard, R.J. 2000, "Enhanced NO inactivation and hypertension induced by a high-fat, refined-carbohydrate diet", *Hypertension*, vol. 36, no. 3, pp. 423-429.

Rodrigo, R., González, J. & Paoletto, F. 2011, "The role of oxidative stress in the pathophysiology of hypertension", *Hypertension Research*, vol. 34, no. 4, pp. 431-440.

Rodrigo, R., Prat, H., Passalacqua, W., Araya, J., Guichard, C. & Bachler, J.P. 2007, "Relationship between oxidative stress and essential hypertension", *Hypertension Research*, vol. 30, no. 12, pp. 1159.

Roggensack, A.M., Zhang, Y. & Davidge, S.T. 1999, "Evidence for peroxynitrite formation in the vasculature of women with preeclampsia", *Hypertension*, vol. 33, no. 1, pp. 83-89.

Romero, F.J., Bosch-Morell, F., Romero, M.J., Jareño, E.J., Romero, B., Marín, N. & Romá, J. 1998, "Lipid peroxidation products and antioxidants in human disease.", *Environmental health perspectives*, vol. 106, no. Suppl 5, pp. 1229.

Rosenblatt, M. 1956, "A central limit theorem and a strong mixing condition", *Proceedings of the National Academy of Sciences*, vol. 42, no. 1, pp. 43.

Sarkar, R., Bhowmick, K., Dey, A., Bhattacharya, R. & Chakraborty, I. 2011, "Oxidative Stress Markers and Antioxidant Status in Human Hypertension", *Al Ameen Journal of Medical Sciences*, vol. 4, no. 4, pp. 339-347.

Sartori-Valinotti, J.C., Iliescu, R., Fortepiani, L.A., Yanes, L.L. & Reckelhoff, J.F. 2007, "Sex differences in oxidative stress and the impact on blood pressure control and cardiovascular disease", *Clinical and experimental pharmacology and physiology*, vol. 34, no. 9, pp. 938-945.

Saydam, N., Kirb, A., Demir, Ö., Hazan, E., Oto, Ö., Saydam, O. & Güner, G. 1997, "Determination of glutathione, glutathione reductase, glutathione peroxidase and glutathione S-transferase levels in human lung cancer tissues", *Cancer letters*, vol. 119, no. 1, pp. 13-19.

Schneider, C., Porter, N.A. & Brash, A.R. 2008, "Routes to 4-hydroxynonenal: fundamental issues in the mechanisms of lipid peroxidation", *Journal of Biological Chemistry*, vol. 283, no. 23, pp. 15539-15543.

Simic, D., Mimic-Oka, J., Pljesa-Ercegovac, M., Savic-Radojevic, A., Opacic, M., Matic, D., Ivanovic, B. & Simic, T. 2005, "Byproducts of oxidative protein damage and antioxidant enzyme activities in plasma of patients with different degrees of essential hypertension", *Journal of human hypertension*, vol. 20, no. 2, pp. 149-155.

Singh, R., Leuratti, C., Josyula, S., Sipowicz, M.A., Diwan, B.A., Kasprzak, K.S., Schut, H.A.J., Marnett, L.J., Anderson, L.M. & Shuker, D.E.G. 2001, "Lobe-specific increases in malondialdehyde DNA adduct formation in the livers of mice following infection with *Helicobacter hepaticus*", *Carcinogenesis*, vol. 22, no. 8, pp. 1281.

Siyad, A.R. 2011, "Hypertension", *Hygeia:: Journal for Drugs and Medicines*, vol. 3, no 1, pp. 1-16.

Tremellen, K. 2008, "Oxidative stress and male infertility—a clinical perspective", *Human reproduction update*, vol. 14, no. 3, pp. 243.

Tsikas, D. 2012, "Analytical methods for 3-nitrotyrosine quantification in biological samples: the unique role of tandem mass spectrometry", *Amino acids*, vol. 42, no. 1, pp. 45.

Tsikas, D., Mitschke, A., Suchy, M.T., Gutzki, F.M. & Stichtenoth, D.O. 2005, "Determination of 3-nitrotyrosine in human urine at the basal state by gas chromatography-tandem mass spectrometry and evaluation of the excretion after oral intake", *Journal of Chromatography B*, vol. 827, no. 1, pp. 146-156.

Tukey, J.W. 1977, *Exploratory data analysis*. Reading, MA: Addison-Wesley Publishing Co., 44.

Turko, I.V. & Murad, F. 2002, "Protein nitration in cardiovascular diseases", *Pharmacological reviews*, vol. 54, no. 4, pp. 619-634.

Upmacis, R.K. 2008, "Atherosclerosis: A link between lipid intake and protein tyrosine nitration", *Lipid insights*, vol. 2008, no. 2, pp. 75.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M. & Telser, J. 2007, "Free radicals and antioxidants in normal physiological functions and human disease", *The international journal of biochemistry & cell biology*, vol. 39, no. 1, pp. 44-84.

Vaziri, N.D. & Rodríguez-Iturbe, B. 2006, "Mechanisms of disease: oxidative stress and inflammation in the pathogenesis of hypertension", *Nature clinical practice nephrology*, vol. 2, no. 10, pp. 582-593.

Vincent, H. & Taylor, A. 2005, "Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans", *International journal of obesity*, vol. 30, no. 3, pp. 400-418.

Walker, A.R.P. 1972, "The human requirement of calcium: should low intakes be supplemented?", *The American Journal of Clinical Nutrition*, vol. 25, no. 5, pp. 518-530.

Wander, R.C., Hall, J.A., Gradin, J.L., Du, S.H. & Jewell, D.E. 1997, "The ratio of dietary (n-6) to (n-3) fatty acids influences immune system function, eicosanoid metabolism, lipid peroxidation and vitamin E status in aged dogs", *The Journal of nutrition*, vol. 127, no. 6, pp. 1198-1205.

White, M., Ducharme, A., Ibrahim, R., Whitton, L., Lavoie, J., Guertin, M., Racine, N., He, Y., Yao, G. & Rouleau, J. 2006, "Increased systemic inflammation and oxidative stress in patients with worsening congestive heart failure: improvement after short-term inotropic support", *Clinical science*, vol. 110, pp. 483-489.

Whitney, E.N. & Rolfes, S.R. (2008). *Understanding nutrition* (10th ed). Wadsworth Publishing. pp.632.

Widmaier, E.P., Raff, H. & Strang, K.T. (2006). *Vander's human physiology the mechanisms of body function* (10th ed.). Boston: McGraw-Hill. pp. 452.

Wiernsperger, N. 2003, "Oxidative stress as a therapeutic target in diabetes: revisiting the controversy", *Diabetes & metabolism*, vol. 29, no. 6, pp. 579-585.

Wood, L., Gibson, P. & Garg, M. 2003, "Biomarkers of lipid peroxidation, airway inflammation and asthma", *European Respiratory Journal*, vol. 21, no. 1, pp. 177-186.

Wu, T., Bechtel, P. & Bower, C. 2009, "Effects of delayed processing of pink salmon (*Oncorhynchus gorbuscha*) by-products on fishmeal quality", *Journal of Aquatic Food Product Technology*, vol. 18, no. 4, pp. 345-359.

Yan, L.J. & Forster, M.J. 2011, "Chemical probes for analysis of carbonylated proteins: A review", *Journal of Chromatography B*, vol. 879, no. 17-18, pp. 1308-1315.

Young, I. & Woodside, J. 2001, "Antioxidants in health and disease", *Journal of clinical pathology*, vol. 54, no. 3, pp. 176.

Zhou, L.C., Xiang, W., Potts, J., Floyd, M., Sharan, C., Yang, H., Ross, J., Nyanda, A.M. & Guo, Z.M. 2006, "Reduction in extracellular superoxide dismutase activity in African-American patients with hypertension", *Free Radical Biology and Medicine*, vol. 41, no. 9, pp. 1384-1391.