MECHANISMS OF SINGLET OXYGEN-DEPENDENT FORMATION OF OZONE, BIOACTIVE LIPID ALDEHYDES, AND AMIDE-TYPE ALDEHYDE ADDUCTS IN BIOLOGICAL SYSTEMS

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Mechanisms of Singlet Oxygen-Dependent Formation of Ozone, Bioactive Lipid Aldehydes, and Amide-Type Aldehyde Adducts in Biological Systems

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this work to firstly to God for enabling me to pursue and successfully complete at this time. To my entire family, my dear wife Lydia Kavinya Kitui, my children Deborah Wafula and Morris George for understanding my absence though physically present. To my parents Bishop Morris Wekesa and Mum Reverent Deborah Wanjala, Dad Geoffrey Kituu and Mum Philomena Kituu for constant prayers and believing in me throughout. To my siblings Eng. Reuben and Melissa, Eng. Erick and Rophina, Bishop, Overseer Joseph and Vivian, Jemimah and CFO Mike, mtua Violet and Dr. Osborne and all my nephews and nieces (Deborah, Joseph Jnr, Reuben Morris, Ryan, Sasha, Blessings, Sharon, Phillip, Alison, Jabali, Cheril and Joy).

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LIST OF ABBREVIATIONS AND ACRONYMS

BHT	2,6-ditertiary butyl- 4-hydroxytoluene
Ch-5α-OOH	Cholestrol-5α-hydroperoxide
Ch-6α-OOH	Cholestrol-6α-hydroperoxide
Ch-6α-OOH	Cholestrol-6β-hydroperoxide
CVD	Cardiovascular disease
CYP2E1	Cytochrome P450 2E1
2,4-DNPH	2,4-dinitrophenyl hydrazine
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
GC	Gas chromatography
GC-MS	Gas chromatography - Mass Spectroscopy
GC-MS-SIM	Gas chromatography - Mass Spectroscopy-Selective ion monitoring
GSH	Glutathione
GSSG	Glutathione disulphide
HEL	N-(hexanoyl)lysine
H_2O_2	Hydrogen peroxide

H_2O_3	Hydrogen trioxide
HILIC	Hydrophilic interaction liquid chromatography
HNE	4-hydroxy-2-nonenal
H-NMR	Proton nuclear magnetic resonance spectroscopy
HOCL	Hypochlorous acid
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography - Mass Spectroscopy
HPNE	4-hydroperoxy-2-nonenal
HPODE	Linoleic acid hydroperoxide
Hs-CRP	Highly sensitive C-reactive proteins
IL-6	Interleucine-6
JBS3	Joint British Societies' consensus recommendations for the prevention of cardiovascular disease
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KCL	Potassium chloride
KH_2PO_4	Potassium orthophosphate buffer
KIRDI	Kenya Industrial Research and Development Institute
LC-ESI-MS	Liquid chromatography electrospray ionization mass spectrometry

LC-MS	Liquid chromatography mass spectrometry
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LPO	Lipid peroxidation
MDA	Malondialdehyde
MPO	Myeloperoxidase
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NCDs	Non-communicable diseases
NDPO2	3,3'-(1,4-naphthylidene) diproprionate
NO	Nitrogen dioxide
Nrf2	Nuclear factor erthroid-2
$^1O_2$	Singlet oxygen
O_3	Ozone
.OH	Hydroxyl radical
-OH	Hydroxyl anion
RAS	Renin-angiotensin system

RNS	Reactive Nitrogen species
ROS	Reactive oxygen species
Secosterol A	3β-hydroxy-5-oxo-5, 6-secocholestan-6-al
Secosterol B	3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde
SOD	Superoxide dismutase
TIC	Total ion chromatogram
TLC	Thin layer chromatography
USDA	United States Department of Agriculture
UV	Ultraviolet light
UVA	Ultraviolet A
WHO	World Health Organization

ABSTRACT

The cholesterol secosterol aldehydes, namely 3β-hydroxy-5-oxo-5, 6-secocholestan-6-al (secosterol A) and its aldolization product 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (secosterol B) are highly bioactive compounds. They have been detected in human tissues and may significantly contribute to the pathophysiology of conditions such as diabetes, certain cancers, atherosclerosis and Alzheimer's disease. Previously, they were considered unique products of cholesterol ozonolysis. Hence they were used as indicators for formation of ozone endogenously. However, the formation of ozone in biological systems has been questioned partly because of inadequate understanding of the mechanisms of its formation. The original mechanism proposes that antibodies or amino acids catalyze the oxidation of water with singlet oxygen to form dihydrogen trioxide (HOOOH). Then HOOOH decomposes to form ozone (O_3) and hydrogen peroxide (H_2O_2). However, in aqueous solutions, HOOOH was found to decompose readily to singlet oxygen and water rather than ozone and hydrogen peroxide. Alternatively it has been suggested that ozone can be formed by first oxidation of amino acids with singlet oxygen. Then by a further reaction of the amino acid oxidation products with singlet oxygen to form zwitterionic polyoxidic species that decompose to form ozone and amino acids or amino acid oxidation products. Because of previous doubts on the occurrence of biological ozone, an alternative mechanism for the formation of the secosterol aldehydes has been proposed. It involves oxidation of cholesterol by singlet oxygen to form cholesterol-5α-hydroperoxide, followed by acid-catalyzed decomposition (Hock cleavage) of the cholesterol-5α-hydroperoxide to secosterol A and subsequent conversion of secosterol A to secosterol B. However, Hock cleavage of cholesterol-5α-hydroperoxide results in the formation of mainly secosterol B and negligible amounts of secosterol A. The secosterol A compound is implicated as the major secosterol in atherosclerotic tissues. Additionally, it was postulated that primary amines such as lysine may catalyze the conversion of cholesterol-5α-hydroperoxide (Ch-5α-OOH), to the secosterol aldehydes. However, no experimental evidence was provided. Therefore, this study tested the hypotheses that (i) reaction of singlet oxygen with amino acids and their oxidation products yields ozone and (ii) amines react with cholesterol-5α-hydroperoxide to form secosterol aldehydes. The first hypothesis was tested by exposing methionine ($C_5H_{11}NO_2S$) and methionine sulfoxide ($C_5H_{11}NO_3S$) to a singlet oxygen-generating system consisting of myeloperoxidase-hydrogen peroxide-halide system in the presence of the ozone 'indicator' molecules, indigo carmine and vinyl benzoic acid. The finding that methionine sulfoxide was more efficient than methionine in converting vinyl benzoic acid and indigo carmine to 4-carboxybenzaldehyde and isatin sulfonate, respectively, supported conversion of methionine sulfoxide to trioxidic anionic species $RS^+(OOO^-)CH_3$ as a precursor of ozone or ozone-like oxidants. The second hypothesis was tested by generating cholesterol-5α-hydroperoxide by the photosensitized oxidation of cholesterol. Then exposed the hydroperoxides to lysine in the presence of 2,6-ditertiary butyl- 4-hydroxytoluene (BHT) to limit free radical reactions. Analysis of the reaction mixtures by electrospray

ionization mass spectrometry revealed the formation of the secosterol aldehydes as well as various types of secosterol-amine adducts including carbinolamines, Schiff's bases and amide-type adducts. The amide-type adducts *in vitro* and *in vivo* contribute to pathophysiological processes such as hexanoyl-lysine. They are also considered biomarkers of lipid oxidation in foods. Their mechanism of formation however is not well understood. Recently it was postulated that such adducts may be formed by the reaction of aldehydes with amines to form Schiff's bases, followed by reaction of the Schiff's bases with hydroperoxides to form unstable peroxide intermediates that rearrange to amide-type adducts and alcohols. However the peroxide intermediate was not detected by liquid chromatography-electrospray ionization mass spectrometry LC-ESI-MS as a direct evidence for this mechanism in this study. Thus, an alternative mechanism was proposed, involving the oxidation of carbinolamine adducts by singlet oxygen. In this case, dioxetane derivatives of cholesterol decompose into triplet carbonyls which transfer some of their energy to triplet oxygen to generate singlet oxygen. Apart from the amine-mediated decomposition of cholesterol hydroperoxide, the analogous amine-mediated decomposition of linoleic acid hydroperoxide was also investigated. Analysis of the products by GC-MS revealed the formation of hexanal, 2-pentyfuran and 2-nonenal. Detection of 2-pentylfuran signified the formation of 4-hydroperoxy-2-nonenal. This is a key precursor of the 4-hydroxy-2-nonenal, a major cytotoxic product of linoleic acid oxidation, and whose mechanisms of formation is of great interest. Another objective of this study was to determine the effect of uric acid on the conversion of linoleic acid hydroperoxides to aldehydic products. Thus, the aldehyde forming reactions were done in the presence of uric acid. Interestingly, uric acid, even without the amines, was found to promote conversion of the hydroperoxides to aldehydes. Thus, the present study obtained evidence for the hypotheses that some amino acids react with singlet oxygen to form ozone and that amines such as lysine mediate the decomposition of cholesterol-5α-hydroperoxide to form secosterol aldehydes, and the analogous conversion of linoleic acid hydroperoxide to hexanal and 4-hydroxy-2-nonenal. Based on identification cholesterol-secosterol aldehyde adducts by ESI-MS spectrometry, a new mechanism for the formation of amide-type aldehydes was proposed. It was also found that uric acid promotes the conversion of lipid hydroperoxides to toxic aldehydes, and this may explain the paradoxical association of hyperuricemia with various physiological disorders, despite its known antioxidant activities.

CHAPTER ONE

INTRODUCTION

1.1 Lipids: Structures, Functions and Reactions

Lipids are a diverse group of water insoluble organic compounds (hydrocarbons), which can be extracted by non-polar solvents from cells and tissues (Smith, 2000). Structurally, lipids may broadly be classified as fatty acids (FAs), triacylglycerols (triglycerides), phospholipids, steroids and terpenes (Christine, 2003, Finley & deMan, 2018). Fatty acids are simple lipids which possess a terminal carboxyl group (COOH) and a hydrocarbon chain (Zarate, *et al.*, 2017, Finley & deMan, 2018). They may be classified as saturated, monounsaturated or polyunsaturated. The polyunsaturated FAs are further divided into two classes; n-3 and n-6 fatty acids (Zarate, *et al.,* 2017). The n-3 or n-6 denotes the first position at which unsaturation occurs when counting from the methyl end of the fatty acid chain.

Fatty acids usually occur as components of other lipids such as triglycerides and phosphoglycerides. Triglycerides consist of a glycerol esterified to three fatty acids, which make up fats and oils of plant and animal origin (Finley & deMan, 2018). They serve as storage and transport form of metabolic energy in mammalian cells. On the other hand, phosphoglycerides and sterols form the major components of biological membranes. Cholesterol is also an intermediate in the synthesis of steroids such as androgens, estrogens, progesterone and adrenocortical hormones (Hu, *et al*., 2010). Cholesterol may occur in tissues in the free form or as esterified to fatty acids. High levels of blood cholesterol have been implicated in atherosclerosis and cardiovascular diseases (Joint British Societies (JBS3) 2014; Balla & Tyihak, 2010; Vella et al., 2015; Jerret et al., 2017). This occurs when lipids such as fatty acids and cholesterol are oxidized through numerous agents such as exposure to metal ions, reactive oxygen species (ROS), and reactive nitrogen species (RNS) among others (Sivanandham, 2011).

Lipids are prone to oxidation in the presence of light, transition metals, or various reactive oxygen species (ROS) such as singlet oxygen, ozone, alkoxyl radicals, and peroxyl radicals (Yin and Porter, 2011). Lipid oxidation in food leads to quality deterioration in terms of the development of rancidity. Moreover, aldehydic lipid oxidation products react with essential nutrients such as lysine and thiamine, thus reducing nutritional value (Gutierez, et al., 2017, Domínguez, et al., 2019). When ingested, such products can also modify important biomolecules such as proteins and DNA, and thus contribute to non-communicable diseases (Ayala, et al., 2014, Onyango, 2017). Such lipid oxidation products do not necessarily have to be ingested to cause harm; they can also be generated in the body, by the same mechanisms that operate in vitro, in foods (Gutierez, *et al*., 2017, Umeno, *et al*., 2013, Onyango, 2018). A previous study reported that in the development of obesity-associated insulin resistance and glucose intolerance in rats, the specific *in vivo* generation of lipid oxidation products by singlet oxygen is a prominent feature of the early stages of this process (Murotomi, *et al* ., 2015; Umeno, *et al*., 2013). In foods, singlet oxygen can be generated by the transfer of some light energy to oxygen molecules when a photosensitizer such as chlorophyll or riboflavin is present in the system (Jung, *et al*., 1995). *In vivo*, various ways for the generation of singlet oxygen through cellular signaling processes have been reviewed (Onyango, 2016b). There has also been a proposal that antibodies or amino acids catalyze a reaction between singlet oxygen and water to form ozone (Wentworth, *et al*., 2002), and that ozone is an important reactive oxygen species in lipid oxidation, and especially that it plays a role in converting cholesterol to highly bioactive secosterol aldehydes that promote atherosclerosis. On the other hand, credible evidence against this mechanism has been reported. An alternative hypothesis that ozone formation involves a reaction between singlet oxygen and amino acids has been proposed, but not tested (Onyango, 2016a). On the other hand, other researchers contend that the secosterol aldehydes are formed by ozone-independent mechanisms, partly because the mechanisms of such 'biological ozone' formation are not well understood. Thus, the current study sought to contribute to an understanding of the mechanisms of biological

ozone formation, which is of relevance to lipid oxidation both in food and *in vivo*, as well as the mechanisms of formation of cholesterol secosterol aldehydes, and whether such mechanisms analogously apply to formation of aldehydes from other unsaturated lipids. In addition, the usefulness of some antioxidants in the prevention of such reactions was considered.

The new information generated will increase the understanding of lipid oxidation reactions *in vitro* and *in-vivo*, pathogenesis of key non-communicable disease conditions and in the development of better nutritional interventions.

1.2 Problem Statement

The mechanisms of formation of 'biological ozone', cholesterol secosterol aldehydes, and other related types of aldehydes, which contribute to the development of physiological dysfunctions such cardiovascular disorders, diabetes, and Alzheimers disease are not well understood.

1.3 Justification

Although there is evidence for the formation of ozone or an ozone-like oxidant which converts cholesterol to atherogenic secosterol aldehydes in living tissues (Wentworth et al., 2002; Wentworth et al., 2003; Yamashita et al., 2008; Tomono et al., 2011; Tyihak et al., 2012), the mechanisms involved are not fully established and understood, in addition to doubts raised about the occurrence and uniqueness of the reactions (Kettle, 2004; Brinkhorst, 2008). The first proposed mechanism; the water oxidation pathway by Wentworth et al., (2002) has been found not to yield ozone from the resultant oxidation products according to Cerkovnic and Plesnicar (2013) as earlier reported. Therefore the detected atherogenic secosterols must have alternative processes leading to their formation in biological systems. However, a recently proposed alternative mechanism involves the reaction of amino acids with singlet oxygen, whose decomposition products yields ozone, the amino acid or amino acid oxidation product (Onyango, 2016b) remains

to be confirmed. An increased understanding of the pathway and reaction mechanism for formation of this oxidant and the atherogenic secosterols in biological systems is worth examination. This is important because these reactions occur in foods and reduce their nutritive value as well as render the foods unsafe for consumption. *In vivo,* the identity of these compounds in atherosclerotic lesions in brain tumor, Alzheimer's patients, cardiovascular disease patients and diabetics owing to these reactions validates why it is critical to understand such processes well. In addition, the effect of key antioxidants such as ascorbic acid, uric acid and tocopherol and amines such as methionine, lysine or methionine sulfoxide (an oxidized amine) on the formation of ozone or the secosterol aldehydes is not fully documented. The lack of better understanding of the effect particular antioxidants or some amines have in ozone generation may limit potential approaches like dietary interventions towards quenching such oxidants in biological systems.

1.4 Objectives

1.4.1 Overall objective:

To determine mechanisms of amino acids catalysed formation of biological ozone, cholesterol secosterol aldehydes, and analogous products from linoleic in the presence of singlet oxygen.

1.4.2 Specific objectives:

i. To test the hypothesis that methionine reacts with singlet oxygen to form ozone
ii. To determine the formation of secosterol aldehyde-adducts during the reaction between lysine and cholesterol-5 hydroperoxide.
iii. To determine whether formation of free aldehydes (hexanal and 2-pentyl furan) occur during the reaction of lysine with linoleic acid hydroperoxides
iv. To determine the effect of uric acid on the formation of hexanal and 2-pentyl furan during the decomposition of linoleic acid hydroperoxides

1.5 Hypotheses:

i. The formation of ozone/ ozone-like oxidant involves a reaction between amino acids and singlet oxygen, rather than catalysis of the oxidation of water by the amino acids.

ii. Secosterol aldehydes and adducts can be formed independently of ozone by a reaction of cholesterol-5α-hydroperoxide with amines such as lysine.

iii. Aldehydic products can be formed by the reaction of linoleic acid hydroperoxides with amines such as lysine without the involvement of radical reactions

iv. Uric acid has no significant effect on the formation of hexanal and 2-penylfuran during the decomposition of singlet oxygen catalysed linoleic acid hydroperoxides.

CHAPTER TWO

LITERATURE REVIEW

2.1 General introduction

Non-communicable diseases (NCDs), also known as chronic diseases are diseases that are not transferrable from person to person directly, but occur as a result of a combination of genetic, physiological, environmental and behavior factors (WHO, 2017). The main types of NCDs include cardiovascular diseases (like heart attacks and stroke), cancers, chronic respiratory diseases such as chronic pulmonary disease and asthma and diabetes (WHO, 2021). NCDs are driven by forces such as rapid urbanization, globalization of unhealthy lifestyles and population ageing (WHO, 2021). These are characterized by unhealthy diets, lack of physical activity and exposure to tobacco smoke and alcohol abuse (WHO, 2021). They result in metabolic risk factors including high blood pressure, high blood sugar, elevated blood lipids and obesity that eventually lead to cardiovascular disease, the principal cause of premature deaths in low and middle income economies (WHO, 2017).

The disease burden caused by non-communicable diseases (NCDs) continues to weigh down the global health budget (WHO, 2021). NCDs claim millions of lives prematurely in low and middle income countries (WHO, 2005). The "Global Action Plan for NCDs 2013-2020", aims at reducing premature deaths from NCDs through banning tobacco and alcohol advertising, promoting healthy diets, disease prevention and increased physical activity (WHO, 2015; Lichtenstein *et al*, 2006; Hill *et al*., 2009). Evidence that atherosclerosis and clinical events are related to modifiable risk factors and that lowering levels of these factors could result in reducing the incidence of metabolic disease (JBS3, 2014) indicates a possible solution. Dietary approaches and lifestyle have been demonstrated to be effective in decreasing cardiovascular morbidity and mortality risk (Lichtenstein *et al*., 2006; Hill *et al*., 2009). However, the role of antioxidants and

dietary supplements needs sufficient evidence for their efficacy before promotion for use in management of cardiovascular disease (Hill *et al*., 2009). Similarly, there is a link between healthy diet and physical activity that could play a key role in managing NCDs. This would require an understanding of the etiology and progression of such disease conditions mainly attributable to reactive oxygen species and the development of oxidative stress.

2.2 Reactive oxygen species; generation and roles in vivo

Reactive oxygen species (ROS) are a group of compounds which are either beneficial or harmful to the body and can be generated endogenously or exogenously (Pizzino, *et al*., 2017). They are generated through irradiation with UV light, X-rays, γ-rays and metal catalyzed reactions. In addition, they are also generated during tissue inflammation and mitochondrial reactions (Kunwar & Priyadarsini, 2011). ROS includes singlet oxygen ($^1O_2$), superoxide anion ($O2^-$), ozone (O_3), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCL) hydroxyl radical ($^{\cdot}OH$), and hydroxide anion (^{-}OH) (Pizzino, *et al*., 2017). *In vivo*, free radicals are produced continuously and are highly reactive with affinity for lipids, proteins and nucleic acids (Sivanandham, 2011). Primary ROS generated within tissues are superoxide, peroxide and hydroxyl radical which have attracted research focus (Kunwar & Priyadarsini, 2011). High amounts of ROS are generated in the liver by cytochrome P450 2E1 (CYP2E1) after alcohol exposure (Yonge & Cederbaum, 2008).

The mitochondrial respiratory chain generates most of the ROS owing to its over 80% utilization of all oxygen in-take by the body (Andreyev, *et al*., 2005). Low concentrations of ROS are essential for gene expression, cellular growth, biosynthesis of molecules such thyroxin, prostaglandin and stimulate growth and development processes (Droge, 2002).

Recently, scientific evidence indicates that ROS and lipid peroxidation (LPO) products have been shown to be capable of acting as signaling mediator and induce adaptive response, up-regulate defense capacity, mainly through nuclear factor erythroid 2-related factor 2 (Nrf2)-Kelch (Niki, 2009, Higdon, *et al*., 2012, Ito, *et al.*, 2010). Immune cells macrophages and neutrophils generate ROS for destruction of invading pathogens (Rosen *et al*., 1995). However, the mechanism of bacterial damage in the phagosome owing to ROS remains unclear. Relevant targets of the phagocytic oxidative burst have still not been clearly identified (Slauch 2011). In addition, the inability to produce singlet oxygen in the laboratory at concentrations that are comparable to amounts generated in the phagosome is yet to be overcome. According to Slauch (2011), perhaps application of molecular and genetic tools available in salmonella could significantly surmount this challenge.

Macrophages and neutrophils contain nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which generates superoxide radical and hydrogen peroxide (Franchini *et al*., 2013). The hydrogen peroxide in turn reacts with chloride to generate hypochlorite which ultimately destroys the pathogens (Franchini *et al*., 2013; Virani, *et al.*, 2008). The NADPH oxidase and the resulting ROS are critical for defense against diseases. Neutrophils additionally express myeloperoxidase (MPO) (Virani, *et al.*, 2008) which in the presence of 'heme' produces hypochlorous acid (HOCl) from hydrogen peroxide and chloride anion (Klebanoff, 2005). MPO a recognized biomarker of atherosclerosis catalyzes key reactions in normal host cell defenses and in inflammation defense (Nambi, 2005). It is secreted from activated phagocytes and is present in human artherosclerotic lessions and low density lipoprotein recovered from human atheroma. The MPO oxidizes tyrosine to tyrosine radical in the presence of hydrogen peroxide (Nambi, 2005). Neutrophils kill pathogens through cytotoxicity using either by HOCl or tyrosine radical (Heinecke *et al*., 1993). Hydrogen peroxide in the presence of free iron or copper ions can yield hydroxyl radical by removing an electron from the participating metal ion (McCord 2004). However, superoxide radical regenerates the metal ions back to their original form making them available to react with hydrogen peroxide (McCord

2004; Wu & Cederbaum 2003). These two reactions account for most of the hydroxyl radical generation in tissues owing to the role of metal ions. The contribution of iron in these reactions is linked to any increases of free iron in cells implying that it directly promotes ROS generation and oxidative stress (Tsukamoto & Lu, 2001).

2.2.1 Oxidative stress and its role in chronic diseases

The imbalance between reactive oxygen species (ROS) and the systems' ability to readily detoxify or repair resulting tissue damage leads to oxidative stress that occurs due to excessive generation of ROS or diminishing levels of antioxidants (Loscalzo, 2004). Oxidative stress results in damage of cellular components like proteins, lipids and deoxyribonucleic acid (DNA) and is believed to have a role in pathogenesis of cancers, cardiovascular diseases, diabetes, atherosclerosis among others (Loscalzo, 2004; Lien *et al*., 2008; Sivanandham, 2011; JBS3, 2014; Mollazadeh *et al*., 2016).

Oxidative stress may cause DNA fragmentation through activated endonucleases as a result of increased levels of calcium ions in cells leading to apoptosis (Zhivotovsky & Orrenius, 2011). Despite oxidative stress being a major cause of complications like in diabetics, Mollazadeh *et al*., (2017), recently demonstrated that pomegranate seed oil significantly decreased oxidative stress in tissues and mitochondrial fractions of diabetic rats and remarkably decreased glucose-induced toxicity, ROS levels and lipid peroxidation in H9c2 cell lines. In another study, Sadeghnia *et al*., (2017), demonstrated that alcoholic extracts of *Terminalia chebula* exhibited neuroprotection and oligoprotection aganist quinolinic acid induced oxidative stress via ROS. Likewise, lipids in biological systems can be subject to attack during such oxidative reactions.

Increasing evidence indicates the role of oxidative damage in chronic diseases. Chen *et al.* (2007) observed that long-term exposure to ozone as an atmospheric pollutant led to significant correlation with increases in lipid peroxidation. According to the findings from this study 8-isoprostane 98-iso-PGF was found to be a good biomarker of oxidative

damage related to air pollution. Long *et al*. (2001) in another study, indicated that ozone induced inflammation and biomolecule oxidation in the lungs, whereas extracellular antioxidant levels were relatively unchanged. Plasma antioxidants like urate, ascorbate, glutathione (GSH) and vitamin E, defend the lungs by reacting with oxidizing agents, hence it was expected that they would decrease upon exposure to ozone and an increase in F2-isoprostanes (lipid peroxidation products).

Exposure to 3ppm of ozone for 6 hours resulted in increase in Broncho alveolar lavage fluid (BALF) neutrophil which indicated inflammation and elevation of BALF F2-isoprostanes (Long *et al*., 2001). Only higher doses of ozone were observed to cause elevation of urate but a decrease in ascorbate. However, there was no effect on other plasma antioxidants upon exposure to ozone (Long *et al*., 2001).

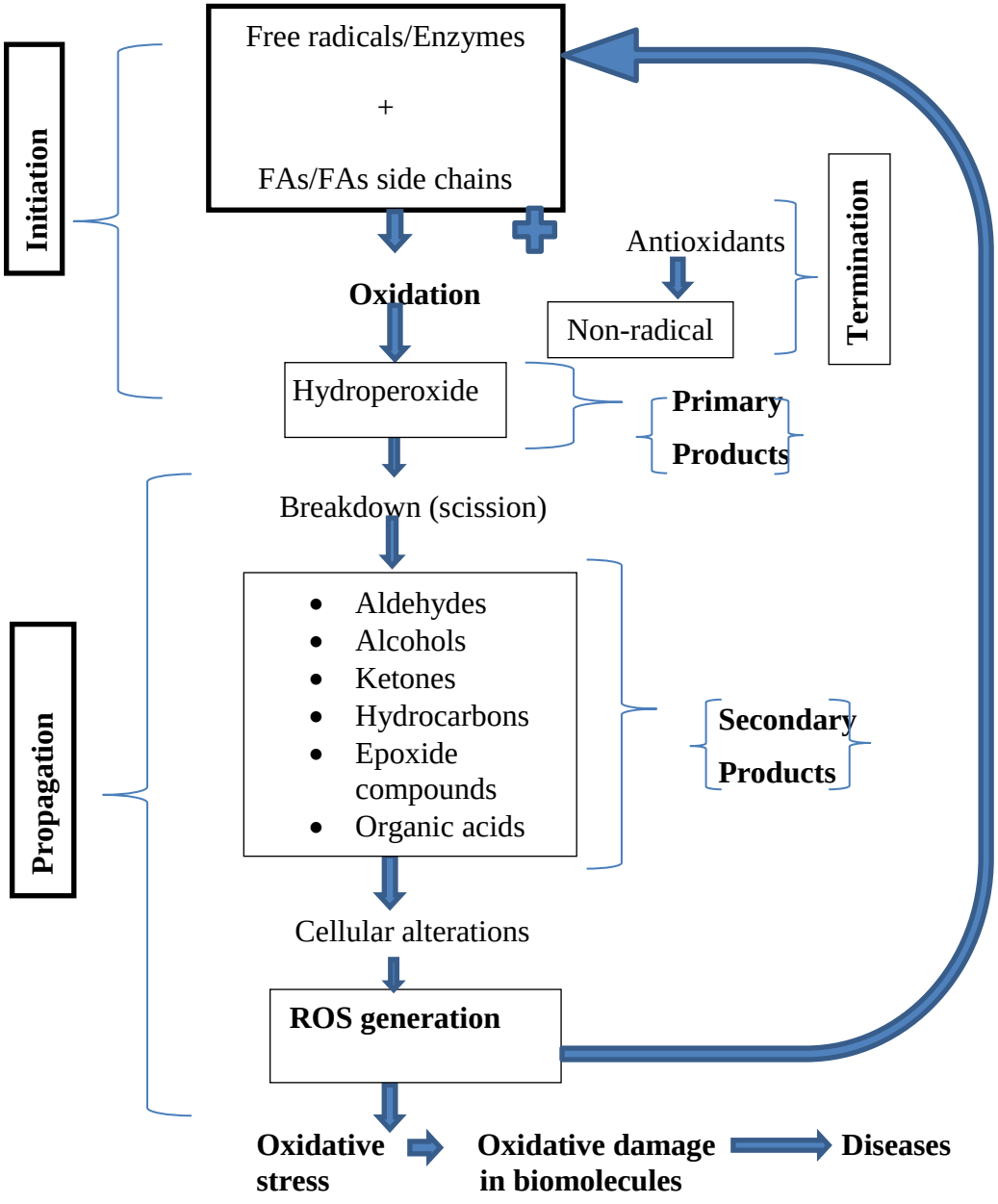
2.2.2 Lipid peroxidation

Unsaturated fatty acids in foods and biological systems may react with oxygen or ROS and become oxidized. Lipid peroxidation (LPO) involves the autoxidation of unsaturated fatty acid esters and sterols (Ayala, *et al*., 2014). In foods, LPO may lead to development of rancidity, loss of essential fatty acids and formation of toxic compounds (Ayala, *et* al., 2014). While, in pharmaceuticals emulsions, LPO's may initiate oxidative stress conditions leading to serious health challenges (Khanum & Thevanayagam, 2017). Increasing evidence indicates that lipid oxidation products have two faces just like ROS and reactive nitrogen species (RNS) (Niki, 2009, Higdon *et al*., 2012). Lipid peroxidation, has been implicated in etiology of various diseases (Niki, 2012). It induces disturbance of fine structure, alteration of integrity, fluidity, and permeability, and functional loss of bio-membranes, modifies low density lipoprotein (LDL) and high density lipoprotein (HDL) to pro-atherogenic and pro-inflammatory forms, and generates potentially toxic products (Niki, 2012). They also exhibit carcinogenesis and mutagenesis. Secondary products of LPO (reactive carbonyl compounds), modify proteins and DNA bases (Poli *et al.*, 2008). Therefore, in the body, LPO is associated

with initiation and progression of disorders such as neurological disorders, certain cancers and cardiovascular diseases (Leonarduzzi *et al.*, 2012, Negre-Salvayre *et al.*., 2010). Model studies have suggested possible beneficial effects of LPO products including antitumor and physiological signaling messenger (Niki, 2012).

Experimentally, increased LPO products have been detected in biological fluids and tissues from patients with these disease conditions as compared with healthy subjects (Niki, 2009, Sayre *et al.*., 2010). The oxidative cleavage of omega 6 unsaturated fatty acids such as linoleic fatty acid generate hexanal (Shahidi, 2001), which is found in mammalian breast milk but also in plasma of cancer patients (Deng *et al*., 2004) and breath condensate of patients with chronic inflammatory lung diseases (Andreoli *et al*., 2003; Corradi *et al*., 2004; Corradi *et al*., 2003). High plasma levels of aldehydes such as nonanal, heptanal and hexanal have been identified in cancer patients (Li *et al.* 2005). The close link of oxidative stress in etiology of tumor genesis may indicate an association to elevated aldehyde concentrations in the breath of lung cancer patients (Li *et al.* 2005). Specific aldehydes may be generated during lipid peroxidation if specific unsaturated fatty acids are present in tumor cell membranes. Such as pentanal as a typical marker of lipid peroxidation in mammalian cells (Eggink *et al*., 2008).

Recently, LPO products have been shown to be capable of acting as signaling mediator and induce adaptive response by up-regulating defense capacity, mainly through nuclear factor erythroid 2-related factor 2 (Nrf2)-Kelch (Niki, 2009, Higdon *et al*., 2012, Ito *et al.*, 2010). Radical scavenging antioxidants such as vitamin C and Vitamin E do not scavenge physiologically important signaling ROS such as hydrogen peroxide and super oxidase, nor inhibit enzymatic lipid oxidation. Therefore these antioxidants may not be potent inhibitors of myeloperoxidase-mediated reactions (Davies, 2011). Even at high concentrations, these antioxidants may likely not impair physiological signaling by ROS and LPO products. LPO in foods and *in vivo* may occur by a free radical mechanism as indicated in Scheme 2.1. (Khanum & Thevanayagam 2017). Cholesterol; an important lipid in mammalian cells may be oxidized by such reactions and by ozone.



Scheme 2.1: Lipid peroxidation process

Adapted from Khanum and Thevanayagam (2017).

2.3 Ozone; Occurrence and Importance

Ozone is a strong oxidant which has been applied extensively in medicine and dentistry (Kumar *et al*., 2014), in food processing (O'Donnel *et al*., 2012) and in numerous industrial processes (Cook, 1982). In the atmosphere the ozone layer prevents dangerous UV rays from reaching the earth's surface (Loscalzo, 2004). However, it has been shown to cause damage to mucosa and respiratory tissues in animals and plant tissues even at low concentrations from 100ppb (Loscalzo, 2004). The exposure to ozone either singly or in combination with other atmospheric pollutants such as diesel exhaust fumes has been linked to induce decrements in lung function (Madden *et al*., 2014). Likewise, individuals with hyper reactive air waves developed decrements to their pulmonary functions when exposed to ozone (Mudway and Kelly 2000).

The discovery of endogenous generation of ozone in biological systems has raised a lot of interest to the possibilities of pathways involved and mechanisms of body protection from any adverse effects (Wentworth *et al*., 2003). This type of oxidant in biological systems could contribute towards the pathogenesis of inflammatory diseases. Currently, inflammation is considered to have a role in the increasing health conditions including autoimmunity, atherosclerosis and ageing related complications. This occurs when ozone cleaves to any compound that contains an alkene or olefin (an unsaturated hydrocarbon) like unsaturated lipids, or oxidized proteins. In addition, ozone reacts with other chemicals to generate more toxic and harmful materials such as hydrotrioxy and hydroxyl radicals. The resultant modified proteins from these reactions may be noted as foreign leading to an autoimmune response in addition to signal amplification of inflammation in tissues. Recent research findings have evidenced a positive association between ozone exposure and incident diabetes in African American women (Jerrett *et al*., 2017).

2.3.1 Mechanisms of ozone generation in biological systems

The identification of cholesterol ozonolysis product 3β-hydroxy-5-oxo-5, 6-secocholestan-6-al (secosterol A) and its aldolization product 3β-hydroxy-5β- hydroxyl-B-norcholestane-6β-carboxaldehyde (secosterol-B) in human atherosclerotic plague and brain tumor tissue was cited as evidence for endogenous ozone formation in human tissues (Wentworth *et al*., 2003). The secosterol A and secosterol B had been thought to be unique to cholesterol ozonolysis within atherosclerotic tissues during carotid endarterectomy confirm ozone production during lesion development (Wentworth *et al*., 2003). *In vitro* activation of these atherosclerotic plagues generated steroids that possessed cytotoxicity, lipid-loading in microphages, and deformation of alipoprotein B, hence participated fully in pathogenesis of atherosclerosis (Wentworth *et al.*, 2003). Separately, Brinkhorst found that cholesterol-5-hydroperoxide obtained by oxidation of cholesterol with singlet oxygen underwent a Hock-cleavage to form secosterol B, and concluded that ozone may not be necessary for formation of the secosterols *in vivo* (Brinkhorst *et al*., 2008). However, it has been confirmed that secosterol A, the major secosterol in atherosclerotic plaques, is only a major product of ozone or 'an oxidant with the chemical signature of ozone' (Wentworth *et al*., 2009).

The generation of ozone or an oxidant with the chemical signature of ozone is still debatable with various research outputs differing on it (Kettle and Winterbourn, 2005). What has not been disputed is the generation of this potent oxidant. Despite the ongoing challenges on differing research hypotheses and outputs, the pathway through which this oxidant is generated has not been clearly established. The pathway through which ozone or ozone like oxidant is formed in the body can answer some of the unresolved research questions about this oxidant. In addition, this pathway can bring closer the utilization of this knowledge in disease prevention or management. The specificity of information could augment the photodynamic therapy for cancer treatment and also frontiers in treatment of drug resistant parasitic infections like malaria. In biological systems, ozone formation has been proposed to occur via the water oxidation pathway where antibodies and /or amino acids act as the catalysts.

2.3.1.1 Antibody catalysed water oxidation pathway

Hydrogen peroxide and ozone were hypothesized to be formed through the antibody-catalyzed water oxidation pathway. Antibodies catalyze the reaction between singlet oxygen and water to give hydrogen peroxide as a frontline in immune defense (Nieva & Wentworth, 2004). Previous research found that antibody-catalyzed ozone formation resulted in increased amounts of hydrogen peroxide (H_2O_2) (more than 500 H_2O_2 molecules) per antibody molecule (Wentworth *et al*., 2001; Wentworth *et al*., 2002). On the other hand, Peng *et al*. (2006) observed ozone and hydrogen peroxide being formed from human leukemia THP-1 monocytes when incubated with human immunoglobulin G and phorbol myristate acetate. The ozone generated was observed to significantly inhibit the accumulation of intracellular lipids chiefly by vinylbenzoic acid than by catalase. Following this path, it can be established that ozone is involved in the pathogenesis of atherosclerosis through the antibody-catalyzed water oxidation pathway more than hydrogen peroxide. The reaction of water with singlet oxygen generates hydrogen trioxide as an intermediate (Wentworth *et al*., 2002), which theoretically either reacts further with singlet oxygen or with another hydrogen trioxide (Cerkovnic and Plesnicar, 2013). In addition, it has been demonstrated experimentally that under aqueous conditions, hydrogen trioxide is highly unstable and decomposes to singlet oxygen and water (Cerkovnic and Plesnicar, 2013). The formation of hydrogen peroxide and ozone through water oxidation pathway was proposed to occur at the hydrophobic site of the poly peptide, where hydrogen trioxide is shielded from water (Wentworth *et al*., 2001) however, up to date it has not been possible to detect the hydrogen peroxide and ozone from the decomposition of hydrogen trioxide (Cerkovnic & Plesnicar, 2013).

Alternative to the water oxidation pathway; the role of amino acid oxidation in the antibody catalyzed formation of hydrogen peroxide is not feasible owing to the high quantity of hydrogen peroxide formed per antibody molecule (Wentworth *et al*., 2001). However, four amino acids; methionine, cysteine, tryptophan and histidine were found

to catalyze ozone formation in the presence of singlet oxygen (Yamashita *et al*., 2008). The common feature of these amino acids is that they are all photo-oxidizable (Wentworth *et al.,* 2001) as evidenced through numerous studies both in free form and as components of proteins (Zhu *et al*., 2004; Pattison *et al.,* 2012; Lundeen *et al*., 2013; Sreethara *et al*., 2013; Amano *et al*., 2014; Liu *et al*., 2014). The reactivity of these amino acids with singlet oxygen largely depends on their position in the protein as exposed residues are readily oxidized while the inaccessible residues remain un-oxidized (Lundeen *et al*., 2013). Despite only a few amino acids being oxidized in the presence of singlet oxygen, they are able to generate adequate ozone and hydrogen peroxide (Sreethara *et al*., 2013). Therefore, it is possible to mention that protein molecules lacking these amino acids are not able to generate ozone.

Conversely, cinnamic acid, resveratrol and formaldehyde have been reported as precursors of biological ozone at molecular level (Tyihak *et al.,* 2013). This was hypothesized through the reaction of formaldehyde with hydrogen peroxide to generate activated formaldehyde and singlet oxygen. The singlet oxygen generated from this reaction then participates in the water oxidation pathway to yield ozone (Tyihak *et al.*, 2013).

Tomono *et al.* (2011) demonstrated that activated neutrophil-like differentiated human leukemia HL60 (nHL-60) cells cultured in a medium containing cholesterol significantly produced secosterol A via myeloperoxidase-dependent generation of singlet oxygen. In a cell-free study, when singlet oxygen was produced in aqueous solutions of immunoglobulins, albumin and 19 amino acids (excluding tyrosine) by ultraviolet A (UVA) irradiation of 6-Formylpterin, formation of ozone occurred in the presence of the proteins and the four amino acids; methionine, histidine, tryptophan and cysteine. Ozone formation was evidenced by conversion of vinylbenzoic acid and indigo carmine to 4-carboxybenzaldehyde and isatin sulfonic acid, respectively (Yamashita *et al*., 2008). In related studies, ozone generation by neutrophils was evidenced by the conversion of indigo carmine to isatin sulfonic acid (Wentworth *et al*., 2002; Babior *et al*., 2003).

These indicators for ozone generation have been challenged when Kettle *et al*., (2004) demonstrated that superoxide generated an equivalent amount of isatin sulfonic acid from indigo carmine just as neutrophils. According to their findings, the bleaching of indigo carmine by neutrophils to isatin sulfonic acid cannot be used as an exclusive indicator to support ozone production in cells. However, Wentworth *et al.*, 2003 showed that both ozone and neutrophils converted vinyl benzoate to 4-carboxybenzaldehyde as evidence for ozone formation.

The oxidative burst of phagocytosing neutrophils due to reduced NADPH oxidase leads to formation of hypochlorous acid, singlet oxygen and hydroxyl radical. However, the antimicrobial activity of ROS is not well elucidated (Wentworth *et al*., 2002; Williams, 2006). Wentworth proposed that neutrophils produce ozone which contributed to bacterial killing where antibodies catalyze the production of ozone from singlet oxygen and water. The mechanisms still remains unclear. Kettle and Winterbourn (2005) challenged the validity of detection of ozone generated basing on their findings that superoxide converted indigo carmine to isatin sulfonic acid and vinyl benzoate to 4-carboxybenzaldehyde as evidence for ozone formation. There is growing evidence to support ozone generation in presence of singlet oxygen *in vivo*. A rare variant of chronic granulomatous disease (CGD); an inherited disorder where phagocytes are unable to kill certain bacteria and fungi produced significant amounts of singlet oxygen but very little superoxide and neutrophils provided a useful model to check oxidative burst (Aussel *et al*., 2011; Slauch 2011). It was found out that superoxide (SOD) mutant mice as compared with wild mice, singlet oxygen was consumed by some reaction that did not result in the production of hydrogen peroxide (Aussel *et al*., 2011). The results from this study clearly points to the validity of the existence of a potent oxidant responsible for the reactions. Findings from this study have since not been challenged implying that they could have provided compelling evidence to support ozone generation *in vivo*. On the other hand, myeloperoxidase (MPO) deficient mice failed to produce hypochlorous acid and singlet oxygen and showed increased susceptibility to infections especially

pneumonia and death when exposed to high doses of bacteria and fungi (Aratani *et al.*, 2006).

The exposure of human neutrophils to quantities of invading pathogens like *E-coli* in ratios of greater than 5:1, initiates amino acid catalyzed oxidant defense system with high bactericidal activity. This indicates that ozone produced by neutrophils is initiated when the host is exposed to high doses of infectious agents (Yamashita *et al.*, 2008). It is worthwhile to consider singlet oxygen mediated amino acid oxidation reaction with the aim of understanding the mechanisms involved in ozone formation.

2.3.1.2 Antibody catalyzed ozone generation by amino acids

Ozone generation was found to occur in a dose-dependent and at comparable levels to the immunoglobulins in the presence of four amino acids (Yamashita *et al.*, 2008). Therefore the residues of these amino acids could be responsible for the production of ozone by antibodies and other proteins. The side chains of all amino acid residues of tryptophan, cysteine and methionine are susceptible to ROS oxidation to result in carbonyls such as aldehydes and ketones. It had earlier been reported that formation of ozone (O_3) and hydrogen peroxide (H_2O_2) by proteins occurred through a pathway that antibodies catalyze oxidation of water by singlet oxygen ($^1O_2$) to form trioxidic species like hydrogen trioxide, which then reacts with $^1O_2$ to form O_3 and H_2O_2 (Wentworth *et al*., 2002; Nyfeller *et al*., 2004). This hypothesis has been challenged owing to the fact that the high quantity of ozone and hydrogen peroxide formed cannot be fully accounted for through the water oxidation pathway, implying that other biological materials are involved (Kettle and Winterbourn, 2004).

On the other hand, methionine, histidine, tryptophan and cysteine easily react with singlet oxygen to form peroxides and other oxidation products (Min & Boff, 2002). In addition, $^1O_2$ inactivates enzymes whose catalytic site contains cysteine or histidine residues, indicating a modification of these amino acid residues (Suto *et al*., 2007). The

accelerated riboflavin-sensitized destruction of ascorbic acid in the presence of histidine and tyrosine suggesting that the intermediate reaction products of amino acid and $^1O_2$ were responsible (Jung *et al*., 1995). Therefore it is possible to note that ozone could be formed from intermediate products of the reaction of $^1O_2$ with amino acids. Moreover, the fact that the sulfur-containing amino acids react with O_3 to produce $^1O_2$ (Kanofsky & Sima, 1991) points to the potential reversibility of O_3 and $^1O_2$ from common intermediaries. A potential mechanism can be speculated for cysteine and methionine that alkyl sulfides react with $^1O_2$ to form a nucleophilic peroxysulfoxide (RSOO-) (Jensen *et al*., 1998). Since $^1O_2$ is electrophilic, (Min & Boff, 2002), it might easily react with the peroxysulfoxide to form a tetroxysulfoxide (RSOOOO-), which could decompose to form ozone and a sulfoxide RSO. Notably, sulfoxides are major products of sulfide oxidation (Jensen *et al*., 1998; Min & Boff, 2002). The sulfoxide can behave as a nucleophile and thus react with $^1O_2$ to form RSOOO-, which could decompose to form ozone and regenerate the sulfide. In this way many molecules of ozone could be generated from a single methionine molecule, which is consistent with the results of Yamashika *et al*., (2008). Interestingly, it has been reported that the products of O_3 reacting with methionine are methionine sulfoxide and $^1O_2$ (Mudd, 1998) and this is likely to form RSOOO- as an intermediate. This intermediate product may therefore be involved in the conversion of $^1O_2$ to O_3 and vice versa, depending on the concentrations of either oxidant. Ozone readily absorbs UV radiation at 254 nm producing H_2O_2 as an intermediate (Munter, 2001) in studies where singlet oxygen is generated by irradiation of amino acids or antibodies. The loss of cyclooxygenase activity by endothelial cells due to formation of H_2O_2 in presence of O_3 (Madden *et al*., 1987) implies that O_3 may also be converted to H_2O_2 by a mechanism not involving irradiation.

2.3.2 Mechanism of action of ozone

Ozone and hydrogen peroxide combine to form peroxone, a potent bacterial and viral inactivator (Merenyi *et al*., 2010). The mechanisms of ozone in neutralizing microorganisms have focused on the oxidation of bacterial lipids and proteins found in bacterial cell membranes and viral envelope, phospholipids, cholesterols and glycoproteins. At molecular level, it has been shown that ozone still performs the same fundamental function.

2.4 Endogenous and exogenous management of reactive oxygen species related health conditions

The body can protect itself against oxidative damage through endogenous or exogenous antioxidants, which even at low concentrations can significantly delay or prevent oxidation in tissues (Kohen & Nyska 2002). The exogenous use of antioxidants through food or supplements to counter oxidative stress is worth exploring. Antioxidants protect the cells against adverse effects of ROS by terminating the chain reaction before vital molecules are damaged through scavenging free radicals or repair of damaged molecules (Loscalzo, 2004; Zhivotovsky & Orrenius, 2011).

Diets rich in fruits and vegetables have been associated with lower cancer rates leading to various theories that their antioxidant content has protective effect against cancer development. Clinically, non-steroidal anti-inflammatory drugs have been demonstrated to inhibit the generation of hypochlorous acid (a ROS) thereby, suppress the oxidative functions of neutrophils (Paino *et al*., 2005). On the other hand, being overweight and/or being obese predisposes the individuals to high incidence of metabolic and inflammatory diseases. Obesity being an independent factor for cardiovascular disease (CVD) and insulin resistance in diabetics is critical in health management. Primarily, the target of managing CVD is lowering of low density lipoprotein cholesterol (LDL-C) through adoption of a therapeutic lifestyle change diet characterized by weight loss and increased

physical activity (Hill *et al*., 2008). Both obesity and metabolic syndrome (MetS) are associated with higher levels of C-reactive proteins (CRP) and insulin resistance as key biomarkers and independent predictors of CVD events. In, obesity elevated CRP and insulin resistance may impede the lipid lowering effects of dietary interventions. Weight loss has been shown to be a successful strategy to reduce CRP and increase insulin sensitivity, but the effects of different macronutrients on inflammation are largely unknown (Hill *et al*., 2008). Bo, *et al*., (2006) demonstrated that type 2 diabetes mellitus (DM), Mets, and inflammation were linked to reduced magnesium and fiber intakes and these associations were reduced by adjustments for each of these nutrients. The prevalence of DM, Mets and highly sensitive C-reactive protein (hs-CRP) > 3mg/L significantly reduced with increases in magnesium and fiber intake. Low magnesium and fiber intakes were linked to hs-CRP > 3mg/L in the entire population under study (Bo, *et al*., 2006). Therefore, high fiber diets that are rich in magnesium could be ideal for reduction of these risk factors across populations.

Despite the mixed evidence of the relationship between fiber intake and control of diabetes, Post *et al*. (2012), evaluated this relationship and demonstrated that fiber supplementation for type 2 DM can reduce fasting blood glucose and glycosylated hemoglobin in patients with type 2 DM. Increasing dietary fiber intake for diets for diabetic mellitus patients could be beneficial for disease management. On the other hand, weight loss and changes in macronutrient content of diets constitute two main approaches in managing insulin resistance according to Reaven, (2005). Weight loss enhances insulin sensitivity among the obese and overweight individuals with insulin resistance, while changes in macronutrient content of diets manages adverse effects of compensatory hyper-insulinemia. The slow and continuous release in the gut of the dietary fiber bound antioxidants influences the health benefits to the host in disease prevention and management (Vitaglione *et al*., 2008).

Dietary patters containing fiber rich foods may offer a protective role in managing diabetes mellitus (Maghsoudi & Azadbakht, 2012). The ‘healthy’, ‘Mediterranean’,

'prudent' and dietary approach to stop hypertension diets were associated with lower risk of hyperglycemia. Separately, Du *et al*. (2010) observed that higher intake of cereal fiber helped the prevention of body weight and waist circumference gain. Similar results have been recorded in dietary fiber being inversely associated with insulin levels, weight gain and other risk factors for cardiovascular disease CVD in young adults (Ludwig *et al*., 1999; Rimm *et al*., 1996; Ascherio *et al*., 1996). This was also collaborated with follow up studies, like 10 years after initial studies. Interestingly, the fiber type (e.g. soluble or insoluble), source (e.g. whole grain, refined grain, vegetable or fruit), or form (e.g. intact, or processed) was not examined. These variables in addition to other biologically active constituents like magnesium and vitamin E may affect insulin response to ingested carbohydrates as well as CVD risk in significant ways. Shai *et al*., (2010) observed that low weight induced by low fat, Mediterranean and low carbohydrate diets over a period of time resulted in significant reduction in coratid atherosclerosis.

Atherosclerosis develops over several decades beginning in youthful years of individuals. It is believed that lipid retention, oxidation and modification provokes chronic inflammation at susceptible sites on the arterial walls (Insull, 2008). Hypertension, diabetes mellitus, obesity, genetic disposition and smoking risk factors accelerate development of atherosclerosis. Although inevitably being a progressive disease, clinically, atherosclerosis can be treated by 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) (Insull, 2008). Clinical use of non-steroid anti-inflammatory drugs has been shown to inhibit hypochlorous acid generation hence suppress oxidative functions of neutrophils (Paino *et al*., 2005). Clinically, ozone therapy has been successfully applied in the treatment of spinal disk herniation as compared to surgical procedures (Bocci, 2005; Bocci *et al*., 2015). Despite nano-medicine being known for anti-cancer therapy, it's potential in clinical diagnosis and treatment of atherosclerosis has been demonstrated (Lobatto *et al*., 2011).

2.5 The role of some tested antioxidants in quenching reactive oxygen species

The body protects itself against oxidative damage through endogenous or exogenous antioxidants. Antioxidants are classified as either enzymatic (protective) and non-enzymatic as indicated in table one. Enzymatic antioxidants act as the first line of body defense against ROS by converting them to less reactive species. They include catalase, glutathione peroxidase and superoxide dismutase (SOD). Secondary defense against generation of ROS is through non-enzymatic antioxidants like alpha-tocopherol, glutathione and ascorbate which scavenge free radicals or chelate metal ions like iron and copper (Seifried *et al*., 2007). Ozone has been shown to react with biomolecules apart from cysteine and methionine and the cysteine-containing glutathione, other biomolecules not containing amino acids, such as uric acid, ascorbic acid, NADH and NADPH to form singlet oxygen (Kanofsky & Sima 1991).

Table 2.1: The antioxidant system

Non enzymatic			**Enzymatic**
Hydro-soluble	**Lipo-soluble**	**Chelating proteins**	
Uric acid, ascorbic acid, glucose, cysteine, cysteamine, tarine, tryptophan, histidine, methionine, Glutathione, plasma proteins.	Vitamin E, Vitamin A, carotenoids, coenzyme-Q, α-lipoic acid, bilirubin, thioredoxin, bioflavonoids, melatonin	Transferrin, ferritin, caeruloplasmin, lactoferrin, haemopessin, albumin	Superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione redox system, reducing equivalents via NADPH and NADH

Adapted from, Bocci (2005).

2.5.1 Ascorbic acid

Ascorbic acid has been reported to promote the decomposition of linoleic acid hydro peroxides to genotoxic aldehydes such as 4-oxo-2-nonenal and 4-hydroxy-2-nonenal (Lee *et al*., 2001) and has also been shown to produce hydroxyl and alkoxyl radicals in the presence of active metals (iron and copper) ions thereby increasing oxidative damage (Jansson *et al*., 2003). In the stomach, ascorbic acid was found to exhibit pro-oxidative properties in the presence of ferrous ions (Kanner *et al*., 2007). At high concentrations, ascorbic acid exhibit pro-oxidative effects in blood cell from healthy donor, as evidenced by ROS and interleukin-6 (IL-6) production (Oliviera *et al*., 2012). Ascorbic acid reacts with O_3 to produce $^1O_2$ (Kanofsky and Sima, 1991) and reacts with $^1O_2$ to produce H_2O_2 as one of its products (Mudd, 1998). In plant cells, ozone challenges the antioxidant protection in the extracellular matrix (Baier *et al*., 2005). Conklin and Barth (2004) found sensitivity to ozone correlated with ascorbate status of the leaf.

2.5.2 Uric acid

Uric acid is the most abundant antioxidant in body fluids (Inoue *et al*., 2003) and estimated to possess 60% of antioxidant capacity of plasma antioxidants (Benzie *et al*., 1996). It has been detected at high concentrations in liver and lungs under oxidative stress (Glantzounis *et al*., 2005) and has been found to inhibit formation of toxic nitric oxide in the stomach (Pietraforte *et al*., 2006). Additionally, uric acid, ascorbic acid and glutathione have been confirmed to react with O_3 (Kermani *et al*., 2006). Uric acid reacts with O_3 to produce high amounts of singlet oxygen (Kanofsky & Sima, 1991) and intermediate products. In the presence of metal ions, and depending on the extent of oxidative reactions, uric acid has been reported to exert both antioxidant and pro-oxidant activities (Bagnati *et al.,* 1999).

2.5.3 Curcumin

Curcumin, a powerful antioxidant found in turmeric, has been shown to have antimicrobial and anti-inflammatory activities especially when it is irradiated with UV light (Aggarwal & Sung, 2009). Irradiation of curcumin produces singlet oxygen however it is equally a powerful quencher of singlet oxygen (Das & Das 2002). It remains to be demonstrated whether the enhanced antimicrobial activity of irradiated curcumin is merely due to singlet oxygen generation or the formation of ozone by reaction of singlet oxygen with curcumin.

2.5.4 Alpha tocopherol (Vitamin E)

Alpha-tocopherol found in vegetables and fish oil has been found to inhibit lipid oxidation by affecting the pathway of lipid hydro peroxides. However, α-tocopherol at high concentrations has pro-oxidant effect such as increased low-density lipoprotein (LDL) oxidation due to tocopheryl radicals (Upston *et al*., 1999). Being a powerful quencher of $^1O_2$ (Kim *et al*., 2009), oxidized α-tocopherol has pro-oxidant properties due to tocopheryl radicals, hydroxyl radical and $^1O_2$ (Kim *et al*., 2007).

There is growing interest in non-enzymatic cholesterol oxidation due to the fact that the resulting oxysterols could be used as non-invasive markers of oxidative stress *in vivo* (Miyoshi *et al*., 2014). Singlet oxygen and ozone are the non-radical molecules involved in non-enzymatic oxidation of cholesterol. The reaction of ozone with cholesterol is very fast at the 5, 6 –double bond to yield 1, 2, 3-trixolane, which decomposes to 3b-hydroxy-5-oxo-5, 6-secocholestan-6-al (secosterol A or 5,6-secosterol) resulting from cleavage of the B-ring and the aldolation product secosterol B. These two have been proposed as specific marker of ozone-associated tissue damage and ozone production *in vivo* (Miyoshi *et al*., 2014). However secosterol A and B can also be generated from singlet oxygen through the Hock cleavage of 5α-hydroperoxy cholesterol or via dioxietane intermediate. Since seco A and B are generated via non-enzymatic routes *in vivo*, they

are ideal biomarkers to indicate oxidative stress pathways and assist in development of pharmacological agents (Miyoshi *et al*., 2014). In addition, cholesterol oxidation, LPO products such as hexanal have been detected in breath of lung cancer patients, which indicates a role as non-invasive determination of inflammation *in vivo* (Fuchs, *et al*., 2010).

2.6 Mechanisms of formation of atherogenic aldehydes

Ozone reacts with cholesterol to form secosterol A. However, singlet oxygen reacts with cholesterol to form cholesterol-5α-hydroperoxide, which easily undergoes acid catalysed Hock cleavage to form secosterol B. Both the secosterols contribute to atherogenesis by different mechanisms. Atherogenic lesions are characterized by accumulation of low density lipoprotein (LDL) through ozone oxidation. This is indicated by increased lipid hydroperoxide concentration, thiobarbituric acid reactive substances, relative electrophoretic mobility (REM) and oxidation-specific immune isotopes. The lipid portion of the LDL oxidized first which made it atherogenic then the protein portion was oxidized last (Horl et al., 2014). Recent detection of stable complexes from glycyrrhizic acid with cholesterol oxidation products indicates additional paths in the struggle against atherosclerosis (Glushchenko et al., 2011). There is therefore a drive into increasing knowledge in understanding pathogenesis, management and possible treatment of arterial disease conditions including dietary interventions.

Given the fact that the role of antioxidants in slowing down the ageing process and prevention of CVD is not comprehensively conclusive, it is therefore important to study them in relation to oxidative stress and any resultant atherogenic compounds. The effects of antioxidants on the formation of secosterol A and B depend on the quenching of singlet oxygen and ozone, respectively. Since the quenching of singlet oxygen may lead to production of ozone and vice versa, it is important to determine the effects of antioxidants on the formation of both secosterols simultaneously.

2.7 Theoretical framework of methodologies and techniques used

The generation of ozone or ozone like oxidants in biological systems was previously not clearly understood. In addition doubts on the formation of cholesterol ozonolysis products being unique to ozone oxidation reactions and no through other mechanisms justified the importance of this study. Analogous reactions involving singlet oxygen catalysed oxidation of linoleic acid as additional pathways to explain and increase the understanding of these reactions and the mechanisms involved. Finally the roles of singlet oxygen, amines such as methionine, methionine sulfoxide and lysine and uric acid as the major antioxidant in the body may have on such reactions had not been fully determined.

2.7.1 Ozone formation reactions

The ozone or ozone like oxidant formation from the reactions of singlet with methionine or methionine sulfoxide was in response to hypothesis one of this study. This was to confirm the alternative mechanism for ozone generation (Onyango 2016) contrary to the water oxidation pathway (Wentworth *et al*., 2002; Wentworth *et al*., 2003; Yamashita *et al*., 2008). Singlet oxygen was generated using the myeloperoxidase-hydrogen peroxide-chloride system (MPO-H_2O_2-Cl) system (Tomono *et al*., 2011; Kiryu *et al*., 1999; Wentworth *et al*., 2002; Babior *et al*., 2003). This was preferred over other singlet oxygen generating reactions because it was at physiological conditions. Singlet oxygen can also be generated by photooxidation using sunlight (Jung *et al.*, 1995), or by ultraviolet light (Regensburger *et al*., 2013; Girroti and Korytowsky, 2019). However these reactions require a photosensitizer in the system a requirement that would affect the composition of the reaction mixture. In this hypothesis, singlet oxygen was generated by the MPO-H_2O_2-Cl in the presence of indigo carmine and 4-vinyl benzoic acid as ozone indicator molecules. The uniqueness of these reactions to ozone alone had been questioned (Kettle 2004). Despite singlet oxygen has the ability to convert indigo carmine to isatin sulfonate but it is not capable of converting 4-vinyl benzoic acid to 4-

carboxy benzaldehyde (Yamashita *et al*., 2008). The indicator molecules are well detected by HPLC reverse phase on C18 column (Yamashita *et al*., 2008; Wentworth *et al*., 2003). It was justifiable to subject the amino acids to the singlet oxygen generating system in presence of the indicator molecules and a control reaction system without the amino acids to check the reactions.

2.7.2 Cholesterol secosterol aldehyde formation

Hypothesis two of the study was that secosterol aldehydes and adducts can be formed independent of ozone but by the reactions of cholesterol with singlet oxygen and amines such as lysine. Cholesterol was irradiated by ultraviolet light and using methylene blue as the photosensitizer to form the cholesterol-5α-hydroperoxide (Regensburger *et al*., 2013). This is a well-known and accepted cholesterol oxidation reaction. The cholesterol-5α-hydroperoxide was subjected to lysine to confirm secosterol aldehyde formation as postulated by Onyango (2017). The reaction mixture was derivatized with 2,4-Dinitrophenyl hydrazine (2,4-DNPH) especially the carbonyls (Wentworth *et al*., 2003; Tomono *et al*., 2011). Derivatization was important to stabilize the compounds prior to their detection using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) (Wentworth *et al*., 2003; Tomono *et al*., 2011). This step was critical such that the detected compounds were from the reaction mixtures and not due to the conditions from the LC-ESI-MS.

2.7.3 Non-radical formation of aldehydes from reactions of linoleic acid hydroperoxides with lysine

Hypothesis three of the study was that aldehydic products can be formed by the reaction of linoleic acid hydroperoxides with amines such as lysine by non-radical reactions. Linoleic acid was irradiated by ultraviolet light and using methylene blue as the photosensor to form the linoleic acid hydroperoxides (Regensburger *et al*., 2013). The linoleic acid hydroperoxides were subjected to lysine in the presence of BHT a radical

scavenger to limit free radical reactions (Chambers *et al*., 2009, Kukuta, *et al*., 2013). The volatile compounds were detected by drawing a sample from the headspace and injecting to gas chromatography (GC) and gas chromatography mass spectra (GC-MS). GC and GC-MS are well known methods for detecting volatile compounds. The compounds chromatograms and their respective mass spectra using the NIST library was used to positively identify them (Chambers, *et al*., 2009; Kukuta, *et al*., 2013).

2.7.4 Non-radical uric acid catalysed conversion of linoleic acid hydroperoxides to aldehydes and alkyl furan

The fourth hypothesis was that uric acid had no significant effect on the decomposition of linoleic acid hydroperoxides in the presence of singlet oxygen. Linoleic acid was irradiated by ultraviolet light and using methylene blue as the photosensitizer to form the linoleic acid hydroperoxides (Regensburger, *et al*., 2013). The linoleic acid hydroperoxides were subjected to uric acid in the presence of BHT a radical scavenger to limit free radical reactions (Chambers, *et al*., 2009, Shoji, *et al*., 2013). The volatile compounds were detected by drawing a sample from the headspace and injecting to gas chromatography (GC) and gas chromatography mass spectra (GC-MS). GC and GC-MS are well known methods for detecting volatile compounds. The compounds chromatograms and their respective mass spectra using the NIST library was used to positively identify them (Chambers, *et al*., 2009, Shoji, *et al*., 2013; Kukuta, *et al*., 2013). Direct involvement of uric acid was checked by detecting allantoin using HPLC reverse phase on C18 column (Kukuta, *et al*., 2013)

CHAPTER THREE

EVIDENCE FOR THE FORMATION OF OZONE OR OZONE-LIKE OXIDANTS BY THE REACTION OF SINGLET OXYGEN WITH SOME AMINO ACIDS

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3.1 Abstract

Antibodies or some amino acids, namely cysteine, methionine, histidine and tryptophan were previously reported to catalyse the conversion of singlet oxygen ($^1O_2$) to ozone (O_3). The original proposed mechanism was that antibodies or amino acids catalyse the oxidation of water molecules by singlet oxygen to yield dihydrogen trioxide (HOOOH). The HOOOH formed would be the precursor of ozone and hydrogen peroxide (H_2O_2). However, because HOOOH is unstable in aqueous solutions because it readily decomposes to form water and singlet oxygen rather than ozone and hydrogen peroxide. Therefore an alternative hypothesis was proposed; that ozone is formed due to the reaction of singlet oxygen with some amino acids to form polyoxidic amino acid derivatives as ozone precursors. Singlet oxygen was generated by the myeloperoxidase-hydrogen peroxide-chloride system in the presence of either methionine or methionine sulfoxide. Ozone indicator molecules, indigo carmine and 4-vinyl benzoic acid were added in the respective reaction systems. A control reaction had the singlet oxygen generating system with the ozone indicator molecules but without the amino acid. The generation of isatin sulfonate and 4-carboxy benzoic acid from the respective reaction systems was detected by HPLC, reverse phase C18 by comparing their retention times with the respective compound standards. Isatin sulfonate and 4-carboxy benzoic acid were detected from the methionine and methionine sulfoxide reaction systems. Only isatin sulfonate was detected in the control. Therefore from this study, evidence that support the alternative hypothesis is presented, that in the presence of singlet oxygen, methionine sulfoxide ($C_5H_{11}NO_3S$), which is an oxidation product of methionine ($C_5H_{11}NO_2S$) was found to promote reactions that can best be attributed to the trioxidic anionic derivative $RS^+(OOO^-)CH_3$ or ozone.

Key words: Singlet oxygen, ozone, antibodies, amino acids, methionine sulfoxide

3.2 Introduction

Ozone (O_3) is a highly reactive gas, which is mainly found in the earth's stratosphere, where it is formed through photolysis of an oxygen molecule (O_2) by solar radiation of below 242 nm, which splits O_2 into two oxygen atoms (O) (Equation 1), followed by reaction of an oxygen atom with an oxygen molecule in a three body reaction (Equation 2), where the third body (M) is often N_2 or O_2 (Bekki & Lefevre, 2009).

$$O_2 \xrightarrow{h\nu} O + O\,(\lambda < 242\,\text{nm}) \qquad (1)$$

$$O + O_2 + M \longrightarrow O_3 + M \qquad (2)$$

Stratospheric ozone plays the vital role of protecting organisms on earth from the harmful effects of solar radiation of 240-320 nm (Bekki &Lefevre, 2009). Some ozone is also formed in the troposphere, mainly through photolysis of nitrogen dioxide (NO_2) to form nitric oxide (NO) and an oxygen atom, followed by reaction of the oxygen atom with O_2 according to Equation 2. Volatile organic compounds such as from car exhaust fumes contribute to formation of NO_2 (Finlayson-Pitts & Pitts, 1993). Tropospheric ozone is regarded as a pollutant which is harmful to the respiratory system and contributes to the pathogenesis of insulin resistance, diabetes and cardiovascular dysfunctions (Balla & Tyihak 2010; Vella *et al*., 2015; Jerret *et al*., 2017; Loscalzo 2004).

Paradoxically, ozone also finds application in alternative medicine, for example in treatment of diabetic ulcers, as recently reviewed (Izaidi *et al*., 2017). Interestingly, ozone (or an oxidant with the chemical signature of ozone) has also been reported to be generated in biological systems involving antibodies, amino acids, formaldehyde, neutrophils, and myeloperoxidase (Wentworth *et al*., 2002; Wentworth *et al*., 2003; Yamashita *et al*., 2008; Tomono *et al*., 2011; Tyihak *et al*., 2012). It has also been reported that endogenous ozone plays a role in the killing of bacteria by neutrophils and some antibiotic compounds (Wentworth *et al*., 2003; Yamashita *et al*., 2008; Tomono *et al*., 2011; Tyihak *et al*., 2012). Such biological ozone formation requires singlet oxygen ($^1O_2$) and is catalysed by antibodies or the amino acids cysteine, methionine, histidine or tryptophan (Wentworth *et al*., 2003; Yamashita *et al*., 2008).

Human myeloperoxidase (MPO) promotes the formation of singlet oxygen by catalysing the conversion of hydrogen peroxide (H_2O_2) to hypochlorous acid (HOCl), followed by

a reaction of the HOCl with H_2O_2 (Equation 3), and this may be the source of singlet oxygen for neutrophil-dependent, antibody-catalysed ozone production by neutrophils (Tomono *et al*., 2011). There are many other sources of singlet oxygen *in vivo*, as recently reviewed (Onyango, 2016a; Onyango, 2017).

$$H_2O_2 \xrightarrow{MPO,Cl^-} HOCl \xrightarrow{HO_2^-} {}^1O_2 + H_2O + Cl^- \qquad (3)$$

Some of the evidence that was presented to support the antibody- or amino acid-catalysed ozone formation included the occurrence of three known ozone reactions, namely the conversion of indigo carmine, 4-vinyl-benzoic acid, or cholesterol to isatin sulfonate, 4-carboxybenzaldehyde or 3-hydroxy-5-oxo-5,6-sechoclestan-6-al (secosterol A), respectively, when the mentioned reactants were incubated with antibodies or amino acids in the presence of singlet oxygen (Wentworth *et al*., 2002; Wentworth *et al*., 2003; Yamashita *et al*., 2008). Although some doubts have been raised concerning the uniqueness of such reactions to ozone (Kettle 2004), no direct evidence against ozone formation has been demonstrated. On the other hand, the antibiotic effect of the oxidant generated by amino acids or antibodies in the presence of singlet oxygen was clearly shown to be distinct from singlet oxygen or hydrogen peroxide, and to be more compatible with ozone or an ozone-like oxidant (Wentworth *et al*., 2003; Yamashita *et al*., 2008). Moreover, ozone produced in plant tissues has reportedly been detected directly by gas chromatography-mass spectrometry in selective ion monitoring mode (GC-MS-SIM) (Balla & Tyihak, 2010).

The mechanisms of biological ozone formation have not been firmly established, although two pathways have been proposed. According to the first pathway, commonly referred to as the water oxidation pathway, antibodies or amino acids catalyse the oxidation of water by singlet oxygen to form dihydrogen trioxide (HOOOH), followed by a not-so-well defined decomposition of the HOOOH to ozone and hydrogen peroxide (Wentworth *et al*., 2002; Wentworth *et al*., 2003; Yamashita *et al*., 2008). However,

dihydrogen trioxide (HOOOH) has been found to readily decompose to singlet oxygen and water, rather than hydrogen peroxide and ozone (Cerkovnic & Plesnicar 2013).

Therefore an alternative hypothesis was proposed, involving the reaction of amino acids with singlet oxygen to form oxidized amino acid derivatives, followed by further reaction of the oxidized amino acid derivatives with singlet oxygen to form organic zwitterionic polyoxidic derivatives which decompose to release ozone, as exemplified in the methionine (**1)** -catalysed ozone formation via methionine persulfoxide **2**, methionine sulfoxide **3** and a trioxyanionic methionine derivative **4** (Scheme 3.1) (Onyango 2016b). Singlet oxygen might also react with methionine persulfoxide **2** to form ozone and methionine sulfoxide **3** via tetroxide intermediate **5** (Scheme 3.1). This mechanism is based on the fact that singlet oxygen is an electrophile, and would thus react with the anionic oxygen atoms in compounds **2** and **3,** and ozone release from intermediates **4** and **5** would be favoured because it results in formation of relatively stable neutral molecules **1** and **3.**

On the other hand, analogous ozone formation from $HOOO^-$, derived from HOOOH would require the energetically unfavourable formation of a hydride anion (H^-). Some reactions involved in the decomposition of ozone by water (Equations 4-7) (Onyango 2016b; Staehelin *et al.,* 1984; Merenyi *et al*., 2010) are also worthy of consideration in that the reactions of ozone with the hydroxide ion or hydroperoxide anion (Equations 4 and 6 respectively) are analogous to the reactions of singlet oxygen with compounds **2** and **3** in Scheme 3.1. It is possible that Equations 4 and 6 are reversible, with $HO4^-$ and $HO5^-$ being precursors of ozone, like compounds **4** and **5** in Scheme 3.1. However, as per Equation 7, $HO5^-$ easily undergoes radical decomposition as well, so that such radical decomposition may be more important under high ozone concentration. On the other hand, in trioxyanion **4**, the positive charge on sulphur likely makes radical decomposition to form radical **6** and superoxide anion less favourable than conversion of **4** to methionine **1** and ozone or methionine sulfoxide **3** and singlet oxygen. In fact, the

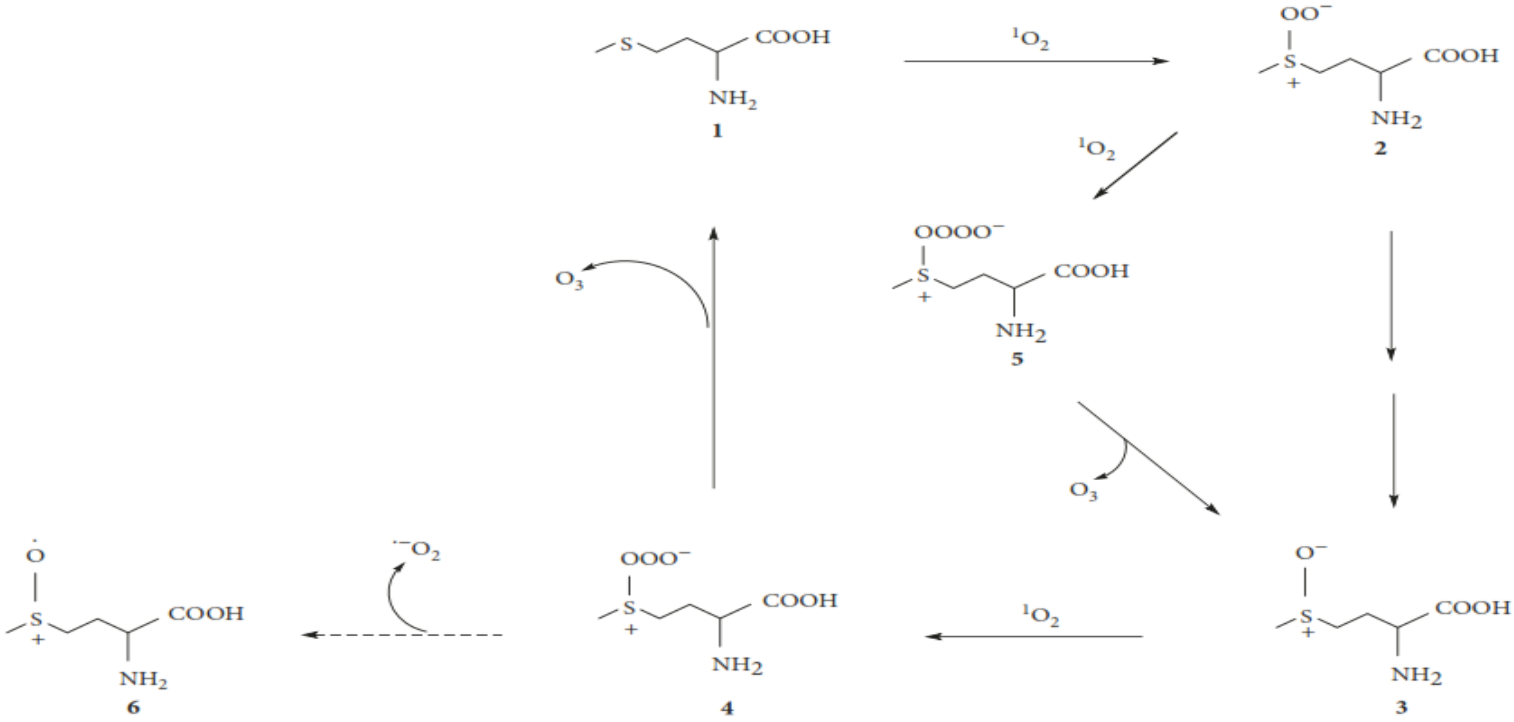
reaction of ozone with methionine **1** was previously found to yield singlet oxygen and methionine sulfoxide **3 (**Kanofsky & Sima 1991; Munoz *et al*., 2001).

$$O_3 + {}^{-}OH \longrightarrow HO_4^{-} \qquad (4)$$

$$HO_4^{-} \longrightarrow HO_2^{-} + O_2 \qquad (5)$$

$$HO_2^{-} + O_3 \longrightarrow HO_5^{-} \qquad (6)$$

$$HO_5^{-} \longrightarrow HO_2^{\cdot} + {}^{\cdot -}O_3 \qquad (7)$$



Scheme 3.1: Previously proposed pathway for methionine-catalysed ozone formation via methionine oxidation products such as methionine persulfoxide 2, methionine sulfoxide 3 and a trioxyanionic derivative 4 (Onyango 2016b), and suggested possibility of ozone and methionine sulfoxide formation from persulfoxide 2 via tetroxide anion 5.

Key: 1-Methionine, 2-Methionine perusulfoxide, 3-methionine sulfoxide, 4-trioxyanionic derivative, 5-tetroxide anion, 6-methionine sulfoxide radical

The present study is the first to directly test and show the role of an amino acid oxidation product, methionine sulfoxide **3** in the formation of ozone. Methioine sulfoxide, rather than any other amino acid oxidation product was used because it is readily available.

3.3 Materials and Methods

3.3.1 Reagents

Human myeloperoxidase (MPO), hydrogen peroxide (H_2O_2), potassium chloride (KCl), methionine, methionine sulfoxide, indigo carmine, isatin sulfonic acid, vinyl benzoic acid, 4-carboxybenzaldehyde, potassium orthophosphate buffer (KH_2PO_4), and acetonitrile were purchased from Sigma Aldrich.

3.3.2 Conversion of indigo carmine to isatin sulfonate in the presence of singlet oxygen and either methionine or methionine sulfoxide

Five (5) units of MPO was dissolved in 1 ml of 100mM potassium orthophosphate buffer (pH 7.4). 0.1 ml of this solution was mixed with 4.4 ml of 100mM KH_2PO_4 (pH 7.4), 100mM KCl, 100μM H_2O_2, 150 μM indigo carmine, and 700 μM of either methionine or methionine sulfoxide was mixed together. The mixture was incubated at 37^{o}C for 1 hour. As a control, a similar reaction without methionine or methionine sulfoxide was done. An aliquot of the reaction mixture was injected to HPLC on a reverse phase C18 column eluted with a solvent consisting of acetonitrile (30%) and 50mM phosphate buffer pH 7.4 (70%) containing 0.1% trichloroacetic acid. Isatin sulfonate and residual indigo carmine were identified by comparing their retention times with respective standards. Peak areas were converted to concentrations by comparison to an isatin sulfonate standard curve (Yamashita *et al*., 2008; Babior *et al*., 2003; Wentworth *et al*., 2002).

3.3.3 Conversion of 4-vinyl benzoic acid to 4-carboxybenzaldehyde in the presence of singlet oxygen and methionine or methionine sulfoxide

This was determined as described in the previous method for the conversion of indigo carmine to isatin sulfonate. However, during the reaction, indigo carmine was replaced by 4-vinylbenzoic acid and during the determination by HPLC, 4-vinyl benzoic acid and 4-carboxybenzaldehyde standards were used instead of indigo carmine and isatin sulfonate standards (Yamashita *et al*., 2008; Babior *et al*., 2003; Wentworth *et al*., 2002).

3.4 Results and Discussion

Singlet oxygen was generated by the myeloperoxidase-H_2O_2-Chloride system (Tomono *et al*., 2011; Kiryu *et al*., 1999). The conversion of indigo carmine to isatin sulfonate was found to occur in control experiment as well as in the presence of methionine and methionine sulfoxide. The system containing methionine gave significantly lower yield than the control, while the methionine sulfoxide system gave higher yield than the control (Table 3.1).

Table 3.1: Yield of isatin sulfonate during the myeloperoxidase-catalysed generation of singlet oxygen in the presence of methionine or methionine sulfoxide.

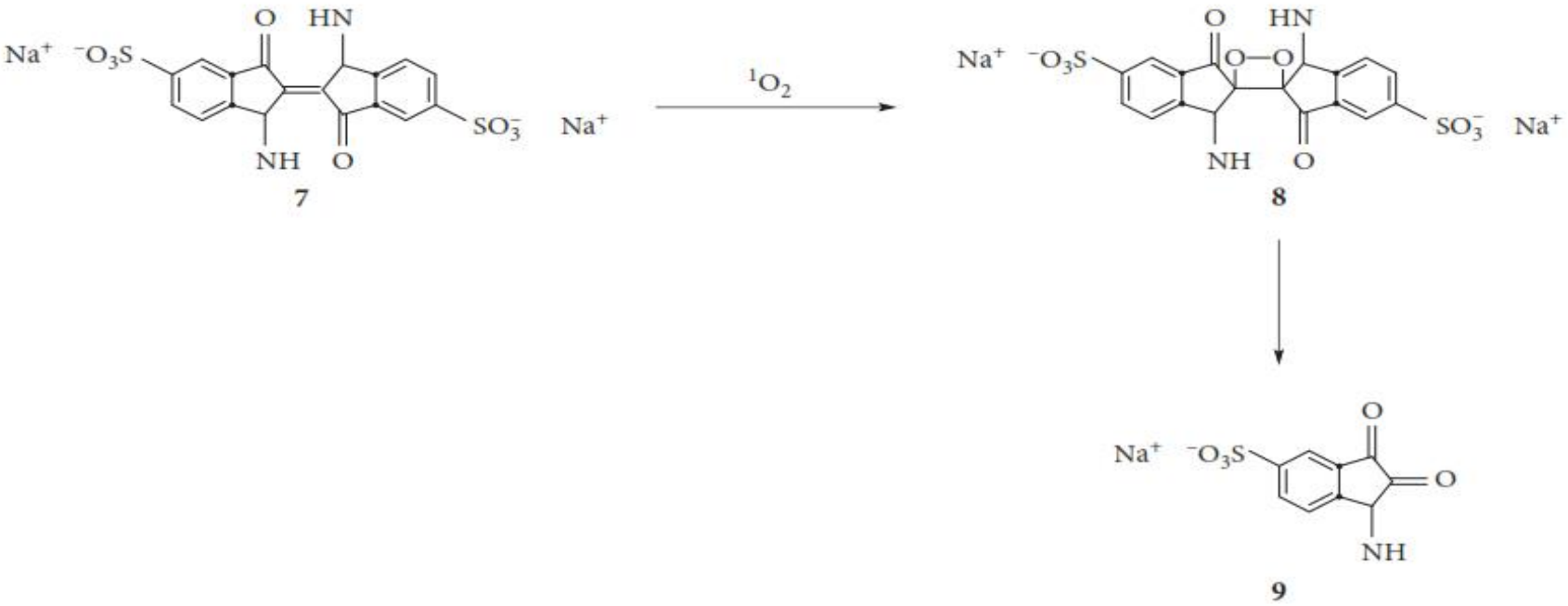
Sample	µM isatin sulfonate
Control	70±0.11
Methionine	64±0.21
Methionine sulfoxide	83±0.26

The conversion of indigo carmine to isatin sulfonate is not an ozone-specific reaction, but it can also be accomplished by singlet oxygen (Wentworth *et al*., 2003; Yamashita *et al*., 2008). Singlet oxygen is expected to convert indigo carmine **7** to dioxetane **8** that decomposes to form two molecules of isatin sulfonate **9** (Scheme 3.2). According to this scheme therefore each of the two molecules of isatin sulfonate incorporates an oxygen atom from singlet oxygen. For ozone-mediated conversion of indigo carmine to isatin sulfonate, however, there is incorporation of an oxygen atom from water, and such incorporation of water-derived oxygen atoms was previously confirmed in studies of antibody or amino-acid catalysed ozone formation (Wentworth *et al*., 2003; Yamashita *et al*., 2008). In the present study, no attempt was made to determine the source of oxygen atoms in the isatin sulfonate molecules. The reduced isatin sulfonate formation in the presence of methionine might be partly due to some physical quenching of singlet oxygen by methionine (Choe & Min, 2006). On the other hand, higher formation of isatin sulfonate in the presence of methionine sulfoxide than in the control experiment is indicative of the involvement of another oxidant beside singlet oxygen, which in previous studies was reported to be ozone or an oxidant with the chemical signature of ozone (Wentworth *et al*., 2003; Yamashita *et al*., 2008).

Incubation of 4-vinyl benzoic acid (**10** in scheme 3.3) in the myeloperoxidase-H_2O_2-chloride system led to minimal formation of 4-carboxybenzaldehyde, which is consistent with a previous finding that singlet oxygen does not convert 4-vinylbenzoic acid to 4-carboxybenzaldehyde (Yamashita *et al*., 2008). This may be understood from the fact that reaction of vinyl aromatics with singlet oxygen more preferably proceeds through a [2+4] cycloaddition to form endoperoxides, rather than [2+2] cycloaddition to form dioxetanes (Posner *et al*., 1987). Therefore, the reaction of singlet oxygen with 4-vinyl benzoic acid **10** should more readily generate, via a [2+4] cycloaddition, the endoperoxide **11**, whose decomposition does not afford 4-carboxybenzaldehyde **12** (Scheme 3.3). Conversely, methionine and methionine sulfoxide generated significant amounts of 4-carboxybenzaldehyde as shown in Figure 3.1, results that further support the involvement of a different oxidant from singlet oxygen. Previous finding have found

that ozone reacts with various unsaturated organic compounds by 1,3-dipolar cycloaddition (Gadzheiv *et al*., 2012; Saito *et al*., 2010), and converts 4-vinyl-benzoic acid to 4-carboxybenzaldehyde (Wentworth *et al*., 2003; Yamashita *et al*., 2008; Babior *et al*., 2003). The finding that methionine sulfoxide (an oxidation product of methionine) promoted 4-carboxybenzaldehyde formation therefore supports the proposal that amino acids promote ozone formation by reacting with singlet oxygen, and the most plausible explanation for the methionine sulfoxide **3**- mediated ozone formation is its further reaction with singlet oxygen to form trioxyanionic intermediate **4** (Scheme 3.1) as explained in the introduction.

Intermediates such as **4** and **5** might, in addition, directly react as ozone-like oxidants. For example, as postulated in Scheme 3, intermediate 5 might undergo a nucleophilic addition to 4-vinylbenzoic acid 10 to form carbanionic intermediate 13, which may convert via dioxetane 14 or primary ozonide 15 to 4-carboxybenzaldehyde 12. Such suggested nucleophilic addition is based on the fact that peroxyanions are very good nucleophiles due to the alpha effect, whereby a lone electron pair in an atom adjacent to the reaction center increases nucleophilicity (McIsaac et al., 1972; Thomsen et al., 2014).



Scheme 3.2: Mechanism of the singlet oxygen mediated conversion of indigo carmine to isatin sulfonate via a dioxetane intermediate.

Key: 7– indigo carmine, 8– dioxetane, 9- isatin sulfonate

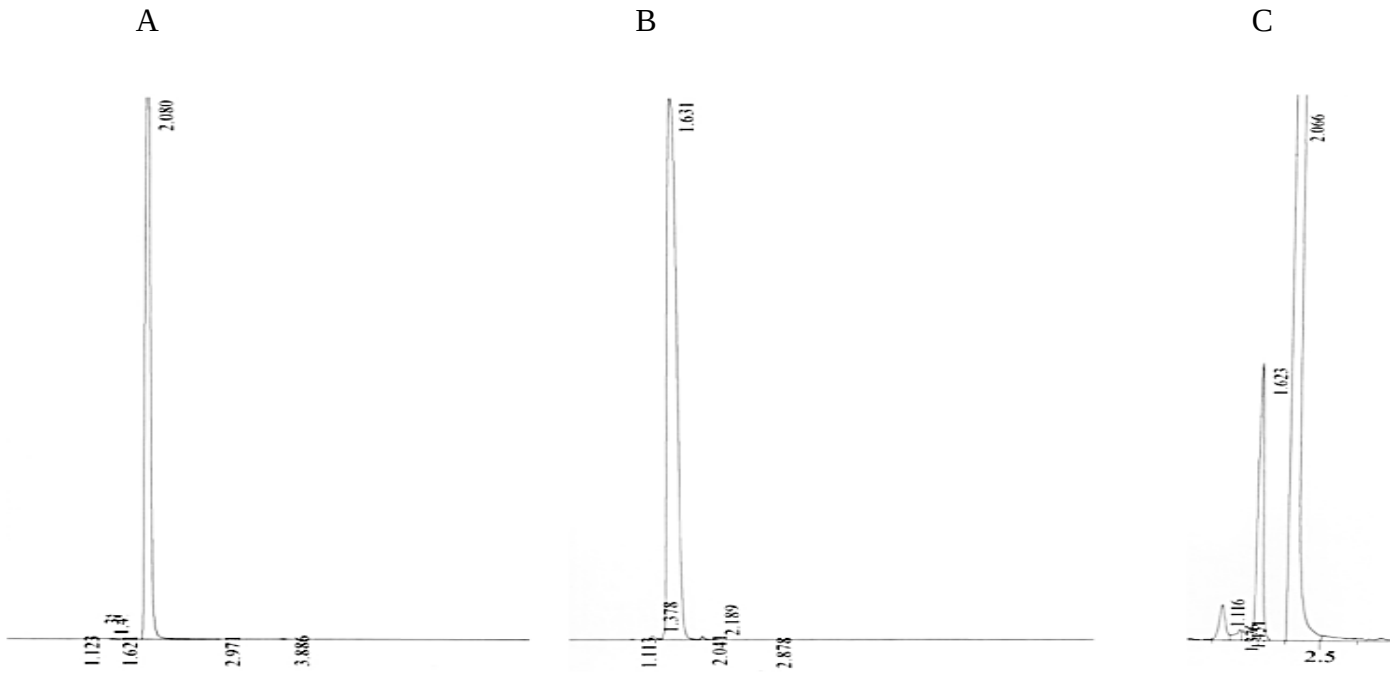
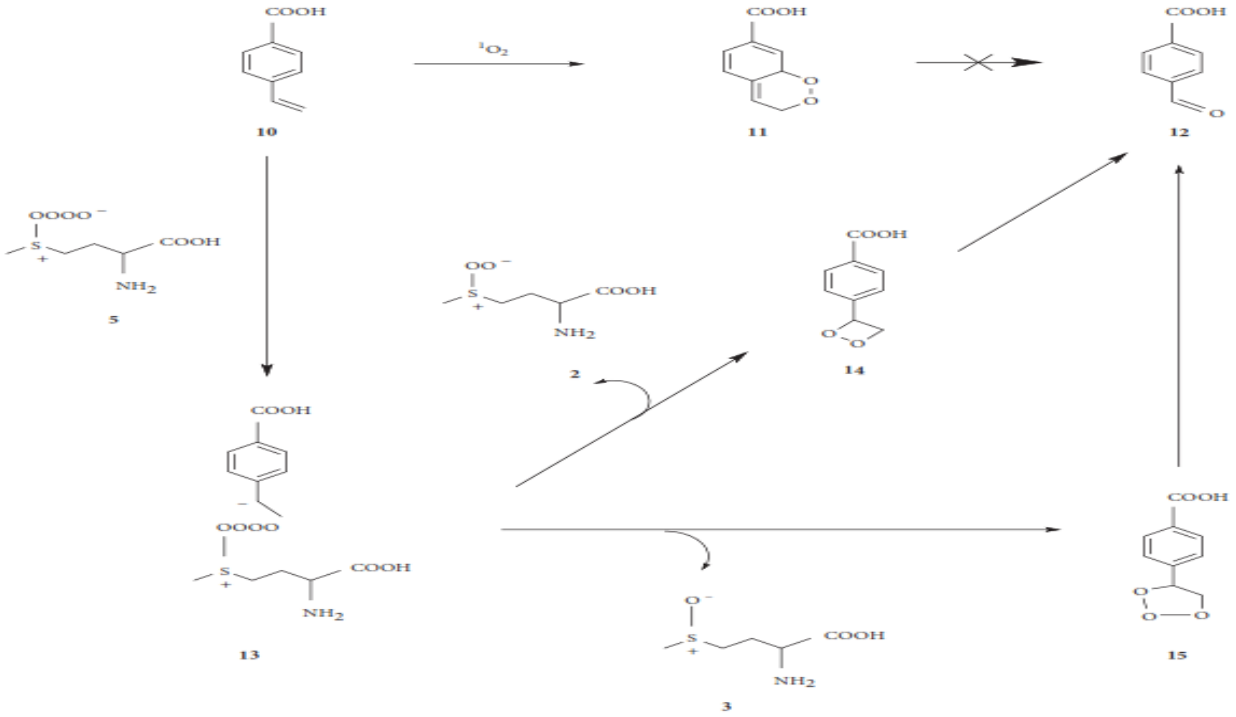


Figure 3.1: HPLC chromatograms of vinyl benzoic acid (A), 4-carboxybenzoic acid (B) and the reaction mixture obtained by incubating vinyl benzoic acid and methionine sulfoxide with a singlet oxygen-generating myeloperoxidase system (C), showing some conversion of vinyl benzoic acid to 4-carboxybenzaldehyde. Incubating methionine sulfoxide with vinylbenzoic acid and the myeloperoxidase system gave a similar chromatogram.



Scheme 3.3: Postulated involvement of polyoxidic methionine derivative 5 as an ozone-like oxidant in the conversion of vinyl benzoic acid 10 to 4-carboxybenzaldehyde 12, via dioxetane 14 or ozonide 15; and the direct reaction of vinyl benzoic acid 10 with singlet oxygen to form peroxide 11 which does not produce 4-carboxybenzaldehyde 12.

Key 2-methionine perusulfoxide, 3-methionine sulfoxide, 5-tetroxide anion, 10-vinyl benzoic acid, 11-peroxide, 12- 4-carboxybenzaldehyde, 13-cabarnionic intermediate, 14-dioxietane, 15-primary ozonide

3.5 Conclusion

Evidence from this study indicates that methionine and its oxidation product methionine sulfoxide, reacts with singlet oxygen to form an ozone-like oxidant. Therefore this provides scientific data in supporting the hypothesis that biological ozone or ozone-like oxidant formation involves the sequential reaction of singlet oxygen with amino acids such as methionine and amino acid oxidation products such as methionine sulfoxide.

CHAPTER FOUR

LYSINE REACTS WITH CHOLESTEROL HYDROPEROXIDE TO FORM SECOSTEROL ALDEHYDE ADDUCTS

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4.1 Abstract

Two cholesterol secosterol aldehydes, namely 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (secosterol A) and its aldolization product 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxyaldehyde (secosterol B) are highly bioactive compounds which have been detected in human tissues and potentially contribute to the development of physiological dysfunctions such as atherosclerosis, Alzheimer's disease, diabetes and cancer. These aldehydes were considered exclusive products of cholesterol ozonolysis, and therefore served as evidence for endogenous ozone formation. However, it was recently postulated that primary amines such as lysine may catalyse their formation from cholesterol-5α-hydroperoxide (Ch-5α-OOH), the main product of the oxidation of cholesterol with singlet oxygen. This involves cyclization of Ch-5α-OOH to an unstable dioxetane intermediate which decomposes to form secosterol aldehydes with triplet carbonyl groups whose return to the singlet state is at least partly coupled to the conversion of triplet molecular oxygen to singlet oxygen. Cholesterol was subjected to photosensitized oxidation using ultraviolet light with methylene blue as the photo-sensor. The generated cholesterol hydroperoxides were exposed to lysine in the presence of the antioxidant 2,6-ditertiary- butyl-4-hydroxytoluene (BHT). The reaction mixtures were analyzed by liquid chromatography-electrospray ionization-mass spectrometry. The secosterol aldehydes were detected and several types of lysine adducts, including carbinolamines, Schiff's bases and amide-type adducts were also detected. From the findings, it is proposed that the amide type adducts, which are major biomarkers of lipid oxidation, are mainly formed by singlet oxygen-mediated oxidation of the carbinolamine adducts.

Key words: Hydroperoxide decomposition, dioxetane, amide-type adducts, LC-ESI-MS

4.2 Introduction

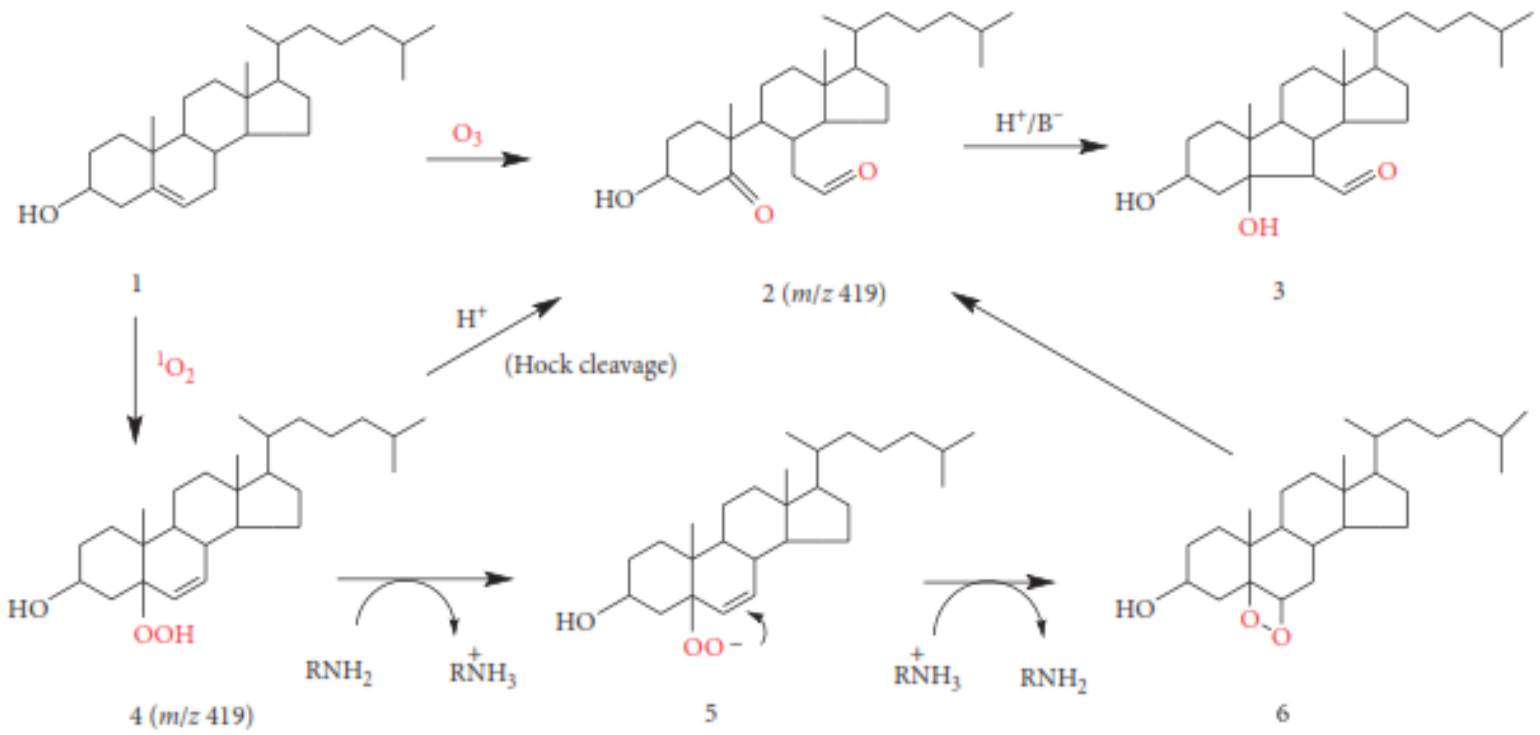
Cholesterol (1 in Scheme 4.1) is an important component of animal cell membranes, but cholesterol oxidation products, formed either in food or *in vivo* have been implicated as contributors to various non-communicable diseases (Staprans *et al*., 2000 ; Soterro *et al*., 2009). Direct evidence for endogenous ozone production in human tissues was cited when cholesterol ozonolysis products, 3β-hydroxy-5-oxo-5,6-sechoclestan-6-al (secosterol A, 2) and the aldolization product 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxyaldehyde (secosterol B, 3) were identified in human atherosclerotic plague and brain tissue of Alzheimer's disease patients (Wentworth *et al*., 2003). Such endogenous ozone production was suggested to involve antibody or amino acid-

catalysed oxidation of water by singlet oxygen (Wentworth *et al*, 2003; Babior *et al*, 2003, Yamashita *et al*, 2009), or more recently through multiple reactions of singlet oxygen with amino acids (Onyango, 2016a; Wanjala *et al*, 2018).

On the other contrary, cholesterol-5α-hydroperoxide **4** (the major product of the reaction of cholesterol with singlet oxygen) was found to be readily converted to secosterol B **3** under acidic conditions. The cholesterol-5α-hydroperoxide **4** was initially converted to secosterol A **2** by Hock cleavage, and the secosterol A **2** rapidly underwent aldolization to form secosterol B **3** (Scheme 4.1). Thus ozone may not therefore be necessary for the formation of cholesterol secosterol aldehydes *in vivo* (Brinkhorst, 2008). However, it was later argued that since ozone largely converts cholesterol to secosterol **2**, which is also the major secosterol aldehyde in atherosclerotic plagues, endogenous ozone rather than Hock cleavage of cholesterol-5α-hydroperoxide **4** should be important for secosterol formation *in vivo* (Wentworth *et al*, 2009). Nevertheless, Tomono *et al*. (2011) found that roughly equal amounts of secosterol A and secosterol B were formed by human myeloperoxidase independently of antibody involvement and suggested that in this case singlet oxygen and possibly another oxidant, but not ozone was involved in both secosterol A and secosterol B formation. In addition, it was recently postulated that lysine (RNH_2 in Scheme 4.1) may catalyse the conversion of cholesterol hydroperoxide **4** to secosterol A **2**, via peroxy anion **5** and dioxetane intermediate **6** (Scheme 4.1) (Onyango, 2017). This was based on an earlier report that lysine residues in proteins directly react with the 13- hydroperoxide of linoleic acid (13-LA-OOH) to form the amide-type adduct, N^{e}-(hexanoyl)-lysine (Kato *et al*, 1999), and the subsequent proposal that lysine initially catalyses decomposition of 13-LA-OOH to hexanal and 12-oxo-9,undecanoic acid, followed by reaction of lysine with hexanal to form the corresponding Schiff's base, and further reaction of the Schiff's base with a molecule of 13-LA-OOH to form the haxanoyl-lysine adduct (Onyango, 2016b).

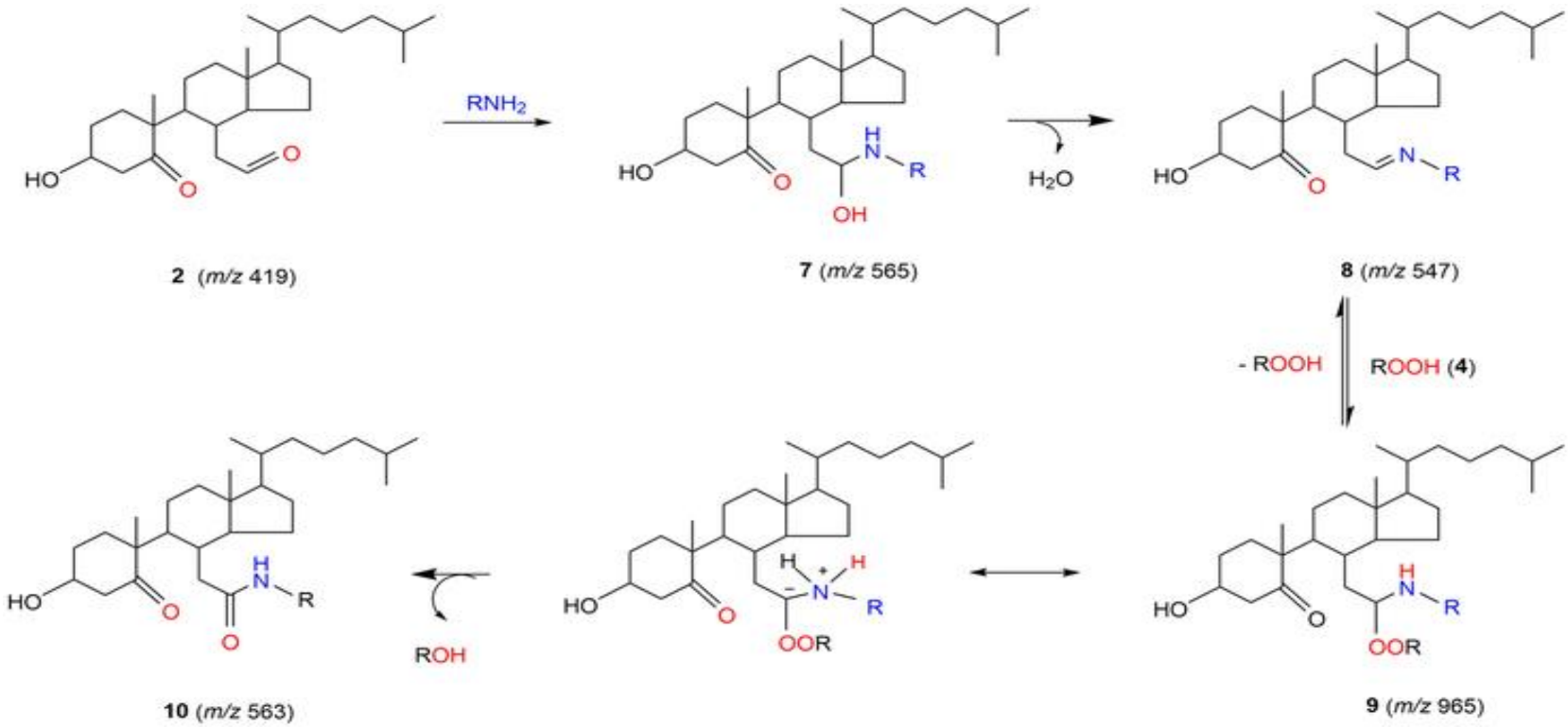
In an analogous manner, if lysine (RNH_2) catalyses conversion of cholesterol-5α-hydroperoxide **4** to secosterol A **2** (Scheme 4.1), the secosterol A **2** should react with

lysine to form an amide-type adduct. It is becoming increasingly evident that such amide-type adducts are readily formed both *in vitro* and *in vivo*, and could have major contributions to pathophysiological processes. For example, formyl lysine adducts formed by the reaction of formaldehyde with lysine residues have been detected in various types of proteins, including histone proteins, and could be involved in epigenetic modifications (Edrissi *et al*, 2012), while hexanoyl lysine and propentofylline adducts are considered as good biomarkers of lipid oxidation in food and *in vivo* (Minato *et al*, 2014a, Minato *et al*., 2014b, Hisaka *et al*, 2009). However, the mechanism of formation of these adducts is not well understood (Kato, 2014). According to the recently postulated pathway for amide-type adduct formation (Onyango, 2016b), secosterol **2** would react with lysine to successively form carbinolamine **7,** Schiff's base **8**, peroxide intermediate **9** and amide-type adduct **10** (Scheme 4.2). Analogously to the conversion of Ch-5a-OOH **4** to secosterol aldehydes **2** and **3** (Scheme 4.1), Cholesterol-6(α & β)-hydroperoxides **11** (Scheme 4.3) formed as minor products of cholesterol photooxidation (Girroti and Korytowsky, 2019) are expected to undergo lysine- catalyzed cyclization to dioxetane **6** and thus also afford secosterol aldehydes **2** and **3** (Scheme 4.3). In this study, the hypothesized lysine-mediated conversion of cholesterol-5α-hydroperoxide to secosterol aldehydes, and formation of adducts (Scheme 4.2) was tested.



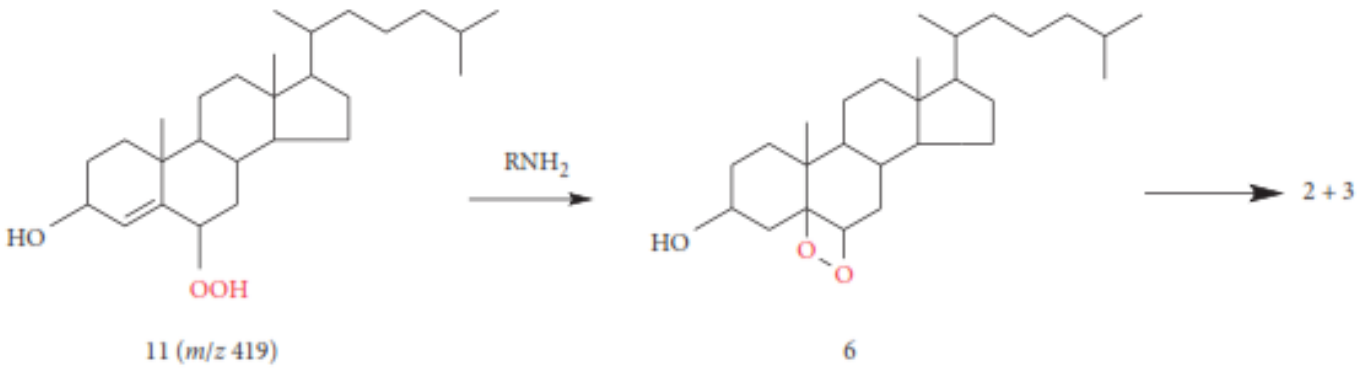
Scheme 4.1: Previously proposed pathways for formation of secosterol aldehydes 2 and 3.

Key: 1-cholesterol, 2–secosterol aldehyde (2) or (A), 3-secosterol aldehyde (3) or (B), 4–cholesterol-5α-hydroperoxide, 5 – peroxide, 6–dioxetane.



Scheme 4.2: Postulated reaction of secosterol aldehyde 2 with an amine (RNH_2) to form amide adduct 10 via carbinolamine 7, Schiff's base 8 and peroxide adduct 9. The expected masses of the protonated molecular ions are given in bracket, when RNH is from lysine.

Key: 2-secosterol aldehyde 2, 7-carbinolamine adduct, 8-Schiff's base, 9-peroxide adduct, 10-amide type adduct



Scheme 4.3: Expected lysine-catalysed conversion of cholesterol-6-hydroperoxide 11 (this structure is general for both 6a and 6b-hydroperoxides) to secosterol aldehydes 2 and 3.

Key: 11-cholesterol-6-hydroperoxide, 6-dioxetane, 2-secosterol 2, secosterol 3.

4.3 Materials and Methods

4.3.1 Reagents

Cholesterol, L-lysine, ethanolamine, formic acid, methylene blue, hexane, 2,4-dinitrophenylhydrazine (DNPH), ethyl acetate, 2-propanol and anhydrous sodium sulphate were purchased from Sigma-Aldrich.

4.3.2 Photosensitized oxidation of cholesterol

Cholesterol (2g) was dissolved in 10 ml of hexane containing 0.27mM methylene blue and irradiated at 10 ^{o}C with ultraviolet light of 366 nm (Funa UV, Light Model SL-800G), from a distance of 2.5 cm for 1 hour. The cholesterol hydroperoxides were purified by column chromatography on silica gel eluted with hexane/ethyl acetate (95:5). The fractions containing the hydroperoxide were confirmed by coloration with potassium iodide. After drying over anhydrous sodium sulfate, the solvent was evaporated *in vacuo* to obtain the cholesterol hydroperoxide (0.9g), which was dissolved in 2-propanol containing a trace of BHT (Regensburger *et al*., 2013).

4.3.3 Reaction of cholesterol-5-hydroperoxide with lysine

L-Lysine was added at 100µM to vials containing cholesterol hydroperoxide in 2-propanol (100 µM) and incubated for 1 hour at 37 ^{o}C. This mixture was filtered through a micron filter, after which a 20µl aliquot was drawn and analyzed by LC-MS (Wentworth, *et al*., 2003).

4.3.4 Derivatization of unreacted carbonyls with DNPH

After reacting lysine with cholesterol-5α-hydroperoxide for 1 hour, 10mM 2,4-DNPH in 0.1% formic acid were added to the reaction mixture and the reaction mixture incubated

at 37 ^{0}C for a further 1 hour prior to analysis by LC-ESI-MS (Wentworth, *et al*., 2003; Tomono *et al*., 2011).

4.3.5 Analysis of products by LC-ESI-MS

LC-MS analysis was done on a Waters 2790 separations module connected to a Micromass Quattro Ultima MS equipped with an electrospray ionization interface (Micromass UK Ltd, Floats Road, Wythenshawe, Manchester, UK). Separation was achieved using Supelco column (150mm*4.6mm*5µm) (Supelco Analytical, North Harrison Road, Bellefonte, USA) eluted with acetonitrile:water (70:30) at flow rate of 0.1ml-0.3 ml/minute. MS data was taken in the positive ion mode. The data was collected and analyzed by Masslynx 4.1 software (Waters, USA) (Wentworth, *et al*., 2003; Tomono *et al*., 2011).

4.4 Results and Discussion

Cholesterol was subjected to photosensitized oxidation in the presence of methylene blue, and the hydroperoxide mixture thus obtained (Ch-5α-OOH and expected small amounts of Ch-6α-OOH and Ch-6β-OOH) was then reacted with lysine, in the presence of the antioxidant 2,6-ditertiary- butyl-4-hydroxytoluene (BHT) to limit free radical reactions. The reaction products were analyzed by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS), which readily gives molecular ion peaks.

The total ion chromatogram (TIC) obtained upon LC-ESI-MS analysis of the reaction mixtures of lysine and cholesterol hydroperoxide indicated the formation of many products. Therefore the extracted ion chromatograms were relied on for detection of the secosterol aldehydes and their adducts. Figures 4.1-4.4 show such chromatograms and matching mass spectra for some of the expected products (Scheme 4.2). These include results for detection of ions attributable to secoseterol aldehyde **2** (m/z 419) (Figure 4.1), carbinolamine **7** (m/z 565.19) (Figure 4.2), Schiff's base **8** (m/z 547) (Figure 4.3) and

amide adduct **10** (563) (Figure 4.4). Analogous adducts were obtained when ethanolamine rather than lysine was reacted with cholesterol hydroperoxide (not shown). Carbinolamines are often considered to be too unstable for detection, and detection of carbinolamine **7** (Figure 4.2) was therefore not quite anticipated.

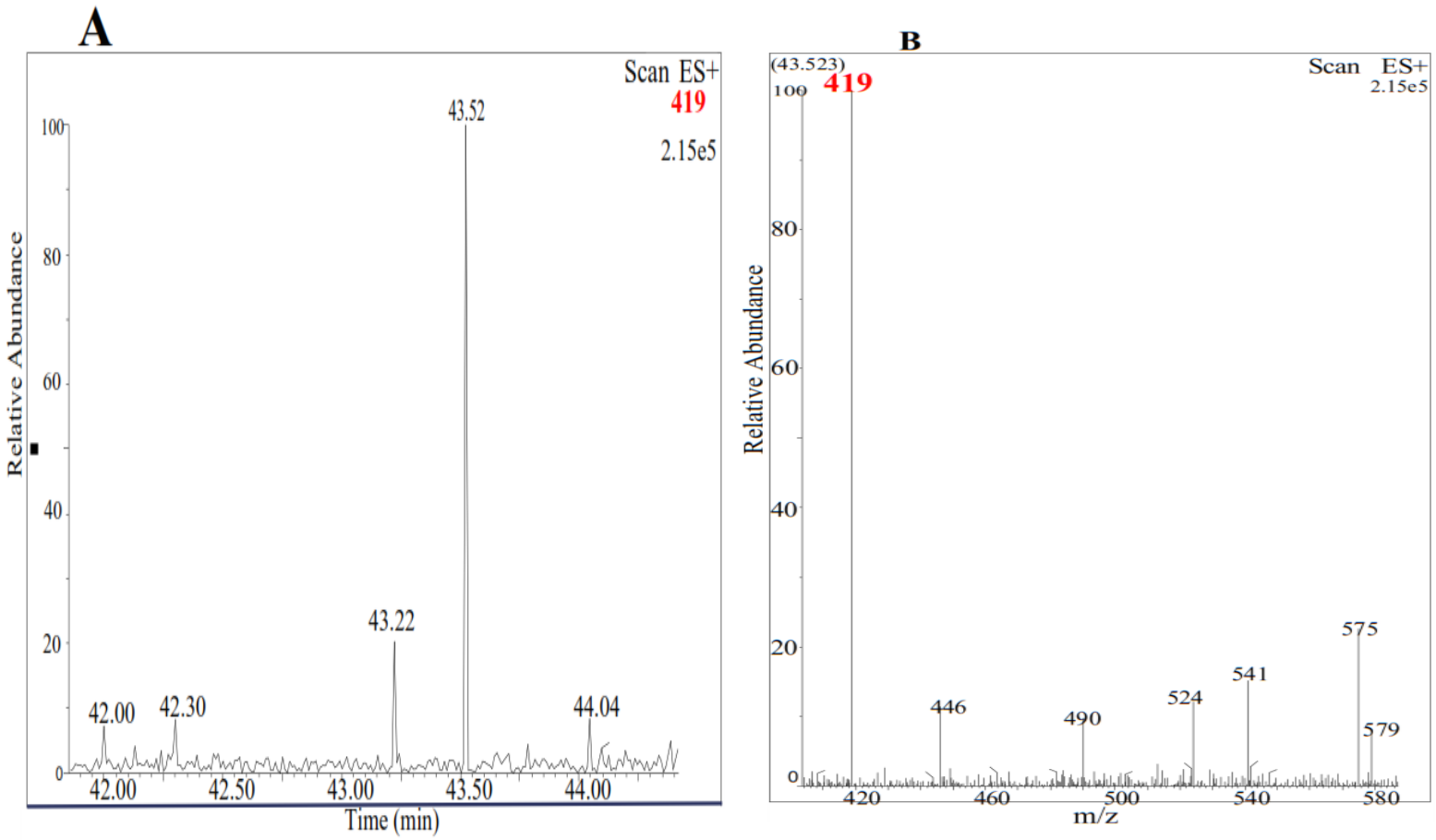


Figure 4.1. Extracted Ion chromatogram (A) and the corresponding mass spectrum (B) based on the protonated molecular ion at m/z 419 that could arise from unreacted cholesterol hydroperoxides 4 or secosterol aldehydes 2 and 3.

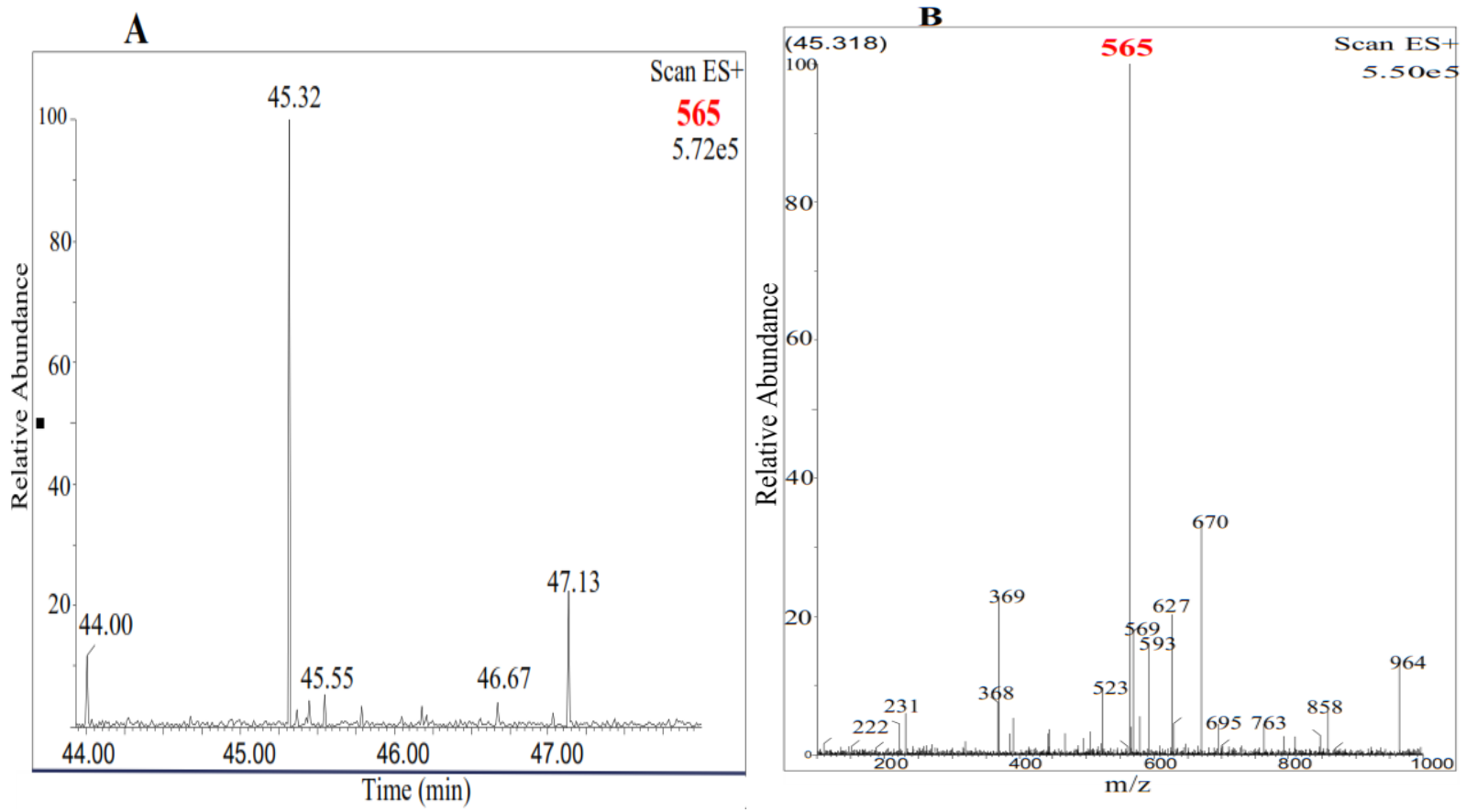


Figure 4.2: Extracted Ion chromatogram (A) and the corresponding mass spectrum (B) based on the protonated molecular ion at *m/z* 565 that could arise from carbinolamine 7 or the corresponding carbinolamines from secosterol aldehyde 3.

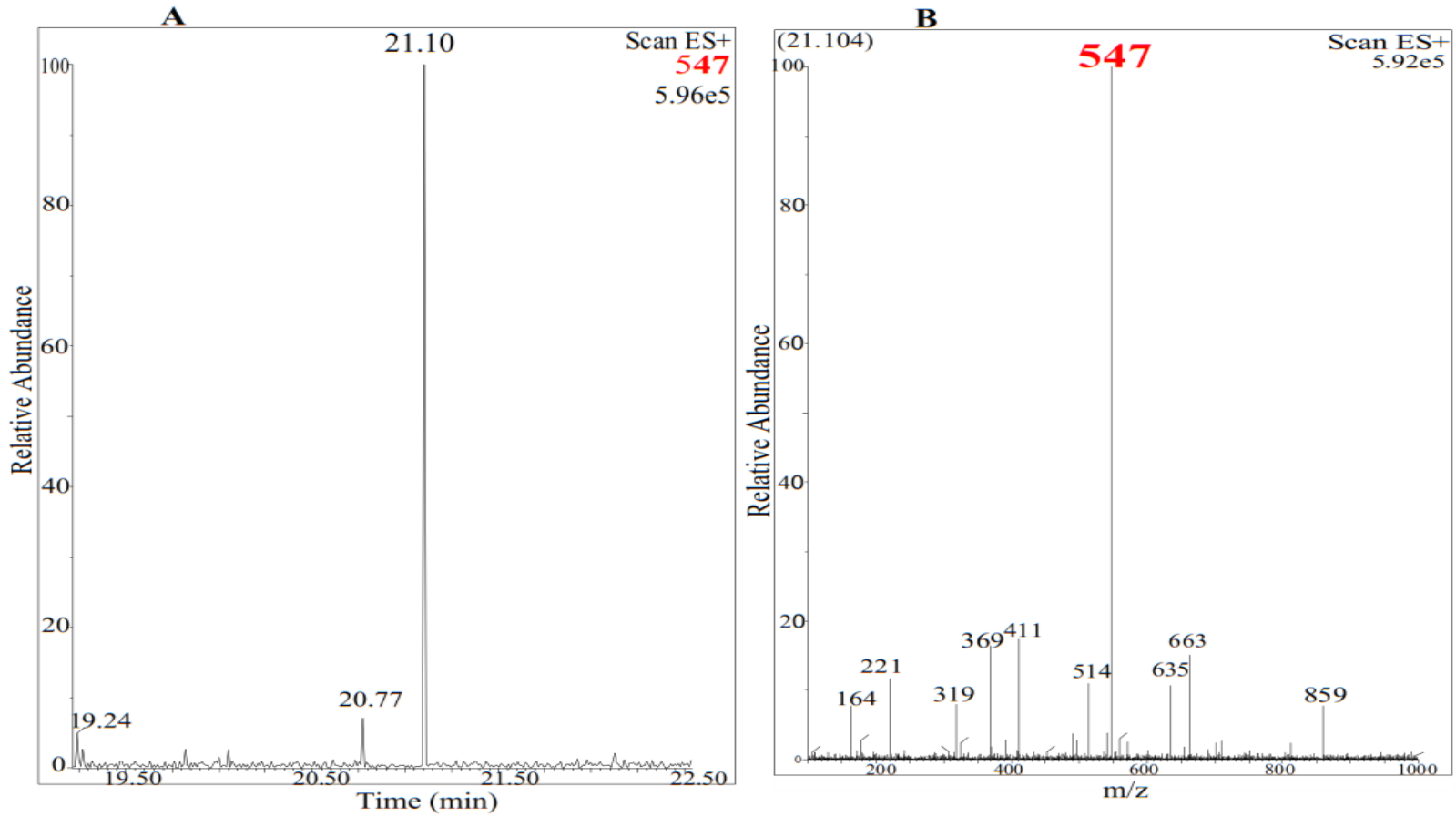


Figure 4.3: Extracted Ion chromatogram (A) and the corresponding mass spectrum (B) based on the protonated molecular ion at *m/z* 547 that could arise from Schiff's base 8 and/or the corresponding Schiff's base derived from secosterol aldehydes 3.

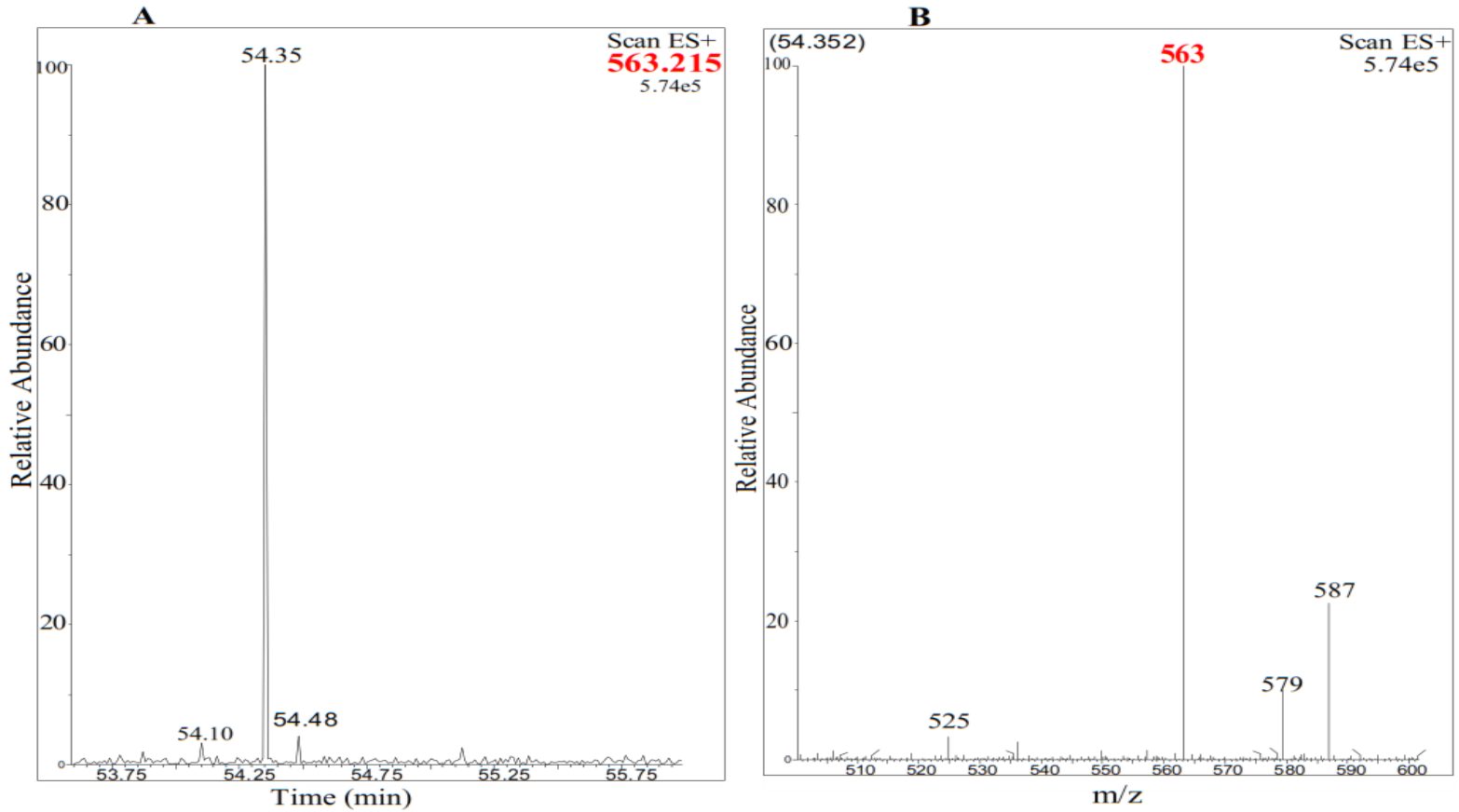
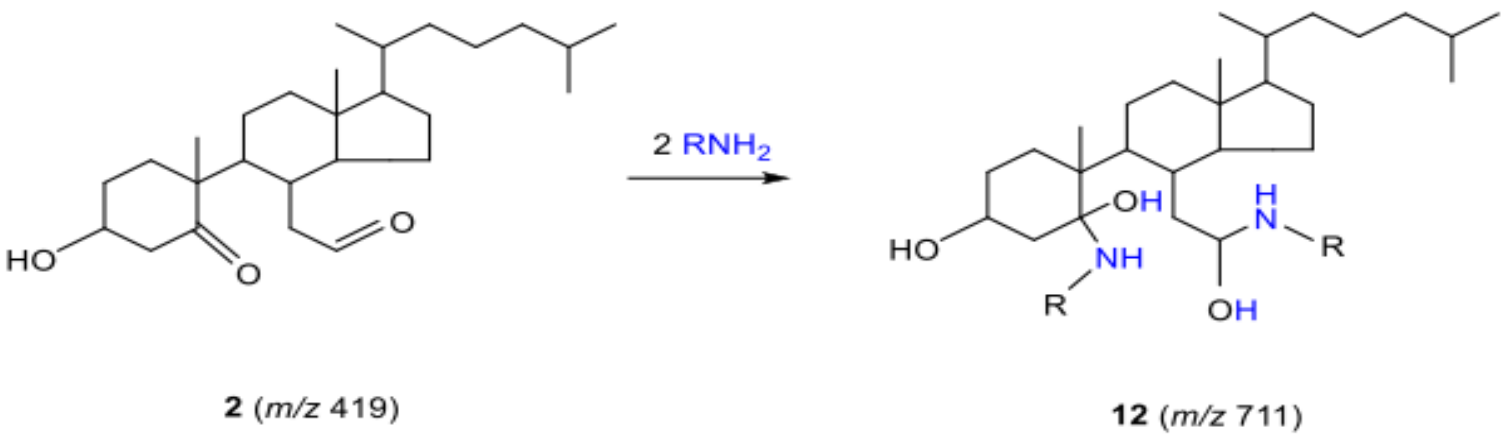


Figure 4.4: Extracted Ion chromatogram (A) and the corresponding mass spectrum (B) based on the molecular ion at *m/z* 563 that could arise from amide adduct 10 or from the corresponding amide adduct from secosterol aldehydes 3.

Nevertheless, there are previous reports on the observation of such species by proton nuclear magnetic resonance (HNMR) and mass spectrometry (Urbansky, 2000). 2,4-Dinitropheyl hydrazine (DNPH), which has a weight of 198.14g/mol is a widely used derivatization agent for aldehydes, and in this study, adducts consistent with the reaction of DNPH with secosterol aldehydes were found to form both the corresponding carbinolamine (*m/z* 617) and the Schiff's base (*m/z* 599) (not shown). It is noted that Figures 4.2-4.4 could also belong to analogous adducts formed from secosterol aldehyde **3**, since lysine is known to catalyse Aldol condensation (Zhang *et al*, 2010), and thus could have promoted some conversion of secosterol **2** to secosterol **3**. A key difference between these two compounds is that secosterol **2** has an additional carbonyl group at C-5, which is lacking in secosterol **3**. Hence, secosterol **2**, but not **3**, can react with two lysine molecules to form an adduct having two carbinolamine moieties, such as dicarbinolamine **12** (Scheme 4.4). Thus, detection of an ion peak at *m/z* 711, attributable to the dicarbinolamine **12** (Figure 4.5), specifically supports the formation secosterol **2**. An analogous dicarbinolamine containing one molecule of lysine and one molecule of DNPH was also obtained at *m/z* 763 (not shown).

The conversion of aldehydes to carbinolamines and Schiff's bases, such as the conversion of secosterol **2** to compounds **7** and **8**, respectively (Scheme 4.2) is well known. Although a molecular ion attributable to amide-type adduct **10** was observed at *m/z* 563.19 (Figure 4.4), none was observed for peroxide **9**. Hence, no direct evidence for the involvement of the latter in formation of **10,** according to Scheme 4.2, was obtained.



Scheme 4.4: Reaction of secosterol aldehyde 2 with two molecules of lysine to form dicarbinolamine 12.

Key: 2- secosterol 2, 12-dicarbinolamine.

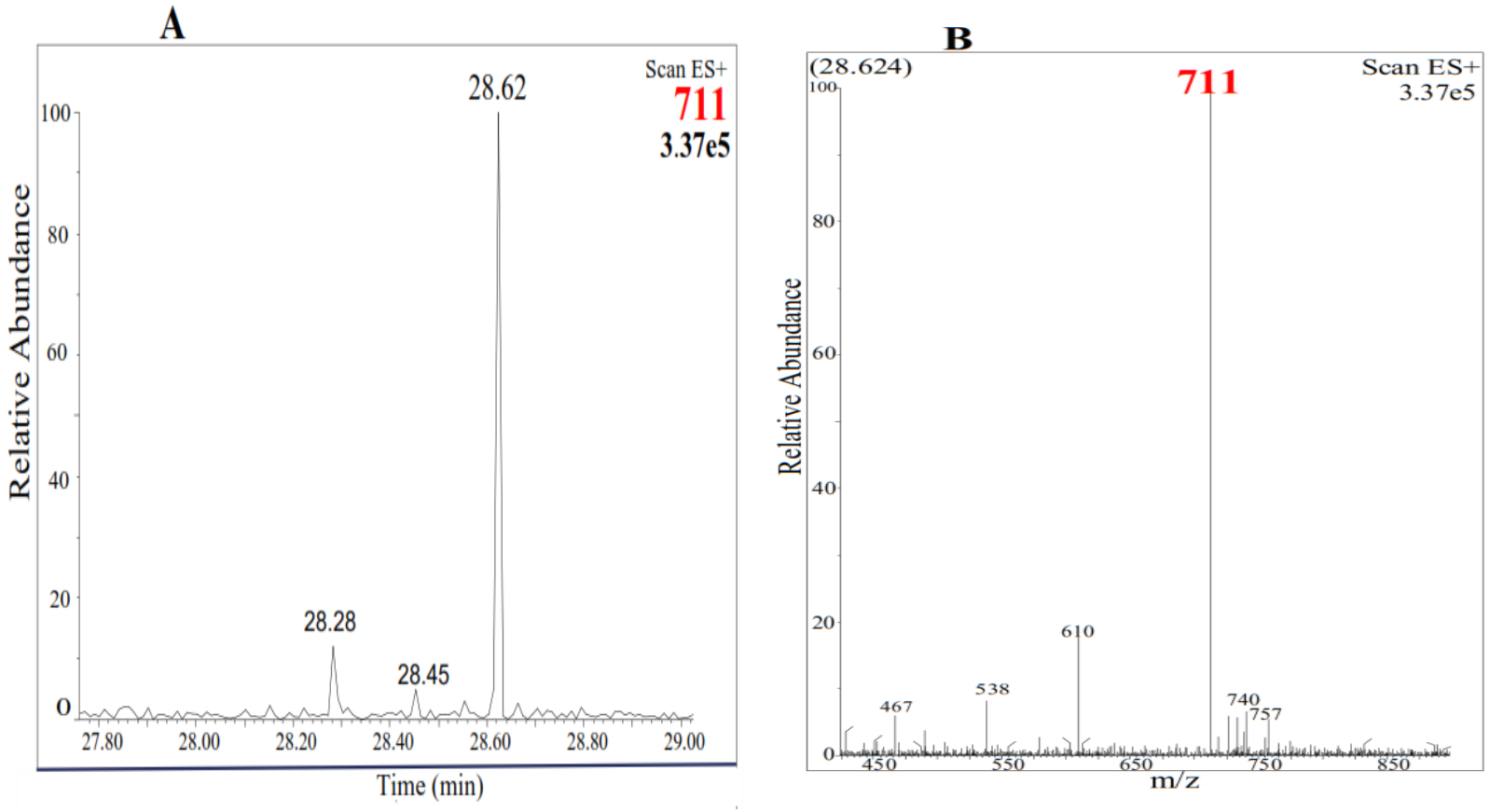
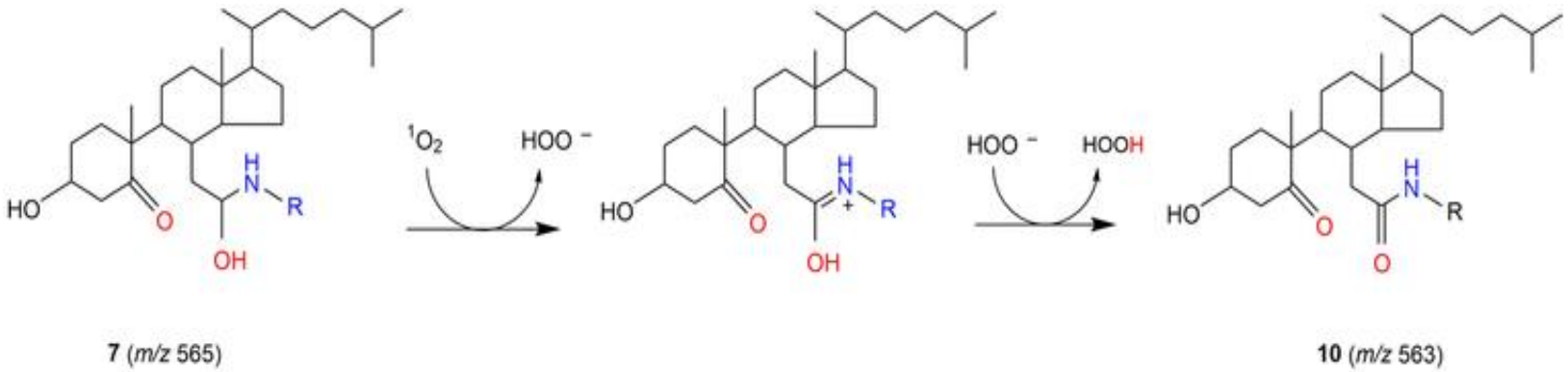


Figure 4.5: Extracted Ion chromatogram (A) and the corresponding mass spectrum (B) based on the molecular ion at *m/z* 711 that could arise from dicarbinolamine 12.

Kato *et al* (1999) found that the reaction of tertiary butyl hydroperoxide with hexanal and lysine did not produce the amide-type adduct, N^{e}-(hexanoyl)lysine, and suggested that aldehyde and hydroperoxide are not directly involved in amide-type adduct formation. Based on this, it was further postulated that linoleic acid hydroperoxide may be converted to a triplet ketone whose reaction with lysine produces the hexanoyllysine adduct (Kato, 2014). On the other hand, Trezl *et al* (1992) found that the reaction of formaldehyde or acetaldehyde with hydrogen peroxide in the presence of lysine led to the formation of triplet state formaldehyde or acetaldehyde, singlet oxygen, and the corresponding amide-type adducts, namely formyl lysine and acetyl lysine. However, they did not propose the mechanism of the reaction of the triplet formaldehyde or acetaldehyde with lysine to generate the amide-type adducts. Interestingly, it has been reported that singlet oxygen readily oxidizes amines to imines (Jiang *et al*, 2009). As suggested in Scheme 4.5, an analogous oxidation of carbinolamine **7** by singlet oxygen may well explain the formation of amide type adduct **10**. This is further consistent with the formation of dioxetane intermediate **6** in Scheme 4.1, because dioxetane decomposition produces triplet state carbonyls (one of the carbonyl groups in secosterol **2** formed by the lysine-mediated pathway in Scheme 1 may be in the triplet state), which transfer some of their energy to molecular oxygen to form singlet oxygen (Miyamoto *et al*, 2012; Onyango *et al*, 2016b). Such a mechanism is also in agreement with the results of Kato *et al* (1999) that the reaction of tert-butyl hydroperoxide with hexanal and lysine did not produce N^{e}-(hexanoyl)lysine, because no triplet carbonyls may be formed in that system, since tert-butyl hydroperoxide cannot cyclize into a dioxetane, unlike the unsaturated linoleic acid or cholesterol hydroperoxides.



Scheme 4.5: Proposed singlet oxygen ($^1O_2$)-mediated conversion of carbinolamine 7 to amide-type adduct 10.

Key: 7-carbinolamine adduct, 10-amide type adduct.

4.5 Conclusion

Lysine was found to react directly with cholesterol hydroperoxides to form secosterol aldehydes and various lysine-secosterol aldehyde adducts including amide-type adducts. The results are consistent with the lysine catalysed cyclization of cholesterol hydroperoxides into a dioxetanes as precursors of the secosterol aldehydes, and singlet oxygen-mediated oxidation of carbinolamines as a major source of the amide-type adducts.

CHAPTER FIVE

FORMATION OF HEXANAL AND 2-PENTYLFURAN DURING THE REACTION OF LYSINE WITH LINOLEIC ACID HYDROPEROXIDES

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5.1 Abstract

Lysine reacts with the 13-hydroperoxide of linoleic acid (13-hydroperoxy-9Z, 11E-octadecadienoic acid, 13-HPODE) to form N^{ε}-(hexanoyl)lysine (HEL), an amide-type adduct. It was recently suggested that the mechanism of this reaction involves an initial lysine-catalysed cyclization of 13-HPODE to a dioxetane that cleaves into hexanal as a precursor of HEL. However, the possible involvement of hexanal in this reaction was previously questioned. According to the same mechanism, other linoleic acid hydroperoxides; 9-hydroperoxy-10E, 12Z-octadecadienoic acid (9-HPODE), 10-hydroperoxy-8E, 12Z-octadecadienoic acid (10-HPODE) and 12-hydroperoxy-9Z, 13E-octadecadienoic acid (12-HPODE) obtained by the photosensitized oxidation of linoleic acid would decompose to form hexanal or (Z)-3-nonenal. Linoleic acid hydroperoxides contribute to off flavour development in lipid rich foods, reduce nutritional value of foods and contribute to pathogenesis of physiological disorders. Linoleic acid hydroperoxides were obtained by the photosensitized oxidation of linoleic acid using ultraviolet light and methylene blue as the photosensitizer. The hydroperoxides were reacted with lysine, and the organic fraction analyzed by gas chromatography-mass spectrometry (GC-MS). Hexanal was detected as a prominent product but (Z)-3-nonenal was not detected. However, 2-pentylfuran, a product of the cyclization of 4-hydroxy-2-nonenal (HNE), a highly cytotoxic aldehyde was detected. A pathway for the conversion of (Z)-3-nonenal to HNE as a precursor of 2-pentylfuran under these conditions is proposed. Thus, evidence for the lysine catalysed conversion of lipid hydroperoxides to various kinds of aldehydic products has been obtained.

Key words: Lipid oxidation, bioactive aldehydes, dioxetane, singlet oxygen, Schiff's base

5.2 Introduction

Lipid oxidation is associated with food deterioration and the pathogenesis of ageing-related physiological disorders such as diabetes due to insulin resistance (Gutierez, *et al*., 2017, Umeno, *et al*., 2013, Onyango, 2018). Lipid hydroperoxides, the primary products of lipid oxidation, decompose into many types of products. Volatile aldehydic products are major contributors to the development of off flavours in lipid-rich foods, while both volatile and non-volatile aldehydes lower the nutritional value of food by reacting with essential nutrients such as lysine and thiamine (Gutierez, *et al*., 2017, Domínguez, *et* al., 2019, Onyango, 2021). When ingested, or generated by lipid oxidation *in vivo*, some of

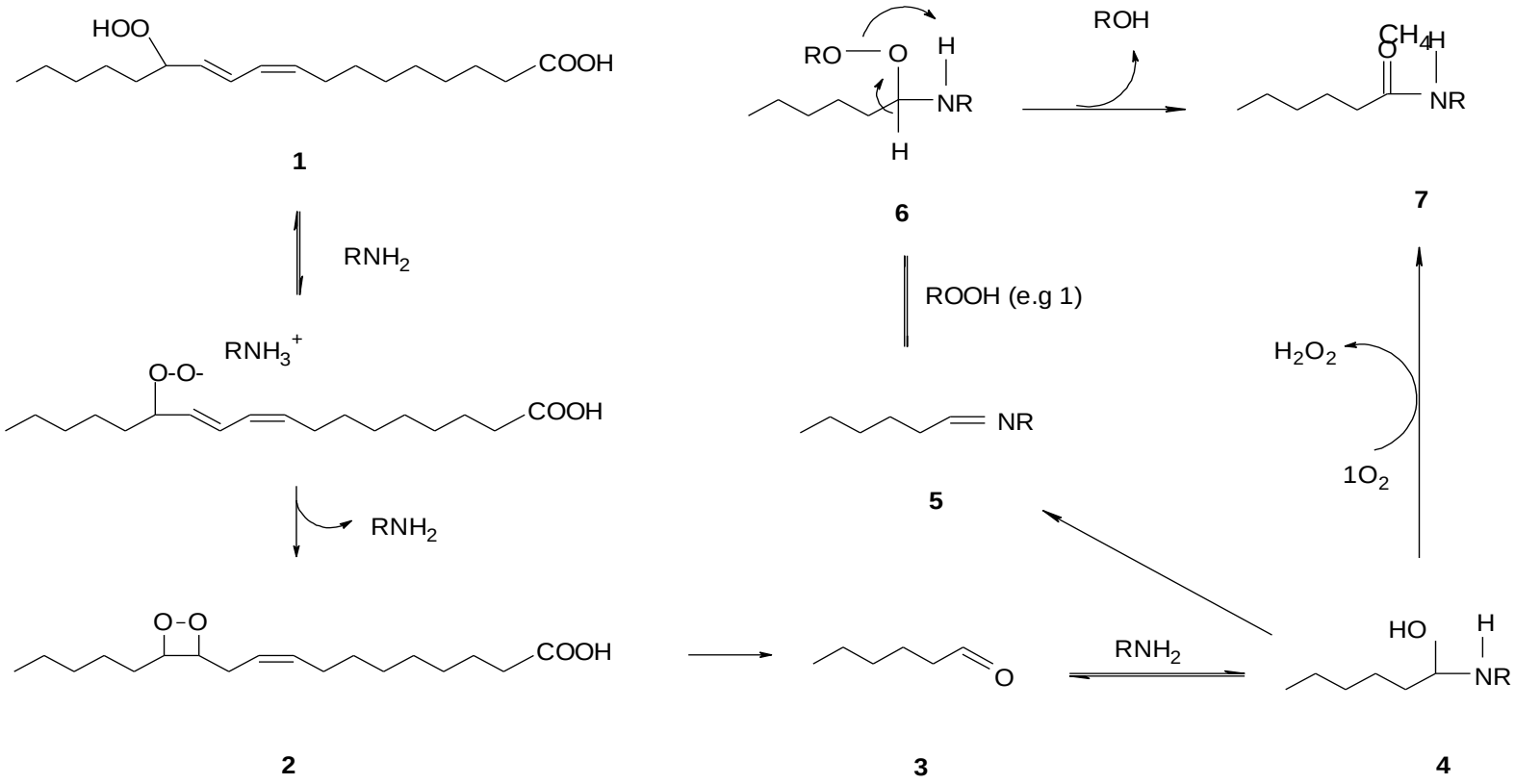
the aldehydic compounds such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) contribute to the pathogenesis of physiological disorders because of biological activities such as mutagenic and cytotoxic properties and induction of cellular oxidative stress (Ayala, *et al*., 2014, Onyango, 2017). An understanding of the mechanisms of formation of lipid oxidation products including the bioactive aldehydes is therefore important for designing strategies to prevent these reactions and thus contribute to food preservation and improved health.

Lipid hydroperoxides can be formed enzymatically or non-enzymatically. Non-enzymatic lipid oxidation occurs by the free radical mechanism (autoxidation) or by the reaction of lipids with singlet oxygen (Domínguez, *et* al., 2019, Onyango, 2017, Minami, *et al*., 2008, Onyango, 2016). The conversion of lipid hydroperoxides (whether formed by autoxidation of by singlet oxygen) to aldehydes is largely regarded to involve decomposition of hydroperoxides to alkoxyl radicals and other radical-dependent reactions (Onyango, 2012). Hence free radical scavengers are often used to prevent the formation of these products. On the other hand, in order to explain a previous finding that lysine reacts with 13-HPODE to form the amide-type adduct, N^{ε}-(hexanoyl)lysine (HEL) (Kato, *et al*., 1999), it was recently postulated that lysine initially catalyses the conversion of 13-HPODE **1** to form a dioxetane **2**, which decomposes to hexanal **3**, followed by reaction of **3** with lysine to form carbinolamine adduct **4**; which converts to Schiff's base **5**, which reacts with another hydroperoxide molecule (ROOH) to form peroxide **6**, which decomposes to form an alcohol (ROH) and HEL **7** (Onyango, 2016) (Scheme 1). If such a non-radical mechanism significantly contributes to the formation of aldehydes such as hexanal **3**, it would be necessary to consider more seriously other types of agents besides radical scavengers for minimizing their formation or their effects in foods or *in vivo*. Moreover, in cells, certain aldehydes promote physiological disorders such as insulin resistance, by inducing oxidative stress through signalling pathways involving activation of the transcription factor nuclear factor kappa B (NF-kB)

and NADPH oxidase, leading to intracellular generation of highly reactive species such as peroxynitrite and singlet oxygen (Onyango, 2016).

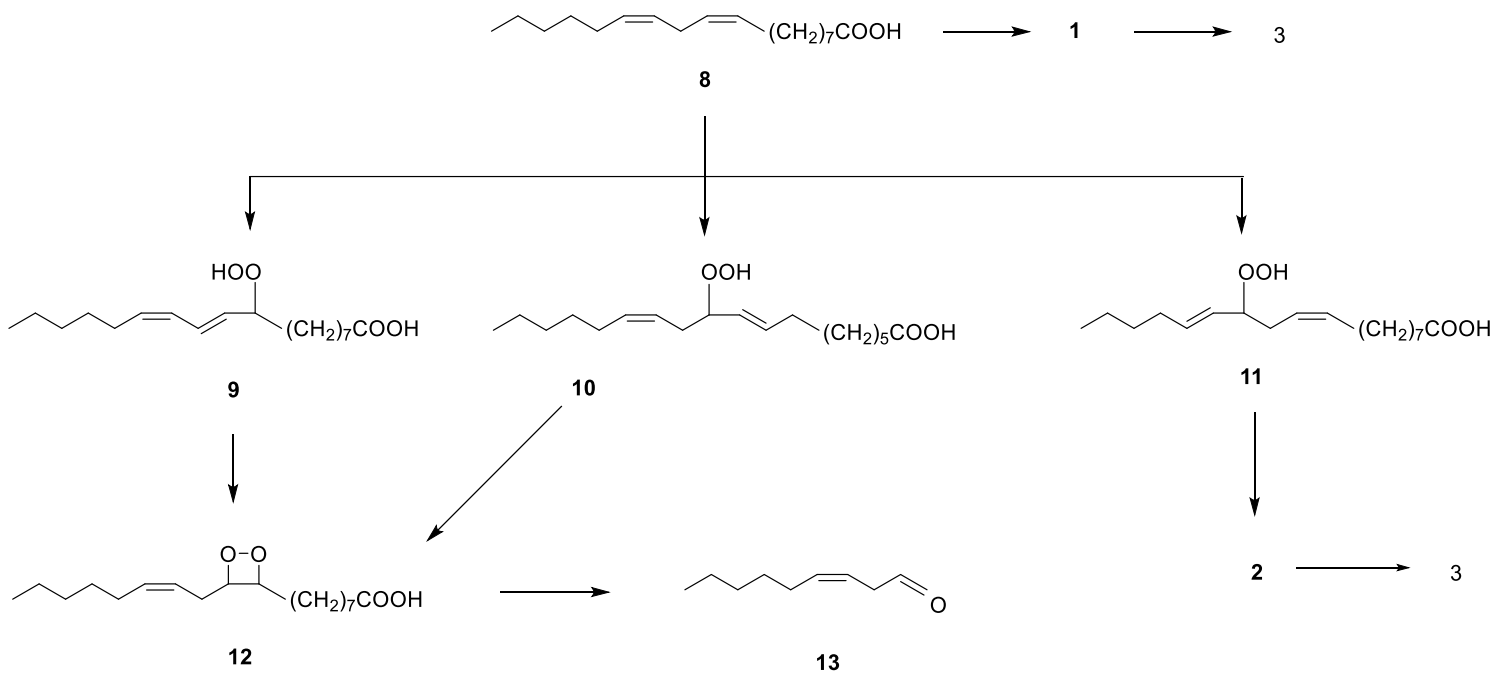
However, based on the finding that a reaction between butyl hydroperoxide, hexanal and lysine did not afford HEL, it was concluded that hexanal is not involved in the formation of HEL during the reaction of lysine with 13-HPODE (Kato, *et al*., 1999). On the other hand, it was recently reported that the reaction of cholesterol-5α-hydroperoxide with lysine leads to formation of cholesterol secosterol aldehyde (analogously to hexanal in Scheme 1) and an amide type adduct between cholesterol secosterol aldehyde and lysine (analogously to HEL) (Wanjala, *et al*., 2020). In that study, cholesterol adducts analogous to adducts **4**, **5** and **7** but not adduct **6** were detected. Thus **6** may be highly unstable, being readily converted back to Schiff's base **5**. Nevertheless, the conversion of **4** to **7** plausibly occurs by a direct oxidation of carbinolamine by singlet oxygen, formed as a result of the transfer of energy from triplet carbonyls (formed during dioxetane decomposition) to triplet oxygen (Wanjala, *et al*., 2020). Interestingly, while HEL was not formed in a system containing hexanal, lysine and tertiary butyl hydroperoxide (Kato, *et al*., 1999), a mixture of hydrogen peroxide (H_2O_2), hexanal and lysine was found to generate HEL (Ishino, *et al*., 2008). A major difference between these two systems is that the reaction of H_2O_2 with aldehydes such as hexanal can generate singlet oxygen (Onyango, 2016, Trezl, *et al*., 1992), while tertiary butyl hydroperoxide cannot generate singlet oxygen by such a mechanism. This supports an important role for singlet oxygen in formation of HEL during lysine catalysed 13-HPODE decomposition.

Thus, the aim of the present study was to determine the formation of hexanal and (Z)-3-nonenal during the reaction of lysine with linoleic acid hydroperoxides obtained by the photosensitized oxidation of linoleic acid as indicated in Scheme 5.1 and Scheme 5.2.



Scheme 5.1: Previously proposed pathways for the formation of hexanoyl-lysine as a product of the reaction of the 13-HPODE1 with lysine (RNH_2).

Key: 1 - 13-hydroperoxy-9Z, 11E-octadecadienoic acid, 2 – dioxetane, 3 – hexanal, 4 – carbinolamine, 5 – Schiff's base, 6 – Peroxide, 7 – amide type adduct



Scheme 5.2: The expected lysine-catalysed conversion of different linoleic acid regioisomers to hexanal 3 or (Z)-3-octenal 13.

Key: 1 - 13-hydroperoxy-9Z, 11E-octadecadienoic acid, 3 – hexanal, 8 - linoleic acid, 9 - 9-hydroperoxy-10E,12Z-octadecadienoic acid, 10 - 10-hydroperoxy-8E, 12Z-octadecadienoic acid, 11 - 12-hydroperoxy-9Z, 13E-octadecadienoic acid, 12 – dioxetane, 13 – (Z)-3-nonenal.

5.3 Materials and Methods

5.3.1 Materials and reagents

Linoleic acid, lysine, methylene blue, hexanal and 2-pentlyfuran were purchased from Sigma Aldrich.

5.3.2 Synthesis of linoleic acid hydroperoxides from pure linoleic acid by photooxidation

Linoleic acid (5g) was dissolved in 10 ml of ethanol containing 0.27mM methylene blue and irradiated at 10 ^{o}C with ultraviolet light of 366 nm (Funa UV, Light Model SL-800G), from a distance of 25 cm for 1 hour. The hydroperoxides were purified by column chromatography on silica gel eluted with hexane/ethyl acetate (95:5). However, no attempt was made to isolate the isomers from one another.

5.3.3 Reaction of linoleic acid hydroperoxides with lysine and detection of volatile compounds

The hydroperoxide mixture (0.6M in diethyl ether) was mixed with L-lysine (0.3M, 0.6M and 1.2M) in sealed 10 ml vials. A control vial had hydroperoxide but not lysine. After shaking the vials for 2 minutes and/or incubating them at 37 ^{o}C for 30 minutes while shaking, 2μL aliquot of the reaction mixture was subjected to GC-MS (electron impact ionization at 70 ev) analysis on Shimadzu GC-MS-QP2010 SE equipped with a BPX5 (SGE Analytical Science) column (30mm*0.25mm*0.25μm), with helium carrier gas at a flow rate of 1mL/min, the column temperature being programmed from 50 ^{o}C (2 min) to 150 ^{o}C (1 min) at 5 ^{o}C/min. Injector and detector temperatures were 240 ^{o}C and 280 ^{o}C, respectively (Chambers *et al*., 2009, Kukuta, *et al*., 2013).

5.4 Results and Discussion

Lysine was found to promote conversion of the fatty acid hydroperoxides to decomposition products despite the presence of the radical scavenging antioxidant BHT. Figure 5.1 shows a typical Total Ion Current (TIC) chromatogram of volatile compounds formed during the reaction of lysine with the mixture of linoleic acid hydroperoxides after incubation in the presence of BHT. At the same time, minimal product formation was detected for a reaction without lysine (not shown). The peaks at retention time 4.45 min and 9.19 minutes were identified as hexanal **3** and 2-pentylfuran, respectively, based on their MS spectra (Figure 5.2) which were identical to the NIST library spectra for these compounds. The mass spectrum for the peak at retention time 8.46 (Fig 5.1) had the NIST spectrum for 2-nonenal as a possible match, but these spectra were not identical. Thus, the identity of this peak requires further confirmation.

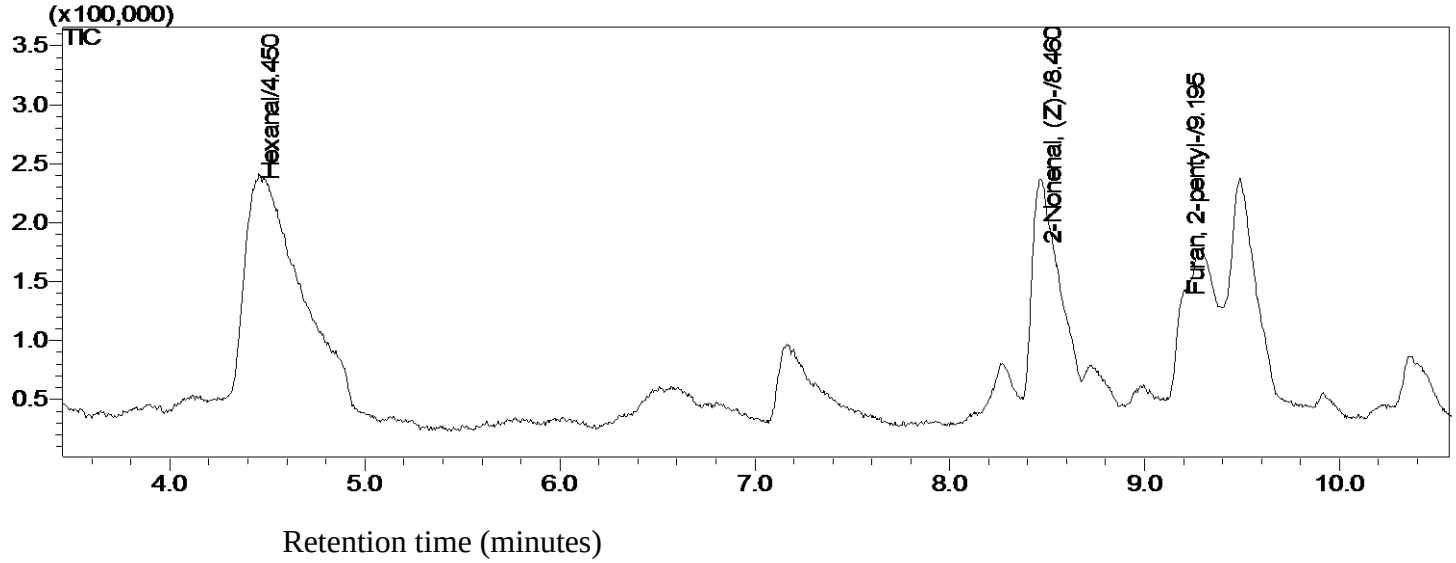
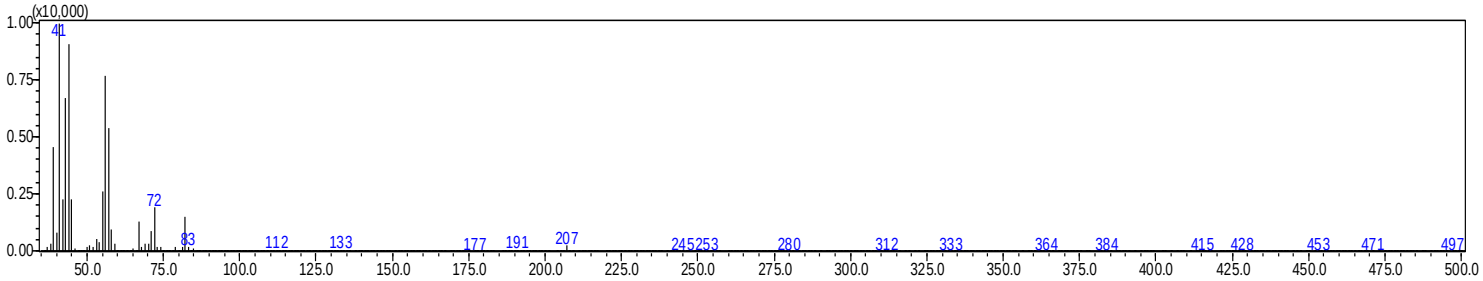
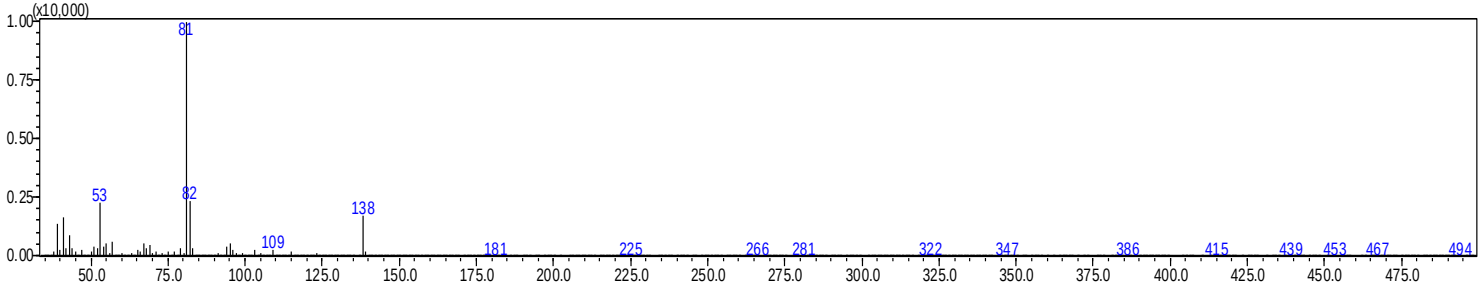


Figure 5.1: TIC chromatogram of the reaction mixture of linoleic acid hydroperoxides with lysine obtained after 30 minutes of reaction, showing peaks for hexanal at retention time 4.45 minutes and pentylfuran at retention time 9.195.

a



b



m/z

Figure 5.2: MS spectra of compounds identified as hexanal (a), and 2-pentylfuran (b) in Figure 5.1.

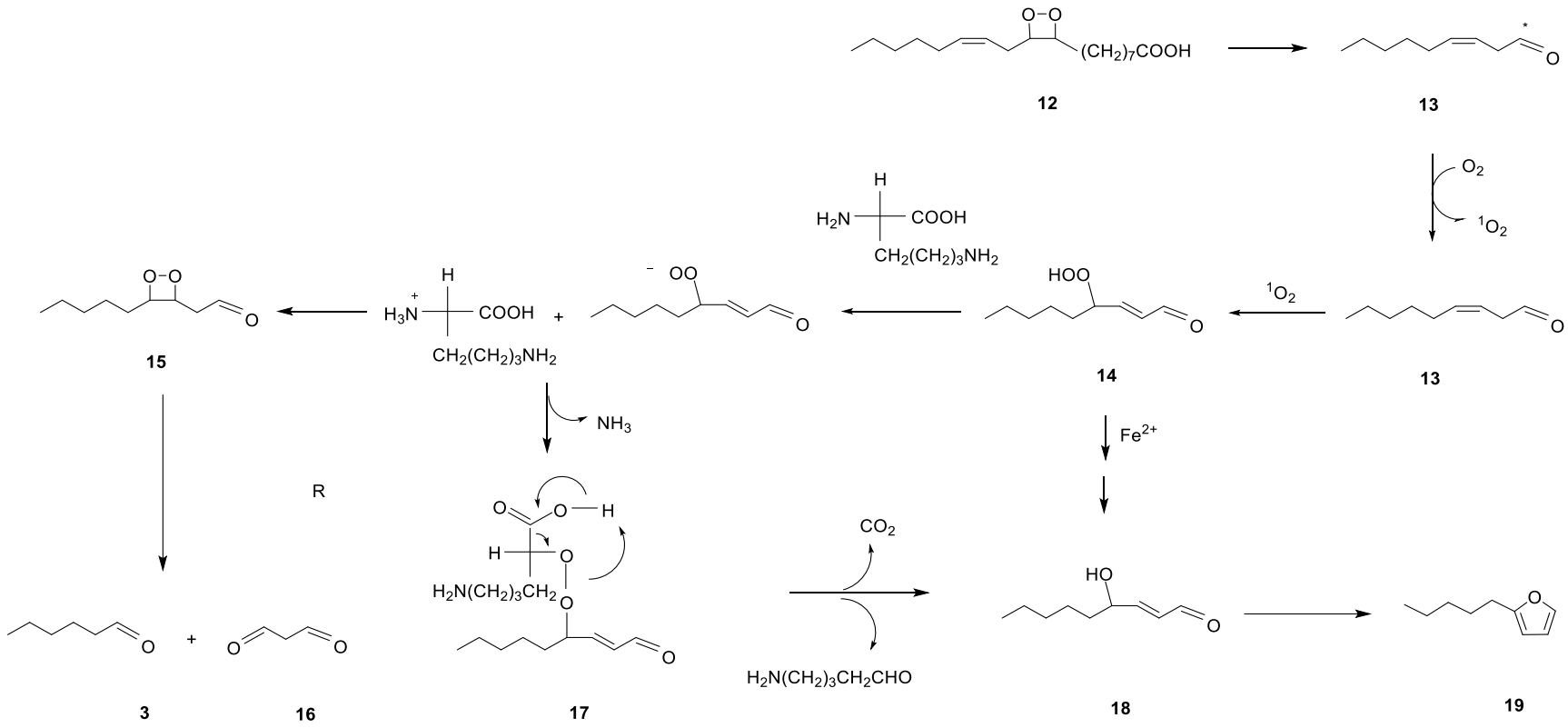
The detection of hexanal **3** from this reaction is consistent with its formation from 13-HPODE **1** and 12-HPODE **11** (Schemes 5.1 and 5.2). The detection of 2-pentylfuran but not the expected (Z)-3-nonenal **13** can be explained by conversion of the (Z)-3-nonenal **13** to 2-pentylfuran, according to Scheme 5.3. This is because, when dioxetanes such as **12** decompose, carbonyl products such as (Z)-3-nonenal **13** are formed in the excited triplet state, which transfers energy to triplet oxygen, thus generating singlet oxygen (Scheme 5.3). In the case of (Z)-3-nonenal **13**, the singlet oxygen is generated in proximity to a double bond, which gives a high probability for the singlet oxygen-mediated conversion of **13** to 4-hydroperoxy-2-nonenal (HPNE, **14)**. A further reaction of HPNE **14** with lysine can generate dioxetane **15,** whose decomposition produces hexanal **3** and malondialdehyde (MDA) **16**.

During lipid oxidation in the absence of lysine, MDA is well known as a product from polyunsaturated fatty acids with at least three double bonds, such as alpha linolenic acid, but not linoleic acid (Onyango and Baba, 2010, Martin-Rubio, *et al*., 2019). However, this product has been found as a major product of the linoleic acid oxidation in the blood, or decomposition of **14** (a major product of linoleic acid) in the presence of lysine (Onyango, 2017, Shimozu, *et al*., 2011), which strongly favours lysine catalysed formation of dioxetane **15** (Onyango, 2017). Malondialdehyde is highly reactive and not so volatile, hence its detection by GC-MS may not have been possible. In a previous study by LC-MS, malondialdehyde-lysine adducts, but not free malondialdehyde, were detected during the decomposition of soybean oil in the presence of lysine (Martin-Rubio, *et al*., 2019).

The reaction of amino acids with hydrogen peroxide (H_2O_2) leads to deamination and decarboxylation of the amino acids to form aldehydes (Yamanaka, *et al*., 1979). Thus, it is likely that the organic hydroperoxide HPNE **14** may undergo an analogous reaction with lysine to form an intermediate **17**, which decomposes to form 4-hydroxy-2-nonenal **18**, as well as 5-aminopentanal **[**$H_2NCH_2(CH_2)_3CHO$], the decarboxylation and

deamination product of lysine (Scheme 3). HNE **18** then cyclizes to form 2-pentylfuran **19** (Spickett, 2013, Onyango, 2012), and this cyclization can also be catalysed by lysine (Adams, *et al*., 2018). Such a mechanism for the conversion of HPNE **14** to HNE **18** is consistent with the recent report that lysine promotes the reduction of hydroperoxides to alcohols and also that there is reduction in the amount of lysine during lipid oxidation (Martin-Rubio, *et al*., 2019).

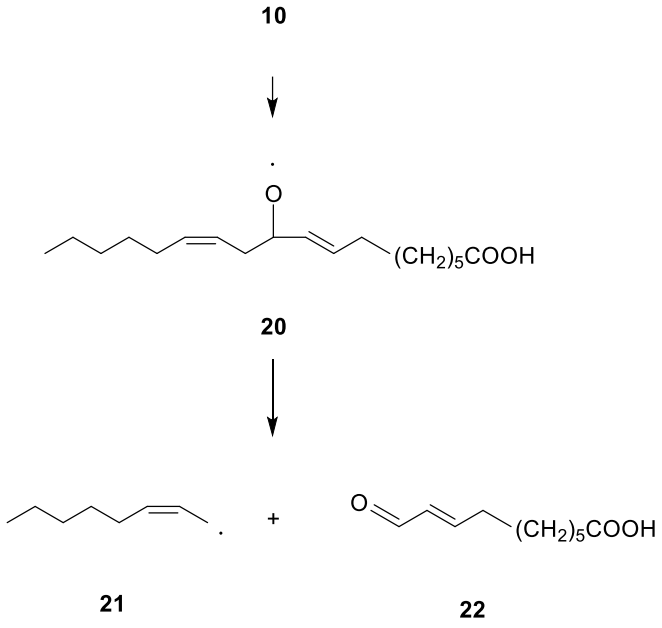
Ferrous ions (Fe^{2+}) can convert HPNE **14** to an alkoxyl radical (not shown), which in the presence of an antioxidants such as BHT would be converted to 4-hydroxy-2-nonenal **18**. However, lysine has been reported to have antioxidant activity through metal chelation and/or radical scavenging (Martin-Rubio, *et al*., 2019, Xu, *et al*., 2018). Hence the metal chelation by lysine might make conversion of HPNE to HNE via an alkoxy radical to be of less importance.



Scheme 5.3. Suggested pathway for the formation of 2-pentylfuran 19 via 3-nonenal and 4-hydroxy-2-nonenal during lysine-catalysed decomposition of 9-HPODE 9 and 10-HPODE 10 via dioxetane 12.

Key: 12-Dioxetane, 13-(*Z*) 3-nonenal, 14-4-hydroperoxy-2-nonenal, 15-dioxetane, 3-hexanal, 16-malondialdehyde, 17-carbonionic intermediate, 18-4-hydroxy-2-nonenal, 19-2-pentylfuran.

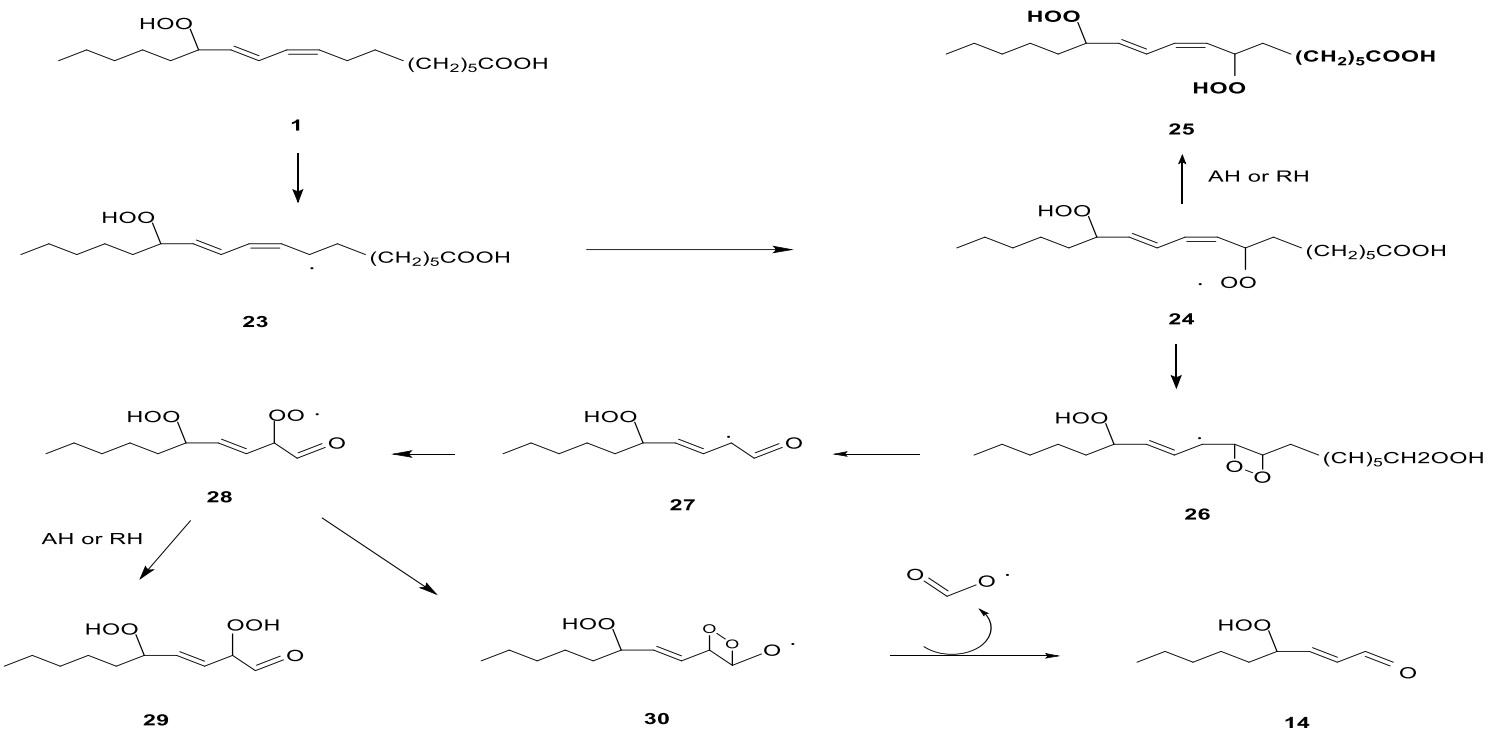
Other reasons why HNE formation by autoxidation is expected to be low under the conditions of our study; although it is perhaps not possible to completely prevent free radical reactions during the decomposition of hydroperoxides are that during autoxidation, (i) even if traces of metal ions were to promote autoxidation, 10-HPODE **10** is expected to undergo Fe^{2+} catalysed conversion to an alkoxyl radical **20** (Scheme 5.4) which readily cleaves to octene radical **21** and 10-oxo-9-decenoic acid **22**, because formation of allylic radicals such as **21** is energetically favourable (ii) similarly, 12-HPODE **11** is expected to be converted to 2-heptenal and an allylic radical (iii) such autoxidative formation of HNE is known to mainly proceed via 13-HPODE **1** and only minimally from 9-HPODE **9** (Schneider, *et al*., 2001, Lee, *et al*., 2001).



Scheme 5.4: The expected fascile conversion of 10-HPODE 10 to octene radical 21 and 10-oxo-9-decenoic acid 22 during autoxidation.

Key: 10-10-hydroperoxy-8E, 12Z-octadecadienoic acid, 20- alkoxyl radical, 21- octene radical, 22-10-oxo-9-decenoic acid

Interestingly, the 13-hydroperoxy-group in 13-HPODE remains intact during the autoxidative conversion of HPODE to of HPNE (Schneider, *et al*., 2001, Lee, *et al*., 2001). Such conversion of HPODE to 4 HPNE occurs readily under conditions in which the 13-HPODE is also converted to 8,13-dihydroperoxy-9Z, 11E-octadecadienoic acid (8,13-HPODE) (Schneider, *et al*., 2001) and several mechanisms have been proposed for it, of which one is illustrated in Scheme 5.5. Thus, HPODE **1** is initially converted via an allylic radical **23** to peroxyl radical **24**, a precursor of 8,13-HPODE **25**. Alternatively, peroxyl radical **24** cyclizes to a dioxetanyl radical **26**, whose decomposition affords carbon centred radical **27**, a precursor of peroxyl radical **28** (Onyango, 2017). The peroxyl radical **28** may abstract a hydrogen to form a dihydroperoxide **29**, or cyclize to a peroxylactonyl radical **30** whose decomposition affords HPNE **14**. An antioxidant such as BHT will promote formation of hydroperoxides such as **25** and **29**, instead of HPNE - forming peroxyl radical reactions. Moreover, decomposition of hydroperoxides such as **25** and **29** in the presence of antioxidants favours conversion of the relevant alkoxyl radicals to alcohols rather than formation of aldehydes (Onyango, *et al*., 2010).



Scheme 5.5: Mechanism for the conversion of 13-HPODE 1 to HPNE 14 under autoxidative conditions. Antioxidants will limit the formation of HPNE by trapping peroxyl radicals to form dihydroperoxides such as 25 and 29. These dihydroperoxides may be further converted via alkoxyl radicals to dihydroxy-derivatives.

Key: 1-8,13-dihydroperoxy-9Z, 11E-octadecadienoic acid, 23-Allylic radical, 24-peroxyl radical, 25-dihydroperoxide, 26-dioxetenyl radical, 27-carbon centered radical, 28-peroxyl radical, 29-dihydroperoxide, 30-peroxylactonyl radical, 14-4-hydroperoxy-2-nonenal

5.5 Conclusion

The formation of hexanal and 2-pentylfuran as volatile products of the reaction between lysine and linoleic acid hydroperoxides, which supports the lysine (or other amine)-catalysed conversion of lipid hydroperoxides to dioxetanes as precursors of bioactive aldehydes and alkyl-furans. Although lysine has been reported to slow down lipid oxidation in food systems, its conversion of hydroperoxides to aldehydes in biological systems might have a different effect, since aldehydes such as 4-hydroxy-2-nonenal (HNE) promote the formation of reactive oxygen species in cells through cell signalling mechanisms. Thus, targeting such signalling mechanisms might be crucial for reducing lipid oxidation *in vivo*

CHAPTER SIX

URIC ACID MEDIATION OF THE CONVERSION OF FATTY ACID HYDROPEROXIDES TO ALDEHYDIC PRODUCTS

Manuscript presented at the "14th JKUAT Scientific, Technological and Industrialization Conference 2019", Uric acid mediation of the conversion of fatty acid hydroperoxides to aldehydic products, Wanjala George Wafula, Onyango Arnold N., Makayoto Moses, Onyango Calvin, Parallel Session 1, SAJOREC. 14th -15th November, 2019.

6.1 Abstract

Uric acid, the final product of purine catabolism is a potent antioxidant but it can also be a pro-oxidant under certain conditions. Linoleic acid hydroperoxides contribute to off flavour development in lipid rich foods, reduce nutritional value of foods and contribute to pathogenesis of physiological disorders. The effect of incubation of fatty acid hydroperoxides with uric acid on the formation of hexanal and pentyl-2-furan as typical aldehydic products of lipid oxidation in the presence of a radical scavenger; 2,6-ditert-butyl-4-hydroxy-toluene (BHT) was therefore examined. Linoleic acid hydroperoxides were obtained by the photosensitized oxidation of linoleic acid using ultraviolet light and methylene blue as the photosensitizer. The hydroperoxides were reacted with uric acid, and the organic fraction analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The involvement of uric acid was checked by the detection of allantoin in the reaction mixtures using by high performance liquid chromatography (HPLC) on reverse phase C18. Hexanal and 2-pentylfuran were detected as products of linoleic acid hydroperoxides decomposition in the presence of uric acid. Allantoin was detected in the reaction mixture. Detection of hexanal and 2-pentylfuran despite of the radical scavenger in the system, indicates that a non-radical reaction mechanism was involved. Detection of allantoin confirmed the involvement of uric acid in the reactions. It is concluded that uric acid decomposes lipid hydroperoxides to aldehydic products by a non-radical mechanism, which is proposed to be analogous to the lysine-mediated heterolytic lipid hydroperoxide decomposition. The potential contributions of this reaction to the negative effects of uric acid on human health are outlined.

Key words: linoleic acid hydroperoxide, uric acid, hexanal, pentyl-2-furan, allantoin

6.2 Introduction

Reactive oxygen species (ROS) have high affinity for lipids, proteins and nucleic acids and may exhibit harmful effects in the body (Sivanandham, 2011, Kunwar & Priyadarsini, 2011). This results in oxidative reactions that facilitate damage of cellular components like proteins, lipids and DNA and is believed to have a role in pathogenesis of cancers, cardiovascular diseases, diabetes, atherosclerosis among others (Loscalzo 2004; Lien *et al*., 2008; Sivanandham, 2011; JBS3, 2014). However, the body can protect itself against oxidative damage through antioxidants, which can delay or prevent oxidation in tissues (Kohen & Nyska 2002). Antioxidants act by terminating the chain reactions through free radical scavenging and/or repair of damaged molecules (Loscalzo 2004; Zhivotovsky & Orrenius 2011). Uric acid and ascorbic acid have been indicated to exert both pro-oxidant and antioxidant roles depending on the reactants and reaction conditions. However, the mechanisms involved and the particular role of uric acid in such reactions has not been conclusively been understood.

Uric acid, the final product of purine catabolism in the human body is generated in diverse sites including the intestines, liver, muscle, kidney, vascular endothelium and adipose tissue (Chaudhary *et al*, 2013; Maiuolo *et al*, 2016). Diets heavy in purine or fructose, or exposure to lead can contribute to hyperuricemia which induces gout when urate crystals accumulate in joints and induce inflammation (Mauiolo *et al*, 2016). Hyperuricemia also contributes to hypertension, insulin resistance, and diabetes (Johnson *et al*., 2013). These effects may at least partly occur as a result of uric acid-mediated activation of the renin-angiotensin system (RAS) in adipocytes and endothelial cells, resulting in the release of angiotensin II and induction of oxidative stress in these cells (Chen & Mehta, 2006; Yu *et al*, 2010; Zhang *et al*, 2015). Angiotensin II signals via Angiotensin Type 1 receptors to induce oxidative and nitrosative stresses that lead to inhibition of insulin signaling (Onyango, 2017).

That uric acid is a major contributor to physiological disorders associated with oxidative stress is somehow paradoxical, because this compound accounts for 60% of the antioxidant activity in the blood, through such mechanisms as chelating ferric ions, quenching singlet oxygen, scavenging free radicals, stabilizing ascorbic acid, preventing hydrogen peroxide-mediated inactivation of extracellular superoxide dismutase, and converting peroxynitrite to a stable nitric oxide donor (Maiuolo *et al*, 2016). Sautin and Johnson (2008) suggested that this paradox may be due to intracellular uric acid acting as an inducer of oxidative stress-generating pathways, for example by inducing interleukin-β (IL−β) processing, while extracellular uric acid acts largely as an antioxidant. Nevertheless, the antioxidant role may not always be true because uric acid has been shown to increase lipid peroxidation under some circumstances, even *in vitro* (Bagnati *et al*, 1999, Patterson *et al*, 2002).

The mechanism by which uric acid promotes the oxidation of low density lipoprotein was also suggested to involve the reduction of Cu2+ to Cu+ (Bagnati *et al*, 1999; Patterson *et al*, 2002). Lee *et al*. (2000) reported that ascorbic acid promotes the conversion of linoleic acid hydroperoxide to genotoxic lipid derived aldehydes, by reducing Fe^{3+} to Fe^{2+} , which convert hydroperoxides to alkoxyl radical precursors of the aldehydes. Thus, uric acid may similarly promote the conversion of hydroperoxides to aldehydes via alkoxyl radicals. On the other hand, the amino acid lysine or other primary amines such as phosphatidylethanolamine mediate the conversion of fatty acid hydroperoxides to aldehydic products including hexanal through a non-radical mechanism (Kato *et al*, 1999; Tsuji *et al*, 2004; Onyango 2016). The present study focused on the possibility of uric acid-mediated conversion of lipid hydroperoxides to aldehydic products by a non-radical mechanism.

6.3 Materials and Methods

6.3.1 Materials

Linoleic acid, hexanal, 2-pentlyfuran, allantoin, uric acid were purchased from Sigma Aldrich, methylene blue, BHT, hexane were analytical grade.

6.3.2 Synthesis of linoleic acid hydroperoxides from linoleic acid by photooxidation

Five grams of pure linoleic acid (99% purity) was dissolved in 10 ml of ethanol containing 0.27mM of methylene blue and irradiated at 10 ^{o}C using ultraviolet light of 366 nm (Funa UV, Light Model SL-800G), from a distance of 2.5 cm for 1 hour. The hydroperoxides were purified by column chromatography on silica gel eluted with hexane:ethyl acetate (95:5) as the mobile phase.

6.3.3 Estimation for complete photooxidation of linoleic acid

The reaction mixture was prepared as described above and exposed to ultraviolet light. The reaction was timed and after every hour of photooxidation 10µl of the mixture was drawn and spotted on silica gel G (Merck, silica gel PF-254) coated plates (0.5mm thick) and eluted with hexane:ethyl acetate (95:5). This was continued upto 2 hours of photooxidation. The reaction mixture after 2 hours of photooxidation was condensed and separated using column chromatography with silica gel (C60) and dried over anhydrous Na_2SO_4 using hexane:ethyl acetate (95:5). Fractions of approximately 1ml of the eluted and separated reactant mixtures were collected in respective collection flasks. From each of these samples, 10µL was spotted on silica gel G (Merck, silica gel PF-254) coated plates (0.5mm thick) and eluted with hexane:ethyl acetate (95:5). The bands of the products were detected under ultraviolet light. Reaction mixtures that did not exhibit evidence of lipid hydroperoxides were discarded. All mixtures from reaction flasks with evidence of linoleic acid hydroperoxides were mixed together and the solvent evaporated *in vacuo.* The total synthesized linoleic acid hydroperoxides were dissolved

in 10ml of hexane, with added BHT and stored under -18^{o}C until the time for oxidative reactions and analysis.

6.3.4 Reaction of linoleic acid hydroperoxides with uric acid and detection of hexanal and 2-pentyl furan

Linoleic acid hydroperoxides (2 ml) were reacted with 100 mM of uric acid or nothing (control) in respective reaction flasks and incubated at 37^{o}C for 1 hour. The organic layer was concentrated *in vacuo*, spotted on TLC plates, eluted with hexane:ethyl acetate (9:1) and observed under UV light (Chambers et al., 2009; Shoji, et al., 2013).

Another set of reactions was done in the same way, except that the reaction flasks were completely sealed to prevent escape of any gases formed. After the reaction, 20µl of the sample or the head space gas was drawn with a syringe and injected directly to a GC (Shimadzu GC-14B, Kyoto, Japan) equipped with a Omega waxTM 530 (Supelco) Fused Silica Capillary column (30mm*0.53mm*0.5µm film thickness at column temperature of (170-230^{o}C). The GC program was: column initial temperature 50^{o}C, time 1 min, rate 5^{o}C/min and column final temperature 170^{o}C and time 2 minutes. Pure hexanal and pentyl-2-furan were used as standards for detection of hexanal in head space (Chambers et al., 2009; Shoji, et al., 2013).

6.3.5 GC-MS for hexanal and pentyl-2-furan

The head space gas generated was injected to GC-MS for confirmatory experiments. Equipment parameters were: omega wax 250 fused silica capillary column (30m*0.25mm internal diameter {ID}*0.25µm, Supelco, USA). Connected to a CP-SiL 8 CB in low bleed column (0.75m*0.25mm I.D. *0.25µm, Varian Inc., Palo Alto, CA, USA) as a transfer line. The carrier gas – helium at a flow rate of 1mL/min, and the injector unit and transfer line temperatures set at 250^{o}C. Oven temperature was kept at 35^{o}C for 2 hours, and then increased by 4^{o}C per minute to 230^{o}C, and maintained at the

temperature for 15 minutes. The mass detector was operated in electron ionization mode (Chambers *et al*., 2009, Kukuta, *et al*., 2013).

6.3.6 Detection and quantification of allantoin by HPLC

From the reaction mixture, 100µl was drawn and injected into HPLC reverse phase with C18 isocratic column was used with acetonitrile:KH_2PO_4 buffer (pH 3.0) mobile phase at a ratio of 80:20, operating at 30^{o}C column temperature, flow rate of 0.1mL/min, detector scanning at 210nm. Pure allantoin was used as a standard. Confirmation of allantoin generation, the reaction mixture was spiked with a known amount of allantoin (Kakuta, *et al*., 2013).

6.4 Results and Discussion

Linoleic acid hydroperoxides were generated from pure linoleic acid by photooxidation. Complete photooxidation of linoleic, separation, concentration and storage of the hydroperoxides was achieved as illustrated in figure 6.1. Sufficient linoleic acid hydroperoxides were prepared to ensure the observations deduced were purely from linoleic acid hydroperoxides. Classically, exposure of linoleic acid to photooxidation systems which generate singlet oxygen results in non-enzymatic lipid oxidation that occurs by the reaction of lipids with the singlet oxygen (Domínguez, *et* al., 2019, Onyango, 2017). This mainly results in mixtures of 9-HPODE and 13-HPODE isomers of the linoleic acid hydroperoxides as shown in scheme 6.1.

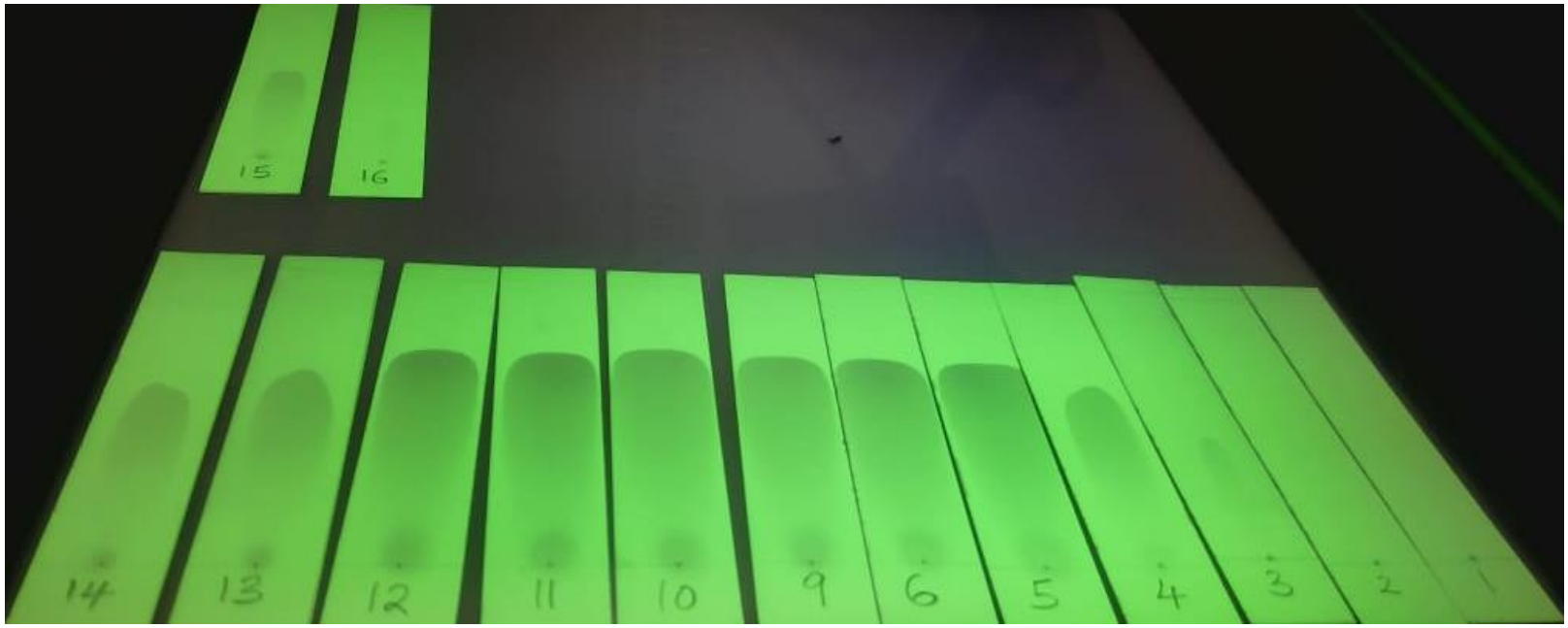
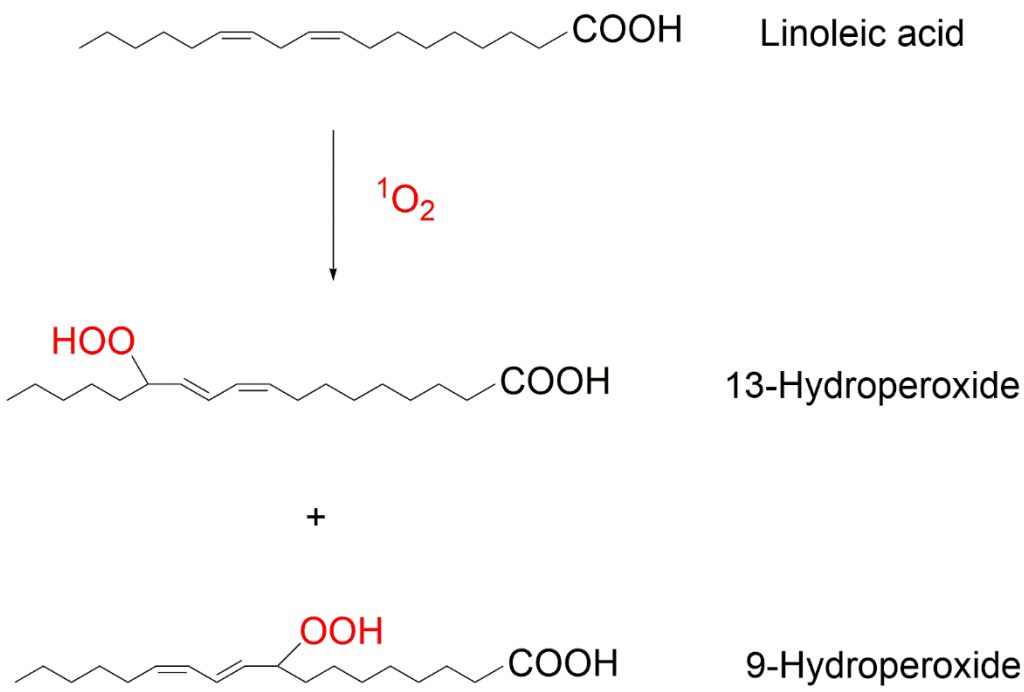


Figure 6.1: The linoleic acid hydroperoxides generated by exposure of pure linoleic acid to UV light with methylene blue as a photosensitizer for 2 hours and maintained at 10°C in a cold water bath.

The reaction mixture was separated by column chromatography on C60 silica gel and dried over anhydrous sodium sulphate. During separation, the eluents approximately 1 ml each were collected in several sterile glass jars. After separation, 10µl of the mixture was spotted on TLC plate, eluted with hexane:ethyl acetate (95:5) and observed under UV light. Glass jars from 3 to 15 with characteristic linoleic acid hydroperoxides were mixed together in a round bottomed flask, evaporated off the solvents, diluted in hexane and frozen at -18°C until time for reactions.

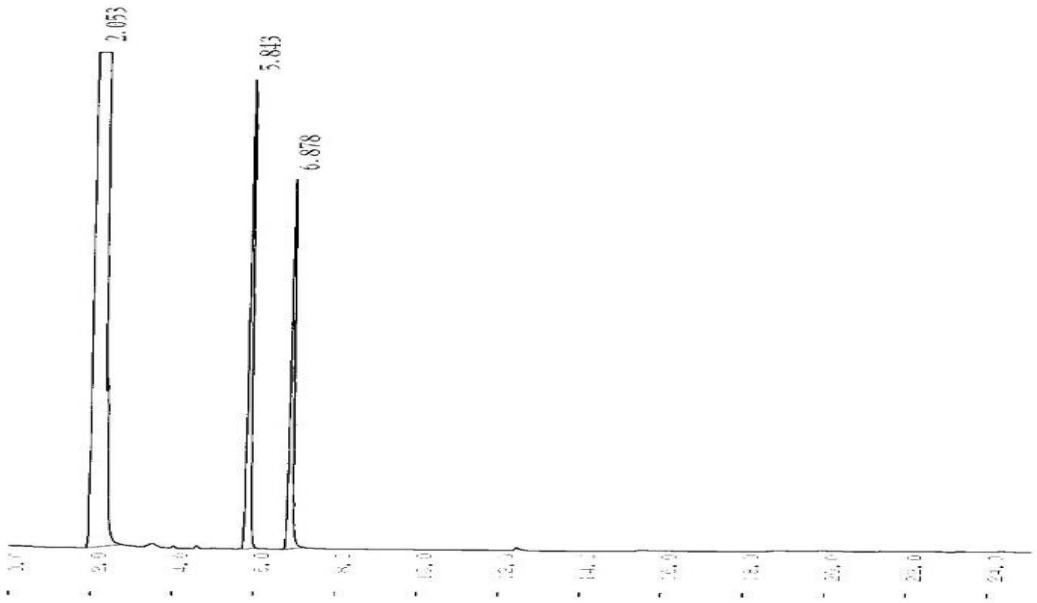


Scheme 6.1: The exposure of linoleic acid to singlet oxygen during photooxidation resulting in generation of mixtures of 13-hydroperoxide and 9-hydroperoxide isomers.

The linoleic acid hydroperoxides were observed to participate in the oxidative reactions. The decomposition reactions were fast, because hexanal gas was detected even after 2 minutes of incubation. Most of the decomposition took place within 1 hour of incubation at 37 ^oC. In a previous study involving lysine catalysed decomposition of linoleic acid hydroperoxides both hexanal and 2-pentylfuran were detected (Wanjala *et al*., 2020). The amino group in lysine is inclined to participate in these reactions due to the positive charge which could be analogous to the uric acid mediated reaction.

A representative GC spectrum obtained upon incubation of the peroxidised oil with uric acid is shown in Figure 6.2, showing the formation of hexanal under the system. The cconfirmatory experiments were conducted and the generated aldehydes were detected

using Shimazdu GC-MS. Hexanal and 2-pentylfuran detected from the uric acid system are shown in Figure 6.3 with their respective spectra shown in Figures 6.4 and 6.5 respectively.



Retention time (minutes)

Figure 6.2: GC chromatogram of the headspace volatiles obtained by uric acid-mediated decomposition of fatty acid hydroperoxides at 37 ºC for 30 minutes. The peak at retention time 6.878 belongs to hexanal.

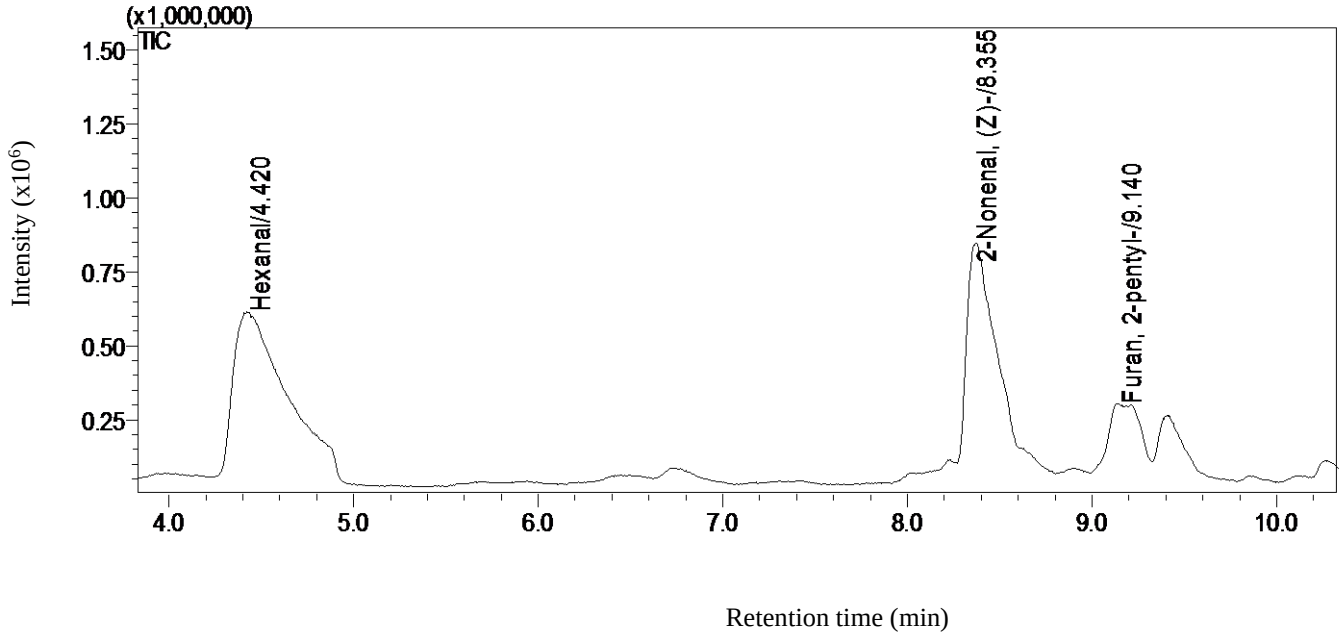


Figure 6.3: GC-MS chromatograms of the head space volatiles obtained by uric acid-mediated decomposition of fatty acid hydroperoxides at 37 ^{o}C for 30 minutes. Hexanal, 2-nonenal and furan, 2-penty were detected and confirmed by comparison using Shimadzu, NIST library.

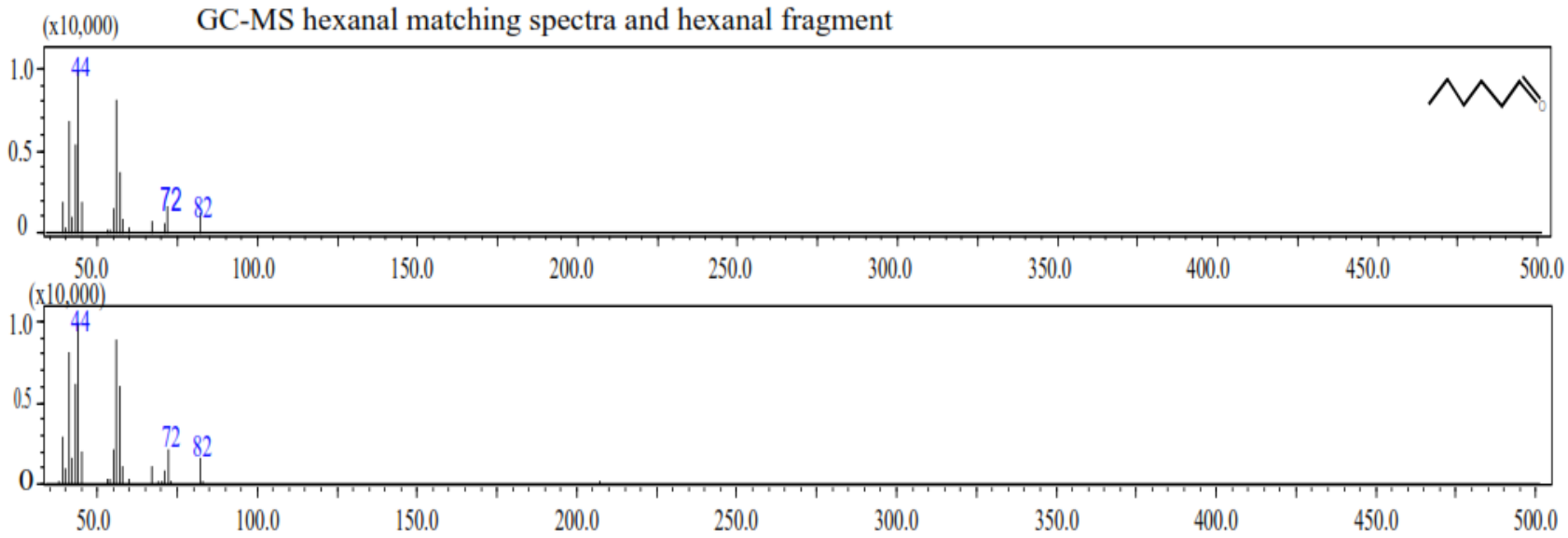


Figure 6.4: GC-MS for hexanal matching spectra from Shimadzu NIST library and sample fragment of volatiles obtained by uric acid-mediated decomposition of fatty acid hydroperoxides at 37 ºC for 30 minutes.

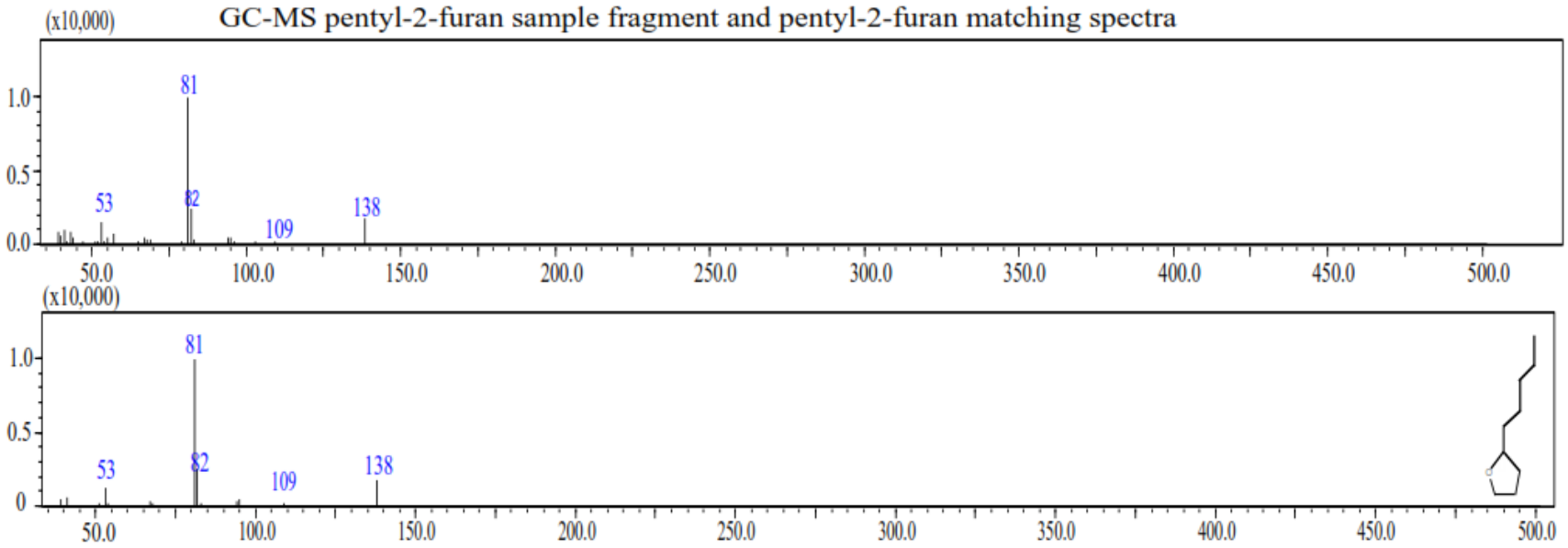
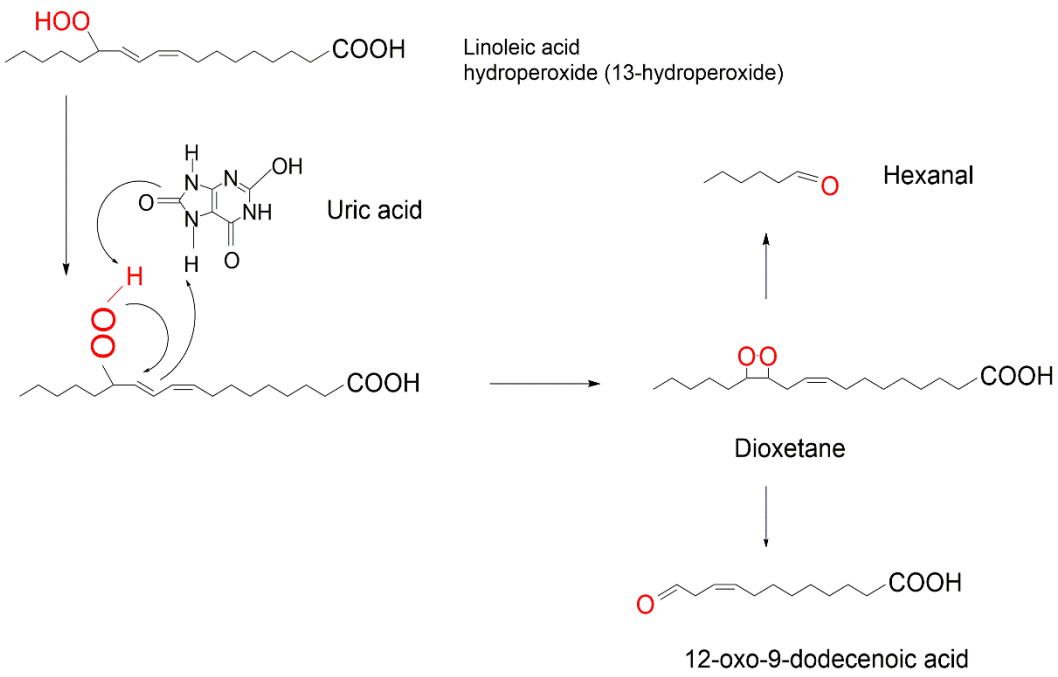
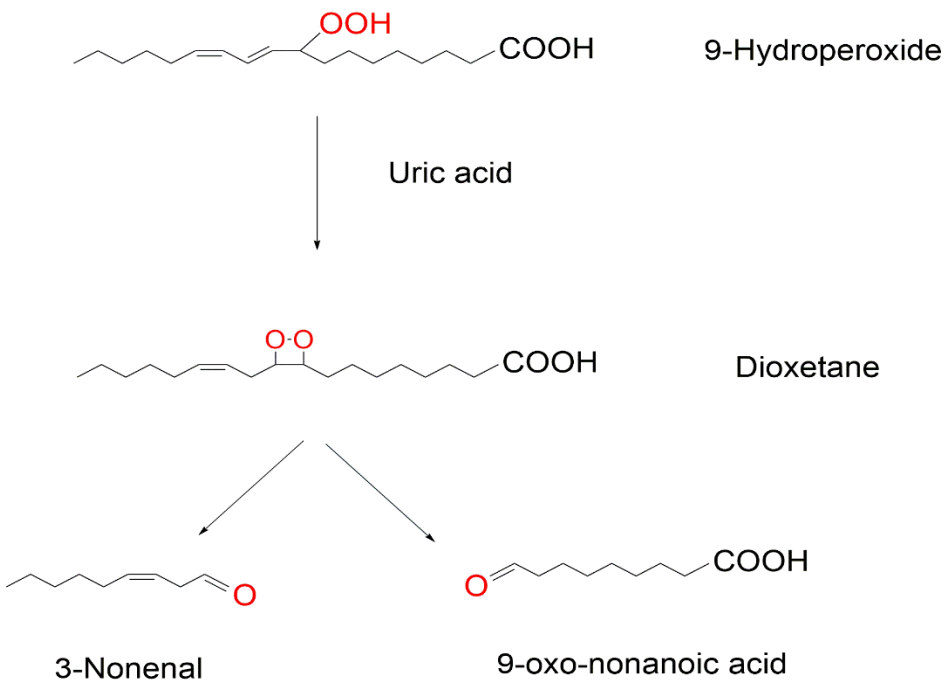


Figure 6.5: GC-MS for pentyl-2-furan matching spectra from Shimadzu NIST library and sample fragment of volatiles obtained by uric acid-mediated decomposition of fatty acid hydroperoxides at 37 ºC for 30 minutes.



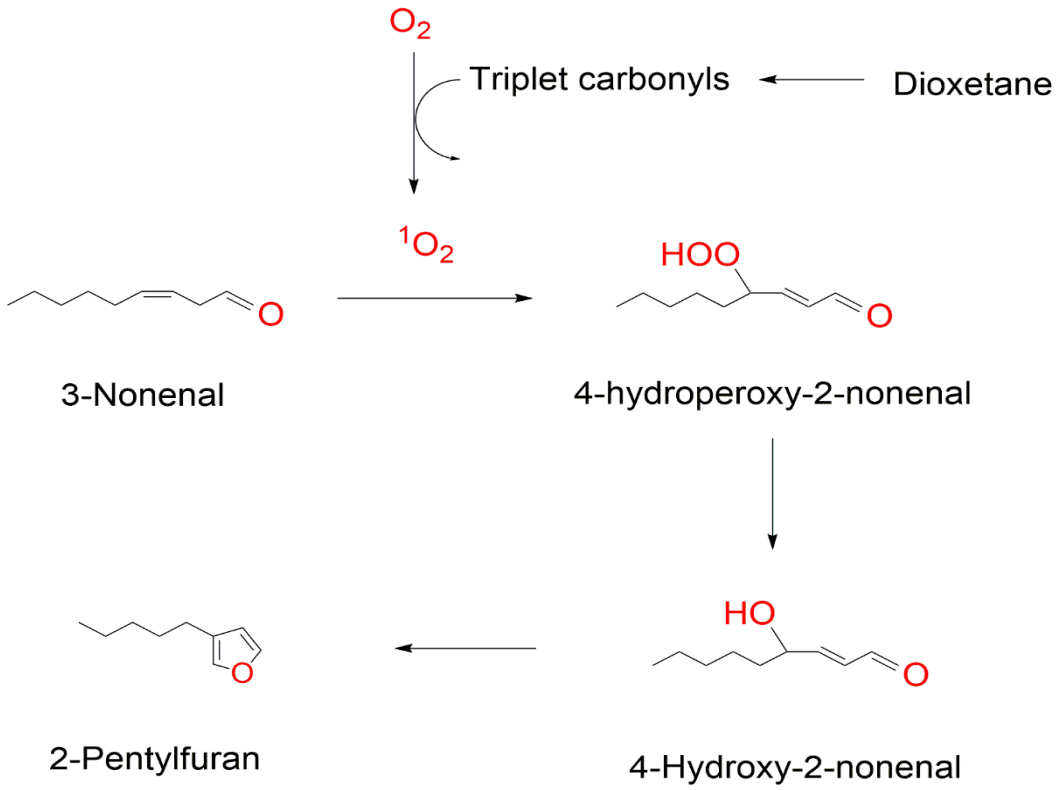
Scheme 6.2: Uric acid catalysed formation of hexanal and 12-oxo-9-dodecenoic acid from 13-HPODE through dioxetane intermediate.

As shown in schemes 6.2 and 6.3, uric acid directly abstracts a hydrogen atom from the 13-HPODE and 9-HPODE which cyclizes to form a dioxetane. This reaction is analogous to the lysine mediated decomposition of linoleic acid hydroperoxide to form a dioxetane (Wanjala, *et al*., 2020). The dioxetane decomposes to hexanal or it can also result in the formation of another aldehyde 12-oxo-9-dodecenoic acid as shown in scheme 6.2. However from 9-HPODE, the dioxetane decomposes to 3-nonenal or 9-oxo-nonanoic acid as shown in scheme 6.3.



Scheme 6.3: Uric acid catalysed formation of 3-nonenal and 9-oxo-nonanoic acid from 9-HPODE through dioxetane intermediate.

Decomposition of dioxetane to triplet carbonyls results in the transfer of energy to molecular oxygen leading to direct oxidation of 3-nonenal by singlet oxygen to form 4-hydroxy-2-nonenal that cyclizes to 2-pentylfuran as illustrated in scheme 6.4.



Scheme 6.4: Dioxetane releases energy that abstracts an atom from molecular oxygen to form singlet oxygen that facilitates conversion of 3-nonenal to 4-hydroperoxy-2-nonenal that looses an oxygen atom to 4-hydroxy-2-nonenal that cyclizes to 2-pentylfuran.

The involvement of uric acid in the reactions was confirmed by the detection of allantoin using reverse phase HPLC. This reaction mixture containing uric acid yielded 134.85±1.55 µg/ml of allantoin with reaction peak at 2.445 minutes as shown in Figure 6.6. To confirm the results another sample was spiked with pure allantoin which yielded the same reaction peak but at elevated intensity at peak time 2.446 minutes as shown in Figure 6.7.

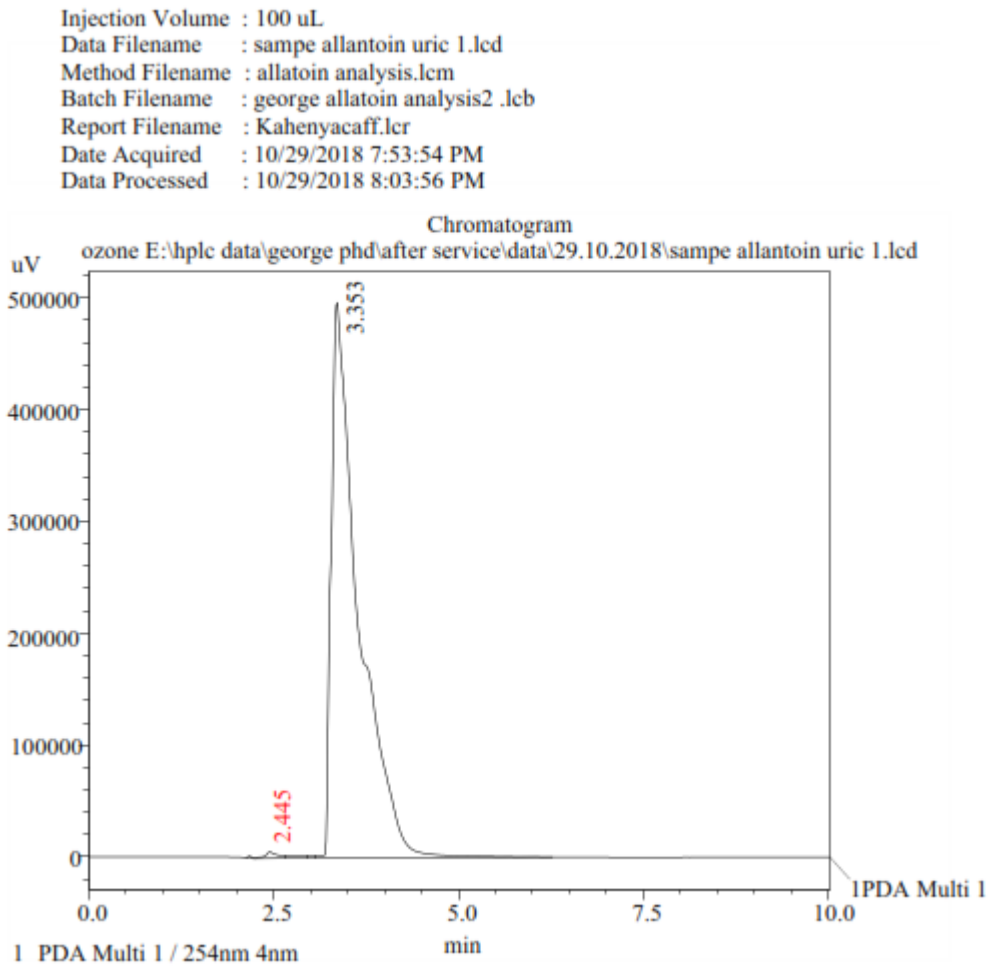


Figure 6.6: HPLC chromatogram of solution mixture obtained by uric acid-mediated generation of allantoin from decomposition of fatty acid hydroperoxides at 37 ^{o}C for 30 minutes. The peak at retention time 2.445 belongs to allantoin.

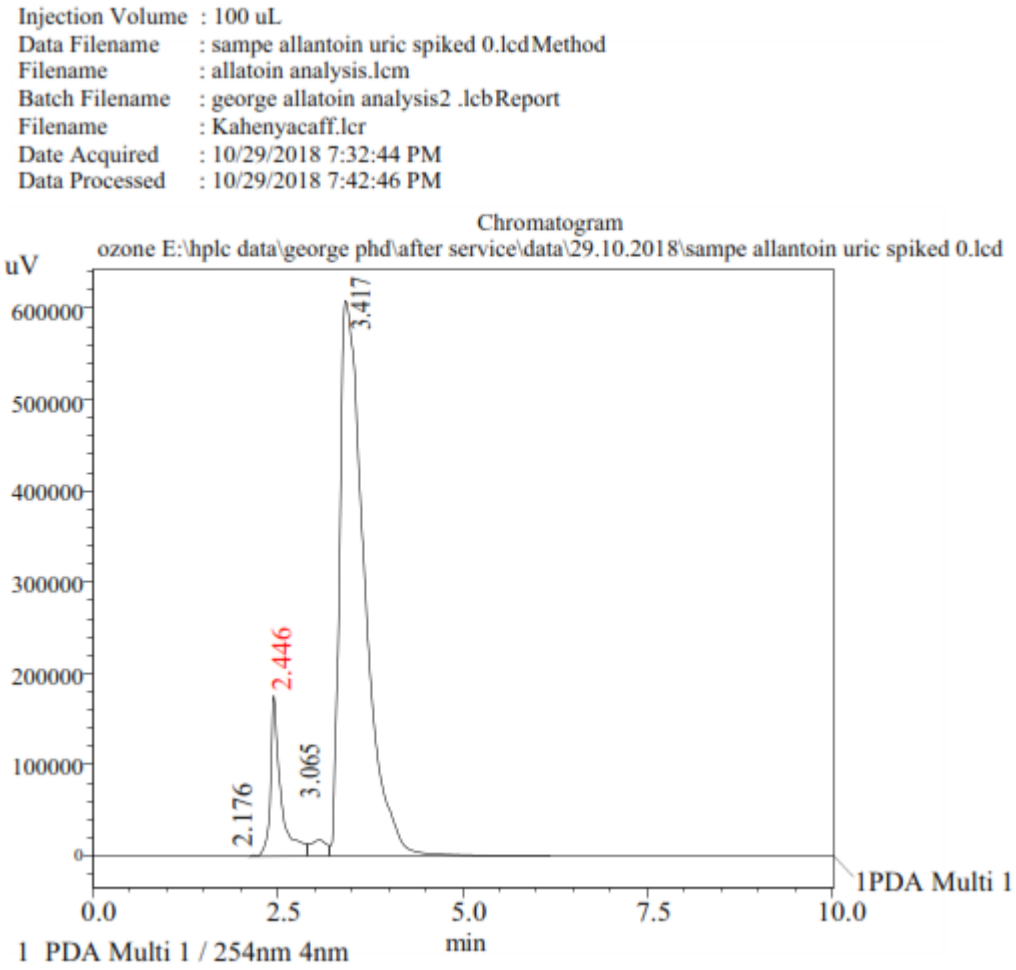


Figure 6.7: HPLC chromatogram of solution mixture obtained by uric acid-mediated generation of allantoin from decomposition of fatty acid hydroperoxides at 37 ºC for 30 minutes and spiked by allantoin. The peak at retention time 2.446 belongs to allantoin.

Previously, uric acid had been demonstrated to exert both antioxidant and pro-oxidant activities during the oxidation of low-density lipoprotein (LDL). Bagnati, *et al.*, (1999), in their study observed pro-oxidant and antioxidant effects of uric acid during copper-induced LDL oxidation. Uric acid exerted LDL oxidation by reducing Cu(II) to Cu(I), making more Cu(I), available for decomposition of the hydroperoxides irrespective of endogenous antioxidants such as α-tocopherol. In the progression of metabolic diseases

like diabetes, there is a marked increase in plasma uric acid. It plays beneficial and harmful roles at the same time. It has been known to reduce lipid oxidation in blood plasma however it has also been cited to participate in cell signaling leading to ROS generation (Sautin and Johnson, 2008; Tait and Green, 2012). Histidine on the other hand has been cited to improve diabetic conditions, but it could also potentially generate ROS in biological systems.

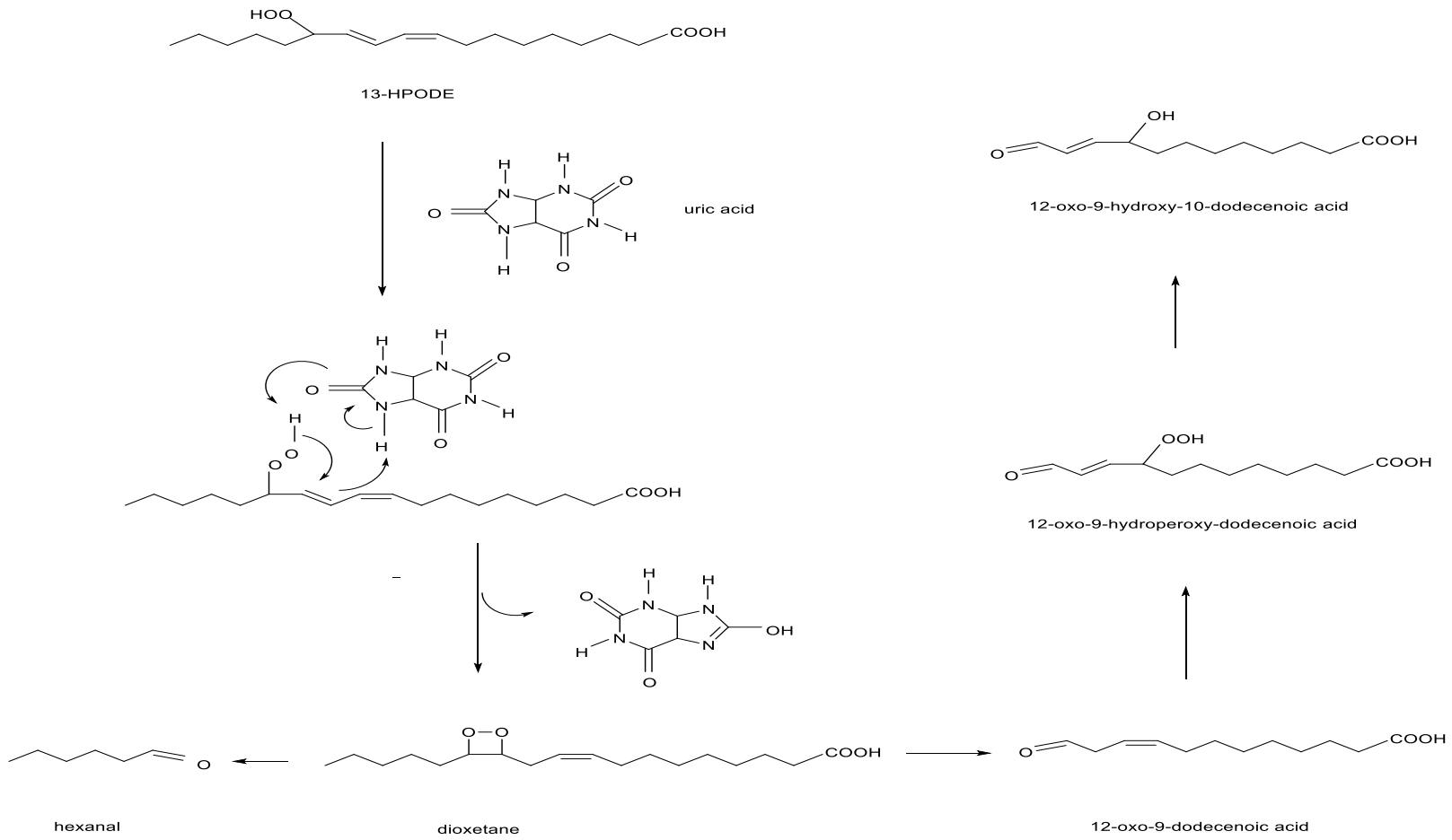
Interestingly, uric acid gave higher hexanal concentrations than an equimolar concentration of lysine from the previous experiment, while the control gave minimal hexanal. The uric acid-mediated conversion of fatty acid hydroperoxides to hexanal was not prevented by the radical scavenger, 2,6-ditert-butyl-4-hydroxytoluene (BHT), confirming that this reaction occurs by a non-radical mechanism. This reaction is proposed to occur analogously to the previously proposed mechanism for the amine-mediated conversion of linoleic acid hydroperoxide to hexanal (Onyango, 2016). As shown in scheme 6.5, the hydroperoxide may donate a proton to the carbonyl oxygen of uric acid, followed by cyclization of the peroxyl anion, and acceptance of hydrogen from the acidic nitrogen of uric acid (scheme 6.5). This is in line with the report that water forms stable complexes with uric acid by donating a proton to the uric acid's carbonyl oxygen and accepting a proton from uric acid's acidic nitrogen (Chandra & Zeegars-Huyskens, 2007).

That more hexanal was detected from uric acid than from lysine containing mixtures may not necessarily mean a greater reactivity of uric acid than lysine, since lysine easily forms hexanoyl-lysine adducts in the presence of hydroperoxides (Kato *et al*, 1999; Onyango, 2016, Wanjala *et al*., 2020). Future characterization of possible uric acid-hexanal adducts will help to explain further the differences in reactivity.

Aldehydic lipid oxidation products including hexanal, 2,4-decadienal and 4-oxo-2-nonenal have been found to accelerate lipid peroxidation (Magoli *et al*, 1980, Saito *et al*, 2011). Thus, uric acid-mediated conversion of lipid hydroperoxides to such products

may be involved in its pro-oxidative effects, which only occur in the presence of preformed hydroperoxides.

According to scheme 6.5, the formation of 12-oxo-9-dodecenoic acid is expected to occur simultaneously with hexanal formation. The 12-oxo-9-dodecenoic acid undergoes further oxidation to 12-oxo-9-hydroperoxy-10-dodenoic acid, which is a precursor of other highly reactive aldehydes such as 9,12-dioxo-10-dodecenoic acid and 12-oxo, 9-hydroxy-10-dodeceoic acid. The formation of such aldehydic compounds in oxidized low density lipoproteins is necessary for some of the bioactivities of the 12-oxo, 9-hydroxy-10-dodeceoic, including platelet prothrombinase activity, interaction with the scavenger receptor that is required for atherosclerotic foam cell formation, and the release of IL-1 β (Ziesenis *et al*, 2001; Ishino *et al*, 2010; Thomas *et al*, 1994). Thus, uric acid may play a role in the 'activation' of oxidized LDL.



Scheme 6.5: Proposed mechanism for the uric-acid mediated conversion of lipid hydroperoxide to hexanal and other aldehydic compounds.

6.4 Conclusion

Uric acid therefore mediated the conversion of 9-linoleic acid hydroperoxides and the 13-linoleic acid hydroperoxide to biologically relevant aldehydes. The uric acid system generated generate hexanal and 2-pentylfuran by a non-radical mechanism. Direct involvement of uric acid in the reactions was confirmed by detection of allantoin in the system. The reactions were rapid which could signify their biological importance. The data generated supports uric acid-catalysed conversion of linoleic acid hydroperoxides to dioxetanes as the precursor of hexanal.

CHAPTER SEVEN

GENERAL CONCLUSIONS AND RECOMMENDATIONS

7.1 General Conclusions

Evidence for the formation of ozone which converts cholesterol to atherogenic secosterol aldehydes in living tissues exist, however, the mechanism for the generation of this oxidant was not well understood. In this study, evidence has been presented that methionine sulfoxide, an oxidation product of methionine, reacts with singlet oxygen to form ozone or an ozone-like oxidant, thus supporting the hypothesis that biological ozone or ozone-like oxidant formation involves the sequential reaction of singlet oxygen with amino acids and amino acid oxidation products. Owing to previous doubts on occurrence of ozone, it was therefore plausible to postulate alternative mechanisms for formation of the secosterol aldehydes.

Under this research study, it was experimentally confirmed that lysine, a primary amine catalysed by a non-radical mechanism the conversion of cholesterol-5α-hydroperoxide (Ch-5α-OOH) to the secosterol aldehydes and several secosterol-amine adducts (carbinolamines, Schiff's bases and amide-type adducts). The amide-type adducts like hexanoyl-lysine are good biomarkers of lipid oxidation in foods and *in vivo* and therefore could additionally have major contributions to pathogenesis of physiological processes. Although the peroxide intermediates were not detected as a direct evidence for the mechanism of formation of the amide-type adducts, an alternative mechanism involving oxidation of carbinolamine adducts by singlet oxygen to dioxetane derivatives as the precursors was illustrated.

On the same vein, similar reactions with linoleic acid hydroperoxides were observed to generate free aldehydes. Hexanal and 2-pentylfuran were detected from the reactions even in the presence of a radical scavenger BHT. This clearly points towards non-radical mechanisms being involved. Detection of 2-pentylfuran signified formation of 4-

hydroxy-2-nonenal, a cytotoxic product of linoleic acid oxidation. Uric acid in particular promoted conversion of lipid hydroperoxides to toxic aldehydes an observation that possibly explains the association of hperuricemia with physiological disorders despite it being an antioxidant. The study therefore has illustrated mechanisms for singlet oxygen mediated ozone formation and outlined mechanisms of toxic aldehyde formation by non-radical mechanism from lipid hydroperoxides and that antioxidants were not able to reverse the reactions once they start but could participate by propagating the reactions. The study signify that it would be crucial to determine the timing for increased promotion of exogenous antioxidant recommendation especially for patients exhibiting potential cholesterol and fatty acid oxidative associated pathophysiological processes. This is because non-radically the increased antioxidant regimes may only worsen the bad situations *in vivo*.

7.2 Recommendations and Future Work

Moreover, owing to the detected revelations on the non-radical nature of the reactions and possible unexpected effects of major antioxidants on the formation of the oxidant and/or the secosterol aldehydes:

- More studies are needed to establish roles that major antioxidants may have on cholesterol oxidation/ozonolysis reactions.
- It is worthwhile to consider pathways of singlet oxygen mediated amino acid and formaldehyde oxidation with the aim of identifying potential steps that could be involved in ozone formation. A key aspect is direct quenching of singlet oxygen from the system may play a critical role and hence more studies on mechanisms targeting this aspect are needed.
- Amines presented interesting participation in ozonolysis and decomposition of lipid hydroperoxides hence more studies are needed to establish their roles in pathophysiological processes.

- The fact that lipid peroxidation has been elucidated to involve a non-radical mechanism, further studies are needed to examine medical preparations (therapeutic agents and nutritional supplements) like syrups and emulsions that contain lipids to check extent of lipid oxidative reactions. The medications and supplements are developed to aid in boosting immunity or remediation of disease conditions but the presence of these reactions poses additional dangers to potential targets.
- Controlled studies involving animal models are needed to increase the continued understanding of ozonolysis and lipid peroxidation reactions in biological systems to assist in early detection such development of newer and non-invasive biomarkers of the disease conditions and improved management of the pathophysiological conditions.
- More studies involving direct food materials to understand the extent and impacts of lipid peroxidation reactions involving foods.

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