FREE RADICAL INDUCED OXIDATIVE DNA DAMAGE.

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

Sabya Farooq B.Sc. (Leicester)

MRC Toxicology Unit

University of Leicester

May 1997.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

All praise is for God only the mistakes have been mine.

This thesis is dedicated to my wonderful parents to whom I owe everything.

ACKNOWLEDGEMENTS

Firstly I would like to thank my supervisor Dr. Peter Farmer for all his advice and supervision throughout the duration of this PhD.

A sincere thank you to Dr Eric Bailey for his help and guidance when I initially started my research.

I am grateful to Dr Helena Hernandez for all the help, advice and support she gave me when I needed it most.

I would also like to thank the following: Mr John Lamb for help and advice with all aspects of mass spectrometry, Dr Ian Podmore for the useful discussions, Dr Michael Festings for the statistical analysis, Dr Gavain Sweetman for all his computer expertise, Dr Karl Herbert and all involved in the collaborative work carried out in chapter 5.

My sincerest thanks to past and present members of the BMI for their friendship and for making even the most difficult days memorable and enjoyable ones.

I am also eternally grateful to **all** the friends I made during my undergraduate and postgraduate studies in Leicester. I believe I am the luckiest person as far as friends are concerned and I thank them for sharing the laughter and the tears.

I would like to thank my sisters Salma, Nazia, Naima and Bushra for all their love and support and to my brothers Ali and Qasim all my love.

Finally I gratefully acknowledge the Medical Research Council for the financial support.

FREE RADICAL INDUCED OXIDATIVE DNA DAMAGE.

Sabya Farooq, MRC Toxicology Unit, Centre for Mechanisms of Human Toxicity, Hodgkin Building, University of Leicester, Leicester LE1 9HN, United Kingdom.

ABSTRACT

Oxidative DNA damage has been implicated in processes such as carcinogenesis, mutagenesis, ageing and cell death. Reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$) are produced in mammalian cells as a result of aerobic metabolism. However excess generation of these species by endogenous or exogenous sources can result in damage to DNA, producing a large number of sugar and base lesions. In order to understand the biological consequences of such free radical induced damage it is essential to characterise and quantitate this damage .

This study describes the establishment of sensitive and specific techniques to chemically characterise and quantitate three markers of oxidative DNA damage, namely: cis-thymine glycol (Tg), 5-hydroxymethyluracil (5-OHMeU) and 8-hydroxyguanine (8-OHG).

Techniques using gas chromatography/mass spectrometry (GC/MS) were established for Tg and 5-OHMeU, following their derivatisation with N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA). Standards of Tg and 5-OHMeU were synthesised, and stable isotopically labelled analogues were prepared as internal standards. Analysis of the DNA was carried out at the base level and therefore required acidic hydrolysis of the DNA in order to release the modified and intact bases. For the quantitation of 8-OHG a novel procedure using high performance liquid chromatography (HPLC) - electrochemical detection (ECD) with guanase incubation of DNA hydrolysates was established.

The established assays were used to quantitate DNA lesions *in vitro* and *in vivo*. *In vitro* dose response curves were established for the three markers upon γ-irradiation of DNA. *In vivo* results of an animal inhalation study indicated there was not a significant increase in oxidative damage upon exposure to crocidolite. An antioxidant supplementation study in humans placental DNA also did not show a significant reduction in levels of the three markers upon supplementation. Comparable background levels of Tg and 5-OHMeU were observed in human and calf thymus DNA, while 8-OHG levels were found to be significantly higher.

LIST OF PUBLICATIONS

Farooq, S., Farmer, P.B. and Bailey, E. (1995) The determination of cis-thymine glycol in DNA by gas chromatography-mass spectrometry. *Brit. J. Cancer.*, **71**, Suppl 24, 60.

Finnegan, M.T., Herbert, K.E., Evans, M.D., Farooq, S., Farmer, P., Podmore, I.D., Lunec, J. (1995) Development of an assay to measure 8-oxoguanine using HPLC with electrochemical detection. *Biochem. Soc. Trans.*, Vol. **23**, No 3, 467-473.

Herbert, K.E., Evans, M.D., Finnegan, M.T.V., Farooq, S., Mistry, N., Podmore, I.D., Farmer, P., Lunec, J.(1996) A novel HPLC procedure for the analysis of 8-oxoguanine in DNA. *Free Rad. Biol. Med.* Vol. **20**, No. 3, 467-473.

Farooq, S., Bailey, E., Farmer, P.B., Jukes R., Lamb, J.H., Hernandez, H, Sram, R., Topinka, J. (1997) Determination of *cis* -thymine glycol in DNA by gas chromatography/mass spectrometry with selected ion recording (SIR) and multiple reaction monitoring (MRM). Submitted for publication.

ABBREVIATIONS

ATP	[γ^{32}P] - adenosine triphosphate
BSTFA	N, O-bis (trimethylsilyl)-trifluoroacetamide
CAT	catalases
CI	chemical ionisation
CuZnSOD	copper zinc superoxide dismutase
dNps	deoxyribonucleoside 3'- monophosphates
dpNps	deoxyribonucleoside 3'-5' bisphosphates
$e^-_{(aq)}$	hydrated electron
ECD	electrochemical detection
EI	electron ionisation
ELISA	enzyme-linked immunosorbent assay
FAB	fast atom bombardment
GC	gas chromatography
Gpx	glutathione peroxidase
GSH	reduced glutathione
GC/MS	gas chromatography/mass spectrometry
GSSG	oxidised glutathione
Gy	gray
HPLC	high performance liquid chromatography
Fapy	formamidopyrimidine
MnSOD	manganese superoxide dismutase
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	mass spectrometry/mass spectrometry
MTBSTFA	N-methyl-N-(*tert*-butyldimethylsilyl) trifluoroacetamide
m/z	mass/charge
NER	nucleotide excision repair
NMR	nuclear magnetic resonance
8-OHA	8-hydroxyadenine
5-OHC	5-hydroxycytosine
8-OHG	8-hydroxyguanine
8-OHdG	8-hydroxydeoxyguanosine
8-OHdGmp	8-hydroxydeoxyguanosine 5' monophosphate
5-OHMeU	5-hydroxymethyluracil
5-OHMedU	5-hydroxymethyldeoxyuridine
5-OHU	5-hydroxyuracil
PPL	^{32}P-postlabelling
PUFA	polyunsaturated fatty acids
RIA	radioimmunoassay
ROS	reactive oxygen species
r.t.	retention time
SD	standard deviation
SIR	selected ion recording
s/n	signal/noise
SOD	superoxide dismutase
TBDMCS	*tert*-butyldimethylchlorosilane

TBDMS	tertiarybutyldimethylsilyl
Tg	thymine glycol
Tdg	thymidine glycol
TdGmp	thymidine glycol 5' monophosphate
TLC	thin layer chromatography
TMP	thymidine 5' monophosphate
TMS	trimethylsilyl
USEIRA	ultrasensitive enzyme-linked radioimmunoassay
UV	ultra violet

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1.

General Introduction

1.0. INTRODUCTION

Oxygen is essential for life, but can be toxic at concentrations higher than 21% (for a review see Balentine 1982). In 1954 Gerschman *et al.* proposed that the damaging effects of oxygen could be attributed to the formation of oxygen radicals. This hypothesis received increasing recognition upon discovery of superoxide dismutase (SOD) by McCord and Fridovich in 1969 and subsequently became known as the superoxide theory of oxygen toxicity (Fridovich 1983). The superoxide theory states that O_2 toxicity is due to excess formation of superoxide and reactive O_2 intermediates of the electron transport chain and that SOD enzymes are antioxidant enzymes that remove O_2^- (Fridovich 1983,1989; Halliwell and Gutteridge 1989).

Oxygen free radicals include the hydroxyl radical ($OH^\bullet$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2^1). Such species are produced continuously as a result of normal aerobic metabolism and by various external factors. These radicals disrupt cell structure and alter cell function by transiently or permanently damaging biological components. The body has evolved its own defences, enzymatic and non-enzymatic, to protect cellular components from oxidative damage but these defences are not completely effective and damage caused by oxygen free radicals occurs. Oxidative stress results when oxygen free radicals are not adequately removed, resulting in severe metabolic dysfunctions and even cell death.

DNA damage is of particular importance due to its role as the genetic material, and its implication in a range of pathologies including ageing and cancer (Totter 1980; Adelman *et al.*. 1988; Ames 1989a; Cerrutti 1985; Halliwell and Gutteridge 1989). Its importance is further supported by the existence of a wide range of enzymes for its repair.

Identifying and monitoring free radical species has proved difficult due to the reactivity and short life span of these species. However, free radical reactions with biological components are increasingly being studied by detecting and monitoring the products of their reactions.

In this thesis, studies on the oxidative DNA lesions thymine glycol (Tg), 5-hydroxymethyluracil (5-OHMeU) and 8-hydroxyguanine (8-OHG) are described. The

aim of this work was to develop sensitive methods for the detection of endogenous levels of these lesions in human DNA samples

This chapter will aim to give a brief introduction to oxygen free radicals (1.1.) and their reactions *in vivo* (1.2.). The mechanisms of preventing and repairing oxidative damage particularly in DNA with respect to disease (1.3.) will also be discussed. Finally a brief description of the different analytical techniques used to detect and quantitate this type of damage will be given (1.4.).

1.1. OXYGEN FREE RADICALS

A free radical may be defined as any species that has one or more unpaired electrons (Halliwell and Gutteridge 1989). Oxygen free radicals are collectively referred to as reactive oxygen species (ROS) to include not only oxygen radicals ($O_2^{\bullet-}$ and $OH^{\bullet}$) but also derivatives of O_2 that do not contain unpaired electrons (H_2O_2 and O_2^{1}). Ironically the diatomic oxygen molecule is itself a free radical containing two unpaired electrons, however due to spin restrictions this free radical is fairly unreactive (Halliwell and Gutteridge 1989, 1990a).

1.1.1. Sources of Reactive Oxygen Species (ROS)

ROS can be produced as a result of exogenous (ionising radiation, redox cycling drugs etc.) and endogenous (aerobic metabolism, phagocytosis) sources.

Exogenous sources.

Exogenous sources such as ionising radiation can lead to the production of ROS. The damaging effects of ionising radiation have been attributed to the damage inflicted upon DNA by ROS particularly $OH^{\bullet}$ (Teoule and Cadet 1978). Ionising radiation can be absorbed directly by DNA, leading to ionisation of the bases, or indirectly by reacting with surrounding cellular water molecules (Steenken 1989). Ionisation of the water molecule results in production of the hydrated electron (e^-_{aq}), $OH^{\bullet}$ and $H^{\bullet}$, all of which react with the bases in DNA (Teoule and Cadet 1978; von Sonntag 1987). This work however will only deal with the reactions of the $OH^{\bullet}$ and the products formed.

It is also believed that many dietary mutagens and carcinogens, e.g. tobacco smoke and other cancer promoters act through the production of ROS (Ames 1983; Joenje 1989; Cerutti 1985).

Endogenous sources.

Endogenous ROS are produced inevitably as a result of normal aerobic metabolism. ROS are produced as by-products of mitochondrial electron transport (see Figure 1.1), oxygen utilising enzymes, peroxisomes, activated phagocytes and other physiological processes (Halliwell and Gutteridge 1989; Ames and Shigenaga 1992; Ames *et al.* 1993a).

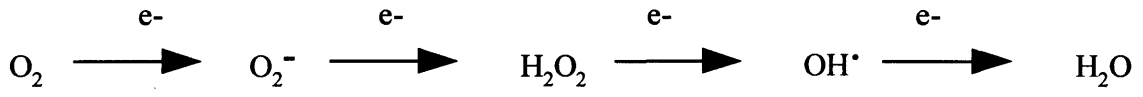


Figure 1.1 Oxidants from normal aerobic metabolism. The formation of superoxide, hydrogen peroxide, and hydroxyl radicals by successive additions of electrons to oxygen. Cytochrome oxidase adds 4 electrons during energy generation in mitochondria (Adapted from Ames and Shigenaga 1992).

The reduction of molecular oxygen to water by the addition of 4 electrons is the major source of energy for most aerobic organisms (Farber 1994). Aerobic cells are generally capable of reducing O_2 to water without the formation of intermediates, however a small fraction (1-5%) of cellular oxygen is reduced by monovalent steps producing intermediate ROS. It is believed that for every 10^{12} oxygen molecules entering a cell each day 1/100 damages protein and 1/200 damages DNA (Acworth and Bailey 1995).

Activated phagocytes also produce ROS (such as H_2O_2, HOCl and O_2^-) in order to defend the body against invading organisms.

1.1.2. Types of ROS

Superoxide Radical (O_2^-)

The poorly reactive O_2^- radical is produced by a 1 electron reduction of oxygen (Figure 1.1.). O_2^- is formed non-enzymatically in almost all aerobic cells (Fridovich 1983; Halliwell and Gutteridge 1989) as a intermediate product in the mitochondrial electron transport chain. In addition O_2^- is generated enzymatically as a result of a number of oxidases including those involved in phagocytosis (Fridovich 1983).

This radical is only reactive in a hydrophobic environment and cannot cross membranes (Takahashi and Asada 1983; Rueff *et al.*. 1990) without the presence of anion channels present only in erythrocytes (Lynch and Fridovich 1978; Halliwell and Gutteridge 1989).

Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide is produced by the addition of a second electron to O_2 producing the peroxide ion, O_2^{2-}. Any O_2^{2-} formed at physiological pH immediately protonates to form H_2O_2 (Halliwell and Gutteridge 1984). In aqueous solution H_2O_2 and O_2 are formed by the dismutation reaction of O_2^- by superoxide dismutase (SOD) (see eqn i) and also by oxidase enzymes such as those involved in phagocytosis. Unlike O_2^-, H_2O_2 resembles water in its molecular structure and readily crosses biological membranes (Halliwell and Gutteridge 1990a)

H_2O_2 can also be reduced to yield $OH^\bullet$ by transition metal ion catalysis, in particular by ferrous and cupric ions (eqns ii and iii). H_2O_2 can be reduced to water by two different enzymes: catalase in peroxisomes and glutathione peroxidase present in mitochondria and in the cytosol (see Section 1.2.1.).

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad \text{(i)}$$

Hydroxyl radical ($OH^{\bullet}$)

The hydroxyl radical is the most toxic of all the ROS and reacts with almost every type of molecule in its vicinity (see section 1.2.). The very short life span and intense reactivity of the $OH^{\bullet}$ radical usually means it reacts at its site of formation (Pryor 1988).

It is usually thought that most of the toxicity of O_2^- and H_2O_2 can be attributed to their metal ion conversion to the highly reactive $OH^{\bullet}$ (Imlay and Linn 1988; Joenje 1983; Halliwell and Gutteridge 1989). Mechanisms proposed have been based on a Fenton type reaction in which transition metal ions such as iron and copper decompose H_2O_2 to produce the $OH^{\bullet}$ (Halliwell and Gutteridge 1989, 1990a).

$$O_2^- + Fe_3^+ \longrightarrow Fe_2^+ + O_2 \quad \text{(ii)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\bullet} + OH^- + Fe^{3+} \quad \text{(iii)}$$

While transition metal ions such as iron and copper exist *in vivo* antioxidant defences ensure they are not freely available and are kept bound to transport or storage proteins (sequestration of metal ions) (Halliwell and Gutteridge 1986,1989,1990a). Transition metal ions that do become available *in vivo* must bind to a biological molecule (e.g. DNA or proteins). Any subsequent reaction with O_2^- and H_2O_2 will lead to formation of $OH^{\bullet}$ upon the biological molecule leading to site specific damage (Halliwell and Gutteridge 1989; Halliwell and Cross 1994). Such damage is very difficult to intercept by $OH^{\bullet}$ scavengers as the site of formation and the site of reaction are too close. Therefore the nature of the damage done by excess ROS *in vivo* depends on the availability and location of metal ion catalysts of $OH^{\bullet}$ formation.

1.1.3. Antioxidants

Aerobes have evolved antioxidant defences in order to protect cellular components from ROS generated *in vivo.* Antioxidants may be defined as any substance having the ability to ' delay or inhibit oxidative damage to a target molecule' (Gutteridge and Halliwell 1994). Among the defences are enzymes such as superoxide dismutase, catalase and glutathione peroxidase (which dispose of H_2O_2 and O_2) and non-enzymatic

defences such as glutathione, ascorbate, α-tocopherols, β-carotene and uric acid (Sun 1990; Halliwell and Gutteridge 1989; Reddy 1990; Halliwell 1994; Halliwell and Cross 1994). Another important aspect of antioxidant defence within cells and extracellularly is the control of metal ion availability for free radical reactions by binding metal ions to proteins (Halliwell and Gutteridge 1989). The evolution of so many overlapping defences is indicative of the importance of preventing or limiting oxidative damage.

In vivo antioxidants exist in balance with ROS (Halliwell and Cross 1994), and there is no reserve of antioxidants in mammals probably due to the useful metabolic functions also performed by ROS (e.g. during phagocytosis). Oxidative stress is said to result when ROS are not efficiently removed as a result of depletion of antioxidants and/or if the formation of ROS is increased beyond the ability of the defences to cope with (Sies 1985). Most organisms can cope with mild oxidative stress and respond to it by inducing synthesis of extra antioxidant defences, but severe oxidative stress however can lead to cell damage and cell death (Sies 1991; Halliwell and Cross 1994).

1.1.3.1. Enzymatic antioxidant defences

Superoxide dismutase (SOD)

SOD was discovered by McCord and Fridovich (1969), and is present in all aerobic organisms. SOD accelerates the dismutation reaction of $O_2^{\cdot}$ to H_2O_2 by ~ 4 orders of magnitude (Halliwell and Gutteridge 1990a). The enzyme dismutases two $O_2^{\cdot}$ molecules to form H_2O_2 and O_2 (eqn i). Four different types of SOD have been found to date. The two types found in eukaryotes are a copper and zinc containing SOD (CuZnSOD) found in cytosol, and a manganese containing SOD (MnSOD) found in mitochondria (presumably to remove O_2 produced by the electron transport chain) (Fridovich 1983,1989).

Excess CuZnSOD has been linked to at least some of the abnormalities of Downs syndrome (Groner *et al.*. 1990).

Catalases (CAT)

CAT exist in most aerobic cells and are responsible for the conversion of H_2O_2 to water and oxygen (eqn iv).

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \quad \text{(iv)}$$

In animals catalase is present in all major organs of the body with especially high concentrations occurring in the liver and erythrocytes (Sun 1990). At the cellular level it is found in the peroxisomes (Sun 1990) in most tissues, and probably serves to remove peroxide generated by oxidase enzymes. CAT consist of four protein subunits containing a heme group bound to its active site (Halliwell and Gutteridge 1989; Sun 1990).

Its affinity for H_2O_2 depends on the concentration of H_2O_2, with affinity increasing at high H_2O_2 concentrations (Gutteridge and Halliwell 1994).

Glutathione peroxidases (Gpx)

Gpx are enzymes which act complementary to catalase in order to remove hydrogen peroxide generated by SOD in cytosol and mitochondria. Gpx catalyse the oxidation of reduced glutathione (GSH) to oxidised glutathione (GSSG) at the expense of H_2O_2 (eqn v) (Halliwell and Gutteridge 1989; Sun 1990).

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O \quad \text{(v)}$$

The Gpx that removes H_2O_2 contains selenium at its active site which is essential for catalytic function.

Antioxidant enzymes function to protect against ROS as summarised in Figure 1.2.

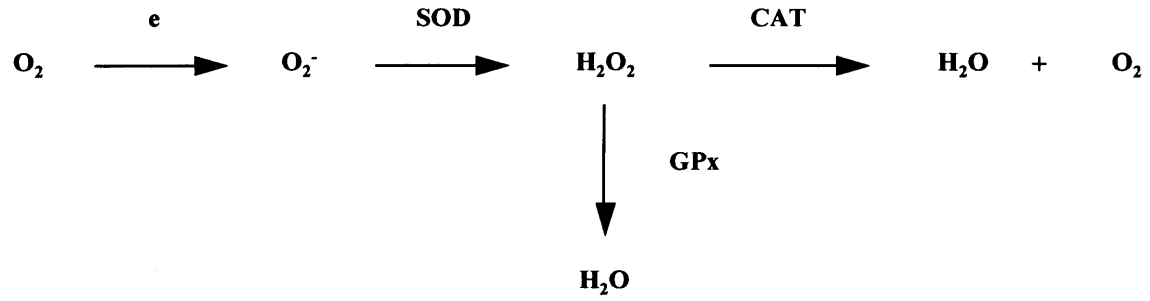


Figure 1.2. Role of enzymatic antioxidant defences in protecting against ROS (Adapted from Sun 1990).

1.1.3.2. Non-enzymatic antioxidants

The non-enzymatic small molecular mass antioxidants include; glutathione, ascorbic acid, α-tocopherol, β-carotene and uric acid. These antioxidants remove free radicals by reacting with them non-catalytically, thereby stopping chain reactions such as lipid peroxidation (Halliwell and Gutteridge 1989, Halliwell 1994).

Glutathione

Glutathione can scavenge $OH^{\bullet}$ and O_2^1 directly as well as being a substrate for Gpx enzymes. Glutathione is involved in the reactivation of some enzymes that have been inhibited by high O_2 concentrations, and is also a co-factor for several enzymes.

Ascorbic Acid

Ascorbic acid cannot be synthesised in the body and therefore must be obtained in the diet, from for example citrus fruits. Ascorbic acid protects more scarce antioxidants such as vitamins A, vitamin E and also essential fatty acids.

α-Tocopherol

Tocopherol is the most important free radical scavenger within membranes and occurs in membranes and lipoproteins. Tocopherol inhibits lipid peroxidation by scavenging peroxy and alkoxy radicals producing a poorly reactive secondary free radical which can be converted back to tocopherol by ascorbic acid.

β-Carotene

β-Carotene is a precursor for vitamin A formation in the intestine. β-Carotene and the carotenoids are found abundantly in green plants and vegetables and are free radical scavengers.

Uric acid

Uric acid is the end product of purine metabolism in man. It scavenges singlet O_2^1, $OH^{\bullet}$, O_3, HOCl and peroxy free radicals and binds metal ions in forms making them unavailable for radical reactions. It is also known to inhibit lipid peroxidation

Since iron and copper are powerful promoters of free radical damage and production of $OH^{\bullet}$, organisms have evolved an antioxidant system of transport and storage proteins to ensure that these metals are rarely 'free'. Iron is stored as ferritin, transported around the body as transferrin or lactoferrin and is also bound to other proteins (haemoglobin, myoglobin) ensuring unavailability of the free ions in solution. Copper is transported around the body as ceruloplasmin. Extracellularly levels of SOD and H_2O_2 removing enzymes are low and therefore metal binding antioxidant ability may be the major antioxidant defence (Halliwell and Gutteridge 1990b).

1.2. CHEMICAL REACTIONS OF ROS AND REPAIR

Most if not all of the toxicity of the ROS has been attributed to the $OH^{\bullet}$ radical, therefore the subsequent sections will deal only with $OH^{\bullet}$, related damage and its implications.

The highly reactive nature of $OH^{\bullet}$ means it is capable of reacting with any biological molecule in its vicinity including lipids, proteins and nucleic acids producing alterations to the structure and function of the cellular components.

1.2.1. Lipid peroxidation

Lipids include a wide range of hydrophobic compounds including fatty acids, phospholipids, steroids and waxes. Fatty acids not only serve as metabolic fuel but are also an integral part of cell membranes where along with phospholipids they regulate membrane fluidity. Lipid peroxidation is the oxidation of polyunsaturated fatty acids (PUFA) in an autocatalytic process leading to the formation of fatty acid hydroperoxides and secondary products including a wide range of aldehyde compounds (for a review see Cheeseman 1993; Halliwell and Gutteridge 1990a; Tappel 1990; Gutteridge and Stocks 1976; Sevanian 1990; Slater 1982,1984). Susceptibility of fatty acids to oxidation increases with increasing number of double bonds (unsaturated fatty acids are particularly susceptible to $OH^{\bullet}$ attack, whereas saturated fatty acids only undergo oxidation under extreme circumstances).

Peroxidation of unsaturated fatty acids makes them more hydrophilic and consequently alters the structure of the membrane, thus altering the normal membrane function (e.g. transporters and receptors). Lipid peroxidation also leads to indirect damage of cellular membranes via the production of reactive products (carbonyl and oxyl radicals). Lipid peroxidation along with other types of oxidative damage is implicated in a wide range of diseases, however is not believed to be the primary mechanism of cellular damage produced by ROS but rather a late stage in cellular injury (Halliwell and Gutteridge 1984).

Lipid peroxidation is considered to occur in 3 stages namely; Initiation, Propagation and Termination (for a review see Cheeseman 1993).

Initiation

Initiation refers to attack by any species sufficiently reactive (e.g. $OH^{\bullet}$) to abstract a H atom from a methylene ($-CH_2-$) group to produce a lipid radical (e.g. $LH^{\bullet}$). The lipid radical produced as a result of $OH^{\bullet}$ attack subsequently rearranges to form a conjugated diene. The C-centred radicals react with oxygen in aerobic conditions to produce lipid peroxy radicals which then react with other PUFA, beginning a new chain of oxidations forming lipid hydroperoxides.

Propagation

Propagation is the initiation of a new chain by lipid peroxyl radicals. Lipid hydroperoxides are not very stable and break down, especially in the presence of transition metal ions, leading to the production of new radicals with the potential of starting new reactions. The breakdown of hydroperoxides is important, as not only are new radicals produced for propagation of lipid peroxidation but non-radical species such as aldehydes are also formed.

Termination

Lipid peroxidation has the potential to destroy all available PUFA if permitted as the reaction is substrate-limited. Termination occurs when favourable conditions permit radical plus radical reactions to halt the lipid peroxidation chain reaction.

1.2.2. Protein damage

Proteins play a major role in almost all biological processes including catalysis (enzymes), transport, co-ordinated motion, recognition (receptors) and the control of growth and differentiation. Oxidants damage proteins as well (Stadtman 1992), leading to protein catabolism which can have disastrous effects on the normal functioning of the organism. For example oxidation at the active site of an enzyme molecule could have huge biological implications. Different forms of protein oxidation can occur including the formation of protein carbonyls, attack on amino acids and the oxidation of sulphydryl groups (Davies 1987; Wolff *et al.*. 1986; Dean 1990).

Protective proteolytic enzymes are known to exist, which hydrolyse oxidised proteins, however these are not sufficient to prevent an age associated increase in oxidised

proteins. Oxidation of proteins has also been implicated with disease. For example Werner syndrome and progeria are two human diseases associated with premature ageing in which oxidised proteins are reported to accumulate at a higher rate than normal (Stadtman 1992).

1.2.3. DNA damage

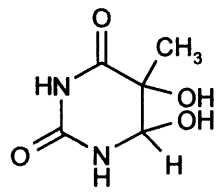
DNA is the genetic code of life. Damage to the genetic material has been shown to disrupt transcription, translation and DNA replication, and to give rise to mutations and ultimately cell death (Ames 1989c; Simic *et al.*. 1989). Endogenous oxidative damage to DNA is believed to be quite extensive with values of 1 modification per 130,000 bases in nuclear DNA (Richter *et al.*. 1988) and 1 modification per 8000 bases in mitochondrial DNA (Richter *et al.*. 1988). The importance of oxidative DNA damage is further supported by the existence of specific enzymes for its repair.

The $OH^{\bullet}$ reacts with DNA to produce base modifications, sugar modifications, strand breaks, abasic sites, and DNA-protein cross links (von Sonntag 1987; Halliwell and Gutteridge 1989; Imlay and Linn 1988; Joenje 1989; Breimer 1988). The spectrum of modifications produced is dependent upon the reactive species involved. In the work described below particular emphasis will be given to the wide range of base modifications produced by ROS.

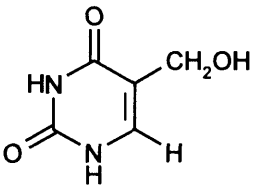
1.2.3.1. Base modifications

The wide array and elevated levels of base modifications found in cancerous tissue is indicative of the important role DNA damage plays in the etiology of cancer and as biomarkers of cancer risk assessment. Steady state levels of oxidatively modified bases are present in DNA with levels being increased *in vivo* and *in vitro* by systems generating ROS (ionising radiation, tumour promoters etc.).

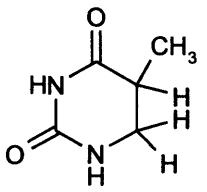
Upon oxidation of DNA approximately 20 modified bases have been reported (see figure 1.3.). The most frequently studied oxidatively modified bases have been 8-OHG, Tg and 5-OHMeU (Nackerdien *et al.* 1992; Teebor *et al.* 1987; Faure *et al.* 1993; Djuric *et al.* 1991a; Frenkel *et al.* 1985; Nagashima *et al.* 1995; Loft *et al.* 1992).



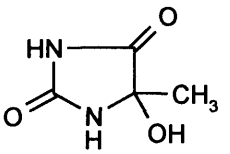
thymine glycol



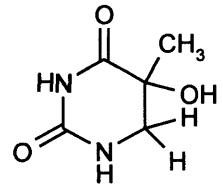
5-(hydroxymethyl)-uracil



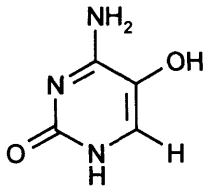
5,6-dihydrothymine



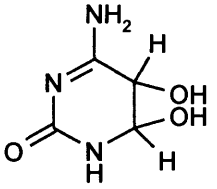
5-hydroxy-5-methyl-hydantoin



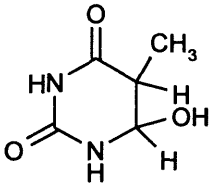
5-hydroxy-6-hydro-thymine



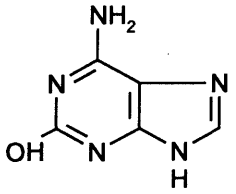
5-hydroxycytosine



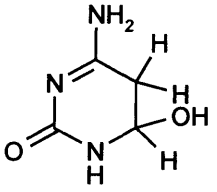
cytosine glycol



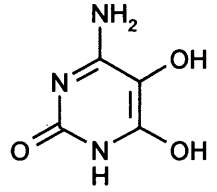
6-hydroxy-5-hydro-thymine



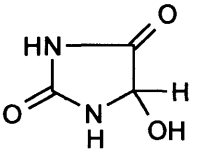
2-hydroxyadenine



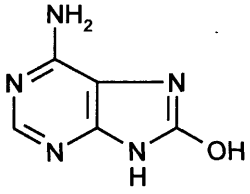
6-hydroxy-5-hydro-cytosine



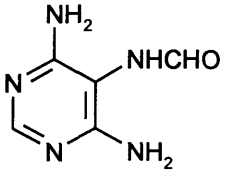
5,6-dihydroxycytosine



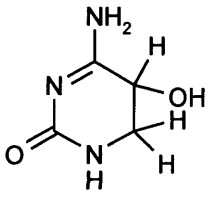
5-hydroxyhydantoin



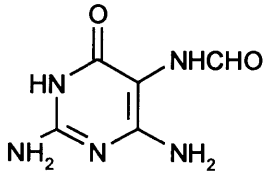
8-hydroxyadenine



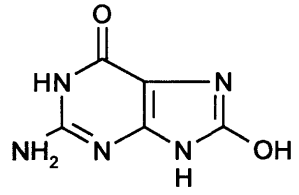
4,6-diamino-5-formamido-pyrimidine



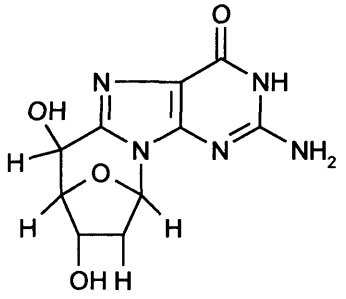
5-hydroxy-6-hydro-cytosine



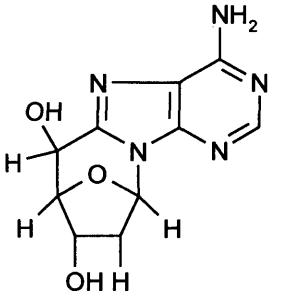
2,6-diamino-4-hydroxy-5-formamidopyrimidine



8-hydroxyguanine



8,5'-cyclo-2'-deoxyguanosine (5'R- and 5'S-)

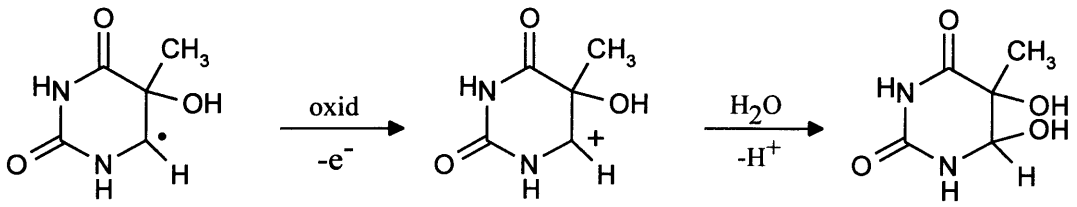


8,5'-cyclo-2'-deoxyadenosine (5'R- and 5'S-)

Figure 1.3. DNA base modifications (Adapted from Dizdaroglu 1993a).

The two main modes of attack of the $OH^{\bullet}$ are addition to the double bonds of bases and H abstraction from the sugar moiety in DNA (Breen and Murphy 1995).

All the heterocyclic bases in DNA are subject to oxidation, however pyrimidines are particularly susceptible. DNA bases undergo ring saturation, ring contraction, ring opening and hydroxylation. The major reaction of the $OH^{\bullet}$ radical is that with pyrimidines at the C5-C6 double bond at diffusion controlled rates (for a review see Dizdaroglu 1993a; Breen and Murphy 1995). The preferred site of attack for the electrophilic $OH^{\bullet}$ is the C5 due to its high electron density. The $OH^{\bullet}$ adds to thymine and cytosine at C5 (60% and 90% respectively) and C6 (30% and 10% respectively) (Fugita and Steenken 1981). Abstraction of an H atom by the $OH^{\bullet}$ from the methyl group of thymine also occurs (~ 10 %) (Fugita and Steenken 1981). Upon reaction of the $OH^{\bullet}$ with the double bonds of pyrimidines, adduct radicals namely 5-OH-6 yl and 6-OH-5 yl radicals, are produced (Dizdaroglu 1993a). The adduct radicals react further to form numerous products. Oxidation of the 5-OH-6 yl radical followed by addition of $OH^{\bullet}$ (or addition of H_2O followed by deprotonation) leads to the formation of Tg (Teoule and Cadet 1978; von Sonntag 1987) (Figure 1.4).

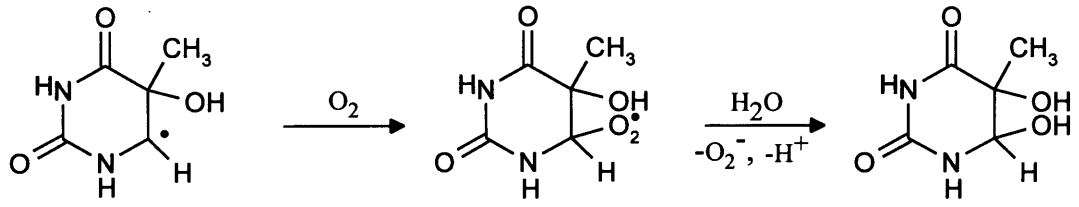


5-hydroxy-6-yl radical

thymine glycol

Figure 1.4. Formation of thymine glycol from the 5-hydroxy-6-yl radical of thymine (Adapted from Dizdaroglu 1993a).

In the presence of oxygen the 5-OH-6 -yl radical reacts with molecular oxygen to yield peroxyl radicals which may eliminate O_2 to also yield Tg (Figure 1.5.) (Teoule and Cadet 1978; Dizdaroglu 1993a). Pyrimidine glycols may be formed in the presence and absence of oxygen (Dizdaroglu 1993a).



5-hydroxy-6-yl radical 5-hydroxy-6-peroxyl radical thymine glycol

Figure 1.5. Formation of thymine glycol from the 5-hydroxy-6-peroxyl radical of thymine (Adapted from Dizdaroglu 1993a).

Purines react with $OH^{\bullet}$ at the C4, C5 and C8 positions producing adduct radicals (von Sonntag 1987; Breen and Murphy 1995; Dizdaroglu 1993a; Cadet 1994). Addition of $OH^{\bullet}$ to the C8 of guanine produces the C8 -OH adduct radical which can be oxidised or reduced (Steenken 1989). The C8-OH adduct radical can be reduced to 7-hydro-8-hydroxyguanine, oxidised to 8-OHG or can undergo opening of the imidazole ring followed by a 1 electron reduction and protonation to form 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGuanine) (Figure 1.6.). Purine and Fapy derivatives may be formed in the absence and presence of oxygen. Products resulting from C4 and C5-OH radicals are not known (Dizdaroglu 1993a).

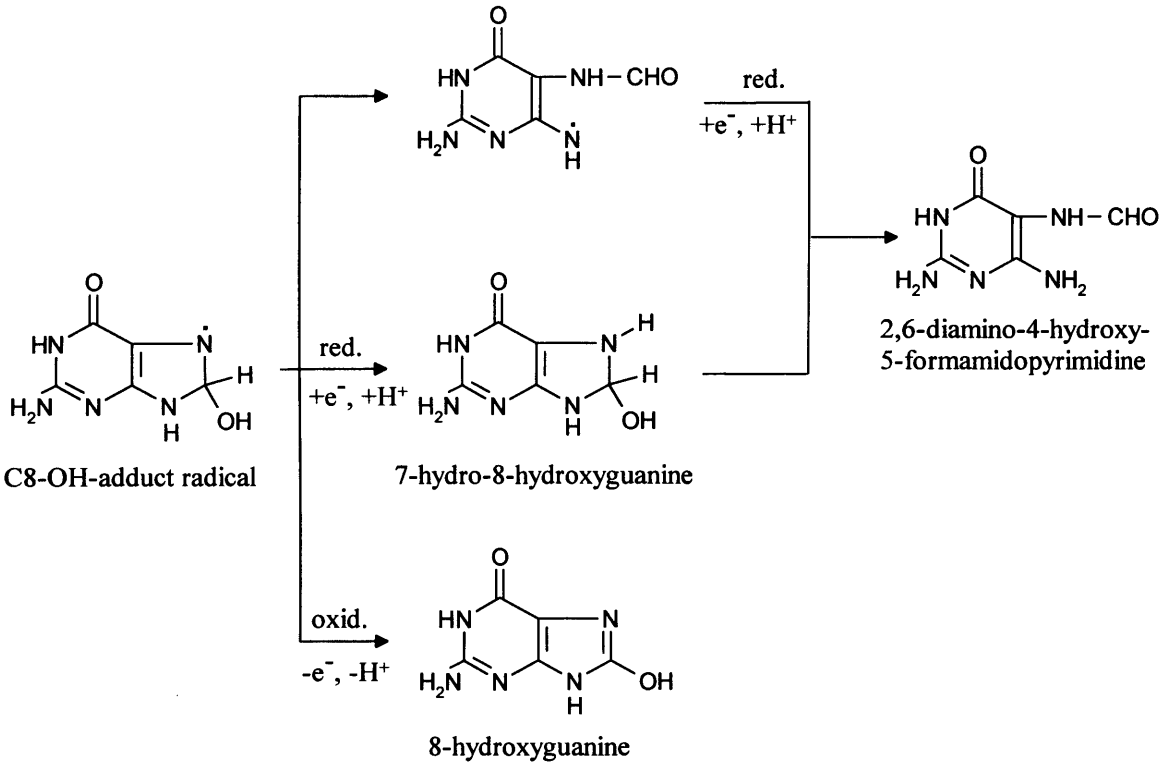


Figure 1.6. Reaction of the C8-OH adduct radical of guanine (Adapted from Steenken 1989).

1.2.3.2. Sugar damage.

A small fraction of $OH^{\bullet}$radicals (up to 20%) reacts with the sugar moiety in DNA (von Sonntag 1987). The $OH^{\bullet}$ can abstract H atoms from any of the 5 carbon atoms producing a C-centred radical (von Sonntag 1987). Oxidative sugar damage leads to sugar fragmentation, base loss and strand breaks (Imlay and Linn 1988; Breen and Murphy 1995). The sugar radicals produced can undergo further reactions. The C4 position is particularly important as the C-H bond is the weakest and the C4 is the most sterically exposed atom to attack (von Sonntag 1987). The C4 centred radical in deoxygenated systems undergoes β-cleavage producing strand breakage, followed by release of an intact base and formation of altered sugars (Dizdaroglu *et al.* 1975). In the absence of oxygen, base radicals can also abstract H atoms from a sugar moiety to yield sugar radicals and strand breakage. In oxygenated systems peroxyl radicals are formed by addition of molecular oxygen to carbon centred sugar radicals which can then undergo cleavage of the C-C bonds producing alkali labile sites (Dizdaroglu *et al.* 1977).

1.2.4. DNA repair

DNA is subjected to continuous degradation processes that if not corrected would interfere with the efficient functioning of the genetic material. As antioxidant defences are not completely effective, repair enzymes exist that destroy free-radical-damaged proteins, remove oxidised fatty acids from membranes and repair free radical damage to DNA (by annealing strand breaks and excising modified bases). It is estimated that over a hundred genes are dedicated to DNA repair emphasising the importance of maintaining the genetic material.

Mechanisms by which organisms cope with DNA damage can be classified into two broad categories: damage tolerance mechanisms and repair mechanisms (see Friedberg 1985 for review). Damage tolerance involves bypassing of a lesion that may block replication resulting in a gap, the new strand being filled by use of the opposite parental strand. Repair of damaged DNA can occur via direct or excision repair mechanisms. Direct repair occurs when a reaction in which DNA damage occurs is reversed, but this is not used in the repair of oxidative damage (Breimer 1991). Excision repair is widely used in the repair of oxidative DNA damage and involves removal and replacement of the damaged or incorrect base. This mode of repair can be separated into nucleotide excision repair and base excision repair (Figure 1.7.).

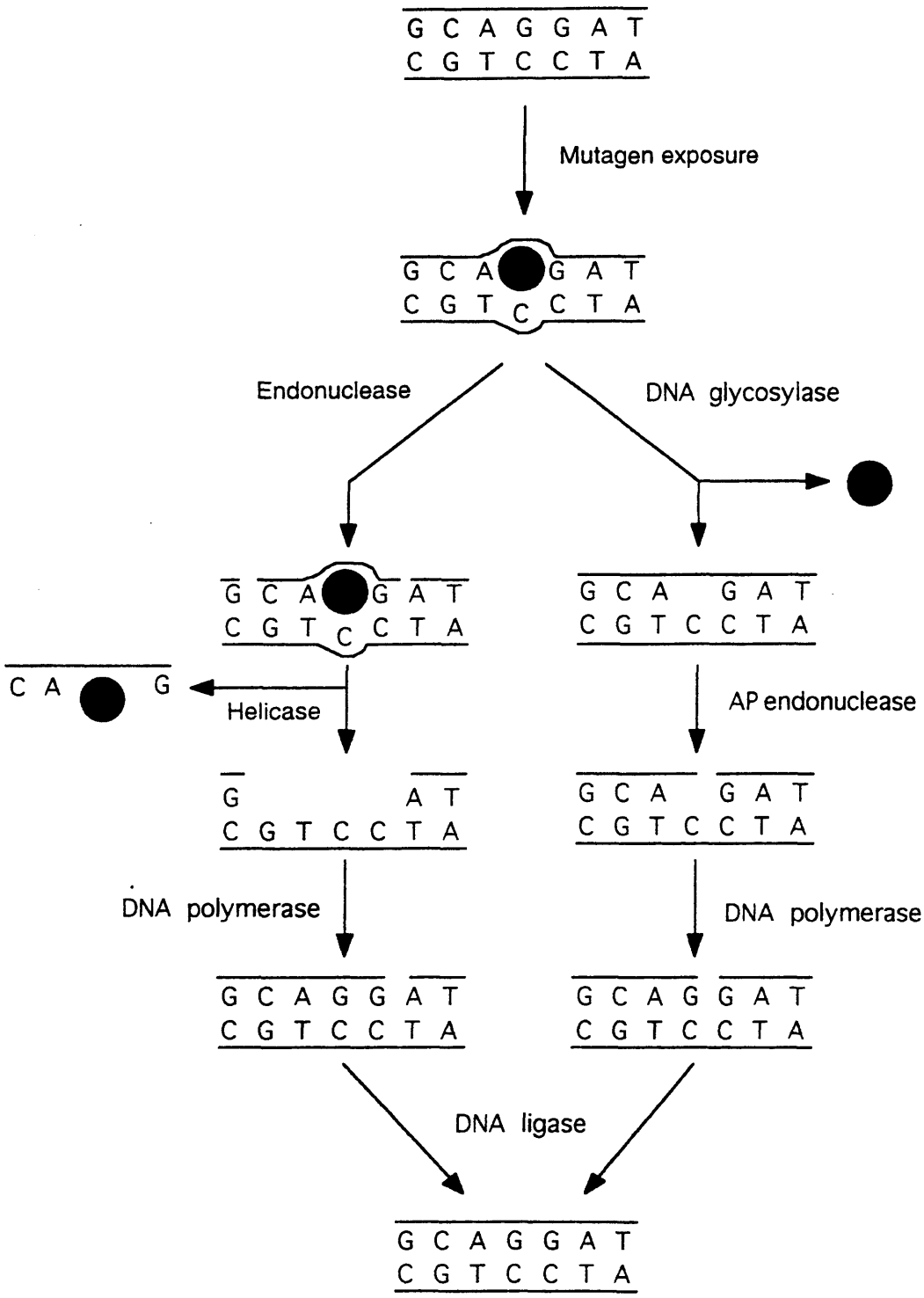


Figure 1.7. Excision repair of DNA damage. (Adapted from Casarett and Doull 1996). The repair pathways called nucleotide excision (left) and base excision (right) remove the damaged region and restore the intact DNA.

1.2.4.1. Nucleotide excision repair

The nucleotide excision repair (NER) pathway is the most general repair mechanism in all organisms and is responsible for the repair of a wide range of damage (Sancar and Tang 1993).

NER is initiated by the action of an endonuclease which nicks the DNA backbone at both sides of the damage. A helicase then removes the damaged oligonucleotide leaving a gap 27 - 29 nucleotides long in eukaryotes. A repair polymerase subsequently fills the gap using the opposite strand as a template. DNA ligase seals the remaining gap in the DNA leading to the restoration of the original DNA sequence (Sancar and Tang 1993; Friedberg 1985).

1.2.4.2. Base excision

During base excision specific DNA glycosylases recognise and remove modified bases from the deoxyribose leaving an abasic site. An apurinic/apyrimidinic endonuclease incises the abasic site and an exonuclease subsequently removes the deoxyribose-phosphate. In comparison to NER the patch repaired is relatively small, usually only a single nucleotide, and as in NER polymerase and ligase action complete the repair process (Friedberg 1985).

1.2.4.3. Repair of oxidative DNA damage

Various oxidative DNA repair enzymes have been found in prokaryotic and eukaryotic organisms (Teoule 1987; Wallace 1988; Myles and Sancar 1989; Demple and Levin 1991; Doetsch 1990; Floyd 1990; Demple and Harrison 1994). The existence of such enzymes is an indication of the occurrence and importance of removing oxidative DNA damage. In addition to their existence repair enzymes have been found to recognise a broad range of overlapping substrate specificities in order to aid fast and efficient removal.

The system of study for DNA repair enzymes has been the bacterium E.coli and most of the damage produced has been as a result of ionising radiation. E.coli has been found to contain a variety of specific glycosylases for the repair of oxidative damage including endonuclease III, 5-hydroxymethyluracil DNA-glycosylase and formamidopyrimidine-

DNA-glycosylase. DNA lesions may be repaired as a result of specific glycosylases or by more general endonuclease enzymes. For example the modified thymine lesion Tg is recognised and repaired in E.coli by endonuclease III (Demple and Linn 1980; Gates and Linn 1977) but could also be repaired via a nucleotide excision repair pathway, the products of repair (Tg and TdG) subsequently being excreted into the urine (Lin and Sancar 1989; Adelman *et al.* 1988). Endonuclease III is also able to recognise a wide range of other oxidised pyrimidine substrates (Breimer and Lindahl 1984). A specific 5-OHMeU-DNA glycosylase has also been found in eukaryotes for the elimination of 5-OHMeU and is different to the Tg repair enzyme (Hollstein *et al.* 1984; Frenkel *et al.* 1985; Teebor *et al.* 1989). The 5-OHMeU-DNA glycosylase enzyme has not been found in bacteria or yeast probably because 5-OHMeU has been found to occur naturally in some bacteria replacing thymine as the fourth base. The formamidopyrimidine-DNA glycosylase enzyme in E.coli has been found to repair oxidised purines including 8-OHG and Fapy derivatives of adenine and guanine (Breimer 1984, Boiteux *et al.* 1992, Loft and Poulsen 1996; Tchou *et al.* 1991).

Various endonucleases such as the Uvr ABC complex in E.coli also recognise and repair (*in vivo* and *in vitro*) oxidative damage such as Tg, 8-OHG and abasic sites by eliminating complete nucleotides in a single step (Lin and Sancar 1989, Kow *et al.* 1990, Czeczot *et al.* 1991).

Analogues of the above E. coli DNA repair enzymes have been identified in mammalian cells (see Table 1.1.). In humans a glycosylase and endonuclease activity for the elimination of 8-OHG and 8-OHdG has also been identified (Bessho *et al.* 1993).

Table 1.1.. Specific glycosylases present in mammalian cells for the removal of oxidative damage (Wallace 1988).

Glycosylase	Repair product
Uracil-DNA glycosylase	Uracil
Hydroxymethyluracil-DNA glycosylase	Hydroxymethyluracil
Hydroxymethylcytosine-DNA glycosylase	Hydroxymethylcytosine
Formamidopyrimidine-DNA glycosylase	Imidazole ring cleaved purines
Hypoxanthine-DNA glycosylase	Hypoxanthine
Redoxyendonuclease	Oxidised pyrimidine products

Note. The repair products stated are not necessarily the **only** product repaired by the glycosylase, but are the products for which repair has been recognised.

From the described mechanisms it can be seen that multiple overlapping repair pathways exist and therefore the fate of an individual lesion is not predictable.

Repair does not provide complete protection against mutagenesis as repair processes may become saturated, or inefficiently repair some kinds of damage. Some lesions may become fixed as mutations before repair or mutations may result during processing of DNA damage i.e. error prone repair (for a review see Friedberg 1985).

1.3. OXIDATIVE DNA DAMAGE AND DISEASE.

Oxygen free radicals and the damage they inflict upon cellular molecules have been implicated in ageing and a wide range of human diseases including cancer (for a review see Halliwell and Gutteridge 1989, Halliwell *et al.* 1992) (Table 1.2.). The role of the ROS in the diseased states however is uncertain; are the free radicals a consequence or cause of the diseases? In the majority of human diseases ROS are produced as a consequence of the tissue injury (Figure 1.8.). For example in rheumatoid arthritis excess production of O_2^- and H_2O_2 and other ROS by phagocytes at sites of chronic inflammation can cause severe damage as seen in the inflamed joints of patients (Halliwell et al. 1992). However in terms of cancer and ageing free radical induced damage is believed to play an important role. This section will briefly examine the role of ROS and oxidative DNA damage in relation to cancer.

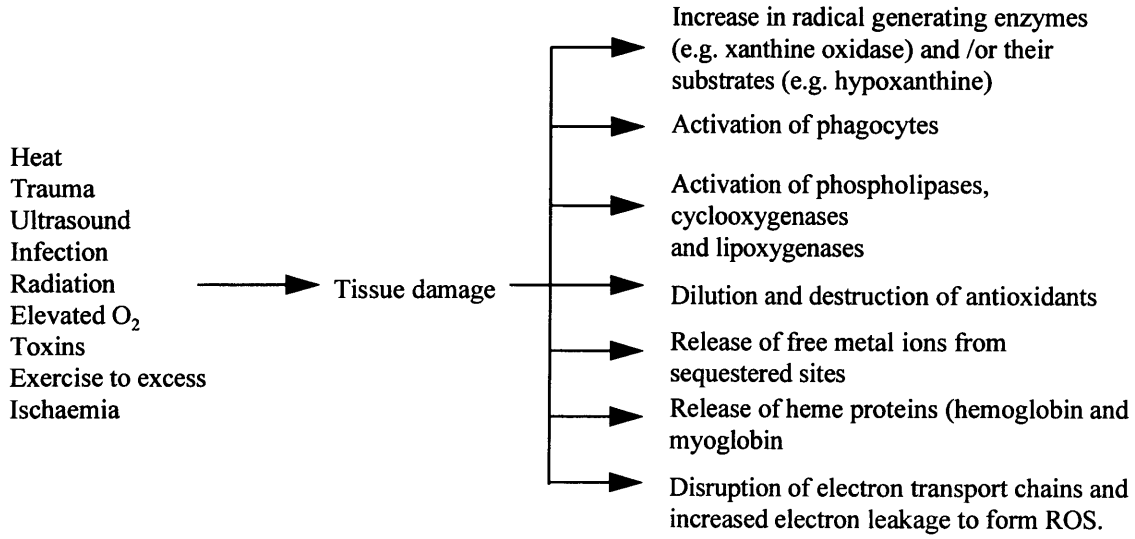


Figure 1.8. Mechanisms by which tissue damage can cause production of ROS and subsequently oxidative stress (Adapted from Halliwell and Cross 1994).

Table 1.2. Clinical conditions in which involvement of oxygen radicals has been suggested (Adapted from Halliwell and Gutteridge 1989).

Inflammatory-immune injury Glomerulonephritis (idiopathic, membranous) Vasculitis (hepatitis B virus, drugs) Autoimmune diseases Rheumatoid arthritis	**Heart and cardiovascular system** Alcohol cardiomyopathy Keshan disease (selenium deficiency) Atherosclerosis Adriamycin cardiotoxicity
Ischaemia-reflow states Stroke/myocardial infarction/arythmias Organ transplantation Inflamed rheumatoid joint Frostbite Dupuytren's contracture ?	**Kidney** Autoimmune nephrotic syndromes Aminoglycoside nephrotoxicity Heavy metal nephrotoxicity (Pb, Cd, Hg)
Brain/nervous system/neuromuscular disorders Hyperbaric oxygen Vitamin E deficiency Neurotoxins Parkinson's disease Hypertensive cerebrovascular injury Neoronal encephalomyelitis and other demyelinating diseases Aluminium overload (Alzheimer's disease ?) Potentiation of traumatic injury Muscular dystrophy Multiple sclerosis	**Gastrointestinal tract** Endotoxic liver injury Pancreatitis Halogentaed hydrocarbon liver injury Diabetogenic action of alloxan NSIAD-induced gastrointestinal tract lesions Oral iron poisoning
Eye Cataractogenesis Ocular haemorrhage Degenerative retinal damage Retinopathy of prematurity Photic retinopathy	**Iron overload** Dietary iron overload (Bantu) Idiopathic haemochromatosis Thalassemia and other chronic anaemias traeted with multiple blood transfusions. Nutritional deficiencies (Kwashiorkor)
Lung Cigarette smoke effects Emphysema Hyperoxia ARDS (some forms) Mineral dust pneumoconiosis Bronchopulmonary dysplasia Oxidant pollutants (O_3, NO_2) Asbestos carcinogenecity Bleomycin toxicity SO_2 toxicity Paraquat toxicity	**Red blood cells** Phenylhydrazine Primaquine, related drugs Lead poisoning Protoporphyrin photoxidation Malaria Sickle cell anaemia Favism Fanconi's anaemia Hemolytic anaemia of prematurity
Alcoholism Inducing alcohol-induced iron overload and alcoholic myopathy	**Ageing** Disorders of premature aging
Radiation injury Nuclear explosions Accidental exposure Radiotherapy Hypoxic cell sensitizers	**Skin** Solar radiation Thermal injury Porphyria Hypericin, other photosensitizers Contact dermatitis
	Drug and toxin-induced reaction

ARDS - adult respiratory distress syndrome. NSAID - non steroidal anti-inflammatory drug.

1.3.1. Cancer.

Carcinogenesis is a multistage process involving genetic and epigenetic changes. The multistage process can be divided into at least three distinct stages; initiation, promotion and progression. Initiation is related to the alteration of DNA, and prevention of this stage may therefore be possible by efficient DNA repair. Promotion however, largely involves the proliferation of an initiated cell, whilst progression is the final stage in which a benign lesion is transformed into a malignant neoplasm (Farber 1982; Pitot and Dragan 1991).

Oxygen free radicals are believed to play an important role in multistage carcinogenesis (Oberley and Oberley 1986; Cerutti 1985). ROS can act as initiators and/or promoters of carcinogenesis, cause DNA damage, activate procarcinogens and alter cellular antioxidant defences. A number of initiators of carcinogenesis have been shown to produce or cause the production of free radical species. Evidence also indicates that tumour promoters such as TPA stimulate ROS production and modify antioxidant levels *in vivo* (for a review see Sun 1990).

The risk of developing cancer is determined in part by genetic predisposition, as well as by exogenous and endogenous factors. Epidemiological studies indicate that most human cancers can be prevented to a significant extent by changes in diet and lifestyle (30% of cancer deaths in 1981 were attributed to smoking and 35% to diet) (Ames *et al.* 1995; Doll and Peto 1981). Apart from exposures to the major exogenous risk factors associated with diet and lifestyle, cancer is believed to become a degenerative disease caused largely by endogenous processes (Ames *et al.* 1995). Oxidation is a major source of endogenous DNA damage *in vivo* (Totter 1980; Ames 1983) and is believed to play a major role in mutagenesis and carcinogenesis. Cancer risk increases with the fifth power of age in humans (approx. 30% have cancer by the end of an 85 year life span) (Ames 1989c; Ames and Shigenaga 1993) and therefore can be considered as a degenerative disease although exogenous factors can increase (cigarette smoking) or decrease cancer (calorie restriction) incidence. Oxidative DNA damage appears to be related to metabolic rate in various species and has also been shown to accumulate with age (Ames and Shigenaga 1992; Fraga *et al.* 1990; Stadtman 1992). Oxidative hits in humans have been estimated at 10,000 per cell per day with lesions and mutations

accumulating with age (Ames *et al.* 1993a; Branda *et al.* 1993). Mitochondrial DNA has more than 10 times the level of oxidative DNA damage than nuclear DNA from the same tissue, mainly due to the close proximity to oxidants generated during mitochondrial electron transport (Richter *et al.* 1988).

DNA lesions if not repaired have a certain probability of giving rise to mutations when the cell divides (Ames *et al.* 1993b). DNA damage and cell proliferation are essential factors in carcinogenesis and agents causing either are considered potential carcinogens. Cell proliferation is a critical factor in mutagenesis, (mutation is not sufficient for carcinogenesis) (Ames 1989a; Farber 1987; Pitot *et al.* 1987). Cell proliferation enables DNA lesions to become mutations and makes single stranded DNA available as a target. If these mutations are related to critical genes such as oncogenes or tumour suppressor genes initiation or progression of carcinogenesis may result. The exact mechanism by which endogenous damage relates to cancer is unclear, yet several plausible mechanisms suggest:

(i) Mutations as a result of oxidative damage to DNA could lead to disruption of cellular functions by altering structural genes and their regulation (ii) High rates of oxidative damage in mitochondrial DNA could result in energy deficiencies leading to deficiencies in maintenance of energy dependent enzymes and possibly cell death (iii) Cell death could cause neighbouring cells to proliferate (iv) Oxidative damage could activate oncogenes (Ames 1989a).

The biomarkers of oxidative DNA damage appear to play a important role in the etiology of cancer and also serve as markers of risk assessment. Increased rates of oxidative modifications or deficient repair have been reported in human cancer tissues (Loft and Poulsen 1996). Elevated levels of oxidatively modified DNA bases have been reported in various cancers from lung, colon, ovary and breast compared to the cancer free surrounding tissue (Malins and Haimanot 1991, Malins *et al.* 1993; Malins 1993; Olinski *et al.* 1992; Jaruga *et al.* 1994). Urinary markers of oxidative damage 8-OHdG have also been found to increase in patients with malignant diseases and in patients receiving radiation or chemotherapeutic treatment (Tagesson *et al.* 1992). Tg and Tdg excretion levels however were found to be similar in neoplastic urine compared to urine from healthy control subjects (Cao and Wang 1993).

Studies of endogenous mechanisms of carcinogenesis are improving greatly, with improvements in sensitive techniques to detect and quantitate oxidative markers in tissue and body fluids paralleling similar achievements in exogenous carcinogenesis through measurements of DNA adducts.

1.3.2. The role of antioxidants.

Antioxidants have been shown to play a important role in multistage carcinogenesis by behaving as anticarcinogens and inhibiting the initiation and promotion stages of cancer (Cerutti 1985; Sun 1990). It is therefore not surprising that altered antioxidant levels have been reported in tumour cells when compared to their normal cell counterparts (for a review see Sun 1990). Tumour cells have been found to nearly always contain low levels of MnSOD, usually low levels of CuZnSOD and almost always low levels of CAT with Gpx levels being highly variable (Oberley and Buettner 1979). The link between altered antioxidant levels and cancer is as yet unknown; are the altered levels a cause or consequence of the disease? Jaruga *et al.* (1994) reported decreased activities of the antioxidant enzymes SOD and CAT in lung cancer tissues and in addition increases in oxidatively modified DNA bases in the same cancerous tissue compared to its cancer free surrounding tissue.

Epidemiological studies also support the protective role of antioxidants indicating reduced risks for cancer associated with a diet rich in antioxidants (Ames *et al.* 1995). However, antioxidant supplementation studies have been less than conclusive. A large scale clinical study involving antioxidant supplements for 30,000 smokers showed no reduced risk of lung cancer following the daily administration of β-carotene or vitamin E (Heinonen *et al.* 1994). Antioxidant supplementations to prevent colon adenoma were also unsuccessful (Greenberg *et al.* 1994), although a combination of vitamin E, β-carotene and selenium reduced the risk of gastric cancer in a high risk population (Blot *et al.* 1993).

In terms of altering rates of oxidative DNA damage supplementation of β-carotene had no effect on the rate of 8-OHdG excretion in smokers (van Poppel *et al.* 1995), neither was 8-OHG excretion effected by daily administration of vitamins C, E and β-carotene. In contrast, however Fraga *et al.* (1991) showed ascorbic acid reduced 8-OHdG formation in sperm DNA.

1.4. MEASUREMENT OF OXIDATIVE DNA DAMAGE.

In order to understand the role of oxidative DNA damage in biological processes and carcinogenesis, it is essential to chemically characterise and quantify DNA lesions.

The most common sources from which DNA is obtained for human studies are white blood cells, placentas, oral biopsies, buccal smears, bladder epithelia, and autopsy tissue. Urinary DNA adducts produced as a result of DNA excision repair enzymes such as Tg, 5-OHMeU, 8-OHG and 8-OHdG have also been detected and quantitated (Cathcart *et al.* 1984; Shigenaga *et al.* 1989; Bianchini *et al.* 1996).

DNA lesions have been detected using ^{32}P-postlabelling (PPL), immunoassay, high performance liquid chromatography (HPLC) with fluorescence or electrochemical detection (ECD) and mass spectrometry (MS). The choice of technique is dependent upon the properties of the lesion under study and the information required. A brief introduction into the existing techniques for detecting oxidative DNA lesions will be given.

1.4.1. Electrochemical detection (ECD)

A method for the detection of oxidative DNA damage has been developed combining HPLC with ECD (Floyd *et al.* 1986). The technique however is limited to electrochemically active modified bases such as 8-OHG (Shigenaga *et al.* 1989), 8-hydroxyadenine (8-OHA) (Berger *et al.* 1990), 5-hydroxycytosine (5-OHC) and 5-hydroxyuracil (5-OHU) (Wagner *et al.* 1992). ECD has been most widely applied to the detection and quantification of the oxidative DNA damage marker 8-OHG assayed as the 2'deoxynucleoside. The 8-OHG lesion has been assayed as the free 2'deoxynucleoside in urine (Shigenaga *et al.* 1989, Fraga *et al.* 1990; Loft *et al.* 1992) and from the DNA of cells exposed to a wide variety of oxidising agents (Takeuchi *et al.* 1994, Fraga *et al.* 1994).

In this procedure DNA is enzymatically digested to yield deoxynucleosides which are subsequently separated by reversed phase HPLC (Floyd *et al.* 1989). The same analysis can be used to measure the unmodified deoxyguanosine by UV absorbance as a reference. More recently the modified base 8-OHG has also been detected and

quantitated using ECD (Herbert *et al.* 1996, see also chapter 5) following acidic hydrolysis of DNA.

Electrochemical detectors are highly sensitive enabling detection of 1 modification per 10^6 nucleosides (Floyd *et al.* 1986; Ravanat *et al.* 1995), and since relatively few components in a mixture are electrochemically active ECD is also considerably selective, and derivatisation of analytes is not required. Limitations of ECD include the lack of structural information available and only analyses of electrochemically active compounds is possible.

1.4.2. ^{32}P-Postlabelling (PPL).

The (PPL) assay developed by Randerath *et al.* (1981) has enabled the detection of DNA damage induced by a wide variety of genotoxins and free radicals (for a review see Watson 1987; Gupta and Randerath 1988). PPL has a number of features which make it a powerful tool for assessing exposure to genotoxic chemicals. Two important aspects of the assay are its sensitivity and versatility, requiring only microgram quantities of DNA, enabling the assay to be performed on small amounts of sample from a variety of sources including human and animal tissues, lymphocytes and cultured cells. In addition PPL can be used to detect a wide range of DNA damage, including unidentified damage, as the knowledge of the structure of the adduct is not necessary for detection purposes. For these reasons PPL is particularly well suited to detecting DNA damage produced as a result of complex mixtures such as tobacco smoke, exhaust fumes and the petrochemical industry. The sensitivity and applicability of the PPL assay to a wide array of genotoxic chemicals makes it an ideal analytical method for studies involving environmental pollution.

The basic procedure (see Figure 1.9.) involves the enzymatic hydrolysis of DNA to deoxyribonucleoside 3'-monophosphates (dNps) using micrococcal nuclease and calf spleen phosphodiesterase. The dNps are then radioactively labelled via T4 polynucleotide kinase and [γ^{32}P] -adenosine triphosphate (ATP).

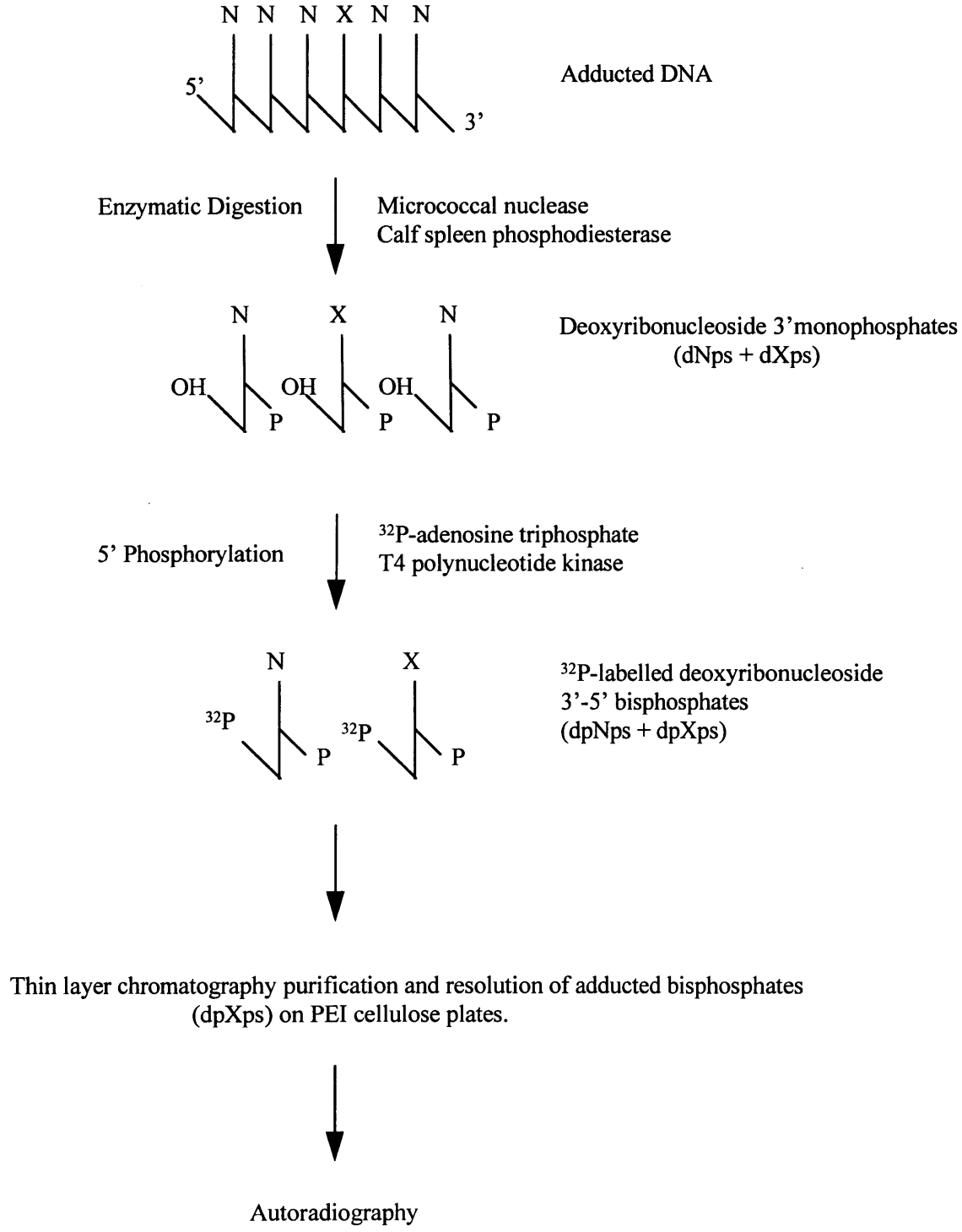


Figure 1.9. Standard procedure for the ^{32}P-postlabelling of DNA adducts (Adapted from Jones and Parry 1992).

The labelled digest containing [5'- ^{32}P] deoxyribonucleoside-3'-5'- bisphosphates (dpNps) is then purified and resolved using anion exchange thin layer chromatography (TLC) or HPLC. The adducts are detected by autoradiography and quantitated by liquid scintillation (~ 40% efficiency) or Cerenkov counting (99% efficiency) (Watson 1987). DNA damage may be seen as diagonal radioactive zones using TLC on autoradiographs indicating a wide array of DNA damage, usually the result of exposure to complex mixtures such as tobacco smoke (Randerath *et al.* 1988).

Several modifications have been incorporated into the original method in order to increase the sensitivity of the assay by eliminating the normal nucleotides and specifically labelling the adducts (Gupta and Earley 1988). Modifications include use of nuclease P1 (Reddy and Randerath 1986) to enrich the adducts, butanol extraction (Gupta 1985) or HPLC (Dunn and San 1988) concentration of the adducts. Gupta and Early 1988 however showed that certain adducts are detected more efficiently with one method than another, and therefore a number of enhancement procedures must be examined in order to find the most appropriate.

The PPL technique is probably the most sensitive technique for DNA adduct analysis to date with the ability to detect one adduct per 10^9-10^{10} normal nucleotides and requiring only a few micrograms of DNA for analysis. The major disadvantage of PPL however is its inability to identify the adducts. This problem can be overcome by co-chromatography with standards, but these are not normally easily accessible. Problems also exist with labelling efficiency as quantification of adducts is based on the assumption that complete labelling of the adducts has occurred. Studies have shown that differences exist in the efficiency of adduct phosphorylation (Hemminki *et al.* 1991). The PPL procedure has been used in human studies to identify for example DNA adducts produced as a result of tobacco smoking in a variety of tissues including heart, lung, placenta, and monocytes (Phillips *et al.* 1988; Everson *et al.* 1986; Randerath *et al.* 1989; Holtz *et al.* 1990).

PPL procedures have also been used for the detection of modified bases produced as a result of oxidative damage. Techniques have been developed for the detection of Tg in γ-irradiated DNA (Weinfeld and Soderlind 1991, Hegi *et al.* 1989) along with techniques for the detection of 8-OHG (Lutgerink *et al.* 1992; Devanboyina and Gupta

1996 ; Podmore *et al.* 1997). Due to structural similarities between the normal DNA base and its modified version assays have taken care to enrich the Tg and 8-OHG from the normal deoxynucleotides and to ensure complete resolution of the DNA lesion from the normal nucleotides. The PPL methods developed have been used for the quantitation of background levels of DNA lesions in calf thymus DNA (Hegi *et al.* 1989) and also basal levels in rat tissues with the highest levels of 8-OHdG being measured in the liver and heart. The assays have reported sensitivities of 1 8-OHdG/10^6 nucleotides (Lutgerink *et al.* 1992; Devanaboyina and Gupta 1996).

1.4.3. Immunochemical

Immunochemical techniques using monoclonal and polyclonal antibodies have been used in the analyses of oxidative DNA damage (for reviews see Shigenaga *et al.* 1994 and Leadon 1988). Antisera specific for DNA lesions can be used in radio immunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), ultrasensitive enzymatic radioimmunoassays (USEIRA) and immunoslotblot techniques (Poirier and Weston 1991). Monoclonal and polyclonal antibodies have been used in immunoaffinity columns to detect the 8-OHG content of urine (Degan *et al.* 1991, Park *et al.* 1992). Despite showing a strong affinity for the modified base, the monoclonal antibody cross-reacts with normal guanine, thus making it unsuitable for assaying 8-OHG levels in DNA. A highly specific monoclonal antibody for Tg has also been developed, which can be used in the presence of normal thymine, with the ability to detect 1 Tg in 200,000 DNA thymines (Leadon and Hanawalt 1983). The Tg monoclonal antibody has been used to measure Tg in oxidised and irradiated DNA from mammalian cells (Kaneko and Leadon 1986, Leadon *et al.* 1988). Polyclonal sera have also been used to detect 8-OHG and 8-OHA and Tg in X-irradiated DNA (Rajagopalan *et al.* 1984; Ide *et al.* 1996).

Immunoassays used for the detection of oxidative damage are inexpensive and sensitive methods which allow for the rapid screening of large numbers of samples with the ability to detect 1 adduct per 10^8 modified nucleotides (Farmer 1994). Limitations of the immunoassays include cross-reactivity, therefore requiring highly specific antibodies as oxidised bases do not differ largely from their unmodified derivatives. In addition only limited antibodies for oxidative damage exist due to the prior lack of

knowledge about the exact nature and structure of DNA lesions required. Further disadvantages of the technique till now have been the requirements for relatively large amounts of sample (e.g. up to 500 μg), however with the arrival of immunoslotblot techniques only a few μg of DNA (as low as 5μg) may be required.

1.4.4. Fluorescence postlabelling.

Fluorescence postlabelling combines the basic idea of postlabelling enzymatically digested DNA by reaction with a fluorophore and fluorescence detection. DNA is enzymatically digested to the nucleoside 5' monophosphate and then fluorescence postlabelled, using compounds such as dansyl chloride, to produce fluorescent nucleotides, which are then separated by HPLC using a fluorescence detector. Fluorescence postlabelling has been used to assay oxidative DNA damage products such as thymidine glycol monophosphate (TdGmp) and 8-hydroxydeoxyguanosine 5'-monophosphate (8-OHdGmp) in X-irradiated DNA (Sharma *et al.* 1990a, b). The assay has been applied to measure background levels in calf thymus DNA of approx 1 pmole of 8-OHdGmp per μg of DNA (Sharma *et al.* 1990b).

The fluorescence postlabelling assay has the ability to detect 1 modified nucleotide/ 10^6 normal nucleotides from 100 μg of DNA for TdGmp and 8-OHdGmp. Further advantages of the fluorescence postlabelling assay include the elimination of the hazards associated with handling and disposing of radioactive material. Limitations of the assay include the lack of structural information available.

1.4.5. Gas Chromatography Mass Spectrometry (GC/MS)

Mass spectrometry (MS) is the most powerful technique used for structural elucidation and quantitation of modified DNA lesions. Various MS ionisation techniques have become increasingly popular for the study of DNA lesions, including fast atom bombardment (FAB), thermospray and electrospray allowing easy detection, without the need for derivatisation. The afore-mentioned techniques are increasingly being used for nucleotide studies; however, for nucleic acid base studies, GC/MS in combination with electron ionisation (EI) or chemical ionisation (CI) are still the techniques of choice.

GC/MS is preferable when higher sensitivity and quantitation of samples is required. This technique has been widely applied for analysis of DNA and chromatin during *in vitro* and *in vivo* studies of oxidative damage, aiding structure elucidation of DNA lesions, (Djuric *et al.* 1991a; Dizdaroglu 1993b; Dizdaroglu *et al.* 1991; Hamberg and Zhang 1995). Detection of DNA adducts is achieved following derivatisation of the modified bases, either released by mild acidic hydrolysis of DNA, extracted from cells or tissues (Dizdaroglu, 1994), or excreted in urine (Bianchini *et al.* 1996; Faure *et al.* 1993). GC/MS offers sensitivity, selectivity, speed and versatility to detect and quantify a large number of lesions in chromatin and DNA in a single analysis.

Due to the sensitivity of the GC/MS technique quantitation of endogenous levels has also been possible and over 11 DNA adducts have been detected in cancerous and cancer free surrounding tissue (Olinski *et al.* 1992; Jaruga *et al.* 1994). Initially quantitation became possible with the use of structurally similar internal standards (Fuciarelli *et al.* 1989; Nackerdien *et al.* 1992). However more recently isotopically labelled analogues have been used for the quantitation of the DNA adducts (Crain and McCloskey 1983; Djuric *et al.* 1991a; Dizdaroglu 1993b). Sensitivities in the range of 1-3 oxidatively modified residues in 10^6 DNA bases can be achieved (Dizdaroglu 1994). However greater sensitivity has been achieved with alkylated DNA adducts using GC/MS.

1.4.5.1. GC/MS Instrumentation.

Gas chromatography (GC) is an ideal separator whereas MS is ideal for identification. The two instruments are combined via an interface with the aim of operating both instruments without degrading the performance of either. An interface is a device used to transfer GC effluent to the MS ion source. It must reduce pressure ($< 10^5$ torr) by removing carrier gas while transferring maximum organic molecules from the GC peak.

The principles behind the operation of the two instruments will be discussed briefly in order to understand the technique of GC/MS.

1.4.5.2. Gas Chromatography (GC)

GC is the technique of separating thermally stable and volatile compounds between a mobile and stationary phase. In capillary GC the mobile phase is an inert gas (such as helium or nitrogen) and the stationary phase is a high molecular weight liquid which is deposited on the walls of a long capillary tubing.

A GC consists of several basic components designed essentially to perform the following functions (see figure 1.10.):

(i) provide a constant flow of carrier gas

(ii) permit the introduction of sample vapours into the flowing gas stream

(iii) contain the appropriate length of stationary phase

(iv) maintain the column at the appropriate temperature (or temperature programme)

(v) detect the sample components as they elute from the column

(vi) produce a signal proportional in magnitude to the amount of each component.

Since the choice of injection modes used in this thesis was split/splitless only these injection modes will be discussed. Samples are injected via a hypodermic syringe through a silicone rubber septum onto a glass liner contained within a metal block. The block is maintained at a sufficiently high temperature in order to instantaneously convert the liquid sample to a "plug" of vapour in order for it to enter the column with the carrier gas. The carrier gas is also conducted into the injector with a flow controlled by pressure regulators. The gas flow affects the speed at which the sample components travel through the column and thus the separation. Injections can then be made in split or splitless mode in order to control the amount of material entering the column. In split mode for example 1 μl of sample may be injected but only say 0.01 μl enters the column with the remainder being vented. This technique prevents column overload and produces symmetrical peak shapes, however a significant amount of the sample is wasted. Splitless injection can be used when dealing with trace levels, therefore enabling the whole sample to enter the column in order to obtain adequate sensitivity.

A large solvent peak is avoided by venting the injector port at the time when essentially all of the sample and most of the solvent have entered the column (30 secs). The sample is carried along by the carrier gas through fused silica capillary columns which provide high inertness, good separation efficiency and high sensitivity measurements. The capillary columns are coiled and mounted inside an oven which allows controlled changes in temperature in order to achieve optimum separation. Different compounds interact differently with the two phases, depending respectively on their solubility in each phase. At the end of the process, separated components emerge in order of increasing interaction with the stationary phase. The least altered component elutes first and the most strongly retained elutes last depending on physical/chemical properties of the components in the sample. A detector (in this case a MS) is situated at the end of the column, and senses the presence of the individual components as they leave the column

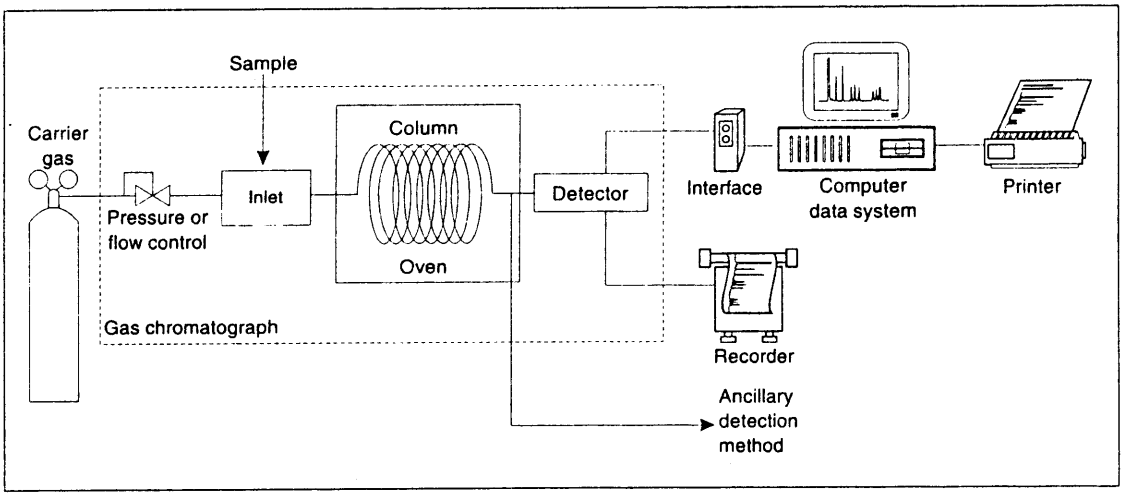


Figure 1.10. Simplified schematic of a gas chromatograph (Hinshaw and Ettre 1994)

1.4.5.3. Mass Spectrometry (MS)

MS is a technique used to separate charged particles according to their mass/charge (*m/z*) ratio. MS can be used to provide qualitative and quantitative information about the atomic and molecular composition of inorganic and organic materials. The main advantages of MS over other analytical techniques is its sensitivity and specificity in identifying unknown compounds or confirming the presence of compounds. The sensitivity of this technique can be attributed to the action of the analyser as a mass/charge filter in reducing background interference and to the sensitive electron multipliers used for detection. The specificity of this technique lies in its ability to produce unique characteristic fragmentation patterns for compounds providing information about molecular weight and structure.

A MS performs three basic tasks (i) produces volatile gaseous ion fragments from the sample (ii) separates these ions according to mass/charge ratio (iii) measures the relative abundance of ion fragments of each mass (See figure 1.11. for the components of a mass spectrometer).

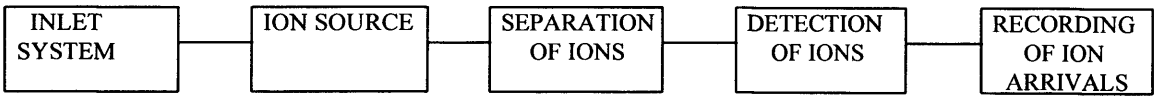


Figure 1.11. Essential features of a mass spectrometer in schematic form (Adapted from Johnstone and Rose 1996).

Samples (solids, liquids or gases) are introduced from the inlet system into the MS which is under high vacuum (< 10^{-5} torr). Ionisation occurs in the ion source and depending on the conditions the charged molecule may fragment producing ions of lower mass than the original precursor molecule.

1.4.5.3.1. Ionisation modes.

There are many types of ionisation modes available and the type of information required and the compound to be analysed determines the choice of mode. Chemical ionisation (CI) tends to give molecular weight information whereas with electron ionisation (EI) fragmentation occurs more frequently, and in some cases structural information can be used for unequivocal identification of compounds. Only EI will be described here, as it was the mode of ionisation used in this study.

Electron ionisation (EI)

In EI mode a beam of high energy electrons is created by heating a filament and attracting the electrons away from the filament to a plate called the trap held at a relative positive potential. The neutral sample molecules are passed into this high energy beam and the resulting interaction results in loss of an electron from the molecule producing a positive molecular ion.

$$M \longrightarrow M^{+\bullet}$$

Depending on the molecule and the ionisation energy, the molecular ion may fragment. For EI, sample molecules must be in the gas phase before ionisation can occur, and therefore this technique may only be applied to the analysis of small (ca < 800 Dalton), thermally stable and volatile molecules.

1.4.5.3.2. Ion separation

The ions produced are repelled out of the ion source into the mass analysing region, which is responsible for separation of the ions, according to their *m/z* ratios. GC/MS: has been most extensively used with 'quadrupole' MS and magnetic 'sector' MS.

Quadrupole mass analysers.

In the quadrupole mass spectrometer ion separation is achieved by passing the ion beam through the centre of four parallel rods to which voltage is applied. The separation of the masses in a quadrupole mass analyser is accomplished by simultaneously applying both a d.c. and a radio frequency (RF) voltage to the rods. The electric field produced is used to filter ions according to their *m/z* ratios.

Magnetic 'sector' mass analysers

A magnetic sector MS can be divided into a single and double focusing instrument. The original MS used single focusing mass analysers in which a magnetic field was used to separate ions according to their *m/z* ratio. As the energy spread of the ions was not corrected this lead to peak broadening and instruments with limited resolution. The double focusing mass analysers mostly used today combine electrostatic and magnetic fields. The electric sector focuses ions of like energies and disperse ions of different energies, while the magnetic sector separates the masses. A mass spectrum is achieved by scanning the magnetic field strength to bring ions with different *m/z* ratio sequentially to focus at the detector.

1.4.5.3.3. Detection

By scanning the field strength all ions are sequentially focused at a single point known as the detector producing a mass spectrum. A scan is defined as one sweep of the field strength e.g. a magnet scanned from high field to low field will focus at the detector ions from high mass to low mass sequentially. The detector therefore detects ions of various intensity at certain times during the scan depending on the range and speed of the scan. The time and intensity data is then sent to a calibrated data system, and converted into masses and intensity normalised to the most abundant ion.

1.4.5.4. GC/MS with Selected Ion Recording (SIR).

GC/MS SIR has the ability to quantify trace levels of compounds in the presence of hundreds of other substances, some of which may be present in concentrations thousands of times higher than that of the analyte. GC/MS SIR has been the technique of choice for the quantitation of a wide array of DNA lesions, due to its combination of increased sensitivity (possible to detect fg levels of certain compounds) and selectivity

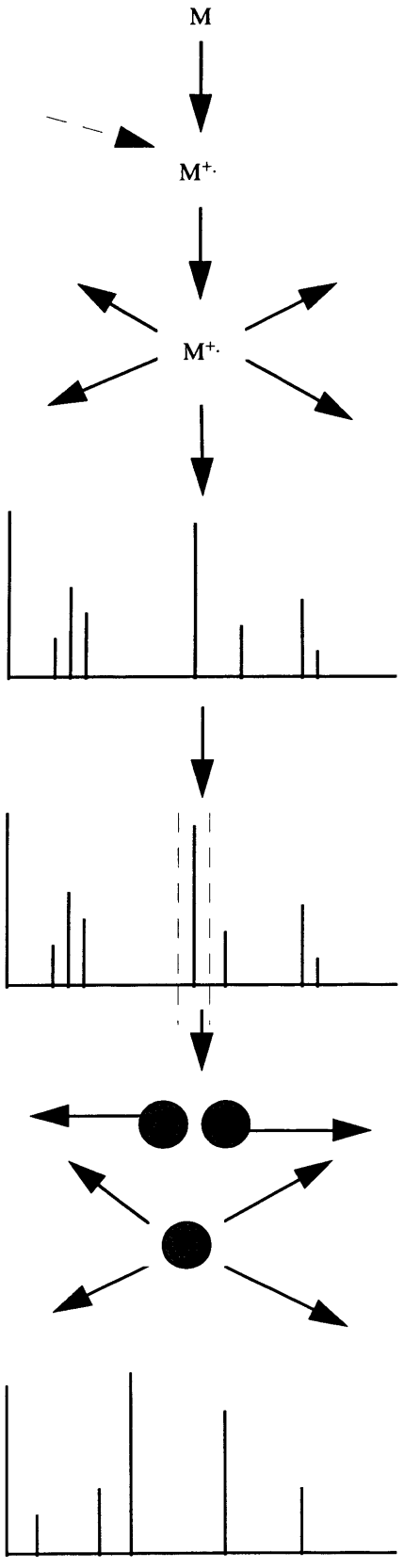
over scan mode. When using SIR mode for identification, knowledge of the mass spectrum and retention time of the analyte is required. In SIR mode only a few preselected ions are detected, thereby increasing the sensitivity of SIR over scan mode 50-500 times depending on the number of ions being monitored and instrumental conditions. Increased selectivity in SIR is obtained as only ions of interest are being monitored. Ions selected for monitoring must be intense representative ions of the molecule to be studied.

Accurate quantification of adducts can be achieved using suitable internal standards (1.4.5.6.), and setting the MS to switch rapidly between the analyte ion and internal standard ion.

1.4.5.5. Tandem mass spectrometry (MS/MS)

MS/MS couples two stages of mass analysis separated by a collision cell to obtain greater specificity and structural information. The first mass analyser is used to select an ion of interest, which is then passed into the collision cell containing an inert gas such as argon. By collision of the ion with the atoms in the cell fragmentation of the ion can be induced. This is known as collision induced decomposition (CID). The fragment ions produced are then analysed in the second mass analyser generating a mass spectrum of the original ion (Figure 1.12.).

In the work described in this thesis MS/MS specifically multiple reaction monitoring (MRM) was used. In MRM the first mass analyser selects ions of a single mass and the second mass analyser also selects ions of a single but different mass to monitor specific fragmentations. Internal standards may also be monitored in MRM by alternating between masses specific to that compound. This technique is analogous to SIR and can be used for increasing the selectivity in detection of compounds in complex or impure mixtures.



Sample molecules

Ionisation and absorption of energy

Fragmentation

Mass analysis

Mass spectrum

Precursor ion

Collision

Dissociation

Product ions

Figure 1.12. Molecule undergoing MS/MS.

1.4.5.6. Quantification using internal standards.

Quantification of DNA lesions using the technique of GC/MS SIR is achieved by the use of an internal standard. An internal standard is a substance used as a reference in quantitative analysis. Isotopically labelled analogues are believed to be the most effective internal standards as they have similar physical and chemical properties as those of the analyte, and therefore mimic its behaviour during the analysis. An isotopically labelled analogue can also be used to confirm identification by correspondence of retention times and to compensate for any losses during sample preparation. Mass spectral fragmentation patterns of the isotopically labelled analogues are usually similar to those of the corresponding unlabelled compounds with ion masses being shifted depending on the isotopic content of the labelled analogue.

The internal standard is added in the early stages of sample preparation, such as in this study prior to DNA hydrolysis, to compensate for possible sample losses during sample preparation. Partial destruction of certain modified bases has been reported during DNA hydrolysis (Nackerdien *et al.* 1992, Djuric *et al.* 1991a) and therefore the use of isotopically labelled analogues of these modified bases ensures accurate quantification as the internal standard would be degraded to the same extent as the analyte. Quantification is achieved by obtaining calibration plots for the response of the mass spectrometer to known quantities of analyte and its labelled analogue internal standard. The ratio of ion currents at selected masses are then plotted as a function of the ratio of analyte to internal standard. A linear relationship should be obtained.

1.4.5.7. Derivatising agents.

Derivatisation is usually a prerequisite for efficient and sensitive GC/MS of compounds possessing functional groups. DNA bases, nucleosides and nucleotides are highly polar and not sufficiently volatile for GC and are therefore converted to volatile derivatives. The choice of derivatising agent to be used is dependent on the chemical nature of the compound and the analytical method to be used. GC/MS analyses of nucleotides, nucleosides and bases have used mainly trimethylsilyl (TMS) and tertiarybutyldimethylsilyl (TBDMS) derivatives.

The usefulness of silylation is due to the ease of reaction with many of the functional groups present in organic compounds. Silyl derivatives are formed by displacement of

the active proton in OH, NH and SH groups. The general reaction for formation of trialkylsilyl derivatives is:

$$R_3Si\text{-}X \quad + \quad R'\text{-}H \quad \longrightarrow \quad R_3Si\text{-}R' \quad + \quad HX$$

All silylation reactions, however are moisture sensitive and therefore are carried out in sealed containers e.g. reactivials. Large excesses of derivatising agent are also usually used in order to minimise problems of interference by moisture or other impurities.

1.4.5.7.1. TMS and TBDMS derivatives

The widespread application of TMS derivatives in GC/MS can be attributed the relative ease of preparation, the good chromatographic properties and the well understood mass spectral fragmentation pattern. TMS mass spectra exhibit diagnostic ions useful in structure elucidation. The molecular ion of TMS derivatives is often either small or absent but molecular weight can be determined using the $[M\text{-}15]^+$ ion produced by loss of a methyl group from the molecular ion.

The TMS derivatives however are less chemically stable than derivatives containing more sterically crowded alkyl substitutes and are therefore more susceptible to hydrolysis.

TBDMS derivatives also have certain advantages including ease of formation, hydrolytic stability, and ionisation properties suitable for SIR and are considered one of the most suitable derivatives for quantitative studies (Halket 1993). Mass spectra of TBDMS derivatives are dominated by $[M\text{-}57]^+$ corresponding to loss of a t-butyl radical which increases precision and reliability of detection in trace analysis. Disadvantages include difficulty in derivatising sterically hindered groups leading to partial derivatives, and increases in molecular weight which may lead to a 2-3 times increase in retention time (r.t.).

A comparison of 5-OHMeU-TBDMS and TMS derivatives by Faure *et al.* (1993) found that the TBDMS derivative provided at least four fold higher sensitivity and precision than the TMS derivatives.

1.4.6. Background levels of oxidative damage.

Despite the abundance of techniques to detect oxidative damage there are still only limited data on endogenous levels of oxidative damage. DNA is constantly being oxidised and repaired resulting in a steady state level of DNA damage. The levels of endogenous background levels is dependent upon the rate of production of ROS (produced during oxidative stress) and efficiency of repair.

A potential complication in the analysis of endogenous levels of oxidised DNA bases is the fact that oxidation of DNA can sometimes occur as a result of some of the procedures used for DNA digestion and analysis. Although the yields of oxidised bases produced during sample work-up may be low, trace amounts are significant because the level of sensitivity provided by chemical techniques is approximately 1 adduct per 10^8 or greater. Thus great care must be taken to avoid artefactual generation of oxidised bases during sample preparation.

Background levels have been quoted for the many different techniques available to detect such damage and are not always consistent (Halliwell and Dizdaroglu 1992). For example determination of 8-OHG by GC/MS shows larger values than those by HPLC with ECD. A comparative study by Ravanat *et al.* (1995) showed that background levels by GC/MS were 50-fold higher than those obtained by HPLC-ECD (see section 5.1.).

Levels of endogenous damage have been calculated by measuring biomarkers of oxidative damage in DNA (extracted from cells or tissue) and urine.

Ames (1989b) have calculated endogenous damage by measuring Tg, Tdg and 5-OHMeU in urine with results indicating that humans excrete 100 nmol/day of the three products representing an average of 10^3 oxidised thymines per cell per day for each of the body's 6×10^{13} cells (Adelman *et al.* 1988; Cathcart *et al.* 1984; Saul *et al.* 1987). The above products are 3 of ~ 20 products of oxidative damage and, therefore

the total number of all types of oxidative hits to DNA per cell per day may be about 10^4 in humans (Ames and Shigenaga 1993).

The levels of background modified bases reported in DNA are presented in Table 1.3. The values presented are just some of a wide range of values reported by the different methods.

Note.

GC/MS analysis of modified bases in DNA may sometimes be expressed in nanomoles of modified base per mg of DNA. These data may be converted into the actual number of base by dividing the figure of nmol bases/mg DNA by 3.14 giving the number of modified bases per 10^3 bases in DNA i.e. 1 nmol/mg DNA corresponds to about 318 modified bases per 10^6 DNA bases (Halliwell and Dizdaroglu 1992).

Table 1.3. Levels of oxidative DNA damage detected in DNA.

Method of detection	DNA lesions	Source	Modification level	References
GC/MS	8-OHG	calf thymus DNA	159-318/10^6 bases	Aruoma *et al.* 1989 a,b, 1991
	8-OHG	human colon DNA	2.71±0.13/10^5 bases	Olinski *et al.* 1992
	Tg	human lung tissue	3-19/10^6 bases	Jaruga *et al.* 1994
	Tg	salmon sperm DNA	0.5±0.1ng/mg DNA	Markey *et al.* 1993
	Tdg	calf thymus DNA	2.73±0.07 pg/μg DNA	Naritsin and Markey 1996
	5-OHMeU	human peripheral nucleated blood cells	mean 9.29±1.89/10^4 T	Djuric *et al.* 1991b
	5-OHMeU	human colon DNA	0.39±0.01/10^5 bases	Olinski *et al.* 1992
HPLC	8-OHdG	DNA from cells	1-5 /10^6 bases	Floyd *et al.* 1990
	8-OHdG	rat nuclear DNA	8-/10^6 bases	Ames 1989b
	8-OHdG	rat mitochondrial DNA	125/10^6 bases	Ames 1989b
	8-OHdG	calf thymus DNA	8-320/10^6	Floyd *et al.* 1989
PPL	8-OHdG	calf thymus DNA	up to 570/10^5 G	Lutgerink *et al.* 1992
	8-OHdG	various rat tissue DNA	87±29-133±49/10^6 nucleotides	Devanoboyina and Gupta 1996
	Tg	calf thymus DNA	10/10^6 T	Hegi *et al.* 1989

1.5. AIMS

The aims of the work described in this thesis were to develop sufficiently sensitive and selective techniques for the quantitation of endogenous oxidative DNA damage in humans. The biomarkers of oxidative damage under study were Tg, 5-OHMeU and 8-OHG. The project began with synthesis of standards and their isotopically labelled analogues. Methods for the quantitation of these markers were developed with investigations into the hydrolysis and derivatisation conditions used. Assays developed were validated on calf thymus DNA and *in vitro* studies using ionising radiation. The established assays were subsequently used to quantitate Tg, 5-OHMeU and 8-OHG in rats exposed to crocidolite (blue asbestos) and in human placental DNA, examining the role of antioxidant supplementation on markers of oxidative damage.

Chapter 2.

Materials and Methods

2.1. MATERIALS

Chapters 3 and 4

Formic acid, N-methyl-N-(t-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) with 1% *t*-butyldimethylchlorosilane (TBDMCS), N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), pyridine and trimethylchlorosilane (TCMS) were purchased from Pierce & Warriner (Chester, UK). 3-[$^2H_3$]-methyl malic acid was a gift (MRC Toxicology Unit, Carshalton, Surrey, UK, I. Bird.)

All solvents were obtained from Fisons (Loughborough, UK); HPLC grade solvents were used for the quantitative assay and analytical grade was employed in the synthesis of the analyte standards. All chemicals were of the highest grade available and obtained from Sigma Chemical Co. Ltd. (Poole, UK) or Aldrich Chemical Co. (Gillingham, UK.).

Male Fischer 344 rats were purchased from Harlan Olac, Bicester, Oxon, 130-150 g and were housed in a flexible film isolator with a 12-h light/dark cycle and food and water ad libitum. UICC crocidolite was used for the rat lung experiments.

SSC Buffer : The buffer was prepared by mixing 150 mM sodium chloride, 15 mM trisodium citrate and 0.1 mM EDTA. The resulting solution had a pH between 7.5-8.0 and was diluted 1:100 with water for dissolving the DNA samples.

Phenol extraction buffer: The buffer was prepared by mixing together 10 mM tris-equilibrated phenol (47.2%), chloroform (47.2%), isoamylalcohol (0.94%) and *m*-cresol (4.7%). Following the addition of 8-hydroxyquinoline (0.1% by weight), 50 mM tris-HCl pH 8.0 was added to the top of the solution to protect it from degradation. The solution was stored at 4°C and protected from light for up to 1 month.

Chapter 5

Guanase (EC 3.5.4.3.) from rabbit liver was obtained from ICN Biomedicals Ltd (Thame, UK). Calf thymus DNA was from Calbiochem Novabiochem UK Ltd (Nottingham, UK). 2-Amino-6,8-dihydroxypurine (8-OHG) was from Aldrich (Gillingham, UK). HPLC grade methanol was obtained from Fisons Ltd (Loughborough, UK). All other chemicals were of the highest grade available and obtained from Sigma Chemical Co. Ltd. (Poole, UK).

2.2. METHODS AND INSTRUMENTATION

2.2.1. Gas Chromatography / Mass Spectrometry (GC/MS)

GC/MS instrumentation comprised a CE8060 gas chromatograph (Carlo Erba, Fisons Instruments, Loughborough, UK) interfaced to a VG Autospec Ultima-Q mass spectrometer (VG Organic, Manchester, UK). Analysis was carried out using a DB-5, 15m x 0.25mm i.d. (0.25 μm film thickness) column (J + W Scientific, Fisons, Loughborough, UK) and a PTE-5, 30 m x 0.25mm, 0.25 μm film thickness column (Supelco, Poole, UK). For both columns, the initial oven temperature was 80°C (1 min) followed by a temperature ramp of 15°C/min to 280°C. Helium carrier column flow rate was 1ml/min. Split injection (1 μl) was employed with split ratios between 10:1 and 25:1 and the injector temperature was 280°C.

All data was obtained using electron ionisation (EI) mode. Electron energy and trap current were 70 eV and 200 μA respectively. The source temperature was 250°C and the transfer line was held at 290°C.

2.2.1.1. Mass spectrometric scan functions

Ions were monitored over a mass range of 700-50 *m/z* units at a resolution of 1000 and scan speed of 1 s/dec. A solvent delay of 5 mins was used.

2.2.1.2. Selective Ion Recording (SIR)

Thymine Glycol

The monitored ions were *m/z* 331.2 for [$^2H_0$] Tg and *m/z* 334.2 for [$^2H_3$] Tg. Dwell and delay times were 40 ms and 10 ms respectively to give a total scan time of 0.1 s. Ions were selected at a resolution of 1000.

5-Hydroxymethyluracil

The monitored ions were *m/z* 427.2 for the unlabelled 5-hydroxymethyluracil and *m/z* 432.2 for the $C_4{}^{13}C_1H_4{}^2H_2{}^{15}N_2O_3$ '[M+5]' 5-hydroxymethyluracil. Dwell and delay times were 40 ms and 10 ms respectively to give a total scan time of 0.1 s. Ions were selected at a resolution of 1000.

Thymine Glycol and 5-Hydroxymethyluracil (combined function)

The monitored ions for Tg were *m/z* 331.2 and *m/z* 334.2 and 427.2 and 432.2 for 5-OHMeU. Dwell and delay times were 40 ms and 10 ms respectively to give a total scan time of 0.1 s. Ions were selected at 1000 resolution. Tg was monitored from 5 to 14:14 mins and 5-OHMeU between 14:15 and 15:00 mins.

2.2.1.3 Multiple Ion Recording

Thymine Glycol

The MRM transitions used were, for [$^2H_0$] Tg, *m/z* 331.2 → *m/z* 288.1 and for [$^2H_3$] Tg 334.2 → 291.1. Dwell and delay times were 300 ms and 20 ms to give a total scan time of 0.64 s. Precursor ions were selected at 1000 resolution and quadrupole resolution was 1.5. Collision energy was optimised prior to calibration and was in the range 5-10 eV. Collision gas was not used.

2.2.2. High Performance Liquid Chromatography (HPLC)

Instrumentation for HPLC consisted of a Beckman 126 solvent module (Beckman Instruments UK Ltd, High Wycombe, UK), a model 507 autosampler, a model 168 diode array detector, and an EG & G Instruments model 400 electrochemical detector. The HPLC was controlled and data analysed using Beckman System Gold Software.

HPLC conditions.

DNA bases were separated by reversed-phase HPLC using a 3 μm ODS Hypersil (150 x 4.6 mm i.d.) stainless steel column (Shandon, Runcorn UK.). Elution was performed isocratically at 1 ml/min with a mobile phase of 40 mM KH_2PO_4, 1 mM EDTA, pH 5.0 containing 1% (v/v) methanol. Samples (50 μl) were injected by autosampler. Detection of native DNA bases was by UV absorbance at 254 nm and of 8-OHG by

electrochemical detection using a glassy carbon working electrode at a potential of +600 mV vs. an Ag/AgCl reference electrode. Both EG & G model 400 and BAS LC-4B (Biotech, Luton, UK) amperometric detectors were also used successfully to quantitate 8-OHG. Peaks were quantified by reference to peak areas obtained from authentic standards. Diode array detection was used to assign probable peak identities following guanase digestions; this was performed on selected peaks over the range 200-350 nm.

2.3. EXPERIMENTS DESCRIBED IN CHAPTER 3

2.3.1. Synthesis of cis-Tg and [$^2H_3$]-cis Tg

cis-Tg was synthesised by the oxidation of thymine with $KMnO_4$ essentially according to the method of Iida and Hayatsu (1970). Thymine (1.0g, 7.9 mmol) was dissolved, in warm water (300 ml) at 50-60°C. After all the thymine had dissolved the solution was cooled in ice/H_2O to 5-10°C, and the pH was adjusted to 8.6 using dropwise addition of NH_4OH (2.5 M, 1ml). $KMnO_4$ (0.07 M, 150ml) was then added to the aqueous thymine solution resulting in the pH rising to 9.3. After 5 minutes saturated H_2SO_3 was added dropwise until MnO_2 was precipitated and the purple colour just disappeared (pH ~ 2.3); the solution was immediately re basified using NH_4OH (2.5 M, ~ 12ml) to pH 8.6.

The solution was then filtered and evaporation was subsequently carried out on a rotary evaporator (~ 40-50°C) down to a few ml. Finally acetone (500ml) was added to the mixture which produced a white precipitate on shaking. The precipitate was removed by filtration and the filtrate concentrated to ~ 5-10 ml using a rotary evaporator (40-50°C). The filtrate was cooled and the product crystallised (yield 63%, m.p. 215°C) (Iida and Hayatsu 1970: 214-216 °C decomposing sharply). Identification and purity was confirmed by NMR (ARX Bruker 250 MHz) determined in [$^2H_2$]O, the spectra were consistent with those reported by Frenkel *et al.* (1981a). Fast atom bombardment (FAB) MS (VG 70-SEQ) in positive ion mode using a glycerol matrix produced the $[M+H]^+$ pseudomolecular ion at *m/z* 161. GC/MS (VG TRIO-1) of the bis-butyldimethylsilyl derivative was also carried out (section 3.2.2.).

The internal standard, [$^2H_3$] cis-Tg was prepared from [$^2H_3$]-thymine (methyl labelled) by a similar procedure to that used for the synthesis of the unlabelled compound.

[$^2H_3$]-Thymine was synthesised from the reaction of [$^2H_3$]-methyl malic acid with urea in oleum solution (acknowledgements to I. Bird). The FAB mass spectrum was consistent with that obtained for the unlabelled compound, with a pseudomolecular ion at *m/z* 164. GC/MS of the bis-t-butyldimethylsilyl derivative was also carried out to confirm the identity of the product (also discussed in section 3.2.2.).

2.3.2. DNA Extraction

The tissue samples were crushed in liquid nitrogen using a mortar and pestle to a fine powder which was transferred to 15 ml polypropylene tubes (Sarstedt) and suspended in 50 mM tris-HCl, 100 mM EDTA, pH 8.0 (5 ml). RNAse (Ribonuclease A, Sigma) (10 µl) was then added to each sample at a concentration of 100 µg per ml of Tris buffer which was subsequently incubated for 30 min at room temperature on a rotating table. Sodium dodecyl sulphate (SDS) (250 µl) was then added at a volume of 0.5% of the total volume in the tube and incubated for a further 30 min at room temperature on a rotating table. The SDS aided in the solubilisation of the tissues and denatured proteins thus inactivating enzymes such as nucleases. Proteinase K (250 µl) was then added at a concentration of 500 µg per ml of the volume in the tube and then samples were incubated with shaking overnight at 37°C.

The samples were extracted with an equal volume of phenol extraction buffer which was mixed on a rotating table for approximately 30 min. The two phases were separated by centrifugation at 3000 rpm for 10 min at 4°C (GPR Beckman centrifuge, Beckmann Instruments Inc., Palo Alto, CA, USA.). The top layers were removed and re-extracted with phenol extraction buffer and further rotated for 10-15 mins and centrifuged as before. To the top layer (aqueous) an equal volume of chloroform:isoamyl alcohol (24:1,v/v) was added and samples were further rotated for 30 min followed by centrifugation at 3000 rpm for 10 min at 4°C. The top layers were carefully removed again making sure not to unsettle the denatured proteins that were present at the interface between the aqueous and organic phases. The DNA was precipitated out using 0.3 M sodium acetate pH 6.5 and 0.8 vol isopropanol (3200 µl). Each tube was gently inverted a few times and the DNA was spooled out and washed in absolute alcohol (ice cold), followed by 70% ethanol in water before resuspending in 1/100 SSC buffer. The purity of the DNA was checked by determining the ratio of the absorbances at 260 and

280 nm and the concentration determined by assuming that $A_{260} = 20$ is equivalent to 1 mg/ml for double stranded DNA (Kontron Uvikon 860 spectrophotometer) (De Groot *et al.* 1994).

2.3.3. Cleaning of glassware

All glassware used for hydrolysis and derivatisation was washed with chromic acid and silylated using a 5% TCMS solution in toluene prior to use.

2.3.4. DNA Hydrolysis

Aliquots of aqueous DNA were dried using vacuum centrifugation (Speedvac, Savant instruments Inc., Farmingdale, US) and then subjected to acid hydrolysis with 500 µl formic acid (60%) under vacuum at 140°C for 40 minutes. The samples were then dried in a vacuum centrifuge prior to derivatisation.

2.3.5. Derivatisation

Derivatisation of samples for GC/MS analyses was carried out using MTBSTFA/1 % TBDMCS and pyridine (1:1, total volume 60 µl) at 60°C for 30 minutes. The derivatised samples were then dried in a vacuum centrifuge and reconstituted in ethyl acetate (20 µl).

2.3.6. HPLC purification of oligonucleotide

Purification of the oligonucleotide was carried out by HPLC (LKB system) using a Techsphore ODS (4.6 x 250 mm) column (HPLC Technology, Macclesfield). Elution was achieved using a linear gradient of 50 mM ammonium formate and methanol (20-40% methanol in 20 mins) at a flow rate of 1 ml/min, with eluate detection being monitored at 254 nm. Samples (100 µl) were injected following a 5 fold dilution of the crude solution from the synthesiser (acknowledgements to Dr G. Sweetman).

2.4. EXPERIMENTS DESCRIBED IN CHAPTER 4

2.4.1. Synthesis of 5-OHMeU

The synthesis was carried out following the procedure of Cline *et al.* (1959). 5-OHMeU was synthesised by suspending uracil (1g, 8.9 mmoles) in potassium hydroxide (0.42M, 0.33g), after which paraformaldehyde (0.33g) was added and the mixture was kept at 50°C for 73 hours.

Following dilution with water (40 ml) the solution was stirred with Sephadex CM (3.33 g) and the mixture filtered to remove resin and a little insoluble product. The filtrate was concentrated on a rotary evaporator to near dryness and recrystallised twice from a acetone and water mixture (2:1) yielding 0.88 g (6.19 mmoles, 70%) of fine white microcrystals melting with decomposition at (270-290°C) (Cline *et al.* 1959: decomposing at 260-300°C).

Identification and characterisation was carried out using FAB MS (VG 70-SEQ) with positive ion mode with a glycerol matrix yielding the $[M+H]^+$ at *m/z* 143, and GC-MS of the tris-t-butyldimethylsilyl derivative (section 4.2.3.).

The internal standard [M+5] 5-OHMeU was prepared from uracil-1,3-$^{15}N_2$ and $^{13}C_1$ $^{2}H_2$ paraformaldehyde by the same procedure to that used for the synthesis of the unlabelled compound (acknowledgements to R. Jukes). The FAB MS (VG 70 SEQ) was consistent with that obtained for the unlabelled compound with a molecular ion at *m/z* 148. GC/MS of the tris-t-butyl dimethylsilyl derivative was also carried out (section 4.2.3.).

2.4.2. Synthesis of 5-OHMedU

The synthesis was carried out using the procedure of Cline *et al.* (1959). To HCl (3 ml) was added deoxyuridine (1 g, 4.38 mmoles) and paraformaldehyde (1 g) and the mixture incubated at 50°C for four days. Deoxyuridine and 5-hydroxymethyldeoxyuridine (5-OHMedU) were detected on a cellulose thin layer chromatogram as U-V spots using t-butyl alcohol, methyl ethyl ketone, formic acid and water (40:30:15:15) as developing solvent.

The mixture was absorbed on a Dowex-1 column (OH form, 100-200 mesh), washed well with water and eluted with HCl (0.1 M). A total of 54 eluate tubes were examined for absorbance at 264 and the appropriate portions were concentrated by vacuum distillation and alternately diluted with ethanol and taken to near dryness until addition of ethyl acetate precipitated the product as a fine powder. The yield was 0.78 g (3 mmoles, 69%), melting point range (177-179°C) (Cline *et al.* 1959: 176-179°C). Identification and characterisation was carried out using FAB MS (VG 70-SEQ) in positive ion mode yielding $[M+H]^+$ at *m/z* 259.

2.4.3. DNA Extraction

DNA extraction was carried out as described in section 2.3.2.

2.4.4. Cleaning of glassware

All glassware was cleaned as described in section 2.3.3.

2.4.5. DNA Hydrolysis

Samples were hydrolysed as described in section 2.3.4.

2.4.6. Derivatisation

Derivatisation of samples for 5-OHMeU analyses was carried out using MTBSTFA/1 % TBDMCS and pyridine (1:1, total volume 60 µl) at 60°C for 60 minutes. The derivatised samples were then dried in a vacuum centrifuge and reconstituted in the solvent stated.

2.5. EXPERIMENTS DESCRIBED IN CHAPTER 5

2.5.1. Cleaning of glassware

All glassware was cleaned as described in section 2.3.3.

2.5.2. DNA Hydrolysis

Aliquots of aqueous DNA were dried using vacuum centrifugation (Speedvac, Savant instruments Inc., Farmingdale, US) and then subjected to acid hydrolysis with 500 µl formic acid (60%) under vacuum at 140°C for 40 minutes. Following acid hydrolysis the DNA hydrolysates were again dried using vacuum centrifugation before being reconstituted in water.

2.5.3. Removal of guanine.

Following reconstitution of dried DNA hydrolysates in water, tris HCl was used to adjust the pH of the hydrolysate to 8. Guanase was subsequently added to the DNA hydrolysates at a level of 1.5 mU/40 µg DNA, followed by incubation at 37 °C for 1 hour. To control samples the same amount of tris HCl was added with substitution of H_2O instead of the guanase. Control samples were also incubated at 37°C for 1 hour.

Undiluted guanase (1.265 units/ml) was added to solutions of bases to 1.5 mU activity/0.5 ml of 5 µM guanine.

CHAPTER 3.

THYMINE GLYCOL

3.1. INTRODUCTION

5,6-Dihydroxy-5,6-dihydrothymine, also known as thymine glycol (Tg), is a marker of oxidative DNA damage. Tg is formed in DNA as a product of oxidative free radical attack on the C(5)-C(6) ethylenic bond of the thymine moiety (section 1.2.3.1).

In DNA it is produced as a major product of ionising radiation (Frenkel *et al.* 1981b; Teebor *et al.* 1987; von Sonntag 1987) and other oxidative stress (Frenkel *et al.* 1981a). Tumour promoters such as TPA have also increased levels of Tg ~ 5 fold (Frenkel *et al.* 1986).

Tg and Tdg have been detected in the urine of various species including humans, (Adelman *et al.* 1988, Cathcart *et al.* 1984; Cao and Wang 1993). The results indicate that humans excrete ca. 32 nmol of the two glycols per day in urine; however output was not seen to increase with age in humans. Tg and Tdg was found to correlate highly with species metabolic rate, consistent with the theory that DNA is a critical target in the ageing process and that larger mammals owe their longer life spans to their lower specific metabolic rates and therefore low rates of oxidative DNA damage. *In vivo* Tg has been found in human cancerous lung tissue and its cancer-free surrounding tissues (Jaruga *et al.* 1994).

Tg has been shown to block DNA polymerases *in vitro* (Ide *et al.* 1985). Repair of this lesion in DNA has been shown to occur via the action of a specific DNA glycosylase (Demple and Linn 1980) and a nucleotide excision repair complex found in E.coli (Lin and Sancar 1989).

Methods for detection and/or determination of Tg have included TLC (Teoule *et al.* 1977), HPLC with UV detection after reduction to thymine (Cathcart *et al.* 1984) or acetylation (Teebor *et al.* 1987), immunochemical methods using polyclonal (West *et al.* 1982) or monoclonal (Leadon and Hanawalt 1983) antibodies, fluorescent postlabelling (Sharma et 1990a), ^{32}P-postlabelling (Weinfeld and Soderlind 1991; Hegi *et al.* 1989; Reddy *et al.* 1991), GC/MS of a methanolysed (Markey *et al.* 1993), or a pentafluorobenzyl-t-butyldimethylsilylated product (Naritsin and Markey 1996) after alkaline cleavage/borodeuteride reduction of DNA, thermospray MS (Berger *et al.* 1992), and laser desorption Fourier-Transform mass spectrometry (Yoshida and Hettich

1994). Despite the abundance of these methods for Tg there is still limited data on background levels of this modified base in human DNA samples.

The purpose of the work described in this chapter was to develop a specific and sensitive method using GC/MS with an isotopically labelled internal standard for Tg determination in DNA. The method was first validated on DNA samples oxidatively modified *in vitro* and then applied to the determination of Tg in animal and human studies. The effect of antioxidant therapy on the level of Tg in human placental DNA was also studied.

3.2. METHOD DEVELOPMENT.

The technique of GC/MS was to be applied to the quantitation of Tg. A study of experimental parameters such as derivatisation and DNA hydrolysis was undertaken and calibration lines for standards and calf thymus DNA were established. The GC/MS parameters for this chapter were as described in section 2.2.1.

3.2.1. Derivatisation.

Most of the previous quantitative GC/MS assays developed for Tg have used the TMS derivative (Dizdaroglu 1994; Nackerdien *et al.* 1992; Fuciarelli *et al.* 1989; Scram 1990). However the use of the TBDMS derivative has also been described for qualitative studies (Dizdaroglu 1984, 1985) due to the advantages of the TBDMS derivatives over the TMS derivatives (refer to section 1.4.5.7.1.). Crain and McCloskey (1983) have described conditions for the formation of the TBDMS derivative of 5-methylcytosine which were subsequently employed by Dizdaroglu (1984) to generate $(TBDMS)_4$ Tg.

Experiments were carried out to repeat the work of Crain and McCloskey (1983), however results indicated that the bis- and tris-TBDMS Tg were also produced in addition to the fully derivatised base. Further heating (60 mins at 120°C) after the derivatisation mixture had been allowed to stand overnight was required in order to convert most of the bis- and tris- derivative to the fully derivatised base. Injection in ethyl acetate, rather than derivatising agent, appeared to cause decomposition of tris- and tetra-TBDMS-Tg to yield the bis-TBDMS derivative (ca. 95% of the detected derivatives). Further optimisation was not pursued as a deuterated internal standard was to be used in the assay. The derivatisation conditions described in the section 2.3.5. yielded the bis-TBDMS derivatives of Tg and its deuterated analogue. The chromatographic properties/instrument response were adequate for quantitation and derivatisation yields were not determined.

It was not possible from the proton NMR spectrum of derivatised Tg, to determine the position of the two TBDMS groups. However, the EI mass spectrum was consistent with derivatisation of the two hydroxyl groups (C5 and C6), evidenced by loss of CONH from the pyrimidine ring via a retro-Diels Alder reaction.

3.2.2. Mass spectrum of Tg

The major higher mass ion in the EI mass spectrum of the TBDMS derivative of [$^2H_0$] Tg is the $[M-57]^+$ ion at *m/z* 331 (Figure 3.1a, Scheme 1). The molecular ion (*m/z* 388) and *m/z* 373 (loss of a methyl radical) were observed but were of low intensity. Consequently, the *m/z* 331 was used for SIR and as the precursor ion for MRM. The MRM product ion , *m/z* 288 [$^2H_0$], is assumed to be due to loss of CONH (-43 amu) from the $[M-57]^+$ ion, based on the EI spectra of pyrimidine bases (McCloskey 1974). Other major ions in the MS spectrum may possibly be attributed to loss of t-butyldimethylsilanol (TBDMS-OH, -132 amu): *m/z* 199 (from *m/z* 331) and m/z 156 (from *m/z* 288). Alternatively, the ion at *m/z* 156 may be the product ion from loss of CONH from the ion at *m/z* 199.

Ions at *m/z* 147, 75 and 73 were attributed to silyl fragments reported previously (Halket 1993). With the exception of the silyl fragments and *m/z* 186, 174 and 160, [$^2H_3$]-Tg produced the expected fragment ions at 3 *m/z* units higher than those observed for [$^2H_0$]-Tg (figure 1.1. b). Monitored channels for the internal standard were *m/z* 334 (SIR) and the transition *m/z* 334 to *m/z* 291 (MRM).

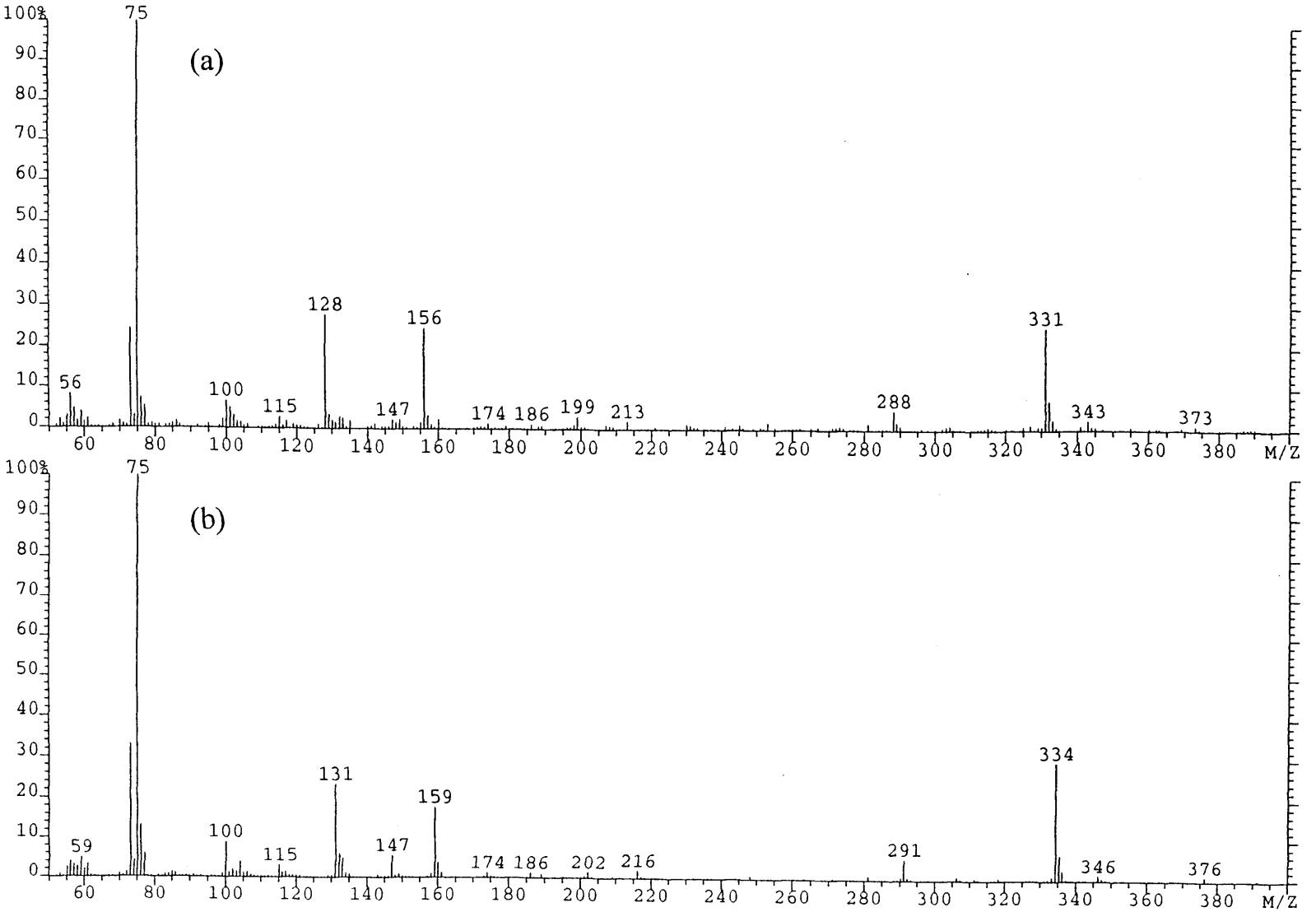


Figure 3.1. EI Mass spectra of bis-TBDMS (a) $[^2H_0]$ and (b) $[^2H_3]$ Tg.

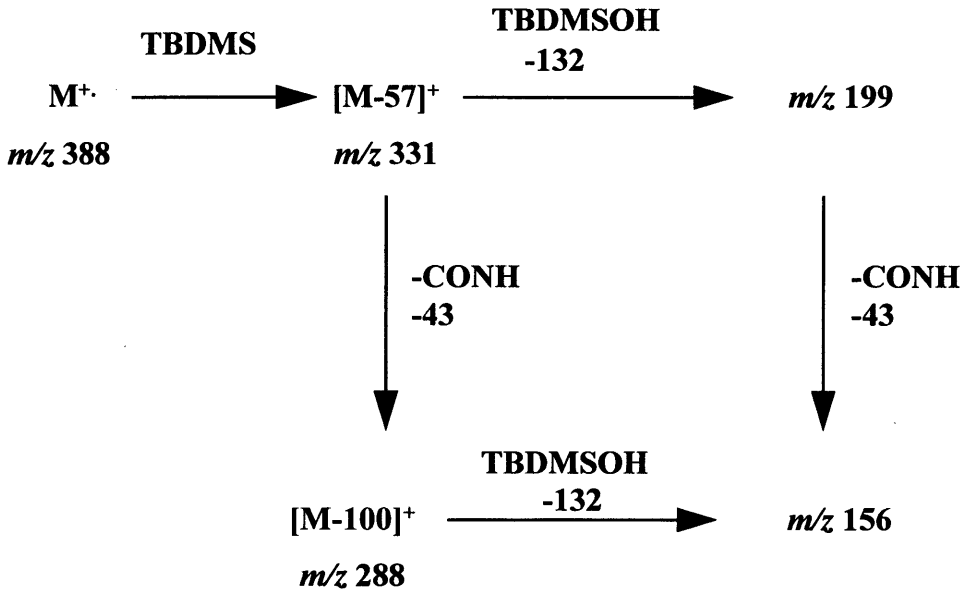


Figure 3.2. Scheme showing the fragmentation pattern of the bis-TBDMS [$^2H_0$] Tg using EI.

3.2.3. Calibration lines.

3.2.3.1. Standards calibration line.

Prior to quantitation of Tg in DNA a calibration line using standards of [$^2H_0$] and [$^2H_3$] Tg was established. Uracil was used with the Tg standards in order to act as a bulk carrier to prevent losses of the small amounts of analyte being handled. Calibration standards utilised uracil (10 μg) to which [$^2H_0$] Tg (0-4 ng), and [$^2H_3$] Tg (2 ng) internal standard were added prior to derivatisation (section 2.3.5). Calibration standards were analysed by GC/MS SIR, and the results are displayed in Figure 3.3.

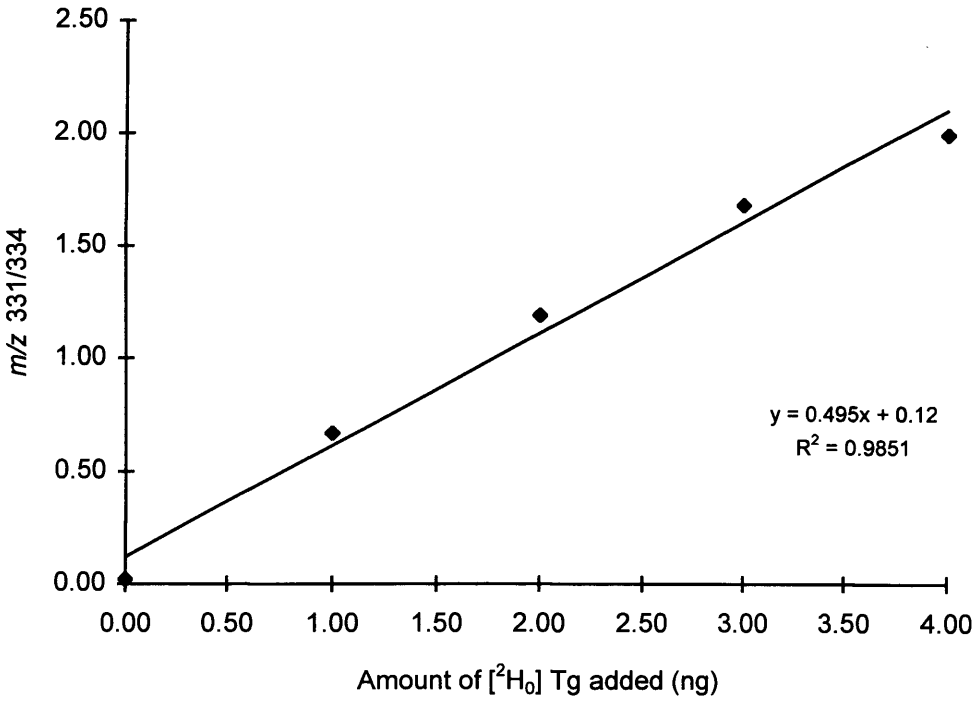


Figure 3.3. Tg standards calibration line.

3.2.3.2. Calf thymus DNA calibration line.

Once a linear calibration line had been achieved with Tg standards a calf thymus DNA calibration line was set up in order to quantitate levels of Tg in calf thymus DNA and eventually in human DNA.

To aliquots of calf thymus DNA (120 μg) was added [$^2H_3$] Tg (2 ng) internal standard and [$^2H_0$] Tg (0-3 ng). DNA samples were hydrolysed and derivatised (section 2.3.4. and 2.3.5.) and analyses were then carried out using GC/MS SIR. From the calibration line a background level of 1.1 ng Tg/mg DNA was calculated.

Results are shown in figure 3.4.

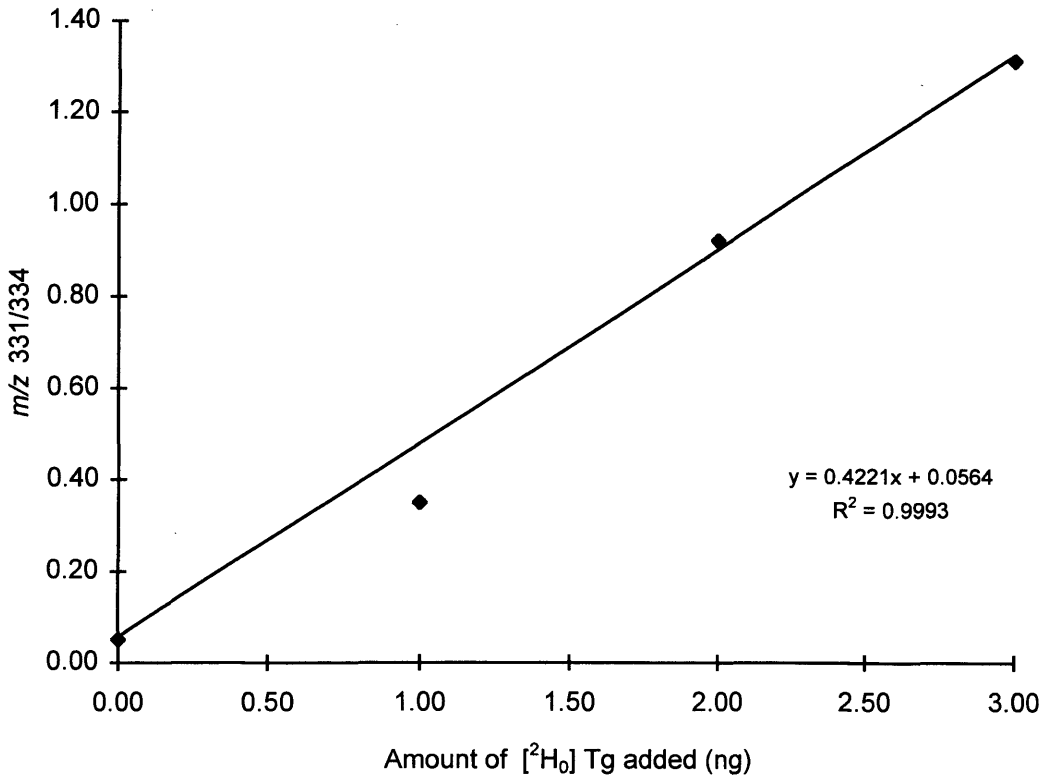


Figure 3.4. Calf thymus DNA calibration line.

3.2.3.3. Calibration line at biologically relevant levels.

A further calf thymus DNA calibration line was set up, containing significantly lower levels of Tg, in order to be comparable to endogenous Tg levels, and thereby to examine the sensitivity of the assay to quantitate endogenous levels of oxidative damage to humans in the form of Tg.

To aliquots of calf thymus DNA (120 μg) was added [$^2H_3$] Tg (2 ng) internal standard and [$^2H_0$] Tg (0-0.4 ng). The levels of Tg being quantitated in this calibration line were equivalent to 0-6 Tg/10^6 DNA bases. Samples were subsequently hydrolysed and derivatised (section 2.3.4. and 2.3.5.) followed by GC/MS SIR analyses.

Results were as shown in Figure 3.5.

Background levels of Tg in calf thymus DNA were calculated to be 0.69 ng Tg/mg compared to a level of 1.1 ng Tg/mg DNA in section 3.2.3.2. This is an example of the variation in background levels of modified bases observed in different batches of calf thymus DNA (see later discussion). A GC/MS SIR trace shows the lowest level calibration standard (Figure 3.6).

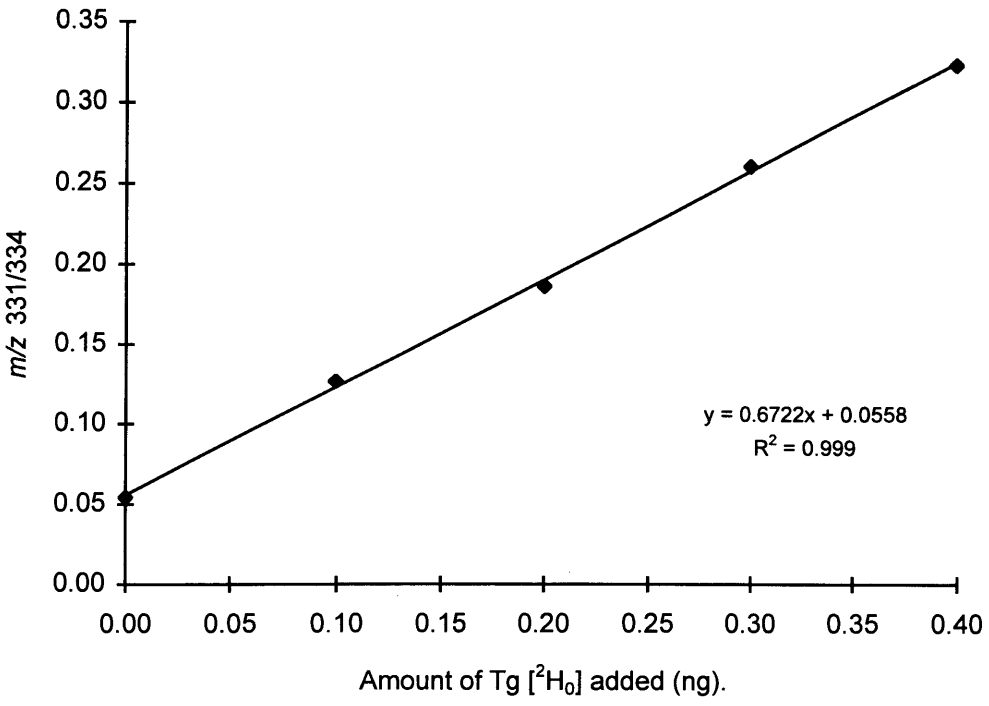


Figure 3.5. Tg calibration line at biologically relevant levels.

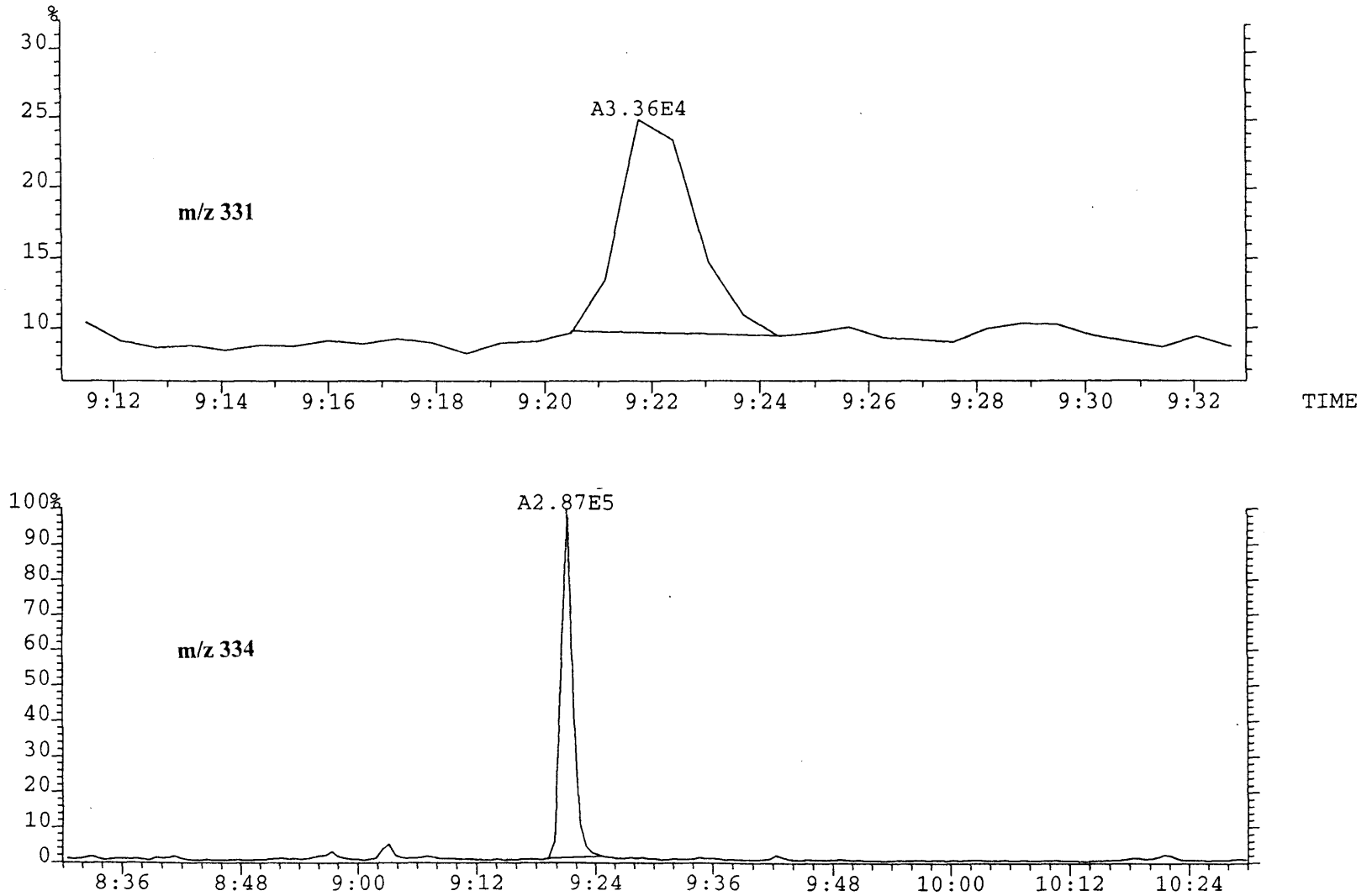


Figure 3.6. GC/MS SIR trace of the 0.1 ng calibration standard (corresponding to the lowest point on the calibration line).

3.2.4. DNA hydrolysis

Accurate measurement of modified bases is dependent upon the complete release of the DNA lesion from the sugar phosphate DNA backbone. The hydrolysis conditions being used were similar to those used by Nackerdien *et al.* (1992). However in order to examine the conditions being used in this assay the hydrolysis parameters were examined further. In order to study the release of Tg the hydrolysis conditions were studied with the use of irradiated and unirradiated calf thymus DNA. The use of irradiated calf thymus DNA as opposed to unirradiated permitted easier analysis and interpretation of results as higher levels of Tg were involved. With the use of unirradiated DNA, while quantitation of low background levels in DNA was possible, relationships due to the effect of the parameters studied were less obvious.

3.2.4.1. Temperature

It had initially been hoped that hydrolysis of DNA could be carried out at lower temperatures (Teoule and Guy 1987) than those being used in the literature. Tg was believed to be easier to remove than thymine, possibly due to its loss of aromaticity. In order to study the release of Tg from DNA as influenced by temperature, irradiated (400 Gy) DNA (100 μg) to which was added [$^2H_3$] Tg (1 ng) was hydrolysed using 60% formic acid at different temperatures for one hour's duration. Following incubation at the different temperatures, samples were derivatised (section 2.3.5.) and analysed by GC/MS SIR.

See figure 3.7. for results.

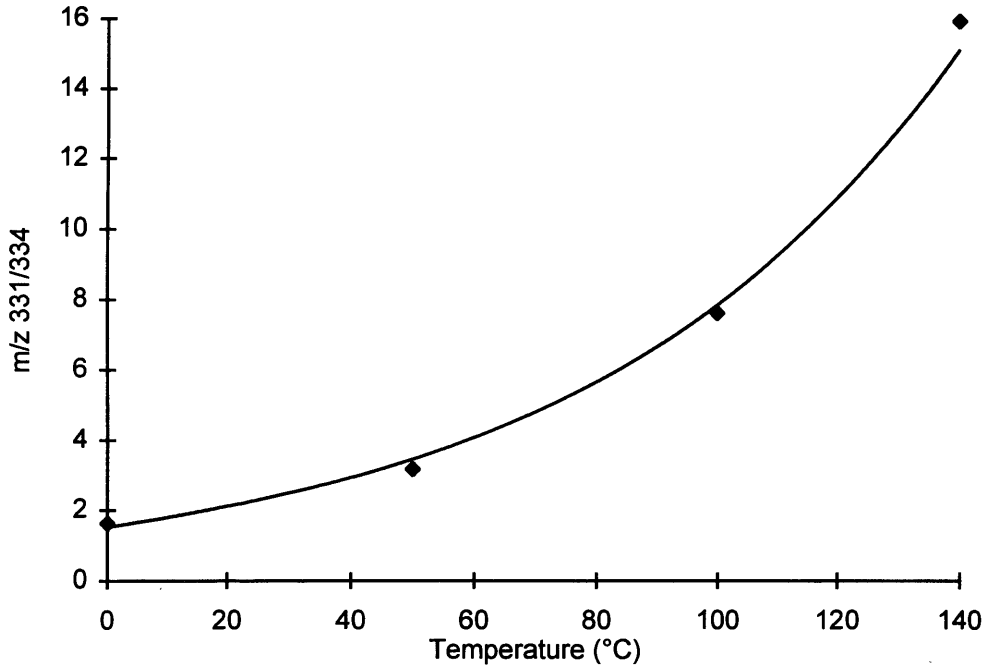


Figure 3.7. Tg release as affected by increasing temperature.

From the shape of the curve it can be seen that Tg release increased with temperature up until 140°C which was the upper limit of the study, therefore not enabling the DNA hydrolyses to be carried out at lower temperatures. A higher hydrolysis temperature was not investigated as literature studies had found 140°C to be sufficient for modified base release from DNA. Therefore the next experiment would examine Tg release over time at 140°C in order to examine if Tg was completely released at this temperature before the need to further elevate temperatures was investigated.

3.2.4.2. Time

An aliquot of the same 400 Gy irradiated calf thymus DNA (100 μg) to which was added [$^2H_3$] Tg (1 ng) sample was hydrolysed using 60% formic acid at 140°C and incubated over different periods of time to determine whether Tg is completely released from DNA at 140°C and after what incubation time. Following derivatisation (section 2.3.5.) samples were analysed by GC/MS SIR.

Results were as displayed in Figure 3.8. The above procedure was then repeated for unirradiated calf thymus DNA. Results were as shown in figure 3.9.

In common with the studies of Nackerdien *et al.* (1992) it was also found that using the temperature of 140°C the hydrolysis reaction was essentially complete between 30-40 minutes following which Tg release reached a plateau and no significantly elevated or decreased levels of Tg were detected over the 120 min study period. This experiment also demonstrates the stability of Tg at 140°C for up to 120 mins using the 60% formic acid conditions being used, as no decrease in Tg yield was observed over the study period.

In conclusion no modifications to the DNA hydrolysis procedure was seen to be required for removal of Tg from the DNA backbone.

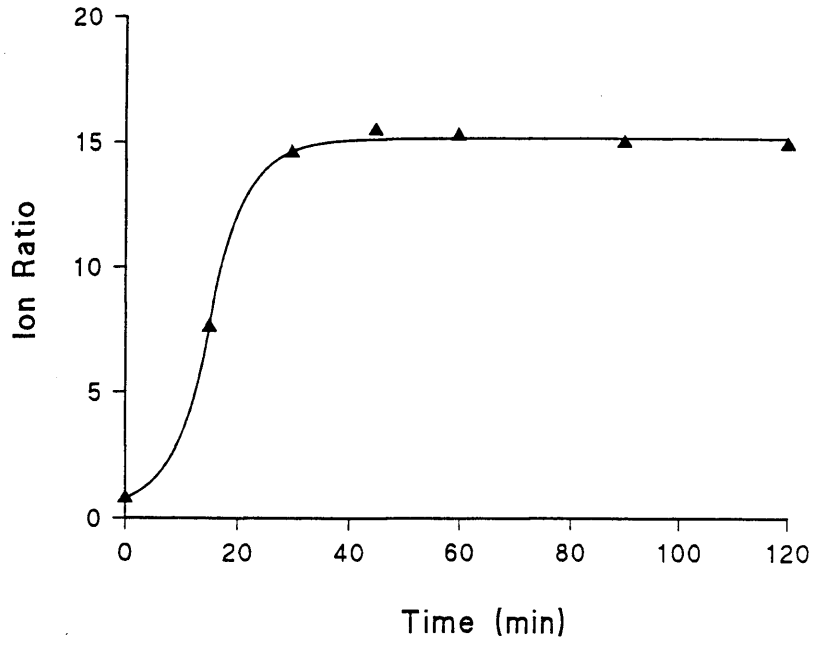


Figure 3.8. Tg release from irradiated calf thymus DNA over 120 minutes.

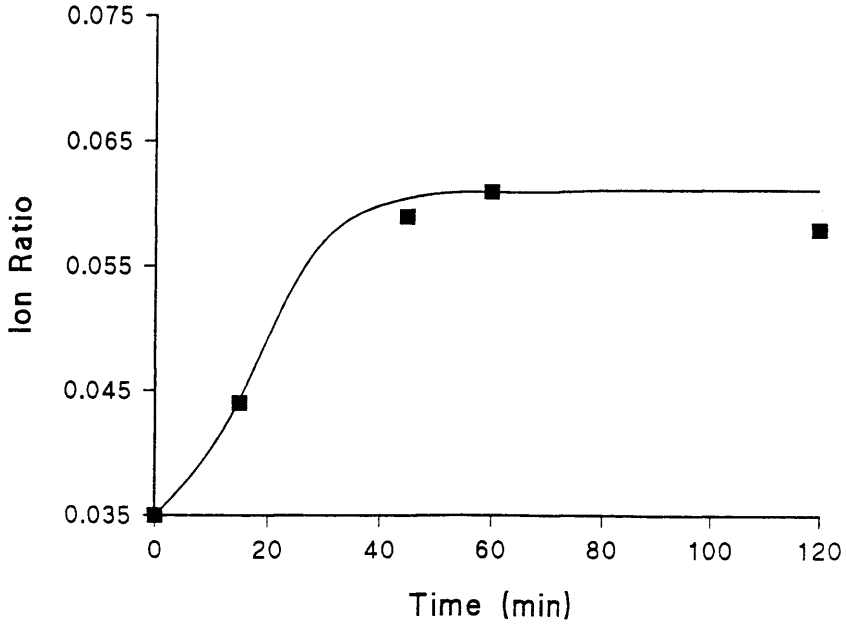


Figure 3.9. Tg release from unirradiated calf thymus DNA over 120 minutes.

3.2.4.3. Increasing amounts of DNA.

To further validate the complete release of Tg from DNA using the conditions outlined an experiment was to be carried out using increasing amounts of irradiated and unirradiated DNA. A linear relationship would further prove that Tg was being completely released from each particular aliquot of DNA with the hydrolysis conditions outlined (section 2.3.4.).

Aliquots (0-100 μg) of ^{60}Co γ-irradiated calf thymus DNA (200 Gy) to which was added [$^{2}H_3$] Tg (1 ng) were hydrolysed and derivatised (section 2.3.4. and 2.3.5.). Analyses of derivatised samples was carried out using GC/MS SIR.

Results are displayed in Figure 3.10.

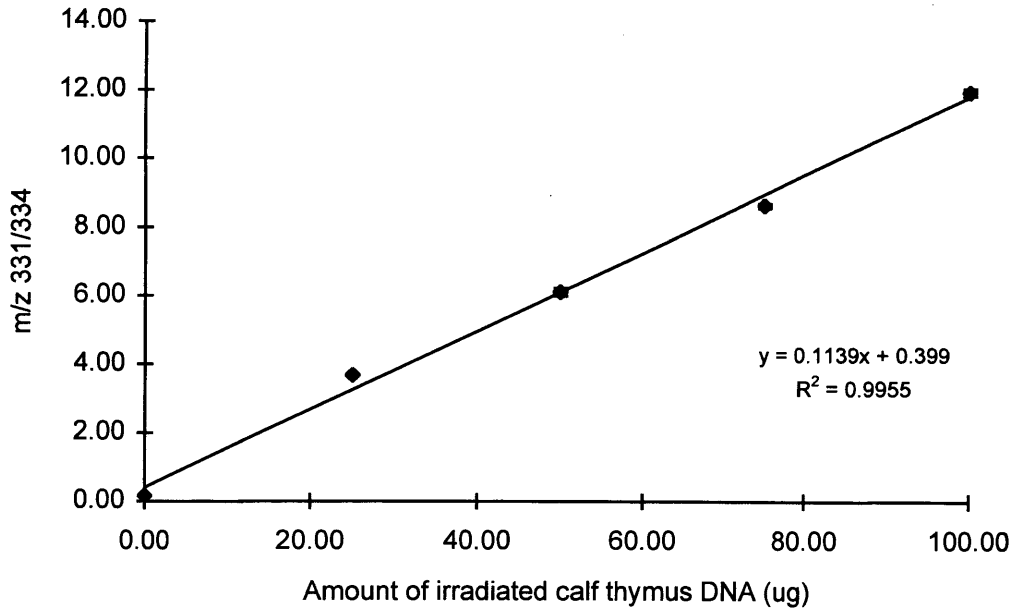


Figure 3.10. Tg release from increasing amounts of irradiated DNA

The above procedure was repeated with the use of unirradiated calf thymus DNA. Results were as shown in Figure 3.11.

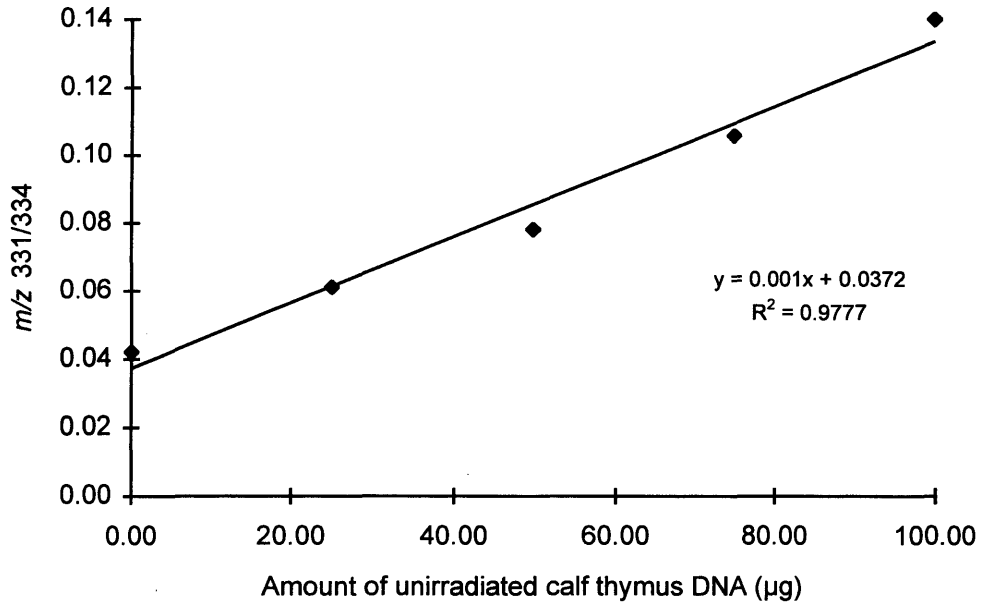


Figure 3.11. Tg release with increasing amounts of DNA analysed.

Linear relationships were observed for Tg release from irradiated and unirradiated calf thymus DNA. Levels of Tg in unirradiated (100 µg) DNA were calculated to be 1.2 ng Tg/mg DNA which is similar to values reported previously (section 3.2.3.2.) and in the literature (Markey *et al.* 1993).

In order to investigate if the background levels were from the DNA or an artefact of the method a oligonucleotide was synthesised.

3.2.4.4. Oligonucleotide

Background levels of modified bases have been found to be present in calf thymus DNA. The source of these 'background' levels however is as yet unknown. Nackerdien *et al.* (1992) however believe that the levels are already present in the calf thymus DNA whereas others believe them to be a result of artefactual production through the hydrolysis and derivatisation procedures. Recently great interest has been shown in the possible use of synthetic oligonucleotides to quantitate any artefactual production during the work-up procedure. In this study a synthetic oligonucleotide was to be taken

through the DNA hydrolysis and derivatisation procedure to study any subsequent formation of Tg. An oligonucleotide containing TTT TTT TT (T_8) was synthesised and subsequently purified by HPLC (see sections 2.3.6.), before being taken through the hydrolysis and derivatisation procedures (sections 2.3.4. and 2.3.5.).

To aliquots of T_8 (50 μg) was added [$^2H_3$] Tg internal standard (1 ng) prior to hydrolysis and derivatisation. Calibration lines were constructed using standards containing calf thymus DNA (50 μg), [$^2H_0$] Tg (0-0.5 ng) and [$^2H_3$] Tg (1 ng). Following hydrolysis and derivatisation samples were reconstituted in ethyl acetate (20 μl). Analysis was carried out using GC/MS SIR.

Results indicated that Tg was present in the oligonucleotide, and in order to verify this the experiment was repeated a further two times with similar results. From the calibration line, levels of Tg were calculated to be 0.0391 Tg/Thymine (~ 4%). Explanations for the possible sources of the Tg included: artefacts from the method, a contribution from the internal standard or the oligonucleotide itself.

3.2.4.5. Increasing amounts of [$^2H_3$] Tg internal standard.

To investigate the method and the contribution of the internal standard an experiment using increasing amounts of [$^2H_3$] Tg was carried out. To calf thymus DNA (75 μg) was added [$^2H_3$] Tg (0-2 ng). A sample containing only [$^2H_3$] Tg (1 ng) and no DNA was also taken through the hydrolysis and derivatisation procedure. As can be seen from the GC/MS trace in Figure 3.12. no measurable contribution could be attributed to the internal standard. Even using twice the level of [$^2H_3$] Tg as used in the above experiment (section 3.2.4.4.) would mean a contribution of < 1%.

It was therefore concluded that the levels of Tg as seen in the T_8 were not a result of the method or internal standard as no evidence of this was seen here. It was possible that the Tg may have been produced during the synthesis of the oligonucleotide.

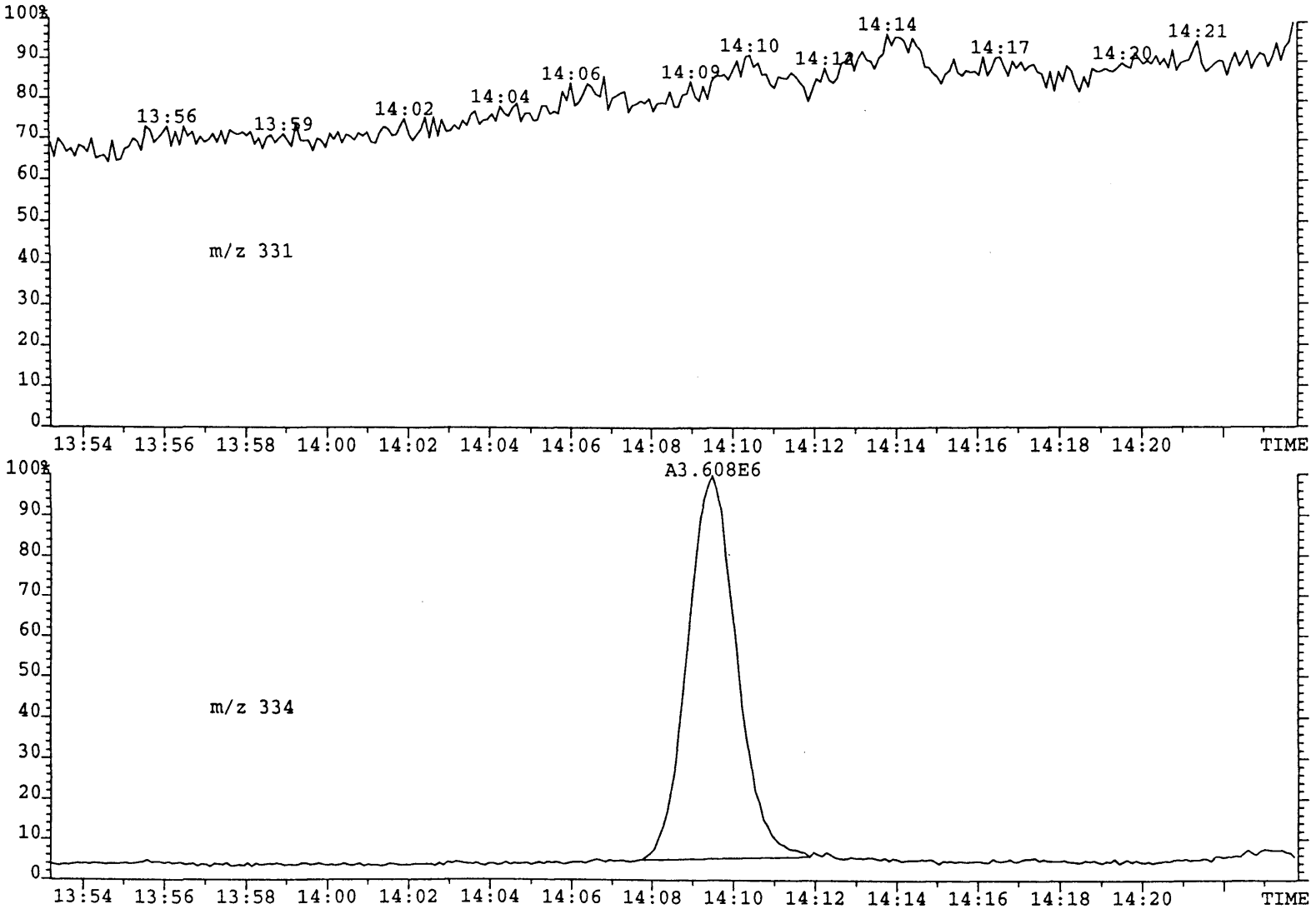


Figure 3.12. A GC/MS SIR trace showing the *m/z* 331 contribution from 2 ng [$^2H_3$] Tg internal standard.

3.2.5. Discussion

Experimental parameters such as DNA hydrolysis and derivatisation were investigated in order to optimise assay conditions. Derivatisation to the bis-TBDMS Tg was successfully achieved and used in this assay. DNA hydrolysis conditions investigated with regard to temperature and time showed Tg to be completely released within 40 minutes using 60% formic acid at 140°C. Tg was also found to be stable to the hydrolysis conditions outlined for upto 120 minutes.

In agreement with other studies background levels of Tg have been detected in calf thymus DNA. The levels detected here in calf thymus DNA are consistent with Tg levels quoted by Markey *et al.* (1993) using GC/MS. Markey *et al.* (1993) investigated variation on background levels in calf thymus DNA and found the levels obtained varied from batch to batch and upon storage. This may explain the variations also seen in the work described here. Variations in Tg levels in calf thymus DNA have been reported with the different techniques for example Hegi *et al.* (1989) have quoted levels of 20 ng Tg/mg DNA using PPL whereas Nackerdien *et al.* (1992) have quoted levels of 8 ng/mg using GC/MS (see chapter 6 for further discussion).

As with any assay the production of artefacts is always a cause for concern. However the importance of using oligonucleotides is appreciated and therefore attempts to quantitate any artefactual production from the method were examined. Contributions of the hydrolysis and derivatisation method to the high levels of Tg in the oligonucleotide are believed to be highly unlikely as no demonstration of this has been seen. However it is believed that oxidation may very well occur during the synthesis of the oligonucleotide, but this may be difficult or impossible to check at 'low' levels without the use of GC/MS hydrolysis and derivatisation procedures. Further work into the area of synthetic oligonucleotides needs to be carried out as the potential of these types of studies cannot be underestimated.

Upon the belief that the levels of Tg quantitated in the oligonucleotide were not a result of the assay the method developed was applied to the quantitation of Tg *in vitro* and *in vivo*.

3.3. APPLICATIONS

3.3.1. *In vitro* studies

3.3.1.1. ^{60}Co γ- irradiation of calf thymus DNA.

Oxidised nucleobases represent one of the main classes of damage induced in DNA by ionising radiation (see section 1.1.1.). Tg is the major species found in aerated irradiated aqueous solutions of thymine (Teoule and Cadet 1978). In the work presented here the GC/MS SIR assay was to be used for the characterisation and quantitation of Tg in calf thymus DNA upon exposure to γ-irradiation in aerated aqueous solution.

Aliquots (1 ml) of a solution of calf thymus DNA in deionised water (1 mg/ml) were irradiated in ^{60}Co-γ source at a dose rate of 1.48 kGy/hr ; the total dose ranged from

(i) 0-100 Gy.

Following irradiation, [$^{2}H_3$]-Tg (1 ng) was added to each aliquot of DNA (100 μg). Samples were taken to dryness by vacuum centrifugation, and subsequently hydrolysed and derivatised (sections 2.3.4. and 2.3.5.). Samples were assayed by GC/MS-SIR.

In order to quantitate the levels of Tg present in ^{60}Co γ-irradiated calf thymus DNA the above described approach was repeated at a dose rate of 1.57 kGy/hr and a dose range of

(ii) 0-400 Gy.

To DNA samples (100 μg) was added [$^{2}H_3$] Tg (1 ng) internal standard. Calibration standards were prepared for quantitation purposes and contained DNA (100 μg), [$^{2}H_3$] Tg (1 ng) internal standard and [$^{2}H_0$] Tg (0-1 ng).

Samples were then hydrolysed and derivatised (sections 2.3.4. and 2.3.5.) before being assayed by GC/MS SIR.

Results and discussion.

Figure 3.13 shows the (i) dose response curve obtained from irradiation of calf thymus DNA using the GC/MS SIR assay. Error bars represent the error involved in repeat analysis of samples one week apart, demonstrating the stability of the TBDMS derivatives used. Absolute levels of Tg were not determined, but the linear response indicates that the increase in Tg from irradiated DNA is linear in the range studied. Figure 3.14 shows a GC/MS trace of the 40 Gy irradiated sample.

Figure 3.15. shows results obtained from (ii) in which dose has been plotted against the total amount of Tg produced in ng/mg DNA

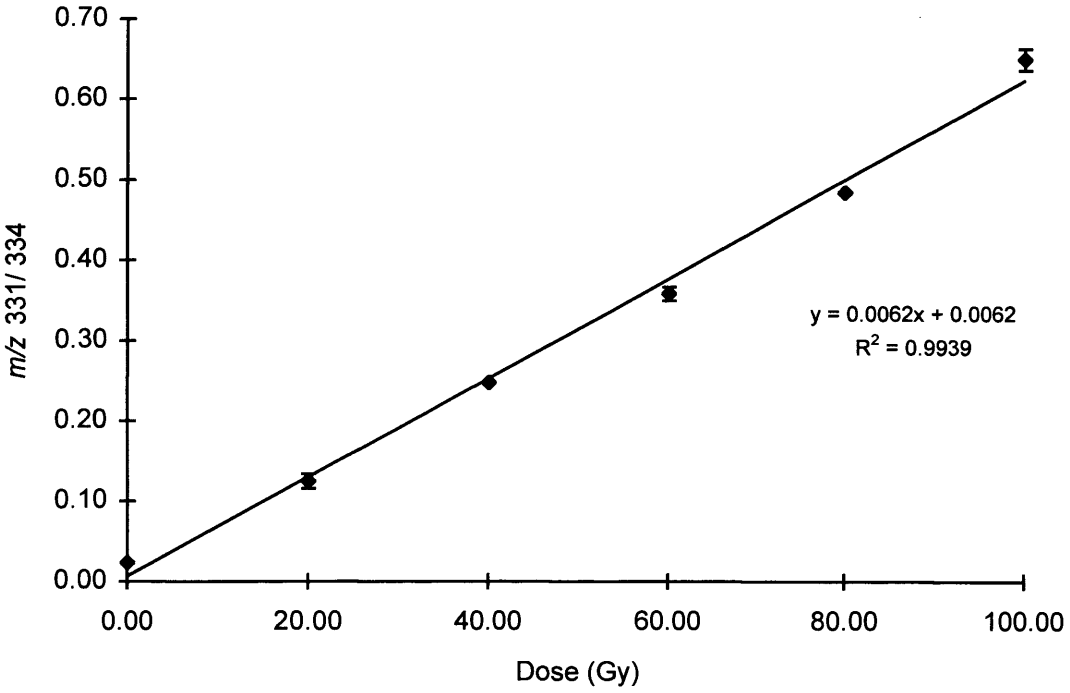


Figure 3.13. Dose response curve of Tg in 0-100 Gy γ-irradiated DNA.

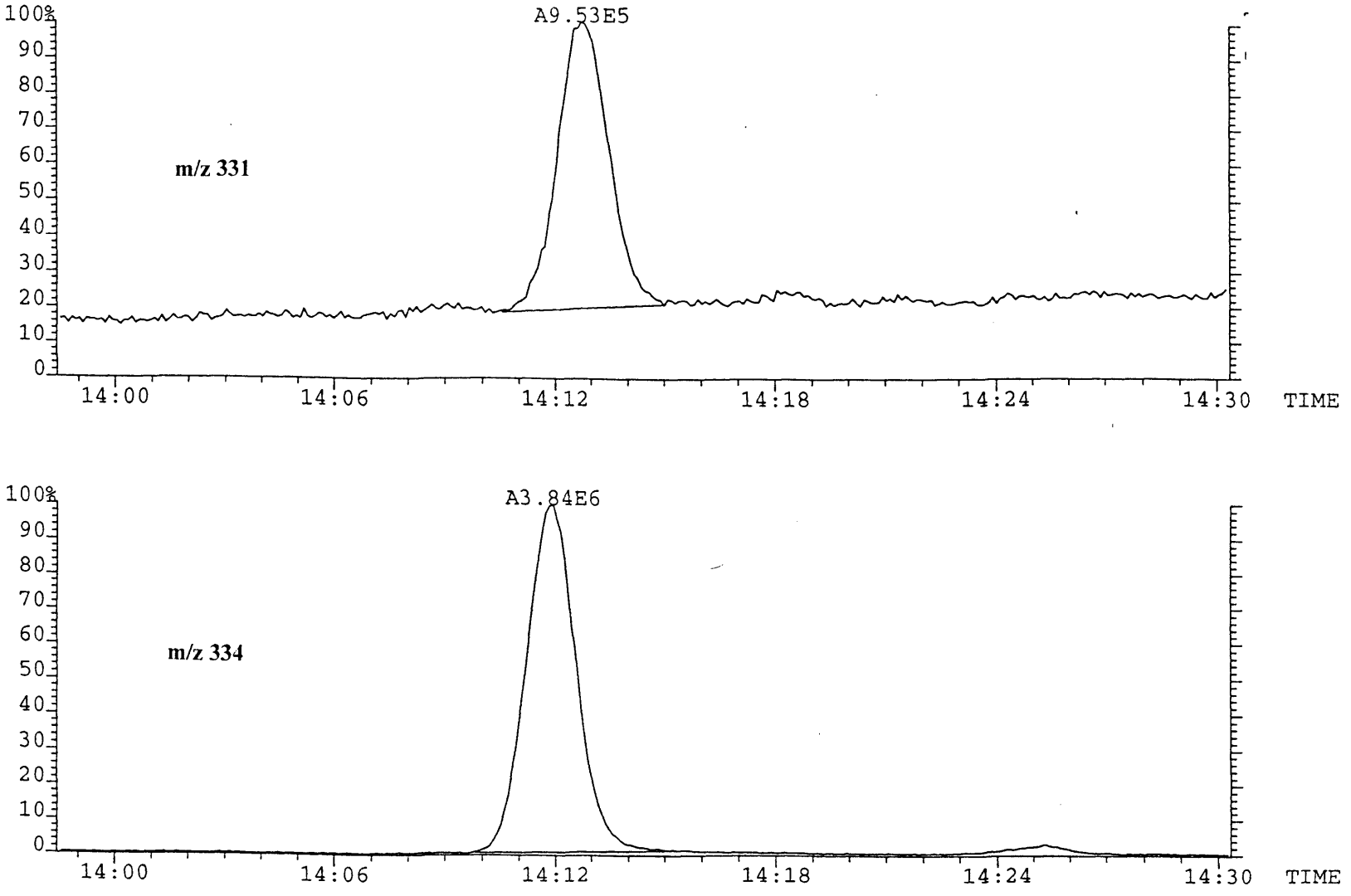


Figure 3.14. A GC/MS SIR trace of irradiated calf thymus DNA (40 Gy).

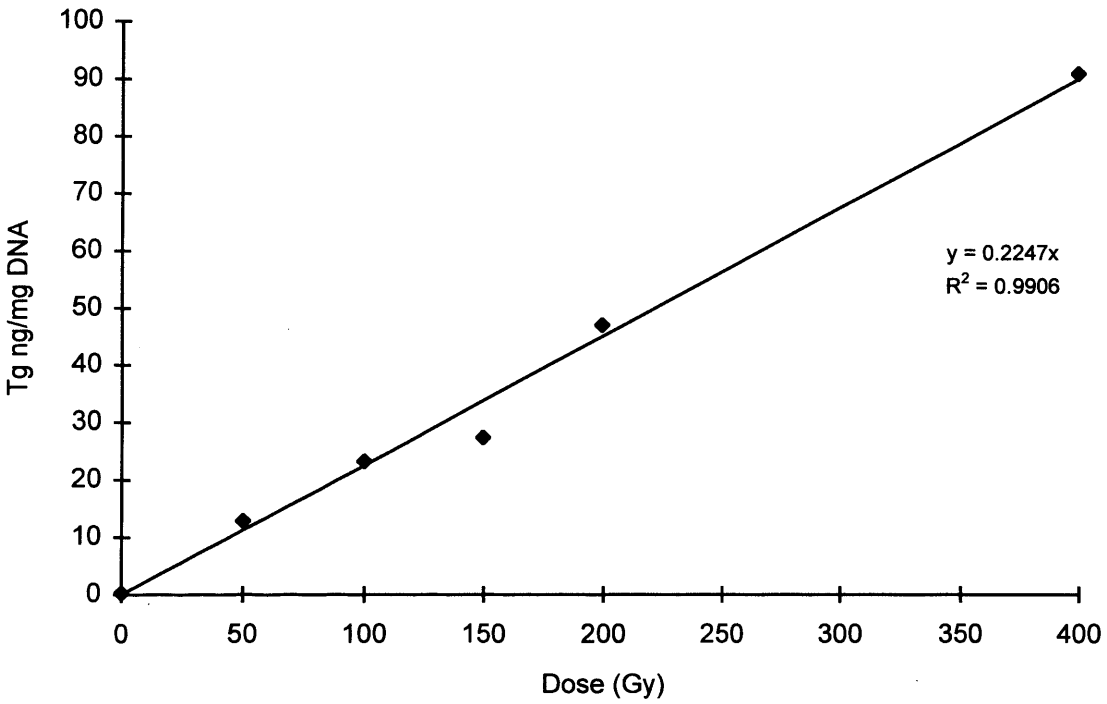


Figure 3.15. Quantitation of Tg in 0-400 Gy γ-irradiated calf thymus DNA calculated from the mean of duplicate analysis.

The experiment was repeated a further two times in which all samples were prepared using exactly the same batch of irradiated DNA and taken through the work-up procedure on three separate occasions. Results were as presented in Figure 3.16.

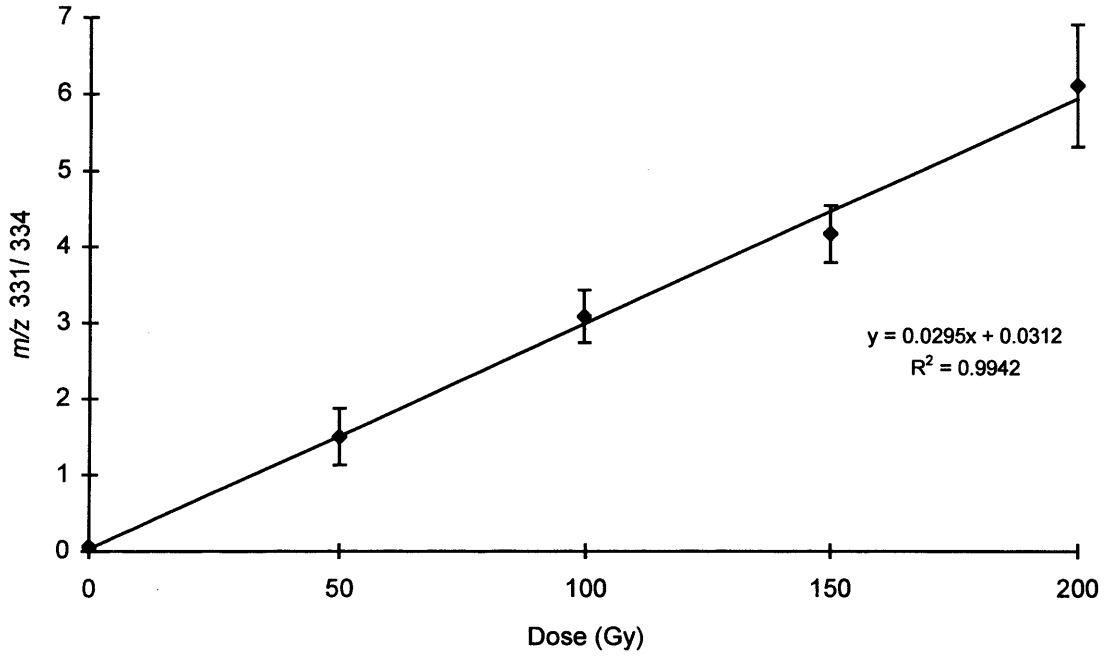


Figure 3.16. Analysis of γ-irradiated calf thymus DNA on three separate occasions.

Linear regression analysis was used to construct calibration plots of ion peak area ratio determined manually versus the amount of added Tg. Total Tg levels in the sample were calculated by adding the 'background' level, determined from the calibration plot. Using this relationship between ion peak area ratio and total Tg levels it was then possible to calculate Tg levels (in units of ng/mg DNA) in calf thymus DNA samples. The dose response relationship observed is in good agreement with similar studies on γ-irradiated DNA (Fuciarelli *et al.* 1989) and chromatin (Nackerdien *et al.* 1992; Dizdaroglu 1993b). Nackerdien *et al.* (1992) obtained linear dose relationships for Tg in γ-irradiated chromatin of human cultured cells in doses up to 420 Gy and at a radiation dose of 116 Gy Tg levels were calculated to be 6.56 ± 0.61 molecules/10^5 DNA bases using structurally similar internal standards. In this study, levels of Tg were calculated to be 4.62 Tg/10^5 bases in DNA irradiated with 100 Gy. Using the technique of postlabelling Hegi *et al.* (1989) detected 400-2700 Tg/10^6 T (159 - 1050 Tg/10^5 DNA bases) in 14-1000 Gy irradiated calf thymus DNA.

3.3.2. *In vivo* studies.

3.3.2.1. The role of antioxidants in preventing oxidative damage.

This study examines the role of antioxidant supplementation in preventing oxidative damage to DNA (for background see section 1.1.3.). Here, levels of Tg in human placental DNA were to be quantitated (for quantitation of 5-OHMeU and 8-OHG see later chapters).

Placental samples were obtained from pregnant women (n=40) resident in Teplice in the Czech Republic. Vitamins were supplemented in 20 subjects who were administered a daily dose of antioxidants (300 mg vitamin C, 200 mg vitamin E and 10 mg β-carotene) from the third month to the end of the pregnancy. The control group (n=20) did not receive any antioxidant supplementation.

Tissue samples were isolated from the middle of the placenta and the DNA isolated from 10% tissue homogenates in the extraction buffer (Tris-HCl, 10 mM, EDTA 100 mM, SDS 0.5%, pH 8.0). Following treatment with Rnases A and T1, and proteinase K, DNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol (as described in section 2.3.2.). Final DNA concentrations were determined by UV spectrophotometry ($A_{260,\ 280}$). Samples were supplied in aqueous solution and kept frozen at –20°C until analysis. Aliquots, containing a total of 75 μg DNA, were taken for analysis. (Gratefully acknowledge Dr R. Sram for collection of placental samples and extraction of DNA).

Quantitative analysis

To DNA samples (75 μg) was added [$^2H_3$] Tg (1 ng) internal standard, following which samples were dried by vacuum centrifugation and subjected to acid hydrolysis and derivatisation (sections 2.3.4. and 2.3.5.). The derivatised samples were dried and reconstituted in ethyl acetate (20 μl). Samples were analysed using GC/MS SIR and GC/MS MS.

Calibration standards utilised calf thymus DNA (75 μg) (from a 1 mg/ml solution in deionised water) to which known amounts of [$^2H_0$] Tg (0-500 pg) and the internal standard [$^2H_3$] Tg (1ng) were added prior to acid hydrolysis and derivatisation. Samples were analysed in batches, up to a total of 6-8 samples and calibration lines were

determined separately for each batch. Calibration standards and samples were assayed on the same day. Linear regression analysis was used to construct calibration plots of ion peak area ratio (determined manually) versus the amount of added Tg. Total Tg levels in the sample were calculated by adding the 'background' level, determined from the calibration plot. Using this relationship between ion peak area ratio and total Tg levels Tg levels (in units of ng/mg DNA) in human DNA samples were calculated.

Results and Discussion.

Initially, the GC/MS SIR assay was employed. However it was found that the SIR scan mode lacked the necessary specificity for reliable quantitation, as can be seen from figure 3.17a, which shows a typical trace from one of the control samples. Numerous (additional) chromatographic peaks were obtained close to the retention time for the analyte and internal standard. This effect was more pronounced in the *m/z* 331 channel. The level of potential artefacts in both the analyte and internal standard channels was assumed to be due to co-extraction of other components with the correct *m/z* values for detection in the monitored SIR channels. Although the extraction and hydrolysis procedures employed for the placental samples were similar to those commonly used for quantitation of constituents of DNA from other tissues by MS, the presence of any co-extracted material from the placenta might reduce the specificity of the assay.

Whilst an improved work-up procedure was desirable, another possibility was to increase the specificity of the mode of detection by using MS/MS detection-specifically, multiple reaction monitoring (MRM). A number of placental samples were analysed by GC/MS-MRM in order to determine whether the level of interferents detected was reduced sufficiently (compared to the SIR traces) to allow quantitation. Figure 3.17b shows the same control sample trace as figure 3.17a. Additional chromatographic peaks were still observed, but at much lower levels relative to the analyte peaks, so that quantitation was possible.

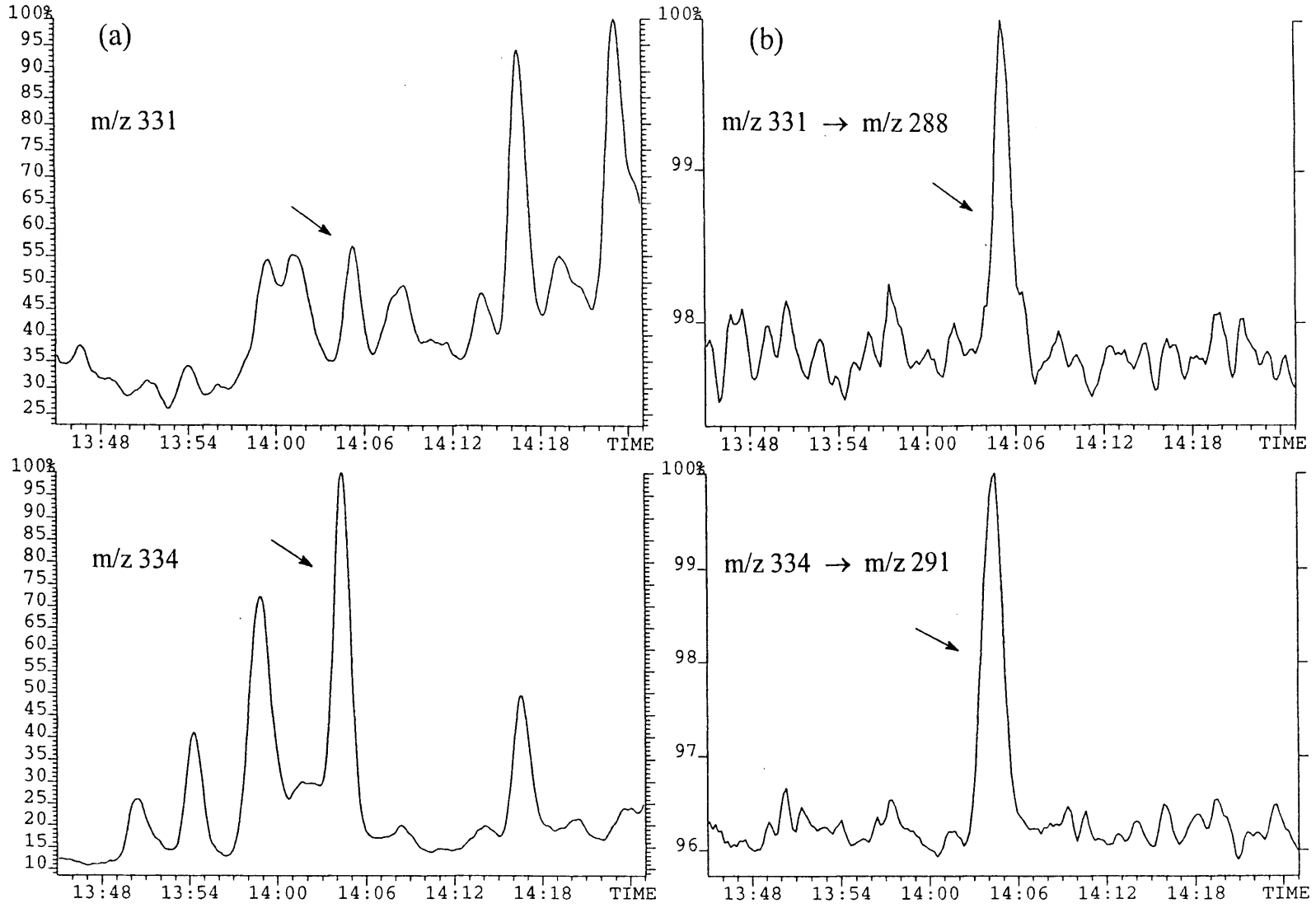


Figure 3.17. Comparison of SIR and MRM scan modes for the same control sample. The retention times of bis-TBDMS [$^2H_0$]-Tg and the internal standard were 14:05 mins and 14:04 mins respectively (indicated peaks). (a) SIR: traces from the monitored channels (b) MRM; traces from the monitored transition channels.

The calibration lines obtained from MRM and SIR scan modes were compared (figure 13.18). From the slope of the lines, it was apparent that MRM response was greater and that background levels of Tg were not significantly different: 2.25 ± 1.29 ng/mg DNA (SIR) and 2.23 ± 0.88 ng/mg DNA (MRM). It is anticipated that the limit of detection for a pure Tg standard would be lower for SIR than MRM, but in assaying samples, the 'limit of detection' is determined by the background level of Tg and the precision with which this level can be measured.

The placental samples were assayed using MRM and the results were as shown in Figure 3.19. The overall results for the two sample groups are also summarised (figure 3.20). In the antioxidant-treated group, three samples did not yield a signal in the analyte channel ($[^2H_0]$); these were not included in the statistical analysis. Given that a background level of Tg was expected, together with the variation of that level between samples, it was concluded that for the three 'zero' samples, the analyte level was below the limit of detection of the assay.

Application of a two sample t-test indicated that there was no significant difference between the control (n=20) and antioxidant (n=17) groups (P = 0.17), which had mean levels of Tg of 5.45 ± 2.98 ng/mg DNA and 4.33 ± 1.58 ng/mg DNA respectively. Other studies which have reported the quantitation of oxidised bases in human samples (cancerous versus normal tissue) levels of Tg were either not significantly elevated or could not be detected (Olinski *et al.* 1992; Jaruga *et al.* 1994).

The placental background level of Tg appears to be unchanged by antioxidant treatment. It has been supposed for some time that antioxidants may reduce the extent of oxidative damage, although the evidence of any extension of lifespan potential is not clear (Scott 1995). Possible explanations as to why there was no significant difference between the control and antioxidant groups could include the impossibility ethically to control the dietary intake of the control subjects. Daily food is enriched with antioxidants and therefore it is impossible to say how much antioxidants the control group received without controlling dietary intake during pregnancy! However the control group did not receive any additional supplementation outside of their daily diet, while the antioxidant treated group did.

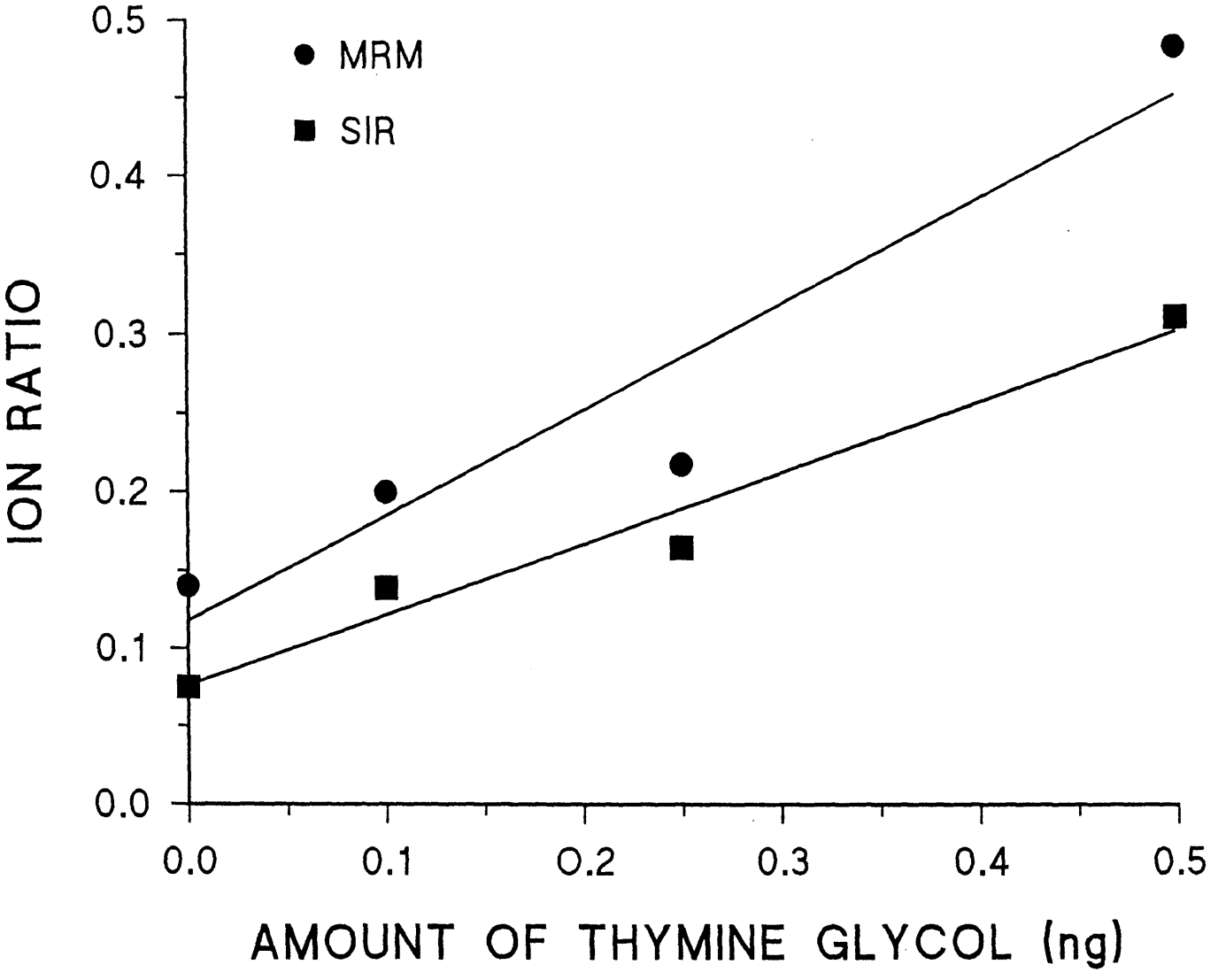


Figure 3.18. Comparison of calibration plots obtained by SIR and MRM with calf thymus DNA.

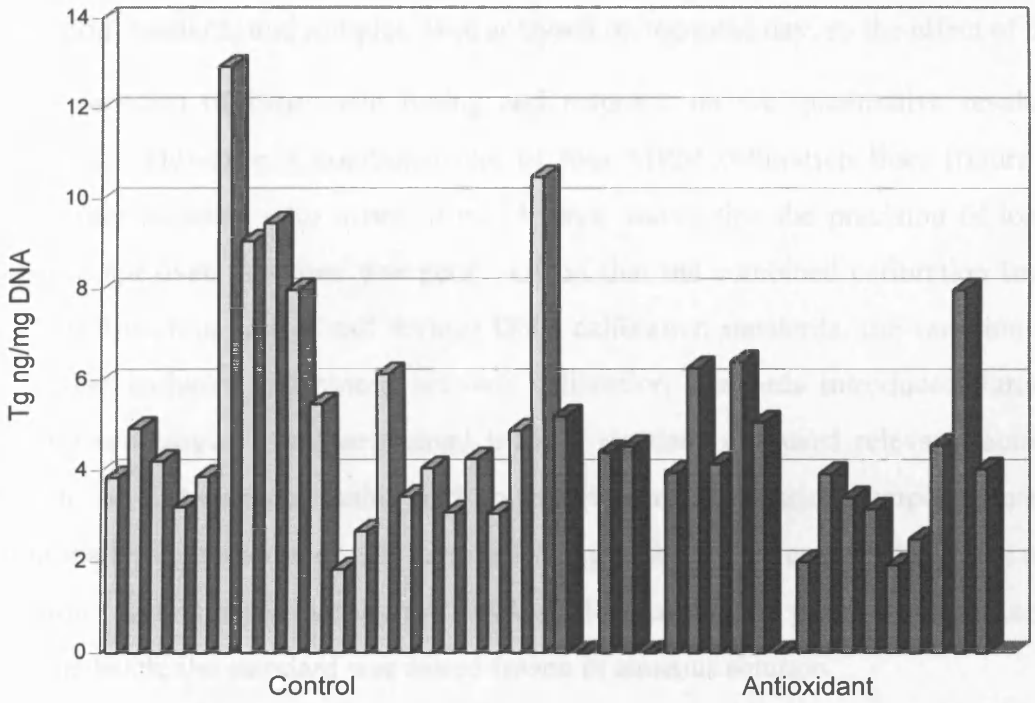


Figure 3.19. Bar graph showing effect of antioxidants on levels of Tg in placental DNA.

Comparison of scan modes

Although MRM has greater specificity than SIR, the MS/MS scan mode does have a number of disadvantages. The limit of detection is higher than SIR due to reduced ion transmission in MS/MS experiments. Additionally, it was seen that ion ratio precision was less than that obtainable by SIR, therefore it is possible that both these factors were important in:

(i) the apparent zero level of Tg in three control samples

(ii) the absence of a significant difference between control and antioxidant samples

A separate calibration line was determined for each batch of placental samples and the calibration standards and samples were analysed on the same day, so the effect of day-to-day variation of instrument tuning and response on the quantitative results was minimised. However, a combined plot of four MRM calibration lines (figure 3.21) which were obtained over a period of 14 days, shows that the precision of ion ratio measurement over this time was poor. Given that the combined calibration line was obtained from four sets of calf thymus DNA calibration standards, the variation in ion ratios also includes differences between calibration standards introduced during the work-up procedure. As a deuterated internal standard was used relevant factors are likely to be instrumental tuning and volumetric precision during sample preparation. Additionally, degradation of calf thymus DNA may well have contributed to the overall precision. Although the calf thymus DNA used for calibration purposes was taken from the same batch, the standard was stored frozen in aqueous solution.

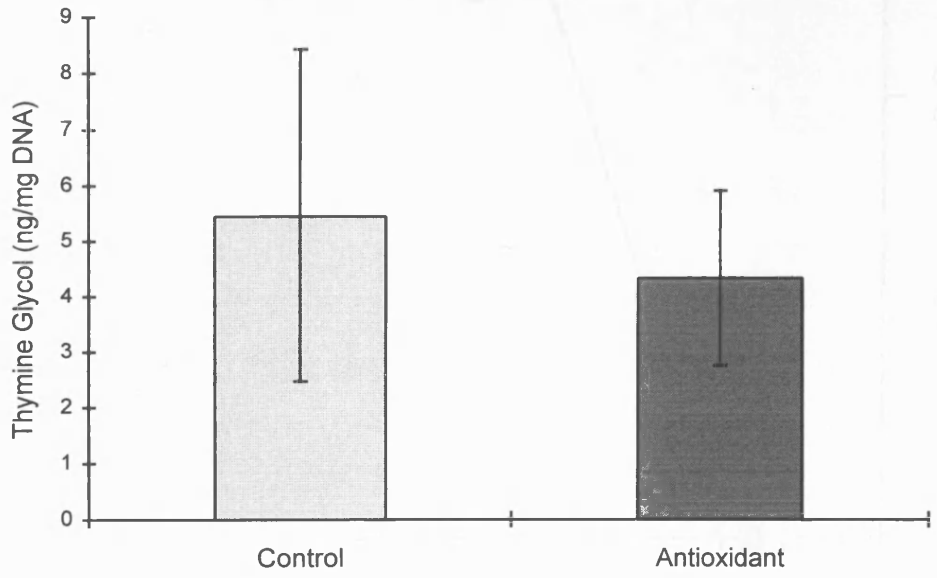


Figure 3.20. Placental DNA samples: antioxidant (n=17) versus control (n=20) group.

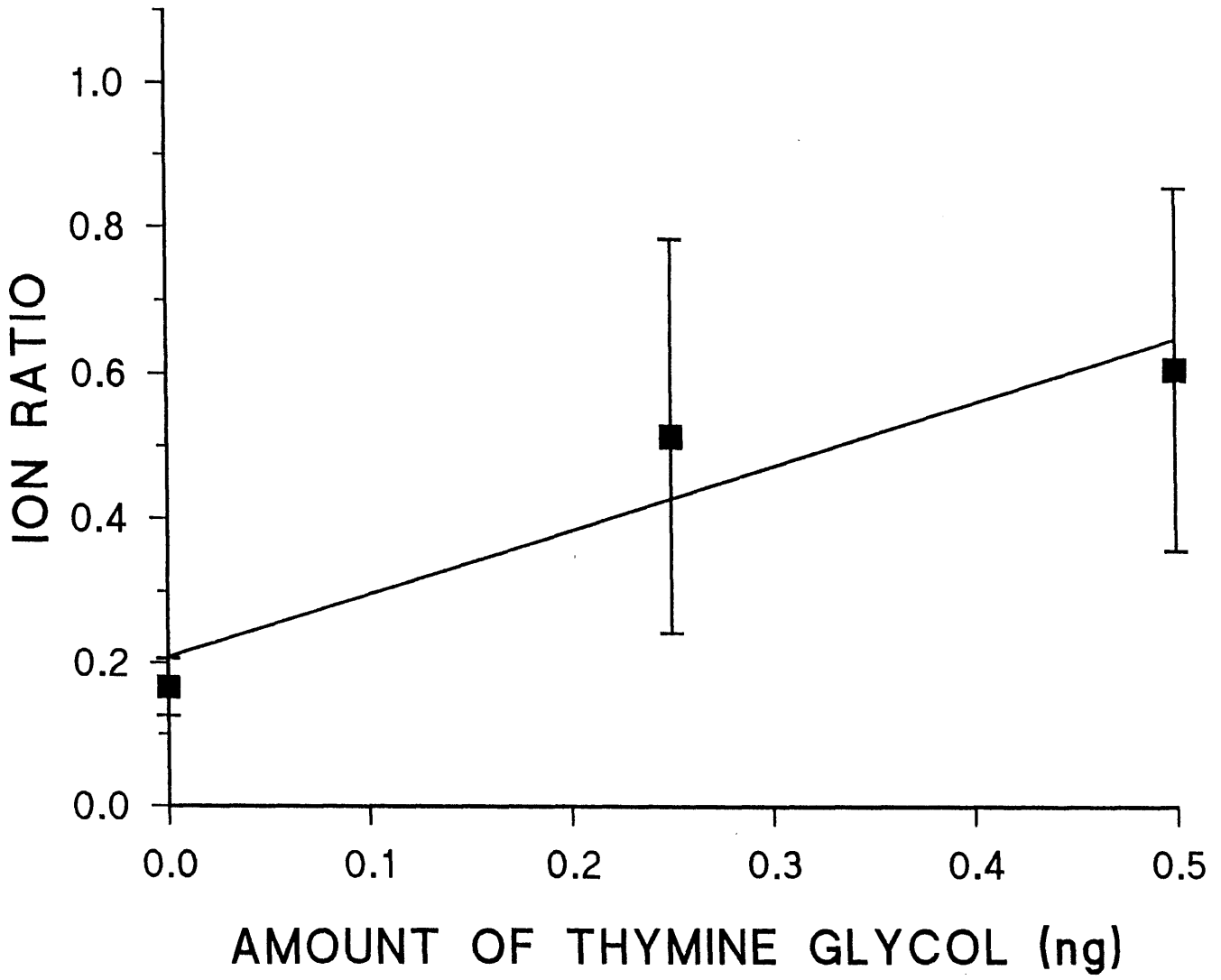


Figure 3.21. Averaged MRM calibration plot. Each point is the mean of four separate sample preparation/calibration experiments, conducted over a 14 day period.

It has recently been reported (Markey *et al.* 1993) that an increase level of Tg in DNA can result on storage in aqueous solution for extended periods of time.

It is not possible to ascertain which aspect of the assay work-up procedure or irreproducibility in ion ratio measurement in MRM had the greatest influence on the overall precision. It is suggested that with MRM there is a greater need for repeat measurement of calibration standards and samples, which may be difficult to achieve due to the length of time required for analysis. From the results presented above, it is apparent that calibration lines need to be obtained for each batch of samples which further increases the overall analysis time. The use of MRM, however, is preferable in terms of assay specificity, especially in the analysis of tissue samples.

3.3.2.2. Effect of crocidolite on oxidative damage in rat lungs.

Crocidolite is believed to be one of the most carcinogenic asbestos fibres known to man, however the mechanism of its carcinogenic action is still unclear. ROS are believed to play an important role in asbestos related damage and possible explanations may include increased release of ROS from neutrophils and macrophages particularly during phagocytosis, and/or increased generation of $OH^{\bullet}$ by crocidolite internalised in cells close to DNA. Crocidolite has also been shown to alter antioxidant levels in the lung including those of MnSoD and glutathione (Weitzman and Graceffa 1984, Mossman and Marsh 1989).

An inhalation study was established in order to investigate the effect of crocidolite on the production of Tg (for quantitation of 5-OHMeU in rat lungs see Chapter 4) and to use the existing assay to quantitate this oxidative marker in rat lung DNA. The study was to be carried out on 72 male, Fischer-344 rats of which 36 were exposed to crocidolite and 36 to air. The proposed dose was 100 mg/m^3 ~ 3000-5000 $fibres/cm^3$ over a period of 3 weeks for 5 days a week 6 hrs a day.

Animals (18 test and 18 control) were sacrificed at the end of the exposure period, the remaining animals (test and control) were allowed a recovery period of 3 weeks, and at the end of this period these rats were also sacrificed.

All animals were killed with a single i.p injection of sagatal containing 300U of heparin, the lungs were then exsanguinated and removed. The DNA was extracted from the

lavaged lungs (section 2.3.2.) and was analysed for Tg and 5-OHMeU. DNA concentrations were determined by UV spectrophotometry ($A_{260,\ 280}$). Samples were stored at – 20°C until analysis (Gratefully acknowledge Dr. A. Clouter for assistance in the crocidolite study).

Quantitative analysis

Prior to analysis of DNA samples the presence and amounts of fibres in lung tissue from two animals in all experimental groups was checked by scanning electron microscopy (SEM). The presence of fibres was confirmed in the exposed group and found to decrease following a three week recovery period, no fibres were found to be present in the control animals (Clouter *et al.* 1997).

Due to time limitations only six DNA samples from each group, namely exposed, exposed/recovery and control, were to be analysed. To DNA samples (70 μg) was added [$^2H_3$] Tg (1 ng) internal standard following which samples were dried by vacuum centrifugation and subjected to acid hydrolysis and derivatisation (sections 2.3.4. and 2.3.5.). The derivatised samples were dried and reconstituted in ethyl acetate (20 μl). Samples were analysed using GC/MS SIR and GC/MS MS.

Calibration standards utilised calf thymus (70 μg) DNA (from a 1 mg/ml solution in deionised water) to which known amounts of [$^2H_0$] Tg (0-1 ng) and the internal standard [$^2H_3$] Tg (1ng) were added prior to acid hydrolysis and derivatisation (sections 2.3.4. and 2.3.5.). Samples were analysed in batches, up to a total of 6-10 samples with random replication. Calibration lines were determined separately for each batch with calibration standards and samples being assayed on the same day. As previously (described in section 3.3.2.2.) linear regression analysis was used to construct calibration plots and subsequently to calculate Tg levels (in units of ng/mg DNA) in rat lung DNA.

Results and Discussion.

The rat lung DNA samples were assayed using MRM (with some samples also being analysed by GC/MS SIR) and the results are shown in Figure 3.2.2.

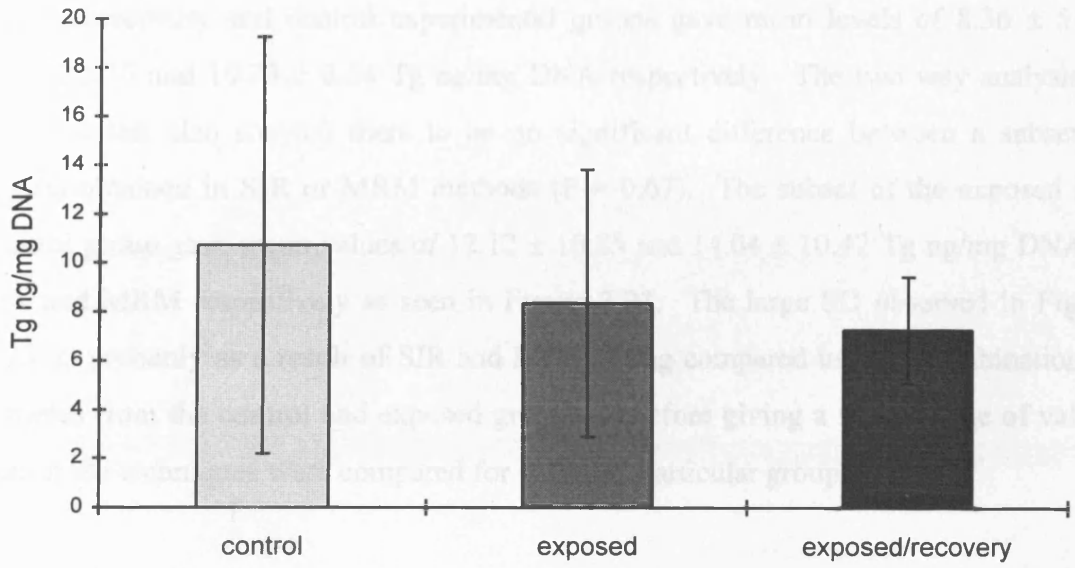


Figure 3.22. Mean Tg levels in rats exposed to crocidolite, exposed/recovery and control rats.

The results were not as expected, with the control group yielding exceptionally high levels of Tg as a result of two particular animal samples which gave significantly higher levels of Tg than the rest of the control group. Further investigations showed that the two control animals with high Tg levels had also shown high levels of extracellular protein. The source of this extracellular protein was probably a result of damaged epithelial cells, as measured in the cell free supernatant of the lavaged lungs (Clouter *et al.* 1997). High levels of extracellular protein are sometimes seen to be indicative of stress or inflammation and possibly an early sign of lung infection (Clouter 1993).

Unfortunately due to the unusual levels of Tg in the control rat lung DNA it was not possible to observe a relationship between Tg and crocidolite exposure. However this experiment did demonstrate the ability of this assay to measure *in vivo* oxidative damage in animals sensitively and specifically. Other studies have reported crocidolite induced increases in the levels of oxidised nucleobases as measured by 8-OHdG

(Takeuchi and Morimoto 1994, Adachi *et al.* 1994) but to date no work on Tg in relation to crocidolite has been published.

Application of a two way analysis of variance test indicated that there was not a significant difference between the crocidolite exposed (n=6) and control (n=6) groups (P = 0.748) using the GC/MS SIR and/or MRM methods. The exposed, exposed/recovery and control experimental groups gave mean levels of 8.36 ± 5.46, 7.28 ± 2.17 and 10.73 ± 8.54 Tg ng/mg DNA respectively. The two way analysis of variance test also showed there to be no significant difference between a subset of results obtained in SIR or MRM methods (P = 0.67). The subset of the exposed and control group gave mean values of 12.12 ± 10.85 and 14.04 ± 10.42 Tg ng/mg DNA in SIR and MRM respectively as seen in Figure 3.23. The large SD observed in Figure 3.23. is probably as a result of SIR and MRM being compared using a combination of samples from the control and exposed groups, therefore giving a wider range of values than if the techniques were compared for only one particular group.

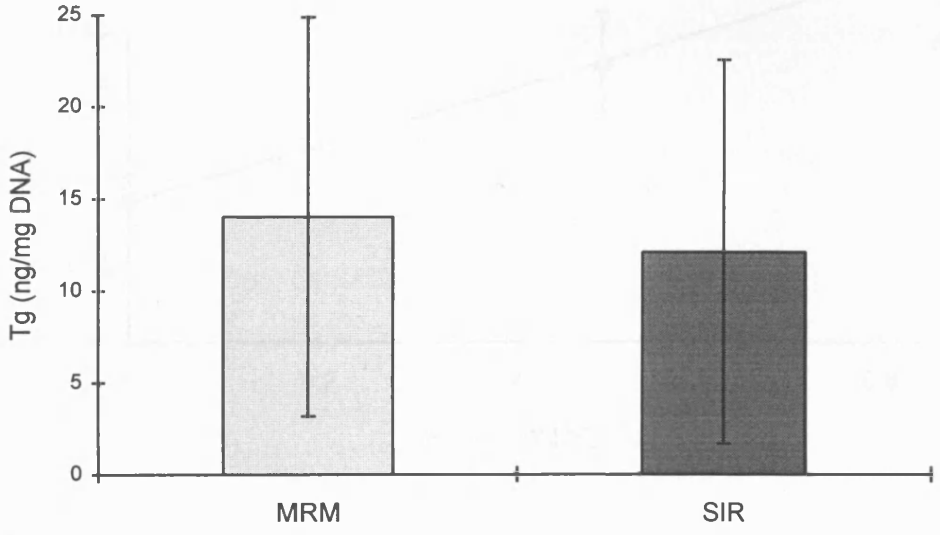


Figure 3.23. Mean levels of Tg (ng/mg) DNA from control and exposed rat groups using SIR and MRM.

Exposed and control group samples were analysed along with calibration standards on 3-4 separate occasions. Application of a paired t test indicated that the exposed and control groups gave consistent results with no significant difference being observed over time.

Calibration lines

A separate calibration line was determined for each batch of rat lung samples and the calibration standards and samples were analysed on the same day, so the effect of day-to-day variation of instrument tuning and response on the quantitative results was minimised. A combined plot of five MRM calibration lines (figure 3.21), shows that the precision of ion ratio measurement over this time was consistent.

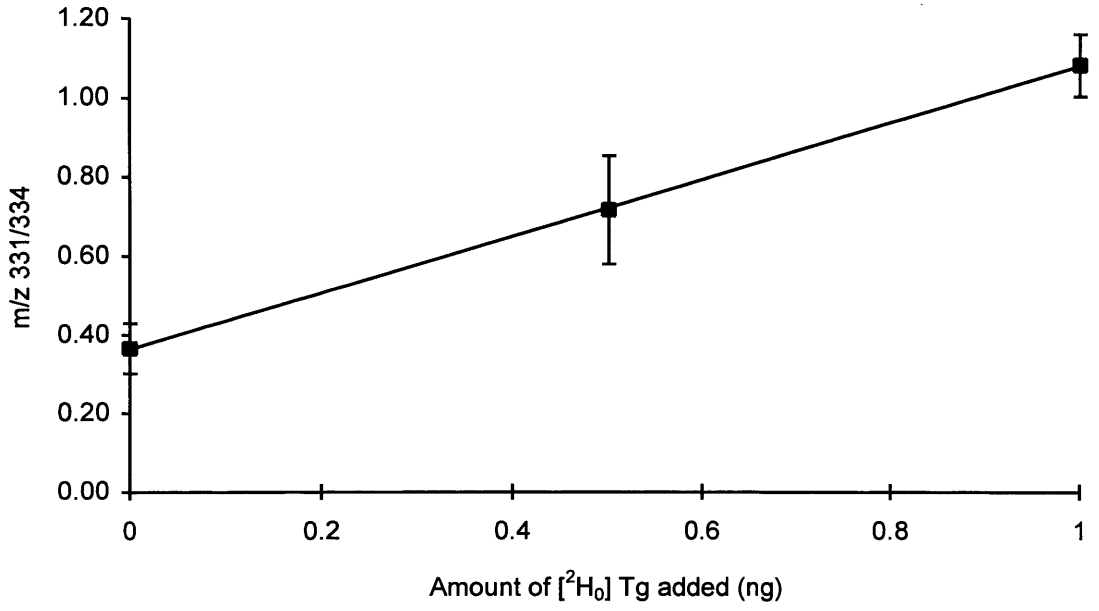


Figure 3.24. Combination of five calibration plots achieved for rat lung DNA samples over a period of two months.

3.4. DISCUSSION

The analytical procedure described, has sufficient sensitivity to detect background levels of Tg in human placental DNA (ca. 5 ng/mg DNA or ca. 1 Tg/10^5 nucleotides). This level of modification is similar to that reported by Hegi *et al.* (1989) using PPL of commercial calf thymus DNA.

In common with Dizdaroglu (1984), it was found that the quantitation of oxidised nucleobases by GC/MS requires a high level of column and injector maintenance. Sensitivity was found to decrease after analysis of approximately twenty hydrolysed DNA samples, which was attributed to the build-up of partially-derivatised nucleobases and the sugar phosphate backbone in the injector and at the top of the column. Removal of approximately 50 cm of column and thorough silylation of the injector liner was required in order to improve sensitivity. It is possible that injection in the derivatising agent would have reduced the frequency of injector/column maintenance. Additionally, the yield of TBDMS-Tg may have increased but it is possible that the tri- and tetra-derivatised base would have been produced. Injection in derivatising agent may also have lead to more derivatised co-extracted material being introduced on to the column. The assay could be improved, both with respect to signal-to-noise ratio and specificity, by introducing an additional clean-up stage to remove (or reduce the amount of) the sugar-phosphate backbone after hydrolysis. This could be achieved for example by the use of a boronate affinity column.

As with any analytical procedure there is the question of whether or not there is any artefactual formation of the analyte during the analytical procedure. However the consistency of our results with those derived from other determinations using techniques other than GC/MS suggests that the production of any significant artefactual production is unlikely. However these parameters may be studied further with the use of synthetic oligonucleotides.

Chapter 4.

5-Hydroxymethyluracil

4.1. INTRODUCTION

5-Hydroxymethyluracil (5-OHMeU) is a product of oxidative DNA damage. This lesion is thought to be produced through $OH^{\bullet}$ attack on the methyl group of thymine, resulting in formation of the 5-methyleneuracil radical (Roti Roti & Cerutti 1974; Roti Roti *et al.* 1974) which subsequently reacts with additional hydroxyl radicals to form the chemically stable 5-OHMeU. Experimentally it has been found that formation of 5-OHMeU is not dependent on DNA conformation since the methyl group of thymine lies in the major groove of the DNA helix and is therefore equally susceptible to hydroxyl attack be it single stranded or double stranded DNA.

This chemically stable derivative of thymine has been produced *in vitro* through the action of ionising radiation on DNA (Teebor *et al.* 1984) and by reacting calf thymus DNA with H_2O_2 and Fe^{2+} (Potter and Djuric 1990, Mouret *et al.* 1991). Singlet oxygen generated in reaction mixtures containing methylene blue and light has also been shown to produce 5-OHMeU (Potter and Djuric 1990).

Endogenously 5-OHMeU has been found to be present in human and rat urine at levels comparable to that of Tg from which a total oxidative damage rate to DNA was estimated as ~ 700 hits per cell per day (Cathcart *et al.* 1984; Adelman *et al.* 1988). Studies have also shown that a decrease in dietary fat can lead to decreased levels of oxidative damage specifically 5-OHMeU in peripheral nucleated blood cells of women at high risk of breast cancer (Djuric *et al.* 1991b). Animal studies in rats fed calorie restricted diets also showed significantly decreased levels of 5-OHMeU in the DNA (Djuric *et al.* 1992). In human studies elevated levels of 5-OHMeU have also been found in human cancerous tissue compared to the cancer free surrounding tissue which had been surgically removed (Olinski *et al.* 1992; Jaruga *et al.* 1994).

5-OHMeU has been shown to be mutagenic in bacterial systems (Cadet *et al.* 1981) and yet 5-OHMeU is also a normal DNA constituent of some Bacillus subtilis phages (Waschke *et al.* 1975). Its harmful potential however to eukaryotic DNA is corroborated by the demonstration of a DNA repair glycosylase enzyme in mouse cells directed specifically against the 5-OHMeU and differing from the DNA repair glycosylase enzyme for Tg (Hollstein *et al.* 1984). The existence of a repair enzyme

also suggests that 5-OHMeU, like Tg may also be formed through endogenous oxidative mechanisms which have been implicated in the etiology of human cancer.

Elucidation of the chemical nature of such DNA lesions at biologically significant levels is required for the assessment of their biological consequences and repair. Various methods for quantitation of 5-OHMeU in DNA are available. 5-OHMeU can be detected by PPL ; however this assay requires chromatographic purification of modified bases prior to PPL (Mouret *et al.* 1991). HPLC assays have also been used to identify 5-OHMeU as the 2'-deoxyribonucleoside 5-hydroxymethyl-2'-deoxyuridine by enzymatically digesting DNA previously labelled with tritiated thymidine (Frenkel *et al.* 1985). MS assays however may be preferable due to the ability to obtain structural information of the analyte. For GC/MS or HPLC/MS pentafluorobenzyl derivatives of 5-OHMeU have been prepared, but require much sample manipulation (Kresbach *et al.* 1989; Annan *et al.* 1989). The GC/MS methodology (trimethylsilylation of the hydrolysed base) has also been used by Dizdaroglu and Bergtold (1986) to quantitate low levels of damage to the base following exposure of DNA to hydroxyl radicals and hydrogen atoms produced by ionising radiation in N_2O-saturated aqueous solutions. Djuric *et al.* (1991a) have also used GC/MS methodology to quantitate 5-OHMeU as the acid hydrolysed base (5-OHMeU) and as the enzymatically hydrolysed deoxynucleoside (5-OHMedU) with the use of an isotopically labelled internal standard.

In this chapter the existing techniques of Dizdaroglu *et al.* and Djuric *et al.* were to be examined and modified in order to combine a sensitive and selective 5-OHMeU assay with that of the existing Tg assay. The combined assay would enable identification and quantitation of the two thymine derivatives with the use of isotopically labelled internal standards. The GC/MS SIR assay would then be used for *in vitro* DNA studies involving photoionisation and γ-irradiation of calf thymus DNA and *in vivo* studies examining the role of fibres and antioxidants in the oxidative damage process.

4.2. METHOD DEVELOPMENT

Given that GC/MS has not been extensively applied to the quantification of 5-OHMeU a study of experimental parameters (such as derivatisation) was carried out. The aim of the method development was to develop an assay for the quantitation of 5-OHMeU and if possible to combine it with the existing Tg assay. The GC/MS parameters are described in section 2.2.1.

4.2.1. Choice of derivatising agent.

Two different approaches were employed :

(i) use of the method of Djuric *et al.* (1991a) 1:1 BSTFA:acetonitrile at 130° C for 20 min.

(ii) use of the same derivatisation conditions as used for Tg i.e. 1:1 MTBSTFA:pyridine mixture at 60° C for 30 min (section 2.3.5.).

Complete reaction was expected to give the tris- derivative in either case.

For both (i) and (ii) the derivatising agent was present at a molar excess of 50, per labile hydrogen, to the amount of 5-OHMeU used which was 1 mg.

GC/MS analysis of the 5OHMeU / BSTFA mixture (i) yielded the fully derivatised base $(TMS)_3$-5-OHMeU with a molecular ion $M^{+\bullet}$ at *m/z* 358. However peak shape was poor and further attempts to optimise conditions were not pursued.

Analysis of the 5-OHMeU/MTBSTFA mixture (ii) yielded two peaks due to the bis- and tris- TBDMS derivatives with $[M-57]^+$ ions at *m/z* 313 and *m/z* 427 respectively. Peak shape was good, yielding symmetrical well defined peaks.

These initial experiments indicated that the TBDMS derivative was preferable, both in terms of chromatographic properties and the possibility of combining both Tg and 5-OHMeU assays.

4.2.2. Optimisation of derivatisation

A series of 5-OHMeU/MTBSTFA mixtures (25 ng/μl) were prepared as described in section 2.3.5. and allowed to react for 30, 60, and 90 minutes at 60° C in an attempt to fully derivatise to the $(TBDMS)_3$ -5-OHMeU.

After a 30 min derivatisation period two peaks were observed due to the bis TBDMS (*m/z* 313, peak retention time 13:05 min) which was ~ 15% of the tris TBDMS peak (*m/z* 427 peak retention time 14:38 min) obtained through complete derivatisation of all the active hydrogen groups. However, after 60 minutes the bis TBDMS (*m/z* 313) peak was ~ 5% of the tris TBDMS (*m/z* 427) and on further heating for 90 min derivatisation was complete (Figure 4.1.). It was considered sufficient to use a 60 min derivatisation time as it would ensure > 95% tris TBDMS derivative and avoid prolonged heating.

MS sensitivity of the derivatised base was evaluated in SIR mode using *m/z* 427 and *m/z* 432 channels. The latter ion was included so that the experiment would be comparable to that expected when monitoring the [M+5] 5-OHMeU internal standard.

Analysis of 500 pg/μl 5OHMeU (30 pg on column) gave a S/N ratio of 10:1 and good chromatographic peak shape. Preliminary analysis indicated that it was possible to detect standards down to 10 pg/μl.

4.2.3. Mass Spectrum of 5-OHMeU

Electron-ionisation (EI) mass spectra of TBDMS derivatives of modified DNA bases provide considerable structural detail that can be used for unequivocal identification. Figure 4.2 shows EI mass spectra for the unlabelled and [M+5] 5-OHMeU TBDMS derivatives. The major higher mass ion in the EI mass spectrum is the $[M-57]^+$ ion at *m/z* 427 for the unlabelled 5-OHMeU derivative (figure 4.2a). The molecular ion *m/z* 484 was observed but was of very low intensity, and consequently the *m/z* 427 ion was used for SIR. Ions at *m/z* 147, 75 and 73 were attributed to silyl fragments (Halket 1993). With the exception of the silyl fragments [M+5] 5-OHMeU produced the expected fragment ions at 5 *m/z* units higher than those observed for the unlabelled 5-OHMeU (figure 4.2b). The monitored channel for the internal standard was *m/z* 432.

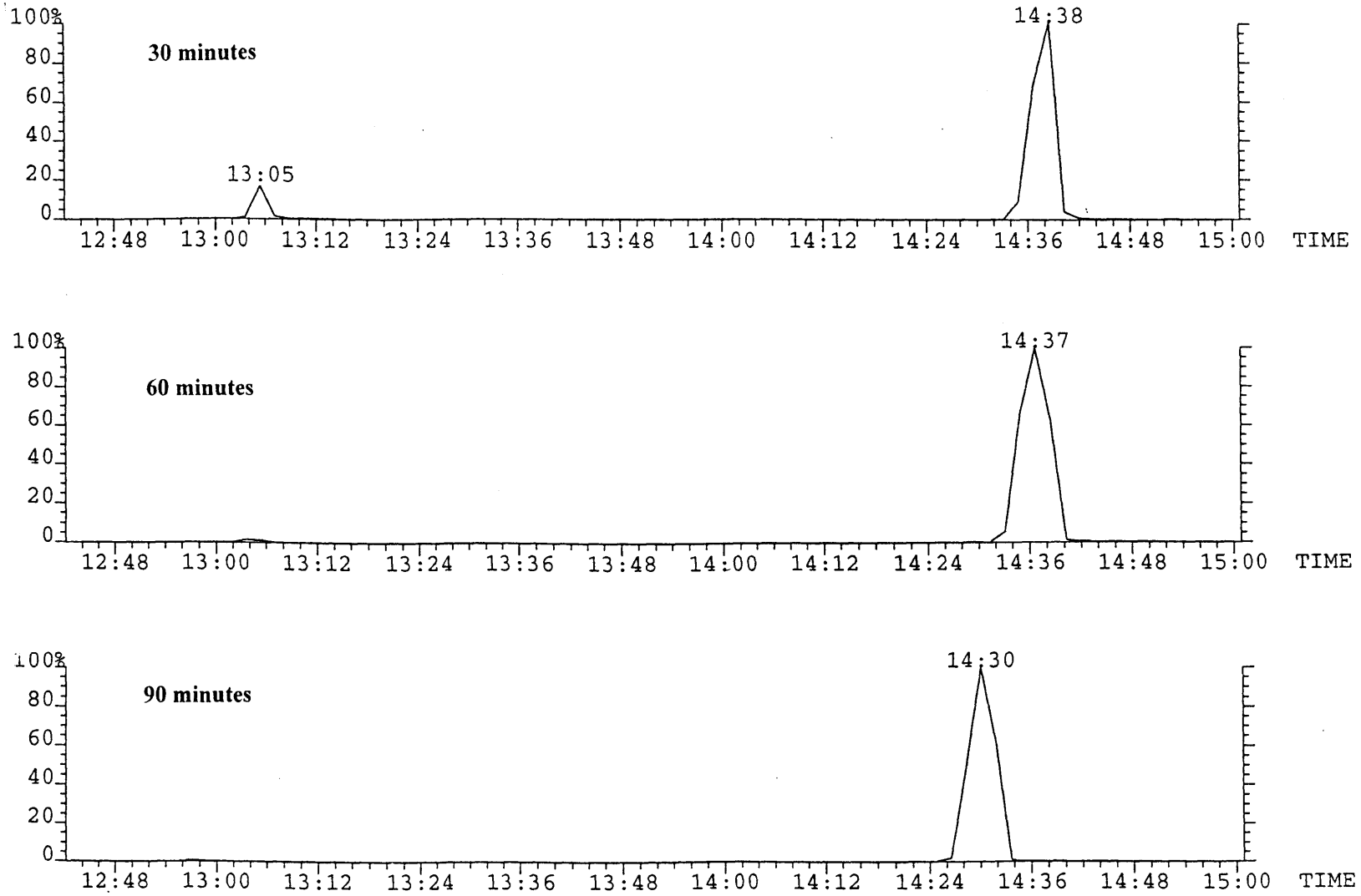


Figure 4.1. 5-OHMeU/MTBSTFA derivatisation for different time periods (the MS for all peaks were identical and the marginal difference in r.t. for the 90 minute reaction did not constitute a different product).

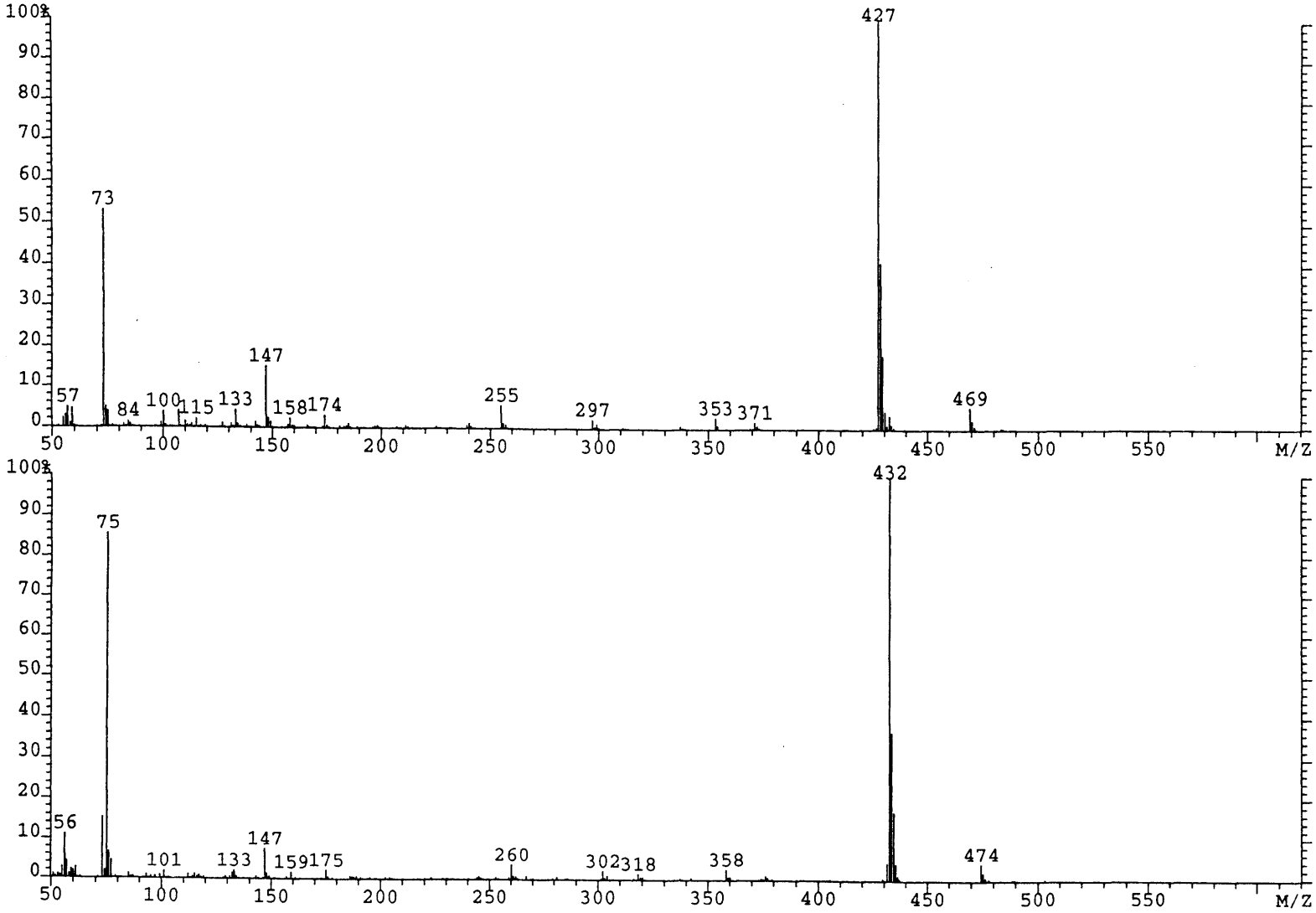


Figure 4.2. EI Mass spectra of tris-TBDMS. a) unlabelled 5-OHMeU b) [M+5] 5-OHMeU

4.2.4. Modification of the Tg derivatisation time and its implications.

In order to combine the Tg and 5-OHMeU assay it was necessary to ascertain the effect, if any, that a 60 min derivatisation period would have on the yield of derivatised Tg, through, for example, losses due to degradation.

A series of experiments were carried out in triplicate using a mixture of 40 μg each of 5-OHMeU and Tg which were derivatised using 1:1 MTBSTFA and pyridine for 30 mins and 60 mins. Mixtures of 40 μg of the internal standards were heated for 30 mins only; all samples were then dried and reconstituted in ethyl acetate before aliquots of the 30 and 60 minute samples were added to the internal standards mixture for quantitation and analysis by GC/MS SIR. Results were as shown in Table 4.1.

The results (table 4.1.) showed that there was no difference between the 30 min and 60 min derivatisation time and, therefore, it would be possible to use a 60 min reaction time when derivatising both Tg and 5-OHMeU.

Table 4.1. Modification of Tg derivatisation.

(a) 30 minute derivatisation of Tg.

Samples	Peak area *m/z* 331 (10^6)	Peak area *m/z* 334 (10^6)	Peak area ratio *m/z* 331/334
1a	2.38	2.14	1.11
1b	4.84	4.30	1.13
1c	3.67	3.30	1.11
2a	6.33	6.09	1.04
2b	4.96	4.66	1.06
2c	5.10	4.77	1.07
3a	4.10	3.44	1.19
3b	2.98	2.43	1.23
3c	5.50	4.36	1.26

(b) 60 minute derivatisation of Tg.

Samples	Peak area *m/z* 331 (10^6)	Peak area *m/z* 334 (10^6)	Peak area ratio *m/z* 331/334
4a	3.68	3.00	1.23
4b	4.42	3.57	1.24
4c	4.88	4.31	1.13
5a	3.96	3.94	1.01
5b	4.31	3.99	1.08
5c	4.96	4.90	1.01
6a	4.45	4.46	1.00
6b	3.56	3.20	1.11
6c	3.93	3.33	1.18

Tg average peak area ratio for 30 minute derivatisation reaction was 1.13 + 0.08 (7% CV).

Tg average peak area ratio for 60 minute derivatisation reaction was 1.11 + 0.09 (8% CV). **Note** a, b and c represent triplicate injections.

4.2.5. Assay Sensitivity

Once the derivatising agent and conditions had been chosen, attempts were made to set up a calibration line using calf thymus DNA. However these were unsuccessful, as the 5-OHMeU in the hydrolysed samples could not be detected. It was possible that this was due to losses resulting from the hydrolysis procedure as earlier derivatised standards which had been analysed previously, did not appear to have deteriorated. Djuric *et al.* (1991a) has reported 5-OHMeU was 60% degraded during DNA hydrolysis.

To test the hypothesis that degradation of 5-OHMeU occurred during hydrolysis two samples containing 1 ng and 2 ng 5-OHMeU were analysed and compared with two samples containing 1 ng and 2 ng 5-OHMeU combined with calf thymus DNA and subjected to the hydrolysis (section 2.3.4.). Following derivatisation (section 2.4.6.) samples were dried and reconstituted in ethyl acetate (20 µl).

Results showed that the 1 ng and 2 ng standards 5-OHMeU samples could barely be detected before the hydrolysis; detection after the hydrolysis proved impossible.

This was in apparent contrast to the sensitivity discussed in section 4.2.2.

The samples analysed in section 4.2.2. however were taken from a 'high concentration' derivatised standard sample (2 ng/µl) which had subsequently been diluted down to lower concentrations of 10- 50 pg/µl. The losses occurring due to adsorption effects are less significant with a higher concentration of derivatised material which is then diluted prior to analysis compared with a dilute solution which is derivatised and then analysed. The data indicated that adsorption was still a problem despite silylation of all glassware used.

It is also possible that injector cleanliness and column condition had deteriorated

In order to improve the sensitivity of the assay 2 modifications were examined

(i) Injecting in derivatising agent

(ii) Use of splitless mode

4.2.5.1. Introducing derivatising agent to the samples

Samples containing 1 ng and 2 ng of both unlabelled and [M+5] 5-OHMeU were derivatised as described earlier (section 2.4.6.). The samples were dried down in an evacuated centrifuge and then reconstituted in 5% derivatising agent MTBSTFA in ethyl acetate (20 μl).

Results showed a significant improvement in sensitivity. The peak area of the 1 ng 5-OHMeU in ethyl acetate was 3.66 x 10^5 (n=2) in comparison to the 1 ng 5-OHMeU sample in 5% derivatising agent which gave a peak area of 1.75 x 10^6 (n=2).

4.2.5.2. Splitless mode

A comparison of sensitivity between the use of injection in derivatising agent in split mode (split ratio 7:1) and, injection in ethyl acetate in split and splitless mode was also made. Two samples of 1 ng unlabelled 5-OHMeU were prepared one of which was reconstituted in 100% derivatising agent and the other which was reconstituted in ethyl acetate. Figure 4.3 shows examples of MS traces with each of the experimental modes and conditions

Table 4.2. lists peak areas of the samples.

Table 4.2. The effect of solvent and GC mode on 5-OHMeU signal.

Sample / analysis mode	Peak area *m/z* 427 (x 10^6) (n=2)	Relative ratio
Ethyl acetate / split	4.64	1
Ethyl acetate / splitless	35.25	8
Derivatising agent / split	177.5	38

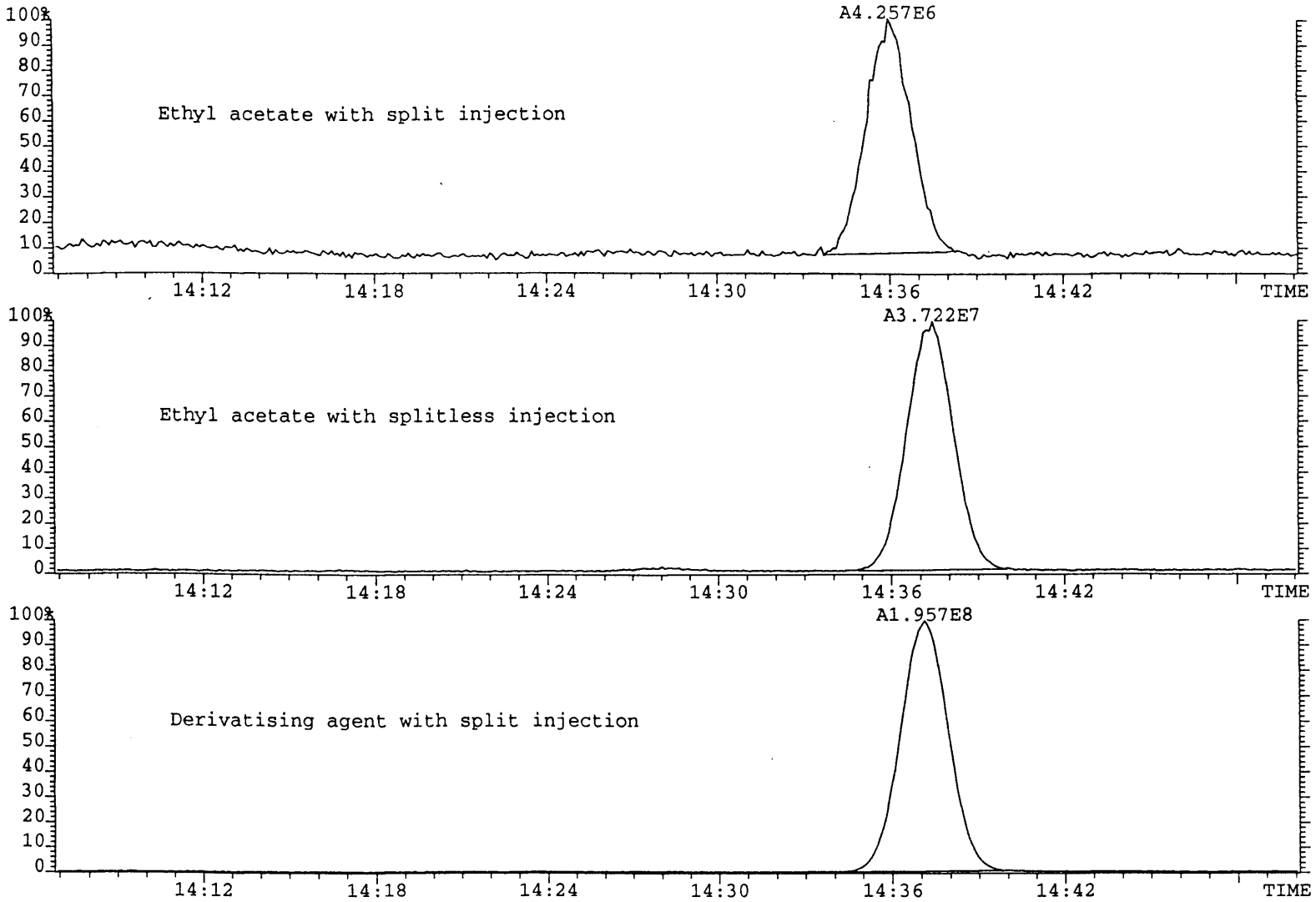


Figure 4.3. GC/MS SIR trace of 5-OHMeU *m/z* 427 analysis using different solvents and GC modes.

4.2.6. Effect of derivatising agent on *m/z* 427 background levels

The use of derivatising agent in the injection mixture however presented a further complication in that a *m/z* 427 ion could be detected in samples to which 5-OHMeU had not been added. Analysis of 1 ng [M+5] 5-OHMeU in 5% derivatising agent (20 µl) using SIR produced a signal in the *m/z* 427 channel at the same retention time as 5-OHMeU, giving a peak area ratio *m/z* 427/432 of 0.136 (n = 2).

Analysis of a 2 ng/µl [M+5] 5-OHMeU had been previously checked and showed isotopic enrichment to be greater than 99%. The sample was then diluted to 50 pg/µl and analysed in SIR in 1% and 5% derivatising agent in ethyl acetate; analyses of the two samples showed little or no difference in peak area ratio and were consistent with the full scan spectrum of the 2 ng/µl sample.

Table 4.3. The effect of derivatising agent concentration on ion intensity.

Sample/% derivatising agent	***m/z* 427 / 432** **n=2**
1 ng [M+5] 5-OHMeU in 1% derivatising agent (1 ml).	0.0024
1 ng [M+5] 5-OHMeU in 5% derivatising agent (1 ml).	0.0030

Analysis of a 2 ng/µl unlabelled and [M+5] 5-OHMeU sample was also carried out in the scan mode which gave peak area ratios *m/z* 427/432 of 1.4 and 1.6 (n = 2).

Peak area ratios did not seem to be consistent. Injection of derivatising agent alone also produced a response in the *m/z* 427 channel. In order to check the variability of this background, triplicate injections of the 5% derivatising agent in ethyl acetate were made in scan and SIR mode which showed that the *m/z* 427 ion originated from the column/injector or the derivatising agent. There was little variability between the three different chromatograms in scan and SIR mode.

It was possible that a deposit of 5-OHMeU had accumulated on the column or in the injection liner which was undergoing derivatisation on injection of MTBSTFA. The amount of derivatising agent was increased to 40% in an attempt to clear this background. An increase in signal in the *m/z* 427 channel was seen following several injections of the 40% derivatising mixture. Further injections of derivatising agent failed to reduce the signal in the *m/z* 427 channel.

Later attempts at analysing derivatising agent on another machine (VG 70 SEQ) showed the derivatising agent to be clean and no evidence of *m/z* 427 was seen in the derivatising agent. Measures were taken to clean the GC system which included repeated cleaning of the injector port, cleaning and silylating the glass liner, removal of the glass wool to reduce surface area for adsorption, using new seals and removing several lengths of column. Despite the measures taken, a 1 µl injection of derivatising agent in SIR showed a *m/z* 427 peak area of 2.71×10^5 (n=2) compared to a sample of 1 ng unlabelled 5-OHMeU giving a peak area of 6.64×10^7 (n=2).

A new column was installed in an attempt to reduce the apparent background level of 5-OHMeU but no difference was observed.

Injection of ethyl acetate did not produce a response and solvent blanks after injection of 50 pg/µl standards (ethyl acetate) were also clear.

Given that thorough cleaning and silylation of the injector port together with installation of a new column had been unsuccessful in eliminating the *m/z* 427 background on injection of derivatising agent, it was decided that ethyl acetate would be used instead as solvent, and a re-evaluation of the sensitivity made.

The source of the *m/z* 427 background was never resolved.

4.2.7. 5-OHMeU calibration line using standards in ethyl acetate (with split injection.)

In order to set up a 5-OHMeU assay the first step had to be to establish a calibration line with the use of standards. Calibration standards contained unlabelled 5-OHMeU (0-1.5 ng) to which was added [M+5] 5-OHMeU internal standard (1 ng). The samples were dried and derivatised as described (section 2.4.6.) before being reconstituted in ethyl acetate (20 µl). GC/MS SIR analysis using split mode produced the results shown in Figure 4.4.

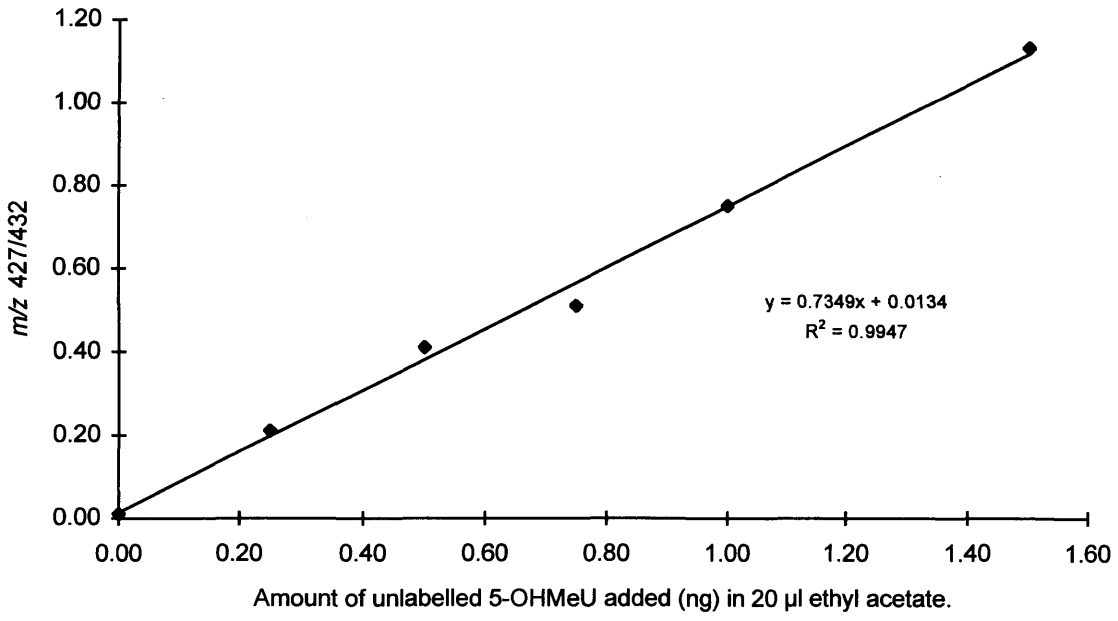


Figure 4.4. 5-OHMeU calibration line containing standards injected in ethyl acetate.

4.2.8. Calf thymus DNA calibration line

Following the establishment of a standard calibration line the approach described in section 4.2.7. was applied to set up a calf thymus DNA calibration line. Calibration samples were prepared using calf thymus DNA (75 μg), to which was added [M+5] 5-OHMeU internal standard (1 ng) and unlabelled 5-OHMeU (0-1.5 ng). All samples were hydrolysed and derivatised (section 2.3.4. and 2.4.6.) and reconstituted in ethyl acetate (20 μl). GC/MS SIR analysis was carried out in split and splitless mode; samples proved difficult to quantitate in both modes. This was in contrast to the successful analyses of calibration standards in section 4.2.7. This may have been due to losses during the DNA hydrolysis procedure. As quantitation was proving impossible in ethyl acetate a reassessment of the situation meant the analyses was repeated with the use of derivatising agent. The samples were subsequently dried in a evacuated centrifuge and reconstituted in 100% derivatising agent (20 μl) and the analyses were repeated in split and splitless mode. In splitless mode, peak shape was poor and peak fronting made quantitation difficult (Figure 4.5.a). Repeat analysis, however, in split injection mode gave considerably cleaner traces and symmetrical peaks which proved easier to quantitate (Figure 4.5.b). Results were as shown in Figure 4.6.

Comparison of the results (Figure 4.6) with the data in figure 4.4. showed an apparent background level. The major sources were believed to be 5-OHMeU already present in the calf thymus DNA and the column background described previously (section 4.2.6.).

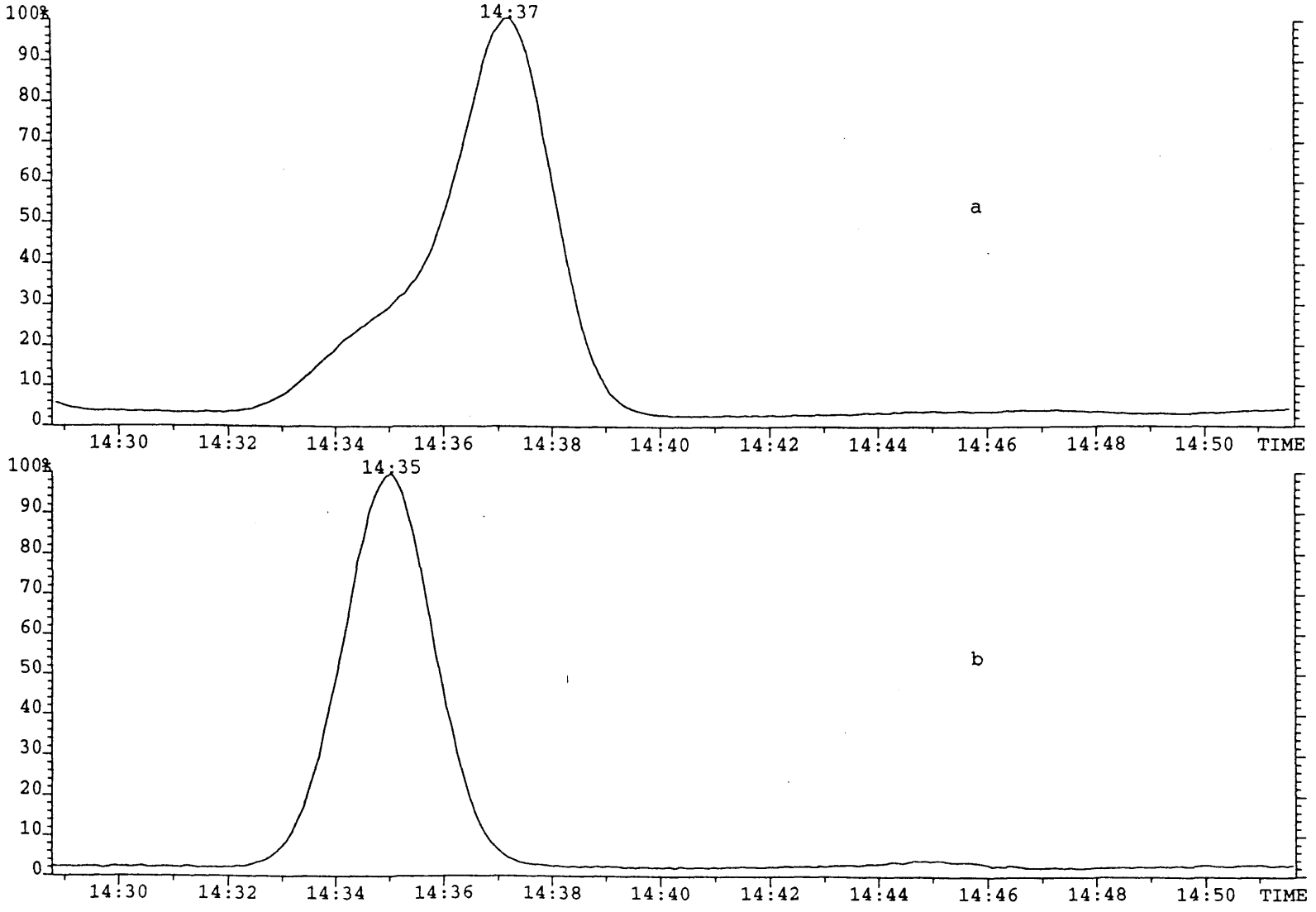


Figure 4.5. GC/MS SIR traces of *m/z* 427 channel in a) splitless and b) split mode in derivatising agent.

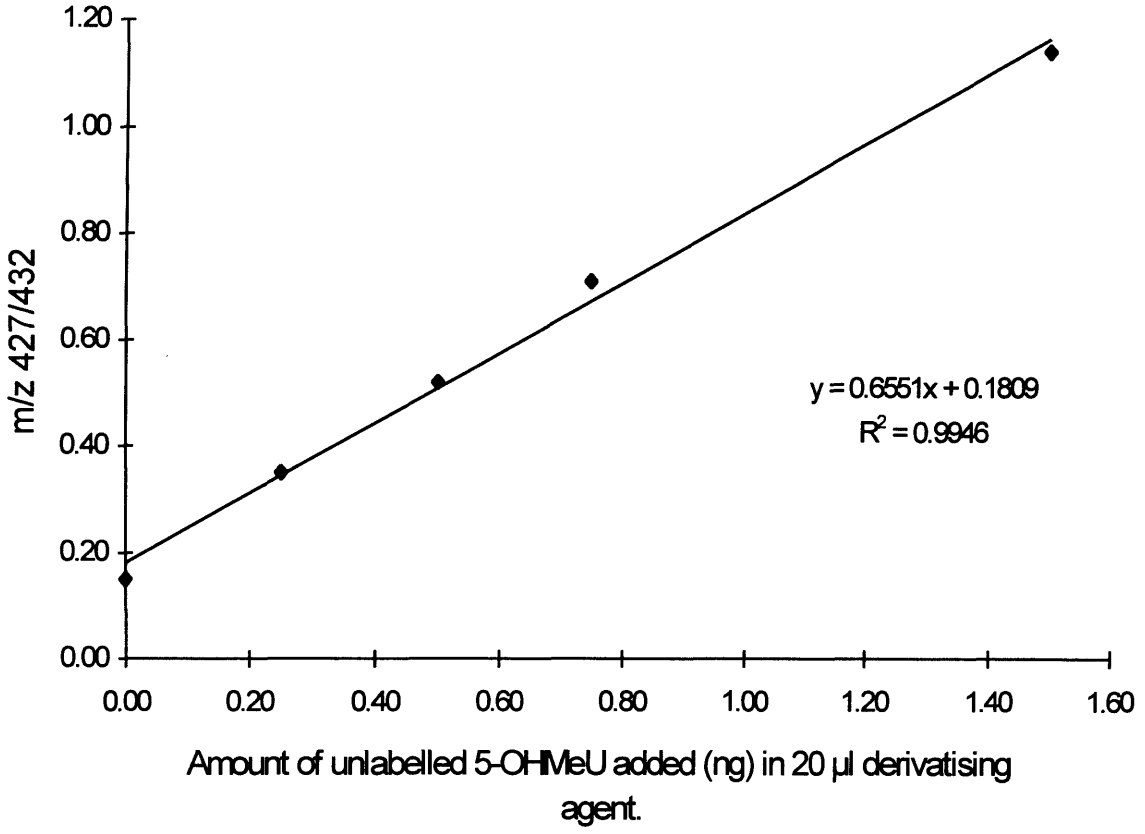


Figure 4.6. Calf thymus DNA calibration line in derivatising agent.

4.2.9. Calf thymus DNA calibration line using analyte internal standard to quantitate background from column.

As the analysis were being carried out in derivatising agent, it was necessary to determine what, if any, effect the *m/z* 427 background (section 4.2.6.) would have on the calibration line and attempt to quantitate it. In order to quantitate the background present from the column, analysis in triplicate of the 5-OHMeU [M+5] internal standard, which had been through the hydrolysis and derivatisation procedure, was performed.

Along with a sample containing [M+5] 5-OHMeU only, calibration line standards were also prepared using calf thymus DNA (100 μg), to which was added 5-OHMeU (0-1 ng) and [M+5] 5-OHMeU internal standard (1 ng). The samples were hydrolysed and derivatised (sections 2.3.4. and 2.4.6.) before being reconstituted in derivatising agent (25 μl). Analysis was carried out using GC/MS SIR.

The results are shown in Figure 4.7.

The [M+5] internal standard (1 ng/25 μl) was analysed in triplicate to quantitate background *m/z* 427 giving a mean peak area ratio (m/z 427/432) of 0.034 (n=3).

From the calibration line (y = 0.859000x + 0.06216) and peak area ratio data obtained for the [M+5] 5-OHMeU samples the *m/z* 427 background could be quantitated.

The total background was calculated to be 0.0724 ng / 100 μg DNA

The apparent background from the column was calculated, using the average of the [M+5] 5-OHMeU peak area ratios (y = 0.034) to be 0.0328 ng/25 μl. Calf thymus DNA was calculated by subtraction from the total to have a 5-OHMeU background of 0.0396 ng/100 μg calf thymus DNA, therefore corresponding to 0.396 ng/mg DNA.

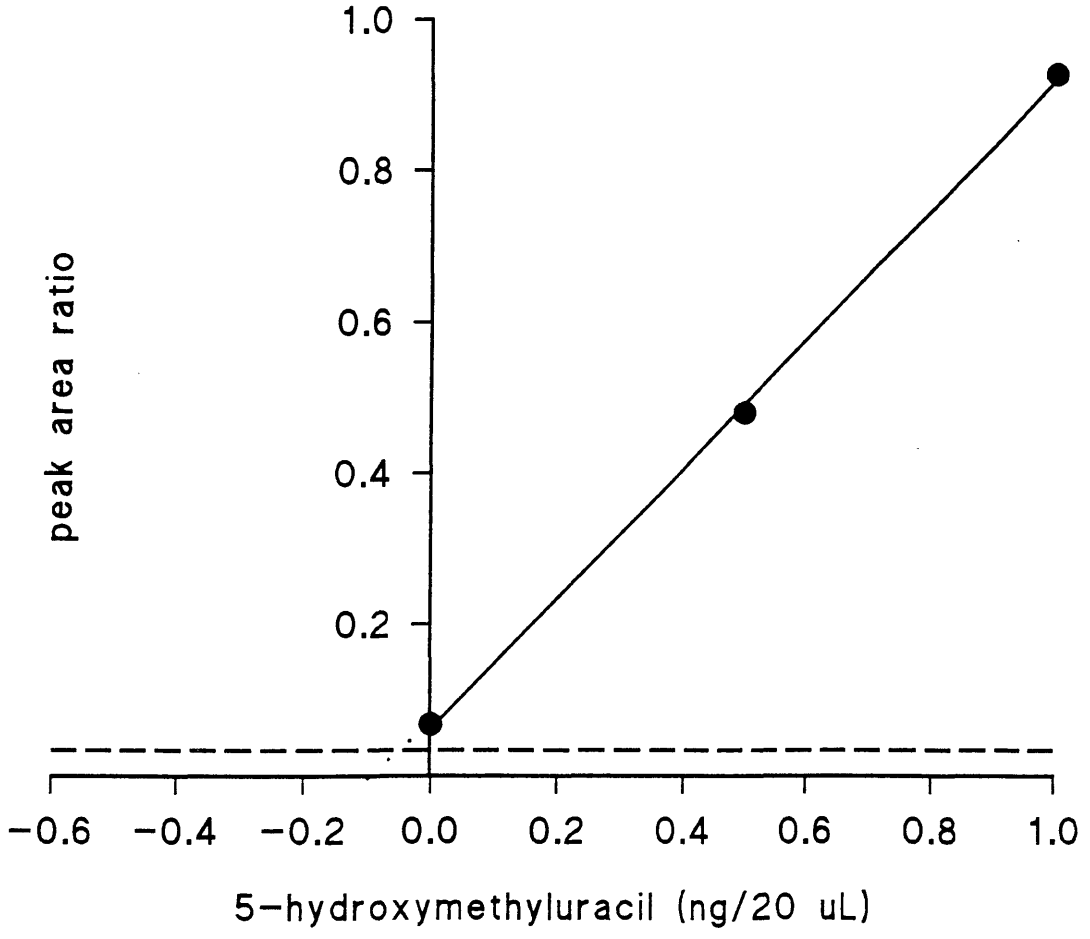


Figure 4.7. Calf thymus DNA calibration line demonstrating contribution from column and DNA background.

4.2.10. Tg and 5-OHMeU calibration line in derivatising agent.

Once linear calibration lines for the 5-OHMeU assay had been established with calf thymus DNA, a combined assay incorporating Tg was established with the use of standards.

Each sample contained [M+5] 5-OHMeU (1 ng) and [$^2H_3$] Tg (2 ng) internal standards and [$^2H_0$] Tg and unlabelled 5-OHMeU (0-1.5 ng). All samples were derivatised (section 2.4.6.) and reconstituted in 100% derivatising agent (20 µl).

Analysis was carried out using GC/MS SIR.

The calibration lines are shown in Figure 4.8. Figure 4.9. shows a MS trace for the Tg and 5-OHMeU (1 ng) combined assay. Retention times for the Tg and 5- OHMeU were 13:30 and 13:56 respectively.

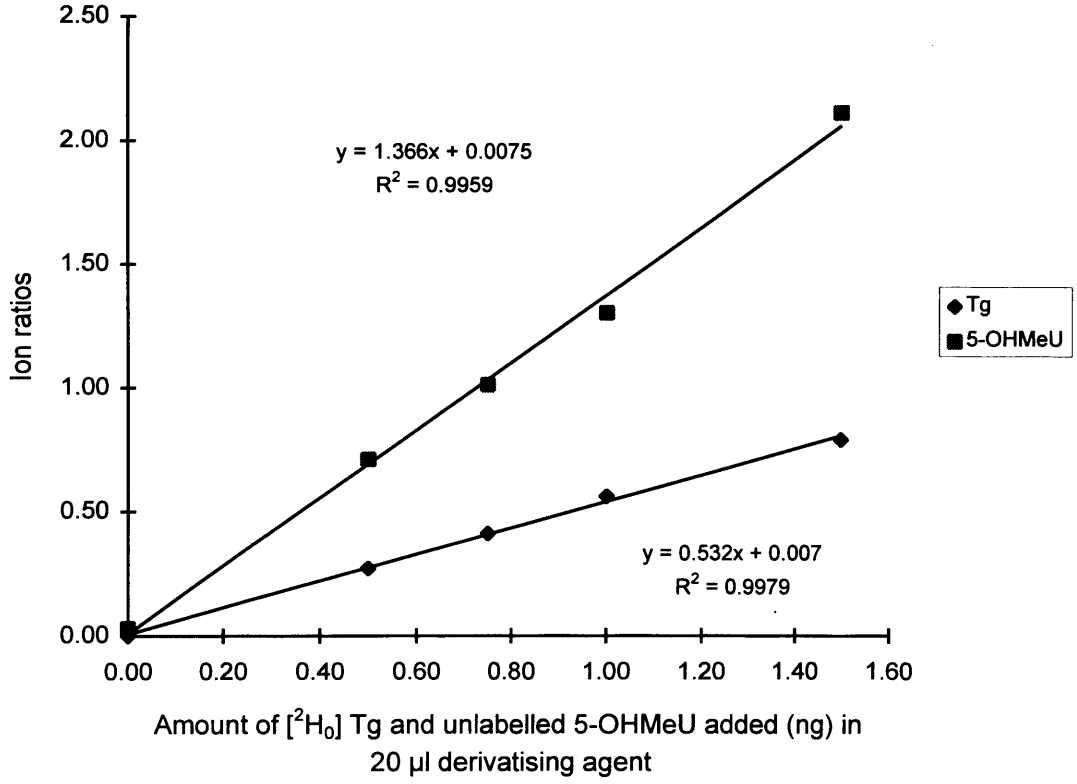


Figure 4.8. Combined standards calibration line for Tg and 5-OHMeU.

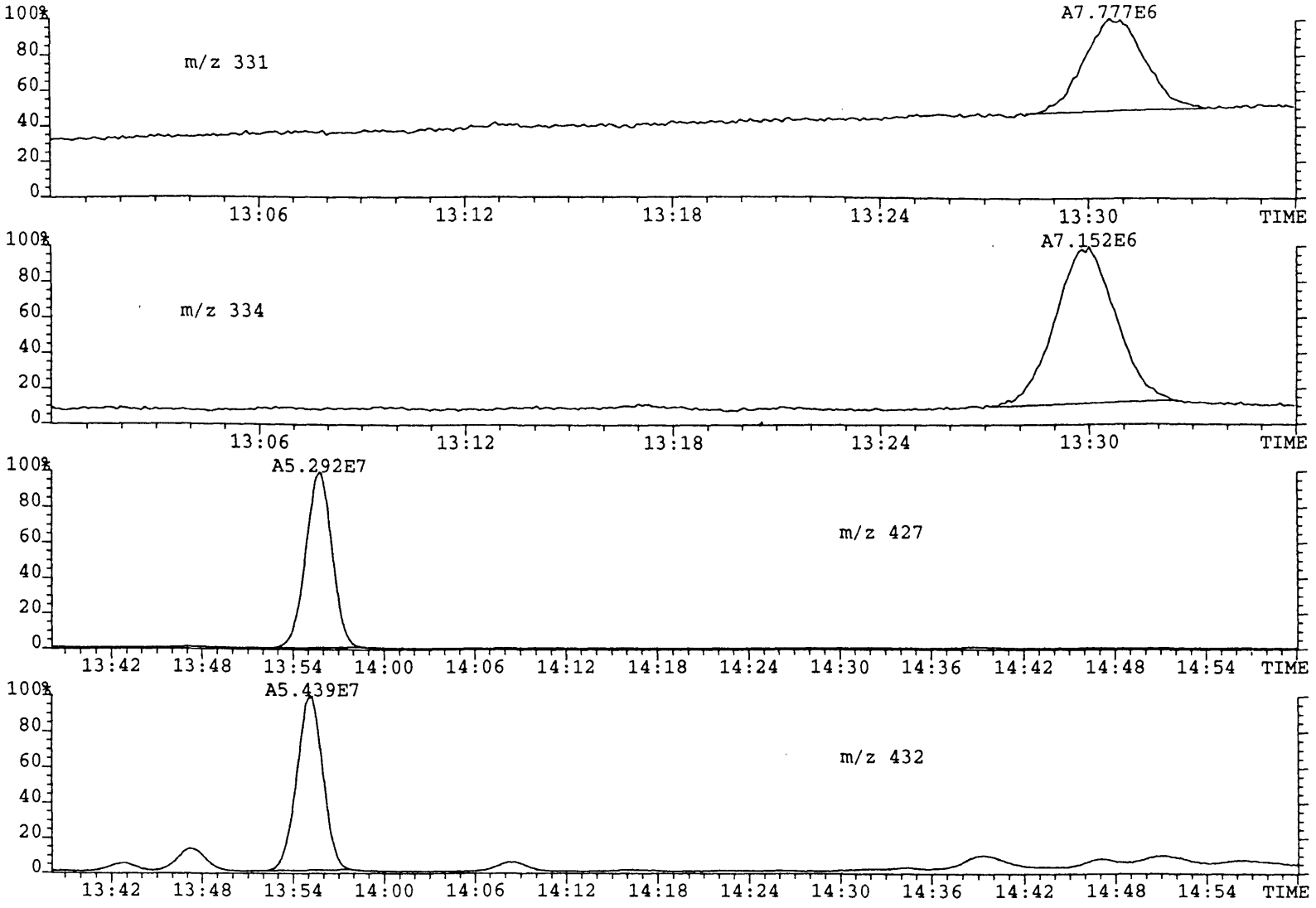


Figure 4.9. GC/MS SIR trace for the Tg (1 ng) and 5-OHMeU (1 ng) combined assay.

4.2.11. 5-OHMeU and Tg calibration lines using standards and DNA.

In this experiment a direct comparison was to be made between simultaneously prepared calibration lines derived from (i) standards alone (ii) standards and calf thymus DNA.

All samples for the standard calibration line (i) contained [$^2H_3$] Tg and [M+5] 5-OHMeU internal standards (1 ng) and [$^2H_0$] Tg and unlabelled 5-OHMeU (0-1 ng).

The samples for the DNA calibration line (ii) contained calf thymus DNA (50 μg), to which was added [$^2H_3$] Tg and [M+5] 5-OHMeU internal standard (1 ng) as well as [$^2H_0$] Tg and 5-OHMeU (0-1 ng). All samples (i) and (ii) were dried in an evacuated centrifuge. The standards samples (i) were subsequently derivatised (Section 2.4.6.) and then reconstituted in 100% derivatising agent (20 μl).

DNA samples (ii) were hydrolysed and derivatised (sections 2.3.4. and 2.4.6.) and reconstituted in 100% derivatising agent (20 μl). The analysis was carried out using GC/MS SIR.

While quantitation of Tg was possible as in section 4.2.10. the s/n ratio in the *m/z* 331 channel made quantitation difficult. Analysis of calibration samples such as the 0.5 ng sample was just about possible but lower biological levels would prove difficult. Figure 4.10 shows a calibration standard (0.5 ng) giving a s/n of 2:1 in the *m/z* 331 channel. Despite changing the column in an attempt to see if the s/n could be improved sufficiently to facilitate quantitation the s/n ratio did not improve. The MS evidence suggested that the Tg di-substituted derivative was being converted to the tri substituted derivative and that the derivatisation reaction was continuing in the presence of derivatising agent. It was thus proving difficult to run the Tg in derivatising agent, and it would be impossible to analyse 5-OHMeU in ethyl acetate. It was concluded that both assays would have to be done separately. Analysis of the DNA calibration standards (ii) was continued monitoring 5-OHMeU only.

Results were as shown in Figure 4.11.

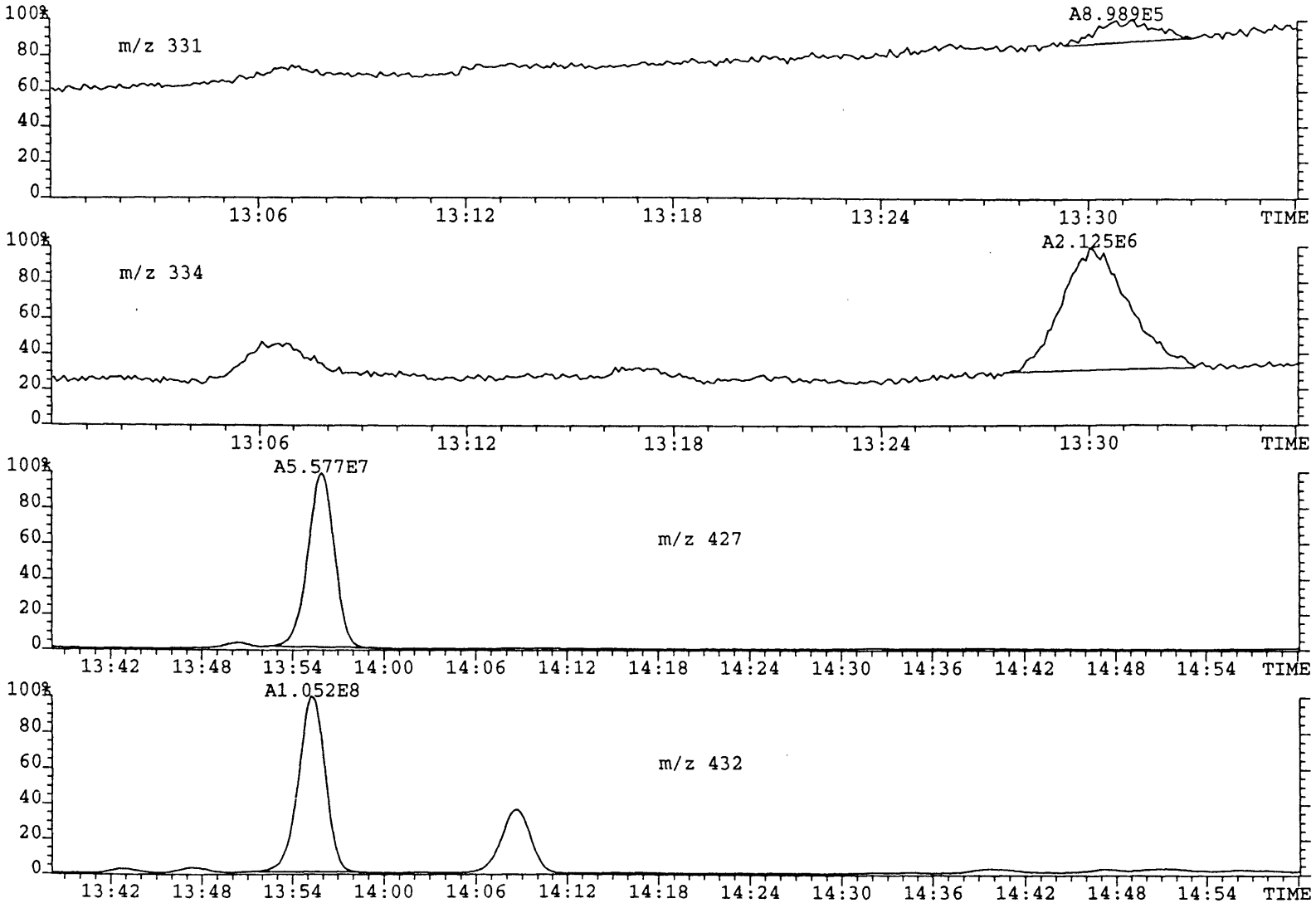


Figure 4.10. Calibration sample (0.5 ng) showing the difficulty encountered quantitating *m/z* 331.

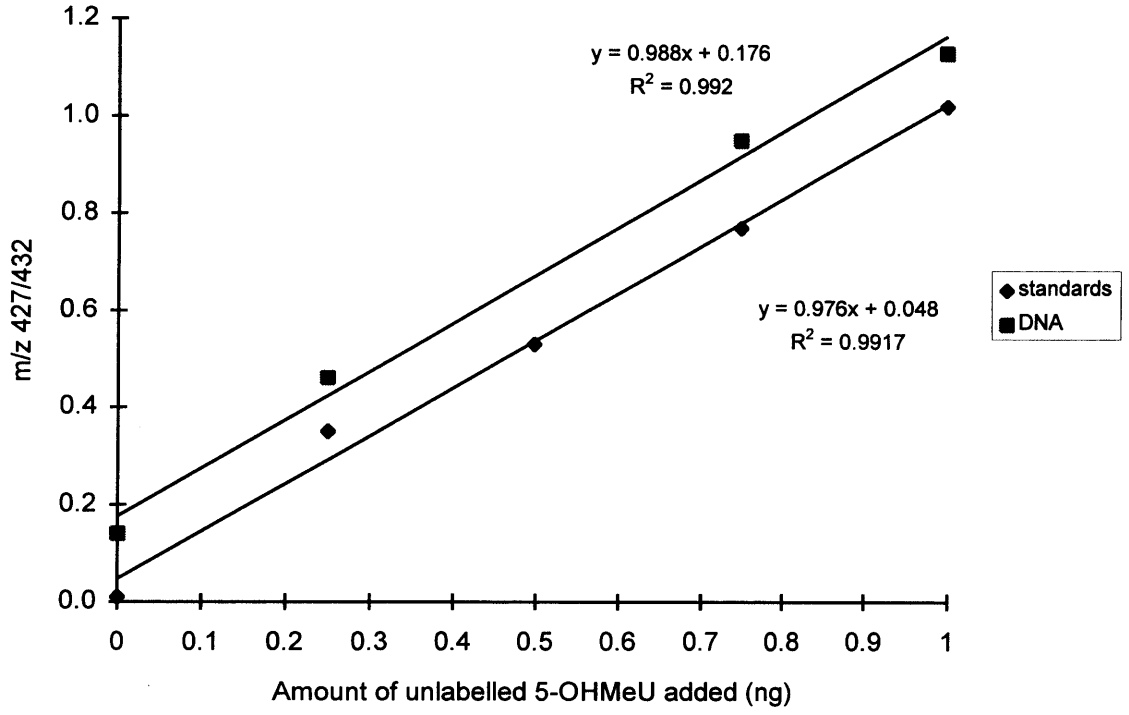


Figure 4.11. Comparison of 5-OHMeU calibration lines: standards vs DNA.

4.2.12. Summary

The conclusion from this work is that the only possible way forward with this assay would be to inject the samples in derivatising agent. Injection with ethyl acetate meant quantification was difficult as one was working with levels close to the limit of detection, and working in the splitless mode did not improve the signal significantly enough to enable quantification of samples after they had been through the hydrolysis stage. Quantification of *in vivo* samples would be impossible using injection with ethyl acetate. The use of the splitless mode also meant putting more contamination on the column and might therefore lead to poor peak shapes. For these reasons the optimal way forward would be to use the split mode with samples in derivatising agent . It was decided that with each analysis [M+5] 5-OHMeU would be used in triplicate to quantitate column background. After this a calibration line would be set up followed by the analysis of samples. The quantifying of samples would still be done in the same way i.e. by determining the amount of 5-OHMeU over background.

4.2.13. Discussion

Column background

The use of derivatising agent was considered necessary to obtain the sensitivity required for this assay. However, the result was a background *m/z* 427, the most likely source of which was accumulated deposits of 5-OHMeU which upon use of derivatising agent was being removed. The result of the *m/z* 427 background meant a 'false' signal from solvent blanks, in this case the MTBSTFA derivatising agent. The presence of this background meant quantitation of 'true' background levels of 5-OHMeU in DNA was difficult, as levels were elevated by a 'false' *m/z* 427 signal. Therefore any background calculated would not only include 'background' levels of 5-OHMeU in the DNA but also column background from the assay.

The only option available after unsuccessfully trying to eliminate this *m/z* 427 background was to quantitate it and work around it. The [M+5] 5-OHMeU internal standard was used in order to quantitate the *m/z* 427 from the background and then to subtract this from the zero value in any calibration line. This method was used successfully and enabled the quantitation of the column background and of DNA samples.

Calibration lines.

Despite the background *m/z* 427 signal, calibration lines were successfully established for standards with and without calf thymus DNA. A method of quantitation to overcome this problem was suggested in section 4.2.9. to enable quantitation of calf thymus DNA. Figure 4.12 shows a combined calibration plot of all the calf thymus DNA calibration lines in this section (n=3). Background levels of 5-OHMeU were calculated to be 0.550 ng/mg DNA using the approach described in section 4.2.9. These levels are comparable to the background levels of Tg quoted in the previous chapter. The contribution of 'background levels can be seen in Figure 4.11. in which standards and calf thymus DNA calibration samples prepared on the same day are compared eliminating any differences in work up procedure or volumetric errors. The differences in the two calibration lines is therefore a result of different levels of 5-OHMeU in the samples (standards and DNA). The 'background' levels of 5-OHMeU quoted in this

study are in agreement with 'background' 5-OHMeU levels in calf thymus DNA quoted by Spencer *et al.* (1994) using GC/MS.

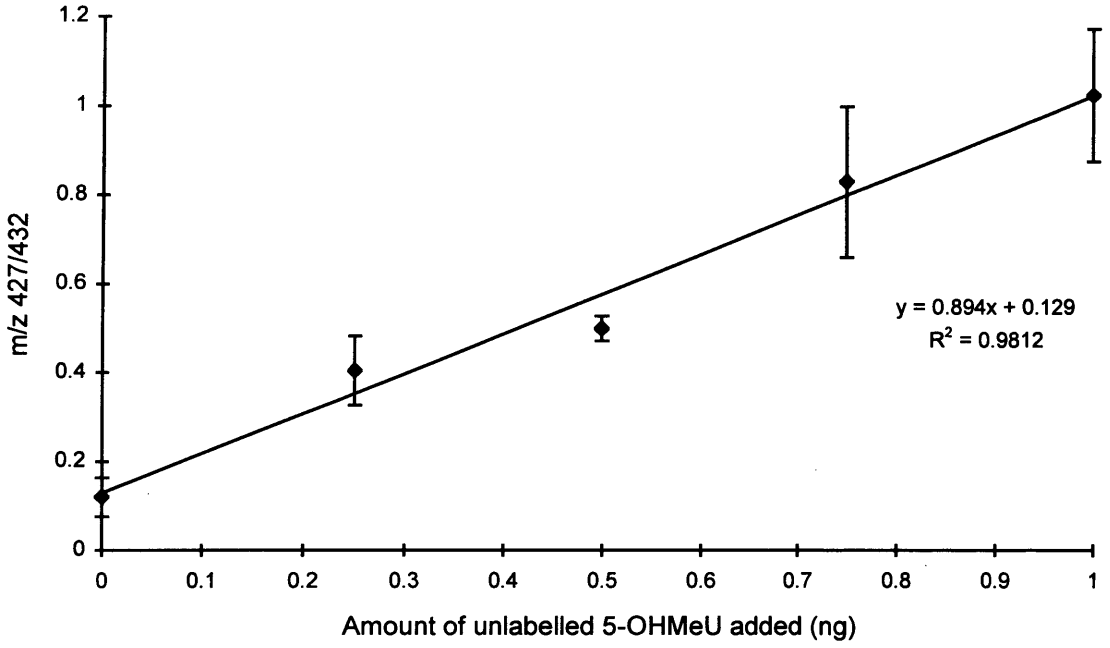


Figure 4.12. Combined calibration plot of all the calf thymus DNA calibration lines in this section (n=3).

4.3. APPLICATIONS

4.3.1. *In vitro* studies

4.3.1.1. Photoionisation of Thymidine 5'-Monophosphate (TMP)

The 5-OHMeU assay was used for *in vitro* studies before any attempts to quantitate samples from *in vivo* studies could be carried out. Here photoionisation of TMP was investigated in which the principal product formed was believed to be 5-OHMeU. Photolysis of the TMP with high intensity UV radiation (248 nm) is believed to result in the successive absorption of two quanta of light. Absorption of this light energy leads to excitation and photoionisation of the bases, leading to the formation of a radical cation and an electron (shown below) (Malone *et al.* 1993).

$$T \longrightarrow T^{+\bullet} + e^{-}$$

Under oxygenated conditions it is believed that the thymine radical cation reacts with oxygen to produce 5-OHMeU.

An aqueous solution of TMP (1mM) was photoionised (0-200 pulses/sec). An oxygenated system was created by bubbling O_2 through the TMP solution prior to photoionisation, and a crude attempt was also made to deoxygenate the TMP by bubbling N_2 through the solution for comparison purposes.

Deoxygenated TMP samples

Preliminary analysis had indicated high levels of 5-OHMeU in the TMP samples, therefore analysis of only the lowest photoionisation doses was carried out (i.e. 0, 1, 5, 10, 20, 40 pulses/sec). To aliquots of TMP (5 μg) was added [M+5] 5-OHMeU internal standard (1 ng).

Samples were hydrolysed and derivatised (Sections 2.3.4. and 2.4.6.) before being reconstituted in 5% derivatising agent in ethyl acetate (20 μl). Analyses were carried out using GC/MS SIR. Results were as shown in Figure 4.13.

Results

Actual 5-OHMeU levels were not calculated, however from the ion ratios it can be seen that there was an increase in 5-OHMeU levels with dose.

The presence of high levels of 5-OHMeU in the deoxygenated TMP system suggest that the solution was not sufficiently deoxygenated, with enough oxygen being present to lead to the production of 5-OHMeU.

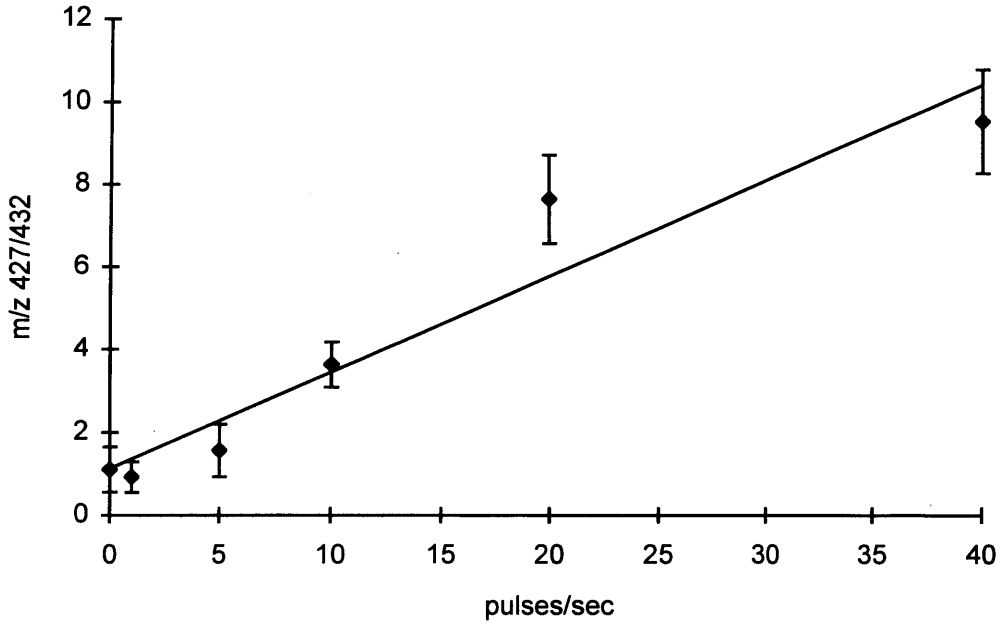


Figure 4.13. Peak area ratios of 5-OHMeU in deoxygenated photoionised solutions of TMP.

Oxygenated TMP samples

In oxygenated TMP samples the levels of 5-OHMeU were expected to be higher therefore the amount of TMP to be used was further reduced. To aliquots of TMP (2 μg) was added [M+5] 5-OHMeU. Calibration samples were prepared containing TMP (2 μg), unlabelled 5-OHMeU (0-2 ng) and [M+5] 5-OHMeU (1 ng) internal standard. The samples were hydrolysed and derivatised (Sections 2.3.4. and 2.4.6.) before being reconstituted in 5% derivatising agent in ethyl acetate (20 μl). Analyses were carried out using GC/MS SIR. Quantitation of actual 5-OHMeU levels (ng/mg TMP) in the photoionised TMP samples were calculated from the calibration line and are shown in Figure 4.14.

Results

From the calibration line constructed actual levels of Tg (ng/mg TMP) were calculated.

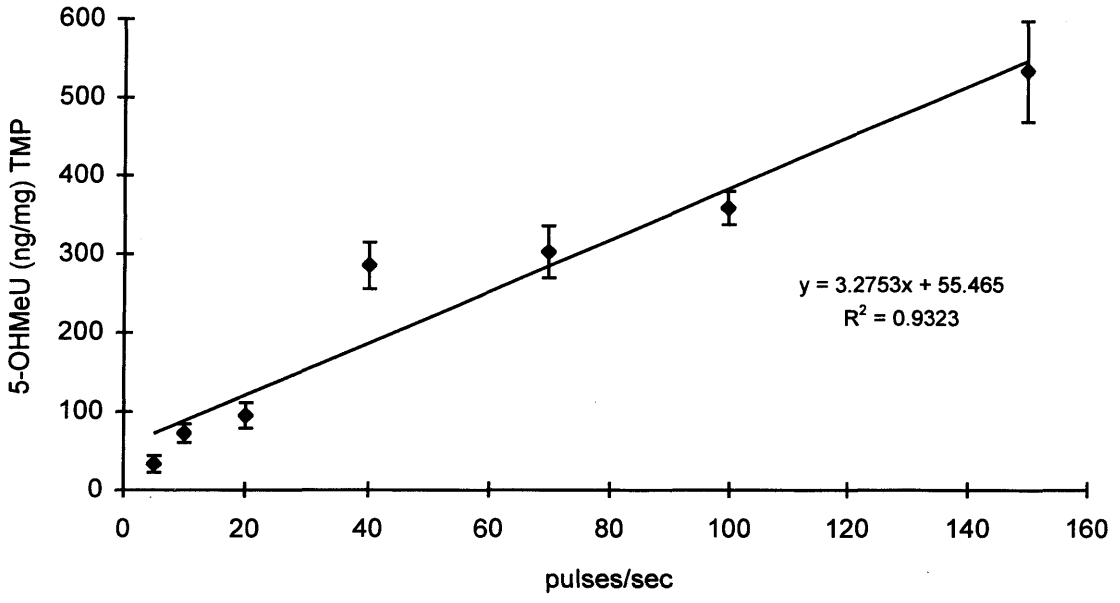


Figure 4.14. 5-OHMeU levels produced in photoionised TMP.

Analyses of the two sets of samples (oxygenated and deoxygenated) suggest increased formation of 5-OHMeU with increasing laser pulses. This is in agreement with other observations of a linear increase in oxidative damage as measured by 8-OHdG (Cullis *et al.* 1996) at low doses of photoionisation. At higher levels 5-OHMeU production would be expected to plateau due to destruction of the product and/or high concentrations leading to other pathways. Actual levels of 5-OHMeU in the deoxygenated samples was not calculated, however from the peak area ratios it appears that the levels were the same or greater than for the oxygenated TMP. A possible explanation would be the crude attempt at removing the oxygen.

4.3.1.2. ^{60}Co γ-irradiated DNA

Aliquots (1 ml) of a solution of calf thymus DNA in deionised water (1 mg/ml) were irradiated in a ^{60}Co-γ source at a dose rate of 1.32 kGy/hr; the total dose ranged from 0-100 Gy. In order to quantitate 5-OHMeU levels in the DNA [M+5] 5-OHMeU (1 ng) was added to each sample. Calibration line samples were also prepared containing calf thymus DNA (50 μg), [M+5] 5-OHMeU (1 ng) and [$^2H_0$] 5-OHMeU (0-2 ng).

All samples were hydrolysed and derivatised (Sections 2.3.4. and 2.4.6.) before being reconstituted in 5% derivatising agent (20 μl). Analysis were carried out using GC/MS SIR.

Results and Discussion

5-OHMeU levels in ^{60}Co γ-irradiated DNA are presented in Table 4.4.

Table 4.4. Levels of 5-OHMeU in ^{60}Co γ-irradiated DNA.

Irradiation (Dose Gy)	5-OHMeU (ng/mg DNA)
0	0.005
20	3.16
40	3.95
60	3.51
80	4.84
100	6.45

5-OHMeU is considered to be a possible quantitative marker of exposure of DNA to ionising radiation (Frenkel *et al.* 1985). This marker of oxidative damage has been characterised and more recently quantitated in DNA exposed to ionising radiation (Frenkel *et al.* 1985, Dizdaroglu and Bergtold 1986, Douki *et al.* 1996). It should be noted that most of the early quantitative work however was carried out without the use of isotopically labelled internal standards.

The levels of 5-OHMeU presented are in agreement with those obtained by Nackerdien *et al.* (1992) also using the technique of GC/MS SIR. At 100 Gy the level of 6.45 ng/mg DNA (1.45 5-OHMeU/10^5) bases is in agreement with the Nackerdien *et al.* (1992) value of 1.89 5-OHMeU/10^5 bases at 116 Gy. Also in agreement with Nackerdien *et al.* (1992) 5-OHMeU levels were found to be approximately a factor of 3 lower than Tg levels (refer to section 3.3.1.3. for Tg levels).

4.3.2. *In vivo* studies.

4.3.2.1. The role of antioxidants in preventing oxidative DNA damage as measured by 5-OHMeU

Placental DNA samples which had previously been examined for Tg (section 3.3.2) were to be analysed for 5-OHMeU in order to compare the levels of the two markers of oxidative damage and observe their relationship if any.

Prior to assaying the placental samples, the calibration method was evaluated. Calibration standards were prepared using calf thymus DNA (70 μg) to which was added unlabelled 5-OHMeU (0-1 ng), together with the [M+5] 5-OHMeU (1 ng) internal standard. Samples were hydrolysed and derivatised (Sections 2.3.4. and 2.4.6.) before being reconstituted in 5% derivatising agent (20 μl). Analyses was carried out in triplicate using GC/MS SIR. Results were as shown in Figure 4.15.

Having demonstrated that the GC/MS SIR assay could be used to quantitate biologically relevant levels of 5-OHMeU in a DNA matrix the method was applied to placental DNA samples.

Analyses of 5-OHMeU in antioxidant treated and control subjects (n=9) DNA was carried out by adding [M+5] 5-OHMeU (1 ng) internal standard to placental DNA (70 μg). Calibration standards and a 5-OHMeU internal standard only sample were also prepared for the quantitation of the levels of 5-OHMeU in the placental DNA and column respectively. Samples were dried in an evacuated centrifuge before being hydrolysed and derivatised (Sections 2.3.4 and 2.4.6.) and reconstituted in 5% derivatising agent (20 μl).

Analyses were carried out using GC/MS SIR because MRM was not possible with analyses involving $(TBDMS)_3$ 5-OHMeU as MS/MS of the *m/z* 427 ion did not give a suitable product ion.

Results

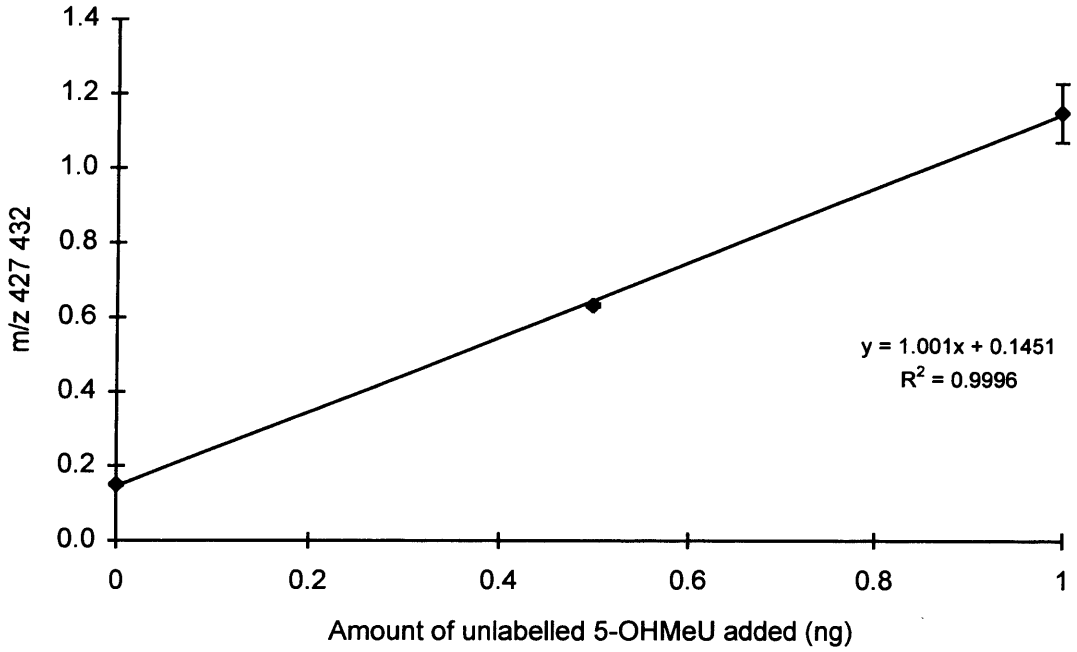


Figure 4.15. DNA calibration line.

Quantitation of placental DNA samples was carried out as previously described in section 3.3.2.2. Figure 4.16. shows a MS trace of one of the antioxidant supplemented placental DNA samples.

Levels of 5-OHMeU in the placental DNA were as shown in figure 4.17.

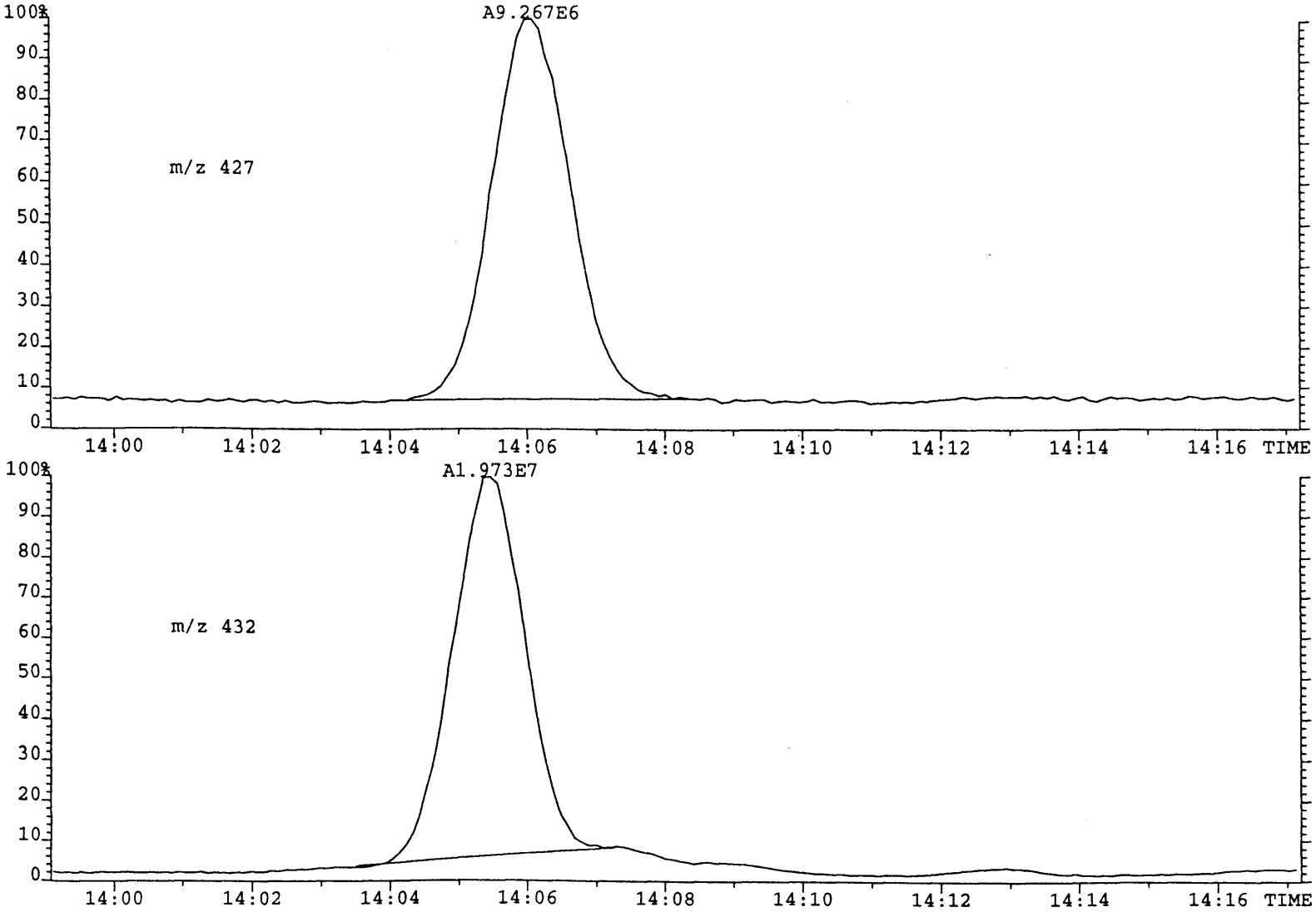


Figure 4.16. GC/MS SIR trace of a of an antioxidant supplemented placental DNA sample.

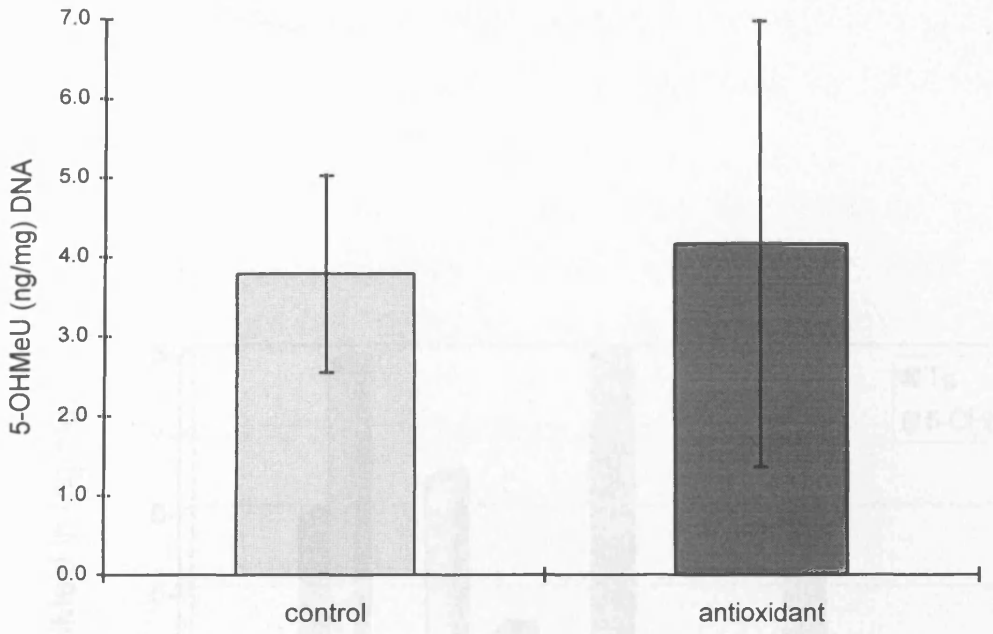


Figure 4.17. Mean levels of 5-OHMeU (ng/mg) DNA in antioxidant treated and control samples.

Application of a two sample t-test indicated that there was no significant difference between the control (n=4) and antioxidant (n=5) groups (P = 0.81) which had mean levels of 5-OHMeU of 3.79 ± 1.24 ng/mg DNA and 4.17 ± 2.81 ng/mg DNA respectively.

Figure 4.18. compares the levels of Tg and 5-OHMeU in placental samples. It can be seen that no particular correlation between the two biomarkers was seen, unlike for the irradiation *in vitro* study. Possible explanations may include the fact that even though the two lesions are structurally similar, *in vivo* two completely separate repair pathways exist for their repair (section 1.2.4.3.). Repair for example could have taken place at different rates and with different efficiencies (See also Chapter 6 for further comparisons of Tg, 5-OHMeU and 8-OHG in placental DNA samples).

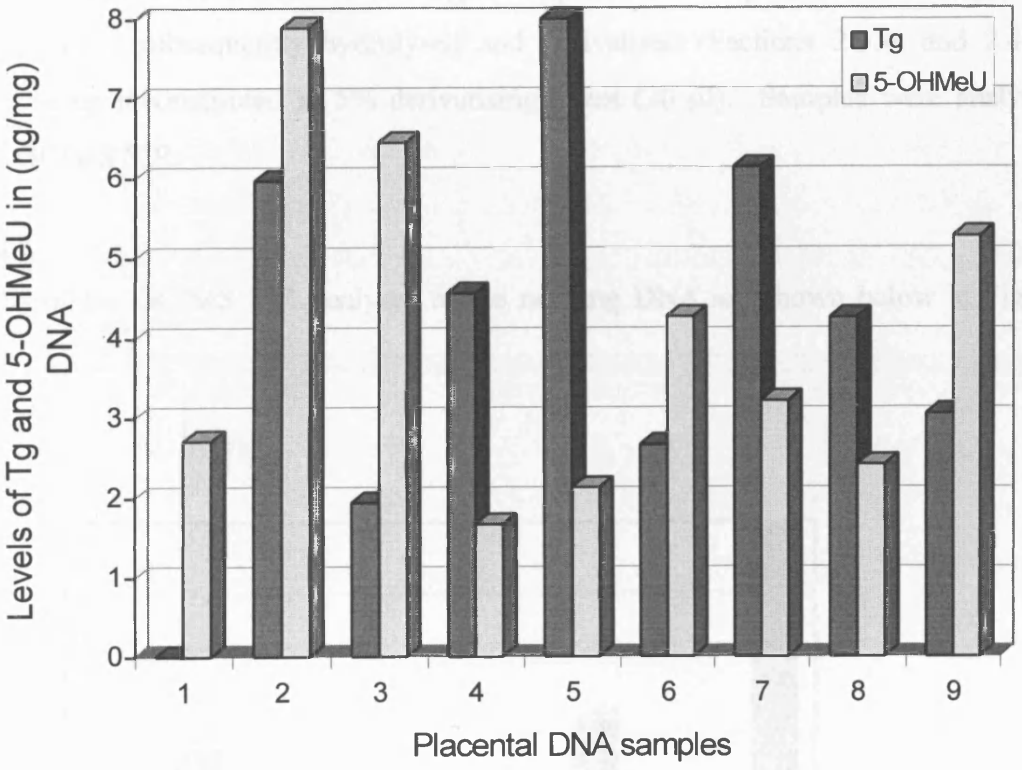


Figure 4.18. Comparison of Tg and 5-OHMeU levels in the same placental DNA samples.

4.3.2.2. Effect of crocidolite on oxidative DNA damage as measured by 5-OHMeU

Rat lung DNA exposed to crocidolite (see section 3.3.2.2. for full details and Tg analysis) was analysed for 5-OHMeU.

Analysis of rat lung DNA was carried out by adding [M+5] 5-OHMeU (1 ng) to the DNA (70 μg). Calibration samples were also prepared by adding [M+5] 5-OHMeU (1 ng) and unlabelled 5-OHMeU (0-1 ng) to aliquots of calf thymus DNA (70 μg). All samples were subsequently hydrolysed and derivatised (Sections 2.3.4. and 2.4.6.) before being reconstituted in 5% derivatising agent (20 μl). Samples were analysed using GC/MS SIR.

Results

Results of the GC/MS SIR analyses of the rat lung DNA are shown below in Figure 4.19.

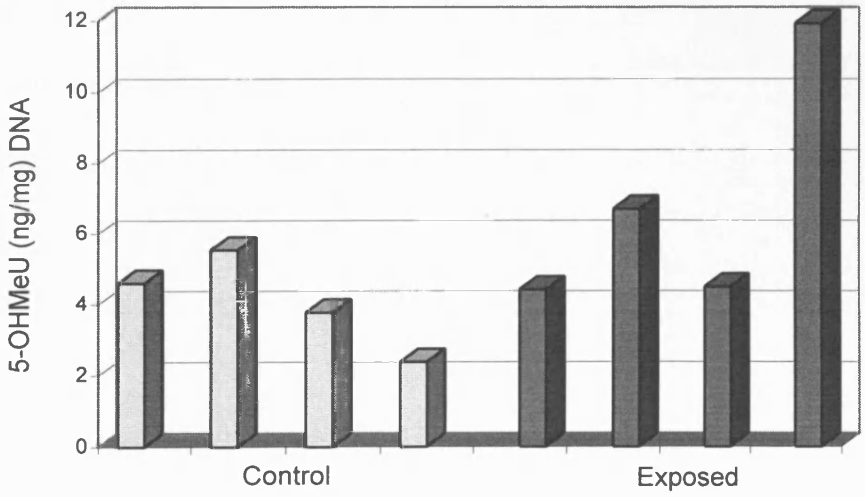


Figure 4.19. 5-OHMeU levels (ng/mg DNA) in crocidolite exposed and control rats.

Application of a one way analysis of variance showed that there was not a significant difference between the control and crocidolite exposed group (P = 0.186) which gave mean 5-OHMeU levels of 4.08 ± 1.34 and 6.89 ± 3.52 ng/mg DNA respectively. Unlike with Tg significantly elevated levels of 5-OHMeU were not observed in the

control group, thus demonstrating the importance of monitoring multiple markers as a measure of oxidative damage.

4.4. DISCUSSION

The assay of 5-OHMeU described in this chapter has sufficient sensitivity to detect down to femtogram levels, making it more sensitive than the Tg assay. Complete derivatisation of the unlabelled 5-OHMeU was achieved using the TBDMS derivatives. As a result a mass spectrum dominated by a intense $[M-57]^+$ was achieved with no other interfering ions. The tris-TBDMS-5-OHMeU yielded well defined symmetrical peaks which even at femtogram levels gave a good s/n ratio. The assay was successfully used to quantitate exogenous and endogenous levels of 5-OHMeU without struggling for sensitivity and signal.

Unfortunately it was not possible to combine the assay with the Tg assay but it is hoped that this is only a matter of further time. Combination of such a sensitive assay for two thymine markers of oxidative damage should be the aim of any future work in this area.

Chapter 5.

8-Hydroxyguanine

5.1. INTRODUCTION

The most studied oxidative DNA base modification is 8-OHG, the C-8 hydroxylation product of guanine. It is believed that 1/100,000 guanine residues in human cellular DNA are oxidised at C-8 (Fraga *et al.* 1990). For reaction of C8-OH adduct radicals refer to section 1.2.3.1.

8-OHG is formed in DNA treated with a variety of reducing agents (Kasai and Nishimura 1984), methylene blue plus light (Floyd *et al.* 1989) and other ROS generating systems such as ionising radiation (Wilson *et al.* 1993). *In vivo,* levels of 8-OHG have been quantitated in urine as a product of repair and in DNA extracted from cells. *In vivo* studies show the amount of 8-OHG in cellular DNA is higher in animals with a higher basal metabolic rate (Shigenaga *et al.* 1989) and is higher in mitochondrial DNA than in nuclear DNA (Richter *et al.* 1988). A comparative study carried out in rats studying 8-OHdG levels in DNA and urine found 8-OHdG to increase with age in DNA and decrease with age in urine reflecting decreased repair (Fraga *et al.* 1990). In relation to disease, elevated levels of 8-OHG have been reported in breast, lung, colon, stomach, ovary and brain human cancer tissue as measured by GC/MS (Malins and Haimanot 1991; Jaruga *et al.* 1994; Olinski *et al.* 1992).

8-OHG is believed to be highly mutagenic due to its ability to hydrogen bond with bases other than cytosine (Shibutani *et al.* 1991). The importance of 8-OHG repair is demonstrated by the existence of mechanisms of cellular repair of this lesion in mammalian and bacterial cells (Tchou *et al.* 1991; Tchou and Grollman 1993).

The 8-OHG lesion is most frequently studied as the oxidised deoxynucleoside 8-OHdG using HPLC with ECD. In this procedure DNA is enzymatically digested to yield deoxynucleosides, these are subsequently separated by reversed phase HPLC. 8-OHG has also been studied by a variety of other methods including GC/MS (Dizdaroglu 1994; Hamberg and Zhang 1995), PPL (Lutgerink *et al.* 1992; Devanoboyina and Gupta 1996; Podmore *et al.* 1997) and immunochemical methods (Degan *et al.* 1991; Park *et al.* 1992; Musarrat and Wani 1994; Yin *et al.* 1995).

The analytical results reported by the different methods however show considerable inconsistencies (for review see Halliwell and Dizdaroglu 1992). For example in calf thymus DNA 'background' levels of 8-320 8-OHdG/10^6 bases have been quoted using HPLC ECD (Floyd *et al.* 1989). In comparison using the technique of GC/MS levels of 8-OHG quoted have been 159-318 8-OHG/10^6 bases (Aruoma *et al.* 1989a, b; Halliwell and Dizdaroglu 1992). Possible explanations for such discrepancies may include incomplete enzymatic hydrolysis in the presence of oxidatively modified DNA for the HPLC analyses, or artefact formation during formic acid hydrolysis and/or derivatisation for GC/MS analyses. A comparative study by Ravanat *et al.* (1995) of GC/MS and HPLC from the same laboratory using the same sample showed that GC/MS levels of 8-OHG were approximately 50-fold higher than those obtained by HPLC ECD. In this study artefactual formation of 8-OHG from guanine was demonstrated in the derivatisation step (Ravanat *et al.* 1995). Although artefactual production of 8-OHG is possible during DNA hydrolysis, this possibility has been investigated and found to be unlikely (Nackerdien *et al.* 1992; Halliwell and Dizdaroglu 1992).

In order to account for some of these methodological variables an assay for 8-OHG using HPLC ECD is presented.

5.2. METHOD DEVELOPMENT

Here a novel procedure for the quantitation of 8-OHG using HPLC ECD was to be developed. Although the quantitation of 8-OHG in the presence of high levels of guanine proved difficult using conventional reverse phase HPLC, a simple incubation of DNA hydrolysates with guanase allowed such analyses. This assay was to combine features of the GC/MS and HPLC ECD techniques. Enzymatic DNA digestion was to be substituted for formic acid hydrolysis and the requirement for derivatisation was eliminated using HPLC ECD.

HPLC instrumentation and methods used were as described in Materials and Methods (section 2.2.2.)

5.2.1. Analysis of 8-OHG in the presence of excess guanine.

With the use of standards the separation of 8-OHG from the other DNA bases was successfully achieved using the conditions outlined in section 2.2.2. However when a excess of guanine was used as expected in a DNA sample the 8-OHG was not resolved from the guanine. Figure 5.1 shows an example of the difficulty in quantitating 8-OHG when a 10 fold molar excess of guanine is used. Analysis of 8-OHG using ECD was therefore impossible due to interference from guanine which is also electrochemically active.

A possible solution would be to remove the excess guanine without affecting the 8-OHG. The reaction catalysed by guanase (guanine deaminase) was therefore investigated using HPLC ECD and UV. Diode array detection was used to confirm that the deamination of guanine by guanase led to the production of xanthine (Figure 5.2.). This was shown by the similar r.t. and by superimposing a xanthine spectrum upon the guanase product peak (Figure 5.3.). Therefore as a result of guanine incubation with guanase at 37°C for 1 hour guanine was deaminated to xanthine and the 8-OHG was sufficiently separated from the xanthine for analysis purposes.

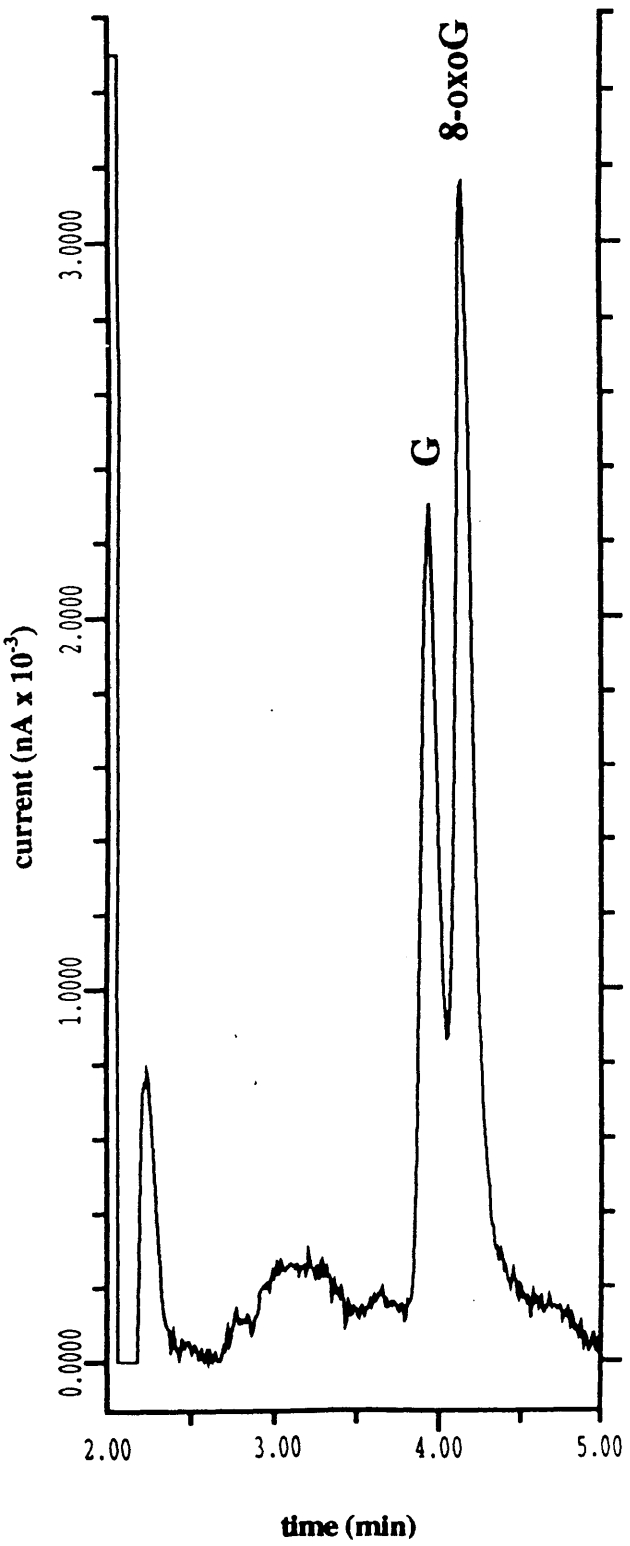


Figure 5.1. Reversed-phase HPLC analysis using ECD at + 600 mV of a solution containing standards of guanine (500 nM) and 8-OHG (40 nM).

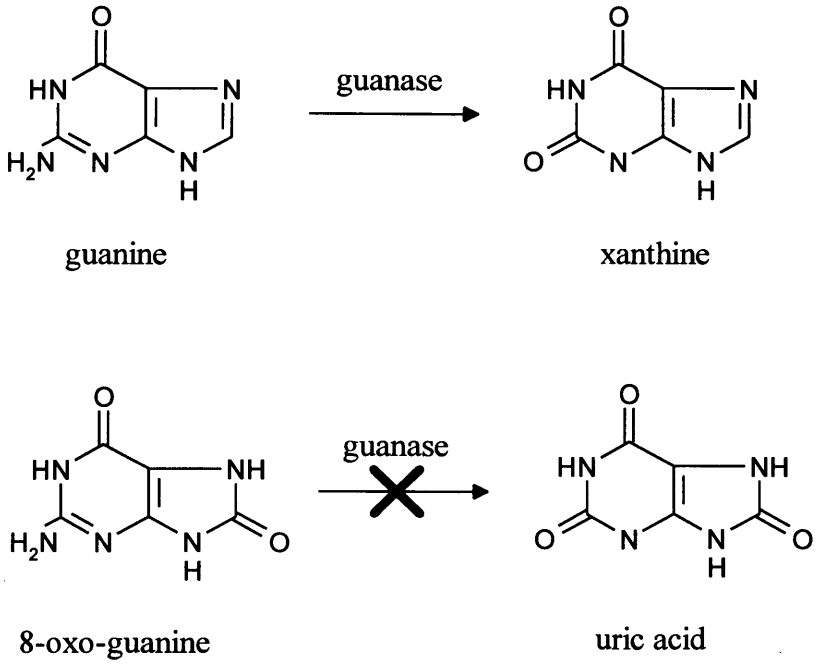


Figure 5.2. The oxidative deamination of guanine by guanase. The formation of uric acid from 8-OHG was not detected by HPLC ECD.

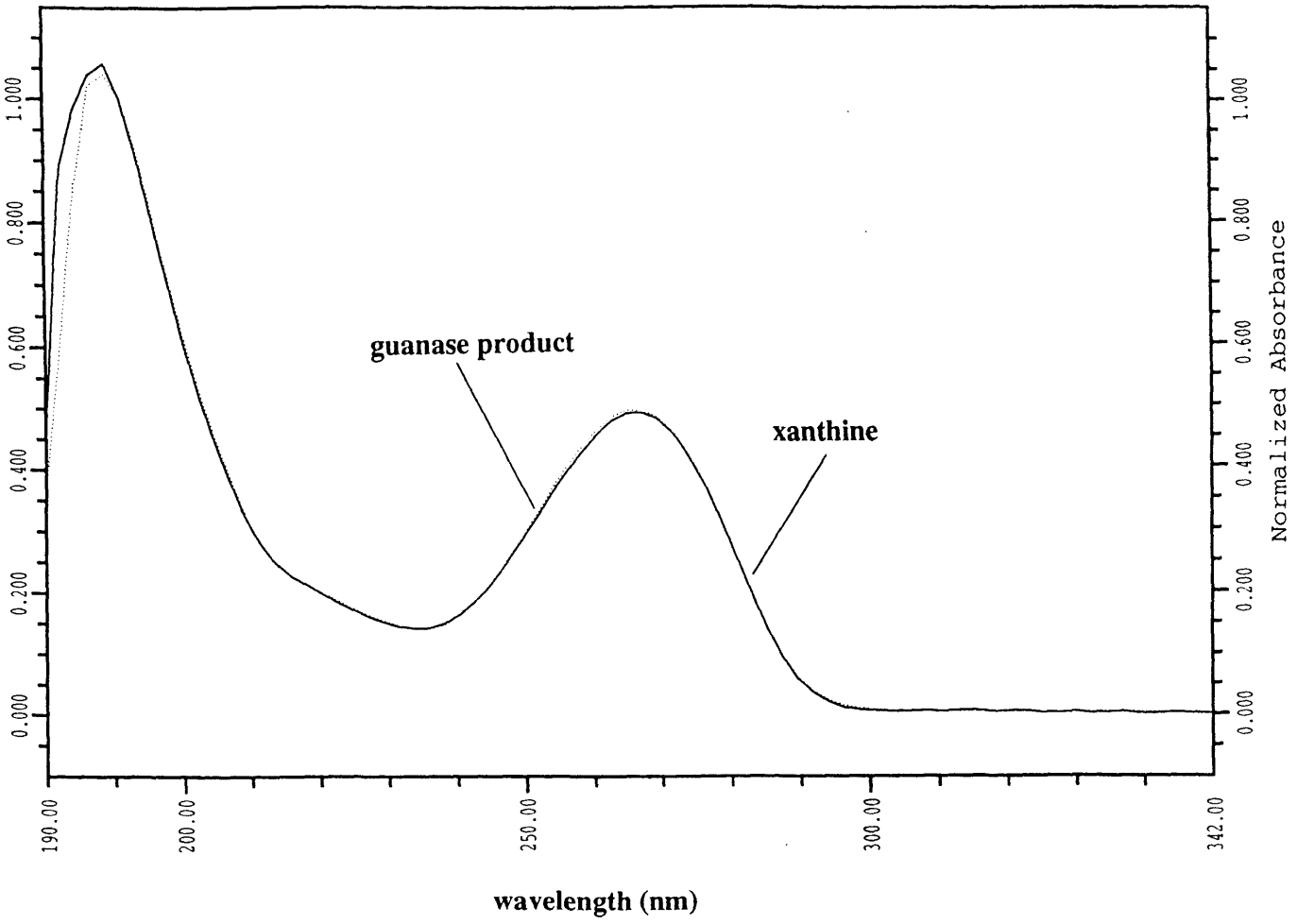


Figure 5.3. A xanthine standard superimposed upon a guanase product peak.

5.2.2. Analysis of 8-OHG in calf thymus DNA

The procedure described in section 5.2.1. was to be repeated using calf thymus DNA. This experiment would demonstrate if any possible conversion of or inhibition by other DNA bases or hydrolysate products occurred. The guanase reaction was to be carried out on aliquots of calf thymus DNA (100 μg). Following DNA hydrolysis (section 2.5.2.) the dried down DNA hydrolysates were divided into two aliquots for guanase and control treatment (section 2.5.3.).

Analyses were carried out using HPLC with UV and ECD detection.

Figure 5.4. demonstrates the difficulty involved in quantitating 8-OHG in the presence of guanine using HPLC ECD, however following guanase treatment 8-OHG analysis was possible.

HPLC UV detection showed that guanine was completely converted to xanthine, whereas cytosine, thymine and adenine were unchanged (Figure 5.5.).

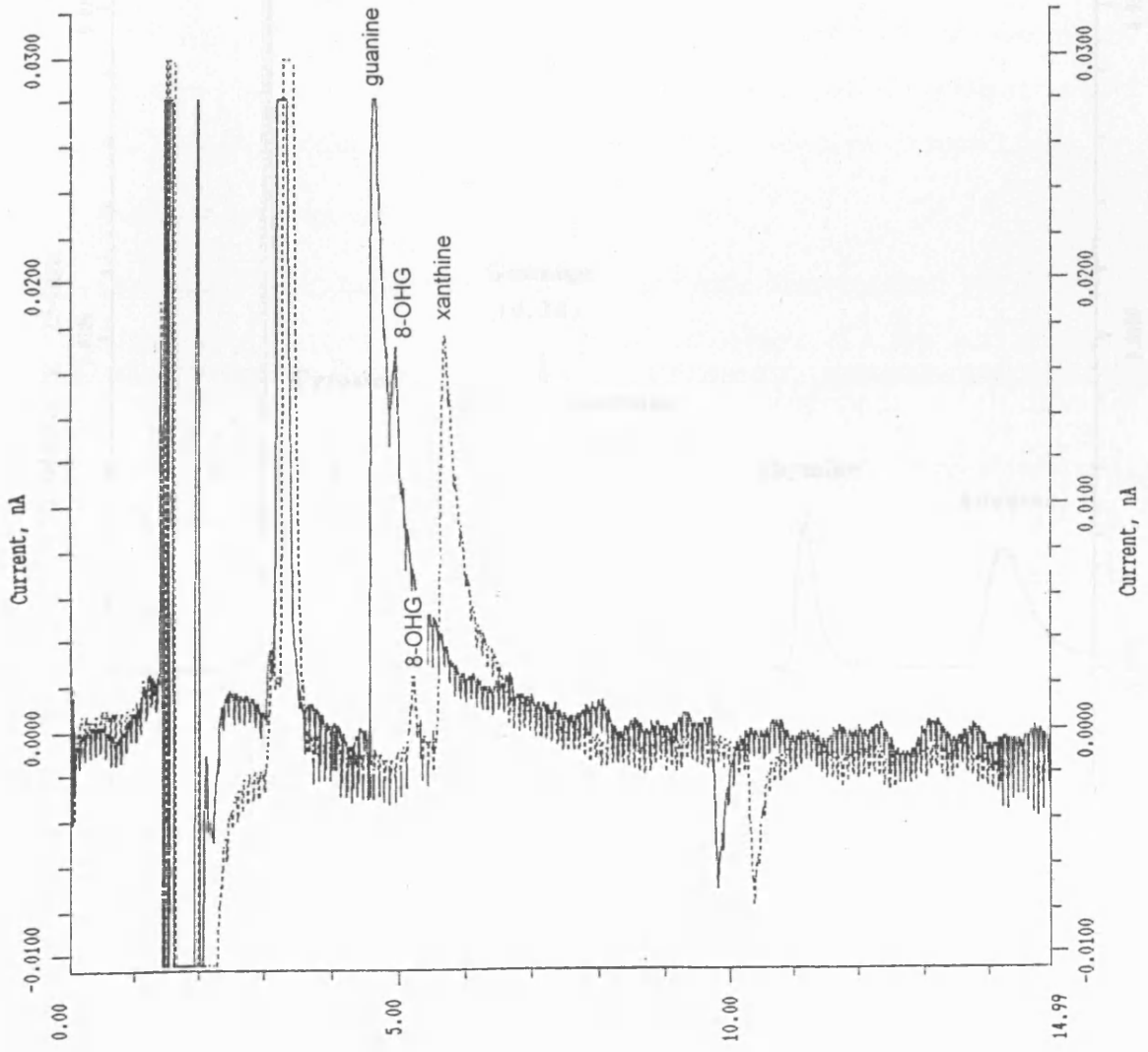


Figure 5.4. Analysis of calf thymus DNA hydrolysate before (—) and after (---) guanase treatment as detected by HPLC with ECD detection.

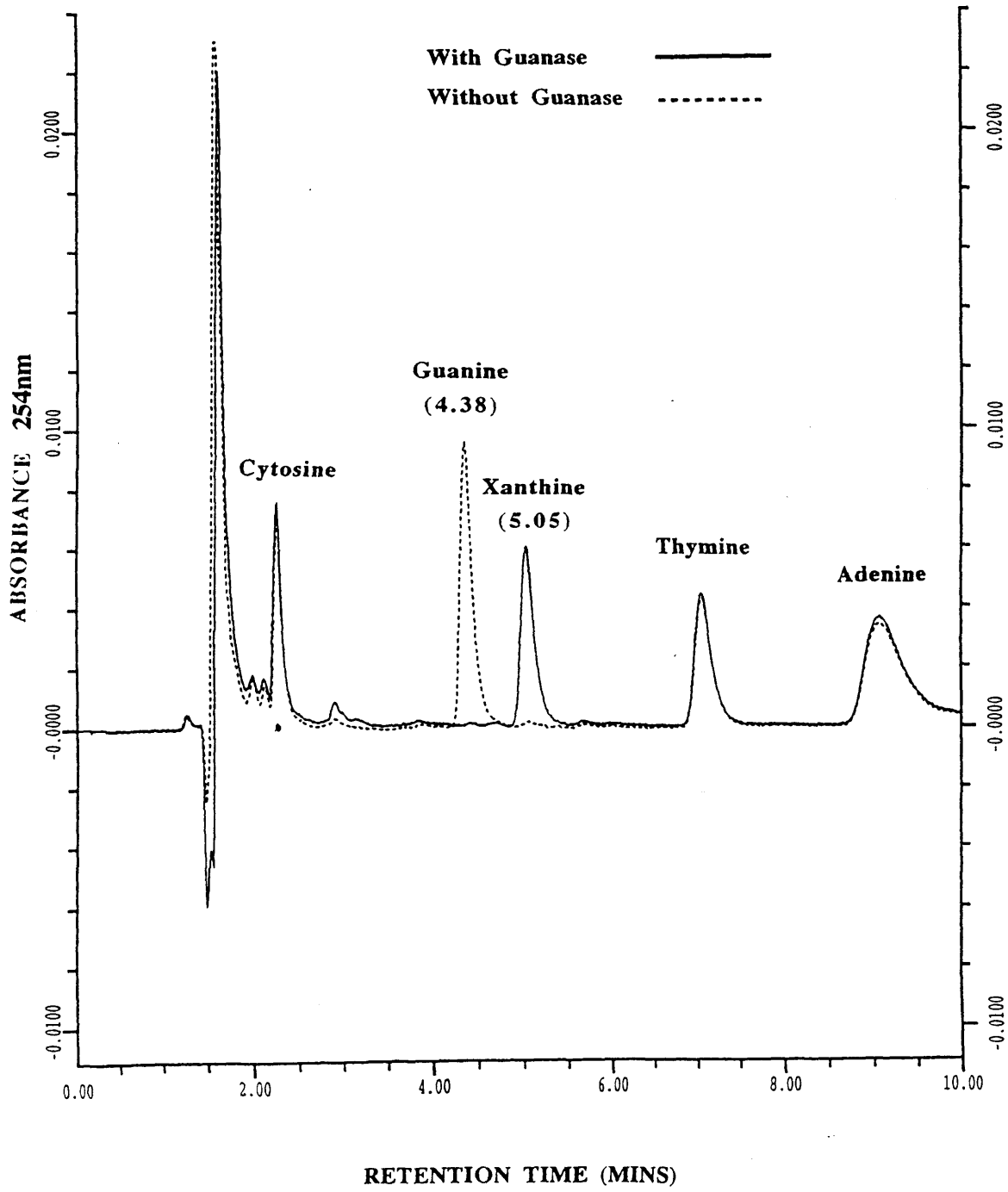


Figure 5.5. Analysis of calf thymus DNA hydrolysate before (----) and after (—) guanase treatment as detected by HPLC with UV detection.

5.2.3. The effect of guanase on 8-OHG

This assay relied on the fact that 8-OHG would not be degraded following guanase incubation. Guanase incubation with 8-OHG was therefore examined in order to eliminate the possibility of any conversion to urate, the hypothetical product of 8-OHG deamination (Figure 5.2.).

Aqueous solutions of 8-OHG were prepared at different concentrations including 6 μM, 970 nM, 480 nM, 240 nM, 60 nM, 19 nM, 10 nM. Two aliquots (80 μl) were taken from each concentration. To one aliquot was added guanase and to another control aliquot was added water followed by incubation at 37°C for one hour (section 2.5.3.).

Samples were analysed using HPLC with ECD.

All chromatograms were checked for the presence of urate, however none was detected in any of the chromatograms. Figure 5.6. shows an example of a uric acid standard superimposed upon a 8-OHG sample following guanase incubation.

The possibility that 8-OHG would be degraded to urate over a longer period of time was also to be investigated. Various concentrations of 8-OHG (10 μM, 1μM, 100 nM, 10 nM) were prepared. The various concentrations were divided into two aliquots (200 μl), the first for guanase treatment and the second as a control. The samples with and without guanase were incubated at 37°C for 1, 2 and 4 hours. Urate was not detected in any of the guanase treated or untreated samples following a incubation period of up to 4 hours with guanase.

The limit of detection for uric acid was < 2 nM at 600 mV.

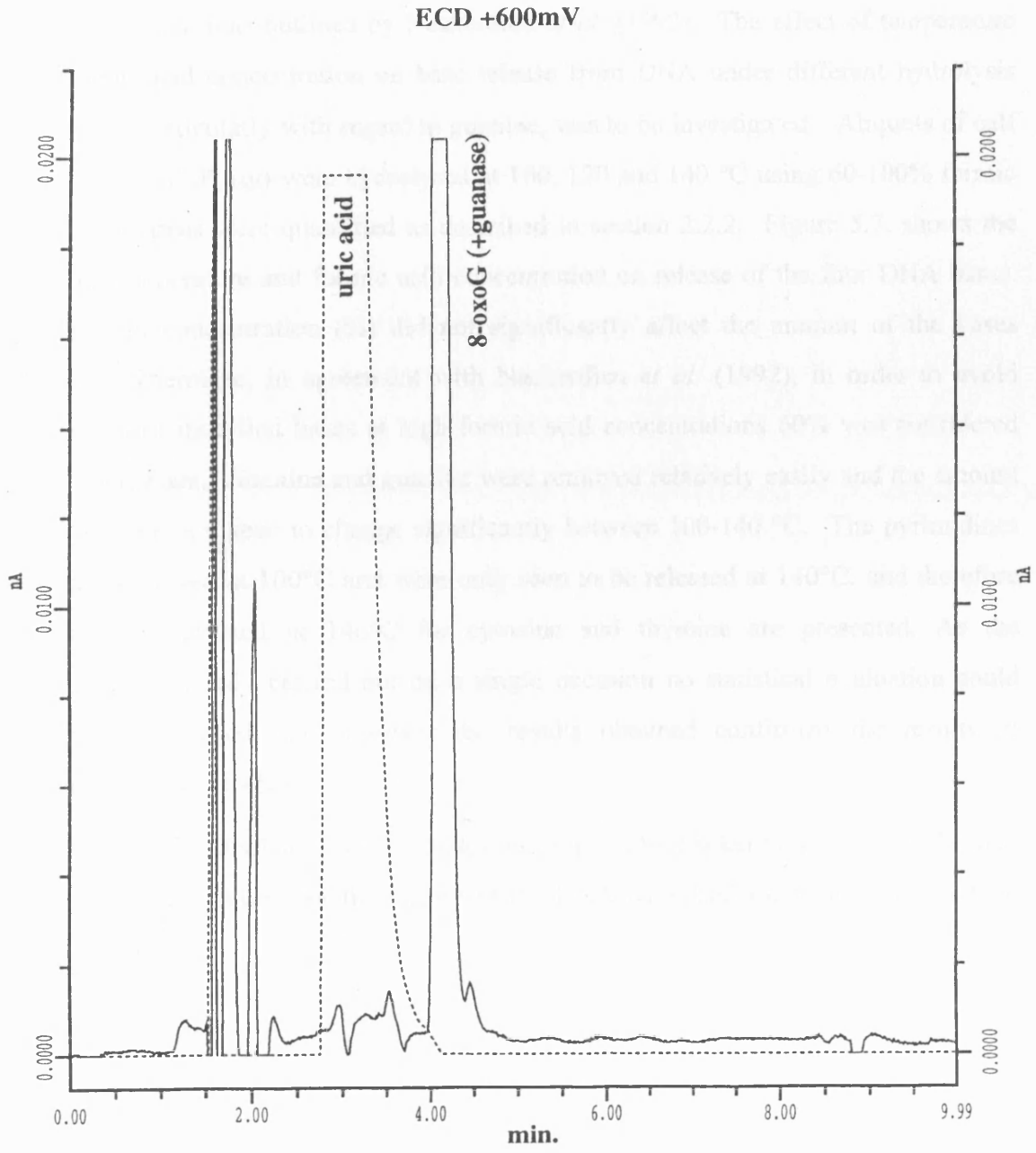


Figure 5.6. Urate standard superimposed on a 8-OHG standard following incubation with guanase for 1 hour.

5.2.4. Effect of formic acid concentration and temperature on base release from DNA.

The following experiment was carried out in order to investigate the optimal DNA hydrolysis conditions outlined by Nackerdien *et al.* (1992). The effect of temperature and formic acid concentration on base release from DNA under different hydrolysis conditions, particularly with regard to guanine, was to be investigated. Aliquots of calf thymus DNA (100 μg) were hydrolysed at 100, 120 and 140 °C using 60-100% formic acid. Peak areas were quantified as described in section 2.2.2. Figure 5.7. shows the effect of temperature and formic acid concentration on release of the four DNA bases. Formic acid concentration (%) did not significantly affect the amount of the bases released. Therefore, in agreement with Nackerdien *et al.* (1992), in order to avoid destruction of modified bases at high formic acid concentrations 60% was considered most appropriate. Adenine and guanine were removed relatively easily and the amount of release was not seen to change significantly between 100-140 °C. The pyrimidines were not removed at 100°C and were only seen to be released at 140°C, and therefore only results obtained at 140°C for cytosine and thymine are presented. As the experiment was only carried out on a single occasion no statistical evaluation could therefore be carried out; however the results obtained confirmed the results of Nackerdien *et al.* (1992).

To conclude, the DNA hydrolysis conditions outlined by Nackerdien *et al.* (1992) were found to be satisfactory for the release of the DNA bases and were therefore used for this study.

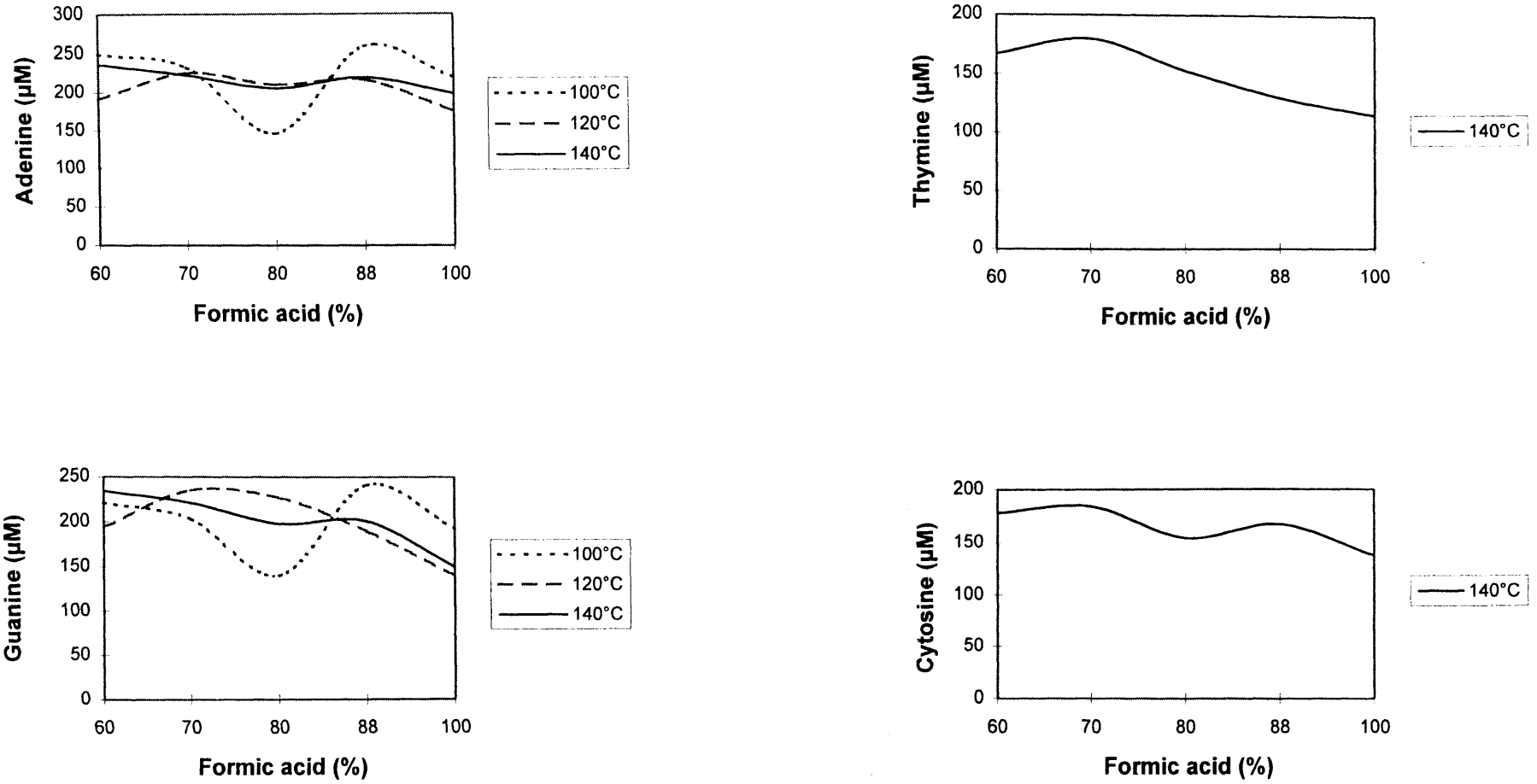


Figure 5.7. Adenine, guanine, cytosine and thymine release from DNA at different temperatures and formic acid concentrations.

5.2.4.1. Effect of formic acid concentration on 8-OHG.

The work of Nackerdien *et al.* (1992) was to be repeated using HPLC ECD to investigate the effect of formic acid concentrations on 8-OHG. This experiment was aimed at determining if 8-OHG was stable under acidic conditions.

An aqueous solution of 8-OHG (1mg/ml) was prepared. Dropwise NaOH was added to the solution in order to dissolve the 8-OHG. The solution was subsequently diluted to 100 nM from which aliquots (100 µl) were added to reactivials and dried in an evacuated centrifuge. The dried 8-OHG aliquots were subjected to the DNA hydrolysis procedure using 50%, 60%, 70%, 80%, 88% and 100% formic acid (500 µl). Following hydrolysis at 140°C for 40 minutes the samples were again dried down and reconstituted in deionised water (100 µl).

A calibration line was established using 10, 50 and 100 nM 8-OHG standards. Samples were analysed using HPLC with ECD.

Results

The results obtained (Figure 5.8.) are in agreement with literature reports of 60% formic acid being the optimum concentration for use in DNA hydrolysis, particularly for liberation of 8-OHG. The use of 60% formic acid appeared to be the least destructive for 8-OHG. While there was no significant variation of 8-OHG with increasing formic acid concentration between 50-80 %, as found by Nackerdien *et al.* (1992), use of higher formic acid concentrations did lead to degradation of 8-OHG. Variations in 8-OHG levels between samples could also be attributed to sample losses occurring during the work up procedure without compensation from an internal standard.

Figure 5.9. shows chromatograms of 8-OHG treated with 60% and 100% formic acid.

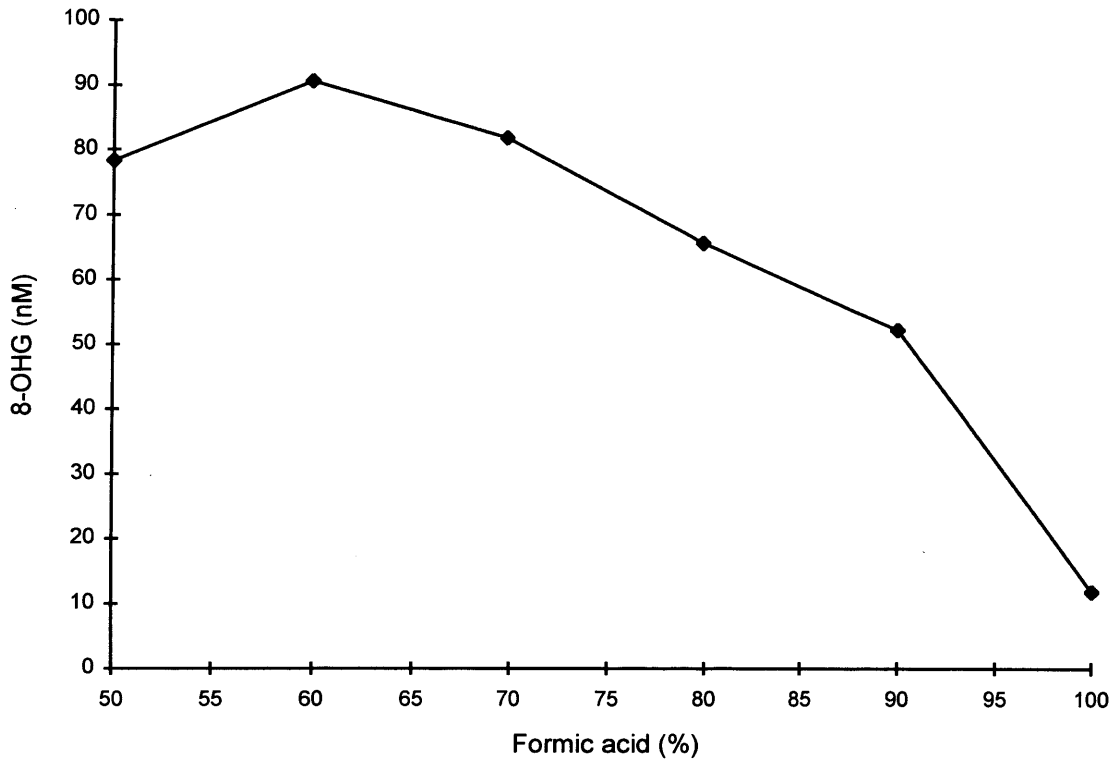


Figure 5.8. Effect of formic acid concentration on 8-OHG standards.

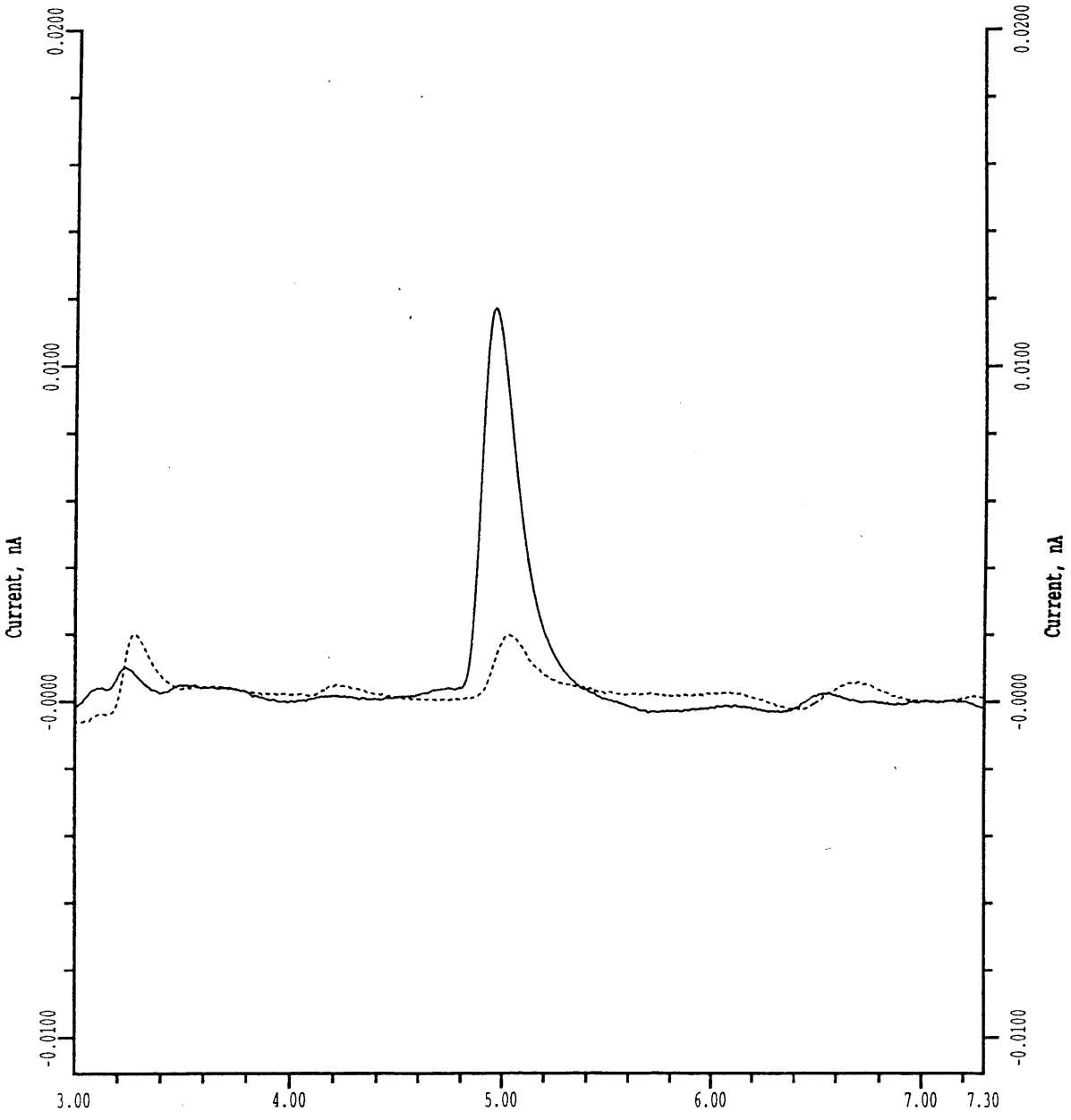


Figure 5.9. HPLC ECD trace of 8-OHG hydrolysed with 60 % (—) and 100% (---) formic acid.

5.2.4.2. Effect of formic acid concentration on guanine.

The experiment described in section 5.2.4.1. was repeated for guanine. An aqueous solution of guanine (50 μM) was prepared. Dropwise NaOH was added to the solution in order to dissolve the guanine. An aliquot (120 μl) of aqueous guanine was to be hydrolysed with formic acid (50%, 60%, 70%, 80%, 88% and 100%) at 140°C for 40 minutes. Following hydrolysis the guanine samples were again dried and reconstituted in deionised water (120 μl).

A calibration line was established using 10, 25 and 50 μM standards. Samples were analysed using HPLC with ECD detection.

Results

Results obtained here (Figure 5.10.) indicate that no significant degradation of guanine was seen with increasing formic acid concentration (50-80%). However particularly high concentrations between 88-100% were destructive. Again 60% formic acid was found to be the optimum formic acid concentration for the least loss of guanine with overall recovery being 84%. This figure would take into account losses occurring during the whole work-up procedure as well as any possible contribution from the formic acid. Therefore in agreement with Nackerdien *et al.* (1992) 60% formic acid was used for DNA hydrolysis for the analysis of 8-OHG. Hamberg and Zhang (1995), using GC/MS with the use of an internal standard, found that 62% formic acid led to removal of 89% of guanine from DNA at 130°C.

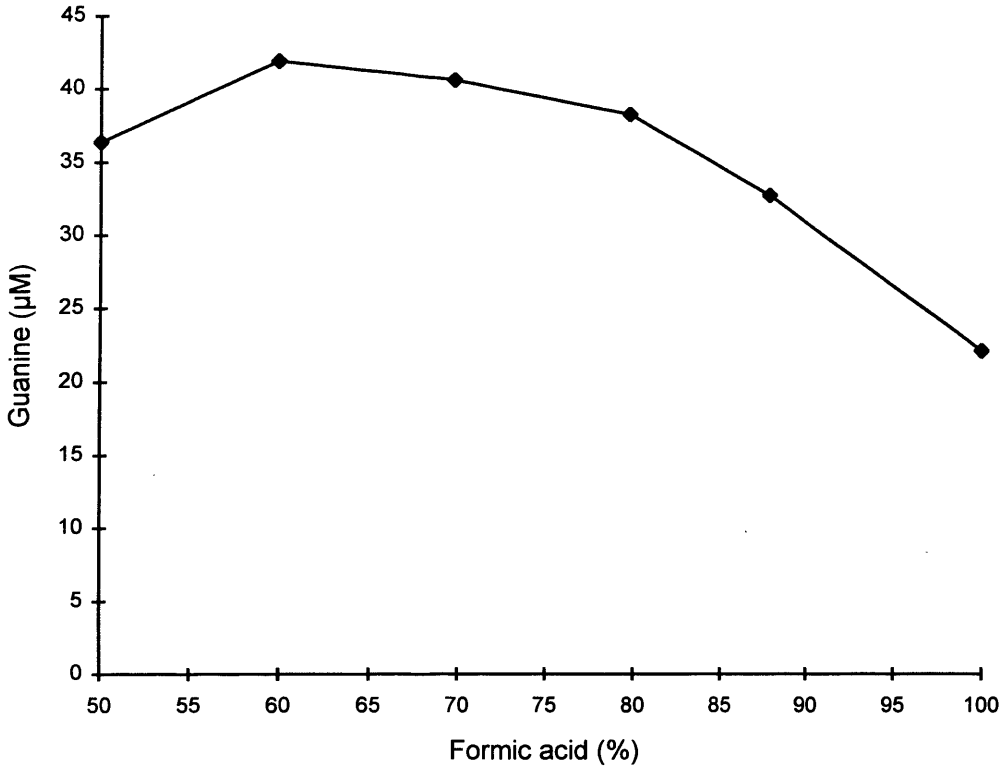


Figure 5.10. Effect of formic acid concentration on guanine.

5.3. APPLICATIONS

5.3.1. Irradiation of calf thymus DNA

The HPLC ECD assay developed was used to quantitate 8-OHG levels in γ-irradiated calf thymus DNA. Aliquots (1 ml) of aqueous solutions of calf thymus DNA (0.5 mg/ml) were irradiated in a ^{60}Co-γ source at a dose rate of 1.70 kGy/hr. The doses given were 0, 20 and 200 Gy. Aliquots of the irradiated solution of calf thymus DNA (120 μg) were hydrolysed (section 2.5.2.) before being dried in an evacuated vacuum centrifuge (two samples were analysed for each radiation dose). The dried samples were reconstituted in deionised water (350 μl). The samples were divided into two, the first for guanase treatment and the second to serve as a control. To the treated sample was added guanase (section 2.5.3.) and to the control water only.

Results

Levels of 8-OHG were calculated as ratios of 8-OHG/guanine by measuring the guanine in the control samples. The ratios were subsequently converted to 8-OHG nmoles/mg or ng/mg DNA . The levels of 8-OHG were as shown in Figure 5.11. The 'background' levels of 8-OHG in the unirradiated calf thymus DNA samples (n=2) were 0.44 nmoles/mg DNA (corresponding to 73.5 ng/mg DNA). These are comparable to the higher values obtained using HPLC ECD and the lower values obtained for GC/MS (Ravanat *et al.* 1995). Levels of 8-OHG were elevated 13 fold in the 200 Gy irradiated sample and are comparable to levels quoted by Fuciarelli *et al.* (1989) using the technique of GC/MS .

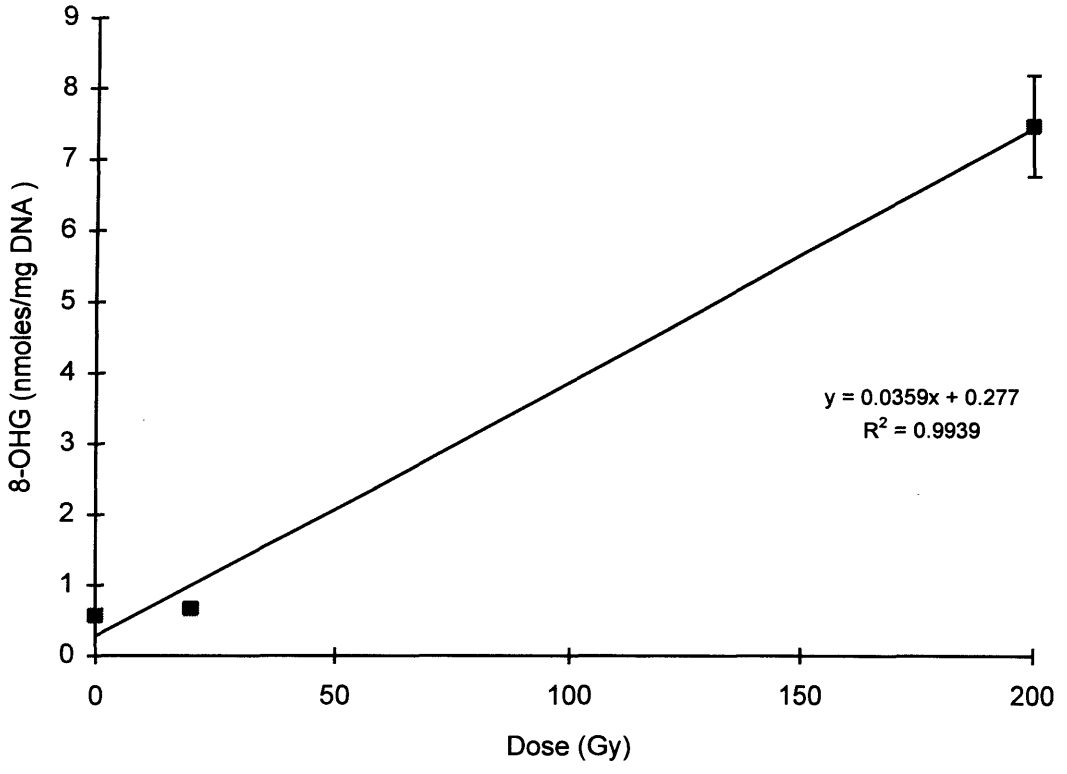


Figure 5.11. Levels of 8-OHG in ^{60}Co γ-irradiated calf thymus DNA.

5.3.2. The role of antioxidants in preventing oxidative DNA damage as measured by 8-OHG.

Human placental DNA from an antioxidant supplementation study which had previously been analysed for Tg and 5-OHMeU (for background see section 3.3.2.) was examined for 8-OHG.

Aliquots of placental DNA (20 μg) (control and treated) were hydrolysed as described in section 2.5.2. Samples were subsequently dried and reconstituted in deionised water (500 μl). The DNA hydrolysates were then divided into two aliquots, the first of which was treated with guanase (section 2.5.3.) and the second served as a control. Samples were analysed in batches of upto 8 with separate calibration lines being determined for each batch. Duplicate injections were carried out for all analyses and the results obtained using the average.

A 8-OHG calibration line was established using 5, 10 and 50 nM standards.

Samples were analysed using HPLC ECD.

Results

The calibration lines (n=3) used for the quantitation of placental DNA samples was as shown in Figure 5.12.

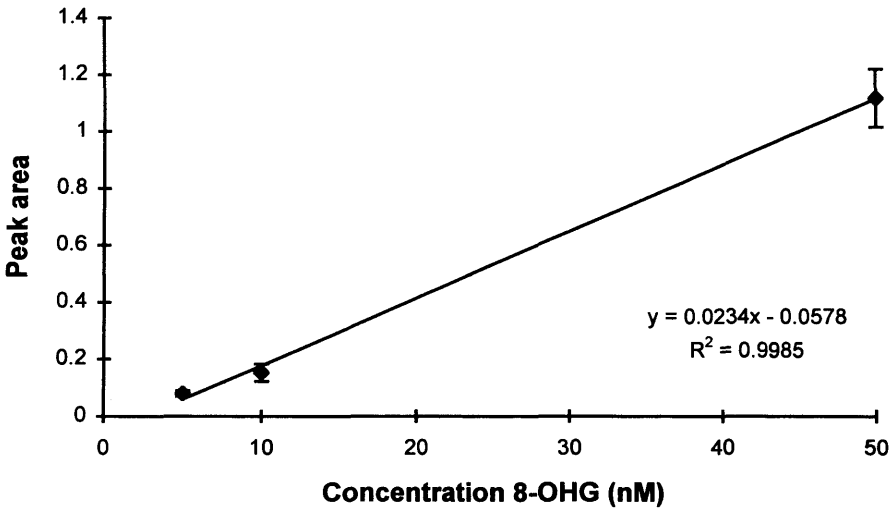


Figure 5.12. Mean of three calibration lines obtained by HPLC ECD for the quantitation of placental DNA samples

From the calibration line levels of 8-OHG were calculated and were as displayed in figure 5.13. An example of a placental DNA HPLC ECD trace is also shown in Figure 5.14.

Application of a statistical analysis of variance indicated that there was no significant difference between the control (n=5) and the antioxidant treated (n=9) groups, which had mean levels 14.11 ± 6.81 and 12.23 ± 3.17 ng/mg DNA respectively. This was in agreement with the lack of relationship observed between the antioxidant and control groups using the Tg and 5-OHMeU as markers. However 8-OHG levels were found statistically to be significantly higher than Tg and/or 5-OHMeU levels obtained for the same samples, as described in chapters 3 and 4. The levels quoted here are in agreement with another study (Yin *et al.* 1995) in which 8-OHdG levels were also calculated from the placental DNA of healthy mothers using HPLC ECD.

For a comparison of these results with the Tg and 5-OHMeU and possible explanations see Chapter 6.

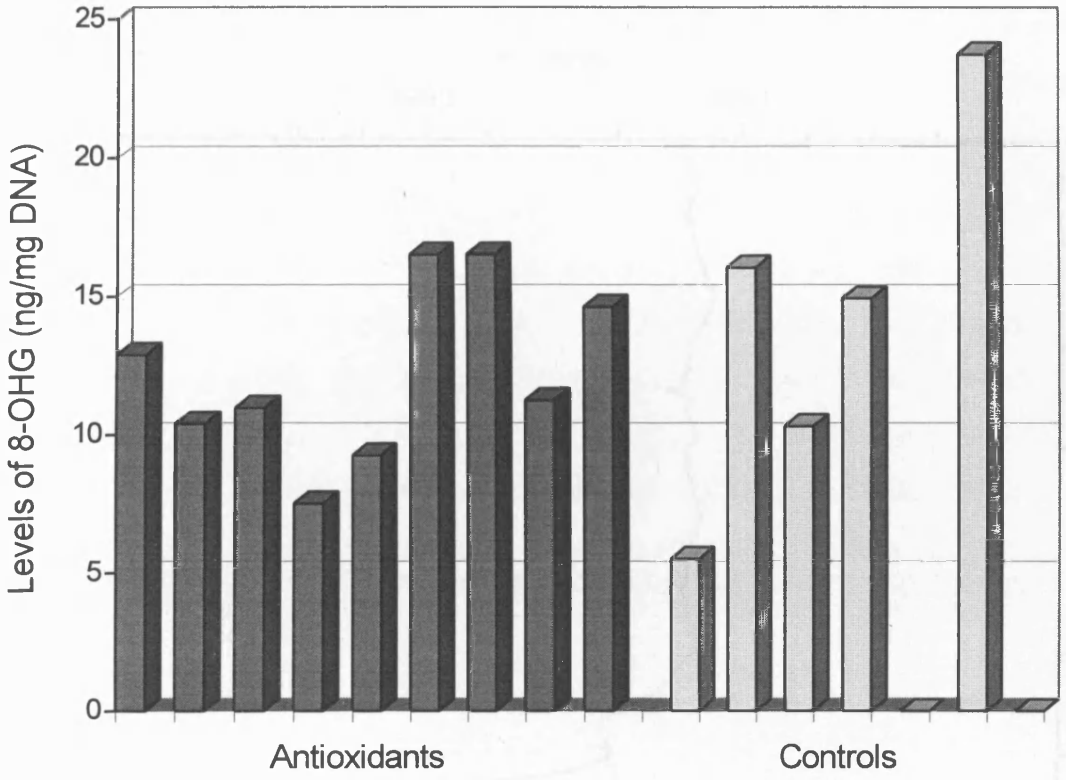


Figure 5.13. 8-OHG levels (ng/mg DNA) in placental DNA samples.

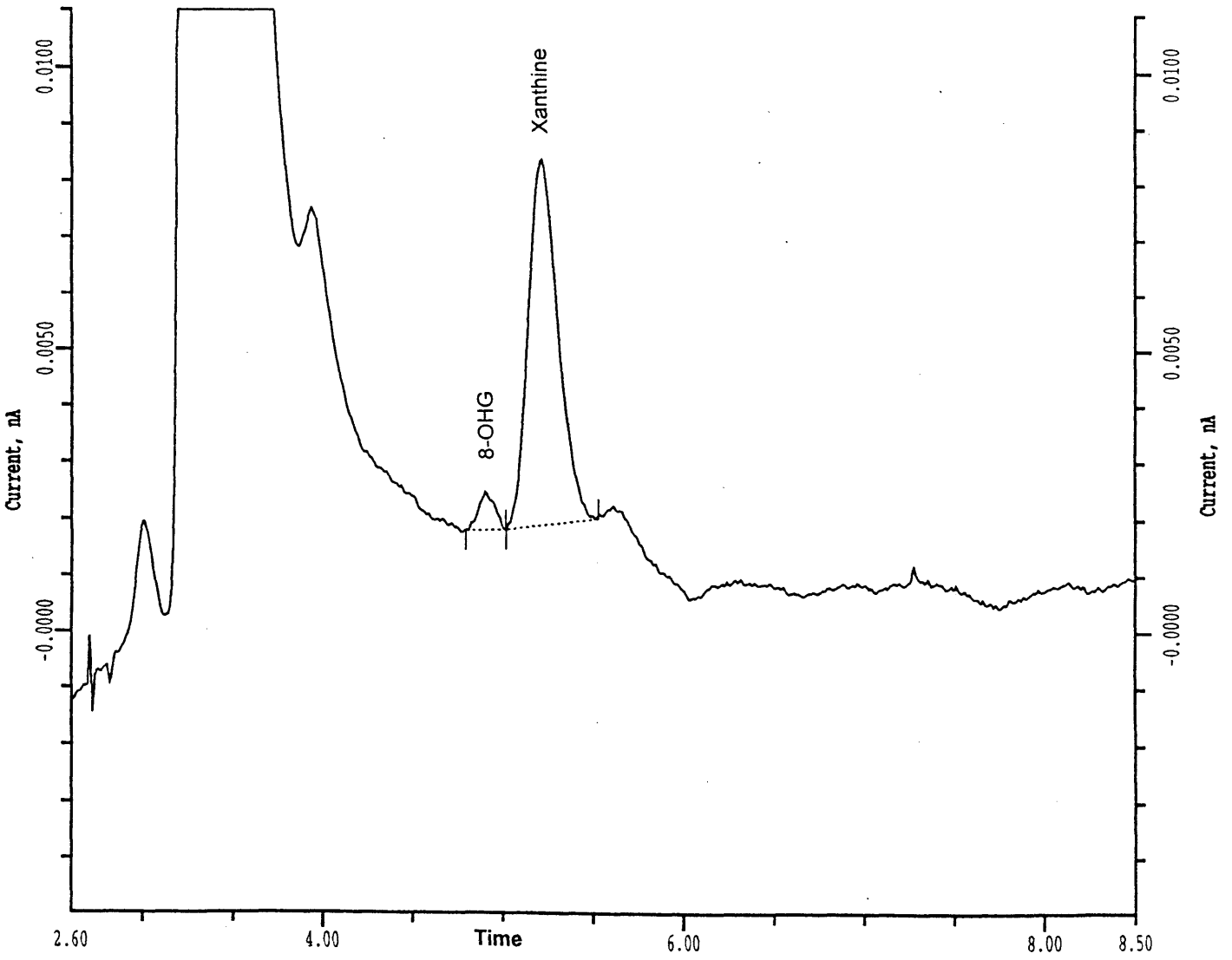


Figure 5.14. HPLC ECD trace of an antioxidant supplemented placental DNA sample

5.4. DISCUSSION

In conclusion an alternative assay for the quantitation of 8-OHG at the base level has been developed. It has been proven to be applicable to the quantitation of exogenous levels and also endogenous 'background' levels in calf thymus and human placental DNA. The method is simple, rapid and sensitive and does not require derivatisation although a simple guanase treatment is necessary.

In all these experiments with guanase, the HPLC peak corresponding to guanine was eliminated, whereas that of 8-OHG was not affected (recovery was > 95%). The limit of detection for 8-OHG using this procedure was calculated to be 5 nM (250 fmol) on column with a s/n of 5:1. The sensitivity of this method is comparable to that obtained for 8-OHdG using HPLC ECD. Floyd *et al.* (1986) quoted a detection limit of 20 fmol (1 8-OHdG/10^6 nucleosides) while Shigenaga *et al.* (1990) quoted detection limits of 5-50 fmol for 40-100 μg DNA. For GC/MS a detection limit of 5 fmol is quoted using 40-100 μg DNA, but analysis of smaller amounts is possible since usually 0.1-0.4 μg of hydrolysed and derivatised material is injected onto the GC column for analysis (Dizdaroglu 1991).

A background level of 73 ng 8-OHG/mg DNA was calculated in calf thymus DNA (n=2). These are comparable to the higher values obtained using HPLC ECD and the lower values obtained for GC/MS (Ravanat *et al.* 1995). However it must be noted that this was only carried out on a single occasion and not investigated using a large sample size and over a period of time. Levels of 8-OHG in human placental DNA were found to be similar to those quoted by Yin *et al.* (1995) using HPLC ECD. The levels of 8-OHG in placental DNA were found to be significantly lower than those obtained for calf thymus DNA. This may support the proposition of Nackerdien *et al.* (1992) that background levels obtained from calf thymus DNA are likely to be present from the DNA itself rather than from the hydrolysis procedure, as significantly lower values have been found *in vivo*.

Further work is required in order to compare 8-OHG levels obtained here with 8-OHdG levels also obtained by HPLC and with 8-OHG levels obtained using GC/MS. Such studies may help resolve differences due to incomplete enzymatic hydrolysis and/or artefact production.

Chapter 6.

Overall Conclusions

6.1. CONCLUSIONS

Oxidative DNA damage has been implicated in carcinogenesis, mutagenesis, ageing and cell death. It is therefore important to chemically characterise and quantitate this form of damage *in vivo*. This thesis describes methods developed and applied to detect and quantitate three markers of oxidative DNA damage at the base level. The assays developed had sufficient sensitivity to detect 'background' levels of oxidative DNA damage in commercial calf thymus DNA and *in vivo* human placental DNA.

The techniques of GC/MS and HPLC ECD were used to detect and quantitate the oxidative markers. GC/MS and HPLC ECD techniques are broadly comparable in sensitivity having the ability to detect 1 modified base/10^6 bases. The exact sensitivity achieved is however dependent upon the instrument and column used. However when quantitating oxidative damage in most cases it is not the absolute sensitivity of the technique that matters but the 'background' levels of base modifications (Lutgerink *et al.* 1992).

Although many techniques for the quantitation of oxidative DNA damage exist there are still only limited studies which have reached the sensitivity required to quantitate endogenous levels of oxidative DNA damage in humans and animals. In this study the technique of choice for detection and quantitation of Tg and 5-OHMeU was GC/MS due to the availability of structural information about the analyte under study. Although many other techniques exist the disadvantages with most of them are the lack of information provided about the analyte. For example even though PPL has been used for the quantitation of oxidative damage and is believed to be the most sensitive technique to date there is no way of identifying the lesion without the use of co-chromatography with standards. With immunochemical techniques cross reactivity of the antibody with normal bases has been shown to lead to elevated levels of modified bases quantitated. Therefore in order to obtain structural information about the lesion under study it may be necessary to compromise sensitivity for a technique that is also highly specific.

While GC/MS of oxidative modified DNA lesions has been carried out for the last 20 years it was not until recently that these techniques reached the sensitivity required to quantitate endogenous levels of oxidative damage in animals and humans. It must also

be noted that the majority of GC/MS studies quantitating DNA lesions *in vitro* and *in vivo* have not used isotopically labelled standards for quantitation purposes but instead used structurally similar internal standards. The danger with this is that since the internal standard and the analyte do not have similar physical and chemical properties they will not necessarily behave in the same way during sample work-up.

It is therefore believed that using GC/MS with the use of an isotopically labelled internal standard as in this study leads to the sensitive and specific quantitation of Tg and 5-OHMeU in DNA. The HPLC ECD technique also used was of comparable sensitivity to GC/MS and highly specific for 8-OHG as only electrochemically active lesions are detected by ECD.

In the work described in this thesis many factors had to be taken into consideration in addition to method development, addressing the issues of artefact production, using the developed assay to quantitate *in vitro* systems in which large amount of oxidative damage were expected but also to ultimately develop techniques that were sensitive enough to quantitate endogenous levels sensitively and specifically. The subsequent sections will describe some of these points briefly and conclude with improvements and future work.

Artefact production

Problems associated with the measurement of oxidative damage are artefact production and/or degradation of the oxidative DNA markers to be studied. For example derivatisation is believed to be the cause of at least some of the elevated levels of 8-OHG measured by GC/MS (Ravanat *et al.* 1995). Alternative derivatisation methods adopted to reduce artefact production include milder derivatisation conditions of purines carried out at room temperature (Hamberg and Zhang 1995) and prepurification of 8-OHG using HPLC or immunoaffinity chromatography prior to derivatisation (Ravanat *et al.* 1995). Hydrolysis of DNA using formic acid has also been shown to degrade some modified bases including Tg and 5-OHMeU (Djuric *et al.* 1991a; Nackerdien *et al.* 1992). However in this study the use of isotopically labelled analogues would compensate for any losses during the work up procedure. Isolation of DNA from cells may also introduce oxidative modifications, particularly if phenol based methods are used (Halliwell and Gutteridge 1989; Claycamp 1992).

The GC/MS work carried out here relied on the use of isotopically labelled analogues which were considered vital for accurate quantitation and for compensation for any losses during the work up. The HPLC ECD method employed eliminated the derivatisation step used in GC/MS believed to lead to artefactual 8-OHG levels. Derivatisation was necessary for Tg and 5-OHMeU analysis using GC/MS however this was carried out at lower temperatures than those being used in the literature, with great care being taken to avoid prolonged heating during derivatisation (section 4.2.2.). The levels obtained as a result of the assays used have been compared to literature values and found to be comparable with the respective methods and techniques used. A great deal of work and interest is continuing into artefact production and into eliminating/minimising steps involving elevated temperatures and harsh chemical treatment. The use of synthetic oligonucleotides may lead to answers regarding artefact production and degradation processes.

Background levels of modified bases in calf thymus DNA

Quantitation of modified DNA base lesions in DNA using GC/MS is relatively recent, and the use of isotopically labelled analogues to ensure accurate quantitation more recent still. Consequently there is limited data available on background levels of these modified lesions in calf thymus DNA or *in vivo* levels in human DNA. The source of 'background' levels is unknown although it is believed that those found in calf thymus DNA are probably present partly as a result of extraction procedures (Nackerdien *et al.* 1992). However some believe 'background' levels to be produced as a result of artefact formation. Although artefact formation is possible, no evidence for this has been provided for DNA hydrolysis (Nackerdien *et al.* 1992), but there is some evidence concerning certain derivatisation conditions leading to artefact formation in purines (Ravanat *et al.* 1995).

The levels obtained in this study have been compared to other GC/MS studies although levels obtained using other techniques such as PPL have also been discussed. Background levels of Tg and 5-OHMeU obtained in calf thymus DNA in this study have been found to average ~ 1.3 ng/mg and 0.55 ng/mg DNA respectively in agreement with Markey *et al.* 1993 and Spencer *et al.* 1994. However variations in Tg and 5-OHMeU levels were seen, which may be attributed to different batches of calf

thymus DNA used and also storage conditions (Markey *et al.* 1993). Tg and 5-OHMeU levels quoted by Nackerdien *et al.* (1992) are ~ 4-6 times higher than levels for the two lesions quoted here. This could be attributed to the high temperatures used in the derivatisation conditions, batch of DNA used or even storage conditions. Background levels of Tg quoted in calf thymus DNA by Hegi *et al.* (1989) using PPL have been ~ 20 ng/mg.

Levels of 8-OHG as measured by HPLC ECD in commercial calf thymus DNA were 75 ng/mg, which were significantly higher than Tg and 5-OHMeU levels, agreeing with the view of this lesion being the most abundant. Nackerdien *et al.* (1992) found 8-OHG levels to be 5 -10 fold greater than Tg and 5-OHMeU respectively using GC/MS of the same aliquot of DNA in a single analysis.

It is difficult to compare the various background levels reported due to the differing techniques and batches of DNA used by different laboratories. It is therefore advisable to carry out inter - laboratory analysis on the same samples for comparison purposes.

The absolute 'background' levels *in vivo* of the products of oxidative DNA damage are also not known, as different methods give different results. In future greater attention must be given to resolving methodological variations and validating the assays before adopting methods as an index of oxidative damage.

Placental DNA samples

Placental DNA samples from an antioxidant supplementation study were quantitated for levels of Tg, 5-OHMeU and 8-OHG (Figure 6.1.). While Tg and 5-OHMeU levels were comparable in the human placental DNA samples levels of 8-OHG were significantly higher in comparison to Tg and 5-OHMeU. However a significant relationship between antioxidant and control groups was not seen for any of the three markers. Possible explanations may include the fact that much of our dietary intake is rich in antioxidants and ethically there is no way of controlling antioxidant intake in a human control group. Another possible explanation may be the difficulty in observing a relationship due to antioxidant supplementation in the presence of high background levels of continuing oxidative damage. This result is in common with other antioxidant supplementation

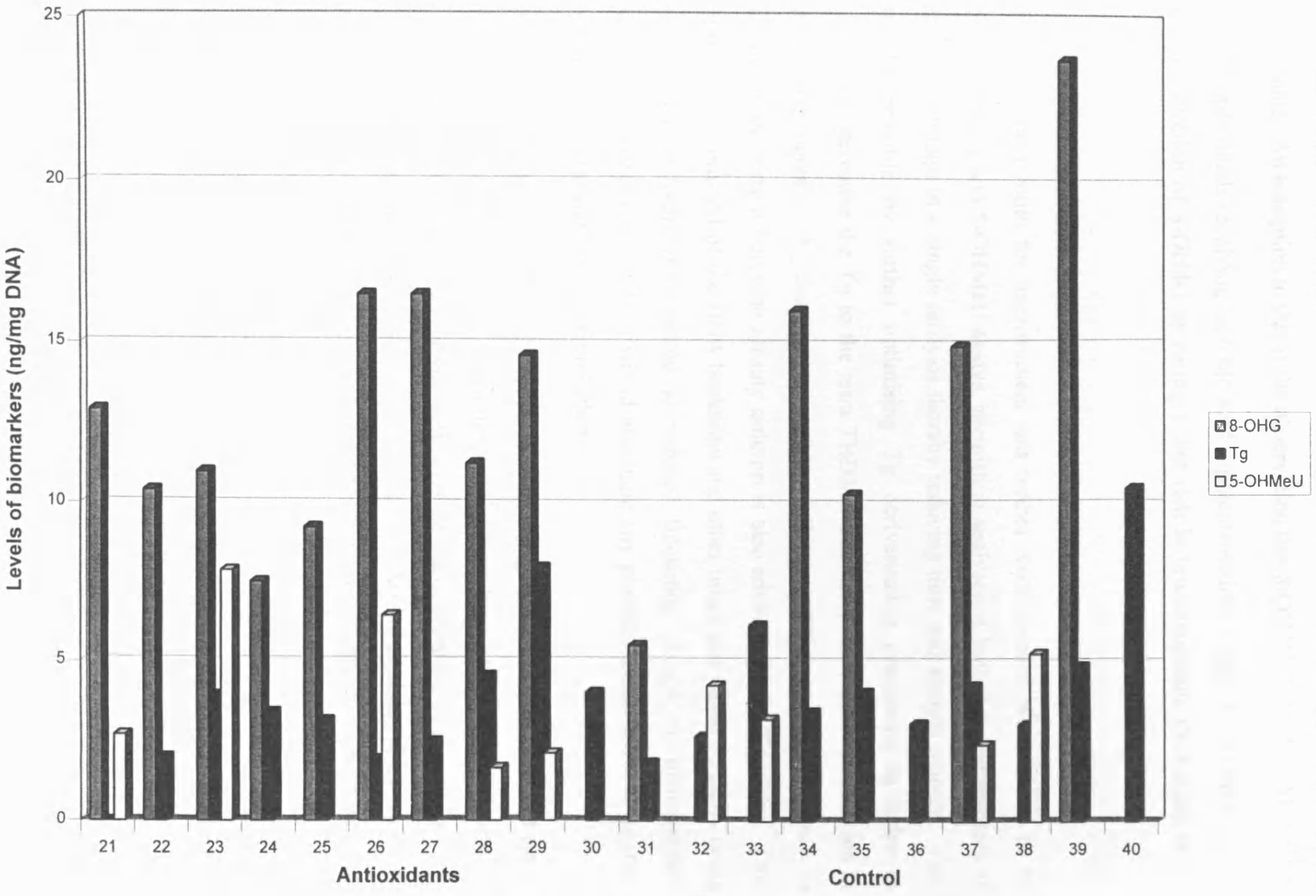


Figure 6.1. Levels of Tg, 5-OHMeU and 8-OHG in placental DNA samples.

studies (van Poppel *et al.* 1995; Witt *et al.* 1992; Heinonen *et al.* 1994) in which no significant reduction in oxidative DNA damage was observed on supplementation of antioxidants. An exception to this is the observation that 8-OHdG was reduced in sperm DNA in individuals receiving ascorbic acid supplementation (Fraga *et al.* 1991) as was urinary excretion of 8-OHdG on eating a diet rich in brussel sprouts (Verhagen *et al.* 1995).

Improvements and future work.

There is always room for improvement and further recommended work would be to combine the Tg and 5-OHMeU assays, permitting analyses of both thymine markers of oxidative damage in a single analysis thereby reducing time and sample amount. This may be possible by further optimising Tg derivatisation conditions in order to completely derivatise the Tg to the tetra TBDMS derivative and carry out analyses in derivatising agent. A clean up procedure following DNA hydrolysis prior to derivatisation using a boronate affinity column is also advised for the Tg assay. This would ensure removal of the DNA backbone and other intact and modified DNA bases released during hydrolysis leading to reduced frequency of column maintenance. Equally and more importantly it would eliminate any possible contribution of artefact formation from the derivatisation procedure.

Comparative studies of both 8-OHG and 8-OHdG using HPLC ECD and 8-OHG using GC/MS are also advised in order to provide explanations for methodological variations. As was attempted in this thesis further work with the use of synthetic oligonucleotides is also required. Synthetic oligonucleotides containing a known amount of the modified base may be used to study any possible artefacts produced during sample work up procedures.

To conclude, which is the most suitable marker for measuring oxidative damage? Great care must be taken in attempting to use levels of any single marker as a quantitative measure of oxidative DNA damage. The reasons for this are that during $OH^{\bullet}$ attack on DNA bases, radicals formed have different fates depending on environmental conditions. Attack of $OH^{\bullet}$ on guanine can lead to formation of 8-OHG by oxidation of the C8-OH adduct radical, but this radical could also lead to the formation of other products such as FapyGua. Therefore it is possible that different amounts of markers

can result from attack of the same amount of $OH^{\bullet}$ on the particular base (Halliwell and Aruoma 1991). For example when aqueous solutions of mammalian chromatin, are exposed to radiation-generated $OH^{\bullet}$, the relative amounts of hydroxypurines and formamidopyrimidines generated is dependent upon the gaseous environment used to saturate the aqueous solution (anoxic conditions favour formamidopyrimidines over 8-hydroxypurines (Gajewski *et al.* 1990)). Therefore it is advisable to look at a spectrum of base products characteristic of $OH^{\bullet}$ attack when studying oxidative DNA damage.

This approach was carried out in this study, where 3 markers of oxidative damage were monitored to in order to study the relationship between antioxidant supplemented and control groups. Care must also be taken in the interpretation of results, as when increased levels of oxidative markers in DNA have been identified, this could mean increased damage but could also mean decreased repair.

References

Acworth, I.N. and Bailey, B. (1995) The handbook of oxidative metabolism. (Abstract)

Adachi, S., Yoshida, S., Kawamura, K., Takahashi, M., Uchida, H., Odagiri, Y., and Takemoto, K. (1994) Induction of oxidative DNA damage and mesothelioma by crocidolite, with special reference to the presence of iron inside and outside of asbestos fiber. *Carcinogenesis* **15**, 753-758.

Adelman, R., Saul, R.L., and Ames, B.N. (1988) Oxidative damage to DNA: Relation to species metabolic rate and lifespan. *Proc. Natl. Acad. Sci. USA* **85**, 2706-2708.

Ames, B.N. (1983) Dietry carcinogens and anticarcinogens. *Science* **22**, 1256-1264.

Ames, B.N. (1989a) Mutagenesis and carcinogenesis : Endogenous and exogenous factors. *Environ. Mol. Mut.* **14**, 66-77.

Ames, B.N. (1989b) Endogenous oxidative DNA damage, aging and cancer. *Free Rad. Res. Comms.* **7**, 121-128.

Ames, B.N. (1989c) Endogenous DNA damage as related to cancer and aging. *Mut. Res.* **214**, 41-46.

Ames, B.N. and Shigenaga, M.K. (1992) Oxidants are a major contributor to aging. *Ann. New York Acad. Sci.* **663**, 85-96.

Ames, B.N. and Shigenaga, M.K. (1993) Oxidants are a major contributor to cancer and aging. In: *DNA and free radicals*, Edited by Halliwell, B. and Aruoma, O.I., Ellis Horwood Ltd, Chichester.

Ames, B.N., Shigenaga, M.K., and Hagen, T.M. (1993a) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA* **90**, 7915-7922.

Ames, B.N., Shigenaga, M.K., and Gold, L.S. (1993b) DNA lesions, inducible DNA repair, and cell division: Three key factors in mutagenesis and carcinogenesis. *Environ. Health Perspect.* **101**, 35-44.

Ames, B.N., Gold, L.S., and Willett, W.C. (1995) The causes and prevention of cancer. *Proc. Natl. Acad. Sci. USA* **92**, 5258-5265.

Annan, R.S., Kresbach, G.M., Giese, R.W., and Vouros, P. (1989) Trace detection of modified DNA bases via moving belt liquid chromatography-mass spectrometry using electrophoric derivatization and negative chemical ionization. *J. Chromatogr.* **465**, 285-296.

Aruoma, O.I., Halliwell, B., and Dizdaroglu, M. (1989a) Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidases. *J. Biol. Chem.* **264**, 13024-13028.

Aruoma, O.I., Halliwell, B., Gajewski, E., and Dizdaroglu, M. (1989b) Damage to the bases in DNA induced by hydrogen peroxide and ferric ion chelates. *J. Biol. Chem.* **264**, 20509-20512.

Aruoma, O.I., Halliwell, B., Gajewski, E., and Dizdaroglu, M. (1991) Copper - ion - dependent damage to the bases in DNA in the presence of hydrogen peroxide. *Biochem. J.* **273**, 601-604.

Balentine, J.D. (1982) *Pathology of oxygen toxicity.* Academic Press, New York.

Berger, M., Anselmino, C., Mouret, J.-F., and Cadet, J. (1990) High performance liquid chromatography-electrochemical assay for monitoring the formation of 8-oxo-7,8-dihydroadenine and its related 2'-deoxyribonucleoside. *J. Liquid Chromatogr.* **13**, 929-940.

Berger, M., Cadet, J., Berube, R., Langlois, R., and van Lier, J.E. (1992) Reversed-phase high-performance liquid chromatography-thermospray mass spectrometry of radiation-induced decomposition products of thymine and thymidine. *J. Chromatogr.* **593**, 133-138.

Bessho, T., Tano, K., Kasai, H., Ohtsuka, E., and Nishimura, S. (1993) Evidence for two DNA repair enzymes for 8-hydroxyguanine (7,8-Dihydro-8-oxoguanine) in human cells. *J. Biol. Chem.* **268**, 19416-19421.

Bianchini, F., Hall, J., Donato, F., and Cadet, J. (1996) Monitoring urinary excretion of 5-hydroxymethyluracil for assessment of oxidative DNA damage and repair. *Biomarkers* **1**, 178-184.

Blot, W.J., Li, J.Y., Taylor, P.R., Guo, W., Dawsey, S., Wang, G.Q., Yang, C.S., Zheng, S.F., Gail, M., Li, G.Y., and *et al.* (1993) Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence and disease-specific mortality in the general population. *J. Nat. Cancer Inst.* **85**, 1483-1492.

Boiteux, S., Gajewski, E., Laval, J., and Dizdaroglu, M. (1992) Substrate specificity of the Escherischia coli Fpg protein (formamidopyrimidine-DNA glycosylase): excision of purine lesions produced by ionising radiation or photosensitization. *Biochem.* **31**, 106-110.

Branda, R.F., Sullivan, L.M., O'Neil, J.P., Falta, M.T., Nicklas, J.A., Hirsch, B., Vacek, P.M., and Albertini, R.J. (1993) *Mut. Res.* **285**, 267-279.

Breen, A.P. and Murphy, J.A. (1995) Reactions of oxyl radicals with DNA. *Free Rad. Biol. Med.* **18**, 1033-1077.

Breimer, L.H. (1984) Enzymatic excision from Gamma-Irradiated polydeoxyribonucleotides of adenine residues whose imidazole rings have been ruptured. *Nucleic Acids Res.* **12**, 6359-6367.

Breimer, L.H. and Lindahl, T., (1984) DNA glycosylase activities for thymine residues damaged by ring saturation, fragmentation or ring contraction are functions of endonuclease III in *Escherichia coli. J. Biol. Chem.* **259**, 5543-5548.

Breimer, L.H. (1988) Ionising radiation-induced mutagenesis. *Br. J. Cancer* **57**, 6-18.

Breimer, L.H. (1991) Repair of DNA damage induced by reactive oxygen species. *Free Rad. Res. Comms.* **14**, 159-171.

Cadet, J. (1994) DNA damage caused by oxidation, deamination, ultraviolet radiation and photoexcited psoralens. In: *DNA adducts: Identification and Biological significance*, 125th Ed., 245-276. IARC Scientific Publications.

Casarett and Doull's Toxicology. (1996) *The basic science of poisons.* Fifth Ed., McGraw-Hill.

Cao, E. and Wang, J. (1993) Oxidative damage to DNA: Levels of thymine glycol and thymidine glycol in neoplastic human urines. *Carcinogenesis* **Vol. 14**, 1359-1362.

Cathcart, R., Schwiers, E., Saul, R.L., and Ames, B.N. (1984) Thymine glycol and thymidine glycol in human and rat urine: A possible assay for oxidative DNA damage. *Proc. Natl. Acad. Sci. USA* **81**, 5633-5637.

Cerutti, P.A. (1985) Prooxidant states and tumour promotion. *Science* **227**, 375-381.

Cheeseman, K.H. (1993) Lipid peroxidation and cancer. In: *DNA and free radicals.* 109-134. Edited by Halliwell, B. and Aruoma, O.I. Ellis Horwood Limited.

Claycamp, H.G. (1992) Phenol sensitization of DNA to subsequent oxidative damage in 8-hydroxyguanine. *Carcinogenesis* **13**, 1289-1292.

Cline, R.E., Fink, R.M., and Fink, K. (1959) Synthesis of 5-substituted pyrimidines via formaldehyde addition. *J. Am. Chem. Soc.* **81**, 2521-2527.

Clouter, A. (1993) Lung clara cells and their reaction to bronchiolar toxins. Ph.D. thesis., University College Cardiff.

Clouter, A., Houghton, C.E., Bowskill, C.A., Hibbs, L.R., Brown, R.C., and Hoskins, J.A. (1997) Effect of inhaled fibres on the glutathione concentration and γ–Glutamyl transpeptidase activity in lung type II epithelial cells, macrophages, and bronchoalveolar lavage fluid. *Inhal. Toxicol.* **9**, 351-367.

Crain, P.F. and McCloskey, J.A. (1983) Analysis of modified bases in DNA by stable isotope dilution Gas Chromatography-Mass Spectrometry: 5-Methylcytosine. *Anal. Biochem.* **132**, 124-131.

Cullis, P.M., Malone, M.E., and Merson-Davies, L.A. (1996) Guanine radical cations are precursors of 7,8-Dihydro-8-oxo-2'-deoxuguanosine but are not precursors of immediate strand breaks in DNA. *J. Am. Chem. Soc.* **118**, 2775-2781.

Czeczot, H., Tudek, B., Lambeert, B., Laval, J., and Boiteux, S. (1991) Escherischia coli fpg protein and UvrABC endonuclease repair DNA damage induced by methylene blue plus visible light in vivo and in vitro. *J. Bacteriol.* **173**, 3419-3424.

Davies, K.J., A. (1987) Protein damage and degradation of oxygen radicals. I General Aspects. *J. Biol. Chem.* **262**, 9895-9901.

De Groot, A.J.L., Jansen, J.G., van Valkenburg, C.F.M., and van Zealand, A.A. (1994) Molecular dosimetry of 7-alkyl- and O^6- alkyl guanine in DNA by electrochemical detection. *Mut. Res.* **307**, 64-66.

Dean, R.T. (1990) Protein damage and repair.: An overview. In: *Oxidative damage and repair. Chemical, Biological and Medical aspects.* 341-347. Edited by Davies, K.J.A. Pergamon Press.

Degan, P., Shigenaga, M.K., Park, E., Alperin, P.E., and Ames, B.N. (1991) Immunoaffinity isolation of urinary 8-hydroxy-2'-deoxyguanosine and 8-hydroxyguanine and quantitation of 8-hydroxy-2'-deoxyguanosine in DNA by polyclonal antibodies. *Carcinogenesis* **12**, 865-871.

Demple, B. and Linn, S. (1980) DNA N-glycosylases and UV repair. *Nature* **287**, 203-208.

Demple, B. and Levin, J.D. (1991) Repair systems for radical-damaged DNA. In: *Oxidative Stress: Oxidants and antioxidants.* 119-154. Academic Press Ltd.

Demple, B. and Harrison, L. (1994) Repair of oxidative damage to DNA: Enzymology and Biology. *Annu. Rev. Biochem.* **63**, 915-948.

Devanaboyina, U. and Gupta, R.C. (1996) Sensitive detection of 8-hydroxy-2'-deoxyguanosine in DNA by ^{32}P-Postlabeling assay and the basal level in rat tissues. *Carcinogenesis* **17**, 917-924.

Dizdaroglu, M., Schulte-Frohlinde, D., and von Sonntag, C. (1975) Strand breaks and sugar release by γ–irradiation of DNA in aqueous solution. *J. Am. Chem. Soc.* **97**, 2277-2278.

Dizdaroglu, M., Schulte-Frohlinde, D., and von Sonntag, C. (1977) γ–Radiolysis of DNA in oxygenated aqueous solution. Structure of an alkali-labile site. *Biosciences* **30**, 826-828.

Dizdaroglu, M. (1984) The use of capillary gas chromatography- mass spectrometry for identification of radiation- induced DNA base damage and DNA base amino acid cross links. *J. Chromatogr.* **295**, 103-121.

Dizdaroglu, M. (1985) Application of capillary gas chromatography- mass spectrometry to chemical characterization of radiation-induced base damage of DNA : Implications for assessing DNA repair processes. *Anal. Biochem.* **144**, 593-603.

Dizdaroglu, M. and Bergtold, D.S. (1986) Characterization of free radical-induced base damage in DNA at biologically relevant levels. *Anal. Biochem.* **156**, 182-188.

Dizdaroglu, M. (1991) Chemical determination of free radical-induced damage to DNA. *Free Rad. Biol. Med.* **10**, 225-242.

Dizdaroglu, M., Rao, G., Halliwell, B., and Gajewski, E. (1991) Damage to the DNA bases in mammalian chromatin by hydrogen peroxide in the presence of ferric and cupric ions. *Arch. Biochem. Biophys.* **285**, 317-324.

Dizdaroglu, M. (1993a) Chemistry of free radical damage to DNA and nucleoproteins. In: *DNA and free radicals*, Edited by Halliwell, B. and Aruoma, O.I.

Dizdaroglu, M. (1993b) Quantitative determination of oxidative base damage in DNA by stable isotope-dilution mass spectrometry. *FEBS Letts.* **315**, 1-6.

Dizdaroglu, M. (1994) Chemical determination of oxidative DNA damage by gas chromatography-mass spectrometry. *Meth. Enzymol.* **234**, 3-16.

Djuric, Z., Luongo, D.A., and Harper, D.A. (1991a) Quantitation of 5-(hydroxymethyl)uracil in DNA by gas chromatography with mass spectral detection. *Chem. Res. Toxicol.* **4**, 687-691.

Djuric, Z., Heilbrun, L.K., Reading, B.A., Boomer, A., Valeriote, F.A., and Martino, S. (1991b) Effects of a low-fat diet on levels of oxidative damage to DNA in human peripheral nucleated blood cells. *J. Nation. Cancer Inst.* **83**, 766-769.

Djuric, Z., Lu, M.H., Lewis, S.M., Luongo, D.A., Chen, X.W., and Heilbrun, L.K. (1992) Oxidative DNA damage levels in rats fed low-fat, high fat, or calorie-restricted diets. *Toxicol. App. Pharmacol.* **115**, 156-160.

Doetsch, P.W. (1990) Repair of oxidative DNA damage in mammalian cells. In: *Oxidative damage and repair. Chemical, Biological and Medical aspects.* 192-197. Edited by Davies, K.J.A. Pergamon Press.

Doll, R. and Peto, R. (1981) The causes of cancer: Quantitative estimates of avoidable risks of cancer in the United States today. *J. Nation. Cancer Inst.* **66**, 1192-1136.

Douki, T., Delatour, T., Paganon, F., and Cadet, J. (1996) Measurement of oxidative damage at pyrimidine bases in γ–Irradiated DNA. *Chem. Res. Toxicol.* **9**, 1145-1151.

Dunn, B.P. and San, R.H.C. (1988) HPLC enrichment of hydrophobic DNA-carcinogen adducts for enhanced sensitivity of ^{32}P-Postlabeling analysis. *Carcinogenesis* **9**, 1055-1060.

Everson, R.B., Randerath, E., Santella, R.M., Cefalo, R.C., Avitts, T.A., and Randerath, K. (1986) Detection of smoking-related covalent DNA adducts in Human placenta. *Science* **231**, 54-57.

Farber, E. (1982) Chemical carcinogenesis, a biological perspective. *Amer. J. Pathol.* **2**, 271-296.

Farber, E. (1987) Possible etiologic mechanisms in chemical carcinogenesis. *Environ. Health Perspect.* **75**, 65-70.

Farber, J.L. (1994) Mechanisms of cell injury by activated oxygen species. *Environ. Health Perspect.* **102**, 17-24.

Farmer, P.B. (1994) Carcinogen adducts : Use in diagnosis and risk assessment. *Clin. Chem.* **40**, 1438-1443.

Faure, H., Incardona, M., Boujet, C., Cadet, J., Ducros, V., and Favier, A. (1993) Gas chromatographic-mass spectrometric determination of 5-hydroxymethyluracil in human urine by stable isotope dilution. *J. Chromatogr.* **616**, 1-7.

Floyd, R.A., Watson, J.J., Wong, P.K., Altmiller, D.H., and Rickard, R.C. (1986) Hydroxyl free radical adduct of deoxyguanosine: Sensitive detection and mechanisms of formation. *Free Rad. Res. Comms.* **1**, 163-172.

Floyd, R.A., West, M.S., Eneff, K.L., and Schneider, J.E. (1989) Methylene blue plus light mediates 8-hydroxyguanine formation in DNA. *Arch. Biochem. Biophys.* **273**, 106-111.

Floyd, R.A. (1990) DNA damage and repair. In: *Oxidative damage and repair. Chemical, Biological and medical aspects.* 175-181. Edited by Davies, K.J.A. Pergamon Press.

Fraga, C.G., Shigenaga, M.K., Park, J., Degan, P., and Ames, B.N. (1990) Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *Proc. Natl. Acad. Sci. USA* **87**, 4533-4537.

Fraga, C.G., Motchnik, P.A., Shigenaga, M.K., Helbock, H.J., Jacob, R.A., and Ames, B.N. (1991) Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc. Natl. Acad. Sci. USA* **88**, 1103-1106.

Frenkel, K., Goldstein, M.S., Duker, N.J., and Teebor, G.W. (1981a) Identification of the cis-thymine glycol moiety in oxidized deoxyribonucleic acid. *Biochem.* **20**, 750-754.

Frenkel, K., Goldstein, M.S., and Teebor, G.W. (1981b) Identification of the cis-thymine glycol moiety in chemically oxidized and γ–irradiated deoxyribonucleic acid by high pressure liquid chromatography analysis. *Biochem.* **20**, 7566-7571.

Frenkel, K., Cummings, A., Solomon, J., Cadet, J., Steinberg, J.J., and Teebor, G.W. (1985) Quantitative determination of the 5-(hydroxymethyl)uracil moiety in the DNA of γ–irradiated cells. *Biochem.* **24**, 4527-4533.

Frenkel, K., Chrzan, K., Troll, W., Teebor, G.W., and Steinberg, J.J. (1986) Radiation-like modification of bases in DNA exposed to tumor promoter-activated polymorphonuclear leukocytes. *Cancer Res.* **46**, 5533-5540.

Fridovich, I. (1983) Superoxide radical: An endogenous toxicant. *Ann. Rev. Pharmacol. Toxicol.* 239-257.

Fridovich, I. (1989) Superoxide dismutases; An adaption to a paramagnetic gas. *J. Biol. Chem.* **264**, 7761-7764.

Friedbarg, E.C. (1985) *DNA repair.* W.H. Freeman and Company.

Fuciarelli, A.F., Wegher, B.J., Gajewski, E., Dizdaroglu, M., and Blakely, W.F. (1989) Quantitative measurement of radiation-induced base products in DNA using gas chromatography- mass spectrometry. *Radiat. Res.* **119**, 219-231.

Fugita, S. and Steenken, S. (1981) Pattern of OH radical addition to uracil and methyl- and carboxyl-substituted uracils. Electron transfer of OH adducts with the N,N,N1,N1-tetramethyl-p-phenylenediamine and tetranitromethane. *J. Am. Chem. Soc.* **103**, 2540-2545.

Gajewski, E., Rao, G., Nackerdien, Z., and Dizdaroglu, M. (1990) Modification of DNA bases in mammalian chromatin by radiation-generated free radicals. *Biochem.* **29**, 7876-7882.

Gates, F.T. and Linn, S. (1977) Endonuclease from Escherichia Coli that acts specifically upon duplex DNA damaged by ultraviolet, osmium tetroxide, acid, or X-rays. *J. Biol. Chem.* **252**, 2802-2807.

Gerschman. K., Gilbert, D.L., Nye, S.W., Dwyer S., Fenn, W.O. (1954) Oxygen poisoning and x-irradiation: a mechanism in common. *Science* **119**, 623-626.

Greeenberg, E.R., Baron, J.A., Tosteson, T.D., Freeman, D.H., Beck, G.J., Bond, J.H., Collachio, T.A., Coller, J.A., Frankl, H.D., Haile R.W., Mandel, J.S., Nierenberg, D.W., Rothstein, R., Snover, D.C., Stevens, M.M., Summers, R.W., and van Stolk, R.W. (1994) A clinical trial of antioxidant vitamins to prevent colorectal adenoma. N. Eng. J. Med. 331, 141-147.

Groner, Y., Elroy-Stein, O., Avraham, K.B., Yarom, R., Schickler, M., Knobler, M., and Rotman, G. (1990) Downs syndrome clinical symptoms are manifested in transfected cells and transgenic mice overexpressing the human Cu/Zn-superoxide dismutase gene. *J. Physiol.* **84**, 53-77.

Gupta, R.C. (1985) Enhanced sensitivity of ^{32}P-Postlabeling analysis of aromatic carcinogen: DNA adducts. *Cancer Res.* **45**, 5656-5662.

Gupta, R.C. and Randerath, K. (1988) Analysis of DNA adducts by ^{32}P-labeling and thin layer chromatography. In: *DNA repair, A laboratory manual of research procedures.* 399-418. Edited by Freidberg, E.C. and Hanawalt, P.C. New York, Marcel Dekker.

Gupta, R.C. and Earley, K. (1988) ^{32}P-adduct assay: comparative recoveries of structurally diverse DNA adducts in the various enhancement procedures. *Carcinogenesis* **9**, 1687-1693.

Gutteridge, J.M.C. and Stock, J. (1976) Peroxidation of cell lipids. *Med. Lab. Sci.* **33**, 281-285.

Gutteridge, J.M.C. and Halliwell, B. (1994) *Antioxidants in nutrition, health and disease.* Oxford University Press.

Halket, J.M. (1993) Derivatives for Gas Chromatography-Mass Spectrometry. In: *Handbook of derivatives for chromatography.* Second Ed., 297-322. Edited by Blau, K. and Halket, J. John Wiley & Sons.

Halliwell, B. (1984) Lipid peroxidation, oxygen radicals, cell damage and antioxidant therapy. *The Lancet* 1396-1397.

Halliwell, B. and Gutteridge, J.M.C. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. (Review article). *Biochem. J.* **219**, 1-14.

Halliwell, B. and Gutteridge, J.M.C. (1986) Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* **246**, 501-514.

Halliwell, B. and Gutteridge, J.M.C. (1989) *Free radicals in Biology and Medicine.* Second Edition Ed., Clarendon Press Oxford.

Halliwell, B. and Gutteridge, J.M.C. (1990a) Role of free radicals and catalytic metal ions in Human disease: An overview. *Meth. Enzymol.* **186**, 1-85.

Halliwell, B. and Gutteridge, J.M.C. (1990b) The antioxidants of human extracellular fluids. *Arch. Biochem. Biophys.* **280**, 1-8.

Halliwell, B. and Aruoma, O.I. (1991) DNA damage by oxygen-derived species. *FEBS Letts.* **281**, 9-19.

Halliwell, B. and Dizdaroglu, M. (1992) The measurement of oxidative damage to DNA by HPLC and GC-MS techniques. *Free Rad. Res. Comms.* **16**, 75-87.

Halliwell, B., Gutteridge, J.M.C., and Cross, C.E. (1992) Free radicals, antioxidants and human disease: where are we now? *J. Lab. Clinic. Med.* **119**, 598-620.

Halliwell, B. (1994) Free radicals, antioxidants, and human disease : curiosity, cause, or consequence ? *The Lancet* **344**, 721-724.

Halliwell, B. and Cross, C.E. (1994) Oxygen-derived species: Their relation to human disease and environmental stress. *Environ. Health Perspect.* **102**, 5-12.

Hamberg, M. and Zhang, L. (1995) Quantitative determination of 8-hydroxyguanine and guanine by isotope dilution mass spectrometry. *Anal. Biochem.* **229**, 336-344.

Hegi, M.E., Sagelsdorff, P., and Lutz, W.K. (1989) Detection by ^{32}P-Postlabeling of thymidine glycol in γ–irradiated DNA. *Carcinogenesis* **10**, 43-47.

Heinonen, O.P., Huttunen, J.K., Albanes, D., Haapakoski, J., Palmgren, J., Pietinen, P., Pikkarainen, J., Rautalahti, M., Virtamo, J., Edwards, B.K., Malila, N., Rapola, S., Jokinen, P., and Karjalainen, A. (1994) The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N. Eng. J. Med.* **330**, 1029-1035.

Hemminki, K., Szyfter, K., Vodicka, P., Koivisto, P., Mustonen, R., and Reunanen, A. (1991) Quantitative aspects of ^{32}P-Postlabeling. In: *New Horizons of biological dosimetry.* 219-228. Edited by Gledhill, B.L. and Mauro, F. Wiley-Liss.

Herbert, K.E., Evans, M.D., Finnegan, M.T.V., Farooq, S., Mistry, N., Podmore, I.D., Farmer, P., and Lunec, J. (1996) A novel HPLC procedure for the analysis of 8-oxoguanine in DNA. *Free Rad. Biol. Med.* **20**, 467-473.

Hinshaw, J.V. and Ettre, L.S. (1994) *Introduction to open-tubular column chromatography.* Advanstar Communications.

Hollstein, M.C., Brooks, P., Linn, S., and Ames, B.N. (1984) Hydroxymethyluracil DNA glycosylase in mammalian cells. *Proc. Natl. Acad. Sci. USA* **81**, 4003-4007.

Holtz, O., Krause, T., Scherer, G., Schmidt-PreuB, U., and Rudiger, H.W. (1990) ^{32}P-Postlabelling analysis of DNA adducts in monocytes of smokers and passive smokers. *Int. Arch. Occup. Environ. Health* **62**, 299-303.

Ida, S. and Hayatsu, H. (1970) The permanganate oxidation of thymine. *Biochim. Biophys. Acta* **213**, 1-13.

Ide, H., Kow, Y.W., and Wallace, S.S. (1985) Thymine glycols and urea residues in M13 DNA constitute replicative blocks *in vitro*. *Nucleic Acids Res.* **13**, 8032-8052.

Ide, H., Kow, Y.W., Chen, B.-X., Erlanger, B.F., and Wallace, S.S. (1996) Properties of antibodies elicited to 7-hydro-8-oxopurines and their reaction with X-irradiated DNA. *Unknown* (Abstract)

Imlay, J.A. and Linn, S. (1988) DNA damage and oxygen radical toxicity. *Science* **240**, 1302-1309.

Jaruga, P., Zastawny, T.H., Skokowski, J., Dizdaroglu, M., and Olinski, R. (1994) Oxidative DNA base damage and antioxidant enzyme activities in human lung cancer. *FEBS Letts.* **341**, 59-64.

Joenje, H. (1983) Oxygen: Our major carcinogen ? *Med. Hypoth.* **12**, 55-60.

Joenje, H. (1989) Genetic toxicology of oxygen.(Review). *Mut. Res.* **219**, 193-208.

Johnstone, R.A.W. and Rose, M.E. (1996) *Mass spectrometry for chemists and biochemists.* Second Edition Ed., Cambridge University Press.

Jones, N.J. and Parry, J.M. (1992) The detection of DNA adducts, DNA base changes and chromosome damage for the assessment of exposure to genotoxic pollutants. *Aquatic Toxicol.* **22**, 323-344.

Kaneko, M. and Leadon, S.A. (1986) Production of thymine glycols in DNA by N-hydroxy-2-naphthylamine as detected by a monoclonal antibody. *Cancer Res.* **46**, 71-75.

Kasai, H. and Nishimura, S. (1984) Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Res.* **12**, 2137-2145.

Kow, Y.W., Wallace, S.S., and Van Houten, B. (1990) UvrABC nuclease complex repairs thymine glycol, an oxidative DNA base damage. *Mut. Res.* **235**, 147-156.

Kresbach, G.M., Itani, M., Saha, M., Rogers, E.J., Vouros, P., and Giese, R.W. (1989) Ester and related derivatives of ring N-pentafluorobenzylated 5-hydroxymethyluracil. Hydrolytic stability, mass spectral properties, and trace detection by gas chromatography-electron-capture detection, gas chromatography-electron-capture negative ion mass spectrometry, and moving belt liquid chromatography-electron-capture negative ion mass spectrometry. *J. Chromatogr.* **476**, 423-438.

Leadon, S.A. and Hanawalt, P.C. (1983) Monoclonal antibody to DNA containing thymine glycol. *Mut. Res.* **112**, 191-200.

Leadon, S.A. (1988) Immunological probes for lesions and repair patches in DNA. In: *DNA repair. A laboratory manual of research procedures.* 311-326. Edited by Freidberg, E.C. and Hanawalt, P.C. New York, Dekker.

Leadon, S.A., Stampfer, M.R., and Bartley, J. (1988) Production of oxidative DNA damage during the metabolic activation of benzo[a]pyrene in human mammary epithelial cells correlates with cell killing. *Proc. Natl. Acad. Sci. USA* **85**, 4365-4368.

Lin, J. and Sancar, A. (1989) A new mechanism for repairing oxidative damage to DNA: (A)BC Exinuclease removes AP sites and thymine glycols from DNA. *Biochem.* **28**, 7979-7984.

Loft, S., Vitisen, K., Ewertz, M., Tjonneland, A., Overvad, K., and Poulsen, H.E. (1992) Oxidative DNA damage estimated by 8-hydroxyguanosine excretion in humans: influence of smoking, gender and body mass index. *Carcinogenesis* **13**, 2241-2247.

Loft, S. and Poulsen, H.E. (1996) Cancer risk and oxidative DNA damage in man. *J. Mol. Med.* **74**, 297-312.

Lutgerink, J.T., Graaf, E.d., Hoebee, B., Stavenuitez, H.F.C., Westra, J.G., and Kriek, E. (1992) Detection of 8-hydroxyguanine in small amounts of DNA by ^{32}P-Postlabeling. *Anal. Biochem.* **201**, 127-133.

Lynch, R.E. and Fridovich, I. (1978) *J. Biol. Chem.* **253**, 4697

Malins, D.C. and Haimanot, R. (1991) Major alterations in the nucleotide structure of DNA in cancer of the female breast. *Cancer Res.* **51**, 5430-5432.

Malins, D.C. (1993) Identification of hydroxyl radical-induced lesions in DNA base structure: Biomarkers with a putative link to cancer development. *J. Toxicol. Environ. Health* **40**, 247-261.

Malins, D.C., Holmes, E.H., Polissar, N.L., and Gunselman, S. (1993) The etiology of breast cancer. Characteristic alterations in hydroxyl radical-induced DNA base lesions during oncogenesis with potential for evaluating incidence risk. *Cancer Letts.* **71**, 3036-3042.

Malone, M.E., Symons, M.C.R., and Parker, A.W. (1993) An EPR study of photoionised thymine and its derivatives at 77K. *J. Chem. Soc. Perkin Trans II* 2067-2075.

Markey, S.P., Markey, C.J., Wang, T.L., and Rodriguez, J.B. (1993) Gas chromatographic-mass spectrometric method for the assessment of oxidative damage to double stranded DNA by quantification of thymine glycol residues. *J. Am. Soc. Mass Spectrom.* **4**, 336-342.

McCloskey, J.A. (1974) *Basic principles in nucleic chemistry.* Edited by PTs'o, P.O. Academic Press, New York.

Mossman, B.T. and Marsh, J.P. (1989) Evidence supporting the role for active oxygen species in asbestos-induced toxicity & lung disease. *Environ. Health Perspect.* **81**, 91-94.

Mouret, J.F., Polverelli, M., Sarrazini, F., and Cadet, J. (1991) Ionic and radical oxidations of DNA by hydrogen peroxide. *Chem. Biol. Interact.* **77**, 187-201.

Musarrat, J. and Wani, A.A. (1994) Quantitative immunoanalysis of promutagenic 8-hydroxy-2'-deoxyguanosine in oxidized DNA. *Carcinogenesis* **15**, 2037-2043.

Myles, G.M. and Sancar, A. (1989) Review : DNA repair. *Chem. Res. Toxicol.* **2**, 197-226.

Nackerdien, Z., Olinski, R., and Dizdaroglu, M. (1992) DNA base damage in chromatin of γ–irradiated cultured human cells. *Free Rad. Res. Comms.* **16**, 259-273.

Nagashima, M., Tsuda, H., Takenoshita, S., Nagamachi, Y., Hirohashi, S., Yokoto,J. Kasai H. (1995) 8-hydroxydeoxyguanosine levels in DNA of human breast cancer are not significantly different from those of non-cancerous breast tissues by HPLC-ECD method. *Cancer Lett.* **90**, 157-162.

Naritsin, D.B. and Markey, S.P. (1996) Assessment of DNA oxidative damage by quantification of thymidine glycol residues using gas chromatography/electron capture negative ionisation mass spectrometry. *Anal. Biochem.* **241**, 35-41.

Oberley, L.W. and Buettner, G.R. (1979) Role of superoxide dismutase in cancer: A review. *Cancer Res.* **39**, 1141-1149.

Oberley, L.W. and Oberley, T.D. (1986) Free radicals, cancer and aging. In: *Free radicals, aging and degenerative diseases*, 325-371. Alan R. Liss, Inc.

Olinski, R., Zastawny, T., Budzbon, J., Skokowski, J., Zegarski, W., and Dizdaroglu, M. (1992) DNA base modifications in chromatin of human cancerous tissues. *FEBS* **309**, 193-198.

Park, E., Shigenaga, M.K., Degan, P., Korn, T.S., Kitzler, J.W., Wehr, C.M., Kolachana, P., and Ames, B.N. (1992) Assay of excised oxidative DNA lesions: Isolation of 8-oxoguanine and its nucleoside derivatives from biological fluids with a monoclonal antibody column. *Proc. Natl. Acad. Sci. USA* **89**, 3375-3379.

Phillips, D.H., Hemminki, K., Alhonen, A., Hewer, A., Grover, P.L. (1988) Monitoring occupational exposure to carcinogens: detection by ^{32}P-postlabelling of aromatic DNA adducts in white blood cells from iron foundry workers. *Mutation. Res.* **204**, 531-541

Pitot, H.C., Goldsworthy, T.L., Moran, S., Kennan, W., Glauert, H.P., Maronpot, R.R., and Campbell, H.A. (1987) A method to quantitate the relative initiating and promoting potencies of heptocarcinogenic agents in their dose-response relationships to altered hepatic foci. *Carcinogenesis* **8**, 1491-1499.

Pitot, H.C. and Dragan, Y.P. (1991) Facts and theories concerning the mechanisms of carcinogenesis. *FASEB J.* **5**, 2280-2286.

Podmore, K., Farmer, P.B., Herbert, K.E., Jones, G.D.D., and Martin, E.A. (1997) ^{32}P-Postlabelling approaches for the detection of 8-oxo-2'-deoxyguanosine-3'-monophosphate in DNA. *Mut. Res.*

Poirier, M.C. and Weston, A. (1991) DNA adduct determination in humans. In: *New Horizons in Biological Dosimetry.* 205-218. Wiley-Liss, Inc.

Potter, D.W. and Djuric, Z. (1990) Comparison of 8-hydroxydeoxyguanosine and 5-hydroxymethyluracil as products of oxidative DNA damage. *Biol. Reac. Inter. IV* 801-803.

Pryor, W.A. (1988) Why is the hydroxyl radical the only radical that commonly adds to DNA ? hypothesis: it has a rare combination of high electrophilicity high thermochemical reactivity and a mode of production that can occur near DNA. *Free Rad. Biol. Med.* **4**, 219-223.

Rajagopalan, R., Melamede, R.J., Laspia, M.F., Erlanger, B.F., and Wallace, S.S. (1984) Properties of antibodies to thymine glycol, a product of the radiolysis of DNA. *Radiat. Res.* **97**, 499-510.

Randerath, K., Reddy, M.V., and Gupta, R.C. (1981) ^{32}P-Labeling test for DNA damage. *Proc. Natl. Acad. Sci. USA* **Vol. 78**, 6126-6129.

Randerath, E., Mittal, D., and Randerath, K. (1988) Tissue distribution of covalent DNA damage in mice treated dermally with cigarette ' tar ': preference for lung and heart DNA. *Carcinogenesis* **9**, 75-80.

Randerath, E., Miller, R.H., Mittal, D., Avitts, T.A., Dunsford, H.A., and Randerath, K. (1989) Covalent DNA damage in tissues of cigarette smokers as determined by ^{32}P-Postlabeling assay. *J. Nation. Cancer Inst.* **81**, 341-347.

Ravanat, J., Turesky, R.J., Gremaud, E., Trudel, L.J., and Stadler, R.H. (1995) Determination of 8-oxoguanine in DNA by Gas Chromatography-Mass Spectrometry and HPLC-Electrochemical Detection: Overestimation of the background level of the oxidized base by the Gas Chromatography-Mass spectrometry assay. *Chem. Res. Toxicol.* **8**, 1039-1045.(Abstract)

Reddy, C.C. (1990) Antioxidant enzymes: An overview. In: *Oxidative damage and repair: Chemical, Biological and Medical Aspects.* 595-601. Pergamon Press.

Reddy, M.V. and Randerath, K. (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-Postlabeling test for structurally diverse DNA adducts. *Carcinogenesis* **7**, 1543-1551.

Reddy, M.V., Bleicher, W.T., and Blackburn, G.R. (1991) ^{32}P-postlabeling detection of thymine glycols: evaluation of adduct recoveries after enhancement with affinity chromatography, nuclease P1, nuclease S1, and polynucleotide kinase. *Cancer Commun.* **3**, 109-117.

Richter, C., Park, J., and Ames, B.N. (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl. Acad. Sci. USA* **85**, 6465-6467.

Roti Roti, J.L. and Cerutti, P.A. (1974) Gamma-ray induced thymine damage in mammalian cells. *Int. J. Radiat. Biol.* **25**, 413-417.

Roti Roti, J.L., Stein, G.S., and Cerutti, P.A. (1974) Reactivity of thymine to γ rays in HeLa chromatin and nucleoprotein preparations. *Biochem.* **13**, 1900-1905.

Rueff, J., Laires, A., Bras, A., Borba, H., and Chaveca, T. (1990) DNA damage and oxygen species. In: *DNA repair mechanisms and their biological implications in mammalian cells.* 171-181. Edited by Lambert, M.W. and Lavai, J. New York, Plenum Press.

Sancar, A. and Tang, M. (1993) Nucleotide excision repair. *Photochem. Photobiol.* **57**, 905-921.

Saul, R.L., Gee, P. and Ames, B.N. (1987) Free radicals, DNA damage, and aging. In: Modern biological theories of aging. 113-129. Edited by Warner, H.R., Butler, R.N., Sprott, R.L. and Schneider, E.L. New York, Raven press.

Scott, G. (1995) Antioxidants the modern elixir ? *Chem. Br.* 879-882.

Scram, K.H. (1990) *Methods in Enzymology.* Edited by McCloskey, J.A. Academic Press.

Sevanian, A. (1990) Lipid damage and repair. In: *Oxidative damage and repair. Chemical, biological and medical aspects.* 543-548. Pergamon Press.

Sharma, M., Box, H.C., and Kelman, D.J. (1990a) Fluorescence postlabeling assay of cis-thymidine glycol monophosphate in x-irradiated calf thymus DNA. *Chem. Biol. Interact.* 107-117.

Sharma, M., Box, H.C., and Paul, C.R. (1990b) Detection and quantitation of 8-hydroxydeoxyguanosine 5'-monophosphate in x-irradiated calf-thymus DNA by fluorescence postlabeling. *Bioch. Biophys. Res. Commun.* **167**, 419-424.

Shibutani, S., Takeshita, M., and Grollman, A.P. (1991) Insertion of specific bases during DNA synthesis past the oxidation damaged base 8-oxodG. *Nature* **349**, 431-434.

Shigenaga, M.K., Gimeno, C.J., and Ames, B.N. (1989) Urinary 8-hydroxy-2'-deoxyguanosine as a biological marker of *in vivo* oxidative DNA damage. *Proc. Natl. Acad. Sci. USA* **86**, 9697-9701.

Shigenaga, M.K., Park, J., Cundy, K.C., Gimenco, C.J., and Ames, B.N. (1990) In vivo oxidative DNA damage: Measurement of 8-Hydroxy-2'-deoxyguanosine in DNA and urine by High-Performance Liqiud Chromatography with Electrochemical detection. *Meth. Enzymol.* **186**, 521-530.

Shigenaga, M.K., Aboujaoude, E.N., Chen, Q., and Ames, B.N. (1994) Assays of oxidative DNA damage biomarkers 8-oxo-2'-deoxyguanosine and 8-oxoguanine in nuclear DNA and biological fluids by high performance liquid chromatography with electrochemical detection. *Meth. Enzymol.* 16-33.

Sies, H. (1985) *Oxidative stress.* London, Academic Press.

Sies, H. (1991) *Oxidative stress II: oxidants and antioxidants.* second Ed., New York, Academic Press.

Simic, M.G., Bergtold, D.S., and Karam, L.R. (1989) Generation of oxy radicals in biosystems. *Mut. Res.* **214**, 3-12.

Slater, T.F. (1982) Lipid Peroxidation. *Biochem. Soc. Trans.* **10**, 70-71.

Slater, T.F. (1984) Free-radical mechanisms in tissue injury. *Biochem. J.* **222**, 1-15.

Spencer, J.P.E., Jenner, A., Aruoma, O.I., Evans, P.J., Kaur, H., Dexter, D.T., Jenner, P., Lees, A.J., Marsden, D.C., and Halliwell, B. (1994) Intense oxidative DNA damage promoted by L-DOPA and its metabolites Implications for neurodegenerative disease. *FEBS Letts.* **353**, 246-250.

Stadtman, E.R. (1992) Protein oxidation and aging. *Science* **257**, 1220-1224.

Steenken, S. (1989) Purine bases, nucleosides and nucleotides: aqueous solution redox chemistry and transformation reactions of their radical cations and e^- and OH adducts. *Chem. Rev.* **89**, 503-520.

Sun, Y. (1990) Free radicals, antioxidant enzymes, and carcinogenesis. *Free Rad. Biol. Med.* **8**, 583-599.

Tagesson, C., Kallberg, M., and Leanderson, P. (1992) Determination of urinary 8-Hydroxydeoxyguanosine by coupled-column high-performance liquid chromatography with electrochemical detection: a noninvasive assay for in vivo oxidative DNA damage in humans. *Toxicol. Meth.* **1**, 242-251.

Takahashi, M. and Asada, K. (1983) Superoxide anion permeability of phospholipid membranes and chloroplast thylakoids. *Arch. Biochem. Biophys.* **226**, 558-566.

Takeuchi, T. and Morimoto, K. (1994) Crocidolite asbestos increased 8-hydroxydeoxyguanosine levels in cellular DNA of a human promyelocytic leukemia cell line HL60. *Carcinogenesis* **15**, 635-639.

Takeuchi, T., Nakajima, M., Ohta, Y., Mure, K., Takeshita, T., and Morimoto, K. (1994) Evaluation of 8-hydroxydeoxyguanosine, a typical oxidative DNA damage, in human leukocytes. *Carcinogenesis.* **15**, 1519-1523.

Tappel, A.L. (1990) Lipid damage and repair: An overview. In: *Oxidative damage and repair. Chemical, Biological and Medical aspects.* 539-543. Pergamon Press.

Tchou, J., Kasai, H., Shibutani, S., Chung, M.-H., Laval, J., Grollman, A.P., and Nishimura, S. (1991) 8-oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. *Proc. Natl. Acad. Sci. USA* **88**, 4690-4694.

Tchou, J. and Grollman, A.P. Repair of DNA containing the oxidatively damaged base, 8-oxoguanine. (1993) *Mut. Res.* **229**, 277-287.

Teebor, G.W., Frenkel, K., and Goldstein, M.S. (1984) Ionizing radiation and tritium transmutation both cause formation of 5-hydroxy-2'-deoxyuridine in cellular DNA. *Proc. Natl. Acad. Sci. USA* **81**, 318-321.

Teebor, G., Cummings, A., Frenkel, K., Shaw, A., Voituriez, L., and Cadet, J. (1987) Quantitative measurement of the diastreoisomers of cis thymidine glycol in gamma-irradiated DNA. *Free Rad. Res. Comms.* **2**, 303-309.

Teebor, G.W., Boorstein, R.J., and Cannon, S.V. (1989) Enzymatic repair of oxidative DNA damage. *Free Rad. Res. Comms.* **6**, 185-187.

Teoule, R., Bert, C., and Bonicel, A. (1977) Thymine fragment damage retained in the DNA polynucleotide chain after gamma irradiation in aerated solutions.II. *Radiat. Res.* **72**, 190-200.

Teoule, R. and Cadet, J. (1978) Radiation-induced degradation of the base component in DNA and related substances-final products. In: *Effects of ionizing radiation radiation on DNA.* 171-203. Edited by Huttermann, J., Kohnlein, W., Toule, R., and Bertinchamps, A.J. Springer, New York.

Teoule, R. (1987) Radiation-induced DNA damage and its repair. *Int. J. Radiat. Biol.* **51**, 573-589.

Teoule, R. and Guy, A. (1987) Quantitative measurements of modified thymine residues in DNA chains by formic acid hydrolysis. *Nucleosid.Nucleotid.* **6 (1&2)**, 301-305.

Totter, J.R. (1980) Spontaneous cancer and its possible relationship to oxygen metabolism. *Proc. Natl. Acad. Sci. USA* **77**, 1763-1767.

van Poppel, G., Poulsen, H., Loft, S., and Verhagen, H. (1995) No influence of Beta Carotene on oxidative DNA damage in male smokers. *J. Nation. Cancer Inst.* **87**, 310-311.

Verhagen, H., Poulsen, H.E., Loft, S., Poppel, G.v., Willems, M.I., and van Bladeren, P.J. (1995) Reduction of oxidative DNA-damage in humans by Brussel sprouts. *Carcinogenesis.* **16**, 969-970.

von Sonntag, C. (1987) *The chemical basis of radiation biology.* Taylor & Francis, New York, NY.

Wagner, J.R., Hu, C.C., and Ames, B.N. (1992) Endogenous oxidative damage of deoxycytidine in DNA. *Proc. Natl. Acad. Sci. USA* **89**, 3380-3384.

Wallace, S.S. (1988) AP endonucleases and DNA glycosylases that recognise oxidative DNA damage. *Environ. Mol. Mut.* **12**, 431-477.

Waschke, S., Reefschlager, J., Barwolff, D., and Langen, P. (1975) 5-Hydroxymethyl-2'-deoxyuridine, a normal DNA constituent in certain Bacillus subtilis phages is cytostatic for mammalian cells. *Nature* **255**, 629-630.

Watson, W.P. (1987) Post-radiolabeling for detecting DNA damage. *Mutagen.* **2**, 319-331.

Weinfeld, M. and Soderlind, K.M. (1991) Postlabeling detection of radiation-induced DNA damage : Identification and estimation of thymine glycols and phosphoglycolate termini. Biochem. 30, 1091-1097.

Weitzman, S.A. and Graceffa, P. (1984) Asbestos catalyses hydroxyl & superoxide radical generation from hydrogen peroxide. *Biochem. Biophys.* **228**, 373-376.

West, G.J., West, I.W.-L., and Ward, J.F. (1982) Radioimmunoassay of a thymine glycol. *Radiat. Res.* **90**, 595-608.

Wilson, V.L., Taffe, B.G., Shields, P.G., Povey, A.C., and Harris, C.C. (1993) Detection and quantification of 8-hydroxydeoxyguanosine adducts in peripheral blood of people exposed to ionizing radiation. *Environ. Health Perspect.* **99**, 261-263.

Witt, E.H., Reznick, A.Z., Viguie, C.A., Starke-Reed, P., and Packer, L. (1992) Exercise, oxidative damage and effects of antioxidant manipulation. *J. Nutrition* **122**, 766-773.

Wolff, S.P., Garner, A., and Dean, R.T. (1986) Free radicals, lipids and protein degradation. *TIBS* **11**, 27-31.

Yin, B., Whyatt, R.M., Perera, F.P., Randall, M.C., Cooper, T.B., and Santella, R.M. (1995) Determination of 8-hydroxydeoxyguanosine by an immunoaffinity chromatography-monoclonal antibody-based ELISA. *Free Rad. Biol. Med.* **18**, 1023-1032.

Yoshida, H. and Hettich, R.L. (1994) Characterization of radiation-induced products of thymidine 3' - monophosphate and thymidylyl (3'-]5') thymidine by High-Presssure Liquid-Chromatography and laser desorption fourier-transform Mass Spectrometry. *Radiat. Res.* **139**, 271