# The Analysis and Identification of Urinary Metabolites of Vitamin E in Man Using Mass Spectrometry and Chemical Synthesis

by

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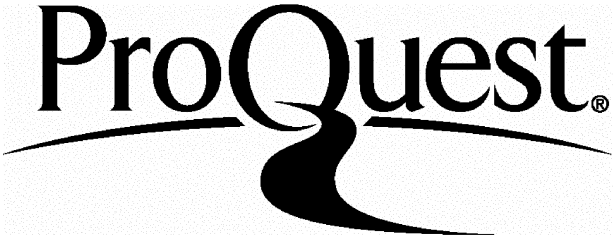

# ABSTRACT

Vitamin E (α-tocopherol) is the major lipid soluble antioxidant *in vivo* and is important for maintaining the integrity of cell membranes. Oxidative stress, defined as an imbalance between oxidants and antioxidants, has been implicated in the aetiology of numerous diseases. There is, therefore, interest in establishing methods to measure oxidative stress. It has been suggested that metabolites of vitamin E such as α-tocopheronolactone (α-TL), with an oxidised chroman ring, may be an indicator of *in vivo* oxidative stress and that the carboxyethyl-hydroxychromans (CEHCs), with a shortened phytyl side chain, may provide a measure of adequate or excess vitamin E status. However, doubts have been raised about the authenticity of α-TL since α-CEHC has been shown to be artefactually oxidised to α-TL in many of the procedures described.

In the course of the current study a relatively simple method using gas chromatography-mass spectrometry (GC-MS) was developed which allowed the reproducible measurement of a wide range of deconjugated vitamin E metabolites in urine. This method was used to study the urinary metabolites produced by normal subjects before and after supplementation with vitamin E. The CEHCs were confirmed as the major urinary metabolites of vitamin E, α-TL was detected and a novel group of metabolites, the carboxymethylbutyl-hydroxychromans (CMBHCs), was also tentatively identified.

A range of conjugated (sulphated and glucuronidated) and free metabolites of vitamin E were synthesised chemically and used to a) confirm the identity of α-CMBHC, b) provide standards for GC-MS and tandem mass spectrometry, c) elucidate the mechanism of artefactual oxidation and to develop new methods for the precise measurement of endogenously produced α-TL and d) investigate the type of conjugation of the various metabolites of vitamin E in human urine.

# ACKNOWLEDGEMENTS

I would especially like to thank my supervisor, Dr. David Muller, for his support and encouragement throughout the project. The financial support provided by Dr. David Muller for the completion of my thesis is also greatly appreciated.

I would like to thank Dr. Andrew Johnson, Dr. Mira Doig, Dr. Sarah Young, Mr Kevin Mills and Dr. Hugh Lemonde for their help in operating and maintaining the mass spectrometers. I would also like to acknowledge the friendship and help I have received from everyone at the Institute of Child Health.

A major part of my project involved the chemical synthesis of standards. Dr. David Madge was very kind in allowing me to work in the chemistry laboratories of the Wolfson Institute of Biomedical Research, even though I had very little previous practical experience of chemical synthesis. The patience and friendship of everyone in the chemistry laboratories is very much appreciated. I would particularly like to express my gratitude to Dr. Guillaume Burtin, without whose friendship, 'motivation' and technical expertise I would not have been able to synthesise the standards.

Thanks are also due to Prof. Peter Clayton, whose critical input at various stages of the project has been invaluable, and Dr. Nasi Mian, who has kindly read through the drafts of my thesis and given his own unique comments and observations over restorative pints of beer.

I would like to express my gratitude to my parents and grandparents for their support throughout my time at university.

Finally, I would like to thank the Engineering and Physical Science Research Council for funding this project.

# PUBLICATIONS

1. Pope SAS, Clayton PT, and Muller DPR (2000) A new method for the analysis of urinary vitamin E metabolites and the tentative identification of a novel group of compounds. *Arch.Biochem.Biophys.* **381**:8-15.

2. Pope SA, Burtin GE, Clayton PT, Madge DJ, and Muller DP (2001) New synthesis of (±)-α-CMBHC and its confirmation as a metabolite of α-tocopherol (vitamin E). *Bioorg.Med.Chem.* **9**:1337-1343.

# PRESENTATIONS

1. 1999 SFRR (Society for Free Radical Research) Europe Summer Meeting – Antioxidants, Adaption, Aging. Dresden, Germany, July $2^{nd}$ – $5^{th}$, 1999.

   Poster presentation: Pope SAS, Clayton PT and Muller DPR. A new method for the analysis of urinary vitamin E metabolites.

2. $11^{th}$ European Fat-Soluble Vitamins Group Meeting. Toulouse, France, March $9^{th}$-$11^{th}$, 2000.

   Oral presentation: Pope SAS, Clayton PT and Muller DPR. The analysis of urinary vitamin E metabolites in man.

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# ABBREVIATIONS

ACN - Acetonitrile

BSTFA – N,O-bis(trimethylsilyl)trifluoroacetamide

CEHC – Carboxy-ethyl-hydroxychroman

CID – Collision induced dissociation

CMBHC – Carboxy-methyl-butyl-hydroxychroman

CV – Coefficient of variation

d – Doublet

Da - Dalton

DMHQ – Dimethyl-hydroquinone

e - Electron

ECD – Electrochemical detection

EI – Electron impact

ER – Endoplasmic reticulum

ESI – Electrospray ionisation

FAB – Fast atom bombardment

GC – Gas chromatography

HCl – Hydrochloric acid

HDL – High density lipoprotein

HPLC – High performance liquid chromatography

HRMS – High resolution mass spectrometry

IDL – Intermediate density lipoprotein

IR – Infra red

LC-MS – Liquid chromatography-mass spectrometry

LDL – Low density lipoprotein

LOO• – lipid peroxyl radical

LOOH – lipid peroxide

LLU-α - Linda Loma University metabolite-α

m - Multiplet

MeOH – Methanol

MS – Mass spectrometry

MS-MS – Tandem mass spectrometry

$^{1}H$ NMR – Proton nuclear magnetic resonance

$^{13}C$ NMR – Carbon 13 nuclear magnetic resonance

PKC – Protein kinase C

PLTP – Plasma phospholipid transfer protein

$PP_2A$ – Protein phosphatase 2A

PUFA – Polyunsaturated fatty acid

ROS – Reactive oxygen species

rt – Room temperature

RT – Retention time

s – Singlet

SPE – Solid phase extraction

t - Triplet

α-TA - α-Tocopheronic acid

THF – Tetrahydrofuran

THQ – α-Tocopherylhydroquinone

α-TL - α-Tocopheronolactone

TLC – Thin layer chromatography

TOH – Tocopherol

TMS – Trimetylsilyl or tetramethylsilane

TQ – α-Tocopherylquinone (benzoquinone)

TQE1 – 5,6-epoxy-α-tocopherylquinone

TQE2 – 2,3-epoxy-α-tocopherylquinone

Trolox – 6-hydroxy-2,5,7,8-tetramethyl-chroma-2-yl-carboxylic acid

α-TTP - α-Tocopherol transfer protein

UV – Ultra violet

VLDL – Very low density lipoprotein

"'Oho," said the fly on the chariot wheel.
"What a dust we do raise."'

Carson McCullers (1917-1967)

# CHAPTER 1

# Introduction


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## 1.1. Background

### 1.1.1. Vitamins

Vitamins are described as any organic substance essential in minute amounts for normal growth and activity of the body and obtained naturally from plant and animal foods (The New Encyclopaedia Britannica, 1990).

The term 'vitamine' was originally used by the Polish chemist Casimir Funk who identified that the anti-beriberi substance in unpolished rice was an amine and since it was vital, he proposed the name vitamine i.e. vital amine. It was later discovered that different vitamins have different chemical properties and functions and that many of them are not amines. However, the term continued to be used, although the final 'e' was dropped. In 1912 Hopkins and Funk advanced the vitamin hypothesis of deficiency – a theory that postulates that the absence of sufficient amounts of a particular vitamin in a system may lead to certain diseases, such as scurvy (vitamin C deficiency) or beriberi (vitamin B1 deficiency) (see The New Encyclopaedia Britannica, 1990). Vitamins can be divided into two groups; water soluble such as vitamins in the B group and vitamin C and fat soluble such as vitamins A, D, E and K.

### 1.1.2. History of vitamin E

Vitamin E was first identified by Evans and Bishop in the 1920's, as a putative dietary factor present in lettuce and wheat germ, that prevented foetal resorption in rats fed a rancid lard diet (Evans and Bishop, 1922; Evans and Burr, 1925). Other animal species, such as chickens, guinea pigs and rabbits also displayed a variety of symptoms, for instance ataxia and muscle weakness, when fed diets deficient in vitamin E (Pappenheimer and Goettsch, 1931; Goettsch and Pappenheimer, 1931).

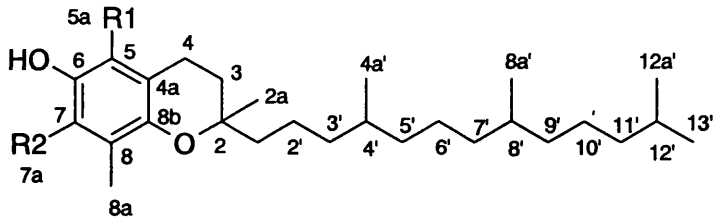
A chemically pure compound with vitamin E activity was first isolated from wheat germ oil by Evans et al. (Evans et al., 1936). It was partially characterised as an alcohol with the chemical formula $C_{29}H_{50}O_2$. The name tocopherol, from the Greek 'to bear offspring', was proposed because of its ability to prevent foetal resorption in rats. The prefixes, α-, β-, γ- and δ-, were used to distinguish between the different biologically active compounds that could be demonstrated in plant oils. Subsequently, the structural formula of α-tocopherol was published by Fernholz (1938).

Initial observations led to the notion that at least part of the function of vitamin E could be attributed to its ability to act as a lipid antioxidant (Olcott and Mattill, 1941). This hypothesis was substantiated further from the observations that diets containing high levels of polyunsaturated fatty acids (PUFAs), which are prone to oxidation, exacerbated vitamin E deficiency (Mackenzie et al., 1941).

### 1.2. Nomenclature and abundance of vitamin E homologues in the diet and body

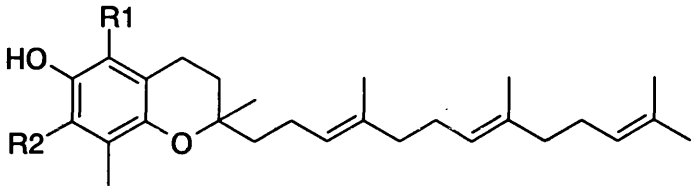
The term vitamin E refers to the tocopherols and tocotrienols, which have saturated and unsaturated phytyl side chains respectively. The α, β, γ and δ-tocopherols and tocotrienols differ in the number and position of methyl groups on the chroman ring (see figure 1.1).

The key structural features of the tocopherols and tocotrienols are the chroman ring and the phytyl side chain. The chroman ring is responsible for the antioxidant properties of tocopherols and tocotrienols, while the side chain is responsible for their lipid-solubility. This combination of lipid-solubility and antioxidant activity makes vitamin E ideally suited to protect lipid molecules from oxidative damage. Indeed, vitamin E has




tocopherols

α : R1=R2=$CH_3$
β : R1=$CH_3$, R2=H
γ : R1=H, R2=$CH_3$
δ : R1=R2=H




tocotrienols

| Name/Configuration | Relative biological activity (%) |
|---|---|
| 2R, 4'R, 8'R-α-tocopherol | 100 |
| 2R, 4'R, 8'S-α-tocopherol | 90 |
| 2R, 4'S, 8'S-α-tocopherol | 73 |
| 2R, 4'S, 8'R-α-tocopherol | 57 |
| 2S, 4'R, 8'R-α-tocopherol | 31 |
| 2S, 4'R, 8'S-α-tocopherol | 37 |
| 2S, 4'S, 8'R-α-tocopherol | 21 |
| 2S, 4'S, 8'S-α-tocopherol | 60 |
| RRR-α-tocopherol* | 100 |
| RRR-β-tocopherol | 50 |
| RRR-γ-tocopherol | 10 |
| RRR-δ-tocopherol | 3 |
| RRR-α-tocotrienol | 30 |
| RRR-β-tocotrienol | 5 |
| RRR-γ-tocotrienol | Not known |
| RRR-δ-tocotrienol | Not known |

**Figure 1.1.** The structures of the tocopherols and tocotrienols and their biological activity in the rat foetal resorption assay (Bunyan et al., 1961; Weiser and Vecchi, 1981; Weiser and Vecchi, 1982)
* RRR = 2R, 4'R, 8'R.

been shown to account for the majority of the lipid soluble antioxidant activity in human blood (Burton et al., 1983; Burton and Ingold, 1986).

Tocopherols have 3 chiral centres and can therefore exist as eight possible stereoisomers. Natural tocopherol is a single isomer, designated RRR, indicating it has the same stereochemistry at every chiral centre (positions 2, 4' and 8' in figure 1.1). Synthetic tocopherol made from trimethylhydroquinone and synthetic isophytol is an equimolar racemic mixture of all 8 different isomers and is therefore designated all-rac-tocopherol.

The principal forms of vitamin E in human and animal diets differ around the world depending mainly on the type of plant oils used. In North American diets RRR-γ-tocopherol is the most abundant form being 2-4 times in excess of RRR-α-tocopherol due to the widespread use of corn and soybean oil (Sheppard et al., 1993). Levels of α- and γ-tocopherol in European diets are similar to those in North America. In contrast, South East Asian diets contain tocotrienols in much higher abundance than European or North American diets due to the routine use of palm oils in cooking. However, generally over 90% of vitamin E in plasma and tissues is RRR-α-tocopherol (Traber et al., 1993). The preferential enrichment with α-tocopherol *in vivo* results from the presence of an α-tocopherol transfer protein (α-TTP), which is discussed below (section 1.3.1). Although α-tocopherol is the major tocopherol in the body, recent evidence (from a study where tissue tocopherol levels were measured in terminally ill or elective surgery patients) indicates that γ-tocopherol levels are higher than originally thought. Indeed, in some tissues such as muscle, skin and adipose, γ-tocopherol levels approach 50% of total vitamin E (Burton et al., 1998).

The biological activity of tocopherols and tocotrienols has been the subject of much argument particularly with regards to the relative activity of natural versus synthetic α-tocopherol found in food supplements. Vitamin E deficiency in rats leads to foetal death and resorption, which can be prevented by addition of vitamin E to the diet. The amount of vitamin E that is required to prevent foetal death and resorption varies widely depending on the tocopherol or tocotrienol given and its stereochemistry. RRR-α-tocopherol is the most potent form of vitamin E in preventing foetal resorption. The relative biological activities of the tocopherols and tocotrienols, assayed using the rat foetal resorption test, are shown in figure 1.1 (Bunyan et al., 1961; Weiser and Vecchi, 1981; Weiser and Vecchi, 1982). The difference in biological activity of natural and synthetic tocopherols and tocotrienols is attributed to their different affinities for α-TTP (section 1.3.1) and depends on the number and position of methyl groups around the chroman ring and the configuration of the asymmetric carbons of the side chain. The configuration of the C-2 carbon is considered to be of particular importance.

### 1.3. Absorption and transport of vitamin E in the body

Vitamin E is absorbed together with other fats from the small intestine (see figure 1.2). It is first solubilised into mixed micelles, formed by bile salts and the hydrolysis products of triglycerides. These micelles transport vitamin E to the brush-border membrane of the enterocytes lining the small intestine, where vitamin E is passed from these micelles across the brush border membrane to the interior of the enterocyte by passive diffusion (Hollander et al., 1975). In the enterocyte, vitamin E is packaged within chylomicrons (lipoprotein particles) which are secreted into the bloodstream via

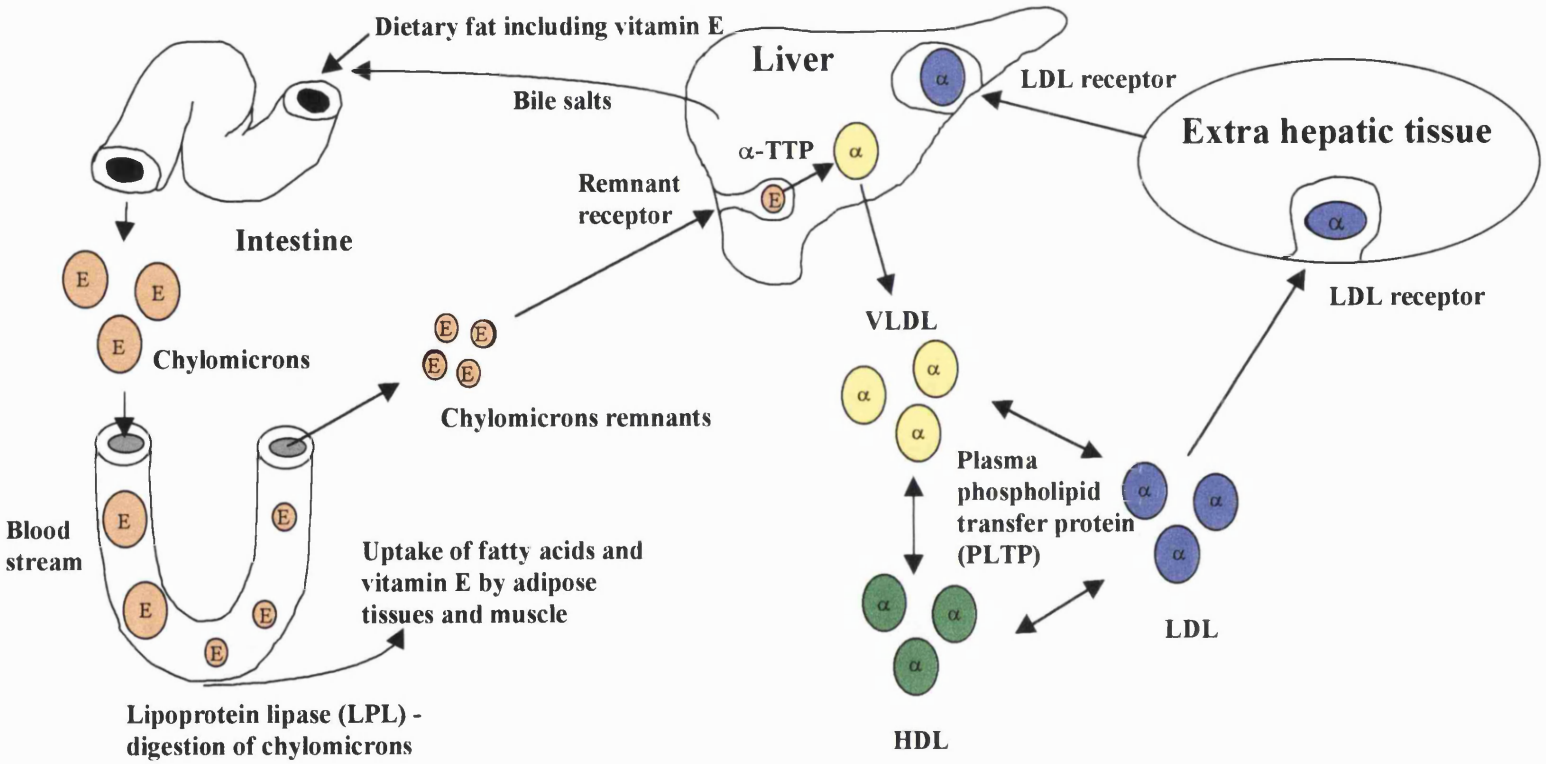



**Figure 1.2.** Absorption, transport and distribution of vitamin E. Vitamin E is absorbed from mixed micelles formed by bile salts and the hydrolysis products of triglycerides. It is then packaged within chylomicrons which are secreted into the bloodstream via the lymphatic system. These chylomicrons are digested by lipoprotein lipase attached to the wall of the capillaries forming chylomicron remnants. Receptor-mediated endocytosis of these remnants allows vitamin E to be taken up by the liver and to some extent by other tissues such as muscle and adipose tissue. Within the liver α-TTP preferentially loads α-tocopherol onto nascent VLDL which is secreted back into the circulation. α-Tocopherol is able to transfer between lipoproteins, possibly catalysed by the plasma lipid transfer protein ( PLTP). These lipoproteins deliver α-tocopherol to extra hepatic tissues and eventually return to the liver to recycle their α-tocopherol load. E = Vitamin E; α = α-Tocopherol.

the lymphatic system. High performance liquid chromatographic (HPLC) analysis of vitamin E within chylomicrons has shown the different forms of vitamin E are incorporated into the lipoprotein particles in similar ratios to those observed in the diet indicating that they are absorbed equally well during these initial steps (Traber et al., 1990). These chylomicrons are digested in the lumen of the capillaries by lipoprotein lipase (LPL), which is bound to the surface of the endothelial lining. The digestion products of chylomicrons (remnant particles) are taken up from the vascular circulation predominantly by liver cells via receptor mediated endocytosis. The high levels of γ-tocopherol in muscle, adipose and skin epithelial cells also suggest these tissues take up a portion of their vitamin E directly from chylomicrons by LPL activity.

During its recycling in the liver, α-tocopherol is secreted back into the bloodstream after its loading onto nascent very low density lipoprotein (VLDL). The preferential loading of different homologues and stereoisomers of vitamin E onto nascent VLDL is determined by an hepatic α-tocopherol transfer protein (α-TTP) which is described in greater detail in section 1.3.1.

α-Tocopherol and lipids within VLDL are able to transfer between different classes of lipoproteins such as low density lipoprotein (LDL), high density lipoprotein (HDL) and intermediate density lipoprotein (IDL). This transfer has been reported to be catalysed by the plasma phospholipid transfer protein (PLTP) (Kostner et al., 1995). Studies using deuterated RRR-α-tocopherol have shown that the α-tocopherol content of plasma chylomicrons increases after ingestion and then, gradually decreases over time (Traber et al., 1990). After chylomicron digestion by lipoprotein lipase, the concentration of α-tocopherol increases first in VLDL and then in the other lipoproteins. The distribution

of α-tocopherol between the various lipoproteins parallels their lipid content i.e. lipoproteins with higher lipid content contain greater amounts of α-tocopherol (Gallo-Torres, 1980). α-Tocopherol is delivered to tissues by either specific receptor-mediated uptake of these lipoproteins by cells via apolipoprotein receptors or direct transfer of lipid-soluble material between membranes. These lipoproteins are continually re-circulated via the liver, illustrating the pivotal role of hepatic α-TTP in maintaining plasma α-tocopherol levels.

### 1.3.1. α-tocopherol transfer protein (α-TTP)

A 31kDa protein with tocopherol binding properties in rat liver was described in 1991 (Sato et al., 1991). cDNA for the human homologue of this protein was later isolated and mapped to chromosome 8q13.1-13.3 (Arita et al., 1995). The human protein consists of 278 amino acids and has 94% homology to the rat protein. This protein, α-tocopherol transfer protein (α-TTP), is responsible for the preferential loading of nascent VLDL particles in the liver with α-tocopherol and lack of functional α-TTP in humans leads to a condition known as ataxia with vitamin E deficiency (AVED), which is discussed in more detail in section 1.4.1. The relative affinities of α-TTP for the different vitamin E homologues were determined by evaluating competition between labelled and non-labelled compounds for transfer between membranes *in vitro*, and the following relative affinities were found: RRR-α-tocopherol taken as 100%; RRR-β-tocopherol, 38%; RRR-γ-tocopherol, 9%; RRR-δ-tocopherol, 2%; RRR-α-tocopherol acetate, 2%; RRR-α-tocopherol quinone, 2%; SRR-α-tocopherol, 11%; R-α-tocotrienol, 12%; S-trolox (a water soluble α-tocopherol analogue; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 9% (Hosomi et al., 1997). There is a good

agreement between these relative affinities and the relative biological activity as judged by the rat resorption-gestation test (see figure 1.1). It appears from the data of Hosomi et al. (1997) and also from other data (Burton and Ingold, 1993; Burton et al., 1998) that the position and number of methyl groups around the chroman ring of the tocopherols and tocotrienols, as well as the stereochemistry at the C-2 position of the phytyl side chain are the most important determinants in the relative rate of transfer of tocopherol-type compounds by α-TTP. Thus, RRR-α-tocopherol has a higher activity than SRR-α-tocopherol because of the difference in stereochemistry at the C-2 position and SRR-α-tocopherol has a higher activity than RRR-δ-tocopherol due to the number and position of methyl groups around the chroman ring, even though it does not have optimal stereochemistry at the C-2 position of the phytyl side chain. Comparison of the excretion rates of metabolites of natural (RRR) versus synthetic (all-rac) α-TOH have also shown preferential retention of the natural stereoisomer (Traber et al., 1998).

Besides liver, the presence of low levels of mRNA for α-TTP has also been reported in the brain, spleen, lung and kidney of rats (Hosomi et al., 1998). Low level expression in these organs may be essential for the transfer and retention of α-tocopherol within these organs.

Recently α-TTP knockout mice have been generated (Terasawa et al., 2000; Jishage et al., 2001). In studies using these knockout mice it was shown that α-TTP was essential for maintaining α-tocopherol concentrations in the circulation and α-tocopherol deficiency increased the severity of atherosclerotic lesions in susceptible mice (Terasawa et al., 2000). It was also shown that α-tocopherol deficiency resulted in a marked reduction in placental labryrinthine trophoblasts, suggesting foetal death and

resorption is, in part, due to the greater susceptibility of the placenta to oxidative stress (Jishage et al., 2001). Interestingly, dietary supplementation with α-tocopherol or a synthetic antioxidant, BO-653, was able to prevent foetal death and resorption indicating the effect was not specific to α-tocopherol (Jishage et al., 2001).

## 1.4. Vitamin E and disease

### 1.4.1. Vitamin E deficiency

A range of vitamin E deficiency syndromes has been described in animal species. Symptoms vary within and between species and include ataxia, growth retardation, foetal resorption, encephalomalacia and myopathy (Pappenheimer and Goettsch, 1931; Dam et al., 1952; Wasserman and Taylor, 1972).

Severe and chronic deficiency of vitamin E in man results in a characteristic neurological syndrome (Muller and Goss-Sampson, 1990) and was first described in patients with severe fat malabsorption as a result of having the condition abetalipoproteinaemia (Muller et al., 1977). These patients lack all lipoproteins except HDL and therefore vitamin E cannot be absorbed or transported. Severe vitamin E deficiency and the same characteristic neurological syndrome has also been observed in patients with severe fat malabsorption caused by other factors, for example, in patients with obstructive jaundice, who lack the bile salts necessary for solubilisation of dietary fats (Elias et al., 1981), in patients with ileal resection (Harding et al., 1982) and occasionally in patients with cystic fibrosis (Elias et al., 1981).

The causal relationship between vitamin E deficiency and neurological dysfunction was confirmed in patients with a specific deficiency of vitamin E but without generalised fat

malabsorption. These patients have a rare condition called ataxia with vitamin E deficiency (AVED) (Ouahchi et al., 1995) resulting from a lack of functional hepatic α-tocopherol transfer protein (α-TTP) which, as discussed above, is necessary for the transfer of α-tocopherol to very low-density lipoproteins (VLDL). This results in impaired secretion of vitamin E back into the circulation and very low tissue and plasma levels of vitamin E.

In all the patient groups with severe vitamin E deficiency the neuropathy is progressive unless treated. Early treatment of vitamin E deficiency, either with intramuscular injection of α-tocopherol, oral supplements of water soluble vitamin E derivatives or large oral doses of α-tocopheryl acetate, prevents the appearance of neurological signs and symptoms. If signs and symptoms are present, treatment invariably halts progression and in some cases can produce objective improvement (Muller et al., 1977; Muller et al. 1983).

The neurological symptoms associated with vitamin E deficiency are thought to stem primarily from oxidative damage to neurons. Therefore, the role of vitamin E has also been investigated in other conditions where oxidative stress has been implicated including some cancers, atherosclerosis and diabetes (Stephens at al., 1996; Gey, 1998; Bursell and King, 1999; Shklar and Oh, 2000; Pryor, 2000). However, conclusive evidence showing that vitamin E supplementation prevents or slows the progress of these conditions is still lacking.

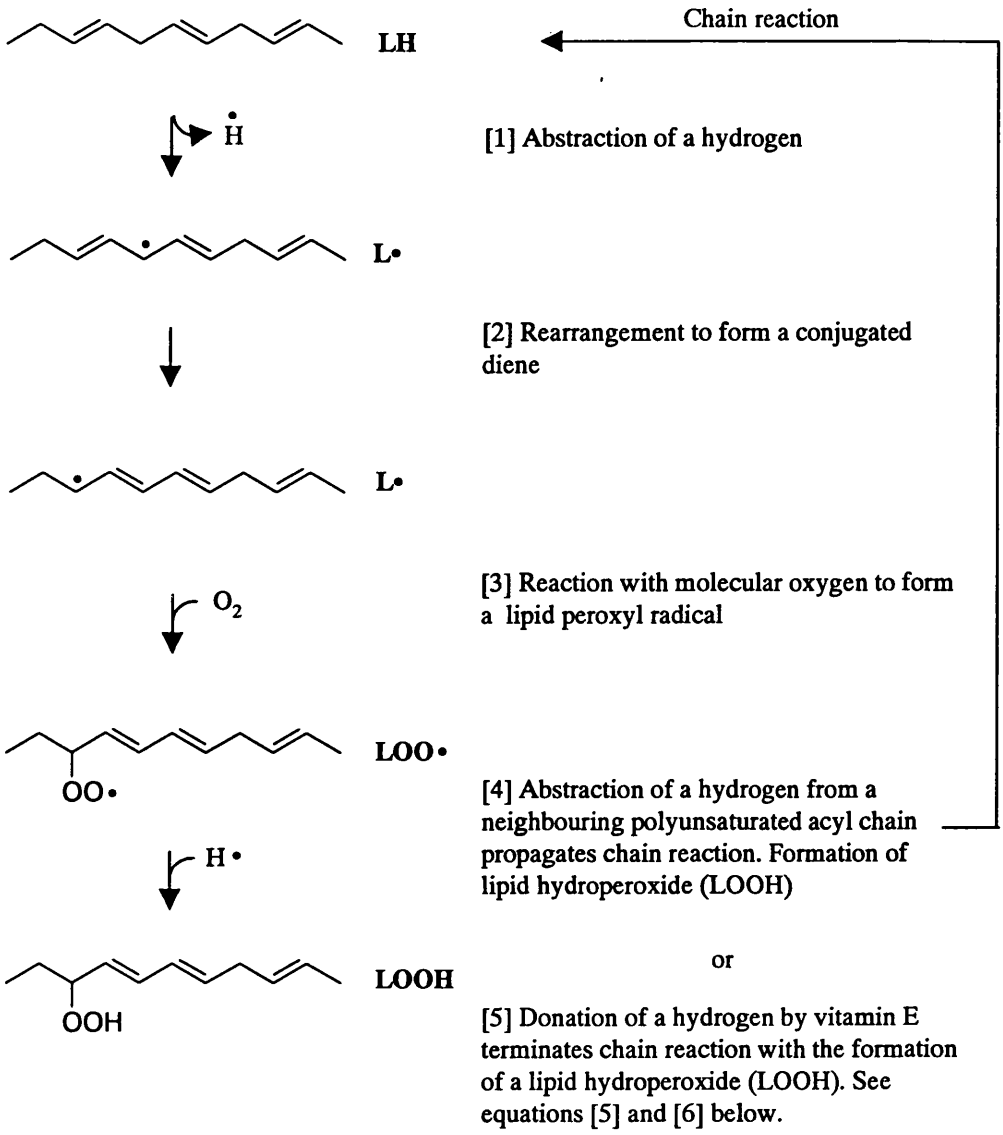
## 1.5. Functions of vitamin E

### 1.5.1. Oxidants, antioxidants and lipid peroxidation

A wide range of highly reactive oxidants are formed in biological systems by chemical reactions involving molecular oxygen. These reactive species are collectively known as reactive oxygen species (ROS) and include hydrogen peroxide ($H_2O_2$) oxygen derived free radicals such as peroxynitrite ($ONOO^-$), superoxide ($\bullet O_2^-$) and the hydroxyl radical ($\bullet OH$) (Halliwell and Gutteridge, 1999).

ROS are produced in normal biological processes such as the respiratory burst of macrophages against invading pathogens, the detoxification of drugs by the cytochrome P450 system and the activity of the mitochondrial respiratory chain (Halliwell and Gutteridge, 1999). They are also generated in biological systems as a result of exposure to a wide range of external factors including pollutants, heavy metals, ultra violet light and other forms of ionising radiation. The generation of these reactive species in an uncontrolled manner causes significant damage to a wide range of biological molecules including DNA, proteins, carbohydrates and lipids. Many of these processes are chain reactions starting with a single initiating radical species, followed by propagation to a large number of target molecules.

The process of fats becoming rancid – also known as lipid peroxidation - is a good example of how ROS can damage biological molecules. After standing at room temperature for prolonged periods, fats become rancid due to chemical reactions with atmospheric oxygen (Figure 1.3). The process involves a free radical chain reaction, which begins with the production of a carbon centred radical of a polyunsaturated fatty acyl chain (L•, [1]). The initial radical (L•), after rearrangement [2], reacts extremely




**Antioxidant reactions of α-tocopherol**

[5] TOH + LOO• ⟶ TO• + LOOH

[6] TO• + LOO• ⟶ NRP + LOOH

**Figure 1.3.** Lipid peroxidation and antioxidant reactions of α-tocopherol (TOH) with lipid peroxyl radicals (LOO•).
NB. One molecule of α-tocopherol (TOH) reacts with two lipid peroxyl radicals to form non-radical products (NRP).

rapidly with molecular oxygen ($O_2$) to form the peroxyl radical (LOO•, [3]). The peroxyl radical (LOO•) can then abstract a hydrogen atom from another polyunsaturated fatty acyl chain (LH) to generate a lipid hydroperoxide product (LOOH) and another carbon centred radical (L•) thereby continuing the chain reaction ([4]).

In order to prevent oxidative damage *in vivo* the body has an array of antioxidant compounds and enzymes, which in healthy subjects are able to scavenge or prevent production of these highly reactive oxidants. These antioxidants function in a variety of different ways and are localised within specific areas of the cell. These antioxidants include enzymes such as catalase, glutathione peroxidase and superoxide dismutase (SOD) as well as small molecules such as the lipid-soluble vitamin E and the water-soluble vitamin C (ascorbate), glutathione and urate. These antioxidants work in redox cycles with each other, eventually converting ROS and free radicals into harmless products. A breakdown in these defences against oxidants or a large increase in the production of oxidants leads to oxidative stress, which has been implicated in the aetiology of numerous diseases.

The biological activity of α-tocopherol has been attributed principally to its ability to act as an antioxidant, which prevents the peroxidation of lipids (reaction [5] and [6], figure 1.3; also see section 1.5.2). Antioxidants can be divided into two broad classes; a) preventive antioxidants, such as ascorbate (vitamin C), that reduce the rate of chain initiation and b) chain breaking antioxidants, such as α-tocopherol, that interfere with one or more of the propagation steps after the initial peroxidative process. α-Tocopherol

is considered to be the principal lipid soluble chain breaking antioxidant *in vivo* (Burton et al., 1983).

### 1.5.2. Vitamin E – reactions with oxidants

Peroxyl radicals are probably the principal oxidants scavenged by α-tocopherol in biological systems. Phenols, such as α-tocopherol, typically trap peroxyl radicals by a two step mechanism as indicated in general terms in figure 1.3 (equations [5] and [6]). First, a peroxyl radical abstracts a hydrogen atom from the phenol to produce a hydroperoxide and a phenoxyl radical ([5]). A second peroxyl radical is then trapped by a simple coupling reaction with the antioxidant phenoxyl radical to form a non-radical adduct (NRP) ([6]). Tocopherol forms the tocopheroxyl radical after abstraction of its hydrogen. The tocopheroxyl radical is unusually stable, owing to resonance stabilisation by the chroman ring, and therefore less likely to propagate the radical chain. However, unlike many phenols, subsequent reactions of the tocopheroxyl radical with peroxyl radicals are complicated and produce a number of non-radical products. Overall, each α-tocopherol molecule is capable of scavenging two radicals.

Numerous model systems have been used to study the antioxidant reactions of vitamin E type compounds. From these studies α-tocopherol has been shown to be the major lipid soluble antioxidant in human blood (Burton and Ingold, 1986). The antioxidant activity of the tocopherols has also been assessed, by measuring their ability to prevent the autoxidation of styrene and are in the order $\alpha > \beta \approx \gamma > \delta$ (Burton and Ingold, 1986). However in some other model systems γ-tocopherol has greater antioxidant activity than α-tocopherol, suggesting there are a number of factors which affect activity including

the oxidant used, the relative concentrations of antioxidant and oxidant as well as the solvent system (Kamal-Eldin and Appelqvist, 1996).

A number of studies have analysed the oxidation products of α-tocopherol in chemical model systems and more recently in mitochondria both *in vitro* and in perfused liver (Ham and Liebler, 1995; Liebler et al., 1996; Ham and Liebler, 1997). These investigations have revealed that two groups of non-radical products are generated after the reaction of α-tocopherol with peroxyl radicals (figure 1.4, (Liebler, 1993)). The first of these groups is the 8a-substituted tocopherones (**7,10**), which upon hydrolysis and rearrangement form α-tocopherylquinone, TQ (**4**). The second group consists of the epoxytocopherones (**8,9**) and their hydrolysis products, the 5,6- and 2,3- epoxy-α-tocopherolquinones, TQE1 and TQE2 (**5,6**) respectively. In order to simplify analysis, these oxidation products are often measured after dilute acid hydrolysis to yield just TQ, TQE1 and TQE2. *In vivo* studies in the perfused rat liver have revealed that α-TQ is the major oxidation product of α-tocopherol and its level increases after oxidative stress. Liebler et al. have shown that α-TQ is present in cell extracts in both benzoquinone and hydroquinone forms and that the ratio of these two forms varies widely, presumably dependent upon the levels of reductants and oxidants within the sample (Liebler et al., 1996; Ham and Liebler, 1997).

The reactions of vitamin E with other biologically important oxidants have also been investigated. Peroxynitrite, which is believed to be produced in the inflammatory response and has been suggested to be important in ischaemic injury and excitotoxicity, has been shown to be efficiently trapped by γ-tocopherol (Hoglen et al., 1997; Hoglen and Liebler, 1999). Although α-tocopherol also reacts with peroxynitrite, γ-tocopherol

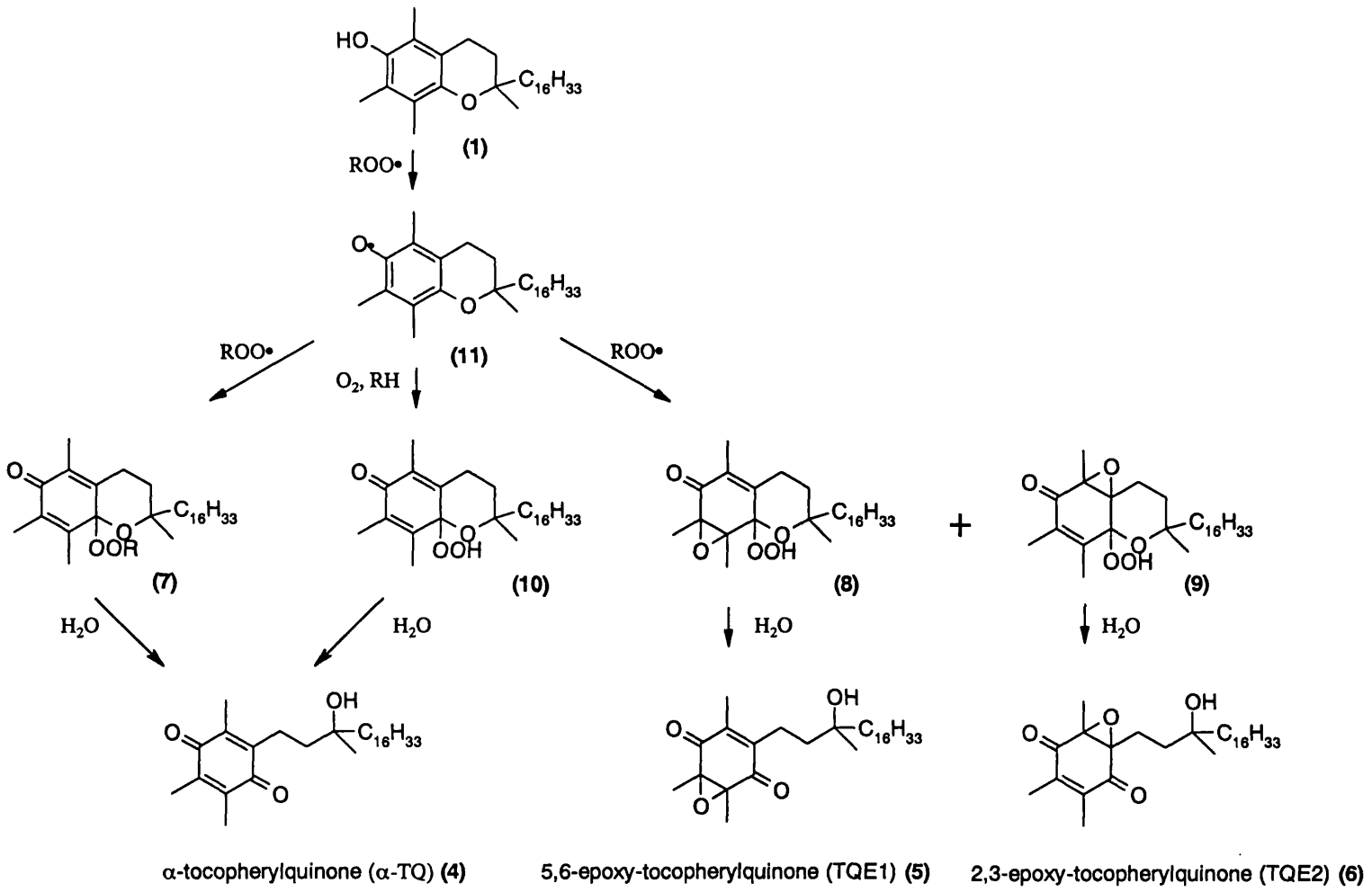



**Figure 1.4.** Reaction of α-tocopherol with peroxyl radicals derived from azo compounds in homogenous solution (Liebler, 1993)

has been shown to inhibit peroxynitrite-induced lipid peroxide formation to a greater extent (Christen et al., 1997). Since γ-tocopherol is the second most abundant tocopherol in the body, this complementary antioxidant role may be of biological importance, especially in tissues where high levels of γ-tocopherol have been observed, such as muscle, adipose and skin.

### 1.5.3. Non-antioxidant functions of vitamin E

Until recently direct evidence of non-antioxidant functions of α-tocopherol was lacking. However, within the past ten years, α-tocopherol has been shown to have a role in cell signalling, especially in relation to protein kinase C (PKC) (Azzi and Stocker, 2000). α-Tocopherol, in contrast to structurally related antioxidants such as trolox and β-tocopherol, is able to indirectly inhibit PKC-α activity by changing its phosphorylation state (figure 1.5). This alteration of PKC's phosphorylation state has been suggested to occur through the known α-tocopherol-mediated activation of protein phosphatase type 2A ($PP_2A$), which in turn dephosphorylates and inactivates PKC (Ricciarelli et al., 1998).

α-Tocopherol (but not related compounds) has been shown to inhibit the proliferation of vascular smooth muscle cells and the transcription of CD36 (a scavenger receptor) and collagenase genes (Azzi et al., 2001). α-Tocopherol is also reported to increase the expression of α-tropomyosin (Aratri et al., 1999) and connective tissue growth factor genes (Azzi et al., 2000). These various effects of α-tocopherol have been attributed to its ability to inhibit the PKC signalling cascade.

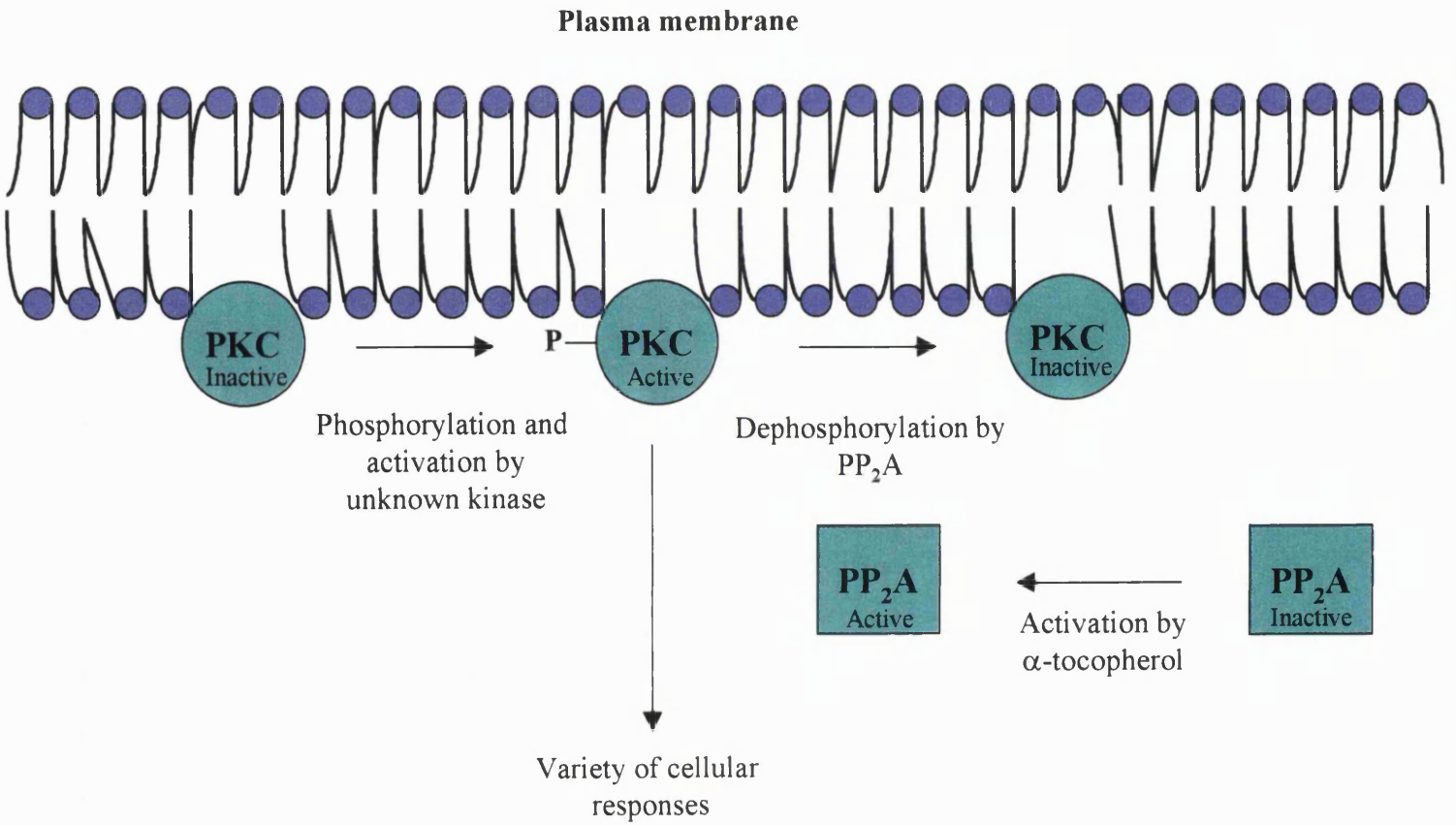



**Figure 1.5.** The activation of protein phosphatase type 2A ($PP_2A$) and inhibition of protein kinase C (PKC) by α-tocopherol and the effect on cellular responses

### 1.5.4. Vitamin E as a pro-oxidant

Although vitamin E is generally considered to function as an antioxidant it has been shown to have pro-oxidative effects *in vitro* under certain conditions. Stocker and co-workers have put forward a hypothesis known as tocopherol-mediated peroxidation (TMP), whereby under certain conditions α-tocopherol acts as a pro-oxidant and increases oxidation of low density lipoprotein (LDL) (Upston et al., 1999).

*In vitro* experiments have shown that under highly oxidising conditions (i.e. between 12 and 16 $Cu^{2+}$ ions per LDL particle) the α-tocopherol present in LDL was consumed rapidly and appeared to act as a conventional antioxidant. After α-tocopherol depletion, the rate of lipid peroxidation increased (Esterbauer et al., 1989). However under milder oxidation conditions than those used by Esterbauer and colleagues, the rate of lipid hydroperoxide (LOOH) formation actually increased in the early stages before α-tocopherol depletion (Bowry and Stocker, 1993). Supplementation with α-tocopherol also increased the rate of lipid hydroperoxide (LOOH) formation and this rate was shown to be greater in the presence of α-tocopherol than immediately after its depletion (Upston et al., 1999). These discrepancies concerning the peroxidation of LDL can be reconciled by the TMP hypothesis, which is described in figure 1.6.

According to the TMP hypothesis, α-tocopherol is proposed to react with the initial oxidant. This initial oxidant, a species such as the peroxyl radical, can interact with surface lipid or α-TOH within LDL. The radical is more likely to react with α-TOH since the peroxyl radical is approximately five times more reactive towards α-TOH despite surface lipids being more abundant than α-TOH. The α-tocopheroxyl radical formed (α-TO•) is 'trapped' within the lipoprotein and its fate is dependent on the

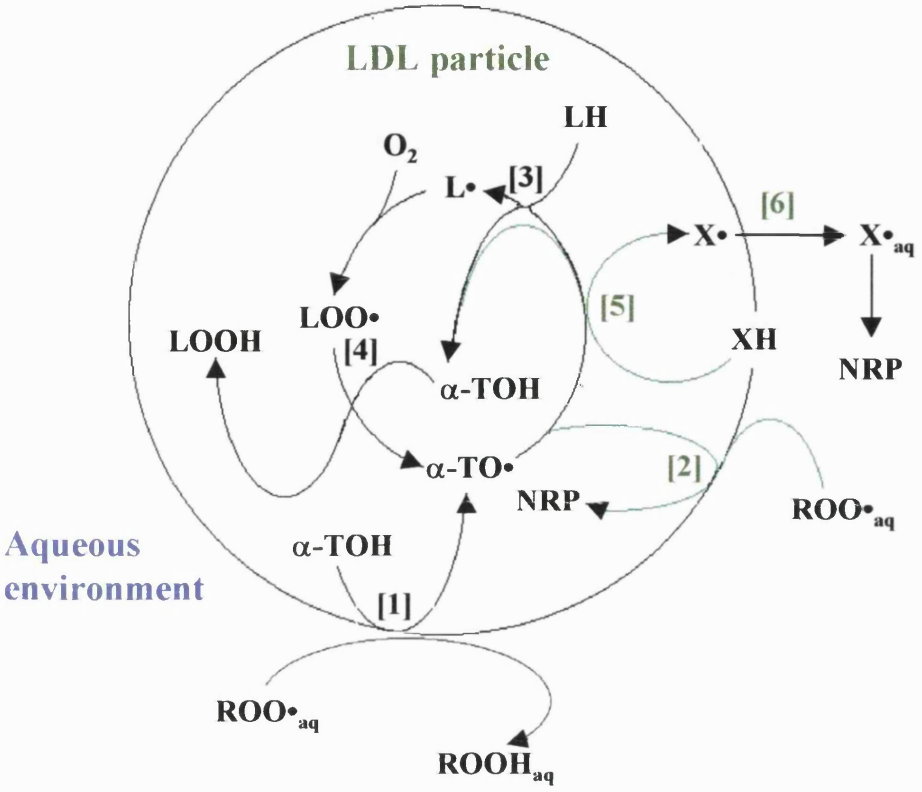



**Figure 1.6.** Tocopherol-mediated peroxidation (TMP). A peroxyl radical (ROO•) preferentially reacts with α-tocopherol (α-TOH), rather than with bisallylic hydrogens (LH) in LDL's core or surface lipids, forming the α-tocopheroxyl radical (α-TO•) within the LDL particle **[1]**. If sufficient ROO• is available, a second ROO• reacts with the LDL particle resulting in termination of lipid peroxidation via radical- radical reaction forming non-radical products (NRP) [2]. However, under conditions of milder radical flux, the relatively stable α-TO• will be isolated within the LDL particle and will react with LH to produce L•, which in turn will react with oxygen to form LOO• **[3]**. This step also regenerates α-TOH. Reaction of LOO• with α-TOH will lead to the formation of lipid hydroperoxide (LOOH) and another α-TO• **[4]**, which will continue the lipid peroxidation chain. This process allows the formation of many molecules of LOOH with little consumption of α-TOH. Co-antioxidants (XH) inhibit TMP by regenerating α-TOH from α-TO• [5]. The co-antioxidant derived radical (X•) may leave the LDL particle by diffusion into the aqueous space where it can react with other radicals or antioxidants to form non-radical products (NRP)[6]. Adapted from Upston et al.(1999).

oxidising conditions present. Under highly oxidising conditions termination reactions are likely to occur owing to interactions between radicals i.e. α-TO• and ROO•. However under milder conditions, such as those used by Stocker et al., α-TO• reacts predominantly with LH, eventually producing LOO• and starting a lipid peroxidation chain reaction (Bowry and Stocker, 1993).

### 1.5.5. Vitamin E recycling

Although it appears that α-TOH is capable of acting as a pro-oxidant *in vitro*, this does not necessarily mean that it occurs *in vivo*. The isolation of α-TO• in a LDL particle allows it to act as an pro-oxidant. However the destruction or elimination of α-TO• by a termination reaction with another radical or its reduction by a co-antioxidant would prevent TMP (**[5]** and **[6]**, figure 1.6). Other co-antioxidants *in vivo* such as ubiquinol-10, ascorbate (vitamin C) and α-tocopheryl hydroquinone (α-THQ) are believed to help to recycle α-TOH from α-TO• and thus prevent TMP.

Addition of ubiquinol-10 to LDL has been shown to increase the resistance of lipids within LDL to peroxidation by ROO• (Thomas et al., 1996). Addition of ubiquinol-10 with α-TOH also reduces the pro-oxidant activity of α-TOH in comparison to when α-TOH is added to LDL alone (Thomas et al., 1996). However the precise role of ubiquinol-10 as a co-antioxidant is unclear because of its low levels in LDL. It is generally present in less than one molecule per LDL particle so to act as an efficient co-antioxidant it would probably need to be able to reduce α-TO• present in more than one LDL particle.

Ascorbate, a ubiquitous aqueous antioxidant, has frequently been proposed to act synergistically with α-TOH. *In vitro* this has been shown by its ability to inhibit lipid peroxidation. This is in part due to its own antioxidant activity but is also due to its reduction of α-TO• (Doba et al., 1985; Kagan et al., 1992) and its concomitant oxidation to the relatively non-reactive ascorbyl radical. Even though ascorbate is highly polar and lipid insoluble it is suggested that it can interact with the chromanol head group of α-tocopherol, which can partition reversibly into the polar region of the lipid bilayer. The ascorbyl radical formed can then be reduced by other aqueous antioxidants and enzymes. However, so far the evidence to suggest that α-TOH is regenerated by ascorbate *in vivo* remains inconclusive (Burton et al., 1990).

A number of reports have also provided evidence that α-tocopherol may be regenerated from the tocopheroxyl radical via NAD(P)H dependent pathways in the mitochondrial (Maguire et al., 1989) and microsomal (Kagan et al., 1990) membranes. These studies have used electron spin resonance (ESR) to monitor tocopheroxyl radicals formed after liposome oxidation. The tocopheroxyl radical signal was suppressed after the addition of NADH and NADPH. However, since the direct antioxidant effects of NADPH-dependent antioxidant factors were not evaluated, the results do not necessarily indicate a tocopheroxyl recycling reaction.

The existence of an α-TOH regeneration mechanism is essential for maintaining its viability as an antioxidant. The epoxides (**5** and **6**, figure 1.4), described earlier as oxidation products of α-TOH, have been suggested to be products of non-antioxidant pathways (see figure 1.7; (Burton et al., 1993)). Epoxide formation is most likely to occur via addition of a peroxyl to either of the ortho carbons **[1]**, followed by

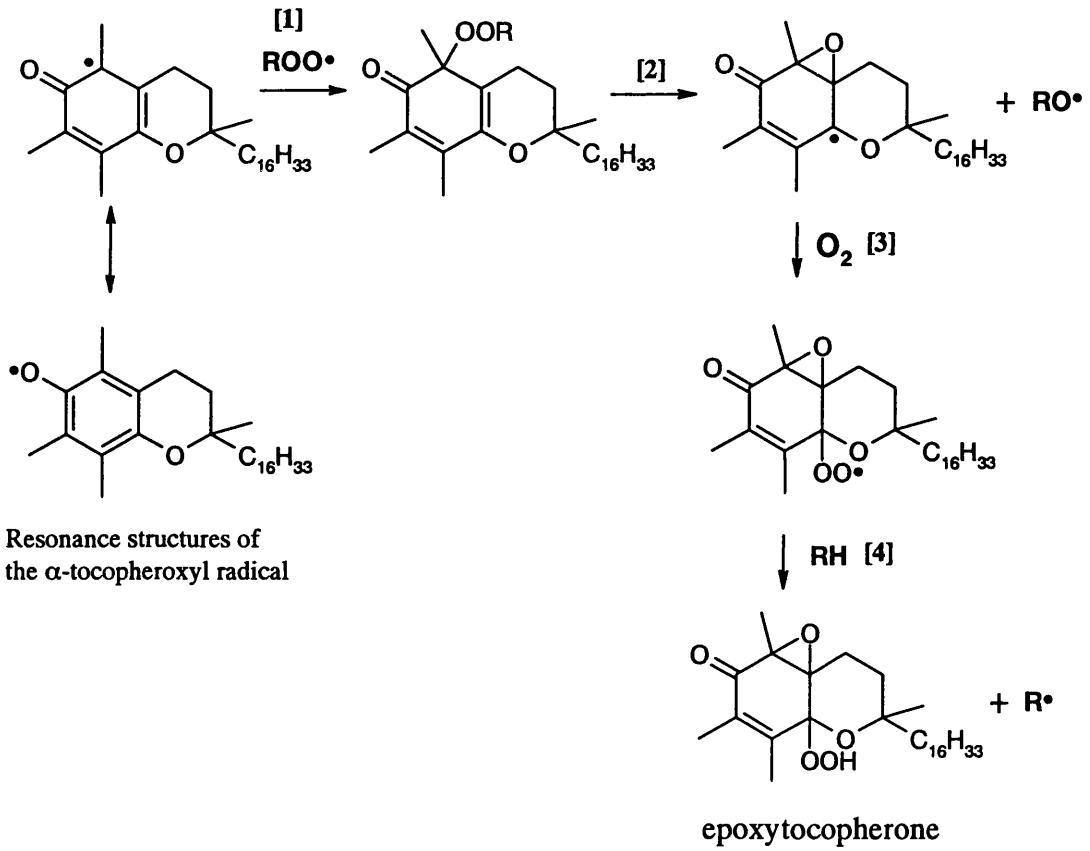



**Figure 1.7.** Epoxide formation - products of non-antioxidant pathways.

decomposition of the adduct to an epoxy carbon-centred radical which is accompanied by the release of an alkoxyl radical (RO•) [2]. Subsequent reaction of this epoxy carbon-centred radical with ambient oxygen results in a peroxyl radical [3], which is able to continue the lipid peroxidation chain reaction [4]. Under this scheme, the reaction of α-TOH with peroxyl radicals will, in fact, continue the chain reaction owing to the release of both an alkoxyl and a peroxyl radical.

Although direct evidence *in vivo* is lacking, a variety of mechanisms must be in place to regenerate α-tocopherol after its reaction with ROS in order to prevent α-tocopherol acting as a pro-oxidant. Interestingly, deuterated α-TQ (α-TQ is the major oxidation product of α-tocopherol) has been shown to be converted to deuterated α-TOH when administered orally to man. Although the yield was low, this was explained by the poor absorption and retention of α-TQ in the body (Moore and Ingold, 1997). α-TQ can also be converted by NAD(P)H:quinone reductase to its hydroquinone form, α-THQ, which can act as an efficient antioxidant (Siegel et al., 1997; Liebler and Burr, 2000).

## 1.6. Metabolism

### 1.6.1. General overview of metabolism

In general, the metabolic breakdown of xenobiotics and lipophilic compounds (catabolism) occurs for two principal reasons. Firstly to reduce the activity or toxicity of compounds, which is especially important with regards to drugs, and secondly to increase the water solubility and thus the rate of excretion of waste compounds.

The body excretes waste products in a number of ways, of which the two most important are:

1) From the liver to the large intestine via the bile and
2) From the kidney via the urine

Many biologically active molecules are lipophilic and remain un-ionised or only partially ionised at physiological pH. These molecules are often bound to plasma proteins and are not readily excreted in the bile or urine, or else are efficiently reabsorbed. Therefore, in order to increase their excretion, hydrophobic compounds such as vitamin E undergo extensive metabolism in order to increase their water solubility. These metabolic reactions occur in a wide range of organs but the liver and the kidney are the primary sites of metabolism before excretion of the water-soluble metabolites in the bile or in the urine.

In general, the greater the lipophilicity of a substrate the more accessible it is to the sites of metabolism, particularly in the liver. Alkyl side-chains, as found in compounds such as vitamin E, are reduced in length by processes such as $\alpha$, $\omega$- and $\beta$-oxidation in the mitochondria or peroxisome. Reduction/oxidation reactions to introduce polar functionalities, including hydroxyl and carboxyl groups, mainly occur in the smooth endoplasmic reticulum (ER) and are catalysed by enzymes such as cytochrome P450 / cytochrome P450 reductase. These polar functionalities are then conjugated with groups such as sulphates or glucuronides to further increase water solubility. Glucuronic acid is most commonly attached to hydroxyl groups and these conjugation reactions are catalysed by a range of glucuronyl transferase enzymes, with broad specificity, in the ER (Meech and Mackenzie, 1997). Sulphate conjugation (mainly of phenol groups), in contrast to glucuronidation, occurs in the cytosol. Once produced,

these polar conjugates are either excreted in the bile directly from the liver or else are taken in the blood to the kidney where they are filtered into the urine.

### 1.6.2. Vitamin E metabolism

Vitamin E metabolites are likely to form two distinct groups. The first of these contains metabolites that are produced after reaction of vitamin E with oxidants while the second contains metabolites that are produced in the liver from excess vitamin E that is not loaded onto nascent VLDL for transport into the circulation.

Vitamin E metabolites are excreted as conjugates but the majority of the research into the metabolism of vitamin E has concentrated on urinary metabolites, after their enzymatic or acidic deconjugation. Urinary metabolites of vitamin E were first reported by Simon et al. (1956a; 1956b). They described two metabolites of α-tocopherol, 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethyl-1,4-benzoquinone (tocopheronic acid, α-TA) and its γ-lactone (tocopheronolactone, α-TL), which were produced by both rabbits and humans (figure 1.8). Enzymatic deconjugation of these metabolites with β-glucuronidase suggested α-TA and α-TL were excreted as glucuronide conjugates. Owing to their quinone structures, α-TL and α-TA were hypothesised to be derived from the known α-tocopherol oxidation product, α-tocopherylquinone (α-TQ) (Simon et al., 1956a; Simon et al., 1956b). It was proposed that these metabolites were produced after reduction of α-TQ to α-tocopheryl hydroquinone (α-THQ), conjugation and then subsequent β-oxidation of the phytyl side chain. Indeed, an unknown conjugate of α-TA, released after acid hydrolysis, was the main metabolite observed after injection of rats with $C^{14}$-α-TQ (Gloor and Wiss, 1966). Oral administration of α-TQ to humans also resulted in the excretion of α-TA in the urine but the level of excretion

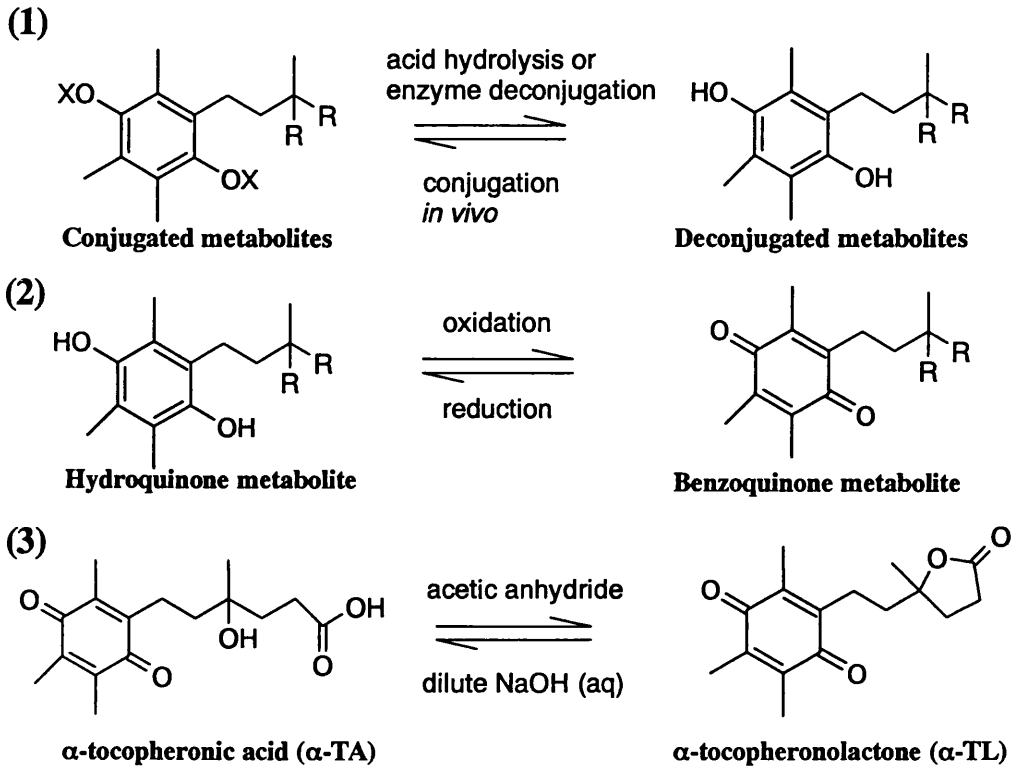



**Figure 1.8.** Urinary α-tocopherol metabolites identified by Simon et al. (1956b).
Two metabolites, α-TA and α-TL, were identified after acid or enzymatic deconjugation of urine extracts (X = Glucuronic acid, (**1**)). Facile oxidation in air of the hydroquinone metabolites lead to the production of their corresponding benzoquinones (**2**). The two metabolites, α-TA and α-TL, were shown to interconvert suggesting the minor metabolite, α-TA, may have been artefactually produced by hydrolysis of the lactone ring of α-TL during the extraction procedure.

only accounted for 0.3% of the original dose (Schmandke, 1965). This low conversion to α-TA was probably because of poor intestinal absorption of α-TQ.

In contrast to the quinone structures of α-TL and α-TA, the major urinary metabolite that was observed after injection of rats with radiolabelled δ-tocopherol had an intact chroman ring. This metabolite was named δ-carboxy-ethyl-hydroxychroman (δ-CEHC) (Chiku et al., 1984) and was proposed to be formed by β-oxidation of the phytyl side chain of δ-tocopherol (figure 1.9). Although δ-CEHC was the major metabolite and accounted for ~ 75% of the radioactivity in urine, another compound was also observed on thin layer chromatography (TLC). This compound did not correspond to the δ-tocopherol equivalent of either α-TA or α-TL but a change in its TLC retention after reaction with diazomethane indicated it had a carboxyl group. However, owing to a lack of isolated material, this compound was not characterised any further.

The α-tocopherol homologue of δ-CEHC was characterised in human urine by Schonfeld et al. (1993) and a more detailed study was carried out by Schultz et al. in 1995. Enzymatic studies indicated α-CEHC was excreted as a sulphate conjugate and since this metabolite was only detected after a daily intake of 50-150mg α-tocopherol, it was proposed to be an indicator of excess vitamin E. Care was taken to use non-oxidative conditions and neither α-TL nor α-TA were observed. Therefore, they proposed that α-TL and α-TA described previously were oxidation artefacts of the experimental procedure. This was shown to be plausible by the oxidative conversion of α-CEHC to α-tocopheronolactone in the presence of air and acid.

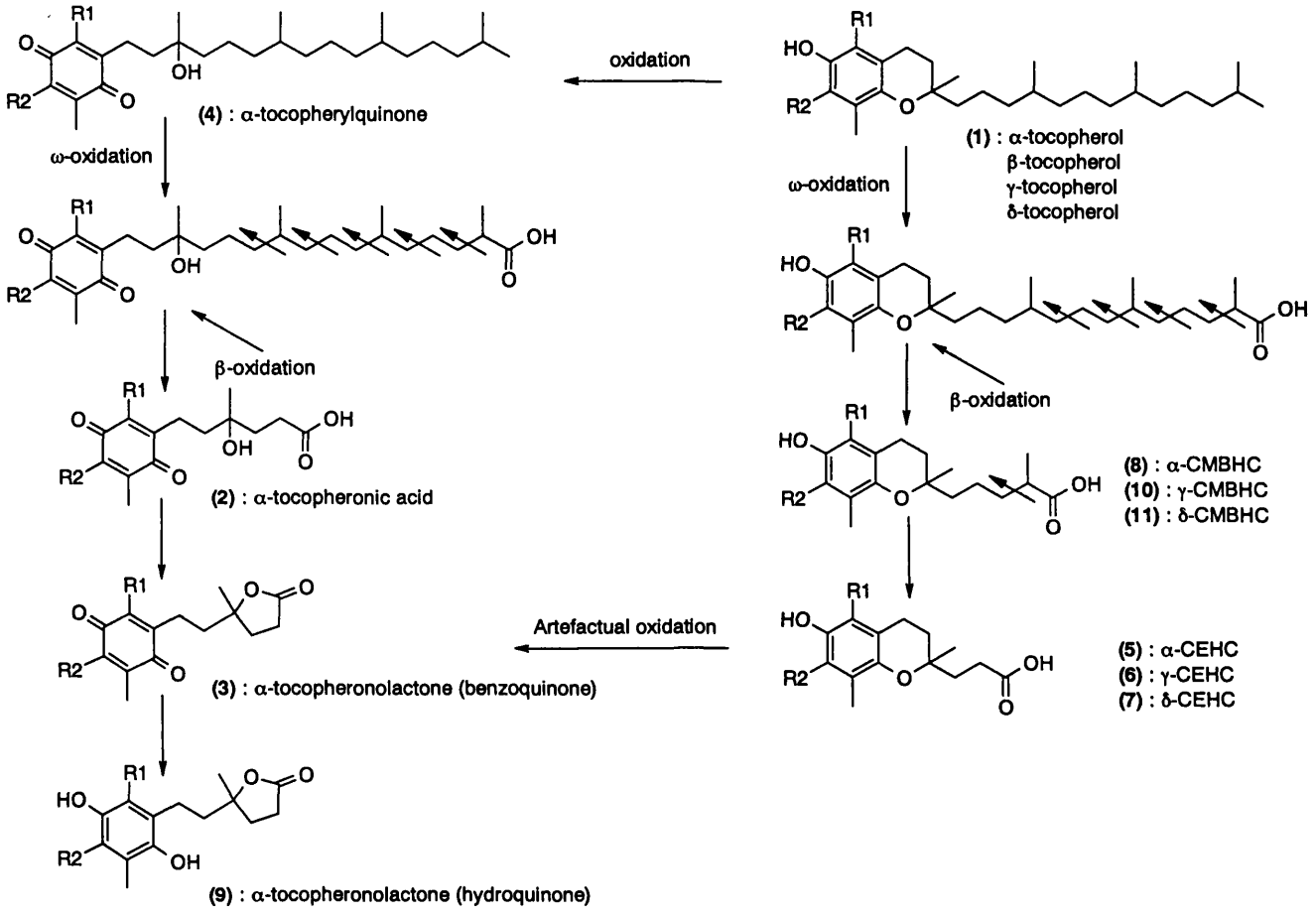



**Figure 1.9.** Overview of vitamin E metabolism showing the presumed β-oxidation of the phytyl side chain. CEHC - carboxy-ethyl-hydroxychroman; CMBHC - carboxy-methyl-butyl-hydroxychroman.

In 1996, Wechter et al. isolated a new endogenous natriuretic factor (a factor that controls the body's pool of extracellular fluid) which was characterised as unconjugated trimethyl-carboxyethyl-hydroxychroman (γ-CEHC) and was therefore presumed to be a metabolite of γ-tocopherol (figure 1.9). Unconjugated γ-CEHC acts as a natriuretic factor by inhibiting the 70 pS ATP-sensitive $K^+$ channel of the thick ascending limb cells of the kidney. The stereochemistry at C-2 of γ-CEHC was assigned as S indicating that no racemisation occurs during side chain metabolism of RRR-γ-tocopherol (Kantoci et al., 1997). By analogy other vitamin E metabolites would be expected to retain their stereochemistry.

The urinary excretion of conjugated γ-CEHC was reported by Traber et al. (1998) and they proposed that CEHC metabolites were synthesised from excess vitamin E in the liver. The relative excretion of differently deuterium labelled synthetic (all rac) and natural (RRR) α-tocopherols as α-CEHC was investigated (Traber et al., 1998) and it was found that α-CEHC derived from synthetic tocopherol was excreted in larger amounts than α-CEHC derived from natural tocopherol. This was proposed to result from the preferential loading of natural as opposed to synthetic α-tocopherol onto nascent VLDL in the liver thus reducing the likelihood of natural α-tocopherol being metabolised to α-CEHC. Contrary to previous evidence by Schultz et al. (1995), α-CEHC was shown to be excreted in the urine at all levels of vitamin E intake and not just when supplementation reached a certain threshold.

Quantitative measurement of urinary γ-CEHC using a deuterium labelled internal standard indicated that γ-tocopherol is inefficiently transferred onto VLDL and is

consequently metabolised to γ-CEHC within a few days of ingestion (Swanson et al., 1999). Daily urinary γ-CEHC excretion was equivalent to about half the daily intake of γ-tocopherol. If other routes of excretion are taken into account this suggests that very little γ-tocopherol is retained in the body. In comparison only a few percent of the daily intake of α-tocopherol is excreted as α-CEHC (Traber et al., 1998). This again emphasises the importance α-TTP in vitamin E metabolism.

Within the last year our group (Pope et al., 2001) and those of Brigelius-Flohe et al. (Schuelke et al., 2000) and Parker et al (Parker and Swanson, 2000; Parker et al., 2000) have described a novel group of metabolites, the carboxy-methyl-butyl hydroxychromans (CMBHCs), which appear to be the precursor of the CEHCs in the side chain metabolism of vitamin E (figure 1.9). These CMBHCs are minor excretion products compared to the CEHCs but are still observed in urine even if subjects have not taken vitamin E supplements. The identification of α-CMBHC and its confirmation using a chemically synthesised standard is described in chapter 3. The tocotrienols, which are metabolised and excreted in the urine as CEHCs (Hattori et al., 2000; Lodge et al., 2001), would also be expected to produce CMBHCs-type metabolites containing a double bond in the side chain, although this has yet to be shown.

Vitamin E metabolites, as mentioned above, are expected to be excreted in the urine as sulphate or glucuronide conjugates. However, no definitive characterisation of the intact conjugates has been reported owing mainly to a lack of standards and suitable techniques to analyse these polar metabolites directly.

## 1.7. Summary and aims of study

Vitamin E was discovered almost 80 years ago as an essential dietary factor that prevented foetal resorption in rats fed a rancid lard diet (Evans and Bishop, 1922; Evans and Burr, 1925). Subsequent research has led to the view that vitamin E has a general role as a lipid soluble antioxidant that helps prevent lipid peroxidation. Vitamin E has, therefore, been proposed to have an important protective role against diseases where increased oxidative damage has been observed. However, recent evidence suggests that the tocopherols and their metabolites may also have important non-antioxidant roles in regulating processes such as protein kinase C signalling cascades and natriuresis (Azzi and Stocker, 2000; Wechter et al., 1996). The metabolism of vitamin E may also be important in regulating its action. Therefore, the full characterisation of vitamin E metabolites and the pathways leading to their formation may help to define the precise roles of vitamin E *in vivo*.

The overall aims of the present study were to develop methods to measure and characterise vitamin E metabolites in urine. To this end it was hoped to:

1) Set up and establish a method to examine all urinary metabolites of vitamin E after deconjugation.
2) Confirm the structure of a previously identified metabolite of α-tocopherol (α-CMBHC) by chemical synthesis.
3) Synthesise a range of unconjugated and conjugated vitamin E metabolites for use as standards.
4) Investigate artefactual production of α-tocopheronolactone.
5) Characterise the conjugated metabolites in urine.

# CHAPTER 2

## Development of a Gas Chromatographic-Mass Spectrometric (GC-MS) Method for the Analysis of Urinary Vitamin E Metabolites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## 2.1. Introduction

In order to study vitamin E turnover and metabolism it was necessary to measure vitamin E metabolites quantitatively in urine. A number of methods have been described in the literature, details of which are shown in Table 2.1. This chapter describes the development of a method for the measurement of the major vitamin E metabolites, i.e. the CEHCs and α-tocopheronolactone.

Vitamin E metabolites are excreted in the urine as sulphate or glucuronide conjugates. The polar nature of these conjugates makes it difficult to analyse them directly using routine techniques such as high performance liquid chromatography (HPLC) or gas chromatography – mass spectrometry (GC-MS). Therefore, in all the methods outlined in Table 2.1, the metabolites were deconjugated either enzymatically or by acid hydrolysis prior to their analysis by HPLC or GC-MS.

The analytical procedure can be divided into 4 main steps. These include 1) extraction of the metabolites from urine, 2) deconjugation of these metabolites, 3) extraction of the deconjugated metabolites and 4) analysis by HPLC or GC-MS. Each of these steps needs to be optimised to produce quantitative and reproducible data. These aspects and the problem of artefactual oxidation of α-CEHC to α-tocopheronolactone will be discussed in this chapter.

**Table 2.1.** Description of methods used for the analysis of vitamin E metabolites

| Authors | Metabolites analysed | Extraction | Deconjugation | Analytical tools and quantitation | Details |
|---|---|---|---|---|---|
| Simon et al., 1956b | α-tocopheronolactone and α-tocopheronic acid. | Acidification (pH 1-2) and ether extraction. Silicic acid-cellite column post deconjugation. | 3N HCl (2 hr, reflux) and β-glucuronidase (5 days, 37°). | UV, paper chromatography. IR.<br><br>No quantitation. | Radiolabelled α-tocopherol was given to rabbits and the urinary metabolites analysed using paper chromatography. Metabolites seen in human urine appeared to be identical. α-tocopheronolactone was shown to be the major metabolite with α-tocopheronic acid the minor. β-glucuronidase released 70-90% of radiolabelled metabolites in rabbit urine. Both seen as benzoquinones. |
| Chiku et al., 1984 | δ-CEHC. | 1) C18 SPE, methanol extraction<br>2) Ethyl acetate extraction of lyophilised urine | 3N methanolic HCl (20 hr, rt, under nitrogen) and sulphatase or β-glucuronidase. | TLC and GC-MS (conditions not given).<br><br>No quantitation. | Radiolabelled δ-tocopherol was given to rats. The urinary metabolites were extracted by three different methods and analysed by TLC (pre and post deconjugation) and GC-MS. δ-CEHC was the major metabolite and enzyme studies showed it was almost exclusively sulphated. |

**Abbreviations**: **HCl**, hydrochloric acid; **UV**, ultra violet; **IR**, infra red; **CEHC**, carboxy-ethyl hydroxychroman; **SPE**, solid phase extraction; **rt**, room temperature; **TLC**, thin layer chromatography; **GC-MS**, gas chromatography-mass spectrometry; **HPLC**, high performance liquid chromatography; **LLU-α**, Loma Linda University metabolite-α; **CMBHC**, carboxy-methyl-butyl hydroxychroman.

Continued.

| Authors | Metabolites analysed | Extraction | Deconjugation | Analytical tools and quantitation | Details |
|---|---|---|---|---|---|
| Schultz et al., 1995 | α-CEHC and α-tocopheronolactone. | Methanol extraction of lyophilised urine. Diethyl ether extraction post deconjugation. | 3N methanolic HCl (20 hr, rt, under nitrogen) and β-glucuronidase/ sulphatase or pure glucuronidase (5 hr, 37°C, under argon). | HPLC with diode array and fluorescence detection, TLC and GC-MS.<br><br>24 hr urine, peak area on HPLC compared to external α-CEHC standard. | α-CEHC was the major metabolite extracted from human urine and was found to be predominantly sulphated by enzyme studies. α-tocopheronolactone was shown to be formed artefactually after bubbling oxygen through an acid solution of α-CEHC. If care was taken to avoid oxygenation during the extraction procedure no α-tocopheronolactone could be detected. |
| Burton et al. (personal communication), 1996 | α-CEHC and α-tocopheronolactone. | Acidification and C18 SPE. | Conc. HCl , rt, overnight under nitrogen. | HPLC with UV detection followed by GC-MS.<br><br>Peak areas on GC-MS compared to deuterated internal standards of α-tocopheronolactone and α-CEHC. | α-CEHC the major metabolite, but small amounts of α-tocopheronolactone detected. Addition of ascorbate to urine samples stops oxidation of α-CEHC to α-tocopheronolactone on storage. |
| Wechter et al., 1996 | γ-CEHC (LLU-α) – unconjugated. | Isopropanol extraction of lyophilised urine. | No deconjugation. | HPLC with UV detection.<br><br>No quantitation. | Unconjugated γ-CEHC was shown to act as a natriuretic factor i.e. controls the body's pool of extracellular fluid. |

**Abbreviations**: **HCl**, hydrochloric acid; **UV**, ultra violet; **IR**, infra red; **CEHC**, carboxy-ethyl hydroxychroman; **SPE**, solid phase extraction; **rt**, room temperature; **TLC**, thin layer chromatography; **GC-MS**, gas chromatography-mass spectrometry; **HPLC**, high performance liquid chromatography; **LLU-α**, Loma Linda University metabolite-α; **CMBHC**, carboxy-methyl-butyl hydroxychroman.

Continued.

| Authors | Metabolites analysed | Extraction | Deconjugation | Analytical tools and quantitation | Details |
|---|---|---|---|---|---|
| Traber et al., 1998 | α-CEHC and γ-CEHC. | Methanol extraction of lyophilised urine. Diethyl ether extraction post deconjugation. | β-glucuronidase/ sulphatase (4 hr, 37°C, under argon). | GC-MS.<br><br>24 hr urine. Peak areas of metabolites compared to Trolox internal standard peak area and response factor. | All-rac as compared to RRR-α-tocopherol was preferentially excreted in the urine as α-CEHC. Large percentage of γ-tocopherol plasma pool excreted daily whereas only a small percentage of α-tocopherol pool excreted. |
| Swanson et al., 1999 | γ-CEHC. | No preliminary extraction. Post deconjugation, acidified (pH 1) and extracted with hexane/t-butyl methyl ether (99:1). | In urine deconjugation with β-glucuronidase (overnight, 30°C, under argon). | GC-MS.<br><br>24 hr urine. Peak areas of metabolites compared to deuterated γ-CEHC internal standard. | γ-CEHC was mainly excreted as a glucuronide conjugate as judged by enzyme studies. Deconjugation in urine appeared to work. No γ-tocopheronolactone observed. |
| Stahl et al., 1999 | α-CEHC and γ-CEHC | After removal of serum lipids, hexane/dichloromethane extraction. | In serum deconjugation with β-glucuronidase (30 min, 37°C, under nitrogen). | HPLC with coulometric detection.<br><br>Peak areas compared to external standards of α-CEHC and γ-CEHC. | First case where metabolites analysed in human serum. Enzyme studies revealed only small amounts of γ-CEHC glucuronide present. About 1/3$^{rd}$ of α-CEHC present in serum glucuronidated. |

**Abbreviations**: **HCl**, hydrochloric acid; **UV**, ultra violet; **IR**, infra red; **CEHC**, carboxy-ethyl hydroxychroman; **SPE**, solid phase extraction; **rt**, room temperature; **TLC**, thin layer chromatography; **GC-MS**, gas chromatography-mass spectrometry; **HPLC**, high performance liquid chromatography; **LLU-α**, Loma Linda University metabolite-α; **CMBHC**, carboxy-methyl-butyl hydroxychroman.

Continued.

| Authors | Metabolites analysed | Extraction | Deconjugation | Analytical tools and quantitation | Details |
|---|---|---|---|---|---|
| Lodge et al., 2000 | α-CEHC and γ-CEHC. | No preliminary extraction. Post deconjugation acidification and diethyl ether extraction. | Deconjugation in urine with β-glucuronidase (4 hr, 37°C). | GC-MS and HPLC with electrochemical detection.<br><br>24 hr urine. Peak areas compared to trolox internal standard and response factors for both HPLC and GC-MS. | First use of electrochemical detection. Sensitivity similar to GC-MS. |
| Parker and Swanson, 2000 | γ-CEHC and γ-CMBHC. | See Swanson et al. above. | See Swanson et al. above. | See Swanson et al. above. | First report of metabolism of γ-tocopherol in cell culture (Hep G2 cells). Also first report of γ-CMBHC in both cell culture and human urine. Interestingly neither α-CEHC nor α-CMBHC produced by Hep G2 cells. |
| Hattori et al., 2000a | γ-CEHC (LLU-α) – unconjugated. | Sample deproteinized and derivatised before C18 SPE. | No deconjugation. | HPLC with fluorescence detection.<br><br>Peak areas compared to derivatised γ-CEHC external standard. | Highly sensitive method which allows the determination of γ-CEHC (LLU-α), and the occurrence of (S)-γ-CEHC shown in rat plasma, urine and bile. Confirms retention of side-chain configuration during metabolism. |

**Abbreviations**: **HCl**, hydrochloric acid; **UV**, ultra violet; **IR**, infra red; **CEHC**, carboxy-ethyl hydroxychroman; **SPE**, solid phase extraction; **rt**, room temperature; **TLC**, thin layer chromatography; **GC-MS**, gas chromatography-mass spectrometry; **HPLC**, high performance liquid chromatography; **LLU-α**, Loma Linda University metabolite-α; **CMBHC**, carboxy-methyl-butyl hydroxychroman.

Continued.

| Authors | Metabolites analysed | Extraction | Deconjugation | Analytical tools and quantitation | Details |
|---|---|---|---|---|---|
| Shuelke et al., 2000 | α-CMBHC and α-CEHC. | See Schultz et al. above. | See Schultz et al. above. | See Schultz et al. above. | Patients with α-tocopherol transfer protein defects showed similar metabolism of α-tocopherol to healthy subjects. α-CMBHC reported. |
| Hattori et al., 2000b | γ-CEHC | See Hattori et al. above. | See Hattori et al. above. | See Hattori et al. above. | γ-CEHC (LLU-α) was produced from γ-tocotrienols as well as γ-tocopherol in rats. Both retain side chain configuration. |
| Parker et al., 2000 | α-CEHC, γ-CEHC, δ-CEHC, α-CMBHC, γ-CMBHC and δ-CMBHC. | See Swanson et al. above. | See Swanson et al. above. | See Swanson et al. above. Peak areas of α- and γ-tocopherol metabolites compared to deuterated α-CEHC and γ-CEHC internal standards respectively. | The side-chain metabolism of tocopherols was shown to be cytochrome P450 3A-dependent and inhibited by sesamin. |
| Lodge et al., 2001 | α-CEHC and γ-CEHC. | See Lodge et al. above. | See Lodge et al. above. | See Lodge et al. above. | α- and γ-tocotrienols are metabolised to α- and γ-CEHC, respectively. |

**Abbreviations**: **HCl**, hydrochloric acid; **UV**, ultra violet; **IR**, infra red; **CEHC**, carboxy-ethyl hydroxychroman; **SPE**, solid phase extraction; **rt**, room temperature; **TLC**, thin layer chromatography; **GC-MS**, gas chromatography-mass spectrometry; **HPLC**, high performance liquid chromatography; **LLU-α**, Loma Linda University metabolite-α; **CMBHC**, carboxy-methyl-butyl hydroxychroman.

## 2.2. Basis of method

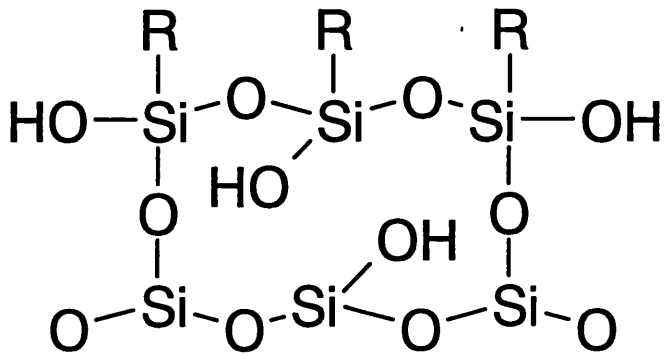
### 2.2.1. Extraction

Extraction of vitamin E metabolites is required both before and after deconjugation. A review of the methods in Table 2.1 reveals that the majority utilise organic solvent extraction. Solid phase extraction, in contrast, has been used less commonly in the extraction of vitamin E metabolites, although it has been used in preference to liquid-liquid extraction in the extraction and separation of many endogenous and xenobiotic metabolites from urine (Gilar et al., 2001).

Solid phase extraction (SPE) is described as "the separation or removal of an analyte or analytes from a mixture of compounds by selective partitioning of the compounds between a solid phase (sorbent) and a liquid phase (solvent)" (Jones Chromatography, 1998). A classical liquid-liquid extraction involves the separation of analyte(s) by the mixing of two immiscible solvents. Liquid-liquid extractions rely on a marked difference in the solubility of the analyte(s) between the two solvents. Problems with liquid-liquid extractions include the relatively large solvent volumes required, the possible formation of emulsions or mixtures resulting from partial miscibility of the solvents, the possible oxygenation and subsequent oxidation of the sample resulting from vigorous shaking and a lack of selectivity. In contrast, solid phase extraction requires small solvent volumes, has inherent immiscibility, can show selectivity for specific functional groups and is applicable to batch processing.

Most solid phase extraction cartridges are based on silica chemistry. Cross-bonded silanols form the basis of the solid phase with changes in the R group affecting specificity (figure 2.1). The most commonly used SPE cartridges are probably C18

**A**



C18, R = $C_{18}H_{37}$

C4, R = $C_4H_9$

**B**

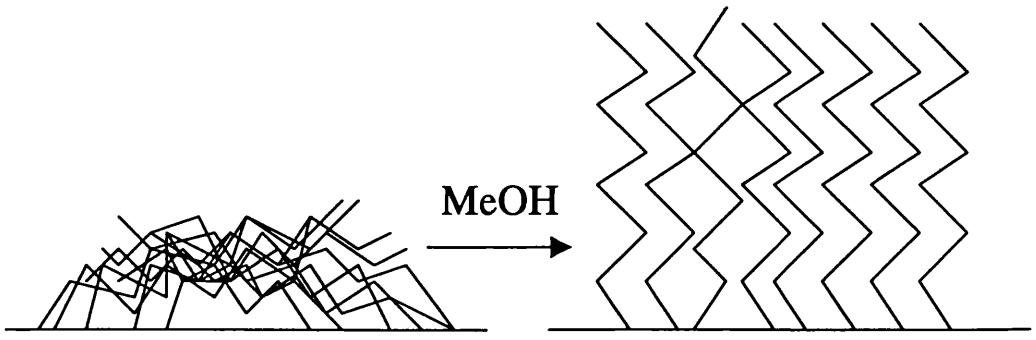


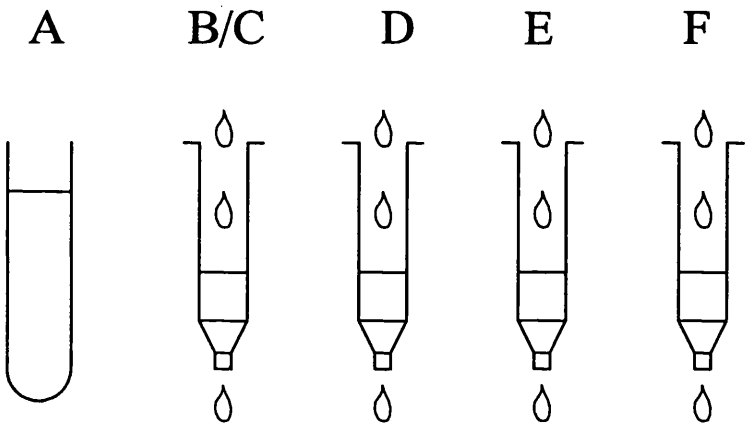

**Figure 2.1. A** The structure of silanol based SPE cartridges. Changes in the R group affect retention and elution characteristics. **B** Priming the cartridges with an organic solvent such as methanol stops aggregation of the hydrophobic side-chains and thereby increases retention of molecules with hydrophobic areas.

SPEs. These consist of the silanol backbone with octadecyl side-chains allowing hydrophobic interactions to occur with the analytes of interest. Sorbents containing other functional groups, which form polar or ionic interactions, are sometimes used to allow greater selectivity.

The six steps involved in a typical solid phase extraction are shown in figure 2.2. The first step is sample pre-treatment (Figure 2.2A). This may involve removal of solid material from the sample, ionisation/deionisation of the sample to increase/decrease interactions with the solid phase or derivatisation. The next step is the solvation or priming (conditioning) of the sorbent bed of the SPE cartridge (Figure 2.2B). This involves adding an organic solvent, such as methanol, to the cartridge so that interactions between the branches of the solid phase are minimised allowing greater interaction with analytes. The hydrophobicity of solid phases such as C18 tends to make the chains aggregate together if they are not first primed. The cartridge is then washed with the solvent that will be used to apply the sample (Figure 2.2C). The sample is then loaded onto the column (Figure 2.2D). Interfering compounds are washed off, often using the same solvent that was used to apply the sample (Figure 2.2E) and then the analytes are eluted using a suitable solvent - often the initial priming solvent (Figure 2.2F). Variations can include sequential elution using greater amounts of organic modifier in order to achieve better separation.

### 2.2.2. Deconjugation

The deconjugation step in the analysis of vitamin E metabolites is required for the removal of sulphate and glucuronide residues, which are attached *in vivo* to the metabolites to facilitate their excretion in urine. Previously this step has been carried out




A. Sample pre-treatment e.g. acidification

B. Column conditioning e.g. MeOH

C. Column equilibration e.g. water

D. Sample application

E. Elution of interfering compounds e.g. water

F. Analyte elution e.g. MeOH

**Figure 2.2.** The six steps involved in a typical solid phase extraction

using rather harsh conditions like acid hydrolysis (Simon et al., 1956a; Gloor and Wiss, 1966). The acid (HCl) hydrolysis reaction under ordinary conditions (i.e. under oxygen) has led invariably to the oxidation of α-CEHC to α-tocopheronolactone and α-tocopheronic acid, and has frequently resulted in a failure to identify α-CEHC as the major metabolite of α-tocopherol (Simon et al., 1956a; Schultz et al., 1995).

Recently the problem of artefactual oxidation has been avoided, either by using short periods of enzymatic deconjugation under an inert gas, such as argon, or by using methanolic HCl that theoretically stops oxidation to α-tocopheronic acid/α-tocopheronolactone by esterifying the carboxyl group of α-CEHC (Chiku et al., 1984; Schultz et al., 1995). The most frequent method of enzymatic deconjugation has been to use a mixture of β-glucuronidase and arylsulphatase in order to remove both glucuronide and sulphate residues. The use of 'pure' enzymes by some groups has been employed to study the types of conjugates present, especially in animal studies where radiolabelled vitamin E has been administered (Chiku et al., 1984). Owing to the difference in polarity between the conjugated and deconjugated metabolites, TLC of the radiolabelled metabolites before and after specific enzymatic deconjugation is a simple way to ascertain the type of conjugates present. However, these studies have not produced unequivocal results. Simon et al. showed 70-90% of α-tocopherol metabolites in the rabbit were released by β-glucuronidase treatment (Simon et al, 1956a; Simon et al., 1956b), whereas Chiku et al. (1984) demonstrated δ-CEHC to be almost exclusively sulphated in the rat. Other work on enzymatic digestion of non-radiolabelled metabolites has also produced unclear results, although the lack of radiolabel makes it difficult to confirm the proportion of conjugates that have been deconjugated (Swanson et al., 1999; Stahl et al., 1999). The differences between the types of conjugates

observed may result from the "impure" nature of the supposedly pure enzyme preparations or the lability of some conjugates under certain conditions. Another reason for anomalous findings may result from metabolite and species differences.

### 2.2.3. Separation and detection

Following a further extraction step, the deconjugated metabolites can be analysed using either HPLC or GC-MS. The choice of GC-MS or HPLC analysis is largely a matter of the equipment available in the laboratory. Nevertheless GC-MS is generally the more sensitive technique, although HPLC can approach this sensitivity if the detection method is suitable, as in the case of electrochemical detection (Lodge et al., 2000) or fluorescence detection of derivatives (Hattori et al., 2000). In this chapter, only GC-MS analysis is discussed.

GC-MS is an established technique for the analysis of complex mixtures and is particularly popular because of its selectivity, sensitivity and versatility. The basic principles underlying GC-MS are relatively simple. Essentially, the sample mixture is separated on a gas chromatography column and each peak eluting from the column is ionised and analysed by the mass spectrometer. This technique allows the separation and quantitation of a large number of different compounds in one sample. The separation of a mixture of compounds is achieved by choosing a suitable column and temperature gradient. It is often necessary to derivatise samples using trimethylsilyl derivatising agents to produce trimethylsilyl (TMS) derivatives so that polar interactions are minimised, hence reducing the temperature needed to elute the metabolites from the column.

GC-MS, unlike gas chromatography alone, also provides important mass spectral information about unknown peaks, which is extremely helpful in the identification of novel compounds. At each instant in time, a mass spectrum is produced from the compound(s) eluting from the column at that time. If the abundances of all the masses in the spectrum at each time point are added, the total ion abundance is obtained and a total ion chromatogram (TIC) can then be produced, which shows the total ion abundance over the entire course of the analysis. If the TIC is complicated because of co-eluting peaks or large contaminating peaks, the data analysis software can be used to produce an extracted ion chromatogram. This allows a specific compound or group of compounds to be visualised by plotting the abundance of ions which are present only in their spectra. An example of an extracted ion chromatogram is shown in figure 2.10, which only shows peaks containing ions of masses specific to urinary vitamin E metabolites.

### 2.2.4. Quantitation

The quantitative estimation of substances by mass spectrometry (MS) is not straightforward because measurements are not exactly reproducible. The response of a sample at the detector is dependent on a number of variables that are difficult to control. The conditions, such as the temperature and pressure in the ion source affect the observed response. For this reason the mass spectrometer must be calibrated with known amounts of the compound under investigation either just before the assay or in a manner which makes the measurement independent of instrument variability.

The two most routine methods for quantitative analysis employ the use of either an external or an internal standard (Figure 2.3). Successively different, known amounts of

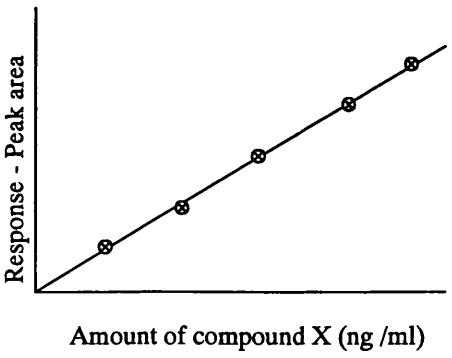
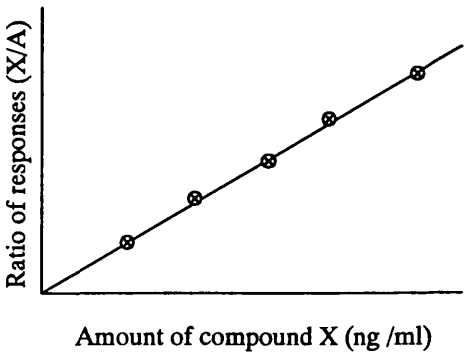
| External standard method | Internal standard method |
|---|---|
| Standard (X) is not added to the sample | Known amount of standard (A) added to sample prior to extraction /derivatisation steps |
| Calibration curve of quantity of standard (X) versus response. | Constant known amount of standard (A) added to various known amounts of the compound to be measured (X). Calibration curve of ratio of responses (X/A) versus quantity of compound X. |
|  |  |
| Response to compound X in sample related to quantity from calibration curve. | Ratio of responses (X/A) in sample related to quantity from calibration curve. |

**Figure 2.3.** A comparison of quantitation methods using external and internal standardisation.

an external standard can be assayed and a plot of signal intensity versus quantity can provide data for a calibration curve. It is an external standard since it is not added to the sample to be analysed. Quantities of the compound in a sample can be determined by relating the signal intensity obtained to the calibration curve. With GC-MS, external standard methods are prone to error resulting from the poor reproducibility of sample volume injections and changes in the condition of the instrument affecting the magnitude of signals.

Using the internal standard approach, a known constant amount of the internal standard is added to increasing amounts of the compound of interest. The different mass spectrometric responses to the varying quantities of the compound under investigation are measured in relation to the internal standard. The ratio of the two responses is plotted against the amount of compound to give a calibration curve. For the actual analysis of a sample of interest, the same amount of internal standard is added and the ratio rather than an absolute value is used to quantitate the compound of interest from the calibration curve.

The internal standard, whether a homologue or an isotopically labelled analogue, may be added to the initial sample before the step-wise analytical procedure. The adoption of this method is also advantageous, since it can account for loss of material in the original sample during the whole procedure from extraction to analysis. In order to achieve the most reliable information, the internal standard should be as similar in structure as possible to the compound of interest.

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble α-tocopherol analogue without the phytyl side-chain, which is structurally close to α-CEHC, is a routinely used standard in both HPLC and GC-MS analyses of vitamin E metabolites. Lodge et al. (2000) showed that the recoveries of trolox and α-CEHC standards added to unsupplemented urine were virtually identical. In some procedures deuterated internal standards have been used for quantitative purposes (Swanson et al., 1999; Parker and Swanson, 2000). Deuterated internal standards are ideal since they account precisely for losses in the work-up procedure and should behave identically in the mass spectrometer.

Typically, various metabolites of vitamin E have been measured in urine samples ranging in volume from 1 to 10 ml. These data have been related to daily levels of vitamin E excretion either by taking 24hr urine samples and/or relating the concentration of metabolites to that of creatinine. Daily creatinine excretion is fairly constant and there is little variation either between days or between subjects. Therefore, it is frequently employed as an index of the urine concentration to enable approximate daily excretion levels of urinary metabolites to be calculated. 24 hr urine samples are theoretically ideal since they allow the measurement of exact daily excretion values. However, the inconvenience and difficulty in obtaining a 24 hr urine often outweigh the advantages.

### 2.2.5. Artefactual oxidation

One of the major considerations in the development of a method to measure vitamin E metabolites is the possibility of errors arising from artefactual oxidation. Schultz et al. (1995) demonstrated that α-CEHC can be oxidised to α-tocopheronolactone in the

presence of acid and oxygen and proposed that any α-tocopheronolactone observed was likely to be the product of artefactual oxidation of α-CEHC during the extraction procedure. They therefore took extra care to exclude oxygen especially during the deconjugation step, which was performed under argon. This modification eliminated the presence of any detectable amounts of α-tocopheronolactone.

Other interventions to reduce artefactual oxidation utilise derivatisation procedures of α-CEHC that prevent its conversion to α-tocopheronolactone. An example of such a derivatisation is the use of methanolic HCl to esterify the carboxyl group of α-CEHC while simultaneously deconjugating (Chiku et al., 1984; Schultz et al., 1995).

## 2.3. Original methods and materials

Figure 2.4 shows the method that was used as a starting point in these studies to measure vitamin E metabolites in urine. The conjugated metabolites were extracted from urine and deconjugated. The deconjugated metabolites were then re-extracted, TMS-derivatised and analysed by GC-MS. Each of these steps required optimisation in order that this multi-step procedure should give quantitative and reproducible yields.

Trolox supplied by Sigma-Aldrich Company Ltd and deuterated $d_9$-α-CEHC and $d_3$-α-tocopheronolactone, kindly supplied by Dr G Burton (Steacie Institute of Molecular Sciences, National Research Council, Ottawa, Canada), were used as standards to optimise and validate the method. Deuterium labelled standards can be distinguished from endogenous metabolites by characteristic mass shifts in their mass spectra. In this case, the molecular ions produced by $d_9$-α-CEHC and $d_3$-α-tocopheronolactone would

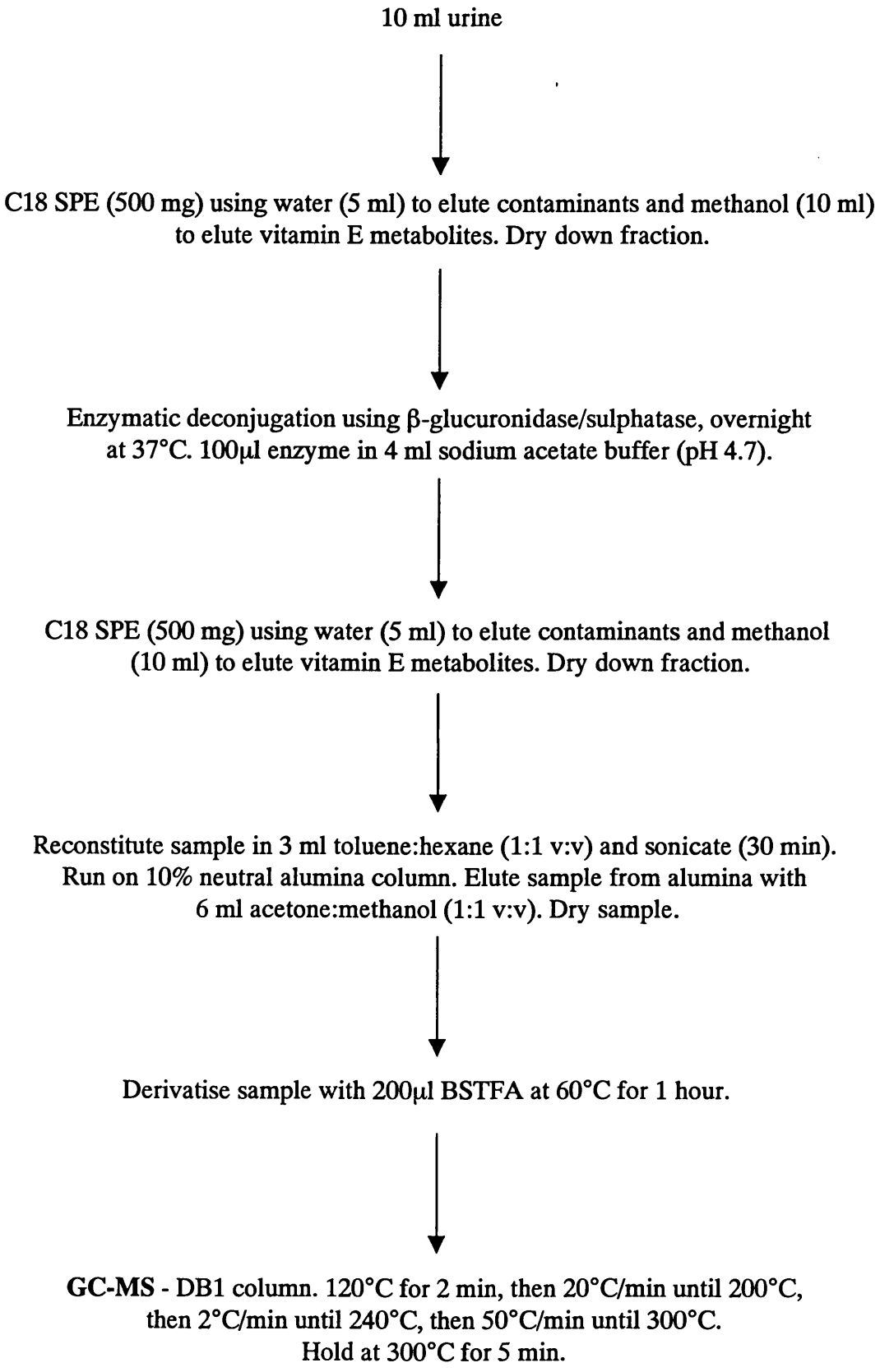



**Figure 2.4.** Method used as starting point for method development.

be 9 and 3 mass units greater than the equivalent ions produced by endogenous α-CEHC and α-tocopheronolactone, respectively.

*GC-MS of deuterated standards:*

20 nmoles of deuterated α-CEHC or α-tocopheronolactone standard was TMS-derivatised with 200µl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, supplied by Pierce and Warriner Ltd.) at 60°C for 1 hour. 2µl of the derivatised mixture was injected (using a splitless technique) onto a DB1 fused silica column (30m, 0.25 mm ID, 0.25µm film thickness) supplied by Jones Chromatography Ltd in a Hewlett-Packard 5890 Series II gas chromatograph linked to a Hewlett-Packard 5970 mass-selective detector and Chem Station data system. The oven was maintained at 120°C for 2 min, then ramped to 200°C at 20°C/min, to 240°C at 2°C/min, to 300°C at 50°C/min and finally held at 300°C for 5 min. The ionisation energy was 70 eV. Scan mode was used and peaks were identified by comparison of their retention time and spectra to known standards and published data.

*GC-MS of urinary vitamin E metabolites:*

Vitamin E metabolites were extracted from 10 ml of urine as detailed in figure 2.4. At this stage no standards were added to the urine. The urine sample was loaded onto a C18 SPE (500mg sorbent mass with a 10ml reservoir supplied by Jones Chromatography Ltd), which had been primed with methanol (5 ml) and water (5 ml). The column was then washed with 5ml water and the metabolites eluted with 10ml methanol. The methanolic extract was evaporated to dryness and was then resuspended in 4ml deionised $H_2O$ to which 200µl sodium acetate (5.0M, pH4.7) and 100µl of β-glucuronidase/sulphatase (β-glucuronidase activity of 100,00 units per ml and

arylsulphatase activity of 7,500 units per ml from Helix pomatia, type HP-2, G7017, Sigma-Aldrich Company Ltd) was added. The sample was incubated at 37°C for 18 hours and then extracted using a second C18 SPE cartridge as described above. The methanolic phase was again dried and the metabolites reconstituted in 3ml toluene:hexane (1:1 v:v) and sonicated for 30 minutes. The organic mixture was then loaded onto 10% neutral alumina and eluted with 6ml acetone:methanol (1:1 v:v). The sample was then dried, derivatised and analysed by GC-MS as described above for the deuterated standards. Extracted ion chromatograms were produced by plotting the abundance of the molecular ions of α-CEHC and α-tocopheronolactone (m/z 422 and m/z 276, respectively).

Modifications to this method and other experimental details are given in the text.

## 2.4. Optimisation of method

### 2.4.1. Preliminary studies

*GC-MS of standards*

TMS-derivatised $d_9$-α-CEHC gave a single peak (B, retention time (RT) = 21 mins; figure 2.5) when analysed by GC-MS and TMS-derivatised $d_3$-α-tocopheronolactone gave two peaks (A and C, RTs = 14 and 23 mins, respectively; figure 2.6). The theoretical structures of these standards are shown in figures 2.5 and 2.6. The spectrum of peak B was compared to the published spectrum of TMS-derivatised $d_0$-α-CEHC (Schultz et al., 1995) and was consistent with a $d_9$-analogue. The molecular ion at m/z 431 and the major fragment ion at m/z 245/246, both containing the 9 deuteriums surrounding the chroman ring, were 9 mass units greater than the corresponding peaks produced by the $d_0$-analogue.

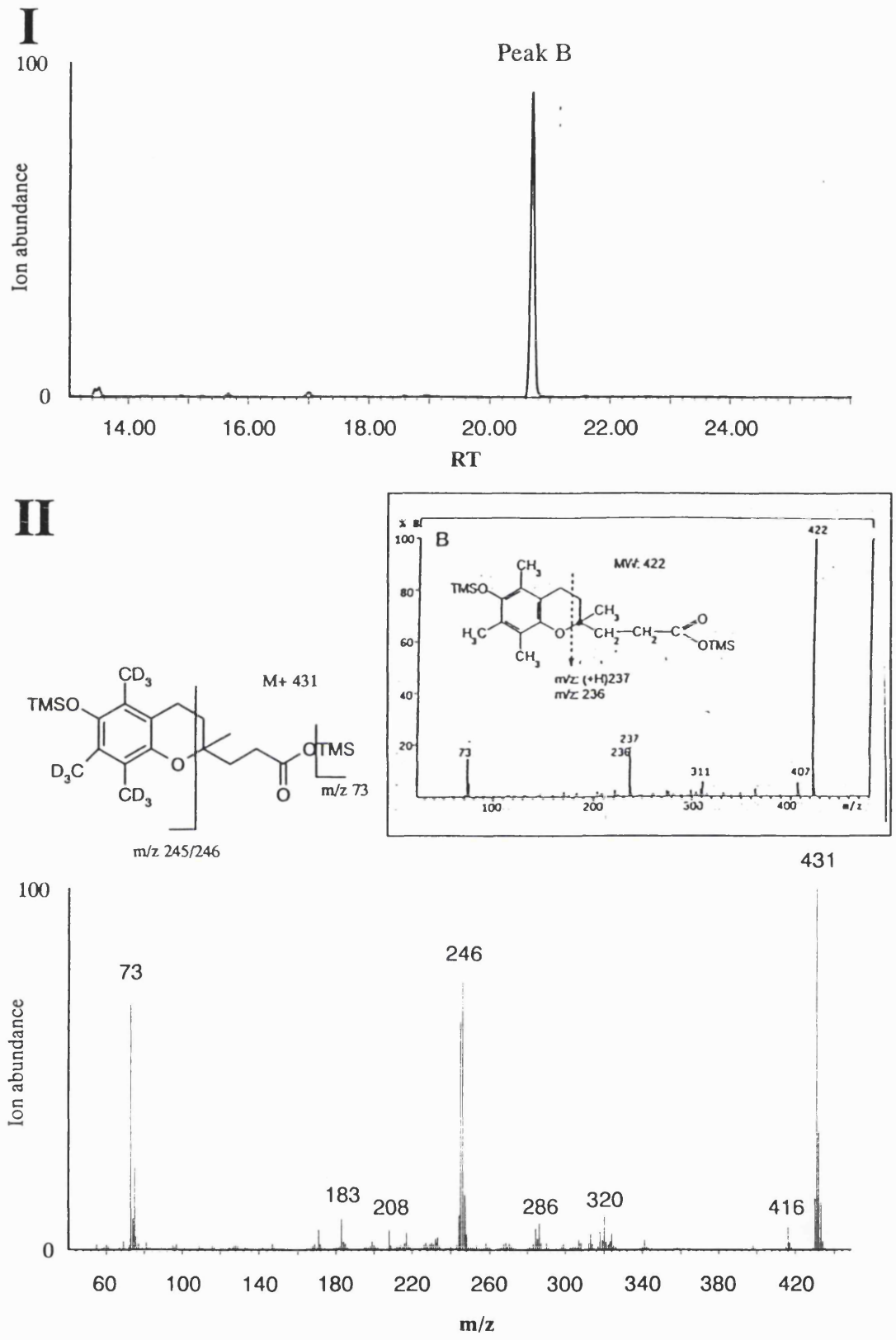



**Figure 2.5.** Chromatogram of $d_9$-α-CEHC standard (**I**) and the corresponding mass spectrum (**II**). The inset shows the spectrum of $d_0$-α-CEHC reported by Schultz et al. (1995). The major fragment ion is produced by cleavage of the chroman ring.

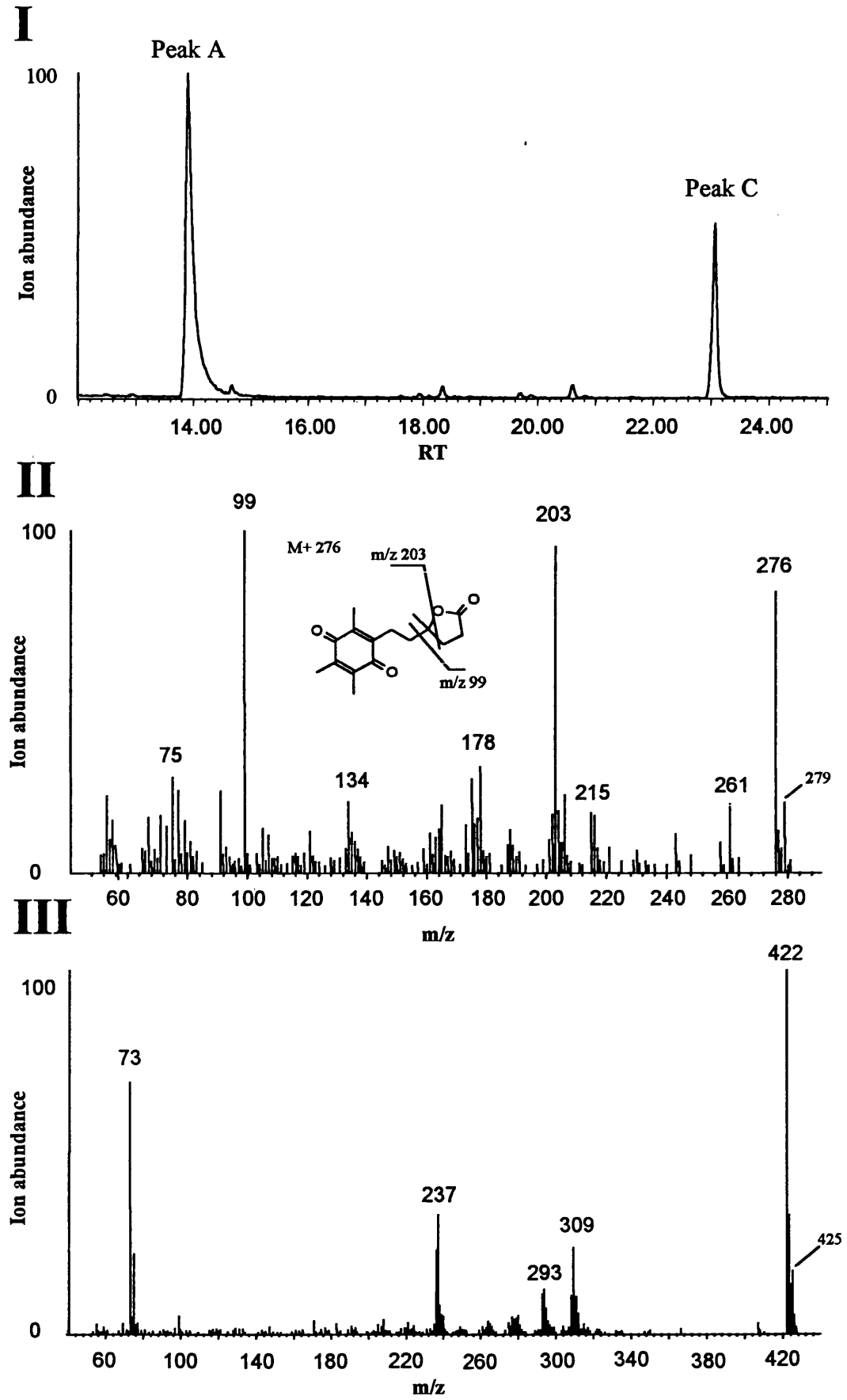



**Figure 2.6.** Total ion chromatogram of $d_3$-α-tocopheronolactone standard (**I**) with spectra of peak A (**II**) and peak C (**III**).
NB. The $d_3$-peaks at m/z 279 and 425 are less than 30% of the abundance of the $d_0$-peaks at m/z 276 and 422.

No reference spectrum of α-tocopheronolactone was available but the spectrum of peak A, containing a molecular ion at m/z 276 and major fragment ions at m/z 99 and 203, was consistent with the structure of $d_0$-α-tocopheronolactone shown in figure 2.6. The spectrum of peak C contained a presumed molecular ion at m/z 422 and major fragment ions at m/z 73, 236/237, and 309 and was very similar to that of $d_0$-α-CEHC (figure 2.5), although the retention time of this peak was 2 minutes greater than that of $d_9$-α-CEHC standard. The expected $d_3$-peaks (m/z 279 and 425) were less than 30% of the size of the corresponding $d_0$-peaks (m/z 276 and 422) in the extracted ion chromatogram indicating that less than 30% of the standard was deuterated (data not shown). The unexpected results obtained on analysis of the $d_3$-α-tocopheronolactone standard are discussed further below.

*GC-MS of urinary metabolites*

Using the procedure outlined in figure 2.4, three peaks, similar to those observed in the standards (A, B and C), were also observed in the urine sample. These were identified as $d_0$-α-tocopherol metabolites by comparison of their retention times and electron impact (EI) mass spectra to the deuterated standards (Figures 2.5 and 2.6). The GC-MS data was re-analysed to produce extracted ion chromatograms, which would pick out the vitamin E metabolites. The ions selected were m/z 276 (molecular ion of the $d_0$-form of peak A) and m/z 422 (molecular ion of the $d_0$-form of peaks B and C). In the extracted ion chromatograms of urine samples, peaks A and C were the major peaks with peak B being the minor peak (Figure 2.7). The spectrum of peak B was consistent with the structure of $d_0$-α-CEHC and the spectrum of peak A was consistent with the structure of $d_0$-α-tocopheronolactone shown in figure 2.6. However, the exact identity of peak C

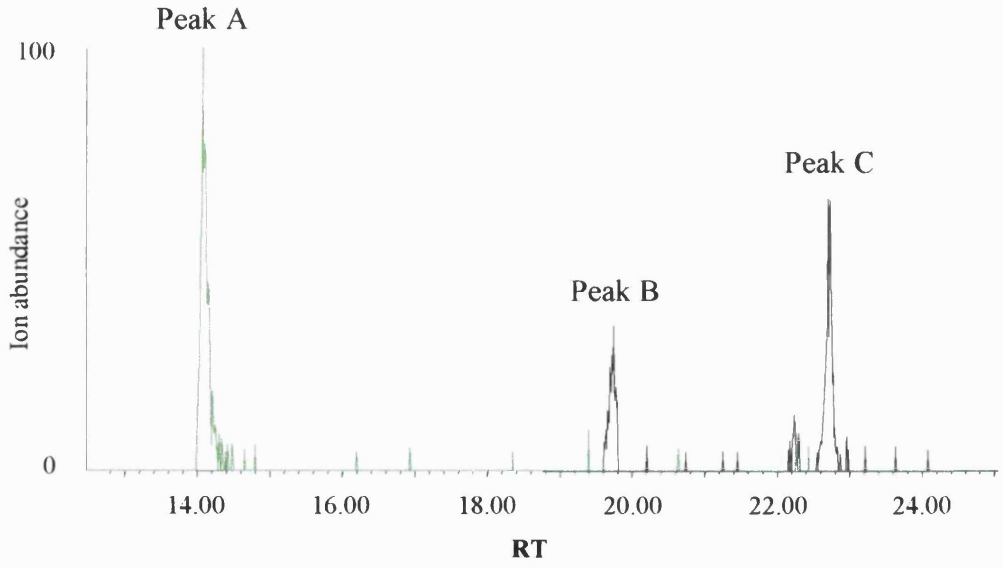



**Figure 2.7.** Extracted ion chromatogram (m/z 276 and 422) of human urine using the procedure outlined in figure 2.4. Peaks **A**, **B** and **C** correspond to the equivalent peaks produced by α-CEHC and α-tocopheronolactone standards (figure 2.5 and 2.6).

was uncertain since it did not appear to correspond to either of the reported α-tocopherol metabolites, α-tocopheronolactone or α-CEHC.

The appearance of peaks A and C in both the standard and urine preparations was puzzling at first. Originally it was proposed that the benzoquinone α-tocopheronolactone standard was able to form enols, thus allowing it to be partially derivatised by BSTFA and therefore producing two peaks. Enols can be identified by reaction with methoxy amine to form their corresponding methoximes (Bournot and Ramirez, 1989) (figure 2.8). However, GC-MS analysis indicated that the keto groups of the benzoquinone did not form methoximes, which indicated that enolisation, was not occurring. The lack of a peak or mass shift after reaction with diazomethane, a reagent that methylates carboxyl groups, also indicated that no carboxyl groups were present in the tocopheronolactone standard. Using this information it was proposed that peak B was α-CEHC, peak A was the benzoquinone form of α-tocopheronolactone and peak C was the hydroquinone form of α-tocopheronolactone. Analysis of the fragmentation patterns agreed with this identification.

The fact that our method showed α-CEHC to be a minor peak in urine compared to α-tocopheronolactone suggested that either artefactual oxidation was a major problem or that α-CEHC was being preferentially lost.

### 2.4.2. Modification of the GC-MS programme

In previous studies no mention has been made of the hydroquinone form of α-tocopheronolactone. α-Tocopheronolactone is described solely as the benzoquinone form (Simon et al., 1956b; Schultz et al., 1995). Hence it was considered that, owing to

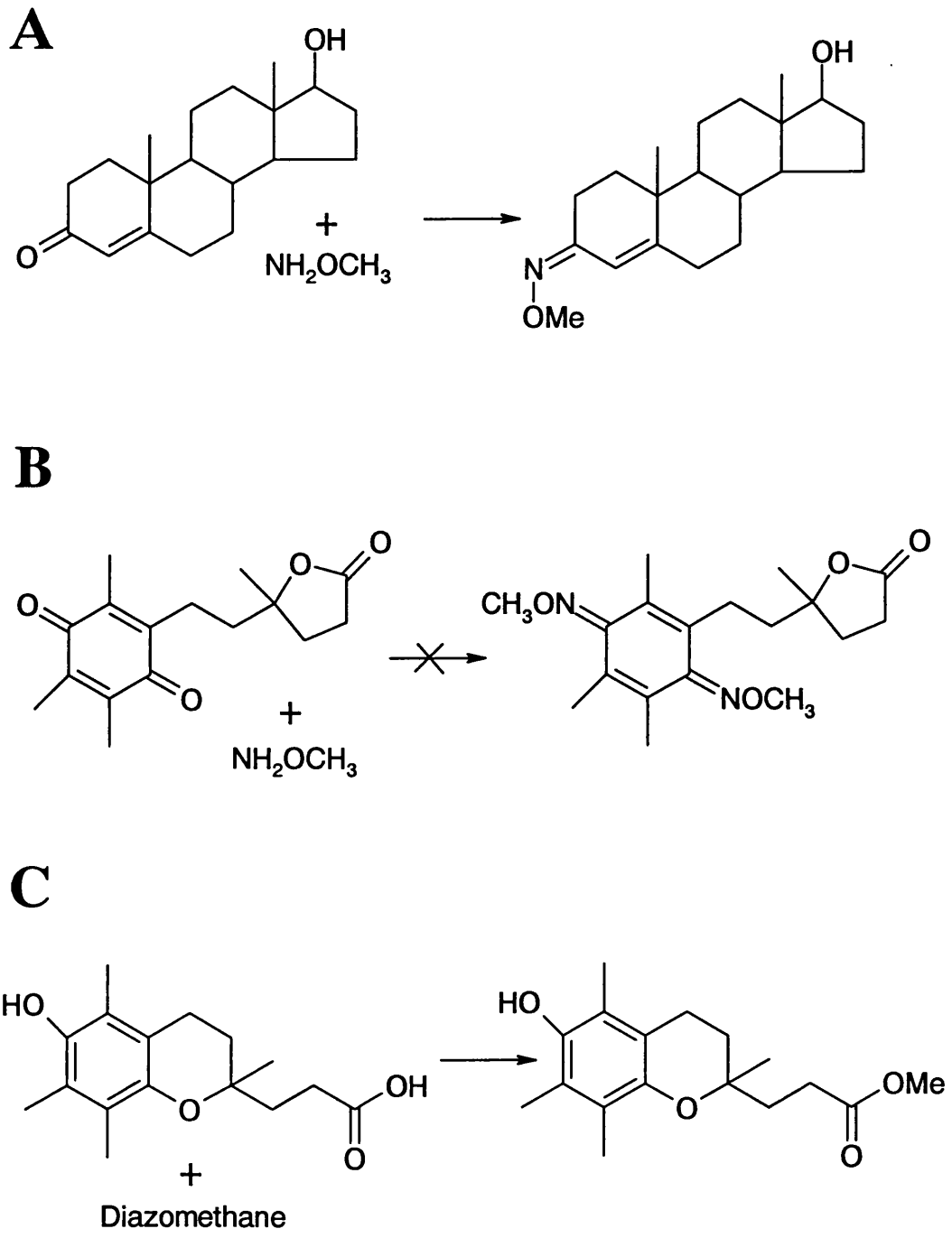



**Figure 2.8.** Use of derivatising agents to probe for functional groups. **A** Formation of the methyl oxime of a steroid to prevent enolization and subsequent silylation (Bournot and Ramirez, 1989). **B** The keto groups of the benzoquinone are unable to enolise and are thus unable to form methyl oximes. **C** Formation of the methyl ester of α-CEHC.

the similarity in the spectra of α-CEHC and α-tocopheronolactone, the hydroquinone form of α-tocopheronolactone may have been missed due to co-elution with α-CEHC. This could result from the shorter run times employed by other groups. This was investigated by using various temperature programmes, including those described in the literature, to investigate whether co-elution was a possibility. However separation of all 3 peaks was achieved under all the conditions (Table 2.2). The data suggested the possibility that either the hydroquinone form of α-tocopheronolactone had been missed by other workers or that it was not produced in the methodology adopted by them.

### 2.4.3. Removal of alumina step

Further work established that α-CEHC was indeed the major α-tocopherol metabolite seen in urine and that the alumina step was responsible for a considerable loss of α-CEHC during the extraction procedure (over 90% of α-CEHC standard was retained on the alumina column). The alumina column also appeared to be responsible for increased oxidation of both α-CEHC and the hydroquinone form of α-tocopheronolactone leading to increased levels of the benzoquinone form of α-tocopheronolactone. Alumina columns have also been reported to cause oxidation of catecholamines (Hugh et al., 1987). After removal of the alumina step only two main α-tocopherol metabolite peaks were observed in urine, α-CEHC and the hydroquinone form of α-tocopheronolactone. The benzoquinone α-tocopheronolactone peak could not be detected above background.

### 2.4.4. Flow rate optimisation and acidification

The procedure detailed in figure 2.9 was used as the basis to optimise the extraction, deconjugation, derivatisation and GC-MS analysis. Trolox and $d_9$-α-CEHC were added to fresh urine and used as standards to help optimise and validate the method.

| Method | GC temperature programme | Retention time Peak A | Retention time Peak B | Retention time Peak C |
|---|---|---|---|---|
| Method employed in our studies | 120°C – 2 min<br>20°C/min → 200°C<br>2°C/min → 240°C<br>50°C/min → 300°C<br>300°C - 5 min<br>Run time = 32.2 min | 13.1 min | 18.3 min | 21.2 min |
| Alternative method 1 | 180°C – 2 min<br>8°C/min → 240°C<br>25°C/min → 300°C<br>300°C – 5 min<br>Run time = 16.9 min | 9.1 min | 11.1 min | 11.7 min |
| Alternative method 2 | 200°C – 2 min<br>5°C/min → 240°C<br>25°C/min → 300°C<br>300°C – 5 min<br>Run time = 17.4 min | 7.6 min | 10.8 min | 11.7 min |
| Swanson et al. (1999) | 200°C – 2 min<br>10°C/min → 240°C<br>25°C/min → 285°C<br>285°C – 8 min<br>Run time = 15.8 min | 6.4 min | 7.9 min | 8.6 min |
| Schultz et al. (1995) | 180°C – 2 min<br>10°C/min → 280°C<br>25°C/min → 300°C<br>300°C – 5 min<br>Run time = 17.8 min | 8.4 min | 10.3 min | 11.3 min |

**Table 2.2.** Comparison of the retention times for the three main metabolite peaks observed in the deuterated standards and in urine using five different GC-MS temperature programmes. No co-elution of the 3 peaks was observed.

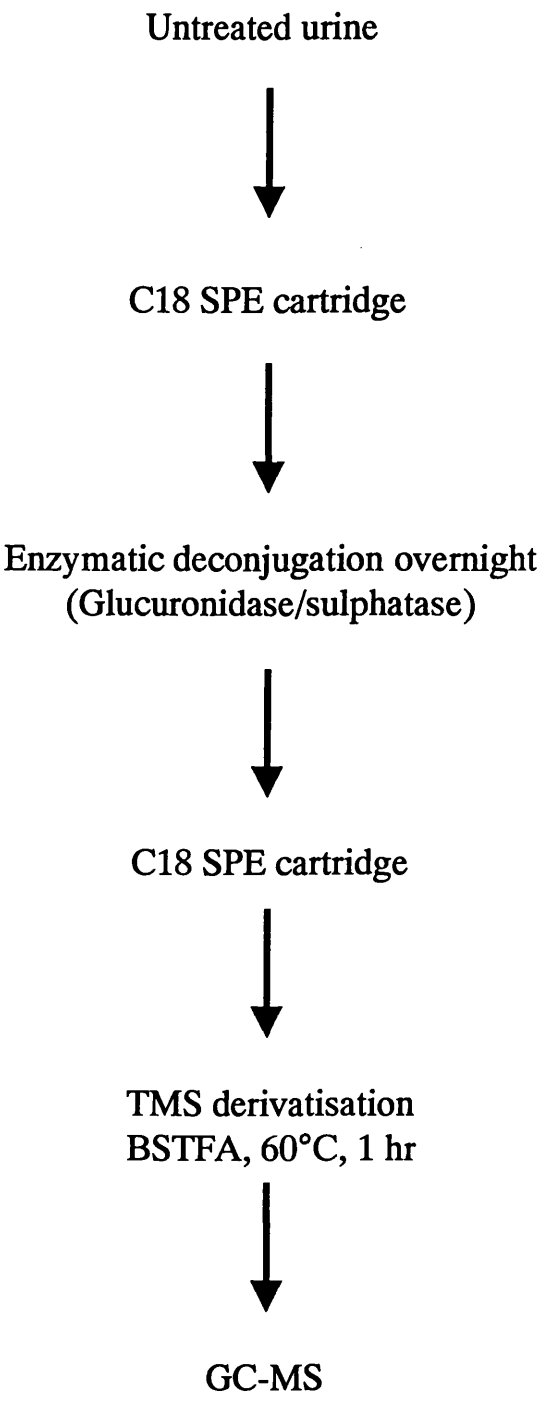



**Figure 2.9.** The initial procedure that was used in order to optimise the method.

The method detailed in figure 2.9 required approximately 5 ml of urine from subjects supplemented with α-tocopherol in order to obtain reasonable peak intensities. Analysis of flow through and wash fractions showed that greater amounts of metabolites were present in these fraction than in the methanol fraction, indicating that the metabolites were not efficiently retained on the C18 SPE cartridge.

Initial modifications were made to standardise flow rates. The cartridges were allowed to elute under gravity (rather than using negative pressure) at a flow rate of approximately 0.5ml/min. Secondly the urine samples were acidified to pH 2.5 with HCl, in order to protonate the metabolites and increase their affinity for the solid phase. These changes resulted in immediate gains in yield of the α-CEHC metabolite, as judged by at least a 5-fold increase in signal intensity/peak area compared to a known amount of derivatised standard ($d_9$-α-CEHC). Further studies indicated that this increase in recovery resulted mainly from the decrease in flow rate rather than the acidification of the sample.

### 2.4.5. Comparison of different SPE cartridges

Different types of SPE cartridges were assessed for their efficiency (table 2.3). These included C18 and C4 SPE cartridges, which consist of carbon chains lengths of 18 or 4 respectively, attached to a silica support, and Oasis SPE cartridges, which consist of a poly(divinylbenzene-co-N-vinylpyrrolidone) co-polymer with both hydrophilic and lipophilic retention characteristics. The assessments were based on the peak area obtained after a known amount of standard in a small volume of methanol (typically 5 μl) was dissolved in water (1 ml) at pH2.5 and extracted using a SPE cartridge. The different SPE cartridges all appeared to give a recovery of $d_9$-α-CEHC of approximately

| Solid phase extraction cartridge | % relative to unextracted $d_9$-α-CEHC (mean and range, n=3) |
|---|---|
| $d_9$-α-CEHC - no cartridge | 100 |
| C4 (Jones Chromatography) | 85 (77-89) |
| C18 (Jones Chromatography) | 83 (71-95) |
| Oasis (Waters Corporation)* | 84 (58-92) |

**Table 2.3.** Comparison of solid phase extraction cartridges. $d_9$-α-CEHC in aqueous solution (pH 2.5) was extracted using various solid phase extraction (SPE) cartridges. The yield was then compared to the same amount of $d_9$-α-CEHC derivatised directly without extraction.

* Oasis are a type of SPE cartridge which are reported to have similar retention characteristics to C18 SPE cartridges but require no priming.

85% compared to the same quantity of standard derivatised directly. This method of comparison is not particularly accurate due to variations in the GC-MS between runs affecting the peak areas, but averaged over triplicates, the results were considered sufficiently reliable for a qualitative assessment of the different SPE cartridges. Although both C18 and C4 cartridges gave similar recoveries, the C4 cartridges were chosen as they gave cleaner extracts, which could be important when a number of compounds of varying concentrations are being analysed in a complex matrix such as urine.

### 2.4.6. Enzymatic deconjugation

Enzymatic deconjugation, using a mixture of β-glucuronidase and aryl sulphatase, was investigated to check whether deconjugation went to completion. There was little difference in the ratios of the urinary metabolites compared to $d_9$-α-CEHC following 2 and 3 hours of incubation at 37°C but there was a slight increase in the concentration of most of the metabolites if the incubation was left for 18 hours at 37°C (Table 2.4). Doubling the amount of enzyme over an 18 hour incubation did not result in any further increase in the ratios of any of the metabolites, indicating that after 18 hours deconjugation had proceeded to completion (Table 2.4).

Deconjugation using methanolic HCl did not methylate all the α-CEHC present, as could be observed by the presence of unmethylated TMS-derivatised α-CEHC. This may have resulted from the presence of water in the methanolic HCl, which led to acid catalysed hydrolysis without subsequent esterification. For this reason enzymatic deconjugation was preferred to methanolic HCl treatment.

| | Ratios relative to $d_9$-α-CEHC | | | |
|---|---|---|---|---|
| Length of digest (hrs) | α-CEHC | α-TL | γ-CEHC | α-CMBHC |
| 2 | 0.94 | 0.09 | 0.29 | 0.16 |
| 3 | 0.89 | 0.14 | 0.33 | 0.21 |
| 18 | 0.99 | 0.22 | 0.46 | 0.21 |
| 18 hr digest using different urine to above | | | | |
| 1 x enzyme | 0.72 | 0.08 | 0.78 | 0.07 |
| 2 x enzyme | 0.70 | 0.13 | 0.76 | 0.06 |

**Table 2.4.** The effect of time and amount of enzyme on enzymatic deconjugation (n=1).
α-TL = α-tocopheronolactone; γ-CEHC = CEHC produced from γ-tocopherol; α-CMBHC = α-carboxy-methyl-butyl hydroxychroman. These metabolites will be discussed in greater detail later in this chapter and also in chapters 3 and 4.

### 2.4.7. Derivatisation

Derivatisation of α-CEHC and α-tocopheronolactone, with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) for 1 hr at 60°C, gave clean chromatograms and good fragmentation spectra following electron impact. Addition of derivatising agent to α-CEHC and α-tocopheronolactone standards just prior to injection gave a very similar response to standards that had been derivatised for one hour, suggesting derivatisation of the metabolites occurred almost immediately.

### 2.4.8. GC-MS Programme

A number of GC-MS parameters were investigated. The two main aspects which can be modified were the temperature programme of the GC oven and whether the MS was used in selected ion monitoring (SIM) or scan mode.

An ideal GC temperature programme is one that affords good separation of the various components in the shortest possible time. The GC temperature programmes used by other researchers in the field are detailed in table 2.2. The majority of these programmes required relatively short run times but the resolution of peaks was poor especially in concentrated urine samples. Therefore, a longer GC temperature programme was chosen since this produced adequate resolution and separation even in concentrated urine samples.

Selected ion monitoring (SIM) is often used to increase sensitivity when analysing low concentrations of compounds in complex mixtures. In normal scan mode, the MS scans the entire mass range focusing on each ion for only a short time. In SIM mode only one or a few selected mass ions are analysed, thereby increasing the time spent in detecting

each mass ion of interest and thereby increasing the sensitivity. SIM mode is, therefore, useful if high sensitivity is required. In the present study it was considered useful to obtain full mass spectra for every peak since it could help in the identification of any unknown peaks.

**2.5. Final method**

Following the optimisation experiments, the following protocol was adopted for the analysis of vitamin E metabolites used throughout the course of this study. (All chemicals and solvents were obtained from Sigma-Aldrich Company Ltd unless otherwise stated.)

50 mg of sodium ascorbate was added to each 30 ml urine sample to prevent oxidation. Trolox (5 nmoles) and $d_9$-α-CEHC (20 nmoles), dissolved in 5 μl of methanol, were added to 0.5ml of fresh urine which was then acidified to pH 2.5 with 3M HCl. The $d_9$-α-CEHC standard was added to monitor any artefactual conversion to $d_9$-α-tocopheronolactone during the procedure. The sample was then loaded onto a C4 solid phase extraction cartridge (SPE) (100mg sorbent mass with a 3ml reservoir supplied by Jones Chromatography Ltd), which had been primed with methanol (1 ml) and deionised water (1 ml, pH 2.5). The cartridge was eluted under gravity. The column was then washed with 1ml deionised water (pH 2.5) and the metabolites eluted with 2ml methanol. The flow rates were maintained at approximately 0.5 ml/min throughout. The methanolic extract was evaporated to dryness under nitrogen at 37°C and was then resuspended in 1ml deionised $H_2O$ to which 50μl sodium acetate (5.0M, pH4.7) and 25μl of β-glucuronidase/sulphatase (β-glucuronidase activity of 100,00 units per ml and arylsulphatase activity of 7,500 units per ml from Helix pomatia, type HP-2, G7017, Sigma-Aldrich Company Ltd) was added. The sample was incubated at 37°C for 18

hours and then extracted using a second C4 SPE cartridge as described above. The methanolic phase was again dried under nitrogen at 37$^{o}$C and the metabolites converted to TMS derivatives with 200µl BSTFA (Pierce and Warriner Ltd) at 60°C for 1 hour. 2µl of the derivatised mixture was injected (using a splitless technique) onto a DB1 fused silica column (30m, 0.25 mm ID, 0.25µm film thickness) supplied by Jones Chromatography Ltd in a Hewlett-Packard 5890 Series II gas chromatograph linked to a Hewlett-Packard 5970 mass-selective detector and Chem Station data system. The oven was maintained at 120°C for 2 min, then ramped to 200°C at 20°C/min, to 240°C at 2°C/min, to 300°C at 50°C/min and finally held at 300°C for 5 min. The ionisation energy was 70 eV. Scan mode was used in preference to single ion monitoring so that full spectra could be obtained to aid the characterisation of any novel or unidentified peaks. Peaks were identified by comparison of their retention time and spectra to known standards and published data. Metabolite and standard peaks were visualised by extracting chromatograms for the following ions:-

Standards -Trolox, m/z 394, 237; $d_9$-α-CEHC, m/z 431, 243; $d_0$-α-tocopheronolactone, m/z 422, 237;

Metabolites - $d_0$-α-CEHC/ tocopheronolactone, m/z 422, 237; $d_6$-α-CEHC/ tocopheronolactone, m/z 428, 243; $d_0$-δ-CEHC, m/z 394, 209; $d_0$-γ-CEHC, m/z 408, 223; $d_0$-β-CEHC, m/z 408, 223; $d_0$-α-CMBHC, m/z 464, 237; $d_6$-α-CMBHC, m/z 470, 243; $d_0$-γ-CMBHC, m/z 450, 223; $d_0$-δ-CMBHC, m/z 436, 209; $d_0$-β-CMBHC, m/z 450, 223. The measurement of these metabolites is discussed in greater detail in chapters 3 and 4.

A typical extracted ion chromatogram (figure 2.10) is described below.

*Quantitation*

Concentrations of metabolites were in general expressed as trolox equivalents per creatinine. Trolox equivalents were obtained by comparing the peak size of the metabolite of interest (molecular ion) to the peak size of a known amount of trolox (m/z 394). Ideally these trolox equivalent values would have been converted, using appropriate calibration curves, into moles. However, this was not possible because of the lack of appropriate standards for each metabolite. Creatinine concentrations were measured on a COBAS analyser using a kit supplied by Roche Products, Ltd.

## 2.6. Results

### 2.6.1. Recovery using radiolabelled rat urine

Urine from rats fed radiolabelled ($C^{14}$) α-tocopherol, kindly supplied by Drs O Froescheis and W Cohn (F Hoffmann-La Roche Ltd), was added to human urine to assess the recovery of the metabolites during the procedure. 100μl of radiolabelled rat urine was added to 2ml of human urine and then extracted and deconjugated as described above. Radioactivity was measured by typically adding 100 - 200μl of the various fractions to 3ml Optiphase Hisafe 3 scintillation fluid (Wallac Scintillation Products Ltd) and counting the dpm using a Wallac 1410 scintillation counter with external standardisation. The mean final recovery of radiolabel after the second solid phase extraction was 98.8% (n=6) with a range of 81.0-115.7%. Assuming rats produced similar or identical metabolites to humans, these results showed that the metabolites of α-tocopherol were efficiently extracted during the procedure.

### 2.6.2. Different Metabolites

Figure 2.10 shows an extracted ion chromatogram of human urine collected 7 days after administration of a single dose of 300mg deuterated $d_6$-RRR-α-tocopheryl acetate (kindly supplied by G Burton). Although standards were only available for α-tocopheronolactone and α-CEHC, the other metabolites were identified by relative retention times, characteristic mass spectra and fragmentation patterns (Chiku et al., 1984; Schultz et al., 1995; Swanson et al., 1999) (see figure 2.5 for the published spectrum of TMS-α-CEHC). The retention times of the CEHCs were expected to be in the order $\delta<\gamma=\beta<\alpha$ because of the known effects of extra methyl groups on the retention of compounds on silica based GC columns (Littlewood, 1970). Oral supplementation with deuterated $d_6$-RRR-α-tocopheryl acetate also helped to confirm identities, as it produced analogous urinary metabolites with mass shifts of 6 daltons (see below and chapters 3 and 4).

Mass spectra of the TMS-esters/ethers of the α-tocopheronolactone and α-CEHC standards are shown in figure 2.11. These isomeric compounds both show a large molecular ion (m/z 422). The major fragment ions are generated by cleavage of weak bonds i.e. those which are separated by a single bond from the double bonds of the unsaturated ring (allylic bonds). Trimethylsilyl ether/ester groups break between the oxygen and silicon atoms producing a typical trimethylsilyl ether peak (m/z 73). The TMS ethers/esters of both α-tocopheronolactone and α-CEHC also show a peak of m/z 237 whose origin, by cleavage of two allylic bonds, is illustrated in figure 2.11. The spectrum of the TMS ether of α-tocopheronolactone also shows a prominent peak of m/z 309, which can be explained by the cleavage of a single allylic bond.

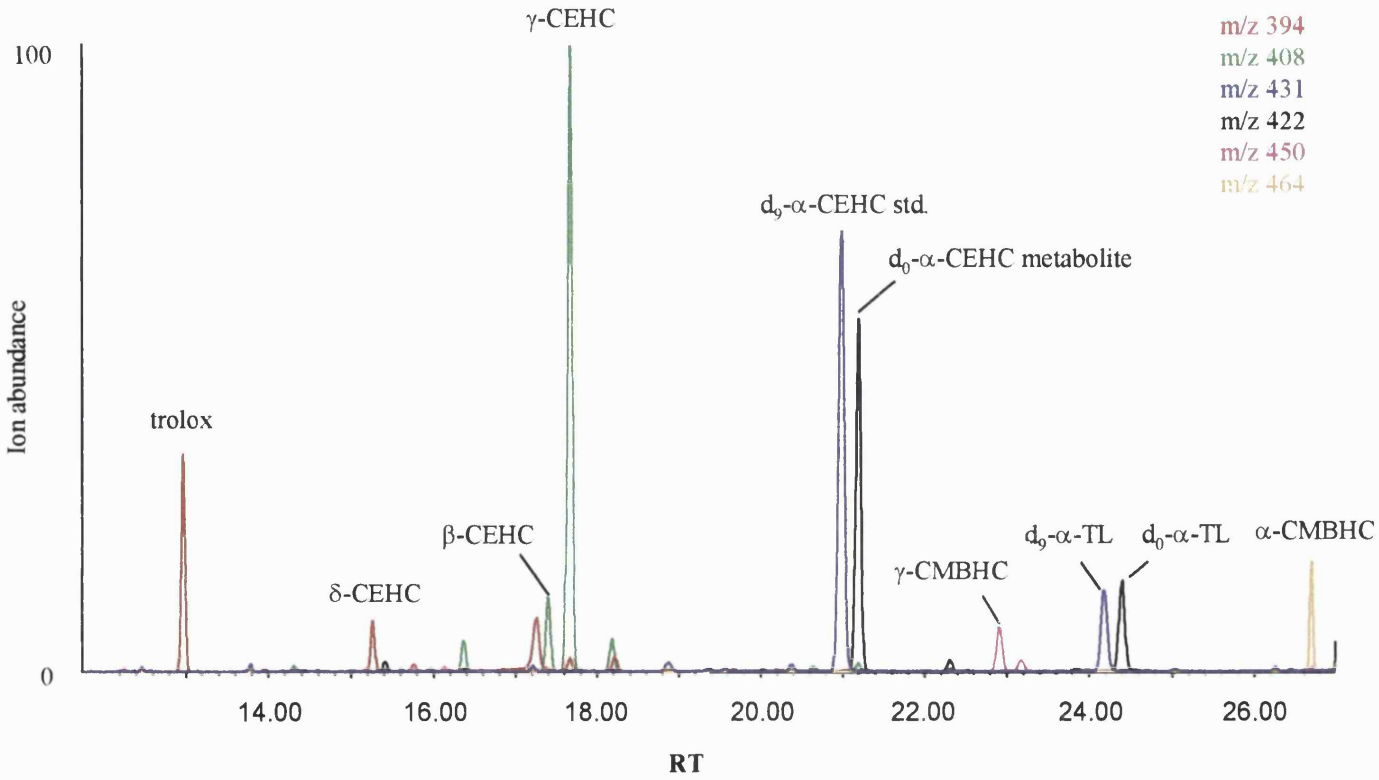



**Figure 2.10.** An extracted ion chromatogram showing the main urinary vitamin E metabolites.

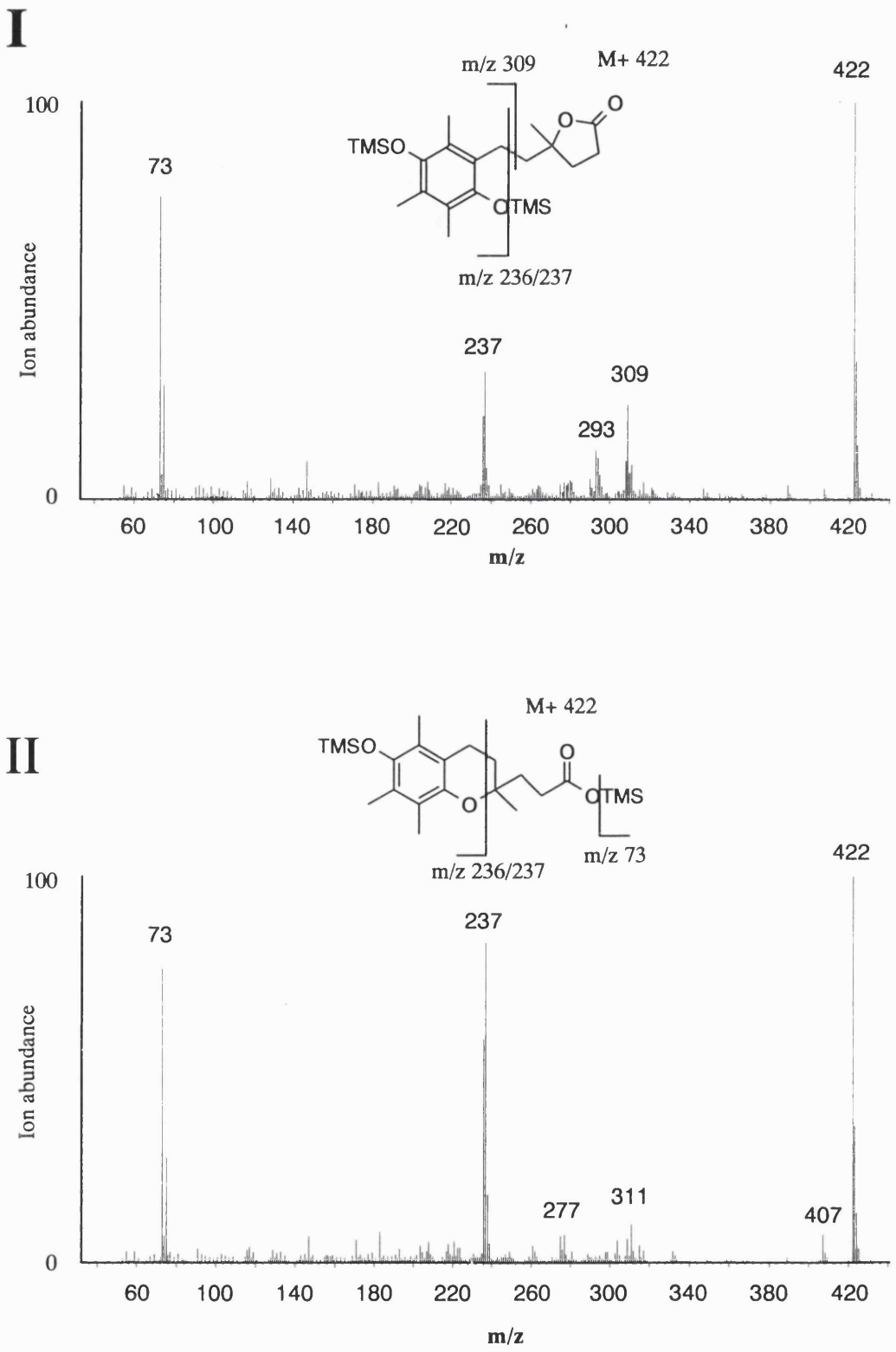



**Figure 2.11.** Mass spectra of α-tocopheronolactone-TMS (**I**) and α-CEHC-TMS (**II**) with fragment and molecular ions shown.

During the development of the method a number of peaks were tentatively identified as novel vitamin E metabolites. These metabolites were observed for all the major tocopherols and displayed mass spectra similar to those of the equivalent CEHCs except the molecular ions were 42 mass units greater. The mass spectral data was consistent with structures identical to the CEHCs but with an extra three carbons in the side-chain. These metabolites were tentatively identified as the carboxy-methyl-butyl hydroxychromans (CMBHCs). The spectra for α- and γ-CMBHC are shown in figure 2.12. Confirmation of the identity, investigation and further discussion of these metabolites is described in chapter 3. The spectra of δ-CEHC and γ-CEHC are shown in figure 2.13.

### 2.6.3. Reproducibility

Since standards were not available for all the metabolites, trolox was used for quantitative purposes. Theoretically, if the method was reproducible, the ratio of the areas of the peaks of interest to trolox would remain constant. Therefore, each peak was given a relative intensity compared to the trolox peak and the reproducibility of these relative intensities, and not the actual amounts of metabolites, was measured. The coefficients of variation (CV) within and between batches of analyses are shown in table 2.5. For the majority of metabolites the within batch CVs were less than 10% (n=6) and between batch less than 20% (n=4 over a 2 week period). α-Tocopheronolactone was an exception, as both the $d_9$-α-tocopheronolactone derived from the $d_9$-α-CEHC and the endogenous unlabelled metabolite showed a much greater CV than the other metabolites for both within and between batches. The possible reasons for this increased variation will now be discussed.

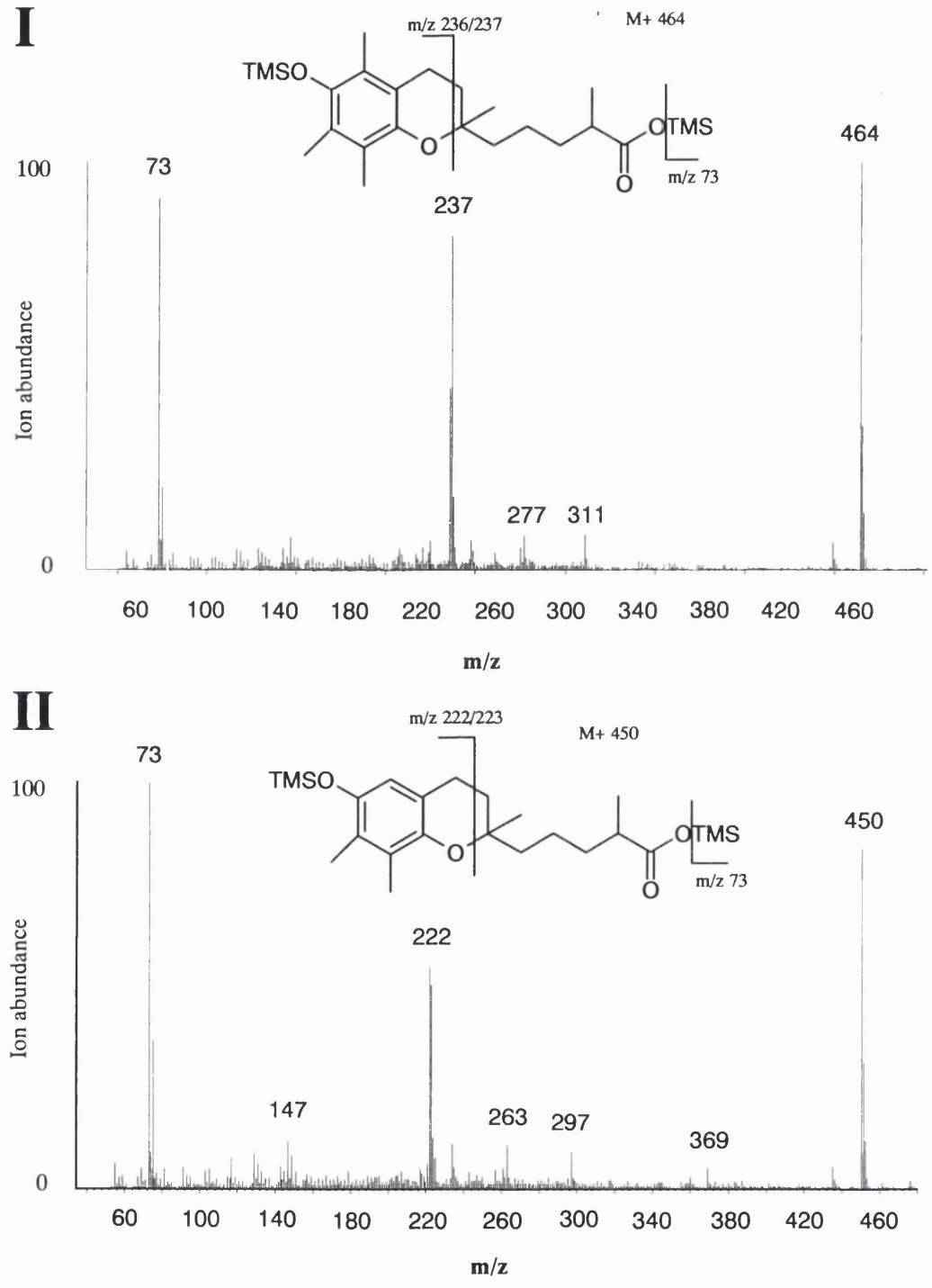



**Figure 2.12.** Mass spectra of α-CMBHC-TMS (**I**) and γ-CMBHC-TMS (**II**).

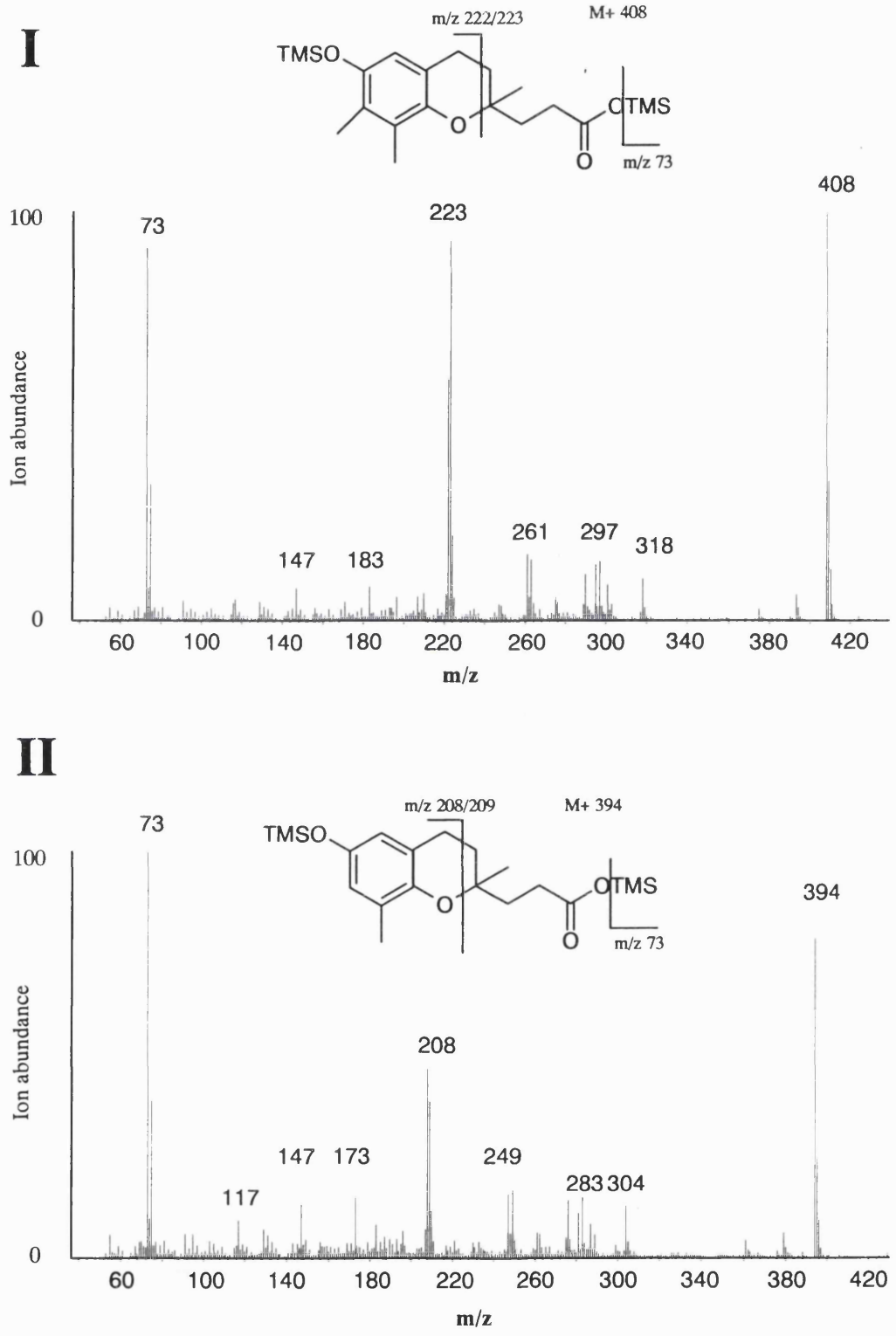



**Figure 2.13.** Mass spectra of γ-CEHC-TMS (**I**) and δ-CEHC-TMS (**II**).

| Metabolite or standard | Within day CV% (n=6) | Between day CV % (n=4, over 14 days) |
|---|---|---|
| $d_9$-α-CEHC (std.) | 4.0 | 17.8 |
| $d_9$-α-tocopheronolactone (std.) | 19.8 | 53.2 |
| $d_0$-α-CEHC (metabolite) | 4.5 | 19.3 |
| $d_0$-α-tocopheronolactone | 21.9 | 64.7 |
| α-CMBHC | 6.8 | 1.6 |
| γ-CEHC | 4.5 | 16.7 |

**Table 2.5.** Reproducibility of the method.

CV = coefficient of variation ; CVs were calculated on full replicate analyses, not on replicates of a silylated sample. Between day CVs were calculated by comparing a pooled urine sample extracted once on four different occasions.

### 2.6.4. Artefactual oxidation

The poor reproducibility of α-tocopheronolactone may result from variable artefactual conversion of α-CEHC to α-tocopheronolactone (Figure 2.14). As described earlier, Schultz et al. (1995) reported almost complete conversion of α-CEHC to α-tocopheronolactone under acidic conditions in the presence of oxygen. Despite trying to keep artefactual oxidation to a minimum by drying under nitrogen, minimising heat and acidity and reducing the number of solvent changes, it was found that when $d_9$-α-CEHC was added to fresh urine, 5 to 10% was converted to $d_9$-tocopheronolactone. However, the ratio of α-tocopheronolactone to α-CEHC was generally greater for the endogenous unlabelled metabolites than for the deuterated compounds ($d_0$-α-TL:$d_0$-α-CEHC / $d_9$-α-TL:$d_9$-α-CEHC gave a mean ratio of 1.82, CV 56%, n=18), which indicated that there may be some endogenous α-tocopheronolactone present in urine. An alternative explanation could be that conjugated α-CEHC might be oxidised to α-tocopheronolactone more easily than the unconjugated form (see discussion). It is of interest to point out that in the present study, tocopheronolactone metabolites from the other tocopherols were rarely detected (see also 4.2.2.3). This suggested that these CEHC metabolites may be more stable to artefactual oxidation than α-CEHC.

Possible causes of artefactual oxidation of α-CEHC to α-tocopheronolactone include the presence of oxygen in air and excess heat. These were investigated by heating $d_9$-α-CEHC standard in methanol, at 60°C for 6 hours in the presence of air. After this time no $d_9$-α-tocopheronolactone could be detected by GC-MS analysis indicating these factors alone are not responsible for causing the artefactual oxidation observed in the urine samples.

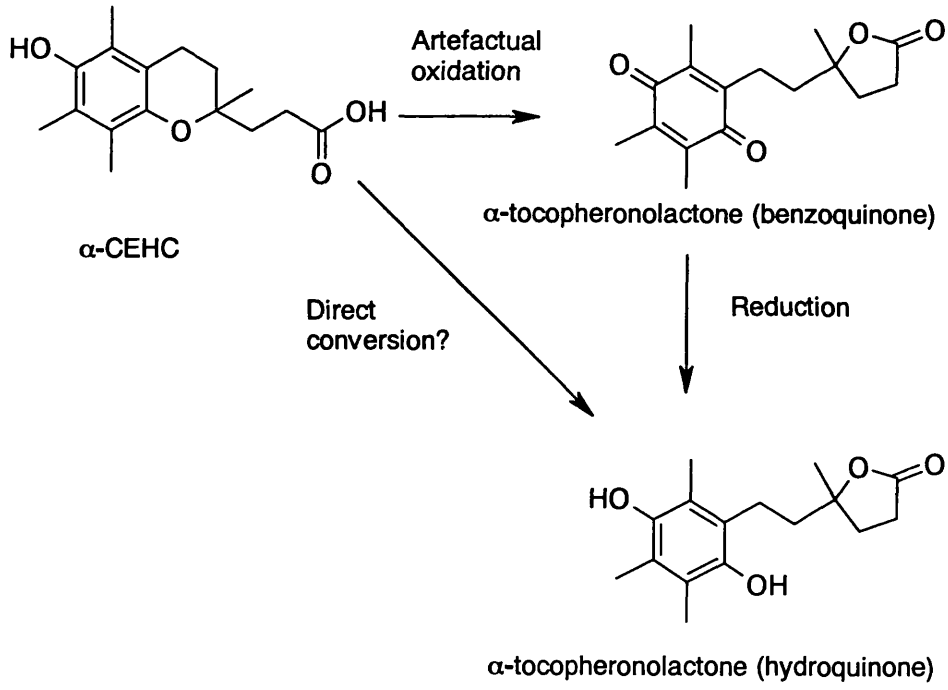



**Figure 2.14.** Artefactual oxidation of α-CEHC to α-tocopheronolactone.

## 2.7. Discussion

The method described above showed reasonable reproducibility for the CEHC and presumed CMBHC type metabolites. However it showed poor reproducibility for α-tocopheronolactone. Addition of $d_9$-α-CEHC standard to fresh urine should be able to give an estimate of the amount of artefactual oxidation which has taken place. However, this assumes that the metabolites experience the same environment as the added standard which may not be the case. The metabolites are expected to be present in the urine in the form of sulphate or glucuronide conjugates whereas the added standard is unconjugated. This difference in polarity may cause the standard to experience a different micro-environment to the endogenous metabolites affecting their relative rates of oxidation. In addition, the fact that the standard is added in a small volume of methanol may also have some effect. The ratio of endogenous α-tocopheronolactone to endogenous α-CEHC is generally greater than that of $d_9$-α-tocopheronolactone to $d_9$-α-CEHC but the trend is not sufficiently consistent to say for sure whether any non-artefactual α-tocopheronolactone is present.

Attempts to derivatise α-CEHC at the carboxyl group were made to prevent formation of the lactone. The use of diazomethane to methylate the carboxyl group prior to deconjugation resulted in drastically reduced levels of the methylated metabolites, perhaps due to methylation of the carboxyl group on the glucuronic acid affecting enzyme recognition. Post deconjugation methylation with diazomethane worked well but since some artefactual oxidation had already occurred during deconjugation, this approach did not prove to be valuable. The use of methanolic HCl seemed more promising owing to its combined deconjugating and esterifying action but as described

above the lack of complete derivatisation meant the method was no better than enzymatic deconjugation.

The oxidation of α-CEHC would be expected to produce a benzoquinone oxidation product similar to that observed for α-tocopherol and other vitamin E type compounds (Liebler, 1993; Yamazaki et al., 1999). The fact that the hydroquinone and not the benzoquinone form of α-tocopheronolactone was observed in our methodology was surprising. There are a number of possibilities to explain this observation. It could be that the α-CEHC was oxidised to the benzoquinone, which was subsequently reduced to the hydroquinone or α-CEHC was converted directly to the hydroquinone.

In conclusion the method developed during the course of this study allowed the reproducible measurement of both CEHC and CMBHC type metabolites. However, owing to variable levels of artefactual oxidation the method was not suitable for accurately measuring concentrations of α-tocopheronolactone. Quantitation could be achieved by using trolox as an internal standard. A typical urinary α-CEHC concentration in unsupplemented urine using this method was between 2 and 20 μmol/l, which compares favourably with published data employing other methods. The sensitivity of this method was of a similar order to that recently reported by Lodge et al. (2000) using HPLC with ECD detection.

# CHAPTER 3

## Identification of a Novel Group of Urinary Vitamin E Metabolites (CMBHCs) and Their Confirmation Using a Chemically Synthesised Standard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## 3.1. Introduction

At the start of the present study the CEHCs and α-tocopheronolactone were the only urinary vitamin E metabolites that had been fully characterised. However, studies where radiolabelled vitamin E had been administered to rabbits and rats suggested that, apart from the CEHCs and α-tocopheronolactone, other metabolites were also excreted in the urine, albeit in small amounts (Simon et al., 1956a; Simon et al., 1956b; Chiku et al., 1984). Chiku et al. (1984) isolated two metabolites by TLC after derivatisation with diazomethane. However only the major metabolite, δ-CEHC, which accounted for 75% of the radioactivity, was fully characterised.

This chapter details an investigation for novel urinary vitamin E metabolites using the GC-MS technique described in the previous chapter. By detailed analysis of fragmentation patterns, the use of deuterated α-tocopheryl acetate supplements and the chemical synthesis of an authentic standard it was possible to fully characterise a novel group of metabolites of vitamin E.*

## 3.2. Initial identification of possible novel urinary vitamin E metabolites

Chromatograms obtained using the extraction procedure and GC-MS method described in chapter 2 were examined for peaks that contained the same fragment ions as those displayed by known vitamin E metabolites. The most common fragment ions displayed by vitamin E metabolites and vitamin E analogues are those produced by cleavage of the chroman ring or the equivalent bonds if the chroman ring has been oxidised as in the case of α-tocopheronolactone and α-tocopherylquinone (Liebler et al., 1996; Liebler etal., 1999; Swanson et al., 1999; Pope et al., 2000) (see figure 3.1).

* A paper describing the work in this chapter has recently been published (Pope et al., 2001).

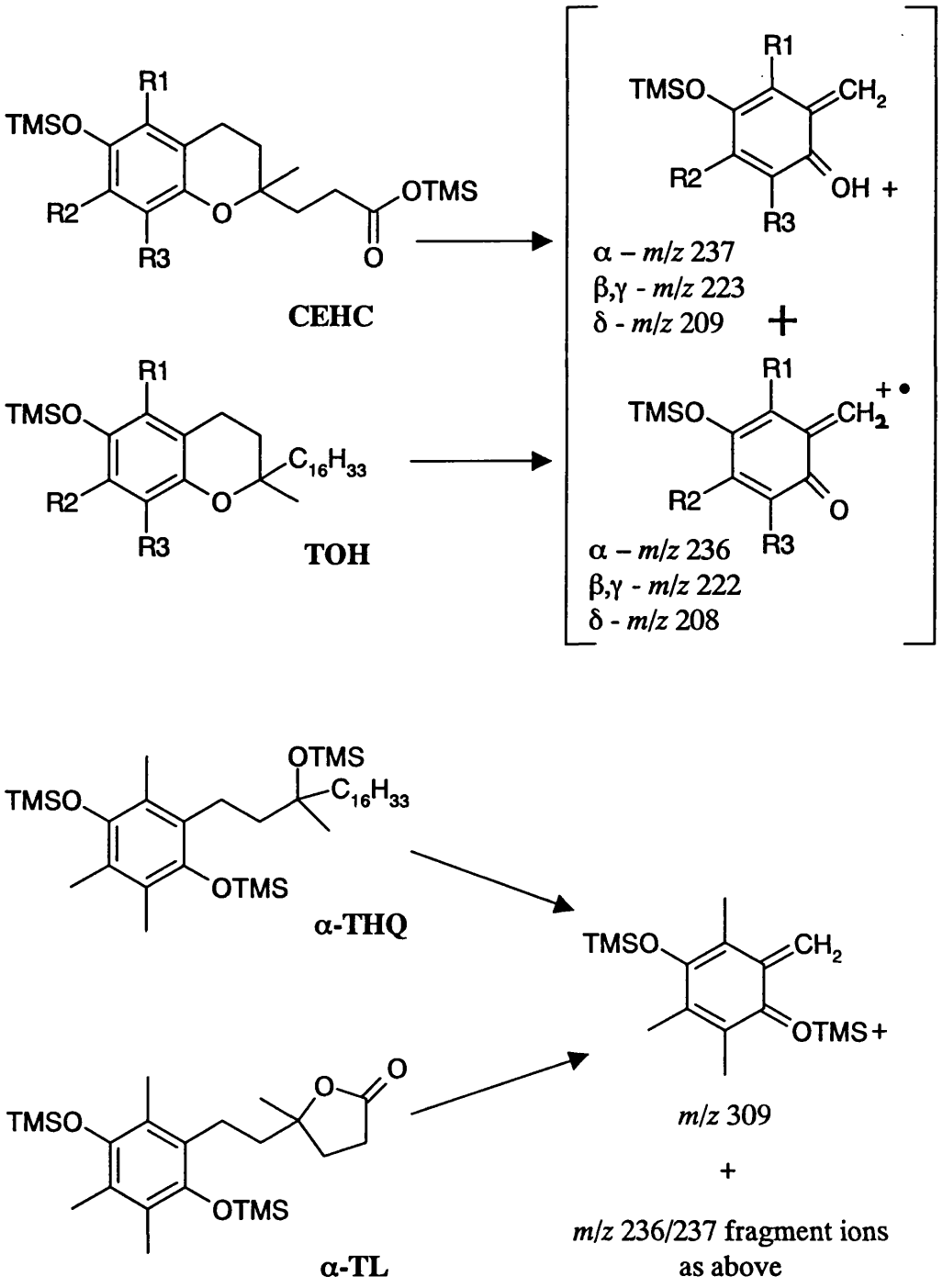



**Figure 3.1.** Major characteristic fragment ions of TMS derivatives of carboxy-ethyl hydroxychromans (CEHCs), tocopherols (TOH), α-tocopheryl-hydroquinone (α-THQ) and α-tocopheronolactone hydroquinone (α-TL).

Using this approach a number of unknown peaks were observed which displayed the major fragment ion, containing the ring structure, of either α-CEHC-TMS (m/z 236/237), α-tocopherylhydroquinone-TMS (m/z 236/237 and 309), α-tocopheronolactone-TMS (m/z 236/237 and m/z 309), γ- and β-CEHC-TMS (m/z 222/223) or δ-CEHC-TMS (m/z 208/209). NB. The fragment ions of α, β, γ, and δ-CEHC differ by 14 or 28 mass units, equivalent to one or two methyl groups respectively. The fragment ions of the benzoquinone form of α-tocopheronolactone, owing to their low mass to charge ratio, were non-selective and were present in numerous peaks.

Figure 3.2 displays the electron impact spectra of three potential vitamin E metabolites, which eluted from the gas chromatography column in the order C, B, A. These spectra display molecular ions which differ from each other by 14 or 28 mass units, equivalent to one or two methyl groups respectively. It is also noteworthy that the molecular and major fragment ion, containing the ring structure, for each metabolite differ by a mass of 227, suggesting they all have the same side chain. These observations supported the notion that these three peaks (A-C) corresponded to homologues of the same metabolite, produced from α-, γ- and δ-tocopherol respectively. The similarity of the spectra in figure 3.2 to those of α-, γ- and δ-CEHC (see figures 2.11 and 2.13, chapter 2) also suggested that these metabolites were related to the CEHCs. The absence of major fragment ions of m/z 309, 295 or 281 made it unlikely that these metabolites were longer side chain precursors of tocopheronolactones (see figure 2.11, chapter 2).

The molecular ion in each case was 42 mass units greater than the equivalent CEHC (i.e. α-homologue versus α-CEHC, γ-homologue versus γ-CEHC etc.), suggesting an

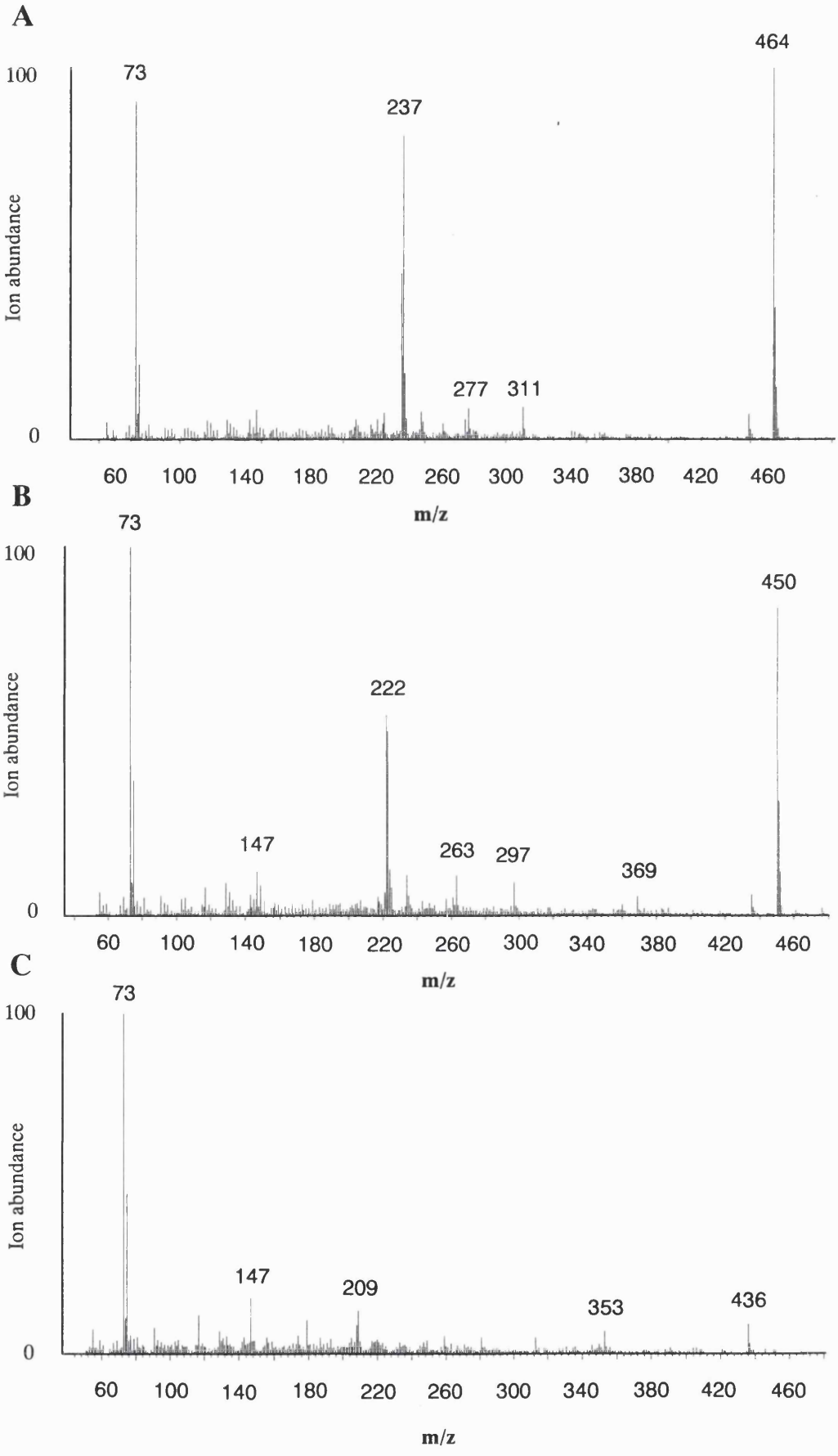


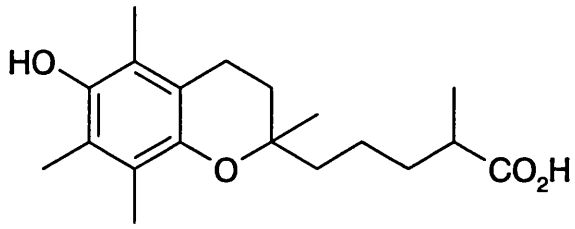

**Figure 3.2**. Spectra of unknown metabolites proposed to derive from **A** α-tocopherol, **B** γ-tocopherol and **C** δ-tocopherol. These metabolites were tentatively identified as carboxy-methyl-butyl hydroxychromans (CMBHCs).

extra three carbons in the structure. Owing to the CEHC ring type fragment ions observed for these metabolites the most likely position of three extra carbons was in the side-chain. Therefore these metabolites were tentatively identified as carboxy-methyl-butyl hydroxychromans (CMBHCs), on the basis of the nomenclature previously used by Weichet et al. (1966) (Figure 3.3). Alternatively these metabolites could be named carboxy-pentyl hydroxychromans CPHCs (Schuelke et al., 2000) or 5' carboxychromans (Parker and Swanson, 2000).

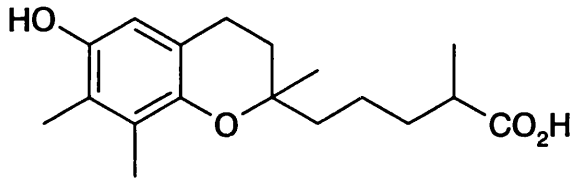
A number of other peaks that displayed fragment ions of m/z 237, 309, 223 or 209 were also observed. However these metabolites did not appear to be members of a group of compounds like the CMBHCs and their spectra were not consistent with the structures of hypothetical vitamin E metabolites. Therefore the majority of these peaks were not thought to be vitamin E metabolites.

### 3.2.1. Use of deuterated RRR-α-tocopheryl acetate to confirm unknown peaks as metabolites of α-tocopherol

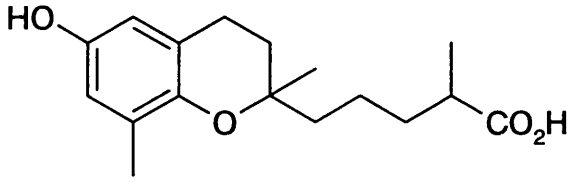
Deuterated $d_6$-RRR-α-tocopheryl acetate was administered to human subjects in order to probe for potential α-tocopherol metabolites. The deuterated analogue contained six deuteriums in the methyl groups surrounding the chroman ring and it was expected to produce a new set of metabolites with a mass shift of 6 for both the molecular ion and the major fragment ion of all urinary α-tocopherol metabolites for a number of days after administration. Using this approach it was hoped to be able to confirm that previously identified metabolites were actually produced from α-tocopherol.




**α-CMBHC**




**γ-CMBHC**




**δ-CMBHC**

**Figure 3.3.** Possible structures of the unknown metabolites tentatively identified as CMBHCs in figure 3.2.

A mass shift of 6 was observed for both the molecular and fragment ions of α-CMBHC after a single oral dose of $d_6$-RRR-α-tocopheryl acetate (figure 3.4). The retention times of $d_0$- and $d_6$-α-CMBHC were very similar but not identical, which is not surprising since $d_9$-α-CEHC and $d_0$-α-CEHC showed similar differences in their retention times (see figure 2.10) and the phenomenon has also been reported for other deuterated compounds (Chapman, 1998). The deuterated species was only detected in urine for about a week after the oral dose and was not detected either before or after this period (figure 3.5). Together these observations confirmed that α-CMBHC was a metabolite of α-tocopherol. By analogy the other metabolites (B and C, figure 3.2) were presumed to be produced from γ- and δ-tocopherol. No equivalent metabolites were observed for β-tocopherol probably owing to the low levels of this tocopherol in the diet.

No mass shift was, however, observed for other unidentified compounds displaying fragment ions consistent with α-tocopherol metabolites (i.e. m/z 236/237 and m/z 309). This suggested that these compounds were either not produced from α-tocopherol or were only produced from α-tocopherol that was present in the body before administration of $d_6$-α-tocopherol i.e. they were produced from the body pool of α-tocopherol rather than from the newly administered α-tocopherol.

### 3.2.2. Plausible structure of novel metabolites based on the biological oxidation of the phytyl side-chain

The side-chain of vitamin E resembles that of a variety of other compounds, which have been shown to be metabolised in the peroxisomes by β-oxidation (Mannaerts and Van Veldhoven, 1995). Examples of these compounds include phytanic acid whose structure

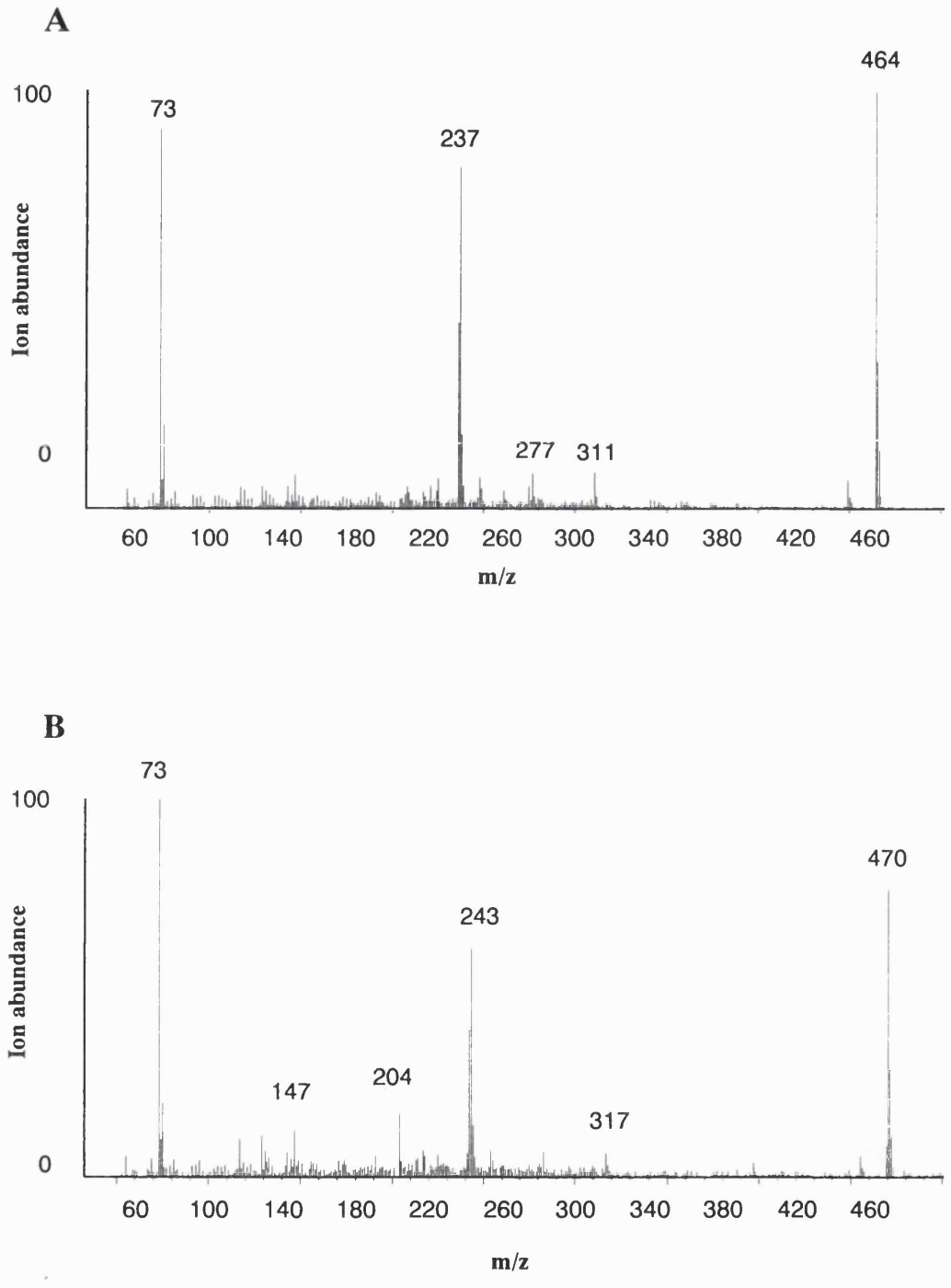
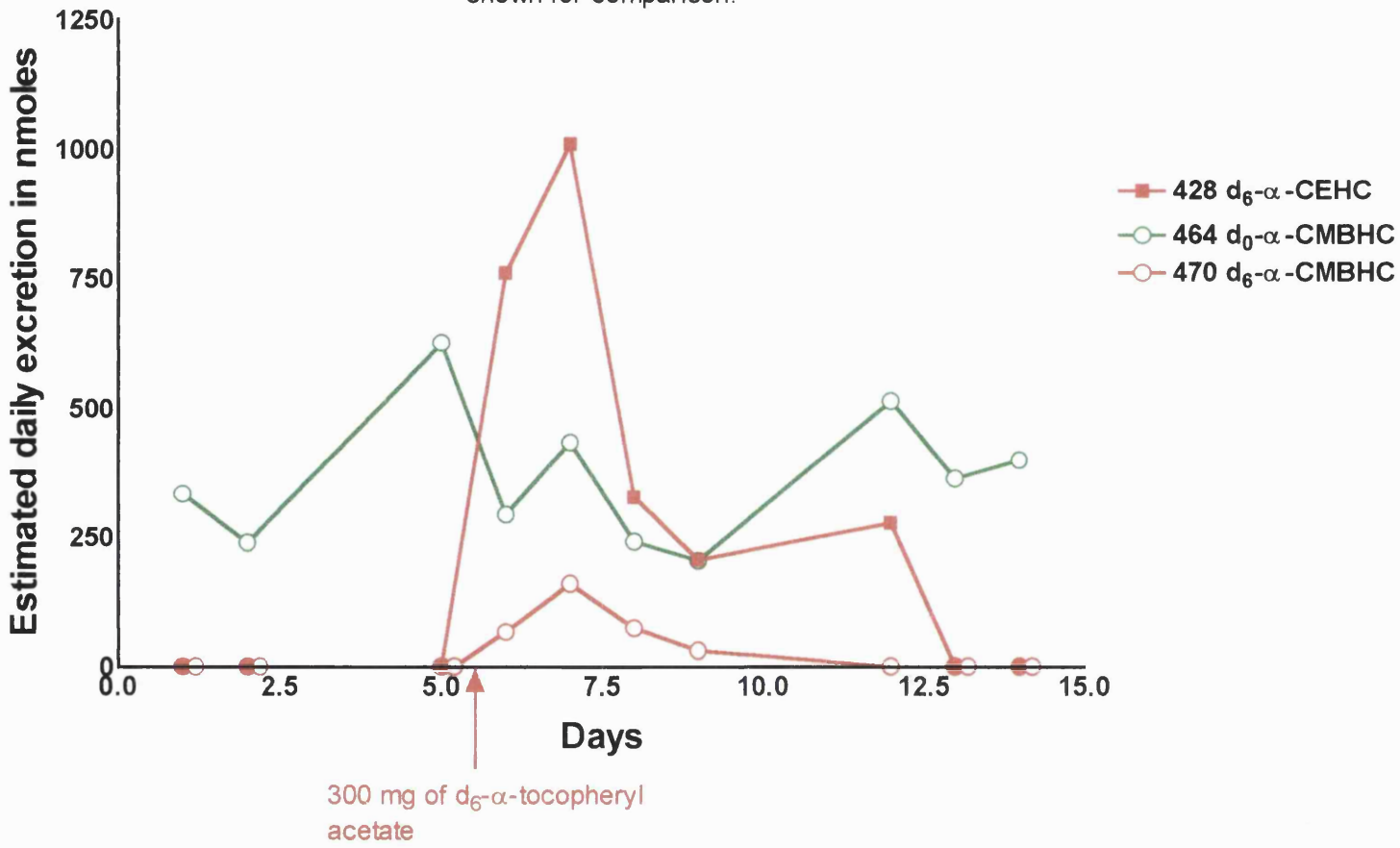



**Figure 3.4.** Spectra of $d_0$-α-CMBHC (**A**) and $d_6$-α-CMBHC (**B**) after supplementation with $d_6$-RRR-α-tocopheryl acetate.

**Figure 3.5.** Excretion of $d_0$- and $d_6$-α-CMBHC before and after a single oral dose of 300 mg $d_6$-RRR-α-tocopheryl acetate. Excretion of $d_6$-α-CEHC is also shown for comparison.

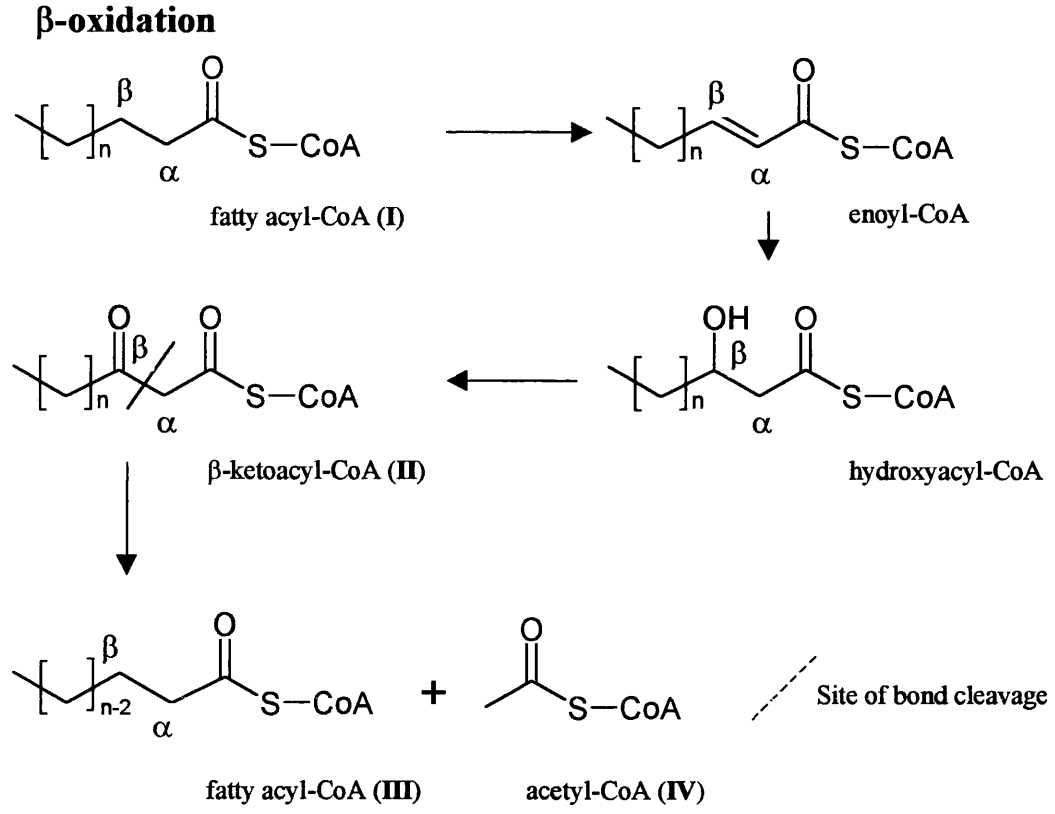


and metabolism is shown in figure 3.6. Phytanic acid initially undergoes α-oxidation and the resulting pristanic acid is converted to its coenzyme A ester. β-oxidation is then able to proceed as shown in figure 3.6. Briefly, stepwise oxidation of the β-carbon of a fatty acyl chain (**I**), via enoyl and hydroxyacyl-CoA, to a ketone intermediate (**II**) allows cleavage of the $C_\alpha$-$C_\beta$ bond in a thiolysis reaction with coenzyme A releasing acetyl CoA (**IV**) and an acyl CoA (**III**) which is two carbons shorter than the starting compound.

Oxidation of the side-chain of vitamin E is thought to proceed via ω-oxidation of the terminal methyl to a hydroxyl group by cytochrome $P_{450}$. This hydroxyl is oxidised to its corresponding carboxylic acid, which is converted to a CoA derivative and oxidised via the β-oxidation pathway, releasing propionyl-CoA and acetyl-CoA. Assuming the phytyl side-chain of vitamin E is oxidised in this way, the CMBHCs are likely metabolic precursors of the CEHCs and might be expected to be excreted in the urine. Other structures, including those with unbranched side-chains, would be unlikely if the side-chain of vitamin E is shortened by conventional β-oxidation.

### 3.2.3. Retention indices

Before the advent of GC-MS, GC data from different laboratories was often compared using retention indices (Littlewood, 1970). Because of differences in column length, temperature programmes and flow rates, absolute retention times alone are not particularly useful in identifying components using GC. Therefore, the retention time relative to a number of standards (retention indices) are often used to compare data.



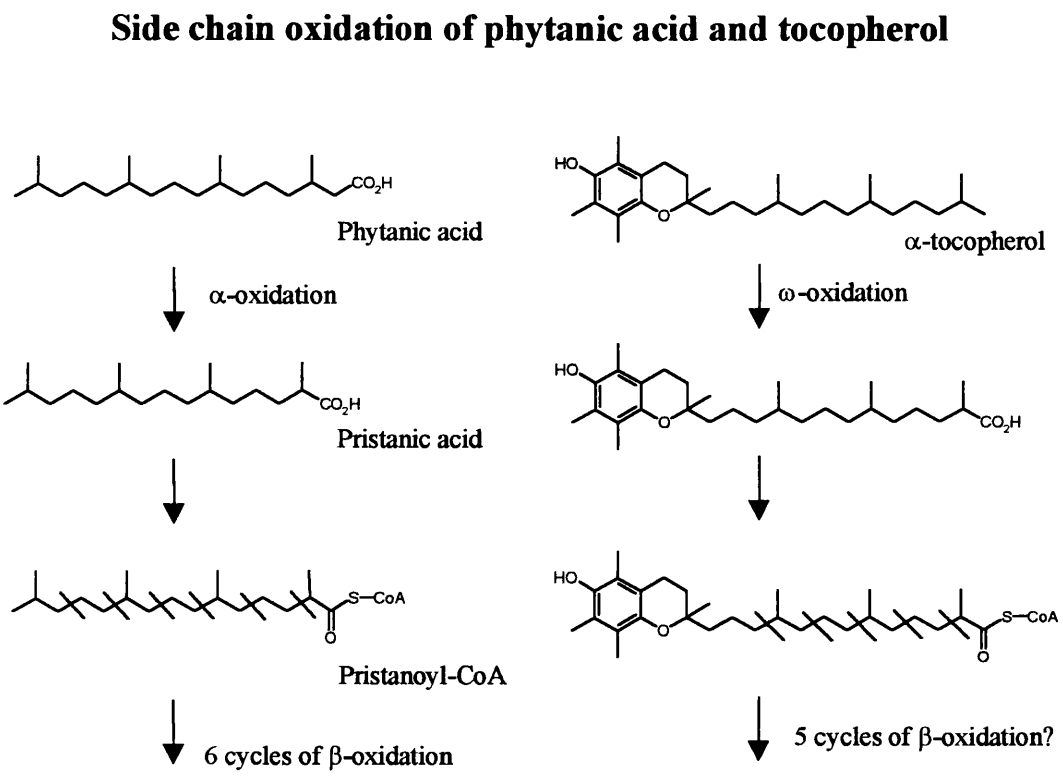



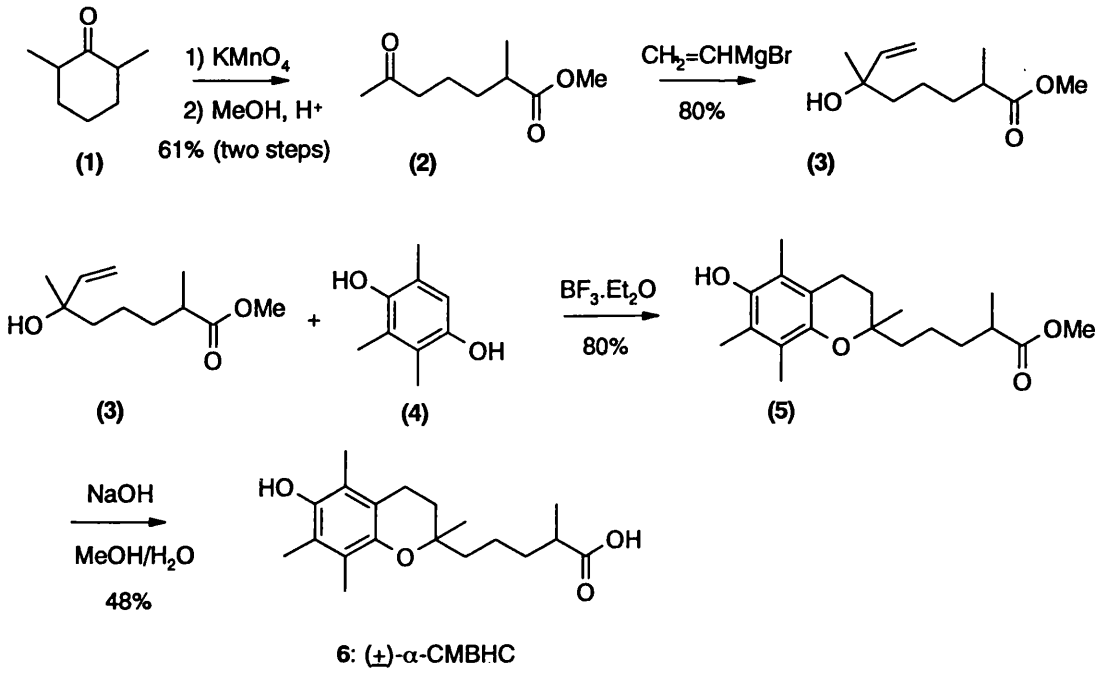

**Figure 3.6.** β-oxidation and its role in the side chain metabolism of phytanic acid and vitamin E.

The most commonly used standards are the alkanes. A series of straight chain saturated alkanes can be analysed and their retention times plotted against the number of carbon atoms. For the alkanes a retention index of 100 is assigned for every methylene unit. Therefore the retention index of octane is 800 while that of decane is 1000. By comparing the retention of an unknown compound to the alkane calibration curve a retention index can be assigned. Retention indices can also be used to probe structural differences between related compounds. Addition of methylene units in a straight chain generally result in an increase in the retention index of 100 for each methylene, while additional methylenes in a branched chain generally result in an increase in retention index of less than 100 for each methylene unit.

In this study, retention indices were used to probe the structure of the proposed α-CMBHC metabolite. The retention index of α-CMBHC was calculated to be 2681, which was 215 greater than α-CEHC. A difference of 300 might have been expected for the addition of three methylene units in a straight chain configuration. Therefore, the observed difference is suggestive of a branched side chain (Littlewood, 1970).

### 3.3. Confirmation of α-CMBHC as a metabolite of α-tocopherol using the chemically synthesised standard

In order to unambiguously confirm the structure of this new metabolite of α-tocopherol, it was decided to synthesise α-CMBHC. The synthesis of α-CMBHC is described in detail in chapter 5 and is shown in scheme 3.1. Briefly, α-CMBHC-methyl ester (**5**) was synthesised as a mixture of diastereoisomers by condensation of trimethylhydroquinone (TMHQ,(**4**)) with the vinylic alcohol (**3**). The methyl ester (**5**) was subsequently saponified using sodium hydroxide to form (±)-α-CMBHC (**6**).




**Scheme 3.1.** Synthesis of (±)-α-CMBHC

Using the synthetic standard (±)-(**6**) in our GC-MS analytical method, it was confirmed that the retention times and mass spectra of the unknown metabolite and synthetic α-CMBHC (**6**) were identical. This was shown by running the synthesised standard and urine extract separately and then in combination to show co-elution of the two peaks and that the spectra were identical (Figure 3.7). It was assumed that the other peaks observed in urine samples with similar mass spectra, but with molecular and fragment ions which were 14 or 28 mass units less than α-CMBHC correspond to γ- and δ-CMBHC respectively. The mass spectrum observed for the presumed γ-CMBHC agrees with that recently reported by Parker and Swanson (2000).

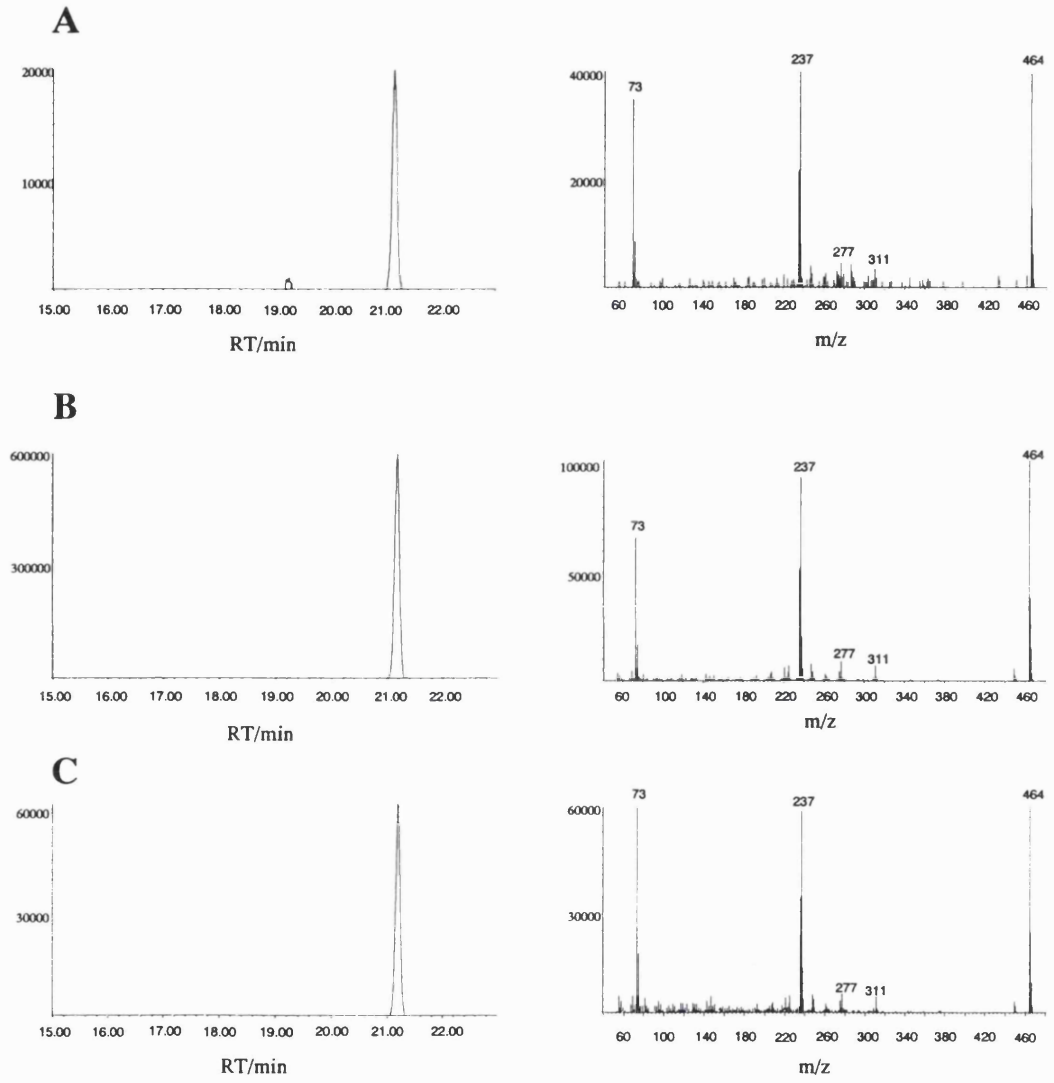



**Figure 3.7. A** and **B** show extracted ion chromatograms (m/z 464) and corresponding mass spectra of silyl derivatives of the urinary metabolite and α-CMBHC standard, respectively. **C** shows the equivalent data for a mixture of α-CMBHC standard and the urinary metabolite, showing co-elution of the two peaks and an identical mass spectrum.

### 3.4. Discussion

A number of unknown metabolites, with fragmentation patterns similar to known vitamin E metabolites, were observed using the GC-MS method described in chapter 2. Administration of $d_6$-α-tocopherol was employed to establish whether any of these were metabolites of α-tocopherol. However only one novel metabolite of α-tocopherol was identified using this method. This metabolite was confirmed to be α-CMBHC by its comparison with a chemically synthesised standard. Other peaks observed using this GC-MS method have mass spectra consistent with the structures of δ- and γ-CMBHC, supporting the notion of a common pathway for the side-chain metabolism of all the tocopherols. The structure of the CMBHCs agrees with the postulated ω- and then β-oxidation of the phytyl side-chain, which is believed to occur in the peroxisome (Mannaerts and Van Veldhoven, 1995). The synthetic procedure that has been developed for (±)-α-CMBHC, could also be used to synthesise the CMBHC metabolites of the other tocopherols from the appropriately methylated hydroquinones.

Owing to the lipophilicity of vitamin E, lipoproteins and transfer proteins are required for the circulation of vitamin E around the body and its transfer between cellular membranes (Traber et al., 1993). At high concentrations the various forms of vitamin E may overload these transport/transfer systems, leading to side-chain shortening of excess vitamin E and urinary excretion of the resulting metabolites, such as α-CEHC and α-CMBHC, in the form of water soluble conjugates (Schultz et al., 1995).

Since the CMBHCs are the probable precursors of the CEHCs, metabolites with longer side-chains, corresponding to the precursors of the CMBHCs, are also possible. However, longer side-chain metabolites may not be excreted in the urine due to their

greater hydrophobicity. It is also possible that the longer side-chain metabolites may only be excreted when the metabolic pathways leading to complete side-chain oxidation are overloaded, which may occur after supplementation with large amounts of vitamin E.

Although the CMBHCs are metabolic products of vitamin E, the possibility that they themselves may have biological activity cannot be ruled out. Recently γ-CEHC, a metabolite of γ-tocopherol, has been shown to act as a natriuretic factor by inhibiting potassium channels in the kidney (Wechter et al., 1996). The synthetic (±)-CMBHC standards could be used in initial investigations of their biological activity. However, it should be pointed out that in the case of *in vivo* studies or enzymatic assays, standards of the naturally occurring pure metabolites would be required. The naturally occurring metabolites would be expected to retain the stereochemistry of the parent compound as has been observed with γ-CEHC (Kantoci et al., 1997). Therefore, synthetic methods allowing tighter control of the chiral centres will have to be employed to synthesise such stereopure standards.

# CHAPTER 4

## Application of the Newly Developed GC-MS Method to the Analysis of Vitamin E Metabolites in Rat and Human Urine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## 4.1. Introduction

The method described in chapter 2 for the measurement of urinary vitamin E metabolites showed good reproducibility for the quantitation of α-CEHC, α-CMBHC and γ-CEHC (Table 2.5). However measurement of α-tocopheronolactone was not reproducible using this method mainly because of the variable artefactual conversion of α-CEHC to α-tocopheronolactone.

The reproducibility data was obtained from measurement of metabolites in human urine samples pooled from a number of subjects. In order to obtain information on the difference in metabolite profiles, especially concerning α-tocopheronolactone, between individual subjects, it was decided to compare the level of urinary vitamin E metabolites in five healthy subjects before and after oral ingestion of $d_6$-RRR-α-tocopheryl acetate (section 4.2). These data would provide information about the baseline levels of the different metabolites, the variation in metabolites produced by different individuals, the effect of a single large dose of α-tocopheryl acetate and the relative levels of α-tocopheronolactone between subjects. The use of deuterium labelled α-tocopheryl acetate also meant that a comparison could be made between the excretion of recently ingested vitamin E (the deuterated supplement) versus endogenous vitamin E (non-deuterated).

In addition to measuring metabolite levels in individual human subjects, it was also of interest to characterise the vitamin E metabolites produced by the rat (section 4.3). Many of the early vitamin E studies were undertaken in rats (Schmandke, 1965; Gloor and Wiss, 1966; Chiku et al., 1984) and radiolabelled rat urine was used to assess the

recovery of α-tocopherol metabolites using our extraction procedure outlined in chapter 2. Therefore, characterisation of the urinary vitamin E metabolites in rat would allow comparison of our data to that of earlier studies and would also allow the reliability of the recovery data obtained in chapter 2 to be assessed. A number of techniques were used to analyse and characterise the rat vitamin E metabolites, including gas chromatography-mass spectrometry (GC-MS), thin layer chromatography (TLC) and scintillation counting.

## 4.2. Urinary vitamin E metabolites in man

### 4.2.1. Materials and methods

Vitamin E metabolites were measured as described in section 2.5 in the first morning urine samples from five healthy volunteers before and after a single oral dose of 300 mg of $d_6$-RRR-α-tocopheryl acetate, which was kindly supplied by Dr. G. Burton (Steacie Institute of Molecular Science, National Research Council, Ottawa, Canada). The oral dose was given at lunchtime on day 5 and urine samples were collected on days 1, 2, 5, 6, 7, 8, 9, 12, 13 and 14. Trolox (3.4 nmole) and $d_9$-α-CEHC (9.3 nmole) were added to 1.0 ml of fresh urine, for quantitation and to measure artefactual oxidation respectively. The amount of metabolite in each sample was related to the urinary creatinine concentration. The creatinine was measured on a COBAS analyser using a commercial kit supplied by Roche Products, Ltd.

*Analysis of data*

Since at the time of analysis, relevant standards were not available for all the metabolites of interest, it was not possible to construct all the appropriate standard curves. Although differences in the ionisation energies of the various metabolites, the

sensitivity of the mass spectrometer for different mass/charge ratios and in the relative abundance of the molecular ion for the different metabolites may weaken the validity of using trolox as a standard, initial experiments demonstrated that quantitation using trolox was adequate. Indeed comparison of α-CEHC quantitation using trolox versus $d_9$-α-CEHC, demonstrated that the use of both standards gave similar molar amounts (see table 4.1).

α-CEHC, α-tocopheronolactone, α-CMBHC, and γ-CEHC were quantitated using trolox as a standard. Trolox equivalents were obtained by comparing the peak size (molecular ion) of the metabolite of interest to the peak size of a known amount of trolox (m/z 394).

Daily excretion rates were calculated for comparison with published data which have often been based on 24 hr urine collection as opposed to morning urine samples. These figures were calculated by measuring the creatinine level of each urine and assuming that excretion of vitamin E metabolites and creatinine was constant throughout the day and that subjects excreted normal amounts (1.4 g) of creatinine (Long, 1971).

### 4.2.2. Results and discussion

#### 4.2.2.1. Excretion of α-CEHC and α-CMBHC after an oral dose of $d_6$-RRR-α-tocopheryl acetate

Figures 4.1-4.5 show the levels of unlabelled and deuterated vitamin E metabolites in the urine of the 5 subjects before and after the oral dose of $d_6$-RRR-α-tocopheryl acetate.

| | Calculated nmoles/ml of urinary metabolite using either trolox or $d_9$-α-CEHC standards | | |
|---|---|---|---|
| | α-CEHC | γ-CEHC | α-CMBHC |
| Sample 1 Trolox | 3.81 | 14.34 | 1.26 |
| Sample 1 $d_9$-α-CEHC | 3.49 | 13.20 | 1.16 |
| Sample 2 Trolox | 2.68 | 8.06 | 0.58 |
| Sample 2 $d_9$-α-CEHC | 2.22 | 6.68 | 0.48 |
| Sample 3 Trolox | 4.90 | 8.67 | 1.60 |
| Sample 3 $d_9$-α-CEHC | 5.04 | 8.93 | 1.65 |

**Table 4.1.** Comparison of trolox and $d_9$-α-CEHC standards for the quantitation of urinary vitamin E metabolites in three different urine samples.

**Figure 4.1.** Subject 1 - Estimated daily urinary excretion of vitamin E metabolites

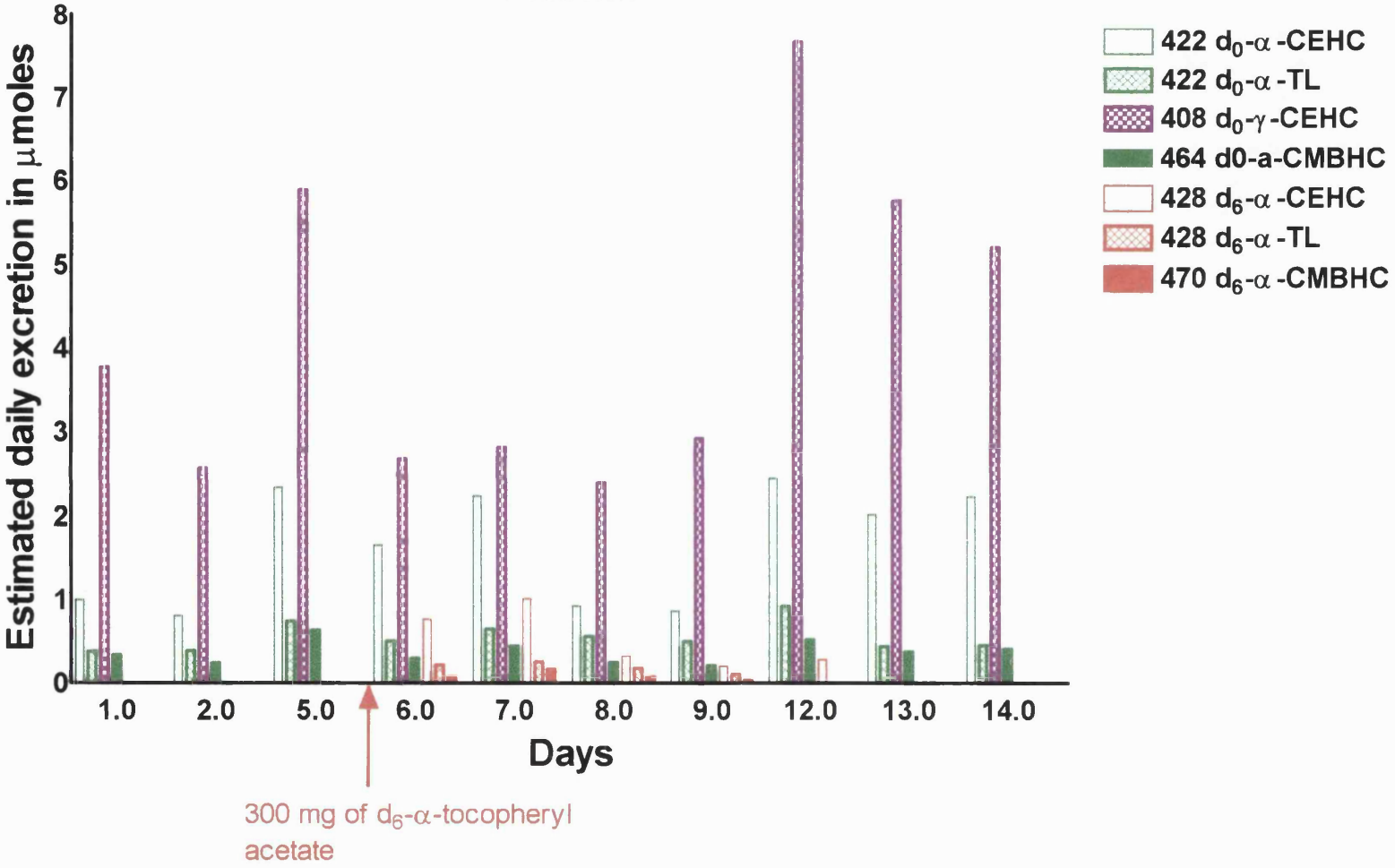


**Figure 4.2.** Subject 2 - Estimated daily urinary excretion of vitamin E metabolites

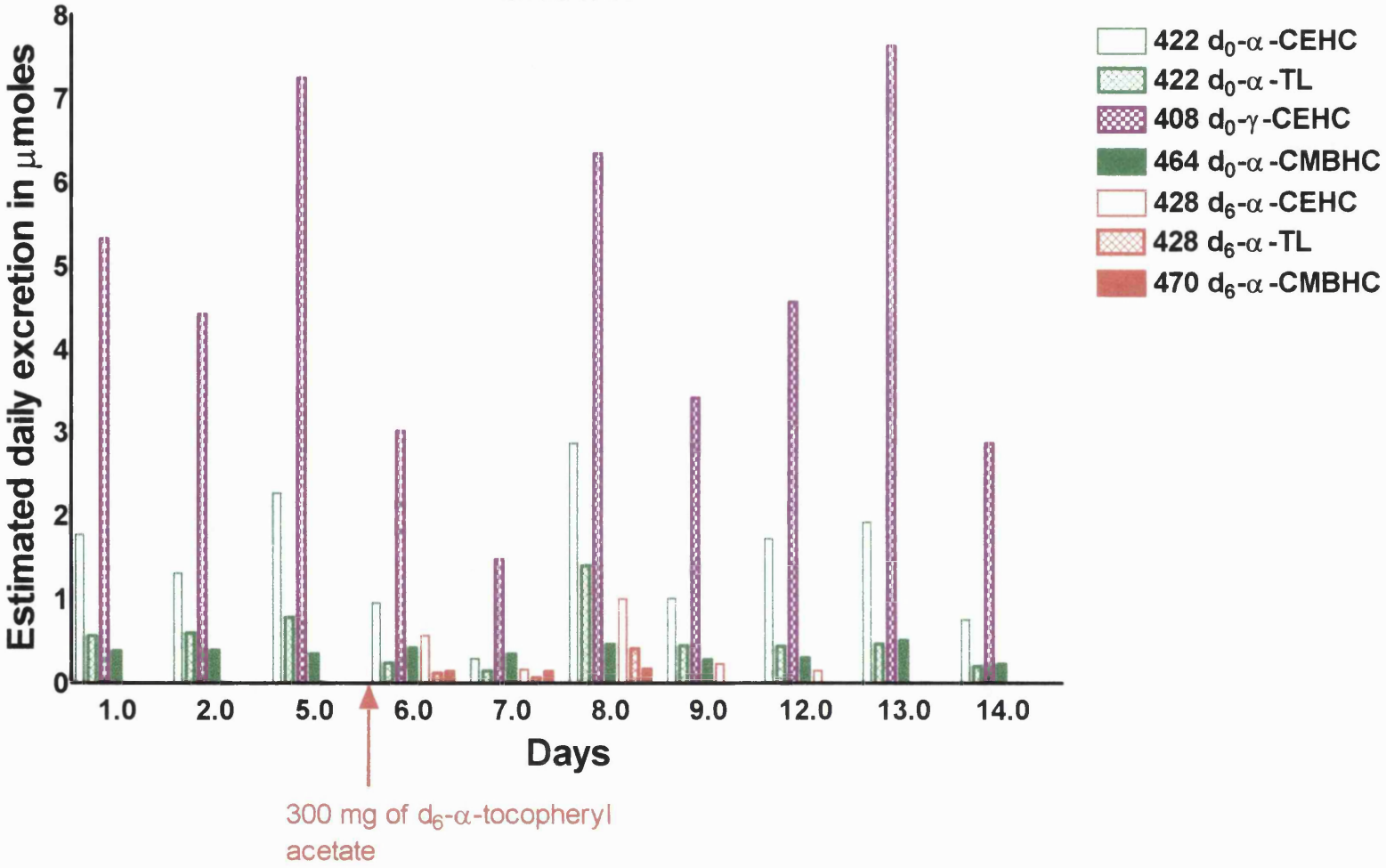


**Figure 4.3.** Subject 3 - Estimated daily urinary excretion of vitamin E metabolites

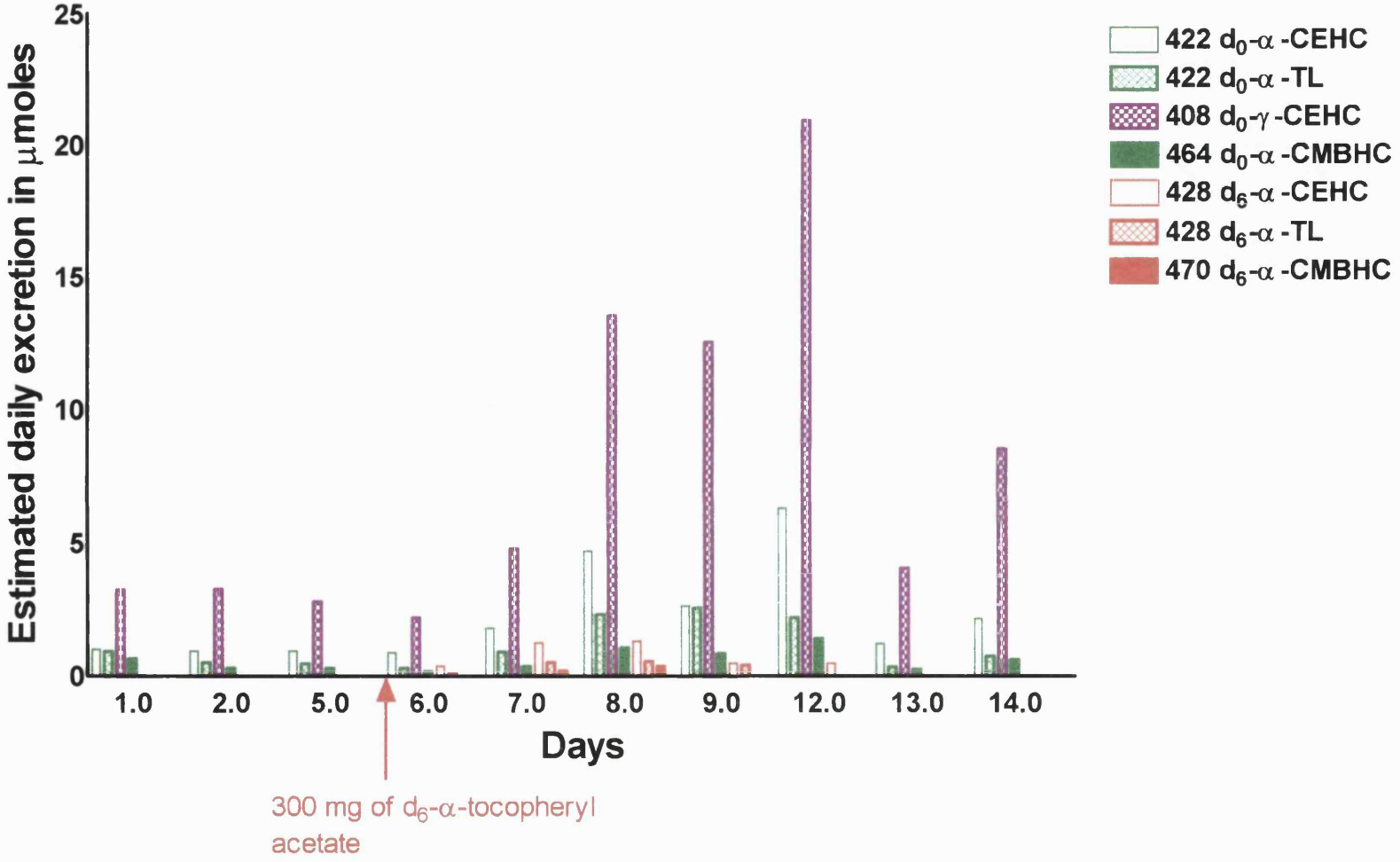


**Figure 4.4.** Subject 4 - Estimated daily urinary excretion of vitamin E metabolites

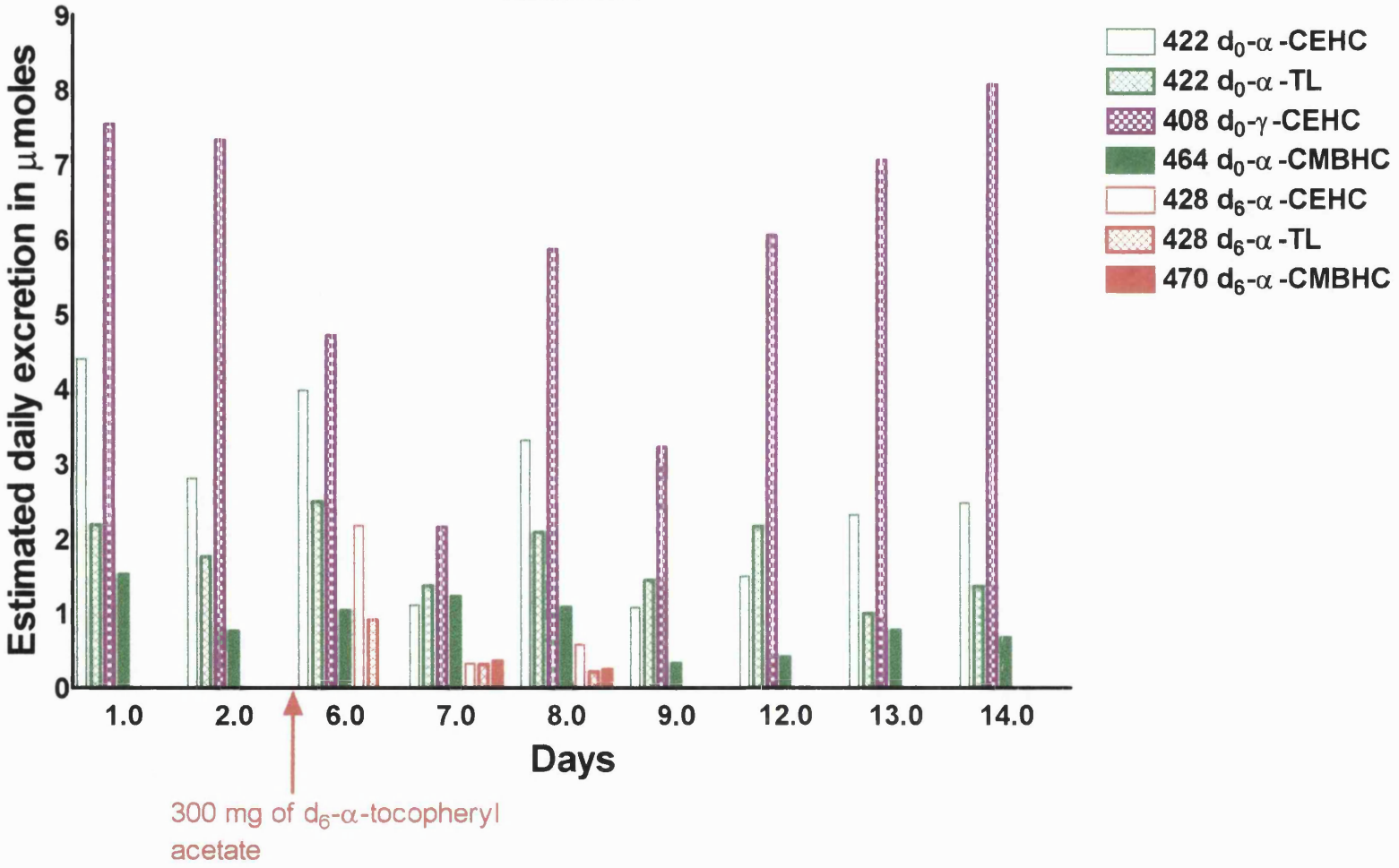
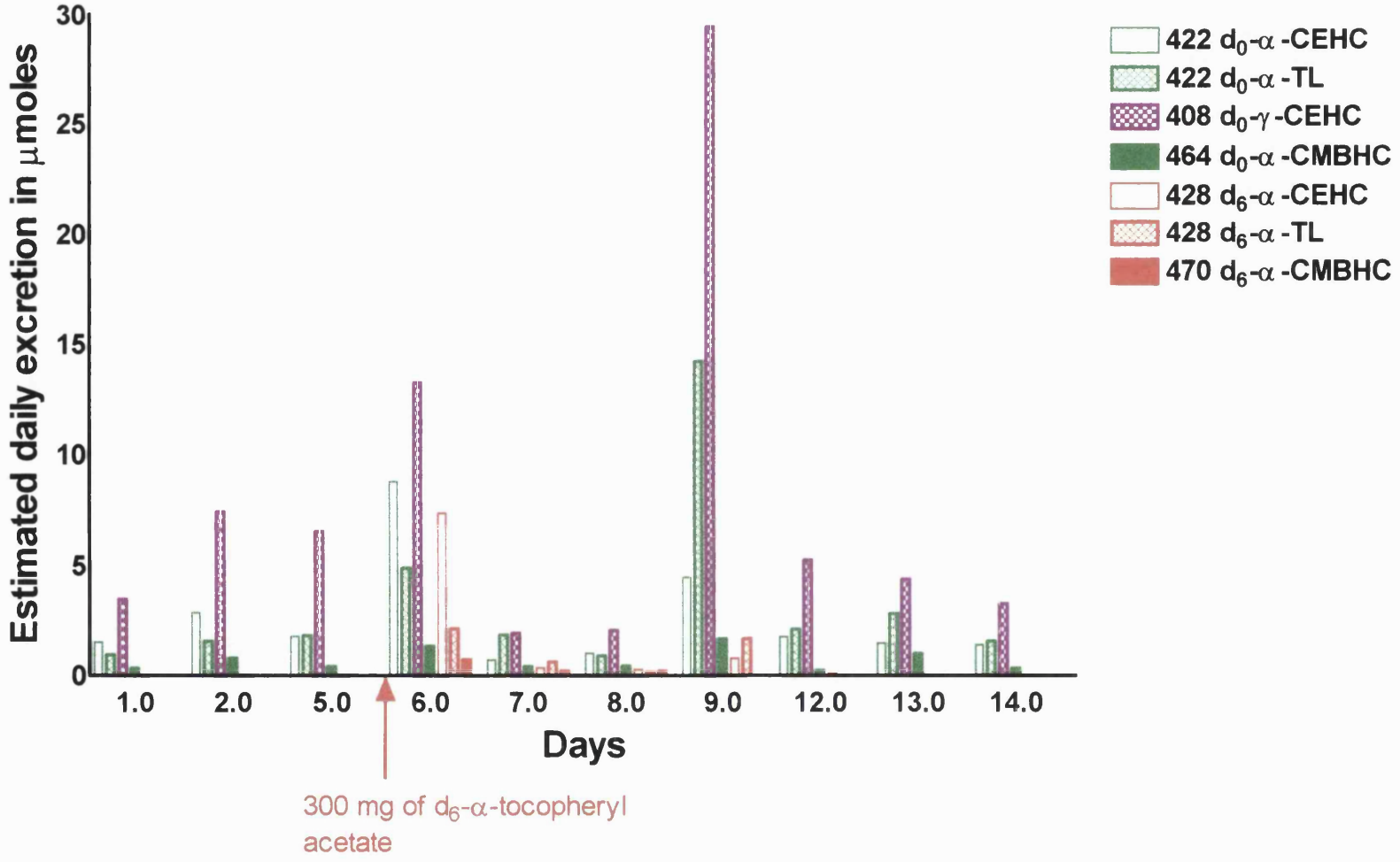


**Figure 4.5.** Subject 5 - Estimated daily urinary excretion of vitamin E metabolites



The level of excretion of undeuterated $d_0$-α-CEHC varied widely among individuals and over the 14 days of the study. The estimated daily excretion of $d_0$-α-CEHC varied from as low as 0.3 μmole per day for subject 2 on day 7 to as high as 8.8 μmole per day for subject 5 on day 6. In general there were five to ten-fold differences between the lowest and highest excretion levels of $d_0$-α-CEHC among individual subjects.

Excretion of $d_6$-α-CEHC started the day following the oral uptake of $d_6$-α-tocopheryl acetate and peaked between days 6 to 8 (1–3 days after the oral dose). At its peak excretion, the amount of $d_6$-α-CEHC varied from 1.0 μmole per day for subject 2 to 7.3 μmole per day for subject 5. The level of $d_6$-α-CEHC excretion did not exceed that of $d_0$-α-CEHC in any of the subjects even though the dose of $d_6$-α-tocopheryl acetate (300 mg) was well above the typical daily dietary intake of α-tocopherol. This could result from either poor absorption of deuterated α-tocopheryl acetate or to its dilution in the total body pool of undeuterated α-tocopherol. $D_6$-α-CEHC could be detected in the urine of subjects for up to a week after the oral intake.

In general, excretion of $d_0$-α-CMBHC varied less than that of $d_0$-α-CEHC although maximal $d_0$-α-CMBHC excretion generally coincided with maximal $d_0$-α-CEHC excretion. $D_0$-α-CMBHC was detected in all urine samples tested and the estimated daily excretion of $d_0$-α-CMBHC varied from 0.15 μmole per day for subject 3 on day 6, to 1.6 μmole per day for subject 5 on day 9. These excretion levels are substantially higher than those reported previously (tables 4.2 and 4.3) and correspond to between 15 and 30% of $d_0$-α-CEHC excretion levels in contrast to the reported 3-6% (Schuelke et al., 2000) (Table 4.2). These differences could result from the higher sensitivity of the

| Authors | Level of urinary metabolite excretion | Other comments |
|---|---|---|
| Swanson et al. (1999) | γ-CEHC 4-30 μmole/day. | Equivalent to 2-12mg of γ-tocopherol excreted daily (10-60% of daily γ-tocopherol intake) |
| Traber et al. (1998) | α-CEHC - 1 μmole/day at baseline.<br>α-CEHC – 1.6 μmole/day after 300 mg supplement.<br>γ-CEHC – 2 μmole/day. | α-CEHC levels peak 2 days after supplementation.<br>γ-CEHC levels consistently higher than α-CEHC.<br>Total all-rac-α-CEHC excreted 2.7 times that of RRR-α-CEHC. |
| Stahl et al. (1999) | 7.1 pmol/ml of α-CEHC in serum at baseline.<br>66.4 pmol/ml of γ-CEHC in serum at baseline.<br>130 pmol/ml of α-CEHC in serum after supplements.<br>86 pmol/ml of γ-CEHC in serum after supplement. | - 43,000 pmol/ml of α-tocopherol in serum<br>- 2,600 pmol/ml of γ-tocopherol in serum<br>- 90,000 pmol/ml of α-tocopherol in serum<br>- 1,200 pmol/ml γ-tocopherol in serum |
| Lodge et al. (2000) | 0.21-1.45 μmol/day α-CEHC – unsupplemented.<br>0.5-5.0 μmol/day γ-CEHC – unsupplemented | 5-25% of metabolites unconjugated |
| Schuelke et al. (2000) | 1 μmol/day healthy subjects α-CEHC<br>α-CMBHC 3-6% of α-CEHC levels<br>36 – 108 μmol/day α-CEHC in AVED patients taking over 1000 mg of α-tocopheryl acetate per day. | 1-3% of α-tocopherol degraded to α-CEHC<br>α-CEHC excretion only increased after plasma α-tocopherol above 30-40 μM. |

**Table 4.2.** Comparison of reported levels of excretion of vitamin E metabolites.

| Authors | Levels of metabolites | Other comments |
|---|---|---|
| Parker et al. (2000b) | γ-CEHC 13.8 μM in urine - unsupplemented<br>γ-CMBHC 0.74 μM in urine – unsupplemented | γ-CMBHC levels were 1-4% of γ-CEHC levels. |
| Hattori et al. (2000b) | Unconjugated γ-CEHC in rat<br>Bile – 6.2 μM<br>Urine – 11.5 μM<br>Plasma – 328 nM = 328 pmol/ ml | Rat plasma γ-CEHC levels higher than humans.<br>Urine levels of γ-CEHC similar between rats and humans. |
| Hattori et al. (2000a) | Unconjugated γ-CEHC upto 5 μM in plasma after supplementation with γ-tocopherol or γ-tocotrienol. | Maximum levels in plasma 9 hours after oral administration . |
| Parker et al. (2000a) | γ-CMBHC about 4% of γ-CEHC levels<br>δ-CMBHC about 3% of δ-CEHC levels<br>α-CMBHC about 20% of α-CEHC levels | Tissue culture data. |
| Lodge et al. (2001) | 1-2% of α-tocotrienyl acetates or<br>4-6% of γ-tocotrienyl acetates excreted as CEHCs in urine. | Large individual differences in the quantity of γ-CEHC excreted |

**Table 4.2.** Comparison of reported levels of excretion of vitamin E metabolites.

| | Calculated μmole/day excretion of urinary vitamin E metabolites | | | |
|---|---|---|---|---|
| | α-CEHC | α-CMBHC | γ-CEHC | γ-CMBHC |
| Range reported in literature | 0.21-1.60 | 0.01-0.08 | 0.5-30 | <1.2 |
| Range reported in this study | 0.2-9.0 | 0.15-1.60 | 1.4-29 | 0.20-2.2 |

**Table 4.3.** Comparison of urinary vitamin E metabolite levels reported in this study and in the literature. See table 4.2 for references.

method reported here, allowing the low levels of α-CMBHC to be accurately measured, or they could be due to differences between the studies, in the size and timing of the α-tocopherol dose, in the dietary levels of vitamin E and in the timing and method of urine collection and handling.

$D_6$-α-CMBHC could only be detected in urine for 3 or 4 days after the oral supplement as opposed to 7 days for $d_6$-α-CEHC. Peak $d_6$-α-CMBHC excretion levels varied from 0.16 μmole per day for subject 2 to 0.70 μmole per day for subject 5. $d_6$-α-CMBHC excretion levels varied from 10-100% of $d_6$-α-CEHC levels compared to 15-30% for undeuterated metabolites. This difference may result from differences in the site of metabolism of the newly administered deuterated compound compared with the existing body pool of undeuterated α-tocopherol. At its peak, excretion of $d_6$-α-CMBHC was generally one third to a half of that of $d_0$-α-CMBHC excretion on the same day.

In general, subjects excreted less than 1% of the $d_6$-α-tocopheryl acetate dose as combined urinary $d_6$-α-CEHC, $d_6$-α-tocopheronolactone and $d_6$-α-CMBHC, over the course of the study. This low level of urinary excretion could result from a number of different factors, including the incomplete absorption of the large oral dose of α-tocopheryl acetate (See Traber et al., 1993, for a review of vitamin E absorption), the efficient retention of α-tocopherol in the body, resulting from the action of α-TTP, and the excretion of deuterated α-tocopherol and its metabolites in the bile. Similar levels of urinary metabolite excretion have also been reported by Schuelke et al. (2000), after large oral doses of α-tocopheryl acetate.

### 4.2.2.2. Excretion of γ-CEHC and other metabolites

Figures 4.1-4.5 also show the excretion of γ-CEHC in comparison to α-CEHC, α-tocopheronolactone and α-CMBHC. γ-CEHC excretion was greater than that of α-CEHC, which would be expected from the high levels of γ-CEHC in the diet and its poor retention in the body. It has been estimated that up to 50% of the daily intake of γ-tocopherol is excreted in the urine as γ-CEHC (Swanson et al., 1999). As a result of differences in dietary intake of γ-tocopherol, it is not surprising that urinary γ-CEHC levels and daily excretion rates varied widely among subjects from 1.4-29 μmole per day. Although levels of conjugated γ-CEHC vary, the excretion of unconjugated γ-CEHC is thought to be regulated because of its action as a natriuretic factor (Wechter et al., 1996).

Other metabolites derived from γ-, δ and β-tocopherol such as γ-CMBHC, β-CEHC, δ-CEHC and δ-CMBHC were also observed although only γ-CMBHC was consistently present in amounts greater than 0.2 μmole per day. γ-CMBHC levels are shown in table 4.3 and varied from about 4-15% of γ-CEHC levels, whereas the levels of α-CMBHC varied from 15-30% of α-CEHC levels. This difference may result from differences in the site and regulation of side chain metabolism of the different tocopherols.

The data obtained in the present study were generally similar to that previously reported (table 4.3). Some differences were noted, especially with regard to α-CMBHC levels, but these probably result from differences in the sensitivity of the various methods, which could affect the measurement of low abundance metabolites such as α-CMBHC, and other differences in the studies as noted above (4.2.2.1).

### 4.2.2.3. Excretion of oxidised metabolites such as α-tocopheronolactone

$D_6$-α-tocopheronolactone, derived from the oral dose of $d_6$-α-tocopheryl acetate, was only observed for 3 or 4 days after the dose, whereas $d_0$- or $d_9$-α-tocopheronolactone, derived from undeuterated α-tocopherol or the added $d_9$-α-CEHC standard, was observed in all urine samples (figures 4.1-4.5 and 4.6-4.10). The presence of $d_9$-α-tocopheronolactone suggested that at least some of the $d_0$- and $d_6$-α-tocopheronolactone was produced artefactually during the analytical procedure. Theoretically, the level of artefactual conversion can be assessed by measuring the amount of $d_9$-α-tocopheronolactone produced.

The amount of $d_0$-α-tocopheronolactone varied widely between samples and was particularly high in some subjects on certain days (e.g. subject 5, day 9; see figures 4.5 and 4.10). The good correlation between the levels of $d_0$-, $d_6$- and $d_9$-α-tocopheronolactone strongly indicates that endogenous levels of oxidants and reductants in the urine and/or the way the sample is handled, during the extraction and analysis procedure, determines the extent of artefactual conversion of α-CEHC to α-tocopheronolactone. However, it should be noted that the relative amount of α-tocopheronolactone compared to the total combined amount of α-CEHC and α-tocopheronolactone was consistently in the order $d_9 < d_6 < d_0$ (figures 4.6-4.10). If α-tocopheronolactone was produced *in vivo* it is likely to be produced in greater amounts from the circulating body pool of α-tocopherol than from short term supplemented α-tocopherol due to the increased chance of it being exposed to oxidants. Therefore, it is not surprising that, as a ratio to combined α-CEHC and α-tocopheronolactone, there was consistently more α-tocopheronolactone produced from long term undeuterated

**Figure 4.6.** Subject 1 - α-tocopheronolactone (α-TL) levels

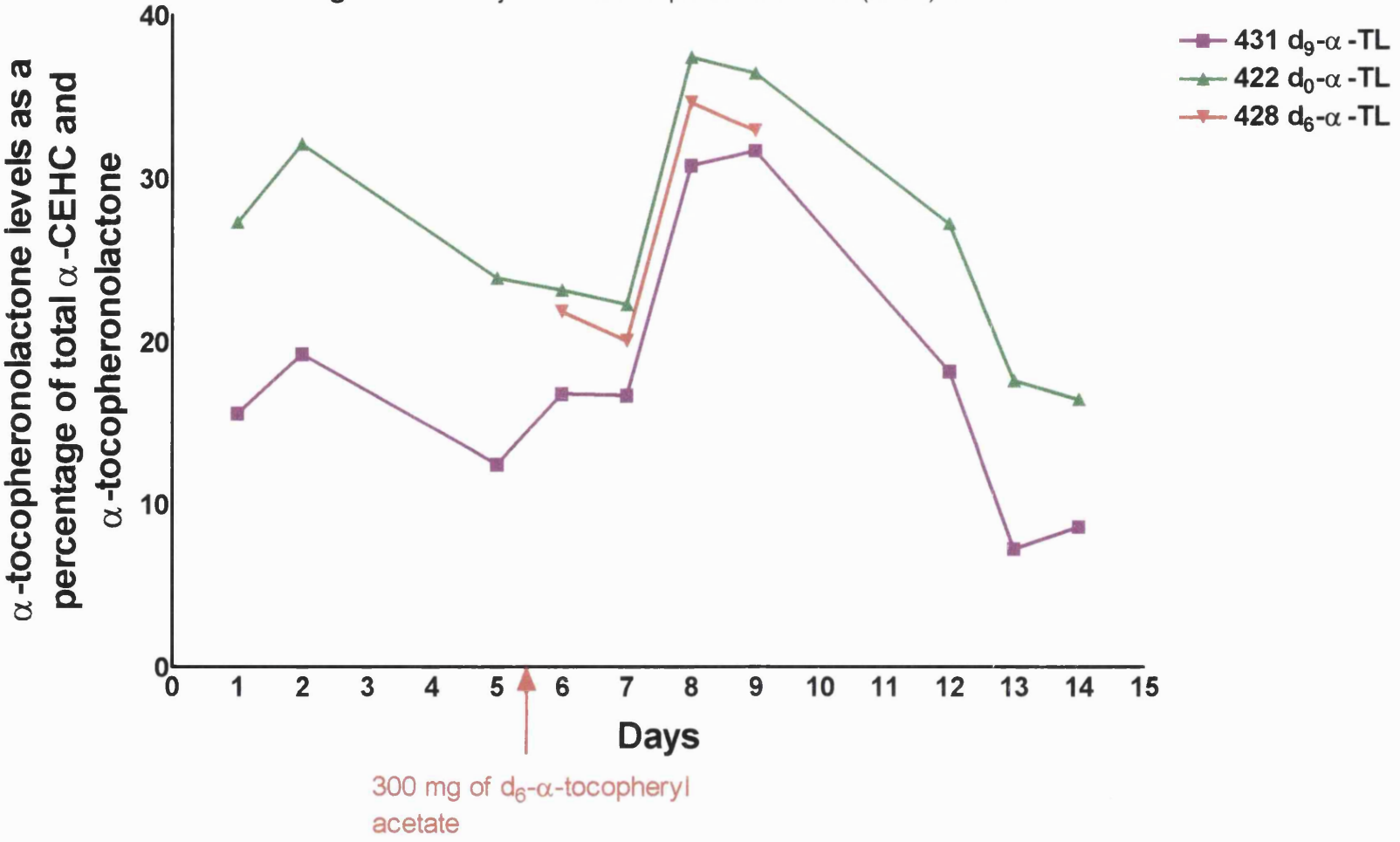


**Figure 4-7.** Subject 2 - α-tocopheronolactone (α-TL) levels

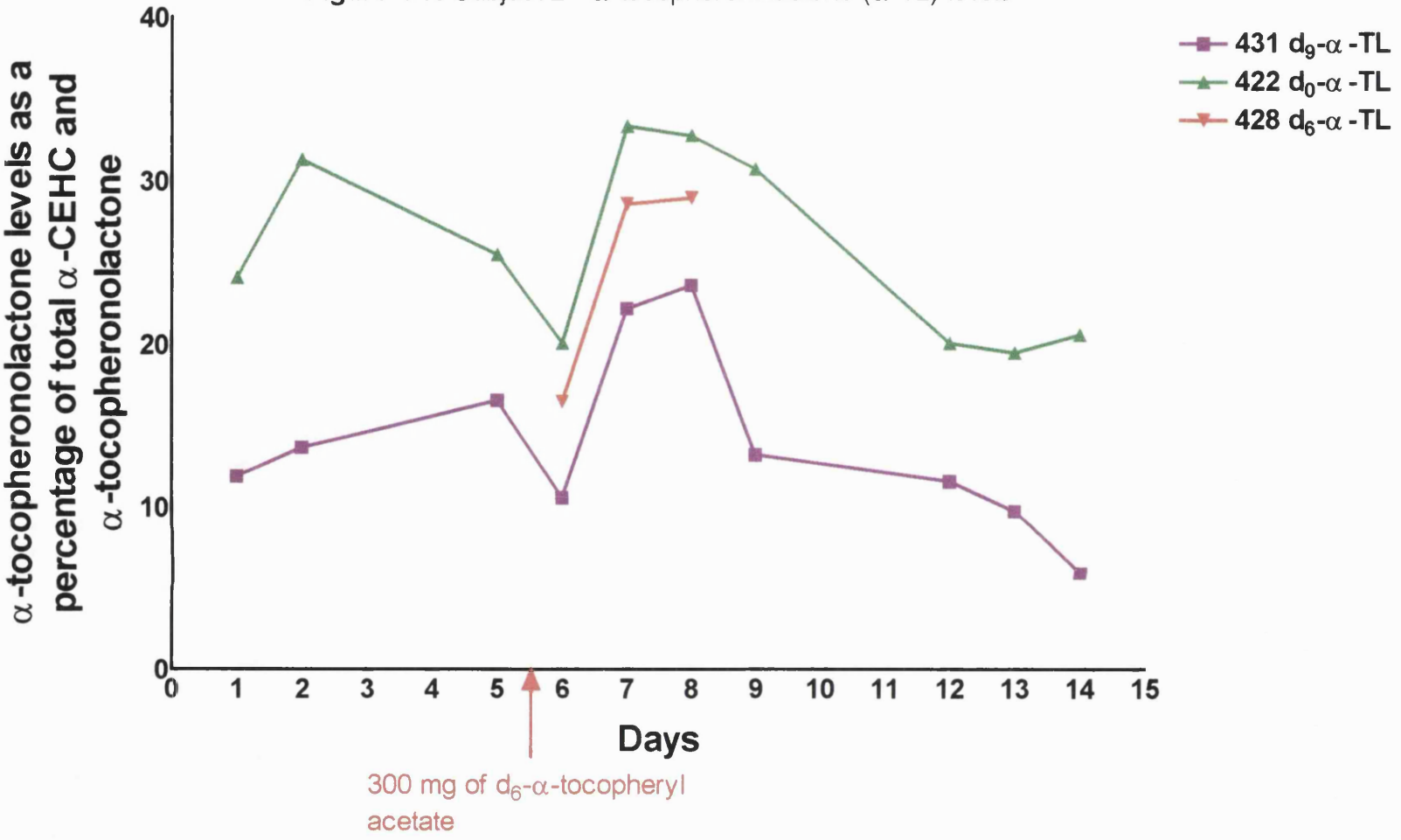


**Figure 4-8.** Subject 3 - α-tocopheronolactone (α-TL) levels

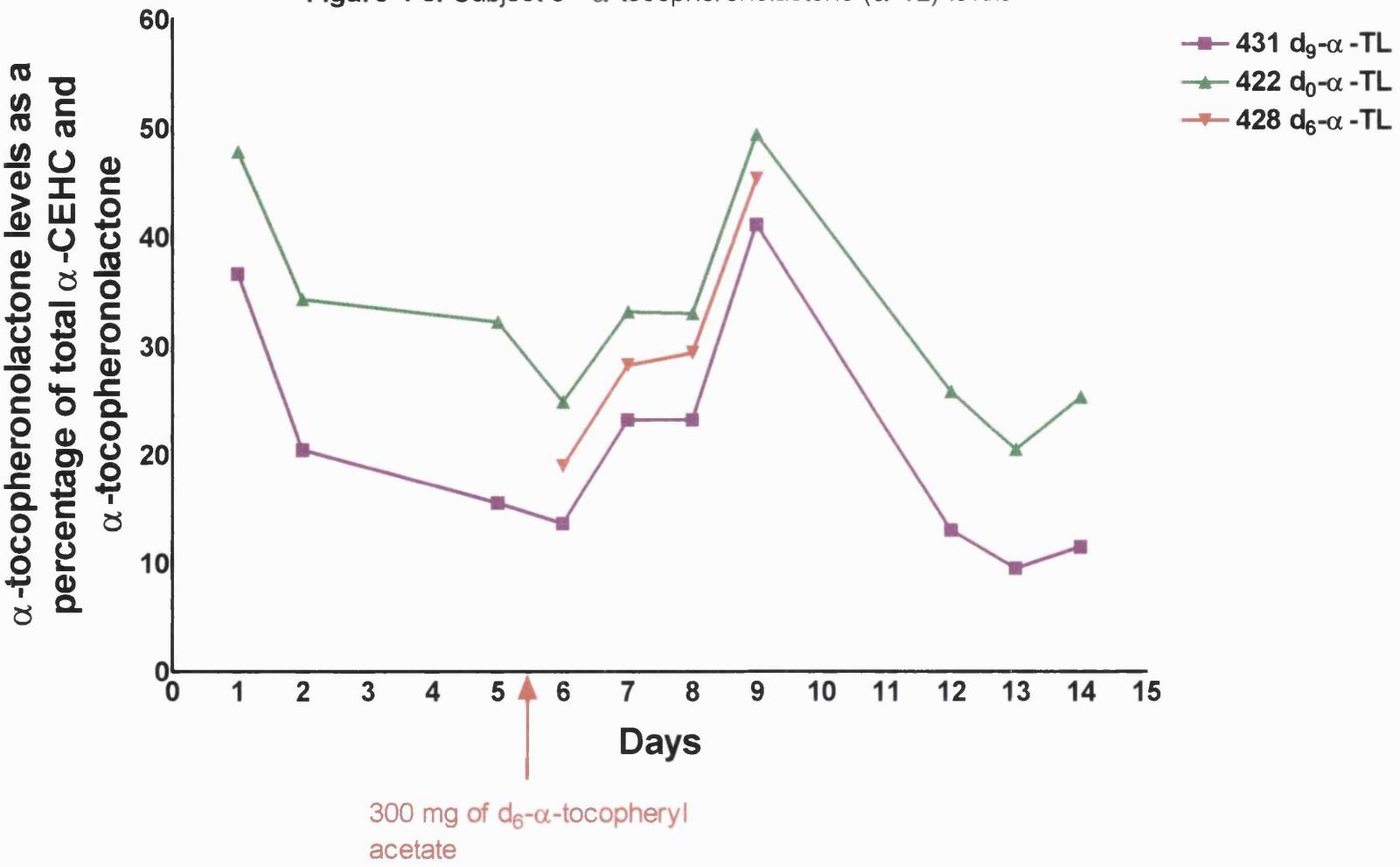


**Figure 4-9.** Subject 4 - α-tocopheronolactone (α-TL) levels

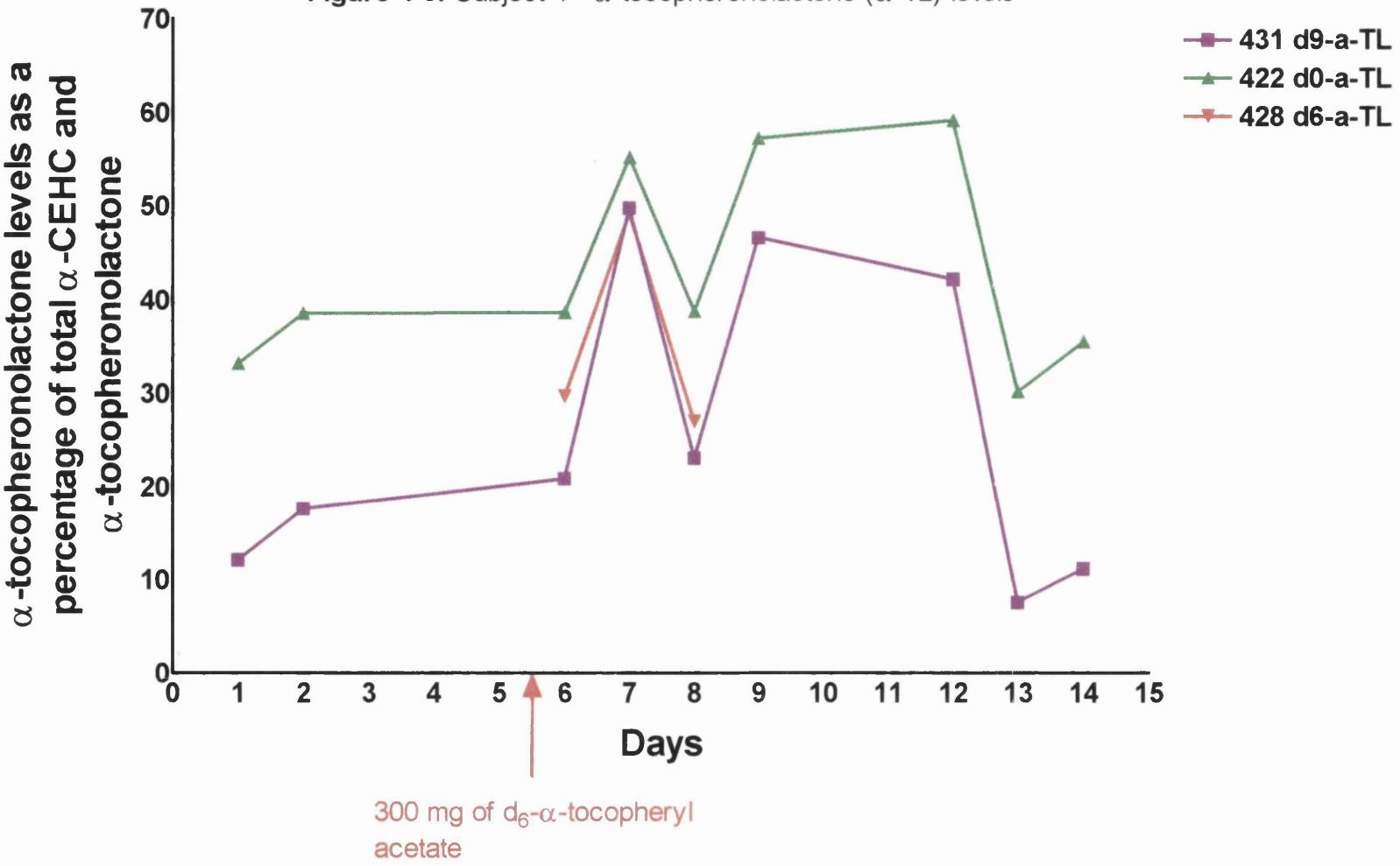
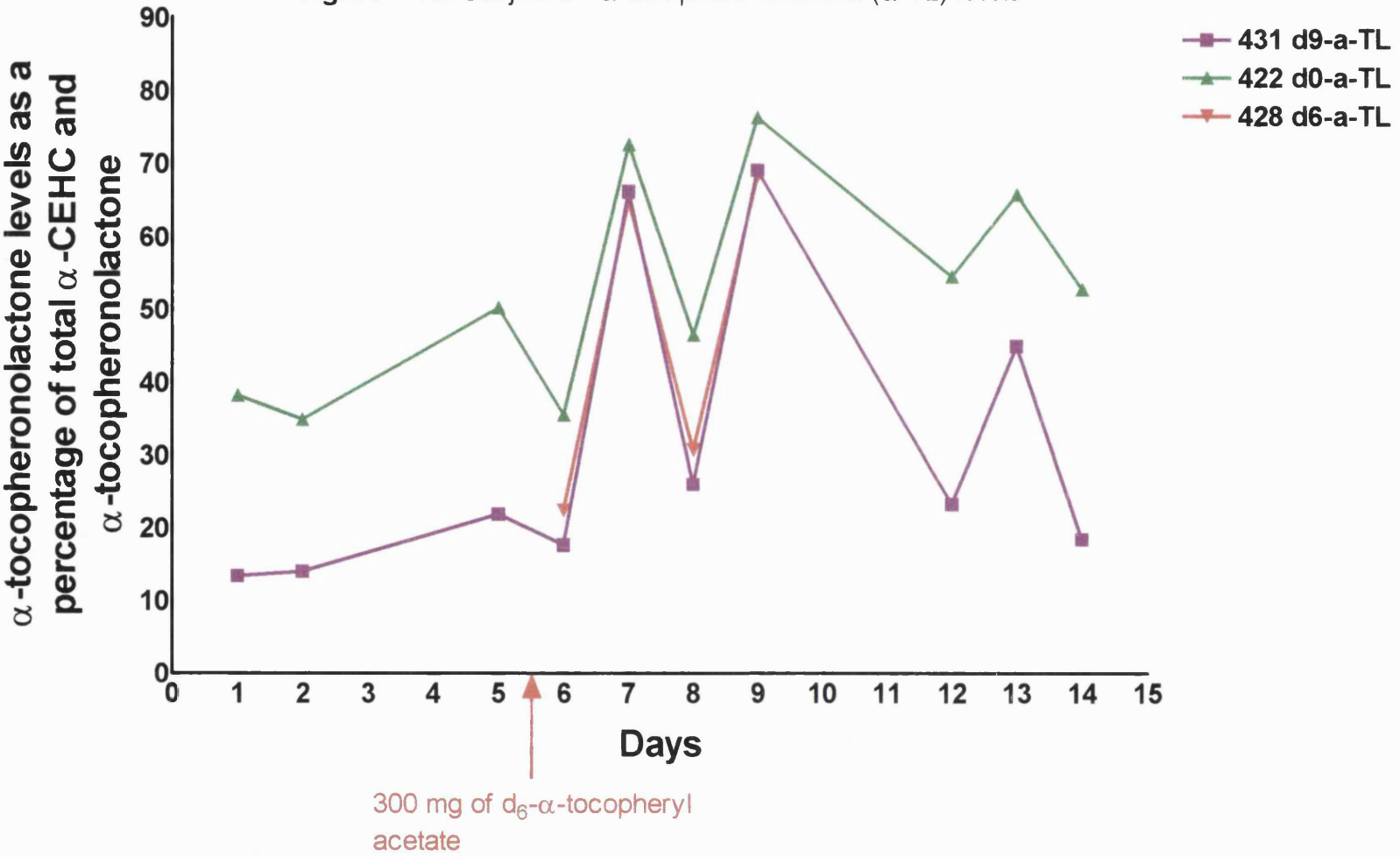


**Figure 4-10.** Subject 5 - α-tocopheronolactone (α-TL) levels



α-tocopherol than from short term $d_6$-α-tocopherol, which has only been in the body for a few days.

Another possible explanation for the different ratios of α-tocopheronolactone to combined α-CEHC and α-tocopheronolactone for the differently deuterated analogues may result from their reactivity. In many chemical reactions deuterated analogues react more slowly than their hydrogenated equivalents. This is usually due to the slower rate of deuterium exchange compared to hydrogen exchange. If deuteriums were exchanging with hydrogens in the conversion of α-CEHC to α-tocopheronolactone there would be a mass shift corresponding to the loss of deuterium. Since the α-tocopheronolactones observed were of the same mass to charge ratio as the equivalent α-CEHCs it is apparent that no deuterium exchange took place. It is possible that differently deuterated analogues have slightly different reactivities. The greater antioxidant activity of α-tocopherol compared to the other tocopherols has been attributed to its extra methyl group(s) surrounding the chroman ring (Kamal-Eldin and Appelqvist, 1996). By analogy extra deuteriums surrounding the chroman ring could reduce the antioxidant activity of α-tocopherol or α-CEHC thus reducing the rate of formation of α-tocopherylquinone or α-tocopheronolactone respectively.

Oxidised α-CMBHC, the longer side chain equivalent of α-tocopheronolactone, was observed in a number of the urine samples, especially those with high levels of α-tocopheronolactone (see figure 4.11 for spectrum). Because of the longer side chain of oxidised α-CMBHC it does not form a lactone ring. Levels of oxidised α-CMBHC

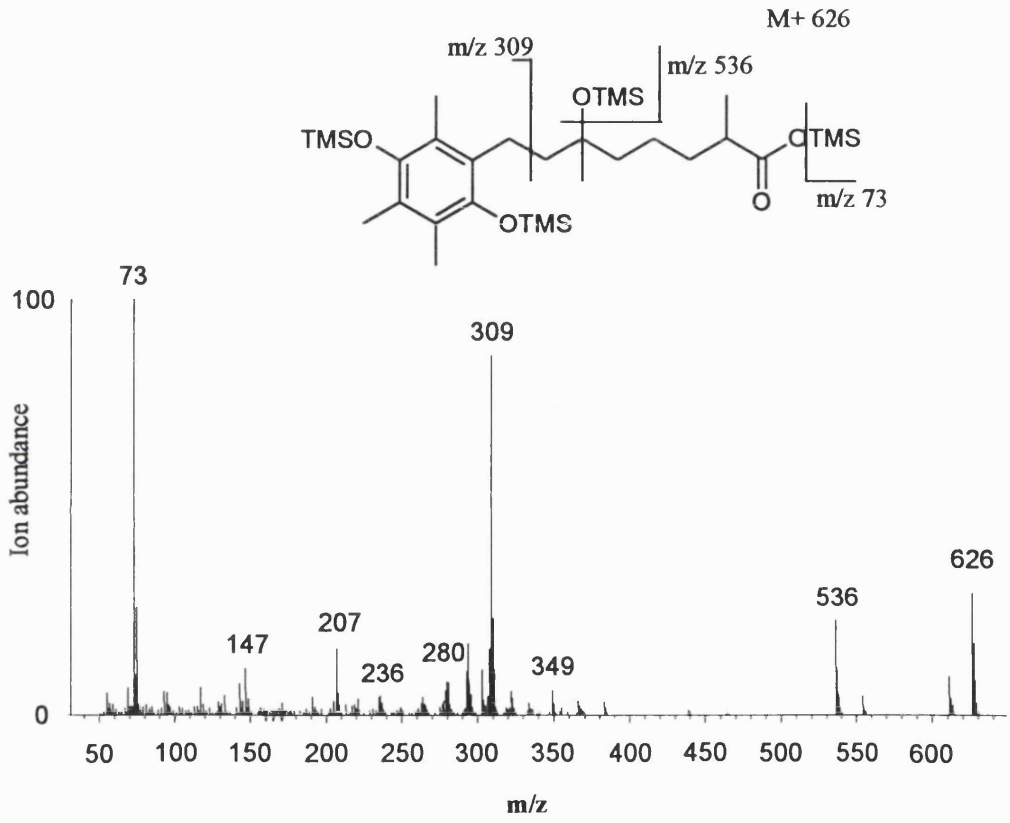



**Figure 4.11.** Spectrum of oxidised α-CMBHC (retention time = 28.7 mins) showing possible fragmentations.

could not be measured accurately as it co-eluted with numerous contaminating compounds towards the end of the GC programme. A longer, more gradual temperature programme would be required to separate oxidised α-CMBHC from contaminating compounds and to allow its quantitation. It would be of interest to measure the relative level of oxidised α-CMBHC to α-CMBHC and compare it to the ratio of α-tocopheronolactone to α-CEHC in the same sample. Theoretically α-CEHC and α-CMBHC are equally susceptible to artefactual oxidation and therefore the two ratios of oxidation product to either α-CEHC or α-CMBHC should be similar. However if it is assumed that α-tocopheronolactone is an *in vivo* metabolite and is produced by a different metabolic pathway to α-CEHC then a difference in the ratios might be expected. However this assumes that the α-tocopheronolactone pathway does not produce oxidised α-CMBHC.

γ-Tocopheronolactone, the γ-tocopherol equivalent of α-tocopheronolactone, was observed in small amounts in only a few urine samples. Since γ-tocopherol is not retained in the body for prolonged periods γ-tocopheronolactone is probably produced by artefactual oxidation of γ-CEHC. The small amounts of γ-tocopheronolactone observed in relation to the high amounts of γ-CEHC probably mean that γ-CEHC is less susceptible to oxidation than α-CEHC. This is in agreement with the known differences in antioxidant activity between γ-tocopherol and α-tocopherol (Kamal-Eldin and Appelqvist, 1996).

#### 4.2.2.4. Differences in vitamin E metabolite profiles between subjects

The differences in the level of metabolites between subjects, most likely results from differences in dietary intakes of vitamin E. However, in this study the dietary levels of the different forms of vitamin E were not measured.

It is interesting to note that subjects 4 and 5 displayed consistently higher levels of artefactual α-tocopheronolactone than the other subjects. This could be due to differences in the natural levels of reductants and oxidants present in the urine samples.

Differences in the levels of CEHC and CMBHC metabolites among subjects may also result from individual variations in the retention of different forms of vitamin E due to α-TTP activity and differences in the expression of metabolic enzymes involved in metabolism of the side chain.

### 4.3. Studies of radiolabelled α-tocopherol metabolites in rat urine

As discussed in chapter 2 rat urine samples containing radiolabelled α-tocopherol metabolites were used to assess the recovery of α-tocopherol metabolites in our extraction method. However the α-tocopherol metabolites in rat urine had not been characterised and, therefore, it was decided to confirm their identity and similarity with metabolites from human urine. The analyses and characterisation of vitamin E metabolites in rat urine were performed using a number of different methods, including scintillation counting, thin layer chromatography and GC-MS.

### 4.3.1. Materials and methods

Urine samples (2 ml) were collected at three different time points (24 hr, 48 hr and 72 hr), from each of three rats after they had received $C^{14}$-α-tocopherol (3 mg or approximately 60 μCi/rat per day). These urine samples were kindly supplied by Drs. W. Cohn and O. Froecheis, Hoffmann-La Roche Ltd., Switzerland. Trolox (6.8 nmol) and $d_9$-α-CEHC (28 nmol) were added to 1.0 ml of each rat urine. The rat urine (0.5 ml) was acidified to pH 2 and extracted and deconjugated as outlined in chapter 2. Aliquots (typically 100 μl) of all fractions, including flow through and wash fractions, were taken at each step of the extraction procedure and counted as described in chapter 2.

Some modifications of the extraction and derivatisation of the samples were found to be necessary and these are described here. Briefly the methanol wash volumes before and after deconjugation were increased from 1 ml to 2 ml in order to elute the radiolabelled metabolites off the cartridge more efficiently. In order to aid solubilisation and TMS-derivatisation of the dried deconjugated fraction, 100 μl of acetonitrile was added in addition to 100 μl of BSTFA and the mixture was heated at 60°C for 1 hour.

Metabolites were hydrolysed either by enzymatic hydrolysis as described in chapter 2 or by acid hydrolysis using 3M HCl at room temperature overnight. Hydrolysed metabolites were separated by thin layer chromatography (TLC) using Whatman silica gel $GF_{254}$ plates (0.25 mm thickness) and a solvent system of ethyl acetate-hexane 6:4 (v/v). The TLC plates were divided into sections and the amount of radiolabelled metabolites in each section was quantitated by scraping off the silica and counting the

radioactivity as described in chapter 2. Non-radiolabelled standards were visualised by exposing the TLC plates to iodine vapour.

### 4.3.2. Results

#### 4.3.2.1. Recovery of radiolabelled metabolites

Initial attempts to extract the radiolabelled α-tocopherol metabolites from rat urine, using the method outlined in chapter 2, resulted in low recoveries (generally < 10%), as judged by the recovery of radioactivity in the final derivatised fractions. Analysis of the radioactivity data of the various fractions indicated only a negligible loss of radiolabelled material in the flow through or wash fractions. It appeared, therefore, that appreciable amounts of metabolites were retained on the solid phase extraction cartridge, since a total of only approximately 30% of the starting radioactivity was observed in all the combined fractions. It was also observed that only a small fraction (30%) of the extracted radioactivity was taken up into the BSTFA derivatisation solvent after drying of the final methanol fraction from the second SPE cartridge.

Modifications to the method, including larger methanol wash volumes and addition of acetonitrile to the BSTFA derivatisation solvent, improved the yields (generally > 50%; see table 4.4), although they still remained lower than those obtained when 100 μl of radiolabelled urine was added to 2 ml of human urine, as described in chapter 2 (>95%). In the absence of information on the actual metabolites present in rat urine it was difficult to ascertain whether this disparity was due to differences in the general characteristics of human and rat urine or whether it resulted from specific differences in the type of metabolites.

| Rat urine sample | Overall % recovery in final derivatised fraction | Total $d_0$-α-tocopherol metabolites as measured by GC-MS (Trolox equivalents) | Radioactivity in final derivatised fraction (DPM) | Radioactivity in final fraction/ total $d_0$-α-tocopherol metabolites (DPM/Trolox equivalents) |
|---|---|---|---|---|
| 1/24 hr | 54.3 | 2.05 | 117,000 | 57,100 |
| 1/48 hr | 49.4 | 1.54 | 172,000 | 111,700 |
| 1/72 hr | 74.6 | 3.37 | 215,000 | 63,800 |
| 2/24 hr | 53.8 | 1.90 | 71,000 | 37,400 |
| 2/48 hr | 49.5 | 2.41 | 117,000 | 48,500 |
| 2/72 hr | 55.8 | 3.62 | 130,000 | 35,900 |
| 3/24 hr | 57.0 | 2.76 | 74,000 | 26,800 |
| 3/48 hr | 54.7 | 2.14 | 138,000 | 64,500 |
| 3/72 hr | 53.3 | 5.14 | 92,000 | 17,900 |

n = 9
Mean = 51,500
CV = 39.3 %

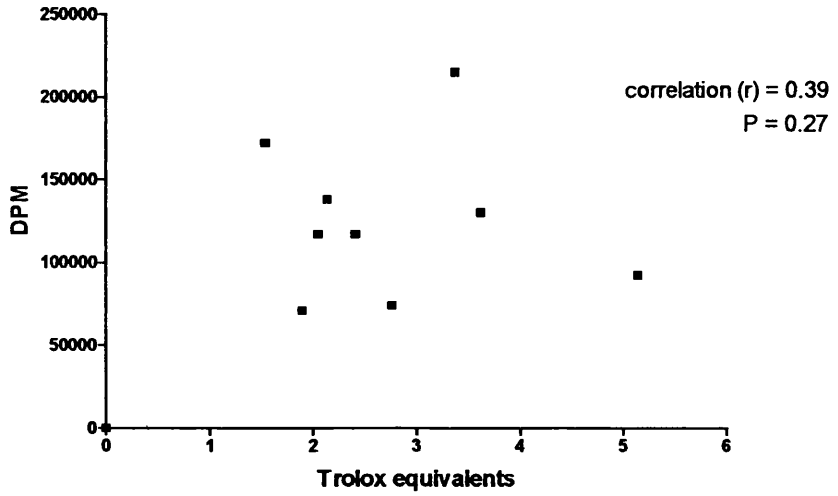



**Table 4.4.** Recovery of radioactivity in the final derivatised fraction and the correlation between radioactivity in this fraction and the amount of α-tocopherol metabolites as measured by GC-MS.

### 4.3.2.2. Metabolites in rat urine

In general, the deconjugated metabolites observed in rat urine were similar to those found in human urine. These included α-CEHC, α-tocopheronolactone, γ-CEHC, γ-tocopheronolactone, β-CEHC and δ-CEHC. However, in contrast to human urine, no CMBHCs were detected, which is surprising since primary rat hepatocyte cultures have been reported to produce α-, γ- and δ-CMBHC *in vitro* (Parker et al., 2000).

The relative levels of the metabolites observed in rat urine are shown in table 4.5 and are different to those observed in human urine. The major metabolites observed in rat urine were β- and γ-CEHC whereas in human urine γ-CEHC was the major metabolite while β-CEHC was only a minor metabolite, owing to the low dietary levels of β-tocopherol. The basal rat diet contained the different tocopherols in the following amounts (per kg of diet) - α-tocopherol, 77.0 mg; β-tocopherol, 1.2 mg; γ-tocopherol, 10.1 mg; δ-tocopherol, 5.0 mg. It is interesting to note the high urinary level of β-CEHC in comparison to the low level of its presumed dietary precursor, β-tocopherol, in the diet. The high level of β-CEHC observed raises the possibility that it could have been produced by demethylation of either α-tocopherol, α-CEHC or one of its precursors. Demethylation is a common method of catabolism of many xenobiotics and drugs, especially if the methyl is attached to a nitrogen, sulphur or oxygen atom (Gibson and Skett, 1994). δ-CEHC was also present in greater amounts in rat urine than expected. This could also be the result of demethylation of α- or γ-tocopherol or their metabolites.

| Rat urine sample | $D_9$-α-CEHC | $D_9$-α-TL | $D_0$-α-CEHC | $D_0$-α-TL | β-CEHC | γ-CEHC | γ-TL | δ-CEHC |
|---|---|---|---|---|---|---|---|---|
| 1/24 h | 2.49 | 2.45 (50.0)* | 1.21 | 0.84 (41.0) | 4.03 | 3.28 | 0.43 (11.6) | 1.72 |
| 1/48 h | 2.39 | 2.12 (47.0) | 0.92 | 0.62 (40.3) | 2.58 | 1.81 | 0.18 (9.0) | 0.93 |
| 1/72 h | 2.64 | 1.42 (35.0) | 2.10 | 1.27 (37.7) | 3.25 | 2.56 | 0.33 (11.4) | 1.26 |
| 2/24 h | 2.90 | 2.27 (44.0) | 1.16 | 0.74 (38.9) | 3.28 | 2.69 | 0.16 (5.6) | 1.40 |
| 2/48 h | 3.14 | 1.82 (36.7) | 1.63 | 0.78 (32.4) | 2.79 | 2.33 | 0.10 (4.1) | 1.10 |
| 2/72 h | 2.75 | 2.18 (44.2) | 2.45 | 1.17 (32.3) | 3.90 | 3.36 | 0.42 (11.1) | 1.66 |
| 3/24 h | 2.47 | 2.69 (52.1) | 1.70 | 1.06 (38.4) | 3.59 | 3.10 | 0.50 (13.9) | 1.58 |
| 3/48 h | 2.81 | 2.38 (45.9) | 1.25 | 0.90 (42.1) | 3.98 | 3.44 | 0.46 (11.8) | 1.71 |
| 3/72 h | 2.70 | 1.60 (37.3) | 3.67 | 1.47 (28.6) | 4.37 | 3.36 | 0.25 (6.9) | 1.57 |

**Table 4.5.** The relative amount of vitamin E metabolites in each rat urine sample.
The amount of metabolite is expressed as a ratio of peak areas compared to the trolox standard (6.8 nmol/ml) and is based on an average of two separate analyses.
*Figures in brackets indicate the amount of α- or γ-tocopheronolactone as a percentage of the total amount of α- or γ-tocopheronolactone and CEHC, respectively.

#### 4.3.2.3. Artefactual oxidation in rat urine

The level of artefactual oxidation in rat urine was greater than that found in human urine (see levels of $d_9$-α-tocopheronolactone in table 4.5). $D_9$-α-tocopheronolactone consistently accounted for 35-50% of the total $d_9$-α-CEHC standard, in comparison to 10-20% in studies of human urine. γ-Tocopheronolactone was also found in all of the rat urine samples, whereas it was only observed in a few human urine samples in which artefactual oxidation of $d_9$-α-CEHC was high. In contrast to human urine samples, the levels of $d_0$-α-tocopheronolactone were generally lower than those predicted by the measurement of $d_9$-α-tocopheronolactone, indicating artefactual conversion of $d_9$-α-CEHC was actually greater than that of endogenous α-CEHC.

In the previous studies of human urine, ascorbate (vitamin C) was added to fresh urine samples to prevent artefactual oxidation. However, ascorbate was not added to the rat urine samples. The high levels of α-tocopheronolactone observed in the rat urine samples could, therefore, either result from the lack of ascorbate or alternatively rat urine may differ from human urine in its relative levels of endogenous reductants and oxidants.

#### 4.3.2.4. Relationship between radioactivity data and the levels of α-tocopherol metabolites

The radioactivity (DPM) measured in each derivatised fraction should correlate to the amount of α-tocopherol metabolites measured in that sample i.e. high radioactivity should correspond to high levels of α-tocopherol metabolites. However, the correlation between the radioactivity data and the amount of α-tocopherol metabolites measured in each sample was poor (CV = 39.3%, n=9; Table 4.4). The recovery of radiolabel in the

final derivatisation solvent varied from 40-80%. However, even though 40-80% of the radiolabelled metabolites were solubilised in the derivatisation solvent, it does not necessarily follow that these metabolites were derivatised or deconjugated. It is conceivable that conjugated metabolites were present in the derivatisation solvent and this may account for the poor correlation between the radioactivity data and the amount of unconjugated α-tocopherol metabolites. Alternatively other unknown α-tocopherol metabolites could be excreted in the rat urine and account for this discrepancy.

#### 4.3.2.5. Deconjugation of metabolites in rat urine

TLC of fractions before and after deconjugation enabled conjugated and unconjugated metabolites to be separated. The TLC plate was divided into sections and the radioactivity of each section was counted and this allowed an estimation to be made of the relative amounts of conjugated and unconjugated metabolites. The finding that, before deconjugation, the majority (>95%) of the radiolabelled metabolites in rat urine remained at the origin after TLC analysis indicated that these metabolites were highly polar and were probably present as sulphate or glucuronide conjugates (figure 4.12).

*Enzymatic hydrolysis of radiolabelled samples*

After enzymatic deconjugation, with the mixed sulphatase/glucuronidase enzyme preparation used for human studies in chapter 2, only approximately 10% of the radiolabelled metabolites moved from the origin, whereas both α-CEHC ($R_f$=0.33) and α-tocopheronolactone benzoquinone ($R_f$=0.56) standards were shown to move from the origin under the same conditions (Figure 4.12). This indicated that the enzymatic deconjugation was inefficient and explained the poor correlation between the radioactivity data and the levels of the metabolites. The activity of the enzyme

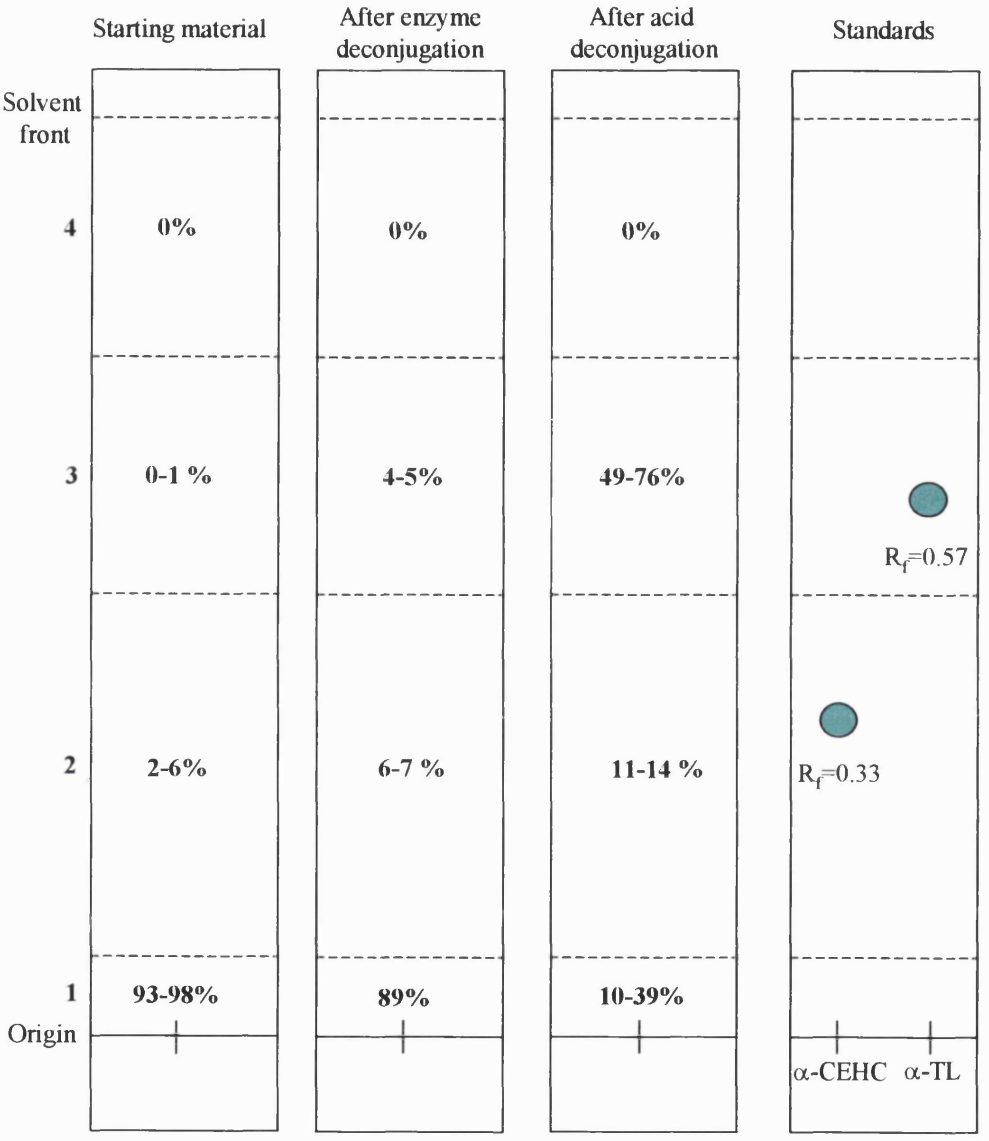



**Figure 4.12.** Thin layer chromatograms of radiolabelled rat urine extracts, showing percentage of total radiolabel in each section (**1-4**) of the chromatogram (n = 2).

preparation was assayed using phenolphthalein glucuronide and nitrocatechol sulphate. Both of these model compounds were efficiently deconjugated by the sulphatase/glucuronidase enzyme preparation, suggesting that either the radiolabelled α-tocopherol metabolites were present as other types of conjugates, that the enzyme activities were inhibited in rat urine or that the metabolites released after deconjugation were more polar than either α-CEHC or α-tocopheronolactone.

*Acid hydrolysis of radiolabelled metabolites*

Acid hydrolysis (3M HCl, overnight at room temperature in the presence of air) cleaved between 60-90% of the conjugates as judged by the amount of radiolabel that moved from the origin (figure 4.12). Lack of complete hydrolysis was probably due to the low temperature used in comparison to others who have used elevated temperatures (70°C, for 2 hr, under $N_2$). The major radiolabelled spot observed after acid hydrolysis corresponded to α-tocopheronolactone, as judged by comparing its $R_f$ value to that of an authentic standard. GC-MS analysis of the acid hydrolysed sample also showed that α-tocopheronolactone was the major metabolite, although α-tocopheronolactone was observed as the TMS-derivatised hydroquinone form by GC-MS as opposed to the benzoquinone form observed on TLC. The benzoquinone and hydroquinone forms can be separated by TLC but the hydroquinone is easily oxidised on silica to the benzoquinone form (see section 5.3.2). The majority of this α-tocopheronolactone was probably produced by oxidation of α-CEHC due to the presence of acid and oxygen during the hydrolysis reaction.

The efficient deconjugation of the radiolabelled metabolites by acid hydrolysis but not by sulphatases or glucuronidases suggested that either differently conjugated forms of metabolites were excreted in the rat compared to human urine or the enzymes were inhibited or inactivated by rat urine.

#### 4.3.2.6. Conjugates present in rat urine

Ideally the conjugated radiolabelled α-tocopherol metabolites should have been characterised by radio-HPLC and tandem mass spectrometry. However, owing to the small amount of sample available, such analyses were not possible. The conjugated metabolites present in rat urine have, however, recently been characterised by Drs. Froecheis and Cohn in Switzerland who provided the samples. They found that the major metabolites were mono-sulphated CEHCs (personal communication). They have also confirmed using radio-high performance liquid chromatography-mass spectrometry (radio-HPLC-MS) before and after enzymatic deconjugation that the metabolites present are resistant to enzymatic deconjugation (personal communication).

## 4.4. Conclusions

From the data obtained by analysis of deuterated human urine samples and radiolabelled rat urine samples, a number of conclusions can be drawn.

1) The levels of metabolites observed in human urine using the methods described in this study are in general agreement with published data although the levels of CMBHCs were found to be greater than that reported in the literature.
2) Artefactual oxidation is a problem in all urine samples but can be estimated by adding deuterated α-CEHC to the sample.

3) Although in general, the type of urinary vitamin E metabolites produced by humans and rats are similar there are some distinct differences.

4) The work of Froecheis and Cohn suggest that mono-sulphated CEHCs are the major conjugates present in rat urine and we have confirmed that they are inefficiently deconjugated by commercially available sulphatase enzymes.

# CHAPTER 5

## The Chemical Synthesis of Unconjugated and Conjugated α-Tocopherol Metabolites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## 5.1. Introduction

Indirect evidence, using sulphatase and glucuronidase enzymes, indicates that vitamin E metabolites are excreted in the urine as sulphate and glucuronide conjugates (Chiku et al., 1984; Swanson et al., 1998; Stahl et al., 1999). However, urinary vitamin E metabolites have only been analysed after enzymatic or acid deconjugation, due to the problems associated with the analysis of polar conjugates and the lack of authentic conjugated standards. The GC-MS and HPLC methods that have been developed to date to measure unconjugated vitamin E metabolites have provided useful information concerning the relative levels and the nature of metabolites (see table 4.3) but, as discussed in chapter 4, problems exist with regards to the efficiency of deconjugation and the production of artefactual α-tocopheronolactone from α-CEHC.

In order to achieve a greater understanding of vitamin E metabolism and to remove the problems associated with measuring deconjugated metabolites, the analysis of conjugated vitamin E metabolites is necessary. Conjugated metabolites can be analysed using electrospray tandem mass spectrometry but characterisation and confirmation of potential metabolites relies upon the availability of authentic conjugated standards. This chapter describes in detail the chemical synthesis of such authentic conjugated standards.

An aim of this study was to investigate the authenticity of α-tocopheronolactone. Therefore, this chapter describes the synthesis of unconjugated α-CEHC (**9**) and α-tocopheronolactone (**12**) (Section 5.3) and the subsequent synthesis of sulphate and glucuronide conjugates of these metabolites (Section 5.4). Analysis of conjugated vitamin E metabolites theoretically eliminates the possibility of artefactual formation of

α-tocopheronolactone and therefore the authenticity of α-tocopheronolactone as an *in vivo* metabolite can be assessed. In addition, we report the synthesis of α-CMBHC which we used to confirm α-CMBHC (**22**) as a metabolite of α-tocopherol, as discussed in chapter 3.

### 5.1.1. Nomenclature

The chemical names used in this and other chapters are those which are most commonly used and do not necessarily conform with IUPAC nomenclature. Below is a list of these commonly used names followed by the official IUPAC name:

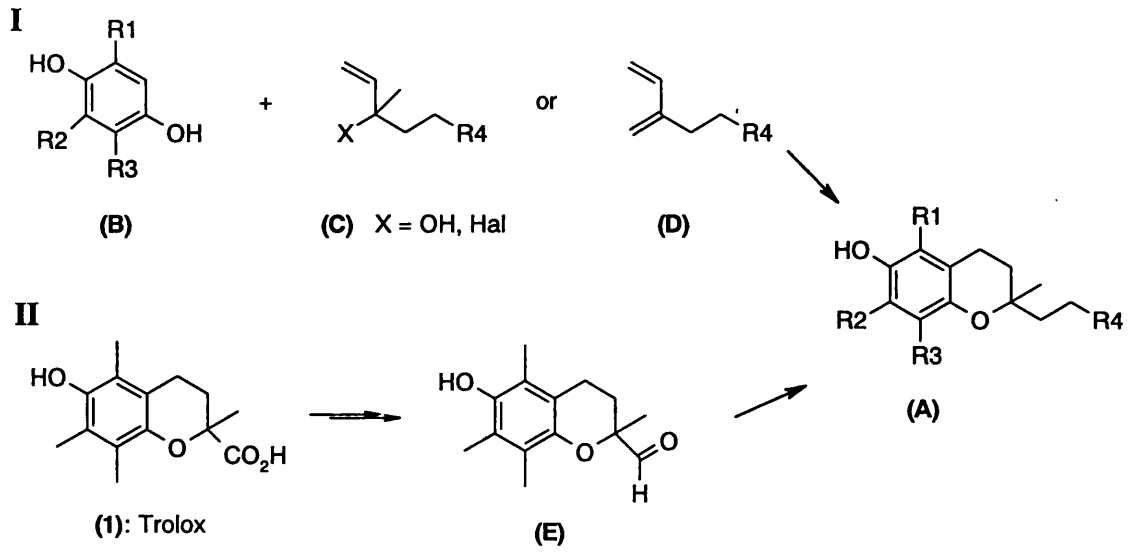
- (±)-2,5,7,8-Tetramethyl-2-(2'-carboxyethyl-2)-6-hydroxychroman (α-CEHC) = (±)-3-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-propionic acid;
- Benzoquinone α-tocopheronolactone = 2,3,5-trimethyl-6-[2-(2-methyl-5-oxo-tetrahydro-furan-2-yl)-ethyl]-[1,4]benzoquinone;
- Hydroquinone α-tocopheronolactone = 5-[2-(2,5-dihydroxy-3,4,6-trimetyl-phenyl)-ethyl]-5-methyl-dihyro-furan-2-one;
- (±)-2,5,7,8-tetramethyl-2(4'-carboxy-4'-methylbutyl)-6-hydroxychroman ((±)α-CMBHC) = 5-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoic acid;
- Trimethylamine (±)-2,5,7,8-tetramethyl-2-(2'-carboxyethyl-2)-6-hydroxychroman sulphate (α-CEHC sulphate) = 3-(2,5,7,8-tetramethyl-6-sulpho-oxy-chroman-2-yl)-propionic acid trimethylammonium salt;
- Trimethylamine α-tocopheronolactone monosulphate = 5-[2-(3,4,6-trimetyl-1/6-sulphooxy-phenyl)-ethyl]-5-methyl-dihyro-furan-2-one trimethylamine salt;
- Methyl 1,2,3,4-tetra-O-acetyl-α/β-D-glucopyranuronate = 3,4,5,6-tetra-acetoxy-tetrahydro-pyran-2-carboxylic acid methyl ester;

- 1-hydroxy-methyl 2,3,4-tri-O-acetyl-α/β-D-glucupyranuronate = 3,4,5-triacetoxy-6-hydroxy-tetrahdro-pyran-2-carboxylic acid methyl ester;
- 2,3,4-tri-O-acetyl-1-O-(trichloroacetimidoyl)-β-D-glucupyranuronate = 3,4,5-triacetoxy-6-trichloroacetimidoyloxy-tetrahydro-pyran-2-carboxylic acid methyl ester;

## 5.2. Previous chemical synthesis of vitamin E type compounds

Over the last fifty years, the chemical synthesis of vitamin E derivatives has attracted interest for their use in biokinetic and metabolic studies and more recently for their potential therapeutic use as inhibitors of platelet aggregation, anticancer agents and as antioxidants of low density lipoprotein (Lahmann and Thiem, 1997; Arya et al., 1998; Uhrig et al., 2000).

Two general approaches (Scheme 5.1) have been reported for the synthesis of vitamin E type compounds (**A**) (Kasparek, 1980). The first is based on the formation of the characteristic chroman ring by using an acid-catalysed Friedel-Craft alkylation of a methylated hydroquinone (**B**) with either a vinylic halide/alcohol (**C**) or a substituted diene (**D**) (Scheme 5.1 (**I**)) (Smith et al., 1939). In the second strategy, a side-chain is attached to a methylated benzopyran aldehyde intermediate (**E**), often derived from commercially available Trolox® (**1**), using a Wittig reaction (Scheme 5.1(**II**)) (Suarna et al., 1997).



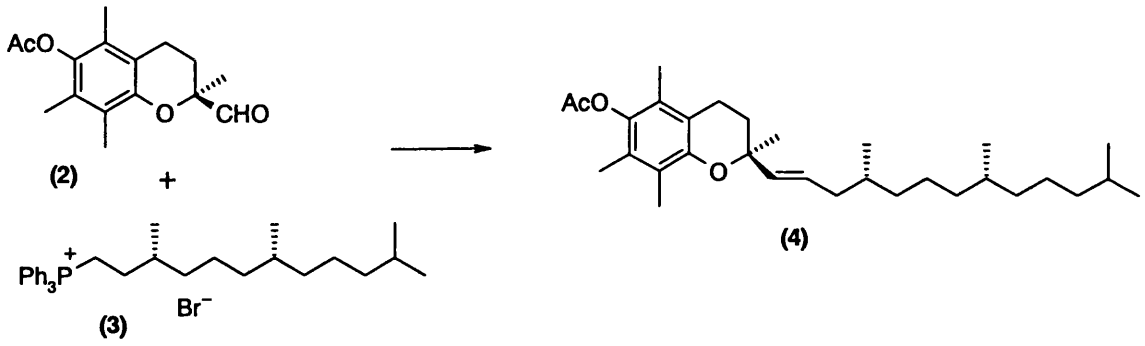

**Scheme 5.1.** The two approaches for the synthesis of vitamin E type compounds

### 5.2.1. Synthesis of α-tocopherol

The two approaches described above for the synthesis of vitamin E type compounds are best illustrated by reference to the synthesis of α-tocopherol (**15**), which has received much attention as it is a major supplement in food and animal feed, with a worldwide annual production of over 10,000 tons (Ullmann's Encyclopaedia of Industrial Chemistry, 1996).

Von Karrer *et al.* (1938) first reported the synthesis of a mixture of (2R, 4'R, 8'R)- and (2S, 4'R, 8'R)-α-tocopherol by the condensation of phytyl bromide, prepared from natural phytol, with trimethylhydroquinone using zinc chloride catalysis. This method was unable to control the stereochemistry at the C-2 position and therefore did not produce enantiomerically pure α-tocopherol.

Subsequently, the synthesis of enantiomerically pure (2R, 4'R, 8'R)-α-tocopherol was reported by Mayer *et al.* (1963) using the Wittig approach and involved the phosphonium salt (**3**) derived from natural phytol and the optically active (chroman-2-yl)-carbaldehyde (**2**) (Scheme 5.2). Subsequent hydrogenation of the unsaturated intermediate (**4**) afforded pure RRR-α-tocopherol (**15**).




**Scheme 5.2.** First total synthesis of (2R, 4'R, 8'R)-α-tocopherol

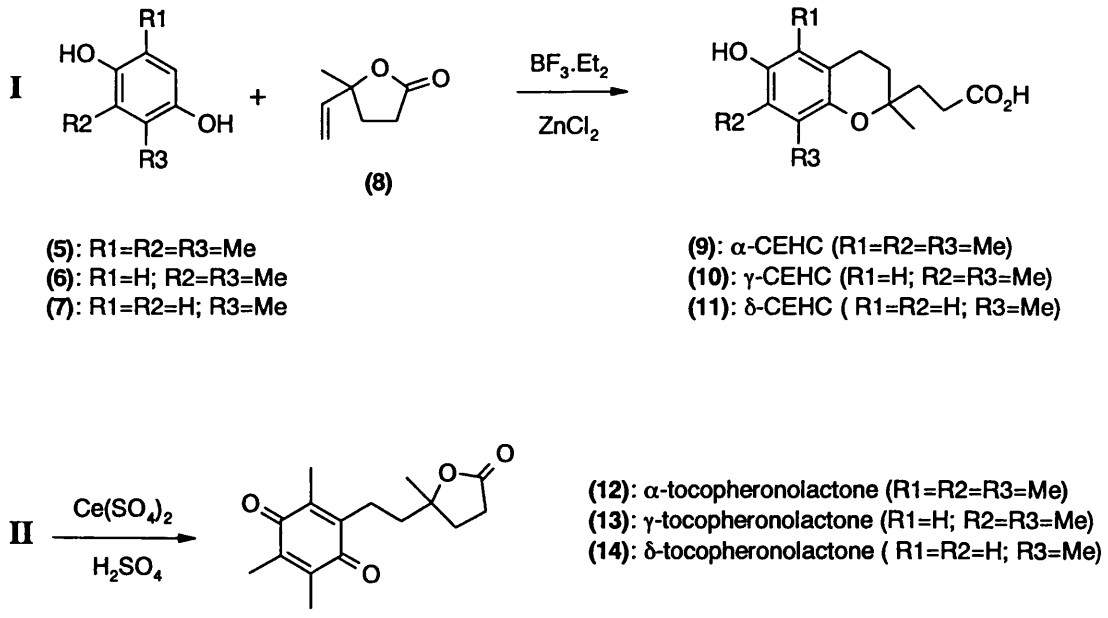
Since these early studies, numerous improvements and developments have been made in the synthesis of both racemic and enantiomerically pure vitamin E type compounds (Scott et al., 1976; Cohen et al., 1979; Ishihara et al., 1996; Bienayme et al., 2000). However, condensation of allylic alcohol with methylated hydroquinone remains the most common method as a result of its relative simplicity (Scheme 5.1 (**I**)).

### 5.2.2. Synthesis of vitamin E metabolites

The initial work on the synthesis of vitamin E metabolites followed soon after the discovery of α-tocopheronolactone (**12**) and α-tocopheronic acid (**37**) in rabbit and human urine in the 1950's (Simon et al., 1956a; Simon et al., 1956b). Weichet *et al*

(1959) reported the synthesis of (±)-α-tocopheronolactone benzoquinone (**3**) and the then unknown α-tocopherol metabolite, (±)-α-CEHC (**9**) (Scheme 5.3). (±)-α-CEHC (**9**) was synthesised by coupling 2,3,5-trimethylhydroquinone (TMHQ) (**5**) with γ-vinylbutyrolactone (**8**), catalysed by a mixture of zinc chloride and boron trifluoride-diethyl etherate complex.

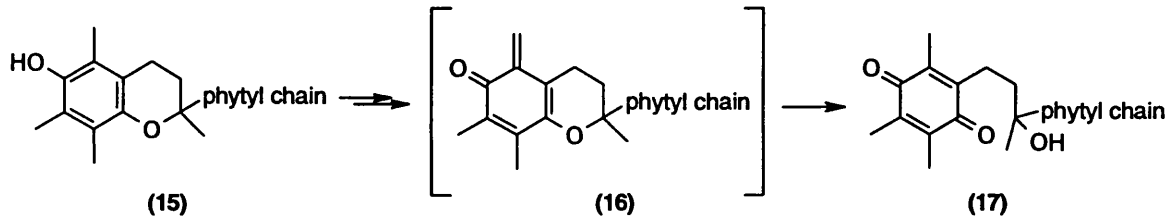
Subsequent oxidation of (±)-α-CEHC (**9**) using cerium-(IV)-sulphate produced α-tocopheronic acid (**37**) which cyclised to α-tocopheronolactone benzoquinone (**12**). Later, Gloor *et al.* (1966), using similar conditions, synthesised (±)-γ-CEHC (**10**) and (±)-γ-tocopheronolactone (**13**) from 2,3-dimethylhydroquinone (**6**) while (±)-δ-CEHC (**11**) and (±)-δ-tocopheronolactone benzoquinone (**14**) were prepared by Chiku *et al.* (1984) from the corresponding monomethylated starting material (**7**) (Scheme 5.3).




**Scheme 5.3.** Syntheses of CEHCs and tocopheronolactones

More recently Kantoci *et al.* (1997) synthesised (±)-CEHCs, (**9**) and (**10**), by condensation of trimethylhydroquinone (**5**) or dimethylhydroquinone (**6**) with (**8**), using only boron trifluoride-diethyl ether complex as catalyst and then converted them to their (±)-tocopheronolactones, (**12**) and (**13**), by treatment with iron (III) chloride.

Another approach for oxidative ring opening of the chroman moiety of α-tocopherol (**15**) was performed by Roseneau *et al.* (1996) by treating the ortho-quinone methide (**16**) intermediate with acidic water (Scheme 5.4). The intermediate (**16**) is produced by bromination of the 5a-methyl of (**15**) followed by dehydrobromination.

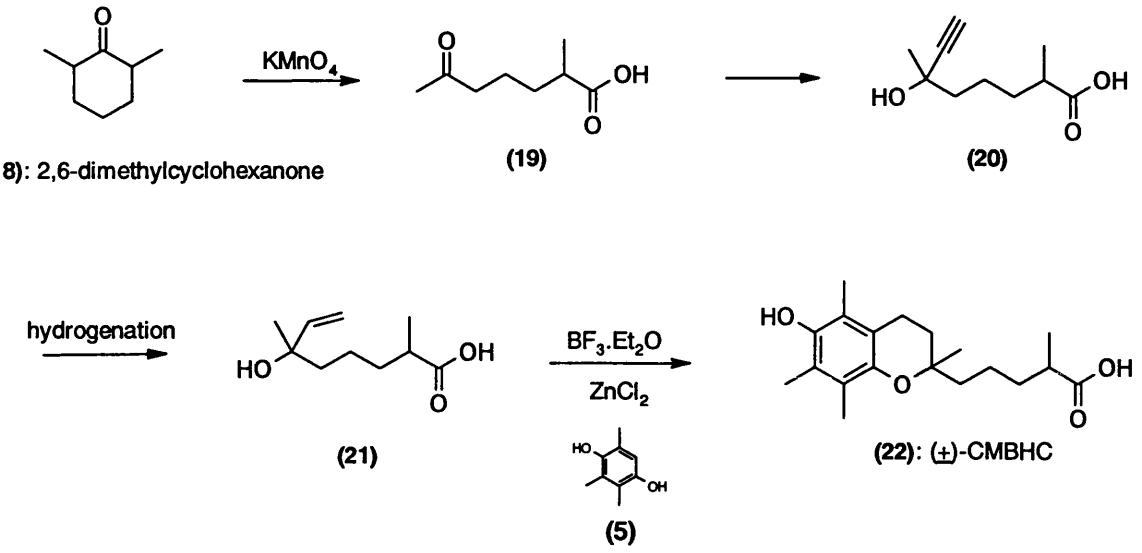



(15) (16) (17)

**Scheme 5.4.** Oxidative ring opening of α-tocopherol (**15**) by $Br_2/H_2O$

In 1966, Weichet *et al.* (1966) reported the synthesis of a number of (±)-α-CEHC analogues with side-chains of varying lengths and structures based on the condensation of an appropriate acyclic allylic alcohol with TMHQ (**5**). In particular, they described the synthesis of the long chain α-CEHC analogue, (±)-α-CMBHC (**22**), which has been confirmed to be an α-tocopherol metabolite during the course of the present study (Chapter 3) (Pope et al., 2000). The CMBHC (**22**) was obtained by condensation of the acid vinyl alcohol (**21**) with TMHQ (**5**) in the presence of a mixture of zinc chloride and boron trifluoride-diethyl ether complex. The heptanoic acid derivative (**21**) was

prepared in three steps from 2,6-dimethylcyclohexanone (**18**) (Scheme 5.5) (Weichet and Blaha, 1966).

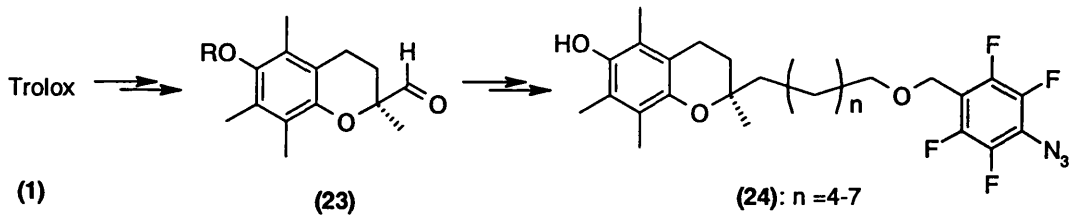



**Scheme 5.5.** Synthesis of (±)-α-CMBHC

### 5.2.3. Synthesis of enantiomerically pure vitamin E derivatives

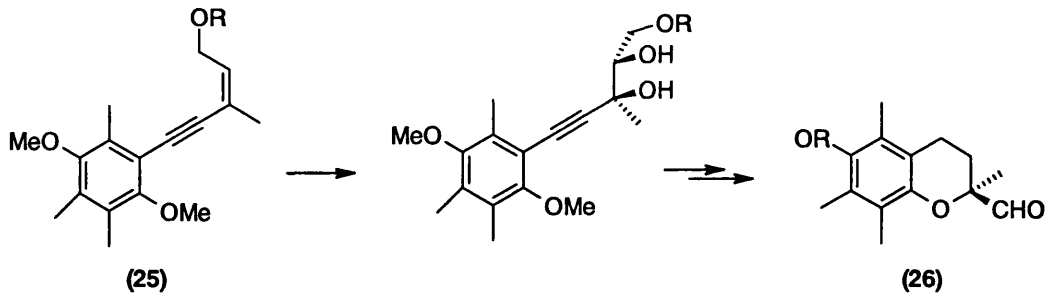
As mentioned above, condensation of methylated hydroquinone with allylic alcohol is the most common way to synthesise vitamin E derivatives. However, as defined stereochemistry at the C-2 centre is important for enzyme assays or *in vivo* studies, synthetic methods allowing tighter control of this chiral centre have been developed. These strategies have included resolution of the products, the use of auxiliaries or enantiomerically pure building blocks, as well as asymmetric oxidation (Tietze and Gorlitzer, 1996; Tietze and Gorlitzer, 1997; Tietze et al., 1999; Trost and Asakawa, 1999).

Many groups have used commercially available *(S)*-Trolox (**1**) as a starting material (Scheme 5.1 (II)). Lei *et al.* prepared the pure α-tocopherol analogue (**24**), containing a photoaffinity label, for use in enzymatic studies (Scheme 5.6)(Lei et al., 1998; Lei and Atkinson, 2000). The tetrafluoroazido benzyl ether (**24**) was synthesised from the *(S)*-Trolox aldehyde (**23**) using a Wittig condensation (Lei and Atkinson, 2000).



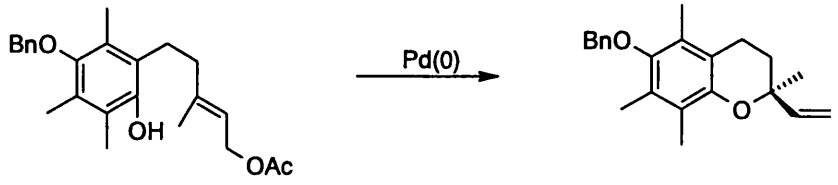

**Scheme 5.6.** Synthesis of a photoaffinity analogue of α-tocopherol from *(S)*-Trolox (**1**).

Over the last few years a lot of work has been done towards the total synthesis of a pure chroman moiety of vitamin E. Tietze and Gorlitzer. (1998) reported the enantioselective synthesis of the pure key aldehyde (**26**) *via* an asymmetric Sharpless bishydroxylation of the unsaturated intermediate (**25**) (Scheme 5.7).



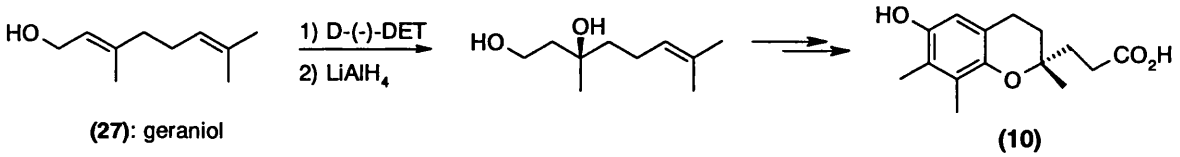

**Scheme 5.7.** Enantioselective synthesis of pure (chroman-2-yl)carbaldehyde intermediate

Palladium catalysed asymmetric allylic alkylation was employed by Trost *et al.* (1999) in their asymmetric synthesis of the chroman ring of vitamin E (Scheme 5.8).




**Scheme 5.8.** Enantioselective synthesis of vitamin E core

Jung and MacDougall (1999) have recently reported the enantioselective synthesis of γ-CEHC (**10**) (also known as LLU-α) from geraniol (**27**) and dimethylhydroquinone monoacetate involving a Sharpless asymmetric epoxidation as a key step (Scheme 5.9).

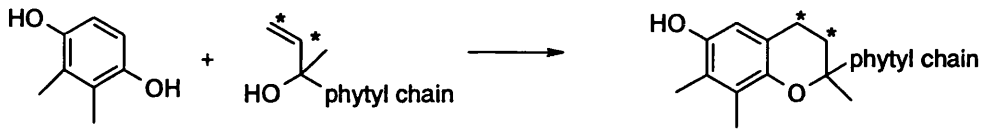



**Scheme 5.9.** Enantioselective synthesis of γ-CEHC (**10**). Abbreviations: D-(-)-DET – diethyl D-tartrate; $LiAlH_4$ – lithium aluminium hydride.

### 5.2.4. Synthesis of labelled vitamin E derivatives

Isotopic labelling of vitamin E and its metabolites has been used successfully to help elucidate the catabolic pathway of vitamin E (Burton and Ingold, 1993) and modifications of the previously reported methods have allowed the synthesis of [$^{14}$C], [$^{3}$H] and [$^{2}$H] vitamin E derivatives.

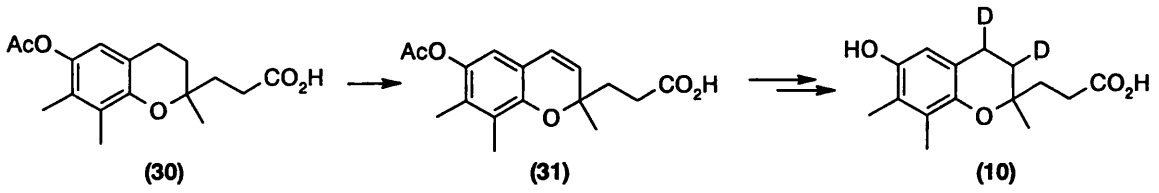
Gloor *et al.* (1966) prepared 3,4-[$^{14}$C]-γ-tocopherol (**29**) by condensation of 2,3-dimethylhydroquinone (**6**) with 1,2-[$^{14}$C]-isophytol (**28**) (Scheme 5.10), while 5a-[$^{14}$C]-methyl α-tocopherol was prepared by Chiku *et al.* (1984) by methylation of γ-tocopherol with [$^{14}$C]-formaldehyde and subsequent reduction.




(6) (28) (29)

**Scheme 5.10.** Synthesis of 3,4-[$^{14}$C]-γ-tocopherol

In order to determine the extent of urinary excretion of γ-CEHC, Swanson *et al.* (1999) developed a new GC-MS method using $d_2$-(±)-γ-CEHC (**10**) as a deuterated internal standard. Compound (**10**) was obtained by oxidation of the acetylated (±)-γ-CEHC (**30**), using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), followed by deacetylation and subsequent catalytic deuteration of the 2,3-dehydro-intermediate (**31**) (Scheme 5.11).

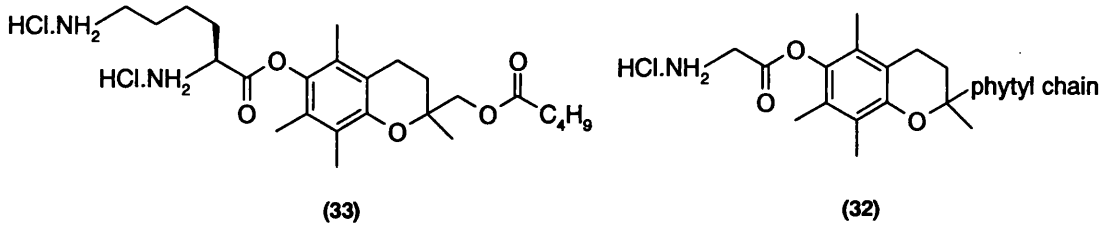



**Scheme 5.11.** Synthesis of $d_2$-(±)-γ-CEHC

### 5.2.5. Synthesis of conjugated vitamin E derivatives

Recently, increased attention has been paid to α-tocopherol and its analogues because of their potential therapeutic role in the treatment of disorders induced by oxygen-derived free radicals. However, a drawback of using this type of compound as a drug is its high insolubility in water, which affects its pharmacokinetics and tissue pharmacodistribution.

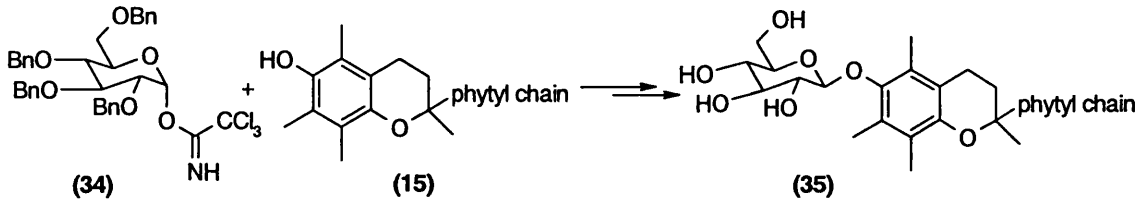
To enhance the hydrophilicity of such compounds and in order to overcome the problem of their restricted bioavailability, Arya *et al.* (1998) synthesised several amino acid conjugates of vitamin E analogues. In particular, they prepared glycine α-tocopherol **(32)** and the lysine conjugate **(33)** (Scheme 5.12) and both were found to have antiproliferative activity against human breast adenocarcinoma cells (Arya et al., 1998).




(33) (32)

**Scheme 5.12.** Lysine and glycine conjugates of vitamin E type compounds

Glycosylation has also been used successfully to increase the water solubility of vitamin E derivatives. Lahmann and Thiem (1997) prepared α-tocopheryl oligosaccharides, and more recently Uhrig *et al.* (2000) synthesised β-D-glucopyranosyloxy-α-tocopherol (**35**) by the reaction of a trichloroacetamide intermediate (**34**) with α-tocopherol (**15**) (Scheme 5.13).




(34) (15) (35)

**Scheme 5.13.** Synthesis β-D-glucopyranosyloxy-α-tocopherol (**35**)

In contrast, the synthesis of glucuronide or sulphate conjugates of vitamin E metabolites has not yet been reported. The next sections describe the synthesis of free α-CEHC, α-tocopheronolactone and α-CMBHC and their glucuronide and sulphate conjugates.

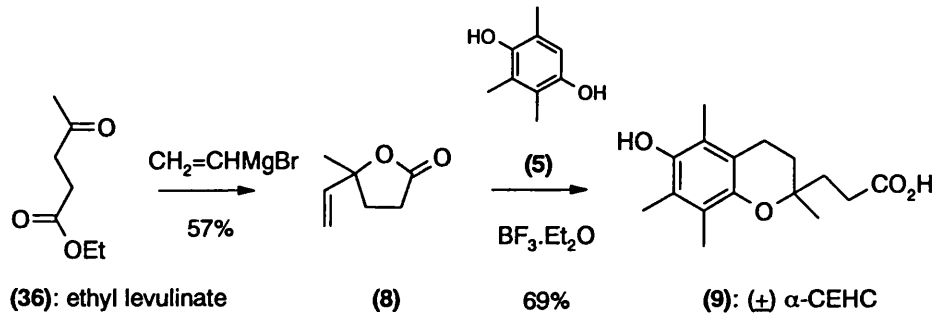
## 5.3. Chemical synthesis of unconjugated vitamin E metabolites in present study

In this study, the α-CEHC (**9**), α-tocopheronolactone (**12**) and α-CMBHC (**22**) standards that were required for electrospray tandem mass spectrometric investigations did not need to be enantiomerically pure. Therefore, the most straightforward synthetic procedures were chosen to synthesise the unconjugated metabolites i.e. condensation of allylic alcohols with methylated hydroquinones (Scheme 5.1 (**I**)).

Experimental details for the various syntheses, including characterisation data, are given in an appendix at the end of this thesis.

### 5.3.1. Synthesis of (±)-2,5,7,8-tetramethyl-2-(2-carboxy-2-ethyl)-6-hydroxychroman (α-CEHC) (9)

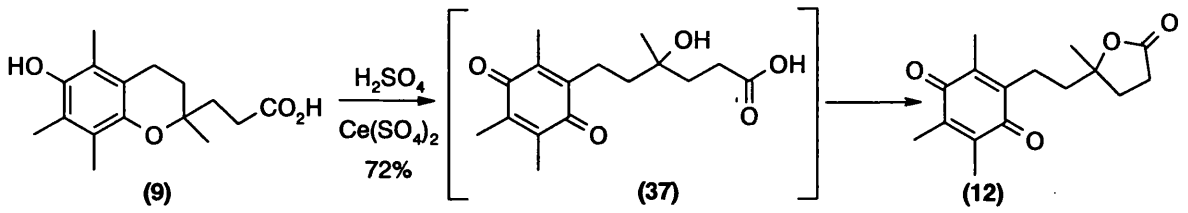
α-CEHC (**9**) was synthesised based on the method developed by Wechter *et al.* (1996) for the synthesis of γ-CEHC (**10**) i.e. from TMHQ (**5**) (instead of DMHQ (**6**)) and γ-methyl-γ-vinylbutyrolactone (**8**) (Scheme 5.14). The lactone (**8**) was formed by condensation of vinyl magnesium bromide with ethyl levulinate (**36**). It was found that the use of an excess of vinyl magnesium bromide allowed the reaction to go to completion, making the purification of the product by distillation under reduced pressure easier. The reaction of intermediate (**8**) with trimethylhydroquinone (**5**), in the presence of boron trifluoride diethyl etherate, gave (±)-α-CEHC (**9**) in a 69% yield.




**Scheme 5.14.** Synthesis of α-CEHC

### 5.3.2. Synthesis of hydroquinone (±)-α-tocopheronolactone (38)

Conversion of α-CEHC (**9**) to the benzoquinone form of α-tocopheronolactone (**12**) was achieved using the method of Weichet *et al.* (1959) (Scheme 5.3 (**II**)). Cerium sulphate dissolved in a mixture of methanol and 5% aqueous sulphuric acid was added to a methanolic solution of α-CEHC (**9**) and the reaction was stirred at room temperature for two hours to give a mixture of an unknown compound and benzoquinone tocopheronolactone (**12**) (Scheme 5.15). However, after leaving the mixture at room temperature overnight, only the benzoquinone (**12**) remained as judged by TLC and EI-MS. It was assumed that the unknown intermediate was tocopheronic acid (**37**), which converted on standing to α-tocopheronolactone (**12**). The benzoquinone form of α-tocopheronolactone (**12**) was obtained in 72% yield.

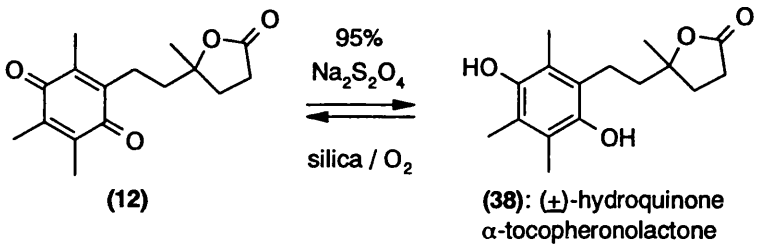



**Scheme 5.15.** Synthesis of (±)-α-tocopheronolactone (benzoquinone)

The conversion of the benzoquinone α-tocopheronolactone (**12**) to the hydroquinone form (**38**) required more attention. In the first instance, reduction of the lactone (**12**) with sodium dithionite appeared to be straightforward (Scheme 5.16) (Kawai et al., 1999). However, TLC appeared to show that the reaction never went to completion with large amounts of benzoquinone (**12**) still present, regardless of the length of time of the reaction and the equivalents of sodium dithionite used. Attempts to separate the benzoquinone (**12**) from the hydroquinone (**38**), using flash chromatography on silica gel, proved impossible even though the two compounds have markedly different polarities. In contrast, the two compounds (**12**) and (**38**) were easily separated using reverse-phase preparative HPLC.

It appears that the hydroquinone (**38**) is particularly unstable and readily oxidises to the benzoquinone (**12**) on contact with silica. This rapid oxidation explains the failure to purify the hydroquinone (**38**) on silica gel columns and also explains the results from the TLC. Once this facile conversion was discovered, synthesis of the hydroquinone (**38**) proved straightforward. The reduction of the benzoquinone (**12**), conducted in a mixture of water and ether in the presence of sodium dithionite, proceeded with almost 100% conversion after only 30 minutes. The product was extracted with ethyl acetate,

concentrated *in vacuo* and stored at 4°C. The hydroquinone tocopheronolactone (**38**) was obtained in 95% yield and HPLC, MS and NMR analyses confirmed its structure and purity.



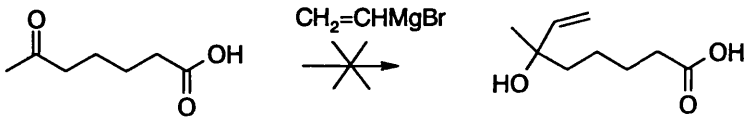

**Scheme 5.16.** Synthesis of (±)-α-tocopheronolactone (hydroquinone) (**38**)

### 5.3.3. Synthesis of (±)-2,5,7,8-tetramethyl-2-(4-carboxy-4-methylbutyl)-6-hydroxy--chroman (α-CMBHC) (22)

The synthesis of α-CMBHC (**22**) was first described by Weichet *et al.* (1966) more than thirty years ago. However, α-CMBHC (**22**) was not proposed as a metabolite of α-tocopherol (**15**) and the study did not include NMR characterisation. The strategy relied upon the condensation of 6-hydroxy-6-vinyl-2-methyl heptanoic acid (**21**) with TMHQ (**5**) using a combination of zinc chloride and $BF_3$-$Et_2O$ as catalysts (Scheme 5.5). The allylic alcohol (**21**) was obtained in three steps by oxidative ring opening of dimethylcyclohexanone (**18**), addition of acetylide to the resulting 6-oxo-2-methylheptanoic acid (**19**) and the final catalytic hydrogenation of the propargylic alcohol intermediate (**20**).

However, in the present investigation it was decided to apply the conditions used in the successful synthesis of α-CEHC (**9**) (Section 5.3.1) to synthesise α-CMBHC (**22**) i.e. condensation of vinyl magnesium bromide onto the ketoacid intermediate (**19**), followed by condensation of TMHQ (**5**) with the resultant vinylic alcohol (**21**) using $BF_3$-$Et_2O$ catalysis.

Since it was decided to use a different synthetic method, the condensation of vinyl magnesium bromide with a commercially available model compound, 6-oxo-heptanoic acid (**39**), was investigated. However, numerous attempts to synthesise the vinylic alcohol intermediate (**40**) failed (Scheme 5.17). One of the major problems resulted from the high water solubility of the acid (**39**), which hindered its extraction.

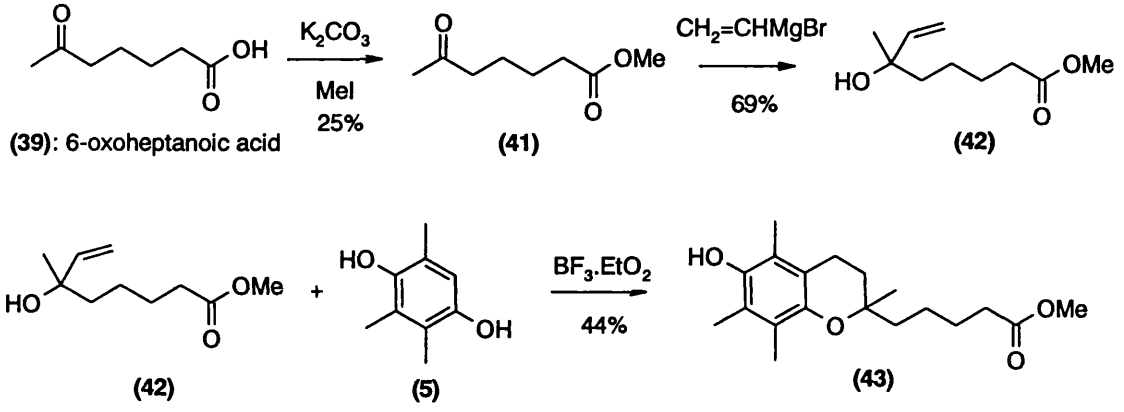



(39) (40)

**Scheme 5.17.** Unsuccessful addition of vinyl magnesium bromide to 6-oxoheptanoic acid

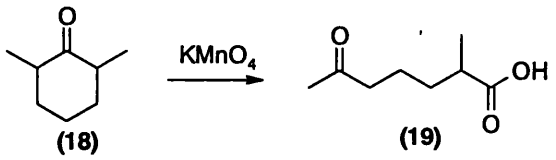
By analogy with the synthesis of α-CEHC (**9**) (Scheme 5.3) it was thought that a lactone intermediate (e.g. **8**) could be necessary for the reaction to proceed. Therefore, the methyl ester of heptanoic acid (**41**) was synthesised in order to encourage lactonisation and also to circumvent the problems of water solubility. The methyl ester (**41**) was produced using methyl iodide and potassium carbonate. Condensation of vinyl magnesium bromide with (**41**) produced the vinylic alcohol (**42**). In contrast to the condensation of vinyl magnesium bromide with ethyl levulinate (**36**), which led to the

butyrolactone (**8**) (Scheme 5.14), the formation of the seven membered lactone, resulting from an intramolecular cyclisation of the hydroxyester (**42**), was not observed. Reaction of TMHQ (**5**) with intermediate (**42**) afforded the carboxybutyl hydroxychroman (α-CBHC) methyl ester (**43**) in 44% yield (Scheme 5.18). From the work on the model compound it was therefore decided to use a methyl ester intermediate in the synthesis of α-CMBHC (**22**).



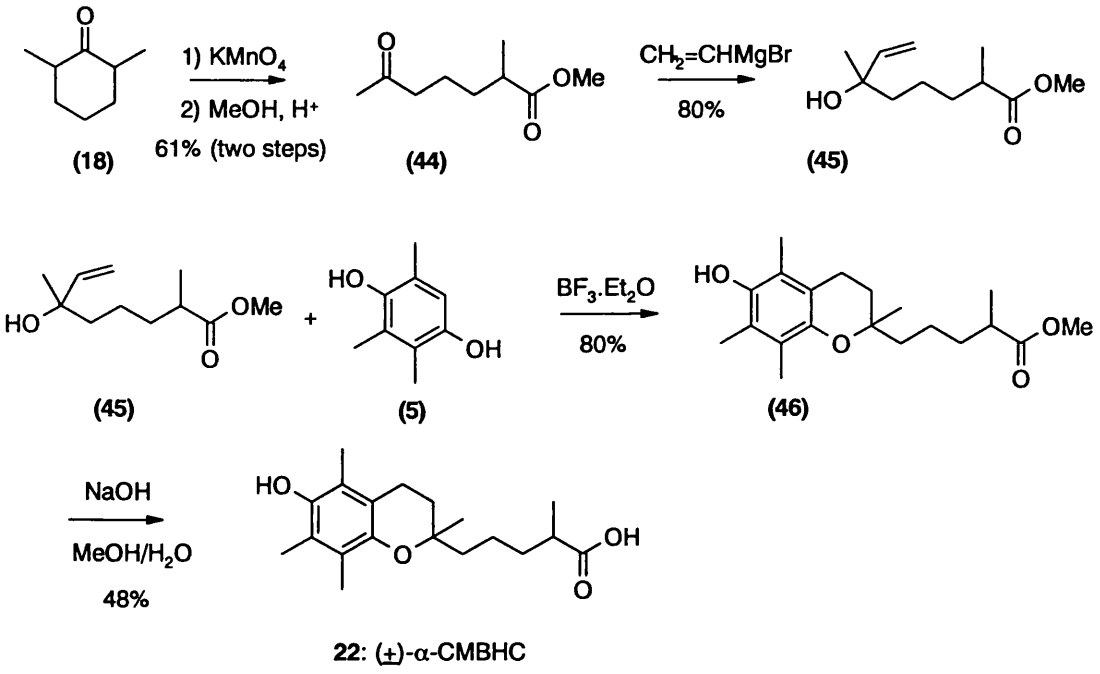

**Scheme 5.18.** Synthesis of α-CBHC methyl ester.

For the synthesis of α-CMBHC (**22**) the methyl branched starting material, 6-oxo-2-methyl-heptanoic acid (**19**), was required (Scheme 5.5). Oxidation of 2,6-dimethylcyclohexanone (**18**) with potassium permanganate following the procedure in the literature (Weichet and Blaha, 1966) yielded 6-oxo-2-methyl heptanoic acid (**19**) (Scheme 5.19).



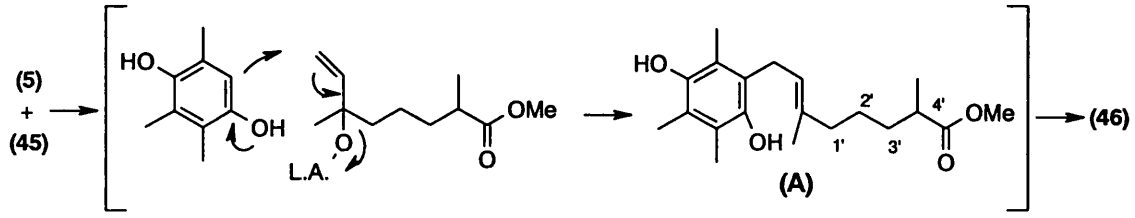

**Scheme 5.19.** Synthesis of 6-oxo-2-methyl heptanoic acid from dimethyl hexanone

The methyl ester (**44**) was obtained by refluxing 6-oxo-2-methyl-heptanoic acid (**19**) in methanol in the presence of sulphuric acid. Vinyl addition onto the ketomethyl ester (**44**) afforded the vinyl alcohol (**45**), which was condensed with TMHQ (**5**) to produce (±)-α-CMBHC methyl ester (**46**) in 80% yield. Saponification of the methyl ester (**46**) using sodium hydroxide in aqueous methanol afforded (±)-α-CMBHC (**22**) in 48% yield (Scheme 5.20).




**Scheme 5.20.** Synthesis of (±)-α-CMBHC

The structure of compound **(22)** was unambiguously assigned using NMR data. Double peaks in $^{13}C$ NMR data indicated that the product **(22)** was present as a mixture of two pairs of diastereoisomers. This was not surprising since the acid-catalysed Friedel-Craft alkylation mechanism, which has generally been proposed for the condensation of vinylic alcohols with hydroquinones, suggests the reaction proceeds without any control of the stereogenic centres (Scheme 5.21) (Wehrli et al., 1971; Ishihara et al., 1996). It was not possible to separate the two diastereoisomers by either HPLC or GC-MS which allowed the mixture to be used as a standard.




**Scheme 5.21.** Proposed formation of (±)-CMBHC (Friedel-Craft alkylation)

## 5.4. Chemical synthesis of conjugated α-tocopherol metabolites

Having synthesised unconjugated α-tocopherol metabolites it was necessary to prepare their glucuronide and sulphate conjugates.

### 5.4.1. Synthesis of glucuronide conjugates

There have been numerous reports of the synthesis of β-glucuronide conjugates (for a review see Stachulski and Jenkins, 1998). This is because many drugs and natural products are glucuronidated prior to excretion in the bile or urine. In addition, a number of these glucuronides, notably morphine glucuronides (Berrang et al., 1997), have been shown to have pharmacological effects which are at least as important as their parent compounds. Of particular interest are a number of reports on the synthesis of glucoside or glucuronide conjugates of vitamin E type compounds (Yoshioka et al., 1991; Lahmann and Thiem, 1997; Uhrig et al., 2000). These reports suggest that, owing to their ease of oxidation, particular care must be taken in the glucosylation or glucuronidation of vitamin E type compounds.

There are a number of possible intermediates that can be used to glucuronidate a substrate of interest. The preparation of common glucuronic acid donors is shown in scheme 5.22, starting with the commercially available D-glucurono-6,3-lactone (**47**).

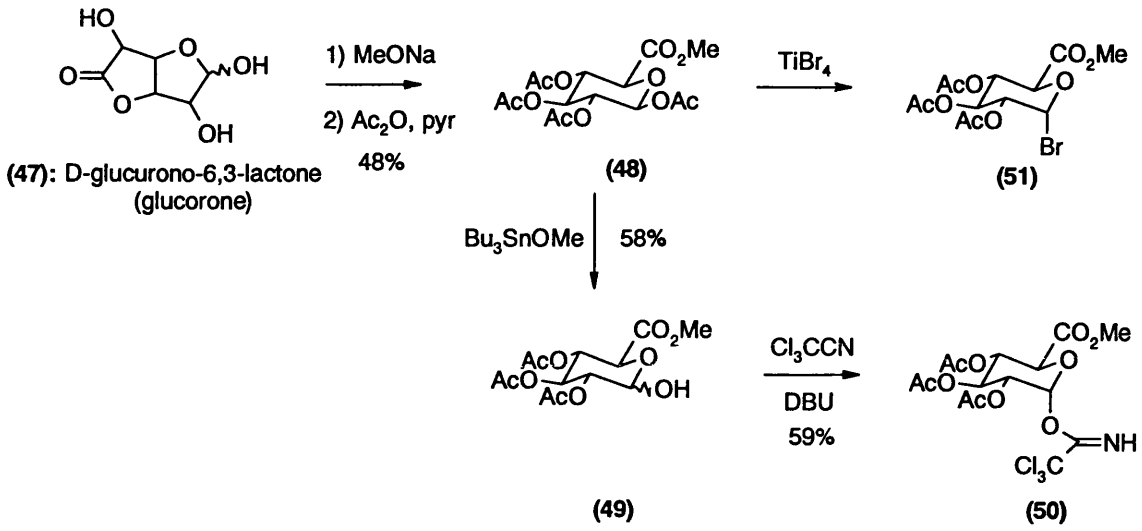
Treatment of the glucuronolactone (**47**) with sodium methoxide produces the glucuronic acid methyl ester, which can be acetylated with acetic anhydride (Bollenback et al., 1955; Leu et al., 1999) to give a mixture of α/β anomers (**48**). These epimers can be easily separated if required by kinetic recrystallisation. In some studies, the β-acetylated anomer (**48**) has been coupled directly to an aglycone moiety (Lahmann and Thiem, 1997; Stachulski and Jenkins, 1998).

From the tetra-acetate sugar (**48**) a number of more reactive intermediates can be made. The hydroxy sugar (**49**) can be prepared from the α/β protected sugar (**48**) using

tributyltin methoxide (Nudelman et al., 1987) and can either be coupled directly to an hydroxy aglycone using Mitsunobu conditions (Laurin et al., 1999) or can in turn be converted to the trichloroacetimidate intermediate **(50)** (Jacquinet, 1990; Brown et al., 1997). Glucuronidation with this intermediate **(50)** is particularly attractive because it has very high β-stereoselectivity and it requires relatively mild catalysis. It has been used in the synthesis of aryl glucuronides (Brown et al., 1997; Ferguson et al., 2000). The common catalysts used for trichloroacetimidate glucuronidation are $BF_3$-$Et_2O$ or more occasionally trifluoromethanesulphonate (TMSOTf) and the reaction is carried out at reduced temperature (Brown et al., 1997; Ferguson et al., 2000).

The other possible intermediate shown in the scheme is the bromo sugar **(51)**. This sugar derivative **(51)** is probably still the most popular glucuronidation intermediate and has been used in the synthesis of both alkyl and aryl glucuronides using the Koenigs-Knorr conditions (Bowering and Timell, 1960; Berrang et al., 1997). The β-bromo sugar **(51)** can be directly synthesised from the tetra-acetate sugar **(48)** using reagents such as titanium bromide or hydrogen bromide. The common catalysts used to couple the bromo sugar **(51)** with an hydroxy aglycone are Ag(I) salts such as $Ag_2O$ or $Ag_2CO_3$. Both the bromo **(51)** and trichloroacetimidate intermediates **(50)** are unstable and care must be taken to avoid exposure to air, heat and water. Storage under desiccation at -20°C is advised.

Once the appropriate intermediate has been coupled to the aglycone, the protected glucuronide can be saponified with sodium or potassium hydroxide in aqueous methanol. The resulting glucuronide can often be re-crystallised from ethanol (Brown et al., 1997).




**Scheme 5.22.** Synthesis of glucuronic acid donors

### 5.4.1.1. Synthesis of glucuronide donors

The glucuronide donors were synthesised as described above in the yields given in scheme 5.22. Briefly, the tetra-acetate sugar (**48**) was made from glucuronolactone (**47**) using sodium methoxide and acetic anhydride followed by two recrystallisations in ethanol in order to isolate the two anomers (**48**). The α-amomer (**48**) was then used in the synthesis of the β-bromo sugar (**51**) using titanium bromide. The residual mixture of anomers (**48**) from the second recrystallisation was used to synthesise the hydroxyl sugar (**49**) by treatment with tin methoxide. Finally the β-imidate intermediate (**50**) was synthesised from the hydroxyl sugar using trichloroacetonitrile and 1,8-Diazabicyclo-undec-7-ene (DBU) (Jacquinet, 1990). No major problems were encountered, except in the synthesis of the bromo sugar (**51**). Attempts at purification were made but the bromo

sugar (**51**) could not be characterised, possibly because it decomposed during the purification procedure. The compound produced was therefore coupled directly to the aglycone without prior purification.

The usefulness and efficiency of the various glucuronide donors were then assessed on a model compound in order to optimise the procedure prior to glucuronidation of the α-tocopherol metabolites.

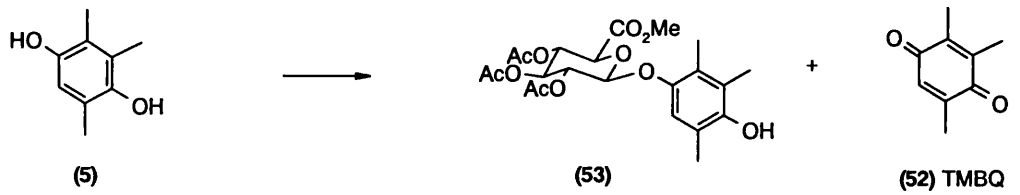
**5.4.1.2. Glucuronidation of trimethylhydroquinone (TMHQ) as a model compound**

Trimethylhydroquinone (TMHQ) (**5**) was chosen as an appropriate model compound because of its similar structure to both α-tocopheronolactone (**38**) and α-CEHC (**9**). Owing to the poor yields obtained using the tetra acetate sugar (**48**), as reported in earlier studies (Yoshioka et al., 1991), initial attempts were made using the hydroxyl sugar (**49**). Coupling of the hydroxyl sugar (**49**) with TMHQ (**5**) using Mitsunobu conditions (Tributylphosphine ($Bu_3P$)/ 1,1'-(azodicarbonyl)dipiperidine (ADDP) in THF, at room temperature (rt) overnight) was tried. However TLC analysis indicated that little or no product was formed and TMHQ was present solely in the oxidised trimethyl benzoquinone form (**52**) (TMBQ).

Glucuronidation using the bromo sugar (**51**) was then attempted. The bromo sugar (**51**) was synthesised and then coupled directly to TMHQ (**5**), without purification. The Koenigs-Knorr method was used and both silver oxide and silver carbonate were tried as catalysts (Berrang et al., 1997; Stachulski and Jenkins, 1998). The TLC data obtained was very similar to that for the Mitsunobu coupling above, with very little or no product spots appearing and the major spot corresponding to trimethyl benzoquinone

(52) (Scheme 5.23). The failure of both these methods, using the hydroxyl (49) and bromo sugar (51), was probably due to the facile oxidation of TMHQ (5) leading to formation of the unconjugatable benzoquinone (52) (Scheme 5.23).

Lahmann and Thiem (1997) suggested that one of the main problems in the synthesis of α-tocopheryl oligosaccharides is the easy oxidation of α-tocopherol to the corresponding open chain quinone and other oxidation products. They, therefore, suggested that methods such as the Koenigs-Knorr glycosylation employing silver salts were not suitable for tocopherol type compounds. In contrast, they successfully used a combination of trichloroacetamide (50) and boron trifluoride diethyl etherate since this did not oxidise tocopherol (Lahmann and Thiem, 1997). Consequently, further attempts at glucuronidation of the model compound were performed employing the imidate (50).



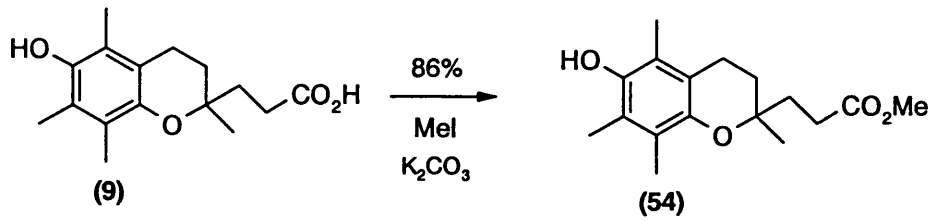

| substrate | glycosyl donor | conditions | products |
|---|---|---|---|
| (5) | (49) | ADDP/$Bu_3P$ (Mitsunobu) | (52) |
| (5) | (51) | $Ag_2CO_3$ (Koenigs-Knorr) | (52) |
| (5) | (50) | $BF_3$. $Et_2O$ | (53), 40 % |

**Scheme 5.23.** Synthesis of TMHQ-glucuronide with various glucuronic acid intermediates

One molar equivalent of the imidate intermediate (**50**) was coupled to TMHQ (**5**) using $BF_3$-$Et_2O$ in dichloromethane at -15°C. The temperature was allowed to rise to room temperature and the reaction left overnight before ethyl acetate extraction and silica gel chromatography. A number of spots were seen on TLC, some of which were probably by-products resulting from the breakdown of the glucuronide donor. Flash chromatography afforded a number of fractions, one of which was shown to be the acyl-TMHQ glucuronide (**53**) by NMR and EI-MS. The benzoquinone (**52**) spot was still present but was not as intense as it had appeared in the previous attempts at glucuronidation. The success of this method in comparison to the other methods using the bromo (**51**) or hydroxyl (**49**) sugars is probably due to the milder catalysis, which prevents complete oxidation of the hydroquinone (**5**) (Scheme 5.23). It is difficult to determine exactly how much oxidation of the aglycone occurs in the reaction mixture, since oxidation also occurs on the silica of the TLC plate and the flash chromatography column. However if the TMHQ (**5**) is dissolved in dichloromethane and the solution spotted at various time periods, a spot corresponding to the hydroquinone is still seen on TLC even after 2 days, whereas no compound (**5**) is seen on TLC after 1 day in the reaction mixture. This suggests that oxidation still occurs in the reaction mixture even under relatively mild catalysis. However, the rate of oxidation is reduced sufficiently to allow partial glucuronidation of the aglycone (**5**). Using this imidate/boron trifluoride method, TMHQ glucuronide (**53**) was produced in reasonable yields (>40%). It was, therefore, decided to use these conditions for the glucuronidation of the α-tocopherol metabolites.

### 5.4.1.3. Synthesis of α-CEHC glucuronide (56)

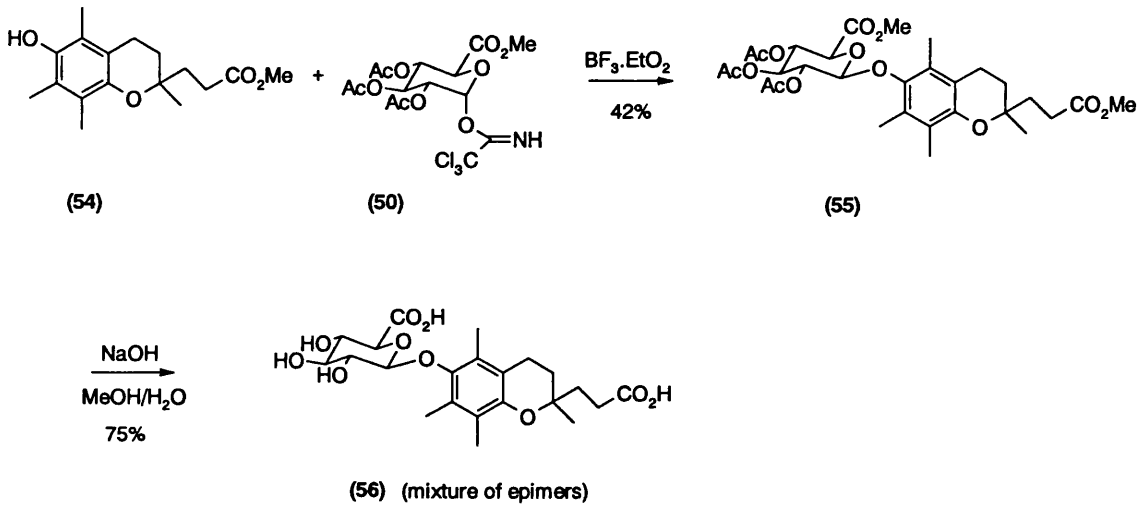
The methyl ester of α-CEHC (**54**) was used in the glucuronidation step in case the carboxyl group interfered with the reaction. The ester (**54**) was synthesised from α-CEHC by treatment with methyl iodide and potassium carbonate (Scheme 5.24). The use of α-CEHC methyl ester (**54**) does not require any additional steps since the final saponification removes the methyl ester along with the other protecting groups on the sugar moiety.




**Scheme 5.24.** Esterification of α-CEHC

The methyl ester of α-CEHC was treated with one molar equivalent of trichloroacetimidate (**50**) and the reaction was allowed to proceed overnight at room temperature (Scheme 5.25). The newly formed protected glucuronide (**55**) was observed on TLC (ethyl acetate/cyclohexane, 50%) as a spot with $R_f$ of ~0.35, which was a similar $R_f$ to that of the protected glucuronide of the TMHQ model compound (**53**). This spot was purified by flash chromatography on silica gel. The appropriate fractions were concentrated and NMR analysis showed that α-CEHC methyl ester glucuronide (**55**) had been produced, although double peaks in $^{13}C$ NMR indicated the presence of a mixture of epimers. Saponification of the protected glucuronide (**55**) by refluxing with

sodium hydroxide in aqueous methanol afforded α-CEHC glucuronide (**56**) (Scheme 5.25).



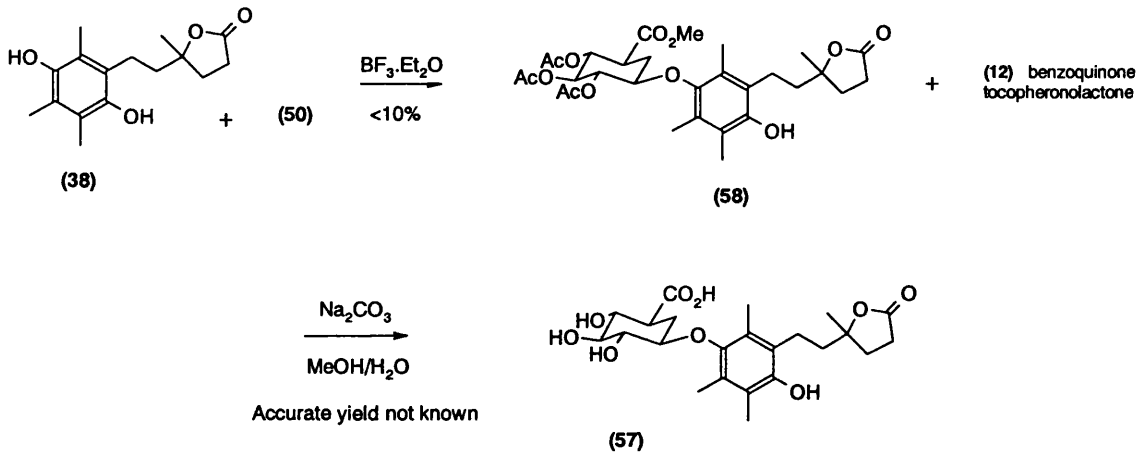

**Scheme 5.25.** Synthesis of α-CEHC-glucuronide using imidate intermediate

The diastereoisomers observed in $^{13}C$ NMR are most likely to be due to differences at the C-2 position of the chroman ring since it has been reported that the imidate coupling is stereoselective, producing only the β-glucuronide (Stachulski and Jenkins, 1998). Despite the presence of a mixture of epimers this synthetic α-CEHC glucuronide was appropriate for use as a standard as shown chapter 6.

### 5.4.1.4. Synthesis of α-tocopheronolactone glucuronide (57)

The glucuronidation of α-tocopheronolactone (**38**) was found to be more problematic than that of α-CEHC (**9**). The imidate method, initially produced only small amounts of protected tocopheronolactone glucuronide (**58**). It was also difficult to separate this

compound from a sugar derivative, which was presumably a breakdown product of the unstable imidate (**50**). These problems probably resulted in part from the oxidation of the hydroquinone to the benzoquinone lactone (**12**) (Scheme 5.16). Over 80% of the oxidised starting material (**12**) could be recovered after flash chromatography but it could not be ascertained whether the oxidation occurred during the reaction or on the silica gel (see section 5.3.2.). In order to improve the yield it was found necessary to use freshly prepared α-tocopheronolactone hydroquinone (**38**) because of the possible oxidation, which could have occurred during storage. The use of freshly prepared hydroquinone (**38**) resulted in the isolation of small amounts of protected α-tocopheronolactone glucuronide (**58**) (Scheme 5.26). Since one molar equivalent of imidate (**50**) was used, a mixture of monoglucuronides would be expected. However, the appearance of single, rather than double peaks in the NMR data, suggested that glucuronidation predominated at one position, although it is difficult to determine from the NMR data which of the two hydroxyl groups is glucuronidated.




**Scheme 5.26.** Synthesis of α-tocopheronolactone glucuronide

In order to prevent hydrolysis of the lactone, saponification of the protected glucuronide (**58**) to the free α-tocopheronolactone glucuronide (**57**) was performed in the presence of sodium bicarbonate instead of the harsher sodium hydroxide treatment (Brown et al., 1997). ESI-MS indicated that the saponification was successful but the availability of only small amounts of product hindered full NMR characterisation.

The synthesis of α-CMBHC-glucuronide was not attempted.

### 5.4.2. Synthesis of sulphate conjugates

In comparison to glucuronidation there is relatively little information available on the synthesis of sulphated conjugates. There have, however, been a number of reviews on sulphate conjugation which proved useful in this study (Roy, 1981; Bergman, 1986; Kaspersen and Van Boeckel, 1987).

There are a number of methods for the synthesis of sulphate esters but the most straightforward are those using commercially available sulphur trioxide complexes. These include dimethylformamide- $SO_3$ (DMF-$SO_3$), pyridine-$SO_3$ and trimethylamine-$SO_3$. All the sulphating reagents are hygroscopic and extra care is required to maintain strict anhydrous conditions during the reaction. Sulphation reactions are carried out in polar anhydrous organic solvents such as pyridine or DMF and generally at room temperature, although higher temperatures are used if there are a number of possible sulphation sites or the sulphation site is hindered. In general, sulphates are easily hydrolysed, especially aryl sulphates, and are most stable when isolated as their salts. The hydrolysis reaction can take place regardless of the pH, but acidic conditions accelerate this reaction. The stability of a sulphate conjugate generally decreases as the

phenol to which the sulphate group is attached increases in acidity i.e. as the phenolate resulting from the cleavage of the sulphate ester becomes more stable (Roy, 1981).

One of the main problems in the production of sulphate conjugates lies in their detection and subsequent purification as a result of their high polarity. Sulphate conjugates are insoluble in most common organic solvents and because of this property they are difficult to purify by routine procedures such as aqueous/organic extractions or flash chromatography on silica gel. Therefore, it was important to devise suitable methods to monitor the progress of the sulphation reactions and to purify the products.

### 5.4.2.1 Preliminary experiments on sulphation reactions

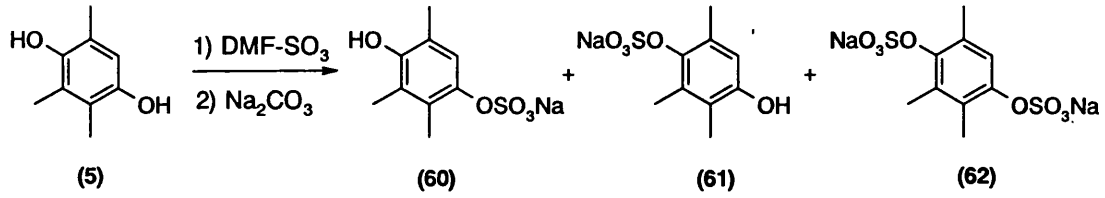
The DMF-$SO_3$ complex was initially chosen as the sulphating agent as it had been used in a recent study to synthesise the neuroactive glyconucleoside disulphate, HF-7 (McCormick et al., 1999). Initial experiments on sulphation were carried out on α-CEHC (**9**) in dimethylformamide at room temperature. TLC analysis (20% methanol in chloroform) indicated that after three hours a new compound, presumably CEHC sulphate (**59**), was formed. HPLC was used to purify the crude reaction mixture after concentration and resolubilisation in methanol. The earliest eluting peak, which presumably corresponded to the polar α-CEHC sulphate (**59**), accounted for only a small percentage of the chromatogram, whereas the sulphate was responsible for an intense spot on TLC. This indicated that either the sulphated α-CEHC (**59**) had been lost during the purification procedure or it had degraded. Following HPLC, the major peak from the sulphation reaction eluted at 10-11 min and was initially presumed to be α-CEHC. However, EI-MS data indicated that the major peak was the methyl ester of α-CEHC (**54**). This suggested that the acidity of the sulphate conjugates caused not only

their own hydrolysis, but also catalysed their esterification as a result of the use of methanol. The problems encountered with α-CEHC (**9**) led to the use of a model compound to optimise the conditions necessary for efficient sulphation.

### 5.4.2.2 Studies on sulphation of TMHQ as a model compound

TMHQ (**5**) and DMHQ (**6**) (Scheme 5.3) were chosen as model compounds in order to determine the effect that methyl groups on the aromatic ring may have on the rate of sulphation. Sulphation of TMHQ (**5**) using the DMF-$SO_3$ complex proceeded at a faster rate than that of DMHQ, as judged by TLC. This suggested that the extra methyl group, which enhances the nucleophilicity of the hydroxy groups, actually encouraged the reaction rather than causing steric hindrance.

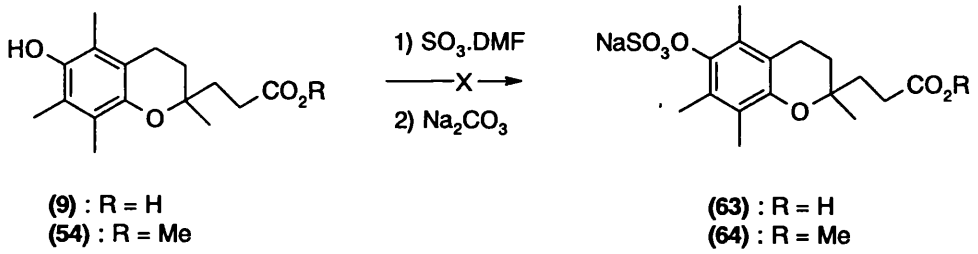
Attempts were then made to prevent the autocatalysed cleavage of the sulphate group, which was observed in the case of α-CEHC (**9**). As mentioned above, the sulphate salts are more stable than their acid forms (Roy, 1981). The TMHQ reaction mixture was therefore treated with anhydrous sodium bicarbonate salt (McCormick et al., 1999), filtered and concentrated to yield the sulphates as their sodium salts. Although some decomposition still occurred, this procedure proved to work reasonably well and allowed the collection of two fractions on HPLC. ESI-MS of these fractions showed that they both contained monosulphate compounds while NMR spectra showed slightly different chemical shifts for the two fractions indicating that they were sulphated on different hydroxyl groups. The fractions correspond to the compounds (**60**) and (**61**). Increasing the amount of sulphating agent and the temperature of the reaction to 60°C facilitated the production of the disulphate (**62**) (Scheme 5.27).




**Scheme 5.27.** Sulphation of TMHQ with DMF-$SO_3$ complex

### 5.4.2.3. Experiments on the sulphation of α-tocopherol metabolites

α-CEHC (**9**) and its methyl ester (**54**) were sulphated using the procedure described above for the model compound (Scheme 5.28). However, HPLC purification of the crude compounds failed to produce sufficient amounts of the sulphated sodium salts (**63**) and (**64**) for full characterisation. Interestingly, the HPLC peaks corresponding to the sulphate derivatives were larger when a mini-preparation of an aliquot of the reaction mixture was carried out than when the whole reaction sample was used. This suggested that concentrated solutions of sulphates may not be compatible with the filters used on the HPLC system.

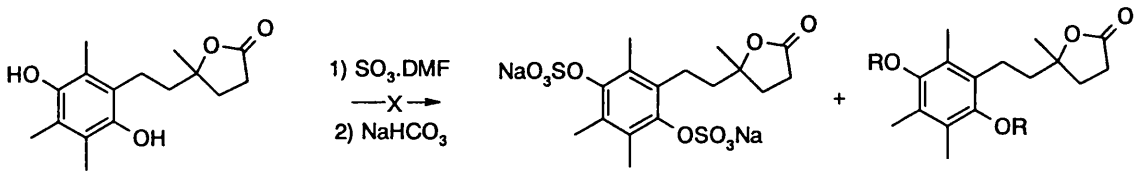



**(9)** : R = H
**(54)** : R = Me

**(63)** : R = H
**(64)** : R = Me

**Scheme 5.28.** Sulphation of α-CEHC and α-CEHC-Me using DMF-$SO_3$ complex

Sulphation of the hydroquinone α-tocopheronolactone **(38)** also produced only small amounts of sulphated conjugates (**65** and **66**) after purification (Scheme 5.29), although TLC and MS-MS analyses confirmed that sulphated derivatives were formed. However preparative scale HPLC could only purify small quantities suggesting that the sulphated compounds were either lost or decomposed during purification. The decomposition of the sulphate derivatives could have occurred for a number of reasons such as the presence of the acid form of the sulphates, traces of trifluoroacetic acid in the HPLC solvent system, the solvents themselves or the filters used on the HPLC system.




**(38)**

**(65)**

**(66)** R = H or $SO_3Na$

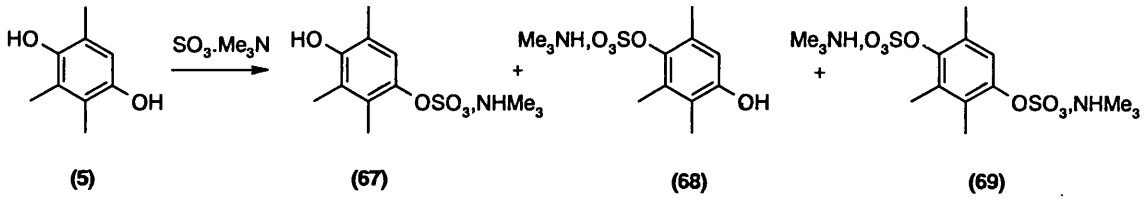
**Scheme 5.29.** Sulphation of α-tocopheronolactone using DMF-$SO_3$ complex

These possibilities were investigated and it appeared that sodium bicarbonate was not forming the sodium salts of the sulphates as expected. The sulphates of the model

compound could be isolated in larger quantities because the reaction was performed on a larger scale owing to the availability of starting materials. The sodium salts were probably only partially formed because of the lack of solubility of the sodium bicarbonate salt in DMF. These experiments led to the suggestion that other sulphating agents such as triethylamine or trimethylamine-$SO_3$ and pyridine-$SO_3$ should be tried. The trimethylamine-$SO_3$ complex is considered particularly useful since it produces sulphates as their trimethylamine salts, which are reportedly stable, soluble in organic solvents such as dichloromethane and can be readily crystallised by the addition of diethyl ether (Dusza et al., 1968).

### 5.4.2.4. Synthesis of TMHQ sulphate (67/68) using trimethylamine-$SO_3$ complex

The trimethylamine salt of TMHQ-$SO_3$ **(67/68)** was prepared relatively easily using trimethylamine-$SO_3$ complex in pyridine at room temperature and the salts were precipitated by the addition of diethyl ether and then filtered. The precipitate was not particularly soluble in dichloromethane so a few drops of methanol were added to ease the resolubilisation. The sulphated derivatives were purified on a very short pre-packed silica gel column, eluting with a 10% to 20% gradient of MeOH/$CHCl_3$. NMR analysis showed a mixture of the two monosulphates **(67)** and **(68)** to be present (Scheme 5.30), as indicated by changes of chemical shift for both aromatic and methyl carbons. Increasing the reaction temperature and the amount of sulphating reagent allowed the disulphate **(69)** to be synthesised. TMHQ sulphates **(67/68)** were produced in good yields (>40%) and the products (trimethylamine salts) appeared to be much more stable than the previously isolated acids or sodium salts. This method was then applied to the sulphation of the α-tocopherol metabolites.



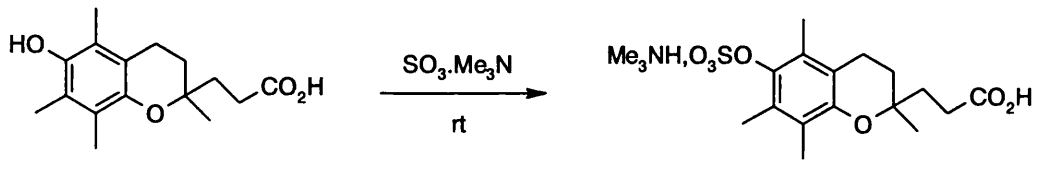

(5) (67) (68) (69)

**Scheme 5.30.** Sulphation of the model compound (**5**) at room temperature (products **67** and **68**) or 60°C (product **69**)

### 5.4.2.5. Synthesis of α-CEHC sulphate (70) and α-CMBHC sulphate using trimethylamine-$SO_3$ complex

α-CEHC sulphate (**70**) was made and purified in the same way as the sulphate of the TMHQ model compound (**67/68**) using 1.1 molar equivalents of trimethylamine-$SO_3$ complex to α-CEHC (Scheme 5.31). The sulphating reagent proved to be difficult to remove from the product but had little effect on the characterisation of the sulphated product (**70**). This should not pose any problems in the use of the product for qualitative purposes.

α-CMBHC sulphate was synthesised in an identical manner to α-CEHC sulphate (**70**). The small quantities of α-CMBHC sulphate meant it could not be fully characterised by NMR but MS data showed that the sulphated derivative was produced.

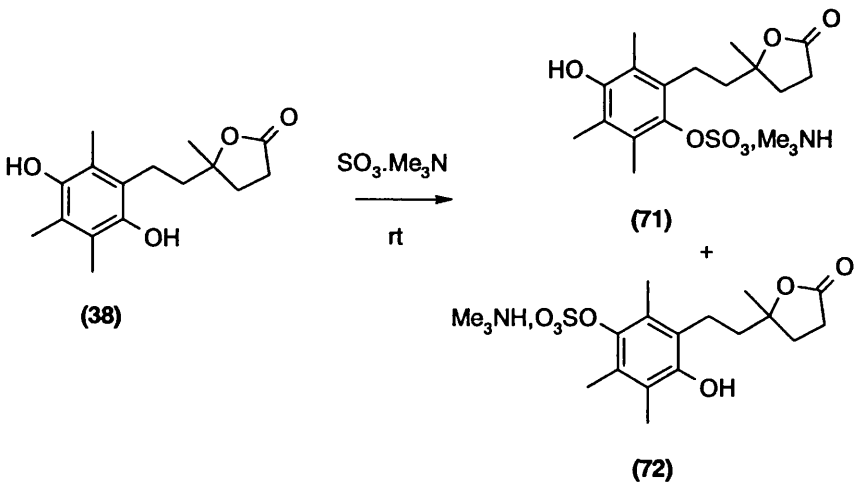



(9) (70)

**Scheme 5.31.** Sulphation of α-CEHC using trimethylamine-$SO_3$ complex

### 5.4.2.6. Synthesis of α-tocopheronolactone sulphate (71/72) using trimethlyamine-$SO_3$ complex

The sulphation of α-tocopheronolactone hydroquinone (**38**), however, caused some problems. The oxidation of hydroquinone (**38**) to benzoquinone (**12**), as encountered in the glucuronidation of α-tocopheronolactone, was probably the principal problem. However, using freshly made hydroquinone α-tocopheronolactone (**38**), small amounts of α-tocopheronolactone sulphate (**71/72**) were synthesised and purified (Scheme 5.32). As for α-tocopheronolactone glucuronide, the lack of sufficient material meant full NMR characterisation could not be performed. However, MS data indicated that only mono-sulphate conjugates were produced and the $^1H$ NMR data indicated that both mono-sulphates (**71** and **72**) were present in similar amounts.




**Scheme 5.32.** Sulphation of α-tocopheronolactone using trimethylamine-$SO_3$ complex

## 5.5. Confirmation of identity of conjugated standards

The identity of each standard was checked using a variety of techniques, including $^{1}H$ and $^{13}C$ NMR, MS, HPLC and IR spectroscopy. This data is given along with other experimental details in the appendix. The identity of the conjugated standards was also checked by GC-MS of the deconjugated compounds following enzymatic deconjugation as detailed below.

*Enzymatic deconjugation and GC-MS:* Approximately 20 μg of the conjugated standards, α-CEHC-glucuronide **(56)**, α-CEHC-sulphate **(70)**, α-CMBHC-sulphate, α-tocopheronolactone-glucuronide **(57)** and α-tocopheronolactone-sulphate **(71/72)**, synthesised as described above, were individually dissolved in 1 ml of de-ionised water. 50 μl sodium acetate (5.0 M, pH 4.7) and 25 μl of β-glucuronidase/sulphatase (type HP-2, G7017, Sigma-Aldrich Company, Ltd.) were then added to each sample. After 18 h at 37°C, the sample was extracted using a C4 solid phase extraction (SPE) cartridge, TMS-derivatised and analysed by GC-MS as described in chapter 2. The resulting gas chromatograms and electron impact (EI) spectra were used to confirm the presence of the appropriate unconjugated compounds i.e. α-CEHC, α-CMBHC or α-tocopheronolactone.

After enzymatic deconjugation, GC-MS revealed that α-tocopheronolactone conjugates produced only α-tocopheronolactone hydroquinone while α-CEHC and α-CMBHC conjugates produced principally α-CEHC and α-CMBHC, respectively, with some α-tocopheronolactone or trace amounts of oxidised α-CMBHC (Figure 5.1). The α-tocopheronolactone and oxidised α-CMBHC observed from the α-CEHC or α-CMBHC

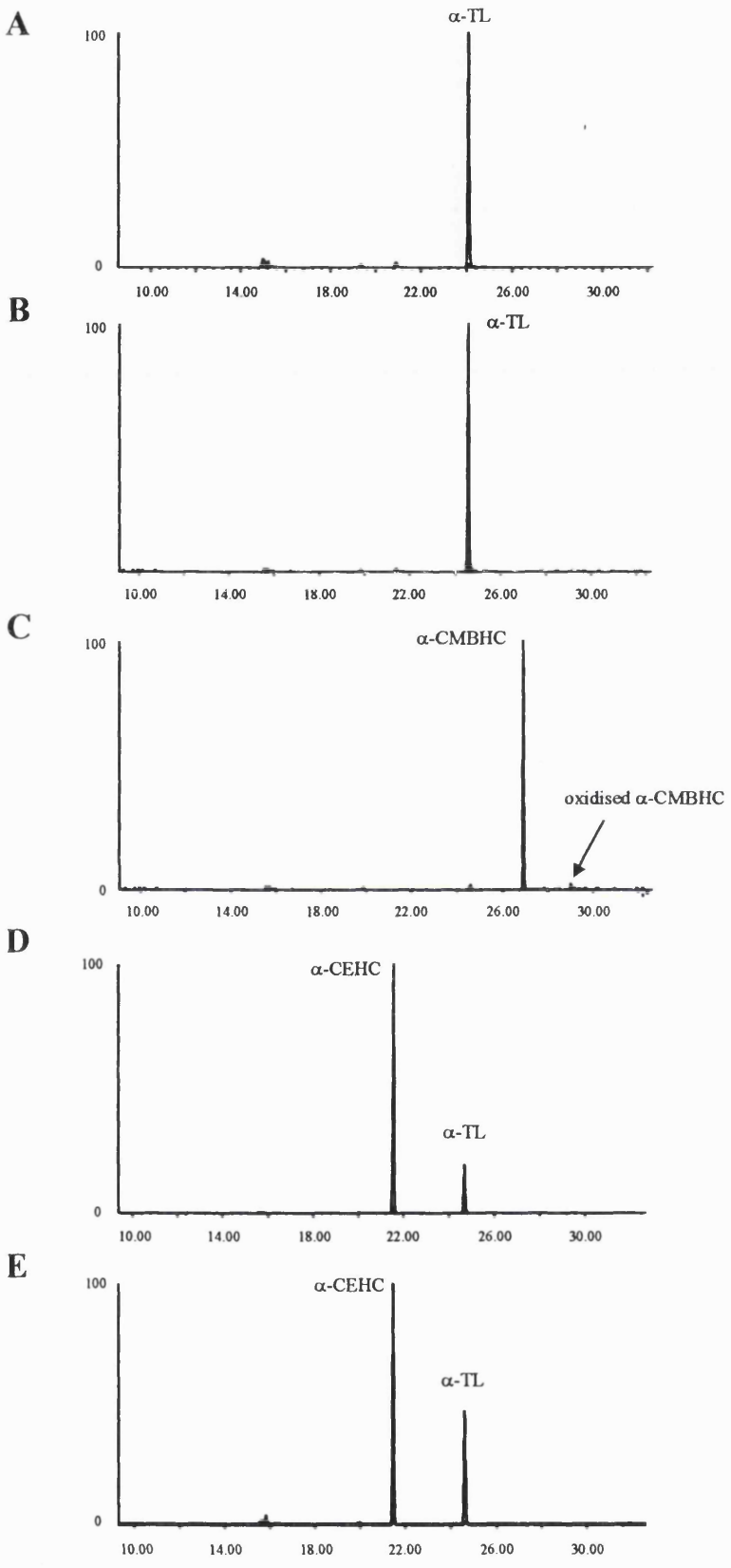



**Figure 5.1.** GC-MS of synthetic conjugated standards after enzymatic deconjugation. **A** α-tocopheronolactone-glucuronide, **B** α-tocopheronolactone-sulphate, **C** α-CMBHC-sulphate, **D** α-CEHC-glucuronide and **E** α-CEHC-sulphate.

conjugate samples were probably formed by artefactual oxidation of α-CEHC and α-CMBHC during enzymatic deconjugation or the subsequent extraction and derivatisation steps, since NMR data of the α-CEHC conjugate standards had not revealed the presence of any α-tocopheronolactone.

### 5.6. Discussion and conclusions

In general, the synthesis of the unconjugated metabolites proceeded well and the only major problem encountered was the facile oxidation of the hydroquinone α-tocopheronolactone **(38)** to the benzoquinone form on silica gel, which hindered TLC analysis and purification of the product. However once this problem was identified, the synthesis of the hydroquinone **(38)** proved relatively straightforward. The facile oxidation of the hydroquinone **(38)** raises interesting questions about what occurs *in vivo* and as an artefact during the extraction of metabolites from urine. In the current GC-MS method for the analysis of vitamin E metabolites, α-tocopheronolactone is detected mainly as the hydroquinone **(38)**. The artefactual oxidation of α-CEHC, referred to in the literature (Schultz et al., 1995), would be expected to form the benzoquinone **(12)**, suggesting that in our method either the hydroquinone is produced *in vivo* or by reduction at some point in the extraction procedure after initial artefactual oxidation. Since the deuterated α-CEHC standard, which was added to urine before extraction, produced deuterated hydroquinone **(38)** it seems that the latter possibility is most likely. It would be interesting to use the synthetic unconjugated standards to study these subtle oxidation/reduction reactions that occur in the urine and during extraction. This would help to elucidate the 'real' metabolites and may also provide some insight as to whether these subtle redox reactions have some regulatory role *in vivo*.

The synthesis of the conjugated metabolites proved more difficult than that of the unconjugated metabolites mainly because of the instability of the starting materials and products and the problems faced in the purification of such polar compounds. Initial attempts illustrated the lability of sulphated derivatives if isolated as their acids, whereas the trimethylamine salts were relatively stable and easier to purify. α-CEHC conjugates were easier to synthesise than the corresponding α-tocopheronolactone conjugates owing to the facile conversion of the hydroquinone to the benzoquinone.

Eventually, unconjugated and mono-sulphate and mono-glucuronide conjugates of both α-CEHC and α-tocopheronolactone and unconjugated and mono-sulphated α-CMBHC were successfully synthesised. The chemical synthesis of these compounds has provided an insight into the chemistry of the individual vitamin E metabolites and will also be useful in further studies to characterise the conjugated metabolites in urine and to study the possible interconversions, which occur *in vivo* and *in vitro*.

# CHAPTER 6

## The Use of Electrospray Tandem Mass Spectrometry for the Analysis of Conjugated Vitamin E Metabolites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## 6.1. Introduction

The studies described in chapter 4, using solid phase extraction and GC-MS to measure vitamin E metabolites in human and rat urine, indicated that there were a number of problems with this method, particularly if the ratio of α-tocopheronolactone to α-CEHC was to be measured accurately. Firstly, the facile artefactual conversion of α-CEHC to α-tocopheronolactone raised doubts about the authenticity of α-tocopheronolactone as an *in vivo* metabolite. Secondly, comparison of acid versus enzymatic deconjugation of α-tocopherol metabolites in rat urine revealed that only a small fraction of the metabolites was released by the sulphatase/β-glucuronidase enzyme preparation. Both of these problems resulted from the necessity to deconjugate the metabolites before GC-MS analysis. A method that allowed the direct measurement of conjugated metabolites, without prior deconjugation or derivatisation, would be superior, as it would reduce the length of the procedure, eliminate the chance of artefactual oxidation and remove variability resulting from differences in efficiency of deconjugation.

In this pilot study, electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) was used to analyse conjugated vitamin E metabolites in human urine, with particular emphasis placed on conjugated α-CEHC and α-tocopheronolactone. The standards synthesised in chapter 5 were used to confirm the identity of the metabolites observed.

## 6.2. Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) is a rapid and sensitive method that is routinely used to permit the identification of polar metabolites in biological samples with minimal sample manipulation (Griffiths et al., 2001).

The basic principles of mass spectrometry and the usefulness of tandem mass spectrometry in identifying and characterising conjugated metabolites are discussed below.

### 6.2.1. Basic principles of mass spectrometry

Mass spectrometers are essentially composed of three parts:

(i) an ion source,

(ii) a mass analyser,

(iii) a detector.

In order to obtain a mass spectrum, ions must be produced in the gas phase. These ions are then accelerated to a specific velocity using electric fields and projected into a mass analyser that separates the ions according to mass. Finally, each charged particle of a particular mass is detected sequentially in time (Figure 6.1).

The appearance of the mass spectra obtained is dependent largely on the ionisation method used. So-called soft or low energy ionisation methods such as electrospray or matrix assisted laser desorption, produce simple spectra which contain peaks corresponding mainly to the masses of molecular ions. In contrast, high energy ionisation techniques, such as electron impact, produce complicated spectra due to the large number of fragment ions produced.

### 6.2.2. Electrospray ionisation (ESI)

High-energy ionisation techniques, such as electron impact (EI), cause decomposition of thermally labile biomolecules, making such techniques unsuitable for the analysis of underivatised biological samples. In contrast, electrospray ionisation (ESI) is able to

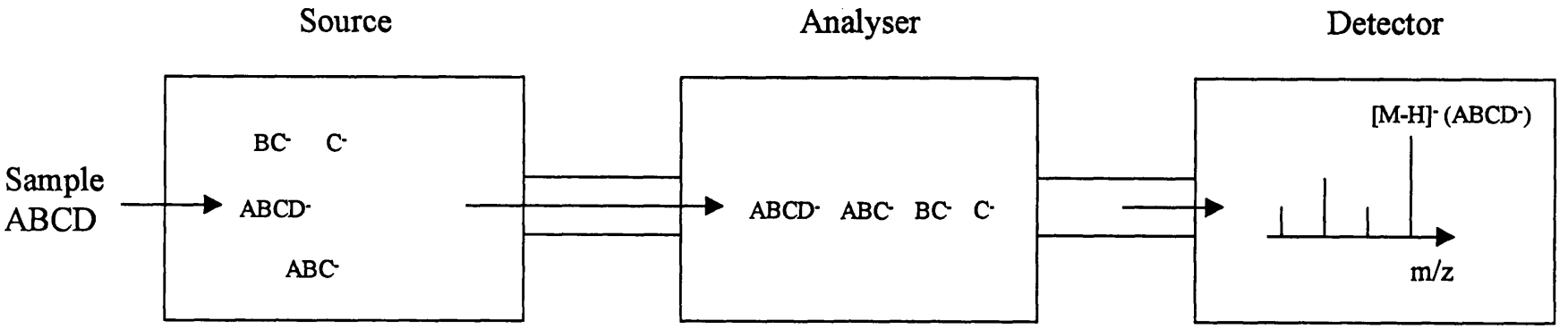


**Figure 6.1.** Diagram of a mass spectrometer. The compound (ABCD) is introduced into the source and ionised; in this case by removal of a proton. During ionisation, some decomposition of the parent compound occurs, producing fragments such as $BC^-$. The ions produced are then passed into the mass analyser, which uses either magnetic and/or electric fields to separate the different ions by their charge-to-mass ratio (m/z). These ions are then sequentially detected in time, producing a mass spectrum. The major or base peak of the spectrum is, in this case, the molecular ion $[M-H]^-$ or $ABCD^-$.

extract non-volatile, thermally labile biomolecules from solutions intact, ionise them and transfer them into the gaseous phase where they may be subjected to mass analysis.

ESI was originally described over 30 years ago (Dole et al., 1968) but the first description of the technique coupled to MS was not until two decades later (Whitehouse et al., 1985). In conventional ESI, the sample is dissolved in a solution and then sprayed through a thin capillary needle, which is maintained at a high voltage (4 kV) (Figure 6.2). At the end of the needle the solution disperses into a mist of highly charged droplets containing the analyte molecules. ESI is able to produce both protonated and deprotonated molecules depending upon the capillary bias. As the charged droplets travel down a pressure and potential gradient towards an orifice in the mass spectrometer-high vacuum system, they desolvate and reduce in size aided by the application of dry gas and/or heat. The surface area of the droplet decreases until either the solvent is completely removed (Dole et al., 1968) or the charge density on the surface of the droplet reaches the so-called Raleigh limit and the ion is ejected (desorbed) (Iribarne and Thomson, 1976; Thomson and Iribarne, 1979). The ion can then be transported to the mass analyser.

An important feature of ESI, as with other soft ionisation methods, is the fact that it produces minimal fragmentation of the molecule of interest following ionisation, allowing its molecular mass to be determined. In certain situations e.g. quantitative mass spectrometry, this may be advantageous but it also has implications for the identification and structural characterisation of molecules within a complex biological sample owing to the isobaric* nature of many biological molecules. It is, therefore, often

* isobaric is used here to mean 'of equal mass'.

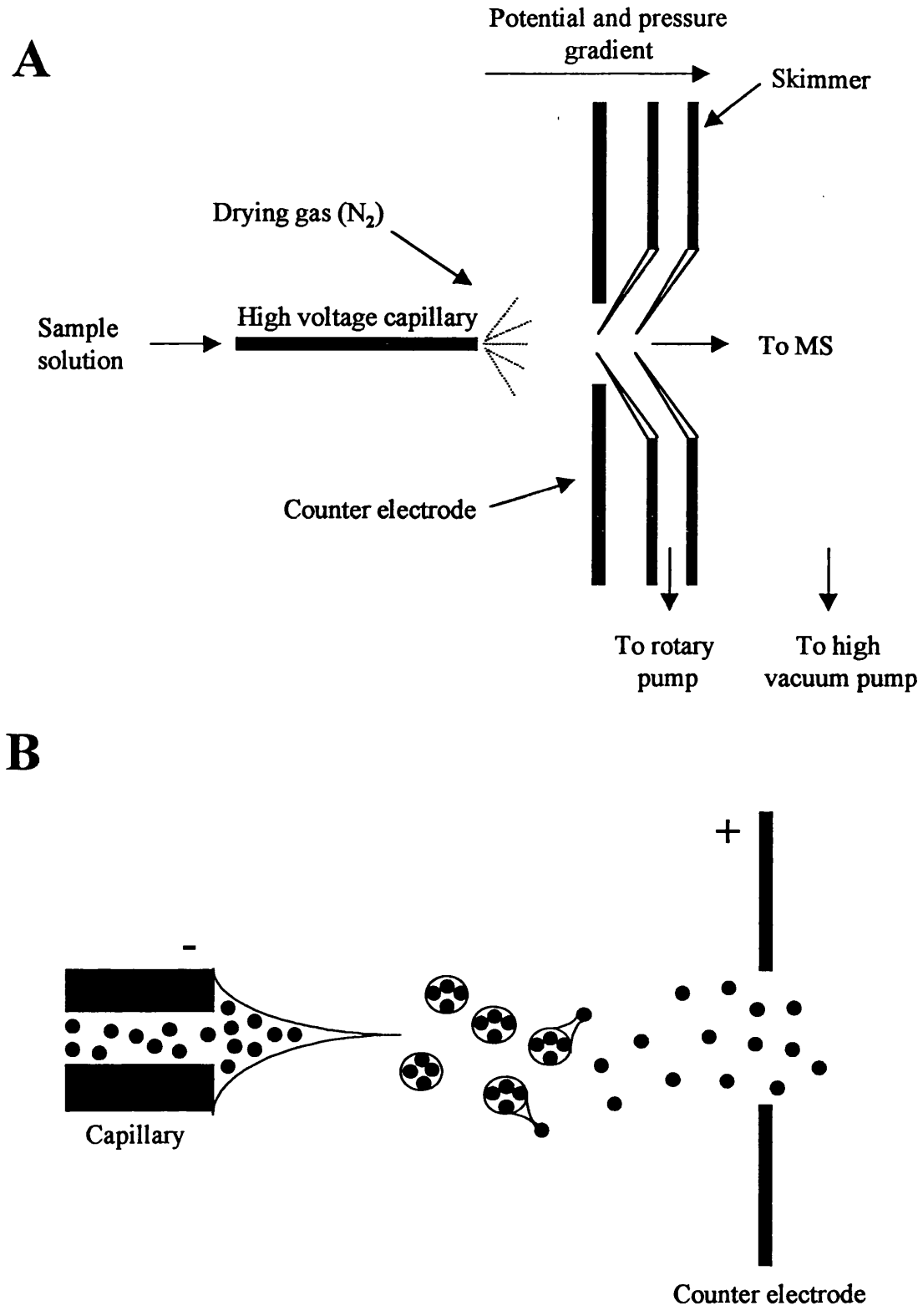


**Figure 6.2. (A)** Features of an ESI source and **(B)** schematic of the ionisation process in negative ion mode.
A high negative potential is applied to the capillary (cathode) and an aerosol of negatively charged droplets is emitted from the tip. Gas-phase ions are formed from the charged droplets through a series of solvent evaporation-Coulomb fission cycles, caused by the action of a drying gas as the charged droplets traverse the skimmers.

necessary to separate the different species present in a biological sample either prior to (e.g. liquid chromatography-mass spectrometry (LC-MS)) or after ionisation (e.g. tandem mass spectrometry (MS-MS))[#] in order to unambiguously identify individual components. In this study tandem mass spectrometry was used to separate and characterise the individual vitamin E metabolites extracted from urine.

### 6.2.3. Basic principles of tandem mass spectrometry

The most commonly used type of tandem mass spectrometer is the triple quadrupole instrument (QQQ), which consists of three quadrupole mass analysers coupled together in series. In a typical tandem mass spectrometric analysis (MS/MS) ions are selected by the first mass analyser (Q1) and focused into a collision region (Q2) preceding a second mass analyser (Q3) (Figure 6.3). The second or middle quadrupole (Q2) performs no mass analysis and serves as a higher pressure gas collision cell, which promotes fragmentation of the ions selected by Q1. The second mass analyser (Q3) detects the fragment ions produced in the collision cell. Therefore, a tandem mass spectrometer not only gives information about the molecular mass of individual components but also allows structural information to be obtained by studying characteristic fragment ions.

### 6.2.4. Possible configurations of a tandem mass spectrometer (Figure 6.3)

The basis of MS/MS is a process known as collision-induced dissociation (CID) (Wu, 2000; Oliveira and Watson, 2000). In this process, ions of a selected mass (precursor or parent ions) are transmitted by the first mass analyser (Q1) into the collision cell, Q2, where they collide with the neutral atoms of an inert gas (usually argon, helium or nitrogen). As a result of these collisions, the internal energy of the parent ion is

[#] Tandem mass spectrometry does not physically 'separate' isobaric components but it allows them to be detected individually due to specific fragmentations.

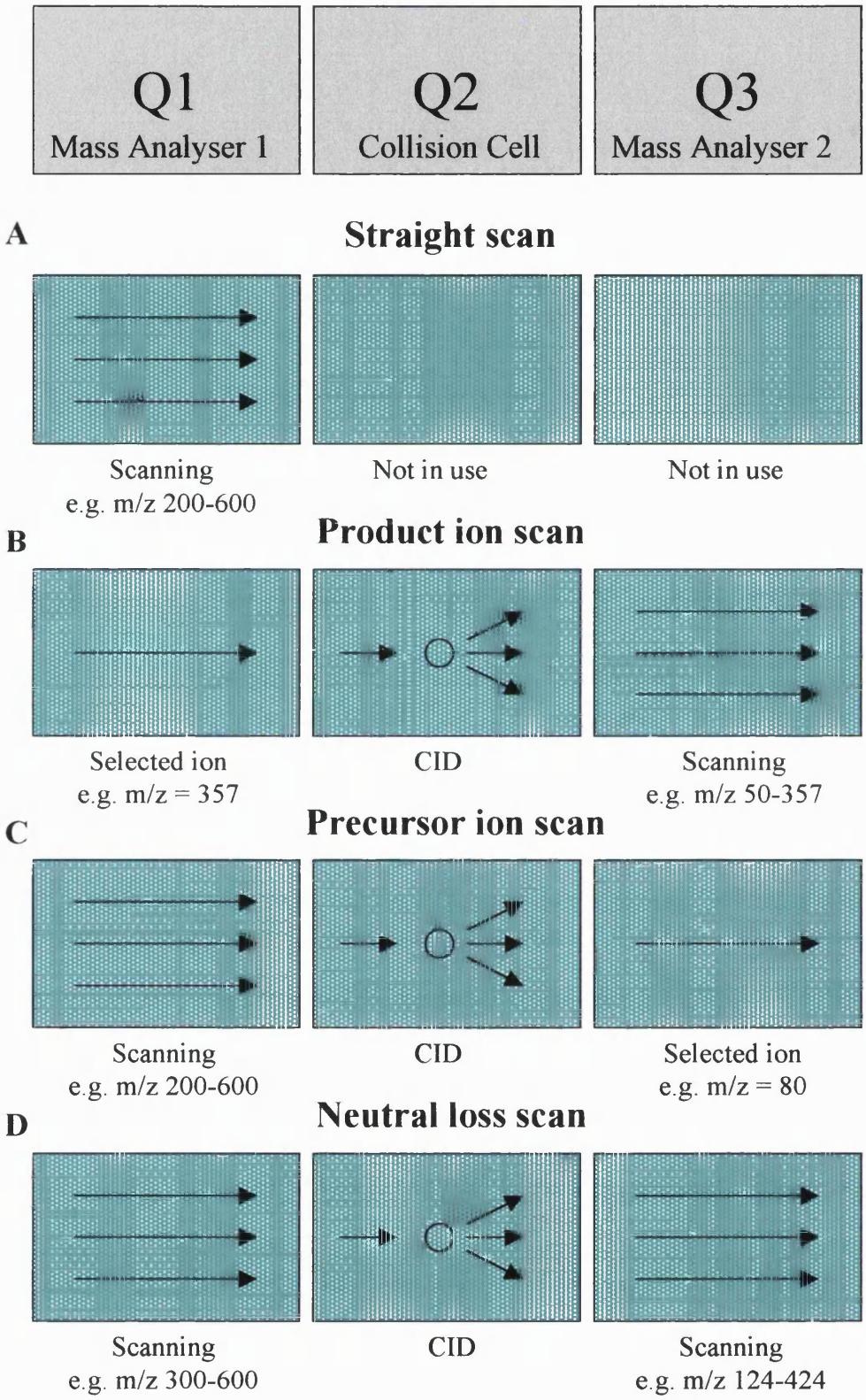


**Figure 6.3.** Examples of possible analysis using a triple quadrupole mass spectrometer. See text for details.
CID = Collision induced dissociation

increased causing the molecule to fragment. The subsequent fragments (product or daughter ions) are then analysed by the second mass analyser, Q3.

The first and second mass analysers (Q1 and Q3) can be operated in either fixed mass mode, whereby only ions of a certain mass are measured, or in scan mode, whereby ions of a range of masses are measured sequentially. By operating Q1 and Q3 in various combinations of fixed mass and scan mode, a number of different types of tandem mass spectrometric analyses are possible.

*Straight scan mode (Figure 6.3A)*

In order to produce a simple spectrum of all the species in a sample, the tandem mass spectrometer can be operated as a simple, single quadrupole mass spectrometer by utilising only the first mass analyser in scan mode. This analysis gives an overview of all the species in a sample and allows fine tuning of the mass spectrometer for further analysis using the collision cell (Q2).

*Product ion scan (Figure 6.3B)*

The simplest reaction in MS/MS is the dissociation of a selected precursor ion into product ions. This reaction can be monitored by selecting one particular ion (e.g. m/z 357) out of the ions generated in the ion source with Q1, transferring that ion to the collision cell and then analysing the product ions with Q3. This enables a characteristic fragment ion spectrum to be produced for each compound of interest.

*Precursor ion scan (Figure 6.3C)*

In a precursor ion scan, Q3 is fixed on one particular fragment ion produced by dissociation of precursor ions in the collision cell (Q2). Scanning of Q1, while Q3 is fixed, allows all precursor ions which fragment to give a specific product ion to be analysed.

A precursor ion scan can be used to detect groups of compounds that produce a common product ion. An example is the analysis of sulphated metabolites by the detection of the characteristic product ion, m/z 80. In this way, if Q3 is set to m/z 80 and Q1 is scanned, the resulting spectrum will display all sulphated metabolites in that sample.

*Neutral loss scan (Figure 6.3D)*

Many fragmentations in the collision cell produce neutral products that cannot be directly analysed by mass spectrometry owing to their lack of charge. However a neutral loss scan, where the two mass analysers, Q1 and Q3, scan simultaneously with a fixed mass difference between them allow neutral losses to be analysed.

An example of the use of a neutral loss scan is in the analysis of glucuronides, which typically fragment with a neutral loss of 176. If Q3 is set to scan 176 mass units below Q1 e.g. if Q1 scans from 300 to 600 and Q3 scans simultaneously between 124 and 424, the resulting spectrum will display all glucuronidated metabolites in the sample.

By combining the information obtained from these different types of tandem mass spectrometric analyses it is possible to identify and characterise even minor metabolites in urine.

In this study, tandem mass spectrometry was used to characterise conjugated metabolites of α-tocopherol in human urine after oral supplementation with deuterated and unlabelled α-tocopheryl acetate.

## 6.3. Materials and methods

*Mass spectrometry*

ESI-MS was performed using a Micromass Quattro 1 triple quadrupole mass spectrometer with an electrospray ionisation source and Masslynx data system (Micromass UK Ltd., Altrincham, UK). The instrument was operated in negative ionisation mode. A 20 μl sample was automatically injected (CMA200, Carnegie, Sweden) into the solvent stream of methanol/water 1:1 (v/v) flowing at a rate of 10 μl/min by means of an LC-9A HPLC pump (Shimadzu, UK). The capillary voltage was maintained at 3.95 kV. The cone voltage was set between 50-150V. The source temperature was held at a constant 80°C and nitrogen was used as the nebulising gas at a flow rate of 20 L/hour.

In collision mode, the collision voltage was set between 20-50 eV and argon was used as the collision gas at a pressure of $2.1 \times 10^{-3}$ mbar. Data was acquired using multiple channel acquisition (MCA) mode over a 2 minute period. Raw data spectra were processed by background subtraction and peak smoothing using the mass spectrometer's data system.

## 6.4. Results

### 6.4.1. Initial studies of conjugated urinary vitamin E metabolites

Urine samples from subjects given large oral doses of RRR-α-tocopheryl acetate were initially used for analysis by ESI-MS/MS. These samples contained high levels of vitamin E metabolites, which aided the detection of conjugated metabolites.

Indirect evidence indicated that vitamin E metabolites are excreted in the urine as sulphate and glucuronide conjugates (Chiku et al., 1984; Stahl et al., 1999; Swanson et al., 1999) (Section 1.6.2). When analysed by negative ion ESI-MS, sulphate and glucuronide conjugates typically give abundant molecular ions [M-H]⁻ and few fragment ions (Yang et al., 1997; Griffiths et al., 1999). The mass spectrometer was, therefore, initially operated in negative ion scan mode, without using the collision cell, in order to identify peaks corresponding to possible conjugated vitamin E metabolites.

Peaks at m/z 357 and 453 were observed, corresponding to the mass of mono-sulphate and mono-glucuronide conjugates respectively, of either α-CEHC or α-tocopheronolactone (Figure 6.4). The isobaric nature of α-CEHC and α-tocopheronolactone conjugates meant they could not be distinguished in simple scan mode. The peak corresponding to the glucuronide of α-CEHC/α-tocopheronolactone was of higher abundance than the corresponding sulphate conjugate, although this may not accurately reflect the relative amounts of the two species in urine because of differences in their ionisation efficiencies.

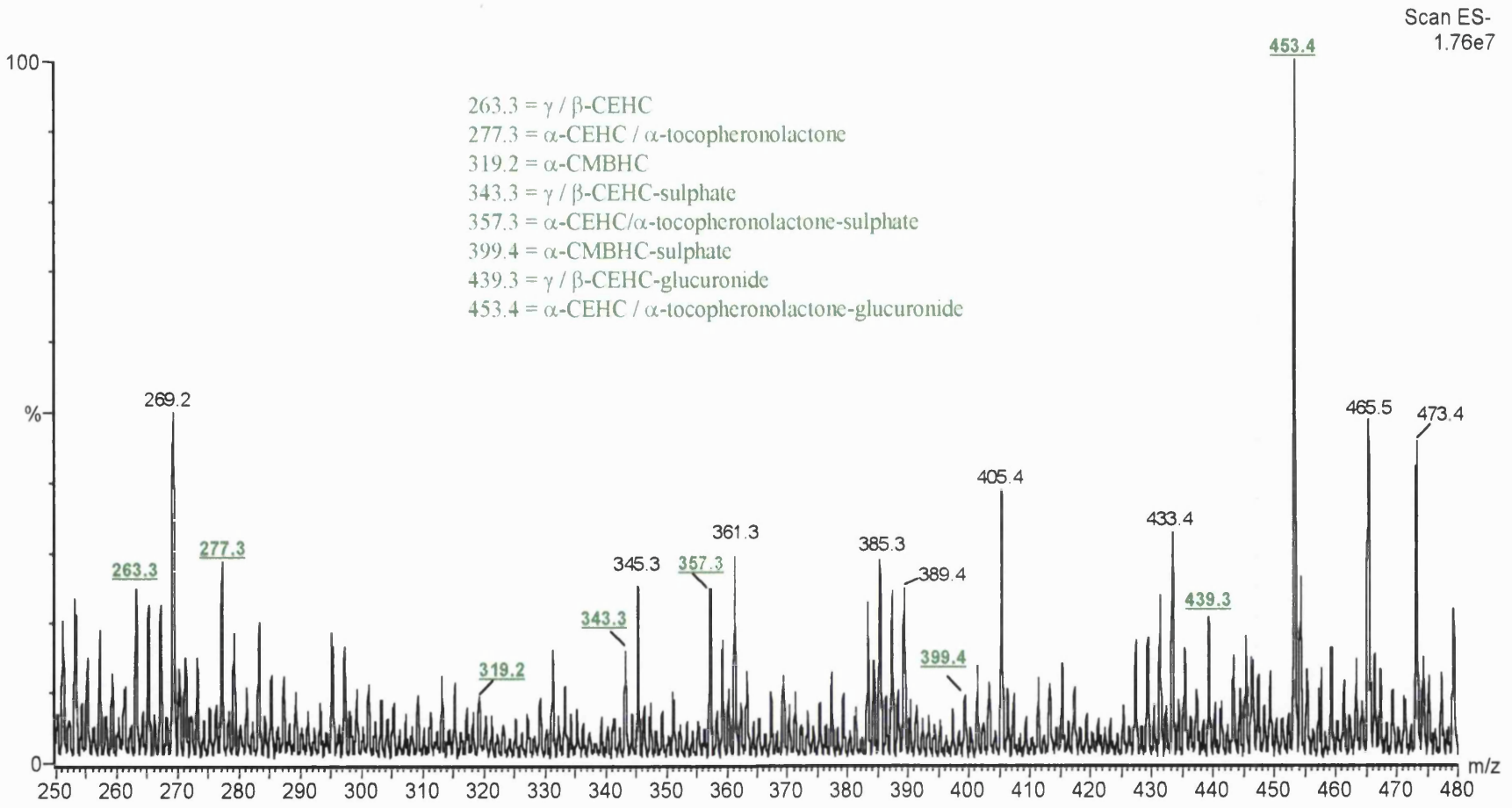


**Figure 6.4.** Negative ion ESI-MS of a urine sample after oral supplementation with α-tocopheryl acetate (1000 mg/d for 3 wk) Peaks corresponding to masses of vitamin E metabolites are underlined and in green.

Peaks at m/z 343 and 399, corresponding to the masses of γ- or β-CEHC- and α-CMBHC-sulphate, respectively, and m/z 439, corresponding to the mass of γ- or β-CEHC-glucuronide, were also observed (Figure 6.4). Peaks corresponding to other conjugated vitamin E metabolites were of lower abundance and were not consistently observed in significant amounts. Others peaks at m/z 263, 277 and 319 corresponded to the masses of unconjugated γ-CEHC, α-CEHC and α-CMBHC respectively.

In order to confirm that these peaks were sulphate or glucuronide conjugates, the mass spectrometer was operated in precursor ion or neutral loss mode (Figure 6.3). In these modes, glucuronide and sulphate conjugates could be specifically analysed by monitoring for a neutral loss of 176 or a negative loss of m/z 80, respectively. When operated in this way, the peak at m/z 453, displayed a neutral loss of 176 and the peaks at m/z 357 and m/z 343 displayed a loss of m/z 80 (figure 6.5). This confirmed that these peaks were glucuronide and sulphate conjugates, respectively. Losses of 176 or m/z 80 were not consistently produced by the other ion peaks shown in figure 6.4, making it unclear whether they were sulphate or glucuronide conjugates.

### 6.4.2. Shift in mass of conjugated metabolites after oral dose of $d_6$-RRR-α-tocopheryl acetate

In order to confirm that the peaks at m/z 453 and m/z 357 corresponded to metabolites of α-tocopherol and not just unrelated isobaric compounds containing glucuronide or sulphate groups, urine samples from subjects who had been given an oral dose of $d_6$-RRR-α-tocopheryl acetate (300 mg) were analysed. After oral supplementation with deuterated α-tocopheryl acetate there was an increase in the intensity of peaks m/z 459

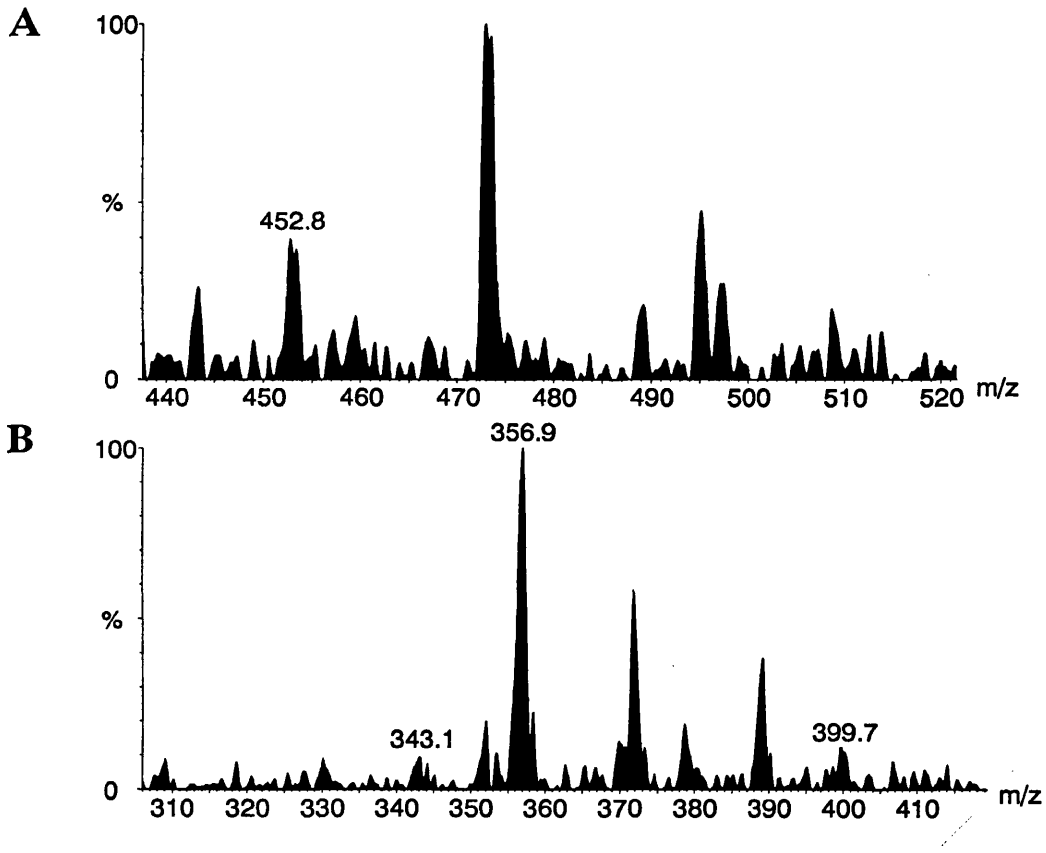


**Figure 6.5.** Negative ion ESI-MS/MS neutral loss and precursor ion spectra after oral α-tocopheryl acetate supplementation. **A** neutral loss of 176 (glucuronides) and **B** precursors of m/z 80 (sulphates). The neutral loss scan indicates that the ion of m/z 453 is a glucuronide and the precursor ion scan indicates that ions of m/z 343, 357 and 399 are sulphates.

and m/z 363, corresponding to the $d_6$-α-CEHC/α-tocopheronolactone glucuronide and sulphate analogues (Figure 6.6). This confirmed that the peaks at m/z 357 and m/z 453 were conjugates of α-tocopherol metabolites. In common with the data obtained after oral supplementation with non-deuterated α-tocopheryl acetate, the peak corresponding to α-CEHC/α-tocopheronolactone glucuronide was of higher intensity than the corresponding sulphate in straight scan mode (data not shown).

### 6.4.3. Use of chemically synthesised standards to confirm identity of conjugated α-tocopherol metabolites

In order to confirm that α-tocopherol metabolites were responsible for the peaks observed at m/z 357 and m/z 453, it was decided to compare the fragment ion or CID spectra obtained from these peaks to those produced by the chemically synthesised conjugated standards described in chapter 5. These conjugated standards of α-CEHC and α-tocopheronolactone were characterised intact by $^{1}H$ NMR, $^{13}C$ NMR, UV, IR and MS and after deconjugation by GC-MS (Chapter 5 and appendix).

Initially the conjugated standards were analysed in straight scan mode to confirm that they had not decomposed and to make sure they were producing molecular ions of sufficient intensity to produce informative fragment ion (CID) spectra. The ESI-MS spectra for the glucuronide standards are shown in figure 6.7 and the sulphate standards in figure 6.8. The details of each spectrum are discussed below.

*α-tocopheronolactone-glucuronide standard (Figure 6.7A):* The major peak observed was at m/z 453 corresponding to α-tocopheronolactone-glucuronide. Peaks at m/z 277

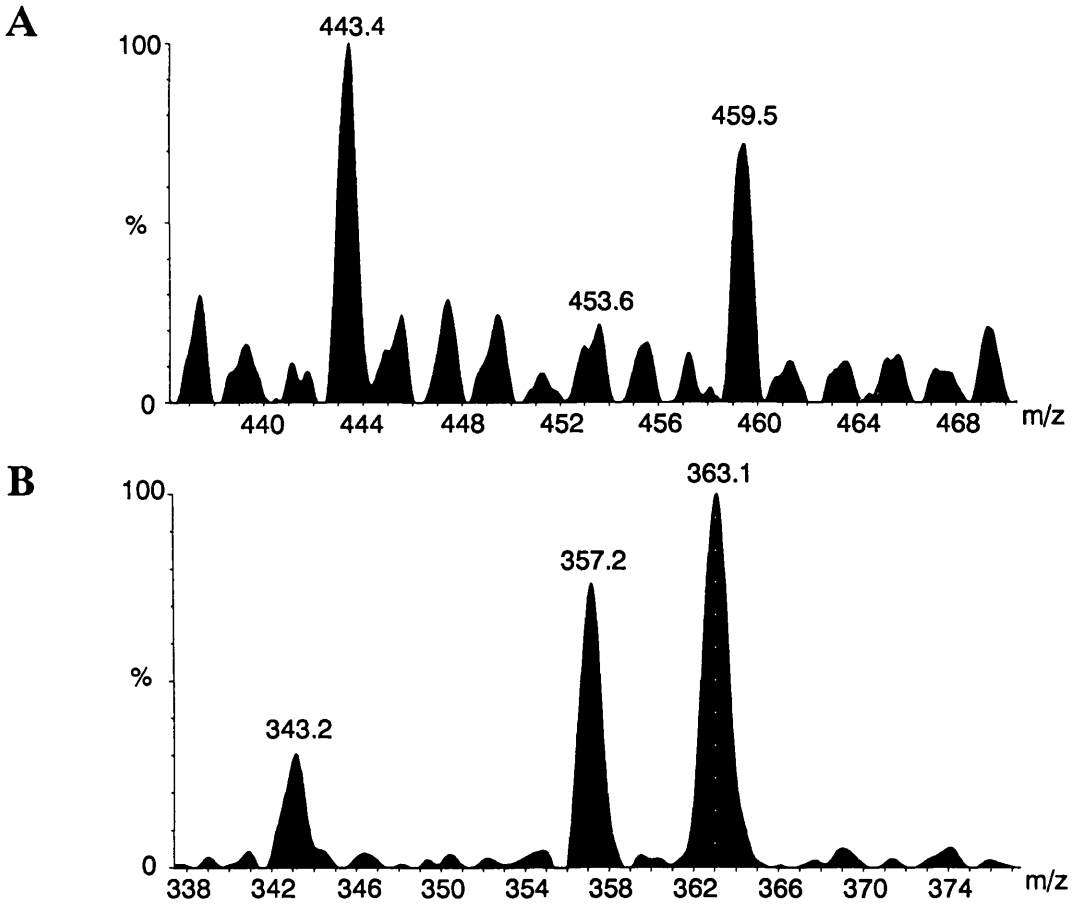


**Figure 6.6.** Negative ion ESI-MS/MS neutral loss and precursor ion spectra after oral $d_6$-α-tocopheryl acetate supplementation. **A** neutral loss of 176 (glucuronides) and **B** precursors of m/z 80 (sulphates). Additional peaks were present when compared to normal urine (figure 6.5). These peaks were greater in mass/charge ratio by 6 Da/e, confirming they were $d_6$-analogues of the natural α-tocopherol metabolites.

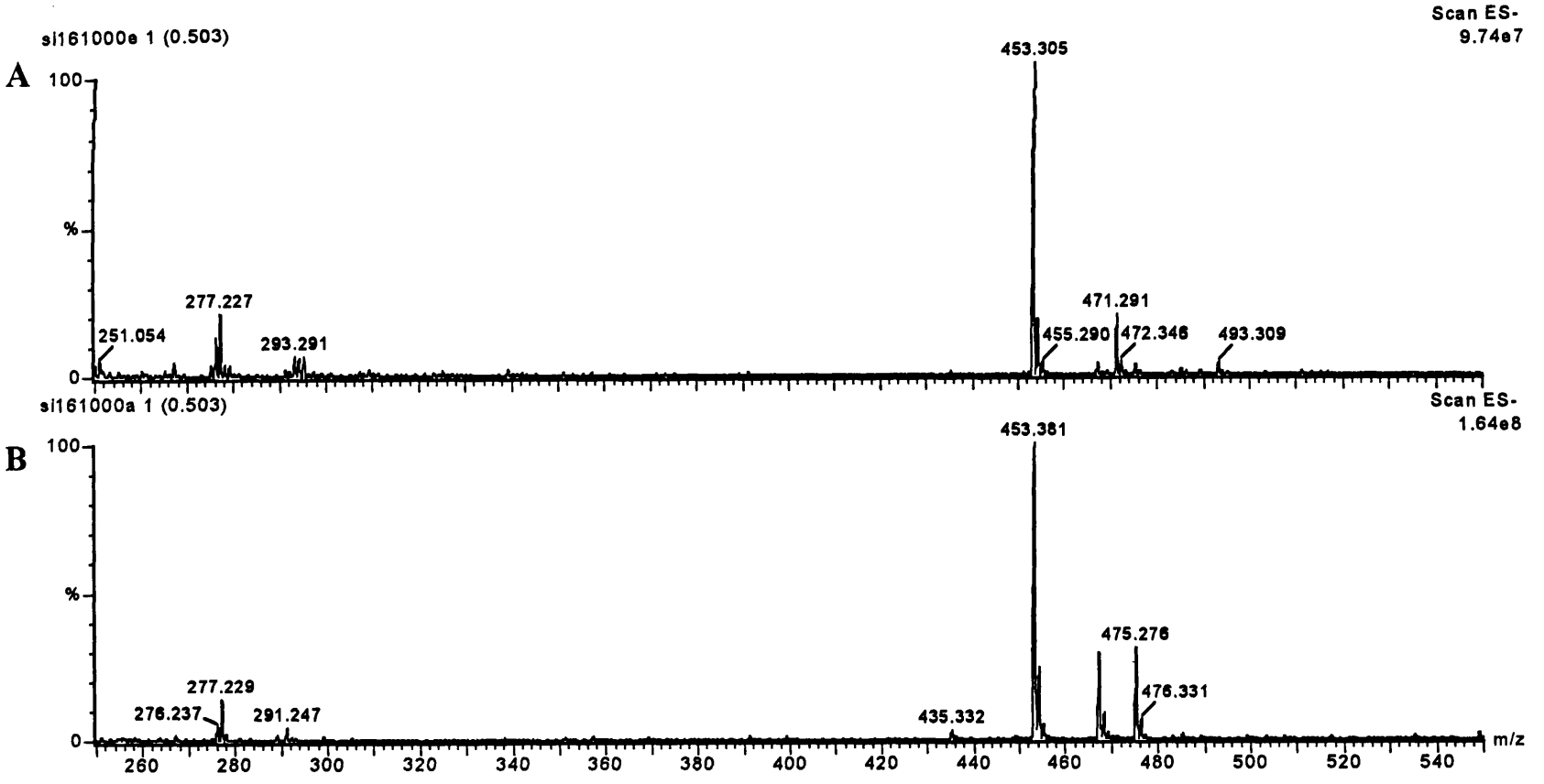


**Figure 6.7.** Negative ion ESI-MS of **A** α-tocopheronolactone-glucuronide and **B** α-CEHC-glucuronide standards

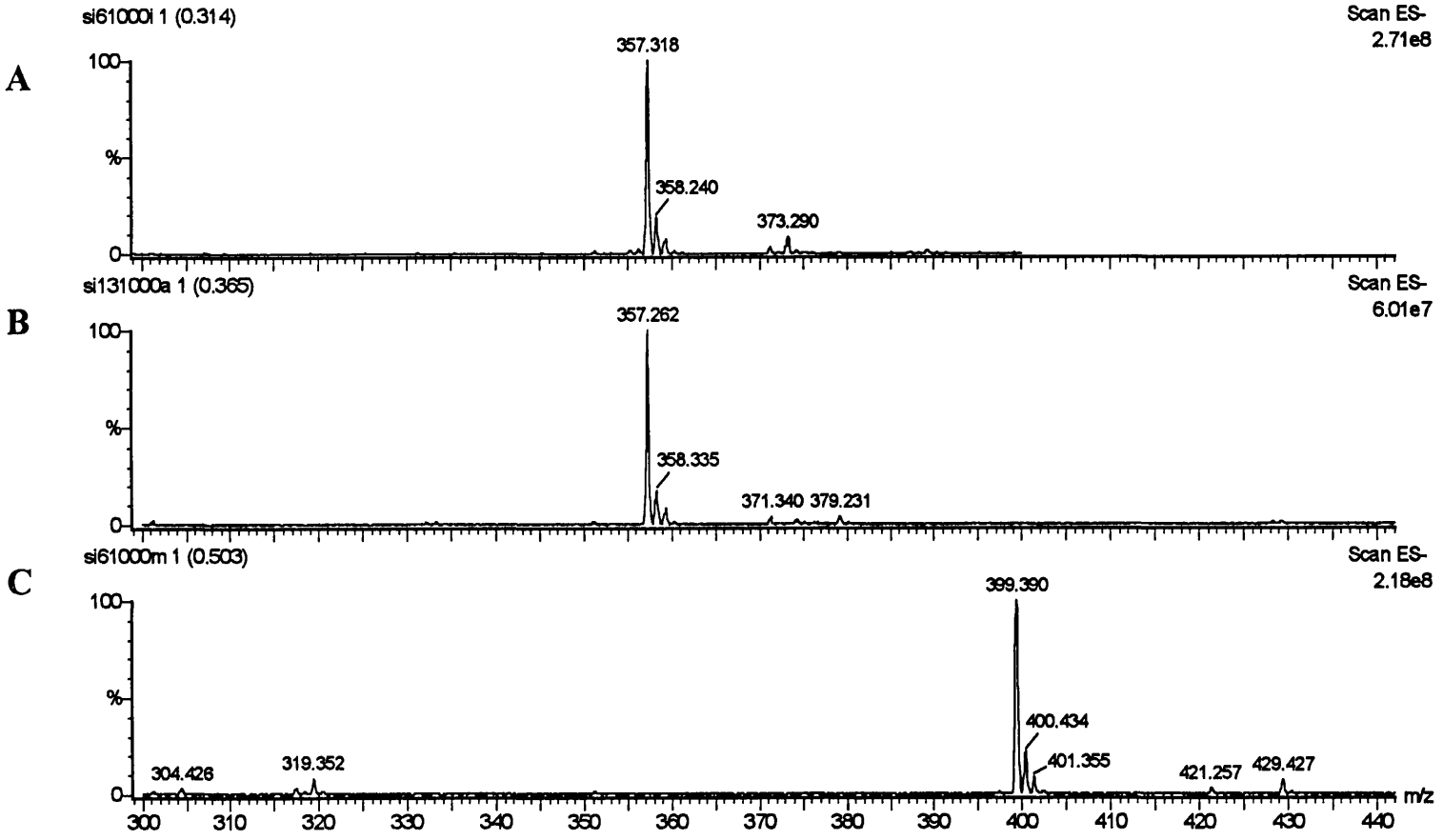


**Figure 6.8.** Negative ion ESI-MS of **A** α-tocopheronolactone-sulphate, **B** α-CEHC-sulphate and **C** α-CMBHC-sulphate standards

and m/z 293 correspond to unconjugated α-tocopheronolactone and α-tocopheronic acid benzoquinone, respectively. The peak at m/z 471 corresponds to α-tocopheronic acid-glucuronide with its corresponding sodium adduct at m/z 493. Both conjugated and unconjugated α-tocopheronic acid were observed in this standard probably resulting from lactone ring opening during saponification of the acyl-protected glucuronide (Chapter 5).

*α-CEHC-glucuronide standard (Figure 6.7B):* The major peak observed was at m/z 453 corresponding to α-CEHC-glucuronide with its corresponding sodium adduct [M-2H+$Na^+$] at m/z 475. A small amount of unconjugated α-CEHC was also observed at m/z 277. The peak at m/z 467, corresponding to the methyl ester of α-CEHC-glucuronide, probably results from incomplete saponification of the esterified intermediate during synthesis of the standard (Section 5.4.1.3).

*α-tocopheronolactone-sulphate standard (Figure 6.8A):* The major peak observed was at m/z 357 corresponding to α-tocopheronolactone-sulphate. A small amount of unconjugated α-tocopheronolactone was observed at m/z 277 (peak not shown). The identity of the minor peak at m/z 373 is unknown, although its mass is consistent with sulphated tocopheronic acid benzoquinone.

*α-CEHC-sulphate standard (Figure 6.8B):* The major peak observed was at m/z 357 corresponding to α-CEHC sulphate with its corresponding sodium adduct [M-2H+$Na^+$] at m/z 379. A small amount of unconjugated α-CEHC was observed at m/z 277 (peak not shown). The minor peak at m/z 371 corresponds to a α-CEHC-sulphate methyl

ester. This species was probably produced by the presence of acidic methanol during synthesis (Chapter 5).

*α-CMBHC-sulphate standard (Figure 6.8C):* The major peak observed was at m/z 399 corresponding to α-CMBHC sulphate. A small amount of unconjugated α-CMBHC was also observed at m/z 319.

Having shown that the standards had not decomposed significantly and contained the appropriate metabolites, fragmentation patterns were then produced from the conjugated standards and compared to those produced from the compounds in human urine with peaks at m/z 357 and m/z 453.

*CID spectra of the $[M-H]^-$ ion from α-CEHC-sulphate standard and α-tocopheronolactone-sulphate standard (m/z 357) (Figure 6.9)*

In order to compare spectra, the same tandem mass spectrometric parameters were used, since small changes in parameters such as collision energy and cone voltage resulted in marked differences in the relative intensity of different peaks. The CID spectra of α-CEHC-sulphate and α-tocopheronolactone-sulphate standards were similar (Figure 6.9 A and B). The major peaks observed in both spectra at m/z 80 and m/z 233 correspond to the loss of sulphate and sulphate plus $CO_2$, respectively. Other common fragment ions include m/z 150, 163 and 277. The proposed structures of these fragment ions are shown in figure 6.11.

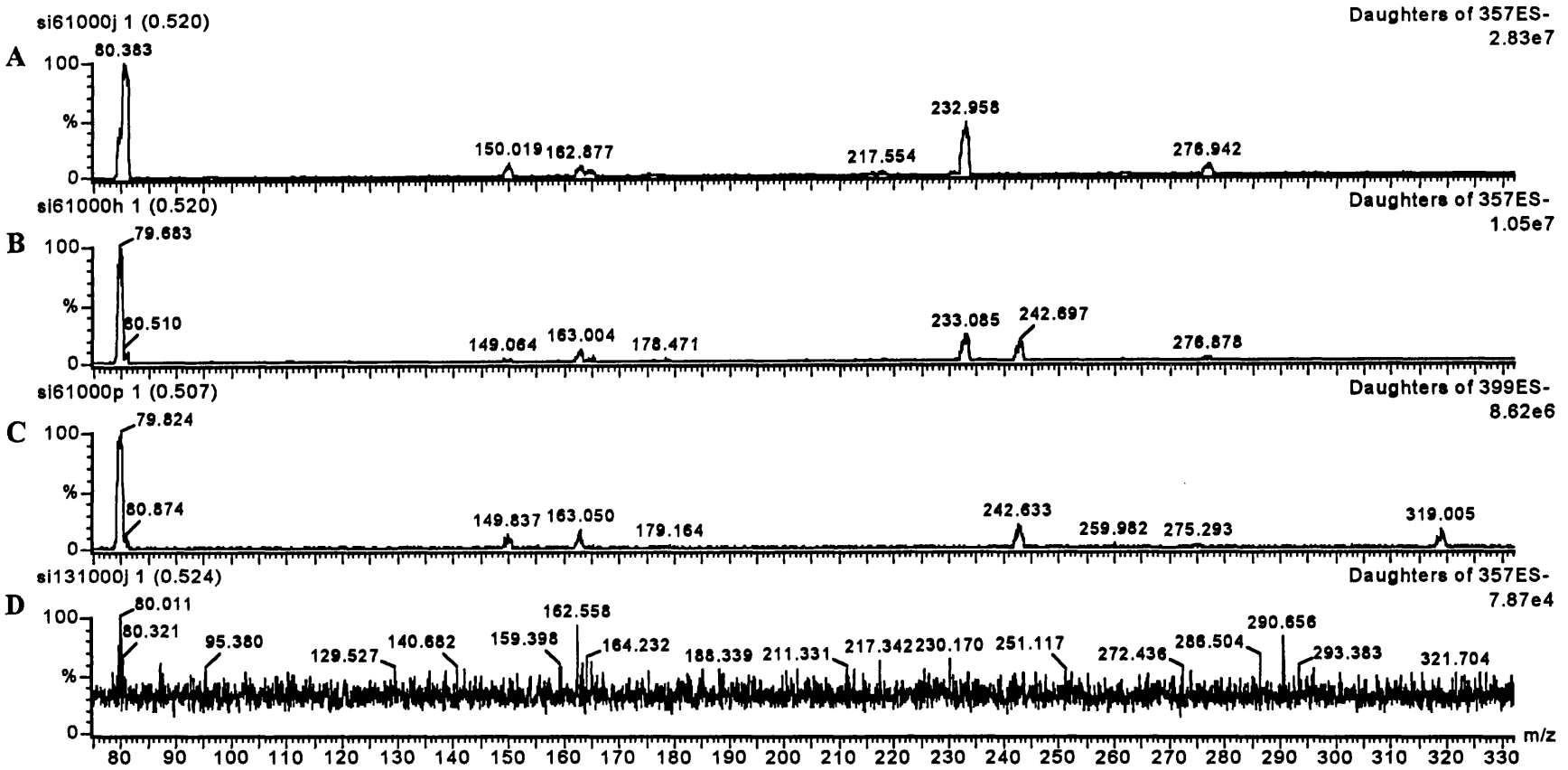


**Figure 6.9.** CID spectra of the $[M-H]^-$ ion from **A** α-tocopheronolactone-sulphate, **B** α-CEHC-sulphate, **C** α-CMBHC-sulphate and **D** sulphated urinary metabolite (m/z 357).

The only major difference between the two compounds was the presence of a peak at m/z 243 in the α-CEHC-sulphate spectrum, which was not present in the α-tocopheronolactone-sulphate spectrum. Even after adjustment of the cone voltage and collision energy, α-tocopheronolactone-sulphate failed to produce this fragment ion. Interestingly, α-CMBHC-sulphate also produced this fragment ion (Figure 6.9 C), indicating it may be specific to compounds with intact chroman rings. The possible origin of this fragment ion is shown in figure 6.11.

*CID spectra of the $[M-H]^-$ ion from α-CEHC-glucuronide standard and α-tocopheronolactone-glucuronide standard (m/z 453) (Figure 6.10)*

The CID spectra of the glucuronide conjugates were more complex than the sulphate conjugates owing to the extensive fragmentation of the glucuronic acid moiety. However, the fragment ions produced by α-tocopheronolactone and α-CEHC conjugates were again very similar with the only significant difference being the presence of a peak at m/z 262 in the α-tocopheronolactone-glucuronide spectrum but not in the α-CEHC-glucuronide spectrum. This fragment is likely to derive from loss of the glucuronide group plus one of the four methyl as shown in figure 6.11.

*Comparison of CID spectra of conjugated standards with those of urinary metabolites*

The CID spectrum of the metabolite observed in human urine (m/z 453) (Figure 6.10 C) was very similar to both the CID spectra of α-CEHC-glucuronide and α-tocopheronolactone-glucuronide standards, despite the higher levels of background noise. However the absence of a significant m/z 262 ion, which was produced by α-tocopheronolactone-glucuronide standard, indicated that the major metabolite in this urine sample was α-CEHC-glucuronide.

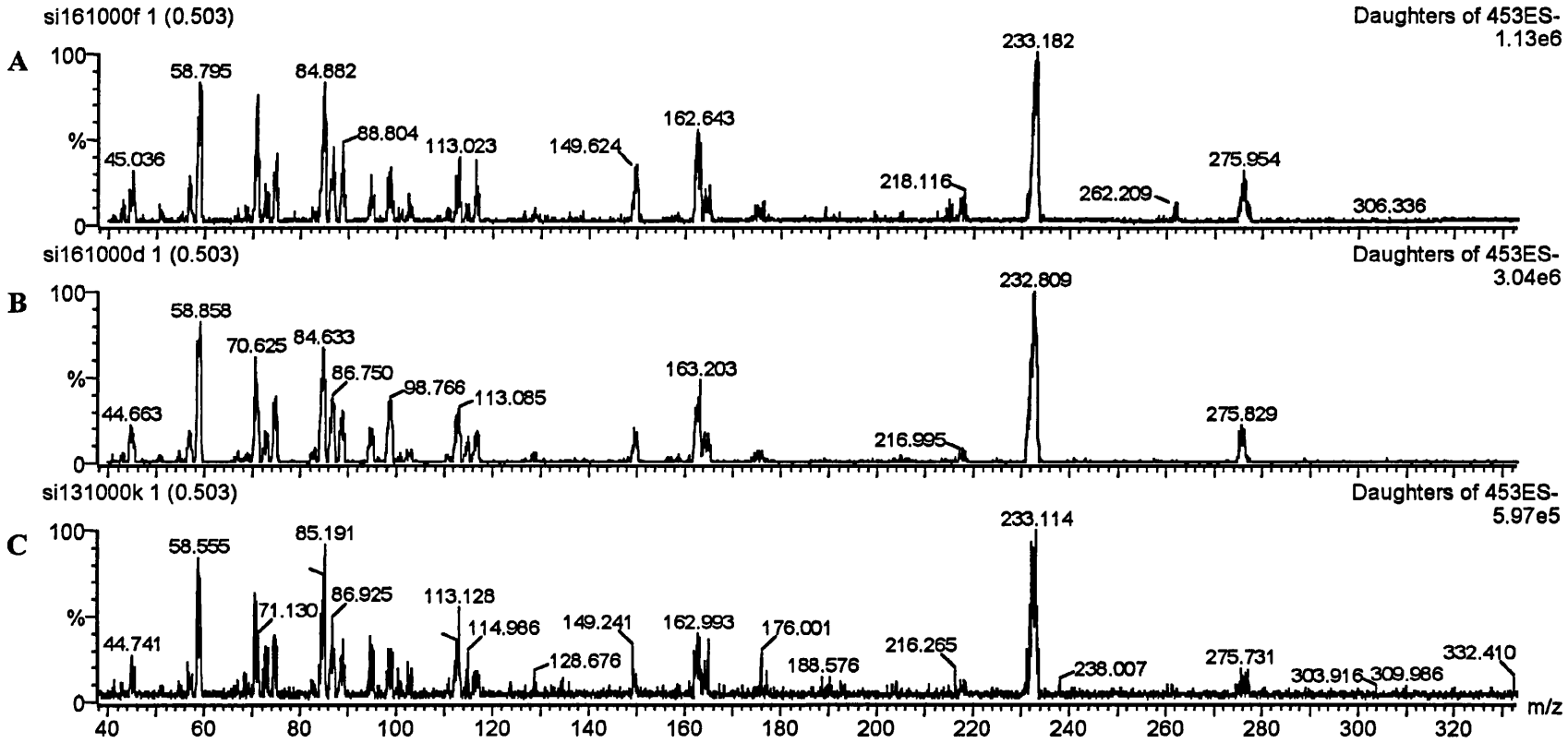


**Figure 6.10.** CID spectra of the $[M-H]^-$ ion from **A** α-tocopheronolactone-glucuronide, **B** α-CEHC-glucuronide, and **C** glucuronidated urinary metabolite (m/z 453).

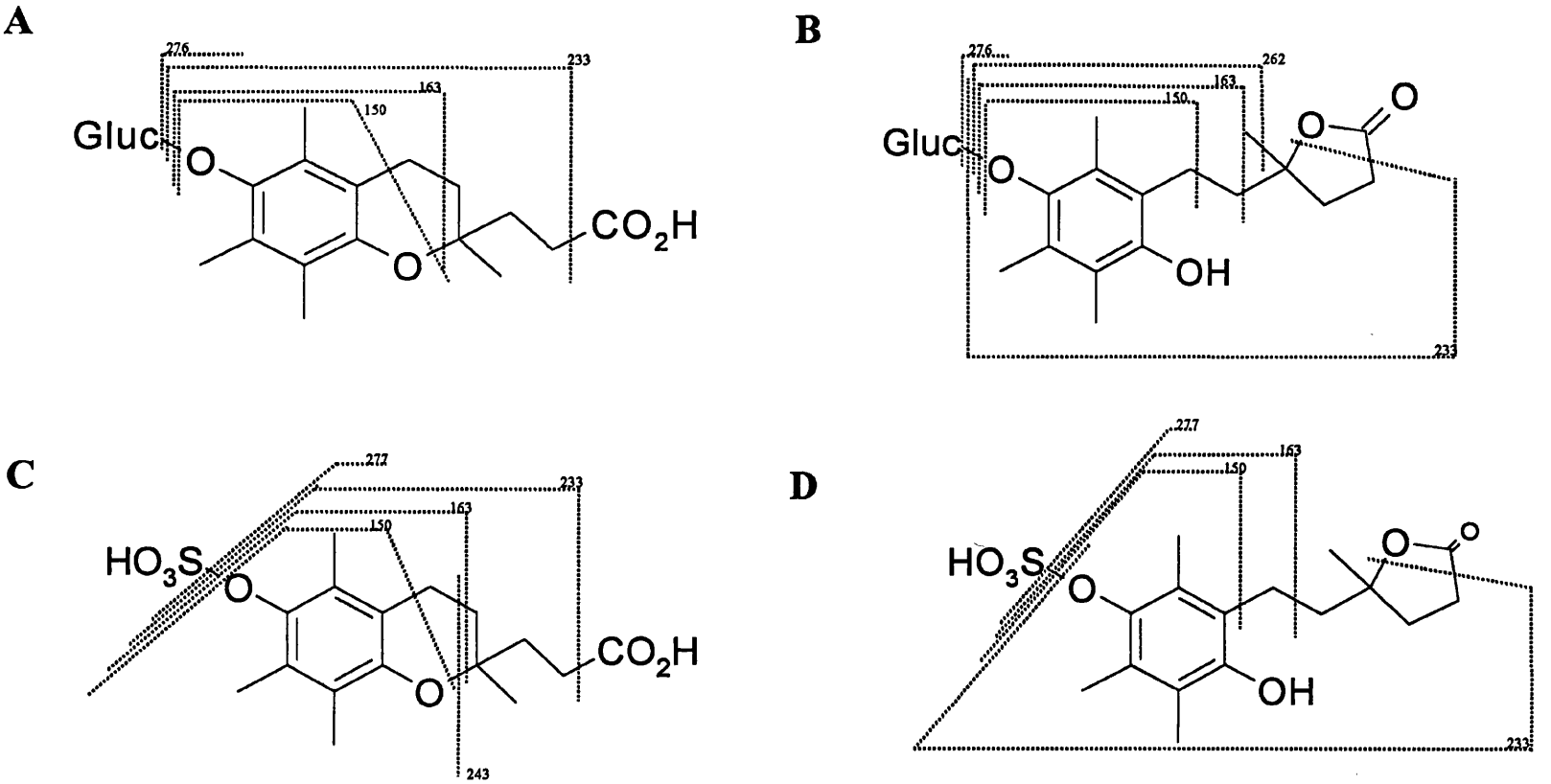


**Figure 6.11.** Possible origins of fragment ions produced by **A** α -CEHC-glucuronide, **B** α–tocopheronolactone-glucuronide, **C** α-CEHC-sulphate and **D** α-tocopheronolactone-sulphate

A similar comparison of the CID spectrum of the sulphated metabolite (m/z 357) with CID spectra of the sulphated standards has so far been hindered by the small amount of sulphated metabolite in urine and the consequent poor quality spectrum obtained (Figure 6.9 D).

### 6.5. Discussion and conclusions

In this study tandem mass spectrometry was used to investigate the conjugated vitamin E metabolites excreted in human urine. Oral supplements of unlabelled α-tocopheryl acetate were given to increase the levels of α-tocopherol metabolites in human urine and subsequent supplementation with deuterated α-tocopheryl acetate confirmed those peaks that corresponded to metabolites of α-tocopherol. After large oral doses of α-tocopheryl acetate, α-tocopherol metabolites (α-CEHC/α-tocopheronolactone) were excreted in the urine as both glucuronide and sulphate conjugates, although the glucuronide peak was more intense than the sulphate peak in the mass spectrum. Assuming that this reflects differences in the relative amounts of sulphates and glucuronides rather than differences in their ionisation efficiencies, it is not surprising that glucuronides are more abundant. Previous studies in humans have demonstrated that at high substrate concentrations, glucuronidation predominates over sulphation, for a range of different substrates, because the enzymes involved in the sulphation process are rapidly saturated (Gibson and Skett, 1994). Small levels of glucuronide and sulphate conjugates of γ- and/or β-tocopherol metabolites were also observed in the urine sample.

Comparison of the CID spectrum of the major urinary metabolite (m/z 453) with conjugated α-tocopherol metabolite standards confirmed its identity as α-CEHC-

glucuronide. Confirmation of the identity of the other presumed metabolites was hindered by the comparatively small amounts of these metabolites in urine.

Initially it was hoped that a study of conjugated urinary vitamin E metabolites would establish the authenticity of α-tocopheronolactone as an *in vivo* metabolite, since conjugation prevents artefact formation. However the isobaric nature of α-CEHC and α-tocopheronolactone conjugates and the similarity in CID spectra hindered their individual identification. The CID spectra of the equivalent synthetic standard of α-CEHC and α-tocopheronolactone conjugates differed in one or two characteristic peaks. However, the high level of background noise in biological samples and the probable low levels of α-tocopheronolactone compared to α-CEHC made it difficult to reliably identify these characteristic peaks.

In order to unambiguously confirm the presence of conjugated α-tocopheronolactone in urine samples, future studies will need to develop more sensitive methods and separate α-tocopheronolactone conjugates from α-CEHC conjugates. Separation of these conjugates may be accomplished either by chemical modification to produce a mass difference or by use of separation techniques such as HPLC prior to mass spectrometry. An example of a simple chemical modification, would be the use of a base to open the lactone ring of α-tocopheronolactone conjugates, converting them to α-tocopheronic acid conjugates and thus increasing their masses by 18. The feasibility of such approaches could be assessed using the chemically synthesised standards used in this chapter.

Having confirmed the effectiveness of these conjugated standards in mass spectrometric studies, they can now be used in further studies looking at the efficiency of enzymatic deconjugation and the possible mechanisms of artefact production. It will, however, be necessary to increase the sensitivity in future mass spectrometric studies to enable the minor, as well as the major metabolites of vitamin E to be fully characterised in urine samples. The latest generation of ESI tandem mass spectrometer has the enhanced sensitivity that should make such analyses possible.

# CHAPTER 7

## Summary and Discussion


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## 7.1. Introduction

Considering that vitamin E was first identified as a natural compound in the 1920's (Evans and Bishop, 1922) and chemically synthesised a decade later (Von Karrer et al., 1938), there is surprisingly little known about its metabolism in mammalian species including man. For example, the full characterisation of all the major and minor metabolites in urine and the biological and physiological factors regulating its metabolism in different tissues and cellular compartments are, as yet, not fully established.

The aim of the present study was to use modern analytical approaches to characterise not only the major urinary metabolites but all possible identifiable metabolites of vitamin E, making use of deuterated α-tocopherol analogues. Characterisation of the different urinary metabolites produced by vitamin E and the development of suitable methods to measure them will be useful in establishing how vitamin E is metabolised and to relate this information to the function(s) of vitamin E and its metabolites *in vivo*.

Evidence from previous studies suggested that metabolites of vitamin E, in common with the majority of metabolites produced from xenobiotics and natural products, are excreted in the urine mainly as sulphate and glucuronide conjugates (Chiku et al., 1984; Swanson et al., 1999; Stahl et al., 1999). Ideally therefore, analytical methods would measure the conjugated metabolites directly, since this would reduce the number of handling steps and give more detailed information about the urinary metabolites. However, the polarity of such conjugated metabolites complicates their extraction, separation and quantitation by conventional techniques. The direct quantitation of any given conjugate also requires pure standards of the appropriate conjugates to be

available. Because of these problems, the conventional analytical approach has depended on hydrolytic cleavage, either by acid or enzyme, and determination of the released deconjugated metabolites by robust and sensitive techniques such as GC-MS. This approach, however, can lead to errors and misinterpretation for several reasons, such as incomplete hydrolysis, lability of the released hydroxylated metabolites and formation of artefacts (Bottcher et al., 1982; Hege et al., 1986).

In the present studies a new method using GC-MS was developed for the measurement of deconjugated urinary metabolites of vitamin E (Pope et al., 2000). Conjugated standards were also synthesised, which enabled the direct determination of conjugated vitamin E metabolites to be made using electrospray tandem mass spectrometry. The results obtained with these different techniques are compared below.

**7.2. GC-MS of deconjugated metabolites**

The use of solid phase extraction and enzymatic hydrolysis, coupled with a GC-MS temperature programme that allowed greater separation of the components in urine, made the method for unconjugated vitamin E metabolites, developed in the course of this study, considerably more sensitive than previous methods using liquid-liquid extraction coupled to HPLC or GC-MS (Schultz et al., 1995; Traber et al., 1998). This increased sensitivity allowed reproducible measurement to be made of both the major and minor metabolites of α, β, γ and δ-tocopherol, whereas previous methods had only been able to measure major metabolites such as α-CEHC and γ-CEHC. Since the development of this GC-MS method, equally sensitive methods have been described by other groups, although these have continued to concentrate on the major metabolites (Stahl et al., 1999; Hattori et al., 2000; Lodge et al., 2000). A comparison of the data

obtained with these methods revealed that in general they all gave similar results (Table 4.2, Chapter 4).

The method developed during the course of this study facilitated the identification of a novel group of vitamin E metabolites, the CMBHCs, which were originally identified due to their similarity in spectra to the CEHCs (Pope et al., 2000). The identity of α-CMBHC was, however, only definitively confirmed by supplementation with deuterated RRR-α-tocopheryl acetate to produce the corresponding deuterated metabolite and by comparison of the urinary metabolite to an authentic chemically synthesised standard (Pope et al., 2001). CMBHCs produced from all the tocopherols can be detected in human urine and these metabolites have also been observed in primary rat hepatocytes in cell culture (Parker et al., 2000), although work presented here (chapter 4) raises doubts as to whether CMBHCs are excreted in rat urine. The reported α-CMBHC levels in human urine vary from 3-6% of α-CEHC (Schuelke et al., 2000) to 15-30% (Chapter 4), which probably results from differences in the extraction and analytical procedures. The structure of CMBHCs, as determined using an authentic chemically synthesised standard, is in agreement with the predicted structure produced by successive rounds of β-oxidation of the side chain of vitamin E.

It is suggested that the formation of the CEHCs and CMBHCs results from the metabolism of excess vitamin E (Schultz et al., 1995; Traber et al., 1998). There is, however, considerable interest in identifying metabolites produced by reactions with oxidants because of their potential use as biomarkers of oxidative stress. α-Tocopheronolactone, a metabolite produced by oxidation of the chroman ring, was detected in low levels in urine samples. α-Tocopheronolactone is, however, considered

by some to be an artefact, produced by the oxidation of α-CEHC during the extraction and/or deconjugation procedures (Schultz et al., 1995). Although, at least some of the α-tocopheronolactone observed in the present study was demonstrated to be artefactual by the partial conversion of a standard of deuterated α-CEHC to deuterated α-tocopheronolactone, the greater levels of unlabelled α-tocopheronolactone produced from endogenous α-tocopherol suggest that it may also be produced *in vivo*. However, α-tocopheronolactone could not be quantitated reproducibly, even when the conversion of $d_9$-α-CEHC to $d_9$-α-tocopheronolactone was used to correct for artefactual conversion.

Estimated daily excretion levels of the various metabolites were calculated and showed good agreement with those reported in the literature (Lodge et al., 2000; Parker and Swanson, 2000; Schuelke et al., 2000) even though untimed morning urine samples, as opposed to 24 hr samples, were used. Mean daily excretion levels of α-CEHC, α-CMBHC, γ-CEHC and α-tocopheronolactone, taking into account artefactual conversion, were 1.0, 0.2, 10 and 0.1 μmole, respectively.

### 7.3. Electrospray tandem MS of conjugated metabolites

Although the GC-MS method gave useful information concerning the general levels and ratios of metabolites, there were a number of important questions that could not be fully answered using this methodology. When the method was applied to rat and human urine samples, a number of observations were noted. First, α-tocopheronolactone was produced artefactually and it could not be measured reproducibly. Secondly, thin layer chromatography of radiolabelled rat urine samples after enzymatic deconjugation indicated that less than 10% of the conjugates were deconjugated by the sulphatase/β-

glucuronidase enzyme. In order to further confirm these findings it was necessary to analyse the conjugated metabolites directly, since the deconjugation and/or extraction procedure were apparently responsible for both the artefactual production of α-tocopheronolactone and for the low yields of deconjugated metabolites in rat urine. To achieve this aim the chemical synthesis of putative or predicted conjugated metabolites of vitamin E was undertaken.

Glucuronide and sulphate conjugates of α-CEHC and α-tocopheronolactone and α-CMBHC-sulphate were chemically synthesised after initially preparing the unconjugated metabolites. The final products were purified and fully characterised using techniques such as NMR, MS, IR and UV to confirm their structures. This is the first time that the synthesis of conjugated vitamin E metabolites has been undertaken.

The conjugated synthetic standards were used to characterise the metabolites of α-tocopherol excreted in human urine by electrospray tandem mass spectrometry. The major conjugate observed in urine from human subjects who had taken large oral supplements of α-tocopheryl acetate was a mono-glucuronide of α-CEHC/α-tocopheronolactone, although a mono-sulphate was also observed in small amounts. Because of the isobaric nature of α-CEHC and α-tocopheronolactone conjugates and the similarity in their fragmentation patterns, the relative contribution of each of these metabolites could not be easily determined using tandem mass spectrometry.

### 7.4. Metabolism of vitamin E

The present study, along with other recent reports (Traber et al., 1998, Schuelke et al., 2000; Parker et al., 2000; Parker and Swanson, 2000; Lodge et al., 2001; Hattori et al.,

2001), provide a more detailed understanding of vitamin E metabolism. However, there are many aspects, such as the specific tissue and cellular sites of metabolism and the mechanisms of regulation of metabolism, which have not as yet been studied in detail. Use of radiolabelled and deuterated vitamin E analogues has enabled vitamin E excretion to be correlated to oral or intravenous vitamin E intake (Chiku et al., 1984; Traber et al., 1998; Pope et al., 2000; Lodge et al., 2001) and this has helped to compare the retention and metabolism of the different forms of vitamin E. The results from these studies have revealed that the liver and in particular the hepatic α-tocopherol transfer protein (α-TTP) have key roles in discriminating between the different forms of vitamin E and in maintaining plasma and tissue α-tocopherol levels. The exact mechanism of α-tocopherol transfer to nascent VLDL by hepatic α-TTP is unknown, but α-TTP has been shown to transfer RRR-α-tocopherol between lipid membranes at a higher rate than other forms of vitamin E (Hosomi et al., 1997). Presumably, vitamin E that is not loaded onto nascent VLDL in the liver is metabolised by the liver or kidney and excreted in faeces via bile or urine.

Measurement of urinary CEHCs has revealed that up to 50% of the daily dietary intake of RRR-γ-tocopherol is excreted in the urine (Swanson et al., 1999) in comparison to about 1% of the daily RRR-α-tocopherol intake (Schuelke et al., 2000). Similar studies using deuterium labelled α-tocopherols has also revealed that all-rac-α-tocopherol is metabolised and excreted in preference to RRR-α-tocopherol (Traber et al., 1998). These results are in agreement with the known differences in affinity of α-TTP for the various forms and stereoisomers of tocopherol and emphasise the importance of α-TTP in the regulation of vitamin E metabolism.

The metabolism of the phytyl side chain of vitamin E is predicted to occur via β-oxidation, following initial ω-oxidation, and this is confirmed by the structures of the CEHCs and CMBHCs (Parker et al., 2000; Pope et al., 2001). Owing to the continual recycling of lipoprotein bound vitamin E by the liver and the action of hepatic α-TTP, the liver is probably the major site of vitamin E side chain metabolism (Traber et al., 1998). This β-oxidation, by analogy with the known metabolism of other compounds with branched side chains, such as phytanic acid, is likely to take place in the peroxisomes (Mannaerts and Van Veldhoven, 1995).

In order to provide a suitable substrate for β-oxidation, the terminal methyl of the vitamin E side chain needs to be oxidised by ω-oxidation. Recent evidence using inhibitors of various cytochrome P450s in rat primary hepatocytes indicates that this ω-oxidation is cytochrome P450 3A dependent (Parker et al., 2000) and is likely to take place in the endoplasmic reticulum. After side chain metabolism of vitamin E in the peroxisomes, the metabolites produced can be conjugated and excreted in the urine or faeces. Studies in rabbits have shown that greater than 70% of an intravenous dose of radiolabelled α-tocopherol was excreted in the faeces within three weeks (Simon et al., 1956a). However, free unconjugated α-tocopherol was found to comprise about 50% of faecal radioactivity indicating that plasma α-tocopherol can be excreted in the faeces without prior metabolism. The urinary radiolabelled metabolites were highly polar conjugates and 70-90% could be released by β-glucuronidase treatment (Simon et al., 1956a). Examination of the conjugates present in human urine in the present study also suggested that the majority of α-tocopherol metabolites were excreted as glucuronide conjugates.

Conjugation of compounds is carried out by a range of enzymes in the Golgi and cytosol (Meech and Mackenzie, 1997). In humans there is a higher capacity for glucuronidation than sulphation, even though the sulphotransferase enzymes have a higher rate constant (Gibson and Skett, 1994). Therefore, after large doses of α-tocopherol, greater amounts of glucuronide than sulphate conjugates would be expected. This is in agreement with the results found in this study using electrospray tandem mass spectrometry and synthetic conjugated standards. In contrast to humans, rats have a higher capacity for sulphation (Gibson and Skett, 1994) which explains the high levels of α-CEHC sulphate observed in rat urine (chapter 4, data from Hoffmann La Roche). The high level of sulphate conjugates in rat urine may also explain the inefficient enzymatic deconjugation of radiolabelled metabolites of α-tocopherol (described in chapter 4), which could result from competitive inhibition of the relatively low amount of sulphatase activity present in the enzyme preparation.

So far only the metabolism and excretion of vitamin E in the liver has been discussed, but the fate of vitamin E once it has been incorporated into tissues is also of interest. Studies comparing the turnover of α-tocopherol in different tissues in rat and guinea pig indicate that tissues such as heart, brain, muscle and spinal cord turnover α-tocopherol at a much slower rate than tissues such as liver, lung, and kidney. Using deuterated RRR-α-tocopherol, Burton et al. reported half-times for the turnover of RRR-α-tocopherol in plasma, liver, muscle and brain of the guinea pig of 3.7, 3.0, 24, and 107 days, respectively (Burton et al., 1990) and for the rat of 6.2, 6.9, 23, and 40 days, respectively (Ingold et al., 1987). Similar results have been obtained in terminally ill human subjects (Burton et al., 1998).

Owing to the relatively low turnover of α-tocopherol in most tissues and the high turnover in liver, a relatively small proportion of urinary vitamin E metabolites are likely to have been produced within tissues such as heart and muscle directly. However, the production of oxidation products of α-tocopherol, such as α-tocopherylquinone (α-TQ), does occur within tissues (Vatassery, 1993; Vatassery et al., 1993; Ham and Liebler, 1995; Ham and Liebler, 1997). The tissue levels of α-TQ, as a percentage of α-tocopherol, are generally less than 1% and have been shown to increase after hyperoxia in rats (Kanazawa et al., 2000). Although the levels of α-TQ are low compared to α-tocopherol, this is not considered to directly reflect the true extent of reactions between α-tocopherol and oxidants. It is thought that the majority of α-tocopherol oxidation products are recycled from radical intermediates, instead of converting to α-TQ, via redox cycles with co-antioxidants such as ascorbate (vitamin C) and glutathione (Liebler, 1993; Upston et al., 1999), although *in vivo* evidence is lacking (see section 1.5.5). However, the accurate measurement of α-TQ and its metabolites may give an indication of the general levels of antioxidants and oxidants and, therefore, of oxidative stress.

Plasma levels of α-TQ have been reported (Vatassery et al., 1993; Vatassery, 1994). If the side chain of α-TQ is metabolised in a similar manner to α-tocopherol it will produce α-tocopheronolactone (Simon et al., 1956b). Although oxidation of α-tocopherol produces the benzoquinone form of α-TQ, this can be converted to the hydroquinone by a diaphorase enzyme (Siegel et al., 1997) allowing the metabolites produced by β-oxidation, such as α-tocopheronolactone, to be conjugated. If it is assumed that plasma α-TQ has similar excretion kinetics to plasma α-tocopherol, then

α-tocopheronolactone levels in urine may be expected to be about 1% of α-CEHC levels. However, α-TQ is likely to be excreted in preference to α-tocopherol, owing to the action of hepatic α-TTP and therefore, urinary α-tocopheronolactone levels may be expected to be greater than 1% of α-CEHC levels.

In the present study, the major portion of α-tocopheronolactone observed appeared to be artefactual, as shown by conversion of the $d_9$-α-CEHC standard to $d_9$-α-tocopheronolactone. If artefactual conversion is taken into account using the deuterated standard, the levels of endogenous α-tocopheronolactone in urine are approximately 5-10% of α-CEHC levels. This would be in general agreement with the estimated levels discussed above, which assumed that plasma α-TQ would be metabolised and excreted at a greater rate than plasma α-tocopherol. However, using the GC-MS method to measure α-tocopheronolactone, even with addition of $d_9$-α-CEHC to the fresh sample, is likely to be prone to error because of the high level of artefact in comparison to the natural metabolite. Measurement of the conjugated metabolites is likely to produce more accurate results, since conjugation of either the carboxyl or phenoxyl group of α-CEHC will prevent α-tocopheronolactone formation. However, analysis of α-CEHC and α-tocopheronolactone conjugates is complicated due to their isobaric nature, as described in chapter 6. Further studies to confirm the presence and accurately measure α-tocopheronolactone conjugates in urine will require their separation from α-CEHC conjugates, either by derivatisation to achieve a mass difference or by HPLC prior to mass spectrometric analysis.

## 7.5. Future work

The investigations, reported in this thesis, on the metabolism of α-tocopherol in humans and rats were performed on urine samples. These findings represent the 'exit metabolic products' from the body. To fully evaluate the kinetics of recycling of vitamin E as well as its intermediary metabolites *in vivo* in the human and rat it is important to carry out parallel investigations on the plasma samples taken from these human subjects and experimental rats.

Once it is established that α-tocopheronolactone is an authentic metabolite and that it can be measured accurately, its use as a non-invasive marker of oxidative stress can be evaluated by measuring the levels of α-CEHC and α-tocopheronolactone in both healthy subjects and in subjects suffering from oxidative stress. Depending on the findings of such investigations, the level of α-tocopheronolactone can be used as a marker of oxidative stress and used to monitor disease progression and the effects of interventions e.g. nutritional supplementation or drugs.

Apart from the authenticity of α-tocopheronolactone there are a number of other questions concerning vitamin E metabolism. For example, little is known about the cellular enzymes involved in vitamin E metabolism as well as differential metabolism in specific tissues and cellular compartments. Studies of vitamin E metabolism in tissue culture have already begun (Parker and Swanson, 2000; Parker et al., 200; Van Houte et al., 2001; Birringer et al., 2001). Such an approach will help to elucidate the mechanisms involved in the intracellular transport of α-tocopherol and α-TQ and their subsequent metabolism and excretion in different cell types, such as hepatocytes, enterocytes, renal epithelial cells, fibroblasts and neuronal cells in culture. Also of

interest would be the studies of metabolites produced by patients with inborn errors of metabolism, such as deficiency of peroxisomal β-oxidation, which will help to identify the sites of and enzymes involved in vitamin E metabolism.

Metabolism of vitamin E could have a key role to play in regulating its action in the body. A number of non-antioxidant roles of vitamin E, and in particular α-tocopherol, have been demonstrated (for a review see Azzi and Stocker, 2000). Recent evidence suggests that α-tocopherol, as opposed to β-tocopherol (which contains one less methyl) has an important role in signal transduction via its effect on protein phosphatase 2A and protein kinase C (Ricciarelli et al., 1998). Since protein phosphatase 2A and protein kinase C are implicated in a wide range of signalling pathways, which are involved in such processes as the regulation of cell growth, this may explain the recently described molecular effects of α-tocopherol (Azzi and Stocker, 2000). The metabolism of α-tocopherol and the other forms of vitamin E could, therefore, be important in regulating these signaling pathways by either increasing or decreasing the activity of the these compounds. The hypothesised demethylation of α-tocopherol to β-tocopherol in rats (see chapter 4) could be of particular importance in regulating the effect of α-tocopherol, since it would be expected to deactivate α-tocopherol. It would therefore be of interest to investigate whether a similar process occurs in human tissues. It would also be interesting to investigate whether the metabolites of vitamin E, and in particular α-tocopherol, also inhibit protein kinase C. Such studies would certainly evaluate whether α-tocopherol and its metabolites have specific molecular functions in addition to α-tocopherols role as a lipid antioxidant.

Other areas where further research is required is in the interconversion of the hydroquinone and benzoquinone forms of both α-TQ and α-tocopheronolactone and that of the urinary metabolites such as α-tocopheronolactone and α-CEHC. A full understanding of these interconversions is not only of interest in elucidating the possible production of artefacts in the analytical procedure but could be important in the possible regulation of bio-active compounds, such as unconjugated γ-CEHC, which has been shown to act as a natriuretic factor controlling the body's pool of extracellular fluid (Wechter et al., 1996).

# APPENDIX – Experimental details


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## A1.1. General materials and methods

All chemicals were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO) unless otherwise stated. Solvents and reagents were used without further purification except tetrahydrofuran (THF) which was dried over sodium. Reactions were monitored by TLC on precoated silica gel plates (Kieselgel 60 $F_{254}$, Merck Ltd.). Purification was performed by flash chromatography using silica gel (particle size 40-63 μM, Merck Ltd.). $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Bruker AMX-300 or a Bruker AMX-400 spectrometer. Chemical shifts are reported as ppm relative to tetramethylsilane as internal standard. Mass spectra were recorded on either a VG ZAB SE spectrometer (electron impact (EI) and fast atom bombardment (FAB)) or a Micromass Quattro electrospray (ESI) LC-mass spectrometer. High resolution mass spectra (HRMS) were recorded on a VG ZAB SE spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrophotometer. Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected.

HPLC was carried out using a Gilson 1100 preparative HPLC system and C18 column. The column was perfused at a flow rate of 20 ml/min with a mobile phase containing solvent A (0.1% trifluroacetic acid (TFA) and 3% propan-2-ol in water) and a linear gradient from 0-97% of solvent B (0.1% TFA and 3% propan-2-ol in acetonitrile) in 11.5 mins.

Examples of NMR data are given where indicated.

## A1.2. Synthesis of unconjugated α-tocopherol metabolites

### A1.2.1. Synthesis of (±)-2,5,7,8-tetramethyl-2-(2'-carboxyethyl-2)-6-hydroxy-chroman (α-CEHC) (9)

***γ-Methyl-γ-vinylbutyrolactone (8) (Kantoci et al., 1997)***

Vinyl magnesium bromide (1M in THF, 70 ml, 70 mmol) was added, dropwise at 0°C whilst stirring, to a solution of ethyl levulinate **(36)** (9.17 g, 64 mmol) in dry THF (10 ml) under nitrogen. The temperature was allowed to increase to room temperature overnight and the mixture was then diluted with an excess of water and acidified with 20% aqueous $KHSO_4$ to pH 4. The mixture was then extracted with ethyl acetate and the organic layer was washed successively with water, saturated aqueous $NaHCO_3$ and brine, and then dried over $MgSO_4$. The organic layer was concentrated under vacuum and the crude product was distilled to give γ-methyl-γ-vinylbutyrolactone **(8)** as a colourless oil (4.6 g, 57%). $^1$H NMR ($CDCl_3$) δ 1.15 (3H, s, $CH_3$), 1.70-1.89 (2H, m, $CH_2$), 2.37-2.43 (2H, m, $CH_2$), 4.79 (1H, d, $J$ = 10.6 Hz, $=CH_2$), 4.94 (1H, d, $J$ =17.3 Hz, $=CH_2$), 5.54 (2H, dd, $J$ =17.3 and 10.9 Hz, =CH); $^{13}$C NMR ($CDCl_3$) δ 26.2, 28.7, 33.7, 85.4, 113.5, 140.1, 176.6; MS (FAB +ve) *m/z* 149 (M+Na+).

***(±)-2,5,7,8-Tetramethyl-2-(2'-carboxyethyl-2)-6-hydroxychroman (α-CEHC) (9) (Kantoci et al., 1997)***

γ-methyl-γ-vinylbutyrolactone **(8)** (470 mg, 3.75 mmol) in dioxane (1 ml) was added, over 3 hours at 110°C whilst stirring, to a solution of 2,3,5-trimethylhydroquinone **(5)** (380 mg, 2.5 mmol) and boron trifluoride diethyl etherate (0.6 ml, 5 mmol) in dioxane (5 ml) under nitrogen. The reaction mixture was cooled to room temperature and diluted with an excess of ethyl acetate. The organic layer was then washed with water, dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by

flash chromatography on silica gel (1-5% methanol in chloroform) to give (±)-2,5,7,8-tetramethyl-2-(2'-carboxyethyl-2)-6-hydroxychroman (α-CEHC) (**9**) as a beige powder (480 mg, 69%). IR ($CH_2Cl_2$) 3700-2600 (acid+hydroxyl), 1700 (carbonyl) $cm^{-1}$; 1H NMR (MeOD) δ 1.20 (3H, s, $CH_3$), 1.82-1.76 (2H, m, $CH_2$), 1.84-2.0 (2H, m, $CH_2$), 2.04 (3H,s, $CH_3$), 2.08 (3H, s, $CH_3$), 2.11 (3H, s, $CH_3$), 2.39-2.47 (2H, m, $CH_2$), 2.58-2.63 (2H, m, $CH_2$); $^{13}C$ NMR (MeOD) δ 12.8 (C5a), 12.9 (C7a), 13.7 (C8a), 22.5 ($CH_2$), 24.5 (C2a), 30.5 ($CH_2$), 33.7 ($CH_2$), 36.4 ($CH_2$), 75.5 (C2), 118.9 (C5), 123.0 (C4a), 124.0 (C7), 125.4 (C8), 147.1 (C6), 147.3 (C8b), 178.7 ($C0_2H$); MS (EI) *m/z* 165 (100%), 278 (35%, $M^+$).

### A1.2.2 Synthesis of α-tocopheronolactone (12/38)

***Benzoquinone α-tocopheronolactone (12)***

Cerium sulphate ($CeSO_4$, 250 mg, 0.75 mmol) in 5% aqueous $H_2SO_4$ (2 ml) was added, at 0°C whilst stirring, to a solution of α-CEHC (**9**) (250 mg, 0.9 mmol) in methanol (75 ml). The reaction mixture was stirred at 0°C for 2 hours, diluted with an excess of water (250 ml) and extracted with ethyl acetate. The organic layer was washed with water, dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (0-3% methanol in chloroform) to give benzoquinone tocopheronolactone (**12**) as a yellow oil (180 mg, 72%). IR ($CH_2Cl_2$) 1766 (lactone), 1636 (quinone); $^1H$ NMR ($CDCl_3$) δ 1.46 (3H, s, $CH_3$), 1.68-1.75 (2H, m, $CH_2$), 2.00 (6H, s, 2x $CH_3$), 2.03 (3H, s, $CH_3$), 2.00-2.20 (1H, m, HC*H*), 2.21-2.28 (1H, m, *H*CH), 2.52-2.67 (4H, m, 2x $CH_2$); $^{13}C$ NMR ($CDCl_3$) δ 12.3, 12.5, 12.7 (3x$CH_3$), 21.6 ($CH_2$), 25.7 ($CH_3$), 29.3, 32.9, 39.5 (3x$CH_2$), 86.5 (C2), 140.7, 140.9, 141.1, 143.3 (4xArC), 176.9 (O-C=O), 187.3, 187.7 (2xC=O); MS (EI) *m/z* 99 (100%), 175, 203, 276 (57%, $M^+$).

**See figure A1.1(A) for $^{13}C$ NMR spectrum of α-tocopheronolactone benzoquinone.**

*Hydroquinone α-tocopheronolactone (38)*

Sodium dithionite ($Na_2S_2O_4$, 1.08 g, 6.3 mmol) in water (10ml) was added, at room temperature whilst stirring, to a solution of benzoquinone α-tocopheronolactone (**12**) (542 mg, 2.0 mmol) in ether (25ml). The reaction mixture was stirred at room temperature for 30 min and the two phases were then separated. The organic layer was washed with water, dried over $MgSO_4$ and concentrated under vacuum to give hydroquinone α-tocopheronolactone (**38**) as an oil (518 mg, 95%). IR ($CH_2Cl_2$) 3410 (hydroquinone), 1751 (lactone); $^1H$ NMR ($CDCl_2$) δ 1.51 (3H, s, $CH_3$), 1.79-1.85 (2H, m, $CH_2$), 2.01-2.12 (2H, m, 2xHC*H*), 2.16 (6H, s, 2x$CH_3$), 2.18 (3H, s, $CH_3$), 2.25-2.35 (2H, m, $CH_2$), 2.65-2.79 (2H, m, $CH_2$); $^{13}C$ NMR (MeOD) δ 13.3, 13.8, 13.9 (3x$CH_3$), 23.5 ($CH_2$), 26.5 ($CH_3$), 30.9, 34.3, 42.2 (3x$CH_2$), 89.9 (C2), 123.5, 124.4, 124.5, 128.3 (4xArC), 147.7, 148.4 (2xC-O), 180.6 (O-C=O); MS (EI) *m/z* 165 (100%), 203, 278 (31%, $M^+$); HPLC – 1 peak, RT 4.1 mins (86%).

**See figure A1.1(B) for $^{13}C$ NMR spectrum of α-tocopheronolactone hydroquinone.**

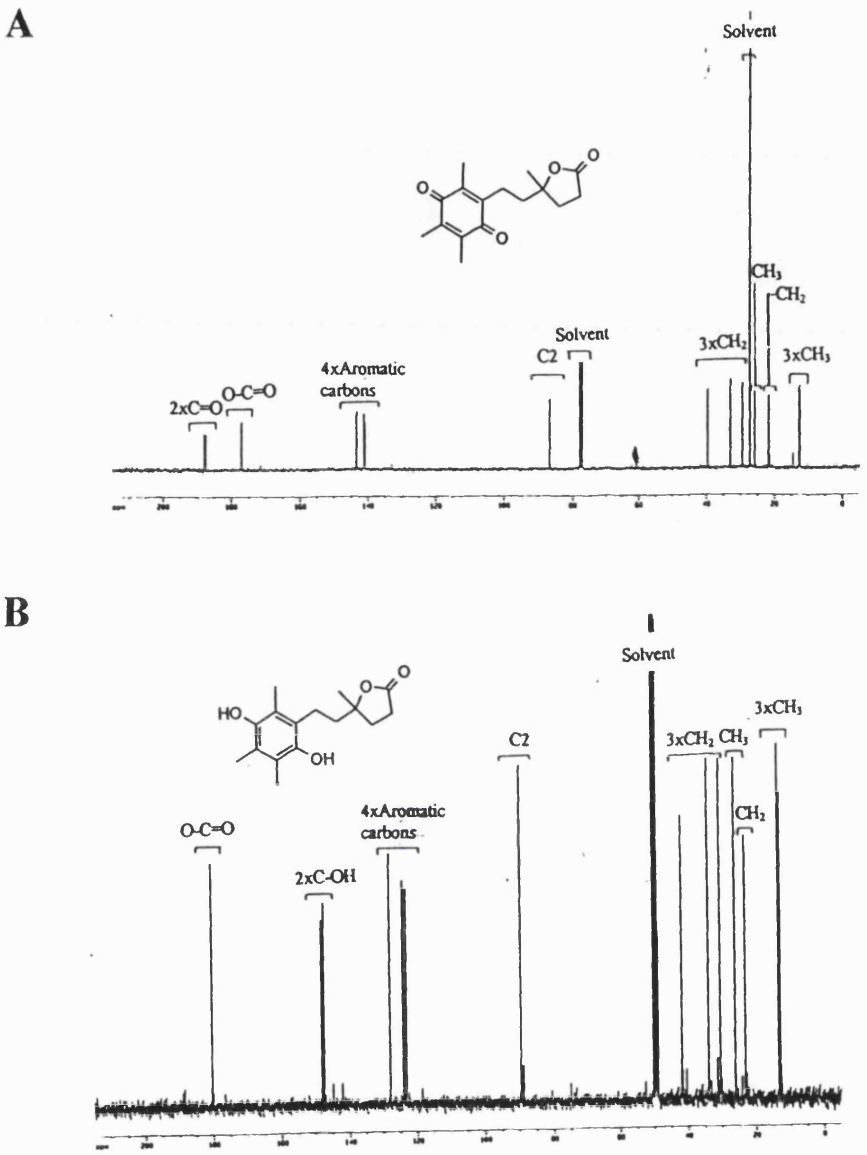


**Figure A1.1.** Comparison of the $^{13}C$ NMR of the benzoquinone (**A**) and hydroquinone (**B**) forms of α-tocopheronolactone. In proton decoupled $^{13}C$ NMR, as above, each chemically distinct carbon has a unique chemical shift value (x-axis). Therefore a compound such as α-tocopheronolactone, which contains 16 distinct carbon atoms, produces a $^{13}C$ NMR spectrum with 16 peaks. The $^{13}C$ NMR spectrum of the benzoquinone (**A**) contains two peaks at a chemical shift of 187 ppm, which correspond to the two C=O carbons. These two peaks are not present in the spectrum of the hydroquinone (**B**) and are replaced by two peaks at a chemical shift of 147 ppm, which correspond to the two C-OH carbons.

**A1.2.3. Synthesis of (±)-2,5,7,8-Tetramethyl-2-(3'-carboxybutyl-2)-6-hydroxy-chroman model compound (43)**

***Methyl 6-oxoheptanoate (41)***

Methyl iodide (4.3 ml, 69 mmol) was added, dropwise at room temperature whilst stirring, to a solution of 6-oxoheptanoic acid (**39**) (1 g, 6.9 mmol) and $K_2CO_3$ (1.44 g, 10.4 mmol) in 25 ml of a 4:1 (v/v) mixture of acetone and dioxane under nitrogen. The reaction was stirred at room temperature overnight, basified with 10% aqueous $K_2CO_3$ and extracted with ethyl acetate. The organic layer was washed with water, dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (1% methanol in chloroform) to give methyl 6-oxoheptanoate (**41**) as an oil (270 mg, 25%). $^1$H NMR ($CDCl_3$) δ 1.61 (4H, t, 2x$CH_2$), 2.13 (3H, s, $CH_3$), 2.32 (2H, t, $CH_2$), 2.44 (2H, t, $CH_2$), 3.66 (3H, s, $OCH_3$); $^{13}$C NMR ($CDCl_3$) δ 23.5, 24.7, 30.1, 34.1, 43.5, 51.8, 174.1, 208.7.

***Methyl 6-hydroxy-6-vinylheptanoate (42)***

Vinyl magnesium bromide (1M in tetrahydrofuran, 0.59 ml, 0.59 mmol) was added, dropwise at 0°C whilst stirring, to a solution of methyl 6-oxoheptanoate (**41**) (85 mg, 0.53 mmol) in THF (2ml) under nitrogen. The reaction was stirred at 0°C for 1.5 hour and then a few drops of deionised water were added. The mixture was then dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (1-3% methanol in chloroform) to give methyl 6-hydroxy-6 vinylheptanoate (**42**) as an oil (69 mg, 69% yield). $^1$H NMR (MeOD) δ 1.26 (2H, d, $CH_2$), 1.39 (1H, m, HCH), 1.52 (3H, m, $CH_3$), 2.15 (2H, d, $CH_2$), 2.34 (2H, m, $CH_2$), 2.53 (1H, m, HCH), 3.67 (3H, s, $OCH_3$), 5.03 (1H, dd, CH=), 5.20 (1H, dd, $CH_2$=), 5.91 (1H, dd, $CH_2$).

*(±)-2,5,7,8-Tetramethyl-2-(3'-carboxybutyl-2)-6-hydroxychroman methyl ester (43)*

Methyl 6-hydoxy-6-vinylheptanoate (**42**) (69 mg, 0.37 mmol) in dioxane (1 ml) was added, at 110°C whilst stirring, to a solution of 2,3,5-trimethylhydroquinone (**5**) (38 mg, 0.247 mmol) and boron trifluoride diethyletherate (60.9 μl, 0.494 mmol) in dioxane (5ml) under nitrogen. The reaction was stirred at 110°C for 3.5 hours and diluted with an excess of ethyl acetate. The organic layer was then washed with water, dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (0-1% methanol in chloroform) to give (±)-2,5,7,8-tetramethyl-2-(3'-carboxybutyl-2)-6-hydroxychroman methyl ester (**43**) as an oil (35 mg, 44%). $^1$H NMR (MeOD) δ 1.23 (3H, s, $CH_3$), 1.45-1.66 (6H, m, 3x$CH_2$), 1.73-1.86 (2H, m, $CH_2$), 2.07 (3H, s, $CH_3$), 2.11 (3H, s, $CH_3$), 2.15 (3H, s, $CH_3$), 2.35 (2H, t, $CH_2$), 2.62 (2H, t, $CH_2$), 3.66 (3H, s, $OCH_3$); $^{13}$C NMR ($(CD_3)_2CO$) δ 12.5, 12.8, 13.5 (3x$CH_3$), 22.1 ($CH_2$), 24.7 ($CH_3$), 24.8, 27.0, 33.2, 35.1, 40.6 (5x$CH_2$), 52.2 (MeO), 75.6 (C2), 118.6, 120.9, 123.3, 123.8 (4xArC), 146.7, 147.1 (2xC-O), 174.8 ($CO_2$Me); MS (EI) *m/z* 165 (100%), 203, 320 (41%, $M^+$).

**A1.2.4. Synthesis of (±)-2,5,7,8-tetramethyl-2(4'-carboxy-4'-methylbutyl)-6-hydroxychroman ((±)-α-CMBHC) (22)**

*6-oxo-2-methylheptanoic acid (19) (Weichet and Blaha, 1966a)*

2,6-dimethyl-cyclohexanone (**18**) (1 g, 7.9 mmol) was added, at room temperature whilst stirring, to a solution of potassium permanganate ($KMnO_4$, 1.76 g, 11.0 mmol) in water (60 ml). The reaction mixture was stirred at room temperature for 12 hours, acidified with 5% aqueous HCl and extracted in ethyl acetate. The organic layer was then washed with water, dried over $MgSO_4$ and concentrated under vacuum. The crude

product was purified by flash chromatography on silica gel (10-40% ethyl acetate in cyclohexane) to give 6-oxo-2-methylheptanoic acid (**19**) as a clear oil (1.0 g, 80%). IR (neat) 3700-2700 (acid), 1704 (carbonyl) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.2 (3H, d, $J$ = 7.2 Hz, CHMe), 1.40-1.49 (1H, m, CHMe), 1.57-1.68 (3H, m), 2,14 (3H, s, COMe), 2.43-2.51 (3H, m); $^{13}C$ NMR ($CDCl_3$) δ 17.1, 21.6, 30.2, 33.1, 39.5, 43.7, 183.0, 209.3; MS (FAB+) *m/z* 113 $[M-CO_2H]^+$, 141 $[M-OH]^+$, 159 $[M+H]^+$.

***Methyl 6-oxo-2-methylheptanoate (44)***

A few drops of concentrated $H_2SO_4$ were added, at room temperature whilst stirring, to a solution of 6-oxo-2-methylheptanoic acid (**19**) (1.0 g, 6.3 mmol) in dry methanol (30 ml) under nitrogen. The reaction mixture was refluxed for 2 hours and then an excess of ethyl acetate was added. The organic layer was then washed successively with saturated aqueous $NaHCO_3$ and deionised water, dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (10-30% ethyl acetate in cyclohexane) to give methyl 6-oxo-2-methylheptanoate (**44**) as an oil (820 mg, 76%). IR (neat) 1727 (carbonyl) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.11 (3H, d, $J$ = 6.8 Hz, CHMe), 1.35-1.45 (1H, m), 1.47-1.70 (3H, m), 2.09 (3H, s, COMe), 2.35-2.45 (3H, m), 3.63 (3H, s, $CO_2Me$); $^{13}C$ NMR ($CDCl_3$) δ 14.1, 17.0, 21.4, 29.7, 33.1, 39.2, 43.3, 51.4, 176.7, 208.2; MS (FAB+) *m/z* 113 $[M-CO_2Me]^+$ 141 $[M-OMe]^+$, 173 $[M+H]^+$.

***Methyl 6-hydroxy-6-vinyl-2-methylheptanoate (45)***

Vinyl magnesium bromide (1M in THF, 6.4 ml, 6.4 mmol) was added, dropwise at 0°C whilst stirring, to a solution of methyl 6-oxo-2-methylheptanoate (**44**) (1 g, 5.8 mmol) in THF (5 ml) under nitrogen. The reaction was stirred at 0°C for 2 hours and then a few

drops of water were added. The mixture was dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (10-30% ethyl acetate in cyclohexane) to give methyl 6-hydroxy-6-vinyl-2-methylheptanoate (**45**) as an oil and a mixture of two diastereoisomers (945 mg, 81%). IR (neat) 3416 (hydroxy) $cm^{-1}$, 1727 (carbonyl); $^1H$ NMR ($CDCl_3$) δ 1.13 (3H, d, *J* = 7.2 Hz, CHMe), 1.27 (3H, s, Me), 1.25-1.70 (6H, m, 3x$CH_2$), 2.37-2.49 (1H, m, CHMe), 3.65 (3H, s, OMe), 5.02 (1H, dd, *J* = 10.6, 1.1 Hz, $CH_2$=), 5.18 (1H, dd, *J* = 17.3, 1.1 Hz, CH=), 5.88 (1H, dd, *J* = 17.3, 10.6 Hz, $CH_2$=); $^{13}C$ NMR ($CDCl_3$) δ 17.3, 21.8, 27.8, 34.3, 39.6, 42.3, 51.7, 73.2, 111.8, 145.3, 177.5; second diastereoisomer 17.4, 28.0, 42.4; MS (FAB+) *m/z* 183 $[M-OH]^+$, 201 $[M+H]^+$.

***(±)-2,5,7,8-tetramethyl-2(4'-carboxy-4'-methylbutyl)-6-hydroxychroman methyl ester (α-CMBHC methyl ester) (46)***

Methyl 6-hydroxy-6-vinyl-2-methylheptanoate (**45**) (830 mg, 4.15 mmol) in dioxane (1 ml) was added, over 3 hours at 110°C whilst stirring, to a solution of 2,3,5-trimethylhydroquinone (**5**) (421 mg, 2.77 mmol) and boron trifluoride diethyletherate (680 µl, 5.5 mmol) in dioxane (15 ml) under nitrogen. The reaction mixture was cooled to room temperature and diluted with an excess of ethyl acetate. The organic layer was then washed with water, dried over $MgSO_4$ and concentrated under vacuum. The crude compound was purified by flash chromatography on silica (10-30% ethyl acetate in cyclohexane) to methyl *(±)-5-(*6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoate (α-CMBHC methyl ester) (**46**) as an oil and a mixture of two diastereoisomers (740 mg, 80%). IR (neat) 3439 (hydroxy), 1727 (carbonyl) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.16 (3H, d, *J* = 6.9 Hz, H4a'), 1.22 (3H, s, H2a), 1.41-1.71 (6H, m, 3x$CH_2$), 1.78 (2H, ddd, *J* = 6.4 12.5 13.4 Hz, $CH_2$), 2.11 (3H, s, H5a), 2.12 (3H, s,

H7a), 2.17 (3H, s, H8a), 2.41-2.50 (1H, m, H4'), 2.60 (2H, t, $J$ = 6.8 Hz, H4) 3.67 (3H, s, OMe); $^{13}$C NMR ($CDCl_3$) δ 11.6 (C5a), 12.1 (C7a), 12.6 (C8a), 17.3 (C4a'), 21.0 (C4), 21.5 ($CH_2$), 23.9 (C2a), 31.8 ($CH_2$), 34.5 ($CH_2$), 39.5 ($CH_2$), 39.6 (C4'), 51.8 (OMe), 74.6 (C2), 117.5 (C5), 119.0 (C4a), 121.6 (C7), 122.8 (C8), 145.0 (C6), 145.6 (C8b), 177.6 (C5'); second diastereoisomer 17.4 (C4a'), 21.6 ($CH_2$), 39.6 ($CH_2$), 39.7 (C4'), 51.9 (OMe), 177.7 (C5'); MS (EI) *m/z* 165 (100%), 334 (72%, $[M]^+$).

***(±)-2,5,7,8-tetramethyl-2(4'-carboxy-4'-methylbutyl)-6-hydroxychroman (α-CMBHC) (22)***

Sodium hydroxide (50 mg, 1.25 mmol) in water (10 ml) was added, at room temperature whilst stirring, to a solution of (±)-2,5,7,8-tetramethyl-2(4'-carboxy-4'-methylbutyl)-6-hydroxychroman methyl ester (**46**) (130 mg, 0.39 mmol) in methanol (10 ml). The reaction mixture was refluxed for 2 hours, acidified to pH 3 with 5% aqueous HCl and extracted with ethyl acetate. The organic layer was then washed with water, dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash chromatography on silica (20-30% ethyl acetate in cyclohexane) to yield (±)-2,5,7,8-tetramethyl-2(4'-carboxy-4'-methylbutyl)-6-hydroxychroman ((±)α-CMBHC) (**22**) as a solid and mixture of two diastereoisomers (60 mg, 48%). IR ($CHCl_3$) 3700-2600 (acid+hydroxy), 1697 (carbonyl) $cm^{-1}$; $^1$H NMR ($CDCl_3$) δ 1.16 (3H, d, $J$ = 6.4 Hz, H4a'), 1.23 (3H, s, H2a), 1.41-1.61 (6H, m, 3x$CH_2$), 1.79 (2H, ddd, $J$ = 6.6 12.1, 13.4 Hz, $CH_2$), 2.12 (6H, s, H5a and H7a), 2.16 (3H, s, H8a), 2.46-2.55 (1H, m, H4'), 2.61 (2H, t, $J$ = 6.4 Hz, H4); $^{13}$C NMR ($CDCl_3$) δ 11.6 (C5a), 12.1 (C7a), 12.6 (C8a), 17.2 (C4'a) , 21.0 (C3'), 21.6 ($CH_2$), 24.0 (C2a), 31.9 ($CH_2$), 34.2 ($CH_2$), 39.7 (C4'), 39.8 ($CH_2$), 74.6 (C2), 117.6 (C5), 119.0 (C4a), 121.5 (C7), 122.9 (C8), 144.9 (C6), 145.7 (C8b), 183.5 ($CO_2H$); second diastereoisomer 17.3 (C4'a), 21.7

($CH_2$), 39.7 ($CH_2$), 39.8 (C4'); MS (EI) *m/z* 165 (100%), 320 (22%, $[M]^+$); HRMS FAB calculated for $C_{19}H_{28}O_4$ (M-H) 319.1908, found to be 319.1881.

## A1.3. Conjugation of the α-tocopherol metabolites

### A1.3.1. Synthesis of glucuronide donors

***Methyl 1,2,3,4-tetra-O-acetyl-α/β-D-glucopyranuronate (48) (Leu et al., 1999)***

Glucurono-γ-lactone (**47**) (5 g, 28.4 mmol) was added whilst stirring to a solution of sodium methoxide (40 mg, 0.74 mmol) in dry methanol (50 ml) under nitrogen. The reaction mixture was stirred at room temperature for 1 hour then concentrated. The viscous residue was dissolved in acetic anhydride (35 ml) and perchloric acid (0.15 ml dissolved in 1ml of acetic anhydride) was added dropwise (taking care that the reaction temperature never exceeded 40°C). The reaction was stirred at room temperature for 24 hours then further perchloric acid was added (50μl). The mixture was stored overnight at 4°C and was then poured onto 100 g of crushed ice and neutralised with sodium bicarbonate. The excess sodium bicarbonate was removed by filtration and the filtrate extracted with chloroform. The organic layer was then washed with water, dried over $MgSO_4$ and concentrated under vacuum. The crude product was recrystallised in methanol to give an anomeric mixture of methyl α/β-1,2,3,4-tetra-*O*-acetyl-D-glucopyranuronate (**48**) as a solid (5.08 g, 48 %). $^1H$ NMR ($CDCl_3$) β-anomer δ 1.88 (3H, s, $CH_3$), 1.91 (6H, s, 2x$CH_3$), 2.06 (3H, s, $CH_3$), 3.61 (3H, s, $OCH_3$), 4.29 (1H, d, J=10 Hz, CH), 4.94-5.12 (2H, m, 2xCH), 5.38 (1H, t, J=10 Hz, CH), 6.25 (1H, d, J=3.5 Hz, CH); α-anomer δ 4.11 (1H, d, J=10 Hz, CH), 5.15-5.26 (1H, m, CH), 5.66 (1H, d, J=7.9 Hz, CH).

*1-hydroxy-methyl 2,3,4-Tri-O-acetyl-α/β-D-glucopyranuronate (49) (Nudelman et al., 1987)*

Tin methoxide ($Bu_3SnOMe$, 1.45 ml, 5.0 mmol) was added whilst stirring to a solution of methyl α/β-1,2,3,4-tetra-O-acetyl-D-glucopyranuronate (**48**) (1.86 g, 4.9 mmol) in THF (15 ml) under nitrogen. The reaction was refluxed for 1.5 hours, cooled and a few drops of 5% aqueous HCl added. The mixture was then extracted with ethyl acetate, dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash chromatography on a silica gel (40-50% ethyl acetate in cyclohexane) to give an anomeric mixture (5:1 α:β mixture) of methyl-2,3,4-tri-O-acetyl-α/β-D-glucopyranuronate (**49**) as a colourless oil (0.95 g, 58 %). $^1H$ NMR ($CDCl_3$) α-anomer δ 2.03 (3H, s, $CH_3$), 2.04 (3H, s, $CH_3$), 2.08 (3H, s, $CH_3$), 3.74 (3H, s, $OCH_3$), 4.07-4.14 (1H, m, OH), 4.58 (1H, d, J=10 Hz, CH), 4.89 (1H, dd, J=3.5, 10 Hz, CH), 5.17 (1H, t, J=10 Hz, CH), 5.53-5.61 (2H, m, 2xCH); $^{13}C$ NMR ($CDCl_3$) α-anomer δ 20.7, 20.7, 20.9 (3xCO-$CH_3$), 53.1 ($CO_2CH_3$), 68.1, 69.4, 69.8, 71.0 (4xCH), 90.3 (C-1), 168.9, 169.9, 170.4 (3x$COCH_3$), 170.5 ($CO_2CH_3$); MS (FAB+) *m/z* 334 (M+H).

*Methyl 2,3,4-tri-O-acetyl-1-O-(trichloroacetimidoyl)-β-D-glucopyranuronate (50) (Brown et al., 1997; Ferguson et al., 2000)*

1,8-Diazabicyclo [5.4.0] undec-7-ene (DBU, 30 µl, 0.2 mmol) was added, at 0°C whilst stirring, to a solution of trichloroacetonitrile (360 µl, 3.6 mmol) and 1-hydroxysugar (**49**) (238 mg, 0.71 mmol) in dry dichloromethane (10 ml) under nitrogen. The reaction was stirred at 0°C for a further 30 minutes and then concentrated. The crude product was purified by flash chromatography on a short silica gel column (30-40% ethyl acetate in cyclohexane) to give methyl 2,3,4-tri-O-acetyl-1-O-(trichloroacetimidoyl)-β-D-glucopyranuronate (**50**) as an oil (200 mg, 59%). $^1H$ NMR ($CDCl_3$) δ 2.01 (3H, s,

$CH_3$), 2.05 (6H, s, 2x$CH_3$), 3.74 (3H, s, $OCH_3$), 4.49 (1H, d, J=10 Hz, CH), 5.14 (1H, dd, J=3.8, 10 Hz, CH), 5.26 (1H, t, J=9.8 Hz, CH), 5.63 (1H, t, J=9.8 Hz, CH), 6.63 (1H, d, J=3.8 Hz, CH), 8.73 (1H, s, NH) ; $^{13}C$ NMR:- δ ($CDCl_3$) 20.6, 20.7, 20.9 (3xCO-$CH_3$), 53.2 ($OCH_3$), 69.1, 69.2, 69.6, 70.6 (4xCH), 90.7 ($CCl_3$), 92.7 (CH), 160.6, 167.3, 169.7, 169.9, 170.1 (3x$COCH_3$ + $CO_2CH_3$ + CONH); MS (FAB+) *m/z* 500/502/504 (M+Na).

### A1.3.2. Synthesis of the glucuronide conjugates

***General procedure for the glucuronidation of α-tocopherol metabolites (Brown et al., 1997)***

Boron trifluoride-diethyl etherate (0.25-1.25 eq.) was added at -15°C (on dry ice) to a stirred suspension of α-tocopherol metabolite (1 eq.) and trichloroacetimidate **(50)** (1 eq.) in dry dichloromethane under nitrogen. The reaction was stirred at -15°C for 1.5 hour before further boron trifluoride-diethyl etherate (0.25-1.25 eq.) was added. The mixture was allowed to warm to room temperature overnight and was then diluted with an excess of dichloromethane, washed successively with saturated aqueous $NaHCO_3$ and water and concentrated. The crude product was purified by flash chromatography on a silica gel column (30-70% ethyl acetate in cyclohexane) to give the protected glucuronide conjugate of the α-tocopherol metabolite.

***Methyl [1-(2,3,5-trimethylhydroquinone)-2,3,4-tri-O-acetyl-β-D-glucopyran] uronate (53)***

The reaction of boron trifluoride-diethyl etherate (15 μl, 0.12 mmol) with 2,3,5-trimethylhydroquinone **(5)** (38mg, 0.25 mmol) and trichloroacetimidate **(50)** (120 mg, 0.25 mmol) in dry dichloromethane (5 ml) gave methyl [1-(2,3,5-

trimethylhydroquinone)-2,3,4-tri-O-acetyl-β-D-glucopyran] uronate (**53**) as a semi solid (45 mg, 40%). $^{13}C$ NMR (MeOD) δ 12.6, 12.7, 16.6 (3x$CH_3$), 21.0, 21.1, 21.5 (3xCO-$CH_3$), 53.3 ($OCH_3$), 69.7, 71.5, 72.4, 72.9 (4xCH), 101.3 (O-C-O), 117.0 (CHAr), 121.1, 123.8, 126.4 (3xArC), 148.6, 148.9 (2xArC-O), 167.4, 169.6, 169.8, 170.6 (3x$COCH_3$ + $CO_2CH_3$); MS (ESI +ve) *m/z* 491 (M+Na).

***(±)-2,5,7,8-tetramethyl-2-(2'-carboxyethyl-2)-6-hydroxychroman methyl ester* (54)**

A few drops of concentrated $H_2SO_4$ was added, at room temperature whilst stirring, to a solution of α-CEHC (**9**) (220 mg, 0.79 mmol) in dry methanol (5 ml) under nitrogen. The reaction mixture was refluxed for 2 hours and then an excess of ethyl acetate was added. The organic layer was washed successively with saturated aqueous $NaHCO_3$ and water, dried over $MgSO_4$ and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (10-30% ethyl acetate in cyclohexane) to give α-CEHC-Me (**54**) as an oil (116 mg, 50%). IR (film), $^1H$ NMR ($CDCl_3$) δ 1.22 (3H, s, $CH_3$), 1.77-2.02 (4H, m, 2x$CH_2$), 2.09 (3H, s, $CH_3$), 2.11 (3H, s, $CH_3$), 2.16 (3H, s, $CH_3$), 2.47-2.53 (2H, m, $CH_2$), 2.59-2.66 (2H, m, $CH_2$), 3.66 (3H, s, $OCH_3$); $^{13}C$ NMR ($CDCl_3$) δ 11.5, 12.0, 12.5 (3x$CH_3$), 20.8 ($CH_2$), 23.5 ($CH_3$), 28.8, 31.9, 34.6 (3x$CH_2$), 51.9 ($CO_2CH_3$), 73.6 (C2), 117.2, 119.3, 121.9, 122.7 (4xArC), 145.2, 145.2 (2xC-O), 174.8 ($CO_2CH_3$); MS (EI ) *m/z* 165 (95%), 292 (100%, $M^+$).

***Methyl [1-(α-CEHC-methyl ester)-O-2,3,4-tri-O-acetyl-β-D-glucopyran] uronate (55)***

The reaction of boron trifluoride-diethyl etherate (50 μl, 0.4 mmol) with α-CEHC methyl ester (**54**) (116 mg, 0.4 mmol) and trichloroacetimidate (**50**) (191 mg, 0.4 mmol) in dry dichloromethane (10 ml) gave methyl [1-(α-CEHC-methyl ester)-O-2,3,4-tri-O-acetyl-β-D-glucopyran] uronate (**55**) (104 mg, 42%). $^1H$ NMR ($CDCl_3$) δ 1.21 (3H, s,

$CH_3$), 1.71-1.92 (4H, m, 2x$CH_2$), 2.01 (3H, s, $CH_3$), 2.04 (6H, s, 2x$CH_3$), 2.10 (3H, s, $CH_3$), 2.12 (3H, s, $CH_3$), 2.16 (3H, s, $CH_3$), 2.42-2.51 (2H, m, $CH_2$), 2.56-2.61 (2H, m, $CH_2$), 3.65 (3H, s, $OCH_3$), 3.67 (3H, s, $OCH_3$), 3.84 (1H, d, J=10 Hz, *CH*$CO_2CH_3$), 4.72-4.77 (1H, m, CH), 5.14-5.35 (3H, m, 3xCH-OAc); $^{13}$C NMR ($CDCl_3$) (epimer peaks in brackets) δ 12.2, 12.9 (13.1), 13.7 (13.8), 20.9, 21.0, 21.4, 23.8 (7x$CH_3$), 27.3, 28.9, 31.7, 34.9 (4x $CH_2$), 52.0, 53.1 (2x$CO_2CH_3$), 69.9, 72.1, 72.6, 72.7 (4xCH), 74.2 (C2), 102.1 (102.2) (O-C-O), 117.7, 123.4, 127.3 (127.6), 128.8 (129.1) (4xArC), 145.7, 148.7 (2xC-O), 167.5, 169.6, 169.9, 170.6, 174.7 (3x*C*$OCH_3$ + 2x$CO_2CH_3$); MS (FAB+) *m/z* 608 (M+H).

***1-(α-CEHC)-β-D-glucopyranosiduronic acid (56)***

Sodium hydroxide (34 mg, 0.85 mmol) in water (5ml) was added whilst stirring to a solution of the methyl [1-(α-CEHC-methyl ester)-O-2,3,4-tri-O-acetyl-β-D-glucopyran] uronate (**55**) (100 mg, 0.16 mmol) in methanol (5 ml). The reaction was refluxed for 2 hours, diluted with water (2 ml), acidified with acetic acid to pH 6 and concentrated. The crude compound was purified by chromatography on a C18 SPE cartridge, eluting with methanol/water (1:1 v/v), to give the glucuronide (**56**) as a yellow semi-solid (95 mg, 75%). $^1$H NMR (MeOD) δ 1.23 (3H, s, $CH_3$), 1.70-1.95 (4H, m, 2x$CH_2$), 2.03 (3H, s, $CH_3$), 2.21 (3H, s, $CH_3$), 2.24 (3H, s, $CH_3$), 2.25-2.45 (2H, m, $CH_2$), 2.50-2.70 (2H, m, $CH_2$), 3.40-3.70 (4H, m, 4xCH), 4.62 (1H, d, J=7.7 Hz, CH); $^{13}$C NMR (MeOD) δ 12.5, 13.7 (13.7), 14.6 (14.7) (3x$CH_3$), 22.1 ($CH_2$), 24.5 ($CH_3$), 28.4, 33.1, 37.5 (3x$CH_2$), 74.2, 75.3, 75.8 (3xCH), 76.1 (C2), 78.1 (CH), 106.6 (O-C-O), 118.9, 123.9, 128.4 (128.5), 130.1 (130.2) (4xArC), 147.9, 149.6 (Ar-O), 180.9, 183.3 (2x$CO_2H$); MS (ESI-) *m/z* 453 (M-H); HPLC – 1 peak, RT=3.0 min (95%); GC-MS

after deconjugation: 2 major peaks, RT=21.0 min (80%, EI-MS *m/z* 422 (100%), 237 (86%)) and RT = 24.1 min (20%, EI-MS *m/z* 422 (100%), 309 (23%), 237 (35%)).

**See figure A1.2 for NMR of α-CEHC-glucuronide.**

***Methyl [1-(α-tocopheronolactone)-2,3,4-tri-O-acetyl-β-D-glucopyran] uronate (58)***

The reaction of boron trifluoride-diethyl etherate (200 µl, 1.6 mmol) with α-tocopheronolactone hydroquinone (**38**) (176 mg, 0.63 mmol) and trichloroacetimidate (**50**) (303 mg, 0.63 mmol) in dry dichloromethane (10 ml) gave methyl [1-(α-tocopheronolactone)-2,3,4-tri-O-acetyl-β-D-glucopyran] uronate (**58**) (56 mg, 15%). $^{1}H$ NMR ($CDCl_3$) δ 1.52 (3H, s, $CH_3$), 1.68-2.00 (4H, m, 2x$CH_2$), 2.02 (3H, s, $CH_3$), 2.05 (3H, s, $CH_3$), 2.12 (6H, s, 2x$CH_3$), 2.15 (3H, s, $CH_3$), 2.17 (3H, s, $CH_3$), 2.27-2.92 (4H, m, 2x$CH_2$), 3.68 (3H, s, $OCH_3$), 3.86 (1H, d, J=9.8 Hz, *CH*$CO_2CH_3$), 4.82 (1H, d, J=7.5 Hz, CH), 5.14-5.36 (3H, m, 3xCH-OAc); $^{13}C$ NMR ($CDCl_3$) δ 13.7, 14.1, 14.9, 15.3, 21.3 (21.4), 21.6, 21.7 (7x$CH_3$), 30.9, 34.3, 41.8 (3x$CH_2$), 54.0 ($CO_2CH_3$), 62.4 ($CH_2$), 71.9, 73.8, 74.1, 74.3 (4xCH), 89.9 (C2), 103.7 (O-C-O), 124.3, 128.4, 129.2, 130.2 (4xArC), 148.5, 151.3 (ArC-O), 169.9, 171.8 (172.1), 172.3, 173.8 (3x*C*$OCH_3$ + $CO_2CH_3$), 180.6 (O-C=O); MS (FAB+) *m/z* 617 (M+Na).

***1-(α-tocopheronolactone)-β-D-glucupyranosiduronic acid (57)***

Sodium carbonate ($Na_2CO_3$, 15 mg, 0.14 mmol) in water (1ml) was added whilst stirring to a solution of the methyl [1-(α-tocopheronolactone)-2,3,4-tri-O-acetyl-β-D-glucopyran] uronate (**58**) (31 mg, 0.05 mmol) in methanol (3 ml). The reaction was refluxed for 5 hours, diluted with water (2 ml), acidified with acetic acid to pH 6 and concentrated. The crude compound was purified by chromatography on a C18 SPE

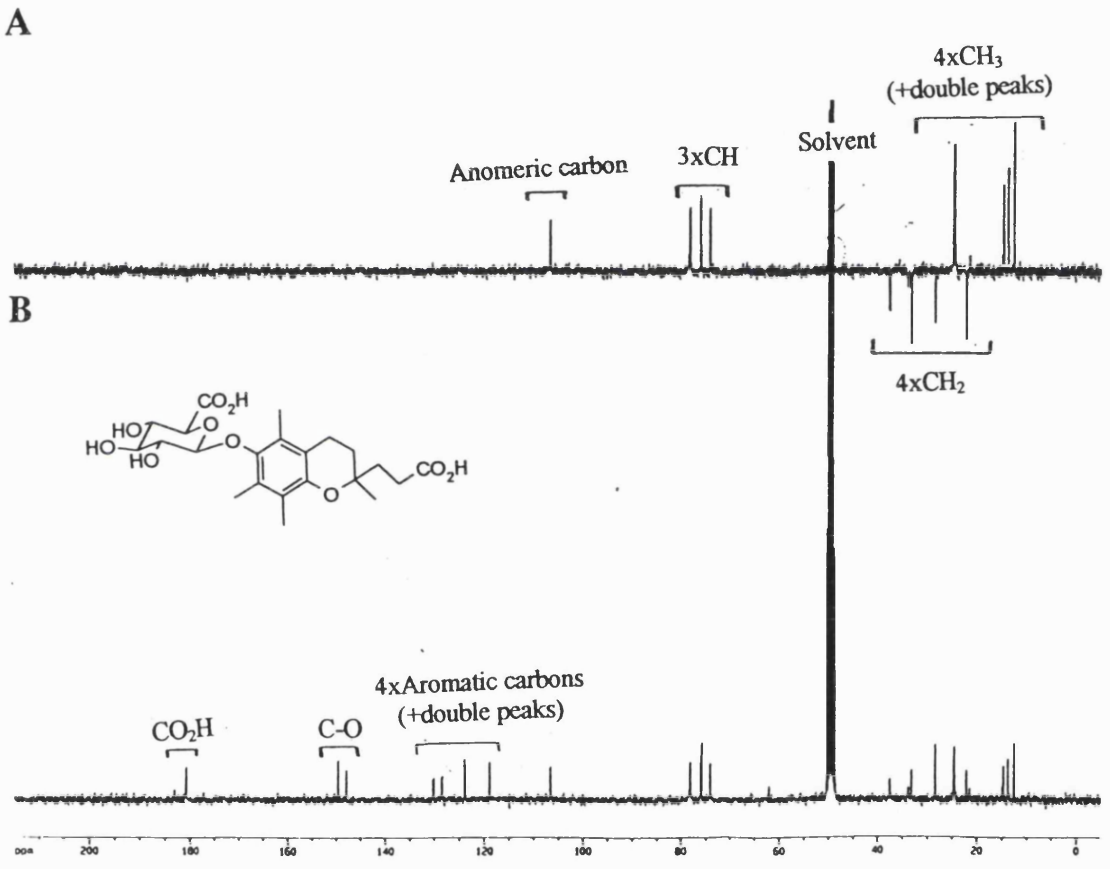


**Figure A1.2.** DEPT (distortionless enhancement by polarisation transfer) $^{13}C$ NMR of α-CEHC-glucuronide.

DEPT spectra allow CH, $CH_2$ and $CH_3$ groups to be discriminated. In spectrum **A** above, peaks for CH and $CH_3$ groups appear above the line whereas peaks for $CH_2$ groups appear below the line. Double peaks in spectra **A** and **B** indicate that α-CEHC-glucuronide is present as a mixture of diastereoisomers.

cartridge, eluting with methanol/water (1:1 v/v), to give the glucuronide (**57**) as an orange-yellow semi-solid (10 mg, 40%). $^1$H NMR (MeOD) δ 1.47 (3H, s, $CH_3$), 1.77-2.13 (4H, m, 2x$CH_2$), 2.09 (3H, s, $CH_3$), 2.23 (3H, s, $CH_3$), 2.27 (3H, s, $CH_3$), 2.33-2.77 (4H, m, 2x$CH_2$), 3.40-3.58 (4H, m, 4xCH), 4.49 (1H, d, J=7.6 Hz, $CH_3$); MS (ESI-) *m/z* 453 (M-H). FAB HRMS: calculated (M-H) 454.1794, found 454.1762 ($^{12}C_{21}{}^{13}C_1H_{30}O_{10}$); GC-MS after deconjugation: 1 major peak, RT = 24.1 min - EI-MS *m/z* 422 (100%), 309 (23%), 237 (35%).

### A1.3.3. Synthesis of the sulphate conjugates

***General procedure for the sulphation of α-tocopherol metabolites (Dusza et al., 1968)***

The α-tocopherol metabolite (1 eq.) in dry pyridine was added whilst stirring to a solution of trimethylamine-sulphur trioxide complex ($Me_3N.SO_3$, 1.1eq.) in dry pyridine under nitrogen. The reaction was stirred at room temperature for 3 hours then the mixture was poured onto an excess of diethyl ether. The precipitate formed was filtered and washed with diethyl ether. The crude product was purified by flash chromatography on a short silica gel column (20% methanol in chloroform) to give the trimethylamine salt of the sulphate.

***Trimethylamine 2,3,5-trimethylhydroquinone monosulphate model compound (67/68)***

The reaction of 2,3,5-trimethylhydroquinone (**5**) (162 mg, 1.1 mmol) with $Me_3N.SO_3$ (163 mg, 1.2 mmol) in pyridine (4 ml) following the general procedure outlined above, produced the two forms of trimethylamine 2,3,5-trimethylhydroquinone monosulphate (**67/68**) as a solid (110 mg, 43%). $^1$H NMR (MeOD) δ 2.11 (3H, s, $CH_3$), 2.18 (3H, s, $CH_3$), 2.21 (3H, s, $CH_3$), 2.23 (3H, s, $CH_3$), 2.29 (3H, s, $CH_3$), 2.33 (3H, s, $CH_3$), 2.85 (18H, s, 6x$NCH_3$), 6.51 (1H, s, CHAr), 7.03 (1H, s, CHAr); $^{13}$C NMR (MeOD) δ 13.3,

13.6, 14.3, 15.5, 17.6, 18.5 (6x$CH_3$), 46.2 (NMe), 56.1 (NMe), 115.9, 123.0 (2xCHAr), 123.1, 124.2, 126.5, 130.1, 131.7, 134.0 (6xArC), 144.6, 145.6 (2xC-O), 152.1, 153.0 (2xC-$OSO_3$); MS (ESI -ve) *m/z* 231 (M-H).

***Trimethylamine (±)-2,5,7,8-tetramethyl-2-(2'-carboxyethyl-2)-6-hydroxychroman sulphate (α-CEHC sulphate) (70)***

The reaction of (±)-2,5,7,8-tetramethyl-2-(2'-carboxyethyl-2)-6-hydroxychroman **(9)** (153 mg, 0.55 mmol) with $Me_3N.SO_3$ (84 mg, 0.60 mmol) in pyridine (4 ml) gave trimethylamine (±)-2,5,7,8-tetramethyl-2-(2'-carboxyethyl-2)-6-hydroxychroman sulphate (α-CEHC $Me_3N$-sulphate) **(70)** as a pale yellow solid (195 mg, 85%). IR 2700-3700 (acid), 1700 (carbonyl), 1232 and 1001 (sulphate) $cm^{-1}$; $^1H$ NMR:- δ 1.24 (3H, s, $CH_3$), 1.77-1.99 (4H, m, 2x$CH_2$), 2.08 (3H, s, $CH_3$), 2.25 (3H, s, $CH_3$), 2.28 (3H, s, $CH_3$), 2.40-2.51 (2H, m, $CH_2$), 2.63 (2H, t, J=6 Hz, $CH_2$), 2.85 (9H, s, 3x$NCH_3$); $^{13}C$ NMR:- δ (MeOD) 12.9, 14.6, 15.5 (C5a, C7a, C8a), 22.2 ($CH_2$), 24.6 (C2a), 30.3 ($CH_2$), 33.1 ($CH_2$), 36.2 ($CH_2$), 76.0 (C2), 119.4, 124.5, 129.8, 131.5 (C5, C4a, C7, C8), 144.5 (C8b), 150.4 (C6), 178.2 ($CO_2H$); MS (ESI -ve) *m/z* 357 (M-H); FAB HRMS: calculated (M-H) 357.1008, found 357.1005 ($C_{16}H_{22}O_4$). GC-MS after deconjugation: 2 major peaks, RT=21.0 min (65%, EI-MS *m/z* 422 (100%), 237 (86%)) and RT = 24.1 min (35%, EI-MS *m/z* 422 (100%), 309 (23%), 237 (35%)).

**See figure A1.3 for $^1H$ NMR spectrum of α-CEHC-sulphate.**

***Trimethylamine α-tocopheronolactone monosulphate (71/72)***

The reaction of α-tocopheronolactone hydroquinone **(38)** (120 mg, 0.43 mmol), $Me_3N.SO_3$ (66 mg, 0.47 mmol) in pyridine (4 ml) following the general procedure gave

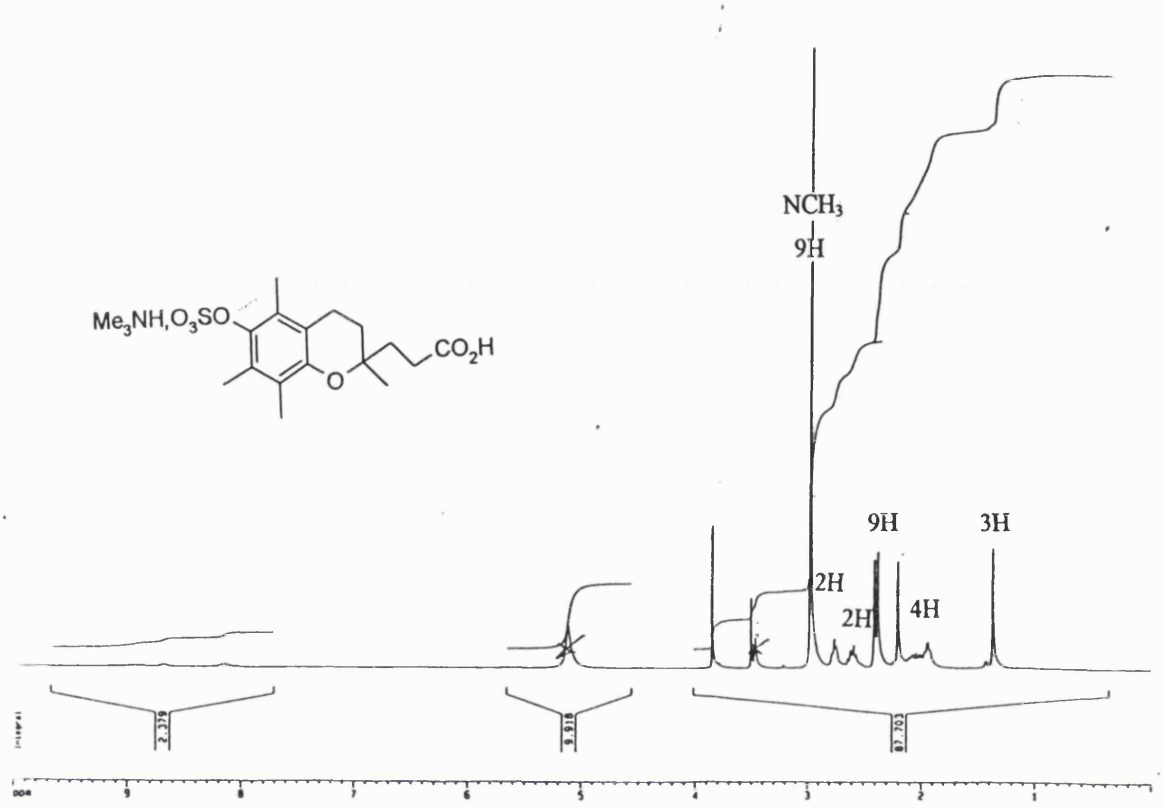


**Figure A1.3.** $^{1}H$ NMR spectrum of α-CEHC-sulphate.

Different types of protons have different chemical shifts but unlike carbons in $^{13}C$ NMR, each proton does not necessarily produce a single peak in $^{1}H$ NMR. Protons or groups of protons can produce singlet (s), doublet (d), triplet (t) or multiplet (m) peaks, depending on their interactions with neighbouring protons. The area under a $^{1}H$ NMR peak, whether a singlet or a multiplet, is proportional to the number of hydrogens which that resonance represents.

In the above spectrum, integration of the large singlet peak at ~2.9 indicates it represents 9 protons and its chemical shift is consistent with the protons of trimethylamine. The other peaks are consistent with sulphated α-CEHC, confirming that the compound is the trimethylamine salt of α-CEHC-sulphate.

trimethylamine α-tocopheronolactone monosulphate (**71/72**) as a dark green semi-solid (16 mg, 13%). IR ($CH_2Cl_2$) 3000-3500 (hydroxyl), 1751 (lactone), 1239 and 1015 (sulphate); $^1$H NMR (MeOD) δ 1.47 (3H, s, $CH_3$), 1.75-2.05 (4H, m, 2x$CH_2$), 2.11 (6H, s, 2x$CH_3$), 2.15 (3H, s, $CH_3$), 2.24 (6H, s, 2x$CH_3$), 2.29 (3H, s, $CH_3$), 2.60-2.95 (4H, m, 2x$CH_2$), 3.30 (18H, s, 2x3x$NCH_3$); MS (ESI -ve) *m/z* 357 (M-H). GC-MS after deconjugation: 1 major peak, RT = 24.1 min - EI-MS *m/z* 422 (100%), 309 (23%), 237 (35%).

**See figure A1.4. for $^1$H NMR spectrum of α-tocopheronolactone-sulphate.**

***Trimethylamine α-CMBHC sulphate (73)***

The reaction of α-CMBHC (**22**) (40 mg, 0.13 mmol) with $Me_3N.SO_3$ (19.3 mg, 0.14 mmol) in pyridine (4 ml) gave trimethylamine α-CMBHC sulphate (**73**) as a yellow semi-solid. MS (ESI –ve) *m/z* 399 (M-H); GC-MS after deconjugation: 2 peaks, RT=26.6 min(98%, EI-MS *m/z* 464 (100%), 237 (92%)) and RT = 28.7 min (2%, EI-MS *m/z* 626 (34%), 536 (24%), 309 (100%)).

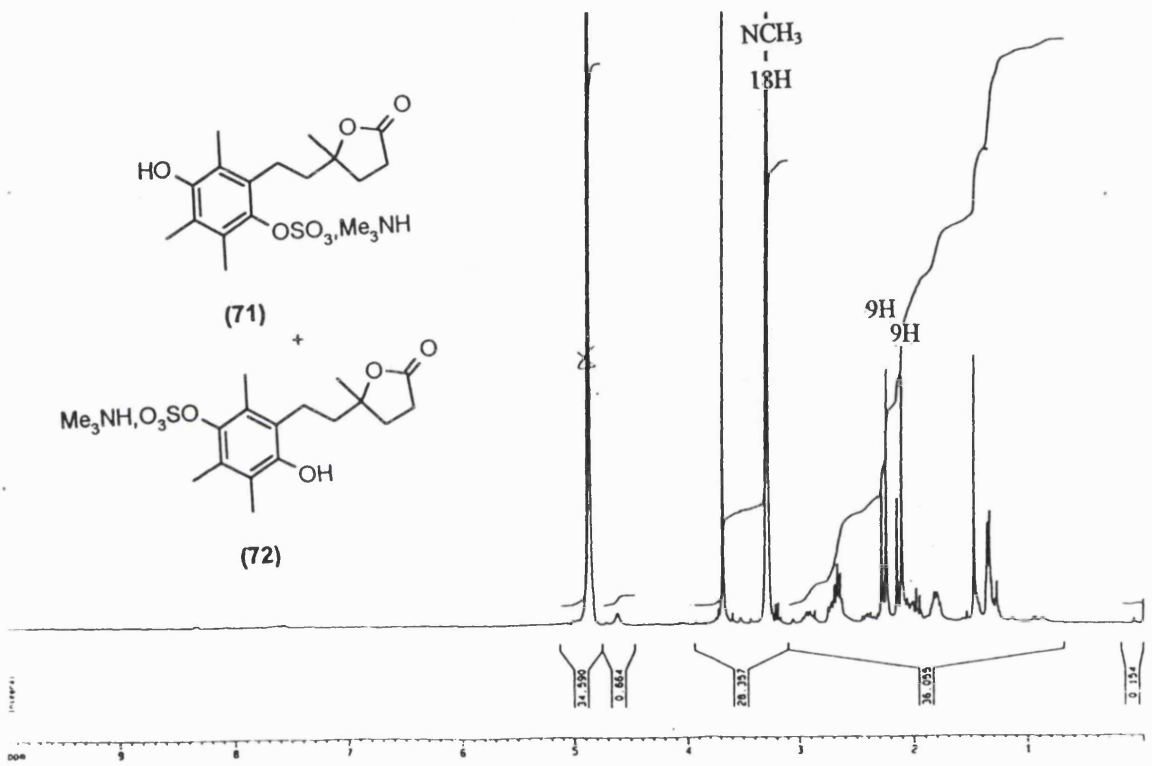


**Figure A1.4.** $^{1}H$ NMR spectrum of α-tocopheronolactone-sulphate.

The $^{1}H$ NMR spectrum above indicates that two isomers of α-tocopheronolactone sulphate are present. Integration of the large singlet at 3.3 ppm indicates that it represents 18 protons, as opposed to the expected 9 protons for a single isomer, and the double set of peaks at 2.11-2.15 and 2.24 -2.29 also indicates that two isomers are present.

# BIBLIOGRAPHY

Aratri E, Spycher SE, Breyer I, and Azzi A (1999) Modulation of alpha-tropomyosin expression by alpha-tocopherol in rat vascular smooth muscle cells. *Febs Letters* **447**:91-94.

Arita M, Sato Y, Miyata A, Tanabe T, Takahashi E, Kayden HJ, Arai H, and Inoue K (1995) Human alpha-tocopherol transfer protein: cDNA cloning, expression and chromosomal localization. *Biochem.J.* **306 ( Pt 2)**:437-443.

Arya P, Alibhai N, Qin H, Burton GW, Batist G, You SXL, and Alaoui-Jamali MA (1998) Design and synthesis of analogs of vitamin E: Antiproliferative activity against human breast adenocarcinoma cells. *Bioorganic & Medicinal Chemistry Letters* **8**:2433-2438.

Azzi A, Breyer I, Feher M, Pastori M, Ricciarelli R, Spycher S, Staffieri M, Stocker A, Zimmer S, and Zingg JM (2000) Specific cellular responses to alpha-tocopherol. *J.Nutr.* **130**:1649-1652.

Azzi A, Breyer I, Feher M, Ricciarelli R, Stocker A, Zimmer S, and Zingg J (2001) Non-antioxidant functions of alpha-tocopherol in smooth muscle cells. *J.Nutr.* **131**:378S-381S.

Azzi A and Stocker A (2000) Vitamin E: non-antioxidant roles. *Prog.Lipid Res.* **39**:231-255.

Badman GT, Green DVS, and Voyle M (1990) A novel synthesis of beta-phenylglucuronides using the mitsunobu reaction - an application of phenolic chromium tricarbonyl complexes. *J.Organomet.Chem.* **388 (1-2)**:117-121.

Bergman A (1986) Synthesis of xenobiotic conjugates, in *Xenobiotic conjugation chemistry* (Paulson G ed) pp 124-158, American Chemical Society.

Berrang B, Brine GA, and Carroll FI (1997) Synthesis of morphine-3,6-di-beta-D-glucuronide. *Synthesis-Stuttgart* **Oct**:1165-1168.

Bienayme H, Ancel JE, Meilland P, and Simonato JP (2000) Rhodium(I)-catalyzed addition of phenols to dienes. A new convergent synthesis of vitamin E. *Tetrahedron Letters* **41**:3339-3343.

Birringer M, Drogan D, and Brigelius-Flohe R (2001) Tocopherols are metabolized in HepG2 cells by side chain omega- oxidation and consecutive beta-oxidation. *Free Radic.Biol.Med.* **31**:226-232.

Bollenback G, Long J, Benjamin D, and Lindquist J (1955) The synthesis of aryl-D-glucopyranosiduronic acids. *J.Am.Chem.Soc.* **77**:3310-3315.

Bottcher J, Bassmann H, Schuppel R, and Lehmann WD (1982) Preparative isolation and purification of urinary conjugates in antipyrine metabolism in man and rat. *Naunyn Schmiedebergs Arch.Pharmacol.* **321**:226-233.

Bournot P and Ramirez LC (1989) Evidence of formation of isomeric methoximes from 20-oxosteroids. *J.Steroid Biochem.* **33**:971-976.

Bowering W and Timell T (1960) Synthesis and characterisation of 2-O-(b-D-glucopyranosyluronic acid)-D-xylopyranose. *J.Am.Chem.Soc.* **82**:2827-2830.

Bowry VW and Stocker R (1993) Tocopherol-mediated peroxidation. The pro-oxidant effect of vitamin E on radical-initiated oxidation of human low-density lipoprotein. *J.Am.Chem.Soc.* **115**:6029-6044.

Brown RT, Scheinmann F, and Stachulski AV (1997) Intermediates for glucuronide synthesis: 7-hydroxycoumarin glucuronide. *Journal of Chemical Research-S* 370-371.

Bunyan J, McHale D, Green, J, and Marcinkiewicz S (1961) Biological potentcies of ε- and ζ-tocopherol and 5-methyltocol. *Brit.J.Nutr.* **15**:253.

Bursell SE and King GL (1999) Can protein kinase C inhibition and vitamin E prevent the development of diabetic vascular complications? *Diabetes Res.Clin.Pract.* **45**:169-182.

Burton GW, Cheeseman KH, Doba T, Ingold KU, and Slater TF (1983) Vitamin E as an antioxidant in vitro and in vivo. *Ciba Found.Symp.* **101**:4-18.

Burton GW, Hughes L, Foster DO, Pietrzak E, Goss-Sampson MA, and Muller DPR (1993) Antioxidant mechanisms of vitamin E and beta-carotene, in *Free Radical: From Basic Science to Medicine* (Poli G, Albano E, and Dianzani MU eds) pp 388-399, Birkhauser Verlag, Basel/Switzerland.

Burton GW and Ingold K (1993) Biokinetics of vitamin E using deuterated tocopherols, in *Vitamin E in health and disease* (Packer L and Fuchs J eds) pp 329-344, Marcel Dekker, Inc., New York, Basel and Hong Kong.

Burton GW and Ingold KU (1986) Vitamin E: application of the principles of physical organic chemsitry to the exploration of its structure and function. *Acc.Chem.Res.* **19**:194-201.

Burton GW, Traber MG, Acuff RV, Walters DN, Kayden H, Hughes L, and Ingold KU (1998) Human plasma and tissue alpha-tocopherol concentrations in response to supplementation with deuterated natural and synthetic vitamin E. *Am.J.Clin.Nutr.* **67**:669-684.

Burton GW, Wronska U, Stone L, Foster DO, and Ingold KU (1990) Biokinetics of dietary RRR-alpha-tocopherol in the male guinea pig at three dietary levels of vitamin C and two levels of vitamin E. Evidence that vitamin C does not "spare" vitamin E in vivo. *Lipids* **25**:199-210.

Chapman JR (1998) *Practical Organic Mass Spectrometry*. Wiley and Sons, Chichester, New York.

Chiku S, Hamamura K, and Nakamura T (1984) Novel urinary metabolite of D-delta-tocopherol in rats. *Journal of Lipid Research* **25**:40-48.

Christen S, Woodall AA, Shigenaga MK, Southwell-Keely PT, Duncan MW, and Ames BN (1997) gamma-tocopherol traps mutagenic electrophiles such as NO(X) and complements alpha-tocopherol: physiological implications. *Proc.Natl.Acad.Sci.U.S A* **94**:3217-3222.

Cohen N, Lopresti R, and Saucy G (1979) A novel synthesis of (2R, 4'R, 8'R)-alpha-tocopherol (vitamin E). Construction of chiral chromans from optically active, nonaromatic precursor. *J.Am.Chem.Soc.* **101**:6710-6715.

Dam H, Prange I, and Sondergaard E (1952) Muscular degeneration (white striation of muscles) in chicks reared on vitamin E deficient, low fat diets. *Acta Path.Scand.* **31**:172.

Doba T, Burton GW, and Ingold KU (1985) Antioxidant and co-antioxidant activity of vitamin C. The effect of vitamin C, either alone or in the presence of vitamin E or a water- soluble vitamin E analogue, upon the peroxidation of aqueous multilamellar phospholipid liposomes. *Biochim.Biophys.Acta* **835**:298-303.

Dole M, Mach L, Hines R, Mobley R, Ferguson L, and Alice M (1968) Molecular beams of macroions. *J.Chem.Phys.* **49**:2240-2247.

Dusza JP, Joseph JP, and Bernstein S (1968) Steroid conjugates IV. The preparation of steroid sulfates with triethylamine-sulfur trioxide. *Steroids* **12**:49-61.

Elias E, Muller DP, and Scott J (1981) Association of spinocerebellar disorders with cystic fibrosis or chronic childhood cholestasis and very low serum vitamin E. *Lancet* **2**:1319-1321.

Esterbauer H, Striegl G, Puhl H, and Rotheneder M (1989) Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radic.Res.Commun.* **6**:67-75.

Evans HM and Bishop KS (1922) On the existence of a hitherto unrecognised dietary factor essential for reproduction. *Science.* **56**:650-651.

Evans HM and Burr GO (1925) The antisterility vitamine fat soluble E. *Proc.Nat.Acad.Sci.* **11**:334.

Evans HM, Emerson OH, and Emerson GJ (1936) The isolation from wheatgerm oil of an alcohol, alpha-tocopherol, having the properties of vitamin E. *J.Biol.Chem.* **113**:319-322.

Ferguson JR, Harding JR, Lumbard KW, Scheinmann F, and Stachulski AV (2000) Glucuronide and sulfate conjugates of ICI 182,780, a pure anti- estrogenic steroid. Order of addition, catalysis and substitution effects in glucuronidation. *Tetrahedron Letters* **41**:389-392.

Fernholz E (1938) On the constitution of alpha-tocopherol. *J.Am.Chem.Soc.* **60**:700-705.

Gallo-Torres HE (1980) Transport and Metabolism, in *Vitamin E: a comprehensive treatise* (Machlin LJ ed) pp 193-267, Marcel Dekker, New York.

Gey KF (1998) Vitamins E plus C and interacting conutrients required for optimal health. A critical and constructive review of epidemiology and supplementation data regarding cardiovascular disease and cancer. *Biofactors* 7:113-174.

Gibson G and Skett P (1994) *Introduction to drug metabolism*. Blackie Academic and Professional, London, Glasgow, New York, Tokyo, Melbourne, Madras.

Gilar M, Bouvier ES, and Compton BJ (2001) Advances in sample preparation in electromigration, chromatographic and mass spectrometric separation methods. *J.Chromatogr.A* **909**:111-135.

Gloor U and Wiss O (1966) Resorption, retention, excretion and distribution of phylloquinone, menaquinone-4 and alpha-tocoperolquinone in comparison with alpha-tocopherol in the rat. *Helv.Chim.Acta* **49**:2590-2594.

Goettsch M and Pappenheimer A (1931) Nutritional muscular dystrophy in the guinea pig and rabbit. *J.Exp.Med.* **54**:145.

Griffiths WJ, Jonsson AP, Liu S, Rai DK, and Wang Y (2001) Electrospray and tandem mass spectrometry in biochemistry. *Biochem.J.* **355**:545-561.

Griffiths WJ, Liu S, Yang Y, Purdy RH, and Sjovall J (1999) Nano-electrospray tandem mass spectrometry for the analysis of neurosteroid sulphates. *Rapid Commun.Mass Spectrom.* **13**:1595-1610.

Halliwell B and Gutteridge JMC (1999) *Free Radicals in Biology and Medicine*. Oxford Univ. Press, Oxford/New York.

Ham AJ and Liebler DC (1995) Vitamin E oxidation in rat liver mitochondria. *Biochemistry* **34**:5754-5761.

Ham AJ and Liebler DC (1997) Antioxidant reactions of vitamin E in the perfused rat liver: product distribution and effect of dietary vitamin E supplementation. *Arch.Biochem.Biophys.* **339**:157-164.

Harding AE, Muller DP, Thomas PK, and Willison HJ (1982) Spinocerebellar degeneration secondary to chronic intestinal malabsorption: a vitamin E deficiency syndrome. *Ann.Neurol.* **12**:419-424.

Harries JT and Muller DP (1971) Absorption of vitamin E in children with biliary obstruction. *Gut* **12**:579-584.

Hattori A, Fukushima T, Hamamura K, Kato M, and Imai K (2001) A fluorimetric, column-switching HPLC and its application to an elimination study of LLU-alpha enantiomers in rat plasma. *Biomed.Chromatogr.* **15**:95-99.

Hattori A, Fukushima T, and Imai K (2000a) Occurrence and determination of a natriuretic hormone, 2,7,8-trimethyl- 2-(beta-carboxyethyl)-6-hydroxy chroman, in rat plasma, urine, and bile. *Anal.Biochem.* **281**:209-215.

Hattori A, Fukushima T, Yoshimura H, Abe K, and Ima K (2000b) Production of LLU-alpha following an oral administration of gamma- tocotrienol or gamma-tocopherol to rats. *Biol.Pharm.Bull.* **23**:1395-1397.

Hege HG, Lietz H, and Weymann J (1986) Studies on the metabolism of propafenone. 3rd Comm.: Isolation of the conjugated metabolites in the dog and identification using fast atom bombardment mass spectrometry. *Arzneimittelforschung.* **36**:467-474.

Hoglen NC and Liebler DC (1999) Products from reaction of peroxynitrite with gamma-tocopherol. *Methods Enzymol.* **301**:483-490.

Hoglen NC, Waller SC, Sipes IG, and Liebler DC (1997) Reactions of peroxynitrite with gamma-tocopherol. *Chem.Res.Toxicol.* **10**:401-407.

Hollander D, Rim E, and Muralidhara KS (1975) Mechanism and site of small intestinal absorption of alpha-tocopherol in the rat. *Gastroenterology* **68**:1492-1499.

Hosomi A, Arita M, Sato Y, Kiyose C, Ueda T, Igarashi O, Arai H, and Inoue K (1997) Affinity for alpha-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs. *FEBS Lett.* **409**:105-108.

Hosomi A, Goto K, Kondo H, Iwatsubo T, Yokota T, Ogawa M, Arita M, Aoki J, Arai H, and Inoue K (1998) Localization of alpha-tocopherol transfer protein in rat brain. *Neurosci.Lett.* **256**:159-162.

Hugh D, Grennan A, Abugila MA, and Weinkove C (1987) Ascorbic acid as an antioxidant in measurements of catecholamines in plasma. *Clin.Chem.* **33**:569-571.

Ingold KU, Burton GW, Foster DO, Hughes L, Lindsay DA, and Webb A (1987) Biokinetics of and discrimination between dietary RRR- and SRR-alpha- tocopherols in the male rat. *Lipids* **22**:163-172.

Iribarne J and Thomson B (1976) On evaporation of small ions from charged droplets. *J.Chem.Phys.* **64**:2287-2294.

Ishihara K, Kubota M, and Yamamoto H (1996) Practical synthesis of (±)-alpha-tocopherol. Trifluoromethanesulfonimide as an extremely active Bronsted acid catalyst for the condensation of trimetylhydroquinone with isophytol. *Synlett* **Nov**:1045-1046.

Jacquinet JC (1990) Syntheses of the methyl glycosides of the repeating units of chondroitin 4- and 6-sulfate. *Carbohydr.Res.* **199**:153-181.

Jishage K, Arita M, Igarashi K, Iwata T, Watanabe M, Ogawa M, Ueda O, Kamada N, Inoue K, Arai H, and Suzuki H (2001) Alpha-tocopherol transfer protein is important for the normal development of placental labyrinthine trophoblasts in mice. *J.Biol.Chem.* **276**:1669-1672.

Jones Chromatography (1998) Solid Phase Extraction Method Development (Technical notes available from Jones Chromatography, Hengoed, UK).

Jung ME and MacDougall JM (1999) First enantioselective total synthesis of the endogenous natriuretic agent LLU-alpha. *Tetrahedron Letters* **40**:6339-6342.

Kagan VE, Serbinova EA, Forte T, Scita G, and Packer L (1992) Recycling of vitamin E in human low density lipoproteins. *J.Lipid Res.* **33**:385-397.

Kagan VE, Serbinova EA, and Packer L (1990) Recycling and antioxidant activity of tocopherol homologs of differing hydrocarbon chain lengths in liver microsomes. *Arch.Biochem.Biophys.* **282**:221-225.

Kamal-Eldin A and Appelqvist LA (1996) The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* **31**:671-701.

Kanazawa H, Miyata C, Nagata Y, Urano S, and Matsushima Y (2000) Determination of alpha-tocopherol and alpha-tocopherylquinone in rat tissues and plasma by high-performance liquid chromatography with electrochemical detection. *Chem.Pharm.Bull.(Tokyo)* **48**:1462-1466.

Kantoci D, Wechter WJ, Murray ED, Dewind SA, Borchardt D, and Khan SI (1997) Endogenous natriuretic factors .6. The stereochemistry of a natriuretic gamma-tocopherol metabolite LLU-alpha. *Journal of Pharmacology and Experimental Therapeutics* **282**:648-656.

Kasparek S (1980) Chemistry of tocopherols and tocotrienols, in *Vitamin E - A comprehensive treatise* (Machlin L ed) pp 7-66, Marcel Dekker, Inc., New York and Basel.

Kaspersen FM and Van Boeckel CAA (1987) Synthesis of glucuronides and sulphates. *Xenobiotica* **17**:1451-1471.

Kawai N, Takao K, and Kobayashi S (1999) Synthetic study of akaterpin: Determination of the relative stereochemistry of the upper decalin moiety with disulfated hydroquinone. *Tetrahedron Letters* **40**:4193-4196.

Kostner GM, Oettl K, Jauhiainen M, Ehnholm C, Esterbauer H, and Dieplinger H (1995) Human plasma phospholipid transfer protein accelerates exchange/transfer of alpha-tocopherol between lipoproteins and cells. *Biochem.J.* **305 ( Pt 2)**:659-667.

Lahmann M and Thiem J (1997) Synthesis of alpha-tocopheryl oligosaccharides. *Carbohydrate Research* **299**:23-31.

Laurin P, Ferroud D, Schio L, Klich M, Dupuis-Hamelin C, Mauvais P, Lassaigne P, Bonnefoy A, and Musicki B (1999) Structure-activity relationship in two series of

aminoalkyl substituted coumarin inhibitors of gyrase B. *Bioorganic & Medicinal Chemistry Letters* **9**:2875-2880.

Lei HS and Atkinson J (2000) Synthesis of phytyl- and chroman-derivatized photoaffinity labels based on alpha-tocopherol. *Journal of Organic Chemistry* **65**:2560-2567.

Lei HS, Marks V, Pasquale T, and Atkinson JK (1998) Synthesis of photoaffinity label analogues of alpha-tocopherol. *Bioorganic & Medicinal Chemistry Letters* **8**:3453-3458.

Leu YL, Roffler SR, and Chern JW (1999) Design and synthesis of water-soluble glucuronide derivatives of camptothecin for cancer prodrug monotherapy and antibody-directed enzyme prodrug therapy (ADEPT). *Journal of Medicinal Chemistry* **42**:3623-3628.

Liebler DC (1993) The role of metabolism in the antioxidant function of vitamin E. *Crit Rev. Toxicol.* **23** :147-169.

Liebler DC and Burr JA (2000) Antioxidant reactions of alpha-tocopherolhydroquinone. *Lipids* **35**:1045-1047.

Liebler DC, Burr JA, and Ham AJ (1999) Gas chromatography-mass spectrometry analysis of vitamin E and its oxidation products. *Methods Enzymol.* **299**:309-318.

Liebler DC, Burr JA, Philips L, and Ham AJ (1996) Gas chromatography-mass spectrometry analysis of vitamin E and its oxidation products. *Anal.Biochem.* **236**:27-34.

Littlewood AB (1970) *Gas Chromatography : Principles, Techniques and Applications.* Academic Press, New York, London.

Lodge JK, Ridlington J, Leonard S, Vaule H, and Traber MG (2001) Alpha- and gamma-tocotrienols are metabolized to carboxyethyl- hydroxychroman derivatives and excreted in human urine. *Lipids* **36**:43-48.

Lodge JK, Traber MG, Elsner A, and Brigelius-Flohe R (2000) A rapid method for the extraction and determination of vitamin E metabolites in human urine. *J.Lipid Res.* **41**:148-154.

Long C (1971) *Biochemists' Handbook.* E. and F.N. Spon Ltd., London.

Mackenzie C, Mackenzie J, and McCollum J (1941) Uncomplicated vitamin E deficiency in the rabbit and its relation to the toxicity of cod liver oil. *J.Nutr.* **21**:225.

Maguire JJ, Wilson DS, and Packer L (1989) Mitochondrial electron transport-linked tocopheroxyl radical reduction. *J.Biol.Chem.* **264**:21462-21465.

Mannaerts GP and Van Veldhoven PP (1995) in *Functions and Biogenesis of Peroxisomes in Relation to Human Disease* (Wanders RJA, Schutgens RBH, and Tabak HF eds) pp 1-27, Royal Academy of Arts and Sciences, Amsterdam, Oxford, New York, Tokyo.

Mayer H, Schudel P, Ruegg R, and Isler O (1963) 67. Uber die Chemie des Vitamins E – Die Totalsynthese von (2R, 4'R, 8'R)- und (2S, 4'R, 8'R)-α-Tocopherol. *Helv.Chim.Acta* **46**:650-671.

McCormick J, Li YB, McCormick K, Duynstee HI, van Engen AK, van der Marel GA, Ganem B, van Boom JH, and Meinwald J (1999) Structure and total synthesis of HF-7, a neuroactive glyconucleoside disulfate from the funnel-web spider Hololena curta. *Journal of the American Chemical Society* **121**:5661-5665.

Meech R and Mackenzie P (1997) Structure and function of uridine diphosphate glucuronyltransferases. *Clinical and Experimental Pharmacology and Physiology* **24**:907-915.

Moore AN and Ingold KU (1997) alpha-Tocopheryl quinone is converted into vitamin E in man. *Free Radic.Biol.Med.* **22**:931-934.

Muller DP and Goss-Sampson MA (1990) Neurochemical, neurophysiological, and neuropathological studies in vitamin E deficiency. *Crit Rev.Neurobiol.* **5**:239-263.

Muller DP, Harries JT, and Lloyd JK (1974) The relative importance of the factors involved in the absorption of vitamin E in children. *Gut* **15**:966-971.

Muller DP, Lloyd JK, and Bird AC (1977) Long-term management of abetalipoproteinaemia. Possible role for vitamin E. *Arch.Dis.Child* **52**:209-214.

Muller DP, Lloyd JK, and Wolff OH (1983) Vitamin E and neurological function. *Lancet* **1**:225-228.

Muller DPR (1997) Neurological Disease, in *Antioxidants in Disease Mechanisms and Therapy. Advances in Pharmacology* (Sies H ed) **38**:557-580, Academic Press.

The New Encyclopaedia Britannica (1990), 15th Edition, **12**:400-403.

Nudelman A, Herzig J, Gottileb HE, Keinan E, and Sterling J (1987) Studies in sugar chemistry. 4. Selective deacetylation of anomeric sugar acetates with tin alkoxides. *Carbohydrate Research* **162**:145-152.

Olcott H and Emerson O (1941) Constituents of fats and oils affecting the development of rancidity. *Chem.Rev.* **29**:257-268.

Oliveira EJ and Watson DG (2000) Liquid chromatography-mass spectrometry in the study of the metabolism of drugs and other xenobiotics. *Biomed.Chromatogr.* **14**:351-372.

Ouahchi K, Arita M, Kayden H, Hentati F, Ben Hamida M, Sokol R, Arai H, Inoue K, Mandel JL, and Koenig M (1995) Ataxia with isolated vitamin E deficiency is caused by mutations in the alpha-tocopherol transfer protein. *Nat.Genet.* **9**:141-145.

Pappenheimer A and Goettsch M (1931) A cerebellar disorder in chicks apparently of nutritional origin. *J.Exp.Med.* **53**:11-26.

Parker RS, Sontag TJ, and Swanson JE (2000) Cytochrome P4503A-dependent metabolism of tocopherols and inhibition by sesamin. *Biochemical and Biophysical Research Communications* **277**:531-534.

Parker RS and Swanson JE (2000) A novel 5 '-carboxychroman metabolite of gamma-tocopherol secreted by HepG2 cells and excreted in human urine. *Biochemical and Biophysical Research Communications* **269**:580-583.

Pope SA, Burtin GE, Clayton PT, Madge DJ, and Muller DP (2001) New synthesis of (+/-)-alpha-CMBHC and its confirmation as a metabolite of alpha-tocopherol (vitamin E). *Bioorg.Med.Chem.* **9**:1337-1343.

Pope SAS, Clayton PT, and Muller DPR (2000) A new method for the analysis of urinary vitamin E metabolites and the tentative identification of a novel group of compounds. *Archives of Biochemistry and Biophysics* **381**:8-15.

Pryor WA (2000) Vitamin E and heart disease: basic science to clinical intervention trials. *Free Radic.Biol.Med.* **28**:141-164.

Ricciarelli R, Tasinato A, Clement S, Ozer NK, Boscoboinik D, and Azzi A (1998) Alpha-tocopherol specifically inactivates cellular protein kinase C alpha by changing its phosphorylation state. *Biochem.J.* **334 (Pt 1)**:243-249.

Rosenau T, Habicher WD, and Chen CL (1996) Novel tocopherol compounds .4. 5-tocopherylacetic acid and its derivatives. *Heterocycles* **43**:787-798.

Roy A (1981) The chemistry of sulphate esters and related compounds, in *Sulphation of drugs and related compounds* (Mulder G ed) pp 6-30, CRC Press.

Sato Y, Hagiwara K, Arai H, and Inoue K (1991) Purification and characterization of the alpha-tocopherol transfer protein from rat liver. *FEBS Lett.* **288**:41-45.

Schmandke H (1965a) Formation of tocopheronlactone from apha-tocopherylquinone in the human organism. *Int.Z.Vitaminforsch.* **35**:321-327.

Schmandke H (1965b) On the conversion of alpha-tocopherol and 2,5,7,8-tetramethyl-2-(beta- carboxyethyl)-6-acetoxychroman to tocopheronolactone in normal and tocopherol deficient organisms. *Int.Z.Vitaminforsch.* **35**:346-352.

Schonfeld A, Schultz M, Petrizka M, and Gassmann B (1993) A novel metabolite of RRR-α-tocopherol in human urine. *Die Nahrung* **37 (5)**:498-500.

Schuelke M, Elsner A, Finckh B, Kohlschutter A, Hubner C, and Brigelius-Flohe R (2000) Urinary alpha-tocopherol metabolites in alpha-tocopherol transfer protein-deficient patients. *J.Lipid Res.* **41**:1543-1551.

Schultz M, Leist M, Petrizka M, Gassmann B, and Brigelius-Flohe R (1995) Novel urinary metabolite of α-tocopherol, 2,5,7,8-tetramethyl-2(2'-carboxymethyl)-6-hydroxychroman, as an indicator of an adequate vitamin E supply. *American Journal of Clinical Nutrition* **62**:S1527-S1534.

Scott JW, Bizzarro FT, Parrish DR, and Saucy G (1976) Syntheses of (2R,4'R,8'R)-alpha-tocopherol and (2R,3'E,7'E)-alpha-tocotrienol. *Helv.Chim.Acta* **59**:290-306.

Sheppard A, Weihrauch J, and Pennington J (1993) Analysis and Distribution of Vitamin E in Vegetable Oils and Foods, in *Vitamin E in Health and Disease* (Packer L and Fuchs J eds) pp 9-31, Marcel Dekker, Inc., New York, Basel and Hong Kong.

Shklar G and Oh SK (2000) Experimental basis for cancer prevention by vitamin E. *Cancer Invest* **18**:214-222.

Siegel D, Bolton EM, Burr JA, Liebler DC, and Ross D (1997) The reduction of alpha-tocopherolquinone by human NAD(P)H: quinone oxidoreductase: the role of alpha-tocopherolhydroquinone as a cellular antioxidant. *Mol.Pharmacol.* **52**:300-305.

Simon E, Gross C, and Milhorat A (1956a) I. The absorption and excretion of D-α-tocopheryl-5-methyl-$C^{14}$-succinate. *J.Biol.Chem.* **221**:797-805.

Simon E, Eisengart A, Sundheim L, and Milhorat A (1956b) II. Purification and characterization of urinary metabolites of α-tocopherol. *J.Biol.Chem.* **221**:807-817.

Smith L, Ungnade H, Stevens J, and Christman C (1939) The chemistry of vitamin E. XVIII. Condensation of phenols and hydroquinones with allylic alcohols, allylic halides, and conjugated dienes. *J.Am.Chem.Soc.* **61**:2615-2618.

Stachulski AV and Jenkins GN (1998) The synthesis of O-glucuronides. *Natural Product Reports* **15**:173-186.

Stahl W, Graf P, Brigelius-Flohe R, Wechter W, and Sies H (1999) Quantification of the alpha- and gamma-tocopherol metabolites 2,5,7,8-tetramethyl-2-(2 '-carboxyethyl)-6-hydroxychroman and 2,7,8-trimethyl-2-(2 '-carboxyethyl)-6-hydroxychroman in human serum. *Analytical Biochemistry* **275**:254-259.

Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, and Mitchinson MJ (1996) Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* **347**:781-786.

Suarna C, Dean RT, and Southwell-Keely PT (1997) Synthesis of alpha-tocopherol analogues. *Australian Journal of Chemistry* **50**:1129-1135.

Swanson JE, Ben RN, Burton GW, and Parker RS (1999) Urinary excretion of 2,7,8-trimethyl-2-(beta-carboxyethyl)-6- hydroxychroman is a major route of elimination of gamma- tocopherol in humans. *Journal of Lipid Research* **40**:665-671.

Terasawa Y, Ladha Z, Leonard SW, Morrow JD, Newland D, Sanan D, Packer L, Traber MG, and Farese RV, Jr. (2000) Increased atherosclerosis in hyperlipidemic mice deficient in alpha - tocopherol transfer protein and vitamin E. *Proc.Natl.Acad.Sci.U.S A* **97**:13830-13834.

Thomas SR, Neuzil J, and Stocker R (1996) Cosupplementation with coenzyme Q prevents the prooxidant effect of alpha-tocopherol and increases the resistance of LDL to transition metal-dependent oxidation initiation. *Arterioscler.Thromb.Vasc.Biol.* **16**:687-696.

Thomson B and Iribarne J (1979) Field induced evaporation from liquid surfaces at atmospheric pressure. *J.Chem.Phys.* **71**:4451-4463.

Tietze LF and Gorlitzer J (1996) Synthesis of enantiopure vitamin E via sharpless bishydroxylation of an enyne. *Synlett* 1041-1042.

Tietze LF and Gorlitzer J (1997) Preparation of enantiopure precursors for the vitamin E synthesis. A comparison of the asymmetric allylation of ketones and the sharpless bishydroxylation. *Synlett* 1049-1050.

Tietze LF and Gorlitzer J (1998) Preparation of chiral building blocks for a highly convergent vitamin E synthesis. Systematic investigations on the enantioselectivity of the sharpless bishydroxylation. *Synthesis-Stuttgart* **Aug**:873-878.

Tietze LF, Gorlitzer J, Schuffenhauer A, and Hubner M (1999) Enantioselective synthesis of the chromane moiety of vitamin E. *European Journal of Organic Chemistry* **No. SI**:1075-1084.

Traber MG, Burton GW, Ingold KU, and Kayden HJ (1990) RRR- and SRR-alpha-tocopherols are secreted without discrimination in human chylomicrons, but RRR-alpha-tocopherol is preferentially secreted in very low density lipoproteins. *J.Lipid Res.* **31**:675-685.

Traber MG, Cohn W, and Muller DPR (1993) Absorption, Transport and Delivery to Tissues, in *Vitamin E in Health and Disease* (Packer L and Fuchs J eds) pp 35-51, Marcel Dekker, Inc..

Traber MG, Elsner A, and Brigelius-Flohe R (1998) Synthetic as compared with natural vitamin E is preferentially excreted as alpha-CEHC in human urine: studies using deuterated alpha-tocopheryl acetates. *Febs Letters* **437**:145-148.

Trost BM and Asakawa N (1999) An asymmetric synthesis of the vitamin E core by Pd catalyzed discrimination of enantiotopic alkene faces. *Synthesis-Stuttgart*1491-1494.

Uhrig RK, Picard MA, Beyreuther K, and Wiessler M (2000) Synthesis of antioxidative and anti-inflammatory drug glucoconjugates. *Carbohydrate Research* **325**:72-80.

Ullmann's Encyclopedia of Industrial Chemistry (1996). VCH Verlag: Gesellschraft.

Upston JM, Terentis AC, and Stocker R (1999) Tocopherol-mediated peroxidation of lipoproteins: implications for vitamin E as a potential antiatherogenic supplement. *FASEB J.* **13**:977-994.

Van Houte H, De Hoffmann E, Van Veldhoven PP, Mannaerts GP, Carchon H, Baes MI, and Declercq PE (2001) Oxidative catabolism of alpha-tocopherol in rat liver microsomes. *Lipids* **36**:367-372.

Vatassery GT (1993) Oxidation of alpha-tocopherol in subcellular fractions from rat brain and its possible involvement in nerve function. *Biochem.Pharmacol.* **45**:2295-2301.

Vatassery GT (1994) Determination of tocopherols and tocopherolquinone in human red blood cell and platelet samples. *Methods Enzymol.* **234**:327-331.

Vatassery GT, Smith WE, and Quach HT (1993) A liquid chromatographic method for the simultaneous determination of alpha-tocopherol and tocopherolquinone in human

red blood cells and other biological samples where tocopherol is easily oxidized during sample treatment. *Anal.Biochem.* **214**:426-430.

Von Karrer P, Fritzsche B, Ringier B, and Salomon H (1938) Synthese des alpha-Tocopheols. *Helv.Chim.Acta* **21**:820-825.

Wasserman RH and Taylor AN (1972) Metabolic roles of fat-soluble vitamins D, E, and K. *Annu.Rev.Biochem.* **41**:179-202.

Wechter WJ, Kantoci D, Murray ED, DAmico DC, Jung ME, and Wang WH (1996) A new endogenous natriuretic factor: LLU-alpha. *Proc.Natl.Acad.Sci.U.S.A* **93**:6002-6007.

Wehrli P, Fryer R, and Metlesics W (1971) Group III metal complexes in the preparation of vitamin E. *J.Org.Chem.* **36**:2911-2912.

Weichet J, Blaha L, and Kakac B (1959) Studien in der gruppe der vitamine K und E VI. Uber die darstellung von 2,5,7,8-tetramethyl-2-(β-carboxy-athyl)-6-hydroxychroman und dessen oxydationsprodukt. *Collection Czechoslov.Chem.Commun.* **24**:1689-1693.

Weichet J and Blaha L (1966a) Studien in der vitamin K und vitamin E reihe. XV. Uber die synthese der 2,6,10,14-tetramethyl-14-hydroxy-hexadecensaure und verwandter verbindungen. *Collection Czechoslov.Chem.Commun.* **31**:2424-2433.

Weichet J, Blaha L, Kakac B, and Hodrova J (1966b) Studien in der vitamin-K und vitamin-E-reihe. XVI. Synthese der „(±)-α-tocopherolsaure" und ihre niedrigeren homologen und derivate. *Collection Czechoslov.Chem.Commun.* **31**:2434-2443.

Weiser H and Vecchi M (1981) Stereoisomers of alpha-tocopheryl acetate - characterization of the samples by physico-chemical methods and determination of biological activities in the rat resorption-gestation test. *Int.J.Vitam.Nutr.Res.* **51(2)**:100-113.

Weiser H and Vecchi M (1982) Stereoisomers of alpha-tocopheryl acetate. II. Biopotencies of all eight stereoisomers, individually or in mixtures, as determined by rat resorption-gestation tests. *Int.J.Vitam.Nutr.Res.* **52(3)**:351-370

Whitehouse CM, Dreyer RN, Yamashita M, and Fenn JB (1985) Electrospray interface for liquid chromatographs and mass spectrometers. *Anal.Chem.* **57**:675-679.

Wu Y (2000) The use of liquid chromatography-mass spectrometry for the identification of drug degradation products in pharmaceutical formulations. *Biomed.Chromatogr.* **14**:384-396.

Yamazaki H, Shibata A, Suzuki M, Nakajima M, Shimada N, Guengerich FP, and Yokoi T (1999) Oxidation of troglitazone to a quinone-type metabolite catalysed by cytochrome P-450 2C8 and P-450 3A4 in human liver microsomes. *Drug metabolism and disposition* **27 (11)**:1260-1266.

Yang Y, Griffiths WJ, Nazer H, and Sjovall J (1997) Analysis of bile acids and bile alcohols in urine by capillary column liquid chromatography-mass spectrometry using fast atom bombardment or electrospray ionization and collision-induced dissociation. *Biomed.Chromatogr.* **11**:240-255.

Yoshioka T, Aizawa Y, Fujita T, Nakamura K, Sasahara K, Kuwano H, Kinoshita T, and Horikoshi H (1991) Studies on hindered phenols and analogues. 5. Synthesis, identification, and antidiabetic activity of the glucuronide of CS-045. *Chemical & Pharmaceutical Bulletin* **39**:2124-2125.

# PUBLICATIONS

Archives of Biochemistry and Biophysics
Vol. 381, No. 1, September 1, pp. 8–15, 2000
doi:10.1006/abbi.2000.1950, available online at http://www.idealibrary.com on IDEAL®

# A New Method for the Analysis of Urinary Vitamin E Metabolites and the Tentative Identification of a Novel Group of Compounds

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Received February 7, 2000, and in revised form May 31, 2000

**There is currently interest in measuring urinary metabolites of vitamin E. It has been suggested that α-tocopheronolactone (αTL), with an oxidized chroman ring, may be an indicator of *in vivo* oxidative stress and 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman (α-CEHC), with a shortened side chain but intact hydroxychroman ring, may provide a measure of adequate or excess vitamin E status. To date, methods in the literature have tended to concentrate on the estimation of single metabolites. We describe the establishment and validation of a relatively simple and reproducible method to extract and quantitate a range of vitamin E metabolites using 0.5 ml of human urine. The vitamin E metabolites were extracted from urine using solid phase extraction cartridges, deconjugated enzymatically, and analyzed using gas chromatography–mass spectrometry. Using this method we have identified αTL and the CEHC metabolites derived from α-, δ-, and γ-tocopherol. In addition we have tentatively identified a novel group of vitamin E metabolites, which are related to the CEHCs but have three extra carbons in the side chain. The possibility of the artifactual oxidation of α-CEHC to αTL during the assay procedure is also discussed.** © 2000 Academic Press

*Key Words:* **urinary metabolites; α-tocopherol; α-carboxyethylhydroxychroman; α-tocopheronolactone; 2,5,7,8-tetramethyl-2(4′-carboxy-4′-methylbutyl)-6-hydroxychroman; oxidative stress; gas chromatography–mass spectrometry.**

An increase in oxidative stress has been implicated in the etiology of an increasing number of disease states (1). In general, however, definitive evidence that oxidative stress is directly involved is lacking. An increase in oxidative stress is the net result of an imbalance between the production of reactive oxygen species (ROS)[2] and their removal by antioxidants. ROS are highly reactive compounds and include oxygen-derived free radicals such as the superoxide and hydroxyl radical or products formed from these radicals such as hydrogen peroxide. Antioxidants are able to prevent free radical formation or terminate their reaction and are, therefore, essential to protect molecules and biological systems from oxidative damage. Vitamin E is one of these antioxidants and is particularly important as (a) it is the major lipid soluble antioxidant *in vivo* (2) and (b) it is frequently administered to patients suspected of suffering from increased oxidative stress.

Vitamin E is a generic term covering the tocopherols and tocotrienols, which have saturated and unsaturated side chains, respectively. Each group has α, β, γ, and δ forms that differ according to the position and number of methyl groups on the hydroxychroman ring. The tocopherols also have three centers of asymmetry and as a result there are eight possible isomers for each tocopherol. When α-tocopherol is synthesized from isophytol and trimethylhydroquinone, all eight isomers are produced in equimolar concentrations producing a mixture termed all-rac-α-tocopherol. Natural α-tocopherol is, however, a single isomer designated RRR-α-tocopherol. RRR-α-tocopherol has the greatest biological activity and is the predominant form *in vivo*, al-

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[2] Abbreviations used: SPE, solid phase extraction; ROS, reactive oxygen species; αTL, α-tocopheronolactone; α-CEHC, 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman; α-TTP, hepatic tocopherol transfer protein; CMBHCs, carboxymethylbutyl-6-hydroxychromans; TMS, trimethylsilyl; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; α-CMBHC, 2,5,7,8-tetramethyl-2(4′-carboxy-4′-methylbutyl)-6-hydroxychroman; CV, coefficient of variation.



0003-9861/00 $35.00

though RRR-γ-tocopherol is the major tocopherol in the diet (3). *In vitro* RRR-γ-tocopherol has 50% of the antioxidant activity of RRR-α-tocopherol but *in vivo* its biological activity is reduced to approximately 10% that of RRR-α-tocopherol (4). The differences in bioavailability and biological activity of these two tocopherols result principally from differences in their handling *in vivo* and in particular reflects the specificity of a hepatic tocopherol transfer protein (α-TTP) (5).

In conditions of increased oxidative stress, the metabolism and turnover of the tocopherols is likely to be increased. Although there have been a number of detailed *in vitro* studies examining the oxidation of α-tocopherol (e.g., Ref. 6), there is surprisingly little known about the metabolism of vitamin E *in vivo* in either normal individuals or those undergoing increased oxidative stress. In the 1950s, two metabolites, α-tocopheronic acid and α-tocopheronolactone (also known as Simon metabolites) were characterized from the urine of animals (7) and man (8) given large oral doses of α-tocopherol. These compounds result from the opening of the hydroxychroman ring as a result of oxidation and subsequent metabolism (presumably β-oxidation) of the side chain. The major portion of these compounds was said to be present in urine as glucuronides. More recently another urinary metabolite of α-tocopherol, i.e., 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman (α-CEHC), with an intact hydroxychroman ring but shortened side chain has been described (9–11). Similar CEHC metabolites of γ- and δ-tocopherol have also been reported in human and rat urine (11–14). There is, however, disagreement in the literature as to whether the Simon metabolites are formed *in vivo* or are produced artifactually during the analytical/extraction procedure (10). There is also disagreement as to whether the metabolites are excreted as glucuronides (14) or sulfates (10).

There is currently interest in measuring these urinary metabolites of vitamin E as it has been suggested that α-tocopheronolactone may be an indicator of *in vivo* oxidative stress and α-CEHC may be a measure of adequate or excess vitamin E status (10). All the published methods have analyzed the deconjugated metabolites, as they are easier to measure than their conjugates. In addition most methods have focused on only one metabolite and very little work has been done comparing the levels of excretion of the different metabolites. In this paper we describe a method that is able to analyze a range of known vitamin E metabolites simultaneously. The method requires small sample and solvent volumes and is relatively quick and simple to perform. In addition we describe the tentative identification of a novel group of vitamin E metabolites, the carboxymethylbutyl-6-hydroxychromans (CMBHCs), with a side chain longer than that of the CEHCs and discuss the problem of artifactual oxidation of α-CEHC to α-tocopheronolactone.

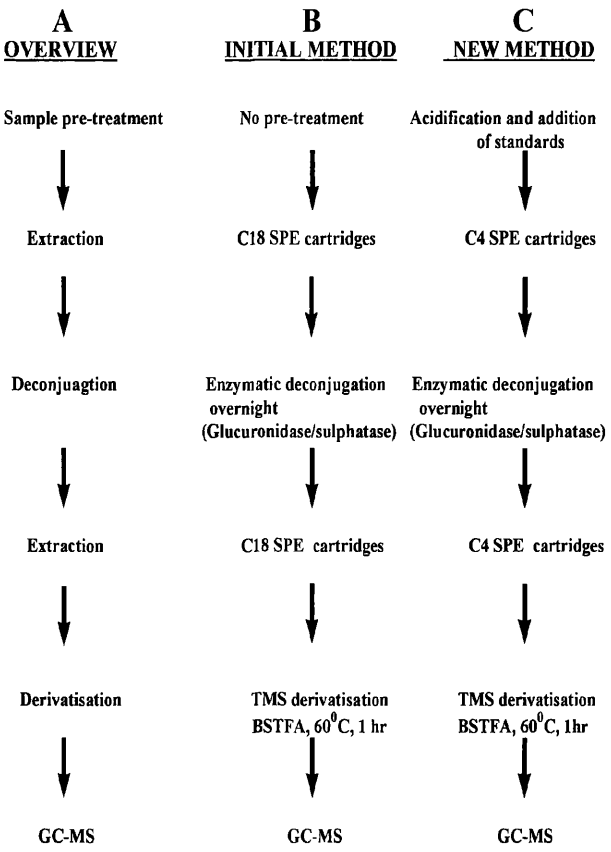


**FIG. 1.** A flowchart displaying the principal steps involved in the analysis of urinary vitamin E metabolites. SPE, solid phase extraction; TMS, trimethylsilyl; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; GC–MS, gas chromatography–mass spectrometry.

## METHOD DEVELOPMENT

An overview of the various steps involved in the extraction and analysis of urinary vitamin E metabolites is shown in Fig. 1A. The conjugated metabolites are extracted from urine and deconjugated. The deconjugated metabolites are then reextracted, derivatized, and analyzed. Each of these steps required optimization to ensure that this multistep procedure gave quantitative and reproducible yields. Trolox (2,5,7,8-tetramethyl-2carboxylic acid-6-hydroxychroman) supplied by Sigma–Aldrich Company, Ltd., and deuterated $d_9$-α-CEHC kindly supplied by Dr. G. Burton (Steacie Institute of Molecular Sciences, National Research Council, Ottawa, Canada) were added to urine and used as standards to optimize and validate the method. We originally commenced with the method outlined in Fig. 1B. This involved C18 solid phase extraction (SPE) cartridges, enzymatic deconjugation, trimethylsilyl (TMS) derivatization, and gas chromatography–mass spectrometry (GC–MS). SPE cartridges were used rather than liquid–liquid extractions, which had previously been employed (10, 11, 14), because they are quick and easy to use, require small sample and wash volumes, and are applicable to batch analysis. Enzymatic deconjugation was chosen, as it is less likely to cause artifacts than the harsher but more rapid acid hydrolysis.

The original method required approximately 5 ml of supplemented urine in order to obtain reasonable peak intensities. Analysis of flow

TABLE I

Comparison of Solid Phase Extraction Cartridges

| Solid phase extraction cartridge | % Recovery[a] (mean and range, $n$ = 3) |
|---|---|
| $d_9$-α-CEHC—no cartridge | 100 |
| C4 (Jones Chromatography) | 85 (77–89) |
| C18 (Jones Chromatography) | 83 (71–95) |
| Oasis[b] (Waters Corporation) | 84 (58–92) |

*Note.* $d_9$-α-CEHC in aqueous solution (pH 2.5) was extracted using various solid phase extraction (SPE) cartridges.

[a] The recovery was compared to the same amount of $d_9$-α-CEHC derivatized directly without extraction.

[b] A hydrophilic–lipophilic balanced copolymer.

through and wash fractions showed that large losses occurred (data not shown). The initial modification was first to standardize the flow rates. The columns were allowed to flow under gravity (rather than using vacuum pressure) at a flow rate of approximately 0.5 ml/min. Second, the urine samples were acidified to pH 2.5 with HCl, in order to protonate the metabolites and increase their affinity for the solid phase. These changes resulted in immediate gains in yield of the α-CEHC metabolite, as judged by an increase in signal intensity/peak area compared to a known amount of derivatized standard ($d_9$-α-CEHC).

Different types of SPE cartridge were then compared (Table I). They all appeared to give a recovery of $d_9$-α-CEHC of approximately 85% compared to samples derivatized directly (again peak areas were used for comparison). The C4 cartridges were chosen, as they gave cleaner extracts, which is important when a number of compounds of varying concentrations are analyzed in a complex matrix such as urine. Enzymatic deconjugation was investigated to check that deconjugation was proceeding to completion. There was little difference in the ratios of the metabolites compared to $d_9$-α-CEHC following 2 and 3 h of incubation at 37°C but there was a slight increase in the concentration of most of the metabolites if the incubation was left for 18 h at 37°C. Doubling the amount of enzyme over an 18-h incubation did not result in any further increase in the ratios of any of the metabolites (data not shown). Derivatization of α-CEHC and α-tocopheronolactone with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 1 h at 60°C gave clean chromatograms and good fragmentation spectra following electron impact. Having refined and validated the extraction, deconjugation, and derivatization, the GC–MS program was optimized to improve the chromatography.

## METHODS

*1. Final method.* The final method (Fig. 1C) used for the studies described below was as follows. Trolox (5 nmol) and $d_9$-α-CEHC (20 nmol) were added to 0.5 ml urine that was then acidified to pH 2.5 with 3 M HCl. $d_9$-α-CEHC was added to monitor any artifactual conversion to $d_9$-α-tocopheronolactone during the procedure. The sample was then loaded onto a C4 solid phase extraction cartridge (100 mg sorbent mass with a 3-ml reservoir supplied by Jones Chromatography, Ltd.), which had been primed with methanol and water (pH 2.5). The sample was allowed to wash through under gravity. The column was then washed with 1 ml deionized water (pH 2.5) and the metabolites were eluted with 2 ml methanol. The flow rates were maintained at approximately 0.5 ml/min throughout. The methanolic extract was evaporated to dryness under nitrogen at 37°C and was then resuspended in 1 ml deionized $H_2O$ to which 50 μl sodium acetate (5.0 M, pH 4.7) and 25 μl of β-glucuronidase/sulfatase (activity of 143,400 units per ml from Helix pomatia (type HP-2, G7017), Sigma–Aldrich Company, Ltd.) were added. The sample was left for 18 h at 37°C and then extracted using a second C4 SPE cartridge as described above. The methanolic phase was again dried under nitrogen at 37°C and the metabolites were converted to TMS derivatives with 200 μl BSTFA (Pierce and Warriner, Ltd.) at 60°C for 1 h. Two microliters of the derivatized mixture was injected (using a splitless technique) onto a DB1 fused silica column (30 m, 0.25 mm i.d., 0.25 μm film thickness) supplied by Jones Chromatography, Ltd., in a Hewlett–Packard 5890 Series II gas chromatograph linked to a Hewlett–Packard 5970 mass-selective detector and Chem Station data system. The oven was maintained at 120°C for 2 min and then ramped to 200°C at 20°C/min, to 240°C at 2°C/min, to 300°C at 50°C/min, and finally held at 300°C for 5 min. The ionization energy was 70 eV. Scan mode was used in preference to single ion monitoring so that full spectra could be obtained to aid the characterization of any novel or unidentified peaks. Peaks were identified by comparison of their retention time and spectra to known standards and published data. Standard and metabolite peaks were visualized by extracting the following ion chromatograms:

Standards—Trolox, *m/z* 394, 237; $d_9$-α-CEHC, *m/z* 431, 246; $d_3$-α-tocopheronolactone, *m/z* 425, 240 (kindly supplied by Dr. G. Burton); $d_0$-γ-CEHC, *m/z* 408, 223 (synthesized by the method of Wechter *et al.* (13), NMR and EI—MS consistent with published data).

Metabolites—$d_0$-α-CEHC/tocopheronolactone, *m/z* 422, 237; $d_6$-α-CEHC/tocopheronolactone, *m/z* 428, 243; $d_0$-δ-CEHC, *m/z* 394, 209; $d_0$-γ-CEHC, *m/z* 408, 223; $d_0$-α-CMBHC, *m/z* 464, 237; $d_6$-α-CMBHC, *m/z* 470, 243; $d_0$-γ-CMBHC, *m/z* 450, 223; $d_0$-δ-CMBHC, *m/z* 436, 209.

*2. Oral loading studies with deuterated vitamin E.* A healthy volunteer took a single oral dose of 300 mg of $d_6$-α-tocopheryl acetate (kindly supplied by Dr. G. Burton) to (a) obtain an extracted ion chromatogram, (b) confirm the identity of presumed α-tocopherol metabolites, and (c) assess the method with urines of varying concentrations. Morning urine samples were taken both before and after the oral dose. Concentrations were in general expressed as trolox equivalents per creatinine, the creatinine being measured (15) on a COBAS analyzer using a kit supplied by Roche Products, Ltd. Trolox equivalents were obtained by comparing the peak size (molecular ion) of the metabolite of interest to the peak size of a known amount of trolox (*m/z* 394).

## RESULTS AND DISCUSSION

### *1. Recovery Using Radiolabeled Rat Urine*

Urine from rats fed radiolabeled [α-$C^{14}$]tocopherol, kindly supplied by Drs. O. Froescheis and W. Cohn (Hoffmann–La Roche, Ltd.), was added to human urine to assess the recovery of the metabolites during the procedure. Radioactivity was measured by typically adding 100–200 μl of the various solutions to 3 ml Optiphase Hisafe 3 scintillation fluid (Wallac Scintillation Products, Ltd.) and counting the disintegrations per minute using a Wallac 1410 scintillation counter with external standardization. The final recovery of radiolabel after the second solid phase extraction was 98.8% ($n$ = 6) with a range of 81.0–115.7%. Assuming rats produce similar or the same metabolites as humans these results show that the metabolites of α-tocopherol were efficiently extracted during the procedure.

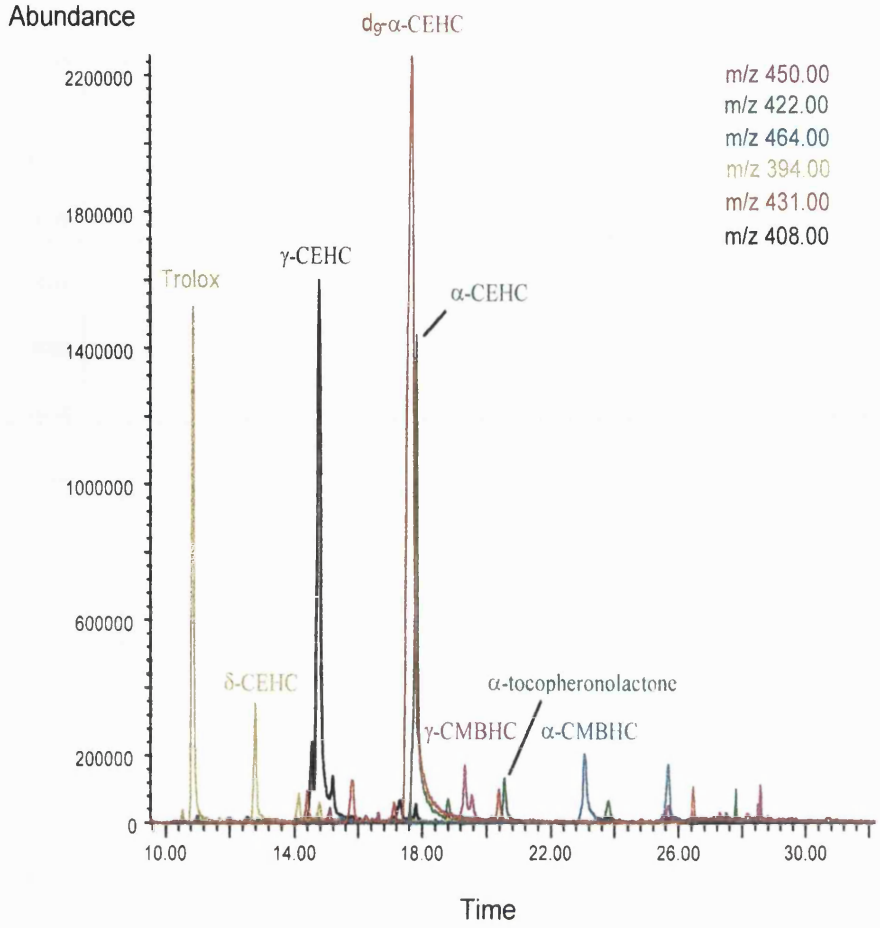


**FIG. 2.** An extracted ion chromatogram of urine collected 7 days after a single oral dose of 300 mg of $d_6$-RRR-α-tocopheryl acetate.

*2. Different Metabolites*

Figure 2 shows an extracted ion chromatogram of human urine collected 7 days after administration of a single dose of 300 mg deuterated $d_6$-RRR-α-tocopheryl acetate. Although standards were only available for α-tocopheronolactone, α-CEHC, and γ-CEHC, the other metabolites were identified by relative retention times and characteristic mass spectra and fragmentation patterns (10, 12). Supplementation with deuterated $d_6$-RRR-α-tocopherol also helped to confirm identities, as it produced analogous urinary metabolites with mass shifts of 6 Da (see below).

Mass spectra of the TMS esters/ethers of the α-CEHC and α-tocopheronolactone standards are shown in Figs. 3A and 3B, respectively. These isomeric compounds both show a large molecular ion (*m/z* 422). The major fragment ions are generated by cleavage of weak bonds, i.e., those that are separated by a single bond from the double bonds of the unsaturated ring (allylic bonds). Trimethylsilyl ether/ester groups break between the oxygen and silicon atoms producing a typical trimethylsilyl ether peak (*m/z* 73). The TMS ethers/esters of both α-CEHC and α-tocopheronolactone also show a peak of *m/z* 237 whose origin, by cleavage of two allylic bonds, is illustrated in Figs. 3A and 3B. The spectrum of the TMS ether of α-tocopheronolactone shows a prominent peak of *m/z* 309, which can be explained by the cleavage of a single allylic bond (Fig. 3B).

Interestingly, we detected only the "reduced" hydroquinone form of α-tocopheronolactone. This may be because (a) low levels of the benzoquinone form coeluted with contaminants at the beginning of the run and were not detected or (b) the benzoquinone form was reduced during the analytical procedure. The hydroquinone form of α-tocopheronolactone is the metabolite expected to be produced after deconjugation of any conjugated α-tocopheronolactone metabolites present in urine while the benzoquinone form is expected to be produced by oxidation of either the hydroquinone form or α-CEHC. The implications of this observation with regard to artifactual oxidation are discussed below.

Figure 4 shows the spectrum of a urinary metabolite (compound C) tentatively identified as

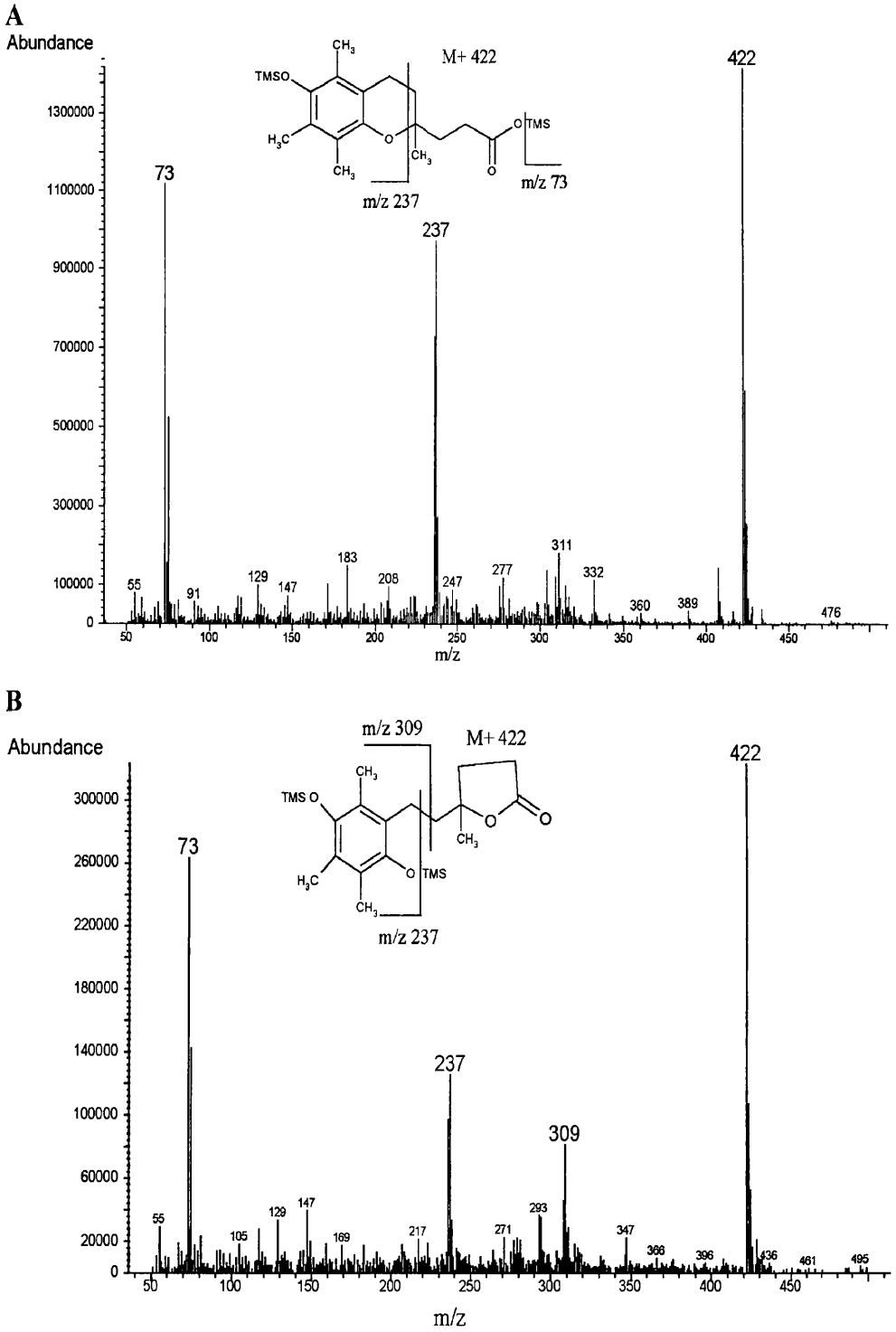


**FIG. 3.** Spectra of TMS–α-CEHC (A) and TMS–α-tocopheronolactone (B).

2,5,7,8-tetramethyl-2(4′-carboxy-4′-methylbutyl)-6-hydroxychroman (α-CMBHC).[3]

Figure 4 also shows the spectrum of compound D, which only appeared in urine after ingestion of $d_6$-α-tocopherol and was tentatively identified as $d_6$-α-CMBHC. The retention times of the two compounds were very similar but not identical, which would be expected for a $d_6$-labeled version of compound C (16). The mass spectrum of compound C contained an ion of *m/z* 237, which was identical to the ion containing the ring structure seen in the spectrum of α-CEHC.

[3] Alternative IUPAC nomenclature would be 5-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methylpentanoic acid.

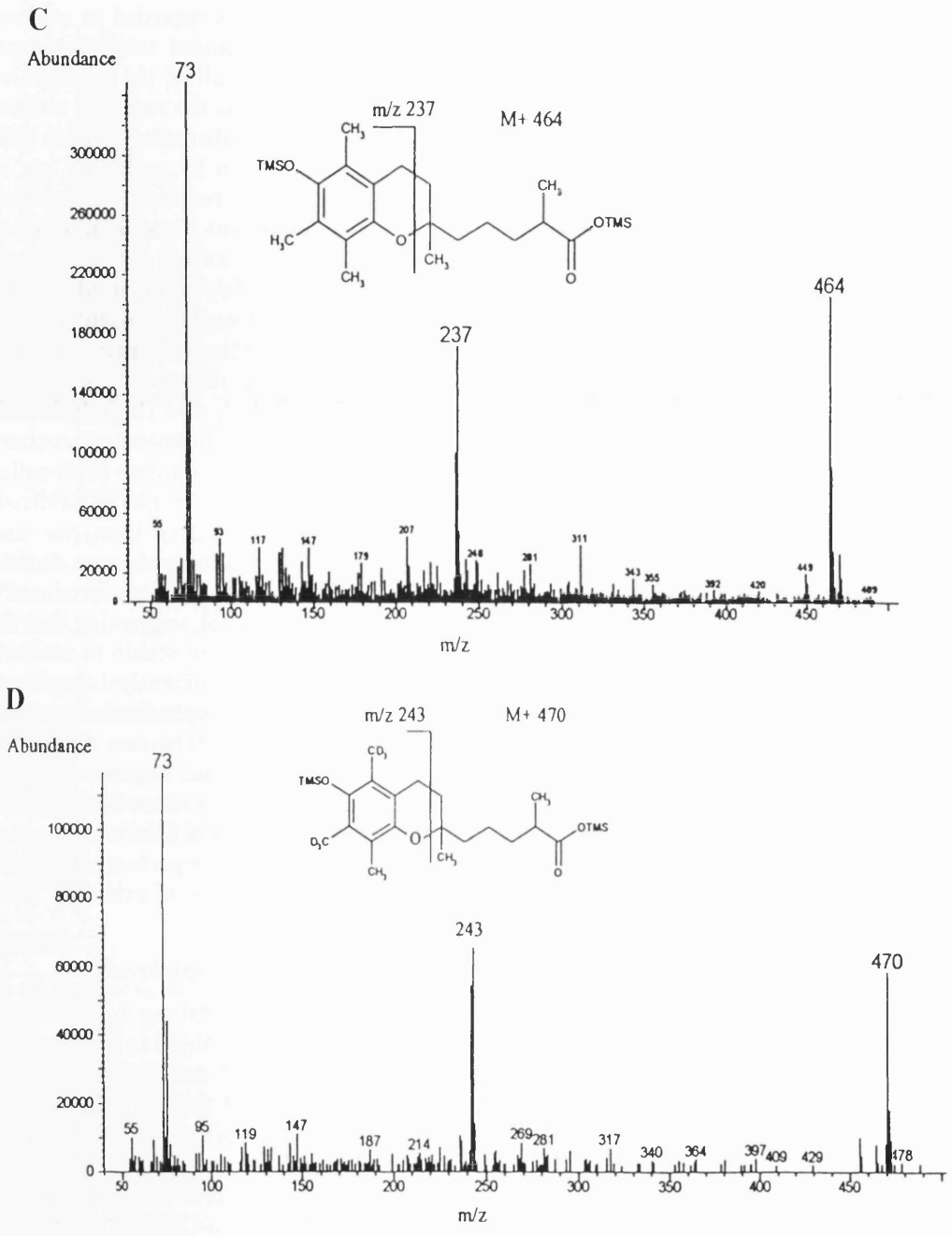


**FIG. 4.** Spectra of $d_0$ TMS–α-CMBHC (compound C) and $d_6$ TMS–α-CMBHC (compound D).

In addition compound D had a ring-containing ion with a mass shift of 6 Da giving an ion of *m/z* 243. These observations provided strong evidence that compound C was a metabolite of α-tocopherol. The molecular ions of compounds C and D were seen at *m/z* 464 and 470, respectively, compared with 422 for α-CEHC. This indicated that they had a side chain with an additional three methylene units. Compound C and α-CEHC had retention indices of 2681 and 2466, respectively. A shift of 2466 to 2766 might have been expected for the addition of three methylene units in a straight chain configuration but the observed difference could be explained if, as is more likely, the side chain is branched (17) (see below). The absence of the ion *m/z* 309 in compound C, which was seen in the spectrum of α-tocopheronolactone, and the fact that compound C had a retention index only slightly longer than that of α-tocopheronolactone (2681 compared to 2575), ruled out the possibility that it was a homolog of α-tocopheronic acid/α-tocopheronolactone with a longer side chain. The longer side-chain homolog would not form lactones so readily due to the greater separation between the carboxyl and hydroxyl groups, but would still be expected to produce a 309 fragment ion. From

TABLE II

Reproducibility of the Method

| Metabolite or standard | Within day CV % ($n$ = 6) | Between day CV % ($n$ = 4) |
|---|---|---|
| $d_9$-α-CEHC (standard) | 4.0 | 17.8 |
| $d_9$-α-Tocopheronolactone | 19.8 | 53.2 |
| $d_0$-α-CEHC (metabolite) | 4.5 | 19.3 |
| $d_0$-α-Tocopheronolactone | 21.9 | 64.7 |
| α-CMBHC | 6.8 | 1.6 |
| γ-CEHC | 4.5 | 16.7 |

*Note.* CV, coefficient of variation of separate complete analyses.

the GC–MS data it was not possible to ascertain conclusively whether compound C had three methylene units in a straight or branched side chain. However, on biological grounds, a branched side chain was more likely, as it would be consistent with a metabolite formed during β oxidation of the α-tocopherol side chain, which probably takes place in the peroxisome (18).

Similar reasoning was used to identify γ-CMBHC, which because it has one less methyl group on the hydroxychroman ring gives a characteristic fragment ion of $m/z$ 223 in place of the 237 ion of the α-tocopherol metabolites.

### 3. *Reproducibility and Artifactual Oxidation*

Since standards were not available for all the metabolites, trolox was used for quantitative purposes. Theoretically, if a method is reproducible, the ratio of the areas of the peaks of interest will remain constant. Therefore, each peak was given a relative intensity compared to the trolox peak and the reproducibility of these relative intensities, and not the actual amounts of metabolites, was measured. The coefficients of variation (CV) within and between batches of separate complete analyses are shown in Table II. For the majority of metabolites the within batch CVs were less than 10% ($n$ = 6) and between batch CVs less than 20% ($n$ = 4 over a 2-week period). α-Tocopheronolactone was an exception, as both the $d_9$-α-tocopheronolactone derived from the $d_9$-α-CEHC and the endogenous unlabeled metabolite showed CVs much greater than those of the other metabolites for both within and between batches.

The poor reproducibility of α-tocopheronolactone may result from variable artifactual conversion of α-CEHC to α-tocopheronolactone and also interconversion between the hydroquinone and benzoquinone forms of α-tocopheronolactone. Schultz *et al.* (10) showed almost complete conversion of α-CEHC to α-tocopheronolactone after bubbling oxygen through a solution of 70 μM α-CEHC in 0.1 M HCl for 24 h at room temperature. This was regarded as evidence that the presence of oxygen caused artifactual oxidation and that the majority or all of the α-tocopheronolactone reported by others was the result of artifactual oxidation of α-CEHC. Despite trying to keep artifactual oxidation to a minimum by excluding air, minimizing heat and acidity, and reducing the number of solvent changes, we found that when deuterated $d_9$-α-CEHC was added to fresh urine 5 to 10% was converted to the hydroquinone form of $d_9$-tocopheronolactone. However, the percentage of α-tocopheronolactone to α-CEHC was generally greater for the endogenous unlabeled metabolites than for the deuterated compounds (by a mean factor of 1.82, CV 56%, $n$ = 18), which indicated that there may be some endogenous α-tocopheronolactone present in urine. An alternative explanation could be that conjugated α-CEHC might be oxidized to α-tocopheronolactone more easily than the unconjugated form. It is of interest that we were unable to detect tocopheronolactone metabolites produced from the other forms of tocopherol, suggesting that these CEHC metabolites may be more stable to artifactual oxidation. Further work is currently being carried out to ascertain whether α-tocopheronolactone is a real metabolite or an artifact of the assay procedure. In particular the interconversion between the benzoquinone and hydroquinone forms of α-tocopheronolactone and their production from α-CEHC need to be studied in order to understand the importance/usefulness of these metabolites as biomarkers of oxidative stress.

### 4. *Load of Deuterated Tocopherol*

A single oral dose of 300 mg $d_6$-α-tocopheryl acetate was given to a healthy subject to confirm the identity of presumed α-tocopherol metabolites and to assess the method with urines of varying concentrations. Morning urine samples were taken on Days 1, 2, and 5 (before the oral dose) and on Days 6, 7, 8, 9, 12, 13, 14, and 15, i.e., after the oral dose. The responses of $d_0$- and $d_6$-α-CEHC and α-CMBHC in terms of trolox equivalents per millimole creatinine are shown in Fig. 5. The levels of CEHC reached a maximum 1 day after supplementation and then decreased toward the original unsupplemented levels. The maximum concentration of $d_0$-α-CEHC was 31 μmol/l using $d_9$-α-CEHC as a standard. The increase in excretion of $d_0^-$ and $d_6$-α-CEHC and $d_0^-$ and $d_6$-α-CMBHC is to be expected since the level of plasma α-tocopherol would be well above the normal range and would cause an overloading of vitamin E transport pathways and in particular an increased competition for binding to hepatic α-TTP. This would be expected to lead to an increased excretion of all forms of vitamin E metabolites, including those derived from RRR-α-tocopherol, which is normally efficiently bound to hepatic α-TTP.

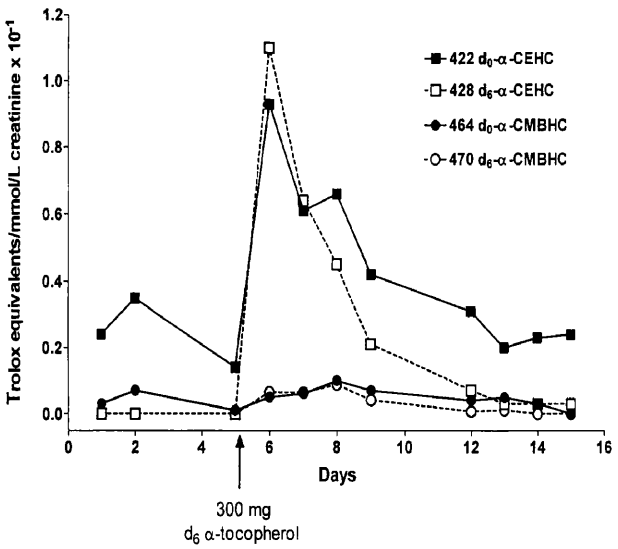


**FIG. 5.** Excretion of urinary $d_0$- and $d_6$-α-CEHC and $d_0$- and $d_6$-α-CMBHC, before and after a single oral dose of 300 mg $d_6$-RRR-α-tocopheryl acetate.

In summary, therefore, we have described a relatively simple and reproducible method for the analysis of a range of vitamin E metabolites in 0.5 ml of human urine. In addition we have tentatively identified a novel group of vitamin E metabolites that are related to the CEHCs but have three extra carbons in the side chain.

## ACKNOWLEDGMENT

S.A.S.P. thanks the Engineering and Physical Sciences Research Council for financial support.

*Note added in proof.* Since submitting this manuscript, the biosynthesis of γ-CMBHC by HepG2 cells and its presence in human urine has been reported. Parker, R. S., and Swanson, J. E. (2000) *Biochem. Biophys. Res. Commun.* **269,** 580–583.

## REFERENCES

1. Halliwell, B., and Gutteridge, J. M. C. (1999) Free Radicals in Biology and Medicine, 3rd ed., Oxford Univ. Press, Oxford/New York.
2. Burton, G. W., Joyce, A., and Ingold, K. U. (1983) *Arch. Biochem. Biophys.* **221,** 281–290.
3. Burton, G. W., Traber, M. G., Acuff, R. V., Walters, D. N., Kayden, H., Hughes, L., and Ingold, K. U. (1998) *Am. J. Clin. Nutr.* **67,** 669–684.
4. Burton, G. W., and Ingold, K. U. (1986) *Acc. Chem. Res.* **19,** 194–201.
5. Kayden, H. J., and Traber, M. G. (1993) *J. Lipid Res.* **34,** 343–358.
6. Liebler, D. C. (1993) *Crit. Rev. Toxicol.* **23,** 147–169.
7. Simon, E. J., Gross, C. S., and Milhorat, A. T. (1956) *J. Biol. Chem.* **221,** 797–805.
8. Simon, E. J., Eisengart, A., Sundheim, L., and Milhorat, A. T. (1956) *J. Biol. Chem.* **221,** 807–817.
9. Schonfeld, A., Schultz, M., Petrizka, M., and Gassmann, B. (1993) *Die Nahrung.* **37,** 498–500.
10. Schultz, M., Leist, M., Petrzika, M., Gassmann, B., and Brigelius-Flohe, R. (1995) *Am. J. Clin. Nutr.* **62,** 1527S–1534S.
11. Traber, M. G., Elsner, A., and Brigelius-Flohe, R. (1998) *FEBS Lett.* **437,** 145–148.
12. Chiku, S., Hamamura, K., and Nakamura, T. (1984) *J. Lipid Res.* **25,** 40–48.
13. Wechter, W. J., Kantoci, D., Murray, E. D., Jr., D'Amico, D. C., Jung, M. E., and Wang, W. H. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6002–6007.
14. Swanson, J. E., Ben, R. N., Burton, G. W., and Parker, R. S. (1999) *J. Lipid Res.* **40,** 665–671.
15. Fabiny, D. L., and Ertinghausen, G. (1971) *Clin. Chem.* **17,** 696–700.
16. Chapman, J. R. (1998) Practical Organic Mass Spectrometry, 2nd ed., Wiley and Sons, Chichester, New York.
17. Littlewood, A. B. (1970) Gas Chromatography: Principles, Techniques and Applications, Academic Press, New York/London.
18. Mannaerts, G. P., and van Veldhoven, P. P. (1995) *in* Functions and Biogenesis of Peroxisomes in Relation to Human Disease (Wanders, R. J. A., Schutgens, R. B. H., and Tabak, H. F., Eds.), pp. 1–27, Royal Netherlands Academy of Arts and Sciences, Amsterdam/Oxford/New York/Tokyo.

Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 1337–1343

BIOORGANIC & MEDICINAL CHEMISTRY

# New Synthesis of (±)-α-CMBHC and Its Confirmation as a Metabolite of α-Tocopherol (Vitamin E)

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Received 13 November 2000; accepted 5 January 2001

**Abstract**—There is currently interest in the metabolism of the various compounds which make up the vitamin E family, especially with regards to the possible use of vitamin E metabolites as markers of oxidative stress and adequate vitamin E supply. A number of vitamin E metabolites have been described to date and we have recently developed a method to extract and quantitate a range of vitamin E metabolites in human urine. During the development of this method a new metabolite of α-tocopherol was identified, which we tentatively characterised as 5-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoic acid (α-CMBHC).[1] Here we describe the synthesis of α-CMBHC as a standard and confirm that it is a metabolite of α-tocopherol. 

## Introduction

α-Tocopherol (**1**) is the major lipid soluble antioxidant in vivo and is a member of the vitamin E family.[2,3] Vitamin E is a generic term describing the tocopherols and tocotrienols, which have saturated and unsaturated side-chains respectively (Fig. 1). Each group has α, β, δ and γ forms which differ in the number and position of methyl groups on the chroman ring. α-Tocopherol (**1**) is the most abundant form in the body accounting for over 90% of the total vitamin E retained, even though γ-tocopherol is generally the most abundant form in the diet.[4,5] Naturally occurring α-tocopherol is a single stereoisomer designated *R,R,R*-α-tocopherol whereas most synthetic supplements are a mixture of the eight possible stereoisomers arising from the three chiral centres.

Over the last 50 years a number of vitamin E metabolites have been described and recently there has been renewed interest in measuring urinary metabolites of vitamin E due to their proposed use as biomarkers of oxidative stress and adequate vitamin E supply.[6] Scheme 1 shows an overview of the proposed metabolism of α-tocopherol. In the 1950's α-tocopheronic acid (**2**) and its lactone, α-tocopheronolactone (**3**), the so-called Simon metabolites, were characterised in the urine of animals and man.[7,8] These metabolites, which result from the ring opening of the chroman moiety, were thought to be products of α-tocopherol oxidation. More recently another α-tocopherol metabolite, 3-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-propionic acid (α-CEHC)[1] (**4**), has been described and found to be the major urinary metabolite of α-tocopherol.[6] α-CEHC (**4**) has an intact chroman ring and was hypothesised to represent excretion of excess α-tocopherol. Analogous CEHC metabolites have also been described for δ- and γ-tocopherol.[9,10] Along with the identification of α-CEHC (**4**) as the major metabolite of α-tocopherol came the hypothesis that the previously identified Simon metabolites were produced by artefactual oxidation of α-CEHC during the extraction procedure.[6] Recently we have reported a longer side-chain metabolite of α-tocopherol that was tentatively identified as 5-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoic acid (α-CMBHC) (**5**) (Scheme 1).[11] The equivalent metabolite of γ-tocopherol has also been described by Parker et al.[12] The metabolites of the various tocopherols would be expected to retain the stereochemistry of the parent compound from which they are derived. For example, metabolites produced from synthetic vitamin E would be present as a mixture of isomers while those

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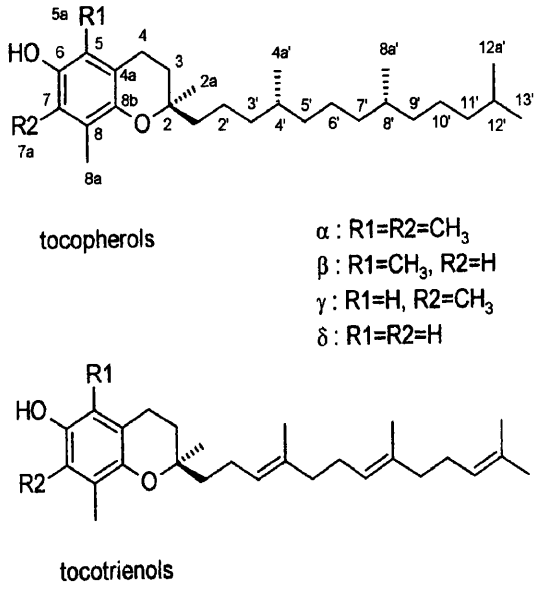



**Figure 1.** The structures of the naturally occurring forms of vitamin E.

produced from naturally occurring vitamin E would be present as single isomers. Indeed, the stereochemistry of γ-CEHC, derived from *R,R,R*-γ-tocopherol, has been shown to be S(+), meaning it is formed without epimerisation at C-2.[13]

In order to unambiguously confirm the structure of this new metabolite of α-tocopherol, we prepared a synthetic standard of α-CMBHC (**5**) and showed that it had GC–MS characteristics identical to those of the urinary metabolite.

## Results

In studies to elucidate the metabolism of α-tocopherol we developed a new extraction procedure and GC–MS method for human urinary vitamin E metabolites.[11] This method, which requires trimethylsilyl-derivatisation of deconjugated metabolites, allowed us to analyse a range of metabolites, including α-tocopheronolactone (**6**) and α-CEHC (**4**). During these studies we observed a minor, late eluting peak in human urine which had a mass spectra displaying a major fragment ion (*m/z* 237) in common with silylated α-CEHC (**4**), but with a molecular ion of *m/z* 464, which is 42 daltons greater than the molecular ion of silylated α-CEHC (Fig. 2). After ingestion of $d_6$-α-tocopherol,[14] this unknown peak increased in size and the mass spectrum showed an increase in mass of 6 daltons for both the molecular ion and the major fragment ion, confirming the unknown peak as a metabolite of α-tocopherol. This unknown peak was tentatively identified as α-CMBHC (**5**) based on the GC–MS data and on the expected side-chain metabolism of α-tocopherol. Although on biological grounds α-CMBHC (**5**) was the most likely structure due to the hypothesised β-oxidation of the phytyl side-chain, other structures could display similar chromatography and mass spectra. The structure of the unknown metabolite has now been confirmed by the unambiguous synthesis of α-CMBHC (**5**).

A synthesis of (±)-α-CMBHC (**5**) was described by Weichet et al. more than 30 years ago.[15] However, this report did not propose α-CMBHC (**5**) as a metabolite of α-tocopherol and did not include NMR characterisation. The strategy relied upon the condensation of 6-hydroxy-6-vinyl-2-methylheptanoic acid (**7**) with 2,3,5-trimethylhydroquinone (**8**) (TMHQ) in the presence of a mixture of zinc chloride and boron trifluoride–diethyl etherate complex. The allylic alcohol (**7**) was obtained in three steps by oxidative ring opening of dimethylcyclohexanone (**9**), addition of acetylide to the resulting 6-oxo-2-methylheptanoic acid (**10**) and final catalytic hydrogenation of the propargylic alcohol intermediate.[16]

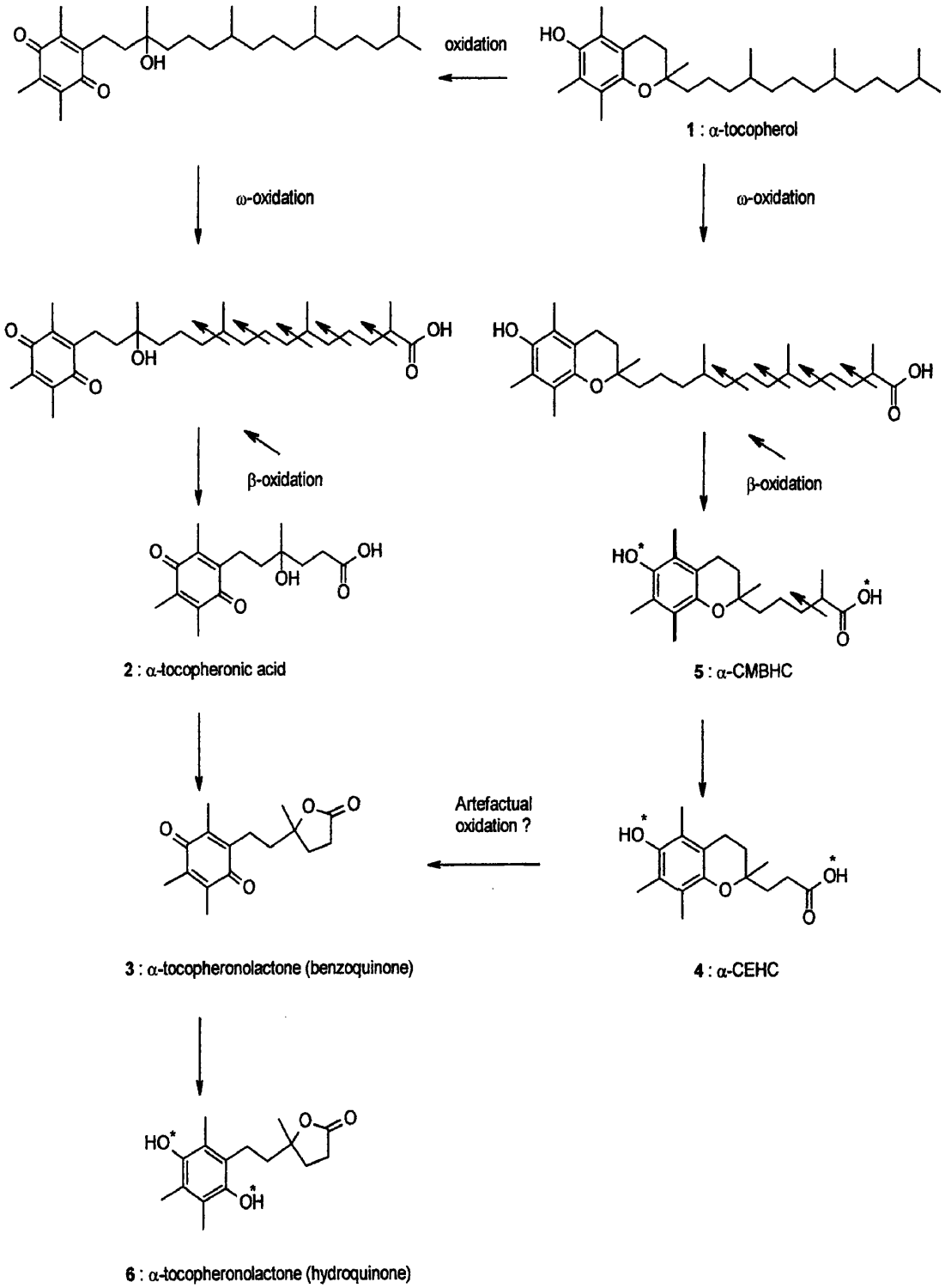
In our synthesis of (±)-α-CMBHC (**5**), the ketoacid (**10**) was prepared in a good yield by treatment of dimethylcyclohexanone (**9**) with potassium permanganate according to the procedure described by Weichet et al.[15] However, we decided to synthesise the key intermediate (**7**) by condensation of vinyl magnesium bromide with 6-oxo-2-methyl-heptanoic acid (**10**) (Scheme 2). Preliminary studies on a commercially available model compound (6-oxo-heptanoic acid) suggested that the Grignard condensation would be more effective on the corresponding methyl ester (**11**). 6-Hydroxy-6-vinyl-2-methyl-heptanoic acid methyl ester (**12**) was thus prepared by condensation of vinyl magnesium bromide with 6-oxo-2-methyl-heptanoic acid methyl ester (**11**), which was obtained from the corresponding acid (**10**). A similar strategy was used by Wechter and Kantoci to prepare the key intermediate, γ-methyl-γ-vinyl-butyrolactone, for their syntheses of α- and γ-CEHC.[10,13]

Condensation of 6-hydroxy-6-vinyl-2-methyl-heptanoic acid methyl ester (**12**) with TMHQ (**8**) was performed according to the conditions described by Kantoci et al.[13] The allylic alcohol (**12**) was added at 110 °C over 3 h to a solution of TMHQ (**8**) and boron trifluoride–diethyl etherate complex in dioxane to give α-CMBHC methyl ester (**13**) in 80% yield. Saponification of the methyl ester (**13**) using aqueous sodium hydroxide afforded (±)-α-CMBHC (**5**).

Using the synthetic standard (±)-(**5**) in our GC–MS analytical method, we confirmed that the retention times and mass spectra for the unknown metabolite and synthetic α-CMBHC (**5**) were identical. This was achieved by running the standard and urine extract separately and then in combination to show co-elution of the two peaks (Fig. 3). We assumed that the other peaks observed in urine samples with similar mass spectra, but with molecular and fragment ions 14 or 28 daltons less, correspond to γ- and δ-CMBHC respectively. The mass spectrum observed for the presumed γ-CMBHC agrees with that reported by Parker et al.[12]

## Discussion

Vitamin E, and in particular α-tocopherol (**1**), is the major fat soluble antioxidant in vivo, protecting cellular membranes and other lipids against oxidative damage caused by oxygen-derived free radicals.[2,3] Owing to the




**Scheme 1.** An overview of the proposed metabolism of α-tocopherol. The metabolites are thought to be excreted in the urine as sulphate or glucuronide conjugates. Possible sites of conjugation are indicated (*).

lipophilicity of vitamin E, lipoproteins and transfer proteins are required to deliver vitamin E around the body and transfer it between membranes.[17] At high concentrations the various forms of vitamin E are likely to overload these transport/transfer mechanisms, leading to side-chain shortening of excess vitamin E and excretion of the resulting metabolites, such as α-CEHC (**4**), in the form of water soluble conjugates.[6] In the present work, we confirmed that α-CMBHC (**5**) is a metabolite of α-tocopherol using a synthetic standard. Other peaks observed using our GC–MS method have mass spectra consistent with the structures of δ- and γ-CMBHC, supporting the idea of a common pathway for the side-chain metabolism of all the tocopherols. The structure of the CMBHCs agrees with the postulated ω- and then β-oxidation of the phytyl side-chain, which is believed to occur in the peroxisome.[18]

Since the CMBHCs are the probable precursors of the CEHCs, metabolites with longer side-chains, corresponding

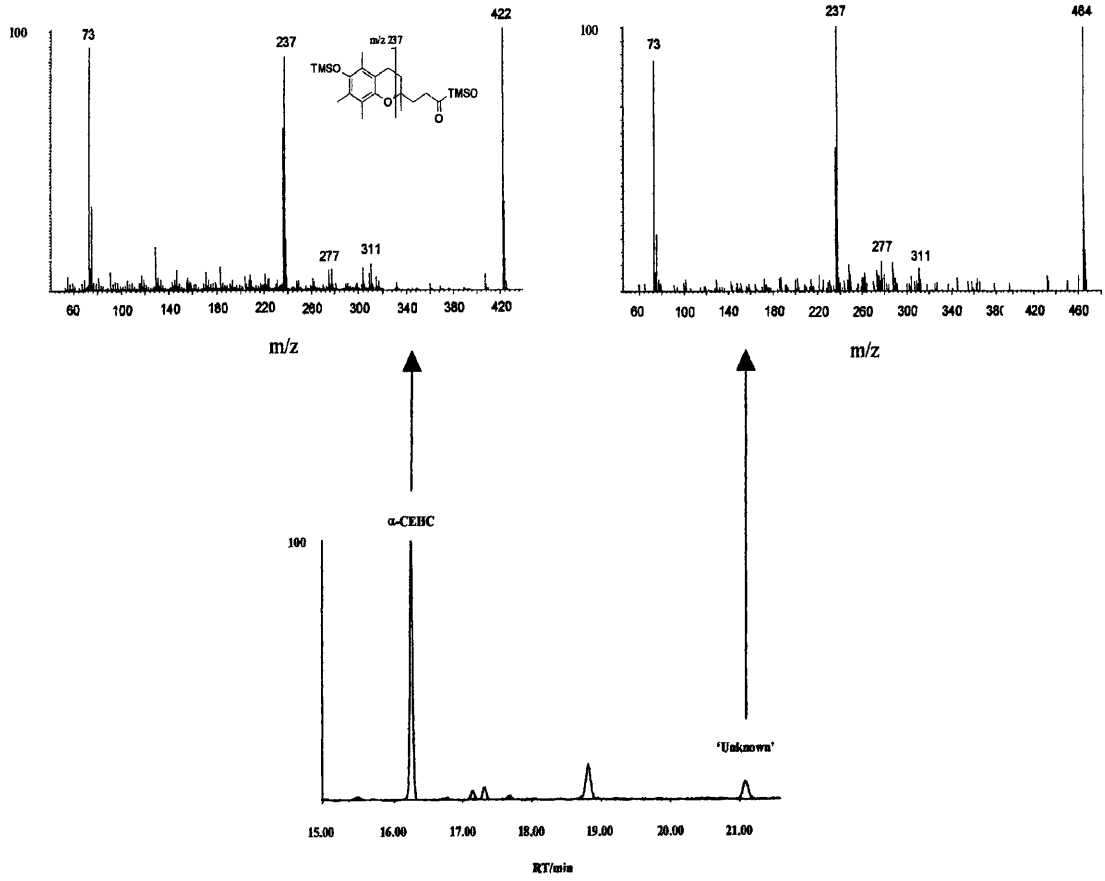
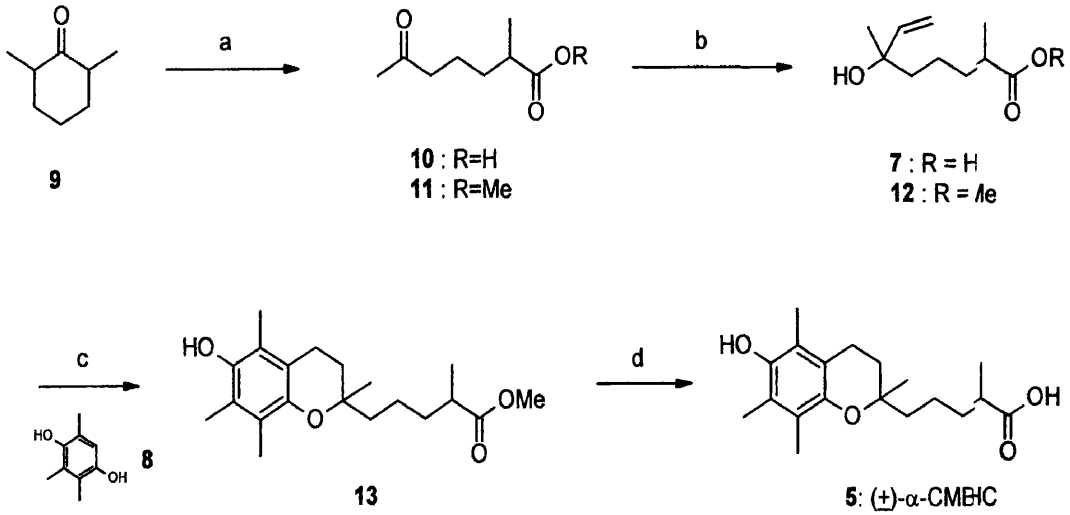



**Figure 2.** Gas chromatogram showing elution of the silyl derivatives of α-CEHC and the 'unknown' metabolite with their corresponding mass spectra.




**Scheme 2.** Synthesis of (±)-α-CMBHC (**5**). (a) (i) $KMnO_4$; (ii) MeOH, $H^+$; (b) $CH_2CHMgBr$; (c) $BF_3.Et_2O$; (d) $NaOH/H_2O$.

to the precursors of the CMBHCs, are also possible. However, longer side-chain metabolites may not be excreted in the urine due to their greater hydrophobicity. It is also possible that longer side-chain metabolites would only be excreted when the metabolic pathways leading to complete side-chain oxidation are overloaded, such as after supplementation with a large amount of vitamin E.

The synthetic pathway we have developed for the preparation of (±)-α-CMBHC, could also be used to synthesise the CMBHC metabolite standards of the other tocopherols from the appropriately mthylated hydroquinones. Although the CMBHCs are metabolic products of vitamin E we cannot rule out the possibility that they may have biological activity themselves. Recently γ-CEHC, a metabolite of γtocopherol, has

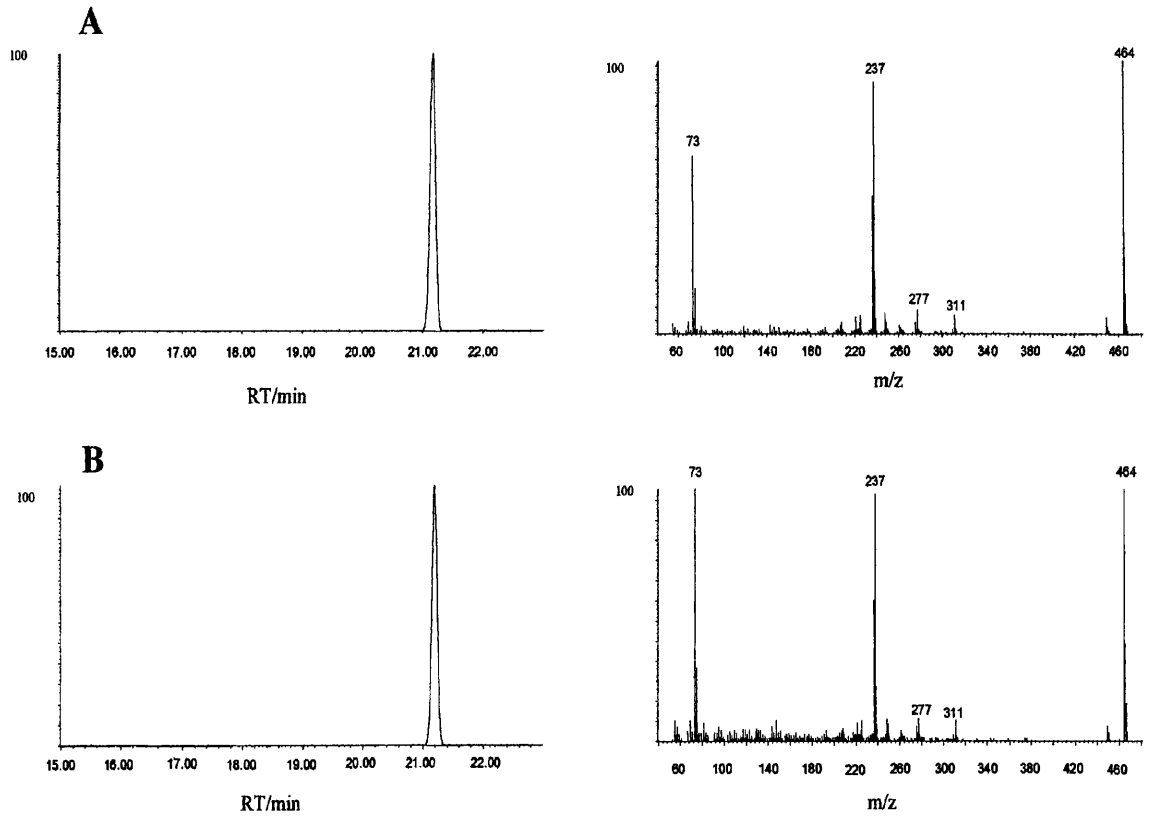



**Figure 3.** A shows an extracted ion chromatogram (*m/z* 464) of the silyl derivative of α-CMBHC standard (**5**) with its corresponding mass spectrum. B shows the equivalent data for a mixture of α-CMBHC standard and the 'unknown' urinary metabolite, showing co-elution of the two peaks and identical mass spectra.

been shown to act as a natriuretic factor by inhibiting potassium channels in kidney.[10] The synthetic CMBHC standards could be used in initial investigations of their biological activity. However, it is worth noting that in the case of in vivo studies or enzymatic assays which require standards of the naturally occurring pure metabolite, synthetic methods allowing tighter control of the chiral centres will have to be used.

## Conclusion

We have developed an efficient strategy for the synthesis (±)-α-CMBHC (**5**) and have used our synthetic material as a standard to confirm that α-CMBHC (**5**) is a minor metabolite of α-tocopherol (**1**) in human urine. (±)-α-CMBHC (**5**) has been synthesised in five steps and 19% overall yield from dimethylcyclohexanone (**9**) and has been fully characterised. This methodology could also be used to synthesise standards of δ- and γ-CMBHC, which both appear to be present in human urine.

## Experimental

### Extraction and analysis of urinary metabolites of vitamin E

The method used for the extraction and analysis of the vitamin E metabolites in this study was the same as that previously described.[11] In summary, the metabolites were extracted from human urine, after acidification and addition of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and $d_9$-α-CEHC standards,[14] using C4 solid phase extraction (SPE) cartridges. Enzymatic deconjugation overnight with mixed β-glucuronidase/sulphatase and re-extraction using a second C4 SPE cartridge gave unconjugated metabolites. Trimethylsilyl (TMS) derivatives were produced using *N,O*-bis-(trimethylsilyl)trifluroacetamide (BSTFA, Pierce and Warriner Ltd) at 60 °C for 1 h. The derivatised mixture (2 μL) was injected (using a splitless technique) onto a DB1 fused silica column (30 m, 0.25 mm ID, 0.25 μm film thickness; Jones Chromatography Ltd) in a Hewlett-Packard 5890 Series II gas chromatograph linked to a Hewlett-Packard 5970 mass-selective detector and Chem Station data system. The oven was maintained at 120 °C for 2 min, then ramped to 200 °C at 20 °C/min, to 240 °C at 2 °C/min, to 300 °C at 50 °C/min and finally held at 300 °C for 5 min. The ionisation energy was 70 eV. $d_6$-α-Tocopherol metabolites were produced in the urine following supplementation with 300 mg $d_6$-α-tocopherol,[14] as previously described.[11] The metabolites and standard peaks were visualised by extracting the following ion chromatograms: Standards—Trolox, *m/z* 394, 237; $d_9$-α-CEHC, *m/z* 431, 246; Metabolites-$d_0$-α-CEHC/tocopheronolactone, *m/z* 422, 237; $d_6$-α-CEHC/tocopheronolactone, *m/z* 428, 243; $d_0$-γ-CEHC, *m/z* 408, 223; $d_0$-α-CMBHC, *m/z* 464, 237; $d_6$-α-CMBHC, *m/z* 470, 243; $d_0$-γ-CMBHC, *m/z* 450, 223; $d_0$-δ-CMBHC, *m/z* 436, 209.

### Synthesis of (±)-5-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoic acid ((±)-α-CMBHC) (5)

**General.** All starting materials were either commercially available or reported previously in the literature unless noted. Solvents and reagents were used without further purification except tetrahydrofuran (THF) which was dried over sodium. Reactions were monitored by TLC on precoated silica gel plates (Kieselgel 60 $F_{254}$, Merck). Purification was performed by flash chromatography using silica gel (particle size 40–63 μM, Merck). $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker AMX-300 spectrometer. Chemical shifts are reported as ppm relative to tetramethylsilane (TMS) as internal standard. The chemical shifts of compounds (**13**) and (**5**) were assigned on the basis of $^1H$–$^{13}C$ NMR correlation and previously published data.[10] Atom numbering follows IUPAC convention. Mass spectra were recorded on either a VG ZAB SE spectrometer (electron impact and fast atom bombardment (FAB)) or a Micromass Quattro electrospray LC-mass spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrophotometer. Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected.

**6-Oxo-2-methyl-heptanoic acid (10).** 2,6-Dimethylcyclohexanone (**9**) (1 g, 7.9 mmol) was added at room temperature to a stirring solution of potassium permanganate (1.76 g, 11.0 mmol) in water (60 mL). The reaction mixture was stirred at room temperature for 12 h, acidified with 5% aqueous hydrochloric acid and extracted in ethyl acetate. The organic layer was washed with water, dried over anhydrous magnesium sulphate and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (10–40% ethyl acetate in cyclohexane) to give (**10**) as a clear oil (1.0 g, 80%). IR (neat) 3700–2700 (acid), 1704 (carbonyl) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.2 (3H, d, $J$=7.2 Hz, CHMe), 1.40–1.49 (1H, m, CHMe), 1.57–1.68 (3H, m), 2.14 (3H, s, COMe), 2.43–2.51 (3H, m); $^{13}C$ NMR ($CDCl_3$) δ 17.1, 21.6, 30.2, 33.1, 39.5, 43.7, 183.0, 209.3; MS (FAB+) $m/z$ 113 $[M-CO_2H]^+$, 141 $[M-OH]^+$, 159 $[M+H]^+$.

**6-Oxo-2-methyl-heptanoic acid methyl ester (11).** A few drops of concentrated sulphuric acid were added at room temperature to a stirring solution of 6-oxo-2-methyl-heptanoic acid (**10**) (1.0 g, 6.3 mmol) in dry methanol (30 mL) under nitrogen. The reaction mixture was refluxed for 2 h and then an excess of ethyl acetate was added. The organic layer was washed successively with saturated aqueous sodium bicarbonate and water, dried over anhydrous magnesium sulphate and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (10–30% ethyl acetate in cyclohexane) to give (**11**) as an oil (820 mg, 76%). IR (neat) 1727 (carbonyl) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.11 (3H, d, $J$=6.8 Hz, CHMe), 1.35–1.45 (1H, m), 1.47–1.70 (3H, m), 2.09 (3H, s, Me), 2.35–2.45 (3H, m), 3.63 (3H, s, OMe); $^{13}C$ NMR ($CDCl_3$) δ 14.1, 17.0, 21.4, 29.7, 33.1, 39.2, 43.3, 51.4, 176.7, 208.2; MS (FAB+) $m/z$ 113 $[M-CO_2Me]^+$ 141 $[M-OMe]^+$, 173 $[M+H]^+$.

**6-Hydroxy-6-vinyl-2-methyl-heptanoic acid methyl ester (12).** Vinyl magnesium bromide (1M in THF, 6.4 mL, 6.4 mmol) was added dropwise at 0 °C to a stirring solution of 6-oxo-2-methyl-heptanoic acid methyl ester (**11**) (1 g, 5.8 mmol) in THF (5 mL) under nitrogen. The reaction was stirred at 0 °C for 2 h and then a few drops of water were added. The mixture was dried over anhydrous magnesium sulphate and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (10–30% ethyl acetate in cyclohexane) to give (**12**) as an oil and a mixture of two diastereoisomers (945 mg, 81%). IR (neat) 3416 (hydroxy), 1727 (carbonyl) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.13 (3H, d, $J$=7.2 Hz, CHMe), 1.27 (3H, s, Me), 1.25–1.70 (6H, m, 3×$CH_2$), 2.37–2.49 (1H, m, CHMe), 3.65 (3H, s, OMe), 5.02 (1H, dd, $J$=10.6, 1.1 Hz, $CH_2$=), 5.18 (1H, dd, $J$=17.3, 1.1 Hz, CH=), 5.88 (1H, dd, $J$=17.3, 10.6 Hz, $CH_2$=); $^{13}C$ NMR ($CDCl_3$) δ 17.3, 21.8, 27.8, 34.3, 39.6, 42.3, 51.7, 73.2, 111.8, 145.3, 177.5; second diastereoisomer 17.4, 28.0, 42.4; MS (FAB+) $m/z$ 183 $[M-OH]^+$, 201 $[M+H]^+$.

**(±)-5-(6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoic acid methyl ester (13).** 6-Hydroxy-6-vinyl-2-methyl-heptanoic acid methyl ester (**12**) (830 mg, 4.15 mmol) in dioxane (1 mL) was added over 3 h at 110 °C to a stirring solution of 2,3,5-trimethylhydroquinone (421 mg, 2.77 mmol) and boron trifluoride diethyletherate (680 μL, 5.5 mmol) in dioxane (15 mL) under nitrogen. The reaction mixture was cooled to room temperature and diluted with an excess of ethyl acetate. The organic layer was washed with water, dried over anhydrous magnesium sulphate and concentrated under vacuum. The crude compound was purified by flash chromatography on silica (10–30% ethyl acetate in cyclohexane) to give (**13**) as an oil and a mixture of two diastereoisomers (740 mg, 80%). IR (neat) 3439 (hydroxy), 1727 (carbonyl) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.16 (3H, d, $J$=6.9 Hz, H4a′), 1.22 (3H, s, H2a), 1.41–1.71 (6H, m, 3×$CH_2$), 1.78 (2H, ddd, $J$=6.4, 12.5, 13.4 Hz, $CH_2$), 2.11 (3H, s, H5a), 2.12 (3H, s, H7a), 2.17 (3H, s, H8a), 2.41–2.50 (1H, m, H4′), 2.60 (2H, t, $J$=6.8 Hz, H4) 3.67 (3H, s, OMe); $^{13}C$ NMR ($CDCl_3$) δ 11.6 (C5a), 12.1 (C7a), 12.6 (C8a), 17.3 (C4a′), 21.0 (C4), 21.5 ($CH_2$), 23.9 (C2a), 31.8 ($CH_2$), 34.5 ($CH_2$), 39.5 ($CH_2$), 39.6 (C4′), 51.8 (OMe), 74.6 (C2), 117.5 (C5), 119.0 (C4a), 121.6 (C7), 122.8 (C8), 145.0 (C6), 145.6 (C8b), 177.6 (C5′); second diastereoisomer 17.4 (C4a′), 21.6 ($CH_2$), 39.6 ($CH_2$), 39.7 (C4′), 51.9 (OMe), 177.7 (C5′); MS (EI) $m/z$ 165 (100%), 334 (72%, $[M]^+$).

**(±) 5-(6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoic acid (5).** Sodium hydroxide (50 mg, 1.25 mmol) in water (10 mL) was added at rt to a stirring solution (±)-5-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoate (**13**) (130 mg, 0.39 mmol) in methanol (10 mL). The reaction mixture was refluxed for 2 h, acidified to pH 3 with 5% aqueous hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous magnesium sulphate and concentrated under vacuum. The crude product was purified by flash chromatography on silica (20–30% ethyl acetate in cyclohexane) to yield (**5**)

as a solid and mixture of two diastereoisomers (60 mg, 48%). Mp 90–92 °C [reported 100–103 °C];[14] IR ($CHCl_3$) 3700–2600 (acid + hydroxy), 1697 (carbonyl) $cm^{-1}$; $^1H$ NMR ($CDCl_3$) δ 1.16 (3H, d, $J$ = 6.4 Hz, H4a′), 1.23 (3H, s, H2a), 1.41–1.61 (6H, m, 3×$CH_2$), 1.79 (2H, ddd, $J$ = 6.6, 12.1, 13.4 Hz, $CH_2$), 2.12 (6H, s, H5a and H7a), 2.16 (3H, s, H8a), 2.46–2.55 (1H, m, H4′), 2.61 (2H, t, $J$ = 6.4 Hz, H4); $^{13}C$ NMR ($CDCl_3$) δ 11.6 (C5a), 12.1 (C7a), 12.6 (C8a), 17.2 (C4′a), 21.0 (C3′), 21.6 ($CH_2$), 24.0 (C2a), 31.9 ($CH_2$), 34.2 ($CH_2$), 39.7 (C4′), 39.8 ($CH_2$), 74.6 (C2), 117.6 (C5), 119.0 (C4a), 121.5 (C7), 122.9 (C8), 144.9 (C6), 145.7 (C8b), 183.5 ($CO_2H$); second diastereoisomer 17.3 (C4′a), 21.7 ($CH_2$), 39.7 ($CH_2$), 39.8 (C4′); MS (EI) $m/z$ 165 (100%), 320 (22%, $[M]^+$); FAB HRMS: calculated (M−H): 319.1908, found 319.1881 ($C_{19}H_{28}O_4$).

## Acknowledgements

The authors would like to thank the Engineering and Physical Sciences Research Council and the Szeben Peto Foundation for financial support.

## References and Notes

1. The names used for the metabolites of vitamin E discussed in this report conform with IUPAC nomenclature. However, several of the compounds mentioned herein are also commonly known by less systematic names. These are as follows: α-CEHC, 2,5,7,8-tetramethyl-2-(2-carboxy-2-ethyl)-6-hydroxychroman; α-CMBHC, 2,5,7,8-tetramethyl-2-(4-carboxy-2-methylbutyl)-6-hydroxychroman. The correct IUPAC names for the other vitamin E metabolites mentioned in the text are: α-tocopheronolactone, 2,3,5-Trimethyl-6-[2-(2-methyl-5-oxo-tetrahydrofuran-2-yl)-ethyl]-[1,4]benzoquinone; γ-CEHC, 3-(6-Hydroxy-2,7,8-trimethyl-chroman-2-yl)-propionic acid; γ-CMBHC, 5-(6-Hydroxy-2,7,8-trimethyl-chroman-2-yl)-2-methyl-pentanoic acid; δ-CEHC, 3-(6-Hydroxy-2,8-dimethyl-chroman-2-yl)-propionic acid; δ-CMBHC, 5-(6-Hydroxy-2,8-dimethyl-chroman-2-yl)-2-methyl-pentanoic acid.
2. Burton, G. W.; Joyce, A.; Ingold, K. U. *Arch. Biochem. Biophys.* **1983**, *221*, 281.
3. Brigelius-Flohe, R.; Traber, M. G. *FASEB* **1999**, *13*, 1145.
4. Burton, G. W.; Traber, M. G.; Acuff, R. V.; Walters, D. N.; Kayden, H.; Hughes, L.; Ingold, K. U. *Am. J. Clin. Nutr.* **1998**, *67*, 669.
5. Bieri, J. G.; Evarts, R. P. *J. Am. Diet. Assoc* **1973**, *62*, 147.
6. Schultz, M.; Leist, M.; Petrizka, M.; Gassmann, B.; Brigelius Flohe, R. *Am. J. Clin. Nutr.* **1995**, *62*, 1527 S.
7. Simon, E. J.; Gross, C. S.; Milhorat, A. T. *J. Biol. Chem.* **1956**, *221*, 797.
8. Simon, E. J.; Eisengart, A.; Sundheim, L.; Milhorat, A. T. *J. Biol. Chem.* **1956**, *221*, 807.
9. Chiku, S.; Hamamura, K.; Nakamura, T. *J. Lipid Res.* **1984**, *25*, 40.
10. Wechter, W. J.; Kantoci, D.; Murray, E. D., Jr.; D'Amico, D. C.; Jung, M. E.; Wang, W. H. *Proc. Natl. Acad. Sci.* **1996**, *93*, 6002.
11. Pope, S. A. S.; Clayton, P. T.; Muller, D. P. R. *Arch. Biochem. Biophys.* **2000**, *381*, 8.
12. Parker, R. S.; Swanson, J. E. *Biochem. Biophys. Res. Commun.* **2000**, *269*, 580.
13. Kantoci, D.; Wechter, W. J.; Murray, E. D.; Dewind, S. A., Jr.; Borchardt, D.; Khan, S. I. *J. Pharmacol. Exp. Ther.* **1997**, *282*, 648.
14. $d_3$-α-tocopheronolactone, $d_9$-α-CEHC and $d_6$-α-tocopherol were all kind gifts from Dr.G.W. Burton, Steacie Institute of Molecular Science, National Research Council, Ottawa, Canada.
15. Weichet, J.; Blaha, L.; Kavac, B.; Hodrova, J. *Collection. Czechoslov. Chem. Comm.* **1966**, *31*, 2434.
16. Weichet, J.; Blaha, L. *Collection. Czechoslov. Chem. Comm.* **1966**, *31*, 2424.
17. Traber, M. G.; Cohn, W.; Muller, D. P. R. In *Vitamin E in Health and Disease*; Packer, L., Fuchs, J., Eds.; **1993**, pp 35–51.
18. Mannaerts, G.P.; Veldhoven, P.P. In *Functions and Biogenesis of Peroxisomes in Relation to Human Disease*; Wanders, R. J. A.; Schutgens, R. B. H.; Tabak, H. F., Eds.; Royal Academy of Arts and Sciences: Amsterdam, Oxford, New York, Tokyo, **1995**, pp 1–27.