Stable isotope studies into the kinetics and bioavailablity of vitamin K in humans

Thesis

Stable isotope studies into the kinetics and bioavailability of vitamin K_1 in humans

A dissertation submitted for the degree of Doctor of Philosophy

Life Sciences Research

The Open University

Medical Research Council, Human Nutrition Research,

Cambridge

Kerry S. Jones, BSc

July 2007

DATE of SUBMISSION: 27 APRIL 2007
DATE of AWARD: 16 AUGUST 2007

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

Stable isotope studies into the kinetics and bioavailability of vitamin K_1 in humans

In Britain, vitamin K_1 (phylloquinone) is the primary form of vitamin K in the human diet and blood. Evidence is accumulating for roles of vitamin K_1 beyond established functions in blood coagulation, particularly in bone metabolism. To aid the determination of recommended intakes vitamin K_1 kinetics and bioavailability were investigated in adult volunteers using stable isotopes.

Methods to measure reliably and accurately the isotopic enrichment of plasma vitamin K_1 using gas chromatography mass spectrometry (GCMS) were developed. Two stable isotope labelled forms of vitamin K_1 (^{13}C and ring-D_4) measured simultaneously disposal kinetics of intravenous doses and absolute absorption of 4 μg oral doses in ten lean, healthy volunteers (1 male and 9 female), aged 22 – 31 y. Isotopic data were fitted to a 2-compartment model with input and output from the sampled (blood plasma) pool, and exchange between it and a remote compartment. Mean half-times for vitamin K_1 disappearance were 0.2 and 2.7 h and mean absolute absorption of oral doses was 13%.

A three-way crossover measured vitamin K_1 bioavailability in twelve lean, healthy volunteers (7 male and 5 female) aged 22 – 49 y. Each volunteer consumed 20 μg of capsulated ^{13}C-labelled vitamin K_1 with one of three test-meals representing convenience, cosmopolitan or animal-oriented diets and balanced for fat, protein and carbohydrate but containing vitamin K_1 in different components. Blood was sampled over 8 h. Relative bioavailability was greater from the convenience meal (relative bioavailability = 1.00), in which most vitamin K_1 was in oils and fats not intact vegetables, compared to either the cosmopolitan (0.46) or animal-oriented (0.29) meals.

These studies demonstrate that stable isotope-based methods successfully measure vitamin K_1 bioavailability and metabolism and their potential for use in establishing recommended dietary intakes.

PREFACE

Declaration

This dissertation is my own work and the outcome does not contain work done in collaboration. No part of the results presented herein has been submitted for a degree or diploma or other qualification at any University.

Publications arising directly from the work presented in this thesis are:

Jones KS, Coward WA and Bluck LJC (2006) Analysis of isotope ratios in vitamin K_1 (phylloquinone) from human plasma by gas chromatography mass spectrometry. *Rapid Communications in Mass Spectrometry* **20** (12) 1894-1898.

Jones KS, Bluck LJC, Wang LY and Coward WA (2007) A stable isotope approach for the simultaneous measurement of vitamin K_1 (phylloquinone) kinetics and absorption. *European Journal of Clinical Nutrition* doi: 10.1038/sj.ejcn.1602859

Jones KS, Bluck LJC, Wang LY and Coward WA (2007) Stable isotope studies to measure vitamin K_1 kinetics and bioavailability. *Proceedings of the Nutrition Society.* In press (meeting abstract).

The bioavailability of vitamin K_1 from meals measured using stable isotopes. Jones KS, Bluck LJC, Wang LY, Stephen AM, Prynne CJ and Coward WA. Manuscript in preparation.

Candidates' contribution to work undertaken during the course of this thesis

Section 2

Performed all method development work

Performed GCMS analysis and maintenance

Wrote ethical application, 'Sample collection for method development work'

Designed, set-up and performed semi-preparative HPLC analysis

Section 3 and 4

Designed study protocols, including formulation of test meals

Prepared oral doses for study

Wrote ethical applications

Recruited volunteers

Coordinated study days and sampled blood

Performed all sample extraction and analysis by GCMS

Performed data analysis

ACKNOWLEDGMENTS

Firstly, at MRC Human Nutrition Research (HNR) I would like to thank Prof. Andy Coward OBE (HNR) and Dr Ann Prentice OBE (HNR) for their initial support in allowing me to pursue this PhD. Further thanks to Dr Coward for his supervision and guidance throughout the course of these studies. Sincere thanks also to Prof. Caroline Pond (Open University) for her supervision, and in particular her effort in reviewing this dissertation. I would also like to thank Dr Les Bluck (HNR) and Dr Alison Stephen (HNR) for their assistance with study design and data interpretation, and reading parts of this thesis.

I am very grateful to my wife and scientific role model, Dr Sophie Moore who has provided both emotional support and practical guidance and had the unenviable task of the first read of each section of this thesis.

Thank you to those people who helped with various aspects of these studies at MRC Human Nutrition Research including in the volunteer suite, Sue Bryant, Kimonie Sturgeon, Dr Mario Siervo and Dr Rosemary Hall. In particular I should thank Dr Laura Wang who performed the HPLC analysis of plasma samples obtained in the volunteer studies. Thanks also to Cheryl Kidney who assisted Laura, and Louise McKenna for her help in analysing the numerous samples from the bioavailability study. I am grateful to Dr Celia Greenberg and Glenda Chandler for their help with the design and nutrient coding of the test meals and analysis of the diet diaries. Thank you to Dr Adrian Mander and Mark Chatfield for statistical advice, and Chris Thane for sharing his knowledge of vitamin K and the Cambridge University medical school library.

I would like to thank my colleagues in the former Stable Isotope Group at MRC Human Nutrition Research, Antony Wright, Dr Sarah Jackson, Mary Pennant and Dr Abdollah Ghavami for their friendship, and other friends and family for their support during the last few years. Finally, I would like to mention the staff of MRC Keneba in The Gambia for providing a welcoming and conducive atmosphere for writing this thesis.

I acknowledge the Food Standards Agency who provided funding for this work (project number N05050).

11th July 2007, MRC Human Nutrition Research, Cambridge

TABLE OF CONTENTS

LIST OF FIGURES

Section 1

Section 2

Section 3

Section 4

LIST OF TABLES

Section 1

Section 2

Section 3

Section 4

APPENDICES

ABBREVIATIONS

%ucOC	% under carboxylated osteocalcin
µg	microgram
µL	microlitre
a	absolute turnover
amu	atomic mass units
AMS	accelerator mass spectrometry
apoA1	apolipoprotein A1
apoB	apolipoprotein B
apoE	apolipoprotein E
AUC	area under the curve
BMI	body mass index (kg/m^2)
BMC	bone mineral content
BMD	bone mineral density
CE	cholesterol esterase
CHO	carbohydrate
CI	chemical ionisation
CO	corn oil
CR	chylomicron remnants
CRS	γ-carboxylation recognition site
CV	coefficient of variation
d	day
D	deuterium (^2H)
Da	Dalton
dc	direct current
DEE	diethyl ether
e	Euler's number (also called Napier's constant)
e.g.	for example
EAR	estimated average [nutrient] requirement
EI	electron ionisation
EV	electron volts
FFQ	food frequency questionnaire
g_1, g_2	exponential slopes comprising exponential curve
Gas 6	growth-arrest specific protein 6
GC	gas chromatography
GCMS	gas chromatography mass spectrometry

Gla	γ-carboxyglutamic acid
Glu	glutamic acid
h	hour
H_1, H_2	coefficients (intercepts) of the separate terms of a complex exponential curve after normalisation of observed intercepts as a fraction of total
HCO	hardened coconut oil
HDL	high density lipoproteins
HDL_2/HDL_3	high density lipoprotein subfractions
HDNB	haemorrhagic disease of the newborn
HEX	hexane
HNR	Human Nutrition Research
HPLC	high performance liquid chromatography
i	input [absorption of oral deuterated vitamin K_1 dose] nmol/min
id	internal diameter
IDL	intermediate density lipoproteins
IS	internal standard
iv	intravenous
k	rate constant of transfer from a pool in terms of fraction of total content moving per unit time
k_{12}, k_{21}, k_{01} etc.	rate constant from transfer to pool 1 from pool 2 etc.
kcal	kilocalories
kg	kilograms
kJ	kiloJoules
LCMS	liquid chromatography mass spectrometry
LDL	low density lipoproteins
LOD	limit of detection
LPFF	lipoprotein free fraction
LRP	low density lipoprotein-related protein
M	molecular ion
MS	mass spectrometry
m/z	mass to charge ratio of ions in mass spectrometer
M+1, M+2... etc	isotopomers of molecular ion
MGP	matrix Gla protein
min	minute
MK-*n*	menaquinones with -*n* isoprenoid subunits
mL	millilitre

MM	mixed micelle (Konakion MM®)
mm	millimetre
MRC	Medical Research Council
MS	mass spectrometry
MUFA	monounsaturated fatty acid
NaTC	sodium taurocholate
NCI	negative ion chemical ionisation
NDNS	National Diet and Nutrition Survey
ng	nanogram
OSO	olive/sunflower seed oil
PCI	positive ion chemical ionisation
pg	picogram
PIVKA-II	protein induced by vitamin K absence (uncarboxylated prothrombin)
PRGP	proline-rich γ-carboxyglutamic acid proteins
PT	prothrombin time
PUFA	polyunsaturated fatty acid
RDA	recommended daily [nutrient] allowance
REC	Research Ethics Committee
RF	radio frequency
RNI	reference nutrient intake
s	second
S/S	split/splitless injector
SCC	Scientific Coordination Committee
SD	standard deviation
SEM	standard error of the mean
SFA	saturated fatty acid
SIM	selected ion monitoring
SO	sunflower seed oil
SPE	solid phase extraction
$T_{½}$	half time: time required for concentration to decline by half
TAG	triacylglycerols
TLC	thin-layer chromatography
TMG	trans-membrane Gla protein
TRL	triacylglycerol-rich lipoprotein
T_t	turnover time
ucOC	under carboxylated osteocalcin
UV	ultra-violet light

v/v	volume per volume
VKD	vitamin K-dependent
VKOR	vitamin K epoxide reductase
VLDL	very low density lipoproteins
w/v	weight per volume
wk	week
wt	weight
y	year

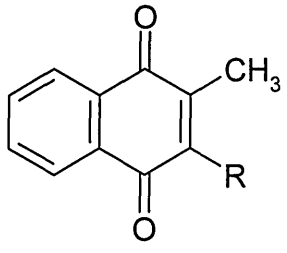
STABLE ISTOPE STUDIES INTO THE KINETICS AND BIOAVAILABILITY OF VITAMIN K_1 IN HUMANS

1 INTRODUCTION

1.1 Vitamin K

Vitamin K is a generic term that refers to a number of related molecules each sharing a similar chemical structure based on a 2-methyl-1,4-napthoquinone ring and possessing an alkyl side chain in position 3 (Figure 1-1). Vitamin K is related to other biologically active compounds, e.g. vitamin E and coenzyme Q families since all contain a quinone-derived ring structure.

Figure 1-1. Molecular structure of the 2-methyl-1,4-napthoquinone ring common to all forms of vitamin K. Forms differ in the length and degree of saturation of the alkyl side chain, R at position 3



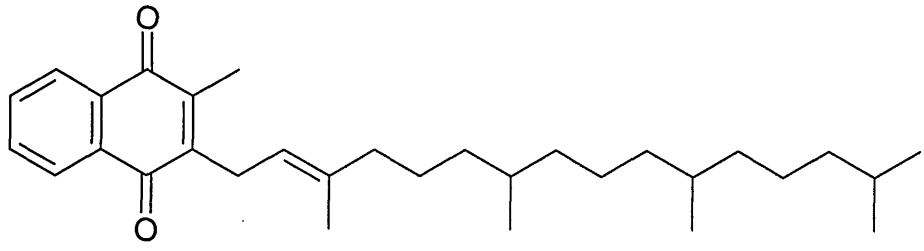
The two natural forms of vitamin K are vitamin K_1, also known as phylloquinone, which is synthesised in plants, and vitamin K_2, or the menaquinones that are produced by bacteria. An additional synthetic form of vitamin K, designated K_3 and known as menadione, comprises only the 2-methyl-1,4-napthoquinone ring. In western populations, the primary dietary form of vitamin K is vitamin K_1 (Schurgers *et al.,* 1999).

1.1.1 Vitamin K_1 – Phylloquinone

Vitamin K_1 is only synthesised in plants where it is closely bound to the thylakoid membranes of chloroplasts. In the chloroplast, vitamin K_1 plays a role in the production of chemical energy, in the form of NADPH, using solar energy. In photosynthesis, a number of cofactors act to transfer electrons along a transfer pathway. One such cofactor, A1, has been identified as two vitamin K_1 molecules (Rustandi *et al.,* 1990; Snyder *et al.,* 1991). It has also been proposed that vitamin K_1 may play the role of a lipid-soluble electron carrier in plant organelle cell membranes, similar to that of coenzyme Q in

animals (Lochner *et al.*, 2003). The vitamin K_1 molecule comprises the 2-methyl-1,4-napthoquinone ring and a partially saturated phytyl side chain at position 3 (Figure 1-2).

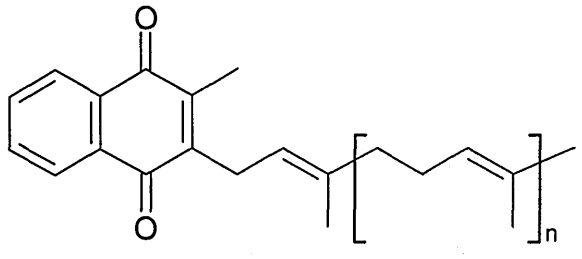
Figure 1-2. Molecular structure of vitamin K_1



1.1.2 Vitamin K_2 – Menaquinones

The term menaquinone describes a number of molecules each comprising the 2-methyl-1,4-napthoquinone ring but differing in the length of the side chain at position 3 (Figure 1-3). The side chain is made up of unsaturated isoprenyl units (marked [...] in Figure 1-3) with the menaquinone being designated by the number of isoprenoids in the form MK-*n*, where *n* equals the number of isoprenoid units, e.g. MK-4 contains 4 isoprenoid units. As with vitamin K_1 in plants, the menaquinones in bacteria act as electron carriers in the production of energy from a variety of substrates (Haddock & Jones, 1977).

Figure 1-3. Molecular structure of vitamin K_2



1.2 Biochemistry of vitamin K

Crucial to identifying suitable markers of vitamin K status, and to investigating relationships between intake, status and health is an understanding of the biochemistry of vitamin K, and its unique biological functions.

1.2.1 Discovery of vitamin K

Vitamin K was discovered during the 1930s, following observations on chicks with hemorrhagic disease. In an early study by Almquist & Stokstad (1935), it was shown that chicks fed meals that had been extracted with ether developed a hemorrhagic disease that could be reversed by adding the extracts back to the diets. Similarly, Dam (1935) demonstrated that symptoms could be suppressed by feeding chicks 'hog liver fat, hemp seed and certain vegetables such as tomatoes and kale, and many cereals'. It was found the vitamin occurs in the 'easily soluble non-sterol fraction of the non-saponifiable matter'. Identified as being similar to vitamin E, Henrik Dam suggested the term Vitamin K after the German word, Koagulation (Dam 1935). He later won the Nobel Prize for his discovery.

1.2.2 Biochemical role of vitamin K

The only known role for vitamin K is to act as a cofactor for the endoplasmic enzyme γ-glutamyl carboxylase that catalyses the post-translational conversion of glutamic acid (Glu) to γ-carboxyglutamic acid (Gla) in vitamin K-dependent (VKD) proteins (Berkner 2000). The amino acid γ-carboxyglutamic acid was first discovered in prothrombin and subsequently in other VKD proteins necessary for hemostasis, including Factor VII, IX and X, protein C, protein S and protein Z (Suttie 1985). Other VKD proteins discovered since include those involved in bone metabolism, osteocalcin and matrix Gla protein (MGP). More recently, a new family of VKD proteins has been identified that have a broad distribution in foetal and adult tissues (Kulman *et al.*, 1997; 2001). This family is divided into two classes; proline-rich γ-carboxyglutamic acid proteins (PRGP), and trans-membrane Gla proteins (TMG). Table 1-1 summarises the currently known VKD proteins and their functions.

Table 1-1. Known vitamin K-dependent proteins and their functions

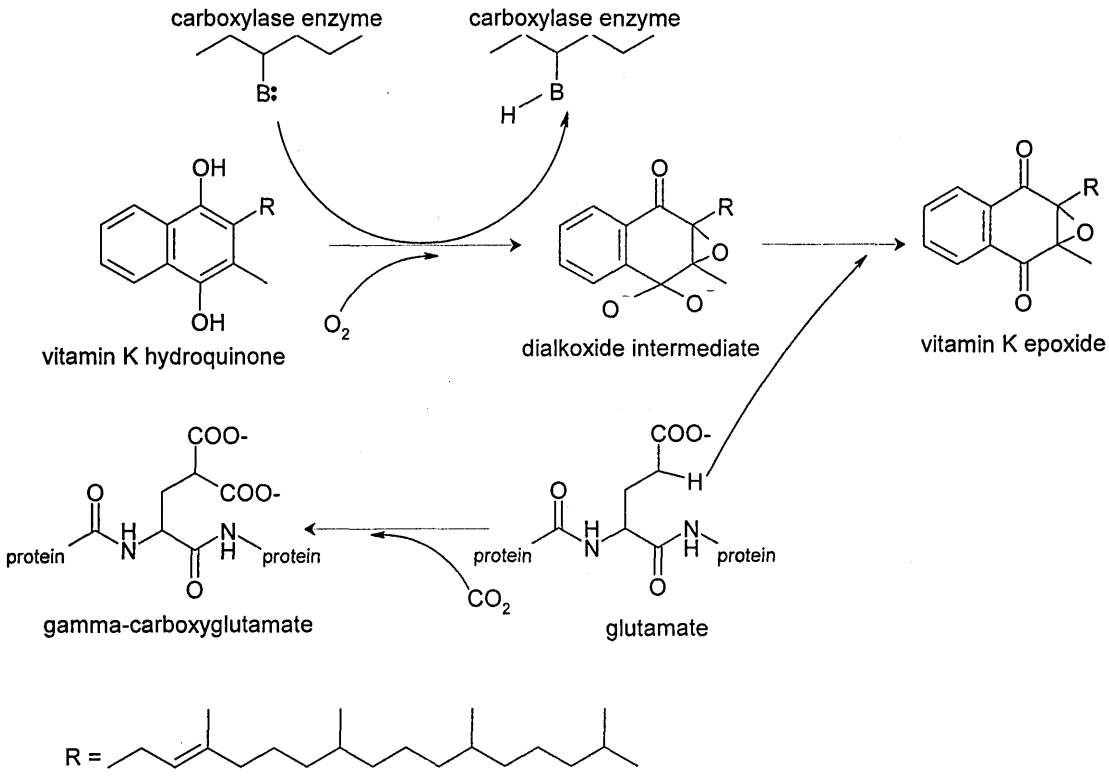
Protein	Function	Reference
Factor II (prothrombin) Factor VII Factor IX Factor X	Blood clotting	Berkner & Runge, 2004; Oldenburg & Schwabb, 2001
Protein C Protein S* Protein Z	Regulation of blood clotting	
Growth-arrest specific 6 (Gas6)	Signal transduction; Cell survival factor; Cofactor in uptake of apoptotic cells by macrophages	Kulman *et al.*, 2001
Osteocalcin Matrix Gla protein	Bone growth and extraosseous calcification	Vermeer 1998
PRGP1 PRGP2	Unknown	Kulman *et al.*, 1997
TMG3 TMG4	Unknown	Kulman *et al.*, 2001

*Protein S is also found in bone

1.2.3 The mechanism of conversion of glutamic to γ-carboxy glutamic acid

The activation of VKD proteins by conversion of Glu residues to Gla residues requires the reduced form of vitamin K, vitamin K hydroquinone, and the enzyme γ-glutamyl carboxylase. However, the exact enzymatic mechanism of VKD protein carboxylation is not fully understood (Berkner & Runge, 2004). The proposed mechanism is based on base-strength amplification sequence (Figure 1-4). In this mechanism, the γ-carboxylase enzyme provides a weak base (a proton acceptor) for the deprotonation of vitamin K hydroquinone. Oxygenation of vitamin K hydroquinone results in the generation of a strong base, possibly the dialkoxide intermediate (shown in Figure 1-4). The resulting strong base extracts a hydrogen atom from the glutamyl residue (Glu) that generates a electron-donating carbanion intermediate (not shown). This reaction is followed by a further reaction with CO_2 and carboxylation (introduction of a carboxylic acid group, COOH) to form the γ-carboxyglutamate product (Dowd *et al.*, 1995; Rishavy *et al.*, 2004).

Figure 1-4. The base amplification model for the carboxylation of glutamic acid. γ-glutamyl carboxylase catalyses the conversion of vitamin K hydroquinone to vitamin K epoxide that extracts a hydrogen atom from glutamic acid. Addition of CO_2 results in the conversion of glutamic acid to γ-carboxyglutamic acid. (B: weak base) (adapted from Rishavy *et al.*, 2004 and Dowd *et al.*, 1995)

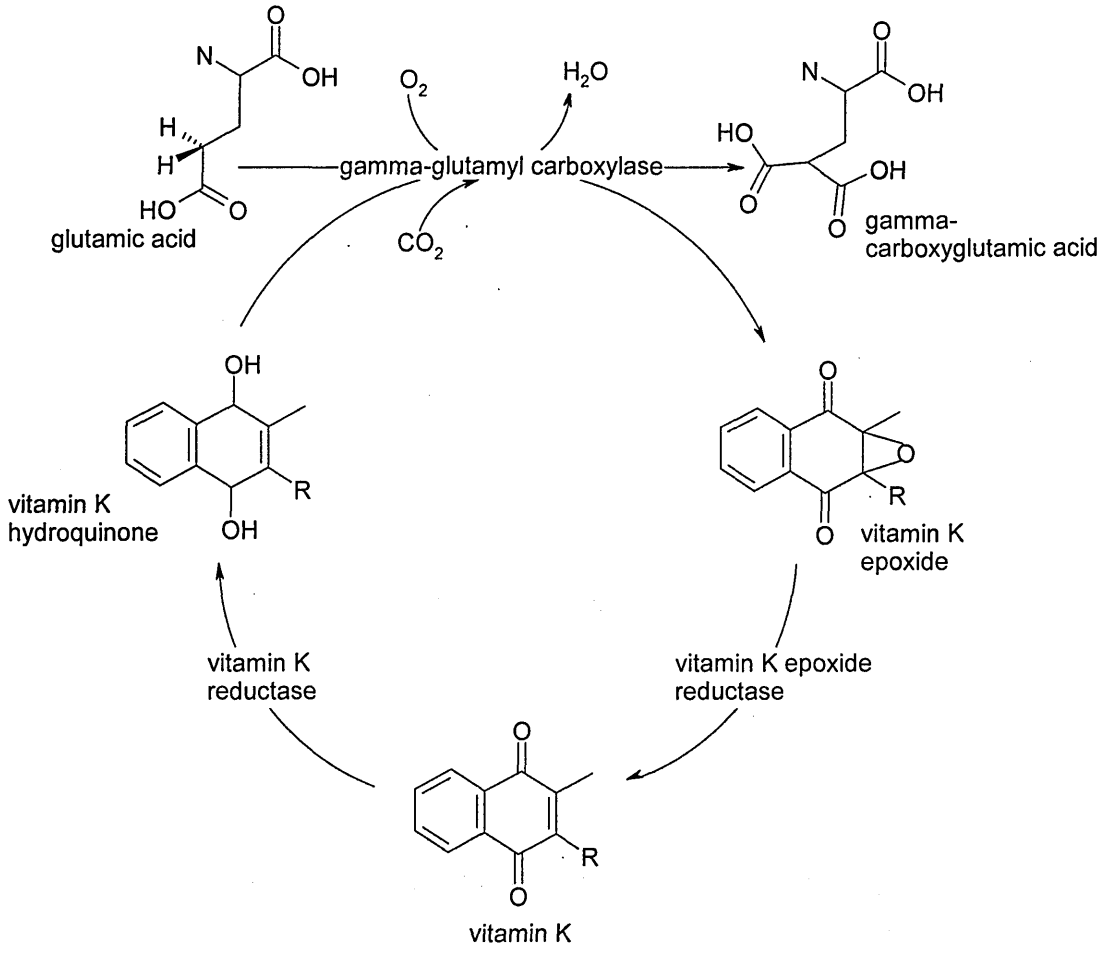


There is some debate with regards to the precise mechanism of the base strength amplification model. It was initially proposed that one cysteine residue of the carboxylase enzyme acted as the weak base, and another served to incorporate CO_2. Subsequent work demonstrated that in fact each of the cysteine residues is required for both epoxidation and carboxylation (Pudota *et al.*, 2000). However, recent evidence has shown that the active residue is not cysteine, but rather histidine acting as a catalytic base (Rishavy *et al.*, 2004). It is suggested that although the cysteine residues are not directly involved in the carboxylation reaction, they may still be important since their modification to serine or alanine causes inactivation of the protein (Tie *et al.*, 2004).

1.2.4 The vitamin K cycle

For every molecule of Glu converted to Gla a molecule of vitamin K 2, 3 epoxide is also produced. The epoxide is converted back to the quinone form of vitamin K via vitamin K epoxide reductase (Furie *et al.,* 1999). Together with the conversion of vitamin K to vitamin K hydroquinone, these steps make up the vitamin K cycle (Figure 1-5). The vitamin K cycle is crucial for the continual carboxylation of VKD proteins since each conversion from Glu to Gla involves one molecule of vitamin K hydroquinone and VKD proteins contain up to 13 Glu residues. Thus, the availability of vitamin K hydroquinone is the limiting factor and crucial for full carboxylation of VKD proteins (Berkner 2005). Additionally, the cycle is important for the maintenance of vitamin K status within tissues, since concentrations are low and vitamin K turnover is relatively fast (Shearer *et al.,* 1974). Vitamin K must be continually recycled to prevent the accumulation of undercarboxylated intracellular VKD proteins and the subsequent secretion of non-functioning, undercarboxylated VKD proteins into the circulation. From studies in rats, it has been estimated that a vitamin K_1 molecule may be recycled between 1000 and 10000 times per day (Uchida & Komeno, 1988).

Figure 1-5. The vitamin K cycle. The cycle is crucial for the maintenance of vitamin K stores to ensure carboxylation of VKD proteins. The product of the carboxylase reaction, vitamin K epoxide, is converted back to vitamin K, and subsequently to the active form, vitamin K hydroquinone. Dietary vitamin K can also enter the cycle



1.2.4.1 Vitamin K-dependent γ-glutamyl carboxylase

The VKD γ-glutamyl carboxylase enzyme catalyses the conversion of glutamic acid to γ-carboxyglutamic acid. The gene coding for VKD γ-carboxylase is found on chromosome 2 and encodes for a protein with a molecular weight of around 94,000 Da (Furie *et al.*, 1999). It has been shown that the carboxylase sequence is found throughout the animal kingdom, including a highly conserved 38-amino acid motif, which suggests this domain is critical for enzyme function (Begley *et al.*, 2000). The high degree of sequence homology throughout the animal kingdom highlights the functional importance of the VKD carboxylase.

1.2.4.2 Vitamin K epoxide reductase

Vitamin K epoxide reductase (VKOR) has two functions. Firstly, it reduces vitamin K to vitamin K hydroquinone, the active cofactor for VKD carboxylase. Secondly, the enzyme reduces vitamin K epoxide, formed during carboxylation of glutamic acid, back to vitamin K (Suttie 1985). VKOR is likely the physiologically important enzyme for recycling vitamin K (Furie *et al.,* 1999) and for the maintenance of vitamin K status. The gene encoding for VKOR has only recently been discovered (Li *et al.,* 2004; Rost *et al.,* 2004a) and the protein purified (Chu *et al.,* 2006). Warfarin works as an anticoagulant by blocking the action of vitamin K epoxide reductase.

A second enzyme, NADPH-dependent quinine reductase is also able to convert vitamin K to vitamin K hydroquinone. This enzyme requires a high concentration of vitamin K and is thus unlikely to be important at physiological concentrations. However, this pathway for the formation of vitamin K hydroquinone is important in the therapeutic use of vitamin K against over anticoagulation, since it is not blocked by warfarin (Berkner & Runge, 2004; Bovill *et al.,* 2004).

1.2.5 Molecular interaction between VKD proteins and the γ-glutamyl carboxylase

VKD proteins are selected for carboxylation via a highly conserved 18 amino acid propeptide incorporating the γ-carboxylation recognition site (CRS). Carboxylation occurs in the endoplasmic reticulum. The conserved region occurs upstream of the Gla domain and, in blood proteins, is proteolytically removed in the Golgi apparatus to form the mature protein (Stanley *et al.,* 1999). Matrix Gla protein is exceptional because it retains the CRS within the mature protein (Berkner & Runge, 2004). It may be expected that, because of the similarities of the propeptides between different VKD proteins, all would have similar affinities for γ-glutamyl carboxylase. However, it has been demonstrated that there is considerable variation in the affinities of the propeptides for γ-glutamyl carboxylase. This wide range could have physiological consequences when vitamin K levels are sub-optimal because it could lead to the preferential carboxylation of some VKD proteins (Stanley *et al.,* 1999).

Following removal of the propeptide region and conformational changes afforded by conversion of Glu to Gla in the Golgi apparatus, the resulting proteins undergo subsequent post-translational modifications including N- and O-linked glycosylation, sulfation, phosphorylation, β-aspartyl hydroxylation and proteolytic cleavage. Mature VKD proteins are either secreted extracellularly (e.g. blood clotting proteins) or secreted to the cell membrane (e.g. PRPG and TMG) (Berkner & Runge, 2004).

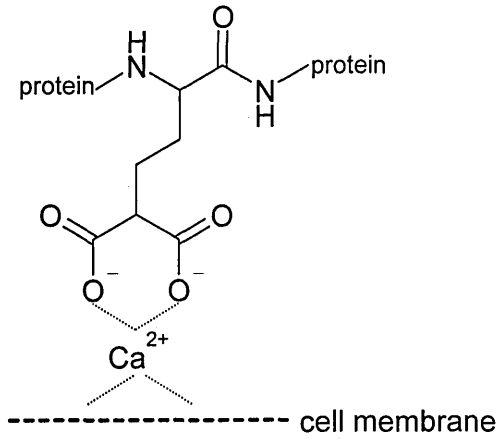
The number of Glu residues converted into Gla residues in the Gla domain of VKD proteins can vary between three (in osteocalcin), five (matrix Gla protein) and 13 in blood coagulation proteins (Berkner & Runge, 2004). In the presence of sufficient vitamin K hydroquinone, and assisted by the vitamin K cycle, secreted VKD proteins are usually fully carboxylated, suggesting that a mechanism exists to ensure complete carboxylation (Berkner & Runge, 2004). It is possible that there is cellular recognition of undercarboxylated forms, at least for some VKD proteins (Hallgren *et al.*, 2002). An alternative mechanism is via 'tethered processivity' whereby all Glu are converted from a single binding between VKD protein and γ-glutamyl carboxylase. In this model, the propeptide remains bound throughout the reaction, while the Gla domain undergoes intramolecular movement to reposition the Glu for carboxylation to Gla (Stenina *et al.*, 2001). The presence of 'chaperones' has also been suggested that may mediate the VKD protein-carboxylase interaction, and which may help override the different affinities of VKD proteins for the carboxylase (Berkner 2005).

1.2.6 The function of γ-carboxy glutamic acid residues

Conversion of Glu to Gla in VKD proteins confers the ability to bind calcium ions (Ca^{2+}). The consequences of Ca^{2+} binding on tertiary structure and properties of the protein depend on the number of Gla residues. In blood coagulation proteins that contain more than 9 Gla residues, the binding of multiple Ca^{2+} ions leads to a structural change in the protein that results in the exposure of a site that binds to phospholipids (Furie *et al.*, 1999). VKD proteins are then able to bind to cell membranes expressing phosphatidylserine via a serine head group of the membrane phospholipid binding to the

Gla domain-bound Ca^{2+} ions (Figure 1-6). This binding is mediated by a number of interactions including calcium coordination, ionic, van der Waals and hydrophobic forces (Huang *et al.,* 2003). Phosphatidylserine exposure on cell surfaces also occurs during cell injury or cell activation (in response to inflammatory signals) and is characteristic of apoptosis. Thus, it is possible that VKD proteins play an important role in other physiological processes (Berkner & Runge, 2004).

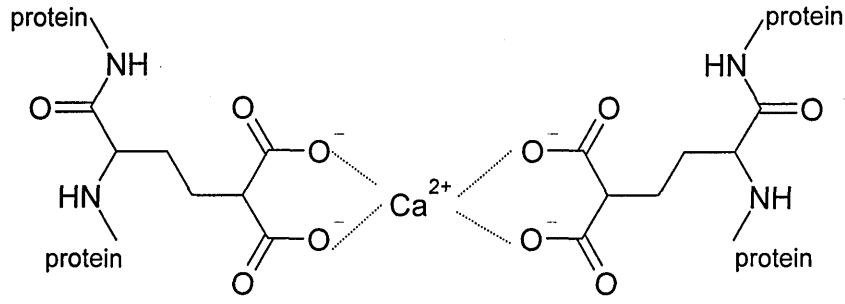
Figure 1-6. Gla residue of VKD protein binding to cell surface through the interaction of Ca^{2+} ions with membrane phospholipids (adapted from Dowd *et al.*, 1995)



In addition, in the blood coagulation proteins, interaction between the Gla residues of VKD proteins and Ca^{2+} ions are believed to provide molecular linkages (Figure 1-7) (Dowd *et al.,* 1995).

In osteocalcin and matrix Gla protein that contain fewer Gla residues, the effect of calcium binding is less well defined. There is evidence that osteocalcin binds to hydroxyapatite (the mineral component of bone) by ionic bonds between the negatively charged protein surface provided by the Gla residues and Ca^{2+} ions interacting with hydroxyapatite (Hoang *et al.,* 2003).

Figure 1-7. Gla residue-Ca^{2+} ion molecular linkage in blood coagulation proteins (adapted from Dowd *et al.*, 1995)

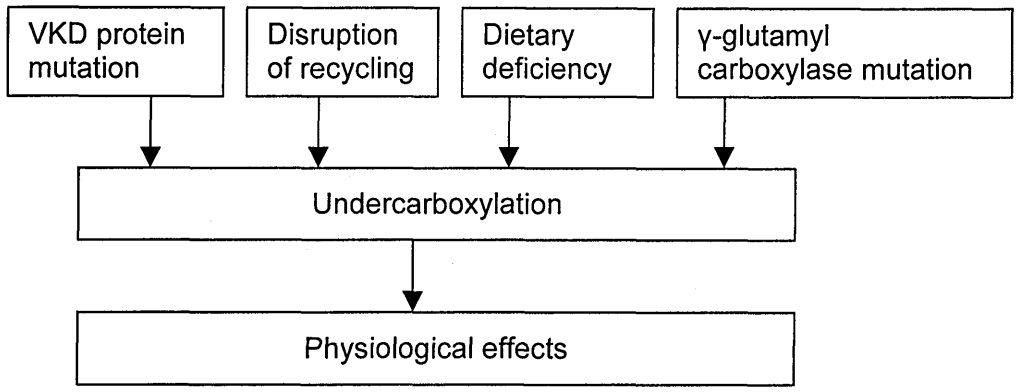


1.2.7 The consequences for health of undercarboxylated VKD proteins

To help understand the potential consequences of vitamin K deficiency and undercarboxylation of VKD proteins it is useful to highlight some molecular and genetic work. A study that selectively mutated Glu residues in VKD proteins yielded prothrombin and protein C that were unable to bind Ca^{2+} ions (Furie *et al.*, 1999) and shows that undercarboxylation results in reduced Ca^{2+} ion binding and low biological activity of the VKD protein (Vermeer *et al.*, 1998).

The release of undercarboxylated proteins into circulation can occur by various means and are summarised in Figure 1-8. A mutation in a single VKD protein can lead to specific disorders affecting, for example blood coagulation. There are a number of specific mutations in genes associated with the VKD coagulation proteins. For example, a mutation in the propeptide γ-glutamyl carboxylase binding site of Factor IX causes haemophilia B. Clinical signs of this condition are haemorrhages, mainly in the muscles and joints (Oldenburg & Schwabb, 2001). The prevalence of mutations in the gene encoding protein C is around 1.5 per 1000 individuals, with mutations found throughout the gene (Oldenburg & Schwabb, 2001). Mutations in other VKD proteins (prothrombin, factors VII and X) are rare.

Figure 1-8. Potential causes of undercarboxylated VKD proteins



Undercarboxylation may also occur through a reduction in the availability of the cofactor vitamin K. The vitamin K cycle can be disrupted by genetic mutation, drug treatment or dietary deficiency. Disruption of vitamin K recycling may exert a universal effect on all VKD proteins resulting in possible bleeding, bone malformation and increased risk of arterial calcification.

Mutations that impact all VKD proteins are rare. A few have been linked to a specific mutation in the gene for γ-glutamyl carboxylase (Brenner *et al.,* 1998; Spronk *et al.,* 2000; Rost *et al.,* 2004b). In a single case, it was reported that hemostatic proteins and osteocalcin were both affected suggesting only one gene encodes for γ-glutamyl carboxylase in all tissues (Spronk *et al.,* 2000). There are also reports of mutations in the vitamin K epoxide reductase enzyme resulting in vitamin K epoxide accumulation (Oldenburg & Schwabb, 2000).

Whilst adults are generally safe from the acute effects of vitamin K deficiency, neonates are at risk of haemorrhagic disease of the newborn (HDNB) partly because of the low concentration of vitamin K in breast milk. As a result, newborns receive a dose of vitamin K shortly after delivery. Where vitamin K deficiency does occur in adults, it is usually the consequence of disease or medical treatment for other conditions. Diseases that interfere with the absorption of vitamin K include forms of biliary obstruction (e.g. gallstones), malabsorption syndromes (e.g. cystic fibrosis, celiac disease, ulcerative colitis and short-bowel syndrome) and liver disease. Lack of dietary vitamin K leading to haemorrhage is also a risk in hospitalised patients receiving total parenteral nutrition (Olson 1985).

Drug therapies that interfere with vitamin K metabolism include the coumarin family of anticoagulants (e.g warfarin) and large doses of salicylates (Olson 1985). The potential effects of vitamin K antagonists was highlighted in infants from mothers on warfarin treatment, who were at risk of a bone defect, chondrodysplasia punctata, also known as fetal warfarin syndrome (Vermeer *et al.,* 1998). The use anti-obesity drugs that inhibit fat absorption may also cause vitamin K deficiency even if it is plentiful in the diet, although any effects are likely to be mild and difficult to detect (see section 1.13.5.1). Some types of antibiotic can also inhibit vitamin K epoxide reductase (Suttie 1995).

There is evidence that warfarin treatment can lead to a competitive state among VKD proteins, resulting in the premature dissociation of undercarboxylated, inactive protein. A similar state could be reached by dietary deficiency. The effects of acute vitamin K deficiency are well known for blood coagulation. However, it is only relatively recently, and with the advent of sensitive techniques, that the relationships between vitamin K intake and markers of status could be investigated.

1.3 Vitamin K deficiency and human health

1.3.1 Blood coagulation

With respect to blood coagulation, vitamin K deficiency is rare in human adults on self-selected diets. The reasons are three-fold; firstly, the wide distribution of vitamin K in different foods (albeit in low amounts); secondly, recycling of the vitamin; and thirdly the potential production of vitamin K by gut bacteria, although the microbial contribution is controversial (see section 1.13.3). Of key relevance for the current investigation is that present dietary recommendations for vitamin K are based solely on the amount of dietary vitamin K necessary to maintain normal plasma concentrations of the VKD blood coagulation proteins (Department of Health 1991).

The first study to investigate the nutritional requirement for vitamin K was performed by Frick *et al.* (1967) with a depletion-repletion study in seven patients all receiving parental nutrition. It was concluded that, for the maintenance of blood coagulation, the minimum daily requirement was 0.03 to 1.5 µg/kg body wt (Frick *et al.,*

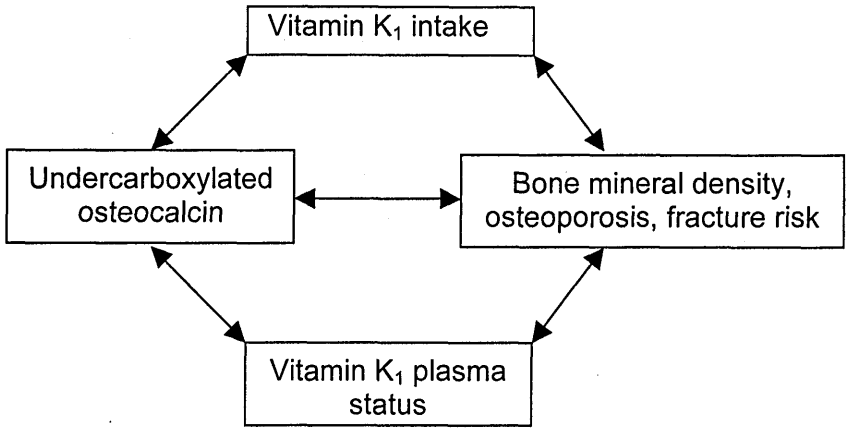
1967). However, this range assumes 100% absorption and thus does not account for variable bioavailability. Since the work of Frick *et al.* there has until recently been little challenge to the 1 µg/kg body wt/d value. While blood coagulation was the only known role for vitamin K, other work provided evidence to support the requirement (Suttie *et al.,* 1988b), while another even suggested it was too high (Allison *et al.,* 1987). The discovery of a requirement for the carboxylation of extrahepatic VKD proteins renewed interest in vitamin K nutrient requirements.

Dietary restriction can reduce circulating vitamin K levels and undercarboxylated coagulation proteins (Suttie *et al.,* 1988b; Ferland *et al.,* 1993; Booth *et al.,* 2003b)), although no clinical manifestations of deficiency have been reported. In some instances, and with perhaps less sensitive assays, little or no vitamin K-related deficiencies were observed in blood coagulation markers (Allison *et al.,* 1987; Ferland *et al.,* 1993) and in particular prothrombin time (Suttie *et al.,* 1988b). Some workers have found prothrombin time sensitive to vitamin K_1 supplementation (Sokoll *et al.,* 1997) while others have not (Binkley *et al.,* 2000) (see section 1.6.2 and 1.6.3).

1.3.2 Bone health

There is now a substantial body of evidence linking vitamin K intake and status to markers of bone health (Figure 1-9). In adults, bone turnover, or remodelling, is a balance between the resorption of old bone and the formation of new bone (Vermeer *et al.,* 2004). The VKD protein, osteocalcin is a frequently used marker of bone turnover, and although its precise role is unclear, it likely acts as a regulator of bone formation. In addition, the VKD proteins, MGP (Price & Williamson, 1985) and protein S (Maillard *et al.,* 1992) have also been implicated in bone metabolism. Described below are a number of studies that provide evidence for the importance of vitamin K in bone health.

Figure 1-9. Summary of evidence for links between vitamin K and bone health



1.3.2.1 Undercarboxylated osteocalcin and bone health

The central link between vitamin K and bone health is the carboxylation of osteocalcin. A number of prospective studies have shown that fracture risk was greater in women with a higher level of ucOC (Szulc *et al.,* 1993; 1996; Vergnaud *et al.,* 1997). Lower bone mineral density (BMD) has been reported in one study in women who had lower carboxylated osteocalcin (Schaafsma *et al.,* 2000), and in another study with higher ucOC, although only in individuals with atherosclerosis (Jie *et al.,* 1996).

1.3.2.2 Vitamin K_1 intake and bone health

A number of epidemiological studies have identified relationships between vitamin K intake and markers of bone health. The largest involved 72,327 women and found that low vitamin K intake increased the risk of hip fracture (Feskanich *et al.,* 1999). A similar conclusion was made in a separate study of 888 US men and women where low vitamin K intake was associated with an increased risk of hip fractures but not low BMD (Booth *et al.,* 2000b). In a subsequent study on the same but enlarged (n = 1,591) US cohort, Booth *et al.*, (2003a) found low vitamin K intake was associated with lower BMD in women, but not in men. However, a more recent study in Denmark in 2016 peri-menopausal women found no relationship between vitamin K intake and either BMD or fracture risk (Rejnmark *et al.,* 2006). Undercarboxylated osteocalcin has also been associated with vitamin K_1 intake in Irish post-menopausal women (Collins *et al.,* 2006) and in a US population (McKeown *et al.,* 2002).

1.3.2.3 *Vitamin K_1 status and bone health*

A lower vitamin K status in patients with fractures compared to controls has been reported in a number of studies. However, many have reported retrospectively (i.e. status was measured after the fracture) and furthermore, and as will be discussed later, a single measure of vitamin K status may not be a reliable measure (see section 1.6). Lower vitamin K plasma status was observed in two studies that compared individuals with hip fracture and spinal crush fractures with controls (Hart *et al.,* 1985; Hodges *et al.,* 1991). One report recognised that lower vitamin K status in the fracture group could be due to a decline in status following the fracture, but concluded this explanation unlikely since the spinal crush fractures represent long-standing, chronic fractures (Hart *et al.,* 1985). In other words, the authors concluded that low plasma vitamin K_1 status contributed to, but was not the effect of the fracture. However, in another study that reported lower vitamin K status in individuals after a fracture compared to controls, it was also found that the time for vitamin K status to return to normal varied depending on the severity of the fracture (Bitensky *et al.,* 1988) suggesting that low vitamin K status was an effect of the fracture. The measurement of vitamin K status after a fracture may not be a good indicator of the relationship between status and fracture risk, whereas prospective studies can provide this information. In a study in 68 haemodialysis patients, lower plasma vitamin K_1 was associated with a greater risk of fracture (Kohlmeier *et al.,* 1995a). A significant positive correlation between vitamin K_1 status and BMD has also been observed (Tamatani *et al.,* 1998). Another recent study in 672 participants has shown an association between low plasma vitamin K and osteoarthitis in hand and knee joints (Neogi *et al.,* 2006). An inverse relationship between undercarboxylated osteocalcin and vitamin K_1 status has been observed in post-menopausal British women, but not in post-menopausal Gambian or Chinese women (Beavan *et al.,* 2005).

1.3.2.4 *Intervention studies*

Intervention studies with vitamin K have investigated both the effect of vitamin K on bone health (fracture risk or BMD) and circulating undercarboxylated osteocalcin

(Weber 2001). The major form of vitamin K in human bone is vitamin K_1 (Hodges *et al.*, 1993; Shearer 1997) but the majority of studies have used vitamin K_2 (typically MK-4), often at pharmacological levels unobtainable from a normal diet (Shiraki *et al.*, 2000; Knapen *et al.*, 2007). However, it is suggested that the effects of MK-4 may be mediated through an alternative mechanism to γ-carboxylation (Shearer 1997).

Two studies have shown an effect of vitamin K_1 supplementation on bone mineral density (Braam *et al.*, 2003; Bolton-Smith *et al.*, 2007) one of which used levels of vitamin K_1 obtainable from the diet (Bolton-Smith *et al.*, 2007). The latter study assigned individuals to one of four groups to receive either 200 µg/d of vitamin K_1, or 10 µg/d vitamin D_3 plus 1000 mg of calcium, or vitamin K_1, vitamin D_3 and calcium together, or a placebo. Bone quality was measured at four sites but only one showed a significant increase in BMD and bone mineral content (BMC), and only in the vitamin K_1+vitamin D_3+calcium treatment group (Bolton-Smith *et al.*, 2007).

The ability of vitamin K_1 supplementation to lower undercarboxylated osteocalcin has been demonstrated in a number of intervention studies, over a range of doses and in men and women of varying ages (Sokoll *et al.*, 1997; Binkley *et al.*, 2000; Booth *et al.*, 2000a; Schaafsma *et al.*, 2000; Binkley *et al.*, 2002; Booth *et al.*, 2003b; Bügel *et al.*, 2007). Sokoll *et al.* (1997) performed a controlled experiment in a metabolic ward in 9 healthy volunteers to assess the effect of a diet containing 100 µg of vitamin K_1, and for 5 d, the effect of increasing intake to 420 µg/d. With supplementation, plasma vitamin K_1 significantly increased and %ucOC significantly decreased. Binkley *et al.*, (2000) studied the effect of 1000 µg vitamin K_1 or placebo for 2 wk in 219 healthy men and women. After 1 wk of supplementation (and sustained through to the 2nd wk) plasma vitamin K_1 increased 10-fold and %ucOC had significantly decreased to around 3% ($P<0.001$). A subsequent study by the same group measured the effect of a range of vitamin K_1 supplementation on %ucOC over 2 wk. It was found that supplementation decreased %ucOC in all supplemented groups (250, 375, 500 and 1000 µg) compared to the placebo. It was concluded that around 1000 µg of vitamin K_1 is required daily to achieve maximal carboxylation of osteocalcin (Binkley *et al.*, 2002). Another study reported a

significant decrease ($P<0.001$) from 44 to 20% ucOC with intake of 200 µg/d after 10 d (Booth *et al.*, 2000a). In a depletion-repletion study by Booth *et al.*, %ucOC was shown to decrease in response to a vitamin K intake of 90 µg/d during the initial stages of the study, however no change was recorded during the depletion (18 µg/d) or repletion (up to 450 µg/d) phases (Booth *et al.*, 2003b). The authors attribute this outcome to the preferential use of vitamin K by hepatic VKD proteins. In a recent study, a 2 y supplementation with either 200 µg/d of vitamin K_1 or vitamin K_1+vitamin D_3+calcium significantly decreased undercarboxylated osteocalcin within 6 month (Bolton-Smith *et al.*, 2007). A crossover study performed by Bügel *et al.* (2007) supplemented 31 post-menopausal women with a placebo, 200 µg/d or 500 µg/d of vitamin K_1 for 6 wk. The volunteers also received 10 µg/d vitamin D_3. Supplementation with 200 µg/d and 500 µg/d did significantly increase total, and decrease undercarboxylated osteocalcin. However, only supplementation with 500 µg/d significantly increased vitamin K_1 plasma concentration (Bügel *et al.*, 2007).

Interestingly, in a study of nutritional interventions to counteract bone loss during space flight, it was reported that vitamin K_1 had a beneficial effect on bone formation markers (serum bone alkaline phosphatase), whereas vitamin D and calcium did not stabilise bone turnover (Heer 2002).

1.3.3 Atherosclerosis

It is now recognized that the processes involved in mineralisation of bone and vascular calcification are similar (Vermeer *et al.*, 2004). There is increasing evidence from human studies linking low vitamin K intake to the development of atherosclerosis (Jie *et al.*, 1995). High intakes of vitamin K_1 have been associated with protection against vascular hardening and loss of arterial elasticity, however these associations were attenuated after adjustment for dietary and lifestyle patterns (Erkkilä *et al.*, 2005a). A subsequent prospective study in 40,087 men concluded that high vitamin K_1 itself is not protective against cardiovascular disease but may be a marker for a 'healthy' diet and lifestyle (Erkkilä *et al.*, 2007). A study examining vitamin K_1 intake and premature coronary artery calcification in a 39 – 45 y old population (n = 807) showed no significant

correlation (Villines *et al.,* 2005). However, it is possible the effect is subtler in this young population and may only become apparent in later life. The relative importance of vitamin K_1 and K_2 is unknown. A population-based study showed no protective effect of vitamin K_1 against cardiac events or aortic atherosclerosis, but an association was observed with vitamin K_2 (Geleijnse *et al.,* 2004). Similar results have been found with rats where warfarin-induced arterial calcification could be blocked by vitamin K_2, but not vitamin K_1 (Vermeer *et al.,* 2004). These observations may be associated with different transport pathways of these two forms of the vitamin (see section 1.13.3).

The mechanism behind the potential relationship between vitamin K_1 and atherosclerosis may involve many of the VKD proteins including the coagulation proteins (during plaque rupture), growth-arrest specific protein (Gas6) (expressed in smooth muscle cells and mediates a number of activities in platelets and the endothelium), osteocalcin (which is up-regulated during calcification) and MGP (Berkner & Runge, 2004). MGP is also up-regulated in smooth muscle cells adjacent to sites of calcification and is known to be an inhibitor of calcification as seen in studies with rats (Berkner & Runge, 2004). Additionally, Keutel syndrome in humans, an autosomal disorder affecting MGP, is characterized by calcification of cartilage (Berkner & Runge, 2004).

1.3.4 Anti-cancer effects

A small number of papers have reported that vitamin K is able to inhibit both the *in vivo* and *in vitro* growth of cancerous cells, however most observations have been made with vitamin K_2 and vitamin K_3 (Lamson & Plaza, 2003). The *in vitro* studies assessing anti-tumour effects of vitamin K_1 on a number of cell lines used unphysiological concentrations. Similarly, in human trials, the amount of vitamin K administered was unphysiological at between 20 and 40 mg/d. In the context of vitamin K intake from the diet, these levels are nearly impossible to achieve and hence the relevance of these studies should be interpreted with caution.

1.3.5 Vitamin K and the nervous system

The vitamin K-dependent growth factor, Gas6 is widely distributed throughout the nervous system. This wide distribution, along with other research demonstrating an indirect but important role for vitamin K in the synthesis of brain sphingolipids, has led to the suggestion that vitamin K is important to the nervous system, particularly during development (Tsaioun 1999). However, further research is required in this area.

1.4 Dietary recommendations for population health

The purpose of dietary recommendations is to ensure that, on a population level, average intakes are adequate for normal health and the maintenance of metabolic function. The estimated average requirement (EAR) is the mean requirement for a chosen level of adequacy. The reference nutrient intake (RNI) or recommended daily allowance (RDA) is ± 2 standard deviations around the mean, and results in a intake value that meets the needs of 97.5% of the population.

1.4.1 Defining adequacy

The requirement for a micronutrient can be defined as an intake level that meets a criterion for adequacy. The extreme criterion of micronutrient deficiency is death, however death resulting from a single micronutrient deficiency is rare. Preventing clinical deficiency, such as impaired blood coagulation, can be regarded as the minimum goal in defining requirements. However, clinical signs of deficiency can be considered an extreme endpoint and thus measures of adequacy with greater sensitivity are required. Such measures should be based on a thorough understanding of the metabolic function of the micronutrient, and have the goal of preserving all micronutrient-related functions. In the case of vitamin K, requirements are typically based on a level of adequacy to prevent disorders of blood coagulation, and were historically assessed by the relatively insensitive prothrombin time test. More sensitive, subclinical conditions can be identified by specific biochemical or functional measures; for example, high levels of undercarboxylated coagulation or bone proteins may be indicative of a subclinical vitamin K deficiency.

These markers of vitamin status, such as plasma concentration or the level of functional proteins (e.g. undercarboxylated osteocalcin), may also provide an indication of tissue stores and hence adequacy. Finally, low urinary excretion of a nutrient or functional marker may reflect low intake and/or a deficiency; urinary γ-carboxyglutamic acid residue is such a marker for vitamin K status.

1.4.2 Establishing requirements

A decision on the criterion or criteria of adequacy is one step on the path to establishing a nutritional requirement. The second stage is to obtain data on what levels of intake affect health and disease through the chosen marker of adequacy.

Since clinical signs of overt vitamin K deficiency are rare, they are in this instance, of little use for setting recommended intakes. For many nutrients, original nutritional requirements were often based on the estimation of mean intakes in an apparently healthy population. Indeed, this method was the basis for the recent increase in the vitamin K_1 RDA for the American population. However, the use of mean intakes in a healthy population is limited by its insensitivity to reflect subclinical deficiency. To be useful, functional markers of subclinical deficiency need to be sensitive to changes in the intake of a specific nutrient. Also required is a solid understanding of biological effects of the nutrient along with sensitive instrumentation to measure these effects. There needs to be a clear link between the nutrient, the marker, and health outcomes.

Undercarboxylated osteocalcin appears to provide a suitable functional marker for the assessment of vitamin K status and possible subclinical deficiency. Osteocalcin is an established marker of bone turnover, and low %ucOC is related in some studies to improved bone health, including clinical outcomes such as fracture rate and BMD. Furthermore, the carboxylation of osteocalcin has been shown to be sensitive to changes in vitamin K_1 intake (see sections 1.3.2. and 1.6.4). However, the biological action of osteocalcin remains uncertain and there are questions over the relevance of undercarboxylated osteocalcin measurements in plasma. Furthermore, recent evidence from work in Rhesus monkeys showed that warfarin-induced vitamin K deficiency had no

effect on BMD or markers of bone turnover (Binkley *et al.*, 2007) casting doubt on the importance of vitamin K in osteoporosis. These concerns notwithstanding, the evidence suggests that osteocalcin carboxylation could be used as an additional functional marker of vitamin K status for the setting of dietary recommendations.

Recommended intakes for vitamin K in the UK and elsewhere are largely based on the requirements to maintain blood coagulation often and were set prior to recognition of the importance of extrahepatic VKD proteins, their functions and their wide distribution in body tissues. In the UK the guideline intake is 1 µg/kg body wt/d (Department of Health 1991). Similarly, the joint German, Austrian and Swiss body, Die Deutsche Gesellschaft für Ernährung e.V., has set recommended intakes for vitamin K as 60 µg/d and 70 µg/d, respectively for women and men aged between 15 y and 51 y, and 65 µg/d and 80 µg/d for women and men aged over 51 y [1]. The physiological functions of vitamin K are now recognised as going beyond its importance in blood coagulation. The nutritional requirements of vitamin K for blood clotting are well-established and are met without difficultly in a normal population, but evidence suggests that requirements may be greater to maintain full carboxylation of extrahepatic VKD proteins, notably osteocalcin. Partly in recognition of the additional requirement of vitamin K for extrahepatic VKD proteins, recommended intakes in the US have been increased to 90 µg/d and 120 µg/d for women and men, respectively (Institute of Medicine 2001). Furthermore, a European Commission report on vitamin K stated that although vitamin K_1 intake of around 1 µg/kg body wt/d is sufficient to meet hepatic requirements it is probably insufficient to fully carboxylate extrahepatic proteins (European Union Scientific Committee on Food 2003). At the same time, recent dietary surveys have shown that more than 50% of people do not meet current recommendations and have revealed temporal declines in vitamin K_1 intake (see section 1.5).

---

[1] http://www.dge.de/modules.php?name=Content&pa=showpage&pid=4&page=14 (accessed 21st February 2007)

The setting of recommended intakes requires evidence from a wide-range of sources and studies including data from both metabolic studies (including depletion-repletion experiments and supplementation interventions) and small population-based and large epidemiological studies. Data are required on food sources of a nutrient and on typical intakes within a population, specifically, how differences in bioavailability from different foods and genetic polymorphisms affect absorption and ultimately status. An understanding of nutrient utilisation to include kinetics of uptake, turnover and storage are also essential.

The focus of the present work was to develop novel, stable isotope-based methods to probe the kinetics of vitamin K_1 absorption, uptake and turnover in humans, and to investigate the bioavailability of vitamin K_1 from different foods to try and improve our understanding of the relationship between vitamin K_1 intake and plasma status.

1.5 Sources and dietary intakes of vitamin K_1

1.5.1 Vitamin K_1 content of foods

Vitamin K_1 is widely distributed throughout the human diet although most foods are poor sources. A summary of the wide-range of foods and their vitamin K_1 content is shown in Table 1-2. The richest sources of vitamin K_1 are green leafy vegetables (e.g. broccoli, kale, spinach) with considerable amounts in other vegetables (e.g. beans, cucumber, cauliflower), which is not surprising since vitamin K_1 is associated with photosynthesis. Indeed it has been shown that the greener outside leaves of a cabbage contain 3 to 6 times more vitamin K_1 than the inner leaves (Ferland & Sadowski, 1992b). The second major source of vitamin K_1 is vegetable oils and margarines, although as with vegetables, there is a broad range of values; rapeseed and soybean oils contain high levels of vitamin K_1, others are intermediate sources (olive, corn oil) whereas some have low amounts (groundnut). Generally, dairy, meat, cereal and fruit sources provide lower amounts of vitamin K_1.

The vitamin K_1 content of the same food can also vary considerably due to geographical location, seasonal variation, and processing and storage factors. For

example, the vitamin K_1 concentration of six individual rapeseed oil bottles varied between 140 and 187 µg per 100 g (CV of 11%) and average pooled values between two sampling time points varied by 13% for oils, and up to 36% in margarines of the same brand (Piironen & Koivu, 2000). In one study, the vitamin K_1 content of 12, 70% fat vegetable oil margarines varied between 0.1 to 61 µg per 100 g (Peterson *et al.,* 2002) and in another study the vitamin K_1 content in six brands varied between 12 and 78 µg per 100 g (Bolton-Smith *et al.,* 2000). These large variations in the vitamin K_1 content of margarine can be attributed to seasonal and market-led variations in the oils used in the formulation of margarine.

Regarding storage, samples of margarine from a single manufacturer taken immediately after production contained 13% more vitamin K_1 then samples tested from shop-bought packs (Piironen & Koivu, 2000). The processing of vegetable oils has been shown not to influence vitamin K_1 concentration, whereas heating caused a small decrease. Daylight and fluorescent light were reported to decrease vitamin K_1 content. After 2 d, fluorescent light decreased vitamin K_1 content by around 50% and daylight by about 90%. Amber glass bottles were shown to offer some protective effect (Ferland & Sadowski, 1992a). The preparation of food will also affect vitamin K_1 intake. For example, peeling of apples and cucumbers decreased the vitamin K_1 content by up to 60% (Koivu-Tikkanen 2001). In addition to the actual variation in vitamin K_1 content between samples and foods, there is analytical variation due to different methods and laboratories.

The assessment of vitamin K in foods is necessary for estimates of dietary intake (Shearer & Bolton-Smith, 2000). The accuracy of food databases is crucial and depends on obtaining precise measurements on truly representative samples, as well as obtaining data on a broad range of foods. A study of the accuracy of two nutrient databases in the United States by direct analysis of the diets revealed that although one showed reasonably close agreement (+/- 22%) another overestimated the amount of vitamin K_1 in the diet by up to 62% (McKeown *et al.,* 2000).

Table 1-2. Vitamin K_1 content of foods (adapted from Shearer *et al.*, 1996)

Concentration range (µg per 100 g fresh weight)			
0.1 – 1.0	1-10	10-100	100-1000
Avocado	Apples	Beans, runner	Broccoli tops
Bananas	Aubergine	Beans, French	Brussel sprouts
Beef, steak	Baked beans	Beans, broad	Green cabbage
Bread, white	Barley	Cabbage, red	Kale
Chicken	Beef, minced	Cauliflower	Lettuce
Cod	Butter	Cress	Parsley
Cornflakes	Carrots	Chick peas	Rapeseed oil
Flour, white	Cheese	Cucumber	Soybean oil
Grapefruit	Chocolate, plain	Greengages	Spinach
Maize	Courgettes	Olive oil, extra virgin	Water cress
Mangoes	Cream	Peas	
Melon	Dates		
Milk	Egg		
Mushrooms	Figs		
Oranges	Grapes		
Parsnips	Leeks		
Peanuts	Liver		
Pineapple	Nectarines		
Pork chops	Oats		
Potatoes	Palm oil		
Rice	Peaches		
Salmon, tinned	Peppers		
Sausages	Plums		
Spaghetti	Rhubarb		
Tuna, tin	Strawberries		
Turnips	Sunflower oil		
Yoghurt	Tomatoes		
	Swede		
	Wheat bran		
	Wholemeal bread		

1.5.2 Dietary intakes of vitamin K_1

Adult intakes in the UK are reported to be between 60 and 80 µg/d (Fenton *et al.,* 1997; Bolton-Smith *et al.,* 1998; Fenton *et al.,* 2000; Thane & Bolton-Smith, 2002; Thane *et al.,* 2006a). It is generally considered that intakes at this level are sufficient to maintain VKD coagulation proteins in a fully γ-carboxylated state (Booth & Suttie, 1998).

Data from the National Diet and Nutrition Survey (NDNS) of 1986-7 in 1,936 individuals, reported that around 48% of British men and women had intakes below the UK guideline intake (Thane & Bolton-Smith, 2002). More recently, data from the NDNS 2000 – 1 survey of adults, showed 66% of men and 52% of women had intakes below the recommended values (Thane & Coward, 2004). Mean intake between the two surveys significantly decreased from 72 to 67 µg/d (Thane *et al.,* 2006a). A similar result was found in Irish adults where 52% had intakes below 1 µg/kg body wt/d (Duggan *et al.,* 2004).

The NDNS and Irish surveys all used intake data collected over 7 d. This protocol is important because of the large variation in foods, and the often-irregular consumption of foods that are very high in vitamin K_1 (e.g. broccoli). Thus when estimating intake, it is recommended that measurements should be taken over at least 5 d (Institute of Medicine 2001). In 1996, data from the US Food and Drug Administration Total Diet Survey were published that are similar to the British and Irish data. Three day dietary records (24 h recall and 2 d food diary) were collected from 2,706 adults (Booth *et al.,* 1996b). The data are presented for four age groups (25 – 30, 40 – 45, 60 – 65 and 70 y +) and intakes ranged from 59 to 86 µg/d for all individuals. Based on the then-current dietary recommendations only individuals in the 25 – 30 y age range were below the recommended values. However, in the US intakes have recently been raised to 120 and 90 µg/d for men and women, respectively (Institute of Medicine 2001). On this basis, all groups were below the adequate intake. A later study that used 14-d food diaries in 4,741 people aged 13 y and above, showed mean intakes of 81 and 73 µg/d for males and females, respectively (Booth *et al.,* 1999b). If the US guideline intake is applied to British

NDNS data then 75% of participants in the 1986 – 7 survey, and 78% from the 2000 – 1 survey had intakes below the cut off values (Thane *et al.,* 2006a).

1.5.2.1 The relative importance of different vitamin K_1 sources

As might be predicted from the relative amounts of vitamin K_1 in foods, the greatest contributor to intake is vegetables. Vegetables contribute between 50% and 70% of dietary vitamin K_1 (Booth *et al.,* 1996b; Thane & Bolton-Smith, 2002; Duggan *et al.,* 2004; Thane & Coward, 2004; Thane *et al.,* 2006a), around half from green leafy vegetables, commonly cabbage, broccoli and spinach. Indeed, in one study spinach, collards (kale), lettuce and broccoli accounted for 34% to 42% of total vitamin K_1 intake in men and women (Booth *et al.,* 1996b). In the NDNS surveys, 19 – 23% of vitamin K_1 was from green leafy vegetables, and 14 – 18% from raw vegetables (mainly lettuce). Interestingly, comparison of the 1986 – 7 and 2000 – 1 survey revealed a decline of the importance of traditional British vegetables (cabbage and Brussel sprouts) as sources of vitamin K_1 (Thane *et al.,* 2006a) that mirrors the overall decrease in vitamin K_1 intake. Other food groups, meat, dairy, fats and oils, and cereals typically contribute around 5% to 10% to total dietary intake of vitamin K_1 (Duggan *et al.,* 2004; Thane *et al.,* 2006a).

These values suggest that vegetables are the major dietary source of vitamin K_1. However, this conclusion assumes that the vitamin K_1 is equally available for absorption between different foods and sources which is unlikely considering the location of vitamin K_1 within plant cell membranes and evidence that suggests greater absorption from oil than from vegetable sources (Booth *et al.,* 2002).

The relative contribution of foods that contain lower amounts of vitamin K_1 but that are eaten in higher quantities should also be considered. For example, a study from Brazil in 115 adults, showed that green leafy vegetables contribute around 30% of total dietary vitamin K_1, as assessed by a food frequency questionnaire. However, based on 24-h recall of diet, kidney beans and soybean oil emerged as important sources of vitamin K_1 contributing around 10% and 30%, respectively (Custódio das Dôres *et al.,* 2007).

1.5.2.2 Non-dietary factors affecting intake of vitamin K_1

An understanding of non-dietary factors that may affect vitamin K_1 intake is useful in identifying groups of a population that may be at risk of dietary deficiency and include age, gender, and geography.

In the US (Booth *et al.,* 1999b), Irish (Duggan *et al.,* 2004) and British surveys (Thane *et al.,* 2006a) vitamin K_1 intake significantly increased with age mainly due to the consumption of green leafy vegetables (Thane *et al.,* 2006a).

In the British NDNS data and a US survey there were significant gender differences when intake was expressed as µg/d however, these differences disappeared when intake was expressed as µg/kg body wt/d (Booth *et al.,* 1999b; Thane *et al.,* 2006a). In a study of 34 adults, the use of 4-d weighed records revealed no relationship between age or gender and vitamin K_1 intake (Booth *et al.,* 1997). In a cohort of 837 men and women that used a food frequency questionnaire (FFQ), daily intake was shown to be higher in women than men (151 µg/d and 115 µg/d, respectively) (McKeown *et al.,* 2002), but again this difference would likely be reduced if expressed per kg of body weight.

There appears to be considerable geographical variation in dietary intakes of vitamin K_1. A study from The Netherlands has estimated intakes in an elderly (55 y and over) population to be 250 µg/d (Schurgers *et al.,* 1999). Although, this value seems very high in comparison to data from other studies, it may be accurate since another study found similar levels in a population of Dutch post-menopausal women (Jie *et al.,* 1996). Higher intakes in the Dutch population have been attributed to their high intake of spinach and broccoli (Thane *et al.,* 2002b). The comparison of two populations of older people showed that people living in Shenyang, China had a vitamin K_1 intake four-times that of people living in Cambridge, UK (285 and 59 µg/d) (Yan *et al.,* 2002). Values from a population in New England, US (McKeown *et al.,* 2002) were considerably higher than those reported in previous US studies. Regional differences have also been observed in the UK with decreasing intakes heading from London and the South-East towards Scotland (Thane & Bolton-Smith, 2002; Thane *et al.,* 2006a).

1.5.2.3 Potential toxicity of high vitamin K_1 intakes

There is no evidence to suggest that high intakes of vitamin K pose any risk. Newborn babies commonly receive milligram doses of vitamin K_1 either orally or intramuscularly against HDNB (Hey 2003). Large doses of vitamin K_1 are also administered in cases of anticogualant poisoning, again with no systematic reports of adverse effects. A number of studies have been performed in human volunteers with milligram oral doses of vitamin K_1 (Lamon-Fava *et al.,* 1998; Binkley *et al.,* 2002). One study reported the supplementation of female athletes with 10 mg/d vitamin K_1 for one month to improve markers of bone status and documented no side effects (Craciun *et al.,* 1998). Vitamin K_2 has also been given in large doses (up to 90 mg) in intervention trials with no reported adverse health effects (Weber 2001). One unpublished report cited in Vermeer *et al.* suggests that vitamin K_1 supplementation (more than 1 mg/d) can contribute to periodontitis (Vermeer *et al.,* 2004). Vitamin K_1 is an essential growth factor for micro-organisms implicated in the progression of periodontal disease (Rawlinson *et al.,* 1998). However, it appears that, unlike vitamin A, there is little risk of vitamin K toxicity, possibly because vitamin K_1 is rapidly excreted and does not accumulate in tissues to the same extent as other fat-soluble vitamins.

1.6 Vitamin K_1 status and its measurement

A number of markers of vitamin K_1 status are available. Some may provide an indication of whole body stores (plasma concentration, urinary Gla excretion), while others may provide information on the vitamin K status of specific tissues, e.g. PIVKA-II for liver and ucOC for bone. However, the relevance of most of the markers of vitamin K status has been questioned due to the lack of evidence for their physiological significance (Institute of Medicine 2001) and whether circulating levels of the markers reflect status at the tissue level. Some of the potential markers are discussed below.

1.6.1 Plasma concentration

Plasma samples are relatively simple to obtain and vitamin K_1 plasma concentration is the most common way to assess vitamin K status. However, this method can be considered a rather indirect and relatively insensitive marker because the biochemical function of vitamin K occurs within tissues and cells (Fell & Talwar, 1998). Furthermore, and as will be discussed in more detail, plasma concentration is highly dependent on recent intake (<12 h) rather than tissue stores (Sokoll *et al.,* 1997).

Due to the low levels of vitamin K found in human plasma, it is only relatively recently that accurate determinations of vitamin K_1 in plasma have been possible. Quantitative analysis of vitamin K_1 in biological tissues is routinely performed using high performance liquid chromatography, usually after post-column reduction and fluorescence detection. Sadowski *et al.* published one of the first studies to measure plasma vitamin K_1 concentration in a reasonable size population with a wide age range (Sadowski *et al.,* 1989). This study measured fasting plasma vitamin K_1 in 396 adults. A normal range was established ranging from 0.29 to 2.64 nmol/L and the geometric mean was 0.87 nmol/L. Plasma concentrations within a population are not normally distributed, but are heavily skewed towards lower values. As a result, geometric means and median values are both used to report plasma vitamin K_1 levels. In a study of 1,154 British adults, the geometric mean was reported as 0.94 nmol/L with 95% of values between 0.1 and 8.72 nmol/L. The higher upper range of values is due the inclusion of non-fasting plasma samples in the analysis (Thane *et al.,* 2006b), although more typical post-prandial values are reported to be in the range 1 – 3 nmol/L (Suttie 1992). The geometric means of plasma vitamin K_1 concentration in 1,042 US adults was 0.57 and 0.92 nmol/L in women and men, respectively (Rock *et al.,* 1999).

The use of plasma vitamin K_1 concentration as a sensitive marker of status has been questioned, since in a number of studies, supplementation with lower levels of vitamin K_1 (25, 50 and 200 µg/d) after depletion had no effect on plasma vitamin K_1 levels (Suttie *et al.,* 1988b; Ferland *et al.,* 1993; Booth *et al.,* 2003b). However, Ferland *et al.* (1993) did observe a 30% increase in plasma vitamin K_1 with 45 µg/d of vitamin K_1 and

Booth *et al.* reported an increase from 0.21 to 0.87 nmol/L after repletion with 200 µg/d for 10 d (Booth *et al.,* 2000a). Plasma vitamin K_1 does respond to recent vitamin K_1 intake (Gijsbers *et al.,* 1996; Garber *et al.,* 1999; Booth *et al.,* 2002) thus is useful for measurements of absorption. Schurgers *et al.* (2004) performed a step-wise supplementation study where volunteers received an increasing supplement on a weekly basis. Plasma concentration was measured at 4 h post-dose at the beginning of each week and a linear relationship between dose (50, 100, 150, 200 or 250 µg) and plasma concentration (2.7 – 8.9 nmol/L) was observed. The response of plasma concentration to intake is short-lived and may explain the contrasting results obtained above. As a result, plasma status may not be suitable as a marker for whole body or tissue status. However, Olson *et al.,* (2002) suggested that plasma levels do relate to body status since they observed a linear relationship between the two ($r=0.6$, $p<0.05$) when body pool sizes were calculated using radiolabelled vitamin K_1. In the study of Schurgers *et al.*, (2004) fasting samples were also collected at the end of each week of supplementation. A linear relationship was still observed although the slope was very shallow with values ranging from 1.1 – 2.9 nmol/L.

There is also some evidence that plasma status may change throughout the day. In six volunteers, plasma vitamin K_1 peaked at 2200 h and decreased to 32% of the maximum by 1000 h, and mirrored TAG concentration (Kamali *et al.,* 2001). Depending on the research question, a potential advantage of using plasma status compared to the other methods listed below is the ability to distinguish between vitamin K_1 and vitamin K_2.

1.6.2 Prothrombin time

Before the advent of methods to measure plasma vitamin K_1 and sensitive methods to measure uncarboxylated proteins, the use prothrombin time (PT) as a marker of vitamin K status was common. Simply, prothrombin time is the time required for a sample of blood to clot. However, it is not a sensitive assay for measuring vitamin K status because normal prothrombin times are still observed even when plasma contains only 50% of the normal amount of prothrombin (Suttie 1992). Early studies with diets

containing <25 µg/d for upward of 20 d have shown conflicting results with some reporting an increase in PT, while others have shown no change (Suttie *et al.,* 1988b), even on a vitamin K-free diet (Allison *et al.,* 1987). Since the advent of sensitive methods to measure vitamin K_1 plasma status, studies have generally shown no significant change in prothrombin time with alterations in vitamin K_1 intake (Suttie 1992; Ferland *et al.,* 1993; Booth *et al.,* 1999a), although one study reported a significant decrease of 1.5% in PT when subjects when repleted with 420 µg/d (Sokoll *et al.,* 1997).

1.6.3 Coagulation proteins

A more sensitive measure of hepatic vitamin K status is undercarboxylated prothrombin (factor II) known as 'protein induced by vitamin K absence' (PIVKA-II). Since prothrombin, along with the other coagulation proteins, are synthesised in the liver, this measure can provide information on liver stores. PIVKA-II has been show to be responsive to reduced vitamin K_1 intake (Suttie *et al.,* 1988b; Ferland *et al.,* 1993; Booth *et al.,* 2003b) and repletion with 200 µg/d (Booth *et al.,* 2000a), 86 µg/d (Booth *et al.,* 2003b) and 50 µg/d (Suttie *et al.,* 1988a), although repletion with intake up to 45 µg/d had no effect (Ferland *et al.,* 1993). In another study, undercarboxylated factor II decreased within 1 wk with supplementation of 100 µg/d and continued to show a linear decrease with supplementation up to 500 µg/d (Schurgers *et al.,* 2004).

Factor VII and protein C activity have also been investigated as potential markers of vitamin K status. Although Factor VII and protein C circulate at much lower concentrations than prothrombin, the half-life is only 6 h (compared to 60 h for prothrombin) (Ferland *et al.,* 1993) thus they were considered potentially useful indicators of current vitamin K status (Institute of Medicine 2001). However, Ferland *et al.,* (1993) reported no change during depletion or repletion in the status of these coagulation proteins.

1.6.4 Undercarboxylated osteocalcin

The level of undercarboxylated osteocalcin (ucOC) is relatively sensitive to changes in vitamin K intake as demonstrated in controlled metabolic trials (Sokoll *et al.,*

1997; Booth *et al.,* 1999a; Binkley *et al.,* 2000) and intervention studies (Bolton-Smith *et al.,* 2007; Bügel *et al.,* 2007). As with undercarboxylated prothrombin as a marker of vitamin K liver status, ucOC primarily provides a measure of vitamin K status of bone. Supplementation with 420 µg/d decreased %ucOC by around 40% within 5 d (from 22% to 13%) (Sokoll *et al.,* 1997), whereas another study observed an approximate 50% decrease with supplementation of 250, 375 and 500 µg/d, and a 75% decrease with 1000 µg/d (with starting values of %ucOC of 6 – 8%) over 1 wk (Binkley *et al.,* 2002). A step-wise supplementation study where volunteers received an increasing supplement on a weekly basis showed no effect of daily intakes of 50, 100, 150, 200 or 250 µg/d incremental, but a significant decrease in %ucOC was observed with 300 µg/d (Schurgers *et al.,* 2004). However, these observations may partly be explained by the gradual increase of vitamin K_1 stores. A depletion-repletion study observed a significant increase in %ucOC (from 35% to 60%) after 90 µg/d for 14 d, but in contrast to Sokoll *et al.,* (1997) failed to observe any significant decrease during step-wise repletion to 450 µg/d, explained by the preferential use of vitamin K_1 by the liver (Booth *et al.,* 2003b). Another study observed a decrease in %ucOC from 44 to 20% after depletion and subsequent repletion with 200 µg/d for 10 d (Booth *et al.,* 2000a).

The usefulness of ucOC as a marker of vitamin K status is supported by evidence linking high %ucOC to physiological outcomes such as low bone mineral density and increased fracture rates. Furthermore, the apparent slower turnover of vitamin K in bone provides a marker of longer-term status (Schurgers *et al.,* 2004). However, concern has been raised as to the usefulness of ucOC as a marker since results from monoclonal antibody kits for measuring ucOC from different manufacturers are reported to provide quite different results (Institute of Medicine 2001). Additionally, the relevance of measuring a non-functional protein has been questioned, since if ucOC makes around 5-10% of total osteocalcin then 90-95% is still fully functional, thus no 'normal' value has been established (Institute of Medicine 2001). In one study, McKeown *et al.* defined high %ucOC as ≥20% and suggested this value as a marker of low vitamin K status. On this

basis, approximately half of 917 men and women had low vitamin K status (McKeown *et al.,* 2002).

1.6.5 Urinary Gla excretion

An indirect method to measure vitamin K status is via the excretion of Gla residues in urine that derive from the metabolism of VKD proteins. Significant reductions (10 – 20% from baseline) in urinary Gla excretion have been induced with intakes between 10 and 100 µg/d (Suttie *et al.,* 1988b; Ferland *et al.,* 1993; Sokoll *et al.,* 1997; Booth *et al.,* 2003b). However, repletion with between 45 to 500 µg/d was not sufficient to raise Gla excretion to baseline levels (Suttie *et al.,* 1988b; Ferland *et al.,* 1993; Booth *et al.,* 2003b). Similarly, supplementation of 420 µg/d and 500 µg/d made no difference to Gla excretion (Booth *et al.,* 1999a; Bügel *et al.,* 2007). Although urinary Gla could provide an indicator of whole body status there are no reference values. Response to variation in vitamin K intake is not consistent, and there is no indication of the relative status of different tissues, although these data may be inferred from the estimated turnover values of vitamin K-dependent proteins. It is estimated that 60% of excreted Gla residues derive from coagulation proteins (Ferland *et al.,* 1993). It has been suggested that urinary Gla is less responsive to short-term changes to vitamin K_1 intake than other markers such as ucOC (Booth *et al.,* 2001).

1.6.6 Urinary vitamin K metabolites

Recently a method for the measurement of the two major urinary metabolites has been published (Harrington *et al.,* 2005; 2007). The methodology was proposed as a non-invasive marker of vitamin K metabolism and its use was demonstrated in subjects before and after supplementation with different levels of vitamin K_1, K_3 (menadione) and MK-4. Values of the aglycone metabolites with side chain lengths of 5 and 7 carbon atoms (Figure 1-12) were expressed relative to creatinine. Whether the method is suitable for detecting small changes in vitamin K status is unknown since supplementation levels were between 1 mg and 50 mg. Additionally, a proportion of vitamin K metabolites are excreted via the bile (Harrington *et al.,* 2005).

1.6.7 Relationships between markers of vitamin K_1 status

No single marker provides a 'gold standard' for the assessment of vitamin K status. It is useful for the interpretation of data on individual markers to understand to what extent they are correlated. However, few studies have provided this comparison. In 263 healthy individuals, %ucOC was inversely correlated with plasma vitamin K_1 ($r=-0.35$, $P<0.001$) and PIVKA-II ($r=-0.15$, $P<0.05$). There was a positive correlation with %ucOC and PIVKA-II ($r=0.27$, $P<0.001$) (Sokoll & Sadowski, 1996). Another study in 219 individuals reported a significant inverse correlation between %ucOC and plasma vitamin K_1 ($r=-0.245$, $P<0.001$) (Binkley *et al.,* 2000) as did Beaven *et al.,* (2005) ($r=-0.57$, $P<0.001$). Tsugawa *et al.,* (2006) reported a significant correlation between plasma vitamin K_1 and ucOC ($r=0.22$, $P=0.001$) in 396 women aged 30 – 79 y. McKeown *et al.,* (2002) also reported an inverse association between %ucOC and plasma vitamin K_1, with the odds of a high %ucOC in those in the lowest plasma vitamin K_1 quintile, 2 and 3 times greater than those in the highest quintile, for women and men, respectively. More recently, a study in 142 haemodialysis patients reported no correlation between plasma vitamin K_1 and %ucOC (Pilkey *et al.,* 2007).

1.6.8 Non-dietary factors affecting vitamin K_1 status

A number of non-dietary factors may affect one or more of the vitamin K status markers. It is necessary to understand which factors may play a role in determining status, so they can be considered in study design and interpretation.

1.6.8.1 Gender

A number of studies have shown that gender has no effect on vitamin K plasma status (Sadowski *et al.,* 1989; Booth *et al.,* 1997; McKeown *et al.,* 2002; Thane *et al.,* 2002a), whereas others have identified a significant gender difference (Rock *et al.,* 1999; Thane *et al.,* 2006b). Thane *et al.*, (2006b) report geometric means of 1.13 and 0.81 nmol/L ($P<0.001$), in 530 and 624, men and women, respectively. Rock *et al.* (1999) report geometric means 0.92 and 0.57 nmol/L in 411 and 631 men and women and that

gender is a significant ($P<0.05$) predictor of plasma status. It has also been shown that the menopause does not affect vitamin K status (Sokoll & Sadowski, 1996).

1.6.8.2 Age

Age has been shown by a number of studies to be related to vitamin K status (Sadowski *et al.,* 1989; Booth *et al.,* 1997; Tsugawa *et al.,* 2006), but not all (McKeown *et al.,* 2002). In 263 adults aged 18 – 85 y, Sokoll & Sadowski (1996) report that plasma levels in males and females were generally constant with the exception of lower levels in the 3rd decade. In the 2000 – 1 NDNS there were no significant differences in vitamin K_1 plasma concentration between individuals in four age groups, with the exception of lower levels in young (19 – 34 y) women (Thane *et al.,* 2006b). Rock *et al.* (1999) reported a 4% change in vitamin K_1 concentration with every decade of life.

The relationship between osteocalcin and age is complicated with numerous conflicting reports. Although total osteocalcin may vary with age, one study showed that when ucOC was expressed as a % of total osteocalcin, many of the age and sex differences were removed (Sokoll & Sadowski, 1996). One study in 219 subjects, reported that %ucOC was higher in young men than older men but there was no difference in women (Binkley *et al.,* 2000). Another study in 396 women (aged 30 – 79 y) showed that ucOC was significantly correlated with age (Tsugawa *et al.,* 2006).

Age was not related to PIVKA-II but differences were observed between young males and young females; urinary Gla excretion was also reported to increase with age (Sokoll & Sadowski, 1996).

1.6.8.3 Plasma lipids

A strong correlation between plasma vitamin K_1 and TAG concentration has been described in a number of population studies (Sadowski *et al.,* 1989; Kohlmeier *et al.,* 1995b; McKeown *et al.,* 2002) and metabolic studies (Dolnikowski *et al.,* 2002; Erkkilä *et al.,* 2004). In a study in 15 individuals, from whom blood samples were taken weekly for 22 wk, vitamin K_1 was positively and significantly correlated with TAG ($r=0.51$, $P<0.001$) (Talwar *et al.,* 2005). This correlation may also explain observations in some studies of

higher plasma vitamin K_1 in the elderly since vitamin K_1 is primarily associated with chylomicrons (Booth & Suttie, 1998). Indeed, in one study in 131 young and 195 elderly individuals, the higher plasma status of vitamin K_1 in the elderly was reversed on correction for TAG, after which the young had proportionally higher levels (Sadowski *et al.,* 1989). This relationship may be expected since, like vitamin E, which is also correlated with TAG (Sadowski *et al.,* 1989), vitamin K is transported by lipoproteins.

In many studies plasma vitamin K_1 has been adjusted for TAG levels in blood on the basis that this may provide a more appropriate marker for vitamin K_1 status by removing inter-individual variation in TAG levels. One study that measured absorption of vitamin K_1 adjusted plasma vitamin K_1 by TAG concentration and found that after adjustment, area under the curve coefficient of variation (CV) was reduced from 43 to 27% (Booth *et al.,* 2002). A further study, noted that normalisation of plasma vitamin K_1 for TAG concentration decreased inter-individual variation in post-prandial concentrations, as indicated by smaller standard deviations (Erkkilä *et al.,* 2004). However, in the study of Talwar *et al.* (2005) where individuals provided fasted samples regularly over 22 wk, correction of plasma vitamin K_1 for TAG, decreased intra-individual variation from 38% to 30% but inter-individual variation increased from 44% to 46%.

If there was a strong relationship between TAG and vitamin K_1 then stronger gender and age differences in vitamin K_1 plasma concentration might also be expected, since TAG are generally higher in males compared to females, and in the elderly (Shearer *et al.,* 1988). This inconsistency suggests that dietary intake is probably a more important factor.

A correlation of plasma vitamin K_1 with cholesterol has also been reported (Cham *et al.,* 1999; Pilkey *et al.,* 2007), although other studies have found no relationship (Erkkilä *et al.,* 2005b).

1.6.8.4 Seasonal variation

Season may affect plasma vitamin K_1 concentration. In the US, higher values have been reported from spring to a maximum in late autumn and a reduction again in

winter (Sadowski *et al.,* 1989). Higher plasma K_1 values have also been found in US populations measured in spring/autumn compared to those sampled during the winter (McKeown *et al.,* 2002). However, another study in Scotland showed no seasonal variation in plasma vitamin K_1 levels (Fenton *et al.,* 2000). The lack of agreement in seasonal effects is probably in large part due to regional and temporal differences in vitamin K_1 content of foods (McKeown *et al.,* 2002).

1.7 The relationship between vitamin K_1 intake and vitamin K_1 plasma status

The measurement of nutrient status is crucial to understanding its function and determining sufficiency and the development of recommended intakes. Since it is not always feasible to directly measure status, nutritional intake data provide indirect measurements of status. Estimates of nutrient intake in a population are assessed using epidemiological data. It is therefore important to understand the relationship between dietary intake and the biochemical marker, commonly plasma vitamin K_1. There are broadly two types of study that can be used to measure the relationship between intake and status, experimental or observational studies.

1.7.1.1 Experimental studies

Experimental studies generally require a period of stay in a metabolic unit where the participants undergo a period of depletion (reduced dietary vitamin K compared to habitual diet) followed by repletion at different levels of intake. Depletion significantly decreases plasma vitamin K_1 concentrations (Suttie *et al.,* 1988b; Ferland *et al.,* 1993; Booth *et al.,* 2003b) although the extent and rate of the decrease has been shown to vary between age groups. Ferland *et al.* (1993) reported that the plasma vitamin K_1 concentration of older individuals decreased more slowly and maintained a higher level than concentrations of the younger age group. Intakes of 10 µg/d decreased levels to 13% of the original values ($p<0.001$) whereas another study showed significant decreases at a relatively high intake of 90 µg/d (Booth *et al.,* 2003b). However, plasma levels decreased no further with an intake of only 18 µg/d possibly suggesting the existence of a deeper pool with slow turnover (Booth *et al.,* 2003b). In a further study in nine individuals,

plasma vitamin K_1 halved between recorded levels at the start of the study and 6 d after consuming 100 µg/d (Sokoll *et al.,* 1997).

Repletion has been investigated over a range of intakes from 15 µg/d to 1000 µg/d. A single study showed no increase in plasma vitamin K_1 at daily doses of 15, 25 and 35 µg/d whereas 50 µg/d produced a small rise but only to 35% of the baseline value (Ferland *et al.,* 1993). A further study showed a small increase in plasma vitamin K_1 (0.43 to 0.57 nmol/L) after increasing intake from 30 to 55 µg/d but a significant increase (0.49 to 1.66 nmol/L) was only recorded when intake rose to 500 µg/d ($p<0.01$) (Suttie *et al.,* 1988b). These data possibly suggest that there is limited capacity for the absorption of single doses of vitamin K, as might be expected if uptake was a saturable, energy-dependent process (Hollander 1973). Binkley *et al.* (2002) reported no difference in concentrations after supplementation with 250, 375 and 500 µg/d for 1 wk. A study in 21 elderly people over 84 d using stepped-repletion found that 86 µg/d and 200 µg/d had no effect on plasma vitamin K_1 concentration. Even on repletion of 450 µg/d plasma vitamin K_1 did not reach pre-study values (Booth *et al.,* 2003b). This observation could be explained by study design since vitamin K was given each morning only, with fasting blood samples taken the following morning (Booth *et al.,* 2003b). However, a similar protocol was used in the study of Ferland *et al.* (1993) where 45 µg/d increased plasma vitamin K_1 by 30%. Suttie *et al.* (1998b) divided the repletion dose between the morning and evening meals but did not observe any change in plasma vitamin K_1 with 50 µg/d. As for the depletion described above, in the study of Ferland *et al.* (1993) older people were observed to increase plasma levels more rapidly during repletion than the young and it was suggested that higher hepatic body stores in the elderly results in a greater resistance to depletion and a greater response to repletion.

1.7.1.2 Observational studies

These involve either large epidemiological studies or smaller studies where intake and status have been measured on a number of occasions. Significant positive relationships have been observed between dietary intake and plasma concentration

(Table 1-3). As may be predicted, the correlations are generally stronger if more measurements of intake and status have been taken (Booth *et al.,* 1997; Bolton-Smith *et al.,* 1998). One study showed no relationship between dietary intake and status (Schurgers *et al.,* 1999) and was possibly a consequence of high intakes in this population since another study showed a plateau effect at higher intakes such that the association between plasma vitamin K_1 and intake was only significant up to 200 μg/d (McKeown *et al.,* 2002). Furthermore, if intakes between two populations with low and high intakes are compared, the correlation is less strong in the population with higher intakes (Yan *et al.,* 2004).

Table 1-3. Observational studies comparing vitamin K_1 intake and vitamin K_1 plasma status

Reference	Population	*n*	Samples	R value	P value
Booth *et al.,* 1997	Adults	34	3 x 4-d weighed record 3 x fasted plasma	0.51	=0.004
Booth *et al.,* 1997	Adults	34	1 x 4-d weighed record 1 x fasted plasma	0.13	0.30
Bolton-Smith *et al.*, 1998	Adults	65	3 x 7-d weighed record 3 x fasted plasma	Men: 0.28 Women: 0.28	<0.05
				Men: 0.45[a] Women: 0.41[a]	<0.001
Schurgers *et al.*, 1999		310	FFQ Fasted plasma	No relationship	
McKeown *et al.,* 2002	Adults	837	FFQ		<0.0001
Thane *et al.,* 2002b	Elderly	1076	4-d weighed record Fasted plasma	Men: 0.18 Women: 0.30	<0.001
Yan *et al.*, 2004	UK	134	7-d food diary	0.29[b]	=0.005
	Chinese	178	FFQ	0.17[b]	=0.03
Thane *et al.,* 2006b	Adults	1,154	7-d weighed record 1 x plasma	Men: 0.26 Women: 0.32	<0.001

[a] After adjustment for TAG

[b] There was no change in the relationship after adjustment for TAG

Abbreviations: FFQ, food frequency questionnaire; TAG, triacylglycerol

Using regression analysis, the variance in plasma vitamin K_1 explained by intake alone is reported as between 6% (Thane *et al.,* 2002a) and 8% (Thane *et al.,* 2006b). Multiple regression analysis showed that intake, season and TAG could explain 11% of plasma vitamin K_1 variation in free-living elderly Britons (Thane *et al.,* 2002a) whilst another study found 44% of the variation was due to vitamin K_1 intake, energy intake and TAG (Bolton-Smith *et al.,* 1998). Booth *et al.* (1997) state that the correlation between intake and status is partly determined by the reproducibility of the measures and as shown above, multiple measurements improved the correlation. Thane *et al.* (2006b) reported that around 20% of plasma vitamin K_1 variation was explained by various biochemical indices (in addition to vitamin K_1 intake) including cholesterol and plasma retinyl palmitate. However, the relevance of including some biochemical indices (e.g. plasma total Fe-binding capacity) is not clear. With the inclusion of other non-dietary factors around 40% of the vitamin K_1 plasma concentration was explained by demographic, lifestyle factors and dietary factors in 1,042 adults in the US (Rock *et al.,* 1999). These studies show that despite the inclusion of numerous factors in the regression models, still less than 50% of the variation in plasma vitamin K_1 can be explained. Kohlmeier *et al.* (1995b) estimated that less than a third of the variation between plasma vitamin K_1 of individuals is due to diet, whereas most of the variation is determined by apoE genotype and TAG metabolism.

1.7.1.3 Summary of intake and status data

As discussed above, plasma status of vitamin K_1 is not well correlated with vitamin K_1 intake, but of interest it is reported to be the strongest of the fat-soluble vitamins (Booth *et al.,* 1997). The strength of the relationship depends on the timing of collection of dietary and biochemical data, since plasma levels are largely determined by recent intake. Thus, the consumption of foods high in vitamin K_1 has a large short-term effect on plasma levels. Based on three, 4-d dietary records, the within-subject variance ratio for intake has been shown to be greater than between-subject variance for all fat-soluble vitamins, but in particular vitamin K_1. For plasma status, only vitamin K_1 of the fat-soluble vitamins had greater within-subject variance than between-subject variance (Booth *et al.,* 1997) and

suggests recent intake is the major factor in plasma concentration. Inter-subject variability is likely a consequence of differences in the uptake and metabolism of vitamin K_1 between individuals, and will be determined by for example, apoE phenotype. Evidence from multiple regression analysis puts the contribution of intake at around 10%, which is probably an underestimate since no correction is made for bioavailability. Probably a major determinant of the intake-plasma relationship within an individual, particularly for vitamin K_1, is bioavailability.

One of the methods for the determination of nutritional requirements is to use the mean intake of an apparently healthy population. Indeed, this method was adopted by the Institute of Medicine for setting of new Adequate Intake levels in the US (Institute of Medicine (IOM) Food and Nutrition Board (FNB) 2001). As a non-invasive method this approach is adequate but more information is required, and in particular a better understanding of the relationship between intake and status.

1.8 Bioavailability

The bioavailability of a nutrient depends on the efficiency of digestion, absorption and utilization, although only digestion and absorption combined can be quantified using plasma response (Schneeman 2004). Bioavailability has recently been defined as 'the fraction of an ingested nutrient that is available for utilization in normal physiologic functions and for storage' (Jackson 1997).

An understanding of nutrient bioavailability is important for setting dietary recommendations and to interpret the relationship between intake and status. Often, bioavailability data is limited and only available for a small number of individual foods. In the case of inorganic nutrients, the development of bioavailability algorithms has been explored, whereby available data are used to develop mathematical models to estimate nutrient bioavailability from different diets (Hunt 1996). Consideration of bioavailability is especially pertinent for vitamin K, where evidence from national surveys suggests intakes are marginal, and evidence is accumulating for a greater nutritional requirement for extrahepatic vitamin K-dependent proteins.

The importance of bioavailability in our understanding of nutrient requirements has been recognised by a number of national bodies. The UK Food Standards Agency lists the development of methods to measure bioavailability as one its key aims under its optimal nutrition research programme (N05)[1], with the objective of providing a scientific basis for population-level recommendations as to the optimal intake for micronutrients. In the US, the need for further studies on the bioavailability of vitamin K has also been recommended by the Institute of Medicine (Institute of Medicine 2001).

1.8.1 Definitions

For pharmacological studies of drugs, bioavailability is determined by the difference between an oral and an intravenous (*iv*) dose, compared by measuring the area under the curve fitted to changes in plasma concentration versus time.

$$\text{Bioavailability} = \frac{[AUC]_{oral} \times dose_{IV}}{[AUC]_{IV} \times dose_{oral}}$$

Where:
AUC = area under the curve
oral = oral dose
IV = intravenous dose

For nutrition research, the term is slightly less rigid and refers more generally to the amount available to the tissues after an oral dose, often measured by plasma concentration. Often in nutrition, relative bioavailability and not absolute bioavailability is calculated.

$$\text{Relative bioavailability} = \frac{[AUC]_{A} \times dose_{B}}{[AUC]_{B} \times dose_{A}}$$

---

[1] http://www.foodstandards.gov.uk/science/research/researchinfo/nutritionresearch/ (accessed 13th March 2007)

1.9 Vitamin K digestion and absorption

Before considering factors that may affect absorption, it is necessary to consider the mechanism of absorption of vitamin K_1. Following ingestion, vitamin K is digested with other fat-soluble vitamins and other lipid components. The gastric emptying of vitamins A and E have been shown to closely follow that of lipids in a study of that measured the vitamins in stomach and duodenum by direct sampling in healthy volunteers (Borel *et al.,* 2001). The primary dietary form of vitamin K, vitamin K_1, is absorbed, as with other non-polar lipids, chemically unaltered, in mixed micelles in the small intestine. Mixed micelles are mainly composed of bile salts, free fatty acids, monoglycerides and phospholipids. Evidence for the importance of bile comes from the comparison of radiolabelled vitamin K absorption between normal, healthy subjects and patients with fat malabsorption disorders (Blomstrand & Forsgren, 1968; Shearer *et al.,* 1974). Once solubized, the components of the mixed micelles are absorbed into the enterocytes via diffusion down a concentration gradient towards the mucosal cell surface. Absorption appears to be similar in both between the proximal and distal small intestine (Hollander *et al.,* 1977). In contrast to the passive absorption mechanism, work by Hollander (1973) using sacs everted from rat gut has suggested that vitamin K is absorbed by an energy-mediated saturable transport mechanism. Later experiments, this time using an *in vivo* technique in rats and a range of vitamin K concentrations, demonstrated saturable kinetics in the micromolar range, further supporting the earlier conclusion (Hollander *et al.,* 1977). However, since these experiments, no laboratory has followed up these observations and no specific mechanism has been published. One explanation may be that there are specific receptors acting as part of a high-affinity, readily-saturated active transport mechanism for vitamin K, which can scavenge low concentrations of the vitamin, coupled with another passive transport route, which predominates at higher concentrations.

Once in the enterocytes, the components of the mixed micelle (including vitamin K_1 and other fat-soluble vitamins) are repackaged into chylomicrons. Chylomicrons are the largest of the lipoproteins and consist of a non-polar, hydrophobic core of cholesterol esters, TAG and other non-polar fat-soluble components. The polar outer layer contains

free cholesterol, phospholipids and apolipoproteins. The main role of chylomicrons is the transport of dietary lipids from the gut in lymph and blood. In the enterocytes, apolipoproteins are synthesised and incorporated into the chylomicrons. From the enterocytes, chylomicrons are secreted into the lymph and subsequently into the venous circulation. In humans, Blomstrand & Forsgren (1968) measured newly absorbed vitamin K_1 in lymph by collection of lymph from the thoracic duct at the neck. Chylomicrons as the major carriers of newly absorbed vitamin K_1 was demonstrated in experiments using tritiated vitamin K_1 that showed around 70% of radioactivity in lymph was associated with the chylomicrons (Blomstrand & Forsgren, 1968; Shearer *et al.*, 1970b). A similar proportion of radioactivity was also reported in chylomicron and lipoprotein fraction of serum at the peak time after administration of ^{3}H-labelled vitamin K_1 in humans (Shearer *et al.*, 1970a). These early studies were performed using radiolabelled vitamin K since no method was available for the measurement of vitamin K_1. More recent studies have confirmed that chylomicrons are the major carrier of newly absorbed vitamin K_1 (Lamon-Fava *et al.*, 1998; Schurgers & Vermeer, 2002; Erkkilä *et al.*, 2004).

1.10 Vitamin K metabolism

Prior to describing the post-prandial transport of vitamin K_1, an overview of general lipoprotein metabolism is required. An understanding of the physiology of vitamin K_1 uptake is necessary to interpret kinetic data and studies.

1.10.1 Lipoprotein metabolism

Figure 1-10 shows the major pathways of lipoprotein metabolism. Once in the blood, chylomicrons pick up the apolipoproteins, apoE and apoC from the high-density lipoproteins (HDL). A major role of HDL is the transfer of apoE and apoC between chylomicrons, very low-density lipoproteins (VLDL) and HDL. TAG are removed from chylomicrons through the action of lipoprotein lipase that is present on the luminal cell surface of adipose and muscle tissue. ApoC is required for the activation of lipoprotein lipase and subsequent hydrolysis of TAG. The remaining particles, known as chylomicron remnants (CR), are transported to the liver. One major pathway for uptake of CR is via

the low-density lipoprotein (LDL) receptor on the surface of hepatocytes via interaction with apoE. Additional pathways include uptake via LDL-receptor related protein (LRP) or sequesterisation by binding to heparan sulfate proteoglycans (Cooper 1997). It is also believed that cholesterol is an essential component of CR uptake, since without cholesterol, CR remain in the plasma (Redgrave 2004). In the liver, CR are further metabolised with removal of cholesteryl esters and remaining TAG. A proportion of these molecules are subsequently packaged into VLDL (Redgrave 2004). VLDL particles synthesised in the liver are the major carrier of plasma TAG to other parts of the body. As in chylomicrons, and after interaction with HDL, the TAG in VLDL are hydrolysed by lipoprotein lipase and the fatty acids absorbed by adipose and muscle tissue. Intermediate-density lipoproteins (IDL), also referred to as β-VLDL, are formed as VLDL remnants and are either taken up by the liver via apoE, or undergo further processing to low-density lipoproteins (LDL). LDL particles are the major carriers of both free and esterified cholesterol. The characteristics of lipoprotein particles are summarised in Table 1-4.

Figure 1-10. Major pathways of lipoprotein metabolism in humans. Newly absorbed lipids leave the intestine and enter circulation via the lymph. Hydrolysis of TAG from chylomicrons occurs in the tissues and the resulting chylomicron remnants are taken up by the liver. VLDL supply lipids to extrahepatic tissues from the liver. IDL are intermediates in the formation of LDL from VLDL and after hydrolysis of TAG. The purpose of HDL is primarily the transport of lipids from the tissues back to the liver

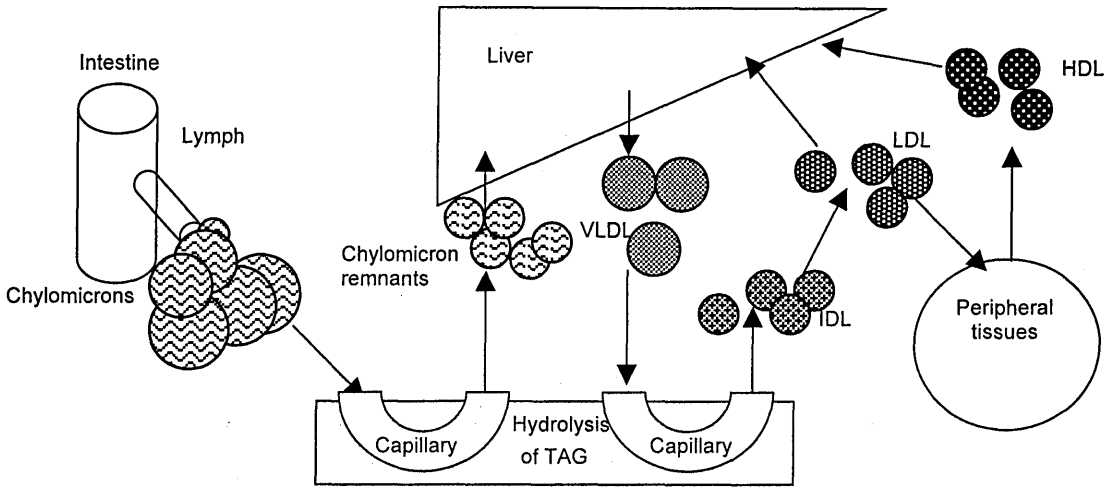


Table 1-4. Classes of lipoproteins, their source, density and function (adapted from Mann & Truswell, 1998)

Class	Source	Density (g/mL)	Function
Chylomicron	Intestine	< 0.950	Transport of exogenous lipids from intestine
HDL_2	Intestine	1.063 - 1.125	Removal and transfer of cholesterol from tissues
HDL_3	Liver and VLDL	1.125 - 1.210	
LDL	VLDL	1.019 - 1.063	Transport of cholesterol to peripheral tissues and liver
IDL	VLDL	1.006 - 1.019	LDL precursor
VLDL	Liver	0.950 - 1.006	Transport of lipids from liver to peripheral tissues

1.10.2 Post-prandial vitamin K_1 transport

The major carriers of vitamin K_1 in the plasma are chylomicrons, chylomicron remnants (CR) and VLDL. Evidence for the association of vitamin K_1 with chylomicrons and CR comes from work by Lamon-Fava and colleagues (Lamon-Fava *et al.,* 1998). In their first study, 15 volunteers were given 1.43 µg/kg body wt (around 100 µg) of vitamin K_1 in a fat-rich milkshake. Samples were taken at 0, 3, 6, 9 and 12 h after ingestion and peak plasma vitamin K_1 was observed at 6 h with 73% found in the TAG-rich lipoprotein (TRL) fraction that includes chylomicrons and VLDL. In the second study, volunteers ingested a higher dose of 50 µg/kg body wt of vitamin K_1, again in a milkshake, and the time course of vitamin K_1 concentration in different lipoprotein fractions in blood plasma was measured. The size of the dose was around 4000 µg for men and 3000 µg for women. The results are summarised in Table 1-5 and show that the majority of vitamin K_1 was carried in TRL. However, the distribution between chylomicrons and VLDL cannot be calculated. The authors postulate that as more chylomicron remnants are taken up by the liver vitamin K_1 is gradually secreted in VLDL (Lamon-Fava *et al.,* 1998). The ratio of IDL to LDL reflects CR clearance and it was reported by Saupe *et al.* that this ratio was a good predictor of vitamin K_1 concentration (Saupe *et al.,* 1993).

Table 1-5. Vitamin K_1 concentrations in plasma and lipoprotein fractions after ingestion of 50 µg/kg body weight of vitamin K_1 (adapted from Lamon-Fava *et al.*, 1998)

	Time after ingestion (h)				
	0	3	6	9	12
Plasma vitamin K_1 (nmol/L)	0.75	1.8[a]	274.6	212.7	146.8
TRL	0.55	189.8 (90.7%)[b]	237.6 (85.8%)	151.3 (76.0%)	83.3 (69.9%)
IDL	0	2.5 (1.2%)	5.2 (1.9%)	8.0 (4.0%)	3.8 (3.1%)
LDL	0	6.5 (3.1%)	16.7 (6.0%)	19.8 (9.9%)	16.8 (14.1%)
HDL	0	9.0 (4.3%)	15.0 (5.4%)	17.7 (8.9%)	13.1 (11.0%)
LPFF[c]	0	1.5 (0.7%)	2.4 (0.2%)	2.4 (1.2%)	2.1 (1.8%)

[a] Value as reported in the publication. However, this is likely a typographical error because 1) it is unrelated to amounts in lipoprotein fractions and 2) sem is ± 26 nmol/L

[b] Percentage total vitamin K_1 in lipoprotein fractions

[c] Lipoprotein-free fraction

Smaller amounts of vitamin K_1 were also found in IDL, LDL and HDL and might deliver vitamin K_1 to parts of the body other than the liver (Lamon-Fava *et al.*, 1998). It should be noted that in this study, volunteers were given very high doses of vitamin K_1 that resulted in plasma concentrations of around 100 times the normal fasting level of vitamin K_1 in plasma. The authors however dismiss this criticism since, in their first study (with a typical dose of 100 µg) a similar percentage of vitamin K_1 was recovered in the TRL fraction.

Further evidence for TRL transport of dietary vitamin K comes from Schurgers & Vermeer (2002) who measured vitamin K_1, MK-4 and MK-9 in volunteers for 48 h after consumption of a meal containing 2 µmol (900 µg) of vitamin K_1. In agreement with Lamon-Fava *et al.*, they found the majority of vitamin K_1 was carried in the TRL fraction (chylomicrons and VLDL) with lower amounts in both LDL and HDL. As a percentage, LDL contained no-more than 17% of the total plasma K_1 and HDL no-more than 18% of

the total, which are slightly higher than maximum amounts at any time point found in LDL and HDL in the study of Lamon-Fava *et al.* (1998) (14 and 11% respectively at 12 h). In the two studies, the amount of vitamin K_1 in LDL and HDL peaked at 6 h (Schurgers & Vermeer, 2002), and 12 h (Lamon-Fava *et al.*, 1998) after ingestion. Appearance in LDL occurs through VLDL metabolism while appearance in HDL could be due to exchange along side lipids and apolipoproteins during chylomicron lipolysis (Lamon-Fava *et al.*, 1998).

The studies of Lamon-Fava *et al.* (1998) and Schurgers & Vermeer (2002) both utilised high levels of vitamin K_1 that might have disrupted normal vitamin K_1 metabolism. Stable isotopes provide a tool to investigate metabolism at physiological levels. In the study of Errikila *et al.* (2004) and to investigate post-prandial vitamin K_1 metabolism, five volunteers were fed intrinsically labelled collard greens containing approximately 400 µg deuterium-labelled vitamin K_1. Blood sampling was performed for up to 216 h after consumption of the labelled vitamin K_1 meal. However, due to reported analytical problems, the lipoprotein distribution profile is reported for total vitamin K_1 only and not labelled vitamin K_1. The results of total vitamin K_1 are in agreement with Lamon-Fava *et al.* (1998); the main carrier of vitamin K_1 was the TRL fraction, with much lower amounts in IDL, LDL and HDL. No vitamin K_1 was detected in lipoprotein-free fraction (LPFF) suggesting the appearance of vitamin K_1 in LPFF in earlier studies was due to the high doses given. Deuterated vitamin K_1 was measured in only the TRL fraction in three subjects. Uptake and disappearance of deuterated vitamin K_1 in the plasma and TRL fraction was reported to be very similar in all three subjects with around 90% of plasma pool being enriched after 6 h suggesting a small pool size. Deuterated vitamin K_1 was no longer detected after 72 h, which points to relatively fast turnover. The report of Errikila *et al.* (2004) supports earlier work that shows the TRL fraction, consisting primarily of chylomicrons, is the main carrier for vitamin K_1. Consequently, the liver must be the primary destination of vitamin K_1. The presence of vitamin K_1, albeit in smaller amounts, in other lipoproteins suggests they may transport vitamin K_1 to the tissues.

1.10.3 Role of Apolipoproteins

Apolipoprotein E directs the fate and uptake of chylomicron remnants (CR). A number of codominant alleles have been found that result in one of six genotypes for apoE, namely E2/E2, E3/E3, E4/E4, E2/E3, E3/E4, E2/E4. The most common genotype is E3/E3, making up around two-thirds of most populations. Approximately one in three people carry the E2/E3 and E3/E4 genotypes, whilst the remaining combinations are very rare (Saupe *et al.,* 1993). Saupe *et al.* found that vitamin K_1 concentration in plasma of haemodialysis patients was related to apoE genotype in the order E2 > E3 > E4, such that individuals with the E2/E2 genotype had the highest vitamin K_1 plasma levels. Similar associations have been found with α-tocopherol and γ-tocopherol (Ortega *et al.,* 2005). Kohlmeier *et al.* reported that apoE genotype is a strong determinant of vitamin K_1 plasma status (Kohlmeier *et al.,* 1995b). Since chylomicron clearance is also slower in individuals with the E2/E2 genotype (Saupe *et al.,* 1993) this finding provides further evidence that chylomicrons are the major vitamin K_1 transporting particles. In contrast, a superior vitamin K_1 plasma status (and lower %ucOC) was reported in healthy older people with the apoE4 genotype in both Britain and China (Yan *et al.,* 2005). More recently, a study in 142 haemodialysis patients reported the opposite result; carriers of the apoE4 gene had the highest %ucOC (Pilkey *et al.,* 2007). These conflicting results are likely due to the relatively small sample sizes, at least for genetic work.

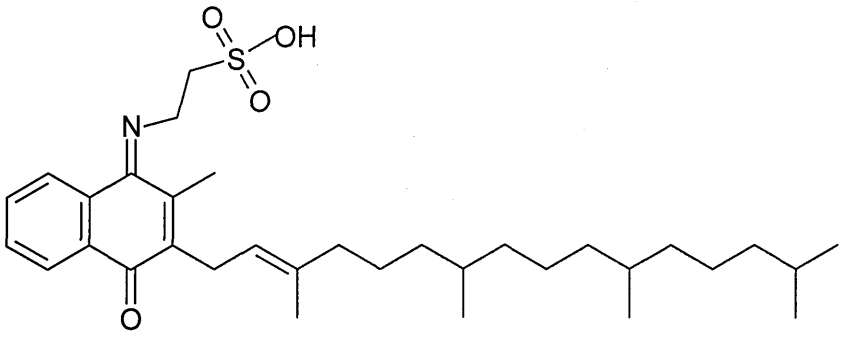
It has also been suggested that apolipoproteins A1 and B can be used to predict plasma concentrations of vitamin K_1 (and vitamin E) and might be important determinants of vitamin K_1 metabolism (Cham *et al.,* 1999). ApoA1 is associated with HDL and is involved in the transfer of cholesterol from tissues. ApoB is involved in chylomicron, VLDL and LDL metabolism and is present in two forms. ApoB100 is produced in the liver, and contains the LDL receptor-binding domain, and thus contributes to the hepatic and peripheral tissue uptake of LDL. ApoB48 is produced in intestinal mucosal cells, is incorporated into chylomicrons, and is involved in hepatic uptake of chylomicron remnants. ApoB48 lacks the part of the apoB100 molecule that is recognised by LDL

receptors so whereas LDL provides cholesterol to peripheral tissues, chylomicron remnants are largely cleared via apoE receptors in the liver (Welty *et al.*, 2004). As a result, the presence and distribution of apoB48 and apoB100 in lipoprotein particles may influence distribution of vitamin K_1 between the liver and extrahepatic tissues.

1.10.4 Vitamin K-taurine conjugate

Petrosian and Haroutounian (2000) have proposed an alterative hypothesis in which taurine forms a water-soluble conjugate with vitamin K (Figure 1-11) that permits a secondary mechanism for the transport of vitamin K and other fat-soluble vitamins. Their hypothesis is based largely on circumstantial evidence, at least for vitamin K, since high concentrations of taurine, with an unknown role, are found in blood platelets and may act as a transporter for vitamin K_1 (Petrosian & Haroutounian, 2000). Alternatively, the water-soluble conjugate may provide a mechanism for the uptake of menaquinones from the colon.

Figure 1-11. Postulated structure of vitamin K_1-taurine conjugate, 2-methyl-3-phityl-1,4,naphtochinolidine taurine. From Petrosian and Haroutounian, 2000



1.10.5 Vitamin K_1 transport and delivery

In contrast to the fat-soluble vitamins A and D, there appears to be no specific mechanism for the transport of vitamin K_1 to other tissues. It seems likely that vitamin K_1 transport is largely determined by the fate of lipoproteins, mainly chylomicrons and CR, and may be partly controlled by the distribution of apoE receptors on the surface of cell membranes (Kohlmeier *et al.*, 1996). The importance of other lipoproteins in the transport of vitamin K_1 is uncertain, although it has been suggested that in a state where vitamin K_1

intake is very low, LDL is the main carrier for vitamin K_1, implying that any vitamin K_1 stores in the liver could be mobilised and transported to other tissues via LDL (Olson *et al.,* 2002). Uptake of vitamin K_1 to the liver is probably highly effective, since functioning hepatic VKD proteins can be maintained even at low vitamin K_1 intakes and plasma status. This effective uptake is consistent with primary CR clearance by the liver (Hussain *et al.,* 1989). Booth *et al.* (2003b) have suggested that plasma vitamin K_1 is preferentially used for hepatic VKD proteins, and only for extrahepatic VKD proteins once liver stores are adequate.

In addition to the liver, bone is also believed to have a high requirement for vitamin K since it is necessary for the VKD proteins, osteocalcin and MGP. In contrast to those of most tissues, cells in bone (in addition to liver and spleen) are in direct contact with the blood and therefore bind lipoproteins in preference to other tissues that are separated from the blood via layers of endothelial cells (Kohlmeier *et al.,* 1996). It is postulated that vitamin K is transferred from circulating lipoproteins to the stromal and mesenchymal stem cells of bone marrow. From the marrow, these bone precursor cells migrate to sites of bone resorption where they form bone tissue. The migration times of these cells determine the rate at which the carboxylation of bone proteins can be influenced by vitamin K supplementation (Kohlmeier *et al.,* 1996). Animal experiments show that a significant proportion of chylomicron uptake is by bone (Hussain *et al.,* 1989). More recently, vitamin K_1-enriched CR (produced *in vivo*) have been used to demonstrate the uptake of CR by osteoblasts *in vitro*, primarily through interaction with LRP receptor and apoE. Furthermore, this study showed that once internalised, vitamin K_1 caused an increase in γ-carboxylation of osteocalcin (Niemeier *et al.,* 2005). Schurgers *et al.* (2002) have suggested, from data showing an inter-subject variability in the relationship between undercarboxylated Factor II and undercarboxylated osteocalcin, that there may be competition between liver and bone for vitamin K_1. Niemeier *et al.* (2005) also commented that LRP uptake of CR might be greater for osteoblasts than for hepatic cells.

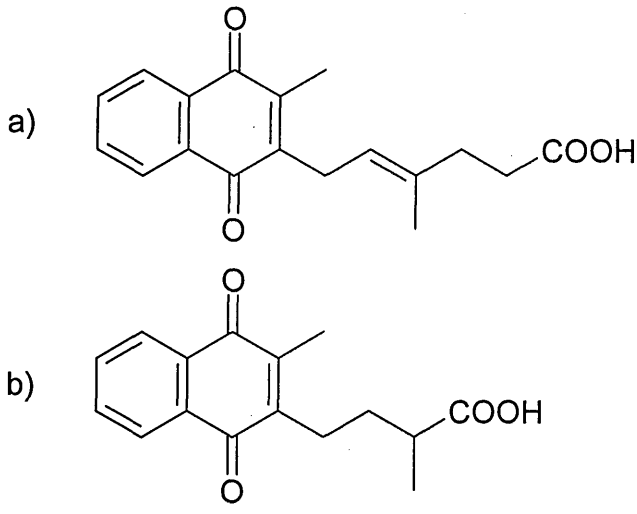
1.10.6 Summary of vitamin K_1 absorption and transport

Vitamin K_1 is absorbed from the small intestine in mixed micelles and is repackaged into chylomicrons before entering the circulation. The TAG-rich lipoprotein fraction is the main carrier of vitamin K_1. The TAG-rich lipoprotein fraction includes chylomicrons and chylomicron remnants that are the main carriers of vitamin K_1 in the post-absorptive state. This fraction also includes VLDL that may be important in the transport of vitamin K_1 to other tissues. Transport of vitamin K_1 to tissues appears to be at the fate of factors affecting the metabolism of chylomicrons and CR, including apoE genotype. Other meal constituents that affect lipoprotein metabolism may also influence the absorption and transport of vitamin K_1, and have consequences for the distribution of vitamin K_1 between hepatic and extrahepatic tissues. For example, a diet rich in PUFA stimulates higher lipoprotein lipase activity resulting in less hepatic uptake and potentially greater delivery to extrahepatic tissues. The importance of other lipoproteins in the transport of vitamin K_1 is unclear.

1.10.7 Catabolism and excretion

Work with radioisotopes (Shearer *et al.,* 1974; Olson *et al.,* 2002) showed that vitamin K_1 was rapidly catabolised to polar metabolites and excreted from the liver in both urine and faeces (from bile). The evidence suggests a greater proportion is excreted in bile than urine (Shearer *et al.,* 1974). Urinary metabolites have been identified as aglycons (Shearer & Barkhan, 1973; Shearer *et al.,* 1974; Harrington *et al.,* 2005) and recently, with the development of a method for their routine analysis, have been suggested as a non-invasive marker of vitamin K status (Harrington *et al.,* 2005) (Figure 1-12).

Figure 1-12. Structures of vitamin K urinary metabolites a) 2-methyl-3-(5'carboxy-3'-methyl-2'pentenyl)-1,4-napthoquinone (7C-aglycone) and b) 2-methyl-3-(3'-3'-carboxymethylpropyl)-1,4-naphthoquinone (5C-aglycone) (adapted from Harrington *et al.*, 2005)



An alternative pathway of vitamin K_1 metabolism has recently been suggested. Vitamin K_1 (and menaquinone-4 and –7) may be converted to menadione during uptake from the gut. It is postulated that menadione is a precursor to menaquinone-4 and is delivered to tissues that do not have a ready supply of vitamin K_1 (Thijssen *et al.*, 2006).

1.11 Vitamin K_1 kinetics and turnover

The study of nutrient kinetics provides information that, together with an understanding of the physiology, leads to a better understanding of metabolism and can contribute to the evidence-base for setting recommended intakes. Kinetic studies provide data on uptake, turnover, the number of kinetically distinct body pools, their masses, and their relative rates of turnover. Additionally, kinetic studies may provide data for more specific studies of nutrient absorption or metabolism; for example, duration and number of samples and dose levels (Gregory III & Quinlivan, 2002). Kinetics can be studied with unlabelled, radiolabelled or stable isotope labelled compounds. The relevance of unlabelled compounds is limited because it is impossible to distinguish between the endogenous nutrient and the dose, and their use is really only applicable to short-term studies using large doses. With a labelled compound, it is possible to measure unambiguously the tracer dose or its metabolites.

1.11.1 Uptake kinetics

Early tracer studies of vitamin K_1 kinetics were hampered by the lack of a suitable methodology to measure plasma levels of vitamin K_1. To overcome this problem, early studies used radiolabelled forms of vitamin K_1, often at pharmacological levels (Shearer *et al.,* 1972; Shepherd *et al.,* 1977; Bjornsson *et al.,* 1979). However, other studies have used more physiologically relevant doses (Shearer *et al.,* 1974; Olson *et al.,* 2002).

Each of the above studies used *iv* doses and describe the kinetics of vitamin K_1 uptake as a model with two compartment characteristics, comprising two exponential functions (relating to two $T_{½}$). Most describe an initial clearance with $T_{½}$ of around 0.5 h followed by second exponential with $T_{½}$ of around 3 h. In contrast, Olson *et al.* (2002) describe a much slower second exponential of 28 h that the authors ascribe to the different time-scales of the experiments (up to 10 h compared to 72 h). However, the differences are more likely a reflection of measurement specificity, since radioactivity in the study of Olson *et al.* (2002) was not categorically associated with vitamin K_1. A recent study using an unlabelled pharmaceutical preparation of vitamin K_1 (Konakion®) also describes two exponentials, one of "initial rapid decrease" followed by $T_{½}$ of 3 h. However, this study, which was investigating pharmacokinetics of Konakion®, used very large doses of vitamin K_1 (22 µM) (Soedirman *et al.,* 1996).

In the study of Olson *et al.* (2002), vitamin K_1 kinetics were measured in five individuals after either a control diet (75 µg vitamin K_1 per d for 1 – 2 wk) or a low vitamin K_1 diet (8 µg per d for 3 – 8 wk). Around 30% to 40% of the total dose was found in urine over 6 d and 30% or 13% in the stools of subjects consuming normal vitamin K_1 or a low vitamin K_1 diet, respectively. These data may suggest a possible mechanism for the retention of vitamin when status is low. Of the total dose, 30% remained unaccounted for after 6 d, suggesting storage in deeper stores such as bone or adipose tissue that are slow to turnover (Olson *et al.,* 2002).

1.11.2 Turnover

The often-quoted 'rapid turnover' of vitamin K_1 largely derives from work by Usui *et al.* (1990) who measured changes in plasma and hepatic vitamin K_1 in 22 patients after either 3 d of vitamin K_1 restriction (5 μg) or a normal hospital diet. Assuming the adult liver weighs 1.2 kg, then the liver pool size of vitamin K_1 decreased from 33.6 nmol (15 μg) to 8.2 nmol (4 μg) within 3 d (Usui *et al.*, 1990). Olson (1999) calculates this decrease to be equivalent to a $T_{½}$ of 1.5 d, which is a turnover time of 2.2 d. The interpretation of turnover values is often complicated by the different expressions. Thus,

Fractional turnover constant (rate of exit of material from the pool)

$= k = 0.693/T_{½}$

Absolute turnover rate (amount of material moving in or out of the pool per unit time)

$= a$ = pool size x k

Turnover time (time required for a quantity of material equal to the pool size to move in and out of the pool)

$= T_t = 1/k = T_{½}/0.693$

Calculation of the turnover time (from the reported $T_{½}$ of the second, slower exponential) from a number of tracer studies provides values that fall into two groups. Firstly, values in the region of 2.6 to 4.9 h (Shearer *et al.*, 1972; Shearer *et al.*, 1974; Shepherd *et al.*, 1977; Bjornsson *et al.*, 1979), and secondly, a value of 39.7 h (Olson *et al.*, 2002) that is consistent with that calculated from Usui *et al.* (1990). Two studies of the clearance of pharmacological doses (22 μM) of vitamin K_1, also reported values that fall into these two groups; 4.3 h (Soedirman *et al.*, 1996) and 36.5 h (Pereira *et al.*, 2005), although the latter study was performed in patients with acute liver dysfunction. Shorter sampling times and high doses have been shown to generate shorter turnover times (Olson *et al.*, 2002). In the study of Olson *et al.* (2002), the slopes of the 2nd exponential of plasma disappearance matched those of the lines for decay of urinary metabolites,

suggesting that both represent the true turnover rate. Furthermore, these are similar to the rates of excretion of urinary metabolites observed by Shearer *et al.* (1972; 1974). However, since Olson *et al.* (2002) did not measure radioactivity unequivocally associated with vitamin K_1, it is likely that the turnover time reflects that of excretory metabolites, as measured in the studies of Shearer *et al.,* as well as menadione (Thijssen *et al.,* 2006). However, the ability to calculate turnover rates from earlier studies was questioned because of the absence of a method for vitamin K_1 quantitation and flooding of the body pool with high doses (Shearer & Barkhan, 1979).

1.11.3 Body pools

Direct tissue analysis can provide some information on vitamin K body stores but analysis in tissues other than plasma is difficult in humans. Liver is a major body store of vitamin K, although only 10% is vitamin K_1, the remainder consisting of menaquinones of various chain lengths (Shearer *et al.,* 1988). Adult values for vitamin K_1 liver stores have been estimated as between 10 and 30 nmol/kg (wet weight) (Shearer *et al.,* 1988; Usui *et al.,* 1990; Thijssen & Drittij-Reijnders, 1996). Bone also has been identified as a major body pool of vitamin K_1 with values in region of 10 nmol/kg (dry weight) (Hodges *et al.,* 1993) and other organs (e.g. heart and pancreas) may make a significant contribution to total vitamin K_1 body stores (Thijssen & Drittij-Reijnders, 1996).

It was not possible to calculate pool sizes from early tracer studies because accurate and sensitive methods did not exist for the quantitative measurements of vitamin K_1. In the study by Olson *et al.*, total body pool size was estimated to be between 17 and 195 µg (38 - 433 nmol (0.3 – 2.2 µg/kg; 0.7 - 4.9 nmol/kg) prior to vitamin K_1 restriction. The pool sizes of vitamin K_1 are believed to be relatively small, especially when compared to the other fat-soluble vitamins (vitamin D, 5 µg/kg; vitamin A, 5000 µg/kg and vitamin E, 40000 µg/kg) (Olson *et al.,* 2002).

1.11.4 Summary of kinetic work

A number of conclusions can be drawn from the above work. Firstly, the evidence suggests the existence of at least two body pools, an initial pool (that can be identified physiologically as plasma) and a second pool (perhaps represented by the liver and other body stores). However, the observation that a significant amount of radioisotope remained in the body after 6 d (Olson *et al.,* 2002), and evidence from tissue measurements, may suggest the existence of a third body pool with slow turnover that has not been identified in studies to date. The apparent absence of active transport of vitamin K_1 to extrahepatic tissues may result in slow kinetics that are difficult to detect given the low plasma concentration and body stores of vitamin K_1. The available evidence from kinetic work and earlier studies on the effect of vitamin K depletion, suggests a relatively fast turnover and limited body stores of vitamin K_1, in contrast to other fat-soluble vitamins.

1.12 The measurement of bioavailability

Bioavailability is commonly assessed through comparison of circulating levels of the compound of interest hours after an oral and an *iv* dose. This approach is often not possible in nutrition research because the nutrient already exists within the measured pool; the use of isotopically labelled compounds however does allow such comparisons. The assessment of relative bioavailability is the common approach, as illustrated in studies that have measured vitamin K bioavailability from foods (Gijsbers *et al.,* 1996; Booth *et al.,* 1999a; Garber *et al.,* 1999; Schurgers & Vermeer, 2000; Booth *et al.,* 2002). Unfortunately, this approach does not readily permit comparisons between different studies because it is not known how much of the nutrient has been absorbed.

1.12.1 Marker selection

When quantifying the bioavailability of vitamin K, a number of markers of status could be measured e.g. plasma vitamin K_1, %ucOC etc. In the studies presented in this thesis, the primary outcome is how much of the vitamin is absorbed from a food or meal and thus the measurement of plasma vitamin K_1 is the most suitable direct measure. The

other markers may be more suitable for longer-term studies where the chronic intake is more related to whole-body status.

1.12.2 Plasma response

Assuming a linear relationship, the amount of vitamin absorbed is reflected in the rise in plasma concentration. This method is applicable to relative bioavailability measurements provided the study is performed over a sufficient period of time to ensure a new steady-state between test doses (Parker *et al.,* 1999). However, this proviso is more relevant to other fat-soluble vitamins than for vitamin K_1 due to their slower turnover.

Plasma responses can be measured after single or multiple doses. Relative absorption of a supplement source of vitamin K and of vitamin K from meals has been measured in a number of single dose experiments (Gijsbers *et al.,* 1996; Booth *et al.,* 1999a; Garber *et al.,* 1999; Schurgers & Vermeer, 2000; Booth *et al.,* 2002). Plasma levels over a 5-d period using a multiple-dosing method has also been applied in the comparison of oil and broccoli diets (Booth *et al.,* 1999a). Although relatively simple, the methodology is limited because plasma response is a function of not only absorption, but also breakdown, tissue uptake and release from body stores. Additionally, it is likely that the large doses used in some of these studies may not behave kinetically in the same way as physiological doses (Yeum & Russell, 2002).

When assessing the absorption of a nutrient, it is common to measure the area under the curve (AUC) as a measure of plasma concentration plotted against time. The mass of the nutrient absorbed is proportional to the area under the curve. Other parameters derived from the curve are the maximum plasma vitamin K_1 concentration (C_{max}) and the time of peak concentration (T_{max}). In studies of nutrients, it is usually necessary to correct the area for the baseline values, although not if a labelled tracer is used. The methodology is best suited to measuring the effects of single doses and has been applied in a number of studies of vitamin K_1 bioavailability (Gijsbers *et al.,* 1996; Booth *et al.,* 1999a; Garber *et al.,* 1999; Schurgers & Vermeer, 2000; Booth *et al.,* 2002).

1.12.3 Post-prandial chylomicron response

In the field of vitamin A research, a method whereby only carotenoids in chylomicrons (or rather the TAG-rich lipoprotein fraction (density <1.006 kg/L) are measured, has been developed (PPC – post-prandial chylomicron response). The rationale behind the method is that, while LDL and HDL transport endogenous carotenoids, newly absorbed carotenoids (as with vitamin K_1) enter the circulation and are transported to the liver in chylomicrons and chylomicrons remnants. Thus, carotenoids in these fractions are primarily exogenous in origin. Although the method could be applied to vitamin K research, it does have a number of short-comings, as outlined by Parker *et al.* (1999). Firstly, since chylomicrons are rapidly metabolised by the action of lipoprotein lipase, CR of a range of sizes are present. As a result, recovery of TAG-rich lipoprotein fraction may vary through differences resulting from centrifugation. The second problem is that during absorption, chylomicron production and clearance are highly variable between individuals (partly due to apoE genotypes). This inter-individual variation makes it difficult to discern differences in absorption (Parker *et al.*, 1999). However, the same would be true for any method that measures fat-soluble vitamin absorption.

1.12.4 Faecal balance studies

In oral-faecal balance studies, the amount absorbed is calculated by the amount ingested minus the amount recovered in the faeces. This method suffers from a number of potential inaccuracies. Firstly, no account is made for losses due to bacterial degradation in the gut. Secondly, there is a lack of discrimination between the amount from normal diet and from the test meal. Thirdly, the results could be largely influenced by inter-individual variation in transit time through the gut. This method has only been applied to vitamin K research with the use of a radiolabelled dose (see section 1.12.6) therefore reducing errors from endogenous contribution.

1.12.5 Depletion / repletion methods

The use of depletion/repletion methods was discussed earlier (section 1.6) in relation to intake and status. The major disadvantage of these studies is the required long stay in a metabolic unit that is both costly in terms of time and money. As discussed previously (section 1.7.1.1), the results from these studies have provided conflicting results as to the dose-response relationship. As with the other methods, only data on relative bioavailability is obtained.

1.12.6 Radioisotopes

Shearer *et al.* (1970a) used [1',2'-$^{3}H_2$] vitamin K_1 and faecal balance measurements to measure the absorption of vitamin K_1 with a meal and concluded that 40 – 50% of the oral dose of vitamin K_1 was absorbed. Later, on the basis that much of the observed faecal radioactivity was associated with metabolites, the estimate of absorption was increased to 80% (Shearer *et al.,* 1974). This study remains the only assessment of absolute vitamin K_1 absorption. Radiolabelled compounds can act as tracers and allow the detection of low amounts of vitamin, however due to perceived health risks, as well as ethical, regulatory and disposal challenges, their use is no longer favoured.

1.12.7 Stable Isotopes

The use of stable isotopes for the measurement of nutrient absorption and metabolism has grown rapidly as the equipment to separate and quantify stable isotopes has become more economical and the availability of labelled compounds has improved. In the fields of vitamin A and E research, the use of stable isotope methodologies is relatively well established but only recently have they been applied to questions of vitamin K metabolism. Stable isotopes have a number of advantages over other methods. Firstly, stable isotopes are safe for use in all population groups. Secondly, it is possible to distinguish between the labelled dose and endogenous molecules and thirdly, it is possible to use low physiological doses that are typical of amounts in food and that do not perturb normal kinetics. Finally, with stable isotopes, it is possible to administer two

labelled forms simultaneously that permits measurement of, for example, absorption and kinetics. In general, there are two approaches for labelling of compounds; intrinsic labelling describes the method of labelling a molecule *in situ*, whereas an extrinsic labelled molecule is synthesised chemically.

1.12.7.1 Intrinsic labelling

Three published studies have used stable isotope methodologies in order to measure vitamin K_1 absorption from food. All have chosen to use intrinsically labelled vegetables. Intrinsic labelling involves growing a plant in isotopically-enriched environment so it takes up labelled precursors. The technique can be applied to both inorganic, e.g. Fox *et al.,* (1991), Harvey *et al.,* (2005) and organic molecules, e.g. Dolnikowski *et al.,* (2002). For organic analysis, deuterated water and ^{13}C labelled CO_2 have been used to label molecules of interest. The use of labelled CO_2 may be preferable since deuterium enrichment over 50 atom percent excess can be deleterious to plant growth and thus only partial labelling can be achieved (Kurilich *et al.,* 2003). The major advantage of intrinsic labelling is that the molecule of interest is labelled and contained within a matrix as it is usually eaten. The converse of course is that the number of foods that can be labelled in this way is limited. A further complication to intrinsic labelling is since there is no control over the extent or position of labelling, multiple isotopomers are formed. The location of the heavier atoms is apparently random, the heavier atoms may be more readily exchanged in chemical reactions or lost during GCMS analysis by fragmentation of the molecule. These problems can, to some extent, be overcome by the use of gas chromatography-combustion-isotope ratio mass spectrometry which quantitatively combusts the molecule after chromatographic separation and prior to analysis by isotope ratio mass spectrometry. This method has better sensitivity and precision for the measurement of isotope ratios but sensitivity in terms of sample concentrations is at least 1000 times lower than that afforded by the use of GCMS (Bier 1997). For vitamin K_1 this consideration is important since concentrations in plasma are low.

1.12.7.2 Application of intrinsic labelling in human feeding studies

The first report of the use of intrinsic labelling applied to vitamin K_1 research was from Dolnikowski and colleagues (Dolnikowski *et al.,* 2002). In this study, broccoli plants were grown hydroponically with the addition of deuterated water during growth of broccoli heads. A single, 23 y old male volunteer took part in the study and was given a breakfast of 115 g of labelled broccoli (containing 168 μg of vitamin K_1 after steaming) together with further food consisting of 850 kilocalories and 31% energy from fat. Blood samples were taken hourly to 5 h, then every 30 min until 8 h and finally at 10, 12, 16, 20 and 24 h. Measurement of labelled vitamin K_1 from broccoli by GCMS gave an isotopomer profile from m/z 452 to m/z 467. The most abundant isotopomer was m/z 458 which corresponded to 14.1% of total labelled vitamin K_1. The pattern of isotopomer proportions was an approximate normal distribution. After 5 h, the isotopomer profile was a mixture of both unlabelled endogenous vitamin K_1 and vitamin K_1 from the labelled broccoli. Because of the limitations of GCMS, not all the heavier ions could be measured except in those samples with the greatest concentration of vitamin K_1. Thus, at the later time points the abundance of the isotopomers that could not be measured were fixed at their ratio to the larger isotopomers in the samples where they could be measured. The authors of this study reported that the labelled vitamin K_1 appeared at 2 h in plasma and that peak enrichment was around 80% between 4 and 6 h. The high levels of enrichment are likely due to the low plasma concentration in the single subject. Interestingly, after a low-vitamin K_1 meal at 5.5 h, total plasma K_1 peaked again at 8 h post-dose although there was no rise in labelled vitamin K_1. These data suggest a) a rise in the label was undetected by GCMS, b) labelled vitamin K_1 had not mixed with endogenous vitamin K_1 and was not released during the post-prandial rise in TAG, or c) that vitamin K_1 in the 5.5 h meal was highly bioavailable and caused a spike in the unlabelled vitamin K_1 plasma concentration.

Later the same group applied this methodology to the study of vitamin K_1 transport in five volunteers after a dose of intrinsically labelled collard greens (Erkkilä *et al.,* 2004).

This study was described in section 1.10.2 in more detail. Vitamin K_1 concentrations in plasma were measured by HPLC and isotope ratios by GCMS.

More recently liquid chromatography mass spectrometry (LCMS) has been applied to the measurement of carotenoids and vitamin K_1 (Kurilich *et al.,* 2003). In this study, kale was labelled with $^{13}CO_2$ by growing the plant in a sealed acrylic box. A single volunteer consumed 400 g of kale (containing 156 nmol (≈ 70 μg) of vitamin K_1) with 30 g of peanut oil. Blood samples were collected hourly during the first day, daily during the first week and then biweekly for the next 5 wk. The analysis of isotope ratios was performed by LCMS with atmospheric pressure chemical ionisation. In this technique, the eluent from the LC is sprayed through a heated vaporiser at atmospheric pressure. The solvent ions are then ionised and transfer the charge to the analyte molecules in a similar way as methane molecules are used in GCMS with chemical ionisation. The base peaks for unlabelled and labelled vitamin K_1 were m/z 451 (due to the additional proton) and m/z 482, respectively, m/z 482 corresponding to the fully labelled vitamin K_1 molecule. In other words, all of the carbon atoms in vitamin K_1 were ^{13}C atoms ($^{13}C_{31}$). The isotopomer proportions were $^{13}C_{31}$ 55%, $^{13}C_{30}$ 27%, $^{13}C_{29}$ 9%, $^{13}C_{28}$ 4% and 0.6% unlabelled.

The bioavailability of vitamin K_1 was estimated at 7%, calculated by multiplying labelled plasma vitamin K_1 concentration by estimated plasma volume (assuming 45 mL plasma per kg body wt) and then dividing this value by total deuterated dose. Using this method, the bioavailability results for three of the subjects from the study by Errikila *et al.* (2004) are 4.7, 6.1 and 4.6% and from Dolnikowski *et al.* (2002) 1.7% (assuming 70 kg body weight since no details are provided).

1.13 Determinants of vitamin K bioavailability

This final section of the introduction considers the determinants of vitamin K bioavailability. A particular emphasis is on dietary factors; including matrix effects, the modulating effects of fat, and inhibitors of bioavailability. Also discussed is the potential contribution to vitamin K sufficiency of both dietary, and endogenous menaquinone production.

1.13.1 Vitamin K_1 isomers

Due to rotation around the double bond of the phytyl chain side-chain, vitamin K_1 exists in two configurations, the *cis*- and the *trans*-isomers. Naturally-occurring vitamin K_1 is found exclusively as the *trans*-isomer but synthetic forms may contain a certain proportion of the *cis*-isomer. The *cis*-isomer may be found in certain foods and supplements due to the addition of the synthetic form. Additionally, the *trans*-isomer can undergo photo-isomerization on exposure to light (Woollard *et al.,* 2002).

Work in rats has shown that although the *cis*-isomer was absorbed it did not have any biological activity as measured by levels of prothrombin. Rats receiving the *cis*-isomer only showed the same prothrombin as vitamin K-deficient control rats, whereas those supplemented with *trans*-vitamin K_1 had normal prothrombin concentrations (Knauer *et al.,* 1975). The authors also demonstrated with ^{3}H-labelled compounds, hepatic retention of the *cis*-isomer, indicating it may not be metabolically active. It is suggested that the *cis*-isomer is unable to act as a substrate to vitamin K epoxidase (Knauer *et al.,* 1975). Other work in rats has shown that the *cis*-isomer had only 1% of the activity of the *trans*-isomer for Factor VII. *In vitro* work has demonstrated that the carboxylase enzyme has no activity with the *cis*-isomer (Suttie 1985).

Since the *cis*-isomer is effectively an inactive form of vitamin K, it is important to consider its contribution to vitamin K intake and adjust recommended values as necessary. A number of foods have been shown to contain appreciable amounts of the *cis*-isomer including oils (10 – 20%), vegetables (<1%) and processed foods (1 – 12%) (Woollard *et al.,* 2002). A recent vitamin K_1 supplementation study reported that the *cis*-isomer accounted for around 12% of vitamin K_1 in tablets (Schurgers *et al.,* 2004).

1.13.2 2',3'-dihydro-vitamin K_1

To improve their shelf-life and other characteristics for food manufacture, vegetable oils are commonly hydrogenated. Hydrogenation of vitamin K_1 results in the saturation of a single 2',3' double bond in the side chain and conversion to 2',3'-dihydro-

vitamin K (Davidson *et al.,* 1996). Two of commonly hydrogenated oils are soybean and rapeseed oil, both good sources of vitamin K (Erkkilä *et al.,* 2005b).

Evidence from work in rats suggests that dihydro-vitamin K_1 is well absorbed and is biologically active, for example reversing warfarin-induced hypoprothrombinaemia (Sato *et al.,* 2003). Dihydro-vitamin K_1 is absorbed by humans and does not appear to affect absorption of vitamin K (Booth *et al.,* 1996a). In humans, a study of the bioavailability of hydrogenated vitamin K_1, as measured by changes in PIVKA-II and ucOC after feeding with either vitamin K_1 or dihydro-vitamin K_1 following vitamin K depletion, showed that dihydro-vitamin K_1 had less biological activity to PIVKA-II and no biological activity to the extrahepatic VKD, osteocalcin (Booth *et al.,* 2000a). These differences are likely due to differences in absorption since plasma concentrations of both forms of the vitamin increased in response to 200 µg/d repletion but the increase in vitamin K_1 was significantly greater than that of dihydro-vitamin K_1 (Booth *et al.,* 2000a).

Dihydro-vitamin K_1 is present in a large range of processed foods (Dumont *et al.,* 2003) and thus it is important to consider its contribution to vitamin K intake and status. This form of vitamin K is thought to make up about 20% of vitamin K_1 intake in the US, with mean intakes for men and women estimated at 19 and 15 µg/d, respectively (Booth *et al.,* 1999b). Dihydro-vitamin K_1 has also been related to elevated ucOC (Erkkilä *et al.,* 2005b). Plasma dihydro-vitamin K_1 has been shown to correlate with both the intake of dihydro-vitamin K_1 and *trans*-fatty acids (mainly from hydrogenated oils) (Erkkilä *et al.,* 2005b). The limited evidence suggests that dihydro-vitamin K_1 may have biological activity but absorption is reduced compared to vitamin K_1. The potential impact of hydrogenated vitamin K_1 is largely unknown but could result in a decrease in vitamin K status. However, recent campaigns for the reduction in the consumption and use of hydrogenated oil in foods may reduce any longer-term concerns.

1.13.3 Menaquinones

Menaquinones (MK-*n*), collectively also known as vitamin K_2, contain a polyisoprenoid side chain of varying length (section 1.1.2). Menaquinones do have

biological activity and can act as co-factors for the γ-glutamyl carboxylation of glutamic acid (Suttie 1995). There are two potential sources of menaquinones, the diet and intestinal bacteria. One exception is MK-4 that can be synthesised from vitamin K_1 and may exert its effect by a pathway additional to carboxylation. The relative importance of menaquinones to vitamin K status remains a relatively unresolved issue and in particular, the relative importance of gut bacteria-derived menaquinones.

1.13.3.1 Dietary menaquinones

Dietary sources of menaquinones are typically fermented food or foods of animal origin. The menaquinone content of fermented foods is thought to derive from the bacterial starter cultures (Shearer 1997) and includes cheese and natto (fermented soybean product). Animal products are another source of menaquinones, especially liver (MK-7 – MK-13). The presence of the short chain menaquinone MK-4 in animal products (cheese, meat, eggs etc.) may be partly due to conversion from the animal food supplement, menadione (Schurgers & Vermeer, 2000). In the typical Western diet, the menaquinones contribute a small proportion of total vitamin K intake (Booth & Suttie, 1998). In a Dutch population, based on a food frequency questionnaire, it was concluded that around 90% of total dietary intake derives from vitamin K_1 (Schurgers *et al.,* 1999).

The potential to absorb menaquinones of dietary origin has been demonstrated in animal models and in human studies. In rats, the injection of ^{14}C-labelled vitamin K into the jejunal loop resulted in absorption values of MK-4, 17%; MK-9, 15% and vitamin K_1, 13% (Uchida & Komeno, 1988). Experiments in humans studying absorption efficiency from different foods of menaquinones MK-7, MK-8 and MK-9, have shown that menaquinones reached a maximum plasma level 10-fold greater than vitamin K_1 (Schurgers & Vermeer, 2000). Subsequent work measuring the absorption of pure vitamin K_1, MK-4 and MK-9 dissolved in oil however, found greater serum levels of vitamin K_1 compared to the menaquinones (Table 1-6). The distribution between lipoprotein fractions and clearance were also very different between vitamin K vitamers. Vitamin K_1 was primarily associated with TRL fraction, whereas MK-4 was equally distributed

between TRL, LDL and HDL. No MK-9 was found in HDL (Schurgers & Vermeer, 2002). The authors conclude that these differences may be due to the different lipophilicity of the vitamin K forms that in turn affect both plasma transport and delivery to target tissues. This work suggests that the dietary menaquinones may in fact contribute to vitamin K status in certain tissues (Schurgers & Vermeer, 2002).

Table 1-6. Comparisons of the appearance of vitamin K_1, MK-4 and MK-9 in plasma following consumption of a vitamin K_1- and menaquinone-enriched meals. Data from Schurgers & Vermeer (2002)

	C_{max} (nmol/L)	T_{max} (h)	Estimated $T_{½}$ (h)
Vitamin K_1	40	4	12
MK-4	17	2.5	12
MK-9	9	5	60

1.13.3.2 Absorption of menaquinones from gut bacteria

Relatively few of the normal intestinal bacteria are major producers of menaquinones (Suttie 1995). The major menaquinone produced in *E. coli* was reported as MK-8 while other bacteria produced mainly MK-10 and MK-11 and less MK-6 -7, -8 and -9 (Conly *et al.,* 1994). It has been estimated that gut flora could provide a potential reservoir of up to 4.5 mg of menaquinones (Conly *et al.,* 1994). Absorption of menaquinones from the gut has been inferred from their presence in liver and other tissues. Liver stores are thought to contain around 90% menaquinones and 10% vitamin K_1 (Shearer *et al.,* 1988; Usui *et al.,* 1990). Bone tissue has been shown to contain, in addition to vitamin K_1, MK-6 to MK-8 (Hodges *et al.,* 1993). As discussed in section 1.6.2 some early vitamin K_1 depletion studies showed little or no effect on prothrombin time and were taken as evidence for the importance of menaquinones. However, and as discussed previously, prothrombin time is a relatively insensitive marker of vitamin K status. Other studies, that used more sensitive makers of vitamin K status have created a sub-clinical deficiency with low levels of dietary vitamin K_1. Clinical reports of antibiotic-induced vitamin K deficiency were also taken as evidence for the importance of bacterially-derived

menaquinones. However, it has been demonstrated that certain antibiotics can disrupt the vitamin K cycle by inhibition of vitamin K epoxide reductase. Furthermore, in many cases vitamin K deficiency can be attributed to poor dietary intakes in seriously ill patients (Suttie 1995). The potential importance of menaquinones, rather than being based on the assumptions above, should be assessed on their function and metabolism, and is largely determined by their bioavailability. The bioavailability of menaquinones is dependent on a number of factors including release from bacteria, uptake from the gut and into the circulation, and utilisation in tissues.

In bacteria, menaquinones are tightly bound to the cytoplasmic membrane (Conly *et al.,* 1994). There is some evidence that both water-soluble and lipid-soluble forms of menaquinones can be released from the bacteria although the majority of this research has been performed with *in vitro* cultures of menaquinone-producing and menaquinone-requiring bacteria (Conly *et al.,* 1994).

In humans, the highest concentration of menaquinones is found in the colon with lower amounts in the terminal ileum. Bile-mediated absorption of colonic menaquinones is unlikely due to the lack of bile in this region of the gut (Shearer 1997). It has been demonstrated in humans that after oral administration, bacterially extracted menaquinones can be absorbed and impact vitamin K status (decreased prothrombin time) (Conly & Stein, 1992). However, the relevance of this experiment must be questioned since oral intake of gut bacteria is not representative of the physiology. Later, the same group used a similar protocol but delivered menaquinones directly to the ileum using a naso-ileal tube. Similarly, they observed an increase in Factor VII and a decrease in prothrombin time and suggested that absorption of menaquinones was possible from the distal small bowel (Conly *et al.,* 1994). It can be summarised that absorption may be possible in the terminal ileum where there are reasonable concentrations of bile salts to mediate absorption (Shearer 1997).

1.13.3.3 Utilisation

In humans, menaquinones constitute the majority of liver vitamin K stores, although vitamin K_1 is the major circulating form. Although MK-6 – 12 are present in liver (Shearer *et al.,* 1988; Thijssen & Drittij-Reijnders, 1996), only MK-4, MK-7 (Tsugawa *et al.,* 2006) and MK–8 have been positively reported in the circulation of normal individuals (Suttie 1995; Shearer *et al., 1988*). Interestingly, the abundance of MK-4, -5, -6 and –7 in bone lipid was found to correlate with decreasing side-chain length (Shearer 1997), further supporting the importance of lipophilicty in distribution of menaquinones. Although certain tissues contain high levels of menaquinones, some evidence suggests that they may not be available for γ-glutamyl carboxylation (Vermeer *et al.,* 1995). In studies in rats, both conventional and germ-free animals showed signs of vitamin K deficiency after 3 d restriction, and liver stores of menaquinones were not decreased (Uchida & Komeno, 1988). In human studies also, the presence of significant liver and bone stores of menaquinones have proved insufficient to prevent signs of vitamin K deficiency (Suttie *et al.,* 1988b).

1.13.3.4 Menaquinone summary

Considerable debate surrounds the question of the relative importance of menaquionones in maintaining vitamin K status. Dietary menaquinones make up only a small percentage of total dietary vitamin K but absorption does appear greater than vitamin K_1 and thus they may provide an additional source of vitamin K. However, the contribution of menaquinones from gut bacteria is probably very small since bioavailability from the gut is low. In addition, the absorption and tissue distribution of menaquinones appears to be related to the length of the isoprenoid side chain and long chain menaquinones appear to accumulate in the liver where they may not be accessible for carboxylation. The situation is different for MK-4 for which there is evidence for tissue specific accumulation (Thijssen *et al.,* 2002), predominantly from the conversion of dietary vitamin K_1 (Thijssen *et al.,* 2006), although the biological activity of MK-4 may be related to a mechanism other than γ-glutamyl carboxylation (Shearer 1997).

1.13.4 Matrix effects

Only a small number of studies using a range of techniques have explored the bioavailabilty of vitamin K_1 from different food sources. These studies have only compared the relative absorption of vitamin K_1 and not absolute absorption.

Three studies have compared absorption parameters after consumption of different test meals or foods. Gjisbers and colleagues (Gijsbers *et al.,* 1996) measured AUC over 24 h (sampling every hour to 10 h and then at 24 h) of 1 mg (2.2 μM) vitamin K_1 as either detergent-solubised concentrate (Konakion®), 227 g boiled spinach with 25 g butter or 227 g boiled spinach only. The crossover study was performed in three men and two women, aged 25 – 45 y. AUC was calculated after subtraction of baseline plasma vitamin K_1 values. Time to peak absorption (T_{max}) was faster for Konakion® compared to either of the meals (4.5 h compared to 6 h), presumably due to extraction efficiency of vitamin K_1 from spinach but possibly influenced by other components present in spinach. The addition of butter with the spinach meal increased both the maximum plasma vitamin K_1 concentration (C_{max}) and AUC compared to spinach alone. In the five subjects, vitamin K_1 from spinach with butter was 26% as available as Konakion® whereas, vitamin K_1 from spinach only was 4% as available. These results suggest a substantial improvement in absorption when fat is consumed with a meal as might be expected considering the uptake mechanism of vitamin K_1. It needs to be noted that the amounts of vitamin K given were high compared to normal intakes. Based on the assumption that the standard Konakion® used in this study had the same absorption as that recorded by Shearer *et al.* (1974) then the authors estimate that 10% of vitamin K_1 from the spinach was absorbed (Gijsbers *et al.,* 1996).

A later study by Garber *et al.* (1999) compared AUC, C_{max}, and $T_{max,}$ of vitamin K_1 absorption from a range of sources. The results from this study are summarised in Table 1-7. Values were measured over 9 h in subjects aged 22 to 30 y. Each source of vitamin K_1 (either spinach, broccoli or lettuce) was consumed with a test meal (one Egg McMuffin® and 240 mL of orange juice) containing 1682 kJ and 27% fat. As in the previous study, AUC were calculated after subtraction of the baseline plasma vitamin K_1

values. In agreement with Gjisbers *et al.*, (1996) absorption from pure vitamin K_1 was greater and faster than from the test meals, the equivalent amount of vitamin K_1 from spinach was only 17% as available as that from a tablet. Using AUC values there was no significant difference between fresh and cooked broccoli and no difference in absorption from lettuce with either 30% or 45% fat (adjusted with corn oil). However, mean absorption from a high-fat lettuce meal reached a higher concentration (2.2 nmol/L compared to 3.7 nmol/L) and was more slowly absorbed than vitamin K_1 from a low-fat lettuce meal. Together with the study by Gijsbers *et al.* (1996) these data suggest that fat is important for the maximal absorption of vitamin K_1, yet the amount of fat may be less important. There was no significant difference found in absorption between the three vegetable sources. These results should be observed with caution since the numbers in each group were very small, mostly comprising only two or three individuals. Additionally it is difficult to compare with other studies since no description of the vitamin K_1 tablet is provided.

Table 1-7. Mean (± SD) absorption parameters of vitamin K_1 from different sources in humans. From Garber *et al.*, (1999)

				Plasma vitamin K_1		
Source of vitamin K_1	Energy from fat (%)	VitaminK_1 dose (μg)	*n*	C_{max} (nmol/L)	T_{max} (h)	AUC (nmol/L.h)
Tablet	27	500	8	9.4 ± 4.7	2.4 ± 0.5	27.6 ± 10.1
Fresh spinach, 150 g	25	495	3	2.9 ± 0.5	4.0 ± 0.0	4.8 ± 1.1
Fresh spinach, 50 g	26	165	4	2.1 ± 0.6	3.5 ± 1.0	2.5 ± 1.1
Fresh broccoli, 150 g	30	214	2	2.1 ± 0.8	5.5 ± 2.1	4.0 ± 0.7
Cooked broccoli, 150 g	30	184	3	2.6 ± 1.6	2.7 ± 0.6	6.3 ± 4.4
Fresh romaine, 200 g	30	179	3	2.2 ± 0.6	5.7 ± 1.2	3.2 ± 1.0
Fresh romaine, 200 g	45	179	3	3.7 ± 1.1	7.7 ± 1.2	4.3 ± 2.0

Schurgers & Vermeer (2000) have compared vitamin K_1 absorption from spinach (400 g) with and without fat (corn oil, 30 g) to absorption from a supplement. Plasma values were measured hourly up to 12 h and then at 24, 48 and 72 h. To correct for baseline values, each volunteer was provided with a vitamin K_1-poor breakfast and these

baseline values were subtracted from the post-test meal values. Each meal contained 3.5 μM (1.6 mg) of vitamin K_1. Unfortunately, few quantitative data are provided but it was shown that T_{max} was faster for the Konakion® meal (4 h) compared to the vegetable meals (6 h). Absorption of vitamin K_1 from vegetables without fat was 5 – 10% compared to absorption from Konakion®, whereas consumption with fat, increased absorption to 10 – 15%. However, this study used very high doses of vitamin K_1 of over 1 mg.

Booth *et al.* (1999a) applied a multiple dosing methodology in a crossover study. During three, 15-d residency periods, 36 subjects consumed a mixed baseline diet containing around 100 μg/d of vitamin K_1. On days 6 to 10 of two of the residency periods, and in addition to the baseline diet, the subjects received two servings per day of either broccoli or vitamin K_1-enriched corn oil. The diets contained around 417 and 377 μg/d, respectively. Plasma vitamin K_1 was measured on days 1, 2, 4, 6, 7, 9, 11, 12, 14, and 16. Using single plasma values, there were significant increases in plasma vitamin K_1 from baseline to both test diets in younger and older subjects. However, on day 11 (final day of test diet), there were no differences between the broccoli and oil diets. AUC data over 5 d also showed a significant increase in both the broccoli and oil diets compared to baseline diet in both younger and older groups. However, only in the older group was there a significant difference between the oil and broccoli diets. This study also measured osteocalcin, %ucOC and urinary Gla. Both diets reduced %ucOC by around 10% (between day 6 and day 11) compared to the baseline diet, but there was no difference between the two diets. There were no significant differences between the three treatments in urinary Gla excretion.

As part of this study, the authors also measured plasma vitamin K_1 concentrations over a 24 h period on day 6 (Booth *et al.,* 1999a) corresponding to the first day of the test diets as mentioned above (baseline, broccoli or oil) (Booth *et al.,* 2002). Table 1-8 summarises the different methods used to assess bioavailability and the observed differences. AUC values over 24 h were significantly greater ($P<0.001$) after the oil diet compared to the broccoli diet, both adjusted and unadjusted for TAG concentration, and in both younger and older adults. However, the measurement of fasting plasma vitamin K_1

after 24 h showed no significant difference between the two treatments in either younger or older groups. The absence of a significant difference between treatments at 24 h post dose is not surprising given the rapid clearance of vitamin K_1 from the circulation and high turnover of the vitamin.

Table 1-8. Summary of methods that assessed absorption of vitamin K_1 from fortified-oil and broccoli in younger and older subjects, and the respective outcomes (Booth *et al.*, 1999a; 2002)

Method	Observed differences in absorption
24 h AUC	Greater from oil than broccoli diet
24 h fasting plasma	No difference between oil and broccoli diets
5 d AUC	Greater from oil diet in older subjects only
Post intervention fasting plasma (d 11)	No difference between oil and broccoli diets

A study by Schurgers *et al.* (2004) although not primarily designed to measure the absorption of vitamin K_1, estimated that vitamin K_1 from broccoli and spinach were only 13 and 29% as available as that from tablet form, based on a single 4 h post dose blood sample. However, given the variation in T_{max} between different sources of vitamin K_1, measurement at a single time point may provide misleading data.

Three studies (see section 1.12.7.2 for detail) have measured absorption of vitamin K_1 from intrinsically labelled vegetables, and although no comparisons were made between matrices they do provide some data on absorption from these sources (Dolnikowski *et al.*, 2002; Kurilich *et al.*, 2003; Erkkilä *et al.*, 2004). In general, values for T_{max} and relationship between dose and C_{max} are in concordance with previous studies. Key information from comparable studies described above is shown in Table 1-9.

Table 1-9. Results summary of single-dose vitamin K_1 bioavailability studies

Reference	T_{max}	Vitamin K_1 source	Dose amount	C_{max}
		Vegetables	(nmol)	(nmol/L)
Gijsbers *et al.*, 1996	6.1	Spinach & butter	2200	7.50
	6.1	Spinach only	2200	2.50
Garber *et al.*, 1999	4.0	Spinach (150 g)	1100	2.02
	3.5	Spinach (50 g)	367	1.19
	5.5	Broccoli (150 g)	476	1.17
	2.7	Cooked broccoli (150 g)	409	1.81
	5.7	Lettuce (with 35% fat)	398	4.81
	7.7	Lettuce (with 45% fat)	398	6.81
Schurgers & Vermeer, 2000	6.0	Spinach (400 g) meal	3500	9.00
	6.0	Spinach & natto meal	3500	9.00
Dolnikowski *et al.*, 2002	5.0	Broccoli	373	2.25
Kurilich *et al.*, 2003	7.0	Kale	156	3.00
Errikila *et al.*, 2004	6.0	Greens	880	11.00
		Supplements	(nmol)	(nmol/L)
Gijsbers *et al.*, 1996	4.0	Konakion®	2200	47.50
Garber *et al.*, 1999	2.4	Tablet	1111	8.50
Schurgers & Vermeer, 2000	4.0	Konakion®	3500	43.00

From these data a number of conclusions can be drawn. Firstly, it can be seen how different types of study can produce different results. For example, from what is know about vitamin K_1 turnover it is perhaps not surprising that no difference was found between two test meals in a 24 h post-dose sample. Secondly, absorption is both faster and greater from artificial forms of vitamin K_1 compared to absorption from meals. Thirdly, absorption from different vegetables is similar and the addition of fat is likely to increase absorption (Gijsbers *et al.*, 1996; Booth *et al.*, 2002) but the amount of fat may be less important (Garber *et al.*, 1999). However, as highlighted by Booth *et al.* (2002) differences in AUC may not be so important if there are no longer term benefits for other markers of vitamin K status (Booth *et al.*, 1999a). A weakness of most of the bioavailability studies described is the sample size and the large inter-individual variation.

1.13.5 Potential inhibitors of vitamin K_1 absorption

The bioavailability of vitamin K_1 may be inhibited by both naturally-occurring and man-made compounds. Inhibition can occur either through an effect on absorption or metabolism.

1.13.5.1 Fat absorption inhibitors

Statins are drugs used to lower cholesterol levels by inhibiting an enzyme necessary for the synthesis of cholesterol. Inhibition leads to the up-regulation of LDL receptors in the liver and increased clearance of LDL from circulation that could affect vitamin K_1 uptake and metabolism. However, there is very little mention in the literature with regard to the potential to decrease plasma vitamin K_1, presumably because any small effect on vitamin K status is outweighed by the benefits of the treatment.

Orlistat (marketed as Xenical by Roche) is a obesity-treatment and works by the inhibition of gastric and pancreatic lipases (Melia *et al.,* 1996), thereby reducing fat absorption. Few studies have been published on the effects of Orlistat on vitamin K bioavailability. A small study in obese adolescents reported a non-significant decrease in plasma vitamin K_1 (McDuffie *et al.,* 2002). A systematic review of Orlistat reported that use of the drug was associated with lower serum fat-soluble vitamin status (O'Meara *et al.,* 2001). However, none of the studies considered vitamin K. One study reported that Orlistat reduced absorption of vitamin E by around 50% but not vitamin A. In this study, each vitamin was given as the acetate ester that must be hydrolysed before absorption, however this enzyme is also inhibited by Orlistat (Melia *et al.,* 1996). It is likely that the use of Orlistat could decrease vitamin K bioavailability but further research is required.

Phytosterols on the other hand, are natural plant-derived inhibitors of cholesterol absorption and are viewed as a relatively simple dietary modification to reduce population cholesterol levels, and ultimately heart disease (Ostlund 2002). Phytosterols are now included in a wide range of foods marketed as beneficial for health, examples include Flora pro-activ® and the Benecol® range of products.

Some studies have shown reductions in α- and β-carotene (after adjustment for cholesterol) of up to 22%, although no decrease in vitamin K_1 plasma levels has been observed (Ostlund 2002). A study comparing two types of phytosterol-enriched margarine to a normal margarine in 15 hypercholesterolemic subjects reported no difference in plasma vitamin K_1 between the three diets after 21 d (Raeini-Sarjaz *et al.,* 2002). A randomised, double-blind, controlled trial was performed in 85 healthy adults who were assigned to one of four groups, three who received phytosterols at three dosage levels from a fat spread and salad dressing and a control group who received no phytosterols. Over an 8-wk period, there was no reduction in plasma vitamin K_1 (Davidson *et al.,* 2001). A year-long study comparing the effects of a plant-sterol enriched spread to a normal spread in 185 volunteers also reported no decrease in vitamin K_1 concentration or osteocalcin carboxylation (Hendriks *et al.,* 2003). The evidence suggests that phytosterol consumption does not interfere with vitamin K_1 absorption, although most studies have relied on fasting plasma status as a marker, which is known to be highly dependent on recent intake. More recently, a study using stable isotope labelled α- tocopherol and β-carotene, showed a 20 and 50% reduction in bioavailability with phytosterol consumption (Richelle *et al.,* 2004). The potential effect of phytosterols on vitamin K bioavailability requires further investigation.

Olestra is an indigestible sucrose polyester that is used as a fat substitute in processed foods. Because it is lipophilic, olestra can interfere with fat-soluble vitamin absorption because of partitioning of vitamins into the olestra rather than mixed micelles (Schlagheck *et al.,* 1997). Evidence for an effect of olestra on fat-soluble vitamin status, and in particular, vitamin K_1 is mixed. A dose response study in 90 individuals reported that olestra did decrease plasma vitamin K_1, however it did not affect other markers of vitamin K status, Gla concentration or undercarboxylated prothrombin (Schlagheck *et al.,* 1997). The authors do comment that the intake of olestra in this study is likely at the higher end of typical intake. A double-blind placebo-controlled trial of olestra that measured undercarboxylated prothrombin, but not plasma vitamin K_1 showed no effect (Koonsvitsky *et al.,* 1997). Data from 403 adults showed that olestra consumption does

not predict circulating vitamin K_1 levels, indeed higher intakes of olestra were associated with higher vitamin K_1 status (Thornquist *et al.,* 2000). Finally, data from over 2000 adults revealed no trend for an effect of olestra consumption on vitamin K_1 status, but those individuals in the highest tertile of olestra consumption had a lower (9%) plasma vitamin K_1 concentration than individuals who consumed no olestra (Neuhouser *et al.,* 2006). Taken together, this evidence suggests that although there is the potential for olestra to decrease vitamin K_1 in plasma status, it is unlikely at typical intakes of olestra.

1.13.5.2 Menaquinones / dihydro-vitamin K_1

Potential inhibitors of vitamin K_1 absorption include menaquinones and dihydro-vitamin K_1. The potential effect of menaquinones was tested in a human feeding study by giving a K_1 meal with and without food containing menaquinones. The results were reported to be similar in each case suggesting no inhibition (Schurgers & Vermeer, 2000). The potential effect of dihydro-vitamin K_1 on vitamin K_1 absorption is largely unknown (Booth & Suttie, 1998) although one study has shown that compared to vitamin K_1, dihydro-vitamin K_1 was less well absorbed and had no effect on bone markers (Booth *et al.,* 2001).

1.13.5.3 Vitamin A and E

Large doses of both vitamins A and E are reported to affect vitamin K status (Olson 1985). Vitamin A may affect absorption of vitamin K (Olson 1985) but there is no evidence for an interaction at physiological levels. Other quinone-derived molecules (such as vitamin E and ubiquinone) have been shown to act as potential competitive inhibitors for vitamin K-dependent carboxylase (Schurgers & Vermeer, 2001) and there are limited reports of vitamin E affecting plasma vitamin K status (Alexander & Suttie, 1999; Mitchell *et al.,* 2001). The evidence comes primarily from animal studies and often with large doses of vitamin E and suggests the effect may be via a metabolic or transport route rather than an effect on absorption (Schurgers *et al.,* 2002).

1.13.6 Fatty acids

The potential of fatty acids to modify vitamin K absorption and metabolism has been demonstrated in a number of studies. *In vitro* studies have demonstrated that polyunsaturated and monounsaturated fatty acids can reduce absorption of vitamin K_1 (Hollander & Rim, 1976). However, in rats only polyunsaturated fatty acids were shown to reduce vitamin K_1 absorption (Hollander *et al.,* 1977). Of three saturated fatty acids tested, only one (short chain butyric acid of 4 carbon atroms) reduced vitamin K_1 absorption (Hollander *et al.,* 1977). It may be possible to influence the primary tissue destination of vitamin K_1 by altering the types of fat consumed. Schurgers & Vermeer (2001) demonstrated in rats that a diet high in saturated fatty acids (SFA) from hardened coconut oil (HCO) led to plasma levels of vitamin K_1 twice as high as from diets lower in SFA and higher in polyunsaturated (PUFA) and monounsaturated fatty acids (MUFA). This rise can largely be attributed to the doubling of plasma TAG since vitamin K_1 plasma status has been shown to correlate with plasma TAG (Sadowski *et al.,* 1989). Rats on a corn oil-enriched diet (CO) showed a significant decrease in vitamin K_1 and TAG concentration, whilst rats on a sunflower oil-enriched diet (SO), showed a slight decrease in TAG but no difference in vitamin K_1 concentration, compared to rats on a low fat diet. To explain the differences between the HCO and CO diets, the authors focus on the potential modulating affect of PUFA on lipoprotein and consequently vitamin K_1 metabolism, primarily the greater activity of lipoprotein lipase on PUFA-rich chylomicrons. However, this explanation doesn't tally with observed differences in the CO and SO-diets that contain similar proportions of fatty acid classes. The SFA content in the SO-enriched diet was 12% compared to 13% in the CO diet. PUFA accounted for 63% of fatty acids in SO-enriched diet and 54% in the corn oil-enriched diet. Therefore, if the hypothesis of the effect of PUFA is correct, it is unclear why the CO diet, but not the SO diet decreased vitamin K_1 concentration. All vitamin K_1-deficient diets were supplemented with around 40 µg of vitamin K_1 per day, whereas the contribution of vitamin K_1 from the oils was 0.14 µg from corn oil and 0.8 µg from sunflower oil. The authors consider this difference negligible

in comparison to the supplemental vitamin K_1 added to the meals, however no consideration is given to possible differences in bioavailability of vitamin K_1 between the various forms. Another study in rats has demonstrated that both a diet rich in *n*-3 and *n*-6 PUFA reduced TAG, however only the fish oil (*n*-3) diet reduced the level of vitamin K-dependent coagulation proteins (Nieuwenhuys *et al.,* 1998).

In humans, evidence for altered vitamin K_1 absorption or metabolism is provided from a crossover feeding study. Twenty-six men spent 2 wk on an adjustment diet and then either a corn oil (CO) or olive/sunflower oil (OSO) diet. The CO diet was considered PUFA-rich since the percent energy of the diet from PUFA was 11 – 13% compared to 7 – 8% in the OSO diet. TAG and vitamin K_1 were lower in the group on the CO diet compared to the adjustment group and the OSO diet. Measurements of blood coagulation showed conflicting results, since prothrombin time showed no difference in any of the diets. Undercarboxylated Factor II was increased in both diets compared to the adjustment diet. %ucOC was increased in both the CO and OSO diets compared to the adjustment diet. Matrix Gla protein was also significantly lower in the CO diet compared to OSO and adjustment diets (Schurgers *et al.,* 2002). The observations are primarily attributed to the elevated PUFA content of the corn oil meal. An alternative explanation may be the vitamin K_1 content of the CO compared to the OSO. Total vitamin K_1 intake for the CO and OSO diets was 291 µg, and contribution of vitamin K_1 intrinsic to the oils was 15.4 µg and 2.8 µg, respectively. The CO and OSO contributed 73% of total fat intake, thus in terms of vitamin K_1 from fats, and with consideration for potential variation in bioavailability, the difference between CO and OSO may be significant and could partly explain the observations. Together, these studies provide some evidence that PUFA-rich diets may reduce plasma vitamin K_1, potentially by affecting absorption and/or transport of vitamin K.

1.13.7 Non-dietary factors

A number of non-dietary, host-related factors may also influence the absorption, uptake and utilisation of vitamin K_1, including genetic factors, gender and age.

1.13.7.1 Nutrient status

Bioavailability of vitamin A is known to be affected by body stores since homeostatic controls can up-regulate absorption and release vitamin A from the liver. There is no evidence for this type of mechanism for vitamin K, although in times of low body stores vitamin K may be preferentially used for carboxylation of the important hepatic blood coagulation proteins (Booth *et al.,* 2003b). Additionally, after a low vitamin K_1 diet Olson *et al.* (2002) reported both a more rapid entry of vitamin K_1 into cells (determined by a decrease in the half-time of the first exponential of the plasma radioactivity decay curve) and a reduction in biliary secretion identified using radiolabelled vitamin K.

1.13.7.2 Genetic factors

The uptake of vitamin K is probably by incorporation into mixed micelles and transfer across enterocytes, as has been discussed in section 1.9, although it has been suggested that absorption may occur through an energy dependent process (Hollander 1973), but little evidence is available to support this theory. If transporters exist then absorption could be affected by genotypic differences in their manifestation. The apoE genotype is known to be important in the clearance of chylomicrons remnants (and hence vitamin K uptake) with the different phenotypes resulting in altered clearance and plasma concentrations of vitamin K_1 (Saupe *et al.,* 1993; Yan *et al.,* 2005).

1.13.7.3 Gender

Epidemiological evidence generally suggests vitamin K status is not related to gender (Sadowski *et al.,* 1989; Booth *et al.,* 1997; McKeown *et al.,* 2002; Thane *et al.,* 2002a). Evidence from metabolic studies also suggests that gender has little impact on the bioavailability of vitamin K_1 (Ferland *et al.,* 1993; Binkley *et al.,* 2000; Booth *et al.,* 2002). Any observed differences in bioavailability may be related to differences in

lipoprotein metabolism and chylomicron clearance, rather than vitamin K_1 absorption. However, a difference in AUC was seen during a 5 d feeding study, where older men had a significantly higher AUC than older women (P=0.008) but there was no difference between younger men and women (Booth *et al.,* 1999a).

1.13.7.4 Age

For age, the observational evidence is mixed with some reporting effect of age on plasma concentration (Sadowski *et al.,* 1989; Booth *et al.,* 1997) while others have recorded no relationship with age (McKeown *et al.,* 2002). Controlled feeding studies have shown that older adults had higher plasma vitamin K_1 levels assessed by single time points and by AUC, compared to the young. This difference was maintained after correction for TAG at single time points but not for AUC (Booth *et al.,* 2002). Ferland *et al.* (1993) reported a 40% higher plasma vitamin K_1 concentration in elderly subjects compared to younger subjects that was maintained during depletion. In addition, urinary Gla did not decrease in the elderly during depletion. However, these differences are likely due to metabolism, rather than absorption. Binkley *et al.* (2000) reported that at baseline and after 1 wk supplementation with 1000 µg of vitamin K_1, serum vitamin K_1 was significantly lower in young (18 – 35 y) compared to old (>65 y) subjects, presumably due to greater TAG concentration with age, although the difference was not reflected in %ucOC.

1.13.8 Summary of factors affecting bioavailability of vitamin K_1

A wide range of factors influence the bioavailability of vitamin K_1. Some are dietary dependent factors that may affect digestion, absorption and/or utilisation, for example dietary fat. Other non-dietary factors such as apoE genotype may also affect observed inter-individual variation. Probably the major intra-individual determinant of the relationship between vitamin K_1 intake and status is the bioavailability of vitamin K_1 from different food sources. However, only a small number of studies have compared vitamin K_1 bioavailability from different foods. These studies have been typically performed in a small number of subjects, using unusual combinations of food, and different methods that

have produced some conflicting results. Thus, further work is necessary to probe factors that may influence vitamin K_1 bioavailability.

1.14 Conclusions

The most important form of vitamin K for meeting the body's vitamin K requirements is vitamin K_1 (phylloquinone). Recent work has demonstrated a role for vitamin K in health beyond its well-established function in blood coagulation, specifically in bone health and cardiovascular disease. A number of studies have found positive associations between both vitamin K intake and status and markers of bone health. Additionally, evidence is emerging for a benefit of increased vitamin K intake for prevention of vascular calcification. Cardiovascular disease is a major consequence of atherosclerosis and is estimated to cost the UK economy around £26 billion per year, around 57% of which is accounted for by direct health care costs [1]. Osteoporosis also has major economic consequences and is reported to cost the NHS and UK government around £1.7 billion per year [2]. Since osteoporosis is primarily a disease of older people, it is particularly pertinent considering the ageing UK population. The number of people aged 50+ y is expected to increase by around 7 million between 2002 and 2031 while the number of people aged over 85 y is expected to double over the same period (Office for National Statistics 2004).

Through an understanding of the biochemistry of vitamin K, it is clear that the benefits of higher vitamin K_1 status result from an increase in the level of fully carboxylated VKD proteins. A number of studies have demonstrated that by increasing vitamin K intake, both plasma levels of vitamin K_1 and levels of undercarboxylated proteins can be improved.

The data suggest that current vitamin K_1 dietary recommendations may not be sufficient for the full γ-carboxylation of some vitamin K-dependent proteins. Furthermore, around half of the UK population does meet the current recommendation. Research is

---

[1] Data from British Heart Foundation: www.heartstats.org (accessed 11th March, 2007)

[2] Data from National Osteoporotic Society: www.nos.org.uk (accessed 11th March, 2007)

required on the relationship between vitamin K_1 plasma status and vitamin K_1 dietary intake, and in particular, how the relationship is determined by bioavailability.

The starting point for studies of absorption should be through a thorough understanding of the kinetics of vitamin K absorption and body pool sizes. At the current time, no studies have applied a stable isotope methodology to the assessment of vitamin K metabolism and previous work using alternative methods has primarily utilised only pharmacological doses.

The focus of this thesis was to develop stable isotope based methods to improve our understanding of vitamin K_1 kinetics and bioavailability. The work had three specific aims:

1) Development of a method to measure isotope ratios of vitamin K_1 in plasma
2) Measurement of vitamin K_1 kinetics and body pool sizes in humans
3) Development and application of methods to measure the bioavailability of vitamin K_1 from food

2 ANALYSIS OF ISOTOPE RATIOS OF VITAMIN K_1 FROM PLASMA

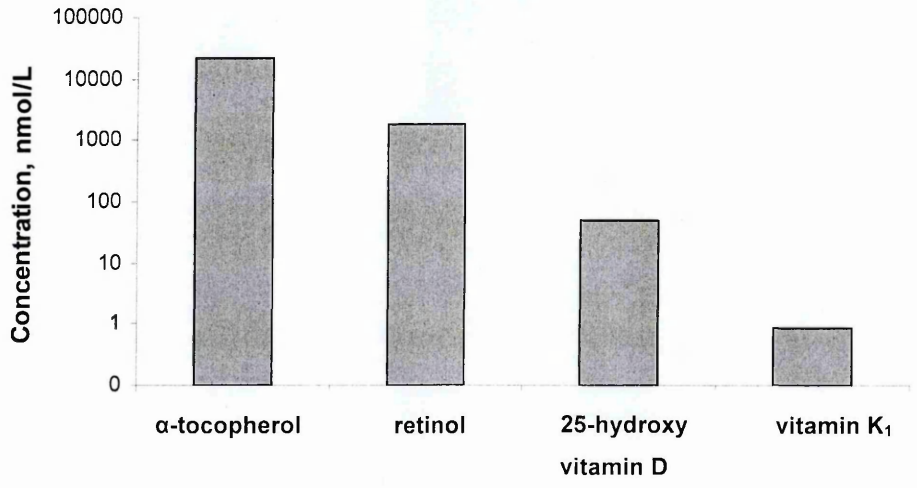
This section addresses the issues relevant to the analysis of vitamin K_1, and focuses primarily on the analysis of isotope ratios that is the major challenge of this work. Stable isotopes and mass spectrometry are described, including a review of work by previous authors. Strategies for the extraction of vitamin K_1 are reported along with the final methodology chosen for use in subsequent studies of vitamin K_1 kinetics (section 3) and bioavailability (section 4) in human volunteers.

2.1 Challenges

Specific challenges for vitamin K_1 analysis from plasma result from its low concentration, interfering plasma lipid components, and the sensitivity of the molecule to degradation. Sample preparation, clean-up and separation of compounds by chromatography are all essential for the analysis of vitamin K_1 (Fauler *et al.*, 2000).

In comparison with other fat-soluble vitamins (Figure 2-1) and co-extracted non-polar constituents of blood plasma, fasting concentrations of vitamin K_1 are low, with typical values between 0.29 and 2.64 nmol/L (Institute of Medicine 2001). The use of large volumes of plasma to achieve the required sensitivity is often not compatible with sensitive separation and detection methods because of high amounts of co-extracted interfering compounds (Fauler *et al.*, 2000).

Figure 2-1. Comparison of mean fasting plasma concentrations of α-tocopherol, retinol, 25-hydroxy vitamin D and vitamin K_1 (adapted from Sadowski *et al.*, 1989)



The major challenge to the extraction and analysis of vitamin K_1 is the presence of lipid components of plasma that are of a similar low polarity and present in much greater concentrations. Many extraction procedures that remove vitamin K_1 also extract other non-polar constituents of plasma. These compounds may mask vitamin K_1 in the chromatogram, interfere with chromatography, or result in rapid loss of chromatographic resolution. Typical concentrations of lipids (and vitamin K_1) are listed in Table 2-1.

Table 2-1. Concentrations of lipids in blood plasma (adapted from Harper, 1963 except for * from Sadowski *et al.*, 1989)

	Concentration in plasma (mg/mL)	
Lipid fraction	Mean	Range
Total lipids	5.8	3.6 - 8.2
Triacylglcyerols	1.4	0.8 - 1.8
Total cholesterol	2.0	1.1 - 3.2
as cholesterol esters	1.4	1.3 - 1.5
Total Phospholipids	2.2	1.2 - 2.9
Vitamin K_1*	0.4 ng/mL	0.1 - 1.2 ng/mL

Vitamin K is sensitive to light, especially blues and ultra-violet, thus procedures need to be performed either with subdued or yellow lighting to minimise degradation. It is reported that, in samples of bone, vitamin K_1 became undetectable after exposure to sunlight for 1 h (Hodges *et al.*, 1993). Degradation is also caused by strong alkalis that can originate from detergents used for glass washing. Thorough rinsing of glassware or heating to > 500 °C is necessary to ensure removal of alkaline agents.

2.2 Measurement of vitamin K by mass spectrometry

Quantitative analysis of vitamin K is routinely performed using high performance liquid chromatography (HPLC) (Fauler *et al.*, 2000). Prior to 1993, no method had been reported to measure vitamin K_1 at physiological levels using gas chromatography mass spectrometry (GCMS). Since then, a small number of reports have described the use of mass spectrometry, with gas chromatography or liquid chromatography, either for the

quantitation of vitamin K_1 or for the measurement of isotope ratios of vitamin K_1 from plasma. The strategies for extraction of vitamin K_1 from plasma prior to mass spectrometric analysis are summarised in Table 2-2. The various GCMS approaches and configurations are detailed in Table 2-3.

Table 2-2. Methods for extraction of vitamin K_1 from plasma prior to analysis with mass spectrometry. All methods used GCMS except for Kurilich et al., (2003) who analysed vitamin K_1 by LCMS

Reference	Plasma volume (µL)	Extraction	Final volume (µL)	Injection volume (µL)	LOD (pg)
Fauler *et al.*, 1996	1000	Add 1 mL water, mix, and stand for 10 min. Add 2 mL hexane, stand for 10 min. Add 4 mL hexane. Centrifuge.	40	10	1
Raith *et al.*, 2000	200	Add 0.2 mL water, 0.6 mL ethanol and 5 mL of hexane. Extract for 15 min. Centrifuge	50	10	1
Dolnikowski *et al.*, 2002	500	Deproteination with ethanol. Extraction with hexane. SPE with silica column. Purification by HPLC.	50	1-2	5
Erkkilä *et al.*, 2004	500	Deproteination with ethanol. Extraction with hexane. SPE with silica column. Purification by HPLC.	50	5	5
Kurilich *et al.*, 2003	500	Deproteination with 500 µL ethanol. Extracted twice with 1.5 mL hexane. Dried under N_2 reconstituted in 200 µL.	200	50	-

Abbreviations: HPLC, high performance liquid chromatography; LOD, limit of detection; SPE, solid phase extraction

2.2.1 Gas chromatography mass spectrometry

Gleispach *et al.* (1993) used GCMS to investigate a number of techniques for the chemical derivatisation of vitamin K_1, including silyation (substitution of an active hydrogen with a silicon-containing group) and acylation (substitution of an active hydrogen atom with an acyl group) after reduction with zinc or hydrogen. However, the authors

concluded that they were unable to find any derivative that was more sensitive than underivatised vitamin K_1, using splitless injection with either electron ionisation (EI) or negative ion chemical ionisation (NCI). The limit of detection (LOD) was reported as 100 pg per injection, although injection volume is not reported. It is important to note that this work was apparently performed with vitamin K_1 standards and not with vitamin K_1 from plasma. Except for the LOD, no information is provided on the working concentrations.

Later, the same group compared three different acyl-derivatives, trifluoroacetyl-, pentafluoropropionyl- and heptafluorobutyryl (HFB)-vitamin K_1. Derivatisation with the *N*-perfluoroacyl anhydride and *N*-perfluoroacid was performed after reduction with zinc. The chosen derivative was heptafluorobutyryl ester since, although all three derivatives provided similar analytical sensitivity, the HFB-derivative was the least impaired by extraneous lipids in the plasma matrix. Vitamin K_1 was purified from plasma only with solvent extraction. Large volumes (10 µL) were injected and the limit of detection was recorded as 1 pg and limit of quantitation as 2 pg (Fauler *et al.,* 1996).

The heptafluorobutyryl derivative-method was then successfully applied to the measurement of vitamin K_1 concentrations in neonates (Raith *et al.,* 2000). However, following a dose of vitamin K_1 to protect against haemorrhagic disease of the newborn (HDNB), neonates have relatively high plasma concentrations of vitamin K_1 and also lower levels of potentially interfering lipid-soluble compounds, particularly when compared with adults (Gleispach *et al.,* 1993). Therefore, it is questionable if this method could be applied to vitamin K_1 in adult plasma.

At the present time, only one methodology has been published that describes the extraction and measurement of isotope ratios of vitamin K_1 from plasma in adults by GCMS (Dolnikowski *et al.,* 2002). The methodology was subsequently applied to the measurement of lipoprotein transport of vitamin K_1 after consumption of labelled vitamin K_1 by human volunteers (Erkkilä *et al.,* 2004). The procedure involves solvent extraction, solid phase extraction, and semi-preparative HPLC prior to isotope ratio analysis by GCMS. Since the method involves two analytical techniques HPLC and GCMS the analysis time per sample is considerable (HPLC run time 24 min, GCMS runtime at least

16 min). Despite the extensive sample preparation, problems with analysis were reported, such that isotopic data from only 60% of subjects were useful for analysis (Erkkilä *et al.*, 2004).

2.2.2 Liquid chromatography mass spectrometry

The use of liquid chromatography mass spectrometry (LCMS) has also been described for the analysis of vitamin K_1 (Kurilich *et al.*, 2003). The use of LCMS is less well established than GCMS for the precise measurement of isotope ratios. The combination of liquid chromatography and mass spectrometry is complicated by the large amount of mobile phase (liquid solvent) entering the vacuum enclosure of the mass spectrometer. However, in theory at least, this method has a number of advantages over GCMS in terms of requirements for sample preparation, particularly in samples with complicated matrices. In this report, plasma samples undergo only solvent extraction prior to analysis by LCMS. The limit of detection is reported as 3 fmol (1.5 pg) (Kurilich *et al.*, 2003).

Table 2-3. Summary of methods for analysis of vitamin K_1 by gas chromatography mass spectrometry (GCMS)

		GC parameters			
Reference	Method	Injector	Column*	Oven	Comment
Gleispach *et al.*, 1993	Derivatised Underivatised EI and NCI	Grob split-splitless	DB5, 15 m 0.25 mm 0.25 µm		Concluded that no derivative was more sensitive than underivatised K_1
Fauler *et al.*, 1996	Heptafluorobutyric anhydride derivative EI	Grob split-splitless	DB5, 15 m 0.25 mm 0.25 µm	160 °C (1 min) 30 °C/min 290 °C (4 min)	SPE provided no advantage over solvent extraction
Raith *et al.*, 2000	Heptafluorobutyric anhydride derivative EI	Grob split-splitless	DB5-MS, 15 m 0.25 mm 0.25 µm	160 °C (1 min) 30 °C/min 290 °C (4 min)	Mostly high concentrations (100-200 ng/ml) in neonates after *iv* dose.
Dolnikowski *et al.*, 2002	Underivatised NCI	Cool on-column	Guard column, 0.5 m 0.53 mm DB5-MS, 30 m 0.25 mm, 0.25 µm	50 - 325 °C at 50 °C/min	
Erkkilä *et al.*, 2004	Underivatised NCI	Cool on-column	Guard column, 0.5 m 0.53 mm HT5, 30 m 0.25 mm, 0.1 µm	50 - 300 °C at 30 °C/min then 10 °C/min to 380 °C	Co-eluting compounds from HPLC did not elute from GC, causing a blockage and unreliable data.

* Column details include: Type, length, diameter, and film thickness. Abbreviations: EI, electron ionisation; NCI, negative ion chemical ionisation; *iv* , intravenous; GC, gas chromatography; HPLC, high performance liquid chromatography; SPE, solid phase extraction

2.2.3 Conclusions

The sum of previous work on vitamin K_1 and gas chromatography mass spectrometry (GCMS) suggested that prior to analysis, extraction of vitamin K_1 from plasma could be achieved with solvent extraction alone, followed by derivatisation. The subsequent pages provide a general introduction to stable isotopes and GCMS and precede a description of the development of a method for the measurement of isotope ratios of vitamin K_1 from human plasma.

2.3 Stable Isotopes

2.3.1 Definition

Stable isotopes are atoms that have a different number of neutrons but the same number of protons and electrons. An additional neutron will increase the atomic mass, whilst one fewer will decrease the atomic mass. It is these differences in atomic mass that can be measured using mass spectrometry and allow the exploitation of stable isotopes to identify particular molecules.

Unlike radioactive isotopes, stable isotopes do not decay. In terrestrial ecosystems, the proportion of stable isotopes is comparatively constant. However, chemical and biological mechanisms can discriminate between stable isotopes. For example, the different photosynthetic pathways between C3 and C4 plants can result in different carbon isotope ratios between these two categories of plants. In addition, discrimination between the isotopes can occur in chemical processes, particularly with hydrogen, since this element has the greatest difference between the two stable isotope forms (protium and deuterium). Reaction rates are frequently slower in samples enriched with deuterium. The properties of the two stable isotopes used in this work are shown in Table 2-4.

Table 2-4. Atomic mass and percent abundance of carbon and hydrogen isotopes[1]

Element		Atomic mass	% abundance in terrestrial ecosystems
Carbon	^{12}C	12.000	98.930
	^{13}C	13.003	1.070
Hydrogen	H	1.008	99.9885
	^{2}H or D	2.014	0.0115

Abbreviations: D, deuterium (^{2}H)

2.3.2 Isotopomers

Molecules of the same chemical composition but with a different abundance of the heavier isotope are known as isotopomers. Because of the natural occurrence of heavier atoms, isotopomers exist naturally, for example, carbon dioxide has six common isotopomers (Table 2-5). Artificially increasing the proportion of one of the rarer isotopomers is the basis for stable isotope tracer studies.

Table 2-5. Major isotopomers of carbon dioxide and their molecular weights [1]

Molecular weight *	Isotopomers	% relative abundance
44	$^{12}C^{16}O_2$	100
45	$^{13}C^{16}O_2$; $^{12}C^{16}O^{17}O$	1.2
46	$^{13}C^{16}O^{17}O$; $^{12}C^{17}O_2$; $^{12}C^{16}O^{18}O$	0.4

* molecular weights are rounded to whole numbers

In mass spectrometric terminology, the ion of the intact molecule is referred to as the molecular ion (M). Isotopomers of the same molecule are referred to as M+1, M+2, M+3 etc, with the number referring to the incremental increase in molecular weight. The chemical composition of a material determines the number and proportion of each isotopomer.

[1] http://www.webelements.com/ (accessed 9th February 2007)

2.4 Mass spectrometry

2.4.1 Introduction

The measurement of isotope ratios is dependent on the separation and detection of molecules using mass spectrometry. This powerful technique is used widely in analytical science and has numerous applications, for example in the detection of very low levels of compounds, identification of unknown compounds, quantifying the level of known compounds and determining chemical structures. Measurements of natural isotopic composition are also widely used in tests for food authenticity, in forensic science, the use of performance-enhancing drugs and also in geochemistry. The artificial enrichment of compounds with stable isotopes for use as tracers, and analysis by mass spectrometry, are frequently used in many fields of chemistry and biology.

2.4.1.1 Ionisation

In order for molecules to be measured by mass spectrometry, the molecule must first be ionised before separation by the mass spectrometer. Ionisation can be achieved in many ways, such as electron ionisation (EI) or chemical ionisation (CI). As a result, it is the mass to charge ratio (m/z) of ions that is measured.

2.4.1.2 Electron ionisation

In electron ionisation (EI), a current passing through a filament produces free electrons. These are accelerated (typically 70 eV) and then used to bombard the analyte molecules, removing an electron and giving the molecule a positive charge. Since the energy of chemical bonds (typically a few eV) is much smaller than the energy of the electrons, bond cleavage in the target molecule is a frequent occurrence, which leads to considerable fragmentation of the parent molecule. This fragmentation produces characteristic patterns in the mass spectra that can act as a fingerprint and aid in positive identification of the molecule. Important terms in the description of mass spectra are the 'molecular ion' that describes the ion corresponding to the molecular weight of the intact

molecule and the 'base peak' that describes the peak with the greatest abundance in the mass spectrum.

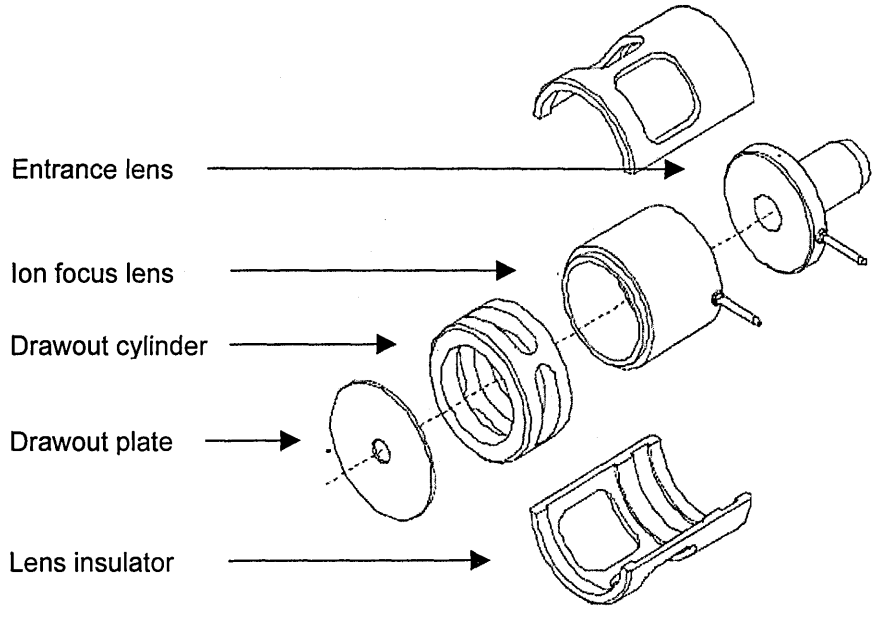
2.4.1.3 Chemical ionisation

Chemical ionisation (CI) is considered a 'softer' form of ionisation and usually results in less fragmentation of the molecular ion. In CI, a reagent gas (often methane) is introduced into the ionisation chamber along with the sample and carrier gas. Most of the electrons emitted from the filament collide with reagent gas molecules forming reagent ions. These ions then react in various ways with sample molecules. There are two important factors to consider with CI. One is the need for an excellent vacuum in the MS chamber since water contamination of reagent gases dramatically decreases sensitivity. Secondly, one must consider the type of ionisation. There are two forms of CI, negative chemical ionisation (NCI) and positive chemical ionisation (PCI). With PCI, the ionised reagent gas ions react chemically with the sample molecules. There are four forms of ionisation in PCI, proton transfer, hydride abstraction, addition and charge exchange. The effect of proton transfer is to produce M+1 ions that may not be compatible with stable isotope work. PCI is not especially sensitive because of the high background due to reagent gas ions, however it is a very soft ionisation method that results in less fragmentation and a greater abundance of the molecular ion. With NCI, the voltage polarities of the analyser are reversed to select negative ions. Ionisation mechanisms can include electron capture, dissociative electron capture, ion pair formation and ion-molecule reactions. NCI can provide excellent sensitivity, however for isotope ratio work there is a greater risk of non-linearity through isotope effects.

2.4.2 Ion focusing

After ionisation, ions are focussed with electrostatic lenses (Figure 2-2).

Figure 2-2. Focussing parts of the Agilent 5973N electron ionisation source



2.4.3 Mass separation

Benchtop GCMS instruments are commonly fitted with quadrupole mass filters. Following beam focussing, the ionised molecules pass to the mass filter and are separated on the basis of their mass to charge ratio (m/z). The quadrupole mass filter consists of four rods. A combination of direct current (dc) and radio frequency (RF) signals are applied to the rods. It is the magnitude of the RF signal that determines which ions can pass through the mass filter. The ratio of dc to RF voltage determines the resolution (widths of the mass peaks). The signals applied to the rods are set to allow ions of only a certain m/z to pass through; the other ions collide with the rods. In a quadrupole instrument, ions with different m/z are not measured simultaneously but rather, via alternation of the signals applied to the rods, pass through the mass filter sequentially. Compared to alternative methods of measuring isotope ratios (e.g. multiple-collector magnetic sector instruments that measure specific ions continuously) the use of quadrupole filter provides lower precision since only a single ion can be measured at any time point.

A quadrupole mass filter can be operated in two modes: full scan or selected ion monitoring (SIM). In full scan a wide range of ions are analysed generating a full fragmentation pattern that is essential for positive identification of the molecule of interest. In SIM, a small number of chosen ions are analysed repeatedly. In this way, greater sensitivity is achieved and better precision is obtained in the measurement of isotope ratios.

2.4.4 Detector

A single detector known as an electron multiplier is used with a quadrupole instrument. Separated ions hit a high-energy diode that releases electrons. The signal is amplified as the electrons cascade through the electron multiplier horn. The limitation is that multiple ions cannot be measured simultaneously, thus the detector must have a fast response and high gain, that rules out the use of a Faraday detector. The integral amplifier of the electron multiplier provides the fast response but lower stability than can be obtained with Faraday cups.

2.5 Sample introduction - gas chromatography

Analysis by mass spectrometry requires that a pure specimen as possible be presented to the ion source. The most common method is by gas chromatography (GC). Together gas chromatography and mass spectrometry (GCMS) provide a flexible and affordable method with excellent selectivity and sensitivity. Gas chromatography describes the separation of volatile molecules on the basis of their affinity for the stationary phase of the capillary column while in a stream of carrier gas, commonly helium or nitrogen. The two most important components of the GC are the injector and the chromatography column.

2.5.1 Injector

There are a number of injector systems available for GC, two of the most common and those considered here are the split/splitless injector and cool on-column injector. They have in common a continual flow of carrier gas through the injector that moves

constituents through the capillary column. The sample to be analysed is dissolved in a solvent and is transferred to the injector via a syringe.

2.5.1.1 Split/Splitless injector

In a split/splitless injector, the sample is injected into a glass liner contained within the heated injector. The sample is vaporised on entry to the liner and transferred to the column by the flow of carrier gas. The shape and size of the liner can be altered depending on the application. Often a small amount of deactivated glass wool is inserted into the liner to improve mixing of the sample and act as a filter to remove non-volatile contaminants that can degrade chromatography. On vaporisation, the volume of the liquid solvent increases thus injection volume is partly limited by the capacity of the liner. Vaporisation volume is dependent on the temperature and pressure of the injector. The crucial step here is efficient transfer of the sample to the column in order to minimise peak broadening and to ensure maximum resolution. During split injection, a set amount of carrier gas is continually purged from the injector, thus only a set portion of the vapour enters the capillary column, but flow through the liner is relatively fast. In splitless injection mode, the purge line is shut for a specified time to allow sample to condense at the top of the column. The purge line is opened at a set time after injection to remove excess solvent before raising the temperature of the column. Split injection has the advantage of reduced peak broadening and increased resolution. The splitless injection method can increase sensitivity but often with peak broadening due the relatively slow flow through the liner and transfer to the column that results in a loss of resolving power.

2.5.1.2 Pulsed split/splitless method

A variation on the split/splitless technique is the pulsed split/splitless method. This option is available on GCs equipped with electronic pressure control (EPC) (or similar systems) to accurately control gas flow rates. During injection, the pressure within the injector is increased to improve transfer to the column. Additionally, because of the higher pressure, larger sample volumes can be injected since the vaporisation volume is reduced.

2.5.1.3 Cool-on column

With a cool on-column injector, the entire sample is injected and directly transferred onto the capillary column. This method can provide high sensitivity and reduce adsorption of analytes on active surfaces present in split/splitless injectors. During injection, the injector and oven are kept below the boiling point of the solvent. If low-boiling point solvents are used, the injector can be further cooled by the use of liquid nitrogen piped around the injector. The major disadvantage of this method is that contaminating non-volatile components are also transferred to the column along with the sample. To partly overcome this problem, and to reduce contamination to the capillary column, a retention gap or guard column can be used that consists of a length of deactivated uncoated capillary that connects the inlet to the capillary column.

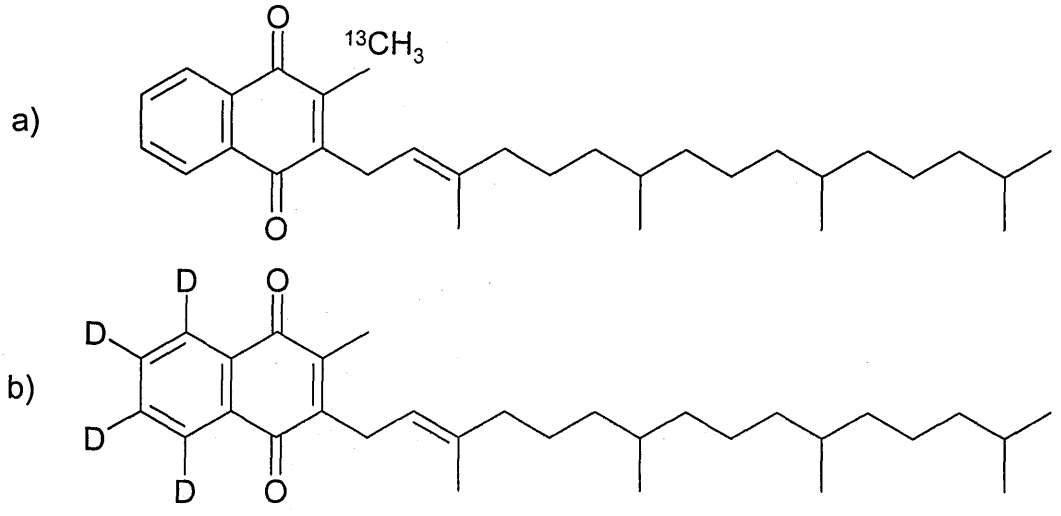
2.5.2 Capillary columns

Capillary columns consist of a fused silica tube with a polyamide coating for strength. The inside of the column is coated with a stationary phase. The characteristics of the phase determine the selectivity of the column. The simplest phases are non-polar and primarily separate compounds on the basis of boiling point. With the addition of phenyl or cyanopropyl units, separation is also determined by polarity. The capillary column is housed within a temperature-controlled oven. By raising the temperature of the oven during analysis, compounds are released from the stationary phase to pass through the column to the detector. The flow of gas through a capillary column can be crucial to the efficient separation of compounds. Helium permits near optimum separation at even relatively high velocities (up to around 50 cm s^{-1}).

2.6 Analysis of standards

Pure unlabelled vitamin K_1 was obtained from Supelco (Dorset, UK). Two stable isotope labelled forms (Figure 2-3) of the vitamin (methyl-^{13}C and ring-D_4) were custom synthesised by ARC Laboratories (Apeldoorn, The Netherlands).

Figure 2-3. Structures of labelled versions of vitamin K_1 a) methyl-^{13}C b) ring-D_4



2.6.1 Preparation of vitamin K_1 standards

Samples of vitamin K_1 were prepared in hexane. Since pure vitamin K_1 is a viscous material, solutions were prepared by the following general method. A volumetric flask was covered in foil and weighed containing a stainless steel spatula. Under subdued-light, a small amount (mg) of vitamin K_1 was transferred to the flask using the spatula. The mass was recorded and hexane was added to around two-thirds of the flask volume while agitating the spatula to ensure dissolution. The spatula was removed and hexane added over the spatula to fill the flask to the correct volume.

2.6.2 GCMS method

All GCMS analysis was performed on an Agilent GCMS system. Sample injection was performed with an Agilent 6890 GC with autosampler and equipped with split/splitless and on-column injector (Agilent Technologies, Stockport, UK). The GC was interfaced to a 5973N inert mass spectrometer equipped with a turbomolecular pump.

2.6.2.1 Injection method

A number of injection methods were investigated, including splitless, pulsed splitless and on-column injection. The advantage of splitless injection over split injection is that more of the sample is transferred to the column. The liner of the injector can

contain a small piece of glass wool that can help filter the sample and prevent less volatile components entering the column. The major advantage of pulsed splitless mode is that greater sample volumes can be injected. With the pulsed splitless method it was possible to inject up to 5 µL whereas with split only up to 2 µL of solvent could be injected. The use of a liner marketed to convert a split/splitless injector into direct injector (equivalent to on-column) was also investigated. This liner tapers to a narrow hole where the analytical column fits tightly into the base of the liner. However, this method was not as sensitive as on-column injection. Of the injection methods, on-column was selected as it provided the greatest sensitivity.

With on-column injection the sample is transferred directly into the column. The use of a retention gap or guard column was found to not only improve peak shape but also permitted the injection of higher sample volumes. Chromatography was performed using a DB5-MS fused-silica capillary column (15 m x 0.25 mm internal diameter (id), 0.25 µm film thickness). The DB5-MS column was connected to a 0.50 m to 5 m length of deactivated fused silica (0.53 mm id) acting as a guard column (retention gap) the other end of which was connected to the on-column injector. Helium was used as the carrier gas, with a flow rate of 1.2 mL/min operating in constant flow mode. Injection volumes were between 1 and 2 µL. Oven conditions were 60 °C for 2 min, ramped at 30 °C/min to 325 °C, then an isothermal hold of 2 min. The transfer line was held at 325 °C.

2.6.2.2 MS parameters

The mass spectrometer was operated in electron ionisation mode as this method minimises the potential for loss of labelled atoms during ionisation processes. The source temperature of the mass spectrometer was set at 230 °C and the quadrupole temperature at 150 °C. The ionisation energy was 70 eV. The analysis of underivatised vitamin K_1 was thus performed under the conditions shown in Table 2-6.

Table 2-6. GC and MS parameters for the analysis of vitamin K_1 standards

GC Parameters	
Injector	On-column, 1 µL injection volume
Column	5 m x 0.53 mm id deactivated fused silica capillary guard column DB5-MS 15 m x 0.25 mm id x 0.25 µm
Column flow	1.5 mL/min
Carrier gas	Helium
Oven profile	Initial: 60 °C for 2 min Ramp: 30 °C/min Final: 325 °C hold 2 min
Transfer line	325 °C
MS Parameters	
Ionisation energy (eV)	70 volts
Source temperature	230 °C
Quad temperature	150 °C

Abbreviations: eV, electron volts; id, internal diameter

2.6.3 GCMS analysis of standards - results

Electron ionisation of vitamin K_1 in the mass spectrometer produced a number of characteristic ions (Figure 2-4). The base peak corresponds to the molecular ion (m/z 450). Other significant ions in this spectrum are m/z 186, 198 and 225.

Figure 2-4. Mass spectrum of unlabelled vitamin K_1

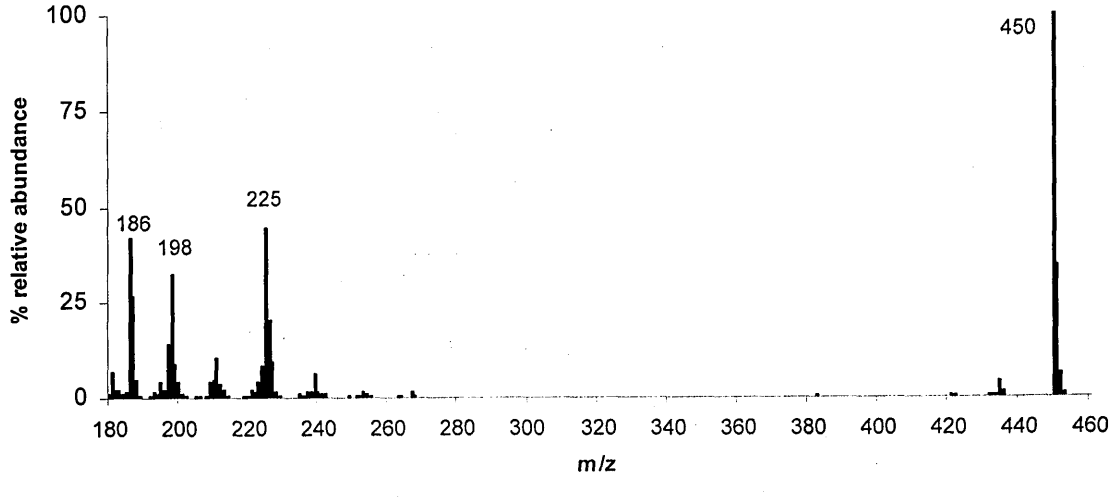


Figure 2-5 and Figure 2-6 show mass spectra under the same MS conditions for the ^{13}C labelled and ring-D_4 forms of vitamin K_1.

Figure 2-5. Mass spectrum of ^{13}C labelled vitamin K_1

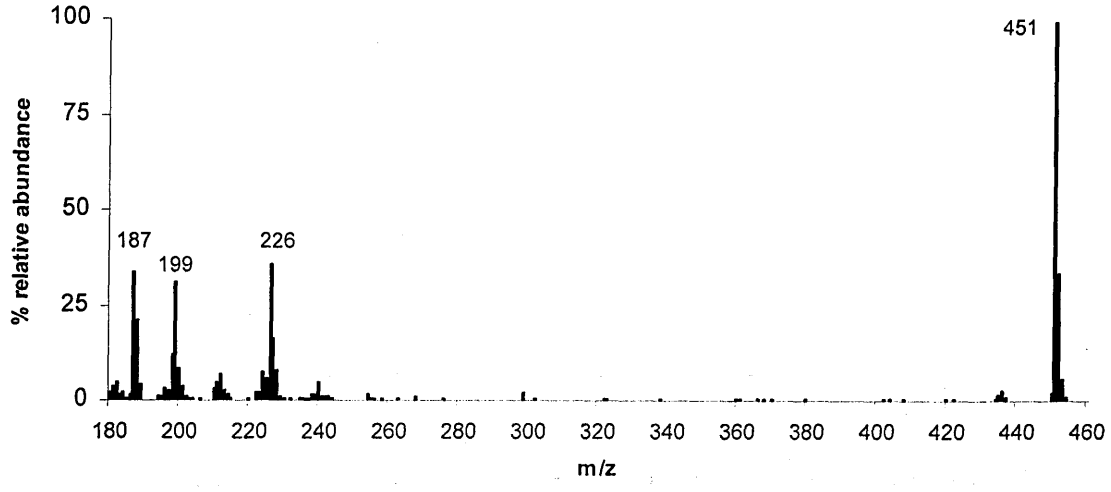
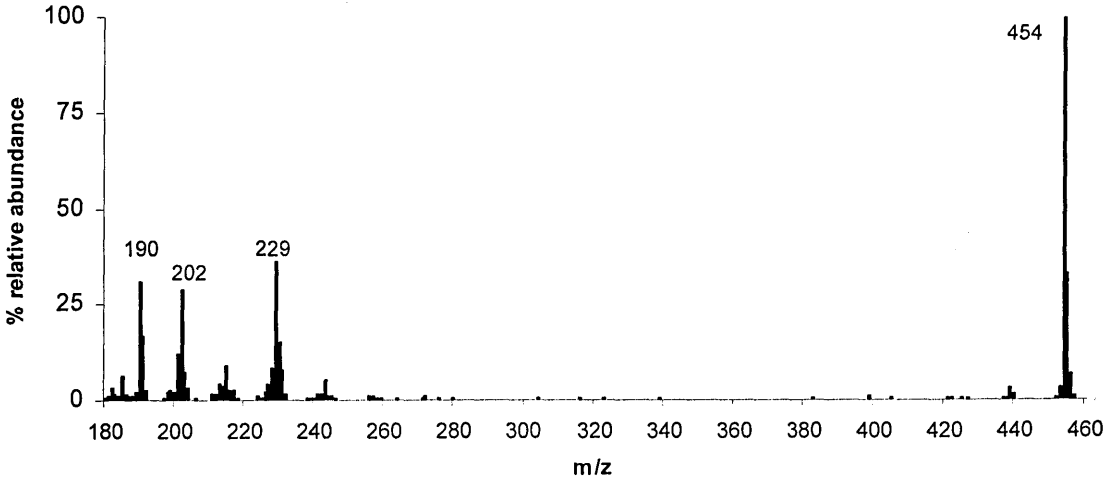


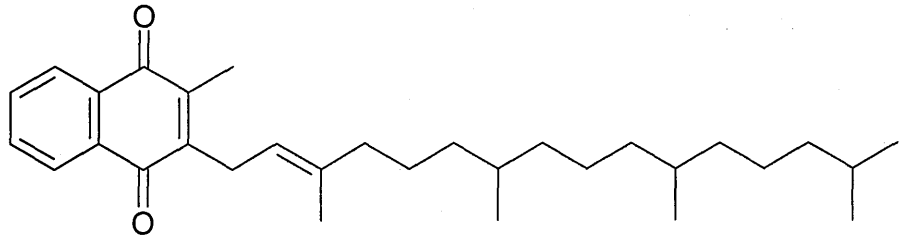
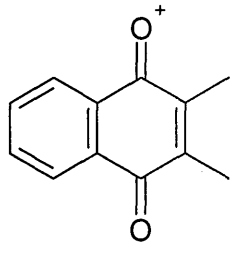
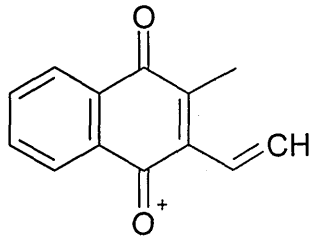
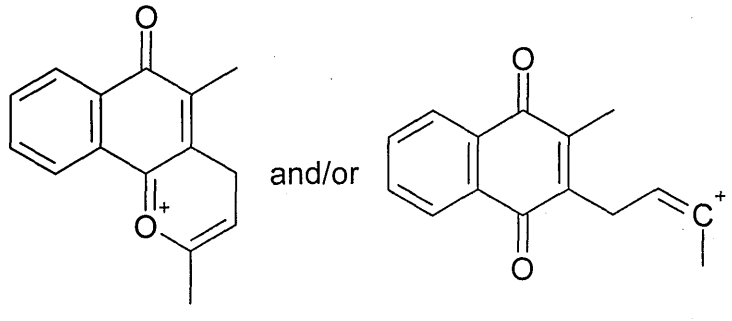
Figure 2-6. Mass spectrum of ring-D_4 vitamin K_1



Comparison of the three mass spectra of the different isotopomers makes it possible to comment on the potential molecular structure of the fragment ions. In the spectra of both labelled species, the three major fragment ions all show the same increase in molecular weight as the parent ion. This demonstrates the fragment ions all contain the same napthoquinone ring structure that includes the labelled atoms. The structures of the fragment ions are shown in Table 2-7 and are provided by the detailed mass spectrometric study of vitamin K_1 by Di Mari *et al.* (1966) who used a number of isotopically labelled vitamin K_1 analogues . Further evidence for m/z 186 and 225 comes from Fauler *et al.* (1996), and from a review of the analysis of non-volatile lipids by mass spectrometry (Murphy *et al.*, 2001). Other smaller ions (< m/z 180) in the mass spectrum

of underivatised vitamin K_1 are probably representative of various parts of the phytyl side-chain and common to each of the three species, characterised by peaks at 14 – 15 mass units apart, representing methylene and methyl units (Di Mari *et al.,* 1966).

Table 2-7. Potential structures of fragment ions of vitamin K_1 after electron ionisation

Molecular structure	Mass
	450
	186
	198
	225

2.6.4 Isotopomer distribution

It is possible to calculate the expected isotopic distribution of a molecule from the known contribution of the heavier isotopes to each element [1]. The theoretical relative abundances (i.e. the analytical response for an isotopomer relative to the response of the most abundant isotopomer) of masses contributing to total vitamin K_1 are shown in Table 2-8. This information is useful to confirm the identity of a peak, especially when working in selected ion monitoring where additional mass spectral information may not be available.

Table 2-8. Theoretical % relative abundance (to M) of isotopomers contributing to total vitamin K_1 abundance

Mass	% relative abundance
450	100.0
451	34.2
452	6.0
453	0.6

2.6.5 Selected ion monitoring

Initial attempts to analyse vitamin K_1 in selected ion monitoring (SIM) resulted in unexpected isotope ratios, however isotope ratios obtained from SCAN mode were of the expected values. There were a number of possible explanations for incorrect isotope ratios including inappropriate mass spectrometer settings (high scan speed, short dwell time, incorrect mass spectrometer tuning), inappropriate peak width, or a dirty ion source. After eliminating these possibilities, the problem was found to be due to the way in which the mass spectrometer obtains data and the differences between SIM and SCAN modes. In SCAN mode, the mass spectrometer scans at 0.1 amu (atomic mass units) increments. Five adjacent data points are smoothed and the centroid mass determined. However, in SIM, data are obtained around a specified window (0.9 amu in low resolution and 0.5 amu in high resolution). If the mass spectrometer is set to monitor a slightly different mass

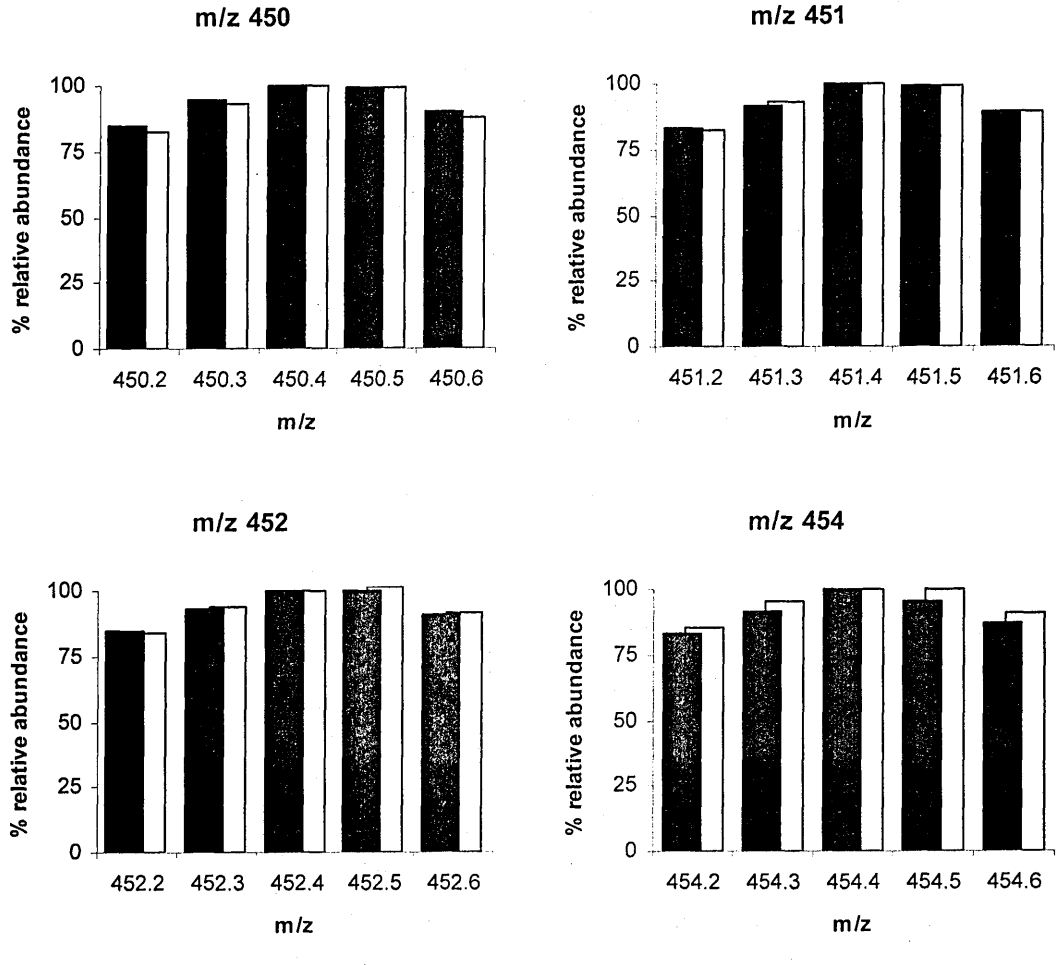
[1] http://www2.sisweb.com/mstools/isotope.htm (accessed 14th April 2007)

(e.g. 450.0) than the actual mass (e.g. 450.4) of a molecule, then peak widths may overlap. This effect was the cause of the incorrect isotope ratios.

2.6.6 Dynamic mass calibration

The problem of incorrect mass assignment can be overcome by performing a dynamic mass calibration (DMC) which allows determination of the centroid mass (the mass of greatest abundance within a single peak or compound). To perform a DMC, a SIM acquisition was performed around the expected centroid mass at m/z spaced 0.1 amu apart. The exact mass of vitamin K is 450.35 thus DMC was performed between 450.2 and 450.6 at 0.1 amu increments. The results show (Figure 2-7) that the centroid mass for each of the isotopomers of m/z, 450.4, 451.4 and 452.4 is at .4, although for the 454.4 isotopomer the abundance is very similar between .4 and .5 (in high resolution). Greater sensitivity was achieved with high resolution.

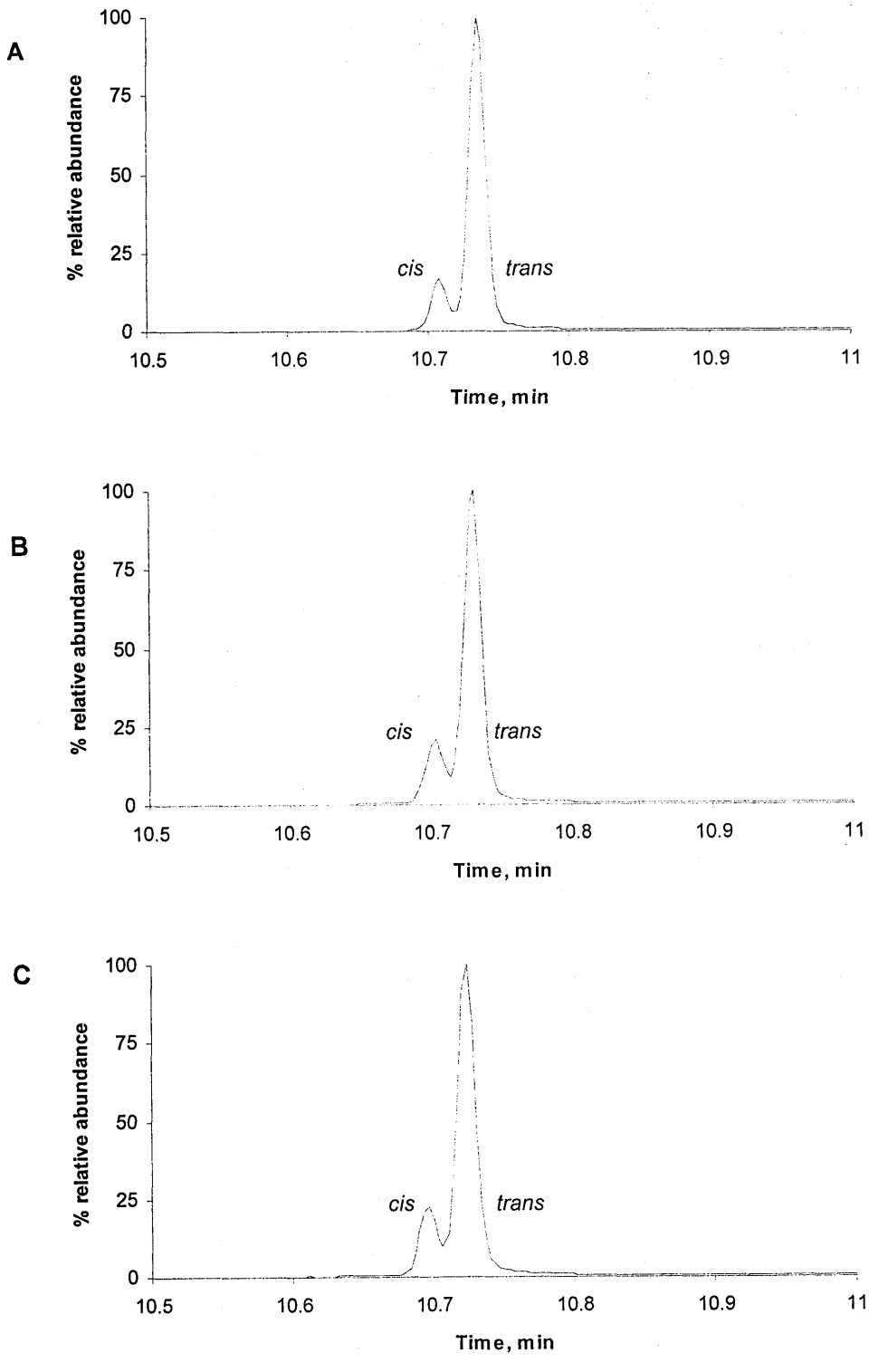
Figure 2-7. Comparison of low resolution (black bars) and high resolution (white bars) and determination of the centroid mass during dynamic mass calibration



2.6.7 Chromatography of vitamin K_1 standards

Figure 2-8 shows the chromatography of vitamin K_1 standards as measured in SIM mode. The double peak is due to the presence of the *cis*- and *trans*-isomers in phytol, used in the synthesis of artificial vitamin K_1 (Fauler *et al.*, 1996).

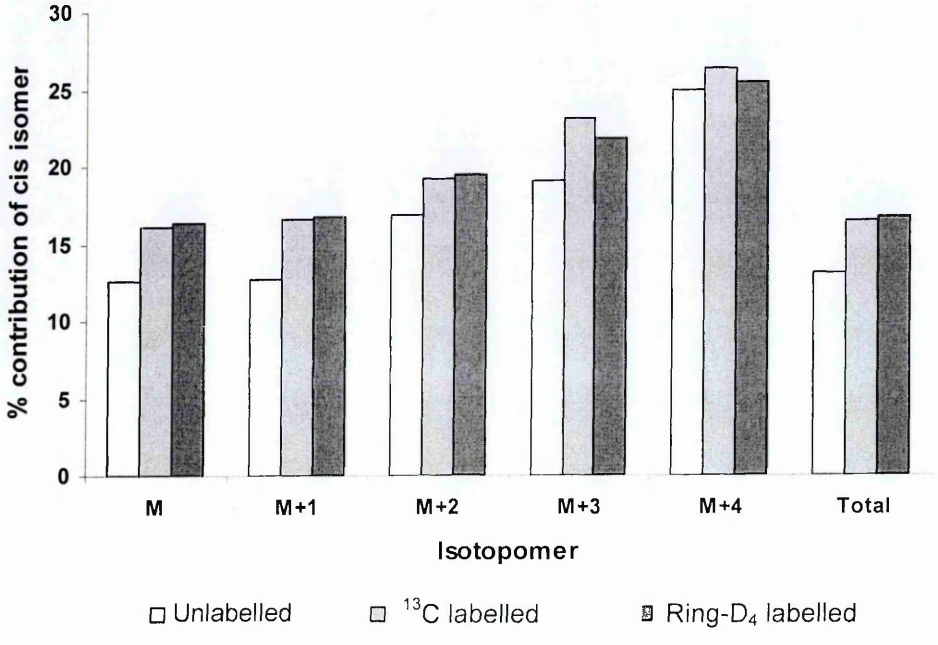
Figure 2-8. Total ion chromatograms of (A) unlabelled, (B) ^{13}C-labelled and (C) ring-D_4 vitamin K_1 standards analysed in SIM mode



2.6.8 Contribution of *cis*- and *trans*-isomers

Figure 2-9 shows the approximate contribution of the *cis*-isomer to total peak area for each of the vitamin K_1 standards for each isotopomer and total ion count. For unlabelled vitamin K_1, the *cis*-isomer contributes around 13% to total vitamin K_1, whereas for labelled standards the *cis*-isomer contributes 16 – 17%. These differences in the *cis:trans* ratio are possibly the consequence of variation in the manufacture and synthesis between suppliers of the unlabelled and labelled forms. Interestingly, in all three standards, the *cis*-isomer had greater relative abundance at the heavier isotopomers. There are a number of possible explanations for this observation. It may be due to the peak integration software. The *cis* and *trans* peaks are not fully resolved and thus each is integrated as far as the valley between to the peaks. The position of the vertical split may be affected by the size of the peak that decreases as the isotopomer number increases. Related to this is the observation that the heavier isotopomers elute slightly earlier than the lighter isotopes. Consequently, the *cis*-isomer contains an apparently higher proportion of the heavier isotopomers overlapping from the *trans*-isomer.

Figure 2-9. Percent contribution of the *cis*-isomer to each ion and total ion count, for unlabelled, ^{13}C, and ring-D_4 labelled standards



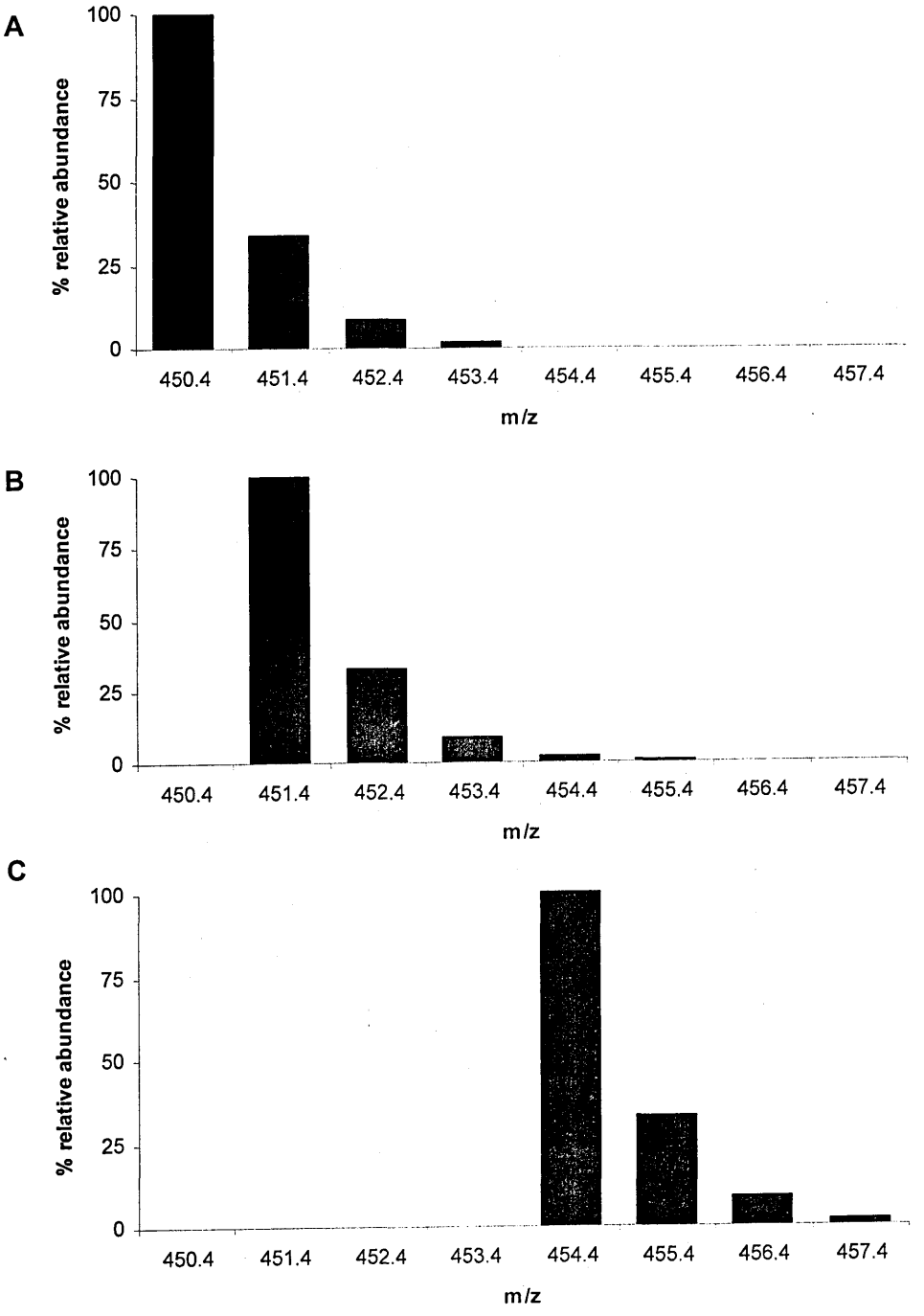
2.6.9 Selected ion monitoring analysis of vitamin K_1 standards

Selected ion monitoring (SIM) of the vitamin K_1 standards allowed accurate determination of the relative isotopomer distribution for each species. Figure 2-10 shows the SIM mass spectra for each standard. The pattern of isotopomer distribution is the same for each species but the m/z is shifted by one mass unit for the ^{13}C-labelled species and by four mass units for the deuterated species. The relative contributions are shown in Table 2-9, and are similar to the theoretical calculated values for unlabelled vitamin K_1 shown in Table 2-8.

Table 2-9. Relative abundance of M to M+4 ions for unlabelled, ^{13}C-labelled and ring-D_4 labelled forms of vitamin K_1

	% relative abundance		
m/z	Unlabelled	^{13}C	Ring-D_4
450.4	100.0		
451.4	33.7	100.0	
452.4	8.4	32.6	
453.4	1.6	8.9	
454.4	0.3	2.0	100.0
455.4		0.3	32.7
456.4			8.6
457.4			1.7
458.4			0.3

Figure 2-10. Selected ion monitoring mass spectra of (A) unlabelled, (B) ^{13}C-labelled and (C) ring-D_4 vitamin K_1 standards



2.6.10 Purity of labelled standards

In order to measure the isotopic purity of the labelled standards, the contribution of each of the ions from each species was divided by the total ion count from m/z 450 to the M+4 of the labelled species. So for the ^{13}C standard:

$$Purity = \frac{\sum \text{m/z } 451-455}{\sum \text{m/z } 450-455} * 100$$

Each standard was measured five times. Isotopic purities for ^{13}C- and ^{2}H-labelled vitamin K_1 were 98.4% and 98.0%, respectively.

2.7 Derivatisation

For separation by GC, compounds must be volatile and thermally stable. In derivatisation, functional chemical groups of the analyte molecule are altered by the addition of other functional groups to improve chromatographic behaviour and suitability for GC analysis. The improvement is the result of any of a number of processes: increased volatility, decreased polarity, improved thermal stability, improved separation, reduced tailing, improved sensitivity and/or increased molecular weight.

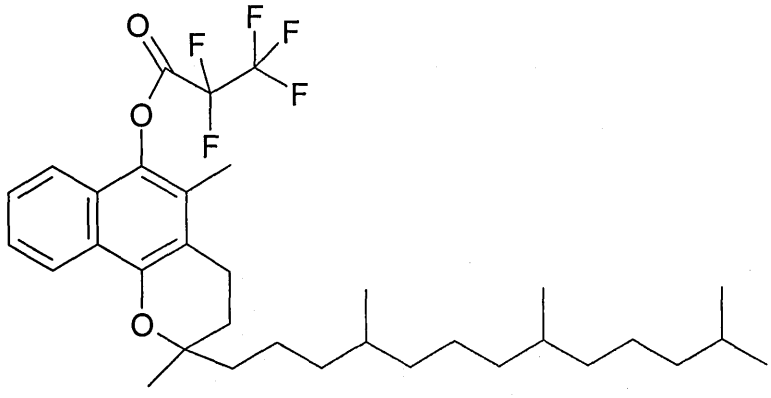
2.7.1 Derivatisation of vitamin K_1

Previous work found that derivatisation with *N*-perfluoroacyl anhydride preceded by reduction with zinc offered improvements in the analysis of vitamin K_1 compared to underivatised vitamin K_1 (Fauler *et al.,* 1996). In this reaction, reduction with zinc is spontaneously followed, under acidic conditions, by cyclization of the phytyl side chain converting the vitamin K-quinol into a chromanol (Langemann & Isler, 1965). The remaining hydroxyl group is converted to the perfluoro ester.

2.7.2 Selection of derivative

Initially, derivatisation was frequently unsuccessful or produced very variable yields. Changes in experimental procedures to include magnetic stirring in conical shaped vials and a change in reagent quality improved reliability of the derivatisation. Previous work favoured the heptafluorobutyryl derivative due to the large increase in molecular weight that removed the compound away from interfering compounds in plasma (Fauler *et al.,* 1996). However, for this work the pentafluoropropionyl derivative was selected (Figure 2-11). Although sensitivity was similar between the two derivatives, some interference was observed in the M+4 region of the heptafluorobutyryl derivative such that it was unsuitable for this work due to use of the ring-D_4 labelled form of vitamin K_1.

Figure 2-11. Molecular structure of the pentafluoropropionyl derivative of vitamin K_1



2.7.3 Derivatisation method

Pentafluoropropionic anhydride (derivatization grade, 99%), pentafluoropropionic acid (Fluka brand, ≥97.0%) and zinc dust (<10 microns) were purchased from Sigma (Sigma-Aldrich Company, Poole, UK) and hexane (Hipersolv) from VWR (VWR International Ltd, Poole, UK).

Derivatisation was performed in conical shaped 3 mL reaction vials (Supelco, Dorset, UK) in a Pierce Reacti-therm heating/stirring block (Perbio Science, Erembodegem, Belgium). 50 µL of pentafluoropropionic anhydride and 25 µL of pentafluoropropionic acid were added to a solution of vitamin K_1 in 1 mL of hexane. The mixture was stirred with a magnetic triangular spin vane and around 100 mg of zinc dust

was added to each sample. Vials were capped and stirred at room temperature. After 2 h, 1 mL of deionised water was added and the samples stirred for a further 5 min. The samples were transferred to disposable glass tubes (12 x 75 mm, Corning brand) and centrifuged for 5 min at room temperature at 1500 *g*. From the upper layer, 700 µL was transferred to amber GC vials. A further 1 mL of hexane was added to the glass tubes and, after centrifugation, removed and combined with the first aliquot. The samples were dried down under N_2 at 40 °C with a Pierce Reacti-therm heating block and Reacti-vap evaporator (Perbio Science, Erembodegem, Belgium) and reconstituted in 20 µL hexane prior to transfer to glass inserts. The derivatised samples were stored at –18 °C until analysis.

2.7.4 Results

Derivatised vitamin K_1 eluted slightly before underivatised vitamin K_1 as a single peak. Isomerisation of the molecule is removed during derivatisation in cyclisation of the phytyl side chain.

2.7.4.1 Total ion spectrum

Mass spectrometry of derivatised vitamin K_1 showed the molecular ion of unlabelled, ^{13}C- and ring-D_4 labelled of vitamin K_1 at m/z 598, 599 and 602, respectively (Figure 2-12, A-C). A second major ion was observed at m/z 333 (m/z 334 and 337 in labelled molecules) corresponding to the fragment ion after loss of the phytyl side chain (Figure 2-13).

Figure 2-12. Mass spectra of derivatised (A) unlabelled, (B) ^{13}C-labelled and (C) ring-D_4 vitamin K_1 standards

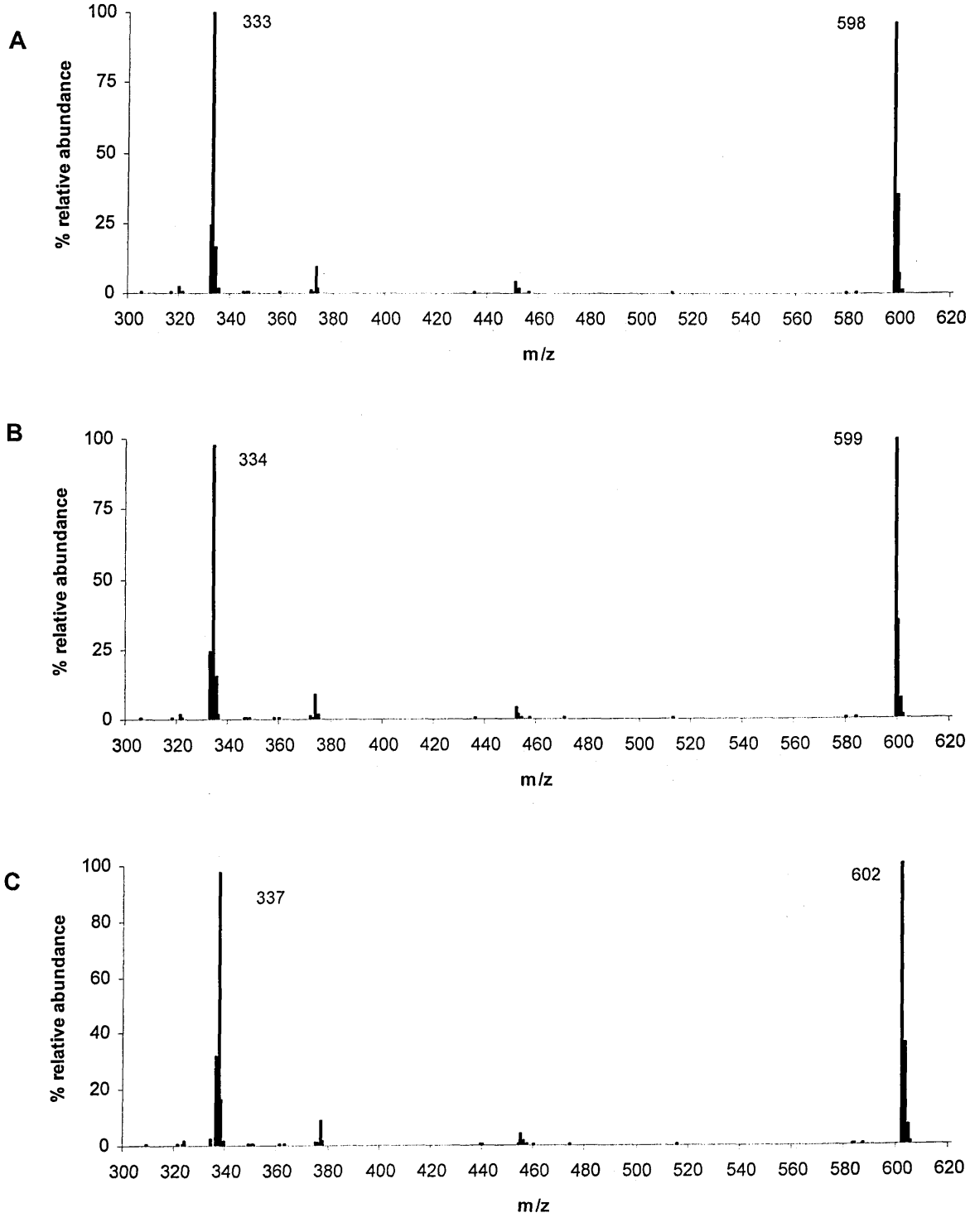
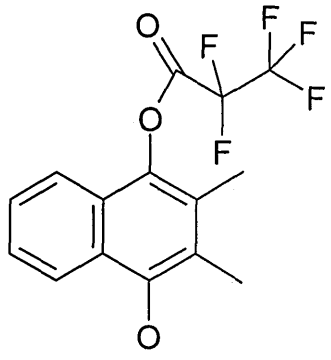


Figure 2-13. Molecular structure of the major fragment of the pentafluoropropionyl derivative of vitamin K_1



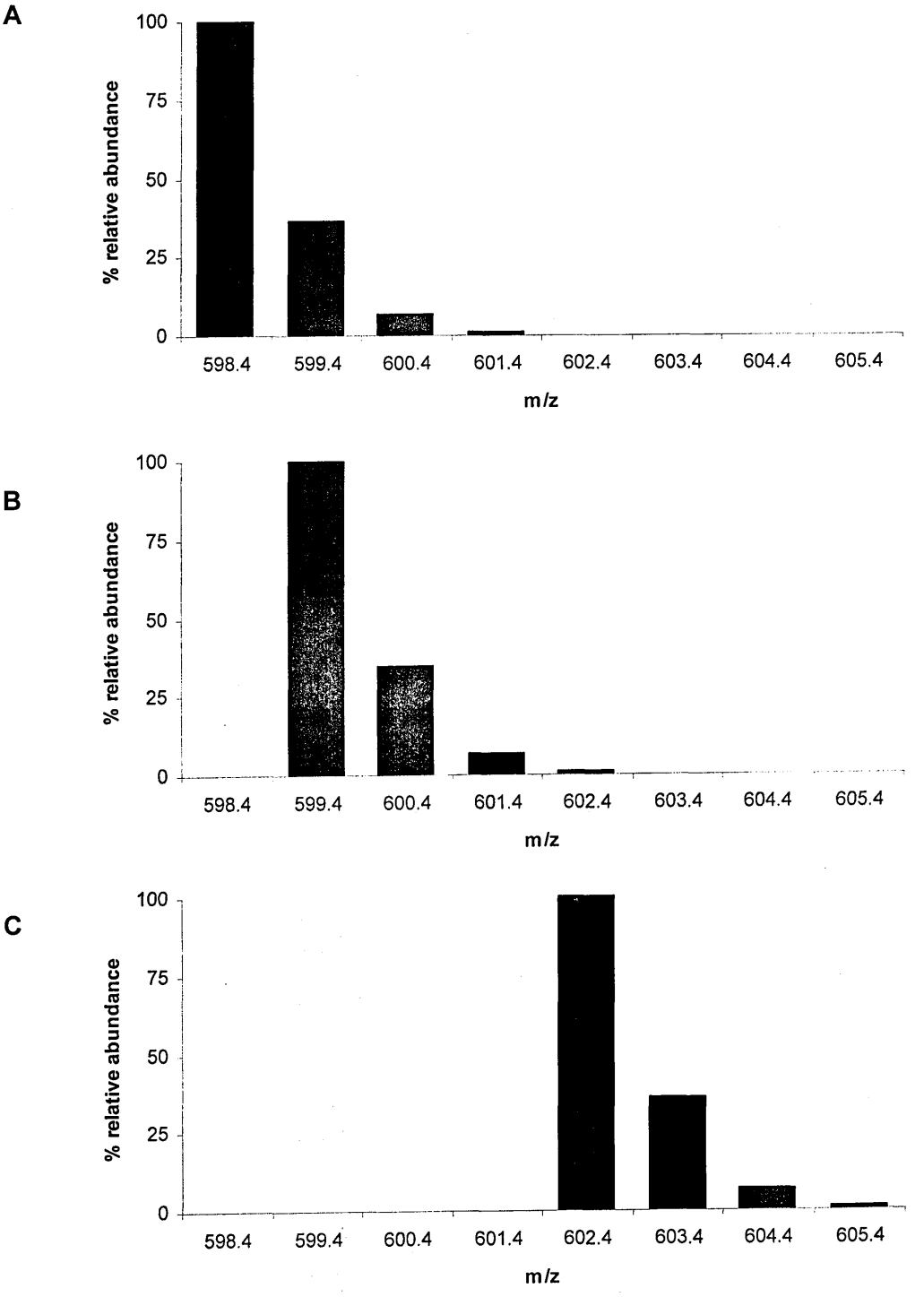
2.7.4.2 SIM analysis

For analysis by SIM, it was necessary to perform a dynamic mass calibration as for the underivatised samples. SIM analysis was subsequently performed at the decimal of 0.4 amu for each mass. The mass spectrum for each species is shown in Figure 2-14. The relative contribution of isotopomers to each species is in shown in Table 2-10.

Table 2-10. Relative abundance of M to M+4 ions for derivatised unlabelled, ^{13}C-labelled and ring-D_4 labelled forms of vitamin K_1

	% relative abundance		
m/z	Unlabelled	^{13}C	Ring-D_4
598.4	100.0		
599.4	36.8	100.0	
600.4	7.1	35.1	
601.4	1.0	7.0	
602.4	0.1	0.9	100.0
603.4		0.1	36.0
604.4			6.9
605.4			1.0

Figure 2-14. Selected ion monitoring mass spectra for derivatised (A) unlabelled, (B) ^{13}C-labelled and (C) ring-D_4 vitamin K_1



2.8 Extraction of vitamin K_1 from plasma

The best clean-up procedure is a compromise between minimising the amount of time required and providing a clean sample for analysis both in terms of maintaining chromatography and ensuring no co-eluting peaks. Saponification is an often-used procedure for the extraction of fat-soluble vitamins, however it was not suitable here because vitamin K_1 is sensitive to strong alkalis. Previous work suggested that deproteination, followed by solvent extraction and derivatisation may provide a method for analysis of vitamin K_1 by GCMS. However, it quickly became clear that this method would not be sufficient for this work due to rapidly deteriorating chromatography and lack of sensitivity. The chosen final extraction procedure required enzyme hydrolysis, solvent extraction and solid phase extraction, prior to derivatisation.

2.8.1 Blood samples

Initial experiments were hindered by the use of pooled plasma from an unknown source that proved unsuitable for method development. As a result an ethical application was submitted to Cambridge local research ethics committee (LREC) for the collection of plasma samples for method development work (Cambridge REC ref: 05/Q0108/30). Potential volunteers were provided with an information sheet (Appendix I) and informed written consent was obtained from all volunteers (Appendix II).

Blood for method development was collected from volunteers into 9 mL EDTA S-monovettes (Sarstedt Ltd, Leicester, UK). Samples were stored on ice and protected from light; samples were centrifuged at 4 °C for 20 min at approximately 2000 *g*. After centrifugation, plasma was removed with disposable plastic Pasteur pipettes and pooled in a single sterile container. After gentle mixing, pooled plasma was removed into 1 mL aliquots and stored at –70 °C until extraction.

Based on data from previous experiments with standards and assuming typical vitamin K_1 plasma concentrations of 0.29 – 2.64 nmol/L it was established that extraction of 1 mL of plasma would be necessary for the measurement of isotope ratios.

2.8.2 Enzyme hydrolysis

Enzyme hydrolysis has been used previously for the extraction of vitamin K from food, in particular milk, oils and infant formulas (Koivu-Tikkanen 2001). However, enzyme hydrolysis has not before been applied to the extraction of vitamin K_1 from plasma. Previous studies measuring carotenoids and vitamin E have used an enzyme hydrolysis step prior to analysis (Yeum *et al.,* 1996; Sommerburg *et al.,* 1997). Lipase was used for the hydrolysis of TAG, releasing glycerol and free fatty acids, and cholesterol esterase for the hydrolysis of plasma cholesterol esters.

2.8.2.1 Method development

A number of factors needed to be considered in the development of an enzyme hydrolysis method, including the amount of substrate, additional components for enzyme activation, buffer concentration and pH. Since it was preferable to perform hydrolysis of TAG and cholesterol esters simultaneously, it was necessary to compromise on the optimum conditions. Lipase and cholesterol esterase had the following definitions:

1) Cholesterol esterase – one unit hydrolyses 1.0 µmol of cholesteryl oleate to cholesterol and oleic acid per min at pH 7.0 at 37 °C in the presence of taurocholate.

2) Lipase – one unit hydrolyses 1.0 micro-equivalent of fatty acid from a TAG in 1 h at pH 7.7 at 37 °C.

As previously shown in Table 2-1, 1 mL of plasma typically contains approximately 1.5 mg of both TAG and cholesterol esters. Sodium taurocholate was used as an activator. The pH of 7.3 was chosen as a compromise between the optimum values for each enzyme.

2.8.2.2 Enzyme hydrolysis procedure

Cholesterol esterase (from *Pseudomonas sp.*, EC 3.1.1.13), lipase (from *Candida rugosa*, EC 3.1.1.3) sodium taurocholate, potassium phosphate monobasic (ACS reagent), and potassium hydroxide pellets (Aldrich brand) and were all obtained from Sigma (Sigma-Aldrich Company, Poole, UK).

Potassium phosphate buffer (0.1 M) was prepared and the pH adjusted to 7.3 by addition of potassium hydroxide pellets. Solutions of cholesterol esterase and sodium taurocholate were prepared by adding buffer to weighed quantities to obtain concentrations of 60 mg per 10 mL and 5 mg per 10 mL, respectively. Lipase (100 mg) was weighed into disposable culture tubes (16 x 100 mm, Corning brand) and to each was added 0.5 mL of both the sodium taurocholate and cholesterol esterase solutions. 1 mL of plasma was added to each tube. The tubes were covered with laboratory film and gently vortexed. They were then incubated in the dark for 2 h in a waterbath set to 37 °C. Every 20 min the samples were gently vortexed to disperse the lipase. After cooling, the samples underwent solvent extraction as described in section 2.8.3.

2.8.3 Solvent extraction

Vitamin K_1 dissolves in a number of solvents, including ethanol, ether, hexane and chloroform. Almost all previous sample preparation methods have used ethanol for deproteination and hexane to extract non-polar components from plasma (Fauler *et al.*, 2000).

2.8.3.1 Solvent extraction procedure

Absolute ethanol (Riedel-de Haën brand, ≥99.8%) was obtained from Sigma (Sigma-Aldrich Company, Poole, UK). Hexane (Hipersolv) was obtained from VWR (VWR International Ltd, Poole, UK). Following enzyme hydrolysis, and after cooling to room temperature, samples were vortexed and divided into two aliquots in disposable culture tubes (16 x 100 mm, Corning brand) for solvent extraction. Deproteination was achieved by the addition of 2 mL ethanol to each aliquot. The samples were then vortexed for 5 min at 1200 rpm (IKA Vortex, Esslab, Essex, UK). Extraction of the non-polar lipid fraction was performed with the addition of 3 mL of hexane and vortexing for 10 min at 1200 rpm. The samples were centrifuged for 10 min at 2000 *g*. The upper layer (3 x 0.75 mL) was removed and transferred to small disposable culture tube (12 x 75 mm, Corning brand). A further 1 mL hexane was added and each sample was vortexed and centrifuged as above, and 1 mL hexane removed and combined with the first extract. In order to reduce the

sample volume prior to solid phase extraction, the samples were dried down under N_2 at 40 °C to a volume of around 300 µL using a Pierce Reacti-therm heating block and Reacti-vap evaporator (Perbio Science, Erembodegem, Belgium).

2.8.4 Additional extraction procedures

For GCMS analysis both solid phase extraction (SPE) and semi-preparative HPLC have been utilised for the purification of vitamin K_1 from plasma. Fauler *et al.* (2000) have reviewed extraction methodologies prior to quantitative HPLC analysis of vitamin K_1 and all have used either SPE with or without semi-preparative HPLC. The use of these extraction methods is considered below.

2.8.5 Solid phase extraction (SPE)

Solid phase extraction is a form of chromatography using a solid stationary phase and a liquid (solvent) mobile phase. The stationary phase is packed into a polypropylene column and held in place by porous frits. The adsorbent is held at the bottom of the column to allow sufficient space for a reservoir of solvent. A wide range of adsorbents are available depending on the application. The generalised SPE procedure involves four steps 1) conditioning of packing material, 2) sample loading, 3) washing and 4) sample elution. Conditioning of the column removes any potential interfering chemicals remaining from manufacture and wets the packing material. After sample loading, the SPE column is washed to remove unwanted compounds from the packing material. The compound of interest remains adsorbed to the packing material. During elution, the compound of interest is more attracted to the solvent than to the packing material. Other, unwanted compounds remain bound to the adsorbent.

The SPE method used here is based on that described by Wang *et al.*, (2004). The method utilises 500 mg silica cartridges. An important consideration was the packing weight since these cartridges were used previously for the extraction of up to 0.5 mL plasma. Advice from manufacturers suggests that compounds to be extracted should not be more than 5% of the mass of the packing in the tube. With 500 mg of sorbent up to 25

mg of compounds can be loaded on the column. Based on the levels of extracted compounds shown in Table 2-1, 500 mg provided sufficient loading capacity.

2.8.5.1 Optimisation of SPE

For quantitative work, it is essential that all vitamin K_1 be eluted from the SPE column. Since the measurement of isotope ratios by GCMS is independent of vitamin concentration (in the absence of fractionation), it was possible to optimise the SPE procedure to minimise the elution of potentially interfering compounds, while ensuring the majority of vitamin K_1 was eluted. The method of Wang *et al.* (2004) requires a solution of 3.5% diethyl ether in hexane for the elution of vitamin K_1. To optimise elution, steps 1 to 3 of the procedure were followed as per Wang *et al* (2004).: (1) the column was washed with 6 mL each of diethyl ether in hexane followed by hexane only, (2) the sample was added and (3) column was then washed with hexane prior to elution. Solutions of 0.4 mg of vitamin K_1 per 1 mL of hexane were prepared. High concentrations were used to permit measurement using a Unicam 5625 UV spectrophotometer (Unicam, Cambridge, UK). Vitamin K_1 was measured at wavelengths 243, 247 and 248 nm (Langemann & Isler, 1965). Solutions of diethyl ether in hexane were prepared as 0.5%, 1%, 1.5%, 2%, 2.5%, 3% and 3.5%. After sample loading, the vitamin K_1 was eluted with each of the solutions in turn, from the 0.5% solution upwards. It was found that maximum concentration in the eluate was reached with 1.5% solution, and thus this solution was chosen for use during the extraction procedure.

2.8.5.2 SPE procedure

Hexane (Hipersolv) and diethyl ether (for chromatography) were obtained from VWR (VWR International Ltd, Poole, UK). The SPE cartridges (Waters Sep-Pak-RC™ 500 mg silica, Hertfordshire, UK) were placed in a 12-port SPE vacuum manifold (Supelco, Dorset, UK) and conditioned with 4 mL 1.5% diethyl ether in hexane and then with 4 mL hexane. Sample extracts were loaded and the sample tubes rinsed with a further 200 µL of hexane. The cartridges were washed with 6 mL of hexane and the vitamin K_1 was eluted with 7 mL 1.5% diethyl ether in hexane. The sample eluate was

collected into disposable glass tubes (13 x 100 mm, Fisher brand) and evaporated to dryness in a vacuum evaporator (Savant, NY, USA) for 30 min with a medium drying rate. The samples were reconstituted in 1 mL hexane, transferred to amber GC vials (Agilent Technologies, Stockport, UK) and stored at –18 °C until derivatisation.

2.8.6 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a powerful and versatile method of separating complicated mixtures of compounds. The main components of a HPLC system are the solvent reservoir, a high-pressure pump, injector, column, detector and, depending on the machine set-up, a sample collector. HPLC columns are typically formed from stainless steel and contain a stationary phase that can be polar (for normal phase chromatography) or non-polar (for reversed phase chromatography). For quantitative or analytical work, columns with a diameter between 1 and 4 mm are used whereas for semi-preparative work, columns of diameter up to 25 mm are available. One or more solvents are used as mobile phase and pumped at high pressure through the column. If a single pump is used the mobile phase is termed isocratic, otherwise a two-pump system allows the use of solvent gradient for greater control of chromatography. A wide-range of detectors are available for HPLC although most are non-specific. These include UV/VIS detectors, fluorescence and electrochemical detectors. The use of mass spectrometers and liquid chromatography were considered in section 2.2.2.

2.8.7 Semi-preparative HPLC

The use of semi-preparative HPLC was investigated to assess if this method provided any advantages over enzyme hydrolysis and SPE. Previous authors have used HPLC to purify plasma samples prior to the analysis by GCMS (Dolnikowski *et al.,* 2002; Erkkilä *et al.,* 2004) and HPLC is commonly used for the purification of vitamin K_1 from food samples.

2.8.7.1 Scale-up from analytical methodology

A scaled-up protocol based on the analytical methodology was used (see section 2.12). Scaling-up was necessary due to the greater volume of plasma to be extracted. The critical changes concerned the HPLC column, namely length, width and pore size. The analytical column was a Thermo BDS Hypersil C_{18}, 15 cm in length, 3 mm wide with 3 µM pore size. For semi-preparative work it was necessary to use a column with a larger pore size due to the greater solvent flow through the column. However, a column identical to the analytical column (with the exception of pore size) was unavailable from Thermo. Thus, the first step in method development was to source a column with the same properties and chromatography as the analytical column, but from a supplier that also offered a 5 µM pore size. For column comparison work the scanning fluorescence detector was used. An ACE-3 C_{18} (10 x 250 mm), 3 µM particle size was sourced (HiChrom, Reading, UK) and vitamin K_1 chromatography compared between the routine analytical system available in our laboratory against a new semi-preparative system. Vitamin K_1 chromatography was similar between the analytical and semi-preparative HPLC systems and with the two different columns. The second step was to scale up from the analytical column to semi-preparative column. Equations to calculate of the size of column required for semi-preparative HPLC were obtained from Waters (the HPLC manufacturer) website.

$$\text{scale up factor} = \frac{(\text{diameter semi prep column})^2 \text{ x length semi prep column}}{(\text{diameter analytical column})^2 \text{ x length analytical column}}$$

The scale-up factor was calculated from analytical sample load (up to 125 µL plasma) and the potential plasma sample volume (2 mL) necessary to provide sufficient vitamin K_1 for GCMS analysis. Thus, the scale-up factor was calculated as 16. A column length of 250 mm was selected. Column diameter was then calculated by rearranging the above equation to:

$$\text{diameter semi prep column} = \sqrt{\frac{\text{scale up factor} \times (\text{diameter analytical column})^2 \times \text{length analytical column}}{\text{length semi prep column}}}$$

Thus,

$$9.3 = \sqrt{\frac{16 \times (3)^2 \times 150}{250}}$$

The semi-preparative column dimensions were finalised as diameter 10 mm, length of 250 mm and a particle size of 5 µM. A disadvantage of using columns with greater particle size is a decrease in column efficiency and resolution. Increasing the column length can provide a small increase in resolution but ultimately resolution is decreased with the scaled-up procedure. Practically, although resolution was decreased, separation of vitamin K_1 from other components was still possible.

2.8.7.2 Semi-preparative HPLC procedure

Initial work concentrated on optimising the mobile phase and injection parameters. The chosen mobile phase was a 10% isocratic solution since at the required flow rates the use of a two-pump gradient approach produced large fluctuations in the baseline. For develop work high concentrations of vitamin K_1 standards were used with the dual gamma absorbance detector.

The final stage was to compare semi-preparative HPLC with enzyme hydrolysis. Eight 1 mL pooled plasma samples underwent solvent extraction and SPE. Four of the samples were previously subjected to enzyme hydrolysis while the remaining four underwent HPLC. Two of each of the samples (non-HPLC and HPLC) were spiked with an additional 500 pg of vitamin K_1. On the semi-preparative HPLC, the retention time of vitamin K_1 was around 22 min and sample was collected with the fraction collector using a time window of 1.5 min. The eluate was dried down under N_2 at 35 °C. All samples were derivatised as described in section 2.7.3 and then analysed by GCMS.

The HPLC was set up as detailed below:

HPLC: Waters 1525EP Binary HPLC Pump with manual injection
Waters 474 Scanning Fluorescence Detector
Waters 2487 Dual λ Absorbance Detector

Solvents: 10% dichloromethane in hexane (with in-line degasser)

Column: ACE-5 C_{18} (10 x 250 mm), 5 µM particle size (p/n ACE-121-2510) with 3 mL/min flow rate

Fraction collector: Waters Fraction Collector III

2.8.7.3 Results

Qualitatively, the HPLC extracted samples appeared slightly 'cleaner' than the enzyme hydrolysis samples. However, all samples had relatively clean chromatograms suitable for isotope ratio measurements. Samples that did not undergo HPLC had a greater abundance than those that were separated by HPLC.

2.8.7.4 Conclusions

Further extraction by HPLC may provide some benefit in terms of a cleaner chromatogram and therefore less gas chromatograph maintenance. However, this advantage is outweighed by the apparent greater losses (possibly from fraction collection or additional drying down step) and additional time and effort.

2.9 Final extraction methodology

The final protocol for the extraction of vitamin K_1 from human plasma utilised enzyme hydrolysis with lipase and cholesterol esterase, solvent extraction and solid phase extraction, prior to derivatisation and analysis by GCMS. For the analysis of plasma samples, the GC parameters were the same as for the standards described above (see Table 2-6) but with the addition of a final temperature ramp of 40 °C/min from 325 °C to 350 °C and a hold time of 5 min to remove contaminating material. This method provided a sufficiently clean sample to measure isotope ratios of extracted vitamin K_1 on

the GCMS. An example chromatogram from plasma is shown in Figure 2-15. A summary of the sample extraction protocol is shown in Figure 2-16.

Figure 2-15. Total ion chromatogram (m/z 598.4 – 602.4) of vitamin K_1 extracted from pooled plasma

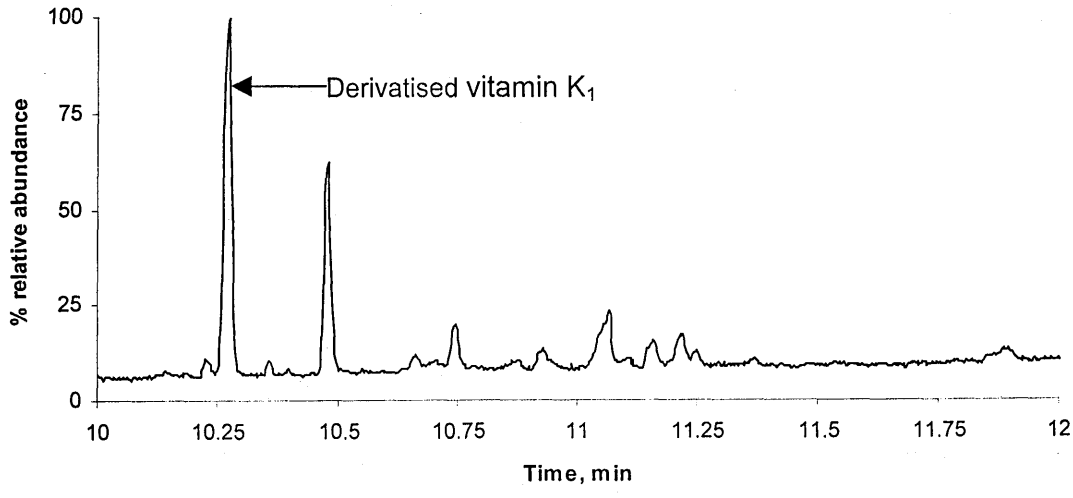
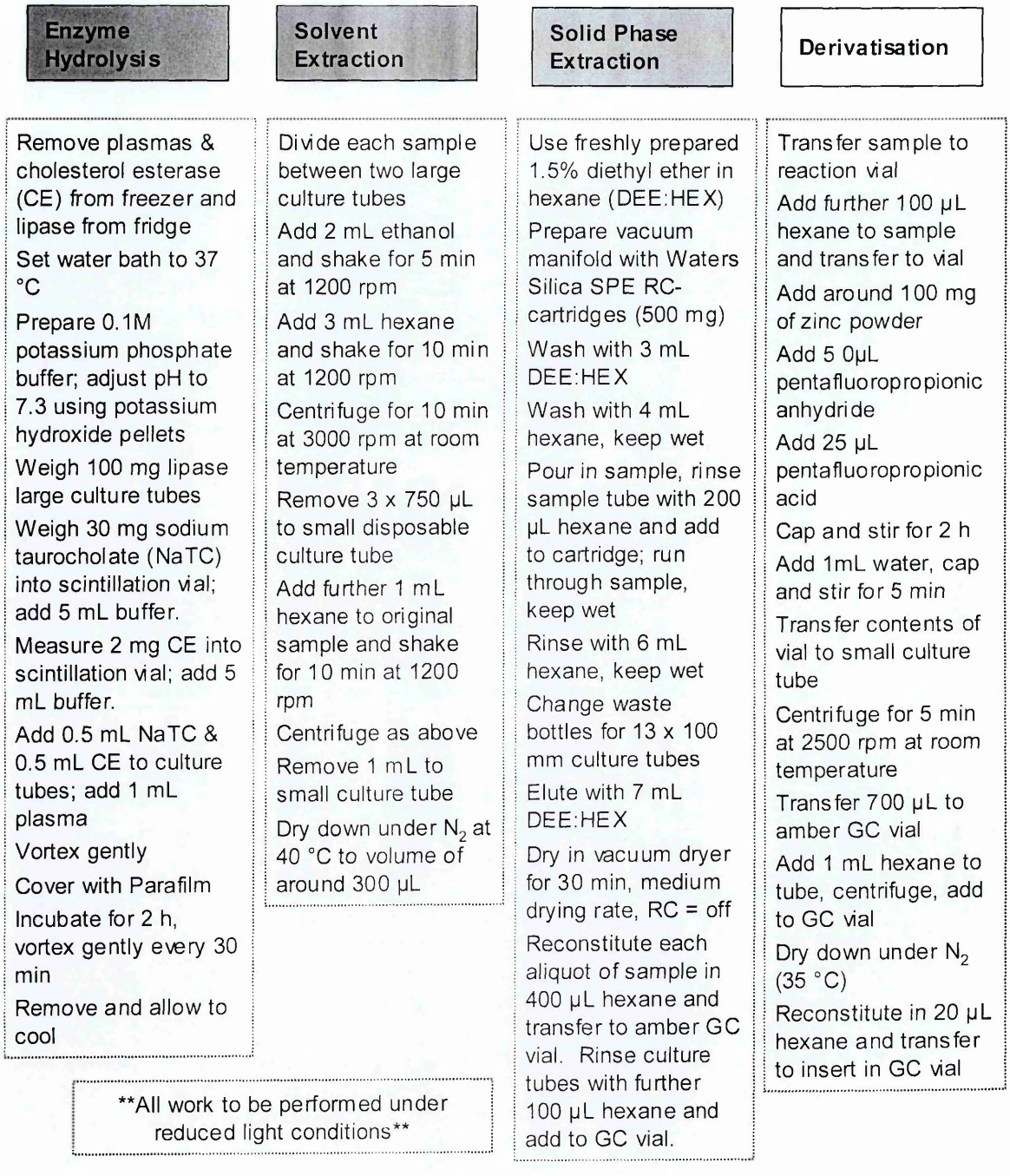


Figure 2-16. The analytical methodology for the extraction and derivatisation of vitamin K_1 from human plasma samples



Abbreviations: CE, cholesterol esterase; NaTC, sodium taurocholate; DEE, diethyl ether; HEX, hexane; SPE, solid phase extraction; GC, gas chromatography

2.10 Method validation and quality

Prior to the analysis of each batch of samples in the subsequent volunteer studies, an underivatised vitamin K_1 standard was analysed to check chromatographic quality and mass spectrometer performance. If chromatographic quality was poor (assessed by the degree of separation of the *cis*- and *trans*-isomers), an approximate 0.5 m length of guard column was trimmed from the injector side. If incorrect isotope ratios or low sensitivity were observed then the mass spectrometer was retuned, and, if necessary, the ion source cleaned. Samples from a single volunteer visit were analysed as a single batch.

2.10.1 Linearity

The testing of linearity is an important part of method validation for tracer methodology and isotope ratio analysis. The linearity of stable isotope measurements was assessed by the volumetric preparation of solutions ranging from 0.3125 to 20% enrichment at a plasma concentration of 1.1 nmol/L. The resulting linear regressions had an R^2 value of 0.997 for the ^{13}C labelled and 0.999 for the ring-D_4 labelled. Concentration dependent linearities were less of a concern in this work because of the narrow range of expected and observed concentrations of vitamin K_1 in plasma.

2.10.2 Precision

Intra- and inter-assay precisions were calculated from multiple analyses of extracted and derivatised pool plasma samples. The coefficient of variation (CV) of injections from the same vial for the M+1/M isotope ratio in plasma was less than 3.5%. The intra-assay CV of samples extracted together and analysed together was less than 2%. Finally, the inter-assay precision for analyses performed on different days was less than 3%. At typical physiological concentrations it was not possible to measure the M+4/M isotope ratio due to the small contribution of the M+4 isotopomer at natural abundance (less than 0.1%).

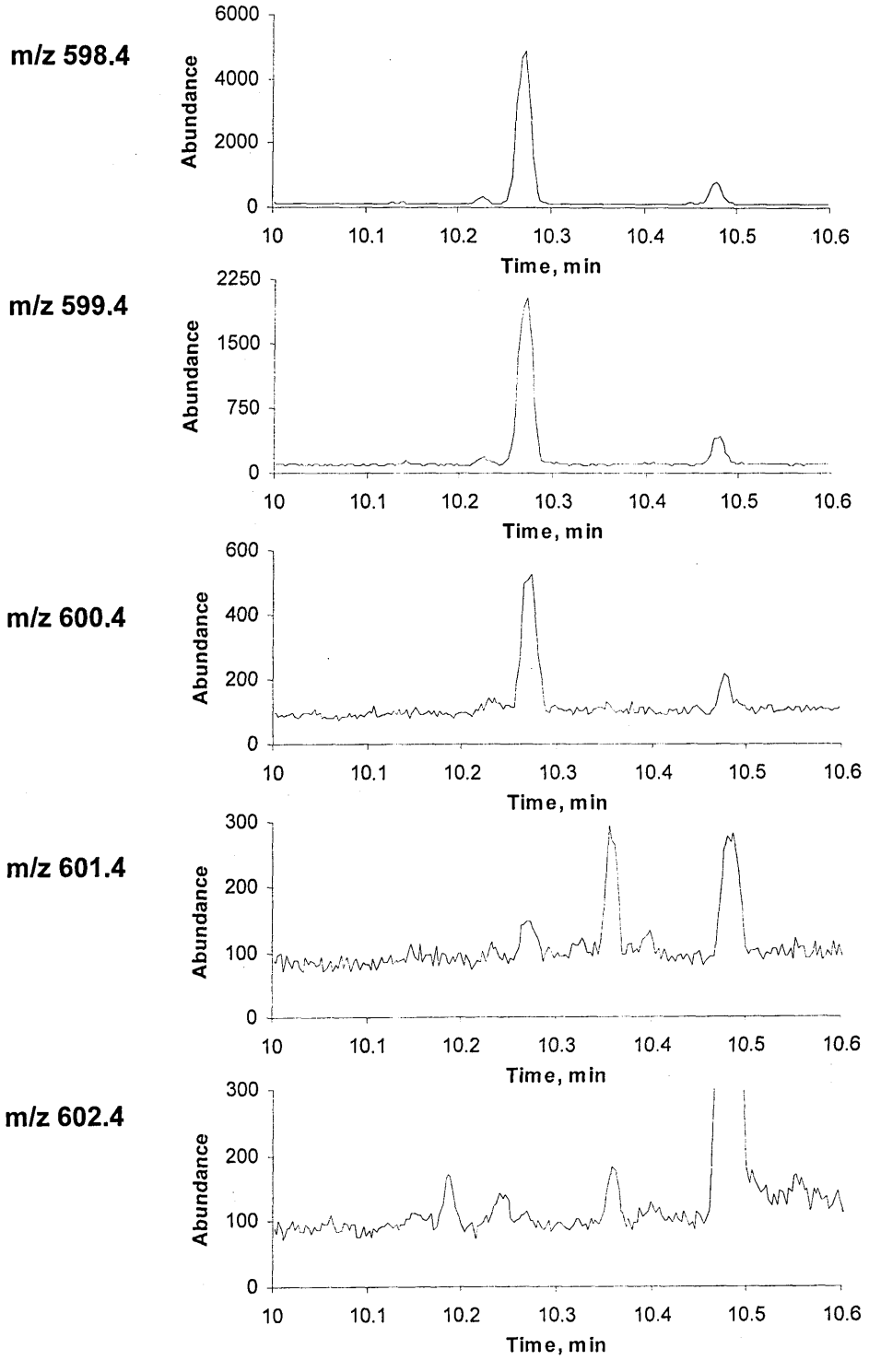
2.10.3 Limit of detection

The limit of detection (LOD) for isotope ratio analysis is often higher than for quantitative analysis since the total abundance is divided amongst the contributing isotopomers. Thus, here the limit of quantitation is more relevant and is estimated to be at least 6 pg per injection (1 µL). With the described methodology this is equal to a plasma concentration of around 0.3 nmol/L. This value can be improved to around 0.15 nmol/L by decreasing the final sample volume to 10 µL.

2.11 Peak integration

Isotope ratios were calculated using the fitting methods of Bluck and Coward (1997) of which a detailed description is presented later in section 3.6.1. Typical chromatograms obtained after extracting ions 598.4 – 602.4 m/z from the cumulative ion counts are shown in Figure 2-17. Note that at physiological concentrations it is not possible to measure m/z 602.4.

Figure 2-17. Extracted ion chromatograms of derivatised vitamin K_1 in plasma



2.12 Quantitative analysis of vitamin K_1 by HPLC

Quantitation of plasma vitamin K_1 was performed using the method of Wang *et al.* (2004). The principle of the method is based on the reduction of vitamin K_1 to its fluorescing product, dihydro-vitamin K_1. The use of a post-column zinc reactor is reported to have 95% conversion rate (Fauler *et al.,* 2000). The method is summarised here.

Absolute ethanol (Riedel-de Haën brand, ≥99.8%) was obtained from Sigma (Sigma-Aldrich Company, Poole, UK). Dichloromethane, hexane, diethyl ether, methanol, acetone, sodium acetate, acetic acid, and zinc chloride were all obtained from VWR (VWR International Ltd, Poole, UK). All solvents were HPLC grade.

2.12.1 Sample extraction

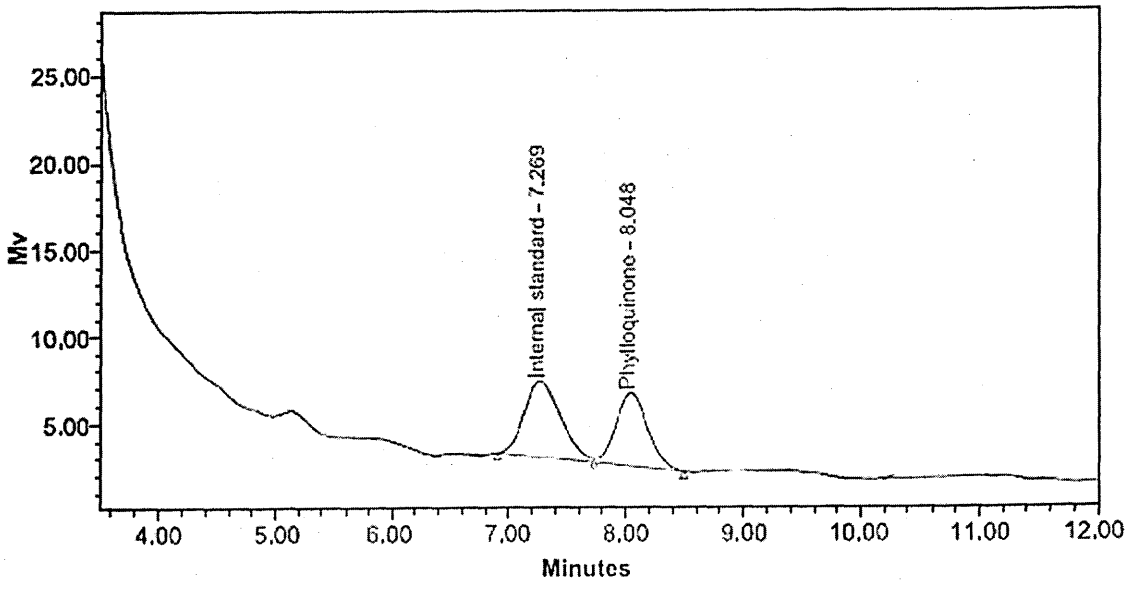
Plasma aliquots of 0.25 mL were made up to 0.5 mL with 0.9% (w/v) of saline and proteins were precipitated with 1 mL of ethanol in large disposable culture tubes (16 x 100 mm, Corning brand). Ethanol for deproteination contained 250 pg of internal standard (IS), a proprietary vitamin K-derivative obtained from Immundiagnostik AG (Bensheim, Germany) and was added to compensate for procedural losses during solvent extraction and SPE. Samples were vortexed for 5 min and 3 mL of hexane added. Samples were then vortexed for 10 min and the upper layer (3 x 0.75 mL) transferred to a clean tube. Solid phase extraction was performed as described in section 2.8.5.2 with the exception that 3.5% diethyl ether in hexane was used to elute vitamin K_1. After evaporation, the sample was reconstituted in 100 µL of solvent, consisting of 25 µL of dichloromethane and 75 µL mobile phase solvent A (see below).

2.12.2 Sample analysis

Analysis was performed on a Waters 2790 separation module with an in-line degasser and a Waters 474 scanning fluorescence detector (Waters, Milford, MA, USA). Separation was performed using a Hypersil BDS-C_{18} column (3.2 x 150 mm), with a 3 µm particle size (Thermo brand, HiChrom, Reading, UK). A guard column was installed in front of the analytical column (Hypersil BDS-C_{18}, 3.2 x 7.5 mm, 5 µm particle size). The

column temperature was kept constant at 22 °C during analysis by a Vydac Model 7956 column temperature controller (Hichrom, Reading, UK). The post-column zinc reactor (for reduction of vitamin K_1) was a stainless steel column (2.1 x 50 mm) packed with zinc dust (<10 microns, Sigma-Aldrich, Poole, UK) and installed between the analytical column and fluorescence detector. In-line graphite pre-filters were installed, one before the guard column and one after the zinc reactor. Fluorescent detection wavelengths were at 244 nm excitation and 430 nm emission. Stepwise gradient elution was performed on the HPLC to separate vitamin K_1 and other plasma components. Solvent A consisted of 99.45% methanol and 0.55% aqueous solution of 2 mol/L zinc chloride, 1 mol/L acetic acid and 1 mol/L sodium acetate. Solvent B was 100% dichloromethane. The solvent gradient was i) 95% solvent A and 5% solvent B for 10 min, ii) 65% solvent A and 35% solvent B for 13 min and iii) 95% solvent A and 5% solvent B for 5 min. The flow rate was 0.6 mL/min and the run time was 28 min. A typical chromatogram from the HPLC is shown in Figure 2-18.

Figure 2-18. HPLC chromatogram of vitamin K_1 extracted from plasma



2.12.3 Data analysis and quality control

Peak areas for the IS and sample vitamin K_1 peaks were measured using the Waters data processing software, Millennium 3.2. For quantitation, a single point calibration standard, with IS, containing the equivalent of 1.25 ng/mL of vitamin K_1 was used. Vitamin K_1 concentration was calculated from the ratios of IS to vitamin K_1 peaks in the samples against the ratio generated from the calibration standard. Quality control was performed by the regular (at least weekly) analysis of vitamin K_1 extracted from plasma samples of known concentration. Intra-assay CVs were around 5% and inter-assay around 12%. The laboratory also participates in the vitamin K external quality assurance scheme (KEQAS) run by the Human Nutristasis Unit at Guy's and St Thomas' Hospital.

2.13 Section 2 conclusions

Until now, only a single method has been published for the extraction and measurement of isotope ratios of vitamin K_1 from plasma with GCMS. This procedure involved laborious sample preparation and required the use of semi-preparative HPLC prior to analysis of isotope ratios by GCMS. Even with extensive sample preparation, problems with the analysis were still reported (Erkkilä *et al.*, 2004). A new method has been presented here that uses enzyme hydrolysis, solvent extraction, solid phase extraction, and subsequent derivatisation and isotope ratio measurement by GCMS of vitamin K_1 from plasma, and that removes the need for semi-preparative HPLC. Stable isotope tracers provide a powerful tool to investigate questions of vitamin kinetics and metabolism. The procedure presented here allows further study of vitamin K_1 absorption and metabolism using stable isotope labelled vitamin K_1 and GCMS.

3 STUDY 1: MEASUREMENT OF VITAMIN K_1 KINETICS AND BODY POOL SIZES

3.1 Background

In the United Kingdom, there is no daily recommended intake for dietary vitamin K_1 but rather a guideline value of 1 µg/kg body wt/d that was set in 1991, (Department of Health 1991) based largely on the requirements to maintain blood coagulation (Frick *et al.,* 1967). More recent evidence suggests a wider function for vitamin K_1 beyond its traditional role in blood clotting (Schurgers & Vermeer, 2001; Binkley *et al.,* 2002; Vermeer *et al.,* 2004). As a result, an Adequate Intake (AI) in the United States has recently been set at 90 and 120 µg/d for women and men, respectively. This value is based primarily on the median intakes in healthy individuals from the third National Health and Nutrition Examination Survey (NHANES III) (Institute of Medicine 2001). The use of mean intakes from large epidemiological surveys is one approach to setting recommended intakes. However, it is assumed that the average intake is appropriate because the population is apparently healthy and does not suffer from any vitamin K_1 deficiency related disease. The method is limited by gaps in our knowledge of the role of a specific nutrient. Additionally, this approach is heavily reliant on both accurate and representative food composition data and the accurate reporting of nutritional intake. Experimental approaches offer an alternative, and can provide data on vitamin K_1 physiology, comprising kinetics and turnover rates, and bioavailability. Kinetic and turnover data provide an estimate of the actual use of vitamin K within the body, without the need for prior knowledge of the physiological functions of vitamin K.

In Western populations, vitamin K_1 is the primary dietary form of vitamin K (Schurgers *et al.,* 1999). Although vitamin K_1 is found in many foods there is uncertainty over the extent of vitamin K_1 availability from the diet. There have been several studies to measure relative availability from measurements of the area under the plasma response curve following oral doses from a variety of sources, including some designated as standards such as Konakion®. (Gijsbers *et al.,* 1996; Booth *et al.,* 1999a; Garber *et al.,*

1999; Booth *et al.*, 2002). However, the results are difficult to compare because a variety of doses have been used, ranging from those typical of a high daily intake but fed in a single meal, to quantities at least an order of magnitude higher, and experimental duration has varied from hours to days.

More reliable data might be expected to emerge from studies that measure absolute amounts of vitamin K_1 absorption. In faecal balance studies, absolute absorption was estimated as around 80% (Shearer *et al.*, 1970a; Shearer *et al.*, 1974). To comprehend fully absorption and to measure absolute vitamin K_1 absorption directly, an understanding of both uptake and disposal kinetics is required.

A number of approaches are available for the measurement of the kinetics of vitamins in humans, including single dose studies with unlabelled compounds, depletion/repletion methods and tracer techniques (Bates *et al.*, 2004). A tracer is a marked form of a substance that can be used to determine kinetic properties of that substance in biological systems. Tracer studies provide a powerful tool since kinetics can be measured at physiological levels using a labelled dose of the compound of interest. Compounds can either be radiolabelled or labelled using stable isotopes. Radioactive tracers have been used to explore the kinetics of vitamin K_1 metabolism but early investigations (Shearer *et al.*, 1970a; Shearer *et al.*, 1972; Shearer *et al.*, 1974; Shepherd *et al.*, 1977) were restricted both by a low specific activity of the tracer dose and the lack of a suitable methodology for the analysis of vitamin K concentrations in plasma. More recently, Olson *et al.* (2002) performed experiments that used physiological levels of radiolabelled vitamin K_1 of much higher specific activity, with high performance liquid chromatography (HPLC) for quantitative measurements, and obtained kinetic data substantially different from those obtained previously.

The use of radioactive tracers is no longer favoured due to ethical considerations. Thus, a major part of this research was to develop the use of a stable isotope methodology to measure vitamin K_1 kinetics in humans. Stable isotope tracers have a number of advantages over radiolabelled compounds. Firstly, they are not a source of ionising radiation and are therefore suitable for use in human studies; secondly, with gas

chromatography mass spectrometry (GCMS), it is possible to positively identify the compound of interest by its mass spectrum while at the same time measuring tracer/tracee molar ratios, and finally, it is possible to simultaneously use more than one tracer (oral and *iv*) to separate the kinetics of absorption and metabolism (Matthews & Bier, 1983; Dainty 2001; Stellaard 2005).

3.2 Study objectives

The three objectives of this study were to:

I. Develop a stable isotope methodology for the measurement of vitamin K_1 kinetics
II. Obtain data on vitamin K_1 kinetics, turnover and body pool sizes in humans
III. Characterise the absorption of an oral dose of vitamin K_1 that can be used in a study to measure vitamin K_1 bioavailability

3.3 Kinetic analysis

Prior to describing the experimental and data analysis methods used in the study, general descriptions of kinetic analysis and compartmental modelling are presented.

3.3.1 Compartments and compartmental modelling

The term compartment does not necessarily relate to a specific physiological entity, but rather comprises any number of physiological components that, kinetically at least, are homogenous (Dainty 2001), although in some circumstances the sampled compartment may be well characterised, e.g. plasma. A compartmental model is a theoretical construct linking any number of compartments with exchange between them. If linear kinetics are assumed, then the fluxes of transfer between compartments are related to the concentration, which leads to a mathematical description of the system which is the sum of a number of exponential terms. The number of exponentials needed to describe the kinetics of transfer is equal to the number of compartments. Thus, the amount of tracer remaining in a system that comprises a single compartment is characterised by a single exponential decay. However, if a semi-logarithmic plot of

remaining tracer against time is non-linear, then the presence of additional compartments in the system is suggested.

3.3.2 Curve peeling, intercepts and slopes

The method of curve peeling, or graphically estimating the intercepts and slope of an exponential, provides a method of estimating the coefficients of equations that describe the kinetic behaviour of a tracer. These terms can then be used to further probe the kinetic parameters of a system. The equation describing the disappearance of tracer from a model with two compartments is written as:

$$q_1 / q_{10} = H_1 e^{-g_1 t} + H_2 e^{-g_2 t}$$

Where:

q1/q10 is the fraction of tracer remaining in pool 1

H_1 and H_2 are the intercepts (coefficients) for each of the exponential terms normalised as a fraction of the total

g_1 and g_2 are the slopes for each of the exponential terms

e = Euler's number (also called Napier's constant)

t = time

3.3.3 Rate constants

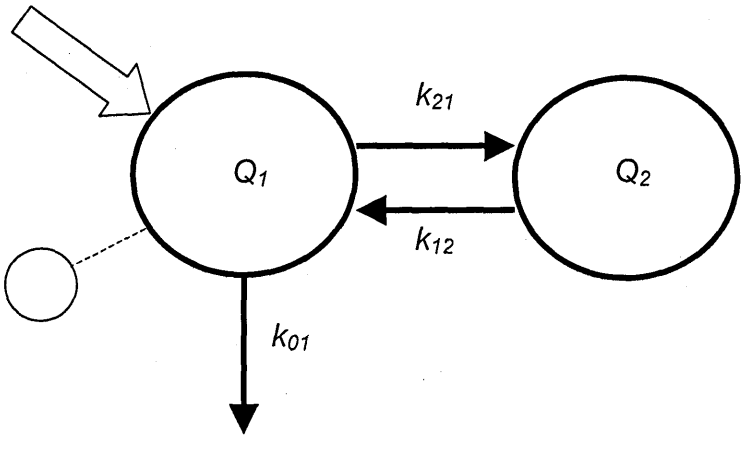
In compartmental models, exchange between pools are expressed in terms of k, defined as a rate constant of transfer from a pool as a fraction of total content moving per unit time. The subscripts refer to the direction of movement with the convention of movement **from** the second digit **to** the first digit. For example, k_{21} is the rate constant for transfer from pool 1 to pool 2. The use of 0 in the subscript denotes movement to outside the system, e.g. k_{01}. Two identical subscript digits denote the sum of all output from that pool, e.g. k_{11} describes movement to both pool 2 and the outside from pool 1. The fractional rate constant k, can also be expressed as F, the actual rate (flux) by incorporating mass in the equation:

$$k = F / Q$$

3.3.4 Two-compartment model for vitamin K_1 kinetics

The model used in the present study was a two-compartment model (Figure 3-1) with output (k_{01}) from the sampled (plasma) pool of size (Q_1), and exchange between it and a remote compartment (Q_2) defined in terms of k_{21} (to 2 from 1) and k_{12} (to 1 from 2). These combine to give two exponential terms for the disappearance of the *iv* dose.

Figure 3-1. Two compartment model of vitamin K_1 kinetics



Q_1 *and* Q_2 = Body pools

k = rate constants

Large open arrow shows entry of *iv* dose

Dashed line indicates sampling pool

3.3.5 Calculating rate constants from intercepts and slopes

Once values for the intercepts and slopes (H and g) are obtained, any and all of the rate constants for a compartmental model can be deduced, including for those pools and rates that have not been sampled directly (assuming there is a steady state for tracee, i.e. that body pools are constant and input is equal to output). A steady-state for tracee is assumed because when using stable isotope tracers, the tracer concentration is derived from the tracer to tracee ratio.

In a two-compartment model with exchange between two pools and input and output from a single pool the following equations apply:

$$k_{11} = H_1 g_1 + H_2 g_2$$
$$k_{01} + k_{21} = H_1 g_1 + H_2 g_2$$

$$k_{11} + k_{22} = g_1 + g_2$$
$$k_{01} + k_{21} + k_{12} = g_1 + g_2$$

$$k_{11} k_{22} - k_{21} k_{12} = g_1 g_2$$
$$(k_{01} + k_{21}) k_{12} - k_{21} k_{12} = g_1 g_2$$

$$k_{12} = \frac{g_1 g_2}{k_{01}}$$

$$k_{01} + k_{21} + \frac{g_1 g_2}{k_{01}} = g_1 + g_2$$
$$k_{21} = g_1 + g_2 - k_{01} - \frac{g_1 g_2}{k_{01}}$$

$$k_{01} + g_1 + g_2 - k_{01} - \frac{g_1 g_2}{k_{01}} = H_1 g_1 + H_2 g_2$$
$$k_{01} = \frac{g_1 g_2}{H_1 g_2 + H_2 g_1}$$

$$k_{12} = \frac{g_1 g_2}{k_{01}}$$
$$k_{21} = g_1 + g_2 - k_{01} - k_{12}$$

The calculations described here refer specifically to the two-compartment model used in the present study, but the same principles apply for any model with two, or more compartments.

3.3.6 Half-times

A common practise when describing the kinetics of a system is to report simply the half-time ($T_{½}$) of disappearance. Half-times are calculated using the slopes of each of the exponentials:

$T_{½} = 1n / g_1$ or

$T_{½} = 0.693 / g_1$

3.4 Considerations for study design

Data on kinetics can be obtained with oral doses of a tracer, but assumptions need to be made about absorption, thereby decreasing the accuracy of the results. Conversely, if we are interested in absorption as the primary outcome then the appearance of tracer in plasma generally underestimates true absorption since, although enrichment in plasma may provide a measure of absorption, it fails to take account of the flux of tracer between body pools. The use of an *iv* tracer, distinguishable from, but otherwise metabolically identical to the oral tracer, provides much additional information that can potentially improve estimates of absorption. The dual isotope-type approach was selected for the current study in order to produce kinetic information that could be applied to establishing the total absorption of oral vitamin K_1 in standard form.

In a typical tracer study, a tracer, either radio- or stable isotope-labelled, but distinguishable from endogenous tracee, is followed after *iv* administration. The rapid (and assumed instantaneous for kinetic modelling) appearance then gradual disappearance of the tracer provides data on kinetic parameters. However, for the purpose of the current study it was deemed undesirable to inject a chemically synthesised form of vitamin K_1 for a number of reasons. Firstly, a sterile, pyrogen-free preparation of the labelled vitamin K_1 could not be guaranteed and secondly, with *iv* administration the risk of an sudden adverse reaction is much greater than with an oral dose. An alternative approach was proposed whereby the volunteer would be given oral doses of ^{13}C-labelled vitamin K_1 over a period of 6 d prior to performing the kinetic study. This intervention had

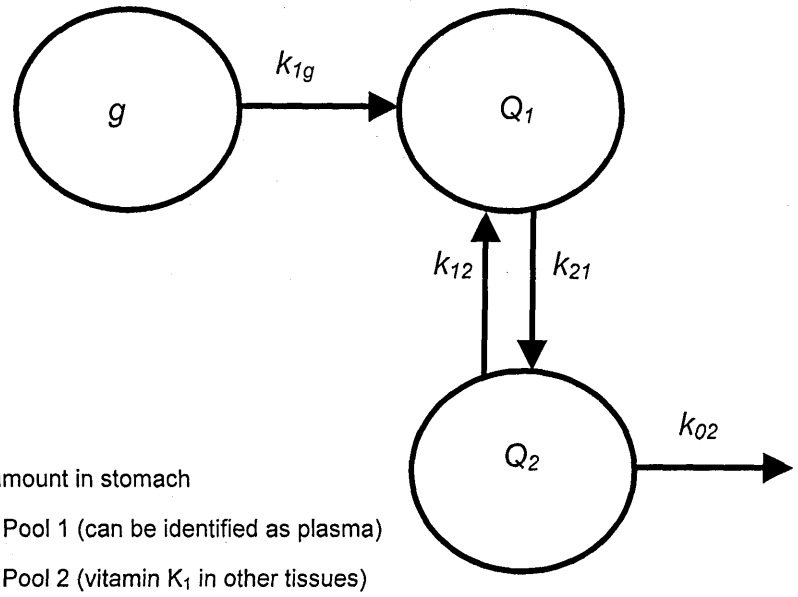
the effect of increasing the ratio of the labelled (tracer) to unlabelled (tracee) vitamin K_1 with the aim of reaching a steady-state (equilibrium) for plasma vitamin K_1 enrichment.

On receiving an *iv* dose of unlabelled vitamin K_1 enrichment rapidly decreased, followed by a gradual increase during which the unlabelled vitamin K_1 is moving from the plasma pool to other body pools and mixing with endogenous vitamin K_1. The observed changes in enrichment are the reverse of the traditional *iv* tracer approach where enrichment would generally increase after *iv* administration, followed by decrease. This approach is feasible since, although enrichment is increasing rather decreasing over the period of measurement, it is the result of the same metabolic processes. It has the additional advantage over the traditional method in that, at the tail end of the experiment enrichment levels are higher, whereas if a labelled tracer was given enrichment would return to near baseline levels. Any differences in enrichment are more easily measured at levels above baseline.

3.4.1 Compartmental model of vitamin K_1 uptake

From previous work by Olson *et al.* (2002), a crude three-compartment model for the absorption of vitamin K_1 absorption and uptake of vitamin K_1 was developed. This model, shown in Figure 3-2, was used to predict the approximate effect of different dose sizes and bioavailability on levels of vitamin K_1 concentration and enrichment, for the oral pre-enrichment, *iv* dose and oral dose for absorption. It is similar to the two-compartment model described in section 3.3.4 but with the addition of third compartment that represents the gut, and which permitted absorption from the gut to be included in the model. The three-compartment model used rate constants derived from the slopes and intercepts presented in Olson's work. Average rate constants from seven subjects in Olson's study were k_{21} – 0.699, k_{12} – 0.113 and k_{02} - 0.035. In the three-compartment model k_{1g} was set at 1. Average pool sizes were calculated as 24 nmol for Q_1 and 121 nmol for Q_2. Turnover was estimated as 47 µg/d (104 nmol/d).

Figure 3-2. Three-compartment model of vitamin K_1 absorption and kinetics



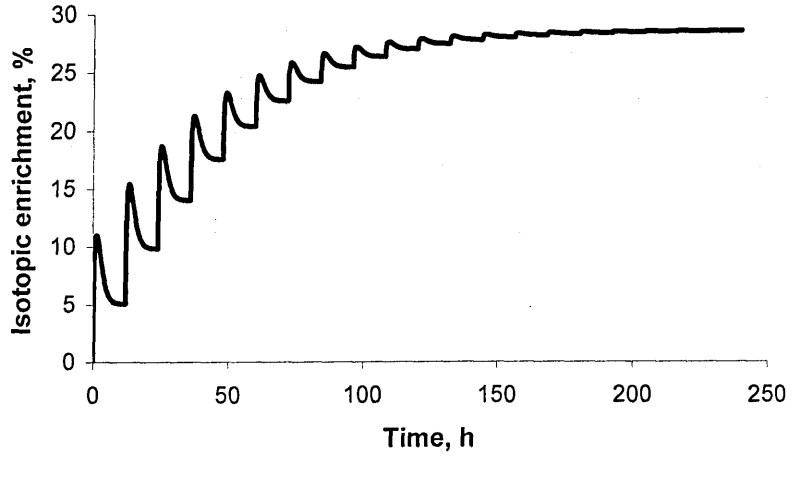
g = amount in stomach

Q_1 = Pool 1 (can be identified as plasma)

Q_2 = Pool 2 (vitamin K_1 in other tissues)

To determine the approximate duration of oral dosing with ^{13}C-labelled vitamin K_1 required to reach a steady-state for enrichment the above rate constants and pool sizes were used. Assumptions were that intake of vitamin K_1 from food was 60 µg/d with a bioavailability of 30%. The labelled dose was 9 µg/d with 80% bioavailability. Enrichment of vitamin K_1 in pool 1 was determined by the relative amounts of unlabelled and ^{13}C-labelled vitamin K_1. Use of this model suggested that a plateau for enrichment would be reached after around 6 d (Figure 3-3) based on kinetics determined by Olson *et al.*, (2002).

Figure 3-3. Modelled effect of oral dosing with 9 µg/d ^{13}C-labelled vitamin K_1 on isotopic enrichment of plasma vitamin K_1



3.5 Study methodology

3.5.1 Choice of tracer

The use of stable isotopes and GCMS allows the simultaneous administration and detection of two or more isotopic species of vitamin K_1. For this dual stable isotope-type study, two forms of vitamin K_1 were custom synthesized by ARC Laboratories, The Netherlands. One was labelled with a single ^{13}C atom in the methyl group of the quinone ring and has a molecular weight one mass unit greater than unlabelled vitamin K_1 (m/z 451). The second form was labelled with four ^{2}H atoms on the quinone ring and has a molecular weight four mass units greater than unlabelled vitamin K_1 (m/z 454) (see Figure 2-3). For this experiment the ^{13}C-labelled species was chosen for oral pre-enrichment and the deuterated species for the measurement of absorption. It was expected that the relatively small changes in enrichment due to the oral dose would be more readily detected, and with the least interference from the unlabelled and labelled forms, by using the deuterated species. To measure absorption at the same time as receiving the *iv* dose, the volunteer received an oral deuterated dose of vitamin K_1. The use of these two different labelled forms of the vitamin allowed the simultaneous determination of both vitamin K_1 kinetics and absorption.

3.5.2 Oral doses

Labelled doses for oral administration were provided to volunteers dissolved in groundnut oil in capsule form. Manufacture of capsules for use in the current study was investigated but since relatively few capsules were required it was not considered economical. Gelatine capsules were subsequently sourced from and donated by Capsulgel (Colmar, France). Although designed primarily for filling with dry powders, the Coni-snap two-piece gelatine capsule was found to be suitable for containing viscous oil without leakage. The delivery of oral vitamin K_1 in gelatine capsules has been described before in a depletion-repletion experiment (Suttie *et al.*, 1988b).

Oral pre-enrichment was achieved using oral doses of 9 µg/d, and from the three-compartment model it was estimated that a steady-state for vitamin K_1 enrichment would

be achieved within 5 to 6 d. The daily doses were prepared as three capsules per day, each containing 3 μg of ^{13}C-labelled vitamin K_1. For the simultaneous measurement of vitamin K_1 absorption from an oral dose, a capsule containing 4 μg of deuterated vitamin K_1 in groundnut oil was prepared. This dose is a considerably lower amount than has been used in previous studies measuring vitamin K_1 absorption.

3.5.2.1 Preparation of oral doses

Oral doses in capsule form were prepared by dissolving labelled vitamin K_1 in groundnut oil. Groundnut oil is known to contain very low levels of endogenous vitamin K_1 (Shearer & Bolton-Smith, 2000). Stock solutions of 40 and 14 mg (^{13}C and ring-D_4, respectively) per 50 mL ethanol were prepared and stored at –18 °C. For the doses given to the volunteers, appropriate volumes of the standard solution were added to groundnut oil to supply 3 μg (6.7 nmol) of ^{13}C-labelled and 4 μg (8.9 nmol) ring-D_4 vitamin K_1 in 0.5 mL of oil. Ethanol was evaporated from the oil by heating at 40 °C under nitrogen with a Pierce Reacti-therm heating block and Reacti-vap evaporator (Perbio Science, Erembodegem, Belgium). A 0.5 mL volume of groundnut oil containing labelled vitamin K_1 was pipetted into the gelatine capsules, which were then sealed. The oil-vitamin K_1 mix and capsules were freshly prepared for each volunteer. After preparation, capsules were stored in amber medicine bottles at 4 °C until use. The vitamin K_1 content of the oil was confirmed by HPLC.

3.5.3 Intravenous tracer

For the *iv* dose, the pharmaceutical preparation of Konakion MM® was chosen. This synthesised form of vitamin K_1 is in a mixed micelle vehicle of glycholic acid and lecithin. Konakion MM® is commonly used as an antidote to anticoagulant drugs of the coumarin type and is used in the treatment of haemorrhage or conditions associated with low blood levels of prothrombin or factor VII.

Konakion MM® was only available in capsules of 10 mg/mL thus it was necessary to dilute an aliquot to the required dosage. Based on previously published kinetics and expected absorption of the oral doses, the first four volunteers were given a 30 μg *iv* dose.

The dilution for *iv* injection was prepared by a research physician as per the instructions below immediately prior to injection.

Instructions for preparation of 30 µg *iv* dose of Konakion MM ®

Amount of vitamin K_1 to be injected: 30 µg in 5 mL

Starting solutions: a) 2 x 50 mL bag 5% glucose in saline

b) 1 mL ampoule of 10 mg/mL Konakion MM®

1. Take 300 µL (3 mg) from a 1 mL Konakion® ampoule
2. Add to first bag of 50 mL 5% glucose solution
3. Remove 5 mL from a second bag of glucose
4. Take 5 mL from first bag and add to second bag containing 45 mL of 5% glucose

   Final concentration of 300 µg in 50 mL
5. Remove 5 mL of this solution containing 30 µg of vitamin K_1 for injection

As detailed below (section 3.8.1), initial analysis of samples from four subjects showed lower than expected isotopic enrichment after the oral pre-enrichment with ^{13}C-labelled vitamin K_1. As a result, the remaining six volunteers were given a 6 µg *iv* dose, prepared as detailed below.

Instructions for preparation of 6 µg *iv* dose of Konakion MM

Amount of vitamin K_1 to be injected: 6 µg in 5 mL

Starting solutions: a) 2 x 50 mL bag of 5% glucose in saline

b) 1 mL ampoule of 10 mg/ml Konakion MM®

1. Take 300 µL (3 mg) from a 1 mL Konakion ampoule
2. Add to first bag of 50 mL 5% glucose solution
3. Remove 1 mL from second bag of glucose
4. Take 1 mL from first bag and add to second bag containing 49 mL of 5% glucose

   Final concentration of 60 µg in 50 mL
5. Remove 5 mL of this solution containing 6 µg of vitamin K_1 for injection

The concentration of the iv doses was measured by HPLC. After preparation and administration of the iv dose, the remaining solution was kept on ice before transfer of around 2 mL to tubes for storage at -70 °C prior to analysis. Only two of the four 30 µg doses were available for analysis and were measured as 33.4 and 31.0 µg. For the group who received the calculated 6 µg dose, the average (± SD) measured dose was 5.8 ± 0.6 µg. One sample of the 6 µg dose was kept on ice for 6 h after preparation and aliquots taken at 1.5, 4.0 and 6.0 h. Measured values were 6.5, 6.0 and 6.4 µg, respectively indicating that the preparation was relatively stable over time and that measured values reflect the actual dose given.

3.5.4 Ethical permission

Following approval by the MRC Human Nutrition Research Science Coordination Committee (SCC), ethical permission for this study was obtained from Cambridge Local Research Ethics Committee (REC ref: 04/001). Informed written constant was obtained from subjects prior to the start of the study (Appendix III).

3.5.5 Study subjects

Since this was a pilot study no formal sample size calculations were performed. Healthy, male or female subjects, aged between 18 and 40 y, were selected. Subjects were selected on the basis that the aim of the study was to investigate vitamin K_1 kinetics under normal physiological conditions. Exclusion criteria were as detailed below.

General health criteria:

Renal, liver or respiratory disease, malignancy, diabetes, pancreatic or gall bladder disease, or any other chronic medical conditions; use of vitamin supplements, smoking, recent heavy bleeding or blood donation, history of anaemia, pregnancy or breast feeding.

Study-specific exclusion criteria:

Bleeding or clotting disorders, gastro-intestinal malabsorption, history of allergies, hayfever, eczema or asthma.

The exclusion of people with a history of allergies was on the basis of a few unconfirmed reports of the occurrence of possible anaphylactoid reactions after the *iv* injection of Konakion MM® (Roche Product Information Sheet).

3.5.6 Volunteer recruitment

The information sheet sent to potential volunteers is attached as Appendix IV. Volunteer recruitment was slow, partly due to the strict exclusion criteria for allergies and a general unease amongst potential volunteers at the prospect of an *iv* injection. From 60 letters sent to individuals registered on the HNR Volunteer Database there were no replies. Thirty-five people responded after a blanket approach through email and poster advertising. Of them, ten agreed to participate, eight were excluded, and the remaining 17 chose not to take part. Volunteers received an honorarium of £40 for completing the study.

3.5.7 Study protocol

At the start of the study volunteers were invited to attend the volunteer suite at MRC Human Nutrition Research (HNR) to have the study explained in full and to provide them with the opportunity to ask any questions. Their weight was measured to the nearest 0.1 kg using Seca 770 digital scales (Seca, Birmingham, UK) and their height was measured to the nearest 0.01 m using a Seca 202 wall-mounted stadiometer (Seca, Birmingham, UK). The volunteer was given a supply of eighteen capsules containing ^{13}C-labelled vitamin K_1 in an amber medicine bottle. The volunteer was asked to take three capsules per day, one with each meal, and to avoid foods containing high levels of vitamin K_1 (green leafy vegetables, broccoli) on day six of study. On days five and six after starting the capsules the volunteer was asked to attend MRC HNR to have single 9 mL fasting blood samples collected by venepuncture.

On day seven the volunteer attended the volunteer suite at MRC HNR having abstained from food and drink (with the exception of water) since the previous evening. An indwelling cannula was inserted into each forearm vein. Two baseline 9 mL blood samples were collected from one cannula and the volunteer then received, through the

other cannula, the *iv* bolus dose of 6 or 30 µg of Konakion MM® (see section 3.8.1 for further explanation). The cannula used to administer the Konakion MM® was removed shortly after the dose had been given. At the same time as receiving the *iv* dose, the participant received an oral dose of 4 µg of deuterated vitamin K_1. This was prepared in the same way as the ^{13}C-labelled vitamin K_1 doses. Subjects were fasted and the capsule taken on an empty stomach with water. Blood samples were taken at 2, 5, 10, 20, 30, 40, 50, 60, 90 min post-dose and then 2, 3, 4, 5 and 6 h post-dose. In order to keep the cannula patent, the cannula was flushed after each blood sample and at regular intervals with 2 mL of saline. Prior to each blood sample, 2 mL of blood was withdrawn and discarded. A total of approximately 170 mL of blood was taken over the course of the day.

After 3 h the volunteer was offered a small, low fat meal (toast with jam with tea or coffee). On completion of sampling the cannula was removed and the volunteer provided with a further meal, after which he/she was free to leave.

3.5.8 Sample collection and analysis

Blood samples were collected into 10 mL syringes and immediately transferred to coded tubes containing EDTA (Sarstedt Ltd, Leicester, UK). The tubes were kept on ice, and protected from light, until centrifugation at 4 °C for 20 min at 2000 *g* to separate the plasma. The plasma was divided into four 1 mL aliquots in 2 mL microtubes (Sarstedt Ltd, Leicester, UK). All samples were stored at –70 °C until analysis.

Extraction of vitamin K_1 from plasma was performed with enzyme hydrolysis, solvent extraction and solid phase extraction as described in section 2.9. Prior to analysis by GCMS, vitamin K_1 was derivatised to the pentafluoropropionyl derivative as detailed in section 2.7. The GCMS was run in SIM mode and measured ions m/z 598.4 to 602.4 (Jones *et al.,* 2006). Total plasma vitamin K_1 concentrations were measured by high-performance liquid chromatography (HPLC) with fluorescence detection after post-column reduction (Wang *et al.,* 2004) (section 2.12).

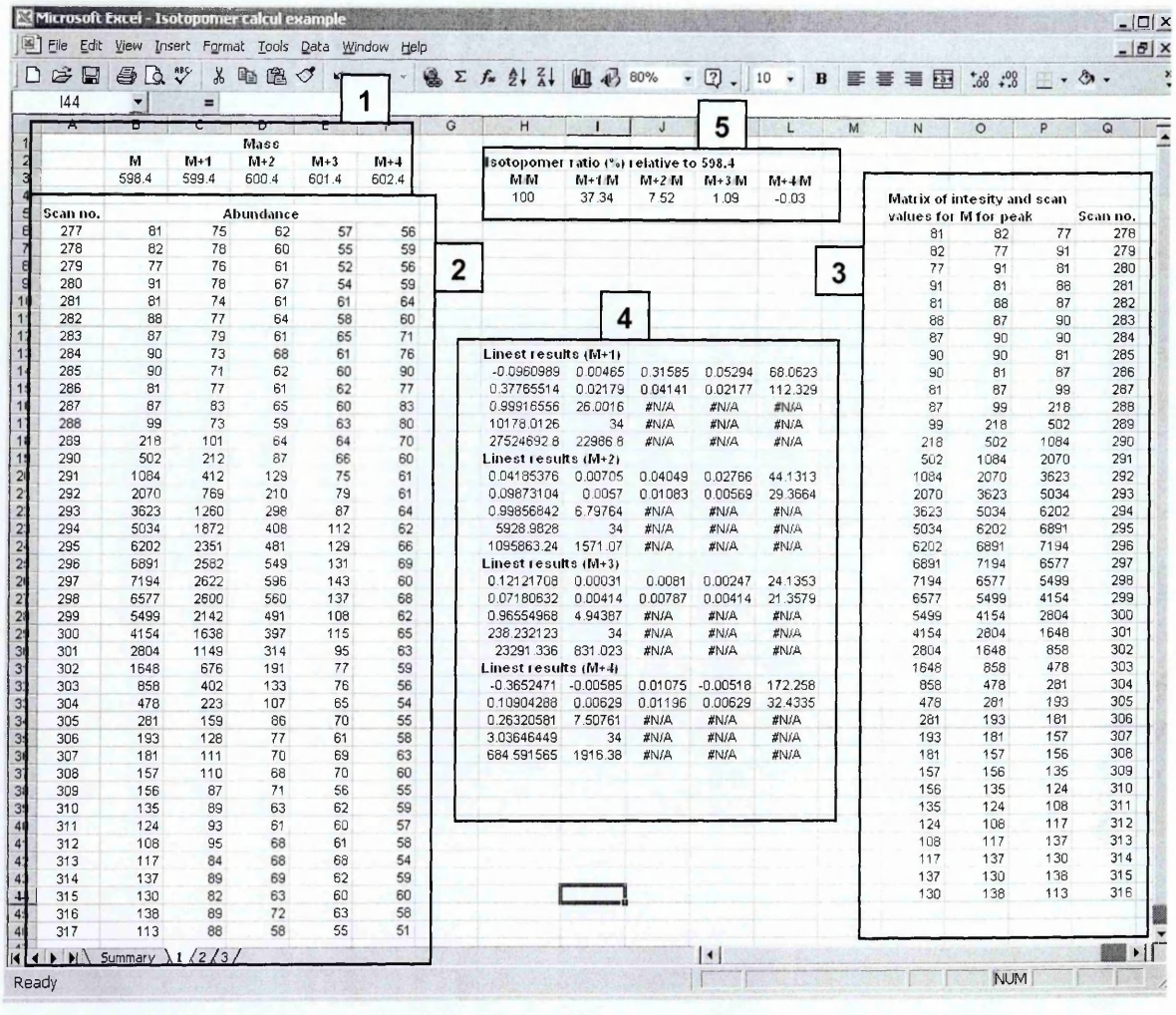
3.6 Data analysis

3.6.1 Isotopomer ratio analysis

Isotopomer ratios were calculated using the fitting techniques described by Bluck and Coward (1997). The method has a number of advantages over alternative peak integration techniques mainly by removing subjective decisions of the operator with regard to peak truncation and assessment of background. The shape of the peak is determined from the species with the greatest intensity, in this case the molecular ion (M), i.e. m/z 598.4, and the remaining species are fitted to this peak shape, thereby eliminating shifts in retention time in the heavier species due to either the sequential nature of mass analysis with a quadrupole instrument, or chromatographic reasons. Practically, the use of this method involves removing raw data files containing intensity and time/scan number data, and copying this information into a template created in Excel. The end-user template is described below (Figure 3-4).

An alternative approach to the measurement of each of the isotopomers as performed, is to only measure the ions of interest, i.e. M+0, M+1 and M+4. Since fewer ions are measured the dwell time can be increased for each ion with an accompanying potential increase in precision. However, monitoring of all isotopomers of the molecule of interest can provide a greater appreciation of any variation in chromatographic or mass spectral quality. This was especially pertinent in this application since the highly concentrated sample led to rapid dirtying of the ion source with the consequent shifts in mass assignment.

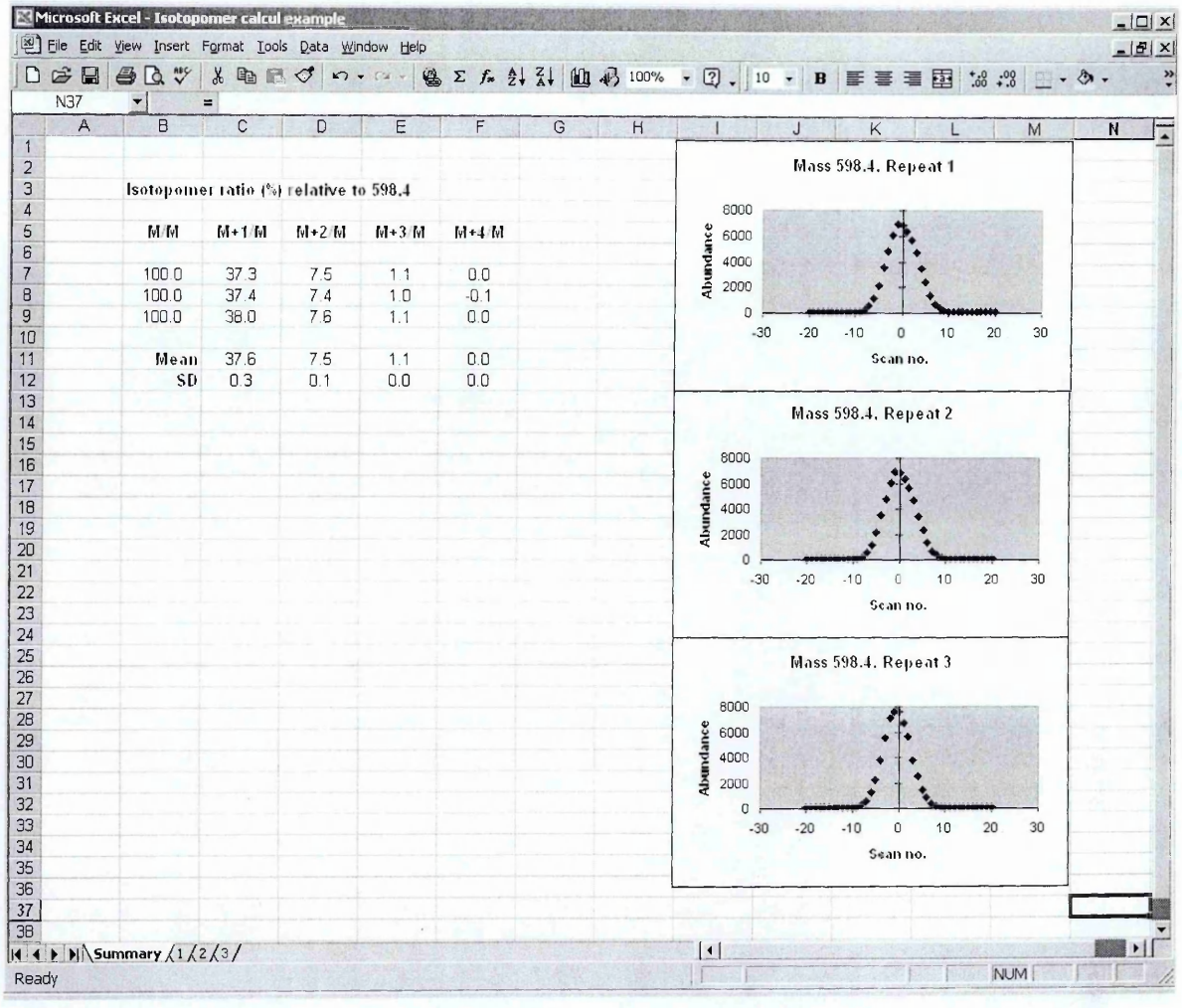
Figure 3-4. Excel worksheet for the calculation of isotopomer ratios



1. Measured isotopomers and respective masses
2. Raw mass abundance data and respective scan number exported directly from GCMS software
3. Matrix of intensities for M (molecular ion). The 1st column contains data for M from the first reading. The 2nd column is the same data but starting with the 2nd reading, and the 3rd column starts from the 3rd reading. The final column is the scan number. This data is used for building the envelope around which the remaining peak shapes are fitted.
4. Multiple linear regression using least squares method, each array corresponding to each isotopomer ratio.
5. The resulting isotopomer ratios (expressed as percentages) obtained from summing the coefficients obtained using the LINEST function in Excel. For example, the 599/598 ratio is obtained from summing values in cells I14, J14 and K14.

The results of triplicate analyses are summarised with graphical representation of the peak shape of the molecular ion (M) and the average and standard deviation for each sample, e.g. Figure 3-5.

Figure 3-5. Summary Excel worksheet of isotopomer calculations



3.6.2 Kinetic methods

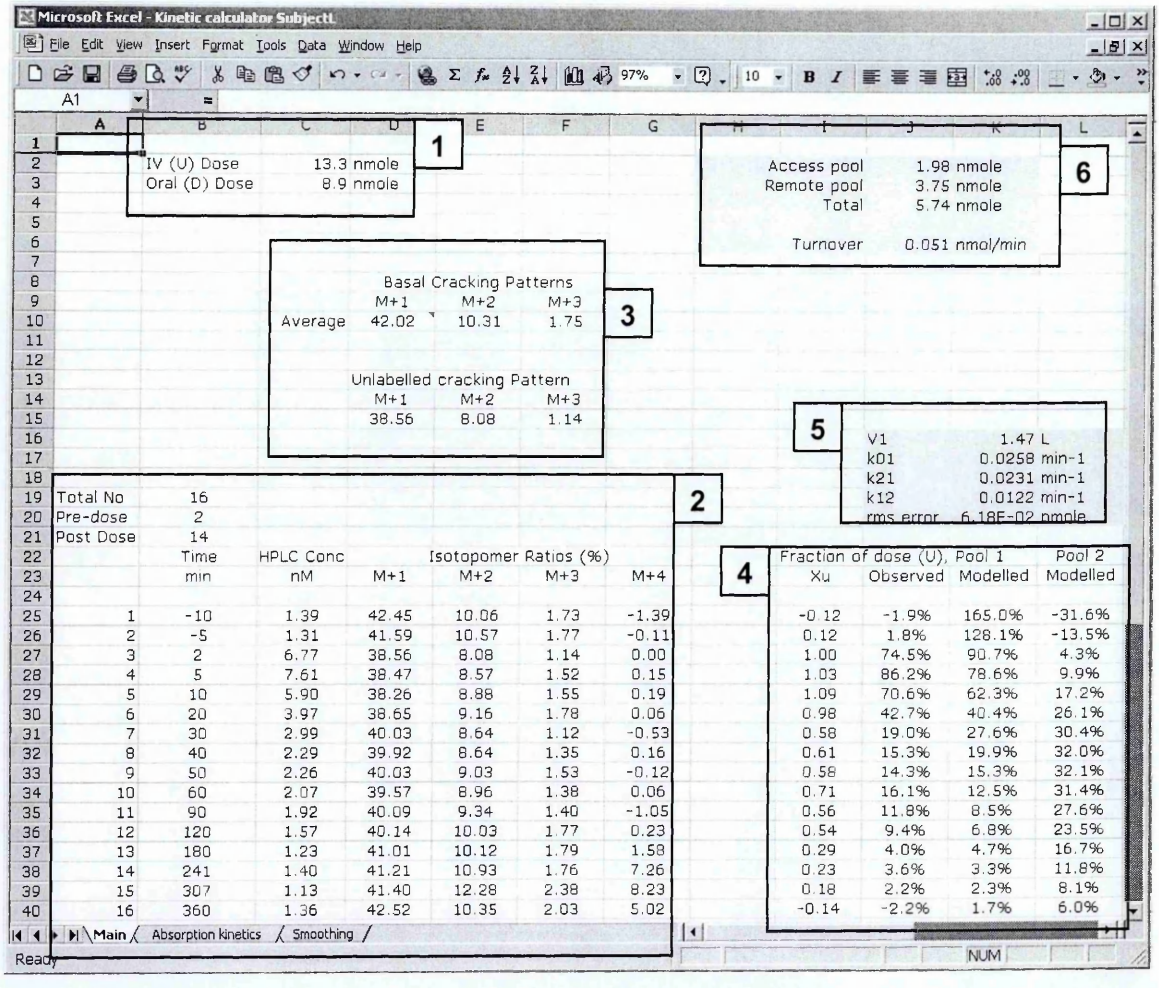
Rather than use the above methods directly, a macro in Excel was utilised that simplifies the calculations necessary to obtain kinetic information. The data were fitted to a two-compartment model, in which, the rate constants combined to give two exponential terms that described the disappearance of the *iv* dose from the plasma (pool 1 or Q_1). Initial estimates of Q_1, k_{21}, k_{12} *and* k_{01} were used to generate the coefficients of the exponentials, and these were compared to the observed values. Using the Solver function in Excel, the initial estimates were then adjusted to minimise the differences between the modelled and observed data using a non-linear method. The 2_comp macro function calculates each of the instantaneous pool sizes of a freely exchanging two-

compartment system defined by its fractional rate constants and initial conditions (personal communication, Dr Les Bluck). It was entered in the form:

_2comp(time, Q1start, Q2start, rate01, rate21, rate02, rate12)

The macro returns a two-element single column array containing the amounts of material found in each compartment at a specified time. Since this function returns an array of values it must be entered as an array formula. In order to generate values for each of the rate constants, as well as pool sizes, a template was constructed in Microsoft Excel for this purpose. The spreadsheet for determining rate constants and pool sizes, is described below and shown in Figure 3-6. Note that bioavailability of the *iv* dose was assumed to be 100%.

Figure 3-6. Excel worksheet for the calculation of vitamin K_1 kinetics



		Time	HPLC Conc	Isotopomer Ratios (%)			
		min	nM	M+1	M+2	M+3	M+4
1		-10	1.39	42.45	10.06	1.73	-1.39
2		-5	1.31	41.59	10.57	1.77	-0.11
3		2	6.77	38.56	8.08	1.14	0.00
4		5	7.61	38.47	8.57	1.52	0.15
5		10	5.90	38.26	8.88	1.55	0.19
6		20	3.97	38.65	9.16	1.78	0.06
7		30	2.99	40.03	8.64	1.12	-0.53
8		40	2.29	39.92	8.64	1.35	0.16
9		50	2.26	40.03	9.03	1.53	-0.12
10		60	2.07	39.57	8.96	1.38	0.06
11		90	1.92	40.09	9.34	1.40	-1.05
12		120	1.57	40.14	10.03	1.77	0.23
13		180	1.23	41.01	10.12	1.79	1.58
14		241	1.40	41.21	10.93	1.76	7.26
15		307	1.13	41.40	12.28	2.38	8.23
16		360	1.36	42.52	10.35	2.03	5.02

Xu	Observed	Modelled	Modelled
-0.12	-1.9%	165.0%	-31.6%
0.12	1.8%	128.1%	-13.5%
1.00	74.5%	90.7%	4.3%
1.03	86.2%	78.6%	9.9%
1.09	70.6%	62.3%	17.2%
0.98	42.7%	40.4%	26.1%
0.58	19.0%	27.6%	30.4%
0.61	15.3%	19.9%	32.0%
0.58	14.3%	15.3%	32.1%
0.71	16.1%	12.5%	31.4%
0.56	11.8%	8.5%	27.6%
0.54	9.4%	6.8%	23.5%
0.29	4.0%	4.7%	16.7%
0.23	3.6%	3.3%	11.8%
0.18	2.2%	2.3%	8.1%
-0.14	-2.2%	1.7%	6.0%

1. Contains the dose values for the *iv* dose and the oral deuterated dose
2. Contains the actual sample times, concentration values and isotopomer values (expressed as % to M). It is necessary to enter the number of pre-dose (baseline) values (cell B20) in order to calculate values in [3].
3. Contains the average basal (after ^{13}C pre-loading and before *iv* dose) isotopomer ratio used in the calculation of *Xu*. The unlabelled isotopomer ratio is equal to that measured immediately after administration of the *iv* dose and is approximately equal to the naturally occurring isotopomer ratio.
4. The first column in this section is equal to the proportion of total vitamin K_1 that is from the *iv* dose (*Xu*) and is calculated by:

$$Xu = \frac{R_{M+1(0)} - R_{M+1(t)}}{R_{M+1(0)} - R_{M+1(u)}}$$

where the subscripts refer to measurements made before the *iv* dose (*0*), after it (*t*) and in the *iv* dose itself (*u*).

In the second column are the observed, experimental values for the fraction of the dose remaining in Pool *1*, calculated by:

$$Xu \times [\text{vit k}] \times (V1 / iv \text{ dose})$$

The 3rd and 4th columns are part of the _2COMP array function described above. The 3rd column contains the equivalent modelled values for Pool *1*, and the final column contains the modelled percentage of the *iv* dose in Pool 2.

5. As described above, the Solver function is used to minimise the difference in the observed and modelled data. The Solver function works to minimise the value in cell K20 (equal to the square root of the sum of the squares in the differences between the observed and modelled values) by adjusting the values for the rate constants k_{01}, k_{21} and k_{12}, and the volume of Pool *1*, *V1*.
6. The final section shows the pool sizes and turnover for this model. The access pool (Pool *1*) is calculated by multiplying total vitamin K_1 concentration at baseline by the modelled volume of Pool *1*. The size of remote compartment (Pool 2, that which is not directly sampled) is calculated by:

$$Q_2 = k_{21} \times Q_1 / k_{12}$$

Total pool size in the measured compartments is the sum of the remote and access pools.

Turnover (F_{01}) is calculated by

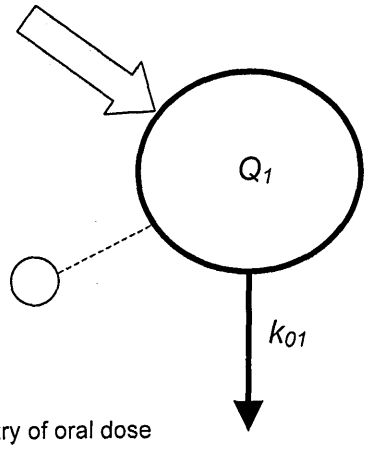
$$F_{01} = k_{01} \times Q_1$$

and provides an actual measure of the amount of vitamin K losses, as opposed to a rate constant (k) that is a fractional loss per unit time.

3.6.3 Calculating absorption

The model for absorption is a simple one-compartment model with total losses as rate k_{01} (Figure 3-7).

Figure 3-7. Model of vitamin K_1 absorption kinetics



Q_1 = Pool 1

Large open arrow shows entry of oral dose

Dashed line indicates sampling pool

3.6.3.1 Calculation method

The characteristics for the oral dose were obtained from the ring-D_4 isotopomer data (R_{M+4}) by deconvolution of appearance of tracer in Q_1 and Q_2. In this case, deconvolution (the mathematical process of separating signals) was used to deduce the input (i) (of the oral dose) from the system response (derived from response to *iv* dose). Using * to denote the deuterated material and i the input (nmol/min), the equations are:

$$\frac{dQ_1^*(t)}{dt} = i(t) - (k_{21} + k_{01})Q_1^*(t) + k_{12}Q_2^*(t) \quad (1)$$

$$\frac{dQ_2^*(t)}{dt} = k_{21}Q_1^*(t) - k_{12}Q_2^*(t) \quad (2)$$

Equation (1) calculates rate of appearance of deuterated material in Q_1 from the rate of input of deuterated material *(i(t))* minus exit from Q_1 $(k_{21} + k_{01})$ and input from Q_2 (k_{12}). Equation (2) calculates the rate of appearance of deuterated material in Q_2 and is equal to input from Q_1 (k_{21}) and exit from Q_2 (k_{12}). Since we cannot sample Q_2, Q_2 * *(t)* was obtained from equations (1) and (2) iteratively using Euler's method[1]. Euler's method is a technique of integration that corrects the slope of the curve at time point intervals to better approximate the real shape of the curve and effectively removes the rate constants to provide an estimate for *i.*

The noisy, observed Q_1 * *(t)* was smoothed by fitting a normal cubic spline, using a roughness penalty approach (see description below) (Green & Silverman, 1994) that also provided an estimate of its first derivative with respect to time. Cumulative absorption was calculated by integration of *i (t)*, and this then summarized as a delay followed by a single exponential term.

3.6.3.2 Data smoothing

The term 'spline' is used to refer to a range of functions for data interpolation (estimation of values in a series between two known values) or data smoothing. The

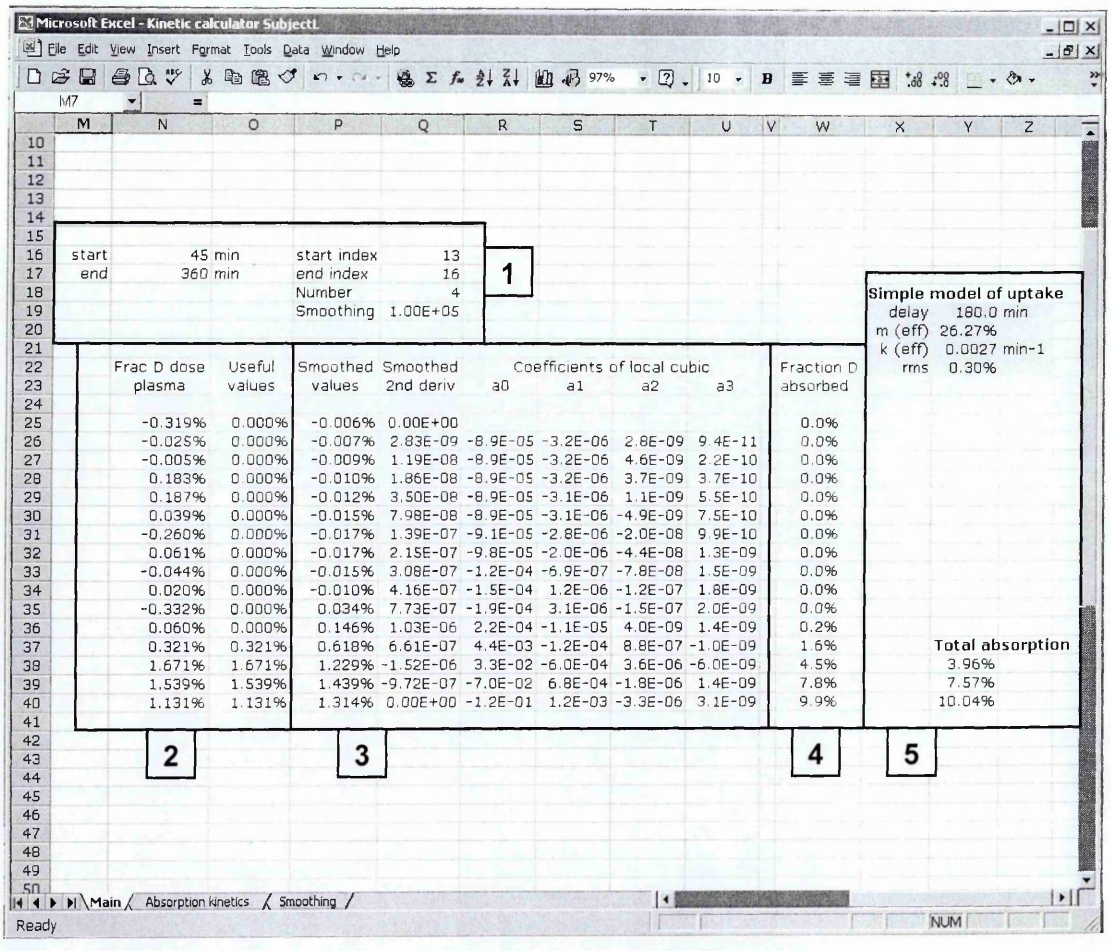
---

[1] http://www.swarthmore.edu/NatSci/echeeve1/Ref/NumericInt/Euler1.html (accessed 22nd March 2007)

simplest spline has degree 0 and is also called a step function. The natural cubic spline has degree 3. Given a dataset of X and Y, it is therefore possible to estimate the values of Y for X's other than those in the sample. Cubic splines are made to be smooth at the known time points (knots) by forcing the first and second derivatives of the function to agree at the knots. The first term measures the closeness to the data and the second penalizes curvature in the function, with the aim to construct a function that balances the twin needs of (1) proximity to the actual sample points, (2) smoothness. So a 'roughness penalty' is defined.

As for the calculation of *iv* kinetic parameters, the calculation of absorption was performed in Excel and is described below and shown in Figure 3-8.

Figure 3-8. Excel worksheet for the calculation of vitamin K_1 absorption parameters



1. A delay is applied to the model in cell N16. The chosen number of useful values is shown in cell Q18, and is user-generated from the entry in cell Q16. The extent of smoothing is determined by the value in Q19 and remained constant for all subjects.

2. The column N contains the calculated fraction of the deuterated dose in plasma [Q_1 * (t)] and is calculated by:

$$X_D = \frac{R_{M+4(t)} \times [vitk] \times V1}{dose}$$

and the next column (O) the values deemed useful. These are subjectively chosen by entering those values to be used in the cells highlighted in box 1 (Q16-18).

3. This part of the spreadsheet contains the smoothing coefficients for each of the data points calculated in the 'smoothing' sheet.

4. The values here contain the cumulative fraction of the dose absorbed at each time point.

5. The Solver function is used to minimise the sum of squares for the corresponding values in columns W and Y (observed and modelled fraction of the dose absorbed). The delay represents the delay from the start of the experiment to appearance of the tracer in plasma. k is the effective absorption rate of the tracer and m is the effective modelled total absorption including extrapolation of the observed absorption curve.

3.7 Results

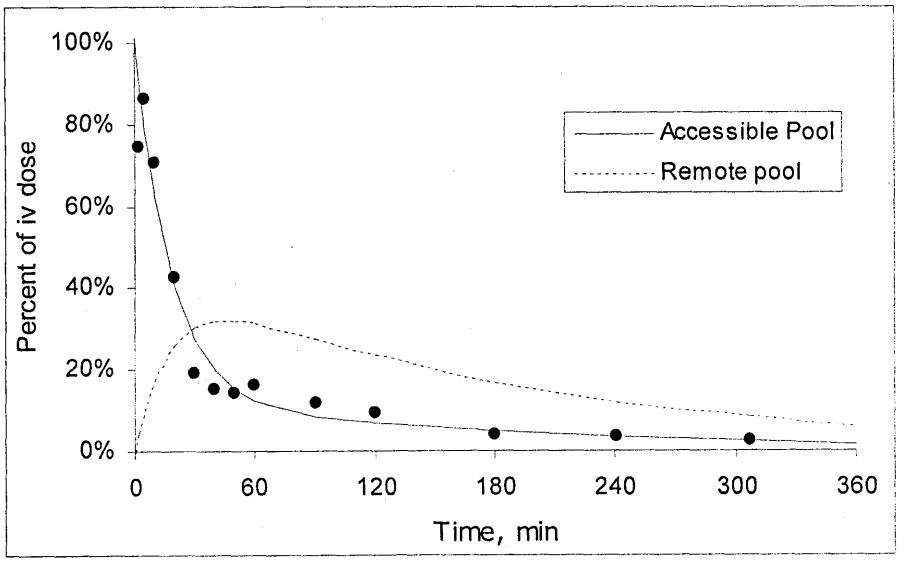
3.7.1 Subject characteristics

The ten subjects (9 women and 1 man) were aged between 22 and 31 y. They had a mean ± SD height of 1.64 ± 0.10 m, body mass of 61.0 ± 10.7 kg and BMI of 22.5 ± 2.4 kg/m^2.

3.7.2 Graphical output

After *iv* administration, the percent of the dose remaining in plasma drops rapidly and slows at around 50 – 60 min, as clearly shown in Figure 3-9 that shows the output from just one of the volunteers. The dashed line shows the modelled entry of the *iv* dose in the remote pool. This chart is plotted directly from the data in columns A, I, J and K of the kinetic calculator spreadsheet Figure 3-6.

Figure 3-9. Graphical representation of output from the two-compartment model

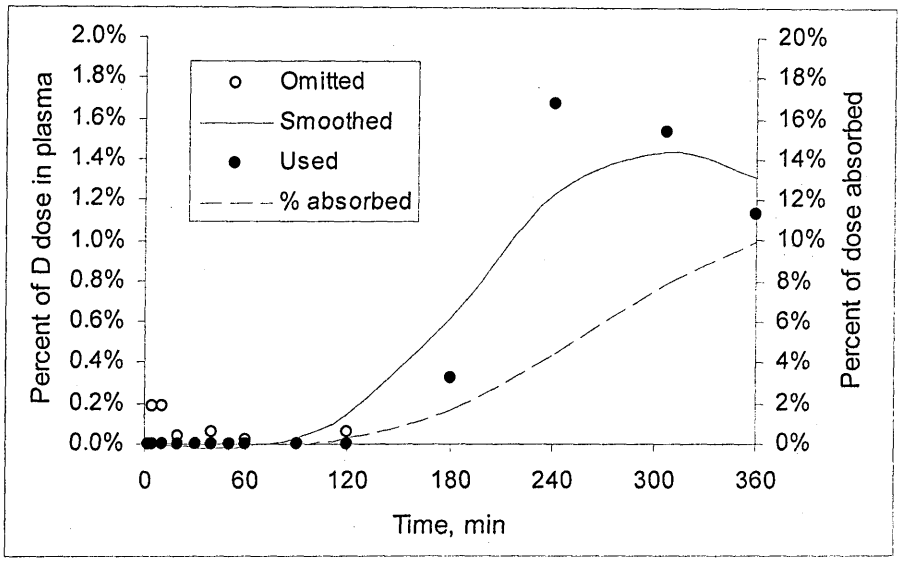


Data points (•) illustrate the observed values, solid line the modelled disappearance of the *iv* dose from the sampled pool (pool 1), and the dashed line the appearance of the *iv* dose in the remote pool (pool 2).

A similar chart is shown in Figure 3-10, displaying a graphical representation of the absorption modelling results. The chart shows observed values, with those in black used for the modelling. The solid line shows the smoothed appearance of tracer (left axis)

while the dashed line is the cumulative absorption (right axis). As with Figure 3-9, this is example is from a single volunteer.

Figure 3-10. Typical output from absorption kinetics model



Solid data points (•) up to and including 120 min, are zero values. Subsequent solid points illustrate the observed values used for the curve smoothing, while the open data points (o) observed values that were not incorporated in the curve smoothing. The solid line shows the modelled appearance of the oral deuterated dose in the sampled pool and the dashed line the cumulative absorption of the deuterated oral dose.

3.7.3 Kinetic parameters

A steady-state for plasma vitamin K_1 enrichment was established by the dosing regime. There were no significant differences between the HPLC data or isotope ratio data obtained on days 5, 6 and 7 (calculated by one-way ANOVA). Average ± SD vitamin K_1 plasma values were 1.40 ± 0.90, 1.21 ± 0.84, 1.09 ± 0.58 nmol/L, on days 5, 6 and 7, respectively. Corresponding isotope ratios (for M+1 / M) were 0.434 ± 0.041, 0.439 ± 0.032 and 0.443 ± 0.031, on days 5,6 and 7, respectively. For the purposes of kinetic analysis only those baseline values obtained on day 7 were used in the calculations.

The kinetic parameters obtained are shown in Table 3-1 and the means plotted in Figure 3-11.

Table 3-1. Kinetic parameters obtained from with a two-compartment model after an *iv* dose of vitamin K_1

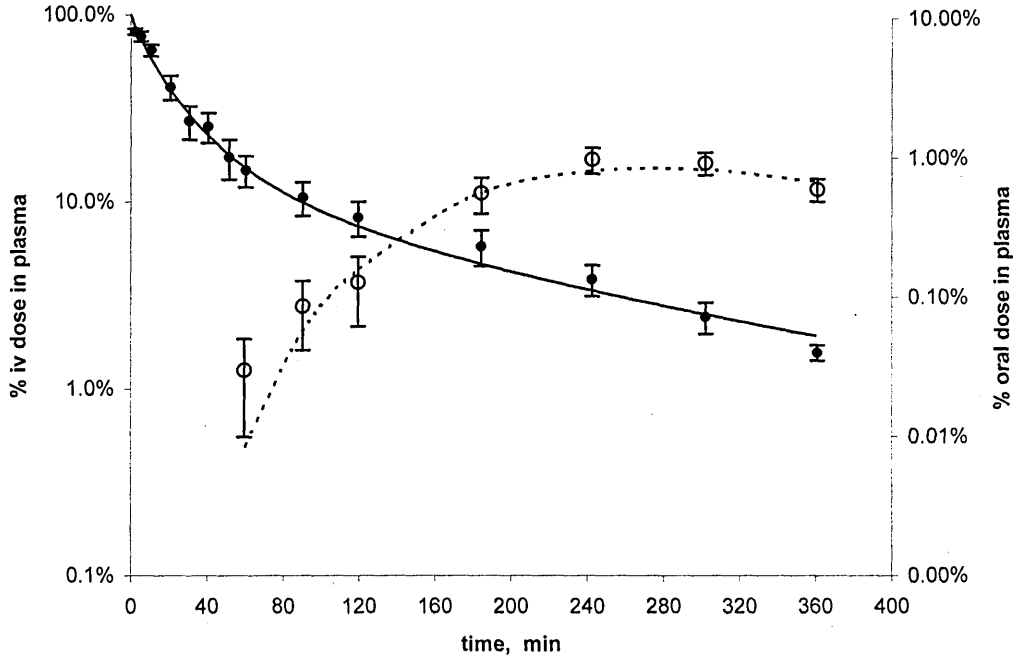
Subject	k_{01}	k_{21}	k_{12}	$T_{½\,(fast)}$	$T_{½\,(slow)}$	Q_1	Q_2	F_{01} [b]
		(h^{-1})			(h)		(nmol)	(µg/kg/d)
A[a]	1.19	0.74	0.50	0.33	2.56	1.1	1.7	0.30
B[a]	1.46	2.66	3.89	0.10	0.89	1.0	0.7	0.18
C[a]	0.89	0.30	0.35	0.53	2.81	2.3	1.9	0.36
F	1.46	1.67	0.22	0.21	6.52	1.6	12.1	0.38
G[a]	4.48	10.10	6.28	0.04	0.47	1.2	1.9	0.40
H	1.78	0.86	0.46	0.25	2.26	0.4	0.8	0.12
I	0.77	1.15	1.11	0.26	2.21	3.0	3.2	0.46
J	2.10	2.03	0.52	0.16	2.70	1.3	5.0	0.41
K	1.44	2.82	0.52	0.15	4.13	1.0	5.2	0.29
L	1.55	1.38	0.73	0.21	2.05	2.0	3.8	0.52
Average	1.71	2.37	1.46	0.22	2.66	1.5	3.6	0.34
SD	1.05	2.84	2.00	0.14	1.69	0.8	3.4	0.12

[a] Subjects who received a 30 µg *iv* dose. The remaining subjects received a 6 µg *iv* dose.

[b] F_{01} (vitamin K loss) was calculated by multiplying k_{01} and Q_1 and is expressed per kg of body weight.

Molecular weight of vitamin K_1 is 450.

Figure 3-11. Summary of *iv* kinetics and absorption of oral dose from all subjects



Semi-logarithmic plot showing plasma clearance of the *iv* vitamin K_1 dose and appearance and clearance of a ring-D_4 oral dose of vitamin K_1 (expressed as percentage of the oral dose in plasma) from all subjects combined: ■ mean % *iv* dose in plasma (n=10); solid line is the mean of modelled data from all subjects. □ mean % oral dose in plasma (n=10); dashed line is the mean of modelled data from all subjects. Error bars are standard errors for observed data points.

3.7.4 Absorption parameters

Results for absorption of the deuterated vitamin K_1 are shown in Table 3-2. Average absorption of the oral deuterated dose was 13%.

Table 3-2. Absorption characteristics after an oral dose of ring-D_4 vitamin K_1

Subject	Dose absorbed (%)	T_{max}[a] (h)
A	10.9	4.0
B	3.4	4.2
C	8.1	5.0
F	25.6	4.7
G	12.1	3.3
H	6.9	5.4
I	2.4	4.7
J	6.4	6.0
K	25.0	5.0
L	26.3	5.1
Average	12.7	4.7
SD	9.4	0.8

[a] T_{max} is the time of peak deuterated vitamin K_1 in plasma as determined from the smoothed curve

3.8 Discussion

Data on the kinetics and bioavailability of vitamins are essential as part of the assessment of recommended intakes. Bioavailability is commonly assessed on the basis of a plasma response to an oral dose. However, relying solely on the appearance of the nutrient in the plasma may lead to inaccurate measurements of bioavailability since no account is taken of metabolic processes occurring post-absorption. The main purpose of the present study was to investigate the kinetics of vitamin K_1 metabolism and at the same time measure the extent to which oral doses are absorbed using stable isotopes.

3.8.1 Chosen methodology

To simultaneously measure the kinetics and absorption of vitamin K_1 the utility of a dual-labelled stable isotope-type technique was investigated. The use of stable isotope

labelled compounds has a number of advantages over radiolabelled tracers. Firstly, they remove the potential negative health effects of exposure to ionising radiation and are therefore suitable for repeated use in human studies. Secondly, with gas chromatography mass spectrometry (GCMS), it is possible to confirm identification of the compound from its mass spectrum and measure the tracer/tracee molar ratio. The converse to this is that the molecule or molecules of interest need to be identified prior to analysis, whereas with radiolabelled compounds, previously unidentified metabolites can be measured. With stable isotope tracers it is also possible to measure simultaneously more than one tracer.

The dual-isotope technique requires one labelled form of the vitamin to be given orally, and the second intravenously. The advantage of this method is the ability to define distribution and disposal of the vitamin after absorption, and hence apply this information to the calculation of absorption.

The labelled vitamin K_1 used in this study was deemed unsuitable for *iv* administration due to practical and regulatory concerns. Other labelled forms of the vitamin suitable for *iv* administration are not easily available. To solve this problem, volunteers in this study were given ^{13}C-labelled vitamin K_1 in capsule form for 6 d. Similar capsule formulations have been used in studies of vitamin K_1 (Suttie *et al.,* 1988b) and vitamin E (Acuff *et al.,* 1994). The aim of dosing for 6 d was to reach a steady-state for plasma ^{13}C-labelled vitamin K_1 enrichment. It was then possible to use tracer doses of injectable unlabelled material for kinetic measurements. This approach is the inverse of that traditionally applied in tracer experiments where following an *iv* dose of tracer and the rapid increase in enrichment, the gradual decrease is used to calculate disposal kinetics. Here, enrichment was increased to a steady-state over a period of 6 d thus allowing the use of unlabelled material as the tracer. Following administration of the unlabelled *iv* tracer, the rapid decrease in enrichment followed by the subsequent increase towards pre-*iv* levels was used to model kinetics. An additional advantage is that when analytical noise is constant (e.g. from electrical noise and column bleed) this approach has the advantage that for later time points (when concentration of vitamin K_1 is lower) the

abundance of the M+1 ion is greater than if enrichment was returning to natural abundance, thereby improving the signal to noise ratio.

After the analysis of samples from the first four subjects it was found necessary to reduce the level of the *iv* dose. With the 30 µg dose, it was found that enrichment levels were not returning to the baseline level over the duration of the experiment, and hence the difference between the maximum and minimum enrichment over the 6 h period was lower than expected. This outcome can be attributed to lower than estimated bioavailability of the vitamin K_1 from the capsule, borne-out in results from absorption of deuterated vitamin K_1 (see below, section 3.9). Further, the total concentration of vitamin K_1 after the *iv* dose was higher than expected due to assumptions of pool volume used in the original calculation of the *iv* dose. As a result, the later subjects received 6 µg of vitamin K_1 intravenously, rather than 30 µg.

Although efforts were made to recruit equal numbers of men and women there was an obvious gender bias in recruitment. It is believed this was due to firstly, a greater interest and awareness of women in nutrition and health and secondly, an apparent reluctance of some male volunteers to submit to an intravenous dose. There is no data to suggest that metabolism of vitamin K_1 is different between men and women, for example, Binkley *et al.*, (2000) showed no significant differences in response to vitamin K_1 supplementation in 219 men and women.

3.8.2 Rate constants and half-times

Previous work to measure vitamin K_1 kinetics has used radiolabelled compounds and the characteristics of the system expressed in terms of the half-times of two exponentials ($T_{1/2(fast)}$ and $T_{1/2(slow)}$) fitted to curves for the disappearance of plasma radioactivity. The radioactivity has to varying degrees been identified as associated with vitamin K_1. The average values of the corresponding parameters found in the present experiments (Table 3-1) most closely resemble the values obtained by Shearer *et al.*, (1972), Shearer *et al.*, (1974), Shepherd *et al.*, (1977) and Bjornsson *et al.*, (1979), rather than those more recently reported by Olson *et al.*, (2002) (see Table 3-3).

Table 3-3. Summary of previous attempts to measure vitamin K_1 kinetics

	Experimental protocol			Kinetic parameters		
Reference	No. of subjects	Dose (µg)	Period (h)	Measurement	$T_{½\,(fast)}$ (h), (range or SD)	$T_{½\,(slow)}$ (h) (range or SD)
Shearer *et al.*, (1972)	3	1000	Up to 96[a]	Lipid soluble radioactivity	0.37[b] (0.33-0.40)	2.3[b] (2.0-2.5)
Shearer *et al.*, (1974)	2[c]	45	7	Pure vitamin K_1 separated by TLC	0.38 (0.35-0.40)	1.8 (1.7-1.8)
Shepherd *et al.*, (1977)	Unknown	700[d]	Unknown	Lipid soluble radioactivity	0.21[e] (±0.03)	3.4[e] (±0.7)
Bjornsson *et al.*, (1979)	4	300	10	Pure vitamin K_1 separated by HPLC	0.43 (±0.13)	2.8 (±0.2)
Olson *et al.*, (2002)	7	0.3	10, then daily	Total plasma radioactivity	1.00 (±0.47)	27.6 (±12.4)
Present study	10	6 / 30	6	Vitamin K_1 enrichment by GCMS	0.22 (±0.14)	2.7 (±1.7)

[a] kinetic parameters are calculated over 6 h

[b] mean values estimated by division by two of the sum of the minimum and maximum values

[c] one subject dosed with warfarin

[d] subjects were given an *iv* dose of 10 µg/kg body weight

[e] average of values for young and elderly patients combined. NB: SEM not SD

3.8.3 Identification of vitamin K_1

In the present experiments, because mass spectrometry was used, the kinetic data was produced from isotopic enrichment unambiguously associated with vitamin K_1. In the case of Olson *et al.* (2002), genuine tracer quantities were used (0.3 µg) but it is unlikely the radioactivity measured represents that only of vitamin K_1. For all samples after 2 h post-dose, only total radioactivity in plasma samples was measured. Prior to 2 h, selected samples were chromatographed, and it is reported that 90% of the radioactivity was associated with the vitamin K_1 fraction (Olson *et al.*, 2002). However, it is likely that over time, the radioactivity was increasingly representative of metabolites rather than vitamin K_1. This conclusion is supported by the earlier work of Shearer *et al.* (1972). In this study, vitamin K_1 was administered intravenously and plasma radioactivity measured in lipid- and water-soluble fractions. If radioactivity was associated only with intact vitamin

K_1 then it would be expected that all radioactivity should have been present in the lipid-soluble fraction. However, at 7 – 8 h post-dose, water-soluble and lipid-soluble radioactivity were about equal and at 24 h the water-soluble component was double the lipid component (Shearer *et al.,* 1972). This work also suggested that although over 6 h radioactivity was identified with two exponentials, over 8 h, the disappearance curve appeared to contain more than two exponentials. Subsequent work by Shearer *et al.*, (1974) showed that when vitamin K_1 was separated from a plasma extract by thin-layer chromatography (TLC), 80% of lipid-soluble radioactivity was associated with vitamin K_1 after 1 h, whereas by 7 h, the proportion had reduced to 40%. These observations show the increasing contribution of lipid-soluble polar metabolites to total radioactivity over time, and suggest that, even at 8 h the true kinetics of disappearance were obscured by the increasing contribution of metabolites, lending further evidence to the questionable results from Olson *et al.* (2002) who measured only total radioactivity. These results stress the requirement of a useable, reliable and robust method for the separation of vitamin K_1.

3.8.4 Timing of samples

In the present study, and in order to ensure proper characterisation of the kinetics, rapid sampling was performed over the expected portion of the fast exponential, and less frequent sampling during the second, slower exponential. This protocol is in contrast to Olson *et al.* (2002), where less frequent sampling may be an additional reason for the observed disparate half-times. Olson *et al.* (2002) sampled at 10 min, and then subsequently at 30 min, 1, 2, 4 and 10 h, and then daily for 7 d. This sampling regimen would make it difficult, if not impossible to detect the initial, fast exponential, and hence the reported $T_{½(fast)}$ of 1 h (Olson *et al.,* 2002), is likely a combination of the $T_{½(fast)}$ and $T_{½(slow)}$ observed in the current study and previously by other workers (Table 3-3).

3.8.5 Body pools

The estimates of pool sizes (Q_1 + Q_2) of 5.1 nmol are low in comparison to values (222 nmol) suggested by direct analysis of tissues (Olson *et al.,* 2002). While Olson's kinetic studies indicated body pool sizes of 1.3 – 2.6 nmol/kg (equivalent to 91 – 182 nmol

for a 70 kg person), this data should be treated with caution because body pool size was determined via the approximation that pool size can be calculated from the intercept of a terminal exponential in kinetic studies where the correspondence of tracer radioactivity to vitamin K_1 abundance was not established.

However, the pool sizes observed in the current study remain low. One explanation may be that modelled estimates of initial plasma pool sizes are low (1.5 nmol). Q_1 is calculated by multiplying actual baseline vitamin K_1 concentration (as measured by HPLC) by the modelled plasma volume. This estimate can be increased upwards if, rather than using modelled plasma volume, pool volume is calculated directly on the basis of body surface area[1]. In this study, average pool size of Q_1 would then be 2.6 nmol. However, total body pool size remains well below previous estimates. In this study, baseline plasma values are around 1 nmol/L, similar to values measured in national surveys (Thane *et al.,* 2006b).

Q_2 is calculated from Q_1 and the rates k_{21} and k_{12}. The value obtained for Q_1 can be considered accurate which suggests the rate constants or model may not represent the true physiology. A possible explanation for low pool size is the presence of additional vitamin K_1 body pools *e.g.* liver and bone (Shearer *et al.,* 1988; Hodges *et al.,* 1993) acting as sinks, or recirculating vitamin K_1 to the plasma at rates insufficient to be detected in the present experiments.

Direct tissue measurements are the most accurate way to determine body pool size. However, the invasive nature of tissue measurements, and the difficulty in using post-mortem samples, results in few published values. The liver concentration of vitamin K_1 is reported to be between 1.1 – 21.3 ng/g (2.4 – 47.3 nmol/kg) liver, based on the analysis of livers of 32 adults post-mortem, with total liver stores between 1.7 and 38.3 µg (3.8 – 85 nmol). Median values were 12.2 nmol/kg, or 17.3 nmol per liver (Shearer *et al.,* 1988). A similar average value of 10.6 nmol/kg is reported from six post-mortem samples

---

1 Plasma volume is assumed proportional to body surface area, with the constant of proportionality 1.41 L/m^2 for women and 1.56 L/m^2 for men (from www.medal.org accessed 30th January 2007)

(Thijssen & Drittij-Reijnders, 1996). Higher values of 28.0 ± 4.3 nmol/kg have been reported from seven surgical patients (Usui *et al.,* 1990).

Direct tissue measurements of vitamin K_1 in bone have revealed average levels in six patients of 10.2 ± 6.4 nmol/kg and 10.7 ± 4.0 nmol/kg (dry weight) in trabecular and cortical bone, respectively (Hodges *et al.,* 1993). Based on a value of 10 kg for the dry weight of a human skeleton (Mitchell *et al.,* 1945), total skeletal pool size may be in the order of 100 nmol. These levels are as much as, if not more than those observed in liver. Combining approximate values for bone, liver and plasma gives a potential body pool size of 120 nmol. Measurements in tissues other than liver and bone suggest a wide distribution of vitamin K_1, for example relatively high concentrations were found in heart and pancreas (9.3 and 28.4 nmol/kg, respectively) and notable concentrations in lung, kidney and brain (1.5, 0.9 and 1.5 nmol/kg, respectively) (Thijssen & Drittij-Reijnders 1996). As a result, total body pool size could be somewhat higher than 120 nmol. To my knowledge, there are no available data on the vitamin K content of adipose tissue, a known store of other fat-soluble vitamins. It is likely that adipose tissue will contain some vitamin K_1 by virtue of the transport of vitamin K_1 in lipoproteins.

3.8.6 Turnover and deductions regarding daily requirements

Early work by Frick *et al.* (1967) on vitamin K requirements in humans estimated the minimal daily requirement to be 0.03 µg/kg/d. An alternative approach to the prediction of daily requirements is to use turnover time. Here, the isotopic data have been interpreted on the basis of a simple two-compartment model in an attempt to provide values for vitamin K_1 turnover (F_{01}) for comparison with current understanding of vitamin K_1 physiology. For a subject in balance, measurement of F_{01} could provide guidance to vitamin K_1 requirements, in which case mean values in the present study are equivalent to 0.34 µg/kg/d. These values are of the same order of magnitude as the current guideline daily intakes for vitamin K_1 in the UK (≥ 1 µg/kg body wt) and AI in the United States (90 and 120 µg for women and men, respectively). Note that the value of 0.34 µg/kg/d takes no account of the bioavailability of vitamin K_1, whereas the recommended intakes have a

'built-in' bioavailability factor. A similar calculation to estimate turnover using Olson's figures provides an approximate turnover value of 0.67 μg/kg/d. Despite the differences in the kinetics between the model presented here and Olson's study, the turnover values are similar. This is due to the large discrepancy in pool sizes as discussed in section 3.8.5. Turnover time from the current study is 3.8 h (see section 1.11.2).

Based on vitamin K_1 in liver, Shearer (1988) suggested that, because requirements were estimated to be greater than body stores, either menaquinones provided a significant contribution to vitamin K needs, or requirements were set too high. An alternative explanation may be that there are other significant body stores of vitamin K, a notion supported by observations of vitamin K in bone.

Bone may be a potential pool of vitamin K_1 because of the requirement of vitamin K_1 for carboxylation of bone proteins and/or the intrinsic lipid content of bone. Turnover may be relatively slow as demonstrated in a recent animal study (Sato *et al.*, 2002). Rats were kept on a vitamin K_1-deficient diet for 17 d and whereas prothrombin time significantly decreased over this time, there was no change in either total osteocalcin or % undercarboxylated osteocalcin (%ucOC) suggesting a slower turnover in bone than liver. However, depletion experiments in humans have shown that markers of bone turnover are a sensitive marker of vitamin K_1 status, since over a 15 d cycle, levels of %ucOC decreased by 28% (Sokoll *et al.*, 1997).

3.8.7 General considerations

There are four conditions that should be met in order to estimate turnover time: specificity of measurement, size of tracer dose, requirement for a steady state, and rapid mixing with the exchangeable pool (Shearer & Barkhan, 1979). These are each discussed below.

3.8.7.1 Specificity of measurement

Previous studies have all used radiolabelled vitamin K_1 tracers. Specificity, or the degree to which the tracer has been positively associated with vitamin K_1, varies from not at all in the case of Olson *et al.* (2002), where plasma radioactivity was measured, to this

study, where vitamin K_1 is unequivocally identified using mass spectrometry. In between these extremes are attempts to use lipid soluble-radioactivity and separation by either thin-layer chromatography (Shearer *et al.,* 1974) or HPLC (Bjornsson *et al.,* 1978). Although the latter two provide chromatographic resolution, absolute and positive association with pure vitamin K_1 cannot be assumed. Disadvantages of TLC are the rather cumbersome methods, chromatographic tailing (and lack of resolution), and exposure to light. The HPLC method described by Bjornsson *et al.*, (1978) does not rely on the direct measurement of radioactivity in vitamin K_1. Rather, lipid radioactivity is extracted from plasma and subjected to HPLC, and the vitamin K_1:vitamin K_1 epoxide ratio used to calculate the amount of radioactivity remaining (Bjornsson *et al.,* 1978). With mass spectrometry, as in this study, it is possible to simultaneously identify and quantify the tracer (in this case, level of enrichment).

Recent work has clearly demonstrated the conversion of oral vitamin K_1 to the structurally similar menaquinone-4 (Thijssen et al., 2006); whereas vitamin K_1 has a single double bond in the side chain, menaquinone-4 has four, and consequently a molecular weight 6 Da lower (444 Da compared to 450 Da). Such similarly related molecules may not have been detected by TLC, although analysis by liquid chromatography has been used frequently for analysis of these two forms of vitamin K (e.g. Schurgers & Vermeer, 2000). Mass spectrometry provides a suitable tool to quantify the extent of this conversion by monitoring ions of both vitamin K_1 and menaquinone-4. This analysis would be possible using the methods described here since the stable isotope label is positioned on the napthoquinone ring, whereas it has been suggested that conversion to menaquinone occurs through initial conversion to menadione, thus any labelled atoms on the side chain many be lost.

An additional consideration is the quantitative measurements. As discussed previously, early studies were not able to measure absolute amounts of vitamin K_1. Methodological problems are still apparent even in Olson's study, where it is recognised that their chosen method of analysis (Ueno & Suttie, 1983) provides values two-fold higher than the method of Sadowski *et al,.* (1989). Thus, there is a lack of confidence in

these measurements. However, in this study there is greater confidence in our quantitative measurements: analysis was performed using the method of Wang *et al.*, (2004), and additionally, all measurements were made under the umbrella of the KEQAS, vitamin K external quality assurance scheme run by Human Nutristasis Unit at Guy's and St Thomas' Hospital. Therefore, this study is likely the only one to date that fully meets the specificity of measurement criterion, both in terms of quantitative and tracer measurements.

3.8.7.2 Size of tracer dose

The size of tracer dose is a challenging problem with vitamin K due to the small pool sizes, especially when compared to other fat-soluble vitamins. A true tracer dose should not perturb endogenous kinetics and should be a physiological size dose, *i.e.* one that could realistically be achieved through the diet. Some of the previous studies (Shearer *et al.*, 1972; Shepherd *et al.*, 1977; Bjornsson *et al.*, 1979) have used doses much higher than obtainable from the diet, and therefore must be considered in the pharmacological range. In this study, the 30 and 6 µg doses produced plasma increments of 37.6 and 6.8 nmol/L, respectively. The 30 µg dose produced plasma values that are not commonly possible to achieve through diet alone, whereas the 6 µg dose produced more physiological values although still on the high-side of typical increments. However, there was no significant difference in kinetic parameters obtained from either dose.

3.8.7.3 Steady-state

The requirement of a steady-state for vitamin K is the third criterion. In this study, measurements of plasma vitamin K status and enrichment showed no significant difference between days 5, 6 and 7 of the experiment. However, the more relevant question is was there a steady-state over the 6 h duration of the experiment? Based on the turnover of vitamin K_1, it may be considered that, even over the relatively short time scale of the experiment, that the tracee was decreasing. The result may be an observed slope that is shallower than the true curve and hence reported half-times might be slower than the true values. However, over the time course of this experiment, vitamin K_1

concentration was also measured thus any deviation from the steady-state was corrected for.

The decision to dose for 6 d in order to reach a steady-state for plasma vitamin K_1 enrichment was taken with the use kinetic values and pool sizes calculated by Olson *et al.,* (2002) as the only suitable and available kinetic data. The actual period required to reach a steady state will be influenced by inter-individual variation in vitamin K metabolism, habitual vitamin K_1 intake and possibly vitamin K status (Olson *et al.,* 2002). In the model, intakes of vitamin K_1 from food were fixed at 60 µg/d but variation of intake both between and within an individual will affect the time taken to reach, and the 'stability' of the steady-state. With free-living individuals it is not possible to control intake, however if an individual's diet was controlled at the same time as dosing then a true steady-state could be established, although the level of enrichment would likely vary between individuals. As discussed previously, the observed levels of enrichment were lower than expected likely due to assumptions in the bioavailability of both vitamin K_1 from food and the labelled dose.

To reach a true steady-state, and notwithstanding the challenges above, then it is likely that because of the putative body pool (bone and adipose tissue) of vitamin K_1 with a slower turnover dosing would be required for a longer period than that used here. A study in rats demonstrated that after 17 d depletion, although liver stores were largely depleted, vitamin K1 in the femur had dropped by only 40% (Sato *et al.,* 2002). It is not possible to comment on how representative the kinetics derived from Olson *et al.* (2002) are of the possible slower body pools since vitamin K and metabolites were measured. For the purposes of the study described here, i.e. characterisation of the fate of an oral dose over the relative-short term to determine absolute absorption, the inability to observe slower turnover would have a minimal influence on the results.

3.8.7.4 Rapid mixing

The final condition that must be met is the rapid mixing of the tracer with exchangeable pools of endogenous vitamin K_1. A complication of working with highly

lipophilic compounds such as vitamin K_1 is the requirement of a carrier for *iv* injection. There is no detailed data on the metabolism of Konakion after *iv* injection, (e.g. lipoprotein partitioning), so there is some uncertainty as to how Konakion behaves compared to absorbed, dietary vitamin K_1 in triacylglycerol-rich lipoproteins. Kinetic results from an *iv* dose of Konakion MM® (Soedirman *et al.,* 1996) are similar to those obtained here and previously reported with radiolabelled, detergent-solubised vitamin K_1 (Shearer *et al.,* 1972; Shearer *et al.,* 1974). Different metabolism of Konakion and absorbed, oral vitamin K_1 would have implications for the kinetic parameters obtained from the model. However, any differences in metabolism (section 3.9.2) are most likely to occur in the initial stages of the model, where the Konakion equilibrates with endogenous K_1 in lipoproteins, and thus is less likely to have a major affect on turnover values calculated from the second, slower exponential.

3.8.8 Potential future studies

Rapid disappearance of vitamin K_1 from plasma, relatively rapid turnover, and sequestering to body pools with slow turnover has meant that previous studies have been unable to measure the kinetics of pure vitamin K_1 much beyond 8 h. Olson *et al.* (2002) performed a kinetic study over a number of days but was most likely measuring predominantly vitamin K_1 metabolites rather than vitamin K_1. In order to further our understanding of vitamin K metabolism and additional body pools, studies are required that measure kinetics over a longer period than has hitherto been attempted.

One solution to the challenge of measuring kinetics in the longer term, is to give higher doses, however one of the difficulties with vitamin K_1 is also the low body pool sizes, and thus a larger tracer dose would not only be unphysiological but may also perturb existing vitamin K_1 kinetics. As a result, the most likely way of recording longer-term kinetics is to increase sensitivity of the measurements to enable monitoring of smaller changes. In future work, a number of approaches may be possible to increase the sensitivity, for example increasing enrichment or using more sensitive equipment. Remaining with the methodology explored in this study, sensitivity to detect long-term

kinetics may be enhanced by increasing the levels of pre-enrichment. The oral pre-enrichment achieved in this study with daily dosing of 9 μg was around 8% above baseline. With average intakes of around 70 μg of vitamin K_1 per day, it would feasible to increase the oral pre-enrichment dose four-fold, while having little affect on total intake. With baseline isotopomer enrichments also increased four-fold, it may be possible to follow the kinetics of vitamin K_1 disposal for longer. Using equipment more sensitive to measure isotope ratios could further enhance our ability to track longer-term kinetics. Gas chromatography-combustion-isotope ratio mass spectrometry is 200 times more sensitive than GCMS in terms of measuring isotope ratios (and has greater precision), but is 1000 times less sensitive in terms of the amount of material needed to observe a peak (Bier 1997). Therefore, this approach is currently not feasible. An alternative equipment-based solution is the use of liquid chromatography mass spectrometry. This approach is currently in its infancy for isotope ratio measurements but may provide a solution in the future and has the potential additional advantage of reducing the need for some sample preparation.

Despite the advantages of using stable isotopes, it may be that the use of radiolabelled vitamin K tracers will have to be revisited. The advantage of radiolabelled compounds is the ability to detect them at very low levels and thus, in theory at least, it should be possible to determine long-term kinetics. However, as demonstrated by the short-comings in Olson's study, there is a requirement for the positive and unequivocal association of radioactivity with vitamin K_1. Establishing this correlation is likely to require laborious sample extraction as well as the use of semi-preparative HPLC, that in the past has either not measured radioactivity in vitamin K_1 directly (Bjornsson *et al.,* 1978; Bjornsson *et al.,* 1979) or not provided sufficient separation and extraction (Erkkilä *et al.,* 2004).

3.8.9 Kinetics summary

The twin objectives of this part of the experiment were 1) develop a stable isotope methodology for the measurement of vitamin K_1 kinetics and 2) obtain data on vitamin K_1

kinetics, turnover and body pool sizes in humans. Both of these objectives have been successfully met using a novel approach of oral pre-enrichment and a non-labelled tracer, thereby circumventing any ethical concerns with regard to the use of chemically synthesised *iv* tracer.

3.9 Absorption

In this study, post-absorptive kinetics were measured following an *iv* dose of Konakion MM® together with enrichment of plasma vitamin K_1 with ^{13}C-labelled vitamin K_1. The resulting kinetic parameters were then used to calculate absolute absorption of an oral dose of ^{2}H-labelled vitamin K_1. Any reservations about the model in this study to fully describe vitamin K_1 disposition within the body over the longer-term, do not however invalidate the usefulness of the method in providing a description of the response to a small, physiologically realistic dose, typical of what might be consumed in a single meal (4 µg), or to the measurement of the amount of it that was absorbed. This study is the first to measure the absorption of an extrinsically labelled form of vitamin K_1 at low levels and, as discussed below, the results are considerably different to a previous estimate of vitamin K_1 absorption (Shearer *et al.,* 1974).

The most commonly used method to measure absorption is relative availability, usually by estimation of the area under the plasma vitamin K_1 response curve when the vitamin is given in either standard or test forms (Gijsbers *et al.,* 1996; Booth *et al.,* 1999a; Garber *et al.,* 1999; Schurgers & Vermeer, 2000; Booth *et al.,* 2002). Such studies measure study-specific relative absorption and as a result it is very difficult to compare between them. Some have measured the relative absorption of vitamin K_1 from a food source compared to a supplement, either in the form of a tablet, (Garber *et al.,* 1999) or as Konakion®, (Gijsbers *et al.,* 1996; Schurgers & Vermeer, 2000), while others have compared different food sources only, albeit fortified oil (Booth *et al.,* 1999a; Booth *et al.,* 2002).

In the present experiments mean absolute absorption was 12.7% with a CV of 74%. The only other previous study to measure absolute absorption of vitamin K_1 in

healthy subjects estimated absorption at around 80% (Shearer *et al.,* 1974). Absorption was assessed by the collection of faecal samples from three volunteers who consumed a 1 mg oral dose of radiolabelled vitamin K_1. Although Shearer's initial estimates of absorption were between 40 and 50%, this value was later increased on the basis that a proportion of radioactivity in faeces was derived from the biliary excretion of metabolites, i.e. radioactivity in faeces was from vitamin K_1 metabolites that had been absorbed and excreted, rather than vitamin K_1 that had not been absorbed at all. This study used high levels of vitamin K_1 to measure absorption; the size of the dose (1 mg) was in the pharmacological, not physiological range. In general faecal-balance studies can suffer from a number of potential inaccuracies, for example no account is made for losses due to bacterial degradation in the gut. However, Shearer *et al.* (1974) concluded there was no significant degradation of vitamin K_1 to polar metabolites by gut bacteria.

It is possible that the low levels of absorption found in the present experiments are a consequence of the form in which the dose was given. For simplicity, the dose was provided in the absence of a test meal. In contrast, Shearer's estimates of 80% were obtained when the standard vitamin K_1 dose was provided in milk with a meal of bread and cheese (Shearer *et al.,* 1970a). The secretion of bile is essential for absorption of vitamin K (Shearer *et al.,* 1974) and it may be in the present study, that a capsule containing only 0.5 mL of oil contained insufficient fat to yield much of a gastric response. Furthermore, gastric emptying effects and possible lack of gastric motility elicited by the capsule may have resulted in the observed low absorption values. Absorption of vitamin E from a supplement was significantly greater when taken with a meal, compared to consumption on an empty stomach (Iuliano *et al.,* 2001). Additionally, low vitamin E absorption from capsules has also been observed when taken without additional fat (Leonard *et al.,* 2004).

There is some evidence to suggest that current estimates of the absorption of other fat-soluble vitamins may be exaggerated. For example, a recent study calculated α-tocopherol absorption of between 10 and 33% (depending on the level of concomitant fat intake). These values are significantly lower than earlier estimates of between 55 and

79% in humans (Bruno *et al.,* 2006) and may be due to the more physiological size doses and method of incorporation of the tracer into the food. β-carotene absorption is reported to vary between 3.5% and 90% (Tyssandier *et al.,* 2003). Direct analysis of the micellar phase of duodenal contents in humans (using a naso-duodenal tube) showed that a maximum of 11% of *all-trans* β-carotene and only 2.5% of *all-trans* lycopene were absorbed from vegetable sources (Tyssandier *et al.,* 2003). Work in rats reported only 10% absorption of vitamin K_1 on a standard diet, and 19% from a high vitamin K_1 diet (supplemented with vitamin K_1 in corn oil) over a period of 5 to 9 d (Koivu-Tikkanen *et al.,* 2000b).

Evidence also exists showing that synthetic forms of vitamin E are less bioavailable than natural forms (Lodge 2005) due to the synthetic form containing eight steoroisomers (Hoppe *et al.,* 2000). Vitamin K_1 exists in two forms due to rotation around the double bond in the phytyl side-chain (Figure 2-3). Although both isomers are believed to be absorbed, only the *trans*-isomer is thought to be biologically active (Knauer *et al.,* 1975; Lowenthal & Vergel Rivera, 1979). The majority of naturally occurring vitamin K exists in the *trans* form (Woollard *et al.,* 2002), whereas our synthetic labelled forms of vitamin K_1 contained around 15% of the *cis*-isomer (Section 2.6.8). Although absorption may be similar for the two isomers, the kinetic and metabolic behaviour of the two forms may be different (Blatt *et al.,* 2004). The contribution of the *cis*-isomer remains an unquantified and potential source of error.

It has been demonstrated in rats that dietary vitamin K_1 is converted to MK-4 after absorption (Thijssen & Drittij-Reijnders., 1994). A possible pathway was originally considered via metabolism of vitamin K_1 to menadione by gut bacteria, subsequent absorption and conversion to menaquinone-4 in the liver. However, from research in rats, gut bacteria have since been shown to be unnecessary in the conversion of vitamin K_1 to menaquinone-4 (Davidson *et al.,* 1998; Ronden *et al.,* 1998). It has recently been shown in a human volunteer study that the conversion to menaquinone-4 occurs via the intermediate conversion to mendione (Thijssen *et al.,* 2006). Furthermore, the conversion is only observed after oral, not subcutaneous administration of vitamin K_1 (Sakamoto *et*

al., 1996; Thijssen *et al.*, 2006). The work by Thijssen *et al.*, (2006) has shown that up to 25% of orally ingested vitamin K_1 is not absorbed intact but is metabolised to menadione, possibly during transfer across the enteroctye. This metabolism would have the effect of decreasing the observed absorption since in the current study only intact vitamin K_1 was measured. If 25% of the dose had been metabolised to menadione then absorption values could be increased to around 17%. Although only a minimal increase, if coupled with the other explanations for low absorption, then absorption could be significantly greater. For future studies, the measurement of changes in menadione and/or menaquinone-4 enrichment may also be worth measuring.

Other sources of potential error in the absorption values derive from the analytical and modelling procedures. Firstly, there remains the possibility that potential slower reaction rates for enriched samples resulted in less conversion to the derivatised form of deuterated vitamin K_1 compared to unlabelled vitamin K_1. Theoretically, this is unlikely to be important in the derivatisation reaction because the deuterated atoms and their chemical bonds to the molecule remain away from the derivatisation reaction sites. Furthermore, during the analytical method development work no obvious discrimination occurred between the two forms, e.g. in the isotope linearity experiments. The second possibility is that assumptions in the model led to an under-estimate of absorption. Potential differences in the handling of the *iv* and oral doses and assumptions of the model are considered below (section 3.9.2).

The value for absorption has a CV of 74% and is not unreasonable compared to other studies of absorption. The inter-individual CV in AUC values to a tablet form of vitamin K_1 was 37% (Garber *et al.*, 1999) and for Konakion 41% (Gijsbers *et al.*, 1996). For more complex meals, values ranged from 17 – 76% (Gijsbers *et al.*, 1996; Garber *et al.*, 1999; Booth *et al.*, 2002). Furthermore, a study measuring α-tocopherol absorption has reported a 40-fold difference in AUC and maximal concentrations after an oral dose in 30 subjects (Roxborough *et al.*, 2000). There are a number of potential physiological and genetic reasons for the variation, including factors influencing gastric emptying, absorption and uptake. For example, the apolipoprotein E genotype has been demonstrated to alter

the transport and status of vitamin K_1 (Saupe *et al.*, 1993; Yan *et al.*, 2005) and other fat-soluble vitamins (Ortega *et al.*, 2005). Studies of vitamin E absorption from capsules have also reported highly variable plasma responses between individuals (Roxborough *et al.*, 2000; Leonard *et al.*, 2004).

This study is unique in magnitude of the oral dose given. Only high doses of between 165 and 1575 µg vitamin K_1 have previously been investigated, whereas average daily intakes in UK adults have been calculated to be around 70 µg (Thane *et al.*, 2006a). Furthermore, plasma response to different levels of vitamin K_1 from the same source (e.g. spinach) may not be linear (Garber *et al.*, 1999). Evidence from intake estimates and their relationship to plasma status suggests the relationship is only linear up to 200 µg (McKeown *et al.*, 2002). Although in a depletion/repletion experiment intakes of up to 450 µg had a linear relationship with vitamin K_1 plasma response after 3 and 6 h (Booth *et al.*, 2003b). Uptake of vitamin K_1 from the gut is thought to be a passive process, however there is also evidence that there may be an active pathway that can be saturated at higher levels (Hollander 1973;1977).

Other attempts to obtain information on vitamin K_1 absorption with the stable isotopically labelled vitamin are restricted to three studies reporting data from intrinsically labelled broccoli (Dolnikowski *et al.*, 2002; Erkkilä *et al.*, 2004) and kale (Kurilich *et al.*, 2003). For the absorption of 70 µg of vitamin K_1 from kale, Kurilich *et al.* (2003) report a value of 7% absorption, based on the amount of dose in the plasma at the time of peak vitamin K_1 concentration. If a similarly crude method is applied to this study, then average absorption values are only around 1%. It is unlikely that absorption from vegetables is actually greater than that from oil, further suggesting the values obtained from this study are low compared to what may have been obtained if the dose had been consumed as a food, rather than a capsule. This finding may have implications for supplement forms of vitamin K_1, or indeed clinical use of oral vitamin K_1.

3.9.1 T_{max}

The time of peak absorption (T_{max}) derived from smoothed values was 4.7 h (SD ± 0.8). In previous studies measuring absorption from a supplement, T_{max} was measured as 2.4 h (Garber *et al.,* 1999) for the tablet form and 4 h for Konakion MM® (Gijsbers *et al.,* 1996; Schurgers & Vermeer, 2000). Our capsule form of the vitamin appeared to be absorbed more slowly than previous supplemental forms, due to either slow transit time due to the minimal stimulation of the gut or delay in release from the capsule. The T_{max} of vitamin K_1 appearance in plasma from food has been measured as between 2.7 h and 7.7 h (Gijsbers *et al.,* 1996; Garber *et al.,* 1999; Schurgers & Vermeer, 2000).

3.9.2 Metabolic handling of *iv* and oral doses

A caveat to this study methodology, and any study investigating the kinetics of vitamin K, could be potential differences in the metabolic handling of the oral and *iv* doses of vitamin K_1. Absorption of vitamin K_1, as with other fat-soluble vitamins, occurs through the formation of micelles in the presence of bile salts in the gut lumen. Once internalised in the enteroctye, vitamin K is packaged into chylomicrons and enters the circulation via the lymph (Shearer *et al.,* 1974; Lamon-Fava *et al.,* 1998; Schurgers & Vermeer, 2002). Any differences in the metabolism of the oral and *iv* forms depends on the fate of vitamin K_1 from the *iv* dose. The *iv* form, Konakion MM®, is a pharmaceutical preparation of vitamin K_1 in which the vitamin is solubised in mixed micelles formulated from glycholate and lecithin. Upon injection, the vitamin K_1 is rapidly released and it is reported that the mixed micelles do not influence the behaviour of the vitamin (Soedirman *et al.,* 1996). Kinetic results from Konakion alone (Soedirman *et al.,* 1996), detergent-solubised radioactive tracers (Shearer *et al.,* 1974) and this study, are all similar. There are no data to suggest that Konakion is or is not metabolised differently to absorbed, dietary vitamin K_1. However, differences may exist between vitamin K_1 uptake from chylomicrons and chylomicron remnants compared to the Konakion formulation since the latter lack any of the intrinsic proteins that lead to lipoprotein uptake, e.g. apoE.

From the absorption curves it was possible to obtain an approximate value for the $T_{½}$ of disappearance of the deuterated oral dose of vitamin K_1. The terminal slope was calculated by regression of the plasma concentration of deuterated vitamin K_1 of the final two or three data points for each subject. The average $T_{½}$ was calculated from division of 0.693 by the slope. Values were obtained for nine of the ten subjects, and the average $T_{½}$ was 2.39 h, not significantly different from 2.66 h derived from the *iv* kinetics (P=0.78). This suggests that there was no difference in the metabolic handling of the oral and *iv* forms of the vitamin. However, the estimate of oral dose $T_{½}$ is very approximate since it based only on two or three data points, thus may not represent the true slope. A better estimate could be obtained with a greater sampling frequency and longer sampling duration.

In the calculations, absorption is calculated on the assumption that the kinetics of the *iv* dose predict the irreversible disposal of the oral dose. Thus, if irreversible disposal occurred at a greater rate than that predicted, then absorption would be under-estimated. However, other data supports the kinetic parameters calculated in this study, while other work suggests a slower rate of irreversible disposal (Olson *et al.*, 2002).

3.9.3 Absorption summary

The aim of the absorption arm of this experiment was 1) design a methodology to measure absorption and 2) obtain values for absorption of an oral dose in a standard form, with the possibility of using this as a standard by which to measure absorption in future studies.

A method was successfully tested and a value was obtained for absolute absorption that included absorbed vitamin K_1 that otherwise would not be identified using traditional AUC methods, due to transfer to other, non-sampled body pools. In conclusion, the absolute absorption of a 4 µg deuterium labelled dose of vitamin K_1 has been calculated by applying post-absorptive kinetics determined from a simultaneous *iv* dose. The percent absorption of the vitamin K_1 dose was measured as 13%. This method may provide a

suitable tool to investigate vitamin K_1 absorption from food sources at levels commonly consumed in a typical Western diet.

3.10 Section 3 conclusions

The kinetics of vitamin K_1 uptake and disposal have been fully characterised over a 6 h period. The results obtained agree with the majority of those published previously where radioactive tracers have been utilised, with the exception of work by Olson *et al.*, (2002). This work clearly demonstrates the importance of having a reliable method for the unambiguous determination of vitamin K_1, such as that described in Section 2. Together with previous work, these results point to the possible existence of, as yet, uncharacterised body pools of vitamin K_1 with slow turnover rates, most likely bone and adipose tissue. Further studies are required to determine longer-term kinetics and turnover rates.

In the present study, it was possible to apply the kinetic parameters obtained to the accurate determination of absorption of vitamin K_1 from an oral dose. Absorption was measured as 13%, a relatively low estimate, possibly due to the absence of a test meal, but also the possibility of conversion to menadione during transition across the enterocyte. This possibility requires further investigation, something that could be possible by measuring changes in menadione enrichment in the plasma samples obtained during this study.

This study has demonstrated the application of a dual stable isotope-type approach to the measurement of fat-soluble vitamin absorption and kinetics, specifically vitamin K_1. In addition, the use of an oral pre-enrichment method to accurately determine kinetics with an unlabelled tracer was demonstrated. Each of these methods provides a basis for the further investigation of vitamin K_1 kinetics, turnover and absorption.

4 STUDY 2: MEASUREMENT OF VITAMIN K_1 BIOAVAILABILITY

4.1 Background

In Western populations, vitamin K_1 is the primary dietary form of vitamin K (Schurgers *et al.,* 1999). Vitamin K_1 is found in a wide range of foods but vegetables contribute at least 50% to total vitamin K_1 intake (section 1.5.1) with the majority from green leafy vegetables. Consequently, green leafy vegetables are considered the major dietary source of vitamin K_1, however recent evidence shows their contribution may be decreasing (Thane *et al.,* 2006a). In the UK, average adult intakes of vitamin K_1 are reported to be between 60 and 80 µg/d (Fenton *et al.,* 1997; Bolton-Smith *et al.,* 1998; Fenton *et al.,* 2000; Thane & Bolton-Smith, 2002b; Thane *et al.,* 2006a). From a recent comparison of data between the 1986 – 7 Dietary and Nutritional Survey of British Adults and the 2000 – 1 NDNS, those participants who had vitamin K_1 intakes below the UK guideline, rose from 47% in 1986 – 7 to 59% in 2000 – 1 (Thane *et al.,* 2006a)

Where intakes may be marginal, and in order to set dietary recommendations, it is important to understand how representative the measurement of status (typically plasma concentration) is of dietary intake. Additionally, defining this relationship is important for identifying groups who may be at risk of nutrient deficiency. Furthermore, based on evidence showing an influence of fluctuating dietary vitamin K_1 intake on anticoagulation treatment (Khan *et al.,* 2004; Kurnik *et al.,* 2003; Schurgers *et al.,* 2004; Couris *et al.,* 2006) data on acute changes in vitamin K_1 plasma concentration in response to different foods are required.

A number of studies have demonstrated a significant and positive relationship between dietary intake and plasma concentration of vitamin K_1, although these relationships are not very strong. For example, a recent paper reported correlations of $r=0.26$ for women and $r=0.32$ for men ($P<0.001$) (Thane *et al.,* 2006a). This may be partly due to plasma status not reflecting chronic intake, although it is probable that bioavailability is also an important factor determining the relationship between intake and

status, particularly in the short term. In common with other fat-soluble vitamins, and as discussed in section 1.8*ff*, bioavailability is a consequence of digestion and absorption. As a result, it is theoretically determined by a number of factors; the food matrix, effects of cooking or processing, other dietary components such as fat which may affect absorption and/or transport of vitamin K_1, and other micronutrients that may enhance or inhibit absorption or transport such as vitamin E or dihydro-vitamin K_1. Differences in the bioavailability of vitamin K_1 between foods could require a reassessment of the relative importance of different sources. However, only a limited number of studies have attempted to measure vitamin K_1 bioavailability. Studies have measured either bioavailability from different foods and/or the effect of fat on absorption. In summary, these studies suggest that absorption is no different between different vegetable sources (Garber *et al.,* 1999) and that the presence of fat may enhance vitamin K_1 absorption (Gijsbers *et al.,* 1996). Studies measuring differences in absorption of vitamin K_1 from oil or vegetable sources have found conflicting results (Booth *et al.,* 1999a; 2002). The varied findings are probably the result of the different methods used and their limitations, particularly the use of protocols where comparisons have to be made between foods or meals given at different times in different subjects and often using relatively large doses of the vitamin. It is also difficult to compare different studies. As described in section 1.12.7, a stable isotope labelled version of a nutrient provides a safe method of investigating bioavailability to obtain information on the relationship between vitamin K_1 dietary intake and plasma status, and the relative importance of different sources of vitamin K_1.

4.2 Study objectives

The two primary objectives of this study were to:

I. Develop a stable isotope methodology for the measurement of vitamin K_1 bioavailability from meals and/or foods

II. Obtain data on the bioavailability of vitamin K_1 from meals and measure absorption of stable isotope dose

4.3 Study design

Section 2 described the development of a method for the measurement of plasma vitamin K_1 enrichment that avoided time-consuming semi-preparative HPLC. This method is reliable and precise for the measurement of isotope ratios of vitamin K_1 in plasma samples. The volunteer study, described in section 3, investigated vitamin K_1 kinetics and absorption of a labelled oral dose in humans. Estimates of absorption and time profiles from this study provided additional data for the development of a further study to measure the bioavailability of vitamin K_1 from food.

Previously, studies measuring bioavailability have generally focussed on a single source of vitamin K_1 (e.g. broccoli or vitamin K_1-fortified oil) often at much higher concentrations than would be present in a typical meal. Although this approach may provide mechanistic information and allow comparison between individual foods, it is unrepresentative of typical intakes or the way in which people commonly consume foods. Furthermore, information is required on which foods or nutrients, when in combination, may inhibit or enhance vitamin K_1 absorption. Therefore, in the current study, volunteers were fed realistic meals containing typical amounts of vitamin K_1.

4.3.1 Meal design

The meals were each designed to reflect a typical dietary intake in the UK. Traditional methods of analysing large amounts of dietary data are often based on the consumption of separate nutrients, and individuals grouped according to their intake of a single nutrient or type of food. Dietary pattern analysis for food consumption provides an alternative statistical approach to the study of isolated dietary components and a basis for the investigation of the interactive effects of different foods on bioavailability. Fahey *et al.*, (2007) have recently described a novel statistical technique for the grouping of individuals into clusters based on the consumption of similar foods. This method was applied to data from the 2000 – 1 NDNS of Adults. In this survey, 958 women and 766 men provided dietary data as 7-d weighed food records. These food data were combined into 25 food groups that were used for the modelling. Dietary pattern analysis identified four dietary

clusters in women and six in men (Fahey *et al.*, 2007). Of these, three showed similar patterns between men and women and were chosen for the design of the meals.

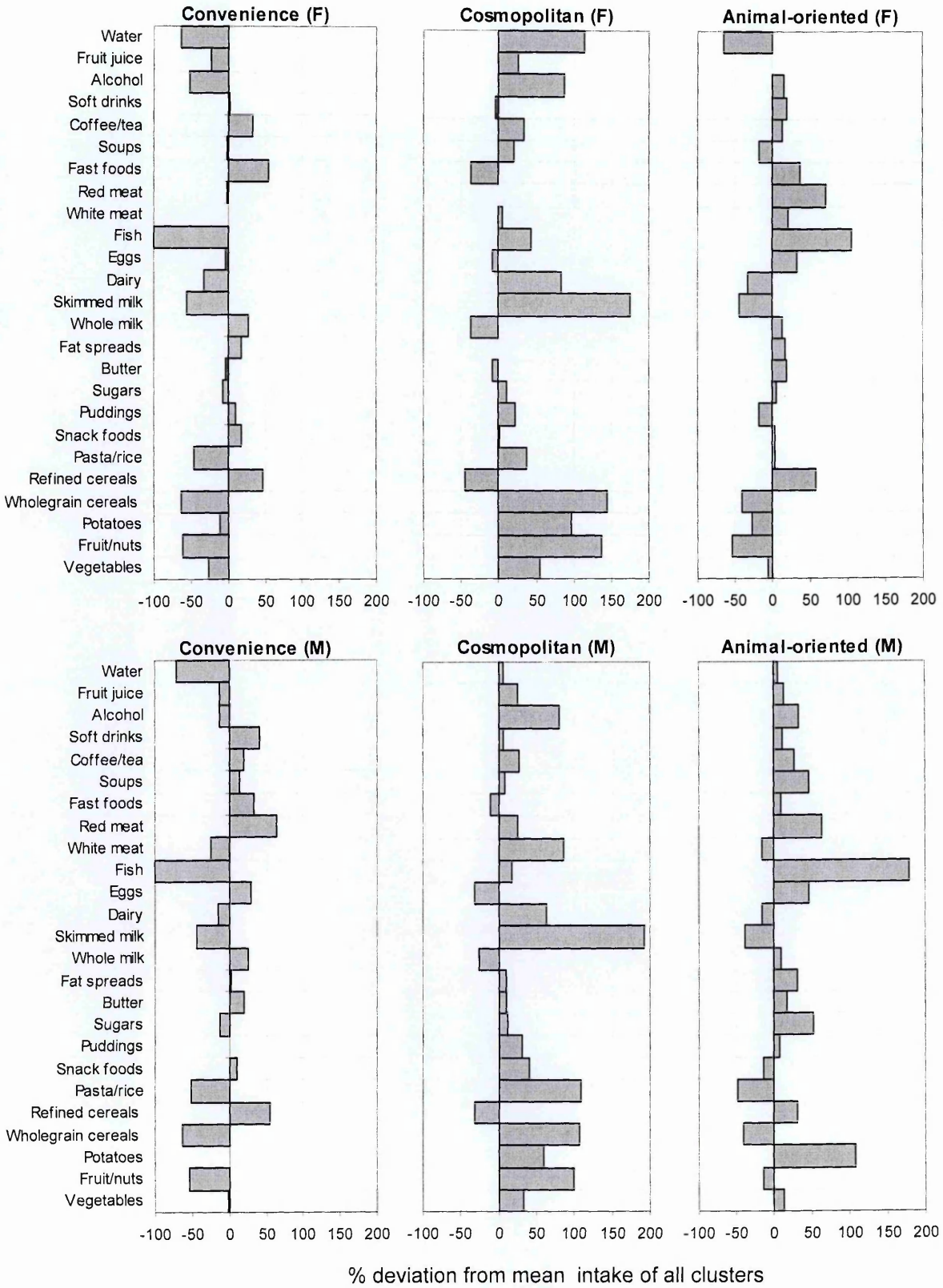
Each cluster was defined by the relative consumption of each of the 25 food groups (Figure 4-1). Based on each cluster's defining characteristics and to aid identification each cluster was given a single descriptive name (Fahey *et al.*, 2007). A danger of the name is that it may lead to assumptions by the reader with regard to the types of food that characterise that group however, the same labels, as defined by Fahey *et al.*, have also been used in this study for the purposes of recognition.

Cluster 1 was characterised by higher than average consumption of fast and snack foods and refined cereals, and lower than average consumption of fruit, vegetables and whole grain cereals. This cluster was labelled as a 'convenience' diet. Cluster 2, the 'cosmopolitan' diet, was characterised by higher than average consumption of fruits, vegetables, whole grains, fish and diary foods. This group had lower consumption of refined cereals, and fast and snack foods. Cluster 3 was distinguished by higher than average consumption of animal products and refined cereals and average consumption of vegetables. This cluster was labelled as 'animal-oriented'.

4.4 Methods

For the bioavailability study, the test meals were designed based on the characteristics of each cluster (Figure 4-1). The clusters represent the intake of each of the food groups and not intake of vitamin K_1. For example, those individuals in the cosmopolitan cluster had a higher intake of vegetables and whole grains compared to individuals in the convenience or animal-oriented clusters. Thus, the cosmopolitan meal contained more of this food group than either the convenience or animal-oriented meals.

Figure 4-1. Predicted mean intakes of food groups in each cluster as the % deviation from the mean food group intake of all clusters, by cluster and sex (F = female; M = male) (adapted from Fahey *et al.*, 2007)



The food group 'dairy' includes cheese, cream, ice cream and yoghurt but excludes those dairy items listed separately (milk and butter). Oils were excluded from the analysis because consumption was low.

The characteristics of each cluster were used to formulate the three different test meals. Thus, the convenience meal was designed to contain foods matching the convenience cluster's defining characteristics: higher than average consumption of refined cereals and fast foods and below average intake of vegetables. Sources of vitamin K_1 in this meal were primarily rapeseed oil and other vegetable oils.

The cosmopolitan cluster was characterised by higher than average consumption of vegetables, wholegrain cereals, fish and dairy foods and thus the meal was based around these characteristics. The main vitamin K_1 source was vegetables.

Those people defined by the animal-oriented cluster consumed red meat and refined cereals in above average amounts, and thus these food groups were prominent in the animal-oriented meal. Their consumption of vegetables was average. The main vitamin K_1 sources in the animal-oriented meal were olive oil and peas.

Actual meal composition is shown in Table 4-1. The convenience meal was a chicken pie served with chips, baked beans and white bread. The cosmopolitan meal was a fish pie (containing green beans and potatoes), served with wholemeal bread. Finally, the animal-oriented meal was a beef lasagne served with white bread. Meals were matched for energy (c. 3200 kJ) and percent energy from protein (20%), fat (40%) and carbohydrate (40%). Dietary composition was estimated using the MRC Human Nutrition Research in-house suite of programs based on McCance and Widdowson's The Composition of Foods, fourth edition (Paul & Southgate, 1978), its supplements (Holland *et al.*, 1988; 1989) and the sixth edition (Food Standards Agency 2002).

Each meal was designed to contain around 40 μg of vitamin K_1. This value was based on the expected plasma vitamin K_1 increment, assuming a relatively low bioavailability, and also on analytical limitations. The major food items contributing to total vitamin K_1 intake are show in Table 4-2. Meals were tested for palatability prior to the start of the study. Sixteen of each meal were prepared in single batches and frozen at -18 °C until use.

Table 4-1. Ingredients and nutrient composition of each test meal designed for the bioavailability study

Convenience	Weight	Energy	Prot	Fat	CHO	Fib	VitK
	g	kJ		g			µg
Chicken, light meat, raw	95	427	22.8	1.1	0.0	0.0	0.0
Flour, self-raising	35	491	3.1	0.4	26.5	1.4	0.3
Margarine, soya based	25	677	0.0	18.3	0.1	0.0	19.5
Milk, whole	65	179	2.1	2.5	3.1	0.0	0.4
Onions	20	20	0.2	0.0	1.0	0.3	0.1
Peas, frozen	16	45	0.9	0.1	1.5	1.1	4.7
Rapeseed oil	9	333	0.0	9.0	0.0	0.0	10.1
Baked beans	70	249	3.6	0.4	10.7	4.8	1.9
Bread, white	40	402	3.3	0.8	19.7	1.5	0.2
Oven chips	60	414	1.9	2.5	18.0	1.7	3.3
Totals	435	3236	38.0	35.1	80.6	10.8	40.5

Cosmopolitan	Weight	Energy	Prot	Fat	CHO	Fib	VitK
	g	kJ		g			µg
Butter	4	121	0.0	3.3	0.0	0.0	0.3
Cheese, Cheddar	20	342	5.1	6.9	0.0	0.0	0.9
Cream, double	25	462	0.4	12.0	0.7	0.0	1.6
Flour, plain	4	58	0.4	0.1	3.1	0.1	0.0
Green beans	84	78	1.5	0.4	2.4	2.4	32.7
Milk, semi-skimmed	50	99	1.8	0.9	2.4	0.0	0.1
Potatoes, boiled	260	796	4.7	0.3	44.2	3.6	2.4
Salmon, raw	45	337	9.1	5.0	0.0	0.0	0.1
Smoked haddock, raw	50	173	9.5	0.3	0.0	0.0	0.0
Bread, wholemeal	65	601	6.1	1.7	27.1	4.8	1.7
Fat Spread, 70% fat	5	130	0.0	3.5	0.0	0.0	0.7
Totals	612	3197	38.5	34.2	79.8	11.0	40.5

Animal-orientated	Weight	Energy	Prot	Fat	CHO	Fib	VitK
	g	kJ		g			µg
Beef, lean, raw	95	490	19.2	4.4	0.0	0.0	0.3
Butter	4	121	0.0	3.3	0.0	0.0	0.3
Flour, plain	4	58	0.4	0.1	3.1	0.1	0.0
Lasagna, boiled	70	298	2.1	0.4	15.4	1.0	0.0
Milk, whole	70	192	2.3	2.7	3.3	0.0	0.4
Olive oil	18	666	0.0	18.0	0.0	0.0	10.4
Onions	20	29	0.2	0.0	1.6	0.3	0.1
Peas, frozen	60	175	3.6	0.6	5.8	4.3	17.4
Tomato puree	10	29	0.5	0.0	1.3	0.0	0.8
Tomatoes, canned	160	110	1.6	0.2	4.8	1.3	9.6
Bread, white	95	955	7.9	1.9	46.9	3.5	0.4
Fat spread, 70% fat	5	130	0.0	3.5	0.0	0.0	0.7
Totals	611	3252	37.8	35.0	82.2	10.5	40.4

Table 4-2. Individual food items contributing more than 3% to total vitamin K_1 content in each test meal

Meal	Meal component	Vitamin K_1 (μg)	% contribution
Convenience (chicken pie)	Margarine	19.5	48.2
	Rapeseed oil	10.1	25.0
	Peas	4.7	11.5
	Oven chips	3.3	8.2
	Baked beans	1.9	4.7
Cosmopolitan (fish pie)	Green beans	32.7	80.7
	Potatoes	2.4	6.0
	Wholemeal bread	1.7	4.2
Animal-oriented (lasagne)	Peas	17.4	43.1
	Olive oil	10.4	25.6
	Tomatoes	9.6	23.8

Soya-based margarine was chosen for its relatively high vitamin K_1 content for the preparation of pastry in the convenience meal. The fat spread used in all three meals was a sunflower-based spreadable fat, lower in vitamin K_1.

4.4.1 Ethical permission

The study protocol was approved by MRC Human Nutrition Research Scientific Coordination Committee (SCC) and Research Governance Committees (RGC). Ethical permission was obtained from Suffolk Local Research Ethics Committee (Suffolk REC 05/Q0102/148). Informed written constant was obtained from all subjects prior to the start of the study (Appendix V).

4.4.2 Sample size

This randomised three-period study compared the absorption of vitamin K_1 from three different meals. There was no directly applicable statistical information from which to calculate sample size but data from previous dose-response studies provided some guidance. These studies indicate an inter-individual coefficient of variation (CV) of 30% for area under the curve (AUC) of plasma dose response (Gijsbers *et al.,* 1996). By using a sample size formula for a normal distribution, it was calculated that twelve subjects

would allow the detection of a 25% difference in AUC at the 5% significance level and with 80% power. The assumption of 30% CV was investigated using sensitivity analysis by recalculating the detectable difference for CVs of 20% and 40%. The detectable differences were 17% and 33%, respectively. This calculation assumed a two-way cross-over design and therefore gave a conservative estimate of the detectable difference, both because it ignored the additional (third) period and did not take into account the likely advantages consequent on providing test and reference materials at the same time and to the same subjects.

4.4.3 Study subjects

It was decided to recruit healthy subjects since the aim of the study was to investigate absorption under normal physiological conditions. Both men and women were recruited, aged between 18 and 65 y, with a BMI between 18 and 25 kg/m^2. Exclusion criteria were split between two categories:

General health exclusions:

Cancer (within previous 10 y), history of heart disease, diabetes, or other chronic medical conditions; anaemia, untreated and elevated blood pressure, recent surgery (minor surgery in the last 6 wk or major surgery in the previous 3 mo) or heavy blood losses (including blood donation in the last 3 mo); history of food intolerance or food allergies, recent rapid weight loss (> 3 kg in previous mo), pregnancy or breast feeding.

Study-specific exclusions - (those conditions that may affect vitamin K_1 absorption or metabolism):

Medication that may interfere with vitamin K_1 absorption or metabolism (laxatives, antibiotics, anti-coagulants, anti-convulsants, bile acid sequestrants, long-term aspirin use, Orlistat, cholesterol-lowering drugs); blood coagulation disorders, known high plasma cholesterol or high triacylglycerols, liver and gall bladder diseases, malabsorption conditions of gastro-intestinal tract, alcohol intake >21 units/wk for women and >28 units/wk for men.

4.4.4 Volunteer recruitment

Volunteers were recruited through the HNR Volunteer Database, and through the email bulletin of the Cambridge University Graduate Union. The information sheet for volunteers is attached as Appendix VI. Volunteers received £90 for full participation in the study.

4.4.5 Preparation of labelled vitamin K_1

For this study, ^{13}C-labelled vitamin K_1 was chosen from the two available labelled forms because, with the expected low concentrations of vitamin K_1, changes in enrichment would be detected with a greater signal to noise ratio on top of existing natural abundance. In contrast, and as noted in section 2.10, the M+4 isotopomer is not detected at natural abundance. A stock solution of 83.8 mg in 100 mL of ethanol was prepared, divided into aliquots and stored at –18 °C. To prepare the dose for volunteers, 48 µL of the standard solution was added to 1 mL of groundnut oil to supply 20 µg (44.4 nmol) of ^{13}C-labelled vitamin K_1 in 0.5 mL of oil. Ethanol was evaporated from the oil by heating at 40 °C under N_2 with a Pierce Reacti-therm heating block and Reacti-vap evaporator (Perbio Science, Erembodegem, Belgium). After vortexing for 1 min, 0.5 mL aliquots of groundnut oil containing the labelled vitamin K_1 were transferred to gelatine capsules (kindly provided by Capsugel, Colmar, France). Capsules were prepared fresh for each subject and stored, refrigerated, in amber medicine bottles.

4.4.6 Study protocol

To ensure that vitamin K_1 intake prior to each study day was similar between subjects, the volunteers were given a meal (pizza) to be consumed the evening prior to each study day containing no ingredients known to be high in vitamin K_1. On three occasions, at least two weeks apart, volunteers were asked to attend the volunteer suite at MRC HNR after an overnight fast. Their weight was measured to the nearest 0.1 kg using Seca 770 digital scales (Seca, Birmingham, UK) and their height was measured to the nearest 0.01 m using a Seca 202 wall-mounted stadiometer (Seca, Birmingham, UK).

An indwelling cannula was inserted into a forearm, and two baseline blood samples were collected into 7.5 mL EDTA S-monovettes® (Sarstedt Ltd, Leicester, UK). The test meal was defrosted overnight and reheated in an electric oven to at least 70 °C for at least 2 min (as per Food Hygiene and Safety protocols) prior to consumption. Meals were weighed after reheating. Immediately before consumption of the test meal the volunteer swallowed the vitamin K_1-containing capsule. To minimise sequence effects, subjects were randomised for the order they received the meals. With three meals, there are six possible combinations, thus two subjects were designated to each sequence. Thirteen, 7.5 mL blood samples were collected at 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0 h after consumption of the meal. A snack (two slices of toast with sunflower spread and jam) was provided after 5 h and water was permitted *ad libertum* throughout the study.

4.4.7 Dietary intake

Volunteers were asked to record a 4-d food diary, completed over one weekend (Saturday and Sunday) and two weekdays. The primary purpose was to allow the determination of the volunteer's typical dietary intake, which may have been important in understanding any large inter-individual observations, or atypical experimental results.

4.4.8 Sample collection and analysis

Blood samples were collected into syringes and immediately transferred to coded tubes containing EDTA S-monovettes (Sarstedt Ltd, Leicester, UK). Samples were stored on ice, protected from light, and within 1 h centrifuged at 4 °C for 20 min at approximately 2000 *g*. The plasma was divided into three 1 mL aliquots and stored in 2 mL microtubes (Sarstedt Ltd, Leicester, UK) at –70 °C until analysis.

Extraction of vitamin K_1 from plasma was performed as described in section 2.9. Derivatisation was performed as in section 2.7.3. The GCMS was run in SIM mode and ions m/z 598.4 to 601.4 were measured. Total plasma vitamin K_1 concentrations were measured by high-performance liquid chromatography (HPLC) with fluorescence detection after post-column reduction (Wang *et al.*, 2004) (section 2.12).

4.5 Analysis of vitamin K_1 in meals

4.5.1 Background

The difficulties encountered in the analysis of vitamin K_1 in food are similar to those for plasma, with typically low levels and co-extraction of lipids in much greater abundance than vitamin K_1. A number of assays have been published for the measurement of vitamin K_1 in foods. Extraction typically required all, or some of solvent lipid extraction, lipid digestion, solid phase extraction and/or semi-preparative HPLC (Booth *et al.,* 1994; Koivu-Tikkanen *et al.,* 2000a; Schurgers & Vermeer, 2000; Indyk *et al.,* 2003). Quantitation has been always performed by HPLC with either electrochemical or fluorescence detection. For quantitative work, internal standards and calibration curves should ideally be used. However, the analytical response for an analyte in a complex sample may not be the same as in a simple standard and quantitation using a calibration curve would require standards that closely match the composition of the sample. For routine analyses, it may be feasible to prepare such standards. However for the requirements of this study, the procedure was deemed too time consuming. As an alternative, the standard addition method was used justified by the accuracy of the results required, the small number of samples and time pressures.

Standard addition requires the unknown sample to be divided into two portions and a known amount of the analyte (a spike) be added to one portion. The two samples are then analysed; the spiked sample shows a larger analytical response than the original sample due to the additional amount of analyte. The difference in analytical response between the spiked and unspiked samples is due to the amount of analyte in the spike, and hence analyte concentration in the original sample can be calculated (see section 4.6.6 for calculations).

4.5.2 Method development

Solvent extraction followed by solid phase extraction, rather than semi-preparative HPLC, was the preferred method. The method described here is modified from Booth *et al.* (1994) and quantitation was achieved using the analytical method for vitamin K_1 in

plasma (Wang *et al.*, 2004). Booth *et al.* (1994) describe the requirement for both silica and C_{18} SPE. Therefore, early attempts focussed on the use of both SPE columns. However, initial experiments revealed significant losses in the extraction. To ascertain where the losses were occurring fractions of eluent were collected from each wash of the SPE columns. Investigation revealed that almost 80% of the vitamin K_1 was lost during the washing phase of the C_{18} column probably due to different brands of SPE columns having different retention characteristics. However, it was found that purification of food samples using only silica SPE provided a sufficiently clean sample and so the extraction was performed with silica SPE only. The full method is detailed below (section 4.5.3).

4.5.3 Extraction and determination of vitamin K_1 in meals

Quantitation was performed by the standard addition method. Chemicals were purchased from VWR (VWR International Ltd, Poole, UK). A sample meal from each batch was defrosted and reheated as for the study subjects, and then liquidised with a kitchen blender with an equal weight of purified warm water. A 30 g portion of the meal-water mixture was homogenised using an IKA Ultra Turrax T25 basic homogeniser (Esslab, Essex, UK). Two grams of the homogenised meal were transferred to a pestle and mortar and ground with 18 g of anhydrous sodium sulphate. One gram of the mixture was weighed into a 50 mL polypropylene centrifuge tube (Sarstedt Ltd, Leicester, UK) and 30 mL of 2-propanol: hexane (3:2 v/v) and 10 mL of purified water were added. At this stage, the spike of 3 ng of vitamin K_1 (Supelco, Poole, Dorset, UK) in 100 µL of hexane was added to half the tubes and to the remaining tubes was added 100 µL of hexane only. The tubes were vortexed for 3 min and then sonicated for 3 min (Microsonix XL2000 model with 1/8 inch tapered microtip (Microsonix, USA)). The tubes were vortexed for a further 3 min and then centrifuged at 2000 *g* for 5 min. From the top layer, 9 mL of hexane was removed and transferred to a disposable culture tube (16 x 100 mm, Corning Ltd., Hemel Hempstead, UK). Excess solvent was evaporated under N_2 at 40 °C. The contents of the tube were reconstituted in 300 µL of hexane and further purified by solid phase extraction using 500 mg silica columns (Sep-Pak-RC™ 500 mg silica, Waters,

Hertfordshire, UK). The columns were conditioned with 4 mL diethyl ether: hexane (96.5:3.5 v/v) and then 4 mL of hexane. The sample was added and washed with 6 mL of hexane before elution with 7 mL of diethyl ether: hexane (96.5:3.5 v/v). The sample eluent was collected into disposable glass tubes (13 x 100 mm, Fisher brand) and evaporated to dryness in a vacuum evaporator (Savant, NY, USA). The samples were reconstituted in 200 µL of dichloromethane and 800 µL of methanol and analysed by high performance liquid chromatography (HPLC) using the same method as for the plasma samples. Extraction and analysis was performed in duplicate.

4.6 Data analysis

4.6.1 Isotopomer ratio analysis

Calculation of enrichments was performed as previously described in Section 3.6.1, by exporting raw data from the Agilent software into Microsoft Excel templates and using the fitting techniques described by Bluck and Coward (1997).

4.6.2 Tracer concentration

Tracer concentration was calculated from the observed isotopomer ratios calculated as described in 4.6.1, and total vitamin K_1 concentration measured by HPLC, using the following equation:

$$\text{Tracer concentration} = \frac{\left(R_{M+1(t)} - R_{M+1(0)}\right)}{1 + \left(R_{M+1(t)} - R_{M+1(0)}\right)} \times C_t$$

Where R_{M+1} is the ratio of the mass M+1 to M and subscripts refer to baseline (0) and subsequent time points (t). C_t is the corresponding total concentration (nmol/L) at each time (min) point .

4.6.3 AUC

Area under the curve (AUC) was used to assess absorption of the labelled vitamin K_1 tracer. AUC was calculated using the trapezoid rule, a method of numerical integration. AUC was calculated using the following equation and units are nmol/L per hour:

$$AUC_{0-8} = \sum \left\{ \frac{C_{t0} + C_{t1}}{2} \times (t_1 - t_0) \right\} + \left\{ \frac{C_{t1} + C_{t2}}{2} \times (t_2 - t_1) \right\} + \ldots$$

Where C_t is the concentration (nmol/L) at each time point and t is each sampling time (min)

These data were used to assess the effect of the meals on the bioavailability of the labelled vitamin K_1 tracer. This 'meal effect' describes meal characteristics e.g. type of fat, nutrient composition, energy density, that may have affected absorption of the labelled vitamin K_1 tracer.

4.6.4 Comparisons of bioavailability

For comparisons of the bioavailability of vitamin K_1 from meals (tracee), a different approach was taken. In each instance, if the absorption profile with time of vitamin K_1 from the meal were the same as that of the tracer then the relationship between them would be equal to unity, after normalisation for the dose given by adjusting concentrations for vitamin K_1 provided as tracer or in the meal. By measuring the slope of regressions between normalised concentration of tracer and tracee from the meal, it was thus possible to measure the relative bioavailability of the vitamin K_1 from each meal compared to the tracer. This 'matrix effect' describes extraction efficiency of vitamin K_1 from the meal.

4.6.5 Statistics

Statistics were performed in Excel (Students *t*-test) or with STATA 9.1 (Stata Corp., Texas, USA). AUC values were checked for normal distribution by observing a quintile-quintile plot.

4.6.6 Calculation of vitamin K_1 in meals

The standard addition method was used. Peak areas were manually integrated using the HPLC software and the amount of vitamin K_1 in the sample calculated using the equation:

$$X_{sample} = \frac{X_{spike} \times A_{unspiked}}{A_{spiked} - A_{unspiked}}$$

Where: X = amount of vitamin K_1

A = peak area

For the measurement of vitamin K_1 in each meal, 0.05 g of food was extracted and thus the total amount of vitamin K_1 within the meal was calculated by:

$$\text{Amount in meal} = X_{sample} \times \frac{\text{Meal weight (g)}}{0.05}$$

4.7 Results

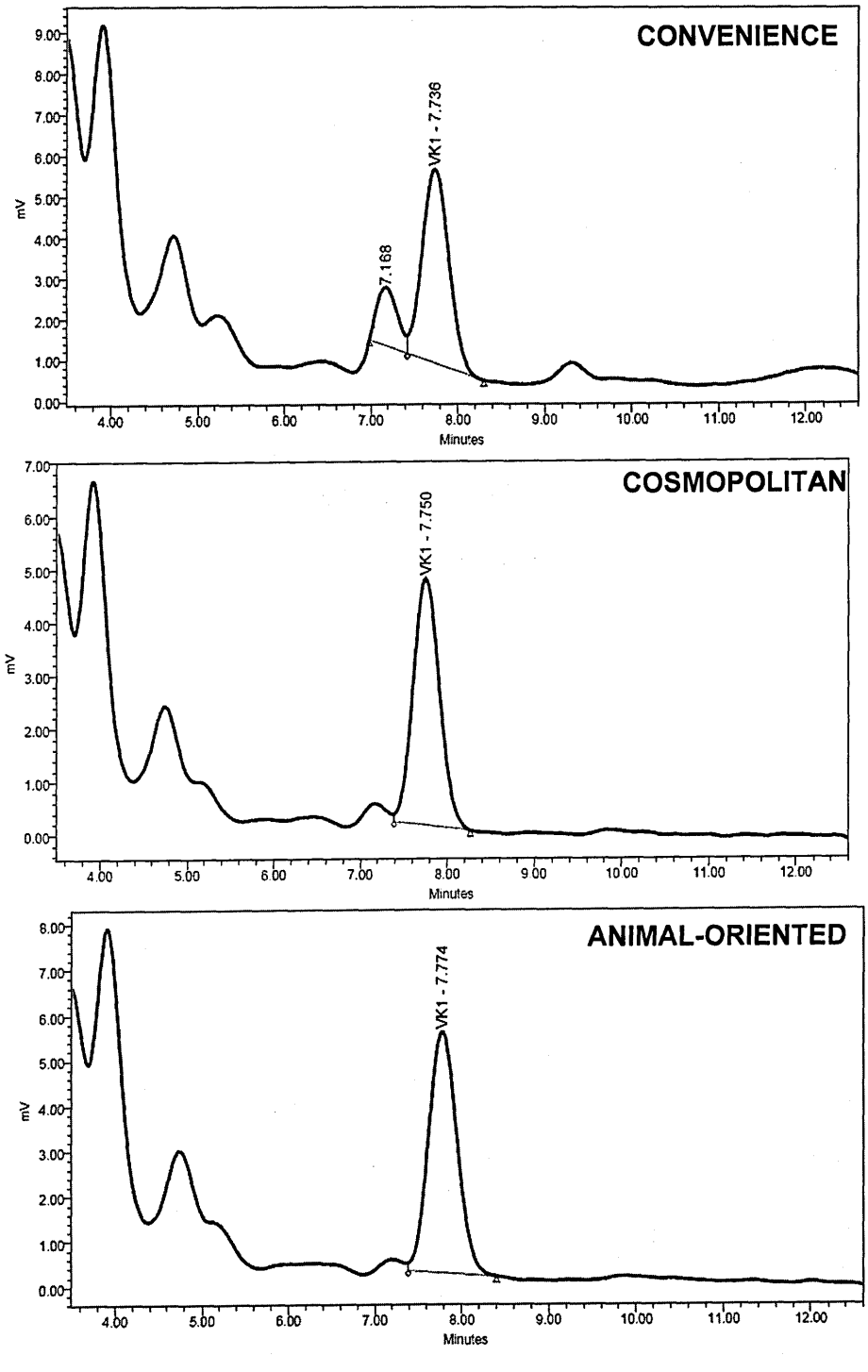
4.7.1 Subject characteristics

The twelve subjects (5 women and 7 men) were aged between 22 and 49 y. They had a mean ± SD height of 1.73 ± 0.09 m, weight of 69.4 ± 9.3 kg, and BMI of 23.1 ± 2.3 kg/m^2. Average baseline plasma vitamin K_1 was 0.35 nmol/L across all 36 visits, with individual's average ranging from 0.12 to 0.74 nmol/L. The average intra-individual coefficient of variation (CV) between the three visits was 48%. Average between subject CV was 78%.

4.7.2 Vitamin K_1 content of meals

The vitamin K_1 content of meals calculated from food composition data was estimated at 40 µg. Average meal weights after cooking and reheating were 423 g, 587 g and 603 g for the convenience, cosmopolitan and animal-oriented meals, respectively. Direct analysis of the meals by HPLC revealed vitamin K_1 content as 19.9 µg, 26.3 µg and 33.0 µg for the convenience, cosmopolitan and animal-oriented meals, respectively. HPLC chromatograms of the extracted 0.05 g food samples are shown in Figure 4-2.

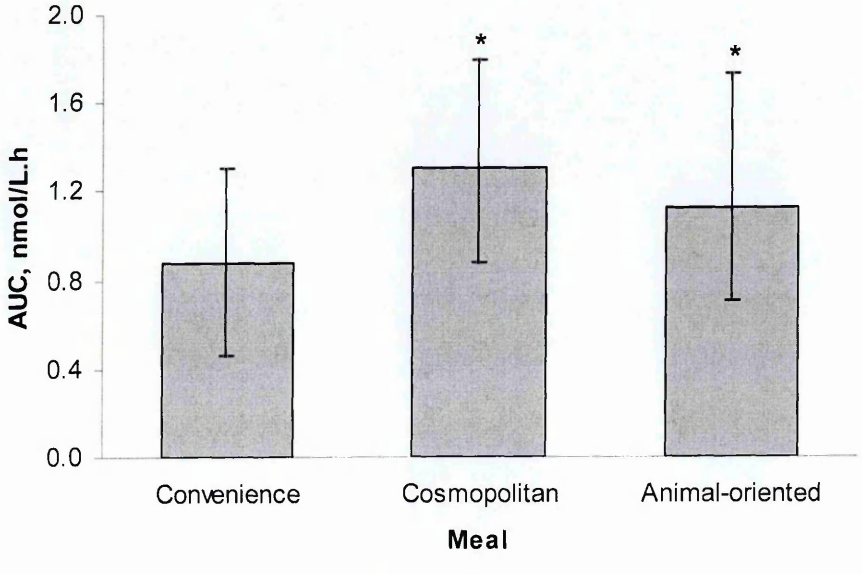
Figure 4-2. Direct output of HPLC chromatograms of food sample analysis



4.7.3 Tracer absorption

Absorption of tracer was assessed by calculating area under the curve. Gender did not affect AUC of tracer within meals calculated using Students *t*-test (convenience P=0.83, cosmopolitan P=0.88, and animal-oriented P=0.81). The mean ± SD of tracer AUC measurements for all subjects were 0.88 ± 0.42, 1.30 ± 0.49 and 1.13 ± 0.60 nmol/L.h for the convenience, cosmopolitan and animal-oriented meals, respectively (Figure 4-3). AUC for tracer measurements were compared using linear regression with fixed effects for meal, subject and period. Significantly less vitamin K_1 tracer was absorbed when consumed with the convenience meal than either the cosmopolitan and animal-oriented meals (P=0.001 and P=0.035, respectively). There was no significant difference between the cosmopolitan and animal-oriented meals (P=0.120). Table 4-3 shows individual AUC measurements calculated from the tracer profiles. Tracer profiles are shown in Figure 4-4. CVs for tracer AUC measurements within meals were between 38 and 53%. Calculation of AUC between 0 – 5 h and 5 – 8 h, tested the possible influence of the 5 h snack on the results. However, differences between meals in absorption of tracer before the snack were similar to differences after the snack.

Figure 4-3. Mean AUC of tracer vitamin K_1 consumed with each of the three test meals in 12 subjects



Each bar represents the mean ± SD AUC of absorption of tracer vitamin K_1 used to measure meal effect on absorption. * indicates significant difference compared to the convenience meal.

Table 4-3. AUC_{0-8} of individual tracer measurements for each test meal

	Convenience	Cosmopolitan	Animal-oriented
Subject	AUC (nmol/L.h)		
A	0.81	1.11	0.85
B	0.64	0.94	0.84
C	1.04	1.71	1.31
D	2.04	1.92	2.44
E	0.94	2.03	1.51
H	0.30	1.11	0.45
I	0.97	0.94	0.96
J	0.51	0.83	0.57
K	0.82	1.02	1.59
L	0.78	1.99	1.49
M	0.69	0.64	0.27
N	1.03	1.40	1.23

Figure 4-4. Time-concentration profiles of plasma tracer for each subject and each meal (convenience o , cosmopolitan Δ , and animal-oriented —□—)

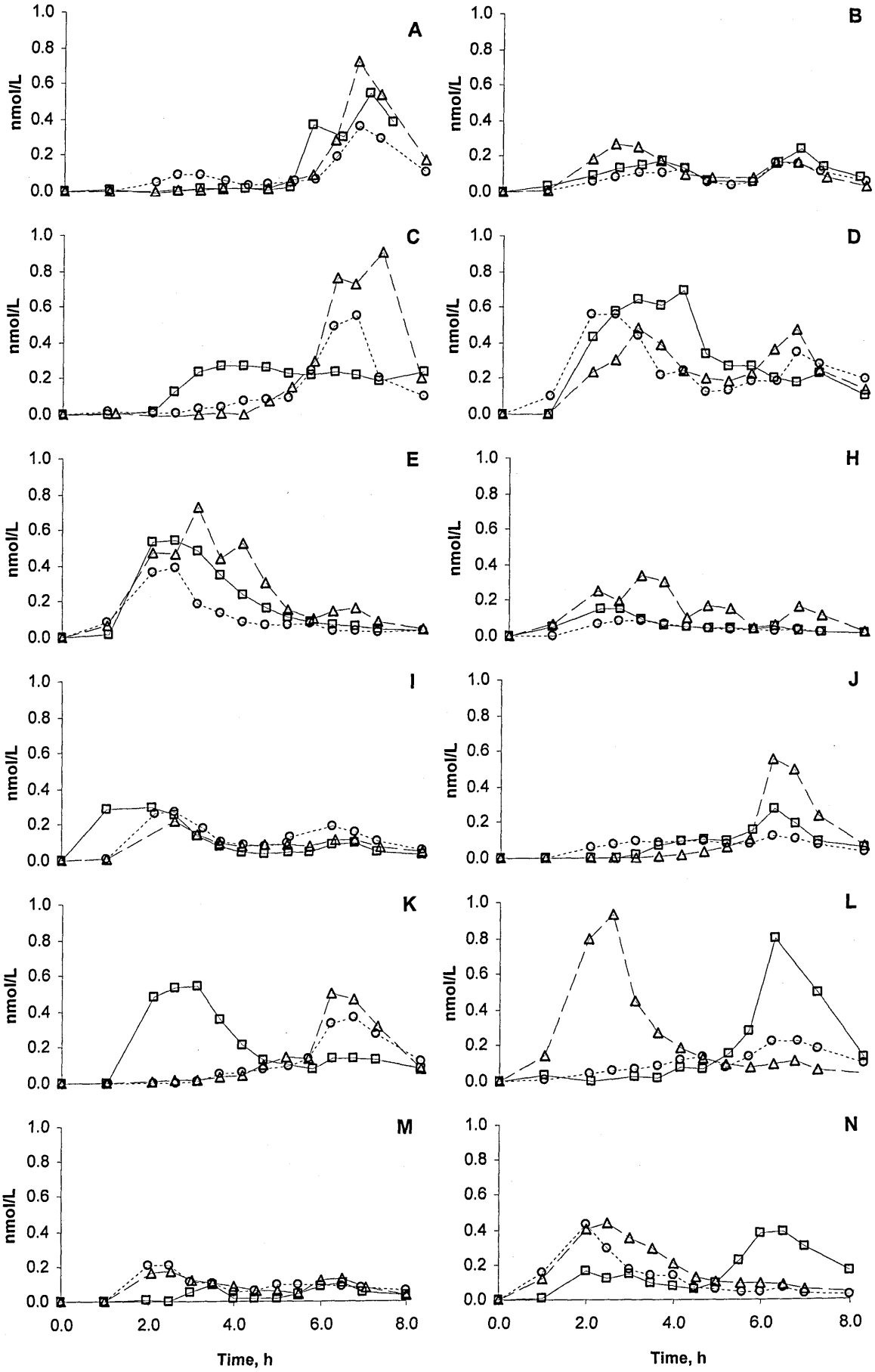
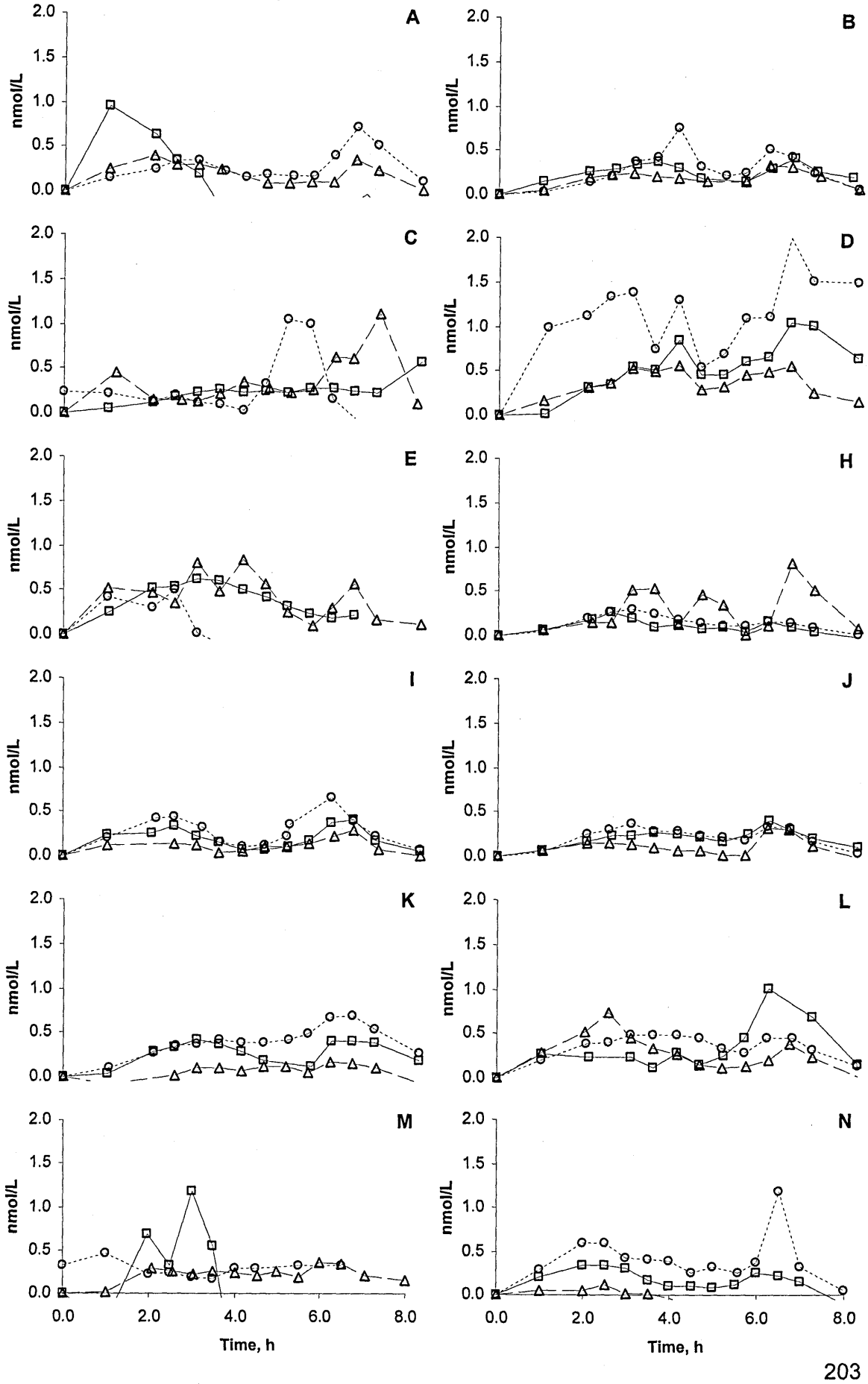


Figure 4-5. Time-concentration profiles of exogenous plasma tracee for each subject and each meal (convenience o , cosmopolitan Δ and animal-oriented —□—)



4.7.4 Bioavailability

Bioavailability (matrix effects) from the meals was assessed based on the relationship between tracer and exogenous tracee (baseline subtracted tracee). Tracer and tracee against time are shown in Figure 4-4 and Figure 4-5. For the regression analysis, concentrations were adjusted for vitamin K_1 in the meals and capsule. Figure 4-6a shows the relationships between tracer and exogenous tracee for subjects A – H, and Figure 4-6b I – N. The slopes of the regressions are shown in Table 4-4. The means ± SD of slopes were 1.88 ± 0.81, 0.59 ± 0.32 and 0.43 ± 0.40 for the convenience, cosmopolitan and animal-oriented meals, respectively.

Final values for total bioavailability from the test meals were calculated by multiplying values for matrix and meal effects. Final relative bioavailability values were then expressed relative to the convenience meal, and were 1.00, 0.46 and 0.29 for the convenience, cosmopolitan and animal-oriented, respectively. Relative matrix and meal effects, and total relative bioavailability values are shown in Table 4-5.

Figure 4-6a. Relationship between tracer and exogenous tracer for Subjects A – H (convenience ○ , cosmopolitan △ , and animal-oriented —□—)

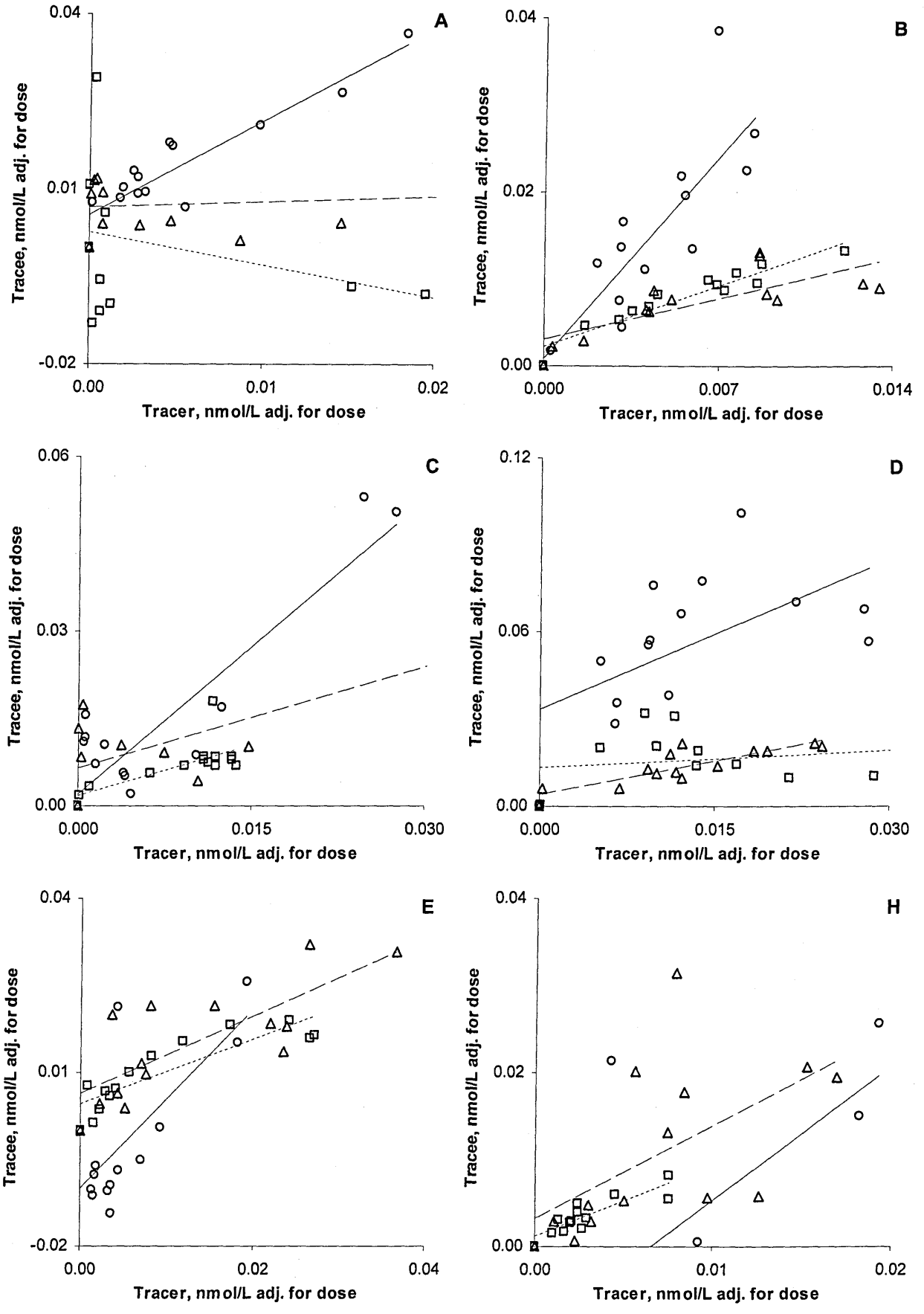


Figure 4-6b. Relationship between tracer and exogenous tracer for Subjects I – N (convenience ○ , cosmopolitan △ and animal-oriented —□—)

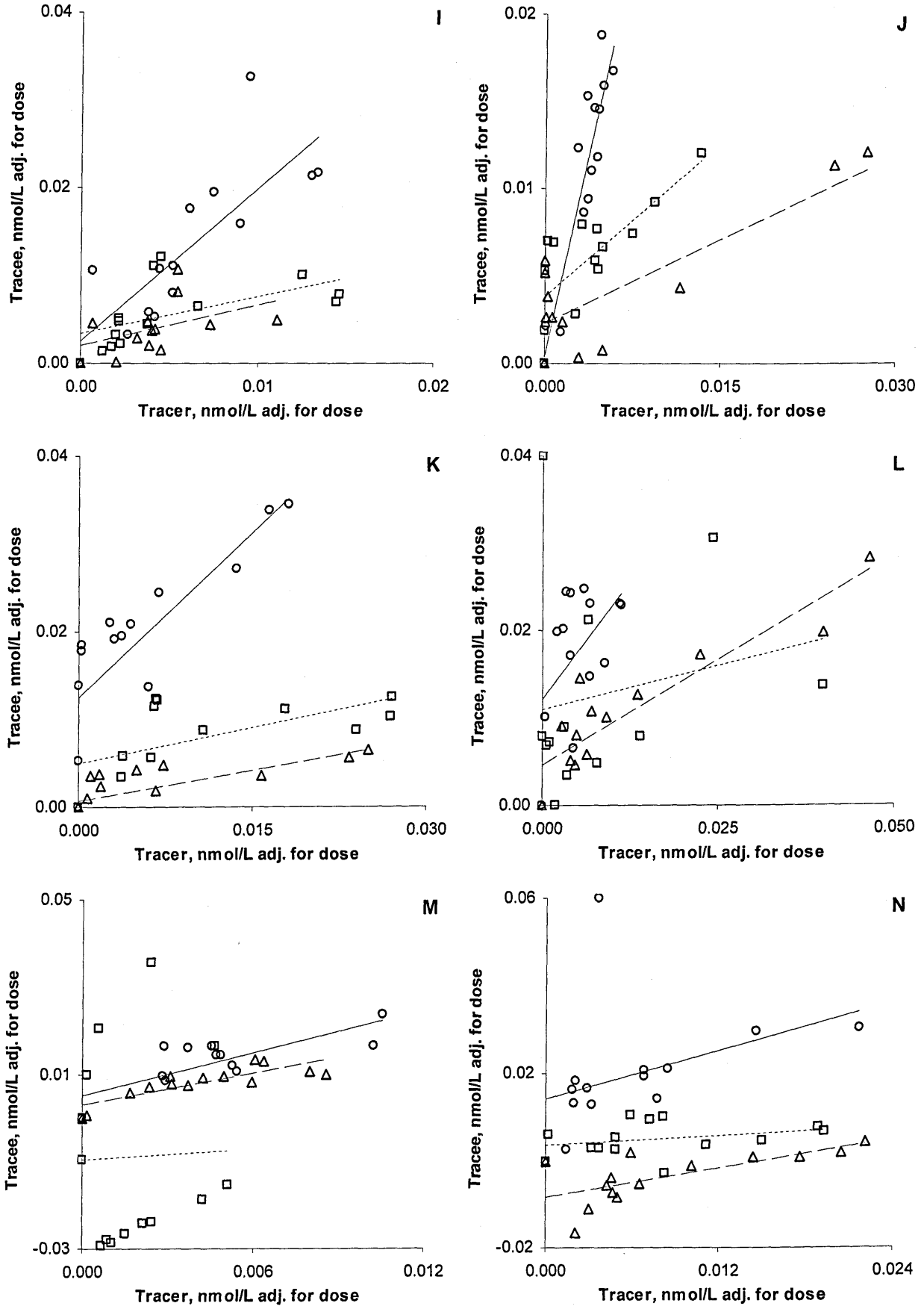


Table 4-4. Slopes of regressions between tracer and exogenous tracee

Subject	Convenience	Cosmopolitan	Animal-oriented
A	1.61	0.1	-0.56
B	3.34	0.67	1.01
C	1.69	0.58	0.58
D	1.73	0.77	0.19
E	1.54	0.67	0.55
H	3.04	1.07	0.82
I	1.73	0.47	0.43
J	3.09	0.32	0.58
K	1.25	0.23	0.28
L	1.09	0.48	0.67
M	1.59	1.19	0.43
N	0.91	0.56	0.17

Table 4-5. Summary of meal and matrix effects and total relative bioavailability of the three test meals

Meal	Meal effect	Meal effect (normalised)	Matrix effect	Matrix effect (normalised)	Total effect
Convenience	0.88	1.00	1.88	1.00	1.00
Cosmopolitan	1.30	1.48	0.59	0.31	0.46
Animal-oriented	1.13	1.28	0.43	0.23	0.29

Meal effect refers to the effect of meal on absorption of tracer vitamin K_1 from the capsule from differences in, for example, fat and nutrient composition and energy density of the meal. Matrix effect refers to the availability of vitamin K_1 from within the food matrix. Values are normalised to the convenience meal.

4.7.5 Dietary intake

The mean daily intakes of macronutrients, vitamin E equivalents and vitamins K_1 for each of the twelve subjects are shown in Table 4-6. Mean vitamin K_1 intakes ranged from 32 to 317 µg/d. Five of the subjects had vitamin K_1 intakes below the UK guideline amount of 1 µg/kg body wt/d and indicated with * in the Table 4-6.

Table 4-6. Average daily nutrient intakes collected from 4-d diet diaries of 12 subjects

	Energy		Protein	CHO	Fat	MUFA	PUFA	SFA	Vitamin E	Vitamin K_1
Subject	(kcal)	(kJ)	(g)			(g)			(mg)	(µg)
A	1831	7718	78	269	56	28	10	17	12.0	317
B	2473	10376	83	263	108	43	20	35	13.4	123
C*	2303	9671	73	238	71	26	9	28	6.2	66
D	1830	7688	63	221	63	19	15	22	14.8	139
E*	1361	5726	71	146	57	21	11	18	7.9	32
H	2808	11784	128	280	139	51	18	51	12.1	248
I	2589	10875	65	294	132	54	16	44	10.8	166
J	2789	11702	86	309	113	34	17	44	16.0	161
K*	1863	7849	77	253	67	21	12	24	9.9	55
L*	2397	10095	81	326	71	23	12	19	10.4	57
M*	1764	7425	54	271	59	18	11	20	9.8	62
N	1897	7958	60	204	73	21	16	23	15.1	90

Abbreviations: kcal, kilocalories; kJ, kilo Joules; CHO, carbohydrate; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids

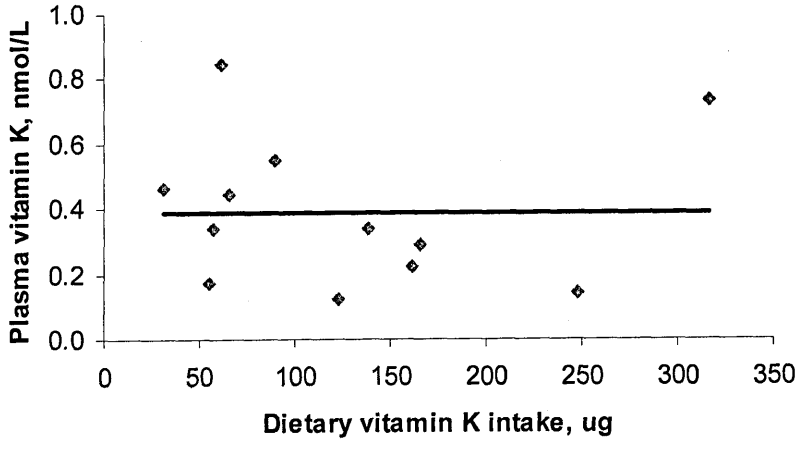
* indicates intake of vitamin K_1 less than the UK guideline amount of 1 µg/kg body wt/d

Vitamin K_1 intake was not significantly correlated with any of the other dietary intake measures. However, subject A appeared as an outlier in a number of the regressions. If Subject A was removed then significant correlations were found between vitamin K intake and the intake of energy (r^2=0.53; P=0.011), protein (r^2=0.43; P=0.029), total fat (r^2=0.76; P<0.001), MUFA (r^2=0.60; P=0.005), PUFA (r^2=0.57; P=0.007) and SFA (r^2=0.80; P<0.001).

Relationships between mean nutrient intakes and average baseline plasma vitamin K_1 (of the three visits for each subject) were also examined. Significant inverse

associations were observed between the plasma vitamin K_1 against intake of energy (r^2=0.37; P=0.04), protein (r^2=0.33; P=0.05), total fat (r^2=0.43; P=0.02), SFA (r^2=0.41; P=0.02) and PUFA (r^2=0.44; P=0.02). Non-significant associations were observed between plasma vitamin K_1 levels and intake of CHO (r^2=0.05; P=0.47), vitamin E (r^2=0.05; P=0.47) and MUFA (r^2=0.28; P=0.07). Notably, there was no association between plasma vitamin K_1 concentration and vitamin K_1 intake (r^2=0.00; P=0.99) (Figure 4-7). Although plasma levels primarily reflect recent intake, the absence of any relationship between vitamin K_1 intake and plasma status is a little surprising. Furthermore, if subject A (the point in Figure 4-7) furthest to the right) is removed then there is even an inverse association.

Figure 4-7. Relationship between dietary vitamin K_1 intake (assessed by 4-d food diary) and plasma vitamin K_1 status (as the average of three fasted blood samples) in 12 subjects



4.8 Discussion

The aims of this study were to develop a stable isotope-based method to measure the relative bioavailability of vitamin K_1, and to obtain data on the bioavailability of vitamin K_1 from meals.

4.8.1 Methodology

The work described in Section 4 on vitamin K_1 kinetics and absorption provided the framework on which to design this bioavailability study. Bioavailability was measured over a single day due to the relatively fast turnover of vitamin K_1 observed in Study 1 and previously (Shearer *et al.,* 1970a; Shearer *et al.,* 1974). In contrast, typical studies on other fat-soluble vitamins, with slower turnover, need to be performed over several days (Brown *et al.,* 1989; Edwards *et al.,* 2001; van Lieshout *et al.,* 2003; Bruno *et al.,* 2005). Additionally, data from the absorption of deuterated vitamin K_1 allowed estimates of both the size of the dose (from the plasma increment in Study 1), and optimisation of the sampling protocol (appearance of the tracer and T_{max}).

The meals were designed to contain 40 µg of vitamin K_1. This value was based on previous work that measured vitamin K_1 absorption from vegetables (Gijsbers *et al.,* 1996; Garber *et al.,* 1999; Booth *et al.,* 2002). Consideration was also made for the expected plasma vitamin K_1 enrichments determined by the relative absorption of the vitamin K_1 tracer and vitamin K_1 within the meal.

The method used ^{13}C-labelled vitamin K_1 as a standard, taken at the same time as a test meal, which could then be used to measure bioavailability by measuring changes in plasma vitamin K_1 isotopic enrichment. Similar procedures have previously been used to study vitamin E (Jeanes *et al.,* 2004) and vitamin A absorption (Edwards *et al.,* 2001; 2002). However, results of the plasma response to the labelled tracer showed significant differences between meals, with less tracer absorbed when consumed with the convenience meal (0.88 nmol/L.h), compared to either the cosmopolitan (1.30 nmol/L.h) or animal-oriented (1.13 nmol/L.h) meals. As a result, it was not possible to use the tracer as a global standard by which to compare the meal bioavailability data. An alternative

approach was taken where relative bioavailability was assessed based on the relationship between the tracer and tracee absorption profiles with time. Consequently, it is possible to consider two separate, but interacting determinants to bioavailability, matrix effects and meal effects. The first determinant, matrix effects, relate to the location of the vitamin K_1 within meal constituents, i.e. whether within vegetable or oil matrix, and the effect of the location on bioavailability. Secondly, meal composition affects the bioavailability of free vitamin K_1. This 'meal effect' is the result of the meal modifying conditions within the gut that may affect digestion and/or absorption, and the potential effect of enhancers or inhibitors of absorption. The absorption of labelled vitamin K_1 measured by AUC was used to determine meal effects, whereas absorption of vitamin K_1 from the meal itself was used to determine matrix effects. A final relative bioavailability value for each of the meals was determined as the product of the matrix and meal effects.

4.8.2 Matrix effects

Using the matrix effects alone, it was observed that absorption of vitamin K_1 from the convenience meal was at least three times that from the cosmopolitan and animal-oriented meals (Table 4-5). Whilst the magnitude of this difference was reduced with inclusion of the meal effect, total relative bioavailability remained greater from the convenience meal.

Greater absorption of vitamin K_1 from the convenience meal may be expected given the sources of vitamin K_1. From the percent contribution of different foods to meal vitamin K_1 content (Table 4-2), more than 80% of the vitamin K_1 in the convenience meal was in fats and oils, not vegetables. In the cosmopolitan meal, only around 10% was in fats, and in the animal-oriented, approximately 20% was in fats. Conversely, the majority of vitamin K_1 in the cosmopolitan and animal-oriented meals was in vegetables where it is tightly bound to the thylakoid membranes, and may be less bioavailable because cell walls and membranes must be digested before absorption.

Although it is generally assumed that fat-soluble vitamin absorption is greater from oil than from vegetables, there have been few direct comparisons using a crossover study

design. Vitamins A and E have been studied more extensively than vitamin K yet there is still a lack of direct comparisons between oil and vegetables matrices. Parker *et al.,* (1999) conclude that carotenoids from oil are probably more bioavailable than those from other food matrices but state that only a few foods have been tested.

Previous studies to compare absorption of vitamin K_1 from oil and vegetables have shown conflicting results. Two papers from a single study are published that compare absorption between fortified-oil and broccoli over different time-periods. In one study, absorption was reported to be significantly greater from oil than from broccoli (Booth *et al.,* 2002) but an earlier study found no difference in absorption between these two sources (Booth *et al.,* 1999a). The different conclusions clearly demonstrate that outcomes can be heavily dependent on the methods used. It is also worth noting that no significant differences were observed in post-intervention samples, only in AUC measurements. This finding may not be surprising when one considers the relatively fast turnover of vitamin K_1. Thus, any differences were evident only a relatively short time after absorption.

No other studies have directly compared absorption of vitamin K_1 between oil and vegetables matrices. Instead, the focus has been on the comparison of vitamin K_1 absorption from different vegetables, with or without the addition of fat. However, these studies have frequently used relatively large amounts of vitamin K_1 (>400 μg) that may not be typical of the levels present in meals. In one study, there were no differences in absorption (measured by AUC) between vegetable sources of vitamin K_1 (spinach, broccoli, and lettuce) but more vitamin K was absorbed when given in tablet form (Garber *et al.,* 1999). Similar results were found in two other studies, where absorption of vitamin K_1 in a detergent-solubised form was greater than that from spinach (Gijsbers *et al.,* 1996; Schurgers & Vermeer, 2000).

A number of assumptions with regard to carotenoid absorption/metabolism have been identified by Tyssandier *et al.* (2003) which can be also applied to vitamin K_1. Firstly, it is assumed that the carotenoids cannot be absorbed when in the vegetable matrix. Secondly, it is assumed that incorporation into mixed micelles is essential for absorption and finally that absorption occurs by passive diffusion across the enterocytes.

These assumptions were tested in human volunteers with the use of naso-gastric and naso-duodenal tubes (Tyssandier *et al.*, 2003). Data from ten volunteers intragastrically fed liquid test meals (with vegetables as the source of carotenoids) showed that carotenoids were present in the fat phase of the stomach contents. This experiment shows that lipid-soluble carotenoids dissolve in lipid droplets. The authors describe the term 'bioaccessibility' defined as 'the ease with which carotenoids are solubised within the mixed micelles from the vegetable matrix' (Tyssandier *et al.*, 2003). This term is comparable to 'matrix effects' as described above. A similar experimental approach could be used to assess matrix effects on vitamin K_1 bioavailability from different foods. Although direct gastric sampling obviously yields appealing and convincing data, such studies are difficult to perform, expensive to run, as well as ethically challenging. Furthermore, the results might not fully reflect bioaccessibility from normally consumed vegetables since the meals used in this study were homogenised (Tyssandier *et al.*, 2003) which breaks up the cells making their contents accessible to digestive enzymes and for absorption.

Data from the present study suggests that vitamin K_1 bioavailability is greater from oil than from a vegetable matrix. This is in agreement with the only other comparable study to compare the two sources (Booth *et al.*, 2002). An observation that plasma vitamin K_1 concentration is highly correlated with α-tocopherol (mainly derived from oils) (Sadowski *et al.*, 1989) also hints at greater contribution of vitamin K_1 from oils than from vegetables. The observed greater bioavailability from oil is likely due to the greater extraction efficiency of vitamin K_1 from this source compared to vegetable matrix where vitamin K_1 is tightly bound to cellular structures.

4.8.3 Meal effects

In contrast to the results above, absorption of tracer vitamin K_1 taken at the same time as the meals was lowest when consumed with the convenience meal. Several possible explanations for this apparent paradox relate to meal characteristics that may affect digestive processes and/or other components that affect vitamin K_1 absorption.

The processes of digestion and absorption of fats and fat-soluble vitamins follows a number of steps. Mechanical grinding and churning of the stomach contents breaks down food matrices and reduces the particle size of the food for release into the duodenum. Following release from the matrix, vitamin K_1 (along with other fat-soluble vitamins) is emulsified in the lipid phase within fat globules. Lipid digestion initiated in the stomach by gastric lipase continues in the duodenum by pancreatic lipase and bile salts and leads to the formation of mixed micelles that contain free fatty acids, monoacylglycerol, phospholipids, cholesterol and fat-soluble vitamins. Micellar components are transferred across the intestinal mucosal into the enterocytes. In the enterocyte, vitamin K_1 is repackaged in chylomicrons that remain the major carrier of post-prandial vitamin K_1 in the circulation (Lamon-Fava *et al.,* 1998; Erkkilä *et al.,* 2004). Circulating levels of vitamin K_1 are largely determined by post-prandial lipaemia. Thus, any aspect of the meal that affects gastric emptying or aspects of lipid absorption/metabolism may influence vitamin K_1 bioavailability. This raises the question was there something about the convenience meal, regardless of the vitamin K_1 content of that meal, that decreased absorption of the tracer vitamin K_1?

4.8.3.1 Gastric emptying

A number of factors that determine gastric emptying and intestinal transit may ultimately affect bioavailability (Schneeman 2004). Meal characteristics that affect gastric emptying or intestinal motility include viscosity, osmolarity, particle size, meal volume, the lipid, protein, carbohydrate and fibre content, and energy density (Low 1990). Slower release of food from the stomach may lead to increased absorption because there is less emulsified food for a constant intestinal surface area. Conversely, if gastric emptying is faster, then there may be less contact of food with the intestine and lower absorption. To minimise differences in gastric emptying between meals, all were balanced for energy, percent energy from fat, protein and carbohydrate, and fibre content. However, the convenience meal was different to the other meals because it was smaller in weight and

volume, but had equal energy, and therefore had an energy density almost 50% greater than both the cosmopolitan and animal-oriented meals.

The higher energy density of the convenience meal may have affected gastric emptying. Evidence suggests that gastric emptying from the stomach is controlled, partly through the energy density of the meal, to maintain a constant supply of energy (from food) into the duodenum; thus a meal with a greater energy density slows gastric release (Hunt 1983) and thereby provides the potential for greater absorption. However, this reasoning doesn't explain the findings of this study, that absorption of the tracer (indicative of meal effects) was lowest in the meal with the greatest energy density. Although this hypothesis could partly explain the greater absorption of the vitamin K_1 from the meal itself, it cannot explain lower absorption of the vitamin K_1 tracer.

That gastric emptying influences bioavailability has also been postulated in a study that measured the effect of two different, but isoenergetic meals, on vitamin E absorption (Jeanes *et al.,* 2004). The study found that more vitamin E was absorbed from a capsule when it was consumed with toast and butter than with cereal and milk. However, the authors do not speculate firstly, how the meals would differentially affect gastric emptying and secondly, a mechanism for how potential differences in gastric emptying would affect absorption.

It is almost certain that gastric emptying is a determinant of the bioavailability of vitamin K_1 and other nutrients. However, the mechanisms are indirect and are a consequence of the meal consumed. It is difficult to state with any confidence that differences in gastric emptying resulted in the observations in the present study.

4.8.3.2 Other factors in absorption

The lipid-water mix of the meal may determine the size of emulsified fat globules released from the stomach and smaller fat globules provide a greater surface area for the action of lipases. Since fat-soluble vitamins are trapped within the fat globule, the rate of lipid hydrolysis may affect fat-soluble vitamin availability (Borel *et al.,* 2001). The test meals used in this study were matched for fat content, although the greater weight and

volume of the cosmopolitan and animal-oriented meals are indicative of a higher water content and the potential for smaller emulsified fat globules to be released from the stomach. However, it has been shown that the size of fat globules did not alter absorption of either vitamin A or E (Borel *et al.,* 2001). It is likely that emulsification affects all the fat-soluble vitamins equally, thus these findings are equally applicable to vitamin K_1. The evidence suggests that emulsification is not an important effect in absorption of vitamin K_1 and is probably not a factor in the differences observed in this study.

The size of mixed micelles may also be a factor in the absorption of fat-soluble vitamins, and is largely dependent on the chain length of fatty acids within the micelle. *In vitro* experiments, using excised sections of rat bowel, showed that longer chain fatty acids reduced vitamin K_1 absorption. The mechanism is uncertain but may be due to increased affinity of the mixed micelle for vitamin K_1 or by reduced diffusion through the unstirred water layer leading to reduced transfer to enterocytes (Hollander & Rim, 1976).

The unstirred water layer, a thin layer of water surrounding the brush border membrane of the microvilli, is important in the absorption of lipids from micellar solutions (Wilson *et al.,* 1971) and may play a role in the absorption of vitamin K_1. Mixed micelles transport the fat-soluble components, including vitamin K_1, through the unstirred water layer to the enterocytes, and the rate of transfer through the layer may affect absorption. Hollander *et al.* (1977), have shown a greater rate of vitamin K_1 absorption in rats when the thickness of the layer was decreased, and suggested that the layer may be a significant barrier to vitamin K_1 absorption. However, it is not possible to comment on what meal factors may influence the thickness of the unstirred water layer.

Although the above examples (fat globule size, micelle size and unstirred water layer thickness) could provide mechanisms for the observed differences in absorption of the tracer vitamin K_1 between meals, in the absence of supporting evidence the hypothesis remains supposition.

4.8.3.3 *Total fat*

Fat is required for vitamin K_1 absorption, however the quantity of fat required, and whether there is an optimum amount is unknown. Fat may influence absorption either by slowing gastric emptying or through the stimulation of bile secretion. In the present study, test meals were balanced for fat so, notwithstanding inaccuracies in the food composition tables, it is unlikely that total fat provides an explanation for the observed results. In other studies, conflicting results have been reported on the effect of total fat. One study found the addition of butter to a meal increased absorption (Gijsbers *et al.,* 1996) whereas another study found no difference in vitamin K_1 absorption from lettuce between a low-fat (30%) or high-fat (45%) meal (Garber *et al.,* 1999).

4.8.3.4 *Fatty acid composition*

There is some evidence to show that the fatty acid composition of a meal can influence both uptake and post-prandial transport of vitamin K_1. Differences in fatty acid composition may affect absorption by changing the physical characteristics of the mixed micelle and/or influencing post-prandial lipid metabolism. *In vitro*, a monounsaturated fatty acid (C18:1), and to a greater extent, a polyunsaturated fatty acid (C18:2), decreased vitamin K_1 absorption possibly through a greater affinity of the mixed micelle for vitamin K_1 decreasing transfer across the enterocyte (Hollander & Rim, 1976). In rats, polyunsaturated fatty acids significantly decreased the rate of vitamin K_1 absorption (Hollander *et al.,* 1977).

More recent studies in humans have shown that diets containing PUFA-enriched (38%) corn oil resulted in lower plasma vitamin K_1 compared with a diet enriched with olive / sunflower oil that contained less PUFA (23%) (Schurgers *et al.,* 2002a). There is some evidence to suggest that meals containing significant levels of polyunsaturated fatty acids can reduce post-prandial lipaemia (Williams 1997). However, other studies have shown no effect of varying MUFA or SFA meal content on TAG responses or, by using retinyl ester as a marker, on chylomicron metabolism (Jackson *et al.,* 1999). Since the presence of vitamin K_1 in plasma is largely controlled by lipid digestion and metabolism,

there is the possibility that fatty acid composition of the meal may affect the transport and metabolism of vitamin K_1.

Table 4-7 shows the fatty acid profile, estimated from food composition tables, for each test meal. The convenience meal had more than two-fold greater PUFA content (37%) than both the cosmopolitan (19%) and animal-oriented meals (14%). Inhibition of vitamin K_1 uptake or altered post-prandial metabolism by the high PUFA convenience meal could explain the lower absorption of vitamin K_1 from the capsule. However, these concepts are contradictory to the results of total bioavailability and could only be possible if considerably more vitamin K_1 was available from the convenience meal to counteract the negative effect of PUFA.

Table 4-7. Fatty acid compositions of test meals expressed as % of total fat content

	Fatty acid		
Meal	Saturated (%)	Monounsaturated (%)	Polyunsaturated (%)
Convenience	26	37	37
Cosmopolitan	52	29	19
Animal-oriented	30	56	14

4.8.3.5 Vitamin E

There is some evidence from studies in rats to suggest that vitamin E may affect vitamin K plasma status (Alexander *et al.*, 1999; Mitchell *et al.*, 2001). However, other studies have shown effects are limited to tissue status, not plasma status both in animal (Tovar *et al.*, 2006) and in human studies (Booth *et al.*, 2004). The available evidence suggests that vitamin E may interfere with vitamin K metabolism rather than absorption (Schurgers *et al.*, 2002a). In the current study, the vitamin E content of each meal was calculated using food composition tables, with estimated values as 11.0 mg, 5.3 mg and 5.8 mg for the convenience, cosmopolitan and animal-oriented meals, respectively. These values could be used to the explain differences in tracer absorption between meals. However, considering the strength of the evidence for vitamin E as an inhibitor of vitamin K_1 absorption, and the levels of vitamin E in the three test meals, it is unlikely that

the observed differences in vitamin K_1 bioavailability between meals could be attributable to vitamin E.

4.8.4 Effects of the meal on gelatin capsule

In the present study, volunteers received the ^{13}C-labelled vitamin K_1 tracer in a gelatin capsule, taken at the same time as the test meal. It is possible that the results obtained from absorption of the tracer were less to do with the influence of the meal on vitamin K_1 *per se*, and more related to meal effects on digestion of the gelatin capsule. Two mechanisms are possible; the meal may have firstly affected the position of the capsule in the stomach and secondly the rate of breakdown of the capsule. However, the possible mechanisms for these effects are unknown. Similarities within individuals in the tracer absorption profiles (see section 4.8.5) suggest the meal did not affect breakdown of the capsule.

4.8.5 Comment on absorption profiles

Tracer concentration-time profiles in Figure 4-4 reveal that in the majority of cases, an individual's profiles show a similar pattern between the three test meals (with the exception of subjects K, L and N). The inter-individual differences of profiles are not related to the meals but rather genetic, metabolic or physiological differences between individuals e.g. absorption of vitamin K_1 across the enterocytes and/or metabolic handling of chylomicrons/vitamin K_1, potentially through apoE genotype.

In a number of individuals tracer vitamin K_1 peaked between either 5 and 8 h, or a second smaller peak was observed, apparently after the snack. Although there was no difference between AUC before and after the snack, the snack may be influencing the absorption profile of vitamin K_1. It may be that in some individuals, the physiological response to the snack releases partially absorbed vitamin K_1 into the circulation. A similar phenomenon has been observed with fatty acids, where fatty acids consumed during a first meal appear in the circulation after ingestion of a second meal a number of hours later possibly through retention in the gastric tissue, or stimulation of hydrolysis of lipids in chylomicrons (Fielding *et al.,* 1996; Maillot *et al.,* 2005). Similarly, it has been suggested

that partially absorbed β-carotene can remain in the enterocytes until a later meal when it is subsequently packaged into chylomicron and released into the circulation (Dueker *et al.,* 2000). An alternative explanation is that vitamin K_1 in the liver is being released in VLDL causing a small rise in vitamin K_1 concentration.

4.8.6 Assessment of methodology

There are a number of techniques available to measure the bioavailability of vitamins in humans, including depletion-repletion methods, oral-faecal balance techniques, and plasma responses, as either AUC or C_{max}. Of the latter two, AUC is the most reliable because it is a consequence of the plasma response over the period of absorption, rather than a single time-point (Hoppe *et al.,* 2000). The advantages and disadvantages of these techniques were considered in section 1.12. The use of stable isotopes is believed to provide the most reliable estimates (van Lieshout *et al.,* 2003) because of the ability to measure only newly absorbed vitamin. A number of approaches are available, particularly if the primary aim is to determine bioavailability from a single food, either alone, or within a test meal, or against a reference dose. Test compounds can be given either at the same time or on separate occasions. Single dose or multiple dosing protocols can be designed. Although there are numerous studies that have investigated vitamin A and E bioavailability, both with and without the use of stable isotopes, only a small number of studies have investigated vitamin K_1 bioavailability. Three of these studies used stable isotopes to measure vitamin K absorption, bioavailability and metabolism (Dolnikowski *et al.,* 2002; Kurilich *et al.,* 2003; Erkkilä *et al.,* 2004) but all with intrinsically labelled vegetables. Although the number of foods that can be labelled in this way is limited, many are vitamin K_1-rich foods e.g. broccoli or kale. Given time and resources it could even be possible to produce labelled vitamin K_1 in rapeseed oil, a relatively good source of vitamin K_1 (Shearer *et al.,* 1996), by exposing rape to deuterated water or ^{13}C-enriched carbon dioxide (Bluck *et al.,* 2002). A precise comparison could then be made between absorption from oil and vegetable as individual food items and/or within meals.

The use of labelled material as a standard to measure absolute bioavailability of vitamin A has been investigated (Edwards *et al.,* 2001;2002) but the results are heavily based on the value for absolute absorption of the standard. In another study, the effect of a meal on absorption of a labelled dose of α-tocopheryl acetate was studied (Jeanes *et al.,* 2004). Edwards *et al.* (2002) used labelled retinyl acetate as an extrinsic reference dose and calculated absorption by assuming 80% absorption of the labelled provitamin A. Absorption was then calculated by dividing baseline-subtracted unlabelled AUC by labelled AUC, and multiplying by the amount of reference dose absorbed (80% of the given dose). However, the use of labelled compounds in this way assumed and/or required that test foods/meals did not affect absorption of the standard. Whilst this assertion was valid for comparison between high and low fat meals, and different food sources of β-carotene (Edwards *et al.,* 2001), meal-effected differences were observed on bioavailability tracer vitamin E (Jeanes *et al.,* 2004).

In the present study, the original aim was to design a method, using a labelled form of vitamin K_1 as a standard, to compare absorption from any combination of foods, rather than individual food items, by observing tracer:tracee ratios. An alternative approach could be to calculate AUC of the tracee (after subtraction for baseline). If the tracer AUC had not been different, it would have been possible to express tracee AUC (after correction for the dose) relative to tracer AUC to obtain values for relative bioavailability. Using tracee AUC values alone would negate the advantages of having the isotope data, hence the described method was chosen. Additionally, with the size of the dose given (less than 40 μg) and the consequent small rise in tracee plasma concentration and coupled with analytical limitations, this approach was not considered the best available.

The advantages of this study compared to previous studies are 1) the crossover design in a relatively high number of subjects and 2) low doses of vitamin K_1, particularly compared to previous studies. An additional benefit of monitoring both tracer and tracee is the separation of meal and matrix effects as discussed above. The tracer data alone

provides interesting results with regard to the effects of the meal on 'free' vitamin K_1, at least within the limitations of the experiment.

4.8.7 Potential improvements to methodology

Potential improvements to the methodology used in the present study include direct injection of the tracer into the meal prior to serving, rather than the use of a capsule to deliver the tracer. This protocol would have ensured the tracer was indeed emptied from the stomach with the meal, and would remove meal effects on degradation of the gelatine capsule and/or absorption of the tracer. The advantage of the capsule however, is the certainty that the entire dose has been consumed.

A major limitation of the current protocol is the observed difference in absorption of the tracer. This problem could be resolved by staggering consumption of the tracer and the tracee, either by a single, bolus dose at sometime prior to consumption of the test meal or subjects could be dosed with tracer in much the same way as in the first volunteer study with the aim of reaching a steady-state for plasma vitamin K_1 enrichment. Consumption of an unlabelled test meal would then result in changes to the tracer:tracee ratio, dependent on how much was absorbed. The advantage of including stable isotopes over simply giving a meal with vitamin K_1 is that low levels of vitamin K_1 could be administered since small changes in enrichment would be more readily detected than changes in absolute concentration.

In the present experiment, samples were collected and AUC calculated over 8 h. In all subjects, this time was sufficient for tracer concentration to be close to baseline levels. In future studies consideration could be made to increase the sampling time to 9 or 10 h to ensure tracer concentration has returned to baseline, as is appropriate for true AUC measurements. The long period of elevated tracer concentration, and/or late appearance of tracer in plasma in this experiment could be a consequence of the large meals. Energy content of the meals was around 3200 kJ thus in future studies consideration for smaller and lower energy meals may be considered.

4.8.8 Vitamin K_1 content of meals and food composition tables

The approach in this study was to measure bioavailability of vitamin K_1 from meals. The three meals in this study were designed using the characteristics of dietary clusters identified in a national nutrition survey. The meals were formulated to contain equal amounts of vitamin K_1, with the major difference between the meals being the sources of vitamin K_1. However, direct analysis of meals revealed variation in the measured value compared to that calculated from food composition tables.

This finding may not be a surprise since the vitamin K_1 content within foods of the same type is known to be highly variable, especially for margarine which was a major source of vitamin K_1 in the convenience meal. For example, a study of the vitamin K_1 content in 70% vegetable oil spread found levels between 0.1 and 60 µg per 100 g (Peterson *et al.,* 2002) and another study of six brands reported values that ranged from 12 to 78 µg per 100 g (Bolton-Smith *et al.,* 2000). Cooking and reheating the meals may reduce the vitamin K_1 content compared to the calculated values (Ferland & Sadowski, 1992a). Furthermore, a comparison of the vitamin K_1 content of ten meals calculated by two nutrient databases compared to direct analysis showed variation of up to 89% (McKeown *et al.,* 2000). It is a common difficulty with food composition analysis to get a fully representative sample of the different sources or brands etc. of a single food. For example, the amount of vitamin K_1 in vegetables depends on maturation, geographical location and by which part of the vegetable is analysed (Ferland & Sadowski, 1992b).

Although the standard addition method for quantitation is not the most precise approach, the results were sufficient for the purposes of this study. Some difficulties were apparent with the chromatogram of the convenience meal in that an interfering peak eluted close to the peak of interest. The presence of an unidentified peak that elutes just before the vitamin K_1 peak during the HPLC analysis of canola oil, soybean oil, margarines and other processed foods (e.g. baked beans) has been reported (Woollard *et al.,* 2002). The convenience meal contained a relatively high proportion of ingredients that could exhibit the mystery peak, and it is therefore possible that the same unidentified peak was present in the analysis of the convenience meal. The result of the interfering peak

may be that the estimation of vitamin K_1 in the convenience meal was an underestimate. However, given the magnitude of the differences in bioavailability, the conclusions are unaffected, even when repeating calculations with the 40 μg theoretical value of vitamin K_1 content in the meal.

4.8.9 Dietary intake data

The difficulties of assessing dietary intake and in particular vitamin K_1 intake are exemplified in the diets analysed in 12 subjects who participated in this study. One particular difficulty is that, although vitamin K_1 is found a wide range of foods, there is a very wide range of concentrations (Shearer *et al.,* 1996). The result is that dietary intakes also have a huge variation, both inter- but also intra-individually, creating a problem when trying to estimate intake since a few foods that contain relatively large amounts may not be eaten regularly. This is demonstrated in the dietary data between individuals which showed that vitamin K_1 intakes can differ by a factor of 10 (Table 4-6) and by a factor of 25 within an individual (data not shown). In terms of assessing typical vitamin K_1 intake, a food frequency questionnaire may provide a better tool, although consideration needs to be made for seasonal variations in intake.

In this study, no significant correlations were observed between the intake of vitamin K_1 and other nutrients. However, subject A appeared as an outlier in a number of the regressions, and the removal of this subject generated significant associations between vitamin K_1 intake and intake of energy, protein, fat and MUFA, PUFA and SFA. This correlation could suggest that oil sources of vitamin K_1 make a substantial contribution to total vitamin K_1 intake.

In these 12 subjects, there was no relationship between vitamin K_1 intake and status. However, to observe such a relationship may be unexpected for a number of reasons. Firstly, the small sample size and secondly, blood samples were not taken at the same time as the dietary data were collected. However, multiple blood sampling may provide some benefit when trying to find relationships between intake and status. Similar conclusions have been drawn by Booth *et al.* (1997) who measured vitamin K_1 intake (with

a 4-d food diary) and status (plasma sample) on three separate occasions. On the first, the regression line between intake and status is almost flat (r=0.13, P=0.30), as in this study, whereas a combination of three sets of data reveal a strong relationship (r=0.51, P=0.004) (Booth *et al.,* 1997).

A number of significant inverse correlations were observed between vitamin K_1 status and other nutrients, including fat, SFA and PUFA, i.e. those individuals with the highest fat intake, had the lowest plasma levels of vitamin K_1. A possible explanation may be that those people who have a high fat diet, perhaps indicative of a less healthy diet, consume less of the foods that contain high amounts of vitamin K, and thus have a lower status. However, results from the bioavailability study suggest that in terms of status, absorption of vitamin K_1 from fat is better than that from vegetables. However, the results from this food diary analysis should not be over-emphasised. The data was obtained from only 12 subjects who completed 4–d food diaries. In addition, vitamin K_1 status was not measured at the same time as the dietary data was collected. The observed significant relationships may be an artefact of performing multiple statistical tests that result in significant findings when in fact there are no significant relationships (type 1 statistical error).

4.8.10 Relationship between vitamin K_1 intake and plasma status

Bioavailability is an important factor in determination of the relationship between intake and status. With good bioavailability data, it may be possible to improve relationships between vitamin K intake and status. Although the relationships between intake and status are often significant, they are not very strong (Booth *et al.,* 1997; Bolton-Smith *et al.,* 1998; Rock *et al.,* 1999; McKeown *et al.,* 2002; Thane *et al.,* 2006b).

The test meals in the present study were formulated based on the characteristic foods consumed by individuals identified in dietary clusters from a national survey. Within each cluster, the relationship between vitamin K_1 intake and status has been investigated. Based on the results from the bioavailability study, one might expect there to be differences in the relationships of intake and status within the three clusters. However,

none were found. Furthermore, adjustment of intakes for each cluster based on the bioavailability values, decreased rather than improved the relationships[1].

There are a number of possible reasons for this observation. Firstly, there is a disparity in the time of collection of data for intake and status measurements. The cluster analysis on which the meals were based was performed on data from the 2000 – 1 National Diet and Nutrition Survey (Fahey *et al.,* 2007). It is reported that for 80% of the participants in this study, a blood sample was collected within 2 wk following completion of the food diary (Thane *et al.,* 2006b). However, since vitamin K_1 plasma status is likely to reflect only the previous one or two days intake, 2 wk is a considerable period. In fact, some blood samples were taken > 100 d after the completion of the diary (Thane *et al.,* 2006b) and with consideration of potential seasonal variation of vitamin K_1 intake (Sadowski *et al.,* 1989; McKeown *et al.,* 2002) the plasma measurement is unlikely to reflect actual intake. Furthermore, the intra-individual variation in plasma vitamin K_1 status is greater than other fat-soluble vitamins (Booth *et al.,* 1997; Talwar *et al.,* 2005), probably reflecting the large variation in intake because of the distribution of vitamin K_1 in foods.

A lack of confidence in sampling provides a second potential confounder for the above results. Blood samples were collected both in the fasting and non-fasting state and at various times of the day (Thane *et al.,* 2006b) thus limiting their capacity to provide uniform comparisons between intake and status.

The third reason could simply be that the test meals do not reflect the clusters of which they are supposed to represent. Previous bioavailability studies have primarily tested single foods, either individually or in combination with untypical food combinations. The meal-based approach more accurately depicts typical consumption of foods in a free-living situation where, components of a mixed-meal may work together to negate effects that may be observed in experiments with individual foods. The cluster analysis provided an objective method by which to design the meals. However, it is obviously not possible to fully represent a typical diet within a single meal. This weakness may explain why the

---

[1] Personal communication with Dr Andy Coward, MRC Human Nutrition Research

conclusions from this bioavailability study do not match with the observation that there is little difference in the intake and status relationships between clusters.

Although data from this bioavailability study suggests there may be differences in the bioavailability of vitamin K_1 between foods and meals, the lack of a significant difference between clusters in terms of the relationship between intake and status suggests that this difference may be negated in an individuals overall diet.

4.9 Comparison of tracer absorption between Study 1 and Study 2

The results from study 1 that measured vitamin K_1 kinetics and absorption showed that only around 13% of small oral dose was absorbed. This is much lower than the only other previous estimate of absolute absorption of vitamin K_1 (Shearer *et al.,* 1974). A possible explanation for low absorption was the absence of a test meal. Together with data from Study 2, it was possible to test this hypothesis by comparing the tracer absorption parameters of C_{max} and AUC, adjusted for size of the dose, in both studies. The C_{max} of tracer absorption in study 1 was 0.008 nmol/L, compared to an average for all meals in study 2 of 0.009 nmol/L. Absorption expressed as AUC over 6 h, also shows similar results in both studies (study 1, 0.020 nmol/L.h; study 2, 0.017 nmol/L.h). This analysis suggests that the absence of the test meal cannot be the primary reason for seemingly low absorption. This evidence, together with no ready metabolic- or model-based explanation, suggests that low absorption from the capsule may be due to the capsule itself. However, gelatine capsules have been used in previous studies of vitamin K_1 (Suttie *et al.,* 1988b) and other fat-soluble vitamins (Dueker *et al.,* 2000) without comment.

4.10 Section 4 conclusions

This study has demonstrated a novel approach to measure vitamin K_1 bioavailability from test meals that may be more relevant to the consumption of foods in a free-living population. Results from absorption of the tracer, as measured by AUC, provided data on the effect of altering the environment in which the vitamin K_1 was absorbed, the 'meal effect'. The results suggested that vitamin K_1 tracer was less well absorbed in the

presence of the convenience meal, than when consumed with the cosmopolitan or animal-oriented meals. The reasons for this observation are unclear but may be related to gastric emptying effects or fat composition affecting absorption and/or metabolism of vitamin K_1, however this is largely conjecture.

Bioavailability of vitamin K_1 from the meal itself (tracee) was assessed based on the relationship between tracer and tracee, and produced the apparently contradictory result that absorption from the convenience was better than either the cosmopolitan or animal-oriented meals. A possible explanation for this observation is that the over-riding factor determining bioavailability is the matrix effect or 'bioaccessibility', since in this study, although absorption of the tracer was lowest when consumed with the convenience meal, overall effects suggest absorption is greatest from the convenience meal.

5 DISCUSSION

The research in this thesis was performed on a background of growing evidence and interest in the wider role of vitamin K in human health. Vitamin K was originally discovered as essential for blood coagulation and current dietary recommendations remain largely based on this function alone. A number of vitamin K-dependant (VKD) proteins have now been discovered that are distributed in different tissues throughout the body, but for some, their precise function remains unknown. Probably the most studied extrahepatic VKD protein is osteocalcin; the presence of undercarboxylated osteocalcin is often used as a marker of vitamin K status. A number of studies have demonstrated that osteocalcin carboxylation is responsive to vitamin K depletion and supplementation. There is also a growing body of evidence that links higher vitamin K status (both plasma and carboxylated osteocalcin) and higher vitamin K_1 intake to clinical markers of bone health, such as fracture risk and bone mineral density. Because of the broader functions of vitamin K, it may be time to reconsider vitamin K_1 dietary recommendations. However, there is already concern the majority of people do not meet the current recommendations and that intakes are decreasing with time (Thane *et al.,* 2006a). In the case of vitamin K_1, where intakes may be marginal, and for the setting of evidence-based dietary recommendations, knowledge of vitamin metabolism and bioavailability are essential. In addition, data on vitamin K_1 bioavailability are also important for the control of coagulation status for individuals on anticoagulation treatment (Couris *et al.,* 2006; Custódio das Dôres *et al.,* 2007). Despite their importance, few studies have investigated the metabolism and bioavailability of vitamin K, partly due to the lack of suitable methodologies.

Unlike the metabolic analysis of drugs, there are considerable difficulties in the study of vitamin metabolism because the compound of interest pre-exists in body pools. The use of tracer techniques, in particular stable isotopes, allows the researcher to distinguish between the labelled tracer dose and endogenous tracee. Stable isotope tracers provide a safe, reproducible and sensitive tool to investigate nutrient bioavailability

and metabolism but have been used only to a limited extent in vitamin K research, particularly in comparison to other vitamins and minerals. The kinetics and bioavailability of vitamin A have been the most extensively researched of all the fat-soluble vitamins. This interest is probably due to world-wide deficiency and subsequent public health importance of vitamin A as well as interest in the many carotenoid precursors of vitamin A. Consequently, numerous studies have investigated the bioaccessibility and bioconversion of vitamin A using tracer techniques (van Lieshout *et al.,* 2003). Similarly, many studies have investigated the relative bioavailability and biopotency of the vitamin E tocopherols owing to its antioxidant and putative cardioprotective role (Lodge *et al.,* 2004).

The use of stable isotopes to investigate vitamin K_1 has been reported only recently (Dolnikowski *et al.,* 2002; Kurilich *et al.,* 2003; Erkkilä *et al.,* 2004) due to the lack of suitable techniques and a low priority on the research agenda. However, the accumulation of evidence for the potentiallly beneficial effects of vitamin K_1, particularly for bone health, have stimulated interest and research into vitamin K_1 bioavailability. A common feature of this thesis is the development and application of stable isotope technologies to questions of vitamin K metabolism and bioavailability.

5.1 Analytical methodologies

Prior to studies in human volunteers, it was necessary to develop a method for the extraction of vitamin K_1 from plasma that provided a sample suitable for gas chromatography mass spectrometry and the measurement of isotope ratios. A number of purification strategies were combined including enzyme hydrolysis, liquid-liquid extraction and solid phase extraction prior to derivatisation. This new method, although somewhat labour intensive, provided an alternative to other reported methods in which analyses were incomplete due to chromatographic difficulties (Erkkilä *et al.,* 2004). Future analytical improvements should focus on reducing sample preparation time. A potential strategy is to use liquid chromatography mass spectrometry (LCMS) that may require less sample clean-up and preparation. Kurilich *et al.* (2003) reported the use of intrinsic labelling and LCMS to measure vitamin K_1 bioavailability. Recently, HPLC with tandem

mass spectrometry has been described for the sensitive measurement of vitamin K_1, MK-4 and MK-7 in human plasma using ^{18}O-labelled standards (Suhara *et al.,* 2005).

Although sensitivity was adequate for these studies, if smaller doses, or longer term kinetics are investigated then improvements are probably necessary. Remaining with GCMS, the use of chemical ionisation may provide some increase in sensitivity but there should be concern for the potential exchange of labelled atoms. For vitamins A and E, the use of gas chromatography-combustion-isotope ratio mass spectrometry (IRMS), with better sensitivity and precision for the measurement of isotopic enrichment, could be appropriate for longer-term experiments. At the current time, this method is not possible for vitamin K_1 due to the limited sensitivity of online IRMS instruments that is typically a 1000 x less than that afforded by GCMS (Bier 1997). An emerging technique for kinetic analysis is accelerator mass spectrometry (AMS) that was originally developed for carbon dating with ^{14}C, but more recently has been applied in the bio-analytical field (Vuong *et al.,* 2004). AMS provides a very sensitive tool for the investigation of absorption and metabolism of vitamins and other phytochemicals (Vuong *et al.,* 2004). Although based on radioactive ^{14}C, very low doses can be used that pose little discernable risk (Vuong *et al.,* 2004). An example of this technique is provided by Dueker *et al.* (2000) who fed intrinsically labelled spinach containing 306 µg of ^{14}C-labelled β-carotene to a single volunteer and measured kinetics over more than 200 d. Sensitivity was in the attomole range (1×10^{-18} mol). Thus, AMS may provide a suitable tool for further investigations of vitamin K kinetics.

A complementary approach to human studies for the measurement of bioavailability is *in vitro* stable isotope methods. Recent *in vitro* work has compared the comparative uptake and metabolism of ^{18}O-labelled vitamin K_1 and MK-4 in human cell lines (Suhara *et al.,* 2006).

5.2 Vitamin K_1 kinetics

In comparison to water-soluble vitamins, there are a number of difficulties associated with tracer studies of fat-soluble vitamins. Studies in which only an oral dose is used to measure kinetics relies heavily on the assumption of the amount of dose absorbed, unless stool samples are also collected. Intravenous administration of fat-soluble vitamins requires a carrier that may not behave kinetically in the same way as an absorbed oral dose. Furthermore, ethical and regulatory difficulties arise if the compound to be injected is isotopically labelled. In this study, a novel approach was taken where, following pre-enrichment of plasma vitamin K_1 with labelled oral vitamin K_1, volunteers received an *iv* dose of a well-established and safe pharmaceutical preparation of vitamin K_1, Konakion MM®. With this methodology, it was possible to obtain kinetic data for all 10 volunteers that were similar to values reported previously from work with radiolabelled tracers (Shearer *et al.,* 1974; Shepherd *et al.,* 1977; Bjornsson *et al.,* 1979). The kinetics of Konakion MM® vitamin K_1 disappearance were resolved into two exponentials with $T_{½}$s of 0.22 and 2.66 h. Olson *et al.* (2002) reported kinetic values considerably different to those published previously, probably due to the measured radioactivity being associated with metabolites rather than vitamin K_1. This discrepancy highlights a major advantage of mass spectrometry; that is the unambiguous identification of vitamin K_1 from the mass spectral information.

The only incongruous value from the kinetic work in this thesis was that calculated for the non-sampled body pool size. In the model, the sampled pool (pool 1) represented the plasma pool, and the non-sampled pool (pool 2), represented other body stores. The calculated size of the pool 2 was much smaller than estimates ascertained from direct sampling of body tissues. In the model, all losses from the system were from pool 1 (k_{01}). An alternative model was investigated with losses to the outside exiting only from the pool 2 (k_{02}), rather than pool 1. However, this alternative model had little effect on the size of pool 2. There is no ready explanation for the observed small size of pool 2, but it may be that the model does not represent the actual physiology. Although the present simple

model was chosen as most likely to represent the known physiology, it is possible, from the same slope and intercepts derived from tracer disappearance, to obtain a number of different models that have different inputs and outputs (Shipley & Clarke, 1972). Additionally, there remains the possibility of other body pools that were not seen in this experiment. Direct measurements suggest bone (Hodges *et al.*, 1993) could contain at least as much vitamin K_1 as liver. This finding is not surprising since vitamin K is necessary for the carboxylation of VKD proteins, osteocalcin and MGP, and recent experiments have demonstrated chylomicron remnant uptake by osteoblasts and a subsequent increase in γ-carboxylation of osteocalcin (Niemeier *et al.*, 2005). Additional kinetic studies are necessary to further characterise the kinetics and turnover of vitamin K_1 over a longer duration.

As discussed in section 3, a caveat to the study methodology is potential differences in the uptake of *iv* and oral forms, that is a consequence of the need for a carrier for *iv* administration. Differences may exist between vitamin K_1 uptake from chylomicrons and chylomicron remnants (CR) compared to the Konakion MM® formulation since the latter lack any of the intrinsic proteins that lead to lipoprotein uptake, e.g. apoE. However, kinetic results from Konakion MM® alone (Soedirman *et al.*, 1996), detergent-solubised radioactive tracers (Shearer *et al.*, 1974) and this study, are all similar. It is known that oral vitamin K_1 is absorbed in mixed micelles from the gut and released into circulation as components of chylomicrons. The $T_{½}$ of initial vitamin K_1 clearance obtained in this thesis is similar to that reported for hepatic chylomicron clearance (0.28 h) (Cortner *et al.*, 1987). These values suggest that uptake of Konakion MM® is representative of uptake of an oral dose absorbed from the gut, at least during the initial period of uptake represented by the fast exponential. Further evidence to suggest no difference between the kinetics of the *iv* and oral doses comes from comparison of the disappearance of the oral dose compared to the *iv* dose (section 3.9.2). The $T_{½}$ for disappearance of the oral dose was not significantly different from the $T_{½}$ of the second, slower exponential of the *iv* dose. Although this calculation is approximate, it suggests that there were no gross differences. A possible future approach for the generation of

vitamin K_1 suitable for *iv* administration could be similar to that used by Niemeier *et al.* (2005) for the generation of vitamin K_1-enriched CR for *in vitro* work. In this method, a single, fasted volunteer was given a fat-rich breakfast with 10 mg of vitamin K_1. After 4 h, a blood sample was taken and the chylomicron fraction separated by ultra-centrifugation. The collected CR were then utilised for *in vitro* experiments (Niemeier *et al.,* 2005). A comparable protocol could be applied for further *in vivo* measurements whereby an individual is given a vitamin K_1-enriched breakfast, a blood sample is taken and chylomicrons separated for analysis and subsequent re-administration to the same volunteer.

5.3 Vitamin K_1 absorption

In the first volunteer study, the absolute absorption of a 4 µg oral dose of deuterated vitamin K_1 was measured as only 13%, considerably lower than the only other previous estimate of around 80% (Shearer *et al.,* 1974). Absolute absorption was calculated by application of the rate constants obtained from *iv* dose to appearance of the deuterated oral dose in plasma. As discussed above, there is no evidence that the kinetics of disappearance of the *iv* dose do not reflect those of the oral dose. If the calculated kinetics were incorrect and if irreversible disposal occurred at a greater rate than that predicted, then absorption would be under-estimated. However, other data supports the kinetic parameters calculated in this study (Shearer *et al.,* 1974; Shepherd *et al.,* 1977; Bjornsson *et al.,* 1978a), while other work suggests a slower rate of irreversible disposal (Olson *et al.,* 2002). An alternative explanation for the low absorption was the absence of a test meal, but it was not supported by data from the second volunteer study. It is possible that the gelatine capsule was the cause of the low absorption, although similar gelatine capsules have been used in previous studies with no reports of lower than expected absorption (Suttie *et al.,* 1988b; Dueker *et al.,* 2000; Jeanes *et al.,* 2004). Thus, there is no obvious explanation for the low bioavailability observed in this study. Further work is necessary to investigate the absolute absorption of vitamin K_1 to provide an

accurate reference for the assessment of bioavailability, and ultimately lead to a better understanding of the relationship between intake and status.

5.4 Bioavailability of vitamin K_1

The second volunteer study presented in this thesis aimed to measure the bioavailability of vitamin K_1 from different meals. The protocol was developed based on data obtained in the first volunteer study that showed a short (<8 h) protocol would be sufficient to measure vitamin K_1 bioavailability. A novel approach was taken for the design of the test meals. A typical method for the measurement of vitamin bioavailability is to use a single test meal that might consist of a simple homogenous food plus a significant source of the vitamin of interest; often less attention is paid to the composition of the whole meal. Whilst this approach may provide mechanistic data, it does not provide a true representation of the normal consumption of foods that may negate the impact of individual foods on bioavailability. Test meals in this study were formulated to reflect typical UK dietary patterns that were recently identified in dietary pattern analysis of data from the 2000 – 1 National Diet and Nutrition Survey of Adults (Fahey *et al.*, 2007). The test meal was consumed with 20 μg ^{13}C-labelled vitamin K_1 as a standard by which to compare bioavailability.

Due to the influence of the meal, absorption of labelled vitamin K_1 from the capsule was different between the meals, with tracer absorption with the conveneince meal significantly lower than tracer absorption with the other meals. The possible reasons include factors related to gastric emptying and fat content and fatty acid profile, which were considered in section 4. The differences in tracer absorption are probably due to a multitude of interactive effects and it is difficult to comment with any certainty on likely explanations for the observation. A similar study that measured the absorption of ^{2}H-labelled α-tocopheryl acetate also reported that the type of meal and the fat content significantly influenced absorption (Jeanes *et al.*, 2004).

Justification for the absence of a test meal in the first experiment is provided by the variable and occasionally complicated nature of the tracer absorption curves from the

second volunteer study. In the bioavailability study, in some instances, appearance of the tracer dose was maximal after 2 h, whereas in others, the peak tracer concentration did not occur until 6 or 7 h. Furthermore, in some individuals two peaks were observed during the 8 h duration of the experiment. Interestingly, the pattern within an individual was usually similar for the three test-meals suggesting that the differences were subject-dependent differences, for example apoE genotype. These differences highlight the potential inaccuracies of using single time point measurements to assess absorption and dose-response relationships, such as used by Schurgers *et al.* (2004). The variation in the absorption profile may be in part due to the relatively large size and energy content of the test-meals.

Relative bioavailability of vitamin K_1 from the meal was assessed based on the relationship between tracer and tracee over the 8 h experiment. The results suggest that absorption of vitamin K_1 from the convenience meal was greater than from the other meals. This finding is in line with the general view that absorption from oil is greater than that from a vegetable source due to greater bioaccessibility (Vermeer *et al.,* 2004).

Further research is necessary to confirm that vitamin K_1 is more bioavailable from fats than from vegetables. The use of labelled vitamin K_1 as a standard to compare unlabelled vitamin K_1 absorption would be possible with adjustments to the methodology used here. Alternative approaches were discussed in section 4 but could include either a stable isotope labelled dose taken a number of hours prior to the test meal to remove meal effects, or oral pre-enrichment as performed in the kinetics study. In each case, bioavailability could be assessed by the change in the isotope ratio before and after consumption of the meal. The advantage of the isotope dose is the ability to detect smaller changes than with plasma concentration alone that would permit lower doses of vitamin K_1 within test meals or foods. The advantages of using an extrinsically labelled dose include control of the extent and positioning of labelling and, with a suitable methodology, the ability to measure bioavailability from a wide range of foods. The alternative method of intrinsic labelling has the advantage of producing labelled vitamin K_1 within the plant matrix. However, there is no control of the positioning of labelling, and

furthermore the requirement to measure multiple ions by mass spectrometry can import additional error. Intrinsic labelling has only been applied to vegetable matrices. An alternative is to produce intrinsically labelled vitamin K_1 in vegetable oil by growing, for example, rapeseed oil in an atmosphere in which carbon dioxide is ^{13}C-enriched or hydroponically with deuterated water. This approach may provide the best available option to determine the relative bioavailability of vitamin K_1 from oils and vegetables.

The use of plasma response provides information on the amount of vitamin K_1 absorbed but longer-term studies are necessary to ascertain the effect of different sources of vitamin K_1 on other tissue markers of vitamin K status. Such studies are difficult and expensive to perform because of requirement to control diets over the longer term.

5.5 Further work

There are potential improvements common to both volunteer studies. Firstly, a better understanding of the contribution of the *cis*-isomer to the outcomes should be investigated. Evidence suggests that although the *cis*-isomer is absorbed it is not biologically active (Knauer *et al.*, 1975). Further work could investigate the absorption of the *cis*-isomer that contributed around 16% to total vitamin K_1 in the administered oral doses. Separation, and thus fraction collection, of the isomers is possible by HPLC (Woollard *et al.*, 2002). From analysis by GCMS of the derivatised forms any difference in the kinetics of uptake could be identified.

The tissue specific accumulation of menaquinone-4 (MK-4) has been demonstrated in rats (Davidson *et al.*, 1998) and humans (Thijssen *et al.*, 1996a). In rats, it has been established that tissue MK-4 is primarily synthesised from dietary vitamin K_1 (Thijssen *et al.*, 1994; Thijssen *et al.*, 1996b) and in humans that MK-4 in breast milk originates from dietary vitamin K_1 (Thijssen *et al.*, 2002). More recently, it has reported that oral, but not subcutaneous, vitamin K_1 supplementation in humans led to an increase in the urinary excretion of menadione (Thijssen *et al.*, 2006). The authors hypothesise that menadione is an intermediate in the conversion of dietary vitamin K_1 to MK-4, and that the conversion to menadione may take place in the enterocytes. MK-4 is then

synthesised from menadione in the tissues, since menadione is able to cross barriers such as the blood-brain barrier (Thijssen *et al.*, 2006). The first study in this thesis that measured absolute absorption only measured vitamin K_1, thus the estimate of 13% absorption could be increased if a proportion of the absorbed dose was converted to menadione.

Further analysis of plasma samples collected in either of the studies described in this thesis could provide additional data on the apparent conversion of vitamin K_1 to menadione. The labelled atoms were positioned on the 1',4,-naphthoquinone structure and thus would be unaffected by side chain removal, and subsequent synthesis to MK-4. The low dose (4 µg) in the first study may preclude analysis of these samples. However, it would be interesting to track the appearance of labelled menadione in the plasma of individuals from the bioavailability study. New methods may have to be devised for menadione analysis by GCMS since, like vitamin K_1, current methods utilise HPLC, although it is likely that MK-4 analysis would be possible using the GCMS method described here with monitoring of the appropriate ions. The development of HPLC-mass spectrometric methods may provide a suitable tool. The use of HPLC-tandem mass spectrometry has recently been described for the sensitive measurement of MK-4 (Suhara *et al.*, 2005).

5.6 Implications of increased dietary recommendations

If dietary recommendations for vitamin K_1 were increased, the question that follows is how to increase the actual intake of individuals? Already, using the current UK recommendations over 50% of individuals fail to met the guideline value and if the higher US values are used then almost 80% of individuals would not reach the cut-off values (Thane *et al.*, 2006a).

A number of strategies could be implemented to improve vitamin K intake. Green leafy vegetables contain the highest amounts of vitamin K_1 and evidence suggests that a decrease in green leafy vegetable consumption is a primary reason for the decrease in overall intakes observed in the UK between 1986 – 7 and 2000 – 1 (Thane *et al.*, 2006a).

Promotion of native, seasonal, green leafy vegetables may contribute to an increase in vitamin K_1 intakes with additional benefits of simultaneously increasing population intakes of vitamin A and folate. The promotion of green leafy vegetables, although relatively simple to implement, may not be the most effective method of increasing vitamin K_1 intakes. Efforts to increase fruit and vegetable consumption through the 'five a day' campaign have had only limited success, with the NDNS 2000 – 1 survey reporting average daily consumption of only 2.8 portions (Swan 2004). In the UK and elsewhere in Europe, a deficit in the consumption of vegetables compared to fruit has been highlighted despite the 'five a day' campaigns (Naska *et al.,* 2000). It has been suggested that fruit and vegetable intake recommendations should be promoted separately (Naska *et al.,* 2000). Furthermore, evidence presented in this thesis and previous data (Booth *et al.,* 2002) show that the bioavailability of vitamin K_1 from green leafy vegetables may be less than that from vegetable oils. Although green leafy vegetables may be high in vitamin K_1, a better strategy for increasing intakes may be to focus on vegetable oils and fats.

If, after consideration for bioavailability, vegetable fats and oils are proven a better source of vitamin K_1, then methods to improve their vitamin K_1 content could be investigated. Further research is required to determine the relative importance of the main sources of vitamin K_1. Vitamin K_1 is rapidly degraded by sun- and fluorescent light (Ferland & Sadowski, 1992a) thus, the vitamin K_1 content of oils could be diminished by inappropriate storage conditions. The majority of vitamin K_1-rich vegetable oils are marketed in clear bottles thus using amber bottles or canned packaging may improve the vitamin K_1 content.

An alternative approach is food fortification. Flour fortification with folic acid has recently been recommended by the UK Scientific Advisory Committee on Nutrition (SACN) to improve the folate intake of young women in order to prevent neural tube defects during early pregnancy (Scientific Advisory Committee on Nutrition 2006). In addition, by law in the UK, margarines are fortified with vitamins A and D to contain similar levels to those naturally contained in butter. The fortification of margarines and oils with

vitamin K_1 would be a feasible strategy to increase vitamin K_1 intake, particularly if further evidence shows that bioavailability from oils is better than that from vegetables.

The third strategy to increase vitamin K_1 intake is supplementation. Any programme of supplementation would target a specific population group. Based on current evidence the best choice might be older woman at risk of osteoporosis. Evidence suggests that older women have a greater level of ucOC and may have a greater requirement for vitamin K compared to younger women (Tsugawa *et al.,* 2006). The strategies outlined above assume that vitamin K_1 alone can have a beneficial effect. However, it has been suggested that the greatest benefit of vitamin K_1 supplementation may only be obtained in combination with vitamin D and calcium (Vermeer *et al.,* 2004) as observed in two long-term (>2 y) supplementation studies (Braam *et al.,* 2003; Bolton-Smith *et al.,* 2007). In the latter study, vitamin K_1 supplementation was performed with 200 μg/d that the authors claim is achievable through dietary modification. However, evidence that relative vitamin K_1 bioavailability from supplements is at least four times that from vegetables (Gijsbers *et al.,* 1996; Garber *et al.,* 1999; Schurgers & Vermeer, 2000) suggests that this assertion is unlikely. Based on current evidence, the relative difficulty of implementing a nationwide scheme may preclude supplementation as an appropriate population strategy.

5.7 Concluding remarks

Stable isotope tracer methods provide a safe and accurate method with which to investigate vitamin kinetics and bioavailability. Within this thesis is described the first method for the analysis of stable isotope labelled vitamin K_1 from plasma by gas chromatography mass spectrometry. The development of this method permitted further study of vitamin K_1 kinetics and bioavailability.

This study was also the first to measure vitamin K_1 kinetics using stable isotopes. The results obtained were similar to those presented in comparable studies of vitamin K_1 kinetics that have used radiolabelled isotopes. Vitamin K_1 disappearance was resolved into two exponentials with a rapid disappearance suggestive of uptake by the liver.

Further studies are required to determine vitamin K_1 kinetics over a longer duration, and using stable isotope methods, the potential impact of vitamin K_1 conversion to menadione and MK-4. The value for absolute absorption of an oral dose of vitamin K_1 calculated using the disposal kinetics of vitamin K_1 was much lower than the only other previous estimate.

In a novel approach, dietary pattern analysis was used to design test-meals for the measurement of vitamin K_1 bioavailability. Consistent with previous studies on vitamin K_1 bioavailability and other fat-soluble vitamins, it was concluded that bioavailability was highest from oil rather than vegetable sources. Greater consideration should be given to the contribution of foods lower in vitamin K_1 but eaten more often and with potential greater bioavailability. Further work is necessary to refine bioavailability methodologies and in particular, for vitamin K_1, a greater knowledge of absolute and relative bioavailability is required.

Prior to any increase in current vitamin K_1 dietary recommendations, further research is necessary. Although the ability of vitamin K_1 to decrease the undercarboxylation of VKD is well established, there is a need for a better understanding of the relationships between undercarboxylated VKD proteins and health, in particular the role of osteocalcin in bone metabolism and the significance of undercarboxylated osteocalcin. If greater vitamin K_1 intake is confirmed as having a beneficial effect on health, then strategies for improving vitamin K_1 intakes and tissue status should be based on a thorough understanding of vitamin K_1 bioavailability.

6 REFERENCES

Acuff RV, Thedford SS, Hidiroglou NN, Papas AM and Odom TA, Jr. (1994) Relative bioavailability of *RRR*- and all-*rac*-α-tocopheryl acetate in humans: studies using deuterated compounds. *American Journal of Clinical Nutrition* **60** (3), 397-402.

Alexander GD and Suttie JW (1999) The effect of vitamin E on vitamin K activity. *FASEB Journal* **40,** 430-431(Abstract).

Allison PM, Mummah-Schendel LL, Kindberg CG, Harms CS, Bang NU and Suttie JW (1987) Effects of a vitamin K-deficient diet and antibiotics in normal human volunteers. *Journal of Laboratory and Clinical Medicine* **110** (2), 180-188.

Almquist HJ and Stokstad ELR (1935) Hemorrhagic chick disease of dietary origin. *Journal of Biological Chemistry* **111** (1), 105-113.

Bates CJ, Jones KS and Bluck LJ (2004) Stable isotope-labelled vitamin C as a probe for vitamin C absorption by human subjects. *British Journal of Nutrition* **91** (5), 699-705.

Beavan SR, Prentice A, Stirling DM, Dibba B, Yan L, Harrington DJ and Shearer MJ (2005) Ethnic differences in osteocalcin γ-carboxylation, plasma phylloquinone (vitamin K_1) and apolipoprotein E genotype. *European Journal of Clinical Nutrition* **59** (1), 72-81.

Begley GS, Furie BC, Czerwiec E, Taylor KL, Furie GL, Bronstein L, Stenflo J and Furie B (2000) A conserved motif within the vitamin K-dependent carboxylase gene is widely distributed across animal phyla. *Journal of Biological Chemistry* **275** (46), 36245-36249.

Berkner KL (2000) The vitamin K-dependent carboxylase. *Journal of Nutrition* **130** (8), 1877-1880.

Berkner KL and Runge KW (2004) The physiology of vitamin K nutriture and vitamin K-dependent protein function in atherosclerosis. *Journal of Thrombosis and Haemostasis* **2** (12), 2118-2132.

Berkner KL (2005) The vitamin K-dependent carboxylase. *Annual Review of Nutrition* **25**, 127-149.

Bier DM (1997). Stable isotope tracers: technological tools that have emerged. In *Emerging Technologies for Nutrition Research: Potential for Assessing Military Performance Capability,* pp 203-213 [Institute of Medicine Committee on Military Nutrition Research]. Washington, D.C.: National Academy Press.

Binkley NC, Krueger DC, Engelke JA, Foley AL and Suttie JW (2000) Vitamin K supplementation reduces serum concentrations of under-γ-carboxylated osteocalcin in healthy young and elderly adults. *American Journal of Clinical Nutrition* **72** (6), 1523-1528.

Binkley NC, Krueger DC, Kawahara TN, Engelke JA, Chappell RJ and Suttie JW (2002) A high phylloquinone intake is required to achieve maximal osteocalcin γ-carboxylation. *American Journal of Clinical Nutrition* **76** (5), 1055-1060.

Binkley NC, Krueger D, Engleke J and Suttie J (2007) Vitamin K deficiency form long-term warfarin anticoagultion does not alter skeletal status in male Rhesus monkeys. *Journal of Bone and Mineral Research* **22** (5), 695-700.

Bitensky L, Hart JP, Catterall A, Hodges SJ, Pilkington MJ and Chayen J (1988) Circulating vitamin K levels in patients with fractures. *Journal of Bone and Joint Surgery* **70** (4), 663-664.

Bjornsson TD, Swezey SE, Meffin PJ and Blaschke TF (1978) Quantitation of radio-labelled vitamin K_1 and vitamin K_1 epoxide in plasma by high pressure liquid chromatography. *Thrombosis and Haemostasis* **39** (2), 466-473.

Bjornsson TD, Meffin PJ, Swezey SE and Blaschke TF (1979) Effects of clofibrate and warfarin alone and in combination on the disposition of vitamin K_1. *Journal of Pharmacology and Experimental Therapeutics* **210** (3), 322-326.

Blatt DH, Pryor WA, Mata JE and Rodriguez-Proteau R (2004) Re-evalution of the relative potency of synthetic and natural α-tocopherol: experimental and clicnical observations. *Journal of Nutritional Biochemistry* **15** (7), 380-395.

Blomstrand R and Forsgren L (1968) Vitamin K_1-^3H in man. Its intestinal absorption and transport in the thoracic duct lymph. *Internationale Zeitschrift für Vitaminforschung* **38** (1), 45-64.

Bluck LJC and Coward WA (1997) Peak measurement in gas chromatographic mass spectrometric isotope studies. *Journal of Mass Spectrometry* **32** (11), 1212-1218.

Bluck LJ, Jones KS, Thomas J, Liggins J, Harding M, Bingham SA and Coward WA (2002) Quantitiative analysis using gas chromatography/combustion/isotope ratio mass spectrometry and standard addition of intrinsically labelled standards (SAIL) - application to isoflavones in foods. *Rapid Communications in Mass Spectrometry* **16** (24), 2249-2254.

Bolton-Smith C, Price RJG, Fenton S, Harrington DJ and Shearer MJ (1998) The relationship between plasma and dietary phylloquinone (vitamin K_1) in Scottish adults. *Proceedings of the Nutrition Society* **57**, 148A.

Bolton-Smith C, Price RJ, Fenton ST, Harrington DJ and Shearer MJ (2000) Compilation of a provisional UK database for the phylloquinone (vitamin K_1) content of foods. *British Journal of Nutrition* **83** (4), 389-399.

Bolton-Smith C, McMurdo MET, Paterson CR, Mole PA, Harvey JM, Fenton ST, Prynne CJ, Mishra GD and Shearer MJ (2007) A two-year randomized controlled trial of vitamin K_1 (phylloquinone) and vitamin D_3 plus calcium on the bone health of older women. *Journal of Bone and Mineral Research.* **22** (4), 509-519.

Booth SL, Davidson KW and Sadowski JA (1994) Evaluation of an HPLC method for the determination of phylloquinone (vitamin K_1) in various food matrices. *Journal of Agricultural and Food Chemistry* **42** (2), 295-300.

Booth SL, Davidson KW, Lichtenstein AH and Sadowski JA (1996a) Plasma concentrations of dihydro-vitamin K_1 following dietary intake of a hydrogenated vitamin K_1-rich vegetable oil. *Lipids* **31** (7), 709-713.

Booth SL, Pennington JA and Sadowski JA (1996b) Food sources and dietary intakes of vitamin K-1 (phylloquinone) in the American diet: data from the FDA Total Diet Study. *Journal of the American Dietetic Association* **96** (2), 149-154.

Booth SL, Tucker KL, McKeown NM, Davidson KW, Dallal GE and Sadowski JA (1997) Relationships between dietary intakes and fasting plasma concentrations of fat-soluble vitamins in humans. *Journal of Nutrition* **127** (4), 587-592.

Booth SL and Suttie JW (1998) Dietary intake and adequacy of vitamin K. *Journal of Nutrition* **128** (5), 785-788.

Booth SL, O'Brien-Morse ME, Dallal GE, Davidson KW and Gundberg CM (1999a) Response of vitamin K status to different intakes and sources of phylloquinone-rich foods: comparison of younger and older adults. *American Journal of Clinical Nutrition* **70** (3), 368-377.

Booth SL, Webb DR and Peters JC (1999b) Assessment of phylloquinone and dihydrophylloquinone dietary intakes among a nationally representative sample of US consumers using 14-day food diaries. *Journal of the American Dietetic Association* **99** (9), 1072-1076.

Booth SL, McKeown NM, Lichtenstein AH, Morse MO, Davidson KW, Wood RJ and Gundberg C (2000a) A hydrogenated form of vitamin K: its relative bioavailability and presence in the food supply. *Journal of Food Composition and Analysis* **13** (4), 311-317.

Booth SL, Tucker KL, Chen H, Hannan MT, Gagnon DR, Cupples LA, Wilson PW, Ordovas J, Schaefer EJ, Dawson-Hughes B and Kiel DP (2000b) Dietary vitamin K intakes are associated with hip fracture but not with bone mineral density in elderly men and women. *American Journal of Clinical Nutrition* **71** (5), 1201-1208.

Booth SL, Lichtenstein AH, O'Brien-Morse M, McKeown NM, Wood RJ, Saltzman E and Gundberg CM (2001) Effects of a hydrogenated form of vitamin K on bone formation and resorption. *American Journal of Clinical Nutrition* **74** (6), 783-790.

Booth SL, Lichtenstein AH and Dallal GE (2002) Phylloquinone absorption from phylloquinone-fortified oil is greater than from a vegetable in younger and older men and women. *Journal of Nutrition* **132** (9), 2609-2612.

Booth SL, Broe KE, Gagnon DR, Tucker KL, Hannan MT, McLean RR, Dawson-Hughes B, Wilson PW, Cupples LA and Kiel DP (2003a) Vitamin K intake and bone mineral density in women and men. *American Journal of Clinical Nutrition* **77** (2), 512-516.

Booth SL, Martini L, Peterson JW, Saltzman E, Dallal GE and Wood RJ (2003b) Dietary phylloquinone depletion and repletion in older women. *Journal of Nutrition* **133** (8), 2565-2569.

Booth SL, Golly I, Sacheck JM, Roubenoff R, Dallal GE, Hamada K and Blumberg JB (2004) Effect of vitamin E supplementation on vitamin K status in adults with normal coagulation status. *American Journal of Clinical Nutrition* **80** (1), 143-148.

Borel P, Pasquier B, Armand M, Tyssandier V, Grolier P, Alexandre-Gouabau M-C, Andre M, Senft M, Peyrot J, Jaussan V, Lairon D and Azais-Braesco V (2001) Processing of vitamin A and E in the human gastrointestinal tract. *American Journal of Physiology: Gastrointestinal and Liver Physiology* **280** (1), G95-G103.

Borel P (2003) Factors affecting intestinal absorption of highly lipophilic food microconstituents (fat-soluble vitamins, carotenoids and phytosterols). *Clinical Chemistry and Laboratory Medicine* **41** (8), 979-994.

Bovill EG, Fung M and Cushman M (2004) Vitamin K and oral anticoagulation: thought for food. *American Journal of Medicine* **116** (10), 711-713.

Braam LA, Knapen MH, Geusens P, Brouns F, Hamulyák K, Gerichhausen MJ and Vermeer C (2003) Vitamin K1 supplementation retards bone loss in postmenopausal women between 50 and 60 years of age. *Calcified Tissue International* **73** (1), 21-26.

Brenner B, Sánchez-Vega B, Wu S-M, Lanir N, Stafford DW and Solera J (1998) A missense mutation in γ-glutamyl carboxylase gene causes combined deficiency of all vitamin K-dependent blood coagulation factors. *Blood* **92** (12), 4554-4559.

Brown ED, Micozzi MS, Craft NE, Bieri JG, Beecher G, Edwards BK, Rose A, Taylor PR and Smith JC, Jr. (1989) Plasma carotenoids in normal men after a single ingestion of vegetables or purified β-carotene. *American Journal of Clinical Nutrition* **49** (6), 1258-1265.

Bruno RS, Ramakrishnan R, Montine TJ, Bray TM and Traber MG (2005) α-Tocopherol disappearance is faster in cigarette smokers and is inversely related to their ascorbic acid status. *American Journal of Clinical Nutrition* **81** (1), 95-103.

Bruno RS, Leonard SW, Park S-i, Zhao Y and Traber MG (2006) Human vitamin E requirements assessed with the use of apples fortified with deuterium-labeled α-tocopheryl acetate. *American Journal of Clinical Nutrition* **83** (2), 299-304.

Bügel S, Sørenson AD, Hels O, Kristensen M, Vermeer C, Jakobsen J, Flynn A, Mølgaard C and Cashman KD (2007) Effect of phylloquinone supplementation on biochemical markers of vitamin K status and bone turnover in postmenopausal women. *British Journal of Nutrition* **97** (2), 373-380.

Cham BE, Smith JL and Colquhoun DM (1999) Interdependence of serum concentrations of vitamin K_1, vitamin E, lipids, apolipoprotein A_1, and apolipoprotein B: importance in assessing vitamin status. *Clinica Chimica Acta* **287** (1-2), 45-57.

Chu P-H, Huang T-Y, Williams J and Stafford DW (2006) Purified vitamin K epoxide reductase alone is sufficient for conversion of vitamin K epoxide to vitamin K and vitamin K to vitamin KH_2. *Proceedings of the National Academy of Sciences* **103** (51), 19308-19313.

Collins A, Cashman KD and Kiely M (2006) Phylloquinone (vitamin K_1) intakes and serum undercarboxylated osteocalcin levels in Irish postmenopausal women. *British Journal of Nutrition* **95** (5), 982-988.

Conly JM and Stein K (1992) The production of menaquinones (vitamin K2) by intestinal bacteria and their role in maintaining coagulation homeostasis. *Progress in Food and Nutrition Science* **16** (4), 307-343.

Conly JM, Stein K, Worobetz L and Rutledge-Harding S (1994) The contribution of vitamin K_2 (menaquinones) produced by intestinal microflora to human nutritional requirements for vitamin K. *American Journal of Gastroenterology* **89** (6), 915-923.

Cooper AD (1997) Hepatic uptake of chylomicron remnants. *Journal of Lipid Research* **38** (11), 2173-2192.

Cortner JA, Coates PM, Le N-A, Cryer DR, Ragni MC, Faulkner A and Langer T (1987) Kinetics of chylomicron remnant clearance in normal and in hyperlipoproteinemic subjects. *Journal of Lipid Research* **28** (2), 195-206.

Couris R, Tataronis G, McCloskey W, Oertel L, Dallal G, Dwyer J and Blumberg JB (2006) Dietary vitamin K variability affects International Normalized Ratio (INR) coagulation indices. *International Journal of Vitamin and Nutrition Research* **76** (2), 65-74.

Craciun AM, J. W, Knapen MH, Brouns F and Vermeer C (1998) Improved bone metabolism in female elite athletes after vitamin K supplementation. *International Journal of Sports Medicine* **19** (7), 479-484.

Custódio das Dôres SM, Booth SL, Aújo Martini L, de Carvalho Gouvêa VH, Padovani CR, de Abreu Maffei FH, Campana ÁO and Rupp de Paiva SA (2007) Relationship between diet and anticoagulant response to warfarin: a factor analysis. *European Journal of Nutrition* **46** (3), 147-154.

Dainty JR (2001) Use of stable isotopes and mathematical modelling to investigate human mineral metabolism. *Nutrition Research Reviews* **14** (2), 295-315.

Dam H (1935) The antihaemorrhagic vitamin of the chick. Occurrence and chemical nature. *Nature* **135**, 652.

Davidson KW, Booth SL, Dolnikowski GG and Sadowski JA (1996) Conversion of vitamin K_1 to 2',3'-dihydrovitamin K_1 during the hydrogenation of vegetable oils. *Journal of Agricultural and Food Chemistry* **44** (4), 980-983.

Davidson MH, Maki KC, Umporowicz DM, Ingram KA, Dicklin MR, Schaefer E, Lane RW, McNamara JR, Ribaya-Mercado JD, Perrone G, Robins SJ and Franke WC (2001) Safety and tolerability of esterified phytosterols administered in reduced-fat spread and salad dressing in healthy adult men and women. *Journal of the American College of Nutrition* **20** (4), 307-319.

Davidson RT, Foley AL, Engelke JA and Suttie JW (1998) Conversion of dietary phylloquinone to tissue menaquinone-4 in rats is not dependent on gut bacteria. *Journal of Nutrition* **128** (2), 220-223.

Department of Health (1991). *Dietary Reference Values for Food Energy and Nutrients for the United Kingdom. Report on Health and Social Subjects No. 41*, London: HMSO.

Di Mari SJ, Supple JH and Rapoport H (1966) Mass spectra of napthoquinones. Vitamin $K_{1(20)}$. *Journal of the American Chemical Society* **88** (6), 1226-1232.

Dolnikowski GG, Sun Z, Grusak MA, Peterson JW and Booth SL (2002) HPLC and GC/MS determination of deuterated vitamin K (phylloquinone) in human serum after ingestion of deuterium-labeled broccoli. *Journal of Nutritional Biochemistry* **13** (3), 168-174.

Dowd P, Hershline R, Ham SW and Naganathan S (1995) Vitamin K and energy transduction: a base strength amplification mechanism. *Science* **269** (5231), 1684-1691.

Dueker SR, Lin Y, Buchholz BA, Schneider PD, Lamé MW, Segall HJ, Vogel JS and Clifford AJ (2000) Long-term kinetic study of β-carotene, using accelerator mass spectrometry in an adult volunteer. *Journal of Lipid Research* **41** (11), 1790-1800.

Duggan P, Cashman KD, Flynn A, Bolton-Smith C and Kiely M (2004) Phylloquinone (vitamin K_1) intakes and food sources in 18-64-year-old Irish adults. *British Journal of Nutrition* **92** (1), 151-158.

Dumont JF, Peterson J, Haytowitz D and Booth SL (2003) Phylloquinone and dihydrophylloquinone contents of mixed dishes, processed meats, soups and cheeses. *Journal of Food Composition and Analysis* **16** (5), 595-603.

Edwards AJ, You C-S, Swanson JE and Parker RS (2001) A novel extrinsic reference method for assessing the vitamin A value of plant foods. *American Journal of Clinical Nutrition* **74** (3), 348-355.

Edwards AJ, Nguyen CH, You CS, Swanson JE, Emenhiser C and Parker RS (2002) α- and β-carotene from a commercial carrot puree are more bioavailable to humans than from boiled-mashed carrots, as determined using an extrinsic stable isotope reference method. *Journal of Nutrition* **132** (2), 159-167.

Erkkilä AT, Lichtenstein AH, Dolnikowski GG, Grusak MA, Jalbert SM, Aquino KA, Peterson JW and Booth SL (2004) Plasma transport of vitamin K in men using deuterium-labeled collard greens. *Metabolism* **53** (2), 215-221.

Erkkilä AT, Booth SL, Hu FB, Jacques PF, Manson JE, Rexrode KM, Stampfer MJ and Lichtenstein AH (2005a) Phylloquinone intake as a marker for coronary heart disease risk but not stroke in women. *European Journal of Clinical Nutrition* **59** (2), 196-204.

Erkkilä AT, Lichtenstein AH, Jacques PF, Hu FB, Wilson PW and Booth SL (2005b) Determinants of plasma dihydrophylloquinone in men and women. *British Journal of Nutrition* **93** (5), 701-708.

Erkkilä AT, Booth SL, Hu FB, Jacques PF and Lichtenstein AH (2007) Phylloquinone intake and risk of cardiovascular disease in men. *Nutrition, Metabolism and Cardiovascular Disease* **17** (1), 58-62.

European Union Scientific Committee on Food (2003). Opinion of the Scientific Committee on Food on the Tolerable Upper Intake Level of Vitamin K. Brussels: European Commission.

Fahey MT, Thane CW, Bramwell GD and Coward WA (2007) Conditional Gaussian mixture modelling for dietary pattern analysis. *Journal of the Royal Statistical Society, Series A, Statistics in Society* **170** (2), 1-18.

Fauler G, Leis HJ, Schalamon J, Muntean W and Gleispach H (1996) Method for the determination of vitamin $K_{1(20)}$ in human plasma by stable isotope dilution/gas chromatography/mass spectrometry. *Journal of Mass Spectrometry* **31** (6), 655-660.

Fauler G, Muntean W and Leis HJ (2000). Vitamin K. In *Modern Chromatographic Analysis of the Vitamins,* pp 229-270 [AP Leenher, WE Lambert and JV Bocxlaer, editors]. New York: Marcel Dekker, Inc.

Fell GS and Talwar D (1998) Assessment of status. *Current Opinion in Clinical Nutrition and Metabolic Care* **1** (6), 491-497.

Fenton ST, Price RJ, Bolton-Smith C, Harrington D and Shearer MJ (1997) Nutrient sources of phylloquinone (vitamin K_1) in Scottish men and women. *Proceedings of the Nutrition Society* **56**, 301A.

Fenton ST, Bolton-Smith C, Harrington D and Shearer MJ (2000) Intra- and inter-individual variability and lack of seasonal variation of plasma phylloquinone (vitamin K_1) for Scottish men and women. *Proceedings of the Nutrition Society* **59**, 32A.

Ferland G and Sadowski JA (1992a) Vitamin K_1 (phylloquinone) content of edible oils: effects of heating and light exposure. *Journal of Agricultural and Food Chemistry* **40** (10), 1869-1873.

Ferland G and Sadowski JA (1992b) Vitamin K_1 (phylloquinone) content of green vegetables: effects of plant maturation and geographical location. *Journal of Agricultural and Food Chemistry* **40** (10), 1874-1877.

Ferland G, Sadowski JA and O'Brien ME (1993) Dietary induced subclinical vitamin K deficiency in normal human subjects. *Journal of Clinical Investigation* **91** (4), 1761-1768.

Feskanich D, Weber P, Willett WC, Rockett H, Booth SL and Colditz GA (1999) Vitamin K intake and hip fractures in women: a prospective study. *American Journal of Clinical Nutrition* **69** (1), 74-79.

Fielding B, Callow J, Owen R, Samra J, Matthews D and Frayn K (1996) Postprandial lipemia: the origin of an early peak studied by specific dietary fatty acid intake during sequential meals. *American Journal of Clinical Nutrition* **63** (1), 36-41.

Food Standards Agency (2002). *McCance and Widdowson's The Composition of Foods*, 6th Summary Edition. Cambridge: Royal Society of Chemistry.

Fox TE, Fairweather-Tait SJ, Eagles J and Wharf SG (1991) Intrinsic labelling of different foods with stable isotope of zinc (^{67}Zn) for use in bioavailability studies. *British Journal of Nutrition* **66** (1), 57-63.

Frick PG, Riedler G and Brogli H (1967) Dose response and minimal daily requirement for vitamin K in man. *Journal of Applied Physiology* **23** (3), 387-389.

Furie B, Bouchard BA and Furie BC (1999) Vitamin K-dependent biosynthesis of γ-carboxyglutamic acid. *Blood* **93** (6), 1798-1808.

Garber AK, Binkley NC, Krueger DC and Suttie JW (1999) Comparison of phylloquinone bioavailability from food sources or a supplement in human subjects. *Journal of Nutrition* **129** (6), 1201-1203.

Geleijnse JM, Vermeer C, Grobbee DE, Schurgers LJ, Knapen MHJ, van der Meer IM, Hofman A and Witteman JCM (2004) Dietary intake of menaquinone is associated with a reduced risk of coronary heart disease: The Rotterdam Study. *Journal of Nutrition* **134** (11), 3100-3105.

Gijsbers BL, Jie KS and Vermeer C (1996) Effect of food composition on vitamin K absorption in human volunteers. *British Journal of Nutrition* **76** (2), 223-229.

Gleispach H, Jorg Leis H, Windischhofer W and Muntean W (1993) A contribution to the measurement of Vitamin $K_{1(20)}$ by mass spectrometry. *Journal of High Resolution Chromatography* **16** (12), 738-740.

Green PJ and Silverman BW (1994) *Monographs on Statistics and Applied Probability (58) Nonparametric Regression and Generalized Linear Models - A roughness penalty approach.* London: Chapman and Hall/CRC.

Gregory III JF and Quinlivan EP (2002) In vivo kinetics of folate metabolism. *Annual Review of Nutrition* **22**, 199-220.

Haddock BA and Jones CW (1977) Bacterial respiration. *Bacteriological Reviews* **41** (1), 47-99.

Hallgren KW, Hommema EL, McNally BA and Berkner KL (2002) Carboxylase overexpression effects full carboxylation but poor release and secretion of factor IX: implications for the release of vitamin K-dependent proteins. *Biochemistry* **41** (50), 15045-15055.

Harrington DJ, Soper R, Edwards C, Savidge GF, Hodges SJ and Shearer MJ (2005) Determination of the urinary aglycone metabolites of vitamin K by HPLC with redox-mode electrochemical detection. *Journal of Lipid Research* **46** (5), 1053-1060.

Harrington DJ, Booth SL, Card DJ and Shearer MJ (2007) Excretion of the urinary 5C- and 7C-aglycone metabolites of vitamin K by young adults responds to changes in dietary phylloquinone and dihydrophylloquinone intakes. *Journal of Nutrition* **137** (7), 1763-1768.

Hart JP, Shearer MJ, Klenerman L, Catterall A, Reeve J, Sambrook PN, Dodds RA, Bitensky L and Chayen J (1985) Electrochemical detection of depressed circulating levels of vitamin K_1 in osteoporosis. *Journal of Clinical Endocrinology and Metabolism* **60** (6), 1268-1269.

Harvey LJ, Dainty JR, Beattie JH, Majsak-Newman G, Wharf SG, Reid MD and Fairweather-Tait SJ (2005) Copper absorption from foods labelled intrinsically and extrinsically with Cu-65 stable isotope. *European Journal of Clinical Nutrition* **59** (3), 363-368.

Heer M (2002) Nutritional interventions related to bone turnover in European space missions and simulation models. *Nutrition* **18** (10), 853-856.

Hendriks HFJ, Brink EJ, Meijer GW, Princen HMG and Ntanios FY (2003) Safety of long-term consumption of plant sterol esters-enriched spread. *European Journal of Clinical Nutrition* **57** (5), 681-692.

Hey E (2003) Vitamin K - what, why, and when. *Archives of Disease in Childhood: Fetal and Neonatal Edition* **88** (2), F80-F83.

Hoang QQ, Sicheri F, Howard AJ and Yang DSC (2003) Bone recognition mechanism of porcine osteocalcin from crystal structure. *Nature* **475** (6961), 977-980.

Hodges SJ, Pilkington MJ, Stamp TC, Catterall A, Shearer MJ, Bitensky L and Chayen J (1991) Depressed levels of circulating menaquinones in patients with osteoporotic fractures of the spine and femoral neck. *Bone* 12 (6), 387-389.

Hodges SJ, Bejui J, Leclercq M and Delmas PD (1993) Detection and measurement of vitamins K_1 and K_2 in human cortical and trabecular bone. *Journal of Bone and Mineral Research* **8** (8), 1005-1008.

Holland B, Unwin ID and Buss DH (1988). *Cereals and Cereal Products: Third Supplement to McCance and Widdowson's The Composition of Foods*, Nottingham: Royal Society of Chemistry Ministry of Agriculture, Fisheries and Food.

Holland B, Unwin ID and Buss DH (1989). *Milk and Milk Products: Fourth Supplement to McCance and Widdowson's The Composition of Foods*, Cambridge: Royal Society of Chemistry Ministry of Agriculture, Fisheries and Food.

Hollander D (1973) Vitamin K_1 absorption by everted intestinal sacs of the rat. *American Journal of Physiology* **225** (2), 360-364.

Hollander D and Rim E (1976) Factors affecting the absorption of vitamin K-1 *in vitro*. *Gut* **17** (6), 450-455.

Hollander D, Rim E and Muralidhara KS (1977) Vitamin K_1 intestinal absorption *in vivo*: influence of luminal contents on transport. *American Journal of Physiology: Endocrinology and Metabolism* **232** (1), E69-E74.

Hoppe PP and Krennich G (2000) Bioavailability and potency of natural-source and *all-racemic* α-tocopherol in the human: a dispute. *European Journal of Nutrition* **39** (5), 183-193.

Huang M, Rigby AC, Morelli X, Grant MA, Huang G, Furie B, Seaton B and Furie BC (2003) Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins. *Nature Structural Biology* **10** (9), 751-756.

Hunt JR (1983) Mechanisms and disorders of gastric emptying. *Annual Review of Medicine* **34**, 219-229.

Hunt JR (1996) Bioavailability algorithms in setting Recommended Dietary Allowances: lessons from iron, applications to zinc. *Journal of Nutrition* **126** (9 Suppl), 2345S-2353S.

Hussain MM, Mahley RW, Boyles JK, Lindquist PA, Brecht WJ and Innerarity TL (1989) Chylomicron metabolism. Chylomicron uptake by bone marrow in different animal species. *Journal of Biological Chemistry* **264** (30), 17931-17938.

Indyk HE, Shearer MJ and Woollard DC (2003). Vitamin K - properties and determination. In *Encyclopedia of Food Sciences and Nutrition,* pp 6032-6038 [B Caballero, L Trugo and P Finglas, editors]. Academic Press.

Institute of Medicine (IOM) Food and Nutrition Board (FNB) (2001). *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*, Washington, D.C.: National Academy Press.

Iuliano L, Micheletta F, Maranghi M, Frati G, Diczfalusy U and Violi F (2001) Bioavailability of vitamin E as function of food intake in healthy subjects: effects on plasma peroxide-scavenging activity and cholesterol-oxidation products. *Arteriosclerosis Thrombosis and Vascular Biology* **21** (10), E34-E37.

Jackson KG, Zampelas A, Knapper JM, Culverwell CC, Wright J, Gould BJ and Williams CM (1999) Lack of influence of test meal fatty acid composition on the contribution of intestinally-derived lipoproteins to postprandial lipaemia. *British Journal of Nutrition* **81** (1), 51-57.

Jackson MJ (1997) The assessment of bioavailability of micronutrients: introduction. *European Journal of Clinical Nutrition* **51** (suppl), S1-2.

Jeanes YM, Hall WL, Ellard S, Lee E and Lodge JK (2004) The absorption of vitamin E is influenced by the amount of fat in a meal and the food matrix. *British Journal of Nutrition* **92** (4), 575-579.

Jie KG, Bots ML, Vermeer C, Witteman JCM and Grobbee DE (1995) Vitamin K intake and osteocalcin levels in women with and without aortic atherosclerosis: a population-based study. *Athersclerosis* **116** (1), 117-123.

Jie KG, Bots ML, Vermeer C, Witteman JC and Grobbee DE (1996) Vitamin K status and bone mass in women with and without aortic atherosclerosis: a population-based study. *Calcified Tissue International* **59** (5), 352-356.

Jones KS, Bluck LJC and Coward WA (2006) Analysis of isotope ratios in vitamin K_1 (phylloquinone) from human plasma by gas chromatography mass spectrometry. *Rapid Communications in Mass Spectrometry* **20** (12), 1894-1898.

Kamali F, Edwards C, Wood P, Wynne HA and Kesteven P (2001) Temporal variations in plasma vitamin K and lipid concentrations and clotting factor activity in humans. *American Journal of Hematology* **68** (3), 159-163.

Khan T, Wynne H, Wood P, Torrance A, Hankey C, Avery P, Kesteven P and Kamali F (2004) Dietary vitamin K influences intra-individual variability in anticoagulant response to warfarin. *British Journal of Haematology* **124** (3), 348-354.

Knapen MHJ, Schurgers LJ and Vermeer C (2007) Vitamin K_2 supplementation improves hip bone geometry and bone strength indices in postmenopausal women. *Osteoporosis International.* doi10.1007/s00198-007-0337-9

Knauer TE, Siegfried C, Willingham AK and Matschiner JT (1975) Metabolism and biological activity of *cis*- and *trans*-phylloquinone in the rat. *Journal of Nutrition* **105** (12), 1519-1524.

Kohlmeier M, Saupe J and Shearer MJ (1995a) Risk of bone fracture in hemodialysis patients is related to vitamin K status. *Journal of Bone and Mineral Research* **10**, S361 (Abstract).

Kohlmeier M, Saupe J, Drossel H-J and Shearer MJ (1995b) Variation of phylloquinone (vitamin K_1) concentrations in hemodialysis patients. *Thrombosis and Haemostasis* **74** (5), 1252-1254.

Kohlmeier M, Salomon A, Saupe J and Shearer MJ (1996) Transport of vitamin K to bone in humans. *Journal of Nutrition* **126** (4), 1192S-1196S.

Koivu-Tikkanen T (2001). Determination of phylloquinone and menaquinones in foods by HPLC. Department of Applied Chemistry and Microbiology. Helsinki, University of Helsinki. (Thesis)

Koivu-Tikkanen TJ, Ollilainen V and Piironen V (2000a) Determination of phylloquinone and menaquinones in animal products with fluoresence detection after postcolumn reduction. *Journal of Agricultural and Food Chemistry* **48** (12), 6325-6331.

Koivu-Tikkanen TJ, Schurgers LJ, Thijssen HH and Vermeer C (2000b) Intestinal, hepatic, and circulating vitamin K levels at low and high intakes of vitamin K in rats. *British Journal of Nutrition* **83** (2), 185-190.

Koonsvitsky BP, Berry DA, Jones MB, Lin PYT, Cooper DA, Jones DY and Jackson JE (1997) Olestra affects serum concentrations of α-tocopherol and carotenoids but not vitamin D or vitamin K status in free-living subjects. *Journal of Nutrition* **127** (8), 1636S-1645S.

Kulman JD, Harris JE, Haldeman BA and Davie EW (1997) Primary structure and tissue distribution of two novel proline-rich γ-carboxyglutamic acid proteins. *Proceedings of the National Academy of Sciences* **94** (17), 9058-9062.

Kulman JD, Harris JE, Xie L and Davie EW (2001) Identification of two novel transmembrane γ-carboxyglutamic acid proteins expressed broadly in fetal and adult tissues. *Proceedings of the National Academy of Sciences* **98** (4), 1370-1375.

Kurilich AC, Britz SJ, Clevidence BA and Novotny JA (2003) Isotopic labeling and LC-APCI-MS quantification for investigating absorption of carotenoids and phylloquinone from kale (*Brassica oleracea*). *Journal of Agricultural and Food Chemistry* **51** (17), 4877-4883.

Kurnik D, Lubetsky A, Loebstein R, Almog S and Halkin H (2003) Multivitamin supplements may affect warfarin anticoagulation in susceptible patients. *Annals of Pharmacotherapy* **37** (11), 1603-1606.

Lamon-Fava S, Sadowski JA, Davidson KW, O'Brien ME, McNamara JR and Schaefer EJ (1998) Plasma lipoproteins as carriers of phylloquinone (vitamin K_1) in humans. *American Journal of Clinical Nutrition* **67** (6), 1226-1231.

Lamson DW and Plaza SM (2003) The anticancer effects of vitamin K. *Alternative Medicine Review* **8** (3), 303-318.

Langemann A and Isler O (1965). Chemistry of Isoprenoid Quinones. In *Biochemistry of Quinones,* pp 90-141 [RA Morton, editor]. London: Academic Press.

Leonard SW, Good CK, Gugger ET and Traber MG (2004) Vitamin E bioavailability from fortified breakfast cereal is greater than that from encapsulated supplements. *American Journal of Clinical Nutrition* **79** (1), 86-92.

Li T, Chang CY, Jin DY, Lin PJ, Khvorova A and Stafford DW (2004) Identification of the gene for vitamin K epoxide reductase. *Nature* **427** (6974), 541-544.

Lochner K, Döring O and Böttger M (2003) Phylloquinone, what can we learn from plants? *Biofactors* **18** (1-4), 73-78.

Lodge JK, Hall WL, Jeanes YM and Proteggente AR (2004) Physiological factors influencing vitamin E biokinetics. *Annals of New York Academy of Sciences* **1031**, 60-73.

Lodge JK (2005) Vitamin E bioavailability in humans. *Journal of Plant Physiology* **162** (7), 790-796.

Low AG (1990) Nutritional regulation of gastric secretion, digestion and emptying. *Nutrition Research Reviews* **3** (1), 229-252.

Lowenthal J and Vergel Rivera G (1979) Comparison of the activity of the *cis* and *trans* isomer of vitamin K_1 in vitamin K-deficient and coumarin anticoagulant-pretreated rats. *Journal of Pharmacology and Experimental Therapeutics* **209** (3), 330-333.

Maillard C, Berruyer M, Serre CM, Dechavanne M and Delmas PD (1992) Protein-S, a vitamin K-dependent protein, is a bone matrix component synthesized and secreted by osteoblasts. *Endocrinology* **130** (3), 1599-1604.

Maillot F, Garrigue MA, Pinault M, Objois M, Théret V, Lamisse F, Hoinard C, Antoine JM, Lairon D and Couet C (2005) Changes in plasma triacylglycerol concentrations after sequential lunch and dinner in healthy subjects. *Diabetes and Metabolism* **31** (1), 69-77.

Mann J and Truswell AS (1998). *Essentials of Human Nutrition*, Oxford, UK: Oxford University Press.

Matthews DE and Bier DM (1983) Stable isotope methods for nutritional investigation. *Annual Review of Nutrition* **3**, 309-339.

McDuffie JR, Calis KA, Booth SL, Uwaifo GI and Yanovski JA (2002) Effects of orlistat on fat-soluble vitamins in obese adolescents. *Pharmacotherapy* **22** (7), 814-822.

McKeown NM, Rasmussen HM, Charnley JM, Wood RJ and Booth SL (2000) Accuracy of phylloquinone (vitamin K-1) data in 2 nutrient databases as determined by direct laboratory analysis of diets. *Journal of the American Dietetic Association* **100** (10), 1201-1204.

McKeown NM, Jacques PF, Gundberg CM, Peterson JW, Tucker KL, Kiel DP, Wilson PW and Booth SL (2002) Dietary and nondietary determinants of vitamin K biochemical measures in men and women. *Journal of Nutrition* **132** (6), 1329-1334.

Melia AT, Koss-Twardy SG and Zhi J (1996) The effect of orlistat, an inhibitor of dietary fat absorption, on the absorption of vitamins A and E in healthy volunteers. *Journal of Clinical Pharmacology* **36** (7), 647-653.

Mitchell GV, Cook KK, Jenkins MY and Grundel E (2001) Supplementation of rats with a lutein mixture preserved with vitamin E reduces tissue phylloquinone and menaquinone-4. *International Journal of Vitamin and Nutrition Research* **71** (1), 30-35.

Mitchell HH, Hamilton TS, Steggerda FR and Bean HW (1945) The chemical composition of the adult human body and its bearing on the biochemistry of growth. *Journal of Biological Chemistry* **158**, 625-637.

Murphy RC, Fiedler J and Hevko J (2001) Analysis of nonvolatile lipids by mass spectrometry. *Chemical Reviews* **101** (2), 479-526.

Naska A, Vasdekis VG, Trichopoulou A, Friel S, Leonhäuser IU, Moreiras O, Nelson M, Remaut AM, Schmitt A, Sekula W, Trygg KU and Zajkás G (2000) Fruit and vegetable availability among ten European countries: how does it compare with the 'five-a-day' recommendation? DAFNE I and II projects of the European Commission. *British Journal of Nutrition* **84** (4), 549-556.

Neogi T, Booth SL, Zhang YQ, Jacques PF, Terkeltaub R, Aliabadi P and Felson DT (2006) Low vitamin K status is associated with osteoarthritis in the hand and knee. *Arthritis and Rheumatism* **54** (4), 1255-1261.

Neuhouser ML, Rock CL, Kristal AR, Patterson RE, Neumark-Sztainer D, Cheskin LJ and Thornquist MD (2006) Olestra is associated with slight reductions in serum carotenoids but does not markedly influence serum fat-soluble vitamin concentrations. *American Journal of Clinical Nutrition* **83** (3), 624-631.

Niemeier A, Kassem M, Toedter K, Wendt D, Ruether W, Beisiegel U and Heeren J (2005) Expression of LRP1 by human osteoblasts: a mechanism for the delivery of lipoproteins and vitamin K_1 to bone. *Journal of Bone and Mineral Research* **20** (2), 283-293.

Nieuwenhuys CM, Béguin S, Offermans RF, Emeis JJ, Hornstra G and Heemskerk JW (1998) Hypocoagulant and lipid-lowering effects of dietary n-3 polyunsaturated fatty acids with unchanged platelet activation in rats. *Arteriosclerosis Thrombosis and Vascular Biology* **18** (9), 1480-1489.

Office for National Statistics (2004). Focus on older people. London, HMSO.

Oldenburg J, von Brederlow B, Fregin A, Rost S, Wolz W, Eberl W, Eber S, Lenz E, Schwaab R, Brackmann HH, Effenberger W, Harbrecht U, Schurgers LJ, Vermeer C and Muller CR (2000) Congenital deficiency of vitamin K dependent coagulation factors in two families presents as a genetic defect of the vitamin K-epoxide-reductase-complex. *Thrombosis and Haemostasis* **84** (6), 937-941.

Oldenburg J and Schwabb R (2001) Molecular biology of blood coagulation. *Seminars in Thrombosis and Hemostasis* **27** (4), 313-324.

Olson RE (1985) The function and metabolism of vitamin K. *Annual Review of Nutrition* **4**, 281-337.

Olson RE (1999). Vitamin K. In *Modern Nutrition in Health and Disease,* pp 363-379 [ME Shils, JA Olson, M Shike and AC Ross, editors]. New York: Lippincott Williams & Wilkins.

Olson RE, Chao J, Graham D, Bates MW and Lewis JH (2002) Total body phylloquinone and its turnover in human subjects at two levels of vitamin K intake. *British Journal of Nutrition* **87** (6), 543-553.

O'Meara S, Riemsma R, Shirran L, Mather L and ter Riet G (2001) A rapid and systematic review of the clinical effectiveness and cost-effectiveness of orlistat in management of obesity. *Health Technology Assessment* **5** (18), 1-81.

Ortega H, Castilla P, Gómez-Coronado D, Garcés C, Benavente M, Rodríguez-Artalejo F, de Oya M and Lasunción MA (2005) Influence of apolipoprotein E genotype on fat-soluble plasma antioxidants in Spanish children. *American Journal of Clinical Nutrition* **81** (3), 624-632.

Ostlund RE (2002) Phytosterols in human nutrition. *Annual Review of Nutrition* **22**, 533-549.

Parker RS, Swanson JE, You CS, Edwards AJ and Huang T (1999) Bioavailability of carotenoids in human subjects. *Proceedings of the Nutrition Society* **58** (1), 155-162.

Paul AA and Southgate DAT (1978). *McCance and Widdownson's The Composition of Foods*, 4th edition. London: HMSO.

Pereira SP, Rowbotham D, Fitt S, Shearer MJ, Wendon J and Williams R (2005) Pharmacokinetics and efficacy of oral versus intravenous mixed-micellar phylloquinone (vitamin K_1) in severe acute liver disease. *Journal of Hepatology* **42** (3), 365-370.

Peterson JW, Muzzey KL, Haytowitz D, Exler J, Lemar L and Booth SL (2002) Phylloquinone (vitamin K_1) and dihydrophylloquinone content of fats and oils. *Journal of the American Oil Chemists' Society* **79** (7), 641-646.

Petrosian AM and Haroutounian JE (2000) Taurine as a universal carrier of lipid soluble vitamins: a hypothesis. *Amino Acids* **19** (2), 409-421.

Piironen V and Koivu T (2000) Quality of vitamin K analysis and food composition data in Finland. *Food Chemistry* **68** (2), 223-226.

Pilkey PM, Morton AR, Boffa MB, Noordhof C, Day AG, Su Y, Miller LM, Koschinsky ML and Booth SL (2007) Subclinical vitamin K deficiency in hemodialysis patients. *American Journal of Kidney Diseases* **49** (3), 432-439.

Price PA and Williamson MK (1985) Primary structure of bovine matrix Gla protein, a new vitamin K-dependent bone protein. *Journal of Biological Chemistry* **260** (28), 14971-14975.

Pudota BN, Miyagi M, Hallgren KW, West KA, Crabb JW, Misono KS and Berkner KL (2000) Identification of the vitamin K-dependent carboxylase active site: Cys-99 and Cys-450 are required for both epoxidation and carboxylation. *Proceedings of the National Academy of Sciences* **97** (24), 13033-13038.

Raeini-Sarjaz M, Ntanios FY, Vanstone CA and Jones PJH (2002) No changes in serum fat-soluble vitamin and carotenoid concentrations with the intake of plant sterols/stanol esters in the context of a controlled diet. *Metabolism* **51** (5), 652-656.

Raith W, Fauler G, Pichler G and Muntean W (2000) Plasma concentrations after intravenous administration of phylloquinone (vitamin K_1) in preterm and sick neonates. *Thrombosis Research* **99** (5), 467-472.

Rawlinson A, Walsh TF, Lee A and Hodges SJ (1998) Phylloquinone in gingival crevicular fluid in adult periodontitis. *Journal of Clinical Periodontology* **25** (8), 662-665.

Redgrave TG (2004) Chylomicron metabolism. *Biochemical Society Transactions* **32** (1), 79-82.

Rejnmark L, Vestergaard P, Charles P, Hermann AP, Brot C, Eiken P and Mosekilde L (2006) No effect of vitamin K_1 intake on bone mineral density and fracture risk in perimenopausal women. *Osteoporosis International* **17** (8), 1122-1132.

Richelle M, Enslen M, Hager C, Groux M, Travazzi I, Godin J-P, Berger A, Métairon S, Quaile S, Piguet-Welsch C, Sagalowicz L, Green H and Fay LB (2004) Both free and esterified plant sterols reduce cholesterol absorption and the bioavailability of β-carotene and α-tocopherol in normocholesterolemic humans. *American Journal of Clinical Nutrition* **80** (1), 171-177.

Rishavy MA, Pudota BN, Hallgren KW, Qian W, Yakubenko AV, Song J-H, Runge KW and Berkner KL (2004) A new model for vitamin K-dependent carboxylation: The catalytic base that deprotonates vitamin K hydroquinone is not Cys but an activated amine. *Proceedings of the National Academy of Sciences* **101** (38), 13732-13737.

Rock CL, Thornquist MD, Kristal AR, Patterson RE, Cooper DA, Neuhouser ML, Neumark-Sztainer D and Cheskin LJ (1999) Demographic, dietary and lifestyle factors differentially explain variability in serum carotenoids and fat-soluble vitamins: baseline results from the sentinel site of the Olestra Post-Marketing Surveillance Study. *Journal of Nutrition* **129** (4), 855-864.

Ronden JE, Drittij-Reijnders MJ, Vermeer C and Thijssen HHW (1998) Intestinal flora is not an intermediate in the phylloquinon-menaquinone-4 conversion in the rat. *Biochimica et Biophysica Acta* **1379** (1), 69-75.

Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hörtnagel K, Pelz HJ, Lappegard K, Seifried E, Scharrer I, Tuddenham EG, Müller CR, Strom TM and Oldenburg J (2004a) Mutations in *VKORC1* cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* **427** (6974), 537-541.

Rost S, Fregin A, Koch D, Compes M, Müller CR and Oldenburg J (2004b) Compound heterozygous mutations in the γ-glutamyl carboxylase gene cause combined deficiency of all vitamin K-dependent blood coagulation factors. *British Journal of Haematology* **126** (4), 546-549.

Roxborough HE, Burton GW and Kelly FJ (2000) Inter- and intra-individual variation in plasma and red blood cell vitamin E after supplementation. *Free Radical Research* **33** (4), 437-445.

Rustandi RR, Snyder SW, Feezel LL, Michalski TJ, Norris JR, Thurnauer MC and Biggins J (1990) Contribution of vitamin K_1 to the electron spin polarization in spinach photosystem I. *Biochemistry* **29** (35), 8030-8032.

Sadowski JA, Hood SJ, Dallal GE and Garry PJ (1989) Phylloquinone in plasma from elderly and young adults: factors influencing its concentration. *American Journal of Clinical Nutrition* **50** (1), 100-108.

Sakamoto N, Kimura M, Hiraike H and Itokawa Y (1996) Changes of phylloquinone and menaquinone-4 concentrations in rat liver after oral, intravenous and intraperitoneal administration. *International Journal of Vitamin and Nutrition Research* **66** (4), 322-328.

Sato T, Ohtani Y, Yamada Y, Saitoh S and Harada H (2002) Difference in the metabolism of vitamin K between liver and bone in vitamin K-deficient rats. *British Journal of Nutrition* **87** (4), 307-314.

Sato T, Ozaki R, Kamo S, Hara Y, Konishi S, Isobe Y, Saitoh S and Harada H (2003) The biological activity and tissue distribution of 2',3'-dihydrophylloquinone in rats. *Biochimica et Biophysica Acta* **1622** (3), 145-150.

Saupe J, Shearer MJ and Kohlmeier M (1993) Phylloquinone transport and its influence on γ-carboxyglutamate residues of osteocalcin in patients on maintenance hemodialysis. *American Journal of Clinical Nutrition* **58** (2), 204-208.

Schaafsma A, Muskiet FA, Storm H, Hofstede GJ, Pakan I and Van der Veer E (2000) Vitamin D_3 and vitamin K_1 supplementation of Dutch postmenopausal women with normal and low bone mineral densities: effects on serum 25-hydroxyvitamin D and carboxylated osteocalcin. *European Journal of Clinical Nutrition* **54** (8), 626-631.

Schlagheck TG, Riccardi KA, Zorich NL, Torri SA, Dugan LD and Peters JC (1997) Olestra dose response on fat-soluble and water-soluble nutrients in humans. *Journal of Nutrition* **127** (8), 1646S-1665S.

Schneeman B (2004) Food factors and gastrointestinal function: a critical interface. *Biofactors* **21** (1-4), 85-88.

Schurgers LJ, Geleijnse JM, Grobbee DE, Pols HAP, Hofman A, Witteman JCM and Vermeer C (1999) Nutritional Intake of Vitamins K_1 (Phylloquinone) and K_2 (Menaquinone) in The Netherlands. *Journal of Nutritional and Environmental Medicine* **9** (2), 115-122.

Schurgers LJ and Vermeer C (2000) Determination of phylloquinone and menaquinones in food. Effect of food matrix on circulating vitamin K concentrations. *Haemostasis* **30** (6), 298-307.

Schurgers LJ and Vermeer C (2001) Corn oil-induced decrease in arterial thrombosis tendency may be related to altered plasma vitamin K transport. *Journal of Lipid Research* **42** (7), 1120-1124.

Schurgers LJ, Shearer MJ, Soute BA, Elmadfa I, Harvey J, Wagner KH, Tomasch R and Vermeer C (2002) Novel effects of diets enriched with corn oil or with an olive oil/sunflower oil mixture on vitamin K metabolism and vitamin K-dependent proteins in young men. *Journal of Lipid Research* **43** (6), 878-884.

Schurgers LJ and Vermeer C (2002) Differential lipoprotein transport pathways of K-vitamins in healthy subjects. *Biochimica et Biophysica Acta* **1570** (1), 27-32.

Schurgers LJ, Shearer MJ, Hamulyák K, Stöcklin E and Vermeer C (2004) Effect of vitamin K intake on the stability of oral anticoagulant treatment: dose-response relationships in healthy subjects. *Blood* **104** (9), 2682-2689.

Scientific Advisory Committee on Nutrition (SACN) (2006). *Folate and Disease Prevention*, London: The Stationary Office.

Shearer MJ, Barkhan P and Webster GR (1970a) Absorption and excretion of an oral dose of tritiated vitamin K_1 in man. *British Journal of Haematology* 18 (3), 297-308.

Shearer MJ, Mallinson CN, Webster GR and Barkhan P (1970b) Absorption of tritiated vitamin K_1 in patients with fat malabsorption. *Gut* **11** (12), 1063-1064.

Shearer MJ, Mallinson CN, Webster GR and Barkhan P (1972) Clearance from plasma and excretion in urine, faeces and bile of an intravenous dose of tritiated vitamin K_1 in man. *British Journal of Haematology* **22** (5), 579-588.

Shearer MJ and Barkhan P (1973) Studies on the metabolites of phylloquinone (vitamin K_1) in the urine of man. *Biochimica et Biophysica Acta* **297** (2), 300-312.

Shearer MJ, McBurney A and Barkhan P (1974) Studies on the absorption and metabolism of phylloquinone (vitamin K_1) in man. *Vitamins and Hormones* **32**, 513-542.

Shearer MJ and Barkhan P (1979) Vitamin K_1 and therapy of massive warfarin overdose. *Lancet* **1** (8110), 266-267.

Shearer MJ, McCarthy PT and Crampton OE (1988). The assessment of human vitamin K status from tissue measurements. In *Current Advances in Vitamin K Research,* pp 437-452 [JW Suttie, editor]. New York: Elsevier.

Shearer MJ, Bach A and Kohlmeier M (1996) Chemistry, nutritional sources, tissue distribution and metabolism of vitamin K with special reference to bone health. *Journal of Nutrition* **126** (4), 1181S-1186S.

Shearer MJ (1997) The roles of vitamins D and K in bone health and osteoporosis prevention. *Proceedings of the Nutrition Society* **56** (3), 915-937.

Shearer MJ and Bolton-Smith C (2000) The UK food data-base for vitamin K and why we need it. *Food Chemistry* **68** (2), 213-218.

Shepherd AMM, Wilson NM and Stevenson IH (1977) Vitamin K pharmacokinetics: response in young and elderly patients. *Clinical Pharmacology and Therapeutics* **21** (1), 117.

Shipley RA and Clark RE (1972). *Tracer methods for in vivo kinetics - theory and applications*, New York: Academic Press.

Shiraki M, Shiraki Y, Aoki C and Miura M (2000) Vitamin K_2 (menatetrenone) effectively prevents fractures and sustains lumbar bone mineral density in osteoporosis. *Journal of Bone and Mineral Research* **15** (3), 515-521.

Snyder SW, Rustandi RR, Biggins J, Norris JR and Thurnauer MC (1991) Direct assignment of vitamin K_1 as the secondary acceptor A_1 in photosystem I. *Proceedings of the National Academy of Sciences* **88** (21), 9895-9896.

Soedirman JR, De Bruijn EA, Maes RA, Hanck A and Grüter J (1996) Pharmacokinetics and tolerance of intravenous and intramuscular phylloquinone (vitamin K_1) mixed micelles formulation. *British Journal of Clinical Pharmacology* **41** (6), 517-523.

Sokoll LJ and Sadowski JA (1996) Comparison of biochemical indexes for assessing vitamin K nutritional status in a healthy adult population. *American Journal of Clinical Nutrition* **63** (4), 566-573.

Sokoll LJ, Booth SL, O'Brien ME, Davidson KW, Tsaioun KI and Sadowski JA (1997) Changes in serum osteocalcin, plasma phylloquinone, and urinary γ-carboxyglutamic acid in response to altered intakes of dietary phylloquinone in human subjects. *American Journal of Clinical Nutrition* **65** (3), 779-784.

Sommerburg O, Zang L-Y and van Kuijk FJGM (1997) Simultaneous detection of carotenoids and vitamin E in human plasma. *Journal of Chromatography B: Biomedical Sciences and Applications* **695** (2), 209-215.

Spronk HM, Farah RA, Buchanan GR, Vermeer C and Soute BA (2000) Novel mutation in the γ-glutamyl carboxylase gene resulting in congenital combined deficiency of all vitamin K-dependent blood coagulation factors. *Blood* **96** (10), 3650-3652.

Stanley TB, Jin D-Y, Lin P-J and Stafford DW (1999) The propeptides of the vitamin K-dependent proteins possess different affinities for the vitamin K-dependent carboxylase. *Journal of Biological Chemistry* **274** (24), 16940-16944.

Stellaard F (2005) Use of dual isotope tracers in biomedical research. *Isotopes in Environmental and Health Studies* **41** (3), 275-286.

Stenina O, Pudota BN, McNally BA, Hommema EL and Berkner KL (2001) Tethered processivity of the vitamin K-dependent carboxylase: factor IX is efficiently modified in a mechanism which distinguishes Gla's from Glu's and which accounts for comprehensive carboxylation in vivo. *Biochemistry* **40** (34), 10301-10309.

Suhara Y, Kamao M, Tsugawa N and Okano T (2005) Method for the determination of vitamin K homologues in human plasma using high-performance liquid chromatography-tandem mass spectrometry. *Analytical Chemistry* **77** (3), 757-763.

Suhara Y, Murakami A, Nakagawa K, Mizuquchi Y and Okano T (2006) Comparative uptake, metabolism, and utilization of menaquinone-4 and phylloquinone in human cultered cell lines. *Bioorganic and Medicinal Chemistry* **14** (19), 6601-6607.

Suttie JW (1985) Vitamin K-dependent carboxylase. *Annual Review of Biochemistry* **54**, 459-477.

Suttie JW, Kindberg CG, Greger JL and Bang NU (1988a). Effects of vitamin K (phylloquinone) restriction in the human. In *Current Advances in Vitamin K Research,* pp 465-476 [JW Suttie, editor]. New York: Elsevier.

Suttie JW, Mummah-Schendel LL, Shah DV, Lyle BJ and Greger JL (1988b) Vitamin K deficiency from dietary vitamin K restriction in humans. *American Journal of Clinical Nutrition* **47** (3), 475-480.

Suttie JW (1992) Vitamin K and human nutrition. *Journal of the American Dietetic Association* **92** (5), 585-590.

Suttie JW (1995) The importance of menaquinones in human nutrition. *Annual Review of Nutrition* **15**, 399-417.

Swan G (2004) Findings from the latest National Diet and Nutrition Survey. *Proceedings of the Nutrition Society* **63** (4), 505-512.

Szulc P, Chapuy M-C, Meunier PJ and Delmas PD (1993) Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture in elderly women. *Journal of Clinical Investigation* **91** (4), 1769-1774.

Szulc P, Chapuy M-C, Meunier PJ and Delmas PD (1996) Serum undercarboxylated osteocalcin is a marker of the risk of hip fracture: a three year follow-up study. *Bone* **18** (5), 487-488.

Talwar DK, Azharuddin MK, Williamson C, Teoh YP, McMillan DC and O'Reilly DSJ (2005) Biological variation of vitamins in blood of healthy individuals. *Clinical Chemistry and Laboratory Medicine* **51** (11), 2145-2150.

Tamatani M, Morimoto S, Nakajima M, Fukuo K, Onishi T, Kitano S, Niinobu T and Ogihara T (1998) Decreased circulating levels of vitamin K and 25-hydroxyvitamin D in osteopenic elderly men. *Metabolism* **47** (2), 195-199.

Thane CW, Bates CJ, Shearer MJ, Unadkat N, Harrington DJ, Paul AA, Prentice A and Bolton-Smith C (2002a) Plasma phylloquinone (vitamin K_1) concentration and its relationship to intake in a national sample of British elderly people. *British Journal of Nutrition* **87** (6), 615-622.

Thane CW and Bolton-Smith C (2002) Dietary intake and sources of phylloquinone (vitamin K_1): regional differences in a national sample of British adults. *Proceedings of the Nutrition Society* **61**, 149A.

Thane CW, Paul AA, Bates CJ, Bolton-Smith C, Prentice A and Shearer MJ (2002b) Intake and sources of phylloquinone (vitamin K_1): variation with socio-demographic and lifestyle factors in a national sample of British elderly people. *British Journal of Nutrition* **87** (6), 605-613.

Thane CW and Coward WA (2004) Dietary intake and sources of phylloquinone (vitamin K_1) according to age of British adults from a recent national survey. *Proceedings of the Nutrition Society* **63** (1), 95A.

Thane CW, Bolton-Smith C and Coward WA (2006a) Comparative dietary intake and sources of phylloquinone (vitamin K_1) among British adults in 1986-7 and 2000-1. *British Journal of Nutrition* **96** (12), 1105-1115.

Thane CW, Wang LY and Coward WA (2006b) Plasma phylloquinone (vitamin K_1) concentration and its relationship to intake in British adults aged 19-64 years. *British Journal of Nutrition* 96 (12), 1116-1124.

Thijssen HHW and Drittij-Reijnders MJ (1994) Vitamin K distribution in rat tissues: dietary phylloquinone is a source of tissue menaquinone-4. *British Journal of Nutrition* **72** (3), 415-425.

Thijssen HH and Drittij-Reijnders MJ (1996) Vitamin K status in human tissues: tissue-specific accumulation of phylloquinone and menaquinone-4. *British Journal of Nutrition* **75** (1), 121-127.

Thijssen HH, Drittij-Reijnders MJ and Fischer MA (1996) Phylloquinone and menaquinone-4 distribution in rats: synthesis rather than uptake determines menaquinone-4 organ concentrations. *Journal of Nutrition* **126** (2), 537-543.

Thijssen HH, Drittij MJ, Vermeer C and Schoffelen E (2002) Menaquinone-4 in breast milk is derived from dietary phylloquinone. *British Journal of Nutrition* **87** (3), 219-226.

Thijssen HHW, Vervoort LMT, Schurgers LJ and Shearer MJ (2006) Menadione is a metabolite of oral vitamin K. *British Journal of Nutrition* **95** (2), 260-266.

Thornquist MD, Kristal AR, Patterson RE, Neuhouser ML, Rock CL, Neumark-Sztainer D and Cheskin LJ (2000) Olestra consumption does not predict serum concentrations of carotenoids and fat-soluble vitamins in free-living humans: early results from the sentinel site of the olestra post-marketing surveillance study. *Journal of Nutrition* **130** (7), 1711-1718.

Tie JK, Jin D-Y, Loiselle DR, Pope RM, Straight DL and Stafford DW (2004) Chemical modification of cysteine residues is a misleading indicator of their status as active site residues in the vitamin K-dependent γ-glutamyl carboxylation reaction. *Journal of Biological Chemistry* **279** (52), 54079-54087.

Tovar A, Ameho CK, Blumberg JB, Peterson JW, Smith D and Booth SL (2006) Extrahepatic tissue concentrations of vitamin K are lower in rats fed a high vitamin E diet. *Nutrition and Metabolism* **3** (29).

Tsaioun KI (1999) Vitamin K-dependent proteins in the developing and aging nervous system. *Nutrition Reviews* **57** (8), 231-240.

Tsugawa N, Shiraki M, Suhara Y, Kamao M, Tanaka K and Okano T (2006) Vitamin K status of healthy Japanese women: age-related vitamin K requirement for γ-carboxylation of osteocalcin. *American Journal of Clinical Nutrition* **83** (2), 380-386.

Tyssandier V, Reboul E, Dumas J-F, Bouteloup-Demange C, Armand M, Marcand J, Sallas M and Borel P (2003) Processing of vegetable-borne carotenoids in the human stomach and duodenum. *American Journal of Physiology: Gastrointestinal and Liver Physiology* **284** (6), G913-G923.

Uchida K and Komeno T (1988). Relationships between dietary and intestinal vitamin K, clotting factor levels, plasma vitamin K, and urinary Gla. In *Current Advances in Vitamin K Research,* pp 477-492 [JW Suttie, editor]. New York: Elsevier.

Ueno T and Suttie JW (1983) High pressure liquid chromatograph-reductive electrochemical deduction analyses of serum *trans*-phylloquinone. *Analytical Biochemistry* **133** (1), 62-67.

Usui Y, Tanimura H, Nishimura N, Kobayashi N, Okanoue T and Ozawa K (1990) Vitamin K concentrations in the plasma and liver of surgical patients. *American Journal of Clinical Nutrition* 51 (5), 846-852.

van Lieshout M, West CE and van Breemen RB (2003) Isotopic tracer techniques for studying the bioavailability and bioefficacy of dietary carotenoids, particularly β-carotene, in humans: a review. *American Journal of Clinical Nutrition* **77** (1), 12-28.

Vergnaud P, Garnero P, Meunier PJ, Breart G, Kamihagi K and Delmas PD (1997) Undercarboxylated Osteocalcin Measured with a Specific Immunoassay Predicts Hip Fracture in Elderly Women: The EPIDOS Study. *Journal of Clinical Endocrinology and Metabolism* **82** (3), 719-724.

Vermeer C, Jie KS and Knapen MH (1995) Role of vitamin K in bone metabolism. *Annual Review of Nutrition* **15**, 1-22.

Vermeer C, Knapen MH and Schurgers LJ (1998) Vitamin K and metabolic bone disease. *Journal of Clinical Pathology* **51** (6), 424-426.

Vermeer C, Shearer MJ, Zittermann A, Bolton-Smith C, Szulc P, Hodges SJ, Walter P, Rambeck W, Stöcklin E and Weber P (2004) Beyond Deficiency: Potential benefits of increased intakes of vitamin K for bone and vascular health. *European Journal of Nutrition* **43** (6), 325-335.

Villines TC, Hatzigeorgiou C, Feuerstein IM, O'Malley PG and Taylor AJ (2005) Vitamin K_1 intake and coronary calcification. *Coronary Artery Disease* 16 (3), 199-203.

Vuong LT, Buchholz BA, Lamé MW and Dueker SR (2004) Phytochemical research using accelerator mass spectrometry. *Nutrition Reviews* **62** (10), 375-388.

Wang LY, Bates CJ, Yan L, Harrington DJ, Shearer MJ and Prentice A (2004) Determination of phylloquinone (vitamin K_1) in plasma and serum by HPLC with fluorescence detection. *Clinica Chimica Acta* **347** (1-2), 199-207.

Weber P (2001) Vitamin K and bone health. *Nutrition* 17 (10), 880-887.

Welty FK, Lichtenstein AH, Barrett PHR, Dolnikowski GG and Schaefer EJ (2004) Interrelationships Between Human Apolipoprotein A-I and Apolipoproteins B-48 and B-100 Kinetics Using Stable Isotopes. *Arteriosclerosis Thrombosis and Vascular Biology* **24** (9), 1703-1707.

Williams CM (1997) Postprandial lipid metabolism: effects of dietary fatty acids. *Proceedings of the Nutrition Society* **56** (2), 679-692.

Wilson FA, Sallee VL and Dietschy JM (1971) Unstirred water layers in intestine: rate determinant of fatty acid absorption from micellar solutions. *Science* **174** (4013), 1031-1033.

Woollard DC, Indyk HE, Fong BY and Cook KK (2002) Determination of vitamin K_1 isomers in foods by liquid chromatography with C_{30} bonded-phase column. *Journal of the American Oil Chemists' Society* **85** (3), 682-691.

Yan L, O'Neill A, Zhou B, Greenberg DC, Prynne CJ, Bolton-Smith C and Prentice A (2002) A comparison of the estimated vitamin K_1 intake in older people from Cambridge, UK and Shenyang, PR China. *Proceedings of the Nutrition Society* **61**, 149A.

Yan L, Zhou B, Greenberg D, Wang L, Nigdikar S, Prynne C and Prentice A (2004) Vitamin K status of older individuals in northern China is superior to that of older individuals in the UK. *British Journal of Nutrition* **92** (6), 939-945.

Yan L, Zhou B, Nigdikar S, Wang X, Bennett J and Prentice A (2005) Effect of apolipoprotein E genotype on vitamin K status in healthy older adults from China and the UK. *British Journal of Nutrition* **94** (6), 956-961.

Yeum K-J, Booth SL, Sadowski JA, Liu C, Tang G, Krinsky NI and Russell RM (1996) Human plasma carotenoid response to the ingestion of controlled diets high in fruits and vegetables. *American Journal of Clinical Nutrition* **64** (4), 594-602.

Yeum K-J and Russell RM (2002) Carotenoid bioavailability and bioconversion. *Annual Review of Nutrition* **22** (1), 483-504.

You are being invited to take part in research work at HNR. Before you decide it is important for you to understand why the research is being done, and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of this study?
We would like to collect samples of blood, urine and saliva from volunteers in order to develop new and/or improved laboratory methods, for training staff in processing procedures and analytical techniques, and for quality assurance and quality control purposes.

As research questions evolve it is necessary to develop new methods for nutrition related research at HNR. It is important that staff are thoroughly trained in all aspects of sample collection, processing and analysis so that everyone can be confident that the data obtained are correct.

Why have I been chosen?
You have been asked to consider donating a sample or samples because you have expressed an interest in our research in the past, or you are currently taking part in another study and are already having blood samples collected, or you have seen a poster. We need men and women over the age of 18 years and will be recruiting up to 100 volunteers in total.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. However, you will still be free to withdraw at any time and without giving a reason. All samples of human biological material obtained will be treated as gifts. Because the samples will be anonymised, you will have no rights to them if you withdraw from the study.

What will happen to me if I take part?
Depending on ongoing research requirements volunteers will be invited to attend the MRC Human Nutrition Research Volunteer suite on one or more occasions, or will be asked to consider donating a small extra sample whilst already at HNR. One or more of the following bodily samples will be collected.

Blood
Up to 10 ml of blood will be taken from your arm by venepuncture and/or a capillary blood sample (literally a few drops) collected from a fingerprick. The sample may be used immediately or pooled and stored for later analysis.

Urine
A complete spot urine will be collected. Some volunteers may be asked to collect a 24 hr urine sample at home (from about 9 am on one morning until the same time the following day). Samples will be frozen and pooled for subsequent analyses.

Saliva
We might ask some volunteers to collect a saliva sample.

What do I have to do?
For most of the measurements we are interested in, there are no restrictions on time of day or eating or drinking. However, in some cases, fasted early morning samples will be required, so we will ask you to come to HNR before breakfast.

What are the possible benefits of taking part?
We are not planning to measure anything in the samples that is clinically relevant, and in many cases the samples will be combined with those from other volunteers.

There will be no direct benefit to you. However, knowledge gained from using your samples will help answer questions related to nutrition research and public health.

What are the possible disadvantages and risks of taking part?
Venepuncture can cause discomfort during the procedure and there is a small risk of bruising afterwards.

What will happen if anything goes wrong?
Any complaints you have about this study should be made to the Unit Manager at MRC HNR, and will be fully investigated. Participants in research carried out at HNR would, with respect to claims against the MRC, be in the same position as if public liability insurance had been taken out.

Will my taking part in this study be kept confidential?
Any information that is collected about you will be kept strictly confidential. The samples collected will be anonymised (given a code number).

What will happen to the study results?
Information obtained will be used to establish new or improved methods. The results from volunteers' samples will not be used directly in a particular study, although they may provide important and valuable information for quality control and quality assurance purposes.

Will I be reimbursed for my time?
In recognition of your time commitment, you will be paid an honorarium of up to £5. Travel expenses will also be paid.

Who is organising and funding the study?
This study is being organised by the Stable Isotope and Nutrition and Health Sections at MRC HNR. The Medical Research Council is funding the research. Members of MRC Human Nutrition Research will be carrying out the study.

Who has reviewed the study?
This study has been reviewed by the Scientific Co-ordination Committee of MRC HNR and by Cambridge Local Research Ethics Committee.

Contact for further information
If you have any further questions then please contact Sue Bryant at MRC HNR on 01223 426356 or email susan.bryant@mrc-hnr.cam.ac.uk

And finally...
Thank you for having taken the time to read this information sheet and for your interest in our work. If you do decide to take part in the study, you will be given a copy of this information sheet and a signed consent form for you to keep.

MRC Human Nutrition Research

Collection of blood, urine and saliva samples from volunteers for use in laboratory method development

Information Sheet for Study Participants

MRC Human Nutrition Research
Elsie Widdowson Laboratory
Fulbourn Road
Cambridge
CB1 9NL

Telephone: 01223 426356
Fax: 01223 437555
http://www.mrc-hnr.cam.ac.uk

Version 1
December 2004

APPENDIX II

MRC Human Nutrition Research

CONSENT BY VOLUNTEER TO PARTICIPATE IN A NUTRITIONAL STUDY

Collection of blood, urine and saliva samples from volunteers for use in laboratory method development

LREC Reference Number: 05/Q0108/30
Name of Lead Investigator: Mr Kerry Jones

Please initial box

1. I confirm that I have read and understand the information sheet (version 1, Dec04) for the above study and have had the opportunity to ask questions. ☐
2. I understand that samples taken as part of the protocol of this study may be stored and used in subsequent nutritional studies. ☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
4. I understand that future research using the sample I give may include genetic research aimed at understanding the genetic influences on nutrition. Since the samples are anonymised this will not have any implications for me personally. ☐
5. I understand that my donated sample is provided as a gift and as such I have no access to the results of any analysis of the sample. ☐
6. I agree to take part in the above study. ☐

Name of volunteer *(Please print)*	Date	Signature
Name of Witness (Must **not** be member of research team) *(Please print)*	Date	Signature
Name of Research Team member *(Please print)*	Date	Signature

APPENDIX III

MRC Human Nutrition Research

CONSENT BY VOLUNTEER TO PARTICIPATE IN A NUTRITIONAL STUDY

Investigation into the availability and metabolism of vitamin K

LREC Reference Number: 04/001
Name of Lead Investigator: Mr Kerry Jones

	Please initial box
1. I confirm that I have read and understood the information sheet dated Nov 2004, Version 2 for the above study and have had the opportunity to ask questions.	
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3. I am willing that my general practitioner is notified of my participation in this research.	
4. I agree to take part in the above study.	

Name of Research Subject *(Please print)*	Date	Signature
Name of Witness to Signature (Must **not** be member of research team) *(Please print)*	Date	Signature
Name of Research Team member *(Please print)*	Date	Signature

3 copies required: top copy for researcher; one copy for volunteer; one copy to be kept with research subject's notes.

Version 01, December 2003

We would like you to consider participating in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of this study?
The purpose of this study is to help us understand how vitamin K is absorbed and used by the body. Vitamin K is a nutrient that has previously been associated primarily with blood clotting, but more recent evidence suggests a much wider role for this vitamin, particularly in bone health.

Why have I been chosen?
We are approaching healthy young men and women.

What will happen to me if I take part?
If you agree to take part, firstly you will be invited to attend the volunteer suite at HNR to have the study explained in full and to provide you with the opportunity to ask any questions. Your height and weight will be measured and you will be given a supply of capsules containing stable isotope labelled vitamin K to take for 6 days.

The labelled vitamin allows us to tell this vitamin apart from the vitamin already present in the body. The label used in this experiment is a stable isotope called carbon13. This is a naturally occurring form of the vitamin that we can detect using sensitive equipment - it is not a radioactive isotope.

On days five and six after first taking the vitamin K, you will be asked to come to HNR so we can take a small blood sample. On day seven we will ask you to spend a full day at HNR where we will investigate further what happens to vitamin K when it enters the body. You will be asked not to have eaten anything since the previous evening and arrive at HNR by 9am. A cannula (a small plastic tube) will be inserted into each forearm vein by someone experienced in this technique. After the cannula is inserted we are able to take blood samples from you, without causing further discomfort.

Firstly we will take two 10ml blood samples so we can measure what your vitamin K levels are at the start of the day. We will then give you an oral dose of another labelled form of the vitamin – again this is a stable isotope but this time the label is a heavier form of hydrogen. This different label will allow us to tell the two labels and the unlabelled form apart from each other. This labelled form is given so we can measure exactly how much of it is absorbed.

At the same time we will give you an intravenous (*i.v.*) dose of vitamin K that is not labelled. This, in combination with the carbon-labelled version of vitamin K will allow us to determine how much vitamin K is in the body and what happens to it after absorption. Over the next six hours, 14 10ml blood samples will be taken. After three hours and at the end of the study you will be provided with a meal. You are allowed to drink water at anytime.

What do I have to do?
You will be asked to consume three vitamin K capsules per day, one with each meal, for six days. The amount of vitamin K in three capsules equals around 10% of a typical daily intake from food. Before blood samples are taken you will be asked not to have eaten anything since the previous evening.

What are the possible risks of taking part?
The only effect of this procedure will be minor bruising from insertion of the cannula. There is a very small risk of an allergic reaction to the *i.v.* dose of vitamin K, although this is extremely rare. In order to minimise this risk you will not be allowed to take part if you have ever had an allergic reaction, eczema, asthma or hay fever. Additionally, a doctor will administer the dose. In the unlikely event of an allergic reaction procedures will be in place to deal with this quickly and safely.

What are the possible benefits of taking part?
Participating in this study would not benefit you directly, but should help with the understanding of how vitamin K and other nutrients are taken up by the body.

What happens at the end of the study?
The blood samples will be analysed to determine how much of the labelled vitamin K dose is present in the blood. This will help us establish how much of the dose of was absorbed by the body and what happened to it once it was absorbed.

Confidentiality – who will have access to the data?
The information we gain will be used for research purposes only and will be treated as confidential. Blood samples will be stored in coded vials. Any personal data will be kept locked away, and will only be accessible by those conducting the study. At the end of the study any personal data will be destroyed.

Will my GP be informed?
We will get in contact with your GP to let them known that you are taking part in a study. Your GP may check to see if there is any reason that you should not take part. We will not inform your GP of the results from the study since they are not clinically relevant.

What will happen to the study results?
The study results may be presented at scientific meetings or published in a scientific journal. Individuals will not be identifiable.

Will I be reimbursed for my time?
We are able to offer £40 for your participation in the study and also any travel expenses.

Who is organising and funding the study?
This study is funded by the Food Standards Agency and the MRC. MRC HNR will be organising and performing the study.

Withdrawal clause
If you decide to go ahead and participate in this study, please remember that you are free to withdraw at anytime without giving a reason.

Contact for further information
If you have any further questions then please contact Kerry Jones at HNR by telephoning 01223 426356.

And finally...
Thank you for having taken the time to read this information sheet. If you do decide to take part in the study then you will be given a copy of this information sheet and a signed consent form for you to keep.

MRC | Human Nutrition Research

Investigation into the Availability and Metabolism of Vitamin K

Information Sheet for Study Participants

Version 02
November 2004

MRC Human Nutrition Research
Elsie Widdowson Laboratory
Fulbourn Road
Cambridge
CB1 9NL

Telephone: 01223 426356
Fax: 01223 437515

http://www.mrc-hnr.cam.ac.uk

APPENDIX V

MRC Human Nutrition Research

CONSENT FORM

Bioavailability of vitamin K from meals

LREC Reference Number: 05/Q0102/148
Name of Chief Investigator: Mr Kerry Jones

	Please initial box
1. I confirm that I have read and understand the information sheet dated October 2005 (version 01) for the above study and have had the opportunity to ask questions.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected.	☐
3. I consent to my general practitioner (GP) being notified of my participation in this research.	☐
4. I agree to take part in the above study.	☐
5. I understand that samples taken as part of the protocol of this study may be stored and used in further nutrition research studies. Samples will only be used in studies that have been approved by the appropriate Ethics Committee.	☐

Name of Volunteer *(Please print)*	Signature	Date
Name of Research Team member *(Please print)*	Signature	Date

We would like you to consider participating in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of this study?
The purpose of this study is to help us understand how much vitamin K is absorbed from different meals. Vitamin K is a nutrient that has previously been associated primarily with blood clotting, but more recent evidence suggests a much wider role for this vitamin, particularly in bone health. The relationship between dietary intake of vitamin K and the amount in plasma is important for deciding on recommended intakes. This study will provide data that will help us understand this relationship.

Why have I been chosen?
We are approaching healthy men and women aged between 18 and 65yrs.

What will happen to me if I take part?
If you agree to take part, you will be asked to complete a four-day food diary. This involves keeping a written record of the types and amount of food you eat over a single four-day period.

You will be invited to attend the volunteer suite at HNR on three occasions. For your evening meal prior to the study day we will provide you with a prepared ready-meal to eat at home. In this way we can be sure that each participant has a low vitamin K meal before the study day.

After your evening meal, you will be asked not to consume anything until you arrive at HNR the following morning between 8am and 9am. We will measure your height and weight. A cannula (a small plastic tube) will then be inserted into a forearm vein by someone experienced in this technique. After the cannula is inserted we will be able to take blood samples from you without causing further discomfort. Prior to consuming a meal, two blood samples will be taken, each of around one and a half teaspoons.

You will then be asked to consume one of three meals:

1) Fish pie
2) Beef lasagne
3) Chicken pie

Each of these meals is of average size and contains around 700kcal and 40 micrograms of vitamin K. This is a normal amount of vitamin K from a meal. With the meal you will be asked to take a capsule containing a small amount of stable isotope labelled vitamin K in groundnut oil.

This is a naturally occurring form of the vitamin that we can detect using sensitive equipment - it is not a radioactive isotope. The labelled form allows us to tell this vitamin apart from the vitamin in the meal and that already present in the body. The label used in this experiment is a stable isotope called carbon-13.

After the meal a further thirteen blood samples will be taken, at half-hourly and hourly intervals, with a final sample at 8 hours. Each sample is equal to one and half teaspoons. The total volume taken per visit is equal to around half a small carton of fruit juice.

You will not be permitted to eat anything until five hours after the first meal, at which time you will be provided with a low-fat, low-vitamin K meal. You are allowed to drink water throughout the study.

After the last sample you will be offered a further meal. You are then free to leave HNR.

On your second and third visits you will be asked to consume the other meals.

What do I have to do?
You will be asked to consume a meal provided by HNR the evening before the study. After this meal you will be asked not to consume any food or drink (except water) prior to coming to HNR.

What are the possible benefits of taking part?
Participating in this study would not benefit you directly, but should help with the understanding of vitamin K absorption from different meals.

What are the possible risks of taking part?
The only effect of this procedure will be minor bruising from insertion of the cannula.

What happens at the end of the study?
The blood samples will be analysed to determine how much of the vitamin K was absorbed by the body. Samples will be stored for possible future analysis.

Confidentiality – who will have access to the data?
The information we gain will be used for research purposes only and will be treated as confidential. Blood samples will be stored in coded vials. Any personal data will be kept locked away, and will only be accessible by those conducting the study.

Will my GP be informed?
We will get in contact with your GP to let them know that you are taking part in a study. Your GP may check to see if there is any reason that you should not take part. We will not inform your GP of the results from the study since they are not clinically relevant.

What will happen to the study results?
The study results may be presented at scientific meetings or published in a scientific journal. Individuals will not be identifiable.

Will I be reimbursed for my time?
We are able to offer £90 for your participation in the study and also reasonable travel expenses.

Who is organising and funding the study?
The Food Standards Agency (FSA) and the Medical Research Council (MRC) are funding this study. MRC HNR will be organising and performing the study.

Withdrawal
If you decide to go ahead and participate in this study, please remember that you are free to withdraw at anytime without giving a reason.

Contact for further information
If you have any further questions then please contact Mr Kerry Jones at HNR by telephoning 01223 426356.

And finally...
Thank you for having taken the time to read this information sheet. If you do decide to take part in the study then you will be given a copy of this information sheet and a signed consent form for you to keep.

MRC Human Nutrition Research

The bioavailability of Vitamin K from meals

Information Sheet for Study Participants

Version 01
October 2005

Kerry Jones

MRC Collaborative Centre for Human Nutrition Research
Elsie Widdowson Laboratory
Fulbourn Road
Cambridge
CB1 9NL

Telephone: 01223 426356
Fax: 01223 437515

http://www.mrc-hnr.cam.ac.uk