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**DIGESTIVE METABOLISM OF GLUCOSINOLATES: A NOVEL
APPROACH USING URINARY MARKERS FOR ESTIMATING THE
RELEASE OF GLUCOSINOLATE BREAKDOWN PRODUCTS IN THE
GASTRO-INTESTINAL TRACT OF MAMMALS**

A thesis submitted for the degree of Doctor of Philosophy

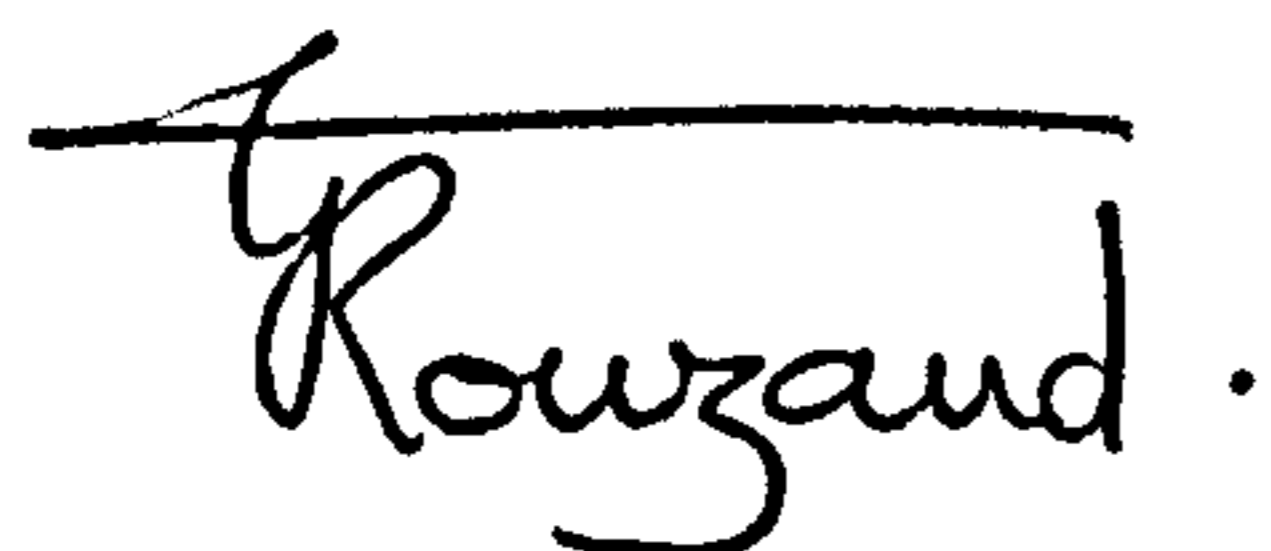
by Gabrielle C.M. Rouzaud

**Robert Gordon University
Macaulay Land Use Research Institute**

April 2001

DECLARATION

I declare that the work contained in this thesis has been composed by myself and that none of the presented material has been previously used in any other submission for an academic award. All sources of information have been specifically acknowledged by reference to the authors.

A handwritten signature in black ink, consisting of a horizontal line above the name 'Rouzaud'.

Gabrielle C.M. Rouzaud

April 2001

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	I
LIST OF FIGURES.....	X
LIST OF TABLES.....	XII
LIST OF APPENDICES.....	XIV
LIST OF ABBREVIATIONS.....	XV
ABSTRACT.....	1
INTRODUCTION.....	2
CHAPTER 1:REVIEW OF LITERATURE	
Metabolic fate of glucosinolates and their breakdown products in mammals.....	4
1.1. Introduction.....	4
1.2. Protective effect of cruciferous vegetables against cancer	5
1.2.1. The epidemiological evidence	5
1.2.2. The phytochemical components of cruciferous vegetables.....	6
1.3. Biochemistry of glucosinolates.....	6
1.3.1. Structure and biosynthesis	6
1.3.2. Measurement of glucosinolates in plant material	8
1.3.3. Distribution	9
1.3.4. Glucosinolate intake by humans	11
1.3.5. Biological properties.....	12
1.4. Myrosinase.....	13
1.4.1. Plant myrosinase	13
1.4.1.1. Biochemistry	13
1.4.1.2. Myrosinase-catalysed hydrolysis.....	16
1.4.1.3. Factors influencing glucosinolate hydrolysis	17
1.4.2. Myrosinase activity in microorganisms	19
1.4.2.1. Myrosinase activity in fungi.....	19
1.4.2.2. Microbial myrosinase activity	20
1.4.3. Myrosinase activity in cells and tissues	24
1.4.4. Metabolic fate of intact glucosinolates <i>in vivo</i>	24

1.5. Breakdown products of glucosinolates	25
1.5.1. Isothiocyanates	26
1.5.2. Nitriles and other minor breakdown products.....	27
1.5.3. Oxazolidinethione	28
1.5.4. Breakdown products of indole glucosinolates	29
1.6. Disposition and metabolism of breakdown products of glucosinolates	30
1.6.1. Isothiocyanates	30
1.6.2. Nitriles and other breakdown products	32
1.7. Fate of glucosinolates and measurement of bioavailability.....	33
1.8. Conclusion.....	36

CHAPTER 2:EXPERIMENT 1

Assesment of the use of urinary mercapturic acids as markers of glucosinolate metabolite release in rats fed a cruciferous diet	37
2.1. Introduction.....	37
2.2. Materials and methods	38
2.2.1. Animals	38
2.2.2. Experimental design.....	39
2.2.3. Treatments.....	41
2.2.4. Diet.....	41
2.2.5. Composition of dosing mixtures.....	43
2.2.6. Urine and faeces collection	44
2.2.7. Analysis.....	44
2.2.7.1. Synthesis of mercapturic acids.....	44
2.2.7.2. Quantification of mercapturic acids	44
2.2.8. Calculation and statistical analysis	46
2.3. Results.....	47
2.3.1. Excretion of mercapturic acids arising from an artificial dose of isothiocyanates	47
2.3.2. Excretion of mercapturic acids arising from a single dose of sinigrin...	49
2.3.3. Excretion of mercapturic acids arising from dietary glucosinolates	50
2.3.4. Diet intake and iron intake	52
2.4. Discussion	52

CHAPTER 3: EXPERIMENT 2

Influence of plant and bacterial myrosinase activity on the metabolic fate of sinigrin and benzyl glucosinolate in the digestive tract of rats harbouring a human faecal flora	57
3.1. Introduction.....	57
3.2. Materials and methods.....	58
3.2.1. Animals.....	58
3.2.2. Maintenance of animals	58
3.2.3. Inoculation of human flora to animals	59
3.2.4. Control of bacterial status	59
3.2.5. Experimental design.....	59
3.2.6. Diet composition and preparation.....	61
3.2.7. Measurement of isothiocyanate release	62
3.2.8. Composition of dosing mixtures.....	62
3.2.9. Sample collection.....	62
3.3. Analysis	63
3.3.1. Analysis of mercapturic acids.....	63
3.3.2. Desulpho-glucosinolate analysis.....	63
3.3.3. Calculations and statistical analysis.....	64
3.4. Results	64
3.4.1. Excretion of mercapturic acids	64
3.4.1.1. Excretion of benzyl and butyl mercapturic acids after ITC dose	64
3.4.1.2. Proportion of benzyl isothiocyanate release from benzyl glucosinolate after BGSL dose	66
3.4.1.3. Excretion of allyl mercapturic acids from ITC dose and BGSL	68
3.4.2. Proportion of glucosinolate excreted in faeces	68
3.4.3. Live weight	69
3.4.4. Daily food intake and daily glucosinolate intake of rats.....	69
3.5. Discussion.....	70

CHAPTER 4:EXPERIMENT 3

Measurement of isothiocyanate release in the intestinal tract of healthy

human volunteers	73
4.1. Introduction.....	73
4.2. Materials and methods.....	74
4.2.1. Experimental design.....	74
4.2.2. Volunteers	74
4.2.3. Vegetables.....	76
4.2.4. Preparation of experimental meal	76
4.2.4.1. Watercress suspension.....	76
4.2.4.2. Cabbage preparation.....	77
4.2.5. Urine collection.....	77
4.2.6. Analysis.....	77
4.2.6.1. Chemicals	77
4.2.6.2. Quantification of isothiocyanates in watercress and mustard	77
4.2.6.3. Quantification of sinigrin in white cabbage:	78
4.2.6.4. Myrosinase activity	78
4.2.6.5. Mercapturic acid analysis	79
4.2.6.6. Calculation of allyl isothiocyanate release from sinigrin	80
4.2.7. Statistical analyses	80
4.3. Results	81
4.3.1. Intake of glucosinolate and isothiocyanate precursors.....	81
4.3.2. Excretion of mercapturic acids	81
4.3.3. Proportion of allyl isothiocyanate release after ingestion of raw and cooked cabbage	84
4.4. Discussion.....	84

CHAPTER 5:EXPERIMENT 4

Urinary excretion of N-acetyl cysteine conjugates following gastric or caecal

delivery of isothiocyanates to rats.....	87
5.1. Introduction	87
5.2. Materials and methods.....	88

5.2.1. Animals	88
5.2.2. Treatments and procedure.....	89
5.2.3. Analysis.....	90
5.2.4. Statistical analysis.....	90
5.3. Results	91
5.4. Discussion.....	94
CHAPTER 6:EXPERIMENT 5	
Influence of a fermentable oligosaccharide on the release of isothiocyanate in the large bowel of rats	
	97
6.1. Introduction.....	97
6.2. Materials and methods.....	99
6.2.1. Description of the experiment.....	99
6.2.2. Animals	99
6.2.3. Diet.....	99
6.2.4. Administration of glucosinolates	101
6.2.5. Collection of gut contents	102
6.2.6. Quantification of non digested glucosinolate in digestive contents.....	102
6.2.7. Quantification of isothiocyanates in digestive contents.....	102
6.2.8. Statistical analysis.....	105
6.3. Results	105
6.3.2. Effect of pH.....	105
6.3.3. Non digested glucosinolates.....	105
6.3.4. Isothiocyanates released in the digestive tract	111
6.3.4.1. Stomach.....	111
6.3.4.2. Small intestine.....	111
6.3.4.3. Caecum and colon	111
6.4. Discussion.....	112
CHAPTER 7: GENERAL DISCUSSION	
	114
7.1. Introduction.....	114
7.2. Experimental approach	115

7.2.1. Relevance of the use of urinary markers.....	115
7.2.2. Application of the approach to isothiocyanates	115
7.2.3. Investigation of the release of nitrile derivatives	116
7.2.4. Limits of the approach	116
7.2.5. Alternative methods to the use of markers.....	117
7.2.6. Relevance of rat studies	118
7.3. Fate of glucosinolates and their isothiocyanate derivatives in the digestive tract.....	118
7.3.1. Isothiocyanate release by plant myrosinase	119
7.3.2. The involvement of the microflora in isothiocyanate release	119
7.3.3. Metabolic fate of non-hydrolysed glucosinolates	121
7.3.4. Degradation of glucosinolates by non-enzymatic autolysis	122
7.3.5. Influence of other components of the diet.....	123
7.3.6. The post-absorptive fate of isothiocyanates.....	124
7.3.7. Digestive metabolism after ingestion of raw vegetables.....	125
7.3.8. Digestive metabolism after ingestion of cooked vegetable.....	126
7.4. Future work.....	128
7.5. Conclusion	130
REFERENCES	132
APPENDICES.....	157
COMMUNICATIONS.....	167

LIST OF FIGURES

Figure 1.1: General structure of glucosinolates.....	7
Figure 1.2: Glucosinolate concentrations in <i>cyrus</i> and <i>maximus</i> varieties of Brussels sprouts.....	10
Figure 1.3: Enzymic hydrolysis of glucosinolates and release of breakdown products.....	17
Figure 1.4: Breakdown products of the indolyl glucosinolate glucobrassicin.....	29
Figure 1.5: Detoxification pathway of isothiocyanates into mercapturic acids in rat and human.....	31
Figure 1.6: Different stages in the pathway of glucosinolates and potential factors which may influence the bioavailability of their breakdown products.....	35
Figure 2.1: Predicted metabolites deriving from studied glucosinolates in the dosing mixtures.....	40
Figure 2.2: Conversion of isothiocyanate to thiourea.....	45
Figure 2.3: Cumulative excretion of mercapturic acids over 48 hours following ITC dose.. ..	48
Figure 2.4: Relationship between excretion of allyl mercapturic acid and butyl mercapturic acid after administration of ISO/CN dose.....	51
Figure 2.5: Proportion of isothiocyanate release from dietary phenethyl glucosinolate.....	51
Figure 3.1: Relationship between cumulative excretion of butyl mercapturic acid and benzyl mercapturic acid after administration of ITC dose.....	65
Figure 3.2: Cumulative excretion of benzyl mercapturic acid excreted after administration of BGSL and ITC dose.....	67
Figure 3.3: Excretion of sinigrin and benzyl glucosinolate in faeces of germ-free rats (Flora-) over 144h after administration of BGSL dose.....	69
Figure 4.1: Experiment timetable.....	75
Figure 4.2: Relationship between the excretion of allyl and phenethyl mercapturic acid when pre-formed allyl and phenethyl isothiocyanate were ingested (meal MUST).....	82
Figure 4.3: Cumulative excretion of urinary markers over 24 hours after ingestion of meals (a) COL, (b) COOK, (c) MUST.....	83

Figure 5.1: Cumulative excretion of mercapturic acids after administration of Gastric and Caecal treatments.....	92
Figure 5.2: Excretion rate of mercapturic acids over 48 hours after administration of isothiocyanates.....	93
Figure 6.1: Average pH in different compartments of the human digestive tract.....	98
Figure 6.2 (a) and (b) : Relationship between the peak areas of phenyl isothiocyanate and allyl isothiocyanate (Figure a) and between phenyl isothiocyanate and benzyl isothiocyanate (Figure b).....	104
Figure 6.3: Effect of diet on caecal pH of rats	106
Figure 6.4: Amount of non-digested sinigrin in the intestinal tract of rats.....	107
Figure 6.5: Amount of non-digested benzyl glucosinolate in the intestinal tract of rats.....	108
Figure 6.6: Amount of allyl isothiocyanate released in the intestinal tract of rats...	109
Figure 6.7: Amount of benzyl isothiocyanate released in the intestinal tract of rats.....	110
Figure 7.1: Proposed metabolic fate of glucosinolates (GLS) and their isothiocyanate derivatives (ITC) after ingestion of cruciferous vegetables.	127

LIST OF TABLES

Table 1.1: Examples of glucosinolates found in <i>Brassicaceae</i>	8
Table 1.2: Examples of glucosinolate-containing <i>Brassicaceae</i>	9
Table 1.3: Biochemical characteristics of myrosinase enzyme from edible cruciferous plants.....	14
Table 1.4: Characterisation of bacterial strains possessing a myrosinase activity....	21
Table 1.5: Examples of nitrile and epithionitrile products derived from glucosinolates	28
Table 2.1: Experimental design.....	41
Table 2.2: Composition of the diet.....	42
Table 2.3: Glucosinolate composition of the cruciferous vegetables and glucosinolate content of the Cruciferous diet.....	43
Table 2.4: Total amount of excreted mercapturic acid markers after administration of ITC dose	49
Table 2.5: Correlation matrix between the excretion of markers coming from isothiocyanates given in ITC dose.....	49
Table 2.6: Excretion of mercapturic acid after administration of ISO/CN and SIN dose.....	50
Table 2.7: Amount of propyl mercapturic acid excreted over 48 hours during feeding of the Cruciferous diet	52
Table 2.8: Food intake on Control and Iron treatments and iron intake in Iron-treated animals.....	53
Table 3.1: Experimental design	60
Table 3.2: Design of the administration of dose mixture to rats.....	60
Table 3.3: Composition of the diets given to rats.....	60
Table 3.4: Glucosinolate composition of Brussels sprouts (<i>Brassica oleracea</i> variety <i>cyrus</i>).....	61
Table 3.5: Cumulative excretion of mercapturic acids over 144h after administration of 25 μ mol of precursor isothiocyanate.....	65
Table 3.6: Proportion of benzyl isothiocyanate released in the digestive tract of rats after administration of 25 μ mol of benzyl glucosinolate.....	66

Table 3.7: Cumulative excretion of allyl mercapturic acids over 144h: with an oral administration of 25 μ mol allyl isothiocyanate (ITC dose) or without administration of allyl isothiocyanate (BGS� dose).....	68
Table 4.1: Experimental design.....	75
Table 4.2: Amount of glucosinolates and breakdown products ingested by volunteers and mean excretion of urinary markers.....	82
Table 5.1: Composition of the pellets given to rats throughout the experiment.....	88
Table 5.2: Excretion of urinary mercapturic acids after administration of 25 μ mol of the related isothiocyanates (μ mol) in Gastric and Caecal treatments.....	91
Table 5.3: Comparison of the rate of excretion between allyl and benzyl mercapturic acids after administration of Gastric and Caecal treatments.....	93
Table 6.1: Allocation of animals to slaughter times.....	100
Table 6.2: Composition of the diet.....	101
Table 6.3: Response factors used for quantification of allyl and benzyl isothiocyanate by SPME-GC analysis.....	103

LIST OF APPENDICES

APPENDIX 1: Synthesis of <i>N</i> -acetyl- <i>S</i> -(<i>N</i> -alkyl thiocarbamoyl)- <i>L</i> -cysteine, dicyclohexylamine salt.....	159
APPENDIX 2: Preparation of urine samples for analysis of mercapturic acids by HPLC	160
APPENDIX 3: Sample of ANOVA table for experiment 2	161
APPENDIX 4a: Questionnaire addressed to applicants for the human study	162
APPENDIX 4b: Determination of score for allocation of volunteers to block	165
APPENDIX 5: Sample of ANOVA table for Experiment 3.....	166
APPENDIX 6a: Sample of ANOVA table for Experiment 4	167
APPENDIX 6b: Sample of ANOVA table for Experiment 4.....	168

LIST OF ABBREVIATIONS

μg	Microgram
μl	Microlitre
μM	Micromolar
μm	Micrometre
μmol	Micromole
A	Aspergillus
amu	Atomic mass unit
AFNOR	French Agency for Normalisation
BGSL	Benzyl glucosinolate
C	Centigrade
CaHPO_4	Calcium hydrogen orthophosphate
cm	Centimetre
COL	Coleslaw
COOK	Cooked cabbage
$\text{CoSO}_4\cdot 7\text{H}_2\text{O}$	Cobalt sulphate
$\text{CuSO}_4\cdot 5\text{H}_2\text{O}$	Copper sulphate
DM	Dry matter
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme linked immuno-sorbant assay
eV	Electron Volt
Fe	Iron
Fe_2O_3	Ferric oxide
$\text{FeSO}_4\cdot 7\text{H}_2\text{O}$	Iron sulphate
Flora-	Germ-free rat
Flora+	Rat harbouring a human faecal microflora
g	Gram
g	gravitation force
GC	Gas Chromatography
GLU	Glutamic acid
GLY	Glycine
h	hour

HPLC	High Performance Liquid Chromatography
INRA	National Institute for Agriculture Research
ISO/CN	Isothiocyanate and cyanide
ITC	Isothiocyanate
IU	International unit
KCl	Potassium chloride
kDa	Kilodalton
kg	Kilogram
kGy	Kilogray
KI	Potassium iodide
kJ	Kilojoule
kP	Kilopascal
l	Litre
LCY	Liquid casein yeast
M	Molar
m	metre
mercapturic acid	<i>N</i> -acetyl-S-(<i>N</i> -alkylthiocarbamoyl)- <i>L</i> -cysteine
Mg	Magnesium
mg	Milligram
MgO	Magnesium oxide
MgSO ₄	Magnesium sulphate
min	Minute
ml	Millilitre
MLURI	Macaulay Land Use Research Institute
mM	Millimolar
mmol	Millimole
MnSO ₄ H ₂ O	Manganese sulphate
MPa	Megapascal
mRNA	Messenger ribonucleic acid
MUST	Mustard
Myro-	Inactivated myrosinase
Myro+	Native myrosinase
NaCl	Sodium chloride
ND	Not determined

NITR	Nitrile
nm	Nanometre
NS	Not significant
p	probability
Pa	Pascal
pH	Potential hydrogen
RGU	Robert Gordon University
s.e.m.	Standard error of mean
SED	Standard error of difference
SIN	Sinigrin
<i>sp.</i>	Species
SPME	Solid phase micro-extraction
T	temperature
t	time
UV	Ultra-violet
UEPSD	Ecology and physiology of the digestive system unit
UK	United Kingdom
USA	United States of America
ZnSO ₄ ·7H ₂ O	Zinc sulphate

ABSTRACT

Digestive metabolism of glucosinolates: a novel approach using urinary markers for estimating the release of glucosinolate breakdown products in the gastro-intestinal tract of mammals.

A thesis submitted for the degree of Doctor of Philosophy

by Gabrielle C.M. Rouzaud

Glucosinolates have been implicated as a mediator of the cancer-protective properties of cruciferous vegetables. Enzymatic hydrolysis of glucosinolates by plant or microbial myrosinase yields a range of metabolites including beneficial isothiocyanates. Little is known about the fate of glucosinolates after their ingestion. Using urinary end-products of metabolism as markers, measurement of the production of isothiocyanates in the digestive tract of monogastric animals has been achieved. Initially, a range of isothiocyanates were administered to rats and their excretion as mercapturic acids was quantified. Relative recovery of different isothiocyanates was found to be consistent and predictable, allowing the use of artificial isothiocyanates as recovery standards in subsequent experiments. Subsequently, the relative influence of plant and bacterial myrosinase on isothiocyanate production was quantified in rats. A proportion of 0.80 (s.e.m. 0.076) of benzyl glucosinolate was hydrolysed to isothiocyanate by plant myrosinase. In the presence of both plant and microbial activity, the proportion of benzyl isothiocyanate release was significantly decreased (0.50 s.e.m. 0.046, $p < 0.01$) suggesting microbial breakdown of isothiocyanates. The approach, adapted for use with human subjects showed that the proportions of allyl isothiocyanate measured after ingestion of raw and cooked cabbage were 0.37 (s.e.m. 0.045) and 0.53 (s.e.m. 0.134) respectively in healthy male volunteers. A further experiment with rats established that isothiocyanate uptake in the distal digestive tract was significantly less than in the proximal intestine (0.12 s.e.m. 0.017 and 0.48 s.e.m. 0.029 respectively), suggesting a potential under-estimation of isothiocyanate release in the distal digestive tract when using urinary markers. Finally, enhancement of bacterial fermentation by addition of inulin to the diet had little influence on isothiocyanate production in the gut. The findings suggested that the formation of the cancer-protective isothiocyanates was significant, *in vivo*, thereby strengthening the evidence for a beneficial effect of cruciferous vegetables for health. The newly developed method opens up possibilities of concurrently exploring the digestive fate of isothiocyanates and the toxicity of carcinogenic compounds.

INTRODUCTION

Reducing the risk of cancer by dietary means has become an important strategy in the field of human health. A wide variety of natural and synthetic compounds present in human diet may promote mutagenesis and carcinogenesis when they are absorbed (Ames, 1992). These are usually present in minimal quantities in the diet. The simultaneous presence of beneficial compounds in the diet is an important factor with regard to cancer prevention as it may counter-balance the toxicity of harmful compounds.

Epidemiological studies have provided convincing evidence of the beneficial effect of a diet rich in fruits and vegetables in reducing cancer incidence (Steinmetz & Potter, 1991). The anti-carcinogenic properties of plant food are thought to be related to the occurrence of secondary plant compounds, also known as phytochemicals. One class of phytochemicals that are currently extensively studied are the glucosinolates and their related hydrolysis products. Studies have investigated their anti-carcinogenic and anti-mutagenic effects on various cellular systems, clarifying the mechanisms underlying their cancer-protective effects (Johnson *et al.* 1994). The determination of dietary exposure and bioavailability of glucosinolates is of primary importance in determining their physiological relevance, particularly in the context of advising consumers on their dietary choice. It is difficult to optimise dietary allowances in relation to cancer protection as little is known of the metabolic fate of glucosinolates *in vivo*. The aim of this research was therefore to identify and quantify the breakdown products of glucosinolates released in the digestive tract under different physiological conditions.

The human colonic microflora is a large ecosystem comprising more than 400 species of bacteria (Macfarlane & Cummings, 1991). Its overall stability results from a complex and highly variable system of metabolic activities (Macfarlane & Gibson, 1994). The interactions between dietary phytochemicals and microflora are not very well described. Only recently has the capacity of a human strain of bacteria to break down glucosinolates into isothiocyanates *in vivo* been studied in detail (Elfoul, 1999).

Confirmation of the ability of the microflora to produce cancer-preventive metabolites in the large bowel may aid the development of preventive nutrition since cancer risks are high in the colon. The possibility of enhancing or optimising this production with other components of the diet could also be important. As a prerequisite to such manipulation, it is important to elucidate the factors which influence the digestive metabolism of glucosinolates. From observations *in vitro*, it is known that damage to the plant matrix or changes in the chemical environment of the hydrolysis may result in the formation of different breakdown products. The preparatory processing of vegetables and the chemical variation induced by intestinal secretions along the digestive tract may also have an influence on the subsequent metabolite release *in vivo*. Consequently, a second objective in this work was to clarify the involvement of plant-related factors, microbial factors and other dietary factors on the pattern of glucosinolate hydrolysis *in vivo*.

CHAPTER 1: REVIEW OF LITERATURE

Metabolic fate of glucosinolates and their breakdown products in mammals

1.1. Introduction

According to the National Food Survey (1998), cruciferous vegetables are one of the most consumed vegetables in the UK (mean intake: 25g/person/day). Cruciferous foods consumed in the human diet include condiments (mustard, horseradish) and brassica vegetables (cabbage, cauliflower, Brussels sprouts, broccoli, turnip, radish and swede). The characteristic pungent and bitter taste of cruciferous vegetables derives from a particular class of thioglucosides, the glucosinolates. Glucosinolates in cruciferous plants have a number of possible biological functions including defence against fungal attack and pests. Based upon the consumption of cruciferous vegetables by humans, the mean daily amount of glucosinolates ingested is 100 $\mu\text{mol/person}$ which constitutes a significant amount in comparison to some micro-nutrients (Sones *et al.* 1984).

Glucosinolates have been the subject of a number of comprehensive reviews (Fenwick *et al.* 1983; Nugon-Baudon & Rabot, 1994; Mithen *et al.* 2000). The scope of the current review will therefore be limited to areas of particular relevance to the current study. Firstly the epidemiological evidence for a relationship between cruciferous vegetable consumption and cancer prevention will be reviewed. The specific involvement of glucosinolates in mediating the health benefits of brassica consumption will be considered. Glucosinolates are precursors of a range of biologically active products. Their release is catalysed by a thioglucosidase enzyme, commonly known as myrosinase. A brief review of this process will be presented together with recent findings on the action of different sources of myrosinases. Finally the nature of the glucosinolate breakdown products and their post-absorptive fate will be discussed. This overview aims to identify the best approach for the measurement of the digestive metabolism of glucosinolates. It also looks at the possible factors which may influence the metabolic fate of glucosinolates *in vivo*.

Much of our current knowledge of glucosinolates is derived from studies on brassicas such as swede and kale or oilseed crops (rape or "Canola" and mustard) since they are important food crops for farm livestock. The overall goal of such research is the production of feed with a high proportion of protein and a low concentration of glucosinolates and glucosinolate derivatives since these latter compounds have detrimental effects on livestock production. Research on cruciferous vegetables produced for human consumption has a different goal. In this regard, research is geared toward the enhancement of flavour and the beneficial health properties of commercial vegetable varieties. As far as possible, this review focuses on research based on vegetables commonly consumed in human diets.

1.2. Protective effect of cruciferous vegetables against cancer

1.2.1. The epidemiological evidence

A wide range of studies have shown an inverse correlation between consumption of vegetables and the incidence of cancer. High variability between studies exists but the overall consistency of results is striking. Among 87 epidemiological studies listed in the literature in 1996, 68 showed a protective effect of cruciferous vegetables against cancer (Verhoeven *et al.* 1996). For other components of the diet the relationship is less clear. Carbohydrates, for instance, were associated with cancer protection in only 18 out of 43 surveys carried out (Steinmetz & Potter, 1991). The epidemiological relationship between consumption of cruciferous vegetables and cancer risk has also been examined more specifically (Verhoeven *et al.* 1996; Watson & Kohlmeier, 1999). A high intake of cruciferous vegetables was associated with a strongly decreased cancer risk for the digestive and respiratory tracts. Evidence for protection against hormone-dependent cancers, such as breast cancer, is weak. To date, however, there have been few epidemiological studies where the effect of cruciferous vegetables has been separated from, or adjusted for, consumption of other vegetables. Experimental data in laboratory animals have confirmed the epidemiological findings. As reported in a recent review, seven studies have shown that a cruciferous vegetable diet led to reduced incidence of chemically-induced cancers at various sites (Van Poppel *et al.* 1999).

1.2.2. The phytochemical components of cruciferous vegetables

Cruciferous vegetables together with allium vegetables are a major source of sulphur-containing compounds in the human diet. The sulphur content of cruciferous vegetables varies between 0.5-4.5 mmol/100g wet weight (Holland *et al.* 1991). Most of the sulphur content of cruciferous vegetables is in the form of glucosinolates, disulphides or dithiol thiones. Cruciferous vegetables also have a high content of anti-oxidant vitamins. They are also a source of minerals such as potassium and magnesium. It is not clear whether the protective effects of cruciferous vegetables is specifically related to thioglucoside compounds but, as the anti-oxidative properties of vitamins are also relevant to cancer-protection, it is likely that the negative correlation between incidence of cancer and consumption of cruciferous vegetables is the result of the joint action of a range of micro-components found in the vegetables. Experimental data give, nevertheless, convincing evidence for a dominant role of glucosinolates. Feeding trials aimed at identifying the mechanism of cancer protection have shown a positive correlation between the amount of glucosinolates and hydrolysis products in the diet and the induction of the enzymes responsible for detoxification of carcinogens (Sparnins *et al.* 1982; McDannel *et al.* 1987; Nijhoff *et al.* 1995; Steinkellner *et al.* 2000). The addition of purified glucosinolates to a non-cruciferous diet in animal feeding experiments led to a similar induction of xenobiotic-detoxifying enzymes (Bradfield *et al.* 1985). A host of studies have also shown a dramatic decrease in the incidence of chemically-induced cancers when glucosinolates or their breakdown products are administered to laboratory animals and these studies have been listed in several reviews (Verhoeven *et al.* 1997; Hecht, 1999; Van Poppel *et al.* 1999). The evidence is thus converging to demonstrate that, among other micro-nutrients, glucosinolates and their derivatives may have a primary role in the mechanism of prevention against cancer.

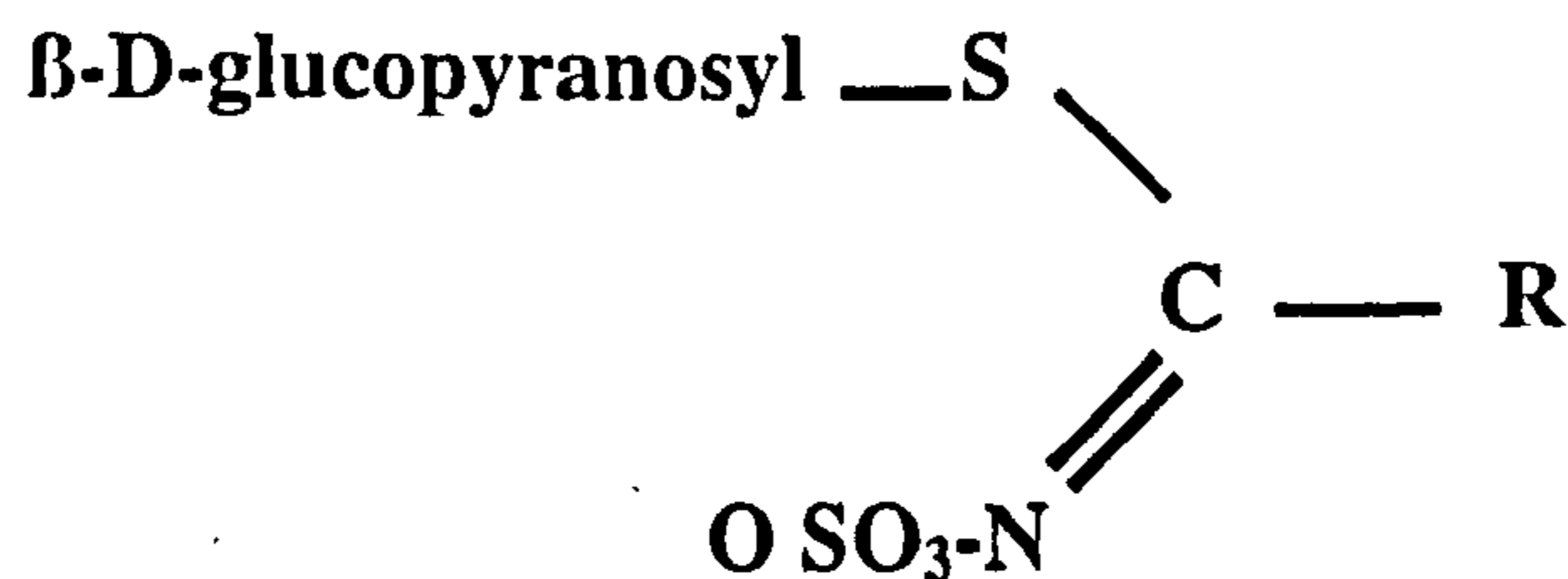
1.3. Biochemistry of glucosinolates

1.3.1. Structure and biosynthesis

Glucosinolates are thioglucoside compounds characterised by a common chemical structure. They consist of a thioglucose grouping, an O-sulphonate group and a side-chain R (Figure 1.1). The side-chain can be either an aliphatic, aromatic or indolyl in nature and approximately 100 glucosinolates have been identified. The structural

elucidation of glucosinolates has been reviewed elsewhere (Fenwick *et al.* 1983). Glucosinolates are usually given a common name in addition to their chemical nomenclature. For instance, prop-2-enyl glucosinolate is generally called sinigrin. Table 1.1 summarises the most important glucosinolates found in the *Brassicaceae*.

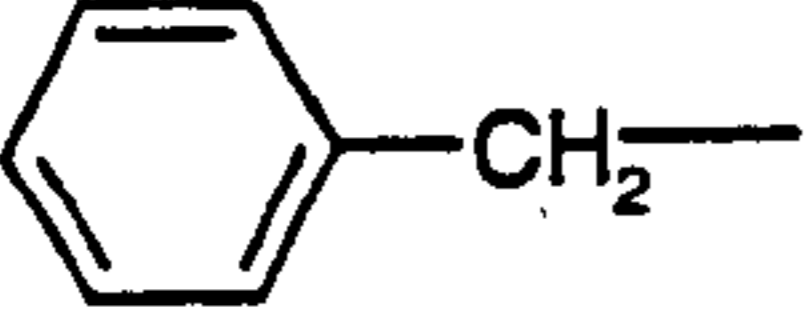
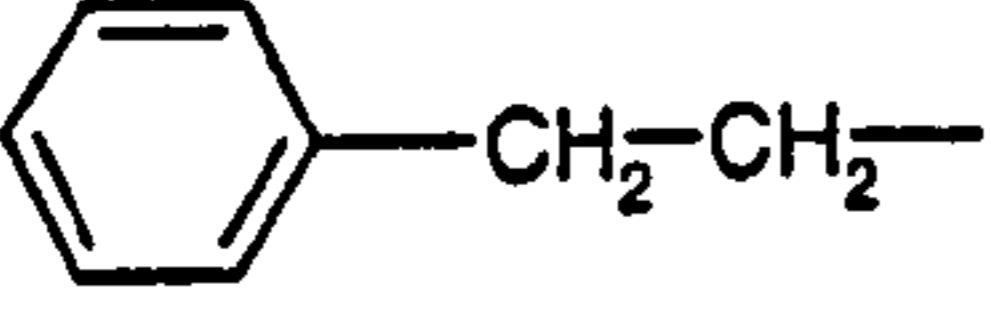
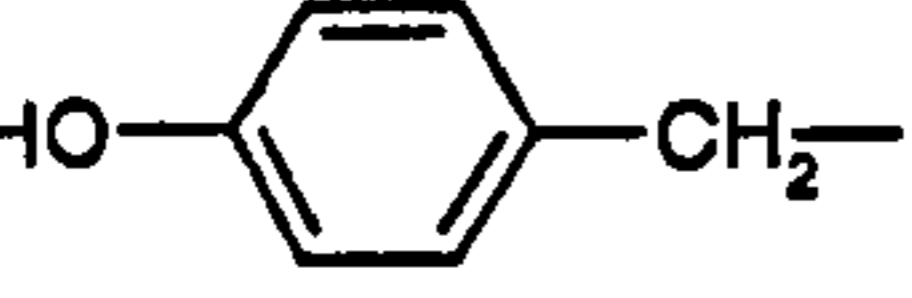
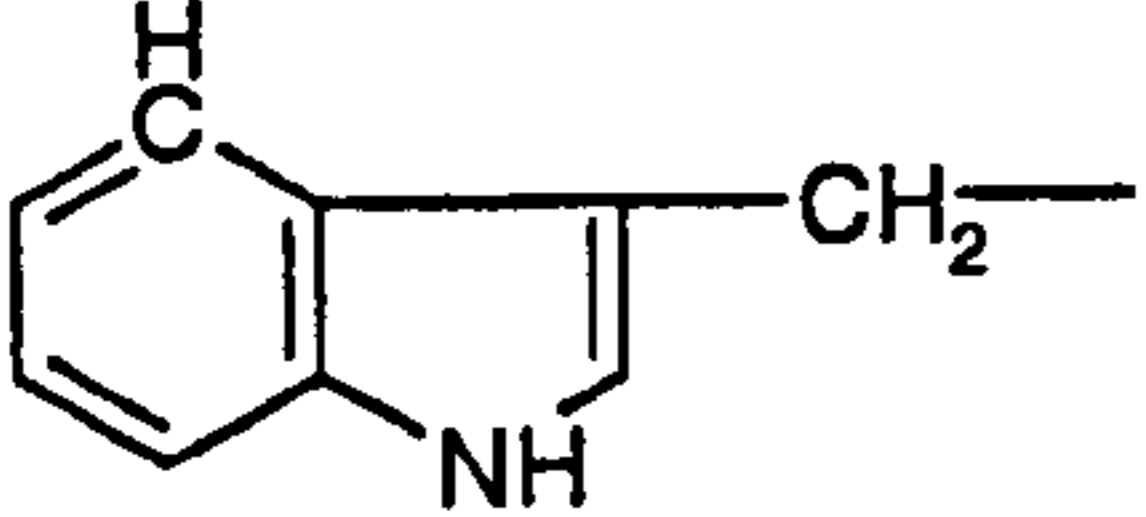
Figure 1.1: General structure of glucosinolates



Glucosinolates are derived from the secondary metabolism of amino-acids in plants. The biosynthetic pathway of glucosinolates has been quite well characterised. Chemical and enzymatic pathways have been detailed in recent reviews (Mithen *et al.* 2000; Wallsgrove *et al.* 2000). Although cross-breeding manipulations have been successfully conducted to develop low-glucosinolate rapeseed varieties, the mechanisms underlying the regulation of glucosinolate synthesis in the plant cell is still unclear. Until now, it has not been possible to artificially manipulate the genome of brassica to selectively increase or repress the production of one specific glucosinolate or one class of glucosinolates.

The exact location of glucosinolates in plant cells is still unclear. A study of horseradish roots reported the glucosinolates to be stored in vacuoles of non-specific cells but since the study relied on observation of lysed cells, it may be that in the intact cells, glucosinolates may also be found in the cytoplasm (Grob & Matile, 1979). Glucosinolates are stored in their anionic form in plant cells. Commercially available purified glucosinolates are generally extracted from plants. They are then associated to cations such as potassium or sodium to form a stable salt. Glucosinolates are generally unstable at temperatures higher than 110°C (VanEtten *et al.* 1966; MacLeod *et al.* 1981) but stable during freezing and freeze-drying (McGregor *et al.* 1983).

Table 1.1: Examples of glucosinolates found in the *Brassicaceae*

Side chain	Glucosinolate	Trivial name
CH ₂ =CH-CH ₂ -	prop-2-enyl	sinigrin
CH ₂ =CH-CH ₂ -CH ₂ -	but-3-enyl	gluconapin
CH ₂ =CH-CH-CH(OH)-CH ₂ -	2-hydroxybut-3-enyl	progoitrin
CH ₃ -S-CH ₂ -CH ₂ -CH ₂ -CH ₂ -	4-methylthiobutyl-	glucoerucin
CH ₃ -SO-CH ₂ -CH ₂ -CH ₂ -CH ₂ -	4-methylsulphinylbutyl-	glucoraphanin
	benzyl-	glucotropaeolin
	2-phenylethyl	gluconasturtiin
	4-hydroxybenzyl-	sinalbin
	indol-3-ylmethyl-	glucobrassicin

Exposure to ultraviolet radiation may accelerate their degradation (Monde *et al.* 1991). The *de novo* synthesis of aromatic glucosinolates and indole glucosinolates as well as their labelled equivalents has been described but the process is expensive and yield is low (Dawson *et al.* 1993). Therefore very few purified compounds are readily and commercially available for biological studies.

1.3.2. Measurement of glucosinolates in plant material

Several analytical methods for the measurement of glucosinolates in plant material have been developed. Early methods relied on the measurement of breakdown products released upon enzymatic hydrolysis including the glucose moiety or the aglucone compounds (Heaney & Fenwick, 1981; McGregor *et al.* 1983). To counteract the potential lack of accuracy inherent in the use of an enzymatic hydrolysis step, direct analysis of non-hydrolysed glucosinolates by HPLC has also been described (Helboe *et al.* 1980; Betz & Fox, 1994; Prestera *et al.* 1996). This method is, however, more suitable for isolation and preparation of glucosinolates than composition analysis. Other analytical methods are also available for structural elucidation of glucosinolates such as mass spectroscopy (Chiang *et al.* 1998). Rapid screening of cruciferous varieties in breeding programmes has employed a recently developed ELISA test (Van Doorn *et al.* 1998). The strengths and weaknesses of

these techniques have been discussed in several reviews (Fenwick *et al.* 1983; McGregor *et al.* 1983; Mithen *et al.* 2000). In an attempt to standardise the analytical procedure and to achieve a meaningful comparison of data between studies, the analysis of individual glucosinolates after a desulphation step has now been adopted as a standard procedure for rapeseed crops within the European Community (Minchinton *et al.* 1982; AFNOR, 1995).

1.3.3. Distribution

Glucosinolates are only found in a restricted number of plant families (Fenwick *et al.* 1983). Among edible plants, they occur predominantly within the family of *Brassicaceae* (Table 1.2). Indian cress (*Tropaeolaceae*), Papaya (*Caricaceae*) and Caper (*Capparaceae*) are the three edible plants outside the *Brassicaceae* family containing glucosinolates. Despite the large number of glucosinolates, most species of vegetables contain only a few individual glucosinolates. The distribution of glucosinolates has been extensively studied in cruciferous plants.

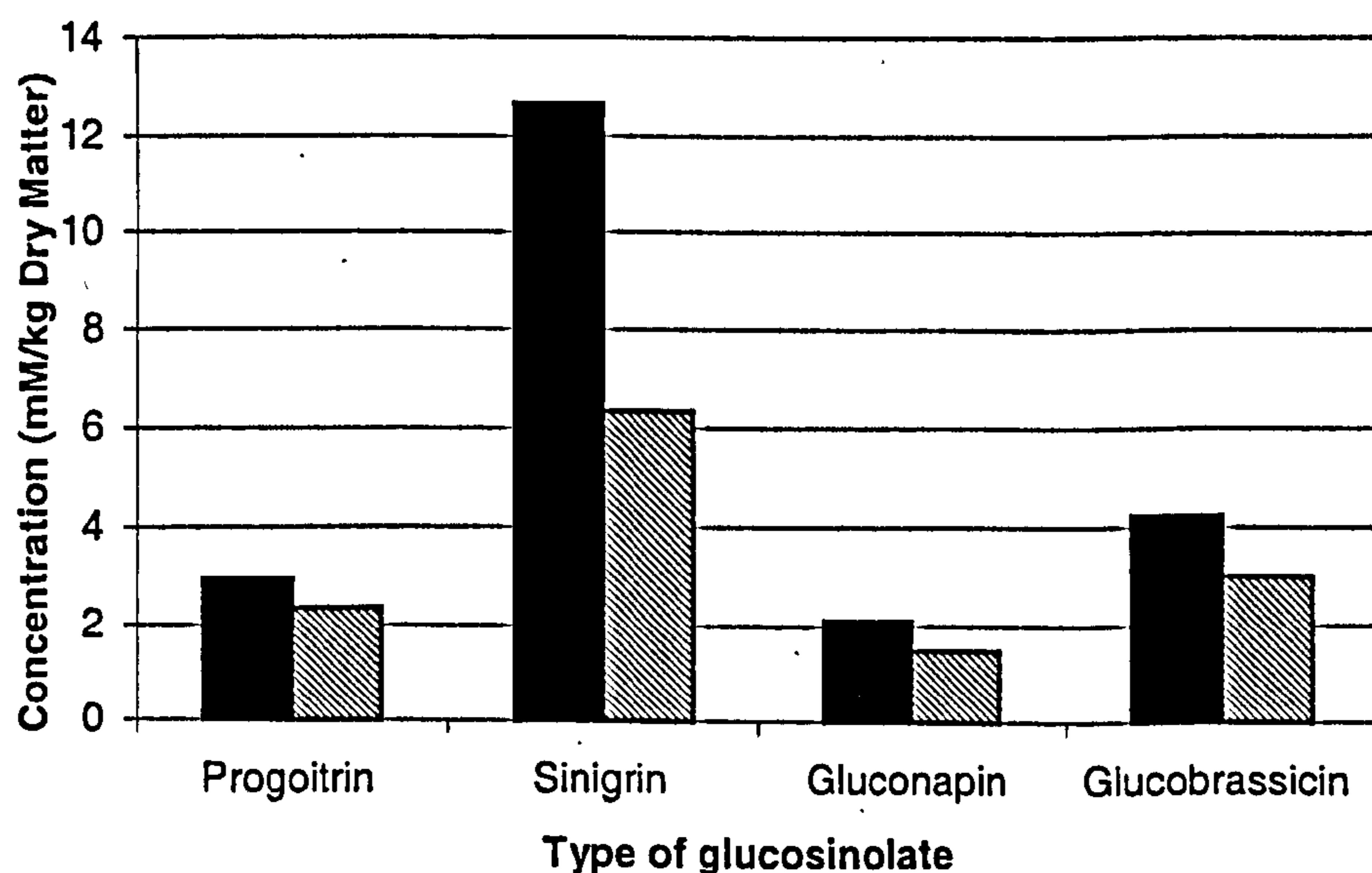
Table 1.2: Examples of glucosinolate-containing *Brassicaceae*

Linnaean name	Common name
<i>Brassica oleracea</i> L.	
<i>gongylodes</i> group	Kohlrabi
<i>capitata</i> group	Red/white cabbage
<i>sabauda</i> group	Savoy cabbage
<i>gemmifera</i> group	Brussels sprouts
<i>italica</i> group	Broccoli
<i>botrytis</i> group var. <i>cauliflora</i>	Cauliflower
<i>acephala</i> group var. <i>millecapitata</i>	Thousand-head kale
var. <i>selensia</i>	Curly Kale
var. <i>sabellica</i>	Collard
<i>Brassica alboglabra</i>	Chinese kale
<i>Brassica chinensis</i>	Chinese cabbage
<i>Brassica campestris</i>	Turnip
<i>Brassica napus</i>	Swede
<i>Brassica nigra</i>	Black mustard
<i>Brassica juncea</i>	Brown mustard
<i>Sinapis alba</i>	White mustard
<i>Armoracia lapathifolia</i>	Horseradish
<i>Wasabi japonica</i>	Wasabi

The distribution of glucosinolates is variable between species (VanEtten *et al.* 1976; Heaney & Fenwick, 1980; Carlson *et al.* 1981; Carlson *et al.* 1987; Kushad *et al.* 1999). Among the Brassica vegetables consumed in the human diet, glucobrassicin is

the most widespread glucosinolate (Nugon-Baudon & Rabot, 1994). Among the aliphatic glucosinolates, progoitrin and sinigrin occur most often. On the other hand, gluconasturtiin (phenethyl glucosinolate) occurs in a restricted number of brassica species, including, for example, watercress. The proportion of indole glucosinolates to aliphatic glucosinolates in root vegetables, such as swede and turnip, is generally higher than in leafy vegetables (Carlson *et al.* 1981; Carlson *et al.* 1987). These large differences in glucosinolate profile have generated research studies on a wide range of cruciferous vegetables as it is not possible to consider one brassica as a model vegetable. Indeed, it is not fully understood whether the beneficial properties of brassicas are related to their full glucosinolate profile or to their content of specific glucosinolates.

Figure 1.2: Glucosinolate concentrations in *cyrus* and *maximus* varieties of Brussels sprouts (Verkerk R., 1998, personal communication). Solid bars correspond to the *cyrus* variety and hatched bars correspond to the *maximus* variety



Within variety, the profile of glucosinolates is generally similar. Broccoli, for instance, is devoid of sinigrin despite a high content of total glucosinolates. The amount of glucosinolates can, however, vary according to the cultivar. For instance, sinigrin is generally present in significant amounts in Brussels sprouts (Carlson *et al.* 1987). The *cyrus* cultivar, however, has a sinigrin concentration twice as high as the *maximus* cultivar (Figure 1.2). Concentrations of glucosinolates also vary within the different parts of plants. They are generally higher in seeds and growing organs than in vegetative tissues (Pihakaski & Pihakaski, 1978). Culture conditions have been

shown to significantly influence glucosinolate content (Heaney & Fenwick, 1980; Rosa *et al.* 1994; Ciska *et al.* 2000). When plants are subjected to stress, concentrations of glucosinolates also change. These factors, together with the multiplicity of methods of measurement of glucosinolates in plant material, make it difficult to compare glucosinolate profiles between vegetables and between studies. However, in any given variety of cruciferous vegetables the relative proportion of individual glucosinolates is relatively stable (Kushad *et al.* 1999).

1.3.4. Glucosinolate intake by humans

Glucosinolate intake is difficult to assess not only due to the large variation in glucosinolate content in vegetables but also the large within-population variation in choices regarding consumption of cruciferous vegetables. Cruciferous vegetable consumption varies markedly with country, season and social group (Sones *et al.* 1984). One more difficulty is the fact that cruciferous vegetables are often processed either industrially or domestically, resulting in further change in glucosinolate content either by hydrolysis during chopping or by leaching in cooking water. These effects have been discussed elsewhere (de Vos & Blijleven, 1988; Mithen *et al.* 2000). The most reliable attempt to estimate the mean daily intake of glucosinolates reported a value of 46.1 mg/day (approximately 100 μ mol/day) when raw vegetables were ingested (Sones *et al.* 1984). This intake was calculated on the basis of the cruciferous consumption in the UK in 1980 and the glucosinolate content of vegetables available commercially at that time. However, the mean daily intake would probably be different now since the consumption of cruciferous vegetables has decreased from 49.7 g/person/day in 1980 to 25 g/person/day in 1998 (National Food Survey, 1999) and the varieties of vegetables available are different. Large within-population variation in the quantity and range of glucosinolates that are ingested is to be expected. For instance, the result of a food frequency questionnaire among 246 Singapore Chinese showed a very high frequency of cruciferous vegetable consumption (345 times per year) and a mean daily intake of 40.6 g (Seow *et al.* 1998). There was, however, a 2.5 fold difference between the low consumer and the high consumer group. The average glucosinolate intake in Singapore is probably much higher than in the Western population. Little is known about the intake of individual glucosinolates. More information on the kind of cruciferous

vegetables ingested and the amount consumed would be necessary for more accurate estimates of glucosinolate composition. Indeed, the wider the range of vegetables eaten, the more varied is the glucosinolate intake. A large variety of glucosinolates ingested may not necessarily be related to a high intake. Conversely, intake of one particular glucosinolate can be very low despite a substantial ingestion of total glucosinolates. Broccoli and cauliflower, for instance, contain very little, or no, sinigrin despite a high content of total glucosinolates (Carlson *et al.* 1987). This information will be necessary to identify whether the beneficial effect of cruciferous vegetables is related to the amount of total glucosinolates ingested, or to specific glucosinolates. In addition, intake of readily available breakdown products as they often occur in condiments must be taken into account. A more detailed knowledge of the cruciferous vegetable consumption habits would be useful for experimental investigations. Indeed, feeding trials using cruciferous vegetables with a wide range of glucosinolates may give qualitative results which provide useful epidemiological information. Quantitative studies, however, need to focus on a restricted number of glucosinolates for which the biological activities have been demonstrated. This information could ultimately lead to selection and commercialisation of vegetable varieties with high health potential.

1.3.5. Biological properties

Regardless of their importance in the defence mechanism of plants, once ingested by animals or humans, intact glucosinolates are thought to have no biological influence *per se*. Germ-free rats given rapeseed meal did not develop the adverse effects generally observed in animals harbouring a viable microflora, suggesting that glucosinolates have to be broken down to generate biological effects *in vivo* (Nugon-Baudon *et al.* 1988). The growth of tumour cells *in vitro* was not affected when they were challenged with purified glucosinolates whereas the related breakdown products showed anti-proliferative effects (Leoni *et al.* 1997). A study *in vitro* on a Chinese hamster ovary cell line has shown that intact sinigrin and gluconasturtiin can have genotoxic effects on cells but the amount of glucosinolates required to obtain these effects, however, is unlikely to occur *in vivo* (Musk *et al.* 1995). Therefore, the hydrolysis of glucosinolates is the primary process leading to the development of a biological response in the host.

1.4. Myrosinase

Myrosinase is a thioglucoside glucohydrolase (EC 3.2.3.1) responsible for the hydrolysis of glucosinolates. Myrosinase activity in plant cells has been well characterised. Recent studies have demonstrated that thioglucosidase activity can also occur in other organisms such as fungi and mammalian gut bacteria (Oginsky *et al.* 1965; Smits *et al.* 1993).

1.4.1. Plant myrosinase

1.4.1.1. Biochemistry

Plant myrosinase is a glycoprotein with a molecular mass varying from 125 to 150 kDa (Bones & Rossiter, 1996). Several isoforms of the enzyme have been isolated from different cruciferous vegetables. Some isoforms coexist within the same plant species such as in the swede (*Brassica napus*) (Bones & Slupphaug, 1989). The biochemical characteristics of the enzyme differ according to the vegetable matrix (Table 1.3). Generally, the optimum activity is at neutral pH although myrosinase activity of *Brassica napus* and *Sinapis alba* was optimum at a pH of 5 (Björkman & Janson, 1972; Bones & Slupphaug, 1989). The optimum activity is at a temperature within the range of 55-75°C (Springett & Adams, 1989; Yen & Wei, 1993). Above this range of temperature, non-reversible inactivation of the myrosinase occurs. These properties are of importance as most cruciferous vegetables are heated at temperatures of around 100°C before ingestion. On the other hand, myrosinase is stable at -20°C which indicates a good stability in frozen vegetables. Activity of myrosinase is also decreased by high pressure which may be used in industrial preparation to preserve vegetable flavours although it is not known whether the inactivation is reversible (Ludikhuyze *et al.* 2000). The purification and amino-acid sequencing of myrosinase has allowed the elucidation of its structure and this is detailed elsewhere (Bones & Slupphaug, 1989). Little is known, however, about the conformation of the catalytic site (Bones & Rossiter, 1996). A family of genes coding

Table 1.3: Biochemical characteristics of myrosinase enzyme from edible cruciferous plants. ND = Not determined, T = temperature.

Vegetables	Number of isoenzymes	Molecular Weight (kDa)	Optimum pH	Optimum Temperature (°C)	Activation factors	Inhibition factors	Reference
Broccoli	ND	ND	6.5-7.0	30	MgCl ₂ +Ascorbic acid Pressure 50-75 MPa	T>50°C Pressure>150 MPa	Ludikhuyze et al 2000
Red and white cabbage	ND	ND	6.0-8.0	60 (red cabbage) 50 (white cabbage)	Ascorbic acid	T>70°C	Wilkinson et al 1984 & Yen et al 1993
Brussels sprouts	ND	ND	6.0-6.5 8.0	50	Ascorbic acid	T>60-90°C pH<5.0	Springett & Adams 1989
Swede	3	154	4.9-5.0	75	Ascorbic acid	ND	Bones & Slupphaug 1989
Mustard	3	151	5.1	60	Ascorbic acid	T>60°C	Bjorkman & Janson 1972
Mustard seed	4	152/125	4.6-4.8	ND	Ascorbic acid	ND	Ohtsuru & Hata 1972

for myrosinase have been characterised and partially sequenced in cruciferous forage (Bones & Rossiter, 1996).

Plant myrosinase is usually stored in the vacuoles of discrete specialised cells called myrosin cells or idioblasts (Thangstad *et al.* 1990). These cells are distributed among the non-specific glucosinolate-containing cells in plant tissues (Bones & Rossiter, 1996). Little is known about the relationship between glucosinolate content and myrosinase occurrence in the plant. Analysis of the edible parts of cruciferous vegetables has not shown a significant correlation between myrosinase activity and glucosinolate content (Yen & Wei, 1993). However, as glucosinolates and myrosinase are involved in the same functions and found in the same family of plants, they are thought to be related in a "myrosinase-glucosinolate system".

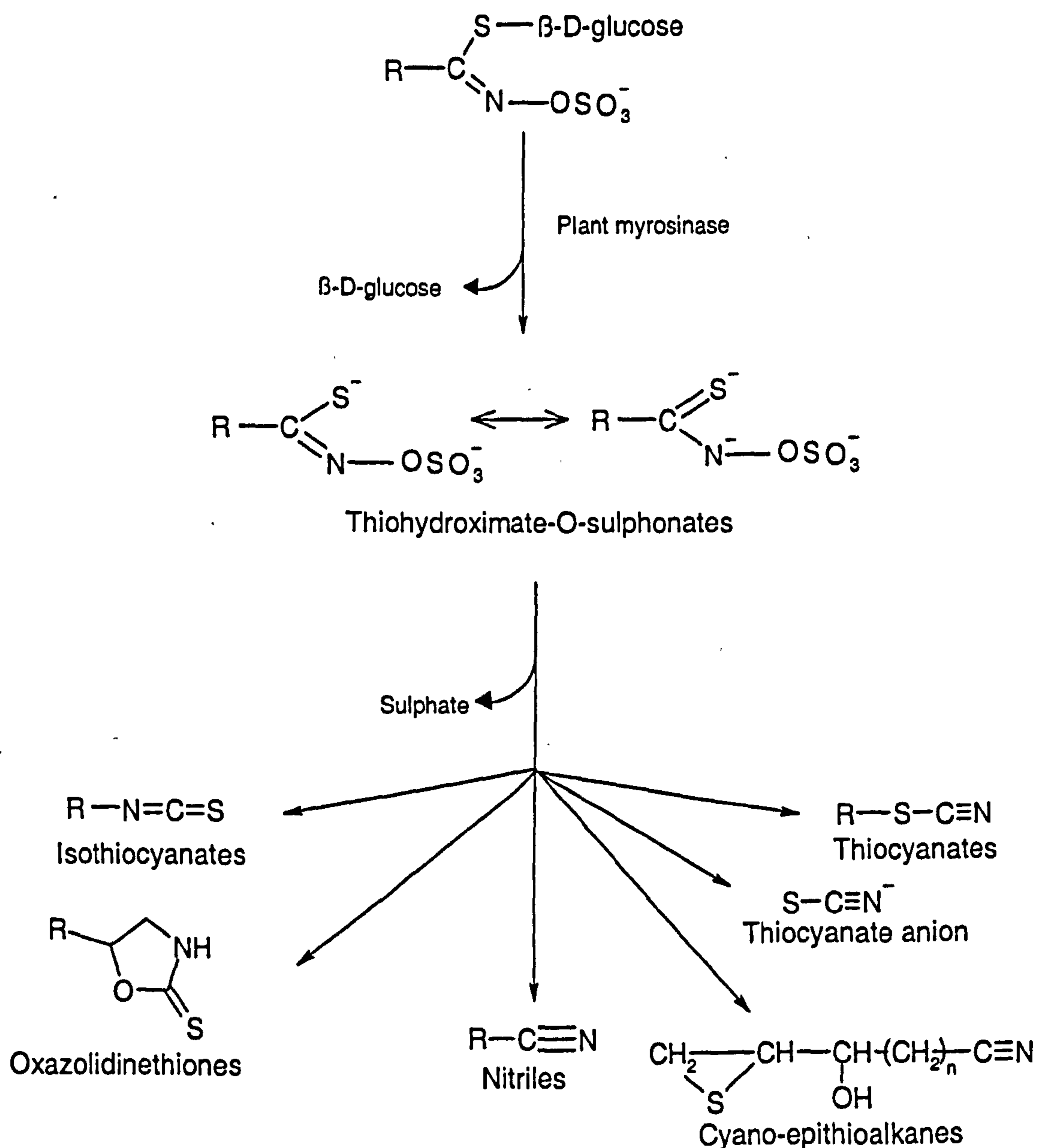
Several studies have focused on the distribution of myrosinase activity, showing large variation according to plant part (Bones, 1990). In white mustard (*Sinapis alba*), for instance, the enzyme activity decreases from storage tissues, where it is at its highest, then in the growing organs and finally in vegetative parts (Pihakaski & Pihakaski, 1978). Myrosinase activity was also found to be higher in the outer leaves of Brussels sprouts than inner leaves, presumably as a defence mechanism against external pathogens (Springett & Adams, 1989). Measured myrosinase activity in different plant tissues also varies with the total concentration of proteins of the considered tissue, the stability of the myrosinase in the myrosin cells and the level of myrosinase synthesis. As a result, myrosinase activity can be highly variable according to circumstances. For instance, upon cell disruption, the synthesis of myrosinase is increased which may result in misleading measurements of myrosinase activity. Several methods of measurement of myrosinase activity have been described, all relying on a similar principle: the enzyme is purified from the plant cell and subsequently incubated with sinigrin as substrate. Methods differ in their approach to the measurement of sinigrin hydrolysis (Palmieri *et al.* 1987). The use of molecular tools, such as mRNA, has allowed an estimation of myrosinase synthesis which potentially facilitates comparison of myrosinase distribution between different tissues or different species of brassicas (Falk *et al.* 1992). Myrosinase expression could not, however, account for the total capacity of myrosinase activity in the cells

as myrosinase can be a stable and long-lived molecule in contrast to other β -glucosidases (Botti *et al.* 1995). This uncertainty in the measurement of myrosinase activity highlights the difficulty in selecting cruciferous vegetables on the single criteria of myrosinase activity.

1.4.1.2. Myrosinase-catalysed hydrolysis

When plant cells are damaged, by food processing for instance, myrosinase is released and interacts with glucosinolates stored in other compartments of the plant cell. Plant myrosinase is responsible for the hydrolysis of the thioglucoside bond of glucosinolates. A proposed mechanism suggests that myrosinase catalysis occurs in two steps (Botti *et al.* 1995; Iori *et al.* 1996). The first step is the formation of an unstable intermediate compound, the thiohydroximate-O-sulphonate (Figure 1.3), immediately followed by the release of glucose. The aglucone moiety is spontaneously transformed to yield sulphate and a wide range of breakdown products including isothiocyanates, nitriles, epithioalkanes, oxazolidinethiones, thiocyanate anions and organic thiocyanates (VanEtten *et al.* 1966). Section 1.5 describes the chemistry of these breakdown products and their metabolic fate. The myrosinases from different plant species are not adapted to a specific set of glucosinolates (Pihakaski & Pihakaski, 1978). Indeed, studies on the mechanism of hydrolysis have shown that the reaction was specific to the thioglucoside bond and to the β -D-glucose moiety, the myrosinase having no binding site to the side-chain (Botti *et al.* 1995). In the absence of a thioglucoside bond, in the desulphoglucosinolate structure for instance, myrosinase-catalysed hydrolysis does not occur (Hanley *et al.* 1990). It is still unclear, however, whether or not myrosinase is involved in the subsequent rearrangement of the intermediate aglucone. The composition of the side-chain on another hand is involved as will be discussed in the following section. Furthermore, it is not known whether the new hydrolysis products formed can regulate myrosinase hydrolysis.

Figure 1.3: Enzymic hydrolysis of glucosinolates and release of breakdown products



1.4.1.3. Factors influencing glucosinolate hydrolysis

Glucosinolate hydrolysis may be influenced by external factors. Ascorbic acid (vitamin C) is an essential activator of myrosinase catalysis (Ohtsuru & Hata, 1979). Cruciferous vegetables have a high content of vitamin C and it is thought that it plays a role in the efficiency of the glucosinolate-myrosinase system. The reported optimum vitamin C concentration varies according to studies and vegetables. An optimum concentration of 5-10 mM for broccoli and cabbage myrosinase has been reported in recent studies but much lower concentrations were suggested in earlier studies (Wilkinson *et al.* 1984; Yen & Wei, 1993; Ludikhuyze *et al.* 2000). These concentrations were derived from experiments where exogenous vitamin C was added to the reaction medium and they do not represent the actual cellular

concentrations found in the vegetable. It is hypothesised that ascorbic acid can bind to a specific site on myrosinase, changing the conformation of the catalytic site which facilitates the hydrolysis. High concentrations (greater than 50 mM) of ascorbic acid lead to an inactivation of myrosinase, probably by competing for the catalytic site (Ohtsuru & Hata, 1979; Bones & Rossiter, 1996).

The presence of myrosinase-binding proteins associated with the cell membrane has been reported (Bones & Rossiter, 1996). Evidence for their specific function and mode of action are still sparse and incomplete (Bones & Rossiter, 1996). Metallic ions, such as Mg^{2+} associated with ascorbic acid, can enhance myrosinase activity (Ludikhuyze *et al.* 2000). Other factors also influence glucosinolate hydrolysis by interacting with the unstable intermediate formed after myrosinase catalysis. An epithiospecifier protein has been shown to facilitate the rearrangement of thiohydroxamate-o-sulphonate to epithionitriles for glucosinolates possessing an unsaturated side-chain. This small protein (30-40 kDa) was found in several edible cruciferous vegetables such as Brussels sprouts, turnip, garden cress, mustard and horseradish (Petroski & Tookey, 1982; MacLeod & Rossiter, 1985). The presence of ferrous ion also enhances the activity of epithiospecifier protein (MacLeod & Rossiter, 1985). The structure of glucosinolates and the nature of the reaction medium also have a strong influence on the rearrangements of the intermediate aglucone into hydrolysis products. For instance, variation in pH in the reaction medium leads to the formation of different breakdown products (Uda *et al.* 1986). At neutral or alkaline pH, isothiocyanates are the predominant products. At an acidic pH, the formation of nitriles predominates. The presence of metallic ions, such as ferrous ions, in the reaction medium enhances the pH effect by increasing nitrile release at the expense of isothiocyanates at both acidic and neutral pH (Tookey & Wolff, 1970; Uda *et al.* 1986). This switch in the pattern of released breakdown products is important in terms of biological effects. Glucosinolates are subject to extreme pH variation and chemical exposure throughout the process of food preparation and digestion. According to whether vegetables are consumed in an acidic form such as pickled cabbage or coleslaw, or in other preparations, the nature of hydrolysis products ingested by consumers may change. Furthermore, after ingestion of the vegetables, the glucosinolates are also exposed to the mineral

concentrations and pH of digestive fluids. These factors may influence the type of hydrolysis products actually available in the digestive tract. In an experiment where rapeseed meal was incubated under *in vitro* conditions simulating digestion in the stomach and small intestine of pigs, the concentration of the breakdown product of progoitrin, oxazolidine thione, was modified according to the origin of the biological fluids (Maskell & Smithard, 1994).

The result of glucosinolate hydrolysis is therefore complex. Although myrosinase hydrolysis is likely to be the major hydrolytic pathway, non-enzymatic hydrolysis may also occur in acidic conditions (Maskell & Smithard, 1994). According to variation in the medium conditions, such as pH, content of ascorbate, presence of bivalent ions, conditions of temperature, pressure and UV and ionising radiation, the myrosinase activity can be enhanced or suppressed and, therefore, the contribution of non-enzymatic hydrolysis changes. It is therefore desirable to expand studies to clarify the conditions that are most likely to be important in particular physiological circumstances.

1.4.2. Myrosinase activity in microorganisms

1.4.2.1. Myrosinase activity in fungi

Several authors have reported the presence of a myrosinase-like activity in fungi. Most of the strains involved are from the *Aspergillus* and *Fusarium* genera (Ohtsuru & Hata, 1973; Smits *et al.* 1993). In the same way as for plant myrosinase, myrosinase from fungi is able to catalyse the breakdown of glucosinolates to isothiocyanates and nitriles (Smits *et al.* 1993). The mechanism of breakdown has not been well described. The nature of released metabolites and the kinetics of hydrolysis seems to differ according to species and to the type of glucosinolates considered (Smits *et al.* 1993). Studies on *A. niger* and *A. clavatus* suggested that the fungal enzyme is located in the cytoplasm and its activity is unstable (Ohtsuru *et al.* 1973; Smits *et al.* 1993). The characterised strains of fungi grow on cruciferous vegetables and have been considered as agents for the detoxification of brassica-based feedingstuffs. No fungus possessing a myrosinase activity has yet been identified in the mammalian microfauna. The involvement of fungal myrosinase

activity, however, is more relevant to ruminant species than human subjects as the human digestive tract does not harbour a constitutive microfauna.

1.4.2.2. Microbial myrosinase activity

The digestive microflora of mammals and birds has been shown to be a potent facilitator of glucosinolate hydrolysis. Myrosinase activity has been demonstrated in digestive and faecal contents of humans, rats, pigs and poultry (Greer, 1962; Slominski *et al.* 1988; Maskell & Smithard, 1994; Michaelsen *et al.* 1994) and in sheep and cattle rumen fluid (Lanzani *et al.* 1974; Wathelet *et al.* 1995). Myrosinase activity has also been reported for bacteria found in soil and food (Tani *et al.* 1974; Brabban & Edwards, 1994; Palop *et al.* 1995).

Several authors have isolated strains of bacteria possessing myrosinase activity from human faeces (Oginsky *et al.* 1965; Rabot *et al.* 1995). Bacterial identification has shown that myrosinase activity occurs in a large variety of bacterial clusters in the dominant and subdominant bacterial populations. Table 1.4 summarises the main genera identified and the substrates used for the characterisation of the myrosinase activity. Anaerobic conditions seem to be more favourable to the expression of myrosinase activity, although some isolated strains can grow in aerobic conditions (Oginsky *et al.* 1965; Tani *et al.* 1974; Rabot *et al.* 1995). This observation may explain the fact that bacterial myrosinase has only been characterised in one strain of bacterium, *Enterobacter cloacae* (Tani *et al.* 1974). The isolated enzyme was smaller than plant and fungal myrosinase (molecular weight 61 kDa). It was found to be intracellular with an optimum activity at neutral pH. These latter characteristics were found to be similar in the human digestive strain of *Paracolobactrum aerogenoides* and in a food-borne strain of bacterium *Lactobacillus agilis* (Oginsky *et al.* 1965; Palop *et al.* 1995). All the studies *in vitro* demonstrated that bacterial myrosinase activity was induced when bacteria grew on a medium supplemented with glucosinolates but myrosinase activity was suppressed when glucose was available (Tani *et al.* 1974; Brabban & Edwards, 1994; Maskell & Smithard, 1994; Palop *et al.* 1995). The optimum induction was obtained when concentrations of glucosinolates were in the concentration range from 5 to 10 mM. Above these concentrations, glucosinolates suppressed microbial myrosinase activity (Tani *et al.* 1974). However,

a recent study *in vivo* on the metabolism of sinigrin by the human intestinal strain *Bacteroides thetaiotaomicron* has not shown any differences in sinigrin degradation when the host animals were fed a glucosinolate-based diet or a standard diet (Elfoul *et al.* 1998). Further evidence *in vivo* is required to establish whether microbial myrosinase induction is an artefact due to conditions *in vitro*.

Table 1.4 : Characterisation of bacterial strains possessing a myrosinase activity

Bacterial strain	Number of identified strains	Source	Substrate used for identification	References
<i>Escherichia coli</i>	4	Human faeces	progoitrin	Oginsky <i>et al.</i> , 1965
<i>Paracolobactrum aerogenoides</i>	1			
<i>Paracolobactrum coliforme</i>	1			
<i>Paracolobactrum sp.</i>	1			
<i>Aerobacter aerogenoides</i>	1			
<i>Proteus vulgaris</i>	1			
<i>Alcaligenes faecalis</i>	1			
<i>Pseudomonas aeruginosa</i>	1			
<i>Bacillus subtilis</i>	1			
<i>Bacillus cereus</i>	1			
<i>Staphylococcus epidermidis</i>	1			
<i>Streptococcus faecalis</i>	1			
<i>Escherichia coli</i>	17	Human faeces	sinigrin	Rabot <i>et al.</i> , 1995
<i>Enterococcus faecalis</i>	1			
<i>Enterococcus faecium</i>	3			
<i>Bacteroides thetaiotaomicron</i>	1			
<i>Peptostreptococcus sp.</i>	3			
<i>Escherichia coli</i>	1	Human faeces	rapeseed	Rabot <i>et al.</i> , 1993
<i>Bacteroides vulgatus</i>	1			
<i>Enterobacter cloacae</i>	1	Human faeces	sinigrin	Tani <i>et al.</i> , 1974
<i>Lactobacillus thermobacterium</i>	1	Chicken intestinal content	progoitrin + sinigrin	Nugon-Baudon <i>et al.</i> , 1990

Unlike plant myrosinase activity, the addition of ascorbate to a suspension of glucosinolate-degrading bacteria *in vitro* was found to have a slight inhibitory effect on microbial myrosinase activity but the evidence is weak and confirmation would be necessary (Oginsky *et al.* 1965; Maskell & Smithard, 1994). In the same way as for plant myrosinase, metallic ions may have an influence on microbial hydrolysis of glucosinolates. Evidence for this relies on observations of animal performance: the addition of copper and ferrous ions to rapeseed meals free of plant myrosinase ameliorated the toxic symptoms generated by glucosinolates in farm animals and in rats (Vermorel & Evrard, 1987). Moreover, the hydrolysis of glucosinolates by the

digestive microflora of pigs *in vitro* was significantly retarded by a copper supplementation (Rowan *et al.* 1991; Maskell & Smithard, 1994). It is, however, difficult to draw conclusions on the direct influence of iron and copper on microbial myrosinase. Iron and copper may have a non-specific influence on the digestive microflora, modifying or suppressing microbial metabolic functions or organisms which in turn modify glucosinolate hydrolysis. Alternatively, metallic ions may interact directly with the glucosinolate precursors or the microbial breakdown products, thus modifying the metabolites and their related effects. The addition of polysaccharides to the diet, such as inulin or tylosin, also favourably modify the microbial degradation of glucosinolates but the specific action of these fibre sources on microbial myrosinase has not been investigated (Maskell & Smithard, 1994; Roland *et al.* 1996).

Studies have reported that the kinetics of microbial myrosinase hydrolysis may differ according to the type of glucosinolates considered. This characteristic was noted *in vitro* for the chicken digestive strain *Lactobacillus thermobacterium* (Nugon-Baudon *et al.* 1990) and could be linked to a variable access of the glucosinolate to the catalytic site or to a difference in the re-arrangement of the aglucone product according to the side-chain. These reactions might involve factors such as myrosinase binding proteins or epithiospecifier protein. The existence of such factors in microorganisms is not known and the presumed mechanisms thus remain speculative. Moreover, the presence of a complex microflora with several bacterial strains possessing myrosinase activity could mask the variation observed for individual strains. This is illustrated by the similarity in time curve degradation of five individual glucosinolates by porcine caecal contents (Maskell & Smithard, 1994). The very low amount of glucosinolates (0.4%) found in caecum, colon and faeces of rats fed a rapeseed meal devoid of plant myrosinase confirmed, nevertheless, that microbial myrosinase is able to hydrolyse a wide range of glucosinolates *in vivo* (Campbell *et al.* 1995).

The nature of the hydrolysis products released by bacterial myrosinase is not very well determined. Rats harbouring different strains of human digestive bacteria showed different toxic symptoms when they ingested rapeseed meal devoid of

myrosinase (Rabot *et al.* 1993). It is likely that bacterial myrosinase leads to the formation of a thiohydroximate-O-sulphonate intermediate and glucose as for plant myrosinase although no direct evidence is available. From results observed in chimaera animals, it can be suspected that the re-arrangements of the aglucone might be different according to the complexity of the microflora and to the host species (Nugon-Baudon *et al.* 1988). Studies on ruminants and rats, using rapeseed or progoitrin, measured the production of 5-vinyl oxazolidine-thione, the breakdown product of progoitrin (Oginsky *et al.* 1965; Lanzani *et al.* 1974; Maskell & Smithard, 1994; Wathelet *et al.* 1995). Isothiocyanates and nitriles derived from gluconapin were detected in incubations with cattle rumen fluid *in vitro* (Wathelet *et al.* 1995). Isothiocyanate derivatives were not detected when rapeseed was incubated with porcine digestive contents (Maskell & Smithard, 1994). A measurable amount of thiocyanates was detected in the excreta of hens fed rapeseed but this result could not be reproduced *in vitro* (Slominski *et al.* 1988).

When the metabolism of glucosinolates by single strains was studied *in vitro*, the release of glucose, subsequently metabolised to lactic acid, was observed with *Lactobacillus* strains (Nugon-Baudon *et al.* 1990; Palop *et al.* 1995). The aglucone released *in vitro* from sinigrin was exclusively allyl isothiocyanate with one strain of *Lactobacillus* (Palop *et al.* 1995) and exclusively allyl cyanide in another study on human faecal bacteria (Maisonneuve, 1995). The absence of allyl isothiocyanate however, does not necessarily mean that it is not released. Indeed, other work has suggested that allyl isothiocyanate can be further metabolised by glucosinolate-degrading bacteria (Palop *et al.* 1995). An illustration of this difference in metabolite release is given by a study on *Bacteroides thetaiotaomicron* (Maisonneuve, 1995; Elfoul *et al.* 1998). *In vitro*, the myrosinase activity of this strain was shown to convert sinigrin to allyl cyanide but no allyl isothiocyanate was measured (Maisonneuve, 1995). Conversely, allyl cyanide could not be found, *in vivo*, but allyl isothiocyanate was detected and quantified (Elfoul *et al.* 1998). Recent studies have confirmed the ability of rat and human microflora to release isothiocyanates *in vivo* (Duncan *et al.* 1995; Elfoul, 1999).

Overall, knowledge on microbial myrosinase is still sparse and sometimes contradictory. The general mechanism of hydrolysis and the breakdown products released seem, however, to be similar to those found with plant myrosinase. The findings highlight the importance of working in conditions where the integrity of microbial activity is preserved. Further studies *in vitro* would be necessary to characterise the microbial myrosinase itself. The use of techniques simulating a human digestive ecosystem *in vivo* seems more appropriate to determine the breakdown products released in physiological conditions. The use of animal models, such as rats harbouring a human flora, may be a suitable tool for the investigation of glucosinolate metabolism by human gut bacteria.

1.4.3. Myrosinase activity in cells and tissues

There is no direct evidence in the literature of myrosinase activity in the intestinal epithelium. One study has reported the existence of a mammalian thioglucosidase but activity with glucosinolates as substrates was not tested (Goodman *et al.* 1959). In a study on germ-free rats where the toxicity of a diet enriched with glucosinolates was investigated, it was demonstrated that in the absence of plant or bacterial myrosinase, glucosinolates did not induce any toxicity, suggesting that mammalian tissue does not possess myrosinase-like enzymatic activity (Rabot *et al.* 1993). These results were corroborated by a more recent study conducted in germ-free rats receiving a substantial dose of sinigrin. In this experiment, breakdown products could not be quantified in the intestinal contents and intact sinigrin was recovered in faeces and urine (Elfoul, 1999).

1.4.4. Metabolic fate of intact glucosinolates *in vivo*

It has been suggested that, in the absence of myrosinase activity, glucosinolates could be absorbed directly by the intestinal tract (Lo & Hill, 1972; Freig *et al.* 1987; Slominski *et al.* 1988; Michaelsen *et al.* 1994). Studies in rats and poultry have measured urinary excretion of intact glucosinolates after ingestion of rapeseed meal (Lo & Hill, 1972; Freig *et al.* 1987). The excretion was much higher in rats (40% of administered dose) (Freig *et al.* 1987) than in poultry (2-3% of administered dose) (Lo & Hill, 1972) suggesting species differences. The absorption of intact glucosinolates is supported by findings demonstrating the passage of glucosinolates

across the intestinal epithelium of rats using an *in vitro* everted intestinal sac method (Michaelsen *et al.* 1994). The study suggested that a passive or facilitated transport may occur and no evidence of active transport through the intestinal mucosa was found. Small concentrations of glucosinolates were detected in blood of poultry (0.5-0.6% of ingested glucosinolates) (Freig *et al.* 1987; Slominski *et al.* 1988). The possibility that glucosinolates may bind to transport proteins, however, make it difficult to measure blood concentrations of glucosinolates accurately (Michaelsen *et al.* 1994). There have been no conclusive attempts to measure intact glucosinolates in human blood to date. From the data available, the extent of intact glucosinolate absorption seems limited *in vivo*. Results obtained so far, however, cannot exclude the possibility that glucosinolates, once absorbed, may undergo further metabolism thereby explaining a wide variation in urinary excretion. A potential accumulation of glucosinolates in the intestinal tissues has been rejected by the everted intestinal sac study (Michaelsen *et al.* 1994). The desulphation of intact glucosinolates by mammalian sulphatase in the digestive tract was also refuted by a study showing an absence of desulphoglucosinolate in urine and faeces of rats administered an oral dose of sinigrin (Elfoul, 1999).

1.5. Breakdown products of glucosinolates

The biological properties of glucosinolates are attributed to their breakdown products. Since the 1970s, the toxicity of glucosinolate breakdown products has been particularly well studied since the consumption of cruciferous plants by livestock has been shown to induce physiopathological disturbances in animals. The mechanism of toxicity is complex since several breakdown products may interact with each other to produce the detrimental effects. The symptoms induced by a specific breakdown product may also differ in different animal species (Fenwick *et al.* 1983). The toxicity of glucosinolates and breakdown products in animals has already been reviewed elsewhere (Duncan & Milne, 1989; Nugon-Baudon & Rabot, 1994). In humans, the occurrence of toxicity symptoms from glucosinolate breakdown products are rare (Michajlovskij *et al.* 1969). In contrast, the involvement of specific metabolites, such as isothiocyanates, in cancer protection has generated a large body of work on their mechanisms of action whereas information is still sparse on other minor metabolites.

1.5.1. Isothiocyanates

Most of the glucosinolates give rise to stable isothiocyanates. These are highly volatile aglucones and confer the typical pungent flavour to brassica vegetables (Fenwick *et al.* 1983). Plants are thought to produce these secondary compounds either as attractants or repellents of insects or pests (Fenwick *et al.* 1983). They also possess a range of medicinal properties and have been employed in the past as antibiotics for the treatments of infections of the respiratory and urinary tracts (Fenwick *et al.* 1983; Mennicke *et al.* 1988).

The anti-cancer properties of isothiocyanates are multiple as they act at several stages in the carcinogenic process (Johnson *et al.* 1994). They act as blocking agents by enhancing the detoxification metabolism of carcinogenic compounds. This chemopreventive effect is currently the most documented area of research and several reviews describe the mechanisms involved (Zhang & Talalay, 1994; Hecht, 1995; Verhoeven *et al.* 1997; Hecht, 1999). Briefly, the primary mechanism involved is the induction of phase II enzymes of the xenobiotic-metabolising system which is the enzymatic complex responsible for the biotransformation of carcinogens (Nugon-Baudon & Rabot, 1994). The enzymes of the phase II system are responsible for the conjugation of foreign compounds to hydrophilic groups so that they are excreted more readily, thereby decreasing their potentially harmful effects. From variation obtained in studies according to the chosen experimental models, the chemical carcinogen, the considered isothiocyanate and the enzymes investigated, it is suspected that isothiocyanates act quite specifically (Zhang & Talalay, 1994; Hecht, 1999). Several naturally occurring glucosinolates have been shown to have significant effect at this stage. They are isothiocyanates derived from aromatic glucosinolates or from methylsulphanylalkyl glucosinolates. Phenethyl isothiocyanate has a specific chemopreventive effect on tobacco-related nitrosamines and is therefore efficient against lung tumorigenesis (Chung, 1992). Benzyl isothiocyanate is able to enhance detoxification enzymes specifically in digestive tissues and exerts a protective effect against polycyclic hydrocarbons and heterocyclic aromatic amines which may be found in food (Wattenberg, 1977; Sparmins *et al.* 1982; Kassie *et al.* 1999). In very low concentrations (from 0.2 to 0.5 μM), 4-methylsulphanylbutyl

isothiocyanate (sulphoraphane), 7-methylsulphinylheptyl isothiocyanate and 8-methylsulphinylloctyl isothiocyanate act as efficient inducers of hepatic detoxification enzymes (Zhang *et al.* 1992; Fahey *et al.* 1997; Faulkner *et al.* 1998; Rose *et al.* 2000). Sulphoraphane also inhibits mammary tumour incidence in rats (Fahey *et al.* 1997). The preventive effect of isothiocyanates was emphasised by studies where the administration of the isothiocyanates after exposure to the carcinogen did not result in a protective effect (Wattenberg, 1977; Hecht, 1999). Other mechanisms of cancer prevention by isothiocyanates include anti-proliferative effects on tumour cell growth and enhancement of apoptosis in tumour cell lines (Musk & Johnson, 1993; Pintao *et al.* 1995; Leoni *et al.* 1997; Kirilin *et al.* 1999). These effects were particularly convincing with allyl isothiocyanate (Johnson, 1993; Smith *et al.* 1998; Kirilin *et al.* 1999; Lund *et al.* 2000; Musk & Smith *et al.* 2000). The action of isothiocyanates is however paradoxical as they can be cytotoxic and genotoxic for cells *in vitro* or at high doses *in vivo* (Bruggeman *et al.* 1986; Musk *et al.* 1995; Nastruzzi *et al.* 1996; Kassie *et al.* 1999). At normal levels of dietary exposure in humans, these effects were not demonstrated. On the contrary, an enhancement of DNA repair was observed (Verhagen *et al.* 1997; Kassie *et al.* 1999; Kassie *et al.* 1999). Studies have also reported that isothiocyanate-conjugates presented the same biological effects as free isothiocyanates although to a lesser extent (Adesida *et al.* 1996; Chung *et al.* 1997). The isothiocyanate-conjugates may, however, act as transporting agents as only free isothiocyanates can cross the cell membrane (Bruggeman *et al.* 1986).

1.5.2. Nitriles and other minor breakdown products

The release of nitriles from glucosinolate hydrolysis has been reported for several cruciferous vegetables. The nitriles derived from glucosinolates are complex organic nitriles (Table 1.5) (Daxenbichler *et al.* 1977). Nitriles produced from the hydrolysis of aliphatic glucosinolates undergo a rearrangement into cyano-epithioalkanes. The formation of epithioalkanes is generally mediated by the epithiospecifier protein (Bones & Rossiter, 1996). The release of nitriles from glucosinolate hydrolysis has been correlated with the toxicity of glucosinolates in livestock. Preferential target organs of nitriles include the liver, kidneys and neural tissues (Duncan & Milne, 1989). The mechanism of toxicity of nitriles released by glucosinolates has not been described but studies on homologous nitriles suggest that the toxicity may be related

to the release of free cyanide (Willhite & Smith, 1981; Farooqui *et al.* 1993). Glutathione conjugation has also been implicated in their toxicity (Pilon *et al.* 1988). Although consumption of nitrile derivatives of glucosinolates may occur when cruciferous vegetables are ingested in their form in coleslaw, the toxicity observed in animals has never been observed in humans. Homologous nitriles have, however, been shown to be neurotoxic and to be inducers of carcinogen (Szabo & Reynolds, 1975; Tanii *et al.* 1993). Nitriles derived from non-indolyl glucosinolates are poor candidates for cancer protection (Nastruzzi *et al.* 2000).

Table 1.5: Examples of nitrile and epithionitrile products derived from glucosinolates

Glucosinolate	Nitriles	Sources	References
Progoitrin	1-cyano-2-hydroxy-3-butene 1-cyano-2-hydroxy-3,4-epithiobutane	Swede	Daxenbichler, 1967
Sinigrin	1-cyano-2,3-epithiopropene allyl cyanide	<i>Brassicaceae</i> plants Cabbage	Cole, 1975 Daxenbichler, 1977
Gluconapin	1-cyano-3,4-epithiobutane	Turnip	Kirk & MacDonald, 1974
Glucotropaeolin	phenylacetonitrile	Garden cress	Cole, 1976
Gluconasturtiin	2-phenylpropionitrile	<i>Brassicaceae</i> plants	Cole, 1975

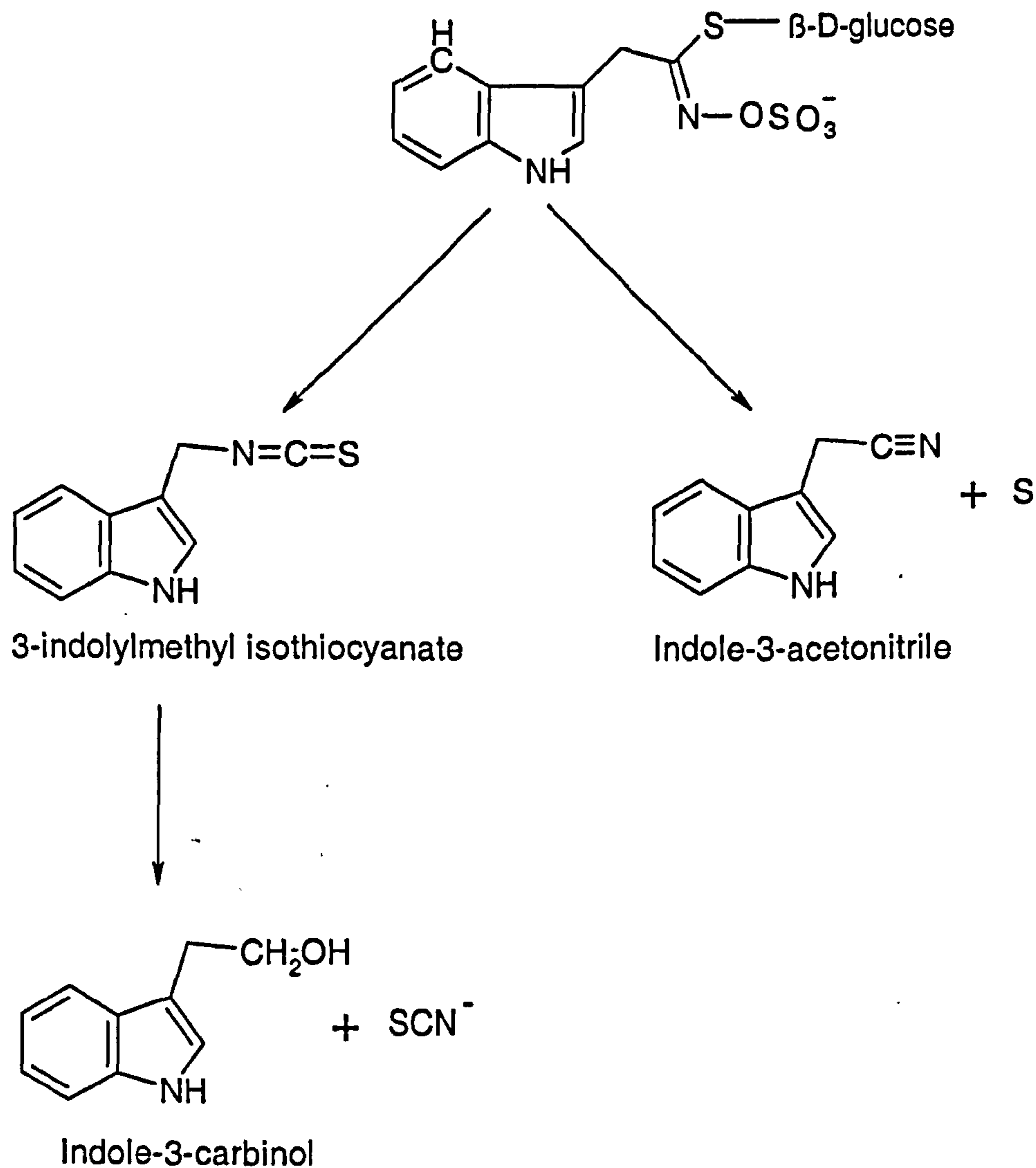
Organic thiocyanates appeared to be released from the hydrolysis of three specific naturally occurring glucosinolates. These are sinigrin, glucotropaeolin, and glucoerucin (Bones & Rossiter, 1996). The formation of these metabolites have been shown in a restricted number of edible plants such as those of the cress family (Fenwick *et al.* 1999). Thiocyanate formation may also result from the post-absorptive metabolism of nitrile derivatives (Pilon *et al.* 1988). Thiocyanates are involved in thyroid disorders and goitre formation in a mechanism similar to goitrin (Duncan & Milne, 1989).

1.5.3. Oxazolidinethione

Oxazolidinethiones derive from the cyclisation of isothiocyanates possessing a hydroxyl group in the 2- position on the R-chain, such as progoitrin which gives rise to an oxazolidine-2-thione commonly known as goitrin. Goitrin has been held responsible for goitre in rats consuming rapeseed meal. The effects of goitrin in laboratory animals have been extensively studied and reviewed (Elfving, 1980;

Duncan & Milne, 1989; Nugon-Baudon & Rabot, 1994) but their relevance in humans is debated. Indeed, the appearance of goitre in humans is restricted to situations where cruciferous vegetable consumption is exceptionally high and iodine intake is low (Michajlovskij *et al.* 1969; Langer *et al.* 1971; McMillan *et al.* 1986).

Figure 1.4: Breakdown products of the indolyl glucosinolate glucobrassicin



1.5.4. Breakdown products of indole glucosinolates

Upon myrosinase hydrolysis, indole glucosinolates also break down to isothiocyanates and nitriles. For instance glucobrassicin is broken down to indole-3-acetonitrile and 3-indolylmethyl isothiocyanate. Due to the presence of the indolyl radical, the isothiocyanate metabolite is further rearranged to a thiocyanate ion and indole-3-carbinol (Figure 1.4). The mechanism of breakdown and the biological effects of indole glucosinolates have been reviewed elsewhere (McDanell *et al.* 1988; Nugon-Baudon & Rabot, 1994). The involvement of indole-3-carbinol in the

chemopreventive effect of cruciferous vegetables has been debated. Its action differs from isothiocyanates as it modulates the effect of phase I enzymes of the xenobiotic-metabolising system but this process may both induce or inactivate the potential carcinogenic compounds (Vermorel & Evrard, 1987; McDanell *et al.* 1988; Zhang *et al.* 1992; Zhang & Talalay, 1994).

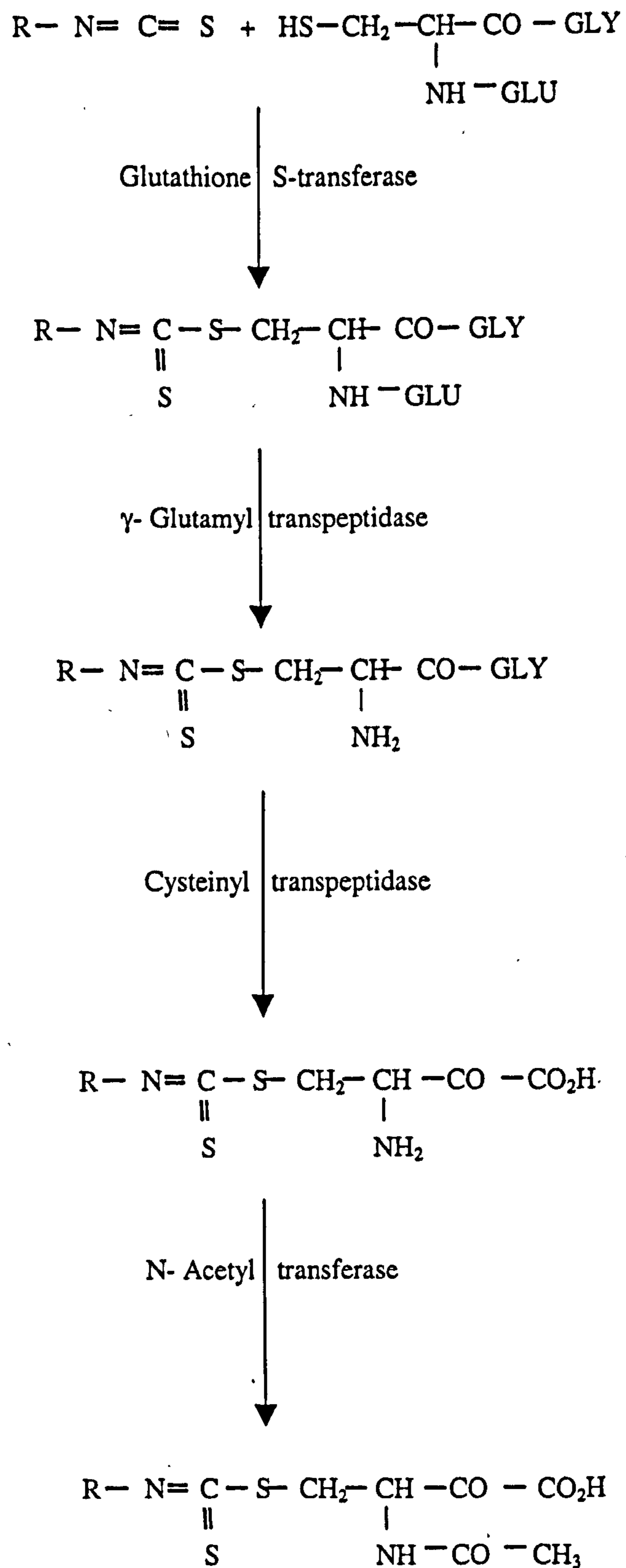
1.6. Disposition and metabolism of breakdown products of glucosinolates

1.6.1. Isothiocyanates

Due to their medical interest, the post-absorptive fate of isothiocyanates has been investigated in several species. The use of radiolabelled isothiocyanates in rats and mice has clarified their metabolism (Brüsewitz *et al.* 1977; Ioannou *et al.* 1984; Bollard *et al.* 1997; Conaway *et al.* 1999). Isothiocyanates are rapidly absorbed by the intestinal tract. A peak of radioactivity in blood occurs approximately three hours after administration (Bollard *et al.* 1997; Conaway *et al.* 1999). Once absorbed by the intestinal tract, isothiocyanates are detoxified, primarily in the liver, where they undergo conjugation with glutathione (Brüsewitz *et al.* 1977). They are subsequently excreted in the urine as their related *N*-acetylcysteine conjugates (*N*-acetyl-S-(*N*-alkylthiocarbamoyl)-L-cysteines) commonly referred to as mercapturic acids (Figure 1.5). The mercapturic acid pathway has been clearly identified as the major route of excretion in rats and humans (Sparnins *et al.* 1982; Mennicke *et al.* 1988). Fifty to 80% of the dose is recovered in the urine within 24h according to the studied isothiocyanate. Isothiocyanates are distributed in the liver, kidneys, lungs and brain but they do not accumulate except in bladder tissues (Bollard *et al.* 1997). Minor excretory pathways are faeces (6 to 9%) and breath (0.1 to 3%). The entero-hepatic pathway is also a variable route of excretion according to the type of isothiocyanate considered and may increase the proportion of faecal excretion (Bollard *et al.* 1997; Conaway *et al.* 1999). Excretion of thiocyanate ion was also detected in the urine of rats after administration of allyl isothiocyanate (Bollard *et al.* 1997).

In humans, studies on the metabolism of isothiocyanates report a metabolism similar to rats. The consumption of garden cress, rich in the benzyl isothiocyanate precursor result in the excretion of the corresponding mercapturic acid; with no other metabolites identified (Mennicke *et al.* 1988). After consumption of brown mustard

Figure 1.5: Detoxification pathway of isothiocyanates into mercapturic acids in rat and human. (Brüsewitz *et al*, 1977). GLY = glycine, GLU = glutamic acid



rich in allyl isothiocyanate, the excretion of the corresponding N-acetyl cysteine conjugate, allyl mercapturic acid was detected (Jiao *et al.* 1994). After consumption of watercress, which is rich in phenethyl glucosinolate, the phenethyl mercapturic acid was measured (Chung *et al.* 1992). A dose-dependent relationship exists between ingestion of isothiocyanates and excretion of the metabolite (Chung *et al.* 1992; Jiao *et al.* 1994; Shapiro *et al.* 1998). The specificity of the mercapturic acid to its precursor and the dose-dependent excretion are interesting characteristics as they suggest the possibility of using the urinary product to trace isothiocyanate uptake. The conversion rate of isothiocyanate to mercapturic acid however may vary. The conversion rate of allyl isothiocyanate was estimated to be 53% (s.e.m 4.05) in humans after ingestion of brown mustard (Jiao *et al.* 1994). In a feeding trial using horseradish, the conversion rate of allyl isothiocyanate was estimated at 42% (s.e.m 1.58) (Shapiro *et al.* 1998).

Other urinary products exist in other species. In guinea-pigs and rabbits, isothiocyanates are excreted as cyclic mercaptopyruvates (Görler *et al.* 1982). In mice, mercapturic acids and cyclic mercaptopyruvates co-exist but the latter is the predominant excretory product (Eklind *et al.* 1990; Bollard *et al.* 1997). In dogs, excretory products are mainly hippuric acids (Mennicke *et al.* 1987). Phenethylamine in the plasma of dogs has also been used as a marker for phenethyl isothiocyanate (Negrusz *et al.* 1998). Phenethylamine, however, is also the main metabolite of the amino acid phenylalanine and therefore is not suitable for quantitative studies of isothiocyanate metabolism (Negrusz *et al.* 1998). N-acetyl cysteine conjugates are easily quantified in urine by HPLC (Mennicke *et al.* 1987). In blood, the measurement of N-acetyl cysteine conjugates is difficult as they are thought to be bound to protein and blood cells (Conaway *et al.* 1999).

1.6.2. Nitriles and other breakdown products

Little is known of the metabolism of nitrile derivatives of glucosinolates. Their metabolic fate may only be assumed from findings with other nitrile compounds. Distribution of radioactive allyl cyanide showed a major excretion in the urine (41%), in expired air (34%) and in faeces (6%) (Farooqui *et al.* 1993). Investigation of the fate of aliphatic nitriles and unsaturated nitriles indicated that metabolic pathways

were complex and variable according to the saturation of the side-chain (Silver *et al.* 1982; Farooqui *et al.* 1993; Markus & Kwon, 1994). Mercapturic acids related to unsaturated acrylonitrile, crotonitrile and cinnamionitrile have been detected in the urine of rats (Van Bladeren *et al.* 1981). A mercapturic acid derivative related to 1-cyano-3,4-epithiobutane, the breakdown product of butenyl glucosinolate, has been found in the urine of rats (Brocker *et al.* 1984). These findings suggest a possible route of excretion for nitrile derivatives from glucosinolates although this has not thus far been investigated.

The enzymic detoxification of nitrile compounds leads in some instances to the formation of cyanide which can be measured in blood (Silver *et al.* 1982; Westley, 1988; Farooqui *et al.* 1993). Further metabolism of cyanide leads to formation of thiocyanate ion which is excreted in urine (Silver *et al.* 1982; Westley, 1988; Farooqui *et al.* 1993). Thiocyanate ion is therefore often used as marker of degradation of cyanogenic compounds (Strugala *et al.* 1995; Carlsson *et al.* 1999). The metabolism of nitriles can also be undertaken by bacteria, suggesting that, *in vivo*, breakdown of nitriles by microflora may occur before absorption (Carter *et al.* 1980; Harris *et al.* 1987).

In investigation of the fate of glucosinolates, measurement of thiocyanate ion can be confusing as a marker of nitrile formation. This is because thiocyanates excreted after ingestion of cruciferous plants may have several origins. They can be formed as direct hydrolysis products of glucosinolates by myrosinase or they can be the metabolic products of indolyl nitriles.

Goitrin has been shown to be excreted mainly in urine of rats in its intact form. A minor excretion of an unknown metabolite was also measured in faeces (Elfving, 1980). The detection of organic thiocyanates in biological fluids remains difficult.

1.7. Fate of glucosinolates and measurement of bioavailability

To understand the balance between the beneficial and detrimental effects of glucosinolates, it is important to determine the bioavailability of the metabolites exhibiting these effects. Bioavailability is generally defined as the proportion of

ingested compound reaching the general circulation and made available for tissue uptake (Fairweather-Tait, 1998). A knowledge of the bioavailability usually encompasses the measurement of three variables; namely the amount of ingested compound, the amount of compound taken up by the intestinal tract and the amount of compound arising in the general circulation (Figure 1.6). In the case of glucosinolate metabolites, there is still a gap of knowledge in the assessment of intestinal uptake and availability of the metabolites in blood. Numerous dietary and host-related variables may interfere at different levels in the metabolic process and modify the final bioavailability of glucosinolate metabolites (Figure 1.6). The administration of vegetables with labelled glucosinolates by humans would be an ideal way of investigation but for technical and ethical reasons this is not possible.

Other approaches must, in consequence, be considered. In this regard, the use of urinary markers is a useful tool. A recent study reported a significant excretion of phenethyl mercapturic acid after ingestion of watercress, a vegetable rich in phenethyl glucosinolate (Getahun & Chung, 1999). The excretion represented 45% of the glucosinolate precursor when watercress was eaten raw and 4.1% when it was cooked. In a different study, the total amount of mercapturic acids excreted in urine was measured after consumption of different cruciferous vegetables (Shapiro *et al.* 1998). In both studies, a wide inter-individual variation was noted but was not accounted for. This variation was attributed to differences in the efficiency of the detoxification process. For this reason, the proportion of markers reported in these studies measured only the minimum amount of isothiocyanates available to the body. Although this demonstrates that the measurement of the end-products of metabolism is a good indicator of isothiocyanate production, the actual intestinal uptake of the metabolites is still unknown. As the key-factor influencing the bioavailability of isothiocyanates and nitriles is their release from glucosinolates, an accurate measurement of this step, independently of the variation occurring during the post-absorptive phase, is paramount for assessing the overall availability.

Figure 1.6: Different stages in the pathway of glucosinolates and potential factors which may influence the bioavailability of their breakdown products

EXTERNAL FACTORS

Glucosinolates content in plant

Food processing

Hydrolysis by plant myrosinase

Interaction with micro-nutrients and other components of the diet

FACTORS RELATED TO INDIVIDUALS

Frequency of consumption of cruciferous vegetables

Mastication

Chemical environment in the digestive tract

Intervention of the microflora

Amount of ingested glucosinolates

Proportion of metabolites available for intestinal cell uptake

Proportion of metabolites transferred to general circulation

Proportion of metabolites exhibiting biological activity

Transport across intestinal wall

Induction of detoxification by xenobiotic metabolizing enzymes

Entero-hepatic circulation

Excretion in urine, breath and faeces

Chemical form

Binding to proteins

Absorption by target organs

Nutritional and physiological status

Exposure to carcinogenic compounds

More recently, studies have applied the use of urinary markers to the specific measurement of isothiocyanate release in the digestive tract of rats. To achieve this, the variability in the post-absorptive fate of isothiocyanates was measured by the administration of an artificial isothiocyanate concurrently to the studied glucosinolate. The recovery of the artificial isothiocyanate allowed the normalisation of the excretion rate for each rat. By this means, the proportion of allyl isothiocyanate released from an oral single dose of sinigrin was found to be 13% in the absence of cruciferous vegetable in the diet and 41% when rats were fed a cauliflower diet (Duncan *et al.* 1997). In a study where rats harbouring a whole human microflora and rats harbouring a single human strain of *Bacteroides thetaiotaomicron* ingested sinigrin, the release of allyl isothiocyanate was 9% and 10% respectively (Elfoul, 1999). This approach has the potential to be of value in investigating metabolism of glucosinolate although existing data is limited and restricted to one isothiocyanate. The application of the method to a larger range of metabolites and to dietary glucosinolates would improve the understanding of glucosinolate hydrolysis *in vivo*.

1.8. Conclusion

Glucosinolates have an interesting status as micro-constituents since they lead either to beneficial or toxic products. Numerous factors can potentially affect the fate of glucosinolates. This literature review stresses the large variability in the glucosinolate distribution in plants. This may be reflected by a wide difference in glucosinolate intake in humans. The existence of several sources of hydrolysis and the crucial influence of environmental factors on the nature of released metabolites have been described but the significance of these results *in vivo* is unclear. Moreover, the extent to which glucosinolate hydrolysis products arise in the gut and are absorbed has not been adequately investigated until now. The improvements of methods of investigation allowing *in vivo* studies where conditions mimic physiological situations would increase the possibilities of exploration and should permit a more comprehensive understanding of the digestive fate of glucosinolates.

CHAPTER 2: EXPERIMENT 1

Assessment of the use of urinary mercapturic acids as markers of glucosinolate metabolite release in rats fed a cruciferous diet.

2.1. Introduction

Isothiocyanates are excreted as their mercapturic acid¹ derivatives in rats (Mennicke *et al.* 1987). These compounds are therefore potentially useful biomarkers for investigation of the metabolic fate of dietary glucosinolates. Mercapturic acids from allyl isothiocyanate, benzyl isothiocyanate and phenethyl isothiocyanate have already been used separately in previous metabolic studies (Brüsewitz *et al.* 1977; Chung *et al.* 1992; Duncan *et al.* 1997). Information obtained from a single isothiocyanate may be misleading since the extent of release of one particular glucosinolate derivative may not be representative of all glucosinolates present in the diet. It is, therefore, important to test a suite of isothiocyanates for which markers are available.

Glucosinolates break down under the action of myrosinase to a number of hydrolysis products of which isothiocyanates are just one group. Nitriles are another important group of glucosinolate breakdown products (Fenwick & Heaney, 1983). To obtain a comprehensive understanding of glucosinolate hydrolysis, it is necessary to follow the formation of several glucosinolate metabolites (isothiocyanates and nitriles for instance) at the same time. As nitriles also undergo conjugation with glutathione (Van Bladeren *et al.* 1981), their recovery as mercapturic acids may also offer a potential tool for measuring nitrile release.

Early studies investigating the digestive fate of glucosinolates have generally used purified glucosinolates given as a single dose to simulate dietary ingestion (Duncan *et al.* 1997; Elfoul *et al.* 1997). Variability in the pattern of metabolite release might occur depending on the mode of ingestion of glucosinolates. Indeed, the kinetics of metabolite release may be different depending on whether the glucosinolates are given as a pure single dose or are present in the diet. These latter compounds are

¹ (N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteines) hereafter referred to as alkyl mercapturic acids for clarity

likely to be absorbed at a much slower rate as they more gradually enter the digestive tract.

The relative proportions of different hydrolysis products which arise is likely to depend on the chemical conditions under which hydrolysis occurs. The presence of metallic ions, such as ferrous ions, has been shown to enhance nitrile production by plant myrosinase at the expense of isothiocyanate formation in experiments *in vitro* (Uda *et al.* 1986). Whether a similar phenomenon occurs in the digestive tract is not known. It may be that the composition of the meal in which brassica vegetables are consumed will influence the profile of breakdown products and hence the ultimate physiological consequences of consuming these vegetables.

Consequently, this experiment was designed to pursue three objectives: (1) to identify the mercapturic acid derivatives of isothiocyanates and nitriles which could be subsequently used in studies *in vivo* using rats, (2) to determine the pattern of glucosinolate degradation when glucosinolates were given as a part of the diet, (3) to investigate whether intake of ferrous ions influenced the profile of primary compounds released during glucosinolate hydrolysis.

Following the conduct of the experiment, the metabolic fate of nitriles by the urinary route was revealed to be insufficiently characterised to attempt a quantification of their excretory products. Analysis of urinary nitrile mercapturic acids was therefore not attempted although inclusion of nitrile compounds in the experiments is fully described.

2.2. Materials and methods

2.2.1. Animals

Twelve male, adult Fischer 344 rats, aged 9 weeks and harbouring a conventional flora (mean weight: 250g, s.e.m. 4.0) (INRA Breeding Unit, Jouy-en-Josas, France), were placed in metabolism cages (Iffa-Credo, Saint Germain sur l'Arbresle, France). Rats were randomly allocated to four groups of three animals (group A, B, C and D). Rats were weighed once a week throughout the experiment. Room temperature was

maintained at 21°C and lights were on a 12:12h light:dark cycle. Food and water were offered *ad libitum*.

2.2.2. Experimental design

Rats in groups A and B acted as control groups (Control treatment) and received pure drinking water whereas rats in group C and D were supplemented with a haem form of iron in their drinking water (Iron treatment, Table 2.1). The experiment was conducted over 30 days divided into two periods. Period 1 involved the measurement of the recovery of isothiocyanates as their mercapturic acid derivatives when they were administered to rats as a single dose. It was also intended to measure the recovery of nitriles as their urinary products in Period 1. During this phase, rats received no glucosinolate in the diet and were administered either with a mixture of isothiocyanates (ITC dose) or nitriles (NITR dose) on two different occasions. Composition of both mixtures corresponded to the expected isothiocyanates and nitriles derived from sinigrin, benzyl glucosinolate, phenethyl glucosinolate and glucobrassicin (Figure 2.1). Groups A and C received the ITC dose first and subsequently the NITR dose whereas the sequence of administration was reversed in groups B and D (Table 2.1).

Period 2 was designed to measure the proportional release of isothiocyanates and nitriles coming from dietary glucosinolates and from dosed sinigrin. Rats were therefore offered a diet containing various glucosinolates but nominally free of sinigrin. Rats were administered on one occasion with a single oral dose of sinigrin (SIN dose). On a different occasion, the expected breakdown products of sinigrin, allyl isothiocyanate and allyl cyanide (ISO/CN dose), were directly administered to the animals to measure their recovery as urinary metabolites. As in Period 1, groups A and C received the SIN dose first and subsequently the ISO/CN dose whereas the sequence of administration was reversed for groups B and D (Table 2.1).

Figure 2.1: Predicted metabolites deriving from studied glucosinolates and included in the dosing mixtures.

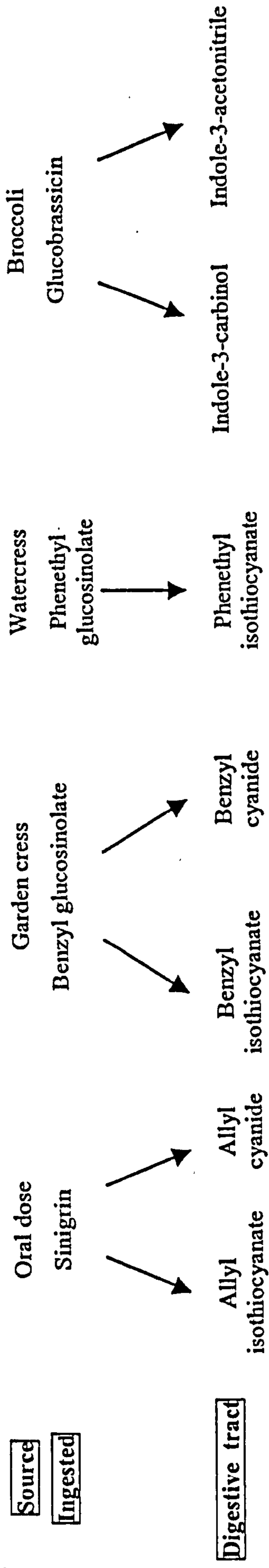


Table 2.1: Experimental design

Period	Diet	Order of administration of the dose			
		Control treatment		Iron treatment	
		Group A (n=3)	Group B (n=3)	Group C (n=3)	Group D (n=3)
Period 1	Standard	ITC	NITR	ITC	NITR
		NITR	ITC	NITR	ITC
Period 2	Cruciferous	SIN	ISO/CN	SIN	ISO/CN
		ISO/CN	SIN	ISO/CN	SIN

Standards to measure recovery rates were also included in the diets and dosing mixtures to allow inter-animal variation in recovery of isothiocyanates and nitriles to be quantified. Butyl isothiocyanate and acrylonitrile were given orally when rats were administered single oral doses. Propyl isothiocyanate and crotonitrile were included in the cruciferous diet.

2.2.3. Treatments

For the **Iron** treatment, a solution of sodium Fe.EDTA (Ferrostrane®, Parke-Davis, Courbevoie, France) was added to drinking water to a final concentration of 30.7 ml.l⁻¹ which corresponded to a concentration of 0.2 g.l⁻¹ iron. A fresh solution was made up daily and given to animals in dark feeding bottles to avoid oxidation due to exposure to light. The amount of water drunk by the animals was measured daily.

2.2.4. Diet

A separate diet was offered in the two experimental periods. Both were semi-synthetic diets, the composition of which simulated a human diet. The control diet (**Standard** diet) was offered to animals during the first phase of the experiment and contained no glucosinolates (Table 2.2). In period 2 of the experiment, a vegetable-containing diet was given to rats (**Cruciferous** diet). This diet was based on a mixture of broccoli (*Brassica oleracea* var. *italica*), watercress (*Nasturtium officinalis*) and garden cress (assumed to be *Lepidium sativum* but later suspected of being *Brassica napus* from the glucosinolate profile) (Table 2.2).

Table 2.2 Composition of the diet (in g.kg⁻¹ Dry Matter)

Ingredients	Diet	
	Standard	Cruciferous
Casein	50.00	50.00
Soya isolate (PP500E, Protein Technologies International)	120.00	84.00
Broccoli	0.00	49.00
Gardencress	0.00	61.00
Watercress	0.00	74.00
Saccharose	50.00	50.00
Mashed potato	290.00	290.00
Maize starch	289.85	168.85
Lard	30.00	30.00
Maize oil	30.00	30.00
Cholesterol	0.15	0.15
Cellulose	60.00	33.00
Mineral additive ^a	70.00	70.00
Vitamin additive ^b	10.00	10.00
Total	1000.00	1000.00
Protein content	171.16	176.7
Energy (kJ. kg ⁻¹ DM)	17514.00	17087.00

^aThe mineral additive includes (g.kg⁻¹DM of diet): CaHPO₄ 30.1, KCl 7.0, NaCl 7.0, MgO 0.735, MgSO₄ 3.5, Fe₂O₃ 0.21, FeSO₄·7H₂O 0.35, MnSO₄·H₂O 0.17, CuSO₄·5H₂O 0.035, ZnSO₄·7H₂O 0.141, CoSO₄·7H₂O 2.8*10⁻⁴ and KI 5.6*10⁻⁴.

^bThe vitamin additive includes (mg. kg⁻¹DM of diet): thiamin 20, riboflavin 15, pantothenic acid 70, pyridoxine 10, myoinositol 150, cyanocobalamin 0.05, ascorbic acid 800, α-tocopherol 170, menadione 40, niacin 100, choline 1360, folic acid 5, p-aminobenzoic acid 50, biotin 0.3, retinol 19800 IU.kg⁻¹diet and cholecalciferol 2500 IU kg⁻¹diet.

The total amount of glucosinolates in the diet was 3.9 μmol/g dry matter of which phenethyl glucosinolate accounted for a proportion of 0.32 (Table 2.3). All three vegetables had undetectable concentrations of sinigrin. Vegetables were purchased from a local greengrocer (Knowles, Aberdeen, UK). They were freeze-dried and ground before inclusion in the diet. Artificial propyl isothiocyanate and crotonitrile were also added to the Cruciferous diet (1.25 μmol of each compound/g diet) as standards to allow correction of post-absorptive isothiocyanate and nitrile recovery. Diets were prepared in pelleted form and stored in double vacuum-bags which were sterilised by γ irradiation at 45 kGy (U.A.R, Villemoisson, France). A period of seven days of habituation to the food was allowed at the beginning of the two feeding periods before the administration of the dosing mixtures. The daily food intake of individual rats was recorded by weighing feeding troughs before and after filling at the same time every day.

Table 2.3: Glucosinolate composition of the cruciferous vegetables and glucosinolate content of the Cruciferous diet. Data expressed in $\mu\text{mol/g}$ Dry Matter.

Glucosinolate	Broccoli	Gardencress	Watercress	Cruciferous diet
Glucoiberin	0.47			
Progoitrin	0.68	52.4		2.02
Epiprogoitrin		1.80		0.20
Sinigrin				
Glucoraphanin	2.08			
Gluconapoleiferin		4.56		0.16
Sinalbin		0.38		
Gluconapin		1.26		
4-hydroxy glucobrassicin	0.42	1.00		0.05
Glucobrassicin		1.20		
Glucotropaeolin			1.79	
Glucobrassicin	0.54	0.83	0.62	0.09
Gluconasturtiine		1.15	24.6	1.27
4-methoxy glucobrassicin	0.41	1.07	0.66	0.10
Neoglucobrassicin	0.68	0.32		0.04
Unknown	0.73			
Total	5.28	66.00	27.6	3.94

2.2.5. Composition of dosing mixtures

After light anaesthesia with diethyl ether, animals were administered the dosing mixtures by a flexible stomach tube. The ITC dose contained 10 μmol each of allyl, benzyl, butyl, phenethyl and propyl isothiocyanate, diluted in 0.5 ml corn oil. The NITR dose contained 10 μmol each of acrylonitrile, allyl cyanide, crotonitrile and benzyl cyanide, diluted in 0.5 ml of corn oil, and 2 μmol each of indole-3-acetonitrile and indole-3-carbinol, diluted in 0.1 ml of 500 ml.l^{-1} ethanol. The ITC dose was administered to rats on day 8 (groups A and C) and day 12 (groups B and D). The NITR dose was administered on day 9 (groups B and D) and day 11 (groups A and C).

The SIN dose contained 10 μmol sinigrin diluted in 0.5 ml water followed by 10 μmol each of butyl isothiocyanate and acrylonitrile, diluted in 0.5 ml corn oil. The ISO/CN dose consisted of 10 μmol each of allyl isothiocyanate, butyl isothiocyanate, acrylonitrile and allyl cyanide, diluted in 0.5 ml corn oil. The SIN dose was administered on day 23 (groups A and C) and day 27 (groups B and D) while the ISO/CN dose was administered on day 24 (groups B and D) and day 26 (groups A and C). Glucosinolates and their breakdown products were flushed into the stomach using an equal volume of the appropriate vehicle.

Sinigrin was purchased from Sigma (Saint-Quentin-Fallavier, France). Allyl, propyl, butyl, benzyl and phenethyl isothiocyanate, acrylonitrile, crotonitrile, indole-3-acetonitrile and indole-3-carbinol were purchased from Aldrich (Saint-Quentin-Fallavier, France).

2.2.6. Urine and faeces collection

Six hours before administering the dose, the metabolism cages were cleaned and the collection receptacles were emptied. Urine and faeces output were collected at the time of the dose administration and subsequently 6, 24 and 48 hours after dosing. Sodium azide (final concentration: 0.2 ml.l⁻¹, Sigma, France) was added to urine collection receptacles to prevent microbial growth.

2.2.7. Analysis

2.2.7.1. Synthesis of mercapturic acids

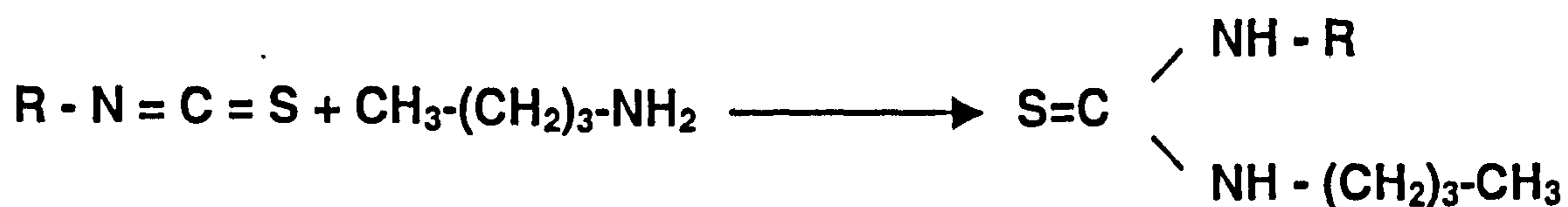
Standards were required for the analysis of mercapturic acid derivatives of isothiocyanates (*N*-acetyl-*S*-(*N*-alkyl-thiocarbamoyl)-*L*-cysteines). The methyl, allyl, butyl, benzyl and phenethyl mercapturic acid derivatives were synthesised as their dicyclohexylamine salts (Mennicke *et al.* 1983, Appendix 1). The purity was checked by HPLC. Melting points were 134-142°C for butyl mercapturic acid (quoted range: 135-138°C), 145-153°C for propyl mercapturic acid and 149-154°C for phenethyl mercapturic acid.

2.2.7.2. Quantification of mercapturic acids

Urine samples were analysed for mercapturic acids by a HPLC method. Samples were prepared according to published methods (Duncan *et al.* 1997, Appendix 2). As mercapturic acids are highly polar and labile molecules, they are not readily extracted from an aqueous medium. At alkaline pH, however, the mercapturic acid derivatives break down to release *N*-acetyl cysteine and the related isothiocyanate. The principle of the method consisted of forming isothiocyanates under alkaline conditions and converting them into the corresponding thioureas by reaction with *n*-butylamine (Figure 2.2). The thioureas were subsequently extracted in diethyl ether. The solvent

phase was finally evaporated and the thioureas resuspended in acetonitrile and analysed by HPLC.

Figure 2.2: Conversion of isothiocyanate to thiourea



In this experiment, 0.2 ml of urine was used. Methyl mercapturic acid (0.3 $\mu\text{mol/sample}$) was used as an internal standard. The inclusion of an internal standard in the analytical procedure allowed correction for extraction and evaporative losses during sample preparation. Samples were analysed in duplicate. HPLC separation was carried out on a Gilson modular HPLC system (Gilson, Villiers le Bel, France) equipped with a 250 mm Lichrospher[®] reversed phase C18 (Merck, Darmstadt, Germany) column. The mobile phase consisted of a gradient of distilled water and acetonitrile. The gradient was programmed as follows: acetonitrile 30 to 70% over 20 min then 70 to 30% over 5 min. The flow rate was 1 $\text{ml}\cdot\text{min}^{-1}$. Eluted peaks were detected by UV absorbance at 240 nm (UV/VIS detector, model 118, Gilson, Villiers le Bel, France). Peaks areas were derived by integration using Gilson 715 system controller software (Gilson, Villiers le Bel, France). Retention times were 5.7 min for methyl mercapturic acid, 10.1 min for allyl mercapturic acid, 11.2 min for propyl mercapturic acid, 14.5 min for butyl mercapturic acid, 16.0 min for benzyl mercapturic acid and 17.8 min for phenethyl mercapturic acid. A standard solution containing 1.5 mM of each synthetic mercapturic acid was analysed identically to test samples and used to determine the detector response factor of studied mercapturic acids relative to methyl mercapturic acid. The individual response factors of standards relative to methyl mercapturic acid were 1.94 (s.e.m. 0.12) for allyl isothiocyanate, 2.13 (s.e.m. 0.15) for propyl isothiocyanate, 2.03 (s.e.m. 0.13) for butyl isothiocyanate, 2.02 (s.e.m. 0.11) for benzyl isothiocyanate and 2.07 (s.e.m. 0.15) for phenethyl isothiocyanate. Mercapturic acid concentrations were calculated by comparing their peak areas to the peak area of methyl mercapturic acid in the test sample corrected by the response factor. Preliminary analysis carried out on urinary

outputs of rats, which had not ingested any glucosinolate or breakdown products, showed no excretion of mercapturic acids or co-eluting substances.

2.2.8. Calculation and statistical analysis

Proportional release of isothiocyanate from its parent glucosinolate was calculated as the ratio of the total amount of urinary mercapturic acid measured after administration of the parent glucosinolate to the estimated potential amount of mercapturic acid excreted if 100% of the parent glucosinolate had been converted to its related isothiocyanate. Isothiocyanate release was thus calculated:

$$\text{Proportion of isothiocyanate release from parent glucosinolate} = \frac{\text{Actual amount of excreted mercapturic acid}}{\text{Potential amount of excreted mercapturic acid given 100\% conversion}}$$

To illustrate the method of calculating the potential amount of excreted mercapturic acid given 100% conversion, the example of allyl isothiocyanate release from a single oral dose of sinigrin is detailed here. The administration of allyl isothiocyanate and butyl isothiocyanate in the ISO/CN dose allowed the calculation of a ratio between the excretion of allyl mercapturic acid and butyl mercapturic acid.

$$\text{Ratio ISO/CN dose} = \frac{\text{Amount of excreted allyl mercapturic acid}_{\text{ISO/CN dose}}}{\text{Amount of excreted butyl mercapturic acid}_{\text{ISO/CN dose}}}$$

This ratio was relatively constant between rats and could, therefore, be used to calculate the potential excretion of allyl mercapturic acid using the excretion of butyl mercapturic acid after administration of the SIN dose:

$$\text{Putative excretion of allyl mercapturic acid}_{\text{SIN dose}} = \text{Ratio}_{\text{ISO/CN dose}} * \text{Amount of excreted butyl mercapturic acids}_{\text{SIN dose}}$$

The actual proportion of allyl isothiocyanate release from sinigrin was therefore:

$$\text{Proportion of allyl isothiocyanate release from sinigrin} = \frac{\frac{\text{Allyl mercapturic acid}_{\text{SIN dose}}}{\text{Butyl mercapturic acid}_{\text{SIN dose}}}}{\frac{\text{Allyl mercapturic acid}_{\text{ISO/CN dose}}}{\text{Butyl mercapturic acid}_{\text{ISO/CN dose}}}}$$

This formula assumes that the amounts of sinigrin, allyl isothiocyanate and butyl isothiocyanate included in the dosing mixture are equimolar. In the case of dietary glucosinolates, the amount of precursor ingested was variable and therefore the amount of excreted mercapturic acid was expressed as a proportion of the amount of precursor ingested. The principle of the calculation was similar to the above example.

Analysis of variance was used to analyse factors influencing the excretion of mercapturic acids and the release of allyl isothiocyanate and phenethyl isothiocyanate from their respective precursors. Factors tested were iron supplementation, rat and dosing occasion. Analyses were performed using Genstat 5 (Lawes Agricultural Trust, 1989).

2.3. Results

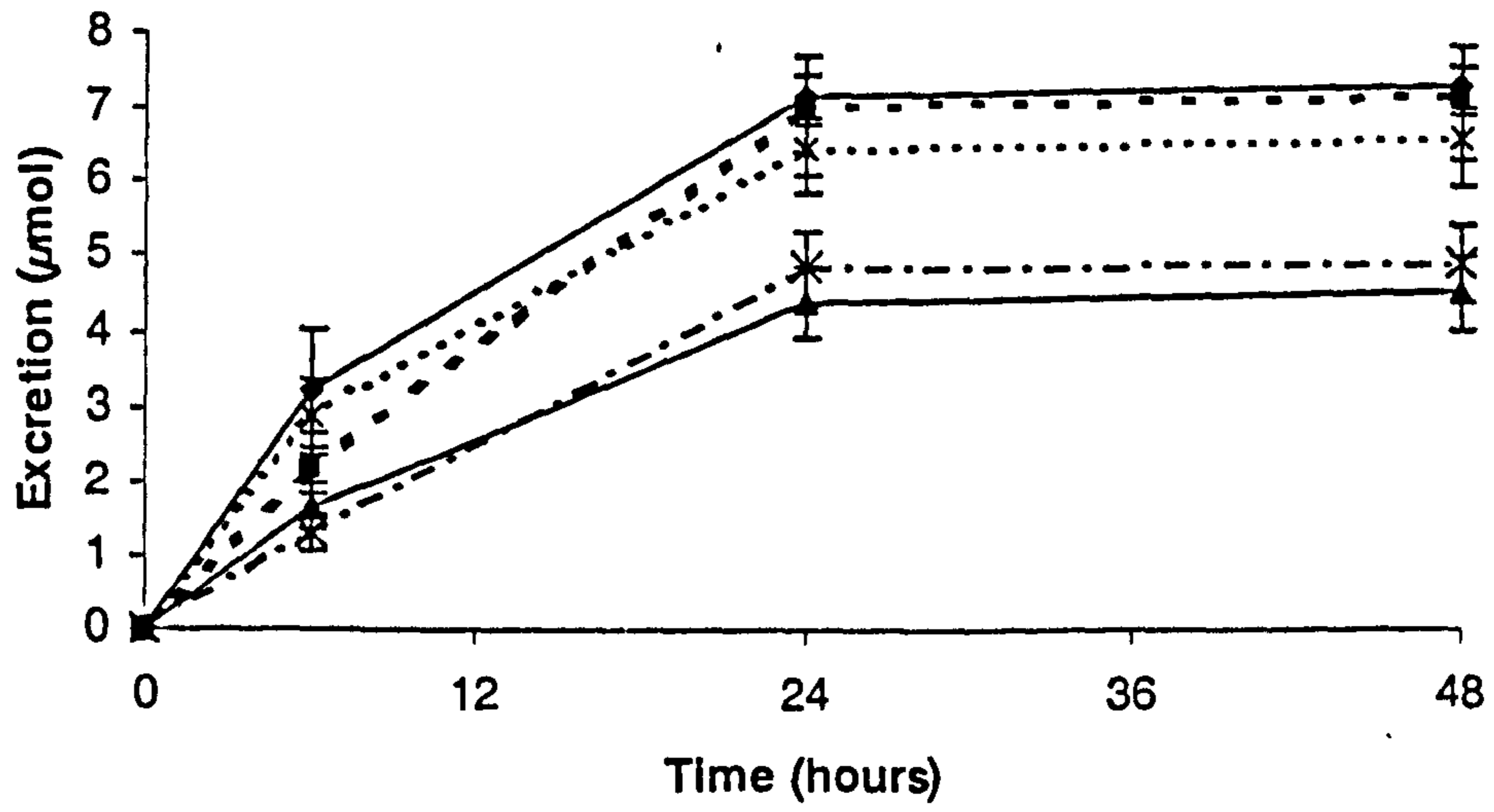
2.3.1. Excretion of mercapturic acids arising from an artificial dose of isothiocyanates

Mercapturic acids were found in the urine of rats for all the isothiocyanates administered during Period 1. A proportion of more than 0.95 of the total amount of mercapturic acids was excreted within 24h (Figure 2.3). The proportion of the isothiocyanates recovered as mercapturic acids varied according to the isothiocyanates considered (Table 2.4). Butyl mercapturic acid and phenethyl mercapturic acid had lower excretory rates than those of allyl mercapturic acid, propyl mercapturic acid and benzyl mercapturic acid. The Iron treatment did not significantly affect the recovery of isothiocyanates as their mercapturic acids (Figure 2.3).

Figure 2.3 : Cumulative excretion of mercapturic acids over 48 hours following ITC dose

◆ allyl mercapturic acid; ■ benzyl mercapturic acid; ▲ butyl mercapturic acid
✱ phenethyl mercapturic acid; * propyl mercapturic acid.

Control treatment



Iron treatment

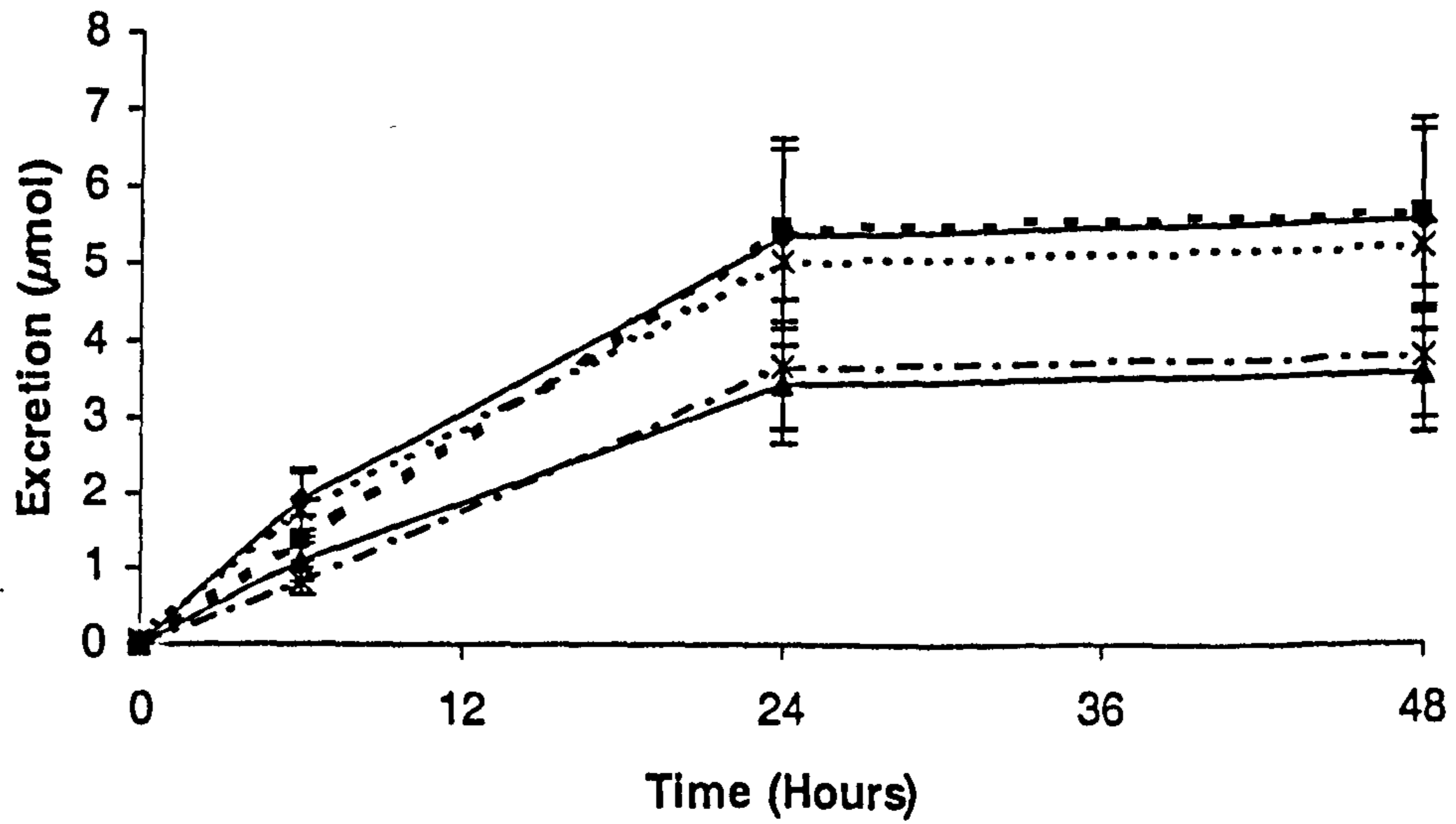


Table 2.4: Total amount of excreted mercapturic acid markers after administration of ITC dose (in μmol)

Treatment Markers	Control		Iron	
	Mean	s.e.m.	Mean	s.e.m.
Allyl mercapturic acid	7.32	0.322	6.67	0.397
Propyl mercapturic acid	6.64	0.292	6.21	0.627
Butyl mercapturic acid	4.52	0.407	4.31	0.372
Benzyl mercapturic acid	7.17	0.608	6.78	0.526
Phenethyl mercapturic acid	4.93	0.504	4.60	0.450

Correlation analysis showed that the amounts of excretion of individual markers of isothiocyanates was highly correlated to each other (Table 2.5). This confirmed that artificial isothiocyanates, such as propyl isothiocyanate and butyl isothiocyanate, are good predictors of the level of excretion of other isothiocyanates arising in the digestive tract and could be used in Period 2 to correct for differences in excretion rates between animals.

Table 2.5: Correlation matrix between the excretion of markers coming from isothiocyanates given in ITC dose.

Markers	Allyl mercapturic acid	Propyl mercapturic acid	Butyl mercapturic acid	Benzyl mercapturic acid	Phenethyl mercapturic acid
Allyl mercapturic acid	1.000				
Propyl mercapturic acid	0.858	1.000			
Butyl mercapturic acid	0.801	0.739	1.000		
Benzyl mercapturic acid	0.815	0.783	0.935	1.000	
Phenethyl mercapturic acid	0.738	0.775	0.900	0.978	1.000

2.3.2. Excretion of mercapturic acids arising from a single dose of sinigrin

The proportion of allyl isothiocyanate and butyl isothiocyanate, recovered as their mercapturic acids after administering the dose ISO/CN, were not significantly influenced by Iron supplementation (Table 2.6). There was a close relationship between recovery of allyl and butyl mercapturic acids ($r^2 = 0.602$, $p < 0.001$, Figure 2.4). The relative recovery of allyl mercapturic acid versus butyl mercapturic acid calculated from the ISO/CN dose was on average 1.37 (s.e.m. 0.098) and this value was not significantly influenced by Iron supplementation. Total excretion of allyl mercapturic acid after administration of the SIN dose was very low (Table 2.6). The proportion of sinigrin converted to allyl isothiocyanate was significantly higher in

Control rats (0.06 s.e.m. 0.011) than in Iron-supplemented rats (0.02 s.e.m. 0.010, $p < 0.001$).

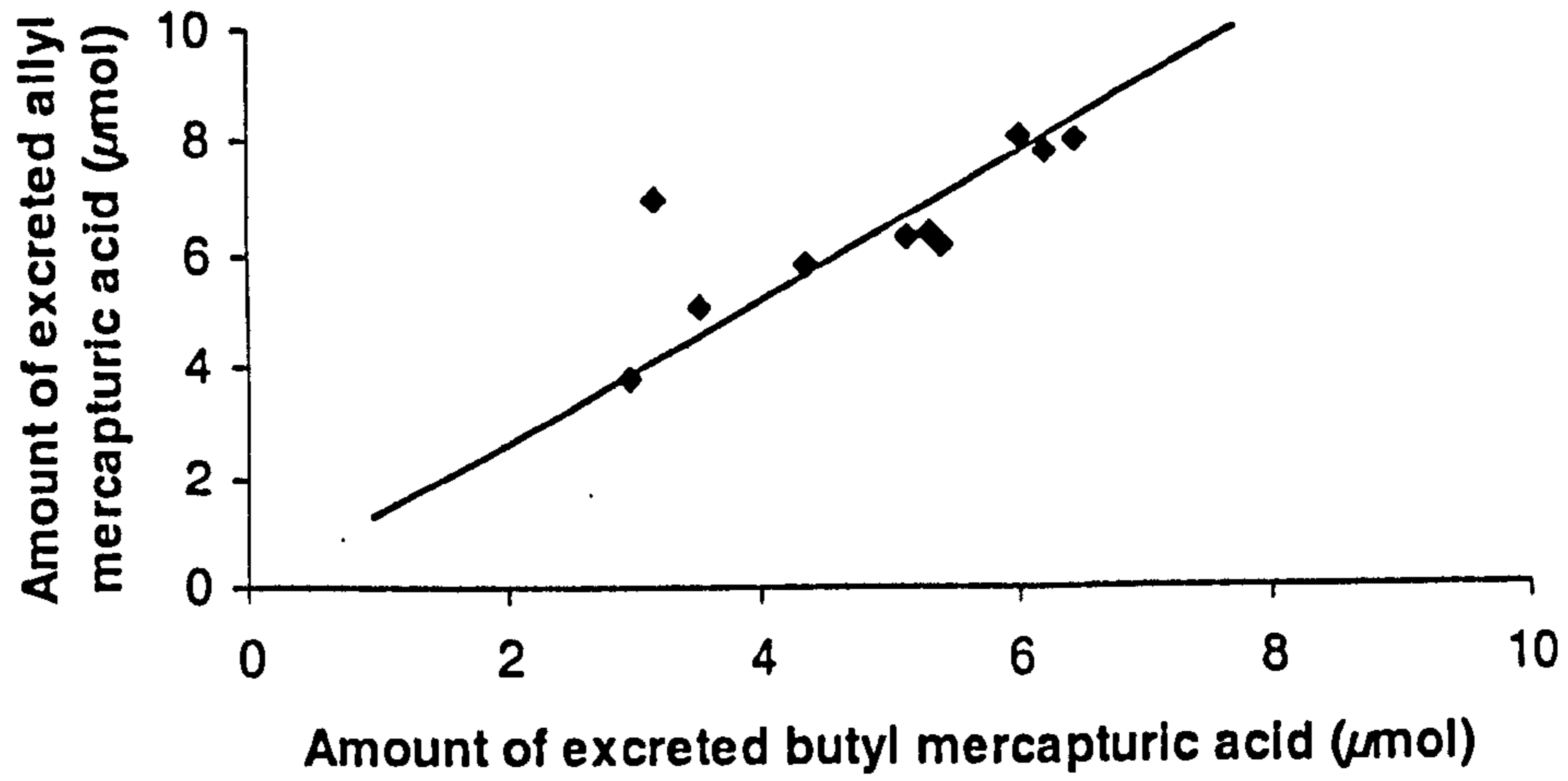
Table 2.6: Excretion of mercapturic acid after administration of ISO/CN and SIN dose (in μmol)

Dose Treatment Markers	ISO/CN				SIN			
	Control		Iron		Control		Iron	
	Mean	s.e.m.	Mean	s.e.m.	Mean	s.e.m.	Mean	s.e.m.
Allyl mercapturic acid	6.57	0.629	6.30	0.634	0.57	0.158	0.12	0.101
Butyl mercapturic acid	4.89	0.610	4.84	0.560	6.41	0.653	4.84	0.939

2.3.3. Excretion of mercapturic acids arising from dietary glucosinolates

While rats were consuming the **Cruciferous** diet (Period 2), only propyl mercapturic acid and phenethyl mercapturic acid were recovered in measurable amounts. The proportion of ingested propyl isothiocyanate excreted as mercapturic acid was only 0.14 (s.e.m.0.031) in **Control** animals and 0.12 (s.e.m. 0.044) in **Iron** treated rats (Table 2.7). This output of propyl mercapturic acid was approximately 5-fold lower than the expected excretion calculated on the basis of the excretory rate of propyl isothiocyanate found for the ITC dose during Period 1 (Table 2.7). Consequently, propyl isothiocyanate incorporated into the diet did not prove to be an effective recovery standard and butyl isothiocyanate was used instead. The relative recovery of phenethyl mercapturic acid to butyl mercapturic acid was estimated from the amount of phenethyl and butyl mercapturic acids excreted after administration of the ITC dose in period 1 as 1.08 (s.e.m.0.031). This value was used to estimate post-absorptive recovery of phenethyl isothiocyanate in Period 2. By this means, the proportion of phenethyl isothiocyanate release from dietary phenethyl glucosinolate was estimated to be 0.53 (s.e.m. 0.040) for **Iron-supplemented** animals and 0.60 (s.e.m. 0.055) for **Control** animals and this difference was significant (Figure 2.5, $p < 0.05$).

Figure 2.4: Relationship between excretion of allyl mercapturic acid and butyl mercapturic acid after administration of ISO/CN dose



Regression equation:
 Benzyl mercapturic acid = $0.825 * \text{propyl mercapturic acid} + 2.44$ $r^2=0.602; p<0.001$

Figure 2.5: Proportion of isothiocyanate release from dietary phenethyl glucosinolate

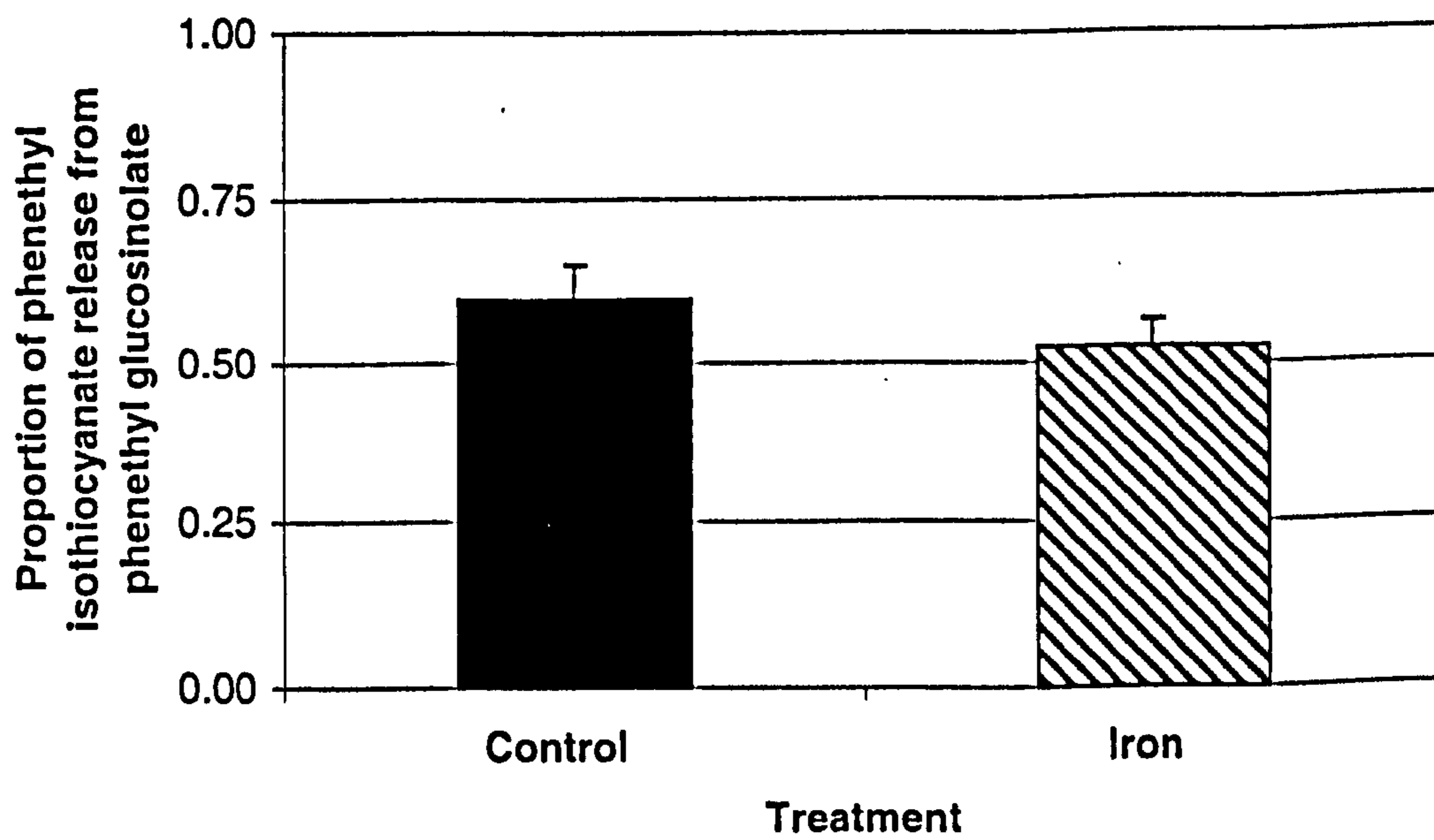


Table 2.7: Amount of propyl mercapturic acid excreted over 48 hours during feeding of the Cruciferous diet

Treatment	Amount of ingested propylisothiocyanate (μmol)		Predicted amount of propyl mercapturic acid in urine (μmol)		Actual amount of propyl mercapturic acid in urine (μmol)	
	Mean	s.e.m.	Mean	s.e.m.	Mean	s.e.m.
Control	36.3	0.88	26.6	1.10	6.1	0.87
Iron	32.8	0.83	21.8	0.76	4.4	0.87

2.3.4. Diet intake and iron intake

Food intake was significantly lower when rats were fed the **Cruciferous** regimen in comparison to the **Standard** one. Food intake was not significantly influenced by iron supplementation in either Period 1 or Period 2 (Table 2.8). In **Iron**-treated rats, the amount of ingested **Iron** was significantly higher with the cruciferous diet than with the standard diet.

2.4. Discussion

This experiment was designed to evaluate urinary end-products which could be potentially used as markers of the digestive metabolism of glucosinolates in further studies. The substantial excretion of urinary mercapturic acids, following administration of isothiocyanates at physiological rates, demonstrated their potential efficacy in the context of dietary studies where the rate of glucosinolate delivery to the gut is likely to be low. This experiment was the first attempt to measure the recovery of a range of isothiocyanates simultaneously. It confirmed previous findings where the recovery of allyl, benzyl and phenethyl isothiocyanate into mercapturic acids ranged from a proportion of 0.37 to 0.80 of the initial dose (Brüsewitz *et al.* 1977; Bollard *et al.* 1997; Conaway *et al.* 1999). These findings open up the possibility of conducting experiments where allyl isothiocyanate, benzyl isothiocyanate and phenethyl isothiocyanate can be quantified concurrently.

Table 2.8: Food intake on Control and Iron treatments and iron intake in Iron-treated animals.

Treatment	Control (n=6)			Iron (n=6)			Sodium Fe.EDTA (mg)					
	Food intake (g)	Glucosinolate intake (µmol)	Phenethyl glucosinolate intake (µmol)	Food intake (g)	Glucosinolate intake (µmol)	Phenethyl glucosinolate intake (µmol)						
Diet	Mean	s.e.m.	Mean	s.e.m.	Mean	s.e.m.	Mean	s.e.m.				
Standard	16.1	0.53	-	-	14.3	0.58	-	-	4.06	0.11		
Cruciferous	14.5	0.23	57.4	1.08	13.1	0.32	51.9	1.18	16.7	0.38	4.53	0.09

Mercapturic acids from isothiocyanates which are not common in plants, such as methyl isothiocyanate, propyl isothiocyanate and butyl isothiocyanate, could be used in animal experiments to quantify variations occurring during the post-absorptive biotransformation of isothiocyanates or as internal standards in the subsequent analysis of samples.

Previous studies of isothiocyanate production from ingested glucosinolates have measured the appearance of mercapturic acids in the urine following ingestion of intact glucosinolates (Chung *et al.* 1992; Shapiro *et al.* 1998; Getahun & Chung, 1999). The isothiocyanate production was not estimated precisely in these studies as the post-absorptive fate of isothiocyanates was not accounted for. To refine the estimates of isothiocyanate production from intact glucosinolates, a new approach has been adopted in the current experiment. On each occasion that intact glucosinolates were administered to animals, an additional isothiocyanate was given concurrently so that the conversion rate of isothiocyanates to mercapturic acids at the time of the measurement could be known. In the current experiment butyl isothiocyanate was used as the recovery standard. The relationship between excretion rate of butyl isothiocyanate and allyl isothiocyanate was used to predict the excretion rate of allyl isothiocyanate arising from hydrolysis of sinigrin. A similar approach was used in estimating release of phenethyl isothiocyanate from phenethyl glucosinolate. The experiment has verified that relative recoveries of a range of isothiocyanates are consistent between animals and are not influenced by dietary manipulations. This increases the confidence with which estimates of isothiocyanate production from intact glucosinolates can be made.

Urinary end-products of nitrile derivatives were not quantified in the current experiment. Although the formation of mercapturic acids from nitriles, such as crotonitrile and acrylonitrile, has been reported (Van Bladeren *et al.* 1981), the high toxicity of these compounds did not allow an inclusion of significant quantities in the dose mixture and in the diet to clearly identify their urinary metabolites. Furthermore, the chemistry of aliphatic nitriles varies notably from that of conjugated nitriles. Urinary excretion may not be the main route of detoxification for nitrile derivatives.

The very low concentrations of benzyl glucosinolate and indole glucosinolates in the cruciferous diet did not allow a measurement of the release of their derivatives as planned in the initial experimental design. The vegetables included in the cruciferous diet were chosen on the basis of typical glucosinolate composition derived from the literature. Subsequent analysis of the glucosinolate content of the vegetables showed that the vegetables, sold as garden cress and used in this experiment, was probably not *Lepidium sativum* but was more likely to have been *Brassica napus*. Substantial amounts of glucobrassicin were present in the broccoli but the level of inclusion of broccoli was relatively low. There was a limit to how much vegetable could be incorporated into the diet without causing digestive problems in the experimental animals. The high fibre content of the diet may also explain the decrease in food intake when rats were fed the cruciferous diet in this experiment. These difficulties highlighted the necessity of using vegetables with a high content of the glucosinolates of interest and a relatively high inclusion rate to allow a significant supplementation of the animal diet while respecting the dietary balance and without impairing the normal food intake by rats.

The low recovery of allyl isothiocyanate arising from the oral dose of sinigrin was surprising as previous studies have shown that a substantial proportion of allyl isothiocyanate was released from an oral dose of 50 μmol of sinigrin (Duncan *et al.* 1997). The initial sinigrin solution was discarded and could not be analysed so a potential error in the actual amount of sinigrin ingested cannot be excluded. Alternatively, the administration of only 10 μmol of sinigrin in this experiment may have resulted in an urinary concentration of allyl mercapturic acid which was at the limits of detection in the chemical analysis.

The high volatility and instability of propyl isothiocyanate may explain the low recovery of propyl mercapturic acid when rats were given the cruciferous diet containing propyl isothiocyanate. The justification for including propyl isothiocyanate in the diet was to account for the gradual delivery of compounds to the digestive tracts in normal feeding circumstances. Other techniques of delivery of propyl isothiocyanate were also tested, such as spraying an isothiocyanate solution on

the pellets given to rats but outcomes were not satisfactory as it was difficult to accurately control the amount of isothiocyanate actually ingested by the animals.

The substantial proportion of phenethyl isothiocyanate arising from dietary phenethyl glucosinolate precursor showed that the isothiocyanate was the major hydrolysis products under the conditions of this experiment. The reduction in isothiocyanate release caused by iron supplementation agrees with studies *in vitro* in which the presence of ferrous ions in the hydrolysis medium tended to favour nitrile production (Tookey & Wolff, 1970; Stoewsand *et al.* 1986). Further analysis of urine samples for nitrile derivatives would be required to confirm this hypothesis.

In conclusion this experiment has demonstrated the validity of new urinary biomarkers for the measurement of isothiocyanate bioavailability in the digestive tract when the precursors were given as part of the diet. This approach shows considerable potential for use in further experiments aimed at characterising the factors influencing glucosinolate hydrolysis.

CHAPTER 3: EXPERIMENT 2

Influence of plant and bacterial myrosinase activity on the metabolic fate of sinigrin and benzyl glucosinolate in the digestive tract of rats harbouring a human faecal flora

3.1. Introduction

The breakdown of glucosinolates is thought to occur at two stages following their consumption by mammals. The first stage occurs when plant cells are disrupted during ingestion of plant. This process exposes glucosinolates to plant myrosinase and hydrolysis occurs to yield several metabolites among which are the isothiocyanates (Cole, 1976). Depending on the activity of plant myrosinase, this hydrolysis may not be comprehensive and intact glucosinolates may reach the lower part of the intestinal tract. Bacteria possessing a myrosinase-like activity may then facilitate a further glucosinolate hydrolysis (Oginsky *et al.* 1965). Glucosinolates may thus be hydrolysed by myrosinase of two different origins and the extent of their respective involvement in glucosinolate breakdown is unclear.

When vegetables are eaten raw, plant myrosinase is still active in the diet. Glucosinolate hydrolysis may then be hypothesised to be mainly due to plant myrosinase and to a lesser extent to microbial myrosinase. Glucosinolate metabolites are then more likely to be absorbed in the upper part of the intestinal tract. On the other hand, when vegetables are processed and cooked, plant myrosinase is inactivated and it can be hypothesised that the contribution of microbial myrosinase to glucosinolate breakdown may increase. Glucosinolate metabolite absorption would then occur primarily in the large bowel.

The objective of this experiment was to measure the respective influence of dietary myrosinase and microbial myrosinase activity on the release of allyl isothiocyanate and benzyl isothiocyanate in the digestive tract of rats. To study the role of microbial myrosinase, two sets of animals differing in microbial status were used. One set harboured a whole human faecal flora (Flora+ treatment) while a second set was germ-free (Flora- treatment). To investigate glucosinolate metabolism due to plant

myrosinase, two diets differing in myrosinase activity were given to rats. One diet contained the plant myrosinase in its native form (**Myro+** treatment) while the other diet contained the same cruciferous material treated to inactivate the plant myrosinase (**Myro-** treatment). Study of the interaction between plant and microbial myrosinase was achieved by alternately offering the two diets to **Myro-** and **Myro+** rats. The proportion of sinigrin and benzyl glucosinolate broken down to their related isothiocyanates was measured using mercapturic acids as markers of metabolism.

3.2. Materials and methods

3.2.1. Animals

Sixteen male adult Fischer 344 rats were used. They were born germ-free and bred in germ-free conditions at the INRA-UEPSD breeding unit. Rats were aged 6-9 weeks at the start of the experiment (mean weight: 163g s.e.m. 2.7). Animals were randomly allocated to **Flora+** and **Flora-** treatments.

3.2.2. Maintenance of animals

To maintain their bacterial status, rats were kept in groups of four animals in four sterile isolators (La Calhène, Vélizy, France). Isolators were sterilised prior to the experiment. Incoming air was filtered by paper filter (Sofitra Poelman, La Garenne, Colombes, France). To avoid bacterial contamination, **Flora+** and **Flora-** rats were housed in different isolators. A positive pressure (50 Pa) was maintained within the isolators. All items introduced into the isolators were sterilised beforehand (Elfoul, 1999). Withdrawal of material and samples was achieved by transfer through a sash which was sterilised after each use or by transfer via a removable germ-free container. Within each isolator, rats were individually housed in metabolism cages (Iffa-Credo, Saint Germain sur l'Arbresle, France). A sterilised diet and autoclaved ultra-filtered water were given *ad libitum* to rats. Rats were weighed once a week throughout the experiment. Room temperature was maintained at 21°C and lights were on a 12:12h light:dark cycle.

3.2.3. Inoculation of human flora to animals

Fresh faeces were obtained from a healthy male subject. Preparation of a faecal suspension was carried out in an anaerobic cabinet to preserve microbial diversity. The fresh faecal flora was homogenised in a Brain Heart Infusion medium (Elfoul, 1999). At day 1, rats from Flora+ treatment were orally administered with 1.0 ml of a 10^{-2} dilution of the fresh faecal suspension.

3.2.4. Control of bacterial status

Throughout the duration of the experiment, the bacterial status of rats in each isolator was checked weekly. To verify the germ-free status of Flora- rats, fresh faeces were collected and diluted in LCY medium (Djouzi, 1995). A microscopic examination of the faecal suspension allowed an initial observation of the absence of bacterial contamination. A further investigation was carried out by inoculating the faecal suspension on culture media adapted to anaerobic bacteria (Djouzi, 1995). After 7 days of incubation at 37°C, the absence of bacterial growth in the culture media confirmed the germ-free status of rats. The implantation of the human flora in Flora+ treated rats was verified by microscopic observation of a fresh faecal suspension. The diversity of bacteria was assessed as well as the level of colonisation.

3.2.5. Experimental design

The experiment was designed as a split plot experiment where the main plots were defined by the microbial status of the rats (Table 3.1). The sequence of offering the diet was allocated to two sub-plots. In one sub-plot, rats were given the Myro+ treatment first and the Myro- treatment second, whereas the sequence of diet treatments was reversed in the second sub-plot. Each period of feeding lasted three weeks. Each period began with a 9-day adaptation phase to allow rats to habituate to the food. On days 10 and 17, rats were administered a dose containing benzyl glucosinolate (BGLS dose) or a mixture of isothiocyanates (ITC dose). The sequence of administration of the dosing mixtures was randomised within each sub-plot of rats (Table 3.2).

Table 3.1 Experimental design

Microbial status	Germ-free		Human Flora	
	A	B	C	D
Isolator				
number of rats	4	4	4	4
Sequence of diets				
Period 1 (3 weeks)	Myro+	Myro-	Myro+	Myro-
Period 2 (3 weeks)	Myro-	Myro+	Myro-	Myro+

Table 3.2 Design of the administration of dose mixture to rats. Grey areas indicate period where the diet Myro+ was offered to the rats. White areas indicate periods when Myro- diet was offered.

Isolator	No of rats	A		B		C		D	
		2	2	2	2	2	2	2	2
Period 1	Day 10	BGLS	ITC	BGLS	ITC	BGLS	ITC	BGLS	ITC
	Day 17	ITC	BGLS	ITC	BGLS	ITC	BGLS	ITC	BGLS
Period 2	Day 10	BGLS	ITC	BGLS	ITC	BGLS	ITC	BGLS	ITC
	Day 17	ITC	BGLS	ITC	BGLS	ITC	BGLS	ITC	BGLS

Table 3.3 Composition of the diets given to rats (in g.kg⁻¹ Dry Matter)

Ingredients	Diet	
	Myro +	Myro-
Casein	50.00	50.00
Soya isolate (PP500E, Protein Technologies International)	90.00	90.00
Brussels sprouts (myro+)	150.00	
Brussels sprouts (myro-)		150.00
Saccharose	50.00	50.00
Mashed potato	230.00	230.00
Maize starch	229.85	229.85
Lard	30.00	30.00
Maize oil	30.00	30.00
Cholesterol	0.15	0.15
Cellulose	60.00	60.00
Mineral additive ^a	70.00	70.00
Vitamin additive ^b	10.00	10.00
Total	1000.00	1000
Protein content	157.00	160.70
Energy (kJ. kg ⁻¹ DM)	16.83	17.08

^aThe mineral additive includes (g.kg⁻¹DM of diet): CaHPO₄ 30.1, KCl 7.0, NaCl 7.0, MgO 0.735, MgSO₄ 3.5, Fe₂O₃ 0.21, FeSO₄7H₂O 0.35, MnSO₄H₂O 0.17, CuSO₄5H₂O 0.035, ZnSO₄ 7H₂O 0.141, CoSO₄ 7H₂O 2.8*10⁻⁴ and KI 5.6*10⁻⁴.

^bThe vitamin additive includes (mg. kg⁻¹DM of diet): thiamin 20, riboflavin 15, pantothenic acid 70, pyridoxine 10, myoinositol 150, cyanocobalamin 0.05, ascorbic acid 800, α-tocopherol 170, menadione 40, niacin 100, choline 1360, folic acid 5, p-aminobenzoic acid 50, biotin 0.3, retinol 19800 IU.kg⁻¹diet and cholecalciferol 2500 IU.kg⁻¹diet.

3.2.6. Diet composition and preparation

The Myro+ and Myro- diets were semi-synthetic diets simulating a human-type diet (Table 3.3). They contained 150g.kg⁻¹ freeze-dried Brussels sprouts (*Brassica oleracea* variety *cyrus*, Novartis Seeds, The Netherlands). Analysis of the glucosinolate content in Brussels sprouts showed that sinigrin accounted for a proportion of 0.54 of the total amount of identified glucosinolates but benzyl glucosinolate was absent (Table 3.4). The total glucosinolate concentration in both diets was 3.6 $\mu\text{mol.g}^{-1}$ of which sinigrin accounted for 1.9 μmol . Diets were pelleted and packed in double-vacuum bags which were sterilised by γ irradiation at 45 kGy (U.A.R, Villemoisson, France). The daily food intake of individual rats was recorded by weighing feeding troughs before and after filling at the same time every day.

Table 3.4: Glucosinolate composition of Brussels sprouts (*Brassica oleracea* variety *cyrus*) ($\mu\text{mol.g}^{-1}$ dry matter)

Glucosinolate	Brussels sprouts
Glucoiberin	1.88
Progoitrin	2.52
Sinigrin	13.0
Gluconapin	1.80
4-OH Glucobrassicin	0.15
Glucobrassicin	3.95
4-OMe Glucobrassicin	0.77
Total	24.1

In the Myro- diet, freeze-dried Brussels sprouts were treated to inactivate myrosinase prior to inclusion in the diet. Inactivation of myrosinase was achieved by soaking 100 g ground, freeze-dried Brussels sprouts in 1 litre of 700 ml.l⁻¹ boiling ethanol for 10 min at 75°C. Ethanol was subsequently evaporated at 60°C, using a rotary evaporator and aqueous residues were removed by oven-drying at 70°C. To check any losses of glucosinolates during the inactivation process, the glucosinolate content of the final batch was measured (method described below) and compared with the glucosinolate content in the initial vegetable material. The activity of myrosinase in non-treated Brussels sprouts was not measured in the current experiment.

3.2.7. Measurement of isothiocyanate release

The procedure used for the measurement of isothiocyanate release in the digestive tract was similar to that in Experiment 1. Briefly, animals were administered on one occasion with a dose containing the glucosinolate precursor (BGSL dose) and on another occasion the isothiocyanates expected to arise in the digestive tract after hydrolysis of the glucosinolate precursors (ITC dose). Butyl isothiocyanate was also given to animals on both dosing occasions as a recovery standard. The rationale for the use of a recovery standard was explained in Chapter 2.

3.2.8. Composition of dosing mixtures

The BGLS dose contained 25 μmol of benzyl glucosinolate (potassium salt, Merck, Darmstadt, Germany) diluted in 0.25 ml ultra-filtered water and sterilised by filtration (Millex-GS 0.22 μm filter, Millipore, Saint-Quentin-en Yvelines, France). The butyl isothiocyanate solution contained 25 μmol of butyl isothiocyanate (Sigma, Saint-Quentin Fallavier, France), diluted in 0.25 ml corn oil (Sigma). The ITC dose contained 25 μmol of allyl isothiocyanate, 25 μmol of benzyl isothiocyanate and 25 μmol of butyl isothiocyanate (Sigma), diluted in 0.25 ml corn oil. Solutions containing isothiocyanates were prepared in sealed-cap vials and autoclaved (20 min, 120°C). Vials of ultra-filtered water and pure corn oil were also prepared and autoclaved for use as flushing solutions. The doses were administered orally using a sterile stainless-steel stomach tube to rats anaesthetised with sterile ether.

3.2.9. Sample collection

The day before each dosing, metabolism cages were cleaned. Urine and faeces were subsequently collected at $t = 0, 6, 24, 48, 72, 120$ and 144h after administration of dosing mixtures. To avoid bacterial degradation of urinary markers, sodium azide (final concentration 0.2 ml.l^{-1} , Sigma) was added to the urine collection receptacles of **Flora+** rats. No sodium azide was added to **Flora-** rats as no bacterial degradation was considered occur. Furthermore, preliminary tests showed that sodium azide did not interfere with the analyses.

3.3. Analysis

3.3.1. Analysis of mercapturic acids

Analysis of mercapturic acids in urine was conducted as described in Chapter 2. A solution of 1.5 mM propyl mercapturic acid (*N*-acetyl-*S*-(*N*-propylthiocarbonyl)-*L*-cysteine) was used as an internal standard. Preliminary analysis showed that a proportion of 0.95 of mercapturic acids were excreted in 48h. Consequently urine samples collected at $t=72, 120, 144$ h were bulked in proportion to their volume prior to analysis.

3.3.2. Desulpho-glucosinolate analysis

Faeces were freeze-dried and ground. Two hundred micro-litres of 1.0 mM phenethyl glucosinolate internal standard was added to 0.2 g of ground faeces. Samples were analysed in duplicate. Extraction of glucosinolates was carried out twice using 700 ml.l⁻¹ boiling methanol to obtain a final volume of 5 ml extract. Extracts were subsequently enzymatically desulphated (Minchinton *et al.* 1982). Separation of desulpho-glucosinolates was carried out on a Gilson modular HPLC system (Gilson, Villiers le Bel, France) equipped with a 250 mm Lichrospher[®] reversed phase C18 (Merck, Darmstadt, Germany) column. The mobile phase consisted of a gradient of distilled water and 200 ml.l⁻¹ acetonitrile programmed as previously described in the literature (Spinks *et al.* 1984). Eluted peaks were detected by UV absorbance at 228 nm (UV/VIS detector, model 118, Gilson, Villiers le Bel, France). Peaks areas were derived by integration using Gilson 715 system controller software (Gilson, Villiers le Bel, France). Retention times were 7.9 min (s.e.m. 0.019) for desulpho-sinigrin, 22.5 min (s.e.m. 0.056) for desulpho-benzyl glucosinolate and 31.6 min (s.e.m. 0.072) for desulpho-phenethyl glucosinolate. Equimolar amounts of 1.0 mM synthetic sinigrin, benzyl glucosinolate and phenethyl glucosinolate, which had been desulphated identically to test samples, were used to determine the detector response factors of measured desulpho-glucosinolates relative to the internal standard. The average response factors calculated for 10 standard samples were respectively 1.53 (s.e.m. 0.067) for desulpho-sinigrin and 1.85 (s.e.m. 0.076) for desulpho-benzyl glucosinolate. Concentrations of desulphosinigrin and desulpho-benzyl glucosinolate

in samples were calculated by comparing their respective peak areas to the internal standard peak area in the test sample corrected by their respective response factor.

3.3.3. Calculations and statistical analysis

The amount of mercapturic acids in the urine was calculated in a similar fashion to Experiment 1. The actual amount of intact glucosinolates excreted in the faeces of rats was obtained by multiplying glucosinolate concentrations by the dry weight of total faeces output. For dietary intake data, daily values were averaged over the 16 days after the adaptation period for each feeding period. Analysis of variance was performed using Genstat 5 (Lawes Agricultural Trust, 1989). To account for the split-plot design of the experiment, a block structure was adopted in the analysis. Isolators, rats within isolator, feeding period within isolator and feeding period within individual rat were the terms used in the block structure. The effects of feeding period and day of dose administration were considered as treatments as their allocation was balanced in the experimental design. The effect of diet, types of dose mixture and microbial status and their interactions were also analysed as treatment effects. With such a structure, the effect of bacterial status was analysed between isolators. The effects of feeding period and diet were studied between feeding periods within isolator. The effects of day of administration and dose mixture were analysed between periods within individual rats (Appendix 3).

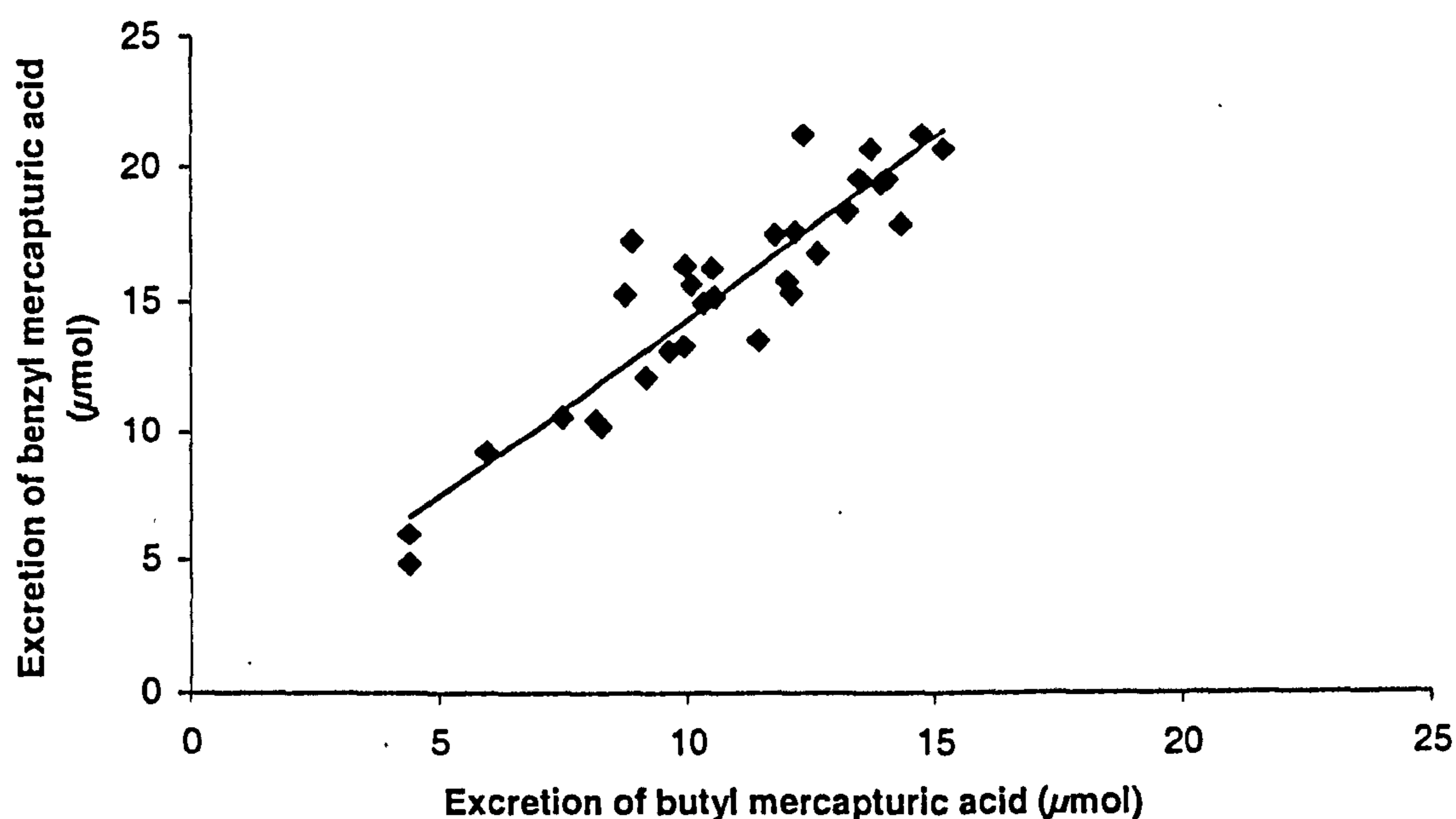
3.4. Results

3.4.1. Excretion of mercapturic acids

3.4.1.1. Excretion of benzyl and butyl mercapturic acids after ITC dose

The proportion of isothiocyanates recovered as their mercapturic acids after administration of 25 μmol of isothiocyanates was 0.41 (s.e.m. 0.018) for butyl isothiocyanate and 0.60 (s.e.m. 0.041) for benzyl isothiocyanate (Table 3.5). The excretion of benzyl mercapturic acid and butyl mercapturic acid was highly correlated ($p < 0.001$, see Figure 3.1) suggesting that concurrently administered butyl mercapturic acid could be used as a good predictor of the recovery of benzyl isothiocyanate as its mercapturic acid.

Figure 3.1: Relationship between cumulative excretion of butyl mercapturic acid and benzyl mercapturic acid after administration of ITC dose.



Regression equation:

$$\text{Benzyl mercapturic acid} = 1.36 \text{ Butyl mercapturic acid} + 0.67$$

$$r^2 = 0.847$$

Table 3.5: Cumulative excretion of mercapturic acids over 144h after administration of 25 μmol of precursor isothiocyanate: NS = Non significant at $p < 0.05$.

Precursor	Diet	Excretion of mercapturic acid				Level of significance
		Bacterial status		Mean	SED	
		Flora+	Flora-			
Benzyl isothiocyanate	Myro+	15.6	18.6	17.1	0.68	NS
	Myro-	12.2	14.1	13.2		
	Mean	13.9	16.4			
	SED	3.51				
	Level of significance	NS				
Butyl isothiocyanate	Myro+	11.0	12.6	11.8	1.20	NS
	Myro-	9.1	10.1	9.3		
	Mean	9.8	11.4			
	SED	2.14				
	Level of significance	NS				

The mean excretion ratio of benzyl mercapturic acid to butyl mercapturic acid was 1.42 (s.e.m. 0.031). Bacterial status, type of diet and sequence of offering the diet had no significant influence on this ratio.

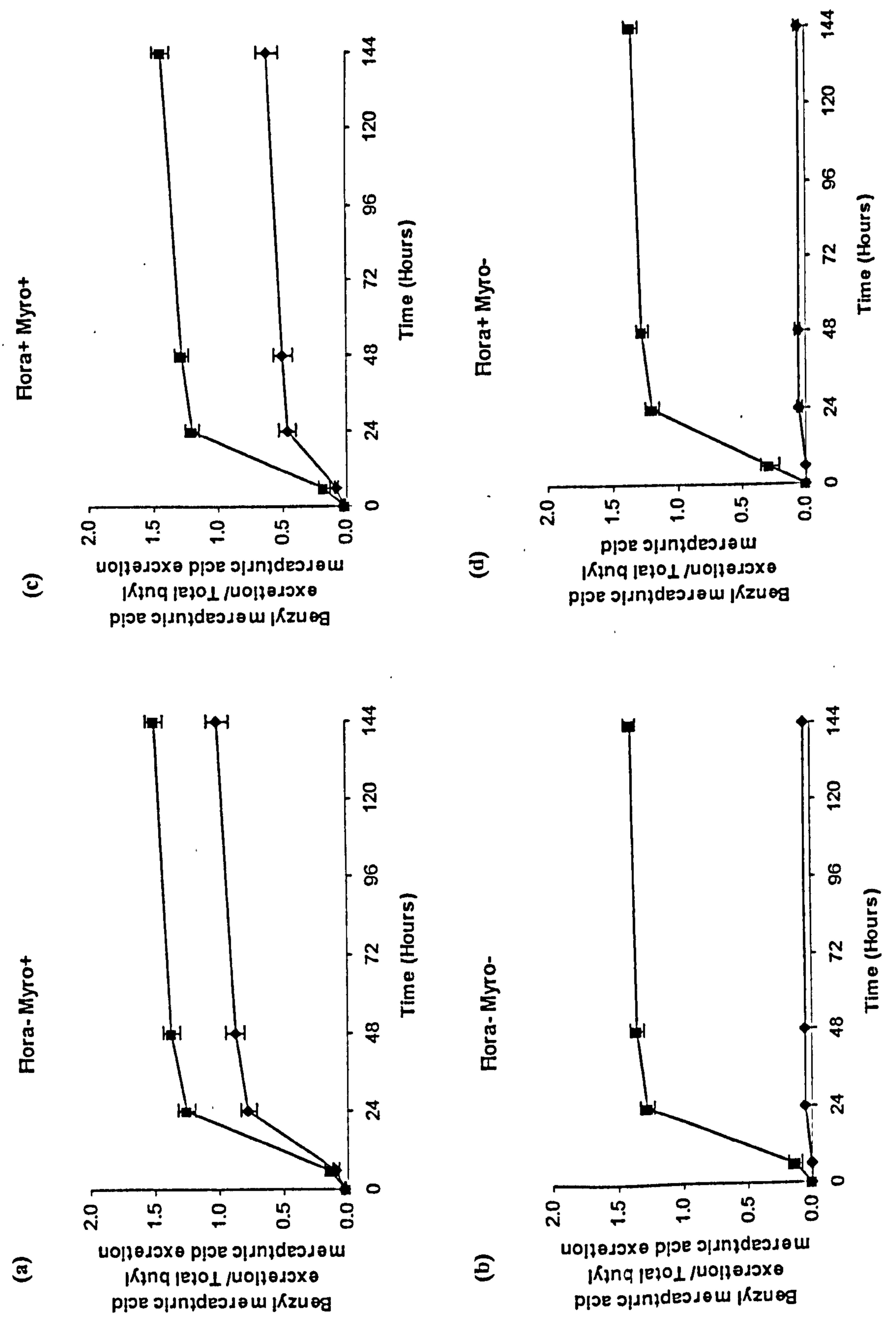
3.4.1.2. Proportion of benzyl isothiocyanate release from benzyl glucosinolate after BGSL dose

Diet had a significant influence on the release of benzyl isothiocyanate with higher estimates when rats were consuming the Myro+ diet than when the Myro- diet was offered ($p < 0.05$) (Table 3.6). The proportion of benzyl isothiocyanate release was higher in germ-free rats (Flora-) than in rats harbouring a microflora (Flora+), ($p < 0.05$) (Table 3.6), indicating a negative effect of the presence of the microflora on the amount of benzyl isothiocyanate available for intestinal uptake. The excretion of mercapturic acid reached a plateau at 48h (Figure 3.2). The proportion of mercapturic acids detected beyond 48 hours contributed for less than 0.2 of the total excretion. The sequence of offering the diet had no significant effect but rats administered with the BGSL dose at day 10 had a significantly higher isothiocyanate release than rats dosed at day 17 ($p < 0.05$).

Table 3.6: Proportion of benzyl isothiocyanate released in the digestive tract of rats after administration of 25 μ mol of benzyl glucosinolate.

Precursor	Diet	Bacterial status		Mean	SED	Level of significance
		Flora+	Flora-			
Benzyl glucosinolate	Myro+	0.50	0.80	0.65	0.0486	$p < 0.05$
	Myro-	0.05	0.04	0.05		
	Mean	0.27	0.42			
	SED	0.0294				
Level of significance		$p < 0.05$				

Figure 3.2: Cumulative excretion of benzyl mercapturic acid excreted after administration of BGS� and ITC dose.
 Data are expressed as a proportion of the total excretion of butyl mercapturic acid. ■ excretion after ITC dose, ◆ excretion after BGS� dose



3.4.1.3. Excretion of allyl mercapturic acids from ITC dose and BGSL

The background level of allyl mercapturic acid excretion was measured from the urinary outputs following the BGSL dose (Table 3.7). The excretion of allyl mercapturic acid was increased after the ITC dose due to the presence of allyl isothiocyanate in the dose (Table 3.7). After both doses, the excretion of allyl mercapturic acid was significantly higher when rats were fed the Myro+ diet than when they were fed Myro- diet ($p < 0.05$) (Table 3.7) but it was not significantly different in the presence or absence of microbial activity.

Table 3.7: Cumulative excretion of allyl mercapturic acids over 144h: with an oral administration of 25 μ mol allyl isothiocyanate (ITC dose) or without administration of allyl isothiocyanate (BGSL dose). NS = Non significant at $p < 0.05$.

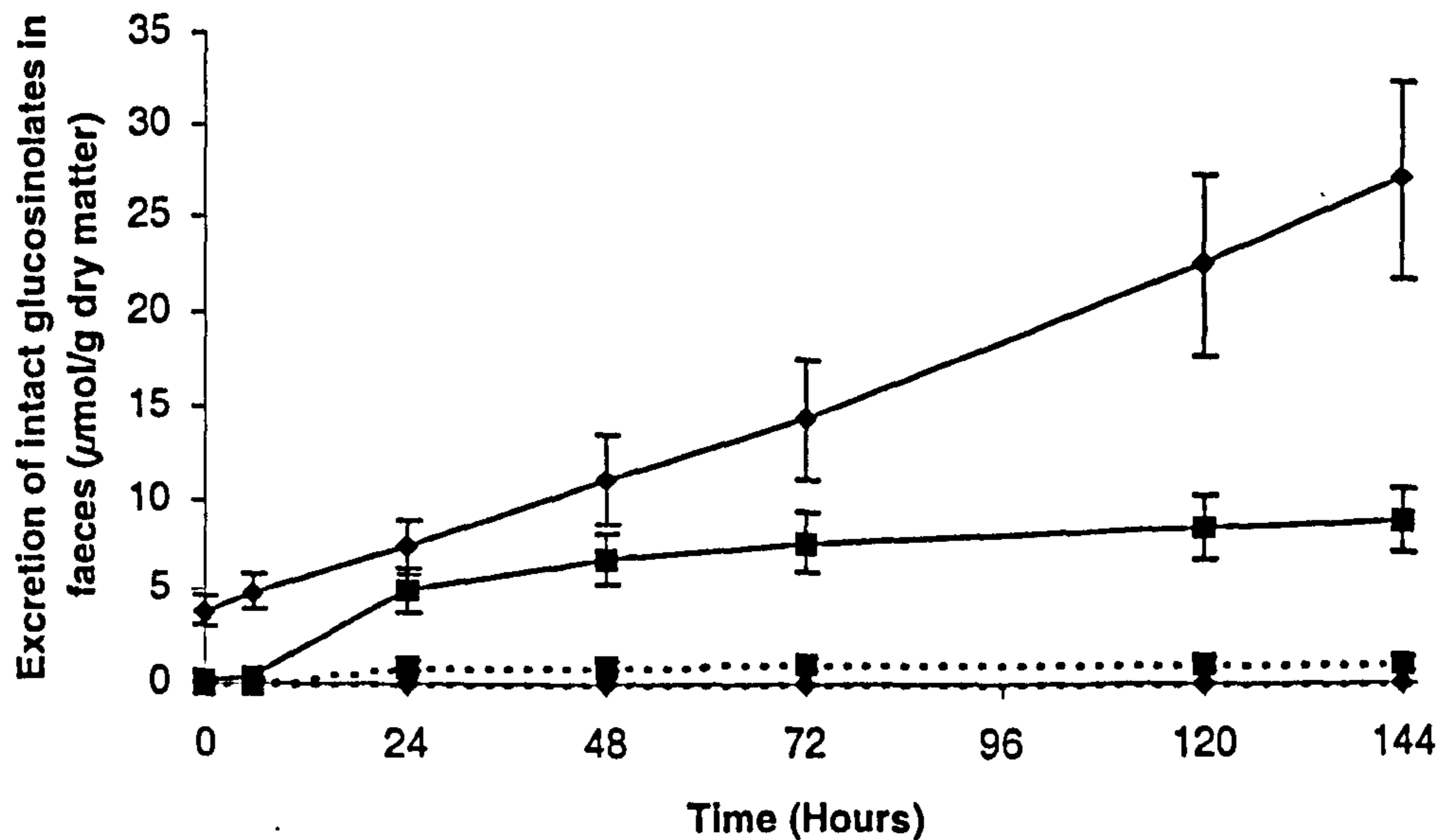
Dose	Diet	Excretion of allyl mercapturic acid				Level of significance
		Bacterial status		Mean	SED	
		Flora+	Flora-			
ITC	Myro+	67.2	58.9	63.3	2.61	$p < 0.05$
	Myro-	16.4	13.2			
	Mean	43.5	34.5			
	SED	5.98				
	Level of significance	NS				
BGLS	Myro+	31.8	42.4	37.1	1.83	$p < 0.05$
	Myro-	4.72	0.84			
	Mean	18.3	21.6			
	SED	2.86				
	Level of significance	NS				

3.4.2. Proportion of glucosinolate excreted in faeces

Faecal excretion of non-digested glucosinolate was measured after administration of the BGSL dose. Intact glucosinolates were only detectable in the faeces of germ-free rats (Flora-). The absence of a measurable concentration of glucosinolates in the faeces of rats harbouring a human flora (Flora+) indicated a strong influence of the presence of the microflora. When plant myrosinase only was active (Flora-, Myro+), the proportion of benzyl glucosinolate recovered in the faeces was 0.04 (s.e.m 0.011). The proportion was less than 0.01 for sinigrin. In the total absence of myrosinase activity (Flora-, Myro-), the faecal recovery was 0.34 (s.e.m. 0.072) for benzyl glucosinolate and 0.07 (s.e.m. 0.018) for sinigrin. Sinigrin was excreted continuously whereas excretion of benzyl glucosinolate reached a peak between 6 and 24 hours

after administration of the oral dose. At $t=72\text{h}$, the cumulative amount of benzyl glucosinolate excreted when the Myro- was offered was 0.86 (s.e.m 0.023) of the total excretion but traces were still detectable after 144h (Figure 3.3).

Figure 3.3: Excretion of sinigrin and benzyl glucosinolate in faeces of germ-free rats (Flora-) over 144h after administration of BGS dose. \blacklozenge sinigrin, \blacksquare benzyl glucosinolate, — Myro-diet, Myro+ diet.



3.4.3. Live weight

Rats gained on average 18.8 g (s.e.m. 1.53) per week and this rate of gain was not significantly influenced by microbial status.

3.4.4. Daily food intake and daily glucosinolate intake of rats

Mean food intake by rats was 16.9 g (s.e.m. 0.34) of pellets per day. Flora- rats ate significantly more than Flora+ rats ($p<0.01$). The amount of Myro+ diet ingested by rats was significantly higher than that of the Myro- diet ($p<0.005$). Food intake was not affected by the sequence of offering the diet. The average intake of sinigrin was 33.1 μmol per day (s.e.m. 0.66).

3.5. Discussion

A fuller understanding of the cancer-protective effects of brassica vegetables requires more information about the fate of glucosinolates from their synthesis in plant cells to their absorption from the gut as breakdown products and delivery to tissues. Studies on the effect of cooking vegetables have provided a better appreciation of the fate of glucosinolates during food preparation (de Vos & Blijleven, 1988). The fate of glucosinolates following ingestion is much less well understood. Plant myrosinase is likely to play a role in glucosinolate hydrolysis following ingestion of brassica vegetables by humans. If vegetables are cooked prior to ingestion, however, intact glucosinolates may reach the lower gut. Upon microbial hydrolysis (Elfoul, 1999), they may exhibit a protective effect on the colorectal epithelium (Lund *et al.* 2000) where the occurrence of cancer is high. This experiment aimed to improve understanding of the relative contribution of plant myrosinase and the myrosinase activity of the microflora.

Preparation of the diets was conducted in such a way as to produce diets which differed only in their activity of plant myrosinase but where an equivalent content of glucosinolates was preserved. The Myro- diet thus represented a cooked vegetable whereas the Myro+ diet was the equivalent of ingesting the vegetable in its raw form. Although Brussels sprouts are not consumed raw in human diets, they offered a good model for this metabolism experiment as various biological effects such as colonocyte apoptosis have been studied using the same variety of vegetables (Smith *et al.* 2000).

The major difference in the production of benzyl isothiocyanate after ingestion of raw and cooked diet confirmed that the activity of plant enzyme plays a major role on the subsequent fate of ingested glucosinolates. The release of isothiocyanate by plant myrosinase, *in vitro*, has been well documented at pH greater than 6.0 (Gil & MacLeod, 1980). The substantial release of benzyl isothiocyanate when rats were fed raw Brussels sprouts confirmed that, *in vivo*, conditions of hydrolysis are favourable for isothiocyanate production after disruption of plant cells. When plant myrosinase only was active, the proportion of dosed benzyl glucosinolate accounted for by isothiocyanate release and faecal excretion was around 0.84. Although other

breakdown products, such as nitriles, were not measured in the current experiment, their release in the digestive tract were probably limited in the conditions of this experiment. This observation promotes support for earlier findings in which the occurrence of toxic symptoms after ingestion of cruciferous vegetables was low (McMillan *et al.* 1986).

The presence of the intestinal microflora led to total degradation of glucosinolates as demonstrated by the essentially zero concentrations of intact glucosinolates in the faeces. This result confirmed the ability of the human microflora to degrade glucosinolates. Several studies have shown a similar effect following incubations of human faeces *in vitro* and using single strains of bacteria (Rabot *et al.* 1995; Getahun & Chung, 1999). The breakdown products resulting from the microbial hydrolysis *in vivo*, however, have not been well characterised. In recent studies, the main microbial metabolite detected *in vivo* following gavage with sinigrin was allyl isothiocyanate. A proportion of approximately 0.10 of sinigrin was converted to allyl isothiocyanate in rats harbouring a human faecal flora, this proportion was 0.15 for rats harbouring a single strain of *Bacteroides thetaiotaomicron* (Elfoul, 1999). Surprisingly, in the current study, the release of benzyl isothiocyanate by microbial myrosinase was relatively low. Moreover, the effect of plant myrosinase was not enhanced by the myrosinase activity of the microflora. Indeed, the presence of an active microflora seemed to reduce the excretion of benzyl mercapturic acid. These observations suggested that the microflora may be able to catalyse both the release of isothiocyanates and their further breakdown to other final products resulting in minimal absorption by the distal digestive tract. When both sources of myrosinase were active, however, the proportion of benzyl glucosinolate broken down to benzyl isothiocyanate found in the current studies (0.50) was similar to previous findings on allyl isothiocyanate release from sinigrin in conventional rats (0.41) (Duncan *et al.* 1997).

Unexpectedly, the excretion of glucosinolates was not complete in the absence of any sources of myrosinase. Only a proportion of 0.34 of benzyl glucosinolate was excreted in faeces. In a parallel experiment, the faecal recovery of sinigrin was in the same order of magnitude (0.28) (Elfoul, 1999). These results suggest a possible

metabolism other than enzymatic hydrolysis for the proportion of glucosinolates (0.66 of administered benzyl glucosinolate) which are not accounted for. Among the possible metabolic pathways are the desulphatation of glucosinolates by the intestinal desulphatase enzymes or the intestinal absorption of intact glucosinolates. The absence of desulphated glucosinolates in faeces of germ-free or human-flora rats given an oral dose of sinigrin rules out an endogenous desulphatation of glucosinolates (Elfoul, 1999). On the other hand, a small excretion of intact sinigrin (0.03 of the initial dose in germ-free rats and 0.04 in animals associated with a human flora) has been measured in the urine of rats administered an oral dose of sinigrin, confirming the potential absorption of intact glucosinolates by the intestinal wall (Elfoul, 1999).

The proportion of allyl isothiocyanate released from dietary sinigrin could not be measured in the current study. Due to the background excretion of allyl mercapturic acid, there was not a strong relationship between the excretion of butyl isothiocyanate and allyl isothiocyanate administered in ITC dose and hence butyl isothiocyanate could not be used to normalise excretion rate of sinigrin. Limiting the background excretion could improve the estimates if animals received the food as a discrete meal instead of *ad libitum*. Nevertheless, the level of excretion of allyl mercapturic acid in urine and intact sinigrin in faeces supported the estimates found with benzyl glucosinolate.

The findings indicated that, in the conditions defined for this experiment, the activity of plant myrosinase *in vivo* was the major factor of influence on the production of isothiocyanates. Myrosinase from raw vegetables induced a substantial and rapid release of isothiocyanates, probably in the upper digestive tract. In contrast, after ingestion of cooked vegetables, the isothiocyanates resulting from microbial hydrolysis tended to be released to a lesser extent and this was probably due to the ability of the microflora to utilise the newly formed isothiocyanates. Consequently, methods of preparation of vegetables may have a determinant role in the cancer-protective effect generally associated with brassica vegetable consumption.

CHAPTER 4: EXPERIMENT 3

Measurement of isothiocyanate release in the intestinal tract of healthy human volunteers.

4.1. Introduction

Glucosinolates may be hydrolysed during and following ingestion by humans to release a range of hydrolysis products. Among the breakdown products, the extent of formation of isothiocyanates in the human digestive tract is of particular interest. Studies on the effects of isothiocyanates in rodents *in vivo* (Stoner *et al.* 1998; Wattenberg, 1977) and on human tumour cell lines *in vitro* (Musk & Johnson, 1993) have not yet fully established whether the rates of isothiocyanate production in the digestive tract is of sufficient magnitude to significantly reduce cancer risks. Indeed, information on the release of the isothiocyanates in the digestive tract of humans is still crude. Isothiocyanate release is likely to be highly dependent on the activity of myrosinase in the vegetable material (Getahun & Chung, 1999). Plant myrosinase, however, can be inactivated by heat when vegetables are cooked, thus modifying the extent of isothiocyanate release. Moreover, the intestinal microflora may influence the proportion of isothiocyanates available for intestinal absorption (Experiment 2). Consequently, the pattern of isothiocyanate release in humans may vary according to individual food habits and to the enzymatic capacity of the intestinal microflora.

The aim of this study was to measure the actual amount of isothiocyanates arising in the digestive tract of healthy human volunteers, in situations similar to normal feeding circumstances. White cabbage, a vegetable commonly consumed in both raw and cooked form, is rich in sinigrin (Sang *et al.* 1984). The conversion of sinigrin to allyl isothiocyanate *in vivo* was therefore compared after consumption of raw and cooked cabbage by volunteers.

Intestinal release of isothiocyanates was measured using mercapturic acids of isothiocyanates as markers of metabolism. The method developed for rats (Experiment 1) was adapted for humans. In human investigations, natural sources of readily available isothiocyanates had to be used to measure the post-absorptive

conversion of isothiocyanates to mercapturic acids. Mustard contains high concentrations of allyl isothiocyanate and no sinigrin (Sang *et al.* 1984). The recovery of allyl isothiocyanate to its mercapturic acid was therefore measured following ingestion of mustard. Watercress is a good source of phenethyl glucosinolate and is essentially free of sinigrin (MacLeod & Islam, 1975). An autolysate of watercress provided a source of phenethyl isothiocyanate (Getahun & Chung, 1999; MacLeod & Islam, 1975). Watercress autolysate was thus offered to volunteers concurrently to mustard or raw or cooked cabbage to correct for variation in excretion rate of mercapturic acids.

4.2. Materials and methods

4.2.1. Experimental design

The experiment involved offering three defined meals to each volunteer in sequence (Figure 4.1). The defined meals included raw cabbage (Treat COL), cooked cabbage (Treat COOK) or mustard (Treat MUST). Meals were separated by periods of 48 hours to allow complete excretion of mercapturic acids. Volunteers were ranked according to their habitual consumption of vegetables (Appendix 4b) and then divided into two blocks of six on the basis of this consumption (high vegetable consumers and low vegetable consumers). Within each block, treatments were allocated to meal-times in two 3x3 Latin Squares with rows comprising meal-times and columns comprising volunteers (Table 4.1). In this way the order of offering meals to subjects was balanced within blocks.

4.2.2. Volunteers

Twelve non-smoking, male volunteers aged 25-39 years were recruited. The average body mass index of all volunteers was 23 (s.e.m= 0.59). Prior to the study, volunteers were asked to complete a questionnaire to assess their general health and their habitual food intake (Appendix 4a). Volunteers signed a consent form before taking part in the trial. Ethical agreement for the study was obtained from the Grampian Research Ethics Committee.

Figure 4.1: Experiment timetable

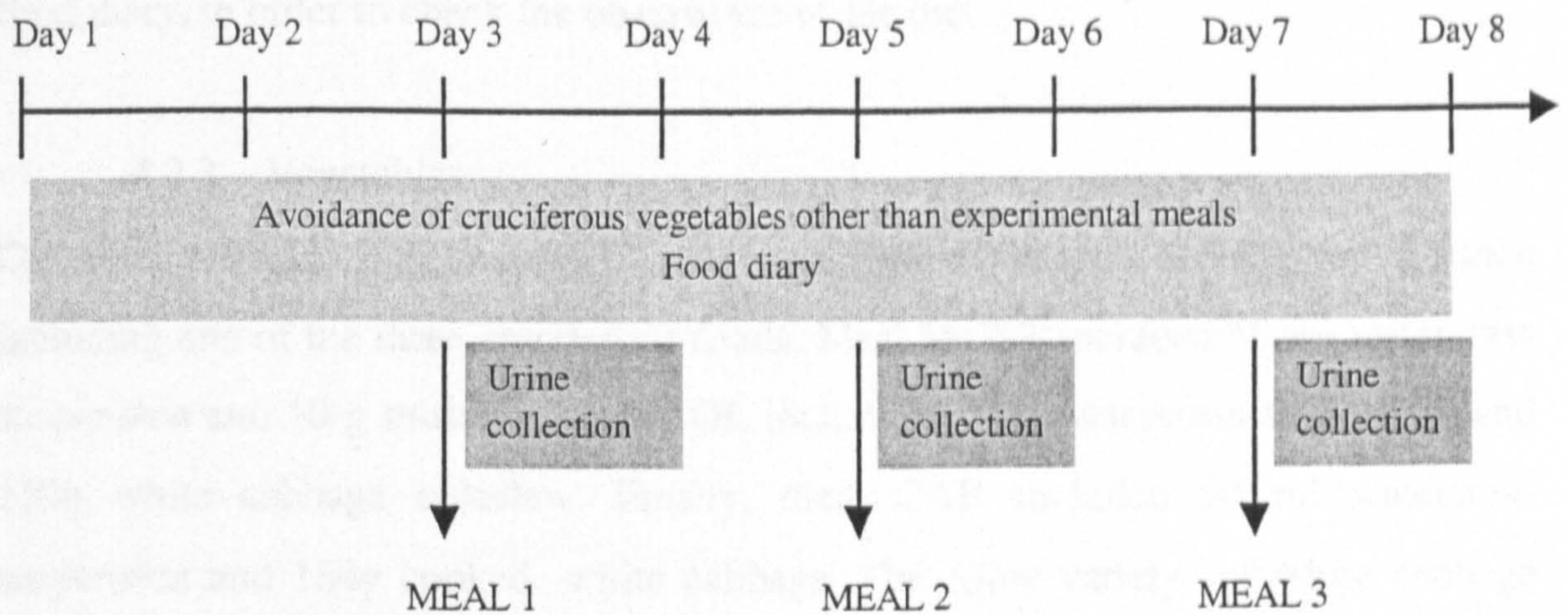


Table 4.1: Experimental design: the allocation of volunteers to blocks was based on the results of a food questionnaire on habitual vegetable consumption.

	Low/normal vegetable consumers					
	1	2	3	4	5	6
Meal 1	CAB	CAB	COL	MUST	COL	MUST
Meal 2	MUST	COL	CAB	COL	MUST	CAB
Meal 3	COL	MUST	MUST	CAB	CAB	COL

	Normal/high vegetable consumers					
	7	8	9	10	11	12
Meal 1	CAB	MUST	MUST	COL	CAB	COL
Meal 2	MUST	CAB	COL	CAB	COL	MUST
Meal 3	COL	COL	CAB	MUST	MUST	CAB

Volunteers were asked to avoid cruciferous vegetables in their diet for two days prior to the study and throughout the period of experiment. They were also asked to keep a food diary, in order to check the observance of the diet.

4.2.3. Vegetables

On three different occasions (t= 0, 48 and 96h) volunteers were given a lunch including one of the three cruciferous foods. Meal MUST included 50 ml watercress suspension and 10 g mustard. Meal COL included 50 ml watercress suspension and 150g white cabbage coleslaw. Finally, meal CAB included 50 ml watercress suspension and 150g cooked white cabbage. The *Kilor* variety of white cabbage (*Brassica oleracea capitata*) was used as it contains a high concentrations of sinigrin. Watercress (*Nasturtium officinalis*, John Hurd, Warminster, UK) and ready-made mustard (Colman, Norwich, UK) were purchased from a wholesale grocer. White cabbage was obtained from Novartis Seeds Ltd, Lancashire, England. In addition to the experimental foods, volunteers were also served an accompanying course composed of chicken fricassee and rice and a sweet course composed of fruit salad and ice cream. The accompaniments were identical for all meals served.

4.2.4. Preparation of experimental meal

4.2.4.1. Watercress suspension

Watercress was freeze-dried immediately after purchase and ground to a powder using a coffee mill. The watercress suspensions were made up by re-suspending 2.0 g of watercress powder in 50 ml water. To improve palatability, 1.2 g of sweetener (Canderel[®], Monsanto plc, England, UK) was added to each suspension. A separate suspension was made up for each volunteer in 100 ml plastic bottles. Three extra portions per day were also prepared for analysis of phenethyl isothiocyanate content. The homogenates were sealed and incubated for two hours at 40°C to allow a complete hydrolysis of phenethyl glucosinolate into phenethyl isothiocyanate.

4.2.4.2. Cabbage preparation

As glucosinolate concentrations vary in different parts of the plant (Pihakaski & Pihakaski, 1978), special precautions were taken when cutting cabbage into portions. Cabbage was cut into an even number of wedges, two adjacent wedges forming a pair. Each wedge was adjusted to 150 g. For the COL meal, wedges of cabbage were individually chopped in a food processor less than 30 min before serving. A salad dressing made of yoghurt and sultana raisins was served with the raw cabbage. Portions of cooked cabbage were micro-waved pair by pair immediately before serving for 4 min at 650 Watts. Subsequently, for each paired portion, one was given to a volunteer whereas the other one was reserved at room temperature until the end of the meal and then kept at -20°C until analysis.

4.2.5. Urine collection

Prior to meal ingestion, volunteers were asked to collect a urine sample. After each meal, urine samples were collected at each micturition over 24 hours by volunteers. The volume of each micturition was recorded by the volunteers and a 15 ml sample from each micturition was collected and kept at -20°C.

4.2.6. Analysis

4.2.6.1. Chemicals

Allyl, butyl, benzyl and phenethyl isothiocyanates were purchased from Aldrich, Dorset, UK. Methanol (analytical grade) and absolute ethanol were purchased from BDH, Poole, Dorset, UK. Dichloromethane and acetonitrile were HPLC grade and purchased from Rathburn, Walkerburn, Scotland, UK. Sinigrin monohydrate, purified from horseradish, was obtained from Sigma, Poole, Dorset, UK. Phenethyl glucosinolate was prepared as a potassium salt by Dr A. Robertson, St Andrews University, Scotland, UK.

4.2.6.2. Quantification of isothiocyanates in watercress and mustard

Phenethyl isothiocyanate concentration in watercress juice and allyl isothiocyanate concentration in mustard were analysed by a modification of published methods (Youngs & Wetter, 1967). For watercress samples, 100 µl of 67 mM benzyl isothiocyanate in absolute ethanol was added as an internal standard to 10 ml of

watercress juice. The juice was extracted twice with 10 ml of dichloromethane using centrifugation at 1000 g to separate the layers. The solvent extracts were pooled and concentrated under an air stream to approximately 1 ml. Each sample was analysed in duplicate by gas chromatography. For mustard sample preparation, 1g of ready-made mustard was added to 10 ml water and 100 μ l of 67mM butyl isothiocyanate (in absolute ethanol) was added as an internal standard. The extraction procedure was the same as for watercress. The extracts were analysed on a gas chromatograph (Philips PU4550, Philips Scientific, Cambridge,UK) fitted with a BPX5 bonded phase capillary column (SGE, Milton Keynes, UK) of 30m length with an internal diameter of 0.52 μ m and a film thickness of 0.2 μ m. Column temperature was isothermal at 90°C for detection of benzyl and phenethyl isothiocyanate and 70°C for detection of allyl and butyl isothiocyanate. Injection and detection temperatures were 200°C. The carrier gas was helium and detection was by flame ionisation. Peak areas were measured on a Spectra-Physics Chromjet integrator (San Jose, California, USA). Retention times and response factors were determined by using 67 mM solutions of isothiocyanates made up in absolute ethanol, diluted 10 times in water and extracted in the same way as for watercress juice. The retention times for allyl, butyl, benzyl and phenethyl isothiocyanates were respectively: 17.2, 19.7, 6.6 and 12.1 min. The response factor for allyl isothiocyanate:butyl isothiocyanate was 0.72 and the response factor for phenethyl isothiocyanate:benzyl isothiocyanate was 1.19. Concentrations of allyl isothiocyanate and phenethyl isothiocyanate were subsequently determined relative to the respective internal standard included in the test sample.

4.2.6.3. Quantification of sinigrin in white cabbage:

Portions of raw and cooked white cabbage, reserved after the experimental meals, were freeze-dried and ground. Analysis of desulpho-sinigrin was conducted as described previously (Chapter 3, Section 3.3.2.).

4.2.6.4. Myrosinase activity

Myrosinase activity was determined for each portion of raw and cooked cabbage using a modification of published methods (Bones & Slupphaug, 1989). The procedure involved a preliminary phase of extraction of the myrosinase from the

vegetable material. A 0.5g sample of freeze-dried and ground cabbage was re-suspended in 20 ml ice-cold distilled water and placed onto an agitator for 1 hour at 4°C. The sample was subsequently centrifuged at 17,000 g for 30 min and the supernatant was dialysed for 48 hours against ice-cold distilled water using pre-soaked dialysis tubing made of cellulose membrane (Sigma, Poole, Dorset, UK). The distilled water was changed every 12 hours. Dialysates were centrifuged at 17,000 g for 30 min and approximately 15 ml of supernatant containing the myrosinase enzyme were collected. To concentrate the myrosinase solution, a 5 ml aliquot of sample was freeze-dried. The myrosinase extract was reconstituted by adding 0.75 ml distilled water. The myrosinase activity was subsequently tested by measuring the glucose released from a known amount of sinigrin incubated with the myrosinase extract. For each myrosinase extract, a 0.25 ml sample was mixed with 0.55 ml citric acid - sodium citrate buffer (0.05M, pH 5.5) and 0.2 ml of 36mM sinigrin. The sample was incubated for 30 min at 37°C. Thereafter, the myrosinase was inactivated by heating the sample at 100°C for 5 min. The sample was allowed to cool and released glucose was measured using a diagnostic kit (A-115, Sigma, Poole, Dorset, UK), adding 1 ml of glucose assay reagent to 0.25 ml of sample. Absorbance was read at 520 nm using a Unicam SP 1800 Ultraviolet spectrophotometer (Unicam, Cambridge, UK) against a blank solution where the enzyme solution was replaced by water. To check for the presence of any residual glucose in the freeze-dried myrosinase extract, glucose concentration was also measured in the myrosinase solutions incubated with water instead of sinigrin. The actual release of glucose was calculated by the difference between glucose release in the absence of sinigrin and glucose release in the sample containing sinigrin.

4.2.6.5. Mercapturic acid analysis

Urine samples were analysed for mercapturic acid derivatives by HPLC (Chapter 2, section 2.2.7.2). Prior to analysis, urine samples were concentrated as follows. A volume of 10 ml urine sample was transferred to a 20 ml glass vial and 200 µl butyl mercapturic acid (1.5 mM in 500 ml.l⁻¹ ethanol) was added as an internal standard. Urine was subsequently freeze-dried and finally re-suspended in 1 ml water. An aliquot of 0.4 ml of the concentrate was used for analysis.

4.2.6.6. Calculation of allyl isothiocyanate release from sinigrin

The proportion of released allyl isothiocyanate after ingestion of meal COOK and COL was calculated as follows. The total amount of allyl and phenethyl mercapturic acid excreted over 24 hours was determined for each meal type. To account for individual differences in the intake of precursor, the yield of mercapturic acids was expressed as proportions of precursor initially ingested (Formulas A and B). The formula used to calculate allyl isothiocyanate is detailed below for meal COL. Similar calculations were used for meal COOK.

Formula A:

$$\text{Molar proportion of allyl mercapturic acid} = \frac{\text{Allyl mercapturic acid}}{\text{Sinigrin}}$$

Formula B:

$$\text{Molar proportion of phenethyl mercapturic acid} = \frac{\text{Phenethyl mercapturic acid}}{\text{Phenethyl isothiocyanate}}$$

$$\text{Proportion of allyl isothiocyanate released from raw cabbage} = \frac{A_{\text{COL}} / B_{\text{COL}}}{A_{\text{MUST}} / B_{\text{MUST}}}$$

4.2.7. Statistical analyses

Analysis of variance was performed on the ratios A/B calculated for meal COOK, COL and MUST. A log transformation was applied to the ratios to conform to the assumption of equal variance required for this analysis. Analysis was carried out using individual volunteers as the block structure. The effects of experimental meals (called *meal* in the analysis), sequence of administration of meals (*day*) and habitual consumption of vegetables (*vegetable*) and the interactions of the three were assessed as treatments. Within the analysis, a contrast was applied for comparison between ingestion of sinigrin and ingestion of allyl isothiocyanate (meals COOK and COL versus meal MUST). A second contrast allowed comparison between ingestion of raw or cooked cabbage (meal COOK versus meal COL). To account for differences in the extent of myrosinase catalysis, myrosinase activity measured in either raw or

cooked cabbage were used as two distinct covariates in the statistical analysis. Within the structure described above, the effect of *vegetable*, the interaction of *meal* with *day* and the three-order interaction of *meal*, *day* and *vegetable* was assessed between volunteers, the main effects of *meal* and *day*, the interaction of *vegetable* with *meal* and the interaction between *vegetable* and *day* were assessed within volunteers (Appendix 5). Statistical analysis was performed using Genstat 5 (Lawes Agricultural Trust, 1989).

4.3. Results

4.3.1. Intake of glucosinolate and isothiocyanate precursors

The difference in sinigrin intake between treatments COL and COOK was significant ($p < 0.001$) (Table 4.2). The intake of phenethyl isothiocyanate was not significantly different between meals (Table 4.2). Myrosinase activity was significantly higher in raw cabbage ($19.1 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$ Dry Matter, s.e.m. 4.60) than in cooked cabbage ($0.85 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$ Dry Matter, s.e.m. 3.19).

4.3.2. Excretion of mercapturic acids

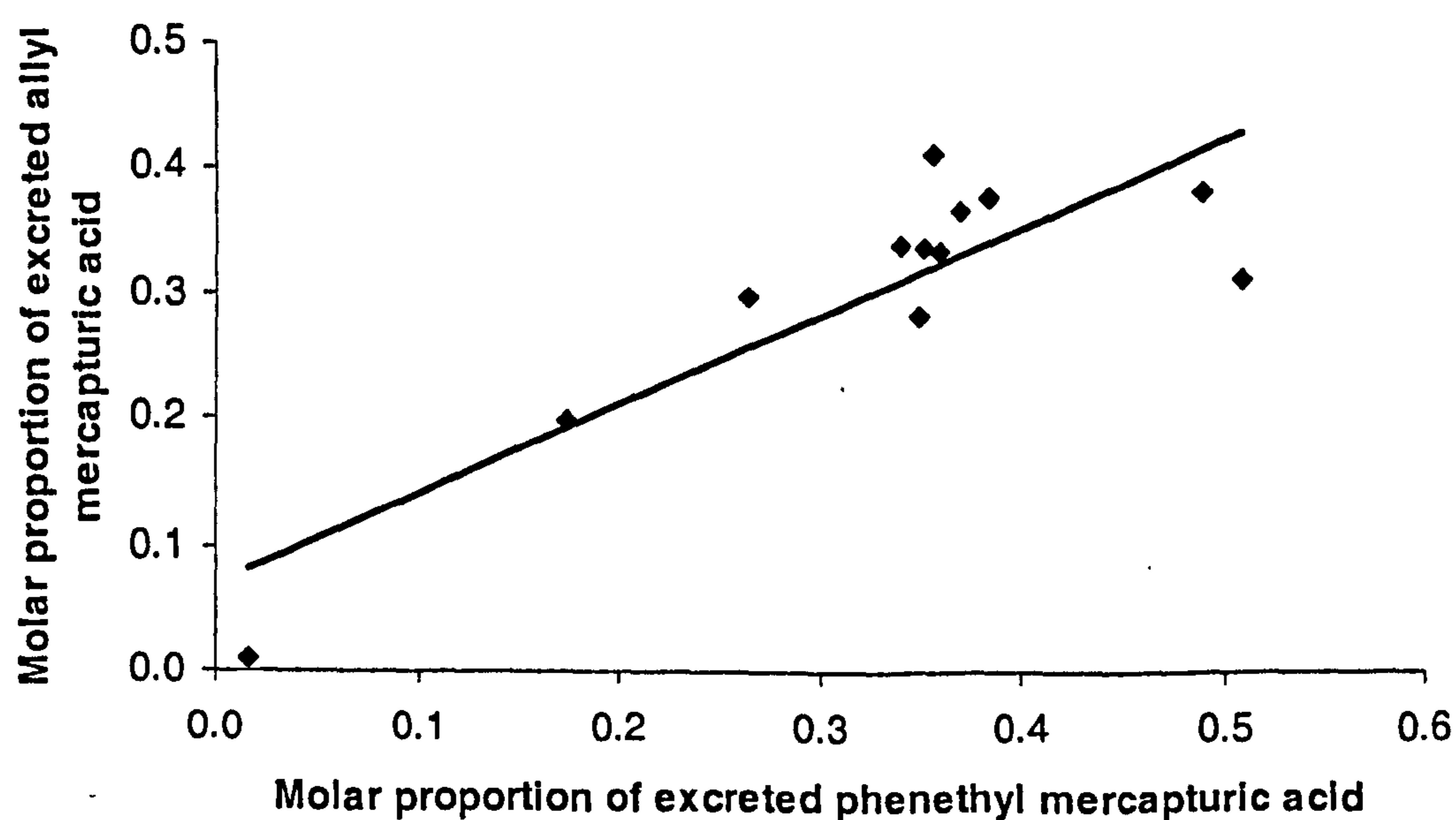
For all meals, the urinary excretion of allyl and phenethyl mercapturic acids was complete within 24 hours (Figure 4.3). The peak of marker excretion occurred within 12 h of ingestion of meals.

There was a significant ($p < 0.001$) positive linear relationship between the excretion of phenethyl mercapturic and allyl mercapturic acid excreted after meal MUST (Figure 4.3, $r^2 = 0.733$, $p < 0.001$). Phenethyl mercapturic acid could therefore be used as a predictor of allyl mercapturic acid excretion after meal COL and COOK. After meal MUST, the recovery of isothiocyanates as their mercapturic acid was 0.33 (s.e.m. 0.038) for allyl isothiocyanate and 0.30 (s.e.m. 0.031) for phenethyl isothiocyanate. There was no significant difference in phenethyl mercapturic acid excretion between the different meals.

Table 4.2: Amount of glucosinolates and breakdown products ingested by volunteers and mean excretion of urinary markers.

Compound	Amount of compound (μmol)					
	Meal COL		Meal COOK		Meal MUST	
	Mean	s.e.m.	Mean	s.e.m.	Mean	s.e.m.
INGESTED						
Sinigrin	163.9	10.91	69.9	5.17	-	-
Allyl isothiocyanate	-	-	-	-	179.8	6.22
Phenethyl isothiocyanate	42.8	2.11	42.7	2.09	42.9	2.13
EXCRETED						
Allyl mercapturic acid	17.9	1.89	16.9	2.71	54.4	6.13
Phenethyl mercapturic acid	15.3	1.96	18.6	1.96	14.1	1.48

Figure 4.2: Relationship between the excretion of allyl and phenethyl mercapturic acid when pre-formed allyl and phenethyl isothiocyanate were ingested (meal MUST)

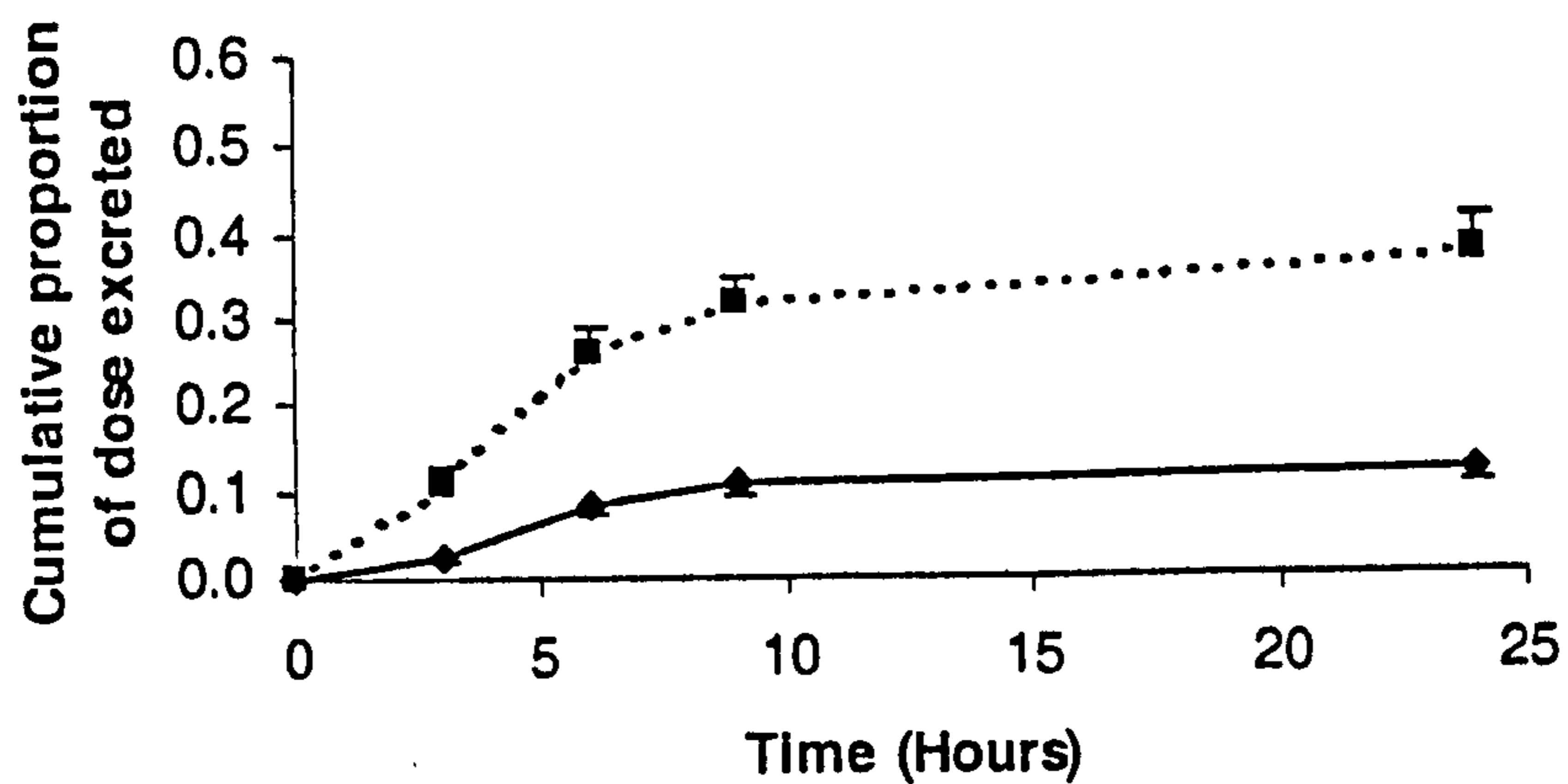


Regression equation:

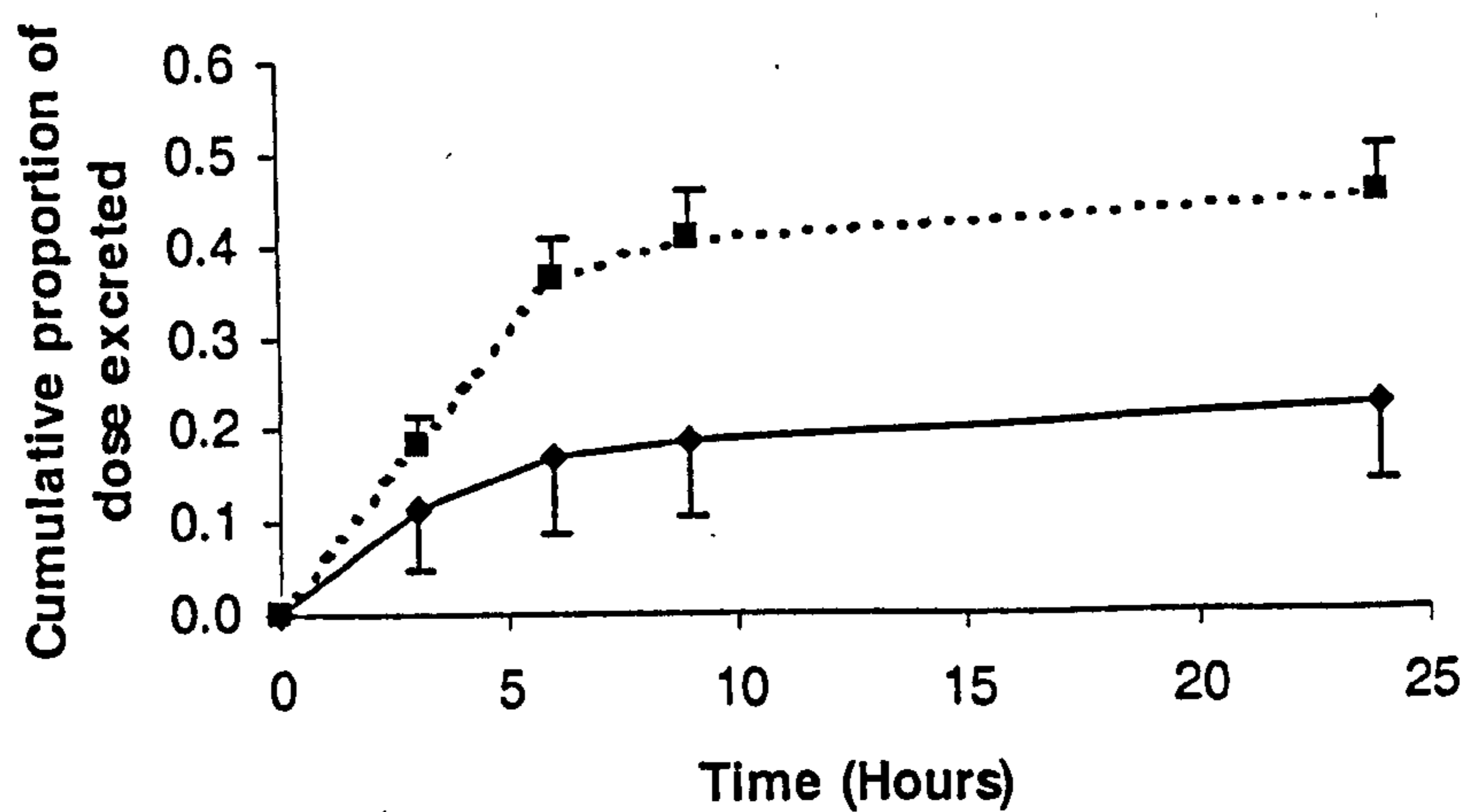
$$\text{Allyl mercapturic acid} = 0.701 \text{ phenethyl mercapturic acid} + 0.071 \quad r^2 = 0.733$$

Figure 4.3: Cumulative excretion of urinary markers over 24 hours after ingestion of meals (a) COL, (b) COOK and (c) MUST. Dotted lines denote phenethyl mercapturic acid, solid lines denote allyl mercapturic acid. Excretion is expressed as a molar proportion of the amount of ingested precursors.

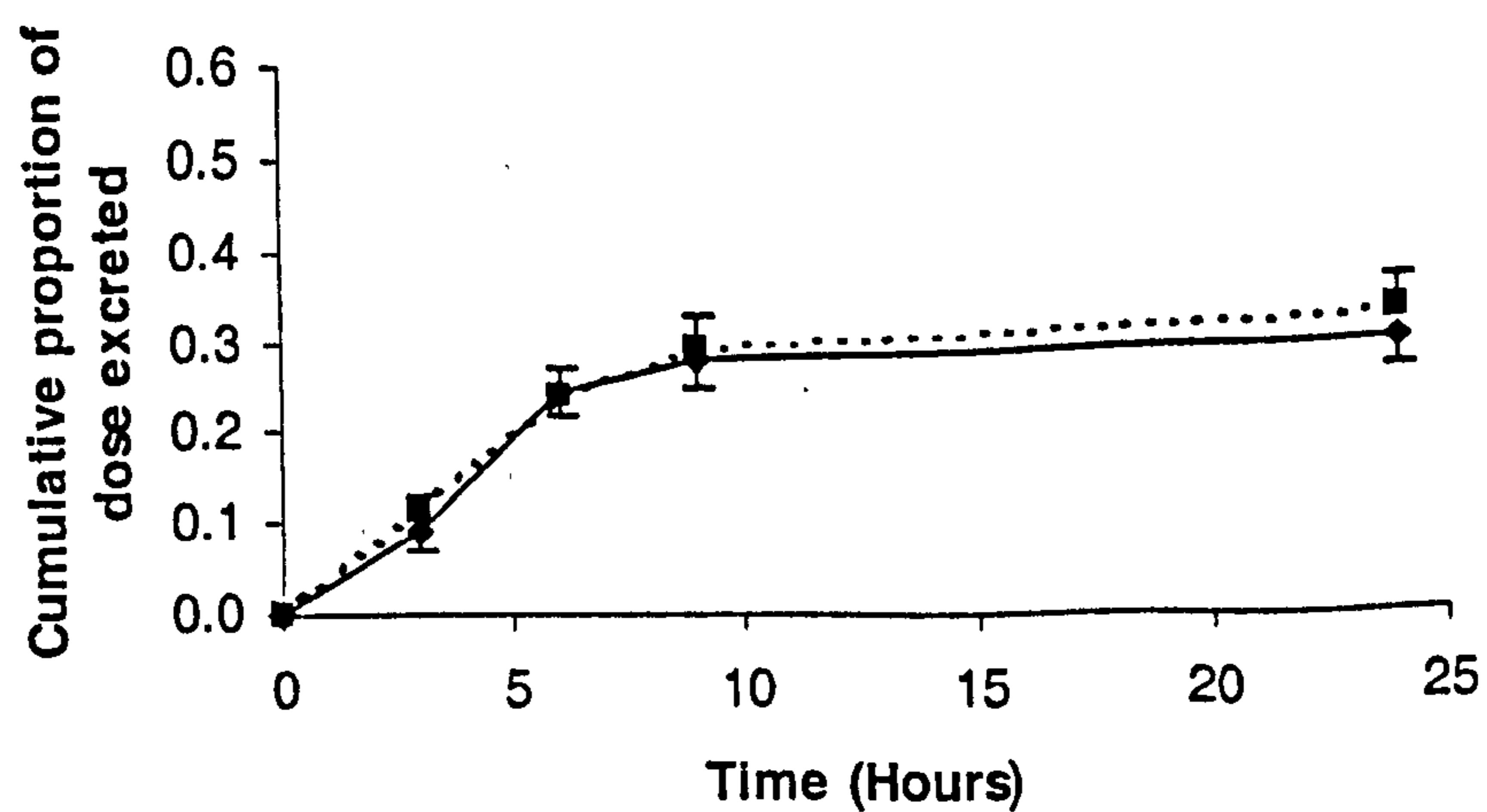
(a) COL meal



(b) COOK meal



(c) MUST meal



4.3.3. Proportion of allyl isothiocyanate release after ingestion of raw and cooked cabbage

The average proportion of sinigrin broken down into allyl isothiocyanate was 0.37 (s.e.m. 0.045) after meal COL and 0.53 (s.e.m. 0.134) after meal COOK but the difference between meals was not statistically significant.

4.4. Discussion

The aim of this experiment was to compare the release of isothiocyanates in two defined nutritional situations where vegetables were eaten either cooked or raw. Heat treatment led to an inactivation of plant myrosinase. This was reflected in the very low value of myrosinase activity in cabbage material subjected to microwave cooking (meal COOK). Previous studies in rats indicated that isothiocyanate release was significantly decreased when plant myrosinase was inactivated (Experiment 2). In the current experiment, the estimates of allyl isothiocyanate excreted after meal COOK and COL were not significantly different. This suggests that the method of food preparation did not significantly modify the release of allyl isothiocyanate.

The proportional release of allyl isothiocyanate found after ingestion of raw cabbage (0.37) was consistent with previous data obtained when cruciferous vegetables were eaten raw. In Experiment 2, the proportion of benzyl isothiocyanate released after consumption of Brussels sprouts by rats harbouring a human flora was 0.50. When conventional rats were given raw cauliflower, allyl isothiocyanate was released to a similar extent (0.41) (Duncan *et al.* 1997). These results suggested that the release of isothiocyanates *in vivo*, may be estimated to be between a third to a half of the amount of aliphatic glucosinolate content of the plant.

The extent of isothiocyanate release after consumption of raw cruciferous vegetables was little affected by variation between individual in the current experiment. Estimates of allyl isothiocyanate release when cabbage was eaten raw were consistent. No differences were found between high consumers of vegetables and individuals with a lower habitual consumption. In contrast, the range of phenethyl

isothiocyanate released after ingestion of raw watercress by healthy volunteers was between 0.30 to 0.67 of glucosinolate precursor in earlier studies but variations in post-absorptive fate were not accounted for (Chung *et al.* 1992).

The release of allyl isothiocyanate after the COOK meal supports the hypothesis that the endogenous microflora was responsible for the hydrolysis of the intact glucosinolates arising in the bowel in the absence of plant myrosinase. The high estimates of isothiocyanate release in COOK meals contrasted with previous findings where glucosinolate conversion to isothiocyanate was found to be 5 to 10-fold lower in the absence of plant myrosinase (Duncan *et al.* 1997; Elfoul, 1999; Getahun & Chung, 1999). The release of isothiocyanates after ingestion of cooked cabbage was highly variable in the current experiment. This variation between individuals suggests that microflora of different individuals may vary in its capacity to release isothiocyanate. Studies on the kinetics of glucosinolate degradation by different human flora would be requested to confirm these findings.

Myrosinase activity of the watercress suspension may have contributed to the observed variation in mercapturic acid excretion after the COOK meal. Myrosinase activity of the ingested watercress juice was not measured in the current experiment. The amount of watercress administered was, however, low in comparison to cabbage and it was assumed that its contribution to the breakdown of sinigrin would be minimal. The myrosinase activity in cooked cabbage was effectively zero rejecting possible bias due to incomplete inactivation, except for one portion of cabbage where myrosinase was still active ($31 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$ Dry Matter). This was accounted for in the subsequent statistical analysis.

Mercapturic acids were detected in the urine of all subjects after administration of the experimental meals except for one subject where the amount of marker detected was very low after each experimental meal (Figure 4.2). The ingestion of a high protein dietary supplement by this volunteer may have interfered with isothiocyanate release as isothiocyanates can bind with proteins (Drobnica & Augustin, 1965).

The original intention was to give volunteers equimolar amounts of the precursor, sinigrin, or allyl isothiocyanate at each experimental meal, the only variable factor being the activity of myrosinase. Preliminary studies on food processing and glucosinolate concentrations in cruciferous vegetables indicated that micro-waving effectively inactivated plant myrosinase (Verkerk, 1999). Furthermore, micro-waving was less likely to induce glucosinolate loss than any other cooking methods (Verkerk, 1999, unpublished). This cooking method was therefore adopted in the current experiment. The content of sinigrin in the cooked portions of cabbage was, however, significantly diminished in comparison with the raw counterparts. A preparation at a higher micro-wave power and with a shorter cooking time might have prevented these losses.

The current experiment has demonstrated that the method of measurement of isothiocyanate release developed in rats could be used in investigations with humans provided that appropriate sources of isothiocyanates and glucosinolates are used. The method enabled a comparison of isothiocyanate release within subjects, allowing an improvement in the accuracy of estimates in comparison with earlier human studies. The experiment suggested that isothiocyanates may be released to similar extents when vegetables are ingested raw or cooked, indicating that the involvement of the microflora in isothiocyanate release may be greater than suggested previously. The large variation observed between individuals after ingestion of cooked vegetables suggests however that the capacity of the microflora with regard to isothiocyanate release may vary according to the subject. It may be that some individuals may benefit more from the cancer-protective effects of brassicas than others according to the properties of their intestinal microflora.

CHAPTER 5: EXPERIMENT 4

Urinary excretion of N-acetyl cysteine conjugates following gastric or caecal delivery of isothiocyanates to rats

5.1. Introduction

Mercapturic acids provide a useful means of estimating production of isothiocyanates in the intestinal tract. This approach has the advantage of being non-invasive, requiring only urine samples. Furthermore, being end-products of metabolism, mercapturic acids are likely to provide a more reliable estimate of isothiocyanate production than direct measurement in digestive fluids. This is because the latter measurements are instantaneous and represent transient concentrations which are subject to variation depending on the relative rates of production and absorption from the digestive tract.

Previous experiments have shown that considerable inter-animal variation exists in the recovery of isothiocyanates as urinary mercapturic acids (Duncan, 1990; Getahun & Chung, 1999). Such variation could potentially reduce the usefulness of urinary mercapturic acids as markers of isothiocyanate release. To quantify inter-individual variation in isothiocyanate recovery, the approach adopted in previous experiments has been to administer homologous isothiocyanates concurrently with parent glucosinolates. The use of such homologous isothiocyanates assumes that a discrete dose of an isothiocyanate delivered instantaneously to the stomach has a similar metabolic fate to isothiocyanates arising from glucosinolate hydrolysis. Such an assumption seems reasonable when hydrolysis of glucosinolates occurs in the upper digestive tract under the action of plant myrosinase as both orally-dosed isothiocyanates and isothiocyanates arising from glucosinolates are likely to be released in the same digestive compartment. Whether the absorptive fate of orally administered isothiocyanates can adequately mimic the metabolic fate of isothiocyanates arising in the lower gut from hydrolysis of glucosinolates by microbial action is less certain. In the latter case, the approach assumes that isothiocyanates are recovered as mercapturic acids to the same extent along the entire intestinal tract. Differences may however exist between the proximal and distal digestive tract. The current experiment was therefore conducted to compare the

recovery of isothiocyanates as their mercapturic acids when they were delivered to the stomach or to the caecum of rats.

5.2. Materials and methods

5.2.1. Animals

Ten male, adult, Fischer 344 rats (mean weight: 353g s.e.m.5.37) (INRA breeding unit, Jouy-en-Josas, France) were placed in individual metabolism cages (Iffa-Credo, Saint Germain sur l'Arbresle, France) and fed a standard diet (Table 5.1) for a period of 6 weeks. The animals were separated into two groups of 5 rats each balanced for body weight. A period of twelve days was allowed before starting administration of treatments to allow the rats to habituate to the cages and to the diet.

Table 5.1: Composition of the pellets given to rats throughout the experiment (g.kg⁻¹ Dry Matter)

Ingredients	Standard diet
Casein	50.00
Soya isolate (PP500E, Protein Technologies International)	120.00
Saccharose	50.00
Mashed potato	290.00
Maize starch	289.85
Lard	30.00
Maize oil	30.00
Cholesterol	0.15
Cellulose	60.00
Mineral additive ^a	70.00
Vitamin additive ^b	10.00
Total	1000.00

^aThe mineral additive includes (g.kg⁻¹DM of diet): CaHPO₄ 30.1, KCl 7.0, NaCl 7.0, MgO 0.735, MgSO₄ 3.5, Fe₂O₃ 0.21, FeSO₄7H₂O 0.35, MnSO₄H₂O 0.17, CuSO₄5H₂O 0.035, ZnSO₄ 7H₂O 0.141, CoSO₄ 7H₂O 2.8*10⁻⁴ and KI 5.6*10⁻⁴.

^bThe vitamin additive includes (mg. kg⁻¹DM of diet): thiamin 20, riboflavin 15, pantothenic acid 70, pyridoxine 10, myoinositol 150, cyanocobalamin 0.05, ascorbic acid 800, α-tocopherol 170, menadione 40, niacin 100, choline 1360, folic acid 5, p-aminobenzoic acid 50, biotin 0.3, retinol 19800 IU.kg⁻¹diet and cholecalciferol 2500 IU kg⁻¹diet.

5.2.2. Treatments and procedure

The experiment was conducted as a cross-over design with the two treatments being administered to each rat in sequence. The **Gastric** treatment consisted of a single dose of 25 μmol each of allyl isothiocyanate and benzyl isothiocyanate administered by oral gavage. The **Caecal** treatment consisted of a single dose of 25 μmol each of allyl isothiocyanate and benzyl isothiocyanate administered directly into the caecum by the rectal route. One group of five rats received the **Gastric** treatment first and the other group received the **Caecal** treatment first. Treatments were administered on day 13 and day 29 of the experiment.

Prior to isothiocyanate administration, animals were anaesthetised by intra-muscular injection of ketamin (Imalgène 1000, 100 mg. kg^{-1} body weight, Merial, Lyon, France). Isothiocyanates doses were diluted in 0.25 ml pure corn oil. The mixture was subsequently flushed with 0.25 ml pure corn oil. When the **Gastric** treatment was given to rats, the isothiocyanate mixture was administered by gavage using a stainless steel stomach tube. When the **Caecal** treatment was given, a teflon catheter (Hasselcath, diameter 5F, length 600 mm, Plastimed, St Leu la Forêt, France) was introduced rectally and positioned in the caecum. The progression of the catheter along the distal digestive tract and the delivery of the dose to the caecum were monitored by X-ray radiography (Fluoroscopic Stenoscop 600, G.E. Medical Systems, Vélizy, France). To avoid chemical interference with the dosing mixture, no radio-opaque contrast chemical was used. The caecum was located on the X-ray image by introducing 0.80 ml air prior to administration of treatment. The air provoked a slight swelling of the caecum which appeared distinctively on the X-ray image. One millilitre of air was also injected after the oil administration to ensure complete flushing of the catheter.

Six hours prior to dosing, urine collection receptacles were emptied and cleaned. After dosing, rats were put back into their respective cages. Total urine output was collected at $t=0$, 6, 24 and 48h after administration of treatments. To avoid bacterial degradation of urinary markers, sodium azide (final concentration 0.2ml.l⁻¹) was added to the urine collection receptacles.

5.2.3. Analysis

The mercapturic acids derived from allyl isothiocyanate and benzyl isothiocyanate were measured by HPLC (Chapter 2, Section 2.2.7.2). Two hundred microlitres of urine were used and butyl mercapturic acid ($0.3 \mu\text{mol/sample}$) was used as the internal standard. The proportion of isothiocyanates converted to their related mercapturic acids for each rat on each dosing occasion was calculated by dividing the cumulative molar amount of mercapturic acid excreted over 48 hours by the molar amount of administered isothiocyanate. The excretion rate was calculated by dividing the amount of excreted mercapturic acid at each urinary output by the duration of urine collection.

5.2.4. Statistical analysis

Cumulative excretion of mercapturic acids was analysed using hierarchical analysis of variance appropriate to a cross-over design. The data were log-transformed to conform to the assumption of constant variance. The analysis was structured so that the effect of dosed compounds (named *product* in the analysis), days of administration (named *day*) and the interaction of the two were nested within animals (*animal*). The effect of Gastric and Caecal treatments (named *site* in the analysis), *day* and *product* and all interactions were assessed within the above structure. The *day.site* interaction was assessed between rats. The effects of *day* and *site* were analysed in the *animal.day* stratum to account for random variation in *day* effect. The effect of *product* and its interactions with other variables were analysed within *day* and within individual animal (Appendix 6a).

To analyse variation in the time-course of mercapturic acid excretion, an excretion rate was calculated for each compound and for each time interval. A log-transformation was applied to the new data set to provide for the assumption of constant variance. Analysis of variance was conducted using a different block structure than described previously to account for temporal variation over the 48 hours of collection. The effect of time of collection (*time*) was nested within *day*, the latter being itself nested within *animal*. The effect of treatments, days of administration and dosed compounds and all interactions were assessed within the above structure. As for the previous analysis, the *day.site* interaction was assessed between rats. The effects of *day* and *site* were analysed in the *animal.day* stratum to

account for random variation in the *day* effect. The difference between *products* and their interactions with other variables was analysed within an individual *animal* having accounted for random variation due to the effect of *time* and *day* (Appendix 6b). All calculations were performed using Genstat 5 (Lawes Agricultural Trust, 1989).

5.3. Results

The extent of urinary excretion of mercapturic acids was greater following Gastric administration than when compounds were given by the Caecal route. This was the case for both allyl and benzyl isothiocyanates (Table 5.2). This difference was observed for all rats except one for which the excretion of mercapturic acids was very low for both isothiocyanates in each treatment. The amount of excreted mercapturic acids was significantly higher at day 29 than at day 13 ($p < 0.05$).

Table 5.2: Excretion of urinary mercapturic acids after administration of 25 μ mol of the related isothiocyanates (μ mol) in Gastric and Caecal treatments. NS: non significant at $p < 0.05$

Excretory product	Treatment		SED	Level of significance
	Gastric Mean	Caecal Mean		
Allyl mercapturic acid	12.26	3.21	0.084	N.S.
Benzyl mercapturic acid	11.91	2.99		
SED	0.212			
Level of significance	$p < 0.001$			

A proportion of 0.9 of the excretion observed occurred within 24 hours (Figure 5.1). The proportions of allyl isothiocyanate and benzyl isothiocyanate converted into mercapturic acids were 0.49 (s.e.m. 0.043) and 0.48 (s.e.m. 0.042) respectively, of the dose initially given in the Gastric treatment. When the Caecal treatment was given, the amount of excreted mercapturic acids represented a proportion of 0.13 (s.e.m. 0.028) of allyl isothiocyanate and 0.12 (s.e.m. 0.020) of benzyl isothiocyanate initially administered to the animals. There was no significant difference between the total amount of excreted allyl mercapturic acid and excreted benzyl mercapturic acid (Table 5.2). The kinetics of excretion indicated that excretion of allyl mercapturic acid was more rapid than excretion benzyl mercapturic acid (Figure 5.2, Table 5.3).

Figure 5.1: Cumulative excretion of mercapturic acids after administration of Gastric and Caecal treatments. ■ allyl mercapturic acid, ◆ benzyl mercapturic acid

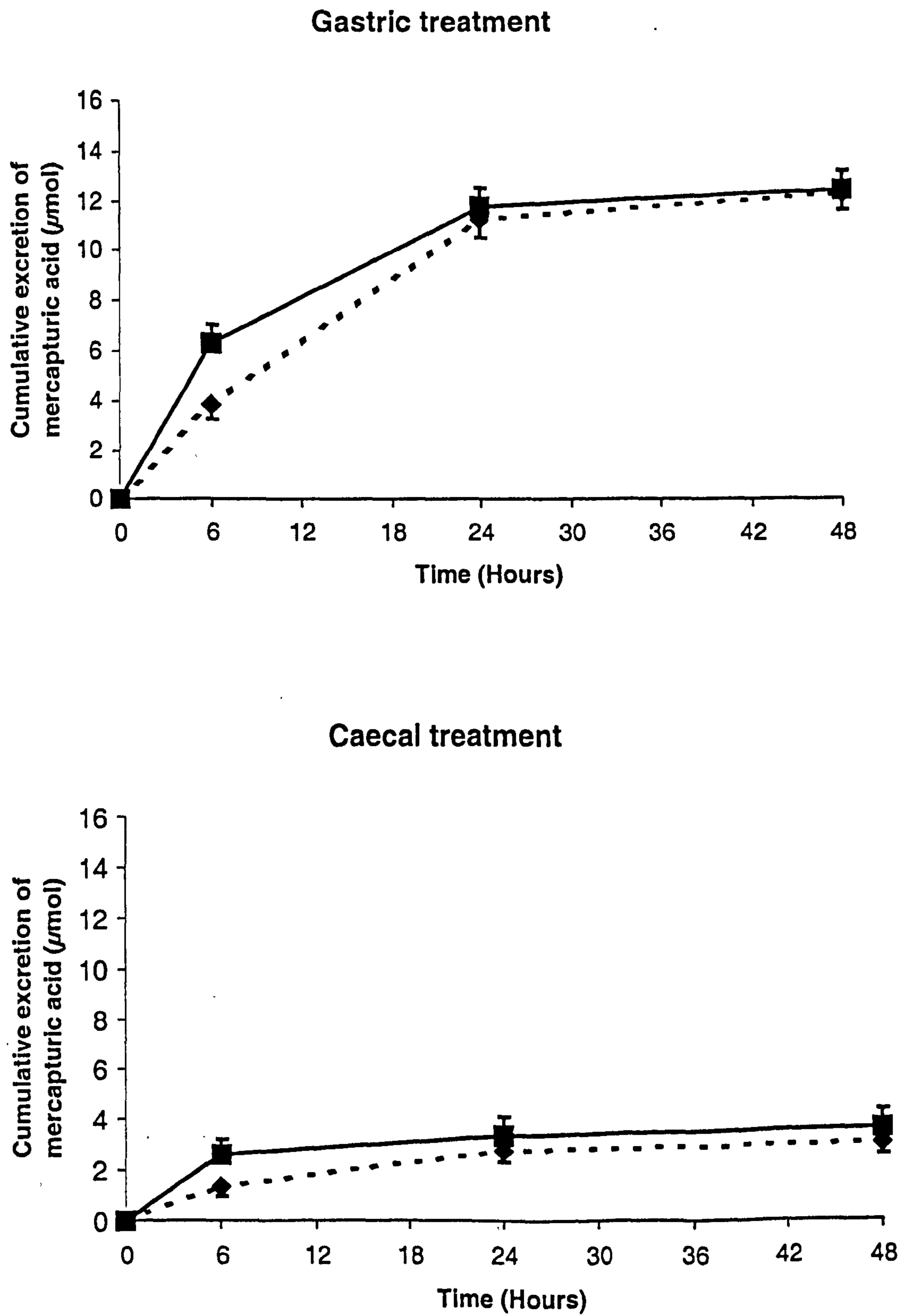


Figure 5.2 Excretion rate of mercapturic acids over 48 hours after administration of isothiocyanates. ■ allyl mercapturic acid ◆ benzyl mercapturic acid

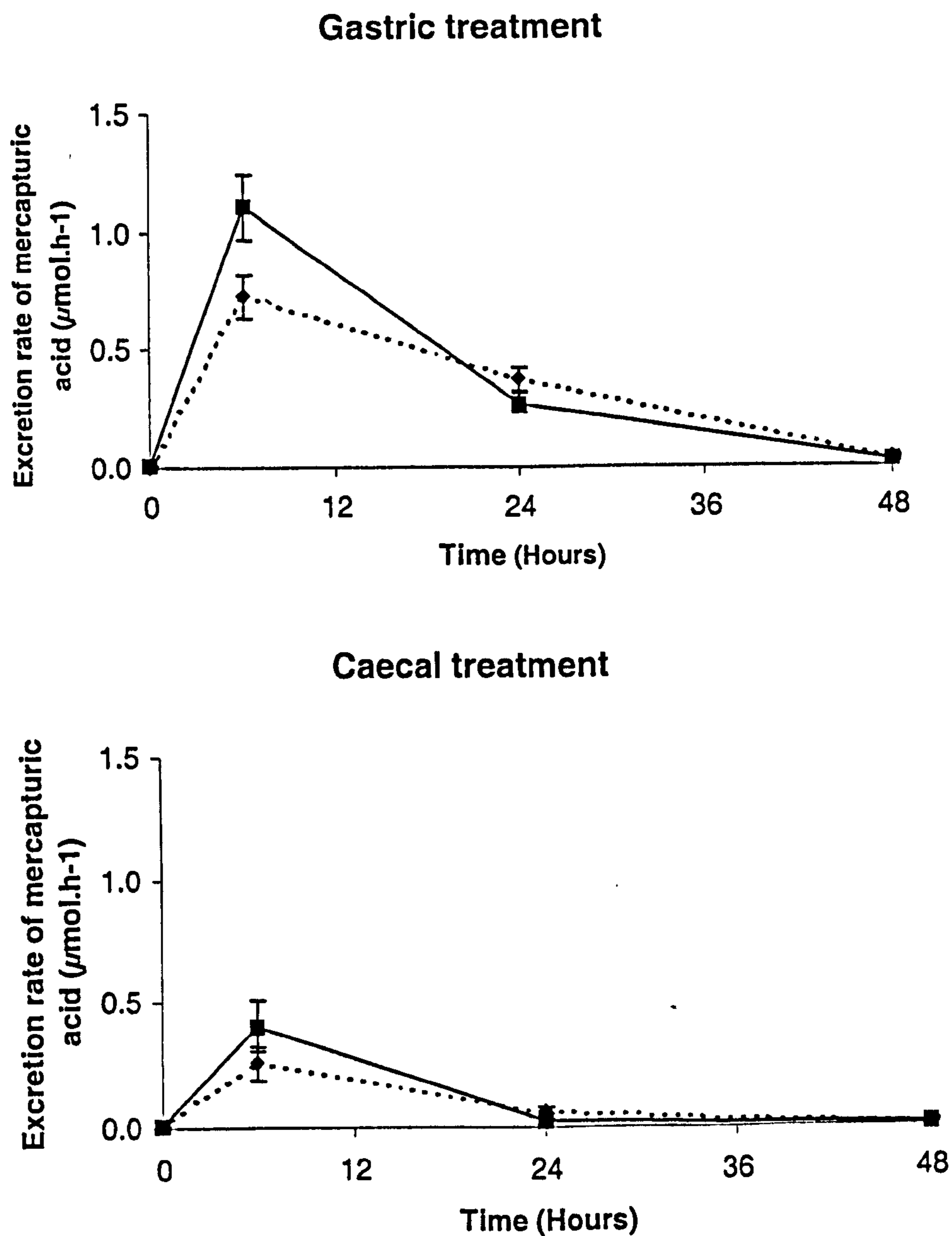


Table 5.3: Comparison of the rate of excretion between allyl and benzyl mercapturic acids after administration of Gastric and Caecal treatments.

Excretory product	Treatment		SED	Level of significance
	Gastric Mean	Caecal Mean		
Allyl mercapturic acid	0.35	0.11	0.064	p<0.01
Benzyl mercapturic acid	0.28	0.084		
SED	0.187			
Level of significance	p<0.01			

5.4. Discussion

This experiment was designed to elucidate whether the extent of absorption of isothiocyanates was similar in the proximal and in the distal part of the intestinal tract. Approximately 0.48 of the isothiocyanate dose was excreted when isothiocyanates were administered orally compared with approximately 0.12 when delivered to the caecum, confirming that the upper intestine is a more efficient site of absorption for isothiocyanates.

Several physiological factors may explain the major differences in recovery of isothiocyanates according to the site of isothiocyanate delivery. The most plausible explanation for the low excretory rate after Caecal treatment is the contribution of caecal microflora to the breakdown of isothiocyanates. The ability of intestinal microflora to break down isothiocyanate was suggested by the outcome of Experiment 2. Recent findings have also shown that amines, a putative breakdown product of isothiocyanates, were released in *in vitro* incubations of a human faecal flora with sinigrin (Rabot *et al.* unpublished). The breakdown of isothiocyanates to amine derivatives has also been shown previously for non-digestive tract bacteria (Tang *et al.* 1972). Nitriles, a further possible metabolite of glucosinolates, are also known to be broken down by intestinal microflora (Duncan & Milne, 1992). The results of the current experiment suggest that microbial microflora may significantly reduce the amount of glucosinolate derivatives available for absorption by the distal digestive tract.

Some constraints inherent to the administration of the Caecal treatment may have played also a role in the low excretion rate of mercapturic acids. The introduction of the catheter rectally may have resulted in transient physiological perturbation of the large intestine. Defecation was observed at the time of the surgery on several rats. Studies using radio-labelled isothiocyanate have shown that a proportion of 0.10 of radioactivity was excreted in faeces when rats were given a dose of isothiocyanate orally (Conaway *et al.* 1999). The delivery of isothiocyanate directly at the caecal site may have increased the faecal excretion of isothiocyanates thereby allowing a smaller recovery of isothiocyanates as mercapturic acids.

An additional factor which may explain the low recovery of isothiocyanates is that colonocytes may not absorb isothiocyanates due to their lipophilic nature. In normal circumstances, the proportion of fat and lipophilic compounds arising in the distal digestive tract is minimal. Fat is mostly absorbed by enterocytes. The formation of micelles from conjugation of fatty molecules with biliary salts increases their solubility and facilitates their hydrolysis and absorption in the upper digestive tract (Jacotot & Le Parco, 1992). The efficiency of absorption of fat and associated compounds by the colonic mucosa may therefore be low. For similar reasons, the presence of high concentrations of corn oil in the caecum may have disturbed the normal course of digestion after administration of the Caecal treatment.

The conjugation of isothiocyanate to glutathione-S-transferase in the cytoplasm of intestinal cells is a further factor which may have affected the recovery of isothiocyanates to mercapturic acids. Glutathione-S-transferase plays an essential role in the conversion of isothiocyanate to mercapturic acids (Brüsewitz *et al.* 1977). Glutathione-S-transferase is distributed along the digestive tract but its activity is generally lower in the distal part than upper part of the digestive tract (Nijhoff *et al.* 1995). The activity of glutathione-S-transferase was not measured in the current experiment. It is therefore difficult to draw definitive conclusions on its influence on observed recoveries.

Regardless of the reasons for lower recovery of isothiocyanates as mercapturic acids following delivery to the caecum, the results have important implications for the use of mercapturic acids as markers of isothiocyanate release in the intestine. The site of hydrolysis of glucosinolates is likely to differ depending on the residual activity of plant myrosinase in ingested food. If myrosinase has been denatured during cooking, parent glucosinolates may pass to the large intestine where they undergo hydrolysis under microbial action. Predicting recovery of isothiocyanates as mercapturic acids using orally administered homologous isothiocyanates is therefore likely to lead to errors in estimates of isothiocyanate release in the lower gut. This suggests that the glucosinolate-degrading capacity of gut microbes using mercapturic acids as markers may have been under-estimated in previous experiments. The low estimates of isothiocyanate release observed in rats harbouring a human flora consuming a myrosinase-free diet must be treated with some caution and alternative methods of

estimating isothiocyanate release from the digestive microflora will need to be developed before drawing definitive conclusions.

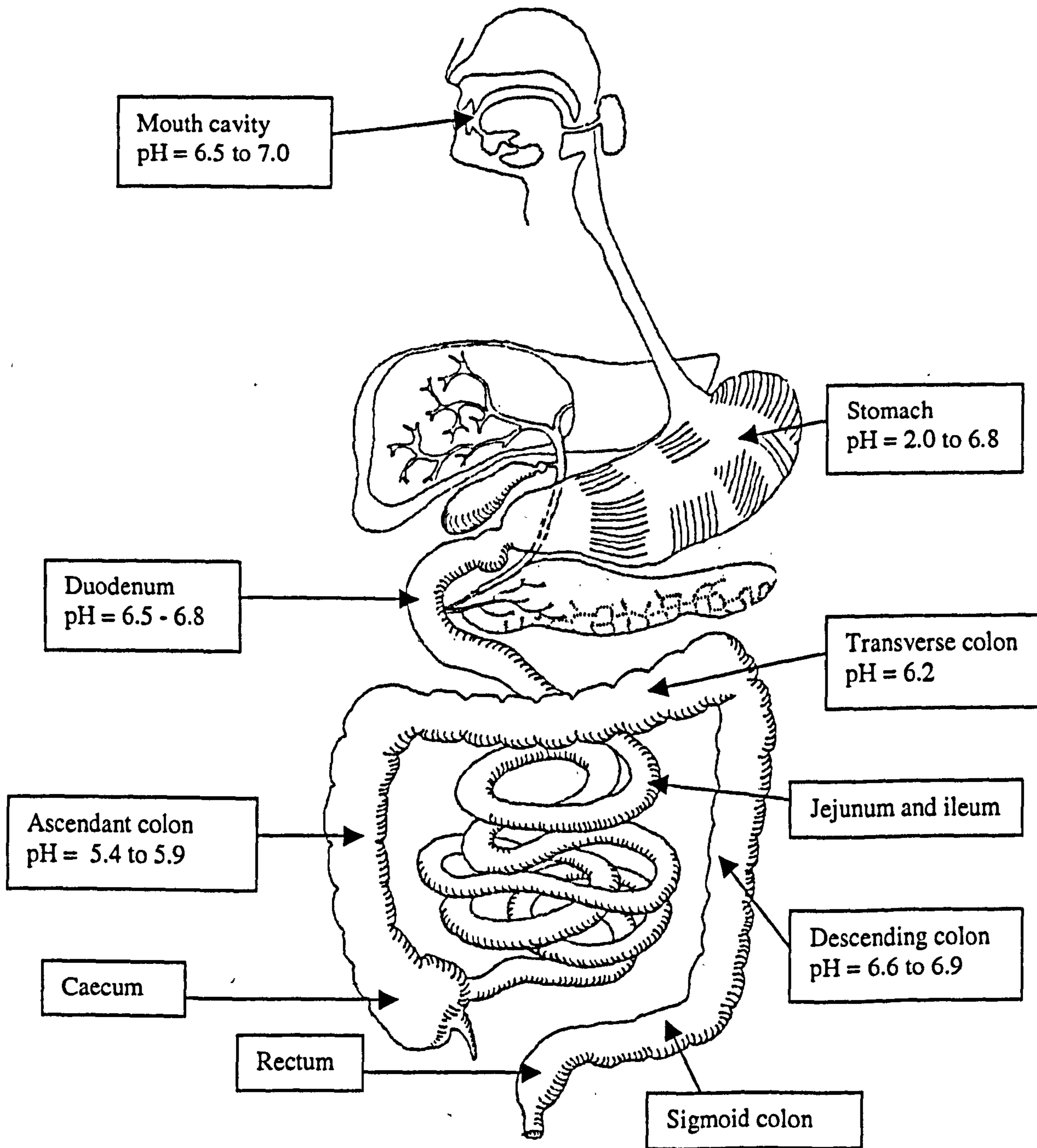
CHAPTER 6: EXPERIMENT 5

Influence of a fermentable oligosaccharide on the release of isothiocyanate in the large bowel of rats

6.1. Introduction

The biological effects of glucosinolates following their ingestion are dependent on both the extent to which they are hydrolysed in the digestive tract as well as the identity of metabolites produced during hydrolysis. *In vitro* studies have shown that pH has a major influence on the profile of metabolites arising during hydrolysis with a low pH tending to favour production of nitriles and a high pH leading to isothiocyanate production. This feature of glucosinolate chemistry has interesting implications when considering the course of hydrolysis in the digestive tract. The pH of the digestive tract varies along its length and is also modified by the transit of the food bolus. Acidic conditions prevail in the stomach whereas more alkaline conditions are found in the small intestine and large bowel (Figure 6.1). Depending on the extent to which plant myrosinase is denatured prior to ingestion of plant material, the site of hydrolysis and hence the pH conditions under which glucosinolates are broken down may vary greatly. Furthermore, more subtle differences in the pH of caecal contents caused by variation in the profile of the end-products of fermentation may influence glucosinolate hydrolysis and hence the biological effects of glucosinolates. Some oligosaccharides, for example, are known to reach the large bowel intact and their fermentation may lead to a more acidic pH in the lower gut. To investigate the influence of variation in the type of fermentation occurring in the lower gut on glucosinolate hydrolysis, an experiment was conducted in which rats were fed on diets with or without added inulin, a fermentable oligosaccharide. The release of isothiocyanates in different portions of the digestive tract following administration of an artificial glucosinolate was directly measured following serial slaughter.

Figure 6.1: Average pH in different compartments of the human digestive tract (from Lecerf, 1996 and Macfarlane & Cummings, 1991)



6.2. Materials and methods

6.2.1. Description of the experiment

To study the effect of a diet enriched in fermentable oligosaccharide *versus* a control diet, two treatments were compared. On one treatment 24 rats were fed a control diet (**Control diet**) whereas on the other treatment, rats were fed a diet enriched with inulin, a water-soluble oligosaccharide (**Inulin diet**). Within each group, animals were distributed into six sub-groups corresponding to six different times of slaughter: $t = 0, 6, 12, 15, 18, 24$. At $t = 0$ h, all the rats were inoculated with a dose containing artificial benzyl glucosinolate and dietary sinigrin. At each time of slaughter, four animals per treatment were sacrificed. As a large number of animals was required, the experiment was carried out in two phases using half the animals from each treatment in each phase.

6.2.2. Animals

Forty-eight germ-free rats were used. Rats were aged 16 to 18 weeks and weighed on average 343 g (s.e.m. 3.2). Five days prior to the experiment, they were inoculated with a 1 ml suspension of human faecal flora (Chapter 3, Section 3.2.4). Animals were maintained in groups of three in conventional cages within two isolators (Table 6.1). Allocation of animals to isolators, to diets and to times of slaughter was balanced for body weight variations.

6.2.3. Diet

The **Control diet** was a semi-synthetic diet simulating a human type diet (Table 6.2). In the **Inulin diet**, the fraction of carbohydrate was reduced and replaced by 105 g.kg⁻¹ DM inulin, (Orafti, Tiemen, Belgium). A seven-day period of adaptation to the food was allowed. On the day of glucosinolate dose administration, rats were fasted for six hours before gavage to avoid any stomach congestion due to the volume of the glucosinolate dose. Access to food was resumed after administration of glucosinolates.

Table 6.1: Allocation of animals to slaughter times

Experimental period	Period 1							
	Isolator 1				Isolator 2			
Isolator number	1	2	3	4	5	6	7	8
Cage number (<i>n</i> = 3 rats per cage)	1	2	3	4	5	6	7	8
Type of diet	Inulin	Control	Inulin	Control	Inulin	Control	Inulin	Control
Day of administration of glucosinolate dose	1	1	3	3	1	1	3	3
Time of slaughter after administration of glucosinolate dose	0, 12, 24h	0, 12, 24h	6, 15, 18h	6, 15, 18h	0, 12, 24h	0, 12, 24h	6, 15, 18h	6, 15, 18h
Experimental period	Period 2							
Isolator number	Isolator 1				Isolator 2			
Cage number (<i>n</i> = 3 rats per cage)	1	2	3	4	5	6	7	8
Type of diet	Inulin	Control	Inulin	Control	Inulin	Control	Inulin	Control
Day of administration of glucosinolate dose	3	3	1	1	3	3	1	1
Time of slaughter after administration of glucosinolate dose	0, 12, 24h	0, 12, 24h	6, 15, 18h	6, 15, 18h	0, 12, 24h	0, 12, 24h	6, 15, 18h	6, 15, 18h

Table 6.2: Composition of the diet (g.kg⁻¹ Dry Matter)

Ingredients	Diet	
	Control	Inulin
Casein	50.00	50.00
Soya isolate (PP500E, Protein Technologies International)	120.00	120.00
Inulin (Orafti)		105.00
Saccharose	50.00	50.00
Mashed potato	290.00	185.00
Maize starch	289.85	289.85
Lard	30.00	30.00
Maize oil	30.00	30.00
Cholesterol	0.15	0.15
Cellulose	60.00	60.00
Mineral additive ^a	70.00	70.00
Vitamin additive ^b	10.00	10.00
Total	1000.00	1000.00

^aThe mineral additive includes (g.kg⁻¹DM of diet): CaHPO₄ 30.1, KCl 7.0, NaCl 7.0, MgO 0.735, MgSO₄ 3.5, Fe₂O₃ 0.21, FeSO₄7H₂O 0.35, MnSO₄H₂O 0.17, CuSO₄5H₂O 0.035, ZnSO₄ 7H₂O 0.141, CoSO₄ 7H₂O 2.8*10⁻⁴ and KI 5.6*10⁻⁴.

^bThe vitamin additive includes (mg. kg⁻¹DM of diet): thiamin 20, riboflavin 15, pantothenic acid 70, pyridoxine 10, myoinositol 150, cyanocobalamin 0.05, ascorbic acid 800, α-tocopherol 170, menadione 40, niacin 100, choline 1360, folic acid 5, p-aminobenzoic acid 50, biotin 0.3, retinol 19800 IU.kg⁻¹diet and cholecalciferol 2500 IU kg⁻¹diet.

6.2.4. Administration of glucosinolates

For each rat, the glucosinolate dose consisted of a suspension of 100 μmol of benzyl glucosinolate (Potassium salt, Merck, Darmstadt, Germany) and 0.8 g of freeze-dried and powdered Brussels sprouts suspended in 5 ml sterile water. Brussels sprouts (variety *cyrus*) were treated to inactivate myrosinase (Chapter 3, section 3.2.6). The main glucosinolate present in the Brussels sprouts was sinigrin (10.3 μmol.g⁻¹ dry matter). The amount of sinigrin present in the glucosinolate dose was therefore 8.2 μmol (the glucosinolate composition of Brussels sprouts is detailed in Chapter 3, Table 3.4). The glucosinolate dose was administered orally using a stomach tube under light ether anaesthesia. Administration of the glucosinolate dose occurred at 21:00 which was two hours into the dark period of the light/dark cycle.

For logistical reasons, it was not possible to administer the glucosinolate dose to all rats simultaneously. The glucosinolate administration was therefore done on two different days. To avoid any confounding effect between day of glucosinolate

administration and time of slaughter, animals were grouped so that animals slaughtered at two consecutive times did not receive the dose on the same day (Table 6.1). The day of administration allocated in Period 1 was reversed for each time of slaughter in Period 2 of the experiment.

6.2.5. Collection of gut contents

Rats were removed from isolators and slaughtered using a lethal dose of carbon dioxide. The digestive tract was removed and wettened using 9 g.l⁻¹ NaCl solution. Stomach, small intestine, caecum and colon were separated. The pH was measured in the caecal contents. The digestive contents of each compartment were separated into three replicate aliquots. Two aliquots were reserved for analysis of non-digested glucosinolates. The rest of the digestive contents was transferred into glass vials (Polylabo, Paris, France) containing a defined volume of 0.1M phosphate buffer solution (pH = 7). Glass vials of 50 ml capacity containing 20 ml buffer solution were used for the caecal contents. Smaller vials of 10 ml capacity containing 4 ml buffer solution were used for digestive contents coming from the other compartments. The vials were capped with airtight septa and sealed caps (Elfoul, 1999). The weight of total digestive contents as well as the weight of each aliquot were recorded for each compartment.

6.2.6. Quantification of non digested glucosinolate in digestive contents

Digestive contents were freeze-dried and ground. Analysis of desulphoglucosinolates by HPLC was performed using previously described methods (Chapter 3, section 3.3.2) except that the amount of starting material varied from 0.025g to 0.1g.

6.2.7. Quantification of isothiocyanates in digestive contents

The quantification of allyl and benzyl isothiocyanates was carried out by gas chromatography using solid phase micro-extraction (SPME). The sample vials were thawed at room temperature and 40 μ l of a 0.25 μ M phenyl isothiocyanate solution was injected through the septum as an internal standard. The sample was thoroughly vortexed to obtain an homogenous solution. A carboxen/polydimethylsiloxane-coated silica fibre (film thickness 75 μ m, Supelco, Saint Quentin Fallavier, France)

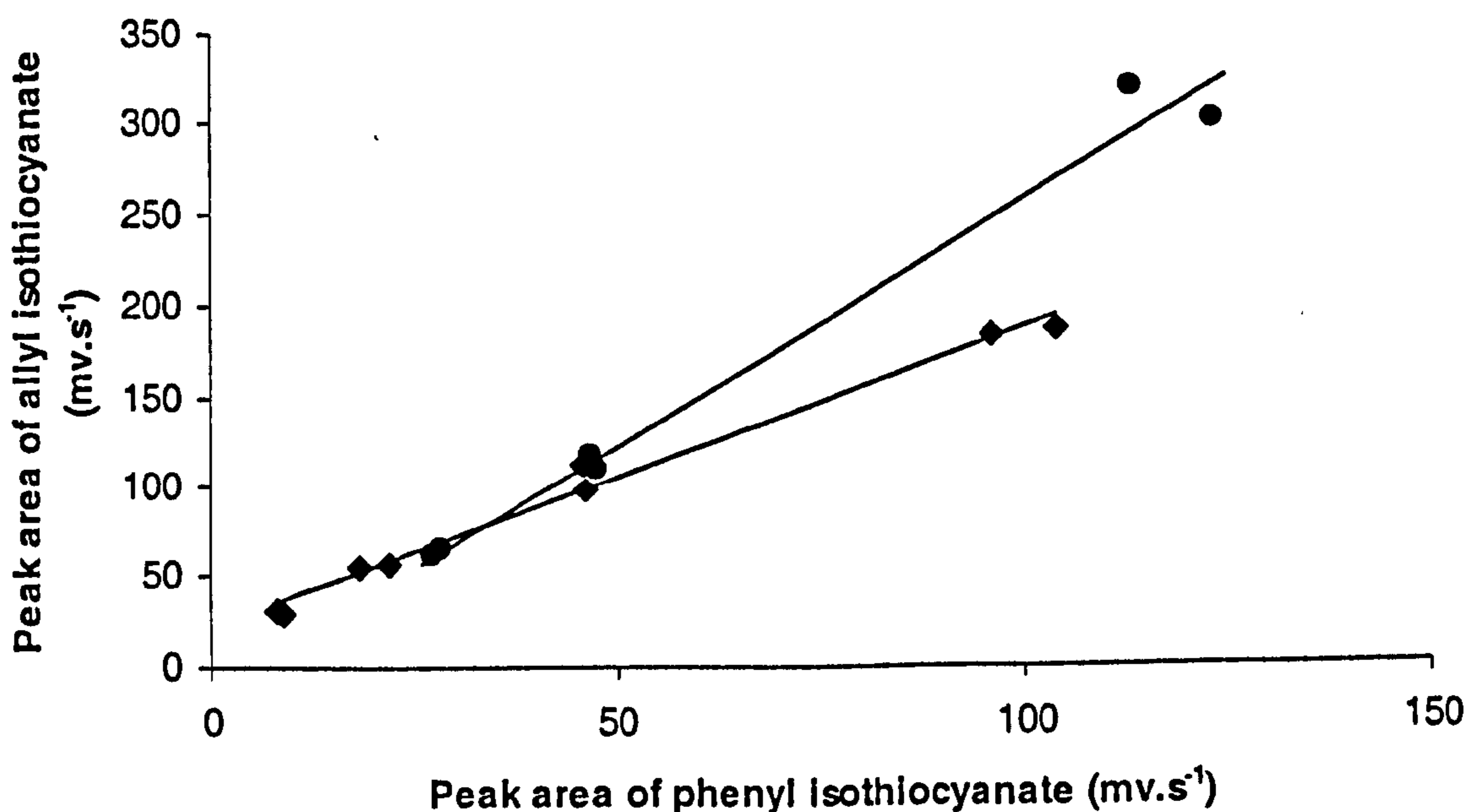
fitted into a protective stainless steel needle, was introduced in the sample vial. The sample vial was placed in a waterbath at 45°C. The micro-extraction fibre was exposed to the headspace of the sample vial for 10 min to allow adsorption of the volatile isothiocyanates. The adsorbed compounds were subsequently desorbed in the splitless injection port of a gas chromatograph (Carlo Erba HRGC 5300, Milano, Italy) during 20 seconds at 250°C. Separation of compounds was carried out using a non-polar capillary column (CP-Sil 8 CB, 25m length, internal diameter: 0.53 mm, film thickness: 2 µm Chrompack, Les Ulis, France). Carrier gas was nitrogen (70 kPa). The temperature gradient was programmed as follows: basal temperature was stable at 45°C for 3 min then temperature increased to 80°C at 6°C.min⁻¹, the rate slowed down to 4°C.min⁻¹ until temperature reached 155°C then the temperature was rapidly increased at 20°C.min⁻¹ to 200°C. Detection was by flame ionisation detection. Detector temperature was set at 250°C. Peak areas were measured on a Shimadzu C-R6A integrator. The retention times for allyl isothiocyanate, phenyl isothiocyanate and benzyl isothiocyanate were 7.1, 18.3 and 24.4 min respectively. Calibrations curves were plotted using equimolar amounts of 2.5, 5 and 10 nmol of allyl, benzyl and phenyl isothiocyanates which were injected in glass vials of identical capacity to sample vials. The response factors were significantly higher in 50 ml glass vials than in the 10 ml vials (Figure 6.2). For this reason, different response factors were used according to the type of vials used (Table 6.3). Amount of isothiocyanates released in the digestive contents were quantified by comparing the peak area of allyl isothiocyanate or benzyl isothiocyanate to the peak area of phenyl isothiocyanate in the sample corrected by the response factor.

Table 6.3: Response factors used for quantification of allyl and benzyl isothiocyanate by SPME-GC analysis. Mean response factor was obtained from the ratio of peak areas of compounds to peak area of internal standard from standard solutions containing 2.5, 5, 10 and 20 nmol of allyl, benzyl and phenyl isothiocyanates.

Compounds	Vial capacity			
	10 ml		50 ml	
	Mean	s.e.m.	Mean	s.e.m.
Allyl isothiocyanate	2.44	0.212	2.62	0.697
Benzyl isothiocyanate	0.32	0.037	0.42	0.267

Figure 6.2 a and b: Relationship between the peak areas of phenyl isothiocyanate and allyl isothiocyanate (Figure a) and between phenyl isothiocyanate and benzyl isothiocyanate (Figure b). Peak areas were obtained by GC following solid phase micro-extraction of standard solutions containing 2.5, 5, 10 and 20 nmol of each compounds. \blacklozenge represents areas obtained for 50 ml capacity vials, \bullet represents areas obtained for 10 ml capacity vials.

(a) Allyl isothiocyanate

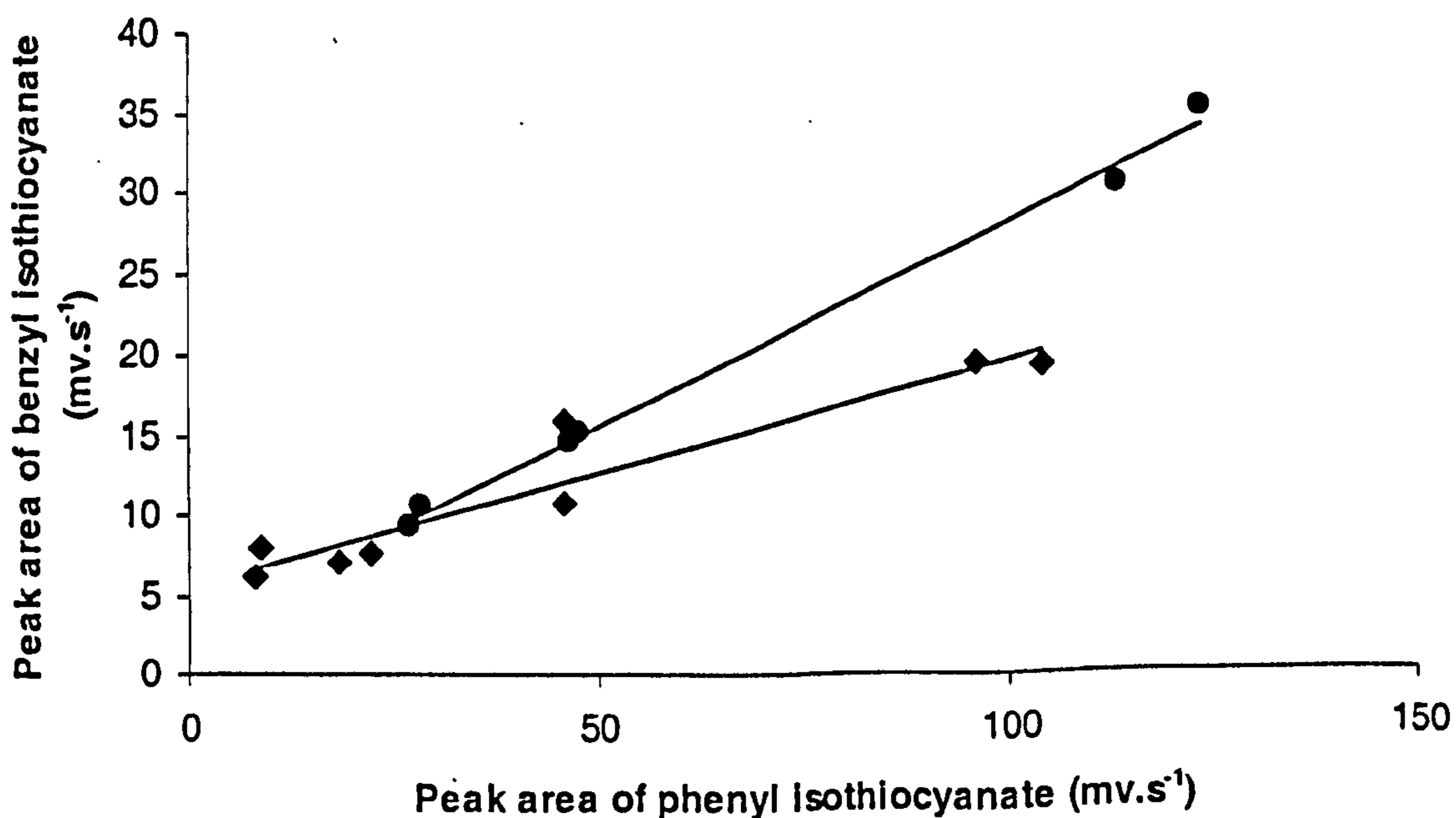


Regression curves:

$$\text{Allyl isothiocyanate (50 ml vial)} = 1.67 \text{ phenyl isothiocyanate} + 21.0 \quad r^2 = 0.987$$

$$\text{Allyl isothiocyanate (10 ml vial)} = 2.72 \text{ phenyl isothiocyanate} - 12.8 \quad r^2 = 0.984$$

(b) Benzyl isothiocyanate



Regression curves:

$$\text{Benzyl isothiocyanate (50 ml vial)} = 0.140 \text{ phenyl isothiocyanate} + 5.67 \quad r^2 = 0.901$$

$$\text{Benzyl isothiocyanate (10 ml vial)} = 0.250 \text{ phenyl isothiocyanate} + 2.86 \quad r^2 = 0.994$$

Identification of chromatographic peaks were confirmed on one sample for each compartment and for each diet using GC-MS (Fisons GC 800 coupled to quadrupole Fisons MD 800) equipped with an acquisition data system INCOS (Finnigan). Compounds were separated on a capillary column (Q2, Quadrex, length: 25m, internal diameter: 0.25 mm, film thickness: 0.25 μ m). Column temperature was programmed as described above for the isothiocyanate analysis. Injection port and interface temperatures were set at 250°C and source temperature was 200°C. Ionisation mode was the electronic impact at 70 eV (emission current: 0.5mA). Masses were scanned from 30 to 400 amu.

6.2.8. Statistical analysis

The method of Residual Maximum Likelihood (Genstat 5, Lawes Agricultural Trust, 1989) was used to analyse the effect of diets and time of slaughter on the sinigrin, benzyl glucosinolate, allyl isothiocyanate and benzyl isothiocyanate concentrations in the digestive tract. Period was used as a block in the analysis. Comparison between diet was tested by compartments. Analysis was restricted to times of slaughter where non-zero concentrations were observed.

6.3. Results

6.3.2. Effect of pH

The pH of caecum contents was significantly more acidic when rats were given the **Inulin** diet (pH = 5.8 s.e.m 0.16) in comparison to their **Control** counterparts (pH = 6.7 s.e.m 0.06) ($p < 0.001$). Caecal pH was the lowest 6 hours after glucosinolate administration (Figure 6.3).

6.3.3. Non-digested glucosinolates

Glucosinolates were found in substantial amounts only within the first twelve hours following the gavage. The kinetics of glucosinolate appearance in the different compartments was similar for both diets. At $t = 0$ h, glucosinolates were found in the upper tract, primarily in the stomach and to a lesser extent in the small intestine (Figure 6.4 and 6.5). The large volume of materials in the stomach of rats immediately after gavage at $t = 0$ h led to an over-estimation of the amount of

glucosinolates in comparison to 100 μmol given by gavage. Six hours after the meal, glucosinolates were detected in the caecum and colon. The major proportion of the glucosinolate dose, however, was still present in the stomach and small intestine. The total amount of non-digested glucosinolates at $t=6\text{h}$ showed high inter-animal variation averaging 1.13 μmol (s.e.m 0.330) for sinigrin and 18.8 μmol (s.e.m. 4.330) for benzyl glucosinolate. Beyond 12 hours, residual glucosinolates were not detected except on one occasion for rats fed the control diet and slaughtered at $t=18\text{h}$. The amount of residual glucosinolates measured in rats fed the **Inulin** diet was not significantly different from rats fed the **Control** diet ($p>0.05$).

Figure 6.3: Effect of diet on caecal pH of rats. Black bars corresponds to rats fed the Control diet, hatched bars corresponds to rats fed the Inulin diet

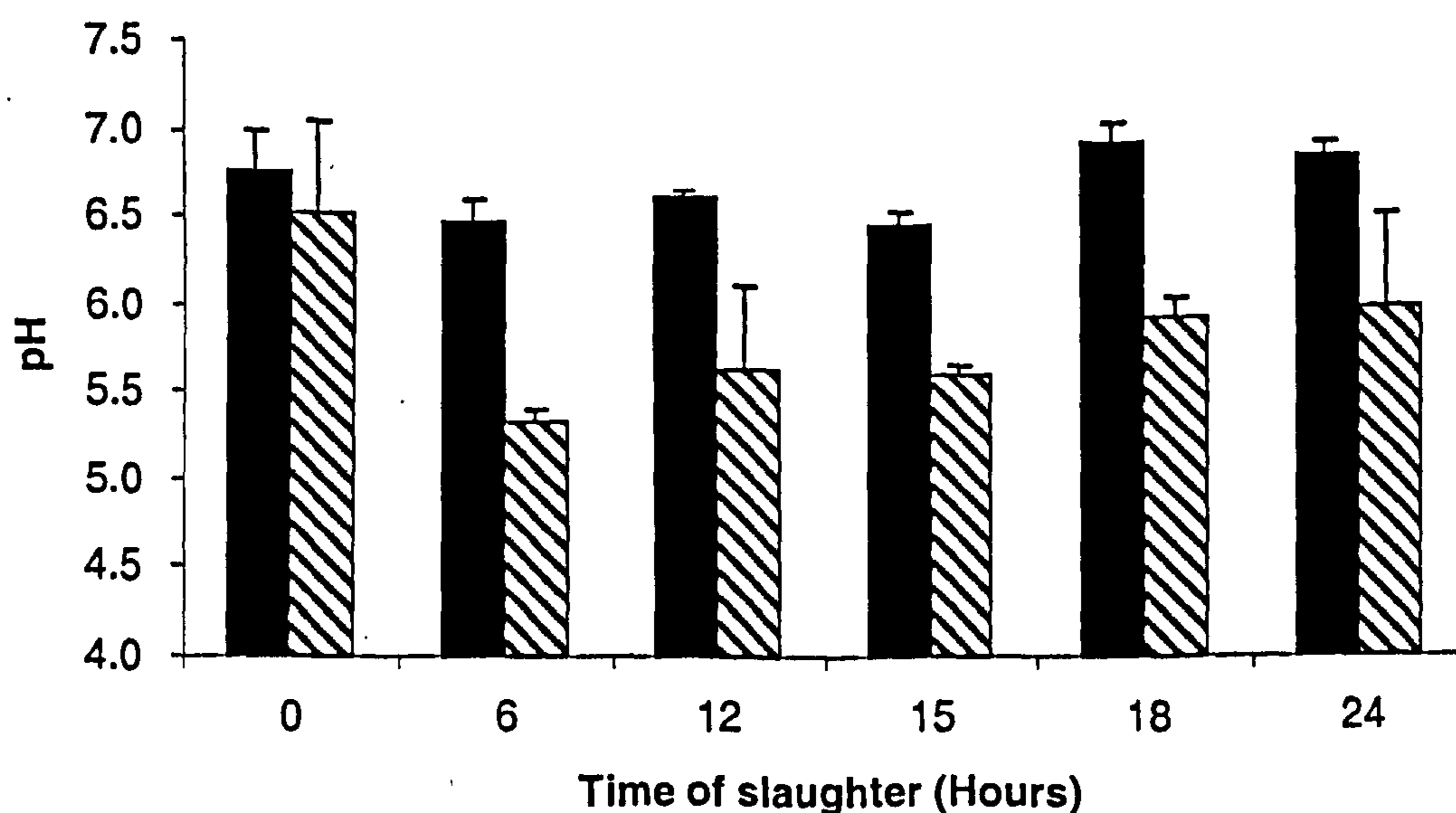

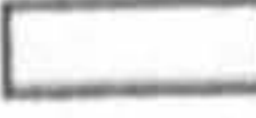




Figure 6.4 Amount of non-digested sinigrin in the intestinal tract of rats.

The digestive compartments represented are: stomach , intestine , caecum , colon 

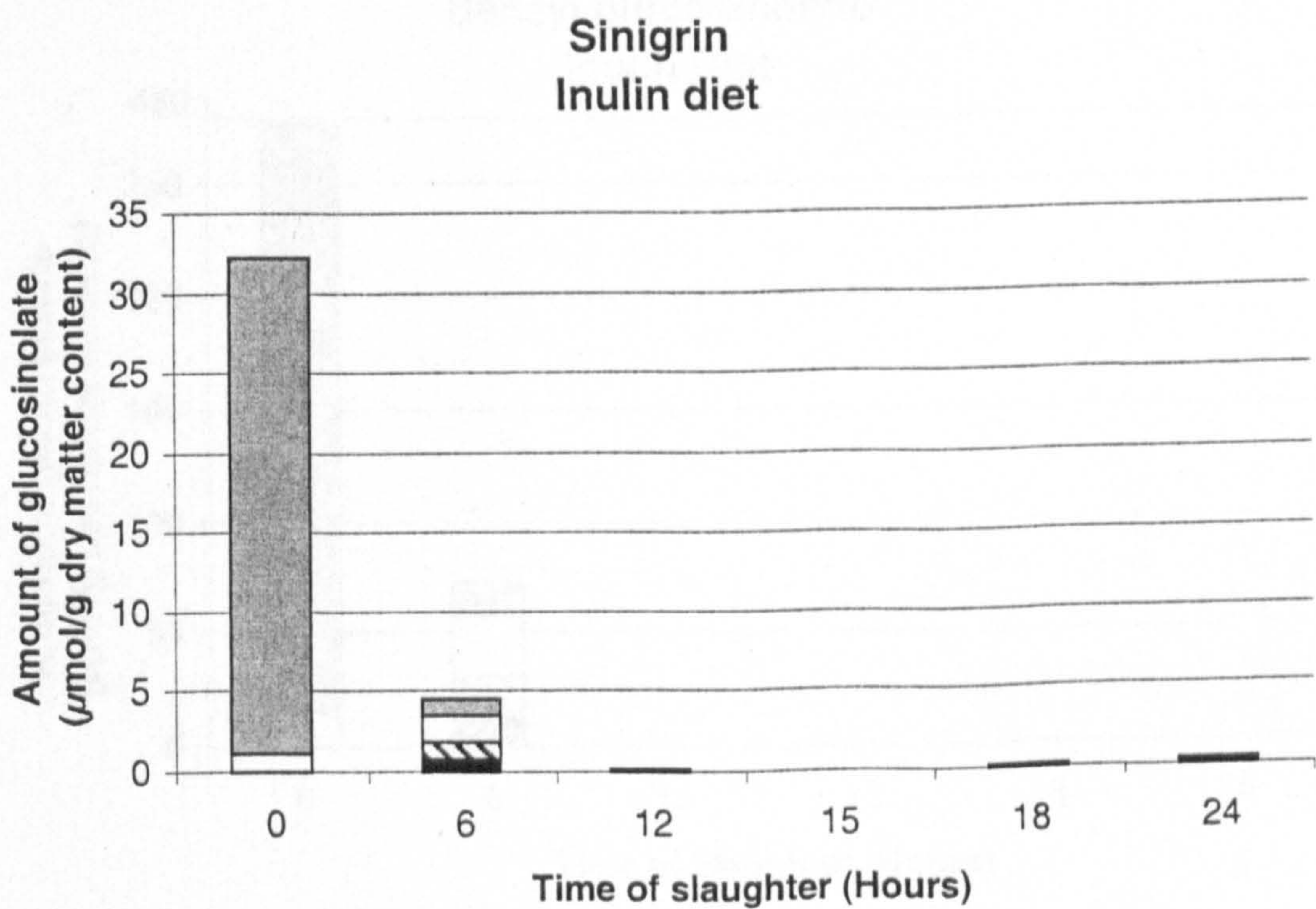
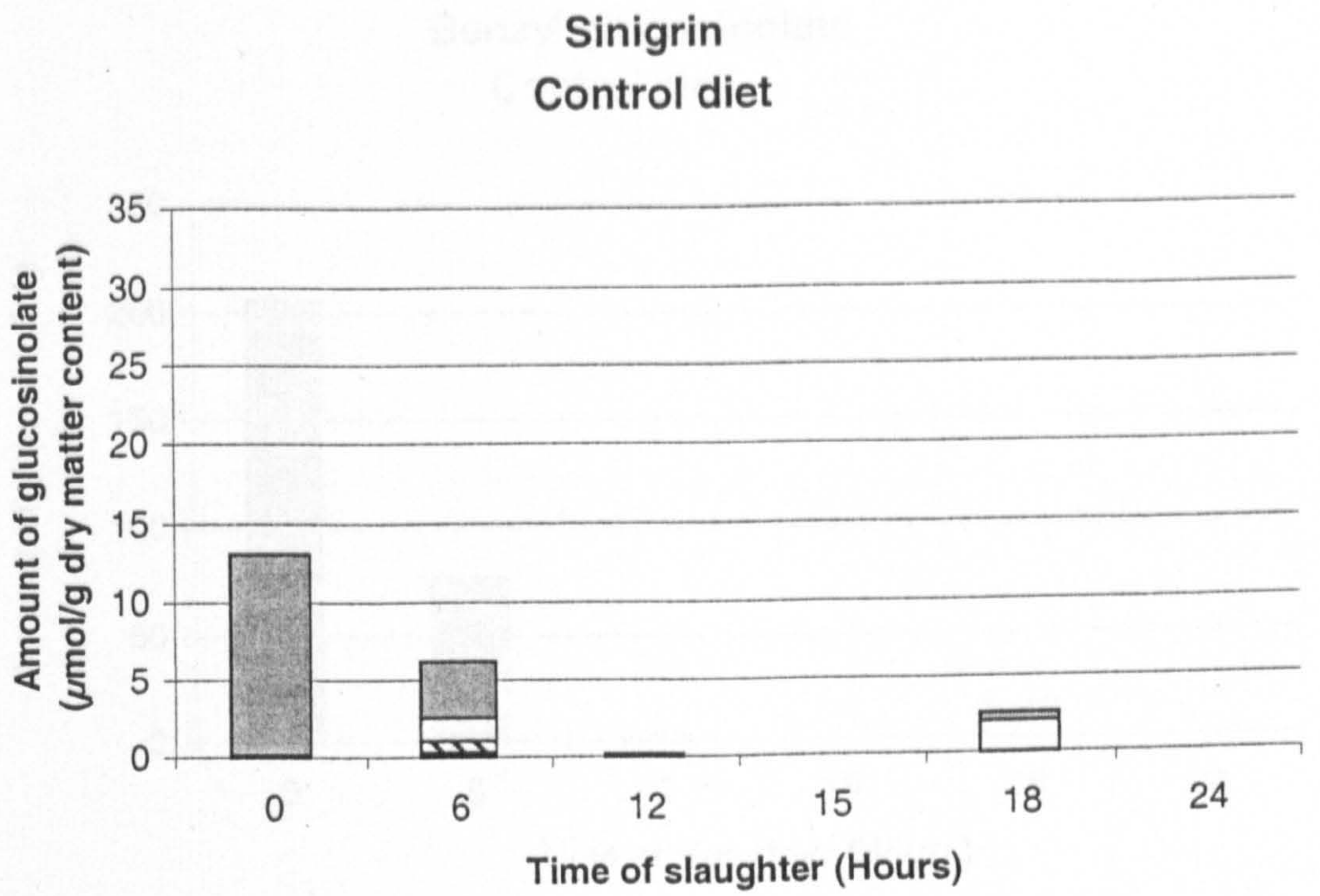

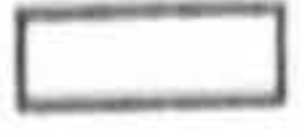
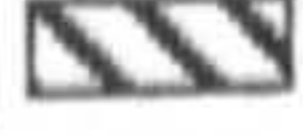



Figure 6.5: Amount of non digested benzyl glucosinolate in the intestinal tract of rats
 The digestive compartments represented are: stomach , intestine , caecum , colon 

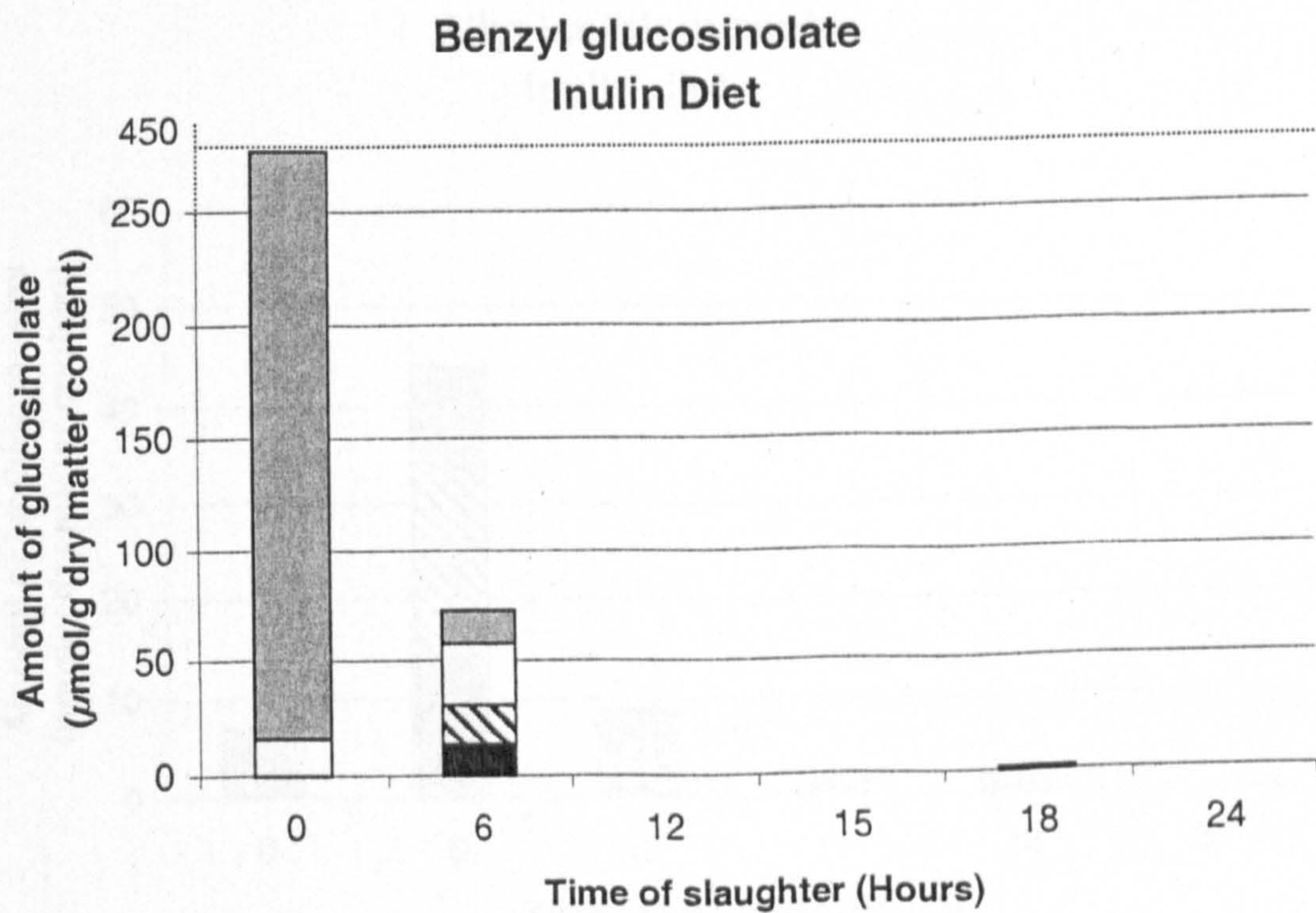
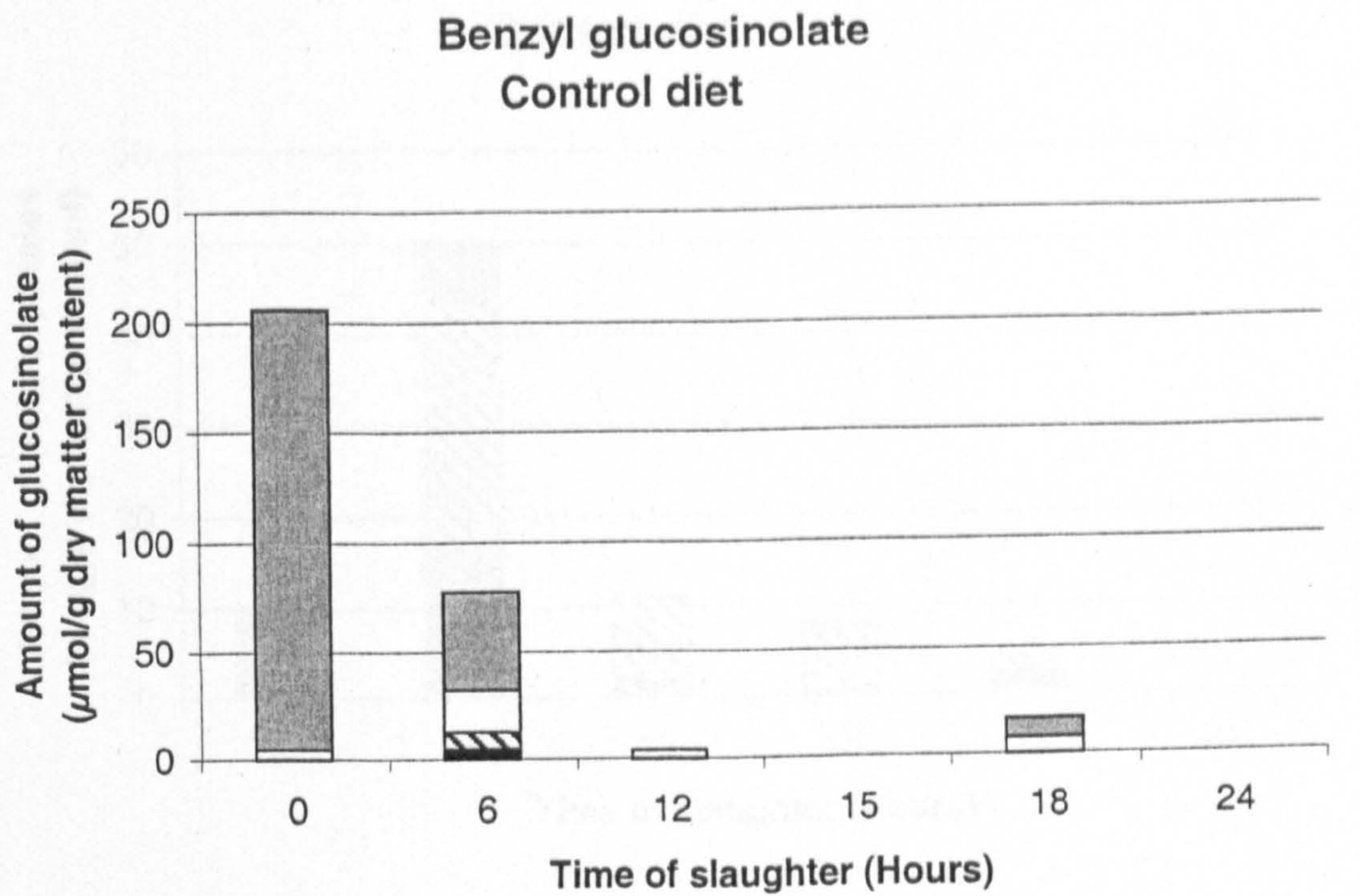
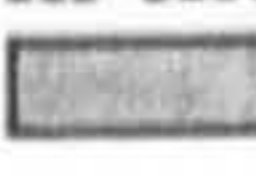
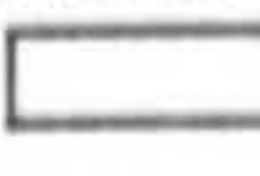




Figure 6.6: Amount of allyl isothiocyanate released in the intestinal tract of rats

The digestive compartments represented are: stomach , intestine , caecum , colon 

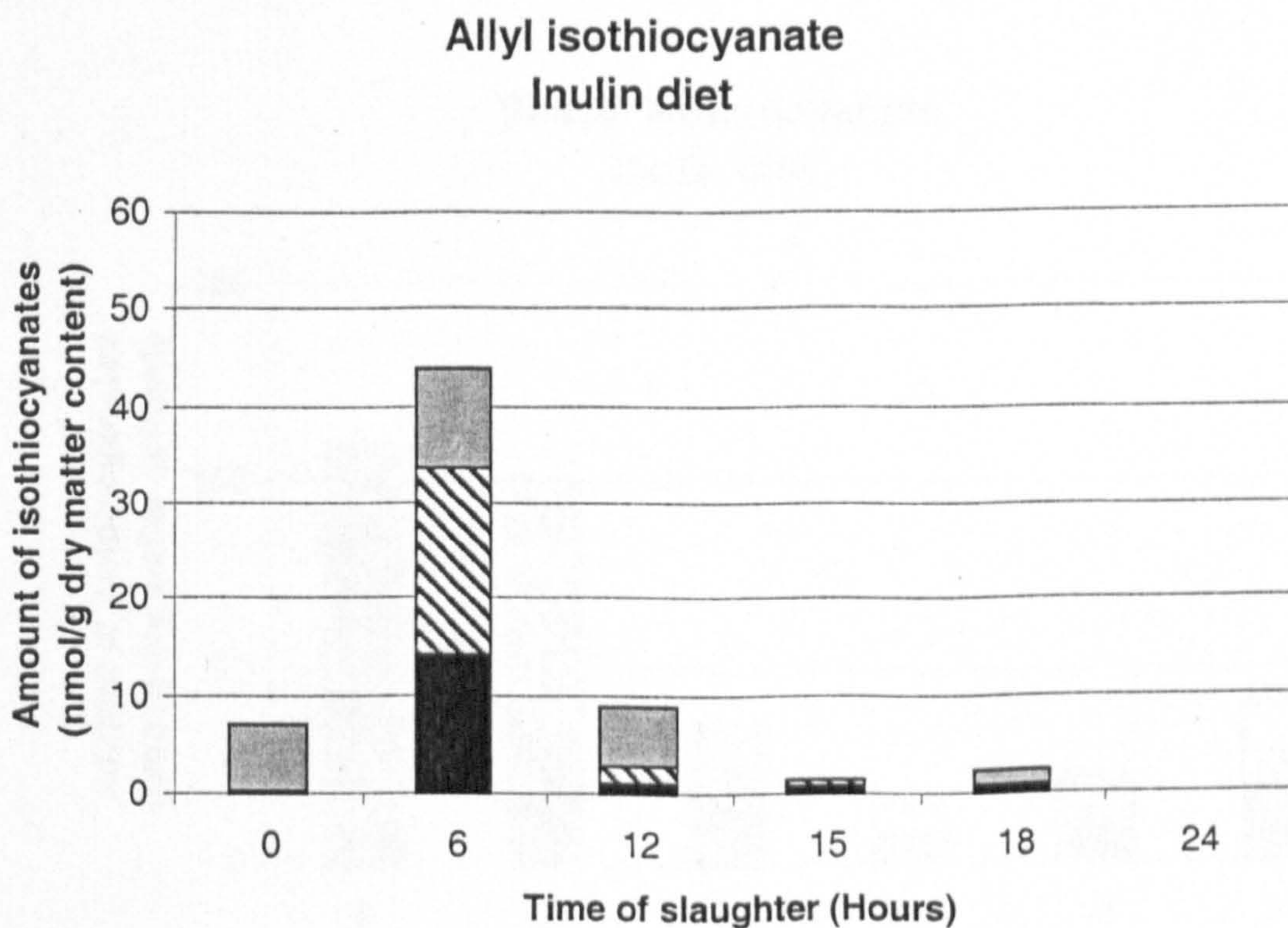
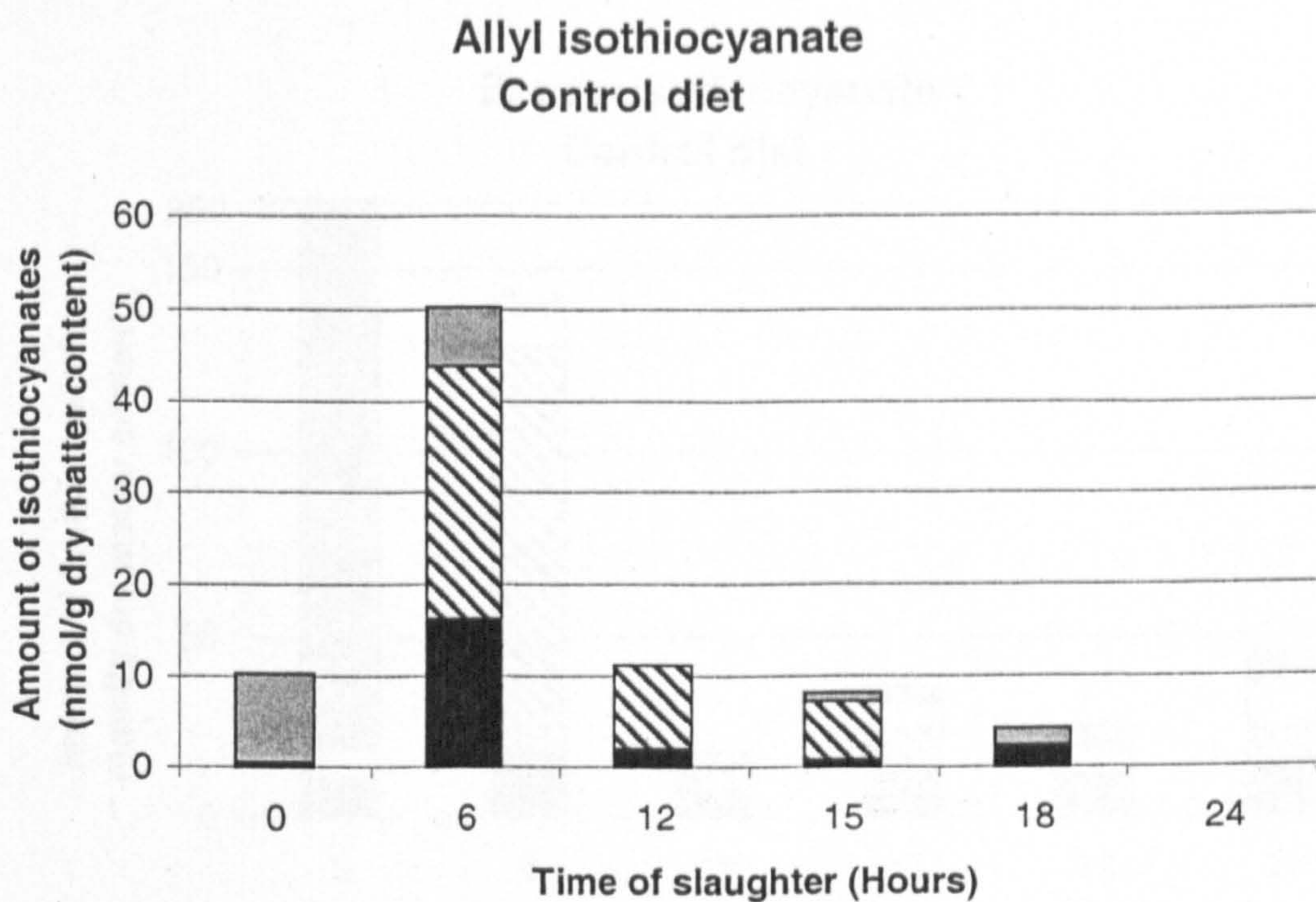

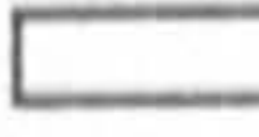


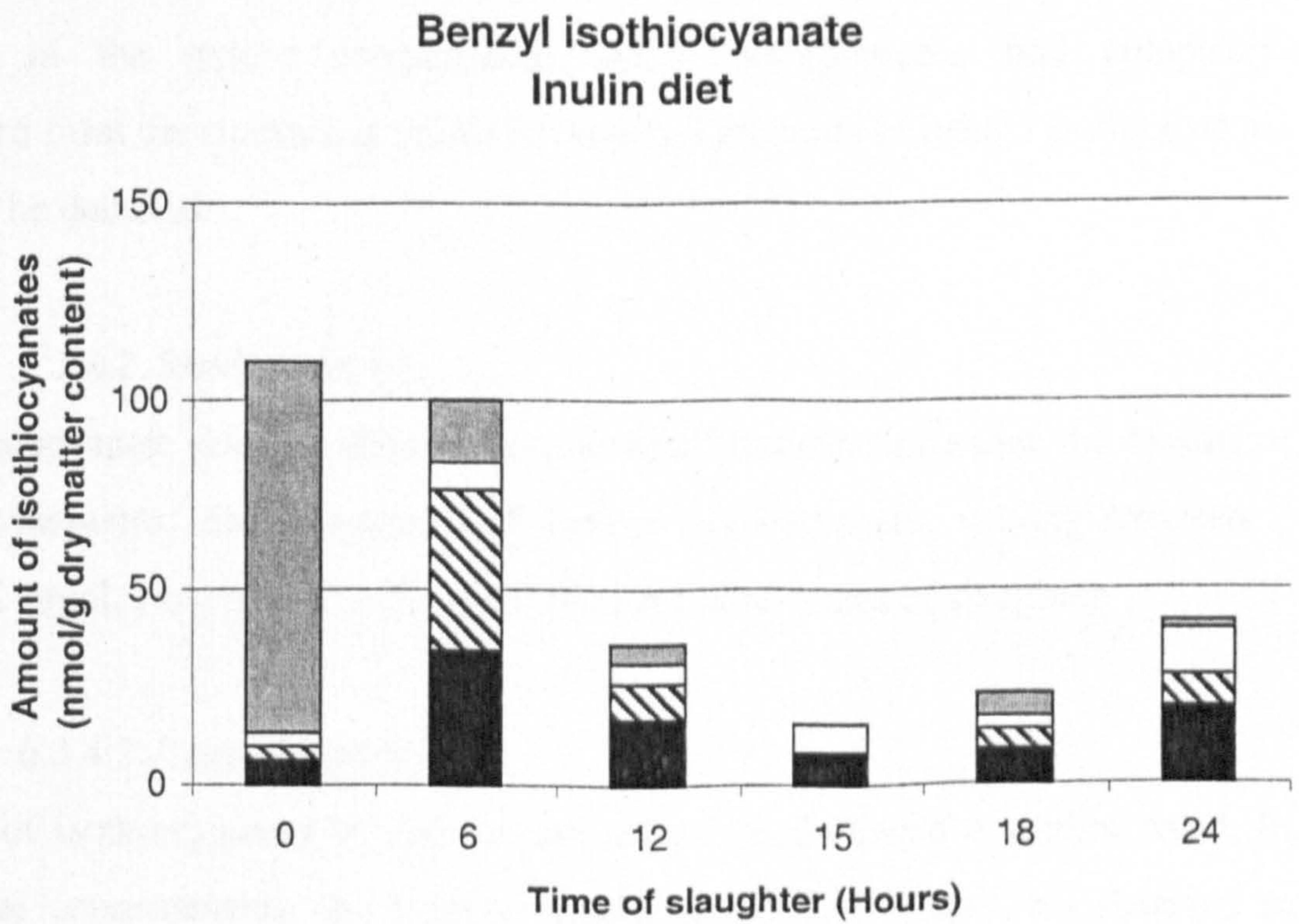
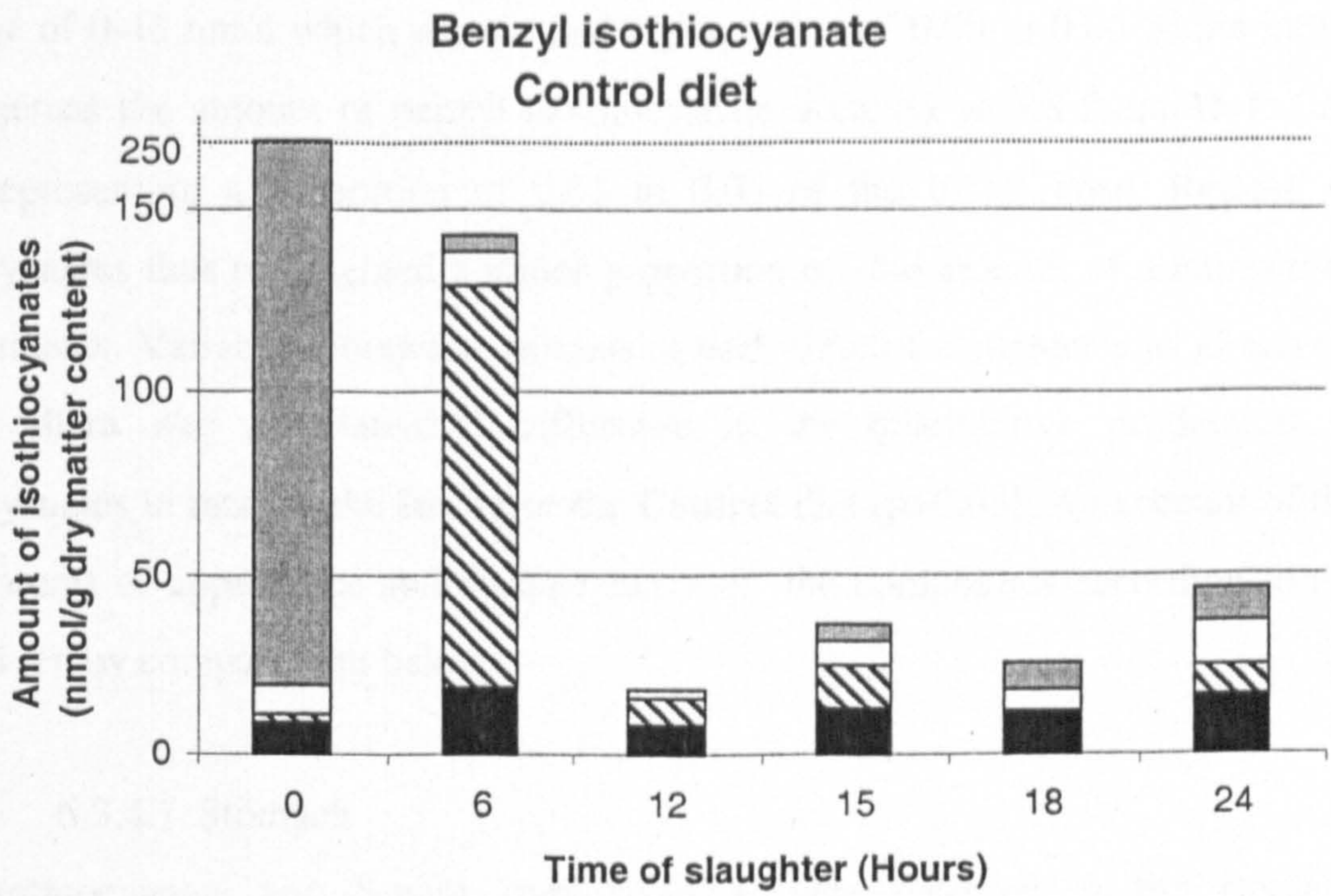


Figure 6.7: Amount of benzyl isothiocyanate released in the intestinal tract of rats

The digestive compartments represented are: stomach , intestine , caecum , colon 



6.3.4. Isothiocyanates released in the digestive tract

The total amount of allyl isothiocyanate detected in the digestive tract of rats was in the range of 0-48 nmol which represented a proportion of 0.00 to 0.06 of the initial dose whereas the amount of benzyl isothiocyanate detected varied from 15 to 258 nmol, representing a proportion of 0.01 to 0.03 of the initial dose. Release of isothiocyanates thus represented a minor proportion of the amount of administered glucosinolates. Variability between animals at each time of slaughter was observed. Overall, there was no statistical difference in the quantitative production of isothiocyanates in rats fed the **Inulin** or the **Control** diet ($p>0.05$). An account of the main features of appearance and disappearance of the compounds according to the diet is given by compartment below.

6.3.4.1. Stomach

Allyl isothiocyanate and benzyl isothiocyanate were detected in the stomach immediately following gavage ($t=0h$), regardless of the dietary treatments. At later times of slaughter ($t=6, 12, 15, 18h$), residual levels of isothiocyanates could be measured in the gastric compartment. Allyl isothiocyanate had completely disappeared from the stomach at $t=24h$ but residual amounts of benzyl isothiocyanate could still be detected.

6.3.4.2. Small intestine

Allyl isothiocyanate was not detected in the small intestine in either the **Inulin** or **Control** treatments. Small amounts of benzyl isothiocyanate, varying between 2 nmol to 12 nmol, were found in the small intestine at all times of slaughter.

6.3.4.3. Caecum and colon

Amounts of isothiocyanates in the caecum and colon followed a similar trend. In these distal compartments, the highest release of isothiocyanates was detected at $t=6h$. At this time of slaughter, the amounts of allyl isothiocyanate found in the caecum were 28 nmol (s.e.m. = 17.3) and 19 nmol (s.e.m. = 10.9) for animals fed **Control** and **Inulin** diets respectively. The amounts of benzyl isothiocyanate found

in the caecum were 110 nmol (s.e.m.= 44.4) and 42 nmol (s.e.m. = 10.7) for animals fed **Control** or **Inulin** diets respectively.

The release of allyl isothiocyanate in animals fed the **Inulin** diet showed an abrupt decrease at t=12h whereas release of allyl isothiocyanate continued over 15 hours in animals fed the **Control** diet, suggesting a more rapid disappearance of the compound in the presence of dietary fibre. The temporal pattern of benzyl isothiocyanate release was similar in animals fed the **Control** or **Inulin** diets. Benzyl isothiocyanate decreased dramatically at t=12h to a residual level. This residual amount remained over the 24h period.

6.4. Discussion

The current experiment provides more evidence of the release of isothiocyanates from glucosinolates in the digestive tract in the absence of plant myrosinase activity. The release of isothiocyanates was very low proportionally to the amount of glucosinolate precursors administered. The amount, however, only represents an instantaneous measurement of isothiocyanate release and does not account for isothiocyanates released and absorbed by the intestinal mucosa. There was no evidence of an influence of diet on the extent of release of isothiocyanate although the fermentation of inulin led to an acidic conditions in the caecal compartment. This may have been because rates of hydrolysis were low or because isothiocyanates were further degraded by bacteria following their release. In a previous study, the addition of inulin to a rapeseed diet was shown to modulate the toxic symptoms induced by glucosinolates in rats harbouring a human faecal flora (Roland *et al.* 1996), suggesting that an enhancement of microbial fermentation may be accompanied by a change in the microbial breakdown of glucosinolates. The current experiment indicated, however, that the addition of a fermentable oligosaccharide to the diet did not significantly change the amount of isothiocyanate available for uptake by the colonic mucosa. The beneficial effect of dietary fibre may involve other mechanisms of action. A measurement of the microbial end-products of isothiocyanates, such as amines, may bring more precise information regarding the modulation of isothiocyanate release by microflora.

Release of isothiocyanate in the stomach may have resulted from a non-enzymic hydrolysis since myrosinase was inactivated in the plant cells. Non-enzymic hydrolysis of glucosinolates has been described *in vitro* in the presence of pepsin and hydrochloric acid (Maskell & Smithard, 1994). The complex chemical interactions occurring in the stomach may favour the hydrolysis of glucosinolate. *In vitro* non-enzymic hydrolysis led, however, to a release of nitrile products but not to isothiocyanates as observed here.

Gastric emptying was slow in the current experiment due to the large volume of Brussels sprouts which presumably caused distension of the stomach and delayed gastric emptying. This explained why six hours after the meal glucosinolates were still present in high amounts in the stomach and intestine. The disturbance in transit time generated by the large amount of digestive contents may also have exaggerated the extent of hydrolysis of glucosinolates in the stomach. In normal circumstances, release of isothiocyanates by non-enzymic hydrolysis may be less dramatic than the production of isothiocyanate observed here.

The current experiment has demonstrated that, in the absence of plant myrosinase activity, several factors may contribute to the formation of isothiocyanates. Contrary to our hypothesis which would have predicted hydrolysis of glucosinolates mainly occur in the distal part of the intestinal tract when vegetables are cooked, the pattern of appearance of isothiocyanates in the current experiment showed that release of isothiocyanate may occur to a significant extent in the proximal intestinal tract. In the absence of plant myrosinase, hydrolysis in the upper tract may occur, probably mainly as a consequence of gastric secretions. Dietary conditions, however, seemed to have little effect on the pattern of isothiocyanate release in the distal digestive tract. A more precise investigation of the balance between microbial release of isothiocyanate and subsequent utilisation of isothiocyanate is required to more fully understand the effect of dietary fibre on the fate of glucosinolates in the lower digestive tract.

CHAPTER 7: GENERAL DISCUSSION

7.1. Introduction

Until now, quantitative data on the breakdown products released from glucosinolates *in vivo* has been sparse. Glucosinolate breakdown products are a diverse group of chemicals with different metabolic fates and a range of biological effects. Their high reactivity makes their measurement in body fluids and tissues difficult. The uptake and the disposition of glucosinolate breakdown products has, therefore, not been well characterised. Crude measurements of human dietary exposure to glucosinolates have been made by assessing the glucosinolate composition and the likely hydrolysis products arising from consumed vegetable material (Sones *et al.* 1984). However, the importance of the hydrolysis environment in influencing the resulting hydrolysis products and recent findings on the involvement of the digestive microflora in glucosinolate breakdown have encouraged the development of more refined techniques. The approach chosen in the series of experiments reported here relies on the use of a combination of methods. Quantitative methods were used to measure glucosinolate concentrations in the plant matrix as well as in digestive fluids and faecal material. By this means, an accurate measurement of the disappearance of glucosinolates was made. In parallel, the formation of hydrolysis products was measured by the use of urinary markers. Additionally, the use of rats with a controlled intestinal microflora enabled the effect of vegetable myrosinase to be distinguished from the effect of the intrinsic microflora. With this comprehensive approach, the extent of hydrolysis was measured in several situations. Subsequently, direct comparisons between the major factors of influence were made. This gave an indication of the most favourable circumstance for optimum dietary exposure to potentially beneficial metabolites.

The first part of this general discussion summarises the different experimental approaches adopted and their advantages and limitations. From the experimental results, a general mechanism for digestive metabolism of glucosinolates is suggested and discussed in the second section. Finally, the unresolved issues deriving from the findings are considered and suggestions made for further research.

7.2. Experimental approach

7.2.1. Relevance of the use of urinary markers

Interest in markers of glucosinolate metabolism and particularly in markers for the cancer-protective isothiocyanates has grown in the past few years. This research interest has been driven by the need to measure the uptake of isothiocyanates by the intestinal tract before assessing the efficacy of these compounds in protecting against cancer. The use of urinary markers as potential tools for non-invasive studies has been investigated in a number of human trials. Several studies have reported the successful measurement of mercapturic acids derived from isothiocyanates after feeding raw or cooked cruciferous vegetables to human volunteers (Chung *et al.* 1992; Jiao *et al.* 1994; Shapiro *et al.* 1998; Getahun & Chung, 1999). The wide inter-individual variation in the data reported so far may have reflected variation in activities of detoxification enzymes rather than a variation in isothiocyanate uptake. The methods used in the series of experiments reported here demonstrate that administration of a homologous isothiocyanate concurrently to the test glucosinolates and isothiocyanates can standardise for the variability in the mercapturic acid recovery. This method was used in studies on rats prior to this work for the measurement of allyl isothiocyanate release from an oral single dose of sinigrin (Duncan *et al.* 1997; Elfoul, 1999). Experiments 1 and 2 of the current studies have shown that it can also be used successfully for the measurement of aromatic isothiocyanates in rats. The measurement of dietary glucosinolate hydrolysis can also be achieved, although in rats the continuous administration of a cruciferous diet reduced the accuracy of the estimates. The work has validated the use of mercapturic acids as markers of isothiocyanate release in humans.

7.2.2. Application of the approach to isothiocyanates

Several assumptions underlie the use of mercapturic acids to estimate isothiocyanate release. The homologous compound used as an internal standard must follow the same metabolic pathway as the studied compound. To be a realistic estimate of the variation in post-absorptive metabolism, the internal standard must be affected by the variation in the glutathione conjugation to the same extent as the studied compound. The results presented here showed that this assumption is satisfied for aliphatic and

aromatic isothiocyanates. The unavailability of other isothiocyanates with demonstrated beneficial properties, such as sulphoraphane, has not allowed the validation of the method to a larger set of compounds during the course of the project but could now be considered in future experiments. On the other hand, isothiocyanates derived from indole glucosinolates, such as glucobrassicin, do not appear to be excreted by the same pathway as aliphatic and aromatic isothiocyanates. The technique is not, therefore, a suitable tool for their study.

7.2.3. Investigation of the release of nitrile derivatives

The attempt to detect urinary markers of nitrile derivatives has not been successful so far. Although knowledge on the disposition of nitrile derivatives is still rudimentary, the method established for isothiocyanates was duplicated for nitriles in Experiment 1 with a view to extending the method to the measurement of nitrile production once their fate had been elucidated. The major problem with studying nitrile metabolism is that all attempts to detect their metabolic fate have been inconclusive except for non-glucosinolate derived nitriles, such as acrylonitrile and crotonitrile (Van Bladeren *et al.* 1981). It is unknown whether they are not formed at all or whether their formation is followed by an immediate degradation to other compounds before their absorption. It has been hypothesised that the nitrilase activity of the microflora may break down nitriles immediately after their formation (Forss & Barry, 1983). Disappearance of allyl cyanide under microbial action has been demonstrated in sheep rumen fluid (Duncan *et al.* 1992).

7.2.4. Limits of the approach

The lipophilic nature of glucosinolate-derived isothiocyanates together with their small molecular weight mean that when they are administered in pure form, they are likely to be absorbed in the upper part of the intestinal tract. The recovery of mercapturic acids from native isothiocyanates measured in this series of experiments represents, therefore, the efficiency of their absorption and detoxification from the proximal gut. In Experiments 2 and 3, it was assumed that measured recoveries were a good estimate of the efficiency of absorption and detoxification along the entire digestive tract. It appeared, however, that the site of isothiocyanate release influenced their absorption (Experiment 4). The conversion of isothiocyanates to mercapturic

acids was found to be significantly lower when isothiocyanates were released in the large bowel than in the upper intestine. Consequently, to achieve an accurate measurement of isothiocyanate release, it would be necessary to assume *a priori*, the site of isothiocyanate release and absorption. Ideally, the recovery of mercapturic acids from isothiocyanates must be measured in the portion of the gut from which the isothiocyanates are absorbed. Isothiocyanates released by plant myrosinase are likely to be absorbed mainly in the stomach and small intestine. The measurement of the recovery as carried out in this study by administering a single oral dose of isothiocyanate would then be adequate. The measurement of the recovery from the lower part of the digestive tract where the isothiocyanate release is likely to occur in the absence of plant myrosinase activity is less straightforward. This would require techniques such as encapsulation of isothiocyanates to bypass their absorption in the small intestine and the choice of a more suitable vehicle than oil which is normally absorbed in the proximal digestive tract. Such a mode of administration was not possible in the experiments described here. The recovery measured in the small intestine was used to calculate isothiocyanate release regardless of their supposed site of release and absorption. Regarding results from Experiment 2, the use of higher recovery values than the actual ones may have led to an under-estimate of isothiocyanate release in the bowel.

7.2.5. Alternative methods to the use of markers

As Experiment 5 focused on isothiocyanate release in the bowel, a direct approach to the release of isothiocyanates in the digestive lumen seemed more appropriate than the mercapturic acid approach for the reasons mentioned above. Attempts to directly measure the formation of isothiocyanates in digestive contents have often been limited by the sensitivity of analytical techniques. The use of an intermediate step of micro-extraction on a solid phase can successfully improve the threshold of detection of isothiocyanates in complex digestive media. The results of Experiment 5 showed that this technique can be a useful complementary tool for investigating isothiocyanate release. As the amounts of isothiocyanates measured represented the instantaneous concentrations of compounds, the information given by this measurement was an integration of the production, degradation and absorption rates of isothiocyanates. This technique can only indicate a strong modification of the

nature of hydrolysis products released. As a first approach to the understanding of the influence of other nutrients, it brought useful qualitative information on the influence of dietary fibre on isothiocyanate release in the bowel.

7.2.6. Relevance of rat studies

The use of rodents as an adequate model for human consumption of vegetables has been questioned by other workers (Ratcliffe *et al.* 2000). Since glucosinolate hydrolysis occurs upon cell disruption, the chewing pattern may be an important factor in determining the subsequent release of metabolites. The process of chewing is less complete in humans than in laboratory rodents and an unknown proportion of cruciferous material may be swallowed intact. Rodents, by gnawing their food, are probably more efficient at damaging plant cells than humans. The extent of hydrolysis observed in rats may be over-estimated in comparison to humans.

Despite the constraints inherent in the feeding pattern of animals, the use of controlled-microflora rats represents a useful model for the study of glucosinolate metabolism. The dynamics of bacterial degradation of glucosinolates may be different according to the microflora harboured by the host. Hence, human flora-associated animals are likely to give more relevant results for extrapolation to humans (Rumney & Rowland, 1992). Furthermore, the similarities in urinary products between rats and humans make the use of rats harbouring a human microflora a good model (Mennicke *et al.* 1983; Mennicke *et al.* 1988).

7.3. Fate of glucosinolates and their isothiocyanate derivatives in the digestive tract

The series of experiments brought new insights to the understanding of glucosinolate metabolism. The data obtained contribute to a better knowledge of the sequence of events determining the fate of glucosinolates and their related isothiocyanates. The experiments provide an indication of the importance of each possible metabolic route when cruciferous vegetables are eaten raw or cooked.

7.3.1. Isothiocyanate release by plant myrosinase

The action of plant myrosinase led to a conversion of around 80% of the ingested benzyl glucosinolates to benzyl isothiocyanate (Experiment 2). This value indicates that in the conditions of Experiment 2, the hydrolysis of glucosinolates *in vivo* is favourable to isothiocyanate production.

It is not clear from the present series of experiments whether hydrolysis by plant myrosinase only occurs in the mouth or is continued in the stomach and distal digestive compartments. The low pH of gastric contents and the presence of gastric peptidases may denature and inactivate myrosinase. On the other hand, as the food gradually fills the stomach, the pH increases and may allow a favourable environment for the plant myrosinase-catalysed hydrolysis to occur. The chewing pattern and the time of transit may influence the amount of glucosinolates which avoid hydrolysis by plant myrosinase.

Studies with labelled compounds have demonstrated that the small intestine is the primary site for isothiocyanate absorption (Conaway *et al.* 1999). Although the rate of isothiocyanate production is very high in the upper digestive tract, the total amount of isothiocyanates arising is not taken up by the intestinal mucosa. Results of Experiment 2 indicate that, in the physiological circumstances tested, when the host harbours a microflora, the proportion of isothiocyanates actually taken up by the intestinal tract is lower than when the microflora is absent. Isothiocyanates released in the upper digestive tract are thus likely to follow several metabolic routes. The rapid absorption of the isothiocyanates by the enterocytes is likely to be the major metabolic pathway. An alternative pathway for isothiocyanates is their passage into the bowel and subsequent metabolism by the resident microflora.

7.3.2. The involvement of the microflora in isothiocyanate release

The findings reported here on microbial myrosinase activity are in accordance with the results of previous studies (Elfoul, 1999). They confirm the ability of the microbial myrosinase to catalyse glucosinolate hydrolysis. This was shown by the absence of even trace amounts of glucosinolates in the faeces of human flora rats fed Brussels sprouts (Experiment 2). Microbial hydrolysis resulted in a minor production

of isothiocyanates. For benzyl glucosinolate, this hydrolysis led to 5% of benzyl glucosinolate being converted to benzyl isothiocyanate in Experiment 2. Other studies have measured a higher release for allyl isothiocyanate derived from sinigrin with values ranging from 10% to 17% according to the type of diet and the microflora (Duncan *et al.* 1997; Elfoul, 1999). Despite the consistency of these results, it is surprising that the proportion of isothiocyanates released is much less efficient than that due to plant myrosinase. One explanation for this effect revealed by the current work is that the actual extent of isothiocyanate production by microbial myrosinase may be masked by the subsequent utilisation of isothiocyanates by the microflora. The hypothesis that the action of microbial activity is additive to the plant myrosinase hydrolysis was refuted in Experiment 2. Furthermore, isothiocyanates delivered to the bowel may be poorly taken up by the intestinal mucosa (Experiment 4). Consequently, the isothiocyanates arising in the bowel either after formation in the upper tract or after the action of the microbial myrosinase may be partially utilised by the microflora. Although more work would be necessary to provide direct evidence of this mechanism, *in vitro* studies indicate that this process is possible and would lead to the formation of amine derivatives (Tang *et al.* 1972, S. Rabot, personal communication).

The question remains as to the fate of hydrolysed glucosinolates which do not form isothiocyanates. The microbial production of other metabolites, such as nitriles, is one possibility. There is little evidence, however, to support this route as a major pathway for glucosinolate degradation by microflora. A very high production of nitriles would be required to account for the 90% of the glucosinolate breakdown products unaccounted for by isothiocyanates. The relatively alkaline pH in the bowel is unlikely to favour nitrile production (assuming that the activity of microbial myrosinase in response to pH variation is similar to that of plant myrosinase). Moreover, allyl cyanide, the nitrile derivative of sinigrin was not found in the digestive contents of rats harbouring a human strain of glucosinolate-degrading bacterium after gavage with an oral dose of sinigrin (Elfoul, 1999). It may be that, in the latter case, nitriles are formed but are immediately broken down by the microflora as seems to occur with isothiocyanates.

To summarise, the outcome of this research suggests that the microflora may have a dual role in isothiocyanate release. It may catalyse release of isothiocyanates and then further degrade them to amines or other breakdown products. The activity of microbial myrosinase may thus be cancelled out by the ability of the microflora to further utilise the breakdown products. Assuming that isothiocyanates are the major hydrolysis product of microbial myrosinase, the following mechanisms may be occurring:

1) When plant myrosinase is inactive, glucosinolates predominantly arise in the bowel. The amount of isothiocyanate release by the microbial myrosinase in these circumstances may exceed the capacity of the microflora to break down the isothiocyanates. The excess of isothiocyanates can therefore be absorbed by the intestinal mucosa. The overall result is a positive yield of isothiocyanates ranging from 5 to 10% of the glucosinolate dose.

2) When plant myrosinase is active, the amount of glucosinolates reaching the bowel is minimal whereas a substantial proportion of pre-formed isothiocyanates enters the digestive tract. In these circumstances, the amount of isothiocyanates deriving from microbial hydrolysis is not sufficient to compensate for the microbial utilisation of isothiocyanates. The overall result may explain the 30% discrepancy observed between germ-free and human-flora animals fed raw Brussels sprouts in Experiment 2.

7.3.3. Metabolic fate of non-hydrolysed glucosinolates

The fact that 66% of the benzyl glucosinolate was not recovered in the faeces of rats in the absence of myrosinase suggested that intact glucosinolates may be absorbed by the intestinal tract (Experiment 2). The evidence for such a phenomenon is still the subject of debate as discussed in Experiment 2. The presence of a small amount of sinigrin in the urine output of human flora rats in similar experiments indicate that it is nevertheless a pathway to take into consideration (Elfoul, 1999). In the present experiments, the extent of urinary excretion of glucosinolates was not measured. Consequently, the actual amount of glucosinolates escaping hydrolysis cannot be accurately estimated. On the other hand, the amount of glucosinolates excreted in the faeces of germ-free rats may give a reasonable estimate of the amount of glucosinolates arriving in the bowel and available for microbial degradation. On the

basis of the recovery of benzyl glucosinolate in faeces of germ-free rats, it can be estimated that approximately 4% of ingested benzyl glucosinolate arrived intact in the bowel when plant myrosinase was active in the specific conditions of Experiment 2. When plant myrosinase was inactive, the proportion of glucosinolates available for microbial degradation in the digestive tract of rats harbouring a human faecal flora was 34% (Experiment 2). A similar figure has been reported (28%) in a similar experiment studying the fate of sinigrin (Elfoul, 1999). There is consistent evidence from this work and previous studies that glucosinolates are not found in faeces when the host harbours a microflora, indicating a total degradation of glucosinolates by the microflora (Experiment 2) (Elfoul, 1999). This observation was true for animals harbouring a single strain of human bacterium or a whole human flora. Hence, the glucosinolate concentration in human stools were not measured in Experiment 3.

7.3.4. Degradation of glucosinolates by non-enzymatic autolysis

In Experiment 2, traces of benzyl mercapturic acid was found in the urine of rats in the absence of any source of myrosinase. A non-catalytic hydrolysis of benzyl glucosinolate was suspected. Direct evidence for glucosinolate autolysis was found in Experiment 5. The formation of allyl isothiocyanate and benzyl isothiocyanate was measured in the stomach of rats immediately after administration of glucosinolates in the absence of myrosinase activity. The proportion of isothiocyanate formed represented a minor part of the dose (0.1% sinigrin and 2.5% benzyl glucosinolate). This mechanism may be due to the acidic content of the stomach. Other studies have shown that glucosinolates carrying an indolic side chain were also prone to autolysis (De Kruif *et al.* 1991). The non-enzymatic degradation of glucosinolates is therefore a possible source of isothiocyanates in the upper digestive tract when plant myrosinase is inactive. The extent of autolysis may vary according to the cooking procedure. A long cooking process may soften the plant cell walls and glucosinolates may then be more exposed to the chemical action of gastric fluids. In contrast, when plant myrosinase is active, enzymatic hydrolysis would predominate and the extent of autolysis is likely to be small.

7.3.5. Influence of other components of the diet

The addition of ferrous ions reduced the proportion of isothiocyanate release when plant myrosinase was active. This was in agreement with findings *in vitro* where the isothiocyanate production by plant myrosinase was inhibited by ferrous ions (Uda *et al.* 1986). Owing to the lack of nitrile markers, it was not possible to verify whether the decrease in isothiocyanate uptake was reflected in an increase in nitrile compounds as observed *in vitro* (Uda *et al.* 1986). It is thus difficult to draw conclusions on the mechanism of this inhibition. Iron metabolism is highly regulated and the capacity for absorption of iron is inversely related to the amount consumed (Fairweather-Tait, 1998). The administration of a continuous dose of ferrous ion may have led to general changes in the physiology of the host. These changes, in turn, may have affected isothiocyanate uptake. In dietary circumstances, changes in the ionic content of digestive contents are likely to be more subtle and may not induce direct effects on myrosinase hydrolysis *in vivo*.

The manipulation of the fermentation in the caecum of human flora rats in Experiment 5 appeared to have relatively little influence on isothiocyanate production in the gut. It can be hypothesised that the increase in fermentative activity influenced the microbial myrosinase activity and the microbial utilisation of glucosinolate breakdown products to the same extent, resulting in no significant change. More detailed work using *in vitro* suspensions of human flora would help to elucidate the actual mechanism.

There are a large number of other components of the diet which may affect the release of isothiocyanates. Protein content may, for instance, be a factor of influence. Isothiocyanates can form chemical bonds with peptides or small proteins (Drobnica & Augustin, 1965). As isothiocyanates are then no longer in a free form, isothiocyanate uptake and disposition may be affected. Vitamin concentration, particularly of ascorbic acid, may also influence isothiocyanate release since vitamin C is an activator of plant myrosinase.

7.3.6. The post-absorptive fate of isothiocyanates

In these experiments, mercapturic acid formation following ingestion of isothiocyanates has been measured in a variety of situations, giving a better insight into the fate of isothiocyanates after their release in the digestive tract. The variation in mercapturic acid recovery from isothiocyanates was high, ranging from 30 to 70 % of the initial dose. There are a number of potential causes of this variability:

The nature of the side-chain may play a role, either at the stage of absorption by the intestinal mucosa, or at the stage of glutathione conjugation. This characteristic seems important in rats. Across all the studies, independently of individual variation, the extent of excretion of allyl and benzyl mercapturic acid was consistently higher than that of propyl, butyl and phenethyl mercapturic acid. In humans, these differences were less apparent.

The site of absorption of isothiocyanates by the intestinal tract has an important influence as detailed in the discussion of the limits of the experimental approach (section 7.2.4).

The role of components of the diet on the efficiency of detoxification may also be important. Ferrous ions, for instance, significantly lowered the excretion rate of mercapturic acid perhaps by inhibiting xenobiotic-metabolising enzymes. All the mercapturic acids were affected to the same extent by this treatment (Experiment 1). It has been speculated that the presence of glucosinolates in the diet may influence the conversion rate of isothiocyanate to mercapturic acids (Duncan *et al.* 1997). Evidence in these experiments does not support this hypothesis. The recovery of isothiocyanates administered as a single dose was not significantly different when rats were fed a glucosinolate-free or a glucosinolate-containing diet (Experiment 1). Similar observations held for the human experiment (Experiment 3). The excretion rate of the internal standard, namely phenethyl mercapturic acid, was not significantly different after three different cruciferous meals for the same subject. Additionally, the habitual consumption of a high or low amount of vegetables did not induce significant differences in recoveries of mercapturic acids.

A proportion of the isothiocyanates released by glucosinolates are not converted to mercapturic acids and presumably follow other excretory routes. These have not been measured in this series of experiments. Three minor pathways can be considered :

1) The excretion of isothiocyanate in faeces. A minor proportion may not be metabolised at all and excreted in the faeces. Traces of faecal allyl isothiocyanate following its oral administration has been found in other studies (Conaway *et al.* 1999; Elfoul, 1999).

2) After conjugation in the liver a minor proportion of isothiocyanate breakdown products may enter the entero-hepatic circulation and be subsequently excreted in faeces as biliary salts (Bollard *et al.* 1997; Conaway *et al.* 1999). This process may explain the secondary peak in isothiocyanates in the digestive content of rats, 24h after the dose was given (Experiment 5).

3) Isothiocyanates are metabolised by tissues which subsequently leads to the excretion of CO₂ in exhaled air (Conaway *et al.* 1999).

Figure 7.1 summarised the different pathways speculated for the fate of glucosinolates.

7.3.7. Digestive metabolism after ingestion of raw vegetables

Data on the fate of glucosinolates after ingestion of raw vegetables have now been extended to three glucosinolates - namely sinigrin, benzyl glucosinolate and phenethyl glucosinolate. When cruciferous vegetables are eaten raw, the plant myrosinase is active. In this circumstance, this series of experiment established that isothiocyanates available for intestinal uptake are released in significant quantities. Isothiocyanates released accounted for 60% of ingested phenethyl glucosinolate derived from watercress (Experiment 1), 50% of benzyl glucosinolate given as an oral dose (Experiment 2) and 37% of sinigrin derived from white cabbage (Experiment 3). Although these value relate closely to each experimental conditions, the fairly consistent results corroborate an earlier study where 41% of sinigrin was found to be converted into isothiocyanates (Duncan *et al.* 1997). This relatively high yield of isothiocyanates, compounds known for their beneficial effects, indicate that glucosinolate metabolism *in vivo* is a favourable mechanism for health protection. The proportion of isothiocyanates measured here presumably reflects primarily

glucosinolate degradation in the upper digestive tract and subsequent uptake in the small intestine (Figure 7.1).

In addition to this main pathway, there is a proportion of isothiocyanates which may not cross the intestinal barrier and may serve as substrate for colonic microflora. In Experiment 2, the proportion of benzyl glucosinolate reaching the bowel intact was estimated around 30%. The microbial myrosinase, although active, may offer only a limited contribution to the overall metabolism of glucosinolates in these conditions.

7.3.8. Digestive metabolism after ingestion of cooked vegetable

In cooked vegetables, plant myrosinase is generally inactive. In these circumstances, the estimates of isothiocyanate release vary considerably with studies. The present studies and those by other authors have estimated that the release of isothiocyanates from glucosinolates varied from 5% for benzyl glucosinolate (Experiment 2) to 10-17% for sinigrin in rats harbouring a human flora (Duncan *et al.* 1997; Elfoul, 1999) (Figure 7.1). Animal experiments thus suggest that the release of isothiocyanates after ingestion of cooked vegetables occurs but it is limited in comparison with the proportion of isothiocyanates released from raw vegetables. This result would not be favourable for an enhancement of the effect of isothiocyanates in the bowel as previously hypothesised. In contrast to the studies in rats, release of allyl isothiocyanate averaged 53% in human volunteers after ingestion of cooked cabbage, suggesting a higher production of isothiocyanates in humans than expected from the findings using rat as animal model. The estimates found in the experiment on human subjects (Experiment 3), however, should be viewed with caution as the presence of a residual plant myrosinase activity in watercress may have exaggerated the extent of glucosinolate hydrolysis.

The large variation among volunteers after ingestion of cooked cabbage would suggest that microflora may catalyse the hydrolysis or induce the breakdown of isothiocyanate to different extents according to individuals. The proportion of isothiocyanate release may thus be less consistent than in the rat studies. The fact that intact glucosinolates pass through the stomach and upper digestive tract may have

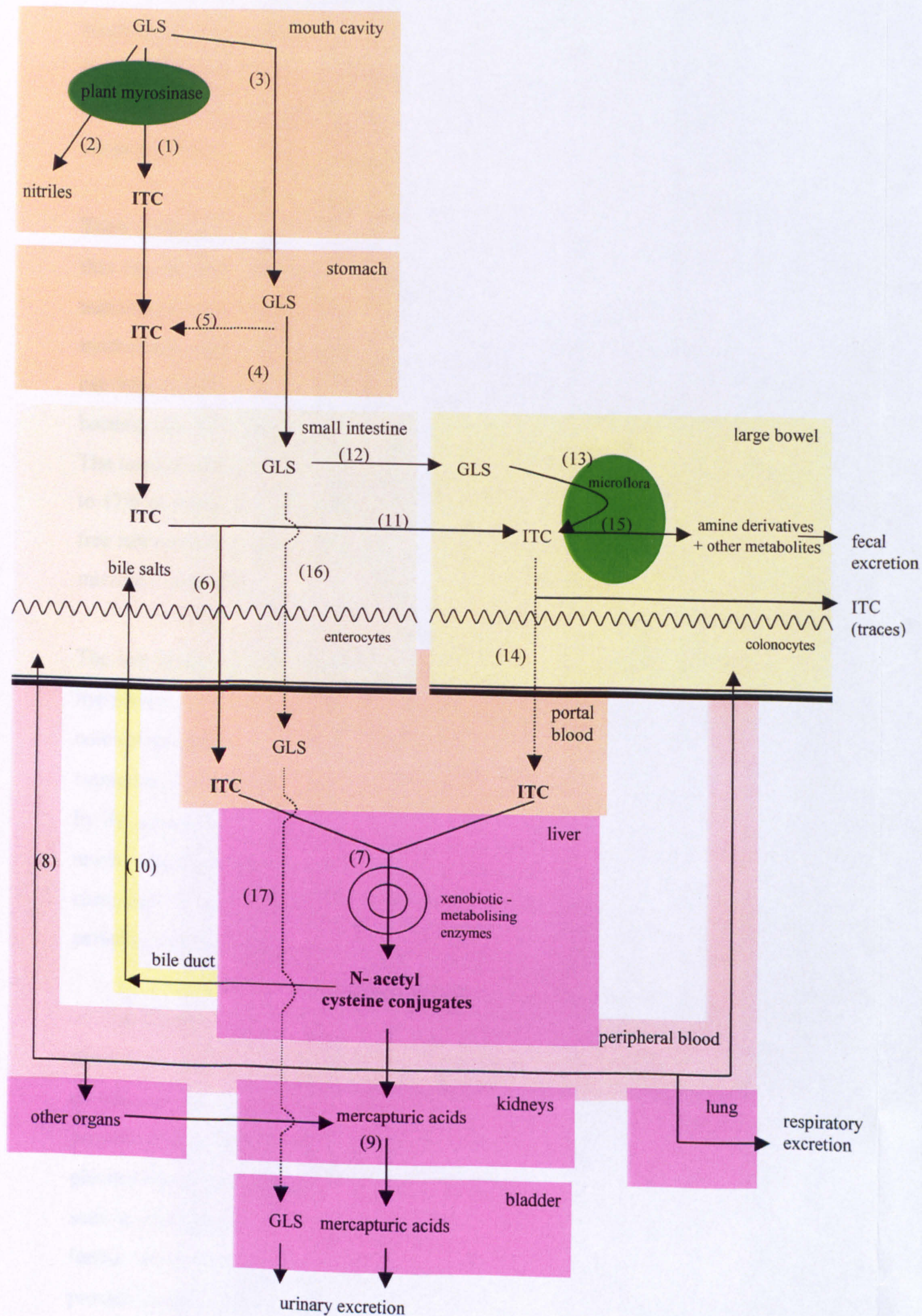


Figure 7.1 Proposed metabolic fate of glucosinolates (GLS) and their isothiocyanate derivatives (ITC) after ingestion of cruciferous vegetables. Plain lines indicate major pathways. Dotted lines indicate minor pathways. The hydrolysis of GLS by plant myrosinase releases primarily ITC (1) or nitriles (2) (Nugon-Baudon & Rabot, 1994). Alternatively, when plant myrosinase is denatured by cooking procedure, GLS may reach the stomach (3) and the small intestine (4) under their intact form as suggested by outcomes of experiment 2 and 5. Results of experiment 5 suggested that non-enzymatic hydrolysis of intact GLS to ITC may occur in the stomach (5). ITC are primarily absorbed in the intestinal tract (6) as demonstrated by studies using radio-labelled compounds (Conaway *et al*, 1999). Activation of the xenobiotic metabolising enzymes by ITC (7) leads to formation of N-acetyl conjugates which are able to circulate to peripheral organs (8) (Brüsewitz *et al*, 1977). The major end-products of ITC metabolism are the mercapturic acids (9). The entero-hepatic circulation has been suggested as a minor pathway of ITC excretion (10) (Conaway *et al*, 1999). A variable proportion of ITC (11) and non-degraded GLS (12) may transit to the large bowel. The quantitative ratio ITC:GLS entering the large bowel may dramatically change whether the vegetables are ingested raw or cooked (experiment 3, 4 and 5). The endogenous microflora can breakdown the GLS arising in the bowel (13) (Campbell *et al*, 1995, experiment 5). Although a microbial production of ITC has been demonstrated (Elfoul, 1999), ITC seem to be poorly available for absorption by the colonic tract (14) (experiment 4). The intestinal microflora may break-down ITC arising in the bowel into end-products such as amine derivatives and other unidentified metabolites subsequently excreted in faeces (15) (Rabot, personal communication, 2000). The possible absorption of intact glucosinolates in the upper intestinal tract following ingestion of cooked vegetables has been suggested from the outcome of experiment 3 (16), although the post-absorptive fate of intact GLS remains unclear (17).

made them susceptible to other routes of metabolism. The release of isothiocyanates by autolysis and the digestive absorption of non-hydrolysed glucosinolate may therefore be an important mechanism for the metabolism of glucosinolates in these circumstances.

These findings highlight an unexpected role for the microflora. Indeed, they suggest that the microflora could be an inhibitor of isothiocyanate absorption, instead of maximising the potential production of beneficial compounds for the host. This mechanism would however not be entirely detrimental for the host. Isothiocyanates can have anti-bacterial effects at high doses (Brabban & Edwards, 1995). Intestinal bacteria may breakdown the isothiocyanates as a defence mechanism against toxicity. The level of isothiocyanate may thus remain low in the bowel. The production of 5% to 17% of isothiocyanates in the bowel would therefore correspond to the amount of free isothiocyanates that is able to cross the colonic mucosa before being exposed to microbial degradation.

The low level of isothiocyanate production in the bowel would tend to weaken the hypothesis that isothiocyanates exert their anti-carcinogenic action on the colonocytes from the intestinal lumen. The isothiocyanates involved in tumour suppression and apoptosis may be transported to the colonocytes as their conjugates by the peripheral circulation (Figure 7.1). This hypothesis would be supported by recent studies where the level of isothiocyanates in the colonic lumen was not correlated to the tumour-suppressing effect in the colonic mucosa (T.K. Smith personal communication).

7.4. Future work

Since this work has extended the use of urinary markers to dietary glucosinolates and to humans, the application of the approach to realistic dietary situations is now possible in nutritional studies. The measurement of isothiocyanate release when glucosinolates are ingested concurrently with potential carcinogenic compounds, such as nitrosamines from cooked meat, would provide an interesting avenue for further work. As the use of markers is a non-invasive approach, the method can also provide useful information in studies on anti-carcinogenic mechanisms. Thus, it

would be possible to directly relate isothiocyanate uptake to induction of detoxification enzymes in tissues, for instance, or to assess anti-mutagenic potential in relation to isothiocyanate formation in the digestive tract. Experiments where both food processing and biological end-points were quantified could potentially provide useful information to develop nutritional guidelines and health policy regarding consumption of cruciferous vegetables.

Mercapturic acids may be less precise indicators of isothiocyanate release in the lower gut. Refinements of the technique would therefore be required for their effective use in this context.

This series of experiments has also highlighted areas of glucosinolate metabolism which require more research. The route of post-absorptive metabolism for non-hydrolysed glucosinolates still remains unresolved. The formation of nitrile breakdown products has not been elucidated. The use of radio-labelled compounds could considerably help investigation in these fields.

Findings still remain unclear on the influence of the microflora on isothiocyanate release. More evidence on the ability of the microflora to degrade isothiocyanates would be required to clarify the hypothesis of a modulating role of the human microbial ecosystem. In this regard, the infusion of radio-labelled glucosinolates and isothiocyanates to suspensions of human microflora *in vitro* may allow identification of the end-products of microbial digestion.

Finally, the present human trial highlighted a potential variation between subjects in the amount of isothiocyanate released in the lower digestive tract. More studies would be required to assess whether myrosinase hydrolysis and isothiocyanate digestion by the microflora have variable capacities among individuals. A confirmation of these observations would indeed suggest that the beneficial effects of consuming brassicas vary according to individuals.

7.5. Conclusion

The successful use of urinary products as markers of isothiocyanates has allowed measurement of isothiocyanate release from glucosinolates as they would normally occur in human diet, that is, when they are present in their plant matrix. The research has also applied the method to humans. The investigation focussed on three glucosinolates, namely sinigrin, benzyl glucosinolate and phenethyl glucosinolate which occur naturally in cruciferous vegetables consumed in human diet. These experiments suggest that, at least for the three glucosinolates studied, the formation, *in vivo*, of cancer-protective isothiocyanates is significant, thereby strengthening the evidence for a beneficial effect of cruciferous vegetables for health. Whether glucosinolate consumption for humans is entirely beneficial remains, however, to be seen since the extent of formation of detrimental nitriles is still unclear.

From these results, it emerged that in a human type of diet the highest release of isothiocyanates is achieved when the plant myrosinase is intact, that is when the vegetables are eaten raw. The cooking process alters the plant myrosinase activity and the influence of the chemical environment and microbial activity may then become important. The involvement of the microflora acting as a secondary catalyst of hydrolysis has been confirmed but new findings revealed that microflora can also modulate the extent of isothiocyanate absorption. A new element must therefore be taken into account when assessing the potential of cruciferous vegetables for the release of beneficial isothiocyanates. The extent and rate of isothiocyanate disposition is not only determined by the hydrolysis step but also by the capacity of bacteria to degrade isothiocyanates.

From a nutritional point of view, these results lead to two interpretations. If further research reveals that the most important factor for cancer protection is the global amount of isothiocyanates arising in the systemic circulation, then the amount of cruciferous vegetables that are eaten raw must be promoted. Focus on enhancing the glucosinolate-myrosinase system in cruciferous vegetables may therefore be of primary relevance. On the other hand, if it is confirmed that protection against colorectal cancer, one of the most widespread cancers, is best achieved by a delivery of isothiocyanates from the lumen of the gut, the emphasis on cooking guidelines for

cruciferous vegetables and increasing the frequency of consumption may achieve a small but regular supply of isothiocyanates to the colon.

This research highlights the difficulty in viewing plant secondary compounds which elicit beneficial effects as isolated micro-constituents that could be used as nutraceuticals. As far as glucosinolates are concerned, this work has demonstrated that the beneficial properties of the compounds rely on a fragile association between the glucosinolate-myrosinase system of the plant and parameters directly associated with the host, among which are the dietary habits and the enzymatic capacities of the endogenous microflora.

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APPENDIX 1:

Synthesis of

N-acetyl-*S*-(*N*-alkyl thiocarbamoyl)-*L*-cysteine, dicyclohexylamine salt.

(adapted from Mennicke *et al*, 1983)

- 1- Two grams *N*-acetyl cysteine are dissolved in 25 ml sterilised water
- 2- Solution is adjusted to pH = 8-9
- 3- Solution is mixed with 15 ml pyridine and maintained at 40°C in a water bath
- 4- Four grams isothiocyanate are dissolved in 10 ml pyridine
- 5- Isothiocyanate-pyridine solution is added to *N*-acetyl cysteine
- 6- Excess of reactives are extracted in 50 ml toluene (5 extractions)
- 7- Aqueous residue is adjusted to pH = 3
- 8- *N*-acetyl-cysteine conjugates are extracted with ethyl acetate (two extractions)
- 9- Ethyl acetate extracts are pooled and washed free of acid with sterilised water
- 10- Ethyl acetate extracts are dried over anhydrous Na₂SO₄
- 11- Residues are washed with petroleum ether and dissolved in 3 ml ethyl acetate
- 12- 0.5 ml dicyclohexylamine are added to residues
- 13- Precipitate is crystallised at -20°C

APPENDIX 2:

Preparation of urine samples for analysis of mercapturic acids by HPLC

(adapted from Duncan et al, 1997)

Standard mix and Internal standard solutions :

The appropriate N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteine, dicyclohexylamine salt is dissolved in 500 ml.l⁻¹ ethanol to a final concentration of 1.5 mM.

Standard solutions are stored at 4°C and remain stable for 12 days

Protocol:

Samples are prepared in duplicate

1. 200 µl of urine or standard mix are added to screw-top test tube
2. 200 µl of internal standard solution are added
3. 600 µl of distilled water are added
4. Thioureas are formed by addition of 50 µl butylamine to test-tubes
5. Test tubes are capped and incubated at 60°C for 30 minutes
6. After cooling (10 min), excess butyl amine is buffered by addition of 300 µl 25% sulfuric acid
7. Thioureas are extracted in 5 ml diethyl ether
8. Ether phase is washed with 1 ml 1M NaOH and 1ml of distilled water (twice)
9. Anti-bumping granules are added to test tube and diethyl ether is evaporated under vacuum at -5°C
10. When evaporated, thioureas are re-suspended in 700 µl 50% acetonitrile before HPLC analysis.

APPENDIX 3:

Sample of ANOVA table for Experiment 2

General Analysis of Variance

BLOCK isolator + isolator.rat + isolator.period + isolator.rat.period

TREATMENTS (period*day)+(diet*dose*status)

COVARIATE "No Covariate"

Variate: butyl mercapturic acid

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
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isolator stratum					
------------------	--	--	--	--	--

status	1				
--------	---	--	--	--	--

Residual	2				
----------	---	--	--	--	--

isolator.rat stratum					
	12				

isolator.period stratum					
-------------------------	--	--	--	--	--

period	1				
--------	---	--	--	--	--

diet	1				
------	---	--	--	--	--

diet.status	1				
-------------	---	--	--	--	--

Residual	1				
----------	---	--	--	--	--

isolator.rat.period stratum					
-----------------------------	--	--	--	--	--

	12				
--	----	--	--	--	--

isolator.rat.period.*Units* stratum					
-------------------------------------	--	--	--	--	--

day	1				
-----	---	--	--	--	--

period.day	1				
------------	---	--	--	--	--

dose	1				
------	---	--	--	--	--

diet.dose	1				
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dose.status	1				
-------------	---	--	--	--	--

diet.dose.status	1				
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Residual	25(1)				
----------	-------	--	--	--	--

Total	62(1)				
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APPENDIX 4a:

Questionnaire addressed to applicants for the human study

CONFIDENTIAL

Volunteer no:.....

QUESTIONNAIRE

This questionnaire is established to provide necessary information for the undertaking of the study entitled: Study on the effect of food processing on the release of anticancer derivatives from cabbage. Data provided by volunteer will remain confidential and will not be used for other purposes or studies. If you do not wish to answer to some questions please specify so or refer to the investigator

1-Personal Detail:

1.1-Name:.....

1.2-Address:.....

1.3-Contact no: home:.....
work:.....

1.4-Age:.....

1.5-Weight:.....

1.6-Height:.....

1.7-Are you a smoker?:(tick as appropriate) Yes No

1.8- Have you been involved in human trial before? Yes No

2-Health condition:

Tick as appropriate :

	Yes	No	Don't know
2.1- Do you consider yourself in a good health?			
2.2- Have you consulted a doctor in the past four weeks?			
2.3- Have you been admitted to the hospital in the past six months?			
2.3-Do you regularly take any medications ?			
2.4-Are you currently under medical treatment for infection or illness?			
2.5- Do you suffer from these diseases?			
Diabetes			
Hypercholesterolaemia			
Crohn's disease			
Kidney insufficiency			
Ulcer			
Food allergy (please specify)			

3-Food consumption:

3.1-Could you remember and list what you have eaten in the past 24 hours?
(List is qualitative: for instance : baked potato, cheese and mixed salad, bag of crisps, chocolate bar)

Breakfast:

Lunchtime:

Tea:

Breaks:

3.2-Are you vegetarian? Yes No

3.3-What would you consider as a your main meal?

Breakfast:
Lunch:
Tea:

3.4-Do you regularly take food supplements: Yes No

If yes, what sort?: (you may tick more than one box)

Vitamin complement Cod liver oil
Minerals Vitamin C
Yeast extract

Others:
specify:.....
.....

3.5- What is your usual consumption of the vegetables below?

Please, tick one box in the "frequency of consumption" part to show how often you have each vegetables listed.

For the "amount per day" box, look at the photographs indicated in brackets and write down the number of the photograph which most closely represents the amount you have a day when you eat this food.

An example of how to complete the table is shown on the dark row:

This person eats broccoli once a week and the portion is as big as picture 525.

Food Eaten	Frequency of consumption										Amount per day eaten
	Never eaten	Once per month or less	Once per fortnight	Number of days per week							Describe amount or photograph
				1	2	3	4	5	6	7	
Example: Broccoli				✓							Picture 52 525
Salad											Picture 61
Cooked vegetables											Picture 62
Coleslaw											Picture 58
Green Cabbage											Picture 53
White Cabbage											Picture 54
Cauliflower											Picture 57
Broccoli											Picture 52
Brussels sprouts											
Watercress											
Sauerkraut											
Mustard											
Horseradish											
Meat (chicken turkey, beef, pork, lamb, etc...)											Picture 41
Fish											Picture 42
Eggs											

3.6-Have you any food aversion?

Yes

No

Don't know

If yes specify:

.....

.....

APPENDIX 4b:

Determination of score for allocation of volunteers to block

$$\text{Score} = \frac{\text{Portion size} * \text{Frequency of consumption}}{\text{Body Mass Index}}$$

Portion size varies from 1 to 8 according to the eaten daily amount indicated by volunteer in the questionnaire

Frequency of consumption varies from 1 to 28 time(s)/month

Body Mass Index = Weight (kg)/ Height² (m)

APPENDIX 5:

Sample of ANOVA table for Experiment 3

General Analysis of Variance (with contrasts) adjusted for covariate
 BLOCK volunteer
 TREATMENTS vegetable*reg (meal;2;mat)*day
 COVARIATE myrosinase activity

Variate: log A/B
 Covariate: myrosinase activity

Source of variation	d.f.
volunteer stratum	
vegetable	1
meal.day	4
contrast1.day	2
contrast2.day	2
vegetable.meal.day	4
vegetable.contrast1.day	2
vegetable.contrast2.day	2
Covariate	1
Residual	1
volunteer.*Units* stratum	
meal	2
contrast1	1
contrast2	1
day	2
vegetable.meal	2
vegetable.contrast1	1
vegetable.contrast2	1
vegetable.day	2
meal.day	4
contrast1.day	2
contrast2.day	2
vegetables.meal.day	4
vegetable.contrast1.day	2
vegetable.contrast2.day	2
Covariate	1
Residual	7
 Total	 35

Appendix 6a:
Sample of ANOVA table for Experiment 4

General Analysis of Variance
 BLOCK animal/(day*product)
 TREATMENTS day*site*product
 COVARIATE no covariate
 ANOVA log excretion

Variate: logexcretion

Source of variation	d.f.(m.v.)
animal stratum	
day.site	1
Residual	8
animal.day stratum	
day	1
site	1
Residual	8
animal.product stratum	
product	1
day.product.site	1
Residual	8
animal.day.product stratum	
day.product	1
product.site	1
Residual	8
Total	39(1)

Appendix 6b:

Sample of ANOVA table for Experiment 4

General Analysis of Variance obtained using GENSTAT 5.

BLOCK animal/day/time
TREATMENTS day*site*product
COVARIATE no Covariate
ANOVA log excretion rate

Variate: logexcr

Source of variation	d.f.(m.v.)
animal stratum	
day.site	1
Residual	8
animal.day stratum	
day	1
site	1
Residual	8
animal.day.time stratum	
	39(1)
animal.day.time.*Units* stratum	
product	1
day.product	1
site.product	1
day.site.product	1
Residual	54(2)
Total	116(3)

COMMUNICATIONS

Rouzaud, G., Duncan, A.J., Rabot, S., Ratcliffe, B., Durao, J., Garrido, S. & Young, S. (2000). Factors influencing the release of cancer-protective isothiocyanates in the digestive tract of rats following consumption of glucosinolate-rich brassica vegetables. In *Dietary anticarcinogens and antimutagens - chemical and biological aspects*, pp 92-95 I.T. Johnson and G.R. Fenwick, editors. Cambridge: Royal Society of Chemistry.

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