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Investigation of the fate of dietary flavonols in humans and rats using HPLC-MS\(^2\) techniques

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MSc. (Med. Sci.)

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A thesis submitted to the Faculty of Medicine, University of Glasgow for the degree of Doctor of Philosophy (Ph D)
Abstract

There is a growing evidence of the potential health benefits of a diet rich in fruits and vegetables. National nutritional guidelines advise the consumption of at least five portions (400 g) of these foods per day with the goal being a reduction in the levels of coronary heart disease (CHD) strokes and certain cancers.

The beneficial properties of fruit and vegetables may be ascribed, in part to the presence of antioxidants and recent attention in this regard has focused on phenolic and polyphenolic compounds. These compounds are present in a wide variety of commonly consumed foods and beverages.

Onions are a rich source of the polyphenolic flavonol quercetin-4′-glucoside. For this compound to have some health effects it must be absorbed and reach target organs in a concentration and form where they can exert an effect. To-date interest has focussed on the levels of the intact quercetin aglycone circulating in plasma and excreted in urine. However, it is now known that quercetin does not circulate in the plasma as the parent compound or the aglycone. However, at the outset of this project, the exact form(s) and concentration of metabolites circulating in plasma were unknown.

The need to know what compounds are actually circulating, and at what concentration, is important if in vitro studies are to be made into the mechanisms by which quercetin could, potentially, exert a health benefit.

The reasons why these issues have not been addressed are due to a number of factors. The main methodology used in studies into absorption, distribution, metabolism and excretion or (A.D.M.E) as it is know in the drug industry, is by use of chromatography coupled to various detection systems. This can range from a simple isocratic single pump linked to a single wavelength absorbance detector, to a gradient pumping system with an autoinjector linked in series to a diode array absorbance detector and mass spectrometer. The latter instruments, although initially expensive are now becoming more affordable.

The original methodology used to determine the level of quercetin in plasma involved hydrolysis of the quercetin conjugates back to the aglycone. The information, which is lost by using this hydrolysis method, is vital if we are to gain a better
understanding of the A.D.M.E process. There have been a large number of feeding studies carried out using onions or the flavonol contained in them. However, very little additional information was gained after the initial investigations.

The objectives of the studies presented in this thesis were to develop methodology to identify and quantify the major metabolites of quercetin in man after ingestion of onions. This would initially require the use of radiolabelled [2-$^{14}$C]quercetin-4´-glucoside fed to rats to facilitate the development of the method. Having successfully developed methods that would work both in rats and in man, it was of great interest to establish the fate of the complete dose of [2-$^{14}$C]quercetin-4´-glucoside in rats.

In Chapter 2 radiolabelled quercetin-4´-glucoside was used as a tracer to follow the metabolism of the compound as it was acted on by the digestive system of the rat. After 1 h 93% of the ingested dose was recovered in the gastrointestinal tract (GIT). Analysis using HPLC with a photodiode array (PDA) detector in series with a radioactivity monitor connected to an electrospray ion trap mass spectrometer facilitated the separation, quantification and partial identification of 18 out of 19 metabolites. The 1 h sample was part of a larger study that investigated the fate of the radiolabelled compounds up to 5 h after dosing. The latter samples formed part of another study not reported on in this thesis.

Having developed the methodology, using the radiolabelled compound, it was then applied to a feed of onions to healthy human volunteers to determine if metabolite detection, identification and quantification could be carried out without the use of the radioactive tracer. In Chapter 3 plasma samples collected 1 h after a feed of onions and urine collection from 0-4 h post feed were used to test if the method could be transferred to a non labelled assay. A total of 22 metabolites plus the parent compound were identified. The metabolic profile of the plasma and urine showed marked differences, again pointing to major post absorption metabolism. The successful transfer of the method from the initial radiolabelled study to the onion feed allowed pharmacokinetic data to be obtained from all plasma samples taken over a 24 h period, along with the 0-24 h urine samples. In Chapter 4 it was seen that the metabolites are both rapidly absorbed and excreted, with plasma levels returning almost back to baseline by 6 h.
The total excretion in urine accounted for 4.5% of the ingested dose. These results were controversial, as the pioneer of this field had published that the elimination half life of quercetin was of the order of 18 h. The differences between the two methods employed are discussed in Chapters 3 and 4.

The fact that only 4.5% could be accounted for in this study, which was in agreement with other studies, leaves the question of what happens to the other 95.5%. It is possible that the potential health benefit attributed to this compound may have nothing to do with the parent compound but could be coming from something in the other 95.5%.

Studies using patients who have undergone an ileostomy have been used to provide further information into what happens to the majority of the dose. By collecting the ileal fluid after a flavonol feed the amount of intact compound can be measured in ileal fluid (Hollman et al., 1995b; Walle et al., 2000). This work and some results from a similar trial study are discussed in Chapter 4, with regard to the process of metabolite absorption and formation.

The only way to follow the parent compound throughout its passage through the body is by use of a labelled compound. In Chapter 5 a second feed of [2-¹⁴C]quercetin-4´-glucoside, which focuses on the overall fate of the compound, has samples collected for up to 72 h. The fate of the dose was monitored both in terms of the level of radioactivity excreted and found in the tissues and also the identity of the radioactive compounds detected in these samples.

In Chapter 5 the results from this study and what impact they could have on quercetin’s potential ability to be the compound responsible for the health benefits are discussed.
# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of contents</td>
<td>5</td>
</tr>
<tr>
<td>List of figures</td>
<td>9</td>
</tr>
<tr>
<td>List of Tables</td>
<td>11</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>12</td>
</tr>
<tr>
<td>Author’s Declaration</td>
<td>13</td>
</tr>
<tr>
<td>Chapter 1 Introduction</td>
<td>16</td>
</tr>
<tr>
<td>1.1 Diet and Health</td>
<td>16</td>
</tr>
<tr>
<td>1.2 Oxidative stress &amp; chronic disease</td>
<td>17</td>
</tr>
<tr>
<td>1.3 Classification of Phenolics</td>
<td>18</td>
</tr>
<tr>
<td>1.3.1 Flavonoids</td>
<td>18</td>
</tr>
<tr>
<td>1.3.1.1 Flavonols</td>
<td>22</td>
</tr>
<tr>
<td>1.3.1.2 Anthocyanidins</td>
<td>23</td>
</tr>
<tr>
<td>1.3.1.3 Flavan-3-ols</td>
<td>23</td>
</tr>
<tr>
<td>1.3.1.4 Flavones</td>
<td>23</td>
</tr>
<tr>
<td>1.3.1.5 Flavanones</td>
<td>24</td>
</tr>
<tr>
<td>1.3.1.6 Isoflavones</td>
<td>24</td>
</tr>
<tr>
<td>1.3.1.5 Non-flavonoids</td>
<td>25</td>
</tr>
<tr>
<td>1.3.1.6 Hydroxybenzoates</td>
<td>25</td>
</tr>
<tr>
<td>1.3.1.7 Hydroxycinnamates</td>
<td>25</td>
</tr>
<tr>
<td>1.3.1.8 Stilbenes</td>
<td>25</td>
</tr>
<tr>
<td>1.4 Dietary Sources and Intake of Phenolics</td>
<td>26</td>
</tr>
<tr>
<td>1.5 Analytical measurement of flavonols</td>
<td>30</td>
</tr>
<tr>
<td>1.6 Absorption of polyphenols</td>
<td>34</td>
</tr>
<tr>
<td>1.6.1 Factors affecting absorption</td>
<td>34</td>
</tr>
<tr>
<td>1.6.2 Glycosylation</td>
<td>34</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------</td>
</tr>
<tr>
<td>1.6.3</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>1.6.4</td>
<td>Esterification</td>
</tr>
<tr>
<td>1.6.5</td>
<td>Matrix effects</td>
</tr>
<tr>
<td>1.7</td>
<td>Bioavailability of flavonols</td>
</tr>
<tr>
<td>1.8</td>
<td>Potential bioactivity</td>
</tr>
<tr>
<td>1.9</td>
<td>Pharmacokinetics of flavonols</td>
</tr>
</tbody>
</table>

Chapter 2  Detection and identification of $^{14}$C-labelled flavonol metabolites by HPLC radio-counting and tandem mass spectrometry in rats

2.1  Introduction

2.2  Material and methods

2.2.1  Animals and sample preparation

2.2.2  Measurement of radioactivity

2.2.3  Extraction of samples

2.2.4  HPLC with diode array detection and tandem mass spectrometry

2.3  Results

2.3.1  Distribution of radioactivity in rat tissues and extracts

2.3.2  Identification of metabolites

2.3.3  Amount of radioactivity incorporated into [2-$^{14}$C] quercetin-4’-glucoside metabolites

2.4  Discussion

2.4.1  Detection system

2.5  Conclusion

Chapter 3  Flavonoid metabolites in human plasma and urine after the consumption of red onions: analysis by HPLC with photodiode array and full scan tandem mass spectrometric detection

3.1  Introduction

3.2  Materials and methods

3.2.1  Study design
3.2.2 Materials  
3.2.3 Extraction of onions  
3.2.4 Extraction of plasma  
3.2.5 Extraction of urine  
3.2.6 HPLC with diode array and MS² detection  

3.3 Results  
3.3.1 Analysis of fried onions  
3.3.2 Analysis of plasma and urine  

3.4 Discussion  

3.5 Conclusion  

Chapter 4 Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions  

4.1 Introduction  

4.2 Methods  
4.2.1 Study design  
4.2.2 Materials and Methods  
4.2.3 Pharmacokinetic analysis of plasma metabolites  

4.3 Results  
4.3.1 Analysis of fried onions  
4.3.2 Qualitative analysis of plasma and urine  
4.3.3 Quantitative analysis of flavonol metabolites in plasma  
4.3.4 Quantitative analysis of flavonol metabolite excretion in urine  

4.4 Total  

4.5 Discussion  
4.5.1 Quantitative analysis of flavonol absorption  

4.6 Qualitative analysis of flavonol absorption  

7
Chapter 5  The fate of [2-¹⁴C] quercetin-4′-glucoside in rats by HPLC radio-counting and tandem mass spectrometry

5.1  Introduction

5.2  Materials and methods

5.2.1  Animals and sample preparation

5.2.2  Materials

5.2.3  HPLC with diode array, radioactivity and MS² detection

5.2.4  Measurement of radioactivity in tissues

5.2.5  Extraction of samples

5.3  Results

5.3.1  Distribution of radioactivity in rat tissues and extracts

5.3.2  Distribution of radioactivity in the GI tract

5.3.2.1  Analysis of the 1 h GI tract samples

5.3.2.2  Analysis of the 6 h GI tract samples

5.3.2.3  Analysis of the 12 h GI tract samples

5.3.2.4  Analysis of the 24 h GI tract samples

5.3.3  Urine analysis

5.3.3.1  Analysis of urine by HPLC-RC-MS²

5.3.3.2  Urine flavonol data

5.3.3.3  Urine phenolic acid data

5.3.4  Faecal analysis

5.3.5  Analysis of plasma

5.3.6  Analysis of kidney

5.3.7  Analysis of liver

5.4  Discussion

5.5  Conclusion

Chapter 6  Summary and conclusion
List of figures

Figure 1-1. Generic structures of the major flavonoids. 18
Figure 1-2. Basic structure and biosynthetic origin of the flavonoid skeleton. 19
Figure 1-3. The Shikimatic acid, pheynlpropanoid & flavonoid biosynthetic pathways. 21
Figure 1-4. The flavonols, kaempferol, quercetin, isorhamnetin and myricetin. 22
Figure 1-5. Structure of naturally occurring flavonols and potential metabolites 41
Figure 2-1. Radiochemical purity of [2-14C]quercetin-4'-glucoside with MS2 of parent ion 49
Figure 2-2. Gradient HPLC-RC traces of 30 x 103 DPM aliquots of aqueous and ethyl acetate extracts from intestine, plasma, liver and kidneys of rats 60 min after ingestion the ingestion of [2-14C]quercetin-4'-glucoside. 53
Figure 2-3. Tandem mass spectra of [2-14C]quercetin-4'-glucoside metabolites. Spectrum 1: MS-MS of ion m/z 667, methylquercetin diglucuronide. Spectrum 2:MS-MS of ion m/z 653 ,quercetin diglucuronide. Spectrum 3: MS-MS of ion m/z 571, methylquercetin glucuronide sulphate. Spectrum 4: MS-MS of ion m/z 557, quercetin glucuronide sulphate. Spectrum 5: MS-MS of ion m/z 491, methylquercetin glucuronide. Spectrum 6: MS-MS of ion m/z 477, quercetin glucuronide. 57
Figure 2-4. Metabolite profile of the plasma and tissue extracts of rats obtained 60 min after the ingestion of [2-14C]quercetin-4'-glucoside. 59
Figure 2-5. HPLC trace of 950 dpm of [2-14C]quercetin-4'-glucoside. 66
Figure 2-6. HPLC trace of ethyl acetate extract showing extracted ion chromatograms of molecular ions of quercetin diglucuronide (653), quercetin monoglucuronide (477) quercetin glucuronide 463 and quercetin 301. (B) MS-MS of peak at 30.2 minutes shows it is not quercetin derived. (C) MS-MS of peak at 44.6 which is quercetin. 67
Figure 2-7. Data dependant MS2 preferentially selects m/z 653 in full scan to carry out MS-MS on which shows the presence of quercetin diglucuronide. 69
Figure 3-1. HPLC absorbance trace of red onion extract at (A) 520 nm and (B) 365 nm 75
Figure 3-2. HPLC absorbance trace at 365 nm of (A) plasma and (B) urine after a feed of red onions 77
Figure 3-3. Traces m/z 477 trace from (A) Urine from onion feed, (B) Urine from onion feed plus quercetin-3-glucuronide and (C) Urine from onion feed plus quercetin-7-glucuronide 84
Figure 3-4. MS2 of m/z 477 (A) quercetin-3-glucuronide and (B) unknown in plasma 90
Figure 3-5. Fluorescence analysis used for quercetin (Hollman et al., 1996a) 93
Figure 4-1. concentration of (A) quercetin-3'-sulphate, quercetin-3-glucuronide (B) a quercetin glucuronide sulphate, isorhamnetin-3-glucuronide and a quercetin diglucuronide in plasma from six human volunteers collected 0-6 h after the ingestion of onions. Data expressed as mean values in nmole/L ± standard error (n = 6). 99
Figure 4-2. Pharmacokinetic plot of quercetin levels in plasma before and after 62mg quercetin Hollman et al (1996). Horizontal lines have been added to show the levels of the T=0 samples.

Figure 4-3. Example of pre and post-meal hydrolysed plasma chromatogram of work by Erlund et al (1999). Quercetin peaks are at 7.80 mins

Figure 4-4. Example of pre and post-meal plasma extract by Jin et al (2004) peak 1 is quercetin

Figure 4-5. HPLC chromatograms of extracts of human plasma taken from Moon et al., (2000). (A). extract of plasma before the trial. (B). extract of plasma after the trial. (C). extract of plasma after the trial and after enzyme hydrolysis.

Figure 4-6. Schematic of the proposed metabolic fate of quercetin-4'-glucoside and quercetin-3,4'-diglucoside as they pass from the lumen of the small intestine into the bloodstream via the liver.

Figure 4-7. Schematic of the proposed metabolic fate of quercetin-3-glucuronide and quercetin-3'-sulphate as they are transported from the small intestine to the liver where they are further metabolised before returning to the bloodstream and being excreted in urine via the kidneys.

Figure 4-8. Modified schematic of proposed quercetin glucoside absorption and metabolism seen in Figure 4-6, based on identity of metabolites in ileal fluid.

Figure 5-1. Metabolism cage with faecal collection tray and urine collection beaker.

Figure 5-2. Level of radioactivity found in G.I. tract segment as percentage of the original dose

Figure 5-3. HPLC radioactivity chromatogram of the 1 and 6 h GI tract samples

Figure 5-4. average urinary excretion of rats over 72 h, error bars are ± SD (n=3).

Figure 5-5. HPLC MS –PDA-radioactivity analysis of 6 h rat urine using the RP-Max method. Peak numbers as used in Table 5-2.

Figure 5-6. HPLC radioactivity chromatograms of compounds in urine using the Polar column method. Peak numbers as used in Table 5-4.

Figure 5-7. Cumulative faecal levels of radioactivity from 72 h rats. ± SD (n=3)

Figure 5-8. level of radioactivity in plasma, ± SD (n=3)

Figure 5-9. Conversion of parent compound as it passes through the GI tract of 1 and 6 h rats. Quantities are as percentage radioactivity found in each segment.

Figure 6-1. Factors affecting the probability of an accurate analysis
List of Tables

Table 1-1. Total polyphenol estimates of (Kuhnau, 1976) taken from (Bravo, 1998) 27
Table 1-2. Flavonoid intake in Denmark taken from Justesen et al 29
Table 1-3. Summary of Human Feeding Studies Involving the Acute Ingestion of a Lightly Fried Onion 42
Table 2-1. Distribution of radioactivity in rats 60 min after ingestion of [2-\textsuperscript{14}C]quercetin-4'-glucoside. 52
Table 2-2. Identification of Metabolites of [2-\textsuperscript{14}C]Quercetin-4'-Glucoside Detected in Rats. 56
Table 2-3. Radioactivity Incorporated into Metabolites in Plasma and Tissues After Ingestion of [2-\textsuperscript{14}C]Quercetin-4'-Glucoside by Rats 60
Table 3-1. HPLC-MS-MS identification of anthocyanins in fried red onions. Peak numbers refer to HPLC trace in Figure 3-1 82
Table 3-2. HPLC-MS-MS identification of flavonols in fried red onions. Peak numbers refer to HPLC trace in Figure 3-1 83
Table 3-3. HPLC-MS-MS identification of quercetin metabolites detected in plasma and urine after the consumption of 270 g of fried red onions by six human volunteers. Peak numbers and HPLC retention times refer to HPLC trace in Figures. 3-2A and 3-2B 87
Table 4-1. Pharmacokinetic parameters of quercetin metabolites in the plasma of human volunteers after the consumption of onions. 100
Table 4-2. Concentration of quercetin metabolites in the urine of human subjects 0-24 h after the consumption of an onion supplement. 101
Table 4-3 Mean levels of quercetin metabolites excreted in the urine of six human subjects 0-4, 4-8 and 8-24 h after the consumption of an onion supplement. 106
Table 4-4. Quercetin metabolites detected in plasma and urine after the consumption of 270 g of fried onions by six human volunteers. Estimates of levels in plasma expressed as nmole/L ± S.E. (n = 6) at peak plasma concentration. Amounts in urine expressed as total amount excreted in nmole ± S.E. over a 24 h period post-ingestion of onions 115
Table 5-1. Distribution of radioactivity in samples, data shown as percentage of administered dose. Data in parentheses are percentage of recovered dose 123
Table 5-2. Compounds detected in the 1 and 6 h G.I. tract, data reported as K dpm and, in parentheses as percentage of the given dose. 129
Table 5-3. Flavonol metabolites found in urine and referenced to Table 5-2GI tract metabolites. 132
Table 5-4. Identification and quantification of radioactive compounds excreted in urine from the 72 h rats. Values reported are as a percentage of the dose, total ± SD 133
Table 5-5 Radioactive compounds excreted in faeces as a percentage of the faecal radioactivity ± SD (n=3) 137
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I would like to thank all those in the Graham Kerr building for making us feel so welcome after we moved here due to the fire in the Bower building.

My old Dad for still managing to be here for me finishing this project. I think he is pleased that the son of a bricklayer from a “scheme” in Clydebank ended up with a PhD.

Last but certainly not least to Trish, the best thing that ever happened to me. For all the help and support she gave me over the years. I admit the writing up took longer than it should have but as she offered to do the cooking while I was writing it sort of took the incentive to get finished away.
Author’s Declaration

The composition of this thesis and the work described within it was carried out entirely by myself unless otherwise cited or acknowledged. The research for this thesis was carried out between January 2003 and January 2007.

Signed……………………………………………….

Bill Mullen

September, 2008
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>A.D.M.E.</td>
<td>Absorption, distribution, metabolism and excretion</td>
</tr>
<tr>
<td><em>a.m.u.</em></td>
<td>Atomic mass unit</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
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<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td><em>MW</em></td>
<td>Molecular weight</td>
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<tr>
<td>MS-MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>MS$^2$</td>
<td>The second scan in MS-MS</td>
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<tr>
<td><em>m/z</em></td>
<td>Mass to charge ratio</td>
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<tr>
<td>n.d.</td>
<td>Not detected</td>
</tr>
<tr>
<td>PDA</td>
<td>Photo diode-array</td>
</tr>
<tr>
<td>RC</td>
<td>Radio chromatography</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SIM</td>
<td>Single ion monitoring</td>
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<tr>
<td>SRM</td>
<td>Selected reaction monitoring</td>
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<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td><em>λ</em> <em>max</em></td>
<td>Wavelength of maximum absorption</td>
</tr>
</tbody>
</table>
Aims of Study

At the outset of this project all I wanted to do was to know more about the absorption, disposition, metabolism and excretion (A.D.M.E.) of the flavonol quercetin-4´-glucoside. In a hypothesis driven environment this was not the accepted way to work. However, to form a hypothesis based on existing knowledge, to me, would be guesswork. I wanted to treat the flavonol as if it was a drug and carry out the sort of testing a drug company would put one of its compounds through.

It was evident that without improvements in the analytical methodology used in A.D.M.E. studies there would be little chance of obtaining the information needed to ascertain if flavonols could have a health benefit.

Therefore I set out the following aims:

1. To develop methods based on HPLC linked to a radioactivity detector and an ion trap mass spectrometer could identify and quantify the radiolabelled flavonol [2-\(^{14}\)C]quercetin-4´-glucoside and its metabolites in rats.

2. To develop, based on the rat work, methods using HPLC linked to diode array absorbance detection and ion trap mass spectrometry to positively identify and quantify onion derived flavonols and their metabolites in man.

3. To quantify flavonols and their metabolites and determine their pharmacokinetic profile in human plasma and urine following a meal of lightly fried onions.

4. To determine the fate (A.D.M.E.) of the total dose of [2-\(^{14}\)C] quercetin-4´-glucoside fed to rats housed in metabolism cages over a time course of 1 to 72 hours.
Chapter 1  Introduction

1.1  Diet and Health

A diet rich in fruits and vegetables has long been recognised to protect against degenerative diseases like coronary heart disease (CHD) and stroke and certain cancers (WHO, 2003). The current dietary guidelines in the UK recommend a daily intake of at least 5 portions (each weighing 80 g). Although the protection afforded by fruits and vegetables can be partly explained by associated lifestyle factors including abstinence from smoking and increased physical activity (Lampe, 1999), specific bioactive dietary constituents are considered to be important to health. These include the known nutrients and also phytochemicals such as flavonoids.

Flavonoids are made up of phenolic and polyphenolic compounds, they are secondary metabolites widespread in the plant kingdom and form an integral part of the human diet with fruits, vegetables, tea and red wine providing an especially abundant source. During the past decade, interest has arisen in these compounds, as there is some evidence to suggest that an increased consumption of phenolic rich foods/beverages may help prevent disease. Polyphenols are reducing agents and their potential health-related properties have been ascribed to their powerful antioxidant abilities, which may protect the body from damaging oxidation reactions, caused by ‘free radicals’ (Kanner et al., 1994).

Dutch investigators measured the levels of the flavonols quercetin, kaempferol and myricetin and the flavones apigenin and luteolin in a number of drinks, fruits and vegetables (Hertog et al., 1992a; Hertog et al., 1993c). This data was used to assess the flavonoid intake of a group of elderly men over a five year period. The flavonoid intake (measured in tertiles) was significantly inversely associated with mortality from coronary heart disease (Hertog et al., 1993a). Onions were one of three foods also associated with the reduction in CHD and strokes in the Zutphen elderly study. The common antioxidant/phytochemical link between the three foods cited, apples and tea being the other two, was the flavonol quercetin. The aglycone quercetin occurs only
rarely in nature and is normally found conjugated to various sugars, most commonly glucose, as is the case for the three foods mentioned here. Although the Zutphen elderly study showed a clear decrease in CHD and strokes, associated with an increase in the consumption of the three aforementioned foods, how this health benefit comes about is unclear. In fact it is a large leap of faith to jump from onions, tea and apples being associated with a health benefit to it then being attributed to a particular compound. Furthermore, the data upon which this epidemiological study was based were measurements of the aglycone of the flavonols in the food and drinks. The need to know the sugar conjugate of the flavonol is of great importance as this can have a major effect on absorption depending on whether hydrolysis occurs in the small intestine to facilitate absorption.

1.2 Oxidative stress & chronic disease

A wide range of chronic diseases have oxidation events as a major component of their pathophysiology and uncontrolled oxidative damage has been implicated in a number of degenerative diseases including cancer, atherosclerosis and chronic inflammatory diseases (Beckman & Ames, 1998a). Harman first proposed the ‘free radical theory of aging’, which stated that free radicals produced during metabolism and other biological reactions cause cumulative cell damage leading to ageing and eventual death (Harman, 1956). This theory has since been transformed into a more general premise which highlights that an over-production of reactive oxygen species (ROS) during normal metabolic processes, or a loss of the protective mechanisms that reduce the ability to withstand oxidative challenge is intricately connected to ageing and lifespan (Beckman & Ames, 1998b).

The association between increased intake of foods containing antioxidants and a reduction in degenerative diseases would seem obvious. But is it the antioxidant property of these compounds that bring about this association or is it some other property? Are all these compounds equally beneficial for health or is it specific compounds for specific diseases? Unless we can find out more about what happens to these compounds in vivo we may never know the answers to these questions.
1.3 Classification of Phenolics

Phenolic compounds are secondary plant metabolites, characterized by having at least one aromatic ring with one or more hydroxyl groups attached. In excess of 8000 phenolic structures have been reported, and they are widely dispersed throughout the plant kingdom (Strack & Wray, 1992). They are synthesized via the shikimate and phenylpropanoid pathways. Phenolics range from simple, low molecular weight, single aromatic-ringed compounds, like gallic acid to large and complex tannins, such as the polymeric proanthocyanidins (Lairon & Amiot, 1999). They can be classified by the number and arrangement of their carbon atoms and are commonly found conjugated to sugars and organic acids. Phenolics can be classified into two groups, the flavonoids and the non-flavonoids.

1.3.1 Flavonoids

![Diagram of flavonoids]

Figure 1-1. Generic structures of the major flavonoids.
Flavonoids are polyphenolic compounds comprising 15 carbons, with two aromatic rings connected by a three-carbon bridge (Figure 1-1). They are the most numerous of the phenolics and are found throughout the plant kingdom. They are concentrated mainly in the epidermis of leaves and the skin of fruits, and they have many important and varied roles as secondary plant metabolites. Their location in plants associates well with their functions, which include UV protection, pigmentation, stimulation of nitrogen-fixing nodules and disease resistance (Koes et al., 1994; Pierpoint, 2000).

The C6-C3-C6 flavonoid structure is the product of two separate biosynthetic pathways (Figure 1-2). The bridge and one aromatic ring, ring B, constitute a phenylpropanoid unit synthesized from phenylalanine, which itself is a product of the shikimic acid pathway. The six carbons of ring A originate from the condensation of three acetate units via the malonic acid pathway. The fusion of these two parts involves the stepwise condensation of a phenylpropanoid, 4-coumaryl CoA, with three malonyl-CoA residues, each of which donates two carbon atoms, in a reaction catalysed by chalcone synthase (Figure 1-2). Tetrahydroxychalcone, the product of this reaction, gives rise to all the other types of flavonoids via the flavonoid biosynthetic pathway (Figure 1-3).

![Figure 1-2. Basic structure and biosynthetic origin of the flavonoid skeleton.](image-url)
The main sub-classes of flavonoids are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins (Figure 1-1). The basic flavonoid skeleton can have numerous substituents. Hydroxyl groups are usually present at the C-4’, 5 and 7 positions. Sugars are very common with the majority of flavonoids existing naturally as glycosides. Whereas both sugars and hydroxyl groups increase the water solubility of flavonoids, other substituents, such as methyl ethers and isopentyl units give flavonoids lipophilic properties.
Figure 1-3. The Shikimatic acid, pheynlpropanoid & flavonoid biosynthetic pathways.

Enzyme abbreviations: PAL-phenylalanine ammonia-lyase; C4H-cinnamate 4-hydroxylase; 4C3H-4-coumarate 3-hydroxylase; CMT-caffeate methyl transferase; 4CL-4-coumarate: CoA ligase; SS-stilbene synthase; CHS-chalcone synthase; CHI-chalcone isomerase; IFS-2-hydroxyisoflavone synthase; IFD-2-hydroxyisoflavanone dehydratase; FNS-flavone synthase; FHT-flavanone 3-hydroxylase; FLS-flavonol synthase; DFR-dihydroflavonol 4-reductase; ANS-anthocyanidin 4-reductase; LAR-leucoanthocyanidin 4-reductase.
1.3.1.1 Flavonols

Flavonols are arguably the most widespread of the flavonoids, being dispersed throughout the plant kingdom with the exception of fungi and algae. Flavonol distribution and the wide structural variations have been comprehensively described (Williams, 1992; Wollenweber, 1992). Within the plant, flavonols such as myricetin, quercetin, isorhamnetin and kaempferol (Figure 1-4) are most commonly found as O-glycosides. Conjugation occurs most frequently at the 3 position of the C ring but 5, 7, 4', 3' and 5' substitutions also occur (Herrmann, 1976). Although the number of aglycones are limited there are numerous flavonol conjugates with more than 200 different sugar conjugates of kaempferol alone (Strack & Wray, 1992). There is information on the levels of flavonols found in commonly consumed fruits, vegetables and beverages (Hertog et al., 1992a; 1993c). However, large differences are often found in the amounts present in seemingly similar produce, possibly due to seasonal variation and varietal differences (Crozier et al., 1997). In addition this information is based on the level of the aglycone present and not on the conjugate, which can have a major effect on the pharmacokinetics and bioavailability of the compound (Hollman et al., 1999; Erlund et al., 2000e).

Figure 1-4. The flavonols, kaempferol, quercetin, isorhamnetin and myricetin.
1.3.1.2 Anthocyanidins

Anthocyanidins, principally as their conjugated derivatives, the anthocyanins, are a group of water-soluble compounds responsible for most floral, fruit and leaf pigmentation in nature (Clifford, 2000). The most common anthocyanidins are cyanidin, found in raspberries (Mullen et al., 2002c), delphinidin in blackcurrants (Rechner et al., 2002), malvidin in red wine (Burns et al., 2002a) and pelargonidin found in strawberries (Mullen et al., 2008b). Two other abundant anthocyanidins are petunidin and peonidin, both found in red wine (Burns et al., 2002a). Naturally occurring anthocyanins are normally found as 3-O-glycosides. Structurally anthocyanins consist of an anthocyanidin bound to one or more sugar moieties, and with a sugar always present at the C3 position and frequently on carbons 7, 3' and 5'. They can also form conjugates with hydroxycinnamates and organic acids such as malic acid and acetic acid, which are both found in red wine (Burns et al., 2002a). Anthocyanins are responsible for the red, blue or violet colour of edible fruits including grapes, plums and berries, with levels increasing during fruit maturation (Peterson & Dwyer, 1998)

1.3.1.3 Flavan-3-ols

Flavan-3-ols range from the simple monomers (+)-catechin and its isomer (-)-epicatechin, to the oligomeric and polymeric procyanidins, more correctly called proanthocyanidins. In addition to forming complexes with other flavan-3-ols, they are hydroxylated to form the gallo catechins, and also undergo esterification with gallic acid. Furthermore, methylation, prenylation and O-glycosylation reactions have all been reported (Porter, 1992). (+)-Catechin and (-)-epicatechin are found in various fruits and vegetables such as apples, pears, grapes and peaches (Arts et al., 2000a), with the highest concentrations of catechins found in tea and red wine (Arts et al., 2000b). Flavan-3-ols are the only flavonoids found in abundance in foods that are not naturally conjugated to sugars.

1.3.1.4 Flavones
Flavones are structurally similar to the flavonols, however they lack oxygenation at position C3. A variety of substitutions are possible providing a wide array of natural and synthetic compounds; these include hydroxylation, methylation, O- and C-alkylation and O and C-glycosylation. Most flavones occur as 7-O-glycosides (Bohm, 1998). Flavone distribution appears to be limited to only a few plant families, with the main flavones in the diet being apigenin and luteolin. They have been identified in celery (apigenin), sweet red pepper (luteolin), parsley and other herbs (Hertog et al., 1992a).

1.3.1.5 Flavanones

Flavanones are mainly represented by taxifolin, naringenin and hesperetin. Flavanones are characterized by the absence of the C2-C3 double bond and the presence of a chiral center at C2. The flavanone structure is highly reactive and they have been reported to undergo hydroxylation, glycosylation and O-methylation reactions. The main dietary source of flavanones is citrus fruit and the most commonly consumed is hesperetin from oranges (Rouseff et al., 1987) in the form of hesperetin-7-rutinoside (hesperidin) and naringenin-7-rutinoside (narirutin). Naringenin is also found in tomatoes and tomato-based products. Fresh tomatoes, especially tomato skin, also contain naringenin chalcone, which is converted to naringenin during processing to tomato ketchup (Krause & Galensa, 1992).

1.3.1.6 Isoflavones

Isoflavonoids are characterised by having the B ring attached at the C3 of the phenylchromane structure. Isoflavonoids are derived from the biosynthetic pathway and can be converted into a wide range of different isoflavonoids including isoflavanones and isoflavonols (Dewick, 1993). Isoflavones are mainly represented by daidzein and genistein. The main dietary source is soybeans and soy products, with soy typically containing around 1 g of genistein and daidzein per kg of wet weight (Mazur et al., 1998; Liggins et al., 2000). These compounds have received much attention due estrogenic activity and to their putative role in the prevention of breast cancer and osteoporosis (Tapiero et al., 2002), which has not been linked to any antioxidant effect.
1.3.1.5 *Non-flavonoids*

The main non-flavonoid phenolics found in the diet are the C₆-C₁ hydroxybenzoates, C₆-C₃ hydroxycinnamates and polyphenolic C₆-C₂-C₆ stilbenes. Phenolic acids exist primarily as conjugates and are rarely found in their acidic forms, often found bound to alcohols, sugars, polysaccharides, or organic acids through ester bonds.

1.3.1.6 *Hydroxybenzoates*

Hydroxybenzoates also referred to as phenolic acids include benzoic acid and derivatives including salicylic acid. Gallic acid is the major hydroxybenzoate and is synthesised from phenylalanine via 3-dehydroshikimic acid. It is found in large quantities in red wine. It can be converted to ellagic acid and a range of gallotannins, with the formation of hydrolysable tannins (polymers of gallic and ellagic acids). Black tea and red wine provide rich dietary sources of gallic acid (Soleas & Goldberg, 1999; Dunfrense & Fransworth, 2001). Fruits such as raspberries contain high concentrations of Sanguuin H6 and Lambertianin C, large hydrolysable tannins with molecular weight 1870 and 2804 respectively (Mullen *et al.*, 2002d; 2003b).

1.3.1.7 *Hydroxycinnamates*

Hydroxycinnamic acids: *p*-coumaric, ferulic, sinapic, caffeic acids and their derivatives are the most important subclass of phenolic acids. Cinnamic acid is produced by the deamination of the amino acid phenylalanine by phenylalanine ammonia lyase (PAL), with *p*-coumaric acid being produced by hydroxylation of cinnamic acid. In addition to being found in their free form hydroxycinnamates are also found esterified to sugars, organic acids and choline (Strack, 1997). Caffeic acid occurs in foods mainly as an ester with quinic acid, chlorogenic acid (5-caffeoylquinic acid). Coffee is a major dietary source of chlorogenic acid in the diet with dietary intakes estimated at 0.5-1 g/day (Clifford, 2004).

1.3.1.8 *Stilbenes*
The stilbene family has a C₆-C₂-C₆ structure and are known to be phytoalexins, a class of antibiotic compounds produced as a part of a plant's defense system against disease. Trans-resveratrol (trans-3,5,4'-trihydroxy stilbene) is synthesised by condensation of 4-coumaroyl CoA with three units of malonyl CoA, each of which donates two carbon atoms, in a reaction catalysed by stilbene synthase. Resveratrol exists as two isomers; cis and trans with trans-resveratrol-3-O-glucoside commonly found in various families of plant tissues such as eucalyptus, spruce, and lily. Grapes, peanuts and their products are considered the most important dietary sources of the resveratrol, with levels of 0.02-1.8 µg/g and 0.6-8 µg/mL reported in peanuts and red wine (Sanders et al., 2000). However, should resveratrol prove to have health benefits the best source would be from the Japanese knotweed Polygonum cuspidatum (Burns et al., 2002b)

1.4 Dietary Sources and Intake of Phenolics

The ubiquitous nature of phenolic compounds in plants ensures that they are found in significant levels in the diet. However, the information available on the levels of phenolics in foodstuff is somewhat mixed. Unlike well-known dietary components, such as vitamin C and E, comprehensive food tables are not available for phenolic compounds. As these compounds, unlike vitamins and minerals, are not essential components of our diet, there are no dietary reference values.

Kuhnau, (1976) used the Folin Ciocalteau method (Singleton & Rossi, 1965) to estimate the total polyphenols in a wide variety of foods consumed in the USA (Table 1-1). Based on these results and the daily food intake tables in the USA a figure of 1.0 to 1.1 g/day of polyphenols was calculated. Hertog et al., (1992b) used a chromatographic method with absorption detection to estimate the aglycone content of flavonols and flavones of 28 vegetables and 9 fruits commonly consumed in the Netherlands. Justesen et al., (1997c) also used this method to estimate the average daily intake of flavonols and flavones in the Danish diet. The figures arrived at by these two groups were 23 and 28 mg/day, well short of Kuhnau’s estimate.

Two reasons for the discrepancy are possible. In any analysis the more selective and specific the measurement is the less chance there is of interference and thereby overestimates being made. Therefore the chromatographic system of the Dutch and
Danish groups would be expected to give a lower result. In addition polymeric procyanidins, which do not chromatograph, anthocyanins and isoflavones would also go undetected thereby giving an actual underestimate.

**Table 1.1. Total polyphenol estimates of (Kuhnau, 1976) taken from (Bravo, 1998)**

<table>
<thead>
<tr>
<th>Food/Beverage*</th>
<th>Total Polyphenols</th>
<th>Food/Beverage*</th>
<th>Total Polyphenols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereals (mg/100 g dm)</td>
<td></td>
<td>Fruits (mg/100 g fm)</td>
<td></td>
</tr>
<tr>
<td>Barley</td>
<td>1200–1500</td>
<td>Blackcurrant</td>
<td>140–1200</td>
</tr>
<tr>
<td>Corn</td>
<td>30.9</td>
<td>Blueberry</td>
<td>135–280</td>
</tr>
<tr>
<td>Millet</td>
<td>590–1060</td>
<td>Cherry</td>
<td>60–90</td>
</tr>
<tr>
<td>Oats</td>
<td>8.7</td>
<td>Cowberry</td>
<td>128</td>
</tr>
<tr>
<td>Rice</td>
<td>8.6</td>
<td>Cranberry</td>
<td>77–247</td>
</tr>
<tr>
<td>Sorghum</td>
<td>170–10,260</td>
<td>Gooseberry</td>
<td>22–75</td>
</tr>
<tr>
<td>Wheat</td>
<td>22–40</td>
<td>Grape</td>
<td>50–490</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grapefruit</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Orange</td>
<td>50–100</td>
</tr>
<tr>
<td>Legumes (mg/100 g dm)</td>
<td></td>
<td>Peach</td>
<td>10–130</td>
</tr>
<tr>
<td>Black grain</td>
<td>540–1200</td>
<td>Pear</td>
<td>2–25</td>
</tr>
<tr>
<td>Chickpeas</td>
<td>75–230</td>
<td>Plum</td>
<td>4–225</td>
</tr>
<tr>
<td>Cowpeas</td>
<td>175–590</td>
<td>Raspberry</td>
<td>37–429</td>
</tr>
<tr>
<td>Common beans</td>
<td>34–280</td>
<td>Red current</td>
<td>17–20</td>
</tr>
<tr>
<td>Green gram</td>
<td>440–800</td>
<td>Strawberry</td>
<td>38–218</td>
</tr>
<tr>
<td>Pigeon peas</td>
<td>380–1710</td>
<td>Tomato</td>
<td>85–130</td>
</tr>
<tr>
<td>Nuts (% dm)</td>
<td></td>
<td>Fruit juices (mg/L)</td>
<td></td>
</tr>
<tr>
<td>Betel nuts</td>
<td>26–33</td>
<td>Apple juice</td>
<td>2–16</td>
</tr>
<tr>
<td>Cashew nuts</td>
<td>33.7</td>
<td>Orange juice*</td>
<td>370–7100</td>
</tr>
<tr>
<td>Peanuts</td>
<td>0.04</td>
<td></td>
<td>660–1000</td>
</tr>
<tr>
<td>Pecan nuts</td>
<td>8–14</td>
<td>Beverages</td>
<td></td>
</tr>
<tr>
<td>Vegetables (mg/100 g fm)</td>
<td></td>
<td>Tea leaves (% dm)</td>
<td></td>
</tr>
<tr>
<td>Brussels sprouts</td>
<td>6–15</td>
<td>Green</td>
<td>20–35</td>
</tr>
<tr>
<td>Cabbage</td>
<td>75</td>
<td>Black</td>
<td>22–33</td>
</tr>
<tr>
<td>Leek</td>
<td>20–40</td>
<td>Tea, cup (mg/200 mL)</td>
<td>50–210</td>
</tr>
<tr>
<td>Onion</td>
<td>100–2025</td>
<td>Coffee beans (% dm)</td>
<td>0.2–10</td>
</tr>
<tr>
<td>Parsley</td>
<td>55–180</td>
<td>Coffee, cup (mg/150 mL)</td>
<td>200–550</td>
</tr>
<tr>
<td>Celery</td>
<td>94</td>
<td>Cacao beans (% dm)</td>
<td>12–18</td>
</tr>
<tr>
<td>Fruits (mg/100 g fm)</td>
<td></td>
<td>Wine (mg/L)</td>
<td></td>
</tr>
<tr>
<td>Apple</td>
<td>27–298</td>
<td>White</td>
<td>200–300</td>
</tr>
<tr>
<td>Apricot</td>
<td>30–43</td>
<td>Red</td>
<td>1000–6000(6500)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Beer (mg/L)</td>
<td>60–100</td>
</tr>
</tbody>
</table>

The method used by Kuhnau is based on the reduction of the Folin reagent causing a colour change. Reducing agents like vitamin C are present in many foodstuffs and this can have a marked and varied effect on the estimates.

Other factors that can contribute to inaccuracy and variation in estimates of dietary intake of phenolics lie in the nature of the foods analysed, as well as the methods used. Seasonal variations can dramatically influence the phenolic contents of fruits and vegetables, as can varietal differences (Crozier et al., 1997; 2000). In the case of onions, (Kuhnau, 1976) the free polyphenol content ranged from 100-2025 mg/100g fresh weight. Whereas varietal variation, white or yellow onions, produced a range from 185 to 650 µmole/g fresh weight.
The estimation of consumption by (Hertog et al., 1993a) applies not to flavonoids but is restricted to only three flavonols and two flavones, and the calculation is based on aglycones rather than conjugates. It is, therefore, almost certainly a serious under estimate of flavonoid ingestion, as would be the Danish intake, calculated using the same method for the same compounds (Justesen et al., 1997a). At the other end of the scale Kuhnau, (1976) estimated the intake in the USA as being over 1000 mg per day for the total polyphenol intake. This figure is based on all phenols being glycosides/conjugates and, due to the previously discussed factors, is likely to be an over estimate of flavonoid consumption.

Berries, along with other fruits can contribute significantly to the dietary consumption of phenolics by their anthocyanin content. A regular daily consumption can readily increase the intake of a variety of phenolics. In addition people who consume large quantities of tea or coffee will also have much higher intake of flavonoids and related compounds. Two of the three aforementioned phenolics were not taken into account in the Dutch and Danish studies. In fact if the dietary sources are examined in the Danish study it is clear that only 6 foods contribute 98% of the estimated phenolic intake (Table 1-2), an unlikely supposition.
In summary, the estimate of the daily dietary intake of polyphenols depends on a number of factors.

1. Which analytical method has been used to obtain data to calculate the content in the foodstuffs.

2. The content of the food can vary from year to year due to climatic factors, over and above seasonal variation.

3. The variety of food can also have a marked influence on the content.

4. The daily dietary intake of these foods is taken from food consumption tables and or diary record.

The problem of estimating the daily intake of polyphenols is only one part of the puzzle. There are also problems with these estimates as flavonoid absorption is dependant on many other factors, which will be discussed in Section 1.6. However, one of the biggest issues in coming to an understanding of polyphenols and health is being able to make accurate measurements of the compounds of interest.

One point that should not be overlooked when examining the accuracy of either the dietary intake tables or the flavonoid content of the food is that this data cannot prove that any particular food does have a health benefit. All they can do is show an

### Table 1-2. Flavonoid intake in Denmark taken from Justesen et al

<table>
<thead>
<tr>
<th>Food</th>
<th>Consumption g/day</th>
<th>Contribution %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td>48</td>
<td>4</td>
</tr>
<tr>
<td>Onion</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Orange</td>
<td>16(^1)</td>
<td>30</td>
</tr>
<tr>
<td>Orange juice</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>Red Wine</td>
<td>67</td>
<td>5</td>
</tr>
<tr>
<td>Tea</td>
<td>200(^2)</td>
<td>29</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>-</td>
<td>4</td>
</tr>
</tbody>
</table>
association with improved health, especially in epidemiological studies. No claim can be made that they are the responsible for it. If we want to be able to prove a compound is responsible for a health effect, more must be known about what happens to it when it is absorbed, what it is metabolised to, what concentration it is in the body at, what tissues it accumulates in and in what form it is excreted.

1.5 Analytical measurement of flavonols

“I often say that when you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind” A quotation from Lord Kelvin.

Measuring the level of quercetin-based compounds in, for example, onions is not a simple task, as outlined in previous section. The most common method used involves a number of processes. Firstlly, extraction using an organic solvent, typically methanol, followed by concentration of the extract and, if deemed necessary purification. An aliquot of the concentrated extract can then be processed by HPLC, to separate it into its individual components. These compounds on elution from the HPLC column pass through various detectors, which can selectively measure the analytes of interest.

The four processes, extraction, purification, separation and detection all have an influence on the outcome of the result. Failure to give sufficient attention to any one of these processes can lead to inaccurate results. This topic will be expanded upon throughout the thesis.

In chromatographic analysis the identity of a compound can be determined only if there is an authentic standard with which to compare it. In chromatography, retention time is a major identifying property of a compound. (i.e. how long it takes a compound to pass through the chromatographic system and produce a peak in the detection system). If a peak in an extract has the same retention time as that of a previously run standard, it can be argued, with some justification, that they are the same compound. Co-chromatography of the standard and the extract can further strengthen the claim.
However, if the extract contains a multitude of compounds there is a high probability that another compound could, by chance, co-elute with the compound of interest.

Although there are only a few flavonol aglycones the number of conjugates of kaempferol is in excess of 200, and a similar number or more, due to the additional hydroxyl group, must be expected for quercetin. This leaves the researcher with a difficult problem. There are no standards available for the majority of these compounds. Therefore, how do they identify and measure the levels present in foods?

One method that has been employed is to use acid hydrolysis to cleave the sugar moiety leaving only the aglycone, which can be readily quantified against a standard obtained from commercial suppliers. This has enabled quasi-quantitative estimates of flavonol content to be obtained for a wide variety of fruit, vegetables and beverages (Hertog et al., 1992a). Such databases, as seen in Table 1-2, have been used in several epidemiological studies to estimate the flavonol intake by different populations. However, while potentially useful in this context, this simplified method of flavonol analysis has limitations.

Studies have shown that, for the same dose, five times more quercetin-4'-glucoside is absorbed than quercetin-3-rutinoside (Hollman et al., 1999). These two compounds make up a major part of quercetin intake in the diet of the western world. Furthermore, the site of absorption would also seem to differ as the time to reach peak plasma concentration is significantly longer for the rutinoside conjugate (Hollman et al., 1999). The glucoside is absorbed in the small intestine, whereas the rutinoside is from the large intestine (Hollman et al., 1995b). What impact this has on the levels and forms of metabolites and catabolites has only recently been ascertained (Jaganath et al., 2006). Such differences could have a major influence on the potential health effects of these flavonols.

Measuring the levels of flavonol metabolites in biological samples after a feed of flavonol glucosides is even more problematic than when measuring the flavonol content of fruit and vegetables. Even the use of authentic standards as a means of identification of metabolites has been called into question. For example, (Aziz et al., 1998) identified quercetin-4'-glucoside in human plasma after a meal of lightly fried onions using HPLC with co-chromatography of a standard. However, it was thought
that quercetin-4'-glucuronide would have very similar chromatographic property to the glucoside and it therefore could in fact be this compound that was being detected in plasma. At the time the analysis was being carried out no standards of quercetin glucuronides were available to test this hypothesis and no chromatographic detection system could uniquely identify them.

Returning to the theme of four processes that affect the probability of a successful outcome of an analysis. With hindsight, the study by Aziz et al., (1998) could have increased the separating powers of the analysis to try and minimise the chance of two compounds co-eluting. However, the gradient reverse phase analysis could separate a far greater number of compounds than the isocratic systems used to analyse the aglycone, though no one so far has suggested that there may be compounds co-eluting with the quercetin peak. Only once standards of the glucuronides or a detection system that could differentiate between the two compounds became available would the problem encountered by Aziz et al., (1998) have become apparent.

With the use of the limited number of standards being called into question, hydrolysis to the aglycone became the standard method of analysis. However, this simplification also leaves many questions unanswered. Does the type of metabolite formed vary from different foods and are certain metabolites more bioactive than others? Moreover, it is still possible to have major errors using this method if insufficient care has been taken with the four processes needed to ensure that the aglycone is free from potential interfering compounds.

Recent advances in analytical instrumentation, in particular tandem mass spectrometers, now allow structural information to be obtained for individual HPLC peaks, so it is possible for instance, to distinguish between quercetin glucuronides and quercetin glucosides without the need for reference compounds. Even if these two compounds co-elute in the chromatographic system they have different molecular weights and therefore would be seen as individual peaks in the mass spectrometer. The selectivity of MS-MS systems is such that two potential metabolites with the same molecular weight, as is the case of methyl quercetin glucoside and quercetin glucuronide, can be detected in the same peak due to their MS² spectra. However, this increase in selectivity comes with a price. In full scan mode the ability to accurately quantify is lost.
Wittig et al., (2001) used a mass spectrometer in SRM mode, this specifically looks for predetermined parent ions fragmenting to known daughter ions, to detected five quercetin monoglucuronides in human plasma after ingestion of 800 g of onions. This would indicate all free hydroxyl groups have been conjugated with a glucuronic acid. Though some doubt has been cast on this possibility by Day et al., (2000), who showed that the C-5 position was not a site for conjugation by human liver extracts. A possible explanation for this result shows one of the limitations of this technique. The mass spectrometer was set up to monitor the fragmentation of the [M+H]$^+$ parent ion at $m/z$ 479 (quercetin glucuronide) to a daughter ion at $m/z$ 303 (quercetin). However, partial fragmentation of a quercetin diglucuronide will produce an ion at $m/z$ 479 which would also then yield an ion at $m/z$ 303. Thus, a quercetin diglucuronide could give a false positive response for a quercetin monoglucuronide. In fact any compound that produces a transition from $m/z$ 479 to $m/z$ 303 will give a positive response in this analysis. This, and other potential sources of misidentification are discussed in detail in Chapter 3.

In a later study by this group (Graefe et al. 2001) no sulphate metabolites were found in plasma after a supplementation with onions. This is in keeping with work reported by Watson & Oliveira, (1999) using a GC-MS method. By chemically synthesising quercetin glucuronides and use of fast atom bombardment (FAB)-MS Moon et al., (2001) were able to identify quercetin-3-glucuronide (Figure 1-5) as being a major metabolite circulating in rat plasma. In another human study twelve metabolites of quercetin were detected in pooled plasma samples after an onion feed (Day et al., 2001). Contrary to the work of Graefe et al., (2001) and Watson & Oliveira, (1999), one of the main metabolites was quercetin-3'-sulphate (Figure 1-5). Identification was achieved by a combination of co-chromatography with standards, enzyme hydrolysis procedures and HPLC-MS in the SIM mode. This facilitated the identification four of the twelve quercetin metabolites, namely a quercetin-diglucuronide, quercetin-3'-glucuronide, 3'-methylquercetin-3'-glucuronide and quercetin-3'-sulphate (Figure 1-5). No quantitative data came from these studies.

The need to have analytical methods to allow identification of the actual metabolites and accurately measure their levels is paramount to being able to understand
the process of absorption, disposition, metabolism and excretion of the compound under investigation.

This topic is of importance and it will be revisited all the way through this thesis.

To take Lord Kelvin’s quote “Only when we can measure it can we say something about it”.

1.6 Absorption of polyphenols

Polyphenols in general and flavonols in particular are poorly absorbed. Measured by urinary excretion in humans Aziz et al., (1998) reported less than 1% recovery of the flavonol dose from onions. If flavonols are to have an impact on health then more must be known about what happens to the absorbed dose.

1.6.1 Factors affecting absorption

The rate and extent of intestinal absorption and metabolism of polyphenols are influenced by a number of factors including their chemical structure, molecular weight, solubility, glycosylation and esterification.

1.6.2 Glycosylation

Most polyphenols (flavonols, isoflavones, flavones, anthocyanins) are found in plants in a glycosylated form and this influences their chemical, physical and biological properties. The sugar moiety makes them much more hydrophilic, thus reducing their ability to passively diffuse across a biological membrane. Therefore, if absorption is to occur in the small intestine either an active transport mechanism must operate or the compound must undergo deglycosylation to allow passive absorption. In the case of flavonols the most noticeable difference is in the case of quercetin-3-rutinoside and quercetin-3-glucoside (Hollman et al., 1999). This is due to the enzymes, lactase phloridzin hydralase (LPH) and or cytosolic β glucosidase (CβG), in the small intestine being unable to hydrolyse the rutinoside sugar from the flavonol. The estimate of flavonoid content in foods being reported as aglycone levels gives no information as to
the type of sugar conjugation. When it is considered that quercetin glucosides from onions are absorbed 5 times more than quercetin-3-rutioside (Hollman *et al.*, 1999) the need for food intake tables that contain information on the sugar conjugate can be appreciated.

### 1.6.3 Molecular weight

High molecular weight (*MW*) compounds including tea theaflavins (*MW* ≥ 568) and proanthocyanidins (*MW* ≥ 578) are unlikely to be absorbed in the GI tract (Donovan *et al.*, 2002). However, the procyanidin dimer B$_2$ has been detected in human plasma following consumption of a flavan-3-ol rich cocoa (Holt *et al.*, 2002). Procyanidin dimers and trimers were also found in rat urine after a feed of grape seed extract (Tsang *et al.*, 2005). The absorption of the ellagitanin punicalagin has also been reported by (Seeram *et al.*, 2006). As the *T$_{max}$* of the ellagic acid metabolite was under 1 h, it can be assumed that initial absorption must have occurred in the stomach and small intestine. However, ellagic acid and urolithin metabolites have of a much lower molecular weight suggesting the punicalagin had been broken down prior to absorption.

### 1.6.4 Esterification

Hydroxycinnamates, such as caffeic acid, are commonly esterified to sugars, organic acids and lipids. Caffeic acid is better absorbed than chlorogenic acid, which is made from an esterification of caffeic acid with quinic acid. Olthof *et al* (2003) reported 95 % intestinal absorption for caffeic acid compared with only 35 % for chlorogenic acid in ileostomy subjects. In an *in vitro* study using an isolated rat intestine absorption of caffeic acid was approximately ten times that of chlorogenic acid (0.115 nmole) (Spencer *et al.*, 1999).

### 1.6.5 Matrix effects

Some studies into the effect of consumption of a flavonol in the presence of another food substance have been investigated. Hollman *et al.*, (1997a) investigated the influence of milk on flavonol absorption with tea and found it had no effect. In a study on what influence milk had on the absorption of flavn-3-ols from milk and dark
chocolate Serafini et al., (2003) found that there was a reduction due to the milk. This paper was quickly criticized by a number of scientists (Halliwell, 2003; Schroeter et al., 2003) which was then replied to in an exchange in the journal Nature (Serafini & Crozier, 2003). The possible conflicting results may be due to the levels of flavan-3-ols in the dose relative to the amounts of protein in the milk. The studies quoted by Halliwell and also by Schroeter used very high doses of flavan-3-ols whereas Serafini used nutritionally relevant ones.

The British tennis meeting at Wimbledon inspired the study into the influence of cream on the absorption of anthocyanins from strawberries. In this study the overall outcome was no effect but the plasma $T_{\text{max}}$ was significantly delayed and 0-2 h urinary excretion was lower in the feed with cream. In keeping with these observations, measurement of plasma paracetamol and breath hydrogen revealed that cream delayed gastric emptying and extended mouth to caecum transit time (Mullen et al., 2008b).

### 1.7 Bioavailability of flavonols

In Section 1.4 the difficulty in obtaining accurate data on the level of polyphenols in food was discussed. Therefore it can be imagined how difficult it is to measure the bioavailability of the onion flavonols quercetin-4´-glucoside and quercetin-3,4´-diglucoside (Figure 1-5). The analytical methods must be highly selective, to prevent overestimates, but also able to detect all metabolites, to prevent underestimates. Quantitative analysis of quercetin in acid hydrolysed human urine collected over a 24 h period after eating a meal of lightly fried onions accounted for only 0.8% of the amount ingested and extrapolations from the amounts present in the bloodstream at peak plasma concentration also yielded a figure of 1.0% (Aziz et al., 1998). These results are in keeping with those obtained by Gugler et al., (1975) at just over 1% after the ingestion of a massive 4 g of the aglycone. Other studies have found 2.6% and 3% for intake of quercetin-3-glucoside and quercetin-4´-glucoside (Table 1-3) respectively Olthof et al., (2000). However, it is evident from the data in Table 1-3 that despite the widely ranging levels reported in plasma that over 95% of the ingested flavonols remain unaccounted for.

In an attempt to investigate the missing flavonols, studies were carried out with ileostomy volunteers. Analysis of ileal fluid after ingestion of onions indicated that 47-
76% of the onion flavonol glucosides were absorbed (Hollman et al., 1995a; Walle et al., 2000). However, the difference between the quantity of flavonols ingested and the level recovered in ileal fluid does not necessarily indicate the amount absorbed. What should have been the conclusion of this study was that 47-76% of the flavonol dose could not be accounted for.

What happens to these compounds when they pass from the small to the large intestine is also under investigation. The large intestine is a major site of flavonol catabolism due to the action of colonic bacteria.

It was reported as long ago as 1956 that the bacteria in the colon break down quercetin, by cleavage of the C-ring to various phenoxyacetic acids (Booth et al., 1956). However, many of these acids are also products of metabolic pathways unrelated to flavonol degradation.

In colonic fermentation experiments (Aura et al., 2002) the faecal microflora rapidly deconjugated rutin, isoquercitrin and a mixture of quercetin glucuronides. Rutin also underwent ring fission and dehydroxylation. This report points out that because the faecal matter used is derived from components of the diet it does contain relatively high background levels of phenolic compounds. This was also evident in the work of Olthof et al., (2003) who fed 20 healthy volunteers chlorogenic acid, rutin and black tea powder in a crossover study. Urine was collected and analysed for 60 potential phenolic acid metabolites using GC-MS. Similar feeds were given to seven volunteers who had had a colostomy and the data obtained from the two groups compared. In the case of rutin, the colostomy volunteers had only traces of phenylacetic acids in urine compared with those of the volunteers with an intact GIT. This implies that the colonic microflora convert flavonols to phenolic acids, which then pass into the circulation and are subsequently excreted in the urine. The measurements of phenolic acids were made against a high background of these compounds. This makes it very difficult to obtain an accurate quantitative picture of flavonol catabolites. The only way to circumvent this problem is to use a labelled substrate.

Samples from a study, in which 3 mg of [2-^14^C]quercetin-4'-glucoside (Hartley et al., 2000) was fed to rats were used to develop a method to identify the quercetin metabolites, formed up to five hours after dosing. In this investigation, reported in
Chapter 3, all of the radiolabelled compounds detected still contained the original flavonol structure with metabolism restricted to methylation, sulphation and glucuronidation.

Chromatographic detection methods rely on the behavioural properties of compounds under investigation remaining constant. One of these properties is retention time another is absorbance spectrum. A change in the compound conjugated to the aglycone will change the retention time. Flavonols have an absorbance spectrum with maxima at around 360 nm. Hydrolysis of the metabolite to the aglycone can restore the retention time property. However, if the digestive process changes the absorbance property then it is no longer possible to detect this compound. What form the “missing” flavonols take and to which extent they are bioavailable remains to be determined. The most obvious approach to answer this question is to feed a radiolabelled flavonol, as mentioned above, but monitor over a longer timescale.

As ethical considerations preclude the use of $^{14}$C-labelled flavonols with humans, such experiments are typically carried out with rats. The one exception to this rule has been a US based study, Walle et al., (2001a). An intravenous and oral intake of 1.85 MBq (50 µCi) [4-$^{14}$C]quercetin was administered to human volunteers in 30 µg and 100 mg of quercetin respectively. It was reported that 23-81% of the radioactivity was exhaled as $^{14}$CO$_2$ over a period of 72 h in both the intravenous and oral experiments. However, the results do not necessarily indicate high levels of absorption as the position of the radiolabel in the flavonol skeleton would allow release of the $^{14}$C-labelled carbonyl group. Fission of the C ring will leave two main catabolites and the carbon at the 4 position, which is likely to form CO$_2$. This study was carried out with the quercetin aglycone, not a dietary flavonol. Although the authors wrote “of key importance is to attempt to define the nature of the radioactivity in plasma”, no further data have been published. A feed with [2-$^{14}$C] quercetin-4’-glucoside to humans would have overcome these problems but as mentioned earlier ethical permission for such a study would not be granted. However, stable isotope experiments with ($^{13}$C)-labelled substrates would allow a great deal of information to be obtained with regard to the compounds we already know or suspect would be formed from feeds of quercetin sugar conjugates.
A study involving a feed of \[\text{[2-}^{14}\text{C]quercetin-4'}\text{-glucoside}\] to rats housed in metabolism cages, for up to 3 days, was carried out. This was to answer some of the questions regarding the bioavailability of quercetin glucosides. Measurement of the radioactivity contained in the urine allowed an estimate of the minimum level of absorption to be obtained. Combined measurement of the radioactivity in the faeces, urine and body tissue gave an estimates of recovery of the dose. A low recovery would have indicated \(^{14}\text{CO}_2\) is a product of catabolism of quercetin-4'-glucoside as found by Walle et al., (2001b) in the feed with \([4^{14}\text{C]}\) quercetin. However, this was not what occurred. In fact, over 70\% of the dose was recovered in urine, 19\% in faeces and the remainder in the liquid used to wash the cages after completion of the experiment. The radioactivity in the cage washes was from both the urine and faeces. The results of this experiment are discussed in Chapter 5. Analysis of urine and extracts of faecal and other tissues by HPLC-MS\(^n\) with on line radio-detection also allowed identification of the metabolite/catabolites of the \([2-^{14}\text{C}]\)quercetin-4'-glucoside. The results of this study are also reported in Chapter 5.

### 1.8 Potential bioactivity

As previously mentioned, methods for the qualitative and quantitative analysis of quercetin conjugates have relied on acid/enzyme hydrolysis of samples followed HPLC analysis of the released quercetin. However, this leaves questions as to whether the actual metabolites present have any biological activity (Manach et al., 1998; Day et al., 2000).

The first attempt to identify the circulating metabolites was reported by Paganga & RiceEvans, (1997) using HPLC and diode array detection. They claimed to have identified quercetin-3-rutinoside in a single analysis of a single plasma sample after an undetermined feed. This and their claims of three other quercetin glucosides, an anthocyanin and an unknown aurone must be treated with some scepticism. It was a single analysis of a single plasma extract and only two volunteers were recruited. The peak seen in the sample was 0.4 min different from that of the quercetin-3-rutinoside standard and no co-chromatography was reported. No internal standard was used in the extraction process. The meal the volunteers consumed was also not reported. Therefore, it must be assumed that it is tomato based if they are looking for quercetin-3-rutinoside.
The meal must also have included a large amount of berries or red wine for an anthocyanin to be found in the plasma. Anthocyanin absorption can be as low as 0.004% of the dose (Wu et al., 2002).

Further work using HPLC with electrochemical detection after hydrolysis of the sample with a specific enzyme pointed to the fact that no quercetin glucosides were present in human plasma after a flavonol supplement (Sesink et al., 2001). Plasma was collected from six human volunteers after feeds with quercetin-3-glucoside and quercetin-4′-glucoside. Samples were extracted and analysed by HPLC with an electrochemical detector, which it was claimed was able to distinguish between flavonol glucosides and glucuronides. No peaks in the chromatogram corresponded with that of the quercetin glucoside standards. However, peaks that co-eluted with quercetin glucuronide standards were present. These peaks disappeared after treatment of the sample with bovine glucuronidase and a peak corresponding to quercetin appeared. Glucuronide standards have been produced enzymatically from incubation of quercetin with pig liver (O’Leary et al 2001). However, they are not commercially available as authenticated standards.

Clearly much progress has been made since Aziz et al., (1998) first attempted to identify the circulating metabolites. However, no method is available that can identify and quantify all the possible metabolites in a single analytical analysis. Without the identity of the main metabolites being known and their concentration in the circulatory system, in vitro studies will not be able to ascertain whether these compounds could have potential health benefits. Knowing the structures of the metabolites in the small intestine, the circulatory system and the forms that are excreted, will allow theories of how they interact with the body to be put forward.
1.9 Pharmacokinetics of flavonols

One way to measure the bioavailability of a compound is to measure the concentration it circulates in the plasma and is excreted into the urine. The majority of the pharmacokinetic studies carried out after feeds of flavonol containing onions and apples or various quercetin glucosides, have all relied on the aforementioned method of simply measuring the quercetin aglycone after acid or enzyme hydrolysis. However, the data obtained by Hollman et al., (1996a) indicated that quercetin is present in plasma for more than 50 h after supplementation. In contrast the study of Aziz et al., (1998), using the same hydrolysis method, showed that the quercetin levels returned to baseline within 24 h.
Table 1-3. Summary of Human Feeding Studies Involving the Acute Ingestion of a Lightly Fried Onion

Supplement or pure compound.

<table>
<thead>
<tr>
<th>Study</th>
<th>Meal (g) onions</th>
<th>Quercetin ingested</th>
<th>Plasma $C_{\text{max}}$ (µmol/L)</th>
<th>Plasma $T_{\text{max}}$ (h)</th>
<th>Urinary excretion (µmol) (% of intake)</th>
<th>Analysis method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hollman et al., (1996a)*</td>
<td>215</td>
<td>206</td>
<td>0.6</td>
<td>2.9</td>
<td>n.q.</td>
<td>A, D</td>
</tr>
<tr>
<td>Hollman et al., (1997a)*</td>
<td>n.q.</td>
<td>225</td>
<td>0.7</td>
<td>0.7</td>
<td>3.2</td>
<td>A, D</td>
</tr>
<tr>
<td>Olthof et al., (2000)*</td>
<td>Q4G</td>
<td>331</td>
<td>5.0</td>
<td>0.6</td>
<td>10.3</td>
<td>A.D.</td>
</tr>
<tr>
<td>Aziz et al., (1998)</td>
<td>300</td>
<td>411</td>
<td>1.3</td>
<td>1.9</td>
<td>3.1</td>
<td>A, D</td>
</tr>
<tr>
<td>Erlund et al., (2000)</td>
<td>Q</td>
<td>166</td>
<td>0.3</td>
<td>4.9</td>
<td>n.q</td>
<td>A.E.</td>
</tr>
<tr>
<td>Graefe et al., (2001)</td>
<td>Q4G</td>
<td>331</td>
<td>7.0</td>
<td>0.7</td>
<td>10.0</td>
<td>A.E.</td>
</tr>
<tr>
<td>Graefe et al., (2001)</td>
<td>160</td>
<td>331</td>
<td>7.1</td>
<td>0.7</td>
<td>14.6</td>
<td>B, E</td>
</tr>
<tr>
<td>Present study</td>
<td>270</td>
<td>275</td>
<td>1.3</td>
<td>0.5-1.0</td>
<td>12.9</td>
<td>C, F</td>
</tr>
</tbody>
</table>

n.q. – not quantified; $C_{\text{max}}$ - maximum concentration of quercetin metabolites in plasma; $T_{\text{max}}$ – time to reach $C_{\text{max}}$. Method of HPLC analysis: A – acid hydrolysis of samples prior to analysis, B – enzyme hydrolysis of samples prior to analysis, C – samples not hydrolysed prior to analysis, D – post-column derivatization and fluorescence detection, E – photodiode array and electrochemical detection, F – photodiode array and full scan MS$^2$ detection. Q4G is quercetin-4´-glucoside, Q is quercetin aglycone. * same laboratory.
In spite of this difference both studies produced similar quercetin peak plasma concentrations at 0.6 and 1.3 µmole/L respectively Table 1-3. Two other groups have carried out studies to investigate the pharmacokinetic characteristics of quercetin conjugates accumulated in plasma after oral feeds (Graefe et al., 1999; Erlund et al., 2000d). As shown in Table 1-3, the Hollman group have carried out three studies (Hollman et al., 1996a; Hollman et al., 1997b); (Olthof et al., 2000). The investigations by Manach et al., (1998) was not a pharmacokinetic study though it did provide peak plasma levels of 0.4 µmole/L and the time required for plasma levels to return to baseline was 20 h. Table 1-3 summarises the results of all these studies.

Detailed examination of the pharmacokinetic profiles obtained in these studies reveals some interesting points. If we examine work from Hollman’s laboratory it would appear that feeds of quercetin glucosides reach peak plasma concentrations of quercetin almost ten times higher than those obtained with apples or onions Table 1-3. However, this is not the case in studies by the Graefe group, where similar peak plasma quercetin levels were obtained in feeds with quercetin-4’–glucoside and onions, containing both quercetin-4’–glucoside and quercetin-3,4’–glucoside. The work by Aziz, Erlund, Manach, and Hollman’s earlier studies, all produced similar results. Graefe and Hollman’s recent studies both appear to show a 10-fold increase in peak plasma quercetin levels. There are always laboratory-to-laboratory differences, even using the same methodologies, and biological variation can also be added to explain such differences. However, in the case of Hollman’s studies, it is hard to believe that the same laboratory could produce results that are an order of magnitude different using the same methodology, without some comment from the author to explain the increase.

The methods employed in these studies rely on there being no interference from unknown compounds that co-chromatograph with the peak of interest. The methodology used by Hollman would appear to be very susceptible to this problem. When relying on a HPLC to separate one compound contained in a complex mixture of compounds, adequate resolving power must be used to minimise the chance of possible interference from other compounds. Especially when the other analytical processes of extraction, purification and identification are not maximised. The isocratic HPLC method used by Hollman, where quercetin elutes after 8 min of a 20 min analysis was claimed to have “adequate resolution”. However, the signal has not returned to baseline before the
The quercetin peak elutes. Therefore, the quantification of quercetin is measured on top of another signal. No sample clean-up is performed on the hydrolysed plasma prior to chromatography. Although it may seem that by carrying out an acid or enzyme hydrolysis the sample has only one compound of interest, which simplifies the analysis, in fact by adding a strong acid or an enzyme mixture to the sample it is increasing the number of potential co-eluting compounds. This problem was seen by Erlund et al., (1999) who reported that without additional purification steps there were compounds from the enzyme preparations from *Helix pomatia* that coeluted with the quercetin peak.

One of the problems with the methods employed is that they can only view the compounds in one dimension. It is not possible for any of the detection systems, whether it is absorbance, fluorescence, electrochemical or mass spectrometry operated in SRM or SIM mode, to determine with absolute certainty if there is another compound co-eluting with the compound of interest.

A re-examination of pharmacokinetic analysis should be carried out using analytical methods developed to accurately identify and quantify individual metabolites not measured in the studies by (Hollman et al., 1996a; 1997c; 1999; Aziz et al., 1998; Erlund et al., 2000c; Olthof et al., 2000) and (Graefe et al., 2001). These methods must allow for the detection of co-eluting compounds and be able to provide quantification even in the presence of such compounds. With this methodology it will be possible to seriously reduce erroneous measurements and overestimates due to interfering compounds and to further our knowledge in A.D.M.E. studies of quercetin glucosides.
Chapter 2  Detection and identification of $^{14}$C-labelled flavonol metabolites by HPLC radio-counting and tandem mass spectrometry in rats

2.1 Introduction

The aim of the work in this chapter was to develop a method to allow the identification and quantification of the intact metabolites formed \textit{in vivo} from the flavonol quercetin-4′-glucoside. This involved the use of [2-$^{14}$C]quercetin-4′-glucoside to allow these metabolites to be followed both in terms of levels found in tissues, by scintillation counting and with regard to their identities and quantities, by mass spectrometry with on-line radioactivity detection.

Epidemiological studies have highlighted a positive correlation between high flavonol intake and a reduction in the risk of contracting coronary heart disease and strokes (Hertog \textit{et al.}, 1995; Duthie \textit{et al.}, 2000). This may reflect the diverse ability of flavonols such as quercetin to show potentially anti-atherogenic effects in model systems. For example, in relation to heart disease some flavonols can prevent the oxidation \textit{in vitro} of low-density lipoprotein to a potentially atherogenic form (Dewhalley \textit{et al.}, 1990), inhibit platelet aggregation and dilate blood vessels (Di Carlo \textit{et al.}, 1999). Potential anticancer effects include the modulation of enzyme activities associated with carcinogen activation and detoxification (Musonda \textit{et al.}, 1997) and the prevention of oxidative DNA damage (Duthie \textit{et al.}, 1997).

If these flavonols, that show protective effects \textit{in vitro}, are to have health effects \textit{in vivo} they must reach the appropriate tissues in concentrations and forms that are effective. However, at the outset of this work the form and concentration of quercetin metabolites circulating in plasma was not known.

Flavonols exist almost exclusively as sugar conjugates in plant-derived foods and beverages, and HPLC is usually the method of analysis in quantitative studies. Unfortunately, very few of the many hundreds of characterised flavonol conjugates are
available from commercial sources for use as reference compounds. To overcome this problem, extracts are subjected to acid hydrolysis and the amounts of flavonol aglycones released, typically myricetin, quercetin, kaempferol and isorhamnetin, are quantified by reverse phase HPLC with detection at 365 nm. This analytical approach, pioneered by Hertog and co-workers in the early 1990’s, (Hertog et al., 1992a) has been used to measure flavonol levels in a wide variety of fruits, vegetables and beverages (Hertog et al., 1992a; 1993b; McDonald et al., 1998; Crozier et al., 2000). Improved sensitivity and selectivity, necessary for the analysis of flavonols in plasma, urine and body tissues can be achieved through the use of either an electrochemical detector Erlund et al., (1999) or post-column chelation with methanolic aluminium nitrate to form highly fluorescent flavonol derivatives (Hollman et al., 1996b).

Irrespective of the detection system used, HPLC of hydrolysed extracts provides no information on the identity of the parent conjugate(s) and, therefore, provides quasiquantitative estimates of flavonol levels. Some investigators have used enzymes, such as glucosidases, glucuronidases and sulphatases, to release flavonol aglycones Erlund et al., (1999; 2000), thereby giving some indication of the type of metabolites present. This approach suffers similar problems to acid hydrolysis and is compounded as the substrate specificities of the enzymes utilised are far from absolute and ensuring complete hydrolysis is not straightforward (Gu et al., 2005b; Donovan et al., 2006).

The HPLC and post-column derivatization of flavonols with methanolic aluminium nitrate, referred to above, provides sensitive and selective detection of flavonols with a free 3-hydroxyl group (Hollman et al., 1996b). A number of studies (Ader et al., 2000; Erlund et al., 2000b) claim to have detected “flavonols and their metabolites” in plasma and urine of human subjects following consumption of pure compounds and flavonol-rich foods. Although there is wide individual variation in absorption, possibly reflecting the type of flavonol, degree of conjugation and assay methodology, the presence of conjugates in body fluids provides necessary evidence for the potential to exert effects in vivo analogous to those observed in model systems. However, the claim of “detecting flavonols and their metabolites” is somewhat misleading. The methodology used by these investigators can quantify quercetin only after hydrolysis has been carried out. Thus any glucuronide conjugates would be detected, but only as an increase in the quercetin concentration from the pre-hydrolysis
results. This is not measuring the actual quercetin metabolite concentration (Hollman et al., 1997b). It was claimed that methyl metabolites would not be detected by this method as they would not hydrolyse.

The methodology used by Aziz et al., (1998) was based on the same detection system as used by Hollman but with a gradient HPLC method run over 40 minutes that would allow the separation of individual metabolites. No acid or enzyme hydrolysis was carried out on the sample prior to analysis. Co-chromatography with the available standards identified the main circulating metabolites in plasma as isorhamnetin-4’-glucoside and quercetin-4’-glucoside.

Graefe et al., (1999) used HPLC-MS\(^2\) to detect the presence of five quercetin glucuronides, but then measured the level of quercetin present after enzyme hydrolysis using HPLC with electrochemical detection.

Of these three studies only Aziz actually attempted to measure the level of intact metabolites present. The results, however, were not in keeping with emerging proposals, based on \textit{in vitro} studies, that during passage through the gut wall flavonol glucosides are subjected to the action of hydrolases and glucuronidases and as a consequence flavonol glucuronides rather than glucosides appear in the bloodstream (Williamson et al., 2000). It has been proposed by Sesink et al., (2001) that the peak identified by Aziz et al., (1998) as quercetin-4’-glucoside was a quercetin glucuronide. It is most likely that the compound seen was not a glucoside, as originally proposed, but it was the first time direct quantification of the circulating metabolites was attempted.

This highlights the problem of the methodology to date. A method that can be used to quantify a compound cannot tell the intact structure of it. The intact structure can be found by another method but this cannot be used to quantify it.

A good analogy is with the Heisenberg uncertainty principle. A simplified interpretation is, the more certain you are of a particles position the less you can say about its energy. The greater the information known about its energy the less is known about its position. However, unlike this principle it is possible, by using two complementary detectors, to determine both types of information at the same time.
The aim of this study was to identify the metabolites of quercetin-4'-glucoside, a major flavonol conjugate in onions (Tsushida & Suzuki, 1995) which are one of the main sources of flavonols in the Dutch diet (Hertog et al., 1993a). Rats were fed [2-\(^{14}\)C]quercetin-4'-glucoside and the radiolabelled components in plasma and body tissues were analysed by high performance liquid chromatography (HPLC) with detection using an on-line radioactivity monitor and a tandem mass spectrometer with an electrospray interface. This methodology can provide structural information on the compounds under investigation thereby eliminating disputes about analytical accuracy and prevent protracted discussions about whether an HPLC peak has been misidentified. Full scan MS\(^2\) is an extremely powerful and flexible tool for the analysis of trace levels of natural products in impure extracts as interpretation of fragmentation patterns, coupled in some instances with knowledge of HPLC retention properties, can facilitate the partial identification of components where reference compounds are not available (Mullen et al., 2002a). When these mass spectra coincide with peaks in the radioactivity detector’s response it can be assumed with some degree of surety that the metabolite present originated from the parent compound in the feed. In addition the level of radioactivity gives a direct measure of the quantity of the compound present.

### 2.2 Material and methods

Synthesis of [2-\(^{14}\)C]quercetin-4'-glucoside (specific activity 3.75 mCi/mmole) was carried out in the Chemistry Department of Glasgow University using a modified version of the method previously reported for the synthesis of [2-\(^{13}\)C]quercetin-4'-glucoside (Caldwell et al., 2000) except that the intermediate ester was not purified by filtration through alumina. The compound was pure by \(^1\)H NMR spectroscopy and only one radioactive species was detected by HPLC-radiocounting (RC).
2.2.1 Animals and sample preparation

After an overnight fast, three male rats of the Rowett Hooded Lister strain (mean weight 430 ± 4 g) were offered 1 g of stock rat feed (CRM, Special Diet Services, Witham, Essex, UK) containing 3.26 mg of [2-14C]quercetin-4′-glucoside to give a dose of 58.5 x 10^6 dpm. Rats consumed 99.3% of the ration within 2 min, as measured by residual radioactivity on the feeding dish. After 60 min, animals were terminally anaesthetised with isofluorane and their gastrointestinal tract, comprising the stomach, small and large intestines and their contents removed intact. The intestines and all other major organs were frozen in liquid nitrogen and lyophilized after which they were ground to a powder using a mortar and pestle and stored at –80°C prior to extraction. All tissues were weighed before and after lyophilization. Blood samples were
centrifuged at 2,000 g and the plasma and red blood cells separated and stored at -80 °C. All tissues and fluids collected are reported in Table 2-1.

2.2.2 Measurement of radioactivity

Ten-mg aliquots of freeze-dried tissue, plasma and red blood cells were treated with 0.5-mL tissue solubiliser (National Diagnostics, Hull, UK) for 3 h at 50 °C in a shaking water bath. With the exception of red blood cells, which were bleached using 1.75 mL of a 25% solution of sodium hypochlorite, the solubilization treatment produced clear solutions and 150 µL aliquots were taken and added to 5 mL scintillation cocktail (Optiflow Safe One, Fisons, Loughborough, UK) before determination of radioactivity using a Wallac 1409 liquid scintillation counter (Pharmacia, Uppsala, Sweden).

2.2.3 Extraction of samples

One g aliquots of freeze-dried tissues were extracted by continuous shaking with 15 mL 50% methanol in 100 mmole/L phosphate buffer (pH 7.0) containing 20 mmole sodium diethyldithiocarbamate. After 30 min the mixture was centrifuged for 20 min at 2000 g. The methanolic supernatant was decanted and the pellet re-extracted a further two times. The three methanolic supernatants were combined and the methanol removed in vacuo. The remaining aqueous phase was adjusted to pH 3.0 and partitioned three times with an equal volume of ethyl acetate. The ethyl acetate extracts were combined and reduced to dryness in vacuo prior to the measurement of radioactivity and analysis. A gentle stream of nitrogen was used to remove residual ethyl acetate from the aqueous phase before it was loaded onto a 2 g C18 Sep Pak cartridge (Waters, Milford, MASS, USA) which was washed with 15-mL distilled water adjusted to pH 3.0 with H2SO4. The cartridge was then eluted with 30 mL methanol to remove polar radiolabelled metabolites. The methanolic extract was dried in vacuo and aliquots taken for radioactivity measurements and analysis.

2.2.4 HPLC with diode array detection and tandem mass spectrometry
Samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, diode array absorbance detector, scanning from 250 to 700 nm and an autosampler cooled to 4°C. (Thermo Finnigan, San Jose, USA). Separation of quercetin metabolites was carried out using a 250 x 4.6 mm i.d. 4 μm Synergi RP-Max column (Phenomenex, Macclesfield, UK) eluted with a gradient over 60 min of 5-40% acetonitrile in 1% formic acid at a flow rate of 1 mL/min and maintained at 40°C. After passing through the flow cell of the absorbance monitor, the column eluate was directed to a radioactivity monitor (Reeve Analytical Model 9701, Lab Logic, Sheffield, UK) fitted with a 500 μL heterogeneous flow cell packed with cerium-activated lithium glass scintillant, after which it was split and 0.3 mL/min was directed to a LCQ DecaXP ion trap mass spectrometer (Thermo Finnigan, San Jose, USA). Analysis with an electrospray interface (ESI) in negative ion mode provided the best limits of detection for quercetin based metabolites. This was carried out using full scan, data dependant MS-MS scanning from m/z 100 to 1000. Capillary temperature was 350°C, sheath gas and auxiliary gas were 60 and 10 units respectively, source voltage was 4 kV.

2.3 Results

2.3.1 Distribution of radioactivity in rat tissues and extracts

Each rat consumed 1 g of stock rat feed containing 58.5 x 10⁶ dpm [2-¹⁴C]quercetin-4’-glucoside. An aliquot of the feed was extracted with methanol and analysed by HPLC-RC-MS-MS which demonstrated that the [2-¹⁴C]quercetin-4’-glucoside was radiochemically pure (Figure 2-1A). Full scan MS analysis was also carried out (Figure 2-1B) which shows a molecular ion [M-H]⁻ at m/z 463. The [M+2-H]⁻ ion at m/z 465, from the ¹⁴C label, can also be seen. Calculated abundance of the M+2 ion is 11.4% from the specific activity of the label. MS-MS was carried out on the m/z 463 molecular ion (Figure 2-1C) rather than the smaller M+2 ion. This yielded a secondary spectrum with the major ion at m/z 301, which corresponds to the aglycone quercetin.
Table 2-1. Distribution of radioactivity in rats 60 min after ingestion of [2-\(^{14}\)C]quercetin-4'-glucoside.

<table>
<thead>
<tr>
<th>Tissue/fluid</th>
<th>Total radioactivity</th>
<th>Concentration of radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine and contents</td>
<td>63184 ± 3902 (93.6%)</td>
<td>2117 ± 106</td>
</tr>
<tr>
<td>Plasma</td>
<td>1644 ± 39 (2.5%)</td>
<td>110 ± 3</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>4 ± 1 (-)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Liver</td>
<td>684 ± 61 (1.2%)</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>Kidneys</td>
<td>468 ± 76 (0.8%)</td>
<td>147 ± 31</td>
</tr>
<tr>
<td>Spleen</td>
<td>6 ± 0 (-)</td>
<td>7 ± 0</td>
</tr>
<tr>
<td>Brain</td>
<td>1 ± 0 (-)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Lungs</td>
<td>38 ± 3 (0.07%)</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>Heart</td>
<td>18 ± 1 (0.03%)</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Muscle</td>
<td>839 ± 113 (1.4%)</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Testes</td>
<td>25 ± 1 (0.04%)</td>
<td>8 ± 1</td>
</tr>
</tbody>
</table>

Radioactivity measured by liquid scintillation counting after dissolving tissues with a solubilizer. Radioactivity expressed as dpm x 10\(^3\) per tissue/fluid per rat ± standard error (n = 3) and in parenthesis as a percentage of the recovered [2-\(^{14}\)C]quercetin-4'-glucoside. Concentration of radioactivity expressed as dpm x 10\(^3\) per g (fresh weight) or per ml.

The sample of rat intestines and contents, obtained 60 minutes after dosing, contained 93.6% of the ingested radioactivity. When fractionated 92% of the recovered radioactivity was associated with the ethyl acetate extract and 8% with the aqueous fraction. Aliquots of both extracts, each containing 30 x 10\(^3\) dpm of radioactivity were analysed by reverse phase HPLC with on line radioactivity detection and data dependant full scan MS-MS. The radioactivity and A\(_{365}\) \(\text{nm}\) chromatograms obtained from the plasma, liver and kidney are illustrated in Figure 2-2. In total there are 18 significant radioactive peaks. It was seen that not all the peaks in the A\(_{365}\) \(\text{nm}\) traces were radiolabelled components, data not shown. The mass spectral data, which are summarised in Table 2-2 facilitated the identification of 17 of the 18 radiolabelled peaks. The relative amounts of these compounds, based on the amount of radioactivity in each peak, are also shown in Table 2-3.
Figure 2-2. Gradient HPLC-RC traces of $30 \times 10^3$ DPM aliquots of aqueous and ethyl acetate extracts from intestine, plasma, liver and kidneys of rats 60 min after ingestion of $[2^{-14}C]\text{quercetin-4'}\text{-glucoside}$.

After passing through the flowcell of a diode array detector (data not shown), the column eluate was directed to a radioactivity monitor operating in heterogeneous mode, after which it was split and 30% directed to a tandem mass spectrometer with an electrospray interface in negative ion mode.
2.3.2 Identification of metabolites

Peak 1 (retention time [R,] 16.7 min) had an [M-H]− at m/z 653 which fragmented to produce ions at m/z 477 (M-176, loss of a glucuronyl unit) and m/z 301, which corresponds with the aglycone quercetin, and results from a further neutral loss of 176, indicating that the metabolite was a quercetin diglucuronide with the glucuronyl moieties attached at different positions on the flavonol ring (Figure 2-3-2). If the two glucuronyl residues had been attached at the same position it is unlikely that an M-176 fragment would have been produced at m/z 477 as it has been shown that anthocyanin disaccharide conjugates fragment with loss of the intact disaccharide unit (Giusti et al., 1999)

Peak 2 (R, 20.6 min) was not identified, as it did not yield mass spectral data.

Peak 3 (R, 21.6 min) had an [M-H]− at m/z 667, 14 mass units higher than the quercetin diglucuronide in peak 1. It fragmented to produce two M-176 fragments at m/z 491 and m/z 315, the latter being the [M-H]− of a methylated analogue of quercetin. Peak 3 therefore contains a methylquercetin diglucuronide (Figure 2-3-1). The most likely methylated quercetin derivative is 3'-methylquercetin, isorhamnetin. However, as a methylation at other positions cannot be ruled out, and there is an absence of reference compounds, the aglycone cannot be identified definitively. MS3 may have been able to show if the methylation was on the A or B ring but MS3 was not available on this instrument at the time of running these samples.

Peak 4 (R, 23.7 min), like peak 1, was a quercetin diglucuronide as it produced an MS-MS spectrum with an [M-H]− at m/z 653, which fragmented to produce m/z 477 (M-176) and m/z 301 (quercetin).

Peak 5 at (R, 24.3 min), like peak 3, was a methyl quercetin diglucuronide as it produce an MS-MS spectrum with a [M-H]− at 667, which fragmented to produce m/z 491 (M-176) and m/z 315 (methyl quercetin)

Peaks 6-8 (R, 25.1, 26.4 and 28.3 min) were quercetin diglucuronides as they all had an [M-H]− at m/z 653 and which yielded fragment ions at m/z 477 and 301.
Peak 9 at R<sub>t</sub> 29.6 min had an [M-H]<sup>-</sup> at m/z 477 which fragmented to produce an M-176 ion at m/z 301 indicating the presence of a quercetin monoglucuronide, probably quercetin-3-glucuronide as it co-chromatographed with a standard of this compound (Figure 2-3-6).

Peak 10 (R<sub>t</sub> 30.3 min) had an [M-H]<sup>-</sup> at m/z 557 which ionised yielding fragment ions at m/z 477 (M-80, loss of SO<sub>3</sub>), m/z 381 (M-176, loss of glucuronyl unit) and m/z 301 (quercetin) (Figure 2-3-4). This metabolite is therefore a quercetin glucuronide sulphate conjugate.

Peak 11 (R<sub>t</sub> 32.6 min) was a methylquercetin glucuronide sulphate as indicated by an [M-H]<sup>-</sup> at m/z 571 that produces an M-80 fragment at m/z 491 which with a further loss of m/z 176 yields an m/z 315 methylquercetin ion (Figure 2-3-3).

Peak 12 (R<sub>t</sub> 34.5 min) was the parent compound quercetin-4'-glucoside. Its mass spectrum had an [M-H]<sup>-</sup> at m/z 463 which fragmented to produce an m/z 301 (quercetin, M-162, loss of a glucosyl unit).

Peaks 13-15 (R<sub>t</sub> 35.6, 36.5 and 37.4 min) all of which have a [M-H]<sup>-</sup> at m/z 491 and fragmented to produce an M-176 ion at m/z 315, are methylquercetin glucuronide regio-isomers (Figure 2-3-5).

Peak 16 (R<sub>t</sub> 37.4 min) was a quercetin glucuronide on the basis of an [M-H]<sup>-</sup> at m/z 477 which fragments yielding m/z 301 (quercetin, M-176, loss of glucuronyl unit)

Peak 17 (R<sub>t</sub> 44.6 min) was quercetin with a [M-H]<sup>-</sup> at m/z 301 m/z.

Peak 18 (R<sub>t</sub> 51.1 min) with a [M-H]<sup>-</sup> at 395 m/z that produces an M-80 fragment at m/z 315 was a methylquercetin sulphate conjugate.

Peak 19 (R<sub>t</sub> 51.2 min) yielded a mass spectrum with ions at m/z 381 [M-H]<sup>-</sup> and m/z 301 (M-80) and was a quercetin sulphate derivative.
Table 2-2. Identification of Metabolites of [2-14C]Quercetin-4'-Glucoside Detected in Rats.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Rt (mins)</th>
<th>Compound</th>
<th>$M^-$ (m/z)</th>
<th>Fragment ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.7</td>
<td>Quercetin diglucuronide</td>
<td>653</td>
<td>477 (M'-GlcUA), 301<a href="M'-GlcUA-GlcUA">Q</a></td>
</tr>
<tr>
<td>2</td>
<td>20.6</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>21.6</td>
<td>Methylquercetin diglucuronide</td>
<td>667</td>
<td>491 (M'-GlcUA), 315 <a href="M'-GlcUA-GlcUA">MQ</a></td>
</tr>
<tr>
<td>4</td>
<td>23.7</td>
<td>Quercetin diglucuronide</td>
<td>653</td>
<td>477 (M'-GlcUA), 301<a href="M'-GlcUA-GlcUA">Q</a></td>
</tr>
<tr>
<td>5</td>
<td>24.3</td>
<td>Methylquercetin diglucuronide</td>
<td>667</td>
<td>491 (M'-GlcUA), 315 <a href="M'-GlcUA-GlcUA">MQ</a></td>
</tr>
<tr>
<td>6</td>
<td>25.1</td>
<td>Quercetin diglucuronide</td>
<td>653</td>
<td>477 (M'-GlcUA), 301<a href="M'-GlcUA-GlcUA">Q</a></td>
</tr>
<tr>
<td>7</td>
<td>26.4</td>
<td>Quercetin diglucuronide</td>
<td>653</td>
<td>477 (M'-GlcUA), 301<a href="M'-GlcUA-GlcUA">Q</a></td>
</tr>
<tr>
<td>8</td>
<td>28.3</td>
<td>Quercetin diglucuronide</td>
<td>653</td>
<td>477 (M'-GlcUA), 301<a href="M'-GlcUA-GlcUA">Q</a></td>
</tr>
<tr>
<td>9</td>
<td>29.6</td>
<td>Quercetin-3-glucuronide</td>
<td>477</td>
<td>301 [Q], (M'-GlcUA)</td>
</tr>
<tr>
<td>10</td>
<td>30.3</td>
<td>Quercetin glucuronide sulphate</td>
<td>557</td>
<td>477 (M'-SO3), 381 (M'-GlcUA), 301 <a href="M'-SO3-GlcUA">Q</a></td>
</tr>
<tr>
<td>11</td>
<td>32.6</td>
<td>Methylquercetin glucuronide sulphate</td>
<td>571</td>
<td>491 (M'-SO3), 315 <a href="M'-SO3-GlcUA">MQ</a></td>
</tr>
<tr>
<td>12</td>
<td>34.5</td>
<td>Quercetin-4'-glucoside</td>
<td>463</td>
<td>301 <a href="M'-Glc">Q</a></td>
</tr>
<tr>
<td>13</td>
<td>35.3</td>
<td>Methylquercetin glucuronide</td>
<td>491</td>
<td>315 <a href="M'-GlcUA">MQ</a></td>
</tr>
<tr>
<td>14</td>
<td>36.5</td>
<td>Methylquercetin glucuronide</td>
<td>491</td>
<td>315 <a href="M'-GlcUA">MQ</a></td>
</tr>
<tr>
<td>15</td>
<td>37.4</td>
<td>Methylquercetin glucuronide</td>
<td>491</td>
<td>315 <a href="M'-GlcUA">MQ</a></td>
</tr>
<tr>
<td>16</td>
<td>37.4</td>
<td>Quercetin glucuronide</td>
<td>477</td>
<td>301 <a href="M'-GlcUA">Q</a></td>
</tr>
<tr>
<td>17</td>
<td>44.6</td>
<td>Quercetin</td>
<td>301</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>51.1</td>
<td>Methylquercetin sulphate</td>
<td>395</td>
<td>315 <a href="M'-SO3">MQ</a></td>
</tr>
<tr>
<td>19</td>
<td>51.2</td>
<td>Quercetin sulphate</td>
<td>381</td>
<td>301 [Q] (M'-SO3)</td>
</tr>
</tbody>
</table>

HPLC-RC retention times, negative ion MS-MS fragmentation patterns and identities of metabolites detected in rat tissues 60 min after oral ingestion of [2-14C]quercetin-4'-glucoside. Peak number refer to peaks in Figure 1. Q – quercetin, MQ –methylquercetin, GlcUA – glucuronyl unit; Glc – glucosyl unit; M$^-$ molecular ion.
Figure 2-3. Tandem mass spectra of [2-¹⁴C]quercetin-4'-glucoside metabolites. Spectrum 1: MS-MS of ion m/z 667, methylquercetin diglucuronide. Spectrum 2: MS-MS of ion m/z 653, quercetin diglucuronide. Spectrum 3: MS-MS of ion m/z 571, methylquercetin glucuronide sulphate. Spectrum 4: MS-MS of ion m/z 557, quercetin glucuronide sulphate. Spectrum 5: MS-MS of ion m/z 491, methylquercetin glucuronide. Spectrum 6: MS-MS of ion m/z 477, quercetin glucuronide.
2.3.3 Amount of radioactivity incorporated into [2-\textsuperscript{14}C] quercetin-4'-glucoside metabolites

The data presented in Figure 2-2 is a qualitative representation of the metabolites found, the identities of which are listed in Table 2-2. The overall levels of radioactivity associated with [2-\textsuperscript{14}C]quercetin-4'-glucoside and its metabolites are presented in Table 2-3. These values were calculated from the peak areas in Figure 2-2, the amount of radioactivity in the ethyl acetate and aqueous extracts from the intestine, plasma, liver and kidneys.

The information in Table 2-3 shows clearly that the majority of the radioactivity was recovered from the intestine and that, within the 60 min period since ingestion, the [2-\textsuperscript{14}C]quercetin-4'-glucoside had undergone extensive metabolism as it represented only 26.2% of the recovered radioactivity as reported in Table 2-3. The major metabolites in the intestine, which comprised the stomach and gastrointestinal tract along with their contents, were quercetin-3-glucuronide (peak 9, 10.6%), quercetin glucuronide sulphate (peak 10, 6.9%), methyl quercetin glucuronide (peaks 13 and 14, 6.5 and 4.9%) quercetin (peak 17, 7.2%). There were two significant peaks both containing two unresolved compounds. Peaks 15/16 (12.2%) contained methyl quercetin glucuronide and quercetin glucuronide and peaks 18/19 (17.9%) contained methyl quercetin sulphate and quercetin sulphate. Although these compounds could be identified by MS\textsuperscript{2} within each peak, radiochemically they appeared as one peak and were therefore quantified as such.

The intestine contained other methylated derivatives, diglucuronides and sulphate conjugates. Quercetin-4'-glucoside and its aglycone quercetin were not detected in extracts from plasma, liver or kidney (Table 3). The intestine contained, in varying amounts, all 18 metabolites of [2-\textsuperscript{14}C]quercetin-4'-glucoside with the exception of quercetin diglucuronide (peak 6) which was detected only in plasma.

The main metabolites in plasma were peak 11 (methylquercetin glucuronide sulphate, 43.6% of radioactivity recovered in plasma), peak 7 (quercetin diglucuronide, 20.5%) and peak 10 (quercetin glucuronide sulphate, 13.8%). The quercetin diglucuronide in peak 7 (33.5%) was the main metabolite in the liver while a range of
low level metabolites were detected in the kidneys, the largest being methylquercetin glucuronide (peak 14, 21.7%).

Figure 2-4 summarizes the types of metabolites that were detected in the rat samples. Diglucuronides were major components in all samples out with the intestine. Plasma had the simplest metabolic profile, having only two major metabolite groups. The kidney and liver contained all the metabolite groups found in the intestine, with the exception of the parent compound.

![Figure 2-4. Metabolite profile of the plasma and tissue extracts of rats obtained 60 min after the ingestion of [2-14C]quercetin-4'-glucoside.](image)

Table 2-3. Radioactivity Incorporated into Metabolites in Plasma and Tissues After Ingestion of [2-14C]Quercetin-4'-Glucoside by Rats

<table>
<thead>
<tr>
<th>Peak number/compound</th>
<th>Intestine</th>
<th>Plasma</th>
<th>Liver</th>
<th>Kidneys</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Quercetin diglucuronide</td>
<td>223.0 (0.8%)</td>
<td>n.d.</td>
<td>20.3 (6.4%)</td>
<td>13.4 (7.4%)</td>
</tr>
<tr>
<td>2. Unknown</td>
<td>223.0 (0.8%)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>8.2 (4.5%)</td>
</tr>
<tr>
<td>3. Methyl quercetin diglucuronide</td>
<td>364.4 (1.3%)</td>
<td>n.d.</td>
<td>40.8 (13.0%)</td>
<td>31.0 (17.0%)</td>
</tr>
<tr>
<td>4. Quercetin diglucuronide</td>
<td>59.8 (0.2 %)</td>
<td>54.5 (6.4%)</td>
<td>n.q.</td>
<td>n.d.</td>
</tr>
<tr>
<td>5. Methyl quercetin diglucuronide</td>
<td>163.2 (0.6%)</td>
<td>n.d.</td>
<td>n.q.</td>
<td>22.7 (12.5%)</td>
</tr>
<tr>
<td>6. Quercetin diglucuronide</td>
<td>n.d.</td>
<td>31.8 (3.7%)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>7. Quercetin diglucuronide</td>
<td>685.4 (2.5%)</td>
<td>174.8 (20.5%)</td>
<td>105.4 (33.5%)</td>
<td>21.4 (11.7%)</td>
</tr>
<tr>
<td>8. Quercetin diglucuronide</td>
<td>152.3 (0.6%)</td>
<td>70.4 (8.2%)</td>
<td>12.6 (4.0)</td>
<td>15.3 (8.4%)</td>
</tr>
<tr>
<td>9. Quercetin-3-glucuronide</td>
<td>2876.6 (10.6%)</td>
<td>18.0 (2.1%)</td>
<td>21.5 (6.9%)</td>
<td>14.4 (7.9%)</td>
</tr>
<tr>
<td>10. Quercetin glucuronide sulphate</td>
<td>1893.3 (6.9%)</td>
<td>118.0 (13.8%)</td>
<td>9.2 (3.0%)</td>
<td>n.d.</td>
</tr>
<tr>
<td>11. Methylquercetin glucuronide sulphate</td>
<td>201.3 (0.7%)</td>
<td>372.3 (43.6%)</td>
<td>40.4 (12.8%)</td>
<td>5.6 (3.1%)</td>
</tr>
<tr>
<td>12. Quercetin-4'-glucoside</td>
<td>7133.9 (26.2%)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>13. Methylquercetin glucuronide</td>
<td>1783.5 (6.5%)</td>
<td>4.5 (0.5%)</td>
<td>42.1 (13.4%)</td>
<td>14.1 (7.7%)</td>
</tr>
<tr>
<td>14. Methylquercetin glucuronide</td>
<td>1323.2 (4.9 %)</td>
<td>3.7 (0.4%)</td>
<td>17.6 (5.6 %)</td>
<td>30.6 (16.8%)</td>
</tr>
<tr>
<td>15/16 Methylquercetin/quercetin glucuronides</td>
<td>3336.8 (12.2%)</td>
<td>6.6 (0.8%)</td>
<td>n.d.</td>
<td>5.7 (3.1%)</td>
</tr>
<tr>
<td>17. Quercetin</td>
<td>1956.1 (7.2%)</td>
<td>n.d.</td>
<td>4.6 (1.5%)</td>
<td>n.d.</td>
</tr>
<tr>
<td>18/19 Methylquercetin/quercetin sulphates</td>
<td>4890.1 (17.9%)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Data expressed as dpm x 10³ and in parentheses as a percentage of the overall level of recovered radioactivity per organ/plasma. n.q. – Identified, but below level of quantification. n.d. – not detected.
2.4 Discussion

The work presented in this chapter was carried out with the aim of developing a method that could positively detect and identify intact metabolites of quercetin-4´-glucoside and also, at the same time, allow quantification of these compounds. This was the first step in the progression to human (A.D.M.E.) studies of flavonols.

The feed of a custom-synthesised, high specific activity $[^{14}C]$quercetin-4´-glucoside to rats was at an amount equivalent to a 70 kg human subject consuming 250 g fresh weight of onions (Tsushida & Suzuki, 1995; Crozier et al., 2000). HPLC with an on-line radioactivity monitor and tandem MS with an electrospray interface was then used to provide detailed identification of radiolabelled metabolites in plasma and body tissues.

Sixty min after feeding $[^{14}C]$quercetin-4'-glucoside to rats, 93.2% of the ingested radioactivity was recovered from the intestine. However, this radioactivity comprised only 26.2% unmetabolised $[^{14}C]$quercetin-4'-glucoside, the remainder having undergone deglycosylation and varying degrees of glucuronidation, sulphation and/or methylation in the process of being incorporated into 18 metabolites. The extent of this metabolism, both in terms of the number of metabolites and the exceptionally high levels of radioactivity incorporated into the metabolites, is on a scale far greater than might have been envisaged from previous feeding studies with unlabelled flavonols. However, as no other studies have attempted to identify and quantify intact metabolites any predictions would have been mere guesswork.

Quercetin glucosides from onion have been reported to be converted to glucuronide conjugates in human intestinal epithelial cells after hydrolysis with β-glucosidase (Day et al., 1998) and conjugation catalysed by uridine-5-diphosphoglucuronyl transferase (UDP-GT) (Cheng et al., 1999). The extensive formation of $^{14}$C-labelled metabolites observed in rat intestine in the present study may occur by similar mechanisms, as rat intestinal mucosal preparations also have cytosolic β-glucosidase activity with broad specificity (Ioku et al., 1998) and UDP-GT activity (Piskula & Terao, 1998). Such transformations are likely to have occurred in the small intestine as substantial quantities of glucuronide and sulphate conjugates of quercetin and methylquercetin accumulated within 60 min of ingestion of $[^{14}C]$quercetin-4´-glucoside.
glucoside. The study here looked at the samples only at 1 h. However, the experiment was run up to 5 h and is reported in Graf et al., (2005). If the radiolabelled flavonol metabolites had reached the large intestine, the available evidence indicates that colonic bacteria would have induced ring fission leading to the formation of compounds such as 4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenyl acetic acid (Kim et al., 1998; Schneider et al., 1999; Justesen et al., 2000) all of which would have retained the $^{14}$C-label and thus have been readily detected.

The results obtained in the present study with rats suggest that absorption of quercetin-4'-glucoside from the gut is relatively poor, as 60 min after consumption only 6.4% of the recovered radioactivity was detected outside the gastrointestinal tract. This is the time after dose that has been shown in human studies to be when the maximum concentration of the compound(s) are circulating in plasma (Hollman et al., 1996a). Moreover, extensive deglycosylation, glucuronidation, sulphation and methylation had occurred and no [2-$^{14}$C]quercetin-4'-glucoside was found in plasma, liver and kidneys. Consequently, cell culture and in vitro studies investigating the effects of this flavonol on systemic cellular processes and gene expression should apply the metabolites rather than the parent compound. Moreover, as 73.8% of the [2-$^{14}$C]quercetin-4'-glucoside was metabolised within 60 min in the intestine, the data indicate that flavonol glucosides are not absorbed per se but undergo extensive metabolism, primarily involving deglycosylation and glucuronidation, prior to passage into the blood stream. It has been proposed that such metabolism occurs during passage across the gut wall (Scalbert & Williamson, 2000; Day et al., 2001; Hollman, 2001). However, the extremely high levels of metabolites in the gut compared to plasma and body tissues suggests that most of the observed metabolism of [2-$^{14}$C]quercetin-4'-glucoside occurs independently of movement into the blood stream. The fact that different spectra of metabolites were detected in plasma, liver and kidneys (Figure 2-4, Table 2-3) and the relatively high proportion of sulphated metabolites in plasma, compared to the intestine, liver and kidneys (Figure 2-4) indicate that some post-absorption metabolism also occurred.

Human liver cells contain both β-glucosidase Day et al., (1998) and UDP-GT activity Day et al., (2000) so further hepatic modification of flavonol metabolites after entering the blood stream cannot be excluded, although in the present study this would
be a relatively minor event compared to metabolism occurring in the gut. Some enterohepatic circulation of the metabolites may have occurred, though it would need to have happened very quickly. It is not possible to identify enterohepatic circulation in this system as the rat does not have a gall bladder and this makes the collection of bile difficult.

On the basis of *in vitro* studies with cell-free systems, it has been proposed that circulating quercetin glucuronides are subject to the action of β-glucuronidase in liver and other human tissues resulting in the release of quercetin (O'Leary *et al.*, 2001). The current *in vivo* study with rats provides no support for this hypothesis as glucuronide conjugates and quercetin represented > 98% and 1.5%, respectively, of the radioactivity recovered from the liver, and the aglycone was not detected in either plasma or kidneys (Table 2-3).

The biological significance of the extensive metabolism of [2-14C]quercetin-4'-glucoside that occurred so rapidly in the intestine of rats, and the relatively small amounts of the quercetin glucuronide, sulphate and methyl conjugates that pass into the blood stream and body tissue, is far from clear. It has been shown that quercetin-3-glucuronide, which was detected as a minor metabolite in the present study (Table 2-3), exerts a considerable inhibitory effect on lipid peroxidation in phospholipid membranes induced by reactive oxygen species and peroxidative enzymes (Shirai *et al.*, 2001). Although the effectiveness of the glucuronide conjugate was less than that of quercetin, it should be noted that the aglycone is not a genuine dietary component. It was also established that quercetin-3-glucuronide exhibited an affinity to phospholipid membranes that was significantly higher than that of ascorbic acid. This may reflect the fact that it is more lipophilic than ascorbic acid but less than the aglycone. On the basis of these observations it was suggested that quercetin glucuronides and other metabolites, claimed to accumulate in plasma after the consumption of food rich in quercetin glucosides, contribute to the antioxidative defences against membranous phospholipid peroxidation occurring *in vivo* (Shirai *et al.*, 2001). However, as around 93.6% of the radiolabelled flavonol consumed, and its metabolites, were found in the stomach and intestines after one hour, the major site of potential protection against oxidative damage must be in the gastrointestinal tract itself.
Quercetin-3-glucuronide has been identified in rat plasma after oral administration of the aglycone (Moon et al., 2001). There are also two reports on the use of HPLC-MS to analyse human plasma after the consumption of onions (Wittig et al., 2001; Day et al., 2001) and neither of these studies were able to obtain full scan mass spectral data on the putative metabolites that were detected. The present study, in which 18 metabolites of quercetin-4'-glucoside were detected, and 17 identified, provides a far more detailed picture of the metabolism and absorption of a flavonol glucoside than any previous reports that have appeared in the literature to date.

2.4.1 Detection system

This is the first study in which the extensive and varied metabolism of quercetin-4'-glucoside, or indeed any other flavonol, has been revealed. The sample analysis, by on line radioactivity detection linked to the mass spectrometer, showed 73.8% of the original compound had been deglycosylated and further metabolised by varying combinations of methylation, glucuronidation and/or sulphation. Full scan MS-MS proved suitable for the identification of all of these metabolites. Further information about the position of the various substituent groups could be achieved by the use of NMR. However, NMR requires not only high sample purity but also the presence of substantially more analyte than the low ng quantities required for MS^2.

HPLC linked to a tandem mass spectrometer is becoming more widely available and provides the most powerful tool currently available for the identification of flavonol conjugates in biological samples. However, a mass spectrometer can be used in many ways depending on the circumstances of the analysis and the samples under study. At one extreme, to increase the sensitivity and selectivity of the analysis, the mass spectrometer can be set up to detect only one ion, selected ion monitoring (SIM). Alternatively when limited availability of sample is not a problem, full scan analysis can provide detailed structural information at the expense of sensitivity. Selected reaction monitoring (SRM) is another mode of analysis that offers high selectivity and sensitivity. However, SRM can be carried out only with triple quadrupole or ion trap mass spectrometers. With these types of mass spectrometers, the “parent ion” of the compound of interest is “focused on or selectively trapped”. The selected ion is then fragmented and a specific fragment ion monitored. In the case of a quercetin
monoglucuronide, the mass spectrometer would be set up, in negative ion mode, to detect the m/z 301 quercetin ion resulting from the fragmentation of the [M-H]⁻ at m/z 477. Wittig et al., (2001) used this method to identify five quercetin monoglucuronide metabolites in human plasma after ingestion of an eye watering 800 g onion supplement. The limitation with SRM is that it can only be used to detect what you already know or suspect to be present in a sample. It is not suitable for the identification of novel metabolites. An advantage of an ion trap mass spectrometer is that there is no loss in sensitivity if a range of ions are selected to be monitored from the fragmentation of the parent ion. For example it would be possible to scan the parent ion of a quercetin monoglucuronide from m/z 477 down to just under m/z 200 with the same level of sensitivity as that if only ion m/z 301 was monitored. This method allows better discrimination of false positive peaks, which may contain additional ions not seen in the compound of interest. This will be discussed later.

Using a triple quadrupole mass spectrometer set up in the positive ionisation mode, (Day et al., 2001) employed LC-MS, as opposed to MS-MS, to identify metabolites of quercetin in human plasma after the consumption of a more manageable 200 g of onions. However, this study did not produce full scan spectra but used the SIM data to support identifications based on chromatographic retention times and enzyme hydrolysis data. In the SIM mode the mass spectrometer was set up to monitor four ions per analysis. Although a total of 12 putative quercetin glucuronide peaks (P1-P12) were suspected to be present on the basis of HPLC-A₃₆₅ nm traces and enzyme hydrolyses, the presence of only three was confirmed by SIM. From our findings one of the compounds in the group P1-P3 may be a methylquercetin diglucuronide. However, the molecular ion of this compound was not monitored so, if present, it went undetected. In addition, as methylquercetin glucoside has the same molecular ion as quercetin glucuronide, the distinct possibility of a misidentification by SIM exists. Choice of the ionisation mode is critical in any mass spectrometric analysis. With our ion trap mass spectrometer the limit of detection for flavonols is at least an order of magnitude better in negative ion mode than positive ionisation and absorbance peaks as small as 3 mAU routinely provide full scan MS-MS data (Figure 2-5).
Analysis by SIM is much less selective than SRM and more likely to produce false positive identifications. Conversely, the secondary fragmentation that operates with both SRM and full scan MS-MS enables identifications to be made that would be missed or be more doubtful when analysed by SIM. The extracted ion chromatograms in, (Figure 2-6) which are taken from the 20-50 min HPLC full scan MS analysis of the ethyl acetate extract in Figure 2-2, are the equivalent of what is produced in a SIM analysis. It can be used to show some of the limitations of SIM analysis when compared to MS-MS. The $m/z$ 653 trace, the [M-H]$^-$ of quercetin diglucuronide, shows only a weak peak at Rt 26.4 min and no fragment peaks are present at either $m/z$ 477, the monoglucuronide or $m/z$ 301, the aglycone (Figure 2-6). This would be poor evidence for the presence of a quercetin diglucuronide. However, the full scan MS-MS spectrum of this ion showed that it is indeed a quercetin diglucuronide (Table 2-3 peak 6).
Figure 2-6. HPLC trace of ethyl acetate extract showing extracted ion chromatograms of molecular ions of quercetin diglucuronide (653), quercetin monoglucuronide (477) quercetin glucoside 463 and quercetin 301. (B) MS-MS of peak at 30.2 minutes shows it is not quercetin derived. (C) MS-MS of peak at 44.6 which is quercetin.

At the other extreme, the ion traces from \textit{m/z} 477 and 301 indicate the presence of a quercetin-based monoglucuronide at Rt. 30.2 min (Figure 2-6). However, full scan MS-MS of the \textit{m/z} 301 fragment revealed that this was not the case as it yielded an MS$^2$ spectrum with major ions at \textit{m/z} 283 and 265, which is quite distinct from the MS$^2$ spectrum of quercetin which has prominent ions at \textit{m/z} 179 and 151 arising from the Retro-Diels-Alder fission of the heterocyclic flavonol ring. In contrast, MS-MS of the small \textit{m/z} 301 peak at Rt 44.6 min Figure 2-6 yielded an MS$^2$ spectrum with major fragment ions at \textit{m/z} 179 and 151, confirming the presence of free quercetin. In addition when the full scan MS-MS of the parent ion \textit{m/z} 477 was examined it contained other ions in additional to that of the aglycone. There should not be any other ions present as
the loss of the glucuronide, like glucosides, occurs at $O$ bond between it and the aglycones (Giusti et al., 1999).

The mass spectrometric method used in this study is known as data dependent MS-MS. The ion trap is set up to select the most significant ion found in each full scan, expel all other ions from the ion trap, then fragment it and scan out the fragment ions thereby yielding a secondary mass spectrum. The secondary spectrum, therefore, contains only ions originating from the “trapped” parent ion. A further refinement to this analytical technique is the ability to program the detector to only look at the same ion a limited number of times (e.g. three) over a set time window, (e.g. 30 s) depending on the peak width in the chromatographic setup. This enables full scan MS$^2$ of individual components in merged peaks to be obtained, as seen in peaks 14 and 15 and also minor peaks in the presence of larger ones, as in peak 13 on the shoulder of peak 12 (Figure 2-2.). A further advantage is that the system can be programmed not to carry out MS-MS on background ions so that “bleed” from the column or the mobile phase can also be rejected from the analysis.

Our study illustrates the power and simplicity of MS-MS analysis for the partial identification of low levels of natural products. This point is well illustrated with the analysis of peak 7 where an uninformative full scan mass spectrum (Figure 2-7) yielded a clean secondary spectrum. The primary mass spectrum, equivalent to that obtained by single stage MS, contained numerous ions and has no diagnostic value. However, fragmentation of the $m/z$ 653 ion produced a daughter spectrum comprised of ions at $m/z$ 477 ([M-H]-176, loss of a glucuronyl unit) and $m/z$ 301 (quercetin, [M-H]-176-176, loss of two glucuronyl units). All the fragments in a secondary spectrum must come from the parent ion and as a consequence the MS-MS spectrum illustrated in Figure 2-7 represents convincing evidence for the presence of a quercetin diglucuronide.

The value of HPLC with full scan MS$^2$ for the structural elucidation of metabolites has also been demonstrated in a recent report on the identification of 17 isoflavone conjugates in the urine of female rats fed on a soya-rich diet (Fang et al., 2002). The daughter mass spectra obtained are in keeping with those seen in the present
study with the initial fragmentation resulting in cleavage of the bond linking the aglycone and the conjugating sugar as outlined by (Giusti et al., 1999). However, this is not the case in the claimed HPLC-MS\textsuperscript{2} identifications of an epicatechin glucuronide and 3′-O-methyl epicatechin glucuronide in rat brains after oral ingestion of high doses of (-)-epicatechin (Abd El Mohsen et al., 2002). In this report the illustrated MS-MS spectra, in marked contrast to the MS-MS spectrum in Figure 2-7, contained 20-30 unexplained ions, some at 10-20% relative abundance and one exceeding 50%. To ignore the presence of these ions and to base identifications on the presence of 3 or 4 known ions, some of very low relative intensity, is a highly subjective interpretation of mass spectral data. The unexplained ions in the MS-MS spectra of (Abd El Mohsen et al., 2002) may
be due to the presence of sizable amounts of co-eluting compound(s) with the same molecular weight or fragment ions, which could impact on the quantitative estimates. The studies of (Wittig et al., 2001) et al. and (Day et al., 2001) and the data presented in this chapter all demonstrate the value of various modes of mass spectrometric detection for the analysis of flavonols and their metabolites.

2.5 Conclusion

The combination of radiolabelled quercetin-4´-glucoside and HPLC RC-MS-MS is an extremely powerful tool in establishing the identity of metabolites present. The level of radioactivity per µL can easily be measured using conventional liquid scintillation techniques. Quantification of the resulting metabolic profile is a simple matter of calculating the percentage each peak makes to the total. The absorption detector, scanning at 365 nm, also saw the majority of the compounds reported. It should be possible in future work using non labelled flavonols to use this detection method to quantify the major compounds present. However, the need for the mass spectrometer to positively identify the peaks in the absorbance trace will still be necessary.

Having established the identity of these metabolites in rats the next step was to use this information to determine whether the methodology used in the rat study could be transferred to use in human studies using flavonol rich foods such as onions. This will be discussed in the following chapter.
Chapter 3  Flavonoid metabolites in human plasma and urine after the consumption of red onions: analysis by HPLC with photodiode array and full scan tandem mass spectrometric detection

3.1 Introduction

In the previous chapter a method was developed using radiolabelled quercetin-4’-glucoside to detect and identify metabolites. A large number of metabolites were detected (19). These were quantified based on the level of radioactivity contained in each peak.

As it is not possible to use radiolabelled compounds in human studies, the aim in this chapter, from the knowledge acquired in Chapter 2, was to develop a method to identify the metabolites using gradient HPLC with MS$^2$ with subsequent quantification by absorbance detection using a photodiode array detector (PDA). Samples from human plasma and urine collected after the ingestion of red onions, which contained high levels of both flavonols and anthocyanins were used to test this method. The samples chosen were those thought to contain the highest levels of the compounds of interest, collected as part of a full bioavailability study (Mullen et al., 2004).

As discussed in Chapter 1 onions are a rich source of flavonols Crozier et al. (1997), in particular quercetin-3,4’-diglucoside, quercetin-4’-glucoside and isorhamnetin-4’-glucoside, (Figure 1-5) Tsushida & Suzuki, (1995) and have been used widely in human feeding studies. Taking the figures from the Danish diet data (Justesen et al., 1997b), onions are a major source of flavonols in the Danish diet accounting for 16% of their daily intake.

In early investigations into the fate of quercetin glucosides after an onion feed, plasma and urine samples were acid hydrolysed and the amount of quercetin released
was then determined by reversed-phase HPLC following post-column reaction with methanolic aluminium nitrate. This method facilitates selective, sub-nanogram fluorescence detection of flavonols with a free 3-hydroxyl (Hollman et al., 1996a). Using this post-column procedure with gradient HPLC, Aziz et al., (1998) detected and quantified fluorescent peaks in unhydrolysed plasma and urine that co-chromatographed with quercetin-4'-glucoside and isorhamnetin-4'-glucoside. As discussed in Chapter 2 results from other studies indicated that quercetin glucuronides rather than the parent glucosides appear in the bloodstream after the consumption of onions (Sesink et al., 2001). It has been suggested that the HPLC peaks identified and quantified by Aziz et al (1998) as flavonol glucosides were probably glucuronide conjugates, which can have very similar HPLC retention times (Day & Williamson, 2001). In-keeping with this possibility, an investigation using HPLC with tandem mass spectrometry in the SRM mode detected five quercetin glucuronides but no glucosides in human plasma after ingestion of an onion supplement (Wittig et al., 2001). However, in this study the MS-MS analysis was carried out separately from the quantitative analysis, which used electrochemical detection. A further study used gradient HPLC-MS and selected ion monitoring to support identifications based on chromatographic retention times and enzyme hydrolysis data. In total, 12 putative quercetin glucuronide peaks were detected in plasma by HPLC-$A_{365nm}$ and selected ion monitoring/co-chromatography identified three of these components as quercetin-3-glucuronide, isorhamnetin-3-glucuronide and quercetin-3'-sulphate (Figure 1-5), (Day et al., 2001). No quantitative data were reported in this study.

The aim in this chapter is to develop a method based on the use of a PDA absorbance detector to quantify metabolites identified by mass spectrometry. If successful this method could be used to produce, for the first time, pharmacokinetic data on the actual metabolites of quercetin.
3.2 Materials and methods

3.2.1 Study design

Six volunteers (4 males and 2 females), who were healthy, non-smokers and not on any medication, participated in this study and gave their written consent. They were aged between 23 and 45 years and had a mean body mass index of 23.7 (range 20.9-27.6). Subjects were required to follow a low flavonoid diet for two days and to fast overnight prior to supplementation. This diet excluded most fruits, vegetables and beverages such as tea, coffee, fruit juices, and wine. On the morning of the study red onions (*Allium cepa*) were skinned, chopped into small slices, and fried for 4 min in margarine. Aliquots of the fried onions were taken for qualitative and quantitative analysis of their anthocyanin and flavonol content.

All subjects consumed 270 g of red onions fried in margarine. Venous blood samples used were those prior to and 1 h post-ingestion. Twelve ml of blood was collected in heparinised tubes at each time point and immediately centrifuged at 4000 g for 10 min at 4°C. The plasma was separated from the red blood cells and 500 µL aliquots were acidified to pH 3 with 15 µL of 50% aqueous formic acid and 50 µL of ascorbic acid (10 mmol) was added to prevent oxidation. The plasma samples were then stored at -80°C prior to analysis. The urine samples used were those collected before and over the initial 4 h period after the consumption of the fried onion supplement. The volume of each sample was recorded prior to acidification to pH 3.0 and the storage of aliquots at -80°C. The study protocol was approved by the Glasgow Royal Infirmary Local Research Ethics Committee.

3.2.2 Materials

Onions were purchased from a local supermarket (Safeway Stores plc, Byres Road, Glasgow G12, UK). HPLC grade methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Scotland). Formic acid was purchased from Riedel-DeHaen (Seeize, Germany) and acetic acid from BDH (Poole, UK). L-(+)-ascorbic acid, quercetin and isorhamnetin-3-glucoside were purchased from Extrasynthese (Genay,
France). AASC Ltd (Southampton, England) supplied quercetin-3,4'-diglucoside, quercetin-4'-glucoside, quercetin-3-glucoside, isorhamnetin-4'-glucoside, malvidin-3'-glucoside and cyanidin-3-glucoside.

[2-14C]Quercetin-4'-glucoside was synthesised in four steps from barium [14C]carbonate (specific activity 3.75 mCi/mmole) by a method previously reported for the synthesis of [2-13C]quercetin-4'-glucoside (Caldwell et al., 2000) except that the intermediate ester was not purified by filtration through alumina. The compound was pure by 1H NMR spectroscopy and only one radioactive peak was detected by HPLC-radio counting.

Quercetin-3-glucuronide was extracted from French beans (Phaseolus vulgaris) and purified by partitioning against ethyl acetate and fractionation using preparative reversed phase HPLC. Quercetin-3'-glucuronide, quercetin-4'-glucuronide, quercetin-7'-glucuronide, quercetin-3'-sulphate, isorhamnetin-3-glucuronide and isorhamnetin-4'-glucuronide were donated by Dr. Paul Needs and Dr Paul Kroon (Institute of Food Research, Norwich, UK).

3.2.3 Extraction of onions

Aliquots of fried onions were taken for quantitative analysis of their flavonol content. Prior to the extraction, they were frozen in liquid nitrogen, lyophilised and powdered. Triplicate samples were extracted as follows: 35 mg of dry powder was homogenized in 3 ml of 70% methanol in water for 1 min using an Ultra-Turrax T 25 (IKA^® Werke, Staufen, Germany). During the homogenization, the samples were kept on ice. The mixture was then centrifuged at 3000 g at 4°C for 15 min. The supernatant was collected and the pellet further extracted and centrifuged twice. The three supernatants were combined and reduced to dryness in vacuo. The dried extract was dissolved in 300 µL of methanol and 1200 µL of 5% formic acid in water, before being centrifuged 25,000 g at 4°C for 10 min. Twenty µL aliquots of the supernatant were analysed by HPLC-MS² (Figure 3-1).
Triplicate samples of plasma collected 1 h after the ingestion of the fried onion meal were treated according to the method of Day et al., (2001). This involved adding 1.5 ml of acetonitrile to 500 µL of plasma. Samples were vortexed for 30 s every 2 min over a 10 min period, before centrifuging the mixture at 4000 g at 4°C for 10 min. The supernatant was collected and the pellet re-extracted as described above but with methanol instead of acetonitrile. Experiments with [14C]quercetin-4'-glucoside and malvidin-3-glucoside showed recoveries of ca. 75% with the initial acetonitrile.
extraction which increased by a further 10-12% with the second methanolic extraction. The acetonitrile and methanol supernatants were combined and reduced to dryness in vacuo. Extracts were then dissolved in 25 µL of methanol plus 225 µL of 1% formic acid in water and centrifuged at 25000 g at 4°C for 10 min prior to the analysis of 100 µL aliquots of the supernatant by HPLC-PDA-MS² on the day of extraction.

### 3.2.5 Extraction of urine

The acidified frozen urine was defrosted, methanol added to make the solution 5% aqueous methanol, which resulted in any precipitated material being redissolved, and 100 µL aliquots were analysed directly by HPLC-MS² without further processing.

### 3.2.6 HPLC with diode array and MS² detection

Samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, PDA absorbance detector, scanning from 250 to 700 nm and an autosampler cooled to 4°C. (Thermo Finnigan, San Jose, USA). Separation was carried out using a 250 x 4.6 mm I.D. 4 µm Synergi Max-RP column (Phenomenex, Macclesfield, UK) eluted with a 60 min gradient of 5-40% acetonitrile in 1% formic acid at a flow rate of 1 mL/min and maintained at 40°C. After passing through the flow cell of the diode array detector the column eluate was split and 0.3 mL/min was directed to a LCQ DecaXP ion trap mass spectrometer fitted with an electrospray interface. (Thermo Finnigan). Analyses utilised the negative ion mode for flavonols and positive ionisation for anthocyanins as this provided the best limits of detection. Analysis was carried out using full scan, data dependant MS² scanning from m/z 100 to 1000. Capillary temperature was 350°C, sheath gas and auxiliary gas were 60 and 10 units respectively, and the source voltage was 4kV.

Additional analyses were carried out specifically for the separation of quercetin-3-glucuronide from quercetin-7-glucuronide. These used the same MS² conditions and negative ionisation, with a 250 x 4.6 mm I.D. 4µm Synergi Polar-RP column
(Phenomenex, Macclesfield, UK), maintained at 40°C, eluted with a 60 min gradient 10-18% acetonitrile in 1% formic acid at a flow rate of 1 ml/min.

Figure 3-2. HPLC absorbance trace at 365 nm of (A) plasma and (B) urine after a feed of red onions

3.3 Results

3.3.1 Analysis of fried onions
Anthocyanins: Gradient reverse phase HPLC with absorbance detection and full scan data dependent MS2 was used to identify and quantify the flavonol and anthocyanin content of the red onion meals. The anthocyanins were monitored at 515 nm and the HPLC trace presented in Figure 3-1 indicates the seven peaks (A1-A7) for which positive ion full scan MS2 data were obtained. All these peaks had a $\lambda_{\text{max}}$ at 515 to 520 nm and their identifications, based on a combination of MS2 fragmentation patterns, co-chromatography and previous characterisation of anthocyanins in red onions (Terahara et al., 1994; Fossen et al., 1996; Donner et al., 1997) are summarised in Table 3-1.

Peak A1 (retention time [t$_R$] - 15.2 min) had a positively charged molecular ion [M+H]$^+$ at m/z 449 which, on MS2 yielded a fragment ion at m/z 287 (cyanidin, M-162, loss of a glucosyl unit). On the basis of the mass spectra data and co-chromatography with a standard, peak A1 was identified as cyanidin-3-glucoside.

Peak A2 (t$_R$ - 16.1 min) had a [M+H]$^+$ at m/z 611 and the MS2 spectrum produced fragment ions at m/z 287 (cyanidin, M-324, loss of a diglucosyl unit). In this system the cyanidin diglucoside, cyanidin-3-sophoroside elutes before cyanidin-3-glucoside as does cyanidin-3,5-diglucoside (Donner et al., 1997; Mullen et al., 2002b). Furthermore, the mass spectrum of cyanidin-3,5-diglucoside contains an additional MS2 fragment ion at m/z 449. However red onions have been shown to contain the disaccharide, cyanidin-3-laminaribioside, which elutes after cyanidin-3-glucoside under similar reverse phase HPLC conditions to those used in the current study (Donner et al., 1997). Based on this earlier identification, its MS2 spectrum and HPLC properties, peak A2 was identified as cyanidin-3-laminaribioside.

Peak A3 (t$_R$ - 17.7 min) had a [M+H]$^+$ at m/z 697 and MS2 produced fragment ions at m/z 449 (M-248, loss of malonylglucoside) and 162 amu lower at m/z 287. On the basis of the mass spectral data, elution order and previously published characterisations (Fossen et al., 1996) this peak was identified as cyanidin-3-(3''-glucosyl-6''-malonylglucoside).

Peak A4 (t$_R$ - 18.5 min) had a [M+H]$^+$ at m/z 535, MS2 produced a fragment ion at m/z 449 (M-86, loss of malonic acid) and at m/z 287 (cyanidin, M-248, loss of malonylglucoside). On the basis of the mass spectral data, elution order and previously published data (Fossen et al., 1996) peak A4 was identified as cyanidin-3-(3''-malonylglucoside).
Peak A5 (t<sub>R</sub> - 20.7 min) was the main anthocyanin in the red onion extract Figure 3-1 and like peak A4 had a [M+H]<sup>+</sup> at m/z 535 and yielded a similar MS<sup>2</sup> fragmentation pattern. On the basis of the concentration of this compound relative to peak 4, the elution order and previously published data (Fossen et al., 1996; Donner et al., 1997) this compound was identified as cyanidin-3-(6"-malonylglucoside).

Peak A6 (t<sub>R</sub> - 21.4 min) had a [M+H]<sup>+</sup> at m/z 697 which on MS<sup>2</sup> fragmented to produce ions at m/z 611 (M-86, cleavage of a malonyl unit) and m/z 287 (cyanidin, M-410, loss of a malonyllaminaribioside unit). The MS<sup>2</sup> spectrum and its elution after cyanidin-3-(6"-malonylglucoside) indicate that A6 was cyanidin-3-(6"-malonyllaminaribioside), a known red onion anthocyanin (Donner et al., 1997).

Peak A7 (t<sub>R</sub> - 24.0 min) had a [M+H]<sup>+</sup> at m/z 549 and MS<sup>2</sup> produced two fragment ions, the first at m/z 463 (M-86, loss of malonic acid) and the second at m/z 301 (peonidin, M-248 loss corresponding to cleavage of a malonylglucoside unit). On the basis of the mass spectral data and HPLC elution profile, A7 was identified as peonidin-3-malonylglucoside, the presence of which in red onions has been reported by (Donner et al., 1997).

The quantities of the individual anthocyanins in the 270 g fried red onion meal consumed by the human volunteers are presented in Table 3-1. The total anthocyanin content of the meal was 77 ± 2 µmole.

**Flavonols:** The flavonols in the red onion extract were analysed using the same gradient HPLC system as employed for anthocyanins but data dependent MS<sup>2</sup> used negative rather than positive ionisation. Figure 3-1 illustrates the HPLC trace obtained at 365 nm and indicates the 10 peaks (F1–F10) for which MS<sup>2</sup> data were obtained. The identifications of these peaks, based on a combination of MS<sup>2</sup> spectra, co-chromatography and the previous identification of flavonols in yellow onions by Tsushida & Suzuki (1995), are summarised in Table 3-2.

Peak F1 (t<sub>R</sub> - 12.9 min) had a negatively charged molecular ion [M-H]<sup>-</sup> at m/z 787 which yielded MS<sup>2</sup> fragments at m/z 625, 463 and 301. The m/z 301 ion is the aglycone quercetin produced by three successive losses of 162 amu each of which
corresponds with the cleavage of a glucosyl unit. Peak F1 was, therefore a quercetin triglucoside and the production of three fragment ions each with a loss of 162 amu indicates that the three sugars are attached at different positions on the flavonol ring.

**Peaks F2 and F3** ($t_R$ –21.5 and 22.8 min) are both quercetin diglucosides with a [M-H]$^-$ at m/z 625 which fragments to produced MS$^2$ ions at m/z 463 and 301. The larger peak, F3 (Figure 3-1), co-chromatographed with quercetin-3,4'-diglucoside which is a major flavonol in onions. The smaller F2 peak was probably quercetin-7,3'-diglucoside, a minor onion flavonol which when analysed by reversed phase HPLC elutes before quercetin-3,4'-diglucoside (Tsushida & Suzuki, 1995).

**Peak F4** ($t_R$ – 23.3 min) yielded a [M-H]$^-$ at 609 and MS$^2$ fragments at m/z 477 and 285. The m/z 285 ion, produced by successive losses of 162 amu, corresponds to the aglycone kaempferol. F4 was, therefore, a kaempferol diglucoside with the sugar moieties attached to kaempferol at different positions. Trace quantities of kaempferol-3,4'-diglucoside have been reported to occur in onions (Tsushida & Suzuki, 1995)

**Peak F5** ($t_R$ – 24.5 min) was a methylquercetin diglucoside yielding [M-H]$^-$ and MS$^2$ fragments at m/z 639 and 477, 14 amu higher than the quercetin diglucoside, F2, because of the additional methyl group. This compound may be isorhamnetin-3,4'-diglucoside which has been identified as a minor onion flavonol (Tsushida & Suzuki, 1995). Its elution after quercetin-3,4'-diglucoside (F3) and kaempferol-3,4'-diglucoside (F4), was in-keeping with this possibility.

**Peak F6** ($t_R$ – 28.5 min) was a quercetin monoglucoside, having a [M-H]$^-$ at m/z 463 which with neutral loss of 162 yielded a MS$^2$ fragment corresponding to quercetin at m/z 301. On the basis of co-chromatography with a reference compound peak 7 was identified as quercein-3-glucoside.

**Peak F7** ($t_R$ – 33.4 min) was a major flavonol in the onion extract (Figure 3-1) and produced a typical quercetin glucoside mass spectrum ([M-H]$^-$ at m/z 463 and a MS$^2$ ion at m/z 301). Co-chromatography established the identity of F7 as quercetin-4'-glucoside. However, peak F7 also contained traces amounts of a methylquercetin glucoside which had a $t_R$ of 33.2 min and co-chromatographed with isorhamnetin-3-glucoside. Both compounds had a [M-H]$^-$ at m/z 477 and a MS$^2$ ion at m/z 315.
Peak F8 ($t_R - 34.7$ min) produced a [M-H]$^-$ at $m/z$ 447 which with a loss of 162 yielded a MS$^2$ at $m/z$ 285. It was, therefore, a kaempferol glucoside, possibly kaempferol-4’-glucoside, which elutes after, quercetin-4’-glucoside (F7) and has previously been identified in onion extracts by (Tsushida & Suzuki, 1995).

Peak F9 ($t_R - 36.2$ min) was identified asisorhamnetin-4’-glucoside on the basis of its mass spectrum [M-H]$^-$ at $m/z$ 477 and a MS$^2$ ion at $m/z$ 315 and co-chromatography with an authentic standard.

Peak F10 ($t_R - 43.2$ min) was identified as the aglycone quercetin on the basis of MS$^2$ and HPLC retention properties. It yielded a [M-H]$^-$ at $m/z$ 301 which fragmented to produced characteristic MS$^2$ ions at $m/z$ 179 and 151 (Mullen et al., 2003a). This identification was confirmed by co-chromatography with a standard.

The total amount of flavonols in the 270 g onion meal was $275 \pm 8.8$ µmole. However, the major components, quercetin-3,4’-diglucoside (107 ± 1.4 µmole), quercetin-4’-glucoside (143 ± 12 µmole) and isorhamnetin-4’-glucoside (11 ± 1.4 µmole), accounted for 95% of the flavonol intake.

### 3.3.2 Analysis of plasma and urine

Plasma collected one hour after ingestion of the fried red onions, and urine excreted 0-4 h after eating the meal, were also analysed by HPLC with diode array and MS$^2$ detection. No peaks were apparent in the 515 nm traces obtained with either urine or plasma. With the sample sizes analysed and the limit of detection at $A_{515\ nm}$, anthocyanins at levels $\geq 0.1\%$ of the amounts ingested would have been detected. This finding is in keeping with other reports on the fate of dietary anthocyanins following absorption. The picture that has emerged is that a variety of anthocyanins appear in urine after supplementation with berries or berry extracts but at best in extremely low concentrations, typically 0.1% or less of the ingested dose (Felgines et al., 2002; Wu et al., 2002).

In contrast to the anthocyanins, sizable quantities of flavonols were present in plasma and urine, corresponding to ca. 2% of the intake, with a total of 23 quercetin-based compounds being detected in the plasma and urine samples. Typical HPLC traces obtained at $A_{365\ nm}$ are illustrated in Figure 3-2 and the identifications based on MS$^2$ spectra are summarised in Table 3-3.
Table 3-1. HPLC-MS-MS identification of anthocyanins in fried red onions. Peak numbers refer to HPLC trace in Figure 3-1

<table>
<thead>
<tr>
<th>Peak</th>
<th>Anthocyanin Name</th>
<th>[M+H]</th>
<th>Amount ingested</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>cyanidin-3-glucoside</td>
<td>449</td>
<td>9.3 ± 0.3</td>
</tr>
<tr>
<td>A-2</td>
<td>cyanidin-3-laminaribioside</td>
<td>611</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>A-3</td>
<td>cyanidin-3-(3''-glucosyl-6''-malonylglucoside)</td>
<td>697</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>A-4</td>
<td>cyanidin-3-(3''-malonylglucoside)</td>
<td>535</td>
<td>0.4 ± 0.02</td>
</tr>
<tr>
<td>A-5</td>
<td>cyanidin-3-(6''-malonylglucoside)</td>
<td>535</td>
<td>48 ± 1.7</td>
</tr>
<tr>
<td>A-6</td>
<td>cyanidin-3-(6''-malonyllaminaribioside)</td>
<td>697</td>
<td>14 ± 0.5</td>
</tr>
<tr>
<td>A-7</td>
<td>peonidin-3-malonylglucoside</td>
<td>549</td>
<td>1.3 ± 0.06</td>
</tr>
</tbody>
</table>

$\mu_t$ - retention time; [M+H]$^+$ - positively charged molecular ion; C – cyanidin; Peo – peonidin; Glc - glucoside unit; Lb, laminaribiose unit; mal - malonyl unit; malGlc - malonylglucoside unit. T - tentative
Table 3-2. HPLC-MS-MS identification of flavonols in fried red onions. Peak numbers refer to HPLC trace in Figure 3-1

<table>
<thead>
<tr>
<th>Peak</th>
<th>retention time</th>
<th>compound</th>
<th>[M-H] ( m/z )</th>
<th>MS² fragment ions ( m/z )</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>12.9</td>
<td>quercetin triglucoside</td>
<td>787</td>
<td>625 ([M-H] -Glc), 463 ([M-H] -Glc-Glc), 301 [Q][([M-H] -Glc-Glc-Glc)</td>
</tr>
<tr>
<td>F2</td>
<td>21.5</td>
<td>quercetin-7,3'-diglucoside</td>
<td>625</td>
<td>463 ([M-H] -Glc), 301 [Q][([M-H] -Glc-Glc)</td>
</tr>
<tr>
<td>F3</td>
<td>22.8</td>
<td>quercetin-3,4'-diglucoside</td>
<td>625</td>
<td>463 ([M-H] -Glc), 301 [Q][([M-H] -Glc-Glc)</td>
</tr>
<tr>
<td>F5</td>
<td>24.5</td>
<td>isorhamnetin-3,4'-diglucoside</td>
<td>639</td>
<td>477 ([M-H] -Glc), 315 [Iso][([M-H] -Glc-Glc)</td>
</tr>
<tr>
<td>F6</td>
<td>28.5</td>
<td>quercetin-3-glucoside</td>
<td>463</td>
<td>301 [Q][([M-H] -Glc)</td>
</tr>
<tr>
<td>F7</td>
<td>33.2</td>
<td>isorhamnetin-3-glucoside</td>
<td>477</td>
<td>315 [Iso][([M-H] -Glc)</td>
</tr>
<tr>
<td>F7</td>
<td>33.4</td>
<td>quercetin-4'-glucoside</td>
<td>463</td>
<td>301 [Q][([M-H] -Glc)</td>
</tr>
<tr>
<td>F8</td>
<td>34.7</td>
<td>kaempferol-4'-glucoside</td>
<td>447</td>
<td>285 [K][([M-H] -Glc)</td>
</tr>
<tr>
<td>F9</td>
<td>36.2</td>
<td>isorhamnetin-4'-glucoside</td>
<td>477</td>
<td>315 [Iso][([M-H] -Glc)</td>
</tr>
<tr>
<td>F10</td>
<td>43.2</td>
<td>quercetin</td>
<td>301</td>
<td>179, 151</td>
</tr>
</tbody>
</table>

\( t_R \) - retention time; [M-H] \(-\) - negatively charged molecular ion; Glc - glucosyl unit; Iso - isorhamnetin; K – kaempferol; Q – quercetin.
The different classes of metabolites that were detected can be summarised as follows:

*Quercetin monoglucuronides.* Three quercetin monoglucuronides were detected, each being characterised by a negatively charged molecular ion [M-H]$^-$ at $m/z$ 477 which on MS$^2$ fragmented with a 176 loss indicative of a glucuronide unit to produce a quercetin fragment at $m/z$ 301. Co-chromatography with reference compounds on the Synergi Max-RP HPLC column facilitated the identification of quercetin-4'-glucuronide, (peak 18) and quercetin-3'-glucuronide (peak 19) (Figure 1-5). However, peak 10, a significant component in both plasma and urine, co-chromatographed with both quercetin-3-glucuronide, quercetin-7-glucuronide (Figure 1-5). A second HPLC system...
using a Synergi Polar-RP column was therefore used to resolve these components (Figure 3-3). Quercetin-7-glucuronide had a $t_R$ of 34.6 min and quercetin-3-glucuronide eluted later with a $t_R$ of 36.2 min. When plasma and urine were analysed using the Polar-RP column, peak 10 co-chromatographed with quercetin-3-glucuronide (Figure 3-3 A and B) and there was no evidence of the presence of quercetin-7-glucuronide (Figure 3-3 C). This finding refutes claims by Spencer et al., (2003) that quercetin-7-glucuronide is one of the major in vivo metabolites of quercetin in humans.

**Quercetin diglucuronides.** Low levels of three quercetin diglucuronides, peaks 1,6 and 9, were present in urine. Each had a [M-H]$^-$ at m/z 653 which yielded MS$^2$ fragments at m/z 477 (M-176) and m/z 301 (M-352, cleavage of two glucuronide units). This indicates that the two glucuronyl units are attached at different positions on the quercetin skeleton. If they have been linked at the same position it is unlikely that a M-176 fragment would have been produced at m/z 477 as it has been shown that anthocyanin disaccharide conjugates fragment with loss of the intact disaccharide moiety (Giusti et al., 1999).

**Methylquercetin monoglucuronides** Two methylquercetin glucuronides were detected, peaks 17 and 20. Which were characterised by mass spectrum with fragment ions at m/z values 14 amu higher than obtained with quercetin glucuronides. Co-chromatography with reference compounds established that peak 17 was 3'-methylquercetin-3-glucuronide (isorhamnetin-3-glucuronide) and peak 20 was 3'-methylquercetin-4'-glucuronide (isorhamnetin-4'-glucuronide) (Figure 1-5).

**Methylquercetin diglucuronides.** Peaks 2 and 4 were identified as methylquercetin diglucuronides on the basis of mass spectra with fragment ions at m/z values 14 amu higher than obtained with quercetin diglucuronides.

**Quercetin.** The plasma of one of the six volunteers contained traces of the aglycone, quercetin, which had a [M-H]$^-$ at m/z 301 and MS$^2$ fragment ions at m/z 179 and 151.

**Quercetin sulphates.** Peaks 22 and 23 had a [M-H]$^-$ at m/z 381 and MS$^2$ yielded a major ion at m/z 301 (M-80) which is in-keeping with the fragmentation of a quercetin sulphate. On the basis of co-chromatography with a reference, compound peak 22 was identified as quercetin-3'-sulphate (Figure 1-5), which was the major quercetin
metabolite in plasma. However, the position of the sulphate group on the quercetin sulphate in peak 23 remains undetermined.

**Quercetin glucuronide sulphates.** Peaks 13 and 14 both produced a \([M-H]\) at \(m/z\) 381 and \(MS^2\) yielded ions at \(m/z\) 477 (M-80, loss of SO₃), \(m/z\) 463 (M-176, loss of a glucuronide unit) and \(m/z\) 301 (quercetin) indicating that both compound are quercetin glucuronide sulphates. Peak 14 was the main quercetin metabolite in the urine of all volunteers.

**Quercetin glucosides.** The plasma of one volunteer contained traces of quercetin-3,4'-diglucoside as well as small amounts of quercetin-3-glucoside and its methylated derivative isorhamnetin-3-glucoside (Figure 1-5). The mass spectra of these compounds are summarised in Table 3-3 and in all three instances identity was established by co-chromatography with the appropriate standard.

**Quercetin glucoside glucuronides.** Peaks 3, 7 and 8 which were detected in urine had a \([M-H]\) at \(m/z\) 639 which on \(MS^2\) yielded ions at \(m/z\) 477 (M-162, loss of glucose), \(m/z\) 463 (M-176, loss of a glucuronide unit) and \(m/z\) 301 indicating that both compound are quercetin glucoside glucuronides.

**Quercetin glucoside sulphates.** Peaks 12 and 15 were characterised by a \([M-H]\) at \(m/z\) 543 which when fragmented produced \(MS^2\) ions at \(m/z\) 463 (M-80, loss of SO₃), \(m/z\) 381 (M-162, loss of a glucoside unit) and \(m/z\) 301 demonstrating the presence of quercetin glucoside sulphates.
Table 3-3. HPLC-MS-MS identification of quercetin metabolites detected in plasma and urine after the consumption of 270 g of fried red onions by six human volunteers. Peak numbers and HPLC retention times refer to HPLC trace in Figures. 3-2A and 3-2B

<table>
<thead>
<tr>
<th>peak</th>
<th>( t_R ) (min)</th>
<th>compound</th>
<th>([\text{M-H}]^-) (m/z)</th>
<th>MS (^2) fragments ions (m/z)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.6</td>
<td>quercetin diglucuronide</td>
<td>653</td>
<td>477([M -H]^-GlcUA), 301 [Q][M -H]^--GlcUA -GlcUA)</td>
<td>urine</td>
</tr>
<tr>
<td>2</td>
<td>20.4</td>
<td>methylquercetin diglucuronide</td>
<td>667</td>
<td>491([M -H]^-GlcUA), 315 [MQ][M -H]^-GlcUA -GlcUA)</td>
<td>urine</td>
</tr>
<tr>
<td>4</td>
<td>22.7</td>
<td>methylquercetin diglucuronide</td>
<td>667</td>
<td>491([M -H]^-GlcUA), 315 [MQ][M -H]^-GlcUA -GlcUA)</td>
<td>urine</td>
</tr>
<tr>
<td>5</td>
<td>22.8</td>
<td>quercetin -3,4*-diglucoside*</td>
<td>625</td>
<td>463([M -H]^-Glc), 301 [Q][M -H]^-Glc -Glc)</td>
<td>plasma</td>
</tr>
<tr>
<td>6</td>
<td>24.8</td>
<td>quercetin diglucuronide</td>
<td>653</td>
<td>477([M -H]^-GlcUA), 301 [Q][M -H]^-GlcUA -GlcUA)</td>
<td>urine</td>
</tr>
<tr>
<td>8</td>
<td>27.0</td>
<td>quercetin glucoside glucuronide</td>
<td>639</td>
<td>477([M -H]^-Glc), 463([M -H]^-GlcUA), 301 [Q][M -H]^-Glc -GlcUA)</td>
<td>urine</td>
</tr>
<tr>
<td>9</td>
<td>27.4</td>
<td>quercetin diglucuronide</td>
<td>653</td>
<td>477([M -H]^-GlcUA), 301 [Q][M -H]^-GlcUA -GlcUA)</td>
<td>urine, plasma</td>
</tr>
<tr>
<td>10</td>
<td>28.4</td>
<td>quercetin -3-glucoside</td>
<td>477</td>
<td>301 [Q][M -H]^-GlcUA)</td>
<td>urine, plasma</td>
</tr>
<tr>
<td>11</td>
<td>28.4</td>
<td>quercetin -3-glucoside*</td>
<td>463</td>
<td>301 [Q][M -H]^-Glc)</td>
<td>plasma</td>
</tr>
<tr>
<td>12</td>
<td>29.6</td>
<td>quercetin glucoside sulfate</td>
<td>543</td>
<td>463([M -H]^-SO_3), 381([M -H]^-Glc), 301 [Q][M -H]^-SO_3 -Glc</td>
<td>urine</td>
</tr>
<tr>
<td>16</td>
<td>33.2</td>
<td>isorhamnetin -3-glucoside*</td>
<td>477</td>
<td>315 [Iso][M -H]^-Glc</td>
<td>plasma</td>
</tr>
<tr>
<td>17</td>
<td>33.1</td>
<td>isorhamnetin -3-glucuronide</td>
<td>491</td>
<td>315 [MQ][M -H]^-GlcUA)</td>
<td>urine, plasma</td>
</tr>
<tr>
<td>18</td>
<td>34.4</td>
<td>quercetin -4'-glucuronide</td>
<td>477</td>
<td>301 [Q][M -H]^-GlcUA)</td>
<td>urine</td>
</tr>
<tr>
<td>19</td>
<td>36.3</td>
<td>quercetin -3'-glucuronide</td>
<td>477</td>
<td>301 [Q][M -H]^-GlcUA)</td>
<td>urine, plasma</td>
</tr>
<tr>
<td>20</td>
<td>37.2</td>
<td>isorhamnetin -4'-glucuronide</td>
<td>491</td>
<td>315 [MQ][M -H]^-GlcUA)</td>
<td>urine, plasma</td>
</tr>
<tr>
<td>21</td>
<td>43.2</td>
<td>Quercetin*</td>
<td>301</td>
<td>179, 151</td>
<td>plasma</td>
</tr>
<tr>
<td>22</td>
<td>47.9</td>
<td>quercetin -3'-sulfate</td>
<td>381</td>
<td>301 [Q][M -H]^-SO_3</td>
<td>urine, plasma</td>
</tr>
<tr>
<td>23</td>
<td>48.3</td>
<td>quercetin sulfate</td>
<td>381</td>
<td>301 [Q][M -H]^-SO_3)</td>
<td>plasma</td>
</tr>
</tbody>
</table>

\( t_R \) - retention time; \([\text{M-H}]^-\) - negatively charged molecular ion; Iso - isorhamnetin; K – kaempferol; MQ – methylquercetin; Glc – glucosyl unit; GlcUA – glucuronyl unit; *indicates compounds detected in the plasma of only one of the six volunteers.
3.4 Discussion

In the previous chapter a method was developed to determine the identity and quantity of flavonol metabolites after a feed of [2-14C]quercetin-4'-glucoside. A total of 19 metabolites were detected and 18 of these identified. In this study 10 of the 18 identified metabolites were also observed. However, the location and relative quantities of the metabolites was different. For example the main plasma peak in the rat study, a methyl quercetin glucuronide sulphate was not found in human plasma. Conversely, the main plasma peak in humans, the quercetin-3'-sulphate was found only in the intestine of the rats. One of the major differences between the rat and human metabolic profiles is the increased number and levels of methyl metabolites. In addition, the rat did not produce any mono sulphate metabolites. A total of 19 quercetin metabolites were found in the plasma and urine of all the volunteers. One volunteer had 4 compounds in plasma that were not detected in samples from any of the other volunteers. These were the aglycone quercetin and 3 of the origin sugar conjugates (Table 3-3).

The present study is the first to report on the use of HPLC with full scan MS$^2$ and absorbance detection to analyse flavonols appearing in human plasma and urine. It provides a much more detailed analysis than was achieved in earlier studies carried out after the ingestion of onions which utilised HPLC with either MS$^2$ in SRM mode (Wittig et al., 2001) or MS in the SIM mode (Day et al., 2001). Both these studies identified the quercetin metabolites with MS in the positive ion mode. However, quantification was made separately using another HPLC system with electrochemical or absorbance detection systems respectively. Negative and positive ionisation with our MS instrumentation provides very similar limits of detection for a standard of quercetin-3-glucoside. However, when a urine sample is spiked with quercetin-3-glucoside, analysis with negative ionisation is about 10 times more sensitive than positive ionisation because there is a much lower background and hence an enhanced signal-to-noise ratio.

During the development of this analytical system it became apparent that no single method for the identification and quantification of quercetin metabolites could be relied upon. It should also be borne in mind when developing a chromatographic system
to investigate novel metabolites that there is sufficient separating capacity to cope with as many potential problems as possible. In our system the earliest eluting major flavonol from the feed, quercetin-3,4′-diglucoside elutes after 22.8 minutes of a 60 minute chromatogram. This is to prevent the more polar diglucuronide metabolites and potential triglucuronides eluting in the void volume peak. Studies presented here have now identified four peaks that elute earlier than quercetin-3,4′-diglucoside.

The importance of having the right chromatographic conditions to adequately resolve peaks is vital when using only retention time data and an absorbance, fluorescence or electrochemical detection system. In fact the less specific the detection system the greater the importance that the chromatographic system is optimised to compensate for the lack of specificity. However, when using a mass spectrometer its identifying power should not be overestimated, even in full scan mode.

As previously discussed the study by Wittig *et al.*, (2001) investigating flavonol levels in human plasma after an 800 g onion feed, five quercetin glucuronides were detected by SRM analysis. This monitored, in positive ion mode, the fragmentation of the [M+H]+ ion at m/z 479 fragmenting to m/z 303, the aglycone of quercetin. Although there are five possible points of glucuronidation of the aglycone only four glucuronide metabolites were formed *in vitro* by UDP-glucuronyl transferase. No sulphates were detected in this study, which is contrary to the findings here (Day *et al.*, 2000) and those of (Day *et al.*, 2000) in another *in vitro* study using human liver cell free extracts. Our analysis found only three such mono glucuronide metabolites. However, the major quercetin diglucuronide (peak 9) and quercetin glucuronide sulphate (peak 14) both produced strong m/z 477 fragment ions in negative ionisation full scan mode. In the selected reaction monitoring method used by Wittig *et al.*, (2001) these fragment ions would have produced a positive response for a mono-glucuronide thus giving a false positive result.

Although full scan MS2 would seem to answer all the problems of identification, over reliance on it can lead to mis-identification. A strong signal for a peak with an m/z of 477 and MS2 predominant fragment ion at m/z 301 would, at first glance, seem strong evidence for a quercetin mono-glucuronide. However, the presence of the molecular ion in the MS2 scan and additional ions at m/z 459 and m/z 415 is not typical of the loss of a
glucuronide moiety (Figure 3-4). It is sometimes forgotten that the absence of ions in a spectra is as important as their presence. Again this compound would have shown up as a false positive in Wittig’s analysis. SIM would also have shown a false positive peak in this analysis, as discussed in the previous chapter. However, use of the absorbance detection system to aid identification can provide vital evidence for potential misidentification. The absorbance spectrum provided further evidence of this compound not being a quercetin metabolite as there was no characteristic flavonol spectrum displaying a 365 nm maxima. The MS$^3$ analysis of this compound is shown in Figure 2-6.

![Figure 3-4. MS$^3$ of $m/z$ 477 (A) quercetin-3-glucuronide and (B) unknown in plasma](image)

The value of full scan data dependant MS$^2$ was seen in the analysis of the “exceptional” volunteer. Quercetin-3-glucoside and quercetin-3-glucuronide were found in the plasma of this volunteer (Figure 3-2). Both compounds eluted at 28.4 mins. and
their absorbance spectra cannot be used to differentiate them. In this case the MS$^2$ capability of our system allowed the identification of both compounds. The relative areas of the molecular ions were used to calculate the concentration of the glucuronide reported. Although MS$^2$ can be used to get around such a problem, if the plasma of all subjects contained both compounds the system would have to be altered to obtain separation of these compounds.

Full scan MS$^2$ also allowed the identification of a further four unexpected metabolites in the plasma of this volunteer, which, like all other metabolites were quantified by the absorbance detector. The use of two detection systems, one to identify and one to quantify the metabolites, in this study prevented possible misidentifications and allowed accurate determination of the quantities present.

Post-column reaction with aluminium nitrate has a better limit of detection, ca. 3 pg on-column (Hollman et al., 1996b) for a standard but 100 pg in an extract (Hollman et al., 1997b), than the absorbance system used in our study. However, it can detect only metabolites with a free three hydroxyl group. Therefore is of no use in studies involving intact metabolites. However, if this method was used along with absorbance detection a check on peak purity would be possible, as the ratio of absorbance peak to the fluorescence peak should also match that of a standard. Thus giving a method of detecting false positives for all but the smallest peaks. A serious practical disadvantage of the fluorescence system is coupling it to a mass spectrometer would be difficult and could prove damaging to the mass spectrometer if the aluminium nitrate was pumped into it.

The absorbance detector used here had a limit of detection of 400 pg injected on column, which proved sufficient to allow measurement of the 18 metabolites of quercetin over the course of the experiment. Using the mass spectrometer in selected reaction monitoring mode we were able to detect and quantify levels as low as 200 pg of quercetin-4′-glucoside. This was used to confirm that the 0 h sample contained levels that were below these detection limits. Samples of 500 µL of plasma proved adequate to allow accurate measurement of metabolites by absorbance detection and full scan dependant MS$^2$ had sufficient sensitivity to allow positive identification of these peaks.
3.5 Conclusion

The data presented here demonstrates the value of gradient HPLC with absorption detection used in conjunction with data dependent full scan MS\(^2\) for the analysis of trace levels of natural products in impure extracts. When reference compounds are available trace quantities of analyte can be identified. In the absence of standards, MS\(^2\) can facilitate a degree of structural elucidation, such as that obtained with quercetin glucuronide sulphates, methylquercetin glucuronides and quercetin diglucuronides (Table 3-3), that would not be possible with traditional single stage MS. This has been discussed in some detail with specific reference to quercetin metabolites in earlier publications (Mullen et al., 2002a; 2003a). Further information on the position and orientation of substituent groups would require the use of NMR. However, this would involve not only extensive sample purification but also a requirement for several orders of magnitude more analyte than the low nanogram quantities required for HPLC-MS\(^2\). With many of the plasma and urinary quercetin metabolites detected in trace amounts in the present study, this would not be a practical proposition. The use of the absorbance detection system provided an accurate method to facilitate the selective quantification of these compounds.

It would appear from the results of previous studies (Hollman et al., 1996a; Erlund et al., 1999; Day et al., 2001; Wittig et al., 2001), and experience gained in developing this method there is no single detection system that can be used to analyse the levels of intact flavonol metabolites in plasma. Although we could use HPLC-PDA-MS\(^2\) in our analysis, expecting that this level of analytical instrumentation is necessary for analysis would be an extreme request. However, some measures when using simpler systems should be taken to reduce the risks of potential errors being made.
In the case of the analytical system employed by Hollman et al., (1996a; 1997) and Erlund et al. (1999) use of a gradient HPLC method would have reduced the risk of any co-elution with interfering compounds. The wish to have a fast isocratic analysis is always superficially attractive but it comes with risks. Furthermore, the post-column fluorescence method may be very selective for flavonols, with only those with a free 3 hydroxyl group fluorescing, but as can be seen in Figure 3-5 there are a multitude of other compounds that also fluoresce when using this method. Hollman states that methylquercetin cannot be measured in this method but this is not due to its inability to react with the aluminium nitrate but due to the large peak that elutes after quercetin, and which has the same retention time as methylquercetin. In brief, the less selective the detection method the greater the need for increased chromatographic separation. A
second detection system can provide additional information that can indicate potential co-elution of compounds. This concept will be discussed in more detail in Chapter 4.

In this chapter a method that can both identify and quantify intact metabolites of quercetin was used to measure these compounds in plasma and urine. In the following chapter this method will be used to generate pharmacokinetic data from plasma collected over 24 h from the same onion feed used in this chapter. Bioavailability and metabolism data will also be discussed.
Chapter 4  Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions

4.1  Introduction

The previous two chapters have described the use of HPLC-PDA-MS\textsuperscript{2}, with and without a radiolabelled standard, to detect metabolites formed in feeds with quercetin-4′-glucoside to rats and humans. Flavonols, like quercetin-4′-glucoside, have had several potential nutritional and health promoting roles in the human body attributed to them (Duthie et al., 2000), but there is still much to be learnt about their bioavailability and, in particular, which metabolites appear in plasma and in what amounts. This information is essential to understanding the potential role of these compounds in reducing coronary heart disease, strokes and possibly cancer, as it is likely that the metabolites do not have the same bioactivity as the parent compound. To gain a full picture of the absorption and metabolism of flavonols it is essential to be able to detect and quantify all the major metabolites in plasma and urine and this requires the use of appropriate analytical methodology such as described in the previous chapters.

Quercetin is the major flavonol in many foods including onions which consistently contain high levels of this flavonol (Crozier et al. 1998). It is now believed that absorption of quercetin glucosides from the gastrointestinal tract involves deglycosylation by luminal lactase phloridzin hydrolase and/or cleavage within the enterocyte by cytosolic β-glucosidase (Day et al. 2003a).

In Chapter 3 the results from the qualitative analysis of a feed Mullen et al., (2004) of 270 g of lightly fried onions to human volunteers was reported. Using HPLC with photodiode array (PDA) and MS\textsuperscript{2} detection, 23 quercetin-based compounds were identified in plasma and urine collected 1 h and 0-4 h, respectively, after ingestion.

This chapter describes an extension of this study with the quantitative analysis of metabolites in plasma and urine collected over a 24 h post ingestion period. The data are
discussed with regard to the potential metabolic pathways that operate within the body following the ingestion of a quercetin glucoside rich meal.

4.2 Methods

4.2.1 Study design

Six volunteers (4 males and 2 females), who were healthy, non-smokers and not on any medication, participated in this study and gave their written consent. They were aged between 23 and 45 years and had a mean body mass index of 23.7 (range 20.9-27.6). Subjects were required to follow a low flavonoid diet for two days and to fast overnight prior to supplementation. This diet excluded most fruits, vegetables and beverages including tea, coffee, fruit juices, and wine. On the morning of the study onions (Allium cepa) were skinned, chopped into small slices, and fried for 4 min in margarine. Aliquots of the fried onions were taken for qualitative and quantitative analysis of their flavonol content.

All subjects consumed 270 g of fried onions. Venous blood samples were taken before (0 h) and 0.5, 1, 2, 3, 6 and 24 h post-ingestion. Twelve ml of blood was collected in heparinised tubes at each time point and immediately centrifuged at 4000 g for 10 min at 4°C. The plasma was separated from the red blood cells and 500 µL aliquots were acidified to pH 3 with 15 µL of 50% aqueous formic acid and 50 µL of ascorbic acid (10 mmole/L) added to prevent oxidation. The plasma samples were then stored at -80°C prior to analysis. Urine was collected before and over 0-4, 4-8 and 8-24 h periods after the consumption of the fried onion supplement. The volume of each sample was recorded prior to acidification to pH 3.0 and the storage of aliquots at –80°C. The study protocol was approved by the Glasgow Royal Infirmary Local Research Ethics Committee.

4.2.2 Materials and Methods

All details were as described in Chapter 3.
4.2.3 Pharmacokinetic analysis of plasma metabolites

Maximum post-ingestion plasma concentration of quercetin metabolites was defined as $C_{\text{max}}$. The time to reach maximum plasma concentration ($t_{\text{max}}$) was defined as the time in hours at which $C_{\text{max}}$ was reached. The elimination half-life for the metabolites in hours was computed by using the following formula $t_{1/2} = \frac{0.693}{K_e}$ where $K_e$ is the slope of the linear regression of the log of 0-6 h plasma metabolite concentrations.

4.3 Results

4.3.1 Analysis of fried onions

Gradient reverse phase HPLC with absorbance detection and full scan data dependent MS$^2$ was used to identify and quantify the flavonol and anthocyanin content of the fried red onion meals. Absorbance at 365 nm and negative ionisation MS$^2$ were used for flavonol analysis. The total amount of flavonols in the 270 g onion meal was 275 ± 8.8 µmole. In keeping with the data of Tsushida & Suzuki, (1995), the major components were quercetin-3,4'-diglucoside, (107 ± 1.4 µmole), quercetin-4'-glucoside (143 ± 12 µmole) and isorhamnetin-4'-glucoside (11 ± 1.4 µmole) (Figure 1-5) which accounted for 95% of the 275 ± 8.8 µmole flavonol intake.

4.3.2 Qualitative analysis of plasma and urine

Plasma and urine samples were analysed by HPLC with PDA and MS$^2$ detection. Flavonol metabolites were present in plasma and urine, corresponding to ca. 4% of the intake, with a total of 23 quercetin-based compounds being detected. Typical HPLC traces obtained at $A_{365}$ nm are illustrated in Figure 3-2 and the identifications based on MS$^2$ spectra and $t_R$ data are summarised in Table 3-3. The use of HPLC-MS$^2$ to identify these quercetin metabolites has been discussed in detail in Chapter 3 and by (Mullen et al., 2004).

4.3.3 Quantitative analysis of flavonol metabolites in plasma
Eleven quercetin metabolites were detected in plasma in quantities that facilitated either their full or partial identification as outlined in Chapter 3 (Table 3-3). Those present in sufficient quantities to enable pharmacokinetic profiles to be obtained were a quercetin diglucuronide \((\text{peak 9})\), a quercetin glucuronide sulphate \((\text{peak 14})\), quercetin-3-glucuronide, isorhamnetin-3-glucuronide and quercetin-3'-sulphate (Figure 1-5). Quercetin-3'-glucuronide and isorhamnetin-4'-glucuronide was present in the plasma of all volunteers in low, non-quantifiable, amounts while other flavonol derivatives, quercetin-3,4'-diglucoside, quercetin-3-glucoside, isorhamnetin-3-glucoside and the aglycone quercetin were detected, albeit in very small quantities, only in the plasma of volunteer 6 (Table 3-3).

The 0-6 h pharmacokinetic profiles of the five major plasma flavonol metabolites are illustrated in Figure 4-1. No quercetin metabolites were present in plasma samples collected at either prior (0 h) or 24 h after supplementation. This was confirmed using the enhanced sensitivity and selectivity of MS\(^2\) in the selected reaction monitoring mode. Pharmacokinetic analyses of the 0-24 h data points are summarised in Table 4-1. The two main metabolites which accumulated in plasma were quercetin-3'-sulphate and quercetin-3-glucuronide. These compounds had a \(C_{\text{max}}\) of 665 ± 82 nmole/L and 351 ± 27 nmole/L, respectively. In both instances \(T_{\text{max}}\) was less than 1 h after the ingestion of the onion supplement (Table 4-1). A quercetin diglucuronide \((\text{peak 9})\) had a similar \(T_{\text{max}}\) (0.80 ± 0.12 h) but a lower \(C_{\text{max}}\) (62 ± 12 nmole/L) than the two main metabolites. The levels of all three metabolites declined after reaching \(C_{\text{max}}\) and they had a similar \(T_{1/2}\) with values of 1.71-2.33 h (Table 4-1). The pharmacokinetic profiles of isorhamnetin-3-glucuronide \((\text{peak 17})\) and quercetin glucuronide sulphate \((\text{peak 14})\) were different to those of the other metabolites illustrated in (Figure 4-1). Isonhamnetin-3-glucuronide had a \(C_{\text{max}}\) of 112 ± 18 nmole/L and a \(T_{\text{max}}\) of 0.60 h and there was a slow rate of decline after \(C_{\text{max}}\) which is reflected in a \(T_{1/2}\) of 5.34 h (Table 4-1). The \(C_{\text{max}}\) of the quercetin glucuronide sulphate \((\text{peak 14})\) was 123 ± 26 nmole/L while its \(T_{\text{max}}\) at 2.5 ± 0.22 h was delayed compared to that of the other metabolites and the \(T_{1/2}\) (4.54 h) was much slower than that observed with quercetin-3'-sulphate, quercetin-3-glucuronide and the quercetin diglucuronide (Table 4-1).
Figure 4-1. Concentration of (A) quercetin-3'-sulphate, quercetin-3-glucuronide (B) a quercetin glucuronide sulphate, isorhamnetin-3-glucuronide and a quercetin diglucuronide in plasma from six human volunteers collected 0-6 h after the ingestion of onions. Data expressed as mean values in nmole/L ± standard error (n = 6).
Table 4-1. Pharmacokinetic parameters of quercetin metabolites in the plasma of human volunteers after the consumption of onions.

<table>
<thead>
<tr>
<th>Metabolite (peak number)</th>
<th>$C_{\text{max}}$</th>
<th>$T_{\text{max}}$</th>
<th>$T_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin-3'-sulphate (22)</td>
<td>665 ± 82</td>
<td>0.75 ± 0.12</td>
<td>1.71</td>
</tr>
<tr>
<td>Quercetin-3-glucuronide (10)</td>
<td>351 ± 27</td>
<td>0.60 ± 0.10</td>
<td>2.33</td>
</tr>
<tr>
<td>Isorhamnetin-3-glucuronide (17)</td>
<td>112 ± 18</td>
<td>0.60 ± 0.10</td>
<td>5.34</td>
</tr>
<tr>
<td>Quercetin diglucuronide (9)</td>
<td>62 ± 12</td>
<td>0.80 ± 0.12</td>
<td>1.76</td>
</tr>
<tr>
<td>Quercetin glucuronide sulphate (14)</td>
<td>123 ± 26</td>
<td>2.5 ± 0.22</td>
<td>4.54</td>
</tr>
</tbody>
</table>

$C_{\text{max}}$ – maximum concentration in plasma expressed in nmole/L. $T_{\text{max}}$ – time to reach $C_{\text{max}}$ expressed in h. $T_{1/2}$ – the elimination half-life of metabolites in h. Data presented as mean values ± standard error (n = 6). Peak numbers as in Figure 3-3

4.3.4 Quantitative analysis of flavonol metabolite excretion in urine

Eighteen flavonol metabolites were detected in urine samples collected 0-4, 4-8 and 8-24 h after the ingestion of red onions (Table 3-3). Six of these compounds, two quercetin diglucuronides (peaks 1 and 6), two quercetin glucoside sulphates (peaks 7 and 8), quercetin-4'-glucuronide and quercetin-3'-sulphate (Figure 1-5), were present in quantities insufficient for routine quantification.

Twelve urinary metabolites were detected in amounts that facilitated quantitative analysis (Table 4-2). These metabolites consisted of quercetin-3-glucuronide, quercetin-3'-glucuronide a quercetin diglucuronide, a quercetin glucuronide glucoside, two quercetin glucuronide sulphates, two quercetin glucoside sulphates, isorhamnetin-3-glucuronide, isorhamnetin-4'-glucuronide and two methylquercetin diglucuronides. The main urinary metabolite present was a quercetin diglucuronide (peak 9) with 2223 ± 417 nmole being excreted over the 24 h period following ingestion of the onion supplement. Substantial amounts of quercetin-3'-glucuronide (1845 ± 193 nmole), isorhamnetin-3-glucuronide (1789 ± 27 nmole) and two quercetin glucuronide sulphates (peak 13, 1384 ± 163 nmole; peak 14, 1229 ± 190 nmole) were also detected.
Table 4-2. Concentration of quercetin metabolites in the urine of human subjects 0-24 h after the consumption of an onion supplement.

<table>
<thead>
<tr>
<th>Metabolites (peak number)</th>
<th>0 - 4 h</th>
<th>4 -8 h</th>
<th>8 -24 h</th>
<th>0-24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin-3-glucuronide (10)</td>
<td>512 ± 101</td>
<td>400 ± 113</td>
<td>n.d.</td>
<td>912 ± 149</td>
</tr>
<tr>
<td>Quercetin-3’-glucuronide (19)</td>
<td>979 ± 220</td>
<td>804 ± 194</td>
<td>62 ± 30</td>
<td>1845 ± 193</td>
</tr>
<tr>
<td>Quercetin diglucuronide (9)</td>
<td>1007 ± 253</td>
<td>942 ± 273</td>
<td>274 ± 98</td>
<td>2223 ± 417</td>
</tr>
<tr>
<td>Quercetin glucuronide glucoside (3)</td>
<td>99 ± 21</td>
<td>64 ± 16</td>
<td>n.d.</td>
<td>163 ± 23</td>
</tr>
<tr>
<td>Quercetin glucuronide sulphate (13)</td>
<td>608 ± 124</td>
<td>566 ± 143</td>
<td>210 ± 73</td>
<td>1384 ± 163</td>
</tr>
<tr>
<td>Quercetin glucuronide sulphate (14)</td>
<td>743 ± 170</td>
<td>418 ± 98</td>
<td>68 ± 50</td>
<td>1229 ± 190</td>
</tr>
<tr>
<td>Quercetin glucoside sulphate (12)</td>
<td>226 ± 73</td>
<td>130 ± 34</td>
<td>35 ± 26</td>
<td>392 ± 60</td>
</tr>
<tr>
<td>Quercetin glucoside sulphate (15)</td>
<td>538 ± 127</td>
<td>257 ± 98</td>
<td>26 ± 11</td>
<td>821 ± 156</td>
</tr>
<tr>
<td>Isorhamnetin-3-glucuronide (17)</td>
<td>767 ± 18</td>
<td>861 ± 10</td>
<td>161 ± 10</td>
<td>1789 ± 239</td>
</tr>
<tr>
<td>Isorhamnetin-4’-glucuronide (20)</td>
<td>451 ± 11</td>
<td>249 ± 2</td>
<td>n.d.</td>
<td>700 ± 114</td>
</tr>
<tr>
<td>Methylquercetin diglucuronide (2)</td>
<td>439 ± 132</td>
<td>475 ± 67</td>
<td>89 ± 69</td>
<td>1003 ± 156</td>
</tr>
<tr>
<td>Methylquercetin diglucuronide (4)</td>
<td>189 ± 49</td>
<td>163 ± 41</td>
<td>74 ± 36</td>
<td>426 ± 99</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>6558 ± 1323</strong></td>
<td><strong>5329 ± 1018</strong></td>
<td><strong>999 ± 267</strong></td>
<td><strong>12886 ± 1038</strong></td>
</tr>
</tbody>
</table>

Mean values expressed as nmole ± standard error (n = 6). Peak numbers as in Table 3-3. n.d. – not detected.

4.4 Discussion

The results of this study have provided, for the first time, detailed quantitative concentrations of metabolites of methyl, glucuronyl and sulfo-conjugates of quercetin in the plasma and urine of human volunteers after ingestion of onions. The pharmacokinetics presented should allow better and more relevant studies of the bioactivity and role of dietary flavonols in disease prevention. Furthermore the data obtained here provides a better insight into the pathways involved with A.D.M.E. of quercetin and provides a platform from which theories on the A.D.M.E. of other flavonoids can be launched.

4.4.1 Quantitative analysis of flavonol absorption
The two major metabolites, quercetin-3'-sulphate and quercetin-3-glucuronide appeared in plasma within 30 min of the ingestion of onions, both had $T_{\text{max}}$ values of under 1 h and $T_{1/2}$ values of 1.71 and 2.33 h respectively. (Figure 4-1, Table 4-1). A quercetin diglucuronide (peak 9) with a lower $C_{\text{max}}$ and similar $T_{\text{max}}$ and $T_{1/2}$ values was also detected. The pharmacokinetic profiles of isorhamnetin-3-glucuronide and a quercetin glucuronide sulphate (peak 14) were somewhat different in that both had a much longer $T_{1/2}$ and the glucuronide sulphate also had a much delayed $T_{\text{max}}$. However, the total contribution of these two compounds to the overall absorption profile was minimal, having no effect on the $T_{\text{max}}$ and only extending the $T_{1/2}$ to 2.61 h. This $T_{1/2}$ is much shorter than similar absorption studies carried out previously Hollman et al., (1996a; 2000a) which, arguably, is a consequence of the enhanced accuracy of analytical data obtained by HPLC-MS$^2$.

The longer $T_{1/2}$ values obtained in these earlier studies are also accompanied by pre-feed plasma that contains conjugated quercetin. This was found after either acid or enzyme hydrolysis. Both of these systems employ a short isocratic HPLC analysis and a non-specific detection system, as can be seen in (Figure 3-5). Erlund et al., (1999) tried a variety of different columns in an attempt to separate the background peak from quercetin and being unable to do so concluded, with some justification, that the peak was, therefore, quercetin. In all our studies we have never encountered such a result. It cannot be due to a lack of sensitivity of our detection system as the level of the background peak is between 20 and 80 ng/mL (Hollman et al., 1996a). Some other factors that give backing to my results come from previously published results Hollman et al., (1996a; 2000); Jin et al., (2004a). As can be seen from Figure 4-2 the level of quercetin present in the pre-feed plasma of Hollman was between 20 and 80 ng/mL. This was after five days on a flavonol free diet. After a meal containing 62 mg of quercetin, plasma levels were back below the starting level within 12 hours with one subject. It is somewhat paradoxical that after five days without flavonols the background levels of quercetin present in plasma would be higher than that found 12 h after a high dose of quercetin.
Figure 4-2. Pharmacokinetic plot of quercetin levels in plasma before and after 62mg quercetin Hollman et al (1996). Horizontal lines have been added to show the levels of the T=0 samples.

Figure 4-3. Example of pre and post-meal hydrolysed plasma chromatogram of work by Erlund et al (1999). Quercetin peaks are at 7.80 mins
Figure 4-4. Example of (A) pre and (B) post-meal plasma extract by Jin et al. (2004) peak 1 is quercetin

The method used by Erlund et al., (1999) is technically more elaborate than that of Hollman et al., (1996b), in that there was a sample purification stage by C$_{18}$ solid phase extraction and partitioning against a dichloromethane/toluene mixture, which was needed to remove a co-eluting peak from the enzyme preparation. The resultant chromatogram (Figure 4-3) is much cleaner than that seen in Figure 3-5 and it seemed the only way to further investigate this work was to try and recreate it and include it into the mass spectrometric method developed in Glasgow. However, Jin et al., (2004b) carried out a similar investigation into flavonols from tea and used HPLC with electrochemical detection. In this method a 40 min gradient was needed to allow separation of quercetin from interfering peaks (Figure 4-4). Furthermore, there was only a 12 h period prior to the feed when quercetin containing drinks were not allowed to be consumed. No conjugated quercetin was found in the pre-feed plasma and although sampling did not occur after 6 h, the quercetin content had fallen to a level comparable to those in the current study (Figure 4-1).

Another study, which adds weight to my explanation was carried out by Moon et al., (2000). Again this was an onion feed to human volunteers and the level of quercetin, after enzyme hydrolysis, was measured by HPLC with electrochemical detection. However, the level of quercetin was also measured before and after the study prior to enzyme hydrolysis. In both pre hydrolysis samples small peaks can be seen at the
retention time of quercetin. In view of our data and that of Jin et al., (2004c) there appears to be no argument with regard to the fact that no free quercetin is found in plasma. Therefore, these interfering peaks could explain the high background seen in other studies.

Figure 4-5. HPLC chromatograms of extracts of human plasma taken from Moon et al., (2000). (A). extract of plasma before the trial. (B). extract of plasma after the trial. (C). extract of plasma after the trial and after enzyme hydrolysis.
Table 4-3 Mean levels of quercetin metabolites excreted in the urine of six human subjects 0-4, 4-8 and 8-24 h after the consumption of an onion supplement.

<table>
<thead>
<tr>
<th>Metabolite (peak numbers)</th>
<th>0 - 4 h</th>
<th>4-8 h</th>
<th>8 -24 h</th>
<th>0-24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylquercetin diglucuronides (2,4)</td>
<td>638 ± 159</td>
<td>648 ± 109</td>
<td>163 ± 79</td>
<td>1449 ± 240</td>
</tr>
<tr>
<td></td>
<td>(43%)</td>
<td>(45%)</td>
<td>(12%)</td>
<td></td>
</tr>
<tr>
<td>Quercetin glucoside glucuronide (3)</td>
<td>99 ± 21</td>
<td>64 ± 16</td>
<td>n.d.</td>
<td>163 ± 23</td>
</tr>
<tr>
<td></td>
<td>(61%)</td>
<td>(39%)</td>
<td>(0%)</td>
<td></td>
</tr>
<tr>
<td>Quercetin diglucuronide (9)</td>
<td>1008 ± 253</td>
<td>942 ± 274</td>
<td>274 ± 98</td>
<td>2223 ± 417</td>
</tr>
<tr>
<td></td>
<td>(56%)</td>
<td>(42%)</td>
<td>(12%)</td>
<td></td>
</tr>
<tr>
<td>Quercetin-3- and 3’-glucuronides</td>
<td>1491 ± 316</td>
<td>1204 ± 301</td>
<td>62 ± 30</td>
<td>2757 ± 322</td>
</tr>
<tr>
<td></td>
<td>(55%)</td>
<td>(44%)</td>
<td>(1%)</td>
<td></td>
</tr>
<tr>
<td>Quercetin glucoside sulphates (12,15)</td>
<td>735 ± 179</td>
<td>490 ± 112</td>
<td>206 ± 167</td>
<td>1431 ± 232</td>
</tr>
<tr>
<td></td>
<td>(52%)</td>
<td>(34%)</td>
<td>(14%)</td>
<td></td>
</tr>
<tr>
<td>Quercetin glucuronide sulphates (13,14)</td>
<td>1366 ± 266</td>
<td>984 ± 225</td>
<td>279 ± 105</td>
<td>2629 ± 282</td>
</tr>
<tr>
<td></td>
<td>(52%)</td>
<td>(37%)</td>
<td>(11%)</td>
<td></td>
</tr>
<tr>
<td>Isorhamnetin-3- and 4’-glucuronides</td>
<td>1218 ± 310</td>
<td>1060 ± 277</td>
<td>163 ± 53</td>
<td>2490 ± 318</td>
</tr>
<tr>
<td></td>
<td>(49%)</td>
<td>(43%)</td>
<td>(8%)</td>
<td></td>
</tr>
</tbody>
</table>

For each time period data expressed as nmole ± standard error (n = 6) and in italicized parentheses as a percent of the total amount excreted over 24 h. Peak numbers as in Table 3-3. n.d. – not detected.

In keeping with the short $T_{1/2}$ values presented in Table 4-1, 92% of the urinary flavonol metabolites were excreted within the first eight hours after ingestion of onions. Table 4-3. Total 0-24 h flavonol metabolite excretion in urine for the individual volunteers were 13.9, 13.7, 10.1, 16.4, 9.6 and 14.0 µmole and the mean value of 12.9 ± 1.1 µmole corresponds to 4.7% of intake. This is in agreement with the level excretion of flavonols in urine after onion consumption by humans reported by Graefe et al. (2001).

One alternative explanation for the presence of actual quercetin conjugates in the $T=0$ samples in the aforementioned studies is the foods the volunteers were allowed to eat during the study. In the study by (Hollman et al., 1996b) the volunteers were given a list of food and drinks to avoid based on the finding of a study of the levels of these
compounds (Hertog et al., 1992a). As discussed in Chapter 1.4 seasonal and varietal variations can account for significant changes in the levels of flavonols in foods. The volunteers may have unwittingly been consuming foods that contained much higher flavonol levels than they thought. As the only measure of flavonol content was by reference to (Hertog et al., 1992a) the actual amount consumed is unknown. In the studies carried out here all flavonol containing foods were, so to speak, off the menu.

4.5 Qualitative analysis of flavonol absorption

The number and varieties of metabolites formed from the two main onion flavonols, quercetin-4’-glucoside and quercetin-3,4´-diglucoside are shown in Table 3-3. The present study cannot provide direct information on the mechanisms involved or the efficiency with which these compounds are hydrolysed and/or enter the enterocyte. It does provide information well beyond that obtained with the classical hydrolysis methods. However, it is evident that following release of the aglycone, quercetin is subjected to glucuronidation, sulphation and/or methylation. The enzymes involved in the production of these metabolites from quercetin, glucuronosyltransferase, sulpharyltransferase and O-methyltransferase, have been found in human intestine (Radominska-Pandya et al., 1998; De Santi et al., 2000; Chen et al., 2003; Murota & Terao, 2003). It is, therefore, feasible that after the initial deglycosylation of the onion quercetin glucosides, all the quercetin metabolites that appear in plasma are the result of conversions occurring in the lumen of the small intestine. The reason for the individual metabolites displaying different pharmacokinetic profiles could be due to differing enzyme specificities and/or varying rates of efflux from the enterocyte into the bloodstream although deposition in body tissues and a slow release in the bloodstream could also be factors of influence. Another possibility is that the major plasma metabolites, quercetin-3'-sulphate and quercetin-3-glucuronide, are produced in the small intestine, pass into the portal vein and are further converted to the more minor components, the quercetin glucuronide sulphate, the quercetin diglucuronide and isorhamnetin-3-glucuronide in the liver as illustrated in Figure 4-6 and Figure 4-7. Human hepatocytes contain glucuronyl-, sulpho- and methyltransferases as well as β-glucuronidase activity (Boersma et al. 2002; O’Leary et al. 2003). Ex vivo incubation of
quercetin-3-glucuronide with human hepG2 hepatoma cells results in cleavage of the glucuronide moiety and the formation of quercetin-3'-sulphate (O’Leary et al. 2003). Further investigation is required to determine if this two step pathway is the way in which the sulphate, the main quercetin plasma metabolite, is synthesized in vivo. A single step sulphation of the aglycone in the enterocyte, as illustrated in Figure 4-6, would appear to be a more straightforward, but not necessarily exclusive, route.

Figure 4-6. Schematic of the proposed metabolic fate of quercetin-4'-glucoside and quercetin-3,4’-diglucoside as they pass from the lumen of the small intestine into the bloodstream via the liver.

Q – quercetin; glc – glucoside; diglc - diglucoside; glcUA - glucuronide; S - sulphate; LPH – lactase phlorizin hydrolase; C-β-G – cytosolic β-glucosidase; UGT – glucuronyltransferase; ST – sulphotransferase.
Quercetin-3-glucuronide and quercetin-3'-sulphate are transported from the small intestine to the liver where they are further metabolised before returning to the bloodstream and being excreted in urine via the kidneys.

Some tentative evidence of the initial stages of metabolism is discussed with regard to results seen in feeds of flavonol meals to ileostomy volunteers. Studies involving volunteers who have undergone an ileostomy can give an insight into the metabolism of quercetin glucosides. In two publications, (Hollman et al., 1995b; 1997c) on the same study, it was claimed that the level of quercetin glucosides absorbed was 52, 17 and 24% after quercetin-rich meals of onions, tea and the quercetin aglycone respectively. Absorption was defined as oral intake minus ileal excretion. However, all measurements were made on hydrolysed samples therefore no information as to the metabolism of the glucosides could be made. In a similar study by Walle et al., (2000) analysis of the ileal fluid prior to enzyme hydrolysis showed that all of the quercetin glucosides from an onion feed, had been hydrolysed. This was using HPLC analysis with absorbance detection. Walle’s conclusion differs from that of Hollman not in the level of quercetin thought to be absorbed but in the fact that glucosides must be hydrolysed to the aglycone prior to absorption from the small intestine. Although positive identification could not be made, two peaks that matched the retention time of quercetin glucuronides, from liver microsome incubations, were seen in Walle’s chromatograms. 

Both studies produced roughly the same results, in terms of what percentage of the dose was recovered in the ileal fluid, but differ in the conclusions that were drawn. Hollman et al., (1995b; 1997c) claimed that quercetin glycosides were more readily absorbed, whereas Walle et al., (2000) stated that all glycoside conjugates were hydrolysed prior to absorption. However, both studies defined the amount absorbed in the same manner, oral intake less ileal recovery, which is not necessarily a true measure of absorption. Not being able to detect a quercetin-based compound does not mean it has been absorbed. It is quite possible that the parent compound has been metabolised to a form that can no longer be recognised by its original properties that is, in Walle’s system no longer having an absorbance maxima at 365 nm or in Hollman’s no longer forming a fluorescent complex with aluminium nitrate.

A study on four ileal volunteers who consumed a flavonol-rich meal of onion soup was undertaken in Glasgow to obtain more information on what was occurring in the small intestine. No amount of persuasion or bribes could convince the ileal volunteers to try the meal of lightly fried onions. Unfortunately due to lack of funding
and ileal volunteers this work could not be completed to a level suitable for publication or to be presented in this thesis. However, the small amount of information available allows for some interesting insights as to what may be occurring in the small intestine and by extrapolation what could be occurring during and post absorption.

The ileal fluid collected over 24 h following consumption of the soup was analysed by HPLC-PDA-MS for the level of quercetin contained in it. The results were in broad agreement with those of Walle et al., (2000) and Hollman et al., (1995b) in that 25% of the flavonol dose was recovered. However, it was also possible to identify the quercetin-based compounds found in the ileal fluid. The two main metabolites peaks were quercetin-3´-glucuronide and quercetin-3´-sulphate, with the quercetin aglycone making up the third largest peak.

This information is of some value to the problem of how and where the metabolites are formed and absorbed. It would seem from these findings the sulphate metabolite is formed in the small intestine and is then transported through the enterocyte into the portal blood stream. The proposed route shown in Figure 4-6 would also require efflux of large quantities of the sulphate out of the enterocyte and into the lumen and hence its presence in ileal fluid. The other main metabolite seen in the ileal fluid is quercetin-3´-glucuronide. This is not one of the metabolites found in quantifiable amount in plasma, though present as a major metabolite in urine. Based on these finding, (Figure 4-6) could be interpreted in a different way as seen in Figure 4-8.
The metabolic profile seen in ileal fluid indicates that the two main compounds formed from onions are both 3′ metabolites. It could be that after deglycosylation the first reaction the aglycone undergoes is to conjugate the 3′ hydroxyl group. It is speculation, but it may be that having an ortho 3′ and 4′ hydroxyl groups on the B ring, which makes quercetin a powerful antioxidant in vitro, (RiceEvans et al., 1997) is something that the body views as being potentially harmful and therefore combats it by conjugation. Support of this speculation would come if other flavonoids which have this 3′,4′ arrangement, were treated similarly. Work by Boersma et al., (2002) demonstrated the human UGT are especially effective in conjugating the 3′,4′ catechol unit of quercetin and luteolin. If other flavonoids which did not have the 3′,4′ arrangement did not undergo such dramatic metabolism, and were better absorbed, this would give further support to this theory.

Although very little is known about the metabolism of flavonoids, other than quercetin, a study into the bioavailability of kaempferol, a 4′ hydroxyl flavonol, found that the 3 glucuronide was the main compound circulating in plasma, with the aglycone
being present too. Urinary excretion also showed the 3 glucuronide to be the main metabolite with putative sulphate and disulphate metabolites. Absorption levels in this study were reported to be 2% from a very low level dose of only 9 mg (Dupont et al., 2004)

Another pair of compounds that could have been used for comparison are the flavones apigenin and luteolin, having the 4´-and 3´,4´-hydroxyl groups, respectively. Unfortunately, as their dietary intake is rather low, few studies have been made into their absorption. In vitro studies with rats by Gradolatto et al., (2004) produced some interesting data. Phase II in vitro metabolism of apigenin showed three monoglucuronide and one mono sulphate conjugate while luteolin produced four monoglucuronide, two mono sulphate conjugates and a methyl metabolite. No information on human metabolites or levels of absorption is currently available. Support of the metabolite is the major compound found in both urine and plasma (Mullen et al., 2008a). With cyanidin-3-glucoside multiple metabolites have been reported to occur in both urine and plasma. (Kay et al., 2005a). Levels of absorption for pelargonidin-3-glucoside range from 1% in the aforementioned study to 1.8% (Felgines et al., 2003), while that of cyanidin-3-glucoside is low at 0.1% intake (Kay et al., 2005b).

The flavanones have two compounds that can be used for comparison. They are hesperetin and naringenin. It is slightly different in this case as hesperetin has a 3' hydroxyl and a 4' methoxy group on the B ring whereas naringenin has only a 4' hydroxyl group. Eriodictyol would have been the ideal compound to compare with naringenin having a 3’,4’ dihydroxyl structure however, no comparable studies could be found. In a study by (Mullen et al., 2008a) a feed of orange juice fortified with 130 µmole of hesperetin-3-rutinoside produced six urinary hesperetin metabolites. The
much lower intake of narigenin-3-rutinoside produced only three metabolites. However, excretion of narigenin was 12% of the intake while that of hesperetin was only 5% for a 10 fold larger dose. This could be due to the dose effect but as other studies agree it suggest that narigenin-3-rutinoside is more bioavailable than hesperetin-3-rutinoside (Erlund et al., 2001; Manach et al., 2005)

Studies involving (-)-epicatechin have shown that it too undergoes substantial metabolism with multiple sulphate, glucuronide and methyl derivatives being found in both plasma and urine (Li et al., 2001; Roura et al., 2005). However, there are no metabolism or bioavailability studies on a mono-hydroxylated flavan-3-ol (epiafzelchin) that can be compared with it.

Of course an alternative view could be that having more hydroxyl groups on the B ring just means there are more sites for conjugation, therefore, more metabolites can be formed. However, unless more studies look at the metabolites formed rather than simply measuring the levels of the aglycone little progress will be made.

Returning to the metabolites in ileal fluid, the quercetin-3′-glucuronide formed in the small intestine, does not appear in the plasma. It must, therefore, either not be absorbed or be the first step in the formation of the diglucuronide or the glucuronide sulphate metabolites, which are both found in plasma. Information on the position of the glucuronide moiety on these two metabolites could help to answer this question.

The $C_{max}$ values and 24 h urinary excretion of the flavonol metabolites (Table 4-2) detected after consumption of onions presents clear evidence of substantial phase II metabolism with many of the major urinary metabolites either not being detected in plasma or being present in low concentrations. For instance, quercetin-3′-sulphate, the main plasma metabolite, was present in urine in only trace quantities while several quercetin glucoside glucuronides and quercetin glucoside sulphates, absent in plasma, were excreted in substantial amounts. The virtual absence of many of these urinary metabolites in plasma indicates that once released into the bloodstream they are rapidly removed by excretion via the kidneys. We assume that most of the observed metabolism occurs in the liver, which contains all the prerequisite enzymes, prior to transport to the kidneys. The possible exception, as illustrated in Figure 4-7, may be the formation of
the glucoside conjugates in the kidneys, which are known to possess β-glucosyltransferase activity (Matern & Matern 1987).

Table 4-4. Quercetin metabolites detected in plasma and urine after the consumption of 270 g of fried onions by six human volunteers. Estimates of levels in plasma expressed as nmole/L ± S.E. (n = 6) at peak plasma concentration. Amounts in urine expressed as total amount excreted in nmole ± S.E. over a 24 h period post-ingestion of onions

<table>
<thead>
<tr>
<th>Metabolite (peak number)</th>
<th>Plasma</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin diglucuronide (1)</td>
<td>n.d.</td>
<td>trace</td>
</tr>
<tr>
<td>Methylquercetin diglucuronide (2)</td>
<td>n.d.</td>
<td>1003 ± 156</td>
</tr>
<tr>
<td>Quercetin glucoside glucuronide (3)</td>
<td>n.d.</td>
<td>163 ± 23</td>
</tr>
<tr>
<td>Methylquercetin diglucuronide (4)</td>
<td>n.d.</td>
<td>426 ± 99</td>
</tr>
<tr>
<td>Quercetin diglucuronide (6)</td>
<td>n.d.</td>
<td>trace</td>
</tr>
<tr>
<td>Quercetin glucoside glucuronide (7)</td>
<td>n.d.</td>
<td>trace</td>
</tr>
<tr>
<td>Quercetin glucoside glucuronide (8)</td>
<td>n.d.</td>
<td>trace</td>
</tr>
<tr>
<td>Quercetin diglucuronide (9)</td>
<td>51 ± 13</td>
<td>2223 ± 417</td>
</tr>
<tr>
<td>Quercetin-3-glucuronide (10)</td>
<td>306 ± 42</td>
<td>912 ± 149</td>
</tr>
<tr>
<td>Quercetin glucoside sulphate (12)</td>
<td>n.d.</td>
<td>393 ± 60</td>
</tr>
<tr>
<td>Quercetin glucuronide sulphate (13)</td>
<td>n.d.</td>
<td>1384 ± 163</td>
</tr>
<tr>
<td>Quercetin glucuronide sulphate (14)</td>
<td>117 ± 12</td>
<td>1229 ± 190</td>
</tr>
<tr>
<td>Quercetin glucoside sulphate (15)</td>
<td>n.d.</td>
<td>821 ± 156</td>
</tr>
<tr>
<td>Isorhamnetin-3-glucuronide (17)</td>
<td>98 ± 17</td>
<td>1789 ± 27</td>
</tr>
<tr>
<td>Quercetin-4'-glucuronide (18)</td>
<td>n.d.</td>
<td>trace</td>
</tr>
<tr>
<td>Quercetin-3'-glucuronide (19)</td>
<td>trace</td>
<td>1845 ± 193</td>
</tr>
<tr>
<td>Isorhamnetin-4'-glucuronide (20)</td>
<td>trace</td>
<td>700 ± 11</td>
</tr>
<tr>
<td>Quercetin-3'-sulphate (22)</td>
<td>539 ± 46</td>
<td>trace</td>
</tr>
</tbody>
</table>

Peak numbers refer to HPLC traces in Figure 3-2 and Table 3-3. n.d. - not detected. Trace - compound detected but not in sufficient amounts for routine quantification. Information on trace levels of metabolites detected exclusively in the plasma of volunteer 6 Table 3-3 are not presented.

The 4.7 % recovery of the ingested flavonol glucosides as metabolites in urine in this study leaves a large amount of the ingested dosed unaccounted for. The most likely fate of these compounds is that they are converted to low molecular weight phenolic acids (Gonthier et al., 2003) most probably 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid and 3-methoxy-4-hydroxyphenylpropionic acid (Olthof et al. 2003). These compounds were not analysed in the current study. They have a low extinction coefficient and a λ\text{max} below 250 nm and as a result are not readily detected.
with a PDA detector and, in addition, they do not ionize readily when subjected to MS with an electrospray interface.

The data obtained in this study reveal that extensive modification of quercetin glucosides occurs following ingestion of onions and the appearance of metabolites in the bloodstream and urine. The metabolic conversions involve a complex combination of deglycosylation, glucuronidation, sulphation, methylation and possibly deglucuronidation and glycosylation steps. Where in the body these events take place and the sequence in which they occur after the initial deglycosylation, is a matter of speculation and a topic that requires further investigation. However, the schematics shown in Figure 4-6, Figure 4-7 and Figure 4-8 are a first step in this process.

While experimentation with human subjects is useful it has it limitations as the analysis of deposition of flavonol metabolites in body tissues such as the liver, kidneys and brain, is not possible for obvious reasons. ex-vivo studies with cultured cells and tissues have their place but it is open to doubt as to whether they reflect the true in vivo systems where the passage of metabolites into and out of cells and organs is likely to be subjected to refined controls. Animal test systems are, therefore, the only direct way in which the true bioavailability of flavonols and other dietary flavonoids and phenolics can be investigated. As demonstrated in recent studies with rats, this is best achieved using radiolabelled substrates as the accumulation of radioactivity in body fluids and tissues can be easily monitored by liquid scintillation counting and the compounds involved identified and quantified using HPLC-MS in combination with an on-line radioactivity monitor (Mullen et al., 2002a; 2003a).

The work in the following chapter will investigate the fate of [2-14C]quercetin-4'-glucoside over a 72 h period to try and determine the fate of this compound in rats. The methodology developed in Chapter 2 will again be used and the animals will be housed in metabolism cages, for the duration of the experiment, to allow separate collection of urine and faeces. The aim is to be able to account for 100% of the given dose by analysis of excretory products and tissues.
Chapter 5  The fate of [2-\textsuperscript{14}C] quercetin-4′-glucoside in rats by HPLC radio-counting and tandem mass spectrometry

5.1 Introduction

In the previous chapters methods were developed to measure the levels and forms of quercetin metabolites after ingestion of onions or quercetin-4′-glucoside in man and in rats. The results of these investigations showed the large number and types of intact metabolites formed post ingestion. However, these studies could provide no information on what happened once these compounds ceased to display the properties used to detect them. The work in this chapter attempts to answer this question.

Previously [2-\textsuperscript{14}C]quercetin-4′-glucoside was used in short term feeding studies with rats to determine the metabolites formed up to 5h after ingestion (Mullen et al., 2002a; Graf et al., 2005). To investigate the fate of the radiolabelled flavonols during their passage through the rat, the experiment must be run for a much longer period of time. To allow for comparison with the original study, a 1 h sample was included in the sampling times of 6 h, 12 h, 24 h, 48 h and 72 h, which were chosen for this study. Housing the rats in metabolism cages for this period of time allowed for the collection of all excreted products except exhaled CO\textsubscript{2}, which has been reported, in an earlier human \textsuperscript{14}C labelled quercetin study by Walle et al., (2001a), to be the major metabolite of quercetin. However, as the quercetin was labelled at the 4 position in the C ring it could not be used to follow the fate of the A or B ring metabolites. The compound used in this study is labelled at the 2 position of the C ring and so will be able to follow the fate of the B ring metabolites (see Chapter 2).

5.2 Materials and methods

5.2.1 Animals and sample preparation

The experimental time points for analysis were 1, 6, 12, 24, 48 and 72 h. At each time point three male Sprague-Dawley rats (Iffa Credo, L'Arbresle, France) weighing 250 g
were used (range 242-256 g.). They were housed individually in stainless steel metabolic cages and had free access to deionised water (Figure 5-1). They were kept under light from 0700 to 1900 h, and the room temperature was 23 ± 1 °C, with constant humidity. They were deprived of food for 16 h before being force-fed by gavage at the beginning of the experiment with a solution of [2-14C]quercetin-4′-glucoside in water/ethanol (92/8) v/v. Rats were handled according to the guidelines of the Committee on Animal Care at the University of Montpellier and NIH guidelines (1). They were anaesthetized with pentobarbital (60 g/L pentobarbital, 60 mg/kg body). Blood was drawn by cardiac puncture with heparin-moistened syringes and plasma and erythrocytes were separated by centrifugation at 2300 g for 10 min at room temperature then stored at -80°C until analysis. The whole body tissues were perfused in situ with chilled 0.15 M NaCl to remove residual blood. Brain, heart, lungs, kidneys, liver, testis, muscle and spleen, were removed and rinsed in saline, blotted dry and immediately frozen in liquid nitrogen and stored at −80 °C until analysis. The gastrointestinal tract was removed intact along with its contents, then separated into stomach, duodenum, small intestine (jejunum and ileum), caecum and colon, immediately frozen in liquid nitrogen then stored at −80 °C until analysis. All tissues were weighed. Faeces and urine were collected from each rat during the time period starting from the force-feeding of labelled compounds to the sacrifice, then stored at −80 °C prior to analysis.

5.2.2 Materials

Methanol and acetonitrile were from Rathburn Chemicals (Walkerburn, Scotland) and were both HPLC grade. Formic acid was purchased from Riedel-DeHaen (Seelze, Germany). Acetic acid was from BDH (Poole, England). L (+)-ascorbic acid and quercetin were purchased from Extrasynthese (Genay, France). Quercetin-3,4′-diglucoside and quercetin-4′-glucoside were from AASC Ltd (Southampton, England).
Hippuric acid, 2, 3 and 4-hydroxyphenylacetic acid, benzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxybenzoic acid, 3-methoxy, 4-hydroxyphenylacetic acid, 3-methoxy, 4-hydroxybenzoic acid, 3,5-dihydroxy, 3-methoxybenzoic acid, 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid and 3-methoxy-4-hydroxyphenylpropionic acid were from Sigma

5.2.3 HPLC with diode array, radioactivity and MS² detection

Samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, diode array absorbance detector, scanning from 250 to 700 nm and an autosampler cooled to 4 °C. (Thermo Finnigan, San Jose, USA). Separation of quercetin metabolites was carried out using a 250 x 4.6 mm i.d. 4 μm Synergi RP-Max column (Phenomenex, Macclesfield, UK) eluted with a gradient over 60 min of 5-40% acetonitrile in 1% formic acid at a flow rate of 1 mL/min and maintained at 40 °C. After passing through the flow cell of the absorbance monitor, the column eluate was directed to a radioactivity monitor (Reeve Analytical Model 9701, Lab Logic, Sheffield, UK) fitted with a 500µL heterogeneous flow cell packed with cerium-activated lithium glass scintillant, after which it was split and 0.3 mL/min was directed to a LCQ DecaXP ion trap mass spectrometer. Analysis with an electrospray interface (ESI) in negative ion mode provided the best limits of detection for quercetin based metabolites. This was carried out using full scan, data dependant MS² scanning from m/z 100 to 1000. Capillary temperature was 350 °C, sheath gas and auxiliary gas were 60 and 10 units respectively, source voltage was 4kV.

Separation of quercetin catabolites was also carried out using the same instrumentation however, separation was achieved using a 250 x 4.6 mm i.d. 4 μm Synergi Polar-RP column (Phenomenex, Macclesfield, UK) eluted with a gradient over 60 min of 5-45% methanol in 0.1% acetic acid at a flow rate of 1 mL/min and maintained at 40 °C. A second chromatographic system using a phenyl based column 250 x 4.6 mm i.d. 4 μm Gemini (Phenomenex, Macclesfield, UK) eluting with the same solvent as the Polar column but running a 12 –20% gradient over 60 minutes was also used to confirm catabolite identification. After passing through the flow cell of the absorbance monitor, the column eluate was directed to a radioactivity monitor (Reeve Analytical Model 9701, Lab Logic, Sheffield, UK) fitted with a 500µL heterogeneous flow cell packed with cerium-activated lithium glass scintillant, after
which it was split and 0.3 mL/min directed to a LCQ DecaXP ion trap mass spectrometer. Analysis using an atmospheric chemical ionisation interface (APCI) in negative ion mode provided the best limits of detection for quercetin catabolites/phenolic acids. This was carried out using full scan, data dependant MS² scanning from m/z 100 to 500. APCI vaporizer temperature was 350 °C, capillary temperature was 140 °C, sheath gas and auxiliary gas were 80 and 40 units respectively, source voltage was 5kV.

5.2.4 Measurement of radioactivity in tissues

Ten-mg aliquots of powdered freeze-dried tissue and 100 µL plasma were treated with 0.5 mL tissue solubilizer (National Diagnostics, Hull, UK) for 3 h at 50 °C in a shaking water bath. Pigmented samples were bleached using 1.75 mL of a 25% solution of sodium hypochlorite. The solubilization treatment produced clear solutions and 150 µL aliquots were taken and added to 5 mL scintillation cocktail (Optiflow Safe One, Fisons, Loughborough, UK) before determination of radioactivity using a Wallac 1409 liquid scintillation counter (Pharmacia, Uppsala, Sweden).

5.2.5 Extraction of samples

The level of radioactivity found in the tissue measurement experiment determined the size of aliquot used for extraction. This ranged from 10mg for stomach samples taken from 1 h time points to 100 mg for 6 h stomach samples. If no radioactivity was found in the tissue no extraction procedure was carried out.

Extraction was by addition of 50% aqueous methanol and continuous shaking. After 30 minutes the mixture was centrifuged at 2000 g for 20 minutes. The methanolic supernatant was decanted and the pellet re-extracted a second time. The extract was reduced to dryness in vacuo and by lyophilization. The residue was re-suspended in the HPLC mobile phase (starting composition) and up to 200 µL injected into the HPLC system.
5.3 Results

5.3.1 Distribution of radioactivity in rat tissues and extracts

Each rat was dosed with 1.84 x 10^7 dpm ± 3.14 x 10^5 of [2-14C]quercetin 4'-glucoside, (specific activity 3.75 mCi/mnmole). The purity of the dose was checked prior to feeding by HPLC-RC-MS^2 which proved that the [2-14C]quercetin-4'-glucoside was radiochemically pure. This was the same procedure used in Figure 2-1.

At the specified time points the rats were sacrificed and the distribution of radioactivity determined. The data obtained are presented in Table 5-1. Recovery of radioactivity was between 80 ± 6% for 1 and 6 h samples and 98 ± 16% SD for 72 h samples. The only areas where substantial levels of radioactivity were detected were the GI tract, urine and faeces. Muscle tissue contained the highest amount out with these samples but this still only amounted for no more than 1% of the dose. When expressed as a dpm per gram the concentration is less than that of any of the major organs e.g. liver, kidney, heart and lungs. These organs only contained trace amounts of the radioactive dose.

The majority of radioactivity was found in the GI tract in the 1 and 6 h samples and from 12 h to 72 h in the urine.

5.3.2 Distribution of radioactivity in the GI tract

Table 5-1 shows the total activity recovered from the whole of the GI tract. However, the GI tract was split into five segments, stomach, duodenum, ileum/jejunum, caecum and colon, that were individually analysed. The distribution of the radioactivity contained in these segments is shown in Figure 5-2.
Table 5-1. Distribution of radioactivity in samples, data shown as percentage of administered dose. Data in parentheses are percentage of recovered dose.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1 hr</th>
<th>6 hr</th>
<th>12 hr</th>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>47 ± 20 (0.3%)</td>
<td>460 ± 144 (2.5%)</td>
<td>9,399 ± 2272 (52%)</td>
<td>11,687 ± 2236 (64%)</td>
<td>11,433 ± 913 (63%)</td>
<td>12,641 ± 3,181 (69%)</td>
</tr>
<tr>
<td>Cagewash</td>
<td>69 ± 17 (0.4%)</td>
<td>776 ± 84 (4.3%)</td>
<td>2,254 ± 1401 (12%)</td>
<td>1,982 ± 1106 (11%)</td>
<td>2,231 ± 877 (12.2%)</td>
<td>1,684 ± 461 (9.2%)</td>
</tr>
<tr>
<td>Faeces</td>
<td>111 ± 96 (0.9%)</td>
<td>0.0 ± 0.0 (0.0%)</td>
<td>1,715 ± 111 (9.4%)</td>
<td>2,077 ± 825 (11.3%)</td>
<td>2,906 ± 984 (13.7%)</td>
<td>3,349 ± 249 (18%)</td>
</tr>
<tr>
<td>Liver</td>
<td>80 ± 19 (0.4%)</td>
<td>95 ± 32 (0.5%)</td>
<td>87 ± 5 (0.5%)</td>
<td>95 ± 4 (0.5%)</td>
<td>94 ± 14 (0.5%)</td>
<td>81 ± 11 (0.4%)</td>
</tr>
<tr>
<td>Kidney</td>
<td>54 ± 17 (0.3%)</td>
<td>98 ± 24 (0.5%)</td>
<td>116 ± 4 (0.6%)</td>
<td>41 ± 6 (0.2%)</td>
<td>51 ± 20 (0.3%)</td>
<td>27 ± 5 (0.2%)</td>
</tr>
<tr>
<td>Brain</td>
<td>2 ± 2 (0.0%)</td>
<td>2 ± 1 (0.0%)</td>
<td>4 ± 3 (0.0%)</td>
<td>4 ± 3 (0.0%)</td>
<td>2 ± 0 (0.0%)</td>
<td>2 ± 2 (0.0%)</td>
</tr>
<tr>
<td>Testes</td>
<td>2 ± 1 (0.0%)</td>
<td>7 ± 1 (0.0%)</td>
<td>4 ± 1 (0.0%)</td>
<td>2 ± 0 (0.0%)</td>
<td>1 ± 0 (0.0%)</td>
<td>1 ± 0 (0.0%)</td>
</tr>
<tr>
<td>GI tract</td>
<td>13,683 ± 767 (74.0%)</td>
<td>13,106 ± 125 (72.2%)</td>
<td>3462 ± 1070 (19.0%)</td>
<td>620 ± 159 (3.5%)</td>
<td>75 ± 53 (0.4%)</td>
<td>51 ± 1 (0.3%)</td>
</tr>
<tr>
<td>Plasma</td>
<td>83 ± 43 (0.5%)</td>
<td>74 ± 10 (0.4%)</td>
<td>40 ± 15 (0.2%)</td>
<td>19 ± 7 (0.1%)</td>
<td>14 ± 5 (0.1%)</td>
<td>0 ± 0 (0.0%)</td>
</tr>
<tr>
<td>Lungs</td>
<td>3 ± 1 (0.0%)</td>
<td>5 ± 3 (0.0%)</td>
<td>1 ± 0 (0.0%)</td>
<td>1 ± 0 (0.0%)</td>
<td>1 ± 0 (0.0%)</td>
<td>0 ± 0 (0.0%)</td>
</tr>
<tr>
<td>Heart</td>
<td>7 ± 4 (0.0%)</td>
<td>8 ± 1 (0.0%)</td>
<td>6 ± 2 (0.0%)</td>
<td>3 ± 1 (0.0%)</td>
<td>2 ± 1 (0.0%)</td>
<td>1 ± 0 (0.0%)</td>
</tr>
<tr>
<td>Muscle</td>
<td>427 ± 249 (2.3%)</td>
<td>408 ± 101 (1.0%)</td>
<td>335 ± 105 (0.8%)</td>
<td>204 ± 64 (0.5%)</td>
<td>156 ± 40 (0.4%)</td>
<td>100 ± 40 (0.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>14,566 ± 1,029 (80 ± 6%)</td>
<td>1,5037 ± 258 (82 ± 14%)</td>
<td>16,869 ± 1,977 (92 ± 11%)</td>
<td>16,735 ± 1,638 (92 ± 9%)</td>
<td>16,949 ± 1,095 (91 ± 6%)</td>
<td>17,938 ± 2,988 (98 ± 16%)</td>
</tr>
</tbody>
</table>
5.3.2.1 Analysis of the 1 h GI tract samples

As it was not possible to identify the flavonols and phenolic acid metabolites in the same analytical conditions, with the exception of hippuric acid, two analyses were carried out. Firstly to identify any flavonol metabolites present. Secondly to identify the potential phenolic acid compounds. Under both conditions it was possible to determine the relative level of radioactivity from each peak.

As can be seen in Figure 5-2, the GI tract from the 1 h rat only contained radioactivity in the stomach, duodenum and ileum/jejunum. This accounted for 74.0 % ± 4.3 of the dose. Analysis of the radioactivity in the stomach samples by HPLC-RA-MS² (Figure 5-3) showed the presence of 5 peaks. Only two of the peaks, which made up over 84% of the dose could be identified. These were the parent compound quercetin-4’-glucoside, 41% ± 6.9 and quercetin 43% ± 5.7, (Table 5-2. Compounds detected in the 1 and 6 h G.I. tract, data reported as K dpm and, in parentheses as percentage of the given dose.). One of the 4 unidentified peaks could be an “oxidised” quercetin compound. It had a [M-H]’ molecular ion at m/z 349, which on fragmentation produced a base ion at m/z 331, a loss of 18, with a smaller fragment at m/z 299 a further loss of 32. This fragmentation pattern could be explained by the presence of a hemiacetal hydrate derivative of quercetin that has reacted with methanol during the extraction process as described by (Dangles et al., 1999). A similar fragmentation pattern was seen by Krishnamachari et al., (2002b) when they reacted quercetin with the peroxyl radical generator 2,2’-azobis-isobutyronitrile.

Analysis of the radioactivity found in the duodenum revealed 8 peaks all of which were identified. The ileum/jejunum contained 11 peaks, 9 of which were identified (Table 5-2).
Figure 5-2. Level of radioactivity found in G.I. tract segment as percentage of the original dose
Figure 5-3. HPLC radioactivity chromatogram of the 1 and 6 h GI tract samples
5.3.2.2 Analysis of the 6 h GI tract samples

Analysis of the 6 h rat GI tract showed the bulk of the radioactivity had left the stomach and duodenum and was distributed through the ileum/jejunum, caecum and colon (Figure 5-3 and Table 5-2), although trace levels of quercetin was detected in the stomach of one of the rats.

The 6 h ileum/jejunum contained 19 metabolites of which 14 were positively identified (Table 5-2). The pattern of metabolites changed dramatically in the caecum with the occurrence of only six radioactive peaks. The same metabolic profile detected in the colon, except for the absence of peak 1 (Table 5-2). Peaks 1, 13 and 28 were not identified. However, the other metabolites were 3,4 dihydroxybenzoic acid (peak 4), 3,4-dihydroxyphenylacetic acid (peak 5) and 3-hydroxyphenylacetic acid (peak 8). Their percentage of the administered dose found in the colon was 6.0%, 31.8% and 31.9% respectively.

What can be clearly seen from the results shown in Table 5-2 is that mono- and di-glucuronidation occurs prior to the compounds being methylated. The two major metabolites in the duodenum of the 1 h sample are both quercetin monoglucuronides, most likely the 3’ and 4’ isomers, from their retention time properties, accounting for almost 50% of the radioactivity. This is also the case in the ileum/jejunum sample. However, in the 6 h sample of the ileum/jejunum the number of methylated metabolites has increased from one in the 1 h sample to six and now accounts for 30% of the radioactivity.

5.3.2.3 Analysis of the 12 h GI tract samples

The 12 h GI tract samples contained lower levels of radioactivity than the 1 and 6 h samples, which made MS identification more difficult. Analysis of the ileum/jejunum showed that hippuric acid was the main compound present along with a
much lower level of 3-hydroxyphenylacetic acid. The caecum and colon contained only 3-hydroxyphenylacetic acid.
Table 5-2. Compounds detected in the 1 and 6 h G.I. tract, data reported as K dpm and, in parentheses as percentage of the given dose.

<table>
<thead>
<tr>
<th>Pno.</th>
<th>Rt</th>
<th>Compound</th>
<th>1 h rat G.I. tract segments</th>
<th>6 h rat G.I. tract segments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stomach</td>
<td>Duodenum</td>
</tr>
<tr>
<td>1</td>
<td>5.5</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6.7</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10.2</td>
<td>Unknown</td>
<td>245 (3.3%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10.5</td>
<td>3,4 dihydroxy benzoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>11.6</td>
<td>3,4 dihydroxy phenyl acetic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>18.3</td>
<td>Hippuric acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>18.8</td>
<td>Quercetin diglucuronide</td>
<td>269 (5.9%)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>19.6</td>
<td>3 hydroxy phenyl acetic acid</td>
<td>337 (4.5%)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>21.6</td>
<td>Methyl quercetin diglucuronide</td>
<td>73 (1.6%)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>22.1</td>
<td>Methyl quercetin diglucuronide</td>
<td>29 (0.6%)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>23.5</td>
<td>Methyl quercetin diglucuronide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>25.4</td>
<td>Quercetin diglucuronide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>26.5</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>27.2</td>
<td>Unknown &quot;oxidised quercetin&quot;</td>
<td>651 (8.7%)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>27.8</td>
<td>Methyl quercetin diglucuronide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>28.0</td>
<td>Quercetin diglucuronide</td>
<td>38 (2.4%)</td>
<td>144 (3.1%)</td>
</tr>
<tr>
<td>17</td>
<td>29.1</td>
<td>Quercetin diglucuronide</td>
<td>202 (4.4)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>29.8</td>
<td>Quercetin diglucuronide</td>
<td>10 (0.6%)</td>
<td>173 (3.8%)</td>
</tr>
<tr>
<td>19</td>
<td>31.2</td>
<td>Quercetin glucuronide</td>
<td>471 (28.9%)</td>
<td>1,072 (23.3%)</td>
</tr>
<tr>
<td>20</td>
<td>32.2</td>
<td>Quercetin glucuronide</td>
<td>195 (12.0%)</td>
<td>504 (11.0%)</td>
</tr>
<tr>
<td>21</td>
<td>34.4</td>
<td>Benzoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>36.6</td>
<td>Quercetin-4'-glucoside</td>
<td>4,356 (41%)</td>
<td>534 (32.8%)</td>
</tr>
<tr>
<td>23</td>
<td>37.5</td>
<td>Methyl quercetin glucuronide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>38.3</td>
<td>Methyl quercetin glucuronide</td>
<td>31 (1.9%)</td>
<td>147 (3.2%)</td>
</tr>
<tr>
<td>25</td>
<td>39.4</td>
<td>Methyl quercetin glucuronide</td>
<td>188 (11.6%)</td>
<td>321 (7.0%)</td>
</tr>
<tr>
<td>26</td>
<td>43.5</td>
<td>Methyl quercetin sulphate glucuronide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>44.3</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>44.3</td>
<td>Quercetin</td>
<td>3,201 (43%)</td>
<td>161 (9.9%)</td>
</tr>
<tr>
<td>29</td>
<td>45.2</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>49.0</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>50.0</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>50.3</td>
<td>Quercetin sulphate</td>
<td>765 (16.5%)</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>54.3</td>
<td>unknown</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3.2.4 Analysis of the 24 h GI tract samples

Trace levels of radioactivity were found in the stomach and duodenum in these samples (Figure 5-2). Analysis of the ileum/jejunum showed the presence of three radioactive peaks. Only hippuric acid could be identified. One of the other radioactive peaks eluted at the same retention time as 3-hydroxphenylacetic acid but could not be identified as it did not ionise. The third peak also had no identifiable ions and eluted at a retention time matching 3,4-dihydroxyphenylacetic acid. The GI tract samples from the later timepoints did not contain sufficient radioactivity to allow identification of the trace quantities of metabolites and catabolites that were present.

5.3.3 Urine analysis

As can be seen from the results in Table 5-1 a small amount of radioactivity is excreted in the urine within one hour. By six hours it has increase to 2.5% of the dose and to 52% after 12 hours. The 24 h sample shows an increase in the level excreted (64%) with no apparent increase by 48 hours. The best picture on excretion rate can be seen in the 72 hour samples. Here urine was collected from these rats at 0-24 h, 24-48 h and 48-72 h thereby giving a complete record from one set of rats. (see Figure 5-4).
Figure 5-4. average urinary excretion of rats over 72 h, error bars are ± SD (n=3).

5.3.3.1 Analysis of urine by HPLC-RC-MS²

As urine contained both flavonol and phenolic acid metabolites analysis had to be carried out with two analytical methods that were used in the GI tract analysis.

5.3.3.2 Urine flavonol data

Urine was analysed using HPLC-RA-MS² as previously described. A total of 20 metabolites were identified (Table 5-3). None of the flavonol metabolites were present in concentrations to allow quantification by radioactivity or absorbance detection. Although it was not possible to quantify the flavonol metabolites the ion current seen in the mass spectrometer at the relevant m/z ratio could be used to show what were the major compounds present. The four major flavonol metabolites found were peaks 10, 11 and 15 which were all methyl quercetin diglucuronides, and peak 12 a quercetin diglucuronide. Peak 12 was one of only three of the 20 urinary flavonol metabolites that was also among the 15 metabolites detected in the G.I. tract (Table 5-2). This would indicate that there is major post-absorption metabolism of flavonols occurring. Peak 3 was a triglucuronide metabolite of quercetin and was detected only in urine.
Analysis of the 24-48 h and 48-72 h urine did not find the presence of any flavonol metabolites.

Table 5-3. Flavonol metabolites found in urine and referenced to Table 5-2GI tract metabolites.

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Rt.</th>
<th>Compound</th>
<th>Table 2 peak No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.8</td>
<td>Quercetin triglucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>2</td>
<td>17.5</td>
<td>Hippuric acid</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>19.0</td>
<td>Quercetin triglucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>4</td>
<td>20.3</td>
<td>Quercetin diglucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>5</td>
<td>21.2</td>
<td>Methyl quercetin diglucuronide</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>22.6</td>
<td>Quercetin diglucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>7</td>
<td>23.5</td>
<td>Methyl quercetin glucoside glucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>8</td>
<td>24.1</td>
<td>Methyl quercetin diglucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>9</td>
<td>25.2</td>
<td>Methyl quercetin diglucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>10</td>
<td>25.7</td>
<td>Quercetin diglucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>11</td>
<td>26.1</td>
<td>Methyl quercetin diglucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>12</td>
<td>28.0</td>
<td>Quercetin diglucuronide</td>
<td>17</td>
</tr>
<tr>
<td>13</td>
<td>28.4</td>
<td>Methyl quercetin sulphate</td>
<td>nd</td>
</tr>
<tr>
<td>14</td>
<td>30.1</td>
<td>Methyl quercetin glucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>15</td>
<td>30.5</td>
<td>Methyl quercetin sulphate</td>
<td>nd</td>
</tr>
<tr>
<td>16</td>
<td>30.9</td>
<td>Methyl quercetin glucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>17</td>
<td>33.3</td>
<td>Methyl quercetin sulphate</td>
<td>nd</td>
</tr>
<tr>
<td>18</td>
<td>34.6</td>
<td>Methyl quercetin glucuronide</td>
<td>nd</td>
</tr>
<tr>
<td>19</td>
<td>39.5</td>
<td>Quercetin sulphate</td>
<td>nd</td>
</tr>
<tr>
<td>20</td>
<td>48.8</td>
<td>Quercetin sulphate</td>
<td>31</td>
</tr>
<tr>
<td>21</td>
<td>53.0</td>
<td>Methyl quercetin</td>
<td>nd</td>
</tr>
</tbody>
</table>

5.3.3.3 Urine phenolic acid data

Analysis of the 1 h and 6 h rat urine samples showed the presence of measurable levels of radioactivity that did not correspond to any flavonol metabolites. However, due to the low levels no positive identity could be made on these compounds. The 12 h, 24 h, 24-48h and 48-72h samples all produced good radioactivity chromatogram and 3 peaks were identified by both mass spectrometric data and co-elution with authentic standards under three different chromatographic methods (RP-Max, Polar and Gemini). The data from the analyses are shown in Figure 5-6 and the quantitative estimates are shown in Table 5-4.
Table 5-4. Identification and quantification of radioactive compounds excreted in urine from the 72 h rats. Values reported are as a percentage of the dose, total ± SD

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
<th>Peak 4</th>
<th>Peak 5 3-HydroxyPAA</th>
<th>Peak 6 Benzoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24h</td>
<td>0.0</td>
<td>2.2</td>
<td>1.2</td>
<td>32.0</td>
<td>11.1</td>
<td>1.2</td>
</tr>
<tr>
<td>24-48h</td>
<td>0.4</td>
<td>0.6</td>
<td>0.9</td>
<td>0.1</td>
<td>4.7</td>
<td>6.1</td>
</tr>
<tr>
<td>48-72h</td>
<td>0.0</td>
<td>0.3</td>
<td>0.7</td>
<td>0.0</td>
<td>5.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Total</td>
<td>0.4 ± 0.3</td>
<td>3.1 ± 1.3</td>
<td>2.8 ± 1.0</td>
<td>32.1 ± 8.3</td>
<td>21.3 ± 7.6</td>
<td>9.4 ± 3.7</td>
</tr>
</tbody>
</table>

Using the data from the 72 h rats, hippuric acid is the largest metabolite with 32.1% of the administered dose being excreted as this. However, it was only excreted during the first 48 h period, only 0.1% was excreted in the later samples. 3-hydroxyphenylacetic acid was the next major metabolite contributing 21.3% of the administered dose. This compound was excreted in all samples. Benzoic acid made up the third largest contributor with 9.4% of the dose. There was very little benzoic acid excreted in the first 24 h period with the bulk appearing in the 24-48 h sample then reducing again in the 48-72 h sample.
Figure 5-5. HPLC MS–PDA-radioactivity analysis of 6 h rat urine using the RP-Max method. Peak numbers as used in Table 5-2.
Figure 5-6. HPLC radioactivity chromatograms of compounds in urine using the Polar column method. Peak numbers as used in Table 5-4.
5.3.4 Faecal analysis

No radioactivity was found in the 1 and 6 h samples. The 12, 24, 48 and 72 h samples all contained significant levels of radioactivity, between 9.4% of the dose in the 12 h sample to 18.4% in the 72 h sample.

The most accurate picture of the rate of faecal excretion of the dose can be seen from the 72 h samples. (see Figure 5-7). The majority of the radioactivity (13%) is excreted in the first 24 hours with a further 4% in the next 24 h period and just over 1% in the final 24 h period.

---

![Radioactivity excreted in faeces](image_url)

**Figure 5-7. Cumulative faecal levels of radioactivity from 72 h rats. ± SD (n=3)**
Analysis of the faecal samples by HPLC-RA-MS² revealed the presence of a number of compounds. Only two peaks were seen in the 12 h sample. The main peak was 3-hydroxyphenylacetic acid, which accounted for 93.7% of the radioactivity present. The other peak was hippuric acid. Again using the 72 h rat, in the 0-24 h samples only 3-hydroxyphenylacetic acid was seen. In the 24-48 h samples 3-hydroxyphenylacetic acid was also the major peak with some hippuric acid in one of the three rats, making up 3.9% of the radioactivity present. A similar picture was seen in the 48-72 h samples with two of the three rats having hippuric acid and one unknown peak present making up 9.6 and 2.2% of the radioactivity present respectively, (see Table 5-5). Peak number as used in Table 5-4

<table>
<thead>
<tr>
<th>Table 5-5 Radioactive compounds excreted in faeces as a percentage of the faecal radioactivity ± SD (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>Peak 1</td>
</tr>
<tr>
<td>Peak 2</td>
</tr>
<tr>
<td>Peak 3</td>
</tr>
<tr>
<td>Peak 4</td>
</tr>
<tr>
<td>Peak 5</td>
</tr>
<tr>
<td>Peak 6</td>
</tr>
<tr>
<td>Peak 7</td>
</tr>
</tbody>
</table>

5.3.5 Analysis of plasma

Radioactivity was rapidly absorbed into the plasma, accounting for 0.45% of the dose in the 1 h sample. The level fell to below half of this by 12 h and was below the limits of detection in the 72 h sample.

Only phenolic acid analysis was carried out on plasma as levels of activity and sample size did not allow for multiple analyses.
Although hippuric acid was found in all plasma samples a radioactive peak co-eluting with hippuric acid was only seen in the 6 h and 12 h samples.

![Radioactivity in plasma](image)

**Figure 5-8. level of radioactivity in plasma, ± SD (n=3)**

### 5.3.6 Analysis of kidney

The level of radioactivity peaked in the kidney at 0.64% of dose in the 12 h sample falling to 0.22% in the 24 h sample. Previous analysis of kidney taken at 1 h had revealed a range of flavonols present. However, our findings here for the 6 h and 12 h kidney show that hippuric acid was the only measurable radioactive compound present in the 6 h sample. The 12 h sample contained three radioactive peaks, hippuric acid was the main peak followed by 3-hydroxyphenylacetic acid and one unknown early eluting peak which matched the retention time of one of the unknown peaks, peak 2 in the urine samples.
5.3.7 Analysis of liver

The level of radioactivity in the liver stayed at a constant level of around 0.5% throughout the experiment. However, analysis of the sample for phenolic acids did not reveal the presence of any of these compounds. The low level of activity did not allow for analysis for flavonols as well as phenolic acids. The liver was the largest organ in the rat and in terms of radioactivity per gram it was lower than that found in the early kidney samples.

No significant levels of radioactivity were found in any other organ or tissue. Muscle did account for 1% of the dose at the 1 h sample but this is more likely due to residual blood. The fact that muscle mass accounts for 25% of the body mass of the rat can be the source of error when only small (10 mg) samples can be solubilized for estimation of radioactive content. This is possibly the case to a lesser extent for the liver samples too.

5.4 Discussion

The data presented here provides the first complete quantitative picture of the fate of [2-\(^{14}\)C]quercetin-4′-glucoside when fed to rats. At least 70% of the dose was absorbed and excreted via the urine while 19% was excreted in faeces. The remainder of the dose was found in the radioactivity washed from the metabolism cages, which could have come from either or a mixture of the urine and faecal material.

Previous attempts to obtain data like this by use of a radiolabelled compound have not been fully successful due to various reasons. Two studies were carried out using radioactively labelled quercetin (aglycone) with the carbon label at the 4 position in the C ring (Walle et al., 2001b; Abrahamse et al., 2005). These studies were limited in their success due to the positioning of the carbon label. During ring fission by colonic bacteria the C ring is opened and the carbon at position 4 is separated from the A and B rings and can therefore only be used to follow the fate of the carbonyl moiety. The study
by Abrahamse et al., (2005) also reported on the fate of $^3$H-labelled (-)-epicatechin in rats. Again this experiment had limited success, as there appears to have been exchange of the tritium label into water during the study. A similar study using both $^3$H-labelled (-)epicatechin and (+)-catechin also reported exchange of the tritium with water in the oral but not the intravenous dose (Catterall et al., 2003). This had occurred in the oral dose within three hours and, as the label was in the 3 position, was believed to be due to bacterial degradation. Unlike the study here very little of the dose was excreted via the urine (2.5%), with the majority being excreted via the faeces within 24 h (84%). A further 5.8% was excreted in faeces between 24-48 h and then trace amounts (0.3% and 0.2%) between 48-72 and 72-96 h. The compounds making up the radioactivity were not investigated. The researchers in this study were aware of the need to administer a nutritionally relevant dose and suggest that high doses may cause presystemic metabolism to become saturated thereby allowing more of the parent compound to pass into the circulatory system.

The fate of (-)-[4-$^3$H]epigallocatechin gallate, EGCg was also investigated (Kohri et al., 2001). The investigators reported no tritiated water in urine of the rats and they found the excretion ratio of bile to urine to be 97:3. The major metabolite, after deconjugation was 4',4''-di-O-methyl EGCg representing almost 15%.

One other study using [4-$^{14}$C]-quercetin was carried out by (Ueno et al., 1983). Although this suffered from the same problem as the studies by Walle et al., (2001b; 2005) the samples collected after 1 and 6 h did contain information that has been an important addition to work reported here, and will be discussed later.

A study into the fate and distribution of the aglycone quercetin in pigs and rats was carried out by (De Boer et al., 2005). They fed non labelled quercetin to rats at doses of 0.1 and 1% of their diet for 11 weeks and also to pigs at a level of 500 mg/kg for 3 days. The two pigs used weighed 122.4 and 138.9 kg. The dose level in both cases was beyond that which could possibly be classed as nutritionally relevant. As onions, one of the richest sources of quercetin, at best contain these compounds at approximately 0.5% w/w one can judge the level of the dose given to rats. In this study they found evidence of quercetin in many organs, especially those that were highly vascularised e.g. lungs followed by the liver and kidney. This could be explained by the
A feed of quercetin, at a dose of 50 mg/kg/d, was given to pigs over a four week period, in an attempt to learn if there was accumulation in tissues (Bieger et al., 2008a). Again this level of dose could not be described as “nutritional”. The finding were that apart from the organs involved in metabolism and excretion quercetin concentrations did not exceed those found in plasma.

There have been very few studies that try to look at the products formed from action of the colonic microflora. Gonthier et al., (2003) fed red wine polyphenols to rats and carried out analysis of phenolic acids in urine. Olthof et al., (2003) fed chlorogenic acid, rutin and black tea to human volunteers and measured the phenolic acids present in urine. Both studies had similar problems in that most of the catabolites formed from these feeds are already present in urine from the normal metabolic functions of the body. It was not possible in these studies to give an accurate estimate for the recovery of the initial dose. Nor did they attempt to estimate the level of intact metabolites in urine.

In contrast in the present study we were able to follow the radioactivity as it progressed through the GI tract, (see Figure 5-2) and also to identify the compounds it was converted into during its passage (see Table 5-2). As would be expected the bulk of the metabolites were formed in the duodenum and ileum in the 1 and 6 h samples. However, what was unexpected was the fact that 43% of the dose in the stomach of the 1 h rat was free quercetin. Along with the fact there were other compounds that no longer displayed flavonol-like properties making up a further 16% of the dose in the stomach. One of these compounds had mass spectral similarities to a proposed “oxidised quercetin” reported by Krishnamachari et al., (2002a). These compounds were no longer present in the duodenum.
Figure 5-9. Conversion of parent compound as it passes through the GI tract of 1 and 6 h rats. Quantities are as percentage radioactivity found in each segment.
It is also apparent from the data represented in Figure 5-9 that after deglycosylation the first metabolites formed are monoglucuronides. From their retention times one of the two must be quercetin-3’-glucuronide. This is the same as human metabolism were the 3’-conjugate is the major glucuronide found in the small intestine of ileal volunteers, as reported in Section 4.6. The pattern of reducing levels of quercetin-4’-glucoside with an increase in glucuronide metabolites is seen in the ileum/jejunum of the 1 h rats, where diglucuronides and methylglucuronides are also detected. Unidentified compounds made up 19% of the dose in the stomach of the 1 h rats and this increased to 26% in the ileum/jejunum. In the ileum/jejunum of the 6 h rats none of the parent compound was found and the appearance of sulphate metabolites was detected.

As previously reported (Mullen et al., 2002a), and in this study, the bulk of the recovered radioactivity, 95 and 84% remained in the GI tract after 1 and 6 h respectively. Very little of the dose would appear to have been absorbed, as measured by levels excreted and circulating in plasma. However, Ueno et al., (1983) were able to show, by using bile duct cannulated rats, that a proportion of the absorbed dose was excreted back into the GI tract as quercetin glucuronides. The dose in parts of this study was a massive 630 mg/kg and some unmetabolised quercetin was excreted in the faecal matter. At a normal, nutritionally relevant dose, quercetin is unlikely to be excreted in faeces intact, and this calls into question whether excretion back into the GI tract via the bile is also unlikely at a lower dose. This highlights the rational behind using such large doses. It is easy to argue that the high dose is needed to be able to detect the target compound. But if the dose is so large that it alters the normal metabolism of the subject, what use are the results? This is a common thyme in many studies including those of (De Boer et al., 2005; Bieger et al., 2008b). and as a consequence their relevance to nutrition and health is unclear.

In the 6 h rat by the time the dose has reached the caecum no intact metabolites are present, and all the radioactivity is made up of phenolic acids. No hippuric acid was found in the GI tract at this time point. However, in the 12 h rat ileum/jejunum, hippuric acid was the only compound detected. Once the radioactivity had passed through to the caecum and colon, 3-hydroxyphenylacetic acid was the main compound present, (see
Table 5-5). The presence of smaller amounts of hippuric acid may be explained by its excretion back into the GI tract via the bile.

Within 24 h over 50% of the dose has been excreted in the urine in the form of phenolic acids. The presence of intact flavonol metabolites is no long seen after the 6 h sample, either as radioactivity or as detected in the mass spectrometer. This agrees with our data found in human feeding studies that excretion of intact metabolites from plasma occurs rapidly post-absorption (Mullen et al., 2006). By 48 h the level excreted has risen to over 60% of the administered dose and by 72 h reached almost 70%.

What is of interest here is the movement of substantial amounts of radioactivity from the GI tract to urinary excretion with no increase in the level of radioactivity found in plasma. This could be explained by the timing of the absorption and excretion falling between the 6 and 12 h samples. However, for such a high proportion of the dose to have been excreted so quickly with no increase in plasma radioactivity levels would seem unlikely. If this is the case then the body must process the colon derived phenolic acids very quickly.

The metabolic profile changed during the course of the experiment. Initially, in the 12 h urine sample hippuric acid was the major metabolite along with 3-hydroxyphenylacetic acid. In the 24 h sample benzoic acid appeared as a major metabolite. [14C]Hippuric acid almost disappeared in the 48 h urine sample where benzoic acid appeared as the second largest peak. No radioactive hippuric acid was detected in the 72 h urine which contained only 3-hydroxyphenylacetic acid and benzoic acid in substantial amounts.

Two possible explanations for this are possible. The first is that hippuric acid is excreted back into the ileum/jejunum and then further broken down into 3-hydroxyphenylacetic acid. The appearance of hippuric acid in the 12 and 24 h ileum/jejunum samples would add weight to this argument. The second possibility is that there was breakdown of the hippuric acid post excretion. Faecal contamination of the urine, which is more likely the longer the experiment progressed, could have occurred. Therefore, the later urinary samples, 24-48 h and 48-72 h could have seen breakdown of hippuric acid to benzoic acid. As no benzoic acid was found outwith the urine it must be formed post absorption, most likely from 3-hydroxyphenylacetic acid. There were up to 3 additional minor metabolites present throughout the time course but their identities are unknown.
5.5 Conclusion

This study gives the most comprehensive picture of the fate of \([2^{-14}C] \text{ quercetin-4’-glucoside}\) in rats to date. At a nutritionally relevant dose, a minimum of 69\% was absorbed and excreted in urine. A further 18\% could be accounted for in faeces. The remaining 10\% was distributed between the urine and faeces and recorded as found in the cage washes. The level of flavonol metabolites was below that which could be measured in the on-line radioactivity detector. However, mass spectrometric analysis revealed their presence. They were rapidly absorbed and excreted in the urine. The parent compound was seen to have been deconjugated in the stomach of the rat within 1 h of receiving the dose. Furthermore, there were compounds present in the stomach that no longer displayed flavonol like properties. The main metabolite excreted was 3-hydroxyphenyacetic acid, accounting for over 32\% of the radioactivity followed by hippuric acid which accounted for just over 21\%. Benzoic made up 10\% with three unknown peaks accounting for a further 6\%. The remaining radioactivity contained in the cage washes was not analysed for the makeup of the radioactive components contained in it.

The radioactivity was rapidly excreted with over 75\% of the dose being excreted in the 12 h sample. In the 72 h rat, by 24 h 86\% had been excreted, by 48 it had increased to 89\% and by 72 h 96\%. The slowing of the excretion rate post 24 h may be due to hippuric acid being excreted into the GI tract via the bile, where it could be further metabolised to benzoic or 3-hydroxyphenylacetic acid before being reabsorbed and excreted.

This is the first quantitative study into the fate of a nutritionally important phytochemical. We were able to determine the total amount absorbed, the nature of the compounds absorbed and in what form they were excreted. The distribution of the compounds within the body tissues was observed and, in this case, there was no sizable accumulation in any organs. This is of great importance, as if this compound was to have some biological effect it would be need to reach a target organ in a concentration sufficient to cause this effect. Almost 70\% of the dose was absorbed as phenolic acids with less than 1\% accounted for by the intact metabolites. It may be that if this
compound does have some positive health benefits the role of phenolic acids may be of some importance, though due to their rapid excretion this may not be the case.

Quercetin is one of the most studied flavonoid and there is a body of associative evidence that it could have some health benefits due to its antioxidant properties. However, as seen in both rat and man the body goes to some lengths to conjugate the hydroxyl groups responsible for its antioxidant properties. In addition the part of the dose absorbed intact is very low and from the results reported here are not retained in the body.

The findings of this study highlight the need for many more of the proposed biologically important compounds to be investigated in a similar manner. Once the major metabolic routes and metabolites formed have been found in animals it would then be possible to carry out studies using $^{13}$C labelled compounds to determine levels of absorption, pharmacokinetic profiles and excretion in humans. It is also clear that rats and man do not produce the same metabolic profile, with the rat producing many more methylated compounds. It may be that the catabolic products formed differ too. But without labelled studies the quantification of metabolite and catabolites formed will remain unknown.
Chapter 6  Summary and conclusion

At the outset of this work the intended aim was to find out what happens to the flavonol conjugate quercetin-4´-glucoside, once ingested. The consumption of food containing quercetin conjugates has been associated with a reduction in the risk of CHD and strokes. Therefore, it would appear a reasonable assumption that a quercetin derived compound must act somewhere in the circulatory system to bring about this effect. Knowing what happens to quercetin after ingestion could make a valuable contribution to finding out if in fact quercetin conjugates play any part in causing the risk reduction. Without this knowledge we will only ever be able to speculate.

There were several issues that, during the course of the work, became central to what I was trying to achieve. Although I will discuss them as separate issues I think they are all linked.

The first and most crucial point is with regard to the methodology used in the analysis (Hertog et al., 1993a). The pioneering work of Hollman, extending Hertog’s method to the investigation of in vivo quercetin, in the mid 1990’s provided the first way of investigating the levels of flavonols in biological matrices. The word pioneering is used because the work was at the forefront in this field of research and could easily be described as “state of the art”. However, almost 15 years later it is still being used as a “gold standard method” and is in fact holding back our knowledge on A.D.M.E. research.

The great advantage of the enzyme hydrolysis method was that the exact standard for generating the calibration line is available. In the methodology I used, many of the mono and diglucuronide conjugates were quantified as quercetin-3-glucuronide equivalents. Therefore it can be argued that the enzymatic method must be more accurate. That is assuming complete hydrolysis of all conjugates has been achieved. From reports in the literature this is not necessarily the case (Gu et al., 2005a; Donovan et al., 2006).

With the increased availability of tandem mass spectrometers and the availability of glucuronide and sulphate standards of quercetin the use of aglycone
measurement should no longer be the method of choice for quercetin ADME studies. We now know that the major plasma metabolite is quercetin-3'-sulphate, which would be detected in the post-column fluorescence system described by Hertog et al. (1992a). As quercetin and (-)-(epi)catechin produce the most complex array of metabolites, other flavonoid A.D.M.E. should not be such a difficult task.

I put forward a theory regarding the possibility that compounds that are powerful antioxidants in vitro are not necessarily welcomed by the host in vivo. While I admit that this is based on a small data set, if all the analysis in this thesis had been carried out using the enzyme hydrolysis method no theory could have been constructed.

One of the other topics I have mentioned in this thesis is with regard to the probability that the results obtained in an analysis are an accurate reflection of what is actually in the sample. This is specifically where quercetin has been found in pre-feed plasma. If it is not the result of non-compliance with dietary instructions, or food being included in the allowed foods that contain measurable amounts of quercetin in them, then there must be an analytical problem.
I have stated that there are four processes that are part of an analysis.

1. Extraction
2. Purification
3. Separation
4. Detection

![Graph showing factors affecting the probability of an accurate analysis]

**Figure 6-1. Factors affecting the probability of an accurate analysis**

If all four of these processes are given adequate attention or weight (Figure 6-1) then the balance probability of obtaining an accurate result is maximised. Failure to pay attention to any one of these can lead to inaccuracies. It is very difficult to formulate what the minimum amount of “attention” must be paid to each of these processes. One level of “attention” may work for one compound in plasma whereas the same approach may not work for a different compound in the same matrix, due to co-elution and a non-selective detection system. However, the more involved the extraction, purification, separation and detection processes, the less likely the probability of having an inaccurate result.
I recently attended the Thermo mass spectrometer manufacturer users meeting, where one speaker told of a problem he had experienced in an analysis of a drug in plasma. The drug company he worked for had been running this validated analysis on a routine basis for some time using HPLC-MS$^2$ in SRM mode. However, a new batch of blank plasma contained a contaminant that co-eluted with the peak of interest and contained ions that matched the fragmentation pattern of the target compound. It being a validated analysis he could not simply adjust the gradient to try and separate the compounds. Being a drug company he was able to add an additional interface to the mass spectrometer that separated compounds based on ion mobility in an electric field, rather than their mass to charge ratios. This achieved the desired result without having to revalidate the analytical method, which I am told, is not a simple procedure.

I think this was a good example of an assay that used a simple protein precipitation extraction procedure with no purification step that was relying on the separation and detection system to ensure the correct result. It worked, in the beginning, due to the lack of interfering compounds, not to the fact that they had been removed by sample purification or been separated from the target compound or not seen due to the selectivity of the detector.

A reduction in attention to one of the processes can be offset, in some occasions, by maximising one or more of the other three. However, it may not always be obvious that there is a contaminant. It also shows that a validated assay is not always accurate.

A simple protein precipitation cannot selectively extract the compound of interest from a plasma sample. No purification steps means the extract must still contain a complex mixture of chemicals. An isocratic chromatographic system cannot separate more than a handful of compounds. Therefore, a non selective detection system cannot be expected to pick out the compound of interest from the other compounds present.

This may be a one sided view of some the analytical methodology used in quercetin aglycone analysis, but I think it is arguably a reflection of what is being carried out. The system I used compensated for the lack of purification by using a comprehensive separation system and two detectors, one for quantification the other to ensure peak purity. The need for the two detectors was seen in the analysis in Chapter 3.
where the mass spectrometer reported a compound that looked, in MS², like a quercetin glucuronide. However, by absorbance detection and MS³ was seen to be a false positive.

In Chapter 5 when analysing phenolic acids in urine, it was necessary to develop a second and third HPLC separation system to augment the already top end reverse phase method linked to the PDA and tandem mass spectrometer. This was because urine is a very complex mixture of compounds and there was no extraction or purification steps in the analysis. Furthermore, these compounds do not have good absorbance spectra that can provide additional information towards their positive identification.

The separation system initially used for flavonols used a C₁₂ column (Synergi RP MAX) with acetonitrile and formic acid. This column was initially chosen due to the separation it gave for a range of flavonol compounds and the low bleed of ions into the mass spectrometer, even at low pH. The column has proven to be stable, in terms of resolution and ion bleed over a period of years. The second used a C₁₈ column (Synergi Polar) this was successful in separating quercetin-3-glucuronide from quercetin-3-glucuronide isomers that did not separate using the C₁₂ system. However, these two systems are very similar in the separation selectivity and therefore a third method using a different selectivity process was used. This used a phenyl phase column (Gemini) which can separate compounds based on aromatic interactions with the stationary phase when using methanol as the organic solvent in the mobile phase.

Having identified the compounds by HPLC-MS² in three different systems the probability of making a misidentification, I believe, is decreased substantially.

The method of choice for phenolic acids is GC-MS. However, if this technique had been used there was no way of linking the radioactivity to the mass spectrometers results. In many of the samples hippuric acid and 3-hydroxyphenylacetic acid were present but not associated with any radioactivity. Therefore the radiolabelled tracer would have lost its advantage.

Evidence for quercetin glucosides having a direct benefit to human health.
Previously I have discussed the probability of an assay providing an accurate result. The analogy could be used with regard to quercetin glucosides having a positive benefit in human health.

The bulk of the evidence that an increased intake of flavonol-rich foods is responsible for reduction in the risk of CHD and strokes is associative. It was an epidemiological study that linked an increased intake of apples, onions and tea with a decrease in the incident of CHD and strokes (Hertog et al., 1993a). There then appears to be a jump in logic to conclude that this effect is due to the common flavonol, quercetin, contained in them. If this theory is correct then quercetin must be absorbed in a form and concentration that can cause such an effect. One of the few in vitro studies that has used a circulatory metabolite to investigate potential health benefits was carried out by Shirai et al., (2001). This was looking at the inhibitory effect of quercetin-3-glucuronide on lipid peroxidation. The lowest concentration used in this study was 20 µmole/L, a one hundred fold increase on the actual level of this compound in plasma as reported in Chapter 4. Based on this finding the probability that this is the mode of action would be very low. The findings in Chapter 4 showed that quercetin is rapidly absorbed and excreted, therefore the time it is available to the body to exert an effect is also minimal.

From the rat work it is clear that the vast bulk of the dose is absorbed as phenolic acids. Although these are still found in urine after 48 hours they seem to move rapidly from the large intestine to the urine with no increase in the level circulating in plasma. It would seem that the phenolic acids are either rapidly excreted into the urine or pass back into the intestine via the bile. So again the probability of these compounds exerting a health effect would appear to be low. Furthermore, radioactivity did not accumulate in any of the organs or tissues of the rat, making it unlikely that it could have any direct effect.

It may be that onions do play a part in reducing the risk factors of CHD and strokes. However, the mechanism by which they do so are far from clear. Onions also contain alkyl cysteine sulphoxides and these could potentially be responsible for the reduction in risk factors. Apples also contain hydroxy-chalcones and black tea also contains theorubigins, it is possible that each of these compounds could contribute to the risk reduction seen by Hertog et al., (1993a) and in fact there is no common link.
If we are serious about wanting to know the mode of action of flavonoids in human health a much more direct approach must be taken. If it is believed that these compounds exert protective effects on health they should be investigated in a similar manner to pharmaceuticals. No drug can reach the market without the manufacturer showing the complete fate and bioavailability of that compound being known. If it is a drug for heart disease the mode of action of the drug must be known and also be shown to reach the target site in a form and concentration that can cause the effect, and the dose required to produce this must also be known. Any drug company that submitted a report to the F.D.A. for approval of a drug based on measurements of the aglycone in plasma would be quickly refused. Maybe we can learn from the way the drug companies must operate and the processes them must comply with for drug approval.

An example of this type of problem is with regard to whether it is procyanidins that are the alleged active ingredients in red wine and cocoa products like dark chocolate. One school of thought claims they are not absorbed the other that there is no technique for extracting and detecting them in plasma. A radiolabelled compound, with the label in a suitable position, would answer this question. Furthermore, if these compounds are to play a role in maintaining epithelial function, animal studies using a radiolabelled compound would also seem like a suitable method to investigate this.

Until there is a change in approach to investigations of diet and human health there probably will be little progress in the field.
Appendices

Reference List


