

## DISPOSITION AND METABOLISM OF [2-<sup>14</sup>C]QUERCETIN-4'-GLUCOSIDE IN RATS

BRIGITTE A. GRAF, WILLIAM MULLEN, STEWART T. CALDWELL, RICHARD C.  
HARTLEY, GARY G. DUTHIE, MICHAEL E.J. LEAN, ALAN CROZIER AND  
CHRISTINE A. EDWARDS

*Plant Products and Human Nutrition Group, Graham Kerr Building, Division of  
Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of  
Glasgow, Glasgow G12 8QQ, UK (B.A.G., W.M., A.C.); Department of Chemistry,  
University of Glasgow, Glasgow G12 8QQ, U.K. (S.T.C., R.C.H.); Rowett Research Institute,  
Greenburn Road, Bucksburn, Aberdeen AB21 9SB, U.K. (G.G.D.); University of Glasgow  
Human Nutrition Section, Division of Developmental Medicine, Queen Elizabeth Building,  
Royal Infirmary, Glasgow G31 2ER, U.K. (M.E.J.L.); University of Glasgow Human  
Nutrition Section, Division of Developmental Medicine, Yorkhill Hospitals, Glasgow G3 8SJ,  
U.K. (C.A.E.)*

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**Corresponding author:**

Dr. C.A. Edwards,

Human Nutrition Section,

University of Glasgow Division of Developmental Medicine,

Yorkhill Hospitals, Glasgow G3 8SJ, U.K.;

Tel: -44-141-201-0709;

FAX: -44-141-201-9275;

E-mail: cae1n@clinmed.gla.ac.uk

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**Abbreviations:** dpm: disintegrations per minute, GI-tract: gastrointestinal-tract, HPLC: high performance liquid chromatography; RC: radiocounting; MS-MS: tandem mass spectrometry.

## Abstract

Quercetin-4'-glucoside is a major flavonol in onions and this study investigated the absorption and fate of radiolabelled quercetin-4'-glucoside in rats. Rats ingested [2-<sup>14</sup>C]quercetin-4'-glucoside and the distribution of radioactivity throughout the body was determined after 0.5, 1, 2 and 5 h. The gastrointestinal tract, liver, kidney and plasma were extracted and radiolabelled components identified and quantified using high performance liquid chromatography with on-line radioactivity detection and tandem mass spectrometry. Two hours after dosing all the [2-<sup>14</sup>C]quercetin-4'-glucoside had been metabolised. Over 85% of the ingested radioactivity was present in the gastrointestinal-tract at all time points with *ca.* 6% being absorbed and present in blood and internal organs, primarily the liver and kidneys. More than 95% of the absorbed radioactivity was in the form of >20 different methylated glucuronated and/or sulphated quercetin conjugates. Five hours after ingestion, the main radiolabelled metabolites were quercetin di-glucuronides in the gut, liver, kidney, and glucuronyl sulphates of methylated quercetin in plasma. The main site of quercetin metabolism appeared to be the gastrointestinal tract. Quercetin metabolites may have a major influence on the gut mucosal epithelium and on colonic disease.

There has been much interest in the role of non-vitamin antioxidants in plant foods and the prevention of heart disease and cancer. Molecules such as quercetin have much greater antioxidant potential *in vitro* than vitamins A, C and E (Rice-Evans et al., 1996; Shi et al., 2001). Epidemiological and laboratory studies suggest that intake of dietary quercetin, which in plants exists almost exclusively as glycoside conjugates, may reduce the incidence of coronary heart disease and certain cancers including colon cancer (Geleijnse et al., 2002; Knekt et al., 2002). Numerous *in vitro* and *ex vivo* experiments suggest the mechanisms may include delayed oxidation of low-density lipoprotein, inhibition of platelet aggregation, anti-inflammatory properties, modulation of enzyme activities associated with carcinogen activation and detoxification, prevention of oxidative DNA damage and modulation of gene expression, apoptosis and malignant transformation (Duthie et al., 2000; Middleton et al., 2000). However, it is still not established whether quercetin glycosides are absorbed from the GI-tract intact or if they are metabolised before, during or after absorption. Studies with ileostomy patients indicated the disappearance of over 50% of quercetin glucosides from onion before the terminal ileum (Hollman et al., 1995; Walle et al., 2000). However, this may have been the result of metabolism in the gut rather than absorption. Less than 2% of the ingested quercetin was found in plasma (Manach et al., 1998; Erlund et al., 2000; Moon et al., 2000) and less than 7% in urine (Graefe et al., 2001). If quercetin glycosides are metabolised in the gastrointestinal (GI) tract, the resultant metabolites may then be absorbed, but equally they may remain in the gut where they could play a role in the maintenance of GI-health due to their close proximity to epithelial cells in the small and large intestine. The activity of potential metabolites may not be the same as the parent compounds and thus it is essential that the exact fate of ingested flavonols be determined.

Quercetin-4'-glucoside is a major flavonol conjugate in onions (Tsushida et al., 1995), and in the present study, [2-<sup>14</sup>C]quercetin-4'-glucoside was fed to rats to investigate the

dynamics of quercetin absorption, metabolite formation and accumulation in body tissues and plasma. We have reported earlier that 1 h after ingestion of  $^{14}\text{C}$ -labelled quercetin-4'-glucoside, only 6.4% of the dose was absorbed from the GI-tract of rats. All absorbed radiolabelled quercetin had undergone metabolic modification (Mullen et al., 2002). However, the fate of the metabolites during the normal passage of food through the small intestine and into the colon was unknown. Consequently, this paper reports on the dynamics of quercetin metabolism absorption and bio-transformation in rat plasma and tissues over a 5 h period after ingestion of  $[2\text{-}^{14}\text{C}]$ quercetin-4'-glucoside.

## Methods

**Chemicals.** [2-<sup>14</sup>C]Quercetin-4'-O-β-D-glucoside (Figure 1), specific activity 138.8 MBq/mmol, was synthesised using procedures based on the previously reported method for the synthesis of [2-<sup>13</sup>C]quercetin-4'-O-β-D-glucoside (Caldwell et al., 2000) except that the intermediate ester was not purified by filtration through alumina. Standards of quercetin and kaempferol were purchased from Sigma-Aldrich Co. Ltd. (Poole, Dorset, U.K.). Isorhamnetin was obtained from Apin Chemicals Ltd. (Abingdon, Oxon, U.K.). A sample of quercetin-3-glucuronide was kindly supplied by Prof. Gary Williamson, Food Research Institute, Norwich, UK. Quercetin-4'-glucoside was generously provided by Dr. T. Tsushida, National Food Research Institute, Ibaraki, Japan. All other chemicals were of analytical grade and solvents were of HPLC grade purchased from Rathburn Chemicals Ltd. (Walkerburn, Peebleshire, U.K.).

**Animals and Oral Administration of [2-<sup>14</sup>C]Quercetin-4'-Glucoside.** After an overnight fast, 12 male rats (Rowett Hooded Lister strain; mean weight 430 ± 4 g) were each offered 1 g of stock rat feed (CRM, Special Diet Services, Witham, Essex, U.K.) containing 3.26 mg (7.6 mg/kg) of [2-<sup>14</sup>C]quercetin-4'-glucoside to give a radioactivity dose of 58.5 × 10<sup>6</sup> dpm. The rats consumed all of the ration within 2 min.

**Sample Collection and Preparation.** After 0.5, 1, 2 and 5 h, three rats were terminally anaesthetised with isoflurane and blood removed by cardiac puncture was collected in heparinised evacuated tubes (Becton Dickinson, Oxford, U.K.). Plasma was obtained by centrifugation at 1500 g for 10 min at 4 °C. The pelleted red blood cells were resuspended in 9 ml of phosphate buffered saline (pH 7.4). Livers were perfused *in situ* with chilled 0.15 M KCl and then removed along with brain, heart, kidney, lung, muscle, spleen, and testes. The GI-tract, including its contents, was removed intact. All samples were frozen

in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$  prior to lyophilization after which they were ground to a powder using a mortar and pestle and again stored at  $-80\text{ }^{\circ}\text{C}$ .

**Measurement of Radioactivity.** Aliquots of the lyophilized tissue, plasma and red blood cells were treated with tissue solubilizer (National Diagnostics, Hull, UK) for 3 h at  $50\text{ }^{\circ}\text{C}$  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. Aliquots of the solubilized tissue were added to scintillation fluid (Optiflow Safe One, Fisons, Longborough, UK) and the radioactivity was determined using a Wallac 1409 liquid scintillation counter (Pharmacia, Uppsala, Sweden).

**Extraction of Samples.** Lyophilized tissues from rats sacrificed at the same timepoint were pooled and extracted as previously described (Mullen et al., 2002). Briefly, aliquots of the pooled lyophilized tissues were extracted three times with 50% methanol in 0.1 M phosphate buffer (pH 7.0) containing 20 mM sodium diethyldithiocarbamate as an antioxidant. After centrifugation, the three methanolic supernatants were combined and the methanol removed *in vacuo*. The remaining aqueous phase was adjusted to pH 3 and partitioned three times with an equal volume of ethyl acetate. The ethyl acetate extracts were combined and reduced to dryness *in vacuo*, then re-suspended and stored in methanol at  $-80\text{ }^{\circ}\text{C}$ . Metabolites present in the aqueous phase were further purified using a  $\text{C}_{18}$  Sep Pak cartridge (Waters, Milford, MA, USA) which was eluted with methanol. The eluent was reduced to dryness *in vacuo*, re-suspended in methanol, and stored at  $-80\text{ }^{\circ}\text{C}$ . To precipitate proteins, plasma was treated twice for 10 min with 2.5 volumes of acetone. After centrifugation the two acetone extracts were combined and reduced to the aqueous phase *in vacuo*, and ethyl acetate and aqueous extracts were obtained as described above. Over 85% of the radioactivity, which was originally present in the tissues/plasma, went into solution during the extraction process. Prior to analysis by HPLC-radiocounting (RC)-tandem mass

spectrometry (MS-MS), the ethyl acetate extracts and the aqueous extracts were combined, and aliquots dissolved in HPLC mobile phase.

**Analysis by HPLC-RC-MS-MS.** Aliquots of plasma and tissue extracts containing *ca.* 30,000 dpm of radioactivity, were analyzed on a P4000 liquid chromatograph fitted with an AS 3000 autosampler and with the initial detection by a UV6000 diode array absorbance monitor scanning from 250 to 700 nm (Thermo Finnigan, San Jose, USA). Separation of the radiolabelled compounds was carried out using a 250 x 4.6 mm i.d. 4  $\mu$ M Synergy RP-Max column (Phenomenex, Macclesfield, U.K.), at 40 °C, eluted with a 60 min gradient of 5-40% acetonitrile in 1% aqueous formic acid at a flow rate of 1 ml/min. After passing through the flow cell of the absorbance monitor, the column eluate was split by 50%, and simultaneously directed to a radioactivity monitor (Reeve Analytical Model 9701, Lab Logic, Sheffield, U.K.), and a Finnigan LCQ Duo tandem mass spectrometer with an electrospray interface operating in negative ion mode and scanning from 150 to 2000 amu.



## Results

**Distribution of Radioactivity in Body Tissues and Plasma.** Distribution of radioactivity in rats was determined at 0.5, 1, 2 and 5 h after the ingestion of [2-<sup>14</sup>C]quercetin-4'-glucoside. At all time points, most (86% - 93%) of the ingested radioactivity was still present in the GI-tract, which included the stomach, small and large intestine, and their contents (Table 1). At 30 min, 7.2 % of the ingested radioactivity was present in internal organs and blood, indicating early absorption from the upper GI-tract. The absorbed radioactivity was distributed throughout all analysed organs, but the amounts present in the spleen, red blood cells and the brain were minute. The maximum amount detected in the brain, for instance, was only 1,000 dpm (8.2 nmoles) which represents 0.0017% of the ingested radioactivity. Highest levels were detected in plasma with a peak concentration of 2.9% of the ingested radioactivity (14  $\mu$ M) at 0.5 h. The total radioactivity present in internal organs and plasma was highest at 0.5 h and decreased thereafter during the time course of the experiment.

**Separation and Identification of Radiolabelled Metabolites.** HPLC-RC analysis of methanolic extracts of the rat feed confirmed that the only radiolabelled compound present was [2-<sup>14</sup>C]quercetin-4'-glucoside and no breakdown products had formed prior to dosing. The HPLC-RC profiles obtained from tissue and plasma extracts revealed a complex pattern of radiolabelled metabolites (Figure 2). Using negative ion, electrospray, tandem mass spectrometry, 22 of 26 detected metabolites were identified as quercetin conjugates with one or two glucuronyl, methyl or sulphate groups attached (Table 2). The use of full scan tandem mass spectrometric fragmentation patterns to identify these quercetin metabolites has been discussed in detail by Mullen et al. (2003). In the present study, eight new radiolabelled

metabolites were detected (peaks 1, 2, 7, 14, 15, 17, 23, 27) along with 19 metabolites identified earlier (Mullen et al., 2002). Peaks 1, 2 and 4 were not identified as they did not yield recognisable mass spectra. Peak 27 was identified as isorhamnetin (3'-*O*-methyl-quercetin) on the basis of its co-elution with an authentic standard and matching UV diode array spectra.

### **Classes of Quercetin Metabolites Detected.**

*Ingested [2-<sup>14</sup>C]quercetin-4'-glucoside:* [2-<sup>14</sup>C]Quercetin-4'-glucoside (peak 18) was present in the GI-tract at 0.5 and 1 h after the meal (Table 3). No intact [2-<sup>14</sup>C]quercetin-4'-glucoside was detected in the kidney and plasma, however the liver contained a very small amount at 0.5 h.

*Quercetin:* Thirty minutes after ingestion of [2-<sup>14</sup>C]quercetin-4'-glucoside, the aglycone quercetin (peak 24) represented 25% of the radioactivity in the GI-tract. At later time points the levels of free quercetin in the GI-tract declined to <8%. No free quercetin was detected in plasma, but small amounts were present in liver and kidney at some time points (Table 3).

*Mono-glucuronides:* Seven different radiolabelled compounds (peak 12, 15, 19, 20, 21, 22 and 23) were identified as glucuronide conjugates of quercetin and methyl-quercetin. Their different retention times indicated different positioning of the methyl and/or glucuronide groups on the quercetin molecule, changing the polarity and the elution behaviour of that molecule. On the basis of co-chromatography with a reference compound and matching mass spectra, peak 12 was identified as quercetin-3-glucuronide, which has been previously identified in rat plasma 1 hour after ingestion of [2-<sup>14</sup>C]quercetin-4'-glucoside (Mullen et al., 2002). Mono-glucuronides were present in all organs, but only at 0.5, 1 and 2 h after the ingestion of [2-<sup>14</sup>C]quercetin-4'-glucoside. After 5 h mono-

glucuronides were found only in the liver in relatively small amounts (Table 3).

*Di-glucuronides:* Nine of the radiolabelled metabolites (peak 3, 5, 6, 7, 8, 9, 10, 11, 14) were identified as di-glucuronide conjugates of quercetin and methyl-quercetin. Due to the fragmentation pattern of these compounds (Table 2) it was assumed that the two glucuronyl moieties were attached at different positions on the flavonol ring (Mullen et al., 2002). Di-glucuronides formed the major group of metabolites in liver and kidney at all timepoints, and in the GI-tract at 5 h. In plasma the quantity of di-glucuronides was highest at 0.5 h and decreased thereafter (Table 3).

*Sulphates:* Five different radiolabelled compounds contained a sulphate moiety. Peaks 13, 16 and 17 were identified as glucuronated and sulphated quercetin or methyl-quercetin. In plasma the quantity of glucuronated sulphates increased over time, and at 5 h 76% of all metabolites present in plasma were glucuronated sulphates. Peak 26 was a quercetin sulphate and peak 25 was a methyl-quercetin sulphate. Both of these sulphated metabolites were found only in the intestine at 0.5, 1 and 2 h (Table 3).

*Un-identified metabolites:* Metabolite peaks 1, 2 and 4 were not identified, as they did not yield recognisable mass spectra. All three metabolites had comparatively short HPLC retention times indicating that they are relatively polar molecules. Metabolite peak 4 appears in small quantities in intestine, liver and kidney. Metabolites 1 and 2 appear only in the intestine 5 h after the meal.

### **Metabolites in Tissues and Plasma.**

*GI-tract:* Thirty minutes after feeding more than half of the ingested radioactivity had undergone metabolic modification. At this early stage, mono-glucuronides, di-glucuronides and sulphated quercetin and/or methyl-quercetin conjugates were detected in the GI-tract together with free quercetin and its methylated derivative isorhamnetin. One and two hours

after the meal, mono-glucuronides formed a major portion of the metabolites. Five hours after the meal the mono-glucuronides had disappeared and di-glucuronides, in particular peak 10, formed the major class of metabolites (Table 3).

*Plasma:* Neither quercetin nor the ingested quercetin-4'-glucoside were detected in plasma at any time point (Table 3). In plasma, the amount of di-glucuronides decreased over the time course of the experiment and mono-glucuronides were present only at 0.5 and 1 h after the meal. However, the amount of glucuronidated sulphates increased steadily and 5 h after the meal glucuronated sulphates form the major group of metabolites in plasma in the form of peak 13, a sulphated quercetin glucuronide, and peak 16 a methylated, sulphated quercetin glucuronide. This latter compound was the predominant metabolite in plasma at 5 h, representing 62% of the radioactivity in plasma at that time point.

*Liver and kidney:* In both liver and kidney the most abundant group of metabolites were di-glucuronides followed by mono-glucuronides and sulphated glucuronides. The amount of di-glucuronides increased steadily over the time of the experiment. Small amounts of free quercetin were detected in the liver, and in kidney. In the liver a small amount of quercetin-4'-glucoside was present at 0.5 h (Table 3).

## Discussion

The biological fate of dietary quercetin was the focus of the present study. To obtain unambiguous data on the dynamics of quercetin absorption, metabolite formation and distribution throughout body tissues and plasma, radiolabelled quercetin-4'-glucoside, a major quercetin conjugate in onion, was synthesised and fed to rats. This paper contains new data on body distribution and biotransformation of [2-<sup>14</sup>C]quercetin-4'-glucoside in rats at 0.5, 2 and 5 h post administration, and includes previously reported data on body distribution and metabolism at 1 h post administration (Mullen et al, 2002).

**Absorption.** The administered dose of 3 mg [2-<sup>14</sup>C]quercetin-4'-glucoside to rats corresponds with the quercetin-4'-glucoside content in 250 g lightly fried onions consumed by a 70 kg human subject (Tsushida et al., 1995; Crozier et al., 2000). Around 6% of the administered radioactivity was absorbed into internal organs and circulating blood, with a peak plasma concentration of 14 µM (2.9% of the administered dose) at 0.5 h. This is in keeping with recent findings in human trials where peak plasma concentrations ranged from 0.74 µM to 7.6 µM following oral ingestion of a dietary relevant dose of onion or quercetin-4'-glucoside (Graefe et al, 2001; Olthof et al, 200; Hollman et al, 1997). In these studies, between 1.4 % and 6.4% of the ingested quercetin dose was found in urine. These results suggest that the relative bioavailability of quercetin from a single dietary relevant dose (i.e. 1-10 mg quercetin/kg body weight) of quercetin-4'-glucoside supplement or from onion ranges from 1-7 %.

In contrast, two studies with ileostomy patients (Hollman et al., 1995; Walle et al., 2000) found that over 50% of the ingested quercetin (in fried onions) disappeared between

oral ingestion and ileostomy effluent collection. As only 5% of this was due to degradation in either the stomach or ileal fluid, it was concluded that the ‘missing quercetin’ must have been absorbed (Hollman et al., 1995). Our data show that up to 44% of the quercetin metabolites in the GI-tract of the rats were methylated. The methylation of quercetin, presumably to isorhamnetin, or tamarixetin, and its further conjugation with glucuronic acid/sulphate groups has been described in a number of human and animal studies (Hollman et al., 1997; Manach et al., 1997, 1999; Moon et al., 2000; Graefe et al., 2001). However, in the ileostomy studies (Hollman et al., 1995; Walle et al., 2000), ileal fluid was analysed for the presence of only quercetin, quercetin glucosides and quercetin conjugates. Metabolic transformation of quercetin to methyl-quercetin and its conjugates might therefore provide an explanation for the “high disappearance” of quercetin in the ileostomy model. Thus, the disappearance of quercetin from the ileostomy fluid may over-estimate its absorption into the body and quercetin bioavailability may be substantially (approx 44%) lower than suggested by the studies of Hollman et al. (1995) and Walle et al. (2000).

High absorption of quercetin has also been reported by Walle et al., (2001) on the basis of a study where human volunteers ingested [4-<sup>14</sup>C]quercetin aglycone. The absorption of radiolabelled quercetin was estimated to be 36–53% of the ingested dose (100 mg) by calculating the “area under the curve” of the total radioactivity present in plasma over a period of 72 h. During this period 4.2 % of the orally ingested radioactivity was excreted in urine, while 52% was exhaled as <sup>14</sup>CO<sub>2</sub>. It was assumed that the exhaled <sup>14</sup>CO<sub>2</sub> originated from the portion of [<sup>14</sup>C]quercetin which was not absorbed from the small intestine but underwent bacterial degradation in the colon. The excretion of radioactivity in urine returned to baseline within 24 h, but the pharmacokinetic profile of total <sup>14</sup>C in plasma showed that sizable amounts of <sup>14</sup>C were still present 24-72 h after ingestion of [<sup>14</sup>C]quercetin. Paradoxically, most supplementation studies with quercetin-rich foods have shown that

plasma quercetin levels return to baseline values within 24 h (Aziz et al., 1998; Erlund et al., 2000; Graefe et al., 2001; Crozier et al., 2000). Therefore, radioactivity detected in plasma after 24 h by Walle et al., (2001) is likely to include  $^{14}\text{CO}_2$ , en route to the lungs for exhalation, together with other radiolabelled products such as hydroxyphenylacetic acids (Aura et al., 2002) and related colonic breakdown products of [ $^{14}\text{C}$ ]quercetin. Consequently, we believe that the “area under the curve” calculations, based on plasma radioactivity levels, used by Walle et al., (2001) to estimate quercetin absorption from the small intestine at 36 – 53%, is probably a substantial over-estimate. Urinary excretion of 4.2% of the ingested radioactivity may better reflect the true absorption of the quercetin supplement.

**Site of Metabolite Formation.** The catalytic activity of drug metabolising enzymes in the small intestine are generally lower than the corresponding values in the liver (Lin et al., 1999). Therefore, it is usually assumed that the liver is the major site for metabolism of xenobiotics such as quercetin (Griffith, 1982; Hackett, 1986). However, from our results we conclude that most of the metabolites must have been formed in the GI-tract. This conclusion is based on following observations: (i) all ingested radiolabelled quercetin-4'-glucoside had undergone metabolic changes within 2 h, and (ii) at all time points most of the radiolabelled compounds were present in the GI-tract (86% – 93% of administered dose). If the liver were the main site of quercetin metabolism, and the metabolites were excreted into the intestine via the bile, unless there was an extremely rapid rate of turnover, the liver should contain much higher levels of radioactivity, at least at one time point. In practice, the liver contained no more than 2% of the ingested radioactivity at any time point. The hypothesis, that quercetin conjugation takes place predominantly in the GI-tract, is supported by Crespy et al, (2003) who perfused rat intestine *in situ* with quercetin and collected intestinal eluent and bile separately. Bile contained only 10% of the quercetin conjugates, intestinal eluent

contained 90% of the quercetin conjugates, indicating that the majority of the metabolism had occurred in the gut not in the liver. Preliminary data by Cermak et al. (2003) supports our conclusion, as it was reported that portal blood of pigs contained exclusively quercetin metabolites, and that quercetin was metabolically transformed before reaching the liver.

A similar mechanism of pre-systemic elimination of xenobiotics through Phase II metabolism and immediate excretion of the metabolites into the lumen of the gut has been described for a range of other drugs (Suzuki and Sugiyama, 2000) and Walgren et al (2000) demonstrated the efflux of quercetin-4'-glucoside across human intestinal Caco-2 cell monolayers by the apical multidrug resistance-associated protein-2 (MRP2).

In the current study, 0.5 h after [2-<sup>14</sup>C]quercetin-4'-glucoside intake, a greater number of metabolites were present in the liver, kidneys and in plasma in comparison with the GI-tract (Table 3). This suggests that following metabolism and absorption from the GI-tract, further methylation and glucuronidation occur in the liver and kidneys, in agreement with previous reports (Moon et al., 2000; Day et al., 2000; O'Leary et al., 2003).

**Groups of Metabolites.** In most previous studies the presence of quercetin metabolites in human and animal plasma was shown indirectly, by releasing free quercetin and/or methyl-quercetin from glucuronyl and sulfate moieties via enzymatic or acid hydrolysis before HPLC analysis (Manach et al., 1999, 1998; Crespy et al., 1999). In the present study using a radiolabelled parent compound and LC-MS-MS analysis with on line radioactivity detection, seven different quercetin mono-glucuronides and nine quercetin di-glucuronides were detected in tissues and plasma together with three glucuronated sulphates and two quercetin sulphates. Glucuronidation of quercetin may occur at different and multiple hydroxyl groups on the quercetin molecule (Day et al., 2000). Isomers of quercetin glucuronide have identical molecular mass, and fragmentation patterns, however can be



separated by HPLC. Assuming that all five hydroxyl groups on the quercetin molecule are possible binding sites for glucuronyl units, and that hydroxyl groups 3' and 4' are potential loci for methylation, eight isoforms of methylated quercetin mono-glucuronides and five isoforms of quercetin mono-glucuronides could theoretically occur. In agreement with our data, Day et al. (2000) and O'Leary et al. (2003) identified seven different quercetin mono-glucuronides (three methylated and four un-methylated isoforms) produced by human liver cell free extracts and human HepG2 cells. Oliveira et al. (2002) and Oliveira and Watson (2000) reported the formation of four different isomers of quercetin mono-glucuronide by rat hepatocytes in suspension and human glucuronyl transferase (UGT) microsomes. Thus, four different laboratories report the same number of isoforms for quercetin mono-glucuronides despite the use of different extraction techniques, chromatographic conditions and equipment, supporting the hypothesis that UGTs selectively conjugate specific hydroxyl groups, most likely at positions 3, 7, 3' and 4' on the quercetin molecule (Boersma et al., 2001; Day et al., 2000). Boersma and co-workers (2001) however also report significant differences of the conjugation rate and conjugation position of UGTs derived from different organs (liver, intestine) and species (human, rat). Individual metabolite isomers may have different biological activities (Day et al., 2000; Yamamoto et al., 1999) therefore species dependent variation in metabolite formation may result in different biological effects.

In the present study the main metabolic end products, 5 h after quercetin-4'-glucoside ingestion, were di-glucuronides in liver, kidney and the GI-tract. In plasma, the main group of metabolites were glucuronated sulphates. Manach et al. (1997) also reported that the main circulating forms of quercetin in rat plasma were sulphated and methylated quercetin glucuronides. Our results clearly show that the metabolite profile in plasma does not reflect the metabolite profile in liver, kidney and the GI-tract.

**Un-identified metabolites.** It has been reported that the microflora in the caecum or colon can form oxidative breakdown products of quercetin (Aura et al., 2002). Five hours after the meal, 21% of the recovered radioactivity from the GI-tract was present in form of metabolite peaks 1 and 2. The identity of these two peaks could not be determined, however it is possible that these two compounds were oxidative breakdown products of quercetin formed by colonic microflora. The present study measured the quercetin absorption and metabolism in the small intestine. Oxidative degradation of quercetin into hydroxyphenylacetic acids and other products would be expected to appear in the caecum and colon of the rat at time points around 5 h after the meal (Brown et al., 1988).

In conclusion, this study supports the view that ~6% of dietary quercetin is absorbed from the GI-tract, at least in rats. Absorbed quercetin occurs in the body almost exclusively metabolised with one or more methyl, sulfate or glucuronide groups attached. From our results, we hypothesise that most of the ingested dietary quercetin is metabolised directly in the GI-tract. The retention of this flavonoid and its metabolites in the GI-tract suggests that the gut epithelium may be one of the main end users of their antioxidant and anti-cancer properties. Animal studies have reported an inverse relationship between quercetin intake and the development of colon cancer (Deschner et al., 1991; Yang et al., 2000). The potential bioactivity of these metabolites needs to be determined. In addition, as this study was stopped at 5 h, more research is needed to determine whether these are the final metabolic products or whether subsequent exposure to the colonic microflora results in further metabolism.

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## Footnotes

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## Legends for Figures

**Figure 1:** Structure of [2-<sup>14</sup>C]quercetin-4'-glucoside.

**Figure 2:** HPLC-RC traces of GI-tract, liver and plasma extracts obtained after oral ingestion of [2-<sup>14</sup>C]quercetin-4'-glucoside by rats. Extracts were analysed by gradient, reverse phase HPLC with on-line radioactivity detection. Parallel to the radioactivity detection, 50% of the column eluate was directed to a tandem mass spectrometer with an electrospray interface in negative-ion mode, for identification of the radiolabelled compounds. The illustrated chromatograms show the number of radiolabelled compounds in the GI-tract at 0.5 h, liver at 0.5 h and plasma at 2 h after ingestion of [2-<sup>14</sup>C]quercetin 4'-glucoside by rats. For HPLC retention times and MS-MS data on these and other metabolite peaks see Table 2

TABLE 1

*Distribution of radioactivity in rats after 0.5, 1, 2 and 5 h after ingestion of [2-<sup>14</sup>C]quercetin-4'-glucoside*

Distribution of radioactivity in rats 0.5, 1, 2, and 5 h after ingestion of [2-<sup>14</sup>C]quercetin-4'-glucoside (58.5 x 10<sup>6</sup> dpm). Radioactivity was measured by liquid scintillation counting after treating tissues/plasma with tissue solubiliser. Radioactivity is expressed as dpm x 10<sup>3</sup> per tissue/fluid per whole rat ± standard deviation (n = 3), and in italicised parenthesis as a percentage of the radioactive dose ingested.

Tissue/fluid	0.5 h	1 h	2 h	5 h
Gastrointestinal tract (including contents)	50,303 ± 4,460 (86.0%)	54,530 ± 4,728 (93.2%)	51,494 ± 8,091 (88.0%)	53,870 (92.1%)
Plasma	1,696 ± 401 (2.9%)	1,644 ± 157 (2.8%)	1,460 ± 35 (2.5%)	982 ± 149 (1.7%)
Red blood cells	7 ± 2 (0.0%)	4 ± 4 (0.0%)	5 ± 2 (0.0%)	5 ± 2 (0.0%)
Kidneys	595 ± 346 (1.0%)	468 ± 295 (0.8%)	104 ± 12 (0.2%)	163 ± 85 (0.3%)
Liver	1,127 ± 623 (1.9%)	684 ± 240 (1.2%)	304 ± 78(0.5%)	155 ± 22 (0.3%)
Spleen	5 ± 1 (0.0%)	6 ± 1 (0.0%)	5 ± 1(0.0%)	18 ± 30 (0.0%)
Brain	0 ± 0 (0.0%)	1 ± 1 (0.0%)	1 ± 2(0.0%)	1 ± 2 (0.0%)
Lungs	25 ± 3 (0.0%)	38 ± 1 0(0.1%)	27 ± 7(0.0%)	14 ± 3 (0.0%)
Heart	15 ± 2(0.0%)	18 ± 2 (0.0%)	16 ± 4(0.0%)	9 ± 3 (0.0%)
Muscle	753 ± 289 (1.3%)	839 ± 310 (1.4%)	471 ± 76(0.8%)	130 ± 51 (0.2%)
Testes	7 ± 3 (0.0%)	25 ± 3 (0.0%)	33 ± 6(0.1%)	24 ± 7 (0.0%)
Total radioactivity absorbed	4,230 ± 1,376 (7.2%)	3,727 ± 405 (6.4%)	2,426 ± 193(4.1%)	1,501 ± 315 (2.6%)
Total radioactivity recovered from rats	54,533 ± 5,569 (93.2%)	58,257 ± 4,804 (99.6%)	53,920 ± 8,143(92.2%)	55,371 (94.7%)

TABLE 2

*Identification of metabolites of [2-<sup>14</sup>C]quercetin-4'-glucoside isolated from rat plasma and tissues*

HPLC retention times, negative ion MS-MS fragmentation patterns and identification of metabolites isolated from rat tissues and plasma after oral ingestion of [2-<sup>14</sup>C]quercetin-4'-glucoside. Peak numbers refer to peaks in Figure 1. ( $R_t$  = retention time, [M-H]<sup>-</sup> = molecular ion, m/z = mass to charge ratio, GlcUA = glucuronyl unit, Glc = glucosyl unit). Comprehensive descriptions of peak identifications are published in Mullen *et al.* 2002.

Peak	$R_t$ (min)	Compound	[M-H] <sup>-</sup> (m/z)	MS <sup>2</sup> fragment ions (m/z)
1	9.30	Not identified		
2	12.04	Not identified		
3	17.22	Quercetin di-glucuronide	653	477 ([M-H] <sup>-</sup> -GlcUA), 301 ([M-H] <sup>-</sup> -GlcUA-GlcUA)
4	20.05	Not identified		
5	21.32	Methylated quercetin di-glucuronide	667	491 ([M-H] <sup>-</sup> -GlcUA), 315 ([M-H] <sup>-</sup> -GlcUA-GlcUA)
6	23.10	Quercetin di-glucuronide	653	477 ([M-H] <sup>-</sup> -GlcUA), 301 ([M-H] <sup>-</sup> -GlcUA-GlcUA)
7	24.24	Quercetin di-glucuronide	653	477 ([M-H] <sup>-</sup> -GlcUA), 301 ([M-H] <sup>-</sup> -GlcUA-GlcUA)
8	24.40	Methylated quercetin di-glucuronide	667	491 ([M-H] <sup>-</sup> -GlcUA), 315 ([M-H] <sup>-</sup> -GlcUA-GlcUA)
9	24.92	Quercetin di-glucuronide	653	477 ([M-H] <sup>-</sup> -GlcUA), 301 ([M-H] <sup>-</sup> -GlcUA-GlcUA)
10	26.27	Quercetin di-glucuronide	653	477 ([M-H] <sup>-</sup> -GlcUA), 301 ([M-H] <sup>-</sup> -GlcUA-GlcUA)
11	27.95	Quercetin di-glucuronide	653	477 ([M-H] <sup>-</sup> -GlcUA), 301 ([M-H] <sup>-</sup> -GlcUA-GlcUA)
12	29.38	Quercetin-3-glucuronide	477	301 ([M-H] <sup>-</sup> -GlcUA)
13	29.54	Quercetin mono-glucuronide sulphate	557	477 ([M-H] <sup>-</sup> -SO <sub>3</sub> ), 381 ([M-H] <sup>-</sup> -GlcUA), 301 ([M-H] <sup>-</sup> -SO <sub>3</sub> -GlcUA)
14	30.26	Methylated quercetin diglucuronide	667	491 ([M-H] <sup>-</sup> -GlcUA), 315 ([M-H] <sup>-</sup> -GlcUA-GlcUA)
15	31.12	Quercetin mono-glucuronide	477	301 ([M-H] <sup>-</sup> -GlcUA)
16	31.77	Methylated quercetin mono-glucuronide sulphate	571	491 ([M-H] <sup>-</sup> -SO <sub>3</sub> ), 395 ([M-H] <sup>-</sup> -GlcUA), 315 ([M-H] <sup>-</sup> -SO <sub>3</sub> -GlcUA)
17	33.16	Quercetin mono-glucuronide sulphate	557	477 ([M-H] <sup>-</sup> -SO <sub>3</sub> ), 381 ([M-H] <sup>-</sup> -GlcUA), 301 ([M-H] <sup>-</sup> -SO <sub>3</sub> -GlcUA)
18	33.87	Quercetin-4'-glucoside	463	301 ([M-H] <sup>-</sup> -Glc)
19	35.01	Methylated quercetin mono-glucuronide	491	315 ([M-H] <sup>-</sup> -GlcUA)
20	35.89	Methylated quercetin mono-glucuronide	491	315 ([M-H] <sup>-</sup> -GlcUA)
21	36.70	Methylated quercetin mono-glucuronide	491	315 ([M-H] <sup>-</sup> -GlcUA)
22	36.74	Quercetin mono-glucuronide	477	301 ([M-H] <sup>-</sup> -GlcUA)
23	38.70	Quercetin mono-glucuronide	477	301 ([M-H] <sup>-</sup> -GlcUA)
24	43.73	Quercetin	301	
25	50.36	Methylated quercetin sulphate	395	315 ([M-H] <sup>-</sup> -SO <sub>3</sub> )
26	50.56	Quercetin sulphate	381	301 ([M-H] <sup>-</sup> -SO <sub>3</sub> )
27	52.38	Isorhamnetin		

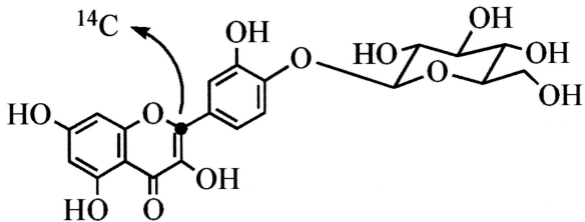
TABLE 3

Quantity of individual radiolabelled metabolites in rat plasma and tissues after oral ingestion of [2-<sup>14</sup>C]quercetin-4'-glucoside

Radiolabelled metabolites identified using HPLC-RC-MS/MS. Quantity of each metabolite expressed in dpm x 10<sup>3</sup> per whole organ or total plasma volume (100 x 10<sup>3</sup> dpm = 12 p mol radiolabelled compound). \* Peaks 21 and 22 indistinguishable in these samples \*\*Peaks 25 and 26 indistinguishable in this sample. GIT: gastro intestinal tract.

Peak	Compound (dpm x 10 <sup>3</sup> / whole organ)	0.5 h				1 h				2 h				5 h			
		GIT	plasma	liver	kidneys	GIT	plasma	liver	kidneys	GIT	plasma	liver	kidneys	GIT	plasma	liver	kidneys
1	Not identified	-	-	-	-	-	-	-	-	-	-	-	-	5716	-	-	-
2	Not identified	-	-	-	-	-	-	-	-	-	-	-	-	5824	-	-	-
3	Quercetin di-glucuronide	-	-	43	38	437	-	44	35	-	-	-	26	-	-	55	66
4	Not identified	905	-	-	20	437	-	-	21	1545	-	-	-	-	-	7	6
5	Methylated quercetin di-glucuronide	-	-	131	94	710	-	89	79	2832	-	15	16	-	-	-	-
6	Quercetin di-glucuronide	-	20	-	18	109	105	-	-	1493	142	9	-	1294	77	66	28
7	Quercetin di-glucuronide	-	75	-	-	-	-	-	-	-	-	-	-	-	31	-	-
8	Methylated quercetin di-glucuronide	-	-	77	72	328	-	-	58	3708	-	-	4	1132	-	-	4
9	Quercetin di-glucuronide	-	105	-	32	-	61	-	-	-	79	21	17	-	31	-	-
10	Quercetin di-glucuronide	1107	509	169	8	1365	337	229	55	7673	218	91	20	35967	21	13	55
11	Quercetin di-glucuronide	2264	180	80	35	328	135	27	39	1854	83	23	4	-	18	-	-
12	Quercetin-3-glucuronide	-	156	-	35	5786	35	47	37	4428	-	-	-	-	-	-	-
13	Quercetin glucuronide sulphate	-	27	77	-	3766	227	20	-	3605	245	25	-	647	138	-	-
14	Methylated quercetin di-glucuronide	-	-	-	-	-	-	-	-	-	-	-	-	-	56	-	-
15	Quercetin mono-glucuronide	-	271	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	Methylated quercetin glucuronide sulphate	-	159	253	14	382	717	87	14	3656	694	61	-	-	611	-	-
17	Quercetin glucuronide sulphate	-	56	-	-	-	-	-	-	-	-	-	-	-	-	3	2
18	Quercetin-4'-glucoside	24648	-	20	-	14301	-	-	-	-	-	-	-	-	-	-	-
19	Methylated quercetin mono-glucuronide	-	19	118	54	3548	8	92	36	3708	-	25	8	-	-	4	-
20	Methylated quercetin mono-glucuronide	1258	36	93	133	2675	7	38	79	3605	-	12	9	-	-	-	-
21	Methylated quercetin mono-glucuronide	-	44	66	42	6659 *	13 *	-	14 *	4737	-	22	-	-	-	-	-
22	Quercetin mono-glucuronide	1660	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	Quercetin mono-glucuronide	-	39	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	Quercetin	12626	-	-	-	3930	-	10	-	3450	-	-	-	3289	-	7	3
25	Methylated quercetin sulphate	-	-	-	-	9771	-	-	-	-	-	-	-	-	-	-	-
26	Quercetin sulphate	3270	-	-	-	**	-	-	-	4686	-	-	-	-	-	-	-
27	Isorhamnetin	2565	-	-	-	-	-	-	-	515	-	-	-	-	-	-	-

Figure 1



[2-<sup>14</sup>C]Quercetin-4'-O-glucoside



Figure 2

