

## Biomarkers for exposure to dietary flavonoids: a review of the current evidence for identification of quercetin glycosides in plasma

A. J. Day and G. Williamson\*

*Diet, Health and Consumer Sciences Division, Institute of Food Research,  
Norwich Research Park, Colney, Norwich NR4 7UA, UK*

Quercetin, a polyphenol with potential health effects, is absorbed by humans and measurement in plasma can be used as a biomarker for intake. However, the chemical nature of the quercetin in blood is still not known, although one possibility is that glucosides are found in an unchanged form from the original food. We propose that the existence of quercetin glucosides in plasma is unlikely, since the metabolic  $\beta$ -glucosidase capacity of the small intestine and of the liver is too great for quercetin glucosides to escape deglycosylation. We critically examine the limited number of studies which purport to detect quercetin glycosides in blood and the current evidence for the absorption of these compounds from the gastrointestinal tract. We emphasise the need for comprehensive identification of circulating compounds, since polyphenol glucuronides, the expected metabolites in plasma, have almost identical chromatographic properties to the glucosides at acid pH. Studies on the nature of quercetin metabolites in plasma are urgently needed so that the proposed biological activities of quercetin can be re-assessed and that a suitable biomarker of exposure can be established.

### Quercetin glucoside: Human metabolism: Deglycosylation: Rutin: Polyphenol

There are many reports in the literature on the possible biological effects of dietary flavonoids such as quercetin. However, there is serious lack of fundamental knowledge on the uptake and metabolism of these molecules in humans. Most biological activities have been measured with free quercetin (the aglycone) because of its ready availability, regardless of the limited amount in the diet compared to the naturally occurring glycosides. Evidence shows that quercetin conjugates have very different activities compared to those of the parent aglycone (Williamson *et al.* 1996; Morand *et al.* 1998). It is, therefore, imperative that the structures of the metabolites are correctly identified in order to test and re-assess biological activities, determine pharmacokinetics and to quantify dietary exposure.

Several studies have now shown that quercetin derivatives are found in the plasma after consumption of flavonoid-rich foods in volunteers (Hollman *et al.* 1997; de Vries *et al.* 1998; Janseen *et al.* 1998; Manach *et al.* 1998; Young *et al.* 1999). Methods to analyse polyphenols in urine and plasma employ gas chromatography, or HPLC with various detectors such as UV spectra, fluorescence, mass spectrometry or electrochemistry. However, results have not been validated between laboratories, and studies are additionally hampered by lack of knowledge on metabolites in humans. It is also very difficult to obtain, use and quantify appropriate standards, especially of the metabolites. Most studies involve enzymic or chemical

pre-treatment to convert quercetin metabolites into the parent aglycone, i.e. free quercetin or methylated quercetin (either isorhamnetin, 3'-methyl; or tamarixetin, 4'-methyl), which can be analysed and quantified chromatographically. Unfortunately information on the chemical nature of the metabolites is lost during pretreatment. Consequently research is now focusing on attempting to identify the quercetin metabolites, including the nature and number of the conjugating groups (methyl, sulphate and glucuronide) and the position of these groups on the polyphenol. Although there is considerable evidence for uptake, the chemical species of quercetin *in vivo* remains unknown. In this paper we review evidence for the absorption of quercetin glycosides and demonstrate the difficulty in distinguishing quercetin metabolites from the parent quercetin glycosides when analysed by HPLC with UV diode array detection.

### Deglycosylation

Flavonols in the diet are glycosylated, and depending on the nature and position of the sugar residue, absorption can occur in the small intestine (Hollman *et al.* 1997, 1999), although the mechanism is not understood. It is possible that a transporter, such as the sodium-dependent glucose transporter (SGLT1), may be able to transport some flavonol glucosides as an interaction has been shown (Gee *et al.* 1998). However, the largest compounds

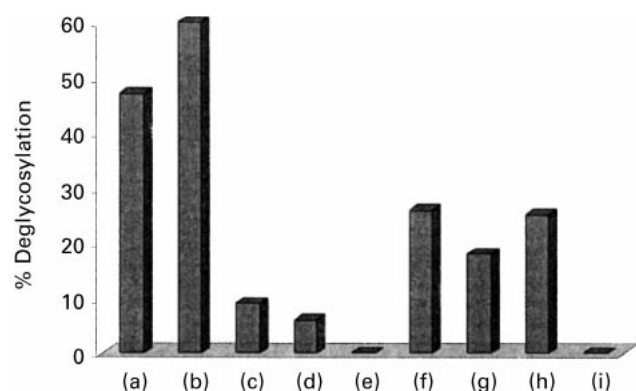
\* Corresponding author: G. Williamson, fax +44 (0) 1603 507723, email gary.williamson@bbsrc.ac.uk

transported by this mechanism (e.g.  $\beta$ -naphthyl glucoside) are smaller than the flavonoid glycosides (Panayotova-Heiermann *et al.* 1996) and phloridzin, a related flavonoid, interacts as a classical inhibitor of SGLT1 (Hirayama *et al.* 1996). High levels of  $\beta$ -glucosidase activity exist in human and rat small intestine epithelial cells (Day *et al.* 1998; Ioku *et al.* 1998) and liver (Day *et al.* 1998). This activity is attributed to a broad-specificity cytosolic  $\beta$ -glucosidase (Lambert *et al.* 1999) present in both tissues, and lactase phloridzin hydrolase (LPH; Day *et al.* 2000), found on the brush-border of the small intestine. If SGLT1 transports the flavonol glucoside, the cytosolic  $\beta$ -glucosidase can hydrolyse most glucosides, although quercetin-3-glucoside and quercetin-3-rutinoside (rutin) are not substrates. LPH can hydrolyse a broad range of flavonoid glucosides as shown in Fig. 1, but rutin was not a substrate for this enzyme. As LPH acts directly within the gut lumen, the released aglycone can diffuse across the gut wall. Within the enterocyte, conjugation of the aglycone will occur rapidly forming mainly the glucuronides and sulphates.

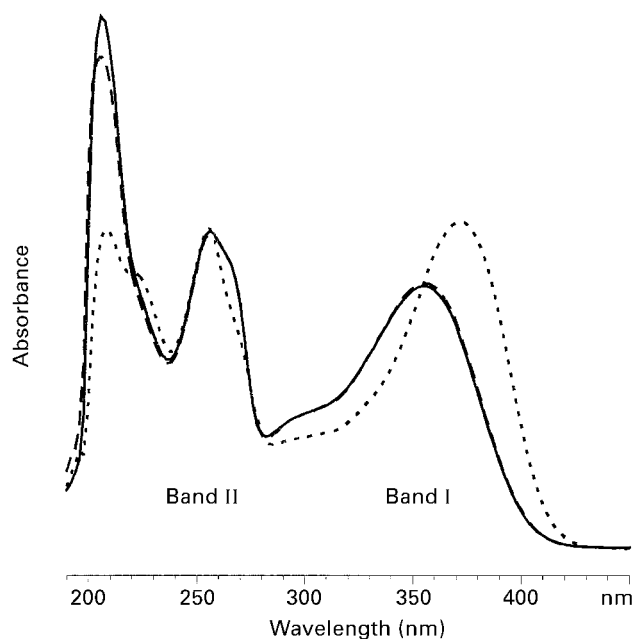
#### Identification by UV absorption spectra

UV spectra provide some detail for the identification of flavonols as shown in Fig. 2 and reviewed extensively by Mabry *et al.* (1970). The flavonol spectrum consists of two bands, I and II; if the 3-hydroxyl group of a flavonol is conjugated, a hypsochromic shift (to a shorter wavelength) occurs. Free quercetin exhibits band I absorption at 370 nm, whereas 3-glycosylation shifts band I to around 355 nm. Fig. 2 also demonstrates that the nature of the sugar substitution does not effect the absorption spectrum, and hence identification of the glucoside or glucuronide based on this evidence is not possible.

Identification of flavonols from biological samples



**Fig. 1.** Deglycosylation of flavonoid glycosides by lactase phloridzin hydrolase (LPH) isolated from sheep small intestine (Rivera-Sagredo *et al.* 1992): (a) quercetin-3-glucoside, (b) quercetin-4'-glucoside, (c) quercetin-3,4'-diglucoside, (d) 3'-methylquercetin-3-glucoside, (e) quercetin-3-rutinoside, (f) genistein-7-glucoside, (g) daidzein-7-glucoside, (h) phloridzin, (i) naringenin-7-rhamnoglucoside. Compounds (100  $\mu$ M) were incubated for 20 min (1  $\mu$ g LPH, 37°C, pH 6, 50 mM sodium phosphate buffer) with the aglycone formed monitored by HPLC with diode array (270 and 370 nm, see Fig. 3).



**Fig. 2.** UV-absorption spectra of quercetin and quercetin conjugates in methanol. ...., quercetin; —, quercetin-3-glucoside; ----, quercetin-3-glucuronide.

becomes a major problem when relying on HPLC data with diode array detection. Not only are physiological levels likely to be near the limit of detection, but the retention times can shift due to residual protein present in samples. Problems in possible misidentification are highlighted by a paper from Paganga & Rice-Evans (1997) in which detection of flavonol glycosides in plasma was suggested by evidence based on HPLC elution times and UV-spectra from on-line diode array. Rutin was identified in the two plasma samples analysed; however, as the retention time shifted 0.4 min between samples, and quercetin-3-sulphate and mixed sulphate-glucuronide conjugates of quercetin and 3'-methylquercetin also elute around this time, positive proof was not provided for the true identification of the circulating flavonol species observed in this study. Furthermore, the concentration of quercetin glycosides (including rutin) is higher (4.57  $\mu$ M) than observed after a meal rich in flavonol glycosides (0.37  $\mu$ M; Manach *et al.* 1998), or after a high intake of onion for several days (1.5  $\mu$ M; Janseen *et al.* 1998). Phloridzin was also tentatively identified in the plasma, which is unlikely due to the low concentration found in the diet (Price *et al.* 1999), the inability to be transported by SGLT1 (Lostao *et al.* 1994) and the rapid deglycosylation and glucuronidation by the small intestine (Mizuma & Awazu, 1998). It is more likely that phloretin glucuronide or some other dihydrochalcone was observed.

#### Identification by elution time

Further research showed the presence of intact flavonol glycosides in human plasma after consumption of onions

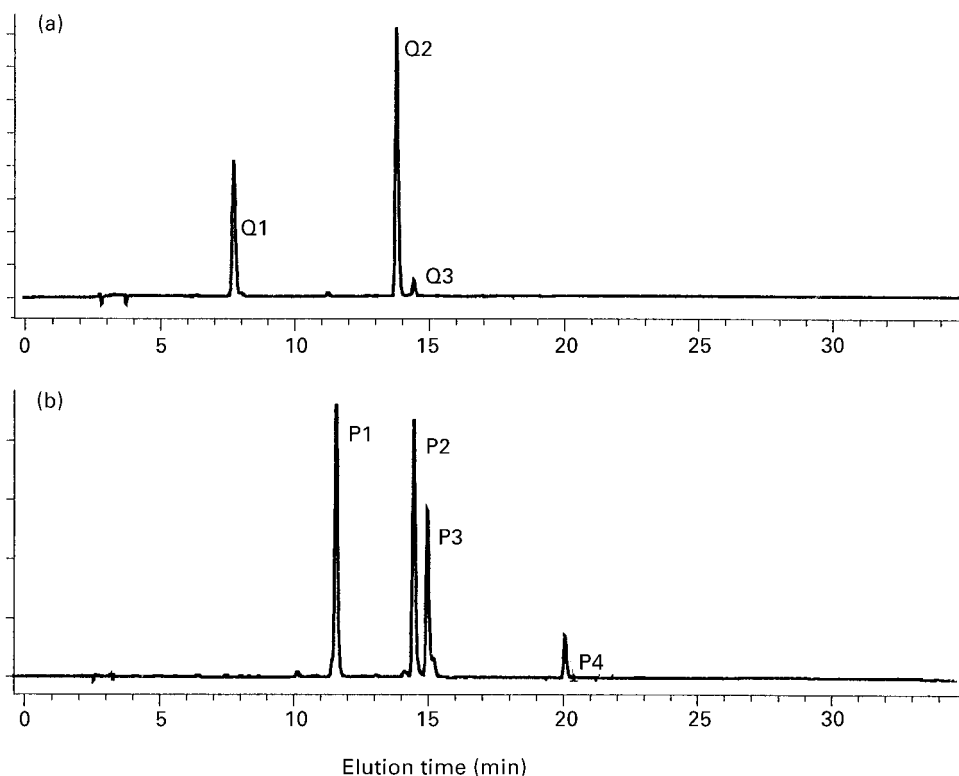
(Aziz *et al.* 1998). Quercetin-4'-glucoside and 3'-methylquercetin-4'-glucoside (both present in onion) were identified by retention time and co-elution with standards. The method employed was based on post-column derivatisation of the flavonol with aluminium for detection by fluorescence (Hollman *et al.* 1996). This method is particularly sensitive and is excellent for identifying low levels of flavonols in biological samples, although a free 3-hydroxyl group for chelation with aluminium is required. Hence, it appears reasonable that the presence of fluorescent-derivatised compounds in the plasma samples are the same as the standard flavonol glucosides eluting at the same retention time. However, no standards of the flavonol glucuronides were compared.

Fig. 3a is an HPLC chromatogram showing the elution profile of quercetin glucosides in a methanol extract of yellow onion (Rijnsburger); the order for elution of the quercetin glucosides was quercetin-3,4'-diglucoside (7.7 min), quercetin-4'-glucoside (13.8 min) and 3'-methylquercetin-4'-glucoside (14.4 min), which is in agreement with others (Park & Lee, 1996). A mixture of quercetin glucuronides can be made by incubation of quercetin with UDP-glucuronosyltransferase (e.g. using rat microsomes) in the presence of UDP-glucuronic acid. Fig. 3(b) shows the elution profile of the quercetin

glucuronides formed, also shown by Morand *et al.* (1998), using the same HPLC conditions as for the onion extract. The retention time of 3'-methylquercetin-4'-glucoside is almost identical to that of quercetin-4'-glucuronide, highlighting the potential problems of misidentification based on retention times without reference to metabolic standards. It should be noted that the three quercetin glucuronides shown in Fig. 3b should fluoresce when derivatised with aluminium as they are not conjugated at the 3-position. Although Aziz *et al.* may well have identified flavonol glucosides in human plasma, the evidence presented in their paper is not sufficient. Mass spectra and/or hydrolysis by a specific  $\beta$ -glucosidase or  $\beta$ -glucuronidase will provide further proof of the compounds circulating in the plasma.

### Absorption of glycosides

Mauri *et al.* (1999) determined rutin in human plasma after subjects consumed tomato puree for 14 d. Evidence was obtained by HPLC coupled to negative-ion electrospray, which produced mass ions of 609 and 300, characteristic of rutin under these conditions, and eluted at the appropriate retention time. This is good evidence, but the published



**Fig. 3.** HPLC chromatogram of (a) methanolic extract of onion; Q1, quercetin-3,4'-diglucoside; Q2, quercetin-4'-glucoside; Q3, 3'-methylquercetin-4'-glucoside, (b) quercetin glucuronides (P1, P2, P3) produced *in vitro* after treatment of quercetin (P4) by UDP-glucuronosyltransferase (UDPGT). UDPGT (5 mg/ml; bovine, Sigma), UDP-glucuronic acid (2 mM; Sigma) and quercetin (100  $\mu$ M) were incubated for 4 h (37°C, pH 7, 10 mM potassium phosphate buffer) followed by analysis by HPLC. Solvents A (water: tetrahydrofuran: trifluoroacetic acid 98:2:0.1) and B (acetonitrile), were run at a flow rate of 1 ml/min, using a gradient of 17 % B (2 min), increasing to 25 % B (5 min), 35 % B (8 min), 50 % B (5 min) and then to 100 % B (5 min). A column clean-up stage maintained B at 100 % (5 min) followed by a re-equilibration at 17 % B (15 min). The column was packed with Prodigy 5  $\mu$ m ODS3 reverse-phase silica, 250 mm by 4.6 mm internal diameter (Phenomenex, Macclesfield, UK). Diode array detection monitored the eluent at 270 and 370 nm.

amount that was detected, 1–5 µg/ml in plasma (from 1.7 mg/d rutin), is too high unless accumulation of rutin in plasma occurred over the period of the study. The actual value for the rutin level in plasma in the study was in the range 0.08–0.3 µM (personal communication; P. Pietta, 2000), although the concentration is still higher than expected based on previous work (0.1 µM after 49 mg/d quercetin mainly as rutin from tea over 3 d; de Vries *et al.* 1998).

Quercetin, but not rutin, was absorbed across the rat intestinal wall during *in situ* perfusion (Crespy *et al.* 1999a), which is not surprising as hydrophilic compounds generally cannot diffuse across biological membranes (Saija *et al.* 1995). Quercetin-3-glucoside was absorbed at approximately double the rate of quercetin in the same system (Crespy *et al.* 1999b), and a rat everted-jejenum model (Gee *et al.* 2000). In both cases quercetin-3-glucoside was not found after passage across the gut. An *in vivo* study with rats also showed quercetin metabolites in the blood but no rutin or quercetin-3-glucoside after oral dosing of the glycosides (Morand *et al.* 2000). Spencer *et al.* (1999), however, found small amounts of rutin and some quercetin-3-glucoside absorbed across an isolated rat perfused intestine. Deglycosylation of quercetin-3-glucoside had occurred, as evident by the aglycone and glucuronide formed, however unlike other flavonoid glucosides tested, a proportion of the glucoside was found in the serosal solution. The differences observed may be accounted for by the experimental model, length of incubation or strain of rat used.

There are several lines of evidence that rutin is absorbed in the colon only after deglycosylation. Rutin, unlike quercetin monoglucosides, did not interact with SGLT1 in the rat (Gee *et al.* 1998). Peak plasma levels of quercetin in human volunteers were observed at 9.3 h after a rutin supplement was taken (Hollman *et al.* 1997), indicating absorption from the colon. In addition, rutin was absorbed in rats at a time corresponding to the colon (Manach *et al.* 1997). Once rutin has reached the large intestine it will be hydrolysed to quercetin by microflora and subjected to further degradation, producing simple phenolics. Thus, it would seem unlikely that rutin *per se* can be absorbed. Baba *et al.* (1981, 1983) could not find intact labelled rutin ( $[^2\text{H}]$ quercetin-rutinoside) in human or rat urine; only ring fission products could be observed. Bourne & Rice-Evans (1998, 1999) were also unable to detect rutin in the urine of humans after consuming high levels of tomatoes. If rutin were absorbed, however, the human  $\beta$ -glucosidase present in the small intestine and liver is not capable of hydrolysing the rhamno-glycosidic bond to the aglycone and hence rutin would be observed in plasma. Biliary excretion of rutin may be the major pathway for elimination of this compound which would account for the lack of detection in the urine.

### Positive identification

In summary, data obtained on the metabolites of quercetin should be closely examined for the reliability of the analytical technique used. In particular, methods

based on UV spectra and retention times can provide useful but incomplete data on positive identification of glucosides compared to the glucuronide. Several approaches could be taken to characterise flavonoid metabolites more clearly.

- (1) Use of specific *highly purified* deconjugating enzymes in combination with UV-diode array: often enzyme preparations used for pretreatment of samples contain a mixture of deconjugating enzymes. Unless controls provide absolute evidence that additional activity is not present, positive identification is not possible. However, recombinant enzymes should be easier to purify to a state which is free of residual activity, and these can be used individually to show the presence of glucosides, glucuronides and sulphates.
- (2) HPLC with coularray detection: on-line electrochemical detection of compounds using a coularray can provide fingerprint-type information on the nature of a compound. The redox potential of the flavonoid will vary depending on the position, number and type of conjugating group. Appropriate metabolic standards will still be required for positive identification, although elimination of parent compounds is more reliable than with evidence based on retention time and UV-absorbance.
- (3) HPLC-MS/MS: tandem mass spectrometry provides the most useful tool currently available for the positive identification of compounds in biological fluids. Under appropriate conditions the mass of the parent metabolite and aglycone are obtained along with specific fragmentation patterns characteristic of conjugation at different positions on the polyphenol.

Furthermore, it is essential to consider the biochemical mechanisms involved in absorption and metabolism when analysing for compounds in biological systems. The ability of flavonoids to be transported into cells, to interact with conjugating or deconjugating enzymes and to be eliminated from the body are important questions that should not be overlooked. For example, although evidence of quercetin glucosides in human plasma has not been established with absolute certainty, the likelihood of significant levels of these compounds being present in plasma is low due to the high activity of human and microbial  $\beta$ -glucosidases. Any intact flavonoid glycosides in the plasma are unlikely to be conjugated further and so the pathway of excretion may be affected. Undoubtedly, however, the flavonols will circulate in the plasma mainly as glucuronide and sulphate conjugates. These metabolites will have different biological effects to the aglycone and parent glycoside, and thus should be the focus of future research.

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