# STUDIES ON THE PRO-OXIDANT CHEMISTRY OF FLAVONOIDS

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# STUDIES ON THE PRO-OXIDANT CHEMISTRY OF FLAVONOIDS

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# Propositions

1. Quinone/quinone methide isomerisation is involved in the pro-oxidant chemistry of catechol B ring flavonoids.
   (*This thesis*).

2. The pH has a significant influence on the chemical electrophilic behaviour of catechol B ring flavonoid quinones/quinone methides.
   (*This thesis*).

3. Pro-oxidant chemistry of quercetin may occur even under reducing cellular conditions.
   (*This thesis*).

4. In spite of the use of flavonoid supplements by consumers, the risk-benefit analysis for these compounds as functional food ingredients or food supplements still needs to be made.

5. Quantitative structure-metabolism studies should not be done under sub-saturating substrate conditions.
   *Holmes et al.*, Xenobiotica 25, 1269-81 (1995), *Bollard et al.*, Xenobiotica 26, 255-73 (1996), *Cupid et al.*, Xenobiotica 26, 157-76 (1996).

6. The anti-oxidant active moiety in flavonoid anti-oxidants varies with the substituent pattern.
   *Jovanovic et al.*, J. Chem. Soc., Perkin Trans. 2, 2497-2504 (1996).

7. The combination of theoretical and experimental approaches in studies on mechanisms of enzyme catalysis yields interesting differences and shows that a combination of the two will provide better answers.
   *Xu et al.*, Biochemistry 40, 12369-78 (2001), *Ridder et al.*, J. A. C. S. 122, 8728-8738 (2000).

8. The term "biological food" indicates a distinction that does not exist.

9. Food modulation aiming at beneficial health effects should consider depletion as well as suppletion of bioactive ingredients.

Propositions belonging to the thesis entitled "Studies on The Pro-oxidant Chemistry of Flavonoids".

Hanem Mohamed Awad
Wageningen, 6 March 2002

*To the soul of my late parents,*

*To Ahmed, Eman and Omar*

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# 1

# Introduction

## 1.1 Flavonoids

Natural polyphenols like flavonoids and their corresponding glycosides are important constituents of fruits, vegetables, nuts, seeds, tea, olive oil and red wine (1,2). Flavonoids are a large class of compounds, ubiquitous in plants, and usually occurring as glycosides. They contain several phenolic hydroxyl functions attached to ring structures, designated A, B and C (Figure 1) (3). Structural variations within the rings divide the flavonoids into several families (Figure 1):

- Flavonols (e.g. quercetin and kaempferol), with the 3-hydroxypyran-4-one C ring.
- Flavones (e.g. luteolin, apigenin and chrysin), lacking the 3-hydroxy group.
- Flavanones (e.g. taxifolin and naringenin), lacking the 2,3-double bond.
- Flavanols (e.g. catechin), lacking the 2,3-double bond and the 4-one structure.
- Isoflavones (e.g. genistein), in which the B ring is located in the 3 position on the C ring.

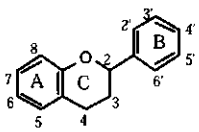
Common glycosylation positions in flavonoids are: the 7-hydroxyl in flavones, isoflavones and dihydroflavones; the 3- and 7-hydroxyl in flavonols and flavanones; and the 3- and 5-hydroxyl in anthocyanidins (4). The sugar most usually involved in the glycoside formation is glucose, although galactose, rhamnose, xylose and arabinose also occur (4), as well as disaccharides such as rutose.

## 1.2 Beneficial effects of flavonoids

On average, the daily Western diet contains approximately 1 g of mixed flavonoids (5), a quantity that could provide pharmacologically significant concentrations in body fluids and tissues (assuming good absorption from the gastrointestinal tract). The anti-oxidative properties of these compounds are often

claimed to be responsible for the protective effects of these food components against cardiovascular disease, certain forms of cancer and/or photosensitivity diseases. Furthermore, beneficial health effects in ageing have also been related to antioxidant action (6-9).

The chemical characteristics of the flavonoids are dependant on their free radical scavenging activity (10-18). This means the flavonoids may inactivate for example reactive oxygen species and prevent the deleterious consequences of their reactions (19). The antioxidant activity of flavonoids is also reflected by their inhibitory effects on lipid peroxidation (20-22), and on LDL oxidation induced by copper ions and macrophages (23,24). Flavonoid antioxidants (25,26) may act not




Flavonoid

| subclasses: | example | R at: 3 | 5 | 7 | 3' | 4' |
|---|---|---|---|---|---|---|
| flavan-3-ol | catechin | OH | OH | OH | OH | OH |
| flavanone | taxifolin | OH | OH | OH | OH | OH |
| | naringenin | - | OH | OH | - | OH |
| flavone | luteolin | - | OH | OH | OH | OH |
| | apigenin | - | OH | OH | - | OH |
| flavon-3-ol | quercetin | OH | OH | OH | OH | OH |
| | kaempferol | OH | OH | OH | - | OH |
| isoflavone | genistein | - | OH | OH | - | OH |

**Figure 1.** Chemical structure of flavonoids.

inhibitory effects on lipid peroxidation (20-22), and on LDL oxidation induced by copper ions and macrophages (23,24). Flavonoid antioxidants (25,26) may act not only by direct quenching of the reactive oxygen species, but also in a variety of other ways including inhibition of enzymes involved in the production of the reactive oxygen species, chelation of low valent ions ($Fe^{2+}$, $Cu^{+}$) able to promote radical formation through Fenton type reactions, and/or regeneration of membrane-bound anti-oxidants such as α-tocopherol (vitamine E) (2,27-34).

### 1.3 Beneficial effects of flavonoids other than anti-oxidant action

In addition to their mode of action as antioxidants, flavonoids may inhibit carcinogenesis by modulation of the metabolism of food-born carcinogens through inhibition and/or induction of phase I and phase II biotransformation enzymes, and by the suppression of the abnormal proliferation of early, preneoplastic lesions. Inhibition of cell proliferation may result from inhibition of various enzymes involved in cellular responses to growth factors, including protein kinase C, tyrosine kinase, phosphatidylinositol 3-kinase and/or the effect of flavonoids on expression of various tumor-related genes including antioxidant protein genes or the tumor suppressor gene p53 (1,35-40). Flavonoids and other plant phenolics are reported to have, in addition to their free radical scavenging activity, multiple biological activities including vasodilatory, anticarcinogenic, antiinflamatory, antibacterial, immune-stimulating, antiallergic, and estrogenic effects, as well as being inhibitors of phospholipase A2, cyclooxygenase, and lipoxygenase, glutathione reductase, and xanthine oxidase (41-54). The flavonoids have also been reported to elicit antiviral activities against HIV, Herpes simplex, influenza virus, and Rhinovirus, and they can act as inhibitors of cyclin-dependent kinases from breast carcinoma cells (55-61). Quercetin has also been shown to mediate the downregulation of mutant p53 in a human breast cancer cell line (62) and other studies indicate that quercetin-induced growth-inhibitory activity in ovarian cancer cells may be mediated by modulation of transforming growth factor beta 1 production (63).

Together these supposed beneficial effects of flavonoids provide the basis for the present rapidly increasing interest for the use of flavonoids as functional food ingredients and/or as food supplements. However, many of the reported health claims have been derived from observational epidemiological studies in which specific diets were shown to be associated with reduced risks on specific forms of cancer, cardiovascular disease, increased action of the immune system, and/or the reduction of stress. Identification of the actual ingredient in a specific diet responsible for the beneficial health effects remains an important bottle-neck for translating observational epidemiology to development of a functional food ingredient. Nevertheless, increased human exposure to polyphenolic flavonoid-type antioxidants can be expected in the near future. Also for flavonoids increased future human exposure regimens induce the

question on their pro-oxidant chemistry, including formation instead of scavenging of radicals and/or reactive electrophilic intermediates.

### 1.4 Risk-benefit concerns

In addition to concerns with respect to the existence of scientific support for the health claims and the proper identification of the active ingredient(s), important toxicological concerns arise. Paracelsus (1493-1541) already indicated toxicity to be a matter of dose, and toxicological risks may arise when daily doses of a compound rise above a certain threshold limit. For specific food enrichments, for example with vitamin A (retinoids), vitamin D, folic acid, selenium, copper and zinc, the margin between the amount functionally required for optimal health and the toxic dose is known to be small and enrichment of food by these ingredients is at present not allowed except for food restauration. For the other vitamins and minerals, including for example the antioxidant vitamins C and E, it is generally assumed that product enrichments will not lead to increases in doses of consumption by more than 4 to 5 times the daily intake. This may change upon increasing use of functional food ingredients in a wide range of food items and/or upon the use of isolated compounds as food supplements. For antioxidant-type functional food ingredients, at higher doses toxic pro-oxidant actions may become of importance.

### 1.5 Pro-oxidant chemistry of *ortho*-dihydroxy substituted aromatics

For a number of *ortho*-dihydroxy substituted aromatic molecules pro-oxidant chemistry has been documented. This holds especially for *ortho*-dihydroxy- (catechol-) type metabolites from estrogens and polycyclic aromatic hydrocarbons (64-66). The possible pro-oxidant toxicity of these catechol-containing compounds has recently been underlined by studies on the mutagenicity of estrogens. Metabolic activation of estrogens to redox active and/or electrophilic quinone/quinone methide-type metabolites has been proposed as one of the mechanisms responsible for the link between estrogen exposure and the risk of developing cancer (64-66). Especially catechol (*ortho*-diol)-type of metabolites resulting from cytochrome P450 catalyzed hydroxylation of estrogens may be involved (Figure 2).

The involvement of catechol-type metabolites has also been outlined to play a role in the metabolic activation of polycyclic aromatic hydrocarbons (Figure 3) (67,68). In addition to the conversion of the dihydrodiol metabolites to diol-epoxides by cytochromes P450, the conversion of the dihydrodiol metabolites of polycyclic aromatic hydrocarbons by dihydrodiol dehydrogenase may result in formation of reactive catechol-type metabolites (Figure 3). These catechol-type metabolites are

suggested to contribute, in addition to the diol epoxides, to the carcinogenicity and toxicity of the aromatic hydrocarbons.

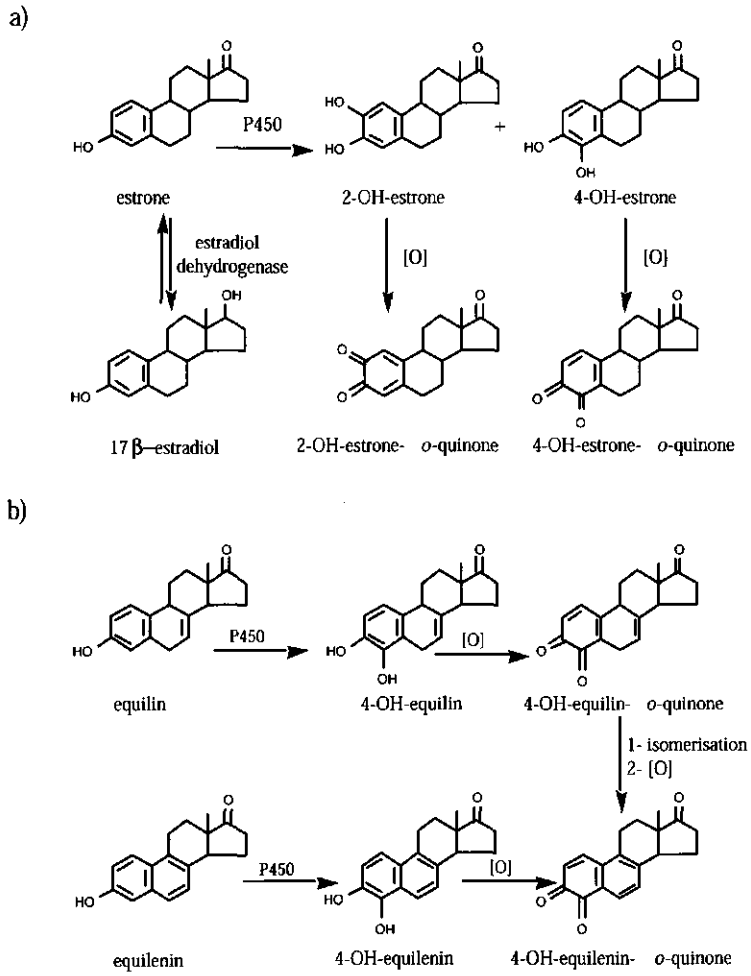



**Figure 2.** Metabolism of a) endogenous and b) equine estrogens to catechol and o-quinone metabolites by cytochromes P450 (65,66).

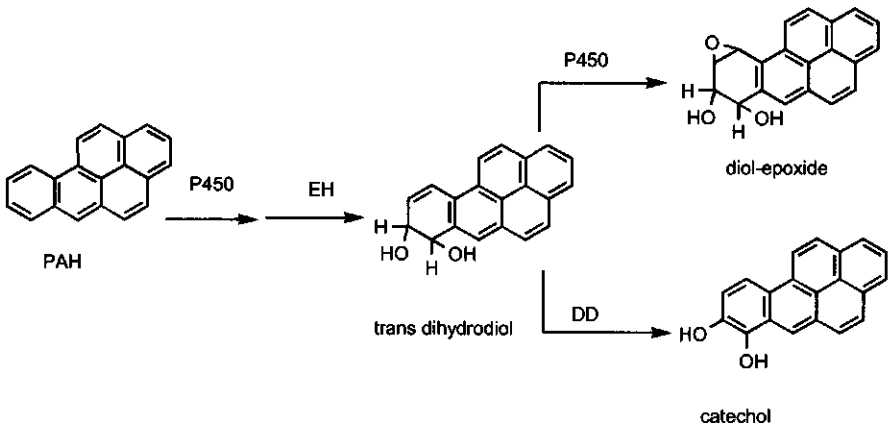



**Figure 3.** Metabolic activation of polycyclic hydrocarbons to catechol metabolites (67,68).

Figure 4 presents the mechanism behind the redox chemistry and alkylating toxicity of catechol-type metabolites. Redox cycling between the catechol and its quinone generates reactive oxygen species able of damaging cellular macromolecules like protein and DNA. In addition, oxidation of the catechols to quinones and their isomeric quinone methides (Figure 4) generates potent electrophiles that could alkylate proteins and DNA.

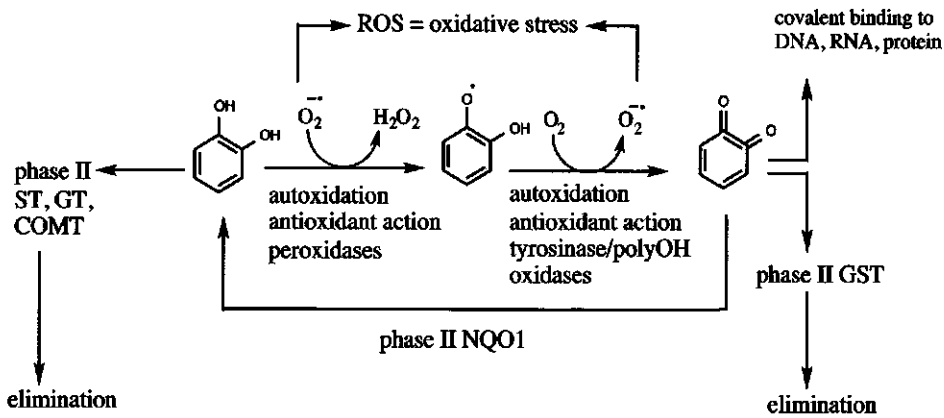



**Figure 4.** Schematic presentation of the redox chemistry and alkylating toxicity of catechol-type metabolites from estrogens and polycyclic aromatic hydrocarbons.

Taking this toxic pro-oxidative behaviour of catechol-type metabolites into account it is of interest that many flavonoids already contain this catechol-type structural element without the requirement for an initial bioconversion step. This especially holds for flavonoids like for example quercetin, taxifolin, luteolin, fisetin and many others containing a 3',4'-dihydroxy structural elements (Figure 1). For these 3',4'-dihydroxyflavonoids their pro-oxidative quinone/quinone methide chemistry is especially of interest because of their increasing use as functional food ingredients and/or food supplements.

### 1.6 Role for catechol pro-oxidant chemistry in the mutagenicity of the flavonoids

More than 70 flavonoids have been tested for mutagenicity in different strains of *Salmonella typhimurium* in the Ames test (69-72). Only aglycone flavonoids exhibited appreciable mutagenic activity (61). MacGregor and Jurd (1978) (69) reported that ten flavonoids, including quercetin, myricetin, kaempferol, tamarixetin and morin, were mutagenic (Table 1). Among the 16 flavonols tested by Nagao *et al.* (1981) (70), all except the 3-alkoxy derivatives were mutagenic. Among these, quercetin, rhamnetin and kaempferol were the most mutagenic to *Salmonella typhimurium* strains TA 98 and TA 100. Among the 22 flavones tested, only one compound, wogonin, showed relatively high mutagenicity (70,73,74). Quercetin and structurally related flavonols (3-hydroxyflavones) are active in both TA 98 and TA 100, the activity being higher in the former. They appear to be activated to DNA-reactive intermediates, probably involving initial oxidation of *ortho*- or *para*-hydroxy groups in the B ring to quinone methide intermediates. A free hydroxyl group at position 3 appears to be essential for this activity. Quercetin with its vicinal hydroxyl groups in the B ring, is mutagenic without metabolic activation. Kaempferol, which has only one hydroxyl group in the B ring, seems to require both an NADPH generating system and microsomes for activity. Also, the activation of kaempferol was more efficient in the presence of Aroclor-induced S-9 fraction than of non-induced S-9 fraction, suggesting the involvement of cytochrome P450 in this mutagenic activation (Table 1). Together the data on mutagenicity of flavonoids indicate that structural features which are essential for mutagenic activity are a flavonoid ring structure with

1) a free hydroxyl group at the 3 position (3-OH),
2) a double bond at the 2,3 position (C2=C3),
3) a keto group at the 4 position (C4=O), and
4) a structure which permits the proton of the 3-hydroxyl compound to tautomerise to a 3-keto compound.
5) Furthermore, especially compounds with free hydroxyl groups at the 3′ and 4′ positions of the B ring exhibit mutagenic activity already without requirement for

metabolic activation. When free hydroxyl groups are not present in the B ring a metabolic activating system is required for induction of the mutagenicity (69).

Together these data pointed at the involvement of quinone methides in the mutagenic activity of flavonoids. In addition to being mutagenic in the Ames test, quercetin displayed mutagenic activity in tester strains of *Escherichia coli* and *Saccharomyces cerevisiae* (75,76). Quercetin and kaempferol were reported to increase the frequency of sex-linked recessive mutations in *Drosophila melanogaster* (77). Thus with respect to the possible pro-oxidant toxicity it is of interest to notice that the mutagenic properties of the flavonoid quercetin have been demonstrated in a variety of bacterial and mammalian mutagenicity tests, and have been related to its quinone/quinone methide chemistry (1,15,69).

**Table 1.** Mutagenicity of selected flavonoids in *Salmonella typhimurium* strain TA98 in the presence of S-9 mix. Data taken from literature (69).

| Flavonoid | characteristics | nanomoles/plate | mutants/plate |
|---|---|---|---|
| quercetin | 3,5,7,3′,4′OH, C2=C3 | 55 | 706 |
| | | 83 | 1055 |
| | | 166 | 2000 |
| luteolin | lacks 3-OH | 166 | 0 |
| | | 1660 | 6 |
| fisetin | lacks 5-OH | 166 | 9 |
| | | 1660 | 26 |
| taxifolin | lacks C2=C3 | 166 | 12 |
| | | 1660 | 119 |
| 5-*O*-methylquercetin | conjugated 5-OH | 166 | 19 |
| | | 1660 | 65 |
| 3-*O*-methylquercetin | conjugated 3-OH | 166 | 7 |
| | | 1660 | 2 |
| 3′, 4′-di-*O*-methylquercetin | conjugated 3′- and 4′-OH | 166 | 33 |
| | | 828 | 15 |
| 3,3′,4′-trihydroxyflavone | lacks 5- and 7-OH | 166 | 6 |
| | | 1660 | 3 |
| kaempferol | lacks 3′-OH | 166 | 400 |
| | | 1660 | 117 |

As regards constituents in foodstuffs, the flavonols quercetin, kaempferol and myricetin, extracted from green tea and black tea, were suggested to account for the mutagenic activity of tea in *Salmonella typhimurium* (78). The fraction containing astragalin extracted from bracken fern was found to be mutagenic using the Ames test (79). Quercetin, kaempferol, isorhamnetin-3-sulfate and quercetin-3-sulfate were

suggested to be the constituents contributing to bacterial mutagenicity in spices and dill seed (80,81). Several authors have proposed that mutagenic activity of red wine and other complex mixtures such as tea in the Ames mutagenicity test is due to flavonols (82,84). However, recent studies using the forward mutation assay, Ara test (l-arabinose resistance test) of *Salmonella typhimurium*, considered to be more sensitive than the Ames test (85), reported that flavonols may not be the only putative mutagens in complex mixtures such as wine (86).

## 1.7 Carcinogenicity of the flavonoids

Based on these positive mutagenicity results in a variety of bacterial as well as mammalian test systems, several studies have investigated the possible carcinogenicity of especially quercetin. Several animal studies reported no tumor initiating activity (87-91). In contrast, Pamukcu *et al.* (1980) (92) reported induction of intestinal and bladder tumors by quercetin in male and female rats. A study from the National Toxicology Program (NTP, 1991) (93) in F344/N rats reported some evidence of carcinogenic activity of quercetin in male rats, based on an increased incidence of renal tubular cell carcinomas. Erturk *et al.* (1985) (94) reported bladder tumors in rats exposed to quercetin. And Dunnick and Haily (1992) (95) reported quercetin to show carcinogenic activity in the kidney of the male F344/N rat.

The mechanism behind this quercetin-mediated toxic effect remains a matter of debate (96). Ito (1992) (96) suggested a possible factor of special interest to be the role of alpha$_{2\mu}$-globulin nephropathy in chemically induced renal carcinogenicity, a nephropathy which is observed selectively in male rats only. Such a hypothesis would be in line with the observations that increased numbers of benign tumors are often observed in male but not female rats (93,95).

Another mechanism which may be of importance for carcinogenicity upon exposure to quercetin is the hypothesis that overloading the organism with quercetin may deplete the cofactor for catechol *O*-methyltransferases, S-adenosyl-L-methionine (SAM), because catechol *O*-methyltransferase metabolism represents an important metabolic pathway for catechol type flavonoids (97,98). This SAM cofactor depletion may affect the methylation of catechol estrogens, thereby providing increased possibilities for estrogen mediated carcinogenesis, because accumulation of catechol-type estrogens in the kidney may stimulate their oxidation to DNA alkylating electrophilic quinones (65,66,99).

## 1.8 Objective of this thesis

Since flavonoid quinone/quinone methides have been suggested as the major metabolites and intermediates responsible for the pro-oxidant toxicity and

mutagenicity of flavonoids, characterisation of flavonoid quinone chemistry is of importance. However, little information is available on the structure and reactivity of these flavonoid oxidation products. Therefore, the objective of this thesis was to investigate the pro-oxidant chemistry of flavonoids and to perform structure activity studies on the chemical behaviour and toxicity of 3',4'-dihydroxy flavonoids with special emphasis on the nature and reactivity of the quinone/quinone methide type metabolites formed. Using the GSH trapping method, HPLC, LC/MS, MALDI-TOF, $^{1}H$ NMR, $^{13}C$ NMR and quantum mechanical computer calculations the quinone/quinone methide chemistry of a series of 3',4'-dihydroxyflavonoids could be characterised. The results provide insight in structure activity relationships for the chemical behaviour and pro-oxidative toxicity of these electrophilic quinone/quinone methide metabolites. The results obtained also illustrate that this quinone/quinone methide chemistry is far from straight forward.

### 1.9 Outline of this thesis

In order to obtain better insight on the pro-oxidant chemistry of flavonoids the pro-oxidant chemistry of quercetin was investigated first. Using the glutathione trapping method and $^{1}H$ NMR the nature and formation of quercetin quinone/quinone methides could be demonstrated (**chapter 2**). In the next step a structure-activity study on the quinone/quinone methide chemistry of a series of 3',4'-dihydroxyflavonoids was performed (**chapter 3**). Using the glutathione trapping method followed by HPLC, $^{1}H$ NMR, MALDI-TOF and LC/MS analysis to identify the glutathionyl adducts, the chemical behaviour of the quinones/quinone methides of the different flavonoids could be deduced. Special emphasis was placed on the regioselectivity of the GSH conjugation and on the structural requirements in the flavonoids necessary for quinone/quinone methide isomerisation. Furthermore the possible pH-dependence of the quinone/quinone methide chemistry of these flavonoids was investigated with special emphasis on the regioselectivity of the glutathione addition as a function of pH (**chapter 4**).

Whereas **chapter 4** describes the mechanism of the pH-dependent chemistry of flavonoid quinones/quinone methides, **chapter 5** focuses on the reversible nature and the stability of the thiol conjugate formation with the flavonoid quinone/quinone methides. Finally **chapter 6** focuses on some initial studies on the possibility to provide data on the biological relevance of the observed quinone/quinone methide chemistry in biological systems.

Finally, the summary and conclusions are presented in **chapter 7**.

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# 2

## Peroxidase-catalyzed formation of quercetin quinone methide glutathione adducts.

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### 2.1 Abstract

The oxidation of quercetin by horseradish peroxidase/$H_2O_2$ was studied in the absence but especially also in the presence of glutathione (GSH). HPLC analysis of the reaction products formed in the absence of GSH revealed formation of at least twenty different products, a result in line with other studies reporting the peroxidase mediated oxidation of flavonoids. In the presence of GSH, however, these products were no longer observed and formation of two major new products was detected. $^1H$ NMR identified these two products as 6-glutathionylquercetin and 8-glutathionylquercetin, representing glutathione adducts originating from glutathione conjugation at the A ring instead of at the B ring of quercetin. Glutathione addition at position 6 and 8 of the A ring can best be explained by taking into consideration a further oxidation of the quercetin semiquinone, initially formed by the HRP-mediated one-electron oxidation, to give the *ortho*-quinone, followed by the isomerization of the *ortho*-quinone to its *para*-quinone methide isomer.

All together the results of the present study provide evidence for a reaction chemistry of quercetin semiquinones with horseradich peroxidase/$H_2O_2$ and GSH ultimately leading to adduct formation instead of to preferential GSH mediated chemical reduction to regenerate the parent flavonoid.

**Keywords:** quercetin; oxidation; semiquinone; *ortho*-quinone; *para*-quinone methide; glutathione adducts

## 2.2 Introduction

Flavonols are a group of naturally occurring compounds which are widely distributed in nature where they are found in glycosylated form primarily in vegetables and fruits (1-3). A number of studies have reported not only anti-oxidant (1-8) but also pro- oxidant (9-11) effects for many of these compounds. Because of its ubiquitous nature, quercetin has been the most widely studied flavonol. Some of its pro-oxidant properties have been attributed to the fact that it can undergo auto-oxidation when dissolved in aqueous buffer (12) resulting in its conversion to semiquinone free radicals, reactive electrophilic *o*-quinones and, due to redox cycling, formation of reactive oxygen species (12-14) (Figure 1). Due to the presence of the C4-keto moiety and the C2-C3 double bond in quercetin, formation of its *ortho*-quinoid type metabolite provides possibilities for isomerisation to quinone methides, known to be even more electrophilic than the quinones, and, like the quinones themselves, capable of forming adducts with various tissue macromolecules (15-16) (Figure 1). The pro-oxidant action of flavonoids as well as the electrophilicity of their *ortho*-quinone and quinone methide-type metabolites is of interest especially in the context of cytotoxicity, mutagenicity and possible carcinogenicity (2). Identification of the oxidation products of this potential antioxidant may therefore provide deeper insight into the mechanism of its toxic pro-oxidant action and may form the basis for new biomarkers for the detection of prooxidant activity of flavonoids.

Several literature studies report on the peroxidase-mediated one-electron oxidation of flavonoids. For quercetin, this one-electron oxidation in peroxidase-mediated transformation has been described to lead to incorporation of oxygen into the flavonoid structure (17-21), followed by enzymatic degradation of the intermediates to substituted benzoic acids. These acids can be further transformed by hydroxylation, decarboxylation, and ring fission reactions (22-24), finally leading to formation of a very complex mixture of polar reaction products (25) (Table I). Furthermore, peroxidase-mediated metabolism of flavonols is known to result in oligo- and polymerisation reactions occurring upon the peroxidase-catalysed one-electron oxidation of the flavonols (10,11). Although peroxidases normally give a one-electron oxidation (10,11), reaction pathways for oxidation of flavonoids often include a second one-electron oxidation and/or dismutation of the flavonoid semiquinone radical, giving rise to quinone-type intermediates (14,26). As a result, one-electron oxidation processes such as, for example, antioxidant action of a

flavonoid and/or its conversion by a peroxidase, in theory, may result in formation of alkylating quinone-type metabolites.

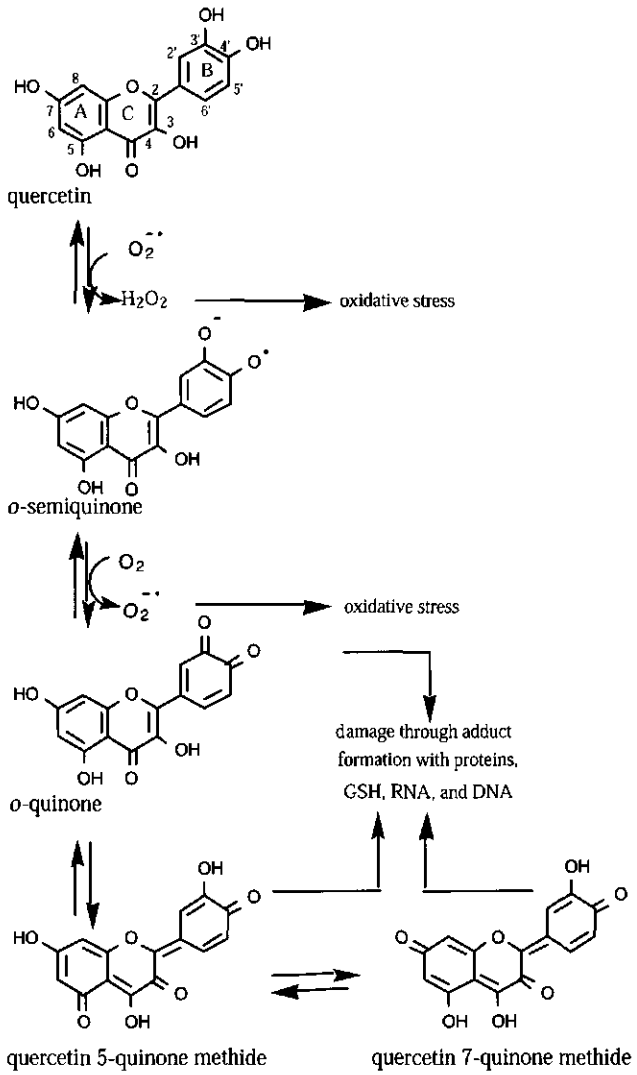
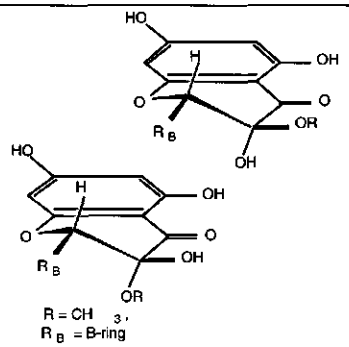
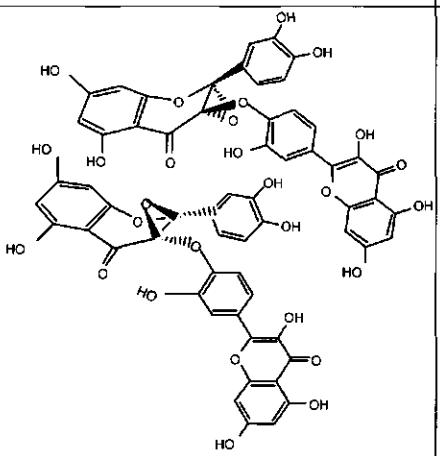
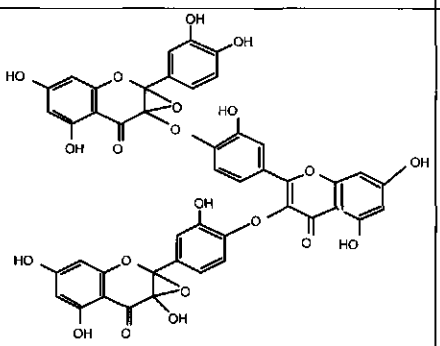
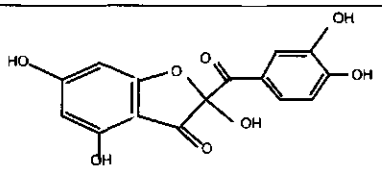



**Figure 1.** The oxidative pathway of quercetin to quercetin semiquinone, quercetin *o*-semiquinone and quercetin *para*-quinone methide indicating the formation of reactive oxygen species and of electrophilic quinone/quinone methides.

The objective of the present study is to investigate whether the one-electron oxidation of quercetin, induced by horseradish peroxidase, would give rise to the formation of quinone-type products. The high reactivity of quinones is known to

**Table 1**-Continued

| Name | Structural formula | Oxidizing system | Reference |
|---|---|---|---|
| 2-(3,4-Dihydroxyphenyl)2-hydro--3,5,7-trihydroxy-3-methoxy-4H-1-benzopyran-4-one (2 isomers) |  | Peroxidase/$H_2O_2$ | 19 |
| 2,3-Epoxy-2-(3,4-dihydroxyphenyl)-3-4O-2-(3-hydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-onyl-5,7-dihydroxy-4H-1-benzopyran-4-one (2 isomers) |  | Peroxidase/$H_2O_2$ | 19 |
| A trimer structurally related to the above compound |  | Peroxidase/$H_2O_2$ | 19 |
| 2-(3,4-Dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone |  | Bulk electrolysis | 14 |

are based on detection at 290 nm. Product peaks were collected and freeze-dried for further analysis by $^1$H NMR. Freeze-dried samples were dissolved in 25 mM potassium phosphate, pH 7.0, made with deuterated water when samples were for $^1$H NMR analysis.

*$^1$H NMR measurements.* $^1$H NMR measurements were performed on a Bruker DPX 400 spectrometer. A 1.5-s presaturation delay, a 70° pulse angle, and a 2.2 s acquisition time (7575 Hz sweep width, 32 K data points) were used. The data were processed using an exponential multiplication of 0.5 or 1.0 Hz and zero-filling to 64 K data points. Resonances are reported relative to HDO at 4.75 ppm.

**2.3 Results**

*HPLC analysis of the one-electron oxidation of quercetin.* Figure **2a** shows the HPLC chromatogram of the incubation of quercetin in the presence of horseradish peroxidase and $H_2O_2$. The results obtained reveal the formation of more than twenty products, with one major product (retention time at 22.6 min and $\lambda_{max}$ 295.1 nm) eluting at a position different from that of quercetin (retention time at 32.2 min and $\lambda_{max}$ 370.1 nm).

Figure **2b** shows the HPLC chromatogram of the aqueous fraction of this incubation remaining after diethyl ether extraction. After this extraction the presence of this major metabolite (at 22.6 min) and also the peak of quercetin (at 32.2 min) are no longer observed.

The HPLC chromatogram of the diethyl ether fraction of this incubation (Figure **2c**) shows the presence of the majority of products, including the one at retention time 22.6 min. This means that the majority of products of the peroxidase-catalysed quercetin degradation partitions preferentially into the ether phase instead of the water phase. Comparing the UV-spectra, it is seen, that the one-electron oxidation products have maximum absorption at lower wavelength than the parent compound, indicating decreased conjugation of the aromatic π-system (spectra not shown).

*HPLC analysis of glutathione adducts of quercetin.* Figure **3a** shows the HPLC chromatogram of the incubation performed in the presence of glutathione. Two major metabolites can be detected with retention times at 17.4 min and 18.5 min and $\lambda_{max}$ at 299.9 nm and 295.1 nm, respectively. Clearly, these two products differ from the oxidation products formed in the absence of glutathione and differ from quercetin as well.

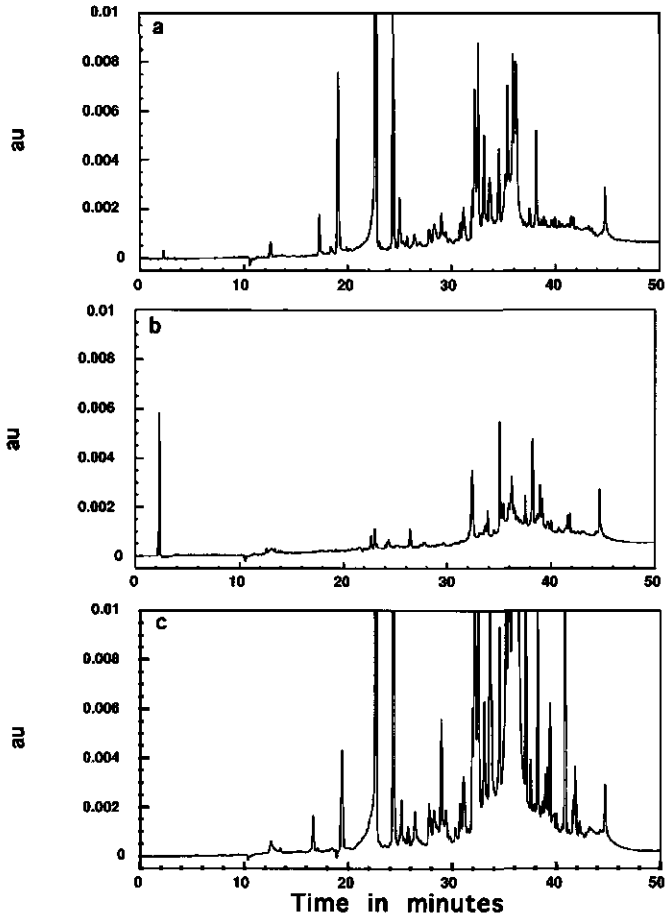



**Figure 2.** HPLC chromatogram of (a) the incubation of quercetin with horseradish peroxidase and $H_2O_2$ in the absence of glutathione, (b) the remaining aqueous layer of the incubation, and (c) the diethylether extract.

The HPLC chromatogram of the aqueous fraction of this incubation (Figure **3b**) shows the presence of the two major metabolites with retention times at 15.6 min and 16.7 min because of the acidic medium. This means that these two metabolites are water soluble.

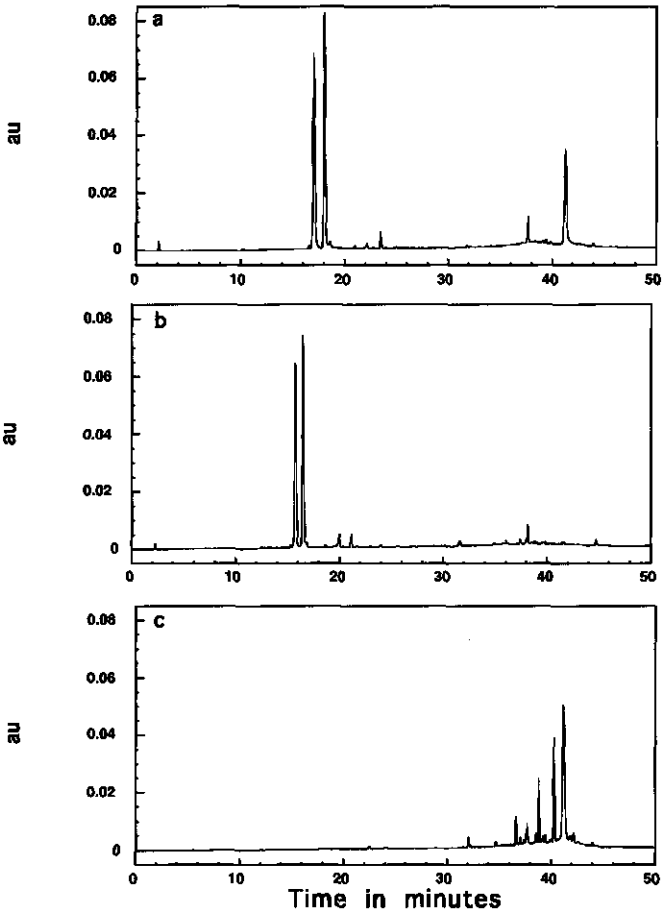



**Figure 3.** HPLC chromatogram of (a) the incubation of quercetin with horseradish peroxidase in the presence of glutathione, (b) the remaining aqueous layer of the incubation, and (c) the diethyl ether extract.

Figure 3c shows the HPLC chromatogram of the diethyl ether fraction of this incubation. The two major metabolites are not extracted from the acidic water phase by diethyl ether.

*$^1$H NMR characterisation of the quercetin-glutathione adducts.* Figures **4a** and **4b** show the $^1$H NMR spectra of the two metabolites collected from HPLC. Identification of the various $^1$H NMR resonances has been done on the basis of the $^1$H NMR chemical shifts and their splitting patterns and on the basis of comparison to literature data for quercetin (14). Table II summarizes the $^1$H NMR characteristics of the two metabolites with HPLC retention time at 17.4 and 18.5 min, measured in 25 mM potassium phosphate in $D_2O$, pD 7.0.

Comparison of the $^1$H NMR spectra of the two quercetin glutathione adducts to the $^1$H NMR spectral characteristics of quercetin (spectrum not shown), clearly reveals the loss of the C6-H and C8-H $^1$H NMR signals as well as the loss of the $^4J_{HH}$ coupling of 1.9 Hz between the H6 and H8 protons. Both metabolites still contain the C5′-H and C6′-H protons reflected by the two doublets with $^3J_{HH}$ = 8.5 Hz in the 6.8-7.3 ppm region, with the resonance of C6′-H also showing the $^4J_{HH}$ coupling of 2.1 Hz with C2′-H. Both metabolites also contain the C2′-H proton giving rise to a signal in the 7.3-7.4 ppm region with a $^4J_{HH}$ coupling of 2.1 Hz with C6′-H. Thus, the $^1$H NMR spectra presented in Figure 4 demonstrate that the two glutathionyl-quercetin adducts detected in the incubation of quercetin with horseradish peroxidase in the presence of glutathione, represent 6-glutathionylquercetin and 8-glutathionylquercetin. This implies glutathione adduct formation in the A ring instead of in the B ring of quercetin, even though the *ortho*-quinone moiety is expected to be formed in the B ring upon oxidation of the 3′-OH, 4′-OH catechol-like motif upon peroxidase-mediated one-electron oxidation of quercetin in the presence of GSH. It also implies that the adducts formed are identical to the adducts we identified previously upon the tyrosinase-mediated two-electron oxidation of quercetin in the presence of GSH (30).

## 2.4 Discussion

In the present study quercetin was incubated with horseradish peroxidase/$H_2O_2$ to generate the one electron oxidized quercetin semiquinone. Previous studies have actually demonstrated the formation of a quercetin *ortho*-semiquinone radical upon the conversion of quercetin by a peroxidase, using stabilisation of the radical by $Zn^{2+}$ complexation (11). In the present study, the incubation of quercetin with peroxidase/$H_2O_2$ was performed in the absence but especially also in the presence of glutathione (GSH). Literature studies generally report a role for GSH in the chemical reduction of the (semi)quinones leading to the parent catechol-type flavonol molecule (32). However, some studies have reported on the ability of GSH to scavenge possible quinone-type reaction products formed during reaction (15, 27-29). HPLC analysis of the reaction products formed in the absence of glutathione revealed formation of at least twenty different products, a result in line with other studies reporting on the

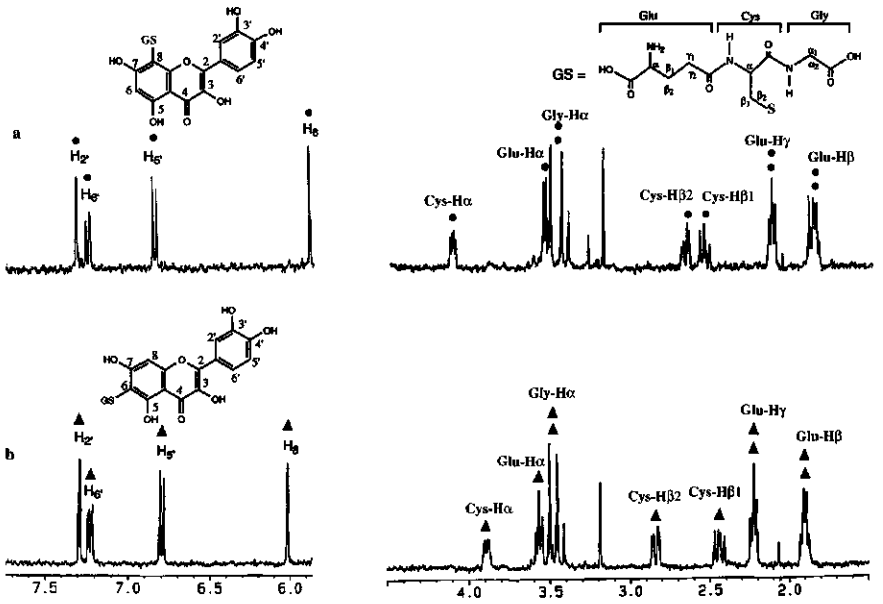
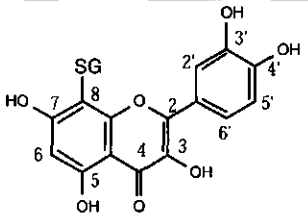
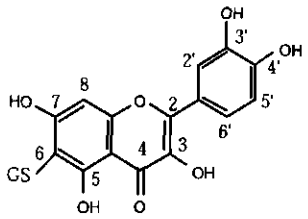



**Figure 4.** $^1$H NMR spectra of the two metabolites formed in the incubation of quercetin with horseradish peroxidase in the presence of glutathione and eluting from the HPLC at 17.4 min (8-glutathionylquercetin) (a) and 18.5 min (6-glutathionylquercetin) (b), both measured in 25 mM potassium phosphate in D2O, pD 7.0. The number of filled circles and filled triangles indicates the number of protons the signal represents.

**Table II.** $^1$H NMR Characteristics of the Two Metabolites with HPLC Retention Times at 17.4 and 18.5 min , Measured in 25 mM Potassium Phosphate in $D_2O$, pD = 7.0

| Structure | NMR characteristics |
|---|---|
|  | 5.88 (s, H6), 6.85 (d, $^3J_{HH}$ = 8.5 Hz, H5′), 7.25 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 2.1 Hz, H6′), 7.32 (d, $^4J_{HH}$ = 2.1 Hz, H2′), 1.86 (m, $^3J_{Hβ-Hα}$ = 6.5 Hz, $^3J_{Hβ-Hα}$ = 6.9 Hz, $^3J_{Hβ-Hα}$ = 9.0 Hz, Glu-Hβ), 2.14 (tr, $^3J_{Hγ-Hβ}$ = 9.0 Hz, $^3J_{Hγ-Hβ}$ = 6.9 Hz, Glu-Hγ), 2.56 (dd, $^3J_{Hβ1-Hα}$ = 9.1 Hz, $^2J_{Hβ1-Hβ2}$ = 14.2 Hz, Cys-Hβ1), 2.68 (dd, $^3J_{Hβ2-Hα}$ = 4.8 Hz, $^2J_{Hβ2-Hβ1}$ = 14.2 Hz, Cys- Hβ2), 3.43 ( d, $^2J_{Hα1-Hα2}$ = 17.3 Hz, Gly-Hα1), 3.53 (d, $^2J_{Hα2-Hα1}$ = 17.3 Hz, Gly-Hα2), 3.55 (tr, $^3J_{Hα-Hβ}$ = 6.5 Hz, Glu-Hα) and 4.12 (m, $^3J_{Hα-Hβ2}$ = 4.8 Hz, $^3J_{Hα-Hβ1}$ = 9.1 Hz, Cys-Hα) (Fig. 4a). |
|  | 6.02 (s, H8), 6.80 (d, $^3J_{HH}$ = 8.5 Hz, H5′), 7.23 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 2.1 Hz, H6′), 7.30 (d, $^4J_{HH}$ = 2.1 Hz, H2′), 1.90 (m, $^3J_{Hβ-Hα}$ = 6.9 Hz, $^3J_{Hβ-Hα}$ = 6.9 Hz, $^3J_{Hβ-Hα}$ = 8.6 Hz, Glu-Hβ), 2.23 (tr, $^3J_{Hγ-Hβ}$ = 8.6 Hz, $^3J_{Hγ-Hβ}$ = 6.9 Hz, Glu-Hγ), 2.44 (dd, $^3J_{Hβ1-Hα}$ = 9.8 Hz, $^2J_{Hβ2-Hβ1}$ = 14.2 Hz, Cys-Hβ1), 2.84 (dd, $^3J_{Hβ2-Hα}$ = 4.2 Hz, $^2J_{Hβ2-Hβ1}$ = 14.2 Hz, Cys- Hβ2), 3.43 ( d, $^2J_{Hα1-Hα2}$ = 17.2 Hz, Gly-Hα1), 3.53 (d, $^2J_{Hα2-Hα1}$ = 17.2 Hz, Gly-Hα2), 3.57 (tr, $^3J_{Hα-Hβ}$ = 6.9 Hz, Glu-Hα) and 3.89 (m, $^3J_{Hα-Hβ2}$ = 4.2 Hz, $^3J_{Hα-Hβ1}$ = 9.8 Hz, Cys-Hα) (Fig. 4b). |

peroxidase mediated oxidation of flavonoids, including kaempferol and quercetin (11,19-25,33,34). Table I summarizes the nature of several of the reaction products reported in the literature to be formed upon the chemical or enzymatic oxidation of flavonoids (11,14,22,31,35-38). The general picture emerging from these studies is that the oxidation of flavonoids is complex, resulting in a wide variety of, in many cases, unidentified reaction products.

Surprisingly, when, in the present study, the reaction between horseradish peroxidase/$H_2O_2$ and quercetin was performed in the presence of GSH, only two major products could be detected on HPLC. These two products were shown to be water soluble, although quercetin itself is relatively insoluble in water surroundings. The products were purified by HPLC and identified by $^1$H NMR analysis as 6-glutathionylquercetin and 8-glutathionylquercetin, representing glutathione adducts originating from glutathione conjugation at the A-ring instead of at the B ring of quercetin quinone.

Previous studies incubating flavonoids with peroxidases in the presence of glutathione have indicated the capacity of GSH to scavenge the flavonoid semiquinone radical, thereby regenerating the flavonoid and generating reactive oxygen species leading to toxicity (11,36). This reaction appeared to be especially efficient for flavonoids like apigenin, naringenin and naringin but not for quercetin (32). Upon oxidation of quercetin by lipoxygenase in the presence of a 4 fold excess of GSH over quercetin, there was no measurable regeneration of quercetin (11). The results of the present study show that upon peroxidase mediated oxidation of quercetin in the presence of GSH two glutathionylquercetin adducts are formed. This provides an explanation for the lack of significant GSH to GSSG oxidation in these previous studies.

Products formed as a result of quercetin oxidation by horseradish peroxidase/$H_2O_2$ in the absence of glutathione were no longer observed in incubations with glutathione. This suggests that the formation of the reaction products in the absence or presence of glutathione proceeds through the same reactive intermediates. The possibility that in the incubation with GSH the products formed without GSH are suppressed by electron transfer from GSH can be excluded, because the $^1$H NMR and HPLC data of a total incubation of quercetin with HRP in the presence of GSH show no indication of significant formation of GSSG (see, for example, Figure 3a in which GSSG should give a peak at 12.9 min).

The nature of the glutathionylquercetin adducts formed in the presence of glutathione provides indirect evidence for the nature of some of these reactive quercetin intermediates. Glutathione addition at positions 6 and 8 of the A ring can best be explained by taking into consideration a further oxidation of the quercetin semiquinone to give the *ortho*-quinone, followed by the isomerization of the *ortho*-quinone to its *para*-quinone methide isomer (Figure 5). In contrast to the *ortho*-quinone, the *para*-quinone methide isomer of quercetin quinone contains electrophilic

character at the C6 and C8 positions in the A ring, providing an opportunity for the formation of the C6- and C8-glutathione adducts (30). It is known that quinone methides are even more reactive than the corresponding *ortho*-quinone isomers (15,28,39). Such an increased reactivity of the quinone methide as compared to the *ortho*-quinone will result in a shift of the equilibrium in favour of the *para*-quinone methide and its C6- and C8-glutathione adducts.

Horseradish peroxidase is known to catalyze one electron substrate oxidation (10,11). The results of the present study indicate formation of the two-electron oxidized quinone/quinone methide followed by its efficient conjugation with GSH. This two-electron oxidizing pathway apparently competes efficiently with the pathway leading to GSH mediated one electron reduction of the semiquinone to regenerate the parent quercetin such as observed for other flavonoids like naringenin, naringin, and asparigin (32). Whether this is due to an efficient second one-electron oxidation step of the quercetin semiquinone radical by horseradish peroxidase, an efficient dismutation of two quercetin semiquinone radicals, and/or the redox potential of quercetin semiquinone, hampering its one electron reduction by GSH, remains to be investigated. Such two-electron oxidation (i.e. two subsequent one-electron oxidation) by HRP have previously been demonstrated for other substrates as well (40,41).

Finally, it is of interest to note that many flavonoid preparations are at present already marketed as herbal medicines or dietary supplements. Results of the present study illustrate that the pro-oxidative behavior of these flavonoids is at present still far from understood. In addition, the results indicate that the behaviour of these compounds in the presence of enzymes like peroxidases may result in formation of reactive alkylating reaction products. Some studies have suggested a role for quinone/quinone methides in the mutagenicity of compounds like quercetin (2,42). The results of the present study provide experimental evidence for the formation of such quinone/quinone methide type metabolites form quercetin upon its enzymatic conversion in one electron oxidation reactions. In addition to the horseradish peroxidase/$H_2O_2$ system, the antioxidant action of quercetin is another process generating quercetin semiquinone radicals. Whether the supposed beneficial effect of quercetin as an antioxidant is also accompanied by formation of the alkylating quinone methides and to what extent this pro-oxidative toxic potential of quercetin should be taken into account during the future development of the compound as a so-called beneficial functional food ingredient, are questions requiring careful examination. This holds especially when taking into account the results of the present study.

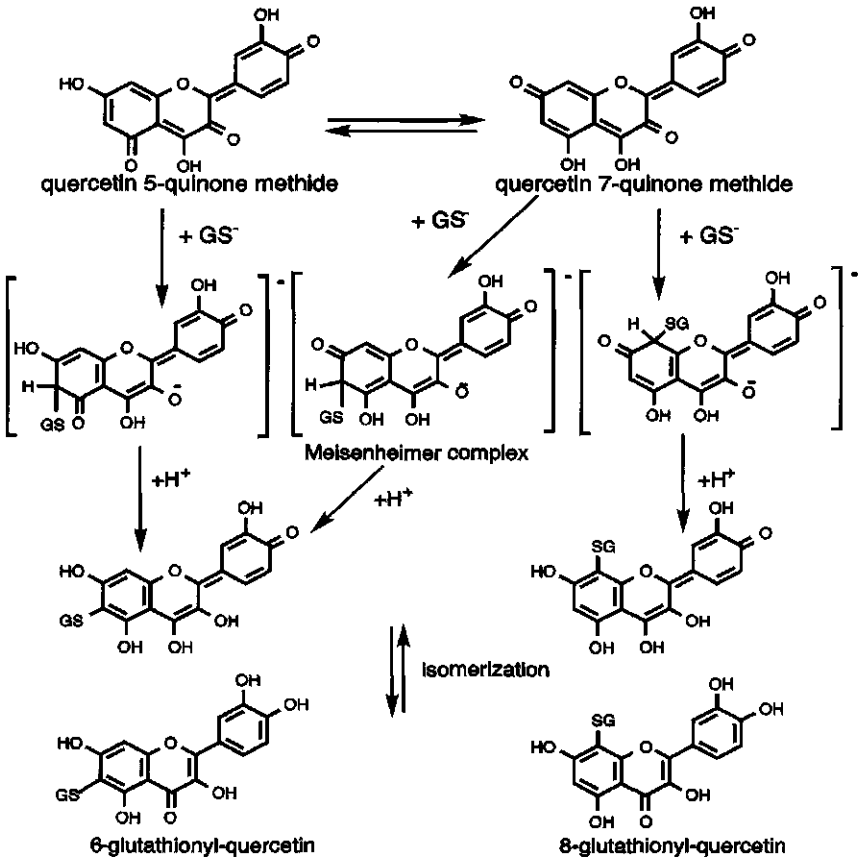



**Figure 5.** A mechanistic scheme for formation of 6- and 8- glutathionyl quercetin from the quercetin quinone methides.

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# 3

## Structure-activity study on the quinone/quinone methide chemistry of flavonoids

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Chemical Research in toxicology **14** (4), 398-408 (2001)

### 3.1 Abstract

A structure-activity study on the quinone/quinone methide chemistry of a series of 3′,4′-dihydroxyflavonoids was performed. Using the glutathione trapping method followed by HPLC, $^{1}$H NMR, MALDI-TOF, and LC/MS analysis to identify the glutathionyl adducts, the chemical behavior of the quinones/quinone methides of the different flavonoids could be deduced. The nature and type of mono- and di-glutathionyl adducts formed from quercetin, taxifolin, luteolin, fisetin, and 3,3′,4′-trihydroxyflavone show how several structural elements influence the quinone/quinone methide chemistry of flavonoids. In line with previous findings, glutathionyl adduct formation for quercetin occurs at positions C6 and C8 of the A ring, due to the involvement of quinone methide type intermediates. Elimination of the possibilities for efficient quinone methide formation by (i) the absence of the C3-OH group (luteolin), (ii) the absence of the C2=C3 double bond (taxifolin), or (iii) the absence of the C5-OH group (3,3′,4′-trihydroxyflavone) results in glutathionyl adduct formation at the B ring due to involvement of the *ortho*-quinone isomer of the oxidized flavonoid. The extent of di- versus mono-glutathionyl adduct formation was shown to be depend on the ease of oxidation of the mono-adduct as compared to the parent flavonoid. Finally, unexpected results obtained with fisetin provide new insight into the quinone/quinone methide chemistry of flavonoids. The regioselectivity and nature of the quinone adducts that formed appear to be dependent on pH. At pH values above the $pK_a$ for quinone protonation, glutathionyl adduct formation proceeds

at the A or B ring following expected quinone/quinone methide isomerisation patterns. However, decreasing the pH below this $pK_a$ results in a competing pathway in which glutathionyl adduct formation occurs in the C ring of the flavonoid, which is preceeded by protonation of the quinone and accompanied by $H_2O$ adduct formation, also in the C ring of the flavonoid. All together, the data presented in this study confirm that quinone/quinone methide chemistry can be far from straight forward, but the study provides significant new data revealing an important pH-dependence for the chemical behavior of this important class of electrophiles.

## 3.2 Introduction

Flavonoids, which are widely distributed in green vegetables, fresh fruits, nuts, seeds, tea, olive oil, and red wine (*1,2*), have recently been identified as a major cancer-preventive component of our diet because of their antioxidative, oxygen radical scavenging, and anti-inflammatory activities (*2-5*). There are also claims that they are anti-artherosclerotic (*6,7*) and, in addition, may provide beneficial health effects in ageing (*1,4*). However, depending on the concentration and OH substituent pattern, these polyphenolic compounds can also act as pro-oxidants (*8-10*). This holds true especially for flavonoids containing a catechol-like 3′,4′-dihydroxysubstituent pattern in their B ring as in, for example, quercetin (Table 1) (*9,10*). This catechol moiety provides possibilities for efficient autoxidation and/or enzymatic one- as well as two-electron oxidation of the flavonoid, all resulting in the formation of semiquinone- and quinone-type metabolites. These semiquinone- and quinone-type metabolites may act as electrophiles binding to cellular macromolecules and may also result in the production of reactive oxygen species through redox cycling (*11,12*). The ability of quinones to redox cycle and create reactive oxygen species and their ability to form covalent adducts with cellular macromolecules are the basis for their potential harmful pro-oxidative effect. The mutagenicity of quercetin is an example of a harmful effect ascribed to the formation of such alkylating quinone-type metabolites (*2,13,14*). Furthermore, the carcinogenic effects of estrogens and polycyclic aromatic hydrocarbons have recently also been linked to the formation of catechol-type metabolites which subsequently (auto)-oxidize to reactive quinones, resulting in similar toxic mechanisms (*11-13,15*).

Previous studies that included incubating flavonoids with peroxidases in the presence of GSH have indicated the capacity of GSH to scavenge the flavonoid semiquinone radical, thereby regenerating the flavonoid and generating oxidized glutathione and reactive oxygen species leading to toxicity (*10,16*). This reaction appeared to be especially efficient for flavones and flavanones containing a phenol-type substituent pattern in their B ring (*16,17*). The GSH oxidizing pro-oxidant

**Table 1.** Substituent patterns and structure characteristics of the flavonoids of importance for the study present here

| | 3′-OH | 4′-OH | 3-OH | C4=O | 5-OH | 7-OH | C2=C3 |
|---|---|---|---|---|---|---|---|
| quercetin | + | + | + | + | + | + | + |
| taxifolin | + | + | + | + | + | + | - |
| luteolin | + | + | - | + | + | + | + |
| fisetin | + | + | + | + | - | + | + |
| 3,3′,4′-trihydroxyflavone | + | + | + | + | - | - | + |

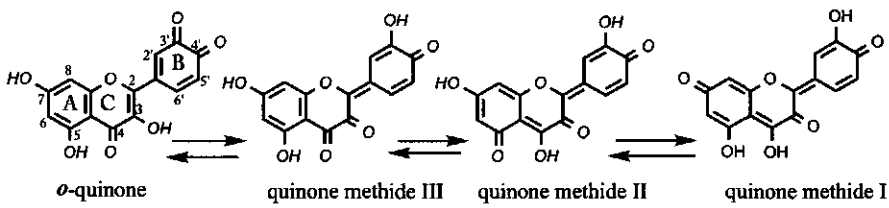


**Figure 1.** Quinone/quinone methide isomerisation of quercetin.

activity of this type of flavones and flavanones seemed to partly correlate with the high one-electron redox potential of the corresponding phenoxyl radicals (*16,18*). Flavonoids containing a catechol-type substituent pattern in their B ring did not co-oxidize GSH when oxidized by horseradish peroxidase (HRP), presumably because of their lower one-electron redox potentials, although it was observed that some GSH depletion did occur without oxygen uptake or GSSG formation (*16,18*). Thus the absence of GSH oxidation upon HRP catalyzed oxidation of quercetin may be due to GSH conjugate formation of the quercetin quinone / quinone methide. Using the glutathione trapping method for scavenging of the reactive and unstable quinoid-type metabolites (*19-21*), we recently identified these glutathione conjugates of the quinoid-type metabolite of quercetin. The same adducts were formed upon one- or two-electron oxidation of quercetin catalyzed by HRP (*22*) or tyrosinase (*23*), respectively. Surprisingly, glutathione addition to the quinoid metabolite of quercetin, in both cases, occurred in the A ring instead of in the catechol containing B ring. The formation of 6-glutathionyl- and 8-glutathionylquercetin pointed at efficient isomerisation of the *ortho*-quinone quercetin metabolite to quercetin quinone methide

isomers (Figure 1). It can be foreseen that this non-enzymatic isomerisation of quercetin *ortho*-quinone to its quinone methides will depend on the structure of the polyphenol compound. Therefore, the objective of this study was to further investigate the quinone/quinone methide chemistry of a series of 3′,4′-dihydroxyflavones using the GSH trapping method. Special emphasis was placed on the regioselectivity of the GSH conjugation and on the structural requirements in the flavonoids necessary for quinone/quinone methide isomerisation, taking into consideration the role of especially the C2=C3 double bond, the C3-OH group and the C5- and C7-hydroxyl moieties of quercetin. Thus, the quinone/quinone methide chemistry of taxifolin, luteolin, fisetin, and 3,3′,4′-trihydroxyflavone was investigated and compared to that previously elucidated for quercetin.

## 3.3 Materials and Methods

*Materials.* Quercetin (toxic, exhibits mutagenic activity) was obtained from Acros Organics (New Jersey, USA). Fisetin (irritant) was from Aldrich (Steinheim, Germany). Luteolin and 3,3′,4′-trihydroxyflavone were from Indofine (Somerville, USA). Taxifolin was obtained from ICN Biomedicals Inc. (Ohio, USA). Horseradish peroxidase was obtained from Boehringer (Mannheim, Germany). Glutathione, reduced form, was purchased from Sigma (St Louis, MO, USA). All substrates were 98-99 % pure. Hydrogen peroxide, potassium hydrogen phosphate, potassium dihydrogen phosphate, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Deuterium oxide was obtained from ARC Laboratories (Amsterdam, The Netherlands). Acetonitrile and methanol were HPLC grade from Lab-Scan, Analytical Sciences (Dublin, Ireland).

*Incubation of flavonoids with glutathione.* To a starting solution of flavonoid (final concentration of 150 μM added from a 10 mM stock solution in methanol) in 25 mM potassium phosphate (pH 7.0) containing glutathione (final concentration of 1.0 mM) was added HRP to a final concentration of 0.1 μM, followed by addition of $H_2O_2$ (final concentration of 200 μM added from a 20 mM stock solution in water). Upon incubation for 8 minutes at 25°C, the incubation mixture was analyzed by HPLC.

*pH-dependence for incubation of fisetin with glutathione.* To a starting solution of fisetin (final concentration of 150 μM added from a 10 mM stock solution in methanol) in 25 mM buffer with a pH varying between 3.5 and 11.0 (prepared according to the literature (*24-26*)), containing glutathione (final concentration of 1.0 mM) was added HRP to a final concentration of 0.1 μM, followed by addition of $H_2O_2$ (final concentration of 200 μM added from a 20 mM stock solution in water). Upon incubation for 8 minutes at 25°C, the incubation mixture was analyzed by HPLC.

*Analytical high performance liquid chromatography.* HPLC was performed with a Waters M600 liquid chromatography system. Analytical separations were achieved using an Alltima C18 column (4.6 mm x 150 mm) (Alltech, Breda, The Netherlands). The column was eluted at 0.7 mL/min with water containing 0.1 % (v/v) trifluoroacetic acid. A linear gradient from 0 to 30 % acetonitrile over the course of 18 minutes was applied, followed by 2 minutes isocratic elution with 30 % acetonitrile. Hereafter, a linear gradient from 30 to 100 % acetonitrile was used over the course of 10 minutes. The percentage of acetonitrile was kept at 100 % for an additional 10 minutes. An injection loop of 10 µL was used. Detection was carried out with a Waters 996 photodiode array detector measuring spectra between 200 and 450 nm. The chromatograms presented here are based on detection at 290 nm. Product peaks were collected and freeze-dried for further analysis by $^{1}$H NMR, MALDI-TOF and LC/MS analysis. Freeze-dried samples were dissolved in 25 mM potassium phosphate (pH 7.0), made with deuterated water when samples were used for $^{1}$H NMR analysis.

*NMR measurements.* $^{1}$H NMR measurements were performed on a Bruker DPX 400 or Bruker AMX 500 spectrometer. A 1.5 s presaturation delay was used, along with a $70^{o}$ pulse angle and a 2.2 s acquisition time (7575 Hz sweep width, 32 K data points, 7°C). The data were processed using an exponential multiplication of 0.5 or 1.0 Hz and zero filling to 64 K data points. Resonances are reported relative to HDO at 4.79 ppm. $^{13}$C NMR measurements were performed in a deuterated methanol/$D_2O$ mixture at 15°C with a dedicated 5 mm $^{13}$C NMR probe (32000 Hz sweep width, 65 K data points, 28000 scans).

*Liquid chromatography/mass spectrometry.* LC/MS analysis was performed to further characterize the peaks in the HPLC elution pattern that could not be identified as one of the reference compounds. An injection volume of 10 µL from the incubation mixture or from the purified metabolite dissolved in potassium phosphate (pH 7.0) was used, and separation of the products was achieved on a 150 mm x 4.6 mm Alltima C18 column (Alltech, Breda, The Netherlands). A gradient from 10 to 30 % acetonitrile in water containing 0.1 % (v/v) trifluoroacetic acid was applied at a flow of 0.7 mL/min over the course of 13 minutes. The percentage of acetonitrile was kept at 30 % for 2 minutes and then increased to 100 % over the course of 2 min. Mass spectrometric analysis (Finnigan MAT 95, San Jose, CA, USA) was performed in the positive electrospray mode using a spray voltage of 4.5 kV and a capillary temperature of 180 °C with nitrogen as sheath and auxillary gas.

*MALDI-TOF-mass spectrometry.* A saturated solution of α-cyano-4-hydroxycinnamic acid in 1% trifluoroacetic acid/49.5% acetonitrile/49.5% $H_2O$ (v/v) mixture was used as the matrix solution. Freeze-dried samples were dissolved in 20 % (v/v) acetonitrile in water. The dissolved samples were diluted 2-1000-flold in the matrix solution. A 1 μL volume of the sample/matrix solution was deposited directly on a welled plate, air-dried, and introduced into the mass spectrometer. Spectra were measured with a Voyager-DE spectrometer (PerSeptive Biosystems) in the positive reflector mode. The MALDI spectra were externally calibrated using bradykinin (monoisotopic *m/z* of 1060.57) and one matrix peak (dimeric α-cyano-4-hydroxycinnamic acid, monoisotopic *m/z* of 379.09).

*Molecular orbital calculations.* Molecular orbital calculations were carried out on a Silicon Graphics Indigo$^2$ workstation using Spartan 5.0 (Wavefunction Inc.). The semiempirical AM1 method was applied (*27*). Geometries were fully optimized. The energy of the highest occupied molecular orbital ($E_{HOMO}$) of the 3′,4′-dihydroxyflavonoids under investigation and their corresponding 2′-glutathionyl adducts was calculated as the parameter to quantify their ease of oxidation, because, following Koopman's theorem $-E_{HOMO}$ equals the ionization potential of a compound. In these calculations, the glutathionyl moiety can be modeled as $CH_3S$ (*28-30*).

### 3.4 Results

*HPLC analysis of glutathione adducts of flavonoids.* Panels a and b of Figure 2 show the HPLC patterns of the incubation of taxifolin with HRP (0.1 and 0.2 μM, respectively) in the presence of glutathione. Using a small amount of HRP, not all taxifolin is converted and only one major metabolite peak ($t_R$ 21.9 min) can be detected eluting at a position different from that of the parent flavonoid. With increasing HRP concentration, all taxifolin is converted upon 8 min of incubation and two major metabolite peaks are formed eluting with retention times of 20.1 min and 21.9 min, respectively.

Panels c and d of Figure 2 show the HPLC patterns of the incubation of luteolin with HRP (0.1 and 0.2 μM, respectively) in the presence of glutathione. In both cases two metabolites can be detected eluting at a position different from that of the parent flavonoid, but the ratio between the two products appears to vary with the amount of HRP added. With a smaller amount of HRP, not all the luteolin is converted and the metabolite with a retention time of 24.6 min appears to be the one initially formed. Increasing the HRP concentration from 0.1 to 0.2 μM changes the ratio of the peak areas of the metabolites eluting at 22.2 min and 24.6 min from 0.5 : 1 to 2 : 1.

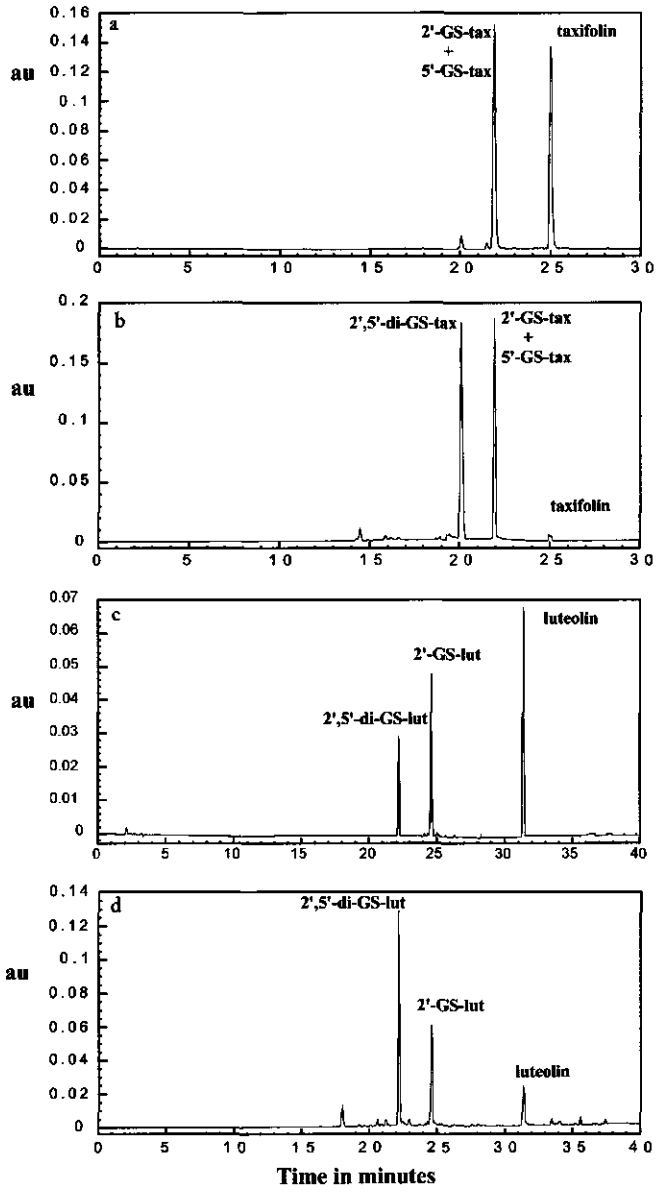


**Figure 2.** HPLC chromatograms of the incubations of taxifolin (a,b) and luteolin (c,d) with 0.1 μM and 0.2 μM HRP respectively in the presence of glutathione at pH 7.0.

Panels a and b of Figure 3 show the HPLC patterns of the incubations of 3,3′,4′-trihydroxyflavone and fisetin with HRP in the presence of glutathione. For 3,3′,4′-trihydroxyflavone, formation of one major metabolite is observed. For fisetin, two major metabolites with retention times of 15.6 min and 16.5 min can be detected eluting at a position different from that of the parent flavonoid. For 3,3′,4′-trihydroxyflavone and fisetin an increase in the HRP concentration from 0.1 to 0.2 μM did not modify the metabolite profile.

*Identification of the glutathionyl flavonoid adducts. (1) Influence of the C2=C3 double bond and the results for taxifolin.* The $^{1}$H NMR spectrum of the peak with a retention time of 21.9 min (Figure 2a) reveals that this peak is a mixture of two metabolites which could be separated isocraticaly using 10 % acetonitrile in water containing 0.1 % (v/v) trifluoroacetic acid into two metabolites showing a peak area ratio of 7 : 3 (HPLC chromatogram not shown). LC/MS analysis of the purified metabolites shows an M+1 peak for both metabolites at *m/z* 610 (data not shown). This indicates the formation of two different mono-glutathionyl adducts. Table 2 presents the $^{1}$H NMR characteristics of taxifolin and these two major glutathionyl adducts formed upon incubation of taxifolin with HRP in the presence of glutathione and collected from HPLC. Comparison of the $^{1}$H NMR data of the major metabolite (70 %) to those of taxifolin (Table 2) (*31,32*) reveals the loss of especially the H2′ signal as well as the loss of the $^{4}J_{H2'-H6'}$ coupling of 2.1 Hz. Splitting patterns and resonances of all other aromatic protons are comparable to those of taxifolin itself. Comparison of the $^{1}$H NMR data of the minor metabolite (30 %) to those of taxifolin (Table 2) (*31,32*) reveals the loss of especially the H5′ signal as well as the loss of the $^{3}J_{H5'-H6'}$ coupling of 8.2 Hz. Splitting patterns and resonances of all other aromatic protons are comparable to those of taxifolin itself. In $^{1}$H NMR measurements, the H2/H3 resonances of taxifolin and of the adducts are in part invisible due to peak overlap with the water resonance. However, varying the temperature from 7 to 45°C gradually reveals these resonances indicating the presence of the saturated C2-C3 bond in all compounds. In addition to the aromatic and the H2/H3 $^{1}$H NMR resonances, the $^{1}$H NMR spectra of the adducts show the $^{1}$H resonances of the glutathionyl side chain (Table 2). On the basis of these $^{1}$H NMR characteristics and the LC/MS data these two metabolites can be identified as 2′-glutathionyl- and 5′-glutathionyltaxifolin respectively.

When the HRP concentration is increased, all the parent compound was converted and an additional major metabolite was observed (Figure 2b). LC/MS analysis of the purified metabolite shows an M+1 peak at *m/z* 915 (data not shown). This indicates the formation of a di-glutathionyl adduct. Comparison of the $^{1}$H NMR

**Table 2.** $^1$H NMR resonances and coupling constants of taxifolin and the glutathionyl adducts of taxifolin (Labeled 2′-GS-tax, 5′-GS-tax and 2′,5′-di-GS-tax)

| | *taxifolin* | | 2′-GS-tax (major, 70%) | | 5′-GS-tax (minor, 30%) | | 2′,5′-di-GS-tax ($t_R$ = 20.1) | |
|---|---|---|---|---|---|---|---|---|
| | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) |
| H3 | 4.60 (d) | $^3J_{H3\text{-}H2}$ = 11.7 | 4.64 (d) | $^3J_{H3\text{-}H2}$ = 11.6 | 4.57 (d) | $^3J_{H3\text{-}H2}$ = 11.8 | 4.45 (d) | $^3J_{H3\text{-}H2}$ = 11.8 |
| H2 | 4.90 (d) | $^3J_{H2\text{-}H3}$ = 11.7 | 5.09 (d) | $^3J_{H2\text{-}H3}$ = 11.6 | 5.26 (d) | $^3J_{H2\text{-}H3}$ = 11.8 | 5.11 (d) | $^3J_{H2\text{-}H3}$ = 11.8 |
| H6 | 5.67 (d) | $^4J_{H6\text{-}H8}$ = 2.2 | 5.71 (d) | $^4J_{H6\text{-}H8}$ = 2.0 | 5.69 (d) | $^4J_{H6\text{-}H8}$ = 2.0 | 5.76 (d) | $^4J_{H6\text{-}H8}$ = 2.4 |
| H8 | 5.75 (d) | $^4J_{H8\text{-}H6}$ = 2.2 | 5.77 (d) | $^4J_{H8\text{-}H6}$ = 2.0 | 5.74 (d) | $^4J_{H8\text{-}H6}$ = 2.0 | 5.79 (d) | $^4J_{H8\text{-}H6}$ = 2.4 |
| H5′ | 6.80 (d) | $^3J_{H5'\text{-}H6'}$ = 8.2 | 6.87 (d) | $^3J_{H5'\text{-}H6'}$ = 8.4 | — | — | — | — |
| H6′ | 6.83 (dd) | $^3J_{H6'\text{-}H5'}$ = 8.2<br>$^4J_{H6'\text{-}H2'}$ = 2.1 | 7.16 (d) | $^3J_{H6'\text{-}H5'}$ = 8.4<br>— | 6.87 (d) | —<br>$^4J_{H6'\text{-}H2'}$ = 2.0 | 7.18 (s) | —<br>— |
| H2′ | 6.93 (d) | $^4J_{H2'\text{-}H6'}$ = 2.1 | — | — | 6.99 (d) | $^4J_{H2'\text{-}H6'}$ = 2.0 | — | — |
| Glu Hβ | — | | 1.79 (m) | | 1.84 (m) | | 1.82 (m) | |
| Glu Hγ | — | | 2.11 (m) | | 2.20 (m) | | 2.13 (m) | |
| Cys Hβ1 | — | | 2.95 (dd) | | 2.96 (dd) | | 2.92 (dd) | |
| Cys Hβ2 | — | | 3.03 (dd) | | 3.19 (dd) | | 3.24 (dd) | |
| Gly Hα1 | — | | 3.32 (d) | | 3.38 (d) | | 3.34 (d) | |
| Gly Hα2 | — | | 3.39 (d) | | 3.44 (d) | | 3.41 (d) | |
| Glu-Hα | — | | 3.45 (m) | | 3.87 (m) | | 3.47 (m) | |
| Cys Hα | — | | 4.15 (m) | | 4.21 (m) | | 4.14 (m) | |

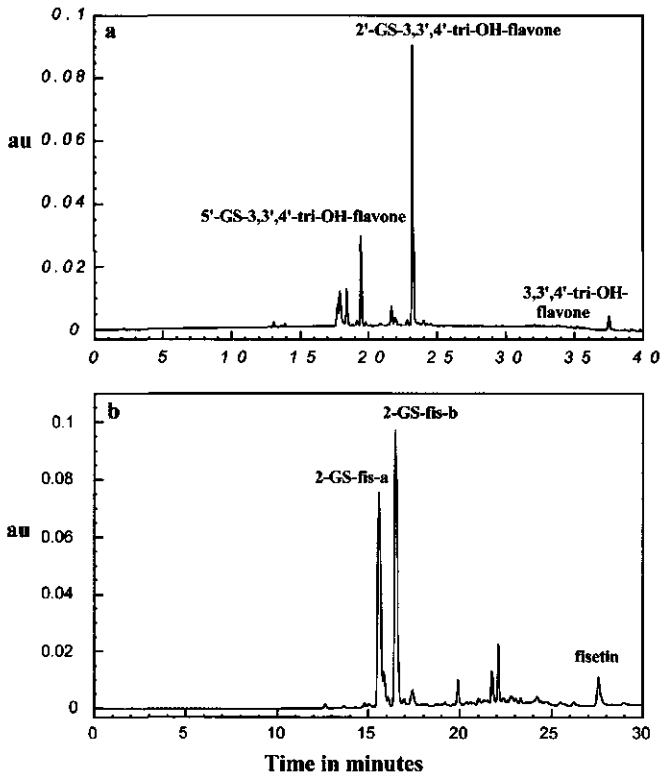


**Figure 3.** HPLC chromatograms of the incubations of (a) 3,3′,4′-trihydroxyflavone and (b) fisetin with HRP in the presence of glutathione at pH 7.0.

data of this additional metabolite (retention time of 20.1 min) to those of taxifolin (Table 2) (*31,32*) reveals the loss of especially the H2′ and H5′ signals as well as the loss of the $^{4}J_{H2'\text{-}H6'}$ coupling of 2.1 Hz and the $^{3}J_{H5'\text{-}H6'}$ coupling of 8.2 Hz. Splitting patterns and resonances of all other aromatic protons are comparable to those of taxifolin itself. On the basis of these $^{1}$H NMR characteristics and LC/MS data this metabolite can be identified as 2′,5′-diglutathionyltaxifolin.

Thus, when the absence of the C2=C3 double bond in the flavonoid prevents extension of the quinoid isomerisation to A ring, the glutathionyl adducts of the oxidized flavonoid are formed in the B ring preferentially at C2′ and subsequently also at C5′.

*(2) Influence of the C3-OH group and the results for luteolin.* Using luteolin the influence of the absence of the C3-OH on the quinone/quinone methide isomerisation was investigated. MALDI-TOF analysis of the purified metabolites from HPLC with retention times of 24.6 and 22.2 min reveals peaks at *m/z* 592 and 897, respectively

(data not shown). This indicates the formation of a mono- and a di-glutathionyl adduct with retention times of 24.6 and 22.2 min, respectively (Figure 2c,d). The di-adduct is the one with the lower retention time (22.2 min) and also the one formed in increasing amounts upon increasing the HRP concentration (Figure 2c,d).

**Table 3.** $^1$H NMR resonances and coupling constants of luteolin and the two glutathionyl adducts of luteolin (Labeled 2′-GS-lut and 2′,5′-diGS-lut)

| | luteolin | | 2′-GS-lut ($t_R$ = 24.6 min) | | 2′,5′-diGS-lut ($t_R$ = 22.2 min) | |
|---|---|---|---|---|---|---|
| | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) |
| H6 | 5.99 (d) | $^4J_{H6\text{-}H8}$ = 1.9 | 6.15 (d) | $^4J_{H6\text{-}H8}$ = 2.0 | 6.10 (d) | $^4J_{H6\text{-}H8}$ = 2.0 |
| H8 | 6.26 (d) | $^4J_{H8\text{-}H6}$ = 1.9 | 6.33 (d) | $^4J_{H8\text{-}H6}$ = 2.0 | 6.31 (d) | $^4J_{H8\text{-}H6}$ = 2.0 |
| H3 | 6.40 (s) | — | 6.29 (s) | — | 6.26 (s) | — |
| H5′ | 6.78 (d) | $^3J_{H5'\text{-}H6'}$ = 8.4 | 6.85 (d) | $^3J_{H5'\text{-}H6'}$ = 7.8 | — | — |
| H2′ | 7.25 (d) | $^4J_{H2'\text{-}H6'}$ = 2.1 | — | — | — | — |
| H6′ | 7.28 (dd) | $^3J_{H6'\text{-}H5'}$ = 8.4 $^4J_{H6'\text{-}H2'}$ = 2.1 | 6.99 (d) | $^3J_{H6'\text{-}H5'}$ = 7.8 — | 7.06 (s) | — — |
| Glu Hβ | — | | 1.73 (m) | | 1.66 (m) | |
| Glu Hγ1 | — | | 1.86 (m) | | 1.80 (m) | |
| Glu Hγ2 | — | | 2.06 (m) | | 1.92 (m) | |
| Cys Hβ1 | — | | 2.80 (dd) | | 2.95 (m) | |
| Cys Hβ2 | — | | 3.13 (dd) | | 3.03 (m) | |
| Gly Hα1 | — | | 3.40 (d) | | 3.33 (d) | |
| Gly Hα2 | — | | 3.42 (d) | | 3.33 (d) | |
| Glu-Hα | — | | 3.46 (m) | | 3.37 (m) | |
| Cys Hα | — | | 3.71 (m) | | 4.19 (m) | |

Table 3 presents the $^1$H NMR characteristics of these two glutathionyl adducts. Comparison of the $^1$H NMR data of these two metabolites to those of the luteolin (Table 3) (*31-33*) reveals the loss of especially the H2′ signal as well as the loss of the $^4J_{H2'\text{-}H6'}$ coupling of 2.1 Hz for the mono-adduct. For the di-glutathionyl adduct, the data indicate the loss of the H2′ and H5′ signals as well as of the $^4J_{H2'\text{-}H6'}$ coupling of 2.1 Hz and the $^3J_{H5'\text{-}H6'}$ coupling of 8.4 Hz. In addition to the aromatic $^1$H resonances, the $^1$H NMR spectra of both luteolin adducts show the $^1$H resonances of the glutathionyl side chain (Table 3). On the basis of these $^1$H NMR characteristics and the MALDI-TOF data, these two metabolites could be identified as 2′-

glutathionylluteolin and 2′,5′-diglutathionylluteolin. Thus, upon the peroxidase mediated oxidation of luteolin, the *ortho*-quinone of luteolin gives rise to glutathionyl adduct formation in the B-ring of luteolin initially at C2′ and subsequently also at C5′.

**Table 4.** $^{1}$H NMR resonances and coupling constants of 3,3′,4′-trihydroxyflavone and the glutathionyl adducts of 3,3′,4′-trihydroxyflavone (Labeled 2′-GS-3,3′,4′-triOH-flavone and 5′-GS-3,3′,4′-triOH-flavone)

| | 3,3′,4′-trihydroxyflavone | | 2′-GS-3,3′,4′-triOH-flavone (major) | | 5′-GS-3,3′,4′-triOH-flavone (minor) | |
|---|---|---|---|---|---|---|
| | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) |
| H5′ | 6.83 (d) | $^{3}J_{H5'-H6'} = 8.5$ | 6.88 (d) | $^{3}J_{H5'-H6'} = 8.3$ | — | — |
| H6 | 7.35 (tr) | $^{4}J_{H6-H8} = 1.3$<br>$^{3}J_{H6-H7} = 7.1$<br>$^{3}J_{H6-H5} = 8.1$ | 7.32 (tr) | $^{4}J_{H6-H8} = 1.2$<br>$^{3}J_{H6-H7} = 7.6$<br>$^{3}J_{H6-H5} = 8.1$ | 7.33 (tr) | $^{4}J_{H6-H8} = 1.4$<br>$^{3}J_{H6-H7} = 8.6$<br>$^{3}J_{H6-H5} = 8.2$ |
| H6′ | 7.58 (dd) | $^{3}J_{H6'-H5'} = 8.5$<br>$^{4}J_{H6'-H2'} = 1.4$ | 6.93 (d) | $^{3}J_{H6'-H5'} = 8.3$<br>— | 7.08 (d) | —<br>$^{4}J_{H6'-H2'} = 1.5$ |
| H8 | 7.65 (dd) | $^{4}J_{H8-H6} = 1.3$<br>$^{3}J_{H8-H7} = 8.5$ | 7.43 (dd) | $^{4}J_{H8-H6} = 1.2$<br>$^{3}J_{H8-H7} = 8.7$ | 7.45 (dd) | $^{4}J_{H8-H6} = 1.4$<br>$^{3}J_{H8-H7} = 8.8$ |
| H7 | 7.68 (tr) | $^{4}J_{H7-H5} = 1.3$<br>$^{3}J_{H7-H6} = 7.1$<br>$^{3}J_{H7-H8} = 8.5$ | 7.62 (tr) | $^{4}J_{H7-H5} = 1.7$<br>$^{3}J_{H7-H6} = 7.6$<br>$^{3}J_{H7-H8} = 8.7$ | 7.62 (tr) | $^{4}J_{H7-H5} = 1.3$<br>$^{3}J_{H7-H6} = 8.6$<br>$^{3}J_{H7-H8} = 8.8$ |
| H2′ | 7.75 (d) | $^{4}J_{H2'-H6'} = 1.4$ | — | — | 7.19 (d) | $^{4}J_{H2'-H6'} = 1.5$ |
| H5 | 8.08 (dd) | $^{4}J_{H5-H7} = 1.3$<br>$^{3}J_{H5-H6} = 8.1$ | 7.97 (dd) | $^{4}J_{H5-H7} = 1.7$<br>$^{3}J_{H5-H6} = 8.1$ | 7.97 (dd) | $^{4}J_{H5-H7} = 1.3$<br>$^{3}J_{H5-H6} = 8.2$ |
| Glu Hβ | — | | 1.52 (m) | | 1.53 (m) | |
| Glu Hγ | — | | 1.85 (m) | | 1.84 (m) | |
| Cys Hβ1 | — | | 2.68 (dd) | | 2.67 (dd) | |
| Cys Hβ2 | — | | 3.03 (dd) | | 3.03 (dd) | |
| Gly Hα1 | — | | 3.25 (d) | | 3.24 (d) | |
| Gly Hα2 | — | | 3.30 (d) | | 3.32 (d) | |
| Glu-Hα | — | | 3.40 (m) | | 3.41 (m) | |
| Cys Hα | — | | 3.82 (m) | | 3.83 (m) | |

*(3) Influence of the hydroxyl moieties in the A ring and the results for 3,3′,4′-trihydroxyflavone and fisetin.* Using 3,3′,4′-trihydroxyflavone and fisetin (3,7,3′,4′-tetrahydroxyflavone) the influence of the two hydroxyl moieties in the A ring was investigated.

LC/MS analysis of the purified metabolites of 3,3′,4′-trihydroxyflavone shows an M+1 peak for both major and minor metabolites at *m/z* of 576 (data not shown). This indicates the formation of two different mono-glutathionyl adducts. Table 4 shows the $^{1}$H NMR characteristics of the major metabolite of 3,3′,4′-trihydroxyflavone collected from HPLC with a retention time of 23.2 min (Figure 3a). Comparison of the $^{1}$H NMR data of this metabolite to those of 3,3′,4′-trihydroxyflavone (Table 4) (*31,34*) reveals the loss of the H2′ signal as well as the loss of the $^{4}J_{H2'-H6'}$ coupling of 1.4 Hz. Splitting patterns and resonances of all other aromatic protons are comparable to those of 3,3′,4′-trihydroxyflavone itself. Comparison of the $^{1}$H NMR data of the minor metabolite with retention time at 19.4 min (Figure 3a) to those of 3,3′,4′-trihydroxyflavone (Table 4) (*31,34*) reveals the loss of the H5′ signal as well as the loss of the $^{3}J_{H5'-H6'}$ coupling of 8.5 Hz. Splitting patterns and resonances of all other aromatic protons are comparable to those of 3,3′,4′-trihydroxyflavone itself. In addition to the aromatic $^{1}$H resonances, the $^{1}$H NMR spectra of both adducts exhibit the $^{1}$H resonances of the glutathionyl side chain. On the basis of these $^{1}$H NMR characteristics and LC/MS data, the glutathionyl adducts can be identified as 2′-glutathionyl- and 5′-glutathionyl3,3′,4′-trihydroxyflavone.

Finally, panels a and b of Figure 4 show the $^{1}$H NMR spectra of the two major metabolites of fisetin collected from HPLC with retention times of 15.6 and 16.5 min (Figure 3b). For these adducts, the $^{1}$H NMR spectra are presented because, in contrast to what was observed for the other flavonoid glutathionyl adducts, they reveal the retention of all the parent compound aromatic protons in both adducts. This indicates the formation of glutathionyl fisetin adducts at a position other than C2′, C5′ and C6′ (B ring) or C5, C6 and C8 (A ring) (Table 5) (*31,34*). LC/MS analysis of the purified metabolites shows an M+1 peak for both metabolites at *m/z* 610 and a second major peak at *m/z* 593. Because the *m/z* value expected for protonated mono-glutathionyl fisetin equals *m/z* 592 the observation of a peak at *m/z* 610 for both metabolites points at formation of mono-glutathionyl adducts which contain an additional $H_2O$ molecule. $^{13}$C NMR spectra of the purified metabolites reveal formation of two $sp^3$ $^{13}$C resonances at 92.0 and 98.4 ppm for the metabolite with a retention time of 15.6 min and at 91.6 and 95.7 ppm for the metabolite with a retention time of 16.5 min which can be assigned to the two $sp^3$ hybridized deshielded C atoms at C2 and C3. In addition, the $^{13}$C NMR spectra of both metabolites each reveal only one resonance in

the carbonyl region (at 189.0 and 189.1 ppm respectively) indicating the presence of only one carbonyl group for each metabolite (data not shown). Together these data point at glutathionyl adduct formation accompanied by $H_2O$ adduct formation, both at the C ring of the fisetin *ortho*-quinone/quinone methide. The fact that two metabolites with similar MS, $^1H$ NMR and $^{13}C$ NMR characteristics are observed points to formation of different (diastereo)isomers of these combined $H_2O$/glutathionyl adducts. A detailed further discussion on the structure of these fisetin metabolites is presented in the discussion. Together, these results point to a reaction of the quinone/quinone methide of fisetin with glutathione which is surprisingly different from the chemistry observed for the other flavonoid quinones.

*pH dependence for formation of glutathionylfisetin adducts.* Panels a and b of Figure 5 show the pH dependence for the formation of the glutathionyl fisetin adducts. At lower pH values (pH ≤ 7.0), the two major metabolites with retention times of 15.6 and 16.5 min are observed and the peak intensities of these metabolites decrease with increasing pH (Figure 5a-**d**). In addition, the formation of a new metabolite with a retention time of 22.1 min could be observed. At higher pH values (pH ≥ 9.5), the two major metabolites formed at lower pH are no longer observed and the metabolite with a retention time of 22.1 min becomes the major metabolite (Figure 5e). The LC/MS analysis of this purified metabolite shows an M + 1 peak at *m/z* 592 (data not shown). Table 5 shows the $^1H$ NMR characteristics of this major metabolite collected from HPLC. Comparison of the $^1H$ NMR data of this metabolite to those of fisetin (Table 5) (*31,34*) reveals the loss of the H2′ signal as well as the loss of the $^4J_{H2'\text{-}H6'}$ coupling of 2.1 Hz. Splitting patterns and resonances of all other aromatic protons are comparable to those of fisetin itself. In addition to the aromatic $^1H$ resonances, the $^1H$ NMR spectrum of the adduct exhibits the $^1H$ NMR resonances of the glutathionyl side chain. On the basis of these $^1H$ NMR characteristics and LC/MS data, the glutathionyl adduct can be identified as 2′-glutathionylfisetin resulting from conjugation in the B ring instead of in the C ring.

*Calculation of the chemical reactivity toward oxidation of the studied 3′,4′-dihydroxyflavonoids and their corresponding 2′-glutathionyl adducts.* Table 6 presents the $E_{HOMO}$ and the relative difference in $E_{HOMO}$ ($\Delta E_{HOMO}$) of the studied 3′,4′-dihydroxyflavonoids and their corresponding 2′-glutathionyl adducts calculated using the semiempirical AM1 method. The results reveal that for luteolin the ease of oxidation of the parent compound and its corresponding 2′-glutathionyl adduct is almost the same and for taxifolin the oxidation of its corresponding 2′-glutathionyl adduct is even more easier than of the parent compound.

**Table 5.** $^{1}$H NMR resonances and coupling constants of fisetin and the three glutathionyl adducts of fisetin (Labeled 2-GS-fis-a, 2-GS-fis-b and 2′-GS-fis)

| | fisetin | | 2-GS-fis-a ($t_R$ = 15.6 min) | | 2-GS-fis-b ($t_R$ = 16.5 min) | | 2′-GS-fis ($t_R$ = 22.1 min) | |
|---|---|---|---|---|---|---|---|---|
| | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) |
| H8 | 6.81 (d) | $^{4}J_{H8\text{-}H6}$ = 1.9 | 6.46 (d) | $^{4}J_{H8\text{-}H6}$ = 2.1 | 6.31(d) | $^{4}J_{H8\text{-}H6}$ = 1.9 | 6.72 (d) | $^{4}J_{H8\text{-}H6}$ = 2.2 |
| H6 | 6.85 (dd) | $^{3}J_{H6\text{-}H5}$ = 8.9 | 6.49 (dd) | $^{3}J_{H6\text{-}H5}$ = 8.7 | 6.46 (dd) | $^{3}J_{H6\text{-}H5}$ = 8.8 | 6.82 (dd) | $^{3}J_{H6\text{-}H5}$ = 8.9 |
| | | $^{4}J_{H6\text{-}H8}$ = 1.9 | | $^{4}J_{H6\text{-}H8}$ = 2.1 | | $^{4}J_{H6\text{-}H8}$ = 1.9 | | $^{4}J_{H6\text{-}H8}$ = 2.2 |
| H5′ | 6.94 (d) | $^{3}J_{H5'\text{-}H6'}$ = 8.5 | 6.71 (d) | $^{3}J_{H5'\text{-}H6'}$ = 8.4 | 6.79 (d) | $^{3}J_{H5'\text{-}H6'}$ = 8.5 | 6.91 (d) | $^{3}J_{H5'\text{-}H6'}$ = 8.4 |
| H6′ | 7.61 (dd) | $^{3}J_{H6'\text{-}H5'}$ = 8.5 | 6.7 (dd) | $^{3}J_{H6'\text{-}H5'}$ = 8.4 | 7.21 (dd) | $^{3}J_{H6'\text{-}H5'}$ = 8.5 | 6.92 (d) | $^{3}J_{H6'\text{-}H5'}$ = 8.4 |
| | | $^{4}J_{H6'\text{-}H2'}$ = 2.1 | | $^{4}J_{H6'\text{-}H2'}$ = 2.3 | | $^{4}J_{H6'\text{-}H2'}$ = 2.1 | | — |
| H2′ | 7.69 (d) | $^{4}J_{H2'\text{-}H6'}$ = 2.1 | 7.23 (d) | $^{4}J_{H2'\text{-}H6'}$ = 2.3 | 7.28 (d) | $^{4}J_{H2'\text{-}H6'}$ = 2.1 | — | — |
| H5 | 7.87 (d) | $^{3}J_{H5\text{-}H6}$ = 8.9 | 7.58 (d) | $^{3}J_{H5\text{-}H6}$ = 8.7 | 7.59 (d) | $^{3}J_{H5\text{-}H6}$ = 8.8 | 7.83 (d) | $^{3}J_{H5\text{-}H6}$ = 8.9 |
| Glu Hβ | — | | 1.81 (m) | | 1.70 (m) | | 1.60 (m) | |
| Glu Hγ | — | | 2.13 (tr) | | 2.01 (m) | | 1.90 (m) | |
| Cys Hβ1 | — | | 2.35 (dd) | | 2.50 (dd) | | 2.72 (dd) | |
| Cys Hβ2 | — | | 2.73 (dd) | | 2.65 (dd) | | 3.07 (dd) | |
| Gly Hα1 | — | | 3.35 (d) | | 3.33 (d) | | 3.34 (d) | |
| Gly Hα2 | — | | 3.40 (d) | | 3.43 (d) | | 3.34 (d) | |
| Glu-Hα | — | | 3.49 (tr) | | 3.49 (tr) | | 3.37 (tr) | |
| Cys Hα | — | | 3.75 (dd) | | 4.04 (m) | | 3.82 (m) | |

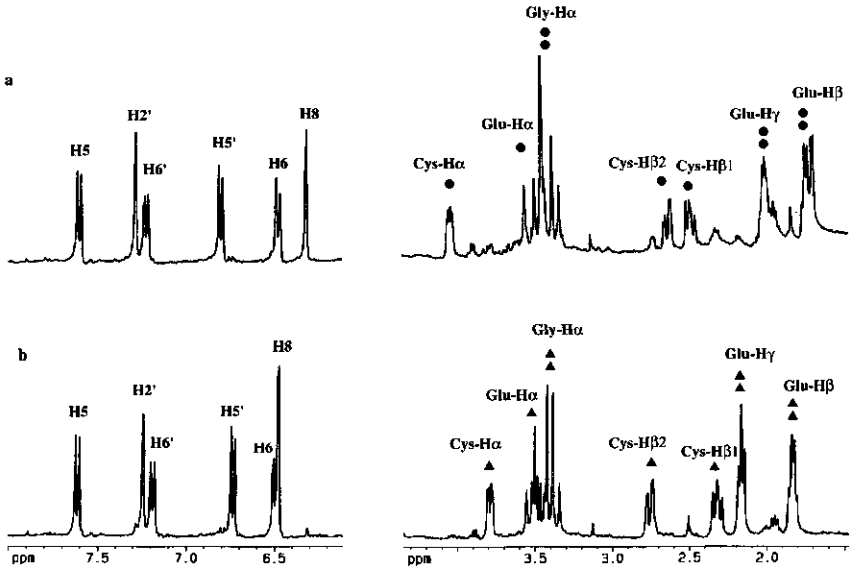


**Figure 4**. Aromatic and aliphatic parts of the $^{1}H$ NMR spectra of the two major metabolites formed in the incubation of fisetin with HRP in the presence of glutathione at pH 7.0 and eluting from the HPLC at 15.6 min (a) and 16.5 min (b) (2-glutathionyl-fisetin, two diastereoisomers) both measured in 25 mM potassium phosphate (pD = 7.0) in $D_2O$.

**Table 6**. Calculated energies of the highest occupied molecular orbital ($E_{HOMO}$) and the relative difference in $E_{HOMO}$ of the investigated 3′,4′-dihydroxyflavonoids and their 2′-glutathionyl adducts, obtained with the AM1 semiempirical method.

| | $E_{HOMO}$ (eV) | | $\Delta E_{HOMO}$ (eV) |
|---|---|---|---|
| Compound | parent | 2′-glutathionyl adduct | |
| quercetin | -8.71 | -8.97 | -0.26 |
| taxifolin | -8.91 | -8.09 | 0.82 |
| luteolin | -9.09 | -9.18 | -0.09 |
| 3,3′,4′-trihydroxyflavone | -8.65 | -8.93 | -0.28 |
| fisetin | -8.65 | -8.92 | -0.27 |

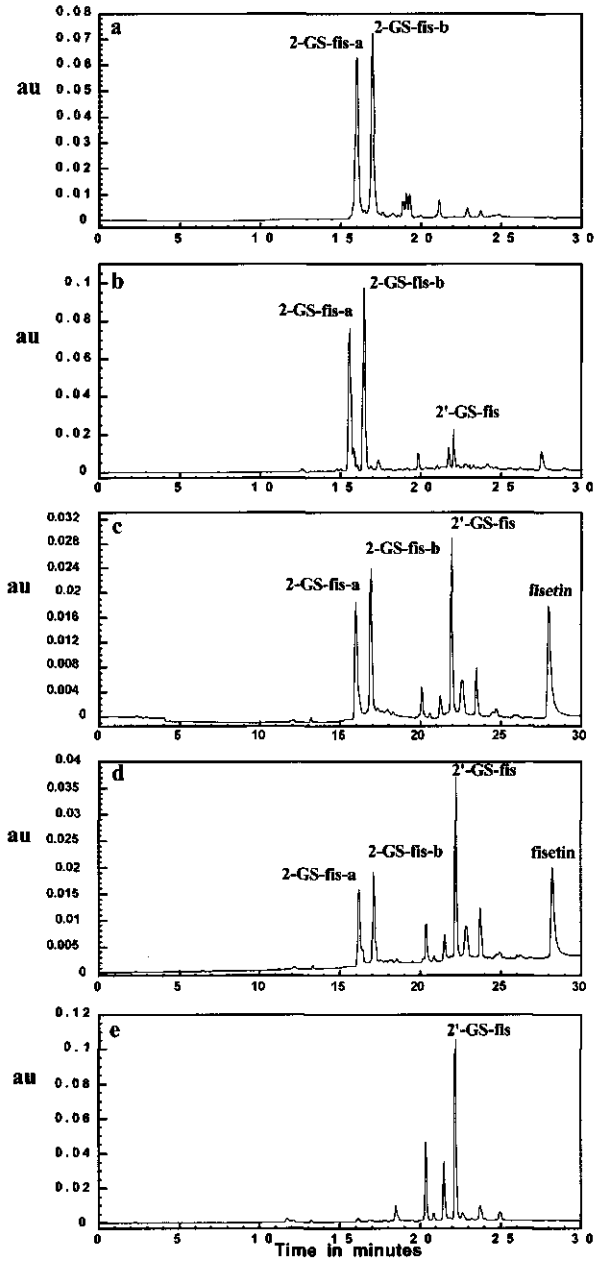


**Figure 5.** HPLC chromatograms of the incubation of fisetin with HRP in the presence of glutathione at a) pH 3.5 b) pH 7.0 c) pH 8.5 d) pH 9.0 e) pH 11.0.

### 3.5 Discussion

Quinone and quinone methides from a variety of natural and synthetic compounds, including 3′,4′-dihydroxyflavonoids and catechol-type metabolites from polycyclic aromatic hydrocarbons, estrogens, and compounds such as the anticancer drug tamoxifen, have been classified as likely candidates for reactive metabolites able to react with cellular macromolecules (*11,13,35*). For 3′,4′-dihydroxyflavonoids, with an intrinsic catechol moiety, their pro-oxidative quinone/quinone methide chemistry is especially important because of their increasing use as functional food ingredients and food supplements (*1,36-38*). Recently, the GSH trapping method (*19-21*) appeared to be an excellent method for investigating the quinone/quinone methide chemistry of the flavonoid quercetin (*22,23*), known to be mutagenic in a variety of bacterial and mammalian mutagenicity tests presumably through its quinone methide-like metabolites (*2,13,14*). The results of the study presented here, in which the quinone/quinone methide chemistry of an additional series of 3′,4′-dihydroxyflavonoids was investigated, provide new insight in the quinone/quinone methide chemistry of 3′,4′-dihydroxyflavonoids.

On the basis of the quinone/quinone methide isomerization chemistry involved in the formation of the A ring type glutathionyl adducts form quercetin *ortho*-quinone/quinone methide (Figure 1), it can be postulated that especially the C2=C3 double bond, the C3-OH group, the C4-keto group, and the C5- and/or C7-OH group are required for efficient quinone methide formation and GSH adduct formation in the A ring instead of in the B ring. In the present study this hypothesis was investigated in more detail.

The formation of 2′-glutathionyltaxifolin is in line with this hypothesis. Although saturation of the C2=C3 double bond, in theory, does not eliminate the quinone methide chemistry of the *ortho*-quinone of taxifolin to extend to the C ring, its conjugation with glutathione appears to be dominated by the *ortho*-quinone isomer resulting in preferential GSH addition at C2′.

With luteolin which lacks the C3-OH group but still contains the C2=C3 double bond, glutathionyl adduct formation was observed preferentially at the C2′ position, giving rise to 2′-glutathionylluteolin. Elimination of the 3-OH prevents quinone methide isomerisation since the rearrangement of the proton of the 3-OH group to generate a 3-keto group (Figure 1) can no longer occur. This restricts the structure of oxidized luteolin to the *ortho*-quinone isomer and, thus, the glutathione addition to the B ring (*14,39*). Preferential formation of 2′-glutathionyl luteolin is in line with the preferential regioselectivity observed for glutathione conjugation of taxifolin *ortho*-quinone. This observation is also in line with the calculations on the various theoretically possible glutathionyl adducts in the B ring of quercetin *ortho*-quinone, showing C2′ in the flavonoid *ortho*-quinone to be more reactive than C5′ or C6′ (*22*). Upon subsequent oxidation of both 2′-glutathionylluteolin and 2′-

glutathionyltaxifolin to their corresponding quinones, glutathione addition at the C5′ is observed, resulting in formation of 2′,5′-diglutathionyl adducts. One may wonder about this tendency of especially 2′-glutathionylluteolin and 2′-glutathionyltaxifolin to compete with the parent flavonoid for HRP oxidation followed by glutathionyl adduct formation, since formation of di-glutathionyl adducts was not observed as readily for any of the other 3′,4′-dihydroxyflavonoids. From time-dependent measurements with luteolin, it follows that especially when the concentration of the 2′-mono-glutathionylluteolin reaches the residual concentration of luteolin itself, formation of the di-adduct starts to compete. Thus, the discrepancy with respect to the ease of di-glutathionyl adduct formation, observed to be relatively favored for especially luteolin and taxifolin, may best be ascribed to the relative tendency of HRP to oxidize the parent compound as opposed to the 2′-mono-glutathionyl adduct. The reason 2′-glutathionylluteolin and 2′-glutathionyltaxifolin oxidation appears to be relatively efficient can be derived from molecular orbital calculations. For luteolin, the calculated ionization potential ($-E_{HOMO}$) and thus the ease of oxidation of the parent compound as compared to the 2′-glutathionyl adduct is almost the same (Table 6). For taxifolin, the calculated ionization potential reveals that the oxidation of 2′-glutathionyltaxifolin is even easier than the oxidation of the parent compound. This explains why especially for luteolin and taxifolin and not for the other model compounds of this study di-glutathionyl adduct formation is readily observed.

With 3,3′,4′-trihydroxyflavone, the lack of the 5-OH group eliminates the intramolecular hydrogen bond of the 5-OH group to the 4-keto and results in formation of an alternative intramolecular hydrogen bond of the proton of the 3-OH group to the oxygen of the 4-carbonyl group (*14,39*). This hydrogen bond interaction of the 3-OH moiety has been reported to hamper the tautomerisation of the 3-OH group to a 3-keto group and, thus, the formation of quinone methides (Figure 1). This explains why, upon elimination of the C5- and the C7-OH moieties, glutathione conjugation is dominated by the *ortho*-quinone, resulting, as for luteolin and taxifolin, in preferential formation of the 2′-glutathionyl adduct of 3,3′,4′-trihydroxyflavone.

Finally, the quinone/quinone methide trapping results of fisetin were different from what could be expected on the basis of the results with the other 3′,4′-dihydroxyflavonoids. The presence of the C2=C3 double bond and the C3-OH group but also the C7-OH group, in theory, may allow the quinoid structural element to extend to the A ring, resulting in a formation of quinone methide I (Figure 1) and providing possibilities for adduct formation at C6 and C8, as in the case of quercetin. However, as in 3,3′,4′-trihydroxyflavone, the lack of the 5-OH group might induce a strong hydrogen bonding of the proton of the 3-OH group to the oxygen of the 4-carbonyl group (*14,39*), which, as for 3,3′,4′-trihydroxyflavone, would hamper the tautomeric shift of the *ortho*-quinone to the corresponding quinone methide (*14*) and would give rise to adduct formation in the B ring, leading to 2′-glutathionylfisetin as a major product. Surprisingly, fisetin quinone behaved quite differently. Two adducts

were formed which were shown to still contain all six aromatic fisetin protons; i.e., H5, H6, and H8 (A ring) and H2′, H5′, and H6′ (B ring) were all still present. This indicates that glutathione addition to fisetin quinone occurs in the C ring. Furthermore, the MS data reveal that both adducts, in addition to the glutathionyl moiety, also contain an additional *m/z* 18 which must be due to an additional $H_2O$ moiety incorporated in the adduct. Especially this latter observation suggests that the reactivity of the fisetin quinone may be initially dominated by a type of reactivity reported previously also for the reaction of quercetin quinone with hydroxyl anions (*40*). This reactivity includes protonation of the quinone followed by hydroxyl ($OH^-$) attack at C2 in the C ring of the flavonoid quinone (Figure 6). This hydration results in the 3,4-flavandione, I. In analogy with anthocyanins, a ring-chain tautomeric equilibrium resulting in the chalcan-trione, II, may exist, which subsequently may lead to formation of the substituted 3(2H)-benzofuranone, III. $GS^-$ addition at C2 of II or III may result in formation of sets of two diastereoisomeric fisetin adducts.

Clearly, $^1H$ NMR and MS data of these substituted 3(*2H*)-benzofuranones and/or chalcan-triones are unable to discriminate between these possibilities, a problem reported previously for full identification of 3,4-flavandione (I) derived additive products (*40*). However, $^{13}C$ NMR spectra which indicate the formation of two $sp^3$ resonances and one carbonyl group for each metabolite clearly reveal formation of the diastereoisomeric glutathionyl adducts of 3(*2H*)-benzofuranone (V) and not those of chalcan-trione (IV) (Figure 6). Nevertheless, the data presented here for fisetin clearly illustrate two important new aspects of flavonoid quinone/quinone methide chemistry. First, the data provide an answer to the question of what causes the differential behavior of fisetin quinone as compared to the other flavonoid quinones with respect to preferential reactivity with water as compared to $GS^-$ addition. Second, the data answer the question of what makes quercetin quinone react with the soft nucleophile $GS^-$ at C6 and C8, but with water ($H^+/OH^-$) at C2. The results of this study clearly indicate that the answers to those questions can be found in the actual reaction conditions and subtle differences in $pK_a$ of the various quinone/quinone methides causing differences in the tendency to actually initiate the protonation and $OH^-$ addition pathway as an alternative for $GS^-$ addition. Based on these considerations, Figure 6 gives possible pathways for the formation of the $GS^-$ /$OH^-$ adducts of fisetin *ortho*-quinone. These pathways explain the unexpected quinone/quinone methide chemistry observed in the study presented here. A competition exists between (i) the reaction of the quinone/quinone methide with the glutathionyl anion ($GS^-$) leading to A or B ring adducts and (ii) a protonation of the quinone/quinone methide leading to water addition as an early event. Differences in the flavonoid structure may be expected to influence this balance in a subtle way. Experiments on the pH-dependent glutathione adduct formation of fisetin confirm the hypothesis that with increasing pH, protonation of the quinone becomes more

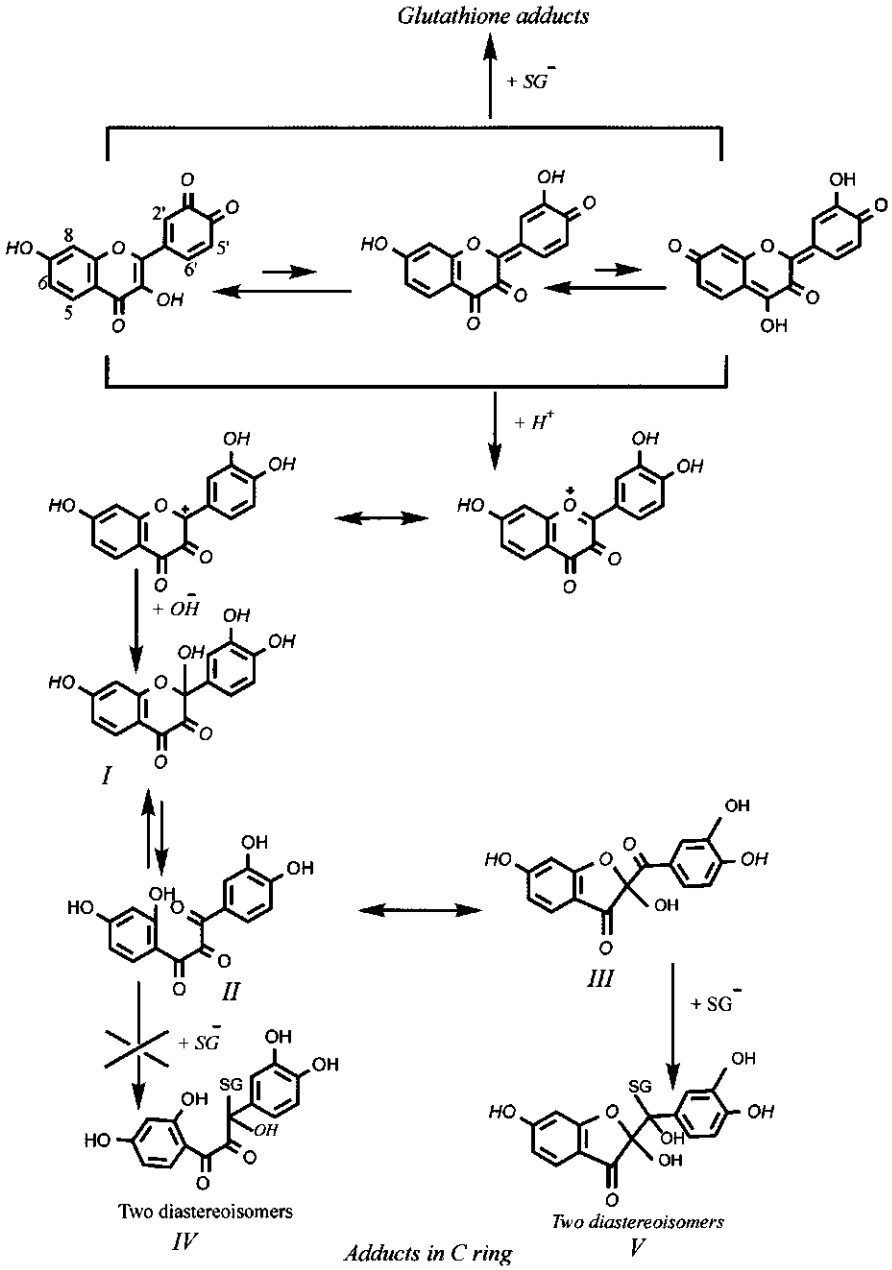


**Figure 6.** Hypothesis for the pH-dependent pathways for formation of the $GS^-$ / $OH^-$ adducts of fisetin *ortho*-quinone.

difficult, increasing the chances of direct glutathione adduct formation of the quinone in the B ring at C2′ as the preferential site for $GS^-$ attack on the quinone. All together, the results of the present study provide significant new insight into the *ortho*-quinone/quinone methide chemistry of flavonoids and show the importance of the pH for the chemistry of this important class of electrophiles. This pH dependency of quercetin and other investigated flavonoids is presently under investigation.

This study identifies the nature of GSH conjugates of several flavonoid quinones. This opens the way for future studies aimed at investigating the formation of these GSH conjugates and their corresponding mercapturic acids in cellular in vitro and in vivo systems. The actual formation of these glutathionylflavonoid quinone adducts and of their corresponding mercapturic acids would represent an in vivo bioactivation pathway of these supposed beneficial functional food ingredients. The formation of quinone-derived mercapturic acids would be comparable to the formation of the glutathionyl and *N*-acetylcysteine conjugates of estrogens (41). Recently, *o*-quinones have been highlighted as active intermediates in the development of cancer (11,21,41-45), since the presence of *o*-quinone-DNA complexes has been confirmed (11,43,44). It has been demonstrated that the *o*-quinones of estrogens react with glutathione, producing N-acetylcysteine conjugates in an in vitro system using rat liver (46,47). In addition, it has been suggested that the urinary levels of mercapturate can be used as a biomarker for exposure to active nucleophilic materials (48-50). Thus, it might be meaningful to determine the urinary levels of these *N*-acetylcysteine conjugates as marker of quinone-induced tumorigenesis. However, the actual detection of these conjugates in urine, for example, requires sensitive detection methods and knowledge on the stability, nature, and chemical behavior of the adducts. The results of this present study indicate the nature of the adducts to be expected upon (auto)oxidation of a series of flavonoids and provide a basis for the future detection of their mercapturic acids in biological samples as possible marker of pro-oxidative flavonoid behavior and toxicity in vivo.

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# 4

## The regioselectivity of glutathione adduct formation with flavonoid quinone/quinone methides is pH-dependent

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### 4.1 Abstract

*In the present study, the formation of glutathionyl adducts from a series of 3′,4′-*dihydroxy flavonoid *o*-quinone/*p*-quinone methides was investigated with special emphasis on the regioselectivity of the glutathione addition as a function of pH. The flavonoid *o*-quinones were generated using horseradish peroxidase and upon purification by HPLC the glutathionyl adducts were identified by LC/MS as well as $^{1}H$ and $^{13}C$ NMR. The major pH effect observed for the glutathione conjugation of taxifolin and luteolin quinone is on the rate of taxifolin and luteolin conversion and, as a result, on the ratio of mono- to di-glutathione adduct formation. With fisetin, 3,3′,4′-trihydroxyflavone, and quercetin, decreasing the pH results in a pathway in which glutathionyl adduct formation occurs in the C ring of the flavonoid, being initiated by hydration of the quinone and $H_2O$ adduct formation also in the C ring of the flavonoid. With increasing pH, for fisetin and 3,3′,4′-trihydroxyflavone glutathione adduct formation of the quinone occurs in the B-ring at C2′ as the preferential site. For quercetin the adduct formation of its quinone/quinone methide shifts from the C ring at pH 3.5, to the A ring at pH 7.0, to the B ring at pH 9.5, indicating a significant influence of the pH and deprotonation state on the chemical electrophilic behaviour of quercetin quinone/quinone methide. Together the results of the present study elucidate the mechanism of the pH-dependent electrophilic

behaviour of B-ring catechol flavonoids, which appears more straight forward than previously foreseen.

## 4.2 Introduction

Flavonoids are widely distributed in higher plants and form a natural component in the human diet. Recent interest in these substances has been stimulated by the potential health benefits arising from, amongst others, the anti-oxidant activity of these polyphenolic compounds (*1*). However a number of studies have reported not only anti-oxidant (*2-9*) but also pro-oxidant (*10-12*) effects of the flavonoids. The most ubiquitous and widely studied flavonoid is quercetin, a B-ring dihydroxylated flavonol. Some of its pro-oxidant properties have been attributed to the fact that it can undergo rapid auto-oxidation when dissolved in aqueous buffer at physiological pH. It has been observed that the rate of auto-oxidation for quercetin is highly pH-dependent and increases with increasing pH (*13*).

With respect to possible pro-oxidant toxicity, it is of interest to note that the mutagenic properties of the flavonoid quercetin in a variety of bacterial and mammalian mutagenicity tests, has been related to its quinone/quinone methide pro-oxidant chemistry (Figure 1). Especially the electrophilicity of the *o*-quinone and quinone methide-type metabolites is of interest in the context of cytotoxicity, mutagenicity and possible carcinogenicity (*3*). Identification of the oxidation products of quercetin and its analogues may therefore provide deeper insight into the mechanism of their toxic pro-oxidant action, and may form the basis for new biomarkers for the detection of pro-oxidant activity of flavonoids. Previous studies that included incubating flavonoids with peroxidases in the presence of GSH have indicated the capacity of GSH to scavenge the flavonoid semiquinone radical, thereby regenerating the flavonoid and generating oxidized GSH and reactive oxygen species leading to toxicity (*12,14*). This reaction appeared to be especially efficient for flavones and flavanones containing a phenol-type substituent pattern in their B ring (*14,15*). The GSH oxidizing pro-oxidant activity of this type of flavones and flavanones seemed to partly correlate with the high one-electron redox potential of the corresponding phenoxyl radicals (*14,16*). Flavonoids containing a catechol-type substituent pattern in their B ring did not co-oxidize GSH when oxidized by horseradish peroxidase (HRP), presumably because of their lower one-electron redox potentials. However, GSH conjugate formation involved their o-quinone/quinone methide metabolites (*17-20*).

We have recently investigated the quinone/quinone methide chemistry of a series of 3′,4′-dihydroxyflavones at pH 7.0 and identified their adducts using the glutathione (GSH) trapping method (*18*). Results obtained revealed that, especially for fisetin, regioselectivity and the nature of the quinone adducts formed, appear to be dependent on

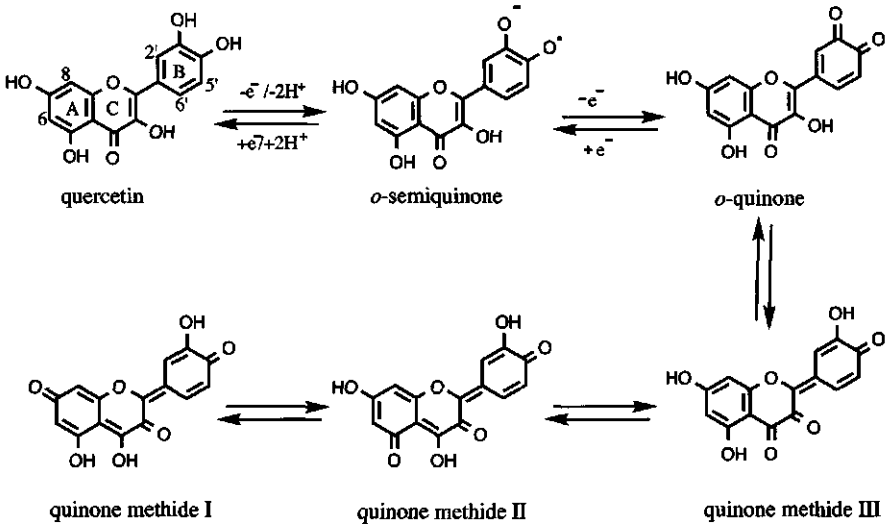



**Figure 1.** Schematic presentation of the formation of quercetin *o*-quinone and its isomerization to quercetin *p*-quinone methides, shifting the alkylating reactivity from the B to the A ring of quercetin. Quercetin = 3,5,7,3′,4′-pentahydroxyflavone, taxifolin lacks C2=C3, luteolin lacks C3-OH and 3,3′,4′-trihydroxyflavone lacks C5-OH and C7-OH.

the pH. With decreasing pH, the site of GSH adduct formation shifted from the B to the C ring (18). The objective of the present study was to further investigate the mechanism of the pH-dependent chemistry of flavonoid quinone/quinone methides. Thus, the pH-dependent quinone/quinone methide chemistry of quercetin, taxifolin, luteolin, and 3,3′,4′-trihydroxyflavone was investigated, using the GSH trapping method, HPLC, $^{1}H$ and $^{13}C$ NMR, and LC/MS analysis to identify the glutathionyl adducts.

### 4.3 Materials and Methods

*Materials.* Quercetin was obtained from Acros Organics (New Jersey, USA). Fisetin was from Aldrich (Steinheim, Germany). Luteolin and 3,3′,4′-trihydroxyflavone were from Indofine (Somerville, USA). Taxifolin was obtained from ICN Biomedicals Inc. (Ohio, USA). Horseradish peroxidase (HRP) was obtained from Boehringer (Mannheim, Germany). Glutathione, reduced form, was purchased from Sigma (St Louis, MO, USA).

All substrates were of 98-99 % purity. Hydrogen peroxide, potassium hydrogen phosphate, potassium dihydrogen phosphate, citric acid, tri-sodium citrate dihydrate, anhydrous sodium carbonate, sodium hydrogen carbonate, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Deuterium oxide was obtained from ARC Laboratories (Amsterdam, The Netherlands). Acetonitrile and methanol were HPLC grade from Lab-Scan, Analytical Sciences (Dublin, Ireland).

*pH-dependent incubation of flavonoids with glutathione.* To a starting solution of flavonoid (final concentration 150 μM added from a 10 mM stock solution in methanol) in 25 mM citrate, phosphate or carbonate, depending on the pH which varied between 3.5 and 11.0 (prepared according to literature) (*19-21*), containing GSH (final concentration 1.0 mM), HRP was added to a final concentration of 0.1 μM, followed by addition of $H_2O_2$ (final concentration of 200 μM added from a 20 mM stock solution in water). Upon 8 minutes incubation at 25°C, the incubation mixture was analyzed by HPLC.

*Analytical high performance liquid chromatography.* HPLC was performed with a Waters M600 liquid chromatography system. Analytical separations were achieved using an Alltima C18 column (4.6 x 150 mm) (Alltech, Breda, The Netherlands). The column was eluted at 0.7 mL/min with water containing 0.1 % (v/v) trifluoroacetic acid. A linear gradient from 0 % to 30 % acetonitrile in 18 minutes was applied, followed by 2 minutes isocratic elution with 30 % acetonitrile. Hereafter a linear gradient from 30 % to 100 % acetonitrile was used in 10 minutes. The percentage of acetonitrile was kept at 100 % for another 10 minutes. An injection loop of 10 μL was used. Detection was carried out with a Waters 996 photodiode array detector measuring spectra between 200 nm and 450 nm. Chromatograms presented are based on detection at 290 nm. Product peaks were collected and freeze-dried for further analysis by NMR, and LC/MS analysis. Freeze-dried samples were dissolved in 25 mM buffer, made with deuterated water when samples were used for NMR analysis.

*NMR measurements.* $^{1}H$ and $^{13}C$ NMR measurements were performed on a Bruker DPX 400 or Bruker AMX 500 spectrometer. A 1.5 s presaturation delay was used, along with a $70^{o}$ pulse angle and a 2.2 s acquisition time (7575 Hz sweep width, 32 K data points). The data were processed using an exponential multiplication of 0.5 or 1.0 Hz and zero filling to 64 K data points. Resonances are reported relative to HDO at 4.79 ppm at 7°C. $^{13}C$ NMR measurements were performed in deuterated methanol/$D_2O$ mixture at 15°C with a dedicated 5 mm $^{13}C$ NMR probe (32000 Hz sweep width, 64 K data points, 28000 scans).

*Liquid chromatography/mass spectrometry.* LC/MS analysis was performed to further characterize the peaks in the HPLC elution pattern. An injection volume of 10 µL from the incubation mixture or from the purified metabolite dissolved in buffer was used and separation of the products was achieved on a 4.6 x 150 mm Alltima C18 column (Alltech, Breda, The Netherlands). A gradient from 10 % to 30 % acetonitrile in water containing 0.1 % (v/v) trifluoroacetic acid was applied at a flow of 0.7 mL/min in 13 minutes. The percentage of acetonitrile was kept at 30 % for 2 minutes and then increased to 100 % in another 2 min. Mass spectrometric analysis (Finnigan MAT 95, San Jose, CA, USA) was performed in the positive electrospray mode using a spray voltage of 4.5 kV and a capillary temperature of 180 °C with nitrogen as sheath and auxillary gas.

**Table 1.** The UV absorption maxima for the investigated flavonoids and their adducts in A, B, and C ring

| Compound | $\lambda_{max}$ |
|---|---|
| taxifolin | 290.4 |
| 2′-glutathionyl taxifolin | 295.1 |
| 5′-glutathionyl taxifolin | 290.4 |
| 2′,5′-diglutathionyl taxifolin | 285.6 |
| luteolin | 252.5 and 347.6 |
| 2′-glutathionyl luteolin | 257.2 and 328.5 |
| 2′,5′-diglutathionyl luteolin | 261.9 and 328.5 |
| quercetin | 252.6 and 370.1 |
| 6-glutathionyl quercetin | 295.1 |
| 8-glutathionyl quercetin | 299.9 |
| 2′,5′-diglutathionyl quercetin | 252.5, 304.6 and 347.6 |
| 2′,5′,6′-triglutathionyl quercetin | 252.5 and 347.6 |
| 2-GS-Q-a | 295.1 |
| 2-GS-Q-b | 295.1 |
| 3,3′,4′-trihydroxyflavone | 247.7 and 361.1 |
| 2′-glutathionyl 3,3′,4′-trihydroxyflavone | 257.2, 285.6 and 352.4 |
| 2′,5′-diglutathionyl 3,3′,4′-trihydroxyflavone | 233.6, 280.9 and 333.3 |
| 2-GS-3,3′,4′-tri-OH-flavone-a | 257.2 and 280.9 |
| 2-GS-3,3′,4′-tri-OH-flavone-b | 257.2 and 280.9 |

### 4.4 Results

*pH-dependent formation of glutathionyl taxifolin adducts.* Figures 2a-c show the pH-dependent formation of the glutathionyl taxifolin adducts. At pH 7.0 (Figure 2b) two major peaks are observed with retention times of 20.4 min and 22.3 min. The first peak

was identified previously as 2′,5′-diglutathionyl taxifolin and the second peak as a mixture of 2′- and 5′-glutathionyl taxifolin (*18*). Table 1 shows the UV absorption maxima for these glutathionyl taxifolin adducts. With pH values increasing from 3.5 to 7.0 to 10.5 (Figures 2a-c) the peak intensity of the 2′,5′-diglutathionyl adduct decreases, and a lower extent of taxifolin conversion is observed. This is in line with previous observations that formation of diglutathionyl flavonoid adducts start to occur when the mono-adduct, because of its concentration and reactivity, starts to compete as a substrate with the parent flavonoid. At pH 10.5 (Figure 2c) metabolite formation was no longer significant. Thus the major effect of increasing pH on HRP mediated taxifolin GSH conjugation is on the rate of taxifolin conversion and as a result, the ratio of mono- to diglutathionyl adduct formation.

*pH-dependent formation of glutathionyl luteolin adducts.* Figures 3a-c show the pH-dependent formation of the glutathionyl luteolin adducts. At pH 7.0 (Figure 3b) two metabolites with retention times of 22.7 min and 25.0 min can be detected which were identified previously as 2′,5′-diglutathionyl and 2′-glutathionyl luteolin (*18*). Table 1 shows the UV absorption maxima for these glutathionyl luteolin adducts. Going from pH 3.5 to 11.0 (Figure 3a-c) the ratio between these two metabolites shift in favor of the mono-adduct due to a decreased overall luteolin conversion. This result is in line with what is observed for the pH- dependent effect on taxifolin quinone chemistry.

*pH-dependent formation of glutathionyl quercetin adducts.* Figures 4a-d show the pH-dependent formation of glutathionyl quercetin adducts. At pH 7.0 (Figure 4b) the formation of two major metabolites with retention times of 17.1 min and 18.1 min, identified previously as 8-glutathionyl and 6-glutathionyl quercetin (*19,20*) is observed. The LC/MS spectra of these peaks obtained with the relatively soft electrospray ionization show in contrast to previous more harsh ionized MALDI-TOF mass spectra, that the 8- and 6-glutathionyl adducts are present in their hydrated forms with M + 1 peaks at *m/z* 626.0. The occurrence of the water addition in the two metabolites clearly follows from the LC/MS analysis, and may result from a peroxidase/$H_2O_2$ catalysed reaction resulting in water addition as reported in the literature (*24*). The UV spectrum of the 6-glutathionyl and the 8-glutathionyl quercetin adducts reveal a shift in their UV absorption maxima from 370.1 nm to 295.1 nm and 299.9 nm respectively reflecting the loss of the conjugation between the ketone of the C ring and B ring (Table 1) (*17,25*). The peak intensities of these 6-glutathionyl and 8-glutathionyl quercetin metabolites decrease with increasing pH (Figures 4c+d). This is accompanied by the formation of at least two new metabolites with retention times of 21.5 min and 23.8 min. At pH $\geq$ 9.5 the hydrated 6-glutathionyl and 8-glutathionyl quercetin adducts are no longer observed and the two new metabolites become the major ones (Figure 4d).

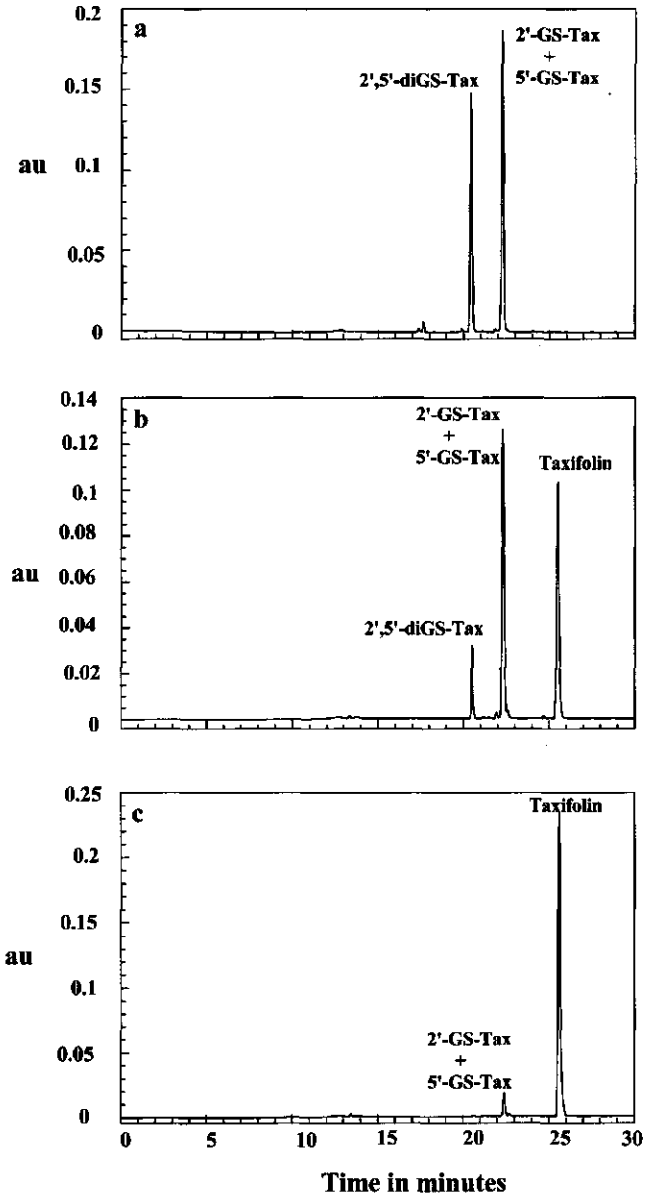



**Figure 2.** HPLC chromatograms of the incubation of taxifolin with HRP in the presence of GSH at (a) pH 3.5 (b) pH 7.0 and (c) pH 10.5.

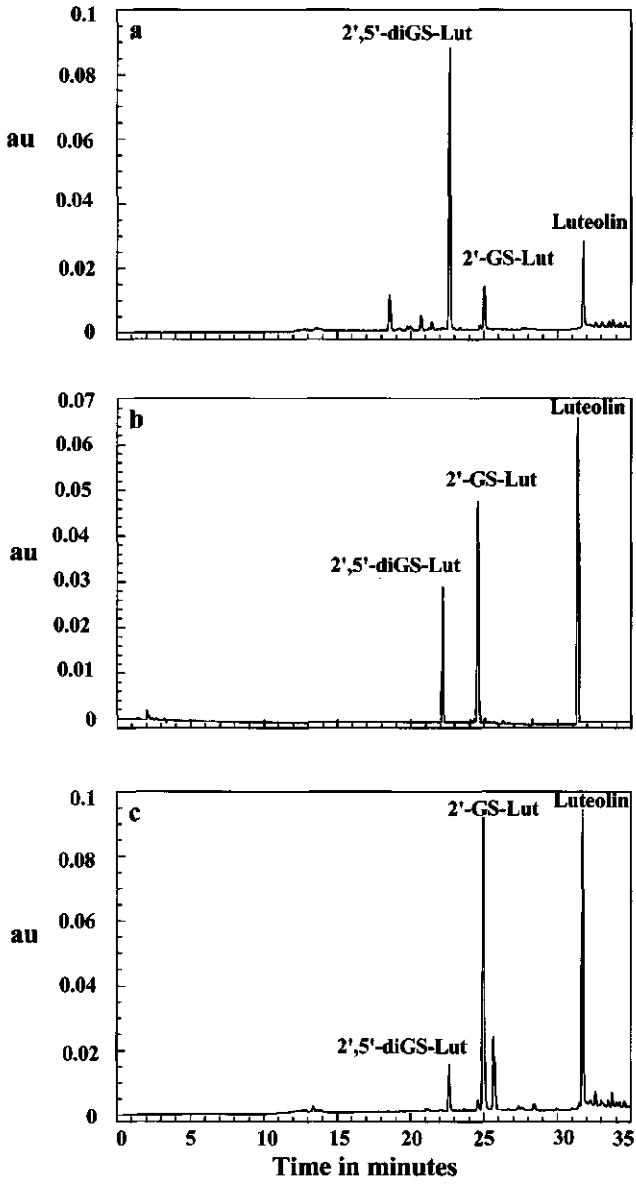



**Figure 3.** HPLC chromatograms of the incubation of luteolin with HRP in the presence of GSH at (a) pH 3.5 (b) pH 7.0 and (c) pH 11.0.

Table 1 shows the UV absorption maxima for these glutathionyl adducts. LC/MS analysis of these metabolites with retention times of 21.5 min and 23.8 min reveals M + 1 peaks at *m/z* 913.0 and 1218.0 respectively. This indicates the formation of a di- and a tri-glutathionyl quercetin adduct respectively. Table 2 shows the $^{1}$H NMR characteristics of these two major metabolites collected from HPLC. Comparison of the $^{1}$H NMR data of these two metabolites to those of quercetin (Table 2) (*19,20,26-28*) reveals the loss of the H2′ and H5′ signal as well as the loss of the $^{4}J_{H2'-H6'}$ coupling of 2.1 Hz and the $^{3}J_{H5'-H6'}$ coupling of 8.5 Hz for the H6′ for the di-glutathionyl adduct. For the tri-glutathionyl adduct the $^{1}$H NMR data indicate the loss of the H2′, H5′ and H6′ signal. In addition to the aromatic $^{1}$H resonances the $^{1}$H NMR spectra of the two adducts shows the $^{1}$H NMR resonances of the glutathionyl side chain. On the basis of these $^{1}$H NMR characteristics and LC/MS data the two metabolites can be identified as 2′,5′-diglutathionyl-quercetin and 2′,5′,6′-triglutathionyl-quercetin resulting from conjugation in the B instead of in the A ring. This glutathionyl addition in the B ring is not accompanied by hydration since the *m/z* signals exactly matche the di- and tri-glutathionyl adduct. At pH values < 7.0 two major peaks with almost the same retention times as the hydrated 6-glutathionyl- and 8-glutathionyl-quercetin metabolites are observed (Figure 4a). Surprisingly, however, the $^{1}$H NMR spectra of these two metabolites are different from those of the metabolites formed at pH 7.0 (Figure 5). The $^{1}$H NMR spectra reveal the retention of all the parent compound aromatic protons in both metabolites formed at pH 3.5, including the protons at C2′, C5′, and C6′ (B ring) and at C6 and C8 (A ring) (Figure 5a+b) (Table 2) (*19,20,26-28*). In addition to the aromatic $^{1}$H resonances the $^{1}$H NMR spectra of the two adducts shows the $^{1}$H NMR resonances of the glutathionyl side chain. LC/MS analysis of the purified metabolites shows an M + 1 peak for both metabolites at *m/z* 626.0. Because the *m/z* value expected for protonated mono-glutathionyl quercetin equals *m/z* 608.0, the observation of a peak at *m/z* 626.0 for both metabolites points at formation of mono-glutathionyl adducts which contain an additional $H_2O$ molecule. The UV spectra of these adducts reveal an absorbance peak at 295.1 for both metabolites and disappearance of the absorbance peak at 370.1 nm for quercetin (Table 1) indicating the loss of the conjugation between the A ring and the B ring (17,25). $^{13}$C NMR spectra of the two purified metabolites (Figure 6, Table 3) reveal formation of two sp$^{3}$ $^{13}$C resonances at 92.5 and 99.2 ppm for the metabolite with a retention time of 17.1 min and at 90.4 and 94.6 ppm for the metabolite with a retention time of 18.1 min which can be assigned to

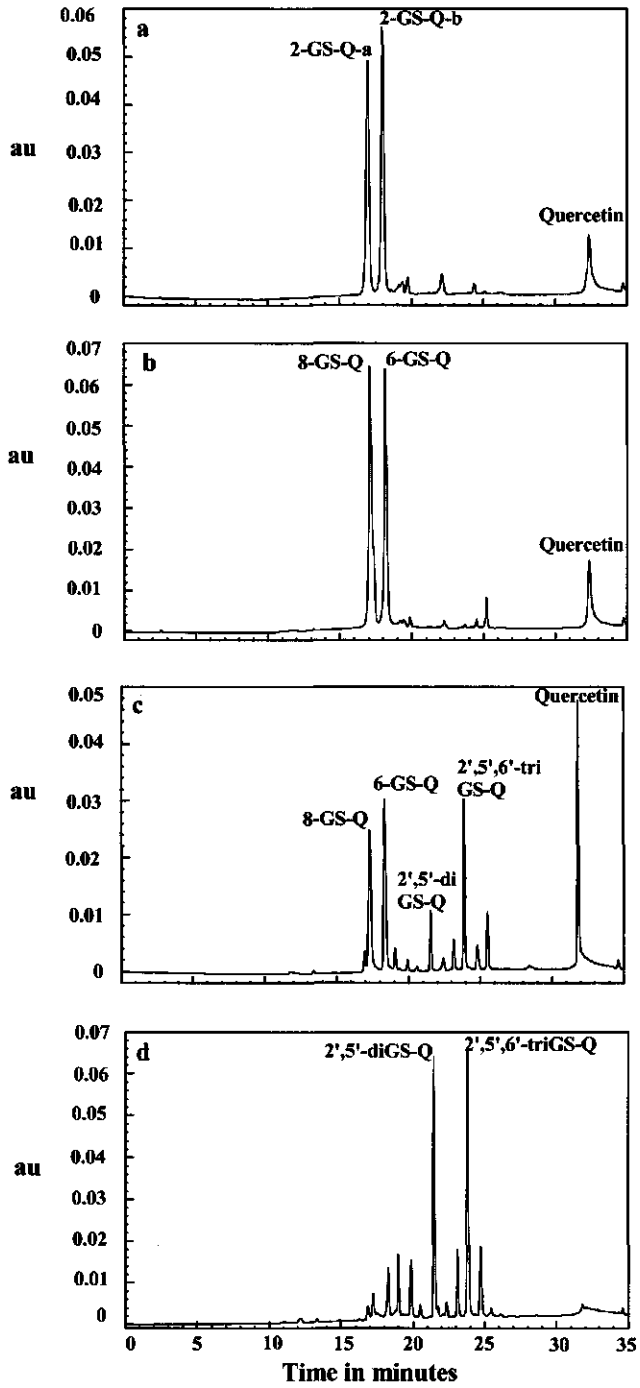



**Figure 4.** HPLC chromatograms of the incubation of quercetin with HRP in the presence of GSH at (a) pH 3.5 (b) pH 7.0 (c) pH 8.5 and (d) pH 9.5.

**Table 2.** $^{1}$H NMR resonances and coupling constants of the glutathionyl adducts of quercetin, labeled 2-GS-Q-a, 2-GS-Q-b, 2′,5′-diGS-Q and 2′,5′,6′-triGS-Q, isolated from HPLC (Figure 4).

| | 2-GS-Q-a | | 2-GS-Q-b | | 2′,5′-diGS-Q | | 2′,5′,6′-triGS-Q | |
|---|---|---|---|---|---|---|---|---|
| | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) |
| H6 | 5.96 (d) | $^{4}J_{H6\text{-}H8} = 2.4$ | 5.99 (d) | $^{4}J_{H6\text{-}H8} = 2.4$ | 6.10 (d) | $^{4}J_{H6\text{-}H8} = 2.1$ | 6.00 (d) | $^{4}J_{H6\text{-}H8} = 2.1$ |
| H8 | 6.00 (d) | $^{4}J_{H8\text{-}H6} = 2.4$ | 6.15 (d) | $^{4}J_{H8\text{-}H6} = 2.4$ | 6.21 (d) | $^{4}J_{H8\text{-}H6} = 2.1$ | 6.20 (d) | $^{4}J_{H8\text{-}H6} = 2.1$ |
| H5′ | 6.77 (d) | $^{3}J_{H5'\text{-}H6'} = 8.4$ | 6.72 (d) | $^{3}J_{H5'\text{-}H6'} \approx 8.4$ | — | — | — | — |
| H6′ | 7.18 (dd) | $^{3}J_{H6'\text{-}H5'} = 8.4$ | 7.15(dd) | $^{3}J_{H6'\text{-}H5'} \approx 8.4$ | 6.90 (s) | — | — | — |
| | | $^{4}J_{H6'\text{-}H2'} = 2.4$ | | $^{4}J_{H6'\text{-}H2'} \approx 2.4$ | | — | | — |
| H2′ | 7.26 (d) | $^{4}J_{H2'\text{-}H6'} = 2.4$ | 7.22 (d) | $^{4}J_{H2'\text{-}H6} = 2.4$ | — | — | — | — |
| Glu Hβ | 1.75 (m) | | 1.82 (m) | | 1.55 (m) | | 1.45 (m) | |
| | | | | | 1.69 (m) | | 1.54 (m) | |
| | | | | | | | 1.67 (m) | |
| Glu Hγ | 2.04 (m) | | 2.17 (m) | | 1.87 (m) | | 1.75 (m) | |
| | | | | | 2.11 (m) | | 1.86 (m) | |
| | | | | | | | 1.99 (m) | |
| Cys Hβ1 | 2.45 (dd) | | 2.31 (dd) | | 2.65 (dd) | | 2.15 (dd) | |
| | | | | | 2.88 (dd) | | 2.67 (dd) | |
| | | | | | | | 2.97 (dd) | |
| Cys Hβ2 | 2.59 (dd) | | 2.74 (dd) | | 3.10 (dd) | | 3.00 (dd) | |
| | | | | | 3.16 (dd) | | 3.10 (dd) | |
| | | | | | | | 3.25 (dd) | |
| Gly Hα1 | 3.48 (d) | | 3.55 (d) | | 3.45 (d) | | 3.49 (d) | |
| Gly Hα2 | 3.53 (d) | | 3.55 (d) | | 3.49 (d) | | 3.49 (d) | |
| Glu-Hα | 3.45 (t) | | 3.51 (m) | | 3.23 (t) | | 3.35 (m) | |
| | | | | | 3.28 (t) | | | |
| Cys Hα | 3.99 (dd) | | 3.78 (dd) | | 3.81 (dd) | | 3.85 (dd) | |
| | | | | | 3.19 (m) | | 3.99 (dd) | |
| | | | | | | | 4.10 (dd) | |

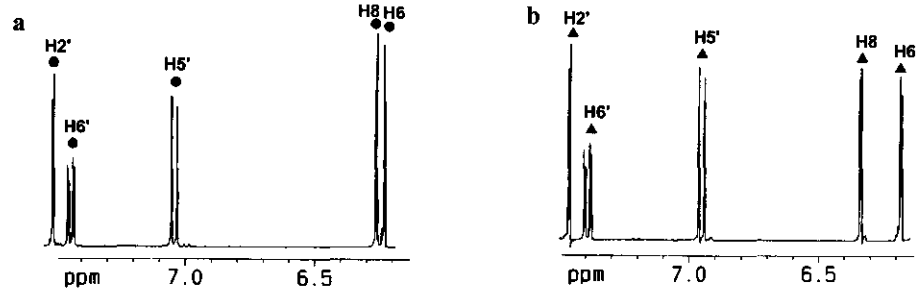



**Figure 5.** Aromatic parts of the $^{1}H$ NMR spectra of the two glutathionyl metabolites, 2-GS-Q-a (a) and 2-GS-Q-b (b), formed in the incubation of quercetin with HRP in the presence of GSH at pH < 7.0 both measured in 25 mM sodium citrate (pD = 3.5) in $D_2O$.

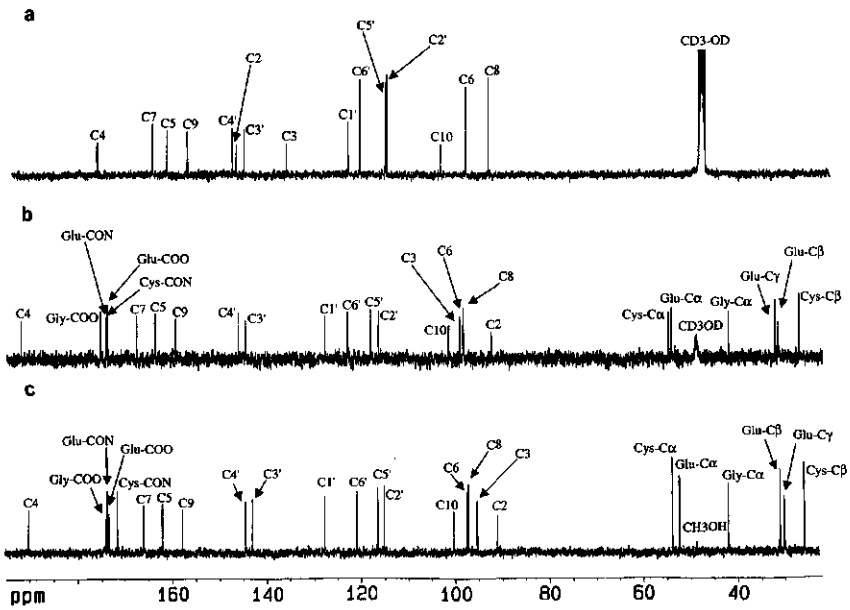



**Figure 6.** $^{13}C$ NMR spectra of a) quercetin and the two glutathionyl metabolites in C ring , 2-GS-Q-a (b) and 2-GS-Q-b (c), formed in the incubation of quercetin with HRP in the presence of GSH at pH < 7.0.

the two $sp^3$ hybridized deshielded C atoms at C2 and C3. In addition, both $^{13}C$ NMR spectra of the metabolites reveal only one resonance in the carbonyl region (at 192.0 ppm and at 190.0 ppm respectively) indicating the presence of only one carbonyl group for each metabolite. Together these data point at glutathionyl adduct formation accompanied by $H_2O$ adduct formation, both at the C ring of the quercetin *o*-quinone/quinone methide similar to what was recently observed for fisetin at low pH values (*18*) and in agreement with the reported literature (28) (see discussion). The fact that two metabolites with similar MS, UV, $^1H$ NMR and $^{13}C$ NMR characteristics are observed points at formation of different (diastereo) isomers of these combined $H_2O$/glutathionyl adducts.

Altogether the adduct formation of quercetin quinone/quinone methide shifts from the C ring at pH 3.5, to the A ring at pH 7.0, to the B ring at pH 9.5, indicating a significant influence of the pH on the regioselectivity of glutathione conjugation to quercetin quinone/quinone methide.

*pH-dependent formation of glutathionyl 3,3′,4′-trihydroxyflavone adducts.* Figure 7a-d show the pH-dependent formation of the glutathionyl 3,3′,4′-trihydroxyflavone adducts. At pH 7.0 (Figure 7c) only one major metabolite with retention time of 23.7 min can be detected which was identified previously as 2′-glutathionyl 3,3′,4′-trihydroxyflavone (*18*). At pH > 7.0 formation of a second major metabolite was observed with retention time at 22.0 min (Figure 7d). LC/MS analysis of this purified metabolite reveals M + 1 peak at *m/z* 881.0. This indicates the formation of a di-glutathionyl adduct. Table 1 shows the UV absorption maxima for these glutathionyl adducts of 3,3′,4′-trihydroxyflavone. Table 4 shows the $^1H$ NMR characteristics of this major metabolite collected from HPLC. Comparison of the $^1H$ NMR data of this metabolite to those of 3,3′,4′-trihydroxyflavone (Table 4) (*18,26,29*) reveals the loss of the H2′ and H5′ signal as well as the loss of the $^4J_{H2'-H6'}$ coupling of 1.4 Hz and the $^4J_{H5'-H6'}$ coupling of 8.5 Hz for the H6′ signal. In addition to the aromatic $^1H$ resonances the $^1H$ NMR spectrum of the adduct shows the $^1H$ NMR resonances of the glutathionyl side chain. On the basis of these $^1H$ NMR characteristics and LC/MS data, this metabolite can be identified as 2′,5′-diglutathionyl-3,3′,4′-trihydroxyflavone. At pH values < 7.0 the peak intensity of the 2′-glutathionyl adduct decreases with decreasing pH values, and the formation of two new major metabolites with retention times of 18.8 min and 19.8 min can be observed. At very low pH value the 2′-glutathionyl adduct is no longer observed and the two new metabolites become the major metabolites detected (Figure 7a). $^1H$ NMR spectra of these two major metabolites of 3,3′,4′-trihydroxyflavone collected from HPLC with retention times of 18.8 min and 19.8 min (Figure 7a) reveal the retention of all parent compound aromatic

**Table 3.** $^{13}$C-NMR chemical shifts of quercetin and the C ring glutathionyl adducts of quercetin labeled 2-GS-Q-a and 2-GS-Q-b.

| | Quercetin | 2-GS-Q-a | 2-GS-Q-b |
|---|---|---|---|
| | Chemical shift (ppm) | Chemical shift (ppm) | Chemical shift (ppm) |
| C2 | 147.4 | 92.5 | 90.4 |
| C3 | 136.7 | 99.2 | 94.6 |
| C4 | 176.7 | 192.0 | 190.0 |
| C5 | 161.9 | 163.8 | 161.7 |
| C6 | 98.6 | 98.6 | 96.8 |
| C7 | 165.0 | 167.7 | 165.6 |
| C8 | 93.8 | 98.5 | 96.5 |
| C9 | 157.6 | 159.4 | 157.4 |
| C10 | 103.9 | 101.6 | 99.7 |
| C1′ | 123.5 | 127.8 | 127.1 |
| C2′ | 115.4 | 116.5 | 114.5 |
| C3′ | 145.6 | 144.7 | 142.5 |
| C4′ | 148.2 | 146.1 | 144.0 |
| C5′ | 115.6 | 118.2 | 115.9 |
| C6′ | 121.1 | 123.0 | 120.3 |
| Cys-Cβ | — | 27.2 | 25.1 |
| Glu-Cβ | — | 31.6 | 29.3 |
| Glu-Cγ | — | 32.2 | 30.2 |
| Gly-Cα | — | 42.2 | 41.3 |
| Glu-Cα | — | 54.3 | 51.7 |
| Cys-Cα | — | 54.9 | 53.2 |
| Cys-CON | — | 173.8 | 171.2 |
| Glu-COO | — | 173.8 | 173.0 |
| Glu-CON | — | 174.1 | 173.4 |
| Gly-COO | — | 175.3 | 173.7 |

protons in both adducts (Table 4). This indicates the formation of glutathionyl 3,3′,4′-trihydroxyflavone adducts at another position than C2′, C5′ and C6′ (B ring) or C5, C6, C7 and C8 (A ring) (Table 4) (*26,29*). The UV spectra of these adducts reveal an absorbance peak at 280.9 nm for both metabolites and disappearance of the absorbance peak at 361.1 nm for 3,3′,4′-trihydroxyflavone (Table 1) indicating the loss of the conjugation between the A ring and B ring (*17,25*). LC/MS analysis of these two purified metabolites reveals a M + 1 peak for both metabolites at *m/z* 594.0. Because the *m/z* value expected for protonated mono-glutathionyl 3,3′,4′-trihydroxyflavone equals 576.0 the

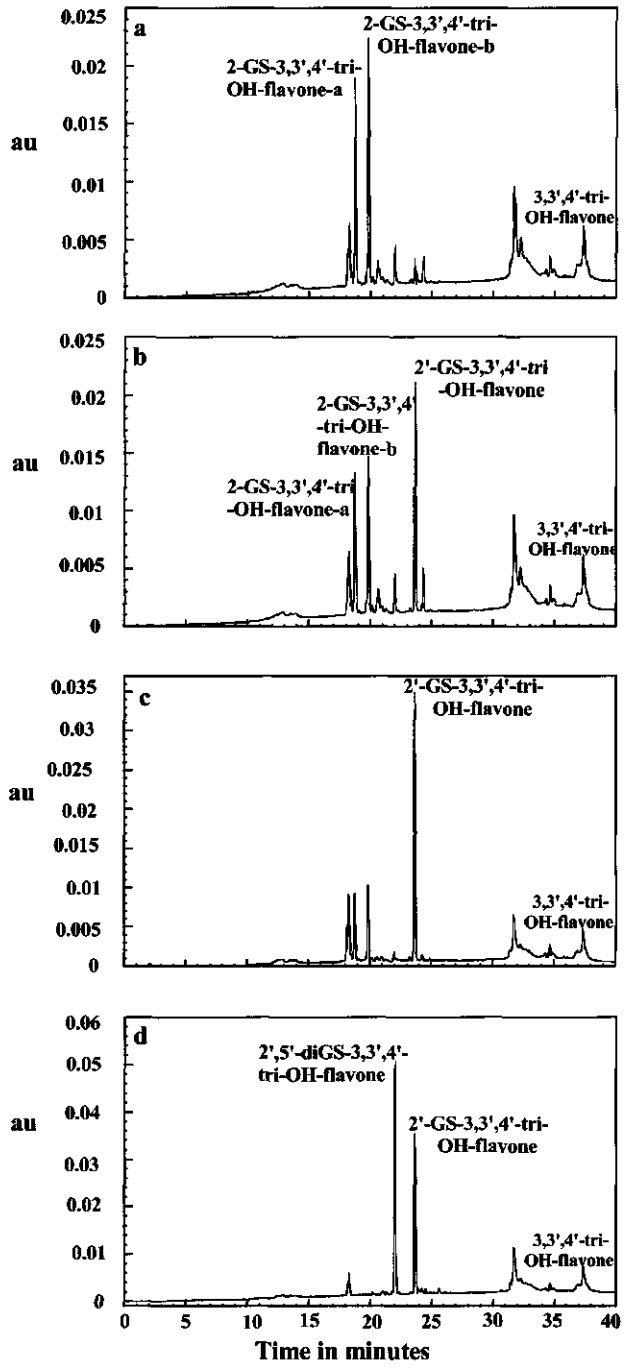



**Figure 7.** HPLC chromatograms of the incubation of 3,3′,4′-trihydroxyflavone with HRP in the presence of GSH at (a) pH 3.5 (b) pH 5.5 (c) pH 7.0 and (d) pH 8.5.

observation of a peak at *m/z* 594.0 for both metabolites points at formation of mono-glutathionyl adducts which contain an additional $H_2O$ molecule. Together these data point at glutathionyl adduct formation accompanied by $H_2O$ adduct formation, both at the C ring of the 3,3′,4′-trihydroxyflavone *o*-quinone/quinone methide similar to what was previously observed for fisetin (*18,28*) and quercetin (above). The fact that two metabolites with similar MS and $^1$H NMR characteristics are observed points at formation of different (diastereo)isomers of these combined $H_2O$/glutathionyl adducts. Together these results point at a reaction of the quinone/quinone methide of 3,3′,4′-trihydroxyflavone with glutathione which is similar to the reaction of fisetin with glutathione discussed previously (*18,28*) and quercetin (above).

**Table 4.** $^1$H NMR resonances and coupling constants of the glutathionyl adducts of 3,3′,4′-trihydroxyflavone, labeled 2-GS-3,3′,4′-tri-OH-flavone-a, 2-GS-3,3′,4′-tri-OH-flavone-b and 2′,5′-diGS-3,3′,4′-tri-OH-flavone, isolated from HPLC (Figure 6).

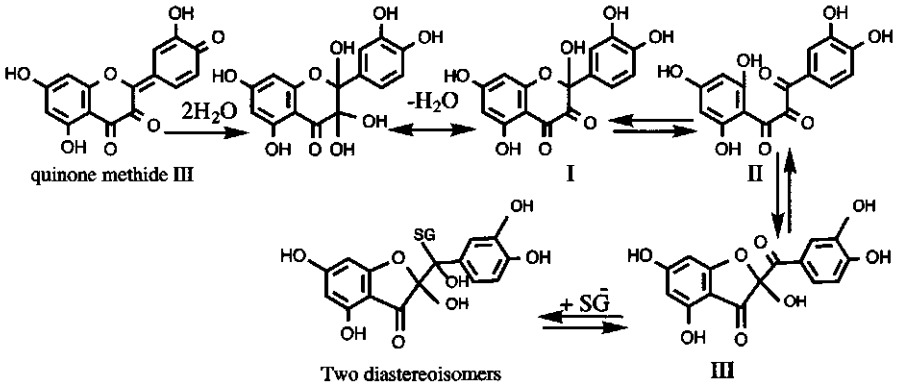
| | 2-GS-3,3′,4′-tri-OH-flavone-a | | 2-GS-3,3′,4′-tri-OH-flavone-b | | 2′,5′-diGS-3,3′,4′-tri-OH-flavone | |
|---|---|---|---|---|---|---|
| | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) |
| H5′ | 6.79 (d) | $^3J_{H5'-H6'} = 8.4$ | 6.74 (d) | $^3J_{H5'-H6'} = 8.4$ | — | — |
| H8 | 7.01 (dd) | $^4J_{H8-H6} = 1.0$ | 7.18 (dd) | $^4J_{H8-H6} = 1.0$ | 7.43 (dd) | $^4J_{H8-H6} = 1.0$ |
| | | $^3J_{H8-H7} = 8.8$ | | $^3J_{H8-H7} = 8.4$ | | $^3J_{H8-H7} = 8.4$ |
| H6 | 7.05 | $^4J_{H6-H8} = 1.0$ | 7.08 (tr) | $^4J_{H6-H8} = 1.0$ | 7.32 (tr) | $^4J_{H6-H8} = 1.0$ |
| | (tr) | $^3J_{H6-H7} = 6.9$ | | $^3J_{H6-H7} = 7.4$ | | $^3J_{H6-H7} = 7.4$ |
| | | $^3J_{H6-H5} = 7.9$ | | $^3J_{H6-H5} = 7.9$ | | $^3J_{H6-H5} = 7.9$ |
| H6′ | 7.24 (dd) | $^3J_{H6'-H5'} = 8.4$ | 7.21 (d) | $^3J_{H6'-H5'} = 8.4$ | 7.00 (s) | — |
| | | $^4J_{H6'-H2'} = 2.0$ | | $^4J_{H6'-H2'} = 2.0$ | | — |
| H2′ | 7.30 (d) | $^4J_{H6'-H2'} = 2.0$ | 7.26 (d) | $^4J_{H6'-H2'} = 2.0$ | — | — |
| H7 | 7.52 (tr) | $^4J_{H7-H5} = 2.0$ | 7.56 (tr) | $^4J_{H7-H5} = 1.5$ | 7.61 (tr) | $^4J_{H7-H5} = 1.5$ |
| | | $^3J_{H7-H6} = 6.9$ | | $^3J_{H7-H6} = 7.4$ | | $^3J_{H7-H6} = 7.4$ |
| | | $^3J_{H7-H8} = 8.8$ | | $^3J_{H7-H8} = 8.4$ | | $^3J_{H7-H8} = 8.4$ |
| H5 | 7.73 (dd) | $^4J_{H5-H7} = 2.0$ | 7.75 (dd) | $^4J_{H5-H7} = 1.5$ | 7.94 (dd) | $^4J_{H5-H7} = 1.5$ |
| | | $^3J_{H5-H6} = 7.9$ | | $^3J_{H5-H6} = 7.9$ | | $^3J_{H5-H6} = 7.9$ |
| Glu Hβ | 1.68 (m) | | 1.81 (m) | | 1.53 (m) | |
| Glu Hγ | 1.94 (m) | | 2.14 (m) | | 1.84 (m) | |
| | | | | | 2.21 (m) | |
| Cys Hβ1 | 2.45 (dd) | | 2.26 (dd) | | 2.64 (dd) | |
| | | | | | 2.95 (dd) | |
| Cys Hβ2 | 2.62 (dd) | | 2.66 (dd) | | 3.10 (dd) | |
| | | | | | 3.23 (dd) | |
| Gly Hα1 | 3.32 (d) | | 3.31 (d) | | 3.30 (d) | |
| Gly Hα2 | 3.42 (d) | | 3.40 (d) | | 3.43 (d) | |
| Glu-Hα | 3.55 (t) | | 3.50 (t) | | 3.38 (m) | |
| Cys Hα | 3.96 (dd) | | 3.79 (dd) | | 3.77 (dd) | |
| | | | | | 4.27 (dd) | |

*Identification of the flavonoid diglutathionyl adducts.* The identification of the diglutathionyl B ring adducts was generally based on the assumption that the remaining singlets in the $^{1}H$ NMR spectra pertain to H6′ (Tables 2 and 3). However, since all B ring proton resonances are close, in theory this single resonance may be ascribed to all three B ring protons, indicating formation of 2′,5′- or 2′,6′- or 5′,6′-diglutathionyl adducts. Therefore additional experiments were performed in which the 2′-glutathionyl adducts of the studied flavonoids were incubated with HRP/$H_2O_2$ in the presence of GSH. This resulted in the formation of metabolites with the same retention times, the same UV spectra, the same mass and the same $^{1}H$ NMR spectra as the compounds identified as the 2′,5′ -diglutathionyl adducts. The formation of these products from the 2′-glutathionyl adducts eliminates the possibility of their identification as 5′,6′-diglutathionyl adducts. Nevertheless their formation from 2′-glutathionyl conjugates may still point at either the 2′,5′- or the 2′,6′-diglutathionyl adducts. Unfortunately, like the H5′ and H6′ resonances in $^{1}H$ NMR, also the C5′ and C6′ $^{13}C$ resonances (see for example Figure 6) appear too close to provide the possibility for unequivocal discrimination of the presence of either a C5′-H5′ or a C6′-H6′ in the diadducts for by example a 2D $^{1}H$-$^{13}C$-correlation spectrum. However, previous studies with taxifolin have unequivocally demonstrated the 2′- and 5′- as most reactive sites in the B ring quinone reflected by formation of 2′ and 5′-mono- but not a 6′-mono-glutathionyl taxifolin metabolite (18). Furthermore adduct formation at C6′ can be expected to be hampered by sterical hindrance. Since for taxifolin isolation of a 5′-mono-glutathionyl adduct was feasible. This isolated 5′-mono-glutathionyl adduct was also incubated with HRP/$H_2O_2$ in the presence of GSH. As for the 2′-mono-glutathionyl adducts this resulted in the formation of a metabolite with the same retention time, the same UV spectrum and the same mass and $^{1}H$ NMR characteristics as the metabolite identified as the 2′,5′-diglutathionyl taxifolin. Based on these data and arguments we have assigned all diglutathionyl adducts as 2′,5′-diglutathionyl adducts.

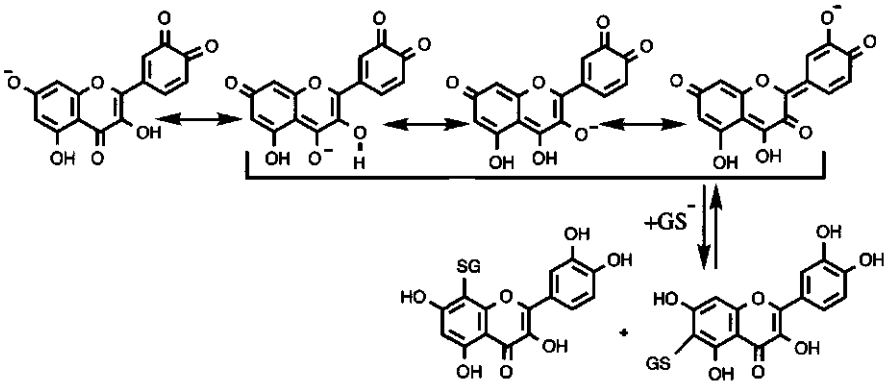
**4.5 Discussion**

In the present study, the formation of glutathionyl adducts from a series of 3′,4′-dihydroxyflavonoid *o*-quinone/*p*-quinone methides was investigated with special emphasis on the regioselectivity of the GSH addition as a function of the pH. The flavonoid *o*-quinones were generated with the use of HRP, an enzyme shown before to catalyse the conversion of flavonoid catechols to their corresponding *o*-quinones (*11,12,12*). The GSH adducts were purified by HPLC and identified by LC/MS and $^{1}H$ and $^{13}C$ NMR analysis. Based on the quinone/quinone methide isomerization chemistry involved in the formation of the A ring type glutathionyl adducts from quercetin *o*-

a) Neutral quinone: C ring adducts




b) Quinone mono-anion: A ring adducts




c) Quinone di-anion: B ring adducts

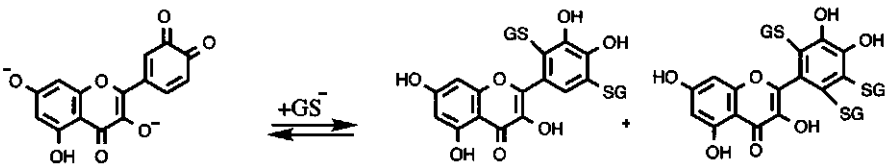



**Figure 8.** Schematic presentation of the mechanism for the pH-dependent formation of the glutathionyl/water adducts of quercetin quinone/quinone methide a) at low pH, b) at neutral pH and c) at alkaline pH. The deprotonation state and substituent pattern of the flavonoid quinone determines its quinone/quinone methide isomerisation but also the regioselectivity of the GSH addition.

quinone/quinone methide (Figure 1), it can be postulated that especially the presence of the C2=C3 double bond, the C3-OH group, the C4-keto moiety and the C5-OH group are required for efficient quinone methide formation and GSH adduct formation in the A instead of in the B ring. Furthermore, in our previous work we have reported that especially for fisetin, regioselectivity and the nature of the quinone adducts formed appear to be dependent on the pH. Depending on pH the site of GSH adduct formation shifted from the B to the C ring of fisetin (*18*). In the present study the pH-dependent chemistry of flavonoid quinone/quinone methide chemistry, was investigated in more detail using other flavonoid model compounds including the most widely studied flavonoid quercetin. This results in a new hypothesis describing the pH dependent shift in the regioselectivity of GSH addition to the flavonoid quinone/quinone methides. Figure 8 schematically presents this model for quercetin.

With quercetin, at low pH, GSH is protonated (thiol form) and thus not nucleophilic enough to compete with the much more abundant water molecules. Water addition to the quercetin quinone/quinone methide takes place at C2=C3 in the C ring (Figure 8a) (*30*). This hydration results in 3,4-flavandione (I) as reported previously (*18,28*). In analogy with anthocyanins, a ring chain tautomeric equilibrium resulting in the chalcantrione (II) may exist, which subsequently leads to formation of the substituted 3(*2H*)-benzofuranone (III). Ultimately, GSH adds to the C=O group at C2 of compound III resulting in formation of a set of two diastereoisomeric glutathionylquercetin adducts in the C ring.

At neutral pH, GSH is partially presented in its highly nucleophilic thiolate form and $GS^-$ addition is preferred over water addition. The quercetin *o*-quinone has lost its most acidic proton at 7-OH followed by an efficient mesomeric equilibrium of the quercetin *o*-quinone mono-anion with its corresponding quinone methide isomers. Quinone methide formation in the A ring is thus favored, which gives rise to glutathionyl adduct formation in the A ring, leading to 6- and 8-glutathionylquercetin adducts (Figure 8b) (*24,30*).

At alkaline pH, the quercetin *o*-quinone has lost its second most acidic proton at 3-OH giving rise to its corresponding quinone di-anion (*30*). The isomerization of quercetin *o*-quinone di-anion to its corresponding *p*-quinone methide is hampered due to the second deprotonation step at 3-OH. As a result the *o*-quinone form prevails, which result in $GS^-$ conjugation in the B ring instead of in the A ring (Figure 8c).

With taxifolin and luteolin the absence of the C2=C3 double bond and/or the 3-OH group hampers the quinone methide isomerization of their *o*-quinone at all pH values and deprotonation states. And, thus, the GSH/$GS^-$ addition is preferentially in the B-ring. Formation of the 2′,5′-diglutathionyl adducts for both taxifolin and luteolin starts to occur when the concentration and reactivity of the 2′-monoglutathionyl-adduct starts to compete as substrate with the parent flavonoid, a process depending on its concentration and ionization potential (*18*). Thus the major effect observed for the pH dependent effect on

HRP mediated taxifolin and luteolin GSH/GS⁻ conjugation in the present study is on the rate of taxifolin and luteolin conversion and, as a result, on the ratio of mono- to di-glutathionyl adduct formation.

In the case of fisetin (*18*), the absence of the 5-OH introduces a strong hydrogen bridge between the C4=O keto and the C3-OH which also prevents isomerization to the quinone methide thereby diminishing possibilities for A ring adducts. C ring addition, however is still observed, probably not starting from the quinone methide III but, as previously indicated, from the quinone itself (*18*). Upon deprotonation of fisetin at alkaline pH adduct formation in the B ring is observed as for all other flavonoid quinone/quinone methides. The pH-dependent chemistry of the quinone of 3,3′,4′-trihydroxyflavone is in line with what would be expected for the trihydroxyflavonoids based on Figure 8. Finally, the results of the present study elucidate the mechanism of the pH-dependent electrophilic behaviour of B-ring catechol flavonoids

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# 5

## Quenching of quercetin quinone/quinone methides by different thiolate scavengers: stability and reversibility of conjugate formation

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(Manuscript in preparation)

### 5.1 Abstract

Oxidation of flavonoids with a catechol structural motif in their B ring, leads to formation of flavonoid quinone/quinone methides which rapidly react with glutathione to give reversible glutathionyl flavonoid adducts. Results of the present study demonstrate that as a thiol scavenging agent for this reaction cysteine is preferred over glutathione and *N*-acetylcysteine. This preferential scavenging by cysteine can be ascribed either to cyclization leading to 1,4-benzothiazine formation or to formation of a stable intramolecular hydrogen bridge both hampering deconjugation. However, the preferential scavenging by cysteine over glutathione reported in the present study is not expected to occur in biological systems, in which detection of the glutathionyl flavonoid conjugates may be hampered by their reversible nature. This, because physiological concentrations of GSH are substantially higher than those of cysteine which was shown to shift the balance in favour of glutathionyl adduct formation. The cysteinyl quercetin adducts, although not showing the reversible nature of the glutathionyl conjugates, appeared nevertheless to be unstable. Thus, as a biomarker for formation in biological systems detection of the glutathionyl or *N*-acetylcysteinyl conjugates should be the method of choice.

### 5.2 Introduction

Natural polyphenols like flavonoids and isoflavonoids and their corresponding glycosides are important constituents of fruits, vegetables, nuts, seeds, tea, olive oil

and red wine (1-3). On average the daily western diet contains about 1 gram of polyphenols (4). The antioxidant properties of these compounds are often claimed to be responsible for protective effects of these food components against cardiovascular disease, certain forms of cancer and/or photosensitivity diseases (1,5-9). In addition it has been proposed that accumulation of oxidative damage is an important contributor to not only pathological conditions but also to the ageing process. Therefore, beneficial health effects in ageing have also been related to antioxidant action (1,5-12). Together these suggested beneficial effects provide the basis for the present rapidly increasing interest for the use of antioxidants as functional food ingredients. As a result, increased human exposure to polyphenolic flavonoid-type antioxidants can be expected in the near future.

Taking into account the facts that many flavonoids already contain in their structure a catechol moiety, a structural element shown to be involved in the carcinogenic potential of steroids and polycyclic aromatic hydrocarbons (13-19), and that quercetin has been reported to be mutagenic in many bacterial as well as mammalian test systems (20-22) even without metabolic activation (20-22), it becomes of interest to investigate the pro-oxidative behaviour and alkylating properties of quercetin and its possible quinone-type metabolites. In a previous study we demonstrated that the incubation of quercetin with tyrosinase, an enzyme capable of catalysing a two-electron oxidation of catechol moieties, in the presence of glutathione (GSH), results in the formation of two GSH adducts, identified as 6- and 8-glutathionylquercetin (23). In subsequent studies it was demonstrated that also one-electron oxidation of quercetin, mediated by horseradish peroxidase, when performed in the presence of GSH, gave rise to formation of the same 6- and 8-glutathionylquercetin adducts (24). This adduct formation in the A instead of the B ring of quercetin provides evidence for the formation of the highly reactive unstable quercetin *para*-quinone methide anion from the originally generated quercetin quinone mono-anion (Figure 1). Time dependent isomerisation of the isolated 6- or 8-glutathionyl quercetin adducts to give an equimolar mixture of both isomers, already pointed at the reversible nature of the adduct formation between the reactive quercetin quinone methide and GSH (23). This reversible nature of GSH conjugation is a well known phenomenon, identified before as a toxicologically relevant mechanism for transport of reactive electrophilic intermediates to sites in the body distinct from the site of their formation (25,26). Due to the high reactivity and instability of the free quercetin quinone methide the reversible nature of the glutathionyl quercetin adducts ultimately leads to loss of the adducts. Clearly this will hamper their detection and the study on quercetin pro-oxidant chemistry in complex biological systems. In contrast to GSH conjugates cysteine conjugates of quinones have been reported not to be of a reversible nature due to especially cyclisation to the corresponding 1,4-benzothiazine (27).

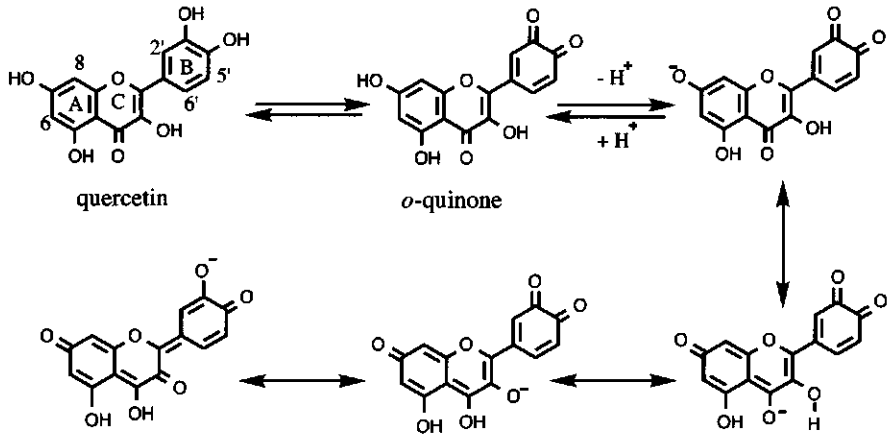


**Figure 1.** Schematic presentation of the formation of quercetin *o*-quinone mono-anion and its isomerisation to its corresponding *p*-quinone methides, shifting the alkylating reactivity from the B to the A ring of quercetin.

In the present study the conjugation of quercetin *para*-quinone methide to GSH was investigated in more detail with special emphasis on the possible reversible nature of the adduct formation, and adduct formation with two other known thiol-based scavengers, cysteine and *N*-acetylcysteine. This, in order to investigate in what way thiol scavenging of the reactive quinone methide type metabolites could be further exploited as a means to detect the oxidative behaviour and quinone methide formation of quercetin in more complex biological systems.

## 5.3 Materials and Methods

*Materials.* Quercetin was obtained from Acros Organics (New Jersey, USA). Glutathione, reduced form, L-cysteine and tyrosinase (EC 1.14.19.1) (from mushroom) were purchased from Sigma (St. Louis, MO, USA). *N*-acetylcysteine was obtained from Aldrich (Steinheim, Germany). Hydrogen peroxide, potassium hydrogen phosphate, potassium dihydrogen phosphate, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Deuterium oxide was obtained from ARC Laboratories (Amsterdam, The Netherlands). Acetonitrile and methanol were HPLC grade from Lab - Scan, Analytical Sciences (Dublin, Ireland).

*Synthesis of quercetin quinone methide thiol conjugates.* To a starting solution of glutathione, cysteine or *N*-acetylcysteine (final concentration of 1 mM) in 25 mM

potassium phosphate pH 7.0 was added tyrosinase to a final concentration of 100 units / ml, followed by addition of 150 μM quercetin, added from a 10 mM stock solution in methanol. Upon 8 minutes incubation at 25°C, the incubation mixtures were analysed by HPLC.

*Preference of quercetin quinone methide thiol conjugate formation.* To a starting solution of different combinations of two out of the three different scavenging thiol reagents (1 mM final concentration each) in 25 mM potassium phosphate pH 7.0 was added tyrosinase to a final concentration of 100 units / ml, followed by addition of 150 μM quercetin, added from a 10 mM stock solution in methanol. Upon 8 minutes incubation at 25°C, the incubation mixtures were analysed by HPLC.

*Time dependent stability of the quercetin quinone methide thiol conjugates.* The purified L-cysteinyl or glutathionyl quercetin adducts (final concentration of 1 mM) were incubated in 25 mM potassium phosphate pD 7.0 at 25°C. The reaction was followed by $^1$H NMR.

*Reversibility of quercetin quinone methide thiol conjugate formation.* The purified glutathionyl quercetin adducts or cysteinyl quercetin adducts (final concentration of 1 mM) were added to 1 mM solution of respectively L-cysteine or glutathione in 25 mM potassium phosphate pD 7.0, either or not containing tyrosinase (final concentration of 100 units/ml). At different time points aliquots of 10 μl were analysed by HPLC, or the whole sample was analysed by $^1$H NMR at 25°C, in order to follow the exchange reaction.

*Analytical high performance liquid chromatography.* HPLC was performed with a Waters M600 liquid chromatography system. Analytical separations were achieved using an Alltima C18 5U column (4.6 mm x 150 mm) (Alltech, Breda, The Netherlands). The column was eluted with water containing 0.1 % (v/v) trifluoroacetic acid, in a linear gradient with 0-30 % acetonitrile, in 18 minutes, followed by 2 minutes isocratic elution with 30 % acetonitrile, followed by 30-40 % acetonitrile from 20 to 25 minutes, 40-60 % acetonitrile from 25 to 28 minutes, 60-100 % acetonitrile from 28 to 30 minutes and 100 % acetonitrile from 30 to 35 minutes. A flow rate of 0.7 ml/min and an injection loop of 10 μl were used. Detection was carried out with a Waters 996 photodiode array detector and performed between 200 nm and 450 nm. Chromatograms presented are based on detection at 290 nm. Product peaks were collected and freeze dried for further analysis by $^1$H NMR, MALDI-TOF and LC-MS analysis. Freeze dried samples were dissolved in 25 mM potassium phosphate pD 7.0, made with deuterated water when samples were for $^1$H NMR analysis.

*$^1$H NMR measurements.* $^1$H NMR measurements were performed on a Bruker DPX 400 spectrometer at 7°C. A 1.5 s presaturation delay was used along with a 70° pulse angle and a 2.2 s acquisition time (7575 Hz sweep width, 32 K data points). The data were processed using an exponential multiplication of 0.5 or 1.0 Hz and zero filling to 64 K data points. Resonances are reported relative to HDO at 4.79 ppm.

*Liquid chromatography/mass spectrometry.* LC/MS analysis was performed to further characterize the peaks in the HPLC elution pattern that could not be identified as one of the reference compounds. An injection volume of 10 µL from the incubation mixture or from the purified metabolite dissolved in potassium phosphate (pH 7.0) was used and separation of the products was achieved on a 150 x 4.6 mm Alltima C18 column (Alltech, Breda, The Netherlands). A gradient from 10 % to 30 % acetonitrile in water containing 0.1 % (v/v) trifluoroacetic acid was applied at a flow of 0.7 mL/min in 13 minutes. The percentage of acetonitrile was kept at 30 % for 2 minutes and then increased to 100 % in another 2 min. Mass spectrometric analysis (Finnigan MAT 95, San Jose, CA, USA) was performed in the positive electrospray mode using a spray voltage of 4.5 kV and a capillary temperature of 180°C with nitrogen as sheath and auxillary gas.

## 5.4 Results

*Quercetin quinone methide thiol conjugate formation.* Figures 2a-c show the HPLC chromatograms of quercetin incubated with tyrosinase in the presence of GSH, *N*-acetylcysteine or cysteine. Incubation in the presence of GSH results in the formation of two major adducts, previously identified as 6-glutathionyl and 8-glutathionylquercetin (23). Incubation with *N*-acetylcysteine results in the formation of one major metabolite peak (Fig 2b). The UV spectrum of this major metabolite peak reveals an absorbance peak at 295.1 and disappearance of the absorbance peak at 370.1 nm of quercetin (Table 1) indicating the loss of the conjugation between the A and B ring (28,29). LC/MS analysis of the isolated metabolite peak reveals an M+1 peak at *m/z* 482.0 and also a second peak at *m/z* 464.0. Because the m/z value expected for protonated mono-*N*-acetylcysteinyl quercetin equals 464.0 these MS data point at formation of a mono-hydrated-*N*-acetylcysteinyl adduct of *m/z* 482.0 which readily loses this additional $H_2O$ molecule. The $^1$H NMR spectrum of the product collected from HPLC reveals that it consists of two compounds. Table 2 shows the $^1$H NMR characteristics of these two metabolites. Comparison of the $^1$H NMR data of these metabolites to those of quercetin (23,24,30-33) reveals the loss of the H8 signal as well as the loss of the $^4J_{H8\text{-}H6}$ coupling of 1.9 Hz for the first metabolite and the loss

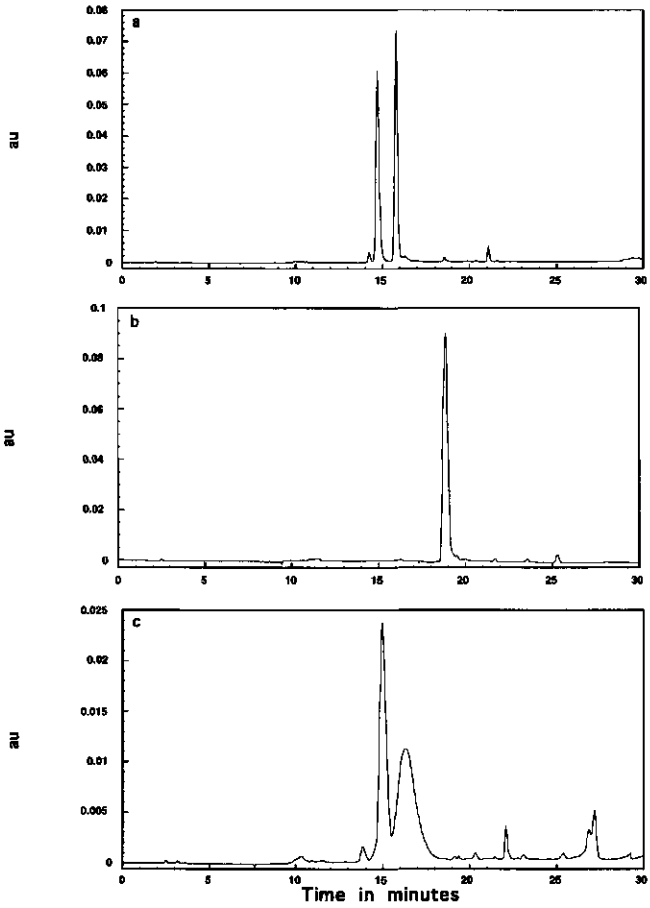


**Figure 2.** HPLC chromatogram of incubations of quercetin with tyrosinase in the presence of (a) glutathione, (b) *N*-acetylcysteine and (c) cysteine.

**Table 1.** The UV absorption maxima for quercetin and its adducts with glutathione, *N*-acetylcysteine and L-cysteine.

| Compound | $\lambda_{max}$ |
|---|---|
| quercetin | 252.6 and 370.1 |
| 6-glutathionyl quercetin | 295.1 |
| 8-glutathionyl quercetin | 299.9 |
| 6- and 8- *N*-acetylcysteinyl quercetin | 295.1 |
| 6-cysteinyl quercetin | 295.1 |
| 8-cysteinyl quercetin | 295.1 |

**Table 2.** $^1$H NMR resonances and coupling constants for quercetin *para*-quinone methide adducts with glutathione, cysteine and *N*-acetylcysteine.

| | 6-glutathionyl | | 8-glutathionyl | | 6-cysteinyl | | 8-cysteinyl | | 6-*N*-acetylcysteinyl | | 8-*N*-acetylcysteinyl | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) | Chemical shift (ppm) | Coupling constant (Hz) |
| H6 | — | — | 6.08 (s) | — | — | — | 5.98 (s) | — | — | — | 6.11 (s) | — |
| H8 | 6.22 (s) | — | — | — | 6.00 (s) | — | — | — | 6.22 (s) | — | — | — |
| H5′ | 6.96 (d) | $^3J_{H5'\text{-}H6'} = 8.5$ | 7.00 (d) | $^3J_{H5'\text{-}H6'} = 8.5$ | 6.77 (d) | $^3J_{H5'\text{-}H6'} = 8.4$ | 6.76 (d) | $^3J_{H5'\text{-}H6'} = 8.4$ | 7.02 (d) | $^3J_{H5'\text{-}H6'} = 8.4$ | 6.99 (d) | $^3J_{H5'\text{-}H6'} = 8.4$ |
| H6′ | 7.38 (dd) | $^3J_{H6'\text{-}H5'} = 8.5$<br>$^4J_{H6'\text{-}H2'} = 2.1$ | 7.39 (dd) | $^3J_{H6'\text{-}H5'} = 8.5$<br>$^4J_{H6'\text{-}H2'} = 2.1$ | 7.16 (dd) | $^3J_{H6'\text{-}H5'} = 8.4$<br>$^4J_{H6'\text{-}H2'} = 2.4$ | 7.10 (dd) | $^3J_{H6'\text{-}H5'} = 8.4$<br>$^4J_{H6'\text{-}H2'} = 2.4$ | 7.42 (dd) | $^3J_{H6'\text{-}H5'} = 8.4$<br>$^4J_{H6'\text{-}H2'} = 2.4$ | 7.40 (dd) | $^3J_{H6'\text{-}H5'} = 8.4$<br>$^4J_{H6'\text{-}H2'} = 2.4$ |
| H2′ | 7.46 (d) | $^4J_{H2'\text{-}H6'} = 2.1$ | 7.48 (d) | $^4J_{H2'\text{-}H6'} = 2.1$ | 7.28 (d) | $^4J_{H2'\text{-}H6'} = 2.4$ | 7.26 (d) | $^4J_{H2'\text{-}H6'} = 2.4$ | 7.50 (d) | $^4J_{H2'\text{-}H6'} = 2.4$ | 7.49 (d) | $^4J_{H2'\text{-}H6'} = 2.4$ |
| Glu Hβ | 2.04 (m) | | 2.00 (m) | | — | | — | | — | | — | |
| Glu Hγ | 2.37 (m) | | 2.28 (m) | | — | | — | | — | | — | |
| Cys Hβ1 | 2.59 (dd) | | 2.69 (dd) | | 2.91 (dd) | | 3.07 (dd) | | 2.66 (dd) | | 2.71 (dd) | |
| Cys Hβ2 | 2.96 (dd) | | 2.81 (dd) | | 3.29 (t) | | 3.77 (dd) | | 2.80 (dd) | | 2.89 (dd) | |
| Gly Hα1 | 3.58 (d) | | 3.57 (d) | | — | | — | | — | | — | |
| Gly Hα2 | 3.67 (d) | | 3.68 (d) | | — | | — | | — | | — | |
| Glu-Hα | 3.72 (t) | | 3.71 (m) | | — | | — | | — | | — | |
| Cys Hα | 4.07 (dd) | | 4.26 (dd) | | 4.05 (dd) | | 3.86 (m) | | 4.18 (dd) | | 4.01 (dd) | |
| *N*-$CH_3$ | | | | | | | | | 2.10 (s) | | 2.26 (s) | |

of the H6 signal as well as the loss of the $^4J_{H6-H8}$ coupling of 1.9 Hz for the second metabolite. In addition to the aromatic $^1H$ resonances the $^1H$ NMR spectra of both adducts show the $^1H$ NMR resonances of the *N*-acetylcysteinyl side chain (Table 2). On the basis of these $^1H$ NMR characteristics UV and LC/MS data these two metabolites can be identified as 8-*N*-acetylcysteinyl and 6-*N*-acetylcysteinyl quercetin with transient/reversible $H_2O$ addition to ring C.

Incubation of quercetin with tyrosinase in the presence of cysteine also results in formation of two major metabolites (Figure 2c). The UV spectra of these adducts reveal an absorbance peak at 295.1 nm for both metabolites and disappearance of the absorbance peak at 370.1 nm of quercetin (Table 1) indicating the loss of the conjugation between the A and B ring (*28,29*). LC/MS analysis of the two purified metabolites from HPLC with retention times at 15.0 min and 16.3 min reveals an M+1 peak at *m/z* 440.0 and also a second peak at *m/z* 422.0 for both metabolites. Because the *m/z* value expected for protonated mono-cysteinyl quercetin equals 422.0 the observation of a peak at *m/z* 440.0 for both metabolites points at formation of mono-hydrated cysteinyl adducts which readily loose this additional $H_2O$ molecule upon mass analysis.

Table 2 shows the $^1H$ NMR characteristics of these two major metabolites. Comparison of the $^1H$ NMR data of these metabolites to those of quercetin (23,24,30-33) reveals the loss of the H8 signal as well as the loss of the $^4J_{H8-H6}$ coupling of 1.9 Hz for the first metabolite and the loss of the H6 signal as well as the loss of the $^4J_{H6-H8}$ coupling of 1.9 Hz for the second metabolite. In addition to the aromatic $^1H$ resonances the $^1H$ NMR spectra of both adducts show the $^1H$ NMR resonances of the cysteinyl side chain (Figure 3, Table 2). On the basis of these $^1H$ NMR characteristics UV and LC/MS data these two metabolites can be identified as 8-cysteinyl and 6-cysteinylquercetin with transient/reversible $H_2O$ addition to ring C.

In MALDI-TOF analysis, the two glutathionyl quercetin adducts reveal formation of an M+1 peak at *m/z* of 608.0 for both metabolites which is identical to the mass expected for protonated mono-glutathionyl quercetin adduct (23). However, the appearance of an additional M+19 peak in LC/MS analysis of both *N*-acetylcysteinyl and cysteinyl quercetin adducts and not in the MALDI-TOF analysis of glutathionyl quercetin adducts which was performed in our previous work (23) led us to perform an additional mass analysis under the less aggresive LC/MS conditions for the glutathionyl quercetin metabolites. The LC/MS data obtained indeed reveal an M+1 peak with *m/z* 626.0 in addition to the *m/z* 608.0 peak for both metabolites. This points at additional incorporation of a water molecule in these C6 and C8 glutathionyl quercetin adducts which is readily lost upon mass analysis. Figure 4 represents the possible structural formulas for the thiol adducts of quercetin quinone/quinone

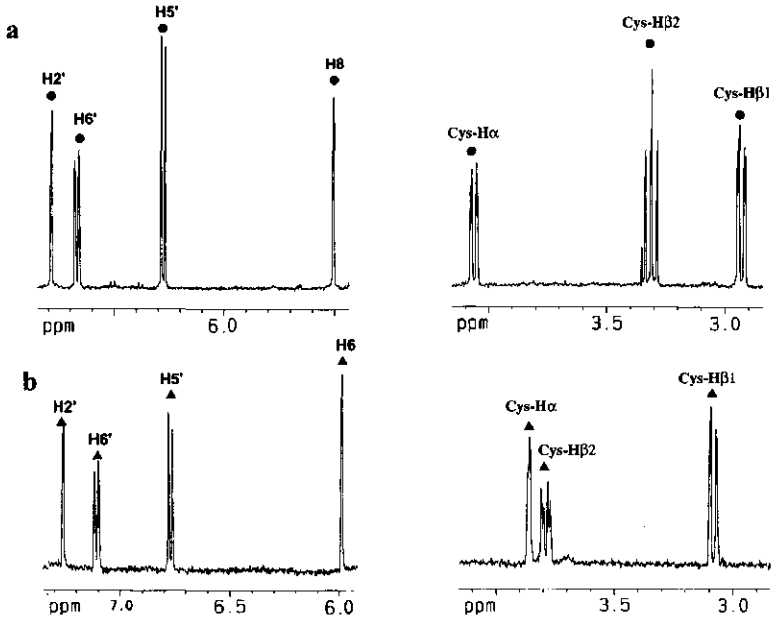


**Figure 3.** Aromatic and aliphatic parts of the $^{1}$H NMR spectra for a) 6-cysteinyl quercetin and b) 8-cysteinyl quercetin revealing the retention of all the cysteinyl aliphatic protons. The resonances ascribed to the 6-cysteinyl quercetin adduct are marked with ●, and those ascribed to the 8-cysteinyl quercetin with ▲.

methide, as well as the reversible addition/loss of the water molecule adduct (34-37). Based on the fact that all B ring aromatic proton resonances and in all cases one A ring aromatic proton resonance are present in the $^{1}$H NMR spectra of the various thiol-quercetin adducts this water addition must be in the C ring as presented in Figure 4. This water addition to the C2=C3 double bond is in line with literature data that report the HRP/$H_2O_2$ or Cu(II)/ROH or sodium periodate/methanol catalysed water addition to the C2=C3 double bond in the flavonoid quercetin. Apparently tyrosinase is capable of catalysing the same reaction.

*Preference of quercetin quinone methide thiol conjugate formation.* In order to establish the preference for conjugate formation with the thiol model compounds, incubations were performed in the presence of 1 mM of combinations of the different scavenging thiol reagents. Table 3 quantifies the relative amounts of the adducts

formed as determined by $^1$H NMR, showing preferential formation of especially the cysteine conjugates.

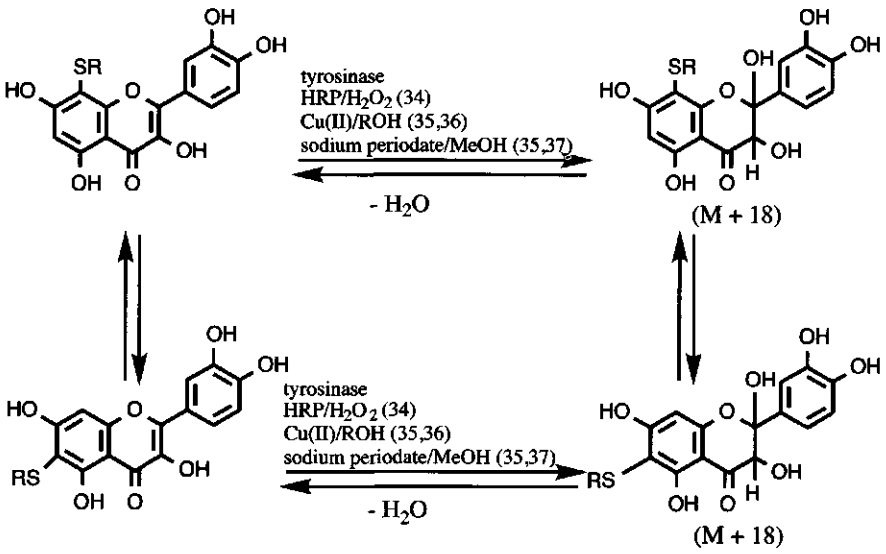


**Figure 4.** Structural formulas for the water-thiol scavenging adducts with quercetin and their reversible water addition equilibria. RS = glutathionyl or *N*-acetylcysteinyl or cysteinyl. The munbers between brackets at the arrows refer to literature references describing the water addition to the C ring of the flavonoid quercetin.

**Table 3.** Ratio of adduct formation in incubations of quercetin with tyrosinase in the presence of equimolar concentrations of different thiol scavenger molecules as determined by $^1$H NMR GSH = glutathione, CYS = cysteine, and NAC = *N*-acetylcysteine.

| Incubation with | ratio of adduct formation | |
|---|---|---|
| GSH + NAC | GSH : NAC | 1.00 : 0.29 |
| GSH + CYS | GSH : CYS | 0.00 : 1.00 |
| NAC + CYS | NAC : CYS | 0.00 : 1.00 |

*Stability of the quercetin quinone methide thiol conjugates.* Incubation of 1 mM of the purified 6-glutathionyl conjugate in time in potassium phosphate buffer pD 7.0 at 25°C was followed by $^1$H NMR. Figure 5a presents the results obtained. In time, a decrease in the relative amount of the 6-glutathionyl adduct is observed, accompanied by formation of a small amount of the free glutathione (amount not shown) (24, figure 3) and an increasing amount (about 30% of total intensity) of the 8-glutathionyl adduct. Loss of some intensity (about 30 after 24 hours) was also observed. These results indicate that in time the C6 adduct isomerizes to give a 55:45% mixture of the C6- and C8-adducts (Fig 5a). When the same experiment was repeated starting with the 8-glutathionyl adduct, formation of a similar isomeric mixture was observed (Fig 5b). These data corroborate but also quantify previous observations on the reversible nature of the glutathionyl quercetin adducts, and reveal the unstable nature of the glutathionyl quercetin adducts.

Similar incubations of the purified cysteine quercetin conjugates (1 mM) were performed and analysed by $^1$H NMR (Figure 5c and d). In contrast to what was observed for the glutathione quercetin adducts the 6-cysteinyl and 8-cysteinyl adduct do not show isomerisation but, instead, the conjugates slowly disappear from the medium (Figure 5c and d). Upon 24 hours incubation of 6-cysteinyl quercetin a loss of about 50% of the intensity was observed (Figure 5c). The 8-cysteinyl adduct disappeared from the incubation at even higher rate showing a 50% reduction already after about 5 hours of incubation (Figure 5d).

*Reversibility of conjugate formation.* Based on the reversible nature of the GSH and not of the cysteine adducts and on the preference for cysteine over GSH conjugation the purified glutathione conjugates of quercetin were incubated with 1 mM cysteine in order to further investigate the possibility for cysteine quenching of the unstable GSH adducts. Figure 6 presents the results from time-dependent $^1$H NMR analysis of this incubation, showing the time dependent conversion of the 6-glutathionyl conjugate first into both the 6- and 8-cysteinyl adduct, finally resulting in an incubation containing the 6-cysteinyl adduct only. When the experiment was repeated with the 8-glutathionyl adduct this also ultimately resulted in an incubation containing only the 6-cysteinyl quercetin ($^1$H NMR spectra not shown). Loss of some intensity due to side reactions is also observed reflected by decreased signal to noise and formation of unidentified metabolites with broad $^1$H NMR resonances reflecting polymerisation products of increased molecular mass (Figures 6c+d). The results obtained corroborate the reversible nature of the GSH adduct formation, providing possibilities for release of the glutathione and an alkylating *para*-quinone methide capable of interacting with other cellular macromolecules but in the present incubations preferentially scavenged by cysteine.

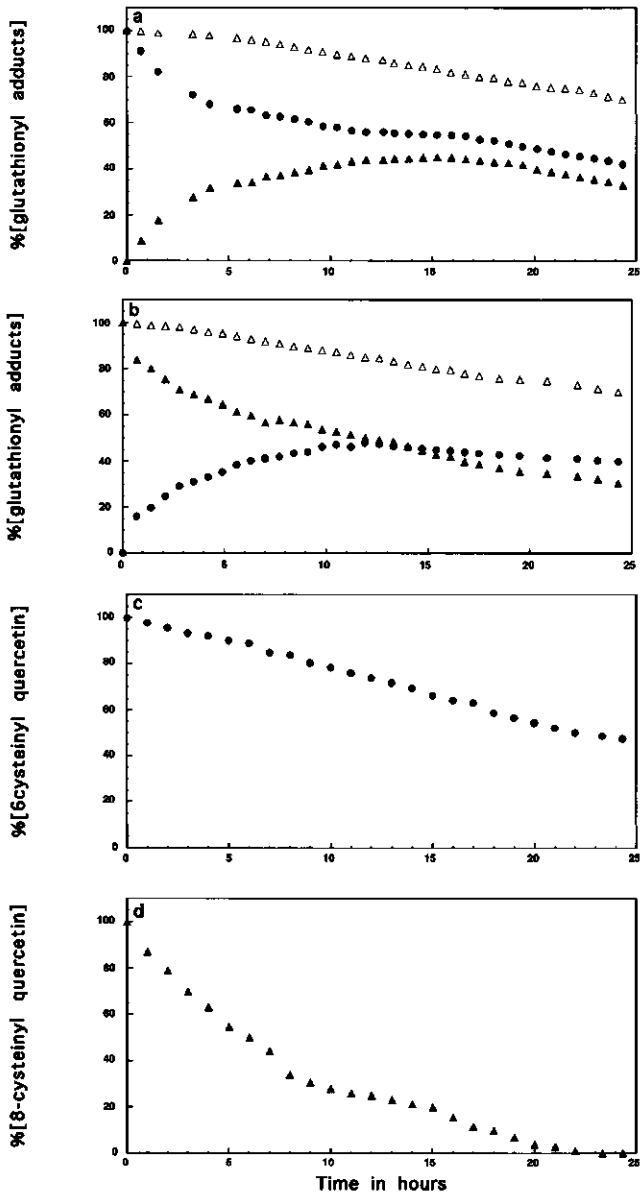


**Figure 5.** Time-dependent incubation of (a) 1 mM of 6-glutathionyl quercetin, (b) 1 mM of 8-glutathionyl quercetin, (c) 1 mM of 6-cysteinyl quercetin and (d) 1 mM 8-cysteinyl quercetin in potassium phosphate pD 7.0 at 25°C. The % of the respective 6-thiol quercetin adducts are presented with ●, and those of the 8-thiol quercetin adducts with ▲. The decrease in the total intensity of the sum of all glutathionyl adducts are presented with Δ.

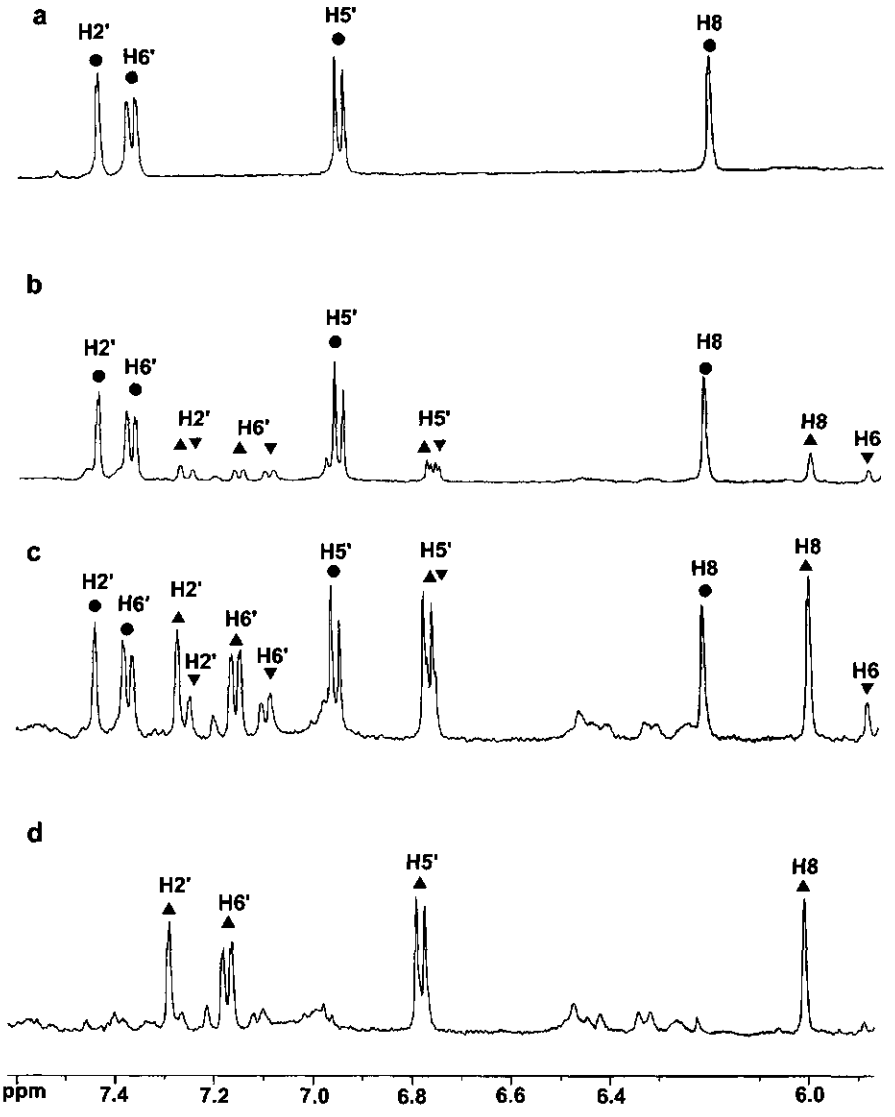


**Figure 6.** Time-dependent $^{1}$H NMR spectra (aromatic region) of the incubation of 1 mM of 6-glutathionyl quercetin with 1 mM of cysteine in potassium phosphate pD 7.0 at 25°C, showing the reversible nature of the adduct formation after (a) 0 min (b) 100 min (c) 180 min and (d) 300 min. The resonances ascribed to the 6-glutathionyl quercetin adduct are marked with ●, and those ascribed to the 6- and 8-cysteinyl quercetin with ▲ and ▼ respectively.

When the reverse experiment was performed, i.e. incubating the purified cysteinyl quercetin adducts with glutathione, no exchange of the thiol moiety was observed. Rather only loss of signal intensity was observed in time (data not shown).

*Influence of thiol concentration.* Finally, because physiological concentrations of GSH are expected to be higher than those of cysteine it was investigated whether the preference for cysteine over GSH would still be observed at relatively higher GSH than cysteine concentrations. Thus, incubations were performed in the presence of combinations of GSH and cysteine (ratio = 10 : 1 and 100 : 1). The results reveal that already at 10 fold excess of GSH over cysteine, the glutathionyl quercetin adducts already dominate the adduct formation completely, eliminates the preferential cysteine over glutathione adduct formation observed at equimolar concentration of the two thiol scavengers.

## 5.5 Discussion

The conjugation of GSH with a variety of chemicals and/or reactive metabolites usually results in the detoxication of the reactive electrophiles and facilitates their excretion into urine as the corresponding mercapturic acids. Recently, however, several examples have been reported where conjugation of quinones with glutathione fails to eliminate their biological or toxicological reactivity (38,39). This can be ascribed to various mechanisms. First of all, quinone thioethers may maintain the ability to redox cycle with the concomitant generation of reactive oxygen species (38,40,41). In addition, quinone thioethers have been shown to be substrates for and inhibitors of a variety of enzymes that utilise either quinones and/or GSH as substrates and cosubstrates (38,42-48). Also it has been reported that the reaction of thiols (GSH and cysteine) with isothiocyanates was readily reversible. As a result, GSH and cysteine can be regarded as transporting agents for the isothiocyanates through the body, and initial detoxication can ultimately result in release of the reactive compound at some other site (25). The results of the present study clearly reveal that this reversible character also holds for the conjugation of quercetin *para*-quinone methide to thiol-based scavengers. Clearly this reversible and reactive nature of the glytathionyl and *N*-acetylcysteinyl quercetin conjugates can be expected to hamper their detection upon formation in complex biological systems (49).

Compared to the glutathionyl and *N*-acetylcysteinyl quercetin adducts the cysteinyl quercetin adducts were shown to be of irreversible nature. This results in preferential cysteine over GSH and *N*-acetylcysteine conjugation of quercetin quinone methide at equimolar concentrations of the different thiol scavengers. The preferential formation of cysteinyl over glutathionyl adducts can not be ascribed to the difference in the concentration of the deprotonated cysteine as compared to the deprotonated GSH since their respective pKa values vary by only 0.33 unites (cysteine $pK_a$ = 8.33

and GSH $pK_a$ = 8.66). The resistance of the cysteinyl adducts to isomerisation can rather be explained by the hypothesis that in case of the cysteinyl conjugates of quercetin, the proton of the $NH_2$ group of the cysteinyl residue is hydrogen-bonded (20,50) to one of the oxygen atoms in the molecule (structures II and III, Figure 7). This would result in decreased chances on deconjugation of the cysteine adducts as compared to adducts with GSH, or *N*-acetylcysteine. Based on this hypothesis it can be anticipated that formation of cysteinyl adducts will also be favored over protein-thiol adduct formation. However, the splitting patterns and chemical shift values for the protons of the cysteinyl moiety in the cysteinyl quercetin adducts differ significantly from those of GSH, the GSH adducts or cysteine itself (Table 2 and Figure 3) (23,24,30). This may point at a structure significantly differ from the adduct itself. As an alternative to intramolecular hydrogen bond formation, it could be suggested that the reaction of cysteine with quercetin quinone/quinone methide involves an intramolecular cyclization and 1,4-benzothiazine formation via an intramolecular 1,4 Michael addition, resulting in structures IV or V followed by the tyrosinase catalysed water addition to the C ring (Figure 7) (27,34,38). This would be a mechanism similar to what has been reported for other quinones, shifting the equilibrium of (de)conjugation in favour of the conjugated form, thereby stabilizing the adduct (27,38). Since such a cyclization requires a free $NH_2$ moiety it would explain why it is observed for the cysteinyl but not for the glutathionyl or *N*-acetylcysteinyl quercetin adducts. This intramolecular cyclisation would also provide an explaination for the observation that formation of the cysteinyl quercetin adducts is irreversible in cintrast to glutathionyl or *N*-acetylcysteinyl quercetin adducts. The nature of the cyclic cysteine adducts could be elucidated as follows. The $^1$H NMR spectra of the cysteinyl quercetin adducts reveal the retention of all the cysteinyl residue aliphatic protons (i.e., Cβ1, Cβ2 and Cα) in both adducts. Especially the presence of the cysteinyl Cα proton in the adducts eliminates the 1,4-benzothiazine IV as a possibility for the final structure. The LC/MS analysis of the two purified metabolites also exclude structure IV as the possible cyclic metabolite. This because the LC/MS data reveal an M+1 peak for both metabolites at *m/z* 440.0 and a second M+1 peak for both metabolites at *m/z* 422.0. Since strucure IV would have M+1 peaks of m/z 402.0 and 420.0 for the water adduct, respectively, the LC/MS data exclude structure IV as the metabolite formed. Alternatively, isomerisation of the initial product form ring closure to intermediate V followed by oxidation and water addition would result in a cyclic product VI which has *m/z* 440.0. Formation of product VI as well as intermediate V may explain the unstable nature of the cysteinyl adducts compaired to the GSH adducts (Figure 5) because similar structures were reported before to be highly unstable (35,37). Based on the unstable nature and the marked changes in the $^1$H NMR of the cysteinyl adducts formation of the cyclic adduct VI seems to be favoured over the explanation suggesting an adduct with a strong intramolecular hydrogen bond (II). Ultimate proof for this choice has to come from

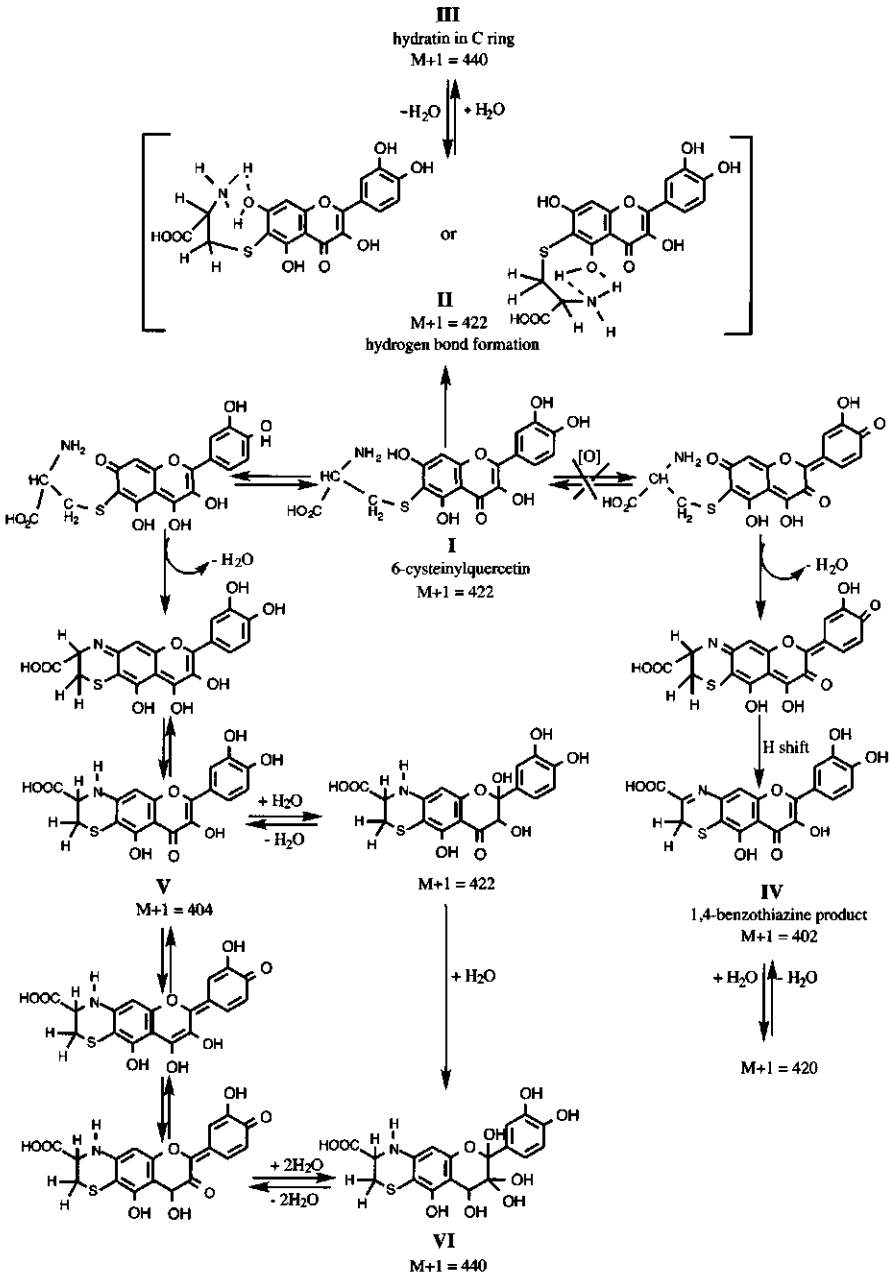


**Figure 7.** Schematic presentation of the possible reaction pathways and the possible cysteinyl quercetin adduct structures in line with the $^{1}$H NMR characteristics and LC/MS data. For details see discussion.

$^{13}C$ NMR analysis which appeared unfortunately to be hampered by the unstable nature of the adducts.

Finally, the preferential scavenging by cysteine over glutathione reported in the present study can not be expected to occur in biological systems, in which detection of the glutathionyl flavonoid conjugates may be hampered by their reversible nature. This, because physiological concentrations of GSH are higher than those of cysteine which was shown to shift the balance again in favor of glutathionyl adduct formation. And also because the cysteinyl quercetin adducts, although not showing the reversible nature of the glutathionyl conjugates, appeared nevertheless to be equally unstable. Thus, as a biomarker for formation in biological *in vitro* and *in vivo* systems detection of the glutathionyl or *N*-acetylcysteinyl conjugates should be the method of choice.

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# 6

# Identification of *o*-quinone/quinone methide metabolites of quercetin in a cellular in vitro system

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## 6.1 Abstract

The formation of the quinone/quinone methide metabolites of quercetin, reflected by the formation of the glutathionyl quercetin adducts as authentic metabolites, was investigated in an *in vitro* cell model (B16F-10 melanoma cells) using the methods previously developed for detection and identification of the glutathionyl adducts. Results of the present study clearly indicate the formation of glutathionyl quercetin adducts in a biologically relevant model. The data obtained also support that the adducts are formed intracellular and subsequently excreted into the incubation medium. Thus, the results of the present study, reveal, for the first time, evidence for the pro-oxidative metabolism of quercetin in a cellular *in vitro* model.

## 6.2 Introduction

Quinone and quinone methides from a variety of compounds, including 3′,4′-dihydroxyflavonoids, catechol-type metabolites from estrogens and polycyclic aromatic hydrocarbons, and compounds like the anticancer drug tamoxifen, have been classified as likely candidates for reactive metabolites able to react with cellular macromolecules (*1-3*). For 3′,4′-dihydroxyflavonoids, with an intrinsic catechol moiety, their pro-oxidative quinone/quinone methide chemistry is especially of importance because of their increasing use as functional food ingredients and food supplements (*4-7*). Recently the glutathione (GSH) trapping method (*8-10*) appeared an excellent method to investigate the quinone/quinone methide chemistry of the

flavonoid quercetin (*11,12*), known to be mutagenic in a variety of bacterial and mammalian mutagenicity tests presumably through its quinone methide like metabolites (*13-15)*

In previous studies we identified the nature of the GSH conjugates of several flavonoid quinones (11,12,16,17). This opens the way for studies investigating the formation of these GSH conjugates and their corresponding mercapturic acids in cellular *in vitro* and *in vivo* systems. The actual formation of these glutathionyl flavonoid-quinone adducts and of their corresponding mercapturic acids would represent an *in vivo* bioactivation pathway of these supposed beneficial functional food ingredients. The formation of quinone-derived mercapturic acids of flavonoids would be comparable to the formation of the glutathionyl and N-acetylcysteine conjugates of estrogens (18). Recently, estrogen-induced *o*-quinones have been highlighted as active intermediates in the development of cancer (1,10,18-22), since the presence of *o*-quinone-DNA complexes has been confirmed (1,20,21). It has been demonstrated that the *o*-quinones of estrogens react with glutathione, producing N-acetylcysteine conjugates in an *in vitro* system (23,24). In addition, it has been suggested that the urinary levels of mercapturic acids can be used as a biomarker for exposure to active nucleophilic compounds pointing at possible risks for quinone-induced tumorigenesis (25-27). However, the actual detection of these conjugates in for example urine requires sensitive detection methods and knowledge on the stability, nature and chemical behavior of the adducts. The results of our previous studies indicate that the flavonoid glutathione adducts, like other GSH adducts have a reversible nature resulting in a limited stability (12). This implies that their detection in body fluid is generally considered difficult due to 1) the relatively low levels of formation of these reactive metabolites and 2) their high reactivity and limited stability (18). However, because the formation of these adducts would represent an important bioactivation pathway of supposed beneficial functional food ingredients, the detection of these GSH adducts as authentic metabolites in cellular *in vitro* or *in vivo* models is certainly of interest. As a first step in the search for the biological relevance of quinone methide-type pro-oxidant chemistry of flavonoids the objective of the present study was to investigate the possible formation of especially quercetin-glutathione adducts as authentic metabolites in an *in vitro* cell model using the methods now developed for detection and identification of the GSH adducts. The *in vitro* system used consists of mouse melanoma cancer cells (B16F-10). These cells were chosen because they have been reported to contain significant levels of tyrosinase (28,29). Tyrosinase was shown before to catalyse flavonoid metabolism to quinone/quinone methide-type metabolites leading to glutathionyl-flavonoid adducts. Thus the B16F-10 melanoma cell line was considered an excellent model system to investigate the possible formation of quercetin-glutathionyl adducts in cells exposed to quercetin.

## 6.3 Materials and Methods

*Materials.* Quercetin was obtained from Acros Organics (New Jersey, USA). Glutathione, reduced form, and tyrosinase (EC 1.14.19.1) (from mushroom) were purchased from Sigma (St. Louis, MO, USA). All substrates were of 98-99 % purity. Potassium hydrogen phosphate, potassium dihydrogen phosphate, citric acid, tri-sodium citrate dihydrate, anhydrous sodium carbonate, sodium hydrogen carbonate, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Acetonitrile and methanol were HPLC grade from Lab-Scan, Analytical Sciences (Dublin, Ireland).

*Synthesis of quercetin quinone methide glutathione conjugates.* To a starting solution of glutathione (final concentration of 1 mM) in 25 mM potassium phosphate pH 7.6 was added tyrosinase to a final concentration of 100 units/ml, followed by addition of 150 μM quercetin, added from a 10 mM stock solution in methanol. Upon 8 minutes incubation at 37°C, the incubation mixture was analysed by HPLC.

*Quercetin exposure and glutathionyl adduct formation.* Mouse melanoma cancer cells (B16F-10) were obtained from ATCC (Manassas, VA). B16F-10 melanoma cells were cultured in Dulbecco's MEM (obtained from Gibco BRL) with 25 mM Hepes, 4500 mg/l glucose, pyridoxine and without sodium pyruvate, supplemented with 10% Fetal Calf Serum and 50 mg/l gentamicin (obtained from Gibco BRL), at 37°C in a humid atmosphere containing 5% $CO_2$. For each experiment, approximately $20*10^4$/mL cells were plated onto a 24-wells tissue cluster (Costar, Cambridge MA, USA) and cultured until a semiconfluent monolayer was obtained (in about 2 days). Cells were exposed to quercetin in quadruplicate for one hour, 6 hours, and 24 hours at 37°C in (a) DMEM medium described above or (b) Hanks balanced salt solution (HBSS without phenol red and $NaHCO_3$, obtained from Gibco) to which $NaHCO_3$ was added to a final concentration of 0.35 g/L. The final volume of the medium was 0.5 mL. The concentration range of quercetin used throughout the experiments was 10, 25, 50, 75 and100 μM. Quercetin was always added from a freshly prepared 200 times concentrated stock solution in DMSO. Exposure to quercetin was performed either in the absence or presence of vitamine C (final concentration of 1 mM) as indicated. Controlled incubations were included containing only the solvent, DMSO, at a final concentration of 0.5% in medium.

*Analytical high performance liquid chromatography.* HPLC was performed with a Waters M600 liquid chromatography system. Analytical separations were achieved using an Alltima C18 column (4.6 x 150 mm) (Alltech, Breda, The Netherlands). The column was eluted at 0.7 mL/min with water containing 0.1 % (v/v) trifluoroacetic acid. A linear gradient from 10 % to 30 % acetonitrile in 12 minutes was applied, followed by 2 minutes isocratic elution with 30 % acetonitrile. Hereafter a linear

gradient from 30 % to 100 % acetonitrile was used in 2 minutes. The percentage of acetonitrile was kept at 100 % for another 3 minutes. An injection loop of 10 µL was used. Detection was carried out with a Waters 996 photodiode array detector measuring spectra between 200 nm and 450 nm. Chromatograms presented are based on detection at 290 nm.

*Liquid chromatography/mass spectrometry.* LC/MS analysis was performed to further characterize the peaks in the HPLC elution pattern. An injection volume of 10 µL from the incubation mixture was used and separation of the products was achieved on a 4.6 x 150 mm Alltima C18 column (Alltech, Breda, The Netherlands). A gradient from 10 % to 30 % acetonitrile in water containing 0.1 % (v/v) trifluoroacetic acid was applied at a flow of 0.7 mL/min in 13 minutes. The percentage of acetonitrile was kept at 30 % for 2 minutes and then increased to 100 % in another 2 min. Mass spectrometric analysis (Finnigan MAT 95, San Jose, CA, USA) was performed in the positive electrospray mode using a spray voltage of 4.5 kV and a capillary temperature of 180 °C with nitrogen as sheath and auxillary gas.

## 6.4 Results

*Incubation of quercetin with tyrosinase in the presence of glutathione.* Figures 1a and b show the HPLC chromatograms of the incubation of quercetin with tyrosinase in the presence of GSH injected either undiluted or 20 times diluted. Formation of two major adducts is observed which were previously identified as 6-glutathionyl- and 8-glutathionyl-quercetin hydrated in their C ring due to tyrosinase catalysed water addition to the C2 and C3 in C ring (11,12,17). Figures 2a and b show the LC/MS for both metabolites which reveal the formation of an M+1 peak at m/z 626.0 for both metabolites and these LC/MS data, together with $^{1}$H NMR characteristics described previously (11,12) identify the nature of the metabolites as indicated in Figure 1. The HPLC patterns presented in Figure 1 are different from those shown in previous studies (11,12) and reveal peaks which appear to be broader than in previous patterns because of using different gradients more suitable for detection of the glutathionyl quercetin adducts in cell line medium which contains several additional compounds in the present study. Also, upon comparison of figure 1a to 1b it can be observed that the HPLC peaks of the GSH adducts broader to even further extent upon a decrease in the concentration of the adducts. This is of importance because the concentration of the glutathionyl adducts in the cellular incubation can be expected to be low.

*Quercetin quinone methide glutathione conjugate formation in a cellular in vitro model.* Figures 3a, b, and c show the HPLC chromatograms of DMEM medium of mouse B16F-10 melanoma cells exposed to 75 µM quercetin for 0 hour, 1 hour, and 6

hours, respectively. Comparison of Figure 3b and c to Figure 3a reveals the occurrence of two broad metabolite peaks with retention times of 9.1 and 11.4 min, which represent exactly the same retention times as those of 8- and 6-glutathionylquercetin under the HPLC conditions applied (Figure 1). The UV spectra of these two metabolites reveal an absorbance peak at 299.9 and 295.1 nm, respectively, and disappearance of the absorbance peak at 370.1 nm of quercetin indicating the loss of the conjugation between the A ring and B ring (30,31). LC/MS analysis This implies that retention times, UV spectra, and mass characteristics of these two metabolites are identical to those of 8- and 6-glutathionylquercetin. Comparison of Figure 3b to Figure 3c reveals that the intensities of the two metabolite peaks decrease in time. After 24 hours (HPLC chromatogram not shown), the presence of these two metabolites as well as of the unreacted quercetin were no longer observed indicating the instability of the metabolites and the full conversion of quercetin under the *in vitro* conditions used.

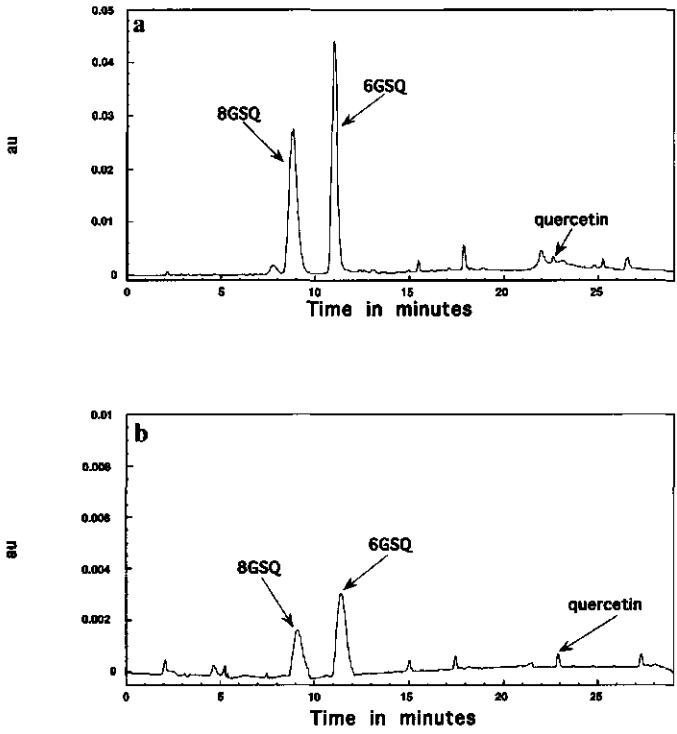


**Figure 1.** HPLC chromatogram of the incubation of a) quercetin with tyrosinase in the presence of glutathione b) the same incubation diluted 20 times in buffer revealing the formation of 6-glutathionyl quercetin (6-GSQ) and 8-glutathionyl quercetin (8-GSQ).

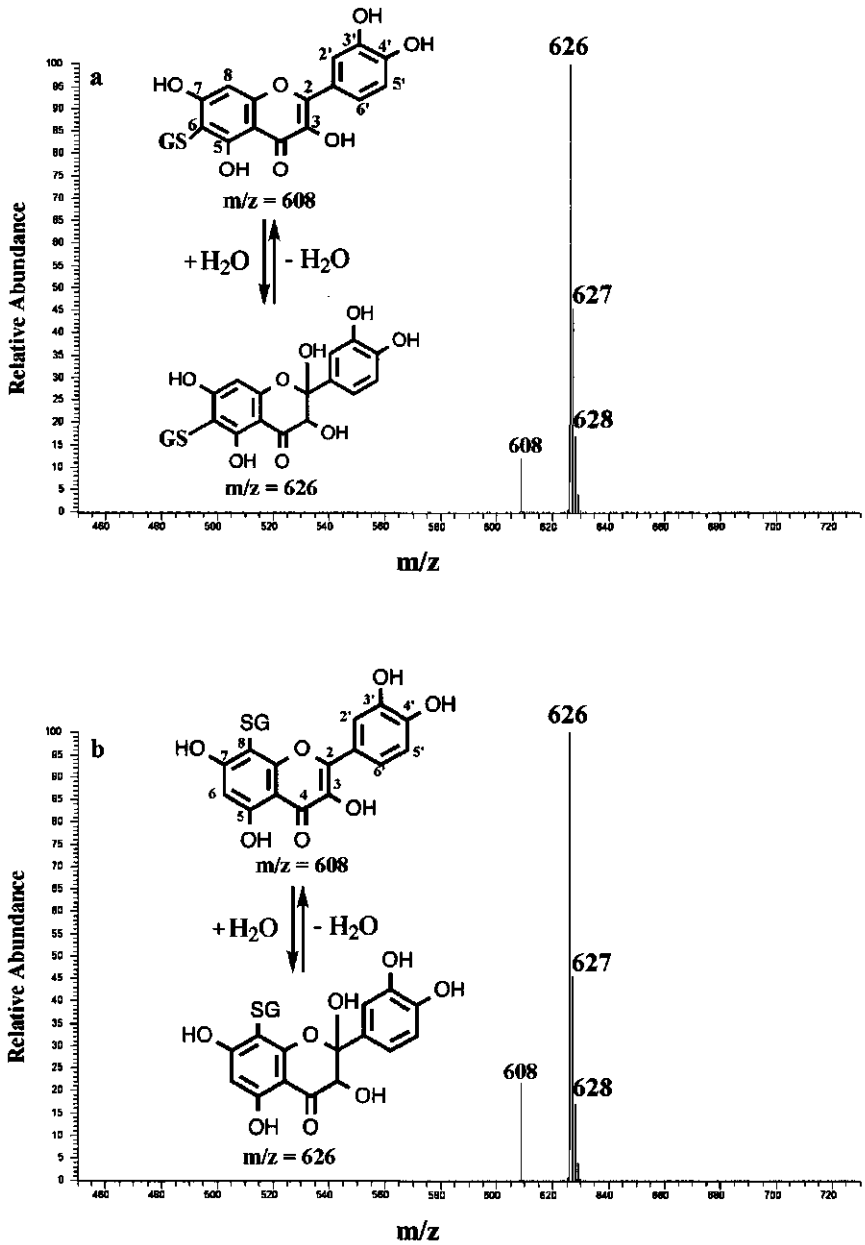


**Figure 2.** Mass spectra of a) 6-glutathionyl quercetin and b) 8-glutathionyl quercetin revealing also the tyrosinase catalysed $H_2O$ addition to the glutathionyl quercetin adducts, which is in line with literature data (17,32).

The intracellularly formed GSH conjugates have been rapidly excreted into the medium since at the time of 1 hour when glutathionyl quercetin concentrations were highest in the medium, no GSH conjugates could be detected by HPLC in the corresponding cellular samples (chromatogram not shown).

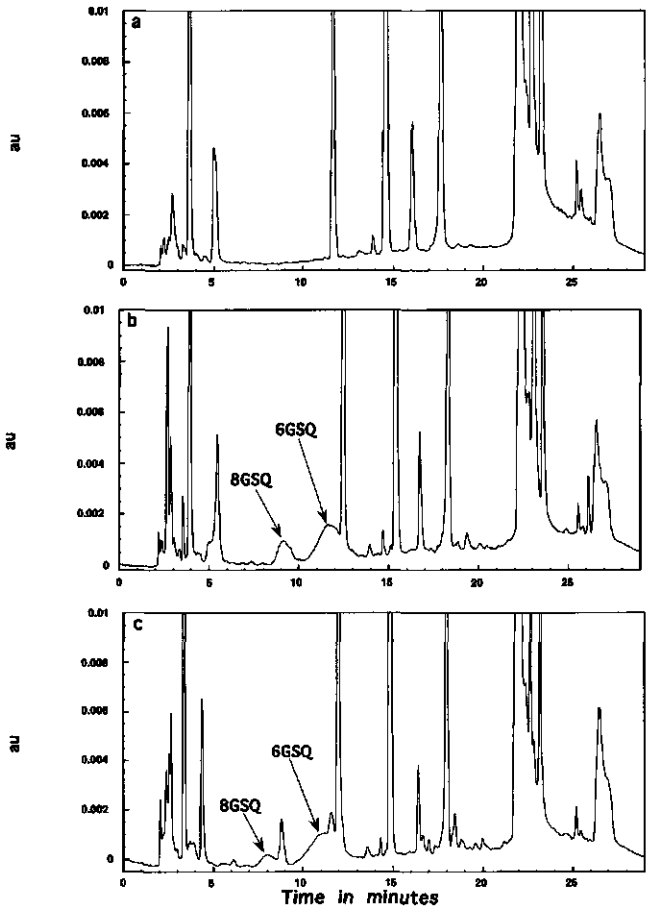


**Figure 3.** HPLC chromatograms of DMEM medium of mouse B16F-10 melanoma cells exposed to 75 μM quercetin for a) 0 hour, b) 1 hour, and c) 6 hours.

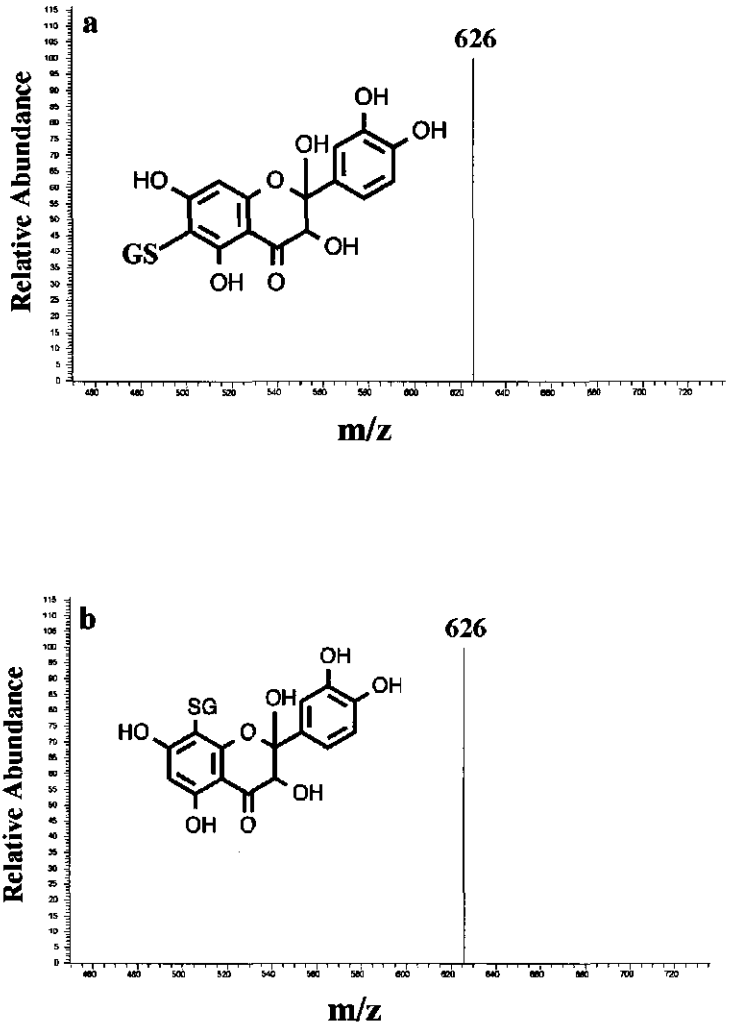


**Figure 4**. Mass spectra of the two metabolites, a) 6-GSQ and b) 8-GSQ,detected in the DMEM medium of mouse B16F-10 melanoma cells during the exposure of 75 μM quercetin for 1 hour.

*Quercetin glutathione conjugation in the presence of vitamin C.* Figure 5 presents the HPLC chromatogram pattern of medium of B16F-10 melanoma cells exposed to 75 μM quercetin for 1 hour in the presence of vitamin C ( final concentration of 1 mM). This experiment was performed to exclude the possible formation of the quercetin quinone/quinone methide and thus the 6- and 8-glutathionylquercetin adducts due to chemical auto-oxidation of quercetin in the incubation medium. Comparison of the results presented in Figure 3b to those in Figure 5 reveal that both in the absence and presence of ascorbate formation of the 6- and 8-glutathionyl quercetin adducts was observed to a similar extent. This supports that the oxidation and glutathione conjugation is not due to an extracellular auto-oxidation process and can be ascribed to intracellular oxidation of quercetin by the tyrosinase present in the melanoma cells. Also the fact that the extracellular medium does not contain free GSH further supports that the 6- and 8-glutathionyl quercetin formation occurs intra- not extracellular pointing at a true metabolic activation pathway.

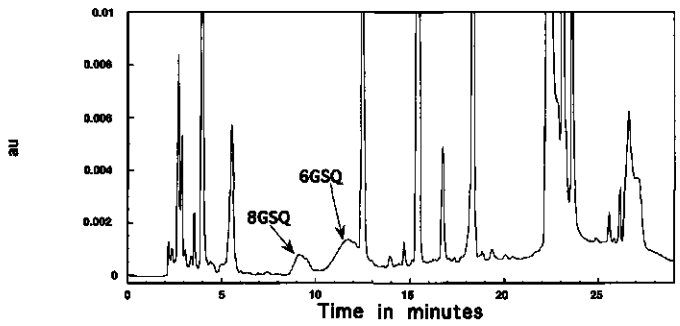


**Figure 5.** HPLC chromatogram of DMEM of mouse B16F-10 melanoma cells exposed to 75 μM quercetin for 1 hour in the presence of vitamin C (final concentration of 1 mM).

## 6.5 Discussion

Quinones represent a class of toxicological intermediates which can create a variety of hazardous effects *in vivo*, including acute cytotoxicity, immunotoxicity and carcinogenesis. The mechanisms by which quinones cause these effects can be quite complex. Quinones are Michael acceptors, and cellular damage can occur through alkylation of crucial cellular proteins and/or DNA. Alternatively, quinones are highly redox active molecules which can redox cycle with their semiquinone radicals,

leading to formation of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and ultimately the hydroxyl radical. Production of ROS can cause severe oxidative stress within cells resulting in the oxidation of cellular macromolecules like lipids, proteins and DNA. Formation of oxidatively damaged bases such as 8-oxodeoxyguanosine has been associated with aging and carcinogenesis (*33,34*). Furthermore, ROS can activate a number of signaling pathways, including protein kinase C and RAS (*33,34*). For 3′,4′-dihydroxyflavonoids, with an intrinsic catechol moiety, their pro-oxidative quinone/quinone methide chemistry is especially of importance because of their increasing use as functional food ingredients and food supplements (*4-7*). Recently the glutathione (GSH) trapping method (*8-10*) appeared an excellent method to investigate the quinone/quinone methide chemistry of a series of 3′,4′-dihydroxyflavonoids including quercetin (*11,12*), known to be mutagenic in a variety of bacterial and mammalian mutagenicity tests presumably through their quinone methide like metabolites (*13-15*). Therefore the objective of the present study was to investigate the possible formation of the quinone/quinone methide metabolites of quercetin reflected by the formation of the glutathionyl quercetin adducts as authentic metabolites in an *in vitro* cell model using the methods previously developed for detection and identification of the GSH adducts.

The cell model chosen consisted of B16F-10 melanoma cells known to contain significant amounts of tyrosinase (28,29). Tyrosinase is often abundant in melanoma cells and the catechol containing flavonoids have been shown to be substrates for tyrosinase, and to deplete GSH and protein thiols in melanoma cells. This effect might be ascribed to *o*-quinone formation although the actual formation of these *o*-quinones or of the glutathionyl conjugates derived from them was not demonstrated (28). Results of the resent study clearly indicate, for the first time, the formation of glutathionyl quercetin adducts in a biologically relevant model provides evidence for the formation of quinone/quinone methide-type metabolites of the flavonoid model compound quercetin. The data obtained also support that the adducts are formed intracellular and subsequently excreted into the incubation medium.

Thus, the results of the present study, reveal for the first time evidence for the pro-oxidative metabolism of quercetin in a cellular *in vitro* model.

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# 7

# Summary and conclusions

## 7.1 Summary

Quinone and quinone methides from a variety of natural and synthetic compounds, including 3′,4′-dihydroxyflavonoids and catechol-type metabolites from polycyclic aromatic hydrocarbons and estrogens have been classified as likely candidates for reactive metabolites, able to react with cellular macromolecules. For 3′,4′-dihydroxyflavonoids, with an intrinsic catechol moiety, their pro-oxidative quinone/quinone methide chemistry is especially important because of their increasing use as functional food ingredients and food supplements. Thus with respect to the possible pro-oxidant toxicity it is of interest to notice that the mutagenic properties of the flavonoid quercetin have been demonstrated in a variety of bacterial and mammalian mutagenicity tests, and have been related to its quinone/quinone methide chemistry. Based on these positive mutagenicity results in a variety of bacterial as well as mammalian test systems, several studies have investigated and reported the possible carcinogenicity of especially quercetin. The possible pro-oxidant toxicity of these catechol-containing compounds has recently been underlined by studies on the mutagenicity of estrogens. Metabolic activation of estrogens to redox active and/or electrophilic metabolites has been proposed as one of the mechanisms responsible for the link between estrogen exposure and the risk of developing cancer. Especially catechol (ortho-diol)-type of metabolites resulting from cytochrome P450 catalyzed hydroxylation of estrogens may be involved. The involvement of catechol-type metabolites has also been outlined to play a role in the metabolic activation of polycyclic aromatic hydrocarbons. Clearly flavonoids like quercetin, luteolin, fisetin and many others already contain the pro-oxidative catechol structural element, without the requirement for an initial bioconversion step.

Since flavonoid quinone/quinone methides have been suggested as the major metabolites and intermediates responsible for the pro-oxidant toxicity and mutagenicity of flavonoids, characterisation of flavonoid quinone chemistry is of importance. However, little information is available on the structure and reactivity of these flavonoid oxidation products. Therefore, the objective of this thesis was to investigate the pro-oxidant chemistry of flavonoids and to perform structure activity studies on the chemical behaviour and toxicity of 3',4'-dihydroxyflavonoids with special emphasis on the nature and reactivity of the quinone/quinone methide type metabolites formed.

**Chapter 1** gives an introduction to the field of flavonoids. In order to obtain better insight on the pro-oxidant chemistry of flavonoids the pro-oxidant chemistry of quercetin was investigated first in **chapter 2.** The oxidation of quercetin by horseradish peroxidase/$H_2O_2$ to generate the one electron oxidized quercetin semiquinone has been studied in the absence but especially also in the presence of glutathione (GSH). HPLC analysis of the reaction products formed in the absence of GSH revealed formation of at least twenty different products, a result in line with other studies reporting the peroxidase mediated oxidation of flavonoids. In the presence of GSH, however, these products were no longer observed and formation of two major new products was detected. $^1$H NMR identified these two products as 6-glutathionylquercetin and 8-glutathionylquercetin, representing glutathione adducts originating from glutathione conjugation at the A ring instead of at the B ring of quercetin quinone. Products formed as a result of quercetin oxidation by horseradish peroxidase/$H_2O_2$ in the absence of glutathione were no longer observed in incubations with glutathione. This suggests that the formation of the reaction products in the absence or presence of glutathione proceeds through the same reactive intermediates. Glutathione addition at positions 6 and 8 of the A ring can best be explained by taking into consideration a further oxidation of the quercetin semiquinone, initially formed by the HRP-mediated one-electron oxidation, to give the *ortho*-quinone, followed by the isomerization of the *ortho*-quinone to its *para*-quinone methide isomers. In contrast to the *ortho*-quinone, the *para*-quinone methide isomer of quercetin quinone contains electrophilic character at the C6 and C8 positions in the A ring, providing an opportunity for the formation of the C6- and C8-glutathione adducts. It is known that quinone methides are even more reactive than the corresponding *ortho*-quinone isomers. Such an increased reactivity of the quinone methide as compared to the *ortho*-quinone will result in a shift of the equilibrium in favor of the *para*-quinone methide and its C6- and C8-glutathione adducts.

All together, the results of this chapter provide evidence for a reaction chemistry of quercetin semiquinone with horseradich peroxidase/$H_2O_2$ and GSH ultimately leading to adduct formation instead of to preferential GSH mediated chemical reduction to regenerate the parent flavonoid.

In **chapter 3** a structure-activity study on the quinone/quinone methide chemistry of an additional series of 3′,4′-dihydroxyflavonoids was performed to provide new insight in the quinone/quinone methide chemistry of 3′,4′-dihydroxyflavonoids. Using the glutathione trapping method followed by HPLC, $^1$H NMR, MALDI-TOF, and LC/MS analysis to identify the glutathionyl adducts, the chemical behavior of the quinones/quinone methides of the different flavonoids could be deduced. The nature and type of mono- and di-glutathionyl adducts formed from quercetin, taxifolin, luteolin, fisetin, and 3,3′,4′-trihydroxyflavone show how several structural elements influence the quinone/quinone methide chemistry of flavonoids. On the basis of the quinone/quinone methide isomerization chemistry involved in the formation of the A ring type glutathionyl adducts from quercetin quinone/quinone methides, it can be postulated that especially the C2=C3 double bond, the C3-OH group, the C4-keto group, and the C5- and/or C7-OH group are required for efficient quinone methide formation and GSH adduct formation in the A ring instead of in the B ring. In line with previous findings, glutathionyl adduct formation for quercetin occurs at positions C6 and C8 of the A ring, due to the involvement of quinone methide type intermediates. Elimination of the possibilities for efficient quinone methide formation by (i) the absence of the C3-OH group (luteolin), (ii) the absence of the C2=C3 double bond (taxifolin), or (iii) the absence of the C5-OH group (3,3′,4′-trihydroxyflavone) results in glutathionyl adduct formation at the B ring due to involvement of the *ortho*-quinone isomer of the oxidized flavonoid. The extent of di- versus mono-glutathionyl adduct formation was shown to be dependent on the ease of oxidation of the mono-adduct as compared to the parent flavonoid. The reason why 2′-glutathionylluteolin and 2′-glutathionyltaxifolin oxidation appears to be relatively efficient can be derived from molecular orbital calculations. For luteolin, the calculated ionization potential ($-E_{HOMO}$) and thus the ease of oxidation of the parent compound as compared to the 2′-glutathionyl adduct is almost the same. For taxifolin, the calculated ionization potential reveals that the oxidation of 2′-glutathionyltaxifolin is even easier than the oxidation of the parent compound. This explains why especially for luteolin and taxifolin and not for the other model compounds of this study di-glutathionyl adduct formation was readily observed.

Finally, unexpected results obtained with fisetin provided a new insight into the quinone/quinone methide chemistry of flavonoids. The regioselectivity and nature of the quinone adducts formed appeared to be dependent on pH. At pH values above the $pK_a$ for quinone deprotonation, glutathionyl adduct formation proceeds at the A or B ring following expected quinone/quinone methide isomerisation patterns. However, decreasing the pH below this $pK_a$ results in a competing pathway in which glutathionyl adduct formation occurs in the C ring of the flavonoid, which is accompanied by $H_2O$ adduct formation, also in the C ring of the flavonoid. All

together, the data presented in this chapter confirm that quinone/quinone methide chemistry can be far from straight forward, but the study provides significant new data revealing an important pH-dependence for the chemical behavior of this important class of electrophiles.

Depending on the results of the pH-dependence of fisetin (**chapter 3**) the formation of glutathionyl adducts from a series of 3′,4′-dihydroxy flavonoid *ortho*-quinone/*para*-quinone methides has been investigated with special emphasis on the regioselectivity of the glutathione addition as a function of pH (**chapter 4**). The flavonoid *ortho*-quinones were generated using horseradish peroxidase and upon purification by HPLC the glutathionyl adducts were identified by LC/MS as well as $^{1}H$ and $^{13}C$ NMR. The major pH effect observed for the glutathione conjugation of taxifolin and luteolin quinones is on the rate of taxifolin and luteolin conversion and, as a result, on the ratio of mono- to di-glutathionyl adduct formation. With fisetin, 3,3′,4′-trihydroxyflavone, and quercetin, decreasing the pH results in a pathway in which glutathionyl adduct formation occurs in the C ring of the flavonoid, being initiated by hydration of the quinone and $H_2O$ adduct formation also in the C ring of the flavonoid. With increasing pH, for fisetin and 3,3′,4′-trihydroxyflavone glutathione adduct formation of the quinone occurs in the B ring at C2′ as the preferential site. For quercetin the adduct formation of its quinone/quinone methide shifts from the C ring at pH 3.5, to the A ring at pH 7.0, to the B ring at pH 9.5, indicating a significant influence of the pH and (de)protonation state on the chemical electrophilic behaviour of quercetin quinone/quinone methides. Together, the results of this chapter elucidate the mechanism of the pH-dependent electrophilic behaviour of B ring catechol flavonoids.

Oxidation of flavonoids with a catechol structural motif in their B ring, leads to formation of flavonoid quinone/quinone methides which rapidly react with glutathione to give reversible glutathionyl flavonoid adducts. Results of **chapter 5** demonstrate that as a thiol scavenging agent for this reaction cysteine is preferred over glutathione and *N*-acetylcysteine. This results from the fact that the equilibrium of conjugate formation with cysteine, in contrast to that for adduct formation with glutathione or *N*-acetylcysteine, is more in favor of adduct formation. In contrast to what has been observed for other aromatic cysteine quinone adducts this preferential scavenging by cysteine can be ascribed either to cyclization leading to 1,4-benzothiazine formation or to formation of a stable intramolecular hydrogen bridge hampering deconjugation. The observation of preferential quercetin quinone/quinone methide scavenging by cysteine over glutathione and *N*-acetylcysteine can not be useful for biomonitoring studies in which detection of the glutathionyl flavonoid conjugates may be hampered by their reversible nature. This because following the results of **chapter 5,** which reveal that although the adduct formation is shifted in

favor of formation of the cysteinyl adducts, these cysteinyl adducts are not stable compared to those of the corresponding reversible unstable glutathionyl adducts. The results of this chapter reveal new insight in the chemistry of quercetin quinone/quinone methide which reveal that in the biological systems, in which the physiological concentrations of GSH are expected to be higher than those of cysteine, the quinone/quinone methide chemistry of the flavonoids should be followed by detecting the corresponding reversible unstable glutathionyl adducts and not the cysteinyl adducts.

In **chapters 2 to 5** we identified the nature of GSH conjugates of several flavonoid quinones. This opens the way for studies investigating the formation of these GSH conjugates and their corresponding mercapturic acids in cellular in vitro and in vivo systems. The actual formation of these glutathionyl flavonoid-quinone adducts and of their corresponding mercapturic acids would represent an in vivo bioactivation pathway of these supposed beneficial functional food ingredients. The results of these chapters indicate that the flavonoid glutathione adducts, like other GSH adducts have a reversible nature resulting in a limited stability. This implies that their detection in body fluid is generally considered difficult due to 1) the relatively low levels of formation of these reactive metabolites and 2) their high reactivity and limited stability. However because the formation of these adducts would represent an important bioactivation pathway of supposed beneficial functional food ingredients, the detection of these GSH adducts as authentic metabolites in cellular in vitro or in vivo models is certainly of interest. As a first step in the search for the biological relevance of quinone methide-type pro-oxidant chemistry of flavonoids the objective of **chapter 6** was to investigate the possible formation of especially quercetin-glutathione adducts as authentic metabolites in an *in vitro* cell model using the methods developed for detection and identification of the GSH adducts. The *in vitro* system used consisted of mouse melanoma cancer cells (B16F-10). These cells were chosen because they have been reported to contain significant levels of tyrosinase. Tyrosinase was shown before to catalyse flavonoid metabolism to quinone/quinone methide-type metabolites leading to glutathionyl-flavonoid adducts. Thus the B16F-10 melanoma cell line was considered an excellent model system to investigate the possible formation of quercetin-glutathionyl adducts in cells exposed to quercetin. Results of this chapter clearly indicate, for the first time, the formation of glutathionyl quercetin adducts in a biologically relevant model. The data obtained also support that the adducts are formed intracellular and subsequently excreted into the incubation medium.

## 7.2 Conclusions

It is of interest to note that many flavonoid preparations are at present already marketed as herbal medicines or dietary supplements. Results of this thesis illustrate that the importance of the pro-oxidant behavior of these flavonoids seems to be underestimated. Indeed, the results indicate that the behaviour of these compounds in the presence of enzymes like peroxidases or tyrosinase may result in formation of reactive alkylating reaction products. Some studies have suggested a role for quinone/quinone methides in the mutagenicity of compounds like quercetin. The results of this thesis provide experimental evidence for the formation of such quinone/quinone methide type metabolites from quercetin upon its enzymatic conversion in one- or two-electron oxidation reactions. In addition, the antioxidant action of quercetin is another process generating quercetin semiquinone radicals. Moreover, the reversible nature of the glutathione adducts derived from quercetin indicate that this usually detoxifying pathway might give rise to unexpected adverse effects of quercetin. Whether the supposed beneficial effect of quercetin as an antioxidant is also accompanied by formation of the alkylating quinone methides and to what extent this pro-oxidative toxic potential of quercetin should be taken into account during the future development of this compound as a so-called beneficial functional food ingredient, are questions requiring careful examination.

The results provide insight in structure-activity-relationships for the chemical behaviour and pro-oxidant toxicity of these electrophilic quinone/quinone methide metabolites. The results obtained also illustrate the unexpected mechanism of the pH-dependent electrophilic behaviour of B ring catechol flavonoids.

The results of this thesis reveal for the first time evidence for the pro-oxidative action of quercetin in a cellular in vitro model. The formation of these GSH flavonoid adducts provides evidence for the actual pro-oxidative formation of reactive quinone type metabolites from B ring catechol flavonoids in a cellular *in vitro* model. Oxidation of the catechols to quinones and their isomeric quinone methides generates potent electrophiles that could alkylate DNA. Interestingly, the structural requirements essential for good antioxidant activity match the requirements essential for pro-oxidant action and quinone methide formation. Altogether, the pro-oxidant behaviour and toxicity of flavonoids and their quinone/quinone methides are far from straight forward and need to be re-evaluated especially with respect to their use as functional food ingredients and/or food supplements.

# Summary

There is currently much interest in the development of functional foods aiming at the prevention of the development of some diseases, for example cancer, by the introduction of selected natural substances at elevated levels into the diet. The rationale for this approach is based especially on epidemiological data that indicate that food items containing such chemicals may reduce the risk of these diseases in humans. Epidemiological studies indicate, for example, that diets rich in fruit and vegetables protect against a variety of diseases, including heart diseases and certain forms of cancer. However, identification of the actual ingredient in a specific diet responsible for the beneficial health effects remains an important bottleneck for translating observational epidemiology to development of a functional food ingredient. The protection against cancer afforded by fruit and vegetables has been attributed to antioxidant micronutrients such as vitamin C, beta-carotene and vitamin E, which may act at many sites, including the stomach, intestine, lung and bladder. However, present scientific attention is focusing as well on the significance of other minor dietary components, notably the flavonoids as protectants against disease. Flavonoids are widespread in nature and are found in considerable quantities in fruits, vegetables, seeds, peel and tubers. The average Western diet may provide up to 1 g of flavonoids per day. Numerous *in vitro* studies show that flavonoids are potent antioxidants and metal chelators. Their potential as anti-inflammatory, antiallergic and antiviral compounds has also attracted attention. These studies provide the basis for the present rapidly increasing interest for the use of flavonoids as functional food ingredients. As a result increased human exposure to flavonoids can be expected in the near future. In shops and at the internet, food and food supplements based on (iso)flavonoids as functional ingredients are marketed. This, although hard scientific data supporting the health claims as well as data allowing a balanced risk-benefit evaluation are lacking. For flavonoids increased future human exposure regimens induce the question on their pro-oxidant chemistry. There is considerable evidence that some flavonoids are mutagenic in both bacterial and mammalian experimental systems. A high incidence of gastric cancer in some human populations has been linked to consumption of wine containing potentially mutagenic flavonoids (Tamura

*et al.*, Proc. Natl. Acad. Sci. USA. 77, 4961-4965, 1980, Hoey *et al.*, Am. J. Epidemiol., 113, 669-974, 1981). Relatively little is understood about either the toxicity or protection afforded by flavonoids in humans.

Since flavonoid quinone/quinone methides have been suggested as the major metabolites responsible for the possible pro-oxidant toxicity and mutagenicity of flavonoids, characterisation of flavonoid quinone chemistry is of importance. However, little information is available on the structure and reactivity of these flavonoid oxidation products. Therefore, the objective of this thesis was to investigate the pro-oxidant chemistry of flavonoids and to perform structure activity studies on the chemical behaviour of 3',4'-dihydroxyflavonoids with special emphasis on the nature and reactivity of the quinone/quinone methide type metabolites formed. Using the GSH trapping method, HPLC, LC/MS, MALDI-TOF, $^{1}H$ NMR, $^{13}C$ NMR and quantum mechanical computer calculations the quinone/quinone methide chemistry of a series of 3',4'-dihydroxyflavonoids could be characterised.

The results provide insight in structure-activity-relationships for the pro-oxidant chemistry of these electrophilic quinone/quinone methide flavonoid metabolites. The results obtained also reveal an unexpected pH-dependent electrophilic behaviour of B ring catechol flavonoids. Furthermore the results of this thesis also reveal, for the first time, evidence for the pro-oxidative chemistry of quercetin in a cellular *in vitro* model. The formation of these glutathionyl-flavonoid adducts provides evidence for the actual pro-oxidative formation of reactive quinone type metabolites from B ring catechol flavonoids in the selected cellular *in vitro* model using melanoma cells. Oxidation of the catechols to quinones and their isomeric quinone methides generates potent electrophiles that could alkylate DNA. Interestingly, the structural requirements essential for good antioxidant activity match the requirements essential for pro-oxidant action and quinone methide formation. Altogether, the pro-oxidant behaviour of flavonoids and their quinone/quinone methides are far from straight forward and need to be re-evaluated especially in the framework of the risk-benefit evaluation of the use of these flavonoids as functional food ingredients and/or food supplements.

# Samenvatting

Er is momenteel veel interesse voor de ontwikkeling van functionele voedingsmiddelen (functional foods), met als doel het voorkomen van het ontstaan van ziekten zoals bijvoorbeeld kanker, via het in verhoogde mate introduceren van geselecteerde natuurlijke bestanddelen in het dieet. De basis voor deze aanpak wordt momenteel met name gevonden in epidemiologische studies die laten zien dat diëten rijk aan specifieke voedselcomponenten of ingrediënten de kans op bepaalde ziekten bij de mens verlagen. Zo geven epidemiologische studies bijvoorbeeld aan dat diëten die rijk zijn aan fruit en groenten beschermen tegen een aantal ziekten zoals hartziekten en bepaalde vormen van kanker. Echter, het identificeren van de belangrijke ingrediënten in het betreffende dieet die het gezondheidsbevorderende effect tot stand brengen is een knelpunt voor het vertalen van de resultaten uit de epidemiologie naar de ontwikkeling van een functioneel voedingsingrediënt.

De bescherming tegen kanker door groenten en fruit is toegeschreven aan antioxidanten zoals vitamine C, beta-caroteen en vitamine E, die op vele plaatsen in het lichaam, zoals de maag, darmen, long en de blaas actief zijn. Wetenschappelijk wordt momenteel veel aandacht besteed aan het mogelijke belang van andere belangrijke dieet componenten, zoals flavonoï den, als beschermende ingrediënten tegen ziekte. Flavonoï den komen in de natuur veel voor, en worden met name in hoge concentraties gevonden in fruit, groenten, knollen en zaden. Het gemiddelde Westerse dieet bevat ongeveer 1 gram aan flavonoï den per dag.

Vele in vitro studies tonen aan dat flavonoï den goede antioxidanten en metaal chelatoren zijn. Daarnaast hebben ze anti-inflammatoire, anti-allergische en anti-virale eigenschappen die van belang worden geacht. Deze bevindingen verschaffen de basis voor de momenteel snel groeiende interesse om flavonoï den te gebruiken als functionele voedingsingrediënten. Als gevolg hiervan zou er in de nabije toekomst een toename in de opname van flavonoï den via het dieet verwacht kunnen worden. In winkels en via het internet worden voedingsmiddelen en voedingssupplementen gebaseerd op (iso)flavonoï den als functionele voedingsingrediënten verkocht. Dit, terwijl zowel de wetenschappelijke onderbouwing voor de gezondheidsclaims als gegevens die een gebalanceerde "risk-benefit" analyse mogelijk maken, nog ontbreken. In het geval van verhoogde toekomstige blootstelling van mensen aan

flavonoï den worden voor de risk-benefit evaluatie vragen van belang rond hun mogelijk pro-oxidatieve chemisch gedrag. Er zijn aanwijzingen dat sommige flavonoï den mutageen zijn in zowel bacteriële als zoogdier *in vitro* test systemen. Een verhoogde mate aan maagkanker in bepaalde humane populaties is in verband gebracht met de consumptie van wijn met daarin mogelijk mutagene flavonoï den (Tamura *et al.*, Proc. Natl. Acad. Sci. USA. 77, 4961-4965, 1980, Hoey *et al.*, Am. J. Epidem., 113, 669-974, 1981). Alles samenvattend is er eigenlijk weinig bekend van de schadelijke maar ook van de gezondheidsbevorderende effecten van flavonoï den.
Omdat flavonoid chinon/chinon methides genoemd zijn als de belangrijkste metabolieten die verantwoordelijk zouden zijn voor de mogelijke pro-oxidatieve toxiciteit en mutageniteit van flavonoï den, is karakterisering van deze pro-oxidant chemie van flavonoï den van belang. Echter er is weinig bekend over de structuur en de reactiviteit van deze flavonoid oxidatie producten. Daarom was het doel van deze studie de pro-oxidant chemie van flavonoï den te onderzoeken en een structuur-activiteits studie uit te voeren naar het chemische gedrag van 3',4'-dihydroxyflavonoï den. Daarbij werd speciale aandacht besteed aan de aard en reactiviteit van de gevormde chinon/chinon methide metabolieten. Met behulp van de GSH-trapping methode, HPLC, LC/MS, MALDI-TOF, $^{1}$H-NMR, $^{13}$C-NMR en kwantum-chemische computerberekeningen kon de chinon/chinon methide chemie van een serie 3',4'-dihydroxyflavonoiden gekarakteriseerd worden.
De verkregen resultaten geven inzicht in de structuur-activteits relaties voor de pro-oxidatieve chemie van de electrofiele chinon /chinon methides metabolieten van de flavonoï den. De resultaten laten ook een onverwacht effect zien van de pH op het electrofiele gedrag van de B-ring catechol flavonoï den. Bovendien laten de resultaten van het proefschrift zien dat zelfs onder reducerende omstandigheden in een cellulair in vitro model (melanoma cellen) de pro-oxidatieve chemie van quercetine van belang kan zijn. Met name de vorming van glutathion-flavonoid conjugaten is een bewijs dat in het gekozen cellulaire model de pro-oxidatieve vorming van reactieve flavonoid chinon/ chinon methide metabolieten is opgetreden. Oxidatie van de catecholen naar chinonen en hun isomere chinon methides genereert electrofielen die DNA kunnen alkyleren. Van belang is dat de structurele randvoorwaarden die een flavonoid een goede antioxidant maken gelijk blijken te zijn aan de structurele kenmerken die essentieel zijn voor pro-oxidant gedrag en chinon methide vorming.
Al met al is de pro-oxidant chemie van flavonoï den en van hun chinon /chinon methides verre van recht toe recht aan gebleken en zou de pro-oxidatieve chemie en de toxiciteit van de flavonoï den in het kader van hun gebruik als functional food ingredienten beter onderzocht en afgewogen moeten worden, rekening houdend met hun mogelijk gezondheidsbevorderende effecten.

## List of Publications

1. Abo-Ghalia, M.H., Shalaby, A.M., El-Eraqi, W.I., and **Awad, H.M.** "Synthesis and comparative anti-phlogistic potency of new proteinogenic amino acid conjugates of 2-[2,6-dichlorophenyl-1-amino] phenyl acetic acid Diclofenac." *Acta Poloniae pharm./Drug Res.*, **55**, 211-221, 1998.

2. Abo-Ghalia, M.H., Shalaby, A.M., El-Eraqi, W.I., and **Awad, H.M.** "Synthesis and comparative anti-phlogistic potency of some new nonproteinogenic amino acid condensed conjugates of Diclofenac." *Amino Acids,* **16**, 425-440, 1999.

3. Abo-Ghalia, M.H., Shalaby, A.M., and **Awad, H.M.** "Acryloyl amino acid conjugates of anticipated anti-allergic activity. Synthesis of N-[cinnamoyl] and N-[3-(2-furyl) acryloyl] amino acid-(4-methylpiperazine) amides." *J. of Association for the advancement of Modelling & Simulation techniques in Enterprises (A.M.S.E.)*, **58**, 9-38, 1998.

4. **H. M. Awad**, M.G. Boersma, J. Vervoort, and I.M.C.M. Rietjens. "Peroxidase-catalysed formation of quercetin quinone methide-glutathionyl adducts." *Archives of Biochemistry and Biophysics,* **378**, 224-233, 2000.

5. **H.M. Awad**, M.G. Boersma, S. Boeren, J. Vervoort, P.J. van Bladeren, and I. M.C. M. Rietjens. "Structure-activity study on the quinone/quinone methide chemistry of flavonoids." *Chemical Research in Toxicology,* **14**, 398-408, 2001.

6. I.M.C.M. Rietjens, **H.M. Awad**, M.G. Boersma, M.L.P.S. van Iersel, J. Vervoort, and P.J. van Bladeren. "Structure activity relationships for the chemical behaviour and toxicity of electrophilic quinones/quinone methides." In: Advances in experimental Medicine and Biology, Vol 500, Biological Reactive Intermediates VI: Chemical and Biological Mechanisms in Susceptibility to and Prevention of Environmental Diseases. P.M. Dansette et al. (Eds), Kluwer Academic / Plenum Publishers New York, Boston (2001) pp 11-21.

7. **H.M. Awad**, M.G. Boersma, S. Boeren, J. Vervoort, P.J. van Bladeren, and I.M.C. M. Rietjens. "The regioselectivity of glutathione adduct formation with flavonoid quinone/quinone methides is pH-dependent." *Chemical Research in Toxicolology,* (2002) (in press).

8. I.M.C.M. Rietjens, M.G. Boersma, L. de Haan, B. Spenkelink, **H.M. Awad**, N.H.P. Cnubben, J. v. Zanden, H. v.d. Woude, G.M. Alink, and J.H. Koeman. "The pro-oxidant chemistry of the natural antioxidants vitamin C, vitamin E, caretenoids and flavonoids." *Environmental Toxicology and Pharmacology,* (2002) (in press)

9. **H.M. Awad**, M.G. Boersma, S. Boeren, H. v.d. Woude, J. v. Zanden, J. Vervoort, P.J. van Bladeren, and I.M.C.M. Rietjens. "Identification of *o*-quinone/ quinone methide metabolites of quercetin in a cellular in vitro system. *FEBS Letters* (submitted).

10. **H.M. Awad**, M.G. Boersma, S. Boeren, J. Vervoort, P.J. van Bladeren, and I.M.C. M. Rietjens. "Quenching of quercetin quinone/quinone methides by different thiolate scavengers: stability and reversibility of conjugate formation." (2002) (in preparation).

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**Hanem**

# CURRICULUM VITAE

The author of this dissertation, Hanem Mohamed Awad was born on December 18$^{th}$, 1969 in Kafer Abou-Naser, Dekernes, El-Dakahlia. In 1991, she obtained the Bachelor of Science degree in chemistry from the Faculty of Science, Mansoura University. She started her post-graduate courses in October 1992 in the Faculty of Science, Mansoura University. Her Master of Science degree was obtained in 1997 from the Department of Chemistry, Faculty of Science, Mansoura University. The topic of the master thesis was "Synthesis of aryl and heteroaryl amino acid and peptide derivatives with anticipated biological activity". From 1993 till 1997 she became *Research Assistant* at the National Research Centre, Tanning Materials & Proteins Department, Dokki, Cairo, Egypt. After finishing her MSc. study, she became Assistant Researcher at the National Research Centre, Tanning Materials & Proteins Dept., Dokki, Cairo, Egypt. In February 1999, she started her Ph-D studies at the Department of Biochemistry of Wageningen University, and the results are summerized in the present dissertation. In March 2002, she will assume a position as a researcher in a post-graduate program in the National Research Centre, Tanning Materials & Proteins Department, Dokki, Cairo, Egypt. Her permanent address will be:

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