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# A synthetic approach to the generation of quercetin sulfates and the detection of quercetin 3′-*O*-sulfate as a urinary metabolite in the rat☆

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**Abstract**—To study the biological effects of quercetin, authentic products of quercetin metabolism are required as standards. The synthesis of quercetin sulfate standards is thus described. Quercetin was reacted with a 10-fold molar excess of sulfur trioxide-*N*-triethylamine, and the products were analyzed by HPLC and mass spectrometry. Four monosulfates and three disulfates were identified, and structural inferences were drawn by $^{1}$H NMR spectrometry of HPLC peak isolates. Analysis of the urine of rats that had received quercetin (1.9 g/kg po) yielded a single peak, which by comparison with the products of the reaction between quercetin and sulfur trioxide-*N*-triethylamine was identified as quercetin 3′-*O*-sulfate.
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## 1. Introduction

Naturally occurring flavonoids and isoflavonoids in the diet are thought to exert beneficial effects on human health.[1] Elucidation of the mechanisms underlying these effects is currently the focus of much research. Epidemiological evidence links diets rich in quercetin (for structure, see Fig. 1) with decreased incidence of cardiovascular and neoplastic diseases.[2–5]

There is accumulating evidence to suggest that polyphenolic phytochemicals including flavonoids are rapidly metabolized in the human or animal organism. Glucuronidation appears to be the most prevalent conjugation pathway for quercetin in rats and humans. Sulfation can occur to form mono- or bis-conjugates or combine with other conjugation pathways to form sulfo-glucuronides and sulfate conjugates of methylated quercetin.[6–8] Most often, the metabolism of polyphenols constitutes a pharmacological deactivation step. Alternatively, metabolites may contribute to the pharmacological or, indeed, toxicological efficacy of the parent polyphenol. Intriguingly, it has recently been shown that the methylated quercetin conjugates isorhamnetin and tamarixetin were much more potent inhibitors of the activity of the enzyme cyclooxygenase-2 (COX-2) than their metabolic precursor.[6] This example illustrates the principle that to rationalize advice as to the potential benefit of consumption of flavonoids as diet constituents or food additives, it is important to clarify the role that their metabolites may play in the pharmacology of the parent substance. Such studies require the availability of authentic standards. Asynthetic approach to furnish quercetin glucuronides led to products, in which four of the five hydroxyl groups were substituted with glucuronyl moieties.[8] In general, phenols not only undergo avid glucuronidation but also metabolic conjugation with activated sulfate to generate *O*-sulfates. The relative prevalence of the different conjugates will depend on the concentration of quercetin, activity of the metabolic enzymes and cell type. Sulfation of quercetin has previously been attempted using a liver extract with activated sulfate and cofactors.[9] This synthesis allowed the generation of one quercetin monosulfate that was

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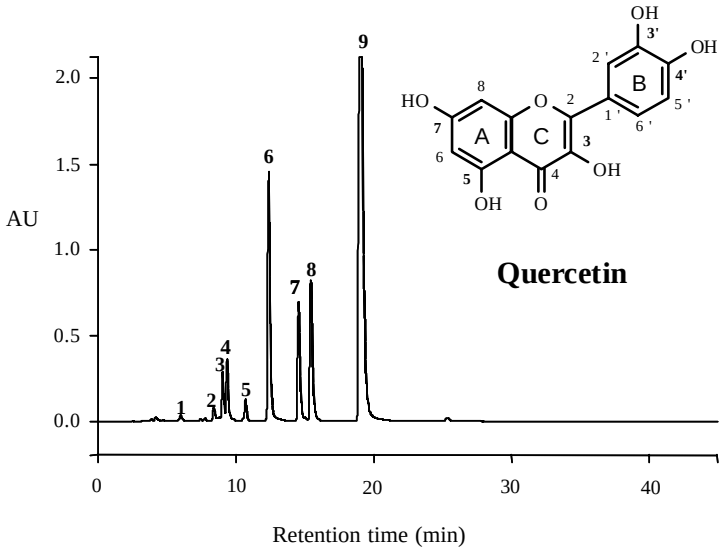


**Figure 1.** HPLC chromatogram of the products of the reaction of quercetin with Esulfur trioxide-*N*-triethylamine. On the basis of mass spectrometric evidence (Table 1) peaks 2–4 are quercetin disulfates and peaks 5, 6, 7 and 8 monosulfates. Peaks 5 and 9 co-eluted with authentic quercetin 3-*O*-sulfate and quercetin, respectively. Also shown is the structure of quercetin. For details of chromatography, see Section 4.

not characterized. Day et al. synthesized quercetin 3′-*O*-sulfate specifically using a chemical synthesis method.[7]

One of the initial objectives was to generate a number of sulfate analogues that would allow comparison in a biological assay. The advantage of a chemical synthetic approach is that the potential yield of product could be even bigger since with biological approaches the enzymes and cofactor concentrations could be a limiting factor.

The aim of the present study was to synthesize authentic quercetin sulfates and to explore in a preliminary fashion their occurrence in biomatrices of rats that have received quercetin.

## 2. Results and discussion

### 2.1. Synthesis

Quercetin was reacted with a 10-fold molar excess of sulfur trioxide-*N*-triethylamine complex, and an extract of the mixture was analyzed by HPLC. The resultant chromatogram (Fig. 1) shows the presence of quercetin (peak 9) and eight peaks that were surmised to be quercetin sulfates. Preliminary studies using a 2-, 4- and 10-fold excess of sulfur trioxide-*N*-triethylamine complex found the 10-fold excess to be optimal, and was used in subsequent studies.

The relative yield of each of the peaks using a 10-fold excess of sulfur trioxide-*N*-triethylamine complex is given in Table 1. The yield varies from 1 to 16% for the monosulfates and 0.8–3.8% for the disulfates. The synthesized sulfates were at least 90% pure (as determined by HPLC), with the major contaminant being the parent molecule, quercetin. The purchased sulfate was >98% pure. Whilst a detailed study with regard to stability was not undertaken, the dried compound, stored at +4 °C, when reconstituted showed no decomposition for a month. Material eluting with each peak was collected individually, and the eluant was evaporated to dryness.

### 2.2. NMR and mass spectrometric data

Isolated peak material was subjected to MS and NMR analyses, the latter after reconstitution in DMSO-$d_6$. Table 1 shows mass spectral inferences with regard to peak identity.

**Table 1.** Molecular ions of species contained in HPLC peaks isolated from a reaction mixture of quercetin with sulfur trioxide-*N*-triethylamine complex

| Fraction number | Rt (min) | Molecular weight | Inference | Yield (%) |
|---|---|---|---|---|
| 1 | 4.5 | n.a. | — | |
| 2 | 8.3 | 462 | Quercetin disulfate | 0.8 |
| 3 | 9.0 | 462 | Quercetin disulfate | 2.6 |
| 4 | 9.3 | 462 | Quercetin disulfate | 3.8 |
| 5 | 10.7 | 382 | Quercetin monosulfate | 1.3 |
| 6 | 12.3 | 382 | Quercetin monosulfate | 16.3 |
| 7 | 14.5 | 382 | Quercetin monosulfate | 8.3 |
| 8 | 15.4 | 382 | Quercetin monosulfate | 10.9 |
| 9 | 19.1 | 302 | Quercetin | — |

Yield of individual sulfate is included in the table.
n.a., the molecular weight could not be assigned.

The materials derived from peaks with numbers 5, 6, 7, 8 and 9 in Figure 1 afforded interpretable $^1$H NMR spectra. Figure 2A and B show spectra for quercetin (peak 9 in Fig. 1) and purchased authentic quercetin 3-*O*-sulfate, respectively. Figures 2C and D depict $^1$H NMR spectra of material contained in peaks 5 and 6, respectively, and Figure 2E the spectrum of a mixture of substances eluting with peaks assigned numbers 7 and 8 in Figure 1 and Table 1. The spectrum of quercetin is characterized by coupling of protons 6-H and 8-H on ring A (Fig. 2A) that appear as a pair of meta-coupled doublets at 6.2 and 6.4 ppm, respectively, with a coupling constant of $J = 2.1$ Hz. The 5′-H on ring B (6.9 ppm) is split by 6′-H and, vice versa, the proton 6′-H is split by 5′H. This ortho-coupling results in a large $J$ value of 8.5 Hz. The 6′-H is split by further long range coupling to 2′-H. Consequently, the 6′-H appears as a doublet of doublets (7.65 ppm, $J = 8.5$ Hz, $J = 2.13$ Hz), and the 2′-H signal appears as a fine doublet (7.75 ppm, $J = 2.13$ Hz). The spectrum of HPLC peak 5 (Fig. 2C) is identical to that of authentic quercetin 3-*O*-sulfate (Fig. 2B) purchased from Extrasynthese (Genay, France). In comparison to the $^1$H NMR spectrum of unconjugated quercetin, the spectrum of this molecule is characterized by an upfield shift of the 2′-H signal. This upfield shift is intriguing, as one might rather expect conjugation at the 3-position to affect the resonance frequency of 6′-H. An analogous observation was made in the analytical comparison of quercetin and quercetin 3-*O*-glucuronide, where the resonance frequency of 2′-H was shifted upfield by glucuronidation at the 3-position.[10] It is likely that the quercetin molecule could allow rotation around the C-2 to C-1′ bond. This rotation is analogous to the C5-C1′ bond rotation in fluoronitropyrimethamine that exists as two slowly interconverting rotamers.[11] NMR evidence for the existence of two rotamers of quercetin could not be generated, although X-ray crystallography data suggest that ring B can be situated at 0° and 180° to the residual structure.[12] The major difference between the spectra of quercetin and HPLC peak 6 is a change in chemical shift of both the 6- and 8-protons (Fig. 2D). The material eluting with HPLC peak 6 might be quercetin 7-*O*-sulfate, based on the similar magnitude of the effect of sulfate substitution on the shifts of 6-H and 8-H. It proved impossible to completely resolve the materials contained in the HPLC peaks 7 and 8 by semi-preparative HPLC. The NMR spectrum of the mixture allowed discrimination between the two components by exploiting the difference in integration (Fig. 2E). The chemical shift of the 2′-H in the spectrum of the material eluting with 7 (Fig. 2E) displays a considerable downward shift in comparison with the equivalent proton in quercetin, compatible with sulfate substitution at the 3′-position. The spectrum of the material eluting with 8 (Fig. 2E) is marked by a large downfield shift of the 5′-H ($\delta$ 0.5 ppm) in comparison to quercetin, suggesting that it is quercetin 4′-*O*-sulfate.

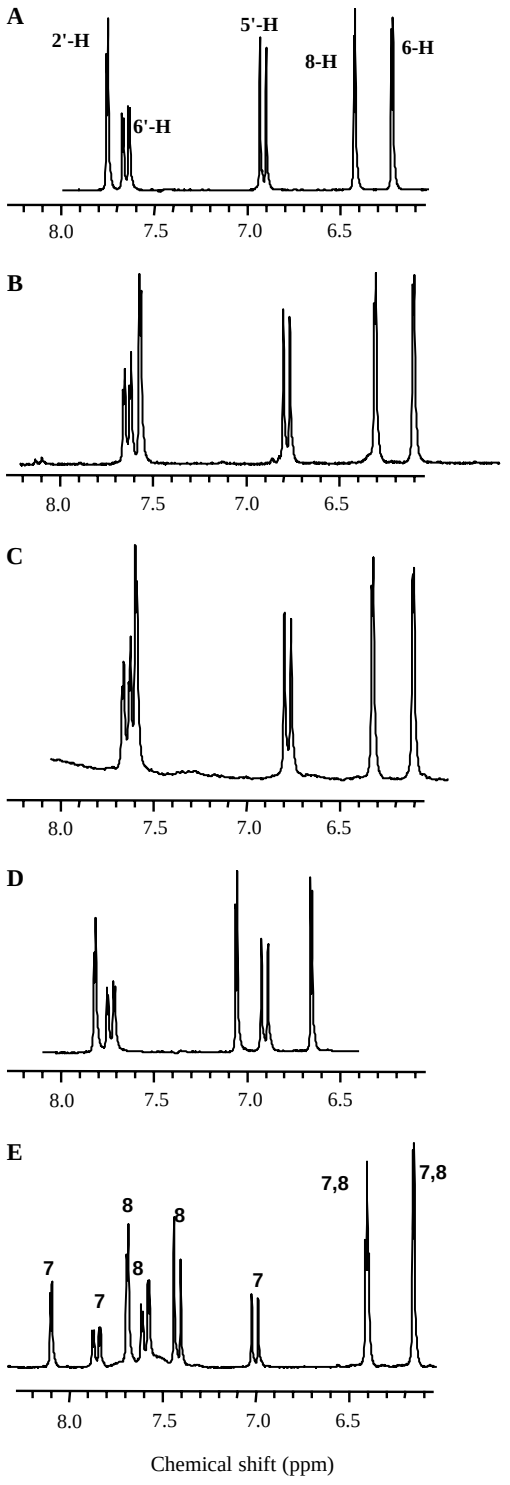


**Figure 2.** $^1$H NMR spectra of quercetin (A), quercetin 3-*O*-sulfate (B) and of peak isolates obtained by HPLC separation of products of the reaction of quercetin with sulfur trioxide-*N*-triethylamine as assigned in Figure 1 and Table 1, as follows: peak 5 (C), peak 6 (D) and a mixture of substances eluting with peaks 7 and 8 (E). For details of reaction and $^1$H NMR spectrometry, see Section 4, for analysis of spectra see Section 2.

## 2.3. Biological data

Urine from rats that had received quercetin (1900 mg/kg po) was analyzed by LC–MS (Fig. 3). Figure 3A shows the urine sample of a rat. There is a single peak, consistent in retention time with material that eluted with peak

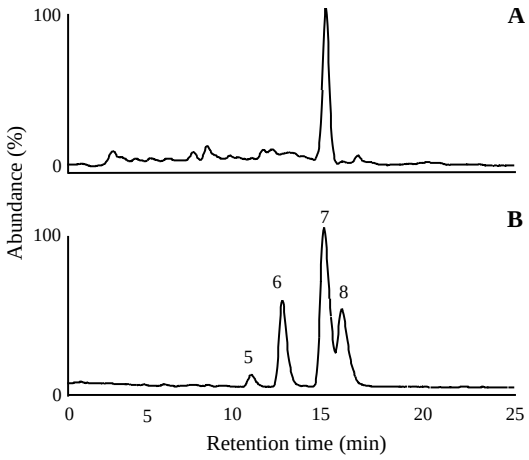


**Figure 3.** LC–MS ion chromatograms (*m*/*z* 381) of extracts of (A) urine collected over 24 h from a rat that had received quercetin (1.9 g/kg by gavage), and (B) products of the reaction of quercetin to sulfur trioxide-*N*-triethylamine. Peak numbers are identical to those in Figure 1 and Table 1. On the basis of mass spectrometric evidence (Table 1), peaks 5, 6, 7 and 8 are monosulfates. Peak 7 co-eluted with authentic quercetin 3′-*O*-sulfate and is seen in the urine of rats treated with quercetin. For details of LC–MS, see Section 4.

7 in the HPLC analysis of the products of the reaction of quercetin with sulfur trioxide-*N*-triethylamine. Figure 3B shows an extracted LC–MS ion chromatogram (*m*/*z* 381) of the extract from the synthesis. Shown are four peaks which from left to right are the 3-*O*-, 7-*O*-, 3′-*O*- and 4′-*O*-sulfates of quercetin. This result suggests that the urinary metabolite was quercetin 3′-*O*-sulfate.

## 3. Conclusions

Polyphenolic phytochemicals are good substrates of metabolic conjugation reactions with activated glucuronic acid or sulfate. The identification of such conjugates often poses an analytical challenge because of the multitude of hydroxy moieties contained in such molecules available for reaction with the conjugating species, furnishing potentially isomeric mono- and poly-conjugates. Quercetin is a suitable example that harbours five OH groups. Whilst quercetin glucuronides have been the subject of investigation,[8,10] there is a fspaucity of information on the chemistry and biochemistry of quercetin sulfates. The results outlined above describe a facile synthetic route to four mono- and three di-sulfates of quercetin. Spectral elucidation suggests monosulfation of quercetin to occur in positions 3, 7, 3′ and 4′. The data did not allow the positional allocation of the sulfate groups with regard to the disulfates, but—in analogy to the preferred *loci* of monosulfation—it is likely that the hydroxy moieties in positions 3, 7, 3′ and 4′, but not the 5 hydroxy, are involved.

The results provide for the first time evidence for the formation of quercetin 3′-*O*-sulfate as the major product of conjugation of quercetin with 3-phospho-adenosine 5′-phosphosulfate in the rat. This finding is closely akin to that in humans, as previous work suggests that quercetin 3′-*O*-sulfate is the most prominent metabolite in human plasma after consumption of a quercetin-containing diet[7] or after iv infusion of pure quercetin.[6]

Many polyphenols are currently being subjected to investigation as potential clinical therapeutic agents. The metabolism of polyphenols often follows a similar path to quercetin, with sulfate, glucuronides and methylated derivatives being generated. It should be possible to use this method for the generation of authentic standards for a whole range of polyphenols and also to understand both mechanistic and metabolic attributes of these interesting molecules.

In conclusion, the work described here helps one with the understanding of the reactivity of quercetin hydroxy moieties vis-à-vis sulfating agents under artificial chemical and biological conditions. Mechanistic studies of bioactive polyphenols exemplified by quercetin tend to concentrate on the parent molecules alone. Many of these polyphenolic phytochemicals undergo avid metabolic conjugation, and one cannot exclude the possibility that such conjugates harbour biological activity. Therefore, the inclusion of such conjugates in pharmacological profiling seems propitious.

## 4. Experimental

### 4.1. Chemicals

Quercetin 3-*O*-sulfate was obtained from Extrasynthese (Genay, France), while all other chemicals and solvents were from either Sigma (Poole, UK) or Fisher (Loughborough, UK). For administration in rats in vivo, quercetin was dissolved in DMSO.

### 4.2. Reaction of quercetin with sulfur trioxide-*N*-triethylamine

To remove any compound-associated water, dry pyridine was added to quercetin (0.5 g, 1.7 mmol) until dissolved. The mixture was rotor evaporated and the procedure repeated twice. The dried quercetin was then dissolved in dioxane (50 mL), and sulfur trioxide-*N*-triethylamine complex (3.1 g, 16.9 mmol) was added under argon to avoid contact with air. The reaction vessel was placed in a water bath (40 °C) for 90 min, after which products of quercetin sulfation precipitated out and stuck to the glass. After incubation, the dioxane was decanted and the conjugates were redissolved in methanol. Aliquots of this solution were diluted in water and methanol, and used for HPLC analysis.

### 4.3. Analysis of rat urine

Four F344 rats (150 g, obtained from Harlan, UK) received quercetin (1.9 g/kg) by gavage. Four control rats received DMSO only. Rats were kept in metabolism cages for 24 h post-administration and urine was collected. Aliquots of urine were mixed with twice the volume of DMSO/methanol (1:4, v/v). The mixture was vortexed and centrifuged (17,000*g*, 15 min). The

supernatant was diluted with water (1:1) and injected onto the HPLC column (injection volume 50 μL).

### 4.4. Analysis of quercetin sulfates by HPLC

The products of the chemical reaction of quercetin with sulfur trioxide-*N*-triethylamine or extracts of urine were separated on a BDS-Hypersil $C_{18}$ column (250 × 4.6 mm I.D., 5 μm particle size, Phenomenex, Macclesfield, UK). The instrument used was a Varian Prostar system (Walton-on-Thames, UK), comprising a UV detector (Model 310), solvent delivery system (Model 230) and an autosampler (Model 410) with a 100 μL injection loop. The gradient elution programme commenced with 75% ammonium acetate (0.1 M, pH 5.15) in methanol, which was followed by a linear decrease over 10 min to 55% ammonium acetate in methanol, and then to 45% ammonium acetate in methanol over a further 20 min, after which the mobile phase remained isocratic for a further 5 min. The instrument was operated with a flow rate of 1.0 mL/min and detection by UV spectrophotometry (375 nm).

A second system was used to fraction-collect the quercetin sulfates from HPLC eluant. A Gilson semi-preparative system (Gilson Inc., Middleton, WI, USA) comprising two pumps and a UV detector with a Rheodyne 7125 manual injector connected to a 500 μl loop was used. Fractions were collected when they eluted from a semi-preparative column (250 × 21.2 mm I.D., 5 μm particle size, Phenomenex, Macclesfield). The chromatographic conditions were as described above, except that the flow rate was 10 mL/min.

### 4.5. Analysis of quercetin sulfates by mass spectrometry and $^1$H NMR spectrometry

LC–MS was carried out using a Micromass Platform mass spectrometer coupled with a Hewlett Packard Series 1100 HPLC system and a UV detector absorbing at 375 nm. The eluant was split 1:7 post column so that approximately 142 μL/min of eluant entered the mass spectrometer. Source temperature was maintained at 110 °C. Cone voltage was 42 V, while the capillary voltage was maintained at 3.82 kV.

Proton ($^1$H) spectra were generated with a Bruker ARX 250 MHz spectrometer, using deuterated dimethylsulfoxide as the solvent.

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