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

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**Abstract**

Blood was collected from six human volunteers 1 h after the ingestion of lightly fried red onions, which are rich in anthocyanins and flavonols. Urine excreted 0–4 h after ingestion was also collected. Plasma and urine were analysed by reversed-phase HPLC with photodiode array and tandem mass spectrometric detection. Twenty-three flavonols as a range of mixed sulphate, methyl, glucuronide and glucoside derivatives of quercetin were detected. The availability of reference compounds facilitated the identification of quercetin-3-glucuronide, quercetin-3′-glucuronide, quercetin-4′-glucuronide, quercetin-3′-sulphate and isorhamnetin-3-glucuronide in samples from all volunteers. Samples from one volunteer also contained trace amounts of quercetin-3,4′-diglucoside, quercetin-3-glucoside, isorhamnetin-3-glucoside and the aglycone quercetin. Despite a high dosage, neither anthocyanins nor anthocyanin metabolites accumulated in either plasma or urine in detectable quantities.


*Keywords:* Red onions; Anthocyanins; Flavonols; Quercetin-4′-glucoside; Quercetin-3,4′-diglucoside; Human plasma and urinary metabolites; HPLC–tandem mass spectrometry

## 1. Introduction

Flavonoids are polyphenolic $C_6$–$C_3$–$C_6$ secondary metabolites with a widespread occurrence in the plant kingdom [1]. Many fruits, vegetables and selected beverages contain substantial quantities. Among the major flavonoids of dietary importance are flavonols, which occur ubiquitously, and anthocyanins, which are glycosylated anthocyanidins that are present in red wines and many commonly consumed fruits and berries [2,3].

Onions, as a rich source of flavonols [4], in particular quercetin-3,4′-diglucoside (Fig. 1, **I**), quercetin-4′-glucoside (**II**) and isorhamnetin-4′-glucoside (**III**) [5], have been used widely in human feeding studies. In early investigations, plasma and urine were acid hydrolysed and the amount of quercetin released was then determined by reversed-phase HPLC followed by post-column reaction with methanolic aluminium nitrate which facilitates selective, sub-nanogram fluorescence detection of flavonols with a free 3-hydroxyl group [6–8]. Using this post-column procedure with high-resolution gradient HPLC, Aziz et al. [9] detected fluorescent peaks in unhydrolysed plasma and urine that co-chromatographed with quercetin-4′-glucoside and isorhamnetin-4′-glucoside. Results from other studies, however, have indicated that quercetin glucuronides rather than the parent glucosides appear in the bloodstream after the consumption of onions [10]. It has been suggested that the HPLC peaks identified by Aziz et al. [9] as flavonol glucosides were probably glucuronide conjugates which can have very similar HPLC retention times [11]. In-keeping with this possibility, an investigation using HPLC with tandem mass spectrometry ($MS^2$) in the selected reaction monitoring mode detected five quercetin glucuronides but no glucosides in human

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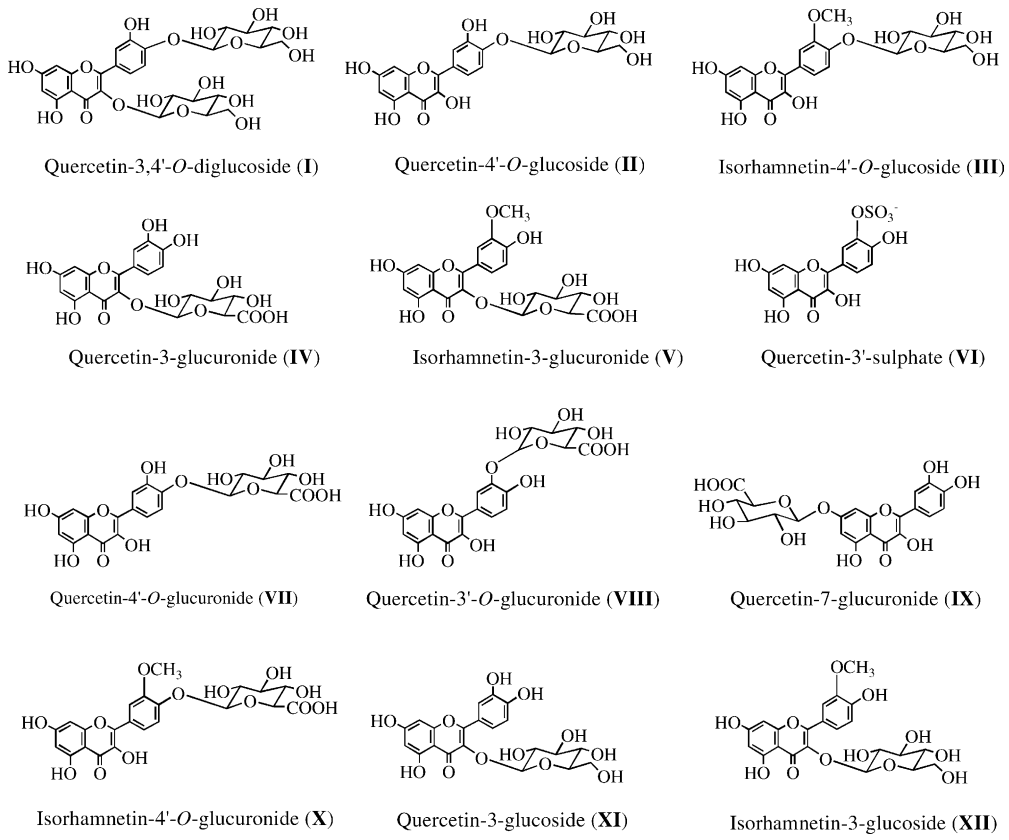


Fig. 1. Flavonol structures.

plasma after ingestion of an onion supplement [12]. A further study used HPLC–MS and selected ion monitoring to support identifications based on chromatographic retention times and enzyme hydrolysis data. In total, 12 putative quercetin glucuronide peaks were detected in plasma by HPLC–$UV_{365\,nm}$ and selected ion monitoring/co-chromatography identified three of these components as quercetin-3-glucuronide (**IV**), isorhamnetin-3-glucuronide (**V**) and quercetin-3′-sulphate (**VI**) [13].

This publication reports on the use of HPLC with photodiode array (DAD–UV) and $MS^2$ detection to analyse human plasma and urine collected after the ingestion of red onions which contained high levels of both flavonols and anthocyanins.

## 2. Experimental

### 2.1. Study design

Six volunteers (four males and two females), who were healthy, non-smokers and not on any medication, participated in this study and gave their written consent. They were aged between 23 and 45 years and had a mean body mass index of 23.7 ± 1.2 (range: 20.9–27.6). Subjects were required to follow a low flavonoid diet for two days and to fast overnight prior to supplementation. This diet excluded most fruits, vegetables and beverages such as tea, coffee, fruit juices, and wine. On the morning of the study red onions (*Allium cepa*) were skinned, chopped into small slices, and fried for 4 min in margarine. Aliquots of the fried onions were taken for qualitative and quantitative analysis of their anthocyanin and flavonol content.

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

### 2.2. Materials

Onions were purchased from a local supermarket (Safeway Stores, Byres Road, Glasgow, UK). HPLC grade methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Scotland). Formic acid was

purchased from Riedel-DeHaen (Seelze, Germany) and acetic acid from BDH (Poole, UK). L-(+)-Ascorbic acid, quercetin and isorhamnetin-3-glucoside were purchased from Extrasynthese (Genay, France). AASC Ltd. (Southampton, England) supplied quercetin-3,4′-diglucoside, quercetin-4′-glucoside, quercetin-3-glucoside, isorhamnetin-4′-glucoside, malvidin-3-glucoside and cyanidin-3-glucoside.

[2-$^{14}$C]Quercetin-4′-*O*-β-D-glucoside was synthesised in four steps from barium [$^{14}$C]carbonate (specific activity 3.75 mCi mmol$^{-1}$) by a method previously reported for the synthesis of [2-$^{13}$C]quercetin-4′-*O*-β-D-glucoside [14] except that the intermediate ester was not purified by filtration through alumina. The compound was pure by $^{1}$H NMR spectroscopy and only one radioactive peak was detected by HPLC-radio counting.

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

### 2.3. Extraction of onions

Aliquots of fried onions were taken for quantitative analysis of their flavonol content. Prior to the extraction, they were frozen in liquid nitrogen, lyophilised and powdered. Triplicate samples were extracted as follows: 35 mg of dry powder was homogenized in 3 ml of 70% methanol in water for 1 min using an Ultra-Turrax T 25 (IKA$^{R-}$Werke, Staufen, Germany). During the homogenization, the samples were kept on ice. The mixture was then centrifuged at 3000 × *g* at 4 °C for 15 min. The supernatant was collected and the pellet further extracted and centrifuged twice. The three supernatants were combined and reduced to dryness in vacuo. The dried extract was dissolved in 300 μl of methanol and 1200 μl of 5% formic acid in water, before being centrifuged at 25,000 × *g* at 4 °C for 10 min. Twenty microlitres aliquots of the supernatant were analysed by HPLC–MS$^2$.

### 2.4. Extraction of plasma

Triplicate samples of plasma collected 1 h after the ingestion of the fried onion meal were treated according to the method of Day et al. [13]. This involved adding 1.5 ml of acetonitrile to 500 μl of plasma. Samples were vortexed for 30 s every 2 min over a 10 min period, before centrifuging the mixture at 4000 × *g* at 4 °C for 10 min. The supernatant was collected and the pellet re-extracted as described above but with methanol instead of acetonitrile. Experiments with [$^{14}$C]quercetin-4′-glucoside and malvidin-3-glucoside showed recoveries of ca. 75% with the initial acetonitrile extraction which increased by a further 10–12% with the second methanolic extraction. The acetonitrile and methanol supernatants were combined and reduced to dryness in vacuo. Extracts were then dissolved in 25 μl of methanol plus 225 μl of 1% formic acid in water and centrifuged at 25000 × *g* at 4 °C for 10 min prior to the analysis of 100 μl aliquots of the supernatant by HPLC–DAD UV–MS$^2$ on the day of extraction.

### 2.5. Urine

The acidified frozen urine was defrosted, methanol added to make the solution 5% aqueous methanol, which resulted in any precipitated material being redissolved, and 100 μl aliquots were analysed directly by HPLC–MS$^2$ without further processing.

### 2.6. HPLC–DAD UV–MS$^2$

Samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, DAD–UV detector, scanning from 250 to 700 nm and an autosampler cooled to 4 °C (Thermo Finnigan, San Jose, USA). Separation was carried out using a 250 mm × 4.6 mm i.d. 4 μm Synergi Max-RP column (Phenomenex, Macclesfield, UK) eluted with a 60 min gradient of 5–40% acetonitrile in 1% formic acid at a flow rate of 1 ml min$^{-1}$ and maintained at 40 °C. After passing through the flow cell of the diode array detector the column eluate was split and 0.3 ml min$^{-1}$ was directed to a LCQ DecaXP ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan). Analyses utilised the negative ion mode for flavonols and positive ionisation for anthocyanins as this provided the best limits of detection. Analysis was carried out using full scan, data dependant MS$^2$ scanning from *m/z* 100 to 1000. Capillary temperature was 350 °C, sheath gas and auxiliary gas were 60 and 10 units, respectively, and the source voltage was 4 kV for negative ionisation and 1 kV for positive ionisation.

Additional analyses were carried out specifically for the separation of quercetin-3-glucuronide from quercetin-7-glucuronide. These used the same MS$^2$ conditions and negative ionisation, with a 250 mm × 4.6 mm i.d. Synergi Polar-RP column (4 μm; Phenomenex, Macclesfield, UK), maintained at 40 °C, eluted with a 60 min gradient 10–18% acetonitrile in 1% formic acid at a flow rate of 1 ml min$^{-1}$.

## 3. Results

### 3.1. Analysis of fried red onions

Gradient reversed-phase HPLC with absorbance detection and full scan data dependent MS$^2$ was used to identify and quantify the flavonol and anthocyanin content of the fried red onion meals. Absorbance at 365 nm and negative ionisation MS$^2$ were used for flavonol analysis. The total amount of flavonols in the 270 g onion meal was 275 ± 8.8 μmol. In keeping with the data of Tsushida and Suzuki [5], the

major components were quercetin-3,4′-diglucoside (**I**) (107 ± 1.4 μmol), quercetin-4′-glucoside (**II**) (143 ± 12 μmol) and isorhamnetin-4′-glucoside (**III**) (11 ± 1.4 μmol) which accounted for 95% of the 275 ± 8.8 μmole flavonol intake.

The anthocyanins were monitored at 515 nm and positive ion full scan $MS^2$ data were obtained. In accordance with previously published work seven anthocyanins were detected [15] and main components in the 270 g meal were cyanidin-3-glucoside (9.3 ± 0.3 μmoles), cyanidin-3-(6″-malonylglucoside) (48 ± 1.7 μmoles) and cyanidin-3-(6″-malonyllaminaribioside) (14 ± 0.5 μmoles). The total anthocyanin content of the onion meal was estimated to be 75 ± 2 μmoles.

### 3.2. Analysis of plasma and urine

Plasma collected one hour after ingestion of the fried red onions, and urine excreted 0–4 h after eating the meal, were also analysed by HPLC–DAD UV–$MS^2$. No peaks were apparent in the 515 nm, obtained with either urine or plasma. With the sample sizes analysed and the limit of detection at $A_{515\,nm}$, anthocyanins at levels >0.1% of the amounts ingested would have been detected. This finding is in keeping with other reports on the fate of dietary anthocyanins following absorption. The picture that has emerged is that a variety of anthocyanins appear in urine after supplementation with berries or berry extracts but at best in extremely low concentrations, typically 0.1% or less of the ingested dose [16,17].

In contrast to the anthocyanins, sizable quantities of flavonols were present in plasma and urine, corresponding to ca. 2% of the intake, with a total of 23 quercetin-based compounds being detected. Typical HPLC traces obtained at 365 nm are illustrated in Fig. 2A and B and the identifications based on $MS^2$ spectra are summarised in Table 1. The different classes of metabolites that were detected can be summarised as follows:

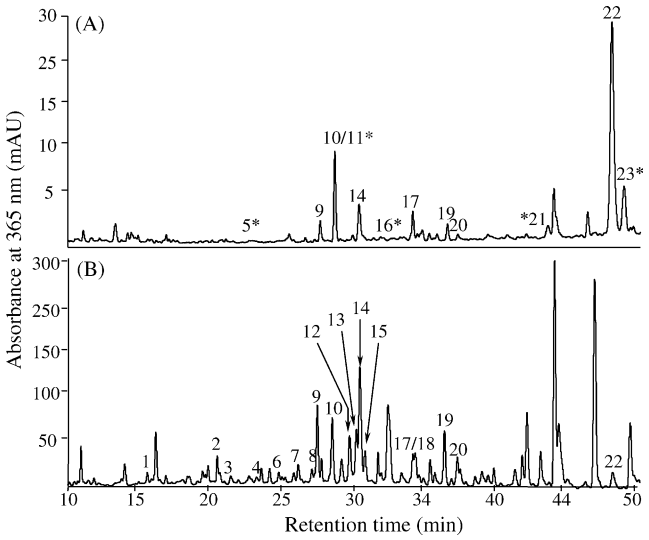


Fig. 2. Gradient reversed-phase HPLC with detection at 365 nm of quercetin metabolites in (A) a plasma extract and (B) urine obtained from a human volunteer after the consumption of fried red onions. For chromatographic conditions see Section 2.6. For identity of peaks 1–23, see Table 1. (*) Indicates peaks detected in samples from only one of six volunteers.

#### 3.2.1. Quercetin monoglucuronides

Three quercetin monoglucuronides were detected, each being characterised by a negatively charged molecular ion ($[M - H]^-$) at *m/z* 477 which on $MS^2$ fragments with a loss of 176 u, corresponding to the cleavage of a glucuronide unit, to produce an ion at *m/z* 301 from quercetin. Co-chromatography with reference compounds on the Synergi Max-RP HPLC column facilitated the identification of quercetin-4′-glucuronide (**VII**) (peak 18) and quercetin-3′-glucuronide (**VIII**) (peak 19). However, peak 10, a significant component in both plasma and urine, co-chromatographed with both quercetin-3-glucuronide (**IV**) and quercetin-7-glucuronide (**IX**). A second HPLC protocol using a Synergi Polar-RP column was therefore used to resolve these components (see Section 2.6). Quercetin-7-glucuronide had a $t_R$ of 34.6 min and quercetin-3-glucuronide eluted later with a $t_R$ of 36.2 min. When plasma and urine were analysed using the Polar-RP column, peak 10 co-chromatographed with quercetin-3-glucuronide and there was no evidence of the presence of quercetin-7-glucuronide. This finding refutes claims by Spencer et al. [18] that quercetin-7-glucuronide is one of the major in vivo metabolites of quercetin in humans.

#### 3.2.2. Quercetin diglucuronides

Low levels of three quercetin diglucuronides, peaks 1, 6 and 9, were present in urine. Each had a $[M - H]^-$ at *m/z* 653 which yielded $MS^2$ fragments at *m/z* 477 ($[M - H]^-$-176) and *m/z* 301 ($[M - H]^-$-352, loss of two glucuronide units). This indicates that the two glucuronyl units are attached at different positions on the quercetin skeleton. If they have been linked at the same position it is unlikely that a M-176 fragment would have been produced at *m/z* 477 as it has been shown that anthocyanin disaccharide conjugates fragment with loss of the intact disaccharide moiety [19].

#### 3.2.3. Methylquercetin monoglucuronides

Two methyl quercetin glucuronides were detected, peaks 17 and 20, which were characterised by a mass spectrum with fragment ions at *m/z* values 14 U higher than obtained with quercetin glucuronides. Co-chromatography with a reference compound established that peak 17 was 3′-methylquercetin-3-glucuronide (isorhamnetin-3-glucuronide, **V**). Peak 20 was tentatively identified as 3′-methylquercetin-4′-glucuronide (isorhamnetin-4′-glucuronide, **X**) on the basis of previous work [13].

#### 3.2.4. Methylquercetin diglucuronides

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

Table 1
HPLC–MS–MS identification of quercetin metabolites detected in plasma and urine after the consumption of 270 g of fried red onions by six human volunteers

| Peak | $t_R$ (min) | Compound | $[M-H]^-$ (*m/z*) | $MS^2$ fragments ions (*m/z*) | Location |
|---|---|---|---|---|---|
| 1 | 15.6 | Quercetin diglucuronide | 653 | 477 ($[M-H]^-$–GlcUA), 301 ($[M-H]^-$–GlcUA–GlcUA) | Urine |
| 2 | 20.4 | Methylquercetin diglucuronide | 667 | 491 ($[M-H]^-$–GlcUA), 315 ($[M-H]^-$–GlcUA–GlcUA) | Urine |
| 3 | 21.5 | Quercetin glucoside glucuronide | 639 | 477 ($[M-H]^-$–Glc), 463 ($[M-H]^-$–GlcUA), 301 ($[M-H]^-$–GlcUA–Glc) | Urine |
| 4 | 22.7 | Methylquercetin diglucuronide | 667 | 491 ($[M-H]^-$–GlcUA), 315 ($[M-H]^-$–GlcUA–GlcUA) | Urine |
| 5 | 22.8 | Quercetin-3,4′-diglucoside[a] | 625 | 463 ($[M-H]^-$–Glc), 301 ($[M-H]^-$–Glc–Glc) | Plasma |
| 6 | 24.8 | Quercetin diglucuronide | 653 | 477 ($[M-H]^-$–GlcUA), 301 ($[M-H]^-$–GlcUA–GlcUA) | Urine |
| 7 | 26.2 | Quercetin glucoside glucuronide | 639 | 477 ($[M-H]^-$–Glc), 463 ($[M-H]^-$–GlcUA), 301 ($[M-H]^-$–GlcUA–Glc) | Urine |
| 8 | 27.0 | Quercetin glucoside glucuronide | 639 | 477 ($[M-H]^-$–Glc), 463 ($[M-H]^-$–GlcUA), 301 ($[M-H]^-$–Glc–GlcUA) | Urine |
| 9 | 27.4 | Quercetin diglucuronide | 653 | 477 ($[M-H]^-$–GlcUA), 301 ($[M-H]^-$–GlcUA–GlcUA) | Urine, plasma |
| 10 | 28.4 | Quercetin-3-glucuronide | 477 | 301 ($[M-H]^-$–GlcUA) | Urine, plasma |
| 11 | 28.4 | Quercetin-3-glucoside[a] | 463 | 301 ($[M-H]^-$–Glc) | Plasma |
| 12 | 29.6 | Quercetin glucoside sulfate | 543 | 463 ($[M-H]^-$–$SO_3$), 381 ($[M-H]^-$–Glc), 301 ($[M-H]^-$–$SO_3$–Glc) | Urine |
| 13 | 30.1 | Quercetin glucuronide sulfate | 557 | 477 ($[M-H]^-$–$SO_3$), 381 ($[M-H]^-$–GlcUA), 301 ($[M-H]^-$–$SO_3$–GlcUA) | Urine |
| 14 | 30.3 | Quercetin glucuronide sulfate | 557 | 477 ($[M-H]^-$–$SO_3$), 381 ($[M-H]^-$–GlcUA), 301 ($[M-H]^-$–$SO_3$–GlcUA) | Urine, plasma |
| 15 | 30.6 | Quercetin glucoside sulfate | 543 | 463 ($[M-H]^-$–$SO_3$), 381 ($[M-H]^-$–Glc), 301 ($[M-H]^-$–$SO_3$–Glc) | Urine |
| 16 | 33.2 | Isorhamnetin-3-glucoside[a] | 477 | 315 ($[M-H]^-$–Glc) | Plasma |
| 17 | 34.1 | Isorhamnetin-3-glucuronide | 491 | 315 ($[M-H]^-$–GlcUA) | Urine, plasma |
| 18 | 34.4 | Quercetin-4′-glucuronide | 477 | 301 ($[M-H]^-$–GlcUA) | Urine |
| 19 | 36.3 | Quercetin-3′-glucuronide | 477 | 301 ($[M-H]^-$–GlcUA) | Urine, plasma |
| 20 | 37.2 | Isorhamnetin-4′-glucuronide | 491 | 315 ($[M-H]^-$–GlcUA) | Urine, plasma |
| 21 | 43.2 | Quercetin[a] | 301 | 179, 151 | Plasma |
| 22 | 47.9 | Quercetin-3′-sulfate | 381 | 301 ($[M-H]^-$–$SO_3$) | Urine, plasma |
| 23 | 48.3 | Quercetin sulfate | 381 | 301 ($[M-H]^-$–$SO_3$) | Plasma |

Peak numbers and HPLC retention times refer to HPLC trace in Fig. 2A and B. $t_R$: Retention time; $[M-H]^-$: negatively charged molecular ion; Glc: glucosyl unit; GlcUA: glucuronyl unit.

[a] Indicates compounds detected only in the plasma of one of the six volunteers.

### 3.2.5. Quercetin

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

### 3.2.6. Quercetin sulphates

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

### 3.2.7. Quercetin glucuronide sulphates

Peaks 13 and 14 both produced a $[M-H]^-$ at *m/z* 381 and $MS^2$ yielded ions at *m/z* 477 ($[M-H]^-$-80, loss of $SO_3$), *m/z* 463 ($[M-H]^-$-176, loss of a glucuronide unit) and *m/z* 301 (quercetin) indicating that both compound are quercetin glucuronide sulphates. Peak 14 was the main quercetin metabolite in the urine of all volunteers.

### 3.2.8. Quercetin glucosides

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

### 3.2.9. Quercetin glucoside glucuronides

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

### 3.2.10. Quercetin glucoside sulphates

Peaks 12 and 15 were characterised by a $[M-H]^-$ at *m/z* 543 which when fragmented produced $MS^2$ ions at *m/z* 463 ($[M-H]^-$-80, loss of $SO_3$), *m/z* 381 (M-162, loss of a glucoside unit) and *m/z* 301 demonstrating the presence of quercetin glucoside sulphates.

## 4. Discussion

The present study is the first to report on the use of HPLC with full scan $MS^2$ to analyse flavonols appearing in human plasma and urine. It provides a much more detailed analysis than was achieved in earlier studies carried out after the ingestion of onions which utilised HPLC with either $MS^2$ with selected reaction monitoring [12] or single stage MS in the selected ion monitoring mode [13]. Both these studies analysed the quercetin metabolites with MS in the positive ion mode. Negative and positive ionisation with our MS instrumentation provides very similar limits of detection for a standard of quercetin-3-glucuronide. However, when a urine sample is spiked with quercetin-3-glucuronide, analysis with negative ionisation is ca. 10 times more sensitive than positive ionisation because there is a much lower background and hence an enhanced signal-to-noise ratio. Signal to noise ratio was also greatly improved for the analysis of anthocyanins by reducing the ionisation voltage. However, although readily detected in onion extracts, the anthocyanin contents of plasma and urine were both below the limit of detection.

The data presented in this paper demonstrate the value of HPLC and data dependent full scan $MS^2$ for the analysis of trace levels of natural products in impure extracts. When reference compounds are available trace quantities of analyte can be identified while in the absence of standards $MS^2$ can facilitate a degree of structural elucidation, such as that obtained with quercetin glucuronide sulphates, methylquercetin glucuronides and quercetin diglucuronides (see Table 1), that would not be possible with traditional single stage MS. This has been discussed in some detail with specific reference to quercetin metabolites in earlier publications [20,21]. Further information on the position and orientation of substituent groups would require the use of NMR. However, this would involve not only extensive sample purification but also a requirement for several orders of magnitude more analyte than the low nanogram quantities required for HPLC–$MS^2$. With many of the plasma and urinary quercetin metabolites detected in trace amounts in the present study, this would not be a practical proposition.

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## References

[1] A. Crozier, Plants Diet and Health, British Nutrition Foundation, Chapman Hall, London, 2003, p. 27.
[2] M. Saltmarch, A. Crozier, B. Radcliffe, in: G. Goldberg (Ed.), Plants Diet and Health, British Nutrition Foundation, Chapman Hall, London, 2003, p. 101.
[3] G.G. Duthie, A. Crozier, in: G. Goldberg (Ed.), Plants Diet and Health, British Nutrition Foundation, Chapman Hall, London, 2003, p. 147.
[4] A. Crozier, M.E.J. Lean, M.S. McDonald, C. Black, J. Agric. Biol. Chem. 45 (1997) 590.
[5] T. Tsushida, M. Suzuki, Nippon Shokuhin Kagaku Kogaku Kaishi 42 (1995) 100.
[6] P.C.H. Hollman, J.M.P. van Trijp, M.N.C.P. Buysman, Anal. Chem. 68 (1996) 3511.
[7] P.C.H. Hollman, J.H.M. de Vries, S.D. Leuuwen, M.J.B. Mengelers, M.B. Katan, Am. J. Clin. Nutr. 62 (1995) 1276.
[8] P.C.H. Hollman, M. van der Gaag, M.J.B. Mengelers, J.M.P. van Trijp, J.H.M. de Vries, M.B. Katan, Free Rad. Biol. Med. 21 (1996) 703.
[9] A.A. Aziz, C.A. Edwards, M.E.J. Lean, A. Crozier, Free Rad. Res. 29 (1998) 237.
[10] A.L.A. Sesink, K.A. O′Leary, P.C.H. Hollman, J. Nutr. 131 (2001) 1938.
[11] A.J. Day, G. Williamson, Br. J. Nutr. 86 (2001) 105.
[12] J. Wittig, M. Herderich, E.U. Graefe, M. Veit, J. Chromatogr. B 753 (2001) 237.
[13] A.J. Day, F. Mellon, D. Barron, G. Sarrazin, M.R.A. Morgan, G. Williamson, Free Rad. Res. 35 (2001) 941.
[14] S.T. Caldwell, A. Crozier, R.C. Hartley, Tetrahedron 56 (2000) 4101.
[15] H. Donner, L. Gao, G. Mazza, Food Res. Int. 30 (1997) 637.
[16] T.K. McGhie, J. Agric. Food Chem. 51 (2003) 4532.
[17] J.M. Cooney, D.J. Jensen, T.K. McGhie, J. Sci. Food Agric. 84 (2004) 237.
[18] J.P.E. Spencer, G.G.C. Kuhnle, R.J. Williams, C. Rice-Evans, Biochem. J. 372 (2003) 173.
[19] M. Giusti, L.E. Rodríguez-Saona, D. Griffin, R.E. Wrolstad, J. Agric. Food Chem. 47 (1999) 4657.
[20] W. Mullen, B.A. Graf, S.T. Caldwell, R.C. Hartley, G.G. Duthie, C.A. Edwards, M.E.J. Lean, A. Crozier, J. Agric. Food Chem. 50 (2002) 6002.
[21] W. Mullen, R.C. Hartley, A. Crozier, J. Chromatgr. A 1007 (2003) 21.