In vitro glucuronidation of kaempferol and quercetin by human UGT-1A9 microsomes

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Abstract Flavonoids are important polyphenolic substances with widespread occurrence in plants and therefore in the human diet. Although considerable work has been done on the pharmacology of flavonoids, the understanding of their metabolism is still incomplete. In this work, the in vitro glucuronidation of the common dietary flavonoids quercetin and kaempferol by human UDP-glucuronosyltransferase microsomes (UGT-1A9) was investigated using HPLC and LC–MS. The two flavonoids were extensively metabolised by this enzyme with four monoglucuronides of quercetin and two of kaempferol being detected after incubation. The presence of a quercetin monoglucuronide in the urine of a volunteer after consumption of *Ginkgo biloba* tablets was demonstrated.

Key words: Quercetin; Kaempferol; Glucuronidation; Liquid chromatography–mass spectrometry; UDP-glucuronosyltransferase 1A9; *Ginkgo biloba*

1. Introduction

A large number of biological activities have been associated with flavonoids, including antiproliferative [1,2], antioxidant [3,4] and antiviral [5]. Also, there is epidemiological evidence of an inverse correlation between flavonoid intake and mortality from coronary heart disease [6] and stroke [7]. Central to the claims for a role of flavonoids in disease prevention, however, is the extent to which they are absorbed, metabolised and excreted as well as the possible biological activities of their metabolites. Early studies on flavonoid metabolism have concentrated on the acid fission products resulting after microbiological cleavage at the heterocyclic ring by intestinal bacteria [8]. However, with the introduction of analytical developments such as liquid chromatography–mass spectrometry (LC–MS) there is growing evidence that glucuronidation is central to flavonoid metabolism and absorption [9], especially for the more hydroxylated ones.

Quercetin and kaempferol (Fig. 1) are common dietary flavonoids and are of particular interest as antioxidants since they possess a 3-hydroxyl group of relatively low oxidation potential which is oxidised irreversibly thus avoiding redox cycling [10]. It has been demonstrated that high amounts of quercetin can be absorbed from onions [11] and that quercetin conjugates possess antioxidant properties [12]. In a recent study we showed that only small amounts of free quercetin and kaempferol could be detected by GC–MS in human urine from unsupplemented volunteers, but pre-treatment with β-glucuronidase significantly increased the levels detected [13], suggesting the presence of glucuronides of these compounds in urine.

A drawback of the study of xenobiotic glucuronidation has always been the lack of UDP-glucuronosyltransferase (UGT, EC 2.4.1.17) selective substrates and inhibitors, which has made difficult the interpretation of data obtained with human liver preparations. An alternative that was made possible only recently is the heterologous expression of UGT encoding cDNAs that allows the production of single enzyme isoforms for metabolism studies.

In this study we found that kaempferol and quercetin are extensively metabolised in vitro by human UGT-1A9 microsomes, with four monoglucuronides of quercetin and two of kaempferol being produced. The characterisation of these metabolites by LC–MS was successfully achieved and the presence of quercetin conjugates in human urine samples taken after consumption of *Ginkgo biloba* tablets (a plant rich in quercetin and kaempferol flavonoid glycosides) is demonstrated.

2. Materials and methods

2.1. Chemicals

Quercetin, kaempferol, uridine diphosphoglucuronic acid (UDP-GluA), 1,1,1-tris-hydroxymethyl ethane, β-glucuronidase (EC 3.2.1.31) and alamethicin were purchased from Sigma-Aldrich Chemical Co. (Poole, Dorset, UK). Solid phase extraction cartridges (Isolute ENV$^+$, 500 mg/3 ml) were purchased from Crawford Scientific (Strathaven, UK). Human UGT-1A9 (EC 2.4.1.17) microsomes from transfected lymphoblastoid cell lines were obtained from Cambridge Bioscience (Cambridge, UK). HPLC grade solvents were obtained from Merck (Poole, Dorset, UK). *G. biloba* tablets had a stated content of 28.8 mg of total flavonoid glycosides and were purchased from Boots (Glasgow, UK).

2.2. In vitro glucuronidation

A 0.5 ml reaction mixture containing 1 mg/ml of microsomal protein, 4.5 mM UDP-GluA in 100 mM Tris buffer pH 7.5 (containing 10 mM $MgCl_2$), 5 μg/ml of alamethicin and 300 μM of quercetin (or 400 μM of kaempferol) was incubated in a 1.5 ml Eppendorf vial at 37°C for 2 h. All incubations were carried out by adding ice-cold microsomal protein to a prewarmed (37°C) mixture of buffer/substrate/UDP-GluA. After initial mixing, incubation mixtures were not agitated. Blanks, to which no enzyme was added, and controls without cosubstrate (UDP-GluA) were run in parallel. The reaction was stopped by adding 0.5 ml of methanol. After incubation the samples were centrifuged at 4000 rpm for 20 min. The supernatant was transferred into a vial and 20 μl injected into the HPLC. Enzyme activity was determined similarly, but final incubation volume was 100 μl, and the incubation time was 3 h 20 min. Activities are expressed here as the amount of substrate consumed and not of product formed.

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2.3. Time course of metabolite production

Samples of kaempferol (400 μM) or quercetin (300 μM) were incubated as described above. At each time point, an aliquot of 50 μl of the incubate was removed and the reaction stopped by adding 50 μl of methanol. The samples were centrifuged at 4000 rpm for 20 min and 20 μl of the supernatant injected. Blank samples (no enzyme added) were run in parallel.

2.4. Hydrolysis with β-glucuronidase

An aliquot of 50 μl from sample incubates was taken and the solvent removed by heating at 70°C and blowing off with a stream of nitrogen. To the residue, 1000 units of β-glucuronidase in 50 mM acetate buffer pH 5 were added, in a final volume of 100 μl. Samples were incubated for 2 h at 37°C. A blank, to which no enzyme was added, was run in parallel. After incubation the samples were centrifuged at 4000 rpm for 20 min and 20 μl of the supernatant directly injected into the HPLC.

2.5. HPLC analysis

High performance liquid chromatography was done using a Thermoseparation Spectra Series P100 isocratic pump coupled with a Knauer variable wavelength detector set at 254 nm. Peak detection and integration was carried out using a Hewlett Packard HP 3395 integrator. Samples were introduced through a rheodyne injector fitted with a 20 μl loop. Separation was achieved using a Phenomenex Luna C18 column (75×4.6 mm, 3 μm). The mobile phase consisted of a mixture of 0.1% trifluoroacetic acid and methanol (6:4, v/v) and the flow rate was 1 ml/min.

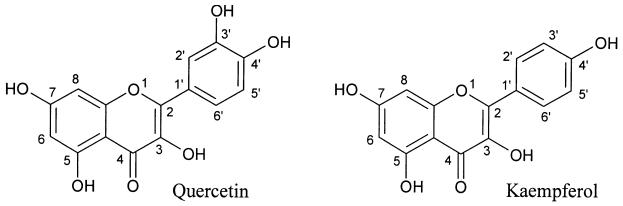

Fig. 1. Chemical structure of quercetin and kaempferol.

2.6. LC–MS

LC–MS experiments were done using a Thermoquest Finnigan Automass multi LC-GC–MS system. Separation was achieved with a Phenomenex Luna C18 column (75×4.6 mm, 3 μm). Samples (20 μl) were injected using a Finnigan Spectra System AS 3000 autosampler and introduced at a flow rate of 0.8 ml/min into the quadrupole mass spectrometer without splitting. The interface was operated in negative ion electrospray mode, with a cone voltage of −45 V. The drying gas temperature was set at 450°C. UV detection was carried out using a Finnigan Spectra System UV 6000 LP photodiode array detector to facilitate peak assignment. Mobile phase consisted of (A) 0.1% acetic acid and (B) methanol. Quercetin metabolites were separated using a linear gradient from 40 to 70% B in 20 min while kaempferol samples were analysed with a gradient from 40 to 88% B in 20 min.

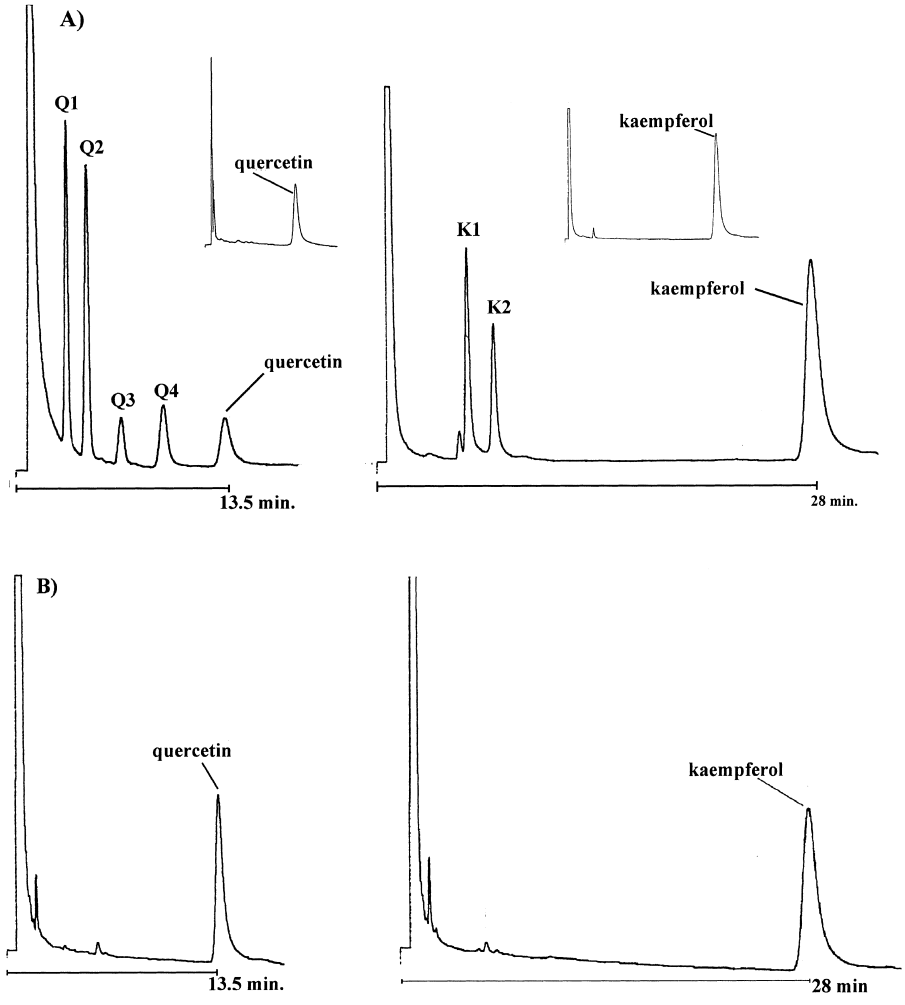

Fig. 2. A: Representative HPLC chromatograms of sample incubates containing 300 μM of quercetin (left) or 400 μM of kaempferol (right) with UGT-1A9 microsomes. Insets are blank incubates. B: HPLC chromatograms of aliquots taken from A after treatment with 1000 units of β-glucuronidase.

2.7. Collection and preparation of urine samples

A urine sample from a human volunteer with no dietary restrictions was taken 4 h after the ingestion of two tablets of *G. biloba*. The urine sample was kept in a freezer (at −20°C) until analysis. For LC–MS analysis a urine aliquot was centrifuged (10 000 × *g*, 5 min) and 5 ml of the supernatant was acidified with 1 ml of 0.3 M hydrochloric acid. Then, 4.8 ml of the acidified urine was extracted using Isolute ENV$^+$ solid phase extraction cartridges (500 mg/3 ml). The cartridges were conditioned by sequentially passing 4.0 ml of HPLC grade methanol and 2.0 ml of 0.05 M hydrochloric acid using a vacuum manifold that gave a flow rate of ca. 3.5 ml/min. After applying the sample, the cartridge was dried and then eluted with 3 × 1.0 ml of methanol. The solvent was blown off under a stream of nitrogen and the residue redissolved in 0.5 ml of methanol. The sample was filtered using a nylon syringe filter (0.45 μm) prior to injection into the LC–MS.

2.8. Statistics

Values are expressed as mean ± S.D. except when otherwise specified. Activities were compared using unpaired two sample *t*-test.

3. Results and discussion

Fig. 2A shows the HPLC chromatograms of samples containing 300 μM of quercetin or 400 μM of kaempferol after incubation with 1 mg/ml of UGT-1A9 microsomal protein for 200 min. Four metabolites of quercetin (Q1–Q4) and two of kaempferol (K1 and K2) are clearly identified and all of them were completely hydrolysed by treatment with β-glucuronidase (Fig. 2B).

The time courses of metabolite accumulation for quercetin and kaempferol (Fig. 3A,B, respectively) were similar and linear with time, with no latency period. The use of alamethicin in the incubation medium did not have any measurable effect on the activity of the enzyme towards the substrates. This is in contrast with claims from the supplier (Gentest, Massachusetts) which reports a two-fold increase on the activity of UGT-1A9 microsomes towards 7-hydroxy-4-trifluoromethylcoumarin with alamethicin.

Under similar conditions, the enzyme metabolised kaempferol and quercetin to a similar extent: 55.12 ± 0.45% and 51.93 ± 1.00% ($n = 4$) respectively. The extent of metabolism was affected by aliquoting the incubation medium, so that the values reported here are from experiments in which the substrates were left undisturbed for the whole incubation period (200 min). Thus, when multiple aliquots were taken (as in the time course experiments, see Fig. 3) the metabolism was comparatively reduced. The activity of UGT-1A9 was slightly but consistently ($P = 0.0001$, $n = 4$) higher towards kaempferol in comparison with quercetin (1083.38 ± 3.20 and 843.95 ± 3.20 pmol/min/mg protein respectively, $n = 4$).

The mobile phase conditions had to be adjusted to carry out LC–MS analysis in the negative ion mode since trifluoroacetic acid inhibited the formation of negative ions by the glucuronide metabolites. LC–MS analysis of the incubates (Fig. 4A,B) revealed that all metabolites consisted of monoglucuronides of the corresponding aglycones. In general the conjugates produced strong molecular ions (*m/z* 478 $[M-1]^-$ and *m/z* 462 $[M-1]^-$ for quercetin and kaempferol respectively) under the conditions used. Also, diagnostic ions corresponding to the quercetin and kaempferol aglycones (*m/z* 301 and *m/z* 285 respectively) formed by the loss of a glucuronic acid moiety $[M-177]^-$ were observed and it was indeed possible to increase the intensity of these ions by increasing the cone voltage. Adduct ions resulting from addition of acetic acid (60 amu) and methanol (32 amu) were also observed (Table 1).

The analysis of a urine sample taken from a human volunteer 4 h after consumption of two tablets of *G. biloba* revealed

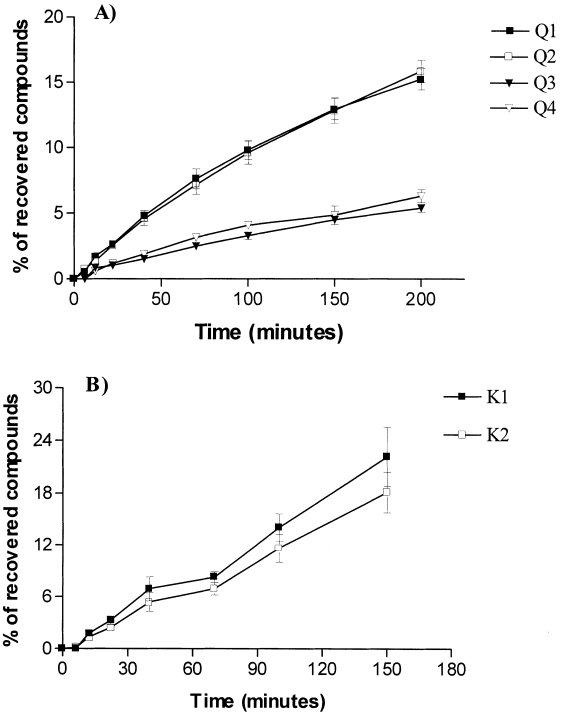

Fig. 3. Time course of metabolite accumulation in the glucuronidation of 300 μM quercetin (A) and 400 μM of kaempferol (B) by human UGT-1A9 microsomes as determined by HPLC. Each point is the mean ± S.E.M. of four experiments.

Table 1
Summary of spectral data for quercetin, kaempferol and their metabolites

Compound	Relevant ions in ES mass spectrum	UV band II maximum (nm)	UV band I maximum (nm)	Shifts in relation to aglycone[a] (nm)
Quercetin	301[b] $[M-1]^-$	254	370	
Metabolite Q1	478[b] $[M-1]^-$, 301 $[M-177]^-$	254	370	
Metabolite Q2	478[b] $[M-1]^-$, 301 $[M-177]^-$	256	354	−16 (band I)
Metabolite Q3	478[b] $[M-1]^-$, 301 $[M-177]^-$	256	366	−4 (band I)
Metabolite Q4	510[b] $[M+MeOH]^-$, 478 $[M-1]^-$, 301 $[M-177]^-$	254	368	
Kaempferol	285[b] $[M-1]^-$	256	364	
Metabolite K1	522[b] $[M+AcOH]^-$, 462 $[M-1]^-$, 285 $[M-177]^-$	256	366	
Metabolite K2	522 $[M+AcOH]^-$, 462[b] $[M-1]^-$, 285 $[M-177]^-$	258	344	−22 (band I)

[a]Shifts of less than 3 nm were not considered significant.
[b]Base ions.

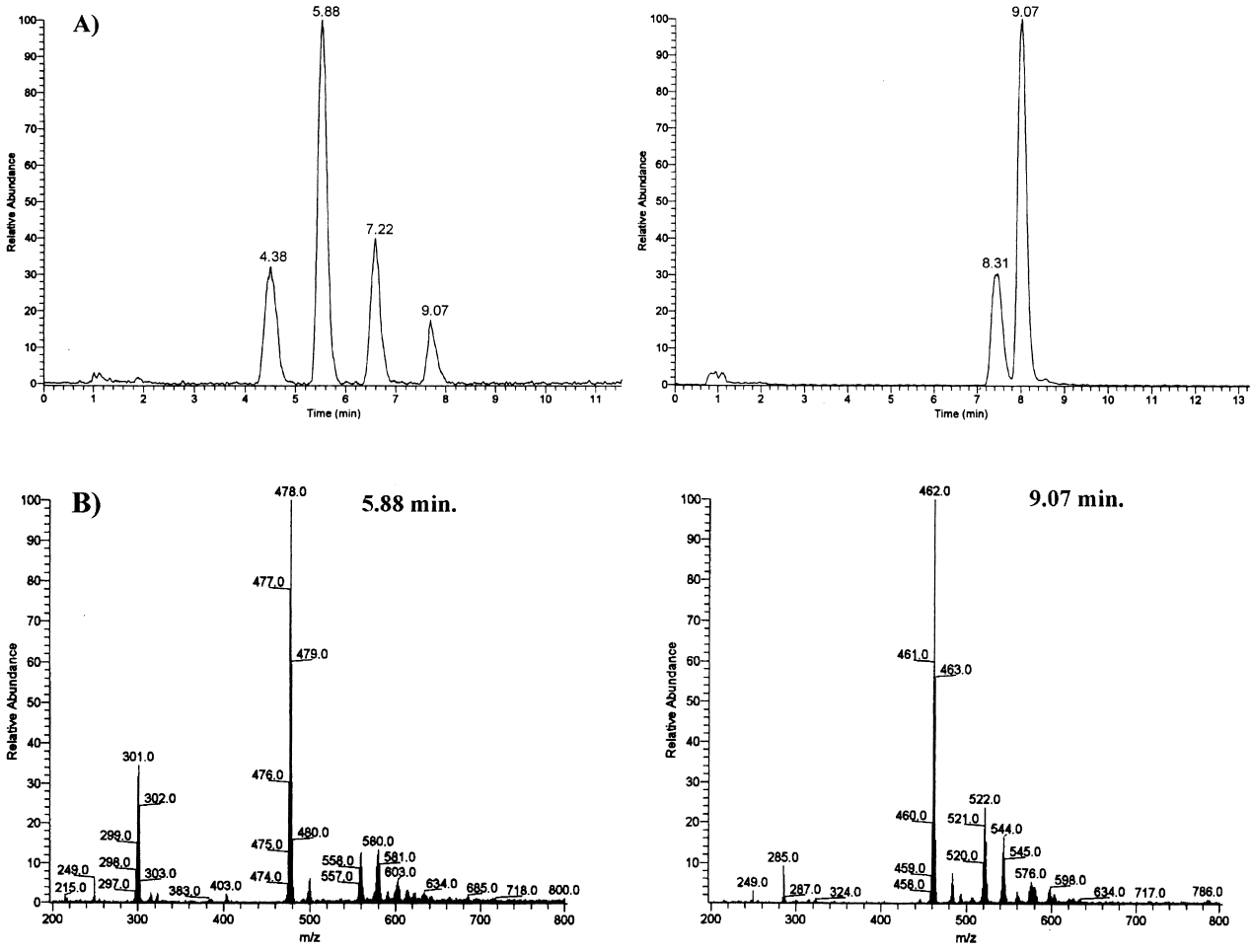

Fig. 4. A: LC–MS analysis of incubation samples of 300 μM quercetin (left) and 400 μM kaempferol (right). The traces are extracted ion profiles (*m/z* 460–461 for kaempferol and *m/z* 477–478 for quercetin) of a single linear scan. B: Typical negative ion electrospray mass spectrum of quercetin (left) and kaempferol (right) glucuronides.

a peak with the same mass and retention time of one of the quercetin glucuronides produced in vitro (Q2) (Fig. 5).

The human UDP-glucuronosyltransferase UGT-1A9 [14] is known to glucuronidate several bulky phenols including some flavonoids, and is probably one key enzyme in the metabolism of xenobiotic compounds [15]. This enzyme isoform is known to be expressed in human liver [16] and oesophagus [17] although little is known about other possible extrahepatic sites, such as intestine. The microsomes used here are from a lymphoblast cell line (AHH-1 $TK^{+/-}$) transfected with human liver UGT-1A9 cDNA by an episomal expression system. Glucuronidation is probably the main route for absorption and metabolism of flavonoids, specially the more hydroxylated ones, such as quercetin and kaempferol [9]. Indeed, it was demonstrated that the intestinal mucosa can glucuronidate flavonoid aglycones before absorption [18]. It thus seems reasonable to say that UGT-1A9 can potentially play a role in the absorption as well as in the metabolism of flavonoid aglycones. Indeed, tissue-specific extrahepatic expression of UDP-glucuronosyltransferases is thought to be part of a complex regulation of xenobiotic metabolism [17].

In this study it was not possible to determine the position at which glucuronidation occurs, but since all conjugates had different retention times we conclude that quercetin has to form at least one conjugate in which one of its hydroxyls from the catechol group is conjugated. Conjugation of the catechol system has been shown to lead to a decrease in the radical scavenging properties of 3′,4′-dihydroxylated flavonoids [3]. The large hypsochromic shifts of band I on the absorption spectra of metabolites K2 and Q2 suggest that these metabolites are the ones in which the glucuronic acid moiety is attached to the 3- position (see Table 1) since this position is much more acidic than the other positions and thus will be appreciably ionised in very dilute acetic acid.

It has recently been suggested that urinary and plasma levels of quercetin and kaempferol reflect short-term flavonoid intake and that the fraction of quercetin excreted in urine is only a small fraction of the amount ingested [19]. The determination of quercetin and kaempferol glucuronides in urine and plasma by LC–MS should thus accurately reflect flavonoid intake. Our results suggest that glucuronidation is indeed the main metabolic pathway for flavonoid metabolism and that UGT-1A9 plays a major role in flavonoid glucuronidation.

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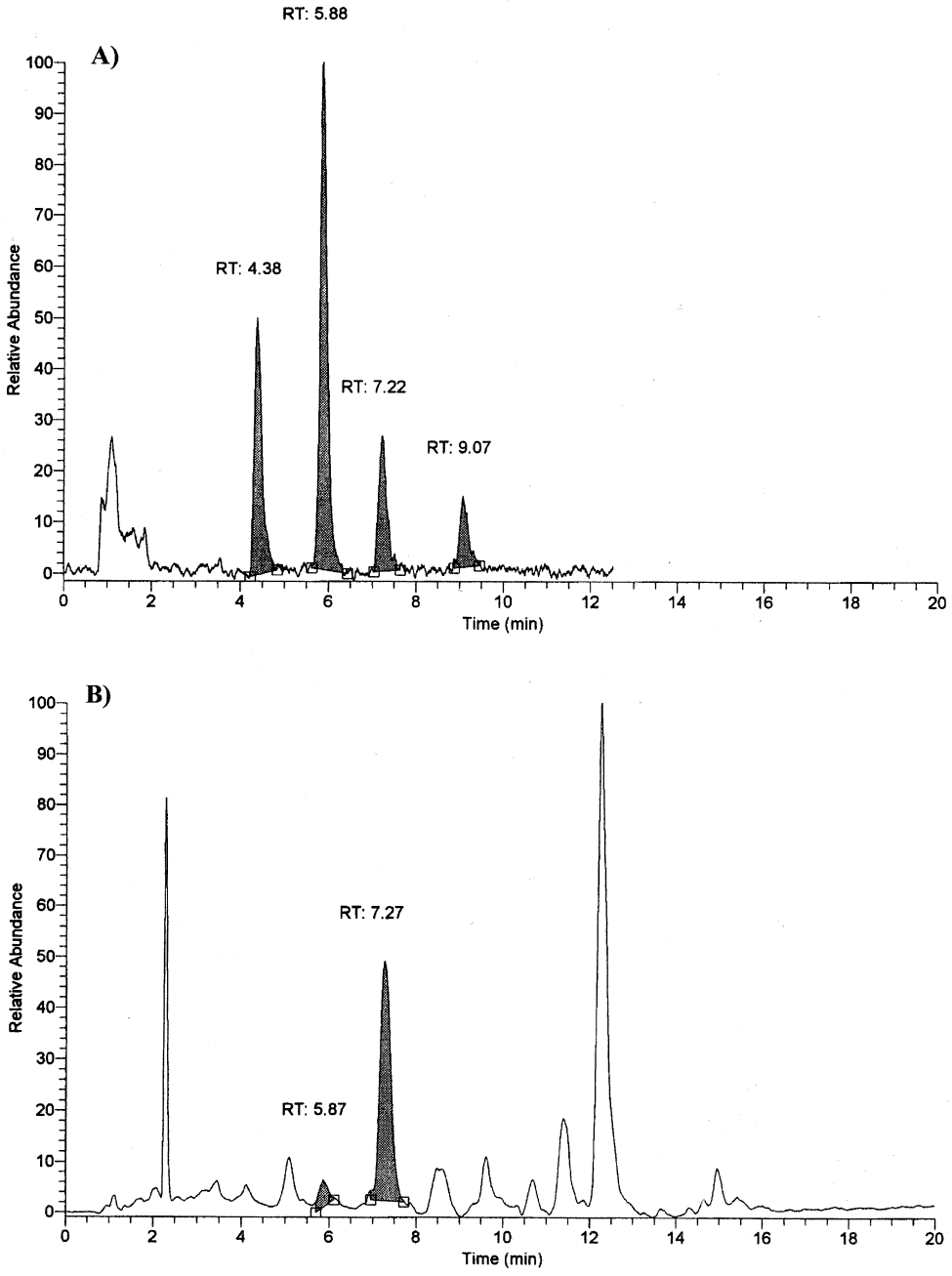

Fig. 5. A: LC–MS of a sample incubate containing 300 μM of quercetin with UGT-1A9 microsomes, showing the four monoglucuronides formed. B: LC–MS trace of a urine sample taken from a volunteer after consumption of two tablets of *G. biloba*. Traces were obtained from a selected ion monitoring scan ($m/z = 478$).

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