Eur Food Res Technol (2008) 227:1069–1076
DOI 10.1007/s00217-008-0821-2

ORIGINAL PAPER

Preparation of quercetin glucuronides and characterization by HPLC–DAD–ESI/MS

Montserrat Dueñas · Hugo Mingo-Chornet · José Joaquín Pérez-Alonso · Romina Di Paola-Naranjo · Ana M. González-Paramás · Celestino Santos-Buelga

Received: 16 October 2007 / Revised: 21 December 2007 / Accepted: 28 December 2007 / Published online: 23 January 2008

Abstract Quercetin is the main flavonol in the human diet, and the most commonly used in studies of biological activity. The major circulating forms of quercetin found in the human plasma after consumption of food containing distinct quercetin glycosides are glucuronides and sulfates. In this work quercetin glucuronides have been obtained from green beans (quercetin 3-glucuronide) and by enzymic synthesis (quercetin 4′-glucuronide) using a modification of the method described by Plumb et al. (Methods in polyphenol analysis, The Royal Society of Chemistry, Cambridge, pp 187, 2003) so as to improve the original low yields of that methodology. The method finally optimised got yields of 19% in the preparation of quercetin 4′-glucuronide, which allows its further isolation for their use in biological assays. In addition, quercetin 3′-glucuronide, 3-glucuronide and a diglucuronide were synthesised with lower yields. The compounds prepared have been employed to perform assays in order to obtain data for their identification by HPLC coupled to photodiode array detection and tandem mass spectrometry. It was observed that the analysis by HPLC–ESI/MS/MS could allow the identification of different quercetin glucuronides based on the presence of some minor key MS^2 fragments.

Keywords Quercetin · Metabolites · Yield · Enzymic synthesis · HPLC–ESI/MS/MS

M. Dueñas (✉) · H. Mingo-Chornet · J. J. Pérez-Alonso · R. Di Paola-Naranjo · A. M. González-Paramás · C. Santos-Buelga
Grupo de Investigación en Polifenoles,
Unidad de Nutrición y Bromatología,
Facultad de Farmacia, Universidad de Salamanca,
Campus Miguel de Unamuno, 37007 Salamanca, Spain
e-mail: mduenas@usal.es

Introduction

Flavonoids are polyphenolic secondary metabolites with a widespread occurrence in the plant kingdom. Among the major flavonoids of dietary importance are flavonols, which occur ubiquitously in many commonly consumed fruits and vegetables. Several epidemiological studies have reported that a high flavonol intake is associated with a reduced incidence of major diseases, such as cancer and cardiovascular disease [2–5]. Quercetin is the main flavonol in the human diet and the most commonly used in studies of biological activity. Much attention has been paid to its antioxidant and free-radical scavenging properties [6] and biological activities, such as antithrombotic and anticarcinogenic activities [7]. Also quercetin is an effective inhibitor of xanthine oxidase and lipoxygenase, enzymes involved in processes such as inflammation, atherosclerosis, cancer and ageing [8, 9]. The biological activity of the flavonols is predicted to be highly dependent on their structure, particularly the availability of hydroxyl groups. It has been indicated that the higher the number of free hydroxyl substituents, the stronger the antioxidant effect, thus glycosylation affecting the antioxidant properties [6, 10, 11]. Quercetin is present in plants and food almost exclusively in the form of β-glycosides that are little affected by the industrial and home processing of the products [12, 13]. However, these type of derivatives would hardly be found as such at biologically significant levels in their sites of action in the organism, since they are largely metabolised in the human body. Some quercetin glycosides can be deglycosylated by endogenous β-glycosidases in the small intestine, such as cytosolic β-glycosidase (CBG) or lactase-phlorizin hydrolase (LPH) [14–16]. In addition, some quercetin glucosides could be transported by epithelial brush border membrane carriers, like sodium-

dependent glucose transporter-1 (SGLT-1) and then deglycosylated in the enterocyte by intracellular β-glycosidases [17–20]. Not all flavonoid glycosides interact with membrane transporters or are potential substrates for β-glycosidases and there are also large inter-individual variations in the activity of these enzymes, making the rate of intestinal absorption highly variable [16]. The absorbed quercetin aglycone is rapidly conjugated firstly in enterocytes and further in the liver [21]. Methylation to isorhamnetin (quercetin 3′-methylether) seems to be an important step in quercetin metabolism, however, the free forms of quercetin and isorhamnetin are hardly detected in plasma leading to the hypothesis that the conjugated metabolites should play a key role in the putative healthy effects associated with dietary quercetin. Around 20 different quercetin-conjugated metabolites have been described [22], being the major circulating forms: quercetin-3-glucuronide, quercetin-3′-sulphate, isorhamnetin-3-glucuronide and an incompletely characterised quercetin-diglucuronide, which are accompanied by minor levels of the 4′-glucuronides of quercetin and isorhamnetin [23–26]. The nature and position of the substitutions will affect the biological activity; therefore, in order to better determine the effects of dietary quercetin, it is necessary to assess the biological activity of its different metabolites and to be able to characterise their profiles in human plasma after consumption of food containing distinct quercetin glycosides. Various methods have been described to obtain conjugates, involving extraction from plants, isolation from blood after consumption of the flavonoid or chemical, enzymic and microbiological synthesis [1, 27–29].

In this work, quercetin glucuronides have been obtained from a plant source and by enzymic synthesis using a modification of the method described in Plumb et al. [1] so as to improve the original low yields of that methodology. Further, the compounds prepared have been employed to perform assays in order to obtain data for their identification by HPLC coupled to photodiode array detection and tandem mass spectrometry.

Materials and methods

Materials

Quercetin was purchased from Sigma–Aldrich (USA). Isorhamnetin was purchased from Extrasynthèse (Genay, France). HPLC-grade methanol and acetonitrile were purchased from CarloErba (Italy) and Merck KGaA (Germany), respectively. Trifluoroacetic acid was purchased from Riedel-de-Haën (Germany). Analytical grade acetic acid glacial and ammonia were purchased from Panreac (Spain). Hepes buffer solution was obtained from Fluka (Switzerland). Polyamide 6 was purchased from Fluka (Germany). UDP-glucosamine sodium salt was purchased from Sigma–Aldrich (Germany). UDP-glucuronic acid was obtained from Fluka (USA).

Enzymic synthesis of quercetin and isorhamnetin glucuronides

Quercetin and isorhamnetin glucuronides were produced enzymatically using pig liver microsomal enzymes with a modification of the methodology described by Plumb et al. [1]. A post-lysosomal fraction was obtained from a pig liver extract which was freshly prepared by the method of Lambert [30], using 12 g of pig liver and a buffer BisTris propane solution (20 mM, pH 7.0), containing sucrose (0.25 M) and KCl (0.15 M). The final supernatant after centrifugation at 4,000*g* during 20 min at 5°C, was mixed with quercetin or isorhamnetin (400 μM). This mixture was incubated at 37°C using different reaction times: 60, 120, 180 and 240 min, in a Hepes buffer (25 mM, pH 5.5 and 7.2) or ultra-pure water, containing in either case 10 mM $MgCl_2$, UDP-glucuronic acid (final concentration 8 mM) and UDP-glucosamine (final concentration 4 mM). The reaction was stopped with 100% methanol containing ascorbic acid (1 mM) and centrifuged for 10 min at 4,000*g* and 5°C. The products obtained were analysed by HPLC–DAD–MS.

Isolation of quercetin glucuronides

Quercetin glucuronides obtained in the synthesis were pre-purified using a C-18 sorbent column and vacuum elution. The column was preconditioned with methanol followed by water. The synthesis extract was previously concentrated under vacuum at 30°C until the methanol added for stopping the synthesis was eliminated. The extract was deposited onto the column and was firstly washed with phosphate buffer (pH 7) and water to remove interferences. Quercetin glucuronides were eluted with MeOH:5% acetic acid (50:50 v/v). This fraction was concentrated under vacuum to be further used for the isolation of compounds by semi-preparative-HPLC in a Waters 600 chromatograph coupled to an UV–VIS model 486 detector. The column was a reversed-phase Supelco Ascentis C18 (5 μm packing, 250 × 10 mm, i.d). Elution conditions were as follows: 3 ml min^{-1} flow rate; room temperature; solvent A, water/acetic acid (95:5 v/v); solvent B, methanol (100%); elution from 15 to 30% B in 10 min, from 30 to 50 in 20 min, and then an isocratic step for 15 min with 50% B followed by washing and re-equilibration of the column. The different glucuronide fractions were concentrated to dryness under vacuum, dissolved in a small amount of water and then freeze-dried. The purity of the quercetin glucuronides compounds was checked by HPLC–DAD and MS.

Isolation of quercetin-3-*O*-glucuronide from a plant source (green beans)

Seeds were removed and the green bean pods were homogenized in 70% MeOH, using a polytron homogenizer (Kinematica, Littau, Switzerland) and then kept for 16 h at −18°C. Subsequently, they were centrifuged for 20 min at 4,000*g* and 5°C. This process was repeated twice, using times of maceration of 4 h to complete a total of 24 h of extraction. The extracts were combined and concentrated under vacuum until the methanol was removed and the resultant aqueous phase was defatted by extraction with *n*-hexane in a Soxhlet apparatus. The defatted extract was fractionated on a polyamide column that had been preconditioned with methanol followed by water and phosphate buffer (pH 7.0, 0.1 M) under vacuum according to Price et al. [13]. The extract was washed firstly with phosphate buffer to eliminate phenolic acids and further with methanol to elute the neutral flavonols (flavonols containing neutral sugars) and with methanol/ammonia (99.5:0.5 v/v) to elute the acidic flavonols (i.e. glucuronides). This last fraction was evaporated under vacuum for posterior purification of the quercetin glucuronide by semipreparative-HPLC using the same protocol above described.

HPLC–DAD–MS analysis

Quercetin glucuronides were analysed using a Hewlett-Packard 1100 series liquid chromatograph. Separation was achieved on an AQUA (Phenomenex, Torrance, CA) reversed phase C18 column (5 µm, 150 mm × 4.6 mm i.d) thermostatted at 35°C. The solvents used were: (A) 0.1% TFA in water, and (B) 100% HPLC-grade acetonitrile. The gradient employed was: isocratic 10% B for 3 min, from 10 to 15% B over 12 min, isocratic 15% B for 5 min, from 15 to 18% B over 5 min, from 18 to 30% B over 20 min and from 30 to 35% over 5 min, at a flow rate of 0.5 ml min^{-1}. Detection was carried out in a diode array detector (DAD), using 370 and 280 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

MS analyses were performed in a Finnigan™ LCQ (Thermoquest, San Jose, CA) equipped with an API source, using an electrospray ionisation (ESI) interface. Both the sheath gas and the auxiliary gas were nitrogen at flow rates of 6 and 1.2 L min^{-1}, respectively. The capillary voltage was 4 V and the capillary temperature 195°C. Spectra were recorded in positive and negative ion mode between *m/z* 120 and 1,500. The mass spectrometer was programmed to do a series of three consecutive scans: a full mass, a MS^2 scan of the most abundant ion in the full mass, and a MS^3 of the most abundant ion in the MS^2, using a normalised energy of collision of 45%.

Results and discussion

Enzymic synthesis of quercetin glucuronides

In vitro glucuronidation of quercetin and isorhamnetin were performed using a freshly prepared post-lysosomal fraction of pig liver, to obtain glucuronides analogous to those formed in vivo. With this aim, minor modifications were introduced in the method described in Plumb et al. [1] in order to try to improve the yields of metabolites obtained when the original protocol was applied. Thus, different pH values (5.5 and 7.2 in Hepes buffer, and 8.3 in water) incubation times ranging from 60 to 240 min were checked. In all cases, similar profile of metabolites was produced (Fig. 1) but with different yields. Four peaks corresponding to glucuronides were formed that in a first approach were identified based on their absorption and mass spectra and elution profile compared to the results previously obtained by other authors [1, 27, 31]. Peak 1 showed λ_{max} at 370 nm and a pseudo-molecular ion $[M-H]^-$ at *m/z* 653. Its MS^2 gave a fragment at *m/z* 477 ($[M-176]^-$, loss of a glucuronide moiety) whose MS^3 produced a signal at *m/z* 301 ($[M-176]^-$, loss of a second glucuronide moiety) corresponding to quercetin. The compound was thus identified as a quercetin diglucuronide. Peaks 2, 3, 4 showed λ_{max} at 365 nm and the same pseudo-molecular ion in negative mode at *m/z* 477. Their MS^2 gave always one fragment at *m/z* 301 ($[M-176]^-$, loss of a glucuronide moiety). These three compounds were respectively assigned to quercetin 3-glucuronide, quercetin 4′-glucuronide and quercetin 3′-glucuronide according to their retention characteristics in HPLC [1]. Peak 5 corresponded to quercetin as showed by its λ_{max} (370 nm) and molecular ion at *m/z* 301. Quercetin monoglucuronides presented a hypsochromic shift of about

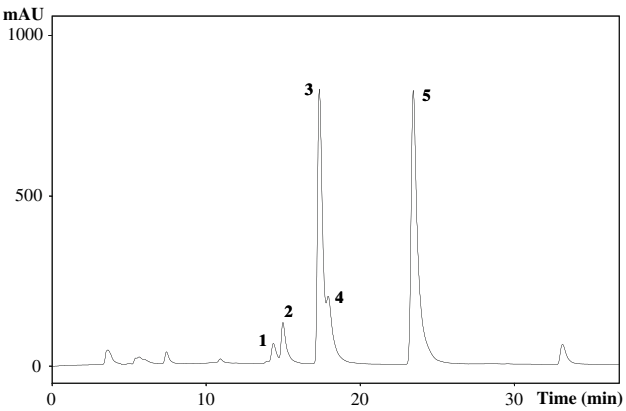

Fig. 1 HPLC chromatogram recorded at 370 nm showing the profile of quercetin glucuronides obtained using a post lysosomal fraction of pig liver after 2 h of incubation. Peak identification: *1* quercetin diglucuronide; *2* quercetin 3-glucuronide; *3* quercetin 4′-glucuronide; *4* quercetin 3′-glucuronide; *5* quercetin

5 nm in their λ_{max} in the visible region of their absorption spectra with regard to the parent aglycone as also found by Justino et al. [32].

Figure 2 shows the levels of quercetin metabolites formed at different pH values using an incubation time of 240 min. A similar distribution was obtained at the three pH values assayed being always quercetin 4′-glucuronide the predominant compound followed by quercetin 3′-glucuronide. No relevant differences were found in the distribution of the different products in the syntheses carried out in Hepes buffer at pH 5.5 and 7.2, with similar yields in the production of quercetin 4′-glucuronide and 3′-glucuronide of around 16 and 5%, respectively. These yields improved when the synthesis was carried out at pH 8.3 in water up to 19% for quercetin 4′-glucuronide and 7% for the 3′-glucuronide. Lower yields were obtained for the other two metabolites: ∼1% for the diglucuronide at all pH values and ∼3% for the 3 glucuronide at pH 8.3. Different incubation times were also checked. Maximum amounts for the 3′and 4′glucuronides were produced at 180 min, whereas better yields of the quercetin diglucuronide and 3-glucuronide were obtained at shorter times of incubation (60 min). At 240 min, the concentrations of all the metabolites slightly decreased probably due to the hydrolysis of the glucuronide moieties, as observed for the increase in the peak of quercetin in the HPLC chromatograms.

In the light of these observations, further syntheses were carried out in water (final pH 8.3 without further adjustment) using an incubation time of 180 min. The use

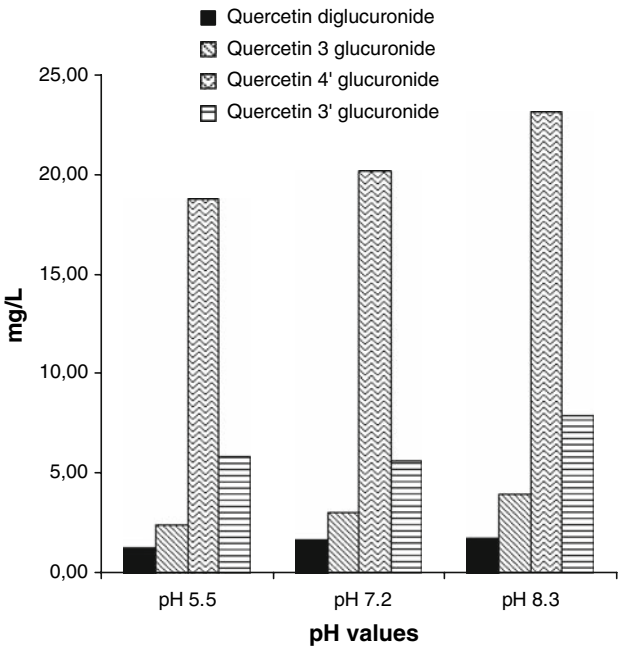

Fig. 2 Quercetin glucuronides concentrations formed at different pH values using an incubation time of 240 min

of water instead of Hepes buffer makes the synthesis much cheaper, which is relevant, taking into account that it is necessary to use large volumes of buffer when sufficient quantities of metabolites have to be prepared for their use in assays of biological activity in in vitro and animal models. Once synthesised, the glucuronides were isolated by semi-preparative-HPLC after pre-purification on a C-18 column. This step was important to clean up crude extracts from side compounds and remove the excess of free quercetin.

Similar methodology was applied using isorhamnetin (3-*O*-methylquercetin) as substrate for the synthesis, with the aim of obtaining isorhamnetin glucuronides. In this case, the yields were much lower than those obtained for quercetin and did not exceed 1% for the majority metabolite (isorhamnetin 4′-glucuronide), which might be either attributed to destabilisation factors involved with regard to the accommodation of the substrate into the active site of the enzyme or by the sterical restrictions imposed by the presence of the methyl group at 3′ position of the aglycone.

A relevant observation was that the yield in the synthesis of glucuronides was highly affected by the microsomal preparations of pig liver used. Freezing and further thawing of the liver notably reduces the yields, especially after a long storage in cold. Thus, it was very important to use recently obtained fresh liver and even in that case great variability could be obtained depending on the animal. Another observation made was that some quercetin was released due to glucuronide cleavage during evaporation of the metabolites fractions catalysed by acetic acid, as also pointed out by Needs and Kroon [28]. In the pre-purification step this could be prevented by substituting acetic acid by water, although the use of the acid could not be avoided for the efficient separation of compounds by semi-prep HPLC. Addition of water to the extracts prior to evaporation reduced glucuronide cleavage, although a second purification by column chromatography might still be necessary in case that some quercetin was released.

Using the optimised protocol, reasonable amounts (yields around 19%) of quercetin 4′-glucuronide could be obtained and purified, as well as some quercetin 3′-glucuronide. However, we failed in the production of satisfactory amounts of the quercetin diglucuronide and quercetin 3-glucuronide, this latter being identified as a major metabolite in the human body. For that reason, we tried its isolation from green beans, in which its presence had been previously described [13]. Extraction of the plant material with 70% methanol followed by fractionation on a polyamide and further isolation by semipreparative HPLC allowed us to obtain that glucuronide with satisfactory yields in an easy and cheaper way (around 7 mg of quercetin 3-glucuronide per kilogram of plant material). The compound isolated also served for the confirmation of the identity of the

peak assigned to quercetin 3-glucuronide in the crude extracts obtained after enzymic synthesis.

ESI/MS–MS analyses of quercetin glucuronides

Different ESI/MS–MS experiments were first performed by direct infusion of the quercetin 3-glucuronide isolated from green bean in the mass spectrometer in positive and negative ion mode, in order to determine the best conditions for the analysis of glucuronides. It was observed that the fragmentation profile obtained in positive ion mode differed according to the collision energy employed. However, more reproducible fragmentation was obtained in negative mode. Furthermore, it was reported that negative ionisation was about ten times more sensitive than positive one for quercetin and its derivatives [25, 33]. Thus, the following assays were carried out using negative ionisation.

Experiments were then carried out with direct infusion in ESI/MS–MS of quercetin 3-glucuronide and quercetin 4′-glucuronide in negative ion mode using increasing collision energies. No fragmentation patterns were obtained that could be useful for the assignment of the substitution position of the glucuronide moiety onto the aglycone, neither on the basis of some characteristic key fragments nor on differences in the relative abundance. Davis et al. [34] using direct-infusion ESI/MS–MS also concluded that collision-induced dissociation (CID) of the deprotonated flavonoid glucuronides did not provide a distinctive fragmentation pattern indicative of a particular site of glucuronidation. In his case only one ion corresponding to the cleavage of the bonds 0 and 3 in the glucuronide residue (*m/z* at 373) as significant product ion (>5% relative abundance) was described. This fragment was not observed in our case, where three fragments that could be attributed to cleavage of the glucuronide moiety were obtained at *m/z* 383, 363 and 341 and the main product ion was always the corresponding to the aglycone at *m/z* 301.

The different glucuronides isolated were then separately analysed by HPLC–ESI/MS–MS with quite different results to those obtained by direct infusion. The results are shown in Table 1. The use of different collision energies yielded common fragments for the quercetin diglucuronide ($[M-H]^-$ at *m/z* 653) at *m/z* 477 $[M-176]^-$ and 301 $[M-352]^-$ corresponding to losses of the two glucuronide units. The successive losses of both moieties indicate that the two glucuronyl units are linked at different positions on the quercetin skeleton. If they were linked at the same position (i.e. constituting a disaccharide) only one fragment corresponding to the aglycone should have been observed [35]. There were differences in the relative abundance among the distribution of the fragment ions at *m/z* 477 and 301; in all cases the abundance of ion at *m/z* 477 was 100%, however the ion at *m/z* 301 presented decreased relative abundances with higher collision energies. A product ion at *m/z* 501 was only observed at 25 and 30% relative collision energies, which was assigned to the Retro Diels-Alder fission (RDA) of quercetin [36], and another fragment at *m/z* 516 appeared at 35 and 45% collision energies, attributed to the loss of 137 amu from the cleavage of two C–C bonds at positions 0/2 of the C ring of quercetin. Although the relative abundance of these fragments was <2%, they are quite interesting since they indicate that the two glucuronide moieties were located on the ring A of the quercetin; although no further information could be obtained to decide about the precise location of each moiety, it can be supposed that they could be located on positions 5 and 7 on that ring.

The main fragment obtained for quercetin 3-glucuronide at all the collision energies was at *m/z* 301 corresponding to the aglycone. The rest of the fragments formed derived from quercetin. Signal at *m/z* 273 would correspond to the loss of the CO group $[M-28]^-$, *m/z* at 257 to the loss of CO_2, and *m/z* at 229 to the loss of both. This latter fragment was only observed at the highest collision energies assayed (40 and 45%). These unusual losses of CO and CO_2 appear to be characteristic of the negative ion mode [36] and the fragment at *m/z* 229 was considered to be characteristic of quercetin [24, 37]. Other characteristic fragments of quercetin are those at *m/z* 151 and 179 corresponding respectively to the A^- ring fragment released after RDA fission and the retrocyclization after fission on bonds 1 and 2 (Fig. 3). The fragment at *m/z* 175 ($[M-126]^-$) is attributed to the loss of a phloroglucinol moiety from the loss of the ring A after cleavage of bonds 1 and 4 in C-ring (Fig. 3). Fragments at *m/z* 255, 229 and 151 have been used by some authors to identify quercetin glucuronides in urine and plasma samples, when its concentration was negligible [22, 24, 25].

Some fragments that did not appear for quercetin 3-glucuronide were observed for the isomers at 3′ and 4′. In particular, signals at *m/z* 343, which corresponds to the cleavage of the bonds 1 and 5 in the glucuronide residue ($[M-134]^-$), and at *m/z* 415 that may be attributed to the elimination of a water molecule from the ion at *m/z* 433. It was observed that the relative abundance of ion at *m/z* 415 increased at higher collision energies, whereas the ion at *m/z* 433 disappeared. The appearance of these fragments suggests that a strong linkage of the glucuronide moiety exists when it is located on the quercetin B ring, thus they could be considered characteristic of substitution of that ring. A distinctive characteristic of quercetin 3′-glucuronide seems to be that a higher number of fragments appear than for the isomers at 3 and 4′positions. The fragment at *m/z* 327 would correspond to the cleavage of bonds 1 and 3 in the quercetin moiety ($^{1,3}A^-$) and can be explained by the mechanism proposed for protonated flavonols by Ma et al. [37], who reported that this loss together with the cleavage of the

Table 1 Principal MS^2 ions (in abundance order) formed in negative ion mode at different collision energies for glucuronide metabolites following HPLC–ESI/MS–MS analysis

EC (%)	Quercetin diglucuronide	Quercetin-3-glucuronide	Quercetin-4′ glucuronide	Quercetin-3′ glucuronide	Isorhamnetin 4′-glucuronide
	Molecular ions	Molecular ions	Molecular ions	Molecular ions	Molecular ions
20	477	301	301	301	315
	494	477	175	459	175
	536	175[a]	477[a]	175	473
	301		343[a]	433	491
	653		257[a]	415	431[a]
				371[a]	
				477[a]	
25	477	301	301	301	315
	301	477[a]	415	371	175
	536	273[a]	175	415	473
	494	179[a]		175	491[a]
	501[a]	175[a]		343[a]	
	653[a]	151[a]		327[a]	
				477[a]	
30	477	301	301	301	315
	301	179	175	459	175
	494	151	343	415	431[a]
	501[a]	273[a]	151[a]	371[a]	
				343[a]	
				327[a]	
				175[a]	
35	477	301	301	301	315
	494	179	343	179	175
	301	151	179	175	357
	516[a]	273	151	459	399
				371	473[a]
				415	
				151	
				327	
				433[a]	
40	477	301	301	301	315
	301	273	343	415	473
	494	175	175	459	175
	536	151	151	179	151
	593	257[a]	415	371	399[a]
	501	229[a]		151	357[a]
				175[a]	
45	477	301	301	301	315
	301	179	343	415	175
	494	151	179	327	431
	516	273	273	179	151[a]
	536[a]	257[a]	151	151	
		229[a]	415	373	
				343	
				175	
				257[a]	

[a] Relative abundance ≤2%

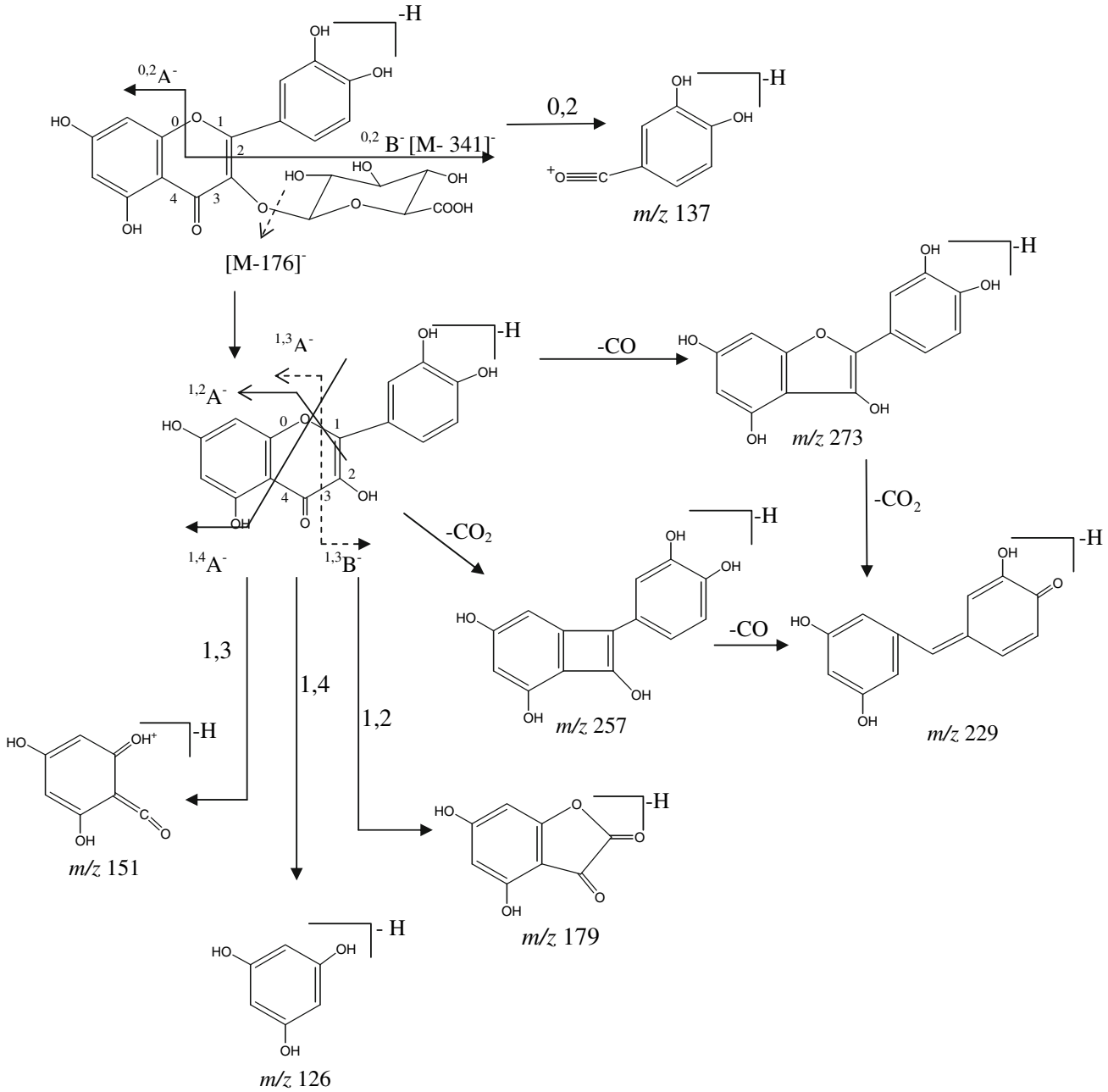

Fig. 3 Main fragmentations of quercetin glucuronides in negative ion mode

bonds 0 and 2 in the flavonol could be a characteristic feature of flavonol aglycones. Fragment at *m/z* 371 may be attributed to the elimination of 2H and cleavage of the bonds 0 and 3 in the glucuronide residue $[M\text{-}104]^-$. Signals at *m/z* 459 and *m/z* 433 would correspond to the loss of water $[M\text{-}18]^-$ and a carboxyl group $[M\text{-}44]^-$, respectively.

Another quercetin metabolite usually identified in plasma and urine is isorhamnetin 4′-glucuronide. It was also enzymatically synthesised and isolated in the same way as quercetin glucuronides and submitted to HPLC–ESI/MS–MS analysis. This compound presented a pseudomolecular ion $[M\text{-}H]^-$ at *m/z* 491, 14 amu higher than quercetin glucuronides. One fragment at *m/z* 315 was obtained at all the collision energies used, with an abundance relative of 100%, corresponding to the aglycone. Other fragments were obtained at *m/z* 473 (loss of a water molecule), *m/z* 431 (loss of CH_3OH and CO, $[M\text{-}60]^-$). Fragments at *m/z* 175 and 151 were also observed as for the quercetin glucuronides. The fragment at *m/z* 175 would correspond to the loss of two CO groups and $C_4H_4O_2$ $[M\text{-}140]^-$, and that at *m/z* 151 is considered to originate from cleavage of bonds 0 and 2 of the of the heterocyclic ring ($^{0,2}B^-$) $[M\text{-}166]^-$.

Both fragments were also observed for the isorhamnetin aglycone by Ma et al. [37].

Conclusion

The results obtained pointed out that it could be possible to draw conclusions about the substitution position of the glucuronides by HPLC–ESI–MS/MS based on some minor product ions released after MS–MS fragmentation. Thus, the observation of a quercetin RDA fission fragment in the case of the diglucuronide allowed locating these moieties on the quercetin A ring. Also, the appearance of a fragment at *m/z* 343, corresponding to the cleavage of the bonds 1 and 5 in the glucuronide residue, can be considered characteristic of glucuronide substitution on ring B. Further, greater number of fragment ions are produced in the case of quercetin 3′-glucuronide compared to quercetin 4′-glucuronide, which allows distinguishing between them.

Acknowledgments The authors are grateful to the Spanish *Comisión Interministerial de Ciencia y Tecnología* (CICYT/AGL 2004-06685-C04-03) for financial support and author M. Dueñas thanks the Spanish "Juan de la Cierva" Program for a contract.

References

1. Plumb GW, O'Leary K, Day AJ, Williamson G (2003) In: Santos-Buelga C, Williamson G (eds) Methods in polyphenol analysis. The Royal Society of Chemistry, Cambridge, pp 187
2. Block G (1992) Nutr Rev 50:207–213
3. Hertog MGL, Feskens EJM, Hollman PCH, Katan MB (1993) Lancet 342:1007–1011
4. Knekt P, Jarvinen R, Reunanen A, Maatela J (1996) Br Med J 312:478–481
5. Yochum L, Kushi LH, Meyer K, Folsom AR (1999) Am J Epidemiol 149:943–949
6. Rice-Evans CA, Miller NJ, Paganga G (1996) Free Rad Biol Med 20:933–956
7. Cook NC, Samman S (1996) J Nutr Biochem 96:66–76
8. Cos P, Ying L, Calomme M, Hu JP, Cimanga K, Van Poel B, Pieters L, Vlietinck AJ, Vanden Berghe D (1998) J Nat Prod 61:70–76
9. da Silva E L, Tsushida T, Terao J (1998) Arch Biochem Biophys 349:313–320
10. Plumb GW, Price KR, Rhodes MJC, Williamson G (1997) Free Rad Res 27:429–435
11. Plumb GW, Price KR, Williamson G (1999) Redox Rep 4:123–127
12. Price KR, Bacon JR, Rhodes MJC (1997) J Agric Food Chem 45:938–942
13. Price KR, Colquhoun IJ, Barnes KA, Rhodes MJC (1998) J Agric Food Chem 46:4898–4903
14. Day AJ, Dupont MS, Ridley S, Rhodes M, Rhodes MJC, Morgan MRA, Williamson G (1998) FEBS Lett 436:71–75
15. Day AJ, Cañada FJ, Díaz JC, Kroon PA, Mclauchlan R, Faulds CB, Plumb FW, Morgan MRA, Williamson G (2000) FEBS Lett 468:166–170
16. Nemeth K, Plumb GW, Berrin JG, Juge N, Jacob R, Naim HY, Williamson G, Swallow DW, Kroon PA (2003) Eur J Nutr 42:29–42
17. Gee JM, DuPont MS, Rhodes MJC, Johnson IT (1998) Free Rad Biol Med 25:19–25
18. Gee JM, DuPont MS, Day AJ, Plumb GW, Williamson G, Johnson IT (2000) J Nutr 130:2765–2771
19. Hollman PCH, Katan MB (1999) Free Rad Res 31:S75–S80
20. Wolffram S, Block M, Ader P (2002) J Nutr 132:630–635
21. Scalbert A, Williamson G (2000) J Nutr 130:2073S–2085S
22. Hong Y, Mitchell AE (2004) J Agric Food Chem 52:6794–6801
23. Day AJ, Mellon F, Barron D, Sarrazin G, Morgan MRA, Williamson G (2001) Free Rad Res 35:941–952
24. Witting J, Herderich M, Graefe EU, Veit M (2001) J Chromatogr B 753:237–243
25. Mullen W, Boitier A, Stewart AJ, Crozier A (2004) J Chromatogr A 1058:163–168
26. Mullen W, Edwards CA, Crozier A (2006) Br J Nutr 96:107–116
27. Day AJ, Bao Y, Morgan MRA, Williamson G (2000) Free Rad Biol Med 29:1234–1243
28. Needs PW, Kroon PA (2006) Tetrahedron 62:6862–6868
29. Williamson G, Barron D, Shimoi K, Terao J (2005) Free Rad Res 39:457–469
30. Lambert N (1989) In: Harris ELV, Angel S (eds) protein purification methods: a practical Approach. IRL Press, Oxford, pp 101
31. O'Leary KA, Day AJ, Needs PW, Wly WS, O'Brien NM, Williamson G (2001) FEBS Lett 503:103–106
32. Justino GC, Santos MR, Canário S, Borges C, Florêncio MH, Mira L (2004) Arch Biochem Biophys 432:109–121
33. Franke AA, Custer LJ, Wilkens LR, Le Marchand LL, Nomura AM, Goodman MT, Kolonel LN (2002) J Chromatogr B-Analyt Technol Biomed Life Sci 777:45–59
34. Davis DB, Needs PW, Kroon PA, Brodbelt JS (2006) J Mass Spectrom 41:911–920
35. Giusti MM, Rodriguez-Saona LE, Griffin D, Wrolstad RE (1999) J Food Agric Chem 47:4657–4664
36. Fabre N, Rustan I, de Hoffmann E, Quetin-Leclercq J (2001) J Am Soc Mass Spectrom 12:707–715
37. Ma YL, Li QM, Van den Heuvel H, Claeys M (1997) Rapid Commun Mass Spectrom 11:1357–1364