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Solid-phase extraction and gas chromatography–mass spectrometry determination of kaempferol and quercetin in human urine after consumption of *Ginkgo biloba* tablets

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Abstract

A method was developed for the quantification of the flavonoids quercetin and kaempferol in human urine using a solid-phase extraction procedure followed by gas chromatography–mass spectrometry. Deuterated internal standards of the analytes were spiked into the samples prior to extraction. The limit of detection of the method was ca. 10 pg on column and precision of the method for quantification in a sample of urine was ±9.40% for kaempferol and ±7.34% for quercetin ($n=6$). The levels of quercetin and kaempferol found in urine samples were only a small fraction of the amount ingested. The treatment of urine samples with β-glucuronidase markedly increased the levels of flavonoids detected, supporting the view that kaempferol and quercetin are eliminated in the urine as glucuronides.

Keywords: Kaempferol; Quercetin

1. Introduction

Flavonoids are a large group of naturally-ocurring polyphenols with a wide range of pharmacological activities [1]. For example, there is evidence that a flavonoid-rich diet inversely correlates with the risk of coronary heart disease [2] and that the antioxidant properties of some flavonoids can protect against certain kinds of cancer [3,4]. Quercetin and kaempferol are very 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 [5].

While a considerable amount of flavonoids are consumed daily in the western diet, little work has been done on the absorption, metabolism and excretion of this class of compounds in humans. Studies of the absorption of flavonoids have been contradictory and there is some debate over whether they are absorbed as aglycones or glycosides, although a recent study was able to demonstrate flavonoid glycosides as normal constituents of human plasma [6]. The metabolism of flavonoids following absorption may have a considerable effect on their biological properties and recently [7], a study suggested that the metabolism of kaempferol and quercetin in

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humans might differ from that observed in vitro using rat liver microsomes where kaempferol was extensively metabolised in vitro into quercetin by hydroxylation of the B ring but the same study failed to detect quercetin in human urine. This suggests that hydroxylation of kaempferol into quercetin might not occur in humans.

A semiquantitative method to estimate flavonoids in human urine following ingestion of *Ginkgo biloba* tablets was recently developed in our lab [8]. The present work describes a improved method for quantifying kaempferol and quercetin in human urine both before and after consumption of *Ginkgo biloba* tablets using deuterated derivatives of these compounds as internal standards and solid-phase extraction for sample preparation.

2. Experimental

2.1. Chemicals

Chemicals were purchased from the following sources: kaempferol, quercetin, sulphatase (EC 3.1.6.1), β-glucuronidase (EC 3.2.1.31), potassium dihydrogen phosphate, acetic acid, sodium acetate, anydrous sodium sulphate, deuterated acetic acid (98% atom %D), deuterated hydrochloric acid (99.5% atom %D), deuterated ethyl alcohol (99.5 atom %D), trifluoroacetic acid (TFA), and deuterated water (99.9% atom %D) from Sigma–Aldrich Chemical Co. (Poole, Dorset, UK). HPLC grade methanol and acetonitrile from BDH–Merck (Poole, Dorset, UK). *N.O*-(bis)trimethylsilyl acetamide (BSA) from Fluka Chemical Co. (Poole, Dorset, UK). *Ginkgo biloba* tablets had a stated content of 28.8 mg of total flavonoid glycosides and were purchased from Boots (Glasgow, UK)

2.2. Preparation of the deuterated internal standards

Deuterated derivatives of kaempferol ($[^2H_4]$-kaempferol) and quercetin ($[^2H_5]$-quercetin) were prepared as follows: 10 mg of either quercetin or kaempferol was dissolved in a mixture of 4 ml of CH_3COO^2H and 4 ml of a 1:4 (v/v) solution of ^2HCl (37% w/w) in D_2O. The solutions were heated in sealed vials at 80°C for four days. The cooled reaction mixtures were evaporated under reduced pressure by adding a small amount of $C_2H_5O^2H$ (3×1 ml). The residue (ca. 8 mg) was extracted with ethyl acetate (2×4 ml); the combined organic extract was dried by passing through a Pasteur pipette filled with anydrous sodium sulphate (ca. 2g) and the solvent evaporated under a stream of nitrogen. The isotopic composition of the deuterated derivatives of kaempferol and quercetin was assessed by NICI GC–MS of their TMS derivatives using selected ion monitoring. Table 1 shows the isotopic peaks for both the internal standards and the undeuterated flavonoids. In the case of quercetin the presence of five silicon atoms in the structure and the large number of carbons means the $M+1$ and $M+2$ ions are abundant.

2.3. GC–MS analysis

A HP5988A GC–MS system was used in the negative ion chemical ionization (NICI) mode with methane as the reagent gas introduced to give an ion source pressure of ca 133.3 Pa. The gas chromatograph was fitted with a Restek RTX-5 capillary column (30 m, 0.32 mm I.D., 0.5 μm) with helium

Table 1
Isotopic data for quercetin, kaempferol, and the obtained deuterated analogues

m/z[a]	Quercetin (%)*	d-Quercetin (%)*
662	22.31	0.51
663	22.74	0.31
664	31.54	0.49
665	16.19	3.89
666	5.64	31.50
667	1.58	63.30
m/z[a]	Kaempferol (%)*	d-Kaempferol (%)*
574	53.68	0.41
575	26.54	1.71
576	13.88	9.73
577	4.52	28.00
578	1.36	35.19
579	–	17.32
580	–	7.62

[a] m/z Values for the isotopomeric TMS derivatives of kaempferol and quercetin.

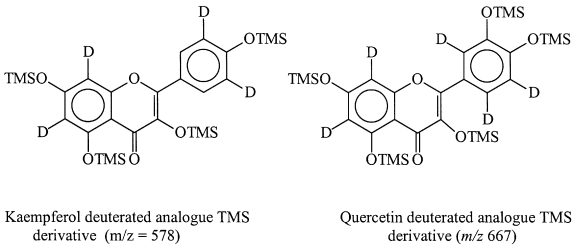
* Data expressed as percentage of total corrected area obtained using selected ion monitoring NICI GC–MS.

as a carrier gas at 68.9 KPa. The injector temperature was set at 250°C and the GC–MS transfer line at 280°C. The oven was temperature-programmed as follows: 160°C (1 min), then at 20°C min^{-1} to 290°C, and to 320°C at 5°C min^{-1}.

Selected ion monitoring was used for quantification. The ions monitored were as follows: m/z 662 and 574 (TMS derivatives of quercetin and kaempferol respectively), m/z 577 and 578 (for the TMS derivative of kaempferol deuterated analogue), and m/z 666 and 667 (for the TMS derivative of quercetin deuterated analogue), see Fig. 1.

2.4. Collection and preparation of urine samples.

Urine samples were collected from five volunteers with no dietary restrictions, before and six h after the ingestion of a single tablet of *Ginkgo biloba.* Urine samples were kept in a freezer (at −20°C). Before analysis, urine aliquots were centrifuged (10 000×*g*, 5 min) and the supernatant taken for analysis. Enzymatic hydrolysis with sulphatase was carried out by adding ten units of sulphatase in 0.25 ml of 1 *M* sodium acetate buffer (pH 5.0) to a 1 ml aliquot of urine and incubating for 1 h at 37°C. Hydrolysis with glucuronidase was carried out by adding 100 units of glucuronidase in 0.25 ml of 1 *M* potassium phosphate buffer (pH 6.8) to 1 ml of urine and incubating for 1 h at 37°C. Glucuronidase-treated samples were acidified with 1 ml of 1 *M* HCl prior to solid-phase extraction (SPE). Acid hydrolysis was carried out by adding 0.5 ml of 3 *M* hydrochloric acid to 1 ml of urine and heating at 80°C for 1 h. The acid-hydrolysed samples were buffered by adding 1 ml of 1 *M* phosphate buffer (pH 6.8) before SPE. Unhydrolysed samples were prepared for extraction by adding 0.25 ml of 1 *M* sodium acetate buffer (pH 5.0) to a 1 ml aliquot of urine. Deuterated internal standards (200 ng) were added to all samples prior to SPE.



Kaempferol deuterated analogue TMS derivative (m/z = 578)

Quercetin deuterated analogue TMS derivative (*m/z* 667)

Fig. 1. Structures of quercetin and kaempferol TMS derivatives and their corresponding deuterated analogues.

2.5. Solid-phase extraction.

Isolute (100 mg ml^{-1}) ENV^{+} cartridges (Crawford Scientific, Strathaven) were conditioned by sequentially passing 1 ml of HPLC grade methanol and 0.8 ml of water through them at a flow-rate of ca. 4 ml min^{-1}. After applying the samples the cartridges were purged with air and were then washed with 1.5 ml (3×0.5 ml) of a solution of 7% methanol in water (adjusted to pH 3.5 with 0.01 *M* TFA) at a flow-rate of ca. 3.5ml min^{-1}. Finally, the cartridges were eluted with 1 ml (2×0.5 ml) of a solution of acetonitrile in water (8:2) at a flow-rate of ca. 4 ml min^{-1}. Samples were blown to dryness with a stream of nitrogen and the residue was derivatized by adding 0.2 ml of BSA and heating the samples in capped vials at 70°C for 30 min. Aliquots of the samples (1μl) were injected into the GC–MS with a 10 μl Hamilton syringe.

2.6. Recovery experiments.

To determine the recovery of kaempferol and quercetin by the SPE method used, 1 ml of distilled water was spiked with 50 ng of both quercetin and kaempferol and extracted using the same SPE procedure used for the urine samples. In order to determine recovery 200 ng of the deuterated internal standards were added either before or after SPE. Recovery was calculated by comparing the ratios of the areas of the deuterated and undeuterated analyte peaks obtained from addition of the internal standards before and after SPE.

2.7. Calibration and quantification.

Calibration curves were prepared for quercetin and kaempferol by mixing varying amounts (0–200 ng) of undeuterated quercetin or kaempferol with fixed amounts (100 ng each) of their deuterated analogues and then derivatizing as described above. In the samples the area ratios between the peaks for quercetin and kaempferol (*m/z* 662 and 574 respec-

tively) and their corresponding deuterated analogues (m/z 666+m/z 667 and m/z 577+m/z 578 respectively) were used for quantification (see Fig. 1) with reference to the calibration curves.

2.8. Statistical analysis.

Significance between treatment groups was verified by means of paired Student's *t*-test.

3. Results and Discussion.

The present work describes a GC–MS method to quantify flavonoids in human urine using deuterated derivatives of kaempferol and quercetin as internal standards. The use of solid-phase extraction prior to analysis markedly improved recovery in relation to a previous work [8]. This improvement in recovery allowed the quantification of quercetin and kaempferol at the low levels present in urine samples taken from subjects before consumption of *Ginkgo biloba* tablets. Thus, the concentration of kaempferol and quercetin from these samples reflect the contribution of an unsupplemented and normal diet.

Fig. 1 shows the deuterated analogues of kaempferol and quercetin. All of the proton positions in the quercetin are activated with respect to exchange with deuterium under acidic conditions and four of the positions in kaempferol are thus activated. From Table 1 it can be seen that it was not possible to achieve complete exchange of all protons and thus quercetin is a mixture of $^{2}H_{4}$ and $^{2}H_{5}$ isotopomers and kaempferol is a mixture of $^{2}H_{3}$ and $^{2}H_{4}$ isotopomers. The potential for crosstalk between the undeuterated compounds and their deuterated isotopomers was acessed and is shown in Table 1. The large numbers of hydrogen and silicon atoms in the structure of the undeuterated compounds result in isotope peaks for the $M+1$ and $M+2$ ions which have a high intensity. However, there was no significant interference with the ions monitored for the deuterated isotopomers.

The response for quercetin and kaempferol was linear over the concentration range examined. The reproducibility of the method was assessed for a β-glucuronidase-treated sample which was analysed as six separate aliquots and which showed a R.S.D. of less than 10% for the amounts of both kaempferol and quercetin (13.00 ng ml^{-1} ±9.40% and 4.45 ng ml^{-1} ±7.34% respectively, $n=6$). Solid-phase extraction using a combined C_{18}, cation and anion-exchanger has been used for the determination of flavonoids in urine [9,10]. In our study, preliminary experiments showed that the use of C_{18} cartridges alone did not allow elimination of endogenous substances that interfered with determination of the analytes. This is in accordance with findings by Ishii et al., [11], who reported the use of a strong anion-exchange cartridge for extraction of naringin and naringenin from human urine instead of C_{18} cartridges which were not effective in removing interferants. For the present study the polymeric phase used (Isolute ENV^{+} cartridges) showed better selectivity than C_{18} cartridges, although complete elimination of interfering endogenous substances was not possible. However, the use of selected ion monitoring allowed baseline resolution for the peaks of interest from any interferants. The recovery of the flavonoids using the present method was affected by the need to remove interfering substances. Consequently, the recoveries obtained were a compromise between retention of the analytes and removal of interfering substances. However, considering that the recovery of phenol reported for C_{18} cartridges is around 50% (HPLC Technology, certificate of analysis), the recoveries obtained for the extracted polyphenols was reasonable and in accordance with the higher retentivity claimed for the polymeric ENV^{+} phase compared with C_{18} phases. Both kaempferol and quercetin showed similar recoveries (mean±S.D., $n=6$): 76.6±10.5% and 73.0±9.8% respectively.

Figs 2 and 3 show typical selected ion profiles for the same urine sample before and after treatment with β-glucuronidase. The traces show the untreated sample with significantly lower levels of quercetin and kaempferol, and consequently with larger background peaks. In both cases however, baseline resolution for the peaks of interest from interferants was achieved.

Fig. 4 shows levels of flavonoids in urine samples before (A) and after (B) consumption of tablets of *Ginkgo biloba*. The levels of flavonoids before consumption of the tablets were significantly lower than those found in samples taken after consumption

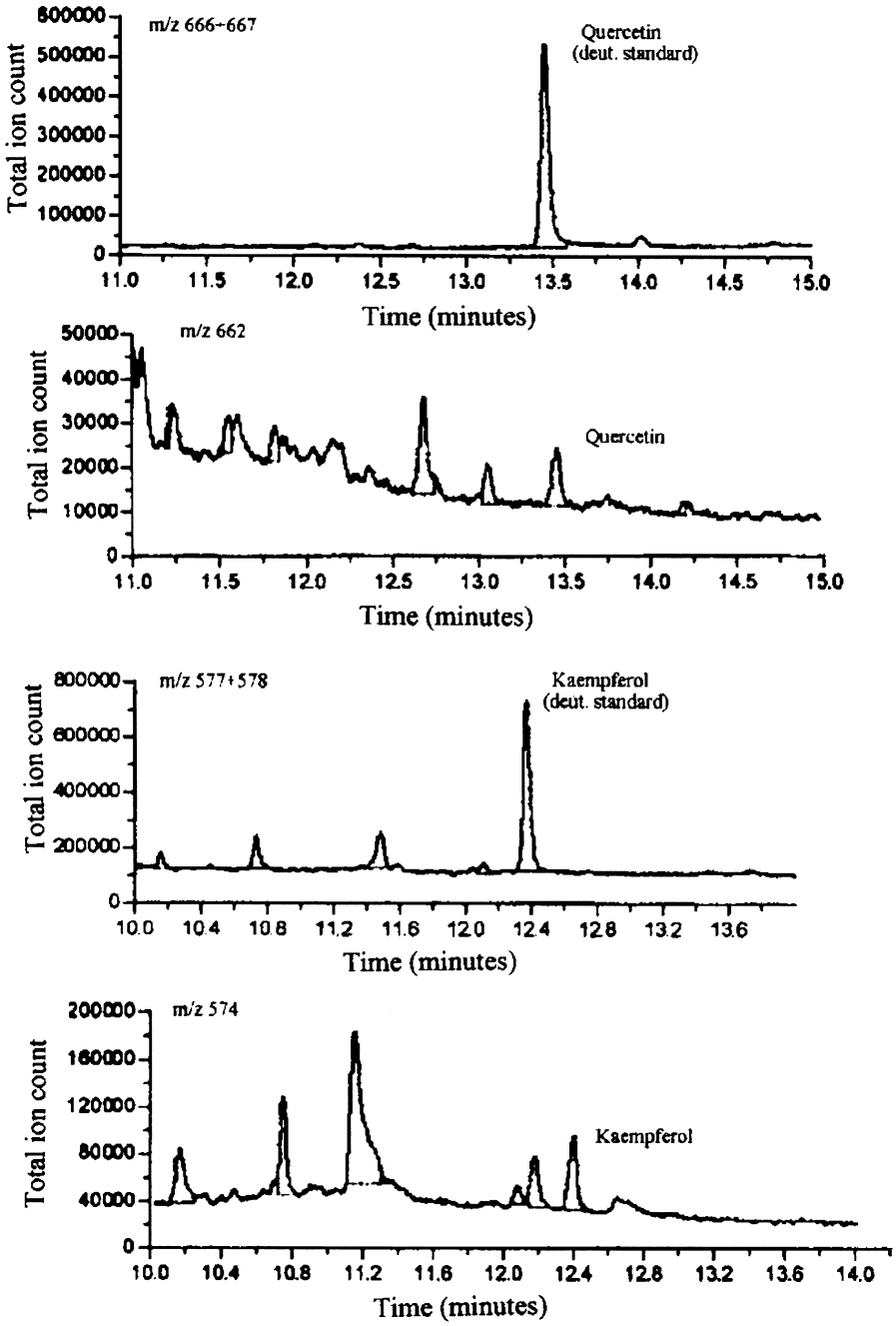


Fig. 2. Typical selected ion profiles obtained from an untreated urine sample containing 200 ng ml^{-1} of deuterated internal standards and 1.8 ng ml^{-1} of quercetin and 5.73 ng ml^{-1} of kaempferol.

of the tablets, but higher amounts of unconjugated flavonoids (specially quercetin) could be detected in comparison to our previous work [8]. Since the levels found before consumption of the tablets reflect mainly the contribution from the diet, variation between individuals is expected. In fact, a recent

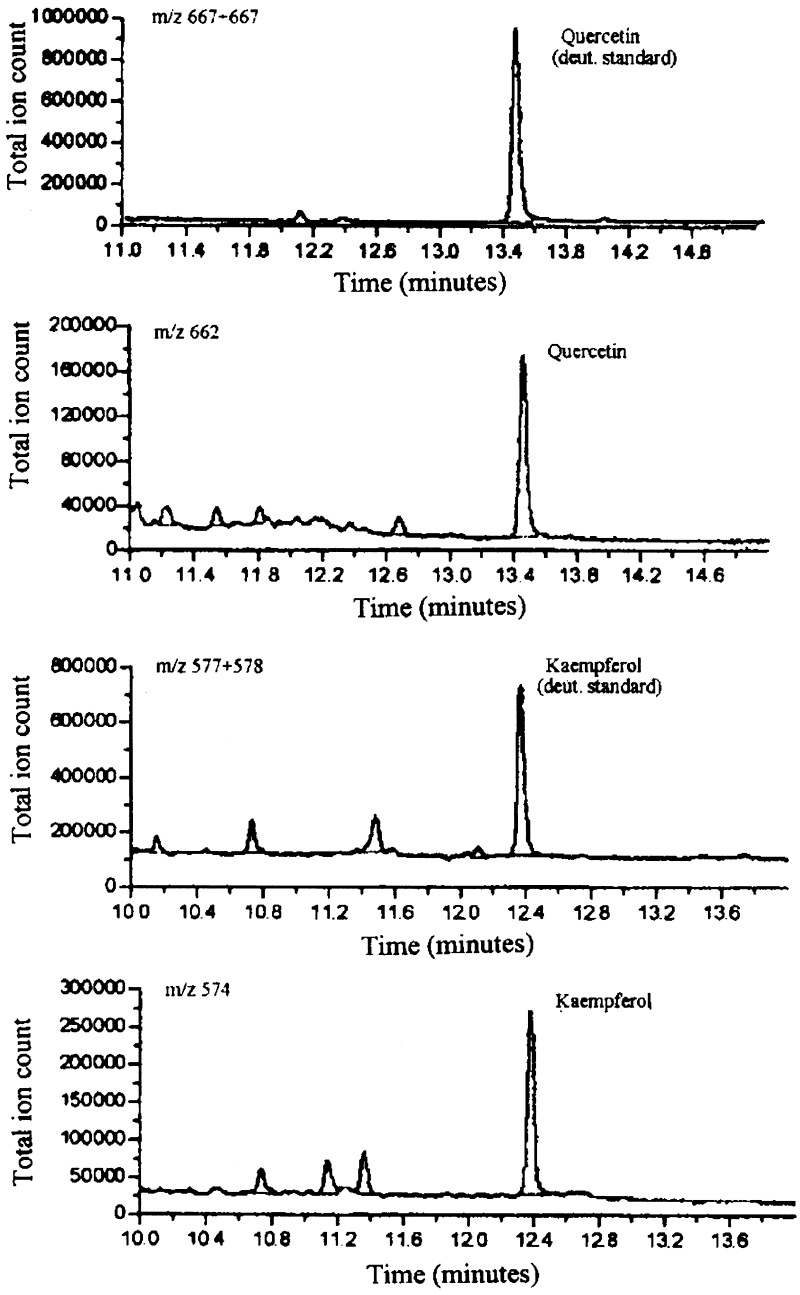


Fig. 3. Typical selected ion profiles obtained from a sample of urine treated with β-glucuronidase containing 200 ng ml^{-1} of deuterated internal standards and 14.82 ng ml^{-1} of quercetin and 25.88 ng ml^{-1} of kaempferol.

study by Hollman et al., [12] showed that the bioavailability of quercetin from several foods is very different, and in all cases, just a small fraction of the ingested amount. In the same study, the maximum bioavailability (from onions) was only 1.39% of the amount ingested. The treatment of the samples with β-glucuronidase significantly ($P<0.05$) increased the amounts of both kaempferol and

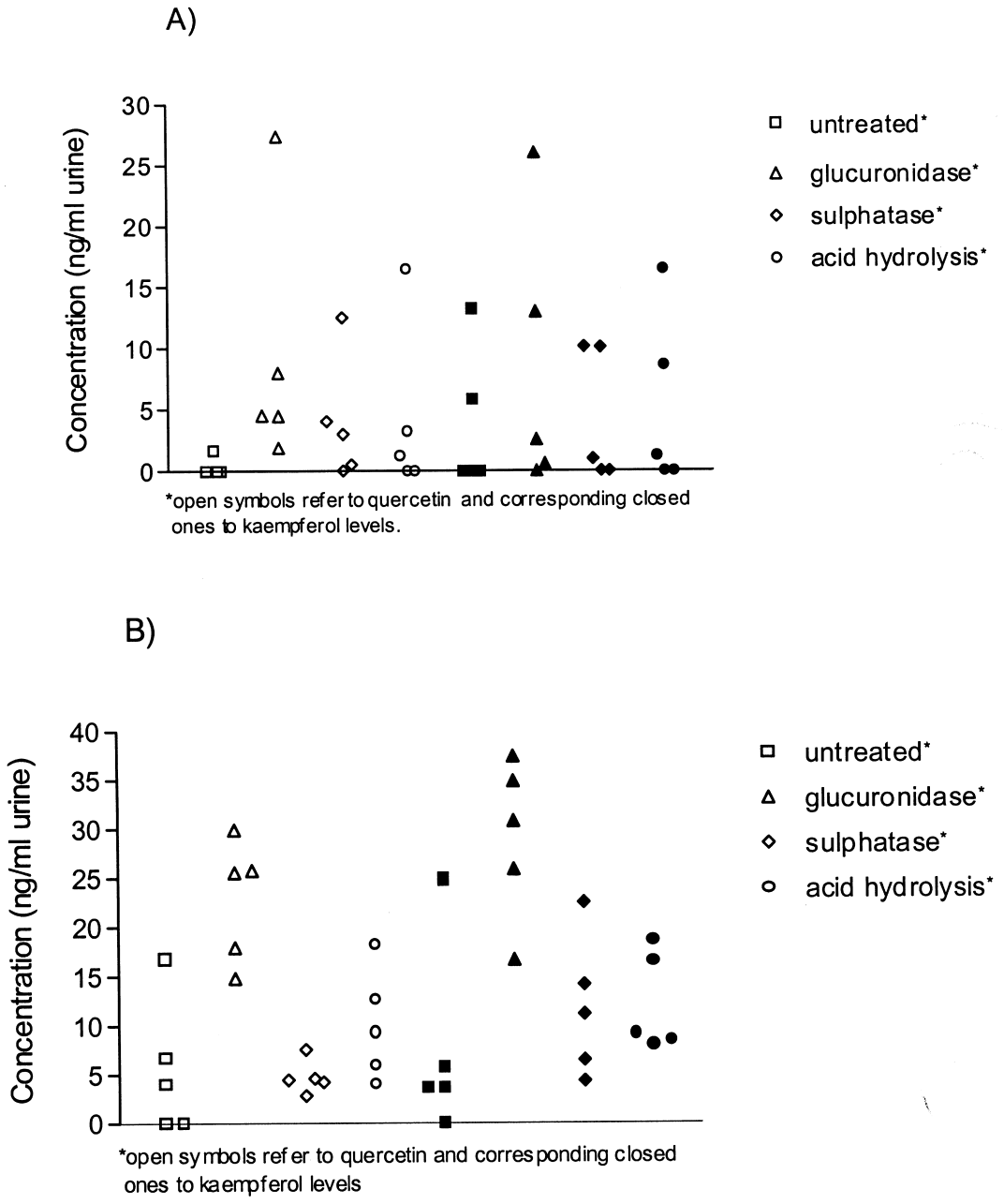


Fig. 4. Levels of quercetin and kaempferol found in urine samples before (A) and after (B) consumption of *Ginkgo biloba* tablets.

quercetin detected (Fig. 4B), suggesting that both flavonoids are excreted mainly as glucuronides. The amounts of quercetin and kaempferol excreted in urine were just a small fraction of the amounts ingested, which is in accordance with previous results [13]. In fact, a recent study [7] using HPLC and LC–MS failed to detect any quercetin in acid-hydrolysed human urine after broccoli consumption and reported very small amounts of kaempferol. Although sulphatase-treated samples showed higher

amounts of kaempferol and quercetin compared with untreated samples, the difference was not statistically significant over the five samples. However, in two of the samples before ingestion of the tablets distinct increases in quercetin were observed following sulphatase treatment suggesting sulphatase conjugation may be a minor route of metabolism. Acid hydrolyzed samples showed significantly ($P<0.05$, $n=5$) higher amounts of quercetin but not kaempferol (Fig. 4B). However, the acid hydrolysis conditions were not optimized with regard to duration and concentration of used. Moreover, acid hydrolysis would be likely to cause losses of analytes since the pyran ring would be fairly susceptible to hydrolysis under these conditions.

The amount of flavonoids in standardised *Ginkgo biloba* extracts (e.g., EGb 761) is usually expressed as ''Ginkgo flavone'' glycoside content. The measure correlates the total amount of aglycones (quercetin, kaempferol, and isorhamnetin) determined after hydrolysis of the extract to the total flavonoid glycoside content. This is done by means of conversion factors that take into account the molecular weights of flavonol coumaroyl ester glycosides. Haslert et al. [14] showed that standardised *Ginkgo biloba* extracts contain an average of 0.39% (w/w) of kaempferol and 0.50% (w/w) of quercetin. It is also known [15] that enriched extracts contain about 24–27% (w/w) of ''Ginkgo flavone'' glycosides. The tablets used in this study contain a stated amount of 28.8 mg of total flavonoid glycosides per tablet, and accordingly an estimated amount of only 0.44 mg of kaempferol and 0.50 mg of quercetin as free aglycones per tablet. Our results suggests therefore that the absorption of orally administered quercetin and kaempferol glycosides is possible, as pointed out previously [6].

Taken together, the results obtained suggest that the general bioavailability of kaempferol and quercetin is low, and that their main pathway of metabolism is through glucuronidation. Considering the spectrum of biological activities exibited by flavonoids and their widely dietary ocurrence there is a need for a more complete understanding of the absorption, metabolism and excretion of this class of compounds. The ingestion of a tablet containing 28 mg of flavonoids had a marked effect on the baseline levels of kaempferol and quercetin in urine suggesting high levels of these compounds are not absorbed from a normal diet. The GC–MS method developed should be of sufficient sensitivity to allow these compounds to be determined in plasma.

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