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Column-switching high-performance liquid chromatographic assay for the determination of quercetin in human urine with ultraviolet absorbance detection

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Abstract

A high-performance liquid chromatographic method is described for the determination of quercetin in human urine using column-switching and ultraviolet (UV) absorbance detection. Urine samples were enzymatically hydrolysed and solid-phase extracted prior to injection onto the high-performance liquid chromatography (HPLC) system. Prior to elution of quercetin and the internal standard, fisetin, from the first column used for sample clean-up, the six-port valve was switched to the second column for analysis with UV detection. Detection of quercetin was accurate and reproducible, with a detection limit of 5 ng/ml. The method was applied to determine the urinary level of quercetin in 120 samples from an intervention study with fruit juice. © 1998 Elsevier Science B.V.

Keywords: Quercetin

1. Introduction

Flavonoids are polyphenolic compounds that occur ubiquitously in plants. The flavonoids mainly occur as glycosides, i.e., bound to sugars [1]. Quercetin is the most abundant flavonoid found in vegetables and fruits and therefore is an important constituent of the human diet [2,3]. Potent biological effects of this flavonoid have been described in vitro and in vivo [4]. In cohort studies, it was recently found that intake of quercetin as determined by a food frequency questionnaire, was inversely correlated with subsequent incidence of coronary heart disease [5,6]. Thus development of a biomarker for exposure to quercetin is important in order to evaluate the potential health effects of this particular dietary component. After ingestion, the flavonoid glycosides are partly hydrolysed by microorganisms in the gastrointestinal tract and the liberated aglycones can be absorbed from the intestinal wall and excreted in the urine and bile as glucuronides and/or sulphate conjugates [7]. Recently, absorption of intact quercetin glycosides in humans has also been inferred [8,9]. In spite a high dietary intake of the flavonoid only very low amounts (0.4–1%) of quercetin have been reported to be excreted in the urine of humans [8–10]. Thus a very sensitive method is required to use urinary quercetin excretion as a feasible biomarker of exposure. So far only one high-performance liquid chromatography (HPLC) method has been described for the detection of quercetin in human urine [11]. The method involves post-column

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derivatization and fluorescence detection. In this paper we report on the development of a column-switching HPLC method for the detection of low levels of quercetin in human urine samples using UV absorbance detection. The usefulness of electrochemical detection (ED) was also assessed. Enzymatic hydrolysis was used to liberate the urinary quercetin from glucuronic acid and/or sulphate conjugates. In addition, the effect of the enzymatic hydrolysis on quercetin glycosides, potentially present in the urine was investigated. The method was applied for a human intervention study, in which the level of urinary quercetin was determined after intake of a mixture of apple and blackcurrant juices [12].

2. Experimental

2.1. Chemicals and materials

Acetonitrile and methanol were of HPLC grade and obtained from Rathburne (Walkerburn, UK). Quercetin, fisetin and rutin (quercetin-3-*O*-rutinoside) were purchased from Aldrich (Steinheim, Germany), quercetin-3-*O*-galactoside (Q-3-Gal) and quercetin-3-*O*-glucoside (Q-3-Glc) were from Apin (Oxon, UK), and β-glucuronidase/arylsulfatase [helix pomatia, 5.5 U/ml β-glucuronidase (=100 000 Fishman units/ml), 2.6 U/ml arylsulfatase (=800 000 Roy units/ml)] from Boehringer Mannheim (Mannheim, Germany). All other chemicals used were of HPLC grade or reagent grade. Solid-phase extraction (SPE) cartridges (Bond Elut, C_{18}, 500 mg) were purchased from Varian (Harbor City, USA).

2.2. Instrumentation

The HPLC system consisted of a Hewlett-Packard (Waldbronn, Germany) 1090 system with three pumps, an automatic six-port column-switching valve, and diode array detection (DAD) system. The outlet from the DAD system was connected via a manual switching valve to a Hewlett-Packard 1049A programmable ED system, with a glassy carbon working electrode and an Ag/AgCl reference electrode. ED was performed at +550 mV versus the Ag/AgCl electrode. The columns used were a Lichrospher RP-select B, RP-8 (125×4 mm, 5 μm) column as column 1 and a Pyrospher RP-18 (125×4 mm, 5 μm) column as column 2 (Hewlett-Packard). Column temperature was maintained constant at 40°C using a thermostatically controlled column compartment. Detection was carried out simultaneously at 290, 375 and 390 nm, with peak scanning between 210 and 600 nm (2-nm step). The mobile phases used were (flow of 1 ml/min): (A) 30% methanol in 0.05% (v/v) acetic acid, pH 3.6, (B) 23.8 m*M* citric acid, 67 μ*M* EDTA, adjusted to pH 2.00 with conc. H_3PO_4, and (C) 100% acetonitrile. A vacuum manifold from Waters (Milford, MA, USA) was used for the SPE.

2.3. Standards

Quercetin and the flavonol fisetin (internal standard, see Fig. 1) were dissolved in DMSO (5 mg/ml) as stock solutions, and stored at −20°C. Spiked urine samples were prepared by addition of quercetin stock solutions to control urine (see Section 2.4), giving final concentrations of 0, 10, 50, 75, 100, 150, 250 and 500 ng flavonoid per 15 ml of urine sample. Each sample was prepared in duplicate or quadruplicate. Fisetin was added as internal standard in an amount of 1 μg per 15 ml urine sample. Calibration curves were generated following enzymatic hydrolysis, SPE and HPLC analysis, as described in Sections 2.5, 2.6 and 2.7.

2.4. Sample collection and preparation

As part of a human intervention study [12], 24 h urine samples were collected after consumption of a 1:1 mixture of blackcurrant juice and apple juice at different levels, up to 1500 ml/day. The intake of quercetin with the juices ranged from 4.8–9.6 mg/

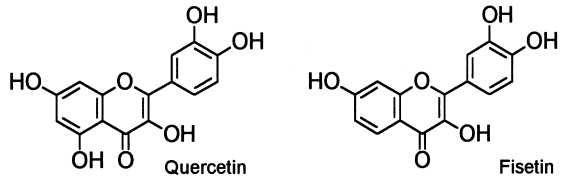

Fig. 1. Chemical structures of quercetin and the internal standard fisetin.

day. The volunteers avoided flavonoid containing foods (fruits, vegetables, wine and tea) one week prior to, and during the intervention study. The pH of the urine samples was adjusted to 3–4 by addition of approximately 50 ml 1 *M* HCl, and aliquots of 250 ml were stored at −20°C until analysed. A baseline urine sample was collected and used as control urine.

2.5. Enzymatic hydrolysis of urine samples

A 15-ml aliquot of urine was bubbled with Ar (g) and adjusted to pH 5 with 0.2 *M* sodium acetate containing 1% ascorbic acid. One μg of fisetin as internal standard was added to the sample. Enzymatic hydrolysis was performed by addition of 100 μl β-glucuronidase/arylsulfatase (0.55 U and 0.26 U, respectively) and the sample was incubated in a sealed vial for 1 h at 37°C under continuous shaking. After hydrolysis, the sample was applied to SPE as described in Section 2.6. The optimal incubation time for the enzymatic hydrolysis, was found by incubating two different urine samples from the human intervention study (No. 1081-13, No. 1085-22) in duplicate for either 1, 2 or 4 h at 37°C and subsequently performing solid-phase extraction and HPLC as described in Section 2.7.

2.6. Solid-phase extraction

A Bond Elut C_{18} cartridge (500 mg) was preconditioned on a vacuum manifold with 5 ml of methanol followed by 5 ml of 1% formic acid. The hydrolysed urine sample was applied to the column with a flow of approximately 5 ml/min. The cartridge was washed with 3 ml 5% methanol, 1% formic acid and eluted with 3 ml 90% methanol (1% formic acid, 1% ascorbic acid) followed by 3 ml pure methanol. The eluates were combined and evaporated to dryness under vacuum. The residue was dissolved in 150 μl 30% methanol, 1% formic acid and the total sample was injected onto the HPLC system.

2.7. HPLC conditions for analysis of urine samples

A schematic diagram of the HPLC system is shown in Fig. 2. The sample was injected onto column 1, using isocratic elution from 0–36 min, with mobile phase A. Between 20–26 min and 29–36 min, the switching valve was shifted from waste position to column 2, thus eluting the target compounds fisetin and quercetin onto the second column. At 36.1–37 min column 1 was washed with pure acetonitrile (C) with the switching valve in waste position. After preconditioning of column 1 from 37.1–42 min with 100% citric acid buffer (B), the switching valve was shifted from waste position to column 2 at 42 min. The flavonoids were eluted from column 2 through the detector, using a gradient of mobile phase B and acetonitrile (C): 48–58 min: 20% C, 68 min: 40% C, 73–75 min: 100% C, 75.1–85 min: 100% B.

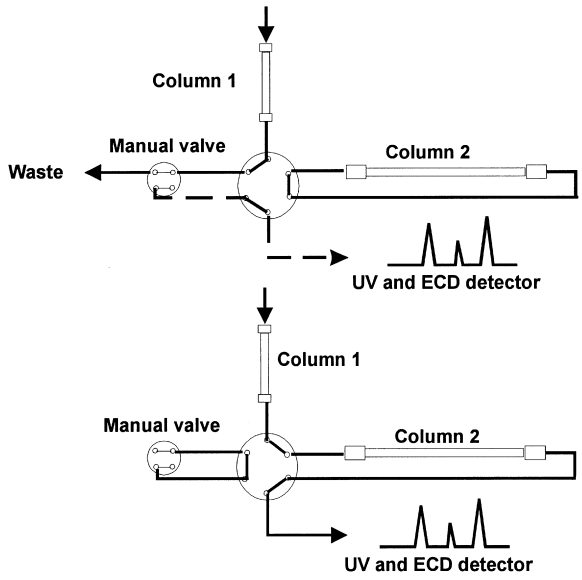

Fig. 2. Schematic diagram of the HPLC system for determination of quercetin in human urine samples.

2.8. Reproducibility and stability

A standard mixture containing 10 ng/μl of fisetin and quercetin was analysed by injecting 100 μl as external standard before and after each series of samples, to ensure the reproducibility of the method. Furthermore, the reproducibility of the retention times of fisetin and quercetin on column 1 was controlled with isocratic elution, using mobile phase A, ensuring that the target compounds elute within

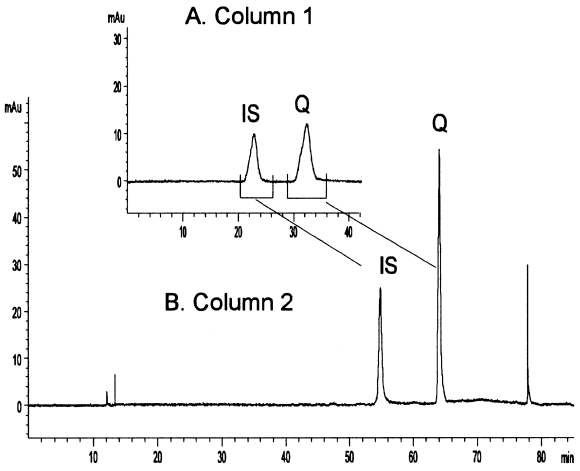

Fig. 3. Chromatograms showing the elution of the internal standard, fisetin (I.S.) and of quercetin (Q) on column 1, using isocratic elution (A). Time-points of column switching indicated with frames. (B) I.S. and Q after elution from column 2, detected at 390 nm.

the time-frames of column-switching (see Fig. 3). This was performed prior to each series of samples by connecting column 1 directly to the detectors, using a manual switching valve (see Fig. 2). When setting up large series of samples, one urine sample was selected as control sample and included repeatedly with each series of samples to ensure reproducibility of the method.

The stability of quercetin in human urine was assessed by spiking control urine samples with 1 μg quercetin per 15 ml sample. The samples were kept at −20°C until analysed. Prior to analysis 1 μg fisetin was added as internal standard.

2.9. Enzymatic hydrolysis of quercetin glycosides

Quercetin glycosides present in apples and blackcurrants [13], might be excreted unchanged in the urine after ingestion of the apple and blackcurrant juices. The influence of the incubation with β-glucuronidase/arylsulfatase on these glycosides, was studied as follows. Five μg Q-3-Gal, 5 μg Q-3-Glc, or 6.5 μg rutin (quercetin-3-*O*-rutinoside) was each incubated in sodium acetate buffer for 1 h in triplicate, with or without the enzyme preparation under the same conditions as described in Section 2.5, but scaled down to a total volume of 1 ml (6.7 μl β-glucuronidase/arylsulfatase used). After incubation, 100 μl of each sample was directly injected onto the 1090 HPLC system, using a 5-μm Purospher RP-18 column (250×4.0 mm, Hewlett-Packard) with a 35-min linear gradient, flow 1 ml/min; Solvent A: 1% aqueous formic acid; solvent B: acetonitrile. 0 min: 10% (v/v) B in A; 20 min: 30% B (v/v) in A; 25 min: 40% (v/v) B in A; 28 min: 60% (v/v) B in A; 30 min: 100% B; and 30–35 min: 100% B. Column temperature was maintained constant at 35°C and detection was carried out simultaneously at 290, 350 and 380 nm, with peak scanning between 210 and 600 nm (2-nm step). A freshly prepared mixture of 5 μg Q-3-Gal, 5 μg Q-3-Glc, 6.5 μg rutin and 3.2 μg quercetin per ml of sodium acetate buffer was used as reference.

3. Results and discussion

3.1. HPLC of urine samples

To obtain sufficient separation of the flavonoid aglycones from interfering compounds in the urine, it was found necessary to use a column-switching method, and to apply as different conditions as possible to the two columns. Various stationary phases were examined, including 3 μm Hypersil BDS, 5 μm Pyrospher RP-18 and 5 μm Lichrospher RP-select B from Hewlett Packard, 5 μm Nucleosil C_6H_5 and 5 μm Nucleosil RP8 from Machery Nagel (Düren, Germany) in different combinations. By selecting a Lichrospher RP-select B, RP-8 column as the first column, the target compounds fisetin (internal standard) and quercetin were eluted onto column 2 with a low percentage of organic solvent. This gave less peak broadening on column 2, a Pyrospher RP-18 column, where a higher percentage of organic solvent was needed to elute the two compounds. It was necessary to use 125×4 mm columns instead of 250×4 mm columns in order to keep the pressure below the limit of the HPLC system (400 bar). To achieve sufficient separation of the flavonoids from interfering compounds in urine, two different organic solvents, methanol and acetonitrile, had to be applied. The aqueous phase was optimized with regard to the signal-to-noise ratio of the ED system. Acetate, phosphate and citric acid buffer, all at pH 2,

were examined and the use of citric acid buffer resulted in the best peak performance and signal-to-noise ratio. By application of isocratic elution on the first column, using aqueous methanol at pH 3.6 (mobile phase A), a very selective elution of the target compounds was achieved. Before the elution of fisetin, and later of quercetin, from column 1, the automatic six-port valve was programmed to switch from waste position to column 2, and back to waste position again. After the elution of the target compounds onto column 2, the more unpolar impurities remaining on column 1 were removed by a quick column wash with 100% acetonitrile, followed by reequilibration with 100% citric acid buffer, with the six-port switching valve in waste position. An acetonitrile gradient (pH 2) was applied to column 2, eluting the compounds through the UV detector and the ED system. The change in pH from column 1 to 2 was necessary to avoid co-elution of impurities from the urine with the target compounds. Typical chromatograms of a blank urine and a urine sample obtained after juice intervention are shown in Fig. 4. The internal standard fisetin and quercetin were highly separated with retention times of 54.5 and 64.3 min, respectively. As seen in the chromatograms obtained at 390 nm (Fig. 4A–C) there are practically no interfering peaks at this wavelength, whereas a higher level of impurities is detected with ED at +550 mV (Fig. 4D). The UV spectrum of quercetin detected in a urine sample, shows good similarity with authentic quercetin (see Fig. 5) and enables positive identification by comparison of the UV spectra. Reproducibility of the retention times of the target compounds on column 1 was crucial for the method, since variations could result in one of the compounds eluting outside the time-frames of column-switching. Thus it was necessary to control the retention times of fisetin and quercetin on column 1 (see Fig. 3), prior to setting up a series of samples, by switching the flow from waste position through the detectors by a manual valve (see Fig. 2) using isocratic elution with mobile phase A.

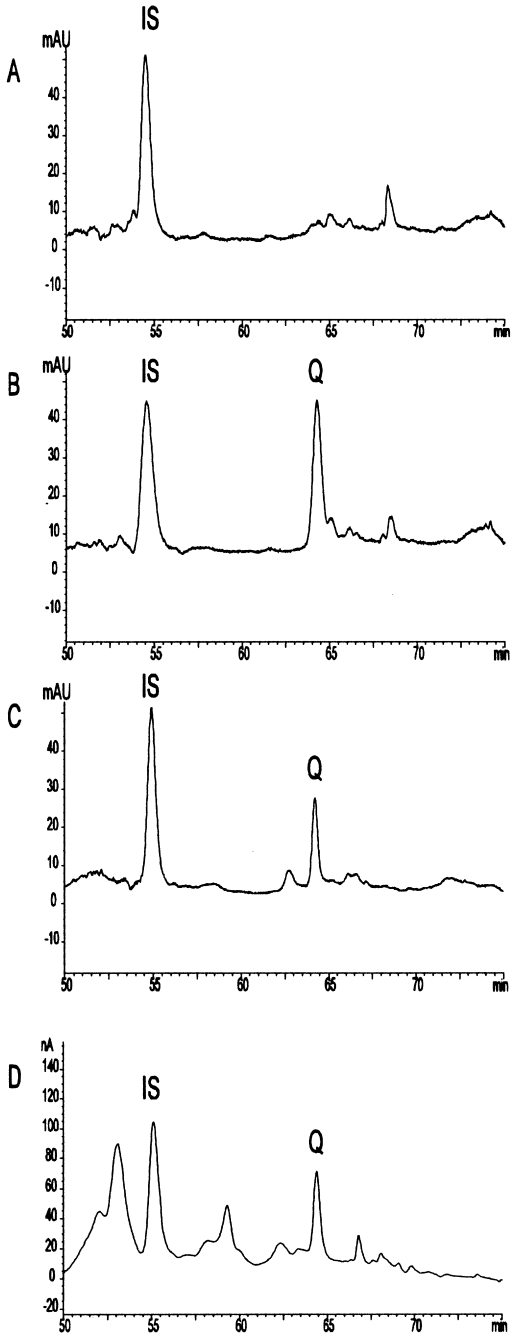

Fig. 4. Chromatograms at 390 nm of (A) control urine containing 1 μg I.S., (B) control urine spiked with 1 μg I.S. and 1 μg Q. Chromatograms of a urine sample collected during intervention study with 1500 ml juice spiked with 1 μg I.S., detected at 390 nm (C) and with ED at +550 mV (D).

3.2. UV absorbance detection and ED

The wavelength used for detection and quantification of fisetin and quercetin was set to 390 nm

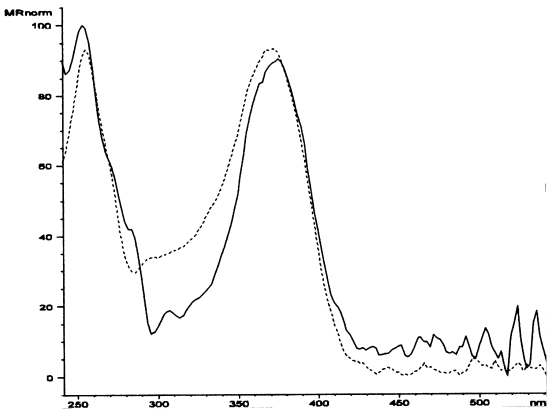

Fig. 5. UV spectrum recorded on-line with DAD of the quercetin peak from a urine sample collected after juice intervention (solid line) compared with authentic quercetin (dotted line).

instead of λ_{max} (~375 nm). This gave a better signal-to-noise ratio and thus a more selective detection of the flavonoids in the urine samples. As seen in Fig. 6, the electrochemical potential giving the peak oxidation of quercetin was achieved at approximately +950 mV vs. the Ag/AgCl electrode. To reduce the intensity of interfering peaks from the urine considerably, it was necessary to use detection at +550 mV vs. Ag/AgCl. The response of the internal standard, fisetin, at this potential was found to be similar to that of quercetin.

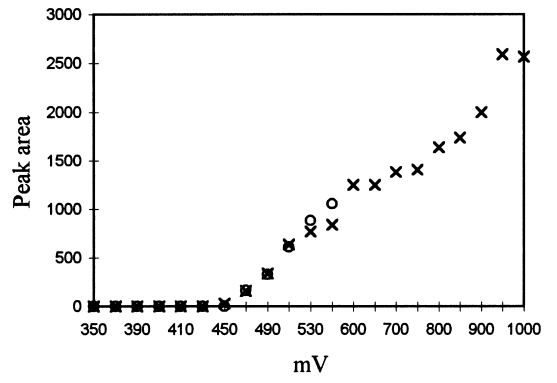

Fig. 6. ED response to 50 ng quercetin (×) and the internal standard, fisetin (○) expressed as peak areas versus the relative potential (mV) of the Ag/AgCl electrode. Fisetin was only determined in the range +350–+550 mV.

3.3. Enzymatic hydrolysis

The optimal conditions for the enzymatic hydrolysis of the urine samples were found by incubating the urine samples with β-glucuronidase/arylsulfatase for 1, 2 or 4 h. The maximum amount of quercetin was recovered after 1 h of incubation, and had decreased to 97±1.2% after 2 h and to 91±4.0% (±S.D., $n=4$) after 4 h. Thus the enzymatic hydrolysis was performed by incubation with the enzyme preparation for 1 h.

Despite the specifications from the manufacturer, the β-glucuronidase/arylsulfatase preparation had completely hydrolysed the three quercetin glycosides after 1 h of incubation (see Fig. 7). The concentration of quercetin glycosides in the incubation mixture corresponded to an urinary excretion of 50% of the maximum dose (9.6 mg) used in the intervention study, in a 24 h urine sample (on average 1500 ml) [12]. This amount was chosen in accordance with the inferred absorption of up to 52% of ingested quercetin glycosides reported by Hollman et al. [8]. Thus the amount of quercetin detected in the hydrolysed urine samples was the total amount of urinary quercetin either free, or bound as glucuronides, sulphates and/or glycosides. The ability of the enzyme preparation to hydrolyse flavonoid glycosides are presently under further investigation.

3.4. Linearity, detection limits, reproducibility and stability

Calibration curves were obtained by enzymatic hydrolysis, SPE and HPLC analysis of spiked control urine samples over the concentration range 50–500 ng/sample (15 ml urine). The equally weighted correlation between peak area determined at 390 nm and the concentration of quercetin was linear, passing through the origin, with a correlation coefficient better than 0.999. The limit of quantification, as determined by spiking of control urine was 5 ng/ml urine for quercetin at 390 nm. The limit of quantification was lowered to about 1 ng/ml by using ED, giving a linear response from 10–500 ng/sample ($r=0.999$). However, in some urine samples from the juice intervention study, interfering peaks were noticed in the ED chromatogram giving difficulties in quantifying the quercetin peak. Thus, the de-

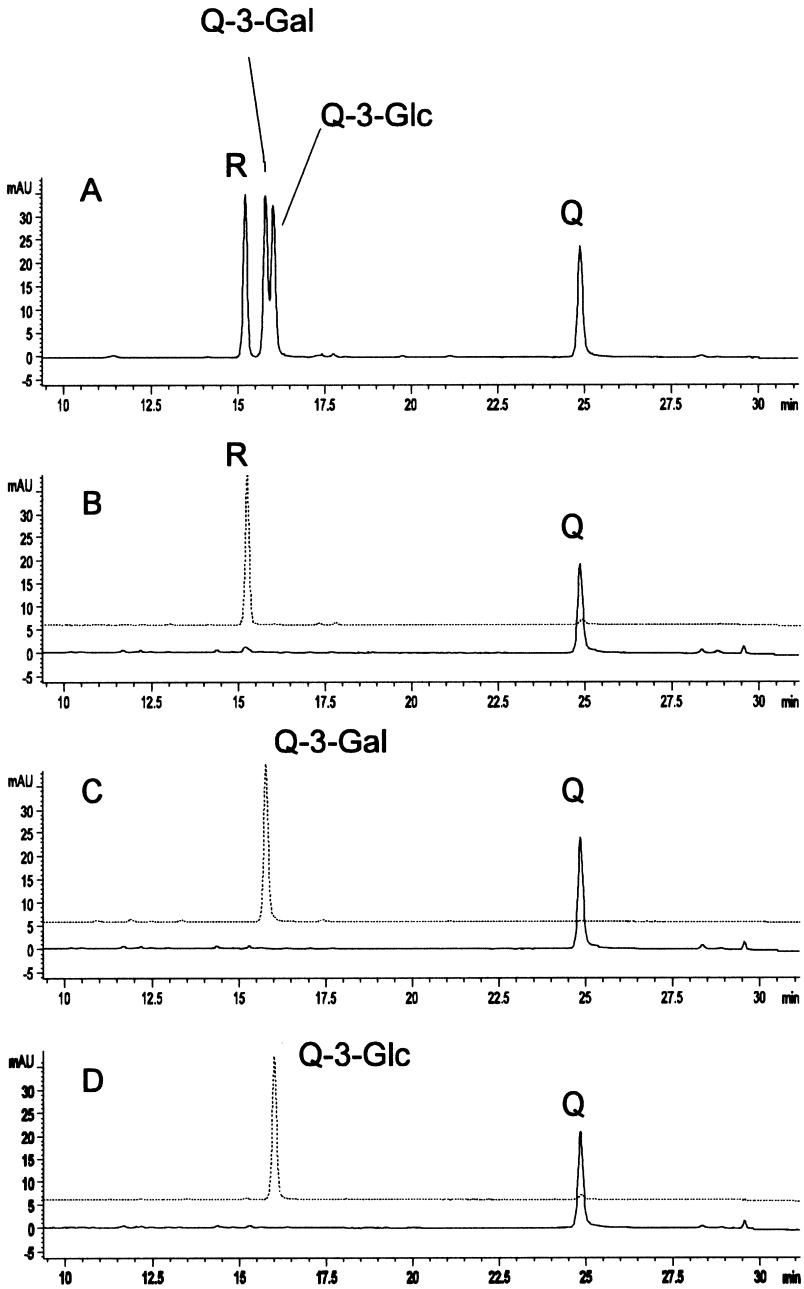

Fig. 7. Chromatograms of quercetin glycosides incubated with (fully drawn line) and without (dotted line) β-glucuronidase/arylsulfatase. (A) Standard mixture of rutin (R), quercetin-3-*O*-galactoside (Q-3-Gal), quercetin-3-*O*-glucoside (Q-3-Glc) and quercetin (Q), (B) incubations of rutin, (C) incubations of Q-3-Gal, (D) incubations of Q-3-Glc.

termination of quercetin in the human urine samples from the intervention study was performed using the UV signal at 390 nm [12]. The reproducibility of the HPLC method was assessed by the external standard mixture of 10 ng/μl of fisetin and quercetin analysed prior to and after each series of samples. The retention times of the external standard showed an inter- and intra-day variation of less than 1% (coefficient of variation, C.V.) and the peak areas determined varied less than 4% (C.V., $n=20$). Quercetin added to urine samples (1 μg/15 ml sample) was found to be stable (defined as ≥95% of initial amount remaining) for at least four months when stored at −20°C.

3.5. Application of the method

The present method was applied for the analysis of more than 120 urine samples [12]. Quercetin was detected in all the urine samples in a dose dependent manner. One of the urine samples (No. 1085-22) kept at −20°C, was included as control sample in each series of analyses over a period of three months. The amount of quercetin detected in this sample was in average 22.3 ng/ml (±1.1 S.D., $n=10$). If the recovery of the internal standard, fisetin in a single sample was found to be lower than 70%, the result was rejected, and a new analysis was performed (about 10% of the samples). Recovery of the internal standard in the final set of analyses was between 70.2–109.9% with an average of 90.1% (±10% S.D., $n=120$). The detected amount of quercetin in each urine sample was individually corrected for analytical loss of the internal standard.

4. Conclusions

The method described in this paper represents a specific and sensitive assay for the determination of quercetin in human urine samples. To our knowledge, this is the first method described to use UV absorbance for the determination of quercetin in human urine. The method was successfully applied to a human intervention study [12], where the dose level of quercetin was similar to or lower than the average daily intake in Denmark [14], and thus more than 10-fold lower than doses used in previous human studies [8,9,15]. The use of SPE and column-switching in the present assay gave a 100-fold concentration of the urine samples, enabling the detection of a very low level of urinary quercetin. This method would therefore be suitable to determine quercetin in human urine samples in general, and urinary quercetin could thus be used as a marker for the dietary exposure to quercetin daily intake of fruit and vegetables.

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