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Identification of quercetin glucuronides in human plasma by high-performance liquid chromatography–tandem mass spectrometry

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Abstract

After intake of food or herbal medicinal products containing quercetin glycosides, the systemic availability of the genuine glycoside, as well as the systemic occurrence of the aglycone or conjugates of this polyphenol has been a matter of dispute. Consequently, we designed this study to develop a reliable method for determination of quercetin and its metabolites. Following consumption of fried onions five different glucuronides of quercetin could be identified in human plasma samples by means of HPLC–UV–MS/MS. Selective determination of the target compounds was achieved by simultaneous UV (254 nm) and MS/MS detection with selected reaction monitoring experiments using positive mode electrospray ionisation. In contrast, neither the free flavonol nor the genuine glycoside could be detected in plasma. Identification of the quercetin glucuronides detected in vivo was confirmed by comparison with authentic reference compounds synthesised enzymatically using glucuronyl transferase from rabbit liver.

Keywords: Quercetin glucuronide; Quercetin; MS/MS; SRM

1. Introduction

Flavonoids are plant polyphenolic compounds present in our daily diet which exhibit potential beneficial properties on human health with regard to the prevention of free radical pathologies such as inflammation and coronary heart diseases [1]. Particularly, the flavonol quercetin has attracted substantial interest in recent years as it is the most abundant flavonoid accumulating in higher plants where it forms glycosides with a large variety of sugars. The intake of quercetin contained in food and herbal medicinal products has been associated with various health benefits and therapeutic or protective effects which are believed to be based on its antioxidant activity [1]. In order to explain these observations, in vitro assays on the antioxidant and radical scavenging capacities were carried out with several flavonoid aglycones [1].

However, the question whether unconjugated quercetin, its genuine glycosides or quercetin glucuronides or sulfates are available in the systemic circulation after consumption of quercetin or quercetin-rich food products in humans is still a matter of dispute [2]. After administration of quercetin or formulations containing quercetin glycosides, only

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quercetin metabolites such as putative glucuronides or sulfates of quercetin or 3-*O*-methylquercetin could be detected in human plasma after enzymatic cleavage of the corresponding conjugates [2]. With regard to quercetin transformations, the intestinal mucosa and the liver are believed to be the major metabolising human organs [3]. Likely, these phase II reactions are catalyzed by enzymes such as UDP-glucuronyl transferases (UGT). Several UGT isoforms exhibiting different substrate specificity have been described from human tissue. Regarding flavonoids with multiple glucuronidation sites such as quercetin, no data are available on substrate specificity of the enzymes and the resulting glucuronidation patterns of individual UGTs [4]. Consequently, we designed this study to develop a reliable analytical method for the determination of quercetin and its metabolites in plasma samples.

To accomplish the selectivity and specificity of detection required to identify quercetin and its metabolites at trace levels in complex biological matrices, preliminary experiments had proved that high-performance liquid chromatography–mass spectrometry (HPLC–MS) techniques were superior as compared to UV or electrochemical detection [5,6]. Following electrospray ionisation (ESI), the compounds under study not only yielded characteristic molecular ions, but could be characterised by further MS/MS experiments from a structural perspective. By means of selected reaction monitoring (SRM) experiments HPLC–MS/MS allowed reliable, selective and sensitive identification of target compounds in multi component chromatographic peaks occurring in complex matrices.

And most important, precursor ion scanning followed by product ion experiments was the key technique to detect and characterise hitherto unknown metabolites in human samples without the need to apply radioactive labelled quercetin. Hence, with this contribution we describe a rapid, specific and highly selective HPLC–UV–MS/MS method for the detection and identification of quercetin glucuronides in human plasma and report on formation and characterisation of these metabolites following UGT-based in vitro biotransformation assays.

The method will be used in an ongoing pharmacokinetic study with human volunteers.

2. Experimental

2.1. Materials

Quercetin dihydrate (M_r=338.23) was obtained from Sigma–Aldrich (Deisenhofen, Germany). Acetone, 85% phosphoric acid, 36% hydrochloric acid, sodium hydroxide, 96% ethanol, 2-mercaptoethanol, 98% trifluoroacetic acid, and gradient-grade methanol were purchased from Merck (Darmstadt, Germany). Trizma hydrochloride buffer, pH 8.0, bovine serum albumin powder, uridine-5′-diphosphoglucuronic acid (UDPGA), uridine-5′-glucuronyl transferase [EC 2.4.1.17], and β-glucuronidase [EC 3.2.1.31] were obtained from Sigma–Aldrich (Deisenhofen, Germany). Magnesium chloride was obtained from Grüssig (Wahrenholz, Germany).

2.2. HPLC–UV–MS/MS system

The HPLC equipment consisted of a Model 140B pump (Applied Biosystems, Bai, Germany). For separation a column (125×2 mm I.D.) packed with Nucleosil 120-5C_{18} (Macherey-Nagel, Germany) was used. The solvents were 0.05% aqueous trifluoroacetic acid (A) and gradient-grade methanol (B). The flow-rate was set at 0.2 ml·min^{-1}.

The gradient profile used for separation was: 0.0–0.6 min, 5–23% B in A, linear; 0.6–1.8 min, 23% B in A, isocratic; 1.8–2.8 min, 23–35% B in A, linear; 2.8–5.0 min, 35% B in A, isocratic; 5.0–6.0 min, 35–50% B in A, linear; 6.0–10.0 min, 50% B in A, isocratic; 10.0–10.6 min, 50–60% B in A, linear; 10.6–15.0 min, 60% B in A, isocratic; 15.0–17.5 min, 60–87% B, linear; 17.5–19.0 min, 87% B in A, isocratic; 19.5–25.0 min, 87–100% B in A, linear.

An UV detector (Knauer, Germany) between column and ESI interface enabled simultaneous monitoring of the UV signal at 254 nm.

Collision-induced dissociation (CID) spectra and SRM experiments were obtained on a TSQ 7000 triple stage quadrupole mass spectrometer (Finnigan MAT, Germany) operated in the positive ionisation mode. ESI-MS/MS parameters were as follows: potential of the ESI source 4.5 kV, capillary temperature 260°C, argon served as collision gas (267 mPa),

the collision energy was 30 eV (SRM_1: m/z 479⇒m/z 303) and 50 eV (SRM_2: m/z 479⇒m/z 229).

2.3. HPLC–photodiode array detection (PDA) system

For HPLC–PDA an autosampler System Gold 502 (Beckman, Germany), a programmable solvent Module System Gold 126 (Beckman), a photodiode array detector Module System Gold 168 (Beckman), and a programmable column oven STH 585 (Gynkotek, Germany) were used.

Separations were carried out with an analytical column (250×3 mm I.D.) Eurospher 100 C_{18} (Knauer). Solvents used were 0.05% aqueous trifluoroacetic acid (A) and gradient-grade methanol (B). The flow-rate was 1.0 ml·min^{-1}.

The gradient profile used for separation was: 0.0–1.2 min, 5–23% B in A, linear; 1.2–3.6 min, 23% B in A, isocratic; 3.6–5.6 min, 23–35% B in A, linear; 5.6–10.0 min, 35% B in A, isocratic; 10.0–12.0 min, 35–50% B in A, linear; 12.0–20.0 min, 50% B in A, isocratic; 20.0–21.2 min, 50–60% B in A, linear; 21.2–30.0 min, 60% B in A, isocratic; 30.0–35.0 min, 60–87% B, linear; 35.0–38.0 min, 87% B in A, isocratic; 38.0–50.0 min, 87–100% B in A, linear.

2.4. UDP glucuronyl transferase assay

A 63.0-ml volume of reaction cocktail containing 20.0 mmol Trizma hydrochloride buffer, pH 8.0, 29.16 μmol quercetin, 3.0 mmol magnesium chloride, 2.3 g bovine serum albumin powder, and 0.2 mmol 2-mercaptoethanol was prepared with water and adjusted to pH 8.0 using hydrochloric acid or aqueous sodium hydroxide solution. At 37°C 88.0 μl of an aqueous 100 mg·ml^{-1} uridine-5′-glucuronyl transferase solution was added to 6.3 ml of the reaction cocktail prior to 211.0 μl of a 30 m*M* UDPGA (Sigma, Germany). The mixture was incubated at 37°C.

In order to stop the enzyme reaction by denaturation of proteins after 60 min, 1.0 ml of 96% aqueous ethanol was added. For separation of denatured protein the reaction cocktail was centrifuged at 7826 *g* for 10 min. The supernatant was analysed by HPLC–PDA and LC–UV–MS/MS.

2.5. Plasma sample preparation from volunteers

The relative systemic availability of quercetin from various formulations containing quercetin or quercetin glycosides was investigated in a pilot study with three healthy volunteers. During the course of the study the subjects were kept on a flavonoid-free diet to avoid interference with other food flavonoids. The study was approved by the local Human Ethics Committee of the University of Rostock, Germany.

One hour after consumption of approximately 800 g stewed onions venous blood samples were collected into EDTA-S-Monovette tubes (Sarstedt, Germany). After centrifugation at 7826 *g* for 10 min 500 μl of plasma was mixed with 20 μl 0.58 *M* acetic acid and 500 μl acetone, vortex-mixed and again centrifuged. This method effectively precipitated proteins without any co-precipitation of analytes and stabilised flavonols and their metabolites. The supernatant was removed and analysed by HPLC–PDA and HPLC–UV–MS/MS.

3. Results

3.1. Ionisation of quercetin

In preliminary experiments we compared ionisation with both ESI and atmospheric pressure chemical ionisation (APCI) in the positive and negative ion monitoring modes under experimental conditions. As a result, the produced ions of quercetin in the ESI positive ion mode showed more abundance and an improved signal-to-noise ratio.

In order to investigate fragmentation behaviour of quercetin, product ions were generated by CID of the molecular ions. The CID spectrum in Fig. 1 shows the characteristic quercetin fragments m/z 229, m/z 153, and m/z 137 [7].

3.2. UGT assays to obtain reference material

The reaction mixture of the UDP glucuronyl transferase in vitro assay was separated on a 250 mm Eurosper analytical column and analysed by HPLC–PDA. In the chromatogram, four additional peaks at retention times (t_R)=25.64, 24.07, 20.70 and 18.44

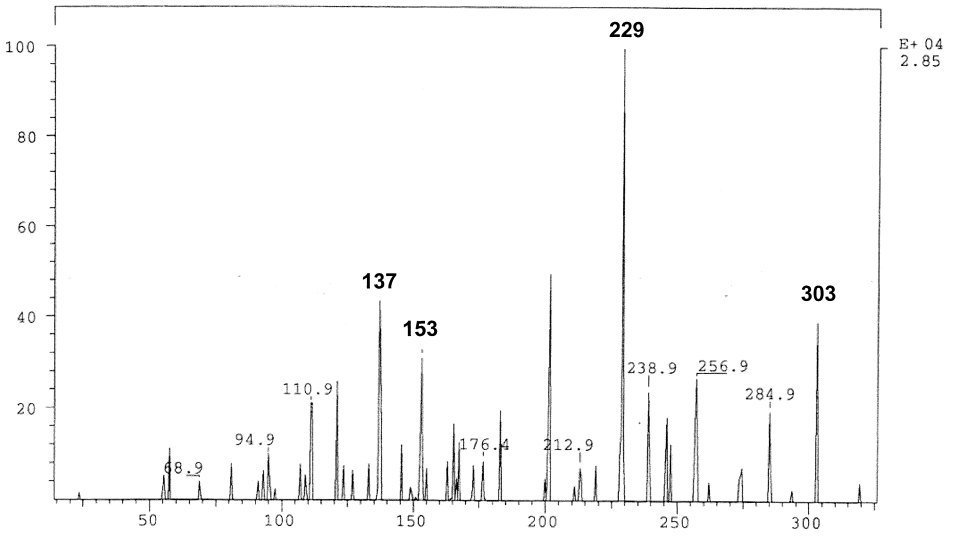

Fig. 1. Product ions spectrum of quercetin (precursor ion m/z 303; $[M+H]^+$).

min were recorded. These compounds represented more polar metabolites as compared to quercetin (t_R=29.3 min) and showed UV–Vis spectra typical for quercetin glucuronides [8]. After incubation with β-glucuronidase all of the quercetin glucuronide peaks disappeared completely and an equivalent increase of the quercetin peak could be observed (Fig. 2). In order to prove these results all samples were subsequently analysed by HPLC–MS/MS. All peaks assigned as quercetin glucuronides showed the expected molecular ion m/z 479.

In order to characterise these putative quercetin glucuronides by tandem mass spectrometry, product ions were generated by CID of the molecular ions. The product ion spectrum (Fig. 3) shows fragments of the quercetin glucuronide eluting at 13.6 min in the positive ESI mode. Besides the precursor ion $[M+H]^+$ (m/z 479), most abundant ions represented the intact aglycone quercetin (m/z 303) together with characteristic quercetin fragments (m/z 229, m/z 153, and m/z 137) [7].

3.3. SRM experiments

Based on the product ion spectra obtained from UGT assay reference material, the following SRM experiments were developed: with SRM_1 neutral loss of hexuronic acid was detected by monitoring the fragmentation from quercetin glucuronide (m/z 479, $[M+H]^+$) to quercetin (m/z 303, $[quercetin+H]^+$) employing 30 eV collision energy. This experiment identified metabolites as glucuronides (or conjugates of isomeric hexuronic acids). A second SRM experiment (SRM_2) was carried out by recording product

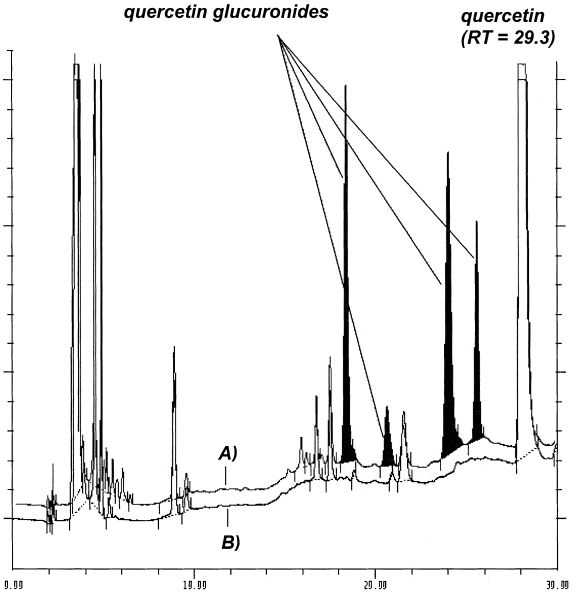

Fig. 2. HPLC chromatograms (UV 254 nm) from samples of in vitro assays prior to (A) and after (B) glucuronidase treatment.

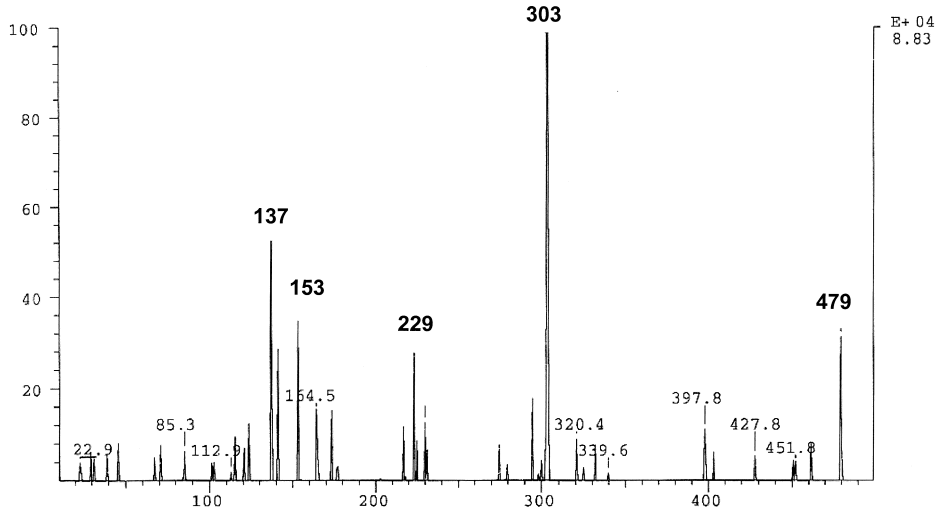

Fig. 3. Product ions spectrum of quercetin glucuronide (precursor ion m/z 479; $[M+H]^+$, t_R=13.6 min).

ions of the quercetin fragment m/z 229 using 50 eV collision energy in order to confirm the identity of the aglycone quercetin. Chromatograms in Fig. 4 (traces 1–3) show the results of SRM experiments with samples from the UGT assay. Consequently, UGT assay data obtained by HPLC–PDA could be confirmed by HPLC–MS/MS applying SRM. Four enzymatically synthesised quercetin glucuronides could be detected by both HPLC–UV (254 nm) and –MS/MS analysis.

In order to investigate whether quercetin glucoside (SRM_3), quercetin sulfate (SRM_4) or the free aglycon quercetin (SRM_5) was present in plasma samples under studies, additional SRM experiments were performed. Experiments SRM_3 and SRM_4 determined the neutral loss of glucose or sulfate at 30 eV by yielding fragmentation to the quercetin aglycon (m/z 303) from quercetin glucoside (m/z 465) or quercetin sulfate (m/z 383), respectively. SRM_5 was performed by recording the fragmentation of the aglycone quercetin (m/z 303) to the fragment ion m/z 229 using 40 eV collision energy.

3.4. Detection of quercetin glucuronides in human plasma

Following consumption of stewed onions, in human plasma samples eight chromatographic peaks representing putative quercetin metabolites were recorded with UV detection at 254 nm with t_R values from 9.0 to 14.0 min (Fig. 4, traces 4–6). The mass chromatogram of SRM_1 showed five conjugates with hexuronic acid at t_R=10.02, 10.8, 11.9, 13.15 and 13.6 min. The SRM_2 data confirmed identification of these quercetin metabolites, and revealed presence of an additional compound at t_R=12.3 min, that yet has to be characterised.

Taken together SRM experiments demonstrated the presence of all five quercetin glucuronides in human plasma in one analysis. The SRM data of both plasma samples and UGT assay are compared in Fig. 4 (traces 3 and 4). At the retention times of the four reference glucuronides obtained from the UGT assay the respective four quercetin metabolites have been detected in the plasma sample. The chromatogram of the plasma sample is showing an additional quercetin glucuronide peak at t_R=13.6 min. One should note, that neither the free aglycone quercetin nor further metabolites such as sulfate conjugates could be detected by HPLC–MS/MS.

4. Conclusions

This study describes simultaneous detection of quercetin conjugates after HPLC separation by means of UV and MS. The method is selective for the detection of quercetin metabolites and allows

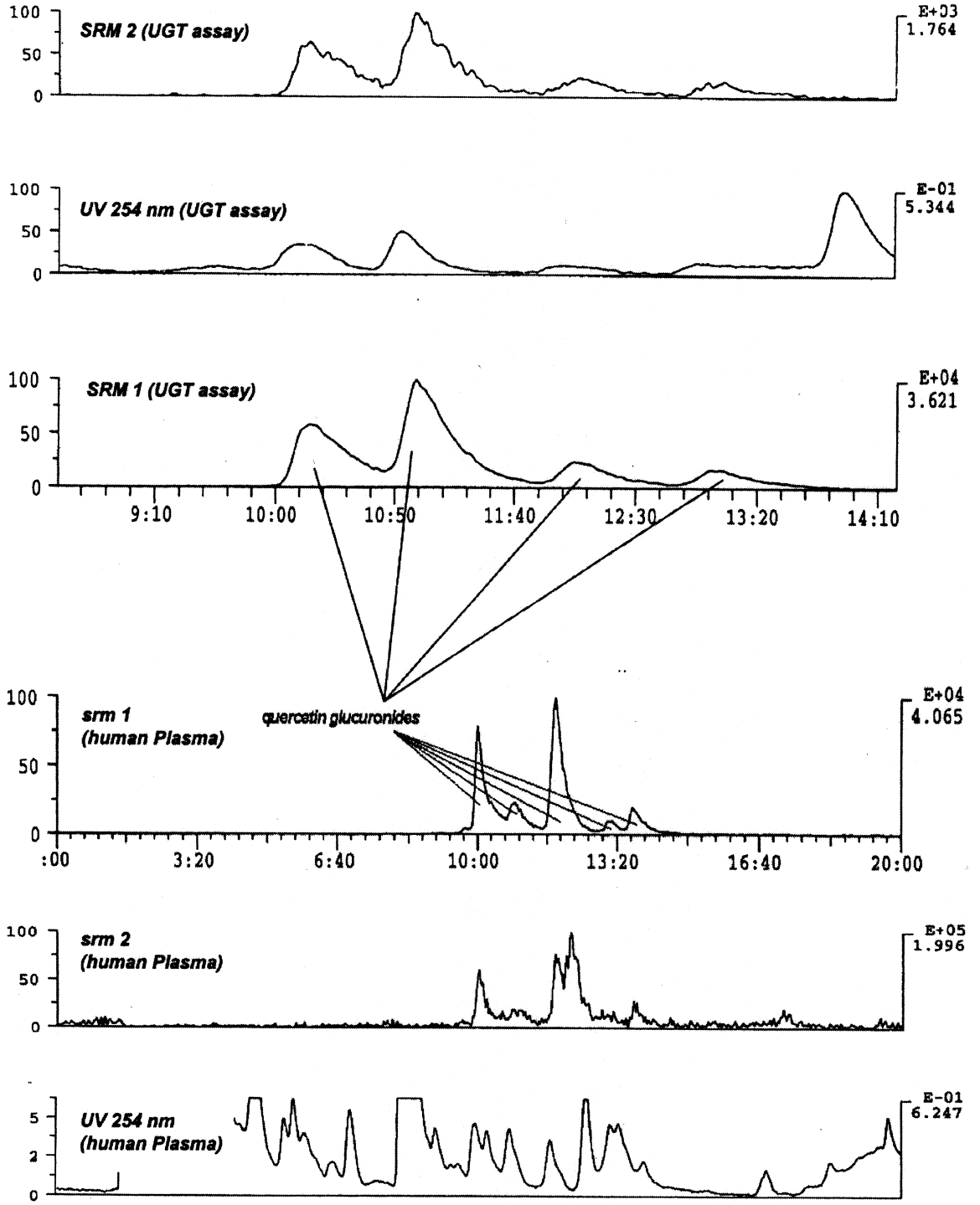

Fig. 4. Traces of HPLC–MS/MS–SRM and HPLC–UV experiments, respectively, with samples from UGT assay (traces 1–3) and samples from human plasma after consumption of onions (traces 4–6).

identification of hexuronic acid conjugates of quercetin. It is particularly useful with regard to the fact that due to similar retention times and UV spectra quercetin glucuronides could easily be mistaken for glycosides. However binding position of the glucuronide moiety could not be determined by MS/MS experiments and requires authentic reference compounds to obtain retention times of the individual regioisomers.

Based on these shortcomings in previously applied analytical methodology, it is still a matter of controversy whether the genuine compound (quercetin glycoside) or any metabolite (phase II conjugate) is systemically available after oral intake [2,4,8–11]. The data obtained in this study suggest that no unconjugated nor genuine glycosidic quercetin could be detected in the systemic circulation in humans after consumption of onions. We also found no evidence for the presence of sulfates, which have been reported from animal experiments as metabolites [12,13]. According to the different number of synthesised quercetin glucuronides by rabbit or human UGTs a difference in substrate specify of UGT between different species is most likely.

The method presented here allows the quantification of the different quercetin glucuronides in principle. However, compared to other methods like fluorescence and electrochemical detection, it is less sensitive. The limit of detection (LOD) using HPLC–MS/MS in the ESI positive mode is about 2 ng on-column compared to an LOD of 0.4 ng on-column employing PDA. Furthermore, as long as no labelled authentic reference compounds of the metabolites are available for isotope dilution experiments, reliability and robustness of quantification by HPLC–MS/MS have to be monitored carefully. Analysing plasma samples by HPLC with electrochemical detection, maximum glucuronide concentrations (c_{max}) in plasma after intake of quercetin glucosides from onions (equivalent to 100 mg quercetin) in 12 healthy volunteers were approximately 7.65 ± 4.83 nmol·ml^{-1}. Further details will be reported elsewhere [14].

Whether the detected quercetin glucuronides represent all five mono glucuronides of quercetin at positions 3,5,7,3′ and 4′, respectively needs further investigation.

With regard to the different positions of glucuronidation significant variance in the antioxidant potential of the individual quercetin glucuronides is most likely. Yet, free quercetin liberated by glucuronidases in the target tissue in vivo could be the most effective compound with regard to antioxidant activity. Ongoing biochemical studies will help to clarify this issue.

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