

Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties

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Abstract Quercetin is one of the most abundant flavonoids in the human diet. This study aimed to determine the plasma concentrations of quercetin in 10 healthy volunteers after the consumption of a complex meal rich in plant products. Quercetin was determined in plasma (2 h before, and 3, 7 and 20 h after the meal), and in a duplicated portion of the meal by HPLC analysis with an electrochemical detection. The amount of ingested quercetin was estimated to be 87 mg. Before the meal, quercetin concentration in hydrolyzed plasmas ranged from 28 to 142 nM. A marked increase was observed 3 h after the meal in all subjects, with a mean concentration of 373 nM (S.E.M. = 61). After 7 h, quercetin concentration in hydrolyzed plasmas decreased and after 20 h basal levels were found again. The antioxidant capacities of quercetin, 3'-O-methylquercetin, and of some of their conjugated derivatives were compared by the measurement of the conjugated dienes resulting from the Cu²⁺-induced oxidation of human LDL. 3'-O-Methylquercetin and conjugated derivatives of quercetin significantly prolonged the lag phase, but the magnitude of their effect was about half that of the aglycone.

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Key words: Quercetin; Flavonoid; Conjugated metabolite; Dietary antioxidant; Low density lipoprotein oxidation; Human

1. Introduction

Quercetin is a powerful antioxidant which is regularly consumed by humans. The quercetin content of foodstuffs is not yet precisely known but the available data display a range of 2–250 mg quercetin/kg wet weight in fruits, 0–100 mg/kg in vegetables (onions being especially rich: 200–600 mg/kg), 4–16 mg/l in red wine, 10–25 mg/l in tea, 3–13 mg/l in fruit juices [1–4]. Quercetin content may vary greatly, depending on the plant varieties, culture conditions, degree of ripeness, as well as on food processing (preservation, peeling, mode of cooking) [5]. From the composition tables, the daily consumption of flavonols plus flavones has been estimated as 3–80 mg, more than 50% of which is quercetin [6–8]. Two recent epidemiological studies support the view that such intake of flavones and flavonols could protect against cardiovascular mortality [6,7]. Quercetin could especially be effective in preventing atherosclerosis and thrombosis by protecting low density lipoproteins (LDL) against oxidation, as well as by lowering the cytotoxicity of oxidized LDL and platelet aggregation. Furthermore, quercetin exhibits antitumor effects in

vitro and inhibits the development of experimental cancers in animal models [9,10].

Few studies have been carried out on the flavonol bioavailability in humans. In 1975, Gugler [11] was unable to detect quercetin, conjugated or not, in urine or in plasma from healthy volunteers after ingestion of 4 g quercetin. During the last years, analytical methods have been greatly improved, and concentrations as low as 10 nM can now be determined in human plasma samples. Hollman et al. [12] have recently reported that the ingestion of 64 mg quercetin (provided as 150 g fried onions) resulted 3 h later in a concentration of 650 nM quercetin in hydrolyzed plasma. Another study [13] performed on two healthy volunteers showed that kaempferol was also absorbed after broccoli intake. Data are still scarce and more studies are needed on the flavonol bioavailability in humans, especially to determine food habits that can increase it.

In parallel, the nature of the flavonol metabolites has to be considered. In vitro studies have established that quercetin is a potent antioxidant, which could affect the activity of numerous enzymes, or even chelate some metals [14]. But quercetin can be metabolized into methylated or conjugated derivatives, as reported in rat [15,16], and no information is available concerning the potential activity of these metabolites.

The present work was performed (i) to determine the plasma concentration of quercetin metabolites in 10 healthy volunteers after the consumption of a complex meal, and (ii) to test the ability of some conjugated derivatives of quercetin and of its 3'-O-methylated form (isorhamnetin) to prevent in vitro oxidation of human LDL.

2. Materials and methods

2.1. Chemicals

Quercetin, UDP-glucuronic acid, UDP-N-acetylglucosamine, UDP-glucuronyl transferase from rabbit liver and β -glucuronidase/sulfatase were purchased from Sigma (L'île d'Abeau, Chânes, France). Quercetin 3-O-sulfate, kaempferol, luteolin and isorhamnetin (3'-O-methylquercetin) were from Extrasynthese (Genay, France).

2.2. In vitro glucuronidation

Quercetin and isorhamnetin glucuronides were obtained by in vitro synthesis. Quercetin or isorhamnetin (100 μ M) were incubated 3 h at 37°C in a HEPES buffer (25 mM, pH 7.4) in the presence of 10 mM MgCl₂, 4 mM UDP-glucuronic acid, 2 mM UDP-N-acetylglucosamine, and 0.12 U/l UDP-glucuronyl transferase. The conjugation rate was about 95%.

2.3. Human study

Ten volunteers (four women, six men) participated in the study. They were between 25 and 54 years of age and their mean body mass index was 23.1 ± 3.0 kg/m². All participants were healthy, did not use any medication and provided informed consent. The protocol

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was approved by the 'Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale' of Clermont-Ferrand.

The subjects followed a quercetin-free diet for 24 h prior to the experimental meal. For this purpose, a list of authorized foods and beverages was given to the participants. At 12.00 h, they were served an experimental lunch (800 g wet weight), containing foods rich in quercetin (onions, salad, tomatoes, french beans, apples, berries, red wine) in combination with quercetin-free foods (chicken, cheese). A duplicate portion of the meal was stored at -80°C for the quercetin composition analysis. An aliquot of wine was collected into vacuum tubes and stored at -20°C .

Venous blood samples from the 10 subjects were collected by venipuncture on heparin, 2 h before, and 3, 7 and 20 h after the experimental meal. Subjects were instructed not to consume any food or beverage containing quercetin after the experimental meal until the last blood sampling.

2.4. Treatment of plasma samples

Flavonoids are unstable when the pH of plasma is higher than 7.4; so, to prevent a drift of the pH due to gradual bicarbonate decomposition, plasmas were acidified with 10 mM acetic acid immediately after blood centrifugation (15 min at $2000\times g$), and stored at -80°C .

A simple treatment procedure was used, to limit the flavonoid losses; namely, plasmas were acidified (to pH 4.9) with 0.1 volume of 0.58 M acetic acid solution and treated for 30 min at 37°C in the presence or in the absence of 5×10^6 U/l β -glucuronidase and 2.5×10^5 U/l sulfatase. Flavonoids were extracted with 2.75 volumes of acetone. After centrifugation, 20 μl of the supernatant was directly injected in the column for HPLC analysis. The percentage of recovery has been estimated to be over 85% using pure quercetin or isorhamnetin in plasma. Analysis were performed in duplicate.

2.5. Treatment of food samples

The duplicate portion of food was lyophilized (15°C , 10^{-2} mbar, 72 h) to obtain a homogeneous powder. This sample and the wine sample were extracted and hydrolyzed with 200 volumes of 1.2 M HCl solution in 50% aqueous methanol (v/v). Extracts were refluxed at 90°C for 2 h, purified on glass filters and subsequently made up to 400 volumes with 50% aqueous methanol.

2.6. Quercetin determination

Quercetin was determined in plasma, wine and food extracts by HPLC analysis coupled with a coulometric detection. The column (150×4.6 mm Inertsil, Interchim, France) was eluted isocratically (1 ml/min) at 30°C with a 30 mM NaH_2PO_4 buffer containing 45% methanol (pH 2.6). Electrochemical detection was performed with a

Coulochem II (Eurosep, France), the potential was set at +150 mV. The limits of detection for quercetin and isorhamnetin were 5 nM and 10 nM respectively.

2.7. Human LDL preparation

A pool of plasmas collected from the 10 subjects before the test meal was used. The plasma LDL fraction was prepared by sequential density ultracentrifugation, in the presence of 1 g/l EDTA. Plasma was adjusted to a density of 1.019 kg/l with solid KBr in sealed tubes (OptiSeal tubes, Beckman, Palo Alto, CA) and centrifuged 2 h at $645000\times g$, 15°C (L90, rotor NVT 90, Beckman). The top fraction (chylomicron+VLDL) was removed and the remaining plasma was adjusted to 1.063 kg/l with solid KBr and centrifuged 2 h at $645000\times g$, 15°C . The top fraction (LDL) was collected and dialyzed 24 h (at 4°C in darkness) against phosphate buffer (0.1 M H_3PO_4 /0.16 M NaCl, pH 7.4).

2.8. Measurement of the oxidative susceptibility of lipoproteins

The final protein concentration (BCA protein reagent kit, Pierce, Interchim, France) in human LDL fractions was adjusted to 50 mg/l. The LDL fraction was supplemented with ethanol (control conditions), quercetin, isorhamnetin, quercetin 3-O-sulfate, which was the sole sulfo-conjugated form of quercetin commercially available, a mix of four quercetin glucuronides or a mix of four isorhamnetin glucuronides obtained by in vitro conjugation of quercetin or isorhamnetin by UDP-glucuronyl transferase. Oxidation was initiated by addition of a freshly prepared CuSO_4 solution (10 μM final concentration) at 37°C . The kinetics of the oxidation of lipoproteins were determined by monitoring continuously the changes in the conjugated diene absorbance (234 nm) at 37°C on an Uvikon 930 spectrophotometer (Kontron, France). Lag time values are means \pm S.E.M. ($n=5$).

3. Results

By the HPLC-ECD analysis of the experimental meal and of the red wine samples, the quantity of ingested quercetin has been estimated as 87 mg, 4.5% of which was provided by the red wine. No kaempferol, luteolin or isorhamnetin could be detected in the meal.

Before the experimental meal, quercetin concentration in plasmas treated with β -glucuronidase/sulfatase ranged from 28 to 142 nM in the 10 volunteers (Fig. 1). A marked increase was observed 3 h after the meal in all the subjects. Quite

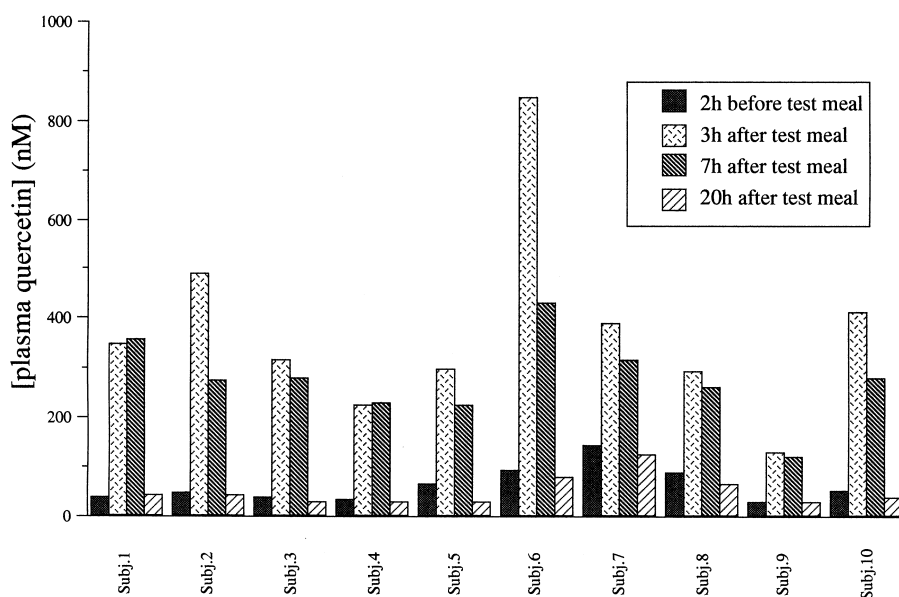


Fig. 1. Quercetin concentration in plasmas treated with β -glucuronidase/sulfatase, from 10 healthy volunteers before and after a meal rich in plant products.

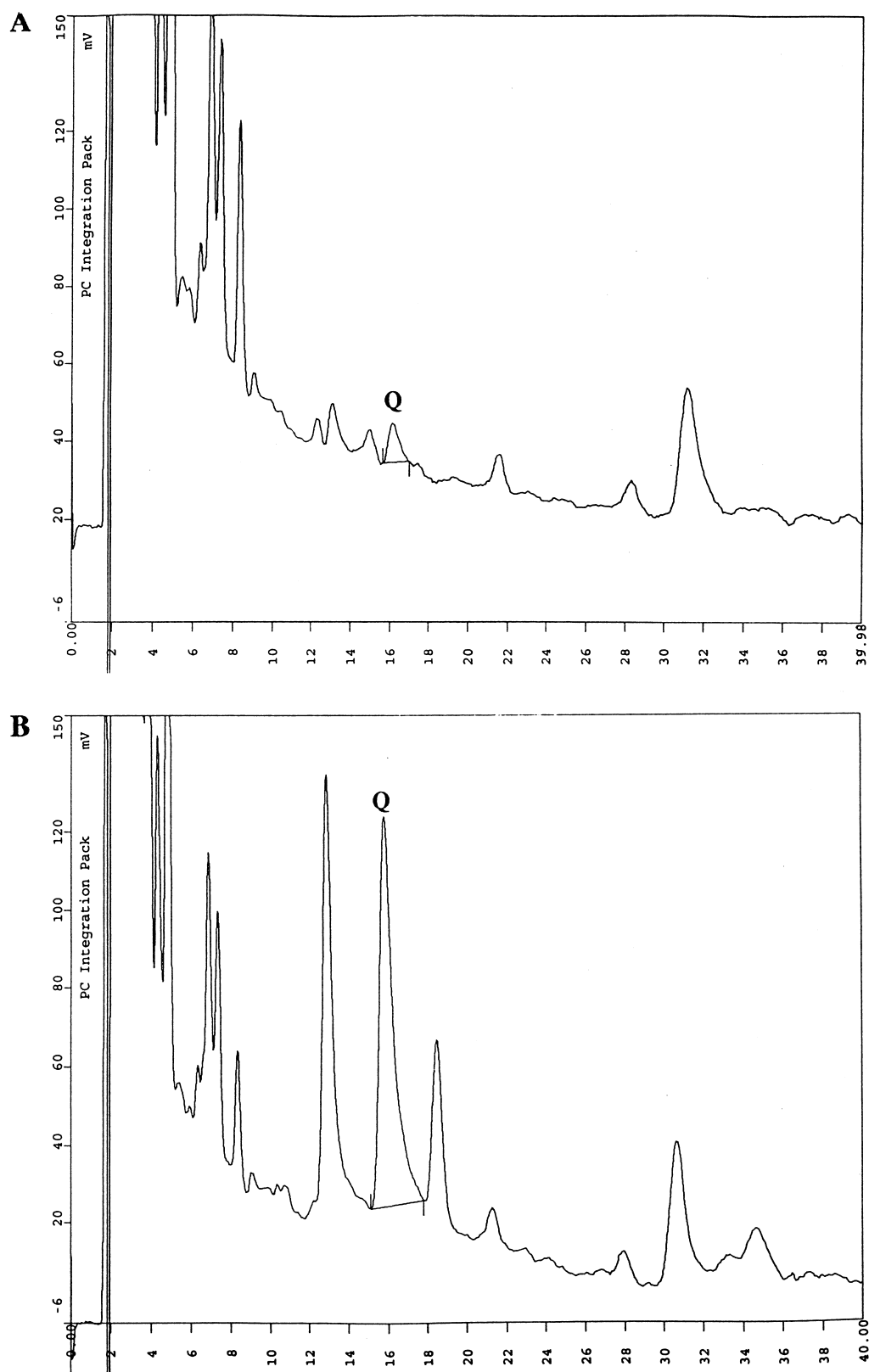


Fig. 2. Representative chromatogram of human hydrolyzed plasma before (A) and 3 h after (B) the experimental meal. Q = quercetin.

homogeneous values were obtained for eight subjects, with a mean concentration of 344 nM (S.E.M. = 29), but a particularly high (843 nM) and a low (129 nM) concentration were observed in subjects 6 and 9 respectively. After 7 h, the quer-

cetin concentrations in hydrolyzed plasmas were similar (for three subjects) or lower than those observed 3 h after the experimental meal, but were still higher than the basal concentrations. 20 h after the test meal, basal concentrations were

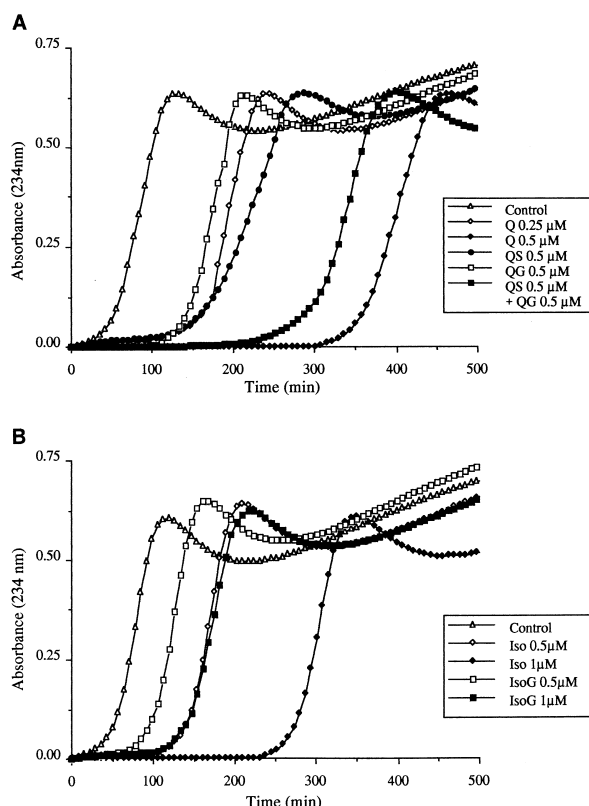


Fig. 3. Inhibition of the Cu^{2+} -induced oxidation of human LDL (measured by conjugated diene formation) by (A) quercetin or its conjugated derivatives (Q = quercetin; QS = quercetin-3-*O*-sulfate; QG = quercetin glucuronides) or (B) isorhamnetin or its conjugated derivatives (Iso = isorhamnetin; IsoG = isorhamnetin glucuronides). Values are from a representative experiment.

found again. It can be noted that the subjects 6, 7 and 8 who had the highest quercetin concentrations in hydrolyzed plasmas before the experimental meal also had the highest concentrations 20 h after the meal. Yet, they were not necessarily those who showed the greatest increase 3 h after the meal. No significant differences could be observed between women (subjects 1–4) and men (subjects 5–10).

Fig. 2 shows typical chromatograms of plasmas, hydrolyzed by β -glucuronidase/sulfatase, from the same subject, 2 h before and 3 h after the experimental meal. The low voltage used (150 mV) was very selective and only a few compounds can be oxidized at such voltage. The increase in the quercetin peak height (retention time 16.1 min) is very clear. Two other peaks, with retention times of 13.2 and 18.4 min, appeared, but were not identified. However, these compounds could not correspond to glycosides, which exhibit lower retention times (<4 min) in such chromatographic conditions. Significant concentrations of isorhamnetin (100–200 nM 3 h after the meal, with a 30% decrease at 7 h, and a total loss after 20 h; retention time 34.5 min) were found in plasma samples from three of the 10 subjects. In the other subjects, no isorhamnetin could be detected (limit of detection 10 nM).

Plasma samples obtained 3 h after the experimental meal were also analyzed by HPLC coupled to UV detection (Hypersil BDS column from Interchim, 27% acetonitrile+73% water/ H_3PO_4 (99.5/0.5), 1.5 ml/min, 370 nm). Both methods gave the same quercetin concentration within a 7% variation,

but the limit of detection was higher with UV than with coulometric detection (150 vs. 5 nM).

We previously reported that, in rats fed a quercetin-supplemented diet, plasma quercetin was completely conjugated with glucuronic acid or sulfate. Similarly, no trace amounts of free quercetin could be detected in human plasma before β -glucuronidase/sulfatase hydrolysis, which implies that all circulating metabolites of quercetin are conjugated derivatives. Thus it was important to test the antioxidant activity of some conjugated forms of quercetin, obtained as described in Section 2. Fig. 3A shows that quercetin increased, in a concentration-dependent manner, the lag phase of conjugated diene formation: $+110 \pm 5$ min at 0.25 μM and $+310 \pm 18$ min at 0.5 μM . When present at 0.5 μM , quercetin glucuronides or quercetin 3-*O*-sulfate also significantly delayed copper-induced lipoprotein oxidation ($+90 \pm 8$ min and $+110 \pm 10$ min respectively); however, the magnitude of their effects was lower than that of the aglycone. The simultaneous presence of 0.5 μM quercetin glucuronides and 0.5 μM quercetin 3-*O*-sulfate resulted in an inhibition of conjugated diene appearance quite similar to that observed with 0.5 μM quercetin. Isorhamnetin, which was recovered in some plasmas, also delayed the oxidation of human LDL ($+90 \pm 5$ min), but was less potent than quercetin (Fig. 3B). As for quercetin, the effect of isorhamnetin glucuronides was lower than that of the aglycone. It could be noted that, even if all the compounds significantly prolonged the lag phase, they had no detectable effect on the rate of lipid oxidation during the propagation phase.

4. Discussion

This present study shows that metabolites of the dietary antioxidant quercetin are found in the blood circulation after consumption of various vegetables and fruits.

The quercetin content of the experimental meal was 87 mg, which can be compared to the 89 mg obtained by Hollman et al. [17] with 150 g fried onions. While this represents about three-fold the mean daily intake estimated in the Northern Europe, it seems quite easy to reach this level of quercetin intake with a Mediterranean diet rich in fresh plant products. This clearly illustrates the great differences of intake that can occur between individuals, depending on their food habits.

The general features of quercetin absorption in rats have been previously described [16,18]. Briefly, the absorbed quercetin is transported bound to albumin, chiefly methylated in the 3' position, and entirely conjugated with glucuronic acid and sulfate. Recent studies have specified that the conjugated forms are mostly glucurono-sulfated derivatives [19]. In spite of the low limit of detection (5 nM) of our HPLC-ECD method, we failed, as in rat plasmas, to detect trace amounts of free quercetin in non-hydrolyzed human plasma samples. Thus plasma quercetin metabolites are also conjugated derivatives in humans. In parallel, it seems that the methylation process is less important in humans than in rats. Although there are probably differences between species, it must be noted that in plasmas from rats fed a 2 g/kg quercetin diet, the isorhamnetin concentration was about four times higher than that of quercetin, whereas in rats fed a lower amount of quercetin (<0.5 g/kg), the isorhamnetin/quercetin ratio was markedly lower (about 2) (data not shown). This suggests that the methylation process could be important only when relatively high quantities of quercetin are ingested, which could

explain why only low amounts of isorhamnetin were found in human plasma.

The quercetin concentrations (about 350 nM) obtained in hydrolyzed plasmas 3 h after the experimental meal were about twice as low as those reported by Hollman et al. [12] after ingestion of onions, but were in the same range as those obtained by the same authors after ingestion of applesauce [20]. It cannot be ruled out that the blood sampling performed 3 h after the meal in our experiment could not exactly correspond to the maximal absorption peak, so that we could slightly underestimate the quercetin concentrations. Nevertheless, the differences between experiments may also result from the nature of the quercetin glycosides present in food, or from the influence of the other constituents of foods. Indeed, the presence of elements liable to bind quercetin (dietary fibers, proteins, etc.), or to solubilize it (alcohol) could modify the kinetics or the extent of its absorption.

With regard to the absorption of glycosides, which are the main forms present in foods, we previously reported that rutin was absorbed more slowly than quercetin in rats since it had first to be hydrolyzed by bacterial glycosidases in the cecum [21]. According to Hollman's work on ileostomy subjects [17], marked differences also exist in humans between absorption of quercetin and of its glycosides: glucosides from onions were better absorbed than quercetin, which was itself better absorbed than rutin. The authors made the assumption that glucosides could be absorbed as such, by a mechanism that would implicate the glucose carrier. However, the presence of quercetin glycosides in plasma is still speculative. The plasma treatment with β -glucuronidase/sulfatase used in the present study and the acid treatment employed by Hollman both hydrolyze glycosides together with glucuronides, so that it is impossible to detect their presence in a native form. Yet, Pa-ganga and Rice-Evans [22], using an array diode detection method, found high concentrations of quercetin glycosides (0.5–1.6 μ M) in plasma samples from non-supplemented subjects. This result is quite surprising and has not been confirmed to our knowledge. Conjugated derivatives could have been mistaken for glycosides, as they exhibit very similar retention times and spectral profiles, as we have checked in our laboratory.

The rapid post-prandial absorption of quercetin indicates that it probably takes place in the proximal part of the intestine. Several hypotheses can be considered: the first one is closely akin to that of Hollman, involving an absorption of the glycoside forms. In this case, the possibility of a rapid conjugation of the glycosides should be examined. The second hypothesis is that quercetin is present in the proximal part of the intestine, resulting from hydrolysis of glycosides during food processing (by release of vegetal glycosidases), or during the first phase of the digestion in the stomach. Moreover, free quercetin could also be present in significant amounts in foods, as has been reported for some onion species [1].

Various flavonoids inhibit the peroxidation of human LDL induced in vitro by cupric ions or macrophages [23,24]. Quercetin is one of the most potent antioxidant polyphenols, according to (i) the unsaturation of its heterocycle, conjugated with the 4-oxo function, (ii) the presence of the *o*-diphenolic part of the B cycle, and (iii) the hydroxyl groups in positions 3 and 5 [25]. The mechanisms involved in preventing LDL oxidation or delaying α -tocopherol consumption are not yet fully established (inhibition of free radical formation, free

radical scavenging, α -tocopherol regeneration), but quercetin could act at the lipid/water interface, since it is not tightly bound to lipoproteins, in contrast to lipophilic antioxidants [26]. The glucuronidation or the sulfation of quercetin modifies its hydrophobicity and the possibilities of electron delocalization so it could be predicted that the antioxidant effect of quercetin conjugates could be different from that of the aglycone. In the present work we demonstrate that some conjugated derivatives of quercetin also significantly delay the Cu^{2+} -induced oxidation of human LDL. Even if the magnitude of this inhibition was about half that measured with the aglycone, conjugated derivatives of quercetin could exert physiological effects, possibly in synergism with other compounds from fruits and vegetables.

In conclusion, the present work shows a marked increase in the plasma concentration of conjugated derivatives of quercetin after consumption of a meal rich in plant products. Quercetin is entirely present as conjugated derivatives, which could modify its biological effects. After 20 h without new quercetin ingestion, plasma concentrations return to quite low basal levels. This implies that beneficial effects of flavonols should depend on a regular consumption of plant foods rich in these polyphenols. Additional studies must be performed to understand the mechanisms of intestinal absorption of flavonols and to determine the exact nature of their metabolites before assessing their actual role on human health.

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