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Dietary quercetin is recovered in rat plasma as conjugated derivatives of isorhamnetin and quercetin

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Quercetin is present in noticeable amount in human diet and this polyphenolic molecule is supposed to exert beneficial effects on human health. However, its intestinal absorption and its metabolic fate in the organism have not received much attention. In the present study, rats were fed a control or a 0.25% quercetin diet, and the plasma, urine, and bile metabolites of the dietary quercetin were analyzed by HPLC. Conjugated derivatives of quercetin and isorhamnetin, a 3'-O-methylated form of quercetin, were identified in the plasma from the rats fed quercetin. After deconjugation, the concentration of aglycones in the plasma reached 120 ± 16 μmol/L, with an isorhamnetin/quercetin ratio of about 5. In bile and in urine, where the 4-oxo-flavonoid concentration were 378 ± 42 and 128 ± 19 μmol/L respectively, conjugated derivatives of quercetin and isorhamnetin, but also of tamarixetin, a 4'-O-methylated form of quercetin were recovered. In plasma, the 4-oxo-flavonoid metabolites are bound to albumin, which induces a bathochromic effect. The bathochromic and chromogenic responses depend on the presence of the unsaturated C2–C3 bound of the C-ring and on the presence of hydroxyl groups on the B-ring. Studies on 4-oxo-flavonoid bioavailability could allow a better understanding of the nutritional effects of various type of plant products. (J. Nutr. Biochem. 7:375–380, 1996.)

Keywords: quercetin; rat; metabolism; isorhamnetin; albumin.

Introduction

Quercetin belongs to the flavonol family of 4-oxo-flavonoids. These compounds, and particularly quercetin, are widely distributed in dietary plants. The daily consumption of 4-oxo-flavonoids in a western diet has been assessed by Kühnau[1] at about 160 mg of quercitrin (PM 464) equivalent, but more recently Hertog et al.[2] have reported a mean intake of 26 mg/d (more than 60% being as quercetin) in the Netherlands. Many studies suggest a possible beneficial effect of flavonoids on human health, owing to their antioxidant properties[3] and their ability to modulate the activity of various enzymes, such as transferases, ATPases, kinases, lipoxygenases, cycloxygenases, and phosphodiesterases.[4] For example, flavonoids could play an important role in the prevention of atherosclerosis, principally by lowering the Low-Density-Lipoprotein peroxidation.[5] On the other hand, flavonoids have been shown to inhibit the growth of various cancer cell lines in vitro,[6–11] and to reduce tumor development in experimental animals.[12–14] Quercetin has been found mutagenic in several in vitro tests[15,16] and thus has been suspected of carcinogenicity. Actually, except for two studies,[17,18] works on this subject rather concluded that quercetin was not carcinogenic in vivo.[19–23] In fact, there is an active metabolism of flavonoids in mammals and thus the physiological impact of native quercetin is questionable.[24] Ueno et al.[25] reported that, after oral administration of a single dose of [^{14}C]quercetin to the rat, at least 20% of quercetin was absorbed from the digestive tract and rapidly excreted into the bile and urine as glucuronide and sul-

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fate conjugates. By feeding 1% rutin (a glycosylated form of quercetin) diet, an accumulation of quercetin metabolites, resulting in a yellow-colored plasma, was observed by Manach et al.[26] It seems thus that in spite of extensive metabolism by the intestinal flora, which give rise to three hydroxyphenylacetic acids,[27] a non-negligible part of the ingested quercetin is absorbed from the digestive tract and further metabolized by the rat tissues. Recently, Xu et al.[28] have shown that, in humans receiving 2 mg isoflavones/Kg body weight (BW), 10 to 20% were absorbed. In fact, to clarify the physiological impact of 4-oxo-flavonoids, it appeared necessary to identify the metabolites present in the plasma, and to describe the modality of their elimination by the urinary or biliary routes.

Methods and materials

Chemicals

Quercetin, hesperetin, kaempferol, naringenin, human and rat albumin (fraction V) and β-glucuronidase/sulfatase were purchased from Sigma (St Louis, MO, USA). Fisetin was from Aldrich (St Quentin, France). Isorhamnetin, tamarixetin, galangin, kaempferid, eriodictyol, luteolin, and taxifolin were from Extrasynthese (Genay, France).

Animals and diets

Twenty male Wistar rats (IFFA-CREDO, L'Arbresle, France) weighing approximately 170 g were randomly divided into two groups. The rats were individually housed in metabolic cages fitted with urine/feces separators, in temperature-controlled rooms (22°C) with a dark period from 2000 to 0800 hr and access to food from 1600 to 0800 hr. The rats were fed semi-purified diets, either the control diet or the same diet supplemented with 0.25% quercetin (namely 8.28 mmol/kg diet) at the expense of wheat starch (*Table 1*). The animals were maintained on the experimental diets for 14 days before sampling. Animals were maintained and handled according to the recommendations of the Institutional Ethic Committee (INRA), in accordance to the decree No. 87-848.

Table 1 Composition of diets

	Control diet	0.25% Quercetin diet
	g/kg dry feed	
Casein	150.0	150.0
Corn oil	50.0	50.0
Wheat starch	730.0	727.5
Mineral mix[1]	60.0	60.0
Vitamin mix[2]	10.0	10.0
Quercetin	0.0	2.5

[1]Mineral mix (UAR, Villemoisson/Orge, France) supplied the following (per kg diet): $CaHPO_4$, 18 g; K_2HPO_4, 3 g; KCl, 6g; $MgCl_2$, 3 g; Fe_2O_3, 3 mg; $MnSO_4$, 150 mg; $CuSO_4.7H_2O$), 0.24 mg; $ZnSO_4.7H_2O$, 120 mg; KI, 0.48 mg.
[2]Vitamin mix (UAR, Villemoisson/Orge, France) supplied the following (per kg diet): thiamin, 20 mg; riboflavin, 15 mg; pyridoxin, 10mg; nicotinamide, 100 mg; calcium panthotenate, 70 mg; folic acid, 5 mg; biotin, 0.3 mg; cyanocobalamin, 0.05 mg; retinyl palmitate, 1.5 mg; DL-α-tocopheryl acetate, 125 mg; cholecalciferol, 0.15 mg; menadione, 1.5 mg; ascorbic acid, 50 mg; myoinositol, 100 mg; choline, 1.36 g.

Sampling procedure

At the time of sampling (14 days after the diet introduction, at 0900 hr), rats were anesthetized with sodium pentobarbital (40 mg/kg BW). Blood (≈6 mL) was then drawn from the abdominal aorta into heparinized tubes.

Bile was collected from four rats of each group. Under anesthesia, the rat abdomen was opened and a micropolyethylene tube was inserted into the bile duct, the other end of the tube being led into a vial. The abdominal wall and skin were sutured, and the bile was collected for an hour.

Urine were collected on 2 consecutive days at the end of the experimental period.

Spectrophotometric studies

The absorption spectra of the plasma samples were performed using an Uvikon 930 spectrophotometer (Kontron, Paris, France). To avoid interference by plasma constituents (especially proteins) in the UV region, the spectrum analysis of flavonoids was limited to the description of Band I absorption. It is generally accepted that band I represents B-ring absorption, in the near-UV and visible region (320–380 nm), and band II the A-ring absorption (around 240 nm).[29] The shift of the absorption band I of pure flavonoids (12.5 μmol/L) to longer wavelengths (bathochromic effect) was studied in a 10 mmol/L phosphate buffer, pH 7.4, in the absence or in the presence of rat or human albumin (62.5 μmol/L).

HPLC analysis

Plasma, bile, and urine samples from rats fed the control or the 0.25% quercetin diets were spiked with 50 μmol/L fisetin, and acidified (to pH 4.9) with 0.1 volume of 0.583 mol/L acetic acid solution. Solutions were treated for 40 min at 37°C in the presence of 2.5×10^6 units/L β-glucuronidase and 1.2×10^5 units/L sulfatase, then treated by 8.5 volumes of acetone and centrifugated. Supernatants were evaporated to a volume equivalent to twice the initial volume of plasma, bile or urine. Recovery data of this method has been checked (>85%) using pure flavonoids (fisetin, quercetin, isorhamnetin, tamarixetin) in plasma, bile, and urine. Spiking samples with fisetin allowed us to adjust the concentrations according to the extraction efficiency in the different biological fluids. For analysis, 20 μl of each preparation was injected in a 12.5 cm RP18 Lichrosorb column (Interchim, France). Elution was performed using water-H_3PO_4 (99.5:0.5) as solvent A and acetonitrile as solvent B, in isocratic conditions (85% A/15% B) for 10 min, then in gradient conditions to 75% A/25% B within 10 min, next isocratically up to 45 min. The flow rate was 1.5 mL/min and the chromatograms were recorded at 370 nm.

Results

Rats fed the semi-synthetic diet supplemented with 0.25% quercetin had a green/yellow-colored plasma which exhibits a peak absorbance at 411 nm (*Figure 1*). A small peak was also detected at 413 nm with plasma from rats fed the control diet. This peak is unlikely to correspond to a flavonol metabolite, because its intensity increases in parallel to the degree of hemolysis; thus, this compound could be oxyhemoglobin, which maximum absorption is 412–415 nm. This result shows that, in spite of extensive breakdown of flavonoids to phenolic acids by the intestinal microflora,[30,31] a part of dietary quercetin is recovered in rat plasma. Due to aromatic nucleus and hydroxyl substituents, flavonoids have a great affinity for proteins, and particularly for albumin. We have previously reported that the binding of quer-

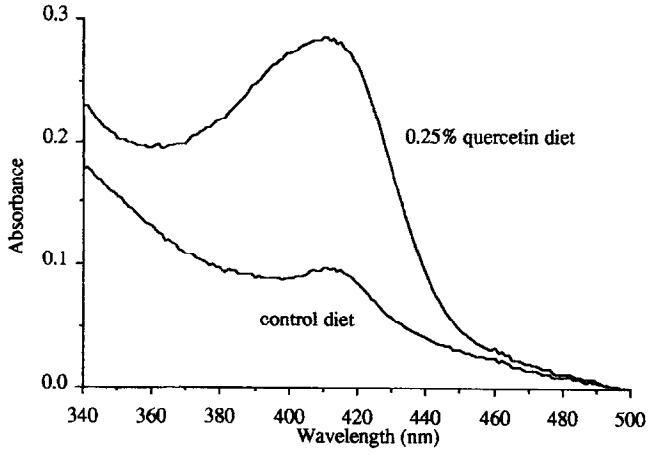
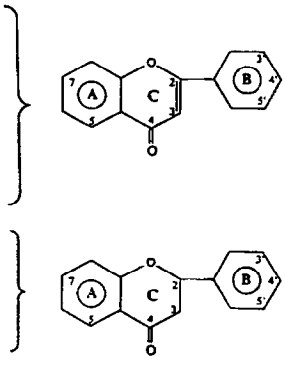


Figure 1 Spectra of diluted plasma from rats adapted for 14 days to a control diet or the same diet supplemented with 0.25% quercetin. The spectra were recorded on plasma samples diluted in 9 vol. of 10 mmol/L phosphate buffer, pH 7.4.

cetin to albumin causes a shift of its maximal absorption to longer wavelengths and that this bathochromic effect increases in parallel to the albumin/flavonoid molar ratio.[26] We have compared the interactions of various flavonoids with rat or human albumin by spectroscopy at pH 7.4 in a 10-mM phosphate buffer (*Table 2*). In the presence of human albumin (albumin/flavonoid molar ratio = 5), the bathochromic and chromogenic effects of isorhamnetin, a 3′-O-methylated form of quercetin, were slightly smaller than those of quercetin. On the other hand, it appears that naringenin, hesperetin, eriodictyol, and taxifolin (which all possess a saturated C-ring) have a very low bathochromic effect in the presence of albumin: the shift of λ_{max} was between +4 nm and +7 nm, depending on the molecule. Luteolin showed a shift of its λ_{max} comparable to that of quercetin (+33 nm), indicating that the lack of the 3-hydroxyl group does not affect the bathochromic effect. Fisetin, which differs from quercetin by the absence of 5-hydroxyl group, presented a bathochromic effect slightly lower (+26 nm versus +33 nm) than that of quercetin. This suggests that there is a relatively modest effect of one hydroxyl deletion on the A-ring on the spectrometric characteristics of the 4-oxo-flavonoids. In contrast, flavonols without hydroxyl group on the B-ring, such as galangin and

Table 2 Bathochromic effect of flavonoids in the presence of human albumin*

FLAVONOIDS	Substitution pattern –OH	Substitution pattern –OCH_3
Flavones		
Luteolin	5,7,3′,4′	-
Flavonols		
Galangin	3,5,7	-
Kaempferid	3,5,7	4′
Kaempferol	3,5,7,4′	-
Tamarixetin	3,5,7,3′	4′
Isorhamnetin	3,5,7,4′	3′
Fisetin	3,7,3′,4′	-
Quercetin	3,5,7,3′,4′	-
Flavanones		
Naringenin	5,7,4′	-
Hesperetin	5,7,3′	4′
Eriodictyol	5,7,3′,4′	-
Flavanonols		
Taxifolin	3,5,7,3′,4′	-



FLAVONOIDS	–Albumin OD_{max}	–Albumin λ_{max}(nm)	+Albumin OD_{max}	+Albumin λ_{max}(nm)	$\Delta\lambda$ (nm)
Luteolin	0.187	368	0.273	400	32
Galangin	0.130	370	0.175	393	23
Kaempferid	0.140	375	0.196	392	17
Kaempferol	0.222	375	0.270	399	24
Tamarixetin	0.195	375	0.219	398	23
Isorhamnetin	0.197	375	0.274	402	27
Fisetin	0.228	372	0.279	398	26
Quercetin	0.196	375	0.279	408	33
Naringenin	0.315	322	0.286	328	06
Hesperetin	0.329	323	0.303	330	07
Eriodictyol	0.228	323	0.255	330	07
Taxifolin	0.331	326	0.285	330	04

*The band I spectra of various flavonoids (12.5 µmol/L) were monitored in a 10 mmol/L phosphate buffer, pH 7.4, in the absence or in the presence of human albumin (62.5 µmol/L); the maximal absorption and the λmax were determined and reported in the present table.

kaempferid, and to a lesser extent those with only one hydroxyl group, such as kaempferol and tamarixetin, show a bathochromic effect definitely lower than that of quercetin (+17 to 24 nm versus +33 nm). Surprisingly, the shift of isorhamnetin spectrum was more important than that of tamarixetin, in spite of the structure similarity of these compounds. This suggests a particular role of the 4′-hydroxyl group in the bathochromic effect in the presence of albumin. These whole data put forward the crucial role of the unsaturated C2–C3 bound of the C-ring of flavonoids in the bathochromic effect observed in the presence of albumin. Moreover, the magnitude of this effect appears to be reinforced by the presence of hydroxyl groups on the B-ring. It must be noted that the chromogenic effect, and to a lesser extent the bathochromic effect, were more intense with human than with rat albumin (data not shown).

To determine the forms, intact quercetin or metabolites, which circulate in plasma, an HPLC analysis was performed. The liver has the capacity to synthesize conjugated derivatives of various molecules, among which some flavonoids,[32,33] by conjugating with a sulphate or a glucuronic acid molecule, and we previously reported that quercetin metabolites are present in rat plasma as glucurono- or sulfoconjugated forms.[26] Therefore the plasmas were treated by β-glucuronidase/sulfatase before HPLC procedure. A plasma chromatogram is presented in *Figure 2A*. The first peak (19.3 min) corresponded to the internal standard fisetin, the second peak eluted at 24.3 min, and it was verified by spiking of the sample with quercetin that it corresponded to native quercetin (data not shown). The third peak eluted at 35.2 min, corresponding to a compound less polar than quercetin. Because it could correspond to a methylated form of quercetin, the plasma sample was spiked with 50 μmol/L pure tamarixetin or isorhamnetin, two methylated derivatives of quercetin. The tamarixetin (3,5,7,3′-tetrahydroxy-4′-methoxyflavone) peak (36.3 min) was clearly distinct from that of the metabolite present in plasma, whereas the isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone) peak superimposed on that of the unidentified metabolite (data not shown). The concentrations of quercetin and isorhamnetin in the plasma from rats fed the 0.25% quercetin diet were determined after deconjugation at 19 ± 3 μmol/L and 101 ± 13 μmol/L respectively, which corresponds to an isorhamnetin/quercetin molar ratio of about 5.

Bile samples from rats fed the control diet or the 0.25% quercetin diet were also analyzed by HPLC after hydrolysis of the conjugated derivatives by β-glucuronidase/sulfatase. Chromatograms corresponding to the quercetin-fed rats exhibited three additional peaks in comparison to those of control rats (*Figure 2B*). Using pure molecules as references, we assigned these peaks to quercetin, isorhamnetin, and surprisingly to tamarixetin, which was not detected in plasma samples. Quercetin, isorhamnetin and tamarixetin concentrations were 139 ± 15, 98 ± 11 and 141 ± 16 μmol/L respectively.

Urine extracts of quercetin-fed rats and control rats were analyzed in the same way. The same metabolites than in bile were recovered in urine from rats fed the 0.25% quercetin diet (*Figure 2C*), and with comparable ratios: urinary quercetin, isorhamnetin and tamarixetin concentrations were 46 ± 7, 38 ± 6, and 44 ± 6 μmol/L respectively.

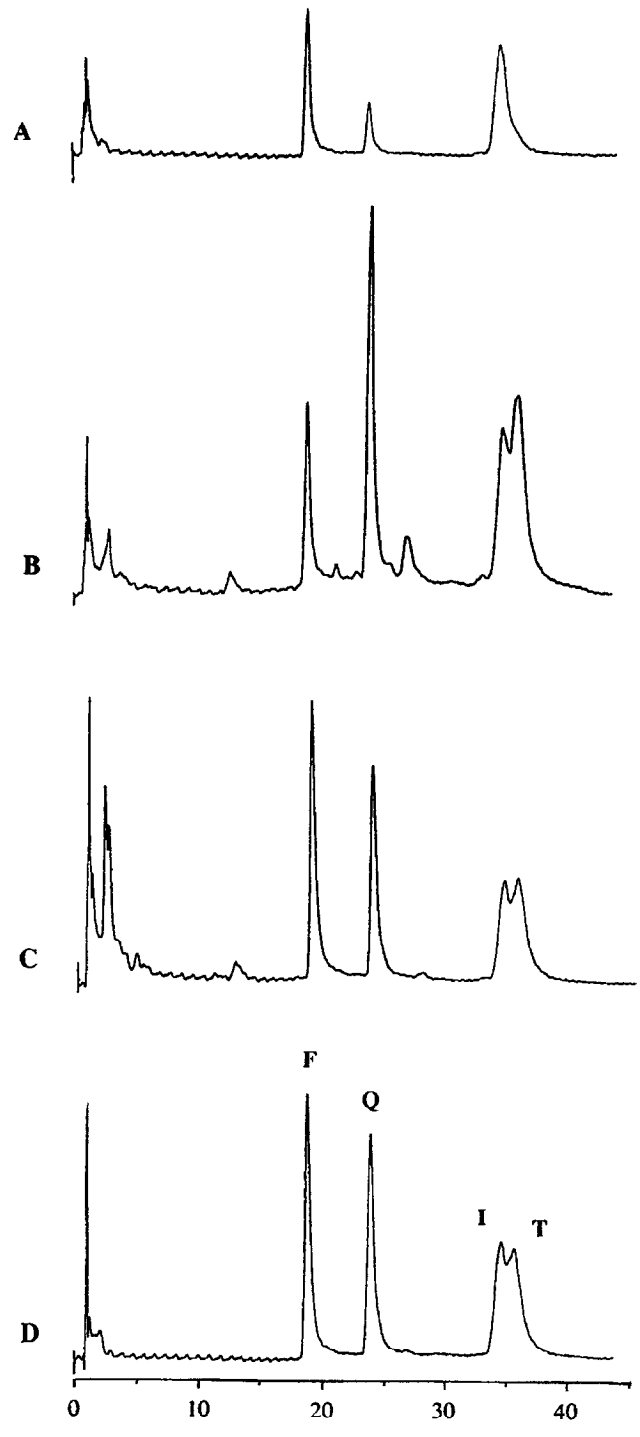


Figure 2 HPLC chromatograms after β-glucuronidase sulfatase treatment (as described in the experimental section). **A)** Plasma from a rat fed the 0.25% quercetin diet. **B)** Bile from a rat fed the 0.25% quercetin diet. **C)** Urine from a rat fed the 0.25% quercetin diet. **D)** Fisetin 50 μmol/L (F), quercetin 50 μmol/L (Q), isorhamnetin 50 μmol/L (I) and tamarixetin 50 μmol/L (T) standards.

Discussion

The present work shows that conjugated forms of quercetin and its 3′-O-methylated derivative are present in plasma at

high concentration (120 μmol/L) in rats fed for 14 days a 0.25% quercetin diet. A comparable result was obtained recently by King et al.[34] with rats receiving a 10-times lower dose of genistein (20 mg/Kg body weight): plasma genistein concentration reached 11 μmol/L 2 hours after oral administration. There is thus a part of the ingested quercetin which escapes the breakdown to phenolic acids by the microflora, and is absorbed from the digestive tract and further metabolized by the rat tissues. We have previously reported that the absorption of quercetin takes place in the large intestine,[26] but the mechanism of absorption of the flavonoids is still uncertain. The question arises whether flavonols depend on a specific transport system for their transfer across the digestive wall. Although quercetin is sparingly soluble in water, it is not particularly lipid-soluble, thus its absorption probably takes place via the portal route (assuming that it has not been modified by intestinal enzymes).

The liver represents a major site for the metabolism of flavonoids: this organ has the capacity to oxidize (introduction of hydroxyl groups), reduce (carbonyl groups), methylate (creation of O-methyl ethers), or conjugate (glucuro- or sulfo-conjugation) flavonoids.[31] The liver could be responsible for the 3′- and 4′-O-methylation and the conjugation of quercetin in the rat, even though a minor role of the intestinal wall and kidneys can not be excluded. Our results confirms those of Ueno et al.,[25] who have previously reported the possibility of 3′- and 4′-O-methylation of quercetin in the rat. On the other hand, Zhu et al.[24] found 3′-O-methyl, but not 4′-O-methyl conjugated derivatives of quercetin in urine of hamsters that have received a intraperitoneal injection of quercetin. The absence of 4′-O-methyl-quercetin may be a particularity of the species. The authors also demonstrated that the hamster kidney catechol-O-methyl transferase was able to methylate quercetin at the 3′-position, with a rate up to three orders of magnitude higher than with catecholamines. The catechol-O-methyl transferase, which catalyses the transfert of methyl groups from S-adenosyl methionine to a variety of substrates, may be the enzyme responsible for the methylation of quercetin. Surprisingly, we found only the 3′-O-methyl-quercetin (isorhamnetin) in the plasma, and the two O-methyl derivatives, 3′ and 4′, in bile and urine. One hypothesis is that once synthesized, the 4′-O-methyl-quercetin (tamarixetin) was preferentially exported to the bile duct. There was also the two methylated forms in the urine, which could be explained by the activity of the renal catechol-O-methyl transferase, or by a very efficient renal clearance of tamarixetin.

Rapid glucuronidation has also been reported in the rat for the flavonoid diosmetin.[33] Moreover, Shimamura et al.[32] have shown that liquiritigenin is metabolized by rat tissues, especially by the liver, to five glucurono and sulfo-conjugated derivatives. The conjugation of quercetin by isolated perfused rat liver was reported by Shali et al.[35] This metabolism could favor the elimination of modified flavonoids by biliary and urinary excretion in the rat. It was interesting to compare the extent of excretion of the conjugated derivatives of quercetin by these two routes. Assuming an average urinary volume of 30 ml/day and a biliary secretion of 1 ml/hr, the excretion was 3.8 and 9 μmol/L for the urine and bile route, respectively. When excreted in the bile, flavonoids are secreted into the duodenum then metabolized by bacteria in the large intestine, which results in the production of fragmentation products and/or the hydrolysis of glucurono- or sulpho-conjugated. The flavonoid metabolites released may be reabsorbed and undergo an enterohepatic cycling, which would favor a high concentration in plasma. Finally, intestinal microflora could convert dietary quercetin or its biliary conjugated derivatives into phenolic acids such as 3,4-dihydroxyphenylacetic, homovanillic and *m*-hydroxyphenylacetic acids, which could themselves be absorbed.

When rats were transferred from a 0.25% quercetin diet to a quercetin-free diet (data not shown), the rate of disappearance of the plasma quercetin metabolites was relatively slow (50% decrease in 48 hr). Yet, the rate of quercetin catabolism and the turnover of its metabolites could be accelerated when rats have a permanent absorption of flavonols.

In fact, no native quercetin—or only trace—circulates in plasma. This may explain the discrepancy between the mutagenicity and the non-carcinogenicity of this molecule.[36] Besides, for the same reason, some results obtained in vitro on the properties of quercetin may be unrelevant in vivo. However, it is noteworthy that quercetin can exert an antioxydant effect at a very low concentration (0.2 μmol/L).[37]

Another point is that the circulating metabolites of quercetin retain the property to bind to albumin, with a bathochromic effect comparable to that of native quercetin. Using an ultrafiltration procedure, we have previously shown that quercetin metabolites are strongly bound to albumin in the blood.[26] Binding of quercetin or its metabolite to albumin may have various consequences on their uptake by tissues, particularly by the liver. Furthermore, the question arises whether the complexation of the circulating metabolite of quercetin to albumin has an effect on the conformation of albumin, by establishing intra- or intermolecular hydrogen bondings. In this view, this could explain the well-known property of flavonoids to decrease capillary permeability.

Few studies have been performed on the flavonoid bioavailability in humans. In human volunteers receiving a 4 g quercetin dose per os, no quercetin or quercetin conjugates were detected in plasma or urine, using a fluorimetric method.[38] It was concluded that the bacterial breakdown of flavonoids to phenolic acids made unlikely any absorption of more than 1% of the oral dose. Nevertheless, in a more recent study, Xu et al.[28] have shown that oral administration of isoflavones (1.3 mg/Kg BW of genistein and daidzein) resulted in an urinary recovery of about 10 to 20% and in a plasma isoflavones concentration of about 2 μmol/L. This bioavailability may be sufficient to exert some health protective effects. However the absorption capacity of flavonols in humans is poorly known, thus it seems particularly interesting to reinvestigate this problem in man to know the fate of dietary flavonols and also to explore the biological interest of this class of micronutriments. The bathochromic effect of flavonols that has been put forward in the presence of albumin could facilitate the investigation of their plasma transport in humans.

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