JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Bound Ferulic Acid from Bran Is More Bioavailable than the Free Compound in Rat

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Ferulic acid (FA) is reported as a good antioxidant absorbed by human or rat but only few data deal with the influence of the food matrix on its bioavailability and with its potential protection against cardiovascular diseases and cancer. Wheat bran is used as a source of ferulic acid, the compound being mainly bound to arabinoxylans of the plant cell walls. Pharmacokinetic profiles of FA and its metabolites are established in rats. Free and conjugated FA quickly appear in plasma, reach a plateau 1 h after intake and remain approximately constant at 1 μM up to 24 h. 2.3% of FA are eliminated in urine. Compared with results obtained after intake of free FA, the presence of FA-arabinoxylans bonds in the food matrix increases the occurrence time of FA in the organism and decreases the level of urinary excretion in 24 h. Nevertheless, sulfated FA is still the main plasmatic form. The antioxidant activity of plasmas of rats fed with a standard diet (containing no FA), pure ferulic acid (5.15 mg FA/kg bw) or bran (4.04 mg FA/kg bw) are measured in an ex vivo test using AAPH as free radical inducer. Plasmas of rats fed with bran show a better antioxidant activity than the control group and the pure FA supplemented group, increasing the resistance of erythrocytes to hemolysis by factors of 2 and 1.5, respectively. These results show the good bioavailability of FA from bran and its potential efficiency to protect organism against pathology involving radical steps of development.

KEYWORDS: Ferulic acid; wheat bran; bioavailability; conjugated metabolites; antioxidant activity

INTRODUCTION

Epidemiological studies have reported a strong benefit of a whole-grain diet toward some causes of mortality and morbidity (e.g., ischemic heart disease, endometrial cancer) (*1*, *2*). Even if the consumption of fibers was clearly associated with a reduction of the death risk, whole-grains also contain many bioactive components including vitamins, lignans, isoflavones, and phenolic acids, which are able to protect the organism. In particular, cereals contain hydroxycinnamic acids bound to arabinosyl chains of plant cell walls. Ferulic acid is the most abundant in cereals following by *p*-coumaric, synapic, and caffeic acids (*3*, *4*). In plant cell walls, ferulic acid can undergo dimerization. The alkaline hydrolysis of rye bran has shown that 8-*O*-4′-diferulic acid was the main dehydrodimer. The daily intake of consumer with a regular intake of cereal products can be up to 100 mg of ferulic acid (*5*).

The interest for these compounds increased in relation to their antioxidant activity (*6*, *7*). Caffeic, chlorogenic, and ferulic acids protect human LDL from oxidation (*8*). Ferulic acid dehydrodimers or bran hemicellulose fragments containing ferulic acid also exhibit good antioxidant activities (*9*−*11*). Nevertheless, to protect the body against atherosclerosis or cancer, these compounds must be absorbed and distributed in tissues, raising up the problem of their bioavailability.

In past years, many papers have investigated the bioavailability of phenolic compounds. All the main classes of phenolics are more or less bioavailable according to their chemical structure (*12*). Data from literature have reported a good absorption of hydroxycinnamic acids including ferulic acid and its dimers (*13*−*16*), but few studies have investigated the influence of the food matrix on their bioavailability (*17*).

The aim of this study was to evaluate the influence of the cell wall linkage on the bioavailability of ferulic acid after an oral short-term intake of wheat bran mixed with a standard diet. Pharmacokinetic profiles of ferulic acid and its conjugated

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10.1021/jf0348323 CCC: $27.50
Published on Web 05/27/2004

Table 1. Composition of the Standard Complete "P14" Powder

element	weight (g/kg)
milk proteins	140
starch	623
saccharose	100
soy oil	40
mineral salts	35
vitamins	10
cellulose	50
choline	2
total	1000

metabolites were established in plasma and urine of rats and these results were compared with the results of a previous study performed after intake of free ferulic acid (*14*). Then, the in vivo effect of the consumption of free or bound ferulic acid was evaluated by measuring the antioxidant activity of the corresponding plasmas of rats.

MATERIALS AND METHODS

Chemicals. Organic wheat bran was a mix of three varieties (Enesco/Trizo/Soisson; 25/60/15; w/w/w) purchased from Moulin Decollogne (Précy sur Marne, France).

Ferulic acid, sulfatase (EC 3.1.6.1) type H-1 from *Helix pomatia*, β-glucuronidase (EC 3.2.1.31) type IX-A from *Escherichia coli*, sodium acetate trihydrate, and ethyl acetate (Chromasolv) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Acetone (Spectronorm grade), hydrochloric acid 37%, and anhydrous sodium sulfate were from Prolabo (Fontenay sous bois, France). Acetonitrile (HPLC grade), methanol (HPLC grade), ethanol (96.2°, analysis grade) and sodium hydroxide (anhydrous pellets) were purchased from Carlo Erba (Val de Reuil, France). Sodium dihydrogenophosphate dihydrate, di-sodium hydrogenophosphate dehydrate, and glacial acetic acid (HPLC grade) were from Fisher Scientific (Elancourt, France). Water was purified with an Elix 3 system (Millipore).

Quantification of Ferulic Acid in Bran. Ferulic acid (FA) occurred in two forms in bran: free and bound to the arabinoxylans of the cell walls. Both forms were quantified according to a procedure adapted from Bonnely et al. (*18*).

Quantification of Free FA. A 10-g sample of sieved ground bran (diameter of particles between 250 and 315 μm) were mixed with 80 mL of ethanol/water (75/25; v/v) for 1 h at room temperature under stirring. The extract was then centrifuged at 20 °C for 20 min at 19 000*g* in a Sorvall ST21 centrifuge. A second extraction was performed under the same conditions with 30 mL of ethanol/water (75/25; v/v). Supernatants were mixed and evaporated to dryness at 50 °C under vacuum and then dissolved in the elution phase of HPLC (acetonitrile/methanol/sodium acetate buffer 50 mmol/L pH 4.8; 10/10/80; v/v/v).

Quantification of Bound FA. The cake obtained from the extraction of free FA was hydrolyzed with 80 mL of 4 M NaOH at 60 °C under N_2 for 4 h. The mixture was then acidified at pH 1 with HCl 37% and extracted three times with ethyl acetate (v/v). The ethyl acetate phases were combined and evaporated to dryness at 50 °C under N_2. The residue was dissolved in 2 mL of methanol and diluted 50 times in the elution phase of HPLC.

Animals and Treatments. Male Sprague Dawley rats, 9 weeks of age, were purchased from Harlan (France). Animals were maintained and handled according to the recommendation of the Institutional Ethic Committee (INRA), in accordance to the European decree no. 86/609/EEC.

Rats ($n = 66$) were housed in individual cages equipped with a system allowing the separation of urine and feces. Animals were kept at 22 ± 1 °C on a 12-h inversed light/dark cycle: day was from 09.00 pm to 09.00 am and night from 09.00 am to 09.00 pm to be fed in an usable mode.

Animals were fed with a complete laboratory diet (P14) (**Table 1**) prepared at INRA (Jouy-en-Josas, France). The P14 diet was an AIN-93M modified diet (*19*). Instead of casein and cystine, this diet contained 140 g of total milk protein per kilogram of diet. Total milk protein is a mixture of casein (85%) and other milk proteins (albumins and globulins).

Two grams of P14 powder mixed with water (1/1; w/w) were supplied between 09.00 am and 09.30 am, and ad libitum between 11.00 am and 06.00 pm for 10 days before experiments. The day of the sacrifice, rats were divided into two groups. The control group (average weight = 338 ± 5 g) received the standard diet, while the second one (average weight = 331 ± 2 g) was fed with the same diet containing 26% of bran, corresponding to 4.04 mg/kg body weight of FA. Six rats were sacrificed before meal and six rats of each group were sacrificed 30 min, 1.5 h, 4.5 h, 7 h, and 24 h after meal. The rats were deeply anaesthetized with an overdose of sodium pentobarbital (48 mg/kg) and exsanguinated by section of the abdominal aorta and vena cava. Blood was collected into heparinized tubes and centrifuged at 3000*g* for 20 min at 4 °C. Then, plasma was aliquoted and frozen at −30 °C until analysis. To collect urine, the bladder was clamped, pierced and rinsed with distillated water. The excreted urine was collected into tubes, mixed with bladder urine and frozen at −30 °C.

In a preliminary experiment, rats had received 5.05 mg/kg body weight of free FA (14). Plasmas and urine had been collected in the same way.

Sampling Procedure. Conjugated metabolites of FA were enzymatically hydrolyzed to identify glucuronidated, sulfated, and sulfoglucuronidated derivates, according to procedures previously described (*14*, *20*).

Plasma. The quantification of sulfoglucuronidated and sulfated FA was performed on 100 μL of plasma incubated at 37 °C in a water bath for 2 h with 20 μL of sulfatase type H-1 solution in 1 M acetate buffer pH 4.9, containing 2.5×10^5 U/L of sulfatase and 7.5×10^6 U/L of β-glucuronidase. After incubation, proteins were precipitated by 250 μL of acetone and removed by centrifugation at 800*g* during 10 min at 20 °C with a Jouan CT 422 centrifuge. The supernatant was filtered on a 0.2-μm Gelmann filter and analyzed by high-performance liquid chromatography (HPLC). For the determination of native FA, a set of samples was incubated without enzymes and treated as described above. The determination of FA glucuronides was realized by incubating at 37 °C for 2 h 100 μL of plasma with 20 μL of phosphate buffer 0.2 M pH 6.8 containing 7.5×10^6 U/L β-glucuronidase type IX-A.

Urine. Two conditions were applied to the samples: first, 90 μL of urine were incubated for 2 h at 37 °C, either with 20 μL of acetate buffer 250 mmol/L pH 4.9 containing or not sulfatase type H-1 (2.5×10^5 U/L sulfatase and 7.5×10^6 U/L β-glucuronidase) and second with 20 μL of acetate buffer 250 mmol/L pH 6.8 containing or not 7.5×10^6 U/L β-glucuronidase type IX-A. After incubation, urine was acidified with 10 μL of HCl 3.7% and extracted twice with 1 mL of ethyl acetate. The organic phases were combined, evaporated to dryness under nitrogen, and then dissolved in 400 μL of methanol and filtered on a 0.2-μm Gelmann filter before HPLC analysis. The concentration of the different metabolites of FA in plasma and urine were calculated from the enzymatically hydrolyzed samples and the non hydrolyzed ones as described by Rondini et al. (*14*).

HPLC Analysis. FA was quantified by high-performance liquid chromatography, using a 600 E Waters system coupled to electrochemical detection (ESA Coulochem II coulometric detector) equipped with a guard cell model 5021 and an analytical cell model 5010 with two analysis electrodes. The electrochemical settings were as follows: guard channel potential −250 mV; channel 1, potential 0 mV and sensitivity 1 μA; channel 2, potential 450 mV and sensitivity varying from 1 to 20 μA. Aliquots of 20 μL were injected on a C_{18} Interchrom column (250 × 4.6 mm i.d.; 10-μm particle size) (Interchim). The elution was performed with acetonitrile/methanol/sodium acetate buffer 50 mmol/L pH 4.8 (10/10/80, v/v/v) at a flow rate of 0.8 mL/min.

A linear calibration curve of FA was established between 0 and 2×10^{-4} mol/L ($R^2 = 0.995$).

Assessment of the Total Antiradical Effectiveness of Plasma. For each group (standard food, standard food + pure FA and standard food + bran), the determination of the antioxidant activity was realized on three plasmas of rats sacrificed 30 min after food intake. Plasmas of groups supplemented with bran or pure ferulic acid were chosen to

Table 2. Intake of Bran and Ferulic Acid for Each Group Fed with P14 + Bran

group	bran intake (g)	ferulic acid intake (mg)
$T + 0.5$ h	0.40 ± 0.13	1.20 ± 0.39
$T + 1.5$ h	0.42 ± 0.10	1.25 ± 0.29
$T + 4.5$ h	0.50 ± 0.01	1.50 ± 0.09
$T + 7$ h	0.49 ± 0.03	1.50 ± 0.01
$T + 24$ h	0.50 ± 0.01	1.43 ± 0.08
mean	0.46 ± 0.04	1.38 ± 0.11

contain a same concentration of total ferulic acid (free + conjugated) and a similar ratio of free and conjugated derivates.

The total antioxidant activity of plasma was evaluated using the KRL biological test based on free radical-induced hemolysis (Laboratoires Spiral, France) (21−23). Control red blood cells without and in the presence of diluted plasma samples were submitted to organic free radicals produced at 37 °C under air atmosphere from the thermal decomposition of 2,2′azobis(2-amidinopropane) dihydrochloride (AAPH). Hemolysis was recorded using a 96-well microplate reader by measuring the optical density decay (Laboratoires Spiral, France). Results were expressed as the time that is required to reach 50% of maximal hemolysis (half-hemolysis time, $t_{1/2}$ in minutes), which refers to the erythrocyte resistance to free radical attack. The antioxidant activity of plasma (AOP) was defined as the percent plasma-mediated increase of the half-hemolysis time of control red blood cells without plasma sample.

Data Evaluation and Statistics. Data of FA and metabolites level in plasma and urine were expressed as mean ± SEM for six rats per time. The statistical significance was calculated using the mathematical derivate of the Student's *t*-test (xlstat 5.1, shareware version). A value of $p < 0.05$ was considered as a significant difference.

The significance at $p < 0.05$ of antioxidant activity values of plasmas was deduced from an ANOVA analysis followed by a Newman Keuls test.

Conversion of the Plasmatic Concentrations Into Percentages of the Intake. To compare the kinetics in plasma after bran and free FA supplementations (the dose of FA being slightly different), the percentage of FA in plasma was calculated, considering that rat blood represents 16.5% of body weight, with a density of 1 and a hematocrit of 0.45 (Gilles Fromentin, personal communication). According to this estimation, a 300-g rat would contain 49.5 mL of blood, equivalent to 27 mL of plasma. Thus, a plasmatic concentration of 1 μmol/L total after bran consumption (4.04 mg/kg of body weight of FA) would represent 0.43% of the intake.

RESULTS

In bran, 95.8% of FA was present in a bound form (3.18 mg/g of dry matter) and only 4.2% on a free form (0.14 mg/g of dry matter). These results are in agreement with the literature, which reports 2.0 mg/g to 4.4 mg/g of total FA (*24*, *25*).

Rats of the bran group have largely consumed the P14 diet with bran: 91.5% of the serving was eaten, corresponding to an average consumption of 0.46 g of bran and 1.38 mg of ferulic acid (**Table 2**).

No FA or conjugated forms were detected in plasma of the control group of rats receiving only P14. In the bran supplemented group, free and conjugated ferulic acids appeared quickly in plasma (**Figure 1**). The concentration of sulfated FA reached a plateau 1 h after intake and remained approximately constant ($p < 0.01$) up to 24 h. Free FA followed a similar kinetic, but the plasmatic concentration was about 10-fold lower (around 0.1 μmol/L). The concentration of glucuronidated FA reached a maximum 30 min after intake, slightly decreased between 0.5 and 4.5 h, and stabilized at a concentration of about 0.35 μmol/L. After 24 h, plasmas of rats still contained more than 1 μmol/L of total FA derivates.

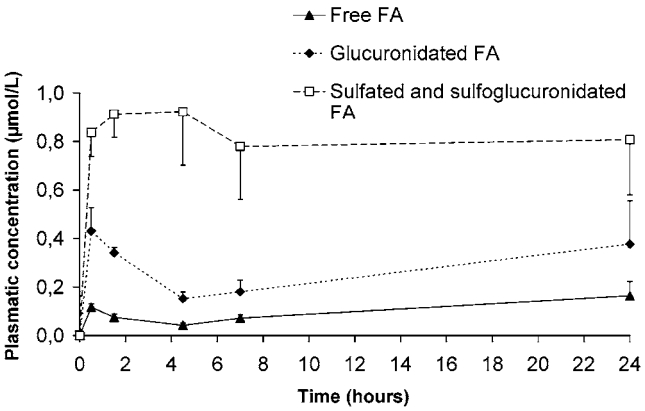

Figure 1. Concentration of native ferulic acid (FA) and metabolites in rat plasma after an oral short-term ingestion of bran providing 4.04 mg/kg (body weight) of ferulic acid. (Values are mean ± SEM, $n = 6$)

Table 3. Ratio of Native Ferulic Acid and Metabolites in Plasma and Urine after Ingestion of Pure Ferulic Acid (5.15 mg/kg body weight) or Bran (Providing 4.04 mg/kg of Body Weight of Ferulic Acid)

	plasma after free ferulic acid intake[a]	plasma after bran intake	urine after free ferulic acid intake[a]	urine after bran intake
free FA	25 ± 3%	9 ± 2%	14 ± 3%	15 ± 2%
glucuronidated FA	17 ± 1%	19 ± 2%	2 ± 1%	11 ± 3%
sulfated or sulfoglucuronidated FA	58 ± 3%	72 ± 3%	84 ± 3%	74 ± 2%

[a] From ref 14.

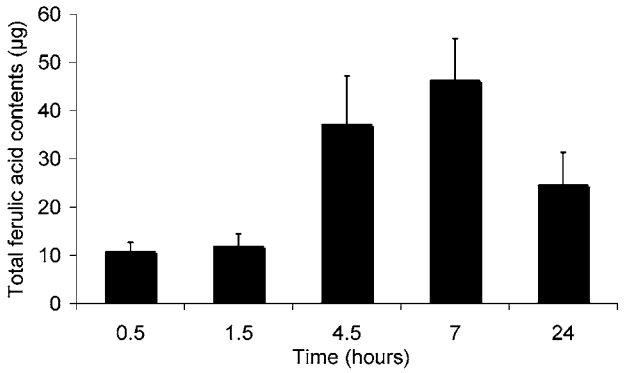

Figure 2. Urinary excretion of free ferulic acid and metabolites after an oral short-term ingestion of bran, providing 4.04 mg/kg (body weight) of ferulic acid. (Values are mean ± SEM, $n = 6$)

Ratios between the different chemical forms did not change in a significant way ($p < 0.05$) between 30 min and 24 h; sulfated FA was the main form, representing 72% of total FA, followed by glucuronidated FA with 19% of the plasmatic form and native FA with only 9% (**Table 3**). The level of free FA was significantly higher (25% of total FA) when rats received the pure compound.

Native and metabolized forms of FA began to be excreted in urine only 30 min after intake of bran (**Figure 2**) and reached a plateau after 4.5 h. The decrease of the FA level in urine at 24 h is quite surprising. Also, several experiments have been made to verify this value. Two urines of rat sacrificed 7 h after meal have been set for 17 h under similar conditions of light and temperature as urines collected after 24 h, to simulate an aging of urine. Nevertheless, the level of total FA before and after aging was still the same, excluding the hypothesis of a degradation of FA during collection and storage. Finally, a new

Table 4. Concentration and Ratio of Native Ferulic Acid (FA) and Metabolites in Plasma Used for the Determination of the Antioxidant Activity (Rat Sacrificed 30 min After Ingestion, $n = 3$) after Ingestion of a Standard Diet Non Supplemented or Supplemented with Pure Ferulic Acid (5.15 mg/kg Body Weight) or Bran (Providing 4.04 mg/kg of Body Weight of Ferulic Acid)

	nonsuplmtd diet	diet suplmtd with pure ferulic acid	diet suplmtd with bran
concentration of total FA (μM)	0	1.4 ± 0.1	1.4 ± 0.2
free FA		4%	5%
glucuronidated FA		7%	16%
sulfated or sulfoglucuronidated FA		89%	79%

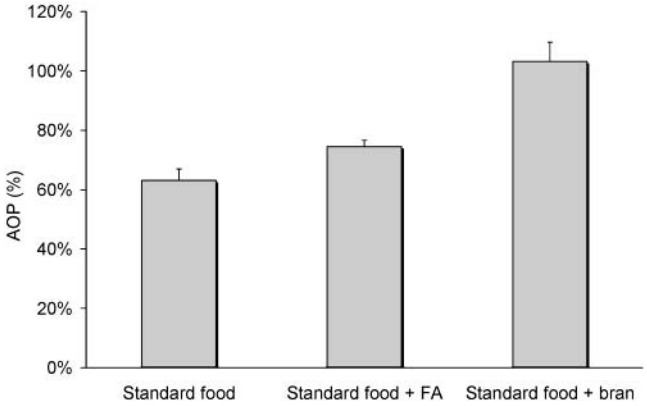

Figure 3. Antioxidant activity (AOP) of plasmas of rats fed with a diet containing no ferulic acid (standard food), pure ferulic acid (standard food + ferulic acid at 5.15 mg/kg body weight), or bran (standard food + bran), providing 4.04 mg/kg (body weight) of ferulic acid.

collection of urine has been made on six rats handled, fed, and sacrificed in the same conditions, but the level of total FA in urine was still around 25 μg. Therefore, the result at 24 h has been included in the calculation of FA content of urine, although the differences between the concentration at 7 and 24 h were significatively different ($p < 0.05$). Then, FA excreted in urine was 46.2 ± 8.7 μg, which only represents 2.6 ± 0.7% of the intake.

The ratio of the different metabolites in urine was also calculated between 0.5 and 7 h after intake (**Table 2**). Sulfated FA was always the main metabolite, representing about 74% of total FA, followed by native FA (15%) and glucuronidated FA (11%). These ratios were quite similar in samples of urine after pure FA consumption.

Three samples of each group (standard food, standard food + pure FA, and standard food + bran) were used to determine the antioxidant activity of plasma after food intake. The plasmas of the control group did not contain any FA. Plasmas of pure FA and bran groups were chosen to contain a similar concentration of total FA (1.4 ± 0.1 μmol/L and 1.4 ± 0.2 μmol/L, respectively) (**Table 4**). They correspond to the plasmas of rats sacrificed 30 min after intake.

Plasmas of rats fed with bran showed a better antioxidant activity than both the non supplemented group and the pure FA supplemented group (**Figure 3**), increasing the resistance of erythrocytes to hemolysis by a factor 2 and 1.5, respectively.

DISCUSSION

Sulfated ferulic acid is the main metabolite recovered in plasma and urine either after bran or pure FA intake. This result demonstrates that the metabolic pathway is the same for free

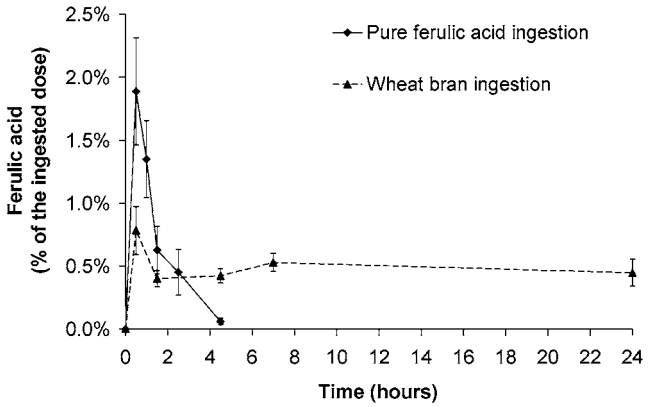

Figure 4. Comparison of the kinetic of ferulic acid and metabolites obtained after a short-term ingestion of free ferulic acid (5.15 mg/kg body weight) or bran, providing 4.04 mg/kg (body weight) of ferulic acid. (Values are mean ± SEM, $n = 6$)

and bound forms of FA. Nevertheless, ratios of conjugated derivates are slightly higher with bran.

Moreover, the kinetics of total metabolites (free + conjugated) in plasma are strongly different (**Figure 4**). If both diets lead to a quick appearance of FA in plasma, its concentration is more than two times smaller 30 min after bran intake (0.8% vs 1.9%) but is still constant for 24 h, while it decreases up to zero after 4.5 h for free FA.

The fast appearance of FA in plasma after intake of pure FA could be explained by a fast absorbance of the compound in jejunum or maybe in stomach, as already shown for quercetin (*26*), genistein, or daidzein, which appear in plasma only 3 min after oral intake (*27*). The hypothesis of an absorption in stomach could be valid if we consider the low molecular weight of FA and the fact that FA is probably under a neutral form at the acid pH of stomach. In the case of bran, the early appearance of few FA may be due to the absorption of free ferulic acid forms, which represent 4.2% of total FA. The hydrolysis of bound FA in the stomach could also be considered, but previous studies reported a low release of FA after incubation of bran with gastric juice (*28*). Thus, free forms of bran should be absorbed quickly, then decreased in plasma, leading to the same profile as that of the pure compound (**Figure 4**). At the same time, additional free FA should be released more slowly by the action of intestinal enzymes. Such enzymes (e.g., esterase or xylanase) are able to hydrolyze more than 20% of hydroxycinnamates linked to arabinoxylans (*29*). These new free forms should be progressively absorbed, allowing the plasmatic concentration to keep constant between 1.5 and 24 h.

The level of the plasmatic concentration around 1 μmol/L of FA may be also maintained by the biliary excretion of FA and its reabsorption in enterocyte. Nevertheless, the weakness (~6% of perfused dose) of FA recovered in biliary duct (*17*) probably failed to fully explain the differences between plasmatic kinetics.

Urinary excretion of FA after bran consumption was 15-fold less than that after intake of the pure molecule, the later representing 43% of the intake dose (*14*) against 2.6%. Such differences of urinary excretion of FA was still observed with a perfusion of rat intestine with pure FA allowing the recovery of 50% of the intake dose and only 3.2% after bran consumption (*17*).

The strong influence of the FA linkage on its bioavailability (extending of the time period in plasmatic compartment and decrease of the urinary excretion)is interesting if we consider the protective effect of hydroxycinnamates. Such a behavior

should probably enhance tissue distribution of FA. A recent paper has clearly shown the presence of quercetin and conjugated metabolites in liver, kidney, muscles, and in a small amount in testes, lungs, and heart after [2-^{14}C]quercetin-4′-glucoside intake (*30*). Radioactivity was also found in heart, lung, and liver after intake of [^{3}H](−)-epigallocatechin gallate, a cancer preventive flavonoid (*31*). Moreover, both of these studies reported strong levels of radioactivity in intestine where food antioxidants could primarily act by protecting the digestive tract.

Changes of the bioavailability depending on the chemical structure have been previously described with flavonols. For example, quercetin was more available when ingested from onions than it was from the pure compound in healthy ileostomic subjects (*32*). In onions, quercetin is mainly glycosylated (e.g., isoquercitrin or spiraeoside), and the glucose moiety may enhance the absorption (*33*).

The erythrocytes test showed that plasma gained a significant protection effect against lysis after consumption of pure ferulic acid or bran by rats. Then, FA and its conjugated metabolites act with a good efficiency against the radical damages. In the same way, it was shown that flavonoid conjugates kept a significant antioxidant activity toward LDL oxidation using copper as inductor (*34*). Plasmas of rats containing glucuronidated or sulfated quercetin were more resistant against copper sulfate-induced oxidation than the control plasma (*35*). FA metabolites and especially glucuronidates reduced in a significant way the formation of conjugated dienes and thus may play a role in atherosclerosis and cardiovascular diseases (*36*).

Nevertheless, the antioxidant activity was better after consumption of bran than pure FA, given that the concentration of total FA and the ratio of metabolites in plasma tested are similar. Such a result underlines that FA cannot fully explained the gain of antioxidant activity of plasma. Hydrolysis of bran could also lead to the release of ferulic acid sugar esters or diferulic acids, which retains an antioxidant activity and which have not been determined (*9*, *11*, *36*). At least, bran may also provide other trace elements acting as antioxidants (e.g., tocopherols or tocotrienols) or some enzymatic cofactors (e.g., Cu, Se, Zn) involved in the metabolism of oxygen reactive species or peroxides.

Considering the kinetic of free FA in blood and the AOP of plasma, we can conclude that a supplementation with wheat bran seems more efficient than a supplementation with pure FA.

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Received for review July 25, 2003. Revised manuscript received April 5, 2004. Accepted April 8, 2004. This work is financially supported by the Ministère de l'Agriculture et de la Pêche (France).

JF0348323