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Bioavailability of *trans*-resveratrol from red wine in humans

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Many *in vitro* studies demonstrated significant biological effects of *trans*-resveratrol. Thus, understanding the rate of intestinal absorption and metabolization *in vivo* of *trans*-resveratrol is the prerequisite to evaluate its potential health impact. Bioavailability studies mainly in animals or in humans using the pure compound at very high doses were performed. In this work, *trans*-resveratrol bioavailability from a moderate consumption of red wine in 25 healthy humans has been studied by three different experiments. The wine ingestion was associated to three different dietary approaches: fasting, a standard meal, a meal with high and low amount of lipids. *Trans*-resveratrol 3- and 4′-glucuronides were synthesized, purified, and characterized as pure standards. Bioavailability data were obtained by measuring the concentration of free, 3-glucuronide and 4′-glucuronide *trans*-resveratrol by high-performance liquid chromatography (HPLC), both with ultraviolet (UV) and mass spectrometry (MS) detection, in serum samples taken at different times after red wine administration. Free *trans*-resveratrol was found, in trace amounts, only in some serum samples collected 30 min after red wine ingestion while after longer times resveratrol glucuronides predominated. *Trans*-resveratrol bioavailability was shown to be independent from the meal or its lipid content. The finding in human serum of *trans*-resveratrol glucuronides, rather than the free form of the compound, with a high interindividual variability, raises some doubts about the health effects of dietary resveratrol consumption and suggests that the benefits associated to red wine consumption could be probably due to the whole antioxidant pool present in red wine.

Keywords: Glucuronides / Liquid chromatography – mass spectrometry / Resveratrol / Wine

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1 Introduction

According to the so-called "French paradox", the lower mortality incidence for cardiovascular pathologies in France than in other countries might be due to the protective effects of red wine consumption. Actually, red wines contain large amounts of polyphenols, a class of compounds able to exert biological activities preventing atherosclerotic pathologies, such as the inhibition of platelet aggregation, the synthesis of proinflammatory and procoagulant eicosanoids, and the inhibition of endotelin synthesis, as evidenced by several *in vitro* studies [1–4]. Among these compounds, *trans*-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is one of the most studied because it is believed to be the main responsible for red wine benefits on human health [5–9]. Numerous *in vitro* studies demonstrated the enormous variety of biological activities associated to this compound [10–22]. It is, therefore, an important issue to establish if free *trans*-resveratrol can be absorbed by the gastrointestinal tract and if it can exert these effects also in living organisms.

Some red wine polyphenols, including *trans*-resveratrol, have been shown to be highly absorbed and rapidly and extensively metabolized by humans through glucuronidation or sulfation reactions in the intestine/liver [23–25]. In particular, Meng and co-workers [24] reported that free and conjugated resveratrol was detectable in human plasma

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Abbreviation: PDA, photodiode array

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after a high-dose administration of the pure compound (0.5 – 1 mg/kg) to fasting subjects but it was not detectable in human plasma after the ingestion of the compound (0.6 – 1.8 mg of total resveratrol) as contained in grape juice. These results suggested the lower bioavailability of resveratrol glycosides in grape juice in comparison to its pure aglycone and/or the possible influence of the matrix sugar content on resveratrol bioavailability. The year before, the composition of the matrix and in particular the alcoholic content had been demonstrated to have no influence on 25 mg oral dose *trans*-resveratrol bioavailability in four healthy subjects [25]. More recently, Walle and co-workers [26] established that the absorption of a dietary relevant 25 mg oral dose of resveratrol was at least 70% and that *in vivo* the molecule was metabolized not only through sulfate and glucuronic acid conjugation of the phenolic groups but also through hydrogenation of the aliphatic double bond, probably due to the intestinal microflora.

The bioavailability studies reported in literature suffer from a drawback, since the amount of free *trans*-resveratrol used as pure compound (25 mg in hydro-alcoholic solution) is too large to be naturally assumed by drinking red wine. Moreover, quite interestingly, although these studies had limited statistically significance because of the few number of subjects involved (3 – 6), their data seem to suggest that resveratrol metabolism and bioavailability is distinctly different in high- or low-dose administrations.

Several animal studies [27 – 29] are available on *trans*-resveratrol absorption and metabolism after administration of red wine while, to the best of our knowledge, no data have been reported on *trans*-resveratrol bioavailability from a moderate consumption of red wine in humans. Similarly, literature is lacking on the possible influence of the type of meal consumed in association with the red wine ingestion on *trans*-resveratrol bioavailability in humans.

The aim of the present study is verifying if the free *trans*-resveratrol or its glucuronides can be found in human serum after a moderate consumption of red wine (300 – 600 mL) in or without association with a meal. For this purpose, the influence of the meal composition, and in particular the effect of lipids, on the absorption of this compound was evaluated. The study was performed on 25 subjects in three different experiments. Blood samples were taken at different time intervals and the serum samples obtained were analyzed by HPLC-UV, HPLC-MS, and HPLC-MS/MS to evaluate the free *trans*-resveratrol and its glucuronide content. The *trans*-resveratrol 4′- and 3-glucuronides, to be used as standards, were obtained in good yields by a simple synthetic strategy starting from resveratrol, in order to directly identify and quantify the resveratrol glucuronides in human serum.

2 Materials and methods

2.1 Chemicals

All chemicals and solvents were of HPLC- or analytical grade; pure *trans*-resveratrol and acetobromo-α-D-glucuronic acid methyl ester were purchased from Fluka (Buchs, Switzerland).

2.2 Syntheses of *trans*-resveratrol 4′-glucuronide and 3-glucuronide

These syntheses were performed using the method described by Learmonth and co-workers [30]. *Trans*-resveratrol (88 mg, 0.4 mmol) was dissolved in 3 mL dry methanol; 1.5 mL of a 0.25 M solution of sodium methoxide was then added dropwise, followed by a solution of acetobromo-α-D-glucuronic acid methyl ester (76 mg, 0.2 mmol) in 0.75 mL dry methanol. The reaction was left under magnetic stirring for 4 h at 40°C in a flask equipped with a condenser. Then 30 mL ethyl ether was added to the solution in order to precipitate the glucuronide derivatives of *trans*-resveratrol. The precipitate was isolated by centrifugation and redissolved in a THF : 1M aq. NaOH 1 : 1 solution (2.5 mL). The mixture was left under stirring for 6 h at room temperature, then the reaction was quenched by adding a 1% solution of acetic acid in methanol. The solvent was evaporated and the crude solid was purified by semi-preparative HPLC (RP C18 column, 250 × 10 mm). The gradient elution was performed by using water (0.2% formic acid) as eluent A and water/acetonitrile 3 : 2 (0.2% formic acid) as eluent B: 0 – 14 min from 70% A to 28% A. Retention time for *trans*-resveratrol 4′-glucuronide: 13.0 min, retention time for *trans*-resveratrol 3-glucuronide: 15.0 min.

2.2.1 *Trans*-resveratrol 3-glucuronide

Yield: 50%. ^{1}H NMR (300 MHz, d_6-DMSO), δ (ppm): 3.2–3.6 (4H, m, non-anomeric C–H sugar), 4.85 (1H, d, J = 7.5 Hz, anomeric C-H sugar), 6.28 (1H, s, C^4–H), 6.52 (1H, s, C^6–H), 6.61 (1H, s, C^2–H), 6.71 (2H, d, J = 8.7 Hz, $C^{3',5'}$–H), 6.81 (1H, d, J = 16.2 Hz, C–H double bond), 6.97 (1H, d, J = 16.5 Hz, C–H double bond), 7.35 (2H, d, J = 8.4 Hz, $C^{2',6'}$-H), 9.54 (2H, br). ESI-MS (MeOH, positive ions), *m/z* 405.3 (MH^+, 15%), 229.2 (resveratrol+H^+, 100%).

2.2.2 *Trans*-resveratrol 4′-glucuronide

Yield: 35%. ^{1}H NMR (300 MHz, d_6-DMSO), δ (ppm): 3.2–3.6 (4H, m, non-anomeric C–H sugar), 4.90 (1H, d, J = 7.5 Hz, anomeric C–H sugar), 5.05 (1H, br. s), 5.27 (1H, br. s), 6.01 (1H, t, J = 1.7 Hz), 6.36 (2H, d, J = 1.7 Hz), 6.84 (2H, d, J = 16.4 Hz), 6.91 (2H, d, J = 16.4 Hz), 6.95 (2H, d, J = 8.7 Hz, $C^{3',5'}$–H), 7.45 (2H, d, J = 8.4 Hz, $C^{2',6'}$–H), 9.16 (2H,

br. s). ESI-MS (MeOH, positive ions), *m/z* 405.3 (MH^+, 15%), 229.2 (resveratrol+H^+, 100%).

2.3 Subjects

All subjects were healthy volunteers, nonsmokers, moderate drinkers. Before the experiments they were subjected to a full physical examination with routine analysis of blood and urine to exclude hepatic and renal diseases, diabetes mellitus, dyslipoproteinemia, and hemopoietic disorders. Moreover, they were asked to sign an informed consent and not to consume alcoholic beverages for one week before the experiment. The main characteristics of the selected populations and the treatments they underwent are reported separately for each experiment.

2.4 Test meals associated to red wine consumption

2.4.1 Experiment 1: *trans*-resveratrol absorption in subjects eating a standard meal with red wine

Ten healthy males, average age 30 years (25 – 40), average body weight 72.4 kg (61 – 90), mean value of body mass index (BMI) 23, were recruited for this experiment. All the subjects ate the same test meal with 300 mL red wine (Lambrusco). The average content of the free *trans*-resveratrol in wine was determined by applying the same method used for the resveratrol determination in plasma (see below) and was found to be 0.82 ± 0.27 mg/L. Thus, during this experiment they ingested 246 μg free *trans*-resveratrol corresponding to an average intake of 3.4 μg/kg b.w. The test meal consisted of "Milanese beef cutlet" (beef, egg, breadcrumbs, fried in maize oil) and chips.

2.4.2 Experiment 2: *trans*-resveratrol absorption in fasting subjects drinking red wine

Five healthy volunteers (1M/4F), average age 29 years (24 – 38), average body weight 58.4 kg (52 – 61), mean value of BMI 20, were selected for this experiment. All subjects were asked to drink before having breakfast 600 mL red wine (Cabernet Franc), containing 3.2 mg/L free *trans*-resveratrol, thus corresponding to a total ingestion of 1.92 mg of the antioxidant compound (average intake 32.9 μg/kg b.w.).

2.4.3 Experiment 3: *trans*-resveratrol absorption by subjects eating two different meals (differing in the lipid content) with red wine

Ten healthy volunteers (3M/7F), average age 31 (24 – 54), average body weight 63.9 kg (52 – 90), mean value of BMI 23, were selected for this experiment. The meals used in this experiment were prepared and cooked with the same ingredients, similar caloric value, and different lipid content. Five subjects belonging to group 1 ate a "fat meal" (1321 kcal and lipid content of 69 g) while the five subjects belonging to group 2 ate a "lean meal" (1200 kcal and lipid content of 3.3 g). The meal was set up with three courses containing pasta and peas as first course, a slice of chicken meat and salad as second course, and a cup of mixed fruits as third course. During the meal all the subjects were asked to drink 600 mL red wine (Aglianico). The wine consumption assured a free *trans*-resveratrol total ingestion of 480 μg corresponding to an average intake of 7.5 μg/kg b.w.

2.5 Blood samples

Blood samples (10 mL) were taken before eating and/or drinking wine (t0, basal value), at 30 min (t30), 60 min (t1h), 120 min (t2h), 240 min (t4h). The blood samples were let to coagulate at room temperature and then centrifuged at $1500 \times g$ for 20 min at 4°C. The serum was collected, transferred to a clean tube, and maintained at –20°C until the analyses were performed (not later than 3 days after).

2.6 Sample preparation

Serum sample extractions were performed, in all the experiments, using the procedure proposed by Zhu and co-workers [31] with some modifications. Briefly, 7 mL ($3.5 \text{ mL} \times 2$) ethyl-acetate and 250 μL of a 0.25 M NaH_2PO_3 aqueous solution were added to 500 μL serum and centrifuged at 4000 rpm for 10 min. 2.5 μL of a methanolic solution of carbamazepine (1 mg/mL) was also added as internal standard. The organic phase was separated, dried under a stream of nitrogen, and redissolved in 200 μL of a 25 mM NaH_2PO_3 solution in water/acetonitrile 70 : 30 v/v. The solution was filtered through 0.45 μm nylon filters and Millipore PLGC ultrafilters (nominal cut-off 10 kDa). Organic layers were collected and dried under N_2 flow. Dried extracts were dissolved in 100 μL methanol before HPLC analysis.

2.7 HPLC-UV analysis

2.7.1 First experiment

The samples collected during the first experiment were analyzed by HPLC (Waters Alliance 2695 separation module) with a RP-C18 column (Jupiter Phenomenex, 5 μm, 300 Å, 250×4.6 mm, $T = 30$°C) under the following conditions: injected volume, 40 μL; isocratic elution with a 25 mM NaH_2PO_3 solution in water/acetonitrile 70 : 30 v/v; flow rate, 1 mL/min; detection at 306 nm (Waters 2487 UV-vis detector). The calibration curve was made by injecting standard

methanolic solutions of *trans*-resveratrol (seven different concentration levels ranging from 2.5 to 2500 ng/mL) added of carbamazepine (ten times more concentrated than resveratrol in each sample). Triplicate injections were made for each different concentration ($r^2 = 0.9971$). The less concentrated sample (2.5 ng/mL) gave a *trans*-resveratrol peak with an S/N ratio of 13.3. By using this calibration curve, each serum sample, added to carbamazepine (2.5 μg in 500 μL serum or wine), was analyzed in duplicate. The presence of *trans*-resveratrol glucuronide was detected by an indirect method, namely by treating the serum samples with β-glucuronidase, as reported in the literature [23]. Samples were then re-analyzed after enzymatic treatment with the method above reported. In this way, the total (free + glucuronidated) *trans*-resveratrol content was determined, allowing to calculate the conjugated *trans*-resveratrol content by comparison with the data previously obtained.

2.7.2 Second and third experiment

The chromatographic analyses in the second and third experiments were carried out using an HPLC-UV-diode array detector (DAD) and a column Phenomenex Prodigy C18 ODS-3 100 Å (5 μm; 4.66 × 250 mm). The mobile phase consisted of water adjusted to pH 2 with acetic acid (solvent A) and a mixture of solvent A: acetonitrile (20 : 80) (solvent B). The flow rate was 1 mL/min and the following stepwise gradient was used: 0.1 min, 10% B; 10 min, 22% B; 15 min, 22% B; 25 min, 100% B; 30 min, 100% B; column returned in 2 min to 10% B and equilibrated for 10 min before injection. Identification of *trans*-resveratrol and glucuronides was carried out by comparison of the spectral features and retention times of the compounds with the authentic standards. Quantification, at the wavelength of 306 nm, was achieved using calibration curves of authentic standard compounds run under the same conditions. This method allowed to identify and quantify in serum samples *trans*-resveratrol-4′-glucuronide, *trans*-resveratrol-3-glucuronide, and *trans*-resveratrol in the free form at retention times of 5.6 min, 7.8 min, 19.8 min, respectively, with a detection limit of 2.5 ng/mL for each compound. The analyses were performed in triplicate.

2.8 HPLC-MS and HPLC-MS/MS analyses

2.8.1 First experiment

In order to confirm the presence of *trans*-resveratrol in human serum samples detected by LC-UV, a confirmatory analysis was also performed by a new LC-photodiode array-(PDA)-MS method. The samples were analyzed by HPLC (Waters Alliance 2695 separation module) with an RP-C18 column (Jupiter Phenomenex, 5 μm, 300 Å, 250 × 3 mm, $T = 30^{\circ}C$) under the following conditions: injected volume, 30 μL; eluent A, water (0.2% HCOOH); eluent B, water/acetonitrile 60 : 40 v/v (0.2% HCOOH); linear gradient elution from 10% to 100% B in 30 min; flow rate, 0.5 mL/min, Waters 996 PDA detector (210–400 nm); Micromass ZMD electrospray mass spectrometric detector (positive ion mode; capillary voltage, 3.5 kV; cone voltage, 30 V; single ion recording at 229 *m/z*). The calibration curve was obtained by measuring the peaks detected by the PDA detector at 306 nm by injecting standard methanol solutions of *trans*-resveratrol (six different concentration levels ranging from 2.5 to 250 ng/mL) and carbamazepine (ten times more concentrated than resveratrol in each sample). Triplicate injections were made for each different concentration ($r^2 = 0.9918$). The less concentrated sample gave a *trans*-resveratrol UV peak (used for quantification) with a S/N ratio of 15.5 and MS peak (used for identification) of 3.6. By using this calibration curve, each human serum sample, added to carbamazepine (2.5 μg in 500 μL serum), was analyzed in duplicate (SD for positive samples < 5 ng/mL).

2.8.2 Second and third experiment

In order to confirm the identification and the quantification of free and conjugated *trans*-resveratrol, a new LC-MS/MS method was also performed. LC-MS/MS analysis was carried out using an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Toronto, Canada) equipped with a Turbo ionspray source with the capillary voltage settled at –4500 V and heated to 100°C. Acquisition was performed in negative ion mode in multiple reaction monitoring (MRM) using the deprotonated molecule of *trans*-resveratrol (*m/z* 227) and resveratrol glucuronide (*m/z* 403) as precursor ions. The analysis with MRM was developed using as declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) the following conditions: –60, –26, –11 and –40, –36, –11 for *trans*-resveratrol (*m/z* 227→184.9) and resveratrol glucuronides (*m/z* 403→227), respectively. The detection limits for the free and the glucuronidated *trans*-resveratrol was 1 ng/mL. The HPLC system consisted of a binary micropump Series 200, automatic solvent degasser, and a Luna C_{18} (3 μm, 100 Å, 1.0 × 250 mm) column. The solvent system consisted of a 10 min linear gradient from 10% to 80% methanol with 0.1% formic acid (phase B) and water with 0.1% formic acid (phase A) as the cosolvent at a flow rate of 200 μL/min. At the end of the gradient, the column was flushed with 100% phase B for 2 min to remove strongly retained compounds before re-equilibration with 10% phase B for 2 min.

3 Results

3.1 First experiment of *trans*-resveratrol uptake: indirect determination of glucuronides

Ten healthy males ate a test meal consisting of "Milanese beef cutlet" (beef, egg, bread-crumbs, fried in maize oil)

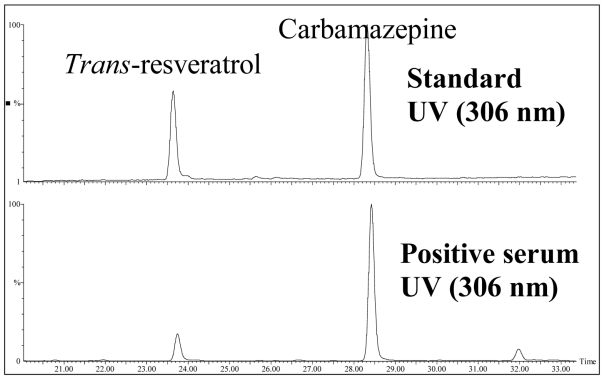

Figure 1. UV chromatograms (306 nm detection) of a standard solution of *trans*-resveratrol and carbamazepine (above, actual concentration of *trans*-resveratrol 25 ng/mL) and positive human serum sample (below).

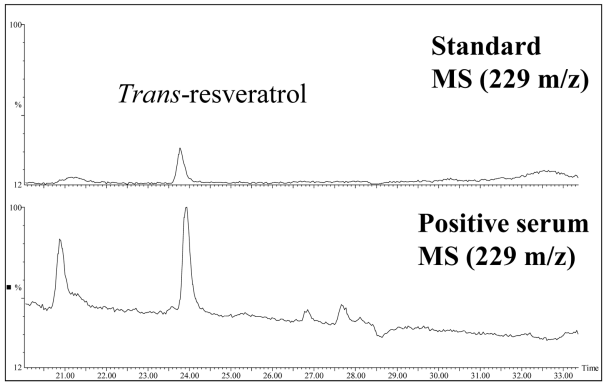

Figure 2. MS chromatograms (*m/z* 229 detection) of a standard solution of *trans*-resveratrol (above, actual concentration of *trans*-resveratrol 25 ng/mL) and positive human serum sample (below).

and chips, together with 300 mL red wine (Lambrusco). In the serum samples taken after 1, 2, or 4 h free *trans*-resveratrol was never detected. In order to confirm the results, a new LC-MS method was developed (with single ion detection at 229 *m/z* for the protonated *trans*-resveratrol, details in the experimental section), but again all human serum samples analyzed with this new method did not gave rise to chromatographic peaks corresponding to *trans*-resveratrol. However, as far as glucuronidated *trans*-resveratrol was concerned, the serum samples of four subjects out of ten showed, after treatment with β-glucuronidase, a peak detected by LC-UV analysis with a retention time compatible with that of *trans*-resveratrol. All positive samples were those collected 1 h after the meal, but the amounts were quite variable, ranging from less than 30 to 160 ng/L (Table 1). The LC-UV-MS method developed by us was again applied for the confirmation analysis. All positive samples resulted in signals detected both by UV and by MS (Figs. 1 and 2), whereas negative samples consistently gave no peaks detectable neither by the PDA nor by the MS detector. As a further confirmation, all positive samples spiked with standard *trans*-resveratrol gave an increased analyte peak in all cases.

3.2 Synthesis of *trans*-resveratrol glucuronides

Since the only serum samples giving positive results in the first experiment were those treated with β-glucuronidase, suggesting that glucuronidated derivatives were mainly present, we synthesized the *trans*-resveratrol 4′-glucuronide and 3-glucuronide, in order to provide suitable standards for the identification and the quantification of the metabolites (Fig. 3). The available synthetic procedures for the synthesis of these derivatives usually follow the strategy of separately synthesizing the two regioisomers by starting from suitable synthons, in order to build the stilbene backbone with the sugar already linked to it [30]. In our case, since we were interested in both regioisomers, we applied a simpler approach for linking the glucuronic acid to the free

Table 1. *Trans*-resveratrol metabolites (3-glu and 4′-glu) found in the serum of the subjects which drank red wine in association to a standard meal

Subjects	T0 (ng/mL)	T30 (ng/mL)	T1h (ng/mL)	T2h (ng/mL)	T4h (ng/mL)
A	n.d.[a)]	n.d.	n.d.	n.d.	n.d.
B	n.d.	n.d.	n.d.	n.d.	n.d.
C	n.d.	n.d.	4′-glu (160)	n.d.	n.d.
D	n.d.	n.d.	n.d.	n.d.	n.d.
E	n.d.	n.d.	3-glu (15)	n.d.	n.d.
F	n.d.	n.d.	n.d.	n.d.	n.d.
G	n.d.	n.d.	3-glu (46)	n.d.	n.d.
H	n.d.	n.d.	n.d.	n.d.	n.d.
I	n.d.	n.d.	n.d.	n.d.	n.d.
J	n.d.	n.d.	4′-glu (168)	n.d.	n.d.

a) Not detected
The amounts found (ng/mL) are given in parentheses.

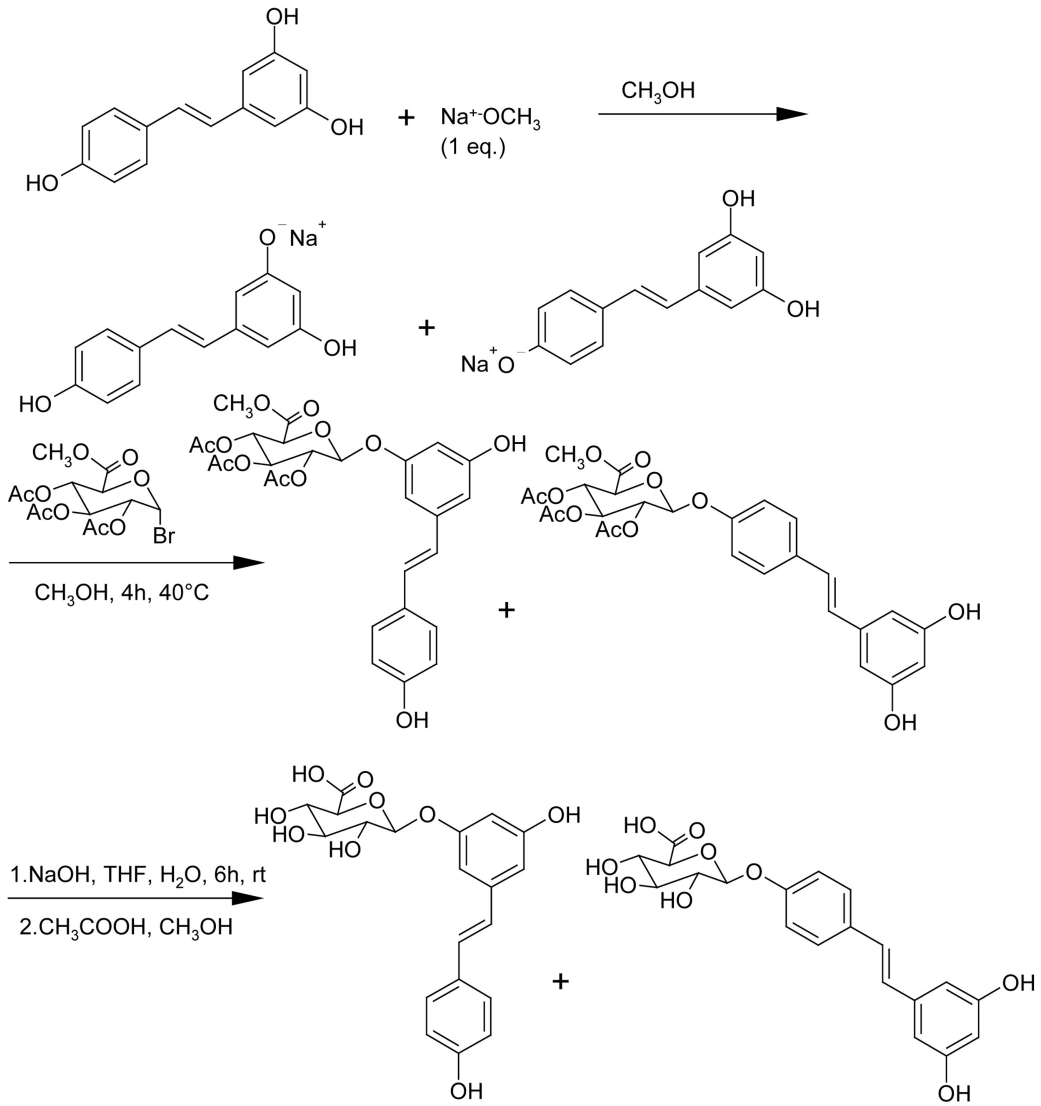

Figure 3. Synthesis of *trans*-resveratrol 4′-glucuronide and 3-glucuronide as a mixture.

resveratrol, in order to obtain the two isomers as a mixture and then separate them by preparative HPLC. Therefore, we reacted the free resveratrol with one equivalent of sodium methoxide, in order to get a mixture of the 4′-deprotonated and of the 3-deprotonated compound. Then acetobromo-α-D-glucuronic acid methyl ester was added to the solution, obtaining a mixture of the acetyl-protected 4′-derivatized and 3-derivatized resveratrol. Basic hydrolysis of the acetyl groups on the sugar gave a mixture of the two desired molecules, which were purified by preparative HPLC (yield: 50% for *trans*-resveratrol 3-glucuronide and 35% for *trans*-resveratrol 4′-glucuronide) and characterized by ^{1}H NMR and ESI-MS. The overall synthetic scheme is reported in Fig. 3.

3.3 Second and third experiment of *trans*-resveratrol uptake: direct determination of glucuronides

In the second experiment, five healthy volunteers were asked to drink, before having breakfast, 600 mL red wine (Cabernet Franc) (Table 2). The HPLC analysis of serum samples of the five subjects showed that only three subjects had absorbed *trans*-resveratrol in a conjugated form. In particular, one subject showed a serum 4′-*trans*-resveratrol-glucuronide content of 487 ng/mL after 30 min, whereas another showed after 1 h a 3-*trans*-resveratrol-glucuronide content of 77.3 ng/mL and after 2 h a 4′-*trans*-resveratrol-glucuronide content of 900 ng/mL; in a third subject, 3-

Table 2. Free *trans*-resveratrol (t-resv) and its metabolites (3-glu and 4′-glu) found in the serum of the fasting subjects which drank red wine

Subjects	T0 (ng/mL)	T30 (ng/mL)	T1h (ng/mL)	T2h (ng/mL)	T4h (ng/mL)
A	n.d.[a)]	t-resv (n.q.[b)])	t-resv (n.q.) 3-glu (77)	t-resv (n.q.) 4′-glu (900)	n.d.
B	n.d.	t-resv (n.q.)	t-resv (n.q.)	t-resv (n.q.)	n.d.
C	n.d.	4′-glu (487)	n.d.	n.d.	n.d.
D	n.d.	n.d.	n.d.	n.d.	n.d.
E	n.d.	n.d.	n.d.	n.d.	n.d.

a) Not detected
b) Not quantifiable
The amounts found (ng/mL) are given in parentheses.

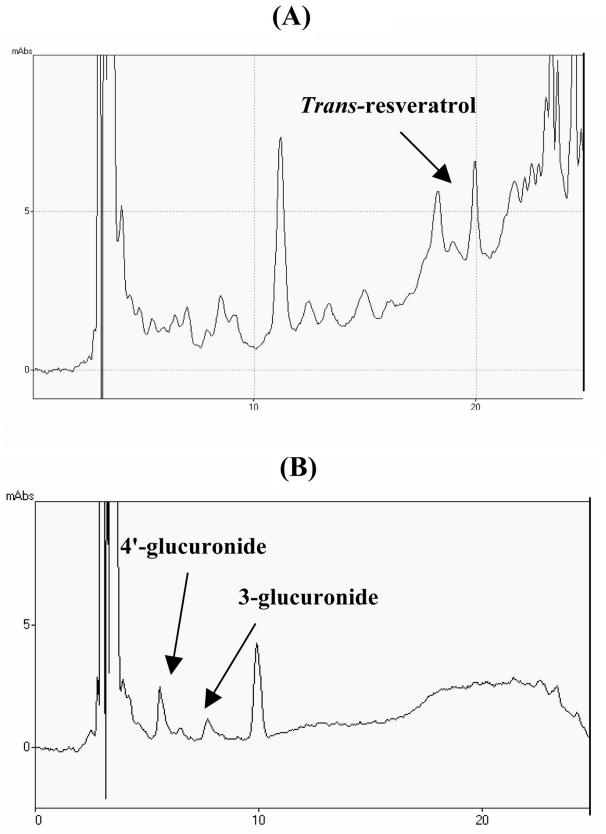

Figure 4. HPLC chromatograms (λ = 306 nm) of (A) *trans*-resveratrol positive serum sample and (B) *trans*-resveratrol-glucuronide positive serum sample.

trans-resveratrol glucuronide after 30 min was detected, but the amount was below the limit of quantification. UV chromatograms of serum samples positive for free *trans*-resveratrol and for *trans*-resveratrol 4′-glucuronide and 3-glucuronide are reported in Fig. 4.

These results were confirmed by those obtained by the newly developed HPLC-MS/MS method. Since this method showed higher sensitivity than the HPLC-UV one (LODs of

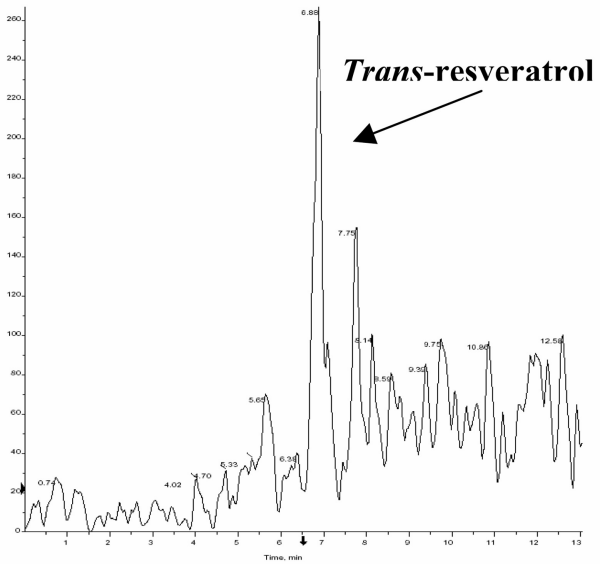

Figure 5. LC-MS/MS chromatogram (*m/z* 227) of *trans*-resveratrol positive serum sample.

1 and 2.5 ng/mL, respectively), it allowed the identification also of the *trans*-resveratrol in its free form, not previously detected with the first set of analyses. In particular, the free compound was found in the serum samples of two subjects after 1 h and 2 h; one of them showed the presence of free *trans*-resveratrol in the serum also after 30 min, although in all cases the amount was below the limit of quantification. An HPLC-MS/MS chromatogram of a serum positive for *trans*-resveratrol is reported in Fig. 5. The results are summarized in Table 2.

In the third experiment ten healthy volunteers were asked to drink red wine (Aglianico) in association with two different kind of meals, containing the same ingredients and with similar caloric value, but different lipid content. In this case, free *trans*-resveratrol was found in a quantifiable amount in the serum of four subjects after 30 min, in a concentration range of 1.1–6.2 ng/mL. Among these four sub-

Table 3. Free *trans*-resveratrol (t-resv) and its metabolites (3-glu and 4′-glu) found in serum of the subjects which drank red wine in association with a "fat" or with a "lean" meal

Subjects	T0 (ng/mL)	T30 (ng/mL)	T1h (ng/mL)	T2h (ng/mL)	T4h (ng/mL)
Fat meal					
A	n.d.[a)]	t-resv (6)	n.d.	3-glu (65)	n.d.
B	n.d.	t-resv (1)	n.d.	n.d.	n.d.
C	n.d.	n.d.	n.d.	n.d.	n.d.
D	n.d.	n.d.	n.d.	n.d.	n.d.
E	n.d.	n.d.	n.d.	n.d.	n.d.
Lean meal					
F	n.d.	t-resv (1)	4′-glu (540)	n.d.	n.d.
G	n.d.	t-resv (1)	n.d.	n.d.	n.d.
H	n.d.	n.d.	n.d.	n.d.	n.d.
I	n.d.	n.d.	n.d.	n.d.	n.d.
J	n.d.	n.d.	n.d.	n.d.	n.d.

a) Not detected
The amounts found (ng/mL) are given in parentheses.

jects, *trans*-resveratrol glucuronides were found only in the serum of two subjects after 2 h and 1 h, respectively. These results are reported in Table 3.

4 Discussion

In Table 4 the three experiments performed in this study were summarized. This is the first study of *trans*-resveratrol bioavailability in humans from a moderate consumption of red wine and using a relevant number of subjects. The data obtained analyzing the serum of the 25 healthy volunteers enrolled for the three experiments reported in this paper showed enormous interindividual differences. In fact, considering all the experiments, in the serum samples of 14 subjects of 25 tested (56%), resveratrol was never found in any form at any time, whereas in 11 of 25 (44%) at least one serum sample was positive for free resveratrol or a glucuronidated derivative.

Among positive serum samples, free resveratrol could be found only 30 min after ingestion in subjects which consumed a meal in association with red wine, whereas it could be found also after a longer time (1 h and 2 h) in fasting subjects. In any case, the detected amounts of free resveratrol were always very low (few ng/mL or less), in many cases below the limit of quantification, and could be confirmed only by HPLC-MS/MS. These results were in accordance to literature data. Goldberg and co-workers [25] reported that 30 min after ingestion of 25 mg *trans*-resveratrol in three different matrices the compound in its free form reached a peak concentration in the serum of 7 μg/L that decreased during the next hours. Trace amounts of free *trans*-resveratrol (< 5 ng/mL) were also detected by Walle and co-workers [26] in plasma of subjects after a 25 mg resveratrol oral dose.

On the other side, both 4′- and 3-glucuronide derivatives were found in several samples, their amounts being quite independent from the time after ingestion of red wine and from the dietary regime associated, but usually being much larger than the amount of free resveratrol, ranging from tens to hundreds ng/mL. This result was expected since the glu-

Table 4. Summary of the three bioavailability studies

n subjects (sex, m.b.w.)[a)]	Total quantity of wine consumed (t-resv. content)	Total amount of free t-resv ingested (average μg/*K*g b.w.)[b)]	Associated meal (group)	Free t-resv (*n* subjects, group, time after drinking)	T-resv metabolites (*n* subjects, group, time after drinking)
10 (M, 72.4 kg)	300 mL (0.8 μg/mL)	0.25 mg (3.4)	"Milanese beef cutlet"	0	4 (1h)
5 (1M/4F, 58.4 kg)	600 mL (3.2 μg/mL)	1.92 mg (32.9)	Fasting	0	1 (1 h and 2 h) 2 (30 min)
10 (3M/7F, 63.9 kg)	600 mL (0.8 μg/mL)	0.48 mg (7.5)	"Fat" meal (G1) "Lean" meal (G2)	2 (G1 – 30 min) 2 (G2 – 30 min)	1 (G1 – 2h) 1 (G2 – 1h)

a) Medium body weight
b) Body weight

curonidation of *trans*-resveratrol by the intestine/liver had been previously reported as an important mechanism in the metabolism of this compound *in vivo* [23, 25]. Moreover, in the present study where it was possible to distinguish between *trans*-resveratrol 4′-glucuronide and 3-glucuronide by a direct identification with authentic standards (five positive samples), three samples were shown to contain the former and two samples were found to contain the latter. Furthermore, the amount of 4′-glucuronide ranged from 487 to 900 ng/mL, whereas the amount of the 3-glucuronide ranged from 65 to 77 ng/mL, indicating that both positions may be glucuronidated, although the 4′-position is likely to be the preferential site of glucuronidation *in vivo*. The data from Meng and co-workers [24] also indicated that both resveratrol positions can be glucuronidated, although in an *in vitro* study performed on human liver microsomes the 3-position seemed to be the preferential glucuronidation site [32]. On the contrary in this study, being the absorption directly studied in human subjects, enzymes coming from different cell lines may be involved in the transformation, thus altering the regioselectivity.

The absence of resveratrol both in free and in glucuronidated form in all serum samples collected 3 – 4 h after wine ingestion could be explained considering literature data which indicate that, when absorbed, resveratrol is rapidly cleared through the glucuronidation and sulfation pathways, and the glucuronide and sulfate derivatives are principally excreted in urines [24–26]. Walle and co-workers [26] have recently reported that 2 h after the administration of a 25 mg *trans*-resveratrol oral dose in six humans, in the plasma of only one subject, resveratrol monosulfate and dihydroresveratrol sulfate were found with an estimated concentration of 124 ng/mL.

In conclusion, the present bioavailability study of *trans*-resveratrol in humans demonstrated for the first time that the compound can be actually absorbed also after a moderate wine consumption (300 or 600 mL). A total number of 25 subjects have been involved in the experiments and it was clearly demonstrated that resveratrol absorption after wine consumption is highly variable. The compound was found in the serum of roughly half of the subjects participating to the experiments, in free or in glucuronidated form and in very different concentrations. A further evidence emerging from the data is that the bioavailability of *trans*-resveratrol absorption associated to wine consumption is generally influenced neither by the meal nor by the kind and/or the quantity of lipids contained in the meal consumed.

The data here reported raises some doubts about the association between the well-known beneficial health effects due to a moderate consumption of red wine and the presence of *trans*-resveratrol. The absolute concentration is really low, there is a high inter-individual variability in the absorption, and a rapid metabolization and clearance of the absorbed compound. The protective effect observed on cardiovascular diseases associated to a moderate consumption of wine (the famous French paradox) could be due to the whole polyphenols contained in wine and not to resveratrol alone, or to reasons different than wine consumption, such as a healthy lifestyle based on a correct dietary regime, practice of sports, and no smoking habit.

5 References

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