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Quantification of *trans*-resveratrol and its metabolites in rat plasma and tissues by HPLC

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ABSTRACT

trans-Resveratrol, a polyphenol from grapes, is being recognized as a bioactive agent with potential beneficial effects on health. However, little is known about its distribution in the organism mainly because of the lack of accurate and precise detection methods. Consequently the aim of the present study was to develop a methodology of extraction and quantification of *trans*-resveratrol and its metabolites in plasma, brain, testis, liver, lungs and kidney by HPLC. To this end, the time of homogenization and liquid extraction were adapted to the different tissues. The methods were validated using homogenized tissues spiked with pure *trans*-resveratrol. The precision (% R.S.D.) ranged from 3.7% in testis to 13.2% in lungs. Recoveries were 98.5 ± 3.2% (liver), 100.1 ± 1.8% (kidney), 96.5 ± 7.6% (lungs), 99.0 ± 0.7% (brain) and 103 ± 2.7% (testicle). The limits of detection ranged from 5.5 nM in testis to 11.2 nM in kidney. After validation, the methods were applied to the assessment of the bioavailability and distribution of *trans*-resveratrol in rats after the intravenous administration of 15 mg/kg. At 90 min, *trans*-resveratrol and its glucuronide and sulfate conjugates were widely distributed in all the tissues studied. The highest concentrations (nmol/g tissue) were found in kidney (resveratrol: 1.45 ± 0.35; glucuronide: 2.91 ± 0.19; sulfate: not detected), and the lowest in brain (resveratrol: 0.17 ± 0.04; glucuronide: not detected; sulfate: 0.04 ± 0.01). In conclusion, accurate and reproducible methods have been described to identify target tissues of resveratrol as a first step to understand its mechanisms of action *in vivo*.

1. Introduction

trans-Resveratrol (*trans*-3,4′,5-trihydroxystilbene, Fig. 1) found in grapes, red wine, peanuts, and various berries is a polyphenol with potential beneficial effects on health. *trans*-Resveratrol holds a wide range of pharmacological properties [1] without harmful effects [2] and is well-known for its antioxidant, anti-inflammatory, analgesic, cardio-protective, neuro-protective, anti-aging and anti-cancer activities [1].

However, our understanding of the bioavailability, metabolism and tissue distribution of this compound is limited. Orally ingested *trans*-resveratrol is extensively metabolized in the enterocyte [3] before its entry into blood and target organs [1]. Currently, the tissue distribution of *trans*-resveratrol has hardly been assessed compared with the number of papers that refers to the plasmatic concentrations of this polyphenol [4]. To improve our understanding of the mechanism of action of *trans*-resveratrol, it is necessary to know if this compound or its metabolites are present in the body and in which organs they are found. Because of the limited information on the distribution available at present, we developed and validated highly sensitive and selective methods for simultaneous determination of *trans*-resveratrol and its metabolites in homogenized rat tissues spiked with this compound. The methods were successfully applied to the assessment of the distribution of *trans*-resveratrol after the intravenous administration of 15 mg/kg to healthy rats.

2. Experimental

2.1. Chemicals and reagents

trans-Resveratrol was chemically pure and purchased from Second Pharma Co., Ltd. (Shangyu, PR China). Acetonitrile and methanol were from J.T. Baker (Deventer, Netherlands) and acetic acid from Scharlau Chemie S.A. (Barcelona, Spain). All these solvents were HPLC grade. Other chemicals used were analytical grade and obtained from Sigma–Aldrich (St. Louis, MO, USA). Water used in all experiments was passed through a Milli-Q water purification system (18 mΩ) (Millipore, Milan, Italy).

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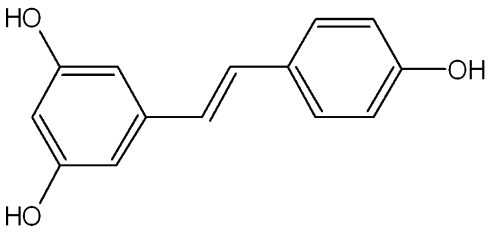

Fig. 1. Chemical structure of *trans*-resveratrol.

2.2. Special precautions

All laboratory procedures involving the manipulations of *trans*-resveratrol were performed in dim light to avoid photochemical isomerization of *trans*-resveratrol to the *cis* form. Stock solutions of 1 mM in ethanol 20% (v/v) *trans*-resveratrol were prepared weekly, and were stored at room temperature, protected from light.

2.3. Instrumentation

An Agilent model 1100 (Agilent Technologies, Palo Alto, USA) gradient liquid chromatograph equipped with an automatic injector, a Synergi® Fusion-RP 80A (250 mm × 4.6 mm; 4 μm) (Phenomenex, Torrance, CA, USA) with a C18 guard column cartridge at 40 °C, and a diode array UV–vis detector, coupled to a ChemStation was used to determine *trans*-resveratrol and its metabolites.

Identification of *trans*-resveratrol conjugates was performed in an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, PE Sciex, Concord, Ont., Canada) equipped with a Turbo IonSpray source in negative-ion mode for monitoring ions of deprotonated molecules $[M-H]^-$.

2.4. Chromatographic conditions and mass spectrometer conditions

The temperature of the column was kept at 40 °C. The flow rate was 1.5 ml/min and injection volume was 100 μl. The mobile phase consisted of two phases, phase A was a 3% acetic acid solution and phase B was a mixture of phase A:acetonitril (20:80, v/v). Separation of *trans*-resveratrol and its metabolites were effected with gradient elution starting at min 0 with 22% solvent B to min 2; 2–6 min, linear gradient from 22 to 30% B; 6–14 min, linear from 30 to 50% B; 14–18 min, increasing to 60% B; 18–25 min, linear from 60 to 100% B and followed by washing and reconditioning the column.

The chromatograms were obtained according to the retention time with detection at 306 nm, at which the absorbance of *trans*-resveratrol presents a maximum. Peak identification of this compound was carried out by comparison of the retention time and its UV spectra (from 200 to 400 nm) with those of a standard. Quantification of *trans*-resveratrol was performed using standard curves constructed after spiking relevant concentrations of this compound in the appropriate sample matrix, either plasma or homogenized tissues. The curves were characterized by regression coefficients of $R^2 = 0.99$ or above. *trans*-Resveratrol glucuronide and sulfate were quantified after the hydrolysis by means of enzymatic treatment.

Mass spectrometry was used to confirm the identity of *trans*-resveratrol conjugates in rat plasma samples after the i.v. administration of 15 mg/kg. The mass spectrometer was operated by electrospray ionization with an ion spray voltage of −5000 V, using nitrogen as the nebulizer gas, 10 (arbitrary units) and curtain gas, 15 (arbitrary units). A standard solution of *trans*-resveratrol was used to optimize the detecting conditions in the presence of LC mobile phase, and were as follows: declustering potential, −50 V; focusing potential, −200 V; drying gas (N_2) heated to 400 °C and introduced at a flow rate of 5000 cm^3/min. Mass spectra were acquired in the 100–450 m/z range.

2.5. Animals

Male adult Sprague–Dawley rats (275–300 g; $n = 18$) were housed in cages ($n = 3$/cage) under controlled conditions of a 12 h light:dark cycle, with a temperature of 22 ± 3 °C and a relative humidity of 40–70%. They received a standard diet (2014 Teklad Global 14%, Harlan, Spain) and water *ad libitum*. No traces of *trans*-resveratrol were detected in the commercial diet, as revealed by the analyses performed using the method of Juan et al. [5]. Handling and killing of rats were in full accordance with the European Community guidelines for the care and management of laboratory animals. The studies were approved by the Ethic Committee of Animal Experimentation of the University of Barcelona. Rats were fasted overnight and anesthetized by intramuscular injection of 90 mg/kg ketamine (Imalgene 1000, Merial Laboratorios S.A. Barcelona, Spain) and 10 mg/kg xylacine (Rompun 2%, Química Farmaceutica Bayer S.A., Barcelona, Spain). All rat manipulations were carried out in the morning to minimize the effects of circadian rhythm.

2.6. Sample preparation

2.6.1. Plasma samples

Resveratrol extraction from plasma samples was carried out on a reversed-phase C18 Sep-Pak Classic Cartridge for manual operation (WAT051910, Waters, Mildford, USA). The method was based on that described previously for human plasma [5] but was adapted to rat samples. Prior to use, the cartridge was conditioned with 4 ml of methanol followed by equilibration with 10 ml of water. 500 μl of plasma was acidified with 15 μl of acetic acid and stirred in the vortex for 2 min. The acidified plasma was slowly loaded onto the cartridge and rinsed with 10 ml of water. *trans*-Resveratrol contained in the cartridge was eluted with 4 ml of methanol. 10 μl of ascorbic acid at 15% was added to the eluted liquid, that was evaporated with a Concentrator 5301 (Eppendorf Iberica S.L., San Sebastian de los Reyes, Spain) at 35 °C to a final volume of 400 μl. Finally, it was placed in a seal amber vial for HPLC analysis.

2.6.2. Tissue samples

1 g of tissue was finely minced with scissors, and placed in a homogenizer vessel. Methanol (80%, v/v) acidified with acetic acid (2.5%, v/v) and 10 μl ascorbic acid (15%, v/v), as antioxidant, were added and tissues were subsequently homogenized. The homogenization process was adjusted to each tissue. Brain and testes were placed in 3 ml of acidified methanol and grinded using a manual glass homogenizer with 30 strokes. An additional 1 ml was used to collect the residues on the glass vase and was added to the initial 3 ml. However, liver, lungs and kidney were homogenized with six short pulses of a Polytron tissue homogenizer (Kinematica AG, Lucerne, Switzerland) using 2 ml of acidified methanol. The homogenizer was cleaned twice with 1 ml of acidified methanol, which were added to the 2 ml making a final volume of 4 ml.

Homogenized samples were transferred to a 10 ml conical glass tube and processed in the vortex for 5 min prior to centrifugation at 3000 × *g* (Megafuge 1.0R, Heraeus, Boadilla, Spain) for 30 min at 4 °C. The supernatant was placed in a clean tube. The residue was extracted two more times with 4 ml of acidified methanol by vigorous agitation in the vortex for 5 min, followed by centrifugation at 3000 × *g* for 30 min at 4 °C. The organic solvent of the supernatants was evaporated to a final volume of 400 μl, and subsequently placed in a seal amber vial for HPLC analysis.

The extraction from the kidney was slightly different. 4 ml were added to the kidney homogenates (final volume 8 ml) and were incubated at 60 °C with constant stirring for 30 min in a hot plate, followed by centrifugation at 3000 × *g* for 30 min at 4 °C. The supernatant was placed in a clean tube. The residue was extracted once more with 4 ml of acidified methanol by vigorous agitation in the vortex for 5 min, and centrifugation at 3000 × g for 30 min at 4 °C. The organic solvent of the supernatants was evaporated to a final volume of 400 μl that was placed in a seal amber vial for HPLC analysis.

2.7. Method validation

The extraction methods were validated according to The United States Pharmacopoeia [6]. Blank plasma and tissues were obtained from rats that were not administered with *trans*-resveratrol. 500 μl of plasma was spiked at the final concentration of 0.1, 0.25, 5, 0.75, 1, 2.5 and 5 μM of *trans*-resveratrol and were stirred in the vortex for 1 min before being extracted as indicated in sample preparation. 1 g of tissue was homogenized with 15 strokes, spiked with 5 nmol of *trans*-resveratrol (final concentration 5 nmol/g tissue), and agitated in the vortex for 2 min. Next, samples were homogenized again with 15 strokes, followed by the same treatment as indicated in Section 2.6.

2.7.1. Precision and accuracy

The precision of the analytical method that measured *trans*-resveratrol in plasma was determined by assaying a sufficient number of samples ($n = 4$–6) at seven different concentrations ranging from 0.1 to 5 μM. For the extraction of *trans*-resveratrol in rat tissues the precision was determined by assaying a sufficient number of samples ($n = 6$) for each tissue at 5 nmol/g and was expressed as the relative standard deviation (% R.S.D.). The intra-day precision was determined by analyzing the spiked samples prepared within a day. The inter-day precision was determined by analyzing the spiked samples prepared on three different days. Peak areas were considered for the calculation of the concentration and establish the precision.

Accuracy was estimated based on the mean percentage of error of the measured concentration (Con_M) to the theoretical concentration (Con_T) according to the equation:

$$\text{Bias}\,(\%) = \left[\frac{Con_T - Con_M}{Con_T}\right] \times 100.$$

2.7.2. Recovery

Recoveries of *trans*-resveratrol from plasma were measured by spiking blank samples at the final concentration of 0.1, 0.25, 0.5, 0.75, 1, 2.5 and 5 μM. The recoveries of *trans*-resveratrol from brain, testes, liver, kidney and lungs were determined by spiking each tissue samples with the final concentration of 5 nmol/g tissue. Absolute recoveries were calculated by comparing the peak area ratio from spiked samples to those of the corresponding concentrations injected directly in HPLC system without extraction.

2.7.3. Sensitivity

The limit of detection (LD) and the limit of quantification (LQ) were calculated by measuring the analytical background response, running six blanks of plasma, brain, testes, liver, kidney and lungs using the maximum sensitivity allowed by the system. The signal-to-noise ratio was used to determine the LD, and it was estimated as the concentration of *trans*-resveratrol in plasma or tissue samples that generated a peak with an area at least three times higher than the baseline noise. LQ was considered to be 10 times superior to the baseline noise analyzed using the maximum sensitivity allowed by the system. The LQ was subsequently validated by the analysis of six samples known to be near the LQ.

2.7.4. Linearity

Spiked plasma samples that contained increasing concentrations of *trans*-resveratrol: 0.1, 0.25, 0.5, 0.75, 1, 2.5 and 5 μM were analyzed according to the procedure described above. For the quantification of *trans*-resveratrol in tissues, calibration curves were constructed spiking liver homogenates with: 0.25, 0.5, 1, 2.5 and 5 nmol/g tissue. Integrated peak areas were plotted against analyte concentration, and linear regression was performed by the least-squares method.

2.8. Method application

trans-Resveratrol was administered intravenously via a tail vein, in overnight fasted rats, as an aqueous solution of 20% hydroxypropyl β-cyclodextrin at the single dose of 15 mg/kg. *trans*-Resveratrol is insoluble in water, so hydroxypropyl β-cyclodextrin, a parentally safe excipient was used to dissolve this polyphenol. Blood was collected from the saphenous vein [7] and placed in Microvette CB300 (Sarstedt, Granollers, Spain) tubes containing EDTA-K_2 as the anticoagulant. Samples were taken at 1, 3, 5, 15, 20, 30, 90, 120, 180, 240, 360, 480 and 720 min. A sparse sampling design including 28 rats, with 4–5 samples per animal and 5–6 values per experimental time was used. Plasma was obtained by centrifugation at 1500 × *g* for 15 min.

For the tissue distribution study of *trans*-resveratrol, a group of six rats was killed at 90 min. Brain, testes, liver, kidney and lungs were rapidly excised, and subsequently perfused with NaCl 0.9% to drain residual blood containing *trans*-resveratrol and their metabolites. Tissues were wiped with filter paper, weighted and immediately frozen in liquid nitrogen, and kept at −80 °C until analysis.

2.9. Hydrolysis of trans-resveratrol glucuronide and sulfate with β-glucuronidase and sulfatase

100 μl aliquots of the processed plasma and tissue samples were incubated with β-glucuronidase (Type L-II, Patella vulgata, Sigma–Aldrich) or sulfatase (Type H-1, Helix pomatia, Sigma–Aldrich). For β-glucuronidase analysis, 5000 U of enzyme was dissolved in 100 μl of 0.2% NaCl at 4 °C and were added to the 100 μl aliquots that were being warmed at 37 °C. Samples were incubated with agitation at 37 °C in the shaking water bath for 30 min. Analysis of *trans*-resveratrol sulfate was performed with 100 U of sulfatase dissolved in 100 μl of 0.2% NaCl at 4 °C and were added to the 100 μl aliquots that were being warmed at 37 °C. Samples were incubated with agitation at 37 °C in the shaking water bath for 180 min. In addition, 100 μl aliquots were incubated with only 100 μl of 0.2% NaCl at the same experimental conditions indicated for the β-glucuronidase or sulfatase assays. These not hydrolyzed samples were taken as controls. After incubation, all samples were centrifuged at 4000 rpm for 15 min at 4 °C and supernatants were analyzed by HPLC as described above.

2.10. Statistical analysis

Data were given as the mean ± S.E.M. *trans*-Resveratrol concentrations were expressed in nmol/g tissue. A commercially available package (Prism version 4.02; GraphPad Software Inc., San Diego, CA) was used for all statistics. Data were evaluated by one-way ANOVA and *post hoc* Tukey's Multiple Comparison tests (Graph Pad Prism). A $P < 0.05$ level was taken as significant.

3. Results

3.1. Sample preparation

For obtaining a better recovery of *trans*-resveratrol from rat plasma, sample acidification was taken into account in order to disrupt polyphenol–protein binding. Therefore, in initial attempts, extractions were performed with plasma spiked with 25 μM of *trans*-resveratrol that was mixed with 20 and 30 μl/ml acetic acid and without acidification. When no acetic acid was added to the plasma, the recoveries were 77.3 ± 1.6% (n = 6). Addition of 20 μl/ml acetic acid improve the recovery to 85.6 ± 2.8% (n = 6) and 30 μl/ml gave a recovery of 96.9 ± 3.4%. Consequently, the addition of 30 μl/ml acetic acid was selected. Using ascorbic acid as an antioxidant was necessary to prevent the degradation of *trans*-resveratrol. Addition of 10 μl of ascorbic acid 15% to the eluted methanol prior to evaporation efficiently protected *trans*-resveratrol and enhanced its recovery to 99.7 ± 2.2% (n = 6). To improve the sensitivity of the analytical method and confirm the detection of low amounts of *trans*-resveratrol, the eluted methanol was concentrated at a final volume of 0.4 ml. A decrease in the recovery was observed when the eluted volumes were concentrated at 250 μl and lower volumes. Recoveries of 85.4 ± 2.4%, 82.1 ± 6.0%, and 75.7 ± 3.0% were observed when the eluted volume was concentrated at 250, 200 and 140 μl, respectively.

The effects of experimental variables were evaluated to develop a rapid, specific, and accurate method for measuring *trans*-resveratrol in different tissues. Consequently, method development was focused on the optimization of sample preparation as well as chromatographic separation. With regard to the complexity of the various biological matrices, different homogenization procedures were attempted. Softer tissues such as brain and testes were adequately grinded with the use of a glass homogenizer. On the other hand, tight tissues were better minced using the Polytron homogenizer. Various extraction solvents, such as acetonitrile, methanol 100%, methanol 80% and methanol 80% acidified with acetic acid 2.5% were tested and the recovery obtained were 40.7 ± 3.3%, 56.7 ± 2.2%, 77.2 ± 4.5% and 88.4 ± 1.0%, respectively. Acidified methanol yielded the best recovery, consequently was chosen to optimize the extractions of *trans*-resveratrol from tissues.

The kidney was processed in first place as the liver, however the recovery was only a 65.4 ± 1.4% (n = 3). Consequently, a different approach to the extraction procedure was attempted. The homogenized kidney was subjected to constant stirring in a hot plate at 60 °C for 30 min and the volume of acidified methanol was doubled. Under these conditions the recovery of *trans*-resveratrol increased to a 100.1 ± 1.8% (n = 6).

The chromatographic conditions were modified from a previous method to detect *trans*-resveratrol in plasma [5]. A change in the column was performed, as well as a different gradient elution, which allowed a sharp peak separation and detection of *trans*-resveratrol conjugates. Three types of RP-HPLC columns, namely Tracer Nucleosil (250 mm × 4 mm; 5 μm), Tracer Excel (250 mm × 4 mm; 5 μm) and Synergi® Fusion-RP 80A (250 mm × 4.6 mm; 4 μm) were tried. Based on the separation efficacy, Synergi® Fusion-RP 80A column was selected to establish this HPLC assay. The presence of acetic acid on the mobile phase markedly retained the sulfate conjugate after the parent compound, and gave superior separation efficiency between resveratrol conjugates.

3.2. Method validation

3.2.1. Selectivity

The specificity of the method was determined by comparing the chromatograms of blank plasma with the corresponding spiked plasma. Typical chromatograms of blank plasma, spiked plasma and a plasma sample taken 15 min after intravenous rat administration are shown in Fig. 2. No interferences from endogenous substances were observed at the retention time of the analyte. A good separation was obtained under the described conditions and *trans*-resveratrol eluted at 11.7 min.

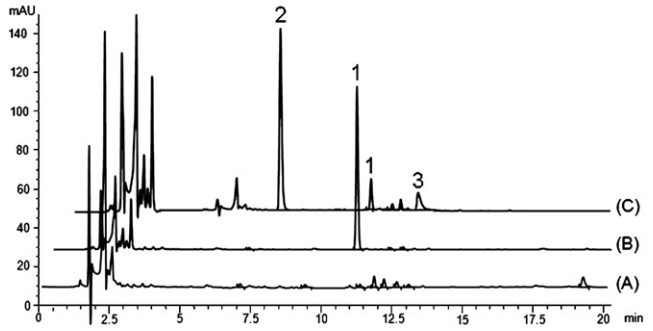

Fig. 2. Representative chromatograms of rat blank plasma (A), blank plasma spiked with 5 μM of *trans*-resveratrol (B) and plasma samples obtained at 15 min after intravenous administration of 15 mg/kg of *trans*-resveratrol (C). Peak 1, *trans*-resveratrol; peak 2, *trans*-resveratrol glucuronide; peak 3, *trans*-resveratrol sulfate.

Chromatograms obtained from blank tissue homogenate of brain, testes, liver, kidney and lungs are shown in Fig. 3A. No significant peak was observed at or near the retention time of *trans*-resveratrol suggesting that the extraction procedure was capable of obtaining highly purified samples which in turn ensured a high selectivity of the HPLC method.

3.2.2. Precision and accuracy

The precision and accuracy data for the analytical procedures are shown in Tables 1 and 2. Intra-day and inter-day precision (% R.S.D.) of the methods were lower than 10% and were within the acceptable limits to meet the guidelines for bioanalytical method validation which is considered to be ≤20%. The accuracy of the both methods was also good with the deviation between the nominal concentration and calculated concentration for *trans*-resveratrol well below the limits of 15%. Precision and accuracy data indicated that the methods to extract *trans*-resveratrol from plasma and tissues are highly reproducible and robust.

3.2.3. Recovery

The extraction recoveries of *trans*-resveratrol were conducted in plasma samples at seven different concentrations (Table 1). The mean recovery in plasma was 97.4 ± 1.3%. Extraction recoveries from different tissues were ranged from 96.5% in the lungs and 103% in the testes, as shown in Table 2. The results pointed out the high extraction efficiency of this procedure.

3.2.4. Sensitivity

Table 3 demonstrates that the sensitivity of the method is adequate to the measurement of *trans*-resveratrol in plasma and different tissues. On the basis of a signal-to-noise ratio of 3, the limit of detection in plasma was 2 nM. The sensitivity could be enhanced by using more volume of plasma or concentrating the processed samples, but the presented method was sensitive enough for the present study. In rat tissue homogenates the average limit of detection was 8.50 pmol/g and the average limit of quantification was 28.4 pmol/g.

3.2.5. Linearity

Linear calibration curves were obtained over the concentration range of 0.01–5 μM for *trans*-resveratrol in rat plasma. A typical calibration plot equation was $y = 100.8x + 1.2$ with a correlation

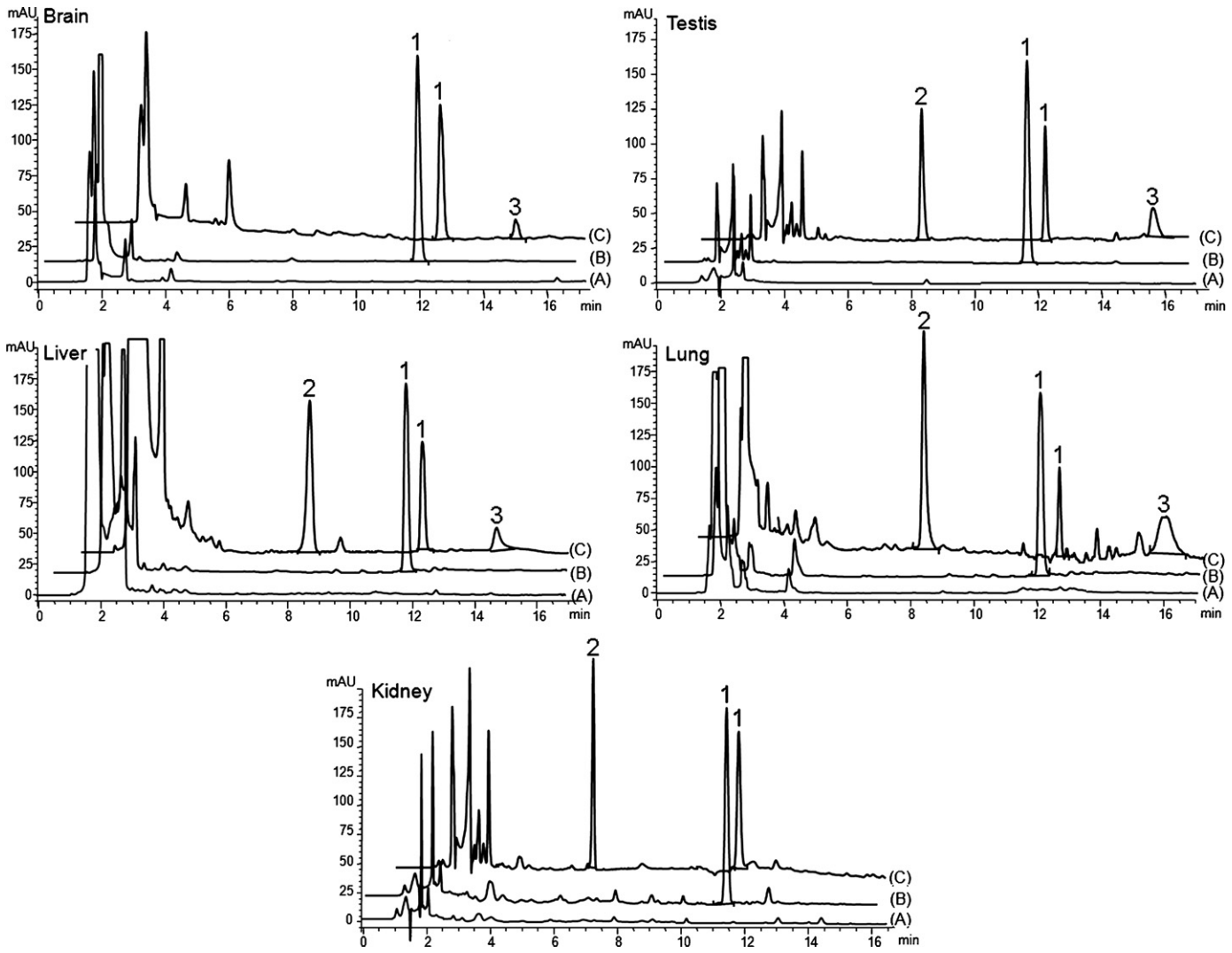

Fig. 3. Representative chromatograms of rat blank tissue (A), blank tissue spiked with 5 nmol/g (B) and tissue samples obtained at 90 min after intravenous administration of 15 mg/kg of *trans*-resveratrol (C) in: brain, testes, liver, lungs and kidney. Peak 1, *trans*-resveratrol; peak 2, *trans*-resveratrol glucuronide; peak 3, *trans*-resveratrol sulfate.

coefficient of 0.9995. For the quantification of *trans*-resveratrol in tissues, calibration curves were constructed in the concentration range of 0.025–5 nmol/g tissue in homogenized liver tissue. A typical calibration plot equation was $y = 102.3x - 4.0$ with a correlation coefficient of 0.999.

3.3. Application of analytical method

3.3.1. Plasmatic levels

After the single intravenous administration of 15 mg/kg of *trans*-resveratrol, plasma samples were collected for the determination of *trans*-resveratrol and its glucuronide and sulfate conjugates. The representative HPLC profile (Fig. 2) was characterized by the peak of *trans*-resveratrol and the presence of two more peaks. The peak with retention time of 7.1 min disappeared after the hydrolysis of the effluent with β-glucuronidase indicating that it corresponded to the glucuronide conjugate. The second peak (retention time 13.9 min) was identified as the sulfate conjugate by means of the hydrolysis with sulfatase. The identification of the peaks was confirmed by MS. *trans*-Resveratrol glucuronide which eluted at retention time of 7.1 min was characterized by the deprotonated molecular ion $[M-H]^-$ at *m*/*z* 403 (Fig. 4A) whereas the resveratrol fragment was observed at *m*/*z* 227. The sulfate conjugate eluted

Table 1
Precision, accuracy and recovery of *trans*-resveratrol in spiked rat plasma samples.

Resveratrol (μM)	Accuracy (% bias)	Precision (% R.S.D.)		Recovery (%)
		Intra-day	Inter-day	
0.1 ($n = 5$)	−0.81	2.01	3.50	100.8 ± 1.2
0.25 ($n = 4$)	1.20	6.98	8.16	98.8 ± 4.7
0.50 ($n = 6$)	−4.52	5.99	8.45	99.2 ± 3.4
0.75 ($n = 6$)	6.02	4.02	3.89	94.0 ± 2.1
1 ($n = 6$)	2.75	3.18	5.38	101.3 ± 3.2
2.5 ($n = 6$)	6.47	2.76	3.70	93.5 ± 2.0
5 ($n = 6$)	6.51	0.75	1.16	93.8 ± 0.5

Table 2
Precision, accuracy and recovery of *trans*-resveratrol in spiked rat tissues with 5 nmol/g.

Tissue	Accuracy (% bias)	Precision (% R.S.D.)		Recovery (%)
		Intra-day	Inter-day	
Testes ($n = 6$)	−2.40	2.59	3.68	103.0 ± 1.7
Brain ($n = 6$)	0.63	6.52	9.93	99.0 ± 0.7
Liver ($n = 6$)	0.80	7.04	8.19	98.5 ± 3.2
Kidney ($n = 6$)	0.40	3.89	4.49	100.1 ± 1.8
Lung ($n = 6$)	3.40	7.94	9.18	96.5 ± 7.6

Table 3
Sensitivity of the extraction method expressed as limit of detection and limit of quantification.

	Limit of detection	Limit of quantification
Plasma (nM)	1.73	5.77
Testes (pmol/g)	5.45	18.19
Brain (pmol/g)	6.54	21.80
Liver (pmol/g)	10.77	35.90
Kidney (pmol/g)	11.21	37.39
Lung (pmol/g)	8.61	28.70

with a retention time of 13.9 min and showed the deprotonated molecular ion $[M-H]^-$ at m/z 307 (Fig. 4B) and also the resveratrol fragment at m/z 227.

The mean plasma concentration versus time profiles for *trans*-resveratrol and its conjugates ($n = 4$–6 for each time point) is presented in Fig. 5. The profile revealed that the elimination of *trans*-resveratrol and its glucuronide and sulfate conjugates were very rapid, but the parent compound was present in plasma for at least 12 h after the intravenous administration. Samples obtained 1 min after the intravenous administration showed that *trans*-resveratrol was the main compound (78.1 ± 10.3 μM). The formation of glucuronide and sulfate conjugates proved to be extremely rapid since they were detected in plasma 1 min after the intravenous administration, with concentrations of 44.1 ± 7.9 and 10.9 ± 1.6 μM, respectively. At 3 min, *trans*-resveratrol glucuronide concentration (40.3 ± 9.9 μM) was higher than the parent compound (30.5 ± 0.1 μM) and *trans*-resveratrol sulfate (13.6 ± 2.4 μM). Unmodified *trans*-resveratrol was quickly cleared and at 15 min its concentration dropped to 8.8 ± 0.5 μM. However, *trans*-resveratrol glucuronide levels were 33.2 ± 6.5 which is 3.8-fold higher than the parent compound. The values of the sulfate conjugate were 7.2 ± 0.5 μM at 15 min and it was quickly cleared from the blood stream since it was not detected in plasma at 90 min. At this time point the concentration of *trans*-resveratrol was 0.18 ± 0.01 μM. At 6 h the level of *trans*-resveratrol dropped to 0.051 ± 0.001 μM, and was still present in plasma at 12 h at 0.025 ± 0.008 μM.

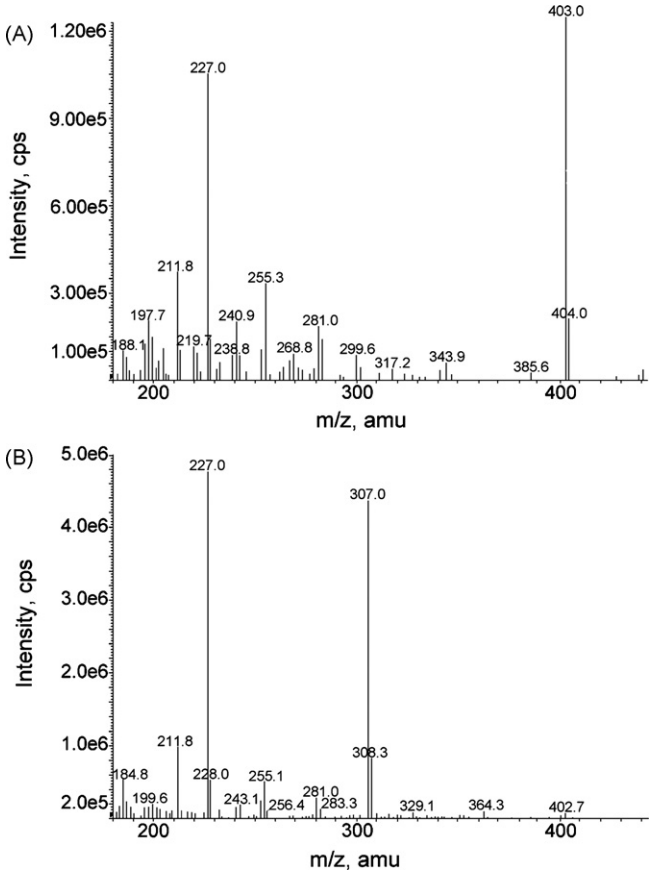

Fig. 4. Full-scan product ion mass spectra of *trans*-resveratrol glucuronide (A) and *trans*-resveratrol sulfate (B).

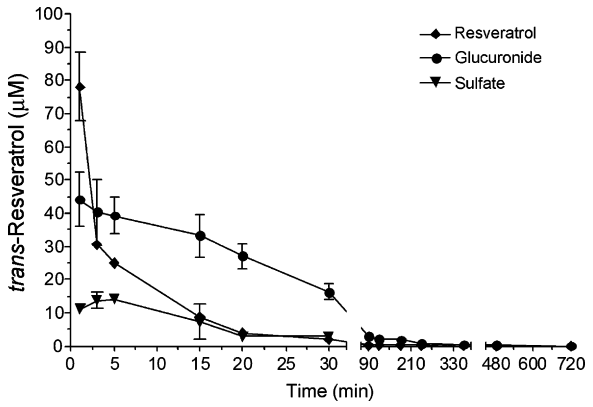

Fig. 5. Plasmatic concentrations after the intravenous administration of 15 mg/kg of *trans*-resveratrol. Values are represented as means ± S.E.M., $n = 4$–6.

3.3.2. Tissue distribution

The developed method was successfully applied to the determination of *trans*-resveratrol and its metabolites in rat tissues. Tissue distribution of *trans*-resveratrol at 90 min is shown in Fig. 6. At 90 min, the higher amount of *trans*-resveratrol was detected in the kidney (1.45 ± 0.35 nmol/g), and lung (1.13 ± 0.34 nmol/g), whereas the tissue extracts from brain (0.17 ± 0.04 nmol/g) and testes (0.05 ± 0.01 nmol/g) contained only moderate amounts of *trans*-resveratrol.

After the intravenous administration of *trans*-resveratrol, this polyphenol was extensively metabolized. Part of *trans*-resveratrol was conjugated with glucuronic acid by UDP-glucuronosyltransferase or with sulfate by sulfotransferase. The higher levels of resveratrol glucuronide were found in the kidney (2.91 ± 0.19 nmol/g), while lower values were observed in testes (0.70 ± 0.03 nmol/g), liver (0.58 ± 0.09 nmol/g) and lungs (0.28 ± 0.02 nmol/g). No resveratrol glucuronide was found in the brain.

The sulfate conjugate was also widely distributed in the organs studied, but in lower levels than resveratrol or the glucuronide.

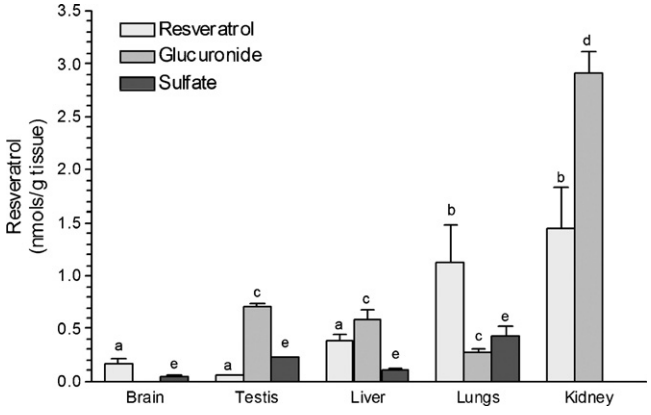

Fig. 6. Concentrations of *trans*-resveratrol and its metabolites in rat tissues at 90 min after intravenous administration of 15 mg/kg of *trans*-resveratrol. Values are means + S.E.M., $n = 4$–6. Within the same compound, means without a common letter differ. Brain: R = S; testis: G > R = S; liver: R = G > S; lung: R > G = S; kidney: G > R. $P < 0.05$.

The higher value was detected in the lungs (0.42 ± 0.10 nmol/g), followed by testis (0.23 ± 0.01 nmol/g), liver (0.11 ± 0.02 nmol/g) and brain (0.04 ± 0.10 nmol/g). *trans*-Resveratrol sulfate was not detected in the kidney, probably due to its rapid urinary excretion.

4. Discussion

Despite the number of studies published on the bioavailability of *trans*-resveratrol [1], our knowledge regarding tissue distribution of this compound is limited, mainly due to the lack of validated methods. Consequently, the aim of the study was to develop an extraction process that allowed the determination and quantification of *trans*-resveratrol and its metabolites in tissues by HPLC with diode array detection.

The extraction of *trans*-resveratrol from tissues was attempted through liquid extraction which is a basic sample preparation method. Bearing in mind that not all the tissues offer the same toughness the homogenization step was optimized in accordance with each organ. Soft tissues such as brain and testes were subjected to manual grinding with a glass homogenizer, whereas tight tissues needed the use of a Polytron homogenizer and longer times. The subsequent extraction step with methanol 80% acidified with acetic acid 2.5% allowed the separation of the analyte from each sample matrix providing an optimum yield and selectivity, so that as few potential interfering species as possible were carried to the chromatographic separation stage.

The chromatographic conditions were modified from a previously validated method [5] changing the Nucleosil C18 column for a Synergi® Fusion-RP 80A. The reason was the co-elution of *trans*-resveratrol glucuronide and sulfate in samples where both metabolites were present. Moreover, the gradient profile was adapted to obtain a sharp peak separation for all the compounds in the different matrixes. By means of the new chromatographic settings *trans*-resveratrol glucuronide and sulfate conjugates from the distribution study were adequately separated and eluted at 7.1 and 14.0 min, respectively. Moreover, *trans*-resveratrol eluted at 11.7 min. It is worth mentioning that the peak of *trans*-resveratrol sulfate was asymmetric in plasma and tissue samples. This peak tailing has been already described for the sulfate conjugate [8,9].

The average extraction recoveries were of 97.3% in the seven concentrations assayed in plasma, and of 99.4% in tissues. The intra-day and inter-day coefficient of variation was within the 15% acceptable limits. The methods were also accurate with a bias of less than 10% at all levels tested. Consequently, the validation of the methods showed good reproducibility, accuracy, precision and recovery in the assays of *trans*-resveratrol in rat plasma and tissues.

Once the methods were established and validated they were applied to the detection of *trans*-resveratrol and its metabolites in plasma and tissues after the intravenous administration of 15 mg/kg. One minute after the intravenous administration *trans*-resveratrol glucuronide and sulfate were observed in rat plasma in a 33% and 8%, respectively indicating the extremely rapid metabolism of this polyphenol. *trans*-Resveratrol represented the 59% of the total circulating resveratrol. However, 2 min later the glucuronide conjugate was the most abundant compound in plasma accounting for a 48% of the total, whereas *trans*-resveratrol represented a 36% and the sulfate conjugate a 16%. Noteworthy, at 20 min *trans*-resveratrol glucuronide percentage increased to 79% while, *trans*-resveratrol represented only a 12%, and the sulfate conjugate a 9%. Therefore, the present results showed that *trans*-resveratrol glucuronide was the predominant conjugate in plasma with a concentration, in average, 4.9-fold higher than that of sulfate from 1 to 30 min, suggesting that glucuronidation was the major metabolic pathway in rats. Moreover, *trans*-resveratrol sulfate was quickly cleared from plasma since it was not detected at 90 min. Our findings are also consistent with previous studies of the plasmatic levels of *trans*-resveratrol after intravenous administration [10,11]. Though, those results were not complete, since either they were restricted to *trans*-resveratrol and its *cis* isomer [11] or only reported the presence of *trans*-resveratrol and its glucuronide conjugate [11]. Our validated method in plasma allowed the detection of *trans*-resveratrol sulfate simultaneously to the glucuronide. The sulfate conjugate displayed a poor chromatographic behavior which is most likely the reason why few observations exists for this compound. In our experimental conditions only these two metabolites were found. Dihydroresveratrol that we have detected in rat intestinal content was not present in plasma (unpublished data).

The present study also shows that *trans*-resveratrol and its glucuronide and sulfate conjugates are widely distributed in the organs, after 90 min of its intravenous administration. *trans*-Resveratrol was mainly distributed in abundant blood-supplied tissues, such as liver, lung and kidney. *trans*-Resveratrol assessment in clearance organs indicate that kidney was the one with the higher concentration of this polyphenol and its glucuronide conjugate. *trans*-Resveratrol sulfate was beyond the limit of quantification which means that it is quickly eliminated once it reached the kidney. Consequently, renal excretion might be one of the major pathways of elimination of *trans*-resveratrol. Conversely, the concentrations of resveratrol and its metabolites were lower in brain and testes. This could be attributed to the presence of different members of the family of ATP-binding cassette (ABC) transporters which excrete xenobiotics from different organs [12,13]. Although recent *in vitro* studies indicated that *trans*-resveratrol can be glucuronidated in the brain [14] it may be efficiently excreted through MRP2 and BCRP transporters. Like the brain, the testes also have a specific barrier, however, only BCRP is included in the interstitial cells, as well as in the Sertoli and Leydig cells [12]. The different locations and presence of efflux transporters, may explain the lower levels of conjugates in the brain and testes compared to other organs.

Despite the numerous studies that have measured the plasmatic levels of *trans*-resveratrol, very few have attempted the tissue distribution of this compound. In support of our work, the tissue distribution after the oral administration of 20 mg/kg of *trans*-resveratrol to rats was reported [15]. *trans*-Resveratrol was the only compound detected in lung, liver, kidney and brain and always below 1 nmol/g tissue. No metabolites were reported neither in tissues nor in plasma and only resveratrol glucuronide was found in urine. In agreement with our analysis, micro-autoradiographic evidence of the distribution of resveratrol-derived radioactivity in mice liver and kidney was previously demonstrated [16]. However, no data on the chemical nature of this radioactivity was provided. The work performed by Sale et al. [17], gave some indications on the distribution of resveratrol and its metabolites in mice administered intragastrically with 240 mg/kg. The distribution of *trans*-resveratrol in different organs in mice supports our results since it was higher in liver, lung and kidney. The authors identified a resveratrol monoglucuronide and *trans*-resveratrol-3-sulfate in liver, lung and kidney samples but not in plasma.

The metabolism of *trans*-resveratrol has been also evaluated after long term feeding of 50 and 300 mg/kg [18]. After 8 weeks of treatment, neither resveratrol nor resveratrol conjugates were detected in plasma, liver and kidney samples of rats receiving the resveratrol 50 mg/kg diet. In the second treatment with 300 mg/kg, free *trans*-resveratrol was neither found in plasma nor in kidney and was only present in liver. Several *trans*-resveratrol conjugates were observed, but only in the 50% of animals administered with 300 mg/kg. However, considering the low recovery in liver and kidney of 32% and 34%, respectively, their results in these organs should be evaluated carefully. Abd El Mohsen et al. [19] described the tis-

sue distribution of [^{3}H]*trans*-resveratrol in rat tissues following oral administration of 50 mg/kg. The authors measured plasmatic levels and tissue distribution at 2 and 18 h, though they only detected *trans*-resveratrol and its glucuronide conjugate, without reporting the presence of the sulfate conjugate, neither in plasma nor in tissues.

5. Conclusion

In conclusion, sensitive liquid–liquid extraction methods followed by HPLC-DAD detection were developed and validated for the quantitative determination of *trans*-resveratrol and its metabolites in rat plasma and tissues. The developed HPLC-DAD methods offered adequate selectivity, linearity, accuracy, precision and recovery. The method has been successfully applied for the study of the plasmatic levels of *trans*-resveratrol and its distribution in organs after the intravenous administration of 15 mg/kg.

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References

[1] J.A. Baur, D.A. Sinclair, Nat. Rev. Drug Discov. 5 (2006) 493–506.
[2] M.E. Juan, M.P. Vinardell, J.M. Planas, J. Nutr. 132 (2002) 257–260.
[3] E. Gonzalez-Pons, M.E. Juan, J. Bolufer, J.M. Planas, J. Physiol. Biochem. 60 (2004) 126.
[4] E. Wenzel, V. Somoza, Mol. Nutr. Food Res. 49 (2005) 472–481.
[5] M.E. Juan, R.M. Lamuela-Raventós, M.C. de la Torre-Boronat, J.M. Planas, Anal. Chem. 71 (1999) 747–750.
[6] The United States Pharmacopoeia, USP31-NF26, 1225, 2008, 752–756.
[7] A. Hem, A.J. Smith, P. Solberg, Lab. Anim. 32 (1998) 364–368.
[8] M.I. Kaldas, U.K. Walle, T. Walle, J. Pharm. Pharmacol. 55 (2003) 307–312.
[9] T. Walle, F. Hsieh, M.H. DeLegge, J.E. Oatis Jr., U.K. Walle, Drug Metab. Dispos. 32 (2004) 1377–1382.
[10] J.F. Marier, P. Vachon, A. Gritsas, J. Zhang, J.P. Moreau, M.P. Ducharme, J. Pharmacol. Exp. Ther. 302 (2002) 369–373.
[11] X. Chen, H. He, G. Wang, B. Yang, W. Ren, L. Ma, Q. Yu, Biomed. Chromatogr. 21 (2007) 257–265.
[12] J. Bart, H. Hollema, H.J. Groen, E.G. de Vries, N.H. Hendrikse, D.T. Sleijfer, T.D. Wegman, W. Vaalburg, W.T. van der Graaf, Eur. J. Cancer 40 (2004) 2064–2070.
[13] S. Dauchy, F. Dutheil, R.J. Weaver, F. Chassoux, C. Daumas-Duport, P.O. Couraud, J.M. Scherrmann, I. De Waziers, X. Declèves, J. Neurochem. 107 (2008) 1518–1528.
[14] N. Sabolovic, T. Heurtaux, A.C. Humbert, S. Krisa, J. Magdalou, Pharmacology 80 (2007) 185–192.
[15] M. Asensi, I. Medina, A. Ortega, J. Carretero, M.C. Baño, E. Obrador, J.M. Estrela, Free Radic. Biol. Med. 33 (2002) 387–398.
[16] X. Vitrac, A. Desmoulière, B. Brouillaud, S. Krisa, G. Deffieux, N. Barthe, J. Rosenbaum, J.M. Mérillon, Life Sci. 72 (2003) 2219–2233.
[17] S. Sale, R.D. Verschoyle, D. Boocock, D.J. Jones, N. Wilsher, K.C. Ruparelia, G.A. Potter, P.B. Farmer, W.P. Steward, A.J. Gescher, Br. J. Cancer 90 (2004) 736–744.
[18] E. Wenzel, T. Soldo, H. Erbersdobler, V. Somoza, Mol. Nutr. Food Res. 49 (2005) 482–494.
[19] M. Abd El-Mohsen, H. Bayele, G. Kuhnle, G. Gibson, E. Debnam, S. Kaila Srai, C. Rice-Evans, J.P. Spencer, Br. J. Nutr. 96 (2006) 62–70.