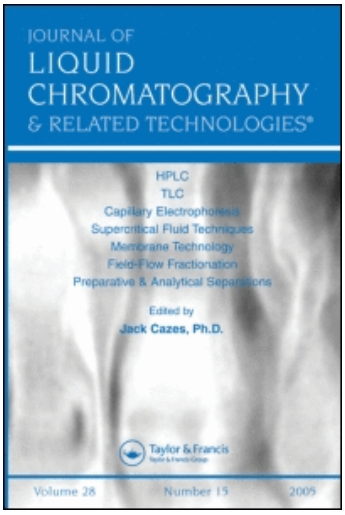

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HPLC Method with UV Detection for the Determination of *trans*-Resveratrol in Plasma

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Abstract: The main representative of phytoalexins (biologically active substances produced by the plant as an immediate immunological response to infections or abiotic stresses) in wine grapes (*Vitis vinifera*) is the stilbene *trans*-resveratrol. The proposed method for the determination of *trans*-resveratrol in blood plasma is based on reversed phase HPLC utilizing UV detection (310 nm), under isocratic conditions (1.0 mL/min), with mobile phase consisting of acetonitrile-phosphate buffer pH 4.8 (30 mM) 25:75 v/v, a C_{18} Novapack 150 × 4.0 mm column (4 μm particle size), and carbamazepine as internal standard (5 μg/mL). Plasma samples (1 mL) are buffered with phosphate buffer (0.4 mL) pH 6.0 and extracted with 3 × 3 mL of ethyl acetate. *trans*-resveratrol is eluted at around 4.6 min, whereas carbamazepine at approximately 9.6 min, yielding a resolution of 4.4. The method appears to be linear within a range of 0.15–4.0 μg/mL ($r = 0.9998$), with good repeatability (%RSD = 0.86) and reproducibility (%RSD = 2.2). Detection and quantification limits were found equal to 0.10 and 0.33 μg/mL, respectively. Accuracy, expressed as recovery determined at two concentrations of 0.3 and 3.0 μg/mL (6-replicates) were found to be 88.3 ± 7.5% and 100.7 ± 0.7%, respectively. The reported method is simple, rapid, accurate, and intended for use to further bioavailability studies, aiming to the development of pharmaceutical dosage formulations of resveratrol for oral administration.

Keywords: *Trans*-resveratrol, HPLC, Plasma, Carbamazepine

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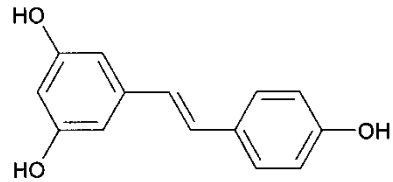
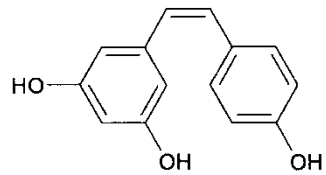
INTRODUCTION

Resveratrol (3,5,4′-trihydroxystilbene, Fig. 1) is a compound found both free and as glycosides in two isomeric forms in a number of plant species and can be present in variable amounts in wines.[1,2] *Trans*-resveratrol is a stilbenic phytoalexin, mainly present in the *Vitis vinifera* (European) vine, where it is distributed on the leaves, bark, seed, and finally in the core of the grape.[3] The highest amount of *trans*-resveratrol is located on the leaves and the bark. Concerning the quantity of *trans*-resveratrol on the seed, the substance is present mainly in the red varieties, while in white varieties total quantity in the grape is usually low. Most of the investigations are focused on the *trans*-isomer, since the physiological activity of the *cis*-form has not yet been elucidated. The *trans*-isomer is transformed to the *cis*-form under UV irradiation.

Phytoalexins are the major type of humoral immuno-response raised by the plant as defense to both biotic and abiotic attacks (stresses).[3] Profound proof for the microbial inhibitive role of *trans*-resveratrol is provided by the fact that the quantities on the bark and leaf are tremendously increasing following an intimate infection by mould or bacteria. Abiotic factors that might increase the amount of *trans*-resveratrol are UV-irradiation, presence of heavy metals on the leaves or root canals, as well as partial damage on the surface of the bark.[4] Along with the increase of *trans*-resveratrol due to a stress, other stilbenic phytoalexins (catechin, epicatechin, rutin, quercetin) increase, enforcing the total humoral response.



Trans-resveratrol



Cis-resveratrol

Figure 1. Structures of *cis*- and *trans*-resveratrol.

Reduced mortality from coronary heart disease (CHD) among moderate consumers of wine is a well-established epidemiologic phenomenon.[5] Moreover, there is significant evidence that people, who regularly drink wine may have lower mortality from CHD than those who turn their preference to other types of alcoholic beverages. This latter event, along with the French paradox, have provoked intense interest to investigate the constituents present in wine (especially the red varieties) that might be responsible for these putative effects.[5–7] Many of the substances that show great anti-oxidant action (including catechin, epicatechin, quercetin, and *trans*-resveratrol) have been proven to protect the human organism from low-density lipoproteins (LDLs) leading to lower mortality from CHD.[8] Biological effects of resveratrol,[9] its pharmacological activity,[10] mechanisms of cardiovascular protection,[5,6,8] as well as its role as a chemopreventive agent,[11–13] have been extensively reviewed in the current literature.

A number of investigations concerning the concentration of *trans*-resveratrol in commercial wines have been conducted.[14–25] The quantitative determination of *trans*-resveratrol has been achieved with HPLC based on fluorimetric,[19,22] electrochemical,[20,21] photodiode array[22] and UV detection,[21] as well as with LC-MS[23,24] and capillary zone electrophoresis.[25] Only a few methods for the determination of *trans*-resveratrol in biological fluids, and especially in plasma, have been published in the literature, based on gas chromatography,[26] gas chromatography with mass selective detection,[27] or HPLC with UV detection.[28,29] The bioavailability of resveratrol in humans, mice, and rats, after oral ingestion of grape juice preparations or pure aglycons,[30] has been recently studied. It was demonstrated that the glycoside forms of resveratrol are absorbed in a lesser extent than the aglycons.[30] An increased interest by many pharmaceutical companies has been recently shown for the development of formulations of *trans*-resveratrol. For bioavailability evaluation, sample handling control conditions have been recently studied to improve stability of wine phenolic compounds in human plasma before analysis, including resveratrol.[31] To carry out bioavailability studies of a formulation under development, a fast, simple, and reliable analytical method for the determination of *trans*-resveratrol in plasma is required.

The aim of this study was to develop and validate an isocratic HPLC method with UV detection for the quantitative determination of *trans*-resveratrol in human blood plasma.

EXPERIMENTAL

Apparatus

Chromatographic analysis was performed with a Waters 600E HPLC modular system, consisting of a control unit (Controller), a pump fitted with a Ternary

Gradient Unit, which was used under isocratic conditions (on manual mode), a Waters model U6K manual injector, and a 20 μL sample loop. A Waters model 486 variable wavelength UV/Vis detector was used, set at 310 nm. Samples were chromatographed on a Waters® Nova Pack C_{18} (150 × 3.9 mm), 4 μm particle size chromatography column. The column was stored in methanol when not in use, at room temperature. The manipulation of chromatograms, as well as the control of the chromatography system was achieved using the Waters Millenium 2010 software.

A Millipore filtration system (Millipore, Bedford, M.A. USA) with type HV Millipore filters (diameter 47 mm, pore size 0.45 μm) was used for degassing mobile phase under vacuum, while helium was purged through mobile phase during the analysis.

Extraction organic phases (ethyl acetate) were evaporated on a Bioblock Scientific isotherm dry bath under nitrogen at 40°C.

A vortex (Genie, model K-550 GE, Scientific Ind. Springfield Mass. 01103) set at speed 4 was used for mixing of plasma samples and standards. The analog pH-meter used was a Metrohm product with a glass combination electrode.

Reagents and Standards

All organic solvents (methanol, acetonitrile, ethyl acetate) were HPLC grade, (Labscan®) and water prepared by Milli-Q Plus® (Millipore Watford, Herts., UK) was used throughout the study.

Trans-resveratrol (*trans*-3,5,4′-trihydroxystilbene) pure substance (purity 99.5%) was purchased from Sigma (St. Louis, MO, USA, code Nr.: R5010) and stored under deep freeze at −20°C protected from direct sunlight and humidity. Under these storage conditions, the pure substance was stable up to two years.

Carbamazepine (5H-dibenz[b,f]azepin-5-carboxamide) was purchased from SIGMA (code Nr.: C4024) and was found very stable when stored in the refrigerator in dark glass containers.

Phosphate buffers of pH 4.8–4.9 (30 mM) for mobile phase and of pH 6.0 (56.8 mM) for extraction, were prepared by dissolving appropriate quantities of $NaH_2PO_4 \cdot 2H_2O$ (Fluka®-analytical grade) in water and adjusting the pH with the addition of NaOH 10 M using the pH meter.

Pooled drug free plasma (blank plasma) was obtained from healthy volunteers, who had not received any medication prior to blood donation, and was used for the preparation of spiked plasma standards.

A stock standard solution of *trans*-resveratrol (1000 mg/L) was prepared in methanol and when it was stored refrigerated and protected from light in an aluminum-covered flask was stable for two weeks. A stock standard solution of carbamazepine (internal standard) was prepared in methanol (1000 mg/L)

and when it was stored refrigerated and protected from light in a dark glass container was stable for up to 40 days.

A series of seven working solutions of various concentrations of *trans*-resveratrol (1.5–40 μg/mL) were prepared daily by appropriate dilutions of stock solutions with water, and stored protected from direct light exposure (*trans*-resveratrol decomposes when exposed to direct sunlight). A standard working solution of carbamazepine (50 μg/mL) was also prepared daily.

Plasma standards for calibration curves were prepared by spiking 1.0 mL aliquots of pooled drug free plasma with 100 μL of the above mentioned aqueous working solutions, to make plasma standards corresponding to the resveratrol concentration range of 0.15 to 4.0 μg/mL.

Calibration graphs based on the peak-area ratio of analyte/internal standard against analyte concentration were prepared for each day of analysis to check linearity and to be used for the calculation of the analyte concentration in the samples.

Procedures

Chromatographic Conditions

The optimized mobile phase consisted of acetonitrile/phosphate buffer pH 4.8–4.9 at a ratio of 25:75 v/v. The mixture, prior to use, was filtered and degassed using helium (degassing cycle 10 min). A flow rate of 1.0 mL/min under isocratic mode was used at ambient temperature, resulting in a pressure of about 160 kg/cm^2.

Extraction Procedure

In 10 mL glass conical tubes with glass stoppers, 1.0 mL of plasma samples or the prepared spiked plasma standards and 100 μL of internal standard aqueous solution 50 μg/mL (5 μg) were added and mixed for 15 s on the vortex. Consequently, 0.4 mL of phosphate buffer pH 6.0 was added to the tube and the whole was mixed for another 15 s on the vortex. Each sample was extracted with 3.0 mL of ethyl acetate with vortexing for 15 s. The sample tube was centrifuged for 5 min at 7000 rpm. After careful removal and transfer of the upper organic layer into a 10-mL conical glass tube, the extraction procedure was repeated two more times with 3.0 mL of ethyl acetate. The combined organic layers were evaporated to dryness at 40°C in the dry bath under a gentle stream of nitrogen. The residue was reconstituted in 500 μL of mobile phase (preconcentration × 2) and an aliquot of about 70 μL was injected onto the HPLC system in order to retrieve results.

RESULTS AND DISCUSSION

Choice of Internal Standard and Detection Wavelength

Since the method involves liquid–liquid extraction, the use of an internal standard is required in order to overcome relevant losses. From the various substances tested, carbamazepine (Fig. 2) was found as optimum due to its stability, adequate absorptivity at detection wavelength, and similar extraction and chromatographic characteristics.

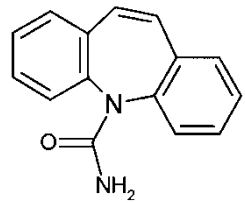
From chromatograms obtained at 306, 310, and 315 nm, the optimum absorptivities were found at 310 nm, being 0.020 and 0.0013 $\mu g^{-1} cm^{-1}$ mL, for *trans*-resveratrol and carbamazepine, respectively.

Choice of the Stationary Phase

Trans-resveratrol is a molecule of medium polarity (three phenolic–OH groups on two benzene rings in combination with its total molecular volume). Thus, solubility of *trans*-resveratrol in polar solvents is low, and increases considerably in medium polarity solvents (methanol, ethanol, ethyl acetate, etc). Therefore, the suitable separation mechanism is reversed phase partition between a polar mobile phase and a non polar stationary phase. A dimethyl-octadecylsilyl bonded silica (C_{18}) Nova Pack (3.9 × 150 mm cartridge, particle size 4 μm) was found to be successful.

Optimization of the Chromatographic Conditions

For the optimization of the chromatographic conditions the so called chromatographic response function (CRF) was used.[32] Various combinations of organic solvents with water were tested but very assymetrical and broadened peaks were obtained. The use of phosphate buffers as the aqueous component improved the peak profile considerably. The best chromatographic response



Carbamazepine

Figure 2. Structure of carbamazepine (internal standard).

(combination of retention times, resolution, and peak symmetry) was achieved using a mobile phase of acetonitrile/aqueous phosphate buffer 30 mM of pH 4.8–4.9 and a ratio of 25:75 v/v, which provides retention times (min) of 4.6 for resveratrol and 9.7 for carbamazepine (IS) and a resolution of at least 4.3. Endogenous plasma compounds extractable with ethyl acetate were eluted before 1.3 min. Figure 3 shows a typical chromatogram of a solution containing *trans*-resveratrol (0.6 μg/mL) and carbamazepine (10 μg/mL) in mobile phase.

Optimization of Liquid Extraction and Evaporation

Ethyl acetate was found the most suitable for this application since: a) solubility of both analyte and IS is very high in this solvent; b) the solvent itself has a low boiling point (volatile); c) it yields a biphasic system with plasma from which both analyte and IS can be extracted quantitatively.

Concerning the pH of extraction, trials showed that at pH below 5, there was significant aggregation of plasma proteins, which led to incomplete extraction of the analyte. At basic pH (9.5 with phosphate buffer) the extraction yield was 90%, whereas at slightly acidic environment (pH 6.0–6.5) the absolute extraction recovery was almost 100%. Thus, it was found optimum to extract plasma samples at pH 6.0, after adjustment with phosphate buffer. For the calculation of the absolute extraction recovery, the peak areas from extracted samples were compared to those obtained

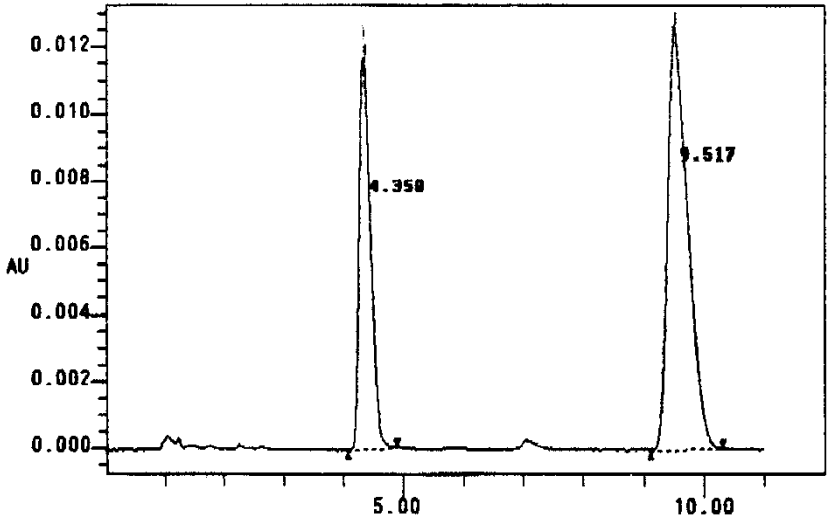


Figure 3. Typical chromatogram of a standard solution containing 0.6 μg/mL resveratrol and 10 μg/mL carbamazepine in mobile phase at 310 nm.

from the direct injection of the corresponding working standards in mobile phase, taking into account a 2 × preconcentration.

The two main factors which were taken into consideration for stability assessment of the analyte and internal standard are exposure to sunlight and temperature. The stability of the analyte and the internal standard during sample storage and treatment was evaluated by exposure to sunlight and temperature. Figure 4 shows a chromatogram of a solution containing 1.8 μg/mL *trans*-resveratrol and 10 μg/mL carbamazepine in mobile phase after exposure to direct sunlight for 15 min, under nitrogen atmosphere. The small peak at 7.0 min represents a degradation product of *trans*-resveratrol, while carbamazepine remains stable (as concluded from the comparison of peak areas). Systematic study of this effect revealed that the optimum conditions for evaporation of the extract is 40°C in a dry bath under nitrogen atmosphere and protection from sunlight.

Figure 5 shows a typical chromatogram obtained from an extracted drug free plasma spiked with *trans*-resveratrol (0.15 μg/mL) and carbamazepine (5 μg/mL). Resolution of peaks in all cases was excellent and no interfering peaks of sample matrix were observed during the analysis of a great number of plasma samples.

Linearity–Detectability

The peak area ratios of resveratrol/IS were linearly related to plasma concentrations of resveratrol, from at least 0.15–4 μg/mL. Regression equations and

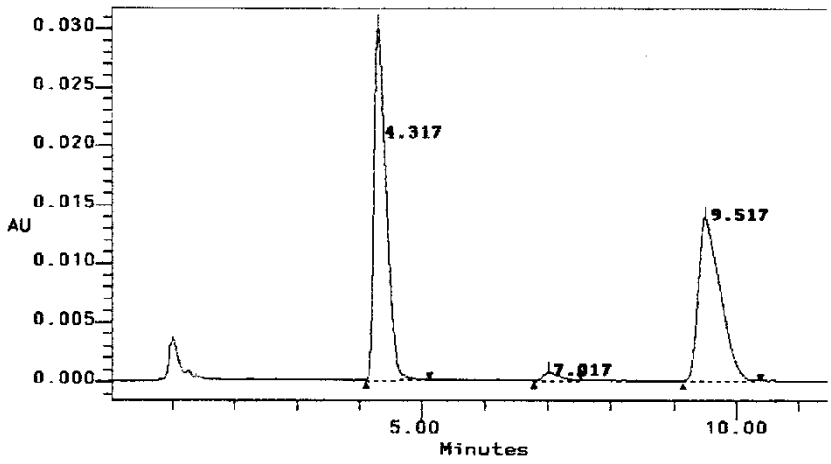


Figure 4. Chromatogram of a standard solution containing 1.8 μg/mL resveratrol and 10 μg/mL carbamazepine in mobile phase at 310 nm after exposure to direct sunlight for 15 min.

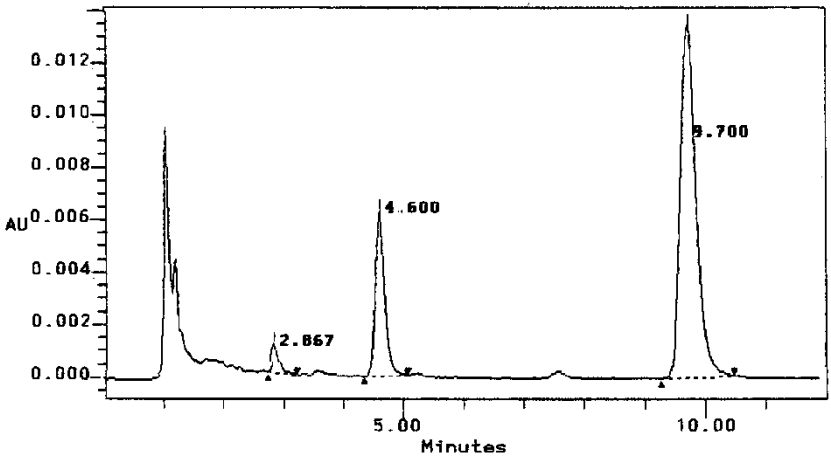


Figure 5. Chromatogram of an extracted spiked plasma standard (resveratrol 0.15 μg/mL, carbamazepine 5 μg/mL). Retention times: 1.3 min, plasma constituents; 2.9 min, residue of extraction solvent; 4.6 min, resveratrol; 9.7 min carbamazepine. Resolution: resveratrol–carbamazepine = 4.9, extraction solvent resveratrol = 3.1.

correlation coefficients of calibration curves for standard solutions containing resveratrol 0.3–8 μg/mL and carbamazepine 10 μg/mL, prepared in mobile phase, as well as for spiked plasma samples (concentration range for resveratrol 0.15–4 μg/mL and carbamazepine 5 μg/mL) are shown in Table 1. The correlation coefficient for each individual standard curve was greater than 0.998 and all intercepts were not statistically different from zero $\{t_{exp} = 2.4 < t_{theor} = 2.6$ (n = 7), for calibration curve of standard solutions in mobile phase and $t_{exp} = 0.15 < t_{theor} = 2.6$ (n = 7) for calibration curve of spiked plasma samples$\}$.

The detection and the quantification limits for the determination of resveratrol in plasma were estimated from the regression equation of the corresponding calibration curve based on the standard deviation of intercepts (3.3 and 10 times the SD, respectively), and were 0.10 and 0.33 μg/mL, respectively (2 × preconcentration).

Extraction Recovery

Relative extraction recovery data are presented in Table 1. For the calculation of the relative recovery of the extraction procedure the ratio of the slopes of plasma calibration curves, and of standard solutions including IS, prepared in mobile phase, was used (taking into account the 2 × preconcentration). Relative recovery % was found to be 92.9 and 101.5% for extraction at pH 9.5 and 6.0, respectively, and therefore pH 6.0 was finally chosen for the extraction procedure of plasma samples.

Table 1. Calibration curves of resveratrol in plasma and in standard solutions prepared in mobile phase with the presence of internal standard and estimation of the relative recovery% through the values of the corresponding slopes (2 × preconcentration)

Calibration curve	Regression equation
Standard solutions in mobile phase (range 0.3–8 μg/mL)	$y = (0.675 \pm 0.012)X + (0.107 \pm 0.045)$ ($r^2 = 0.9987$)
Spiked plasma standards Extraction pH = 9.5 (range 0.15–4 μg/mL)	$y = (1.254 \pm 0.043)X - (0.144 \pm 0.072)$ ($r^2 = 0.9965$)
Spiked plasma standards Extraction pH = 6.0 (range 0.15–4 μg/mL)	$y = (1.371 \pm 0.021)X - (0.007 \pm 0.045)$ ($r^2 = 0.9988$)
Relative recovery% (pH 9.5) = 92.9% Relative recovery% (pH 6.0) = 101.5%	

Relative recovery % = (slope of plasma calibration curve: 2)/slope of calibration curve of standard solutions prepared in mobile phase.

y = peak area ratio of analyte/internal standard

Precision and Accuracy

Repeatability of the chromatographic system was tested by the injection of a standard solution containing 2 μg/mL resveratrol and 10 μg/mL carbamazepine (n = 6). The peak areas of resveratrol and carbamazepine showed RSD% equal to 1.1 and 0.30, respectively, while the peak area ratios of resveratrol/IS showed RSD% equal to 0.86 %.

Reproducibility of extraction procedure, from plasma samples spiked with resveratrol at 0.3, 1, and 3 μg/mL is presented in Table 2 (RSD%

Table 2. Reproducibility of extraction procedure and CV% for resveratrol from spiked plasma samples (n = 6)

Spiked plasma Standards[a] (μg/mL)	PAR ± SD	RSD%
0.3	0.309 ± 0.022	7.3
1.0	1.0 ± 0.014	2.2
3.0	3.516 ± 0.025	0.72

PAR: Peak area of extracted analyte to peak area of extracted internal standard.

[a]All spiked plasma standards contained carbamazepine (internal standard) at 5 μg/mL.

Table 3. Within run precision and accuracy for the determination of resveratrol in spiked plasma samples (n = 6)

Concentration added (μg/mL)	0.3	1.0	3.00
Concentration found, Mean ± SD (μg/mL)	0.265 ± 0.020	1.04 ± 0.013	3.022 ± 0.022
RSD%	7.5	1.25	0.73
Relative error% (Er %)	−11.7	4.0	0.73

from means of six experiments). For the calculation of the reproducibility of extraction procedure the ratio of peak areas of analytes to that of the internal standard was used. Over all calibration curve RSD% ranged between 0.72 and 7.3.

Precision and accuracy of the method were evaluated by analyzing plasma samples (n = 6) spiked with resveratrol at 0.3, 1, and 3 μg/mL, and the results are presented in Table 3. The peak areas of extracted resveratrol and carbamazepine showed RSD% equal to 2.7 and 0.81, respectively, while the peak area ratios showed RSD% equal to 2.2 at 1.7 μg/mL spiked plasma standards. Within-run RSD% ranged from 0.7 to 7.5% for over all calibration curve. At the low concentration tested (0.3 μg/mL), equal to the quantitation limit of the method, the within run RSD% and the relative error were relatively high.

CONCLUSIONS

The analytical method developed in this report provides a simple, rapid, and accurate procedure for the quantitation of *trans*-resveratrol in human plasma. Nevertheless, the proposed method seems to be less sensitive than previously reported methods, which show detection limits for resveratrol in animal plasma samples equal to 0.015–0.02 μg/mL.[26,28] However, the proposed method can be used for monitoring resveratrol plasma levels in bioavailability studies. Since there are not many reports in the literature concerning the plasma levels of resveratrol in humans, the method developed can be further modified to correspond to the desirable concentration range.

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