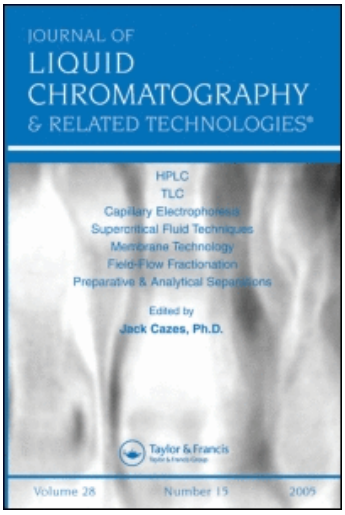

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Abstract: This paper reports the results of a study performed to develop a rapid and straightforward chromatographic method for the identification and quantification of *trans*-resveratrol in commercial *trans*-resveratrol containing dietary supplements. The method employs an Alltech Platinum EPS 100 A C-18 column operated under isocratic elution mode with a 70:30 (v/v) water-acetonitrile mixture as the mobile phase, at flow rate of $1.0\,mL\,min^{-1}$. Peak detection is performed at 306 nm with limit of detection (LOD) of $0.27\,\mu g\,mL^{-1}$ and limit of quantification (LOQ) of $0.95\,\mu g\,mL^{-1}$ and linear calibration graph within the concentration range of 2.63 to $10.53\,\mu g\,mL^{-1}$. Other data related to the successful method validation include representative linear regression equation and correlation coefficient of the calibration graph for quantification of *trans*-resveratrol, as well as the results of replicate analysis of real samples and of a recovery study, carried out to evaluate precision and accuracy, respectively. The method is successfully applied to study the stability of *trans*-resveratrol in methanol solution under various storage conditions and to analyse commercial preparations of *trans*-resveratrol containing dietary supplements.

Keywords: Dietary supplements, Method validation, RP-HPLC, *Trans*-resveratrol, *Trans*-resveratrol isomerization

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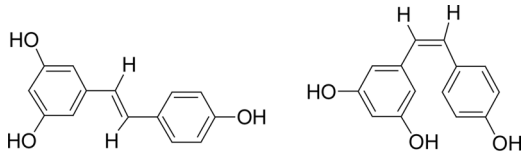
INTRODUCTION

Resveratrol (3,5,4'-trihydroxystilbene) is a phenolic compound belonging to the class of stilbenes that is present in a relatively limited number of plant species, including grape wine, Vitis vinifera,[1] in which it was observed to occur as a response to fungal infection or injury.[2] Grape and red wines are the major dietary sources of resveratrol and recent studies have associated the moderate consumption of red wine with the reduction of the risk of coronary heart disease (CHD).[3,4] Resveratrol, as other phenolic compounds, is a potent antioxidant and epidemiological studies have suggested a direct correlation between high phenolic compounds intake and reduced CHD mortality, by suppressing the oxidation of low density lipoprotein.[5] Resveratrol may occur in the *trans*- and *cis*-isomeric forms (Figure 1), with numerous reports suggesting *trans*-resveratrol to be the most bioactive form of this molecule.[6,7] Clinical studies have reported evidence that *trans*-resveratrol may exhibit a great number of cell protective actions, such as antimutagenic,[8] antiestrogenic,[9] and anti-inflammatory.[10] The chemopreventive and chemotherapeutic properties of *trans*-resveratrol against cancer have been investigated, evidencing the ability of this stilbene at inhibiting cellular events associated with carcinogenesis, including tumor initiation, promotion, and progression.[11,12]

The *trans*-isomer of resveratrol is easily converted to the *cis*-form under exposure to UV light.[13] Usually, plant tissues contain primarily *trans*-resveratrol, whereas processed plant extracts may contain higher proportions of the *cis*-isomer.[14,15] For example, in the grape skin of Vitis vinifera *cis*-resveratrol is rarely detected, whereas both isomers are usually identified in red wine, where the presence of *cis*-resveratrol has been attributed to the photochemical isomerization of the *trans*-form during the winemaking process.[16] Therefore, most of the analytical methods reported in literature are usually committed to the identification and quantification of *trans*-resveratrol. The majority of these methods are based on high performance liquid chromatography



***trans*-resveratrol** ***cis*-resveratrol**

Figure 1. Chemical structure of *trans*- and *cis*-resveratrol.

(HPLC) with UV,[17,18] fluorescence,[19] electrochemical,[20] and mass spectrometry (MS)[12,21] detection. However, gas chromatography (GC),[22] capillary electrophoresis (CE),[23] and micellar electrokinetic chromatography (MEKC)[24] have also been employed.

The use of complementary or alternative beneficial products for human health is growing worldwide, and the supposed health beneficial effects of *trans*-resveratrol has recently increased the demand of consumers for resveratrol-containing functional foods, which are primarily marketed as an herbal or dietary supplement in the form of pills, capsules, powders, and extracts from raw botanical sources (i.e., grape seeds/skins; Japanese knotweed Polygonum cuspidatum).[25] The growing interest of the market for resveratrol containing dietary supplements has prompted us to develop a rapid and robust method for the identification and quantization of *trans*-resveratrol in commercial preparations. The method has been developed with the additional purpose of further investigating the stability of *trans*-resveratrol in methanol solution under various storage conditions. The method was developed using a reversed-phase HPLC column operated under isocratic elution mode.

EXPERIMENTAL

Materials and Methods

Chemicals and Samples

Distilled water, further purified by a Milli-Q Water Purification System (Millipore, Bedford, MA, USA), was used to prepare all solutions and eluents; HiPerSolv HPLC grade acetonitrile was purchased from BDH (Milan, Italy); HPLC grade Chromasolv methanol, was obtained by Fluka (Milan, Italy); *trans*-resveratrol was purchased from Sigma (Milan, Italy); single use Anatop 10 LC, 0.2 μm inorganic membrane filters were obtained by Whatman (Springfield, UK). The *trans*-resveratrol containing dietary supplements, Activin Plus® and Acutil Senior®, were purchased from Phoenix Srl (Peschiera Borromeo, Milan, Italy) and Angelini Acraf S.p.A. (Rome, Italy), respectively.

Equipment

The experiments were performed with a Gilson (Middleton, WI, USA) Liquid Chromatograph consisting of a Model 305 solvent delivery pump, a Model 805 manometric module, a Model 115 variable wavelength UV

detector, and a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 20 μL sample loop. Chromatograms were recorded and processed by a Gilson Model 712 System Controller Software. An Alltech Platinum EPS 100 A C-18 5 μm column (250 × 4.6 mm ID) was supplied by Alltech Italia Srl, (Sedriano, Milan, Italy).

Analytical Conditions

The identification of the isomeric forms of resveratrol was performed on the basis of their retention times. A 10.53 $\mu g\,mL^{-1}$ stock solution of *trans*-resveratrol was prepared by dissolving the weighted amount of the analyte in the proper volume of methanol. This solution was diluted with methanol to obtaining five working solutions covering the concentration range 2.63 to 10.53 $\mu g\,mL^{-1}$. Quantification of *trans*-resveratrol was performed by the external standard method using a five point regression curve of the UV absorption data collected at 306 nm. The peak of *cis*-resveratrol was identified by its retention time, with the new peak appearing after UV irradiation at 365 nm of the *trans*-resveratrol solution. As *cis*-resveratrol is not commercially available, the calibration graph for this compound was obtained at 285 nm (maximum absorption for *cis*-resveratrol) with the same solutions used for the *trans*-isomer, after exposure of the solution to UV light at 365 nm for 60 min, which is the time necessary to determine the conversion of at least 90% of the *trans*- to *cis*-isomer. The quantity of this compound was ascertained on the basis of the decrease in the *trans*-isomer following UV irradiation.[14] The concentrations of the standard solution previously reported for t*rans*-resveratrol were obviously corrected for the percentage of conversion obtained.[26]

Sample Preparation

The evaluation of the stability of *trans*-resveratrol in methanol solution was carried out by preparing a stock solution of 5.59 $\mu g\ mL^{-1}$ *trans*-resveratrol in methanol that was divided in several aliquots, which were stored in dark glass vials, covered with aluminium foil. All aliquots were stored in the dark, part at room temperature and the others at −20°C. Aliquots of each sample were taken at every 10 min and *trans*-resveratrol was quantified by the developed HPLC method. A weighed amount of each commercial preparation was dissolved in the proper volume of methanol, in a volumetric flask protected from light in order to obtain solutions containing *trans*-resveratrol within the concentration range of 2.63 to 10.53 $\mu g\,mL^{-1}$. Sample solutions were sonicated for 90 min at room temperature and then filtered through a 0.2 μm single use inorganic membrane filter, before being injected onto the HPLC column.

RESULTS AND DISCUSSION

Initial phases of the investigation were focused on the optimization of the chromatographic conditions required to obtain selective resolution of *trans*- and *cis*-resveratrol by reversed phase HPLC, using a C_{-18} column eluted under isocratic conditions with mobile phase consisting of a water-acetonitrile mixture. The influence of the content of acetonitrile into the hydro-organic mobile phase on the retention factor of *trans*-resveratrol is depicted by the plot displayed in Figure 2. As expected, the retention factor of *trans*-resveratrol decreased with increasing the content of acetonitrile into the mobile phase.

With the mobile phase consisting of a 70:30 (v/v) water-acetonitrile mixture, a solution of *trans*-resveratrol in methanol irradiated with UV light at 365 nm for 30 min displayed a chromatogram with two resolved peaks, whose average retention times of quintuplicate runs were 7.78 and 10.02 min, respectively, with RSD better than 0.30% (see Figure 3 and Table 1). The peak eluting at retention time of 7.78 min was confirmed to be *trans*-resveratrol, since its retention time was identical to that of the authentic standard solution of *trans*-resveratrol. The *cis*-isomer of resveratrol was identified by comparison of the retention time of the additional peak observed after UV irradiation at 365 nm of the stock solution of *trans*-resveratrol. This additional peak was drastically smaller than that of *trans*-resveratrol due to the molar adsorptivity of the *cis*-isomers at 306 nm, which is about 3.4 times lower than that of the *trans*-form.[13]

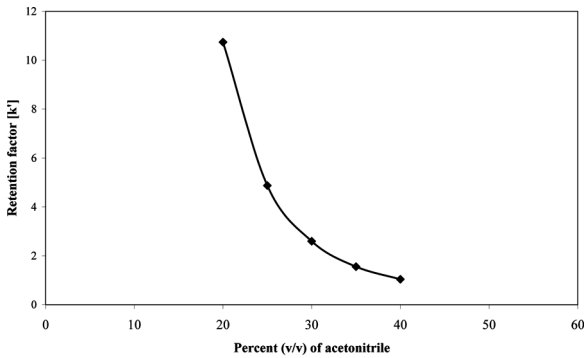


Figure 2. Variation of the retention factor of *trans*-resveratrol as a function of the percent (v/v) of acetonitrile into the mobile phase consisting of water-acetonitrile mixture. Column, Alltech Platinum EPS 100 A packed with 5 μm C_{-18} stationary phase (250 × 4.6 mm ID); flow rate, 1.0 mL min^{-1}; sample size and concentration, 20 μL and 5.26 μg mL^{-1}, respectively; UV detection at 306 nm, 0.1 absorption units full scale; retention times, minutes.

Figure 3. Separation of *trans*- and *cis*-resveratrol in a $5.59\,\mu g\,mL^{-1}$ *trans*-resveratrol solution exposed to UV light at 365 nm for 30 min. Column and experimental conditions as in Figure 2, except percent of acetonitrile into the mobile phase of 30% (v/v).

The limit of detection (LOD) was determined from the amount of *trans*-resveratrol required to give a signal-to-noise ratio of 3 and was $0.27\,\mu g\,mL^{-1}$, whereas the limit of quantification (LOQ), defined as the lowest concentration giving a signal-to-noise ratio of 10, was $0.95\,\mu g\,mL^{-1}$. The calibration graph for quantitative analysis was constructed by plotting the concentration of the standard solutions of *trans*-resveratrol as a function of peak area, which showed good linearity in the concentration range 2.63 to $10.53\,\mu g\,mL^{-1}$. The representative linear regression equation was $y = 32475x - 4165.8$ with a significant correlation coefficient (0.99985).

Table 1. Intra-day and inter-day repeatability of the retention time (t_R) of *trans*- and *cis*-resveratrol

	Intra-day repeatability			Inter-day repeatability		
Analyte	t_R^a (min)	SD (min)	RSD (%)	t_R^b (min)	SD (min)	RSD (%)
trans-resveratrol	7.78	0.02	0.23	7.65	0.07	0.90
cis-resveratrol	10.01	0.03	0.30	9.96	0.11	1.10

[a]Average value of quintuplicate analysis carried out within the same day.
[b]Mean value of five chromatographic runs per day over a period of three consecutive days.

Table 2. Intra-day and inter-day repeatability of the peak area of *trans*- and *cis*-resveratrol

	Intra-day repeatability			Inter-day repeatability		
Analyte	Peak area[a] (arbitrary units)	SD	RSD (%)	Peak area[b] (arbitrary units)	SD	RSD (%)
trans-resveratrol	263667.73	2531.25	0.96	264012.18	4805.02	1.82
cis-resveratrol	24442.5	322.6	1.32	24085.46	551.16	2.29

[a]Average value of quintuplicate analysis carried out within the same day.
[b]Mean value of five chromatographic runs per day over a period of three consecutive days.

The repeatability and precision of the method were assessed by analyzing 5 repeated times a 5.59 μg mL^{-1} standard solution of *trans*-resveratrol before and after exposition to UV light for 30 min, during the same day and over a period of three days. Retention times and peak areas were employed to evaluate intra-day and inter-day repeatability (see Tables 1 and 2). In the latter case, the measured values, representing the means of 5 determinations per day and per analyte, were used for the evaluation of the overall inter-day precision for the method. The chromatogram displayed in Figure 3 and data reported in Table 1 show that the two isomeric forms of resveratrol were completely resolved in about 10 min with highly repeatable retention times. Also satisfactory was the intra-day and the inter-day repeatability of the detector response (see Table 2).

The developed method was applied to monitor the conversion of *trans*-resveratrol into its *cis*-form when exposed to UV light. The experiment was carried out preparing a 5.26 μg mL^{-1} solution of *trans*-resveratrol in methanol, which was irradiated for 150 min at 365 nm. Aliquots of the irradiated solution were subjected to HPLC analysis at different time intervals to monitor the isomerization and *cis*-resveratrol formation by evaluating the decrease of the concentration of *trans*-resveratrol. The results of this study are reported in Figure 4, showing that after 60 min more than 90% of *trans*-resveratrol was converted into its *cis*-isomer, as it has been previously reported.[14]

A further study was performed to evaluate the stability of a solution of *trans*-resveratrol in methanol stored in the dark at −20°C and at room temperature. The experiments were carried out by analyzing, by the developed method, aliquots of a stock solution of *trans*-resveratrol stored in the dark both at room temperature and at −20°C. As it has been reported by Wang et al.,[14] the concentration of *trans*-resveratrol was unvaried for up to five days storage at −20°C and no conversion

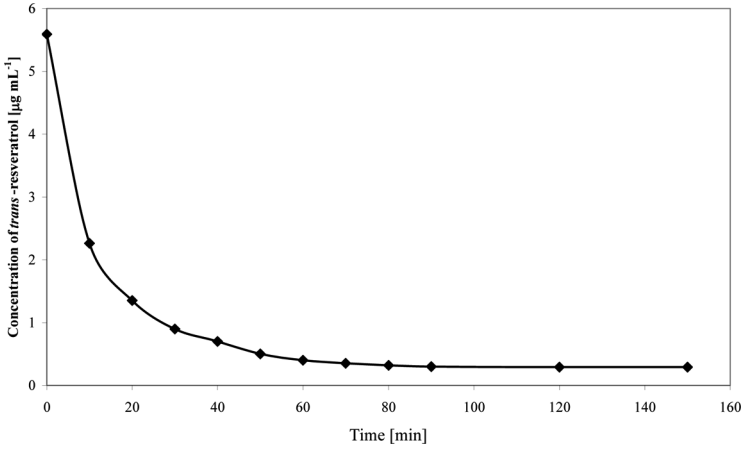


Figure 4. Evaluation of the conversion of *trans*-resveratrol into its *cis*-isomer by the decrease of the concentration of *trans*-resveratrol as a function of the time of irradiation with UV light at 365 nm of a 5.26 μg mL^{-1} *trans*-resveratrol solution in methanol.

of the *trans*- to *cis*-isomer was observed. However, Wang[14] reported a slight decrease of the concentration of *trans*-resveratrol for solutions stored in the dark at 4°C that was not observed in our investigation, which evidenced that also at 4°C the concentration of *trans*-resveratrol was practically unaffected up to five days storage. On the other hand, the concentration of *trans*-resveratrol varied from 8.96 to 6.10 μg mL^{-1} when the above solution was exposed to the laboratory day-light for 6 h at room temperature.

The proposed method was than applied for the identification and quantification of *trans*-resveratrol in two commercially available *trans*-resveratrol containing dietary supplements (see Fig. 5). Each sample was treated as reported in the Experimental section and *trans*-resveratrol was identified and quantified by the proposed method. The content of *trans*-resveratrol in the pills of Acutil Senior® (labeled amount, 5 mg/pill) and in the capsules of Activin Plus® (labeled amount, 0.5 mg/capsule) determined in quintuplicate analysis resulted to be 5.26 ± 0.8 mg/pill and 0.54 ± 0.02 mg/capsule, respectively.

In order to determine the accuracy of the method a recovery study was performed. Known amounts of *trans*-resveratrol were added to each commercial preparation and the resulting spiked samples were subjected to the entire analytical method. Three different amounts of *trans*-resveratrol, corresponding to 80, 100, and 120% of the labeled content, were added. All samples were injected five times and an average

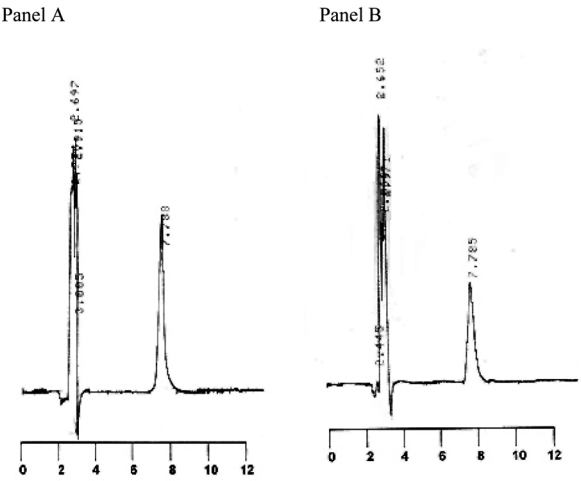


Figure 5. Chromatograms of samples of Acutil Senior® (panel A) and Activin Plus® (panel B) analyzed by the proposed method. Chromatographic conditions as in Figure 3.

of the response peak areas was the basis for the found concentration. The recoveries were calculated on the basis of the difference between the total concentration determined in the spiked samples and the concentration observed in the non-spiked samples. Data reported in Table 3 show that the overall recovery ranged between 98, 87 (±4.05), and 106.70% (±2.98), indicating that the method is adequately accurate and precise. The accuracy of the method was further evaluated by quintuplicate analysis of samples containing known amounts of *trans*-resveratrol, which resulted in RSD values lower than 5.0%.

Table 3. Recovery of *trans*-resveratrol added to each of two commercial preparations at levels corresponding to 80, 100, and 120% of labeled amounts (*l.a.*)

Commercial preparation	Recovery[a] (%)			Overall[b] (%)
	80% (*l.a.*)	100% (*l.a.*)	120% (*l.a.*)	mean ± SD
Acutil senior	101.01	94.20	101.4	98.87 ± 4.05
Activin plus	108.8	108.02	103.30	106.70 ± 2.98

[a]Each commercial preparation was assayed five times before and after addition of *trans*-resveratrol and average of results is presented.
[b]Obtained by pooling all recovery data. SD, standard deviation.

CONCLUSIONS

Reversed phase high performance liquid chromatography, using a C_{-18} column operated under isocratic elution mode with a 70:30 (v/v) water-acetonitrile mixture as the mobile phase, has emerged as being useful to develop a versatile procedure for the rapid resolution and quantification of *trans*- and *cis*-resveratrol. The method has been successfully validated and efficiently applied to monitor the conversion of *trans*-resveratrol into its *cis*-form when exposed to UV light and when stored in the dark either at room temperature or at −20°C. The method is highly reproducible, the quantification of *trans*-resveratrol is linear over a useful concentration range, and the results of the recovery studies show good accuracy. The method can be successfully applied to identify and quantify the *trans*-isomer of resveratrol in *trans*-resveratrol containing dietary supplements commercialized either as pills or as capsules, which can be analyzed without sample pretreatment, except dissolution of the commercial preparation in the proper volume of methanol to obtain solutions of concentration within the range of linearity of the calibration graph.

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