# Development and validation of an RP-HPLC method for quantification of *trans*-resveratrol in the plant extracts

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*Abstract*

New, simple, cost effective, accurate and reproducible RP-HPLC method was developed and validated for the quantification of *trans*-resveratrol in the extracts of grape exocarp and seeds. The method has proved to be simpler and faster than available methods. Methanol was used as a mobile phase with a flow rate of 1.0 $cm^3$ $min^{-1}$, while the quantification was effected at 306 nm. The separation was performed at 35 °C using a $C_{18}$ column. The results showed that the peak area response was linear in the concentration range of 1–40 μg $cm^{-3}$. The values of *LOD* and *LOQ* were found to be 0.125 and 0.413 μg $cm^{-3}$, respectively. The antioxidant activity of the extracts was determined using DPPH assay. The ability of DPPH radicals inhibition decreases in the following order: the extract of grape exocarp > *trans*-resveratrol standard > the extract of grape seeds.

*Keywords*: validation, RP-HPLC, *trans*-resveratrol, grape extract, antioxidant activity.

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Resveratrol (3,5,4'-trihydroxystilbene) presents a bioactive compound that was extracted as a white powder from the roots of *Veratrum grandiflorum* in 1940 [1]. Then, it was detected in more than seventy species of plants, including grapes [2], blueberries, cranberries, mulberries, peanuts, white pine and corn. Although the two isomeric forms of resveratrol can be found in the nature (Fig. 1), *trans*-resveratrol is the most commonly used isomer during investigation, because *cis*-isomer is not commercially available due to its stability. After effect of UV light or natural daylight, *trans*-isomer is transformed to *cis*-isomer [3].

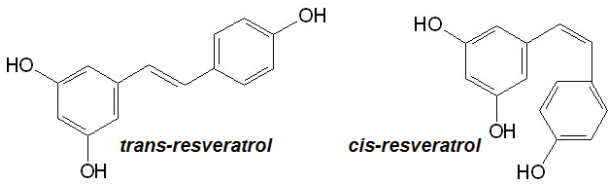


*Fig. 1. The structures of trans- and cis-resveratrol.*

Based on the pharmacological studies, it can be concluded that resveratrol act as: antioxidant [4], inhibitor of cyclooxygenase and lipid modifications [5], inhibitor of LDL oxidation and platelet aggregation, vasodilator [6, 7] and antiviral agent [8]. Furthermore, resveratrol is able to inhibit transcriptional activation of carcinogen-activating enzyme CIP1A1, thereby preventing the occurrence of cancer in the initiation stage [9].

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For the determination of *trans*-resveratrol in wines, there are many described analytical methods, such as gas chromatography-mass spectrometry (GC–MS) [10], capillary electrophoresis (CE) [11] and high performance liquid chromatography-mass spectrometry (HPLC-MS) method [12,13]. GC-MS coupled methods commonly require derivatization with bis(trimethylsilyl) trifluoroacetamide in order to improve the evaporation of analyzed compounds [14,15]. The extraction and derivation procedures require a considerable time and may cause the conversion of *trans*- to *cis*-resveratrol. Additionally, the lack of these methods is a low sensitivity. The content of resveratrol was also determined in the biological samples by GC–MS methods [16,17]. An advantage of HPLC analysis in compared with other methods is due to detection of lower concentrations of resveratrol and its metabolites [18–24]. Also, the separation of resveratrol isomers in wine is possible using HPLC method [25]. Based on the literature search, the HPLC method for simultaneous determination of the resveratrol content and other compounds (*e.g.*, phenolic compounds in the plant extracts or metabolites in the biological samples) are mainly developed. The combined solvents such as methanol, acetic acid, water, acetonitrile, etc. were used as the mobile phase in many cases [26–29].

Given the state of the literature, the aim of this study was to develop simple, precise, accurate and validated RP-HPLC method for the determination of the *trans*-resveratrol content in the extracts of grape exocarp and seeds in the presence of other bioactive compounds. These plant materials were used because they represent the main source of this bioactive compound. The developed analytical method was validated in

accordance with ICH guidelines and USP requirements [30,31].

## EXPERIMENTAL

*Chemicals and reagents*. *trans*-Resveratrol (>99.4%) (Dr. Ehrenstorfer GmbH, Augsburg, Germany), methanol HPLC grade (LGC Standards, Merkatorstabe, Wesel, Germany), ethanol (96%) analytical grade (Zorka Pharma-Hemija d.o.o., Šabac, Serbia), 2,2-diphenyl-1--picrylhydrazyl (DPPH, Sigma Chemical Company, Saint Louis, USA).

*Plant materials*. The used red grape was of variety "Cardinal" from Southeastern Serbia. Plant materials (exocarp and seeds) were dried at room temperature and then disintegrated in laboratory mill.

*Procedure of resveratrol extractions*. About 2 g of the disintegrated plant materials (grape exocarp and seeds) were transferred into the round-bottom flask of 100 $cm^3$. After adding 96% ethanol in the solid to liquid ratio of 1:15 (*m*/*V*), the mixture was put under reflux at the boiling temperature of solvent for 90 min. The extraction temperature was maintained using a water bath. After extraction, the solid matrix was separated from the liquid by vacuum filtration. Then, the extract was evaporated under reduced pressure on the rotary evaporator at 60 °C until semi-solid consistency. The extract was dried to constant mass in a desiccator.

*HPLC analysis*. The development of method was performed using an Agilent 1100-Series HPLC system. It consisted of a DAD detector and Agilent 1100-Series autosampler (Waldbronn, Germany). The system was controlled and data analyses were tested by Agilent HPLC Data Analysis software. The assays were reproduced using another LC system for repeatability. This system contained an Agilent 1100-Series binary pump and Agilent 1100-Series DAD detector (Waldbronn, Germany). The detector was set and the peak areas were integrated automatically by the computer using the Agilent HPLC Data Analysis software program. RP-HPLC analysis was performed by isocratic elution with a flow rate of 1.0 $cm^3$ $min^{-1}$. Methanol was used as a mobile phase, which was filtered through a 0.45 μm millipore filter (Econofilters, Agilent Technologies, Germany) before injecting into the system. The injected volume of samples was 20 μL, and the detection wavelength was 306 nm. The separation was carried out at 35 °C using a Supelco $C_{18}$ column (250 mm×4.6 mm, 5 μm), Agilent Technologies, USA.

*Preparation of samples*. The stock solutions of *trans*-resveratrol and extracts were prepared by dissolving 12.5 mg of standard and 50 mg of the dried extracts in 25 $cm^3$ of methanol, respectively. From these solutions, the aliquot of 1 $cm^3$ was transferred into the flask of 10 $cm^3$ and filled to the mark with mobile phase. The samples were sonificated for 15 min, filtered through a cellulose membrane of 0.45 μm (Econofilters, Agilent Technologies, Germany). The volume sample of 20 μl was injected into the HPLC system. The identification of *trans*-resveratrol in the extracts was performed by comparison of the retention times and UV spectra with *trans*-resveratrol standard. The samples of standard solution and extracts were stored in the dark to avoid oxidative degradation and isomerization of *trans*-resveratrol to *cis*- form.

### Analytical method validation

*Linearity*. The series of *trans*-resveratrol standard solutions were prepared in the range from 1–100 μg $cm^{-3}$ to establish a linearity of the proposed method.

*Accuracy*. The different concentrations of *trans*-resveratrol (10, 20 and 30 μg $cm^{-3}$) were prepared from independent stock solution and then analyzed ($n$ = 10). Accuracy was assessed as a percentage accuracy and mean recovery (%). In order to provide an additional support to the accuracy of the developed assay method, the method of standard addition was employed. It involves the addition of different concentrations of pure *trans*-resveratrol (1, 3 and 5 μg $cm^{-3}$) to the known pre-analyzed sample.

*Precision*. Inter-day, intra-day and inter-instrument variation were studied to determine the intermediate precision of the proposed analytical method. Three different concentrations of *trans*-resveratrol (10, 20 and 30 μg $cm^{-3}$) were analyzed for three days (three times, $n$ = 3) to study the intra-day variation. The same procedure was followed for three different days to study the inter-day variation ($n$ = 10). One set of different concentrations was reanalyzed by proposed method using another HPLC Agilent 1100-Series system to study inter-instrument variation ($n$ = 10).

Limit of detection (*LOD*) and limit of quantitation (*LOQ*). *LOD* and *LOQ* were determined based on the solutions of *trans*-resveratrol used to construct the calibration curve. *LOD* and *LOQ* were calculated as 3.3 $\sigma/S$ and 10$\sigma/S$, respectively, where $S$ is the slope of the calibration curve and $\sigma$ is the standard deviation of regression equation intercept ($n$ = 10).

*Robustness*. A full factorial design was used for investigation of method robustness, where the temperature and flow rate of mobile phase were used as the independent variables. The temperature and the flow rate were in the range of 33–37 °C, *i.e.,* 0.8–1.2 $cm^3$ $min^{-1}$, respectively. In accordance with the design matrix 9 experimental runs were performed and repeated 3 times. Statistica version 8.0 (StatSoft Inc., Tulsa, OK, USA) was used to generate the experimental designs, statistical analysis and regression model.

*DPPH assay*. The series of different concentrations of the extracts and *trans*-resveratrol standard were prepared by dissolving the stock solutions (2.0 mg $cm^{-3}$). The ethanolic solution of DPPH radicals with the con-

centration of $3\times10^{-4}$ mol dm$^{-3}$ (1 cm$^3$) was added into the investigated solution (2.5 cm$^3$). The absorbance of samples was measured at 517 nm in compared to 96% ethanol after incubation of 30 min with DPPH radicals. The incubation was performed in the dark at room temperature. Under the same conditions, the absorbance of the diluted ethanolic solution of DPPH radical (1 cm$^3$ was diluted with 2.5 cm$^3$ of ethanol) was determined. The inhibition of DPPH radicals was calculated using the following equation:

$$\text{Inhibition of DPPH radicals (\%)} = \\ = 100 - \left[ \left( A_U - A_B \right) \left( \frac{100}{A_K} \right) \right] \quad (1)$$

where $A_U$ – absorbance of the sample treated with the solution of DPPH radicals, $A_B$ – absorbance of the untreated sample with the solution of DPPH radicals, $A_K$ – absorbance of the diluted ethanolic solution of DPPH radicals [32,33].

## RESULTS AND DISCUSSION

### Optimization of HPLC method

RP-HPLC method was developed and validated for the quantification of *trans*-resveratrol in the plant extracts. The chromatographic conditions were optimized to provide a good performance of the assay. The different stationary phases like $C_{18}$ and $C_8$ were tested during method optimization. The satisfactory separation was achieved on a Supelco $C_{18}$ column (250 mm×4.6 mm, 5 µm) using methanol as a mobile phase. The effects of various organic modifiers on the peak properties (peak height, peak area, peak symmetry, retention time, etc.) and response function were observed. A maximum absorption of *trans*-resveratrol was detected at 306 nm, and this wavelength was chosen for monitoring its content in the samples. A chromatogram of the *trans*-resveratrol solution at 20 µg cm$^{-3}$ is presented in Fig. 2. The peak of *trans*-resveratrol at the retention time of 2.535 min was confirmed based on the UV spectrum.

The column efficiency was considered based on peak asymmetry (*As*), height equivalent to a theoretical plate (*HETP*), number of theoretical plates (*N*) and retention time ($t_r$). The calculated value of *HETP* was 0.042, *i.e.*, the value of *N* was 5958. The asymmetry peak of 1.4 indicates that the peak is not ideally symmetric and that it is not a Gauss's peak. Having in mind the fact that $W_{ab}$ is lower than $W_{bc}$, there is the presence of some interactions between the stationary phase and the investigated compound. Most practical researchers strive that the asymmetry peak to be lower than 1.5.

### Validation

The obtained results for construction of the calibration curve can be fitted as follows: $A_{306}$ = 111.75*c* (µg cm$^{-3}$) + 123.95. The linearity of the constructed curve was noticed in the concentration range of 1–40 µg cm$^{-3}$ ($r$ = 0.9994). The high value of regression coefficient indicates a good fitting of the curve. The precision of fitting was further confirmed based on standard error (*S.E.*) at 95% confidence interval for the values of intercept (0.114) and slope (0.456).

In order to determine the accuracy of the proposed method, the different levels of *trans*-resveratrol concentrations: lower concentration (*LC*, 10 µg cm$^{-3}$), intermediate concentration (*IC*, 20 µg cm$^{-3}$) and higher concentration (*HC*, 30 µg cm$^{-3}$) were prepared from independent stock solutions and analyzed ($n$ = 10). Accuracy was assessed as the percentage relative error and mean recovery (Table 1).

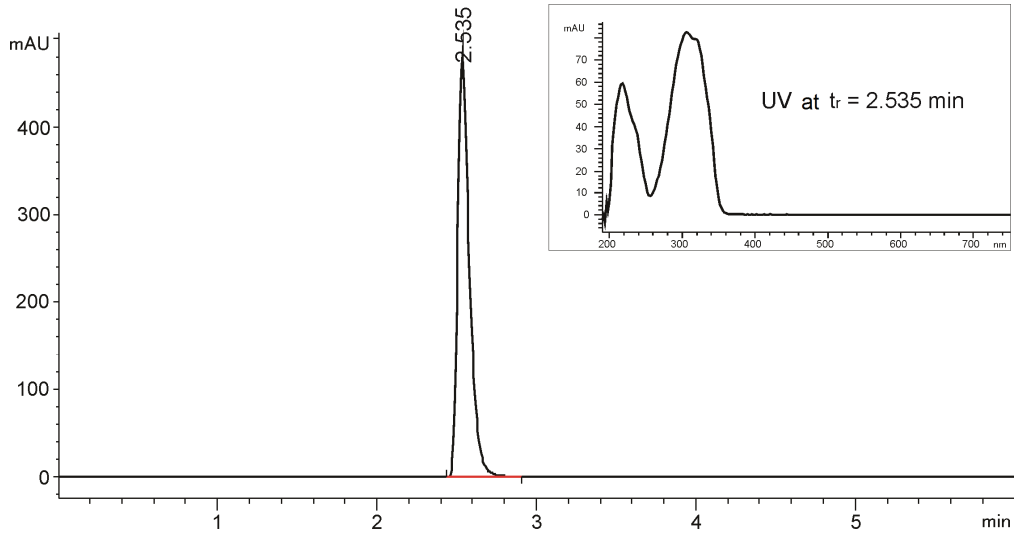


*Fig. 2. Chromatogram of the standard solution of trans-resveratrol (20 µg cm$^{-3}$) at 306 nm.*

*Table 1. Accuracy and precision data for the developed method (n = 10)*

| Level, μg cm$^{-3}$ | Predicted concentration, μg cm$^{-3}$ | | Mean recovery, % | Accuracy, % |
|---|---|---|---|---|
| | Mean (±*SD*) | *RSD* / % | | |
| 10 | 10.29±0.24 | 0.71 | 102.90 | 2.90 |
| 20 | 19.47±0.51 | 0.62 | 97.35 | –2.70 |
| 30 | 29.39±0.41 | 0.49 | 97.96 | –2.03 |

The excellent mean recovery values, close to 100%, and their low standard deviation values (*RSD* < 1.0) represent high accuracy of the analytical method. The validity and reliability of the proposed method were further assessed by recovery studies *via* standard addition method. The mean recoveries (*RSD*) for the concentration of 20 μg cm$^{-3}$ are shown in Table 2.

These results revealed that any small change in *trans*-resveratrol concentration in the solution could be accurately determined by the proposed analytical method.

Precision was determined by studying the repeatability and the intermediate precision. The precision was determined using the same *trans*-resveratrol concentration levels taken in the accuracy study.

The results of repeatability studies (Table 3) indicate the precision under the same operating conditions over a short interval of time and the inter-assay precision. The intermediate precision expresses within-laboratory variations in different days and with different instruments. In the intermediate precision study, *RSD* values were not more than 2.0% in all cases (Table 3). *RSD* values of the proposed analytical method were well within the acceptable range. These values indicate the excellent repeatability and intermediate precision of the method.

The calculated *LOD* and *LOQ* values of *trans*-resveratrol were 0.125 and 0.413 μg cm$^{-3}$, respectively.

*Stability of solution*. The different concentrations of *trans*-resveratrol (10, 20 and 30 μg cm$^{-3}$) were used to determine the stability of the test solution (20 μg cm$^{-3}$). The change in *trans*-resveratrol concentration was monitored during 24 and 48 h. Test and standard solutions were stored in autosampler vials at ambient temperature. The obtained results for test and standard solutions are shown in Table 4.

*Robustness of method*. The robustness of method is investigated in the framework of method development as a concept of quality by design. The aim of the robustness of the developed RP-HPLC method is to examine the variation which might be expected in routine use of this method. A full factorial design with two variables at three levels was used for monitoring the robustness of the developed method [34–37]. The peak area ($Y_1$), retention time ($Y_2$), number of theoretical plates ($Y_3$) and peak asymmetry ($Y_4$) were defined as the response, while the column temperature ($x_1$) and flow rate of mobile phase ($x_2$) were used as the independent variables. The column temperature and flow rate of mobile phase were analyzed in the range of 33–37 °C, *i.e.*, 0.8–1.2 cm$^3$ min$^{-1}$, respectively. A polynomial equation that can be used for investigation of the interaction between linear and quadratic terms in the equation was used for modeling the experimental data. The terms with p-values higher than 0.05 are the statistically insignificant and they were excluded from the polynomial equation. The equations that present the effect of the process variables on the observed response, it can be presented in the following way:

*Table 2. Determination of trans-resveratrol by standard addition method (n = 10); concentration: 20 μg cm$^{-3}$*

| Pure drug added, μg cm$^{-3}$ | Total drug found ± *SD*, μg cm$^{-3}$ | *RSD* / % |
|---|---|---|
| 0 | 20.17±0.3 | 100.85±0.6 |
| 1 | 20.89±0.3 | 99.47±0.5 |
| 3 | 23.11±0.4 | 100.48±0.6 |
| 5 | 24.91±0.4 | 99.64±0.5 |

*Table 3. System precision study (n = 10)*

| Concentration, μg cm$^{-3}$ | Estimated concentration, intra-day reproducibility (*RSD* / %, *n* = 10) | | | Intra-instrument reproducibility (*RSD* / %, *n* = 10) |
|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | |
| 10 | 8.15 (0.81) | 10.74 (0.57) | 10.41 (0.44) | 9.09 (0.89) |
| 20 | 20.86 (0.05) | 20.32 (0.30) | 19.78 (0.10) | 20.83 (1.43) |
| 30 | 29.59 (0.10) | 28.66 (0.84) | 30.13 (0.12) | 28.99 (1.58) |

Peak area: $Y_1 = 2531.2 - 510.6x_2 - 52.1x_2^2$
($r^2$ = 1.000) (2)

Retention time: $Y_2 = 2.6 - 0.004x_1 - 0.5x_2 - 0.05x_2^2$
($r^2$ = 1.000) (3)

Number of theoretical plates:
$Y_3 = 6825 - 784.6x_1^2 - 3173.8x_2 - 949.4x_2^2 +$
$+1151.2x_1x_2 + 928.3x_1x_2$
($r^2$ = 0.998) (4)

Peak assymetry:
$Y_4 = 1.87 - 0.11x_2 - 0.15x_2^2 -$
$-0.15x_1x_2 - 0.07x_1x_2$ ($r^2$ = 0.921) (5)

*Table 4. Stability of trans-resveratrol solutions*

| Time elapsed, h | Sample | $c$ / μg cm$^{-3}$ | Recovery, % |
|---|---|---|---|
| Initial | st | 20.00 | 100.0 |
| 24 | | 19.51 | 97.55 |
| 48 | | 20.42 | 102.1 |
| Initial | 1 | 10.00 | 100.0 |
| 24 | | 9.81 | 98.1 |
| 48 | | 10.34 | 103.4 |
| Initial | 2 | 20.00 | 100.0 |
| 24 | | 20.88 | 104.4 |
| 48 | | 19.20 | 96.0 |
| Initial | 3 | 30.00 | 100.0 |
| 24 | | 29.01 | 96.7 |
| 48 | | 30.57 | 101.9 |

The high values of correlation coefficient indicate a good agreement between experimental and predicted values. In all case this value is almost equal to 1. The highest impact on the peak area has the flow rate of mobile phase, while the effect of column temperature is statistically insignificant. The retention time, number of theoretical plates and peak asymmetry mainly depend on the flow rate of mobile phase. Based on the obtained data, it can be concluded that unlike the change in column temperature, the significant change in flow rate can be impact the performance of the system.

A functional dependency between flow rate of mobile phase and column temperature is presented in Fig. 3. In this case, a number of theoretical plates were used as a response. The previous conclusion that the flow rate of mobile phase most impacts the number of theoretical plates was confirmed based on that three-dimensional diagram. Also, it can be noticed that the column temperature is not a statistically significant parameter and does not impact the system performance.

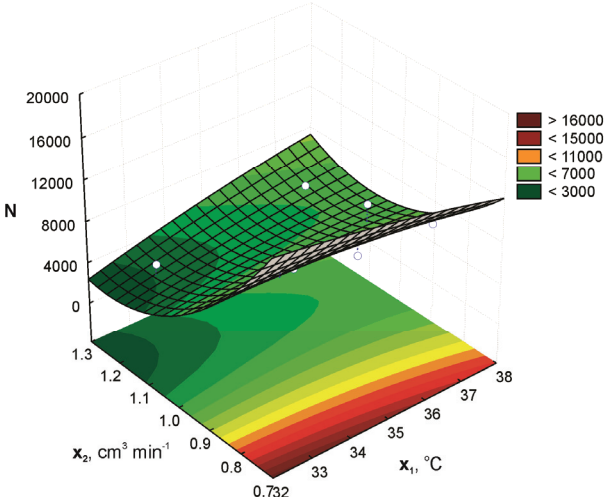


*Fig. 3. The effect of column temperature ($x_1$) and flow rate of mobile phase ($x_2$) on number of theoretical plates (N).*

## Application of the method for analysis of the plant extracts

In order to apply the developed and validated RP-HPLC method, the ethanolic extracts of red grape exocarp and seeds were prepared. The two solutions of the obtained extracts were dissolved and two solutions were analyzed in order to identify and quantify *trans*-resveratrol. The chromatograms of the extracts solutions are presented in Fig. 4. The presence of *trans*-resveratrol in the extracts of seeds and exocarp was identified at the retention times of 2.523 and 2.525 min, respectively. The peaks of *trans*-resveratrol were confirmed by comparison of the UV spectra at these retention times with the UV spectrum of *trans*-resveratrol standard. Based on the regression equation of calibration curve, the content of *trans*-resveratrol in the extracts of exocarp and seeds was found to be 208.88 mg and 277.63 mg calculated per 100 g of the dried extracts, respectively.

The RP-HPLC method was presented as suitable for analysis of *trans*-resveratrol in the plant extracts due to simpler composition of the mobile phase and shorter time of analysis in compared with available methods [26–29].

## Antioxidant activities of *trans*-resveratrol and grape extracts

In this study, DPPH assay was used for determination of antioxidant activities of *trans*-resveratrol standard and grape extracts. The incubation time has the impact on inhibition of DPPH radicals and thereby the antioxidant activity is better observed. Due to this reason, all samples were incubated for 30 min before the absorbance measurement.

The effect of concentration of the samples on inhibition of DPPH radicals is presented in Fig. 5. It can be noticed that the inhibition of DPPH radicals increases

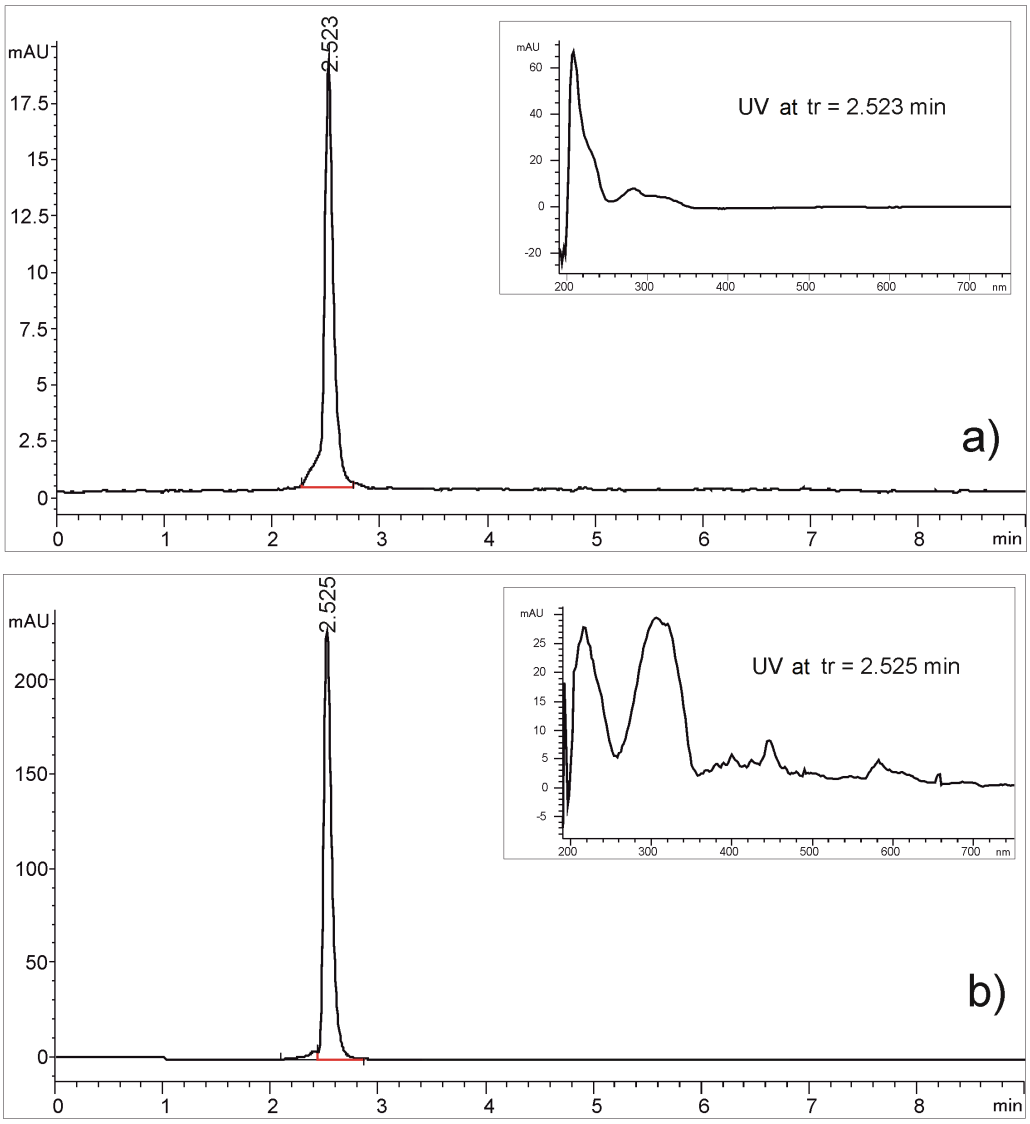


*Fig. 4. Chromatogram of the extracts of: grape seeds (a) and grape exocarp (b) at 306 nm.*

with increasing the concentration of *trans*-resveratrol standard (6.25–800 μg cm$^{-3}$). The maximum inhibition of DPPH radicals (93.48%) was achieved at the concentration of *trans*-resveratrol standard of 0.4 mg cm$^{-3}$ after incubation of 30 min. In the literature, the inhibition of DPPH radicals of 14% was achieved at the concentration 250 μM [38], while the concentration of 5.7 mM inhibited 21.16% of DPPH radicals in this study.

The obtained results indicate that the extract of grape seeds has better antioxidant activity compared to the extract of grape exocarp at similar concentrations of the samples. The inhibition of DPPH radicals of 96.06% for the grape seeds extract was achieved at the concentration of 0.125 mg cm$^{-3}$, while the inhibition of 99.47% was at 2.000 mg cm$^{-3}$ of the extract of grape exocarp. Thus, the antioxidant activity of the extract of grape seeds is lower about 16 times in compared with the extract of grape exocarp, *i.e.*, about 3 times lower in compared with *trans*-resveratrol standard.

$EC_{50}$ values of the samples were determined empirically from diagrams given in Fig. 5. The highest value ($EC_{50}$ = 0.44 mg cm$^{-3}$) was obtained for the extract of grape exocarp, and something lower value ($EC_{50}$ = = 0.066 mg cm$^{-3}$) for *trans*-resveratrol standard. The lowest value ($EC_{50}$ = 6.77 μg cm$^{-3}$) was calculated for the extract of grape seeds. The obtained values indicate that the extract of grape exocarp has the lowest ability of DPPH radicals inhibition in compared with the extract of grape seeds and *trans*-resveratrol standard. The ability of the extract of grape seeds to better "scavenge" DPPH radicals than *trans*-resveratrol standard and the extract of grape exocarp is probably the result of the presence of other additional compounds that also show the antioxidant activity. The flavonoids and

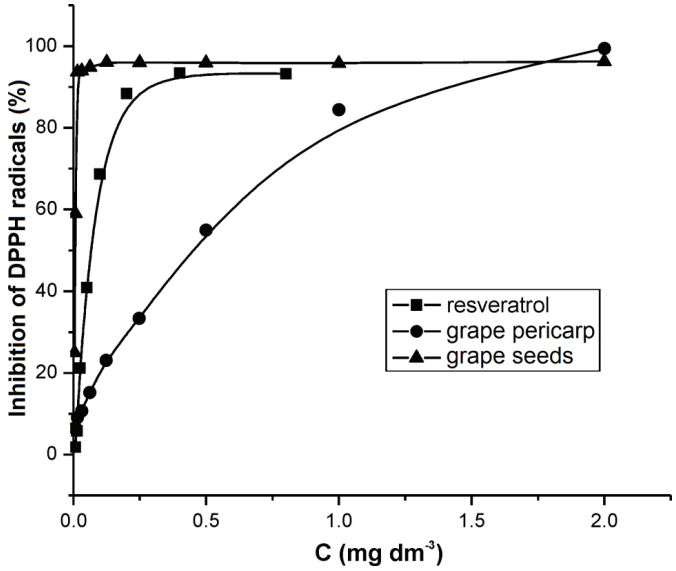


*Fig. 5. Antioxidant activity of trans-resveratrol standard (■), the extract of grape exocarp (●) and the extract of grape seeds (▲).*

phenolic acids commonly belong to the group of these compounds.

## CONCLUSION

The proposed RP-HPLC method is presented as a simple, rapid, accurate, precise and economic analytical method. It can be used for the routine analysis of *trans*--resveratrol in the red grape extracts and other plant extracts containing *trans*-resveratrol. This method has advantages due to fast analysis and simpler composition of the mobile phase in compared with the other HPLC methods for monitoring the content of *trans*-resveratrol in the plant extracts. The results of robustness study indicate that the proposed method stays stable even after the nominal changes in the column temperature. The antioxidant activity of the extract of grape seeds was higher than the extract of grape exocarp probably due to the presence of additional bioactive compounds with expressed activity.

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## IZVOD

### RAZVOJ I VALIDACIJA RP-HPLC METODE ZA KVANTIFIKACIJU *TRANS*-RESVERATROLA U BILJNIM EKSTRAKTIMA

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U ovom radu razvijena je i validirana nova, jednostavna, isplativa i ponovljiva RP-HPLC metoda za kvantifikaciju *trans*-resveratrola u ekstraktima egzokarpa i semena grožđa. Metoda se pokazala jednostavnijom i bržom u odnosu na ostale dostupne metode. Kao mobilna faza korišćen je metanol pri protoku 1,0 $cm^3$ $min^{-1}$. Separacija je postignuta na 35 °C primenom $C_{18}$ kolone. Kvantifikacija *trans*-resveratrola vršena je na 306 nm. Rezultati su pokazali da je konstruisana kalibraciona kriva linearna u opsegu koncentracija 1–40 μg $cm^{-3}$. Sračunate vrednosti *LOD* i *LOQ* iznosile su 0,125 i 0,413 μg $cm^{-3}$, redom. Antioksidativna aktivnost ektrakata određena je primenom DPPH testa. Sposobnost inhibicije DPPH radikala smanjuje se sledećim redosledom: ekstrakt egzokarpa grožđa > standard *trans*-resveratrola > ekstrakt semena grožđa.

*Ključne reči*: Validacija • RP-HPLC • *trans*-Resveratrol • Ekstrakt grožđa • Antioksidativna aktivnost