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Analysis and Quantification of *trans*-Resveratrol in Wines from Alentejo Region (Portugal)

Nuno Ratola[1], *Joaquim Luís Faria*[2] *and Arminda Alves*[1]*

[1]LEPÆ – Departamento de Engenharia Química, Faculdade de Engenharia da Universidade do Porto, Rua Dr. Roberto Frias, P-4200-465 Porto, Portugal

[2]Laboratório de Catálise e Materiais, Departamento de Engenharia Química, Faculdade de Engenharia da Universidade do Porto, Rua Dr. Roberto Frias, P-4200-465 Porto, Portugal

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Summary

A simple procedure for determination of *trans*-resveratrol in wines from Alentejo region delimited appellation (Portugal) is described and validated. A set of 47 red and 21 white wines was analysed by direct injection in high performance liquid chromatograph with UV detector. A detection limit of 0.06 mg/L was achieved. Global uncertainty associated with the results, according to EURACHEM/CITAC rules, ranged from 16.33 to 27.15 %. *Trans*-resveratrol was detected in all red wines and in 8 white wines. The amount was consistently higher in the red wines (up to 2.64 mg/L), when compared to the white wines (never exceeding 0.19 mg/L).

Key words: *trans*-resveratrol, wines, HPLC, method validation

Introduction

While cure for cancer escapes continuous research efforts in developed countries, one in every four persons, on average, is likely to suffer from this mortal illness. From the chemical point of view, some molecules have been identified because of their roles as protective agents. Special attention has been given to *trans*-resveratrol (*trans*-3,4',5-trihydroxystilbene) due to its proved relation with anti-initiation, anti-promotion and anti-progression activities in malignant tumours (*1*) and its recognition as a chemotherapeutic agent in humans, acting upon leukaemia and breast carcinoma cells (2). *Trans*-resveratrol is a phytoalexin of a phenolic nature, belonging to the stilbene family, which was detected in grapes, wine, peanuts, soy, tea and other plants (*3*), as well as in rat and human blood and urine after oral administration (*4,5*).

Wine, particularly red wine, is the main source of introduction of *trans*-resveratrol in the human diet. The attention of the scientific community was primarily triggered by a study on a phenomenon known as the »French Paradox« (*6*), in which residents from Toulouse (France) were found to have a low cardiovascular disease incidence, despite a diet prone to such events. Red wine is markedly present in such diet and *trans*-resveratrol was identified as one of the elements responsible for the observed beneficial activity. Later it was found to be present in significant amounts in grape skin (*7,8*) and in certain wines (*9*).

Red wine generally contains higher amounts of *trans*--resveratrol than white wine. This is presumably due to the longer extraction time during contact between grape skin and juice in the production of red wine. Wines of *rosé* type do have intermediate values between red and white wine (*10,11*), which is in line with the previous conclusion.

* Corresponding author; Phone: ++351 22 50 81 883; Fax: ++351 22 50 81 449; E-mail: aalves@fe.up.pt

Variations on the contents of *trans*-resveratrol from different sources of the same kind of wine appear to be related to the wine-making process, rather than to the source itself (*12–14*). In spite of this, average temperature of the region is reported as an important factor which seems to be responsible for some observed fluctuations within a given variety (*15*).

Since the pioneer work of Siemann and Creasy (*9*), several authors have identified and quantified *trans*-resveratrol and other stilbenes in wines from many different regions and countries. Regarding Portugal, some results for local wines can be found in a global survey on *trans*-resveratrol concentrations in worldwide commercial wines (*15*). Only two works focusing on Portuguese wines can be found in literature (*16,17*), but they are limited to specific regions and do not include the wines from the Alentejo region. In order to increase the database of Portuguese wines, more studies are required.

The quantification methods of resveratrol (Fig. 1) are evolving very rapidly. A simple, fast and reliable method is still being sought. Early works used HPLC with UV detection (*9,18*) or a fluorimetric detector (*19,20*) and more recently GC-MS (*21*). In these analyses sample pre-treatment was always required, which is a time-consuming procedure. Direct injection without pre-treatment or post-derivatisation was attempted using a HPLC with electrochemical detection (*22*), and GC-MS (*23*), which saves the time of analysis while maintaining high sensitivity. Recent works refer to capillary electrophoresis separation (*24,25*), HPLC with diode array UV/VIS detection (*26,27*) and reverse phase HPLC (*28*), as well as techniques of solid phase micro-extraction (SPME) followed by GC-MS (*29*).

In the present study, a technique of easy implementation in quality control laboratories is described, based on direct injection in HPLC with UV detection. It was decided to focus on the determination of the *trans*-isomer (Fig. 1a), in detriment of the *cis*-isomer (Fig. 1b), due to greater importance of the former in terms of therapeutic properties.

The aim is the validation of an expedite method of analysis, assessing the global uncertainty of the sample results, besides the elucidation of *trans*-resveratrol contents of Portuguese wines from Alentejo wine region (trying to infer differences between sub-regions). The novelty is the special focus on the global uncertainty, according to EURACHEM/CITAC rules (*30*), in order to provide meaningful information about the significance of the numbers obtained.

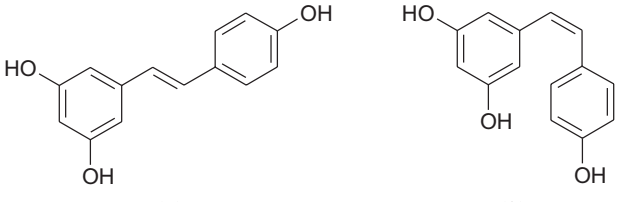

Fig. 1. Chemical structure of (a) *trans*- and (b) *cis*-resveratrol (alias 3,4′,5-trihydroxystilbene)

Materials and Methods

Reagents and chemicals

The *trans*-resveratrol standard (99 % GC) was purchased from Sigma-Aldrich Co. Methanol (Chromasolv for HPLC) was obtained from Riedel-de Haën, acetonitrile (LiChrosolv for HPLC) from Merck and glacial acetic acid (100 % p.a.) from Pronalab. All other chemicals were of *pro analysis* grade. Twice distilled and demineralised water was used for preparation of the aqueous solutions.

Standard solutions

A stock solution of 20 mg/L of *trans*-resveratrol was prepared in a 12 % alcoholic (aqueous) solution. The solid standard was initially dissolved in a minimum volume of ethanol, before the addition of water, in order to guarantee a complete dissolution. Immediately prior to analysis, a set of standards with 0.1, 0.3, 0.5, 0.7 and 1.0 mg/L were prepared from the stock solution, diluted in a 12 % alcoholic (aqueous) solution. Special care was taken in relation to the degradation of the standard solutions, keeping them protected from air and light exposure.

Sample handling

In this study 68 samples of commercial wines (47 red and 21 white) from Alentejo region were analysed. Red wines were blended with the varieties Trincadeira, Moreto, Tinta Caiada, Aragonez and Castelão, and white wines with the varieties Roupeiro, Antão Vaz, Rabo de Ovelha, Perrum and Arinto. All samples were diluted (1:3) in a 12 % alcoholic (aqueous) solution, filtered through Schleicher and Schuell filters (0.45 μm) and kept from light at 4 °C. Although the most common type of isomerisation is *cis*- to *trans*-, special care was taken in order to avoid exposure to light and thermal heating of the wine samples and standards. Several works do refer the influence of light on *trans*-resveratrol oxidative degradation and *trans*- to *cis*- conversion, which can occur in relatively short periods of time (*11,14,19*).

Comparing the results using three different procedures assessed the need of an initial step of substrate extraction from the wine sample. First a liquid-liquid extraction (using *n*-hexane as extracting solvent) was performed before the injection of the sample in the chromatograph. Second procedure consisted of a solid phase extraction (using C18 mini-columns) before injection. Finally, in the third procedure the wine sample was simply diluted in a 12 % alcoholic (aqueous) solution and filtered, before chromatographic analysis.

HPLC procedure

Each standard and sample was injected in duplicate in a Knauer chromatograph with UV detection, equipped with a C18 Lichrokart column from Merck (250 × 4.6 mm, with 5-μm particles) and pre-column with the same stationary phase. Mobile phase consisted of a water/acetonitrile/acetic acid mixture (volume ratio 70/29.9/0.1), with a flow rate of 1.0 mL/min. Injection volume was 20 μL. Detection was performed at a 310 nm wavelength. Run time was 15 min. Quantification was based on the

peak area, using the external standard method, with results integrated and displayed by a Hitachi D-2500 Chromato-Integrator.

Results and Discussion

Analytical methodology

The liquid-liquid extraction and solid phase extraction procedures for sample treatment led to a lower detection limit, and failed to give a good reproducibility. A coefficient of variation of about 30.0 % was obtained for independent extractions of the same sample. On the other hand, direct injection of the diluted sample gave a coefficient of variation of 9.99 % (corresponding to 12 samples with an average content in *trans*-resveratrol of 0.18 mg/L), for a detection limit of 0.06 mg/L, (calculated by the sum of the intercept and three times $s_{y/x} = [\Sigma(y_i - y_{icalc})^2/(n-2)]^{1/2}$, where y_i are experimental values and y_{icalc} are calculated by the calibration curve). For the latter procedure the recovery average was (110±14) %, using standard addition of 0.5 mg/L *trans*-resveratrol to a random universe of 15.0 % of all analysed samples.

Based on these results, the direct injection procedure was adopted despite the potential increase of interference in the initial stages of each chromatogram and less favourable detection limit. These setbacks can be explained by the fact that the detection is made at 310 nm, where several substances present in wines absorb. Any extraction procedure performed would reduce such interference proneness, allowing a more stable baseline and, consequently, a lower detection limit. Whenever doubts in the identification of the peak for *trans*-resveratrol arose in consequence of interferent peaks in its vicinity, the technique of standard addition to the sample (at different concentrations) was employed to confirm *trans*-resveratrol peak identity.

However, the chromatograms of the standards are free from interferences, with the peaks of the solvent and *trans*-resveratrol clearly separated (Fig. 2a). Regarding the wine samples it is noticeable that in red wines (Fig. 2b) the interferences are more significant than those in white wines (Fig. 2c), in spite of the lower amounts found in the latter samples.

The determination of the *trans*-resveratrol alone proved to be accurate using a simple procedure, without sample pre-treatment other than dilution and filtration, allowing for a less than 15-minute analysis run time.

The global uncertainty associated with sample results obtained by different analytical methodologies is usually not given in the literature data, making a reliable comparison between methods quite difficult. In fact, no similar studies were found for *trans*-resveratrol determination. Many innovating analytical methodologies are described and validated without consistent comparison against other methods. A simple way to overcome the difficulty of assessing comparisons, either by absence of reference materials or by difficulties to find available inter-laboratorial studies in specific areas, is the estimation of the global uncertainty, according to EURACHEM/CITAC (*30*). This estimation accounts for the most significant sources of uncertainty that are thought to interfere in the final result.

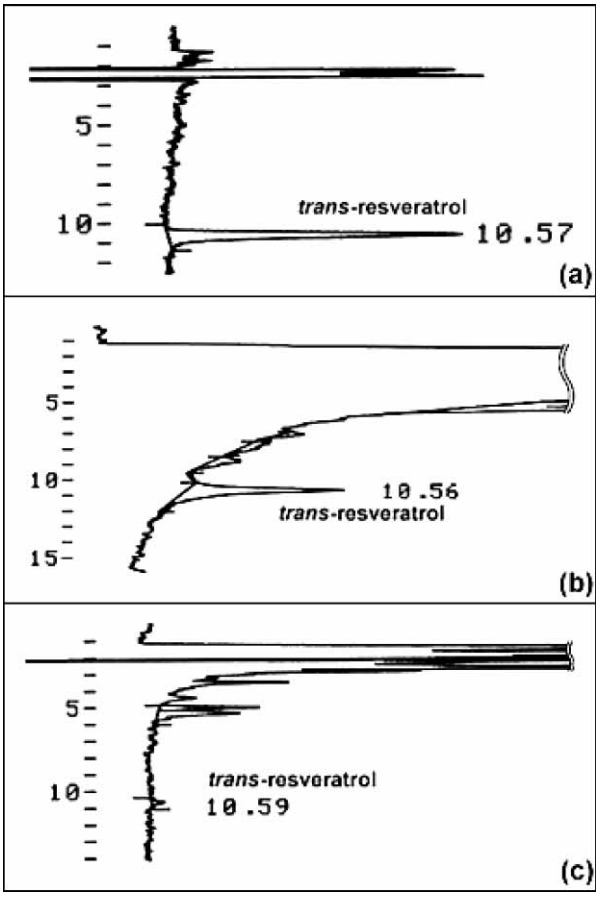

Fig. 2. Chromatogram for: (a) a *trans*-resveratrol standard solution; (b) a red wine sample; (c) a white wine sample

In this work, the contributions to uncertainty were ascribed to: (*i*) the uncertainty in the preparation of standards; (*ii*) the uncertainty of the calibration curve; (*iii*) the uncertainty in the precision of the method; and (*iv*) the uncertainty in the accuracy. Expressing each term of relative uncertainties (Table 1), it can be concluded that the calibration curve is the higher source of uncertainty, the weight increases when the concentration decreases. If concentration is above 0.25 mg/L, the global uncertainty is around 16.3 %, increasing to 27.2 % below 0.10 mg/L (Fig. 3).

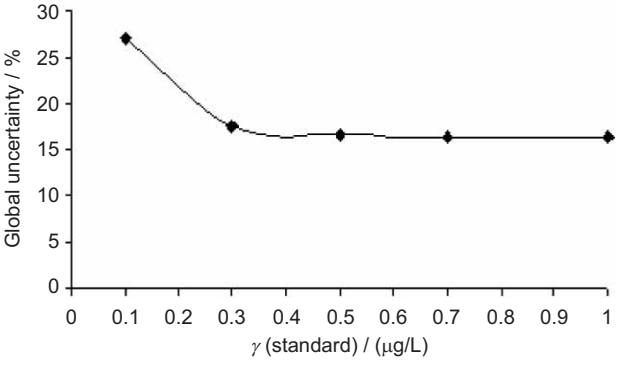

Fig. 3. Global uncertainty as a function of *trans*-resveratrol standards concentration

Table 1. Components used for global uncertainty calculation

Components of global uncertainty	Formula	Legend
Standard preparation (U_1=u_{st})	$u_{st} = \sqrt{\sum_i \left(\frac{\Delta m_i}{m_i}\right)^2}$	Δm_i – error associated to a measurement; m_i – measured value
Calibration curve determination (U_2=s_{xo}/x_o)	$s_{xo} = \frac{s_{y/x}}{b}\left\{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2\sum(x_i - \bar{x})^2}\right\}^{1/2}$	s_{xo} – concentration standard deviation, calculated from the calibration curve
Precision (U_3=u_p/x_o)	$u_p = \frac{s}{\sqrt{n}}$	s – standard deviation for precision assays; n – number of assays
Accuracy (U_4=u_e)	$u_e = \frac{[Max(\eta) - Min(\eta)]}{\sqrt{n}\cdot Average(\eta)}$	η – values for recovery assays; n – number of assays
Global Uncertainty (U)	$U = \sqrt{U_1^2 + U_2^2 + U_3^2 + U_4^2}$	

Quantification of trans-resveratrol in Portuguese wines

In the work of de Revel *et al.* (*16*), Portuguese red wines from different regions had *trans*-resveratrol contents from 0.3 to 4.5 mg/L and white wines had values ranging from 0.13 to 0.51 mg/L. A more recent report (*17*) describes the analysis of 46 red wines with average concentrations of *trans*-resveratrol of 1.3 mg/L (maximum 5.7 mg/L) and 74 white wines with average concentrations of 0.6 mg/L (maximum 2.1 mg/L), respectively.

In our study, the occurrence of *trans*-resveratrol was confirmed in all of the 47 red wines analysed, with concentrations ranging between 0.13 and 2.64 mg/L. As for the white wines, only 8 of the 21 tested revealed the presence of the compound, and only 4 of those had concentrations above the detection limit. The highest value for white wines was 0.19 mg/L, and on average close to 0.100 mg/L.

The samples were taken from the Alentejo region in Portugal, characterised by extremely high temperatures in the summer and reduced rainfall during almost all the maturation season. The wines came from eight different delimited sub-regions (Fig. 4). The impossibility to obtain a correlation between the *trans*-resveratrol contents and the origin of the particular wine (according to a given sub-region) limits a possible distinction between them on this basis. However, the number of wine samples for each region was not large enough to establish reliable conclusions (Table 2).

Table 2. *Trans*-resveratrol content for red and white wines of 1999, according to each delimited region of Alentejo

Region	Number of red wines	*trans*-resveratrol* mg/L	Number of white wines	*trans*-resveratrol* mg/L
Portalegre	6	1.24 ± 0.81	–	–
Borba	1	0.26	–	–
Redondo	3	0.59 ± 0.34	–	–
Reguengos	7	0.92 ± 0.34	1	n.d.
Évora	9	0.47 ± 0.20	2	n.d.
Moura	11	0.55 ± 0.35	8	0.1 ± 0.03 [a]
Granja	10	0.81 ± 0.85	9	n.d.
Vidigueira	–	–	1	0.19

* average ± standard deviation
n.d. non-detected (detection limit = 0.06 mg/L)
[a] 5 wines out of 8 below the detection limit

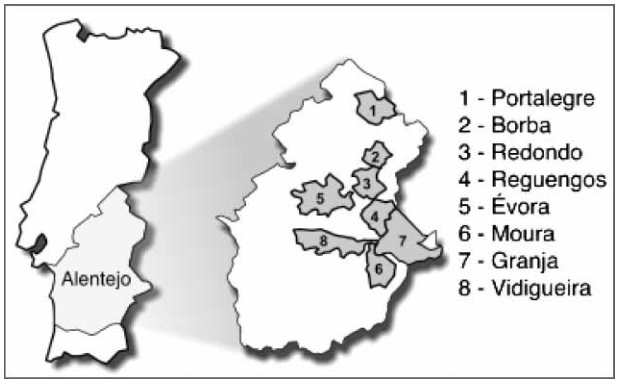

Fig. 4. Alentejo delimited regions and their geographic location in Portugal

Attempts to correlate *trans*-resveratrol levels with some chemical characteristics of the wines analysed including volumetric alcoholic content (VAC), reduced sugars, total acidity and pH, show that these parameters are rather constant, irrespective of the *trans*-resveratrol content in either red (Fig. 5) or white (Fig. 6) wines. This probably means that there is no relationship between *trans*-resveratrol and such parameters. This is reasonable to assume, considering its chemical thermal stability. However, a correlation with other biochemical factors is not excluded, affecting namely the photochemical development of this compound in the grapes during growth by exposure to light and its subsequent transfer to the wine, throughout the winemaking process.

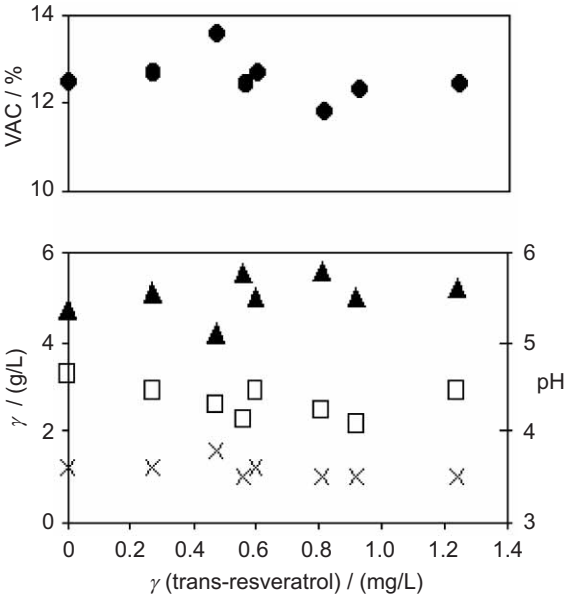

Fig. 5. Comparison between *trans*-resveratrol, VAC (●), reduced sugars (□), total acidity (▲) and pH (×) levels in red wines of Alentejo region

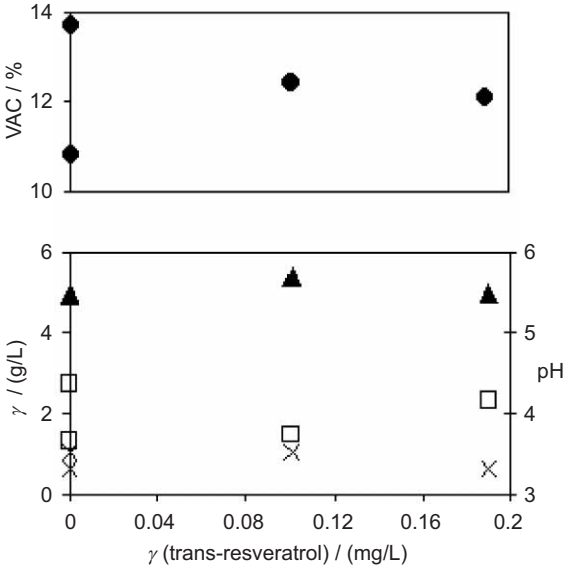

Fig. 6. Comparison between *trans*-resveratrol, VAC (●), reduced sugars (□), total acidity (▲) and pH (×) levels in white wines of Alentejo region

Conclusion

This work enabled the implementation of an easy and reliable methodology for *trans*-resveratrol analysis in wines. The use of direct injection in HPLC with UV detection allows the screening of a large number of samples in a reduced time span, as required for statistical analysis. For concentrations of above 0.25 mg/L, the global uncertainty of the method is around 16.3 %, increasing to 27.2 % below 0.10 mg/L, which is acceptable for such concentration ranges.

As expected, the levels of *trans*-resveratrol are clearly higher in red wines, most of the white wines being actually under the detection level of the method (0.06 mg/L). The levels of *trans*-resveratrol found here are comparable to those found by other authors (*15–17*), which demonstrates the suitability of this very simple analytical technique. The somehow higher values in *trans*-resveratrol contents found here can be taken as a positive characteristic of the wines from Alentejo region in relation to what can be considered as a hale and hearty diet, as established by the »French Paradox«. Not surprisingly, the values found for wines within the same region are quite disperse, which is in line with the collected evidence that *trans*-resveratrol content is a function of many variables, including local and environmental factors in the vineyard, but also the grape variety, and the wine processing technique.

From a public health point of view, the values obtained may suggest that the physiological effects on the consumers of Portuguese wine resulting from the daily intake of stilbenes can be of some significance. However, it should be taken in consideration that *trans*-resveratrol only represents a percentage of all stilbenoid compounds present in wine.

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Analiza i kvantifikacija *trans*-resveratrola u vinima iz pokrajine Alentejo (Portugal)

Sažetak

Opisan je i potvrđen jednostavan postupak za određivanje *trans*-resveratrola u vinima iz pokrajine Alentejo (Portugal). Analizirano je 47 crvenih i 21 bijelo vino izravnim ubrizgavanjem u HPLC s UV-detektorom. Granica detekcije iznosila je 0,06 mg/L. Ukupna je nepouzdanost rezultata, prema pravilima EURACHEM/CITAC, bila od 16,33 do 27,15 %. U svim crvenim vinima i u 8 bijelih vina nađen je *trans*-resveratrol. Količina *trans*-resveratrola bitno je veća u crvenim (do 2,64 mg/L) nego u bijelim vinima (vrijednosti nikada nisu bile veće od 0,19 mg/L).