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Rapid method for resveratrol determination by HPLC with electrochemical and UV detections in wines

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Abstract

The study describes an improved version of the reverse-phase-HPLC method. To determine free resveratrol isomers, the method uses isocratic elution and electrochemical detection with the use of a glassy working electrode at a potential of 0.75 V. The effects of important factors – such as the isomerization of standard resveratrol solution in diffused daylight and its dependence on temperature or resveratrol isomerization in wine samples – were investigated. The equilibrium between *trans*- and *cis*-isomer was achieved after a 5-h exposure to the daily diffused light and it was not significantly influenced by the temperature (30–60 °C). Linearity was obtained in the concentration range from 0.01 to 10 mg l^{-1}. The detection limit ($S/N = 3$) was 3 μg l^{-1} for *trans*-resveratrol and 15 μg l^{-1} for *cis*-resveratrol. The *trans*- and *cis*-resveratrol were determined in selected Czech red and white wines and in the extract from *Vitis vinifera* plant. The concentration of *trans*-resveratrol ranged from 0.7 to 11 mg l^{-1}, that of *cis*-resveratrol from 0.6 to 5.1 mg l^{-1}.

Keywords: Resveratrol; Isomerization; Wine; *Vitis vinifera*; HPLC; Electrochemical detection

1. Introduction

Flavonoids and phenolic acids are widely present in higher plants and form a part of the human diet. As well as other plant phenolics, they have been reported to have multiple biological effects such as antioxidant or antimicrobial activities (Burns et al., 2000). Resveratrol (Fig. 1), as a polyphenol, is an antioxidant and a free radical scavenger.

Grapes and wines contain large amounts of phenolic compounds, mostly flavonoids. At high concentrations of 1000–1800 mg l^{-1}, a large part of phenolics found in wines may act as antioxidants (Kanner, Frankel, Granit, German, & Kinsella, 1994). Resveratrol (*trans*-3,5,4′-trihydroxystilbene) has been identified as the major active compound of the stilbene phytoalexins. It exists as *trans* and *cis* isomers, both of which are present in biological materials in wide concentration range. The wave of interest in monitoring the presence of resveratrol in wine arose when epidemiological studies showed an inverse correlation between the consumption of red wine and the incidence of cardiovascular diseases – the so-called French paradox. Resveratrol has antibacterial and antifungal properties and induces platelet hypoaggregation in rats; it protects the liver from lipidic peroxidation and inhibits the oxidation of low-density lipoproteins (LDL), modulating their metabolism. The *cis*-isomer have potential anticancer activity, as do the *trans* isomers, by inhibiting protein-tyrosine kinase (Jayatilake et al., 1993). More recently, the carcinopreventive activity of resveratrol has been proved (Palomino, Gómez-Serranillos, Slowing, Carretero, & Villar, 2000). Consequently it has not been clear yet whether biological activity of *cis* form differs from *trans* isoform. There has also been interest in the presence of resveratrol in wine in connection with its protective effects against heart diseases.

Resveratrol was firstly detected in *Vitis vinifera*, where it is accumulated in leaf tissue in response to mechanical injury and exposure to UV light, aluminium

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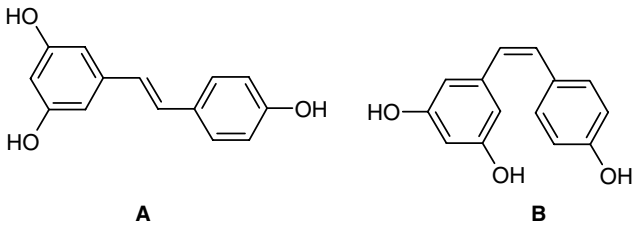

Fig. 1. Structures of the *trans*- isomer (**A**) and *cis*-isomer (**B**) of resveratrol.

chloride (Adrian, Jeandet, Breuil, Levite, Deborg, & Bessis, 2000) or to fungal attack, mainly by *Botrytis cinerea* and *Plasmospora viticola*. Irradiation of plant tissue with UV light has important effects on phenolic metabolism. UVB light irradiation seems to be associated with an increased concentration of the enzyme responsible for flavonoid biosynthesis, flavonoids being able to act as UV screens preventing the UV-induced damage in the genetic material of plant cells (Cantos, García-Viguera, De Pascual-Teresa, & Tomás-Barberán, 2000; Dong, Mitra, Koostra, Lister, & Lancaster, 1995).

During the last decade, several methods have been developed. The most widely used methods for quantification of resveratrol are gas chromatography (GC)-coupled with MS (Goldberg, Karumanchiri, Ng, Yan, Diamandis, & Soleas, 1995a, 1995b; Soleas, Goldberg, Ng, Karumanchiri, Tsang, & Diamandis, 1997), capillary electrophoresis (CE) (Arce, Tena, Rios, & Valcárcel, 1998; Chu, O'Dwyer, & Zeece, 1998) and high-performance liquid chromatography (HPLC) (McMurtrey, Minn, Pobanz, & Schultz, 1994; Vrhovsek, Eder, & Wendelin, 1995). These methods generally involve extraction and derivatization that require extensive precautions such as creating exclusive nitrogen environment or ensuring protection from UV light. Generally, HPLC methods use a C18 normal phase- or a reverse phase column. They have also been used for a direct analysis of resveratrol when the separation was coupled with photodiode array detection (Goldberg, Tsang, Karumanchiri, Diamandis, Soleas, & Ng, 1996; López, Mártínez, Del Valle, Orte, & Miró, 2001) – either UV or electrochemical detection (McMurtrey et al., 1994; Vrhovsek et al., 1995).

The high sensitive fluorimetric detection (Jeandet et al., 1997; Viñas, López-Erroz, & Marín-Hernández, 2000) of stilbenes – which is more specific than the UV detection (Goldberg et al., 1995a, 1995b; Lamuela-Raventós & Waterhouse, 1993; Vrhovsek et al., 1995) – was subsequently used to determine the content of resveratrol in grapes and wine. The LC with electrochemical detection (McMurtrey et al., 1994; Zhu, Huang, Cregor, Long, Kissinger, & Kissinger, 2000) has proved to be at least as selective and as sensitive to resveratrol and other phenolic compounds in natural sources as detections based on the fluorescence detection (McMurtrey et al., 1994; Melzoch, Filip, Buckiová, Hanzlíková, & Šmidrkal, 2000).

In this contribution we present completely new experimental set up based on classical HPLC method with acetonitrile as a mobile phase that was established due to easier and more accurate detection of phenolic compound – resveratrol isolated from Czech red and white wines and also from plant extracts, particularly *Vitis Vinifera*. We coupled reverse-phase HPLC not only with UV detection but also with electrochemical detection. This experimental set up allows direct injection of samples without any prior purification. It leads to significant time reduction of analyses. Another advantage is sufficient separation and quantification of *trans* and *cis* resveratrol isoforms even though its concentration limits are very low.

This effective analytical method may be used universal for identification and quantification of phenolic compounds in wines and plants extracts.

2. Material and method

2.1. Resveratrol

Trans-resveratrol was synthesized in the crystalline form and at a purity of 99% (Šmidrkal, Filip, Melzoch, Hanzlíková, & Buckiová, 2001). *Cis*-resveratrol was obtained after a 10-h exposure of a standard *trans*-resveratrol ethanolic solution (100 $mg\,l^{-1}$) to the diffused daylight (it was examined by NMR). Under these conditions, 80% of *trans*-resveratrol was converted into the *cis*-isomer.

2.2. Samples

Samples of the raw plant material included *Vitis vinifera* and commercial red and white wines, harvested between 1986 and 1999, and produced in the Bohemian (Most, Velké Žernoseky, Litoměřice, Roudnice) and Moravian (Čejkovice, Hodonín, Dubňany, Lednice) vineyard regions. The samples were obtained directly from the wineries. The wines were made from varieties commonly growing in these regions (blue grapes: Blaufränkisch, Pinot noir, Saint Lawrence, Cabernet sauvignon, Cabernet moravia, Zweigeltrebe, Laurot, Tintet, Neronet, Merlot; white grapes: Erilon, Rubikon, Hibernal).

2.3. Extraction

The samples of the grapes, rachises and leaves were extracted in 80% (v/v) ethanol solution. The plant material was used in its fresh state after grinding. The published results refer to the dry matter. The extraction

was carried out in darkness at a temperature of 20 °C and lasted 24 h. Consequently, the liquid was separated from solids by filtration.

2.4. Calibration

Resveratrol was dissolved in ethanol at a concentration of 1 mg l^{-1} and stored away from direct light at 4 °C until used. The standard ethanolic solution of resveratrol was diluted and reference solutions in the "working" range from 10 μg l^{-1} to 10 mg l^{-1} were obtained. These solutions were analyzed and the corresponding peak areas were compared the concentration of resveratrol injected. Twenty microlitre of each solution was injected into the HPLC. The concentration of resveratrol was calculated from the peak areas using the normalization method.

2.5. HPLC analysis

Trans- and *cis*-resveratrol were determined by the HPLC method using the TSP 3500 liquid chromatograph (TSP, USA) equipped with TSP 4100 UV detector (TSP, USA) and the HP 1049 electrochemical detector with glassy working electrode (Hewlett–Packard, USA) at a potential of 0.75 V, coupled to the Apex Data Station (Apex, Czech Republic). The sample was injected with the Rheodyne valve (Cotati, USA) into the column filled with the stationary 120-5-C18 reverse-phase Nucleosil (250 × 4 mm, 5 μm; Supelco, USA), with the pre-column (10 × 4 mm) packed with the same stationary phase. The separation was carried out at room temperature. The isocratic elution at a flow rate of 1.0 ml/min used the mobile phase of 25% acetonitrile, 0.1% H_3PO_4 and NaCl ($c = 5$ mmol/l) in demineralized water. After each analysis, the column was rinsed with mobile phase for 10 min. Identification and quantification of resveratrol isomers were carried out by the internal or external standard techniques.

3. Results and discussion

3.1. UV detection

The *trans*-resveratrol spectrum's maximum ranged from 306 to 320 nm (Fig. 2). The spectrum of *cis*-resveratrol reached its maximum at 295 nm (Fig. 2). For the detection of both isomers of resveratrol, the optimal wavelength of 306 nm was used.

3.2. Electrochemical detection

Then, the optimal potential for resveratrol determination was sought. The optimal potential is to be understood as a potential at which the resveratrol peak is sufficiently high, the limit of detection minimal (= it is possible to determine a very low resveratrol content) and the ratio of the peak height to the noise maximal (= maximal peak height and minimal noise) (Table 1). The working potential was optimized using a standard resveratrol 50% ethanolic solution. The value of 0.75 V proved to have the best potential with very good selectivity aspect.

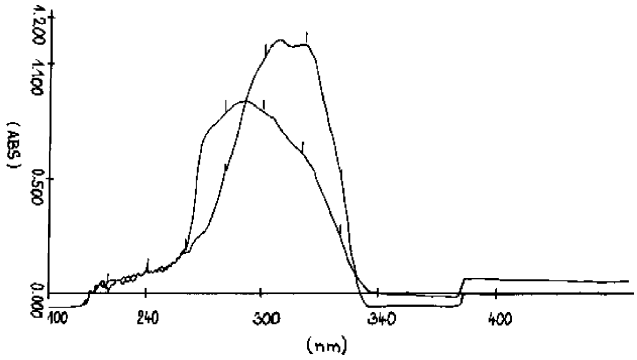

Fig. 2. UV spectrum of *trans*- and *cis*-resveratrol.

3.3. HPLC method validation

3.3.1. Linearity and detection limit

The linearity of response was examined by analyzing solutions in a range of concentration between 0.01 and 10 mg l^{-1}. The correlation coefficient of the linear regression of the standard curves was greater than 0.999 for both detectors. *Trans*-resveratrol calibration curves equations of electrochemical detection (Eq. (1)) and of UV detection (Eq. (2)) were (see below):

$$c_{ec} = 0.0008 * x_{ec} - 0.151 \quad (1)$$

$$c_{uv} = 0.0065 * x_{uv} + 0.0376 \quad (2)$$

(c_{ec}, c_{uv} – concentration of *trans*-resveratrol (mg l^{-1}); x_{ec}, x_{uv} – peak area (mV s)).

We could see that the calibration curve was linear. The above range of concentrations covered more than 95% of red and white wines and of vine extracts. The range of *cis*-resveratrol isomer could not be strictly predetermined because the isomer was obtained by the exposure of the pure *trans*-isomer solution to the diffused daylight. However, the range was confirmed by the fact that the sum of both of the isomers after irradiation was equal to the initial concentration of *trans*-isomer.

Detection limits (LOD) were determined using progressively lower concentrations for a signal/noise ratio of 3:1 ($S/N = 3$) with an injection volume of 20 μl. The limits of detection were lower than 30 μg l^{-1} (306 nm) and 3 μg l^{-1} (0.75 V) for *trans*-resveratrol and 100 μg l^{-1} (306 nm) and 15 μg l^{-1} (0.75 V) for *cis*-resveratrol.

Table 1
Optimization of electrochemical potential of working glassy electrode for resveratrol assay

Potential (mV)	Peak height *trans*- (mV)	Peak height *cis*- (mV)	Total height (mV)	Noise (mV)	Detection limit (*trans*-, *cis*-resveratrol) (mV)	Height noise ratio
0.10	0	0	0	0.0050	0.0100	0
0.30	0	0	0	0.0050	0.0100	0
0.50	0	0	0	0.0050	0.0100	0
0.60	0.260	0	0.260	0.0050	0.0100	52
0.70	0.572	0.017	0.589	0.0060	0.0120	98
0.75	0.615	0.100	0.715	0.0041	0.0082	174
0.80	0.608	0.031	0.639	0.0048	0.0095	133
0.90	0.713	0.145	0.858	0.0046	0.0184	186
1.00	0.513	0.339	0.852	0.0043	0.0085	198
1.10	0.348	0.415	0.763	0.0048	0.0095	159
1.20	0.281	0.584	0.685	0.0046	0.0092	188

Table 2
Results of recovery experiments for real samples ($n = 3$)

Samples	*trans*-Resveratrol original amount	*trans*-Resveratrol added amount	*trans*-Resveratrol found	Recovery (%)	RSD (%)
Red wine	4.653 ($mg\,l^{-1}$)	1.000 ($mg\,l^{-1}$)	5.576 ($mg\,l^{-1}$)	98.63	1.45
White wine	1.216 ($mg\,l^{-1}$)	0.500 ($mg\,l^{-1}$)	1.670 ($mg\,l^{-1}$)	97.35	2.16
Vine leaf	3.654 ($mg/kg^{-1}_{d.m.}$)	1.950 ($mg/kg^{-1}_{d.m.}$)	5.357 ($mg/kg^{-1}_{d.m.}$)	97.18	1.89
Rachis	368.2 ($mg/kg^{-1}_{d.m.}$)	145.0 ($mg/kg^{-1}_{d.m.}$)	498.7($mg/kg^{-1}_{d.m.}$)	98.75	2.64
Grape berry	0.312 (mg/kg^{-1}_{berry})	0.150 (mg/kg^{-1}_{berry})	0.479 (mg/kg^{-1}_{berry})	101.3	3.04

3.3.2. Recovery

The recovery for *trans*- and *cis*-resveratrol were determined by adding known amounts of resveratrol to red and white wines and to vine extract and by performing quadruplicate assays before and after addition. The recovery was $97.2 \pm 4.3\%$ (Table 2).

3.3.3. Repeatability

The repeatability of the analytical method was tested by the repeated injection of the diluted standard ethanolic solution of *trans*- and *cis*-resveratrol ($n = 6$). The relative standard deviation (RSD) from the peak area or from the estimated concentration was found to be 2.3% for *trans*-resveratrol and 5.1% for *cis*-resveratrol. To set the RSD Dean-Dixen and Grubbs tests were used.

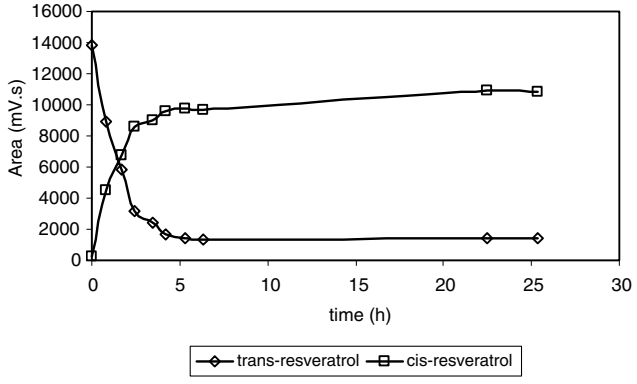

Fig. 3. *Trans*- and *cis*-resveratrol isomeration during exposition in diffused daylight. (Resveratrol concentrations were determined by HPLC with electrochemical detection).

3.4. Stability of standard resveratrol solution

The initial analyses of the standard resveratrol solution in the 40% ethanol showed that resveratrol, when exposed to light, undergoes changes relating to the *trans*- and *cis*-resveratrol ratio. In the solutions exposed to the daily-diffused light, the peak area of *cis*-resveratrol grew whereas that of *trans*-resveratrol became smaller. It was therefore necessary to find out the dependence of these changes on time until equilibrium is reached. Fig. 3 shows the *cis*/*trans*-isomer equilibrium reached after a 5-h exposition to the daily-diffused light. The equilibrium has moved towards *cis*-isomer. The peak area of *cis*-isomer, determined by EC detection, is 85% larger than that of *trans*-resveratrol. Moreover, the table shows that after a 3.5-h exposition, the resveratrol solution contained – apart from the *cis*- and *trans*-isomers – two unknown substances. The above-mentioned isomeration was under way at the room temperature (See Fig. 3).

3.5. Dependence of the trans-resveratrol isomeration on temperature

The dependence on temperature was measured at various temperatures (6, 20, 30, 40 and 50 °C). The resveratrol samples were analyzed after 24 h. The standard *trans*-resveratrol solution – the concentration of 10

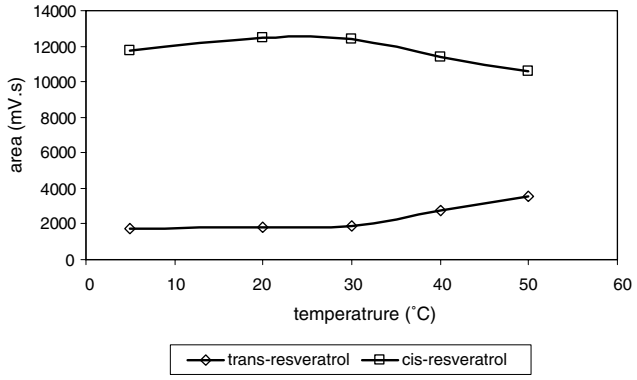

Fig. 4. Effect of temperature on the equilibrium of *trans*- and *cis*-resveratrol isomers in the resveratrol solution exposed by diffused daylight. (Resveratrol concentrations were determined by HPLC with electrochemical detection.)

$mg\,l^{-1}$ – was used and was kept away from light. The standard *trans*-resveratrol solution is not highly dependent on the changes in temperature – its *cis*/*trans* isomer ratio stays more or less the same.

With the temperature rise, the *trans*-resveratrol peak area of the equilibrated resveratrol solution grew at the expense of the *cis*-isomer (Fig. 4).

3.6. Resveratol in wines

The number of wines analyzed was approximately 300. All of them originated from the Bohemian and Moravian vineyard regions and were produced by local wineries (Tables 3, 4). Only free forms of resveratrol were analyzed. Contents of resveratrol in red wines showed relatively high variability. The concentration of *trans*-resveratrol ranged from 0.7 to 11 $mg\,l^{-1}$, that of *cis*-resveratrol from 0.6 to 5.1 $mg\,l^{-1}$. The total amount reached up to 15 $mg\,l^{-1}$. The concentration in white wines is usually lower. The results reflect the differences in the technology of white and red wine production. The total resveratrol content ranged from 0.2 to 0.9 $mg\,l^{-1}$. The tested red wines had comparatively higher resveratrol concentrations than the wines made from the grapes that grow in more hospitable climates of sunnier southern localities, e.g., California, Spain or Italy. The Czech wine-growing regions are among the northernmost winegrowing localities in Europe, which is the reason for their lower degree of ripeness when compared to the grapes from southern regions. The higher content of resveratrol in Czech grapes mirrors the unfavorable climatic conditions and higher microbiological pressure, which are greater in northern Bohemia.

3.7. Changes in the trans- and cis-resveratrol ratio in the opened wines

The wines were analyzed immediately after they were opened (0 h), and again after 48 h and after 17 days. After being opened and analyzed, the wine was resealed and stored in the original bottle at room temperature in diffused daylight until it was analyzed again. Table 5

Table 3
Resveratrol content in red wine originating from theBohemian and Moravian vineyard regions

Vineyard region *Locality*	Variety of vine	Year of harvest	*trans*-Resveratrol ($mg\,l^{-1}$)	RSD (%; $n = 3$)	*cis*-Resveratrol ($mg\,l^{-1}$)	RSD (%; $n = 3$)
Mostecká	Saint Lawrence	1998	1.035	2.73	0.781	3.89
Most	Saint Lawrence	1996	2.358	2.41	1.351	2.58
	Zweigeltrebe	1998	0.916	3.01	1.356	3.85
	Zweigeltrebe	1996	0.925	3.12	1.025	2.65
	Pinot noir	1998	1.322	2.25	2.149	2.15
	Pinot noir	1996	1.450	2.44	1.324	2.68
Žernosecká	Saint Lawrence	1998	1.100	2.61	0.712	3.52
Velké Žernoseky	Saint Lawrence	1996	1.752	2.38	1.012	2.41
	Pinot noir	1998	2.012	2.65	1.562	2.85
Žernosecká	Saint Lawrence	1998	5.565	2.16	1.213	3.11
Litoměřice	Zweigeltrebe	1998	2.141	2.50	1.456	2.96
	Pinot noir	1996	2.650	2.32	1.114	2.98
Roudnická	Saint Lawrence	1998	4.850	1.35	1.523	2.74
Roudnice nad Labem	Saint Lawrence	1996	4.961	1.85	1.741	2.66
	Pinot noir	1998	6.253	1.26	2.806	2.45
Mutěnická	Blaufrankisch	1998	1.784	3.45	1.402	2.79
Čejkovice	Blaufrankisch	1996	1.521	3.26	1.443	2.81
	Blaufrankisch	1992	3.042	2.06	2.152	2.55
	Blaufrankisch	1986	2.685	2.85	0.683	3.82

Table 4
Resveratrol content in leaves, rachises and grape berries of *Vitis vinifera* and wines originating from the Mikulov vineyard region, vintage 1999

Variety of vine	Vine leaf				Rachis				Grape berry		
	trans-($mg/kg^{-1}_{d.m.}$)	RSD (%)	*cis*-($mg/kg^{-1}_{d.m.}$)	RSD (%)	*trans*-($mg/kg^{-1}_{d.m.}$)	RSD (%)	*cis*-($mg/kg^{-1}_{d.m.}$)	RSD (%)	*trans*-(mg/kg^{-1}_{berry})	RSD (%)	*cis*-(mg/kg^{-1}_{berry})
Cabernet Sauvignon	5.012	2.1	1.321	3.12	7.056	2.41	Traces		0.723	3.15	Traces
Pinot noir	1.642	2.35	1.234	3.45	13.42	1.85	Traces		2.312	3.04	Traces
Laurot	4.422	2.22	1.625	3.05	15.23	2.02	2.012	2.21	5.841	2.75	Traces
Tintet	3.652	2.34	Traces		440.1	1.86	6.845	2.65	0.303	2.99	Traces
Neronet	9.941	2.01	Traces		210.3	1.96	2.31	2.97	0.702	3.11	Traces
Merlot	7.156	2.11	3.085	3.42	15.4	2.34	1.843	3.42	0.703	3.21	Traces
Erilon[a]	44.32	1.12	2.211	3.14	180.4	1.12	9.913	3.11	0.442	2.78	Traces
Rubikon[a]	15.41	1.42	3.612	2.79	6.041	2.01	Traces		0.201	3.14	Traces
Hibernal[a]	5.420	2.13	1.344	3.66	63.23	1.35	3.402	2.78	0.324	3.23	Traces

[a] White grapevine varieties.

Table 5
Changes in the *trans*- and *cis*-resveratrol ratio in Saint Lawrence opened wines

Locality	Year of harvest	Ratio *trans*/*cis*		
		in 0 h	After 48 h	After 17 days
Litoměřice	1998	4.45	2.63	0.52
Roudnice	1998	2.84	1.93	0.55
	1996	3.32	2.67	0.92
Žernoseky	1998	1.51	1.31	0.54
	1995	1.72	1.3	0.66
Hodonín	1997	4.75	2.49	0.47
Most	1998	1.79	1.3	0.45
	1996	1.00	0.87	0.42

shows figures relating to the Saint Lawrence variety. The wines of this variety came from various Czech regions and vineyards. The figures reflect a fall in the value of the *trans*/*cis* resveratrol ratio after 48 h. This means that the peak area of *cis*-resveratrol grows. It is therefore estimated that, due to the activity of the antioxidant

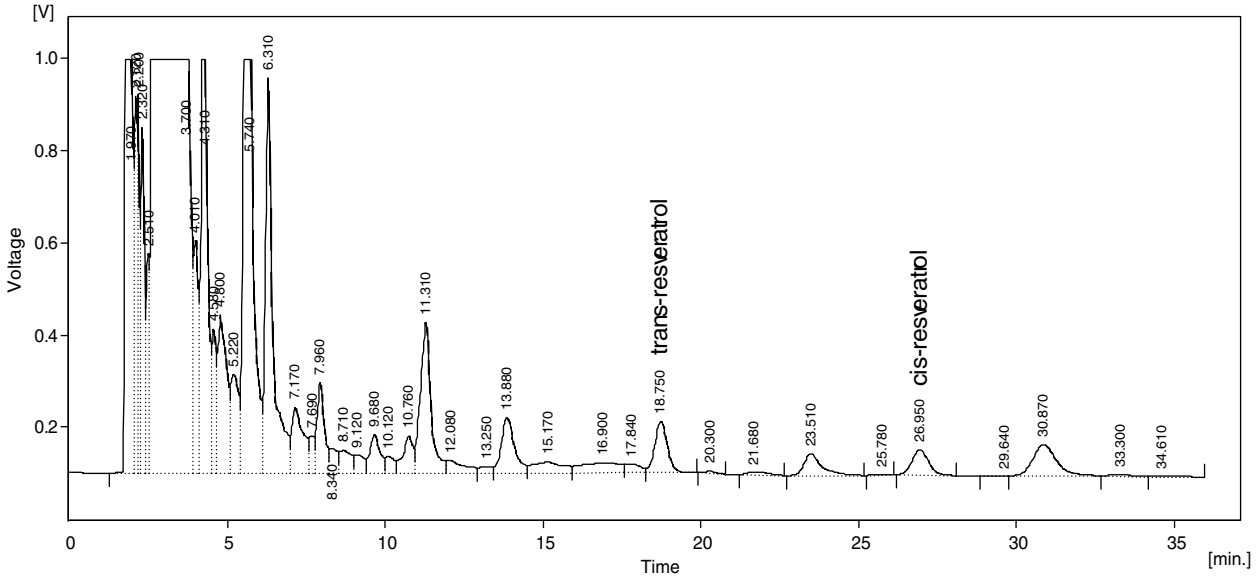

Fig. 5. Chromatogram of Pinot noir wine.

complex present in wines of the Saint Lawrence variety, it either takes longer for the *trans*- and *cis*-isomers to reach equilibrium or that the ratio at which equilibrium is reached changes. After 17 days, the phenomenon becomes even more obvious. In some case, the concentration of *trans*- and *cis*-resveratrol is higher than at the beginning. This rise in concentration can be associated with the hydrolysis of resveratrol glucosids.

3.8. Resveratol in Vitis vinifera extracts

Different parts of *V. vinifera* contain various concentrations of resveratrol. The concentration of *trans*- and *cis*-resveratrol in the vine leaves was relatively high, ranging from 2.8 to 46 mg/$kg_{d.m}$, in rachis it rose to a value of 490 mg/$kg_{d.m.}$. The *cis*-resveratrol concentration in the grapes was very low, this isomer being produced mainly during the fermentation process. The content of *trans*-resveratrol varied and reached up to 5.8 mg/kg_{berry} (Table 4).

There is no major difference between the resveratrol content in the leaf, rachis and grapes of *Vitis vinifera*. The cardinal difference between the resveratrol concentrations in the white and in the red wines, however, is caused by the wine-making technology. The technology used is very important in the initial steps during which resveratrol is released from the grape skin. The Czech wineries use both traditional and modern vinification methods, including reductive technologies or the addition of enzymes, used in the extraction of the colouring and aromatic compounds. We suppose that it is the climate, which is the predominant factor in the concentration of resveratrol in vines and wines. No significant relation to the vintage was found.

4. Conclusion

We have described a simple and sufficient method for the analysis of resveratrol. The HPLC assay brings several advantages. First, the samples are injected directly, without any prior preparatory procedures. The second advantage is the isocratic elution with a good separation of both isomers of resveratrol and of other compounds. The third advantage one is the short analysis time.

The applied method could be used to analyze natural samples such as white and red wines or plant extracts. In said conditions, interference of resveratrol isomers with other compounds related to the preservation of the purity of determination did not occur (Fig. 5). The analysis comprised more than 300 kinds of wine, coming from different Czech wineries, and 350 vine extracts. Moreover, the distribution of resveratrol isomers in *Vitis vinifera* was also investigated.

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