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Content of *trans*-resveratrol, *trans*-ε-viniferin and *trans*-δ-viniferin in young spring grapevine canes – the influence of samples drying

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Abstract: The main objective was to study the effect of sample preparation on the content of *trans*-resveratrol, *trans*-ε-viniferin and *trans*-δ-viniferin in young spring grapevine canes. The samples of six varieties of *Vitis vinifera* L. ('Hibernal', 'Malverina', 'Kolor', 'Fioletovij Augustovskij', 'Grüner Veltliner' and 'Blaufränkisch') were either lyophilised or slow dried at room temperature (three months), extracted and analysed by HPLC using DAD and FLD detectors and by LC-MS. The presence of *trans*-δ-viniferin was confirmed by comparison with *trans*-δ-viniferin prepared by dimerization of *trans*-resveratrol using laccase and its structure was verified by LC-NMR. The slow drying of the samples at room temperature enables the synthesis of a significant amount of stilbenes in all the studied varieties of *V. vinifera* canes resulting in a higher stilbene content compared to the lyophilised samples. The results of this study show the importance of the method of drying the cane sample before the extraction and analysis.

Keywords: viniferins; *Vitis vinifera;* grapevine cane; lyophilization; room temperature drying; LC-MS

Trans-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a phytoalexin produced by several plants in response to injury or attack by pathogens such as bacteria or fungi. This compound is usually accompanied in plants by other related stilbenoids like pterostilbene, pinosylvin and piceid, but also by its oligomers known as viniferins (Rivière et al. 2012). Besides other plant *trans*-resveratrol and its derivatives are present in wine, and it is believed that these substances can be linked to the French paradox. The French paradox means that the French enjoy a relatively low incidence of coronary heart disease and a relatively long lifespan, despite a diet high in saturated fats. The proposed explanations include the consumption of wine, specifically red wine, alcohol, and resveratrol, an antioxidant in wine (Lippi et al. 2010). Therefore, this particular compound has been studied intensively in recent decades. Langcake and Pryce (1976; 1977a) demonstrated, for the first time, that *trans*-resveratrol is a phytoalexin which is synthesised in grapevine

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leaf tissue after fungal infections and/or UV light irradiation. These authors also identified oxidation products of *trans*-resveratrol as *trans*-ε-, α-, β- and γ-viniferin as a dimer, trimer, tetramer, and a highly polymerised oligomer, respectively (Langcake, Pryce 1977b). The structures of *trans*-resveratrol and some of its oligomers mentioned in our study are shown in Figure 1.

The content of *trans*-resveratrol and other stilbenes in various part of grapevine plants have been described in numerous publications. The content of *trans*-resveratrol in grapevine leaves can vary between 1.0–20.9 mg/kg of fresh weight (f.w.), while it only reaches up to 2.5 mg/kg of f.w. in berries (Bábíková et al. 2008; Balík et al. 2008). Its content at the termination of the lateral shoots bearing young leaves varies between 7.0–15.6 mg/kg of f.w., while can reach even several hundred mg/kg of f.w. in the pedicels and rachises (Balík et al. 2008). Also, the stem bark of the grapevine contains stilbenes (Choi et al. 2010). Wang et al. (2010) found that the content of *trans*-resveratrol in small young grapevine plants varies between 0.2 and 16.5 mg/kg of f.w., where the highest content was found in the stems and the lowest was found in the leaves. Nevertheless, grapevine canes represent the richest source of stilbenes. The content of *trans*-resveratrol in one-year-old canes cut in the early spring, can reach several thousand mg/kg of dry weight (d.w.) (Rayne et al. 2008; Zhang et al. 2011; Vergara et al. 2012; Soural et al. 2015; Piñeiro et al. 2016). Other dominant stilbenes accompanying *trans*-resveratrol in grapevine canes are usually *trans*-ε-viniferin and r2-viniferin (Soural et al. 2015; Tříska et al. 2017). Recently, a comparative analysis of the stilbene concentration in grapevine shoots (Guerrero et al. 2020) and a reference list of phenolic compounds including stilbenes in grapevine roots, woods, canes, stems and leaves (Goufo et al. 2020) was published.

On the other hand, *trans*-δ-viniferin, a resveratrol dehydrodimer, described by Pezet et al. (2003) is a major *trans*-resveratrol dimer synthesised in the leaves of *Vitis vinifera* plants (Chasselas variety) under *Plasmopara viticola* infections and its content increased further by UV irradiation. An infestation by *P. viticola* can lead to an increase in the

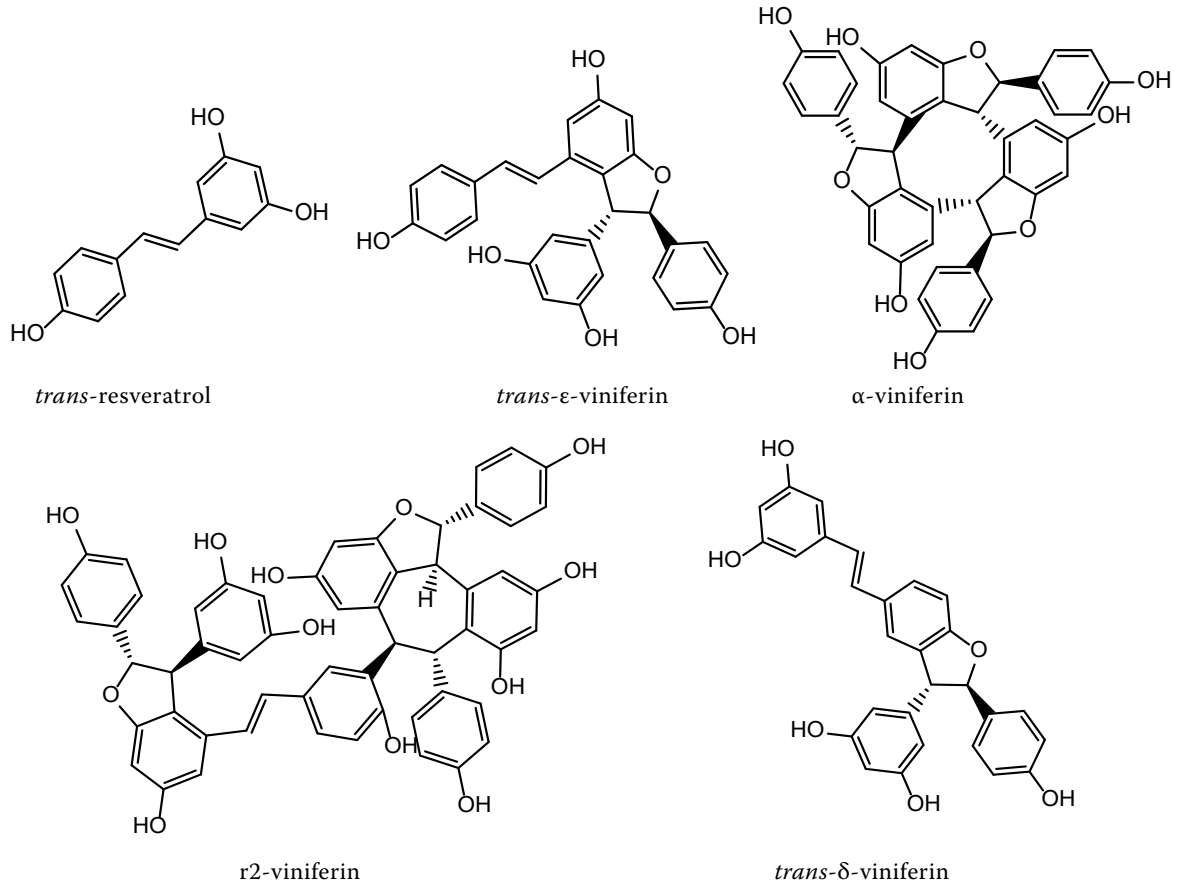

Figure 1. The structure of *trans*-resveratrol and some of its oligomers

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stilbene levels (among others *trans*-ε-viniferin and *trans*-δ-viniferin) in the leaves of different varieties *V. vinifera* and *V. riparia* as well (Boso et al. 2012).

When the Cabernet Sauvignon variety was infected by *Botrytis cinerea*, the content of *trans*-δ-viniferin increased in the leaves and flowers of the infected plants (Timperio et al. 2012). Landfeld et al. (2015) reported an increase in the *trans*-resveratrol content in the berries skin after UV irradiation, where the most pronounced effect was observed in the 'Grüner Veltliner' white variety compared to the 'Blaufränkisch' blue variety. Beside UV irradiation, ozonisation can also increase the stilbene content as evidenced by a study of the stilbene content in Superior white table grapes berries. Ozonisation seems to be an effective method as the amount of piceatannol (3',4',3,5-tetrahydroxy-*trans*-stilbene) and *trans*-δ-viniferin increased. The increase of *trans*-resveratrol and *trans*-ε-viniferin amount was even more pronounced (González-Barrio et al. 2006). The content of stilbenes (Vergara et al. 2012) and other phenolic compounds (Cebrián et al. 2017) also increases during the drying process as evidenced for one-year-old grapevine canes drying over two months at room temperature. Gorena et al. (2014) reported a five-fold increase in the stilbenoid concentration in 'Pinot Noir' during the grapevine cane storage for up to eight months, while this effect does not occur in grapevine canes that are frozen, lyophilised, or ground. It generally means that the corresponding metabolic processes are active even after harvesting and during the drying process.

This study is focused on the analysis of *trans*-resveratrol, *trans*-ε-viniferin and *trans*-δ-viniferin in young spring grapevine canes, the dependence on the samples drying procedure and a comparison to the stilbene content in one-year-old canes.

MATERIAL AND METHODS

Plant material. Samples of *Vitis vinifera* L. 'Hibernal' (white inter-specific variety), 'Malverina' (white inter-specific variety), 'Grüner Veltliner' (white variety) and 'Blaufränkisch' (blue variety) were obtained from the wine region in Moravia. The vineyards were in the Mendeleum area of the University (vineyard Na Valtické, Lednice) at an altitude of 176 metres a.s.l. The location is open and well sunlit. The land is mainly flat, slopping slightly to the south-east.

V. vinifera samples of the L. blue variety 'Kolor' and blue variety 'Fioletovij Augustovskij' were obtained from the Bohemian region at Karlštejn. The Research Station for Viticulture Karlštejn manages grape vineyards on difficult terraced terrain, 256 m a.s.l.

In July 2015, canes that grew in spring ("young spring grape canes") were collected; the foliage was immediately removed. One part of the young spring grape canes was lyophilised after collection and a second part was dried at room temperature for three months in darkness. Two types of dried plant samples were obtained in this way. Both dried plant materials were then cut to length at around 0.2 cm.

Chemicals and reagents. Standards of *trans*-resveratrol (purity > 99 %) and *trans*-ε-viniferin (purity > 95 %) were purchased from Sigma-Aldrich (Prague, Czech Republic) and used as external standard solution in pure methanol. Methanol LiChrosolv, gradient grade for LC, was purchased from Merck (Prague, Czech Republic), acetonitrile Optima LC/MS was obtained from Fisher Scientific (Pardubice, Czech Republic), *ortho*-phosphoric acid p.a. was obtained from Fluka (Prague, Czech Republic), ethyl acetate gradient grade and formic acid was obtained from Sigma-Aldrich (Prague, Czech Republic). The laccase from *Trametes versicolor* was purchased from Sigma-Aldrich (Prague, Czech Republic).

Sample preparations. The collected canes were dried either in darkness for three months at room temperature (20 °C) or lyophilised. Both dried plant materials were then cut to length at around 0.2 cm.

The cut grapevine canes (0.5 g) were extracted for 165 min using 100 % methanol (6 mL) at 50 °C as described in the literature, e.g., (Soural et al. 2015; Tříska et al. 2017). After extraction, the mixture was centrifuged at 3 500 rpm for 10 min at 20 °C. The supernatant was transferred into a calibrated tube, 1 mL of fresh solvent was added to the solid residue, mixed and this new mixture was centrifuged again. This rinsing procedure was then repeated. As a result, the sample was extracted only once and the residue was washed two times with solvent. All the supernatants were combined and the final volume of the supernatant mixture was adjusted to 5 mL. Each sample was prepared in triplicate.

HPLC and LC/MS methods. The samples were analysed by High Performance Liquid Chromatography (HPLC) and Liquid chromatography–mass spectrometry (LC-MS) using methods described in the literature (Soural et al. 2015, Tříska et al. 2017). An HP 1050 (Ti-series, Hewlett Packard, Palo Alto,

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USA) HPLC system equipped with a G1315B diode array detector (DAD, Agilent, Santa Clara, USA) and a G1321A fluorescence detector (FLD, Agilent, Santa Clara, USA) was utilised. A Luna C18(2) column, 150 × 2 mm, 3 μm particles, 100 Å pore size (Phenomenex, Torrance, USA) was used. For the LC-MS analysis, an LCQ Accela Fleet (Thermo Fisher Scientific, San Jose, USA) with atmospheric pressure chemical ionisation in a positive mode (+ APCI) was employed. A mixture of acetonitrile/water/*o*-phosphoric acid (or formic acid for LC-MS) was used as the mobile phase. Mobile phase A was composed of 5 % of acetonitrile + 0.1 % of *o*-phosphoric acid (or formic acid), mobile phase B was a mixture of 80 % of acetonitrile + 0.1 % of *o*-phosphoric acid (or formic acid). The gradient elution was as follows: mobile phase B increased from 20 % to 80 % within 20 min and then from 80 % to 100 % within 5 min. The flow rate was set to 0.25 mL/min (for the LC-MS, 0.4 mL/min) and the column temperature was set to 25 °C. The spectral data were collected in a range of 190–600 nm. The quantification of the *trans*-resveratrol was performed by HPLC-DAD according to the calibration curve of *trans*-resveratrol and the viniferins were quantitated according to the calibration curve of *trans*-ε-viniferin. Limits of detection: *trans*-resveratrol (LOD 0.056 μg/mL, LOQ 0.187 μg/mL), *trans*-ε-viniferin (LOD 0.158 μg/mL, LOQ 0.525 μg/mL). An HPLC-Fluorescence detector with an excitation wavelength of 315 nm, an emission wavelength of 395 nm, and scanning the emissions in a range of 300–600 nm was used to confirm the stilbenes.

LC-NMR. A commercial HPLC system (Dionex UltiMate 3000, Thermo Fisher Scientific, San Jose, USA) with a 250×4.6 mm HPLC column (Luna C18(2), Phenomenex, 5 μm particles, 100 Å pore size) was employed. Fifty microlitres (50 μL) of the concentrated methanol solution was injected into the HPLC. The separation was performed by isocratic elution (system ACN–D_2O, 45:55) and was monitored at 220 nm and by the on-flow ^{1}H NMR detection. The ^{1}H Nuclear Magnetic Resonance (NMR) observations were conducted on a Varian INOVA 500 MHz spectrometer (Varian Inc., Palo Alto, USA) equipped with an HCN triple resonance (60 μL active volume) microflow probe at 22 °C. Standard NMR software VnmrJ 4.2 (Agilent Technologies, version 4.2., 2014) was used for the data collection. The separation and NMR detection were conducted at ambient temperature (22 °C). The ^{1}H NMR data were collected in the on-flow mode employing Water suppression Enhanced through T1 effects (WET) multiple frequency solvent suppression (Smallcombe et al. 1995). The signals of the residual solvents were suppressed using one scout scan prior to the whole data collection. The ^{1}H NMR spectrum was referenced to the signal of acetonitrile (δ = 2.00 ppm). The data acquisition over 1 s of acquisition time covering the spectral width of 6 kHz followed after a 90° radiofrequency (RF) pulse (3.4 μs), four transients were accumulated in each spectrum. No relaxation delay was employed. A detailed analysis of the chromatographic peaks was performed in the stop-flow mode. The ^{1}H NMR spectra were accumulated over 1 000 scans with 2 s of acquisition time and 1 s of relaxation delay. The assignment of the NMR signals was confronted with the predicted ^{1}H NMR spectrum using the current version of the NMR predictor software (Mestrelab Research S.L., NMR program MestReNova), this comparison also showed good agreement to the experimental spectrum. *Trans*-δ-viniferin: ^{1}H NMR: δ (ppm) 7.41 (d, 1H, H-2'), 7.23 (d, 2H, H-2, H-6), 7.20 (bs, 1H, H-6'), 7.05 (d, 1H, H-7'), 6.90 (d, 1H, H-3'), 6.84 (d, 1H, H-8'), 6.83 (d, 2H, H-3, H-5), 6.49 (bs, 2H, H-10', H-14'), 6.22 (bs, 1H, H-12), 6.19 (bs, 3H, H-10, H-14, H-12'), 5.54 (d, 1H, H-7), 4.48 (d, 1H, H-8).

Synthesis of *trans*-δ-viniferin. The dimerization of the *trans*-resveratrol was performed using the laccase from *Trametes versicolor* according to Bhusainahalli et al. (2012) and Gazavezzotti et al. (2015). Laccase (2.5 mg) was dissolved in 1 mL of acetate buffer (0.1 mol/L), then 1 mL of the *trans*-resveratrol solution (2 mg/mL in methanol) was added. The reaction ran at the laboratory temperature in the dark for 18 h on a laboratory shaker. The procedure was slightly modified by the dissolution of the samples in 7 mL of distilled water and extraction by 2 mL of ethyl acetate and then several times by 1 mL ethyl acetate. The ethyl acetate extracts were evaporated under a stream of nitrogen, the residue was dissolved in 1 mL of methanol and used for the NMR analysis and the comparison experiments.

RESULTS AND DISCUSSION

The young spring grapevine canes were collected from the Moravian (4 varieties) and Bohemian (2 varieties) wine growing regions of the Czech Republic. The six grape varieties in this study have different char-

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acteristics. Specifically, they are the standard *V. vinifera* variety 'Blaufränkisch' (blue traditional variety), 'Grüner Veltliner' (the most cultivated white variety), 'Kolor' (newer "teinturier", i.e., colour blue variety) and the resistant varieties 'Hibernal' (the most cultivated white PIWI variety), 'Malverina' (the first Czech white PIWI variety) and 'Fioletovyj Augustovskij' (newer early blue PIWI variety). On the contrary, all the varieties show more growth, higher fertility and vine production, which were essential for this experiment. One part of the plant material was lyophilised immediately after collection and the second part was dried in darkness at room temperature for three months. The dry material was cut, ground and extracted into methanol and finally analysed by HPLC. Figure 2 shows the profile and HPLC separation of the substances in the 'Malverina' young spring grapevine cane extract using a diode-array detector (DAD) and fluorescence detector (FLD). The FLD detector is much more sensitive and especially more selective for the detection of stilbenes, so we use both detectors when studying and searching for new substances. After their determination, we use a DAD for their quantification. While the dominant stilbenes in the one-year-old grapevine canes were identified as *trans*-resveratrol, *trans*-ε-viniferin and r2-viniferin (Soural et al. 2015), the young spring grapevine canes also contain, besides *trans*-resveratrol (peak 1) and *trans*-δ-viniferin, (peak 2) another stilbene (peak 3) which is not r2-viniferin. The unknown substance has a higher fluorescence than *trans*-ε-viniferin, but a very similar UV-VIS spectrum as *trans*-ε-viniferin (Figure 3).

The compound was tentatively assigned to *trans*-δ-viniferin according to its retention time, molecular mass and MS spectrum fragmentation. In order to confirm the assignment, *trans*-δ-viniferin was synthesised by the enzymatic dimerization of *trans*-resveratrol using laccase (Bhusainahalli et al. 2012). The prepared *trans*-δ-viniferin was properly characterised and the obtained data corresponded well with the data obtained from the grapevine cane samples. The final confirmation was based on a detailed MS and NMR spectra analysis (Figures 3 and 4). The mass spectrum of *trans*-δ-viniferin (Figure 3, APCI in a positive mode) shows fragments at *m*/*z* 437 (neutral loss of water) and a fragment typical for viniferins at *m*/*z* 361 (loss of phenol ring, 94 Da) (Zhang et al. 2019). The 1H NMR spectrum with the assigned structure of *trans*-δ-viniferin is depicted in Figure 4. The structure elucidation was solely based on the 1H NMR experiment and its comparison with the literature data (Huang et al. 2000; Pezet et al. 2003). The 13C NMR experiment could not be performed to further confirm the compound structure due to the low concentration. Based on the data in the literature, it is obvious that *trans*-δ-viniferin can be synthesised in larger quantities under stress impact in *V. vinifera* plants (Pezet et al. 2003; Jean-Denis et al. 2006; Timperio et al. 2012). It can be found in different parts of the grapevine plants, but usually in a negligible amount (Flamini et al. 2015). A very low content (almost trace amount) of *trans*-δ-viniferin in the young spring grapevine cane varieties Cabernet Sauvignon, 'Pinot Noir' and Tintorera (sampling period July-August 2016) has only been

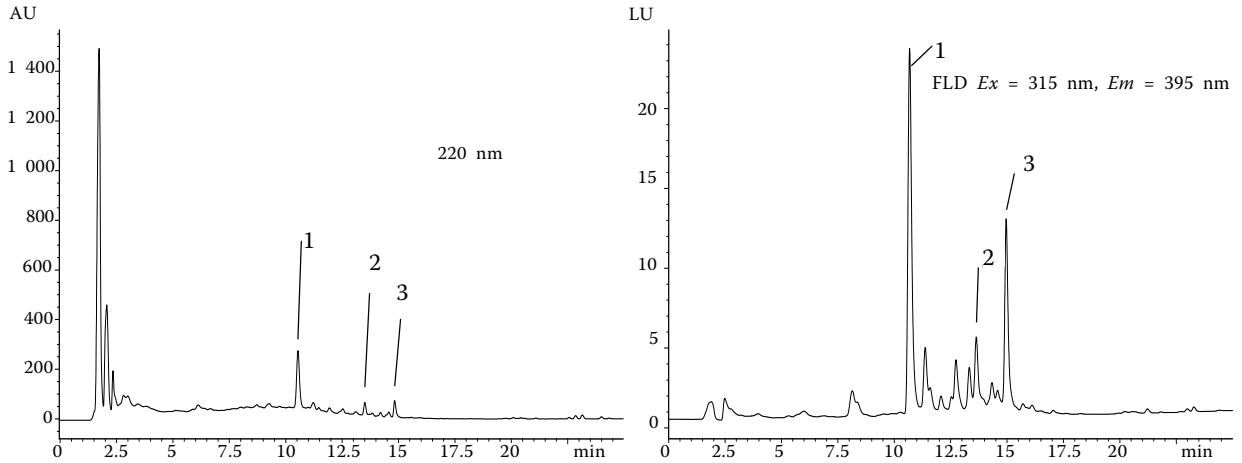

Figure 2. High Performance Liquid Chromatography separation of the extract of the 'Malverina' young spring grapevine canes (a diode-array detector and fluorescence detector)

Grapevine canes were dried at room temperature

1 – *trans*-resveratrol; 2 – *trans*-ε-viniferin; 3 – *trans*-δ-viniferin; AU – absorption units; LU – light units

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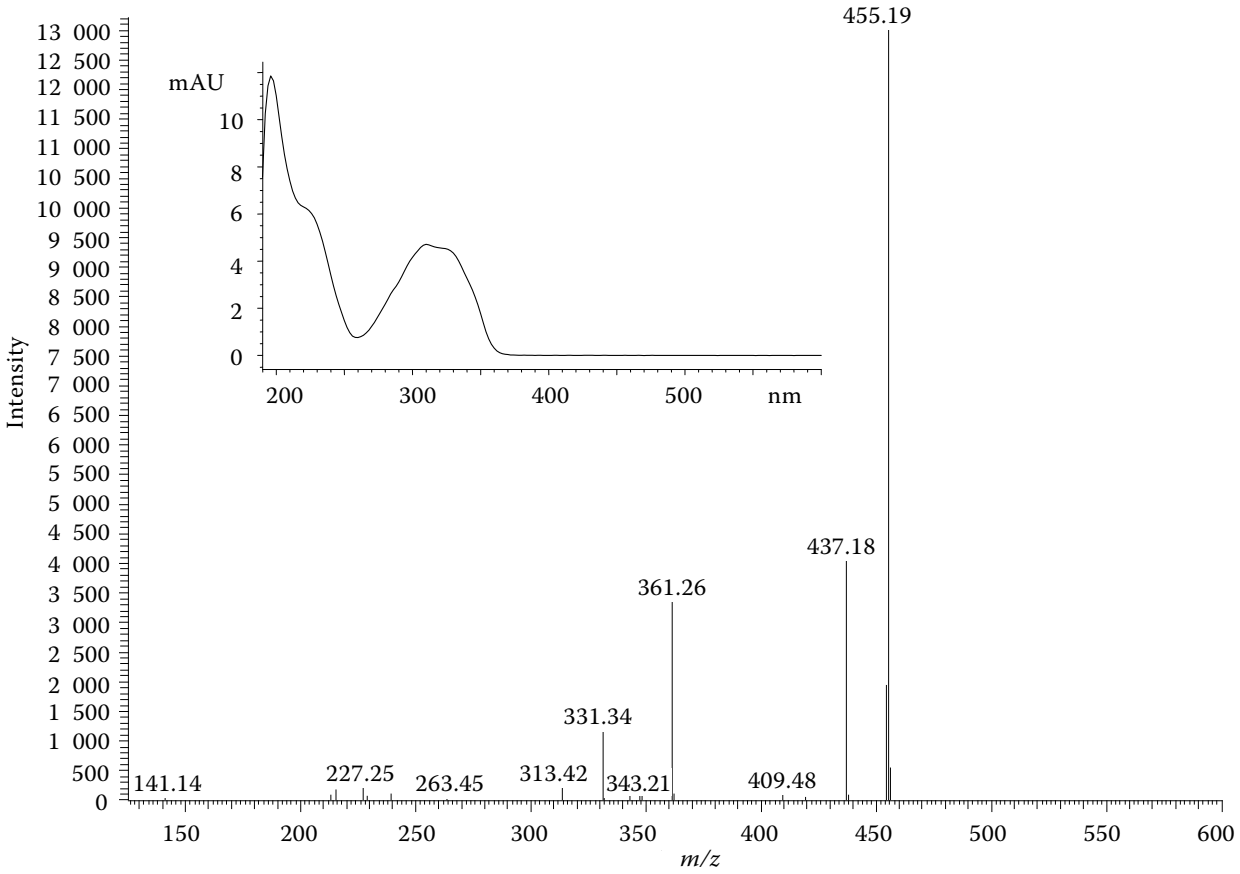

Figure 3. UV-VIS and mass spectra (APCI in a positive mode) of *trans*-δ-viniferin

described by Sáez et al. (2018). Plant material dried by lyophilisation only contained a very small amount of stilbenes (Figure 5) compared to the material dried at room temperature. The amount of *trans*-resveratrol was found under the method detection limit in all the lyophilised samples. The amount of *trans*-ε-viniferin and *trans*-δ-viniferin was found under the method detection limit for the variety 'Malverina', 'Kolor' and 'Fioletovij Augustovskij' and for *trans*-δ-viniferin also for variety 'Blaufränkisch'. Only a small amount of *trans*-ε-viniferin (1.22 mg/kg) was found for the variety 'Hibernal', while 8.09 mg/kg was found for the variety 'Grüner Veltliner' and was found 5.9 mg/kg for the variety 'Blaufränkisch'. The amount of *trans*-δ-viniferin was only 1.94 mg/kg in the variety 'Hibernal' and 0.77 mg/kg in the 'Grüner Veltliner' variety in the lyophilised samples.

In contrast to lyophilised samples, the highest concentration of *trans*-δ-viniferin was found in the 'Hibernal' and 'Malverina' varieties (38.9 and 40.8 mg/kg of d.w., Figure 4) dried at room temperature, but *trans*-resveratrol is still the most dominant stilbene in all the samples dried at room temperature. The highest *trans*-resveratrol level was found in the 'Blaufränkisch' blue variety (313.2 mg/kg of d.w.). However, all the blue coloured varieties were expected to have higher levels of *trans*-resveratrol than the white varieties. A higher value was expected for 'Kolor'. An exception for the white varieties was 'Malverina', with a relatively high value of *trans*-resveratrol, but this is known for this variety (Bábíková et al. 2008). The higher content of *trans*-ε-viniferin in the standard variety of 'Grüner Veltliner' was also interesting, which only proves the fact that the concentration of this compound tends to be higher in white varieties (white varieties have higher levels of *trans*-ε-viniferin and *trans*-δ-viniferin which is opposite to blue varieties where *trans*-resveratrol prevails).

The standard deviations are indicated on the bars in Figure 5 and it is clear that the contents of the substances in the lyophilised samples and in the samples dried at room temperature are different and the difference is statistically significant. Obviously, fast drying procedures, as lyophilisation in this case, can completely stop the corresponding metabolic

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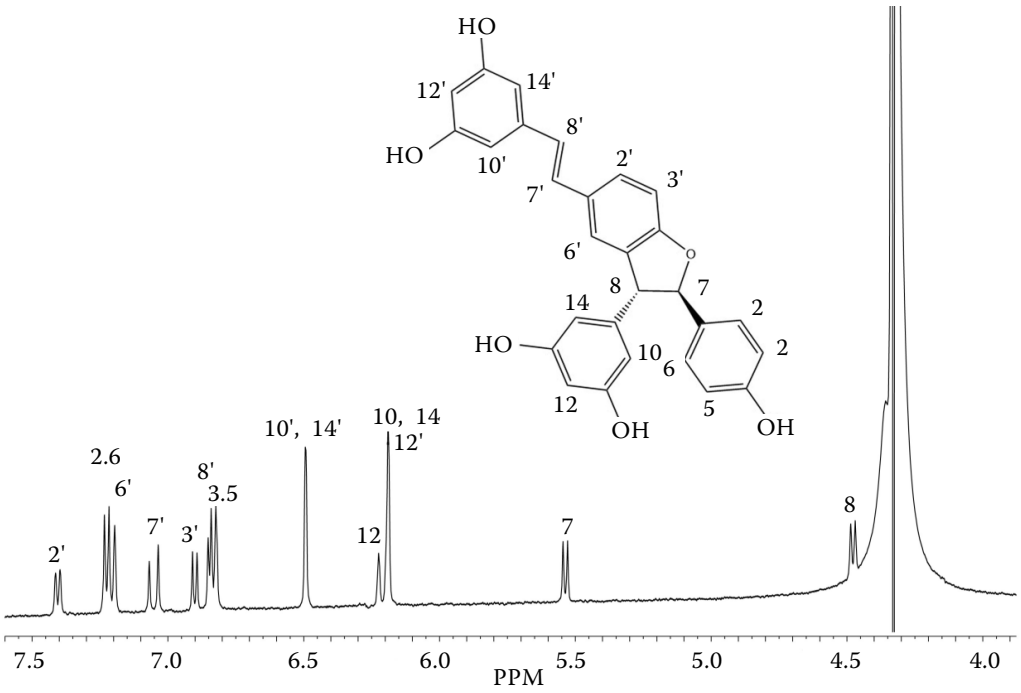

Figure 4. ^{1}H NMR spectrum of *trans*-δ-viniferin

processes. This observation is in agreement with the literature (Vergara et al. 2012). The total amount of stilbenes found in the samples dried at room temperature is in a range between 150–400 mg/kg of d.w. (Figure 5). The overall content found in the young spring grapevine canes was found in an order of magnitude smaller than in the one-year-old grapevine canes that can achieve several thousand mg/kg of d.w. (Soural et al. 2015). It is interesting to note that *trans*-δ-viniferin was found also in Brazilian wines and its amount reached a value of 22.4 mg/L in the variety 'Merlot' (Vitrac et al. 2005).

If we summarise our measured data and the literature data concerning the content of stilbenes in the young spring cane, which are very scarce, and the data concerning one-year-old canes, it is clear

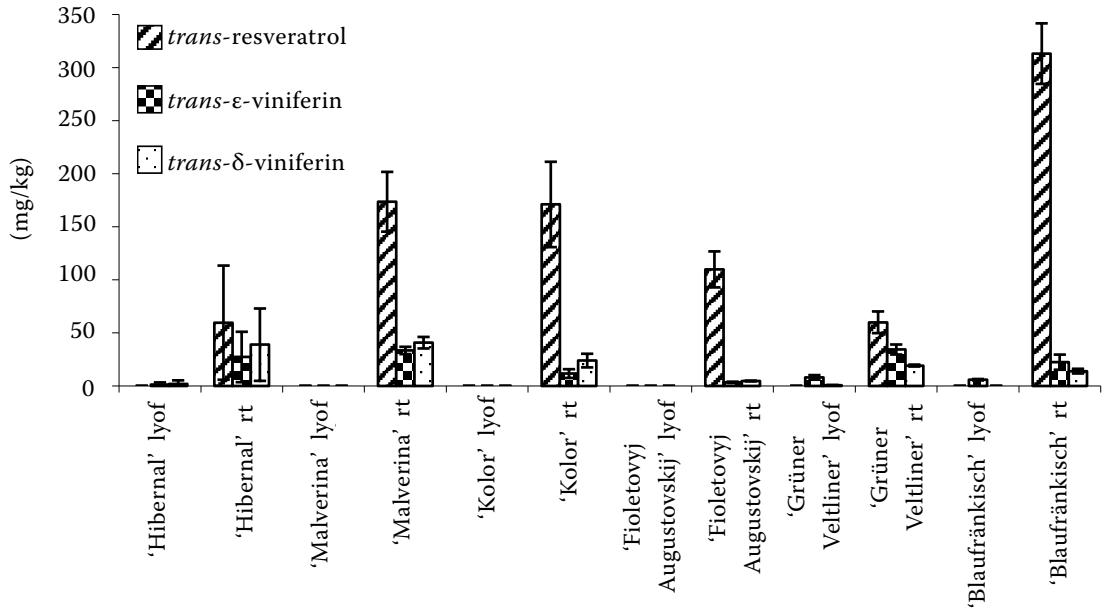

Figure 5. The content of the stilbenes in spring young grapevine canes prepared under different drying methods
Lyof – lyophilisation; rt – room temperature

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that there is a significant difference in the content of *trans*-δ-viniferin and r2-viniferin. In the young spring cane of the studied varieties, the *trans*-δ-viniferin is contained in concentrations comparable to concentrations of *trans*-ε-viniferin and its concentration gradually, depending on the age of the canes, decreases, while the concentration of *trans*-ε-viniferin increases, probably related to the grapevine cane transition from endodormancy to ecodormancy (Rätsep et al. 2021). In contrast, r2 viniferin does not occur in the young spring canes and begins to appear in the one-year-old canes (e.g., Soural et al. 2015) and its amount gradually increases towards the roots (Gabaston et al. 2018). Therefore, there is a very interesting possibility of using young spring canes as the raw material to obtain *trans*-δ-viniferin, resp. its mixtures with *trans*-ε-viniferin. If we consider the current acreage of the three most widespread varieties in the Czech Republic from the studied varieties (159 ha of 'Grüner Veltliner', 105 ha of 'Hibernal' and 16 ha of 'Blaufränkisch') and the yield of the canes for the season, approx. 9.5 tonnes per ha (Balík 2021), then, for example, we could theoretically obtain 39 kg of *trans*-δ-viniferin from the 'Hibernal' variety only. Currently, the use of *trans*-δ-viniferin is intensively studied, for example, in the food industry as an antimicrobial agent against foodborne pathogens (Mattio et al. 2019).

CONCLUSION

The presented results show the effect of the drying process on stilbene production in grapevine canes harvested in spring. Lyophilisation, a fast drying procedure, can completely stop the metabolic processes involved in stilbene production. However, slow drying at room temperature enabled the synthesis of a significant amount of stilbenes in all six studied varieties of *V. vinifera*. The results show that a post-harvest treatment can have a similar effect in *V. vinifera* as stress induced by a pathogen infection or UV irradiation. On the other hand, it was found that, beside the major *trans*-resveratrol, *trans*-δ-viniferin is produced in the spring grapevine canes in amount comparable with *trans*-ε-viniferin, being the highest in the inter-specific varieties 'Hibernal' and 'Malverina'. The stilbene composition found in the spring grapevine canes was noticeably found to be different when compared to the one-year-old grapevine canes where *trans*-resveratrol is accompanied by *trans*-ε-viniferin while *trans*-δ-viniferin is detected only in trace amounts.

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