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# Purification of resveratrol from vine stems

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**Abstract.** A modified method for preparative purification of *trans*-resveratrol (*trans*-3,4′,5-trihydroxystilbene) from vine stems is presented. It includes two new procedures added to the known scheme of resveratrol separation from ligneous organs of vine by ethanol–water extraction: (1) treatment of ethanolic solution of vine stem stilbenoid extract with diethyl ether for precipitation of a dark brown matter from the extract; (2) preparative column chromatography on polyamide carrier for separation of resveratrol from the crude ethanolic extract. As a result, 93.8% resveratrol concentrate with 0.7% of its dimer viniferin impurity was obtained. Resveratrol and viniferin contents in the stems of various frost-hardy hybride cultivars of *Vitis vinifera* grown open air in Estonia ('Hasaine Sladki', 'Jubilei Novgoroda', *Vitis vinifera* cv., 'ES 12-7-98', 'Marechal Joffre', 'Zilga') were determined for the plant material collected in July and in October. Lignified stems collected in October turned out to be an excellent source for producing vine stem stilbenoid (resveratrol and viniferin) concentrates for use as healthy ingredients in functional food for preventing heart diseases and atherosclerosis.

**Key words:** resveratrol, stilbenoids, viniferin, vine stems, *Vitis vinifera*.

## INTRODUCTION

Resveratrol (*trans*-3,4′,5-trihydroxystilbene), a natural polyphenolic antioxidant compound, can be found in various plant materials such as grape berry skins [1, 2], peanuts [3, 4], roots of Japanese knotweed [5], and rhubarb [6]. Among foodstuffs the main source of resveratrol is red wine, and its moderate dietary consumption is considered to explain the so-called "French paradox" that in some parts of France the death rate caused by heart diseases is remarkably low

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in spite of high consumption of fats. Indeed, according to numerous published data, resveratrol is cancer chemopreventive and it acts against coronary artery diseases and atherosclerosis (for reviews see [7–10]). In addition, resveratrol has recently been found to activate sirtuins and increase cell survival by stimulating SIRT1-dependent deacetylation of p53 [11], which may open new lines for its use as sirtuine activator.

It is known that resveratrol is synthesized in plants as phytoalexin in response to various stress factors such as UV-radiation, mechanical injuries, and, specifically in vine grapes, to *Botrytis* infection [1, 2], which makes grape skin a main source of resveratrol in wine. However, it has been shown recently that, in fact, hydroxystilbenes are a constituent part of the vine plant and there is much more resveratrol in its stems and leaves than in grape berries [12, 13]. Methods for purification of resveratrol and its dimer ε-viniferin from vine stems have been worked out [14]. This allows us to consider the woody parts of vine a potential feedstock for producing concentrated extracts of hydroxystilbenes for use as healthy ingredients of functional food to reduce risks for heart diseases and atherosclerosis.

In the present work we studied *trans*-resveratrol contents in the stems of various hybride cultivars of *Vitis vinifera* grown in Estonia, and worked out a modified method for preparative purification of resveratrol from vine stems.

## MATERIALS AND METHODS

### Chemicals

*trans*-Resveratrol, 99% pure, was from Sigma. Acetonitrile, purity 99.9+, was from Rathburn Chemicals Ltd. Methanol was a standard preparation of 99.9% purity. High quality 96.6% ethanol was used. Diethyl ether was a standard preparation for medical use. Polyamide carrier (Polyamide Woelm for Column Chromatography) was from Woelm Pharma, Germany. Deionized water (Barnstead Easypure RF, 18.3 MΩ/cm) was used throughout the work.

### Analytical procedures

For identification of stilbene derivatives in the methanolic extracts of vine, an Agilent LC-MS 1100 Series HPLC system instrument consisting of an auto-sampler, binary pump, column thermostat, and diode array and MS/MS detectors was used. The column temperature was 30 °C. The sample volume injected was 5 μL and the flow rate was 0.5 mL/min. Elution was in linear gradient mode of two solvents: A – 0.1% formic acid in water and B – 0.1% formic acid in acetonitrile.

Quantitative measurements at $\lambda = 306$ nm were performed on a Merck-Hitachi HPLC instrument, Model D-6000 (LH-4250 UV-VIS detector, L-6200 pump, L-5025 column thermostat) with column Alltech GmbH, Platinum 100A EPS, $250 \times 4.6$ mm, with particle size 5 μM. The column temperature was 30 °C. The

sample volume injected through Rheodyne was 20 μL. The flow rate was 1.0 mL/min in isocratic elution mode with either 25/75 or 40/60 v/v acetonitrile/ water. In gradient mode, solvent A was methanol and B was 0.5% formic acid in water. The computer program Merck-Hitachi HPLC Manager, Version 2 was used.

Calibration curves for the quantitation of *trans*-resveratrol in absorption units *versus* concentration were built up.

## Extractions

Three sets of plant material, marked as A, B, and C, were used in the study.

**A**: Woody stems of *Vitis vinifera* cv. grown at Harku in the garden of M. Haga were collected on 15 January 2002, and dried in a thermostat at 45 °C for 24 h. Then the samples were powdered in a coffee mill and macerated with methanol, 5 mL per g of the powdered material for 72 h at room temperature in darkness. After filtration, the extracts were kept in a refrigerator at 4 °C for use in analyses. Additionally, stems from the same plant were collected on 4 April 2002, and processed as described above, with a difference that the samples were macerated with 80% (v/v) ethanol.

**B**: Stems from the same plant at Harku as well as from several hybrid cultivars of *Vitis vinifera* from Räpina ('Marechal Joffre', 'ES 12-7-98', 'Hasaine Sladki', 'Zilga', 'Jubilei Novgoroda', all from the garden of J. Kivistik) were collected on 15 July 2002, and dried at room temperature for one month. Then the samples were powdered in a coffee mill and macerated with methanol or ethanol, 5 mL per g of the material for 72 h at room temperature in darkness. After filtration, the dry matter was macerated once more with the same amount of the solvent, and the two extracts were combined. Until use in analyses, the extracts were kept in a refrigerator at 4 °C.

**C**: Stems from the same plants from Harku and Räpina as in set **B** were collected on 26 October 2002, and dried for two months at room temperature. Then the samples were powdered in a cutting mill pulverisette-15 (Fritsch GmbH, Germany). The powders were additionally dried at 45 °C in a thermostat for 24 h and macerated twice for 72 h with 96.6% ethanol, analogously to set **B**. The second extraction added 10–15% stilbenoids to the amount obtained at the first extraction. The extracts were stored in a refrigerator at 4 °C.

## Purification

In preparative purification of resveratrol from vine stems, the powdered stems of several cultivars (*Vitis vinifera* cv., 'Hasaine Sladki', 'Zilga', 'Jubilei Novgoroda') were mixed (total 2790 g) and macerated twice with 96.6% ethanol, 5 mL per g of the material as described above. Then the ethanol solution was evaporated to dryness in a rotational evaporator at 45 °C resulting in 119 g of brown powder. The powder was transferred to 790 mL of 80% (v/v) ethanol and centrifuged 15 min at 5000 rpm for the separation of the insoluble matter that did not contain any component with optical activity at 306 nm (when dissolved in

96.6% ethanol). After the evaporation of the supernatant to dryness, 11.6 g of resveratrol/viniferin powder was obtained. Our attempts to decrease the amount of a dark brown matter in the resveratrol/viniferin preparation by subsequent dissolving it in 50% (v/v) ethanol as well as by water treatment as described in [14] were not successful and resulted in loss of a considerable amount of resveratrol/viniferin.

Gromova et al. [15] used diethyl ether in the extraction of resveratrol from the bark of *Pinus sibirica*. However, we found the dry extract of vine stems obtained from its solution in 80% ethanol to be practically insoluble in diethyl ether. On the other hand, by adding diethyl ether to the soluton of the extract in 96.6% ethanol, at the ethanol–ether ratio 1 : 4, an easily separable precipitate of the brown matter appeared in the amount of 30% of the weight of the ethanol–dissolved matter. As there were no components with optical activity at 306 nm in the precipitate, the described diethyl ether treatment turned out to be an effective method for decreasing the content of the dark brown matter in ethanolic extracts of vine stems without loss of stilbenoids.

For the separation of resveratrol from the crude extract, its ethanol–ether solution was evaporated to dryness and the resulting powder was dissolved in 96.6% ethanol to have resveratrol/viniferin concentration around 10 mg/mL, and the solution was subjected to column chromatography on a polyamide carrier (Polyamide Woelm). A $1.57 \times 60$ cm column was equilibrated with water or 40% (v/v) ethanol. Methanol–water or ethanol–water linear gradient elution was used and stilbenoids were monitored by optical density at 306 nm.

## RESULTS AND DISCUSSION

In Fig. 1 a representative chromatogram of the reversed-phase HPLC analysis of vine stem ethanolic (80% v/v) extracts by diode array detection at 309.1 nm is shown, and in Fig. 2 the APCI negative-ion mass spectra of the individual compounds in the mixture are presented.

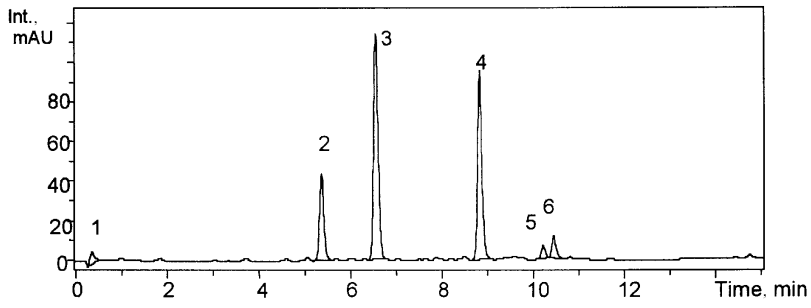


**Fig. 1.** HPLC chromatogram (at $\lambda = 309.1$ nm) of vine stem ethanolic (80% v/v) extract. Compound 2 – piceatannol; compound 3 – *trans*-resveratrol; compound 4 – viniferin. "Wintered" stems from *Vitis vinifera* cv. collected in April 2002 were used.

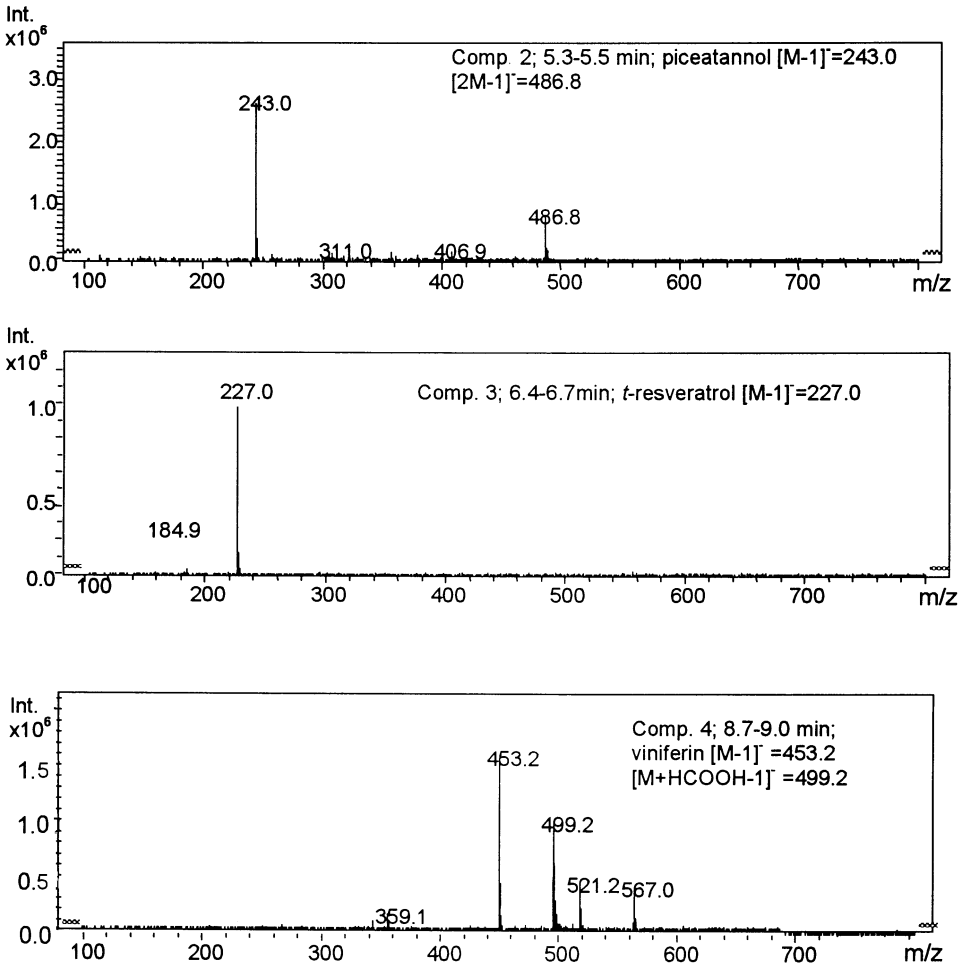


**Fig. 2.** APCI negative-ion mass spectra of stilbenoids in vine stem ethanolic extract corresponding to HPLC chromatogram in Fig. 1. In the spectrum of compound 4, 521.2 should correspond to one of the isomers of tri-OH-tri-MeO-flavone glucoside and 567.0 to its formic acid complex, 359.1 $[M-1]^-$ belongs to the aglycon of this flavone glucoside.

Three main stilbenoid compounds can be identified in the extract: piceatannol (**2**), resveratrol (**3**), and its dimer viniferin (**4**). The MS detection pointed also to possible trace amounts of resveratrol sulphate (at 1.9 min) and piceid (at 5.1 min) in the extract. Peak (**5**) may possibly be one of the isomers of tri-OH-tri-MeO-flavone glucoside by MS; peaks (**1**) and (**6**) were mixtures of some minor components.

It should be noted that piceatannol, in a comparable amount with resveratrol, was present only in the *Vitis vinifera* cv. "wintered" stems collected in early April. In all other samples only two stilbenoid compounds, resveratrol and viniferin, appeared in reversed-phase HPLC chromatograms. Quantitative data

on the contents of stilbenoids in the stems of the studied *Vitis vinifera* hybride cultivars are shown in Table 1.

It can be seen from Table 1 that the highest content of both resveratrol and viniferin was found in "autumn" stems of 'Hasaine Sladki' (set **C**, the material collected in October). In the total stilbenoid concentration (resveratrol+viniferin), the ethanolic extracts of 'Hasaine Sladki' stems exceeded those of 'Jubilei Novgoroda', *Vitis vinifera* cv., and 'ES 12-7-98' 1.6 times, 'Marechal Joffre' 3, and 'Zilga' 4 times. As a plant for resveratrol biosynthesis, 'Hasaine Sladki' deserves special attention. In the literature, the highest value of the stilbenoid content in vine has been reported for *Vitis vinifera* cv. Merlot stems if dried in open air at room temperature for three months, 1.9 $mg/g_{d.m.}$ of resveratrol and 1.2 $mg/g_{d.m.}$ of viniferin [14]. In most cases the contents of resveratrol/viniferin in various lignified grapevine organs including stems have been found to be more than 10 times less [12–14].

The concentration of resveratrol per dry matter in 'Hasaine Sladki' stems is closely comparable with its high concentration in the roots of *Rheum rhaponticum* [6]. From the practical point of view, production of resveratrol concentrates from rhubarb roots is, however, complicated by the presence of anthraquinones of different physiological activity in the roots. As no undesirable side-effects have appeared in using vine shoots and leaves in culinary art for ages, this plant should be the best source for producing stilbenoid concentrates for use as healthy ingredients in functional food.

In the preparative purification experiment we used a mixture of stem powders of 'Hasaine Sladki', 'Jubilei Novgoroda', *Vitis vinifera* cv., and 'Zilga', 351 g, 1215 g, 600 g, and 624 g, respectively. The first three were chosen because of their high stilbenoid content; 'Zilga' was included for its excellent winterhardiness. As

**Table 1.** Content of stilbenoids in the stems of some *Vitis vinifera* hybride cultivars grown in Estonia

| Plant material | Concentration, $mg/g_{d.m.}$ | |
|---|---|---|
| | Resveratrol | Viniferin |
| Set **A** (collected in January 2002) | | |
| *Vitis vinifera* cv. | 1.8 | 0.8 |
| Set **B** (collected in July 2002) | | |
| *Vitis vinifera* cv. | 1.2 | ≤0.1 |
| 'Hasaine Sladki' | 0.3 | <0.1 |
| 'Marechal Joffre' | 0.1 | <0.1 |
| 'Zilga' | 0.1 | <0.1 |
| Set **C** (collected in October 2002) | | |
| 'Hasaine Sladki' | 4.7 | 1.5 |
| 'Jubilei Novgoroda' | 2.9 | 1.0 |
| *Vitis vinifera* cv. | 2.8 | 1.1 |
| 'ES 12-7-98' | 2.4 | 1.1 |
| 'Marechal Joffre' | 1.6 | 0.3 |
| 'Zilga' | 1.1 | 0.5 |

described in Materials and Methods, by extraction with 96.6% (v/v) ethanol and further treatment with 80% ethanol according to [14], 11.6 g of resveratrol/viniferin preparation was obtained as a brown powder. The extract was further dissolved in 96.6 % ethanol and treated with 4-fold excess of diethyl ether for decreasing the content of the dark brown matter by its precipitation from the solution, and then evaporated to dryness yielding 8.12 g of resveratrol/viniferin powder.

Before column chromatography on polyamide carrier, the extract powder was freshly dissolved in 597 mL 96.6% ethanol. The HPLC profile of the solution at 306 nm is shown in Fig. 3. Two main peaks in the chromatogram represent 58.7% resveratrol (**3**) and 32.4% viniferin (**4**). The concentrations of resveratrol and viniferin in the solution were 8.77 mg/mL and 4.84 mg/mL, respectively.

Figure 4 illustrates separation of resveratrol from viniferin in column chromatography on a polyamide carrier. For resveratrol, fractions 52–60 and for viniferin fractions 65–76 were combined giving 105.3 and 140.4 mL of methanolic solutions, respectively. After solvent evaporation, 30.8 mg of slightly brownish powder was obtained for resveratrol preparation and 21.7 mg for viniferin preparation. Thus, the column recovery of stilbenoids was 77%, and the overall yield of the resveratrol/viniferin product in the preparative experiment with 2790 g of the mixed stem-powder of four cultivars used was 2.4 mg from 1 g of dry matter. By reversed-phase HPLC analysis, resveratrol preparation was 93.8%, with 0.7% viniferin impurity. Viniferin preparation was 77.8%, with 7.1% resveratrol impurity. The reversed-phase HPLC chromatogram of the final product of resveratrol is shown in Fig. 5.

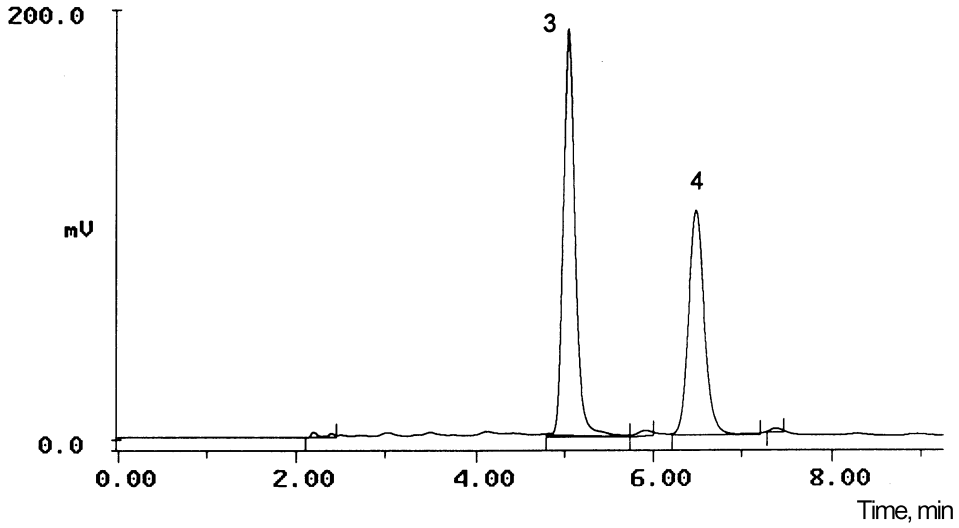


**Fig. 3.** Reversed-phase HPLC profile at 306 nm of the ethanolic extract from a mixture of stem powders of *Vitis vinifera* hybride cultivars Hasaine Sladki, Jubilei Novgoroda, *Vitis vinifera* cv., and Zilga. **3** – *trans*-resveratrol; **4** – viniferin.

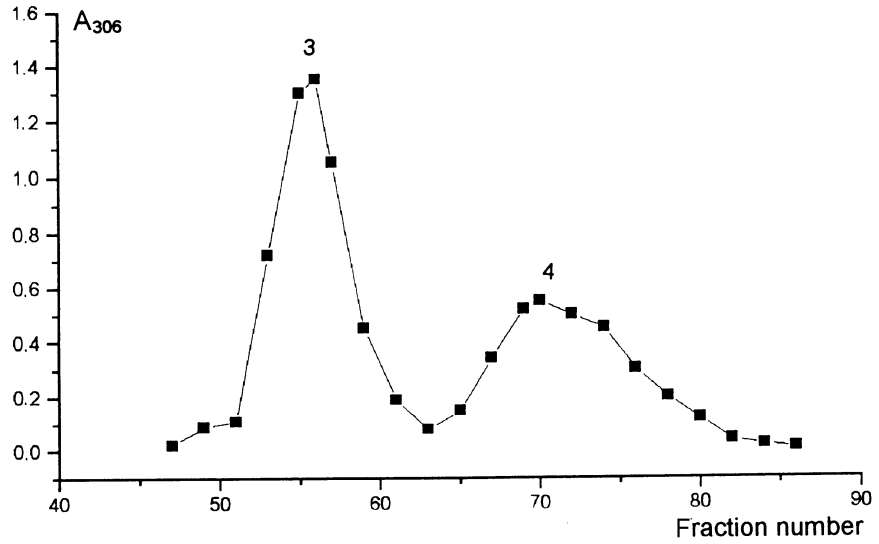


**Fig. 4.** Column chromatography profile of *trans*-resveratrol separation from viniferin in vine stems ethanolic extract on Polyamide Woelm. Column 1.57 × 60 cm, fraction volume 11.7 mL; linear gradient elution with 300 mL water + 320 mL methanol, and further from fraction 50, elution with methanol; 5 mL of ethanolic solution with the resveratrol/viniferin content 13.6 mg/mL was applied. **3** – *trans*-resveratrol; **4** – viniferin.

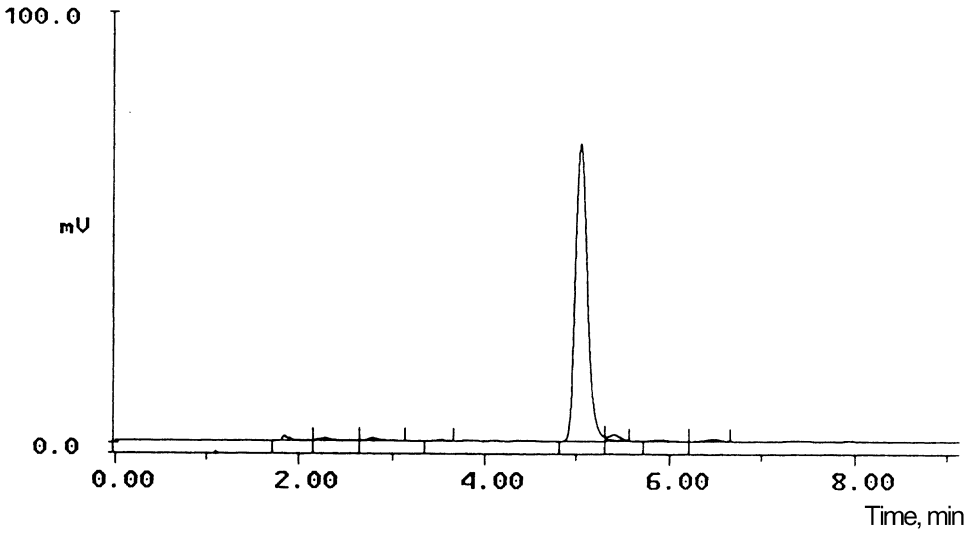


**Fig. 5.** Reversed-phase HPLC profile of ethanolic solution of *trans*-resveratrol purified from vine stems by ethanolic extraction, diethyl ether treatment, and column chromatography on Polyamide Woelm.

In conclusion, vine stems of several *Vitis vinifera* hybride cultivars grown in the open air in Estonia are an excellent source of dietary stilbenoids, resveratrol, and viniferin, for use as healthy ingredients in functional food. By combining ethanolic extraction with precipitation of impurities by diethyl ether and column chromatography on polyamide carrier, resveratrol with a small content of viniferin can be separated from lignified vine stems.

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## Resveratrooli eraldamine viinapuuväätidest

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On esitatud viinapuuväätidest resveratrooli kontsentraadi eraldamise preparatiivne meetod, milles etanooli ja vee seguga ekstraktsiooni on täiendatud taimmaterjali etanoolsete ekstraktide dietüüleetriga töötlemisega pruuni segava komponendi hulga vähendamiseks preparaadis ning kolonnkromatograafiaga polüamiidkandjal resveratrooli eraldamiseks tema dimeerist viniferiinist. Resveratrooli sisalduseks kontsentraadis saadi 93,8%, viniferiini lisand oli 0,7%.

On määratud resveratrooli ja viniferiini sisaldus suvistes (juulis kogutud) ja sügisestes (oktoobris kogutud) väätides mitmetel Eestis avamaal kasvatatavatel külmakindlatel viinapuusortidel: ‘Hasaine sladki’ (Varajane sinine), ‘Jubilei Novgoroda’, ‘Marechal Joffre’ ja ‘Zilga’. Sügisel kogutud puitunud väädid on sobiv materjal stilbenoidide (resveratrool ja viniferiin) kontsentraadi valmistamiseks. See on kasutatav funktsionaaltoidu komponendina südame- ja veresoonkonna haiguste profülaktikas.