Biologically active agents as part of extracts of grape leaves and vine and method of their extraction

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Abstract. The paper experimentally assesses the characteristics of grape raw materials (vines, leaves) in terms of quantitative and qualitative composition of polyphenols - the main functional biologically active ingredients of grape processing products. For obtaining alcoholic extracts, vines and leaves of grape were pre-dispersed and extracted using the ethanol. The extraction was carried out at room temperature for several months until an equilibrium concentration of polyphenols was obtained. The extracts were separated by leaking off on a sieve. The qualitative and quantitative composition of polyphenols in alcohol extracts was determined by the HPLC method. The analysis of the obtained data shows that a broad array of polyphenols appropriate for grape pomace, seeds and stalks (flavones, flavan-3-ols, hydroxy-cinnamic, oxybenzoic acids, stilbenes, oligomeric and polymeric procyanidins) is present in vine and leaves.

1. Introduction
As of today, biologically active substances and medicinal products based on herbal raw materials are commonly used. Of particular interest are polyphenolic substances, represented by groups of compounds such as stilbenoids, lignans, phenolic acids, phenolalcohols, flavonoids [1, 2]. The stilbenoid group counts more than 400 compounds that can be found in the chemical composition of plants such as moss, vascular spore plants, as well as grapes, peanut fruits, blueberry fruits, Siberian pine wood, Norway spruce roots, palm seeds.

According to their chemical structure, the stilbenoid class belongs to the group of phenylpropanoids. The stilbenoid nucleus consists of a 14-carbon skeleton composed of phenolic rings linked by an ethylene bridge. In plants, polyphenols, including resveratrol, are present as precursors - 3-O-β-glucosides. Other conjugated forms of resveratrol contain 1 to 2 methyl groups, sulfogroups or fatty acid residues. Compounds with at least two hydroxyl groups in the molecular structure have well-defined antioxidant properties. A natural analogue of resveratrol, which has four hydroxyl groups, is piceatannol (3, 4, 3’, 5’-tetrahydrostilbene). Stilbene – an associated structure of resveratrol consists of two phenolic rings with double bonds at the 3, 4 and 5 carbon positions forming the trihydrostilbene structure. Although the availability of double bonds in the structure of the resveratrol molecule facilitates transitions into cis- and trans-isomers, the trans-isomer form is more stable for this polyphenol [3]. Stilbenoids, in particular, resveratrol, are formed in response to emergence of factors such as ultraviolet radiation (UV), chemical irritants, mechanical damage, exposure to microorganisms, although stilbenoids have long been noted for their useful properties in protecting plants against fungal infection. For example, exposure to grey mould of grape (Botrytis cinerea)
results in the increase of the synthesis of stilbentase with its subsequent accumulation at the site of exposure to the pathogen. Stilbene biosynthesis occurs in the presence of stilbene synthase. Moreover, the precursor molecules in the formation of hydrostilbenes are malonyl coenzyme (CoA) and p-coumaroyl, which are present in plants [4].

Resveratrol is one of the most common representatives of the stilbenoid group. It is a secondary metabolite, a natural phytoalexin (3, 5, 4’ - trihydroxystilbenoid) possessing biological activity. Resveratrol was first discovered in 1940 by Japanese scientist M. Takaoka and isolated from Veratrum grandiflorum (white hellebore) [5]. Resveratrol is produced in many plants (grapes, peanuts, mulberries, conifer needles, pinecones) as a response to the infectious effects of infection and protection against ultraviolet radiation. It is contained in more than 30 plant species; some plants, such as Polygonum cuspidatum (Japanese knotweed) and Pinus spp. constructively accumulate large amounts of stilbenes. However, most studies carried out on cells and leaves, sprouts of peanut, grape, pine, have shown that stilbenes are present only in very low amounts under normal conditions. But they accumulate strongly in response to a wide array of biotic and abiotic stresses, such as exposure to UV light, treatment with chemicals as a result of increased transcription of their biosynthetic genes [6,7].

In grape skins, resveratrol is contained in an amount of 50-100 mg/g, and in red wine it was found in the range of 0.2 mg/l to 7.7 mg/l [8, 9]. The papers [10, 11] determined the presence in red leaves of autochthonous grape varieties cultivated in the Rostov region of technologically significant amounts of phenolic substances, among which a powerful antioxidant - resveratrol was found. The wild Amur grape (Vitis amurensis), which grows in the Far Eastern Taiga, is known to be particularly rich in resveratrol. The effect of transformation of plant cells with the rolB gene from A. rhizogenes on stilbenes biosynthesis was studied for the first time. In a V. amurensis cell culture actively expressing the rolB gene, the production of trans-resveratrol by the cells was 111.5 mg/liter medium, or 1431.3 µg/g of raw cell weight (2.32% of dry weight of cells), which is substantially higher than previously reported data for resveratrol-producing plant cell cultures [12].

Red grape wines of different countries contain different amounts of resveratrol. The concentration of resveratrol depends on the variety and growing area of the grapes. The Pinot Noir grape from the Mediterranean region and the West Pacific Coast of California (USA) has a resveratrol concentration of 4.4 to 5.3 mg/l. Cabernet Souvignon grape from California (USA) has a concentration of 0.9 mg/l, which is two times less than the same factor of the grape grown in Australia. In red wines from Bordeaux, France, resveratrol concentration amounts to 3.9 mg/l [13,14]. Resveratrol is also found in vines, stalks and seeds of grape; the maximum stilbene content in grape berries does not exceed 5.5 mg/kg; in vines and stalks 1,900-3,200 mg/kg are expressed in terms of dry weight of raw material [15].

2. The purpose of the study
With the purpose of experimental determination of the stock of total polyphenols, including stilbenoids in grape raw material (vines, leaves) and determination of the qualitative and quantitative composition of the phenolic complex in the obtained alcoholic equilibrium extracts of grape raw materials, samples of raw materials from different zones of grape growing in the Black Sea region: the western piedmont-coastal zone, the piedmont zone, the Southern coast, the Eastern coastal zone were harvested.

3. Materials and methods
Grapevine samples were harvested in the autumn-winter period of 2019 and 2020, leaves in the autumn period of 2019, 2020 during the grape harvest.

- vine, during pruning of grape varieties: Tsitronny Magaracha, Pinot-noir (Sevastopol); Biyas Aibatly, Pervenets Magaracha, white Kokur, Sauvignon vert, Golubok, Bastardo magarachski (village of Vilino, Bakhchysarai district, ampelographic collection of “Magarach”); Aligote, Rkatsiteli,
Cabernet-Sauvignon (village of Vilino, Bakhchysarai district); Pinot-noir (city of Bakhchysarai); Tsitronny Magaracha (Southern Coast, city of Alushta);
- grape leaves, of green, yellow, red varieties of Cabernet Sauvignon (Southern Coast, city of Alushta).

For obtaining alcoholic extracts, vines and leaves of grape were pre-dispersed with a Bosch AXT Rapid 2000 grinder to a particle size of 2-11 mm, after which an aqueous-alcoholic extractant with a volume fraction of 95% ethanol at a solid-liquid ratio of 1:3 was poured onto them. The extraction was carried out at room temperature for several months until an equilibrium concentration of polyphenols was obtained. The extracts were separated by leaking off on a sieve.

The mass concentration of phenolic substances in the extracts was determined by the Folin-Ciocalteu colorimetric method, according to Р. 4.1.1672–2003 [16].

The qualitative and quantitative composition of polyphenols in alcohol extracts was determined by the HPLC method using an Agilent Technologies chromatography system (model 1100) with diode matrix detector [17]. Calculation of quantitative content of individual components was performed using calibration graphs of the dependence of the peak area on the concentration of the substance, plotted on the solutions of individual substances. Anthocyanins content was determined expressed in terms of malvidin-3-O-glucoside chloride. Catharic acid content was determined expressed in terms of caffeic acid, polymeric and oligomeric procyanidins content is expressed in terms of (+)-D-catechin. All determinations were carried out in three replications. As standards, gallic acid, caffeic acid, (+)-D-catechin, malvidin-3-O-glucoside chloride, dihydroquercetin, isoquercitrin (Fluka Chemie AG, Switzerland) and trans-resveratrol, (-)-epicatechin, syringic acid (Sigma-Aldrich, Switzerland) were used. The results were processed using standard methods of mathematical statistics.

4. Results and Discussion

The dynamics of the extraction of phenolic substances from Cabernet-Sauvignon grape leaves (green, yellow, red leaves), grape leaves (red) and Cabernet Sauvignon vine using a food grade extraction agent with 95% ethanol volume fraction is shown in Figures 1, 2.

![Figure 1](image-url)  
**Figure 1.** Dynamics of the extraction of phenolic substances from Cabernet-Sauvignon grape leaves.
Figure 2. Dynamics of the extraction of phenolic substances from Cabernet-Sauvignon grapevine.

Table 1. Composition of the phenolic complex of Cabernet Sauvignon grape leaves

| Component name, in mg per 1 kg of dry weight | Leaves: green, yellow, red | Leaves: red |
|--------------------------------------------|----------------------------|-------------|
| (+)-D-Catechin                             | 440.5                      | 1028.5      |
| (-)-Epicatechin                            | 254.9                      | 588.6       |
| Syringic acid                              | 11.3                       | 30.3        |
| Caftaric acid                              | 4193.3                     | 5191.9      |
| Coutaric acid                              | 234.1                      | 255.2       |
| p-Coumaric acid                            | 56.4                       | 122.4       |
| Quercetin-3-O-glucoside-7-O-glucuronide    | 14467.6                    | 12937.6     |
| Quercetin-3-O-glucoside                    | 12798.4                    | 11214.6     |
| Isohammetin-3-O-glucoside                  | 1431.5                     | 1884.3      |
| Kaempherol-3,7-di-O-glucoside              | 2663.3                     | 2065.9      |
| Quercetin                                  | 40.2                       | 38.9        |
| Kaempherol                                 | 8.3                        | -           |
| Atocyanins sum                             | 177.5                      | 2687.9      |
| Trans-Resveratrol                          | 91.7                       | 31.3        |
| Oligomeric proanthocyanidins               | 3430.4                     | 5023.3      |
| Polymeric proanthocyanidins                | 21271.1                    | 36863.5     |
| Total content of phenolic substances, HPLC method, g/kg dry weight | 61.6 | 80.0 |
| Mass concentration of phenolic substances, Folin-Ciocalteu method, g/kg dry weight | 37.1 | 55.5 |
| Trans-resveratrol content of the sum of phenolic substances by HPLC in % | 0.15 | 0.04 |
Table 2. Composition of the phenolic complex of white grape varieties

| Component name, in mg per 1 kg of dry weight | White grapes |
|---------------------------------------------|-------------|
| Tsrtneny Magaracha | Algote | Pervenets Magaracha | Kokur | Sauvign green | Rkatsiteli |
| Gallic acid | 6.7 | 18.8 | 24.1 | 18.2 | 14.0 | 12.4 |
| Ellagic acid | 7.6 | 21.7 | 150.5 | 85.8 | 80.2 | 18.8 |
| (+)-D-Catechin | 99.3 | 335.0 | 736.6 | 919.3 | 748.3 | 165.2 |
| (-)-Epicatechin | 304.1 | 496.0 | 735.6 | 683.6 | 302.6 | 164.5 |
| Quercetin-3-O-glucoside-7-O-glucuronide | - | 9.1 | 18.8 | 19.9 | 30.9 | 6.5 |
| Quercetin-3-O-glucoside | - | - | 15.1 | - | 75.3 | - |
| Quercetin | - | 5.0 | 20.4 | 19.2 | 26.4 | - |
| Kaempherol | 1.4 | - | 7.9 | 9.0 | 6.4 | - |
| Trans-Resveratrol -4'-O-glucoside | 13.4 | 30.7 | 11.3 | 11.8 | 6.8 | 16.3 |
| Trans-pieced | 36.0 | 11.6 | 5.0 | 5.4 | 8.1 | 3.5 |
| Piceatannol | 63.9 | 128.7 | 27.1 | 27.3 | 32.7 | 8.6 |
| Trans-Resveratrol | 222.8 | 203.9 | 72.5 | 85.6 | 79.0 | 12.0 |
| ε-Viniferin | 1547.3 | 2196.9 | 2943.8 | 999.6 | 2051.3 | 453.8 |
| Unidentified stilbenes | 308.6 | 441.1 | 480.6 | 317.2 | 376.8 | 130.5 |
| Sum of all stilbenes | 2191.9 | 3013.0 | 3540.4 | 1446.8 | 2554.7 | 624.7 |
| Oligomeric proanthocyanidins | 1875.5 | 2635.6 | 2681.5 | 2650.1 | 1645.9 | 1059.3 |
| Polymeric proanthocyanidins | 3638.8 | 6353.6 | 7150.6 | 6313.5 | 480- | 4772.2 |
| Total content of phenolic substances, HPLC method, g/kg dry weight | 8.13 | 12.89 | 15.08 | 12.17 | 10.28 | 6.82 |
| Mass concentration of phenolic substances, Folin-Ciocalteu method, g/kg dry weight | 6.07 | 8.43 | 10.87 | 10.53 | 7.30 | 5.34 |
| Trans-resveratrol content of the sum of phenolic substances by HPLC in % | 3.22 | 1.58 | 0.48 | 0.70 | 0.77 | 0.18 |

In the alcoholic extracts of grape leaves and vines, the qualitative and quantitative composition of polyphenols was determined. According to the results of Tables 1, 2, 3 it was found that oxybenzoic acids, flavones (quercetin, kaempferol, quercetin-3-O-glucoside-7-O-glucuronide), flavone-3-ols (catechin and epicatechin), stilbene substances - trans-resveratrol, oligomeric procyanidins, polymeric proanthocyanidins are present in leaves and vines of grape. In alcoholic extracts of grapevines, in addition to trans-resveratrol, its derivatives (trans-piceid, trans-resveratrol-4'-O-glucoside, ε-viniferin and pikeatannol) were revealed. Among white grape varieties, the highest percentage of trans-resveratrol, in relation to the sum of polyphenols, was found in the vine of the Tsitronny Magaracha grape - 3.22%, and among the grapes of red varieties, the highest analogous index was for the perennial vine of Pinot-Noir variety, which amounted to 41.3%. It was found that the content of stilbene substances in white and red grapevines varies from 0.6 g/kg to 20.7 g/kg of dry weight, including trans-resveratrol from 0.012 g/kg to 14 g/kg of dry weight. That by an order of magnitude exceeds its concentration in grape leaves or berries and makes it possible to use the vine as a promising source of stilbenes substances.
### Table 3. Composition of the phenolic complex of red grape varieties

| Component name, in mg per 1 kg of dry weight | Red grapes |
|---------------------------------------------|------------|
|                                             | Golubok   | Bastardo | Pinot-noir (annual vine) | Pinot-Noir (perennial vine) | Cabernet-Sauvignon |
| Gallic acid                                 | 7.3       | 12.8     | 4.5                     | 13.3                        | 16.7               |
| Ellagic acid                                | 18.6      | 33.4     | 6.9                     | 21.7                        | 12.8               |
| (+)-D-Catechin                              | 364.0     | 483.6    | 257.0                   | 108.3                       | 278.3              |
| (-) - Epicatechin                           | 464.9     | 561.0    | 554.4                   | 546.6                       | 436.9              |
| Quercetin-3-O-glucoside-7-O-glucuronide     | 13.3      | 7.7      | 3.5                     | 25.0                        | 4.3                |
| Quercetin-3-O-glucoside                     | 53.8      | 11.5     | 5.0                     | 5.8                         | -                  |
| Quercetin                                   | 13.9      | -        | 3.7                     | -                           | -                  |
| Kaempferol                                   | 5.1       | -        | 2.9                     | 12.0                        | -                  |
| Trans-Resveratrol -4'-O-glucoside           | 5.6       | 7.0      | 24.1                    | 37.9                        | 4.4                |
| Trans-pieced                                | 18.3      | 4.3      | 24.6                    | 90.5                        | 3.6                |
| Piceatannol                                  | 22.4      | 13.1     | 71.3                    | 522.5                       | 44.2               |
| T Trans-Resveratrol                         | 50.8      | 28.4     | 1253.1                  | 13984.7                     | 69.6               |
| η- Viniferin                                 | 2734.0    | 904.1    | 2818.3                  | 4078.0                       | 1042.9             |
| Unidentified stilbenes                      | 461.8     | 171.0    | 756.8                   | 1981.2                       | 290.9              |
| Sum of all stilbenes                        | 3292.9    | 1128.0   | 4948.1                  | 20694.8                     | 1455.5             |
| Oligomeric proanthocyanidins                | 2011.8    | 1062.4   | 1488.5                  | 3360.9                       | 1060.8             |
| Polymeric proanthocyanidins                 | 4507.1    | 4358.2   | 3210.5                  | 9036.6                       | 4820.1             |
| Total content of phenolic substances, HPLC method, g/kg dry weight | 10.75 | 7.66 | 10.48 | 33.82 | 8.09 |
| Mass concentration of phenolic substances, Folin-Ciocalteu method, g/kg dry weight | 10.75 | 7.66 | 10.48 | 33.82 | 8.09 |
| Trans-resveratrol content of the sum of phenolic substances by HPLC in % | 0.47 | 0.37 | 12.0 | 41.3 | 0.86 |

### 5. Conclusion

Given the importance of grape stilbenic polyphenols as natural antioxidants, possessing cardioprotective activity and anticarcinogenic activity [2], it seems an actual task to obtain more detailed experimental information on the localization, technological stock, methods of effective extraction of stilbenic polyphenols from grape leaves and vines.

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