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Resveratrol extraction and analysis methods from different plant parts

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Abstract

Resveratrol (3,5,4'-trihydroxystilbene) is a natural polyphenol and a phytoalexin that has been intensively studied in the last few years. It was extracted for the first time from white hellebore in 1940 but only in 1992 the interest for resveratrol increased for its cardio-protective effects. A lot of plants have been found containing resveratrol but nowadays it is most extracted from grape skins, grape seeds, grape juice and wine. The quantity of resveratrol can be increased by ultrasonication, infection with fungal pathogens, $AlCl_3$ treatment and ultra violet irradiation. The extraction is performed by using solid-liquid extraction, liquid-liquid extraction, pressurized liquid extraction-solid phase extraction and supercritical carbon dioxide extraction and the analysis is performed using chromatography: high performance liquid chromatography coupled with a diode array detector, a fluorimetric detector or a mass spectrometer and gas chromatography coupled with a mass spectrometer.

Keywords: resveratrol, grape, wine, extraction, HPLC

1. Introduction

Plants have been used from the beginning of humanity. They were the only methods of maintaining people healthy and to heal them. Nowadays natural remedies are used to prevent and treat diseases. This doesn't mean that we are traveling back in time, but that plant based remedies increase life quality.

Over the years there were extracted a lot of compounds that have proven to be useful for humanity. They had nutritional value, they were involved in preventing and treating diseases.

Some of the natural compounds were found several years ago but they were highlighted later. Resveratrol is one of these compounds [1].

It was first isolated from The roots of white hellebore (*Veratrum grandiflorum* O.Loes) in 1940 [1,2] by The japonese researcher Michio Takaoka [2]. Later, in 1963 resveratrol was isolated from roots of japanese knotweed [3] (*Poligonum cuspidatum* Siebold et. Zucc), a plant used in traditional Chinese and Japanese medicine [1,2,4].

The interest for resveratrol increased in 1992 when it was proven that red wine has cardio-protective effects. This was explained by The French paradox phenomenon [2,4]. French people had a lower incidence of coronary heart diseases than in other countries evin though they had high-fat diets [1,2]. This happened because of The regular consumption of red wine, which contains a great amount of resveratrol [1,5].

In 1997 researchers discovered that resveratrol has the ability to inhibit carcinogenesis at multiple stages. Meanwhile there were found also anti-inflamatory [2,6] and anti-oxidant activity [2,7,8].

Starting with The year 2003 The interest in resveratrol grew, when it was discovered that resveratrol is an activator of SIRT1, capable of mimicking The effects of calorie restriction [2,9,10]

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and increasing lifespan in lower organisms [2]. Until now , resveratrol is a promising candidate as a mimetic for calorie restriction in humans [9].

2. Chemical structure

Resveratrol ($C_{14}H_{12}O_3$ - 3,5.4' - trihydroxystilbene) is a stilbene derivative and a polyphenolic compound. It is a phytoalexin because of its synthesis in plants after exposure to biotic or abiotic stress [11].

This compound can be found in different forms: stereoisomers, trans- and cis- resveratrol (Fig.1) and in its glycosilated form named piceid (resveratrol-3-O-β-D-glucoside) (Fig.1) [1].

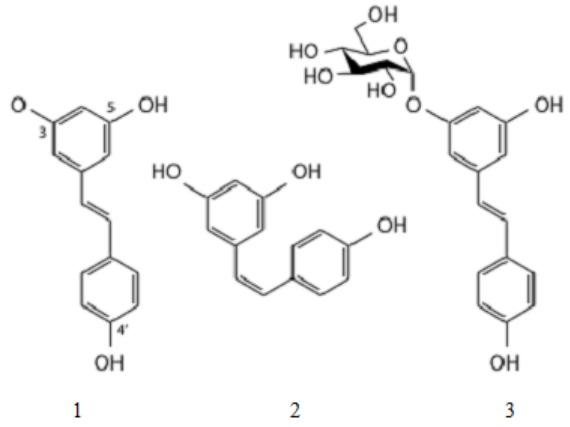

Figure 1. Chemical structures of: 1.trans- resveratrol, 2. cis- resvertrol, 3. resveratrol-3-O-β-D-glucoside [http://allnaturalnutritionalproducts.com/wp-content/uploads/2012/10/resveratrol-diagrams1.png]

Both stereoisomers have similar effects, but trans-resveratrol is investigated more because it's stability and because of the isomerisation to exposure to UV light [1].

3. Working material

Nature provides us with a lot of material to work with when it comes to the extraction of different natural compounds.

Reveratrol can be found in many plant species and vegetable foods like Japanese knotweed, peanuts, species of *Vaccinium*, black spruce, peanuts, indian blackberry, hop (beer), mulberry, elder berry,jackfruit and vine products [1, 12-16].

A lot of vegetable foods have been found containing different amounts of resveratrol (Table1).

Resveratrol is most extracted from the fruit of *Vitis vinifera* L.(vine) and derivates: grape skins, grape seeds, grape juice and wine. The amount of resveratrol extracted depends on many factors: product of extraction, grape variety, geographic region, agronomic factors, climatic factors, plant stress conditions and oenological practices [11].

The highest average level of trans- resveratrol was found in Pinot Noir wine from France. The second highest average level was found in Spanish and Italian Pinot Noir wine [3]. The lowest average level of trans- resveratrol was found in Zinfandel variety wine made in USA [3].

Disregarding of the region, the five highest levels of resveratrol were found in (Table2).

In Romania the quantity of resveratrol was determined from 30 samples of red wine from Oltenia (Table 3) [17].

Table 1. Quantity of resveratrol in vegetable foods [12-16]

Species	**Product**	**Extracted resveratrol**
Arachis hypogaea L.	Peanuts	1,93- 4,04µg/g
Artocarpus heterophyllus Lam.	Jackfruit	0,87-3,56 µg/g
Humulus lupulus L.	Hop (beer)	66,74 µg/L
Morus rubra L.	Mulberry	50,61 µg/g
Sambucus nigra L.	Elder berry	1,9-2,1µg/g
Syzygium cumini L.	Indian blackberry	11,9-34,87 µg/g

Table 2. Highest levels of trans-resveratrol [3]

Grape variety	**trans-Resveratrol (mg/L)**
Pinot Noir	0,7-6,5(3.6+/-2.9)
St. Laurent	1,4-5,0(3.2+/-1.8)
Marzemino	0,9-5,1(3.0+/-2.1)
Merlot	0,2-5,4(2.8+/-2.6)
Blaufrankisch	1,3-3,9(2.6+/-1.3)

Table 3: Levels of trans-resveratrol in Romanian wines [17]

Grape variety	**trans-Resveratrol (mg/L)**
Cabernet Sauvignon	1,513+/-1.12
Merlot	2,944+/-1.89
Pinot Noir	2,192+/-0.79
Feteasca Neagra	1,998+/-1.52
Syrah	1,817+/-2.27
Negru de Dragasani	1,569+/-0.04

The interest in resveratrol, extracted and analyzed from grapes, grape juice or wine is increasing due to the high accessibility, the wide spreading of vine yards and the great amount of extractible resveratrol.

4. Extraction

4.1. Increasing the quantity of resveratrol

4.1.1. Ultrasonication. Post harvest ultrasonication, cleaning for 5 minutes and incubation for 6 hours in the dark at 25ºC resulted in increasing amount of resveratrol by 1.15-1.53 times in grape juice [18].

4.1.2. Infection with fungal pathogens. Leafs, shoots and flowers of grapevines at different stages (pre-bloom, bloom and post-bloom) were inoculated with conidia of *Botrytis cinerea* by spraying [20]. The infection is characterized by higher levels of trans- resveratrol [19,20] and the enhancement of resistance to pathogens [20].

Trans- resveratrol has been shown to enhance resistance to other panthogens, such as *Plasmopara viticola*, *Phomopsis viticola* and *Rhizopus stolonifer* [20,21].

4.1.3. $AlCl_3$ treatment. *In vitro* cultures of grapevine with additional elicitor agent ($AlCl_3$) in their medium were initiated. The specific medium for vine multiplication was supplemented with different 1% $AlCl_3$ solution doses. They showed an increase of resveratrol according to the used dose of the elicitor agent [21].

4.1.4. UV-C irradiation. The grape plants used were pre-cultured in a controlled environment glass greenhouse at 25ºC in the day time and 18ºC in the night time and 65% relative humidity. The plants were cut and stored at -80ºC after freezing in liquid nitrogen.

Grape plants were irradiated by UV-C light at 254 nm for 10 minutes at 15 cm distance. After that they were incubated in the dark at 25ºC with relative humidity of 80% for 60 hours. At several incubation periods plants were removed from incubation and frozen in liquid nitrogen and then stored at -80ºC [23].

4.2. Extraction methods

4.2.1. Solid - liquid extraction. This sort of extraction is performed on grape skins. 4g of grape tissue was firstly ground to fine powder under liquid nitrogen and then was extracted by 100 mL methanol with agitation at room temperature under darkness for 48 hours. After centrifugation 10,000 x g for 15 minutes, the supernatant was evaporated at a temperature that is lower than 35ºC to dryness and recovered with distilled water. 20 mL of the aqueous extract was extracted further three times

with 20 mL of ethyl acetate at room temperature under darkness. The organic phases were combined, evaporated to dryness at less than 35ºC. The residue was recovered by 2 mL of ethanol in water (50%) and stored at -20ºC until analysis [23].

The extraction could have been performed by ethyl acetate extraction, centrifugation, supernatant evaporation and recovery with ethanol in water, without the methanol extraction [22].

4.2.2. Liquid - liquid extraction. Wine makes the object of this extraction. First the volume of wine is reduced to 1/7 of the original volume to obtain a wine concentrate. Then, the obtained concentrate is mixed with an organic solvent and stirred at 200rpm at room temperature for 2 hours. The two phases were decanted in separatory funnels during 24 hours to ensure efficient separation.

The organic solvents were evaporated under vacuum at a bath temperature of 80ºC. preliminary studies had shown that pure trans-resveratrol was not degraded at this temperature.

Prior to analysis, the dried organic extracts were dissolved in ethanol 95% [24].

4.2.3. Pressurized liquid extraction - solid phase extraction. The grape samples were lyophilised untill reaching a constant weight (loss of aprox. 80% from the original weight) and then triturated and kept at - 20ºC until the extraction.

First extraction phase was in three cycles of 5 min, at 40ºC and 40 atm of pressure, using water as a solvent. Then an extraction/elution stage was performed using methanol at 50ºC and 40 atm for 3 cycles of 5 minutes each. The 3 fractions were united and an aliquot was taken for analysis [25].

4.2.4. Supercritical carbon dioxide extraction. The samples used are freshly pressed grapes that were dried in an oven at 60ºC for 48 hours in order to obtain a dry solid that was grinded to obtain adequate particle sized samples.

The extraction was carried out in an Isco extractor. The equipment consists of one extractor, 2 µm filters and a thermostatic system. The solvent was introduced by syringe pumps to provide constant pressure.

4,6 g of sample were introduced into the extractor. After they reached 20ºC the extractor was pressurized with carbon dioxide and ethanol. The extraction time was 3 hours. The extracts were collected in glass tubes containing methanol and analyzed [26].

5. Chromatographic analysis

5.1. HPLC

5.1.1. HPLC-DAD/Fluorimetric detector. High Performance Liquid Chromatography is the most commonly used quantification method.

Analysis are performed using an HPLC system that has a diode array detector (DAD) [24,26-31], and an additional fluorescence detector [27-29], a pump and an automatic sampler [24,26,27,29,30].

The solid phase is represented mostly by reverse phase C18 columns [24,26-31] that can be coupled with a pre-column [24,30] or a C18 guard cartridge [28]. The column is thermostated at 20-40ºC [24,28,30,31].

The mobile phase is represented by: water, acetic acid and acetonitril [24,30] or water, methanol and acetic acid [26,28,31] or water, methanol and acetonitrile [31] or acetonitril and acetic acid [29]. The injection volume is 10 or 20 µL [24,26-31].

5.1.2. HPLC-MS. The determination was performed using an HPLC system coupled with a mass spectrometer [32]. The mass spectrometer was equipped with an electrospray ionisation source [32-34]. The temperature of the ion trap as maintained at 250ºC. Helium was introduced into the trap at an evaluated pressure ($6x10^{-6}$ mbar) [33] and nitrogen [33,34] was used for drying and as nebulizing gas at a 30 psi pressure and 10L/min flow rate [33].

5.2. GC-MS

The analysis was performed using a BP-5 type column (30m x 0.25 mm i.d., d_f: 0.25µm) operated at a constant helium flow at 1.2 mL/min. The GC oven was programmed at 90ºC for 1 minute, and for 15 minutes up to 280ºC. The temperature of the injector was maintained at 280ºC. The injection volume was 1-2µL. transfer line, electron impact ionization source and trap temperatures were set at 285,180 and 120ºC. The helium dumping gas flow was fixed at 2.5mL/min [35].

The mass spectrometer was operated in the electron impact ionization mode (70eV). The MS spectra were acquired in the m/z range between 150 and 450 a.m.u., using a filament emission current of 50μA. The most intense ions in the spectra were m/z 228, 270 and 312 a.m.u. [35].

6. Conclusion

Resveratrol is a stilbene that can be found in two stereoisomeric forms that have similar effects. trans-Resveratrol is investigated more because of its increased stability.

Nature provides us with a lot of working material, so resveratrol can be extracted from a lot of plants. It is most extracted from *Vitis vinifera* L. fruit and its derivates: grape skins, grape seed, grape juice and wine.

The extracted quantity depends on the product of extraction, grape variety, geograhyc region, agronomic factors, climatic factors and plant stress conditions. It can be increased by using post harvest ultrasonication, plant infection with fungal pathogens, *in vitro* $AlCl_3$ treatment and UV-C irradiation.

The extraction methods are based on the product of extraction and they can be: liquid-liquid extraction, solid-liquid extraction, pressurized liquid extraction- solid phase extraction, supercritical carbon dioxide extraction.

The analyses are based on chromatography. The High Performance Liquid Chromatography is the most commonly used and can be coupled with diode array detectors, fluorescence detector or with a mass spectrometer. Gas chromatography can be coupled with mass spectrometry as well.

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Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

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