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Elicitation of *trans*-resveratrol by laser resonant irradiation of table grapes

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ABSTRACT Table grapes were irradiated with UV nanosecond laser pulses in searching for resonant photo-elicitation of *trans*-resveratrol, a known antioxidant compound naturally produced by grapevines and other plants. To this end, the irradiation time as well as the wavelength dependence of the induced *trans*-resveratrol content was investigated by comparing the elicitation level of this compound at two laser wavelengths. One wavelength was selected right at the maximum of the absorption band (302.1 nm, the resonant wavelength for this compound) while the second was selected (300 nm, a non-resonant wavelength) such that *trans*-resveratrol absorption is negligible. It was found that the resonant irradiation enhances the resveratrol content in grapes by up to six times more than that of non-resonant irradiation, the rest of the conditions being the same. This work demonstrates how selective laser excitation of fruits can open new possibilities for the development of functional foods with enhanced nutritional and beneficial properties.

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1 Introduction

An important claim in human dietary habits concerns the necessity of increasing the intake of vegetables and fruits [1, 2] because they are rich in beneficial ingredients such as for example polyphenols [3–6]. An example of such compounds is *trans*-resveratrol (3, 5, 4′-trihydroxystilbene), a known antioxidant compound naturally produced by vines and other plants as a self-defense agent acting against pathogens' attack. This compound has attracted considerable attention because it has shown important health benefits, namely because of its antioxidant, anti-inflammatory, estrogenic, cardioprotective, chemotherapeutic and anticarcinogenic properties, as has been widely reviewed [7–12].

The lack of a proper level of intake of such phenolic compounds present in fruits and vegetables has attracted a great interest in the development of functional foods to overcome the mentioned dietary needs by resorting to different methodologies, namely: (a) eliminating undesirable compounds, (b) adding new ingredients to modify for example organoleptic properties, (c) increasing the active compounds' bioavailability or (d) increasing natural health-promoting compounds by post-harvest induction of these phenolic compounds. Within the last category a significant number of studies have been published devoted to biotic or abiotic *trans*-resveratrol induction in table grapes, UV irradiation being one of the most extensively investigated both in vine leaves [13, 14] and in grapes [15–19]. The enhancement of the *trans*-resveratrol content in wine has been investigated by using UV-irradiated grapes for the wine elaboration [20] and by directly irradiating the wine [21]. Recently, it has been reported that UV irradiation is more efficient than ozone treatments in increasing the *trans*-resveratrol content in grapes with short treatments [22].

A good correlation has been reported between resveratrol production (as induced by UV-C elicitation) and gray mould resistance [23], a black mould caused by *Rhizopus stolonifer* on several table grape varieties. On the other hand, Nigro et al. have shown that UV irradiation can reduce post-harvest decay of table grapes [24] and, although these authors studied only the time evolution of the damaged grapes and no chemical analysis was performed, the correlation between the enhancement of the natural resistance of the grapes observed and the elicitation of the resveratrol content demonstrated by the former authors is clear.

Regarding the election of the wavelength used for the irradiation, most of the previously published works do not specify the reasons for choosing one or another wavelength. The most widely used are 340 nm (UV-B) and 254 nm (UV-C), while the UV absorption spectrum of *trans*-resveratrol in ethanolic solution shows a wide band from 280 to 360 nm with the maximum of absorbance at 306 nm.

The use of a tuneable laser for the irradiation allows using photons of resonant energy to produce the absorption through real electronic states of the molecule under study, and hence to involve a molecule-specific parameter in the absorption process, significantly increasing the absorption yield. Generally speaking, aromatic compounds show absorption bands in the UV mainly associated with the UV bands of benzene. Of relevance for the present investigation is the benzene $\tilde{a} - \tilde{X}$ transition, extended over the 340–300 nm region [25]. In previous works we have determined that the resonant wavelength for *trans*-resveratrol is 302.1 nm [26]. In this work we investigated the effect on the *trans*-resveratrol elicitation via UV irradiation using the resonant wavelength of this compound (i.e. its 'resonant elicitation') as compared with the irradiation with a close but non-resonant wavelength.

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Therefore, in view of the mentioned *trans*-resveratrol induction using UV light we were prompted to investigate the wavelength dependence of the *trans*-resveratrol elicitation. Specifically, our aim was to investigate the induced *trans*-resveratrol content in table grapes as a function of the resonant character of the UV excitation. For this purpose we measured the resveratrol induction level by laser excitation of table grapes at two distinct wavelengths, the rest of the conditions being identical. One wavelength, λ_{on}, was selected right at the maximum of the absorption band (see Fig. 2, below) and the second wavelength, λ_{off}, was selected such that *trans*-resveratrol absorption is negligible compared to that of λ_{on}.

2 Materials and methods

2.1 *Reagents and standards*

HPLC-grade methanol, from Romil (Barcelona), ethanol, from Sigma-Aldrich, glacial acetic acid from Carlo Erba (Milan, Italy) and purified water with a Milli-Q system from Millipore (Milford, MA, USA) were used. Also, a *trans*-resveratrol standard (99%) from Sigma-Aldrich was used.

Standard solutions: a 50 μg mL^{-1} stock solution in ethanol was prepared. Working standard solutions were prepared by diluting the stock solution in ethanol. The standard solutions as well as the samples were stored at -4 °C in darkness.

2.2 *Samples and irradiation treatments*

Red grapes (*Vitis vinifera*, Red Globe variety) were directly purchased from the market at the usual mature ripening stage for commercialization and no additional cleaning was performed. To minimize effects of different maturity stages between bunches, they were cut into several moieties and each one was incorporated into the groups. The grapes were removed from the bunch by means of a sharp cutter leaving the peduncle attached to the berry in order to minimize dehydration of the samples. The irradiation was carried out by placing the individual grapes over the external crown of a disc of 30-cm diameter. Twenty-four grapes were uniformly distributed at each run with a separation of 15° between two consecutive samples. During the irradiation the disc was turning at 6° /min in order to obtain a uniform irradiation of all the samples. The treatments were performed at room temperature.

For the laser irradiation, a frequency-doubled dye laser (Continuum ND60) was used with a system (INRAD-AT-III-UV) for frequency doubling the output of the dye laser with a doubling crystal (BBO-TST), allowing us to scan the output from 235 up to 365 nm. The diameter of the laser beam (6 mm) was opened up to 15 mm by means of a divergent lens (focal length = -4 m) aiming at having a higher irradiation zone on the grapes. The laser fluence was 0.141 kJ/m^2 with 5-ns pulses running at a frequency of 10 Hz.

Two different wavelengths were used for the irradiation: 302.1 nm for the resonant wavelength and 300.0 nm for the non-resonant one (see below for details). Four sets of irradiation times were selected for each of the two different wavelengths: 15, 30, 45 and 60 min. Three replicas of six grapes each were obtained for the different irradiation conditions by repeating three times the whole experiment. Also, three replicas of six non-irradiated grapes were included in the experiment as a control.

Immediately after the irradiation, the grapes were peeled and the skin was triturated during 5 min by means of a mill (IKA-ANALYTICAL MILL A 11 BASIC), allowing us to obtain a particle size of 1 μm. Then, the skin powder was poured into vials with 60 mL of ethanol. The vials were maintained at 4 °C during 4 weeks before analysis to ensure enough time for *trans*-resveratrol extraction.

2.3 *Chromatographic method*

The chromatographic system consisted of the following components: a liquid chromatograph using a PU-1580 pump and a LG-1580-02 gradient controller from Jasco (Tokyo, Japan), a 154 Gastorr vacuum system, a Rheodyne 77251 injection valve furnished with a 20 μL loop, a UV-1575 spectrophotometric detector and a Worwin 1.50 version workstation (LC-2000 Plus Series Jasco). Separation was carried out at room temperature using a Teknokroma Kromasil C_{18} column (150 × 4.6 mm^2), 4 μm and a guard column (2 × 4.6 mm^2), 5 μm of the same stationary phase. Membranes (Millipore) of 0.45 μm were used to filter the mobile phase. A vacuum pump (Barna, USA) was also used.

The chromatographic separation was carried out using a linear gradient as mobile phase with methanol–acetic acid–water (10 : 2 : 88 v/v) as solvent A and methanol–acetic acid–water (90 : 2 : 8) as solvent B at a flow rate of 1.0 mL min^{-1} using the gradient conditions shown in Table 1.

A wavelength of 306 nm characteristic for *trans*-resveratrol HPLC (High Performance Liquid Chromatography) analysis was used for the spectrophotometric detector. The chromatographic peak was identified by comparing retention times of samples with that of the standard and confirmed with the one obtained from a grape sample directly exposed to sunlight during a day in order to complete the *trans–cis* resveratrol isomerization.

The external standard technique was used for the quantification of *trans*-resveratrol in the samples. The standard and samples were injected in a similar composition to the mobile phase. The external standard calibration curve was established by plotting the area of the peak against different concentrations from 0.5 to 10 μg mL^{-1} of *trans*-resveratrol. The analytical characteristics for the standard were lineal in the studied range with adjusted R-squared of 99.99%. A detection limit (LD = Oo + 3*$s_{y/x}$) of 0.13 μg mL^{-1} and a quantification limit (LQ = Oo + 10*$s_{y/x}$) of 0.44 μg mL^{-1} were obtained.

Time, min	Solvent B, %
0.0	15
5.0	35
10.0	35
20.0	100
25.0	100
28.0	15

TABLE 1 Linear gradient, at a flow rate of 1.0 mL, used for the separation of *trans*-resveratrol by RP (reverse phase)-HPLC-UV

3 Results and discussion

The mobile phase and the flow rate were the same those used previously for the analysis of *trans*-resveratrol in grapevine leaves [27], but the linear gradient program for separation and identification of *trans*-resveratrol in grape samples was slightly modified because of the different matrix (Table 1). The analysis time was 30 min, and the *trans*-resveratrol peak emerged at 10.7 min. The samples were prepared using the same sample volume and water–acetic acid solution (at pH 2.6). All the extracts were analyzed using the optimal conditions indicated above and the samples were ramdomized and identified only by a number.

Figure 1 shows a chromatogram obtained by HPLC and UV spectrophotometric detection at a wavelength of 306 nm corresponding to a control sample (non-irradiated grapes). For this sample a concentration of $3 \pm 1\,\mathrm{mg\,L^{-1}}$ of *trans*-resveratrol in the ethanolic extract was calculated on the basis of the calibration curve previously determined.

As stated before, two different wavelengths were used for the irradiation of the samples: 302.1 nm (resonant wavelength) and 300.0 nm. Figure 2 shows the REMPI (Resonance Enhanced MultiPhoton Ionization) spectrum of *trans*-resveratrol previously obtained by our group showing both wavelengths, including an inset with the structural formula of this compound.

Figure 3 shows the differences in two chromatograms obtained from samples irradiated during the same time and conditions except that one was irradiated with 300.0 nm (A) and the other with the resonant wavelength of 302.1 nm (B). In the chromatogram A, obtained from a sample irradiated during 45 min with the non-resonant wavelength, only a little enhancement of the *trans*-resveratrol peak can be noticed in comparison with the one in the control sample shown in Fig. 1; in contrast, the chromatogram B clearly shows the increase in the *trans*-resveratrol content obtained just by irradiating with the resonant wavelength of the compound of interest.

All the extracts were analyzed using the optimal conditions indicated above. The results are shown in Table 2. They have been plotted in Fig. 4, in which the *trans*-resveratrol content of the samples irradiated with the resonant wavelength (top) and with the non-resonant one (bottom) is shown vs. the irradiation time. It can be noticed how the resonant irradiation produces a clear elicitation of the resveratrol biosynthesis, which reaches a six-fold increase for the samples irradiated during 45 min in comparison with the initial concentration; in contrast, grapes irradiated at 300.0 nm show no significant variation in their *trans*-resveratrol content.

The observed enhancement is of the same order of magnitude as was found in most of the works recently published for the UV elicitation in mature grapes. Also, it is important to note that the enhancement of the *trans*-resveratrol content in the irradiated grapes was monitored immediately after the irradiation took place. In all the works previously published there was necessary an induction time ranging from 18 h [13] to 72 h [16] in order to reach the maximum *trans*-resveratrol content and the effect was negligible during the first hours after the irradiation. This explains why there is no appreciable enhancement in the *trans*-resveratrol content for the grapes ir-

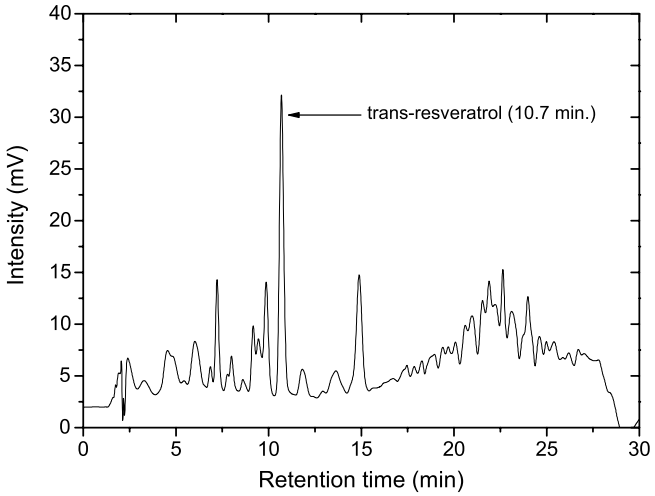

FIGURE 1 Chromatogram of a control sample showing the *trans*-resveratrol peak by HPLC-UV

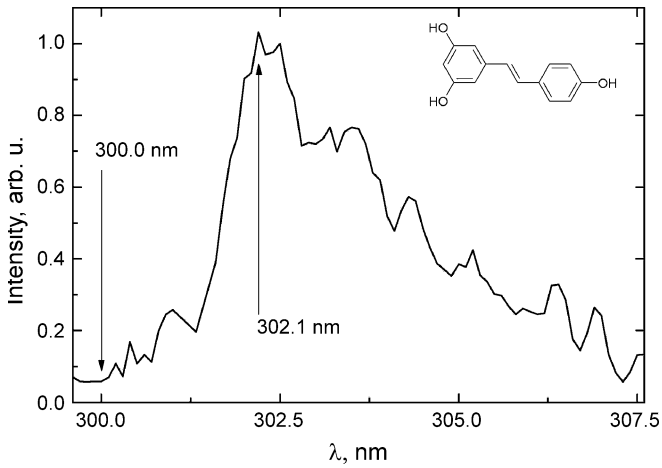

FIGURE 2 REMPI spectrum of *trans*-resveratrol with the two wavelengths used for the experiments clearly indicated. The *inset* shows the structural formula of the compound

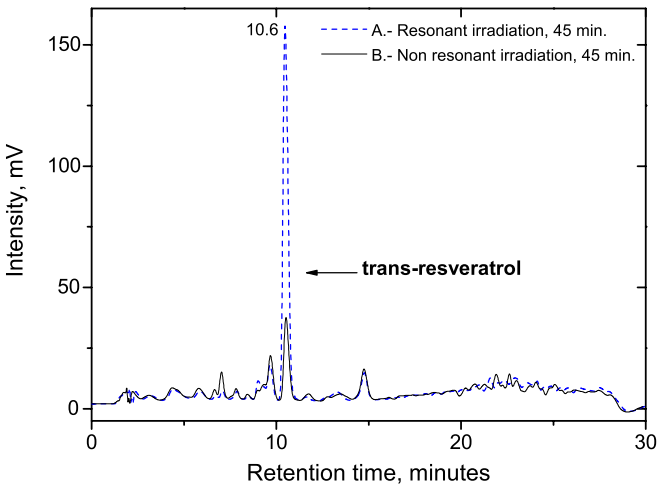

FIGURE 3 Chromatograms obtained by HPLC-UV from two samples irradiated during 45 min. (A) With 302.1 nm, the resonant wavelength, (B) with 300.0 nm, a non-resonant wavelength

Run	λ, nm/t, min	[] (mg L^{-1})	[]$_m$ (mg L^{-1})	SD (mg L^{-1})
1	Control	2.9		
2	Control	4.8	3.43	1.19
3	Control	2.6		
4	NR/15	4.6		
5	NR/15	4.5	4.03	0.89
6	NR/15	3.0		
7	R/15	2.4		
8	R/15	1.6	2.27	0.61
9	R/15	2.8		
10	NR/30	3.2		
11	NR/30	2.1	2.73	0.57
12	NR/30	2.9		
13	R/30	7.1		
14	R/30	8.2	6.33	2.34
15	R/30	3.7		
16	NR/45	6.7		
17	NR/45	2.1	3.73	2.57
18	NR/45	2.4		
19	R/45	19.0		
20	R/45	14.6	21.3	8.09
21	R/45	30.3		
22	NR/60	3.5		
23	NR/60	3.6	3.56	0.06
24	NR/60	3.6		
25	R/60	2.0		
26	R/60	1.2	2.40	1.44
27	R/60	4.0		

λ/t: wavelength, nm/exposure time, min
[]: concentration
[]$_m$: mean concentration
SD: standard deviation

TABLE 2 Concentration of *trans*-resveratrol in the samples analyzed by RP-HPLC-UV. R stands for the resonant wavelength (300.1 nm) and NR for the non-resonant wavelength (300.0 nm). The irradiation time is expressed in minutes

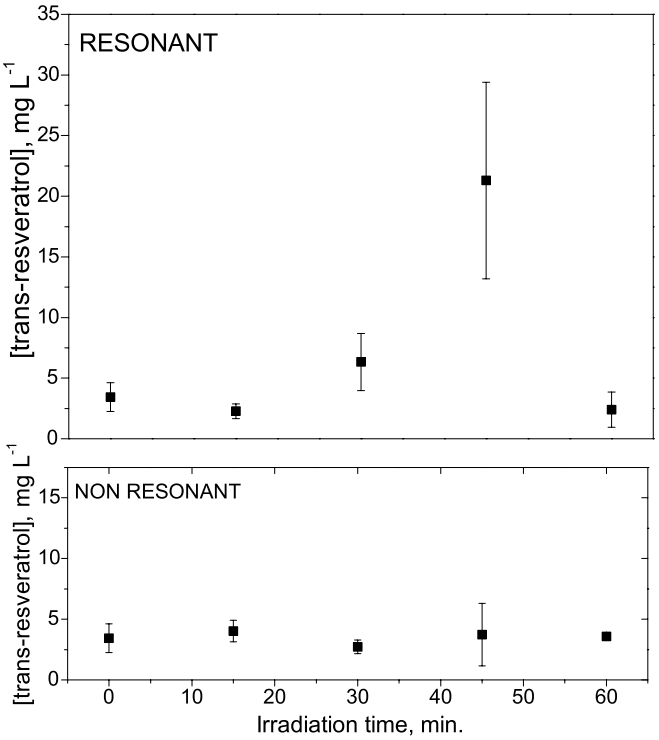

FIGURE 4 Content of *trans*-resveratrol vs. time of irradiation for the samples irradiated with the resonant wavelength (*top*) and a non-resonant one (*bottom*)

Experimental time (min)	Irradiation time (s)	Accumulated number of laser pulses	Accumulated fluence (kJ/m^2)
15	7.2	72	10.15
30	10.8	108	15.22
45	15.0	150	21.15
60	18.0	180	25.38

TABLE 3 Accumulated laser fluence calculated for each irradiation time

radiated at 300.0 nm. Experiments are in progress in order to find the optimal conditions for the induction period after laser resonant irradiation and therefore to obtain the maximum resonant elicitation of *trans*-resveratrol in grapes.

With respect to the apparently long time of irradiation presented in this work in comparison with the previously published ones, it must be said that due to the spatial arrangement of the experimental set-up used as previously described, the real time of irradiation for each individual grape must be considered far shorter. For example, the real time of irradiation for the grapes maintained on the turning disc during 45 min has been estimated (taking into account only the geometrical factors) as approximately 2.5 min. Moreover, the frequency of the pulsed laser used for the irradiation was 10 Hz, i.e. at each second the grapes were exposed to photons of only 10 pulses of 5 ns each. Table 3 shows the values obtained for each experimental time after these geometric and frequency corrections were taken into account.

Despite this very short irradiation time, the main factor to be considered in the case of pulsed laser irradiation is the incident power, which is far higher than those obtained with UV lamps or cw lasers. Table 3 shows the accumulated laser fluence as a function of the experimental time; it was calculated by multiplying the accumulated number of pulses (also given in the table) by the fluence of the laser (0.141 kJ/m^2).

Regarding the results obtained for the grapes irradiated at 302.1 nm during 60 min in which the *trans*-resveratrol content was even below the initial one, this trend (obviously at different time scales) is similar to the one observed in other experiments aiming at enhancing the *trans*-resveratrol content in grapes by other external stresses such as anoxic treatments of the grapes [28] or infection with *Botrytis cinerea* both in grapes [29] and in leaves [30]: in all these cases there was found a period of time in which the *trans*-resveratrol content increased, followed for a sudden reduction of its concentration. At present we still do not have any clear explanation for this result, although it could be due to degradation of the compound by the laser energy.

It is well described that *trans*-resveratrol is synthesized as an end-product of the phenylpropanoid pathway by con-

densation of one molecule of 4-coumaroyl-CoA with three malonyl-CoA units in a reaction catalyzed by stilbene synthase (STS). As has been stated in the introduction, one of the stressing agents that can catalyze this synthesis is UV irradiation, but in the case of post-harvested grapes (out of the plant) this reaction can continue only up to the depletion of the precursors. Therefore, the temporal evolution of the *trans*-resveratrol showing an initial increase up to a maximum followed by a post-maximum decline might result from the competition of two processes: a first one in which UV light induces a photochemical change of a given precursor into *trans*-resveratrol and a second one dominated by *trans*-resveratrol photodissociation. As the precursor concentration (inside the irradiated grapes) is finite, the yield of the photoinduced *trans*-resveratrol should level off at some time, after which only the *trans*-resveratrol photodissociation process should remain, explaining then the observed post-maximum decline.

4 Conclusions

The UV irradiation treatment implemented in the present investigation has proved to significantly enhance the (endogenous) *trans*-resveratrol content in grapes. The main difference with previously published works is the use of the resonant wavelength of the compound (302.1 nm) for the irradiation. The use of photons of resonant energy to produce the absorption through real electronic states of the molecule significantly increased the absorption yield, producing an important effect on the photoinduced *trans*-resveratrol level in the grapes.

The enhancement was optimum (six-fold) for 45 min of irradiation with 302.1 nm and it was of the same order of magnitude as most of the previously published values. A second important difference is that in previously reported work an induction time period was allowed to reach the maximum *trans*-resveratrol content and the effect was negligible during the first hours after the irradiation. As mentioned, in this work, the samples were prepared for analysis immediately after irradiation in order to detect the direct effect of resonant elicitation, which has now been demonstrated for the first time.

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