



Analytical Methods

Determination of stilbenes in Sicilian pistachio by high-performance liquid chromatographic diode array (HPLC-DAD/FLD) and evaluation of eventually mycotoxin contamination

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Abstract

In this work, we have investigated about the presence of several natural stilbenes in 12 samples of pistachios harvested from 10 different farms of Sicily (Bronte and Agrigento). At the same time, we have evaluated the relation between the stilbenes synthesis and the possible contamination of mycotoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus*. We have found two types of stilbenes in the samples of pistachios examined: *trans*-resveratrol and *trans*-resveratrol-3-*O*- β -glucoside (*trans*-piceid). Their concentration ranged from 0.07 to 0.18 mg/kg (av. = 0.12 ± 0.03 mg/kg) for *trans*-resveratrol, from 6.20 to 8.15 mg/kg (av. = 6.97 ± 0.55 mg/kg) for *trans*-piceid and from 6.38 to 8.27 mg/kg (av. = 7.09 ± 0.54 mg/kg) for total resveratrol.

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1. Introduction

Stilbenes are a family of many compounds that, in the last decade, have attracted the attention of researchers due to their wide range of biological activities. One of the most relevant and extensively studied stilbenes is resveratrol (*trans*-3,4',5-trihydroxystilbene) (Fig. 1a), a phytoalexin present in grapes and its derivatives as red wines, grape juice (Adrian et al., 2000; Vitrac et al., 2005; Wang, Catania, Yang, Roderick, & van Breemen, 2002) and other foods, which is capable of acting as cancer chemopreventive agent (Burns, Yokota, Ashihara, Lean, & Crozier, 2002). The function of stilbenes consists in defending plants

against many abiotic and biotic factors such as UV irradiation, injuries, cold, heat, pathogen microorganism's attack, and growth of molds (Langcake & Pryce, 1976, 1977). These molds such as *Aspergillus flavus* and *Aspergillus parasiticus*, what is more, produce mycotoxins (Aflatoxin B1, B2, G1, G2), which are a potent risk for human health, and are potent carcinogens. In particular, the AFB1 is the most potent hepatocarcinogen known in mammals (Hadidane et al., 1985; McGlynn et al., 2003). The European Community has set limits of AFB1 to 2 μ g/kg and of total AFs (B1 + B2 + G1 + G2) to 4 μ g/kg for the pistachio nuts (Regulations CE N. 1525/98). Several in vitro and in vivo studies have shown that *trans*-resveratrol (*trans*-3,4',5-trihydroxystilbene) (Fig. 1a) inhibits cellular events associated with cancer initiation, promotion and progression (Jang et al., 1997). Moreover, it has been associated with reduced cardiovascular diseases by inhibiting or

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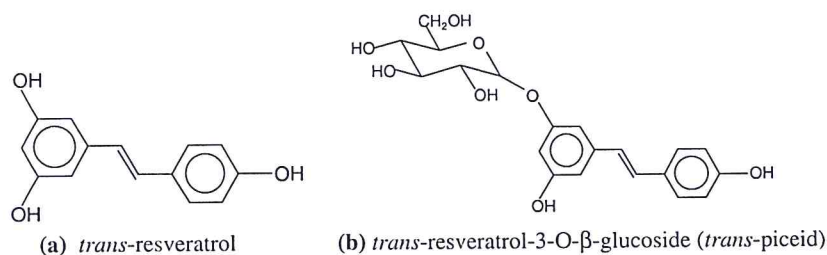


Fig. 1. (a) *trans*-Resveratrol (b) *trans*-resveratrol-3-*O*- β -glucoside (*trans*-piceid).

altering platelet aggregation and coagulation, or modulating lipoprotein metabolism (Arichi et al., 1982; Chung, Teng, Cheng, Ko, & Lin, 1992; Kimura, Okuda, & Arichi, 1985; Kimura, Okuda, & Kubo, 1995). *trans*-Resveratrol-3-*O*- β -glucoside (*trans*-piceid) (Fig. 1b) is another interesting stilbene endowed with antioxidant properties similar to *trans*-resveratrol (Bavaresco, Fregoni, Cantù, & Trevisan, 1999; Fauconneau et al., 1997; Frankel, Kanner, German, Parks, & Kinsella, 1993; Frankel, Waterhouse, & Kinsella, 1993). *trans*-Piceid is usually present in the grapes berries (Korhammer, Reneiro, & Mattivi, 1995; Waffo Tegu, Decendit, Vercauteren, Deffieux, & Mérellon, 1996; Waterhouse & Lamuela-Raventos, 1994). Resveratrol has been detected in several plants such as edible peanuts (*Arachis hypogaea* L.), peanut products (Ibern-Gomez, Roig-Perez, Lamuela-Raventos, & de la Torre-Boronat, 2000; Lee et al., 2004; Sanders, McMichael, & Hendrix, 2000; Sobolev, Cole, Dorner, & Yagen, 1995; Sobolev & Cole, 1999; Tokuşoğlu, Ünal, & Yemiş, 2005) and in pistachios varieties grown in Turkey (Tokuşoğlu et al., 2005). The pistachio plant (*Pistacia vera* L.) is capable of growing in prohibitive condition such as the lava rocks. Pistachio is a unique nut that is not destroyed in the industrial making process (roasting, salting and packaging). The *P. vera* L. crop in Sicily is one of the most important commercial crops; in Italy, the cultivation of pistachio is done only in Sicily, and particularly in Bronte and Agrigento areas. The Sicilian pistachio is highly appreciated for its taste property and is mainly used as a confectionery, as a dessert ingredient (sweet pastry) and in the manufacturing of ice-cream.

Aflatoxin (AFs) contamination in pistachio varies drastically and very much depend on relative humidity and temperature during harvesting and storage period. The Sicilian climate favours the growth of molds and consequently a probable contamination of AFs in pistachio nuts. At present there are no data on stilbenes content and mycotoxins contamination in Sicilian pistachios.

The aim of this work was to identify stilbene molecules in twelve samples of Sicilian pistachio by using liquid chromatography diode array detection (HPLC-DAD) and a fluorescence detector (FLD). Moreover, to check that stilbenes synthesis was not induced from aflatoxins contamination, we have evaluated the absence of AFs (Aflatoxin B1 and total Aflatoxins).

2. Materials and methods

2.1. Materials

trans-Resveratrol was purchased from Sigma (St. Louis, MO, USA); *trans*-resveratrol-3-*O*- β -glucoside (*trans*-piceid) was purchased from Apin Chemicals Ltd. (Great Britain); *cis*-resveratrol and *cis*-piceid were obtained after the exposure of *trans*-resveratrol and *trans*-piceid UV irradiation; after 12 h of exposure, 80% of *trans*-resveratrol and *trans*-piceid was converted to the respective *cis*-isomers.

Acetonitrile, methanol and ethanol of HPLC-grade were obtained from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Aluminiumoxide (Al_2O_3) 90 standardized was obtained from Merck (Darmstadt, Germany). Econo-column (Poly-Prep chromatography column) was obtained from Bio Rad Laboratories, Roma. Accubond^{II} SPE ODS C18 (59 μm , Agilent Technologies, West Lothian, UK). Phosphate buffer was prepared with potassium dihydrogen phosphate ACS-ISO for analysis (Carlo Erba Reagents, MI, Italy) at 0.02 M and adjusted to pH 3.0 with orthophosphoric acid 85% ACS-ISO for analysis (Carlo Erba Reagents, MI, Italy), and later it was filtered with nitrocellulose 0.45 μm , 47 mm gridded (Millipore, Bedford, MA, USA). Phosphate buffered saline (PBS), pH 7.4, was prepared with potassium chloride (0.20 g/l), potassium dihydrogen phosphate (0.20 g/l), disodium hydrogen orthophosphate (1.16 g/l) and sodium chloride (8.0 g/l). The pH was adjusted to 7.4 with HCl (0.1 mol/l) or NaOH (0.1 mol/l). Pyridinium hydrobromide perbromide (PBPB) was used for post-column derivatization. Immunoaffinity column (IAC) was used for the preparative and purification phase of aflatoxins B1, B2, G1 and G2. AFs standards for the experiments were purchased from Sigma Chemical Company, USA. The *n*-hexane (technical grade) and the reagent for PBS and PBPB were obtained from Carlo Erba Reagents (MI, Italy).

2.2. Samples

Pistachio samples analysed in this work are obtained from the plants of pistachio belonging to the Anacardiaceae family, genus *Pistacia*, species *P. vera* L., variety

“Napoletana” or “White”. In Bronte *P. vera* plants are generally in the form of clutches in the plant of *P. terebinthus* L. which naturally grows in this area; in contrast, in Agrigento area, *P. terebinthus* L. does not grow spontaneously but it is planted, clutches with *P. vera* and cultivated. The Bronte pistachio is cultivated to a height ranging from 500 to 800 m; in contrast, Agrigento pistachio is cultivated to a height of 400 m. The Agrigento pistachio samples were picked up by machinery, while the Bronte pistachio samples were traditionally picked up by hand. All twelve samples were harvested in September 2005 during the maturation phase. After harvesting the pistachio were parted from external fleshy part, called “mallo”, by hand (small production), or by machinery (large production). All samples were dried in the sun and stored in transpiring bags, at room-temperature in a ventilated place.

2.3. Analysis of stilbenes

2.3.1. Sample preparation

All pistachio samples were shelled by hand and immediately analysed. They were analysed three times. Sample extraction procedure was carried out according, in part, to the method described by Sanders et al., 2000. Each sample was milled and 7 g of it was extracted with 70 ml of ethanol/water (80/20, v/v) by a Polytron homogenizer at high speed. The homogenizer mixture was filtered through a glass microfibre filter (Whatman GF/A) and the filtrate was transferred to a 100 ml volumetric flask, diluted to volume with ethanol/water (80/20, v/v). An aliquot (8 ml) of the filtrate was passed through the clean-up column composed of an Econo-column (Poly-Prep chromatography column), packed with 1.0–1.2 ml of a mixture of Al₂O₃ and Accubond^{II} SPE ODS C18 (59 µm, Agilent Technologies, West Lothian, UK). The column was washed with 2 ml of extracting solvent and diluted to 10 ml. Of this 5 ml was dried under nitrogen gas at 30 °C and then it was dissolved with 1 ml methanol/water (50/50, v/v), followed by filtration using 0.20 µm cellulose membrane filter (Minisart RC 25 Sartorius, Germany). All steps were carried out under yellow light to protect light-sensitive *trans*-resveratrol and its glucoside.

2.3.2. HPLC analysis

The liquid chromatograph used was an HPLC system Agilent Technologies (Palo Alto, CA) Series 1100, constituted by a quaternary pump equipped with a microdegasser, thermostat autosampler, thermostat column compartment and DAD detector. To increase the sensitivity of the procedure, an Agilent 1100 Series fluorescence detector set at 325/390 nm (excitation/emission) was also used for qualitative analysis of *trans*-resveratrol and *trans*-piceid. The column was a reversed-phase Luna ODS C18 (Phenomenex), 250 × 4.6 mm ID, 5 µm. The mobile phase was made with solvent A (acetonitrile) and solvent B (phosphate buffer, pH 3.0). We have developed

Table 1
Gradient elution 1

Time (min)	Solvent A (%)	Solvent B (%)
5.00	10	90
8.00	20	80
10.00	20	80
13.00	40	60
25.00	40	60
27.00	60	40

Table 2
Gradient elution 2

Time (min)	Solvent A (%)	Solvent B (%)
5.00	10	90
8.00	20	80
19.00	20	80
30.00	40	60
33.00	60	40
50.00	60	40

two methods which differ in the gradient mobile phase. The first gradient and the second gradient are composed as reported, respectively, in Tables 1 and 2. Both gradients were used to separate the *trans*-resveratrol and the *trans*-piceid.

The flow rate of mobile phase was 1 ml/min and the temperature of the column was 25 °C. The injection volume was 50 µl. Each sample was injected three times. For both gradients, the experimental conditions were the same, whereas the only different consisted in the ratio of the solvent (A and B) during the elution. The wavelength was set at 325 nm that was the maximum absorbance of both stilbenes. To check the peak purity, the eluates were monitored with a photodiode array detector (DAD), with a wavelength range of $\lambda = 220$ –600 nm. The absorption spectra of each peak were compared with the absorption spectra of the standard and these were identified when the match factor was >995. The t_R of *trans*-resveratrol ranged from 17.92 to 18.03 min and 28.89–30.10 min, respectively, with gradient elution 1 and gradient elution 2; for *trans*-piceid the t_R ranged from 15.30 to 15.49 min and from 17.93 to 18.09 min using, respectively, gradient elution 1 and gradient elution 2. In Fig. 2 the chromatographs of a sample eluted with gradients 1 and 2 are depicted.

2.3.3. Calibration

Standards of *trans*-resveratrol and of *trans*-piceid were employed at different concentrations to construct a calibration curve. Both standards were diluted in methanol/water (50/50, v/v), the concentrations ranging from 0.05 to 3.0 mg/l (0.05, 0.25, 0.50, 1.0, 1.5, 2.0, 3.0 mg/l). The curves had a good linearity; the correlation coefficient for *trans*-resveratrol was 0.99927 and for *trans*-piceid was 0.99769.

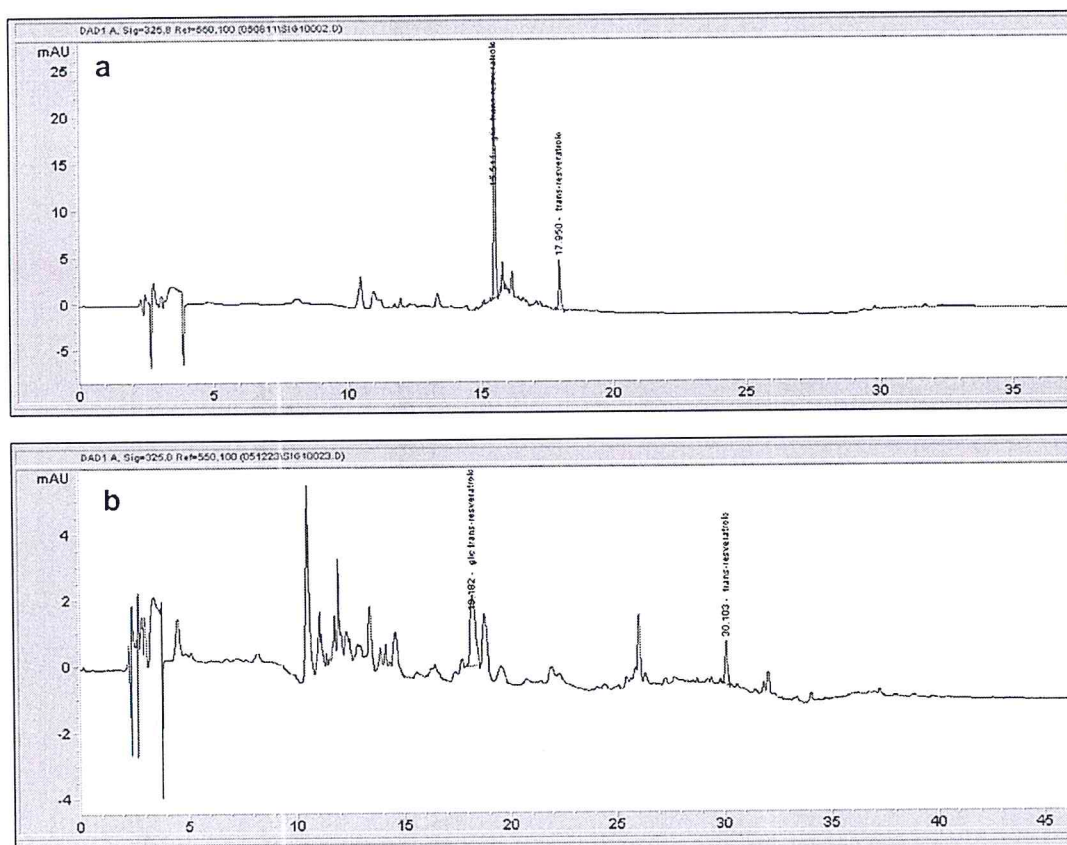


Fig. 2. Chromatograph of *trans*-resveratrol and *trans*-piceid with gradient 1 (a) and gradient 2 (b).

2.3.4. Recovery analysis, analytical precision, LOD and LOQ

Peaks identity was obtained by comparing the peaks retention time and the absorption spectra with that of the pure standards. The presence of the stilbenes is confirmed only when the match between the absorption spectra of the peak analysed and the pure standard peak was >95.

The recovery factors for *trans*-resveratrol and *trans*-piceid were determined at four different concentrations (between 0.05 and 8 mg/kg) spiking the samples of pistachio with pure standards. Each concentration was tested six times before and after addition. The mean *trans*-resveratrol recovery averaged 75.5% and for *trans*-piceid it averaged 91.0%. This was connected probably to the greater solubility of the *trans*-piceid in the solvent extraction of ethanol/water (80/20, v/v).

The precision of the analytical method for both stilbenes was determined by spiking one sample at five different concentrations (0.5, 1.5, 3.0, 6.0, and 8.0 mg/kg), by performing six replication analyses for each concentration.

The limit of detection (LOD) was estimated by multiplying the SD by a factor of 3 (signal-to-noise ratio of 3), while the limit of quantitation (LOQ) was considered to be five times the SD. The lowest detected value of both stilbenes in pistachio was 0.01 mg/l which was significantly different

from zero ($P < 0.01$), under the analytical conditions used and at the best wavelengths of 325 nm.

2.4. Analysis of aflatoxin

2.4.1. Sampling

Samples were taken exactly according to the sampling procedure of European Law (CE) N. 401/2006 for sampling of pistachio nut. The pistachio nut consignments were usually about 5.0 ton. Following the taking of one sample weighing 18 kg (60 incremental samples, each 300 g), it was mixed and divided into two sub-samples.

2.4.2. Samples preparation

The pistachio samples were homogenized appropriately to give a paste, using a high speed blender, called "slurry". Approximately, 50 g of the homogenized test portion was put into a 500 ml conical flask and to it were added 5 g of sodium chloride, 200 ml of extraction solvent, consisting of methanol and water (8/2, v/v), and 100 ml of *n*-hexane. This mixture was blended for 3 min with a high speed blender. The extract was filtered using a paper filter, 24 cm in diameter, with two separated layers, carried on with the lower phase, and diluted with 60 ml of PBS. This diluted sample extract was added to the reservoir connected to the conditioned immunoaffinity column. The filtrate passed

through the IAC at a flow rate of 3 ml/min (approximately one drop per second). The IAC was washed with 15 ml of PBS at a flow rate of 3 ml/min and dried by passing air through the IAC for 10 s.

The eluates were collected in a two-step procedure. The first time 0.50 ml of methanol was applied on the IAC, and was allowed to pass through by gravity, 1 min later a second portion of 0.75 ml of methanol was applied. Then, air was pressed through the column for collecting most of the applied elution solvent, it was then filled to a volume of 5 ml with water. This solution was used for HPLC analysis directly if appeared clear; on the contrary, the solution was filtered through a disposable filter (0.45 µm).

2.4.3. HPLC analysis

The AFs were separated by isocratic reverse-phase HPLC at 40 °C with water/methanol/acetonitrile (54/29/17, v/v/v). The flow rate was 1 ml/min. The column was a phenomenex C18 (250 mm × 4.6 mm × 5 µm). The injection volume was 200 µl. The post column derivatization was made with PBPB using a flow rate of 1 ml/min for the mobile phase and 0.30 ml/min for the reagent. The excitation wavelength was 365 nm and emission was 435 nm.

2.4.4. Calibration curve

The calibration curves were prepared using the mixed AFs standard solution. These solutions for AFB1 and AFG1 ranged from 0.5 ng/g to 4.5 ng/g and for AFB2 and AFG2 ranged from 0.1 ng/g to 0.9 ng/g.

2.4.5. Spiking procedures

For the determination of the recovery, the spiking level was within the calibration range (mean value). The spiking solution was added to the sample without AFs and the spike was left in the dark overnight.

2.5. Statistical analysis

The statistical analysis were done by using MedCalc statistical program.

3. Results

Twelve pistachio samples were analysed for their content in *trans*-resveratrol and *trans*-resveratrol-3-*O*-β-glucoside (*trans*-piceid): six were harvested from Sicilian farm situated in the Bronte area and six harvested from the Agrigento area. All pistachio samples belonged to a variety called “Napoletana” or “White”. We observed that all the examined samples contained *trans*-resveratrol and its glucoside. The concentration of *trans*-resveratrol ranged from 0.07 to 0.18 mg/kg, the *trans*-piceid content was in the range 6.20–8.15 mg/kg and the content of total resveratrol was from 6.38 to 8.27 mg/kg (Table 3).

No *cis* isomers of both stilbenes were detected in the analysed samples. The mean content for *trans*-resveratrol

Table 3

Concentration of *trans*-resveratrol and *trans*-piceid in pistachio samples

Sample	Origin	Year of harvest	<i>trans</i> -Resveratrol-3- <i>O</i> -β-glucoside (mg/kg)	<i>trans</i> -Resveratrol (mg/kg)	Total resveratrol (mg/kg)
1	Racalmuto	2005	6.67	0.18	6.85
2	Racalmuto	2005	6.32	0.13	6.45
3	Favara	2005	6.98	0.10	7.08
4	Raffadali	2005	6.75	0.09	6.84
5	Agrigento	2005	7.18	0.13	7.31
6	Agrigento	2004	6.20	0.18	6.38
7	Bronte	2005	7.21	0.13	7.34
8	Bronte	2005	7.21	0.07	7.28
9	Bronte	2005	7.60	0.09	7.69
10	Bronte	2005	8.15	0.12	8.27
11	Bronte	2005	6.93	0.09	7.02
12	Bronte	2005	6.50	0.09	6.59

was 0.12 ± 0.03 mg/kg, for *trans*-piceid was 6.97 ± 0.55 mg/kg and for total resveratrol was 7.09 ± 0.54 mg/kg.

The concentration of *trans*-piceid was markedly higher than that of *trans*-resveratrol in all samples examined ($P < 0.01$). This result was similar to that obtained generally in the case of red grapes and its derivatives (wine, juice) (Mark, Nikfardjam, Avar, & Ohmacht, 2005; Romero-Perez, Ibez-Gomez, Lamuela-Raventos, & de la Torre-Boronat, 1999). Indeed, the amount of *trans*-piceid in red wine may be more than ten times greater than its aglycone *trans*-resveratrol (Vitrac, Monti, Varcauteren, Deffieux, & Merillon, 2002).

No significant difference in stilbenes content was observed on comparison of the samples of pistachio of Bronte and Agrigento areas.

Each sample of pistachio nuts examined for the presence of AFs was devoid of these mycotoxins. The amount of AFB1 was <0.1 µg/kg and the amount of total AFs (B1 + B2 + G1 + G2) was <0.4 µg/kg.

4. Discussion

Our research has shown that Sicilian pistachio nuts contain *trans*-resveratrol and its glucoside. Recently, Tokuşoğlu et al. have reported the presence of *trans*-resveratrol in Turkey peanuts and pistachio varieties (Tokuşoğlu et al., 2005). However, in Tokuşoğlu's study, *trans*-piceid was not detected. *trans*-Piceid is a form of resveratrol important for human health because it is converted in a free form (*trans*-resveratrol) by human small intestine and liver β-glucosidase activity after ingestion (Day et al., 1998).

Moreover, the absence of AFs in these samples proves that the synthesis of stilbenes was not induced from mold infections such as *A. flavus* and *A. parasiticus*.

In conclusion, the Sicilian pistachio results an excellent source of stilbene compounds (*trans*-resveratrol and *trans*-piceid) and the absence of mycotoxin demonstrates that is a healthy food.

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