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Full Length Research Paper

Evolution throughout the ripening in grape fruit skins: High performance liquid chromatography-diode array detector (HPLC-DAD) detection of trans-resveratrol

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***Trans*-resveratrol (3, 5, 4' – trihydroxy-trans-stilbene) is a stilbene naturally present in a number of plant families. Berry skins of grape (*Vitis vinifera* L.), Alicante, Black Malvasia, Nerello and Prunesta cultivars were analyzed at weekly intervals for *trans*-resveratrol production at five ripening stages during the last five weeks of maturation, from August 26th to September 23th. Analysis was carried out using the high performance liquid chromatography-diode array detector (HPLC-DAD) technique after fractioning of *trans*-resveratrol through a 500 mg C_{18} column (Solid Phase Isolation – SPI technique). A continuous decrease in *trans*-resveratrol content in all cultivars was observed during ripening. Alicante had the highest *trans*-resveratrol content, showing a decrease of 46% from the first to last sampling, while Black Malvasia produced the lowest amount of *trans*-resveratrol, 4 to 5 times less than Alicante at each sampling date.**

Key words: Antioxidants, grape, phenols, stilbenes, *trans*-resveratrol, high performance liquid chromatograph (HPLC).

INTRODUCTION

The increase in food quality information has led to a cultural evolution among consumers. Food quality can be defined as the capacity of a product to answer consumer needs and can be characterized by numerous aspects: chemical, physical, microbiological, nutritional, hedonistic and economical. One of the most important aspects of food quality is related to nutraceutical and functional activity. The term “functional food” originated in Japan in the 1980s, and is defined as “any food or ingredient that has a positive impact on an individual’s health, physical performance or state of mind in addition to its nutritive value” (Hardy, 2000; Goldberg, 1994). The increasing interest for nutraceuticals and functional foods is also related to the awareness that chemical pharmaceutics are always expensive, superfluous and sometimes unsafe and have dubious benefits (Bagchi, 2006). Fruits are among the most important foods that naturally contain functional biomolecules like polyphenols. Trans-resveratrol is a polyphenolic component of the stilbenes group, (3, 5, 4' - trihydroxy-trans-stilbene) and its structure and metabolism have been widely studied (Guiso et al., 2002; Deak and Falk, 2003; Halls and Yu, 2008; Szekeres et al., 2010). Its formation is caused by a response of plant tissues to external stress (Frémont, 2000; Hammerschmidt, 2004) and the berry skins of red grape contain this minor but important substance. In several studies, trans-resveratrol, a cardioprotective component, has been associated with many beneficial effects on human health (Frémont, 2000; Dawn, 2007) and cardiovascular benefits have also been observed among the Greek population (Kopp, 1998). An important role was also found in diabetes with insulin regulation (Szkudelska and Szkudelski, 2010) and beneficial effects have also been observed for anti-inflammatory arthritis with experiments carried out on animals (Elmali et al., 2007). Beneficial effects have been described for

skeletal muscles, increasing glucose transport, restraining protein degradation, improving strength and endurance and protecting from oxidative injury (Dirks Naylor, 2009). In addition, studies have shown that trans-resveratrol, as a natural antioxidant, plays an important role in chemoprevention (Schneider et al., 2000; Savouret and Quesne, 2002; Dong, 2003; Goswami and Das, 2009). The trans-resveratrol concentration in grape skins and wines is influenced by a number of factors including the geographical area of production, climateage of the wine, grape cultivar, fungal infection and ultraviolet irradiation (Roggero and Garcia-Parrilla, 1995; Roggero, 1996; Roldán et al., 2003; Moreno-Labanda et al., 2004).In Northern Italian red wines, Recioto (sweet) and Amarone (dry) produced from the same grape cultivar in the same geographical area and with the same technical proce-dures by processing dried grapes recorded a 0.05 to 0.40 and 0.05 to 0.80 mg/L trans-resveratrol content for Recioto and Amarone, respectively (Celotti et al., 1996). In Czech red wines from the Bohemian and Moravian regions, a trans-resveratrol content of between 0.92 and 6.25 mg/L was observed (Kolouchová-Hanzlíková et al., 2004). In French red wines, the resveratrol concentration in young and nine-month old wines was analyzed with the highest content generally found in the young wines (Roggero and Archier, 1994). In Hungarian wines, a maximum of 10.4 mg/L was recorded with a relatively higher content in the warm “Villany” Region (Southern Hungary) and a lower content in wines produced in the cold and humid “Eger” Region (Montsko et al., 2010).

Despite a wide interest in grape cultivation and wine production in the Calabria Region (Southern Italy), little information has been found regarding the chemical characteristics and the correct time of harvest of the grape cultivars of this region, discriminating between industrial and technological maturity. Industrial maturity time is characterized by a higher concentration of sugars in the fruit and therefore leads to a higher theoretical degree of alcohol in the wine while technological maturity is reached when the grapes have the optimum chemical composition for obtaining a wine with specific characteristics (Robredo et al., 1991; Jin et al., 2009). Although, several studies have focused on the trans-resveratrol concentration in red wines, relatively few have examined the trans-resveratrol content in berry skins, but no data has been found on the trans-resveratrol content in the berry skins of Calabrian grape cultivars. The aim of this paper was to study the evolution of the trans-resveratrol content in berry skins of 4 red grape cultivars which are widely cultivated on the Tyrrhenian side of the Province of Reggio Calabria, South West Calabria (Southern Italy) during ripening, in order to define the moment of maximum accumulation of this antioxidant. For trans-resveratrol quantification, a solid-phase isolation (SPI) coupled with a HPLC analysis was studied in UV-visible by diode array detector (DAD). No previous data regarding this antioxidant exists for this geographical area.

MATERIALS AND METHODS

Chemicals

Trans-resveratrol standard was purchased from Sigma (Milan, Italy) and a C_{18} Sep-Pak separation column was purchased from Millipore (Milan, Italy). All other reagents were purchased from Merck, Darmstadt (Germany).

Sampling

Four red grape cultivars, Alicante, Black Malvasia, Nerello and Prunesta were grown in vineyards at 80 meters above sea level in the Bagnara Calabra-Scilla area, a coastal location in the province of Reggio Calabria (Southern Italy). The area is characterized by a favourable climate for grape cultivation with rainfall also occurring in summer from the middle of August at regular intervals and especially at night. In this contest, Black Malvasia, Nerello and Prunesta are autochthonous cultivars, Alicante is allochthonous. Temperatures are mild and there are no sudden changes between day and night. Twenty-five grape plants per cultivar, 18 years old in 2009, with similar growth and production characteristics were selected in the vineyard. Plants were selected and labeled in mono-cultivar groves with the same microclimatic conditions. Each mono-cultivar grove was at least 700 meters from the others. Plants were well managed, uniform in size and had no nutrient deficiency or pest damage and were spaced in adjacent rows 1.2 m apart and 1.5 m apart within rows. For all cultivars, cordons were supported by two wire mounted on a trellis at 1.0 m above the vineyard floor. No irrigation was applied to the area during the course of this study. All cultivars were fertilized with 60 kg N ha^{-1}, 5 kg P ha^{-1} and 50 kg ha^{-1} per year. Briefly, no differences there were in applied measures or agrochemicals or climate between individual vineyards. Five samplings were carried out for each cultivar with grapes harvested on the following weekly basis: August 26th, September 2th, 9th, 16th and 23th of the 2009 to 2010 crop year. The fifth sample was conducted at industrial ripeness (Robredo et al., 1991; Jin et al., 2009). Berries were harvested when fully ripe (that is, at the moment when they possessed the required industrial maturity) (Robredo et al., 1991; Di Stefano and Cravero, 1991). Two hundred berries/cultivar were randomly hand-picked from plants and separated into three lots: small (0.00 to 1.00 g), medium (1.01 to 2.00 g), and large (2.01 to 3.00 g) according to their weight. From the three lots, ten sub-lots of 10 berries were formed, each containing small, medium and large berries. At this point, the three most similar sub-lots by weight were chosen to carry out analysis in three replicates. Berries were neither washed nor cleaned before peeling.

Extraction of trans-resveratrol from berry skin

Berries of the three most similar sub-lots were longitudinally cut with a bistoury into halves and the skin was removed within 4 h of harvest. Extraction was conducted according to the Di Stefano and Cravero method (1991) modified as follows: the skins of ten berries were quickly placed in a 25 ml extracting buffer solution (pH=3.2), from a 1 L buffer solution consisting of 200 ml de-ionized water, 5 g tartaric acid, 120 ml ethanol (95% type), 2 g sodium metabisulfite, 22 ml of a 1 N sodium hydroxide solution and de-ionized water up to the 1 L volume. Phenolic compounds were extracted from the skin in darkness for 24 h by means of the above-mentioned extracting buffer solution at a temperature of 25°C. The extracting solution of the four cultivars was separated from the skin by decanting and was then stored in a freezer (-10°C) until analysis.

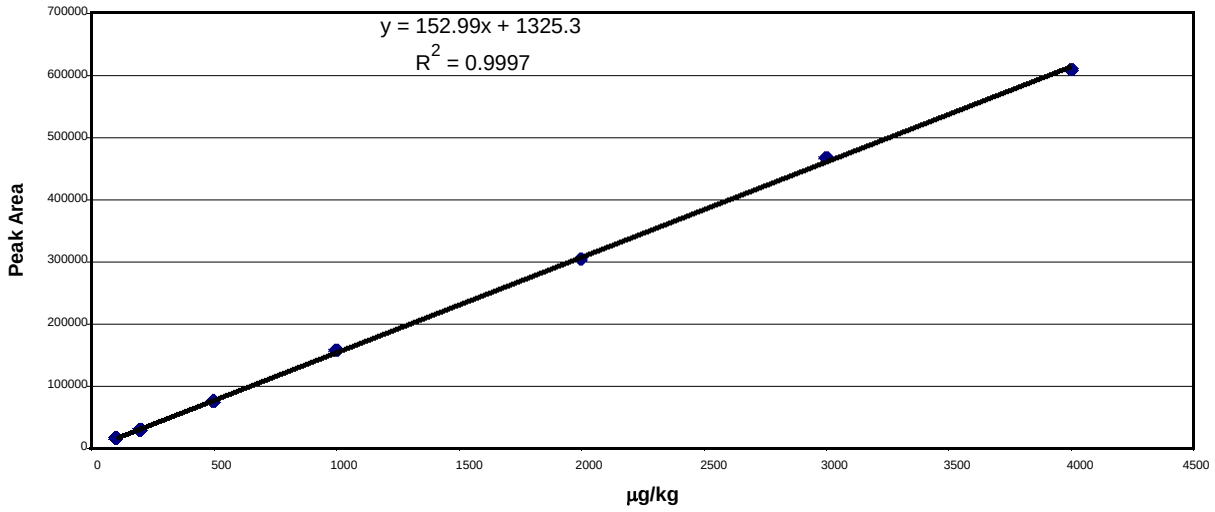

Figure 1. Linear response of 100 to 4,000 µg/kg for *trans*-resveratrol as external standard, after analysis by HPLC-DAD.

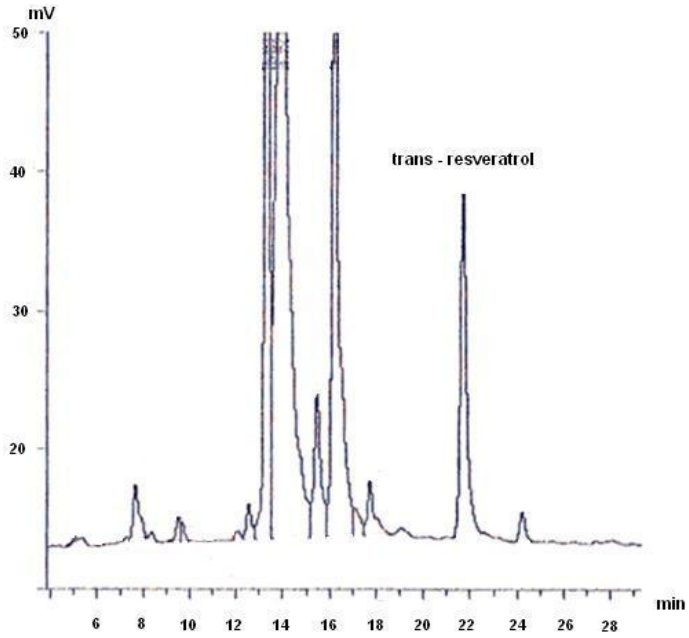

Figure 2. HPLC-DAD chromatogram showing *trans*-resveratrol in grape berry skin.

Solid-phase isolation of trans-resveratrol

The Mattivi (1993) and Kallithraka et al. (2001) methods, adapted for grape skin, were used for the solid-phase isolation of trans-resveratrol. A 5 ml quantity of phenolic extract was adjusted to pH 7 with a 1N NaOH solution. The neutralized extract was quantitatively transferred into a 10 ml volumetric flask and filled up to volume with de-ionized water. A 5 ml volume was placed in a 500 mG C_{18} column.

After adsorbing the sample was washed with 3 ml of a pH 7 buffer solution and 5 ml of de-ionized water, respectively. The eluted washing solution was discarded. Trans-resveratrol was eluted with 40 ml of ethyl acetate. After eluting, the sample was dried by means of a rotary evaporator and a flow of nitrogen. At this point, the dried sample was dissolved in 1 ml of methanol (HPLC grade) in order to prepare the injecting solution.

Chromatographic analysis

Trans-resveratrol was identified by comparison of retention times with standard and by enrichment of skin extracts with authentic sample. For quantitative analysis and response factor calculation, the external standard method was used with trans-resveratrol as the authentic sample. Figure 1 shows the linearity of the response correlation coefficient (R^2) for a range of standards from 100 to 4,000 µg/L which was found to be 0.9997.

The chromatographic detection of trans-resveratrol was conducted using the Roldán et al. (2003) and Kolouchovà-Hanzlìkovà et al. (2004) methods modified by using a Knauer Instrument, consisting of a Smartline Pump 1000 and a Smartline UV detector 2600, equipped with a Lichroshper C_{18} 100 mm × 5 µm pre-column and a ODS Hypersil 200 mm × 2.1 mm × 5 µm separation column (Figure 2). Eluent A, H_3PO_4 10^{-3} M; eluent B, CH_3CN. Program: linear gradient from 0% A to 60% A in 35 min; flow 0.8 ml/min; operating temperature 30°C; detection 310 nm; injection volume 20 µl.

Statistical analysis

"Statistica" software was used for statistical analysis (ANOVA One-way) and Duncan's multiple range tests ($p \leq 0.05$) was used for comparison of means. Excel software was used to build the Figures 1 and 3 and to calculate the SD.

RESULTS AND DISCUSSION

Data of the sample analysis of berries at fifth sampling about physico-chemical characteristics are shown in Table 1 as mean ± standard deviation of ten replicates. The highest °Brix and the lowest TA were found in Black Malvasia. The pH value was similar for all cultivars. Nerello had the lowest °Brix and the highest TA.

Generally was found that the higher the °Brix, the lower the TA. Trans-resveratrol content was expressed as µg/kg

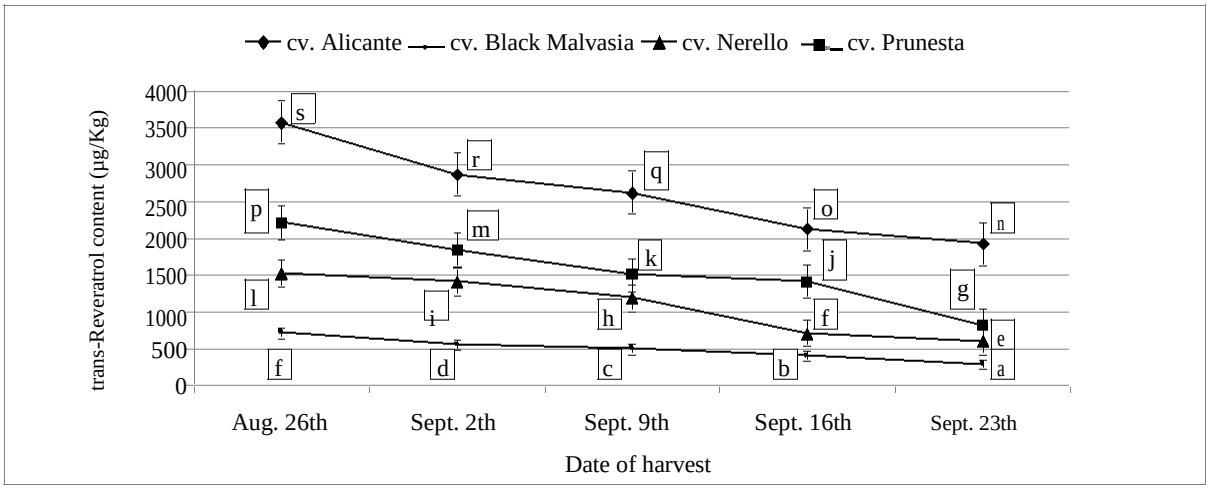

Figure 3. HPLC-DAD analyses of *trans*-resveratrol on fresh berry skins during grape ripening. The results are mean values of three independent experiments ± SD. Data followed by different letters are significantly different by Duncan's multiple range test (P≤0.05).

Table 1. Titratable acidiy (TA) as grams tartaric acids equivalents per liter, °Brix and pH.

Cultivar	TA (g/L) ± SD	°Brix ± SD	pH ± SD
Alicante	7.0 ± 0.64	22.9 ± 0.40	3.5 ± 0.02
Prunesta	8.1 ± 0.70	22.0 ± 0.45	3.7 ± 0.03
Nerello	8.3 ± 0.72	21.8 ± 0.57	3.6 ± 0.02
Black Malvasia	6.9 ± 0.58	23.0 ± 0.51	3.5 ± 0.04

The results are mean values of ten independent experiments ± SD. Data were measured at the fifth sampling.

of the studied stilbene contained in fresh berry skin. Analysis of variance showed a high significant difference between the four cultivars and dates of harvest with a variation coefficient of 2.08% and Duncan's test shows 19 separated homogeneous groups (Figure 3). Analysis of trans-resveratrol evidenced a decreasing trend for all cultivars from the first to the fifth sampling. Of all the cultivars, Alicante showed by far the highest trans-resveratrol content in all sampling dates, ranging from 3,573 µg/kg recorded on August 26th to 1,917 µg/kg recorded after 5 weeks at the industrial ripeness grade, resulting in a decrease of 46% during the five sampling dates. Similar data (2,980 µg/kg) were found by Li et al. (2008) in ripe fresh berry skin of Takasuma cultivar grown in the Institute of Botany of the Chinese Academy of Science. In contrast, Black Malvasia showed the lowest content in all harvest dates, 715, 553, 498, 400 and 286 µg/kg, measured on August 26th, September 2th, September 9th, September 16th and September 23th, respectively, decreasing by 60%. The same decrease (60%) was found in the Nerello cultivar, the third highest for total trans-resveratrol content, varying from 1,521 µg/kg on August 26th, to 607 µg/kg on September 23th. In Prunesta berry skins the highest trans-resveratrol decrease was observed during ripening, 63% of the total content, from 2,209 µg/kg at the first sampling date to 812 µg/kg at the fifth sampling date. This cultivar had the second highest trans-resveratrol content of those studied. In the last sampling date, Prunesta had a very similar trans-resveratrol content (812 µg/kg) to that found by Liu et al. (2013) in fresh berry skins of Saint-Emilion cultivar grown in China (880 µg/kg).

The trans-resveratrol content of berry skins of Black Malvasia grown in South West Calabria (Southern Italy) in the first week of September (500 to 550 µg/kg) was similar to that of ripe fresh berry skin in Tano red cultivar (600 µg/kg) grown in the Institute of Botany of the Chinese Academy of Science (Li et al., 2008). Other Authors have found a 3, 5, 4' – trihydroxy-trans-stilbene content varying between 32,000 µg/kg (Tempranillo cultivar) to 245,000 µg/kg (Bobal cultivar) measured on dry weight of berry skins (Navarro et al., 2008). Pascual-Martí et al. (2001) have found, in dried grape skin, a 3,5, 4' – trihydroxy-trans-stilbene content ranging between 48,000 µg/kg (Cabernet cv) and 172,000 µg/kg (Tempranillo cv).

All Calabrian grape cultivars showed a trans-resveratrol content in fresh berry skins lower than Merlot,

Cabernet Sauvignon, Colorino del Valdarno e Montepulciano cultivars grown in Tuscany (Central Italy), (Iacopini et al., 2008).

Esna-Ashari et al. (2008) reported a trans-resveratrol content in red grape cultivars ranging between a not detectable amount and 5.52 mg/kg calculated on whole berry; more importantly, it appeared that the second major trans-resveratrol content was found in a cultivar named Italia (5.48 mg/kg) out of a pool of 147 cultivars grown in Iran.

Conclusion

Trans-resveratrol has been found to be an important functional bio-molecule with beneficial effects on human health as a cardioprotective substance. The aims of this work was to verify the efficacy of the SPI-HPLC-DAD technique applied to this analysis and to quantify the trans-resveratrol content in red skin of four grape cultivars grown in South West Calabria (Southern Italy) during ripening. The SPI-HPLC-DAD technique applied to red berry skin analysis was successful in extracting and quantifying trans-resveratrol. Analysis of trans-resveratrol in the berry skin of red grapes cultivated in the South West Calabria (Southern Italy) evidenced a decreasing trend for all the studied cultivars during ripening. Significant differences were found in the trans-resveratrol content among four cultivars grown in the same area. These differences proved to be interesting elements for the characterization of cultivars without climatic or agronomic interference. A higher degree of grape ripeness leads to a lower trans-resveratrol content. Alicante (that is, the allochthonous cultivar) was found to be with the highest trans-resveratrol content among those studied. As all the plants were cultivated with identical agronomical and climatic conditions and as an identical sanitary state was observed, the different results for trans-resveratrol content are mainly due to the genetic characteristics of each cultivar and to the relationship between cultivar and environment (that is, soil and climate).

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