Comparative Study of the Resveratrol Content of Twenty-one Italian Red Grape Varieties

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INTRODUCTION

The phytoalexin trans-3,5,4’-trihydroxystilbene (trans-resveratrol) has been reported in grape skins (Creasy & Coffee, 1988; Jeandet et al., 1991) and in wines (Sienmann & Creasy, 1992; Goldberg et al. 1995; Okuda & Yokotsuka, 1996; Ratola et al., 2004; Vitrac et al., 2005; Naugler et al., 2007). The content of stilbenes in the final product depends mainly on the grape variety (Gatto et al., 2008), but a variety of other factors, like the climate, soil (Bavaresco et al., 2005), canopy management (Bavaresco et al., 2008) and the occurrence of pathogen infections can influence the synthesis of these molecules (Bavaresco et al., 1997; Romero-Perez et al., 2001). Since stilbenes are located mainly in the grape skin, winemaking practices have a great effect on the extraction of these compounds from pomace into the wine (Jeandet et al., 1995a; Okuda & Yokotsuka 1996; Vrhovsek et al., 1997; Gambuti et al., 2004; Atanackovic et al., 2012). For this reason, the content of trans-resveratrol in red wines is much higher than in white ones, although no significant differences have been found in the corresponding grapes (Jeandet et al., 1995a; Okuda & Yokotsuka, 1996; Romero-Perez et al., 2001).

In the last twenty years there has been a great deal of interest in the presence of trans-resveratrol in wine because of its beneficial effects on human cardiovascular health, derived from its ability to inhibit platelet aggregation (Varache-Lembèche et al., 2000) and LDL oxidation (Fauconneau et al., 1997), and to produce endothelial nitric oxide-dependent vasorelaxation in vivo (Li et al., 2000). With regard to cancer, trans-resveratrol inhibits the proliferation of tumour cells and has a cancer-chemopreventive potential, inhibiting cellular events associated with the three major stages of carcinogenesis (Jang et al., 1997). However, the concentration of trans-resveratrol is too low to affect the physiology of consumers who consume wine in moderation, and the benefits of the wine consumption on human health therefore are under debate. On the other hand, the physiological effects attributable to the presence of this compound in wine could be affected by the content of other resveratrol derivatives, like cis-resveratrol and resveratrol glucoside isomers, which have some functional properties similar to trans-resveratrol (Chung et al., 1992; Jayatilake et al., 1993). Cis-resveratrol does not occur naturally in grapes and is generally formed by exposure to UV light (Soleas et al., 1995), whereas both the cis- and the trans-isomers of 3-β-glucoside of resveratrol (piceid) have been detected in the grape skin (Waterhouse & Lamuela-Raventos 1994). The contribution of the piceids could be important, as the content of resveratrol glucosides in berry skins is two to four times higher than that of the aglycon forms for the

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Stilbene extraction

For each sample, a sub-sample of 40 berries was used. The skins were removed manually from frozen berries, then weighed and freeze-dried. One gram of freeze-dried skins was treated as reported by Sun et al. (2006), with some modifications. Briefly, the berry skins were introduced into 40 mL of methanol containing 50 µL of hydrochloric acid and 250 µL of internal standard (trans-hydroxyl stilbene, 200 µg/mL in ethanol). After homogenisation for 1 min with Ultraturrax, the samples were maintained for 48 h in closed containers under stirring at room temperature in the dark. The extract containing polyphenols was obtained by centrifugation (5 000 x g, 5 min) and the solid residue was washed twice with 5 mL of methanol. The washing solutions were added to the first supernatant and the mixture was filtered with a 0.2 µm PTFE syringe filter (Advantec, Milano, Italy). After adding 2 mL of water, the extract was almost completely evaporated to dryness under vacuum at 35°C. The dense residue obtained was suspended in 20 mL of water and stilbene compounds were extracted twice for 15 min with 10 mL of ethyl acetate. The upper solvent layer containing stilbenes was recovered carefully. The organic phase was dried by the addition of anhydrous sodium sulphate and filtered through Whatman 589/3 paper. The ethyl acetate fraction was completely evaporated to dryness under vacuum at 35°C. The residue was dissolved in 2 mL of methanol and 50 mM of formic acid (ratio of 1:1) and then centrifuged at 14 000 x g for 10 min. Each grape sample was extracted in duplicate. All organic solvents and acids were obtained from Carlo Erba (Italy).

HPLC analysis

Stilbenes were separated in a C18 Lichrospher column (4 mm x 250 mm, 5 µm, Agilent Technologies, Milano, Italy) using an HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Dual Band UV detector (Waters Corporation, Milford, MA, USA). The mobile phase consisted of 50 mM of formic acid (solvent A) and methanol (solvent B). The gradient program was 0 to 10%B in 3 min, followed by 10 to 30%B in 5 min, 30 to 44%B in 35 min, 44 to 55%B in 2 min, 55 to 75%B in 15 min and 75 to 100%B in 1 min. After washing for 2 min with solvent B, the column was re-equilibrated with solvent A. The flow rate was 1.0 mL/min. The injection volume was 20 µL and the column temperature was set to 40°C. Detection was performed at 306 and 285 nm for trans- and cis-isomers respectively. The concentration of individual stilbenes was quantified at the base of the peak area and calibration curves were derived from the commercially available standards of trans-piceid, trans-piceatannol, trans-resveratrol and trans-
hydroxystilbene between 5 and 40 µg/mL. All the stilbene standards were obtained from Extrasynthese (Genay Cedex, France). Cis-isomers were obtained by exposure of the corresponding trans-molecules to UV light for 1 min and, for their quantification, the extinction coefficient of the trans forms was assumed.

Statistical analysis
Statistical analysis was performed with XLSTAT-Pro 7.1 Software. Data (three replicates for each variety) were analysed with one-way ANOVA and the Tukey multiple comparison test was used to compare the means when significant differences were found in the variance analysis. Linear correlation among variables was measured with Pearson’s coefficient.

RESULTS AND DISCUSSION
The extraction of resveratrol and related stilbenes from grape berries is a difficult task, mainly due to their low content. In this case, a protocol for the quantitative extraction of stilbenes from grape skins was used (Sun et al., 2006). It included the extraction of total polyphenols with acidified methanol, followed by liquid-liquid extraction with ethyl acetate to separate stilbenes from a large amount of other phenolic compounds. In addition to the protocol of Sun et al. (2006), a fixed amount of trans hydroxystilbene was added as internal standard before the extraction procedure. The average recovery of the internal standard, calculated from 18 different extractions, was 96.60 ± 3.93 %. The grapes used in this study were treated in order to avoid any fungal infection and, therefore, to measure only the basal synthesis of stilbenes. In fact, if any infection is present in the bunch, the selection of only healthy berries is not sufficient to guarantee the measurement of the basal level of stilbenes, because, as previously reported (Jeandet et al., 1995b), resveratrol is present predominantly in non-infected fruits close to the infected area.

The stilbene content was expressed in µg/g of skins (fresh weight) to better compare the varietal response and to avoid the dilution effect due to different berry sizes. The trans-resveratrol content of different red wine grape varieties showed high variability, ranging from 19 to 508 µg/g (average value of 169 µg/g) in the berry skins (Fig. 1). These results are in the same order of magnitude as those previously published for this stilbene compound. Shi et al. (2003) reported an average content of trans-resveratrol of 65.67 µg/g in grape skins. Even selecting clusters in the same vineyard and berries having the same sugar content, replications were sometimes quite different within the same variety, as in the case of Gamay, Teroldego, Lambrusco and Merlot grapes, for example. This confirms the high berry-to-berry variability in resveratrol due to the large number of factors influencing its synthesis.

International varieties (Merlot, Pinot noir, Cabernet Sauvignon and Gamay) showed resveratrol concentrations close to the average content. Merlot grapes, however, presented a higher resveratrol content than Cabernet Sauvignon, which agrees with previous data (Romero-Perez et al., 2001). Some Italian autochthonous varieties showed a high concentration of this compound, like Barbera, Schiava gentile, Corvina and Marzemino, whereas others, like Primitivo and Croatina, contain very low amounts. Comparing the resveratrol and anthocyanin contents in berry skins (data not shown), no significant correlation was found, despite the fact that the enzymes involved in their synthesis, stilbene synthase and calchone synthase respectively, can compete for the same substrate, as reported previously (Jeandet et al., 1995c).

Cis-resveratrol was detected in the berry skins at very low concentrations (data not shown), and only in grape varieties having a high content of the trans-isomer. This confirms that this molecule is not synthesised in grapes but is the result of the isomerisation of the trans-isoforms promoted by UV light during extraction (Soleas et al., 1995).

FIGURE 1
Content of trans-resveratrol in skins of 21 different red grape varieties (Lambrusco FF: Lambrusco a foglia frastagliata). Data is expressed as µg/g of fresh skins. Each extraction was performed in duplicate.
The monoglycosides of resveratrol are probably the more represented stilbenes in grapes (Regev-Shoshani et al., 2003), and both the cis- and trans-isomers have been identified in grape berries and wines. In the present work, the glycosylated forms of resveratrol were detected in almost all the varieties (except for Montepulciano).

The trans-piceid content in berry skins ranged from not detected to 1 196 µg/g (average value of 260 µg/g) (Fig. 2), in agreement with data previously published showing that the piceid concentration in grapes is generally higher than that of resveratrol (Romero-Perez et al., 2001; Sun et al., 2006). It can be assumed that the concentration of the glycosylated form is related to that of the corresponding aglycone. However, the correlation coefficient between trans-piceid and trans-resveratrol, even though significant (p < 0.05), was only 0.457. In fact, the Franconia variety, with a resveratrol content close to the average value, showed the highest content of trans-piceid. In contrast, Schiava gentile, with one of the highest contents of trans-resveratrol, presented a very low concentration of trans-piceid. This indicates that

FIGURE 2
Content of trans-piceid in skins of 21 different red grape varieties (Lambrusco FF: Lambrusco a foglia frastagliata). Data is expressed as µg/g of fresh skins. Each extraction was performed in duplicate.

FIGURE 3
Content of cis-piceid in skins of 21 different red grape varieties (Lambrusco FF: Lambrusco a foglia frastagliata). Data is expressed as µg/g of fresh skins. Each extraction was performed in duplicate.
the synthesis of the different stilbenes in grape skins could depend on different pathways.

Cis-piceid is also present in high amounts in grape skins (Fig. 3), ranging from not detected to 551 µg/g (average value of 205 µg/g). Both cis- and trans-isomers were previously detected in V. vinifera cell cultures, grapes and wines, but the cis/trans ratio was highly variable (Waterhouse & Lamuela-Raventos 1994; Waffo Teguo et al., 1998; Ribeiro de Lima et al., 1999; Bavaresco & Fregoni, 2001). In the 21 red wine grape varieties studied, the correlation coefficient between the two piceid isomers was 0.736 (p < 0.05). Franconia, even though it had large variability between replicates, was still one of the varieties with the highest content of this glycosylated form of resveratrol in berry skins, followed by Negroamaro, Schiava gentile, Barbera and Marzemino. Among international varieties, Merlot showed a higher content of both cis- and trans-glycosides than Cabernet Sauvignon, confirming previous data (Romero-Perez et al., 2001).

In some varieties it was also possible to detect a minor stilbene, trans-piceatannol (Fig. 4). Curiously, Montepulciano, which was devoid of both piceid isomers and showed a low content of trans-resveratrol, was the variety with the highest mean content of piceatannol (72.1 µg/g). On the other hand, Barbera, which contained large quantities of resveratrol and piceids, also showed a high content of piceatannol (69.9 µg/g). Even at low concentrations, piceatannol can play an important role in contributing to health properties, because the additional hydroxyphenyl group in the B ring (catechol structure) significantly increases the trapping effect of free radicals and antioxidative properties. Piceatannol doses of 25 µg/kg show antiarrhythmic and cardioprotective properties in rats (Hung et al., 2001).

CONCLUSIONS
In the survey of 21 red wine grape varieties, including most of the autochthonous Italian cultivars, a large variability was found in stilbene content. Some varieties, such as Barbera, Franconia, Negroamaro, Corvina and Marzemino, showed a very high content of both resveratrol and its monoglycosides in the berry skins. In particular, Barbera grapes, which also contain a relatively high concentration of trans-piceatannol, could be an interesting variety as a source of health-promoting molecules. On the other hand, other varieties, such as Montepulciano, Dolcetto, Croatina, Refosco and Primitivo, seem to synthesise very small amounts of stilbenes.

LITERATURE CITED


