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Modelling of the evolution of phenolic compounds in berries of “Italia” table grape cultivar using response surface methodology

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Abstract

The aim of this work was to determine the phenolic profile of *Vitis vinifera* L. cv. “Italia” table grapes during ripening, as influenced by the harvest date and berry heterogeneity. The results showed that this cultivar is rich in phenolic compounds with health-promoting properties, particularly at early harvest stage (341 and 178 mg/kg berries of total phenols in the skin and pulp, respectively). Caftaric acid was the most abundant compound in the skin (28.95 – 51.93 mg/kg), while *p*-coumaroyl-glucose was the highest in the pulp (6.39 – 17.18 mg/kg). Low levels of resveratrol (0.11 - 0.29 mg/kg) were found in the skin starting from the 14th day of harvest. Response surface methodology (RSM) was used to model the evolution of phenolic compounds in berries during ripening. The regression models were highly significant for protocatechuic acid, catechin, epicatechin and *t*-resveratrol in the skin, and total hydroxycinnamoyl tartaric acids in the pulp ($R \geq 0.80$). This modelling could be a tool that would permit better exploitation of maximum accumulation of phenolic compounds in the vineyard by selecting the most suitable combination of sampling date and berry density. An adequate sampling strategy could be implemented to increase the content of specific bioactive phenolic compounds according to consumer preference, thus promoting the health-promoting quality of fresh table grapes and ready-to-eat fruit salads.

Keywords: Table grapes; *Vitis vinifera*; HCTA index; Ferulic acid; Epicatechin; Resveratrol; Ripening; Berry density; HPLC-MS/MS; Food composition; Food analysis

1 Introduction

Table grapes are among the most valuable sources of phenolic compounds (Brat et al., 2006). These compounds are probably responsible for most of the beneficial effects of grapes with respect to a number of chronic diseases, including atherosclerosis, cardiovascular diseases, neurodegenerative disorders and aging (Iriti and Faoro, 2009; Xia et al., 2010; Pezzuto, 2008). Recently, Carrieri et al. (2013) studied selected table grape varieties and showed that the ability of the grape skin extracts to inhibit the synthesis of the tissue factor involved in the pathogenesis of thrombotic diseases is correlated to the phenolic composition.

The main phenolic classes found in table grapes are phenolic acids, anthocyanins, proanthocyanidins and stilbenes, each distributed differently within the grape tissues (Liang et al., 2008; Iriti and Faoro, 2009). In particular, hydroxycinnamic acids are located mainly in the grape skin and flesh, whereas anthocyanins and resveratrol just in the skin, and proanthocyanidins in both

skin and seeds (Iriti and Faoro, 2009). Anthocyanins and resveratrol are reported to be the most bioactive grape compounds (Xia et al., 2010). Several studies on the health-promoting properties of table grapes have already compared the phenolic composition of different cultivars using grapes purchased from the market or harvested at maturity, whereas others have examined the effect of in-field and/or postharvest treatments on the phenolic profile (Baiano and Terracone, 2011; Cantos et al., 2002; Lago-Vanzela et al., 2011; Capriotti et al., 2012; Crupi et al., 2013; Rolle et al., 2013). In most of the cited cases, polyphenols were determined by using liquid chromatography coupled with UV and mass-mass spectrometric detection systems, providing useful structural information for the identification of these compounds.

The phenolic content changes during grape ripening according to the different regulation of the enzyme activity involved in the mevalonate pathway, as is the case of resveratrol, whose concentration seems to decrease from veraison to harvest (Singh Brar et al., 2008; Iriti and Faoro, 2009). As already pointed out by other authors, berry maturity stage at harvest can greatly affect the overall quality of table grape berries in terms of texture, colour and chemical composition (Baiano and Terracone, 2011; Parpinello et al., 2013; Rolle et al., 2015). However, in the vineyard the grapes do not ripen homogeneously. Each cluster of the vine and also each berry of the cluster can ripen at different rates depending on the position, environmental factors, and management of viticultural practices. As a consequence, a high berry heterogeneity can be found from the beginning of the ripening process until the moment of the harvest (Kontoudakis et al., 2011; Río Segade et al., 2013). All the quality traits are strongly linked to the in-field berry variability, which can have a great influence on the consumer acceptance (Rolle et al., 2015). Recently, it has been shown that berry densimetric sorting and size can be applied to separate berries with different chromatic characteristics, texture parameters, aromatic profiles and phenolic composition (Rolle et al., 2015; Río Segade et al., 2013). Gallo et al. (2015) also highlighted that the agronomical practices can influence the composition of primary metabolites of table grapes and proposed the NMR spectroscopy (an advanced analytical technique) in complement or as an alternative to traditional determination of °Brix, titrable acidity, and so on to discriminate among the applied practices.

Table grape berries can be consumed fresh, processed as juice or added to salads, drinks and desserts. Because of their many uses and increased consumer demand, the presence of table grapes in the market should last as long as possible (if not all year round), while care is taken to preserve their quality attributes.

Given that the health-promoting properties of table grapes play a significant role in determining their overall quality, and also increasingly influence the consumer choices, the main aim of this work was to investigate when the highest content of phenolic compounds in *Vitis vinifera* cv. “Italia” table grape berries is reached, particularly for the production of ‘ready-to-eat’ fruit salad. For this aim, not only the evolution of phenolic compounds during ripening of the berries was studied, but also a model was developed to assess the combined effect of grape density and harvest date using response surface methodology (RSM). The selection of the most suitable harvest time and berry density to achieve the maximum accumulation of phenolic metabolites during ripening is a novel aspect with relevance for the fresh-cut industry. The study was carried out on cv. Italia, one of the most popular Italian seeded white table grape varieties, originally bred in 1911 by crossing Bicane and Muscat Hamburg (Baiano and Terracone, 2011).

2 Materials and methods

2.1 Grape sampling and selection

Vitis vinifera L. cultivar “Italia” (Italia, hereafter) table grapes were harvested in 2012 from a vineyard located in Trinitapoli (BT province, Puglia region, Southern Italy, N 41.1872, E 16.0079). The vineyard was established in 2007 on a clay loam soil high in mineral elements and with moderate organic matter content; 6% Ca⁺⁺; and pH 8.1. Cv. Italia was grown on 140 Ru stock and trained onto an overhead “tendone” trellis (Apulia type). Vines were pruned to 4 canes and spurs, with average number of 35 shoots per vine and 1.7 clusters per shoot. The vineyard was managed according to viticultural practices common for the growing area, including winter mineral nutrition with guanito (6 q/ha), shoot and bunch positioning, basal leaf removal, spring-summer fertigation, and irrigation with seasonal volume of about 2000 m³/ha by drip irrigation.

Grapes were harvested for five consecutive weeks, from August 22 to September 18. Single berries and small groups of three to five berries were randomly taken from all parts of the clusters. Once in the lab, broken berries were discarded and the single berries with attached short pedicels were densimetrically sorted by flotation in different saline solutions, ranging from 190 to 80 g/L sodium chloride (NaCl) concentration, following the procedure described by Rolle et al. (2012). Afterwards, the respective berry groups were weighed to obtain the distribution percentages of berries into density classes (Figure 1). In order to evaluate grape heterogeneity and model the compositional differences, the most represented (according to weight percentage) five density classes were considered. The berry groups corresponding to different density classes and sampling points were treated separately for all subsequent analyses.

2.2 Chemicals

Ultrapure water was produced using Milli-Q equipment (Merck Millipore, Darmstadt, Germany). Protocatechuic acid, caftaric acid, *p*-coumaric acid, procyanidin B1, procyanidin B2, catechin, caffeic acid, epicatechin, ferulic acid, rutin hydrate, isoquercitrin, *t*-resveratrol, Folin-Ciocalteu reagent, formic acid, citric acid, tartaric acid, malic acid, glucose, fructose and methanol were purchased from Sigma-Aldrich (Milan, Italy).

2.3 Determination of technological ripeness

For each sample, two sets of 50 berries each were taken ($n=2$) and manually crushed and centrifuged to obtain the respective juice. The juice was used for determining titratable acidity, organic acids and reducing sugars. Titratable acidity (TA) was determined as defined by International Organization of Vine and Wine (OIV, 2011) method and expressed in g/L tartaric acid. Sugars (SSC, as sum of glucose and fructose), citric acid, tartaric acid, and malic acid were determined by HPLC following the protocol described by Giordano et al. (2009), and the contents were expressed in g/L. SSC/TA ratio was calculated as the ratio between the sugar content and titratable acidity values, both expressed in g/L (OIV, 2008).

2.4 Extraction and determination of phenolic compounds from berry skin and pulp

For each sample, three sets of ten berries each ($n=3$) were taken, weighed, and peeled using a laboratory spatula (Di Stefano and Cravero, 1991). The pulp was collected in a flask containing 100 mg of Na₂S₂O₅ to avoid oxidation, diluted 9:1 (w/w) with H₂SO₄ 5 mol/L to avoid tartaric precipitation, homogenized using a Ultra-Turrax T10 (IKA Labortechnik, Staufen, Germany) at 6000 rpm for 1 min, and centrifuged in a PK 131 centrifuge (ALC International, Milan, Italy) for

15 min at 3000 g (Rolle et al., 2013; Rolle et al., 2015). The resulting solution was used for pulp analysis. The skins were carefully cleaned from pulp residuals and immediately immersed in 25 mL hydro-alcoholic buffer solution prepared with 12% v/v ethanol, 5 g/L tartaric acid, 2 g/L Na₂S₂O₅, and adjusted to pH 3.2 with NaOH 1 mol/L. The skins were homogenized using an Ultra-Turrax T25 (IKA Labortechnik) at 8000 rpm for 2 min and then centrifuged for 15 min at 3000 g (Rolle et al., 2013; Rolle et al., 2015). The supernatant was used for skin analysis. Skin and pulp extracts were stored at -20°C until analysis.

2.4.1 Spectrophotometric determinations

All the spectrophotometric determinations were done with a UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) using plastic (Vis) or quartz (UV) cuvettes, with an optical path of 10 mm.

Total phenol index was evaluated, after sample purification, using the Folin-Ciocalteu assay (Singleton and Rossi, 1965; Di Stefano and Cravero, 1991). A quantity of 200 µL (skin extract) or 500 µL (pulp extract) was diluted 4-fold with H₂SO₄ 0.05 mol/L (to reduce the ethanol content) and then run through a Sep-Pak C18 SPE cartridge (Waters Corporation, Milford, MA, USA). Phenolic compounds were eluted with 4 mL of methanol, which were collected in a 20 mL volumetric flask. Then 1 mL of Folin-Ciocalteu reagent was added to the flask and, after 4 min, 4 mL of Na₂CO₃ 10% w/v, followed by water to bring the total volume to 20 mL. After 90 min, the absorbance at 750 nm was measured against a blank prepared substituting the sample with water. The results were expressed as mg catechin/kg berries using external calibration.

The hydroxycinnamoyl tartaric acid (HCTA) index of the pulp extracts was determined by ten-fold dilution with water and measuring absorbance at 325 nm. The results were expressed as mg caffeic acid/kg berries using external calibration (Di Stefano and Cravero, 1991).

2.4.2 HPLC-PDA – ESI-MS/MS determinations

The preparation of the skin extracts for HPLC analysis consisted in the 2% addition of H₃PO₄ 1 mol/L to the extract, filtration using 0.20 µm PTFE filters, and then injection. For the preparation of the pulp extracts, 2 mL of the extract were diluted 4-fold with H₂SO₄ 0.05 mol/L and purified using the aforementioned Sep-Pak C18 cartridges procedure. Phenolic compounds were eluted with 4 mL of methanol, which was evaporated to dryness under an N₂ stream (Glas-Col, Terre Haute, IN, USA) at 0.5 mL/min flow-rate and 35 °C in a 150 rpm moving plate. The residue was redissolved in 1 mL of 0.1% v/v formic acid in water, filtered using 0.20 µm PTFE filters and then analysed as described by Belviso et al. (2014) with modifications as follows.

A Thermo-Spectra System HPLC (ThermoFisher Scientific, Waltham, MA, USA) was used, consisting of a P2000 binary gradient pump, SCM 1000 degasser, AS 3000 automatic injector and Finnigan Surveyor PDA Plus detector (PDA) coupled in tandem with an API 3200 QTRAP LC/MS/MS system using a Turbo V source (Applied Biosystem Sciex, Foster City, CA, USA). The ChromQuest software (version 5.0) was used for instrument control and UV-data collection and processing, while the Analyst software (version 1.6) was used for MS/MS analysis. The separation was achieved on a Kinetex 5 µm Phenyl – Hexyl 100A 150 × 4.6 mm analytical column (Phenomenex, Castel Maggiore, Italy) equipped with a SecurityGuard™ cartridge system (Phenomenex). The mobile phase was composed of solvent A (0.1% formic acid in ultrapure water) and solvent B (methanol). The flow rate was set at 1 mL/min and the injection volume was 10 µL. The elution program was as follows: 85% A kept in isocratic for 5 min, decreased to 75% A in 1 min and kept in isocratic for 15 min, decreased to 65% A in 1 min and kept in isocratic for 10 min, and increased to 85% A in 1 min and kept in isocratic for 5 min. PDA spectra were recorded in full

scan mode in the λ range from 220 to 600 nm and quantification was carried out using the external standard calibration method. The results were expressed as mg compound/kg berries. Coumaric acid, *t*-ferric acid and *p*-coumaroyl-glucose were quantified as *p*-coumaric acid.

The ion source was operated in the negative ion mode using the following conditions: ion spray voltage, -4500 V; turbo spray ion source temperature, 550 °C; collision gas, high; curtain gas, 30 psi; interface heater, on; ion source gas 1, 30 (arbitrary units); ion source gas 2, 20 (arbitrary units). Nitrogen was used as the nebulizer, heater, curtain and collision gas. Masses were recorded in the *m/z* range of 100-700 amu using the enhanced mass spectrum (EMS) scan experiment with a declustering potential (DP) of -30 V and an entrance potential (EP) of -10 V. Product ions (MS/MS) were generated according to the information dependent acquisition (IDA) mode, with a threshold of 50000 cps and a collision energy (CE) of -30 eV and were collected in the enhanced product ions (EPI) scan mode.

2.5 Response surface methodology experimental design

A two-factor experimental design was carried out, where the first factor consisted of the sampling date (X_1 , 5 points), and the second factor of the selected density class (X_2 , as juice sugar content, 5 density classes). Two- as opposed to one-dimensional models make it possible to better simulate all processes involved during grape ripening because of the nonlinear nature of events (Valipour et al., 2015). For each parameter studied, the average results of each sampling date-density class combination were considered, and then analyzed to fit the following second-order polynomial model:

$$Y = a + bX_1 + cX_2 + dX_1^2 + eX_1X_2 + fX_2^2 ,$$

where Y is the predicted variable, and X_1 and X_2 are the independent variables. The equations were calculated using the Statistica 7.0 package (Statsoft Inc., Tulsa, OK, USA) and expressed as surface plots using RSM (Khuri and Mukhopadhyay, 2010).

2.6 Statistical analysis

The Tukey-b test at $p < 0.05$ was performed to establish significant differences by one-way analysis of variance (ANOVA) using the SPSS Statistics software package version 19.0 (IBM Corporation, Armonk, NY, USA).

3 Results and discussion

3.1 Evolution of chemical parameters with harvest date in the vineyard

Table 1 shows the evolution of the contents of organic acids, the SSC/TA ratio and the contents of skin and pulp phenolic compounds in Italia table grapes with the harvest date. The later the harvest, the lower the content of malic acid, which decreased significantly; whereas citric acid decreased significantly only between the first and the second harvest. Tartaric acid content was not significantly affected by the harvest date.

The SSC/TA ratio increased regularly with the delay of the harvest date (Table 1), except for the last week. Other studies have reported a similar trend for the SSC/TA ratio as well as some similarities in the acidic profile of the juice from Italia berries harvested on a unique date and sorted by flotation to verify the possible grape-ripening effect (Río Segade et al., 2013).

According to the OIV resolution VITI 1/2008, “Standard on Minimum Maturity Requirements for Table Grapes” (OIV, 2008), table grapes are considered to be ripe when the SSC/TA ratio (where SSC is expressed as g/L, and TA as g/L tartaric acid) is greater than 20. As shown in Table 1, Italia berries met the OIV ripeness threshold for all sampling dates.

The sensory quality of table grapes depends primarily on sugar content, titratable acidity, the organic acid composition and the balance among these factors (Muñoz-Robredo et al., 2011; Piazzolla et al., 2016). However, for consumer acceptance, other important quality criteria such as phenolic composition (because of its health-promoting implications) must also be considered (Xu et al., 2017).

Total phenol index was higher in the skin than in the pulp. In the skin, it ranged from the maximum of 341 mg/kg berries on the first sampling date (day 0) to the minimum of 244 mg/kg berries on day 14, while in the pulp it remained almost unchanged during ripening (158-178 mg/kg). HCTA index varied from 125 mg/kg berries (day 0) to 94 mg/kg berries (day 21). These values are slightly higher than those reported by Río Segade et al. (2013) for the same variety at the same SSC/TA ratio, probably because of the different vineyard and seasonal effects. Baiano and Terracone (2011) also found a lower HCTA content (22 mg/L of pulp juice) and, in this case, the difference could be related to berry maturity.

Table 1 shows also the quantitative skin and pulp phenolic composition of the Italia table grape. A total of 14 compounds were found: protocatechuic acid (skin), caftaric acid (skin and pulp), procyanidin B1 (skin), catechin (skin), coumaric acid (skin and pulp), caffeic acid (pulp), *t*-ferulic acid (pulp), procyanidin B2 (skin), epicatechin (skin and pulp), *p*-coumaroyl glucose (pulp), ferulic acid (pulp), rutin (skin), isoquercitrin (skin) and *t*-resveratrol (skin). Table 2 shows the retention times (R_t), wavelengths of maximum UV absorption (λ_{max}), negative pseudomolecular ions ($[M - H]^-$), MS/MS products, identification method, and the presence of the detected compounds in the skin and/or pulp. Hydroxycinnamic acids (caffeic and ferulic acids) and their derivatives (caftaric, coumaric and *t*-ferulic acids) are common in grapes. Coumaric and *t*-ferulic acids were identified by comparing mass spectral and UV data with those of literature (Hollecker et al., 2009; Lago-Vanzela et al., 2011; Rebello et al., 2013), while the identification of caffeic, ferulic and caftaric acids was achieved by injection of the corresponding analytical standards. Hydroxybenzoic acids (protocatechuic acid), monomeric flavan-3-ols (catechin and epicatechin), dimeric B-type procyanidins (procyanidin B1 and B2), flavonols (rutin and isoquercitrin) and stilbenes (*t*-resveratrol) were also identified by comparing mass spectral and UV data with those of analytical standards. Peak 10 ($\lambda_{max}=315$ nm; $[M - H]^- = 325$ m/z; MS/MS=163, 145 m/z) was tentatively identified as *p*-coumaroyl glucose on the basis of literature data (Rebello et al., 2013).

At different sampling dates, the content of protocatechuic acid in the skin of Italia table grapes increased from 0.10 mg/kg berries (day 7) to 0.21 mg/kg berries (day 21). This compound was also determined by Boido et al. (2011) in the berry skin of *Vitis vinifera* L. cv. “Tannat”, with contents ranging from 0.9 mg/kg berries (20 days after veraison) to 1.9 mg/kg berries (at harvest). There are numerous *in vitro* and animal studies which suggest an important role of protocatechuic acid in the control of oxidative stress and inflammation, but clinical studies are still needed to warrant its clinical use (Semaming et al., 2015).

Among hydroxycinnamic acid derivatives, caftaric acid was the most abundant compound in both skin and pulp, followed by coumaric acid and *t*-ferulic acid. The compound tentatively identified as *p*-coumaroyl-glucose was found in the pulp at contents comparable to those of caftaric acid (6.39–17.18 mg/kg berries). In other varieties, this compound was also detected in the skins (Boido et al., 2011; Rebello et al., 2013). Caftaric and coumaric acids were located mainly in the skin, in agreement with Lago-Vanzela et al. (2011), showing the highest ratios of pulp to skin on the last

sampling date (data not shown); *t*-feraric acid was detected only in the pulp. Other authors found it in the skin as well (Montealegre et al., 2006), but with a resolutely higher proportion in the pulp than in the skin (Lago-Vanzela et al., 2011). Boido et al. (2011) found contents of 6.9 and 3.4 mg/kg grape, for caftaric and coutaric acids respectively, in cv. “Tannat” skins at harvest.

Because of the relatively high contents of caftaric acid (>37 mg/kg) and coutaric acid (>2 mg/kg), especially on the first three sampling dates (the contents significantly decreased as the sampling date advanced), even a moderate consumption of cv. Italia berries, including the skin, could contribute to providing health benefits. In fact, hydroxycinnamates contribute to many biological activities and seem to be easily bioavailable after ingestion (Lee et al., 2014). Small contents of free forms of caffeic (0.03-0.42 mg/kg) and ferulic acids (<0.01-0.13 mg/kg) were found in the pulp of the cv. Italia berries. Baiano and Terracone (2011), who studied the phenolic profile of the same table grape cultivar, and detected caffeic acid only in the pulp, in accordance with the results of our study. The contents significantly decreased from day 14 for caffeic acid and from the first sampling date for ferulic acid. Gómez-Alonso et al. (2007) did not find caffeic and ferulic acids in the berry skin of cv. “Cencibel” (*Vitis vinifera* L.), but did find it in the corresponding wine.

Among flavan-3-ols, procyanidin B1 and catechin were the most abundant compounds in the skin, ranging from 2.92 to 6.46 mg/kg berries and from 3.83 to 9.07 mg/kg berries, respectively, followed by procyanidin B2 (0.15-0.97 mg/kg berries) and epicatechin (0.10-0.24 mg/kg berries). All compounds showed higher contents on the first sampling dates than at the end of ripening. In the pulp, only epicatechin was detected on the first sampling date (0.21 mg/kg berries). Crupi et al. (2015) reported contents (mg/kg berry, skin and pulp) of 4.0, 5.7 and 6.5 for procyanidin B1, catechin and procyanidin B2, respectively, in 2009 and 4.6, 7.3 and 7.7 in 2010 for Italia grapes, whereas epicatechin was not detected. The same authors also showed that Italia grapes contain relatively high quantities of catechin with respect to other table grape cultivars. Katalinić et al. (2010) investigated the skin phenolic composition of 14 *Vitis vinifera* L. cultivars, and the following ranges were reported (mg/kg grape) for single flavanols: 1.30–18.13 for procyanidin B1, 0.73–4.57 for catechin, 0–7.13 for procyanidin B2 and 0–2.22 for epicatechin. Bioactivities such as anticancer, free radical scavenging, antibacterial, anti-inflammation and antioxidant have been attributed to catechin, epicatechin and procyanidins, which in addition exhibited a rapid absorption into plasma after ingestion (Xia et al., 2010).

Isoquercitrin prevailed among flavonols (3.52-15.41 mg/kg), displaying the highest content in the skin on sampling day 21. Rutin ranged from a minimum value of 3.12 mg/kg berries at day 7 to a maximum value of 4.09 mg/kg berries at the day 21. The contents of isoquercitrin found in this study were in the range reported in literature for the skin of the same cultivar (Capriotti et al., 2012). Indeed, Cavaliere et al. (2008) found 19.5 mg isoquercitrin/kg skin. Crupi et al. (2015) reported contents of isoquercitrin from 9.3 to 11.9 mg/kg and of rutin+quercetin-3-*O*-glucuronide from 11.0 to 12.9 mg/kg, in the grape berry (flesh+skin), as the main flavonols. In this last study, Italia grape was compared with other white table grapes and discriminated thanks to its high content of isoquercitrin. In a study performed on 344 European (*Vitis vinifera*) cultivars evaluated for two consecutive years, the mean contents of isoquercitrin and rutin were 0.014 and 0.003 mg/g grape, respectively (Liang et al., 2011). Quercetin seems to have an important role in reducing the risk of cancer and cardiovascular diseases (Murakami et al., 2008; Egert et al., 2009). However, in foods this compound is usually found bound to sugar molecules, and the type of sugars influences its bioavailability and thus its bioactivity in the human body. Because cv. Italia berries were particularly rich in isoquercitrin, they could be taken into consideration as an interesting source of this bioactive compound.

Increasing quantities of *t*-resveratrol, between 0.11 and 0.29 mg/kg, were found from sampling day 14. The highest content was found at the end of the ripening period (day 28). In agreement with

these results, other researchers showed similar progressive resveratrol accumulation during berry ripening in the absence of biotic and abiotic stimuli (Gatto et al., 2008; Versari et al., 2001). To our knowledge, resveratrol has not yet been reported in the polyphenolic profile of cv. Italia berries. Liang et al. (2011) found resveratrol contents in the range of 0–0.012 mg/g berries for white table grapes, while Katalinić et al. (2010) reported an average *t*-resveratrol content of 0.24 mg/kg berries in the skins of white wine grape cultivars. Iacopini et al. (2008) found contents of resveratrol between 0.7 and 25.5 mg/100 g in red grape skins of different *V. vinifera* genotypes, suggesting that these differences depend on the grape varieties and on the fact that plants produce stilbenes as a defence mechanism against mould infections and physiological stress. Among polyphenols, resveratrol has certainly been the most studied compound for its health benefits. It is considered a cardioprotective and chemopreventive agent because of its many biological activities (Frémont, 2000). However, the health benefits of grape consumption must be explained in a synergistic manner with other phenolic compounds with similar properties.

3.2 Modelling of chemical composition evolution in the berries during ripening

In order to evaluate in-field grape variability, the distribution of Italia berries in different density classes was represented as a percentage for the five sampling dates. Figure 1 shows a Gaussian bell-shaped distribution at all the sampling points, which demonstrates a significant heterogeneity in the degree of grape ripeness at each sampling date. These results confirmed the variability observed in wine grapes during the ripening process (Kontoudakis et al., 2011). In the Italia cultivar, a more homogeneous distribution was observed at the beginning and the end of the ripening process, because the berries were grouped into fewer density classes. In contrast, these berries were more heterogeneously distributed at intermediate stages of ripening, as it was necessary to select a higher number of density classes to achieve the same representativeness. The lower dispersion was also observed in wine grapes collected one week after veraison, and the highest dispersion corresponded to berries sampled three weeks after veraison (Kontoudakis et al., 2011). In any case, it is important to take into account that the average sugar content of the berries (belonging to the most abundant density classes) corresponded to the average sugar content found in the vineyard at a given sampling time. Such heterogeneity may have a strong impact on the chemical composition of the berries.

A two-factor experimental design was used to model the evolution of the chemical composition during ripening. The second-order polynomial equations obtained for the different chemical parameters, and the respective correlation coefficients (*R*) are shown in Table 3. The two independent variables had almost exclusively linear effects on the predicted responses (quadratic effects were generally 100 times smaller than linear effects), and the interaction effect was very small (X_1X_2 coefficient ranging from 0.025 to 3.9E-05). Figures 2–4 represent the surface plots obtained using RSM for the most significant regressions according to the correlation coefficient ($R \geq 0.80$). Figure 2 shows a three-dimensional representation of the response surface to better display the effects of both the sugar content and of the sampling time on the chemical parameters that define the grape technological ripeness. The RSM model can significantly predict citric acid content as well as the SSC/TA ratio, because the high correlation coefficients ($R > 0.90$) suggest goodness-of-fit for the models proposed. The highest concentration of citric acid corresponded to the berries with the lowest sugar contents that were sampled at the beginning of the ripening process, and the citric acid content decreased as ripening progressed (Figure 2a). However, the relationship with the sugar content was negative for citric acid at the beginning of sampling, while it was positive at the end of the ripening process. The highest values of SSC/TA ratio were associated with a combination of the highest sugar content and the latest sampling dates; the sugar content effect was stronger than the sampling date effect (Figure 2b). A previous study found that significantly lower contents of

citric acid, as well as higher values for the SSC/TA ratio, were obtained for the Italia berries with higher sugar content (Río Segade et al., 2013).

Figure 3 shows the modelling of the evolution of protocatechuic acid, catechin, epicatechin and *t*-resveratrol contents in the skin of Italia berries with different sugar content levels during ripening. The changes observed were significant because the results were adequately fitted into second-order polynomial models ($R = 0.81-0.88$). The highest protocatechuic acid content was found in berries with the lowest sugar content when sampled at the end of the ripening process (late harvest, Figure 3a). In fact, the positive effect of sampling day was particularly strong for those berries with the lowest sugar contents. Nevertheless, the greatest abundance in catechin and epicatechin was achieved in berries with the highest sugar content that were sampled at the beginning of the observed period (days 0 and 7; Figures 3b, 3c, 3d). Although lower catechin and epicatechin contents were generally obtained when decreasing the sugar content or delaying the sampling date, relatively high catechin contents were also found in berries with the lowest sugar content at any sampling date (Figure 3b). Conversely, the positive effect of the sugar content was more evident in the resveratrol content than the effect of the sampling date (Figure 3d). A small positive effect of the sampling date when the berries had the lowest sugar content was observed for resveratrol as well as for epicatechin (Figure 3c). The lowest contents of these compounds were obtained in berries with intermediate sugar contents, particularly on the latest sampling dates for catechin and epicatechin (Figures 3b, 3c), and at the beginning of the ripening process for protocatechuic acid and *t*-resveratrol (Figures 3a, 3d). Regarding the content of *t*-resveratrol, a study performed on red wine grapes revealed that the evolution during ripening depends mostly on the cultivar and season (Moreno et al., 2008), but a progressive accumulation was observed in several grape varieties (Gatto et al., 2008; Versari et al., 2001). To our knowledge, resveratrol has not previously been determined in Italia table grapes.

Figure 4 represents the modelling of the effect of sugar content and harvest date on the HCTA index in the pulp, and the high correlation coefficient shows goodness-of-fit for the models proposed ($R = 0.85$). The highest values of HCTA index corresponded to the first sampling dates, particularly for sugar contents between 110–220 g/L, whereas the lowest values were associated with a combination of the latest sampling dates and the highest sugar contents (Figure 4a). At any sampling date, an increasing trend of HCTA index was observed with the increase in the sugar content up to 160 g/L, and then it decreased. In general, the value of HCTA index decreased with delaying the sampling date. Another study carried out on Italia berry pulp showed that the values of the HCTA index increased by increasing the berry density, but the differences were not significant (Río Segade et al., 2013). However, such an increase was significant for other table grape cultivars such as Muscat Hamburg (Rolle et al., 2015).

Once the minimum ripeness threshold established by OIV resolution VITI 1/2008 for table grapes ($SSC/TA > 20$) has been attained, the maximum contents of catechin, epicatechin and *t*-resveratrol in Italia berry skins can be obtained from the berries rich in sugars at the beginning of the ripening process. The maximum abundance in protocatechuic acid in skins requires a late harvest date and berries with low sugar contents. On the other hand, the maximum contents of HCTA in the pulp can be found at early harvest, but only by selecting berries with sugar contents ranging from 110 to 220 g/L.

4 Conclusions

A detailed knowledge of grape phenolic compound profiles during ripening makes it possible to determine the best harvest date in order to exploit the maximum accumulation of specific bioactive compounds. Modelling the evolution of the chemical composition of table grapes during ripening,

taking into account the simultaneous effects of sugar content and harvest date, can provide relevant results when harvesting programs aimed at improving their phenolic compound composition are being implemented. Density sorting of table grape berries takes advantage of in-field grape variability, because both harvest date and sugar content are significantly related to parameters influencing sensory (SSC/TA ratio) and health-promoting qualities (specific bioactive phenolic compounds). Early harvested Italia berries with relatively high sugar content (higher than 225 g/L) were rich in catechin, epicatechin and *t*-resveratrol, whereas those having intermediate sugar content (ranging from 110 to 220 g/L) showed more abundant HCTA. On the other hand, late-harvested berries with low sugar content showed the highest levels of protocatechuic acid in skins. Depending on which compound is required, an adapted sampling strategy could be implemented so as to increase that particular compound.

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Highlights

- The evolution of polyphenols during ripening of “Italia” table grapes was studied
- This cultivar is rich in health-promoting polyphenols particularly at early harvest
- Caftaric acid was abundant in the skin whereas *p*-coumaroyl-glucose was in the pulp
- Low contents of resveratrol were found in the skin from the 14th day of harvest
- Grape density and harvest date were modeled to improve polyphenol accumulation

Table 1. Evolution of chemical parameters in Italia table grape, according to the harvest date (from Aug 22 to Sept 18).

Sampling date (days)	Sign ^c	Day 0	Day 7	Day 14	Day 21	Day 28
Juice composition (g/L except for SSC/TA)^a						
Citric acid	***	1.1 ± 0.1 b	0.3 ± 0.1 a	0.2 ± 0.1 a	0.2 ± 0.1 a	0.2 ± 0.1 a
Tartaric acid	ns	6.0 ± 0.3	5.8 ± 0.2	5.7 ± 0.2	5.5 ± 0.2	5.9 ± 0.3
Malic acid	**	1.7 ± 0.1 c	1.6 ± 0.1 bc	1.5 ± 0.1 ab	1.4 ± 0.1 ab	1.3 ± 0.1 a
SSC/TA	***	27.4 ± 1.2 a	36.1 ± 1.5 b	45.3 ± 1.9 c	54.8 ± 2.3 d	46.1 ± 2.0 c
Skin phenolic compounds (mg/kg berries)^b						
Total phenol index (as catechin)	***	341 ± 23 c	306 ± 10 b	244 ± 7 a	298 ± 10 b	279 ± 13 b
Protocatechuic acid	***	0.14 ± 0.01 b	0.10 ± 0.01 a	0.14 ± 0.01 b	0.21 ± 0.01 d	0.17 ± 0.01 c
Caftaric acid	***	43.10 ± 1.16 b	51.93 ± 2.12 c	44.49 ± 2.48 b	29.85 ± 2.50 a	34.75 ± 2.95 a
Procyanidin B1	***	5.75 ± 0.64 cd	6.46 ± 0.51 d	4.59 ± 0.11 b	4.91 ± 0.16 bc	2.92 ± 0.27 a
Catechin	***	8.28 ± 0.52 c	9.07 ± 0.72 c	7.19 ± 0.08 b	6.86 ± 0.20 b	3.83 ± 0.48 a
Coutaric acid	***	3.06 ± 0.14 c	3.18 ± 0.15 c	2.81 ± 0.15 c	1.99 ± 0.11 a	2.35 ± 0.18 b
Procyanidin B2	***	0.97 ± 0.01 c	0.84 ± 0.02 b	0.83 ± 0.02 b	0.94 ± 0.02 c	0.15 ± 0.01 a
Epicatechin	***	0.24 ± 0.03 c	0.21 ± 0.01 c	0.18 ± 0.01 b	0.17 ± 0.01 b	0.10 ± 0.01 a
Rutin	***	4.04 ± 0.04 d	3.12 ± 0.07 a	3.74 ± 0.12 c	4.09 ± 0.17 d	3.48 ± 0.05 b
Isoquercetin	***	7.82 ± 1.40 b	3.52 ± 0.67 a	6.35 ± 1.04 ab	15.41 ± 2.88 c	8.74 ± 1.76 b
<i>t</i> -Resveratrol	***	nd	nd	0.11 ± 0.01 a	0.15 ± 0.02 a	0.29 ± 0.03 b
Pulp phenolic compounds (mg/kg berries)^b						
Total phenol index (as catechin)	ns	178 ± 12	162 ± 5	158 ± 3	160 ± 12	161 ± 8
HCTA index (as caffeic acid)	***	125 ± 6 c	114 ± 5 b	107 ± 3 b	94 ± 4 a	105 ± 5 ab
Caftaric acid	ns	12.12 ± 2.88	13.87 ± 0.22	12.87 ± 1.85	7.53 ± 1.69	11.43 ± 6.67
Coutaric acid	***	0.65 ± 0.13 b	0.69 ± 0.01 b	0.61 ± 0.07 b	0.07 ± 0.01 a	0.23 ± 0.13 a
Caffeic acid	***	0.35 ± 0.01 bc	0.42 ± 0.01 c	0.33 ± 0.02 bc	0.03 ± 0.01 a	0.23 ± 0.12 b
<i>t</i> -Fertaric acid	ns	0.33 ± 0.13	0.35 ± 0.01	0.26 ± 0.08	0.23 ± 0.07	0.21 ± 0.12
Epicatechin	/	0.21 ± 0.01	nd	nd	nd	nd
<i>p</i> -Coumaroyl-glucose	***	17.18 ± 1.05 d	15.41 ± 1.05 cd	10.76 ± 1.99 b	6.39 ± 1.46 a	11.72 ± 2.29 bc
Ferulic acid	***	0.13 ± 0.01	nd	nd	nd	0.01 ± 0.01

All data are expressed as average value ± standard deviation. ^an = 2. ^bn = 3. ^cSign: **, *** and "ns" indicate significance at $p < 0.01$, 0.001 and not significant, respectively, for the differences among sampling dates. Different Latin letters within the same row indicate significant differences (Tukey-b test; $p < 0.05$). SSC/TA = Reducing sugars (expressed as g/L)/titratable acidity (expressed as g/L tartaric acid). HCTA = hydroxycinnamoyl tartaric acids. nd = not detected.

Table 2. Chromatographic and spectroscopic characteristics (retention times, R_t ; UV maxima, λ_{max} ; negative pseudomolecular ions, $[M-H]^-$; product ions, MS/MS) of phenolic compounds detected in Italia table grape and their presence in skin and/or pulp (x).

Peak	R_t (min)	λ_{max} (nm)	$[M-H]^-$ (m/z)	MS/MS (m/z)	Tentative identification [§]	Skin	Pulp
1	4.1	260, 294	153	—	Protocatechuic acid ^a	x	
2	5.45	327	311	179, 149, 135	Caftaric acid ^a	x	x
3	6.7	278	577	451, 425, 407, 289	Procyanidin B1 ^a	x	
4	7.8	280	289	245, 203	Catechin ^a	x	
5	8.65	313	295	163, 119	Coutaric acid ^b	x	x
6	9.23	323	179	135	Caffeic acid ^a		x
7	9.85	327	325	193	<i>t</i> -Fertaric acid ^b		x
8	10.16	278	577	451, 425, 407, 289	Procyanidin B2 ^a	x	
9	11.16	280	289	245, 203	Epicatechin ^a	x	x
10	15.25	315	325	163, 145	<i>p</i> -Coumaroyl-glucose ^b		x
11	17.64	322	193	175, 147, 134	Ferulic acid ^a		x
12	28.6	354	609	463, 301	Rutin ^a	x	
13	29.05	354	463	301	Isoquercetin ^a	x	
14	29.7	306	227	185, 143	<i>t</i> -Resveratrol ^a	x	

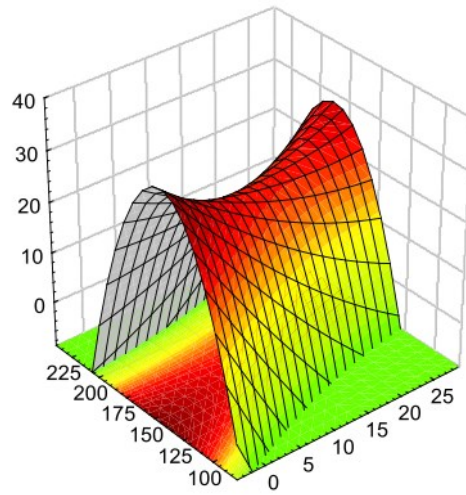
[§]:Identification method - ^a: comparison with reference standards; ^b: tentative identification with literature data.

Table 3. Correlation coefficients and values of second-order polynomial model by response surface methodology.

Parameter (Y)	Correlation coefficient	Variable coefficient					
		Intercept	X ₁	X ₂	X ₁ ²	X ₁ X ₂	X ₂ ²
Citric acid	0.91	1.10	-0.130	5.10E-03	8.58E-05	5.16E-04	-4.34E-05
Tartaric acid	0.68	11.0	0.154	-0.0704	1.44E-03	-1.08E-03	2.33E-04
Malic acid	0.71	0.808	-0.0898	0.0121	-9.82E-05	4.25E-04	-3.83E-05
SSC/TA	0.98	-17.2	-0.181	0.362	-0.0300	7.49E-03	-2.75E-05
Skin phenolic compounds							
Total phenols index	0.73	1230	0.148	-11.6	0.134	-0.0244	0.0357
Protocatechuic acid	0.83	0.782	0.0231	-8.62E-03	1.30E-05	-1.14E-04	2.73E-05
Caftaric acid	0.55	160	2.48	-1.64	0.0283	-0.0210	5.67E-03
Procyanidin B1	0.73	20.1	0.202	-0.204	-8.43E-04	-1.47E-03	6.90E-04
Catechin	0.81	18.9	0.314	-0.158	-6.17E-03	-1.57E-03	5.51E-04
Coutaric acid	0.71	12.0	0.191	-0.128	2.95E-03	-1.75E-03	4.49E-04
Procyanidin B2	0.64	8.01	0.227	-0.100	-1.06E-03	-1.21E-03	3.38E-04
Epicatechin	0.88	0.742	0.0162	-9.33E-03	2.53E-04	-1.80E-04	4.03E-05
Rutin	0.69	27.8	0.823	-0.368	3.48E-03	-5.56E-03	1.37E-03
Isoquercitrin	0.66	17.7	-0.427	-0.179	3.10E-04	3.18E-03	6.64E-04
<i>t</i> -Resveratrol	0.85	1.40	6.17E-03	-0.0231	9.57E-04	-1.74E-04	9.32E-05
Pulp phenolic compounds							
Total phenols index	0.55	219	-2.79	-0.643	0.0722	-9.15E-05	2.49E-03
HCTA index	0.85	50.7	-2.40	0.989	0.0616	-5.96E-04	-3.15E-03
Caftaric acid	0.45	-13.9	-1.90	0.467	-0.0103	0.0131	-1.96E-03
Coutaric acid	0.61	0.783	-0.0835	5.07E-03	-7.15E-04	5.43E-04	-4.11E-05
Caffeic acid	0.52	0.625	-7.70E-03	-3.11E-03	2.49E-04	-3.91E-05	9.81E-06
<i>t</i> -Fertaric acid	0.69	0.702	-0.0445	1.49E-04	-5.25E-04	3.66E-04	-2.10E-05
<i>p</i> -Coumaroyl-glucose	0.70	50.5	-0.242	-0.447	0.0242	-4.19E-03	1.50E-03

X₁ = sampling days. X₂ = sugar content. SSC/TA = Reducing sugars (expressed as g/L)/titratable acidity (expressed as g/L tartaric acid). HCTA = hydroxycinnamoyl tartaric acids.

W%



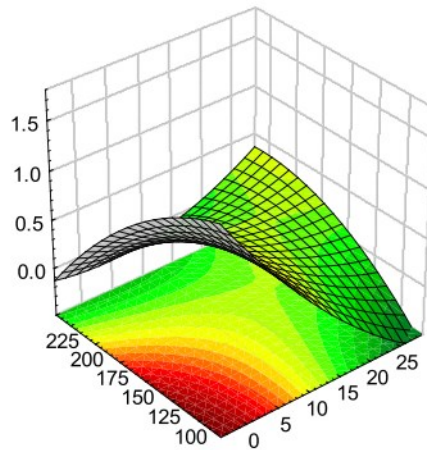
Total sugars (g/L)

Sampling days

Figure 1. Modeling of the distribution percentage in weight (W%) of Italia table grape berries with different sugar content (g/L) during ripening.

Citric acid
(g/L)

a

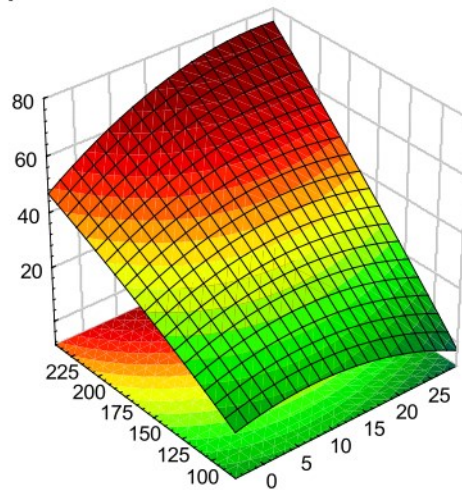


Total sugars (g/L)

Sampling days

SSC/TA

b

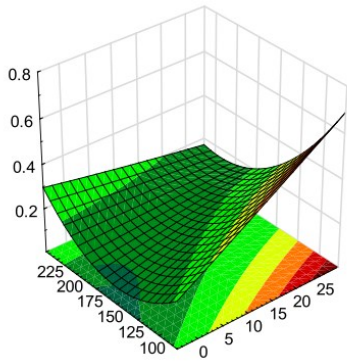


Total sugars (g/L)

Sampling days

Figure 2. Modeling of (a) citric acid content (g/L) and (b) SSC/TA ratio of Italia table grape berries with different sugar content (g/L) during ripening.

Protocatechuic acid
(mg/kg)

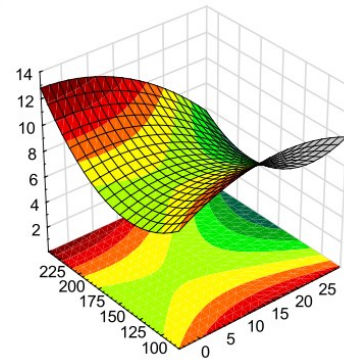


Total sugars (g/L)

Sampling days

a

Catechin
(mg/kg)

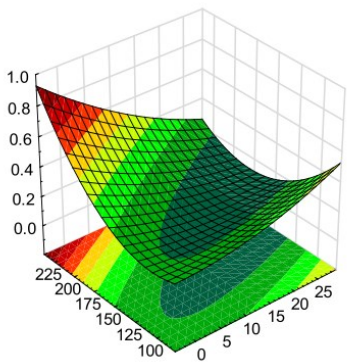


Total sugars (g/L)

Sampling days

b

Epicatechin
(mg/kg)

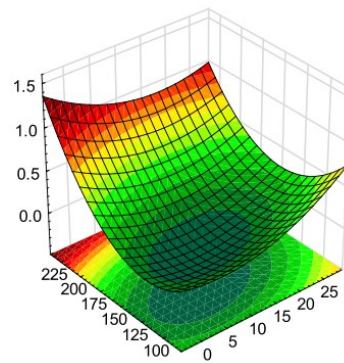


Total sugars (g/L)

Sampling days

c

t-Resveratrol
(mg/kg)



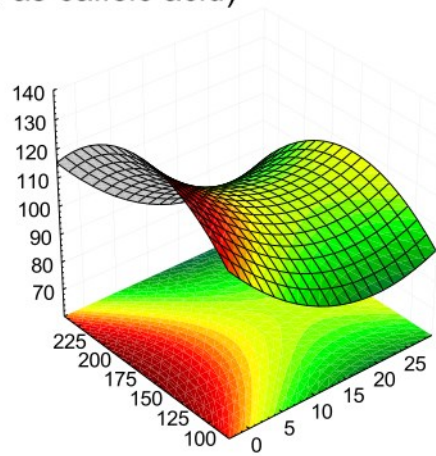
Total sugars (g/L)

Sampling days

d

Figure 3. Modeling of (a) protocatechuic acid, (b) catechin, (c) epicatechin, and (d) *t*-resveratrol contents (mg/kg) in the skin of Italia table grape berries with different sugar content (g/L) during ripening.

HCTA index
(mg/kg, as caffeic acid)



Total sugars (g/L)

Sampling days

Figure 4. Modeling of HCTA index in the pulp of Italia table grape berries with different sugar content (g/L) during ripening.