JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Relationship of Changes in Rotundone Content during Grape Ripening and Winemaking to Manipulation of the 'Peppery' Character of Wine

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ABSTRACT: Biosynthesis of the sesquiterpene rotundone in Vespolina grapes during berry ripening was investigated over two consecutive seasons, revealing that the compound accumulates from veraison to harvest and reaches relatively high concentrations (up to 5.44 μg/kg). Rotundone levels up to 1.91 μg/kg were also found in clones of Gruener Veltliner, a white grape variety known to give 'peppery' wines. These concentrations are higher than those reported for Syrah grapes and are similar to the levels found in some plants. Rotundone was shown to accumulate almost exclusively in berry exocarp, suggesting that skin contact during winemaking could be used to modulate the peppery character of red wine. However, rotundone yield after the winemaking process was relatively low. Indeed, only 10% of the rotundone present in grapes was extracted during fermentation, and only 6% was recovered in bottled wine. The results presented in this work provide key knowledge for manipulation of the peppery character of wine in order to optimize the intensity of this characteristic wine aroma.

KEYWORDS: *Vitis vinifera*, rotundone, GC-MS/MS, grape ripening, sesquiterpenes, winemaking

INTRODUCTION

Of the hundreds of molecules that could potentially contribute to the varietal flavor and aroma of wine, including esters, alcohols, and sulfur compounds, only a handful of impact compounds, conferring a distinctive aroma on their own, have been reported.[1,2] Some of these belong to the chemical class of isoprenoids, in particular, monoterpenes. These molecules play a significant role in grapes and wine, to the extent that the distinction between "aromatic" grape varieties (such as Muscat, Malvasia, Gewurztraminer, Riesling) and "neutral" grape varieties (which are the vast majority) has historically been based on the presence of geraniol, linalool, and nerol above the sensory threshold in grapes. The 'Muscat character' can be sensorially appreciated in both grapes and wine and is an important positive trait for the cultivation of table grapes. Research recently succeeded in identifying the causal single nucleotide polymorphism responsible for this character that could be used for marker-assisted breeding programs.[3] Monoterpenols are also considered to be important for the varietal character of wines and can be used for varietal and clonal discrimination.[4−6] Extensive studies on the composition of grape juice and wine have led to the identification of many monoterpenoids and norisoprenoids. However, the knowledge available on higher terpenoids such as sesquiterpenoids in these matrices is still very limited.

Some authors have investigated the sesquiterpene content of grape varieties such as 'Baga',[7] Syrah,[8] Riesling, and Cabernet Sauvignon,[9] reporting the presence of an unexpectedly wide range of compounds. Sesquiterpenes are synthesized via the mevalonate (MVA) pathway localized in the cytosol, and their biosynthesis could be induced by methyl jasmonate treatment in grapevine cell suspension.[10,11] The remarkable diversity of sesquiterpenes is generated by the large family of sesquiterpene synthases (TPS-a) recently discovered in grapes, each producing multiple substrates.[12] Nevertheless, the contribution of this great number of sesquiterpenes to wine flavor and aroma is still unclear.

Recently, a very interesting sesquiterpene ketone, called rotundone (Figure 1), was identified as being responsible for the peppery aroma in grapes, wine, herbs, and spices.[13] This compound is considered to be one of the most interesting aroma compounds ever reported, because it is associated with the aroma of the most widely used spice in the world, pepper (*Piper nigrum*) and with the 'peppery' aroma of grapes and wine. Due to its very distinctive aroma and low sensory threshold (16 ng/L in red wine, 8 ng/L in water), rotundone is a potent aroma and is one of the few known impact compounds in wine.[13] This guaiane-like compound was first found in Syrah, Mourvèdre, and Durif wines produced in Australia, at concentrations of up to 145 ng/L,[13] whereas a recent study[14] showed that rotundone was present in higher (up to 561 ng/L) concentrations in Schioppettino and Vespolina red wines and in Gruener Veltliner white wines, produced in Europe.

One of the *Vitis vinifera* cultivars that gives very 'peppery' wines is Vespolina. This variety, also called Ughetta di Canneto, is a red grape autochthonous to northwestern Italy, and it is mainly cultivated in the Piedmont region (in the provinces of Novara, Vercelli, and Varese). Its use is also widespread in the area of Oltrepò Pavese (Lombardy). This grape variety is moderately vigorous and has good basal fertility. Vespolina is well suited to

Received: February 26, 2011
Accepted: April 22, 2011
Revised: April 21, 2011
Published: April 22, 2011

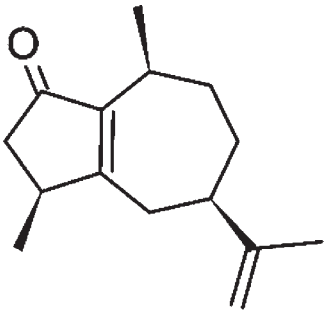

Figure 1. Chemical structure of (−)-rotundone.

Guyot and cordon farming. The cluster is of average size, with a cylindrical−pyramidal shape, and is basically straggly. The budding and flowering time for this cultivar is around average (respectively around the second 10 days in April and the second 10 days in June), whereas veraison is medium-early (first half of August); ripening takes place in mid/late September.

Vespolina produces high-quality wines with floral and spicy nuances and a clear 'peppery' note. In the Oltrepò Pavese area it is present in most of the denominations of origin, but together with other cultivars: in DOC "Bonarda dell'Oltrepo Pavese" it may be present up to a maximum level of 15%, whereas in DOC "Buttafuoco" and DOC "Oltrepo Pavese" it may represent up to 45%; it is also present in DOC "Casteggio" up to a maximum content of 35%. In the Piedmont area it is often present as a complementary variety, together with Nebbiolo.

Rotundone concentration in must and wine may depend on several factors such as cultivar, region, and mesoclimate. It is assumed that the intensity of its characteristic aroma could be modulated by careful management of the vine and winemaking techniques. However, at the moment there is a lack of basic knowledge about this important substance. The aims of this work were to develop a method for analysis of rotundone in grapes, adapted from an existing method optimized for wine, to analyze the kinetics of its biosynthesis and accumulation during ripening of Vespolina grapes, and to estimate its distribution in berry skin and mesocarp and its extractability and fate during the red winemaking process.

MATERIALS AND METHODS

Chemicals. Rotundone and d_5-rotundone were synthesized using the methods described by Mattivi et al.[14] and Siebert et al.,[15] respectively. All reagents required for the synthesis were purchased from Sigma (St. Louis, MO) and used as received. Acetone, *n*-pentane, ethyl acetate, ethanol, and methanol were of high-purity grade and were purchased from VWR International (Milan, Italy). Ultrapure water from a Milli-Q system (Millipore, Bedford, MA) was used in this study.

Grapes. The Sangiovese grapes used for preparation of the calibration curves were collected in 2010 at maturity from experimental vineyards located in San Michele all'Adige, Italy.

Vespolina grapes were sampled from an experimental vineyard belonging to the Azienda Agricola Bisi located in Cascina San Michele at San Damiano al Colle, Italy (latitude 45° 1′ N, longitude 09° 20′ E, about 200 m average altitude), during the 2009 and 2010 seasons. This vineyard is on a slope of about 30%, and the rows are oriented east−west; the soil is clay-calcareous, and it is managed with spontaneous cover crop between the rows; spring weeding is carried out on the rows. The form of farming adopted is cordon, with a planting density of 6000 plants/ha. Thanks to the collaboration between the University of Milan and Vitis Rauscedo, this grape genotype will be homologated with the name "UNIMI-Vitis Vesp. 1".

Samples of about 500 g of grape berries were collected randomly from different grapevines at different times during grape ripening. In 2009 samples were taken at the following times: 50% veraison, or half-veraison, which can be defined as the time when 50% of the grapes had changed color; 100% veraison, corresponding to the time when all of the grapes had changed color; 21 days after 100% veraison; and harvesting. In 2010, two additional times were chosen for sampling between 100% veraison and harvest at weekly intervals. Furthermore, an over-ripe sample was collected 14 days after harvesting. Because the experimental vineyard was located partly on the hillside and partly at the foot of the hill, differences in grape maturity between the top and bottom of the vineyard could be noted, thus requiring sampling from the two zones to be carried out separately. After collection, the samples were immediately stored at −20 °C until analysis.

Three Gruener Veltliner clones were provided by the Walek winery and were grown in the Thermen region (Austria) in 2009. The rows in the vineyard were oriented north−south, and the form of farming adopted was simple Guyot with a planting density of 4000 plants/ha. Clones A1-2 and A1-3 were selected at the Agricultural Secondary School of Krems (Austria), whereas clone A1-5 was selected at the Federal Institute for Viticulture and Pomology in Klosterneuburg (Austria). All clones were originally collected in the Steiermark region (Austria) and identified as Gruener Veltliner genotypes using SSR analysis and ampelography. All clones grown in the vineyard were on Kober 5BB rootstocks.

Wines. Experimental wines were produced from Vespolina grapes collected during 2009 and 2010 from the hillside and the foot of the hill. The grapes were destemmed and crushed. Batches of 40 L were prepared after addition of 100 mg/L of $K_2S_2O_5$. Alcoholic fermentation conditions were as follows: inoculation with 400 mg/L Zymaflore F15, addition of 200 mg/L of Tanin VR Supra and 200 mg/L of Thiazote, all from Laffort (Bordeaux, France); fermentation was carried out at 20 °C; manual punching occurred three times a day for the first three days and every 12 h in the following days. After 9 days of maceration, the wine was separated from the pomace. Malolactic fermentation was induced by inoculation of lactic bacteria Viniflora (Christian Hansen, Hørsholm, Denmark). After 12 days, the wines were separated and filtered through depth filter sheets Beco KD3 (Begerow, Langenlonsheim, Germany) and Sartopure PP2 1.2 μm filters (Sartorius Stedim, Aubagne, France). Must samples were collected for analysis during the fermentation process at the following times: prefermentation, cap formation, end of fermentation, wine separation, and final bottled wine.

Must Analysis for Rotundone Content. Before analysis, all of the samples were centrifuged at 5400*g* for 10 min and analyzed using the method developed previously.[14] The analytical method is based on the use of solid phase extraction (SPE) to separate rotundone from other components of the matrix and to allow its concentration before analysis, coupled with a GC separation of only 30 min with selective quantification of rotundone using tandem mass spectrometry in multiple reaction monitoring (MRM) mode with d_5-rotundone as internal standard. This protocol was shown to provide the desired sensitivity and selectivity for routine analysis of rotundone in both white and red wines.

Briefly, after the addition of 200 μL of d_5-rotundone (100 μg/L) as internal standard to 100 mL of must, the samples were subjected to SPE on Isolute Env+ (Biotage, Uppsala, Sweden) cartridges (1 g, 6 mL volume) previously conditioned with 10 mL of *n*-pentane/ethyl acetate (4:1), then 10 mL of methanol, and finally 20 mL of model wine solution (12% ethanol and 4 g/L of tartaric acid buffered to pH 3.2). The samples were percolated through the cartridges, which were then washed with water (10 mL) and eluted with 20 mL of *n*-pentane/ethyl acetate (9:1). After elution, the samples were concentrated to dryness in an EZ-2 (GeneVac, Ipswich, U.K.). The residues were dissolved in 1 mL of

ethanol, and 13 mL of deionized water was added. The solutions were analyzed in duplicate using the SPME GC-MS/MS method.

SPME GC-MS/MS Method. GC analysis was performed on a Trace GC Ultra gas chromatograph coupled with a TSQ Quantum Tandem mass spectrometer upgraded to the XLS configuration. The DuraBrite IRIS ion source with prefilter was installed to improve the performance of the spectrometer. The system was equipped with a Triplus autosampler (Thermo Electron Corp., Waltham, MA).

SPME, GC separation, and MS/MS detection were performed as previously described.[14] Data acquisition and analysis were performed using the upgraded Xcalibur Workstation software version 2.1 supplied by the manufacturer.

Extraction and Quantification of Rotundone from Whole Berries, Exocarp, and Mesocarp. Frozen grape berries (−20 °C) were deseeded, avoiding removal of flesh, immediately transferred into liquid nitrogen, and ground using an analytical mill (IKA, Staufen, Germany). Twenty-five grams of the frozen grape powder samples was extracted with 50 mL of acetone. After vortexing for 1 min, the suspensions were left at room temperature for 1 h, mixing thoroughly every 10 min. The extracts were filtered by gravity on glass wool, and the solvent was carefully evaporated under vacuum using a rotary evaporator set at 40 °C. The aqueous residues (ca. 20 mL) were diluted to 100 mL with a model wine solution and centrifuged at 5400*g* for 10 min. After the addition of 200 μL of d_5-rotundone (100 μg/L) as internal standard, the samples were subjected to SPE as described for must samples and analyzed using SPME GC-MS/MS.

For rotundone localization studies, frozen berries were allowed to soften for 20 min at room temperature before the exocarp (skin) was separated from the mesocarp (flesh). The skin and flesh obtained from 50 g of berries were weighed, frozen in liquid nitrogen, and ground in a mill. The powder was extracted in 50 mL of acetone for 1 h and processed as described above.

For rotundone quantification in grapes and must, calibration curves were prepared using extracts of Sangiovese grapes, a variety lacking the 'peppery' aroma. The calibrator standards were prepared by spiking the grape extracts with rotundone concentrations between 5 and 150 ng (five calibrators, two replicates). Along with the calibration curves, zero samples (grape extract spiked only with the internal standard) were also analyzed. Good linearity was achieved in the tested concentration range. In fact, the linear regression equation was $y = 0.138 \pm 0.004x + 0.232 \pm 0.242$ with a coefficient of determination (R^2) of 0.993. All samples were analyzed in duplicate. The repeatability of the extraction process was evaluated by processing five aliquots of the same grape powder sample and quantifying the rotundone content. The limit of detection (LOD) and the limit of quantification (LOQ), defined as the concentrations giving S/N ratios of 3:1 and 10:1, respectively, were calculated in grape extracts spiked with 10 ng/L of rotundone. The LOD was 1.5 ng/L and the LOQ was 5 ng/L, values similar to those reported previously for wine.[14]

Statistical Analysis. ANOVA was performed using Statistica 9.1 software (StatSoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Quantitative Extraction of Rotundone from Grape Samples. Quantitative analysis of rotundone in grapes required the development of an efficient method of extraction that could allow maximum recovery of the compound from the matrix. We decided to use frozen grape powder instead of frozen berries to increase the surface of extraction and speed the process. Because previous work from Siebert et al.[15] suggested that rotundone recovery could be affected by the presence of seeds, we decided to use in our experiments powder obtained from deseeded grapes.

We reasoned that the use of a less polar solvent such as acetone could maximize the extraction efficiency. Thus, we extracted 25 g of homogeneous powder of Vespolina grapes with 50 mL of acetone for 1 h at room temperature (five repetitions) obtaining a 3.2-fold higher concentration of rotundone compared to the extraction of the same amount of grape powder for 24 h with 50 mL of model wine (five repetitions), conditions similar to those used by Siebert and co-workers.[15] The calculated amount of rotundone extracted using acetone was 3.02 ± 0.15 μg/kg, with a CV% of 5.1; while using model wine, we calculated a rotundone content of 0.95 ± 0.14 μg/kg with a CV% of 14.4. These results indicated that extraction with acetone is definitely faster and more efficient compared to model wine and provides more consistent results as well.

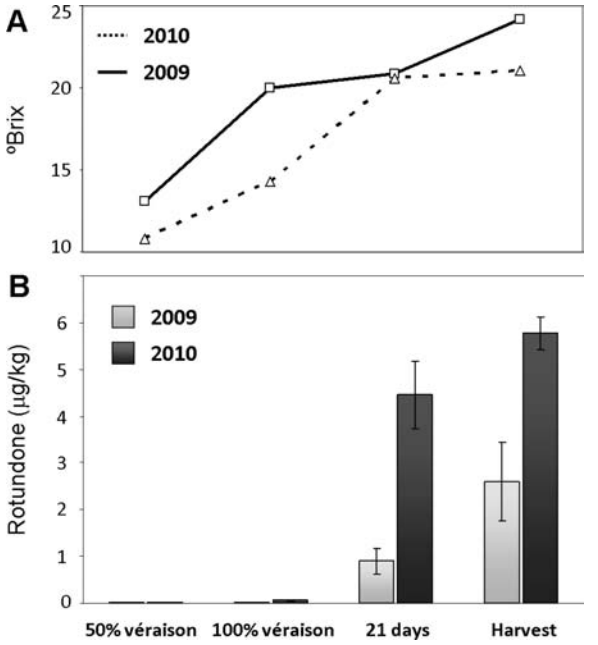

Figure 2. Berry ripening and rotundone accumulation during 2009 and 2010.

In a time course study, in which Vespolina grape powder was extracted at room temperature with acetone for 1, 3, and 4 h, we did not observe any significant increase in rotundone content, suggesting that extraction was already complete after only 1 h (data not shown). The recovery of rotundone during sample preparation was evaluated by spiking acetone extracts of neutral (rotundone-free) Sangiovese grape powder with known amounts of rotundone. A recovery of >90% was observed.

Rotundone Accumulation during Grape Ripening. To get an insight into the kinetics of rotundone accumulation during grape berry ripening, we focused on the stages of veraison and harvest of Vespolina grapes. The term veraison refers to the change in color of grape berries, indicating transition from berry growth to berry ripening. The change of color is also associated with sugar accumulation and softening of the berries followed by an increase in pH and an accumulation of polyphenols and flavor compounds.[16] However, the onset of veraison does not occur uniformly in all berries, but depends on exposure of the berries and clusters to warmth.

In our study, we followed the kinetics of rotundone accumulation during berry ripening in 2009 and 2010. ANOVA for the repeated-measures factor, that is, the concentration of rotundone analyzed in duplicate in grapes at different points in time (50 and 100% veraison, 21 and 34 days after veraison) with two

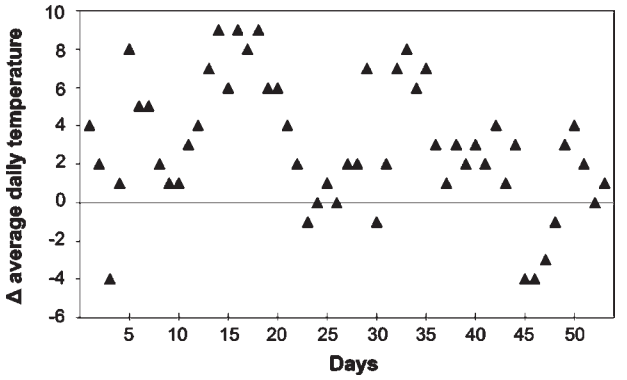

Figure 3. Day-by-day differences in the average daily temperatures in 2009 and 2010 (Δ = 2009 − 2010) during berry ripening.

between-group factors, the vineyard (high and low) and year (2009 and 2010), suggested that there was a common trend for the kinetics of rotundone accumulation ($p < 0.001$), which was influenced in our experiment both by the vineyard and year ($p < 0.001$) and by their interaction ($p = 0.003$).

The data obtained (Figure 2A) indicated that the ripening process occurred differently during the two years. In particular, in 2010 the sugar levels were lower than in 2009. This difference can be probably ascribed to mesoclimatic differences between the two years. In Figure 3, the day-by-day differences in average daily temperature between 2010 and 2009 during the ripening period of Vespolina grapes (between August and September) are given. The plot clearly shows that in 2010, when sugar accumulation was lower, only for 7 days was the average daily temperature higher than in 2009 in the period considered (53 days). Furthermore, we recorded 10 rainy days in 2010 and only 5 in 2009.

In this study, we collected grape samples from half-veraison, the time when 50% of the grapes were changing color, to harvest in 2009 and from half-veraison to over-ripeness in 2010, analyzing their rotundone content using the SPE-SPME-GC-MS/MS method. As shown in Figure 2B, in both years rotundone started to accumulate from the onset of veraison, especially from 100% veraison, when all of the berries had changed color, and continued until harvest. The total amount of rotundone detected at the same time points was higher in 2010 than in 2009. It is well-known that one of the most important factors influencing the varietal aroma of grapes is the stage of ripening. In particular, both free and conjugated forms of terpenoids are mainly accumulated in grapes during ripening.[17] Several studies have shown that terpenoids are more abundant toward harvest than during early berry development. Wilson et al.[18] showed that the levels of terpenes in Muscat berries increased with sugar accumulation, in some cases reaching peak levels in the over-ripe fruit. Sesquiterpenoids, in particular, were shown to accumulate from veraison to maturity and to remain constant until postripe in *V. vinifera* L. cv. 'Baga' berries.[7] However, the recent work of Kalua and Boss[9] on Riesling and Cabernet Sauvignon grapes reported a significant decrease in sesquiterpenoid concentration toward harvest, in contrast with the previous observations. Nevertheless, the accumulation of sesquiterpenoids toward maturity is supported by the expression pattern of two sesquiterpene synthases, (+)-valencene and (−)-germacrene synthases, which were found to be expressed only in the later stages of berry development in Gewürztraminer grapes.[19] Curiously, valencene synthase was also found to be expressed during Cabernet Sauvignon flower development.[20] It is

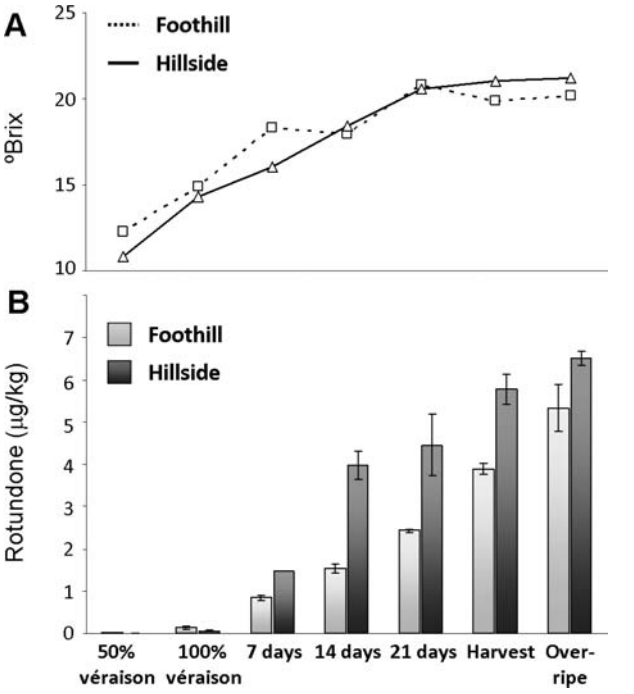

Figure 4. Comparison between grapes collected from vines growing on the hillside and at the foot of the hill during 2010.

worth considering that the guaiane sesquiterpenes are expected to be synthesized via two cyclization reactions, the first yielding a germacrene intermediate as germacrene A[21] and the second generating the guaiane product. Although guaiane-like terpenes are very common in nature, only a few enzymes producing guaianes as secondary reaction products have been described,[22−25] and many questions regarding biosynthetic sequence and backbone modifications, such as hydroxylation and glycosylation, remain unanswered.

Because our data were collected over only two years and from only one vineyard, it is impossible to draw general conclusions about the effect of temperature on rotundone evolution, but it is likely that higher levels are accumulated in cooler vintages and sites. This is consistent with the observation that there are 'peppery' vineyards that consistently produce 'peppery' wines, especially in cooler years.[8,26]

The experimental vineyard from which we collected the Vespolina grape samples is partly located on a very steep hillside and partly on a plain, at the foot of the hill. Because we expected a certain degree of variation in berry ripening between the two sites due to differences in mesoclimate (especially exposure to sunlight and wind), soil, and water availability, we analyzed samples from the two parts of the vineyard separately. Although sugar accumulation took place to the same extent in the two areas of the vineyard (Figure 4A), rotundone levels increased at a lower rate in the vines located on the plain and did not reach the same levels at harvest as in the samples collected from the hillside (Figure 4B). In the samples from the hillside rotundone reached concentrations of 2.77 ± 0.57 μg/kg in 2009 and 5.44 ± 0.33 μg/kg in 2010, whereas in the samples from the foot of the hill it accumulated to 1.42 ± 0.53 μg/kg in 2009 and to 3.67 ± 0.12 μg/kg in 2010. Further increases in rotundone levels were observed in samples collected in 2010 at the over-ripe stage (6.13 ± 0.16 μg/kg).

To investigate the extent of rotundone accumulation in white grape varieties at commercial harvest, we analyzed three different

Table 1. Rotundone Concentration and Yield[a] during the Winemaking Process

	rotundone content				
	grapes (μg/kg)	must (ng/L)	end of fermentation (ng/L)	separation (ng/L)	final wine (ng/L)
2009 hillside	2.77	32	388 (9.8%)	344 (8.7%)	242 (6.1%)
2009 foothill	1.42	10	194 (9.6%)	153 (7.6%)	101 (5.0%)
2010 hillside	5.44	281	na[b]	1109 (14.3%)	503 (6.5%)
2010 foothill	3.67	214	na[b]	986 (18.8%)	370 (7.0%)

[a] The yield, shown in parentheses, was calculated considering that 1 kg of grapes produced 0.7 L of must (70%). [b] na = not analysed.

clones of Gruener Veltliner currently used for the production of wine with 'peppery' notes and shown to contain rotundone.[14] We found that the different clones accumulated different amounts of rotundone. In particular, clone A1-2 reached a concentration of 0.86 ± 0.06 μg/kg, clone A1-3 a concentration of 0.54 ± 0.04 μg/kg, and clone A1-5 a concentration of 1.91 ± 0.013 μg/kg. A 4-fold difference in the content of a key aroma in grapes collected in the same vineyard suggests the presence of significant variability between clones, which deserves future research.

Localization of Rotundone in Grape Berries. Unlike other plants producing terpenes, grapes lack any specialized anatomical structure for the storage of these compounds. Thus, the accumulation of the terpenes in grape berries mainly occurs as glycosylated forms in the exocarp cell vacuoles, although some terpenoids may also be present as free volatiles[27] and in the mesocarp.[28]

To date, information concerning sesquiterpene localization in grape berries is still lacking, and apart from nerolidol and farnesol, no other sesquiterpene glycoconjugates are known to be present in grapes.[29,30] Hence, we decided to investigate the site(s) of rotundone accumulation in the berry, because this information could provide indications for improving the 'pepperiness' of wine, for instance, through extended skin contact during winemaking.

In a first experiment, we separated the exocarp from the mesocarp of Vespolina berries collected at harvest and containing 1.05 μg/kg rotundone. The two fractions were ground in liquid nitrogen and extracted with acetone to quantitatively extract the compound from the two matrices. Analysis of the samples showed that only a small amount of rotundone (3.8%) was released from the mesocarp or was probably present as a consequence of skin disruption during sample preparation, whereas the fraction containing the skins contained the majority of the compound. In fact, the value of 7.7 ng/g of skin obtained corresponded to 96.2% of the total extracted rotundone (the weight of the skin was 12.5% of the berry weight). Similar results were also obtained from analysis of Gruener Veltliner grapes, in which 98.9% of the total rotundone was found in the skin.

Kinetics of Rotundone Extraction during Alcoholic Fermentation. The complex chemistry of wine aroma is only partly due to the molecules present in grapes. Most of the aroma compounds are instead the result of the many different processes occurring during fermentation. It is known that several compounds undergo chemical rearrangement due to pH changes, some are released from odorless precursors through acidic or enzymatic hydrolysis, and some originate from yeast and bacterial metabolism.

In this study, we investigated the release of rotundone during must fermentation in the presence of the skin (fermentation−maceration) from grape juice to wine. Two batches of wine were prepared from grapes collected from the hillside and from the foot of the hill in 2009. Rotundone levels were monitored at five stages: prefermentation, cap formation, end of fermentation, wine separation, and final bottled wine.

At the prefermentation stage, a small amount of rotundone was already present in the must, indicating that it was released after disruption of the grapes during mechanical crushing. Its concentration dramatically increased from cap formation to the end of the fermentation, reaching 12−15 times the initial concentration. However, the amount of rotundone detected at this stage was in the range of 9.6−9.8% of the total rotundone present in the grapes used for winemaking, assuming a must yield of 70% (e.g., 1 kg of grapes provides 0.7 L of must).

Because rotundone is a very hydrophobic molecule (predicted Log K_{ow} = 4.98), it is reasonable to assume that its extraction from the skins is improved by the formation of alcohol during the process. The alcohol levels in the wine produced in 2009 reached 13.1% (hillside) and 12.6% (foothill) at the end of the fermentation process. However, after separation of the wine from the skins, a significant amount (10−30%) of the compound was lost, probably because it was bound to the particles removed (marc and lees). Additional loss also occurred following wine filtration, bringing the rotundone content in the final wine to about 50−60% of the amount extracted during the fermentation process. Analysis of the final bottled wine revealed that only a small amount (5.0−6.1%) of the rotundone present in grapes was found in the final product. The experiment was repeated with similar outcomes in 2010, and the results are summarized in Table 1.

The rotundone concentration detected in Vespolina grapes at harvest was very high. Its levels ranged from 1.42 ± 0.53 μg/kg in the samples from the foot of the hill collected in 2009 to 5.44 ± 0.33 μg/kg in the samples from the hillside collected in 2010. Such levels are higher than the maximum level of 0.62 μg/kg reported for Syrah grapes.[30] However, we observed that the yield of the winemaking process was relatively low. Indeed, only about 10% of the rotundone present in grapes was extracted during fermentation, and only 5.0−7.0% was recovered in bottled wine. Our experiment of rotundone extraction from frozen grape powder demonstrated that acetone is required for quantitative extraction, providing 3.2 times more rotundone compared to model wine. On the basis of these findings, we reckon that the low yield at the end of the fermentation process could be mainly attributed to the hydrophobicity of the molecule, which is poorly extracted by an aqueous solution containing about 12% of ethanol, like wine. Nevertheless, the contribution of several factors, such as back-binding to marc and lees, winery materials, and/or filtration/clarification treatments to the low recovery, is still under investigation. Furthermore, formation of stable adducts between carbonyl compounds and bisulfite, called hydroxyalkylsulfonic acids (HASAs) has also been reported in wine.[31] Because rotundone contains a keto group, formation in wine of HASAs involving this compound cannot be excluded a priori. Thus, more studies are required to evaluate whether or not their formation occurs and, if that is the case, their impact on the

recovery yield in the final wine. The results presented in this work provide key knowledge for manipulation of the 'peppery' character of wine to maximize the intensity of this distinctive aroma. An understanding of when rotundone accumulation occurs is critically important to obtain higher levels of the compound in wine. Our observations indicate that mesoclimate influences accumulation of the compound, hence affecting the 'pepperiness' of grapes. Analysis of three different Gruener Veltliner clones grown in the same vineyard also indicates that the levels of rotundone accumulation could be a clonal trait. Rotundone accumulates almost completely in berry exocarp and is released in only a low percentage in wine during the fermentation process. Thus, long skin contact would contribute to enriching 'peppery' notes in wine. However, a significant amount of the compound is lost during separation and filtration processes, suggesting that attention should be paid to the choice of the wine filtration and fining methods. To date, we have no evidence that can suggest or exclude the existence of a conjugated form of rotundone, but we can conclude that Vespolina grapes as well as Schioppettino and Gruener Veltliner[14] and other very 'peppery' varieties can contain rotundone at levels typical of some herbs such as basil and thyme.

This work provided also a rationale for the traditional use of very 'peppery' grape varieties, such as Vespolina, in blends to increase or confer the 'peppery' aroma to wine.

AUTHOR INFORMATION

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Funding Sources

This research was partly supported by the ADP 2010 project funded by the Autonomous Province of Trento. L.C. was supported by the MetaQuality project, funded by the Autonomous Province of Trento (Italy).

ACKNOWLEDGMENT

We thank Graziano Manenti for producing the experimental wines at the Vitis experimental winery in Rauscedo, Italy. We also thank Azienda Agricola Bisi for hosting the experiment in its vineyards.

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