ORIGINAL RESEARCH ARTICLE

Monoterpenoids and norisoprenoids in Italian red wines

Davide Slaghenaufi, Leonardo Vanzo, Giovanni Luzzini, Panagiotis Arapitsas, Matteo Marangon, Andrea Curioni, Fulvio Mattivi, Paola Piombino, Luigi Moio, Andrea Versari, Arianna Ricci, Susana Rio Segade, Luca Rolle and Maurizio Ugliano

1 Department of Biotechnology, University of Verona, 37134 Verona, Italy
2 Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, 35020 Legnaro, Italy
3 Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, 38123 Povo, Italy
4 Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach, 38010 San Michele all’Adige, Italy
5 Department of Agricultural Sciences, Division of Vine and Wine Sciences, University of Napoli Federico II, 83100 Avellino, Italy
6 Department of Agricultural and Food Sciences, University of Bologna, 47521 Cesena, Italy
7 Department of Agricultural, Forest and Food Sciences, University of Torino, 10095 Grugliasco, Italy
8 Department of Wine, Vine and Beverage Sciences, School of Food Science, University of West Attica, Ag. Spyridonos str, Egaleo, 12243 Athens, Greece

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ABSTRACT

This work aimed to investigate the occurrence of different monoterpenoids and norisoprenoids in 10 monovarietal Italian red wines (Aglianico, Cannonau, Corvina, Montepulciano, Nebbiolo, Nerello, Primitivo, Raboso, Sangiovese and Teroldego) from 12 regions across Italy. The wines were produced in their terroirs following the oenological practices and legislation of the respective appellation. Data showed that different wine varietals were characterised by peculiar profiles of monoterpenoids and norisoprenoids. Montepulciano and Cannonau wines showed highest levels of nerol, terpinolene, α-terpineol, limonene and of the norisoprenoids β-damascenone, α-ionone, TPB, TDN and vitispirane. Aglianico, Corvina, Nebbiolo and Primitivo were characterised by relatively high levels of linear monoterpane alcohols such as linalool and geraniol. Sangiovese samples from Tuscany could be differentiated from those of Romagna, with the former characterised by a higher content of α-terpinene and the latter of γ-terpinene. The monoterpane 1,4-cineole was present in Montepulciano, Raboso and Sangiovese at potentially relevant olfactory levels, its occurrence associated with higher content of terpinen-1-ol acting as a potential precursor under wine acid conditions.

KEYWORDS: monoterpenoids, norisoprenoids, red wine, SPME-GC-MS
INTRODUCTION

Wine is a very complex matrix containing over 1000 volatile compounds (Polášková et al., 2008). Among these, terpenoids represent a large class of compounds with a skeleton formed by isoprene units (2-methylbuta-1,3-diene) generally joined in a head-to-tail fashion (Wallach, 1887). Based on the increasing number of isoprene units, terpenoids can be classified into hemiterpenoids, monoterpenoids, sesquiterpenoids, diterpenoids, sesterterpenoids, triterpenoids and carotenoids (IUPAC, 1997).

In plants, the biosynthesis of the isoprenic building blocks can occur in both cytoplasms via the mevalonic acid (MVA) pathway or in the plastid organelles through the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Laule et al., 2003).

Some terpenoids are very important for table grape and wine perceived sensory quality as they can contribute to its aroma. Monoterpene alcohols such as linalool and geranial are particularly interesting because they contribute to the aroma of the wine with pleasant notes such as floral and citrus, which characterise aromatic grape varieties such as Muscat, Gewürztraminer and Riesling (Jackson, 2008; Marais, 1987; Rapp, 1990). A large diversity among the cultivars is expected, given that the terpenoid synthase gene (VvTPS) is one of the largest gene families producing secondary grape metabolites contributing to berry and wine flavour and other biological functions such as defence and resistance (Martin et al., 2010). Monoterpenes are considered part of wine varietal aroma and it has been proposed to classify grape varieties into Muscat (total monoterpenes > 6 mg/L), non-Muscat but aromatic (total monoterpene concentration 1–4 mg/L) and neutral varieties (total monoterpenes concentration < 1 mg/L) (Mateo and Jiménez, 2000). Accordingly, almost all red grape varieties belong to the third group and are commonly considered neutral varieties.

However, there is a growing interest in the potential contribution of terpene compounds to the aroma of red wines. It has been shown that over time the monoterpenoids can undergo acid-catalyzed reactions (Skouroumounis and Sefron, 2000) that lead to the formation of new potent odorant compounds such as 1,8-cineole and 1,4-cineole (Fariña et al., 2005; Slaghenaufi and Ugliano, 2018). Thanks to their low odour thresholds, cineoles and other cyclic monoterpoids with p-methane structures seem to contribute to the fresh and minty notes found in certain red wines even at the low concentrations of red wines from non-aromatic varieties (Antalick et al., 2015; Fariña et al., 2005; Picard et al., 2016; Picard et al., 2017; Luzzini et al., 2021; Slaghenaufi et al., 2020a).

Norisoprenoids are aroma compounds derived from the enzymatic oxidative cleavage—operated by the carotenoid cleavage dioxygenases (CDD)—of the carotenoids (C40). They are therefore ubiquitous in red and white wines. Due to their very low odour threshold, some of them can play a major role in wine aroma. Among the most studied norisoprenoids, there are β-damascenone, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), E-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB) and vitispiranes that can contribute to wine bouquet with aroma notes of cooked apple, petroleum, tobacco and camphor, respectively (Black et al., 2015).

Among wine-producing countries, Italy is characterised by an extremely vast ampelographic heritage, with over 500 cultivars registered in the Italian National Catalogue of Grapevine Varieties (Lacombe et al., 2011). However, few studies have focused on the chemical characterisation of wines obtained from these varieties. Recently some comparative studies among the main Italian red wines have been done focusing on the phenolic (Giacosa et al., 2021) and non-volatile metabolomic profile (Arapitsas et al., 2020) as well as on sensory characteristics such as astringency (Pittari et al., 2020; Piombino et al., 2020). However, little is known about their content in monoterpenoids and norisoprenoids, despite the well-documented relevance of these compounds to perceived wine aroma (Black et al., 2015). Such knowledge would be of central importance in developing specific viticultural and winemaking protocols and strategies aimed at expressing the aroma identity of Italian wines, also in the current context of climate change which demands reconsidering established viticultural and winemaking practices.

The main objective of this study was to characterise the profile of monoterpenoids and norisoprenoids of ten varieties of red wine, among the most important in Italian ampelography.

MATERIALS AND METHODS

1. Reagents

Octan-2-ol (97 % purity), linalool (97 %), terpinen-4-ol (≥ 95 %), α-terpinol (90 %), nerol (≥ 97 %), geranial (98 %), β-citronellol (95 %), p-cymene (99 %), teripolene (≥ 85 %), γ-terpine (≥ 97 %), limonene (97 %), 1,8-cineole (99 %), 1,4-cineole (≥ 98.5 %), β-damascenone (≥ 98 %), α-phellandrene (95 %), 3-carene (≥ 95 %), α-ionone (90 %), α-terpinen (≥ 95 %), β-myrcene (≥ 90 %), supplied by Sigma Aldrich (Milan, Italy). 1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN) with 80 % of purity was supplied by Synchem UG & Co (Felsberg, Germany). Sodium chloride (≥ 99.5 %) was supplied by Sigma Aldrich (Milan, Italy).

2. Wines

A total of 96 monovarietal red wines from 10 different grape varieties and vintage 2016 were collected directly from commercial wineries across different regions of Italy. The wines produced from the following ten grape varieties were considered: 10 Aglianico (AG), 8 Cannonau (CAN), 7 Corvina (CO), 6 Montepulciano (MON), 11 Nebbiolo (NE), 11 Primitivo (PR), 11 Teroldego (TE), 10 Raboso del Piave (RAB), 19 Sangiovese including 7 from Tuscany (SAT) and 12 from Romagna (SAR) and 3 Nerello Mascalese (NER). Wines were obtained from a single grape variety fermented in stainless-steel vats and they were not in contact with oak wood. Sampling was done before malolactic fermentation.
The samples were collected in early 2017, adjusted at 50 mg/L of free SO$_2$ and bottled using Select Green 500 synthetic closures (Nomacorc SA, Thimister Clermont, Belgium). The bottles were stored in the dark, at constant temperature (T = 14 °C), until the analysis in June 2018.

3. Terpenoids and norisoprenoids analysis

Terpenoids and norisoprenoids were analysed by the SPME-GC-MS method, following the procedure described by Luzzini et al. (2021). An amount of 5 µL of internal standard 2-octanol (4.2 mg/L in ethanol) was added to 5 mL of wine diluted with 5 mL of deionised water in a 20 mL glass vial. An amount of 3 g of NaCl was added prior to GC-MS analysis. Samples were equilibrated for 1 min at 40 °C. Subsequently, SPME extraction was performed using a 50/30 µm divinylbenzene–carbon–polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco, Bellafonte, PA, USA) exposed to sample headspace for 60 min. GC-MS analysis was carried out on an HP 7890A (Agilent Technologies) gas chromatograph coupled to a 5977B quadrupole mass spectrometer, equipped with a Gerstel MPS3 autosampler (Müllheim/Ruhr, Germany). Separation was performed using a DB-WAX UI capillary column (30 m × 0.25, 0.25 µm film thickness, Agilent Technologies) and helium (60.0 grade) was used as carrier gas at 1.2 mL/min of constant flow rate. GC oven was programmed as follows: started at 40 °C for 3 min, raised to 230 °C at 4 °C/min and maintained for 20 min. The mass spectrometer was operated in electron ionisation (EI) at 70 eV with ion source temperature at 250 °C and quadrupole temperature at 150 °C. Mass spectra were acquired in synchronous Scan (m/z 40-200) and SIM mode. Samples were analysed in random order. The calibration curve was prepared for each analyte using seven concentration points and three replicate solutions per point in red wines. An amount of 5 µL of internal standards 2-octanol (4.2 mg/L in ethanol) was added to each calibration solution, which was then submitted to SPME extraction and GC-MS analysis as described for the samples. Calibration curves were obtained using Chemstation software (Agilent Technologies, Inc.) by linear regression, plotting the response ratio (analyte peak area divided by internal standard peak area) against concentration ratio (added analyte concentration divided by internal standard concentration). Retention indices, quantitation and qualifying ions are reported in Table S1.

4. Statistical analysis

Statistical analysis was performed using XLSTAT 2017 (Addinsoft SARL, Paris, France). Shapiro-Wilk test was used to verify the normal distribution of the data. Due to the non-normal distribution, the Kruskal–Wallis test was used to determine statistical differences between wines with a significant level of α = 0.05. Dunn’s post-hoc test was performed using the statistical package XLSTAT 2020.1.3. (Addinsoft SARL, Paris, France). The hierarchical cluster analysis heatmap was performed using MetaboAnalyst v. 5.0 (http://www.metaboanalyst.ca, accessed on 15 January 2022), created at the University of Alberta, Edmonton, AB, Canada (Xia et al., 2015). After the uploading to the MetaboAnalyst platform, the dataset was scaled using the autoscaling option, and then we proceeded without any data transformation or missing values estimation. The selected parameters for the Heatmap plot were Ward for the clustering method and Euclidean distance for the distance measure. Different colours and gradients were associated with the scale and combination for each group

RESULTS AND DISCUSSION

A total of 16 monoterpenoids and 5 norisoprenoids were analysed in the 96 monovarietal wines (Table 1). Among the monoterpenoids analysed, on average, the most abundant was β-citronellol (7.81 µg/L), followed by linalool (5.26 µg/L), α-terpineol (4.91 µg/L), geraniol (3.85 µg/L) and terpinene-4-ol (2.29 µg/L). All other monoterpenoids were present at a concentration on average lower than 2 µg/L. Among norisoprenoids, TDN and vitispirane were the most abundant, with average values of 11.4 µg/L and 29.2 µg/L, respectively (the latter being ‘semi-quantitative’).

To identify compounds able to discriminate wines, a non-parametric test (Kruskal–Wallis) was used. All compounds showed a p-value < 0.0001 (Table 1) except nerol, with a p-value of 0.026 and α-terpinein showing a p-value of 0.001, indicating that the vast majority of the compounds analysed were significantly different in at least one wine type. The heatmap in Figure 1 provides an overview of how the samples were clustered according to their volatile profile. Despite a certain overlap, the samples of several varieties were clustered rather well with each other, highlighting the high variety-related diversity existing across Italian red wine appellations... This was particularly evident for Montepulciano, Raboso, Teroldego, Cannonau, Sangiovese di Romagna and Sangiovese di Toscana, which showed distinctive profiles of monoterpenoids and norisoprenoids. In detail, Montepulciano samples were clustered together, with two samples diverging and forming a group of their own. Cannonau wines were grouped together by forming the closest group to Montepulciano. This result is in agreement with LC-MS-based metabolomics analysis made on the same wine sample (Araptitas et al., 2020), where it was observed that Montepulciano and Cannonau wine samples were characterised by a higher amount of terpenes diglycosides. Raboso and Teroldego also formed distinctive clusters. Interestingly, Sangiovese wines were separated into two different groups based on their origin (Sangiovese from Romagna and Sangiovese from Tuscany), suggesting the existence of volatile patterns related to geographical origin.

Araptitas et al. (2020) reported relatively minor differences in non-volatile metabolomics profiles of Sangiovese wines of this same sample set according to geographical origin, while significant differences in astringency attributes were observed by Piombino et al. (2020). Canuti et al. (2019) observed differences in volatile profiles, including monoterpenoids and norisoprenoids, between Sangiovese wines produced in California and Italy. A cluster containing the majority of Primitivo and Nebbiolo samples was also formed, indicating
similarities in the terpenoids and norisoprenoids profiles of these two wine types.

Volatile compounds that were mostly associated with differences are further detailed in Figures 2–6.

The cluster containing Montepulciano samples was characterised by a high level of 1,4-cineole (Figure 2), α-phellandrene, α-terpinene, terpinolene (Figure 3), terpinene-4-ol, terpinene-1-ol (Figure 4), p-cymene and α-terpineol. Two Montepulciano samples were particularly rich in 1,8-cineole, γ-terpinene, limonene and 3-carene. The bicyclic terpenoids 1,8-cineole and 1,4-cineole were reported to contribute, respectively, to the eucalyptus/mint odours (Hervé et al., 2003) and hay, dried herbs and blackcurrant aromas (Antalick et al., 2015). Slaghenaufi and Ugliano (2018) reported that 1,8-cineole and 1,4-cineole could be formed by acid hydrolysis during wine ageing; however, they could also be found in young wines generally at lower concentrations. From this point of view, the concentrations of 1,4-cineole found in Montepulciano samples are quite high considering that the wines analysed were not aged, suggesting that this compound could be a good marker of Montepulciano. Furthermore, 1,4-cineole could significantly contribute to the aroma of Montepulciano wines, as in the analysed samples, the concentrations systematically exceed the olfactory threshold of 0.66 µg/L (Antalick et al., 2015). As shown in Figure 2, also Raboso samples were characterised by a high concentration of 1,4-cineole, above the odour threshold, whereas Sangiovese showed an average content of 0.40 µg/L, close to the odour threshold, with three samples exceeding the threshold. Even for Sangiovese and Raboso, this could be a varietal marker and play a role in the aroma of these wines. Interestingly, for Montepulciano and Raboso of this same set a high relative contribution of the unripe astrignency sub-quality was highlighted (Piombino et al., 2020) and Montepulciano also showed a correlation with vegetal odours (Pittari et al., 2020).

The cluster formed by Cannonau samples was close to the Montepulciano cluster, showing relatively high levels of nerol, terpinolene, β-citronellol, α-terpineol and limonene, but also of the norisoprenoids β-damascenone, α-ionone, TPB, TDN and vitispirane (Figure 5).

A large group containing the majority of Aglianico, Corvina, Nebbiolo Sangiovese di Toscana and Primitivo samples were characterised by a high content of linear monoterpenoid alcohols like linalool and geraniol (Figure 6). Corvina was confirmed to be characterised on average by a higher content of linalool compared to other red wines (Slaghenaufi et al., 2019; Slaghenaufi et al., 2021). Linalool and geraniol have an odour threshold of 25 (Francis and Newton, 2005) and 30 µg/L (Sánchez-Palomo et al., 2017), respectively, and both can contribute to the floral odour notes of wines. In the present dataset, linalool, geraniol and also citronellol varied largely across different samples, suggesting the existence of multiple factors affecting their concentration in red wines. Although in no case, the concentrations detected attained the odour threshold of individual compounds, floral odour attributes have been often mentioned in relationship to monovarietal Italian red wines (Moio, 2016), which might be due to the additive effect between odorants like terpenes (Ferreira et al., 2021).

Nerello, a minor variety from Sicily, showed a terpene profile with the highest average concentration of β-citronellol, a linear monoterpenol whose odour is reminiscent of citrus fruits and

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β-Citronellol is mainly formed in wine by the reduction of geraniol catalysed by yeast during alcoholic fermentation (Ugliano et al., 2006; Slaghenaufi et al., 2020b). Nerello was also characterised by a higher level of TPB (Figure 5), an ageing-related norisoprenoid reported to contribute to tobacco aroma notes in wine (Janusz et al., 2003; Slaghenaufi and Ugliano, 2018). It has been previously suggested that TPB should be absent in red wines due to its reactivity towards polyphenols; however, recent works on wines from Corvina grapes suggested that TPB was also present in red wines (Slaghenaufi and Ugliano, 2018; Slaghenaufi et al., 2020a). The results of the present work are further confirmation of the presence of TPB in red wines of all the varieties studied.

As for the differences between the two Sangiovese groups, these could be mostly ascribed to the different relative proportions of two terpinene isomers, namely α-terpinene and γ-terpinene (Figure 3).

Although most terpenes did not attain above-threshold levels, recent interest concerning the importance of terpenes to red wine aroma pointed out that terpenes have a moderately low odour threshold; for example, the monoterpenoid alcohols linalool and geraniol can act as precursors to more potent
aromatic terpenes. Slaghenaufi and Ugliano (2018) indicated that the potent mint, balsamic, hay-like cyclic terpenes 1,4- and 1,8-cineoles could originate from several different terpene compounds under wine mild acidic conditions. Exploring the relationship existing in the present dataset between cineoles and various possible precursors, a strong correlation ($R^2 = 0.809$) was observed between terpinen-1-ol and 1,4-cineole (Figure 7). We hypothesise that, in a wine acid environment, terpinene-1-ol could form a tertiary carbocation promoting then ring closure via ether-bound formation, leading to 1,4-cineole (Figure 8). The reaction may occur similarly to the reaction involving terpinene-4-ol as starting precursor (Slaghenaufi and Ugliano, 2018). 1,4-Cineole was also correlated with p-cymene ($R^2 = 0.657$), possibly because both compounds share various intermediates in their chemical formation from monoterpenes, including compounds such as terpinen-4-ol, α-terpinene and terpinolene (Slaghenaufi and Ugliano 2018). The complex dynamic equilibrium involving monoterpenoids made it complicated to find other significant linear correlations; however, further possible chemical relationships among compounds cannot be excluded and should be studied with a specific experiment.

Finally, a correlation was found between vitispirane and TDN ($R^2 = 0.636$), probably due to common precursors and biochemical conditions of formation (Knapp et al., 2001; Kwasniewski et al., 2010).
CONCLUSION

Significant differences among wines of different varietal origin and produced in a single vintage were observed in relation to their terpenoid and norisoprenoid content. Compounds such as 1,4-cineole and 1,8-cineole, linalool, β-citronellol and β-damascenone could be associated with specific clusters of wine categories, whereas the content of γ-terpinene and α-terpinene distinguished Sangiovese from two different geographical areas. Altogether, these results provide a first survey of the levels of various varietal volatile terpenes and norisoprenoids present in red wines of major Italian appellations, providing useful information for the development of dedicated viticultural and winemaking production approaches. Considering that some of the compounds with great discriminating capabilities are also affected by fermentation and ageing, the influence of these variables deserves further investigation. To this point, the data reported herein indicate a strong correlation between terpinen-1-ol and 1,4-cineole, suggesting the existence of a carbocation-mediated reaction mechanism leading to the formation of the bicyclic terpene. Such a mechanism would be relevant to aroma evolution during prolonged ageing, which will need to be investigated, especially in the context of wine types that were particularly associated with 1,4-cineole, such as Montepulciano.
FIGURE 8. Proposed 1,4-cineole formation pathway from terpinen-1-ol.

The data pool presented in this study represents a first attempt to characterise the varietal volatile profile of the large Italian wine heritage. Considering that terpenes and norisoprenoids are strongly impacted by viticultural conditions (e.g., clone, bunch sun exposure, irrigation) as well as by oenological variables (e.g., maceration conditions, yeast strain, fermentation nutrients), the varietal profiles highlighted in this study will provide several clues to develop dedicated viticultural and oenological strategies. This is of great importance not only for the expression of varietal and terroir factors that are highly sought after by consumers but also in the current context of climate change, requiring extensive revisitation of consolidated production practices.

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