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Titolo della tesi

"Metabolic modifications occurring during maturation in Ribolla Gialla grapes, and implication of vineyard site, cluster thinning, and harvest time on the composition and the sensory evaluation of sparkling wines"

in co-tutela con Fondazione Edmund Mach

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ABSTRACT

Ribolla Gialla (*Vitis vinifera* L.) is an indigenous white grape variety cultivated in northeastern Italy, in Slovenia and on the Ionian Islands in Greece, where it is known as Rebula and Robola, respectively, and can be considered as one of the most promising for producing high-quality monovarietal sparkling wines. However, due to its neutral aromatic potential, the manipulation of wine quality thus mainly depends on the application of viticultural or oenological practices. For instance, cluster thinning is a commonly adopted viticultural technique for selective removal of excessive clusters and allows calibrated vine productivity with increased accumulation of metabolites in the fruit. Modulating of wine grapes yield will therefore activate the intrinsic changes in basic berry composition (including soluble solids, organic acids, pH phenolics and anthocyanins), resulting from thinning treatment, while also changing the rate of grape ripening. Consequently, monitoring the ripening of the grapes is important mainly to determine the ideal harvest time. As this study shows, different harvest times have a significant impact on chemical composition of wines, which can lead to different sensory characteristics. The objective of this research work was therefore to produce a comprehensive study of discussed viticultural measures in the production of monovarietal Ribolla Gialla sparkling wine from two different locations. The position of vineyard site should not be neglected, as it is an integral part of the terroir, that can be defined as an ecosystem, in a given place, including factors like climatic conditions, cultivar and rootstock, geography and topography, as well as soil characteristics like mineral nutrition and water supply. For this purpose, the multi-targeted approach was adopted, using different analytical techniques (GC-MS, UPLC-MS, and FTIR) to investigate the aromatic characteristics of the sparkling wines obtained, including their volatile organic composition, lipid compounds, and the metabolites of aromatic amino acids, which play a key role in the organoleptic and sensory properties of wine. The findings of this study could provide a sort of guideline, intended for winegrowers and professional experts, designed to facilitate the decision about the level of production, or to help out determine the optimal harvest time, by taking into account changing climatic conditions.

GENERAL INTRODUCTION

1 ABOUT RIBOLLA GIALLA

The *Vitis vinifera* L. cv. Ribolla Gialla is an autochthonous white grape variety grown most prominently in the northeastern Italy. According to the literature, the first mentions of this ancient variety date back in 1409 (Peterlunger et al., 2004), with later recorded ampelographic descriptions being made in 1844 (Vertovec, 1844). Records shows that in 1982, there were only 93 ha cultivated with Ribolla Gialla in Italy, but nowadays, this area has increased to 1,159 ha, due to the strong demand for highly appreciated sparkling wine. This is also the reason why this variety is considered one of the economically relevant local cultivars in Italian region Friuli Venezia Giulia (Crespan et al., 2020).

Figure 1: The cluster of Ribolla Gialla (adopted from http://ribollagialla.org/).

There is a considered amount of literature, investigating the genetic origin of Ribolla Gialla and its genetic associations with Rebula and Robola, traditionally cultivated in southwestern Slovenia and Kefalonia Island in Greece, respectively (De Lorenzis et al., 2013). In addition, sympatric relationships with other cultivars (e.g., Gouais Blanc, Schiava and Glera) have been studied previously, suggesting a common geographical origin (Crespan et al., 2020; Imazio et al., 2016; Rusjan et al., 2010). However, only a small number of studies focused on compositional characterization of grapes and wines produced from Ribolla Gialla. Due to the low concentrations of grape-derived aroma compounds, it is classified by some authors among a neutral varieties (Bavčar et al., 2011). Namely, based on total free monoterpene concentration, the grape cultivars can be divided into three general groupings: neutral varieties with very low concentrations, aromatic cultivars with 1–4 mg/L

of monoterpenes and Muscat types with more than 6 mg/L of free monoterpenes (Lin et al., 2019; Mateo & Jiménez, 2000). Since monoterpenes are mainly linked to sugar moieties in the grape juice and wines without any olfactive activity, the acidic or enzymatic hydrolysis of terpene glycosides can occur when certain winemaking techniques are applied. One of such is for example the use of skin contact of crushed grapes under controlled condition, which allows the enhancement of both free and glycosided forms of volatile compounds, deriving from grapes in must and wines. Thus, in the work of Bavčar et al. (2011), the authors have investigated the effect of pomace maceration and whole berry maceration during alcoholic fermentation, on the concentration of free volatile varietal and fermentative aroma compounds from Ribolla Gialla. Similarly, Bavčar et al. (2016) exploited the impact of the freezing of pomace before pressing and the freezing of whole grapes before pressing on free aromatic compounds and sensory attributes. Another way to increase the complexity of Ribolla Gialla wine and thus avoid a rather poor beverage bouquet is to perform a mixed-culture fermentation. A study of such an approach has been conducted by Dashko et al. (2015), where the fermentative performance of non-conventional yeast strains has been compared to the one of *Saccharomyces cerevisiae*. However, none of the aforementioned studies examined the influence of viticultural practices on the chemical composition of Ribolla Gialla wines.

2 GRAPE BERRY DEVELOPMENT AND RIPENING

The fruit quality characteristics and quality attributes of grape juice and, subsequently, wine, depend on physical and chemical composition of grape berries at harvest. Namely, as berries ripen, they undergo a multitude of physical and chemical changes, although many changes and processes important to fruit quality also occur long before ripening begins (Keller, 2010). In general, berry development consists of two successive sigmoidal growth periods, interrupted by a lag phase, as presented on the Figure 2. Therefore, for ease of description, the fruit development will be divided into three stages: herbaceous phase (I), lag phase (II) and ripening (III).

2.1 HERBACEOUS PHASE

The first stage (stage I) starts at bloom and terminates after approximately sixty days. During this stage, the berry is formed, and rapid cell division occurs, which causes an exponential increase in berry size. The seeds embryos starting to be produced as well. The accumulation of the principal solutes such as tartaric and malic acid arise mainly in skin and flesh, respectively, where tartaric acid appears to accumulate during the initial stages of berry development, and malic acid accumulates just prior the veraison (Kennedy, 2002). At the same time, hydroxycinnamic acids are synthesized in grapes, which is involved in browning reactions and is a precursor to volatile phenols, and also tannins from both skins and seeds are accumulated (Conde et al., 2007; Kuhn et al., 2014).

2.2 LAG PHASE

The lag transition stage (stage II) is distinguished by a pause in berry growth, during which however, seed embryos start to grow rapidly. The duration of this stage is specific to the cultivar and to the fluctuations of the temperatures (Smart & Sinclair, 1976); however its end correspond to the end of the herbaceous phase of the fruit (Conde et al., 2007). At the start of the lag stage, berries have reached at least half of their final size. This stage is also characterized by the beginning of the synthesis of anthocyanins, soluble flavonoid compounds that provide color to red grape varieties. Consequently, this is the stage, where the veraison takes place. Moreover, the sucrose, originating from the leaves, reaches the fruits through phloem followed by a hydrolytic process that forms glucose and fructose (Robinson & Davies, 2000). The process of softening coincides with the beginning of the sugar accumulation, and then continue to the ripening stage. Namely, softening occurs as a consequence of reduced mesocarp cell turgor, due to the gradual disassembly of the mesocarp cell walls (Keller, 2010).

2.3 RIPENING PHASE

Following the 5 to 10 day lag, the third stage of development (stage III) starts with veraison and involves important morphological and physiological changes, such as accelerated anthocyanin accumulation (in the colored grapes), enhanced accumulation of soluble solids, decreased acidity, turgor reduction and berry enlargement (Serrano et al., 2017). One of the compounds which content is significantly reduced during ripening stage is malic acid. During this stage malic acid is exported from vacuole and so used for respiration, while sugars are instead imported in vacuoles. This can be achieved via two pathways: in cytosol where NADP-malic enzyme regulates the malic acid or malate breakdown producing the pyruvate and CO_2, or in mitochondria, where a subsequent action of malate dehydrogenase degrade malate into oxaloacetate, or, alternatively, NAD-malic enzyme oxidize the malate to pyruvate (Conde et al., 2007). In addition, it has been found that the metabolism of malic acid can be correlated with the climate, as cool regions typically produce grapes with higher concentration of malic acid, while the grapes grown in warmer regions tend to have lower acidity. In contrast to the process of the malic acid breakdown, the level of tartaric acid usually remains almost constant after veraison (Conde et al., 2007).

The post-veraison period is characterized also by an intensive accumulation of hexoses in the vacuoles of mesocarp cells (Conde et al., 2007). At first, the production of sucrose occurs in the mature grapevine leaves via the photosynthetic carbon assimilation and is subsequently transported to the berries in the phloem. This is followed by a conversion of sucrose into glucose and fructose, with studies reporting two possible pathways of transformation. One pathway suggests that sucrose is transported into the vacuole where the cleavage to fructose and glucose is caused by an invertase, or alternatively, the sucrose is inverted by an invertase, present in the apoplast or in the cytoplasm and the resulting hexoses

are transported into the vacuole by membrane hexose transporters (Robinson & Davies, 2000). Subsequently, the grape berries tend to accumulate the glucose and fructose steadily during the course of stage III, whereby the ratio of both hexoses is roughly 1:1 in the most *V. vinifera* cultivars, while this ratio varies from 0.47 to 1.12 in wild species (Conde et al., 2007; Kuhn et al., 2014).

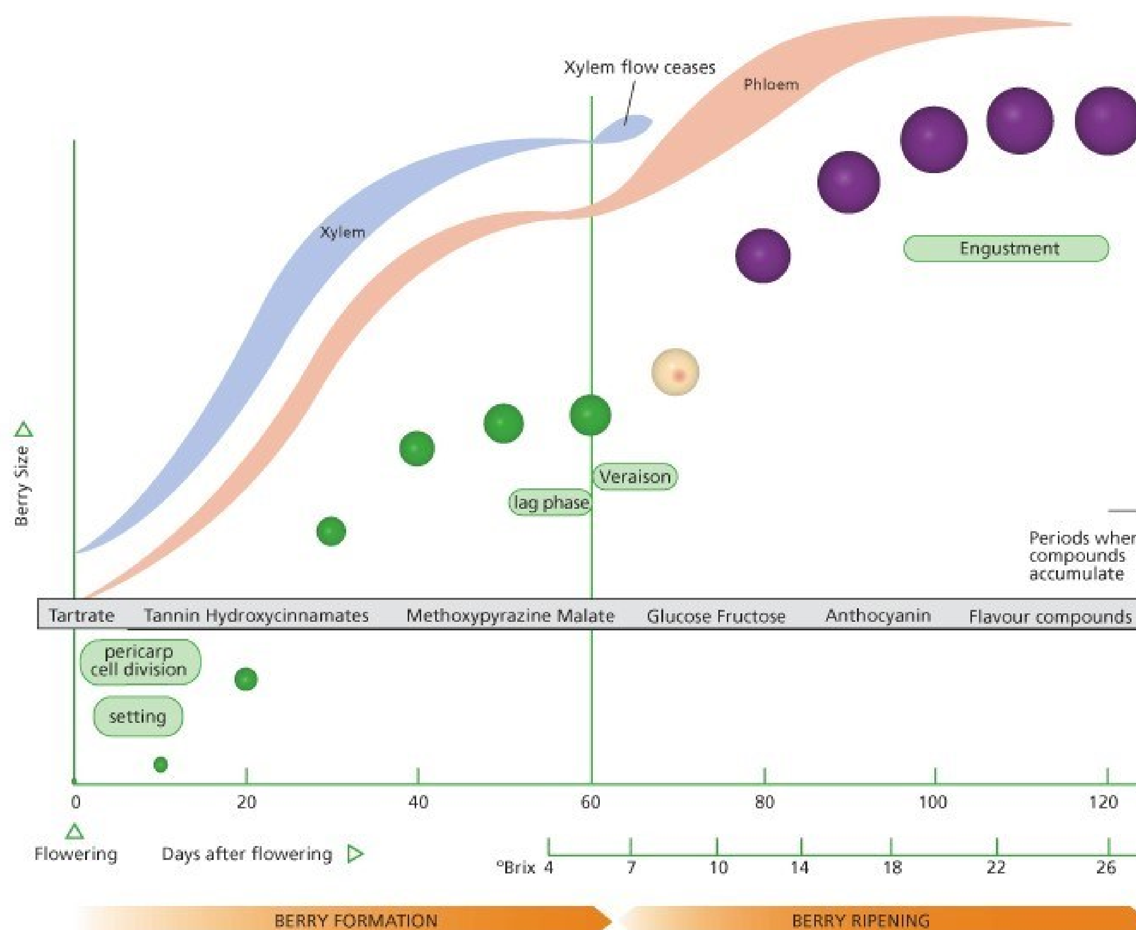

Figure 2: Grape berry development and ripening process (adopted from Kennedy, 2002).

3 PRIMARY AND SECONDARY METABOLITES IN GRAPES

In addition to the plants primary metabolites that are involved in normal growth, development and reproduction of plant species, the secondary metabolites are known to play crucial role in physiological functions, such as adaptation to environmental conditions (Lewinsohn et al., 2001), the enhancement of resistance to pests and disease related microorganisms (Harborne, 2001), and the determination of the quality of the food attributes (Ali et al., 2010). Thus, in this section, the development of volatile organic compounds (VOCs), lipids and metabolites of aromatic amino acids (AAA) in grapes is addressed, as these groups of substances were studied in depth as a part of our studies. Nevertheless, the

compounds derived from grape berries are important contributors to the sensory attributes of wine.

3.1 VOLATILE ORGANIC COMPOUNDS

Grape berries contain hundreds of compounds that could potentially contribute to the aromatic profile of wine. In the pulp and skin of the berries are located the most important grape aroma substances, such as monoterpenes, C13-norisoprenoids, benzenoid compounds and polyfunctional sulfured compounds, present in both free and bound form (Flamini & Traldi, 2010). During alcoholic fermentation, these compounds can be subjected to minimal or no transformation, thus reflecting a particularity of grape cultivar and emphasizing the quality and regional character of the wines (Conde et al., 2007).

3.1.1 Monoterpenes

Monoterpenes (C10 class of terpenes) are recognized as key odorants associated with the varietal (or primary) aromas of certain white wines. Their contribution to the floral, rose-like, coriander, green, citrus and herbaceous aroma is often related with the aromatic grapes, such as Muscat, Malvasia, Gewürztraminer and Riesling (Flamini & Traldi, 2010; Mateo & Jiménez, 2000), although they are also present at low concentrations in simple-flavored varieties (Conde et al., 2007). Among the free monoterpenes, the alcohols appear to be most prevalent, including linalool, geraniol, and nerol, together with the pyran and furan forms of the linalool oxides (Mateo & Jiménez, 2000). Depending on climatic conditions and the processing method of grape juice, to this group of compounds we can include also citronellol, α-terpineol, hotrienol, nerol oxide, myrcenol, the ocimenols and several other oxides, aldehydes and hydrocarbons (Conde et al., 2007). However, in the most cases the glycosidically conjugated forms prevail over the non-glycosylated forms of individual monoterpenes and polyols, despite the fact that they do not make direct contribution to the aroma of the grape (Mateo & Jiménez, 2000). These can be hydrolyzed into free forms by acids or enzymes (Williams et al., 1982). As regards the acidic hydrolysis, it has been shown to be strongly dependent on pH value, which can induce a molecular rearrangement of the monoterpenols (Maicas & Mateo, 2005). On the other hand, the enzymatic release of free aromatic compounds from natural glycoside precursors implies to more "natural" production of wine aroma and occurs in two successive steps: firstly, α-l-rhamnosidase, α-l-arabinosidase or β-d-apiosidase make the cleavage of the terminal sugar and rhamnose, arabinose or apiose and the corresponding β-d-glucosides are released; subsequently liberation of monoterpenols takes place after action of a β-d-glucosidase (Gunata et al., 1988; Mateo & Jiménez, 2000). Most previous studies have focused on investigating the accumulation of monoterpenes in the final stage of grape ripening, where it was mainly found that the levels of monoterpenes increase during grape ripening (Dunlevy et al., 2009). However, more recent results have showed that the level of production of terpenes varies from pre-veraison phase, through the lag phase and finally to the post-veraison phase (Kalua

& Boss, 2009; Yue et al., 2020). Thus, the total monoterpene content peaked in the first stage of grape berry formation in the Cabernet Sauvignon and Shiraz variety (Kalua & Boss, 2009; Zhang et al., 2016), while the maximum level of terpenes was reached at the intermediate-ripe stage in Macabeo, Airén, Chardonnay, and Fernão-Pires grapes (Coelho et al., 2007; Savoi et al., 2020). Consistent with the full ripeness of the grapes, the highest level of monoterpenes was found in Pinot noir and Bimeijia varieties (Fang & Qian, 2006; Yang et al., 2011). This suggest that the synthesis and accumulation of monoterpenes differ among grape cultivars, however, they can be also affected by agronomic practices, climatic conditions and water supply (Yue et al., 2020).

3.1.2 Norisoprenoids

The listed factors can influence the content of another group of chemical compounds, important for the aromatic profile of the wine – norisoprenoids. This family of compounds derives from oxidative degradation of carotenoids (C40) and consists of 18, 15, 13, 11, 10, 9 and 8 carbons, among which the C13-norisoprenoids have the greatest influence on the aromatic profile in grape berries. In contrast to the monoterpenes, the free forms of C13-norisoprenoids are present in small amounts, therefore non-volatile bound glycosylated conjugates predominate among them (Williams et al., 1992). Despite large quantity of C13-norisoprenoids identified in the grapes, a relatively small number of these proved to have a high odor impact. These include compounds such as β-damascenone, β-ionone, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), vitispiranes and actinidiols, which evoke rose-like, sweet, kerosene, resinous–eucalyptus–like and woody notes in the wine (Dunlevy et al., 2009; Flamini & Traldi, 2010). The production of these apocarotenoids is directly dependent on the amount of their respective carotenoid precursors formed during fruit development. However, although the carotenoids are largely accumulated before veraison, the production of the resulting norisoprenoids does not peak until late in ripening (Keller, 2010). Moreover, recent studies suggest that this trend varies between individual compounds. Yuan and Qian (2016) noticed that the increasing content of β-damascenone, TDN and vitispirane was in accordance with carotenoid breakdown of lutein, β-carotene, neochrome a and neoxanthin during Pinot noir grape berry development, whereas the changes for α-ionone and β-ionone were not obvious. In addition, norisoprenoids concentration may be also affected by cluster light exposure. Since the pre-veraison grape berries are more photosynthetically active, the sun exposure results in conversion of epoxyxanthophylls to de-epoxidized xanthophylls, which are considered the putative precursors of TDN and vitispirane (Figure 3). Greater exposure to the sun thus leads to higher proportion of de-epoxidized xanthophylls, which consequently leads to the different yield of C13-norisoprenoids in the post-veraison stage (Kwasniewski et al., 2010).

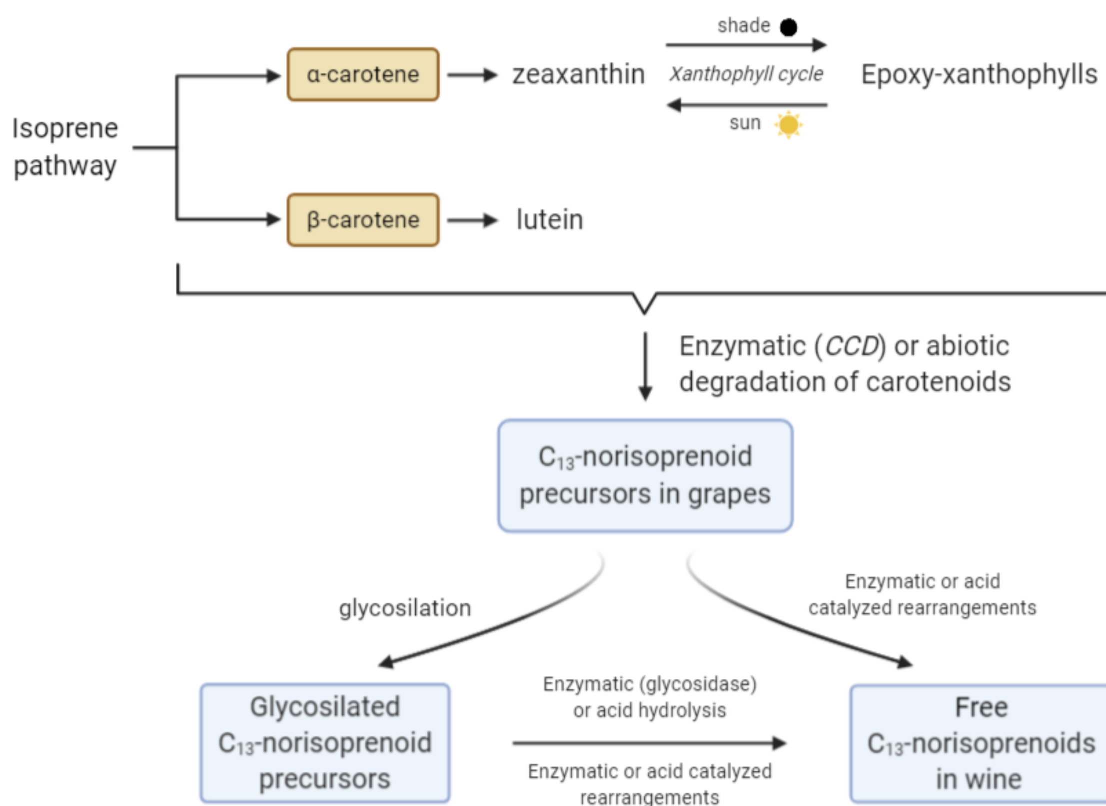

Figure 3: The biotransformation of carotenoids to C13-norisoprenoids according to the Baumes et al., 2006; Yuan & Qian, 2016.

3.1.3 LIPOXYGENASE COMPOUNDS

Other volatile compounds formed in grape berries are also aliphatic substances, that originate from fatty acid oxidation and amino acid degradation. C6 and C9 alcohols and aldehydes, derived from membrane lipids, formed via the aerobic activity of lipoxygenase (LOX) on polyunsaturated fatty acids (PUFAs). (Oliveira et al., 2006). This pathway is constituted of a series of enzymes that oxidize and cleave fatty acids to yield aldehydes, that can be subsequently reduced to alcohols and esterified (Dunlevy et al., 2009). In order to facilitate the production of C6 compounds, the free forms of linoleic and α-linolenic acids are released from phospholipids, fatty acyl-CoA esters, diglycerides and triglycerides, with acyl hydrolase. Released free fatty acids are then oxidized, forming hydroperoxides through the action of the LOX. Once the hydroperoxides are formed, the hydroperoxide lyase catalyzes the cleavage of fatty acid hydroperoxides – resulting in production of volatile hexanal, from hydroperoxide of linoleic acid, and *cis*-3-hexenal and *trans*-2-hexenal from hydroperoxide of α-linolenic acid (Oliveira et al., 2006). Isomerase can inter-convert the two hexenals and alcohol dehydrogenase can catalyze the reversible reduction of aliphatic aldehydes to alcohols (i.e., 1-hexanol, *cis*-3-hexenol and *trans*-2-hexenol). In general, the C6 alcohols and aldehydes contribute to the herbaceous and green characters to grapes and wine, while their associated acetate esters and ethyl *trans*-hex-3-enoate have floral and fruit

aroma descriptors. Alcohol acetyl transferase (AAT) catalyzes the formation of acetate esters through acetyl-CoA derivatives.

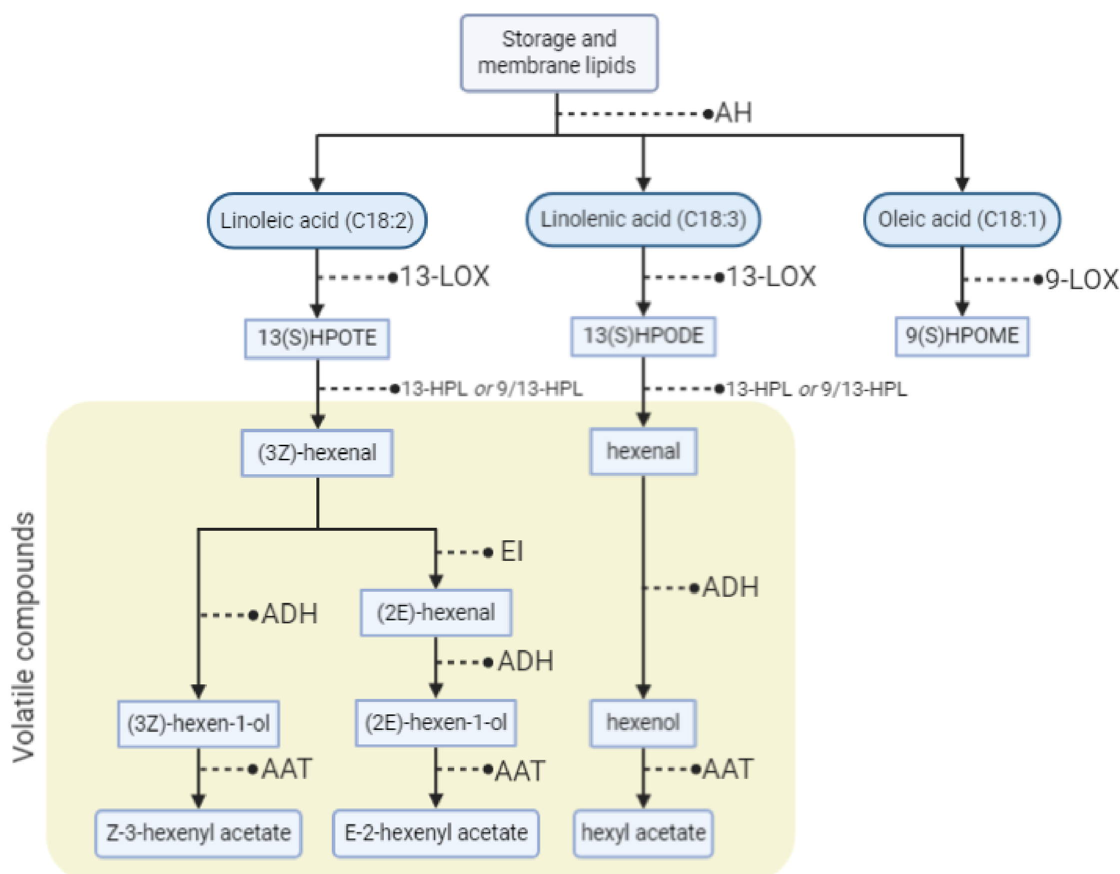

Figure 4: Biosynthesis of lipid derived volatile compounds in grapes (adapted from Dunlevy et al., 2009).

3.2 LIPID COMPOUNDS

Due to their biological functions, lipids are important constituents of plant cells and animal cells. They are essential molecules, since they possess functions to constitute cellular membranes in biological organisms that provide hydrophobic barriers to separate cellular compartments, serve as an optimal mix to facilitate transmembrane protein function, act as a source of precursors for lipid second messengers during signaling, and provide the storage and/or supplement of fuel for biological functions (Della Corte et al., 2015; Han, 2016; Kim, 2020). The plant membranes are composed mainly of lipids that possess a hydrophilic, polar head attached to a glycerol backbone and a hydrophobic tail, consisted of two fatty acids. The core building block of fatty acids is a hydrocarbon chain with a carboxyl group (-COOH) located on its terminal end. Based on the chain length of fatty acids, they are classified as short-chain (aliphatic tails of up to 5 or even 7 carbons), medium-chain (aliphatic tails of 6–8 up to 12–14 carbons), long-chain (aliphatic tails of 13–18 up to 22 carbons), or very long-chain fatty acids (aliphatic tails longer than 22 carbons; > C_{22}). Moreover, the division of

fatty acids can be additionally made according to the saturation of their aliphatic chains. In saturated fatty acids (SFA) all carbon-carbon linkages reveal single bonds, while in unsaturated fatty acids (UFA) some carbons are matched by one or more double bonds, in particular are the basic building blocks of more complex lipids (Reszczyńska & Hanaka, 2020).

The current classification determinates that the lipids can be classically divided in eight categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides (Figure 5). Moreover, each of these classes can be additionally subdivided into distinct classes and sub-classes (Fahy et al., 2009). Among the main classes of lipids, glycerolipids, sphingolipids and sterols appear to be the most important constituents of the plant membranes. Lipids are important organic compounds also in the grapes where their concentration ranges from 0.15% to 0.24% on the fresh weigh basis (Gallander & Peng, 1980). It is important to note that the lipid constitution is not evenly distributed in grape berry, being of minor importance in skins and pulp, while a higher concentration is present in the grape seeds. For instance, in *V. vinifera* L. cv. Cabernet Sauvignon grapes it has been shown that phospholipids were predominant in skins, and neutral lipids were predominate in seeds, while glucolipids, phospholipids and neutral lipids (e.g., cholesteryl esters, triglycerides, and fatty acids) were found in Condour grape skins and pulp (Higgins & Peng, 1976; Miele et al., 1993). Grape seed oil is additionally rich in unsaturated fatty acids such as linoleic and oleic acid. Due to its antioxidant properties is highly valuable for use in food, pharmaceutical and cosmetic industries (Sabir et al., 2012). In the grape skin, the major phytosterol is β-sitosterol, accounting 86–89% of the total phytosterols. It has been shown that the concentration of β-sitosterol decreases towards the end of grape ripening (Le Fur et al., 1994). In grape berries, the concentration of long-chain fatty acids appears to be predominant over the short-chain fatty acids (SCFAs), whereupon UFAs represent the major components of the total lipid fraction. The most abundant UFA is linoleic acid (C18:2), followed by oleic acid (C18:1) and linolenic acid (C18:3) (Pérez-Navarro et al., 2019; Santos et al., 2011).

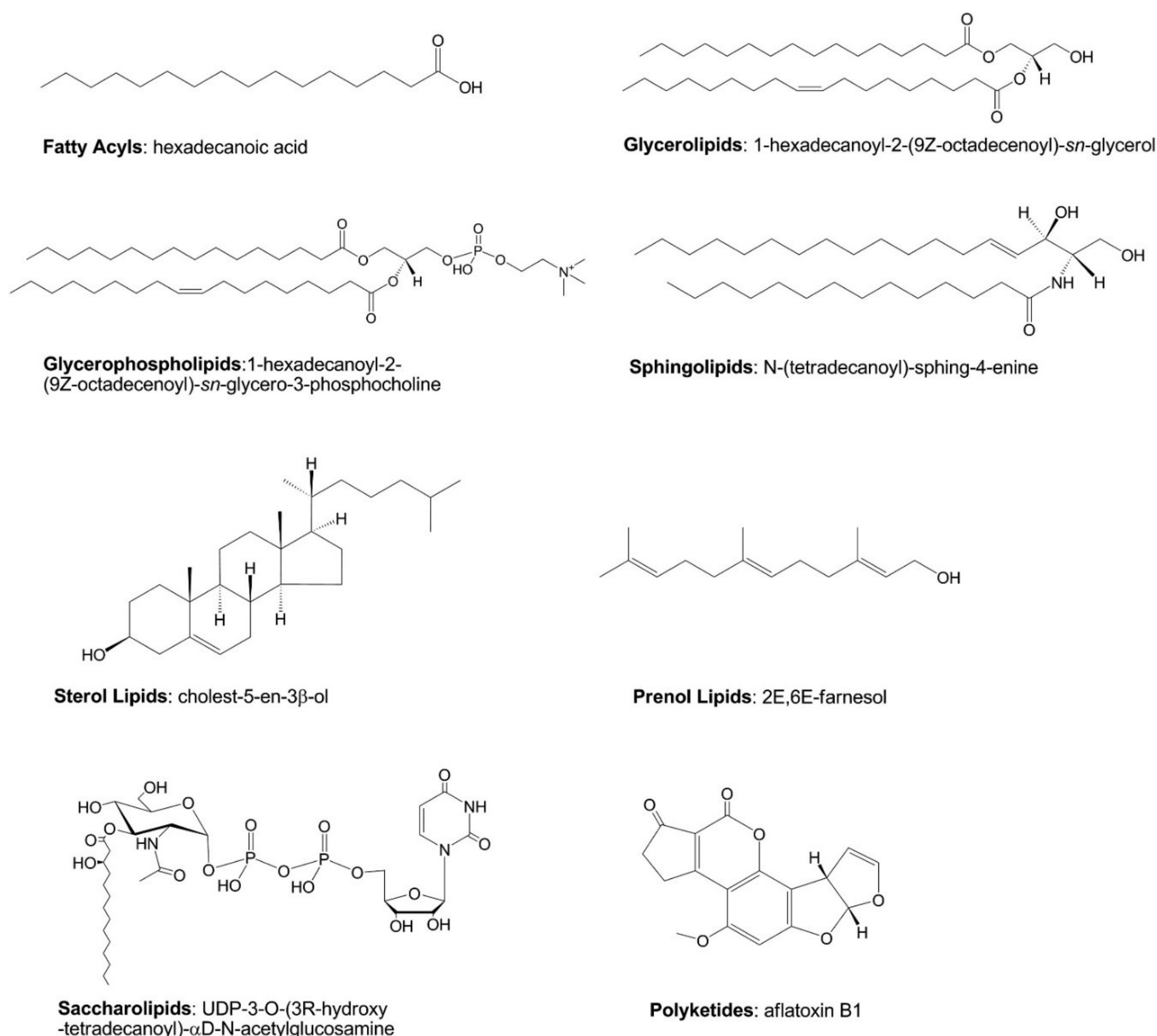

Figure 5: Examples of lipid categories with respective representative structures (adopted from Fahy et al., 2011).

3.2.1 Oenological significance of lipids

Grape lipids are essential important factors in oenology since they are considered essential nutrients whose availability is capable of modulating the yeast metabolism and thus affect the yeast alcoholic fermentation. They maintain the membrane build of yeast and promote their growth, mainly UFAs and sterols that increase the cell membrane fluidity (Pérez-Navarro et al., 2019). Sterols also allow yeast cells to withstand the increasing ethanol concentration during the alcohol fermentation, which decrease the risk of sluggish and stuck fermentations (Tesnière, 2019). Moreover, it has been proved that in the presence of ergosterol, the lag phase of the yeast growth was shortened. The compositional variation of lipids depends also on winemaking technology, which is of major importance in the case of white and rosé winemaking, where short contact with the grape skins can represent lipids as a limiting factor. However, the inability of *S. cerevisiae* cells to acquire complex lipids (i.e.,

phospholipids and glycerolipids) from the extracellular medium makes them highly dependent on exogenous sources of fatty acids. Free fatty acids are therefore cleaved from more complex lipid species through lipolytic activity (Dyer et al., 2002; Tumanov et al., 2015).

As it was previously described, the oxidation of polyunsaturated fatty acids α-linolenic acid and linoleic acid via lipoxygenase-hydroperoxide lyase pathway, leads to forming the C6 and C9 alcohols and aldehydes such as *trans*-2-hexenal, *trans*-2-nonenal and *cis*-2-hexenol that are related with green and herbaceous odors in wine (Ju et al., 2021). Additionally, increasing free fatty acids (FFAs) concentration through lipolysis of grape juice lipids, such as oleic acid and linoleic acid, resulted in a decrease of the concentration in the 3-mercaptohexyl acetate, a volatile thiol that is responsible for the distinctive tropical fruit notes in Sauvignon Blanc wines (Tumanov et al., 2018). Conversely, the excessive addition of linolenic acid in grape must leads to an increase in 4-methyl-4-mercaptopentan-2-one and 3-mercaptohexan-1-ol content, thus enhancing tropical fruit notes (Pinu et al., 2014). High phytosterol concentration are resulting in low acetic acid content, which could be related to variations in acetyl-CoA requirement for lipid synthesis, which is likely low in the presence of exogenous lipids such as phytosterols. In addition, phytosterol content had a similar effect on the production of isobutanol and isoamyl alcohol. Impairment of the activity of alcohol acetyltransferases (that convert higher alcohol molecules into the corresponding esters) likely explained this result, *ATF1* expression being repressed by lipids (Tesnière, 2019). Finally, the phytosterol content appears to be positively correlated with the final ethyl ester concentration: these volatile compounds being produced through lipid metabolism. Rollero et al. (2015) observed that the formation of ethyl esters seems more dependent on the availability of fatty acids, their precursors, than on the expression of those genes encoding acyl-coenzyme A/ethanol O transferases.

In addition to aroma, lipids play an important role also on other organoleptic properties of wine. The foam of a sparkling wine is a key parameter of its quality, and the main characteristic differentiating sparkling wines from the still wines. Foam properties, particularly foam height and foam stability, are significantly influenced by the chemical composition of wine, which include lipids. Among these, the relationship between fatty acids and foam properties is the most researched. Namely, the amphipathic properties of SCFAs, where one part of the molecules has an affinity for the nonpolar media (air interface) and the other part has an affinity for polar media such as water that is the main constituent of beverages, makes lipids a good candidates for involvement in foaming of carbonated beverages (Kemp et al., 2019). The addition of octanoic and decanoic fatty acids to wines had a negative effect on the foam stability time, but it positively influenced foam collar height (Maujean et al., 1990). However, in the study of Dussaud et al. (1994), the authors observed an adverse effect on bubble lifetime only when the alcohol concentration was below 5% of ethanol whereas at higher alcohol levels (11.3% v/v) the foam behavior was mainly governed by the ethanol. Additionally, linolenic acid and palmitic acid were,

respectively, the best indicators of foam stability and foam height in base wines and sparkling wines, respectively, both having a positive influence (Martínez-Lapuente et al., 2018; Pueyo et al., 1995). Moreover, certain studies have reported that not only FFAs have a significant effect on wine foaming, but also their esterified forms. Thus in a work on Cava sparkling wines, the free fatty acids C8, C10 and C12 resulted to be negatively correlated with foamability, while the esters of hexanoic, octanoic and decanoic acids expressed a positive association with foam formation (Gallart et al., 2002). It was also shown that monoacylglycerols of palmitic and stearic acids and glyceryl ethylene glycol fatty acid derivatives were surface active compounds, preferentially partitioned by the sparkling wine foam rather than the liquid phase, allowing the inference of their role as key components in the promotion and stabilization of sparkling wine foam (Coelho et al., 2011; Martínez-Lapuente et al., 2018).

3.3 AROMATIC AMINO ACIDS AND THEIR METABOLITES

Nitrogen-containing compounds are important sources of nutrients for yeast during alcohol fermentation, and the nitrogen content of grape must greatly impacts fermentation rate (Keller, 2010). The majority (50–90%) of the nitrogen is present in the form of free amino acids, and the rest is made up mostly of proteins, ammonium, and nitrate. The transport of nitrogenous compounds into berry occurs already before veraison via xylem (in the form of glutamine and nitrate), but also after veraison via the phloem (mostly as glutamine) throughout ripening (Keller, 2010). Enzymatic activity of aminotransferases in the grape berry, converts glutamine into other amino acids. The most predominant amino acids in musts are proline and arginine which together account for 60–70% of the amino acids in mature grape berries, followed by valine and alanine (Bell & Henschke, 2005).

In addition to the predominant amino acids in the grape berry, L-phenylalanine (PHE), L-tyrosine (TYR) and L-tryptophan (TRP) are also of particular importance, since they are not only essential components of protein synthesis, but they also serve as precursor for a wide range of secondary metabolites that are important for plant growth, aromatic properties of wine as well as for human nutrition and health (Tzin & Galili, 2010). The listed amino acids are also defined as aromatic amino acids (AAA). The AAA pathway consist of the shikimate pathway, where chorismate plays a role of a common precursor compound, and individual post-chorismate pathways leading to the formation of PHE, TYR and TRP (Maeda & Dudareva, 2012). These pathways are found in bacteria, fungi, plants, and some protists but are absent in animals, therefore, the AAAs metabolism is vital for many biological functions of higher importance also for human. For instance, TRP can be metabolized to the neurotransmitter serotonin (SER) (Hikosaka, 2010), the neurohormone melatonin (MEL) (Hikosaka, 2010), and the sleep regulator tryptophol (TOL) (Feldstein et al., 1970). TYR can be converted by lactic bacteria into another neurotransmitter tyramine that can cause an increased heart rate and higher blood pressure (Keller, 2010).

In wine, most of the amino acids do not exhibit a direct effect on the aromatic profile, as their concentration is usually 1–2 order of magnitude below the taste threshold (Waterhouse et al., 2016). However, proline and glutamate are amino acids, whose concentrations are high enough to approach their threshold, and at the same time impart the sweet and umami taste, respectively (Keller, 2010). However, the compounds that are synthesized because of amino acid metabolism of yeast, can significantly affect the organoleptic properties of wine. Depending on the order in which they are consumed, the sources of nitrogen in alcoholic fermentation are classified into three groups: lysine is defined as prematurely consumed, PHE belongs to the early consumed group, while TYR and TRP belong to the late consumed group of amino acids (Álvarez-Fernández et al., 2019). Via the Ehrlich catabolic pathway, the amino acids TRP, PHE and TYR produce the aromatic higher alcohols TOL, phenylethanol, and tyrosol (TYL), respectively. However, excessive concentration of these higher alcohols can result in a strong, pungent smell and taste, whereas lower levels contribute to the wines flowery character (Swiegers et al., 2005). In addition, TRP pathway in yeast also enables a direct transformation of the odorless metabolites into flavor-active compounds, such as methyl mercaptan and indole via tryptophanase (Álvarez-Fernández et al., 2019). Additionally, chemical reactions occurring during the wine aging process can indirectly affect the aromatic profile of the wine. Namely, TRP and its metabolites, especially kynurenine and indole-3-acetic acid (IAA) are considered to be potential precursors of an aroma compound, 2-aminoacetophenone (2-AAP), which occurs upon oxidative degradation of IAA. Consequently, the amount of 2-AAP in wine can be formed in wine after the sulfonation by co-oxidation of sulfite to sulfate (Christoph et al., 2000). A significant increase of 2-AAP may cause formation of "untypical aging off-flavor" (UTA), predominately in *V. vinifera* white wines, and this phenomenon is related with the development of a floor polish-like flavor (Engin, 2015). In addition, the IAA is also known as hormone auxin, usually associated with growth during the early stages of fruit development and later during the ripening. IAA concentrations have been claimed to be low and relatively constant throughout the berry development (Symons et al., 2006). On the other hand, PHE and TYR catabolism during fermentation produces the aromatic ester 2-phenyl acetate (related to rose, honey and flowery odors), phenyl acetic acid *p*-OH-phenyl acetic acid, phenyl pyruvate, and *p*-OH-phenyl-pyruvate (Styger et al., 2011). Despite the fact that aroma characters appear to be largely dependent on the yeast strain used during the fermentation and winemaking techniques, the actual differences in TRP and IAA concentrations of musts and wines, are attributed more upon the soil type, and therefore the terroir characteristics (Engin, 2015; Maslov et al., 2005). Lately, it has been demonstrated that the three amino acids and the metabolites related to the TRP metabolism can be helpful in the discrimination of monovarietal wines (Arapitsas et al., 2020).

4 VITICULTURAL PRACTICES AND SPARKLING WINE QUALITY

A plethora of factors such as variety, clone, planting density, pruning method, local climate and soil type play an important role in achieving optimal fruit quality at harvest of grapes destined for sparkling wine production (Jones et al., 2014). However, in contrast to the considerable amount of literature, outlining the relationship between vineyard management and still wine quality, much less has been researched in relation to sparkling wines (Pozo-Bayón et al., 2004). This is of particular importance, since sparkling wine production benefits from relatively low pH, high titratable acidity (TA), and low soluble sugars. These parameters are considered as the principal fruit quality criteria for determining harvest date and knowledge of these parameters leads to implementation of vineyard management strategies, which are essential for producing high-quality sparkling wine.

4.1 CLIMATE AND SOIL FACTORS

Vineyard location has been described as one of the key factors influencing the character of sparkling wines (de la Presa-Owens et al., 1998). Thus, the sugar accumulation and its composition can be strongly affected by environment or *terroir* that can be defined as an interactive cultivated ecosystem, in a given place, including climate, soil, water supply and the vine (van Leeuwen, 2010). Incidences of adverse weather conditions during the growing season can have disastrous results for the wine quality. For instance, due to excessive rainfall conditions prior the harvest, the vineyards can be exposed to the outbreaks of some economically important fungal diseases, such as downy mildew (*Plasmopara viticola*), ripe rot (*Colletotrichum gloeosporioides*), white rot (*C. petrakii*), black rot (*Guignardia bidwellii* f. *muscadinii*), brown spot (*Pseudocercospora vitis* (Lev)), and grey mold (*Botrytis cinerea*) (Du et al., 2015). In a recent study on sparkling wines from cool-climate Champagne region, it has been observed that under premise of guaranteed grape health, delaying harvest can improve base wine protein content and therefore the foamability of sparkling wines, despite the increased risk of developing various fungal diseases, associated with *B. cinerea* (Liu et al., 2018).

In addition to the precipitation, temperature is of paramount importance for the synthesis and accumulation of the grape metabolites. During the twentieth century, the global mean temperature increased by 0.89 °C, a phenomenon that led to a change in the base climate for the world’s main wine-growing regions. The impact of this change on viticulture is important reflecting the grapevine phenology, an increase in must alcoholic potential, and a decrease in total acidity, less predictable size and quality of grape yields, earlier ripening of the grapes with associated color and aroma profile alternations, and subsequently, the modification of well-known wine sensory profiles (Irimia et al., 2018). Under cool conditions, photosynthesis, sugar production and growth are often limited, while in hot regions, temperatures regularly exceed the photosynthetic optimum during a large part of the day. Moreover, the transport of assimilates via phloem is inhibited by prolonged

periods above 40 °C, probably due to temporary blockage of sieve plate pores by callose (Keller, 2010). However, studies have shown that sugar accumulation is influenced by temperature in the first two phases of the berry development (stage I and II), while during the stage III, the temperature has no major effect on it (Buttrose & Hale, 1971). Grape sugars appear to accumulate most rapidly in the ambient temperature range from 20–30 °C, whereby a sufficient soil moisture must be guaranteed and the residual nutritional factors are not limiting (Keller, 2010). It is generally considered that in warmer sparkling wines region, grapes destined for sparkling wines production need to be harvested earlier, in order to ensure low pH and high acidity (Zoecklein, 2002). Moreover, temperatures also decrease with latitude. For instance, in mountainous regions, Guyot (1997) reported that temperature decreases by 0.65 °C for every 100 m in altitude. However in a work on changes in Prosecco sparkling wine aroma profile at two different altitudes, it resulted that the highest altitude (380 m above sea level) was warmer than the lowest site (200 m above sea level) (Alessandrini et al., 2017). The higher site was also characterized by more abundant concentration of monoterpenes, such as geraniol, geranic acid, *cis*-8-hydroxy-linalool, linalool, and 7-hydroxy-geraniol, while the amount of C13-norisoprenoids was comparable in both vineyard sites, with the most representative compounds 3-oxo-α-ionol, and vomifoliol (Alessandrini et al., 2017).

In addition to meteorological parameters, the soil type, and its physical aspects, such as depth and texture can greatly affect vine vigor and subsequently the development of the fruits. In fact, the soil depth and clay fraction determine water supply and aeration (Jones et al., 2014; Tardaguila et al., 2011). In the study, where several table wines were considered, the results showed that the soil type can have a direct or indirect impact on berry growth, sugar accumulation, and anthocyanin concentration, and it was possible to distinguish between Merlot, Cabernet Sauvignon, and Cabernet franc. However, soil type appeared to be less important factor for total acidity and pH of the grape juice (van Leeuwen et al., 2004). The same authors also came to the conclusion that the effect of soil on vine development and berry composition can be explained by their influence on water status, since an early water deficit induced early shoot growth cessation and reduced berry size, which in turn led to increased sugar concentration and accelerated ripening and synthesis of anthocyanins (Cornelis van Leeuwen et al., 2004). Similarly, Coelho et al. (2009) studied the impact of three type of soils (clay-calcareous, sandy, and clayey) on the volatile profile of sparkling wines. The authors concluded that the wines produced from clay-calcareous and clayey soils resulted richer in terms of total volatiles followed by the wines from sandy soil. Monoterpenoids, sesquiterpenoids and C13-norisoprenoids were expressed in greater concentrations in wines from clay-calcareous soil. (Coelho et al., 2009). This could be due to the better capacity of water retention and volumetric wetness in clayey soils, compared to sandy soils.

4.2 VITICULTURAL AND VINEYARD MANAGEMENT PRACTICES

In order to modify fruit zone microclimates, such as sunlight exposure of the clusters, fruit zone temperature, and fruit zone air circulation, the basal defoliation can be considered as one of the most common viticulture management practices, used for improvement of wine aromas (Wang et al., 2018). Wolf et al. (1986) applied this technique on Chardonnay wines in New York, which led to increased amount of soluble solids and lowered concentration of TA. Therefore, the basal removal can be widely used to advance the berry ripening, especially in cool regions with high humidity and common rainfall, in order to increase the sunlight exposure and cluster temperature (Frioni et al., 2017). Moreover, Bubola et al. (2019) demonstrated that hand leaf removal significantly increased the concentration of varietal thiol 3-sulfanylhexan-1-ol, monoterpenes, β-damascenone and esters, which directly reflected in the more intense positive odor notes, such as fruity, floral, and tropical in Istrian Malvasia wines. Conversely, in New Zealand vineyards, the fruit zone leaf removal reduced TA, and the herbaceous/pyrazine-like character, while the level of terpene aroma volatiles increased in Sauvignon Blanc grape juices and wines (Smith et al., 1988). Namely, varietal aromas of wine mainly derive from grape and therefore are subjected to environmental factors. Timing of basal defoliation also plays an important role in the concentration of varietal aromas in wine. Wang et al. (2018) have shown that pre-veraison defoliation can induce an increase in β-damascenone and linalool as well as a reduction in 3-isobutyl-2-methoxypyrazine. The ripeness of the grapes is also related to this, as the grape maturity can influence the production of aliphatic alcohols and esters, which are considered as fermentative aromas (1-hexanol, β-phenylethanol, 2-phenylethyl acetate, decanoic acid, and ethyl octanoate) (Wang, He, Pan, et al., 2018). On the other hand, in the case of Pinot noir grapes it was found out that early leaf removal may represent an alternative to veraison application, especially because of some positive indications regarding color improvements (Lemut et al., 2013). Moreover, flavonols were found to respond significantly to leaf removal, while anthocyanins were affected to an intermediate extent (Lemut, et al., 2011).

The winegrowers often seek to maximize the crop level, which is concurrently adequate to obtain the targeted grape composition and wine quality. In response to the general belief that high crop loads can lead to inferior quality of grapes and wine (Reynolds, 2010), the cluster thinning has become a widely used method to control the crop load of vine by simply removing whole clusters after the berries have set to achieve desired goal (Rutan et al., 2018). It is also performed to equilibrate vines with excessive crop level in comparison to its vegetative capacity (such as in high-yielding cultivars; e.g., Sangiovese, Montepulciano, Trebbiano, etc.) (Frioni et al., 2017; Kliewer & Dokoozlian, 2005). Nevertheless, several studies have confirmed the positive impact of cluster thinning on grape and wine quality, where the authors were focusing primarily on the concentration of soluble solids and anthocyanin content (Guidoni, et al., 2002; King et al., 2015; Xi et al., 2018). In the case of high-quality sparkling wines as champagne, the yields are prescribed by the Appellation d'Origine Contrôlée, which determines whether the crop removal is necessary (Jones et al.,

2014). Similar measures are applied also for cava that is Spanish sparkling wine of high quality with Protected Designation of Origin (PDO) produced by the Champenoise method (Izquierdo-Llopart & Saurina, 2019; Pozo-Bayón et al., 2004). However, it is not clear, whether the lower yields of grapes, destined for the sparkling wine production actually leads to higher quality, since the low crop level causes accelerated ripening of the grapes, and decrease in titratable acidity with simultaneous increase in pH, leading to overripe fruit, which is enhanced by the warm conditions (Jones et al., 2014). Furthermore, the impact of reduced crop level was extensively studied on colored grapevine varieties in correlation with wine phenolic composition, while only a limited number of studies were devoted on exploring the impact of reduced yield on the aromatic composition of wines. Bubola et al. (2020) have shown that restricting the yield in vineyard at the expense of obtaining better quality of white wines led to the decrease of ethyl esters, presumably because of lower and more adequate vine vigor. On the other hand, crop level did not obtain a consistent effect on monoterpenes, as well as on C13-norisoprenoids, including β-damascenone (Bubola et al., 2020). Similarly, the amount of ethyl esters was higher in thinned samples in the study on Syrah cultivar, contributing a fruity sensory properties in wine samples (Condurso et al., 2016). Particularly, ethyl-2-methylbutanoate and ethyl-3-butanoate enhanced the desired strawberry-like aroma in Syrah wines (Condurso et al., 2016). Among the primary aromas, the monoterpenes and sesquiterpenes expressed higher amount in the samples after the cluster thinning. In addition, the study on Riesling icewines grapes found them to be responsive to cluster thinning (Bowen & Reynolds, 2015). However, certain studies reported that cluster thinning had a limited effect on the volatile composition of wines. The results of a recent study on Pinot gris, Riesling, Cabernet franc, and Cabernet Sauvignon showed that crop level had little impact on the sensory properties of these cultivars (Luna et al., 2017).

The fact that fruit removal is a costly exercise leads winegrowers to consider whether the crop load reduction at veraison produces a significant increase in fruit and sparkling wine quality to justify the cost (Jones et al., 2014). The amount of crop load removal is also related to this issue. Recommendations in Champagne region are that removal of 30% of the fruit will result in a less than 0.5% increase in potential alcohol, whereas removing from 30 to 50% could increase potential alcohol between 0.5 and 1.5% (Jones et al., 2014). As it was shown in malvasia samples, the wines obtained from low crop level expressed higher amount of certain C6 alcohols and volatile fatty acids, while the concentrations of esters were slightly increased in samples with higher crop level. Moreover, high cluster level vines were considered more balanced in terms of the yield to shoot growth ratio (Bubola et al., 2020). In another study, the authors compared the effect of cluster thinning versus berry thinning that reduced grape yield per vine by around 40% versus 20%, respectively (Gil et al., 2013; Sivilotti et al., 2020). Cluster thinning thus led to wines with a significantly higher ethanol content, increased anthocyanin and polysaccharide concentration, while berry thinning led to wines with significantly higher total polyphenol index, flavonol, and proanthocyanidin, and with lower TA (Gil et al., 2013).

AIM OF THE PhD PROJECT

Due to its limited area of cultivation in northeastern Italy throughout the history, the winemakers often blended Ribolla Gialla with other local varieties in the winemaking process. This also stems from the fact that Ribolla Gialla has been mostly planted on hillside vinayards with limiting yield, but nowadays the growing area with Ribolla Gialla is increasing, which is evidenced by the fact that from 1982 until today, the area planted with Ribolla Gialla has increased from 93 ha to 1,159 ha, due to the strong demand for highly appreciated sparkling wine. As a result, this variety has become one of the economically relevant local cultivars in Friuli Venezia Giulia region, which also meant an increase in research interest in this variety. However, to the best of our knowledge, no previous study has examined the chemical composition of sparkling wines, produced from Ribolla Gialla cv. On the other hand, only a handful of studies were dealing with the improvement of aromatic potential through various oenological processes in Ribolla Gialla still wines, but no previous work contributed to the current understanding of the influences of climate, environment, and viticultural practices on fruit quality, and how this affects the quality of sparkling wine. Therefore, the aim of this study was to investigate how various viticultural measures affect the chemical composition of grapes and wines from Ribolla Gialla variety.

This was achieved by performing four sub-studies discussing the following main topics each presented in a separate chapter:

- *Chapter 1*: chemical caracterization of thirty-three commercial sparkling Ribolla Gialla wines from different areas of Friuli Venezia Giulia region;
- *Chapter 2*: analysis of the volatile, non-volatile and sensory profile of sparkling wines produced after the application of cluster thinning treatment in two vineyard sites and in three harvest seasons;
- *Chapter 3*: optimal harvest time decision for Ribolla Gialla sparkling wines, based on chemical and sensory analysis;
- *Chapter 4*: monitoring of Ribolla Gialla grape ripening in different vintages and the impact of cluster thinning on the quality of grapes.

Therefore, the aim of this work is to characterize the chemical composition of grapes and wines from Ribolla Gialla variety, after the implementation of various viticultural measures.

1

COMPOSITIONAL CHARACTERIZATION OF COMMERCIAL SPARKLING WINES FROM cv. RIBOLLA GIALLA PRODUCED IN FRIULI VENEZIA GIULIA[1]

[1] This chapter has been reprinted* from:

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PREFACE

Ribolla Gialla (*Vitis vinifera* L. cv.) is an autochthonous white grape variety, whose production is limited mainly on the area between Northeastern region in Italy (Friuli Venezia Giulia) and western part of Slovenia (Brda). It is considered as one of the most economically relevant local grapevine cultivars; therefore, its cultivation is constantly increasing and is vastly used for the production of sparkling wines. The increased interest of growers in the production of wines from the Ribolla Gialla simultaneously led to increased research activities related to this variety. As a result, a number of studies have focused on exploring its genetic origins (Crespan et al., 2020; De Lorenzis et al., 2013; Rusjan et al., 2010), while the remaining studies were mostly concerned with addressing the improvement of the aromatic potential of still wines, using different enological procedures (Bavčar et al., 2016; Bavčar et al., 2011). However, none of these research works devoted to the more complex metabolic exploration of sparkling wines produced from the Ribolla Gialla variety. Thus, the work presented in the Chapter 1 aimed to thoroughly investigate the chemical composition of thirty-three Ribolla Gialla sparkling wines from different DOC (Denominazione di Origine Controllata) districts of Friuli Venezia Giulia region. This screening sought to determine the main characteristics of sampled wines, focusing on the basic chemical composition parameters, with an emphasis on multi-targeted analysis of volatile compounds, lipid content and the amount of aromatic amino acid metabolites present in the samples. The sum of all, significantly influences the development of positive or negative organoleptic properties of wine.

It has been previously reported that the wines produced from Ribolla Gialla are characterized by a relatively high content of esters, which contribute to fruity odor, but they lack grape-derived aroma compounds. Similarly, the terpenes in the present study did not appear to be particularly characterizing the aroma profile of sparkling wines, whereas the presence of volatile esters and β-damascenone proved to be the predominant. It has been accepted that esters contribute to the fruity aroma of young wines, since they are mainly enzymatically synthesized by yeast during the alcoholic fermentation. However, the amount of certain esters increases during aging process and their content can be also modulated by lactic acid bacteria during malolactic fermentation (Antalick et al., 2014). This process occurs during aging of wines on the lees after second fermentation. Namely, sparkling wines can be produced by different methods, whereby the base wine is left to undergo a second alcoholic fermentation in bottles (Traditional method) with prolonged contact with the lees or in stainless tanks (Charmat method) where the *sur lies* aging tends to be as short as possible. Although Ribolla Gialla is generally produced by the Charmat method with no contact with the lees after the completion of secondary fermentation, the appearance of aging esters and the altered ratio of malic acid and lactic acid possibly indicated the occurrence of malolactic fermentation in some samples.

Beside polysaccharides, proteins and tartrates, the wines lees are constituted also by yeast cells, resulting from autolysis that takes place during fermentation of sparkling wine and later on during the aging process. The autolytic activity of the yeast cells is manifested in increased release of intracellular substances that exert a major impact on the sensory characteristics of the wine. Lipids represent a wide class of compounds among these substances, although an important part of lipids is also contributed by the solid tissues of the grapes. Fatty acids composition, for instance, is considered to be highly variable, depending on the environmental factors as well as on the fermentation conditions, where they may be present in free or bound form as a result of ethyl esterification (Tesnière, 2019). Fatty acid ethyl esters can therefore directly contribute to the flavor of wine, while the ratio of saturated and unsaturated free fatty acids affects the development of “greenish” C6 aldehydes and alcohols. Moreover, together with the saturation degree, the length of the fatty acid chain also influences the effervescence and persistence of foam, which are differential attributes of sparkling wines and are therefore paramount in any assessment quality. Thus, the predominant amount of saturated fatty acids over unsaturated in Ribolla Gialla sparkling wines could favor a more stable foam.

The metabolomic analysis of commercial sparkling wines from Ribolla Gialla was completed with the adoption of targeted LC-MS based method for characterization of aromatic amino acid metabolites. When present in low quantities, the biosynthetic products of tryptophan can have a positive effect on the aromatic properties of wine, while excessive transformation of essential amino acids into higher alcohols via Ehrlich pathway is reflected in pungent smell and taste. Additionally, the sulfonation of indoles can catalyze their degradation and accelerates the formation of 2-aminoacetophenone, an aroma compound that causes an atypical aging off-flavor in *V. vinifera* wines (Hoenicke et al., 2002). A significant amount of these precursor compounds was detected in Ribolla Gialla sparkling wines, which could eventually lead to a deterioration of the aromatic profile during the aging phase.

The lack of information on qualitative characteristics of sparkling wines from Ribolla Gialla variety was therefore the main guide in the formation of this chapter. The findings of the presented study could thus form the basis of any further investigation related to the optimization of different practices in order to maximize the aromatic potential.

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ORIGINAL PAPER

Compositional characterization of commercial sparkling wines from cv. Ribolla Gialla produced in Friuli Venezia Giulia

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Abstract

Ribolla Gialla (RG) is a white grape variety used in the production of high-quality sparkling wines. It is cultivated in a limited area between Friuli Venezia Giulia (FVG—Northeast Italy) and Slovenia; for this reason, there is little information about the composition and chemical characteristics of the sparkling wines produced. This work used different analytical approaches (FTIR spectroscopy, UHPLC–MS/MS, liquid–liquid extraction, SPE and SPME–GC–MS) to characterize thirty-three commercial sparkling RG wines from different areas of FVG. The characteristics included the overall volatile profile and content of terpenes, C_{13}-norisoprenoids, lipids, and metabolites of aromatic amino acids. The aroma profile of RG wines was mainly characterized by fermentative esters and β-damascenone, whereas other norisoprenoids and varietal aromas were below the odor threshold. Appreciable amounts of certain fatty acids were found (e.g., palmitic acid), which could be potentially correlated with greater foam stability. However, high concentrations of aromatic amino acids metabolites highlighted a higher risk of developing atypical aging defects.

Keywords Ribolla Gialla · Sparkling wine · Volatile compounds · Aromatic amino acids metabolites · Lipids

Introduction

Ribolla Gialla is a promising white grape variety that has recently been used for the production of premium sparkling wines, which have received good appreciation in international wine markets. Ribolla has been cultivated since ancient times in Northeastern Italy (in the Friuli Venezia Giulia region), as well as Slovenia and the Ionian Islands (Kefalonia), where it is known as Rebula and Robola, respectively [1]. A certain number of studies have been carried out on the genetic identity and characterization of Ribolla grapes [1–4], but very few studies have dealt with the metabolomic profile of the wines [5, 6]. In addition, none of them have focused on the aromatic characteristics of sparkling wines obtained from Ribolla grapes, including their volatile organic compound (VOC) content, lipids, polyphenols, and metabolites of aromatic amino acids. All of these compounds are important because they may be linked with positive and negative aspects of wine quality.

VOCs are fundamental for determining the sensory characteristics of wine and its profile. These compounds are classified into four groups [7]: (i) primary grape VOCs, which are present in the cells of grapes; (ii) secondary grape aroma, which is formed during the pre-fermentation phases (crushing, pressing, and skin contact) or by thermal, chemical, and enzymatic reactions in must; (iii) fermentation bouquet, which includes aroma compounds that form during alcoholic fermentation; (iv) and maturation bouquet, which refers to the aroma compounds that develop when aging the wine. Primary aroma is particularly important for the production of certain wine typologies, such as white sparkling wines. Furthermore, different aroma compounds may be produced after fermentation and aging that further affect the aroma profile of the final wine, which are mainly due to pre-fermentative processes and yeast metabolism (e.g.,

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C6 compounds, norisoprenoids, fatty acids, alcohols, esters, carbonyl compounds) [8–11].

Moreover, yeast autolysis takes place during the aging of sparkling wine, which leads to the release of substances in the medium. Lipids represent a wide class of compounds among these substances [8]. Fatty acids may have a significant impact on the sensory properties of the wine. A large part of the fatty acids originates from the firm tissues of the grapes, but the greatest amount is formed during alcoholic fermentation. Therefore, fatty acids may be present in wine in free or bound forms as ethyl esters. Both forms directly contribute to the flavor of the wine, while unsaturated fatty acids such as oleic, linolenic, and linoleic acids act as precursors of C6 aldehydes and alcohols with herbaceous notes, and are critical for yeast growth during fermentation [12]. Another part of the sensory assessment of sparkling wine is the relationship between the foaming properties and lipid compounds. Therefore, it has been reported that medium-chain free fatty acids C8, C10, and C12 are negatively correlated with foamability, while the ethyl esters of hexanoic, octanoic, and decanoic acids have a positive effect on the formation and stability of the foam [13].

One of the essential aromatic amino acids, tryptophan (TRP) and its metabolites, especially indole-3-acetic acid (IAA), are considered to be potential precursors of 2-aminoacetophenone (2-AAP), which is an aroma compound that causes an atypical aging off-flavor (ATA) in *Vitis vinifera* wines. The off-flavor is described with aroma descriptors such as "acacia blossom," "furniture polish," "wet wool," "mothballs," or "fusel alcohol," which are combined with a loss of the typical bouquet of the grape variety [14]. Depending on the wine matrix, the detection threshold of 2-AAP varies from 0.5 to 1.5 μg L^{-1}. Strongly aromatic wines are able to integrate more than 1.5 μg L^{-1} of 2-AAP, while those characterized by poorer aroma profile might be rejected as tainted by ATA with less than 0.5 μg L^{-1}. It is generally accepted that the ultimate cause of ATA development in white wines is a stress reaction in the vineyard triggered by drought, nutritional deficiency, and other viticultural factors, such as the time of harvest and leaf removal [14, 15].

Most papers on sparkling wines focus on the characterization of internationally known varieties with a worldwide distribution [16–19], but there are few studies on local or less relevant cultivars. Therefore, the aim of this work was to investigate the composition of Ribolla Gialla sparkling wines produced in different DOC (Denominazione di Origine Controllata) districts of Friuli Venezia Giulia region (Northeast Italy), to identify the most characterizing compositional traits, and tracing a common compositional profile for Ribolla wines. The wines were characterized for their chemical composition (pH, titratable acidity, residual sugars and alcoholic strength), volatile profile (overall profile of aroma compounds, free and bound terpenes, and norisoprenoids), and content of lipids and metabolites of aromatic amino acids. The results are critically discussed with the aim of defining a common metabolomic profile of Ribolla Gialla sparkling wines.

Materials and methods

Reagents and materials

Hydrogen peroxide (30% w/w), ethanol (96% v/v), ACS-grade hydrochloric acid (37%), anhydrous sodium sulfate and citric acid were purchased from Carlo Erba Reagents (Milan, Italy). Sodium chloride (99.5%) was obtained from Honeywell Fluka (Morris Plains, New Jersey). HPLC-grade solvents dichloromethane, *n*-pentane, and methanol, LC–MS-grade methanol, acetonitrile, 2-propanol, chloroform, formic acid, ammonium formate, ethyl heptanoate, 1-heptanol, 2-octanol, ethyl hexanoate-d_{11}, 3-(2-hydroxy ethyl)-indole, kynurenic acid, D-tryptophan methyl ester, L-tyrosine-ethyl ester, *N*-acetyl tyrosine-ethyl ester, and 3,5-di-*tert*-4-butylhydroxytoluene (BHT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). C_7–C_{30} *n*-alkane solution in *n*-hexane was purchased from Supelco (Bellefonte, PA, USA), while cholesterol-d_7 and octadecanoic acid-d_3 were obtained from CDN Isotopes (Quebec, Canada). The chemical standards used for the determination of aromatic amino acids metabolites and lipid molecules were purchased from Aldrich-Fluka-Sigma S.r.l. (Milan, Italy), except for tryptophol-2-sulfonate (TOL-SO_3H), indole-lactic acid-2-sulfonate (ILA-SO_3H), and indole-acetic acid-2-sulfonate (IAA-SO_3H) that were synthesized as described by Arapitsas et al. [23]. Finally, the glycosidase preparation (Rapidase Revelation Aroma) used for the determination of bound monoterpenes was obtained from Oenobrands (Montellier, France).

Wine samples

Local wineries located in the Northeast Italian region of Friuli Venezia Giulia provided thirty-three commercial Ribolla Gialla sparkling wines, representative of the regional production. The locations of the wineries are shown in Fig. 1. For all samples, one single bottle was provided.

Basic analysis of wine samples

The basic quality control parameters (reducing sugars, alcoholic strength, total acidity, volatile acidity, pH, malic, lactic and tartaric acid) were determined by FTIR spectroscopy with a Winescan™ FT-120 instrument (FOSS, Hillerød, Denmark). Wines were pre-filtered by vacuum filtration on Whatman 1 filter paper (purchased from Sigma-Aldrich, St.

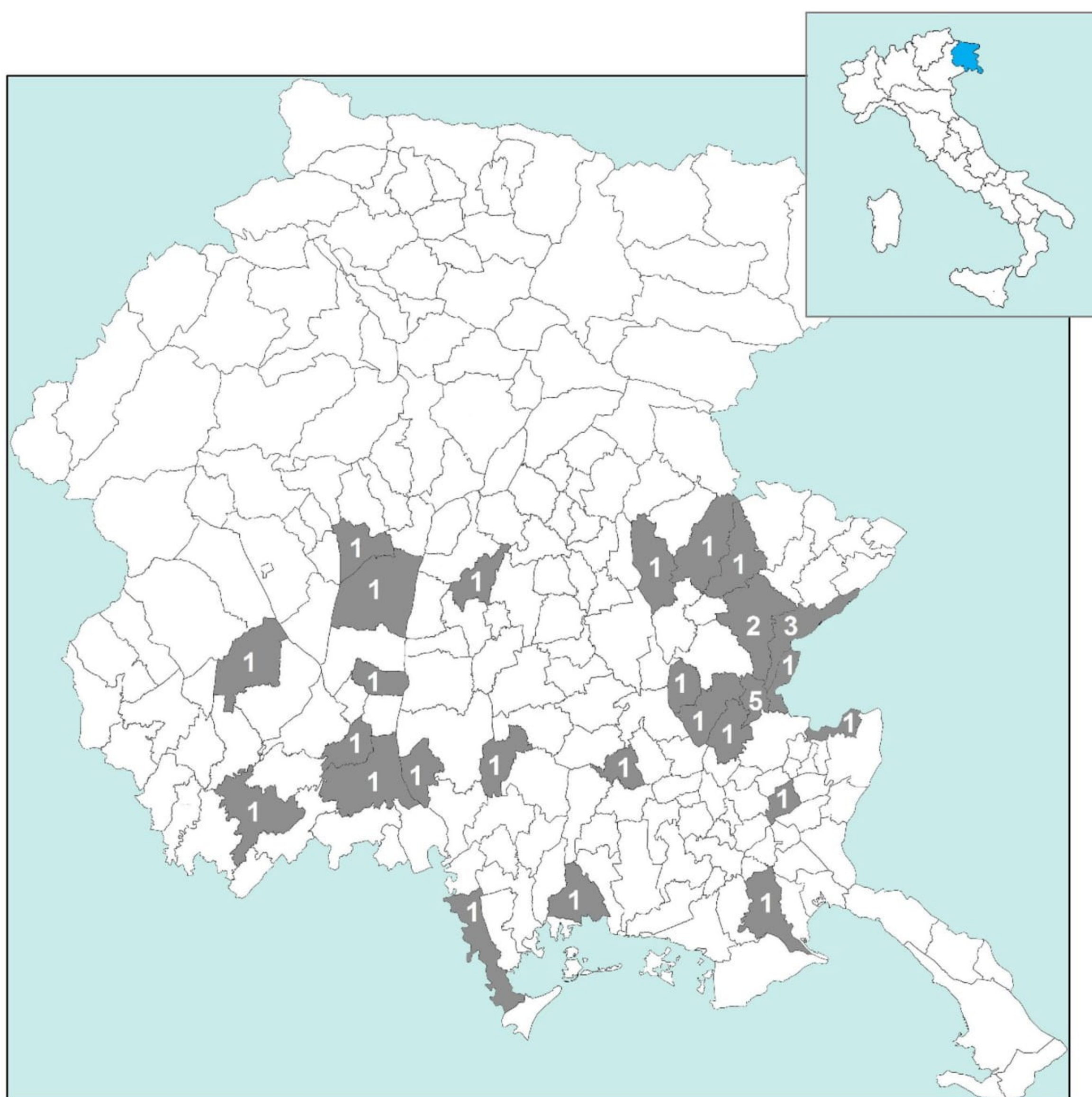

Fig. 1 Geographical position of Friuli Venezia Giulia region. Gray areas mark the municipalities in which the wineries that supplied wine samples are located. The number of samples/wineries for each municipality is also reported

Louis, MO, USA) to eliminate carbon dioxide. All of the samples were analyzed two times, and the mean value of the two measurements was considered for the data analysis.

Determination of volatile compounds

The volatile compounds in the commercial wines were determined using three different extraction methods: liquid–liquid extraction (LLE), solid-phase extraction (SPE) and solid-phase microextraction (SPME). Samples were degassed before analysis by placing them in an ultrasonic bath (Falc Labsonic, Treviglio, Italy) for 2 min.

LLE–GC–MS

LLE–GC–MS was used for analyzing non-varietal aromas. Volatile compounds were extracted as reported by Loira et al. [20] by mixing 5 mL of wine with 5 mL of a 30%

(w/v) sodium chloride solution and 200 µL of internal standard (ethyl heptanoate, 422 mg L^{-1} in 96% v/v ethanol). The mixture was subjected to three extractions using 2.5 mL of pentane:dichloromethane (2:1 v/v) each. The organic phase was collected in a Pyrex tube, dehydrated with anhydrous sodium sulfate, and concentrated under nitrogen flow to a final volume of about 1 mL.

Extracts were analyzed on a GCMS-QP-2010 system (Shimadzu, Kyoto, Japan). Volatile compounds were separated on a J&W DB-Wax capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) provided by Agilent Technologies Inc. (Santa Clara, CA, USA) using the operating conditions described by Loira et al. [20]. Electron impact mass spectra were recorded at 70 eV and volatile compounds were tentatively identified by comparison of their mass spectra and retention times with those of standard compounds or by comparison of the mass spectrum with those reported in the Wiley 6, NIST 21, and NIST 107 mass spectrum libraries (provided by the manufacturer). Linear retention indices were also calculated according to the retention times of *n*-alkanes and compared with those reported in literature. Semi-quantitative analysis was based on the internal standard method, considering a response factor equal to 1.00.

SPE–GC–MS

SPE–GC–MS was used for the evaluation of terpenes and C_{13}-norisoprenoids in free and bound form. Such molecules were extracted on Isolute® 500-mg, 6-mL, C_{18} SPE cartridges (Biotage, Uppsala, Sweden) according to the method reported by Comuzzo et al. [21]. Briefly, 100 µL of internal standard (1-heptanol, 312 µg mL^{-1} in 96% v/v ethanol) was added to 100 mL of degassed wine and loaded onto the SPE cartridge previously conditioned with 25 mL of methanol and 25 mL of Milli-Q grade water. Sample loading was followed by a washing step with 150 mL of Milli-Q water and the elution of free varietal aromas was carried out with 25 mL of pentane:dichloromethane (2:1 v/v). The eluate was dehydrated with anhydrous sodium sulfate and concentrated under nitrogen flow to a final volume of about 1 mL.

Bound aromas were eluted from the same cartridge with 25 mL of HPLC-grade methanol. The eluate was collected in conical tubes and evaporated to dryness in a Univapo 100 H (Uniequip, Planegg, Germany) vacuum centrifuge. The residue was resuspended in 5 mL of citrate buffer (0.2 M, pH 5.00), then, 200 µL of glycosidase preparation (25 g L^{-1} in Milli-Q water) was added. Samples were stored for 15 h at 40 °C for allowing enzymatic hydrolysis, and then transferred in a 10 mL volumetric flask. Internal standard (1-heptanol, 100 µL) was added and the terpenes and norisoprenoids released after enzyme treatment were extracted using LLE as described above [20]. Extracts were finally concentrated under nitrogen stream to a final volume of approx. 1 mL.

GC–MS analysis followed as reported for LLE–GC–MS analysis [20], for both free and bound fraction.

SPME–GC–MS/MS

SPME–GC–MS/MS was the third analytical approach used; it allowed the detection of both varietal and non-varietal aroma compounds. Degassed wine (1 mL) was spiked with 50 µL of 2-octanol at 2.13 mg L^{-1} (IS) in ethanol and placed in a 20-mL headspace vial containing 1.5 g of sodium chloride. Two technical replicates were prepared for each sample, along with a blank sample containing only sodium chloride. GC analysis was performed using a Trace GC Ultra Gas Chromatograph coupled with a TSQ Quantum Tandem Mass Spectrometer, which was upgraded to the XLS configuration and equipped with a Triplus autosampler (Thermo Fisher Scientific, Waltham, MA, USA). The method used was adopted by Carlin et al. [22]. Samples were incubated for 5 min at 35 °C, and volatiles were extracted for 20 min with a 2-cm-long 50/30-µm coated divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber (Supelco, Sigma-Aldrich, St. Louis, MO, USA). After solid-phase microextraction, the fiber was desorbed for 3 min at 250 °C in the GC system with the injector set in splitless mode. The fiber was reconditioned between each sample at 270 °C for 7 min. Helium was used as a carrier gas at a flow rate of 1.2 mL min^{-1}.

The GC oven was equipped with a 30 m × 0.25 mm VF-WAXms column (Agilent Technologies Inc., Santa Clara, CA, USA) with a film thickness of 0.25 µm. The oven temperature was held for 2 min at 40 °C after the injection, ramped at 6 °C min^{-1} up to 250 °C, and held for 5 min. The ion source was set at 230 °C, and electron impact mass spectra were recorded at 70 eV. Data acquisition and analyses were performed using the Xcalibur Workstation software supplied by the manufacturer.

Determination of aromatic amino acids metabolites by UHPLC–MS/MS

Sparkling wine samples were filtered at 0.22 µm by a Millex-GV filtration unit (Merc, Darmstadt, Germany) and directly collected in 2-mL HPLC amber vials. The analyses were performed on an Acquity UHPLC system provided with an autosampler and coupled with a XEVO TQMS mass spectrometer equipped with an electrospray source (Waters Corporation, Milford, MA, USA). Metabolites of aromatic amino acids were separated at a flow rate of 0.4 mL min^{-1} on a 1.8-µm 150 × 2.1-mm Waters Acquity HSS T3 column (Waters Corporation), which was conditioned at 40 °C. A linear gradient of water containing 0.1% formic acid (solvent

A) and 0.1% formic acid in acetonitrile (solvent B) was used according to the conditions reported by Arapitsas et al. [23]. The injection volume was 10 µL, and the wine samples were stored in the autosampler at 6 °C during analyses.

The MS conditions included a capillary voltage of 3.5 kV in positive mode and − 2.7 kV in negative mode. The ion source temperature was set at 150 °C, while the desolvation temperature was 500 °C. Nitrogen was used as the cone gas with a flow rate of 50 L h^{-1}. The data were processed using Waters MassLynx (version 4.1) and TargetLynx software (Milford, MA, USA).

For quantitative analysis, calibration curves were constructed with standard compounds as reported by Arapitsas et al. [23], with the exception of sulfonated indole 3-lactic acid glucoside (ILA-GLU-SO_3H), and indole 3-lactic acid glucoside (ILA-GLU), that were quantified as indole 3-lactic acid (ILA).

Analysis of lipid molecules by UHPLC–MS/MS

Lipid analysis was performed as described by Della Corte et al. [24] with slight modifications. An aliquot (3 mL) of degassed wine was introduced in a 50-mL Falcon tube and spiked with 30 µL of a methanolic solution containing cholesterol-d_7 and octadecanoic acid-d_3 (IS) at concentrations of 1.01 mg mL^{-1} and 1.002 mg mL^{-1}, respectively. Lipids were extracted two times in 21 mL of a chloroform–methanol solution (2:1 v/v) containing BHT (10 mg L^{-1}). The total lower lipid-rich layer was collected in 100-mL flasks, and the solvent was evaporated to dryness using a rotary evaporator. The samples were reconstituted in 300 µL of acetonitrile/2-propanol/water (65:30:5 v/v/v) and filtered (0.22 µm) into 2-mL HPLC amber vials for UHPLC–MS/MS analysis.

UHPLC separation was performed on a Dionex 3000 chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an autosampler and coupled with an API 5500 triple-quadrupole mass spectrometer (Sciex, Concord, Vaughan, ON, Canada), which was provided with an electrospray ion source (ESI). Lipids were separated on a 2.7-µm 150 × 2.1-mm RP Ascentis Express column (Sigma-Aldrich, St. Louis, MO, USA) set at 55 °C. The injection volume was 5 µL, and the samples were stored in an autosampler at 10 °C during the analyses. The mobile phase and chromatographic conditions are those described by Della Corte et al. [24], and the flow rate was 0.260 mL min^{-1}.

The spray voltage of the ESI source was set at 5500 V for positive mode and − 4500 V for negative mode, and the source temperature was 250 °C. The nebulizer (Gas 1) and heater gas (Gas 2) pressures were set at 40 and 20 psi, respectively. Ultra-high-purity nitrogen (99.999%) was used as both a curtain gas and collision gas at 20 and 9 psi, respectively. Instrument control and data acquisition were performed by Analyst software (Applera Corporation, Norwalk, CT, USA), and the data were processed using MultiQuant, version 2.1 (Sciex, Concord, Vaughan, ON, Canada). For quantitative analysis, calibration curves were constructed with standard compounds, as previously described by Della Corte et al. [24].

Statistical analysis

Statistica for Windows Version 8 (Statsoft, Tulsa, OK, USA) was used to calculate means, standard deviations (SDs), coefficients of variation (CVs), minimum values (MIN), and maximum values (MAX) of the different analytical parameters determined for wine samples.

Coefficient of variation was calculated as the ratio of the standard deviation to the average value of each parameter.

Results and discussion

Basic quality parameters

Table 1 shows the results related to the basic chemical composition of the wines. The mean values, SDs, and CVs show that the samples are very homogeneous concerning alcoholic strength, titratable acidity, tartaric acid, pH, and even volatile acidity, which shows quite low values despite a slightly higher CV. This relatively homogeneous composition is probably related to the fact that the sparkling wines analyzed are all DOC productions and were manufactured in fulfillment of the standards reported in the DOC production guidelines.

However, some differences that are more relevant from a practical point of view were observed for residual sugars (min–max range 6–19 g L^{-1}), malic acid (0.01–3.79 g L^{-1}), and lactic acid (0.00–2.75 g L^{-1}). These differences could be related to the application of different winemaking protocols

Table 1 Chemical composition of Ribolla Gialla commercial wines ($n = 33$)

Parameter	Mean ± SD	MIN	MAX	CV
Alcoholic strength (% v/v)	11.68 ± 0.64	10.40	12.70	0.05
Reducing sugars (g L^{-1})	10.64 ± 2.91	6.00	18.87	0.27
Titratable acidity (g L^{-1})	5.94 ± 0.48	5.00	7.25	0.08
Volatile acidity (g L^{-1})	0.31 ± 0.06	0.20	0.48	0.21
pH	3.22 ± 0.12	3.03	3.51	0.04
Malic acid (g L^{-1})	2.08 ± 0.93	n.d.	3.79	0.45
Lactic acid (g L^{-1})	0.26 ± 0.63	n.d.	2.75	2.45
Tartaric acid (g L^{-1})	2.83 ± 0.36	2.30	3.93	0.13

SD standard deviation, *MIN* minimum value, *MAX* maximum value, *CV* coefficient of variation, *n.d.* not detected

by the producers, leading to different styles of wine for Ribolla Gialla DOC, even if they are in agreement to the DOC production guidelines. In regard to sugars, most of the wines analyzed showed a residual sugar content of less than 12 g L^{-1} or between 12 and 17 g L^{-1}. According to the European Commission Regulation (EC) n. 607/2009 [25], these wines are classified as brut and extra dry, respectively. Only one sample had higher sugar content (about 19 g L^{-1}) and was categorized as sec (or dry).

The variations of malic and lactic acid highlight the different ways of managing malolactic fermentation (MLF), which are probably connected with the different storage times of the wines on yeast lees after refermentation. Most of the wines did not contain lactic acid in appreciable amounts, which was probably due to the lack of malolactic fermentation. MLF generally occurs during aging on the lees because of the positive effect that they have on promoting the growth of lactic acid bacteria [26]. For this reason, most winemakers would consider Ribolla Gialla sparkling wine as a fresh and young wine according to the data.

Ribolla Gialla is generally refermented via the Martinotti/Charmat short method, which involves refermentation in stainless-steel autoclaves without contact with the lees at the end of refermentation. However, six wine samples had a higher content of lactic acid. This accounted for the increase of the CV calculated for lactic acid in Table 1 and was combined with the decrease of malic acid concentration. This evidence may be related to the manufacture of certain Ribolla Gialla wines, with some products being kept in contact with the lees after refermentation in an autoclave. Such *sur lies* aging may be prolonged for up to several months (2–4 months on average). In addition, certain winemakers also produce Ribolla Gialla by the traditional refermentation method in bottles.

Wine aroma profile

Table 2 shows the results of the qualitative and quantitative determination of the aroma compounds detected in wine samples by the different analytical techniques. Concerning non-varietal aromas, a total number of fifty-eight volatiles were tentatively identified in the wines, including acids, alcohols, esters, C6 compounds, diols, and carbonyls.

Fatty acids

Fatty acids are some of the most representative volatiles in the wines analyzed, and generally, they are described as having cheese, rancid, and fatty notes [27, 28]. If their amounts are higher than the odor detection threshold (ODT), they may negatively affect the organoleptic characteristics of the wines. In the samples analyzed, the average concentration of fatty acid was often close and sometimes higher than their ODT. Nevertheless, their odor activity value (OAV), which is the ratio between the concentration and ODT, was in the range of 1–4 for many of the compounds analyzed. The exception was octanoic acid, which is the most representative fatty acid in the volatile composition of Ribolla wines (OAV ≈ 30). These results suggest that fatty acids might have minor relevance in the aromatic characteristics of Ribolla Gialla. Acetic acid (which is responsible for the typical vinegar off-flavor) was found to be lower than ODT in all samples analyzed. Interestingly, the variability among the samples (i.e., the CVs) for fatty acids and volatile compounds in general (Table 2) was higher than that detected for the basic parameters in Table 1. This highlights a certain level of differentiation among Ribolla Gialla DOC wines, which is probably related to a number of variables, such as those typically connected with the development of wine aromas. Concerning fatty acids, for instance, the differences among the samples might have resulted from the different origin of the grapes used, the amount of lipid substances in the musts, the diverse winemaking conditions, and the yeast strains used for fermentations [29].

Higher alcohols

Higher alcohols (HA) are also important volatiles among the compounds listed in Table 2. They are produced by yeasts during alcoholic fermentation as products of amino acid metabolism [11]. Their contributions to wine aroma vary from honey, rose, and floral characters (2-phenylethyl alcohol and benzyl alcohol) to pungent and solvent-like smells (1-propanol, 1-butanol, 2- and 3-methyl-1-butanol) [30], and the effects depend on their concentration [9]. The average amounts of HA observed in the wines analyzed were generally found to be lower than their ODT except for 2- and 3-methyl-1-butanol and 2-phenylethanol (ODT: 40 and 10 mg L^{-1}, respectively). This composition may be linked with the yeast strains used, as well as the winemaking conditions adopted and the amino acidic composition of the grapes. The presence of significant amounts of 2-phenylethanol is very interesting for its fresh rose-like odor [31], even if its OAV range was about 1–6.

Fermentative esters

Esters are the most representative class of volatile compounds found in Ribolla Gialla sparkling wines. Isoamyl acetate (3-methyl-1-butanol acetate), ethyl hexanoate, ethyl lactate, ethyl octanoate, diethyl succinate, and 2-phenylethyl acetate showed the highest concentrations. The compounds eluted at higher retention times (e.g., diethyl malate) presumably had a minor impact on the aroma profile because of their low volatility. Some esters, such as ethyl butanoate and ethyl hexanoate, were close to or higher than their ODT

Table 2 Non-varietal aroma compounds detected in Ribolla Gialla commercial sparkling wines ($n=33$) by different analytical approaches

Compounds	AM	IM	Mean ± SD	MIN	MAX	CV	ODT (μg L^{-1})	
Acids								
Acetic acid	LLE	MS RI S	2624 ± 1206	1000	6388	0.46	200,000	[35]
2-Methylpropanoic acid	LLE	MS RI S	376 ± 168	n.d.	877	0.45	230	[30]
Butanoic acid	LLE	MS RI S	423 ± 126	218	812	0.30	10000	[35]
3-Methylbutanoic acid	LLE	MS RI S	228 ± 242	n.d.	1103	1.06	250	[30]
Hexanoic acid	LLE	MS RI S	4196 ± 1298	2111	7817	0.31	3000	[35]
Heptanoic acid	LLE	MS RI	56 ± 70	n.d.	224	1.24	–	
Octanoic acid	LLE	MS RI S	6923 ± 2390	2878	15,070	0.35	500	[47]
Nonanoic acid	SPME	MS S	454 ± 96	239	708	0.21	–	
Decanoic acid	LLE	MS RI S	872 ± 736	n.d.	4043	0.84	1000	[47]
Benzoic acid	SPME	MS S	5 ± 3	2	17	0.61	–	
Dodecanoic acid	SPME	MS S	3 ± 2	1	9	0.67	–	
Hexadecanoic acid	LLE	MS RI	2065 ± 2072	n.d.	7714	1.00	–	
Alcohols								
2-Methyl-1-propanol	LLE	MS RI	8784 ± 2825	5569	18,165	0.32	40,000	[35]
1-Butanol	LLE	MS RI S	210 ± 103	n.d.	515	0.49	40,000	[48]
2- and 3-methyl-1-butanol	LLE	MS RI S	125,666 ± 24,151	85,297	190,584	0.19	40,000	[30]
3-Ethoxy-1-propanol	LLE	MS RI	172 ± 349	n.d.	1651	2.03	–	
2-Phenylethanol	LLE	MS RI S	23,645 ± 12,214	9954	63,961	0.52	10,000	[35]
C6 compounds								
1-Hexanol	LLE	MS RI S	957 ± 231	603	1443	0.24	2500	[30]
trans-3-hexen-1-ol	SPME	MS S	3 ± 1	2	9	0.41	1000	[30]
trans-2-hexen-1-ol	SPME	MS S	7 ± 6	n.d.	35	0.84	–	
cis-3-hexen-1-ol	LLE	MS RI S	39 ± 118	n.d.	646	3.01	400	[30]
Hexanal	SPME	MS S	2 ± 7	n.d.	42	3.19	5	[49]
trans-2-hexenal	SPME	MS S	4 ± 1	1	8	0.36	82	[50]
Diols								
2,3-Butanediol	LLE	MS RI	2987 ± 1079	1497	5601	0.36	–	
1-2 Propandiol	LLE	MS RI	674 ± 250	258	1216	0.37	–	
Esters								
2-Methyl-1-propanol acetate	SPME	MS S	0 ± 0	n.d.	1	1.07	–	
Ethyl butanoate	SPME	MS S	44 ± 12	18	64	0.26	20	[35]
3-Methyl-1-butanol acetate	LLE	MS RI	1182 ± 1168	n.d.	5908	0.99	30	[35]
Methyl hexanoate	SPME	MS S	1 ± 0	n.d.	2	0.38	–	
Ethyl hexanoate	LLE	MS RI S	554 ± 268	132	1165	0.48	65	[47]
Hexyl acetate	LLE	MS RI	8 ± 32	n.d.	171	3.94	2	[49]
Ethyl lactate	LLE	MS RI	20,976 ± 33,732	1589	141,330	1.61	60,000	[30]
Methyl octanoate	SPME	MS S	3 ± 1	1	6	0.39	200	[49]
Ethyl octanoate	LLE	MS RI S	433 ± 326	n.d.	982	0.75	580	[47]
3-Methylbutyl lactate	SPME	MS S	1 ± 0	n.d.	1	0.30	–	
Ethyl-2-hydroxy-4-methylpentanoate	SPME	MS S	6 ± 3	1	14	0.46	–	
Methyl decanoate	SPME	MS S	0 ± 0	n.d.	1	0.54	4	[50]
Ethyl 3-hydroxybutanoate	LLE	MS RI	42 ± 87	n.d.	295	2.04	–	
Ethyl decanoate	LLE	MS RI S	36 ± 80	n.d.	307	2.25	200	[47]
Methyl ethyl succinate	SPME	MS S	96 ± 54	25	283	0.56	–	
3-Methylbutyl octanoate	SPME	MS S	5 ± 2	2	9	0.35	125	[51]
Diethyl succinate	LLE	MS RI	2555 ± 1849	263	8391	0.72	100,000	[30]
Ethyl 9-decenoate	SPME	MS S	9 ± 11	1	50	1.15	–	
Methyl salicylate	SPME	MS S	5 ± 6	1	29	1.31	40	[49]
2-Phenylethyl acetate	LLE	MS RI S	136 ± 341	n.d.	1707	2.50	250	[35]

Table 2 (continued)

Compounds	AM	IM	Mean ± SD	MIN	MAX	CV	ODT (μg L^{-1})	
Ethyl dodecanoate	LLE	MS RI	57 ± 125	n.d.	456	2.20	–	
Diethyl malate	LLE	MS RI	5544 ± 4199	679	18,639	0.76	760,000	[30]
Ethyl tetradecanoate	LLE	MS RI	57 ± 140	n.d.	504	2.48	–	
Diethyl 2-hydroxypentanedioate	LLE	MS RI	605 ± 406	n.d.	1472	0.67	–	
Ethyl hexadecanoate	LLE	MS RI S	1069 ± 1764	n.d.	6185	1.65	–	
Ethyl hydrogen succinate	SPME	MS S	57 ± 30	19	125	0.52	1,000,000	[30]
Carbonyl compounds								
3-Hydroxy-2-butanone (acetoin)	LLE	MS RI S	599 ± 311	162	1230	0.52	150,000	[51]
Furfural	SPME	MS S	29 ± 25	7	138	0.86	770	[30]
Benzaldehyde	SPME	MS S	14 ± 28	4	164	2.05	350	[49]
3,4-Dimethyl benzaldehyde	SPME	MS S	1 ± 0	n.d.	2	0.34	–	
Others								
Dihydro-2-methyl-3(2*H*)-thiophenone	SPME	MS S	4 ± 3	1	11	0.59	–	
Dihydro-2(3*H*)-furanone (γ-butyrolactone)	LLE	MS RI S	691 ± 204	316	1237	0.30	1000	[30]
Methionol	LLE	MS RI	232 ± 145	n.d.	623	0.62	1000	[51]

Concentrations are expressed in μg L^{-1}

AM analytical method, *IM* identification method (S comparison of mass spectra and retention time with those of standard compounds, *RI* comparison of order of elution with those reported in literature, *MS* comparison of mass spectra with those reported in mass spectrum libraries), *SD* standard deviation, *MIN* minimum value, *MAX* maximum value, *CV* coefficient of variation, *ODT* odor detection threshold, *n.d.* not detected

(20 and 65 μg L^{-1}, respectively) in all the samples analyzed. Others such as isoamyl acetate, 2-phenylethyl acetate, and hexyl acetate were detected in only certain wines with concentrations higher than their ODT. Compounds such as ethyl hexanoate and isoamyl acetate were found to have significant OAVs (e.g., up to 18 for ethyl hexanoate, 85 for hexyl acetate, and approximately 200 for isoamyl acetate). This observation is very interesting from a sensory point of view, because esters generally confer floral, fresh, and fruity notes (rose, banana, pear, green apple) to the wines [30, 32, 33], which are generally recognized as typical in Ribolla wines. For this reason, esters appeared to be an important component of the volatile profile of Ribolla Gialla.

Aging esters

Among esters, ethyl lactate and diethyl succinate are considered "aging esters" (AE), and their content in wines generally increases during aging and after malolactic fermentation [31, 34]. The same consideration is true for diethyl malate, but this compound is normally detected less frequently in wines. The concentration of AE was generally found to be lower than the ODT in most of the wines analyzed. However, in some samples, ethyl lactate had higher concentrations than its odor threshold (60 mg L^{-1}) in accordance with the amount of malic and lactic acid. In fact, the highest concentration of these esters was observed in the same products in which malolactic fermentation was hypothesized to have taken place (i.e., the wines where malic acid was not detected), as shown in Table 1. This may confirm that certain Ribolla Gialla sparkling wines are produced with a more or less prolonged period of *sur lies* aging or by refermenting them in bottle by the traditional method.

Carbonyls, diols and C6 compounds

Carbonyls, diols, and C6 compounds appeared not to have an important contribution to the aromatic characterization of the samples, and in most of the cases, they were detected at levels below their ODT. Hexanal was found to overcome its ODT in some samples, but at the low concentrations detected, it may or may not contribute to the fresh vegetal notes of Ribolla wines.

Terpenes

Table 3 reports the detected varietal aromas (terpenes and C_{13}-norisoprenoids) in free and bound form in the samples. Terpenes are well known to confer floral odors to wines [31]. In Ribolla, they were especially found in the free form, but in most cases, their average concentrations were below their ODT with a few exceptions for linalool and geraniol. Although it was present below its ODT, α-terpineol was the most abundant free terpenic alcohol in the wines. Interestingly, even if Ribolla Gialla cannot be considered as an aromatic variety, the concentrations detected for α-terpineol and linalool were higher than those normally observed in certain international white varieties, such as Pinot blanc,

Table 3 Free and bound terpenes and norisoprenoids detected in Ribolla Gialla commercial sparkling wines ($n=33$) by different analytical approaches

Compounds	AM	IM	Mean ± SD	MIN	MAX	CV	ODT (μg L^{-1})	
Free terpenes								
β-Myrcene	SPME	MS S	0 ± 1	n.d.	7	3.56	–	
Limonene	SPME	MS S	2 ± 1	1	4	0.33	200	[49]
cis-Linalool oxide (furanic)	SPE	MS RI S	4 ± 4	n.d.	12	0.88	6000	[30]
trans-Linalool oxide (furanic)	SPE	MS RI S	1 ± 2	n.d.	9	1.61	6000	[30]
Linalool	SPE	MS RI S	10 ± 15	n.d.	80	1.51	50	[30]
Terpinen-4-ol	SPME	MS S	1 ± 0	1	2	0.34	340	[49]
α-Terpineol	SPE	MS RI S	23 ± 34	n.d.	194	1.49	250	[51]
β-Citronellol	SPE	MS RI S	2 ± 4	n.d.	18	1.81	100	[35]
Nerol	SPME	MS S	1 ± 1	n.d.	6	0.80	60	[52]
Geraniol	SPE	MS RI S	4 ± 10	n.d.	39	2.87	30	[51]
Geranic acid	SPME	MS S	7 ± 4	2	23	0.65	–	
Free C_{13}-norisoprenoids								
β-Damascenone	SPE	MS RI	5 ± 6	n.d.	28	1.27	7	[38]
3-Oxo-α-ionol	SPE	MS RI	10 ± 11	n.d.	42	1.11	–	
Riesling acetal	SPME	MS S	4 ± 2	n.d.	7	0.49	–	
Vitispirane (isomer 1)	SPME	MS S	4 ± 3	2	14	0.68	800	[40]
1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN)	SPME	MS S	5 ± 6	1	28	1.19	20	[40]
Vitispirane (isomer 2)	SPME	MS S	14 ± 9	3	37	0.61	800	[40]
Bound terpenes								
Nerol	SPE	MS RI S	3 ± 4	n.d.	20	1.54		
Geraniol	SPE	MS RI S	13 ± 13	n.d.	51	1.03		
1-Hydroxylinalool	SPE	MS RI	2 ± 4	n.d.	15	1.99		
Geranic acid	SPE	MS RI	8 ± 9	n.d.	36	1.13		
Bound C_{13}-norisoprenoids								
3-Hydroxy-β-damascenone	SPE	MS RI	6 ± 10	n.d.	50	1.83		
trans,trans-2,6-Dimethyl-2,6-octadiene-1,8-diol (Z8-hydroxygeraniol)	SPE	MS RI	11 ± 13	n.d.	56	1.15		
3-Oxo-α-ionol	SPE	MS RI	40 ± 32	n.d.	135	0.81		
3-Oxo-7,8-dihydro-α-ionol (blumenol C)	SPE	MS RI	16 ± 22	n.d.	103	1.38		
3-Hydroxy-7,8-dihydro-β-ionol	SPE	MS RI	1 ± 2	n.d.	7	2.02		

Concentrations are expressed in μg L^{-1}

AM analytical method, *IM* identification method (S comparison of mass spectra and retention time with those of standard compounds, *RI* comparison of order of elution with those reported in literature, MS comparison of mass spectra with those reported in mass spectrum libraries), *SD* standard deviation, *MIN* minimum value, *MAX* maximum value, *CV* coefficient of variation, *ODT* odor detection threshold, *n.d.* not detected

Pinot Gris, Chardonnay, and Sauvignon blanc [19]. Also, β-citronellol was found at lower levels with respect to its ODT (100 μg L^{-1}) [35] and it showed similar average values to those of some Riesling wines [19].

Considering the glycosylated forms, only geraniol was significantly detected as both free and bound terpenol with a prevalence of the latter (combined) form of the molecule. According to other experiments, geraniol (with minor traces of nerol) was the only terpenic alcohol normally found in Ribolla Gialla grapes [36]. For this reason, in Ribolla Gialla, linalool, α-terpineol, and β-citronellol may be formed from such terpenic alcohols during grape processing. Citronellol, for instance, is reported to be produced by *S. cerevisiae* from geraniol and nerol during alcoholic fermentation, and other formation pathways have also been suggested for linalool (from geraniol) and α-terpineol (by cyclization of nerol) [37]. For both free and bound geraniol, the average values were generally lower than the ODT reported for the alcohol, but some of the samples showed significant levels of total geraniol (Table 3). Considering that the bound form generally prevails for this terpenol, one interesting way to improve the volatile profile of these wines might be the application of techniques (i.e., addition of enzymes with β-glucosidase activity on must or base wine) to increase the release of free

geraniol from its glycosides in the production process of Ribolla Gialla sparkling wines.

C_{13}-norisoprenoids

In contrast to terpenes, which did not appear particularly characteristic for the aroma profile of the wines, some C_{13}-norisoprenoids were detected in appreciable concentrations (Table 3). TDN (1,1,6-trimethyl-1,2-dihydronaphthalene) and β-damascenone, for instance, overcame their sensory thresholds in different samples. Norisoprenoids are generally not connected to specific aromatic grapevine varieties and generally have a low odor threshold. The ODT of β-damascenone is 0.04–0.06 μg L^{-1} in a model dilute alcohol solution [31], while a relatively wide range of ODTs was found in wine: Sefton et al. [38] reported 0.14 μg L^{-1} in deodorized white wine, 0.85–2.10 μg L^{-1} in deodorized red wine, and 7.00 μg L^{-1} in red wine. The presence of β-damascenone may be particularly interesting for Ribolla wines for not only the relatively high concentrations found but also because it is reported to modify the sensory perception of some esters. Escudero et al. [39] found that the addition of low levels of β-damascenone (0.85 μg L^{-1}) to a solution of esters increased the fruity notes of the mixture, while higher levels (3.5 μg L^{-1}) accounted for the development of strong raisin/dry-plum odors.

The amount of norisoprenoids is also dependent on the winemaking conditions. In general, β-damascenone is observed at higher concentrations in young wines [38], whereas for TDN, a positive correlation between its occurrence in wine and aging was reported [40]. Their odor descriptors range from floral (e.g., vitispiranes) and fruity-honey scents (β-damascenone) to notes of kerosene and petrol (for TDN) [41]. The presence of TDN among the compounds listed in Table 3 may represent further evidence about the aging of certain Ribolla Gialla sparkling wines after refermentation.

Another interesting aspect related to norisoprenoids is the high concentration observed for some of them in bound form, particularly 3-oxo-α-ionol and 3-hydroxy-β-damascenone, which are the most representative compounds. As for terpenes, Ribolla Gialla appeared to have an unexpressed aromatic potential for norisoprenoids. Thus, from a technical point of view, the optimization of winemaking process to maximize the release of this bound aromatic potential might be an interesting way to improve the quality of Ribolla Gialla sparkling wines.

Metabolomic fingerprint of aromatic amino acids metabolites and indoles

The second group of metabolites analyzed included aromatic amino acids and their catabolites, as shown in Table 4. There were twenty-two compounds detected, among which the sulfonated derivates of ILA, tryptophol (TOL), ILA-GLU, and IAA are presented. Sulfonation is the addition of a sulfonic acid group ($–SO_3H$) to an organic compound and is a widespread industrial process used in a diverse range of products. It also has a major function in modulating biological activities that are known to occur in wine, which involve several metabolites such as polyphenols and indoles [23]. By the Ehrlich pathway in yeast, the essential amino acids TRP, phenylalanine (PHE), and tyrosine (TYR) produce aromatic higher alcohols, such as TOL, and tyrosol (TYL), which at high concentrations leads to a strong, pungent smell and taste, while lower

Table 4 Aromatic amino acids metabolites detected in Ribolla Gialla commercial sparkling wines ($n=33$). Concentrations are expressed in mg L^{-1}

Compound[a]	Mean ± SD	MIN	MAX	CV
ILA-SO_3H	8.53 ± 10.31	0.66	54.20	1.21
TOL-SO_3H	3.73 ± 2.55	0.23	12.49	0.68
TYL	2.55 ± 0.97	0.93	4.79	0.38
TYR	1.13 ± 0.49	0.08	1.98	0.43
ILA-GLU-SO_3H[b]	0.53 ± 0.37	0.12	1.79	0.70
PHE	0.51 ± 0.26	0.03	0.99	0.51
TRP-EE	0.44 ± 0.21	0.11	0.89	0.48
TOL	0.29 ± 0.27	0.01	1.08	0.93
ABA	0.22 ± 0.11	0.02	0.46	0.50
IAA-SO_3H	0.20 ± 0.07	0.13	0.39	0.35
ABA-GLU	0.13 ± 0.05	0.04	0.26	0.38
TRP	0.13 ± 0.10	0.01	0.39	0.77
ILA-GLU[b]	0.10 ± 0.05	0.01	0.25	0.50
TYR-EE	0.08 ± 0.03	0.02	0.13	0.38
ILA	0.03 ± 0.03	n.d.	0.12	1.00
KYNA	0.02 ± 0.01	n.d.	0.04	0.50
AA	0.01 ± 0.06	n.d.	0.33	6.00
IAA-ASP	4.41 ± 2.87	n.d.	12.35	0.65
N-TYR-EE	3.86 ± 2.11	n.d.	9.63	0.55
KYN	2.42 ± 1.98	n.d.	9.24	0.82
IAA	1.75 ± 1.63	n.d.	8.84	0.93
N-TRP-EE	1.08 ± 0.81	n.d.	3.47	0.75

[a] *ILA-SO_3H* indole-lactic acid-2-sulfonate, *TOL-SO_3H* tryptophol-2-sulfonate, *TYL* tyrosol, *TYR* tyrosine, *ILA-GLU-SO_3H* sulfonated indole 3-lactic acid glucoside, *PHE* phenylalanine, *TRP-EE* tryptophan-ethyl ester, *TOL* tryptophol, *ABA* abscisic acid, *IAA-SO_3H* sulfonated indole 3-acetic acid, *ABA-GLU* glucoside of abscisic acid, *TRP* tryptophan, *ILA-GLU* indole 3-lactic acid glucoside, *TYR-EE* tyrosine-ethyl ester, *ILA* indole 3-lactic acid, *KYNA* kynurenic acid, *AA* anthranilic acid, *IAA-ASP* indole 3-acetic acid conjugate with aspartic acid, *N-TYR-EE* *N*-acetyl-tyrosine-ethyl ester, *KYN* kynurenine, *IAA* indole 3-acetic acid, *N-TRP-EE* *N*-acetyl-tryptophan-ethyl ester

[b] Quantified as ILA

SD standard deviation, *MIN* minimum value, *MAX* maximum value, *CV* coefficient of variation, *n.d.* not detected

levels are positively reflected in the floral aroma of wines [23].

Sparkling wines are well known to have much lower amounts of TRP than other wines, which is probably due to the second fermentation [23]. TRP values for the commercial samples ranged from 0.01 to 0.39 mg L^{-1}, with a mean value of 0.13 mg L^{-1}, similarly occurred for its ethyl ester (0.44 mg L^{-1} mean value). TOL-SO_3H had higher amounts among all the other metabolites of aromatic amino acids with a mean value of 3.73 mg L^{-1}. The unsulfonated form of TOL varied from 0.01 to 1.08 mg L^{-1} in all samples. This could mean that the sulfonated/unsulfonated TOL ratio that favored TOL-SO_3H may also give rise to further products similar to 2-AAP.

ILA (0.03 mg L^{-1}) and ILA-SO_3H (8.53 mg L^{-1}) pairing showed similar behavior to the previously described TOL and TOL-SO_3H pairing. Arapitsas et al. [23], found that young red wines and rosé wines had a much higher concentration of ILA and especially ILA-GLU, which is synthesized by plants [42]. This was probably due to maceration with the skins during winemaking. In contrast, white and especially sparkling wines had the lowest concentrations due to soft pressing and secondary fermentation, which necessitate further nitrogen consumption.

IAA-SO_3H was detected at concentrations between 0.13 and 0.39 mg L^{-1}, while its parent compounds were detectable at very low concentrations. Hoenicke et al. [43] showed that the sulfonation of indoles in a model wine solution could be responsible for their degradation and the formation of aromatic aminobenzenes such as 2-AAP, which are responsible for some of the heavy aromatic characteristics of white wines. On the other hand, the structural similarity of TOL to IAA could also cause the detection of 2-AAP or other similar aromatic compounds in wine. Because of the lack of anthocyanins and the low flavanol content in white wines, the sulfonation of indoles can increase the risk of developing an atypical off-flavor. Thus, the sparkling wines produced from the Ribolla Gialla variety might have a higher tendency to develop atypical aging defects, which could also be promoted by inappropriate storage temperature.

Metabolomic fingerprint of lipids

Table 5 shows the twenty-six lipid compounds found in the samples of commercial sparkling wines according to UHPLC–MS–MS. Most of the compounds found are saturated long-chain fatty acids (LCFAs, more than 12 carbon atoms), although the mid-chain fatty acids (MCFAs, 4–12 carbons) and their esters have a major influence on the organoleptic properties of wine. In addition to the fact that lipids are an integral part of solid grape tissues, they are also an important building block in wine yeasts, where the majority of LCFAs are esterified with glycerol or glycerophosphate to form mono-, di-, and tri-acylglycerides or glycerophospholipids, respectively [44].

Table 5 Lipid compounds detected in Ribolla Gialla commercial sparkling wines ($n=33$)

Compounds	Mean ± SD[a]	MIN[b]	MAX[c]	CV[d]
Fatty acids				
Palmitic acid	9.24 ± 13.52	5.21	11.49	1.46
Stearic acid	6.49 ± 9.32	4.43	8.40	1.44
Myristic acid	0.31 ± 0.84	0.11	0.50	2.68
Oleic acid + *cis*-vaccenic acid	0.19 ± 0.32	0.12	0.26	1.72
Arachidic acid	0.15 ± 0.40	0.08	0.26	2.70
cis-11-Eicosanoic acid	0.11 ± 0.03	0.10	0.12	0.28
Heptadecanoic acid	0.06 ± 0.14	0.04	0.10	2.19
Myristoleic acid	0.05 ± 0.04	0.05	0.06	0.78
Linoleic acid	0.04 ± 0.08	0.02	0.06	2.29
Lignoceric acid	0.03 ± 0.16	n.d.[e]	0.09	5.52
Behenic acid	0.02 ± 0.08	0.01	0.05	4.21
Palmitoleic acid	0.02 ± 0.36	n.d.	0.14	20.00
Linolenic acid	0.01 ± 0.04	0.00	0.03	8.00
Sterols				
Ergosterol	0.18 ± 0.97	0.03	0.35	5.54
Lupeol	0.10 ± 1.10	0.01	0.48	10.89
Glycerolipids				
1-Linoleoyl-rac-glycerol	0.02 ± 0.02	0.01	0.02	1.18
1-Oleoyl-rac-glycerol	0.01 ± 0.02	0.01	0.02	1.43
Glyceryl tripalmitoleate	0.01 ± 0.09	n.d.	0.04	6.43
Fatty acid esters				
Ethyl stearate	0.14 ± 0.18	0.10	0.17	1.31
Ethyl palmitate	0.13 ± 0.22	0.07	0.17	1.64
Ethyl oleate	0.03 ± 0.05	0.02	0.05	1.47
Ethyl linoleate	0.01 ± 0.01	0.00	0.01	2.00
Methyl palmitate	0.08 ± 1.20	n.d.	0.50	15.19
Methyl stearate	0.04 ± 0.36	n.d.	0.15	9.73
Methyl oleate	0.01 ± 0.06	n.d.	0.03	6.67
Triacylglycerols				
Tripentadecanoin	0.01 ± 0.04	n.d.	0.02	5.00

Concentrations are expressed in mg L^{-1}

SD standard deviation, *MIN* minimum value, *MAX* maximum value, *CV* coefficient of variation, *n.d.* not detected

The two most abundant LCFAs detected in the samples were palmitic acid (C16:0), which ranged from 5.21 to 11.49 mg L^{-1} with a mean value of 9.24 mg L^{-1}, and stearic acid (C18:0), which ranged from 4.43 to 8.40 mg L^{-1} (mean value: 6.49 mg L^{-1}). Previous studies showed that after the beginning of second fermentation, which is after the inoculation of the medium with yeast, the concentration of saturated fatty acids C16:0 slightly increased, while the proportion of C18:0 remained constant. However, after the end of the growth phase of the yeasts, the distribution of both saturated fatty acids decreased exponentially [45].

Unlike saturated fatty acids (SFA), the values of unsaturated fatty acids (UFA) were lower in the wine samples (*cis*-11-eicosanoic acid 0.11 mg L^{-1}, myristoleic acid 0.05 mg L^{-1}, linoleic acid 0.04 mg L^{-1}, palmitoleic acid 0.02 mg L^{-1}, and linolenic acid 0.01 mg L^{-1}). UFAs also had a much smaller proportion than SFAs. Because of their membrane fluidity, wine yeasts are able to modify the UFA/SFA ratio in their membrane composition in response to stress conditions, such as the presence of ethanol or cooler fermentation conditions under which fermentation takes place, especially for white wines. Normally, the UFA/SFA ratio is close to 1:1, but a deficiency of UFA leads to impaired biosynthesis of phospholipids and consequently stuck fermentations [12].

Similarly, Pueyo et al. [46] studied the effect of the total contents of linolenic acid and palmitic acid as compounds that best define foam stability in wines and foam height in the cavas, respectively. They found a positive correlation between these compounds and the measured properties, meaning that the wines with a greater amount of these fatty acids in the lipidic fraction could form more foam with high stability than those with lower concentrations. Since the analyzed samples in the present study contained a greater concentration of palmitic acid compared to the linolenic acid, it can be expected that foam formation of the Ribolla Gialla sparkling wines will be increased, which is a key parameter for determining the quality of sparkling wines.

In conclusion, Ribolla Gialla sparkling wines showed homogeneous characteristics concerning basic parameters (e.g., sugar content, alcoholic strength, pH, and titratable acidity) as well as their aroma composition. Sparkling Ribolla wines are generally characterized by low levels of free terpenols, and their aroma seems to be mostly characterized by the development of volatile esters and β-damascenone during fermentations, storage, and processing. The presence of these compounds is probably connected with the fresh and fruity notes that normally characterize the sparkling wines produced from Ribolla grapes.

A minor amount of malolactic fermentation was found, which reflects that two distinct wine styles coexist for Ribolla sparkling wines. The first characterizes young wines normally produced by refermentation in stainless-steel autoclaves (Martinotti/Charmat short refermentation method), and the second is based on a more or less prolonged aging period on refermentation lees in an autoclave or bottles (according to the traditional refermentation method). The analysis of lipid molecules and metabolites of aromatic amino acids highlighted other interesting features of Ribolla sparkling wines from a practical point of view. Ribolla Gialla shows a high ratio between saturated (e.g., palmitic acid) and unsaturated fatty acids (e.g., linolenic acid), which may lead to higher foam height in the sparkling wines produced and represents one of the key quality features of sparkling wines in general. In contrast, due to the high amounts of certain aromatic amino acids metabolites, Ribolla Gialla could be prone to the formation of atypical aging aromas. Considering that this specific Italian product has only recently been appearing in wine markets, further investigations should carefully consider these results to properly address the production practices and techniques towards producing high-quality products, thus increasing the local and international competitiveness of Ribolla Gialla sparkling wines.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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CORRECTION

Correction to: Compositional characterization of commercial sparkling wines from cv. Ribolla Gialla produced in Friuli Venezia Giulia

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In the original publication of the article, Table 4 has been published with an error. The concentration of the last 5 compounds was given in mg/L instead of µg/L.

The correct Table 4 is given in this correction.

The original article has been updated.

Table 4 Tryptophan metabolites detected in Ribolla Gialla commercial sparkling wines

Compound	Mean ± SD	MIN	MAX	CV
ILA-SO_3H	8.53 ± 10.31	0.66	54.20	1.21
TOL-SO_3H	3.73 ± 2.55	0.23	12.49	0.68
TYL	2.55 ± 0.97	0.93	4.79	0.38
TYR	1.13 ± 0.49	0.08	1.98	0.43
ILA-GLU-SO_3H	0.53 ± 0.37	0.12	1.79	0.70
PHE	0.51 ± 0.26	0.03	0.99	0.51
TRP-EE	0.44 ± 0.21	0.11	0.89	0.48
TOL	0.29 ± 0.27	0.01	1.08	0.93
ABA	0.22 ± 0.11	0.02	0.46	0.50
IAA-SO_3H	0.20 ± 0.07	0.13	0.39	0.35
ABA-GLU	0.13 ± 0.05	0.04	0.26	0.38
TRP	0.13 ± 0.10	0.01	0.39	0.77
ILA-GLU	0.10 ± 0.05	0.01	0.25	0.50
TYR-EE	0.08 ± 0.03	0.02	0.13	0.38
ILA	0.03 ± 0.03	n.d.	0.12	1.00
KYNA	0.02 ± 0.01	n.d.	0.04	0.50
AA	0.01 ± 0.06	n.d.	0.33	6.00
IAA-ASP*	4.41 ± 2.87	n.d.	12.35	0.65
N-TYR-EE*	3.86 ± 2.11	n.d.	9.63	0.55
KYN*	2.42 ± 1.98	n.d.	9.24	0.82
IAA*	1.75 ± 1.63	n.d.	8.84	0.93
N-TRP-EE*	1.08 ± 0.81	n.d.	3.47	0.75

Concentrations are expressed in mg L^{-1} or, where indicated, in µg/L (*)

ILA-SO_3H indole-lactic acid-2-sulfonate, *TOL-SO_3H* tryptophol-2-sulfonate, *TYL* tryptophol, *TYR* tyrosine, *ILA-GLU-SO_3H* sulfonated indole-3-lactic acid, *PHE* phenylalanine, *TRP-EE* tryptophan-ethyl ester, *TOL* tryptophol, *ABA* abscisic acid, *IAA-SO_3H* sulfonated indole 3-acetic acid, *ABA-GLU* glucoside of abscisic acid, *TRP* tryptophan, *ILA-GLU* indole 3-lactic acid glucoside, *TYR-EE* tyrosine-ethyl ester, *ILA* indole 3-lactic acid, *KYNA* kynurenic acid, *AA* anthranilic acid, *IAA-ASP* indole 3-acetic acid conjugate with aspartic acid, *N-TYR-EE* N-acetyl-tyrosine-ethyl ester, *KYN* kynurenine, *IAA* indole 3-acetic acid, *N-TRP-EE* N-acetyl-tryptophan-ethyl ester, *SD* standard deviation, *MIN* minimum value, *MAX* maximum value, *CV* coefficient of variation, *n.d.* not detected

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2

CLUSTER THINNING AND VINEYARD SITE MODULATE THE METABOLOMIC PROFILE OF RIBOLLA GIALLA BASE AND SPARKLING WINES[2]

[2] This chapter has been reprinted* from:

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PREFACE

Due to its neutral aromatic potential, the winegrowers of Ribolla Gialla variety are enforced to look for various ways to increase the content of aromatic compounds present in wine for the needs of the market. The most convenient way to perform this would be to manipulate the secondary aromas. These compounds include mainly higher aliphatic alcohols, ethyl esters and acetates formed from yeasts during alcoholic fermentation. Contents of these compounds in wine are linked to the winemaking processes used, related to the fermentation temperature, yeast strain type, nitrogen level and other nutrients in must available for yeast to perform successful fermentation, clarification of wine and similar. However, all these procedures very little affect the wine primary aroma compounds, which are also defined as varietal aroma compounds and represent the typical aroma of the grapes noted in wines. The free forms of these compounds may contribute directly to odour, while non-volatile forms sugar-bound conjugates are the most abundant. The hydrolysis of these glycoconjugates by acids, enzymes or while wine aging, can yield odour-active aglycones such as terpenes, C13-norisoprenoids, benzene derivates, and aliphatic alcohols (Zalacain et al., 2007). For this reason, the authors of previous studies focused mainly on practices that enhance the presence of free and glycosided forms of volatile compounds in wine throughout pre-fermentation maceration and other alternative skin contact techniques (Bavčar et al., 2016; Bavčar et al., 2011). On the contrary, some researchers aimed to intensify the aroma profile by modifying the inoculated yeast strains (Dashko et al., 2015). However, none of the previous work has explored the impact of viticulture practices on a common metabolomic profile of Ribolla Gialla sparkling wines.

Generally, the fruit quality parameters, such as lower pH, higher titratable acidity, and lower soluble sugars are considered desirable for grapes destined for sparkling wine production. These parameters can be achieved by adjusting the viticultural management, such as determining vine density, pruning system, canopy management, and harvest method and in addition also with altering the cluster temperature and exposure to incident light and yield manipulation. The latter factor can be influenced by cluster thinning, that is a widely accepted agronomic practice adopted in the vineyard with the aim to regulate source/sink ratio and to increase the accumulation of the secondary metabolites (Alem et al., 2019). Moreover, it is generally considered that lower yield leads to higher quality. However, if the crop level is too low, the fruit may become overripe, especially in warm seasons (Jones et al., 2014). The difference of cultivar, climate, vineyard location, rootstock and other factors may lead to conflicting results regarding the appropriate level of cluster thinning. Recommendations in Champagne are that 30% of the fruit removal will result in a less than 0.5% increase in potential alcohol, whereas removing from 30 to 50% could increase potential alcohol between 0.5 and 1.5% (Jones et al., 2014). This is on the other hand inevitably connected with higher total soluble solids and lower titratable acidity as a consequence of cluster thinning treatment.

In addition to studying the impact of crop yield on basic wine parameters, there is a considerable amount of literature dealing with crop removals as a quality tool for red grape cultivars, responding in increased anthocyanins and phenolics (Sivilotti et al., 2020), which resulted in enhanced wine color and astringency (Avizcuri-Inac et al., 2013; Chapman et al., 2004). Nevertheless, only a limited number of studies examined the effect of bunch removal on the aroma composition of wines (Alem et al., 2019), with contrasting results obtained. For instance, some of these studies reported amplification of monoterpenes and esters after bunch removal (Condurso et al., 2016; Rutan et al., 2018; Talaverano et al., 2017), while other authors argued that crop level had little impact on the volatile profile of wines (Bowen & Reynolds, 2015; Bubola et al., 2020; Moreno Luna et al., 2018). Interestingly, no previous work has studied the effect of cluster thinning on the lipid composition of sparkling wine, and very few studies have addressed the impact of this viticultural practice on the content of aromatic amino acid metabolites.

Unlike most scientific publications that are dealing with the impact of cluster thinning on the quality of still wines, the aim of this sub-study was to investigate the effect of aforementioned viticultural practice on the chemical properties of monovarietal sparkling wines from locally important Ribolla Gialla variety. The experiment was expanded over the course of three harvest seasons. In addition, this work also dealt with the influence of the vineyard position on the final quality of wine, since grapes from hillside vineyards should in principle lead to better wine quality, due to limiting yields. The results have shown that the vintage had the greatest influence on the differentiation of the wine samples. Only after normalization of this factor it was possible to observe a minimal positive effect of cluster thinning treatment on the volatile composition in both vineyard sites. However, a slightly higher amount of metabolites associated with aromatic amino acids was present in the flat vineyard which could be due to meteorological factors. Sensory analysis of the wines also confirmed the results of targeted metabolomic profiling which meant that no significant differences were observed regarding the cluster thinning. On the other hand, a contrasting effect appeared evident by comparing the thinning effect in the two vineyard sites.

Article

Cluster Thinning and Vineyard Site Modulate the Metabolomic Profile of Ribolla Gialla Base and Sparkling Wines

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Abstract: Depending on the vineyard location, cluster thinning (CT) may represent an effective tool to obtain the desired grape composition and wine quality. The effect of 20% cluster thinning on Ribolla Gialla (*Vitis vinifera* L.) sparkling wine aroma, lipid compounds, and aromatic amino acid (AAA) metabolites composition was studied for three consecutive seasons in two vineyards located in the Friuli Venezia Giulia region, Italy. In the examined sparkling wines, the vintage meteorological conditions exhibited significant influences on the metabolic profile of the samples. Data were normalized by season, and the impact of the CT treatment was evaluated for each vineyard site separately. Crop removal showed a limited positive impact on aroma compounds in sparkling wines from vineyards located in the valley. Concerning the AAA compounds, their concentration was higher in the vineyard at the foot of the hills. Cluster thinning resulted in a drop in concentration, reducing the risk of atypical aging. Despite minor differences according to targeted metabolome profiling, the sensory analysis confirmed the effects of the CT treatment in the valley floor vineyard. Reducing crop in this site, where the yield was higher, promoted a moderate improvement of Ribolla Gialla sparkling wine. In contrast, for wine produced in the vineyard at the foot of the hills, the sensory analysis indicated a preference for wines from the unthinned control samples. Overall, the study indicates that cluster thinning is a viticultural technique that could potentially improve the quality of Ribolla Gialla sparkling wines, but only in situations of excessive grape production.

Keywords: Ribolla Gialla; sparkling wine; cluster thinning; vineyard site; volatile organic compounds; lipids; aromatic amino acids; sensory analysis

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1. Introduction

Among the indigenous white varieties cultivated in northeastern Italy, Ribolla Gialla (*Vitis vinifera* L.) is one of the most promising for producing high-quality monovarietal sparkling wines. However, due to its neutral aroma potential, some authors in previous studies focused mainly on enological practices that enhance the presence of free and glycosided forms of volatile compounds in wine throughout pre-fermentation maceration and other alternative skin contact techniques [1,2]. On the contrary, some other researchers tried to intensify the aroma profile by modifying the inoculated yeast strains [3]. To the best of our knowledge, none of the previous works have explored the impact of viticulture practices on a common metabolomic profile of Ribolla Gialla sparkling wines.

Cluster thinning is a widely used agronomic practice adopted in the vineyard with the aim to regulate the source/sink ratio, thus increasing the accumulation of the secondary

metabolites [4]. In most cases, cluster thinning induces faster grape ripening, leading to higher total soluble solids, and lower titratable acidity [5]. There is a considerable body of literature dealing with crop removal as a quality tool for red grape cultivars, responding with increased anthocyanins and phenolic concentration [6], which finally promoted enhanced wine color and astringency [7,8]. Nevertheless, only a limited number of studies examined the effect of cluster removal on the aroma composition of wines [4], with contrasting results. For instance, some of these studies reported amplification of monoterpenes and esters after cluster thinning [9–11]. Other authors argued that the crop level had little impact on the volatile profile of wines [12–14]. For each grape variety, various productivity and crop loads need to be targeted to optimize grape composition. This means that such viticultural practice results positively when applied in conditions of over-crop or low crop load. At the same time, it could be ineffective or detrimental when the vines already stand in equilibrium [15]. Moreover, in the case of grapes destined for sparkling wine production, although lower sugar levels and higher titratable acidity (TA) are desirable, the excess yield could negatively affect the biosynthesis of secondary metabolites, such as aroma precursors. Thus, a yield level must be matched to obtain the best compromise between a target sugar accumulation and the biosynthesis/composition of aroma precursors in grape berries at harvest. In sparkling wine production, the yield level is normally higher to meet lower sugar accumulation, but the correct yield range needed to optimize the Ribolla Gialla sparkling wine quality still must be identified. Higher acid levels in fruit are desirable for sparkling wines since flowery and fresh aromas are preferred over greener flavors. Therefore, it can be misleading to expect lower yields in the vineyard to lead to higher wine quality, especially in warm vintages [16]. Nevertheless, a study carried out on Cavas made from *V. vinifera* cv. Parellada showed that the panel preferred wines from grapes grown in low-yielding vineyards [17]. However, despite enhancing perceived wine quality due to the increase of aroma constituents (e.g., monoterpenes) in low crop levels [18], it appears that there are conflicting data on whether the cluster thinning practice contributes positively to sparkling wine quality [16].

To our knowledge, no previous studies investigated the effect of cluster thinning on the lipid profile of sparkling wines. Lipids are important constituents in the membrane structure; they are responsible for stress adaptation and act as signaling molecules. They are also essential nutrients, and their availability can have major effects on yeast alcoholic fermentation [19]. Fatty acids represent the most important group of substances of this group of compounds, as the number of carbon atoms in their chains and the degree of saturation largely determine the effect on the wines' organoleptic properties. Since fatty acids can also be released during winemaking fermentation, they may be present in wine in free or bound forms as ethyl esters, and they contribute with fruity characters [20]; on the contrary, a higher concentration of unsaturated fatty acids (UFAs) than saturated fatty acids (SFAs), acts as a precursor of C6 aldehydes and alcohols, causing the herbaceous flavor in the wine. Moreover, previous studies reported that medium-chain fatty acids (MCFAs) C8, C10 and C12 were negatively correlated with the foamability of sparkling wines, while the ethyl esters of C6, C8 and C10 fatty acids appeared to stabilize the foam [21]. Additionally, Pueyo et al. [22] described a positive relationship between foam stability and the total content of unsaturated linolenic acid. In contrast, saturated palmitic acid was positively related to the height of the foam collar in Cava sparkling wines.

To avoid sluggish and stuck fermentation, the course of successful fermentation kinetics in sparkling wine production can also depend on the availability of certain nitrogen compounds. Through the Ehrlich pathway, the yeasts use tryptophan (Trp), phenylalanine (Phe) and tyrosine (Tyr) to produce higher alcohols, such as tryptophol (Tol), phenyl ethanol and tyrosol (Tyl), respectively. At high concentrations, such compounds may cause forming pungent notes to both taste and smell. In contrast, at low concentrations, their contribution is more related to floral notes [23]. Through the Trp pathway, the indole metabolites can be generated. During wine aging, they participate in chemical reactions as precursors for forming other aroma substances, such as 2-aminoacetophenone (2AA) [24]. Additionally,

the sulfonation of those indoles can facilitate forming off-flavors called untypical aging aroma [25]. Compared to table wines, the Trp level in sparkling wines is usually lower because two fermentations are carried out [26].

Most scientific publications in this field deal with the impact of cluster thinning on the quality of still wines, but very few have addressed how this vineyard practice affects the chemical properties of monovarietal sparkling wines. In this regard, the locally important Ribolla Gialla variety is under-researched. In addition, this study also dealt with the influence of the vineyard location on the final quality of wine from Ribolla Gialla, as in different soil conditions, yield and grape quality can be significantly affected. Therefore, this multi-targeted study was designed to monitor the changes in the composition of volatile organic compounds (VOCs), aromatic amino acid (AAA) metabolites and lipids in base and sparkling wines obtained in two different vineyards sites and with two cluster thinning treatments. Sensory analysis of the wines was performed for complementary quality assessment.

2. Results

2.1. Weather Conditions

The three seasons considered in the present investigation showed several differences in terms of temperatures and rain. However, they could be considered representative of the recent meteorological trend of the Friuli Venezia Giulia region (Supplementary Materials Figure S1).

Considering all the meteorological data together, the seasons 2017 and 2019 were similar in terms of temperatures. In terms of rainfall, the summer part of the vegetative cycles revealed similarities between the years 2018 and 2019. The particular meteorological behavior of the three seasons was responsible for some of the differences in the yield and compositional parameters described in the following paragraphs.

2.2. Yield and Basic Grape Quality Parameters

Yield parameters were significantly affected by all treatment, site, and season factors (Table 1). Starting with the thinning treatment, as expected, a significant reduction in the number of clusters and yield was recorded in the case of CT vines, slightly offset by a non-significant increase of the cluster weight. Moving on to the effect of the site, the average values calculated for the FG and FCO vineyards highlighted a significantly higher number of clusters and yield in the first location. The higher number of clusters registered in FG during the three seasons was counterbalanced by reducing the average cluster weight. Moreover, seasonal factors also affected the yield parameters, according to the meteorological characteristics described above. Thus, the mean number of clusters was significantly higher in the year 2018, accounting for a parallel difference in the yield parameter, partially offset by reducing the average cluster weight. On the contrary, the lack of rain that characterized the maturation period in 2019 significantly affected the cluster weight. In 2017, the same parameter was higher because of the abundance of water during the ripening period. To our knowledge, the high yield registered in the season 2018 must be considered exceptional not only for Ribolla Gialla but also for all the other varieties grown in the Friuli Venezia Giulia region [27]. This result occurred because of higher bud fertility and the meteorological course of the season that ensured optimal temperatures and rain distribution. No interaction among factors was ascertained for the yield parameters.

Alongside the production data, the technological maturation parameters of the grapes were also measured. The results revealed that cluster thinning did not significantly impact grape maturation parameters than untreated vines (UNT), even if a non-significantly higher Brix and a lower value of TA were recorded. Concerning the production site, also, in this case, Brix and TA were not significantly different. Still, the grapes of the FCO location highlighted tendentially higher values of both parameters. The lower TA analyzed in FG grapes could be explained by the different meteorological conditions of the site, even if it must be considered that the grapes were harvested 4-to-7 days earlier in the FCO

location. This advance could account for the higher values of the TA analysis. The value of pH was instead significantly higher in the FG location, as it is inversely correlated with the TA [28]. A lower accumulation of total soluble solids (TSS) in the grape berries was observed in 2018 due to the higher yield of the vines. On the contrary, TA and pH were not significantly different between the seasons. Still, higher values of the former parameter were recorded in 2017 and 2018 as a result of the already mentioned higher yield that delayed the maturation dynamics.

Table 1. Yield and basic grape parameters of Ribolla Gialla grape subjected to the cluster thinning in two vineyard sites and in seasons from 2017–2019.

Parameter	Treatment (T)			Site (S)			Year (Y)				Y × T	S × T	Y × S	Y × S × T
	UNT	CT	Sig. F [1]	FG	FCO	Sig. F	2017	2018	2019	Sig. F				
N° clusters/vine	32.89 a [2]	25.20 b	***	35.18 a	22.91 b	***	27.13 b	31.99 a	28.02 b	**	ns	ns	ns	ns
Cluster weight (g)	190.67	200.20	ns	180.45 b	210.43 a	*	206.96 a	198.32 ab	181.03 b	***	ns	ns	ns	ns
Yield (kg/vine)	6.40 a	4.69 b	***	6.36 a	4.73 b	***	5.38 b	6.26 a	5.00 b	***	ns	ns	ns	ns
Yield (t/ha)	20.11 a	14.75 b	***	18.92 a	15.94 b	***	16.90 b	19.66 a	15.74 b	***	ns	ns	ns	ns
TSS (°Bx)	17.44	17.98	ns	17.61	17.81	ns	18.03 ab	16.79 b	18.32 a	***	ns	ns	ns	ns
TA (g/L) [3]	6.94	6.74	ns	6.66	7.03	ns	6.61	7.11	6.81	ns	ns	ns	ns	ns
pH	3.25	3.27	ns	3.30 a	3.22 b	***	3.27	3.27	3.24	ns	ns	ns	***	ns

[1] Data were analyzed by three-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Tukey's HSD test ($p < 0.05$). [2] Different letters (a, b) identify significantly different means. UNT—untreated control; CT—cluster thinning; FG—Friuli Grave; FCO—Friuli Colli Orientali. [3] TA—titratable acidity expressed in tartaric acid.

2.3. *Wine Basic Compositional Parameters*

The alcohol content of the sparkling wines was affected by both the treatment and the season. At the same time, similar values were shown when comparing the vineyard sites (Table 2). In detail, cluster thinning had a significant, positive impact on the alcohol level, according to the accumulation of TSS in the same year. Between the seasons, the lowest values of the same parameter were recorded in 2018, with no differences between the remaining two seasons. Vineyard site and year significantly affected titratable acidity, and no effects were revealed regarding the thinning treatment. As far as the comparison between the two vineyard sites is concerned, the TA parameter was in line with grapes analysis, meaning that wines from the FG district contained a significantly lower concentration of TA compared to the wines from FCO. Additionally, the highest amount of TA was found in the wines obtained in 2017 (7.68 g/L) and 2019 (7.51 g/L), and the lowest in 2018 (7.10 g/L). Furthermore, the thinning treatment and the location factor showed no significant effect on the pH value. At the same time, seasonal differences between 2017 and 2019 were reported. Although the interaction between all factors revealed significant trends of pH between locations and treatments between the seasons, the range of variation of such parameter was limited and probably not affecting the overall quality of the wines.

Table 2. Characteristics of Ribolla Gialla sparkling wine composition at different cluster thinning levels, two vineyard sites and in season from 2017–2019.

Parameter	Treatment (T)			Site (S)			Year (Y)				Y × T	S × T	Y × S	Y × S × T
	UNT	CT	Sig. F [1]	FG	FCO	Sig. F	2017	2018	2019	Sig. F				
Alcohol (% v/v)	10.94 b [2]	11.41 a	***	11.23	11.14	ns	11.51 a	10.42 b	11.62 a	***	ns	ns	ns	ns
Reducing sugars (g/L)	0.99 b	1.26 b	*	1.07	1.17	ns	0.20 c	1.85 a	1.32 b	***	ns	ns	ns	ns
TA (g/L) [3]	7.50	7.36	ns	7.21 b	7.65 a	**	7.68 a	7.10 b	7.51 ab	**	ns	ns	ns	ns
pH	3.16	3.14	ns	3.16	3.14	ns	3.17 a	3.15 ab	3.12 b	*	**	ns	ns	**

[1] Data were analyzed by three-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated busing Tukey's HSD test ($p < 0.05$). [2] Different letters (a, b) identify significantly different means. UNT—untreated control; CT—cluster thinning; FG—Friuli Grave; FCO—Friuli Colli Orientali. [3] TA—titratable acidity expressed in tartaric acid.

2.4. *Volatile Profile of Base Wines and Sparkling Wines*

Sixty-two and sixty-six compounds, present in Ribolla Gialla base (Supplementary Materials Table S1) and sparkling wines (Supplementary Materials Table S2), respectively, were semi-quantified and separated according to their chemical classes (monoterpenes, norisoprenoids, aldehydes, alcohols, esters, acids, and ketones) and the three-ways ANOVA was applied to investigate the main effects of crop level, location of the vineyard and season.

In the case of base wines (Supplementary Materials Table S1), the CT treatment significantly affected only a very scarce number of individual aroma compounds compared to the control. Among monoterpenes, linalool was the only significantly increased compound after the thinning treatment. Similarly, the concentration of isoamyl alcohol and *cis*-3-hexenol was significantly enhanced by CT, together with the total concentration of alcohols. Concerning the other aroma classes, norisoprenoids and aldehydes were nearly unaffected by the CT treatment. At the same time, a non-significant increase in esters and reduction of acids and ketones occurred.

On the other hand, more metabolites were affected by vineyard location and mostly by the season. Several compounds revealed higher concentrations in FCO wines, including sensory-important linalool and limonene. Among norisoprenoids, β-damascenone contributed the most to the significant differences between sites and seasons. Concerning the groups of fermentation-derived compounds, an increased concentration of alcohols and aldehydes was predominantly observed in the wines originating from the FCO location. Similar findings apply to most esters, acids, and ketones, apart from methyl ethyl succinate, diethyl succinate, ethyl 9-decanoate and 2-methylthiolan-3-one significantly prevailed in the wine samples produced from the FG vineyard located in the valley floor. The statistical analysis revealed many differences related to the seasonal factor, mostly caused by the meteorological peculiarities. The concentration of most of the examined compounds was higher in the year 2017, while the lowest values were found in the season 2018 because of both the higher yield and the lower accumulation of sugars in berries.

As in the base wines, the location and mostly seasonal factor had a significant effect on the volatile profile of sparkling wines (Supplementary Materials Table S2). However, the products obtained after the secondary fermentation showed a higher degree of sensitivity to the CT treatment, with a significant increase of the concentration of citronellol, linalool and β-myrcene by 13%, 9% and 14%, respectively, than UNT. Among the aldehydes, *trans*-2-hexenal and acetaldehyde increased significantly in CT sparkling wines. A similar effect was also observed in the case of alcohols, where isoamyl alcohol, 2-phenylethanol and isobutanol accounted for the most important higher alcohols found in sparkling wines. Ethyl lactate, diethyl succinate, and ethyl-9-decanoate were shown to be the most affected esters in a positive manner. The composition of acids was similar between base wines and sparkling wines. The effect of thinning concerned mainly the concentrations of acetic acid and benzoic acid.

Significantly higher concentrations of primary aromas of monoterpenes (citronellol, linalool, and nerol) and C-13 norisoprenoids (β-damascenone and 1,1,6-trimethyl-1,2-dihydronapthalene (TDN)) were detected in the sparkling wines originating from the FCO vineyard. The FCO wines were also characterized by higher production of acetaldehyde, n-hexanol, *trans*-3-hexenol, and acetic acid. At the same time, the concentration of esters was almost unaffected by the location.

In addition to the results of the three-way ANOVA, which showed the strong effect of the vintage and only a few significant differences between treatments and locations, the main aim of the experiment was to ascertain if a yield reduction could promote an increase of aroma compounds in base and sparkling wines. To eliminate the season effect, data were normalized by season and location, and thereafter one-way ANOVA was applied (see materials and methods for details) to understand the pure effect of cluster thinning. To better show the treatment effect, data were presented in a heatmap as log2-fold change (CT/UNT) separately for FCO and FG and for the base and sparkling wines (Figure 1).

Figure 1. Heatmaps represent log2-fold change (CT/UNT) of the volatile compounds in the FG and FCO vineyard sites and in the base (**A**) and sparkling wines (**B**), separately. Blue and red boxes indicate lower and higher concentrations in CT, respectively. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between treatments after one-way ANOVA. Heatmaps were created based on the averaged values from all three vintages.

The results obtained for base wines (Figure 1A) highlighted the significantly higher effect of cluster thinning in the FG vineyard site, with increased concentration, especially for alcohols (methionol, isoamyl alcohol, 3-methyl-1-pentanol, and 2-phenylethanol), aldehydes (hexanal and furfural), and esters (ethyl acetate, isoamyl lactate, methyl decanoate, isoamyl octanoate, and ethyl hydroxybutanoate). On the contrary, a significant reduction of geraniol, β-damascenone, and total C-13 norisoprenoids was shown as an effect of thinning treatment in the case of the FCO base wines. Only 3-methyl-1-pentanol and 3-methylbutyric acid showed a significant increase in CT wines compared to the control.

Similar results were also obtained for the sparkling wines (Figure 1B), and their trend was mostly in line with what was just explained for base wines. In addition to groups of alcohols, aldehydes, and esters, citronellol, linalool, and β-myrcene from monoterpenes increased significantly in the FG samples after CT. A significantly higher concentration of vitispirane was detected in the same samples, together with *trans*-2-hexenal and *cis*-3-hexenol. Contrary to the described results, only ethyl-2-OH-4-methylpentanoate and nonanoic acid appeared to have significantly higher concentrations in UNT wines from the lowland FG vineyard site. Furthermore, the aroma composition of sparkling wines from the FCO vineyard revealed certain similarities with base wines. Even in this case, the number of statistically significant differences was noticeably smaller, with generally lower levels of substances present in the CT samples. Therefore, only α-terpineol, 3-methyl-1-pentanol, and ethyl-9-decanoate were statistically impacted by cluster thinning.

2.5. Lipid Profile of Base Wines and Sparkling Wines

In Supplementary Materials Tables S3 and S4, eighteen and nineteen lipid compounds were found by UHPLC/MS-MS analysis in base and sparkling wines. Most of the compounds analyzed are represented by fatty acids. At the same time, other molecules of interest belonged to the group of glycerolipids, sterols, fatty esters and prenols. Two compounds were found in higher concentration in both base and sparkling wines, in detail C16 palmitic acid and C18 stearic acid, both belonging to the group of long-chain fatty acids (LCFAs).

The analysis of variance performed on the lipids in base wines (Supplementary Materials Table S3) showed no significant impact of cluster thinning, even if small differences in concentration in favor of CT appeared in most cases. Minimal differences were also observed by comparing both wine-growing locations. It turned out that only long-chain saturated myristic acid and polyunsaturated linolenic acid were present in significantly higher concentrations in FG and FCO base wines, respectively. Even in the case of lipids, a more significant impact of the season was ascertained for fatty acids and sterols, and in general lower concentrations were reported in 2018 and 2019, while higher concentration characterized the first season of the trials.

Moving on to sparkling wines (Supplementary Materials Table S4), the analysis of variance showed that the cluster thinning treatment moderately affected the lipid concentrations, as only unsaturated palmitoleic acid appeared to be significantly different from the control (0.41 mg/L to 0.31 mg/L). However, the percentage variation between CT and UNT samples was, in general, moderate, except for lupeol, where the concentration decreased by 50% in wines after thinning. In addition, palmitoleic acid together with ergosterol was the only compound significantly different between locations. Due to low concentrations, most of the analyzed lipids did not show any trend related to the vineyard site. Furthermore, in this case, a strong impact of the seasonal factor was ascertained, with a higher concentration of lipids in the last year of the trial.

As previously described for the VOCs, one-way ANOVA was performed on normalized data for the two locations separately to better understand the effect of cluster treatment. As for the base wines (Figure 2A), not much impact was ascertained since only 1-monopalmitoeoyl-rac-glycerol and palmitoleic acid resulted statistically different for FG and FCO wines, respectively. In addition, lupeol was the only prenol analyzed, and its concentration was found to be consistently higher in UNT wines in both vineyard sites,

with a higher value in the FG site located on the valley floor, although the difference did not appear to be statistically significant. Conversely, two UFAs (palmitoleic acid and oleic acid + *cis*-vaccenic acid), two SFAs (stearic acid and lignoceric acid), 1-oleoyl-rac-glycerol, and ergosterol were significantly reduced by cluster thinning in the case of FCO sparkling wines, apart for 1-monopalmitoleoyl-rac-glycerol, whose concentration increased in CT wines produced in FG (Figure 2B). Moreover, ethyl stearate was additionally quantified in sparkling wines, and its concentration appeared to decrease with CT. By excluding few exceptions, the heatmap of Figure 2B more clearly shows that the effect of the CT treatment was greater in wines produced in the lowland FG area compared to the FCO wines.

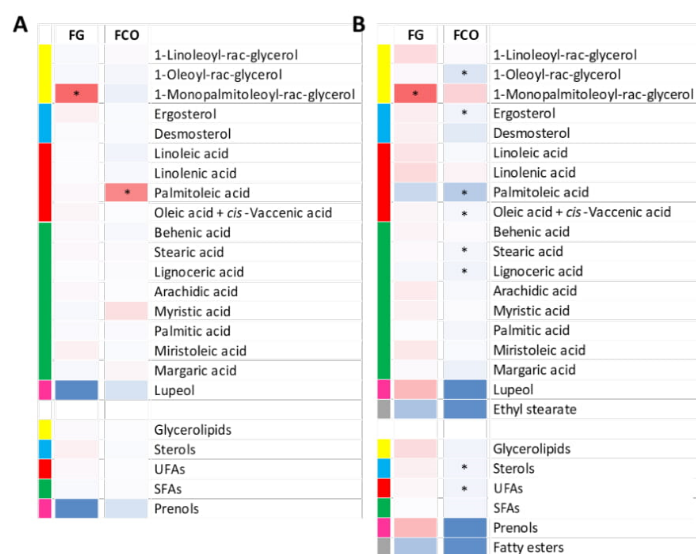

Figure 2. Heatmaps represent log2-fold change (CT/UNT) of the lipid compounds in the FG and FCO vineyard sites and in the base (**A**) and sparkling wines (**B**), separately. Blue and red boxes indicate lower and higher concentrations in CT, respectively. Asterisks indicate significant differences ($p < 0.05$) between treatments after one-way ANOVA. Heatmaps were created based on the averaged values from all three vintages.

2.6. Aromatic Amino Acid Metabolites Profile in Base Wines and Sparkling Wines

The results of AAA metabolites analysis with abscisic acid (Aba) and its glucoside (Aba-Glu) revealed a poor separation between control and CT samples in the base wines (Supplementary Materials Table S5). This being said, only N-acetyl-L-tyrosine ethyl ester (N-Tyr-EE) and indole 3-lactic acid glucoside (Ila-Glu) belonged to the class of compounds that were significantly and positively affected by the thinning treatment, as their concentrations increased in CT samples by 41% and 27%, respectively. N-acetyl serotonin (N-SER), on the other hand, decreased significantly after crop removal from 0.50 mg/L to 0.42 mg/L. In general, the amount of amino acids presents in our samples, such as Tyr and Phe, did not change by comparing UNT and CT samples, except for Trp. Furthermore, a comparison between the two locations also yielded mixed results. Tyl proved to be the most abundant compound in the FG samples, and the same was also true for Ila-Glu, phenyl lactic acid (Ph-LA), and Aba. In the FCO samples Tyr, Phe, kynurenic acid (KYNA), an indole 3-acetic acid (IAA) prevailed. The vast majority of AAA metabolites showed their statistical significance in the season factor, which leads to a greater number of interactions between year and location factors.

In sparkling wines (Supplementary Materials Table S6), the thinning treatment increased the concentration of Tyr (+40%) and N-Tyr-EE (+46%) in the CT samples, while the

Trp-EE (−30%) and IAA (−39%) were significantly higher in the UNT samples. With respect to crop removal, the concentration variability was highest for those four compounds. From the results, an increase of KYNA, Tol, Ph-LA and Aba in CT was furthermore evident. At the same time, the rest of the AAA metabolites were higher in the UNT samples. Interestingly, most of the compounds prevailing in the FCO base wines maintained this ratio even after secondary fermentation, except for the phenethyl alcohols OH-Tyl and Tyr. The results of the analysis of the sparkling wine mostly coincided with the results of the base wine analysis, except for tyrosine ethyl ester (Tyr-EE), which scored 2.5 times higher concentration in FCO sparkling wines compared to the base wines, and sulfonated tryptophol (Tol-SO3H), which was additionally detected in sparkling wines. The content of Tol-SO3H was also significantly higher in the FCO wines. Considering the year factor, its high impact has been demonstrated again, as most of the analyzed compounds showed a great statistical significance. However, the only three-way interaction was identified for Tyr and Phe ($p < 0.001$ and $p < 0.05$, respectively).

The results of the subsequent one-way ANOVA of AAA metabolites on transformed data (see Materials and Methods for details) are presented in Figure 3. By considering the vineyard sites separately, the thinning treatment in base wines (Figure 3A) resulted in a significant increase of Trp and Aba in the FG site. In contrast, the amount of N-Tyr-EE increased significantly in the FCO location. A mild increase of KYNA was shown in the FG vineyard, whether a non-significant growth of Ila-Glu was noticed in FCO. Moreover, the FG wines showed a lower concentration in UNT for N-SER and glucoside of Aba. By observing the results obtained after sparkling wines analysis (Figure 3B), only KYNA, N-Tyr-EE and phenylacetic acid (Ph-AA) resulted in statistical significance in the FG samples. However, KYNA and N-acetyl Tyr-EE only appeared to be in higher concentration in the CT wines. Meanwhile, it appeared that CT samples presented an elevated concentration of a group of compounds, including Tyr, N-Tyr-EE, Ph-AA, and Ila, as against the UNT samples.

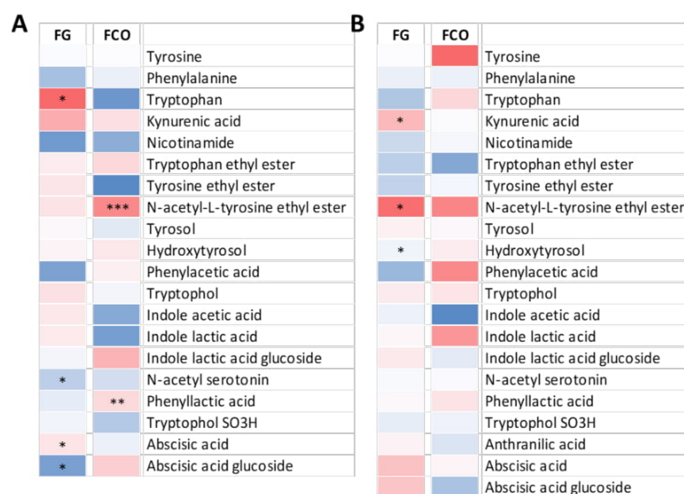

Figure 3. Heatmaps represent log2-fold change (CT/UNT) of the aromatic amino acid metabolites in the FG and FCO vineyard sites and in the base wines (**A**) and sparkling wines (**B**), separately. Blue and red boxes indicate lower and higher concentrations in CT, respectively. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) between treatments after one-way ANOVA. Heatmaps were created based on the averaged values from all three vintages.

2.7. Sensory Attributes

The sensory analysis results were normalized by the panelist before any processing and are reported in radar plots (Supplementary Materials Figures S2, S3 and Figure 4). Furthermore, in this case, a large effect of the season appeared, with the wines produced in the last season being preferred by the panel. By considering the overall effect of cluster thinning, the effects on most descriptors were negligible, with significantly lower values only for the dry vegetable character (Supplementary Materials Figure S2). On the contrary, greater differences emerged regarding different organoleptic descriptors relative to the grape production areas (Supplementary Materials Figure S3). In particular, the sparkling wines produced on the FCO site were rewarded by general pleasantness.

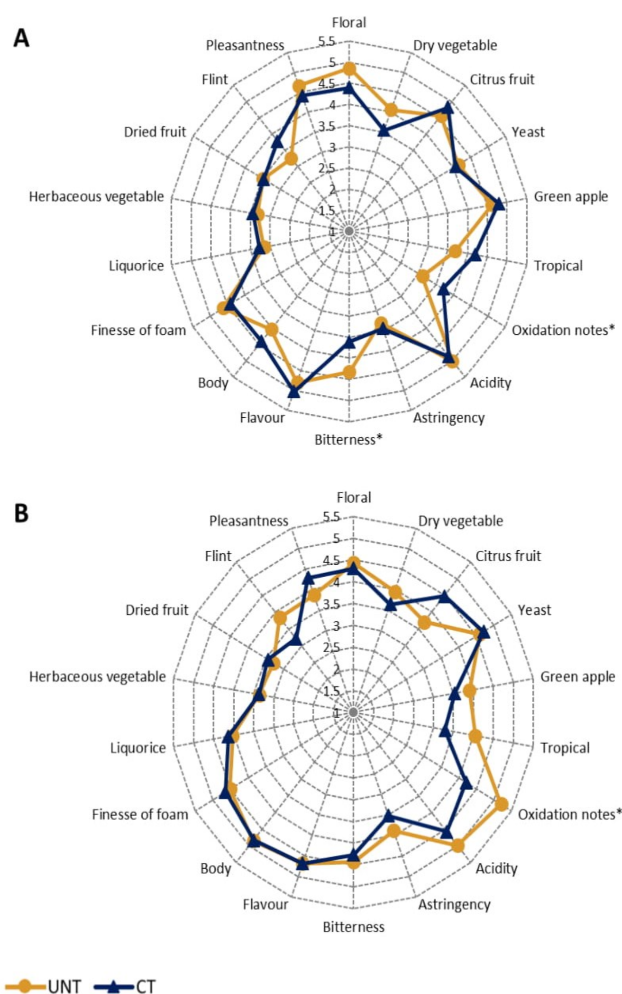

Figure 4. Effect of cluster thinning on the organoleptic characteristics of Ribolla Gialla sparkling wines in FCO (**A**) and FG (**B**). Average values were obtained from 2017–2019. Yellow and blue lines represent untreated (UNT) and treated (CT) samples, respectively. Asterisks (*) indicate statistical significance ($p < 0.05$) for each sensory attribute.

Furthermore, to show the effect of cluster thinning on the sensory properties of sparkling wines, data were standardized by season and site and processed through one-way ANOVA. Figure 4A shows the three-year average of the panel evaluation of the sparkling wines in the FCO area. Although not statistically significant, a higher preference for the overall pleasantness was given to the UNT wines, mostly due to significantly lower oxidation notes coupled with higher intensities of the floral and dry vegetable sensorial component. On the other hand, the mouthfeel descriptor of bitterness was significantly higher in the same UNT wines. On the contrary, CT showed a positive effect on the general pleasantness of the wines produced in the FG site (Figure 4B). Compared to the FCO location, the FG sparkling wines produced with UNT grapes were judged to be significantly more oxidized, and a higher astringency was reported. On the other hand, at the olfactory level, the CT sparkling wines showed a non-significant increase in the citrus sensation and a reduction in the tropical and green apple notes, which was combined with lower intensities of acidity perceived at mouthfeel level.

2.8. Multivariate Analysis of Sparkling Wines

The PCA biplot of all three groups of compounds detected in the sparkling wines is presented in Figure 5; looking at the spatial distribution, most of the wine samples from the 2017 harvest are located on the positive side of the PC1. Those from 2018 are present in the third quadrant. The samples from 2019 are entirely positioned in the second quadrant of the PCA biplot. After data normalization, the cluster thinning effect was observed in the FG and FCO locations independently.

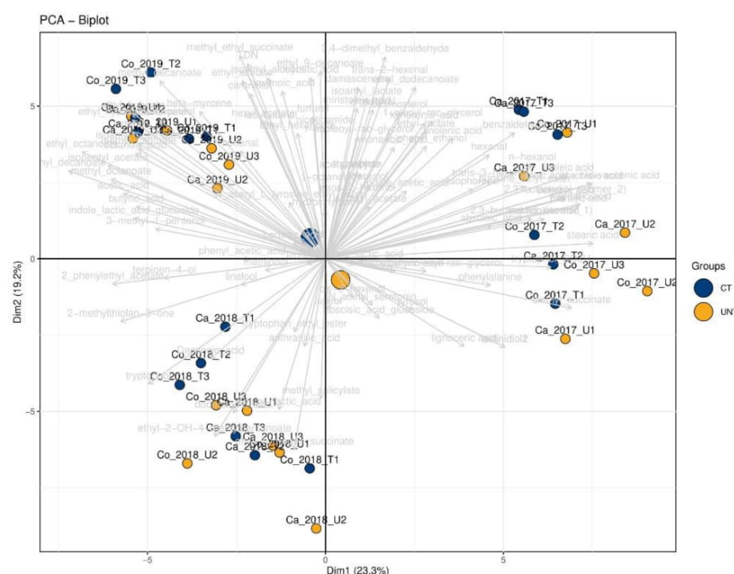

Figure 5. Principal component analysis biplots of sparkling wines from both vineyard sites. Small blue and yellow dots represent CT and UNT samples, respectively, and larger dots represent the centroids of respective samples. Light gray lines represent the loadings of the analyzed metabolites. Each sample label indicates the location, treatment, harvesting year and biological replicate.

In the sparkling wines from the FG vineyard (Figure 6), the first two components accounted for 56.4% of the data explained variance, with 36% and 20.4% contribution of PC1 and PC2, respectively. Treatments were well separated, with all UNT wines mostly located on the negative side of PC1 and associated with dry fruit, tropical, oxidation notes, astringency green apple, dry vegetable, and bitterness. UNT wines were also characterized by actinidiol (isomer 2) and 1-octanol. Most CT wines were located on the positive side of

PC1; they were characterized by floral, yeast, citrus fruits, body, and overall pleasantness. Pleasantness was located in the fourth quadrant, together with *trans*-3-hexenol, methyl caproate, and hexyl acetate. The rest of the ethyl esters were in the first quadrant, coupled with higher alcohols and aldehyde furfural.

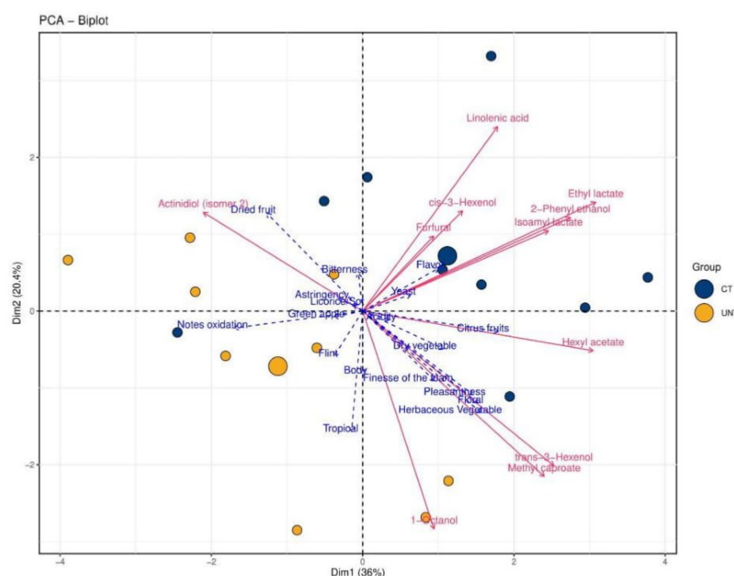

Figure 6. Principal component analysis biplots of sparkling wines from FG. Small blue and yellow dots represent CT and UNT samples, respectively, and larger dots represent the centroids of respective samples. Red lines represent significant loadings of volatiles, lipids, and AAA metabolites; dotted blue lines represent the loadings of sensory descriptors.

As far as the sparkling wines from the FCO parcel are concerned, the first two PCs accounted for 73.1% of the data explained variance, with 56.1% and 17% contributions of PC1 and PC2, respectively (Figure 7). In this case, the treatments were not clearly separated. However, most of the CT wines were located on the positive side of PC1 and characterized by both negative (licorice/sotolone) and positive descriptors (citrus fruits, body, and tropical). They were correlated with most ethyl esters and monoterpenes, such as β-myrcene and geraniol. The vast majority of the UNT wines were on the negative side of PC1. The samples were associated with dried fruit and dry vegetables, as well as oxidation notes, acidity, flavor, and astringency to a less extent, and finally also with overall pleasantness. Palmitic acid and 2,3-butanediol (isomer 2) were positioned in the same quadrant as excluding pleasantness and more negative sensory descriptors. In contrast, anthranilic acid was associated with herbaceous vegetable and foam finesse.

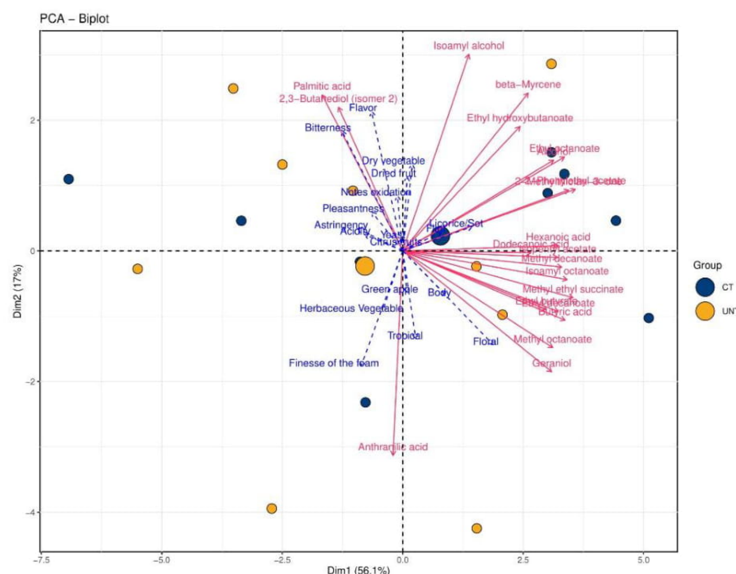

Figure 7. Principal component analysis biplots of sparkling wines from FCO. Small blue and yellow dots represent CT and UNT samples, respectively, and larger dots represent the centroids of respective samples. Red lines represent significant loadings of volatiles, lipids, and AAA metabolites; dotted blue lines represent the loadings of sensory descriptors.

3. Discussion

As already pointed out in the introduction, the effect of cluster thinning can be significantly different depending on the initial crop level. Thus, in over-crop conditions, the elimination of a certain percentage of clusters significantly improves grape quality composition, while the result could be negligible when grapevines stand in equilibrium. Moreover, the meteorological course of the seasons investigated promoted a significant impact on the biosynthesis of aroma precursors and thus on wine aromas, requiring a seasonal normalization of the data to better understand the effect of cluster thinning and location. The application of cluster thinning promoted increased VOCs concentration in the sparkling wines from the FG location. At the same time, the effect was minimal for the FCO site.

Among the monoterpenes, cluster thinning positively affected the concentration of citronellol, linalool and β-myrcene in the FG sparkling wines, in agreement with other findings [9,10,29]. Although our results show a lower concentration, it turned out that linalool and citronellol were among the most abundant terpenes in the commercial Ribolla Gialla sparkling wines [30]. Considering the effects of cluster thinning in the two locations separately, only vitispirane, among the C-13 norisoprenoids, was increased by the technique in FG sparkling wines, which contrasts with previous studies in which authors mostly found increased β-damascenone [10,31].

The CT wines from the FG location also showed higher concentrations of one of the essential sensory carbonyl compounds, acetaldehyde, which could be due to differences in Brix between locations, since acetaldehyde is considered a leakage product of the alcoholic fermentation by yeast, where sugar represents the primary substrate [32]. In this location, negligible effects of cluster thinning were ascertained on the concentration of aldehydes, in agreement with the findings of Moreno Luna et al. [14].

Concerning alcohols, cluster thinning affected 7 out of 12 compounds. The contribution of higher alcohols to wine aroma may vary from the more pleasant honey, rose, and floral attributes (e.g., 2-phenylethanol, predominantly present in the CT wines) to pungent

aromas [33]. Nevertheless, the pair of higher aliphatic alcohols isobutanol and isoamyl alcohol present in the CT wines can suppress the fruity notes, but not leather, animal aromas, and therefore, play a negative role in wine aroma quality [34]. Again, cluster thinning promoted a higher concentration of alcohols when applied to the FG grapevines.

The group of esters was the most representative class of volatile compounds in this study. Two different groups of esters were dominant: the acetates of ethanol and higher alcohols and the so-called fatty acid esters that are synthesized by esterification of fatty acids with ethyl alcohol. Furthermore, for this group of compounds, cluster thinning did promote a significant increase in the FG wines, in base and mostly in sparkling wines. For instance, the concentration of isobutyl acetate was the most elevated among all the FG thinned wines. Interestingly, the same compound appeared to be an important discriminator for sparkling wines produced from the Manzoni Bianco grape variety [35]. It contributes to the sweet fruit aroma in wine [36].

The effect of cluster thinning was further researched also by analyzing the lipid composition of the samples obtained from the sparkling wines. In contrast to the base wines, the monounsaturated C16:1 palmitoleic acid appeared to be statistically impacted by both treatment and location. UFAs are required for *Saccharomyces cerevisiae* to grow under anaerobic conditions. Together with oleic acid (C18:1), palmitoleic acid represents the main UFA of *S. cerevisiae* [37]. The yeast autolysis, which occurred after the secondary fermentation, could, therefore, increase the amount of palmitoleic acid in the sparkling wines. While the amount of C16:1 in the sparkling wines from the FG site appeared to be higher in the control wines, the concentration of linolenic acid (C18:3) was higher in the CT wines. This compound represents one of the major components of the total lipids in grapes [38]. It is released into the grape juice, where acts as a substrate for lipoxygenase and hydroxyperoxide lyase activities, which are responsible for forming C6-aldehydes and alcohols, which contribute to the "green flavor" [39].

Fatty acids are not only important for the aroma properties of wine, but they also actively influence tactile perceptions in wines, which also includes wine foaming [40]. Different forms of fatty acid molecules can affect the stability of the foam or foam collar height in the sparkling wine in different ways. In a study on Portuguese sparkling wines [41], it was reported that the presence of surface-active monoacylglycerols of palmitic and stearic acid promoted and stabilized sparkling wine foam, while Gallart et al. [21] discovered a positive correlation between foamability and the presence of esterified fatty acids. In our case, the overall production of fatty acids and their derivatives (e.g., fatty esters) decreased in the CT Ribolla Gialla wines, regardless of location, which could consequently lead to reduced foaming or a destabilized foam collar.

Additionally, monoacylglycerols are also used in the food industry as stabilizers of foams and emulsions, so they are expected to have a certain effect on the foam properties of sparkling wines [42]. Among those, the concentration of 1-monopalmitoyl-ra-glycerol (monopalmitin) has significantly increased after the thinning treatment in FG wines. This glycerolipid is foremost present in the grape skins and seeds [43,44], but it can also be found in wine due to yeast autolysis [45].

Finally, ergosterol and desmosterol were detected among sterols. Ergosterol, in particular, is especially important, as it helps to preserve the structural integrity of yeast membranes in stressful environmental conditions, with an emphasis on yeast ethanol tolerance [46]. It may also stimulate producing esters, higher alcohols and volatile fatty acids in white wines [47]. However, our results showed a decreased number of sterols in CT sparkling wines, especially from FCO, which could lead to sluggish or stuck fermentation, which is more common during the white winemaking process, as the process of clarification can lead to heavy lipid losses [19].

In base wines, the concentration of ethyl ester of N-Tyr-EE, glucoside of Ila-Glu and of N-SER was significantly higher in the case of CT wines. N-Tyr-EE and N-SER are fermentative products of the yeast. However, their amount in the wine is determined by the number of amino acids found in grapes, such as Tyr and Trp. Moreover, N-Tyr-EE

plays an active role as a Trp synthase inhibitor in regulating Trp synthesis and metabolism in yeast [48]. The Ila aglycon of hydrolyzed Ila-Glu can react with SO2 and produce the sulfonated indole Ila-SO3H. This could be of particular importance, as the sulfonation of indoles could lead to forming 2AA, which is correlated with the atypical aging off-flavor [24,25]. Different winemaking procedures, as well as wine aging, can significantly affect the amount of Ila-Glu present in wines. Arapitsas et al. [26] reported a higher concentration of Ila-Glu in young wines than sparkling wines.

A further investigation carried out in our study by employing one-way ANOVA showed some interesting results when comparing the effect of cluster thinning in two different locations. Namely, the amount of Trp in wines obtained from the FG wine-growing location was significantly higher in the CT samples than the UNT ones. This reduces the risk of atypical aging aroma formation since Trp is directly connected with the synthesis of 2AA [25]. Compared to the other white wines, Ribolla Gialla is characterized by a lower concentration of Trp compared to Malvasia [49] and Chardonnay [50], while to study Tudela et al. [51], the Trp content ranged from 6.5–574.9 μg/L in Cava sparkling wines, in line with our results.

In sparkling wines, the cluster thinning treatment led to increased the concentration of Tyr and the ethyl ester of N-acetyl-L-Tyr, while for most of the other compounds, there was a significant reduction, especially for indoleacetic acid (IAA), the ethyl ester of Trp and for Phe. The sulfonation process of IAA is largely similar to the sulfonation of Ila, described previously. As it turned out, also IAA-SO3H could promote forming aromatic aminobenzenes in wines (2AA). Such an outcome is more probable in white wines and sparkling wines compared to red wines [26]. Therefore, from the results presented in our study, it emerges that cluster thinning represents an effective tool to reduce this component by avoiding atypical aging problems. By comparing the previous results of commercial sparkling wines from the Ribolla Gialla variety [31], it has been observed that the concentration of N-acetyl-L-Tyr and IAA was higher in wines already present on the market. At the same time, the content of free Tyr and Trp-EE appeared to be greater in CT and UNT sparkling wines, respectively.

Olfactory and taste attribute rated in sparkling wines showed that significantly lower values emerged in the CT samples only for the dry vegetable sensory descriptor, considering the overall cluster thinning treatment. Furthermore, the attributes of citrus and green apple were related to the sparkling wines produced from the vineyard site close to the hills. Several authors associate the green apple descriptor with ethyl esters in wine (e.g., ethyl hexanoate), whose concentration prevailed in the FCO wines [52,53], while Alessandrini et al. [54] demonstrated that terpenes were more abundant in grapes from the high vineyard site, which agrees with our results. When the cluster thinning effect was analyzed in two separate vineyard sites, in the FCO location, overall pleasantness was assigned to the UNT samples due to their more pronounced floral aroma. Still, simultaneously, the same samples were characterized by increased bitterness of the wines, to which some esters may contribute [55]. On the contrary, general pleasantness was attributed to the CT sparkling wines from the FG vineyard site. This was most likely due to the higher amount of monoterpenes. However, the fact that oxidative notes were detected in the UNT wines also contributed to this result. According to Mayr et al. [56], the most important contributors to the oxidative off-flavor were found to be methional and 2-phenylacetaldehyde, in addition to long-chained aldehydes like *trans*-2-nonenal, *trans*-2-octenal, *trans*-2-hexenal, as well as benzaldehyde, furfural, and hexanal and some alcohols, such as 1-octen-3-ol and eugenol.

4. Materials and Methods

4.1. Chemicals and Reagents

HPLC-grade solvents dichloromethane, n-pentane, and methanol, LC–MS-grade methanol, acetonitrile, 2-propanol, chloroform, formic acid, ammonium formate, ethyl heptanoate, 1-heptanol, 2-octanol, ethyl hexanoate-d11, 3-(2-hydroxy ethyl)-indole, kynurenic

acid, d-tryptophan methyl ester, L-tyrosine-ethyl ester, N-acetyl tyrosine-ethyl ester, and 3,5-di-tert-4-butylhydroxytoluene (BHT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). C7–C30 n-alkane solution in n-hexane was purchased from Supelco (Bellefonte, PA, USA), while cholesterol-d7 and octadecanoic acid-d3 were obtained from CDN Isotopes (Quebec, QC, Canada). The chemical standards used to determine aromatic amino acid metabolites and lipid molecules were purchased from Aldrich-Fluka-Sigma S.r.L. (Milan, Italy), except for tryptophol-2-sulfonate (Tol-SO3H), indole-lactic acid-2-sulfonate (Ila-SO3H), and indole-acetic acid-2-sulfonate (IAA-SO3H) that were synthesized as described [26].

4.2. Vineyard Sites

The experiment was carried out during 2017, 2018 and 2019, in two different commercial vineyards of Ribolla Gialla, located in two different DOC districts of the FVG region. The first vineyard chosen is located in Corno di Rosazzo at the foot of the hills, sited in the Friuli Colli Orientali and Ramandolo (FCO) district (46°00′19.1″ North; 13°26′30.6″ East; elevation 94 m a.s.l.), with a planting density of 3367 plants/ha (2.7 m between the rows and 1.1 m within the row). The vineyard soil was classified as Eutric Cambisols and was characterized by a silt-clay-loam texture with no coarse. In such soil, the root penetration is limited to a depth of about 100–150 cm due to the lack of oxygen during the rainy periods of the growing season, as there is inadequate water drainage. During the summer season, the vines suffer from periods of water stress, as there is no possibility of irrigation. The second vineyard was located south of the town of Casarsa della Delizia, on the plain of Friuli Grave (FG) district (45°55′21.9″ North; 12°50′54.2″ East, 35 m a.s.l.). The planting density was 3086 plants/ha (2.7 m between rows and 1.2 m within the row). In this area, the typical soil was classified as Cutanic Luvisols. It was characterized by a silt-clay-loam texture with a low presence of skeleton. Unlike the first vineyard site, root deepening in this soil was not limited since there is adequate water drainage, even at-depth. Therefore, the conditions of anoxia are easily avoided. In addition, the water stress situations were resolved during the summer season thanks to using a drip irrigation system. In both vineyards, the clone used was the VCR 100 (Vivai Cooperativi Rauscedo, Italy) grafted onto the Kober 5BB rootstock. The training system adopted is a single arched Guyot.

4.3. Vine Treatments and Harvest of the Grapes

A completely randomized experimental design was set up with three replicates for each treatment compared on both vineyard sites. To optimize the choice of plants used, an evaluation of the number of clusters/vine was made in each season to correctly select the plants to use in the experiment, standardize the production where necessary, and decide the number of clusters to be removed in the cluster thinning and in the untreated plots. The treatments were applied as follows: (untreated, where the standardized production was maintained on plants (UNT), and a thinning treatment, where 20% of the production was removed from the plants at veraison stage (CT). In the vineyard in Corno di Rosazzo, CT was applied on 27 July 2017, 19 July 2018, and 02 August 2019. In the vineyard located in Casarsa della Delizia, the same treatment was performed on 4 August 2017, 23 July 2018, and 05 August 2019.

During both established experiments, the meteorological data were recorded by the ARPA–OSMER weather stations of Cividale del Friuli and San Vito al Tagliamento (ARPA FVG–OSMER, http://www.meteo.fvg.it/ (accessed on 20 May 2021)).

Grape samples were collected at harvest point from each parcel and kept in a portable fridge until arrival in the laboratory. Each sample was manually squeezed. The musts obtained were manually squeezed and used to evaluate soluble solids, titratable acidity, and pH.

4.4. Vinification of Base Wines

For each vineyard, approximately 30 kg of grapes × plot were harvested manually and immediately transported to the experimental winery of the University of Udine. Rotten berries were removed, and the rest of the grapes were pressed at 2 bar in an A20 pneumatic press (Grifo Marchetti, Piadena, Italy). The 15–18 L of must obtained from each vineyard plot was added to 80 mg/L of $K_2S_2O_5$ and placed in a 50 L glass carboy, used as a fermentation vessel, to undergo the first fermentation. All the carboys were immediately inoculated with 400 mg/L of selected *Saccharomyces bayanus* commercial yeast strain Mycoferm IT07 (Ever, Pramaggiore, Italy), prepared according to the instructions of the manufacturer. The vessels were closed with airlocks to eliminate CO_2 produced during fermentation and left at 20 °C. The fermentative activity was monitored every two days by measuring the sugar concentration of the musts. Once the concentration dropped below 10 g/L, the fermenting musts were transferred into smaller, 15 L glass carboys until alcoholic fermentation was finished. Subsequently, the base wines produced were maintained at 4 °C for about two weeks to allow the tartaric stabilization and the sedimentation of the lees.

4.5. Secondary Fermentation of Sparkling Wines

To produce sparkling wine with the Martinotti–Charmat method, 7 L of each base wine obtained was transferred into a stainless-steel keg. The wine was supplemented with 18 g/L of sucrose (needed to provide a 4.5 bar pressure) and 400 mg/L of the same *S. bayanus* yeast strain, used for the primary fermentation. Compared to the base wine, the preparation of the yeast cell suspension was slightly modified. Following rehydration in water at a temperature of 35–38 °C, an aliquot of base wine and a sucrose solution were added to better adapt the yeast to fermentative conditions. The autoclaves were sealed, saturated with carbon dioxide to avoid oxidations, and maintained at 18 °C. During fermentation, the consumption of sugars and the pressure increase were monitored continuously until the fermentation was completed after ca 40 days. Thereafter, the sparkling wine was cold stabilized at 4 °C for two weeks and bottled using an isobaric bottling machine. The sparkling wines were then stored under controlled conditions until the analysis was performed. Before the chemical analysis, all the samples were degassed for 2 min and kept at 4 °C until the extraction procedures.

4.6. Enological Parameters

Alcoholic strength (% *v/v*), reducing sugars (g/L), titratable acidity (g/L tartaric acid) were determined as reported in Voce et al. [31], using FTIR spectroscopy with a WineScan™ FT-120 instrument (FOSS, Hillerød, Denmark).

4.7. Volatile Compound Analysis

In a 20 mL headspace (HS) vial, a wine sample (1 mL) was spiked with 50 µL of 2-octanol (2.13 mg/L in ethanol) as internal standard (IS) and added to previously introduced 1.5 g of sodium chloride. Extraction of volatiles was performed by headspace solid-phase microextraction (HS–SPME) using 2 cm long 50/30 µm coated divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Sigma-Aldrich, Milan, Italy). GC–MS analysis was performed by Thermo Trace GC ultra gas chromatograph coupled to a Thermo Quantum XLS mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a PAL combi-xt (CTC, Zwingen, Switzerland) autosampler with an SPME option. The fiber conditioning, microextraction regime, and the configuration of the mass spectrometer were set up as previously reported by Carlin et al. [57]. Briefly, solid-phase microextraction was followed by fiber desorption in the splitless mode (3 min at 250 °C), and the fiber was reconditioned between each sample (7 min at 270 °C). The column installed in the GC oven was 30 m × 0.25 mm VF-WAXms, with a 0.25 µm film thickness (Agilent Technologies Inc., Santa Clara, CA, USA). After the sample injection, the oven temperature was initially maintained at 40 °C for 2 min and gradually

ramped at 6 °C/min up to 250 °C (sustained for 5 min). The detection was carried out by electron impact mass spectrometry (MS) in total ion current (TIC) mode, using ionization energy of 70 eV and ion source temperature of 230 °C. Using ThermoXcalibur software (1.1.1.03, Thermo Scientific, Milan, Italy), the semiquantitative analysis was carried out. The final concentration of detected compounds was expressed as µg/L of the standard internal 2-octanol, considering a response factor equal to 1. Where applicable, the linear temperature-programmed retention indices were calculated (Supplementary Materials Table S7) for a series of n-alkanes (C7–C30) and compared with those reported in literature [58–86].

4.8. Lipid Compound Analysis

Lipid analysis was performed as previously described by Della Corte et al. [43], with minor modifications. A 0.5 mL aliquot of the wine sample was introduced in a 20 mL glass HS vial, with 1.5 mL of methanol, 3.0 mL of chloroform and 1.25 mL, containing BHT (500 mg/L) as an antioxidant substance, and 1.25 mL of H_2O Milli-Q. The solution was spiked with 10 µL of stearic acid d3 (100 µg/mL) as IS. Lipids were extracted two times, and the total lower lipid-rich layer was collected and evaporated to dryness under N_2. Afterward, the samples were reconstituted in 300 µL of acetonitrile/2-propanol/H_2O Milli-Q (65:30:5 *v/v/v*), containing cholesterol d7 (1.0 µg/mL) and filtered (0.22 µm) into 2 mL HPLC amber vials with a glass insert.

UHPLC separation was performed on a Dionex 3000 chromatograph (Thermo Fisher Scientific, Waltham, MA, USA), coupled with an API 5500 triple-quadrupole mass spectrometer with an ESI source (Sciex, Concord, Vaughan, ON, Canada). Lipids were separated on a 2.7 µm, 150 × 2.1 mm RP Ascentis Express column (Sigma-Aldrich, Milan, Italy), set at 55 °C. The injection volume was 5.0 µL, and the samples were maintained in an autosampler at 10 °C during analyses. The mobile phase and chromatographic conditions are those described by Della Corte et al. [43]. Instrument control and data acquisition were performed by Analyst software (Applera Corporation, Norwalk, CT, USA), while data processing was carried out by MultiQuant, version 2.1 (Sciex, Concord, Vaughan, ON, Canada).

4.9. Aromatic Amino Acid Metabolites Analysis

The UHPLC-MS/MS analysis was carried out on a Waters Acquity UPLC system (Milford, MA, USA) according to Arapitsas et al. [26] with slight modifications. First, the base and sparkling wine samples were filtered at 0.22 µm by a Millex-GV filtration unit (Merc, Darmstadt, Germany) directly into 2 mL MS certified amber vials and 20 µL of the IS (10 mg 3-nitrotyrosine in 10 mL of MeOH) was added. For the separation, 1.8 µm particle size and 150 × 2.1 mm Waters Acquity HSS T3 column (Milford, MA, USA) were used. Mobile phase A consisted of H_2O containing 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. The flow rate was 0.4 mL/min, while the column was kept at 40 °C. The injection volume was 10 µL, and the samples were stored in the autosampler at 6 °C during the analysis. Waters Xevo TQ (Milford, MA, USA) triple-quadrupole mass spectrometer with an electrospray (ESI) source was coupled to the UPLC system to perform the MS analysis. The Waters TargetLynx tools of the MassLynx 4.1 software were used for data processing. Standard solutions at eleven different concentrations were used to construct the calibration curves as adopted from Arapitsas et al. [26], except for sulfonated indole 3-lactic acid glucoside (Ila-Glu-SO3H) and Ila-Glu, which were quantified as indole 3-lactic acid (Ila).

4.10. Sensory Analysis

About nine months after bottling, the sparkling wines were tasted each year separately by a panel of local oenologists and producers, as well as researchers and students from the University of Udine. The median number of ten panelists was calculated, considering the three years. In the first part of the sensory evaluation, some of the wines being tasted

were used to prepare the scorecard, i.e., to determine the list of sensory descriptors that characterized the sparkling wines from Ribolla Gialla. Therefore, the final list consisted of eighteen attributes: eleven referred to aroma (floral, dry vegetable, citrus fruit, yeast, green apple, tropical, oxidation notes, licorice, herbaceous vegetable, dried fruit), seven were mouthfeel attributes (acidity, astringency, bitterness, flavor, body, the finesse of foam, flint) and overall pleasantness referred to global perception. These attributes were used to develop the scorecard used by the panel for olfactory, tactile and taste characterization of the experimental wines. Samples were presented to the judges in three replicated samples each, distributing them according to a balanced, randomized service order over three subsequent sessions. Each panelist received six wines per session and was asked to evaluate a continuous 10 cm linear scale, measuring the length from the beginning of the line for each sensory attribute and sample. Sensory data were normalized twice, by panelist and by season (z-transformation) and recalculated back using the average and the standard deviation of the three-season dataset.

4.11. Statistical Analysis

Three-ways ANOVA was carried out using 106 JMP® software (JMP 7.0, SAS Institute Inc., NC, USA) to compare the effect of cluster thinning and to assess the difference between different vineyard sites and vintages (*p* reported). When the test was significant, the averages were separated using the Student–Newman–Keuls test ($p < 0.05$). Before the data analysis, the missing values of the volatile compounds, aromatic amino acid metabolites and lipids were imputed with a random value between zero and LOQ, using a custom R script [87]. The multi-exploratory analysis was performed using principal component analysis (PCA) to evaluate the association between each group of chemical compounds and sensory variables. The R package FactoMineR v2.3 [88] was used to perform the PCA, and the packages factoextra v1.0.7 [89] and ggplot2 v 3.3.2 [90] were used to extract and visualize the result. By processing the data, a vintage effect initially emerged as dominant after the PCA analysis.

Still, to examine the impact of cluster thinning separately for the two locations, the data from all three vintages were subsequently normalized by season using the z-scale transformation, and log2 (CT/UNT) was represented in heatmaps to highlight the fold change increase/reduction of concentration in CT samples than UNT. Moreover, the significance of loadings between VOCs, lipids and aromatic amino acid metabolites was ascertained through factor analysis for each vineyard location to reduce the number of chemical parameters used in PCA. Thus, PCA analysis was repeated for each vineyard location separately, considering the just mentioned significant parameters together with sensory scores.

5. Conclusions

In this work, we presented the effect of cluster thinning on developing global metabolite profiles during producing monovarietal sparkling wine of the Ribolla Gialla variety. Considering the meteorological conditions during which the experimental part of this study took place, it was confirmed that the vintage had the greatest influence on the differentiation of the samples. After normalizing the results, it was possible to examine the effects of cluster thinning separately for the two production sites. The thinning of the grape clusters thus showed a minimal positive effect on the volatile composition, where the higher concentration of varietal aroma compounds (citronellol, linalool and β-myrcene) were present in the sparkling wines produced from the FG vineyard located in the valley floor. A similar outcome was achieved for the C13-norisoprenoid group of substances, where vitispirane prevailed in the FG samples. The effect of cluster thinning additionally caused the increase in metabolites associated with aromatic amino acids. In contrast to the volatile compounds, the AAA compounds, such as Trp, were predominantly present in the flat FG vineyard, which could be due to high yield and higher amount of rainfall.

At the level of organoleptic analysis, however, sparkling wines did not show any significant differences regarding overall pleasantness. At the same time, a strong effect of the production location emerged. By comparing the thinning effect in the two vineyard sites, a contrasting effect appeared in favor of the CT wines from FG and in favor of the UNT samples from the FCO site.

To sum up, cluster thinning caused slightly different effects in the two locations examined, highlighting that there may be a yield threshold above, which the wine metabolome can be significantly changed, with obvious repercussion also on the perceived organoleptic sparkling wine quality.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/metabo11050331/s1, Figure S1: Meteorological data from 01 April to 30 September in the San Vito al Tagliamento—FG (A–C) and Cividale del Friuli—FCO (D, E, F) locations during 2017 (A, D), 2018 (B, D) and 2019 (C, F); Figure S2: Effect of cluster thinning on the organoleptic characteristics of Ribolla Gialla sparkling wines. The average values were obtained from 2017–2019 and FG-FCO vineyard sites; Figure S3: Effect of production site on the organoleptic characteristics of Ribolla Gialla sparkling wines. The average values were obtained from 2017–2019 of both UNT and CT samples; Table S1: Impact of cluster thinning treatment, vineyard site and harvest season on the volatile profile of Ribolla Gialla base wines; Table S2: Impact of cluster thinning treatment, vineyard site and harvest season on the volatile profile of Ribolla Gialla sparkling wines; Table S3: Impact of cluster thinning treatment, vineyard site and harvest season on the lipid profile of Ribolla Gialla base wines; Table S4: Impact of cluster thinning, vineyard site and harvest season on the lipid profile of Ribolla Gialla sparkling wines; Table S5: Impact of cluster thinning, vineyard site and harvest season on the aromatic amino acid metabolites profile of Ribolla Gialla base wines; Table S6: Impact of cluster thinning, vineyard site and harvest season on the aromatic amino acid metabolites profile of Ribolla Gialla sparkling wines; Table S7: Retention indices and identification method used for VOCs analysis in base wines and sparkling wines.

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Article

Cluster thinning and vineyard site modulate the metabolomic profile of Ribolla Gialla base and sparkling wines

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Supplementary material

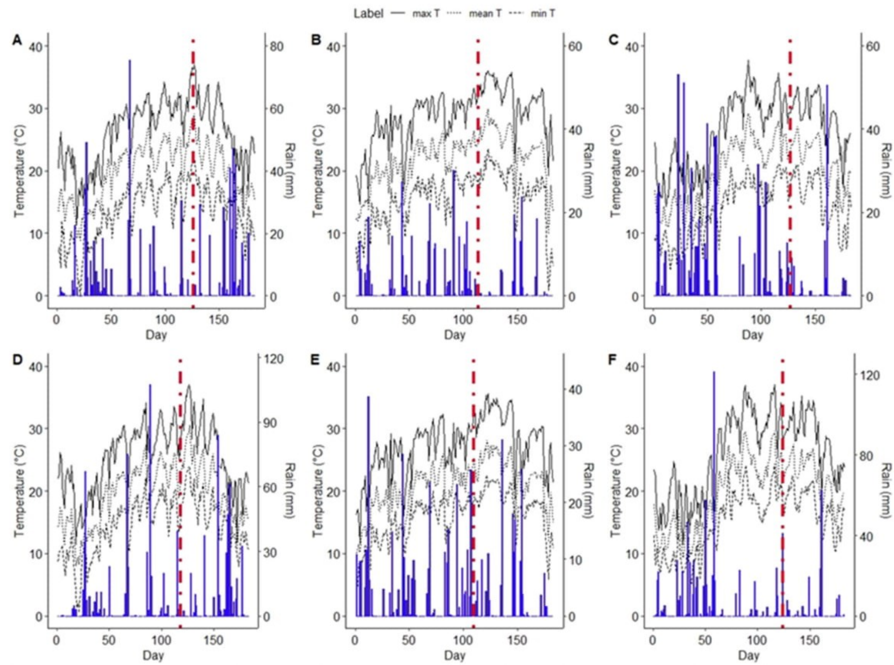

Figure S1. Meteorological data from 01 April to 30 September in the San Vito al Tagliamento—FG (**A**, **B**, **C**) and Cividale del Friuli—FCO (**D**, **E**, **F**) locations during 2017 (**A**, **D**), 2018 (**B**, **D**) and 2019 (**C**, **F**). Scattered lines represent min, mean and max temperatures while blue histograms represent rainfall. The dashed red line highlights the timing of cluster thinning in the different seasons.

Table S1. Impact of cluster thinning treatment, vineyard site and harvest season on the volatile profile of Ribolla Gialla base wines.

Compound	Treatment (T)			Site (S)			Year (Y)				Y×T	S×T	Y×S	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
Monoterpenes	18.87	19.26	*ns*	18.83	19.30	*ns*	21.21a	19.44ab[2]	16.54b	***	*ns*	*ns*	*	*
β-myrcene	0.31	0.33	*ns*	0.29	0.35	*ns*	0.59a	0.20b	0.17b	***	*ns*	*ns*	*ns*	*ns*
Limonene	1.31	1.40	*ns*	1.24b	1.47a	**	0.57c	1.37b	2.13a	***	*	*ns*	*ns*	*
Linalool	3.76b	4.26a	*	3.81b	4.22a	*	4.58a	4.77a	2.69b	***	*	**	*	**
Geraniol	7.35	6.30	*ns*	6.68	6.96	*ns*	7.88a	5.99b	6.59ab	*	*ns*	*ns*	*ns*	*ns*
Citronellol	3.70	3.81	*ns*	3.66	3.85	*ns*	4.75a	3.54b	2.98b	***	*ns*	*ns*	***	*ns*
Nerol	1.51	1.57	*ns*	1.52	1.56	*ns*	1.53	1.65	1.43	*ns*	**	*ns*	**	**
Terpinen-4-ol	0.24	0.28	*ns*	0.25	0.27	*ns*	0.30a	0.30a	0.19b	**	*ns*	*ns*	*ns*	*ns*
α-Terpineol	0.79	1.31	*ns*	1.38	0.72	*ns*	1.01	1.62	0.51	*ns*	*ns*	*ns*	*ns*	*ns*
Norisoprenoids	13.29	12.88	*ns*	11.20b	14.97a	***	19.68a	11.76b	7.82c	**	*ns*	*ns*	***	*ns*
Vitispirane	0.37	0.47	*ns*	0.44	0.40	*ns*	0.70a	0.22b	0.34b	***	*ns*	*ns*	*ns*	*ns*
TDN	0.67	0.69	*ns*	0.66	0.70	*ns*	0.64	0.75	0.65	*ns*	*ns*	*ns*	*ns*	*ns*
β-Damascenone	11.95	11.46	*ns*	9.87b	13.54a	***	17.88a	10.53b	6.71c	***	*ns*	*ns*	***	*ns*
Actinidiol (isomer 1)	0.17	0.14	*ns*	0.12	0.20	*ns*	0.23a	0.18a	0.06b	*	*ns*	*ns*	*ns*	*ns*
Actinidiol (isomer 2)	0.12	0.12	*ns*	0.11b	0.14a	**	0.23a	0.08b	0.06c	***	*ns*	*ns*	*	*ns*
Aldehydes	116.94	131.80	*ns*	113.25b	135.49a	*	181.26a	98.29b	93.56b	***	*ns*	*ns*	**	*ns*
Hexanal	0.43	0.69	*ns*	0.45	0.68	*ns*	1.02a	0.34b	0.33b	***	*ns*	*ns*	**	*ns*
trans-2-Hexenal	84.88	89.28	*ns*	91.43	82.73	*ns*	108.56a	71.83b	80.85ab	*	*ns*	*ns*	*ns*	*ns*
Nonanal	14.63	26.22	*ns*	6.41b	34.44a	**	45.71a	10.51b	5.05b	***	*ns*	*ns*	**	*ns*
Benzaldehyde	6.16	5.51	*ns*	4.66	7.00	*ns*	10.99a	4.26b	2.25b	**	*ns*	*ns*	*ns*	*ns*
3,4-Dimethyl benzaldehyde	0.69	0.60	*ns*	0.36b	0.93a	*	0.49b	1.17a	0.28b	*	*ns*	*ns*	*	*ns*
Acetaldehyde	9.71	9.01	*ns*	9.51	9.20	*ns*	13.42a	9.97a	4.68b	**	*ns*	*ns*	*ns*	*ns*
Furfural	0.44	0.49	*ns*	0.44	0.50	*ns*	1.08a	0.21b	0.12b	***	*ns*	*ns*	*ns*	*ns*

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Table S1. (Continued)

Compound	Treatment (T)			Site (S)			Year (Y)				Y×T	S×T	Y×S	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
Alcohols	2388.08b	2591.65a	*	2335.76b	2643.97a	***	1829.44b	1382.90c	4257.26a	***	*ns*	*ns*	***	*ns*
n-Hexanol	81.63	83.19	*ns*	63.33b	101.49a	***	157.24a	41.29b	48.70b	***	*ns*	*ns*	***	*ns*
trans-3-Hexenol	1.55	1.61	*ns*	1.14b	2.02a	***	2.59a	1.06b	1.09b	***	*ns*	*ns*	***	*ns*
1-Octanol	10.27	9.46	*ns*	9.65	10.08	*ns*	9.25	11.39	8.96	*ns*	*ns*	*ns*	*ns*	*ns*
Isobutanol	102.81	98.72	*ns*	90.30b	111.22a	*	85.49b	118.73a	98.06b	**	*ns*	*ns*	*	*
Methionol	4.78	5.12	*ns*	4.36b	5.54a	***	6.62a	4.10b	4.13b	***	*ns*	*ns*	***	*ns*
Isoamyl alcohol	1462.30b	1595.95a	**	1431.83b	1626.42a	**	658.32b	645.54b	3283.53a	***	**	*ns*	**	*ns*
3-Methyl-1-pentanol	7.10	8.36	*ns*	6.50b	8.96a	***	7.15	8.06	7.99	*ns*	*ns*	*ns*	***	*ns*
2,3-Butanediol (isomer 1)	16.85	17.59	*ns*	18.64	15.80	*ns*	21.42a	13.12b	17.13b	**	*ns*	*ns*	*ns*	*ns*
2,3-Butanediol (isomer 2)	4.35	5.15	*ns*	5.26	4.24	*ns*	5.71	4.45	4.09	*ns*	*ns*	*ns*	*ns*	*ns*
cis-3-Hexenol	696.46b	766.49a	*	704.75	758.20	*ns*	875.65a	535.17c	783.60b	***	*ns*	*ns*	***	*ns*
Esters	2286.64	2414.24	*ns*	2363.73	2337.15	*ns*	4111.28a	1809.03b	1131.00c	***	*ns*	*ns*	*ns*	*ns*
Ethyl acetate	93.05b	112.05a	*ns*	86.36b	118.74a	**	127.85a	67.37b	112.44a	***	*ns*	*ns*	*ns*	*ns*
Ethyl butyrate	27.66	30.36	*ns*	28.01	30.01	*ns*	32.00	26.89	28.13	*ns*	*ns*	*ns*	*ns*	*ns*
Isopentyl acetate	208.71	226.90	*ns*	212.83	222.78	*ns*	310.17a	151.91b	191.33b	***	*ns*	*ns*	*ns*	*ns*
Hexyl acetate	50.03	47.89	*ns*	41.03b	56.89a	*	114.19a	13.12b	19.58b	***	*ns*	*ns*	***	*ns*
Methyl caproate	0.44	0.41	*ns*	0.45	0.40	*ns*	0.36	0.43	0.49	*ns*	*ns*	*ns*	*ns*	*ns*
Ethyl hexanoate	705.59	733.38	*ns*	713.18	725.79	*ns*	974.87a	726.63b	456.96c	***	*ns*	*ns*	*ns*	*ns*
Ethyl lactate	3.24	3.50	*ns*	3.33	3.40	*ns*	4.70a	2.49b	2.92b	***	*ns*	*ns*	***	*ns*
Methyl octanoate	1.26	1.29	*ns*	1.21	1.34	*ns*	2.15a	1.04b	0.64b	***	*ns*	*ns*	*ns*	*ns*
Ethyl octanoate	687.39	743.26	*ns*	679.43	751.23	*ns*	1641.87a	306.94b	197.17b	***	*ns*	*ns*	*ns*	*ns*
Isoamyl lactate	0.65	0.73	*ns*	0.56b	0.82a	***	0.52b	0.83a	0.72a	***	*ns*	*ns*	***	*ns*
Methyl decanoate	0.12	0.12	*ns*	0.11	0.13	*ns*	0.26a	0.04b	0.06b	***	*ns*	*ns*	*ns*	*ns*

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Table S1. (Continued)

Compound	Treatment (T)			Site (S)			Year (Y)				Y×T	S×T	Y×S	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
Ethyl decanoate	180.36	179.37	*ns*	184.99	174.73	*ns*	387.03a	99.53b	53.03b	***	*ns*	*ns*	*ns*	*ns*
Isoamyl octanoate	3.23	3.65	*ns*	2.77b	4.10a	*	5.85a	3.21b	1.25c	***	*ns*	*ns*	*ns*	*ns*
Methyl ethyl succinate	128.68	118.34	*ns*	153.74a	93.28b	**	102.55b	267.94a	0.04c	***	*ns*	*ns*	**	*ns*
Diethyl succinate	60.91	64.43	*ns*	70.35a	54.99b	*	156.40a	28.99b	2.63c	***	*ns*	*ns*	*ns*	*ns*
Ethyl 9-decanoate	56.68	61.14	*ns*	101.15a	16.67b	***	104.12a	58.89b	13.72c	***	*ns*	*ns*	**	*ns*
Ethyl-2-OH-4-methylpentanoate	1.39	1.28	*ns*	1.20b	1.48a	**	1.77a	1.48b	0.75c	***	*ns*	*ns*	***	*ns*
2-Phenylethyl acetate	58.91	61.97	*ns*	64.23	56.65	*ns*	108.44a	38.28b	34.60b	***	*ns*	*ns*	*ns*	*ns*
Ethyl hydrogen succinate	3.94	4.40	*ns*	3.53b	4.80a	*	5.87a	6.18a	0.44b	***	*ns*	*ns*	***	*ns*
Methyl salicylate	9.21	5.66	*ns*	9.00	5.87	*ns*	19.98a	1.88b	0.46b	**	*ns*	*ns*	*ns*	*ns*
Ethyl hydroxybutanoate	5.20	6.26	*ns*	6.28	5.18	*ns*	10.34a	4.98b	1.87c	***	*ns*	*ns*	*ns*	*ns*
Acids	1702.88	1551.69	*ns*	1489.00	1765.56	*ns*	2533.89a	732.68 c	1615.27b	***	*ns*	*ns*	*ns*	*ns*
Acetic acid	36.27	36.28	*ns*	32.45	40.10	*ns*	16.89b	46.92a	45.02a	***	*ns*	*ns*	*	*
Butyric acid	5.38	5.56	*ns*	5.06	5.88	*ns*	5.28	6.28	4.85	*ns*	*ns*	*ns*	*ns*	*
3-Methylbutyric acid	18.15	19.16	*ns*	17.03b	20.27a	*	23.79a	19.80b	12.37c	***	*ns*	*ns*	*	*ns*
Hexanoic acid	106.63	105.41	*ns*	102.06	109.98	*ns*	154.54a	65.16c	98.36b	***	*ns*	*ns*	*	*ns*
Octanoic acid	462.97	453.73	*ns*	436.30	480.41	*ns*	729.19a	230.62c	415.25b	***	*ns*	*ns*	*ns*	*ns*
Nonanoic acid	32.07	25.98	*ns*	28.99	29.07	*ns*	6.24b	6.34b	74.51a	***	*ns*	*ns*	*ns*	*ns*
Decanoic acid	1016.65	882.67	*ns*	842.94	1056.38	*ns*	1557.00a	346.49c	945.50b	***	*ns*	*ns*	*ns*	*ns*
Benzoic acid	6.75	5.99	*ns*	5.02	7.72	*ns*	15.44a	2.62b	1.06b	*	*ns*	*ns*	*ns*	*ns*
Dodecanoic acid	18.00	16.90	*ns*	19.15	15.75	*ns*	25.53a	8.46b	18.36ab	*	*ns*	*ns*	*	*
Ketones	17.75	19.38	*ns*	18.24	18.89	*ns*	20.08	15.45	20.16	*ns*	*ns*	*ns*	*ns*	*ns*
2-Methylthiolan-3-one	2.23	2.42	*ns*	2.81a	1.84b	*	1.99	2.79	2.20	*ns*	*ns*	*ns*	*ns*	*ns*
Isophorone	15.52	16.96	*ns*	15.43	17.05	*ns*	18.1	12.67	17.96	*ns*	*ns*	*ns*	*ns*	*ns*

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[1] Data were analyzed by three-ways ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).
[2] Different letters (a, b, c) identify significantly different means. UNT, untreated control; CT, cluster thinning; FG, Friuli Grave; FCO, Friuli Colli Orientali.
All the concentrations are expressed in μg/L as IS 2-octanol.

Table S2. Impact of cluster thinning treatment, vineyard site and harvest season on the volatile profile of Ribolla Gialla sparkling wines.

Compound	Treatment (T)			Site (S)			Year (Y)				Y×T	S×T	Y×S	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
Monoterpenes	24.54	25.54	*ns*	25.57	24.51	*ns*	21.44b	22.21b	31.48a	***	*ns*	***	*ns*	*ns*
β-Myrcene	0.70b[2]	0.80a	**	0.74	0.75	*ns*	0.70b	0.70b	0.83a	**	*ns*	*ns*	*ns*	*ns*
Limonene	2.32	2.45	*ns*	2.50	2.27	*ns*	2.36	2.40	2.41	*ns*	*ns*	*ns*	*ns*	*ns*
Linalool	4.55b	4.95a	*	4.59b	4.90a	*	4.49b	5.11a	4.64b	*ns*	*ns*	*	*ns*	*ns*
Geraniol	7.85	7.27	*ns*	7.33	7.80	*ns*	8.18	7.13	7.38	*ns*	*ns*	*	*ns*	*ns*
Citronellol	1.41b	1.60a	*	1.37b	1.64a	**	1.44b	1.19c	1.88a	***	*ns*	**	*ns*	**
Nerol	3.04	2.96	*ns*	2.80b	3.20a	***	2.34c	2.66b	4.00a	***	*ns*	***	*	**
Terpinen-4-ol	0.54	0.55	*ns*	0.52	0.57	*ns*	0.45b	0.61a	0.57a	**	*ns*	*ns*	*ns*	*ns*
α-Terpineol	4.13	4.97	*ns*	5.73a	3.38b	***	1.47b	2.42b	9.77a	***	*ns*	***	*ns*	*ns*
Norisoprenoids	16.17	16.63	*ns*	15.16b	17.63a	***	18.00a	11.92b	19.28a	***	*ns*	***	*ns*	*
Vitispirane	0.62	0.79	*ns*	0.76	0.66	*ns*	0.81	0.60	0.72	*ns*	*ns*	*ns*	*ns*	*ns*
TDN	0.99	1.08	*ns*	0.92b	1.15a	**	0.95b	0.40c	1.75a	***	*ns*	*ns*	*ns*	*ns*
β-Damascenone	13.62	13.88	*ns*	12.57b	14.93a	***	15.21a	10.00b	16.05a	***	*ns*	***	**	**
Actinidiol (isomer 1)	0.40	0.37	*ns*	0.39	0.39	*ns*	0.44a	0.40b	0.32c	***	*ns*	***	*ns*	*ns*
Actinidiol (isomer 2)	0.53	0.50	*ns*	0.52	0.51	*ns*	0.59a	0.53b	0.43c	***	*ns*	***	*ns*	*ns*
Aldehydes	417.83b	474.89a	*	423.12	469.60	*ns*	499.58a	360.28b	479.23a	***	*ns*	*ns*	*ns*	*ns*
Hexanal	0.09	0.07	*ns*	0.08	0.09	*ns*	0.12a	0.05c	0.08b	***	*ns*	*ns*	*ns*	*ns*
trans-2-Hexenal	388.74b	441.41a	*	394.47	435.68	*ns*	464.22a	334.23b	446.77a	***	*ns*	*ns*	*ns*	*ns*
Nonanal	3.24	2.92	*ns*	3.01	3.15	*ns*	2.86b	2.49b	3.89a	*	*ns*	*	*ns*	*ns*
Benzaldehyde	6.69	7.00	*ns*	6.42	7.27	*ns*	8.75a	5.28c	6.51b	***	*ns*	*ns*	*ns*	*ns*
3,4-Dimethyl benzaldehyde	1.43	1.52	*ns*	1.43	1.52	*ns*	1.69a	1.00b	1.73a	***	*ns*	*ns*	*ns*	*ns*
Acetaldehyde	16.18b	20.48a	**	16.14b	20.52a	**	20.41	16.14	18.44	*ns*	*ns*	*ns*	*ns*	*ns*
Furfural	1.47	1.49	*ns*	1.59	1.38	*ns*	1.53a	1.09b	1.82a	***	*ns*	*ns*	*ns*	**

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Table S2. (Continued)

Compound	Treatment (T)			Site (S)			Year (Y)				Y×T	S×T	Y×S	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
Alcohols	3678.56b	4086.47a	**	3791.62	3973.41	*ns*	3975.01a	3538.87b	4133.67a	**	*ns*	*ns*	*ns*	*ns*
n-Hexanol	75.74	79.19	*ns*	61.63b	93.30a	***	144.07a	30.64c	57.68b	***	*ns*	***	*ns*	*ns*
trans-3-Hexenol	1.76	1.88	*ns*	1.36b	2.28a	***	3.04a	1.02c	1.40b	***	*ns*	***	*ns*	*ns*
1-octanol	11.20	10.44	*ns*	10.74	10.90	*ns*	10.99	10.39	11.09	*ns*	**	**	*ns*	*ns*
Iso-butanol	106.05b	119.85a	*	109.58	116.32	*ns*	110.03b	95.94b	132.87a	***	*ns*	*ns*	*ns*	**
Methionol	2.31	2.85	*ns*	2.76	2.40	*ns*	2.24	2.84	2.66	*ns*	*	*ns*	*ns*	*ns*
Isoamyl alcohol	2749.83b	3073.61a	**	2845.52	2977.92	*ns*	2893.35b	2669.83b	3171.99a	**	*ns*	*ns*	*ns*	*ns*
3-Methyl-1-pentanol	9.13b	10.86a	**	10.03	9.96	*ns*	8.90b	10.59a	10.50a	*	*ns*	*ns*	*ns*	*ns*
2,3-Butanediol (isomer 1)	5.55a	2.44b	*	3.32	4.66	*ns*	10.39a	0.59b	1.00b	***	*ns*	*ns*	*	*ns*
2,3-Butanediol (isomer 2)	2.22a	1.23b	*	1.58	1.87	*ns*	3.87a	0.46b	0.85b	***	*ns*	*ns*	*	*ns*
cis-3-Hexenol	0.29b	0.51a	*	0.47	0.32	*ns*	0.45	0.38	0.36	*ns*	*ns*	*ns*	*ns*	*ns*
trans-2-Hexenol	1.04	1.49	*ns*	1.05	1.48	*ns*	1.39	1.13	1.27	*ns*	*ns*	*ns*	*ns*	*ns*
2-Phenylethanol	714.77b	784.12a	**	745.10	753.79	*ns*	788.14	716.56	743.64	*ns*	*ns*	*ns*	*ns*	*ns*
Esters	2667.36	2978.17	*ns*	2912.35	2733.18	*ns*	2484.72b	2539.83b	3443.76a	***	*ns*	*ns*	*ns*	*ns*
Ethyl acetate	299.39	347.16	*ns*	298.65	347.9	*ns*	307.33b	237.68c	424.81a	***	*ns*	*ns*	*ns*	*ns*
Ethyl butyrate	42.62	46.24	*ns*	46.86	42.00	*ns*	36.59b	41.44b	55.26a	***	*ns*	*ns*	*ns*	*ns*
Isopentyl acetate	189.33	193.35	*ns*	205.63	177.05	*ns*	138.94b	189.43c	245.65a	***	*ns*	*ns*	*ns*	*ns*
Hexyl acetate	22.09	21.81	*ns*	25.96a	17.93b	*	20.84ab	16.46b	28.53a	*	*ns*	***	*ns*	*ns*
Methyl caproate	0.58	0.63	*ns*	0.60	0.61	*ns*	0.48b	0.57b	0.76a	***	*ns*	*ns*	*ns*	*ns*
Ethyl hexanoate	947.62	1044.37	*ns*	1020.51	971.48	*ns*	1000.85	940.15	1046.99	*ns*	*ns*	*ns*	*ns*	*ns*
Ethyl lactate	15.40b	17.84a	**	17.04	16.20	*ns*	18.74a	14.52c	16.60b	***	*ns*	**	*ns*	*ns*
Methyl octanoate	3.06	3.17	*ns*	3.18	3.05	*ns*	2.41b	3.26a	3.68a	***	*ns*	*ns*	*ns*	*ns*

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Table S2. (Continued)

Compound	Treatment (T)			Site (S)			Year (Y)				Y×T	S×T	Y×S	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
Ethyl octanoate	587.02	659.98	*ns*	627.28	619.72	*ns*	462.69c	627.70b	780.11a	***	*ns*	*ns*	*ns*	*ns*
Isoamyl lactate	2.31	2.40	*ns*	2.31	2.40	*ns*	2.46a	2.12b	2.49a	**	*ns*	*	*ns*	*ns*
Methyl decanoate	0.28	0.31	*ns*	0.28	0.30	*ns*	0.23b	0.22b	0.43a	***	*ns*	*ns*	*ns*	*ns*
Ethyl decanoate	168.27	188.86	*ns*	183.12	174.01	*ns*	27.45c	168.00b	340.25a	***	*ns*	*ns*	*ns*	*ns*
Isoamyl octanoate	2.47	2.59	*ns*	2.55	2.51	*ns*	1.91b	2.54a	3.14a	**	*ns*	*ns*	*ns*	*ns*
Methyl ethyl succinate	106.51	121.36	*ns*	117.71	110.17	*ns*	109.76b	81.40c	150.65a	***	*ns*	*ns*	*ns*	*ns*
Diethyl succinate	89.89b	102.10a	**	96.81	95.18	*ns*	144.32a	98.95b	44.71c	***	*ns*	*ns*	*ns*	*ns*
Methyl decanoate	0.28	0.31	*ns*	0.28	0.30	*ns*	0.23b	0.22b	0.43a	***	*ns*	*ns*	*ns*	*ns*
Ethyl decanoate	168.27	188.86	*ns*	183.12	174.01	*ns*	27.45c	168.00b	340.25a	***	*ns*	*ns*	*ns*	*ns*
Isoamyl octanoate	2.47	2.59	*ns*	2.55	2.51	*ns*	1.91b	2.54a	3.14a	**	*ns*	*ns*	*ns*	*ns*
Methyl ethyl succinate	106.51	121.36	*ns*	117.71	110.17	*ns*	109.76b	81.40c	150.65a	***	*ns*	*ns*	*ns*	*ns*
Diethyl succinate	89.89b	102.10a	**	96.81	95.18	*ns*	144.32a	98.95b	44.71c	***	*ns*	*ns*	*ns*	*ns*
Ethyl 9-decanoate	118.88b	148.53a	**	185.20a	82.21b	***	151.24b	15.40c	234.47a	***	*ns*	***	*ns*	*ns*
Ethyl-2-OH-4-methylpentanoate	3.21	2.94	*ns*	3.05	3.10	*ns*	2.62b	3.68a	2.92b	***	*ns*	*ns*	*ns*	*ns*
2-Phenylethyl acetate	43.50	47.62	*ns*	48.41	42.71	*ns*	37.47b	51.25a	47.96a	**	*ns*	*ns*	*ns*	*ns*
Ethyl hydrogen succinate	16.76	19.68	*ns*	19.74	16.7	*ns*	12.69b	35.27a	6.69b	***	*ns*	*ns*	*ns*	*ns*
Methyl salicylate	5.38	3.91	*ns*	4.65	4.64	*ns*	3.58b	7.58a	2.79b	**	*ns*	*ns*	*ns*	*ns*
Ethyl hydroxybutanoate	2.01b	2.51a	*	2.06	2.46	*ns*	1.14b	1.62b	4.02a	***	*ns*	*ns*	*ns*	*
Ethyl dodecanoate	0.78	0.83	*ns*	0.76	0.85	*ns*	0.97a	0.58b	0.86a	***	*ns*	*	*ns*	*ns*
Isobutyl acetate	1.02	1.69	*ns*	1.27	1.43	*ns*	1.56	1.15	1.34	*ns*	*	*ns*	*ns*	*ns*
Acids	2280.49	2267.72	*ns*	2296.39	2251.83	*ns*	1840.70b	2718.65a	2262.97ab	**	*ns*	*ns*	*ns*	*ns*
Acetic acid	33.76b	41.79a	*	33.27b	42.28a	**	21.45c	36.02b	55.85a	***	*ns*	*	*	*ns*
Butyric acid	7.30	7.17	*ns*	7.45	7.01	*ns*	6.08c	7.34b	8.29a	***	*ns*	*ns*	*ns*	*ns*

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Table S2. (Continued)

Compound	Treatment (T)			Site (S)			Year (Y)				Y×T	S×T	Y×S	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
Butyric acid	7.30	7.17	*ns*	7.45	7.01	*ns*	6.08c	7.34b	8.29a	***	*ns*	*ns*	*ns*	*ns*
Hexanoic acid	120.51	124.46	*ns*	121.49	123.47	*ns*	124.04	115.83	127.59	*ns*	*ns*	*ns*	*ns*	*ns*
Octanoic acid	455.15	466.16	*ns*	460.77	460.55	*ns*	388.98b	443.06b	549.94a	***	*ns*	*ns*	*ns*	*ns*
Nonanoic acid	49.37	43.05	*ns*	45.50	46.92	*ns*	61.10a	20.42b	57.11a	**	*	*ns*	*ns*	*
Decanoic acid	1548.92	1517.93	*ns*	1558.47	1508.38	*ns*	1185.85b	2005.13a	1409.30b	**	*ns*	*ns*	*ns*	*ns*
Benzoic acid	2.63	3.22	*ns*	3.00a	2.85b	*	2.84	2.99	2.94	*ns*	*ns*	*ns*	*ns*	*ns*
Dodecanoic acid	43.63	44.51	*ns*	47.19	40.95	*ns*	30.19b	68.46a	33.55b	***	*ns*	*ns*	*ns*	*ns*
Ketones	139.98	147.75	*ns*	145.05	142.68	*ns*	159.06a	134.54b	137.98b	**	*ns*	*ns*	*ns*	*ns*
2-Methylthiolan-3-one	2.30	2.71	*ns*	3.21a	1.80b	***	0.83c	3.79a	2.89b	***	*ns*	***	*ns*	*ns*
Isophorone	137.68	145.03	*ns*	141.84	140.88	*ns*	158.24a	130.75b	135.09b	**	*ns*	*ns*	*ns*	*ns*

[1] Data were analyzed by three-ways ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).
[2] Different letters (a, b, c) identify significantly different means. UNT, untreated control; CT, cluster thinning; FG, Friuli Grave; FCO, Friuli Colli Orientali.
All the concentrations are expressed in μg/L as IS 2-octanol, except *cis*-3-hexenol, *trans*-2-hexenol, and isobutyl acetate that are reported in ng/L.

Table S3. Impact of cluster thinning treatment, vineyard site and harvest season on the lipid profile of Ribolla Gialla base wines.

Compound	Treatment (T)			Site (S)			Year (Y)				Y×T	S×T	Y×S	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
Glycerolipids	0.26	0.26	*ns*	0.25	0.26	*ns*	0.27	0.24	0.26	*ns*	*ns*	*ns*	*ns*	*ns*
1-Linoleoyl-rac-glycerol	0.23	0.23	*ns*	0.22	0.23	*ns*	0.23	0.21	0.24	*ns*	*ns*	*ns*	*ns*	*ns*
1-Oleoyl-rac-glycerol	0.02	0.02	*ns*	0.02	0.02	*ns*	0.02a[2]	0.02b	0.02a	**	*ns*	*ns*	*ns*	*ns*
1-Monopalmitoleoyl-rac-glycerol	0.01	0.01	*ns*	0.01	0.01	*ns*	0.01	0.01	0.01	*ns*	*ns*	*ns*	*ns*	*ns*
Sterols	0.30	0.30	*ns*	0.29	0.31	*ns*	0.32a	0.27b	0.31a	*	*ns*	*ns*	*ns*	*ns*
Ergosterol	0.28	0.27	*ns*	0.27	0.28	*ns*	0.29a	0.25b	0.29a	*	*ns*	*ns*	*ns*	*ns*
Desmosterol	0.03	0.03	*ns*	0.03	0.03	*ns*	0.03a	0.02b	0.03a	*	*ns*	*ns*	*ns*	*ns*
Fatty acids UFA	4.66	4.71	*ns*	4.67	4.71	*ns*	6.26a	3.83b	3.98b	***	*ns*	*ns*	*ns*	*ns*
Linoleic acid	0.28	0.27	*ns*	0.27	0.28	*ns*	0.36a	0.23b	0.24b	***	*ns*	*ns*	*ns*	*ns*
Linolenic acid	0.03	0.03	*ns*	0.03a	0.03b	*	0.03a	0.03b	0.03ab	**	*ns*	*ns*	*ns*	*ns*
Palmitoleic acid	0.14	0.16	*ns*	0.15	0.16	*ns*	0.23a	0.11b	0.11b	***	*ns*	*ns*	*ns*	*ns*
Oleic acid + *cis*-Vaccenic acid	4.21	4.25	*ns*	4.22	4.24	*ns*	5.64a	3.46b	3.59b	***	*ns*	*ns*	*ns*	*ns*
Fatty acids SFA	154.72	152.89	*ns*	152.80	154.81	*ns*	179.54a	142.44b	139.44b	***	*ns*	*ns*	*ns*	*ns*
Behenic acid	0.65	0.64	*ns*	0.64	0.64	*ns*	0.73a	0.60b	0.60b	***	*ns*	*ns*	*	*ns*
Stearic acid	45.70	45.97	*ns*	45.38	46.30	*ns*	54.75a	42.66b	40.10b	***	*ns*	*ns*	*ns*	*ns*
Lignoceric acid	0.34	0.34	*ns*	0.34	0.34	*ns*	0.37a	0.34ab	0.31b	*	*ns*	*ns*	*ns*	*ns*
Arachidic acid	2.24	2.26	*ns*	2.22	2.28	*ns*	2.76a	1.95b	2.03b	***	*ns*	*ns*	*ns*	*ns*
Myristic acid	1.55	1.60	*ns*	1.49b	1.66a	**	1.60b	1.33c	1.79a	***	*ns*	*ns*	*	*ns*
Palmitic acid	103.31	101.18	*ns*	101.84	102.66	*ns*	118.31a	94.73b	93.70b	***	*ns*	*ns*	*ns*	*ns*
Miristoleic acid	0.47	0.47	*ns*	0.46	0.48	*ns*	0.49a	0.42b	0.49a	*	*ns*	*ns*	*ns*	*ns*
Margaric acid	0.45	0.44	*ns*	0.44	0.45	*ns*	0.53a	0.40b	0.41b	***	*ns*	*ns*	*ns*	*ns*
Prenols	0.15	0.09	*ns*	0.09	0.14	*ns*	0.11	0.13	0.11	*ns*	*ns*	*ns*	*ns*	*ns*
Lupeol	0.15	0.09	*ns*	0.09	0.14	*ns*	0.11	0.13	0.11	*ns*	*ns*	*ns*	*ns*	*ns*

[1] Data were analyzed by three-ways ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).
[2] Different letters (a, b, c) identify significantly different means. UNT, untreated control; CT, cluster thinning; FG, Friuli Grave; FCO, Friuli Colli Orientali.
All the concentrations are expressed in mg/L.

Table S4. Impact of cluster thinning, vineyard site and harvest season on the lipid profile of Ribolla Gialla sparkling wines.

Compound	Treatment (T)			Site (S)			Year (Y)				Y×T	S×T	Y×S	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
Glycerolipids	0.27	0.27	*ns*	0.27	0.27	*ns*	0.23b[2]	0.24b	0.33a	***	*ns*	*	*ns*	*ns*
1-Linoleoyl-rac-glycerol	0.23	0.23	*ns*	0.23	0.23	*ns*	0.20b	0.21b	0.28a	***	*ns*	*ns*	*ns*	*ns*
1-Oleoyl-rac-glycerol	0.03	0.03	*ns*	0.03	0.03	*ns*	0.03ab	0.02b	0.04a	*	*ns*	*ns*	*ns*	*ns*
1-Monopalmitoleoyl-rac-glycerol	7.23	8.47	*ns*	6.35	9.35	*ns*	4.76b	5.62b	13.16a	*	*ns*	*ns*	*ns*	*ns*
Sterols	0.33	0.33	*ns*	0.34	0.32	*ns*	0.29b	0.27c	0.44a	***	*	*	*ns*	*ns*
Ergosterol	0.30	0.30	*ns*	0.31a	0.29b	*	0.26b	0.24c	0.40a	***	*ns*	*	*ns*	*ns*
Desmosterol	0.03	0.03	*ns*	0.03	0.03	*ns*	0.03b	0.02b	0.04a	***	*ns*	*ns*	*ns*	*ns*
Fatty acids UFA	6.02	5.81	*ns*	5.84	5.99	*ns*	6.07a	5.45b	6.22a	***	*ns*	*	*ns*	*ns*
Linoleic acid	0.32	0.33	*ns*	0.33	0.33	*ns*	0.29b	0.31b	0.37a	***	*ns*	*ns*	*ns*	*ns*
Linolenic acid	0.03	0.03	*ns*	0.03	0.03	*ns*	0.03b	0.03b	0.04a	***	*ns*	*ns*	*ns*	*ns*
Palmitoleic acid	0.41a	0.31b	**	0.33b	0.40a	*	0.55a	0.33b	0.21c	***	*ns*	*ns*	*	*
Oleic acid + *cis*-Vaccenic acid	5.25	5.14	*ns*	5.16	5.23	*ns*	5.20b	4.79c	5.60a	***	*ns*	*	**	*ns*
Fatty acids SFA	205.53	198.55	*ns*	203.76	200.32	*ns*	202.31b	184.40c	219.42a	***	*ns*	*ns*	*ns*	*ns*
Behenic acid	0.81	0.81	*ns*	0.81	0.80	*ns*	0.76b	0.71b	0.96a	***	*ns*	*ns*	*ns*	*ns*
Stearic acid	62.61	60.38	*ns*	61.88	61.11	*ns*	60.79b	56.35c	67.35a	***	*ns*	*ns*	*ns*	*ns*
Lignoceric acid	0.46	0.44	*ns*	0.45	0.45	*ns*	0.49a	0.42b	0.45b	***	*ns*	*ns*	*ns*	*ns*
Arachidic acid	2.81	2.80	*ns*	2.85	2.77	*ns*	2.68b	2.50c	3.24a	***	*ns*	*ns*	*ns*	*ns*
Myristic acid	1.84	1.81	*ns*	1.81	1.84	*ns*	1.76b	1.68b	2.04a	***	*ns*	*ns*	*ns*	*ns*
Palmitic acid	135.95	131.28	*ns*	134.92	132.31	*ns*	134.88b	121.84c	144.12a	***	*ns*	*ns*	*ns*	*ns*
Miristoleic acid	0.49	0.50	*ns*	0.51	0.49	*ns*	0.41b	0.41b	0.68a	***	*ns*	*ns*	*ns*	*ns*
Margaric acid	0.56	0.53	*ns*	0.54	0.56	*ns*	0.55b	0.50c	0.60a	***	*ns*	*ns*	*ns*	*ns*
Fatty esters	16.29	9.70	*ns*	9.45	16.54	*ns*	24.22a	9.98b	4.79c	*	*ns*	*ns*	*ns*	*ns*
Ethyl stearate	16.29	9.70	*ns*	9.45	16.54	*ns*	24.22a	9.98b	4.79c	*	*ns*	*ns*	*ns*	*ns*

(Continues on the next page)

Table S4. (Continued)

Compound	Treatment (T)			Site (S)			Year (Y)				Y×T	S×T	Y×S	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
Prenols	0.14	0.11	*ns*	0.14	0.11	*ns*	0.11ab	0.07b	0.18a	*	*ns*	*ns*	*ns*	*ns*
Lupeol	0.14	0.11	*ns*	0.14	0.11	*ns*	0.11ab	0.07b	0.18a	*	*ns*	*ns*	*ns*	*ns*

[1] Data were analyzed by three-ways ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).
[2] Different letters (a, b, c) identify significantly different means. UNT, untreated control; CT, cluster thinning; FG, Friuli Grave; FCO, Friuli Colli Orientali.
All the concentrations are expressed in mg/L except 1-monopalmitoleoyl-rac-glycerol, fatty esters, and ethyl stearate that are reported in μg/L.

Table S5. Impact of cluster thinning, vineyard site and harvest season on the aromatic amino acid metabolites profile of Ribolla Gialla base wines.

Compound[3]	Treatment (T)			Site (S)			Year (Y)				Y×S	Y×T	S×T	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
TYR	3.06	3.15	*ns*	2.51b[2]	3.70a	**	4.15a	2.36b	2.89b	***	***	*ns*	*ns*	*ns*
PHE	0.99	0.89	*ns*	0.46b	1.42a	***	2.00a	0.35b	0.45b	***	***	*ns*	*ns*	*ns*
TRP	0.26	0.30	*ns*	0.27	0.29	*ns*	0.20	0.36	0.29	*ns*	*ns*	ns	*	*
KYNA	6.09	8.24	*ns*	4.96b	9.38a	**	9.66a	1.50b	10.79a	***	*	*ns*	*ns*	*ns*
NIC	0.39	0.25	*ns*	0.32	0.32	*ns*	0.28	0.26	0.41	*ns*	*	*ns*	*ns*	*ns*
TRP-EE	0.27	0.33	*ns*	0.30	0.30	*ns*	0.23b	0.39a	0.27b	***	*	*	*ns*	***
TYR-EE	6.50	5.50	*ns*	6.62	5.38	*ns*	10.91a	2.85b	4.51b	***	*ns*	*ns*	*ns*	*ns*
N-TYR-EE	0.28b	0.43a	**	0.36	0.34	*ns*	0.41a	0.26b	0.39a	*	**	*ns*	*ns*	*ns*
TYL	14.08	14.08	*ns*	15.26a	12.90b	**	15.26	13.10	14.02	*ns*	**	*ns*	*ns*	*ns*
OH-TYL	0.34	0.38	*ns*	0.38	0.34	*ns*	0.36b	0.27c	0.45a	***	**	*ns*	*ns*	***
Ph-AA	0.28	0.24	*ns*	0.26	0.26	*ns*	0.28	0.20	0.29	*ns*	*ns*	*ns*	*ns*	*ns*
TOL	0.35	0.39	*ns*	0.40	0.33	*ns*	0.27b	0.39a	0.46a	**	**	*ns*	*ns*	*ns*
IAA	0.49	0.41	*ns*	0.36b	0.54a	*	0.60a	0.34b	0.43b	*	*ns*	*ns*	*ns*	*ns*
ILA	0.39	0.34	*ns*	0.40	0.32	*ns*	0.47	0.30	0.30	*ns*	*ns*	*ns*	*ns*	*ns*
ILA-GLU[4]	0.99b	1.21a	**	1.21a	0.99b	**	0.74c	1.14b	1.38a	***	***	*ns*	**	***
N-SER	0.50a	0.42b	**	0.48	0.44	*ns*	0.42	0.48	0.46	*ns*	*ns*	*ns*	*ns*	*
Ph-LA	0.64	0.69	*ns*	0.72a	0.62b	**	0.54b	0.71a	0.76a	***	***	*ns*	*	*ns*
TOL-SO_3H	0.42	0.37	*ns*	0.42	0.38	*ns*	0.72a	0.27b	0.20b	***	*ns*	*ns*	*ns*	*ns*
ABA	0.39	0.42	*ns*	0.44a	0.37b	*	0.56a	0.31b	0.34b	***	***	*ns*	*ns*	*ns*
ABA-GLU	0.40	0.39	*ns*	0.33	0.46	*ns*	0.41	0.34	0.43	*ns*	*	*ns*	*	*ns*

[1] Data were analyzed by three-ways ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).
[2] Different letters (a, b, c) identify significantly different means. UNT, untreated control; CT, cluster thinning; FG, Friuli Grave; FCO, Friuli Colli Orientali.
[3] (TYR) Tyrosine; (PHE) Phenylalanine; (TRP) Tryptophan; (KYNA) Kynurenic acid; (NIC) Nicotinamide; (TRP-EE) Tryptophan ethyl ester; (TYR-EE) Tyrosine ethyl ester; (N-TYR-EE) N-acetyl tyrosine ethyl ester; (TYL) Tyrosol; (OH-TYL) Hydroxytyrosol; (Ph-AA) Phenyl acetic acid; (TOL) Tryptophol; (IAA) Indole 3-acetic acid; (ILA) Indole 3-lactic acid; (ILA-

GLU) Indole 3-lactic acid glucoside; (N-SER) N-acetyl serotonin; (Ph-LA) Phenyl lactic acid; (TOL-SO3H) Tryptophol-2-sulfonic acid; (ABA) Abscisic acid; (ABA-GLU) Abscisic acid glucoside.
[4] Quantified as ILA.
All the concentrations are expressed in mg/L.

Table S6. Impact of cluster thinning, vineyard site and harvest season on the aromatic amino acid metabolites profile of Ribolla Gialla sparkling wines.

Compound[3]	Treatment (T)			Site (S)			Year (Y)				Y×S	Y×T	S×T	Y×S×T
	UNT	CT	*Sig. F*[1]	FG	FCO	*Sig. F*	2017	2018	2019	*Sig. F*				
TYR	3.62b[2]	5.26a	**	3.80b	5.07a	*	4.50	4.59	4.23	*ns*	*	*	**	***
PHE	2.68	2.36	*ns*	1.48b	3.56a	***	4.32a	2.43b	0.81c	***	***	*ns*	*ns*	*
TRP	0.34	0.28	*ns*	0.31	0.31	*ns*	0.35a	0.20b	0.38a	**	*	*	*	*ns*
KYNA	7.80	8.50	*ns*	5.32b	10.98a	**	12.32a	2.34b	9.78a	***	*	*ns*	*ns*	*ns*
NIC	0.48	0.39	*ns*	0.45	0.42	*ns*	0.46a	0.21b	0.63a	***	*	*ns*	*ns*	*ns*
TRP-EE	0.40a	0.21b	*	0.28	0.33	*ns*	0.24	0.40	0.29	*ns*	*ns*	*ns*	*ns*	*ns*
TYR-EE	14.99	12.41	*ns*	9.94b	17.47a	***	24.65a	6.49b	9.98b	***	**	*ns*	*ns*	*ns*
N-TYR-EE	0.29b	0.53a	*	0.39	0.43	*ns*	0.40	0.32	0.52	*ns*	*ns*	*ns*	*ns*	*ns*
TYL	15.25	15.65	*ns*	14.59	16.31	*ns*	16.78	15.80	13.77	*ns*	*ns*	*ns*	*ns*	*ns*
OH-TYL	0.31	0.31	*ns*	0.24b	0.38a	***	0.31ab	0.26b	0.35a	**	***	*ns*	*ns*	*ns*
Ph-AA	0.34	0.31	*ns*	0.34	0.31	*ns*	0.24	0.34	0.39	*ns*	*ns*	*ns*	**	*ns*
TOL	0.49	0.54	*ns*	0.51	0.51	*ns*	0.33c	0.73a	0.48b	***	*ns*	*ns*	*ns*	*ns*
IAA	0.43a	0.24b	*	0.33	0.33	*ns*	0.44	0.21	0.35	*ns*	*ns*	*ns*	*ns*	*ns*
ILA	0.32	0.41	*ns*	0.31	0.42	*ns*	0.40	0.36	0.33	*ns*	*ns*	*ns*	*ns*	*ns*
ILA-GLU[4]	1.31	1.27	*ns*	1.46a	1.13b	**	0.95b	1.37a	1.56a	***	*ns*	*ns*	*ns*	*ns*
N-SER	0.44	0.42	*ns*	0.47	0.39	*ns*	0.46	0.42	0.41	*ns*	*ns*	*ns*	*ns*	*ns*
Ph-LA	0.84	0.89	*ns*	0.86	0.88	*ns*	0.73b	1.15a	0.72b	***	**	*	*ns*	*ns*
TOL-SO_3H	0.60	0.53	*ns*	0.41b	0.72a	***	1.00a	0.44b	0.26b	***	*ns*	*ns*	*ns*	*ns*
2AA	0.38	0.36	*ns*	0.47a	0.27b	**	0.33b	0.48a	0.30b	*	*	*ns*	*ns*	*ns*
ABA	0.51	0.59	*ns*	0.58	0.53	*ns*	0.83a	0.43b	0.40b	***	***	*ns*	*ns*	*ns*
ABA-GLU	0.41	0.35	*ns*	0.38	0.39	*ns*	0.42	0.42	0.31	*ns*	*ns*	*ns*	*ns*	*ns*

[1] Data were analyzed by three-ways ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).
[2] Different letters (a, b, c) identify significantly different means. UNT, untreated control; CT, cluster thinning; FG, Friuli Grave; FCO, Friuli Colli Orientali.
[3] (TYR) Tyrosine; (PHE) Phenylalanine; (TRP) Tryptophan; (KYNA) Kynurenic acid; (NIC) Nicotinamide; (TRP-EE) Tryptophan ethyl ester; (TYR-EE) Tyrosine ethyl ester; (N-TYR-EE) N-acetyl tyrosine ethyl ester; (TYL) Tyrosol; (OH-TYL) Hydroxytyrosol; (Ph-AA) Phenyl acetic acid; (TOL) Tryptophol; (IAA) Indole 3-acetic acid; (ILA) Indole 3-lactic acid; (ILA-

GLU) Indole 3-lactic acid glucoside; (N-SER) N-acetyl serotonin; (Ph-LA) Phenyl lactic acid; (TOL-SO3H) Tryptophol-2-sulfonic acid; (2AA) 2-Aminoacetophenone; (ABA) Abscisic acid; (ABA-GLU) Abscisic acid glucoside.
[4] Quantified as ILA.
All the concentrations are expressed in mg/L.

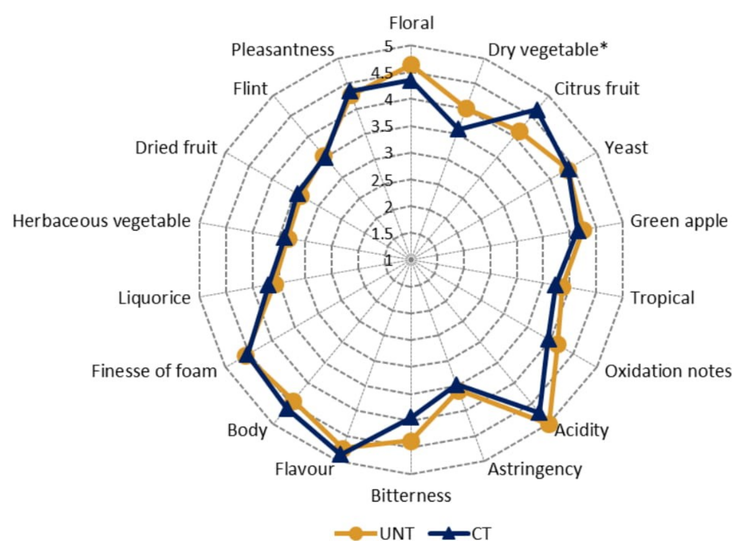

Figure S1. Effect of cluster thinning on the organoleptic characteristics of Ribolla Gialla sparkling wines. The average values were obtained from 2017–2019 and FG-FCO vineyard sites. Yellow and blue line represent untreated (UNT) and treated (CT) samples, respectively. Asterisks (*) indicate statistical significance ($p < 0.05$) for each sensory attribute.

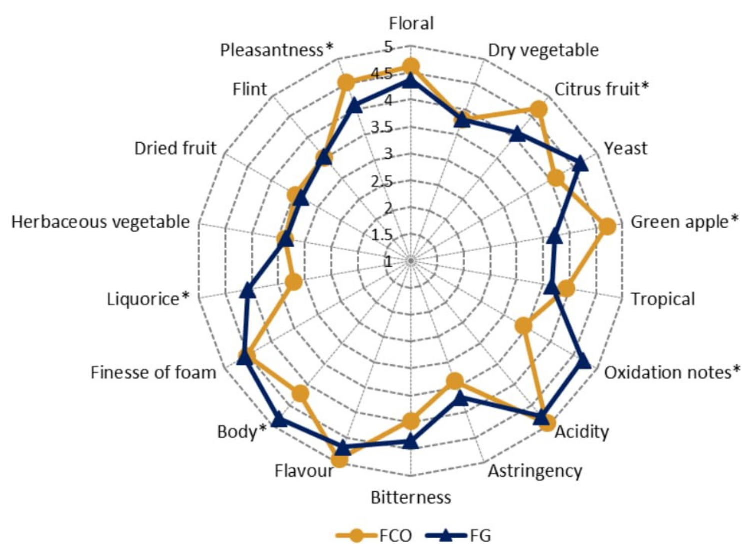

Figure S2. Effect of production site on the organoleptic characteristics of Ribolla Gialla sparkling wines. The average values were obtained from 2017–2019 of both UNT and CT samples. Yellow and blue line represent Friuli Colli Orientali (FCO) and Friuli Grave (FG) samples, respectively. Asterisks (*) indicate statistical significance ($p < 0.05$) for each sensory attribute.

Table S7. Retention indices and identification method used for VOCs analysis in base wines and sparkling wines.

Compound	RT (min)	RI_{exp}	RI_{lit}	IM	Literature
Monoterpenes					
β-Myrcene	8.59	1159	1173	MS RI STD	[62]
Limonene	9.32	1187	1204	MS RI STD	[59]
Linalool	17.60	1546	1555	MS RI STD	[60]
Geraniol	23.28	1876	1850	MS RI STD	[61]
Citronellol	21.81	1766	1777	MS RI STD	[62]
Nerol	21.73	1761	1777	MS RI STD	[63]
Terpinen-4-ol	18.69	1600	1618	MS RI STD	[64]
α-Terpineol	20.54	1696	1679	MS RI STD	[65]
Norisoprenoids					
Vitispirane	17.15	1524	1505	MS RI	[86]
TDN	21.44	1745	1732	MS RI	[60]
β-Damascenone	22.81	1833	1857	MS RI STD	[66]
Actinidiol (isomer 1)	24.65	2005	-	MS	-
Actinidiol (isomer 2)	24.88	2018	-	MS	-
Aldehydes					
Hexanal	6.55	1081	1072	MS RI STD	[67]
trans-2-Hexenal	10.73	1242	1235	MS RI STD	[68]
Nonanal	14.32	1392	1397	MS RI	[69]
Benzaldehyde	17.08	1520	1507	MS RI STD	[70]
3,4-Dimethyl benzaldehyde	22.23	1789	1790	MS RI	[71]
Acetaldehyde	1.72	549	-	MS	-
Furfural	15.86	1463	1460	MS RI	[72]
Alcohols					
n-Hexanol	13.40	1353	1358	MS RI STD	[70]
trans-3-Hexenol	13.63	1363	1374	MS RI STD	[65]
1-Octanol	17.81	1556	1562	MS RI	[70]
Iso-butanol	7.14	1104	1114	MS RI STD	[73]
Methionol	20.88	1715	1711	MS RI	[74]
Isoamyl alcohol	10.11	1218	1209	MS RI STD	[75]
3-Methyl-1-pentanol	12.76	1325	1316	MS RI STD	[74]
2,3-Butanediol (isomer 1)	17.41	1537	1529	MS RI	[76]
2,3-Butanediol (isomer 2)	18.16	1574	1583	MS RI	[75]
cis-3-Hexenol	14.57	1403	1382	MS RI STD	[76]
trans-2-Hexenol	15.06	1426	1420	MS RI STD	[73]
2-Phenylethanol	24.08	1906	1923	MS RI	[80]

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Table S7. (Continued).

Compound	RT (min)	RI_{exp}	RI_{lit}	IM	Literature
Esters					
Ethyl acetate	2.69	892	889	MS RI	[77]
Ethyl butyrate	5.52	1040	1025	MS RI STD	[58]
Isopentyl acetate	7.60	1122	1120	MS RI STD	[59]
Hexyl acetate	11.46	1272	1295	MS RI STD	[60]
Methyl caproate	9.28	1185	1180	MS RI STD	[61]
Ethyl hexanoate	10.53	1234	1241	MS RI STD	[62]
Ethyl lactate	13.18	1343	1355	MS RI	[63]
Methyl octanoate	14.26	1390	1387	MS RI	[64]
Ethyl octanoate	15.24	1434	1453	MS RI STD	[65]
Isoamyl lactate	18.04	1568	1570	MS RI	[81]
Methyl decanoate	18.60	1596	1604	MS RI	[82]
Ethyl decanoate	19.48	1641	1643	MS RI STD	[60]
Isoamyl octanoate	19.85	1660	1654	MS RI STD	[66]
Methyl ethyl succinate	19.36	1635	1641	MS RI	[83]
Diethyl succinate	20.16	1677	1679	MS RI	[83]
Ethyl 9-decanoate	20.46	1692	1708	MS RI	[84]
Ethyl-2-OH-4-methylpentanoate	17.51	1542	1547	MS RI	[67]
2-Phenylethyl acetate	22.72	1825	1832	MS RI STD	[68]
Ethyl hydrogen succinate	31.69	-	-	MS	-
Methyl salicylate	21.99	1775	1765	MS RI STD	[70]
Ethyl hydroxybutanoate	22.51	1806	-	MS	-
Ethyl dodecanoate	22.80	1832	1850	MS RI	[85]
Isobutyl acetate	5.42	1036	1020	MS RI STD	[72]
Acids					
Acetic acid	15.66	1453	1437	MS RI STD	[70]
Butyric acid	19.26	1630	1598	MS RI STD	[72]
3-Methylbutyric acid	20.05	1671	1657	MS RI	[70]
Hexanoic acid	23.28	1876	1857	MS RI STD	[62]
Octanoic acid	26.86	-	-	MS STD	-
Nonanoic acid	28.54	-	-	MS STD	-
Decanoic acid	30.13	-	-	MS STD	-
Benzoic acid	32.47	-	-	MS	-
Dodecanoic acid	33.17	-	-	MS	-
Ketones					
2-Methylthiolan-3-one	17.16	1524	1510	MS RI	[78]
Isophorone	18.00	1566	1600	MS RI	[79]

RT, retention time in min; RI_{exp}, experimentally determined retention index; RI_{lit}, retention index reported in the literature; IM, identification method (MS, comparison of mass spectra with those reported in mass spectrum libraries; RI, comparison of order of elution with those reported in literature; STD, comparison of mass spectra and retention time with those of standard compounds).

3

DETERMINING THE RIGHT HARVEST TIME TO IMPROVE THE QUALITY OF SPARKLING WINES FROM RIBOLLA GIALLA L. cv.[3]

[3] This chapter is a part of a manuscript in preparation.

1 INTRODUCTION

In addition to a balanced production level, the timing of grape harvest is another crucial factor to be considered in the winemaking process (Šuklje et al., 2019). Luna et al. (2017) even argued that keeping a full crop with not abundantly extended harvest date have a greater impact on wine quality than reducing crop level. This was best seen on the sugar concentration, where an extended harvest date lead to increase of Brix more than crop reduction with commercial harvest (Luna et al., 2017). A sufficient accumulation of sugars in grapes throughout ripening is necessary, not only to achieve the desired alcohol levels in the wine but also to guarantee the biosynthesis of the aromatic precursors. It has been previously shown that the delayed harvest increased the content of varietal aromas, esters, aldehydes and alcohols in Pinot Gris and Riesling wines, but at the same time, the concentration of volatile acids and green odour related compounds decreased. As a result, sensorial analysis significantly distinguished the wines according to the time of harvest, preferring those from delayed harvest (Moreno Luna et al., 2018). In addition, the existing studies were mostly dealing with the influence of harvest date on wines, obtained from colored grape varieties, where the impact of harvest timing on the quality of sparkling wines is neglected, resulting in the lack of literature. In study dealing with Cabernet Sauvignon wines, the researchers reported that fermentative aroma compounds, with volatile esters, dimethyl sulfide, glycerol and mannoproteins, increased in parallel with must sugar concentration, whereas isobutyl methoxypyrazine, C6 alcohols and hexyl acetate decreased as ripening progressed (Bindon et al., 2013). Conversely Šuklje et al. (2019) found out that Shiraz wines from early harvest date were characterized by an abundance of C5 and C6 lipoxygenase derived compounds, norisoprenoids and sulfur-containing compounds, while the accumulation of acetate esters was observed in wines produced from the more mature grapes. It has been proven that harvesting in later dates increases the concentration of sesquiterpene rotundone (Caputi et al., 2011; Geffroy et al., 2014), while in Grenache wines produced from the most mature grapes, the increase of certain esters has been observed, together with the decrease of acetaldehyde (Arias et al., 2019). Moreover, authors have reported a positive correlation between the maturity of the grapes and increased concentration of terpene compounds (Arias et al., 2019; Marais, 2017).

For winegrowers, the sugar concentration, total acidity, and pH value are the principal fruit criteria used for determining harvest dates for sparkling wine. However, a delay of harvest is linked to an increase in Brix due to the reduction in berry weight because of dehydration process (Moreno Luna et al., 2018). In addition, a great impact on the harvest timing depends also to growing season variability and climatic conditions. A temperature drop and higher precipitation can create ideal situation, where the fungal infections by *Botrytis cinerea* can occur. Since the skin of the riper grapes becomes softer and more elastic (Elmer & Michailides, 2007), such grapes are more susceptible to infection with *Botrytis cinerea*, therefore the grape-growers from Champagne tend to advance the harvest dates in order to ensure the quality of the grapes (P.-H. Liu et al., 2018). Similarly, the winemakers

of Cava sparkling wine also tend to harvest the grapes earlier, since warmer conditions can cause faster ripening of the grape pulp. This results in increased pH value as well as sugar and protein concentration, which does not necessarily lead to improved foamability of sparkling wines (Esteruelas et al., 2015). However, it has been shown previously, that proteins are not the only substances on which foaming properties of sparkling wines depend. Several authors have thus described the connection between lipid content, in particular fatty acid composition, and the foamability of sparkling wines (Gallart et al., 2002; Pueyo et al., 1995; Pueyo et al., 2000). Linolenic (C18:3) and linoleic acid (C18:2) are the major components of the total lipids in grape berries, therefore, it is important how their content changes during the vegetative growth of grapes (Pérez-Navarro et al., 2019). These two fatty acids are also susceptible to oxidation on the presence of lipoxygenase, which can give rise to herbaceous organoleptic defects later in wine, caused by lower-molecular-weight compounds known as oxylipins (Pilati et al., 2014; Zamora et al., 1985). However, in the study of Macabeo grapes, the authors have shown, that there was no direct correlation between the content of linoleic and linoleic acid, lipoxygenase and C6 aldehydes during the vegetative stages (Iglesias et al., 1991). On the contrary, it is not completely clear how the composition of fatty acids behaves during the vegetative cycle of grapes. Some authors claimed that the alternations of the total lipid content occur from the veraison to the end of the ripening, while others reported no variation in saturated fatty acid and linoleic acid concentrations in unripe and ripe grapes (Barron et al., 1989). Since these compounds can be extracted into the fermentation medium during winemaking process, it is important to see how their presence influence the overall quality of the wine.

Tryptophan (TRP) and its metabolites, especially indole-3-acetic acid (IAA), the most common naturally occurring plant auxin plant hormone, are considered to be potential precursors of 2-aminoacetophenone, an aroma compound which is responsible for the untypical aging off-flavor (Hoenicke et al., 2000). This particular off-flavor is considered to be detrimental, as characterize the wines with odor taints like naphthalene, floor-polish, washing-soap or acacia-blossom (Hoenicke et al., 2000). Ruiz-Rodríguez et al. (2017) were comparing the levels of tryptophan during grape ripening. The tryptophan levels decreased for the reference sample during ripening period, while an additional date for post-harvest study showed that grapes on vines produced 12% higher levels of tryptophan. However, according to the Hoenicke et al. (2001) the amounts of bound IAA and free and bound TRP in grapes increased significantly with the stage of maturity, which confirms that the nitrogen or amino acid contents of the grape musts and wines increase with the ripeness of the harvested grapes. Therefore, it could be expected that the wines from later harvest stage will be more prone to develop the untypical aging off-flavor (UTA), but Hoenicke et al. (2000) claimed that UTA appearance is not directly correlated to the amount of IAA present in the must or wine but it is more likely to be connected with a nitrogen deficiency of the harvested grapes. Considering the sparkling wines, it has been shown, that they can contain much lower amount of TRP compared to the other wines, which could be the consequence of the secondary fermentation or the earlier harvest time (Arapitsas et al., 2018).

In order to target optimal grape ripeness and maximize positive attributes of the wine produced, it was additionally decided to experiment with different harvest dates to determine, whether an extended harvest date might have a greater positive organoleptic impact and lead to larger increases in important odor-impact compounds.

2 MATERIALS AND METHODS

2.1 HARVEST OF THE GRAPES AND MICROVINIFICATIONS OF THE BASE WINES AND SPARKLING WINE

The harvest timing trial was carried out during the 2017–2019 harvest period, only in the vineyard of Corno di Rosazzo (46° 00' 19.1" North; 13° 26' 30.6" East; elevation 94 m a.s.l.), following sequential harvests on the untreated vines where the production was standardized. The vineyard site is described in the detail in the previous *Chapter 2*. The first harvest was set when a minimum compromise was reached between the accumulation of sugars and the level of titratable acidity of the grapes. The second harvest was established about one week after the first, and the third harvest was positioned 4–7 days after the second harvest based on meteorological conditions of each growing season. The third harvest was carried out only in 2018 and 2019, since in 2017 the excessive level of rain prevented the harvest of healthy grapes. More detailed information on each harvest performed is presented in Table 1. The sampling of the grapes and subsequent vinification were carried out as reported in *Chapter 2*.

Table 1: Harvest dates of Ribolla Gialla grapes according to the vintage year.

Harvest	Vintage		
	2017	2018	2019
H1	29 Aug	21 Aug	10 Sep
H2	05 Sep	29 Aug	17 Sep
H3		04 Sep	24 Sep

H1, harvest 1; H2, Harvest 2, H3, Harvest 3

2.2 CHEMICAL CHARACTERIZATION

All the chemicals and reagents that have been used for the chemical characterization of wine samples are listed in *Chapter 2*, together with the methods of extractions and analysis performed, described in detail. Briefly, VOCs have been extracted using HS–SPME technique, and the analysis was performed using a GC–MS method, with Thermo Trace GC Ultra gas chromatograph, coupled to a Thermo Quantum XLS mass spectrometer. Additional parameters for fibre conditioning, microextraction regime, chromatographic and mass spectrometric conditions were based on the work of Carlin et al. (2016). Secondly, the extraction of lipid molecules, UHPLC separation and MS detection was adopted from Della Corte et al. (2015). The chromatograph used was Dionex 3000 from Thermo Fisher

Scientific, while the mass spectrometer was API 5500 triple-quadrupole from Sciex. Finally, the UHPLC-MS/MS analysis of aromatic amino acid metabolites was carried out on a Waters Acquity UPLC system, coupled to Water Xevo TQ triple-quadrupole mass spectrometer. All the instrumental parameters were according to Arapitsas et al. (2018).

2.3 SENSORY ANALYSIS

The sparkling wines were assessed in all three vintages by a panel (as described in the *Chapter 2*), and the same list of eighteen sensory descriptors was also used for the evaluation. In all three seasons, the wines have been presented to the tasters anonymously, by dividing three replicas in three subsequent sessions, completely randomized. All the data collected were subsequently normalized for each taster.

2.4 STATISTICAL ANALYSIS

In order to assess the difference between different harvest timings in three consecutive seasons, the two-way ANOVA was performed on 106 JMP® software (JMP 7.0, SAS Institute Inc., NC, USA). The *p* value was set at 0.05 and the values bellow were considered as statistically significant, and the means were consequently separated using Student-Newman Keuls test ($p < 0.05$). By using a custom R script (R Core Team, 2020), the missing values of volatile compounds, lipids and aromatic amino acid metabolites were imputed with a random value between zero and LOQ. Principal Component Analysis (PCA) was then performed, with the data previously scaled using Z-transformation. The R packages FactoMineR v 2.3, factoextra v 1.0.7 and ggplot v 3.3.2 were used to perform the PCA analysis and to visualize the results (Husson et al., 2020; Kassambara & Mundt, 2020; Wickham et al., 2020).

3 RESULTS

3.1 RIPENING TREND OF GRAPES FROM VERAISON TO HARVEST

The maturity parameters, such as total soluble solids (TSS), titratable acidity (TA) and the pH value were analyzed by two-way ANOVA to compare the differences between two (Table 2) or three harvest timings (Table 2). Considering two harvest timings (H1 versus H2) in all three harvest seasons, the results did not reveal any significant difference for TSS, TA and pH value. However, the trend indicated that H1 was characterized by a higher TSS and TA content and consequently a lower pH value. The year of harvest appeared to be significant only in case of TSS, where the highest value (18.43 °Brix) was evident in the first year of experimental trial, followed by 2019 (18.12 °Brix) and 2018 (17.12 °Brix). However, there was a strong substantial interaction effect between harvest timing and year for TA and pH value.

Table 2: The effect of two harvest timings on basic composition of Ribolla Gialla grape in season from 2017–2019.

Parameter	Harvest timing (H)			Year (Y)				H×Y
	H1	H2	*sig. F*[a]	2017	2018	2019	*sig. F*	
Total soluble solids (°Brix)	17.96	17.83	*ns*	18.43a[b]	17.12b	18.12ab	*	*ns*
Titratable acidity (g/L)[c]	6.84	6.56	*ns*	7.02	6.49	6.58	*ns*	**
pH	3.24	3.26	*ns*	3.29	3.24	3.22	*ns*	**

[a] Data were analyzed by two-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when the differences were significant, the means were separated using Tukey's HSD test ($p < 0.05$).
[b] Different letters (a, b) identify significantly different means. H1, first harvest, H2, second harvest.
[c] Expressed in tartaric acid.

Results showed that when comparing the effects of three harvest times (Table 3), later dates resulted in non-significant increase of TSS, while the values of TA and pH varied inconsistently. The TSS increased from H1 to H3 for 0.31 °Brix, while the content of TA appeared to be the highest in H1, following by H3 and finally H2. Harvest season had once again a greater impact on the amount of TSS, as 2019 was characterized by a significantly higher sugar content compared to the previous year. The higher TA content in 2019 and complementary lower pH lead to significant interaction between harvest timing and harvest season for these two parameters.

Table 3: The effect of three harvest timings on basic composition of Ribolla Gialla grape in season from 2018–2019.

Parameter	**Harvest timing (H)**				**Year (Y)**			**H×Y**
	H1	**H2**	**H3**	***sig. F*[a]**	**2018**	**2019**	***sig. F***	
Total soluble solids (°Brix)	17.54	17.71	17.85	*ns*	17.27b	18.13a[b]	*	*ns*
Titratable acidity (g/L)[c]	6.78	6.30	6.66	*ns*	6.45	6.70	*ns*	*
pH	3.20	3.26	3.23	*ns*	3.25	3.21	*ns*	**

[a] Data were analyzed by two-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when the differences were significant, the means were separated using Tukey's HSD test ($p < 0.05$).
[b] Different letters (a, b) identify significantly different means. H1, first harvest, H2, second harvest; H3, third harvest.
[c] Expressed in tartaric acid.

3.2 WINE BASIC COMPOSITIONAL PARAMETERS

When comparing the H1 and H2 (Table 4), the results of extended harvest showed an expected slight increase of sugar concentration, which was in line with the alcohol content analyzed in the samples of sparkling wines. However, stronger statistical significance was observed for the TA ($p < 0.01$), compared to the pH value ($p < 0.05$) and these two parameters followed the principles of harvest time, as the content of TA in H1 compared to H2 decreased from 7.80 g/L to 6.92 g/L, respectively. The seasonal factor had the biggest influence on all basic parameters. The highest concentration of reducing sugars in 2017 was correlated to the highest content of produced alcohol in sparkling wine samples.

Additionally, no abnormalities were observed with regard to complementary TA and the pH value. Interestingly, no interaction was observed between the harvest timing and the harvesting season, for any of the basic parameters.

Table 4: Characteristics of Ribolla Gialla sparkling wine composition at two different harvest times in seasons from 2017–2019.

Parameter	Harvest timing (H)			Year (Y)				H×Y
	H1	H2	*sig. F*[a]	2017	2018	2019	*sig. F*	
Reducing sugars (g/L)	1.23	1.30	*ns*	0.48b	1.96a	1.35a	***	*ns*
Titratable acidity (g/L)[c]	7.80a[b]	6.92b	**	7.58ab	6.82b	7.68a	*	*ns*
pH	3.12b	3.21a	*	3.20a	3.23a	3.07b	**	*ns*
Alcohol (% v/v)	11.25	11.49	*ns*	11.95a	10.76b	11.40ab	**	*ns*

[a] Data were analyzed by two-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated busing Tukey's HSD test ($p < 0.05$).
[b] Different letters (a, b) identify significantly different means. H1, first harvest; H2, second harvest.
[c] Expressed in tartaric acid.

The effects of additional third harvest were analyzed in Table 5. The content of reducing sugars (RS) resulted to be the only parameter where the differences between H1, H2 and H3 were not statistically significant. Moreover, the second extended harvest resulted in a lower content of RS compared to H2 (1.70 g/L versus 1.83 g/L). In addition, the H3 sample produced the most alcohol, which further suggests the inconsistency of the result when comparing the alcoholic strength with the sugar level in the sparkling wines, probably due to insignificant trend of differences in Brix between H1 and H3 in grape samples. As for TA, the concentration of this parameter turned out to be lower with the extended harvest date. Conversely, the pH value increased proportionally with every subsequent harvest date. The comparison between two vintages, where the third harvest was carried out, resulted in increased RS concentration in 2018 and a concomitant decrease in alcoholic strength. Strong statistical significance ($p < 0.001$) characterized the TA and pH value. The winegrowing season 2019 thus differed from the previous season in higher TA content (7.54 g/L versus 6.57 g/L) and ultimately low pH (pH 3.10 versus pH 3.27). As already observed when comparing only two harvest dates, there was no statistically significant interaction between harvest date and harvest season.

Table 5: Characteristics of Ribolla Gialla sparkling wine composition at three different harvest times in seasons from 2018–2019.

Parameter	Harvest timing (H)				Year (Y)			H×Y
	H1	H2	H3	*sig. F*[a]	2018	2019	*sig. F*	
Reducing sugars (g/L)	1.49	1.83	1.70	*ns*	1.91a	1.43b	*	*ns*
Titratable acidity (g/L)[c]	7.57a[b]	6.93b	6.66b	***	6.57b	7.54a	***	*ns*
pH	3.12b	3.18ab	3.26a	*	3.27a	3.10b	***	*ns*
Alcohol (% v/v)	10.96b	11.20ab	11.80a	*	11.07	11.56	*ns*	*ns*

[a] Data were analyzed by two-way ANOVA (ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001), and when differences were significant, the means were separated busing Tukey's HSD test (p < 0.05).
[b] Different letters (a, b) identify significantly different means. H1, first harvest; H2, second harvest; H3, third harvest.
[c] Expressed in tartaric acid.

3.3 VOLATILE PROFILE OF BASE WINES AND SPARKLING WINES AFTER TWO HARVEST TIMINGS

The results of the volatile profiling in the base and sparkling wines after two subsequent harvests are presented in Table 6. All the compounds were separated into seven chemical classes (monoterpenes, norisoprenoids, aldehydes, alcohols, esters, acids, and ketones). The two-ways ANOVA was applied in order to investigate the main effects of extended harvest date and winegrowing season.

From the results of the base wines (Table 6) it can be observed that the concentration of volatile compounds on general increased in wines from H1 to H2. Among eight monoterpenes only citronellol contributed significantly to sample separation between the harvest dates. Moreover, citronellol was together with linalool and geraniol the most abundant among the monoterpenes. β-damascenone appeared to be the most abundant among the norisoprenoids class of compounds with concentrations 9.41 and 14.55 μg/L in H1 and H2 base wine samples, respectively. Together with one isomer of actinidiol they resulted to be only norisoprenoids, where the difference between H1 and H2 was statistically significant. Additionally, seven aldehydes were identified and quantified in the base wine samples, among which the nonanal, 3,4-dimethyl benzaldehyde and furfural were found to be increased significantly in H2 wines. The most abundant amongst the rest of the carbonyl compounds was *trans*-2-hexenal, whose concentration represented 70% of the total aldehyde composition. The most important sensory carbonyl substance, acetaldehyde, decreased in H2 compared to the H1 (9.50 μg/L to 7.25 μg/L). The average concentration sum of alcohols prevailed among all the compounds. Except 1-octanol, isoamyl alcohol and 2,3-butanetiol alcohol, all the remaining alcohols showed strong statistical significance (p < 0.001) in differentiating H1 from H2. In terms of content, the third most concentrated group proved to be esters. Interestingly, only four of the twenty-one esters contributed to the statistical distinction between reference harvest and an extended one. Apart from the general trend, the abundances of methyl ethyl succinate, diethyl succinate and ethyl-2-OH-4-methylpentanoate

decreased in wines from H1 to H2. Similarly, 2-methylthiolan-3-one was characterized by the same trend of decreasing concentration, while for acids the additional harvest date also meant an increase in concentration. Finally, several significant interactions between harvest timing factor and harvest season have been observed, which is most likely due to the seasonal differences, that appeared to have strong influence.

Season 2018 was therefore characterized with high amount of monoterpenes, considering the other two vintages. However, only a few individual compounds followed this pattern (e.g., linalool and α-terpineol), while the amount of the remaining monoterpenes was distributed among the remaining two years in a randomized manner. The highest concentration of β-myrcene was detected in 2017, which was in line with the sum of all norisoprenoids. Among the aldehydes, all substances showed a higher concentration in 2018, except for the acetaldehyde and furfural, which were most produced in 2017. The quantity of isoamyl alcohol resulted to be by far most synthesized in 2019 vintage, which consequently affected the sum of higher alcohols, when comparing all three vintages. The domination of C6, C8 and C10 fatty acids and their ethyl esters in wines from 2017, lead to significantly higher concentration of total esters and acids in the same year.

Sparkling wines (Table 6) generally followed the trend of increased amount of volatile compounds after extended harvest, with a few exceptions. In particular, the total concentration of monoterpenes has decreased in H2 wines, which is due to the reduced concentration of α-terpineol, the most abundant terpene compound. On the contrary, the amount of citronellol increased, following the results from the base wines. β-damascenone and hexanal were the most significant contributors to the increase of norisoprenoids and aldehydes, respectively in H2 wines. Considering the higher alcohol content, the abundance of n-hexanol, *trans*-3-hexanol and isobutanol in wines significantly increased from H1 to H2. A comparison of the additional harvest for fermentative esters did not show uniform results, which was probably related to secondary fermentation. The total amount of esters decreased from 2957.84 μg/L in H1 to 2845.35 μg/L in H2. However, the compounds with most significant effect proved to be ethyl-9-decanoate, ethyl-2-OH-4-methylpentanoate and ethyl hydroxybutanoate with only the latter increasing in H2 wines. The amount of acetic acid increased significantly in H2 wines, while the higher amount of total acids characterized the H1 samples.

As regards different winegrowing seasons of sparkling wines, the results obtained showed significantly higher presence of monoterpenes in 2019, which was true also for α-terpineol and nerol, while other important terpenes (e.g., linalool and geraniol) resulted to have higher influence in the second year of the experiment. Similar observation emerged also for the norisoprenoids, where total amount was significantly higher in the 2019 ($p < 0.001$), together with β-damascenone and 1,1,6-trimethyl-1,2-dihydronapthalene (TDN). Compared to the remained seasons, the year 2017 was characterized mainly by a higher content of aldehydes, in particular benzaldehyde and furfural, and quantitatively significant *trans*-2-hexanal. Similarly, the sum of all alcohol compounds was higher in the first year;

however, the only significant compounds resulted to be methionol and 3-methyl-1-pentanol, and the highest concentration of both was found in 2018. The vast majority of substances that belong to the ester group proved to have a significant impact on the volatile profile of sparkling wines, from 2017 season, although it turned out that the wines from 2019 contained higher concentration of acetate esters. Moreover, in the present study, acids exhibited inconsistent behavior in relation to the harvest, where acetic acid dominated in the wines from 2019, while 3-methylbutyric acid was predominantly present in wines form 2017. Similarly, the concentration of 2-methylthiolan-3-one ketone prevailed in 2018, while 2017 was characterized with higher abundance of isophorone.

Table 6: Impact of two harvest timings and harvest season on the volatile profile of Ribolla Gialla base wines and sparkling wines.

Compounds	Base wines								Sparkling wines							
	Harvest timing (H)			Year (Y)				H×Y	Harvest timing (H)			Year (Y)				H×Y
	H1	H2	*sig. F*[a]	2017	2018	2019	*sig. F*		H1	H2	*sig. F*[a]	2017	2018	2019	*sig. F*	
Monoterpenes	19.25	20.4	*ns*	19.30ab	23.95a	16.22b	*	*ns*	24.86	24.73	*ns*	21.32b	22.40b	30.66a	***	**
β-myrcene	0.28	0.34	*ns*	0.54a	0.23b	0.16b	***	*ns*	0.74	0.76	*ns*	0.69	0.75	0.81	*ns*	ns
Limonene	1.23	1.40	*ns*	0.52c	1.44b	1.99a	***	*	2.53	2.33	*ns*	2.60	2.45	2.24	*ns*	ns
Linalool	4.33	4.67	*ns*	4.03b	7.00a	2.48b	**	*ns*	4.61	4.92	*ns*	4.28b	5.59a	4.43b	**	ns
Geraniol	6.24	6.59	*ns*	6.36	6.26	6.62	*ns*	*ns*	7.03	7.64	*ns*	8.48	6.88	6.64	*ns*	ns
Citronellol	3.43b[b]	4.48a	***	5.25a	3.83b	2.78c	***	***	1.28b	1.90a	**	1.52	1.47	1.79	*ns*	ns
Nerol	1.55	1.87	*ns*	1.48b	2.13a	1.51b	**	*ns*	2.77	3.12	*ns*	2.08b	2.39b	4.36a	***	*
Terpinen-4-ol	0.27	0.47	*ns*	0.26	0.68	0.18	*ns*	*ns*	0.55	0.62	*ns*	0.44b	0.70a	0.61ab	*	ns
α-terpineol	1.93	0.57	*ns*	0.86	2.38	0.51	*ns*	*ns*	5.35[b]	3.43b	**	1.22b	2.16b	9.78a	***	ns
Norisoprenoids	10.78b	16.01a	**	17.96a	13.10b	9.13c	**	*ns*	15.04b	19.16a	***	16.98b	12.25c	22.06a	***	ns
Vitispirane	0.49	0.35	*ns*	0.55a	0.40ab	0.30b	*	*ns*	0.81	0.75	*ns*	0.88	0.76	0.69	*ns*	ns
TDN	0.65	0.69	*ns*	0.58	0.77	0.67	*ns*	*ns*	0.96	1.10	*ns*	1.00b	0.37c	1.71a	***	ns
β-damascenone	9.41b	14.55a	**	16.44a	11.44b	8.05b	**	*ns*	12.35b	16.46a	***	14.14b	10.30c	18.77a	***	ns
Actinidiol (isomer 1)	0.12b	0.32a	*	0.19b	0.41a	0.06b	**	**	0.39	0.36	*ns*	0.41	0.35	0.38	*ns*	ns
Actinidiol (isomer 2)	0.11	0.11	*ns*	0.19a	0.08b	0.06c	***	*	0.53	0.48	*ns*	0.55	0.46	0.50	*ns*	ns
Aldehydes	110.05	134.69	*ns*	144.66	121.71	100.73	*ns*	*ns*	414.6	439.39	*ns*	472.76a	367.96b	440.25ab	*	ns
Hexanal	0.38	0.50	*ns*	0.54a	0.56a	0.21b	***	*ns*	0.07b	0.09a	*	0.12a	0.04c	0.08b	***	**
trans-2-hexenal	89.8	95.01	*ns*	111.9	75.75	89.57	*ns*	*ns*	384.77	407.36	*ns*	436.31a	336b	415.88a	*	ns
Nonanal	5.86b	25.02a	***	10.79b	31.24a	4.30c	***	***	2.81	2.89	*ns*	2.86	2.81	2.89	*ns*	ns
Benzaldehyde	3.67	5.50	*ns*	6.76	4.86	2.14	*ns*	*ns*	6.55	7.11	*ns*	8.98a	5.4b	6.11b	***	ns
3,4-dimethyl benzaldehyde	0.37b	0.85a	***	0.58b	0.96a	0.29c	***	***	1.44	1.57	*ns*	1.77a	1.22b	1.52a	**	ns
Acetaldehyde	9.50	7.25	*ns*	13.01a	8.01b	4.10c	**	*ns*	17.27	18.71	*ns*	20.68a	21.29a	12.00b	*	ns
Furfural	0.46b	0.56a	*	1.08a	0.32b	0.12c	***	**	1.69	1.66	*ns*	2.05a	1.20c	1.77b	***	**
Alcohols[c]	2.29b	2.71a	*	1.97b	1.69b	3.84a	***	**	3.86	4.14	*ns*	4.19	3.87	3.94	*ns*	*
n-hexanol	63.96b	133.78a	***	195.87a	46.27b	54.48b	***	***	63.55b	115.11a	***	169.95a	33.63c	64.41b	***	***
trans-3-hexenol	1.26b	2.45a	***	2.87a	1.54b	1.15b	***	***	1.5b	2.58a	***	3.24a	1.39b	1.49b	***	***

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Table 6: (Continued)

Compounds	Base wines								Sparkling wines							
	Harvest timing (H)			Year (Y)				H×Y	Harvest timing (H)			Year (Y)				H×Y
	H1	H2	*sig. F*[a]	2017	2018	2019	*sig. F*		H1	H2	*sig. F*[a]	2017	2018	2019	*sig. F*	
1-octanol	8.90	10.95	*ns*	8.64	12.46	8.67	*ns*	*ns*	9.97	10.39	*ns*	9.21	10.49	10.85	*ns*	ns
iso-butanol	90.80b	143.60a	***	97.65b	163.87a	90.07b	***	***	120.21b	137.17a	*	130.86	132.89	122.32	*ns*	*
Methionol	3.98b	7.61a	***	7.77a	6.14b	3.48c	***	***	2.86	2.91	*ns*	1.90b	3.50a	3.25a	**	ns
Isoamyl alcohol	1406.49	1548.16	*ns*	705.15b	772.51b	2954.31a	***	**	2900.42	3078.49	*ns*	3046.59	2898.87	3022.89	*ns*	*
3-methyl-1-pentanol	6.41b	10.00a	***	8.37a	9.54a	6.71b	**	***	9.97	10.38	*ns*	10.37ab	11.34a	8.83b	*	ns
2,3-butanediol (isomer 1)	19.29	19.88	*ns*	24.43a	17.06b	17.27b	*	*ns*	2.13	6.74	*ns*	7.86	4.80	0.64	*ns*	ns
2,3-butanediol (isomer 2)	5.72	5.30	*ns*	6.39	6.13	4.01	*ns*	*ns*	1.13	3.04	*ns*	3.58	1.99	0.68	*ns*	ns
2-phenylethanol	685.58b	830.05a	*	913.45a	659.79b	700.19b	**	**	757.99	774.49	*ns*	809.75	778.45	710.51	*ns*	ns
cis-3-hexenol									0	0	*ns*	0	0	0	*ns*	ns
trans-2-hexenol									0	0	*ns*	0	0	0	*ns*	ns
Esters	2439.1	2398.17	*ns*	4297.29a	1927.41b	1031.21c	***	*ns*	2957.84	2845.35	*ns*	2725.04b	2681.00b	3298.73a	*	ns
Ethyl acetate	89.12b	128.01a	**	154.58a	79.30b	91.82b	**	*ns*	304.77	377.51	*ns*	353.20	286.52	383.71	*ns*	ns
Ethyl butyrate	29.78	30.77	*ns*	34.96	30.36	25.50	*ns*	*	48.65	43.98	*ns*	38.87	46.16	53.91	*ns*	ns
Isopentyl acetate	222.08	214.89	*ns*	303.93a	187.50b	164.02b	*	*ns*	205.33	188.86	*ns*	142.92b	218.70a	229.67a	*	ns
Hexyl acetate	43.64	48.29	*ns*	100.90a	15.91b	21.10b	***	*ns*	26.10	18.49	*ns*	16.60b	19.38ab	30.90a	*	ns
Methyl caproate	0.45	0.55	*ns*	0.48	0.51	0.51	*ns*	*ns*	0.65	0.62	*ns*	0.55	0.67	0.68	*ns*	ns
Ethyl hexanoate	738.62	728.41	*ns*	1007.10a	760.54b	432.9c	**	*ns*	1076.26	1041.3	*ns*	1149.92	1006.32	1020.09	*ns*	ns
Ethyl lactate	3.36	3.50	*ns*	5.34a	2.31b	2.65b	***	**	17.54	16.32	*ns*	21.79a	13.28b	15.70b	***	ns
Methyl octanoate	1.20	1.63	*ns*	2.52a	1.17b	0.56c	***	*ns*	3.19	3.24	*ns*	2.82	3.31	3.50	*ns*	ns
Ethyl octanoate	714.09	797.53	*ns*	1729.55a	342.68b	195.2b	***	*ns*	626.8	588.22	*ns*	456.06c	603.72b	762.76a	**	ns
Isoamyl lactate	0.48b[b]	0.76a	**	0.56b	0.83a	0.48b	**	***	2.35	2.40	*ns*	2.74a	2.04c	2.35b	***	ns
Methyl decanoate	0.10	0.16	*ns*	0.31a	0.04b	0.04b	***	*ns*	0.27	0.34	*ns*	0.32a	0.22b	0.37a	*	ns
Ethyl decanoate	187.65	197.98	*ns*	428.45a	101.84b	48.15b	***	*ns*	165.52	177.88	*ns*	28.87c	180.24b	306.00a	***	ns
Isoamyl octanoate	2.89	4.06	*ns*	5.90a	3.46b	1.06c	***	*ns*	2.38	2.79	*ns*	1.93	3.08	2.74	*ns*	ns
Methyl ethyl succinate	146.96a	99.97b	*	112.93b	257.44a	0.03c	***	**	113.48	139.29	*ns*	154.06a	86.32b	138.76a	*	ns
Diethyl succinate	71.89a	56.43b	**	162.56a	27.27b	2.64c	***	*ns*	101.85	95.01	*ns*	152.01a	100.09b	43.20c	***	ns

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Table 6: (Continued)

Compounds	Base wines								Sparkling wines							
	Harvest timing (H)			Year (Y)				H×Y	Harvest timing (H)			Year (Y)				H×Y
	H1	H2	*sig. F*[a]	2017	2018	2019	*sig. F*		H1	H2	*sig. F*[a]	2017	2018	2019	*sig. F*	
Ethyl 9-decanoate	105.34a	9.30b	***	104.15a	56.09b	11.71b	**	*	185.88[b]	78.35b	***	145.49b	13.30c	237.55a	***	***
Ethyl-2-OH-4-methylpentanoate	1.16	1.22	*ns*	1.59a	1.25a	0.73b	**	*ns*	3.12a	2.71b	*	2.34b	3.33a	3.07a	***	ns
2-phenylethyl acetate	65.57	56.97	*ns*	107.3a	46.65b	29.87b	***	*	49.25	42.64	*ns*	36.86b	54.87a	46.11ab	**	ns
Ethyl hydrogen succinate	3.43	4.05	*ns*	5.56a	5.36a	0.30b	**	*ns*	17.86	18.56	*ns*	12.22b	32.22a	10.20b	**	ns
Methyl salicylate	4.98	6.85	*ns*	16.27a	0.98b	0.49b	***	*ns*	4.12	3.19	*ns*	3.11	4.14	3.72	*ns*	ns
Ethyl hydroxybutanoate	6.31	6.84	*ns*	12.34a	5.92b	1.46c	***	*ns*	1.78b	2.75a	*	1.53b	2.39ab	2.88a	*	ns
Ethyl dodecanoate									0.71	0.89	*ns*	0.82	0.71	0.87	*ns*	ns
Isobutyl acetate									0	0	*ns*	0	0	0	*ns*	ns
Acids	1212.23	1728.44	*ns*	2429.58a	674.37b	1307.05b	***	*ns*	2102.98	2089.01	*ns*	1831.29	2226.04	2230.67	*ns*	ns
Acetic acid	30.18	40.17	*ns*	15.50b	50.18a	39.85a	**	*ns*	31.48b	41.67a	*	22.68b	39.68a	47.36a	***	ns
Butyric acid	4.10	5.23	*ns*	4.35	6.83	2.81	*ns*	*ns*	7.46	7.24	*ns*	6.92	7.41	7.73	*ns*	ns
3-methylbutyric acid	15.71	18.95	*ns*	22.78a	22.60a	6.62b	***	*	19.69	19.48	*ns*	22.35a	20.63a	15.77b	***	***
Hexanoic acid	103.92	111.19	*ns*	151.17a	70.66c	100.82b	***	**	121.26	125.93	*ns*	130.71	119.38	120.70	*ns*	ns
Octanoic acid	432.10	457.51	*ns*	687.30a	227.17c	419.95b	***	*	470.16	461.55	*ns*	430.87	455.01	511.67	*ns*	ns
Nonanoic acid	24.22	43.23	*ns*	2.93b	19.38b	78.87a	*	*ns*	46.97	47.03	*ns*	91.17a	16.81b	33.04b	***	ns
Decanoic acid	588.50	1024.57	*ns*	1521.45a	254.76b	643.41b	**	*ns*	1357.36	1340.11	*ns*	1089.74	1506.74	1449.74	*ns*	ns
Benzoic acid	2.18	4.88	*ns*	5.96	2.82	1.81	*ns*	*ns*	3.20	3.04	*ns*	3.48	2.97	2.91	*ns*	ns
Dodecanoic acid	11.32	22.71	*ns*	18.15	19.98	12.92	*ns*	*	45.40	42.95	*ns*	33.37	57.41	41.75	*ns*	ns
Ketones	18.69	21.63	*ns*	26.44a	15.82b	18.21b	**	*	146.89	154.22	*ns*	173.50a	152.10b	126.06c	***	*
2-methylthiolan-3-one	2.98a	2.20b	**	2.29b	3.57a	1.92b	***	*	3.27	2.61	*ns*	0.91c	5.35a	2.57b	***	ns
Isophorone	15.71	19.42	*ns*	24.15a	12.25b	16.29b	**	*	143.62	151.61	*ns*	172.59a	146.75b	123.5c	***	*

[a] Data were analyzed by two-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).
[b] Different letters (a, b, c) identify significantly different means. H1, first harvest; H2, second harvest.
All the concentrations are expressed in µg/L as IS 2-octanol.

3.4 VOLATILE PROFILE OF BASE WINES AND SPARKLING WINES AFTER THREE HARVEST TIMINGS

The results of two-way ANOVA for three harvest timings in base wines are presented on Table 7. The concentration of total monoterpenes decreased in wines from H1 to H3. However, the only significant compounds were β-myrcene and citronellol and their concentration increased as harvest date extended. The norisoprenoids results did not show any consistent trend, correlated with harvest timing, as the total amount of these increased from H1 to H2, but subsequently decreased when comparing H2 to H3. Vitispirane significantly contribute to the differences in harvest time, but the concentration with prolonged harvest date gradually decreased. Aldehydes showed similar behavior as total norisoprenoids, and the same was also true for individual compounds belonging to this class of compounds (e.g., nonanal, 3,4-dimethyl benzaldehyde, and acetaldehyde). Iso-butanol, 3-methyl-1-pentanol and 2-phenylethanol were among the compounds, where the concentration differed statistically from first to the third harvest. They were characterized by an increase in concentration as a function over time, while methionol differed in its inconsistency (H1, 2.92; H2, 7.32 and H3, 6.96 µg/L). Higher variability of results was observed also in the case of esters. Compounds such as ethyl 9-decanoate and isoamyl lactate contributed to this phenomenon, as their concentration increased from H1 to H2 and then decreased at an additional harvest date. Contrary to previous observations, a gradual decrease in the concentration was also observed in this case. All three ethyl succinates confirmed this result. The statistically significant total acid content corresponded to an anomalous drop in concentration from H2 to H3. This also included all analyzed fatty acids, other than C8 octanoic acid.

The inspection of the seasonal factor showed that the amount of varietal aroma compounds was in general significantly higher in wines, produced in the 2018, and similar observation was noted for aldehydes, with the exception of *trans*-2-hexanal, which negatively contributes to the aromatic profile of wines. In contrast to varietal aromas, 2019 was characterized by a much higher alcohol content than the year before (1812 µg/L for 2018 versus 4012 µg/L for 2019). This was largely due to the fact that much more isoamyl alcohol was produced in wines from 2019. Except for ethyl acetate, the abundance of all remaining esters was showed in the 2018 wines. Among them ethyl octanoate, isoamyl lactate, isoamyl octanoate, methyl ethyl succinate, diethyl succinate, ethyl hydrogen succinate and ethyl hydroxybutanoate showed the highest significance ($p < 0.001$). Acids prevailed in base wines from 2019, with the most contributing C6, C8, C9 and C10 fatty acids. Finally, 2-methylthiolan-3-one resulted to be dominant in the wines from 2018, while the opposite was true for isophorone.

The impact of three harvest timings on volatile composition of sparkling wines is presented on Table 7. Citronellol and α-terpineol contributed significantly to sample separation between the three harvest dates. However, lack of consistency in the pattern of

monoterpene behavior was noted within this chemical group itself. Thus, for example, the concentration of citronellol increased moderately from H1 to H3, while α-terpineol experienced some abnormalities. A similar trend was observed also for the main norisoprenoids (β-myrcene and TDN), where the initial increase in concentration was followed by a decrease in the wines, produced from the third harvest. Considering the carbonyl class of compounds, the number of statistically significant compounds was low, as only furfural and 3,4-dimethyl benzaldehyde resulted to differ during each consecutive harvest. However, both compounds exhibited a decreasing trend with increased grape maturity in the H3 sparkling wines. With regard to fermentative substances as alcohols and esters, it was interesting to note that extremely few compounds were related to extended harvest date in statistically significant manner. In fact, the only two compounds that were able to distinguish between the three harvest timings were hexyl acetate and ethyl hydroxybutanoate. The relative abundance of acetic acid, 3-methylbutyric acid, octanoic acid, nonanoic acid, benzoic acid and isophorone could be described as H3 > H2 > H1, while for the rest of acids and ketonic compounds, inconsistent trend was observed.

General overview on the results of different winegrowing seasons has shown several significant differences between 2018 and 2019. Some of the monoterpenes were present to a greater extent in wines from 2019 (citronellol, α-terpineol and nerol), while linalool was the significant compound whose higher concentration characterized the wines of 2018. β-damascenone and TDN followed the majority of terpenoids and were therefore more present in the last year of viticultural trial. Trans-2-hexenal appeared to be the most abundant in samples from 2019, which was essentially the case for all other aldehydes except acetaldehyde. For alcohols, the total amount resulted to be in favor of the 2018 wines, although the difference for the most abundant isoamyl alcohol was minimal between the two years (3081 µg/L versus 3056 µg/L for 2018 and 2019, respectively). The higher amount of esters in 2019 was mostly due to significant differences in ethyl acetate, ethyl decanoate, methyl ethyl succinate and ethyl 9-decanoate. Absence of unambiguous results of volatile acids concentration were also characteristic when comparing the seasonal effect. Namely, 3-methylbutyric acid and dodecanoic acid resulted to be more abundant in 2018, while nonanoic acid dominated in samples from 2019. Significantly higher consistency was therefore found in ketones, where both compounds (2-methylthiolan-3-one and isophorone) appeared to be supreme in sparkling wines, produced in 2018.

Table 7: Impact of three harvest timings and harvest season on the volatile profile of Ribolla Gialla base wines and sparkling wines.

Compound	Base wines								Sparkling wines							
	Harvest timing (H)				Year (Y)			H×Y	Harvest timing (H)				Year (Y)			H×Y
	H1	H2	H3	*sig. F*	2018	2019	*sig. F*		H1	H2	H3	*sig. F*[a]	2018	2019	*sig. F*	
Monoterpenes	20.23	19.94	17.56	*ns*	22.21a	16.28b	**	*ns*	28.11	24.94	27.77	*ns*	23.49b	30.39a	**	*ns*
β-myrcene	0.16b[b]	0.23a	0.26a	*	0.26a	0.18b	**	*ns*	0.76	0.80	0.88	*ns*	0.81	0.82	*ns*	*ns*
Limonene	1.59	1.84	1.75	*ns*	1.45b	2.01a	**	*ns*	2.53	2.17	2.75	*ns*	2.69	2.27	*ns*	*ns*
Linalool	4.32	5.16	3.01	*ns*	5.82a	2.51b	***	*ns*	4.94	5.07	6.01	*ns*	6.09a	4.59a	*	*ns*
Geraniol	6.38	6.51	6.13	*ns*	6.46	6.21	*ns*	*ns*	7.06	6.46	6.62	*ns*	7.12	6.31	*ns*	*ns*
Citronellol	3.31b	3.30b	4.13a	*	3.96a	3.21b	**	*ns*	1.46b	1.80ab	2.03a	*	1.56b	1.97a	*	*ns*
Nerol	1.78	1.87	1.58	*ns*	2.02a	1.46b	**	*ns*	3.35	3.40	3.38	*ns*	2.46b	4.29a	***	*ns*
Terpinen-4-ol	0.30	0.55	0.21	*ns*	0.51	0.2	*ns*	*ns*	0.63	0.69	0.73	*ns*	0.73	0.63	*ns*	*ns*
α-terpineol	2.40	0.48	0.49	*ns*	1.74	0.51	*ns*	*ns*	7.37a	4.57b	5.37b	*	2.04b	9.50a	***	*ns*
Norisoprenoids	8.42	13.81	10.73	*ns*	13.14a	8.83b	*	*ns*	15.28b	19.02a	16.29b	*	12.07b	21.66a	***	*ns*
Vitispirane	0.40a	0.30ab	0.24b	*	0.31	0.31	*ns*	*	0.83	0.62	0.63	*ns*	0.71	0.67	*ns*	*ns*
TDN	0.69	0.74	0.71	*ns*	0.76	0.67	*ns*	*ns*	0.95b	1.13a	0.98b	*	0.38b	1.66a	***	*
β-damascenone	7.19	12.3	9.50	*ns*	11.59a	7.73b	*	*ns*	12.61b	16.46a	13.97b	**	10.22b	18.48a	***	*ns*
Actinidiol (isomer 1)	0.08	0.38	0.22	*ns*	0.40a	0.06b	*	*ns*	0.38	0.34	0.30	*ns*	0.33	0.36	*ns*	*ns*
Actinidiol (isomer 2)	0.06	0.08	0.07	*ns*	0.08a	0.06b	*	*ns*	0.51	0.46	0.41	*ns*	0.44	0.48	*ns*	*ns*
Aldehydes	86.41b	136.03a	124.12a	*	125.94	105.11	*ns*	*ns*	391.81	416.4	410.85	*ns*	381.45	431.25	*ns*	*ns*
Hexanal	0.31	0.46	0.42	*ns*	0.57a	0.23b	*	*ns*	0.06	0.06	0.08	*ns*	0.04b	0.09a	**	*ns*
trans-2-hexenal	72.28	93.04	94.46	*ns*	79.77	93.42	*ns*	*ns*	365.59	386.29	376.84	*ns*	349.37b	403.11a	*	*ns*
Nonanal	3.95b	31.59a	17.23ab	*	30.96a	4.22b	**	*	2.77	2.93	3.59	*ns*	2.81	3.38	*ns*	*ns*
Benzaldehyde	3.08	3.93	3.96	*ns*	5.11a	2.20b	***	*ns*	5.76	5.74	6.63	*ns*	5.93	6.16	*ns*	*ns*
3,4-dimethyl benzaldehyde	0.27c	0.97a	0.73b	***	1.02a	0.30b	***	***	1.31b	1.43b	1.72a	*	1.32	1.65	*ns*	*ns*
Acetaldehyde	6.39	5.73	7.00	*ns*	8.11a	4.63b	***	*ns*	14.68	18.62	20.34	*ns*	20.77	14.99	*ns*	*ns*
Furfural	0.13b	0.31a	0.32a	***	0.39a	0.12b	***	***	1.64a	1.33b	1.66a	*	1.21b	1.87a	***	*ns*
Alcohols	2.54b	2.99a	3.21a	*	1.82b	4.01a	***	**	3.82	3.99	4.35	*ns*	4.11	4.01	*ns*	*ns*
n-hexanol	48.47	52.27	51.39	*ns*	47.1	54.32	*ns*	*ns*	49.39	48.65	50.68	*ns*	35.65b	63.50a	***	*ns*
trans-3-hexenol	1.25	1.45	0.97	*ns*	1.40	1.04	*ns*	*ns*	1.46	1.42	1.11	*ns*	1.32	1.34	*ns*	*ns*

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Table 7: (Continued)

Compound	Base wines								Sparkling wines							
	Harvest timing (H)				Year (Y)			H×Y	Harvest timing (H)				Year (Y)			H×Y
	H1	H2	H3	*sig. F*	2018	2019	*sig. F*		H1	H2	H3	*sig. F*[a]	2018	2019	*sig. F*	
1-octanol	9.69	11.44	10.17	*ns*	12.42a	8.45b	**	*ns*	10.8	10.54	10.08	*ns*	10.61	10.34	*ns*	*ns*
iso-butanol	93.66b	160.28a	191.96a	***	193.96a	103.3b	***	***	121.47	133.74	152.77	*ns*	138.99	133.00	*ns*	*ns*
Methionol	2.29b	7.32a	6.96a	***	7.24a	3.81b	***	***	3.28	3.47	3.71	*ns*	3.91	3.07	*ns*	*ns*
Isoamyl alcohol	1784.50b	1942.32ab	2106.37a	*	821.93b	3066.86a	***	**	2889.75	3032.02	3284.91	*ns*	3081.71	3056.08	*ns*	*ns*
3-methyl-1-pentanol	5.56b	10.68a	10.11a	***	10.68a	6.88b	***	***	9.96	10.21	11.11	*ns*	11.84a	9.02a	**	*ns*
2,3-butanediol (isomer 1)	17.38	16.95	15.85	*ns*	17.48	15.97	*ns*	*ns*	0.63	4.82	1.16	*ns*	3.42	0.99	*ns*	*ns*
2,3-butanediol (isomer 2)	5.51	4.63	4.28	*ns*	5.73	3.88	*ns*	*ns*	0.65	2.03	0.91	*ns*	1.53	0.86	*ns*	*ns*
2-phenylethanol	572.01b	787.98a	816.98a	*	703.15	748.16	*ns*	**	739.51	749.45	843.36	*ns*	823.78a	731.10b	*	*ns*
cis-3-hexenol									0	0	0	*ns*	0	0	*ns*	*ns*
trans-2-hexenol									0	0	0	*ns*	0	0	*ns*	*ns*
Esters	1541.11	1417.5	1528.66	*ns*	1959.39a	1032.13b	**	*ns*	3043.35	2936.38	3067.9	*ns*	2824.89	3206.87	*ns*	*ns*
Ethyl acetate	74.35	96.76	137.13	*ns*	83.41	122.08	*ns*	*ns*	288.31	381.92	473.96	*ns*	309.67b	453.12a	*	*ns*
Ethyl butyrate	25.63	30.24	30.33	*ns*	32.63	24.83	*ns*	*ns*	50.51	49.56	47.72	*ns*	47.25	51.28	*ns*	*ns*
Isopentyl acetate	174.11	177.42	186.72	*ns*	204.92	153.91	*ns*	*	227.02	221.35	228.01	*ns*	235.51	215.41	*ns*	**
Hexyl acetate	21.84	15.16	15.01	*ns*	17.76	16.91	*ns*	*	29.75a	20.53b	17.53b	*	20.11	25.09	*ns*	**
Methyl caproate	0.51	0.51	0.49	*ns*	0.54	0.46	*ns*	*ns*	0.73	0.63	0.59	*ns*	0.68	0.61	*ns*	*ns*
Ethyl hexanoate	614.2	579.23	592.61	*ns*	783.02a	407.68b	**	*ns*	1057.43	968.98	931.35	*ns*	1018.65	953.19	*ns*	*ns*
Ethyl lactate	2.11	2.84	2.57	*ns*	2.35	2.67	*ns*	*	14.93	14.06	15.9	*ns*	13.55b	16.38a	*	*ns*
Methyl octanoate	0.76	0.96	1.24	*ns*	1.41a	0.56b	**	*ns*	3.58	3.23	3.05	*ns*	3.41	3.17	*ns*	*ns*
Ethyl octanoate	241.43	296.44	323.74	*ns*	371.72a	202.69b	***	*ns*	714.73	651.74	722.52	*ns*	659.39	733.27	*ns*	*ns*
Isoamyl lactate	0.44b[b]	0.86a	0.80a	**	0.92a	0.48b	***	**	2.18	2.21	2.26	*ns*	2.09b	2.34a	*	*ns*
Methyl decanoate	0.04	0.04	0.04	*ns*	0.04	0.04	*ns*	*ns*	0.29	0.30	0.27	*ns*	0.24b	0.33a	*	*ns*
Ethyl decanoate	90.06	59.93	88.85	*ns*	113.56	45.67	*ns*	*ns*	232.43	253.82	220.15	*ns*	194.75b	276.18a	*	*ns*
Isoamyl octanoate	1.68b	2.84ab	3.92a	*	4.53a	1.09b	***	*	2.74	3.09	2.93	*ns*	3.35	2.49	*ns*	*ns*
Methyl ethyl succinate	169.56a	87.91b	70.77b	**	218.79a	0.04b	***	**	107.23	117.85	106.23	*ns*	93.21b	127.66a	*	*ns*
Diethyl succinate	20.00a	9.92b	8.28b	**	22.82a	2.64b	***	**	76.01	67.27	79.67	*ns*	103.78a	44.86b	***	*ns*

(Continues on the next page)

Table 7: (Continued)

Compound	Base wines								Sparkling wines							
	Harvest timing (H)				Year (Y)			H×Y	Harvest timing (H)				Year (Y)			H×Y
	H1	H2	H3	*sig. F*	2018	2019	*sig. F*		H1	H2	H3	*sig. F*[a]	2018	2019	*sig. F*	
Ethyl 9-decanoate	63,84a	3,95b	11,79b	**	38.90	14.16	*ns*	*	153.30	97.55	118.84	*ns*	12,08a	234,38b	***	*ns*
Ethyl-2-OH-4-methylpentanoate	0.89	1.09	1.08	*ns*	1.26a	0.79b	*	*ns*	3.43	2.98	3.11	*ns*	3.14	3.21	*ns*	*
2-phenylethyl acetate	33.23	43.29	44.67	*ns*	47.65a	33.14b	**	*	51.91	49.07	58.07	*ns*	58.03a	48.00b	*	*ns*
Ethyl hydrogen succinate	1.76	3.90	3.76	*ns*	6.00a	0.28b	***	*ns*	19.37	23.05	23.09	*ns*	35.35a	8.32b	***	*ns*
Methyl salicylate	0.70	0.77	0.92	*ns*	1.16	0.43	*ns*	*ns*	4.70	3.15	7.82	*ns*	7.00a	3.45b	*	*
Ethyl hydroxybutanoate	3.96	3.42	3.97	*ns*	6.00a	1.56b	***	*ns*	2.10b	3.17ab	4.12a	*	2.95	3.31	*ns*	*ns*
Ethyl dodecanoate									0.70	0.88	0.70	*ns*	0.71	0.81	*ns*	*ns*
Isobutyl acetate									0	0	0	*ns*	0	0	*ns*	*ns*
Acids	669.49b	1311.93a	949.92ab	*	722.36b	1231.87a	*	*ns*	2219.97	2236.73	2229.39	*ns*	2355.47	2101.92	*ns*	*ns*
Acetic acid	36.76	53.27	47.41	*ns*	47.79	43.83	*ns*	*ns*	35.64b	51.40a	59.43a	**	43.60	54.05	*ns*	*ns*
Butyric acid	3.81	5.82	5.62	*ns*	6.84a	3.33b	**	*ns*	7.59	7.54	7.58	*ns*	7.61	7.53	*ns*	*ns*
3-methylbutyric acid	10.69	18.53	18.79	*ns*	23.28a	8.73b	***	*ns*	17.62	18.78	19.92	*ns*	21.47a	16.08b	***	*ns*
Hexanoic acid	83.76	87.72	83.39	*ns*	72.56b	97.35a	***	**	121.30	118.78	120.14	*ns*	123.15	117.00	*ns*	*ns*
Octanoic acid	304.99	342.12	353.29	*ns*	252.05b	414.89a	***	**	481.88	484.8	513.26	*ns*	465.72	520.91	*ns*	*ns*
Nonanoic acid	35.14	63.11	53.74	*ns*	17.48b	83.85a	*	*ns*	23.94b	25.91b	47.32a	*	18.50b	46.28a	***	*ns*
Decanoic acid	190.29	707.88	371.48	*ns*	280.18	566.26	*ns*	*ns*	1478.42	1478.05	1406.70	*ns*	1606.93	1301.85	*ns*	*ns*
Benzoic acid	1.15	3.47	2.62	*ns*	2.94	1.89	*ns*	*ns*	2.89	2.98	3.37	*ns*	3.26	2.90	*ns*	*ns*
Dodecanoic acid	2.89b	30.00a	13.59b	*	19.25	11.73	*ns*	*ns*	50.68	48.49	51.68	*ns*	65.23a	35.33b	**	*ns*
Ketones	17.54	16.49	18.09	*ns*	16.09	18.66	*ns*	*	133.68	144.48	155.07	*ns*	156.66a	132.17b	**	*ns*
2-methylthiolan-3-one	3.34a	2.15b	2.41b	**	3.18a	2.09b	**	*	4.44	3.48	4.24	*ns*	5.34a	2.76b	***	*ns*
Isophorone	14.20	14.34	15.67	*ns*	12.91b	16.57a	*	*ns*	129.25	141.00	150.83	*ns*	151.32a	129.40b	*	*ns*

[a] Data were analyzed by two-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).
[b] Different letters (a, b) identify significantly different means. H1, first harvest; H2, second harvest; H3, third harvest.
All the concentrations are expressed in µg/L as IS 2-octanol.

3.5 LIPID PROFILE OF BASE WINES AND SPARKLING WINES AFTER TWO HARVEST TIMINGS

By observing the results presented in Table 8, it can be observed that only one additional harvest date did not affect in significant manner any of the analyzed lipid compounds, present in base wines and sparkling wines, respectively. Regardless, the results in base wines appeared to be inconsistent, as the total amount of abundant saturated and unsaturated fatty acids (UFAs) increased slightly, but at the same time, the concentration of some of the most predominant individual compounds decreased after the additional harvest date. In particularly this was true in the case of saturated stearic acid. In the case of sparkling wines, such a trend was less noticeable, which could be due to secondary fermentation and additional release of lipids into the fermented medium.

Consequently, more substances were expected to be statistically significant when comparing individual harvest seasons. Due to the low detected concentrations of glycerolipids and sterols in base wines samples, there was no significant difference between winegrowing seasons. However, the samples from the first year of established viticultural experiment showed the highest amount of UFAs, followed by the season 2019 and lastly 2018. Similarly, the concentration of UFAs prevailed in 2017, only that in this case, the differences between individual years were higher. Palmitic acid resulted to be the most abundant among all SFAs in all three years, followed by stearic and arachidic acid. The amounts of miristoleic and lignoceric acid, found in the base wines were similar.

The increased number of statistically significant compounds when comparing the three different harvest seasons were observed also in the case of sparkling wines (Table 8). On contrary with base wines, the concentration of glycerolipids differed from one harvest season to another, especially for 1-linoleoyl-rac-glycerol and 1-oleoyl-rac-glycerol. Similar behavior was noted also when comparing the results of sterols, where the amount of ergosterol and desmosterol predominated in the wines from 2019. The concentration of palmitoleic acid was highest in 2017, however, the remaining UFAs proved to be predominant in the 2019, which was additionally confirmed by the total amount of UFAs. Interestingly, all the SFAs resulted to be statistically significant, and the vast majority of the compounds prevailed in 2019 growing season. Lignoceric acid, however, differed from this point of view, as its amount was found to be slightly higher in 2017. Compared to the base wines, where eighteen compounds were analyzed and quantified, in the sparkling wines this number increased to nineteen, with a presence of ethyl stearate. However, no statistically significant differences have been observed regarding its span over the three vintages.

Since in base wines and in sparkling wines no lipid compound emerged as statistically significant for harvest timing as a factor, no interaction was observed between the harvest timing and harvest season.

Table 8: Impact of two harvest timings and harvest season on the lipid profile of Ribolla Gialla base wines and sparkling wines.

Compound	Base wines								Sparkling wines							
	Harvest timing (H)			Year (Y)				H×Y	Harvest timing (H)			Year (Y)				H×Y
	H1	H2	sig. F[a]	2017	2018	2019	sig. F		H1	H2	sig. F	2017	2018	2019	sig. F	
Glycerolipids	0.27	0.26	*ns*	0.26	0.25	0.28	*ns*	*ns*	0.26	0.26	*ns*	0.23b	0.21b	0.34a	***	*ns*
1-linoleoyl-rac-glycerol	0.24	0.23	*ns*	0.23	0.22	0.25	*ns*	*ns*	0.22	0.22	*ns*	0.2b	0.18b	0.28a	***	*ns*
1-oleoyl-rac-glycerol	0.02	0.02	*ns*	0.03	0.02	0.02	*ns*	*ns*	0.03	0.03	*ns*	0.02b	0.02c	0.04a	***	*ns*
1-monopalmitoleoyl-rac-glycerol	0.01	0.00	*ns*	0.01	0.00	0.00	*ns*	*ns*	0.01	0.01	*ns*	0.00	0.01	0.02	ns	*ns*
Sterols	0.31	0.31	*ns*	0.31	0.28	0.33	*ns*	*ns*	0.31	0.33	*ns*	0.30b	0.25b	0.42a	***	*ns*
Ergosterol	0.28	0.28	*ns*	0.29	0.26	0.30	*ns*	*ns*	0.29	0.31	*ns*	0.27b	0.23c	0.38a	***	*ns*
Desmosterol	0.03	0.03	*ns*	0.03	0.02	0.03	*ns*	*ns*	0.03	0.03	*ns*	0.02b	0.02b	0.04a	***	*ns*
Fatty acids UFA	4.76	4.67	*ns*	6.14ab	3.75c	4.25b	***	*ns*	5.81	6.06	*ns*	5.65b	5.39b	6.77a	***	*ns*
Linoleic acid	0.27	0.29	*ns*	0.35a	0.23b	0.26b	***	*ns*	0.32	0.31	*ns*	0.28b	0.28b	0.38a	***	*ns*
Linolenic acid	0.03	0.03	*ns*	0.03	0.03	0.03	*ns*	*ns*	0.03	0.03	*ns*	0.03b	0.03b	0.04a	***	*ns*
Palmitoleic acid	0.17	0.14	*ns*	0.25a	0.10b	0.12b	***	*ns*	0.33	0.40	*ns*	0.52a	0.32b	0.25b	***	*ns*
Oleic acid + cis-Vaccenic acid	4.29	4.21	*ns*	5.52a	3.38c	3.85b	***	*ns*	5.13	5.32	*ns*	4.82b	4.76b	6.10a	***	*ns*
Fatty acids SFA	153.55	153.63	*ns*	176.73a	136.4c	147.65b	***	*ns*	193.91	197.50	*ns*	187.48b	177.48b	222.15a	***	*ns*
Behenic acid	0.64	0.63	*ns*	0.70a	0.59c	0.63b	***	*ns*	0.79	0.78	*ns*	0.71b	0.70b	0.95a	***	*ns*
Stearic acid	45.73	45.32	*ns*	54.18a	40.98b	41.41b	***	*ns*	59.34	59.84	*ns*	56.69b	53.75b	68.32a	***	*ns*
Lignoceric acid	0.35	0.33	*ns*	0.36	0.35	0.32	*ns*	*ns*	0.44	0.46	*ns*	0.48a	0.42b	0.46a	***	*ns*
Arachidic acid	2.27	2.26	*ns*	2.78a	1.93b	2.09b	***	*ns*	2.72	2.74	*ns*	2.49b	2.40b	3.30a	***	*ns*
Myristic acid	1.69	1.69	*ns*	1.57	1.60	1.92	**	*ns*	1.83	1.85	*ns*	1.65b	1.71b	2.14a	**	*ns*
Palmitic acid	101.94	102.47	*ns*	116.14a	90.14c	100.34b	***	*ns*	127.78	130.83	*ns*	124.58b	117.63b	145.72a	**	*ns*
Miristoleic acid	0.48	0.47	*ns*	0.48	0.44	0.51	*ns*	*ns*	0.48	0.47	*ns*	0.38b	0.38b	0.65a	***	*ns*
Margaric acid	0.45	0.45	*ns*	0.52a	0.38c	0.44b	***	*ns*	0.53	0.54	*ns*	0.5b	0.49b	0.61a	*	*ns*
Prenols	0.12	0.15	*ns*	0.10	0.19	0.10	*ns*	*ns*	0.11	0.11	*ns*	0.08b	0.09b	0.15a	*	*ns*
Lupeol	0.12	0.15	*ns*	0.10	0.19	0.10	*ns*	*ns*	0.11	0.11	*ns*	0.08b	0.09b	0.15a	*	*ns*
Fatty esters									0.01	0.01	*ns*	0.01	0.01	*n.d.*	*ns*	*ns*
Ethyl stearate									0.01	0.01	*ns*	0.01	0.01	*n.d.*	*ns*	*ns*

[a] Data were analyzed by two-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).

[b] Different letters (a, b, c) identify significantly different means. H1, first harvest; H2, second harvest. All the concentrations are expressed in mg/L.

3.6 LIPID PROFILE OF BASE WINES AND SPARKLING WINES AFTER THREE HARVEST TIMINGS

Table 9 report the results of three consecutive harvest dates in 2018 and 2019 winegrowing seasons, for base wines and sparkling wines, respectively. As it has been already observed previously in the case of two harvest timings, only harvest year appeared to be significant factor, while none of the analyzed lipids resulted to be statistically significant when comparing different harvest dates.

Concerning the base wines, 1-linoleoyl-rac-glycerol contributed most to the fact that the concentration of glycerolipids prevailed in the 2019 harvest season, compared to 2018. In the class of sterols, it was similarly observed, that ergosterol exhibited as a compound with the highest concentration in the most recent vintage, while the significant difference of desmosterol between two years, appeared to be much lower. Although it is characterized as one of the most important UFAs in grapes, the palmitoleic acid showed no significant difference between two consecutive winegrowing seasons, while remain of the UFAs prevailed in samples from 2019. Myristic acid, palmitic acid, miristoleic acid and margaric acid emerged as SFAs, whose concentration was higher in 2019, and the same trend was followed in the rest of SFAs (behenic acid, stearic acid, lignoceric acid and arachidic acid). The only pentacyclic triterpenoid lupeol did not appeared as notably important compound to differentiate the two vintages. In addition, no significant interaction between harvest date and season has been observed.

As far as sparkling wines are concerned, it should be noted, that all lipid compounds were present in a higher concentration in the wines from 2019. It has been showed that amount of monolinolein (1-linoleoyl-rac-glycerol) exceeded monoglyceride 1-oleoyl-rac-glycerol, and both substances distinguished from 1-monopalmitoleoyl-rac-glycerol that was only non-significant glycerolipid. Notable dissimilarity in concentration content was observed among sterols, where ergosterol appeared as predominant over desmosterol. Similar to the base wines, only palmitoleic acid proceed to be statistically insignificant, compared to the other UFAs. Nevertheless, the only significant interaction between both studied factors, belonged to linoleic acid. Compared to 2018, the total concentration of saturated fatty acids increased by 23% in 2019. Most credit for this can be attributed to palmitic acid, which has been recognized as the most abundant SFA in sparkling wine samples. The only ethyl ester that has been analyzed by UPLC-MS/MS method resulted statistically significant; however, the concentration appeared to be too low, therefore a zero sensory effect is highly likely. Finally, the amount of lupeol from 2018 to 2019 nearly doubled.

Table 9: Impact of three harvest timings and harvest season on the lipid profile of Ribolla Gialla base wines and sparkling wines.

Compound	Base wines								Sparkling wines							
	Harvest timing (H)				Year (Y)			H×Y	Harvest timing (H)				Year (Y)			H×Y
	H1	H2	H3	*sig. F*[a]	2018	2019	*sig. F*		H1	H2	H3	*sig. F*	2018	2019	*sig. F*	
Glycerolipids	0.26	0.26	0.24	*ns*	0.24b[b]	0.27a	***	*ns*	0.28	0.28	0.29	*ns*	0.22b	0.35a	***	*ns*
1-linoleoyl-rac-glycerol	0.23	0.24	0.21	*ns*	0.21b	0.25a	**	*ns*	0.23	0.24	0.26	*ns*	0.19b	0.30a	***	*ns*
1-oleoyl-rac-glycerol	0.02	0.02	0.02	*ns*	0.02b	0.02a	**	*ns*	0.03	0.03	0.03	*ns*	0.02b	0.04a	***	*ns*
1-monopalmitoleoyl-rac-glycerol	0.01	*n.d.*	*n.d.*	*ns*	*n.d.*	*n.d.*	*ns*	*ns*	0.01	0.01	0.01	*ns*	0.01b	0.01a	*ns*	*ns*
Sterols	0.3	0.31	0.29	*ns*	0.27b	0.32a	***	*ns*	0.34	0.33	0.36	*ns*	0.25b	0.43a	***	*ns*
Ergosterol	0.27	0.28	0.26	*ns*	0.25b	0.30a	***	*ns*	0.31	0.3	0.33	*ns*	0.23b	0.39a	***	*ns*
Desmosterol	0.03	0.03	0.03	*ns*	0.02b	0.03a	**	*ns*	0.03	0.03	0.03	*ns*	0.02b	0.04a	***	*ns*
Fatty acids UFA	3.96	4.04	4.17	*ns*	3.81b	4.31a	***	*ns*	5.92	6.26	6.41	*ns*	5.48b	6.92a	***	*ns*
Linoleic acid	0.24	0.25	0.24	*ns*	0.23b	0.26a	*	*ns*	0.35	0.34	0.38	*ns*	0.29b	0.42a	***	**
Linolenic acid	0.03	0.03	0.03	*ns*	0.03b	0.03a	**	*ns*	0.03	0.03	0.04	*ns*	0.03b	0.04a	***	*ns*
Palmitoleic acid	0.11	0.11	0.14	*ns*	0.12	0.12	*ns*	*ns*	0.25	0.29	0.31	*ns*	0.32a	0.25b	*ns*	*ns*
Oleic acid + *cis*-Vaccenic acid	3.58	3.65	3.76	*ns*	3.43b	3.89a	***	*ns*	5.29	5.60	5.68	*ns*	4.85b	6.20a	***	*ns*
Fatty acids SFA	141.49	142.57	141.17	*ns*	137.93	145.56	*ns*	*ns*	196.85	203.73	202.04	*ns*	180.12b	221.62a	***	*ns*
Behenic acid	0.60	0.61	0.61	*ns*	0.60	0.62	*ns*	*ns*	0.83	0.79	0.84	*ns*	0.69b	0.95a	***	*ns*
Stearic acid	41.3	41.09	40.21	*ns*	40.7	41.03	*ns*	*ns*	60.67	62.42	61.11	*ns*	54.8b	68.01a	***	*ns*
Lignoceric acid	0.34	0.32	0.33	*ns*	0.35	0.32	*ns*	*ns*	0.43	0.43	0.44	*ns*	0.41b	0.45a	*	*ns*
Arachidic acid	2.02	2.00	1.93	*ns*	1.9	2.07	*ns*	*ns*	2.79	2.96	2.89	*ns*	2.46b	3.31a	***	*ns*
Myristic acid	1.71	1.8	1.81	*ns*	1.63b	1.92a	**	*ns*	1.92	1.92	1.80	*ns*	1.72b	2.04a	*	*ns*
Palmitic acid	94.64	95.84	95.42	*ns*	91.93b	98.67a	*	*ns*	129.15	134.13	133.86	*ns*	119.16b	145.6a	***	*ns*
Miristoleic acid	0.47	0.48	0.45	*ns*	0.43b	0.51a	***	*ns*	0.53	0.51	0.55	*ns*	0.39b	0.67a	***	*ns*
Margaric acid	0.41	0.41	0.41	*ns*	0.39b	0.43a	*	*ns*	0.53	0.56	0.55	*ns*	0.50b	0.60a	*	*ns*
Prenols	0.15	0.15	0.10	*ns*	0.15	0.11	*ns*	*ns*	0.13	0.07	0.14	*ns*	0.08b	0.15a	*	*ns*
Lupeol	0.15	0.15	0.10	*ns*	0.15	0.11	*ns*	*ns*	0.13	0.07	0.14	*ns*	0.08b	0.15a	*	*ns*
Fatty esters									0.01	0.01	0.01	*ns*	0.01a	0b	***	*ns*
Ethyl stearate									0.01	0.01	0.01	*ns*	0.01a	0b	***	*ns*

[a] Data were analyzed by two-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).
[b] Different letters (a, b, c) identify significantly different means. H1, first harvest; H2, second harvest; H3, third harvest.
All the concentrations are expressed in mg/L.

3.7 AROMATIC AMINO ACID METABOLITES PROFILE OF BASE WINES AND SPARKLING WINES AFTER TWO HARVEST TIMINGS

The results of two harvest timings on aromatic amino acid metabolites (AAA) in base wines and sparkling wines are presented in Table 10. In base wines, the total amount of compounds detected and quantified was 20, while an additional compound was discovered in the samples of sparkling wines.

As regards the base wines analysis, the results did not show clear advantages of an extended harvest date, compared to the normal harvest. The results were accordingly inconsistent, as it turned out that additional harvest date increased the concentration of only a certain number of statistically significant substances. The most important compounds have thus proved to be ethyl ester of tryptophan and tyrosine, phenylacetic and phenyllactic acid, indole lactic acid with complementary bound glucoside form and the degraded product of tryptophan, tryptophol. On contrary, the list of significant compounds, where their amount decreased from H1 to H2 was shorter, since only phenylalanine and kynurenic acid were congregated in this group.

Further analysis of the results in base wines revealed significant influence on the content of AAA compounds. For two essential amino acids (tyrosine and phenylalanine) and abscisic acid it was shown that their concentration gradually decreased from 2017 to 2019. The concentration of tryptophan ethyl ester, tryptophol, indole lactic acid glucoside and phenyllactic acid peaked in second winegrowing season (2018), while the drop in concentration tended to be inconsistent in 2017 or 2019. In case of kynurenic acid, it was interestingly to observe a notable decrease in concentration from 2017 to subsequent 2018 (from 10.27 mg/L to 1.14 mg/L), followed by an increase in 2019 (9.35 mg/L). Similar situation occurred for N-acetyl-L-tyrosine ethyl ester and important auxin indole acetic acid.

Despite the greater number of quantified compounds in sparkling wines (Table 10), the number of those statistically different from H1 to H2 was lower compared to the base wines. While tyrosine ethyl ester showed a decreased concentration in extended harvest date, the extension in harvest positively affected on the amount of hydroxytyrosol, tryptophol, indole lactic acid glucoside and anthranilic acid. Inconsistency of the remained results in H1 and H2 has led to difficult prediction of any trend for negligible compounds. For example, the decrease of tyrosol from H1 (16.00 mg/L) to H2 (13.00 mg/L) was related also with decreased concentration for phenylalanine and tryptophan as well, while the prolonged harvest date effected positively the tyrosine as remained precursor for some bioactive compounds.

In terms of harvest season as a factor for AAA metabolites in sparkling wines, they were mostly in line with those of the base wines. Namely, a systematic decrease in concentration from 2017 to 2019 winegrowing season was identified in tyrosine and phenylalanine, as well as in sulfonated tryptophol and abscisic acid. High statistical significance ($p < 0.001$) was additionally observed also for ethyl ester of tyrosine and

hydroxytyrosol, whereby these two substances predominated in 2017. For kynurenic acid, indole lactic acid glucoside and indole acetic acid, the accumulation in sparkling wines was most intense in the last year of viticultural experiment. Moreover, the effect of harvest timing depended on the effect of the harvest season in the case of phenylalanine, OH-tyrosol, phenylacetic acid and tryptophol.

Table 10: Impact of two harvest timings and harvest season on the aromatic amino acid metabolites of Ribolla Gialla base wines and sparkling wines.

Compound	Base wine								Sparkling wine							
	Harvest timing (H)			Year (Y)				H×Y	Harvest Timing (H)			Year (Y)				H×Y
	H1	H2	*Sig. F*[a]	2017	2018	2019	*Sig. F*		H1	H2	*Sig. F*[a]	2017	2018	2019	*Sig. F*	
Tyrosine	3.47	2.78	*ns*	4.20a	2.76b	2.41b	**	***	6.54	6.92	ns	10.36a	5.88b	3.95c	***	ns
Phenylalanine	1.35a[b]	0.82b	*	2.38a	0.50b	0.37b	***	**	3.49	3.15	ns	5.99a	3.15b	0.82c	***	*
Tryptophan	0.25	0.16	*ns*	0.21	0.17	0.23	ns	ns	0.30	0.25	ns	0.18	0.34	0.31	ns	ns
Kynurenic acid	10.77a	3.06b	***	10.27a	1.14b	9.35a	***	***	10.34	3.40	ns	9.37a	1.51b	9.72a	*	ns
Nicotinamide	0.34b	0.59a	*	0.23b	0.49ab	0.66a	*	*	0.36	0.35	ns	0.34	0.20	0.52	ns	ns
Tryptophan ethyl ester	0.25b	0.39a	***	0.22b	0.56ab	0.16b	***	***	0.30	0.26	ns	0.21	0.16	0.47	ns	ns
Tyrosine ethyl ester	3.05b	7.87a	***	9.77a	4.55b	2.06b	***	**	18.17a[b]	10.59b	*	25.68a	6.83b	10.64b	***	ns
N-acetyl-L-tyrosine ethyl ester	0.40	0.31	*ns*	0.52a	0.26b	0.30b	**	**	0.56	0.40	ns	0.73	0.35	0.37	ns	ns
Tyrosol	13.2	15.25	*ns*	15.52	13.39	13.76	ns	*	16.01	13.70	ns	16.82	15.70	12.04	ns	ns
Hydroxytyrosol	0.28	0.24	*ns*	0.25	0.27	0.27	ns	*	0.38b	0.55a	***	0.65a	0.26c	0.48b	***	*
Phenylacetic acid	0.19b	0.39a	**	0.40a	0.17b	0.31ab	*	ns	0.29	0.48	ns	0.43	0.31	0.42	ns	**
Tryptophol	0.34b	0.61a	***	0.28c	0.68a	0.46b	***	***	0.51b	0.61a	*	0.36b	0.92a	0.40b	***	*
Indole acetic acid	0.42	0.33	*ns*	0.54a	0.18b	0.41ab	*	ns	0.34	0.38	ns	0.28b	0.19b	0.60a	*	ns
Indole lactic acid	0.34b	0.60a	*	0.35	0.38	0.68	ns	ns	0.50	0.34	ns	0.31	0.42	0.53	ns	ns
Indole lactic acid glucoside	0.82b	1.60a	***	0.79c	1.65a	1.18b	***	*	1.06b	1.75a	***	1.08c	1.46b	1.68a	***	ns
N-acetyl serotonin	0.40	0.37	*ns*	0.38	0.42	0.37	ns	ns	0.38	0.41	ns	0.39	0.40	0.40	ns	ns
Phenyllactic acid	0.59b	0.70a	**	0.57b	0.69a	0.68a	**	***	0.91	0.83	ns	0.71b	1.16a	0.74b	**	ns
Tryptophol SO3H	0.33	0.43	*ns*	0.66a	0.30b	0.18b	***	ns	0.75	0.60	ns	1.22a	0.43b	0.36b	***	ns
Abscisic acid	0.39	0.44	*ns*	0.47a	0.43a	0.34b	*	*	0.54	0.56	ns	0.63a	0.59a	0.41b	***	ns
Abscisic acid glucoside	0.49	0.29	*ns*	0.46	0.35	0.37	ns	ns	0.39	0.52	ns	0.25	0.58	0.53	ns	ns
Anthranilic acid									0.26b	0.60a	**	0.43	0.52	0.35	ns	ns

[a] Data were analyzed by two-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).
[b] Different letters (a, b, c) identify significantly different means. H1, first harvest; H2, second harvest.
All the concentrations are expressed in mg/L.

3.8 AROMATIC AMINO ACID METABOLITES PROFILE OF BASE WINES AND SPARKLING WINES AFTER THREE HARVEST TIMINGS

The addition of third harvest date was investigated in Table 11 for base wines and sparkling wines, respectively. In the base wines, it has been observed, that third harvest date increased concentration of tyrosine and its derivatives as ethyl ester, and N-acetyl-L-tyrosine ethyl ester, as well as phenylalanine, tryptophol, glucoside of indole lactic acid and phenyllactic acid. All listed compounds appeared to be statistically significant. However, the extension of harvest season affected certain compounds also in the opposite direction, where a moderate decrease has been observed from H1, over H2 to H3. Bioactive product of tryptophan, kynurenic acid therefore faced a drop in concentration.

When considering a harvest season as one of the factors in two-way ANOVA for the base wines (Table 11), several compounds resulted as significantly different. It has been shown that except for the kynurenic acid, all remain compounds expressed higher concentration in 2018 compared to 2019. That was also true for some less relevant compounds that did not contribute significantly to the differences between 2018 and 2019. Moreover, ethyl esters of TRP and TYR appeared to have higher concentration in 2018, compared to the last year of viticultural experiment. Significant interaction between extended harvest date and harvest season has been observed for several compounds. Tryptophol appeared to be the substance with highest significance ($p < 0.001$), followed by kynurenic acid, tyrosine ethyl ester and tyrosol ($p < 0.01$).

By observing the results of sparkling wines (Table 11), it could be seen, that concentration of tyrosine, hydroxytyrosol and indole lactic acid glucoside increased with the extended harvest date, while for kynurenic acid and anthranilic acid the results appeared to be inconsistent. The concentration of the latter peaked in the H2 wines, while the concentration of kynurenic acid appeared to be the highest after H3, even though the increase of concentration was not uniform from H1 to H3. The trend for the remaining insignificant compounds from H1 to H3 wines, resulted to be inconsistent as well.

By analyzing the effect of the harvest season on the AAA metabolites in sparkling wines, it was observed that vintage season had the greatest impact for phenylalanine, kynurenic acid, hydroxytyrosol, tryptophol, and abscisic acid ($p < 0.001$); however, there was no uniform result to indicate whether these compounds were present in higher concentration in 2018 or in 2019 winegrowing season. Additionally, in wines, produced in 2018, tyrosine and phenyl lactic acid resulted were present in higher concentration, compared to the wines from 2019, while the reverse was true for nicotinamide and tryptophan ethyl ester. A significant interaction between harvest date and harvest season was observed for hydroxytyrosol, phenylacetic acid, kynurenic acid and indole acetic acid.

Table 11: Impact of three harvest timings and harvest season on the aromatic amino acid metabolites of Ribolla Gialla base wines and sparkling wines.

Compound	Base wines								Sparkling wines							
	Harvest timing (H)				Year (Y)			H×Y	Harvest timing (H)				Year (Y)			H×Y
	H1	H2	H3	*Sig. F*[a]	2018	2019	*Sig. F*		H1	H2	H3	*Sig. F*[a]	2018	2019	*Sig. F*	
Tyrosine	2.32b[b]	2.86b	4.56a	*	4.15a	2.34b	*	*	4.88b	5.56b[b]	8.14a	*	8.04a	4.56b	*	*ns*
Phenylalanine	0.32b	0.55ab	1.11a	*	0.98a	0.35b	*	*	2.11	1.95	1.66	*ns*	3.09a	0.81b	***	*ns*
Tryptophan	0.23	0.17	0.20	*ns*	0.23	0.17	*ns*	*ns*	0.37	0.27	0.34	*ns*	0.37	0.29	*ns*	*ns*
Kynurenic acid	7.46a	3.02b	2.52b	***	1.15b	7.52a	***	**	6.88a	4.00b	10.20a	**	1.31b	11.61a	***	**
Nicotinamide	0.43b	0.73a	0.20b	**	0.40	0.50	*ns*	*	0.43	0.28	0.50	*ns*	0.19b	0.62a	**	*ns*
Tryptophan ethyl ester	0.25	0.47	0.44	*ns*	0.59a	0.19b	***	*ns*	0.30	0.29	0.28	*ns*	0.17b	0.41a	*	*ns*
Tyrosine ethyl ester	2.19b	4.42b	12.38a	*	10.81a	1.84b	**	**	11.6	6.57	16.76	*ns*	8.42	11.67	*ns*	*ns*
N-acetyl-L-tyrosine ethyl ester	0.24b	0.32ab	0.46a	*	0.32	0.36	*ns*	*ns*	0.49	0.21	0.39	*ns*	0.40	0.33	*ns*	*ns*
Tyrosol	12.39	14.76	15.52	*ns*	14.59	13.86	*ns*	**	13.22	14.35	16.21	*ns*	16.39	13.14	*ns*	*ns*
Hydroxytyrosol	0.31	0.22	0.30	*ns*	0.26	0.30	*ns*	*ns*	0.30b	0.42a	0.49a	**	0.25b	0.56a	***	***
Phenylacetic acid	0.17	0.31	0.41	*ns*	0.22	0.38	*ns*	*ns*	0.29	0.38	0.21	*ns*	0.30	0.33	*ns*	**
Tryptophol	0.38b	0.77a	0.79a	***	0.83a	0.46b	***	***	0.68	0.67	0.75	*ns*	1.00a	0.43b	***	*ns*
Indole acetic acid	0.33	0.26	0.33	*ns*	0.26	0.35	*ns*	*ns*	0.42	0.47	0.45	*ns*	0.35	0.48	*ns*	*
Indole lactic acid	0.33	0.73	0.48	*ns*	0.38	0.64	*ns*	*ns*	0.44	0.44	0.22	*ns*	0.38	0.42	*ns*	*ns*
Indole lactic acid glucoside	1.02b	1.81a	2.05a	**	2.01a	1.24b	***	*	1.38c	2.08b	2.29a	***	1.78	2.02	*ns*	*ns*
N-acetyl serotonin	0.42	0.37	0.41	*ns*	0.40	0.40	*ns*	*ns*	0.37	0.41	0.43	*ns*	0.41	0.39	*ns*	*ns*
Phenyllactic acid	0.60b	0.77ab	0.98a	*	0.87	0.69	*ns*	*	0.98	0.86	1.24	*ns*	1.23a	0.89b	*	*ns*
Tryptophol SO3H	0.19	0.30	0.45	*ns*	0.39	0.23	*ns*	*ns*	0.44	0.32	0.43	*ns*	0.46	0.31	ns	*ns*
Abscisic acid	0.38	0.38	0.36	*ns*	0.44a	0.31b	***	*	0.51	0.44	0.48	*ns*	0.58a	0.39b	***	*ns*
Abscisic acid glucoside	0.49	0.22	0.34	*ns*	0.30	0.40	*ns*	*ns*	0.47	0.57	0.54	*ns*	0.52	0.55	*ns*	*ns*
Anthranilic acid									0.31b	0.56a	0.15b	***	0.39	0.29	*ns*	*ns*

[a] Data were analyzed by two-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).
[b] Different letters (a, b, c) identify significantly different means. H1, first harvest; H2, second harvest; H3, third harvest.
All the concentrations are expressed in mg/L.

3.9 SENSORY ATTRIBUTES

The results of sensory analysis are reported in radar plots (Figure 1–2). Sparkling wines were assessed in all three vintages by a commission of tasters made up of technical oenologists, agronomists, researchers, and students. In the three vintages, the wines have always been submitted to tasters anonymously, dividing the replicas in three successive sessions in a randomized manner. In a first data processing, the three-year averages (2017–19) of the organoleptic descriptors for the first two harvest times were then calculated (Figure 1). As can be seen from the results, the general pleasantness of the wines of the second harvest was better than the wines produced with the grapes harvested earlier. At the level of individual descriptors, it clearly emerged that the wines of the postponed harvest were characterized by more marked notes of dry vegetable, yeast, and oxidative notes. Additionally, a reduction in citrus and green apple hints has been observed. In terms of taste and retro nasal olfaction, no substantial differences emerged as regards licorice and herbaceous vegetables. The sparkling wines of the later harvest showed more intense notes related to acidity and astringency, flavor, and body, while on the contrary the bitterness and the hints of flint have diminished.

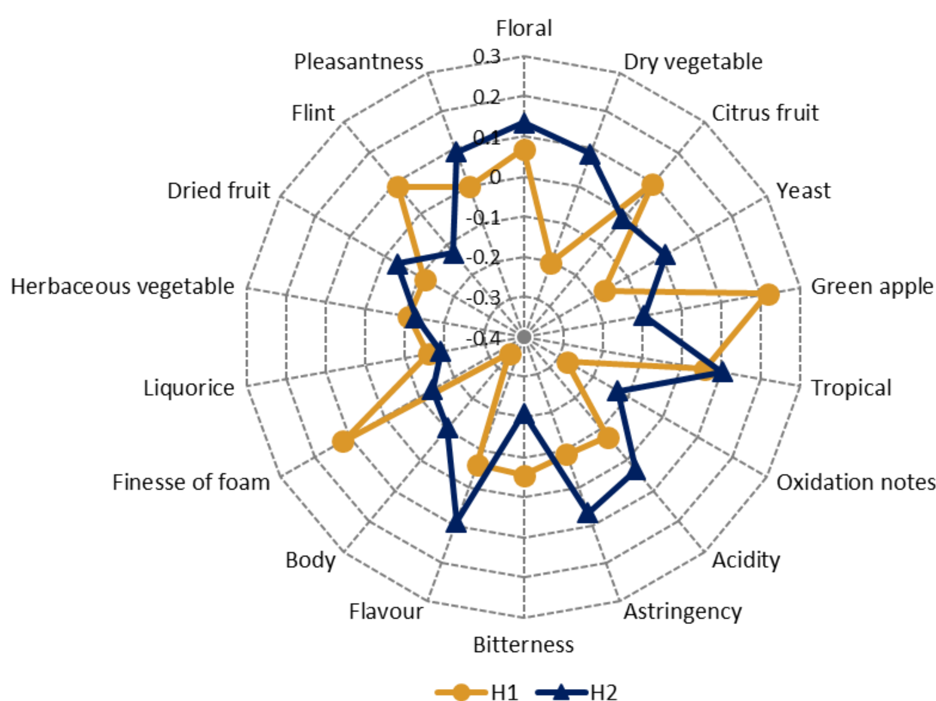

Figure 1: Effect of two harvest dates on the organoleptic characteristics of Ribolla Gialla sparkling wines (average values were obtained from 2017–2019). Yellow and blue line represent first (H1) and second (H2) harvest, respectively.

In a second analysis, the averages of the different descriptors in the 2018 and 2019 vintages were calculated, in order to compare the results of three subsequent harvest dates

(Figure 2). The results have shown that the differences between the averages of the H1 and H2 have remained more or less similar to what it was highlighted when comparing only two harvest dates from three-year average, while the differences are more evident for the wines of the H3. It was clear from the start that the postponement of the harvest was excessive what regards to general pleasantness, and probably the beginning of rot on the grapes had negative repercussions on the quality of sparkling wines. In fact, compared to the data of the first two harvests, higher values of the hints of oxidation notes, of the body and of dried fruit emerge, which in the case of sparkling wines are to be considered negative. Furthermore, at the olfactory level, the floral, citrus, green apple and tropical scents were reduced, which instead should be present to give freshness to the wine. On a gustatory and retro nasal smell level it can be observed that licorice and herbaceous vegetables did not show any significant differences between the three vintages, while notes of dried fruit emerged in more intense manner, penalizing the flavor and acidity.

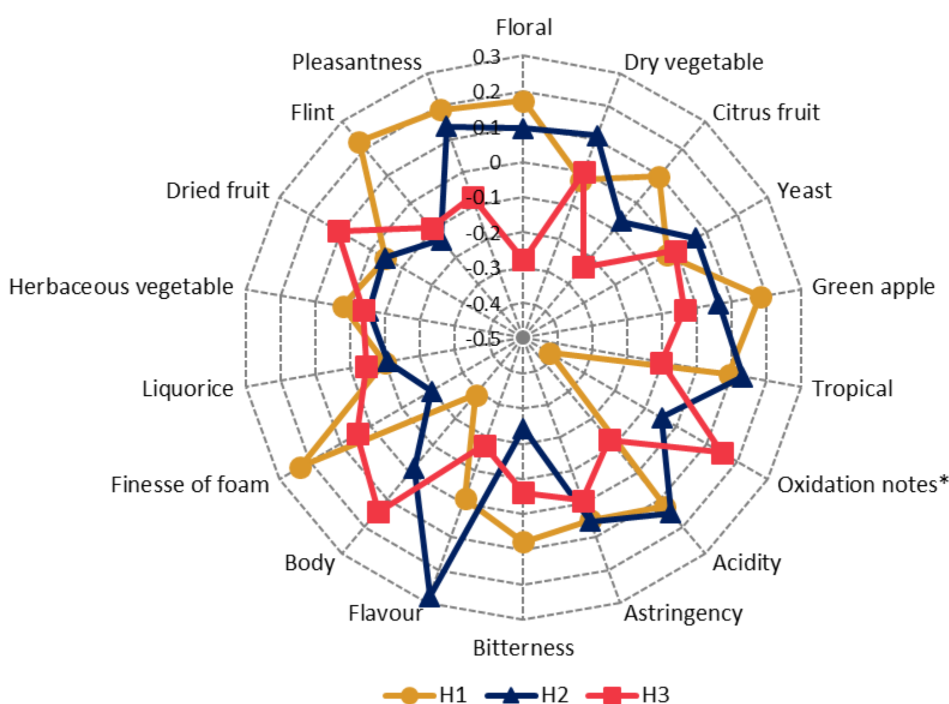

Figure 2: Effect of three harvest dates on the organoleptic characteristics of Ribolla Gialla sparkling wines (average values were obtained from 2018–2019). Yellow and blue and red line represent first (H1), second (H2) and third (H3) harvest, respectively. Asterisks (*) indicate statistical significance for each sensory attribute.

3.10 MULTIVARIATE ANALYSIS OF BASE WINES AND SPARKLING WINES

In order to inspect the overall impact of harvest date on the wine chemical composition, a Principal component analysis (PCA) was generated for base wines and sparkling wines, separately (Figure 3–6). The compounds that emerged as statistically significant for harvest timing factor after a two-way analysis of variance were taken into consideration and their

concentration was z-scaled. Considering only sparkling wines, all sensory attributes were added to the PCA as the supplementary quantitative variables, in addition to the selected chemical parameters.

In base wines, where only two harvest dates were performed from 2017–2019, the first two components (PCs) accounted for 46.3% of the data set variability, with PC1 comprising 28.4% and PC2 17.9% (Figure 3). The results demonstrated that wines from different harvests can be clearly separated based on their chemical composition of volatile compounds and AAA metabolites, as no lipid compound emerged as statistically significant after the two-way ANOVA. Samples from the different harvest dates (H1 versus H2) were separated along PC2 with all H1 wines on the negative side of PC2 and associated with some grape-derived aroma compounds (e.g., citronellol and β-damascenone), together with certain ethyl esters and kynurenic acid, related from metabolism of tryptophan. Most of the H2 wines were positioned on the positive side of PC2 and were mainly associated with AAA metabolites. However, the season remain to have a strong impact, as three sub-clusters of each harvest time were observed. Therefore, in the H2 wines from 2018 were located in the second quadrant of the PCA biplot, paired with tryptophol, tryptophane ethyl ester, phenyllactic acid, phenylalanine, and indole-3-acetic acid, while the 2017 and 2019 samples

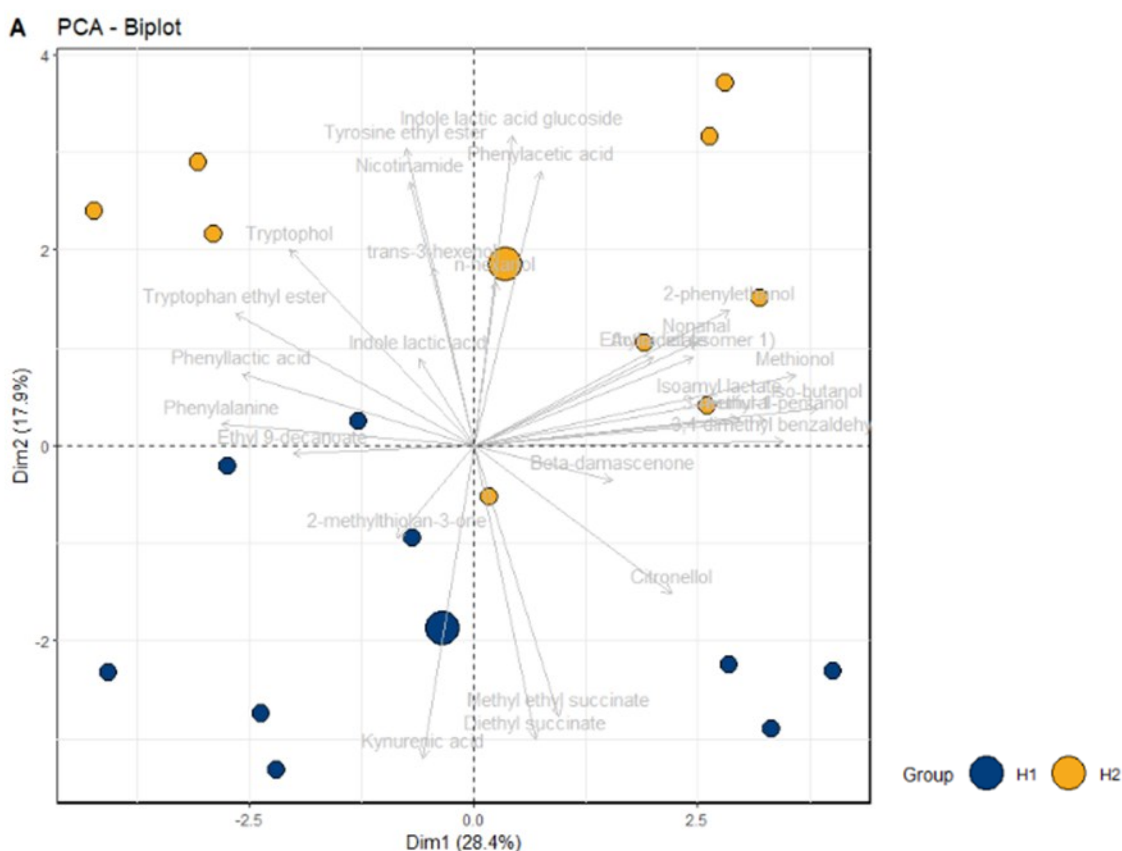

Figure 3: Principal component analysis biplots of base wines from two harvest dates. Small blue and yellow dots represents H1 and H2 samples, respectively, and larger dots represents the centroid values of respective samples. Grey lines represents significant loadings of volatiles and AAA metabolites.

were located in the first quadrant of PCA and were correlated with carbonyl substances and fermentative higher alcohols.

Considering the base wines that were obtained from three harvest timings, the first two PCs accounted for 65.1% of the variability in the data, with PC1 comprising 45.5% and PC2 19.6% (Figure 4). All three harvest dates were separated relatively well especially the difference between H1 and H3 was well evident. All H1 wines were positioned on the positive side of PC1 and were characterized by norisoprenoids (vitispirane) and monoterpenes (citronellol), but also with fatty dodecanoic acid. The positive correlated variables, such as certain ethyl esters, higher alcohols and, aldehydes and AAA metabolites (nicotinamide) were positioned in the same direction as H2 wines from 2018 season, while H2 wines, produced in 2019 were located on the negative side of PC1, along with the samples of H3 wines. The latter were positively associated with essential amino acids (tyrosine and phenylalanine) and their fermentative derivates (tyrosine ethyl ester and tryptophol), while the H2 wines were characterized by ethyl decanoate and isoamyl alcohol.

Figure 4: Principal component analysis biplots of base wines from three harvest dates. Small blue, yellow and red dots represents H1, H2 and H3 samples, respectively, and larger dots represents the centroid values of respective samples. Grey lines represents significant loadings of volatiles and AAA metabolites.

In the H2 sparkling wines (Figure 5) the first two PCs accounted for 57.9% of the variability, with PC1 comprising 41.7% and PC2 16.2%. The normal harvest and the extended harvest were separated well, with all H1 wines located on the negative side of PC1 and associated with citrus fruit aroma descriptors, as well as green apple and herbaceous vegetable. The rest of the descriptors were correlated with mouthfeel attributes (bitterness, astringency acidity and finesse of the foam). Chemical attributes, such as α-terpineol, ethyl 9-decenoate, tyrosine ethyl ester and ethyl-2-hydroxy4-methyl pentanoate were correlated as well with H1 wine samples. On the contrary, the overall pleasantness was related to the H2 wines, together with tropical, floral, dried fruit and body sensory descriptors. However, some negative attributes were also assigned together with H2 wines (oxidation notes and licorice). This duality of the results was most likely related to the chemical compounds that were present in H2 wines. According to the PCA analysis, H2 samples were correlated with hexanal and acetic acid, affecting the negative character of wines, as well as with β-damascenone, that contribute to the floral aroma of wines.

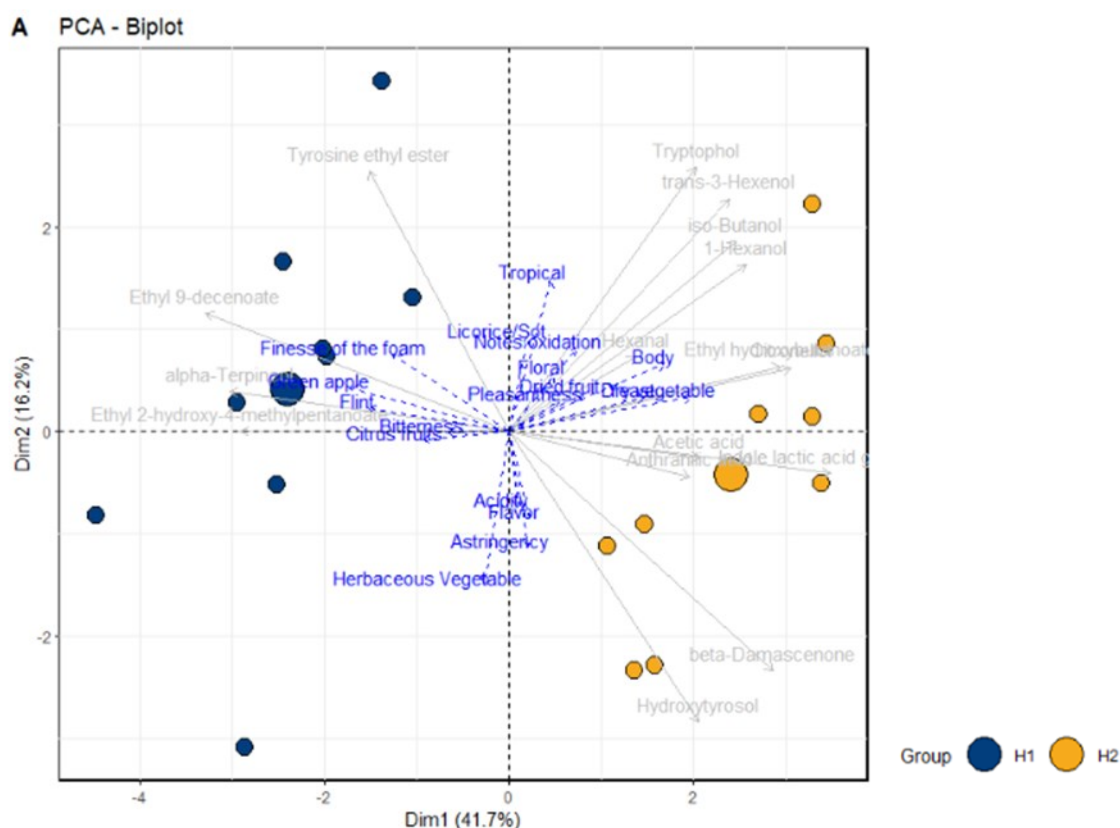

Figure 5: Principal component analysis biplots of sparkling wines from two harvest dates. Small blue and yellow dots represents H1 and H2 samples, respectively, and larger dots represents the centroid values of respective samples. Grey lines represents significant loadings of volatiles and AAA metabolites, while blue lines represents the loadings of sensorial descriptors.

Concerning the three-harvest timing experiment, over a period of two years (Figure 6) it was observed, that the first two PCs accounted for 54.6% of the variability in the data set,

with PC1 comprising the 32% and PC2 22.6%. All three harvest dates were separated well; all H1 wines were located on the negative side of PC1 in the second quadrant. H1 wines were correlated with finesse of the foam, flint, and bitterness, as well as with kynurenic acid and furfural. However, the general pleasantness appeared to correspond with H1 sparkling wine samples. The H2 group of samples resulted to be less homogeneous, since they were widespread over PC1, but remained on the negative side of PC2. Interestingly, primary volatile compounds were positively correlated with H2 wines (e.g., β-damascenone), with floral sensory descriptor. Nevertheless, notes of oxidation, astringency and dry vegetable were also correlated with H2 samples. Finally, the samples of additional third harvest date were located in the first quadrant of PCA and correlated with dried fruit, liquorice, and body as sensory descriptors. Tyrosine, nonanoic acid, and, interestingly, citronellol were also correlated to this samples.

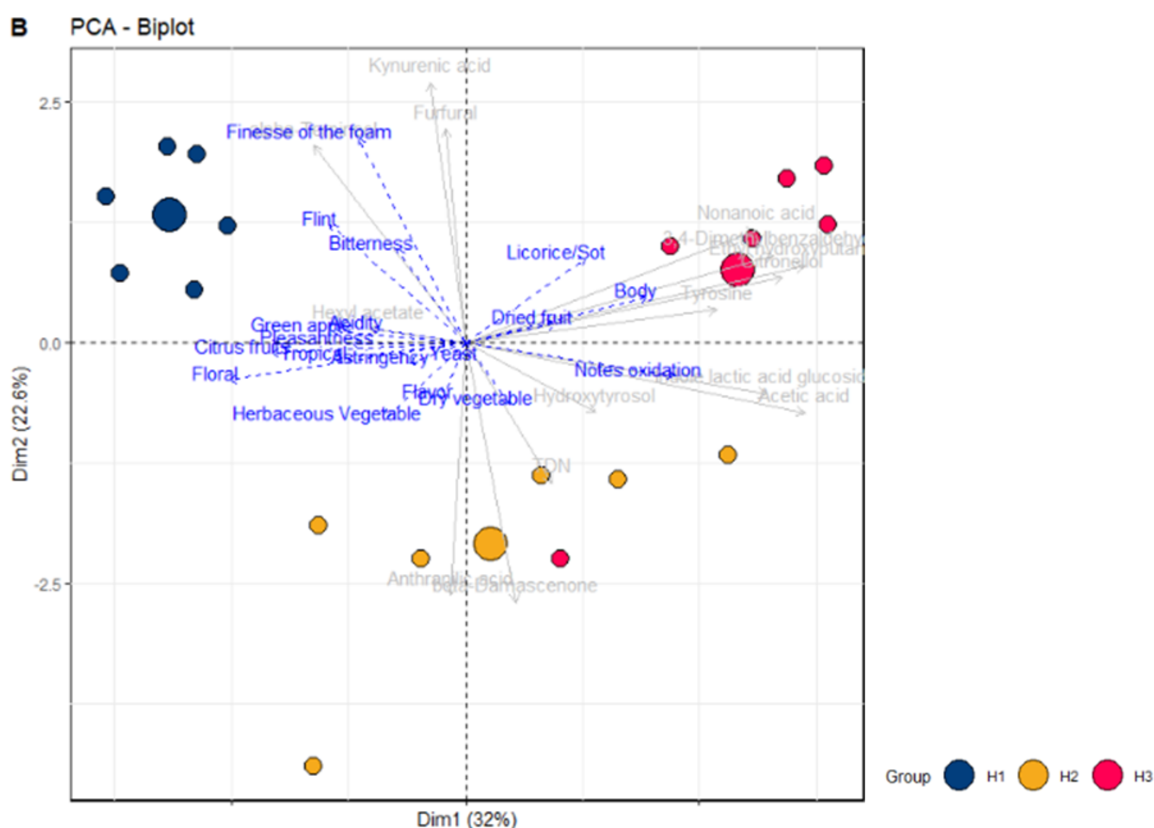

Figure 6: Principal component analysis biplots of sparkling wines from three harvest dates (B). Small blue, yellow and red dots represents H1, H2 and H3 samples, respectively, and larger dots represents the centroid values of respective samples. Grey lines represents significant loadings of volatiles and AAA metabolites, while blue lines represents the loadings of sensorial descriptors.

4 DISCUSSION

4.1 BASIC GRAPE QUALITY PARAMETERS

The amount of TSS, TA and pH value represent one of the most important parameters in determining the harvest date for the sparkling wines (Jones et al., 2014). During the grape maturation, the amount of TA decreases with a simultaneous increase of TSS concentration (Bowen & Reynolds, 2015), which corresponded with our study. However, our results showed that the concentration of TSS did not increased from H1 to H2 in the three-year average. Moreover, when observing the effect of seasonal factor, the discrepancies between averaged amounts of TSS in the grapes have been also observed, while a constant increase in TSS concentration from H1 to H3 was noted in the two-year average. Therefore, it can be considered, that the ripening of the grapes can be significantly influenced by a meteorological conditions, especially in the last points of grape ripening, as the berries can absorb rainwater more quickly and dilute the TSS content (Lijavetzky et al., 2012). From meteorological data obtained it was clearly seen that the summer months in 2017 and 2019 were particularly dry with high average temperatures, compared to the 2018, so the lowest TSS concentration in this year was excepted. Moreover, Costa et al. (2020) reported that there is a weak negative correlation between precipitation quantity and the amount of TA and vice versa for pH value, which was in accordance with our results, taking into consideration 107.4 mm and 106.4 mm of rainfall in July and August 2018, respectively. Also in 2017, the data of the second harvest was probably influenced by the dilution of the juice of the berries, since the September of the first year was characterized by a high amount of the precipitation (345.4 mm versus 45.2 mm and 114.2 mm in 2018 and 2019, respectively).

4.2 WINE BASIC COMPOSITIONAL PARAMETERS

The alcoholic strength in sparkling wines, obtained from delayed harvests when comparing both timing trials, resulted to be higher than expected, however, the relationship between RS and alcohol content did not show major deviations, compared to the grape maturity trend. Wine pH and TA however did not show any clear trend with grape maturity, especially when three harvest timings were tested. Moreover, the trend of the differences between the H1 and H2 harvest relating to the 2017 vintage was similar to what was found in the two subsequent years. However, lower concentration of TA in several cases could be most likely caused by cold stabilization at which the wines were subjected once finished the fermentation. It is well known, that cold stabilization can lead to a decrease in the concentration of tartaric acid in the wines, which in turn means a decrease in TA and an increase in pH (Bindon et al., 2013).

4.3 VOLATILE PROFILE OF BASE WINES AND SPARKLING WINES

4.3.1 Monoterpenes

The presence of terpenes on volatile aroma of wine is of great importance, as they are generally associated with floral, sweet fruit and citrus aromas (Zhao et al., 2019). In our study, eight monoterpenes were found. The postponement of harvest by one week from H1 to H2 led to an increase in the concentration of all monoterpenes apart from α-terpineol in base wines, while the further delay in harvesting was positive in the case of limonene, citronellol and β-myrcene, but negative for the others. In general, our findings therefore turned out to be in accordance with other studies, where an increase of grape monoterpenes was correlated with sugar accumulation in grapes (Bowen & Reynolds, 2015; Zhao et al., 2019). However, one of the most distinctive contributors to the floral aroma of wines is linalool, and it is well known, that its concentration increases until optimal grape maturity, followed by an immediate fall in concentration (Marais & van Wyk, 2017). Therefore, this could be the reason that the amount of linalool decreased right after grapes entered into the final week of ripening. In addition to linalool, geraniol appeared to be equally important in base wines, but its concentration similarly began to decline in H3 wines. This probably occurred due to the increased geraniol reductase activity towards the end of the ripening, which can produce high levels of citronellol (Luan et al., 2005). In fact, H3 wines were characterized with higher content of citronellol compared to the previous two harvest dates. In connection with the seasonal effect on the monoterpene content, it turned out that monoterpenes reached significantly higher values in 2018 season, which was considered as the warmest in the three-year average. Similar relationship was described previously by Pons et al. (2017), where higher temperatures appeared to be beneficial on the aromas and aroma precursors of fruity and floral nuances, which are characteristic of terpenes.

In sparkling wines, the concentration of monoterpenes appeared to be higher than in base wines for limonene, linalool, 4-terpineol, nerol and above all for α-terpineol. The reason for this could be the protonation of linalool, which causes the formation of monoterpenes in wine conditions (Šuklje et al., 2019). Although the hydrolysis of glycosidic linkages and thereby the release of free volatile compounds has not been the aim of this study, it is known that this process is facilitated during wine ageing However, the extended harvest date resulted in increasing concentrations of citronellol and geraniol and decreasing concentration of limonene. Additionally, a higher concentration of monoterpenes could be observed in the 2019 season for sparkling wines, which suggests that environmental factors also play a major role in the synthesis of volatile compounds. The positive influence of UV light (Song et al., 2015) and higher environmental temperatures (Pons et al., 2017) on the production of free terpenes were previously studied, and given that 2019 was characterized as a year with the greater number of days above 30 °C, this could contribute to a higher monoterpene content in wines.

4.3.2 Norisoprenoids

The concentration of norisoprenoids tends to accumulate during ripening, and starts to degrade once grapes reach the full maturity (Waterhouse et al., 2016). This can be directly applied on Ribolla Gialla base wines and sparkling wines, where the amount of norisoprenoids increased when comparing the three-year average and only two harvest dates. However, by adding the third harvest timing, the amount of all norisoprenoids decreased in H3 base wines and sparkling wines, except for vitispirane. The same observation of decreasing amount of norisoprenoids with maturity has been confirmed in other studies, especially in the case of β-damascenone and 1,1,6,-trimethyl-1,2-dihydronapthalene (TDN). (Šuklje et al., 2019; Versini et al., 2002). β-damascenone was recognized as the most abundant C13-norisoprenoid in sparkling wines, and its concentration nearly doubled the concentration, detected in the commercial sparkling wines from Ribolla Gialla variety, which is promising for the development of fruity-honey scents (Voce et al., 2019). Since the norisoprenoids are aromatically inactive at the beginning of the winemaking process, their release from glycosides might enhance the differences between separate harvest stages. The evidence for such chemical rearrangement can be evident in the increased amount of norisoprenoids in sparkling wines, compared to the base wines. Similar to the monoterpenes, increased sunlight exposure seem to encourage the development of carotenoids, and consequently increase the levels of norisoprenoids in finished wine (Waterhouse et al., 2016). Additionally, higher temperatures promote the synthesis of norisoprenoids, as it was seen in the case of Glera grapes (Alessandrini et al., 2017). Therefore, in sparkling wines, it was possible to observe that the total amount of norisoprenoids was higher in the warm season 2019, while the results of base wines reported contrary.

4.3.3 Aldehydes

The formation of aldehydes is mostly associated with lipoxygenase (LOX) pathway, where the breakdown of unsaturated fatty acids leads to the production of aldehydes, as well as alcohols and carboxylic acids (Moreno Luna et al., 2018). In the base wines and in the sparkling wines analyzed, not many compounds pertaining to the class of aldehydes were found, and among these, *trans*-2-hexanal was the most abundant. Generally, postponing the harvest led to an increase in the concentration of aldehydes, especially in the case of base wines and by comparing H1 to H2. When a third harvest date was added in 2018 and 2019 season, a substantial drop in concentration was observed. This reduction in fact coincided with a drastic decrease of alcohol dehydrogenase activity (ADH), an enzyme that is involved in the LOX pathway (OuYang et al., 2015). The decreased amount of *trans*-2-hexanal is ultimately desirable because aldehydes contribute to the herbaceous note in wine aroma profile, which turns out to be detrimental and undesirable to the consumer. Moreover, the occurrence of alcohols and aldehydes, that are originated enzymatically from unsaturated fatty acids is related to the leafy grassy, a herbaceous odor initially attributed to leaves included when the grapes were mechanically harvested (Herraiz et al., 1990).

4.3.4 Alcohols

In this study, twelve higher alcohols were detected which does not rank them among the most numerous, yet the expressed concentration was considered one among the most important. In certain studies where the authors compared the effect of sequential harvest timings, higher alcohols accounted even for 86% of the total volatile compounds (Zhao et al., 2019). An average increase in the concentration of alcohols in base wines with extended harvest date can be noted in our study, especially in the case of methionol, 3-methyl-1-pentanol and isobutanol. An increased amount of isobutanol is able to suppress fruity and woody notes in wine, but not leather/animal/ink nuances, therefore aliphatic higher alcohols play a negative role on wine aroma quality (de-la-Fuente-Blanco et al., 2016). Environmental stresses, in particular water deficiency, can activate the ADH activity, which is responsible for catalyzing the reduction of aliphatic aldehydes to alcohols (Moreno Luna et al., 2018). This resulted to be in conflict with our observations, since the concentration of n-hexanol was higher in the 2017, where June was characterized with abundant amount of precipitation (274.7 mm), compared to other two seasons (73.7 mm versus 37.5 mm for 2018 and 2019, respectively). In the case of sparkling wines, the differences were more limited when comparing the three sequential harvest dates. However, by taking into consideration only two harvests, spanned over three seasons, it turned out that concentration of C6 alcohols (e.g., hexanol and *trans*-3-hexenol), derived from LOX pathway, increased in the later stage of ripening. However, conflicting results have been previously published, arguing that concentration of *trans*-3-hexenol and its isomer have decreased in wines from the latest harvest (Antalick et al., 2015; Fang & Qian, 2012; Šuklje et al., 2019).

4.3.5 Esters

The esters that most influenced the aromatic potential of base wines and sparkling wines were sub-divided into acetates of ethanol and higher alcohols (e.g., ethyl acetate, isopentyl acetate and hexyl acetate) and esters that are product of fatty acid metabolites and ethanol (e.g., ethyl octanoate). Acetyl-CoA and higher alcohols are considered the main precursors, from which the acetate esters are synthesized (Zamora et al., 1985). Therefore, an increased Brix in grapes from later harvest dates results in the enhanced production of ethanol and higher alcohols and consequently leads to increased levels of acetate esters (Moreno Luna et al., 2018). Water stress can further stimulate the formation of these compounds which can be detrimental for overall wine quality, but only in case of excessive concentration of ethyl acetate (Zamboni et al., 2008). The delayed harvest showed an increased value for a large number of esters in base wines, where ethyl acetate, isopentyl acetate and hexyl acetate were showing an increasing trend towards the latest harvest date, due to their statistical insignificance. On the contrary, the concentrations of hexyl acetate, methyl-ethyl-succinate, diethyl-succinate and ethyl-9-decanoate appeared to decrease. Moving on to sparkling wines, the later harvests have shown in many cases a decreasing trend in the concentration of esters. However, it is important to underline, that major ethyl esters of fatty acids and

higher alcohol acetates are strictly fermentative compounds, produced by wine microorganisms (Pons et al., 2017). Therefore, the results show large differences in ester content between base wine and sparkling wines. The loss of esters during the second fermentation and subsequent ageing period is a consequence of chemical hydrolysis and thermodynamical instability. Despite this, the results of sparkling wines showed that the concentration of diethyl succinate increased compared to the base wines, which was in accordance with the results reported by Ubeda et al. (2019). Moreover, researchers have found that in dry hot seasons, aggravated sunshine and daytime temperature on berry cluster could be the main affecting factor for causing a reduction in the levels of C6-derived esters (He et al., 2020). This is also true for the present study, since 2018 and 2019 seasons were characterized by high average temperatures, which was due to the large number of days with temperatures above 30 °C. For instance, the amount of ethyl hexanoate in 2017 was 1007 μg/L, whether 761 and 433 μg/L were measured in 2018 and 2019, respectively.

4.3.6 Acids

Considering the concentration of volatile acids in our study, the effect of the harvest time had a different effect on the composition of base wines and sparkling wines. In the case of base wines, the extended harvest date increased the concentration of all acids, especially dodecanoic, decanoic and benzoic, especially in the case of the second harvest. However, these finding were not compatible with the results of Moreno Luna et al. (2018), where the delayed harvest reduced the amount of hexanoic, octanoic and decanoic acid. Fortunately, as far as sparkling wines are concerned, the later harvests did not greatly modify the concentration of these acids, which potentially affect the wine aroma with negative notes of sweat, cheese and rancid fat (Ferreira et al., 2000). Moreover, when only two harvest timings were studied in our case, the increased amount of acetic acid was produced in the extended harvest date, and in addition, it was observed that the concentration of acetic acid was the highest in 2019. It is well known that acetic acid is considered as a byproduct of microbial metabolism, therefore an extensive rainfall or higher Brix in grapes can lead to enhanced production of acetic acid (Arias et al., 2019; Chidi et al., 2018). This was contrary to our results, as the 2019 season was not characterized as very wet season, and the TSS amount did not predominate. However, our findings could be consistent with the results of Casassa et al. (2013) who reported that diminished TA led to increased acetic acid in association with extended harvest date in Merlot wines.

4.4 LIPID PROFILE OF BASE WINES AND SPARKLING WINES

4.4.1 Fatty acids

As regards lipid composition in base wines and sparkling wines, it has been observed that the delayed harvest date did not contribute to any significant difference, when analyzing two or three harvest timings. Instead, the results showed that the seasonal factor affected

most of the nineteen lipids analyzed, especially in the final sparkling wines. In the research of the Arita et al. (2017) where the authors were investigating the lipidome characteristics of Pinot Noir and Japanese Koshu grape berries, it has been shown, that fatty acid molecules in Pinot Noir grape berries had more double bonds than those in Koshu variety. The increase in number of double bonds in a fatty acid molecule is observed, when plant is placed in a low-temperature growing region (Graham & Patterson, 1982). In particular, the berries from cold regions showed higher content of linoleic acid than berries cultivated in warm regions (Arita et al., 2017). However, these processes are only relevant to the conditions, when the vine is frequently exposed to temperatures below 0 °C during the wintertime, as this can affect the growth and development of grapevine dormant buds, leaves and flowers in the sequential growing season. Even though that our study did not cover winter temperature measurements, changes in the fatty acid composition were still evident. It turned out that the concentration of saturated fatty acids (SFAs) overcame the concentration of unsaturated fatty acids (UFAs) in all three years of experiment. The sum of average concentrations of SFA and UFA was highest in the last year of established viticultural experiment (season 2019), followed by 2017, while 2018 was characterized with the lowest amount of both lipid classes. A similar observation was shown also for polyunsaturated fatty acids (PUFAs), which are considered to be the major components of the total lipids in grapes (Pérez-Navarro et al., 2019). The latter class include linolenic acid (C18:3) and linoleic acid (C18:2). Previous studies have shown that the content of SFA and UFA is highly affected by the ripening period of grape variety as well as climate factors. Tociu et al. (2017) argued that the harvesting time is an important factor for the ripening of the grapes, therefore the amount of Mamaia grape seed oil increased with the delayed harvest. Moreover, the authors reported increasing values of mono-unsaturated fatty acids (MUFA) in the years with high precipitation during ripening period. Conversely, the concentration of PUFA content was higher and the SFA content appeared to be lower in the dry years. (Tociu et al., 2017). In our case, the summer temperatures of 2019 increased above average values as compared to the other two vintages, however, the ratio between SFA and UFA remained unchanged in favor of a higher SFA concentration. The amount of oleic acid (C18:1) was the highest in 2019 season, but the palmitoleic acid (C16:1) appeared to have the highest content in 2017, which at the same time proved to be the rainiest season. The ratio between SFA and UFA composition is important in the wines, as both have an impact to the aroma characteristics of wine. Liu et al. (2019) reported that low concentration of linoleic acid enhanced the productions of certain free fatty acids (e.g., octanoic and decanoic acid), while the oleic acid promoted the isoamyl acetate biosynthesis. Interestingly, a low amount of linolenic acid had no effect on acetate esters production, while higher supplementation promoted the production of C6 alcohols (1-hexanol) and higher alcohols (isobutyl alcohol and 2,3-butanediol). Our results proved to be in accordance with this case, since the 2018 season was characterized with the lowest amount of linolenic acid, which consequently led to lower LOX activity and lower C6 alcohol production.

The ratio between UFAs and SFAs can also have major influence on the wine foaming in the sparkling wines. Several studies have therefore described the positive correlation between foamability and the presence of fatty acids or their derivatives (Coelho et al., 2011; Gallart et al., 2002). While the free fatty acids C8, C10 and C12 are considered to be negative contributors to the foamability, their ethyl esters were found to be positively correlated to the occurrence of the foam in the sparkling wines (Gallart et al., 2002). In one study on cavas, the authors have described that the total content in unsaturated linolenic acid and saturated palmitic acid were the compounds that affected the most foam properties of wines. The linolenic acid was positively correlated with foam stability, while the palmitic acid exhibited stronger relation to foam height. While palmitic acid resulted to be the most abundant among SFA, this was not true for linolenic acid concentration, compared to the other UFA substances in our study. Nevertheless, both compounds were higher in the season in 2019, therefore it can be expected, that the wines from that particular vintage will be characterized with positive descriptors, related to the foam.

4.4.2 Sterols

The deprivation of sterols in fermenting medium can lead to sluggish or stuck fermentation; an event that is more likely to occur during the white winemaking process due to the clarification process (Tesnière, 2019). Our results showed that the sterol concentration was increased in the 2019, both in base wines and sparkling wines, irrespectively of the harvest timing trial. Sterols are very important integral components of the membrane bilayer and one of their primary roles is to maintain the membrane homeostasis by developing stress resistance in plant cells (Rogowska & Szakiel, 2020). Many valuable crop plants are frequently exposed to drought stress, which is highly detrimental for the agricultural industry and has been an increasing problem in recent years due to the global climate change (Rogowska & Szakiel, 2020). In grape berries, the prolonged water deficit stress resulted in smaller berries, but this decrease in size was also associated with an increase in cuticular wax content, composed mainly from sterols and triterpenoids (Dimopoulos et al., 2020). The concentration of lupeol, a triterpenoid analyzed in our study exhibited an increased concentration in 2019, which could be also due to the high temperatures that characterized that vintage season. Another observation regarding the concentration of sterols was that the concentration of ergosterol (a major sterol that is found in lower eukaryotic membranes) that has increased from base wines to the sparkling wines, probably due to the secondary fermentation. Consequently, the yeast cells were less susceptible to ethanol stress; in fact, *S. cerevisiae* cells were able to produce higher ethanol content in sparkling wines, due to enhanced ethanol tolerance and higher concentration of ergosterol in the fermentative medium.

4.5 AROMATIC AMINO ACID METABOLITES PROFILE OF BASE WINES AND SPARKLING WINES

The results presented regarding the tryptophan metabolites highlight a substantially positive role in the extended harvest date. The comparison of three harvest moments in the two-year period from 2018-2019, exhibited a significant effect on the composition of the AAA metabolites, when passing from the first to the second harvest. Interestingly, the tryptophan did not show any significant differences, when comparing from H1 to H2 and finally to H3. Moreover, the trend in concentration has proven to be quite inconsistent. This contradicts the results of some other authors who reported that the amount of free and bound TRP in the grapes and wines increased significantly with the stage maturity (Hoenicke et al., 2001). However, together with L-tryptophan, L-phenylalanine and L-tyrosine represent the AAA, derived from the shikimate pathway and they all serve as a precursors of numerous products in plants (Maeda & Dudareva, 2012). Despite very few significant changes regarding the amount of TRP in the different harvest dates, our results for TYR and PHE showed that their concentration increased in the samples of base wines with an extended harvest date. This observation was in line with the fact that the nitrogen or amino acid contents of the grape, musts and wines increase with the ripeness of the harvested grapes (Hoenicke et al., 2001). Concomitantly with an increase of the amino acid TYR in the base wines, the concentration of its catabolites tyrosine ethyl ester (TYR-EE), N-acetyl-tyrosine ethyl ester (N-TYR-EE), and tyrosol (TYL) also increased. TYR-EE and N-TYR-EE are products of alcoholic fermentation by *S. cerevisiae*, and they play an important role in the yeast mechanism and thus in fermented food quality. Namely, it has been previously observed, that N-TYR-EE actively participate in the inhibition of TRP synthesis and metabolism in the yeast, as well as mediator of the production of tryptophol (TOL). (Álvarez-Fernández et al., 2019). This may be crucial for aromatic profile of the wines, since an off-flavor is known to be formed in the white wines and is associated with the aroma compound 2-aminoacetophenone (2-AAP), which formation is stimulated by the precursors TRP and indole-3-acetic acid (IAA). This off-flavor is often described by aroma descriptors such as acacia blossom, furniture polish, wet wool, mothball and fusel alcohol and usually leads to the loss of a typical bouquet of the grape variety. (Arapitsas et al., 2018; Hoenicke et al., 2002; Hoenicke et al., 2001). Additionally, it has been previously reported that high yield of the grapevine may be related to the occurrence of untypical ageing flavor, however its development is even more influenced by the dry-stress of the vines, caused by low rainfall or intensive solar radiation (Hoenicke et al., 2001). Since in our study the 2-AAP has not been identified, it was observed that IAA concentration predominated in sparkling wines during the 2019 harvest season that has been characterized as one of the driest in the three-year average. The location of the vineyard, where the experiment has been carried out may have also contribute to the increased IAA concentration, since there was less possibility of irrigation in the event of drought, which could further contribute to the dry stress.

The seasonal factor, on the other hand, also affected the remaining compounds. One of these was abscisic acid (ABA). When three different harvest dates were studied, the accumulation of ABA was most intense in 2017 winegrowing season for base wines and sparkling wines. By comparing three harvest times it has been observed, that earlier 2018 season have been characterized with higher concentration of ABA. According to the Jiang (2002), water stress not only induces ABA accumulation, but it also causes an enhancement in the generation of reactive oxygen species (ROS) and antioxidant defenses. In correlation with our meteorological data, the accumulation of ABA in 2018 is reasonable since this season was the hottest of the last 20 years and the sum of accumulated rain in 2018 was the lowest. However, our results regarding the 2017 season are not in line with the literature since the cumulative rain in 2017 was the highest.

Further analysis of the obtained results for AAA metabolites also showed the accumulation of the glycosidic form of indole lactic acid (ILA-GLU) with the extended harvest time in base wines and in sparkling wines. Higher concentration of ILA-GLU at later stage of maturity was associated with higher Brix. However, when the glycosidic bond is hydrolyzed in wine, the producing ILA can react with the present SO_2 to deliver the sulfonated ILA-SO_3H (Arapitsas et al., 2018). The degradation of ILA-GLU and its consequent sulfonation could be responsible for the formation of the aromatic aminobenzene 2-AAP, which in turn could lead to emergence of UTA flavor in wine. Similarly, the increase of tryptophol concentration (TOL) was observed in the base wines, especially when comparing the H1 with H2 wines. The high level of sulfonation of TOL in young white wines indicate, that TOL-SO_3H may also give rise to further products similar to 2-AAP (Arapitsas et al., 2018), although the difference in concentration of TOL-SO_3H in H1 and H2 base wines did not appear to be statistically significant.

Lastly, the phenylacetic acid (PhAA) also appeared to show the tendency towards accumulation in the later stage of the harvest. The synthesis of PhAA is correlated with the occurrence of the grape sour, which often occurs towards the end of grape ripening, when the grape skin becomes thinner and more susceptible to the damages due to the microorganism infections. High quantity of precipitations and relatively high temperatures may facilitate the development of grape sour rot (Pinar et al., 2017). When the damaged berries are not removed prior the winemaking process, this could contribute to development of sweet-like, honey off-odor (Campo et al., 2012). Thus, it was shown in our results that the concentration of PhAA was the highest in 2018 winegrowing season, which was characterized by a large amount of precipitation in July and August, which could encourage the occurrence of sour rot and at the same time increase the concentration of PhAA.

4.6 SPARKLING WINE SENSORY ANALYSIS

The sensory attributes of obtained sparkling wines showed that the general pleasantness was assigned to the wines from second harvest, when only two harvest times were compared. The wines from the postponed harvest were characterized by dry vegetable, yeast, and

oxidative aromas, which, however, negatively affects the overall aroma of the wine. Certain aldehydes can cause the phenomenon of oxidative aroma in wines (Mayr et al., 2015), which was confirmed by the present study, as the total concentration of aldehydes predominated in H2 wines. In general, the addition of third harvest time did not greatly affect the results, where subsequent harvesting therefore meant an increased assessment of the perception of oxidative aroma in wines, which could be related to rising content of nonanal, 3,4-dimethyl benzaldehyde, and furfural. This proved to be partly in line with the study of Zhao et al. (2019), where the authors found out the total aldehyde and ketone contents in sequential harvest wines increased, compared to the control samples. Interestingly, the score for citrus and floral sensory descriptors decreased with each additional harvest. The appearance of these aromas is largely due to increasing concentration of monoterpenes and esters during ripening (Bowen & Reynolds, 2015; Ubeda et al., 2020; Zhao et al., 2019).

5 CONCLUSIONS

This work confirmed the importance of determining the harvest time of grape, intended for the production of quality sparkling wines. As far as the composition in volatile compounds is concerned, the results have shown a significant advantage in the transition from first to the second harvest time, which resulted in enhanced production of esters, while no encouraging result emerged in the third harvest. In certain cases, the additional third harvest even meant a deterioration in the aromatic profile of the sparkling wines. In fact, the addition of an extra harvest date caused the increase of acetic acid, C8 and C9 volatile fatty acids.

The lipid composition of base wines and sparkling wines was not affected by the harvest time. However, the lipid content varied depending on seasonal factors. Thus, the hot season of 2019 was associated with higher content of SFAs, in particular palmitic acid, which could positively affect the foam height of sparkling wines.

At the level of tryptophan metabolites, it has been clearly shown that the extension of harvest date, is not necessarily correlated with the formation of UTA substances that could compromise the quality of sparkling wines.

Lastly, the sensory evaluation of sparkling wines appeared to be in accordance with the chemical analysis, since it has revealed that the wines from the second harvest were rated as most pleasant, while wines from the later harvest were evaluated as the least attractive from olfactory/gustatory point of view.

4

MONITORING OF TARGETED METABOLIC PROFILE OF GRAPES FROM RIBOLLA GIALLA, DESTINED FOR MONOVARIETAL SPARKLING WINE PRODUCTION DURING RIPENING AND AFTER CLUSTER THINNING APPLICATION[4]

[4] This chapter is a part of a manuscript in preparation.

1 INTRODUCTION

The metabolic composition of grapes encompasses many compounds that ultimately affect the quality of the grapes. Consequently, the physical and chemical composition of grapes at harvest is of paramount importance for the fruit quality characteristics and the quality attributes of wine or grape juice that is produced from the fruit (Keller, 2010). The changes in the metabolic composition of grapes begin long before the ripening process starts, however, just as the grapes ripen, they undergo a multitude of both physical and chemical changes (Keller, 2010b). The first and foremost noticeable change during the ripening of the grapes, is the alternation of skin color and the softening of the berry, which coincides with the beginning of sugar accumulation in the grape. Therefore, it is important to emphasize that sugar concentration, along with total acidity and the pH represent the main fruit quality criteria for determining harvest dates for sparkling wine production, which ultimately affects the final quality of wine (Jones et al., 2014). Current trends show that less alcohol-rich wines are preferable among the consumers, therefore, it has been previously suggested that sparkling wines should contain 10–11 % (v/v) of alcohol in order to give raise the desired complexity while maintaining the same freshness, which is characteristic of sparkling wines. For comparison, the desired level of maturity of grapes at the beginning of harvest, intended for the production of champagne, is determined at 9% v/v of alcohol (Jones et al., 2014). It follows that the fruity maturity of grapes, destined for sparkling wine production is considered to be optimally ripe at approximately 18–20 °Brix (Keller, 2010a, 2010b). However, ripening of the grapes can be also manipulated with certain viticultural techniques. One of these is cluster thinning which directly affects the yield. The effects of this technique were extensively presented in *Chapter 2*; however, it is worth emphasizing that lower yield can lead to higher quality of the wines, but it can also simultaneously cause overripe fruit, especially in warm seasons (Jones et al., 2014).

The primary metabolites therefore have a major influence on the ripening of the grapes and the subsequent physico-chemical properties of the produced wine; however, the secondary metabolites are equally of exceptional importance. Namely, lipids are actively involved in the plethora of biological functions, as structural, signaling and energy molecules (Della Corte et al., 2015; Subramaniam et al., 2011). Moreover, their contribution to the nutritional value of foods where they can act as biomolecule regulators of various biological processes, related to cardiovascular and other chronic diseases, plays an important role in living biological systems (Fahy et al., 2011). The vast majority of the lipid molecules can be found in the membranes of plant cells and together represent 5–10% of the dry weight of vegetative plant cells (Della Corte et al., 2015). In grapes this quantity slightly increases, as the proportion of lipids ranges 0.15–0.24% on the fresh weight basis (Gallander & Peng, 1980). Lipids are differently distributed in the grape berry. Most of these hydrophobic molecules are therefore found in the grape seeds, whereas in skins and pulp they account for a small proportion (Pérez-Navarro et al., 2019). Moreover, in grape berries lipids can be implicated in determining the characteristic aroma of grapes, in addition to influencing the

aromatic composition of wines as precursor of some odor-active compounds or being odor-active themselves. For example, previous studies have shown that lipoxygenase activity appears to be higher during the semi-ripe stage, which could lead to the formation of C6 and C9 aldehydes and corresponding alcohols, such as *trans*-2-hexenal, *trans*-2-nonenal and *cis*-2-hexenol, which eventually leads to the synthesis of unwanted herbaceous aroma later in the wine (Iglesias et al., 1991; Šuklje et al., 2019). In the winemaking technology, lipids are considered to be a vital nutrient for the yeast, and their availability can drastically affect alcoholic fermentation capacity.

Grape volatile compounds are the primary contributors to fresh and fruity note of wines (Sánchez-Palomo et al., 2005). The vast majority of compounds for this kind of aroma derives from grape skins, where concentration of terpenes, C13-norisoprenoids, benzene derivatives and aliphatic alcohols is the highest (Ribéreau-Gayon et al., 2006). Grape volatile composition therefore varies based on grape variety, viticultural practices, climatic and biological factors. When the grape aroma compounds are present as free volatiles, they may directly contribute to odor, but they can be also bound sugar conjugates that are initially nonvolatile. The glycosidic conjugates are able to transform into odor-active form when they undergo acid or enzyme hydrolysis, by releasing free volatile substances and thus contributing to the complexity of wine aroma (Vilanova et al., 2012). The terpenoids and C13-norisoprenoids are often described as the varietal volatile compounds, present as free or glycosylated form and whose flavor characterizes fresh berries, musts, and wines of many different genotypes. Other grape volatiles are ascribed to the chemical classes of benzenoids, aliphatic aldehydes and alcohols and lipid derivates. C6 and C9 lipid-derived substances are produced as a consequence of wound stress in plants, especially at the crushing phase of the berries and therefore represent the majority of varietal pre-fermentative grape volatile compounds (Carlomagno et al., 2016). Most of the volatile compounds are synthesized in the period from veraison until harvest. However, previous studies reported that the concentration of notable aroma components are not subjected to continuous accumulation until the very date of harvest, but they are produced during the first period of growth and then they start to decline during the fruit ripening. An example for these compounds are terpenes, where the concentration appear to accumulate during the herbaceous phase and then decline steadily during fruit ripening (Hashizume & Samuta, 1999; Vilanova et al., 2012).

Another group of important nutrients for yeast during the alcoholic fermentation are the nitrogen-containing compounds. From 50–90% of the nitrogen is present in the form of free amino acids in grapes, among which arginine and proline together account 60–70% of the amino acids in mature grape berries (Keller, 2010). Previous investigations reported that the arginine can be found in the skin, pulp and seeds throughout fruit development (Lamikanra & Kassa, 1999). However, the accumulation of arginine depends greatly on the cultivars, since for Chardonnay and Cabernet Sauvignon the accumulation appears to cease at veraison, whereas for Müller-Thurgau it may not begin until veraison. On the contrary,

previous studies have discovered, that most of the proline is accumulated after veraison and the accumulation seemed to continue in the ripening process. (Keller, 2010b). At very high concentrations, proline induce a sweet taste in wines, whereas arginine and phenylalanine cause bitter taste. However, the presence of amino acids in grapes does not necessarily mean that their presence in wine will significantly affect the aromatic profile, since amino acids are present in wine at concentrations that are too low to have any sensory impact. Aromatic amino acids such as tryptophan, phenylalanine and tyrosine are synthesized from shikimate pathway and they have a great influence on the sensory properties of wines. Namely, tryptophan and its metabolites, especially the well-known phytohormone indole-3-acetic acid, are considered to be potential precursors of 2-aminoacetophenone, a molecule that causes atypical aging defect in wine (Hoenicke et al., 2001).

It is therefore clear, that the listed groups of compounds found in grapes have a great direct or indirect influence on the quality of the produced wines. Therefore, it is reasonable to expect that winegrowers will resort permitted viticultural measures to improve the quality of grapes destined for the production of sparkling wines. This work thus focuses in the first part on monitoring the ripening of Ribolla Gialla grapes, in order to determine how the concentration of lipids, volatile compounds and aromatic amino acid metabolites change with increasing Brix rate. The second part of the work aimed to investigating how cluster thinning affects the development of metabolite profile in individual stages of grape ripening in two different vineyard sites. This sub-study thus represents an important contribution to the lack of the research regarding the viticultural practices for sparkling wine production, which can have particularly great meaning for the local winegrowers that cultivate Ribolla Gialla variety.

2 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Chemicals acetonitrile (ACN, LC-MS grade), 2-propanol (IPA), methanol (CH_3OH, LC-MS and HPLC grade), choloform ($CHCl_3$), 3,5-di-tert-4-butylhydroxytoluene (BHT), sodium chloride (NaCl), citric acid, ascorbic acid and 3-nitrotyrosine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (HCOOH) and ammonium formate (NH_4COOH) additives for LC-MS analysis were purchased from Fluka-Sigma S.r.l. (Milan, Italy). C7–C30 n-alkane solution in n-hexane was purchased from Supelco (Belle- fonte, PA, USA), while cholesterol-d7 and octadecanoic acid-d3 were obtained from CDN Isotopes (Quebec, Canada). All aqueous solutions, including the HPLC mobile phase were prepared with water purified using a Milli-Q system (Milipore, Vimodrone, Milan, Italy). Pure chemical standards used for determination of volatile compounds, lipid molecules and aromatic amino acid metabolites were obtained from Aldrich-Fluka-Sigma S.r.l. (Milan, Italy) and Avanti Polar Lipids (Alabaster, AL, USA), except for tryptophol-2-sulfonate

(TOL-SO_3H), indole-lactic acid-2-sulfonate (ILA-SO_3H), and indole-acetic acid-2-sulfonate (IAA-SO_3H) that were synthesized as described by Arapitsas et al. (2018).

2.2 VINEYARD SITES AND EXPERIMENTAL DESIGN

The experiment was carried out from 2018–2019 harvest seasons in two different vineyard sites of Ribolla Gialla in Friuli Venezia Giulia region in North-Eastern Italy. The characterization of both commercial vineyards, the implementation of the viticultural experiment and the meteorological measurements are described in more detail in *Chapter 2*. Additionally, in 2018 and 2019 winegrowing seasons, the changes in the content of individual groups of metabolites were monitored by regular sampling of the grapes, which took place approximately every seven days at veraison stage, only in FCO vineyard site. Table 1 represents the sampling dates of untreated and thinned vines for the cluster thinning experiment.

Table 1: Dates of sampling points for the cluster thinning trial.

Point	FCO		FG	
	2018	2019	2018	2019
1	24-Jul	06-Aug	30-Jul	05-Aug
2	31-Jul	13-Aug	03-Aug	13-Aug
3	07-Aug	20-Aug	10-Aug	20-Aug
4	13-Aug	27-Aug	17-Aug	27-Aug
5	21-Aug	03-Sep	30-Aug	03-Sep
6	29-Aug	10-Sep		10-Sep
7	03-Sep			17-Sep

2.3 SAMPLE PREPARATION AND BASIC ANALYSIS

From each parcel, grape samples were collected and transferred in the laboratory, where berries with pedicels were separated randomly from different parts of grape cluster and frozen at –80 °C. A certain quantity of frozen grapes without pedicels was homogenized under liquid nitrogen with a IKA A11 homogenizer to generate ~ 30 g of powder as previously described by Gika et al. (2012). Despite the fact that the contact between grape skins and seeds is minimal during the sparkling wine production, the samples were divided in two parts: homogenized whole grape berries were intended for the study of physiological aspects of fruit ripening, while the berries where the seeds were removed prior the homogenization were analyzed from the enological perspective.

The basic analysis was performed as reported in *Chapter 2*.

2.4 VOLATILE COMPOUND EXTRACTION AND ANALYSIS

A 2.50 g of each sample powder was introduced in a 20 mL headspace vial, together with 2.0 g of NaCl and 20 mg of citric and ascorbic acid consecutively. Finally, 2.50 mL of Milli-Q purified water was added, and the samples were spiked with 25 µL of 2-octanol (2.13 mg/L in ethanol) as an internal standard. Extraction of volatile organic compounds (VOCs) was performed by headspace solid-phase microextraction (HS-SPME). The fibre conditioning, microextraction regime, and the configuration of the GC-MS analysis was set up as previously reported Carlin et al. (2016) and reported previously in the *Chapter 2*. The semi-quantitative analysis was carried out where the final concentration of detected compounds was expressed as µg/L of the internal standard 2-octanol, considering a response factor equal to 1.

2.5 LIPID COMPOUNDS EXTRACTION AND ANALYSIS

Extraction of lipids from the homogenized grapes followed the protocol of Della Corte et al. (2015) with minor modifications. A 1.5 mL of CH_3OH was added to a previously weighted samples (0.5 ± 0.005 g) and vortexed for 30 s. Additionally, 3 mL of $CHCl_3$ with butylated hydroxytoluene (BHT 50 mg/L) were added and spiked with 10 µl of octadecanoic acid-d3 as an internal standard (100 µg/mL). After one-hour extraction in an orbital shaker, 1.25 mL of water was unified the samples and centrifuged at 3600 rpm for 10 min. The lower chloroform phase was collected into the new 20-mL glass vial, while 2 mL of $CHCl_3$/CH_3OH/Milli-Q H_2O was joined to the remaining of extraction mixture and centrifuged again (3600 rpm, 10 min). Again, the chloroform infranatant was removed and combined with the previous one. The solvent was evaporated under the stream of N_2 to dryness and the samples were reconstituted in 300 µL of ACN/IPA/H2O (65:30:5 v/v/v) containing the IS cholesterol-d7 (1 µg/mL). To remove any solid particles of plant material that may be present after drying, the reconstituted samples were once again centrifuged (5 min) and the supernatant was finally transferred to a glass insert, adaptive for 2-mL amber HPLC vials.

The UHPLC separation (UHPLC Dionex 3000, Thermo Fisher Scientific Germany) and the conditions of triple-quadrupole mass spectrometer (API 5500, Applied Biosystems/MDS Sciex, Toronto, Canada) were adopted by Della Corte et al. (2015) and are described in the Chapter 2. Quantification was carried out by constructing the calibration curves as reported by Lukić et al. (2019) and data were expressed as mg/kg after normalization on the basis of the internal standard octadecanoic acid-d3.

2.6 AROMATIC AMINO ACID METABOLITES EXTRACTION AND ANALYSIS

Into 15-mL amber vials, 1 g of each sample was weighed and 50 µL of the freshly prepared internal standard (10 mg 3-nitrotyrosine in 10 mL of CH_3OH/H_2O 1:1 v/v) was added. Afterwards, 4 mL of 80% CH_3OH was united to the grape powder, and the extraction

mixture was vortexed for 1 min at room temperature (RT). Samples were placed on an orbital shaker (Grant Bio Rotator PTR-60) for 15 min at RT. Subsequently, the samples were centrifuged at 0 °C and 4000 g for 10 min, followed by transferring the supernatant into a 10-mL flask. The second extraction was performed by adding another 4 mL of 80% CH_3OH. followed by homogenization on vortex mixer for 1 min and 15-min extraction on an orbital shaker. After the centrifugation step (0 °C, 4000 g, 10 min) both fractions were unified in 10-mL flask and the remaining volume was supplemented with Milli-Q water. The final solution was filtered with the 0.2 µm PTFE filters into a 2 mL amber vial (MS certified).

The UHPLC-MS/MS analysis was performed on a Waters Acquity UPLC system (Milford, MA, USA) coupled with Waters Xevo TQMS (Milford, MA, USA). The separation, detection and quantitation of the aromatic amino acid metabolites were performed according to the Arapitsas et al. (2018) and are reported in the *Chapter 2*.

2.7 STATISTICAL ANALYSIS

For inspection of the grape ripening trend of the grapes in terms of lipids, volatile compounds and tryptophan metabolites, the analytical results were subjected to the Pearson correlation analysis. Additionally, one-way ANOVA was used to determine, whether there was difference between each sampling point, followed by Tukey's post-hoc pairwise comparison (95%). Similarly, one-way ANOVA was performed for analyzing the effects of cluster thinning on the grapes in two different vineyard sites, where the p value was set at 0.05. In order to have a general overview of all the samples, a principal component analysis (PCA) was carried out. The one-way ANOVA and the PCA analysis were performed in R (R Core Team, 2020).

3 RESULTS

3.1 EFFECT OF GRAPE RIPENING ON CONVENTIONAL PARAMETERS OF GRAPES

The ripening trend was monitored in the seasons 2018 and 2019, and the evolution of total soluble solids (TSS), titratable acidity (TA) and pH value is presented in Figure 1A–C. It can be immediately observed, that in 2018 winegrowing season, the sugar accumulation curve reached the plateau at the penultimate sampling point, whereas in 2019 the slow-down of sugar accumulation occurred in the third penultimate point (Fig. 1A). The sudden decrease in the sugar level during the ripening of grapes in the 2018 season is mainly due to the amount of precipitation. Namely, in the first 12 days of August, the total amount of precipitation was 13.9 mm, followed by 36.4 mm of rain the next two days. Similar fluctuations in the concentration of TA and pH can be noticed for the same growing season.

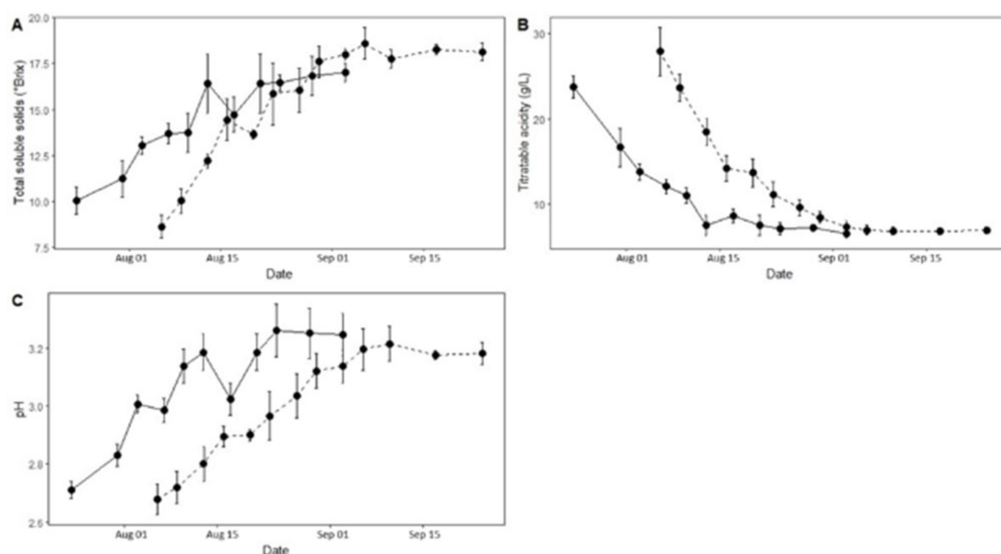

Figure 1: Maturity indices (total soluble solids, A; titratable acidity, B; pH value, C) measured throughout the ripening stage on Ribolla Gialla control grapes from 2018 (solid line) and 2019 (dashed line) season in FCP vineyard site. Each point represents mean value of five different replicates for each sampling point.

3.2 FFECT OF GRAPE RIPENING ON THE VOLATILE COMPOSITION

In total, forty-six compounds were analyzed in the grapes (Appendix F) without differences between samples of whole grape berries and samples, where the seeds have been removed. Analyzed compounds were further divided into nine groups: monoterpenes, norisoprenoids, aldehydes, alcohols, esters, acids, ketones, furans, and phenols.

When considered only the samples with seeds (Figure 2), the total concentration of VOCs for each group of substances, predominated in the 2018 winegrowing season. In addition, each of the chemical groups was characterized by positive correlation, depending on the ripening of the grapes in 2018, while in 2019 this was in line only for monoterpenes, aldehydes, and acids. Among the monoterpenes and norisoprenoids, only limonene and β-damascenone were analyzed in the samples of grapes, respectively. While for β-damascenone the significant correlation was positive in both years (2018, r = 0.81; 2019, r = 0.54), the similar trend for the terpene was evident only in the second year of grape ripening. Aldehydes resulted to be the most numerous groups of compounds analyzed by the HS-SPME-GC-MS method. In both years, the correlation of the whole aldehydes group appeared to be positive (2018, r = 0.4; 2019, r = 0.7), with a higher concentration in 2018. However, not all compounds belonging to this group have followed this trend, where the amount of volatile compounds increased with grape ripening. Examples of compounds with a strong significant negative correlation ($p < 0.001$) in both years are *trans*-2-heptenal, pentanal, octanal, *trans*-4-oxohex-2-enal and *trans*-2-decenal. On the contrary, the significant positive correlation resulted for the compounds as 2-hexenal and *trans*-2-hexenal. The concentration of both compounds in 2019 exceed the amount present in 2018, and in addition, both compounds were the most quantitatively important in terms of aldehydes. The

correlation between alcohols and the sugar composition in the grapes with seeds appeared to be significant only in 2019 harvest season (r = –0.36, $p < 0.01$). The negative relationship was observed in the most abundant alcohol (1-hexanol), where the correlation was negative in both vintages (2018 versus 2019; r = –0.76 versus r = –0.35 with $p < 0.001$). Mixed correlation model between the two years was present in the group of esters, ketones, furans, and phenols, with the 2018 year of monitoring for volatile compound changes depending on the Brix level being negatively correlated. While *trans*-2-hexenoic acid resulted negatively correlated with sugar accumulation over the ripening period, the hexanoic acid and acetic acid reported a negative correlation in 2018, while the opposite was found in the 2019 harvest season. Interestingly, the majority of analyzed ketones in the whole grape samples showed a negative correlation, except 2,3-octanedione, which showed a significant positive correlation in 2019 (r = 0.49, $p < 0.001$).

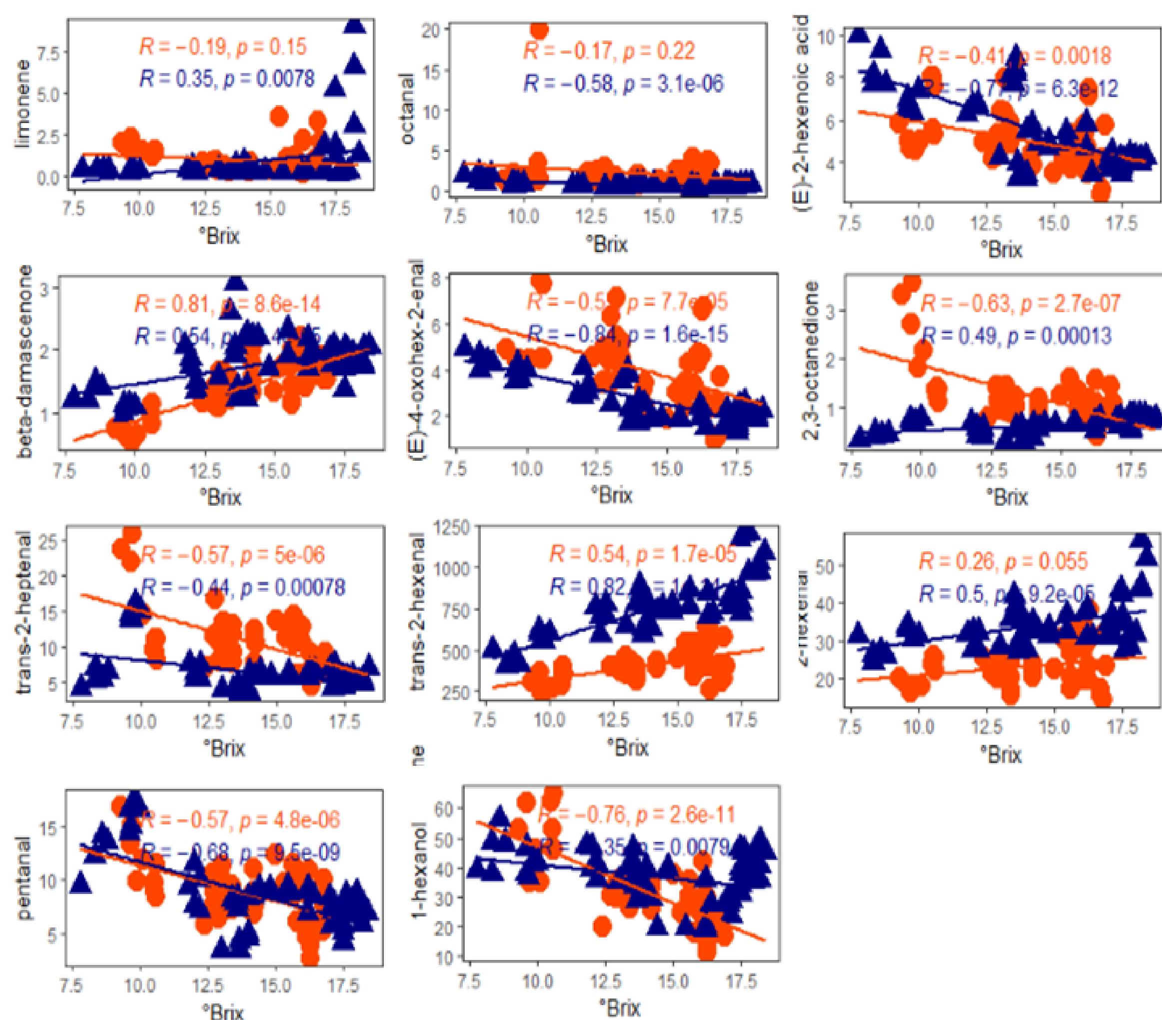

Figure 2: The scatter plot with the calculated Pearson coefficient of volatile molecules in control grape berries (with seeds) in 2018 (red) and 2019 (blue) harvest season, from FCO vineyard site.

In Figure 3, the results of correlation analysis are presented for the grapes without seeds. The behavior of monoterpenes and norisoprenoids substances was different as compared to the whole berry samples, as the trend of accumulation of monoterpenes was negatively correlated in 2019 (r = –0.44, $p < 0.001$) while in the 2019 the correlation resulted to be positive (r = 0.42, $p < 0.001$), which was in line with the norisoprenoids. Similarly, the correlation for the group of aldehydes appeared to be positive as it was in the previous analysis; however, it can be observed that the concentration of total aldehydes was higher in the 2019 compared to the 2018 harvest season. Within the aldehyde group, the compounds with the highest concentration were again 2-hexenal and its *trans* isomer.

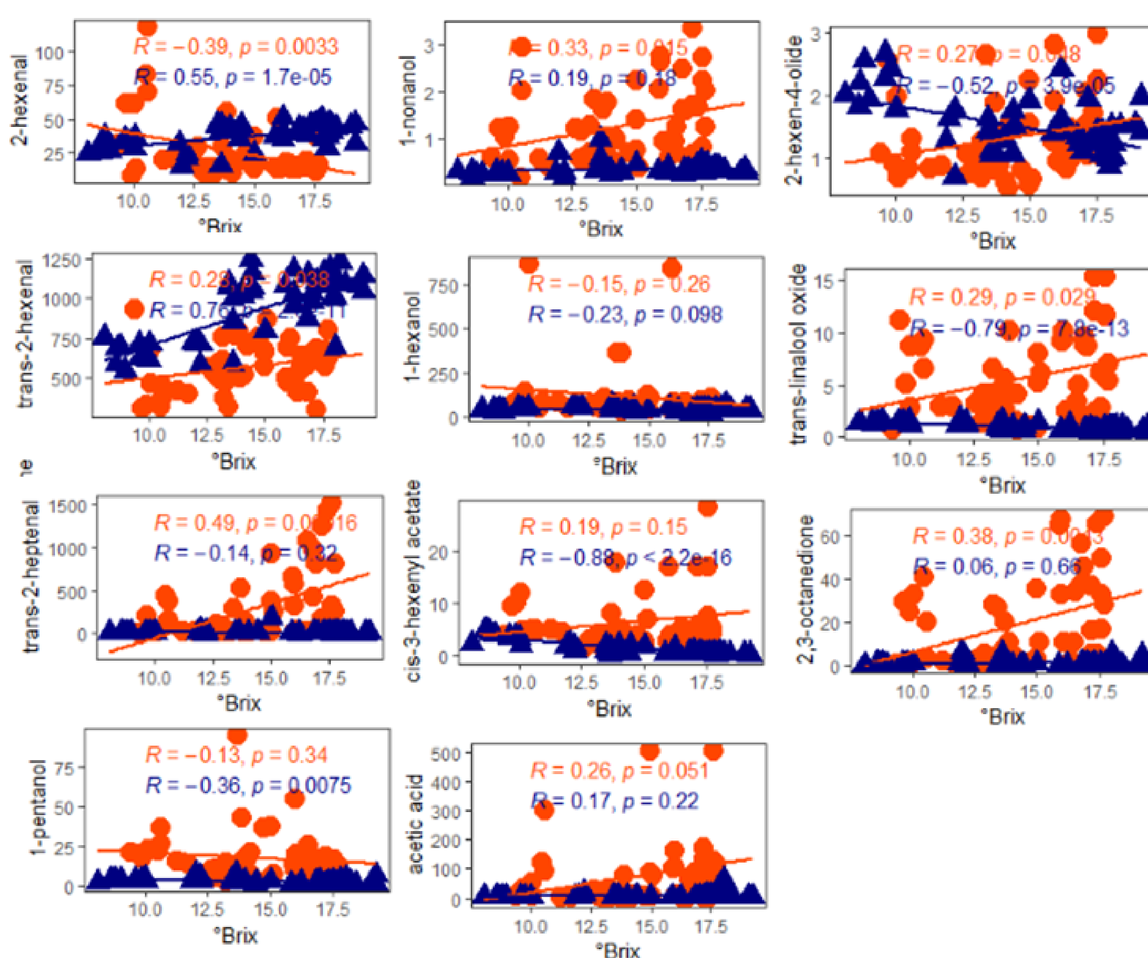

Figure 3: The scatter plot with the calculated Pearson coefficient of volatile molecules in control grape berries (with removed seeds) in 2018 (red) and 2019 (blue) harvest season, from FCO vineyard site.

trans-2-Hexenal was significantly positively correlated to Brix in both winegrowing seasons ($p < 0.001$), while 2-hexenal showed less uniformity (2018, r = –0.39; 2019, r = 0.55), similarly as *trans*-2-heptenal (2018, r = 0.49; 2019, r = –0.14). The samples without seeds also reported a negative correlation with alcohol concentration in the two consecutive years. Lower coherence rate between 2018 and 2019 also resulted in alcohol compounds, as

for most of them the correlation was found negative in the second year of grape ripening, and opposite in the first year, 2018. The pure model of increasing or decreasing alcohol concentration in both vintages was expressed only in samples 1-pentanol (2018 versus 2019; $r = -0.13$, $p = ns$ versus $r = -0.36$, $p < 0.05$), 1-hexanol (2018 versus 2019; $r = -0.15$, $p = ns$ versus $r = -0.23$, $p = ns$) and 1-nonanol (2018 versus 2019; $r = 0.33$, $p < 0.05$ versus $r = 0.19$, $p = ns$). Strong negative correlation resulted to be characterizing the group of esters, acids, ketones, and furans in both vintages. For acetate ester that has been analyzed in the grapes with removed seeds, a strong correlation was ascertained in 2018 and 2019 ($p < 0.001$), while the *cis*-3-hexenyl acetate resulted to have a significant negative correlation only in the 2019 ($r = -0.88$, $p < 0.001$). Subsequently, acetic acid was identified as the most important of the analyzed acids in terms of the amount detected. As regards the analysis of ketones in grape samples, it was evident that their concentration tended to decrease with a more advanced ripening stage. The total quantity of furans was consisted of 2-hexen-4-olide, *cis*- and *trans*-linalool oxide. The significant negative correlation ($p < 0.001$) was characteristic for all three substances in both years, with concentration prevailing in 2018.

3.3 EFFECT OF GRAPE RIPENING ON THE LIPID COMPOSITION

Since the grapes ripened at different rates between the two vintages, the Figure 4–5 presents the results of the correlation analysis for the single lipid compounds in whole berries and in seedless samples, respectively. In the whole berries, twenty-six compounds were successfully analyzed and quantified, while in samples where the seeds were previously removed from grape berries twenty-four lipid substances were characterized. All lipid compounds were subsequently divided into the following groups: sterols, unsaturated fatty acids (UFA), saturated fatty acids (SFA), glycerolipids, glycerophospholipids, prenols and fatty esters.

In general, it was noticed that the whole berry samples from 2019 harvest season contained higher concentration of lipids, with the exception of sterols, oleanolic acid and the saturated lignoceric fatty acid, that predominated in 2018. However, certain discrepancies were also observed within individual groups of lipids, in which maturation did not always proceed in the same direction. The sum of sterols presented negative correlation in both years, although the correlation was significant only in the year 2018 ($p < 0.05$). Furthermore, in both harvest seasons, uvaol, stigmasterol, campesterol, and brasicasterol showed a homogeneous negative correlation with increasing Brix concentration, while desmosterol appeared to be only compound, whose concentration increased with grape ripening. Among UFAs, the only significant compound in both harvest years was linoleic acid, and its concentration increased with the sugar level in the grapes (2018 versus 2019; $r = 0.38$, $r = 0.33$). In contrast, the concentration of SFAs exceeded the concentration of UFAs, whereby a concentration gap was observed between the two years. It was possible to note, that only arachidic and miristoleic acid were significantly positively associated with grape ripening in both harvest seasons, while the amount of lignoceric acid decreased significantly during

grape ripening in both years. As regards quantitatively important compounds (i.e., palmitic, and stearic acid), mixed models of increasing either decreasing were characteristic in both years. The group of glycerophospholipids resulted as one of the most homogeneous in term of comparison in both years, as the concentration appeared to be significantly positively correlated with the ripening of the grapes. That was in line with 1,2-dioleoyl-sn-glycero-3-phosphocholine (2018 versus 2019; r = 0.79, r = 0.46) and 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (2018 versus 2019; r = 0.68, r = 0.55). Both fatty esters (ethyl linoleate and ethyl stearate) analyzed resulted to be positively correlated in 2018 while the concentration of both compounds was shown to decrease in 2019, although this trend was not significant.

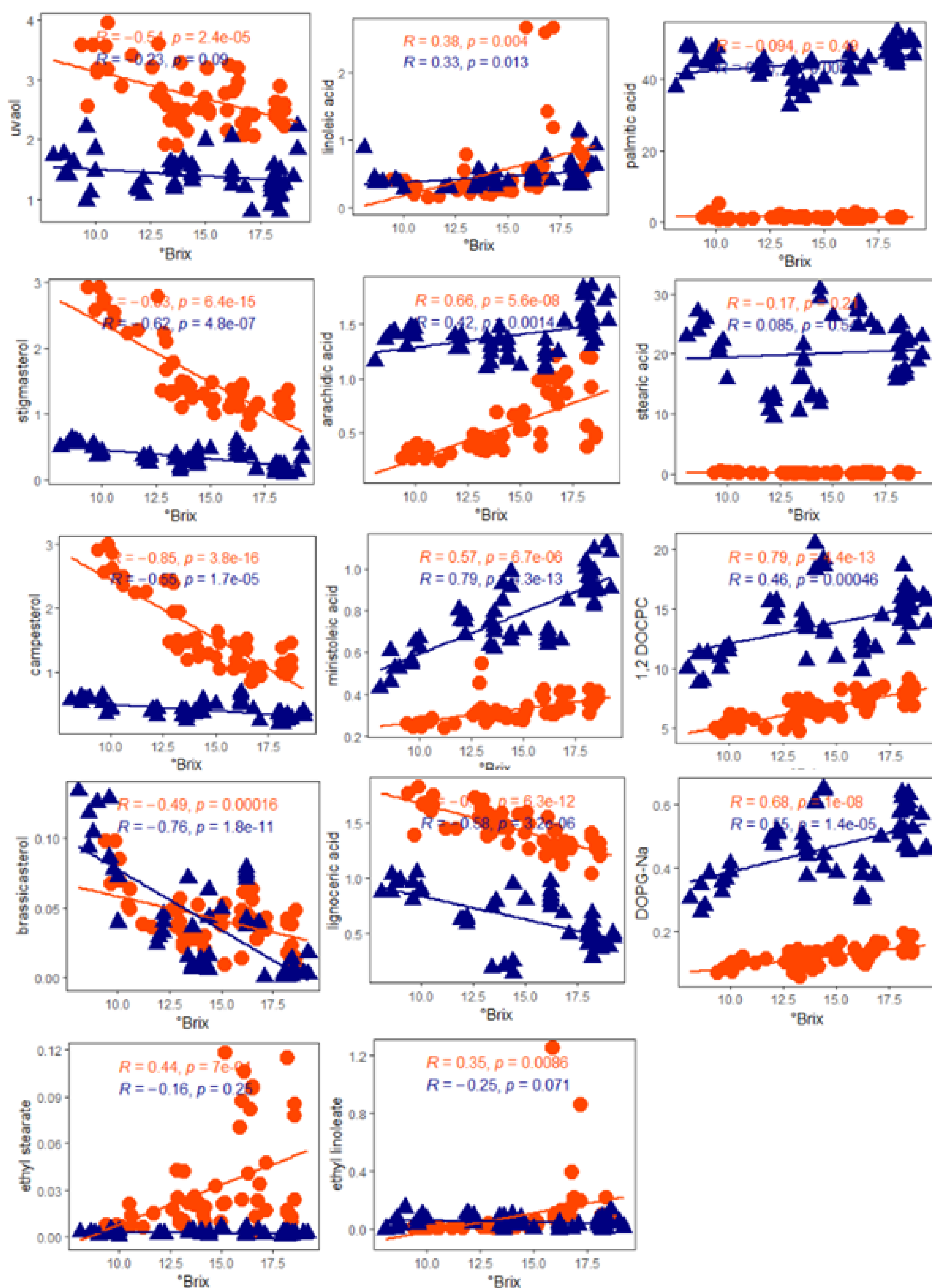

Figure 4: The scatter plot with the calculated Pearson coefficient of lipid molecules in control grape berries (with seeds) in 2018 (red) and 2019 (blue) harvest season, from FCO vineyard site.

Conversely, in the samples where the seeds were removed prior the extraction procedure (Figure 5), the sterols appeared to be the only group of compounds, where the concentration was more abundant in the 2019 over 2018 growing season. This was most probably related to the concentration of uvaol, the most abundant sterol. However, the concentration of uvaol in 2018 harvest season resulted to be decreasing during grape maturation (r = -0.64; $p < 0.001$), while for the samples from the following year was positively correlated to the increase of Brix in the grapes (r = 0.32; $p < 0.05$). Moreover, all sterols from 2018 samples showed a strong negative correlation ($p < 0.001$), while only desmosterol (r = 0.41; $p < 0.01$) proved to be significant in 2019 in addition to uvaol. Compared to grapes with seeds where more compounds from the group of UFAs appeared to be characterized with significant correlation, this only applied to linoleic acid (r = 0.35; $p < 0.01$). Among the SFAs, a significant positive correlation was observed in case of gondoic acid (2018 versus 2019, r = 0.76, $p < 0.001$ versus r = 0.29, $p < 0.05$), while lignoceric acid was negatively correlated in both harvest seasons (2018 versus 2019, r = –0.73, $p < 0.001$ versus r = –0.32, $p < 0.05$). Negative correlation was characterized also for behenic acid and margaric acid, while arachidic and miristoleic acid were positively correlated; however, the correlation for those SFAs was significant only in 2018. 1-oleoyl-rac-glycerol was the only glycerolipid, being characterized by a significantly positive correlation in both harvest seasons. Strong positive correlation was observed also for the remained two glycerolipids. As for the grapes with seeds, glycerophospholipids again showed a positive correlation compared to the level of Brix in 2018 (r = 0.75, $p < 0.001$), as well as in 2019 (r = 0.28, $p < 0.05$). Finally, the only compound that proved to express a significant correlation was oleanolic acid from 2018 season.

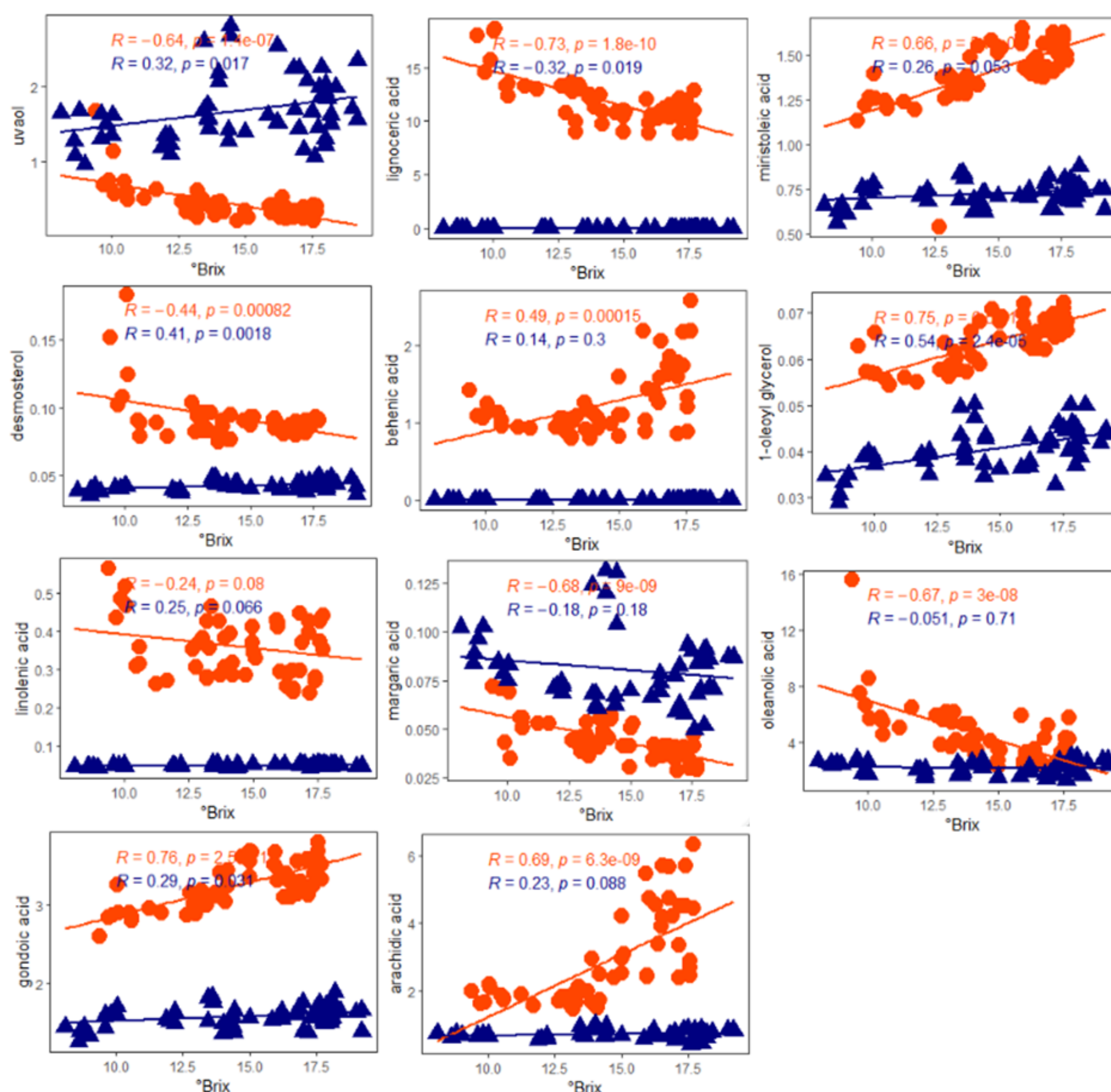

Figure 5: The scatter plot with the calculated Pearson coefficient of lipid molecules in control grape berries (with removed seeds) in 2018 (red) and 2019 (blue) harvest season, from FCO vineyard site.

3.4 EFFECT OF GRAPE RIPENING ON THE AROMATIC AMINO ACID METABOLITES

The results of the correlation analysis in the grape samples with seeds is represented in Figure 6. As regard tyrosine, we can observe a positive correlation in both harvest seasons, but significant only in 2018 ($p < 0.001$). As regards the concentrations, the tyrosine level in 2018 steadily grew from the beginning of monitoring, while in 2019 this increase was less evident. A similar observation was verified for phenylalanine, where the 2018 growing season was characterized with r = 0.5 ($p < 0.001$), while a medium strength of association (r = 0.49, $p < 0.001$) characterized the 2019. The concentration of tryptophan was higher in 2019. However, for the same year, a small and non-significant correlation was observed. In case of the glucoside of indole lactic acid (ILA-GLU), a significant large level of association

was noted in both harvest seasons. Additionally, the concentration of ILA-GLU in 2019 increased very steadily during the course of grape ripening, with a minimal final increment near harvest. Finally, abscisic acid (ABA) also shown a very small change in concentration in 2019, despite a constant negative correlation in both harvest seasons (2018 versus 2019; r = –0.7, $p < 0.001$ versus r = –0.62, $p < 0.001$).

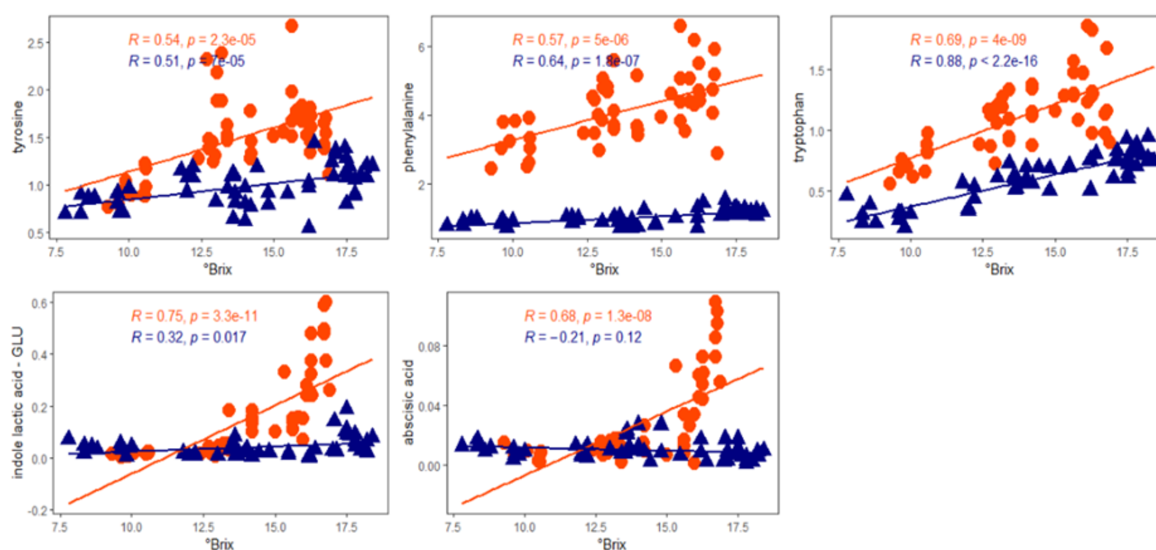

Figure 6: The scatter plot with the calculated Pearson coefficient of aromatic amino acid metabolites in the samples of the control grape berries with extracted grape seeds in 2018 (red) and 2019 (blue) harvest season, from FCO vineyard site.

Figure 7 on the contrary, reports the scatter plot with Pearson correlation analysis in the grape berries, where the seeds were previously extracted out of the grape berry. The results were in accordance with the previous analysis to a certain extent. Namely, the positive correlation between increasing level of Brix and the amount of essential amino acids was noted. In Tyr, Phe and TRP that meant a positive correlation, where the coefficient r was above 0.5 in 2018 and in 2019 harvest season. Despite the fact that the difference in concentration between 2018 and 2019 was slightly larger in the case of phenylalanine, all three amino acids reported a significant correlation with Brix ($p < 0.001$). The scatter plots of ILA-GLU and ABA turned out to be similar in the samples of grapes without seeds. In 2019, the positive correlation characterized the ILA-GLU (r = 0.32, $p < 0.05$), while the correlation for the same harvest season for ABA resulted to be negative and non-significant (r = –0.21).

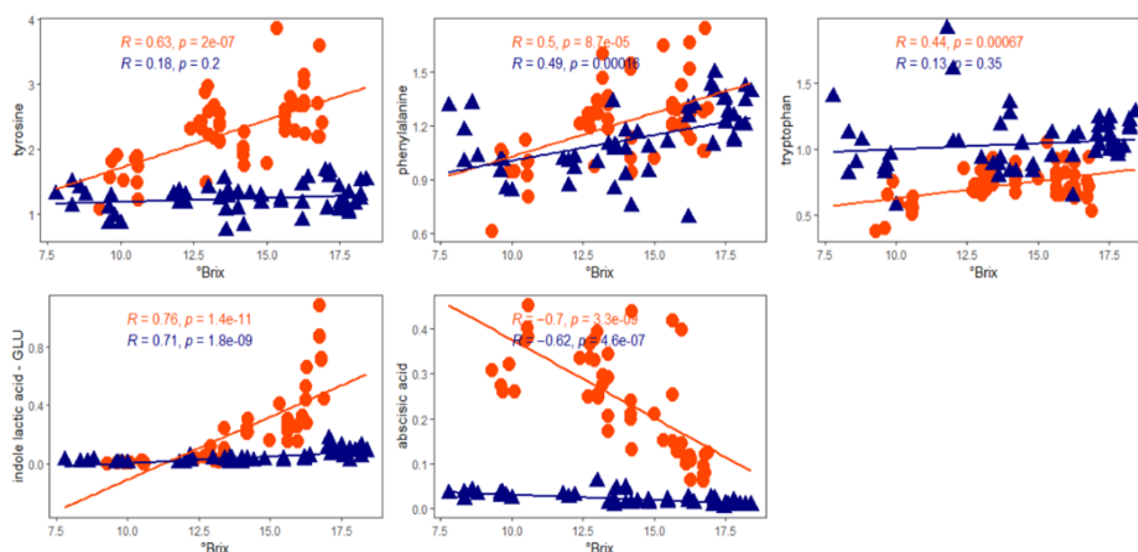

Figure 7: The scatter plot with the calculated Pearson coefficient of aromatic amino acid metabolites in control grape berries (with seeds) in 2018 (red) and 2019 (blue) harvest season, from FCO vineyard site.

3.5 EFFECT OF CLUSTER THINNING ON THE VOLATILE COMPOSITION OF GRAPES

Very few significant changes were observed when considering the cluster thinning treatment in the samples of the whole grape berries from FG vineyard site in 2018 harvest season (Figure 8). Alcohols 1-hexanol, *cis*-2-hexenol, and aldehydes hexanal, *trans*-2-hexanal and 2,4-hexadienal reported the highest concentration. For explicit substances (e.g., β-cyclocitral), it has been observed that the concentration in CT samples increased significantly, compared to the UNT samples in the second point of ripening, following by the drop in concentration.

Statistical analysis of the data in 2019, revealed more significant compounds, compared to the first year of the study in the FG vineyard site (Figure 9). However, this did not necessarily mean clearer differences between UNT and CT samples. Similarly to 2018, where several molecules reported a statistically higher content of CT in point 2, in 2019 this trend expanded also to the penultimate, sixth point of ripening of the grapes. This, in turn, led to a final higher content of mainly alcohol group of compounds, namely in CT samples. Among all the analyzed compounds, it was again confirmed that the C6 related compounds (aldehydes and alcohols) predominated in the CT and UNT samples. However, the concentration of the most abundant compound, *trans*-2-hexenal doubled, compared to the previous year.

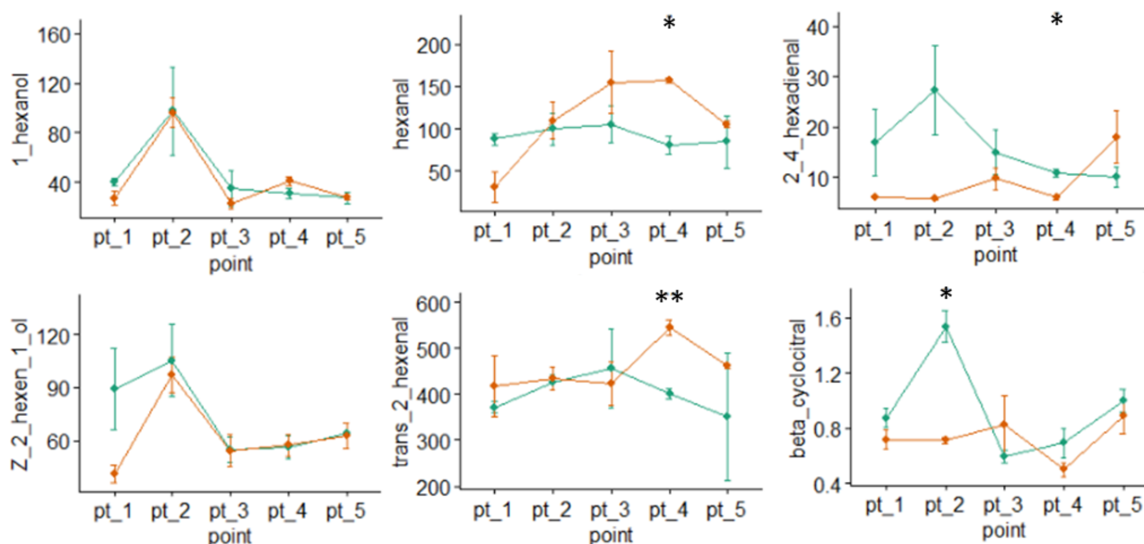

Figure 8: Evolution of volatile compounds in untreated (red) and 20% thinned (green) grape samples (with seeds) from FG in 2018 harvest season.

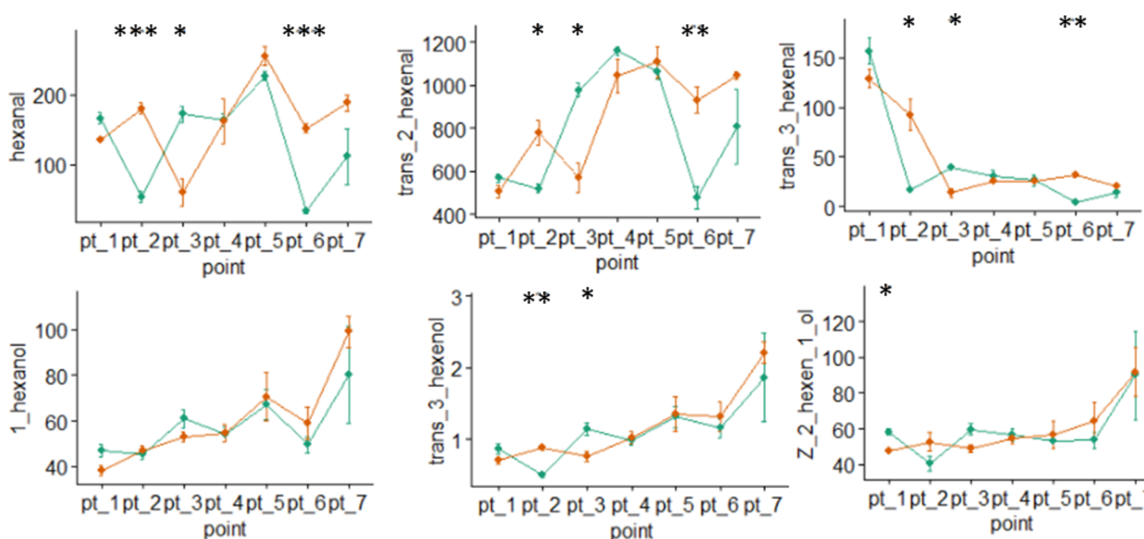

Figure 9: Evolution of volatile compounds in untreated (red) and 20% thinned (green) samples of grapes with seeds from FG in 2019 harvest season.

As regards the evolution of VOCs in the 2018 from FCO vineyard site (Figure 10), it appeared that the yield reduction through cluster thinning affected several compounds. The CT treatment therefore increased the concentration of pentanal, acetaldehyde and ethyl acetate. However, it is interestingly to notice, that this year was characterized with a higher content of compounds present in the UNT samples. Statistically significant changes were most obvious when comparing the C6 compounds (*trans*-2-hexenol, *trans*- and *cis*-3-

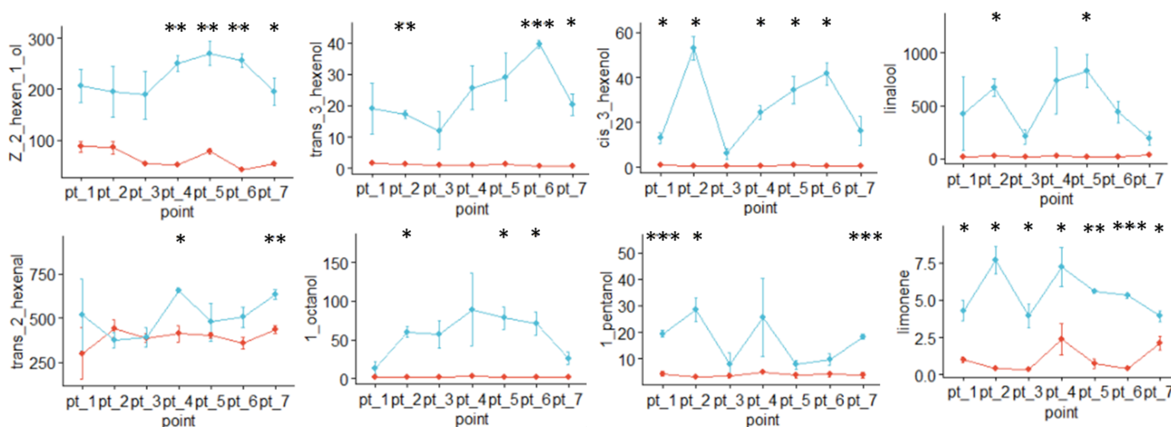

Figure 10: Evolution of volatile compounds in untreated (blue) and 20% thinned (red) grape samples (with seeds) from FCO in 2018 harvest season.

hexenol, *trans*-2-hexanal), but also in cases of some other primary alcohols (1-pentanol and 1-octanol) and even in the group of monoterpenes (limonene and linalool). The accumulation of β-damascenone resulted to be constant until the fifth point in the CT samples, followed by the sudden decrease in concentration.

As regards the following harvest, the FCO grape samples revealed fewer significant differences when comparing UNT to the CT samples (Figure 11). The content of *trans*-2-hexanal in the CT samples was higher than UNT throughout the maturation monitoring, but the concentration coincided in the last point with control grapes. The results generally showed a decrease during the ripening, even in the case of 1-hexanol, where initially the concentration of CT samples appeared to be statistically higher, which later decreased over time and finally and eventually equated with the UNT.

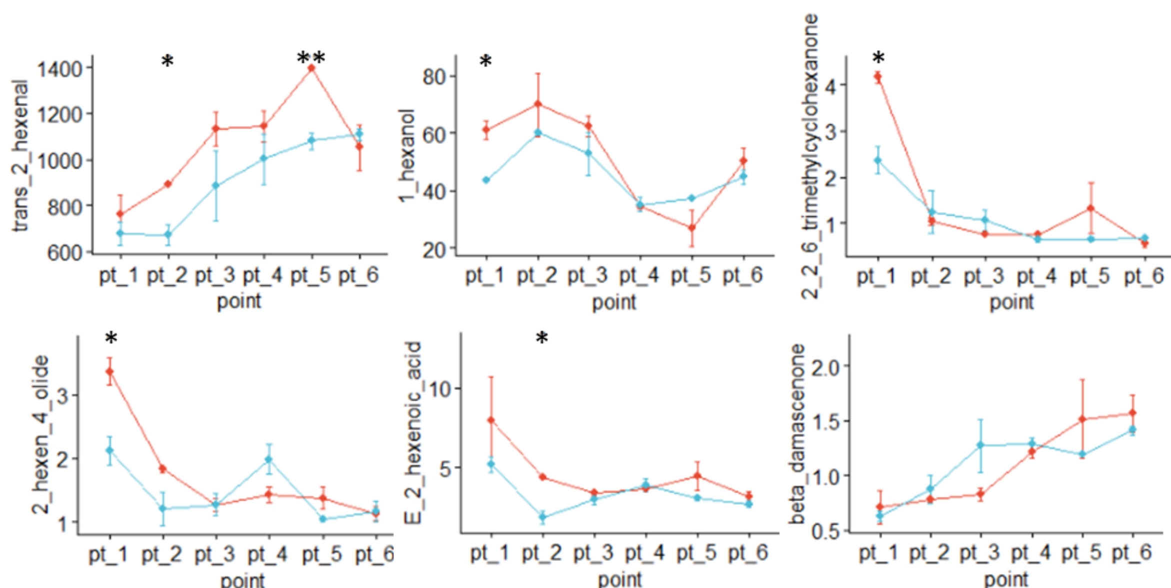

Figure 11: Evolution of volatile compounds in untreated (blue) and 20% thinned (red) grape samples (with seeds) from FCO in 2019 harvest season.

Similar observation was made for 2,2,6-trimethylcyclohexanone, 2-hexen-4-olide and *trans*-2-hexenoic acid. Accumulating trend of β-damascenone resulted to be similar to the previous harvest season, while the concentration of linalool appeared to be higher in the UNT samples in the last point of maturation.

The samples without seeds from FG presented on the Figure 12 showed similar results as samples of while grapes in 2018. UNT berries were mostly characterized by a higher concentration compared to the CT grapes. Nevertheless, there were few statistically significant differences during the maturation course that could confirm this. According to what already shown in case of whole berry samples, most of the differences of *trans*-2-hexenal, linalool, furfural, acetaldehyde *cis*-3-hexenal and *cis*-3-hexyl acetate between UNT and CT samples, were found at the second sampling point. Interestingly, both *cis*- and *trans*-linalool oxide showed strong statistical significance in the penultimate point of the grape maturation, and in both cases, the concentration resulted to be higher in the CT samples.

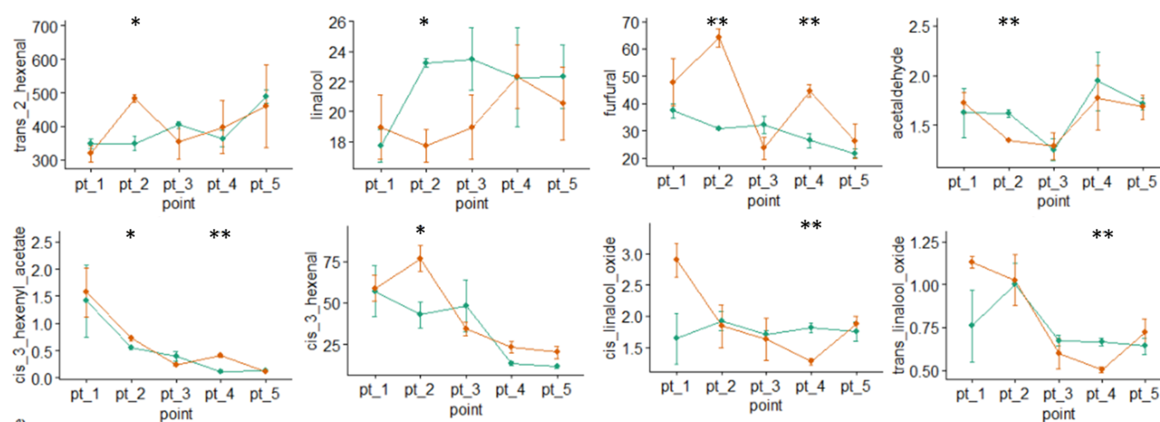

Figure 12: Evolution of volatile compounds in untreated (red) and 20% thinned (green) grape samples (with removed seeds) from FG in 2018 harvest season.

Due to the inconsistent results, the cluster thinning treatment showed poor effects also in the 2019 winegrowing season (Figure 13). Some compounds, such as furfural and heptanal reported a significantly higher concentration in the last point of sampling in UNT samples, while opposite situation was true for 1,5-octadien-3-ol, *trans*-2-nonenal, and 2,3-octanedione. Quantitatively important molecules (i.e., *trans*-2-hexenal) showed no discrepancies between CT and UNT samples and, in addition, the concentration of both samples was shown to accumulate through the grape ripening. The trend in increasing concentration was also observed in β-damascenone. Conversely, a downward trend in concentration was seen for substances as *cis*-3-hexenol, 2-ethyl-1-hexenol, and to some extent also linalool.

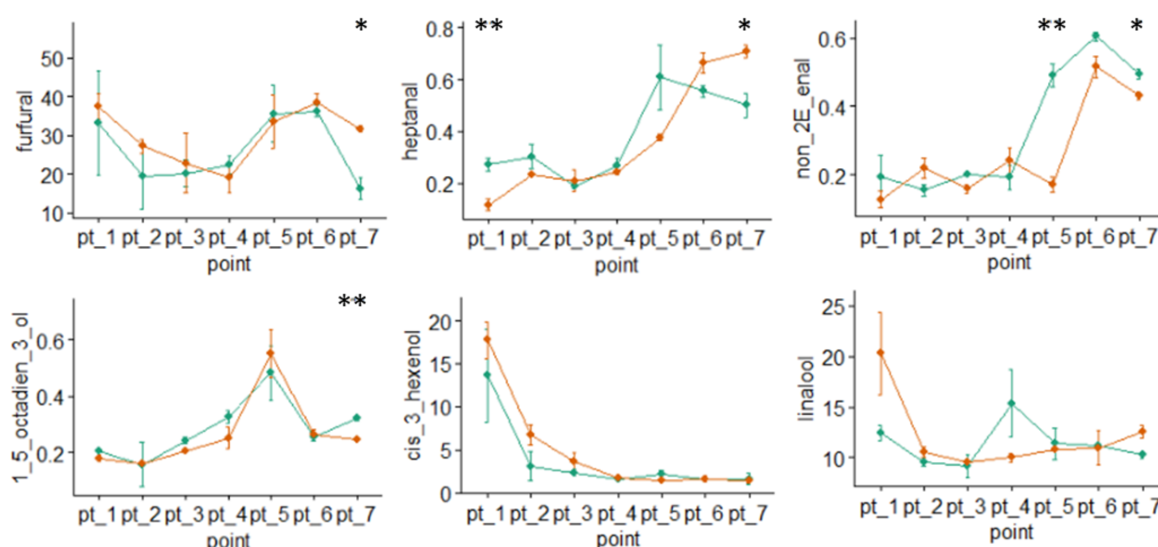

Figure 13: Evolution of volatile compounds in untreated (red) and 20% thinned (green) berry samples (with removed seeds) from FG in 2019 harvest season.

As compared with the results where seeds were not removed from the grapes in the same year, the distinction between CT and UNT samples in the FCO vineyard was less evident, due to similar concentration in most of the analyzed compounds (Figure 14). Ethyl acetate showed strong statistical significance in CT samples in the last point grape sampling, while an opposite effect was ascertained in case of hexanoic acid. A unique floral character is a consequence of monoterpene contribution to grape and wine; therefore it is interesting to observe, that the amount of linalool initially predominated in untreated samples.

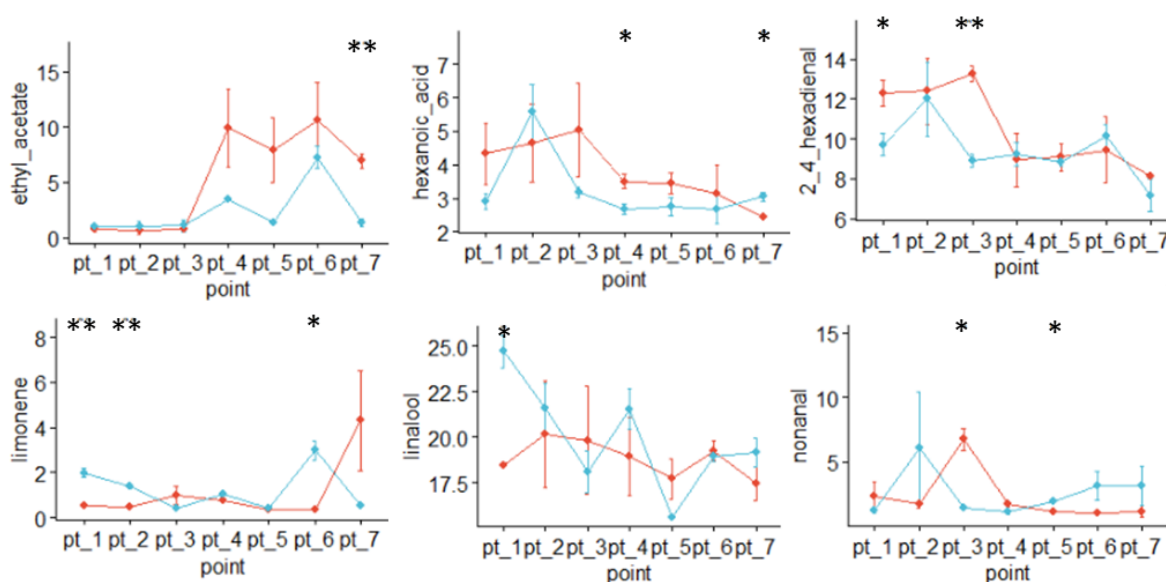

Figure 14: Evolution of volatile compounds in untreated (blue) and 20% thinned (red) berry samples (with removed seeds) from FCO in 2018 harvest season.

Similar observation was made for the limonene, but compared to the linalool, its amount started to increase over the time, which eventually led to a predominance of concentration in CT samples.

The results from the 2019 season showed more statistically significant differences among UNT and CT samples of grape berries where seeds were removed (Figure 15). Significant changes characterized predominately the group alcohols (1-octen-3-ol, 1,5-octadien-3-ol, 2-ethyl-1-hexanol, 1-pentanol, 1-hexanol and 1-nonanol), with higher concentrations in case of CT samples. Similar behavior was noted in the group of certain aldehydes (hexanal, pentanal, trans-3-hexanal, 2-hexanal and *trans*-2-nonanal); however, the concentration of such compounds in CT in the last sampling time was rarely higher than in the UNT. This applied also to both monoterpenes analyzed, limonene and linalool.

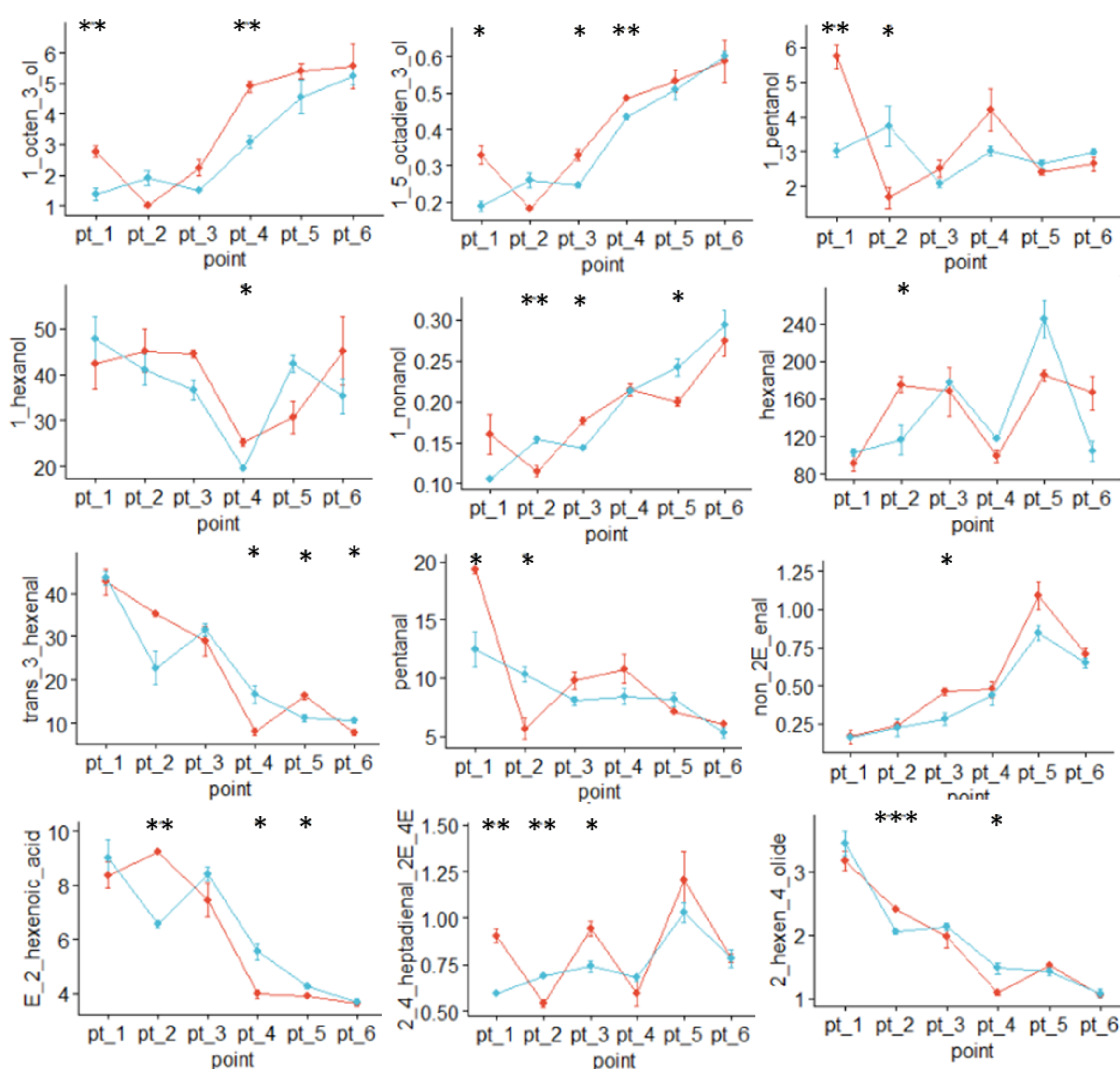

Figure 15: Evolution of volatile compounds in untreated (blue) and 20% thinned (red) berry samples (with removed seeds) from FCO in 2019 harvest season.

Finally, the concentration of *trans*-2-hexanoic acid was significantly higher in CT only in the second point of sampling, followed by a gradual decrease.

3.6 EFFECT OF CLUSTER THINNING ON THE LIPID COMPOSITION OF GRAPES

The evolution of lipid compounds in grapes with seeds is represented in the Figure 16. As regards cluster thinning, it has been generally observed that the treatment in FG vineyard site in both studied years did not provide much significant changes as compared to the control samples, which ultimately reflected in a very similar lipid accumulation, during the last points of grape ripening. Main differences appeared in the year of 2019, where the final maturation point resulted in a concentration drop in thinned samples. This was mainly the case for majority of saturated fatty acids, such as palmitic, stearic, miristoleic and myristic acid. On contrary, several statistically significant differences during the maturation were shown in the samples from the FCO vineyard site, especially in the 2018 winegrowing season. Namely, the results have showed that the cluster thinning treatment affected positively glycerophospholipids and most sterols, with the exception of β-sitosterol, stigmasterol and campesterol, the concentration of which was higher in the control samples. During the ripening of the grapes and after that, changes in the lipid content likewise occurred for miristoleic and myristic acid, both of which predominated in the CT samples. Stearic and palmitic acids resulted the most important saturated fatty acid, while the amount of C16 long-chain saturated palmitic acid resulted to be comparable between treatments in the last point of the maturation, and the C18 stearic acid appeared to have significantly higher concentration in 2019. Due to the high variability of some compounds, it was therefore difficult to identify any upward or downward trend in the concentration, as it turned out towards the end of maturation. One of the most noticeable differences therefore emerged in the 2019 in FG vineyard site where the amount of SFAs (arachidic, stearic, lignoceric and margaric acid) increased towards the last sampling point, together with campesterol.

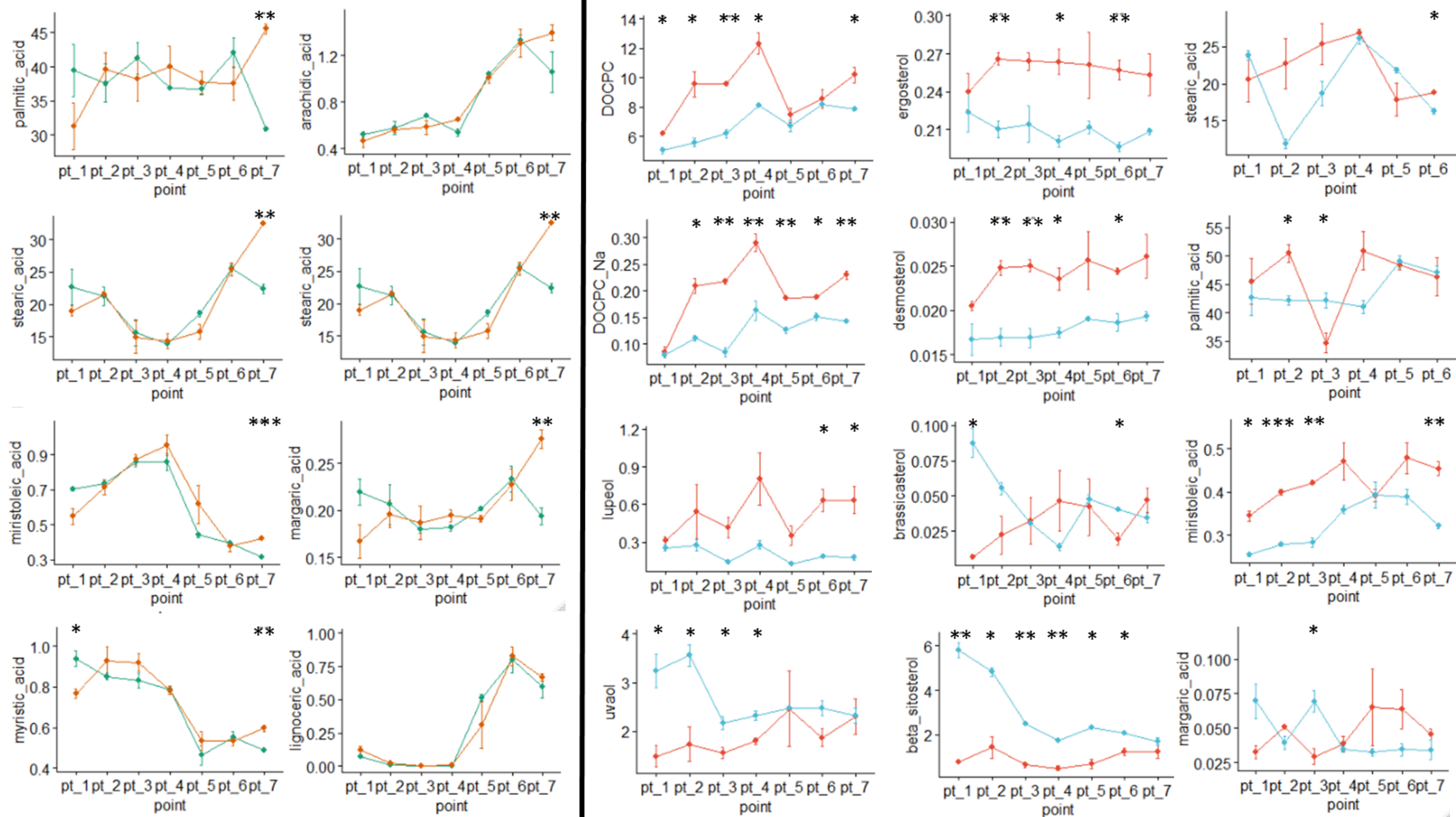

Figure 16*:* Evolution of lipid molecules in untreated (red) and 20% thinned (green) grape samples (with seeds) from FG (left) and FCO (right) in 2019 harvest season.

As regards the grape samples with extracted seeds (Figure 17), similar fluctuations in the concentration of lipid molecules were observed, as in the case of the whole grape berries. In addition, the second most obvious general observation was also that samples with removed seeds were characterized by a lower content of lipid substances, compared to the whole-berry samples. However, the extraction of the seeds positively affected the concentration of analyzed phospholipids, in particular 1,2-dioleoyl-sn-glycero-3-phosphocholine, as these samples were shown to achieve higher values, especially in 2018. In the same year, the samples from the FG vineyard reported also significant differences in the first point of ripening as regard 1-monopalmitoleoyl-rac-glycerol, oleanolic acid, myristic acid and campesterol, followed by rapid decrease in the concentration of the listed compounds. Again, the most significant differences were found in samples from FCO, where the concentration of SFAs in thinned samples predominated and increased additionally towards the end of the maturation. These differences were most typical for 2018, while control and treated samples from the following year appeared to be more uniform, leading to a smaller number of statistically significant differences between UNT and CT samples. Interestingly, the concentration of stigmasterol, campesterol and β-sitosterol resulted to be much higher in UNT samples in the first points of maturation, followed by a drastic reduction and final equalization of quantity as in CT samples. The same was true for oleanolic acid. In contrast, CT samples had higher concentration of both detected phospholipids from the first point of monitoring grape ripening. As mentioned before, cluster thinning treatment had much less impact in 2019 winegrowing season; however, 1-monopalmitoleoyl-rac-glycerol, along with margaric and behenic acid was shown to predominate in UNT samples at the end point of maturation.

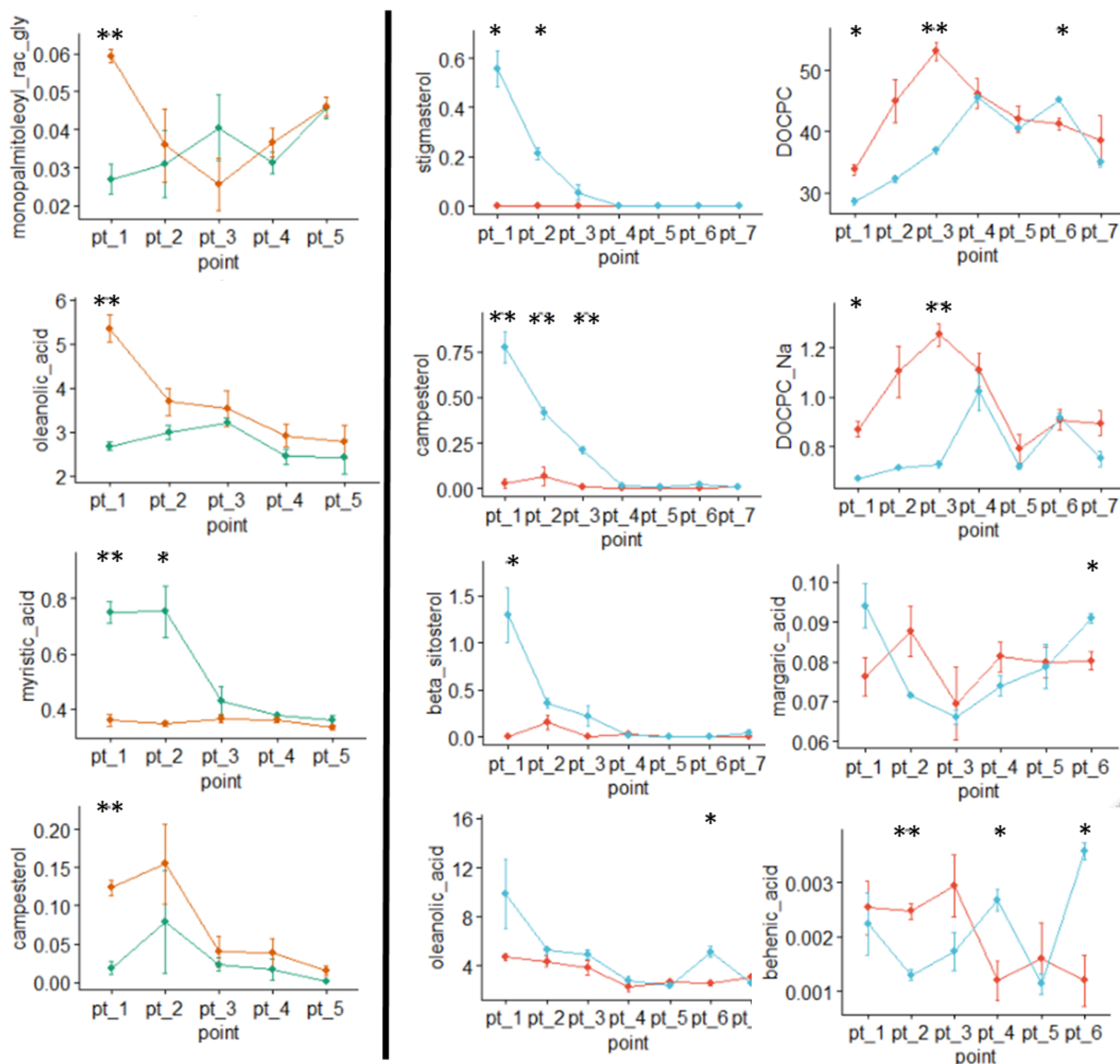

Figure 17: Evolution of lipid molecules in untreated (red) and 20% thinned (green) grape samples (with removed seeds) from FG (left) and FCO (right) in 2019 harvest season.

3.7 EFFECT OF CLUSTER THINNING ON THE AROMATIC AMINO ACID METABOLITES OF GRAPES

Figure 18 represents the results of the aromatic amino acids from both harvest seasons in the plain vineyard site of FG. It has been observed that the results of AAA metabolites appeared more inconsistent in the second harvest season, therefore there are also less significant changes, compared to 2018. The latter season was generally characterized by an increase in the concentration in tyrosine, phenylalanine, and glucoside of indole lactic acid, while the content of abscisic acid declined constantly from the first sampling point onwards; therefore, the difference in the first four points appeared to be statistically significant, with a predominant concentration in case of the CT samples. As for ILA-GLU, it was possible to observe at first a moderate accumulation in CT and UNT samples, followed by abrupt increase in concentration.

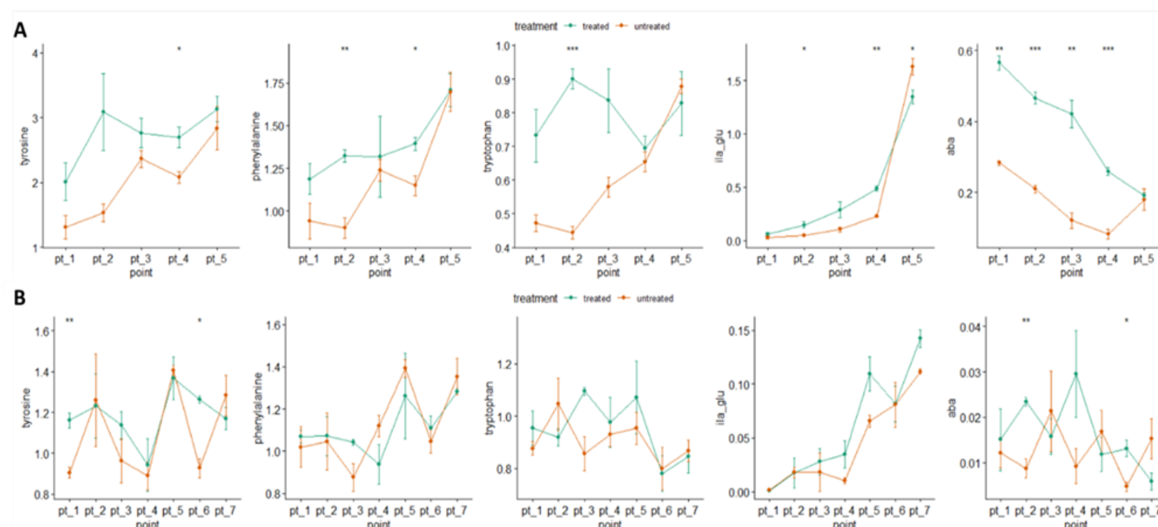

Figure18: Evolution of aromatic amino acids compounds in untreated (red) and 20% thinned (green) berry samples (with seeds) from FG in 2018 (A) and 2019 (B) seasons.

The grape samples from FCO vineyard site (Figure 19) revealed similar results as in FG vineyard. This means that in 2019, inconsistencies were observed in terms of the analyzed aromatic amino acids (tyrosine, phenylalanine, and tryptophan). The first season thus reported an increased concentration of all three amino acids in the CT samples. The second and third sampling point were critical, as the concentration of TYR, PHE and TRP began to decline, but they still managed to maintain a higher concentration compared to the control samples. The yield reduction obtained with cluster thinning had the most marked effect on ILA-GLU and ABA levels in the 2018 samples. For both substances, the concentration was higher in treated samples and, in case of ILA-GLU, was increasing over the time, while ABA decreased.

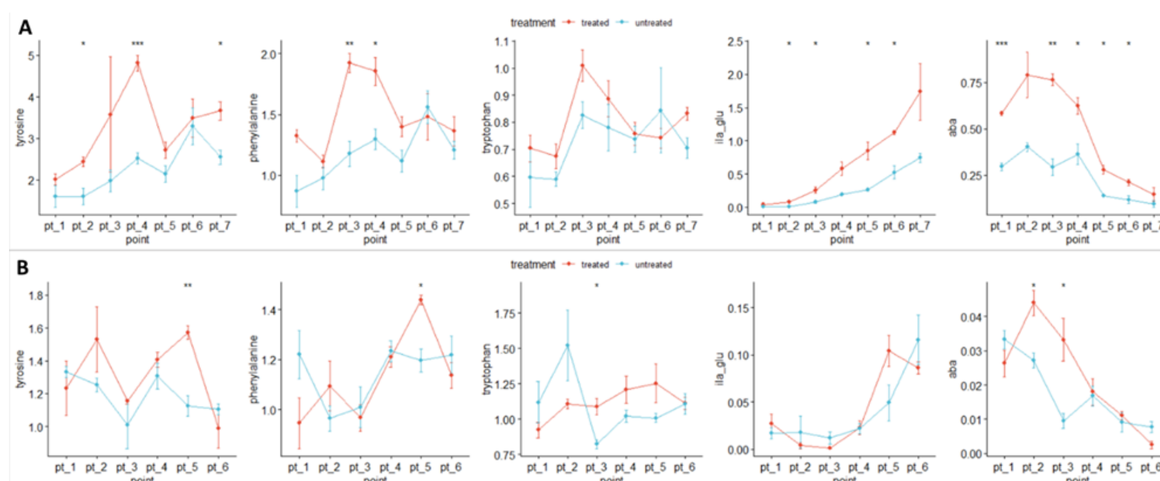

Figure 19: Evolution of aromatic amino acids compounds in untreated (blue) and 20% thinned (red) berry samples (with seeds) from FCO in 2018 (A) and 2019 (B) seasons.

The behavior of grape samples with seeds removed, in the FG vineyard site (Figure 20), was similar to whole-berry samples, with the difference that in 2018 only a few significant differences between control and thinned samples were ascertained. Thus, the largest differences between UNT and CT samples occurred in the second half of sampling, concerning the ILA-GLU ($p < 0.05$) and ABA ($p < 0.01$). Moreover, the concentrations of both compounds were considered to be accumulating during maturation. Additionally, it turned out that there were fewer significant differences when comparing the analyzed amount of aromatic amino acids TYR, PHE and TRP.

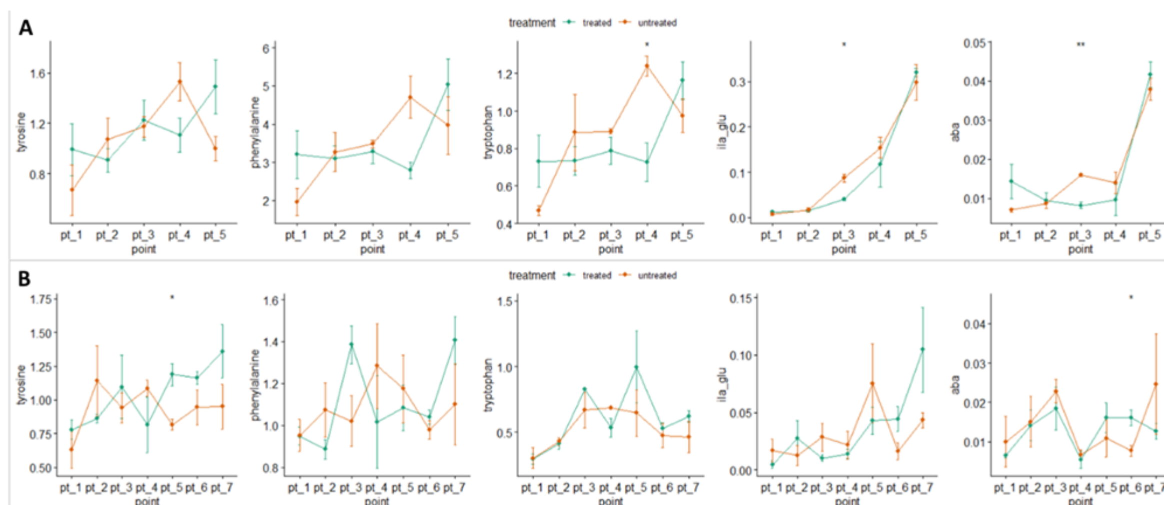

Figure 20: Evolution of aromatic amino acids compounds in untreated (red) and 20% thinned (green) berry samples (with removed seeds) from FG in 2018 (A) and 2019 (B) seasons.

Finally, the evolution of aromatic amino acid from FCO are presented on the Figure 21. As it has been observed, the lack of significant differences between UNT and CT samples

led to relatively homogeneous results in both seasons observed. In 2018 the significant difference characterized TYR and PHE at the beginning of the ripening monitoring, while a significant difference in the ABA concentration resulted to be greatest at the penultimate maturation point in CT samples, followed by significant drop in concentration.

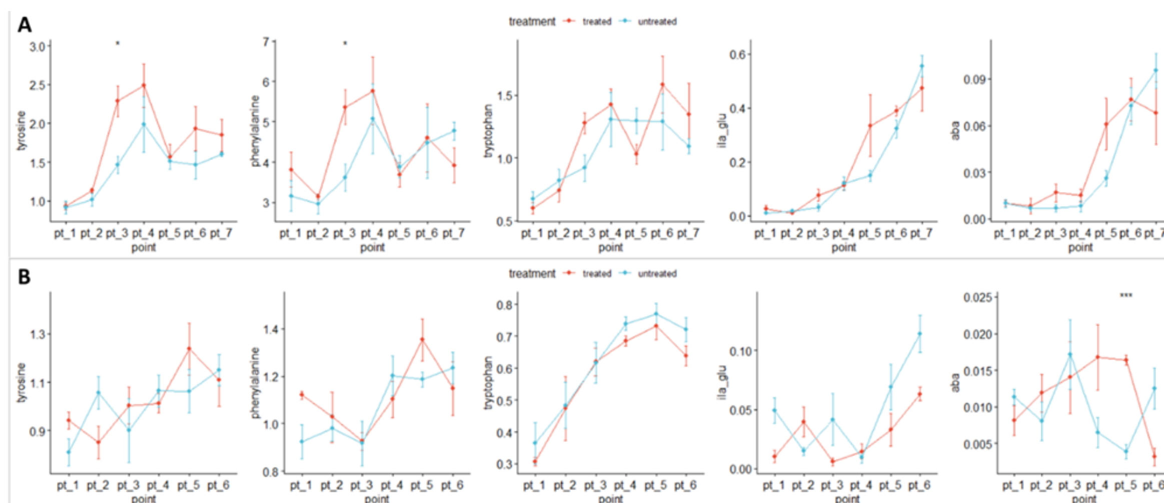

Figure 21: Evolution of aromatic amino acids compounds in untreated (blue) and 20% thinned (red) samples of seedless grapes from FCO in 2018 (A) and 2019 (B) harvest season.

4 DISCUSSION

In addition to monitoring of the classical parameters that determine the ripeness of the grapes (e.g., maturation index, the ration between soluble solids and titratable acidity and the pH value) the analysis of other secondary metabolites can be used for evaluation of optimal harvest timing for sparkling wine production (Jones et al., 2014). Moreover, the use of a variety of viticultural practices could significantly affect the rate of grape ripening and the accumulation of metabolites up harvest. Thus, cluster thinning represents a good example of common viticultural technique of modulating the source-sink balance to improve berry quality and elevate the level of secondary metabolites (Alem et al., 2019). Both aspects have been therefore studied in the present work on Ribolla Gialla variety.

4.1 BASIC GRAPE QUALITY PARAMETERS

The amount of TSS, TA and pH value represent one of the most important parameters in determining the maturation progress and thus determining the optimal harvest date of grapes, destined for the sparkling wines production (Jones et al., 2014). During the grape maturation, the amount of TA decreases with a simultaneous increase of TSS concentration (Bowen & Reynolds, 2015), which corresponded with our study. However, our results showed sudden decrease in the sugar level during the ripening of grapes in the 2018 season, which could be due to the meteorological conditions. Namely, in the first 12 days of August, the total amount of precipitation was 13.9 mm, followed by 36.4 mm of rain the next two

days in the FCO vineyard site. Finding a balanced grape maturity is of paramount importance, as optimal harvest time maximizes the aromatic potential of the grapes. Immature fruits thus produces wines that are green or grassy in aroma, while overripe fruit can produce a base wine that is excessively varietal or assertive (Zoecklein, 2002). In accordance with our results, authors have previously considered a range from 18–20 °Brix as optimally ripe from sparkling wine production (Keller, 2010), although in certain winegrowing regions where Champagne and Cava are produced, the viticultural regulations are dictating the harvest parameters. Thus, for example, in the Champagne region, the potential alcohol content of grape berries generally varies from 8% v/v to 11% v/v (Liu et al., 2018).

4.2 VOLATILE COMPOUNDS METABOLISM

Aroma is an important aspect of the fruit quality and can have a major effect on the development of varietal aromas in wines. Several families of compounds are thus responsible for the primary aroma of fruits, such as esters, terpenes, alcohols and others (Lund & Bohlmann, 2006). This part of our study was therefore focused on studying the evolution of free aromas with berry development in Ribolla Gialla variety, and the effects of cluster thinning treatment on this free fraction of grape volatile profile.

4.2.1 Lipoxygenase derived compounds

The formation of C6 compounds is linked to the concentration of UFAs, found predominately in grape seeds. These derive from the lipoxygenase (LOX) pathway and usually have a negative effect on fruit quality due to their herbaceous characters (Wu et al., 2020). When seeds are damaged, during crushing, linoleic acid can be oxidized to hexanol, which is converted by yeast to hexyl acetate, characterized by odor of fruit, apples or herb (Keller, 2010). However, certain authors reported that the concentration of free C6 compounds during berry development may be more dependent on enzyme activity and specificity than the content of substrates (Kalua & Boss, 2009; Kalua & Boss, 2010). From our results, it was clear that the most abundant compound from LOX pathway was *trans*-2-hexanal, which corresponded to the findings of other studies in Riesling and Cabernet Sauvignon grapes (Kalua & Boss, 2010). Early studies reported that the content of *trans*-2-hexanal and hexanal significantly increased after veraison, followed by a decrease in the late harvest (Kalua & Boss, 2009). Our results are in line with these findings since the concentration of both compounds were positively correlated with berry development in 2018 and in 2019 seasons. In addition to C6 aldehydes, the HS-SPME-GC-MS analysis also revealed the presence of C6 alcohols, among which the most abundant were 1-hexenol, *trans*-2-hexenol, and *trans*- and *cis*- isomers of 3-hexenol. For all listed compounds, it has been observed, that their concentration tends to decrease with increasing Brix content. This was in line with previous conducted studies, where the authors reported a significant decrease of *cis*-3-hexenol and *trans*-2-hexenol with grape maturation (Antalick et al., 2015;

Šuklje et al., 2019). Moreover, the authors reported no linear contribution of *trans*-2-hexenol, hexanol and corresponding aldehydes to the formation of hexyl acetate. However, the initial abundant concentration of *trans*-2-hexenyl acetate gradually decreased between sequential harvests, suggesting its direct contribution with its precursor *trans*-2-hexenol (Šuklje et al., 2019). A similar relationship occurred in our samples, where the decreasing amount of *cis*-3-hexenol led also to decreasing content of *cis*-3-hexenyl acetate.

In addition to grape ripening, the effect of cluster thinning was investigated on the content of C6 compounds. Only significant differences between the CT and UNT samples in our study were observed in the FCO vineyard. This was probably due to the meteorological conditions in this vineyard site as well as the higher presence of UFA substances as precursors. In contrast with control, the samples of thinned Jumeigui grapes resulted in the altered concentration of *trans*-2-hexenal, *cis*-3-hexenal and *trans*-2-hexenol, being hexanal the most abundant (Xi et al., 2020). This was partly in accordance with our results, since only *trans*-2-hexenal resulted to achieve higher concentration in grapes after the thinning treatment, while the remaining compounds reached higher concentration in control grape samples (e.g., *trans*-2-hexenol, 2-ethyl-1-hexenol, *cis*- and *trans*-3-hexenol). Moreover, in the samples of Ribolla Gialla grapes, the hexanoic acid and *trans*-2-hexanoic were notable, however, their concentration was lower in thinned grapes, confirming the findings of Condurso et al. (Condurso et al., 2016). The largest concentration belonged to the acetic acid. The formation and biosynthesis of this compound in grapes is associated with bacterial spoilage in *Botrytis cinerea*-infected grapes, and the high amount of produced acetic acid acceptable in icewines (Vilela-Moura et al., 2011).

4.2.2 Terpenes and norisoprenoids

In addition to the LOX derived compounds that were accounted for the largest amount in our samples, terpenes and norisoprenoids are the most important constituents in the grape aroma profile, as they contribute to the aroma profile providing floral and/or citrus odors as monoterpenes, or floral and fruity notes as C13-norisoprenoids (Luo et al., 2019). Although C13-norisoprenoids are only present at low concentrations, their sensory thresholds are usually low, which is of particular importance for Ribolla Gialla sparkling wines, as β-damascenone has been found to be one of the main contributors to the aromatic profile of this wine (Voce et al., 2019). In grapes, limonene and linalool were only analyzed monoterpenes, while β-damascenone was the only norisoprenoid. As regards this last compound, the results have unequivocally showed that its accumulation was positively associated with grape ripening, which also coincided with previous study, performed on Shiraz, Cabernet Sauvignon, Riesling, Chardonnay and Pinot Gris varieties (Luo et al., 2019). On the other hand, it was extremely difficult to determine an unambiguous trend in the increase or decrease in linalool concentration during grape ripening. Wu et al. (Wu et al., 2020) suggested that the levels of terpenes become low before the softening, followed by re-accumulation. Sudden decrease in free terpenes or their complete disappearance before the

veraison in neutral and non-Muscat aromatic varieties was additionally reported by other authors (Kalua & Boss, 2009; Kalua & Boss, 2010). Additionally, it was assumed that the free terpenes tends to be released from the bound forms in the early stages and converted into their bound forms in the more mature stages (Wu et al., 2020).

Previous studies reported that cluster thinning significantly increased the concentration of free and glycosylated volatile terpenes in Sauvignon Blanc berries, with the highest concentration revealed in thinned grapes one week before veraison (Kok & Rastilantie, 2005; X. Xi et al., 2020). Moreover, cluster thinning significantly enhanced the concentrations of terpenes at harvest in Jumeugui grapes (Xi et al., 2020). However, our results differed from previous publications, as the concentrations in thinned samples of linalool and limonene did not differ significantly from the controls in most cases. The only exception was ascertained in the winegrowing season 2018 in the FCO vineyard site, where the concentration of terpenes in UNT samples resulted to be higher than in CT samples. In addition to the seasonal factor that could influence such a discrepancy between the results, the occurred difference was probably due to the intensity of cluster thinning, since in previous studies the authors performed 50% cluster thinning, compared to our experimental design, where only 20% of clusters were removed (Kok & Rastilantie, 2005; X. Xi et al., 2020).

4.3 LIPID METABOLISM

4.3.1 Free fatty acid composition

The relationship between lipid molecules and grape-ripening indices is important, as the concentration of long-chain unsaturated fatty acids can affect the development of herbaceous aroma development in must and wine (Barron et al., 1989). In the analyzed grape samples, the highest concentration was represented by the free fatty acids. The major saturated fatty acids found in the whole grape berries were palmitic acid (C16:0), followed by stearic (C18:0), arachidic (C20:0) and lignoceric acid (C24:0), in agreement with previous studies (Pérez-Navarro et al., 2019). A similar composition with added gondoic acid was also observed in the grape samples where seeds were removed before extraction. The concentration of listed FAs is most abundant in the grape skins as previously revealed from different *Vitis vinifera* grape cultivars, while in grape seeds the UFAs like linoleic acid (C18:2) and oleic acid + *cis*-vaccenic acid (C18:1) and linolenic acid (C18:3) appeared to be predominant (Gallander & Peng, 1980; Pérez-Navarro et al., 2019). However, in our results the concentration of oleic acid + *cis*-vaccenic acid resulted to be more abundant in comparison to the linoleic and linolenic acid, and its concentration decreased in samples (with removed seeds) by an average of 12% compared to the whole-berry samples. Pérez-Navarro et al. (2019) revealed that an important amount of SFAs is also present in the grape seeds, with palmitic acid, stearic acid and behenic acid being the most abundant ones. However, from our results it can be noticed, that the harvest season had a large impact on the amount of fatty acids analyzed in the grapes, since the concentration of UFAs and SFAs

in grape berries (with removed seeds) dominated in 2018, while the amount of fatty acids in whole grapes appeared to be higher in the subsequent season. According to the study on the Mamaia grapes seed oil (Tociu et al., 2017), the seasons with high temperature and high precipitation during ripening period are characterized with higher amount of monounsaturated fatty acids (MUFAs), while in the dry years, the amount of SFAs resulted to be lower compared to the higher content of polyunsaturated fatty acids (PUFAs). Thus, our findings are in line with these results, as 2019 was characterized with higher amount of precipitation and lower average temperature, compared to the 2018. Moreover, the study of lipid profile of eleven grape cultivars grown in Japan, showed that the berries from colder regions were characterized by higher content of linoleic acid than berries cultivated in warmer regions (Arita et al., 2017; Yunoki et al., 2005). In addition, the gene expression of omega-3 fatty acid desaturase can decrease the amount of linoleic acid, leading to an increase of linolenic acid.

Overall, the berry concentration of fatty acid increased during ripening, in accordance with previous studies (Millán et al., 1992). While studying the changes in the fatty acid composition during the ripening of Pedro Ximénez grapes, the authors found that the proportion of UFAs decreased, which was most notable when comparing the linoleic acid content of unripe and ripe grapes. Conversely it has been shown that the concentration of SFAs (e.g., palmitic acid and stearic acid) increased with ripening (Agudelo-Romero et al., 2013; Millán et al., 1992). However, this was not completely in accordance with our results, as only in 2019 season was possible to observe a positive trend in accumulation of SFAs. Very few differences when cluster thinning was applied led to similar conclusions, although a significant difference between UNT and CT samples in 2019 for SFAs, implying that perhaps later harvest timing could lead to a clearer increase in SFAs content. Nevertheless, the authors studied the lipid composition of seeds oil from Tempranillo and Cencibel variety, and they found no significant differences in fatty acid composition when oil was extracted from grape seeds collected before veraison or when they were almost mature (Rubio et al., 2009). In addition, Bombai et al. (2017) reported that palmitic acid concentration was recognized as the lowest at harvest, while the stearic acid varied during the ripening displaying the lowest value at harvest. Therefore, it can be speculated that cultivar can also play a major role in the accumulation of fatty acids in grapes.

4.3.2 Sterols

The phytosterols (plant sterols) have been described as bioactive molecules since they can contribute to reduce cholesterol in humans (Millán et al., 2015). Over the years, the authors have successfully analyzed several sterols in grapes that are predominantly located in the grape skin and seeds. Thus, the most important sterols found are β-sitosterol, campesterol, stigmasterol and lanosterol, with β-sitosterol being the most important, accounting for 86–89% of the total detected phytosterols (Le Fur et al., 1994). This was in accordance also with our study, where, in addition to β-sitosterol, the most abundant was

campesterol and stigmasterol, while lanosterol was not present in Ribolla Gialla grapes. According to Le Fur et al. (1994), the evolution of β-sitosterol content in Chardonnay grape skins showed a decrease during the last stage of ripening and similar behavior characterized other two sterols analyzed. This observation was in line with our results, were all the main sterols analyzed were negatively correlated to the increasing Brix value in whole-berry samples, as well as in berry samples where seeds were removed. However, the particularly negative correlation characterized only samples from 2018, which could be due to the higher concentration in that year. Meteorological conditions and abiotic stresses can therefore have a major impact on the content of sterols in plants. Previous studies have found that during exposure to low temperatures, the level of UFAs and sterols in plant cells membranes increases, which consequently changes the membrane fluidity from fluid state to rigid gel form (Rogowska & Szakiel, 2020). Moreover, in *Oryza sativa* cultivars, the increase in sterols levels and their esters was proportional to the duration of dehydration stress (Kumar et al., 2015, 2018). Given that 2018 winegrowing season was marked as one of the hottest, it could mean that the water stress caused an increase of sterol concentration in grapes that season (Calderan et al., 2021). To our knowledge, there is very scarce number of studies that address the impact of cluster thinning technique on sterol content in grapes. Nevertheless, our experiment showed that in 2018 sterol concentration differed statistically between UNT and CT samples. Interestingly, the sterol concentration was initially higher in the control sample but decreased during grape ripening, which was consistent with the result described above.

4.3.3 Glycerolipids, glycerophospholipids and triterpenoids

The analysis of glycerolipids, glycerophospholipids and triperpenoids in several *V. vinifera* grape cultivars, revealed that glycerolipids and glycerophospholipids can be found in grape skins and grape seeds, while terpenoids, such as oleanolic acid are present predominantly in the skins (Pérez-Navarro et al., 2019). The same authors have found a higher concentration of 1,2-dioleoyl- sn-glycero-3-phosphocholine and 1,2-dilinoleoyl-sn-glycero-3-phosphocholine in grape seeds in comparison to the grape skins, which was not in line with our results, since both substances predominated in the samples without seeds. According to Della Corte et al. (2015) the glycerophospholipids represented one of the most abundant lipids in the whole berries, where the amount of 1,2-dilinoleoyl-sn-glycero-3-phosphocholine ranged from 77–328 mg/kg. Our results showed that the whole berries of Ribolla Gialla contain a lower concentration of 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (average value 38 mg/kg), but it was nevertheless found to be the highest glycerophospholipid. It is well known that glycerolipids are composed of mono-, di- and tri-substituted glycerols, the best-known being the fatty acids triesters of glycerol, called triglycerides. The latter have been analyzed in study of Barron and Santa Maria (1990), where it was shown that linoleo-oleo-palmitin and triolein were significantly correlated with the ripening factor. Our results agreed with these findings since the concentration of

glycerolipids appeared to be positively correlated with increasing amount of Brix in Ribolla Gialla grapes.

The compounds that are belonging to the group of triterpenoids are predominately present in the cuticle of grape berries, which forms the hydrophobic coating on the epidermis and act as a barrier to prevent an excessive water loss, prevent the restriction of leaching of organic and inorganic compounds from internal tissues and protect against biotic and abiotic environmental stresses. Triterpenoids are formed by the C30 precursor squalene, and are essentially divided into tetracyclic and pentacyclic triterpenoids (Pensec et al., 2014). The most abundant triterpenoid analyzed in our study appeared to be oleanolic acid and ranged in our samples from 5.2–30.3 mg/kg, which was less than found in some previous results published by Pérez-Navarro (2019). However, it has been shown that the amount of oleanolic acid decreased over the ripening period, which was also true when the cluster thinning treatment was applied. This observation was in accordance to the results of Pensec et al. (2014) where authors attributed this decrease to an increase in the level of aliphatic constituents of cuticular waxes and, subsequently, by the dilution of oleanolic acid in the grape wax extract mass. Similarly, the decrease in triterpenoid during fruit development was observed also in other fruits (i.e., in sweet cherry) (Peschel et al., 2007), whether selected fruits from *Rosaceae* family showed an increasing accumulation of triterpenoids (Dashbaldan et al., 2020).

4.4 AROMATIC AMINO ACID METABOLISM

Beside sugar concentration, the nitrogen composition of grape berries are key determinants of must composition, which is consequently essential for yeast growth (Garde-Cerdán et al., 2018). Not only that amino acids affect significantly the fermentation kinetics, but are also considered as precursors of important volatile compounds in wines, which are formed during the alcoholic fermentation (Bell & Henschke, 2005). Besides viticultural practices, soil management and nitrogen type, the grape maturity and cultivar are considered as the most influential factors in the content of amino acids that accumulate in grape berries tissues (Garde-Cerdán et al., 2009). One of the most important nitrogen compounds are also aromatic amino acids, such as TRP, PHE and TYR, that represent an important source of secondary metabolites and consequently influence the aroma and flavor of produced wine (Cordente et al., 2019). In our study, all three compounds exhibited positive correlation with the ripening of the grapes. This finding was in accordance with previously conducted study on the Grenache grapes, where PHE and TRP showed a progressive increase in their concentration during grape ripening presenting their maximum content at 25 °Brix. TYR on the other hand showed the same pattern of evolution but its maximum content was reached at 20 °Brix (Garde-Cerdán et al., 2018). Given the fact that the maximum sugar level in Ribolla Gialla grapes was determined 19.40 °Brix, it is difficult to predict whether this value already reached the maximum accumulation of aromatic amino acids, especially since grapes are intended for sparkling wine production and are therefore harvested at lower Brix values.

However, the trends that has been obtained after the cluster reduction showed that in 2018 season in FG vineyard site, the amount of PHE, TYR and PHE in the last sampling point did not express a decrease in concentration, therefore it could be expected that the grapes of Ribolla Gialla could reach full amino acid maturity in the later stages of ripening. Moreover, in a study carried out by Ruiz-Rodríguez et al. (Ruiz-Rodríguez et al., 2017) on Verdejo grapes, the authors showed that the application of nitrogen as a cultural practice, led to higher levels of TRP at the harvest day when compared to other cultural techniques. Additionally, greater significant differences between UNT and CT samples were observed when comparing the composition of ILA-GLU and ABA. Especially in 2018 harvest season, it turned out that the concentration of ILA-GLU and ABA gradually increased and decreased throughout maturation, respectively.

5 CONCLUSIONS

All the samples were collected when the accumulation of sugars had reached the highest level, and in the subsequent harvests, there were no further accumulation of grape solids in berries; in parallel, TA has not undergone significant reductions between harvests.

The results of volatile compounds confirmed the findings from previous studies, where it was reported that the concentration of β-damascenone accumulates during the ripening of the grapes. Moreover, the content of “greenish” hexanal and *trans*-2-hexanal increased accordingly with the berry development, along with complementary C6 alcohols. On the other hand, the meteorological characteristics of the seasons conditioned the effect of CT treatment, as it turned out that higher amount of *trans*-2-hexanal was detected only in samples from FCO vineyard site.

The results obtained also provided clear evidence of differences in lipid composition from different berry tissues. Grapes with extracted seeds were characterized by higher levels on saturated fatty acids and prenol lipids. Differences in accumulation during the ripening and concentration discrepancies between UFAs and SFAs can be considered responsible for further development of aromatic compounds, that characterize herbaceous aromas and tropical notes. Furthermore, it is possible to expect that less C6 compounds will be formed in the grapes from 2019 as a result of lower C18:3 concentration.

CONCLUDING REMARKS

The concluded study provided some useful insights into the potential capabilities of Ribolla Gialla variety for the production of monovarietal sparkling wines. Various analytical techniques have been combined into so-called multitargeted approach that has enabled us to perform the measurements of chemically defined groups of metabolites, which consisted of volatile organic compounds, lipid substances, and aromatic amino acid metabolites. All of listed classes of compounds have a significant effect on the overall aromatic character of the wine and may be linked with positive or negative aspects of wine quality.

As known from the literature, the vineyard, surrounding environment, climatic characteristics, and the application of viticultural practices can significantly influence the aromatic profile of wine. The aim of this work was therefore to understand how all these aspects affect the metabolic catabolites in Ribolla Gialla grapes, and to outline some indications for optimized quality of grapes, destined for sparkling wine.

- ❖ The study on the commercially available sparkling wines from Ribolla Gialla showed a homogeneous characteristics concerning basic parameters, despite the fact that the samples originated from different production areas of Friuli Venezia Giulia region, and that some of them were produced by the Champenoise method, with prolonged aging period on refermentation lees in bottles. Low levels of free terpenols were substituted with relatively high presence of volatile esters and β-damascenone, and thus contributed to fresh and fruity notes. Commercial wines also exhibited a high ratio between saturated and unsaturated fatty acids, which may positively affect the foam height. In contrast, due to the high amounts of certain aromatic amino acids metabolites, Ribolla Gialla could be prone to the formation of atypical aging aromas.

- ❖ The application of cluster thinning in two vineyard sites, followed by sparkling wine production based on Charmat method resulted only in few significant differences, when log2 analysis was applied for each production site separately. Vineyard that was suited at the foot of the hill produced higher concentration of citronellol, linalool and nerol, together with β-damascenone and TDN. The results of sensory analysis confirmed findings regarding volatile compounds, although the production level did not affect overall pleasantness. Moreover, separation of samples was noticed based on the season according to the principal component analysis. In contrast to volatile compounds, the tryptophan and its derivates were predominantly present in the flat side vineyard, presumably due to the higher yield.

- ❖ The sequential harvesting experiments have highlighted a significant advantage in the transition from first to second harvest date, which occurred a week after the technological ripeness of grapes, which was particularly well evident from enhanced production of esters. The majority of these compounds are known to contribute to fruity aromas. The addition of a later harvest in most cases led to a significant loss of a typical Ribolla Gialla bouquet and to the accumulation of acetic acid and C8 and

C9 volatile fatty acids. Sensory analysis appeared to be in accordance with volatile composition of wine, as the overall pleasantness characterized samples from second harvest, while the oxidation notes and dried fruit aromas characterized the third harvest date. These results thus indicate significant and coherent modulations of wine aroma profile in relation to grape harvest date.

❖ In terms of grape ripening, the results obtained showed a higher level of saturated fatty acids (e.g., palmitic, stearic, lignoceric and arachidic acid) where the grape seeds were extracted from the berry, suggesting a clear evidence of differences in lipid composition from different berry tissues. Higher concentration of unsaturated fatty acids in whole berries could potentially have an effect on the sensory characteristics of wines since they are precursors of C6 and C9 alcohols and aldehydes that are responsible for herbaceous aromas and thiols related to tropical notes. Additionally, the altitude of the vineyard had a great impact on the expression of *trans*-2-hexanal with thinning treatment.

The presented results can thus have a great practical importance. Such detailed and comprehensive profiling of multiple classes of compounds could serve winegrowers as a sort of guideline, designed to facilitate the decision about the level of production, or to help out determine the optimal harvest time, by taking into account changing climatic conditions. Nevertheless, our experimental results showed that seasonal factor is critical to the development of many flavor and quality traits in grapes and wine. Finally, these findings may also serve to emphasize the peculiarities that are typical for the certain territory and thus further enhance the identity of the wine.

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APPENDIX

Appendix A: Analysis of volatile compounds in the base wines and sparkling wines from 2017–2019 period.

Compound	Base wine			Sparkling wine			RT (min)	RI_{exp}	RI_{lit}	IM	Literature
	2017	2018	2019	2017	2018	2019					
Monoterpenes	21.21 ± 3.46	19.44 ± 3.83	16.54 ± 1.54	21.44 ± 3.40	22.21 ± 3.54	31.48 ± 3.93	-	-	-	-	
β-myrcene	0.59 ± 0.14	0.20 ± 0.06	0.17 ± 0.06	0.70 ± 0.12	0.70 ± 0.11	0.83 ± 0.09	8.59	1159	1173	MS RI STD	1
Limonene	0.57 ± 0.06	1.37 ± 0.38	2.13 ± 0.41	2.36 ± 0.71	2.40 ± 0.56	2.41 ± 0.22	9.32	1187	1204	MS RI STD	2
Linalool	4.58 ± 0.58	4.77 ± 1.26	2.69 ± 0.37	4.49 ± 0.80	5.11 ± 0.56	4.64 ± 0.43	17.60	1546	1555	MS RI STD	3
Geraniol	7.88 ± 2.19	5.99 ± 1.37	6.59 ± 1.53	8.18 ± 1.80	7.13 ± 1.75	7.38 ± 1.03	23.28	1876	1850	MS RI STD	4
Citronellol	4.75 ± 1.15	3.54 ± 0.83	2.98 ± 0.43	1.44 ± 0.51	1.19 ± 0.20	1.88 ± 0.33	21.81	1766	1777	MS RI STD	5
Nerol	1.53 ± 0.35	1.65 ± 0.34	1.43 ± 0.15	2.34 ± 0.77	2.66 ± 0.18	4.00 ± 0.33	21.73	1761	1777	MS RI STD	6
Terpinen-4-ol	0.30 ± 0.10	0.30 ± 0.13	0.19 ± 0.04	0.45 ± 0.10	0.61 ± 0.12	0.57 ± 0.08	18.69	1600	1618	MS RI STD	7
α-terpineol	1.01 ± 0.11	1.62 ± 2.38	0.51 ± 0.06	1.47 ± 0.29	2.42 ± 1.54	9.77 ± 4.02	20.54	1696	1679	MS RI STD	8
Norisoprenoids	19.68 ± 4.22	11.76 ± 2.72	7.82 ± 0.99	18.00 ± 4.84	11.92 ± 1.16	19.28 ± 2.22	-	-	-	-	
Vitispirane	0.70 ± 0.27	0.22 ± 0.16	0.34 ± 0.11	0.81 ± 0.21	0.60 ± 0.25	0.72 ± 0.35	-	-	-	MS	
TDN	0.64 ± 0.21	0.75 ± 0.18	0.65 ± 0.11	0.95 ± 0.21	0.40 ± 0.07	1.75 ± 0.36	21.44	1745	1732	MS RI	3
β-damascenone	17.88 ± 4.10	10.53 ± 2.79	6.71 ± 1.02	15.21 ± 4.51	10.00 ± 1.10	16.05 ± 1.93	22.81	1833	1857	MS RI STD	9
Actinidiol (isomer 1)	0.23 ± 0.05	0.18 ± 0.23	0.06 ± 0.01	0.44 ± 0.06	0.40 ± 0.06	0.32 ± 0.05	17.15	1524	-	MS	
Actinidiol (isomer 2)	0.23 ± 0.05	0.08 ± 0.02	0.06 ± 0.01	0.59 ± 0.08	0.53 ± 0.08	0.43 ± 0.07	-	-	-	MS	
Aldehydes	181.26 ± 35.84	98.29 ± 32.79	93.56 ± 39.71	499.58 ± 107.12	360.28 ± 47.31	479.23 ± 71.33	-	-	-	-	
Hexanal	1.02 ± 0.87	0.34 ± 0.20	0.33 ± 0.25	0.12 ± 0.04	0.05 ± 0.02	0.08 ± 0.02	6.55	1081	1072	MS RI STD	10
trans-2-hexenal	108.56 ± 38.33	71.83 ± 22.44	80.85 ± 39.88	464.22 ± 103.32	334.23 ± 43.69	446.77 ± 70.14	10.73	1242	1235	MS RI STD	11
Nonanal	45.71 ± 52.18	10.51 ± 17.28	5.05 ± 0.79	2.86 ± 0.70	2.49 ± 0.75	3.89 ± 1.59	14.32	1392	1397	MS RI	12
Benzaldehyde	10.99 ± 9.59	4.26 ± 0.44	2.25 ± 0.44	8.75 ± 1.52	5.28 ± 0.67	6.51 ± 1.19	17.08	1520	1507	MS RI STD	13
3,4-dimethyl benzaldehyde	0.49 ± 0.07	1.17 ± 1.43	0.28 ± 0.04	1.69 ± 0.29	1.00 ± 0.21	1.73 ± 0.26	22.23	1789	1790	MS RI	14
Acetaldehyde	13.42 ± 5.92	9.97 ± 5.93	4.68 ± 1.17	20.41 ± 5.76	16.14 ± 3.80	18.44 ± 6.38	1.72	549	1418	MS RI	
Furfural	1.08 ± 0.19	0.21 ± 0.10	0.12 ± 0.04	1.53 ± 0.49	1.09 ± 0.29	1.82 ± 0.45	15.86	1463	1460	MS RI	15
Alcohols	1829.44 ± 162.66	1382.90 ± 425.05	4257.26 ± 364.58	3,975.01 ± 459.96	3,538.87 ± 447.90	4,133.67 ± 340.64	-	-	-	-	
n-hexanol	157.24 ± 69.80	41.29 ± 6.73	48.70 ± 9.96	144.07 ± 61.78	30.64 ± 5.81	57.68 ± 11.63	13.40	1353	1358	MS RI STD	13
trans-3-hexenol	2.59 ± 1.60	1.06 ± 0.31	1.09 ± 0.24	3.04 ± 1.81	1.02 ± 0.31	1.40 ± 0.31	13.63	1363	1374	MS RI STD	16
1-octanol	9.25 ± 2.08	11.39 ± 3.94	8.96 ± 1.01	10.99 ± 2.65	10.39 ± 2.10	11.09 ± 1.05	17.81	1556	1562	MS RI	13
Isobutanol	85.49 ± 7.66	118.73 ± 51.22	98.06 ± 14.69	110.03 ± 9.79	95.94 ± 24.25	132.87 ± 24.61	7.14	1104	1114	MS RI STD	17
Methionol	6.62 ± 1.69	4.10 ± 2.84	4.13 ± 1.25	2.24 ± 0.94	2.84 ± 0.78	2.66 ± 1.42	20.88	1715	1711	MS RI	18
Isoamyl alcohol	658.32 ± 56.22	645.54 ± 217.85	3283.53 ± 308.22	2,893.35 ± 378.88	2,669.83 ± 357.51	3,171.99 ± 278.07	10.11	1218	1209	MS RI STD	19
3-methyl-1-pentanol	7.15 ± 1.09	8.06 ± 4.55	7.99 ± 1.25	8.90 ± 1.33	10.59 ± 2.27	10.50 ± 1.62	12.76	1325	1316	MS RI STD	18
2,3-butanediol (isomer 1)	21.42 ± 5.02	13.12 ± 4.64	17.13 ± 4.86	10.39 ± 8.12	0.59 ± 0.21	1.00 ± 0.80	17.41	1537	1529	MS RI	20
2,3-butanediol (isomer 2)	5.71 ± 1.24	4.45 ± 2.81	4.09 ± 1.33	3.87 ± 2.23	0.46 ± 0.16	0.85 ± 0.28	18.16	1574	1583	MS RI	19
cis-3-hexenol	875.65 ± 95.98	535.17 ± 166.62	783.60 ± 95.70	788.14 ± 72.43	716.56 ± 89.11	743.64 ± 70.05	14.57	1403	1382	MS RI STD	20

(Continues on the next page)

Appendix A: (Continued)

Compound	Base wine			Sparkling wine			RT (min)	RI_{exp}	RI_{lit}	IM	Literature
	2017	2018	2019	2017	2018	2019					
trans-2-hexenol	-	-	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	15.06	1426	1420	MS RI STD	17
2-phenylethanol	-	-	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-	-	MS	
Esters	4111.28 ± 872.83	1809.03 ± 605.63	1131.00 ± 184.11	2,484.72 ± 434.64	2,539.83 ± 562.89	3,443.76 ± 379.35	-	-	-	-	
Ethyl acetate	127.85 ± 27.98	67.37 ± 15.88	112.44 ± 48.22	307.33 ± 83.07	237.68 ± 36.49	424.81 ± 118.22	2.69	892	889	MS RI	20
Ethyl butyrate	32.00 ± 6.95	26.89 ± 11.94	28.13 ± 5.01	36.59 ± 11.60	41.44 ± 10.44	55.26 ± 5.74	5.52	1040	1025	MS RI STD	21
Isopentyl acetate	310.17 ± 93.44	151.91 ± 72.68	191.33 ± 54.92	138.94 ± 52.32	189.43 ± 53.75	245.65 ± 43.18	7.60	1122	1120	MS RI STD	22
Hexyl acetate	114.19 ± 49.23	13.12 ± 7.06	19.58 ± 9.71	20.84 ± 11.91	16.46 ± 8.86	28.53 ± 15.78	11.46	1272	1295	MS RI STD	23
Methyl caproate	0.36 ± 0.08	0.43 ± 0.24	0.49 ± 0.17	0.48 ± 0.17	0.57 ± 0.17	0.76 ± 0.11	9.28	1185	1180	MS RI STD	20
Ethyl hexanoate	974.87 ± 208.33	726.63 ± 270.60	456.96 ± 70.43	1,000.85 ± 233.88	940.15 ± 263.97	1,046.99 ± 130.17	10.53	1234	1241	MS RI STD	17
Ethyl lactate	4.70 ± 1.07	2.49 ± 1.01	2.92 ± 0.50	18.74 ± 3.65	14.52 ± 2.44	16.60 ± 2.48	13.18	1343	1355	MS RI	24
Methyl octanoate	2.15 ± 0.44	1.04 ± 0.60	0.64 ± 0.10	2.41 ± 0.50	3.26 ± 0.92	3.68 ± 0.39	14.26	1390	1387	MS RI	13
Ethyl octanoate	1,641.87 ± 365.54	306.94 ± 77.67	197.17 ± 32.83	462.69 ± 99.99	627.70 ± 135.98	780.11 ± 86.74	15.24	1434	1453	MS RI STD	19
Isoamyl lactate	0.52 ± 0.08	0.83 ± 0.40	0.72 ± 0.22	2.46 ± 0.29	2.12 ± 0.30	2.49 ± 0.23	18.04	1568	-	MS	
Methyl decanoate	0.26 ± 0.12	0.04 ± 0.02	0.06 ± 0.02	0.23 ± 0.04	0.22 ± 0.06	0.43 ± 0.09	18.60	1596	1604	MS RI	25
Ethyl decanoate	387.03 ± 181.71	99.53 ± 94.73	53.03 ± 13.38	27.45 ± 9.74	168.00 ± 49.29	340.25 ± 85.25	19.48	1641	1643	MS RI STD	17
Isoamyl octanoate	5.85 ± 1.69	3.21 ± 2.53	1.25 ± 0.28	1.91 ± 0.63	2.54 ± 0.88	3.14 ± 0.66	19.85	1660	1654	MS RI STD	9
Methyl ethyl succinate	102.55 ± 45.16	267.94 ± 121.08	0.04 ± 0.02	109.76 ± 18.05	81.40 ± 23.54	150.65 ± 31.01	19.36	1635	-	MS	
Diethyl succinate	156.40 ± 29.43	28.99 ± 15.58	2.63 ± 0.54	144.32 ± 18.20	98.95 ± 12.60	44.71 ± 5.27	20.16	1677	1679	MS RI	26
Ethyl 9-decanoate	104.12 ± 81.63	58.89 ± 70.79	13.72 ± 5.73	151.24 ± 83.42	15.40 ± 4.97	234.47 ± 97.08	20.46	1692	1708	MS RI	27
Ethyl-2-OH-4-methylpentanoate	1.77 ± 0.23	1.48 ± 0.62	0.75 ± 0.19	2.62 ± 0.39	3.68 ± 0.46	2.92 ± 0.37	17.51	1542	-	MS	
2-phenylethyl acetate	108.44 ± 34.77	38.28 ± 7.79	34.60 ± 6.96	37.47 ± 9.75	51.25 ± 8.28	47.96 ± 7.87	22.72	1825	1832	MS RI STD	19
Ethyl hydrogen succinate	5.87 ± 1.25	6.18 ± 3.64	0.44 ± 0.74	12.69 ± 4.22	35.27 ± 11.45	6.69 ± 6.57	-	-	-	MS	
Methyl salicylate	19.98 ± 23.87	1.88 ± 2.12	0.46 ± 0.16	3.58 ± 1.19	7.58 ± 5.23	2.79 ± 2.39	21.99	1775	1765	MS RI STD	28
Ethyl hydroxybutanoate	10.34 ± 2.33	4.98 ± 3.06	1.87 ± 0.48	1.14 ± 0.35	1.62 ± 0.69	4.02 ± 1.04	22.51	1806	-	MS	
Ethyl dodecanoate	-	-	-	0.97 ± 0.28	0.58 ± 0.06	0.86 ± 0.15	-	-	-	MS	
Isobutyl acetate	-	-	-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	5.42	1036	1020	MS RI STD	22
Acids	2533.89 ± 569.68	732.68 ± 289.49	1615.27 ± 828.09	1,840.70 ± 222.97	2,718.65 ± 821.02	2,262.97 ± 461.47	-	-	-	-	
Acetic acid	16.89 ± 2.05	46.92 ± 11.62	45.02 ± 21.18	21.45 ± 3.81	36.02 ± 9.93	55.85 ± 17.46	15.66	1453	1437	MS RI STD	16
Butyric acid	5.28 ± 1.66	6.28 ± 1.12	4.85 ± 2.64	6.08 ± 1.48	7.34 ± 1.31	8.29 ± 0.57	19.26	1630	1598	MS RI STD	29
3-methylbutyric acid	23.79 ± 2.29	19.80 ± 4.83	12.37 ± 6.85	20.17 ± 4.35	19.41 ± 2.50	18.40 ± 2.01	20.05	1671	1657	MS RI	30
Hexanoic acid	154.54 ± 25.33	65.16 ± 10.09	98.36 ± 13.36	124.04 ± 15.27	115.83 ± 19.38	127.59 ± 7.58	23.28	1876	1857	MS RI STD	5
Octanoic acid	729.19 ± 96.68	230.62 ± 64.93	415.25 ± 42.59	388.98 ± 90.31	443.06 ± 71.54	549.94 ± 59.43	-	-	-	MS STD	
Nonanoic acid	6.24 ± 4.75	6.34 ± 4.82	74.51 ± 57.00	61.10 ± 51.35	20.42 ± 8.79	57.11 ± 26.79	-	-	-	MS STD	
Decanoic acid	1,557.00 ± 521.60	346.49 ± 271.29	945.50 ± 765.57	1,185.85 ± 168.96	2,005.13 ± 734.01	1,409.30 ± 433.95	-	-	-	MS STD	
Benzoic acid	15.44 ± 20.35	2.62 ± 0.45	1.06 ± 1.70	2.84 ± 1.09	2.99 ± 0.56	2.94 ± 0.61	-	-	-	MS	
Dodecanoic acid	25.53 ± 21.61	8.46 ± 11.25	18.36 ± 17.83	30.19 ± 16.62	68.46 ± 17.20	33.55 ± 9.79	-	-	-	MS	
Ketones	20.08 ± 3.08	15.45 ± 4.23	20.16 ± 8.59	159.06 ± 27.02	134.54 ± 16.86	137.98 ± 11.36	-	-	-	-	
2-methylthiolan-3-one	1.99 ± 0.41	2.79 ± 1.29	2.20 ± 0.82	0.83 ± 0.19	3.79 ± 1.55	2.89 ± 1.10	17.16	1524	1510	MS RI	31
Isophorone	18.10 ± 2.97	12.67 ± 4.48	17.96 ± 8.60	158.24 ± 26.99	130.75 ± 16.98	135.09 ± 10.92	18.00	1566	1600	MS RI	32

Concentrations are expressed in µg/L ± standard deviation for each analyzed compound. RT, retention time reported in min; RI_{exp}, experimental retention index; RI_{lit}, literature retention index; IM, identification method (MS, comparison of mass spectra with those reported in mass spectrum libraries; RI, comparison of order of elution with those reported in literature; STD, comparison of mass spectra and retention time with those of analytical standard compounds)

Reported literature to determine RI_{lit}

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Appendix B: Analysis of lipid compounds in the base wines and sparkling wines from 2017–2019 period.

Compound	Base wines			Sparkling wines		
	2017	2018	2019	2017	2018	2019
Glycerolipids	0.27 ± 0.05	0.24 ± 0.03	0.26 ± 0.02	0.23 ± 0.02	0.24 ± 0.03	0.33 ± 0.05
1-linoleoyl-rac-glycerol	0.23 ± 0.04	0.21 ± 0.02	0.24 ± 0.02	0.20 ± 0.03	0.21 ± 0.03	0.28 ± 0.04
1-oleoyl-rac-glycerol	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.02	0.02 ± 0.00	0.04 ± 0.00
1-monopalmitoleoyl-rac-glycerol	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
Sterols	0.32 ± 0.06	0.27 ± 0.04	0.31 ± 0.01	0.29 ± 0.03	0.27 ± 0.02	0.44 ± 0.04
Ergosterol	0.29 ± 0.06	0.25 ± 0.03	0.29 ± 0.01	0.26 ± 0.03	0.24 ± 0.02	0.40 ± 0.04
Desmosterol	0.03 ± 0.01	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.04 ± 0.00
Fatty acids UFA	6.26 ± 0.48	3.83 ± 0.25	3.98 ± 0.18	6.07 ± 0.46	5.45 ± 0.25	6.22 ± 0.61
Linoleic acid	0.36 ± 0.05	0.23 ± 0.02	0.24 ± 0.02	0.29 ± 0.03	0.31 ± 0.03	0.37 ± 0.05
Linolenic acid	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
Palmitoleic acid	0.23 ± 0.07	0.11 ± 0.02	0.11 ± 0.03	0.55 ± 0.20	0.33 ± 0.07	0.21 ± 0.05
Oleic acid + *cis*-Vaccenic acid	5.64 ± 0.46	3.46 ± 0.24	3.59 ± 0.18	5.20 ± 0.33	4.79 ± 0.21	5.60 ± 0.56
Fatty acids SFA	179.54 ± 11.47	142.44 ± 8.12	139.44 ± 8.77	202.31 ± 16.15	184.40 ± 11.91	219.42 ± 13.79
Behenic acid	0.73 ± 0.05	0.60 ± 0.04	0.60 ± 0.03	0.76 ± 0.04	0.71 ± 0.05	0.96 ± 0.08
Stearic acid	54.75 ± 3.18	42.66 ± 2.91	40.10 ± 3.10	60.79 ± 4.61	56.35 ± 3.34	67.35 ± 4.29
Lignoceric acid	0.37 ± 0.06	0.34 ± 0.02	0.31 ± 0.02	0.49 ± 0.04	0.42 ± 0.03	0.45 ± 0.04
Arachidic acid	2.76 ± 0.14	1.95 ± 0.14	2.03 ± 0.18	2.68 ± 0.18	2.50 ± 0.13	3.24 ± 0.29
Myristic acid	1.60 ± 0.12	1.33 ± 0.29	1.79 ± 0.12	1.76 ± 0.18	1.68 ± 0.12	2.04 ± 0.20
Palmitic acid	118.31 ± 8.76	94.73 ± 5.39	93.70 ± 5.79	134.88 ± 11.54	121.84 ± 8.70	144.12 ± 9.33
Miristoleic acid	0.49 ± 0.10	0.42 ± 0.06	0.49 ± 0.02	0.41 ± 0.03	0.41 ± 0.03	0.68 ± 0.07
Margaric acid	0.53 ± 0.04	0.40 ± 0.03	0.41 ± 0.04	0.55 ± 0.07	0.50 ± 0.04	0.60 ± 0.05
Prenols	0.11 ± 0.10	0.13 ± 0.12	0.11 ± 0.09	0.11 ± 0.12	0.07 ± 0.07	0.18 ± 0.10
Lupeol	0.11 ± 0.10	0.13 ± 0.12	0.11 ± 0.09	0.11 ± 0.12	0.07 ± 0.07	0.18 ± 0.10
Fatty esters	-	-	-	0.02 ± 0.03	0.01 ± 0.00	0.00 ± 0.00
Ethyl stearate	-	-	-	0.02 ± 0.03	0.01 ± 0.00	0.00 ± 0.00

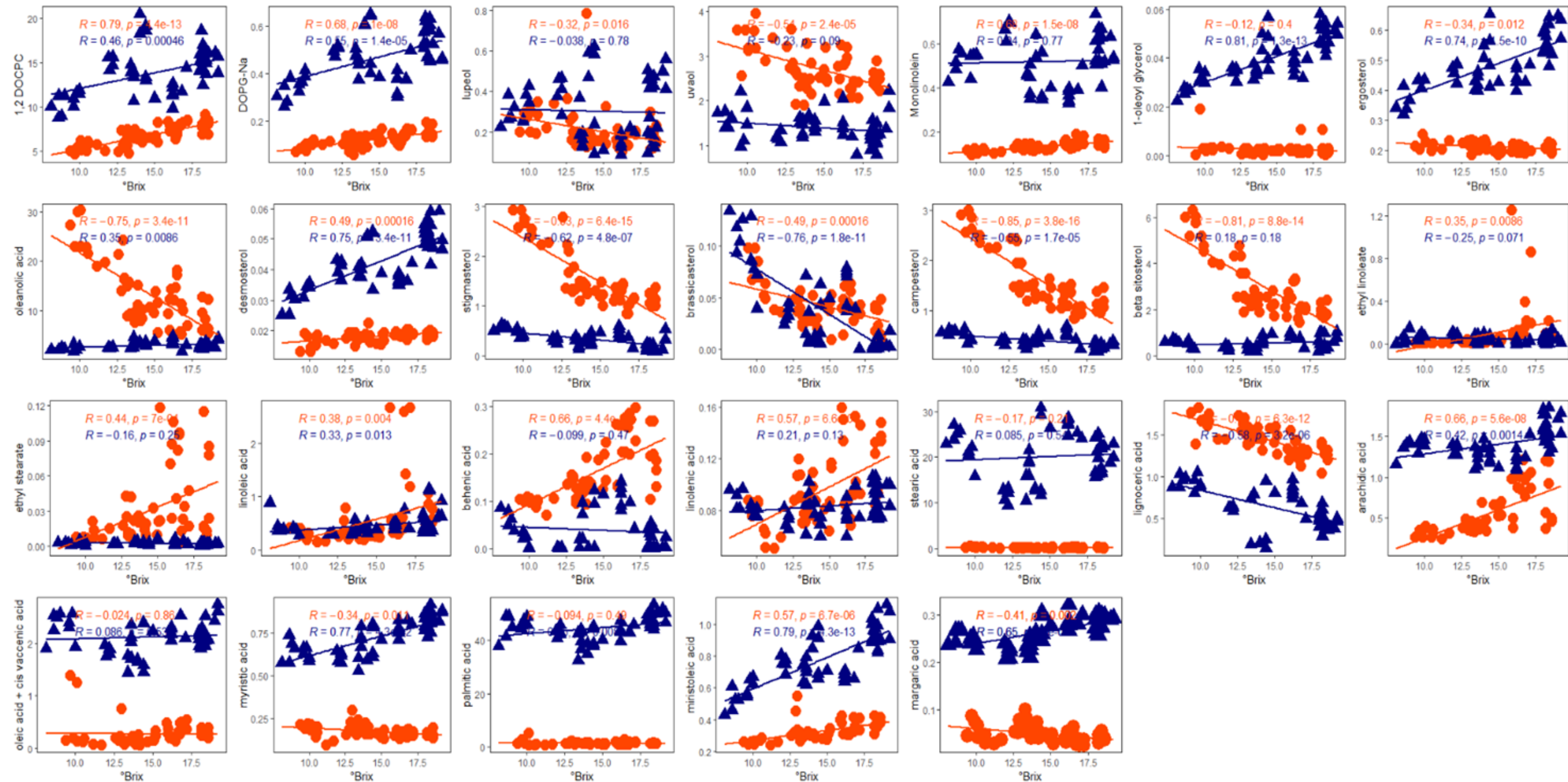
Concentrations are expressed in mg/L ± standard deviation for each analyzed compound.

Appendix C: Analysis of aromatic amino acid metabolites in the base wines and sparkling wines from 2017–2019 period.

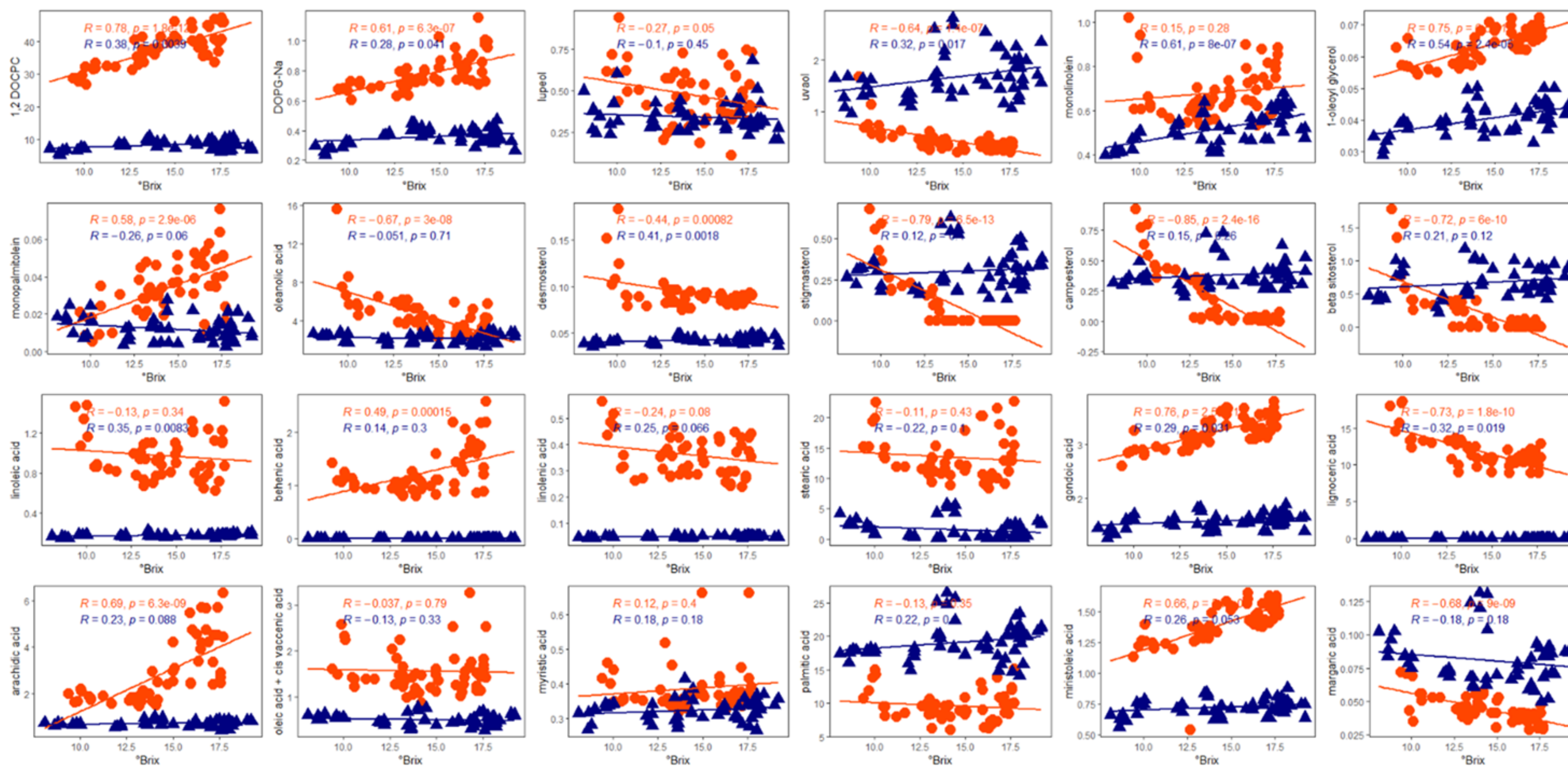
Compund	Base wines			Sparkling wines		
	2017	2018	2019	2017	2018	2019
TYR	4.25 ± 1.98	2.61 ± 1.38	3.04 ± 1.41	4.50 ± 4.48	4.90 ± 2.04	4.38 ± 1.59
PHE	2.03 ± 1.57	0.52 ± 0.60	0.62 ± 0.52	5.11 ± 4.25	2.66 ± 1.24	0.85 ± 0.39
TRP	0.25 ± 0.24	0.36 ± 0.32	0.32 ± 0.29	0.43 ± 0.30	0.25 ± 0.24	0.42 ± 0.23
KYNA	10.55 ± 7.10	1.53 ± 0.71	10.53 ± 5.34	12.48 ± 9.11	2.34 ± 0.82	10.03 ± 4.50
NIC	0.37 ± 0.28	0.29 ± 0.26	0.45 ± 0.31	0.46 ± 0.27	0.22 ± 0.06	0.63 ± 0.31
TRP-EE	0.34 ± 0.26	0.43 ± 0.20	0.34 ± 0.25	0.27 ± 0.21	0.47 ± 0.38	0.34 ± 0.28
TYR-EE	12.20 ± 7.46	3.47 ± 3.59	4.24 ± 3.07	24.64 ± 9.88	6.49 ± 1.83	10.92 ± 8.06
N-TYR-EE	0.42 ± 0.23	0.28 ± 0.16	0.42 ± 0.20	0.45 ± 0.35	0.38 ± 0.25	0.54 ± 0.29
TYL	15.26 ± 1.09	13.10 ± 5.12	14.22 ± 1.80	17.94 ± 3.97	15.80 ± 4.44	13.76 ± 4.26
OH-TYL	0.41 ± 0.24	0.32 ± 0.22	0.46 ± 0.14	0.31 ± 0.18	0.26 ± 0.06	0.36 ± 0.09
Ph-AA	0.38 ± 0.28	0.21 ± 0.21	0.31 ± 0.25	0.27 ± 0.22	0.41 ± 0.28	0.43 ± 0.29
TOL	0.36 ± 0.26	0.46 ± 0.26	0.45 ± 0.10	0.34 ± 0.12	0.73 ± 0.11	0.48 ± 0.08
IAA	0.61 ± 0.23	0.38 ± 0.29	0.43 ± 0.24	0.46 ± 0.34	0.30 ± 0.29	0.37 ± 0.30
ILA	0.47 ± 0.26	0.30 ± 0.15	0.31 ± 0.19	0.46 ± 0.23	0.45 ± 0.33	0.39 ± 0.29
ILA-GLU	0.77 ± 0.36	1.14 ± 0.32	1.48 ± 0.61	1.02 ± 0.45	1.38 ± 0.31	1.58 ± 0.25
N-SER	0.45 ± 0.13	0.52 ± 0.14	0.49 ± 0.09	0.46 ± 0.22	0.45 ± 0.17	0.47 ± 0.18
Ph-LA	0.54 ± 0.11	0.72 ± 0.33	0.80 ± 0.24	0.75 ± 0.22	1.17 ± 0.30	0.75 ± 0.19
TOL-SO3H	0.72 ± 0.37	0.35 ± 0.35	0.20 ± 0.05	1.02 ± 0.37	0.51 ± 0.34	0.27 ± 0.28
ABA	0.56 ± 0.23	0.32 ± 0.12	0.34 ± 0.09	0.87 ± 0.43	0.43 ± 0.16	0.40 ± 0.10
ABA-GLU	0.43 ± 0.26	0.38 ± 0.22	0.44 ± 0.26	0.46 ± 0.29	0.42 ± 0.27	0.31 ± 0.22
AA	-	-	-	0.36 ± 0.13	0.48 ± 0.32	0.33 ± 0.22

Concentrations are expressed in mg/L ± standard deviation for each analyzed compound.
(TYR) Tyrosine; (PHE) Phenylalanine; (TRP) Tryptophan; (KYNA) Kynurenic acid; (NIC) Nicotinamide; (TRP-EE) Tryptophan ethyl ester; (TYR-EE) Tyrosine ethyl ester; (N-TYR-EE) N-acetyl tyrosine ethyl ester; (TYL) Tyrosol; (OH-TYL) Hydroxytyrosol; (Ph-AA) Phenyl acetic acid; (TOL) Tryptophol; (IAA) Indole 3-acetic acid; (ILA) Indole 3-lactic acid; (ILA-GLU) Indole 3-lactic acid glucoside; (N-SER) N-acetyl serotonin; (Ph-LA) Phenyl lactic acid; (TOL-SO3H) Tryptophol-2-sulfonic acid; (ABA) Abscisic acid; (ABA-GLU) Abscisic acid glucoside, (AA) Anthranilic acid.

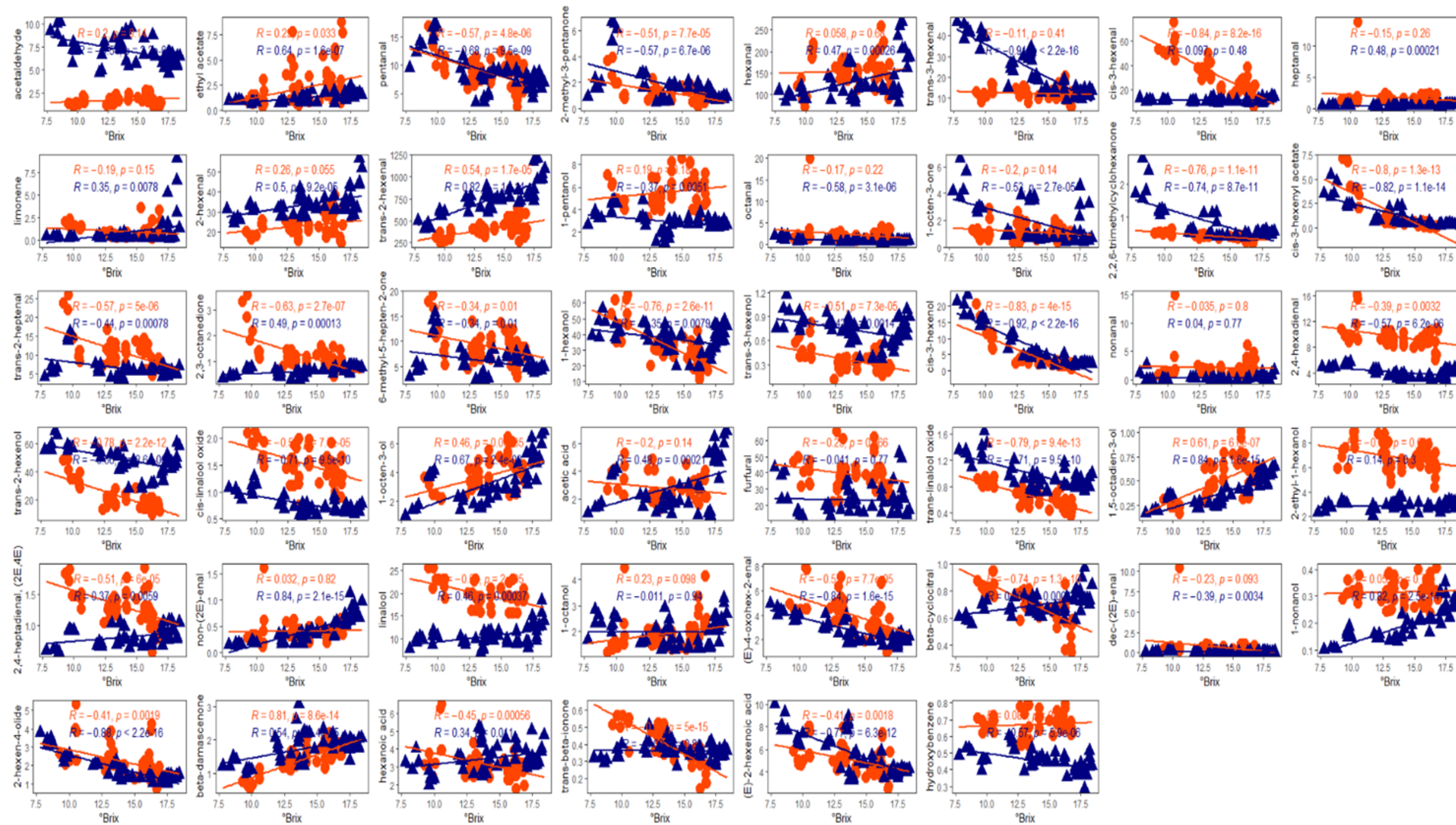
Appendix D: The scatter plot with the calculated Pearson coefficient of lipid molecules in grape berries (with seeds) in 2018 (red) and 2019 (blue) harvest season. 1,2 DOCPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPG-Na, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt.



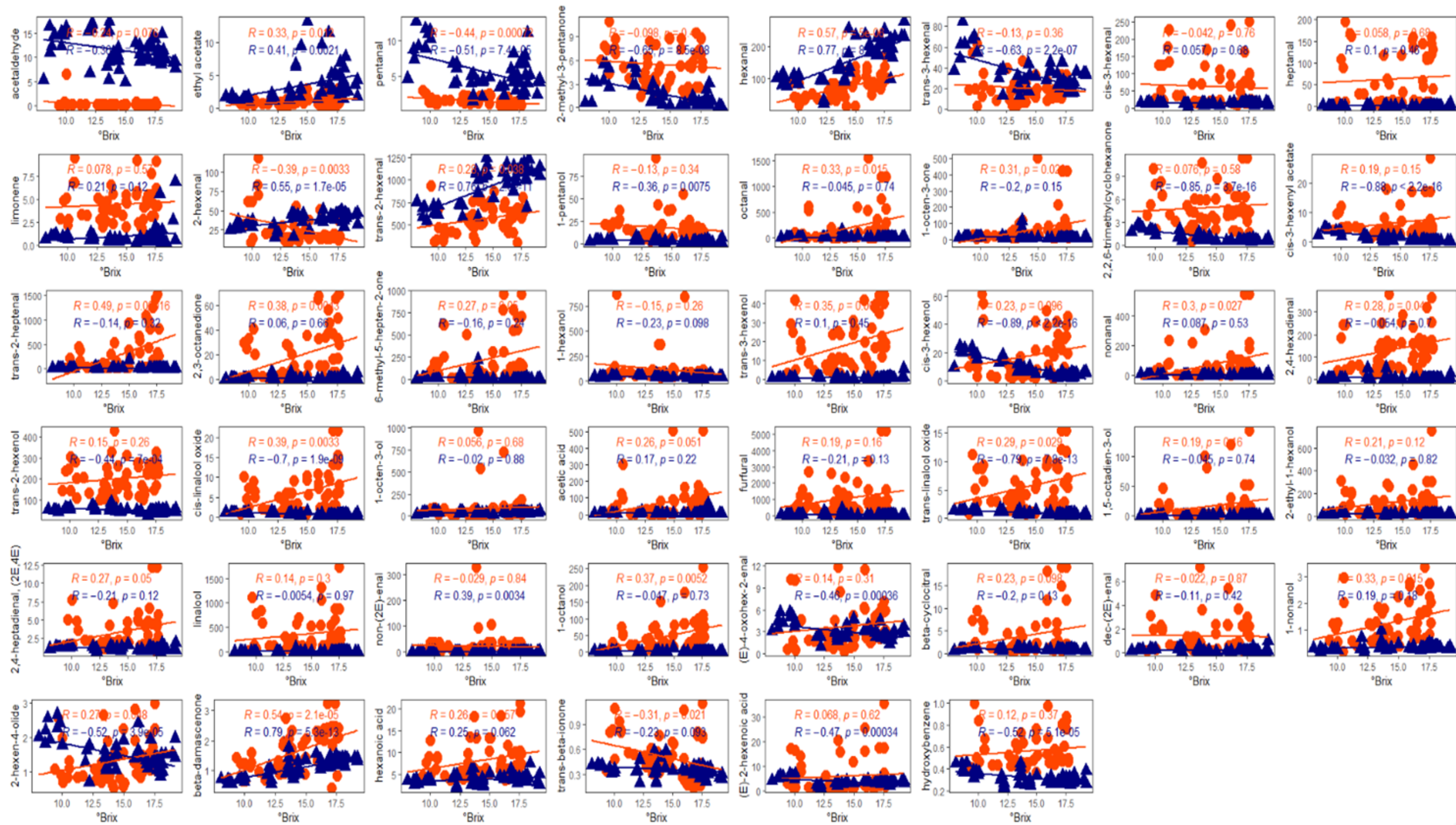
Appendix E: The scatter plot with the calculated Pearson coefficient of lipid molecules in grape berries (with removed seeds) in 2018 (red) and 2019 (blue) harvest season. 1,2 DOCPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPG-Na, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt.



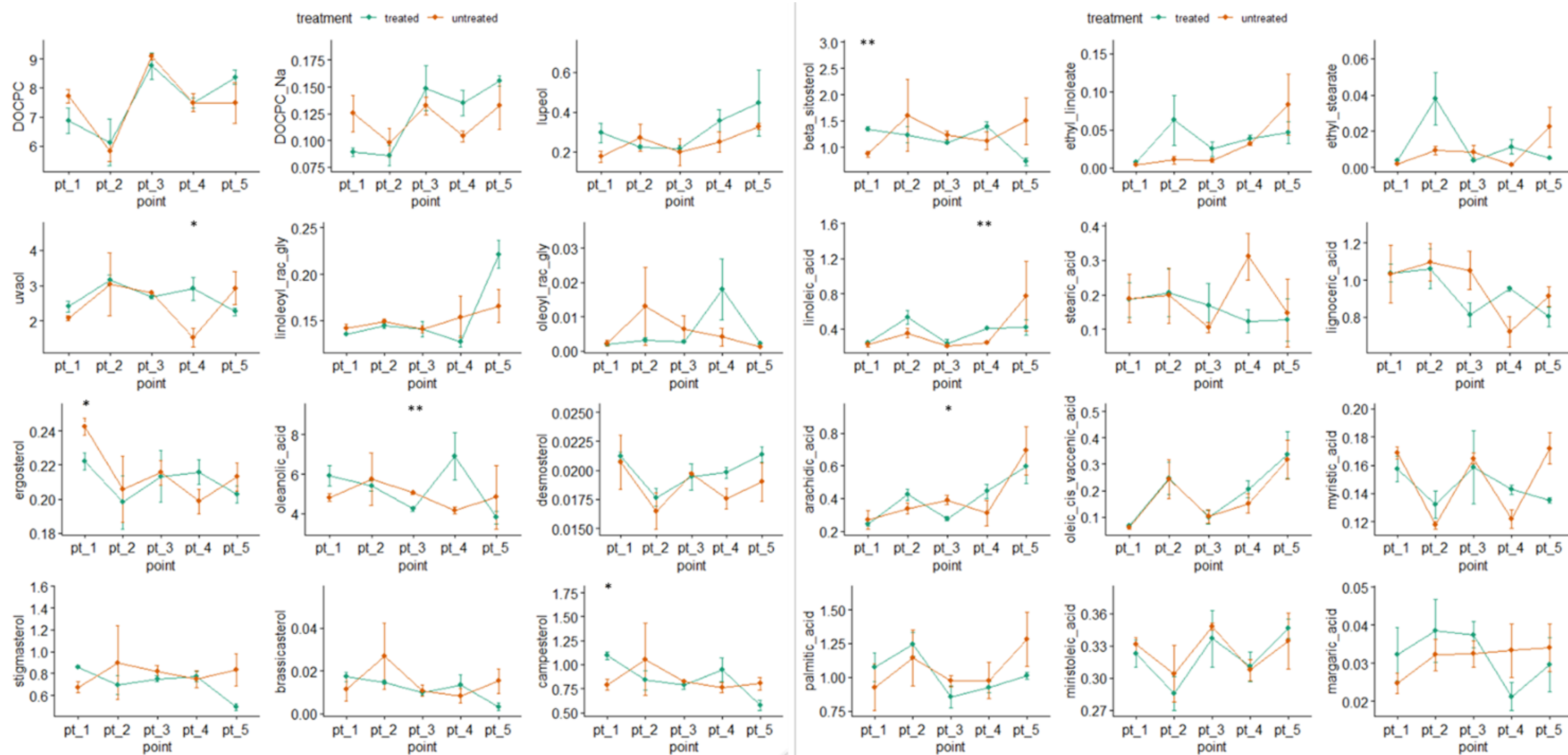
Appendix F: The scatter plot with the calculated Pearson coefficient of volatile molecules in grape berries (with seeds) in 2018 (red) and 2019 (blue) harvest season.



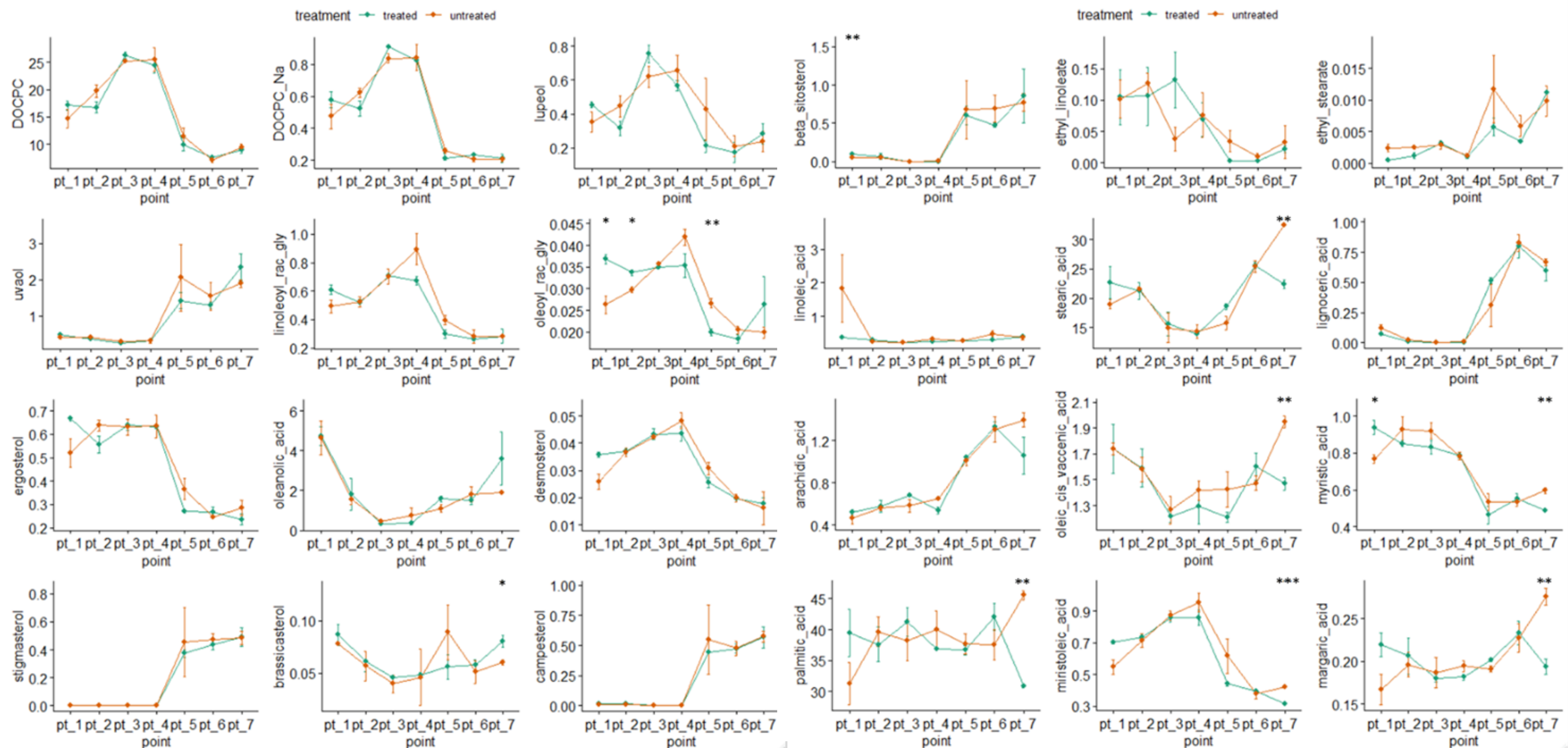
Appendix G: The scatter plot with the calculated Pearson coefficient of lipid molecules in grape berries (with removed seeds) in 2018 (red) and 2019 (blue) harvest season.



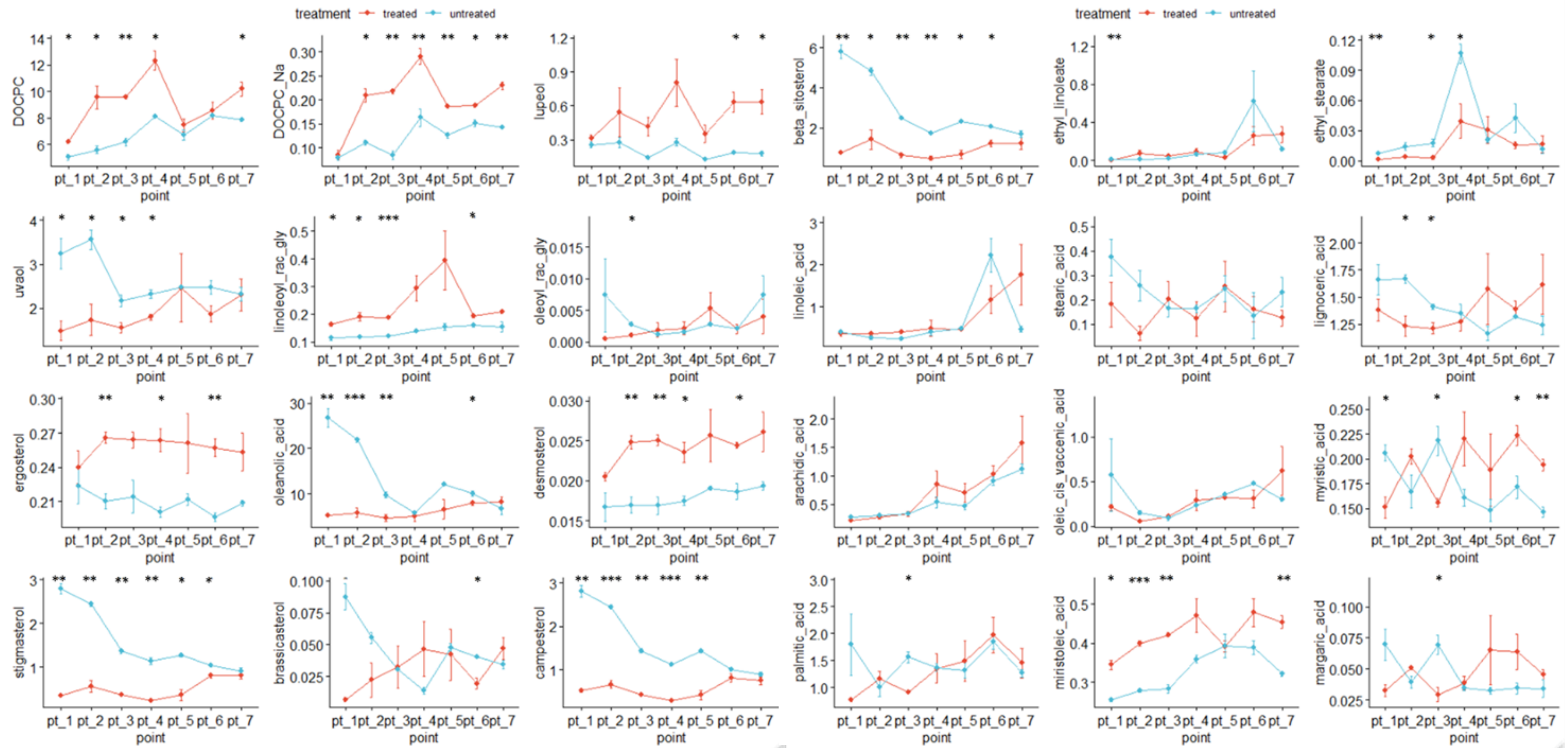
Appendix H*:* Evolution of lipid molecules in untreated (red) and treated (green) grape samples (with seeds) from FG in 2018 harvest season.



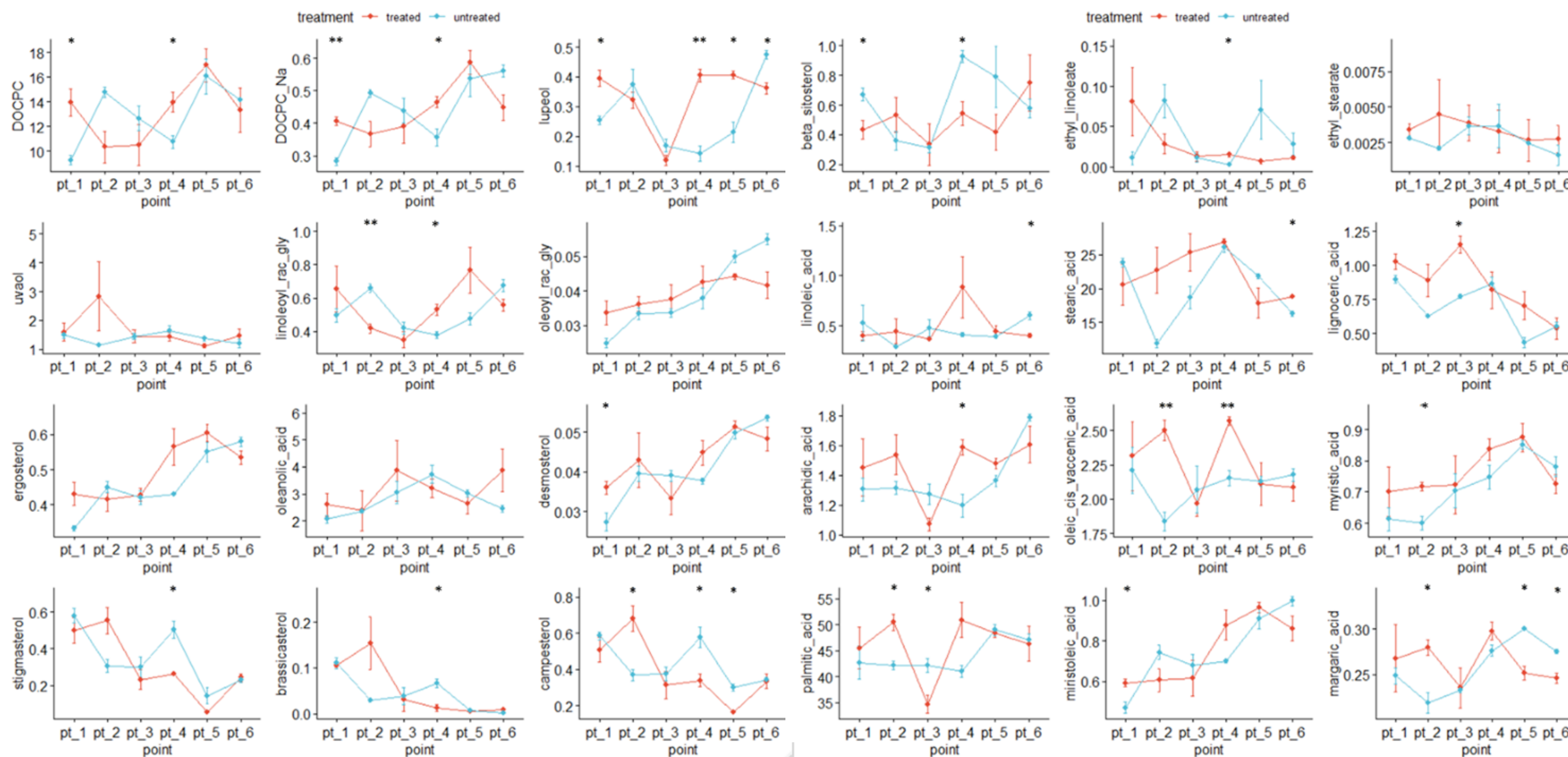
Appendix I*:* Evolution of lipid molecules in untreated (red) and treated (green) grape samples (with seeds) from FG in 2019 harvest season.



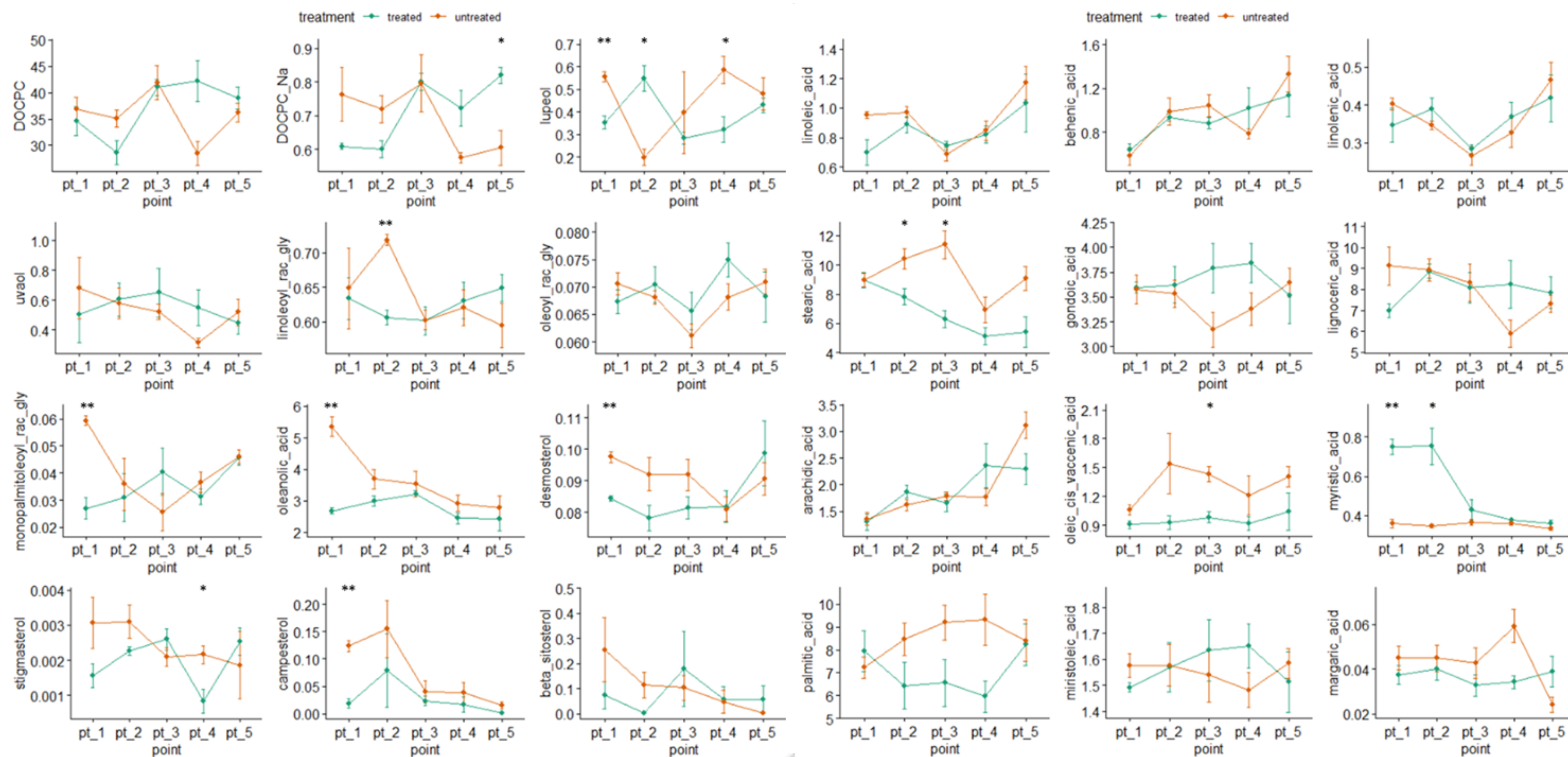
Appendix J: Evolution of lipid molecules in untreated (blue) and treated (red) grape samples (with seeds) from FCO in 2018 harvest season.



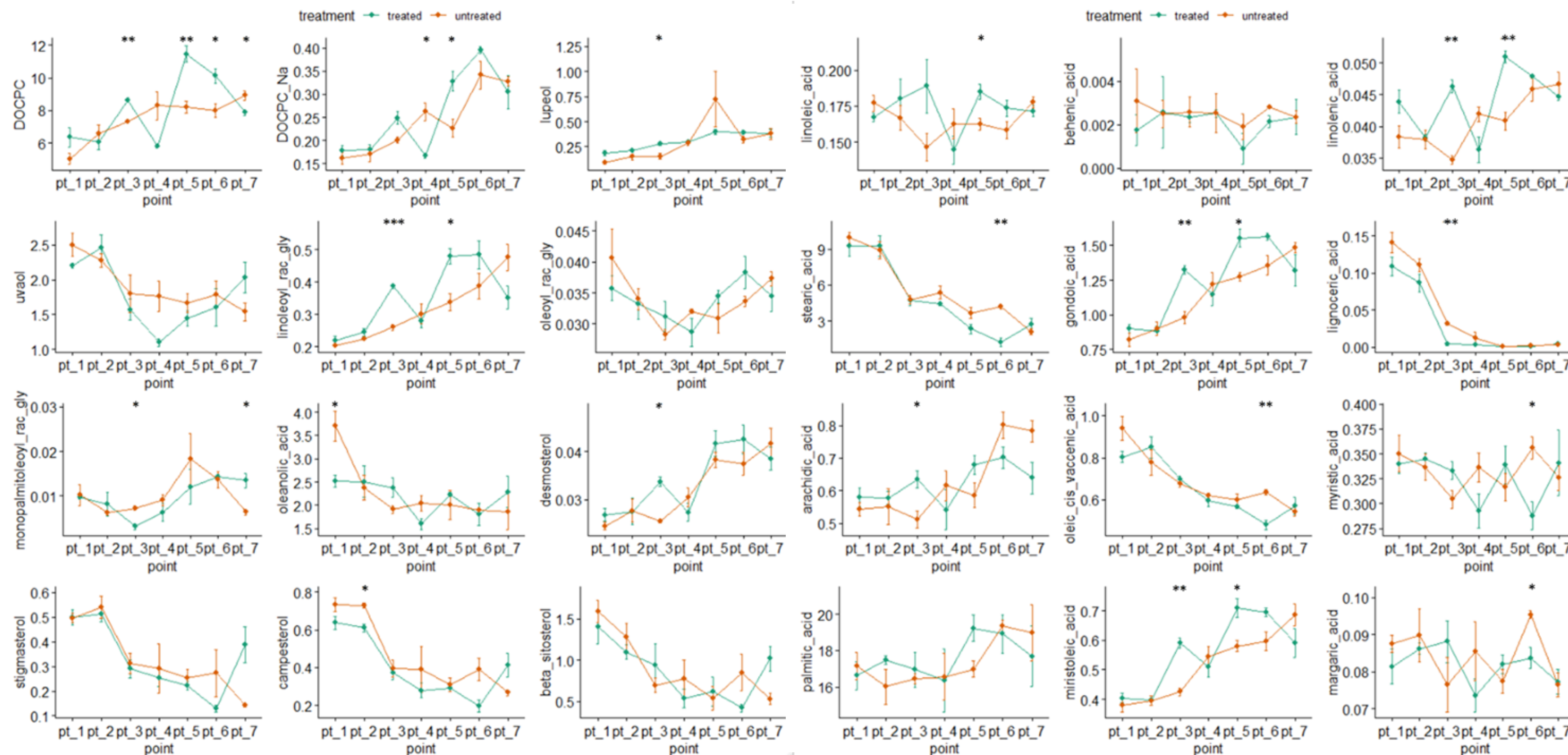
Appendix K: Evolution of lipid molecules in untreated (blue) and treated (red) grape samples (with seeds) from FCO in 2019 harvest season.



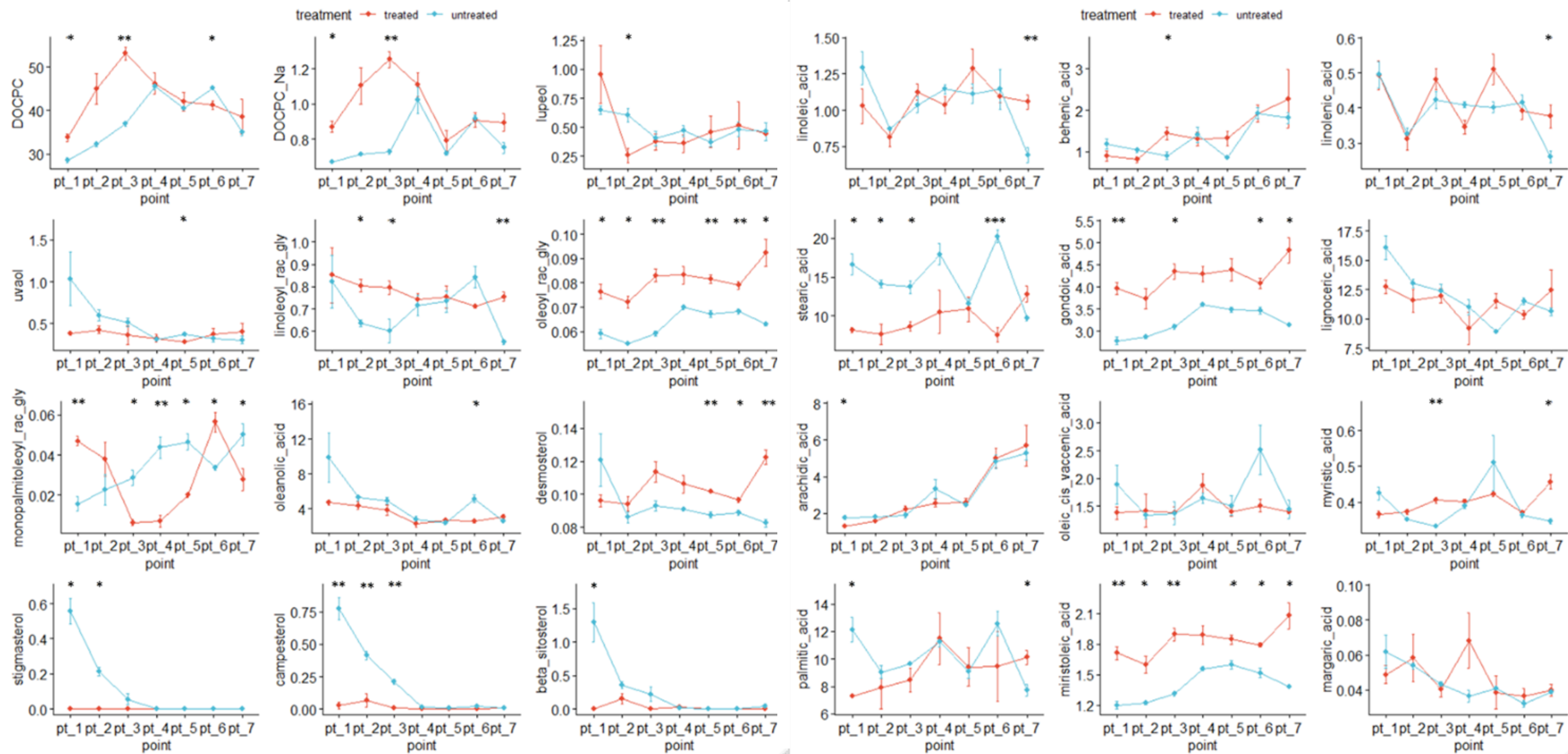
Appendix L: Evolution of lipid molecules in untreated (red) and treated (green) grape samples (with removed seeds) from FG in 2018 harvest season.



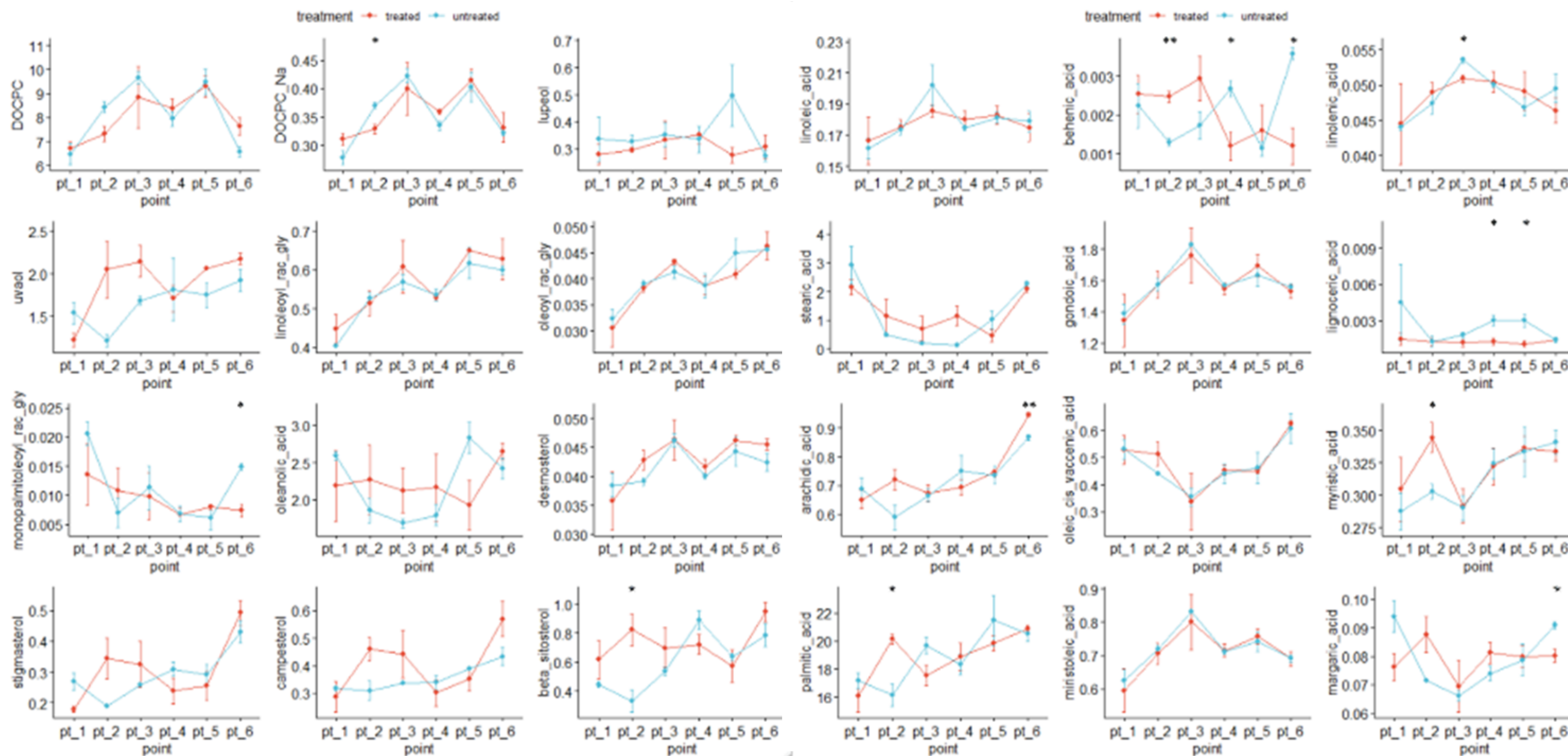
Appendix M: Evolution of lipid molecules in untreated (red) and treated (green) grape samples (with removed seeds) from FG in 2019 harvest season.



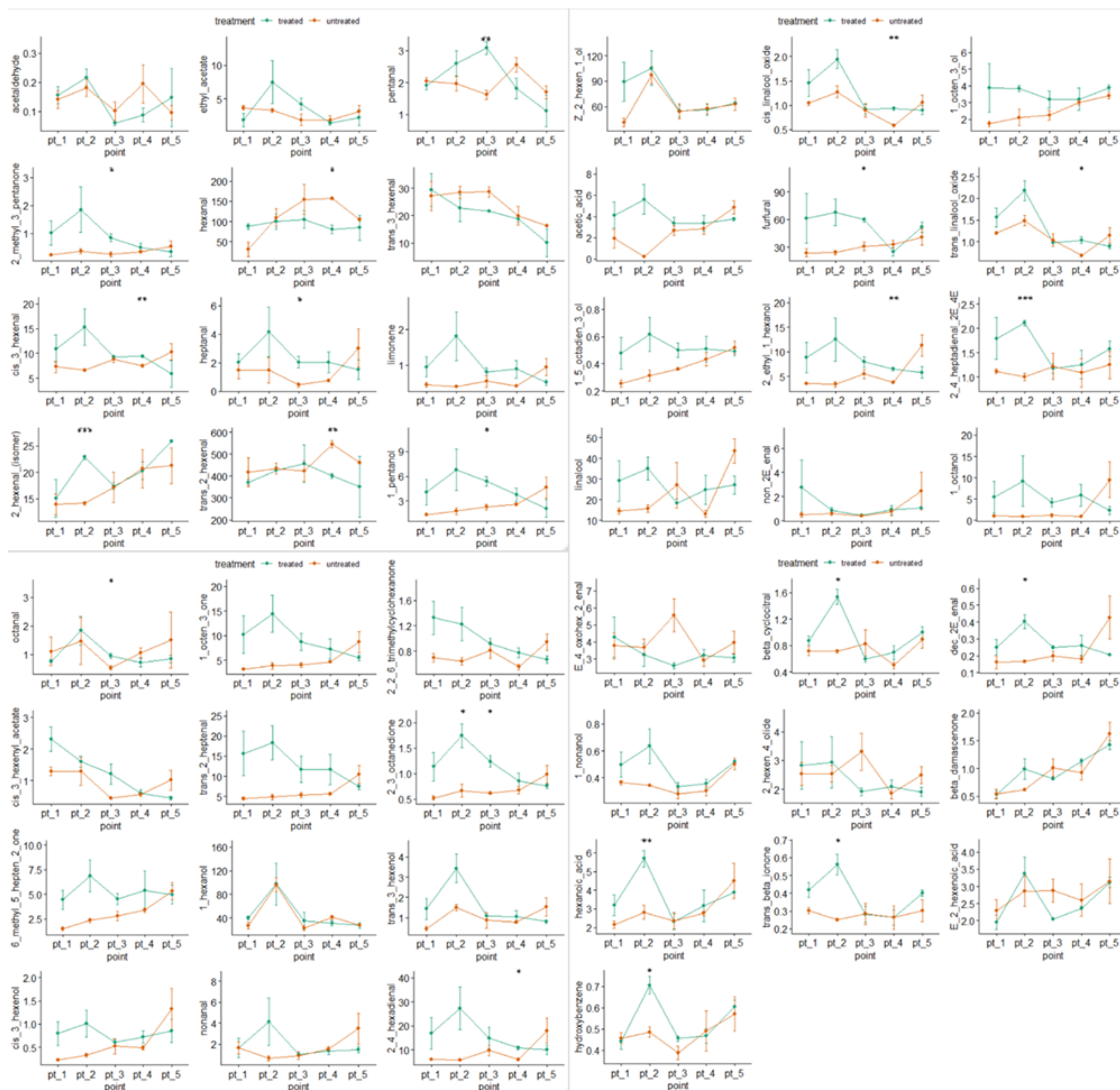
Appendix N: Evolution of lipid molecules in untreated (blue) and treated (red) grape samples (with removed seeds) from FCO in 2018 harvest season.



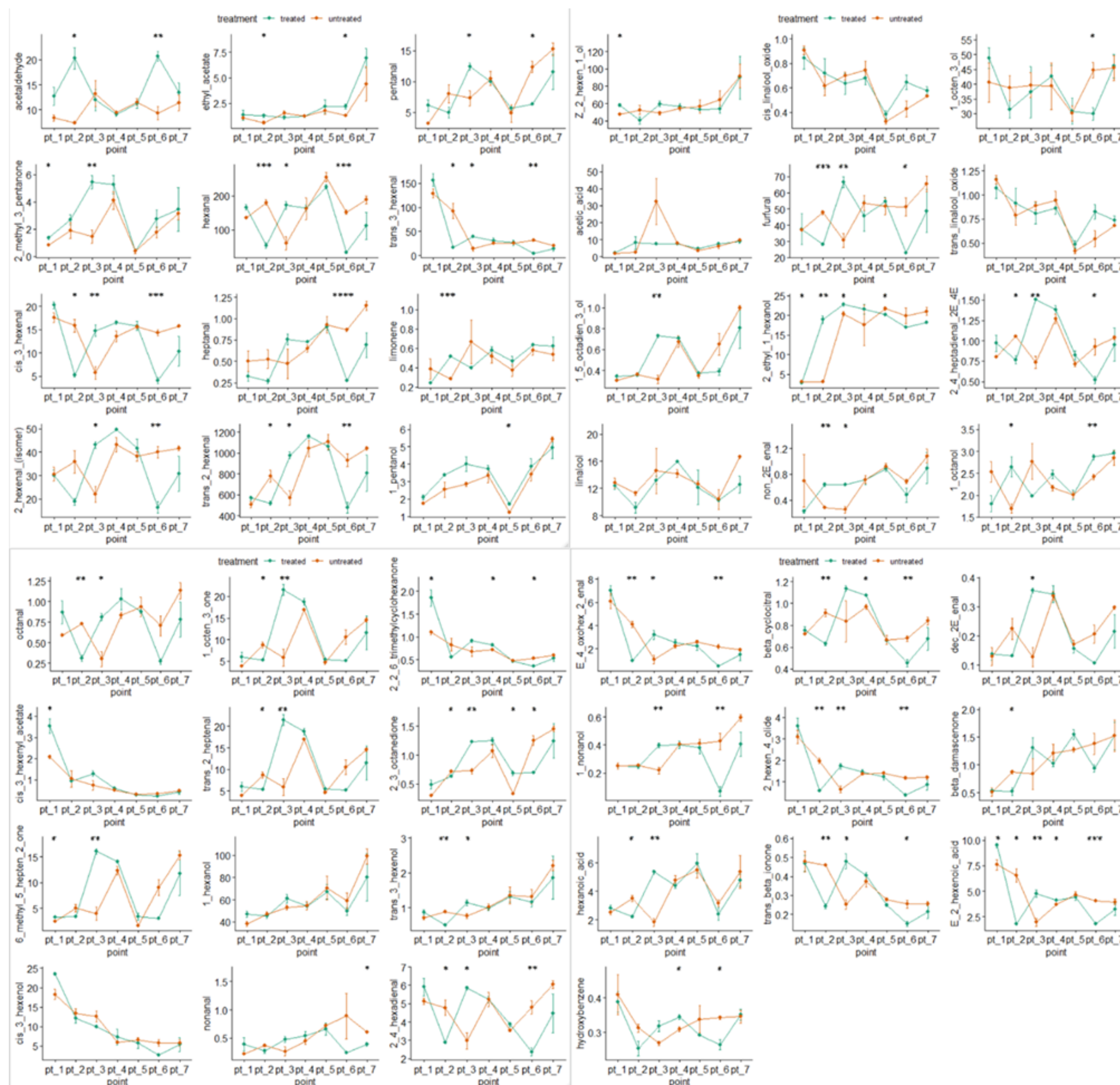
Appendix O: Evolution of lipid molecules in untreated (blue) and treated (red) samples of grapes without seeds from FCO in 2019 harvest season.



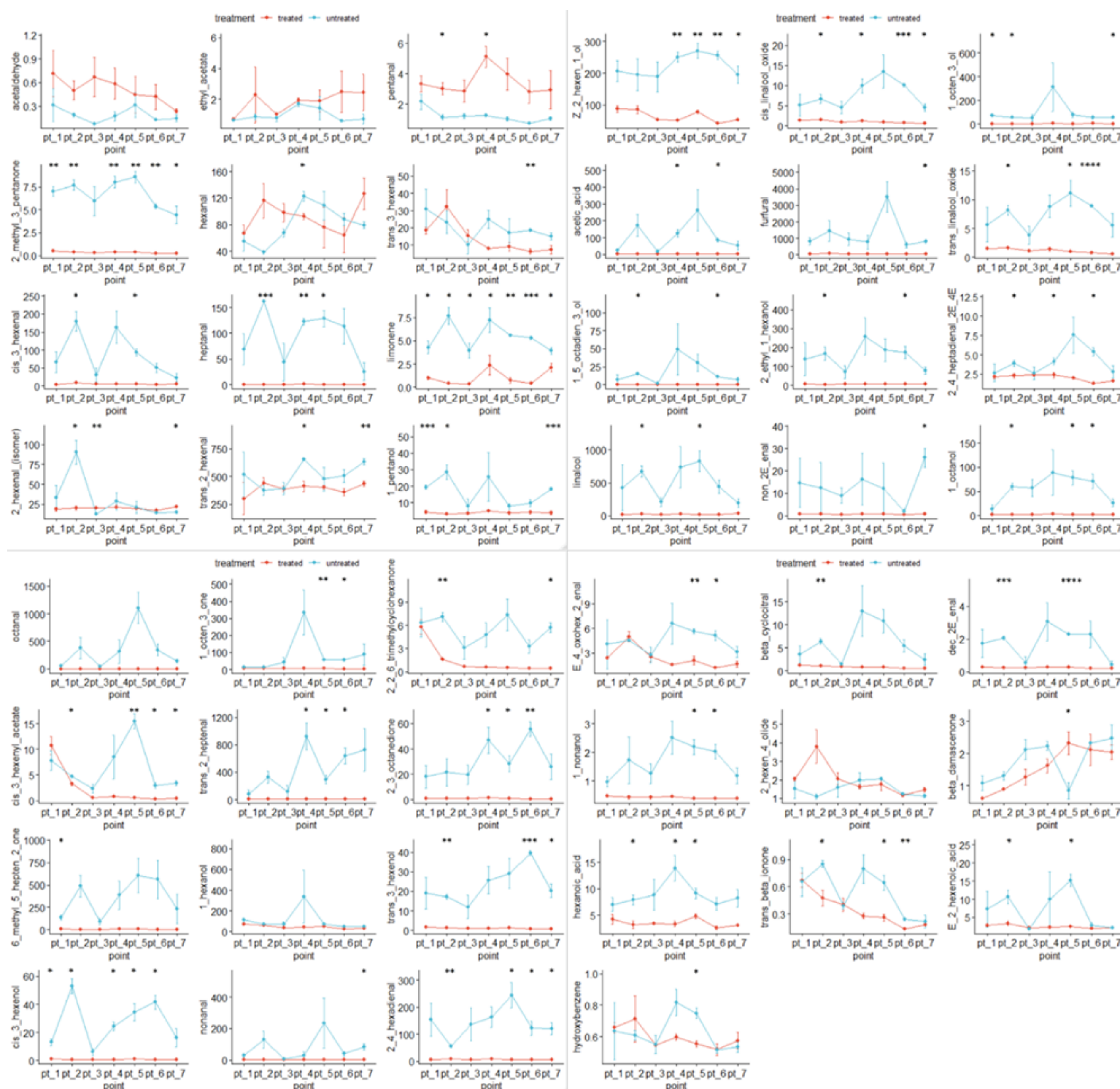
Appendix P: Evolution of volatile compounds in untreated (red) and treated (green) grape samples (with seeds) from FG in 2018 harvest season.



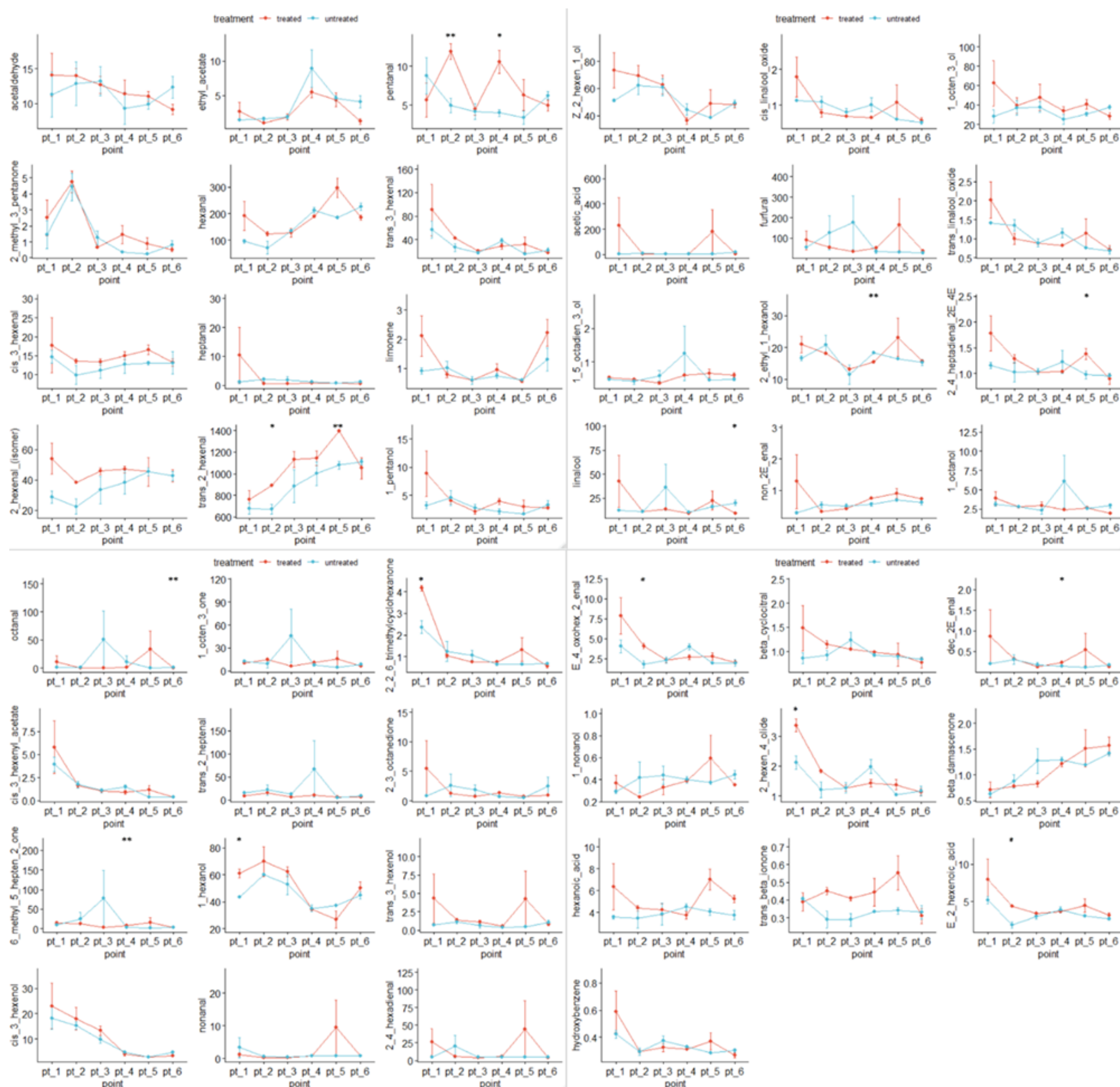
Appendix Q: Evolution of volatile compounds in untreated (red) and treated (green) samples of grapes with seeds from FG in 2019 harvest season.



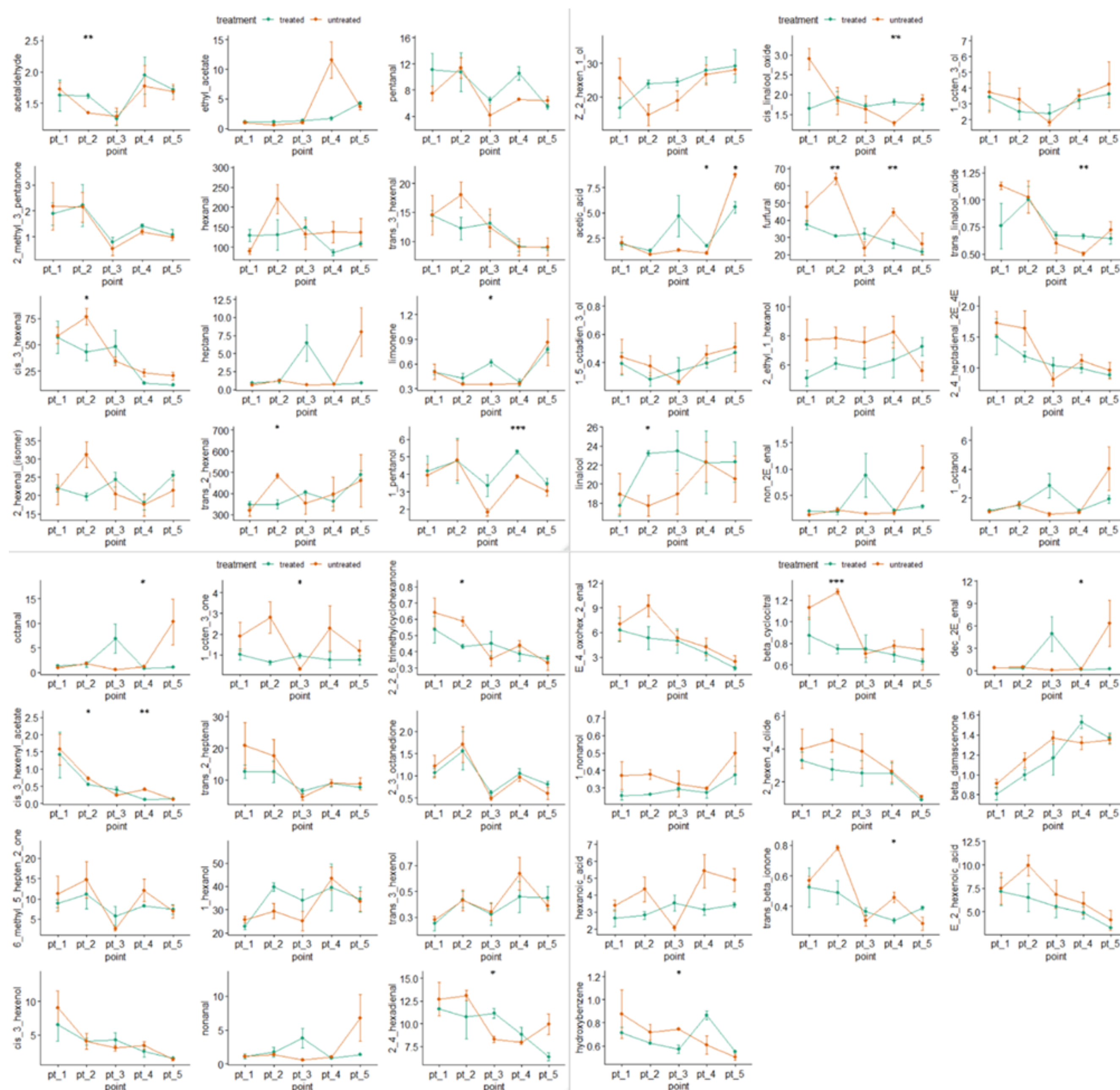
Appendix R: Evolution of volatile compounds in untreated (blue) and treated (red) grape samples (with seeds) from FCO in 2018 harvest season.



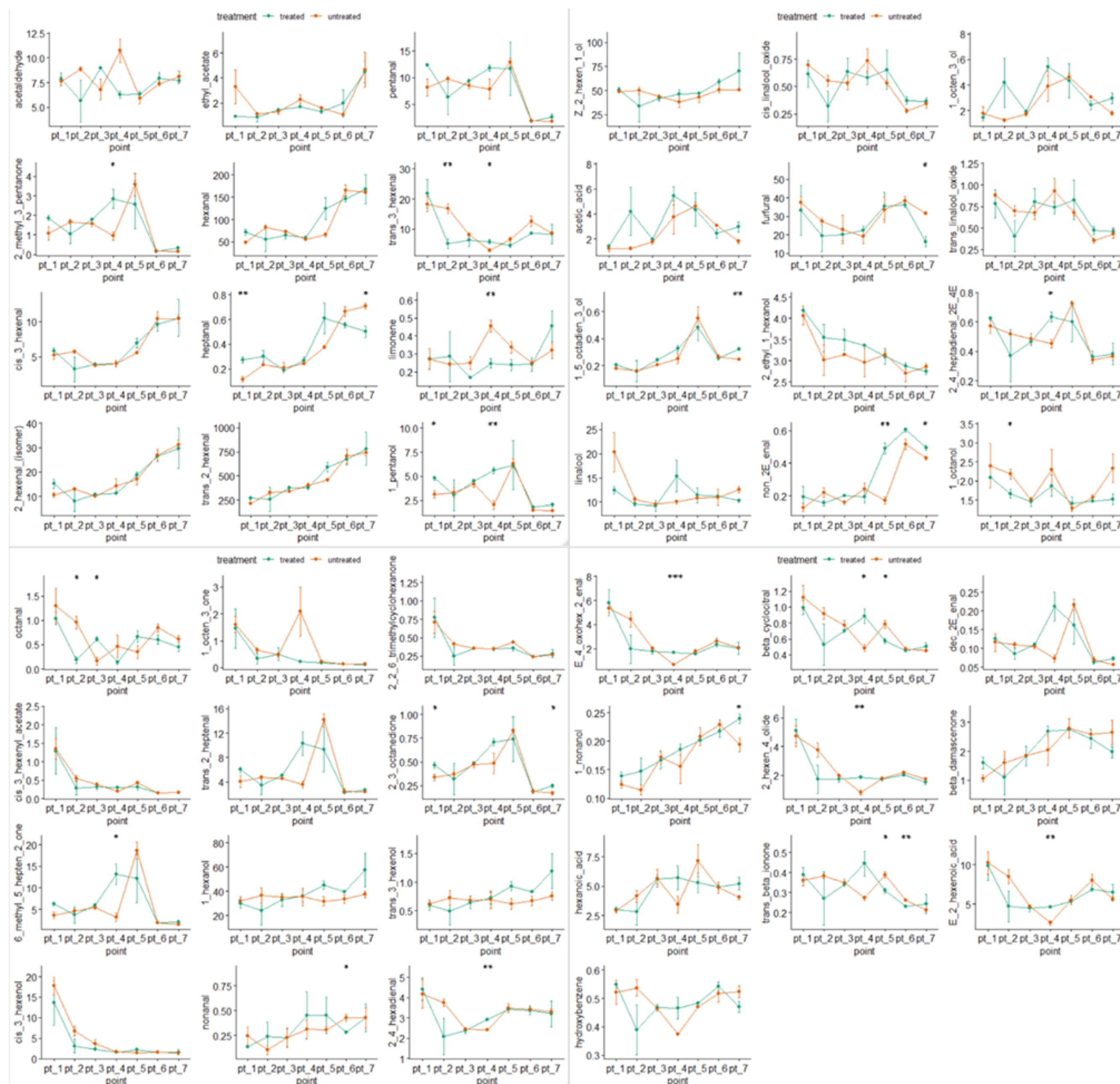
Appendix S: Evolution of volatile compounds in untreated (blue) and treated (red) grape samples (with seeds) from FCO in 2019 harvest season.



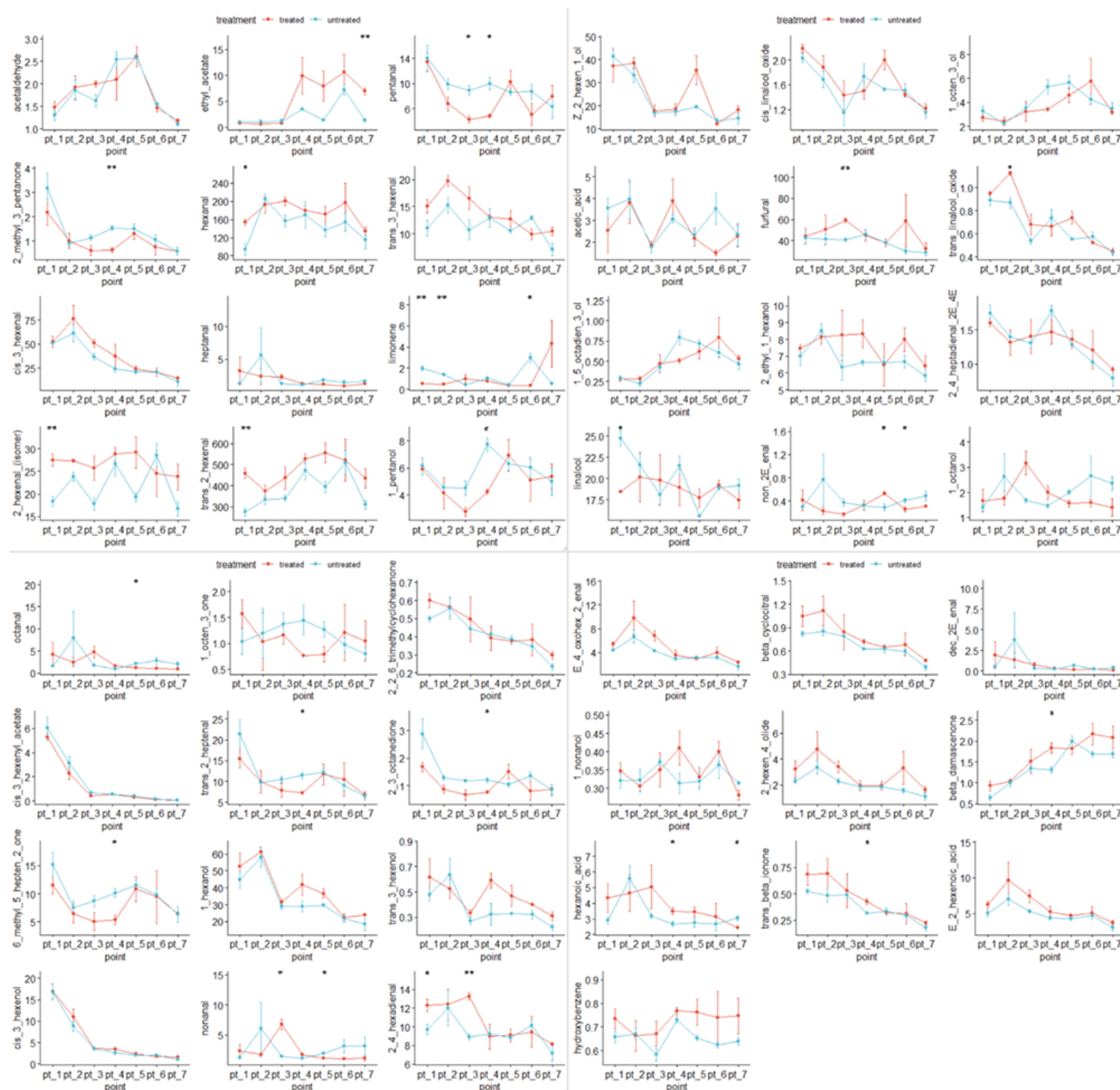
Appendix T: Evolution of volatile compounds in untreated (red) and treated (green) grape samples (with removed seeds) from FG in 2018 harvest season.



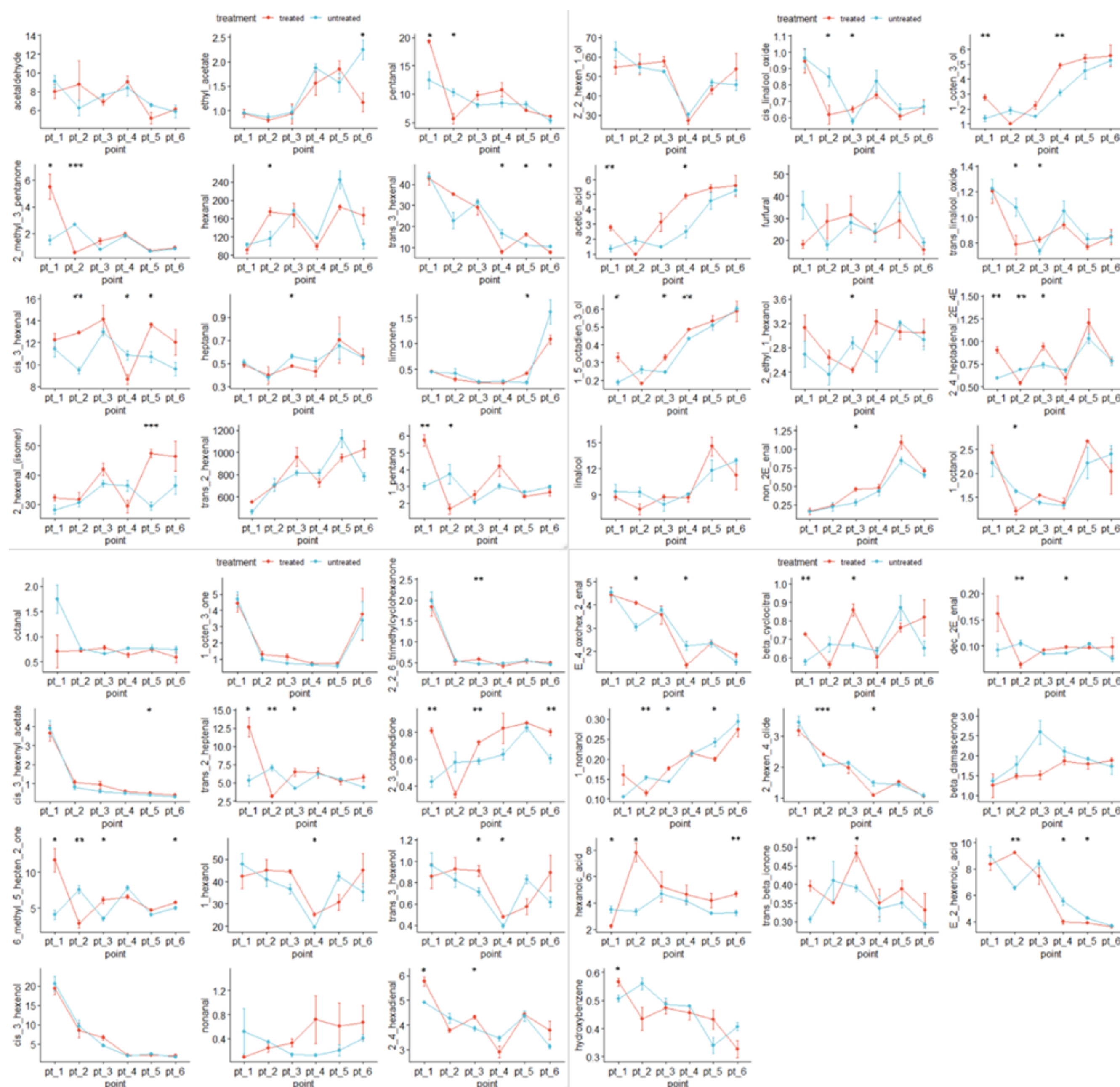
Appendix U: Evolution of volatile compounds in untreated (red) and treated (green) berry samples (with removed seeds) from FG in 2019 harvest season.



Appendix V: Evolution of volatile compounds in untreated (blue) and treated (red) berry samples (with removed seeds) from FCO in 2018 harvest season.



Appendix W: Evolution of volatile compounds in untreated (blue) and treated (red) berry samples (with removed seeds) from FCO in 2019 harvest season.



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